Metabolism of biogenic amines in animals and man with particular reference to manic depressive illness

Thesis
Presented for the Degree of
Doctor of Science
in the University of Edinburgh
by
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MB, ChB University of Aberdeen, 1957
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Research Experience

1962-1965 Mental Health Research Fund Fellowship. Held in Department of Pharmacology, University of Edinburgh. Work on cerebral metabolism in animals in Dr. T. B. B. Crawford's laboratory and in man in the Royal Edinburgh Hospital.

1965-1970 MRC Clinical Scientific Officer, MRC Brain Metabolism Unit, Department of Pharmacology, University of Edinburgh and Royal Edinburgh Hospital.

1970 to date Assistant Director, MRC Brain Metabolism Unit.
Animal Studies

Section I

Tryptophan metabolism in animals in relation to its role as a precursor of the 5-hydroxyindoles. A loading dose of the amino acid is given to increase the turnover of 5-hydroxytryptamine. Using this technique the turnover of intermediate metabolites also increases and points of drug action on this pathway may be more easily discerned.

   (with G. W. Ashcroft and T. B. B. Crawford).

   (with G. W. Ashcroft and T. B. B. Crawford).

   (with A. T. B. Moir).


Section II

The estimation of tryptamine in tissues including brain: the effects of administration of L-tryptophan and a monoamine oxidase inhibitor.

   (with G. W. Ashcroft, T. B. B. Crawford and R. Loose).
Section III

Studies on the metabolism of 5-hydroxytryptamine to 5-hydroxytryptophol.

   (with A. T. B. Moir, H. W. Reading and I. M. Ritchie).

   (with H. W. Reading and I. M. Ritchie).

Section IV

Studies in hydroxytryptamine metabolism with especial reference to the functional state of the neuron using \( ^3 \text{(H)} \) tryptophan as a precursor.

   (with A. Padjen and M. Randic).

   (with I. M. Ritchie and M. H. T. Roberts).

    (with P. J. Shields).

    (with P. J. Shields).

    (with N. B. Thoa and J. Axelrod).

    (with N. B. Thoa and J. Axelrod).
Section V

Review articles on 5-hydroxyindoles.

   (With G. W. Ashcroft and A. T. B. Moir).

   (with A. T. B. Moir, G. W. Ashcroft, T. B. B. Crawford and H. C. Guldberg).


Catecholamine metabolism in animals

Section VI

Sulphate conjugate formation in brain with particular reference to the glycol metabolites of noradrenaline. Paper II uses the technique of intraventricular injection of radioactive sodium sulphate to label endogenous and exogenous phenolic compounds.

   (with R. F. Sugden).

   (with I. M. Ritchie).

Section VII

One measurement of catechol glycol metabolites of noradrenaline by gas chromatography. The effects of lesions, electrical and self-stimulation.

   (with D. S. Walter).
(with G. W. Arbuthnott, J. E. Christie, T. J. Crow and D. S. Walter).

(with G. W. Arbuthnott, J. E. Christie, T. J. Crow and D. S. Walter).


Section VIII

An attempt to relate the experimental production of central noradrenergic supersensitivity by determining the activity of the enzyme adenyl cyclase.


Studies in Man

Section IX

Estimation of the metabolites of 5-hydroxytryptamine and dopamine in human cerebrospinal fluid in psychiatric patients, mainly in the affective illnesses.

(a) Without drug administration

26. 5-Hydroxyindole compounds in the cerebrospinal fluid of patients with psychiatric or neurological diseases. The Lancet (1966) II, 1049-1052.


(b) The use of precursor loading with L-tryptophan in man and the subsequent examination of lumbar cerebrospinal fluid for the content of 5-HIAA.


Section X

Review articles on the aetiology of affective illness with especial reference to the MRC Brain Metabolism Unit's contribution.


Section XI

A methodological paper for the estimation of 4-hydroxy-3-methoxy-phenylglycol in urine.

(with F. T. Antun and I. A. Pullar).

Section XII

Studies of 5-hydroxyindoles in man in relation to sleep, drugs, amniotic fluid and the carcinoid syndrome.

(with I. Oswald, G. W. Ashcroft, R. J. Berger, J. I. Evans and V. R. Thacore).

(with J. le Gassicke, G. W. Ashcroft, J. I. Evans, I. Oswald and E. B. Ritson).
(with G. W. Ashcroft, F. Knight, E. J. McDougall and J. L. Waddell).

(with A. E. H. Emery, J. B. Scrimgeour and M. Johnstone).


(with T. B. B. Crawford and G. W. Ashcroft).

41. Some observations on the metabolism of indoles in two patients with the carcinoid syndrome. Gastroenterol. (1965) 48, No. 6, 745-752.
(with T. B. B. Crawford, G. W. Ashcroft and A. N. Smith).

(with J. D. Kinloch, J. N. Webb and J. Zeitlin).
Nature and extent of contribution of applicant

ANIMAL EXPERIMENTS

Section I  The author played a major role in the design of the experiments, the development of methods and in terms of bench work 50% of that required for papers 1, 2, 3 and 30% of paper 4.

Section II  The author contributed about 70% to the bench work and was responsible to a large extent for the development of methods and the experimental design. The methods were evolved by the author.

Section III  The bench work and development of methods was shared with I. M. Ritchie in these papers and the experimental design with the other workers.

Section IV  The author designed most of the experiments in this section. Bench work varied from 20% papers 10 and 11, which was mostly development of methods, and 90% in papers 12 and 13.

Section V  The author had a minor contribution in papers 14 and 15 but was sole author of paper 16.

Section VI  The author was responsible for the design of the experiments of papers 17 and 18 and 40% of the bench work in these papers.

Section VII  The author designed experiments of papers 19 and 22 and collaborated with the other workers on papers 20 and 21. Bench work in this section was minimal the gas chromatographic methods being developed by D. S. Walter.

Section VIII  The ideas and design of these experiments and method development were the author's although actual bench work was minimal.

STUDIES IN MAN

Section IX  Most of the patients in paper 6 were examined by the author who did 50% of the bench work of paper 28. For papers 27 and 29 the author was responsible for the diagnosis and rating of almost all the patients when ill and recovered. The experimental design of these studies was shared with G. W. Ashcroft.

Section X  The author was responsible for review papers 30, 32 and 33. The writing of 31 was shared with G. W. Ashcroft and I. A. Pullar but with the major part produced by G. W. Ashcroft.

Section XI  The author was responsible for the design of the experiments and 20% of the bench work.
Section XII

Papers 35, 36 and 37. The design was shared with the other authors and 50% of the bench work with G. W. Ashcroft.
Papers 38 and 39. Design shared with other authors. The methods were completed by a technician (I. M. Ritchie).
Paper 40. The design was shared with the other authors, 90% of the bench work was by the author.
Paper 41. The design was shared by the other authors, bench work with G. W. Ashcroft and T. B. B. Crawford.
Paper 42. Author responsible for bench work in connection with 5OH indoles, the design shared with other authors.

The material in papers 1, 2 and 5 was presented previously within the PhD degree to this University.
Acknowledgements

I wish to acknowledge the help of the many people with whom I have worked to produce these papers especially to Mrs. Isobel Ritchie for her excellent technical assistance. I would also particularly like to thank Dr. George W. Ashcroft for his stimulating and ebullient collaboration over the years.
SECTION I
5-HYDROXYINDOLE METABOLISM IN RAT BRAIN. A STUDY OF INTERMEDIATE METABOLISM USING THE
TECHNIQUE OF TRYPTOPHAN LOADING—I

METHODS

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The discovery of the highly active biogenic amines in discrete areas of the brain (AMIN, CRAWFORD and GADDUM, 1954; VOGT, 1954; CARLSSON, 1959) has led to the suggestion that they may be concerned with brain function in these areas. The finding that many psychotherapeutic agents alter the metabolism of the amines (PLETSCHER, 1963) has stimulated the interest both of pharmacologists and of psychiatrists and many attempts have been made to study their metabolism in animals and man.

In vitro techniques using tissue homogenates do not take into account the spatial distribution of enzymes and substrates in vivo and there are advantages in using the intact animal. In experiments in vivo measurements of amine concentrations are of limited usefulness since they do not distinguish between different types of drug action, e.g. the fall in brain levels of 5-hydroxytryptamine (5-HT) produced by reserpine as a result of interference with storage, and the fall produced by α-methyldopa by interference with amine synthesis (PLETSCHER, 1963). Attempts have been made to extend the information obtainable from in vivo studies (a) by measuring not only the amines, but also amine precursors and metabolites, e.g. 5-HT and 5-hydroxy-indol-3-yiacetic acid (5-HIAA) (ASHCROFT and SHARMA, 1962) and (b) by administering a ‘loading dose’ of a precursor to increase the concentration of intermediate metabolites. Previous investigations with the latter technique include studies of the effect of tryptophan administration on tryptamine levels in animal tissues (HESS, REDFIELD and UDENFRIEND, 1959), of tryptophan administration on tryptamine and 5-HT levels in rat brain (HESS and DOEPPNER, 1961) and of 5-hydroxytryptophan (5-HTP) administration on 5-HIAA levels in rabbit brain (ROOS, 1962).

The present paper describes an attempt to combine ‘loading’ with the measurement of precursors, amines and metabolites. Metabolites in the 5-hydroxyindole pathway were determined in the brains of rats killed at varying times after intraperitoneal injection of tryptophan. The aim was to obtain a picture of the pathway in vivo under load. Investigations of the influence of certain drugs on these parameters are reported in the accompanying paper (ECCLESTON, ASHCROFT and CRAWFORD, 1965).

METHODS

Animals. Male Wistar rats 150-180 g (mean weight 170 g) were used. For 10 days prior to the experiment the animals were maintained on ‘Blue Cross’ diet 41 B (Rank). They were housed in...
groups of three and remained in these groups during the experiment, the tissues and blood from each group being pooled for the various estimations; the animals were transferred to the laboratory on the evening before the experiment. Food, but not water, was removed from the cages 24 hr before the experiment.

**Tryptophan loading.** A suspension of L-tryptophan (140 mg/ml) was prepared for administration by grinding first with a few drops of Tween 80 and then diluting with further trituration in 0.9% NaCl. This was given intraperitoneally in amounts equivalent to tryptophan 800 mg/kg body weight (Hess et al., 1959). Injected rats were killed by decapitation at various time intervals up to 8 hr after loading.'

Blood was collected from the neck wounds into a polythene tube containing heparin (0.2 ml of 1,000 I.U./ml) and stored at −15° until estimations were carried out. In one experiment, 5 ml blood was centrifuged for 5 min at 2,500 rev/min to obtain plasma.

**Preparation of brain extracts.** Solvents of Analytical Reagent grade and deionized distilled water were used throughout. The brains were dissected out as rapidly as possible, the cerebellum removed and the group of three brains weighed. They were homogenized in an all-glass homogenizer in 40% (v/v) acetic acid, 1 ml/g brain tissue. To the homogenate, a further 1·0 ml/g 20% acetic acid was added and the homogenization repeated. The homogenate was transferred to a 25 ml glass-stoppered measuring cylinder and, after the addition of a few drops of 2-octanol to break the froth, the volume was recorded and the homogenate was allowed to stand at 4° for at least 30 min. After thorough mixing, a 4.0 ml portion of the homogenate was pipetted into a 35 ml glass-stoppered centrifuge tube and to this was added 24 ml acetone at 4° to precipitate protein. After mixing, the extract was kept at −15° for 45 min and then the precipitate was removed by centrifugation. A 24 ml portion of the supernatant fluid was stored overnight in a glass-stoppered tube at 4°.

The following day, the acetone was evaporated off by means of a jet of nitrogen and the bulk of the lipid removed from the remaining aqueous phase by extraction with 16 ml, followed by 8 ml, light petroleum (b.p. 40–60°C). After addition of ascorbic acid, (0·1 ml of 50 mg per 100 ml, freshly prepared) the aqueous phase was evaporated to damp dryness under reduced pressure (external temp. 55°). During this evaporation, sufficient acetic acid remains in the aqueous extract to maintain the pH at 3–4, a pH at which the 5-hydroxyindoles are most stable. The evaporation, under these conditions, should take less than 10 min.

In each experiment, two additional portions of one of the homogenates were carried through the above extraction procedure. To the first was added 0·8 μg each of 5-HT, 5-HTP and 5-HIAA before protein precipitation to serve as internal standards throughout the procedure. To the second specimen, the three 5-hydroxyindoles were added to the final residue from the extraction process in amounts sufficient to give visible colourations on the developed chromatogram (see below).

**Paper chromatography**

The final residue was transferred in 0·2 ml followed by 0·1 ml 80% (w/v) methanol containing ascorbic acid (50 mg per 100 ml) to a 5 cm wide paper chromatogram (Whatman No. 1 paper—see Fig. 1) by replicate applications using a capillary tube to give a 1 cm band. During application, the solvent was removed in a continuous stream of nitrogen. The ascending chromatogram was developed in butanol-1:1:1 acetic acid:water (12:3:5 by vol.) in a nitrogen atmosphere at room temperature overnight (15–16 hr solvent run of 24–25 cm).

The developed chromatogram was dried in a glass tank, through which was passed a stream of nitrogen. The positions of the 5-OH indoles were established from the marker chromatograms (see below). Appropriate sections of the sample chromatogram were eluted with 4·2 ml 0·1 N-H₂SO₄ by immersion and gentle agitation in glass-stoppered tubes. After 15 min the paper strips were removed, the volume of each eluate recorded and, if necessary, made up to 4·0 ml with 0·1 N-H₂SO₄. Conc. HCl (2·0 ml containing ascorbic acid 50 mg per 100 ml) was added and the samples read against a standard of 5-HT in a Farrand recording spectrophotofluorimeter (Ashcroft, Crawford, Binns and MacDougall, 1964). The relative fluorescences of standard solutions of 5-HT, 5-HTP and 5-HIAA were determined.

**Localization and identification of the 5-OH indole compounds in a chromatogram of brain extract.** This was achieved (a) by location with reference to a parallel chromatogram of authentic 5-HT, 5-HTP and 5-HIAA and (b) by precise location of the 5-OH indoles on the brain extract chromatogram by fluorimetric analyses of sequential segments of the chromatogram.

It was found that the Rf values of 5-HT, 5-HIAA or 5-HTP applied in brain extracts and applied in pure solution were not identical, the greatest disparity occurring with the amine (Table 1). Routinely, therefore, the positions of these substances in chromatograms of brain extracts were determined from a parallel chromatogram of a similar brain extract, to which had been added 5-HT, 5-HIAA and 5-HTP in amounts sufficient to permit easy visualisation of the indoles by (a) the characteristic pinkish fluorescence in u.v. light, while the chromatogram still retained a small amount of acetic acid from
Table 1.—$R_f$ values for 5-hydroxyindole compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Indole applied to chromatogram in pure solution</th>
<th>$R_f$</th>
<th></th>
<th>Indole applied to chromatogram in brain extract</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Hydroxytryptophan</td>
<td></td>
<td>0.26</td>
<td></td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td></td>
<td>0.43</td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>5-Hydroxyindol-3-ylacetic acid</td>
<td></td>
<td>0.70</td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
</tbody>
</table>

Ascending paper chromatography in butan-1-ol:acetic acid:water (12:3:5 by vol.)

the chromatographic solvent (Mitoma, Weissbach and Udenfriend, 1955) and (b) the colour reaction with Ehrlich's reagent (Rodnight, 1956).

While 5-HTP and 5-HT were separated by several cm, only about 2 cm separated the upper boundary of the 5-HT area from the lower boundary of the 5-HIAA area. It was, therefore, essential that the division between these two areas should be accurately located. Templates were made up from paper strips on which were marked the proposed sections of the chromatogram (Fig. 1). To allow for slight differences in the extent of solvent flow in the extract and 'marker' chromatograms, six such templates were prepared, varying in length by 0.5 cm; the shortest being 1 cm less than, and the longest 1 cm greater than the distance from the origin to the solvent front from the 'marker' chromatogram. The positions of the proposed cuts were marked on each template at the appropriate points calculated from the $R_f$ values on the 'marker' chromatogram and the length of the template strip. The dried chromatogram of a brain extract was aligned with the template appropriate to the solvent front position. The appropriate sections were cut and dropped into the eluting fluid as quickly as possible; nine consecutive regions were analysed. The presence of 5-OR indoles in those sections of the chromatograms postulated to contain none would point either to poor separation, or to the presence of an unexpected 5-OR indole, but there were no detectable amounts of 5-HT, 5-HIAA or 5-HTP in the eluates of those sections.

Measurement of tryptophan in brain extracts and whole blood. The method of Hess and Udenfriend (1959), as applied by Guroff and Udenfriend (1962), was used.

Measurement of 5-HTP in plasma. To 20 ml plasma were added 0.4 ml acetic acid, and the mixture left at 4° for 30 min before adding 18 ml acetone at 4°. The further preparation of the extract and the subsequent chromatography was then carried out as for the brain extracts. The strip of the chromatogram corresponding to the 5-HTP marker was eluted and the 5-HTP assayed spectrofluorimetrically.

Confirmation of the identity of substances in the '5-HT' and '5-HIAA' sections of chromatograms of brain extracts. In a few experiments, two additional methods of identification were used: (a) confirmation of the basic or acidic nature of the 5-hydroxyindole compounds, using solvent extraction procedures on the eluted compounds and (b) comparison of biological and fluorescence assays.

(a) Solvent extraction. In one experiment, the eluates from the chromatogram strips corresponding to the marker 5-HT and 5-HIAA positions were subjected to the solvent extraction procedures, whilst in a second experiment only the 5-HIAA strips were treated in this way. The concentration of 5-hydroxyindoles in the eluates was first assayed fluorimetrically in 3 N HCl. The eluates in 3 N HCl were then saturated with NaCl and extracted with diethyl ether, using a modification of the method of Udenfriend, Titus and Weissbach (1955) described by Ashcroft and Sharman (1962) for the estimation of 5-HIAA, and the acid 5-hydroxyindole compounds measured spectrofluorimetrically.

The aqueous phase, after the ether extraction, was adjusted with 20% NaOH (w/v, Analar) to pH 10, using phenolphthalein as internal indicator and basic 5-hydroxyindole compounds were extracted and measured, using the method of Bogdanski, Pleitscher, Brodie and Udenfriend (1956).

The concentration of basic and acidic 5-hydroxyindole compounds in the original eluates were calculated by reference to internal standards carried through the extraction procedure.

(b) Comparison of biological and fluorescence assay. Extracts were prepared from the brains of animals killed 2 hr after loading with tryptophan 800 mg/kg and also of control animals. The appropriate strips for '5-HIAA' and '5-HT' from the developed chromatogram were placed in glass tubes and dried in vacuo in a desiccator containing NaOH pellets, in order to remove any traces of acetic acid. The strips were eluted with 0.9% (w/v) NaCl and the eluates subjected to fluorescence assays, carried out as described above; and to biological assays using the isolated rat uterus preparation (Amin et al., 1954).

RESULTS

Recovery experiments. Table 2 shows the results of the recovery experiments of 5-HT, 5-HIAA, 5-HTP added to brain homogenate. Recoveries of 5-HTP were lower
Fig 1.—Left: Diagram of 'strip' chromatogram of 5-hydroxyindole compounds developed in butan-1-ol: acetic acid: water (12: 3: 5 by vol.). Indoles located by the colour reaction with Ehrlich’s reagent.

Right: Template defining positions of strips for sample chromatogram.

Table 2.—Recoveries of 5-hydroxyindoles from brain homogenates

<table>
<thead>
<tr>
<th>5-Hydroxytryptamine</th>
<th>5-Hydroxyindol-3-ylacetic acid</th>
<th>5-Hydroxytryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td>87 ± 6.5 (9)</td>
<td>96 ± 10.9 (8)</td>
<td>55 ± 6.9 (9)</td>
</tr>
</tbody>
</table>

0.8 μg of each of the 5-hydroxyindoles was added to the homogenates.

* Mean ± standard deviation (no. of experiments).
than those for 5-HT or 5-HIAA, the loss occurring during protein precipitation. The reported estimates of the concentration of 5-HP, 5-HT and 5-HIAA in brain samples have been corrected for the mean recovery of each indole obtained during this series of experiments. Recovery of 5-HP from plasma in a single experiment was 67%.

Identity of substances in '5-HT' and '5-HIAA' sections of chromatogram. (a) A comparison of direct estimation of 5-hydroxyindoles in the eluates from '5-HT' and '5-HIAA' strips and estimates of basic and acidic 5-hydroxyindole compounds in the strips, obtained by the solvent extraction techniques, are shown in Table 3. There was good quantitative agreement between pre- and post-extraction estimates and no basic 5-hydroxyindole found in the '5-HIAA' strip, or acidic 5-hydroxyindole in the '5-HT' strip. (b) A comparison of the fluorimetry and biological assay of eluates from '5-HT' and '5-HIAA' strips is given in Table 4. No biological activity was detected in the

Table 3.—Identification of 5-hydroxyindole compounds in eluates from chromatograms of brain extracts by solvent extraction for acidic and for basic compounds

<table>
<thead>
<tr>
<th></th>
<th>'5-HT Strip'</th>
<th>'5-HIAA Strip'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>Extractable</td>
</tr>
<tr>
<td></td>
<td>fluorescence</td>
<td>basic 5-OH indole</td>
</tr>
<tr>
<td></td>
<td>estimate</td>
<td>(µg 5-HT per eluate)</td>
</tr>
<tr>
<td>0.94</td>
<td>0.92</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>0.54</td>
<td>0.40</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

Table 4.—Identification of 5-hydroxyindole compounds in eluates from chromatograms of brain by a comparison of estimates obtained by bioassay and fluorescence techniques

<table>
<thead>
<tr>
<th>Brain sample from rats after</th>
<th>'5-HT' section of chromatogram</th>
<th>'5-HIAA' section of chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT equivalents, µg/ml eluate</td>
<td>5-HIAA µg/ml eluate</td>
</tr>
<tr>
<td></td>
<td>Fluorimetry</td>
<td>Bio-assay</td>
</tr>
<tr>
<td>No treatment L-tryptophan 800 mg/kg intraperitoneally 2 hr before</td>
<td>0.10</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>0.30</td>
</tr>
</tbody>
</table>

In the '5-HT' strip, biological assay gave higher results (0.07–0.08 µg higher) than fluorescence assay, both in unloaded and tryptophan loaded animals. These results indicate the presence of a second substance with a biological activity (as measured on the rat uterus) equivalent to 0.07–0.08 µg 5-HT per ml eluate, the concentration being unaltered by tryptophan loading. No attempts have been made to investigate the discrepancy.
Uptake of amino acids by brain tissue. The uptake of 5-HTP into brain has not been studied, but Table 5 illustrates the relationship between whole blood and brain concentrations of tryptophan. This relationship would not be expected to be a simple one, as factors such as binding of tryptophan to plasma protein and uptake by red cells (McMenamy, Lund and Oncley, 1957) make calculation of the concentration of freely diffusible plasma tryptophan impossible from the figures given for whole blood. However, it is postulated that any significant changes in the tissue uptake of tryptophan would be reflected in changing blood/brain ratios.

The blood/brain ratio is seen to increase rapidly during the first half-hour after loading, as a result of the failure of brain uptake processes to keep pace with the increase in blood tryptophan levels. Between 1 and 2 hr, the ratio decreases as brain levels continue to rise whilst blood levels fall. At 2 and 4 hr, the ratio is lower than in the unloaded animal, as the fall in brain levels is slower than that in blood. By 8 hr, the ratio is again returning to normal.

The presence of 5-HTP in rat plasma following tryptophan administration. Evidence was obtained which indicated the presence of measurable amounts of 5-HTP in the plasma of animals following tryptophan loading (Table 6). None was detectable in the plasma of control animals.

Concentrations of 5-hydroxyindoles in rat brain following tryptophan administration. Following administration of tryptophan to rats in a dose of 800 mg/kg, the brain concentration of 5-HT showed a rise (Table 7) from a mean control concentration of 0.3 μg/g to a maximum of 0.6 μg/g within 1 hr. This concentration was maintained during the following 3 hr, after which it returned to the control value within a maximum of 8 hr after the tryptophan administration. The 5-HIAA, with a control level of 0.24 μg/g also increased in concentration, but, unlike the 5-HT, the concentration of the acid did not remain at a constant maximum level during the 1–3 hr interval after loading, but continued to increase, reaching 1.09 μg/g 4 hr after loading. Although the same concentration was found also at 6 hr after the tryptophan administration, we have not examined whether the 5-HIAA was maintained at this level over the 4–6 hr interval, or whether the concentration did, in fact, reach a higher but relatively unsustained maximum during this period. No 5-hydroxytryptophan was detectable in the brain samples at any time before or after the tryptophan administration.

DISCUSSION

The major metabolic pathway of 5-hydroxyindoles in the brain is 5-hydroxytryptophan → 5-hydroxytryptamine → 5-hydroxyindol-3-ylacetic acid. The analytical technique permitted the estimation of these three substances in a single sample of tissue or plasma and by combining this technique with tryptophan loading it was possible to build up a dynamic picture of the formation and metabolism of 5-hydroxytryptamine in the rat brain.

Limiting step in the synthesis of 5-HT. The blood/brain barrier prevents uptake of 5-HT from blood into brain tissue; hence the first step in the rise in concentrations of 5-HT and 5-HIAA in brain following tryptophan loading must be either the hydroxylation of tryptophan in the brain tissue, or the uptake into brain of 5-HTP produced elsewhere in the body. Cooper and Melcer (1961) deduced that 5-HTP must be transported in the plasma from an extracerebral site, but, since the amino acid had not been detected in the blood, they suggested that it might be carried in bound form.
Table 5.—Tryptophan concentration in rat blood and brain at various times after intraperitoneal administration of L-tryptophan

<table>
<thead>
<tr>
<th>Time after tryptophan (hr)</th>
<th>Blood tryptophan (μg/ml)</th>
<th>Mean blood tryptophan (μg/ml)</th>
<th>Brain tryptophan (μg/g)</th>
<th>Mean brain tryptophan (μg/g)</th>
<th>Blood/brain ratio</th>
<th>Mean blood/brain ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (No tryptophan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19-2</td>
<td>17-0</td>
<td>15-9</td>
<td>17-4</td>
<td>7-1</td>
<td>6-9</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>890</td>
<td>1220</td>
<td>1037</td>
<td>177</td>
<td>146</td>
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<td>480</td>
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</tr>
<tr>
<td>8</td>
<td>300</td>
<td>265</td>
<td>288</td>
<td>210</td>
<td>219</td>
<td>215</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation. Dose of tryptophan, 800 mg/kg.
G. W. ASHCROFT, D. ECCLESTON and T. B. B. CRAWFORD

Table 6.—Concentration of 5-hydroxytryptophan in rat plasma at various times after intraperitoneal injection of L-tryptophan

<table>
<thead>
<tr>
<th>Time after tryptophan (hr)</th>
<th>Plasma 5-hydroxytryptophan (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (no tryptophan)</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>0.5</td>
<td>0.13</td>
</tr>
<tr>
<td>1</td>
<td>0.075</td>
</tr>
<tr>
<td>2</td>
<td>0.075</td>
</tr>
<tr>
<td>4</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Results are from a single experiment. Each result represents an estimate on pooled plasma from three rats. Dose of tryptophan, 800 mg/kg.

Table 7.—5-Hydroxyindole concentration in brain tissue at various time intervals after intraperitoneal injection of L-tryptophan in rats

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>No. of experiments</th>
<th>Concentration ± s.d. (µg/g)</th>
<th>5-HT</th>
<th>5-HIAA</th>
<th>5-HTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (no tryptophan)</td>
<td>4</td>
<td>0.32 ± 0.06</td>
<td>0.24 ± 0.015</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>3</td>
<td>0.49 ± 0.04</td>
<td>0.45 ± 0.03</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0.62 ± 0.04</td>
<td>0.57 ± 0.06</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.59 ± 0.14</td>
<td>0.84 ± 0.13</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0.59 ± 0.13</td>
<td>1.09 ± 0.15</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.46</td>
<td>1.00</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>0.32 ± 0.08</td>
<td>0.42 ± 0.18</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Dose of tryptophan, 800 mg/kg. Result expressed in µg 5-hydroxyindole/g brain.

In our experiments, the presence of 5-HP in plasma was detected, but only after tryptophan loading. Recent work by GAL, POCZIK and MARSHALL (1963) and by GRAHAME-SMITH (1964) has demonstrated the ability of brain tissue to 5-hydroxylate tryptophan. It is, therefore, conceivable that the 5-HTP serving as a precursor of 5-HT in brain is derived from tryptophan both cerebrally and peripherally.

The failure of 5-HTP to accumulate in brain tissues, the concentration of 5-HT and 5-HIAA having reached steady levels, would indicate that hydroxylation of tryptophan is the rate limiting step in the production of 5-HT, but a 'feed-back' mechanism from accumulated 5-HT or 5-HIAA limiting the rate of hydroxylation in an extracerebral site, or in the brain itself is not ruled out.

Limiting concentrations of 5-HT and 5-HIAA and possible existence of metabolic pools. The concentration of 5-HT reached a maximum of approximately 0.6 µg/g brain 1 hr after loading with tryptophan, following which the concentration remained at this level for the next 3 hr. The 5-HIAA levels showed no such tendency to level out at this time, but continued to rise, reaching an apparent maximum 4 hr after loading.

There are at least two possible explanations. Firstly, the presence of a 'pool,' or store, of 5-HT with a limited capacity estimated at 0.6 µg/g brain. This having filled,
all the amine subsequently produced would overflow on to sites of destruction, with a continued rise of 5-HIAA. An alternative explanation for the sustained maximum concentrations of 5-HT might be the existence of a limiting supply of 5-HP, owing to either a maximum limiting rate of formation as a result of saturation of the decarboxylating enzyme, or to a 'feed-back' mechanism operating on this enzyme. Given this constant supply of 5-HP, with rapid decarboxylation to 5-HT, then the concentration of 5-HT will rapidly rise to a plateau, the level of which will depend upon the establishment of an equilibrium between synthesis, storage and breakdown to 5-HIAA. The 5-HIAA will then continue to rise, possibly reaching a plateau at a later time (there is the suggestion of such a plateau between 4 and 6 hr). The level reached by 5-HIAA will depend on the relative rates of formation from 5-HT and removal, which, in this case, will involve passage from brain tissue into blood and C.S.F.

A study of 5-OH indole metabolism in brain by the load technique has been described here, but obviously more information can be obtained using modifications of the technique, such as the use of drugs which block enzymes in the pathway. Several such studies are described in the following paper.

The techniques of extraction and separation used in these studies have been deliberately chosen to allow their application to tissue other than brain and to body fluids, such as C.S.F. and plasma.

No detailed comment has been made on the behavioural effects noted after loading the rats with tryptophan as no objective measure of these effects was used. However, it should perhaps be mentioned that within a few minutes of injection spontaneous activity was reduced and it was often possible to place the rats in abnormal postures, e.g. recumbent on their hind legs with their backs propped against the side of the cage. These effects persisted for about 4 hr after administration of 800 mg/kg tryptophan.

SUMMARY

A sensitive technique for the assay of the 5-hydroxyindole compounds was devised, involving separation by paper chromatography followed by elution and fluorimetric assay. The specificity of the analytical procedure was determined. Concentrations of tryptophan were measured in blood and brain tissue of the rat and the concentrations of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid were determined in the brain at times up to 8 hr after loading with tryptophan, 800 mg/kg.

No 5-hydroxytryptophan was detected in the brain after tryptophan loading, although it appeared in measurable amounts in the plasma. The 5-hydroxytryptamine in brain rose rapidly to a level which remained constant over a period of hours. The 5-hydroxyindol-3-ylacetic acid in brain also increased rapidly, reaching a maximum at a time later than 5-hydroxytryptamine. The significance of these findings is discussed.

Acknowledgements—The authors are grateful to Professor W. L. M. Perry for his advice, interest and criticism and to Miss A. Urquhart for preparation of the figures.

REFERENCES


GRAHAM-SMITH D. G. (1964) Biochem. J. 92, 52 P.


5-HYDROXYINDOLE METABOLISM IN RAT BRAIN. 
A STUDY OF INTERMEDIATE METABOLISM USING 
THE TECHNIQUE OF TRYPTOPHAN LOADING—II 
APPLICATIONS AND DRUG STUDIES

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The Pharmacology Department, Edinburgh University, Edinburgh 

(Received 12 October 1964)

The investigation of 5-hydroxytryptamine metabolism in the rat brain, combining the 
technique of tryptophan loading with the measurement of intermediate metabolites, 
has been described in the preceding paper (Ashcroft, Eccleston and Crawford, 1965). 
This paper is concerned with applications of this technique. A study was made of the 
effect of varying the dose of tryptophan and also of the effects of administration of 
drugs known to block enzymes concerned in the metabolism of tryptophan.

METHODS

Male Wistar rats (160 180 g) were deprived of food for 16-18 hr before tryptophan loading 
treatment and transferred to the laboratory on the evening prior to the experiment. Water was 
continuously available before and during the experiment. A group of three animals was used for 
each treatment and time interval, the animals being killed by decapitation. Analyses were carried 
out on pooled tissues and the body fluids (Ashcroft, Eccleston and Crawford, 1965).

Drug treatments. (a) Tryptophan was administered intraperitoneally as a suspension, 140 mg/ml 
in 0.9% sodium chloride, in doses of 400, 800 and 1,600 mg/kg body weight and for each dose level 
a group of animals was killed 1, 2 and 4 hr after the injection. (b) a-Methyldopa was administered 
as a suspension, 140 mg/ml in 0.9% saline, in a dose of 800 mg/kg, followed 1 hr later by tryptophan 
800 mg/kg, both substances being given intraperitoneally. The animals were killed at 0.5, 1, 2, 4, 6, 
and 8 hr after the tryptophan was given. As a control, a further group of animals, to which no tryptophan 
was given, was killed 1 hr after administration of a-methyldopa. (c) Iproniazid phosphate 25 mg/ml 
in 0.9%, saline was given by intramuscular injection in a dose of 25 mg/kg, daily for 5 days, the time 
of the last dose being 1 hr before injecting 800 mg/kg tryptophan intraperitoneally. The animals 
were killed at the same time after tryptophan administration as in (b). Animals treated for 5 days 
with iproniazid, but not tryptophan, were used as controls.

Analytical procedures. The concentrations of 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine 
(5-HT) and 5-hydroxyindol-3-vlacetie acid (5-HIAA) in brain and 5-hydroxytryptophan in plasma 
were estimated by the methods described in the preceding paper. In the course of the experiments, 
the results of the application of the analytical procedure to extracts of brain from animals treated with 
a-methyldopa before tryptophan administration indicated the presence of 5-HTP. Supplementary 
evidence of the identity of 5-HTP was obtained as follows. The appropriate section of the paper 
chromatogram of brain extract was eluted in 2 ml 20%, (v/v) acetic acid. The eluate was evaporated 
to dryness under reduced pressure (external temperature 55°). The residue, dissolved in 0.2 ml 
80%, methanol (v/v) containing 50 mg per 100 ml ascorbic acid, was applied to a 5 cm wide Whatman 
No. 1 paper strip and chromatographed in parallel with 5-HTP in the solvent system composed of 
aqueous NaCl 0.1 M: acetic acid (100: 1 by vol.) in an atmosphere of nitrogen. Eluates from sequential 
1 cm strips of the developed chromatogram, dried in a stream of nitrogen, were made in 0.1 N-H/SO 
(4.0 ml). After addition of 2.0 ml conc. HCl containing 50 mg per 100 ml ascorbic acid, each eluate 
was examined spectrophotofluorimetrically. In this way, the sample chromatogram was shown to

* Dr. Eccleston is working on a Medical Research Council Personal Research Grant to Professor 
W. L. M. Perry. Dr. Ashcroft on a Mental Health Research Fund Senior Fellowship.

Abbreviations used: 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindol-3-vlacetie acid.
contain material with the fluorescence characteristics of a 5-hydroxyindole (max. actuation 300 m\(\mu\), max. fluorescence 550 m\(\mu\) uncorrected) located solely at the same position on the chromatogram as authentic 5-HTP. The small amount of material available prevented further characterisation tests. Tryptophan in blood and in brain homogenates was estimated by the method of Hiss and Udenfriend (1959), as applied by Ashcroft and Udenfriend (1962).

RESULTS

(a) Effect of varying the dose of tryptophan. The 5-HT concentration in brain rose from a control level of 0-32 \(\mu\)g/g to a maximum of 0-7 \(\mu\)g/g (Table 1). Alteration of the amount of tryptophan administered within the range examined did not influence the maximum level but only its duration of the maintenance.

**Table 1. Concentrations of 5-HT, 5-HIAA and 5-HTP in rat brain tissue following intraperitoneal injection of varying doses of L-tryptophan**

<table>
<thead>
<tr>
<th>Dose of tryptophan mg/kg</th>
<th>Time after tryptophan hr</th>
<th>5-HT (\mu)g/g</th>
<th>5-HIAA (\mu)g/g</th>
<th>5-HTP (\mu)g/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control</td>
<td>0.32 (\pm) 0.06 (4)*</td>
<td>0.24 (\pm) 0.015 (4)*</td>
<td>0.05 (4)*</td>
</tr>
<tr>
<td>400</td>
<td>1</td>
<td>0.62</td>
<td>0.61</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.69</td>
<td>1.13</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.41</td>
<td>0.54</td>
<td>0.05</td>
</tr>
<tr>
<td>800</td>
<td>1</td>
<td>0.62 (\pm) 0.04 (3)*</td>
<td>0.57 (\pm) 0.06 (3)*</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.59 (\pm) 0.14 (4)*</td>
<td>0.84 (\pm) 0.13 (4)*</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.59 (\pm) 0.13 (3)*</td>
<td>1.09 (\pm) 0.15 (3)*</td>
<td>0.05</td>
</tr>
<tr>
<td>1,600</td>
<td>1</td>
<td>0.67</td>
<td>0.61</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.61</td>
<td>1.00</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.61</td>
<td>1.13</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Except where stated, results are from a single experiment. Each result represents an estimation on the pooled brains of three rats.

* Mean conc. \(\pm\) standard deviation (no. of experiments) quoted from Ashcroft et al. (1965).

The 5-HIAA concentration in brain also increased from a control level of 0-24 \(\mu\)g/g to a maximum of 1-1 \(\mu\)g/g, the rate of rise apparently being similar to that of 5-HT over the first hour. Similarly the 5-HIAA appeared to rise to a maximum concentration not correlated to the tryptophan dose, but this was reached later than with 5-HT and the absolute rise was greater than that for the amine. Again, only the duration of the maintenance of the maximum concentration was dependent on the dose of tryptophan administered.

No 5-HTP was detected in the brain at any time after the tryptophan administration.

The effect of varying the dose of tryptophan on the concentrations of tryptophan in rat blood and brain is shown in Table 2.

(b) Effect of pre-treatment with \(\alpha\)-methyldopa followed by tryptophan loading. (1). Figure 1a shows the increase in the concentration of 5-HT and 5-HIAA in brain, occurring after treatment with tryptophan alone (ASHCROFT et al., 1965) but this increase was not observed in animals treated with \(\alpha\)-methyldopa before the tryptophan administration (Fig. 1b). Another 5-hydroxyindole metabolite, considered to be 5-HTP on the basis of its behaviour on paper chromatography, appeared in measurable quantities in the brain extracts.
5-Hydroxyindole metabolism in rat brain - II

### Table 2. Tryptophan Concentrations in Rat Blood and Brain at Various Time Intervals after Intraperitoneal Injection of Various Doses of L-Tryptophan

<table>
<thead>
<tr>
<th>Dose of Tryptophan (mg/kg)</th>
<th>Time after Tryptophan (hr)</th>
<th>Blood Tryptophan (μg/ml)</th>
<th>Brain Tryptophan (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None* Control</td>
<td>1</td>
<td>17.4 ± 1.4 (3)</td>
<td>73 ± 0.62 (3)</td>
</tr>
<tr>
<td>400</td>
<td>1</td>
<td>305</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>150</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>33</td>
<td>16.4</td>
</tr>
<tr>
<td>800*</td>
<td>1</td>
<td>785 ± 82.5 (3)</td>
<td>216 ± 11.8 (3)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>587 ± 75.8 (3)</td>
<td>307 ± 38.8 (3)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>288</td>
<td>215</td>
</tr>
<tr>
<td>1600</td>
<td>1</td>
<td>1800</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1350</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>820</td>
<td>545</td>
</tr>
</tbody>
</table>

Except where stated, each result represents an estimation on the pooled blood or brains of three rats.

* Mean ± standard deviation (no. of experiments) quoted from Ascroft et al. (1965).

(2) The blood and brain concentrations of tryptophan (Table 4) must be compared with those following administration of tryptophan alone, and this comparison can be made by expressing the results as a blood/brain ratio for tryptophan (Table 5). This ratio is increased in the α-methyl-dopa group, compared with the untreated groups, during the first 2 hr after tryptophan administration, although the difference reaches statistical significance (P < 0.02) only in the 1 hr groups.

(3) Plasma levels of 5-hydroxytryptophan were found to be lower in animals pre-treated with α-methyl-dopa (Table 6).

(c) Effect of pre-treatment with iproniazid on the concentrations of 5-hydroxyindole metabolites in brain following tryptophan administration. There was an increase in the the brain 5-HT concentrations to a level higher than that reached following administration of tryptophan alone (Table 7; Fig. 1c). The concentration of 5-HT in the brains of animals receiving only the iproniazid also showed an increase over that of untreated animals.

In contrast, pre-treatment with iproniazid reduced the rise in concentration of 5-HIAA in brain occurring after tryptophan loading (compare Fig. 1a and 1c).

At no time in these experiments was 5-HTP detected in the brain extracts.

In a single experiment, it was found that pre-treatment with iproniazid did not appear to affect the tryptophan concentrations in brain or in blood at the various times subsequent to tryptophan administration.

**DISCUSSION**

Varying the dose of tryptophan from 400 to 1,600 mg/kg did not alter the maximum concentrations which 5-HT and 5-HIAA reached and maintained in brain. At the higher doses, however, these concentration maxima were maintained for longer periods.
It was suggested in a previous paper (Ashcroft et al., 1965) that the limiting concentration of 5-HT following tryptophan loading might result either from the presence of a 'pool' or store of 5-HT of limited capacity, or from the presence of limiting supply of 5-HTP. The present results showing the same maxima for 5-HT and for 5-HIAA when different tryptophan loads were administered give evidence against a limited store for 5-HT as the sole factor for, if this were the case, the concentration of 5-HIAA would be expected to rise to higher levels when a larger dose of tryptophan was administered. The results indicate that the hydroxylation of tryptophan is the rate-limiting step in the biosynthesis of 5-HT and that at all doses of
tryptophan administered the maximum rate of formation is reached, either as a result of saturation of the hydroxylase by excess of substrate or as a result of a 'feed back' mechanism from accumulation of metabolites.

Effect of 3-methyldopa

Several points of action on the 5-hydroxyindole metabolic pathway in brain have been suggested for 3-methyldopa. The situation has been reviewed by Sharman and Smith (1962), who suggested the following possibilities:—1. Inhibition of the decarboxylation of 5-HTP; 2. Inhibition of the uptake of amino acid precursors into brain; 3. Displacement or release of 5-HT by 3-methyldopa, or its metabolites; 4. Inhibition of amine oxidase by 3-methyldopamine, a metabolite of 3-methyldopa. A fifth possible mechanism, inhibition of tryptophan hydroxylase, has been suggested by Pletscher, Burkard and Gey (1964).

Inhibition by 3-methyldopa of the decarboxylation of dopa by dopa decarboxylase was demonstrated in vitro by Sourkes (1954). It has subsequently been shown to inhibit the synthesis of 5-HT by inhibition of 5-HTP decarboxylase (Westermann, Balzer and Knill, 1958). Sharman and Smith (1962) measured total, acidic and basic 5-hydroxyindoles in rat brain following administration of 3-methyldopa and demonstrated the appearance of neutral and/or amphoteric 5-hydroxyindolic material, probably 5-HTP, whose concentration was arrived at by subtracting the sum of basic and acidic 5-hydroxyindoles from the total 5-hydroxyindole concentration. The present technique, however, enables direct estimation of 5-HTP at concentrations as low as 0.025 µg/ml in plasma, or 0.05 µg/g in brain.

In animals treated with tryptophan alone 5-HTP appears in the plasma but does not reach detectable concentrations in brain tissue. However, following tryptophan loading in the animals pre-treated with 3-methyldopa, 5-HTP appeared in brain in concentrations increasing with time (Table 3, Fig. 1b), while 5-HT and 5-HIAA failed to increase in concentration. These results are consistent with the view that 3-methyldopa inhibits the decarboxylation of 5-HTP in vivo, resulting in the accumulation of...
Table 3. 5-Hydroxyindole Concentrations in Brain Tissue of 2-Methyldopa Pre-Treated Rats at Various Times after L-Tryptophan Loading

<table>
<thead>
<tr>
<th>Time after L-tryptophan (hr)</th>
<th>5-hydroxytryptamine</th>
<th>5-hydroxyindol-3-ylacetic acid</th>
<th>5-hydroxytryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 1</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>0.25</td>
<td>0.32</td>
<td>0.24</td>
</tr>
<tr>
<td>(2-Methyldopa alone)</td>
<td>0.22</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td>1</td>
<td>0.22</td>
<td>0.24</td>
<td>0.27</td>
</tr>
<tr>
<td>2</td>
<td>0.18</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>0.12</td>
<td>0.17</td>
<td>0.19</td>
</tr>
<tr>
<td>8</td>
<td>0.21</td>
<td>0.21</td>
<td>0.19</td>
</tr>
</tbody>
</table>

The rats were treated with 800 mg/kg 2-methyldopa 1 hr before administration of 800 mg/kg tryptophan.

* Mean ± standard deviation (no. of experiments) quoted from Ashcroft et al. (1965).
### Table 4: Tryptophan Concentration in Blood and Brain of Rats Pre-Treated with 5-Methyl-Dopa at Various Times After L-Tryptophan Loading

<table>
<thead>
<tr>
<th>Time after tryptophan (hr)</th>
<th>Blood tryptophan (µg/ml)</th>
<th>Mean blood tryptophan (µg/ml)</th>
<th>Brain tryptophan (µg/g)</th>
<th>Mean brain tryptophan (µg/g)</th>
<th>Blood brain tryptophan ratio</th>
<th>Mean blood-brain tryptophan ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.6 18.0 22.0 19.0</td>
<td>18.7</td>
<td>8.0 5.5 7.2 7.0</td>
<td>6.9</td>
<td>1.95 3.27 3.06 2.71</td>
<td>2.75 0.58</td>
</tr>
<tr>
<td>(2-Methyl-Dopa alone)</td>
<td>95.0 7.250 970</td>
<td>1057</td>
<td>118 164 117</td>
<td>133</td>
<td>8.05 7.62 8.29</td>
<td>7.99 0.31</td>
</tr>
<tr>
<td>0.5</td>
<td>600 760 870</td>
<td>743</td>
<td>147 180 200</td>
<td>176</td>
<td>4.08 4.22 4.35</td>
<td>4.22 0.14</td>
</tr>
<tr>
<td>1</td>
<td>610 615 670</td>
<td>632</td>
<td>152 276 246</td>
<td>225</td>
<td>4.01 2.23 2.54</td>
<td>2.99 0.92</td>
</tr>
<tr>
<td>2</td>
<td>290 250</td>
<td>270</td>
<td>168 226</td>
<td>197</td>
<td>1.73 1.10</td>
<td>1.42</td>
</tr>
<tr>
<td>8</td>
<td>250</td>
<td>25.0</td>
<td>13.6</td>
<td>13.6</td>
<td>1.84</td>
<td>1.84</td>
</tr>
</tbody>
</table>

The rats received 800 mg/kg 2-methyl-Dopa 1 hr before administration of tryptophan, 800 mg/kg.

* Mean ± standard deviation.
the substrate (5-HTP) before the block and in the fall in concentration of the metabolites (5-HT and 5-HIAA) beyond it.

Despite the increase in brain concentrations of 5-HTP, the plasma levels rose no higher in the α-methyldopa pre-treated animals than in those given tryptophan alone. The brain concentrations (μg g) in the α-methyldopa groups from 1 hr onwards reached higher levels than the plasma concentrations (μg ml). This unexpected result has two possible explanations. Firstly, amino acid uptake into brain appears to be an active process (LAJTHA, 1964) and it may be possible, if plasma levels are maintained and if the amino acid is not further metabolised (in this case decarboxylation of 5-HTP is at least partially blocked), for uptake to proceed against a concentration gradient and for 5-HTP to accumulate in the brain cells. This explanation assumes that 5-HTP is formed by hydroxylation of the tryptophan at extra-cerebral sites, as suggested by COOPER and MILLER (1961) and is transported to the brain in the plasma. A second possibility is that 5-HTP is formed in the brain by hydroxylation of tryptophan, and evidence for this has recently been produced in vitro by GAI, POZIK and MARSHALL.
(1963) and in vitro by Graham-Smith (1964). In this case, in the x-methylidopa pre-treated animals, hydroxylation would proceed in the brain with accumulation of 5-HTP locally, due to inhibition of the decarboxylation mechanism. With the facts available, it is impossible to decide between the two alternatives.

The active process of amino acid uptake into brain tissues shows such characteristics as stereospecificity and competition for uptake between amino acids within the same subgroup (Chirigos, Greengard and Udenfriend, 1960). Guroff and Udenfriend (1962) demonstrated in vitro the inhibition of uptake of tyrosine into rat brain produced by pre-treatment with tryptophan, using the blood/brain ratio for tyrosine as an index of uptake. In the present experiment, during the first 2 hr after administration of tryptophan, the blood/brain ratios for tryptophan were higher in animals pre-treated with x-methylidopa than in those treated with tryptophan alone (Table 5). However, the difference reached statistical significance ($P < 0.02$) only in the 1 hr groups. These results suggest that x-methylidopa inhibits the uptake of tryptophan into brain. No direct evidence for an effect of x-methylidopa on 5-HTP uptake into brain can be advanced, although the active uptake of this amino acid by rat brain slices has been established (Shanberg and Giarman, 1960) and the inhibition of its uptake by x-methylidopa in rat brain slices has been demonstrated by Smith (1963).

There was no evidence that x-methylidopa, or its metabolites (Carlsson and Lindqvist, 1962), was exerting a reserpine-like or an amine oxidase inhibitory action, mechanisms 3 and 4 suggested by Sharman and Smith (1962). The failure of 5-HIAA to show a rise in concentration speaks against a reserpine-like action (Ashcroft and Sharman, 1962) and the lack of an increase in the 5-HT levels is contrary to expectation if amine oxidase inhibition was occurring. Although such mechanisms are not completely excluded, in view of the reduced synthesis of the 5-HT brought about by x-methylidopa, it is unlikely that they contribute to any marked extent. It is of interest that a reserpine-like effect has been suggested to account for the sustained decrease of cerebral noradrenaline after x-methylidopa administration (Hess, Connamacher, Osaki and Udenfriend, 1961).
An inhibitory action on tryptophan 5-hydroxylase has been postulated recently by Pletscher et al. (1964) to account, in part, for the effect of \( \alpha \)-methyldopa on cerebral 5-hydroxyindole metabolism. Two observations in our experiments are consistent with, but not conclusive of, such an action. Firstly, the accumulation of 5-HTP in the brain after pre-treatment with \( \alpha \)-methyldopa and tryptophan loading did not equal or surpass the combined increase in 5-HT or 5-HIAA observed following tryptophan alone. This is not the result that might be expected if decarboxylase inhibition was the sole mode of action of \( \alpha \)-methyldopa. Secondly, the plasma concentrations of 5-HTP have been found in preliminary experiments to be lower in tryptophan loaded animals pre-treated with \( \alpha \)-methyldopa than in similar animals given tryptophan alone (Table 6).

**Effect of iproniazid**

Iproniazid has long been known to inhibit the enzyme monoamine oxidase (Zeller and Barsky, 1952) and to produce an increase in the concentration of endogenous 5-HT in rat brain (Udenfrnd, Weissbach and Bogdanski, 1957). In our experiments, animals loaded with tryptophan when pre-treated with this inhibitor showed a rise in concentration of 5-HT in brain to 1-27 \( \mu \)g/g, considerably higher than that seen in animals treated with tryptophan alone (0-6 \( \mu \)g/g). The 5-HIAA did not rise to 1-1 \( \mu \)g/g as occurred in the animals treated with tryptophan alone, but reached a much lower plateau concentration, which was then sustained for 3 hr. It would appear that even with this high dose of iproniazid, cerebral monoamine oxidase inhibition is not complete. No effects of iproniazid on the uptake of tryptophan into brain, or on the decarboxylation of 5-HTP, were demonstrated.

The actions of the drugs used in this work on the levels of brain amines have already been investigated extensively (see Pletscher, 1963). The studies reported here demonstrate how the tryptophan load technique can be used to obtain a dynamic profile of the 5-hydroxyindole metabolic pathway in brain. It makes possible an examination of each point of action of a drug, studying simultaneously uptake of amino-acid precursors, synthesis and breakdown of the amine.

**SUMMARY**

Tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindole 3-ylacetic acid were determined in the rat brain at different times after administering L-tryptophan.

\( \alpha \)-Methyldopa treatment before tryptophan loading prevented the increases in 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid which occur following tryptophan administration alone, and also resulted in the appearance of the amine precursor, 5-hydroxytryptophan; it may also interfere with the uptake of tryptophan from plasma into the brain. Iproniazid treatment before tryptophan loading increase the concentration of 5-hydroxytryptamine and diminished the rise of 5-hydroxyindole 3-ylacetic acid, with no apparent effect on the uptake of tryptophan or the decarboxylation of 5-hydroxytryptophan in the brain.

**Acknowledgements** The authors are grateful to Professor W. L. M. Perry for his advice, interest and criticism and to Miss A. Urot Hart for preparation of the figures. They are also indebted to Messrs. Merck, Sharp & Dohme for liberal supplies of \( \alpha \)-methyldopa.
THE EFFECTS OF PRECURSOR LOADING IN THE CEREBRAL METABOLISM OF 5-HYDROXYINDOLES

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Pharmacology Department, University of Edinburgh

(Received 13 October 1967)

Abstract—Using either tryptophan or 5-hydroxytryptophan as the precursor, and examining the metabolites in whole rat brain and in brain regions of dog, the pattern of metabolites resembled that found under physiological conditions only after tryptophan administration. From these and other observations on the cerebral 5-hydroxyindoles the main conclusions are firstly, that there are regional differences within brain in storage, turnover or metabolic fate of 5-HT. Secondly, that the normal pathway appears to be well localized biochemically with linking of its succeeding steps, and thirdly, that turnover through the system is normally controlled by intracerebral tryptophan 5-hydroxylase in both rats and dogs although there are differences between the species in the cerebral metabolism of 5-HT.

Since the discovery of the presence of the biologically active amine serotonin, 5-hydroxytryptamine (5-HT), in discrete regions of brain (AMIN, CRAWFORD and GADDUM, 1954), many attempts have been made to elucidate the normal metabolic pathways of this amine and their modification by drug action. There is no doubt that the source of brain 5-HT is tryptophan (UDENFRIEND, TITUS, WEISSBACH and PETERSON, 1956). UDENFRIEND, CREVELING, POSNER, REDFIELD, DALY and WITKOP (1959) showed that tryptamine is not hydroxylated to 5-HT in vivo, while on the other hand, BOGDANSKI, WEISSBACH and UDENFRIEND (1957) showed that the administration of 5-hydroxytryptophan (5-HTP) led to an increase in the level of 5-HT in brain.

These observations, which have since frequently been confirmed, establish that the synthesis of intracerebral 5-HT proceeds from tryptophan via the intermediate metabolite 5-HTP even though this metabolite is not normally detectable in the brain or plasma. The site of the hydroxylation of tryptophan remained uncertain.

In 1957 DALGLIESH and DUTTON suggested that tryptophan was hydroxylated extracerebrally and that the cerebral synthesis of 5-HT depended upon the rapid entry of 5-HTP into brain and its subsequent but immediate decarboxylation to 5-HT. COOPER and MELCER (1961) showed that rat small intestine possessed an enzyme capable of 5-hydroxylating tryptophan and supported the above hypothesis. An enzyme capable of 5-hydroxylation of tryptophan was also found to be present in rat liver by RENSON, WEISSBACH and UDENFRIEND (1962) who demonstrated, however, that it had a poor affinity for tryptophan as a substrate and were able to identify it as phenylalanine hydroxylase. These authors therefore thought that it was likely to have little functional importance in 5-HT synthesis in vivo.

The demonstration that cerebral tissue can hydroxylate tryptophan both in vivo (GAL, POCZIK and MARSHALL, 1963; GAL, MORGAN and MARSHALL, 1964) and in vitro (GRAHAME-SMITH, 1964) makes it probable, however, that brain 5-HT is derived from the hydroxylation of tryptophan occurring mainly if not wholly in the brain. Supporting evidence was provided by WEBER and HORITA (1965) who showed
that the rise in cerebral 5-HT in rats obtained after intraperitoneal injection of tryptophan was unaffected by evisceration and that perfusion of a cerebral hemisphere with a tryptophan solution gave rise to an elevated 5-HT concentration on the perfused side only. Despite this, Gal, Morgan, Chatterjee and Marshall (1964) concluded, from an analysis of the cerebral 'turnover' rates of 5-HT, after intracerebral or intraperitoneal administration of tryptophan, that the cerebral hydroxylation of tryptophan, was too slow to account for all cerebral 5-HT, and that this must be supplemented from an extra-cerebral source of 5-HTP.

By studying the changes in the concentrations of 5-hydroxyindoles in whole rat brain and various brain regions of dog after tryptophan and 5-HTP administration in vivo, we have deduced that intracerebral tryptophan hydroxylation is the primary factor controlling cerebral 5-HT formation.

METHODS

Rats. Male Wistar rats (160–200 g) were deprived of food, but not water, for 16–18 hr before the experiment. In the tryptophan loading experiments rats were used in groups of three and the brain and plasma of each group were pooled for analysis. The tryptophan was injected intraperitoneally in a solution of 10mg/ml in 0-9% saline.

In the experiments using loads of L-5-HTP, D,L-5-HTP and 5-hydroxyindol-3-ylacetic acid (5-HIAA) the analysis were done on the tissues of single animals.

The rats were killed by decapitation, the brains rapidly dissected out and the cerebellum removed. The blood was collected from the neck wound and a portion was centrifuged immediately, if plasma estimations of the hydroxyindoles were to be performed.

Dogs. The animals used were adult mongrels of both sexes. They were provided with water but no food for 8–12 hr before the experiment. The drugs were given intravenously at a concentration of 10 mg/ml in 0-9% saline, in doses of L-tryptophan 50 mg/kg, and D,L-5-HTP and L-5-HTP 10 mg/kg.

Each dog was anaesthetised with intravenous sodium thiopentone. A 5 ml sample of CSF was withdrawn from the cisterna magna, and the animal 'bled out'. The brain was removed and dissected and the parts required were homogenized, immediately after weighing, in acetic acid in preparation for subsequent biochemical analyses. The method of brain dissection was described by Eccleston, Ashcroft, Moir, Parker-Rhodes, Lutz and O'Mahoney (1968).

Biochemical determinations. Estimations of 5-HTP, 5-HT and 5-HIAA in plasma and brain were carried out by the method of Ashcroft, Eccleston and Crawford (1965). Quantities of 0.8 µg of each of 5-HTP, 5-HT and 5-HIAA were added to one of two portions of whole rat brain homogenate or of homogenate of dog hindbrain or cerebellum before precipitation with acetone. These samples served as internal standards for the whole procedure and the results were corrected appropriately by adjusting to 100 per cent recovery. The concentrations of 5-hydroxyindoles found in rat or dog plasma were also corrected to 100 per cent by comparison with internal standards added to duplicate portions of the appropriate plasma samples. Plasma 5-HT concentrations were not estimated as they varied greatly even in control data due to a variable platelet content of the plasma. Tryptophan in blood and brain were estimated by the method of Hess and Udenfriend (1959) as applied by Guroff and Udenfriend (1962).

5-HIAA in CSF was determined by the method of Udenfriend, Titus and Weissbach (1955)—as modified by Eccleston et al. (1968).

Identification procedures. The routine paper chromatograms employed in the separation of the 5-hydroxyindoles (Ashcroft et al., 1965) would have measured 5-hydroxytryptophol as 5-HIAA and 5-HIAA sulphate as 5-HTP. Their identity was however further confirmed by ascending paper chromatograms in the following systems. Sodium chloride 8% (w/v)-acetic acid (100:1, v/v) and iso-propyl alcohol-ammonia 0-88-water (85:5:15, by vol.)

RESULTS

Rats

The effects of small doses of tryptophan. Eccleston, Ashcroft and Crawford (1965) found the maximum brain concentration of 5-HT, after intraperitoneal.
The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles

Tryptophan loading to be about 0.68 μg/g. Increasing the load led only to prolongation of the duration of this maximum concentration. Even with very low doses (Table 1) a transient rise in the brain 5-HT concentration to near maximum occurs. The maximum 5-HT concentrations occur earlier than the peak 5-HIAA concentrations.

Table 1.—Concentrations of Tryptophan in Rat Blood and of Tryptophan, 5-HT and 5-HIAA in Rat Brain Tissue

<table>
<thead>
<tr>
<th>Tryptophan dose (mg/kg)</th>
<th>Time after injection (hr)</th>
<th>Blood tryptophan μg/ml</th>
<th>Brain tryptophan μg/g</th>
<th>Brain 5-HT μg/g</th>
<th>Brain 5-HIAA μg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(6)</td>
<td>18.2 ± 1.8</td>
<td>5.6 ± 1.7</td>
<td>0.38 ± 0.11</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>39</td>
<td>43</td>
<td>0.53</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>57</td>
<td>37</td>
<td>0.63</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26</td>
<td>19</td>
<td>0.46</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>(mean of 2 exps.)</td>
<td>135</td>
<td>50</td>
<td>0.64</td>
<td>0.39</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
<td>74</td>
<td>29</td>
<td>0.56</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>61</td>
<td>36</td>
<td>0.50</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21</td>
<td>7.4</td>
<td>0.37</td>
<td>0.28</td>
</tr>
</tbody>
</table>

5-HTP could not be detected in the brains (< 0.07 μg/g). 5-HTP and 5-HIAA could not be detected in plasma (< 0.03 μg/ml).

Control values are means ± s.d. (number of experiments).

It seemed probable that, if the rate-limiting step in the metabolic pathway was the hydroxylation of tryptophan, the value of the total 5-hydroxyindoles (5-HT + 5-HIAA) might be the best index of the turnover rate. Consequently the gram molal concentrations of 5-HT and 5-HIAA were summed for each dose and time and the results compared graphically (Fig. 1) in comparison with similar data derived from the results of Eccleston et al. (1965). It is clear that the initial rates of rise of the total 5-hydroxyindoles are almost identical within the range of tryptophan loads 50–1600 mg/kg.

The effects of small doses of 5-HTP. In these experiments doses of L-5-HTP were approximately equal in their effects to twice the dose of D,L-5-HTP. The results for the latter only are quoted here.

After a load of 10 mg/kg D,L-5-HTP given intraperitoneally there was no apparent rise in 5-HT concentration in the brain, but a large rise in the concentration of 5-HIAA, so that the total 5-hydroxyindole metabolites ([5-HT] + [5-HIAA]) showed an initial rate of rise comparable to that obtained after tryptophan loads (Fig. 1). At the same time it became possible to detect 5-HTP in the brain (Table 2). Larger doses of 5-HTP (20 mg/kg) produced even more striking changes (Fig. 1). A dose of 20 mg/kg given intraperitoneally (Table 3) produced only a moderate rise in the brain 5-HT concentration to 140 per cent of the control concentration but the concentration of 5-HIAA in brain rose rapidly to values of more than 650 per cent of the control concentration. Thus it would appear that 5-HTP is capable of passing the blood–brain barrier and of being metabolized to 5-HIAA without greatly increasing the amount of 5-HT detectable in brain.

In contrast with tryptophan loading the initial rate of rise of total 5-hydroxyindole metabolites caused by 5-HTP increases with dose (Fig. 1). (This relationship may
Fig. 1.—Concentrations (n-moles/g) of total 5-hydroxyindoles ([5-HT] + [5-HIAA]) in rat brain after administration of different doses of L-tryptophan (TRP.) and D,L-5-HTP intraperitoneally. The figures shown for the 400-1600 mg/kg doses of tryptophan were calculated from the data of Eccleston et al. 1965.

Dose administered:

- D,L-5-HTP 10 mg/kg (mean values of 3 expts.)
- D,L-5-HTP 20 mg/kg (mean values of 3 expts.)
- L-tryptophan 50 mg/kg (mean values of 3 expts.)
- L-tryptophan 100 mg/kg (mean values of 2 expts.)
- L-tryptophan 400 mg/kg (mean values of 3 expts.)
- L-tryptophan 800 mg/kg (mean values of 3 expts.)
- L-tryptophan 1600 mg/kg

Table 2.—Concentrations of 5-hydroxyindoles in rat plasma and brain following intraperitoneal injection of 10 mg/kg of D,L-5-HTP

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>Plasma 5-HT</th>
<th>Plasma 5-HIAA</th>
<th>Brain 5-HT</th>
<th>Brain 5-HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>8.6 ± 1.6</td>
<td>1.7 ± 0.8</td>
<td>0.23 ± 0.07</td>
<td>0.44 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>4.3 ± 1.1*</td>
<td>1.9 ± 1.6*</td>
<td>0.11 ± 0.11</td>
<td>0.34 ± 0.12</td>
</tr>
<tr>
<td>1</td>
<td>0.68 ± 0.30</td>
<td>0.54 ± 0.48</td>
<td>0.03 ± 0.06</td>
<td>0.38 ± 0.19</td>
</tr>
<tr>
<td>2</td>
<td>0.04 ± 0.03</td>
<td>0.11 ± 0.15</td>
<td>N.D.</td>
<td>0.42 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>0.30 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>
The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles 1097

Table 2b

Concentrations corrected† Brain

<table>
<thead>
<tr>
<th></th>
<th>5-HTP</th>
<th>5-HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-14</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>0-07</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>0-02</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>N.D.</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

Mean concentrations of 5-hydroxyindoles in brain and plasma μg/g or ml ± s.D.

Mean of 3 experiments except* when only two were performed.

N.D. Not detected in any experiment (level of detection = 0.07 μg/g).

† For explanation see section on 5-HIAA administration.

Table 3.—Concentrations of 5-hydroxyindoles in rat plasma and brain following intraperitoneal injection of 20 mg/kg of D,L-5-HTP

Table 3a

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>Plasma</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HTP</td>
<td>5-HIAA</td>
</tr>
<tr>
<td>0-5</td>
<td>17:0 ± 8:7*</td>
<td>3:5 ± 2:4</td>
</tr>
<tr>
<td>1</td>
<td>7:1 ± 4:4</td>
<td>3:0 ± 0:5</td>
</tr>
<tr>
<td>2</td>
<td>4:1 ± 3:2</td>
<td>3:6 ± 3:4</td>
</tr>
<tr>
<td>4</td>
<td>0:22 ± 0:03</td>
<td>0:31 ± 0:21</td>
</tr>
</tbody>
</table>

Table 3b

Concentration corrected Brain

<table>
<thead>
<tr>
<th></th>
<th>5-HTP</th>
<th>5-HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:26</td>
<td>1:12</td>
<td></td>
</tr>
<tr>
<td>0:38</td>
<td>1:55</td>
<td></td>
</tr>
<tr>
<td>0:21</td>
<td>1:09</td>
<td></td>
</tr>
<tr>
<td>0:10</td>
<td>0:82</td>
<td></td>
</tr>
</tbody>
</table>

Mean concentrations of 5-hydroxyindoles in brain and plasma μg/g or/ml ± s.D.

Mean of three experiments except* when only two were performed.

† For explanation see section on 5-HIAA administration.

be linear (Fig 2). This is a reflection in vivo that the new rate limiting step of this system is not yet saturated at these dose levels of 5-HTP.

5-HIAA administration. 5-HTP can be metabolised to 5-HT and 5-HIAA extracerebrally and these metabolites do not normally penetrate the blood–brain barrier (AXELROD and INSCOE, 1963; ROOS, 1962). However, the very high plasma levels produced by loading and extracerebral metabolism may contribute to the concentrations of 5-HTP and 5-HIAA apparently found in brain tissue by the contamination of the brain homogenates with plasma.
Fig. 2.—Concentrations of total metabolites ([5-HT] + [5-HIAA]) (n-moles/g of brain tissue), 30 min after varying doses of D,L-5-HTP intraperitoneally (corrected for plasma contamination). Single estimate ○, Mean X, S.E.M. 1, number of estimates ( ).

Rats were given 20 mg/kg of 5-HIAA by intraperitoneal injection in an attempt to find the degree to which plasma concentrations of 5-HIAA influence the concentrations found in brain.

If we assume that no 5-HIAA enters brain itself, then we can deduce that, since the plasma will occupy a constant fraction of the 'brain' samples, the increase in 'brain 5-HIAA' should be a constant fraction of the plasma 5-HIAA. The results are shown in Table 4. Excluding the 2 hr result (which is of very doubtful accuracy), the ratio is indeed relatively constant at a mean value of 0·01, implying that the plasma occupies about 1 per cent of the volume of the brain samples. This value is in good agreement with the calculations of brain capillary whole blood volume made by Sokoloff (1961), namely 0·4—1·2 per cent, based on autoradiography after 131I-labelled albumin injection, especially since Sokoloff took special precautions to exclude large vessels from his samples.

Using the estimated value of 1 per cent of the samples as being occupied by plasma the results of the brain concentrations (Tables 2a and 3a) have been corrected appropriately (Tables 2b and 3b). The corrections make little difference to the estimates
of cerebral 5-HIAA but reduce the estimates of 5-HTP in brain by 30–50 per cent. Blood corpuscles may well contain 5-HTP (Gershon and Ross, 1966a) also the marked fluorescence formed in the region of the brain capillary endothelium after 5-HTP administration to rats (Bertler, Falck and Rosengren, 1963) may well be partially due to 5-HTP. Thus it is possible that even less of the 5-HTP apparently found in brain is actually present in cerebral tissues.

**Table 4.—The concentrations of 5-HIAA in rat brain tissue and plasma following intraperitoneal injection of 5-HIAA (20 mg/kg)**

<table>
<thead>
<tr>
<th>Time in hr</th>
<th>Brain 5-HIAA μg/g</th>
<th>Plasma 5-HIAA μg/ml</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0·24 ± 0·08</td>
<td>0·06</td>
<td></td>
</tr>
<tr>
<td>0·25</td>
<td>0·78</td>
<td>54·2</td>
<td>0·010</td>
</tr>
<tr>
<td>0·5</td>
<td>0·62</td>
<td>29·2</td>
<td>0·013</td>
</tr>
<tr>
<td>1</td>
<td>0·33</td>
<td>20·4</td>
<td>0·005</td>
</tr>
<tr>
<td>2</td>
<td>0·40</td>
<td>1·04</td>
<td>0·156</td>
</tr>
</tbody>
</table>

* Ratio—this is the increase in the 5-HIAA concentration in brain tissue from control values to the concentration of 5-HIAA in plasma.

**Dogs**

It was suggested by Tozer, Neff and Brodie (1966) that in normal animals a steady state will apply to the system.

\[
\text{Synthesis } \rightarrow \text{5-HT} \xrightarrow{K_1} \text{5-HIAA} \xrightarrow{K_2} \text{Elimination}
\]

and that if there is no alternative route in the system

\[
K_1 \cdot [5-HT] = K_2 \cdot [5-HIAA]
\]

where \(K_1\) and \(K_2\) are equal to ‘efflux constants’ for 5-HT and 5-HIAA and [5-HT] and [5-HIAA] are normal molal concentrations. From this it was suggested that if the ratio \([5-HT]/[5-HIAA] = K_0/K_1\) was found to be constant in different parts of brain, then it could be inferred that the turnover times in these regions were similar and that variation in the concentrations of 5-hydroxyindoles reflected the concentration of serotonergic units. They also reported evidence favouring this in that their calculations of \(K_2\) were similar for whole brain, brain stem and rest of brain in rats.

While the inferences made from their data might well have been correct, we have found evidence, by analysing the data of the concentrations of 5-hydroxyindoles in normal dog brain (Eccleston et al., 1968) that this ratio of molal concentrations varies in different regions of dog brain (Table 5). Such a finding shows that there are regional differences in brain in the metabolism of 5-HT: However, it cannot pinpoint which of the individual rate constants making up the composite ‘efflux constants’ is responsible or even whether \(K_1\) or \(K_2\) is involved in each case. The differences may even be caused by the variable utilisation of an alternative metabolic route.

It was thought that in view of the information obtained from the experiments with rats where the hydroxylation of tryptophan appeared limiting, that the molal concentration of \([5-HT] + [5-HIAA]\) might give the best indication of controlling mechanisms (Table 6).
Control dogs

The total molal concentration of 5-hydroxyindoles ([5-HT] + [5-HIAA]) in different regions of the brains of six normal dogs are presented in Table 6 (calculated from the data of ECCLESTON et al., 1968). It would appear from the statistical analysis that there is an extremely significant agreement as to the existence of an order in which the concentrations of 5-hydroxyindoles in these regions may be ranked. The best estimate of the 'true' rank order may be given by the rank order of the total ranks for each area (KENDAL, 1948) or where there is equal weight as in this case by the rank order of the mean values.

Table 6.—Total 5-hydroxyindoles, concentration in different regions of the brains of six control dogs

<table>
<thead>
<tr>
<th>Region</th>
<th>Dogs</th>
<th>Rank of Mean</th>
<th>Mean of ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Midbrain</td>
<td>8.4</td>
<td>11.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>8.8</td>
<td>10.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Thalamus</td>
<td>7.6</td>
<td>4.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Caudate</td>
<td>3.1</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>3.4</td>
<td>1.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>3.2</td>
<td>4.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Cortex</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Concentrations of total 5-hydroxyindoles ([5-HT] + [5-HIAA]) in n-moles/g. N.D. Neither 5-HT nor 5-HIAA detected (< 0.2 n-moles/g of each).

The relative amounts of 5-HT and 5-HIAA are presented in the companion paper of ECCLESTON et al. (1968).

The concentrations in the different regions of brain in each dog were assigned rank values and the coefficient of concordance (KENDAL, 1955) was calculated and its level of significance determined from table for the value $F, W = 0.899, P < 0.001$.

Tryptophan administration to dogs. Table 7 presents the total molal concentrations of ([5-HT] + [5-HIAA]) in the same regions of dog brain at varying times after the intravenous administration of 50 mg/kg of tryptophan (calculated from the data of ECCLESTON et al., 1968). The statistical analysis of the order of the concentrations of total 5-hydroxyindoles in these regions, throughout the entire tryptophan load effect, shows extremely good correlation with the order found in control dogs, and and it can be seen from Table 8 that this correlation is not just one of order but is of interval type.
These observations fit well with the idea that the metabolism of such a precursor load is being controlled by the normal physiological rate determining processes.

5-HTP Administration to dogs. Following the intravenous administration of D, L-5-HTP 10mg/kg a high concentration of 5-HTP was found in the different brain regions (which would not have a significant contribution from plasma contamination) together with a rise in both 5-HT and 5-HIAA (Table 9). 5-HT became detectable in the cortex and cerebellum. The caudate, the area with highest decarboxylase activity, showed the greatest increase in 5-HT and 5-HIAA concentration.

<table>
<thead>
<tr>
<th>Region</th>
<th>1 hr</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>14.4</td>
<td>25.2</td>
<td>37.1</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>13.4</td>
<td>18.2</td>
<td>16.9</td>
</tr>
<tr>
<td>Thalamus</td>
<td>7.3</td>
<td>8.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Caudate</td>
<td>4.9</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>7.2</td>
<td>7.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>9.7</td>
<td>12.9</td>
<td>5.4</td>
</tr>
<tr>
<td>Cortex</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Concentrations of total 5-hydroxyindoles ([5-HT] + [5-HIAA]) in n-moles/g of brain tissue following intravenous administration of 50 mg/kg of l-tryptophan. N.D. Neither 5-HT nor 5-HIAA detected (<0.2 n-moles/g each).

The relative amounts of 5-HT and 5-HIAA are presented in the companion paper of Eccleston et al. (1968).

The different brain regions were assigned rank values as described for Table 6 and the coefficient of concordance calculated on the total data W = 0.949, P < 0.001).

Coefficients of Concordance were also calculated for the replicates at 1, 2 and 4 hr. 1 hr W = 0.968, 2 hr W = 0.968 and 4 hr W = 0.947, P < 0.001 for all times.

Ranking the total of the ranks assigned to each region at each time or ranking the mean values gives the same orders and these show perfect correlation between times. It can be seen from a comparison of the means of ranks of Tables 6 and 7 and from the rank orders of their mean values that there is a high degree of correlation between the two tables.

One dog had 10 mg/kg L-5-HTP administered intravenously and the brain analysed after 1 hr. Many of the values of metabolites obtained are approximately double those obtained with the same dose of D, L-5-HTP. Variation between animals and in experimental estimates precludes any attempt to establish a definite relationship between the L-isomer and its racemic mixture without many replicates of each.

The results found, however, would be compatible with the formation of the cerebral metabolites of 5-HTP from its L-isomer alone.

The ranks of 5-HT concentrations and the total molal concentration of ([5-HT] + [5-HIAA]) in the brains of dogs 1 and 2 hr after intravenous 5-HTP loading exhibit significant rank orders which correlate very well with the order of decarboxylase activity (Table 9) and are different from the order exhibited by the total molal concentration of ([5-HT] + [5-HIAA]) in normal dogs (Table 6) or during intravenous tryptophan load (Tables 7 and 8). The rank order of ([5-HT] + [5-HIAA]) in the dog examined 4 hr after intravenous 5-HTP presumably represents a change towards the physiological pattern. While correlation of these concentrations of
Table 8.—The concentrations of total 5-hydroxyindoles in different regions of dog brain, before and following intravenous administration of L-tryptophan

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>1 hr</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>78 ± 20%</td>
<td>83 ± 10%</td>
<td>77 ± 45%</td>
<td>95 ± 21%</td>
</tr>
<tr>
<td>Thalamus</td>
<td>44 ± 24</td>
<td>39 ± 10</td>
<td>38 ± 21</td>
<td>34 ± 13</td>
</tr>
<tr>
<td>Caudate</td>
<td>30 ± 9</td>
<td>25 ± 8</td>
<td>24 ± 12</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>39 ± 13</td>
<td>37 ± 12</td>
<td>25 ± 11</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>39 ± 5</td>
<td>48 ± 21</td>
<td>42 ± 21</td>
<td>57 ± 5</td>
</tr>
</tbody>
</table>

The results from Tables 6 and 7 are here expressed as percentages of the concentration of ([5-HT] + [5-HIAA]) in the midbrain region of each dog. Mean values ± s.d.

Table 9.—The concentration of 5-hydroxyindoles in different regions of dog brain after intravenous 5-HTP administration

<table>
<thead>
<tr>
<th>Regions</th>
<th>Control mean values</th>
<th>L-5-HTP</th>
<th>D,L-5-HTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>2 hr</td>
<td>4 hr</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP</td>
<td>N.D.</td>
<td>4-88</td>
<td>1-64</td>
</tr>
<tr>
<td>5-HT</td>
<td>0-84</td>
<td>2-45</td>
<td>1-53</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0-92</td>
<td>2-70</td>
<td>1-75</td>
</tr>
<tr>
<td>Rank of total metabolites</td>
<td>(1)</td>
<td>(3)</td>
<td>(2)</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP</td>
<td>N.D.</td>
<td>2-42</td>
<td>1-41</td>
</tr>
<tr>
<td>5-HT</td>
<td>0-81</td>
<td>2-60</td>
<td>1-45</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0-54</td>
<td>2-68</td>
<td>1-25</td>
</tr>
<tr>
<td>Rank of total metabolites</td>
<td>(2)</td>
<td>(2)</td>
<td>(3)</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP</td>
<td>N.D.</td>
<td>3-38</td>
<td>2-19</td>
</tr>
<tr>
<td>5-HT</td>
<td>0-34</td>
<td>0-56</td>
<td>0-66</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0-40</td>
<td>0-85</td>
<td>0-95</td>
</tr>
<tr>
<td>Rank of total metabolites</td>
<td>(3)</td>
<td>(6)</td>
<td>(4)</td>
</tr>
<tr>
<td>Caudate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP</td>
<td>N.D.</td>
<td>1-89</td>
<td>0-85</td>
</tr>
<tr>
<td>5-HT</td>
<td>0-32</td>
<td>3-69</td>
<td>2-01</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0-18</td>
<td>2-11</td>
<td>1-94</td>
</tr>
<tr>
<td>Rank of total metabolites</td>
<td>(6)</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP</td>
<td>N.D.</td>
<td>5-31</td>
<td>2-54</td>
</tr>
<tr>
<td>5-HT</td>
<td>0-44</td>
<td>1-42</td>
<td>0-77</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0-18</td>
<td>0-92</td>
<td>0-65</td>
</tr>
<tr>
<td>Rank of total metabolites</td>
<td>(5)</td>
<td>(4)</td>
<td>(5)</td>
</tr>
<tr>
<td>Hindbrain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HTP</td>
<td>N.D.</td>
<td>5-86</td>
<td>3-18</td>
</tr>
<tr>
<td>5-HT</td>
<td>0-31</td>
<td>1-05</td>
<td>0-65</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0-37</td>
<td>1-20</td>
<td>0-47</td>
</tr>
<tr>
<td>Rank of total metabolites</td>
<td>(4)</td>
<td>(5)</td>
<td>(6)</td>
</tr>
</tbody>
</table>
Concentrations of 5-HTP, 5-HT and 5-HIAA (μg/g of brain tissue) in different regions of dog brain after the intravenous administration of 10 mg/kg of 5-HTP (L or D,L form).

The corresponding mean concentrations in control dogs from Eccleston et al. (1968).

Total metabolites—rank of the total molal concentration of \((\text{5-HT} + \text{5-HIAA})\) N.D. Not detected < 0.05 μg/g of 5-HTP, 5-HT or 5-HIAA in brain < 0.03 μg/ml of 5-HTP or 5-HIAA in plasma.

* Decarb. = 5-HTP decarboxylase activity in μg of 5-HT formed/g of brain tissue/hr as reported by Bogdanski et al. (1957).

† M.A.O. = Monoamine oxidase activity in μg of 5-HT destroyed/g of brain tissue/hr as reported by Bogdanski et al. (1957).

The concentrations of the total 5-hydroxyindole metabolites \((5\text{-HT} + 5\text{-HIAA})\) 1 and 2 hr \( \text{d,L-5-HTP} \) and 1 hr \( \text{L-5-HTP} \) (Table 7), were ranked and their coefficient of concordance calculated \(W = 0.955\) \(P < 0.001\).

The concentrations of 5-HTP in the different areas of the brains of these dogs were also ranked and their coefficient of concordance calculated \(W = 0.956\) \(P < 0.001\).

Both these parameters thus have significant rank orders. Their best rank order was taken to be that of the rank totals.

The correlation coefficients of these rank orders, with the order of decarboxylase activity found in these areas of brain by Bogdanski et al. (1957), were calculated by the method of Kendall (1955).

\[
\tau((5\text{-HTP}), \text{Decarb.}) = 0.785\ P = 0.0028
\]

\[
\tau((5\text{-HT}) + 5\text{-HIAA}), \text{Decarb.}) = 0.837\ P = 0.0014^\dagger
\]

‡ Siliotto, (1947).

Thus it would seem that these concentrations are well correlated with decarboxylase activity and that their levels in discrete areas of brain tissue would be dependent on the decarboxylase activity of these areas. It is possible that the same findings could have been made if the different areas of brain had different capacities for the rate of uptake of 5-HTP which had a similar rank order to decarboxylase activity. However a Kendall partial rank correlation coefficient was calculated excluding any direct influence of such differences in the rate of 5-HTP uptake into brain.

\[
\tau((5\text{-HT} + 5\text{-HIAA}) \text{Decarb.}, (5\text{-HTP})) = 0.658
\]

Similarly partial rank correlation coefficient excluding the direct influence of decarboxylase activity and total \((5\text{-HT} + 5\text{-HIAA})\) were calculated.

\[
\tau((5\text{-HT} + 5\text{-HIAA}) (5\text{-HTP}), \text{Decarb.}) = 0.294
\]

\[
\tau((5\text{-HTP}) (\text{Decarb.}), (5\text{-HT} + 5\text{-HIAA})) = 0.522
\]

Interpretation of such correlations would suggest that the correlations of 5-HTP concentrations and of \((5\text{-HT} + 5\text{-HIAA})\) concentrations with decarboxylase activity are the controlling correlations and obscure any direct relationship between 5-HTP and \((5\text{-HT} + 5\text{-HIAA})\).
hydroxyindoles in the brains of dogs 1 and 2 hr after loading can be seen to be of an interval type its association with decarboxylase activity is of an order type only. However, partial rank analysis of these parameters would suggest that these are direct, significant correlations. Thus it would seem probable that differences in 5-HP concentration in the various regions represent differences in the rate of utilisation controlled by decarboxylase activity and that there may be no real differences in the rate of uptake of 5-HTP into brain; similarly that the step limiting the rate of production of the subsequent metabolism is now the decarboxylase activity.

**DISCUSSION**

It has been accepted for some time that stores of cerebral 5-HT are derived from dietary tryptophan and that the rate limiting step in its formation must lie between systemic tryptophan and intracerebral 5-HT. This leaves the following possibilities for the rate limiting step in metabolism:

1. Entry of tryptophan into brain.
2. Hydroxylation of tryptophan, (a) extracerebrally (b) intracerebrally.
3. Entry of 5-HTP into brain.
4. Decarboxylation of 5-HTP.
5. Intracerebral storage of 5-HT.

From previous (Eccleston et al., 1965) and present data on tryptophan loading in rats it has been shown (a) that concentrations of brain tryptophan increase with increasing dosage of tryptophan (b) that 5-HTP never becomes detectable in brain even with very large loads (c) that the initial rate of rise of the total molal concentration of total 5-hydroxyindoles ([5-HT + [5-HIAA]]) is the same over a very wide dose range (50-1600 mg/kg) (Fig. 1). This indicates that cerebral 5-hydroxyindole metabolism is limited by hydroxylation under these conditions. Two additional points were noted from previous data (a) that with very high doses of tryptophan 5-HTP could be detected in small amounts in the plasma and (b) that when plateau levels for 5-HT and 5-HIAA were produced after a single large dose of tryptophan the 5-HT/5-HIAA ratio was smaller than the ratio in control brain. These findings could be due to either a progressive filling of the stores of 5-HT towards saturation with increasing overspill of 5-HT directly on to monoamine oxidase, or to 5-HTP derived from extracerebral hydroxylation of tryptophan by-passing the storage mechanism for 5-HT, thus providing a progressively increasing contribution to the brain concentration of 5-HIAA.

Small doses of 5-HTP which could produce an initial rate of rise of total metabolites equivalent to the maximum that could be produced on tryptophan loading gave only an increase in 5-HIAA and none in 5-HT. Thus it seems clear that all 5-HT normally found in rat brain is derived via the intracerebral hydroxylation of tryptophan and that this enzyme normally limits its synthesis.

The effect seen in the whole rat brain sample is the resultant of the metabolism of different brain regions. This could have been due to one or more regions which like the caudate nucleus in the dog normally have low rates of hydroxylation (small rise of total hydroxyindoles on tryptophan loading) but high rates decarboxylation (large rise of metabolites in 5-HTP loading) (Tables 6–9 and Eccleston et al., 1968).

In dogs regional differences in the storage, or the rate or the pathway of metabolism of 5-HT can be seen to exist (Table 5). These results cannot merely be explained
by an alternative pathway for the metabolism of 5-HT, other than to 5-HIAA, unless one assumes that the balance between such a metabolite and 5-HIAA varies in different regions, as otherwise the molal ratio of [5-HT]/[5-HIAA] would still have remained constant under steady state conditions

\[
\frac{[5-\text{HT}]}{[5-\text{HIAA}]} = K_a \frac{K_2}{K_1}.
\]

(Where \(K_a\) would be a constant determined by the proportion of 5-HT being metabolised to the acid.) One cannot analyse or explain these regional differences in metabolism further at present other than point out possible causative mechanism as mentioned above.

It is interesting to speculate at this point that such a pathway as is envisaged above might involve the metabolism of 5-HT to 5-hydroxytryptophol, a pathway demonstrated for rat brain in vitro (Eccleston, Moir, Reading and Ritchie, 1966). This pathway might act as a route of elimination or as a controlling shunt mechanism producing localised intracellular alterations in the redox capacity of coenzymes in the vicinity of stored 5-HT (Moir, 1967). Tryptophan 5-hydroxylase has been shown to be a localised intracellular enzyme which is associated with 5-HT stores and requires a reduced coenzyme (Gal, Armstrong and Ginsberg, 1966; Graham-Smith, 1967) it is possible, therefore, that this initial controlling step in 5-hydroxyindole metabolism may be coupled with the route of metabolism of 5-HT to its acidic or alcohol metabolite through localised alterations in the redox environment.

Analysis of regions of brain at intervals following tryptophan loads in dogs show that although there was an absolute increase of 5-HT and 5-HIAA in all regions the pattern of metabolism remained unchanged (apart from hindbrain\(^1\)). Small doses of 5-HTP in dogs can give rise to significant rises in brain 5-HT but what is very striking is that the pattern of metabolism is not normal (Tables 6–9) and that now the order of total metabolite concentration correlates very well with the order of the decarboxylase activity in dog brain reported by Bogdanski et al., 1957. From the analysis of the data (Table 9) it would appear that the decarboxylase activity is controlling both the concentrations of 5-HP and total metabolites found in brain. Thus although cerebral 5-HT can be formed from extracerebrally produced 5-HTP it can be seen (especially when one considers the status of the caudate nucleus) that again it is extremely likely that only tryptophan which is hydroxylated intracerebrally contributes to the stores of 5-HT in brain.

Some interesting species differences are apparent from our loading experiments and show that limiting steps in metabolism depend on the species studied as well as the type of precursor load. In both rats and dogs only the L-isomer of 5-HP appeared to be taken into brain, thus it would seem likely that uptake into brain will be controlled at these dose levels mainly by an active transport process. However there are obvious species differences in the uptake mechanism. Firstly, the uptake of 5-HP into dog brain would appear to be more efficient (Brain/plasma ratio for 5-HP is 5–20 times greater in dogs than in rats, and also the ratio of the increase of all 5-hydroxyindoles in brain to the concentration of plasma 5-HP is also greater).
Secondly, the relationship of the rate of increase of metabolism to the dose of 5-HTP administered to rats shows a very flat exponential rise. It is probable because of the doubtful significance of 5-HTP found in rat brain that this flat exponential rise found for the rate limiting step is due to the rate of entry of 5-HTP into brain.

It is possible that some of the differences in uptake between the species might be due to 5-HTP and tryptophan using the same carrier system into brain. Thus it is interesting to note that our normal blood tryptophan levels for control rats were much higher than the values for control dogs.

The differences seen between the species in the incorporation of 5-HTP into 5-HT stores in brain is probably due to species differences in the intracellular localization of 5-HTP decarboxylase. Species differences in the intracellular distribution of this enzyme were reported by McCaman, Rodriguez de Lores Arnaiz and De Robertis (1965) and although dog brain was not studied, rat brain was shown to have a particularly high activity of this enzyme associated with the supernatant fraction of centrifuged homogenates.

From the evidence and arguments which have been presented it will be obvious that to incorporate our findings into a cohesive whole it is necessary to expand the conventional concept of the 5-hydroxyindole pathway to allow for functional localization. One can therefore envisage that cerebral 5-hydroxyindole is best represented...
by two pathways with multiple intercommunicative compartments (Fig. 3). It is probably relevant at this point to consider how on the basis of such concepts we envisage the metabolism of an 'unphysiological' load like 5-HTP.

We consider that the most likely possibility is that when 5-HTP is presented systemically it may be taken up into brain and be decarboxylated generally in the cell cytoplasm. The 5-HT formed will be subsequently oxidatively deaminated fairly readily. The normal physiological metabolism may involve the hydroxylation of tryptophan at localized sites intraneuronally which allow 5-HT when formed in the vicinity of the 5-HT stores to have a better chance of being incorporated therein. It is one extension of the above idea that 5-HTP when presented or derived systemically may well be transaminated (Haavaldsen, 1962; Tangen, Fonnum and Haavaldsen, 1965) to 5-hydroxyindol-3-ylpyruvic acid (which could probably not be differentiated from 5-HIAA by our present techniques) and would thus not contribute to 5-HT stores.

Our finding of differences between control animals and 5-HTP loaded ones in the inter- or intracellular compartmental distribution of 5-HT and its subsequent metabolism is well supported by the findings of Green and Sawyer (1964) who noted poor recoveries of 5-HT in rat brain of 5-HTP loaded animals as opposed to recoveries of 5-HT from normal brain. Also the recent studies of Bulat and Supek (1967) with intravenous tritiated 5-HT confirm that what little 5-HT does reach cerebral tissue after such a non-physiological load does not become stored but is deaminated rapidly to 5-HIAA.

Aghajanian and Bloom (1967) have shown that following intraventricular injection of tritiated 5-HT in rats the retention of 'firmly bound label' correlates well with the areas known to have high endogenous levels of 5-HT and is predominantly associated with 'nerve endings'. These experiments confirm rather than contradict our findings as although their experiments use an unphysiological loading technique the analysis by autoradiography measures only that part of the total tritiated 5-HT localized with 'bound' 5-HT as any 'non-bound' [3H]5-HT or [3H]5-HIAA would be removed by the procedures prior to measurement. However, unless an analytical technique takes some account of the changes in the distribution of 5-HT between its intracellular compartments (ultra-centrifugation, histochemistry or autoradiography), valid interpretations based on estimates of total pool 5-HT when using loads other than tryptophan must be difficult. A similar criticism can be applied to the work of Gershon and Ross (1966 a, b) who administered [14C]5-HTP by peripheral injection.

In the clinical sphere 5-HTP appears to have been accepted as a physiological precursor of 5-HT and has been given in the investigation (Coppen, Shaw and Malleson, 1965) and treatment (Kline and Sacks, 1963; Kline, Sacks and Simpson, 1964) of depression; in the treatment of schizophrenia (Brengelmann, Pane and Sandler, 1959; Klee, Bertino, Goodman and Aronson, 1960) and more recently in the treatment of Down's syndrome (Bazelon, Pain, Cowie, Hunt, Houck and Mohammed, 1967). In view of the fact that low doses of 5-HTP probably do not influence intraneuronal concentrations of 5-HT in normal sites many of the conclusions drawn from these studies should be viewed with circumspection. On the other hand, in that we have shown in this paper that the normal pattern of 5-hydroxyindole metabolism is maintained after a loading
dose of tryptophan, it is worthwhile considering the administration of tryptophan as one of the best means of investigating 5-HT metabolism in man.

Acknowledgements—The authors wish to acknowledge the help and advice of Prof. W. L. M. Perry and Dr. G. W. Ashcroft, and also Mrs. Isobel Ritchie for her technical assistance.

REFERENCES

A COMPARISON OF 5-HYDROXYINDOLES IN VARIOUS REGIONS OF DOG BRAIN AND CEREBROSPINAL FLUID

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Abstract—Normal values for the concentration of 5-HT, 5-HIAA and tryptophan are established in various regions of the dog brain. After administration of tryptophan by intravenous injection the rise and fall of 5-HT and 5-HIAA were estimated at 1, 2 and 4 hr. Best fit quadratic regression curves obtained by computer programme were fitted to the data. Similar tryptophan doses were given to dogs and the 5-HIAA concentration estimated in the cisternal CSF. Quadratic regression curves fitted to these values show that the concentration of 5-HIAA in CSF reflects the changes of 5-HIAA in the brain and in particular in the brain stem. α-Methyl dopa pretreatment blocked the rise of 5-hydroxyindoles in brain and CSF and appeared to inhibit tryptophan hydroxylase as well as decarboxylase.

INTRODUCTION

Tryptophan has been shown to be a precursor of 5-HT in the brain (Udenfriend, Titus, Weissbach and Petersen, 1956). When the amino acid was administered to rats there was a rise in the brain in the concentration of the amine 5-HT and 5-HIAA, the acid metabolite (Ashcroft, Eccleston and Crawford, 1965). Previous workers (Ashcroft and Sharman, 1962) had found a correlation between the changes in the concentration of 5-HIAA in the caudate nucleus and its concentration in CSF in dogs, following the administration of reserpine, and in previous studies in this laboratory an attempt was made to determine the same sort of relationship between 5-HIAA in several brain regions and CSF following the oral administration of tryptophan in dogs (Eccleston, 1966). The concentration of the acid metabolite was found to rise in CSF as well as in the brain. The rise was, however, inconsistent between experiments owing to the variable rate of absorption of the amino acid.

In the present experiments the tryptophan was administered intravenously to obviate the variable rate of absorption from the gut. The present work aimed firstly to investigate the effect of intravenous tryptophan on brain tryptophan and 5-hydroxyindole derivatives, and on 5-HIAA in the CSF, secondly to determine the effect of α-methyl dopa, an inhibitor of aromatic L-amino acid decarboxylase, on these changes and thirdly to see whether the pattern of changes produced by α-methyl dopa shed any light on its mode of action.

Abbreviations used: α-methyl dopa, α-methyl 3,4-dihydroxyphenylalanine; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindol-3-acetic acid; 5-HTP, 5-hydroxytryptophan.
METHODS

Animals. Adult mongrel dogs of both sexes were used, food being withdrawn 8 hr prior to the experiment, water being available ad libitum.

Drug administration. Tryptophan was administered intravenously over 2-3 min in the conscious dog in 0·9% saline solution (10 mg/ml) in a dose of 50 mg/kg.

α-Methyl dopa was given intravenously (over 2-3 min) in 0·9% (w/v) saline solution (20 mg/ml) in a dose of 50 or 100 mg/kg, 30 min prior to the injection of tryptophan. The animals were killed at 1, 2 and 4 hr after administration of the tryptophan. Dogs given α-methyl dopa alone (100 mg/kg) were killed at 0·5, 1·5, 2·5 and 4·5 hr after the injection. Six untreated dogs served as controls.

Brain dissection. The animals were anaesthetised with intravenous sodium thiopentone and, after intravenous injection of 1 ml heparin solution (5000 units/ml), were bled out by the introduction of a polythene cannula into the femoral artery. The skull was opened by means of a vibrating electric saw, the dura incised, and the whole brain rapidly removed. The cerebellum was removed in one piece and the brain was bisected longitudinally in the median sagittal plane. Each half was then dissected accordingly to LM, Liu and Moffit (1960).

The dissected brain regions were weighed and homogenised in 1 vol. 40% (v/v) and 2 vol. 20% (v/v) acetic acid in an all-glass homogeniser.

Cerebrospinal fluid

Acute experiments. In the dogs which were killed, after the administration of the anaesthetic and prior to bleeding out, the dog's head was flexed and 5 ml of CSF was withdrawn from the cisterna magna with a polythene syringe.

Chronic experiments. Four dogs were used over a period of several months. During each experiment three dogs were given tryptophan (50 mg/kg) intravenously as described above. At various times after the administration of the amino acid, and under anaesthesia, 5 ml of CSF were withdrawn from the cisterna magna. The 1-hr sample was withdrawn from the first dog, and 2-hr sample from the second dog and the 4-hr sample from the third dog. The fourth dog served as a control, CSF being withdrawn without prior administration of tryptophan. The experiment was repeated at 2 weekly intervals until each dog had served as either control or the donor of the 1-, 2- or 4-hr CSF sample (four experiments). This latin square design of the experiment allows for detection of between animal and between experiment variation. The latin square was repeated with tryptophan alone and again to include the pretreatment of the animals with α-methyl dopa (50 mg/kg) 30 min prior to the administration of tryptophan. The control dog in this experiment was treated with α-methyl dopa alone and the CSF withdrawn 30 min later.

Analytical procedures

5-Hydroxyindoles in brain and plasma. These were measured as described by Ashcroft et al. (1965). Quantities of 0-8 μg of pure 5-HT, 5-HIAA and 5-HTP were added to homogenates of cortex or cerebellum and taken through the analytical procedures.

The recoveries were as follows:

\[ 5\text{-HTP} \, 52\pm1\% \pm \, 2\cdot6 \, (18) \]
\[ 5\text{-HT} \, 68\pm4\% \pm \, 2\cdot4 \, (26) \]
\[ 5\text{-HIAA} \, 70\pm6\% \pm \, 3\cdot5 \, (26) \]

Mean ± s.e.m. (number of observations)

5-HIAA in CSF. This was estimated by a modification of the method of Ashcroft and Sharman (1962).

Tryptophan in blood and brain and CSF. These were estimated by the method of Hess and Udenfriend (1959) as modified by Guroff and Udenfriend (1962).

RESULTS

Tryptophan

Tryptophan concentrations in blood, brain and CSF. The concentration of tryptophan in the brain of normal animals varied between regions (Table 1). Analysis of variance showed this difference to be significant \( P < 0·05 \). The regions for each dog were arranged in a rank order for the concentration of tryptophan. The coefficient of concordance for the five dogs for this ranking suggests that it does not arise by chance \( P < 0·05 \). The best estimate of descending rank order of concentration is
<table>
<thead>
<tr>
<th>Region</th>
<th>Tryptophan and derivatives</th>
<th>Control</th>
<th>1 hr</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>Tryptophan</td>
<td>6.84 ± 2.09(5)</td>
<td>32.3 ± 7.3</td>
<td>39.5 ± 4.3</td>
<td>10.0 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.84 ± 0.14(6)</td>
<td>1.83 ± 0.46</td>
<td>1.78 ± 0.47</td>
<td>1.16 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.92 ± 0.19(6)</td>
<td>1.76 ± 0.47</td>
<td>3.33 ± 1.10</td>
<td>1.79 ± 0.26</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Tryptophan</td>
<td>7.56 ± 2.08(5)</td>
<td>34.7 ± 6.3</td>
<td>3.6 ± 5.5</td>
<td>6.9 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.81 ± 0.21(6)</td>
<td>1.71 ± 0.20(2)</td>
<td>1.62 ± 0.3</td>
<td>1.40 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.53 ± 0.21(6)</td>
<td>1.03 ± 0.24(2)</td>
<td>1.99 ± 1.23</td>
<td>1.33 ± 0.09</td>
</tr>
<tr>
<td>Thalamus</td>
<td>Tryptophan</td>
<td>7.82 ± 1.85(5)</td>
<td>33.5 ± 5.5</td>
<td>36.7 ± 4.3</td>
<td>9.6 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.34 ± 0.14(6)</td>
<td>0.74 ± 0.22</td>
<td>0.58 ± 0.14</td>
<td>0.43 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.40 ± 0.22(6)</td>
<td>0.64 ± 0.25</td>
<td>1.15 ± 0.58</td>
<td>0.45 ± 0.13</td>
</tr>
<tr>
<td>Caudate</td>
<td>Tryptophan</td>
<td>5.12 ± 1.68(5)</td>
<td>31.9 ± 8.4</td>
<td>31.0 ± 12.3</td>
<td>8.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.32 ± 0.04(6)</td>
<td>0.59 ± 0.05</td>
<td>0.53 ± 0.14</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.19 ± 0.08(6)</td>
<td>0.26 ± 0.03</td>
<td>0.57 ± 0.19</td>
<td>0.38 ± 0.12(2)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Tryptophan</td>
<td>6.22 ± 1.69(5)</td>
<td>35.6 ± 4.7</td>
<td>35.6 ± 4.7</td>
<td>8.3 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.44 ± 0.13(6)</td>
<td>0.65 ± 0.09</td>
<td>0.65 ± 0.09</td>
<td>0.40 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.19 ± 0.08(5)</td>
<td>0.52 ± 0.23</td>
<td>0.52 ± 0.23</td>
<td>0.36 ± 0.1</td>
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<tr>
<td>Hindbrain</td>
<td>Tryptophan</td>
<td>6.92 ± 1.69(5)</td>
<td>33.3 ± 6.5</td>
<td>43.0 ± 3.8</td>
<td>10.5 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.31 ± 0.06(6)</td>
<td>0.75 ± 0.19</td>
<td>0.52 ± 0.20</td>
<td>0.47 ± 0.08</td>
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<tr>
<td></td>
<td>5-HIAA</td>
<td>0.37 ± 0.15(6)</td>
<td>0.97 ± 0.52</td>
<td>1.42 ± 0.45</td>
<td>1.05 ± 0.14</td>
</tr>
<tr>
<td>Cortex</td>
<td>Tryptophan</td>
<td>7.53 ± 1.32</td>
<td>34.6 ± 5.3</td>
<td>38.4 ± 2.5</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D.†</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Tryptophan</td>
<td>8.54 ± 3.5(5)</td>
<td>44.6 ± 13.3</td>
<td>45.2 ± 10.5</td>
<td>11.7 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D. (4)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>N.D. (4)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CSF (Cisternal)*</td>
<td>Tryptophan</td>
<td>0.78 ± 0.40(7)</td>
<td>4.93 ± 3.28(6)</td>
<td>4.36 ± 2.15(8)</td>
<td>1.50 ± 0.74(8)</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.028 ± 0.09(5)</td>
<td>0.055 ± 0.02(8)</td>
<td>0.071 ± 0.02(8)</td>
<td>0.054 ± 0.013(8)</td>
</tr>
<tr>
<td>Blood*</td>
<td>Tryptophan</td>
<td>10.1 ± 2.8(23)</td>
<td>42.8 ± 13.3(25)</td>
<td>21.8 ± 8.7(15)</td>
<td>15.5 ± 5.1(8)</td>
</tr>
</tbody>
</table>

Concentration of tryptophan, 5-HT and 5-HIAA (µg/g) in various regions of dog brain in control animals and following the intravenous administration of tryptophan (50 mg/kg).

Means ± s.d. of three animals unless otherwise indicated. 5-HTP not detected (<0.05 µg/g) in any region.

* This includes data obtained during acute and chronic experiments.

† N.D. = <0.05 µg/g.

5-Hydroxyindoles in brain and CSF
as follows: cerebellum, thalamus, hypothalamus, hindbrain, midbrain, hippocampus and caudate.

Following administration of tryptophan the concentration of the amino acid in blood showed an exponential fall. The concentration of the amino acid rose in CSF and all the brain regions examined (Fig. 1, Table 1). Analysis of variance of the concentration of tryptophan at all times after tryptophan loading showed no significant
difference between regions. When α-methyl dopa (50 mg or 100 mg/kg) was administered prior to tryptophan the peak concentration for tryptophan in brain occurred earlier than in animals treated with tryptophan alone, and fell more rapidly (Tables 2 and 3).

5-Hydroxyindoles in brain

*Treatment with tryptophan alone.* The estimates for the concentration of these substances in control animals are shown in Table 1. There was a marked variation in the concentration, both between regions and between animals. Midbrain and hypothalamus showed the highest concentration of both 5-HIAA and 5-HT. Neither of these compounds was detected in either cerebellum or cortex. 5-HTP was not detected (<0.05 μg/g) in any region.

Following the administration of tryptophan the concentration of 5-HIAA and 5-HT rose in all regions of brain where they were initially present (Table 1, Fig. 2).

*Pretreatment with α-methyl dopa.* Treatment with α-methyl dopa alone (100 mg/kg)
### Table 2: Tryptophan Metabolites after Administration of l-Tryptophan in Animals Pretreated with α-Methyl Dopamine

<table>
<thead>
<tr>
<th>Region</th>
<th>Tryptophan and derivatives</th>
<th>Controls 0 hr</th>
<th>α-Methyl dopa alone 0-5 hr</th>
<th>1-5 hr</th>
<th>α-Methyl dopa + Tryptophan 2-5 hr</th>
<th>4-5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>Tryptophan</td>
<td>6.8 ± 1.5</td>
<td>4.2</td>
<td>38.8</td>
<td>11.7</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>l-tryptophan</td>
<td>0.88 ± 0.1 (6)</td>
<td>0.89</td>
<td>0.82</td>
<td></td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.92 ± 0.18</td>
<td>0.78</td>
<td>0.75</td>
<td></td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.49</td>
<td>7.2</td>
<td>1.52</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Tryptophan</td>
<td>7.6 ± 2.1 (5)</td>
<td>7.0</td>
<td>48.6</td>
<td>9.3</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.81 ± 0.21</td>
<td>0.63</td>
<td>0.80</td>
<td>0.88</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.53 ± 0.21</td>
<td>0.85</td>
<td>0.34</td>
<td>1.08</td>
<td>0.60</td>
</tr>
<tr>
<td>Thalamus</td>
<td>Tryptophan</td>
<td>7.8 ± 1.9 (5)</td>
<td>4.9</td>
<td>36.0</td>
<td>10.5</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.34 ± 0.14 (6)</td>
<td>0.34</td>
<td>0.18</td>
<td>0.18</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.45 ± 0.22 (6)</td>
<td>0.45</td>
<td>0.09</td>
<td>0.44</td>
<td>0.61</td>
</tr>
<tr>
<td>Caudate</td>
<td>Tryptophan</td>
<td>5.1 ± 1.7 (5)</td>
<td>4.5</td>
<td>36.9</td>
<td>11.2</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.32 ± 0.0 (4)</td>
<td>0.19</td>
<td>0.20</td>
<td>0.38</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.19 ± 0.08 (6)</td>
<td>0.26</td>
<td>0.35</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Tryptophan</td>
<td>6.2 ± 1.7 (5)</td>
<td>5.0</td>
<td>39.8</td>
<td>12.4</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.44 ± 0.13 (6)</td>
<td>0.26</td>
<td>0.26</td>
<td>0.34</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.19 ± 0.08 (5)</td>
<td>0.13</td>
<td>0.10</td>
<td>0.28</td>
<td>0.23</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>Tryptophan</td>
<td>5.9 ± 1.7 (5)</td>
<td>5.1</td>
<td>40.2</td>
<td>13.3</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.31 ± 0.06 (6)</td>
<td>0.19</td>
<td>0.40</td>
<td>0.35</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.37 ± 0.15 (6)</td>
<td>0.28</td>
<td>0.89</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>Tryptophan</td>
<td>7.5 ± 1.3 (3)</td>
<td>5.5</td>
<td>35.2</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D. † (3)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>N.D. (3)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Tryptophan</td>
<td>8.5 ± 3.5 (5)</td>
<td>6.0</td>
<td>79.2</td>
<td>9.9</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D. (4)</td>
<td>N.D.</td>
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<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>N.D. (4)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CSF (Cisternal)</td>
<td>Tryptophan</td>
<td>0.78 ± 0.40 (7)</td>
<td>1.15 ± 0.73 (4)</td>
<td>5.7 ± 1.23 (3)</td>
<td>2.55 ± 1.73 (4)</td>
<td>1.13 ± 0.30 (4)</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.028 ± 0.009 (8)</td>
<td>0.038 ± 0.011 (5)</td>
<td>0.037 ± 0.011 (4)</td>
<td>0.047 ± 0.009 (4)</td>
<td>0.041 ± 0.016 (5)</td>
</tr>
<tr>
<td>Blood</td>
<td>Tryptophan</td>
<td>10.1 ± 2.8 (23)</td>
<td>8.5 ± 3.1 (19)</td>
<td>35.0 ± 12.2 (15)</td>
<td>20.7 ± 9.4 (10)</td>
<td>11.9 ± 3.4 (5)</td>
</tr>
</tbody>
</table>

*Mean ± s.d. (no. of observations). 5-HTP not detected (<0.05 μg/g) in any region.
†N.D. = <0.05 μg/g.
<table>
<thead>
<tr>
<th>Region</th>
<th>Tryptophan and derivatives</th>
<th>Controls</th>
<th>0.5 hr</th>
<th>α-Methyl dopa Alone</th>
<th>1.5 hr</th>
<th>2.5 hr</th>
<th>4.5 hr</th>
<th>α-Methyl dopa + Tryptophan</th>
<th>1.5 hr</th>
<th>2.5 hr</th>
<th>4.5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>Tryptophan</td>
<td>6.8 ± 2.1(5)*</td>
<td>4.2</td>
<td>8.2</td>
<td>8.3</td>
<td>4.4</td>
<td>71.5</td>
<td>19.8</td>
<td>10.8</td>
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<tr>
<td></td>
<td>5-HT</td>
<td>0.84 ± 0.14(6)</td>
<td>1.17</td>
<td>0.98</td>
<td>0.88</td>
<td>0.42</td>
<td>1.20</td>
<td>0.92</td>
<td>0.54</td>
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<td>5-HIAA</td>
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<td>0.79</td>
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<td>1.44</td>
<td>0.63</td>
<td>1.01</td>
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<td>1.16</td>
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<td>Hypothalamus</td>
<td>Tryptophan</td>
<td>7.6 ± 2.1(5)</td>
<td>7.3</td>
<td>11</td>
<td>7.9</td>
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<td>51.9</td>
<td>23.7</td>
<td>12.3</td>
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<td>5-HT</td>
<td>0.81 ± 0.21(6)</td>
<td>0.62</td>
<td>0.63</td>
<td>0.41</td>
<td>0.42</td>
<td>0.77</td>
<td>0.45</td>
<td>0.51</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.53 ± 0.21(6)</td>
<td>0.49</td>
<td>0.58</td>
<td>0.56</td>
<td>0.26</td>
<td>0.79</td>
<td>0.77</td>
<td>0.86</td>
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<tr>
<td>Thalamus</td>
<td>Tryptophan</td>
<td>7.8 ± 1.9(5)</td>
<td>4.8</td>
<td>8.0</td>
<td>6.5</td>
<td>4.3</td>
<td>48.9</td>
<td>19.6</td>
<td>9.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.34 ± 0.14(6)</td>
<td>0.10</td>
<td>0.24</td>
<td>0.18</td>
<td>0.14</td>
<td>0.24</td>
<td>0.20</td>
<td>0.26</td>
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<tr>
<td></td>
<td>5-HIAA</td>
<td>0.40 ± 0.22(6)</td>
<td>0.28</td>
<td>0.72</td>
<td>0.39</td>
<td>0.32</td>
<td>0.44</td>
<td>0.26</td>
<td>0.32</td>
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<tr>
<td>Caudate</td>
<td>Tryptophan</td>
<td>5.1 ± 1.7(5)</td>
<td>4.9</td>
<td>8.0</td>
<td>7.7</td>
<td>3.7</td>
<td>49.4</td>
<td>15.6</td>
<td>11.6</td>
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<tr>
<td></td>
<td>5-HT</td>
<td>0.32 ± 0.04(6)</td>
<td>0.26</td>
<td>0.27</td>
<td>0.15</td>
<td>0.31</td>
<td>0.26</td>
<td>0.32</td>
<td>0.33</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.19 ± 0.08(6)</td>
<td>0.26</td>
<td>0.24</td>
<td>0.02</td>
<td>0.16</td>
<td>0.16</td>
<td>0.25</td>
<td>0.20</td>
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</tr>
<tr>
<td>Hippocampus</td>
<td>Tryptophan</td>
<td>6.2 ± 1.7(5)</td>
<td>4.6</td>
<td>6.8</td>
<td>7.2</td>
<td>3.7</td>
<td>39.5</td>
<td>16.2</td>
<td>9.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.44 ± 0.13(6)</td>
<td>0.17</td>
<td>0.39</td>
<td>0.24</td>
<td>0.18</td>
<td>0.34</td>
<td>0.38</td>
<td>0.35</td>
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<tr>
<td></td>
<td>5-HIAA</td>
<td>0.19 ± 0.08(5)</td>
<td>0.12</td>
<td>0.28</td>
<td>0.11</td>
<td>0.98</td>
<td>0.29</td>
<td>0.11</td>
<td>0.17</td>
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<tr>
<td>Hindbrain</td>
<td>Tryptophan</td>
<td>6.9 ± 1.7(5)</td>
<td>4.5</td>
<td>8.6</td>
<td>7.0</td>
<td>4.8</td>
<td>49.3</td>
<td>20.3</td>
<td>10.0</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>0.31 ± 0.06(6)</td>
<td>0.10</td>
<td>0.40</td>
<td>0.18</td>
<td>0.14</td>
<td>0.19</td>
<td>0.26</td>
<td>0.26</td>
<td></td>
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<tr>
<td></td>
<td>5-HIAA</td>
<td>0.37 ± 0.15(6)</td>
<td>0.23</td>
<td>0.84</td>
<td>0.34</td>
<td>0.27</td>
<td>0.51</td>
<td>1.11</td>
<td>1.23</td>
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<tr>
<td>Cortex</td>
<td>Tryptophan</td>
<td>7.5 ± 3.3(3)</td>
<td>3.9</td>
<td>13.9</td>
<td>7.9</td>
<td>3.7</td>
<td>50.3</td>
<td>15.8</td>
<td>10.6</td>
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</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D.† (3)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td></td>
<td>5-HIAA</td>
<td>N.D. (3)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Tryptophan</td>
<td>8.5 ± 3.5(5)</td>
<td>4.0</td>
<td>—</td>
<td>7.6</td>
<td>4.5</td>
<td>54.2</td>
<td>18.4</td>
<td>11.2</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D. (4)</td>
<td>N.D.</td>
<td>—</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>N.D. (4)</td>
<td>N.D.</td>
<td>—</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF (Cisternal)</td>
<td>Tryptophan</td>
<td>0.78 ± 0.40(7)</td>
<td>1.2</td>
<td>1.3</td>
<td>1.0</td>
<td>—</td>
<td>8.6</td>
<td>2.9</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>0.028 ± 0.009(8)</td>
<td>0.038</td>
<td>0.043</td>
<td>0.020</td>
<td>—</td>
<td>0.034</td>
<td>0.025</td>
<td>0.055</td>
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<tr>
<td>Blood</td>
<td>Tryptophan</td>
<td>10.1 ± 2.8(23)</td>
<td>10.2</td>
<td>8.8</td>
<td>8.4</td>
<td>7.5</td>
<td>38.6</td>
<td>23.0</td>
<td>13.8</td>
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</tr>
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</table>

Concentration of tryptophan, 5-HT and 5-HIAA (μg/g) in various brain regions following intravenous administration of α-methyl dopa (100 mg/kg) alone and followed 30 min later by intravenous administration of L-tryptophan (50 mg/kg).

* Mean ± S.D. (no. of observations). 5-HTP was detected in midbrain and hippocampus at 1.5 hr in a concentration of 0.13 and 0.16 μg/g respectively following the administration of both tryptophan and α-methyl dopa.

† N.D. = <0.05 μg/g
results in a slight rise in 5-HIAA with a fall in 5-HT in most regions (Table 3). Pre-treatment with either 50 or 100 mg/kg α-methyl dopa prior to tryptophan blocked the usual large rise in the concentration of 5-HT and 5-HIAA seen with tryptophan alone. The maximum rise of 5-HIAA after α-methyl dopa tended to occur later than the 2-34 hr mean for tryptophan alone (Tables 2 and 3). This was particularly noticeable in brain stem and parallels the changes seen in 5-HIAA in CSF under the same conditions of drug administration (Fig. 3).

Following the dose of 100 mg/kg α-methyl dopa prior to tryptophan, 5-HTP appears in small concentrations in both midbrain and hippocampus at 1 hr after precursor loading. 5-HTP was not detected in plasma (<0.025 μg/ml) of animals treated with either tryptophan or α-methyl dopa alone or after a combination of the two.

5-HIAA in CSF

In dogs used repeatedly for CSF sampling the concentration of 5-HIAA rose in CSF to reach a peak at about 2 hr after administration of tryptophan (Fig. 3, I, II and III). The second latin square (II) resembles the first (I) but the mean concentration of the acid was lower. The prior administration of α-methyl dopa blocked the large rise of the acid metabolite (Fig. 3, III) and delayed the time of peak concentration.
Statistical section

5-HIAA in CSF. The three latin squares, two for the values of 5-HIAA after tryptophan administration (I and II), and one for α-methyl dopa and tryptophan (III) were treated as regression problems and in each case a quadratic regression curve (Fig. 3) was fitted by computer programme of the general form:

\[ y = a + bx + cx^2 \]

where \( x \) = time and \( y \) = response.

It was found that in all three quadratics an acceptable fit was obtained. The average value for \( y \) on square I and II is significantly different (\( P < 0.005 \)). Thus some unexplained factor has arisen between experiments I and II to depress the mean concentrations of 5-HIAA. The ‘form’ of the curves for squares I and II is, however, very similar as is shown by the regression coefficients \( b \) and \( c \).

It is possible to pool the regression coefficients (the \( b \)'s and \( c \)'s) of curves I and
II and their variances, and to compare them against the corresponding values for curve III. The quadratic regression coefficients of curve III are significantly different from the mean values of I and II.

\[
\begin{align*}
& b_3 = 11.05 \text{ is significantly different from } b = 38.17 \text{ (average of } b_1 \text{ and } b_2) \quad P < 0.01 \\
& C_3 = -1.55 \text{ is significantly different from } C = 8.02 \text{ (average of } C_1 \text{ and } C_2) \quad P < 0.01
\end{align*}
\]

In view of the statistical interdependence of the regression coefficients, these parameters of curve III were tested against the pooled results of curves I and II simultaneously using the Generalised T test. The results were highly significantly different (\( P < 0.01 \)). Curve III has been 'flattened' as compared with I and II.

The variance about regression curve III (30.55) is significantly less than the pooled variance for I and II (186). Thus the response appears also to have been stabilised.

**DISCUSSION**

Price and West in 1960 showed a regional variation in the concentration of tryptophan in dog brain. Our results also show a regional variation with a significant rank order which, however, differs from that presented by Price and West.

The control group of six dogs showed a wide variation in the concentration of 5-HT and 5-HIAA between the regions examined (Table I). The relationship of the concentration of 5-HT between the various regions is largely in agreement with those of previous studies (Amin, Crawford and Gaddum, 1954; Bogdanski, Weissbach and Udenfriend, 1957), although the absolute concentrations differ markedly. The reason is probably the methodological differences in the techniques employed by the various workers to determine the concentration of the amine (Eccleston, 1966).

It has been suggested that the turnover rate for catecholamines varies from region to region (Iversen and Glowinski, 1966). One might also anticipate similar differences between areas for the turnover rate of 5-HT. That the concentration of 5-HIAA might be used to assess the turnover depends on the assumption that a negligible amount of the acid is derived from alternative metabolic pathways such as transamination of 5-HP; that 5-HT is not metabolised to other products which rapidly leave the brain such as 5-hydroxytryptophol, and that the egress of the acid, probably a process involving active transport (Guldberg, Ashcroft and Crawford, 1966) is uniform for all regions.

Best fit quadratic curves (see statistical section) for the changes in concentration of 5-HIAA after tryptophan loading were fitted by computer programme to the available data for the various brain regions (Fig. 2, IV–IX). Although at best they are approximations, the rate of rise (the slope of the curve at zero time given by the coefficient of \( x \)) would be an index of the increased rate of synthesis of 5-hydroxyindoles. Comparing the rates of rise of 5-HIAA in the different regions after tryptophan loading we see that only caudate and hippocampus are similar, the others differ, midbrain and hypothalamus having the highest rate of rise. The efficiency of a drug which blocks hydroxylation, probably the limiting step in the synthesis of 5-HT, could be determined by the change in this coefficient for total 50H indoles (Moir and Eccleston, 1968) when a drug is used in combination with tryptophan loading.
When the quadratic regression curves for the various regions of brain are examined, certain regions appear similar. When, using a 'generalised' $T$ test, hindbrain and thalamus, midbrain and hypothalamus, caudate and hippocampus were compared statistically, only with caudate and hippocampus could the same quadratic curve be fitted to both sets of data. The rest differed significantly from each other. This would tend to indicate that the various regions have different turnover rates for 5-HT apart from caudate and hippocampus.

Following tryptophan loading the concentration of tryptophan and 5-HIAA rose in cisternal CSF. The experiment was conducted in a Latin square design to detect between animal and between experiment variation. In each Latin square there was found to be no significant variation between animals nor between experiments. However, the mean rise of 5-HIAA was found to be significantly different between the two Latin squares for tryptophan alone (Fig. 3, I and II). The reason for this was not readily apparent. Apart from the first constant the form of the quadratic regression curves were remarkably similar. Although no direct statistical comparison between the curves of the various brain regions and those for cisternal CSF are possible, those for brain stem shows the closest resemblance to CSF. The time to achieve maximum concentration of 5-HIAA after tryptophan loading did not differ significantly between the various regions of brain and had a mean value of 2-34 hr. The corresponding value for the CSF in square I and II was 2-34 and 2-33 hr respectively, indicating that the CSF mirrors fairly closely in time the events in the brain.

$\alpha$-Methyl dopa was used in these experiments to produce a change in the pattern of response to a tryptophan load. Previous investigators have found the drug to be a decarboxylase inhibitor in vitro (Sourkes, 1954) and in vivo (Sharman and Smith, 1962). Its status as a tryptophan hydroxylase inhibitor in the brain is disputed. Roos and Werdinius (1963) related the fall in 5-HIAA and 5-HT in brain after $\alpha$-methyl dopa to the inhibition of hydroxylase. Gal, Morgan and Marshall (1965) were unable to demonstrate inhibition of tryptophan hydroxylase in rat brain by $\alpha$-methyl dopa following an intracerebral injection of $^{14}$C-labelled tryptophan.

In these experiments when $\alpha$-methyl dopa alone is administered, apart from midbrain, there is a fall in 5-HT but a rise in 5-HIAA. This can be interpreted as the result of a release of 5-HT with or without an inhibition of the synthesis of the amine. The possibility of the release of 5-HT by $\alpha$-methyl dopa was first suggested by Sharman and Smith (1962). In the present experiments the rise in the concentration of both 5-HT and 5-HIAA after tryptophan is blocked in all regions by $\alpha$-methyl dopa. The inhibition is, however, incomplete. There does not seem to be a greater inhibition of the synthesis of 5-HT up to 2 hr after tryptophan with 100 mg/kg of $\alpha$-methyl dopa compared with 50 mg/kg. However, at 4 hr the 5-HIAA and 5-HT is higher in the animals treated with the smaller dose of $\alpha$-methyl dopa suggesting that the inhibition has not persisted as long as it does with 100 mg/kg. Unlike rats under similar experimental conditions, 5-HP' does not appear in significant amounts in any brain region (Eccleston, Ashcroft and Crawford, 1965). Small quantities are however detected in midbrain and hippocampus. It is interesting to note that the hippocampus has relatively low levels of decarboxylase activity and one would anticipate more complete decarboxylase inhibition by the drug in this region. That 5-HP did not accumulate in large quantities in spite of the fall in the synthesis of 5-HT and 5-HIAA would tend to indicate inhibition at the hydroxylating enzyme.
Blood levels of tryptophan are significantly lower following pretreatment with \( \alpha \)-methyl dopa, while brain levels appear higher initially but show a dramatic fall at 2 hr. This would indicate a general increase in tissue uptake of the amino acid with subsequently a more rapid metabolism to products other than 5-hydroxyindoles.

In CSF the rise of 5-HIAA after tryptophan is inhibited by \( \alpha \)-methyl dopa and the quadratic regression curve (III) differs significantly from those obtained with tryptophan alone. The form of the curve resembles the change found in brain stem. The time of maximum rise of 5-HIAA is delayed to 3-56 hr and is difficult to explain. It is possible that \( \alpha \)-methyl dopa, or one of its metabolites, interferes with the efflux of the 5-HIAA from the brain. The variability about the curve for 5-HIAA is also greatly diminished and may be due to a limitation of the range of response of one of the enzymes to tryptophan, presumably the hydroxylase.

These results show that the changes in the concentration of the metabolite 5-HIAA in CSF after tryptophan loading and when modified by \( \alpha \)-methyl dopa pretreatment reflect changes in the concentration of the metabolite in brain, particularly the brain stem. In man a study of the metabolite in CSF may be used as an indirect method of investigating cerebral 5-hydroxyindole metabolism.

Acknowledgement—The authors wish to acknowledge the help and advice of Professor W. L. M. Perry.

REFERENCES

SECTION II
SOME OBSERVATIONS ON THE ESTIMATION OF TRYPTAMINE IN TISSUES

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Hess, Redfield and Udenfriend (1959) reported the presence of tryptamine in brain under certain circumstances. They did not detect it in the brains of normal animals (rat, guinea pig and dog), but found the amine in the brains of guinea pigs treated with L tryptophan, or with a combination of L tryptophan and the amine oxidase inhibitor, iproniazid. The method, solvent-extraction followed by fluorescence assay, was used subsequently by other workers (Green and Sawyer, 1960; Hess and Doeppner, 1961) to determine the tryptamine content of brain under various experimental conditions.

During a study of the effect of tryptophan administration in rats on the concentration of 5-hydroxyindole compounds in whole brain, Ashcroft, Eccleston and Crawford (1965), using a method involving separation by paper chromatography, attempted to examine in parallel the levels of tryptamine in the brain. Using this technique, they were unable to detect tryptamine in the brains of untreated animals, while in animals to which tryptophan had been given only very small amounts of the amine were detected. The present paper presents an investigation of the discrepancy between these results and those previously reported, together with a critical re-evaluation of the solvent-extraction technique of Hess and Udenfriend (1959). A description of a modification of the method of Hess and Udenfriend (1959), incorporating ion-exchange chromatography, is presented.

METHODS

Animal treatment. The animals were killed by decapitation and the blood collected from the neck wound into a polythene tube containing heparin (0·2 ml; 100 I.U./ml). The contents of the tube were mixed by gentle shaking.

Iproniazid phosphate (60 mg/ml in saline) was administered by intraperitoneal injection to 200 g guinea pigs in groups of three, in a dose of 150 mg/kg. Sixteen hours later, L-tryptophan (800 mg/kg) was given by the intraperitoneal route to the animals in a 2 ml saline suspension, prepared as described by Hess et al. (1959). The animals were killed in groups of three at 20, 40, 60, 80 and 120 min following the administration of tryptophan. Further groups of guinea pigs were given L-tryptophan by the same route, without pre-treatment with iproniazid, and killed at comparable times following the administration of the amino acid. A group of untreated animals and a group treated 16 hr previously with iproniazid, but without injection of tryptophan, were examined as controls.

Preparation of tissue and plasma extracts. Plasma: The blood was centrifuged at 2500 revs/min for 10 min and the plasma separated off. A 2 ml portion of the plasma was diluted to 10 ml with deionized distilled water. Zinc sulphate solution (2 ml, 10% w/v) was added with mixing. The proteins were precipitated by the addition of 0·2 ml 20% (w/v) NaOH, when the tube was immediately inverted gently 3 times to ensure adequate mixing. After standing for 10 min, the tube was centrifuged for 5 min at 2500 revs/min and the supernatant fluid transferred to a 10 ml beaker. After adjustment of the pH to 7·5 (glass electrode) with 0·2 N-NaOH, the solution was then ready to pass through an ion-exchange column.
Brain and liver: The brains were dissected out as rapidly as possible and the group of three brains weighed. These were then homogenized in an all glass homogenizer in 0.1 N HCl (2 ml/g brain tissue). The homogenate was transferred to a 50 ml glass-stoppered measuring cylinder and the volume recorded. The homogenate was stored at -15° prior to use (no longer than 12 hr).

To a 5 ml portion of the homogenate, diluted to 9 ml with deionized distilled water in a glass-stoppered centrifuge tube, was added 1.0 ml 1 N perchloric acid and the contents of the tube mixed by repeated inversions. After standing for 10 min, the precipitated proteins were sedimented by centrifugation at 2500 revs/min for 10 min. The supernatant fluid was transferred to a 10 ml beaker and the pH adjusted to 7.5 (glass electrode) by dropwise addition, with constant stirring, of 5 N and then 0.2 N-KOH. The contents of the beaker were transferred to a 10 ml centrifuge tube and centrifuged at 2500 revs/min for 5 min. The supernatant fluid was separated from the precipitated potassium perchlorate and passed through an ion-exchange column.

A weighed portion (about 3 g) of liver was removed from each animal and the pooled tissue subjected to the same analytical procedures as brain.

Estimation of tryptamine. 1. Solvent-extraction technique of Hess and Udenfriend (1959): In brief, the method (Method 1) consists of the extraction into benzene of tryptamine from alkaline tissue homogenates. The benzene extract is washed once with 0.1 N NaOH and the tryptamine extracted into 0.1 N H₂SO₄. Cyclization by heating the tryptamine with formaldehyde and hydrogen peroxide leads to the formation of norharman, which has a characteristic fluorescent spectrum and allows quantitative assay by spectrophotofluorimetry.

2. Paper chromatography: This was carried out as part of a study of the effect of tryptophan administration (800 mg/kg) on the concentration of 5 hydroxy-indole compounds in rat brain. The preparation of the brain extracts and subsequent chromatography was as described by Ashcroft et al. (1965). The appropriate eluates from the chromatogram were assayed for tryptamine and tryptophan, using the norharman fluorescent technique (Hess and Udenfriend 1959).

3. Modification of Method of Hess and Udenfriend (1959). (a) Ion-exchange column chromatography: The method (Method 2), a modification of the procedure of Oates (1961) is dependent on the adsorption of tryptamine on a column of suitable cation exchange resin under conditions in which tryptophan is not adsorbed. The tryptamine is eluted from the column with strong acid. A column, 70 mm × 7 mm diameter, of Amberlite ion-exchange resin C.G. 50 type 1, 100–200 mesh, a weak cation-exchange resin, was employed. Before use, the resin was treated as follows. It was first stirred for 30 min with 3 vol. N HCl and the acid was decanted off and the resin washed by decantation with distilled deionized water until no chloride was detectable in the supernatant fluid on addition of a few drops 2.5% (w/v) silver nitrate solution. The resin was stirred twice with 3 vol. 3 n-NH₄OH for 30 min. The NH₄OH was decanted off and the resin washed repeatedly with distilled water until the pH of the supernatant fluid was 9. The resin was finally treated with 0.2 M ammonium acetate buffer, pH 7.5, until the buffer pH remained unchanged after standing in contact with the resin for at least 12 hr. The resin was stored in this buffer solution. After preparation of the resin column, 5 ml 0.02 M ammonium acetate buffer, pH 7.5, were passed through before applying the sample solution to be chromatographed.

(b) Chromatography of extracts. Deproteinized extracts of brain, liver, or plasma (8–10 ml) were passed through the column at approximately 0.5 ml/min. Then 15 ml 0.02 M ammonium acetate buffer, pH 7.5, followed by 4 ml 0.1 N H₂SO₄ were passed through the column at approximately 0.5 ml/min and the effluents discarded. Tryptamine was eluted from the column by the passage of 6 ml N H₂SO₄ at 0.2 ml/min, all of the eluate being collected.

Estimation of tryptamine in the column eluate by the 'norharman' procedure. The column eluate was adjusted to pH 11 (universal indicator paper) by the dropwise addition of 10 N NaOH and shaken with 20 ml benzene for 5 min. The tryptamine was returned to aqueous solution by shaking for 5 min with 6.0 ml 0.1 N H₂SO₄. To a 5–5 ml portion of the acid extract was added 0.2 ml 18% (w/v) formaldehyde and the mixture heated in a covered tube for 20 min after addition to the solution of 0.2 ml 5% (w/v) H₂O₂. The fluorescence intensity of the norharman thus formed from tryptamine was determined, using an Amico-Bowman spectrophotofluorimeter.

Calculation of results. Norharman shows a maximum fluorescence at 440 mμ, with two activation maxima at 310 mμ and 360 mμ (Hess and Udenfriend, 1959). The tryptamine content of the eluate was determined from the fluorescence intensity reading at 360 mμ (activation); 440 mμ (fluorescence), in comparison with that obtained from 1.0 μg tryptamine added to another portion of the brain homogenate and processed in parallel with the sample. A blank for the norharman reaction was determined from the norharman procedure on the eluate from pure solutions run through the column.

Calibration of Method 2. Since the inclusion of the ion-exchange chromatography step appeared to give a sensitive and specific method for the estimation of tryptamine, an experiment was performed to calibrate the method. Amounts of tryptamine, from 50 100 μg, were added to 5 ml
portions of brain homogenates and the quantity of tryptamine estimated by the method. The results (Fig. 1) are found to be linear in this range and an amount as low as 30 mμg/g of brain is measurable.

The fluorescence from the brain sample without tryptamine added is the same as that from pure solutions of reagents taken through the procedure.

Recoveries. The mean recoveries of 1 μg quantities of tryptamine added to 5 ml brain homogenate and carried through the procedure was 47·9 per cent (s.d. 6·9; eight estimations). The mean recoveries of 0·5 μg quantities of tryptamine in 2 ml plasma carried through the procedure was 54·2 per cent (s.d 8·4; eleven estimations). These recoveries are low because of the relatively poor partition of tryptamine between benzene and the aqueous phase at pH 11.

Estimation of tryptophan in brain, liver and plasma. These estimations were carried out by the method of Hess and Udenfriend (1959), as applied by Guroff and Udenfriend (1962).

RESULTS

Failure to detect tryptamine in extracts of rat brain using paper chromatographic separation and fluorescence assay

Paper chromatography gave inconsistent recoveries of internal standard and the fluorescence of the norharman was masked by interfering fluorophores. Attempts to detect tryptamine in the brains of animals killed after pre-treatment with tryptophan, 800 mg/kg, showed only low concentrations of the amine (>0·2 μg/g at 1 hr). However, despite the difficulties, the discrepancy between the results obtained by this method and those by the solvent-extraction technique appeared to be real ones.
A comparison of tryptamine estimations in guinea pig brain and liver by the solvent-extraction method of Hess and Udenfriend (1959) and the modified method as described above.

Homogenates of liver and brain were made from the tissues of animals as described above. A 3·0 ml portion of each homogenate was examined by method 1, the solvent-extraction procedure of Hess et al. (1959), and a 5·0 ml portion by the modified technique (method 2). The results (Table 1) show a discrepancy between the values for the concentration of tryptamine in the tissues obtained by the two methods. Table 2 shows the high concentration of tryptophan found in these tissues and plasma at the times the animals were killed.

Investigation of discrepancy of estimated tryptamine concentrations between the two methods.

(a) Effect of high concentrations of tissue tryptophan on the estimates of tryptamine. Brain homogenates were prepared from control guinea pigs. L-Tryptophan (250 µg/g) was added to the homogenate and this was analysed in triplicate portions by method 1. This homogenate, with added tryptophan, was also analysed in triplicate by the modified procedure. The results showed that in method 1, tryptophan was carried over to the final acid phase and converted to norharman. Tryptamine was therefore apparently present in a concentration of 0·6 µg/g. No tryptophan was found to be carried through the column chromatography procedure (method 2).
Some observations on the estimation of tryptamine in tissues

(b) Identification by thin-layer chromatography of indolic substances in final extracts from the two methods. 1. Extract from method of Hess and Udenfriend. A benzene extract of 18 g pooled whole brains from guinea pigs treated with tryptophan and iproniazid and killed 2 hr following administration of the amino acid was prepared as described by Hess and Udenfriend (1959). To the benzene phase 1 ml 40% (w/v) acetic acid was added, and the benzene removed in a stream of nitrogen to near dryness at 55°.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (min)</th>
<th>Brain Mean</th>
<th>Brain Range</th>
<th>Liver Mean</th>
<th>Liver Range</th>
<th>Plasma Mean</th>
<th>Plasma Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>7.5 (3)†</td>
<td>6.6–8.7</td>
<td>11.9 (2)</td>
<td>11.7–12.0</td>
<td>15.6</td>
<td>13.4–17.8</td>
</tr>
<tr>
<td>Iproniazid</td>
<td>0</td>
<td>8.1 (3)</td>
<td>5.8–10.5</td>
<td>12.8 (2)</td>
<td>11.0–14.6</td>
<td>17.0</td>
<td>15.2–18.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>20</td>
<td>86 (1)</td>
<td></td>
<td></td>
<td></td>
<td>670 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>112 (1)</td>
<td></td>
<td></td>
<td></td>
<td>670 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>128 (1)</td>
<td></td>
<td></td>
<td></td>
<td>600 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>138 (1)</td>
<td></td>
<td></td>
<td></td>
<td>465 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>189 (2)</td>
<td>158–220</td>
<td>700* (1)</td>
<td>784* (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan +</td>
<td>20</td>
<td>147 (3)</td>
<td>95–176</td>
<td>1015 (2)</td>
<td>950–1080</td>
<td>860 (3)</td>
<td>730–1000</td>
</tr>
<tr>
<td>Iproniazid</td>
<td>40</td>
<td>170 (3)</td>
<td>95–220</td>
<td>1188 (2)</td>
<td>525–1850</td>
<td>848 (3)</td>
<td>345–1280</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>210 (3)</td>
<td>200–230</td>
<td>995 (2)</td>
<td>660–1330</td>
<td>796 (3)</td>
<td>600–940</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>232 (3)</td>
<td>176–290</td>
<td>935 (2)</td>
<td>850–1020</td>
<td>823 (3)</td>
<td>670–1000</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>264 (4)</td>
<td>210–324</td>
<td>800 (3)</td>
<td>570–950</td>
<td>738 (3)</td>
<td>560–850</td>
</tr>
</tbody>
</table>

Concentration of tryptophan in guinea pig brain, liver and plasma at varying time intervals after the intraperitoneal administration of L-tryptophan (800 mg/kg), with and without pretreatment for 16 hr with iproniazid phosphate (150 mg/kg). Results are given in μg/g tissue.
* Pooled tissues from six animals.
† Figures in parenthesis indicate number of experiments with groups of three animals.

This extract contained considerable amounts of lipid and was unsuitable for direct application to thin-layer chromatograms. Initial paper chromatography with isopropanol–25% (w/v) ammonia–water (17:1:3) was used to purify the extract. Appropriate strips of the paper chromatogram were eluted with 20% (w/v) acetic acid. These eluates were evaporated and the residues taken up in 80% (w/v) methanol and applied to two thin-layer plates of silica gel G, one being developed in butanol–acetic acid–water (12:3:5) and the other in methyl acetate–isopropanol–25% (w/v) ammonia (9:7:4).

The developed plates were sprayed with a cinnemaldehyde reagent (Jepson 1963). Two substances were detected in the extract chromatograms which behaved like authentic tryptophan and tryptamine (Table 3).

2. Extract produced by modified technique. Homogenate from 36 g pooled brains of guinea pigs treated with iproniazid and killed at 2 hr following the administration of L-tryptophan (800 mg/kg) were analysed by the method 2. To the benzene extract of the amine containing eluate was added 1 ml 20% acetic acid and the mixture evaporated to near dryness, as described above. Portions of the extract were applied to thin-layer plates of silica gel G, using 0.1 ml 80% methanol, and developed in three solvent systems (Table 4). The plates were dried and visualized with cinnemaldehyde, or ultraviolet fluorescence following spraying with Prochazka reagent (Stahl 1965). In the extract chromatograms, material corresponding to authentic tryptamine, but not to tryptophan, was detected.
Table 3.—Thin-layer chromatographic identification of indolic substances in brain extracts treated by method 1

<table>
<thead>
<tr>
<th>Preliminary paper chromatography solvent</th>
<th>Silica gel solvent</th>
<th>Tryptophan marker in brain extract</th>
<th>Indole 1</th>
<th>Indole 2</th>
<th>Authentic tryptamine marker in brain extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol-acetic acid-water (12:3:5)</td>
<td>Methyl acetate–isopropanol–ammonia (45:35:20)</td>
<td>0·32</td>
<td>0·29</td>
<td>0·66</td>
<td>0·66</td>
</tr>
<tr>
<td>Isopropanol–35% ammonia–water (85:15:5)</td>
<td>Butanol–acetic acid-water (12:3:5)</td>
<td>0·38</td>
<td>0·38</td>
<td>0·47</td>
<td>0·47</td>
</tr>
</tbody>
</table>

$R_f$ values of the indolic substances in two solvent systems in silica gel G found in the final extract of 18 g of brain from animals treated with iproniazid 150 mg/kg and tryptophan 800 mg/kg in method 1 (Hess et al., 1959), following preliminary purification by paper chromatography.

Table 4.—Thin-layer chromatographic analysis of brain extracts treated by the modified method

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$R_f$ value</th>
<th>Tryptamine marker in brain extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl acetate–isopropanol–25% ammonia (9:7:4)</td>
<td>0·68</td>
<td>0·68</td>
</tr>
<tr>
<td>Butanol–acetic acid–water (12:3:5)</td>
<td>0·50</td>
<td>0·50</td>
</tr>
<tr>
<td>Chloroform–methanol–acetic acid (15:4:1)</td>
<td>0·20</td>
<td>0·20</td>
</tr>
</tbody>
</table>

$R_f$ values of indolic substance in three solvent systems in silica gel G found in the benzene extract of the amine fraction from the ion-exchange column after extraction of 9 g of brain of guinea pigs treated with iproniazid 150 mg/kg and tryptophan 800 mg/kg.

Table 5.—Estimation of tryptamine in brain homogenate from guinea pigs, treated with tryptophan and iproniazid, by three methods

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Method 1 (µg/g)</th>
<th>Method 2 (µg/g)</th>
<th>Method 3 (µg/g)</th>
<th>Tryptophan in benzene extracts from method 1.* (µg/g)</th>
<th>Conc. tryptophan in brain (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan alone</td>
<td>0·70</td>
<td>0</td>
<td>0</td>
<td>0·62</td>
<td>294</td>
</tr>
<tr>
<td>Tryptophan plus iproniazid</td>
<td>0·89</td>
<td>0·20</td>
<td>0·18</td>
<td>0·61</td>
<td>300</td>
</tr>
</tbody>
</table>

The animals were given iproniazid, 150 mg/kg, 16 hr before receiving tryptophan, 800 mg/kg. Method 1, solvent-extraction method of Hess et al. (1959). Method 2, modified method incorporating ion-exchange column chromatography. Method 3, solvent-extraction method followed by column chromatography. * Tryptophan estimated by column chromatography in the benzene extracts of Method 1.
Column chromatogram of extract of brain produced by the method of Hess and Udenfriend (1959). The final acid extract as prepared by the solvent-extraction method has two components which give the norharman reaction. The first is eluted from the column in the fraction in which the authentic tryptophan is contained, and the second in fraction containing authentic tryptamine. Quantitatively (Table 5), the addition of the two components could account for the apparent tryptamine estimated by the solvent-extraction technique.

Estimation of plasma tryptamine. The content of tryptamine in plasma was estimated by the modified method. The results (Fig. 2) show the amine to be present in plasma in low concentrations after the administration of iproniazid and tryptophan.

Fig. 2.—Concentration of tryptamine in guinea pig brain and plasma, as estimated by the modified procedure of Hess et al. (1959) (Method 2) at varying time intervals after the intraperitoneal administration of L-tryptophan (800 mg/kg) with pretreatment for 16 hr with iproniazid phosphate (150 mg/kg). Each point represents the mean of three experiments.

The levels never greatly exceed those found in brain at the same time. Following administration of tryptophan alone, tryptamine was found only at 2 hr in a concentration of 0.03 μg/ml.

DISCUSSION

The method described here is basically that reported by Hess et al. (1959) with the essential additional step of ion-exchange chromatography. In our hands, the original procedure of Hess et al. (1959) gave falsely high values for the estimates of tryptamine in the brains of animals which had been given a tryptophan load, with or without pretreatment with an amine oxidase inhibitor. The error was traced to a transfer of a portion of the brain tryptophan into the benzene phase and finally into the acid extract, in spite of all efforts to prevent any mechanical carry over. Since tryptophan yields a fluorophore identical in characteristics with that obtained from tryptamine, the estimates of the latter were too high.

The presence of tryptophan in the tryptamine extracts for fluorimetry was demonstrated in several ways. Tryptophan was added to homogenates of brain from normal
animals, which contained no detectable tryptamine, to give an amino acid concentration similar to that found in the brains of animals treated with tryptophan. Processed by the method of Hess et al. (1959) such normal brain homogenates apparently contained 0-6 µg/g of tryptamine. The acid extract obtained by the method of Hess et al. (1959) from the brains of animals treated with tryptophan, after prior administration of an amine oxidase inhibitor, was passed through an ion-exchange column and norharman-producing material was found in two eluates, one of which would have been expected to contain tryptophan and the other tryptamine. The norharman-yielding material in the two fractions accounted for the total apparent tryptamine in the original acid extract (Table 5). Finally, it was demonstrated by thin-layer chromatography on silica gel, using two solvent systems, that the acid extracts contained indolic substances with Rf values identical to those of authentic tryptophan and tryptamine (Table 3).

These techniques of identification were reduplicated for the final extract of the modified method of Hess et al. (1959) (method 2). Tryptophan added to normal brain tissue was found not to reach the final stage of this procedure. Thin-layer chromatography in three solvent systems (Table 4) of the final extract showed only one indolic component with Rf values identical with those of authentic tryptamine.

There are inconsistencies in the literature regarding the changes in the level of brain tryptamine following the administration of L-tryptophan, with and without amine oxidase inhibitors. Hess et al. (1959) failed to detect the amine in the brain of normal rats, guinea pigs or dogs. However, 2 hr after administration of tryptophan to guinea pigs, in a dose of 800 mg/kg, they found tryptamine to be present in brain in a concentration of 1-4 µg/g. Pretreatment with iproniazid, 150 mg/kg, prior to administration of tryptophan, resulted paradoxically in a fall in the concentration of the amine to 0-8 µg/g. In a later paper, Hess and Doepfner (1961), in similar experiments using the same method of estimation but a smaller dose of tryptophan (200 mg/kg), found 0-14 µg/g of tryptamine in the brains of rats 2 hr after administration of the amino acid. Pretreatment of these animals with iproniazid (150 mg/kg) prior to the dose of tryptophan led to an increase in the brain tryptamine to 0-37 µg/g at 2 hr.

In our investigations, using the modified procedure for the estimation of tryptamine, the amine was found in brain only after treatment with both amine oxidase inhibitor and L-tryptophan (Table 1; Fig. 2). The amine was detected at 60 min in low concentrations and rose slowly over the 2 hr period of the experiment. Liver, on the other hand, had detectable levels of tryptamine after treatment of the animals with amine oxidase inhibitor alone. Combining the drug with administration of tryptophan produced very high concentrations (Table 1). Plasma tryptamine levels became detectable at 20 min after tryptophan and iproniazid, the levels still rising at 2 hr. These results did not demonstrate a high plasma to brain gradient in the concentration of tryptamine.

Tryptamine may be formed in brain by the decarboxylation of tryptophan. The enzyme L-aromatic amino acid decarboxylase capable of effecting this conversion is present in brain (Lovengberg, Weissbach and Udenfriend 1962). The tryptamine may then be converted by monoamine oxidase and aldehyde dehydrogenase to indol-3-ylacetic acid. The failure to detect tryptamine in concentrations which can be estimated by the technique described may be due to its rapid destruction by amine oxidase, in the absence of any storage mechanism. Only when this enzyme is inhibited
by iproniazid, and under conditions of increased turnover along the tryptamine pathway, is there a rise in the amine to detectable levels.

The other possibility is that the tryptamine detected in brain is synthesized at other sites, transported in the plasma, and penetrates into brain across the blood-brain barrier. TEDESCHI, TEDESCHI and FELLOWS (1959) injected tryptamine intravenously into rats and noted the onset of convulsions and tremors. They found that these effects were potentiated in duration and severity by amine oxidase inhibitors, and postulated that tryptamine penetrated brain and stimulated 5-HT receptors. GREEN and SAWYER (1960) repeated this procedure and at the same time estimated tryptamine in brain. They were able to show increases in the brain concentration of tryptamine, following the intravenous administration of 5 mg/kg of the amine (0.173 ± 0.019 μg/g) (mean ± s.e.m.). However, each rat received at least 1000 μg of tryptamine by intravenous injection. The animals were killed 75 sec after this dose and it would seem probable that at this time the plasma–brain concentration gradient for tryptamine was extremely high. Under these conditions, it is not surprising the amine penetrated the blood–brain barrier. The concentration of tryptamine in blood was not, however, estimated during the course of these experiments. Penetration into brain, excluding active transport, would depend on lipid solubility and ionization at body pH, and on these considerations tryptamine should penetrate the blood–brain barrier.

Indol-3-ylacetic acid present in brain might well be an index of tryptamine turnover. In preliminary investigations, the acid has been detected in brain after tryptophan loading.

**SUMMARY**

The method of Hess and Udenfriend (1959) for the estimation of tryptamine in tissues, is demonstrated to allow the carry over of tryptophan to the final extract, resulting in falsely high values for the amine. A modification of the original method is described, incorporating ion-exchange chromatography, which gives complete separation of these components. Using this method, tryptamine is found neither in the brain of normal guinea pigs, nor in animals treated with L-tryptophan. It is found only when L-tryptophan is administered following pretreatment with an amine oxidase inhibitor.

**Acknowledgements**—We wish to thank Professor W. L. M. Perry for his help and advice and Miss Alice Urquhart for valuable technical assistance.

**REFERENCES**


SECTION III
THE FORMATION OF 5-HYDROXYTRYPTOPHOL IN BRAIN IN VITRO

BY

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Kveder, Iskric & Keglevic (1962) described the formation of 5-hydroxytryptophol from 5-hydroxytryptamine (5HT) in vivo in rats. The alcohol was isolated as conjugates from urine, and distinguished from 1'-N-acetyl 5-hydroxytryptamine by paper chromatography. Feldstein & Wong (1965) demonstrated the enzymatic conversion of 5HT to 5-hydroxytryptophol in rat liver. The alcohol appears also to be a major metabolite of the amine in platelets (Bartholini, Pletscher & Bruderer, 1964).

The isolation of the alcohol from bovine pineal gland by Mclsaac, Farrell, Taborsky & Taylor (1965) led us to attempt the synthesis of 5-hydroxytryptophol from 5HT in rat and subsequently human brain homogenates.

METHODS

Reagents

5-hydroxytryptamine creatinine sulphate was obtained from Koch-Light Ltd., Colnbrook, and 5-hydroxyindolacetic acid from Roche Products, Hertfordshire. The authenticated sample of 5-hydroxytryptophol was a gift from the National Institutes of Health, Bethesda.

Preparation of brain homogenates

Wistar strain rats of either sex weighing 180-220 g were used. The animals were killed by decapitation and the brain was quickly taken out. After removal of the cerebral hemispheres and cerebellum the remainder of the brain was homogenized in 25 vol. 0.25M sucrose at 4°; 1 ml. of the homogenate was preincubated in a metabolic shaking incubator for 15 min at 37°. The reaction was started by the addition of 80 μg of 5HT in 1 ml of 0.5M phosphate buffer, pH 7.4 (Feldstein & Wong, 1965), containing 500 μg of one of the pyridine nucleotide co-enzymes (nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide reduced (NADH2), nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide phosphate reduced (NADPH2)). The reaction was stopped at varying intervals by the addition of 0.1 ml conc. HCl. Human brain material obtained at necropsy from an adult female was examined in a manner similar to that described above—that is, samples of midbrain and cerebral cortex were dissected, homogenized in 25 vol. 0.25M sucrose at 4° and incubated with 5HT, etc.

Estimation of 5-hydroxytryptophol, 5-hydroxyindol-3-yl acetaldehyde and 5-hydroxyindol-3-yl acetic acid (5HIAA)

The analyses were a modification of the method reported by Feldstein & Wong (1965). Duplicate incubation mixtures were pooled and centrifuged at 3,000 rev/min for 7 min. After decantation the pH of the supernatant was adjusted to 7.4 (glass electrode) using 5N NaOH, and passed through
a 10 × 0.7 cm column of CG—50 ion-exchange resin (B.D.H. Ltd.) in the ammonium form at pH 7.4 prepared as described by Eccleston, Ashcroft, Crawford & Loose (1966). This took up the majority of the residual 5HT. The acid and neutral metabolites were washed through the column with 1 ml 0.02M pH 7.4 acetate buffer. The effluent was collected, salt saturated and acidified by the addition of 0.1 ml conc. HCl. The neutral and acidic components were then extracted into 20 ml freshly distilled peroxide-free diethyl ether. The acid component was back extracted into 5 ml salt-saturated 0.2M borate buffer, pH 10, and 1 ml of the extract was used for determination of the acid. To duplicate 1 ml aliquots of the buffer extract was added 3 ml N/10 H$_2$SO$_4$ and 2 ml conc. HCl containing ascorbic acid 100 mg/100 ml, for control estimations. The hydroxyacid was estimated in a Farrand spectrophotofluorimeter with the activation spectrum emission set at 550 m.$\mu$. An activation maximum is obtained at 295 m.$\mu$. After addition to the ether phase of 0.1 ml methanol containing 10 mg/ml ascorbic acid and 0.1 ml 20% (v/v) acetic acid, the ether was evaporated under a stream of nitrogen and the residue (about 0.1 ml) applied under nitrogen to Whatman No. 1 paper strips as described by Ashcroft, Eccleston & Crawford (1965). The chromatogram was developed in 8% (w/v) aqueous NaCl: glacial acetic acid, 100:1 by volume in an atmosphere of nitrogen. Initially, excess authentic 5-hydroxytryptophol was added to a sample to act as marker and the spot was developed with cinnamaldehyde reagent. Subsequently, the 5-hydroxyindoles were localized by cutting a 0.5 cm longitudinal strip from one of the paper chromatograms, spraying with acid acetone (conc. HCl:acetone 5% v/v), and viewing under ultraviolet light. 5-hydroxytryptophol separates from 5-hydroxyindolacetaldehyde under these conditions. The 5-OH indoles were assayed by cutting paper strips containing 5-hydroxytryptophol and 5-hydroxyindolacetaldehyde and eluting into 4 ml 0.1N sulphuric acid. The amount of

Fig. 1. Separation of 5-hydroxytryptophol and 5-hydroxyindolacetaldehyde by paper chromatography. A sample of the extract containing the neutral indole components was run on Whatman No. 1 paper in the solvent system 8% w/v aqueous NaCl: glacial acetic acid. 100:1. The indoles were assayed after elution from strips cut from the paper, as described in the text.
5-hydroxyindole present in each eluate was determined by spectrophotofluorimetry as described above.

The separation of the compounds was confirmed by taking 4 x 0.5 cm strips from the paper in the region between the alcohol and the aldehyde (determined under ultraviolet light), eluting into 4 ml. of 0.1N H$_2$SO$_4$ and estimating the 5-hydroxyindoles by spectrophotofluorimetry.

**Identification of 5-hydroxyindoles**

(a) Thin layer chromatography

Extracts of rat brain homogenates after incubation with 5HT and NADPH$_4$ and with the acidic 5-hydroxyindoles removed (as described), were developed in three solvent systems on thin layer plates of silica gel H (Merck) (300μ). The 5-hydroxyindoles were visualized by spraying first with acid acetone and observing under ultraviolet light, followed by spraying with cinnamaldehyde reagent. Comparisons of Rf were made against extracts containing additional authentic 5-hydroxytryptophol. Two major 5-hydroxyindole components were found among the neutral 5-hydroxyindole fractions on thin layer chromatograms. The first behaved like authentic 5-hydroxytryptophol. The second gave a brown colour reaction with the aldehyde spray reagent 2:4 dinitrophenylhydrazine (0.5 g in 100 ml. 2N HCl) and was presumed to be 5-hydroxyindolacetaldehyde. Chromatographic properties of the components are shown in Table 1. The fraction containing acidic 5-OH indoles (pH 10 buffer) was acidified to pH 1, salt-saturated and the acids were extracted into ether. The ethereal extract was taken to dryness after the addition of acetic acid and ascorbic acid, as described above. Chromatography on thin layers of Silica Gel H showed that the single component of the acidic fraction had an Rf value identical with that of authentic 5HIAA.

(b) Spectrophotofluorimetry

The two neutral components were found to have characteristic 5-hydroxyindole fluorescence during an excitation scan showing maximum fluorescence at 295 mμ when emission was set at 550 mμ. Authentic 5-hydroxytryptophol was found to have only 37.3% fluorescence when compared with an equimolar solution of 5HT. (83.3 μg/ml.)

(c) Spectral properties

5-hydroxytryptophol purified and isolated by paper partition chromatography from the neutral fraction of an extract made after incubation was eluted from the paper with spectrscopically pure ethyl alcohol. The ultraviolet spectrum of this solution was examined in a Unicam SP 800 recording spectrophotometer. The E$_{max}$ of this solution at 278 mμ was 5,950 and at 302 mμ, 4,140.

**Recoveries** (before identification)

Recoveries of 2.5, 5 and 10 μg of authentic 5-hydroxytryptophol taken through the procedure were low (57.2, 42.5 and 44.5%, respectively). 5-hydroxytryptophol was not detected in pH 10 buffer used to extract acids. 5HIAA, 10 μg, taken through the procedure gave a recovery of 50.6%. 5HIAA was not detected in the final paper chromatogram. 5HT did not interfere with the estimations at any stage.

**pH-Activity Curve**

Incubations were continued for 1 hr at 37° in 0.5M phosphate buffer at varying pH values from 5.4 to 9.4 (Fig. 2).

**Time course of formation of metabolites**

Using optimal pH and coenzyme conditions, incubations were continued over a 4 hr period to determine the curves for the time course of the formation of the 5-OH indole components (Fig. 3).
**Table 1**

Thin-Layer Chromatography on Silica Gel H of the Neutral Components in the Ether Phase

<table>
<thead>
<tr>
<th>Solvent System: Ethyl Acetate/Chloroform, 3:1 (v/v)</th>
<th>RF values of components</th>
<th>Acid/acetone reagent + ultraviolet inspection</th>
<th>Cinnamaldehyde reagent</th>
<th>2:4-dinitrophenylhydrazine reagent</th>
<th>Component identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.92</td>
<td>0.83</td>
<td>—</td>
<td>Light brown→ blue-grey on standing</td>
<td>Deep pink on standing</td>
<td>Yellow on standing</td>
</tr>
<tr>
<td>0.5</td>
<td>0.423</td>
<td>Yellow-green fluorescence</td>
<td>Pink</td>
<td>Pink</td>
<td>Urea?</td>
</tr>
<tr>
<td>0.15</td>
<td>0.292</td>
<td>Yellow-green fluorescence</td>
<td>Light blue</td>
<td>Faint brown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solvent System: Ethyl Acetate</th>
<th>RF values of components</th>
<th>Acid/acetone reagent + ultraviolet inspection</th>
<th>Cinnamaldehyde reagent</th>
<th>2:4-dinitrophenylhydrazine reagent</th>
<th>Component identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.94</td>
<td>—</td>
<td>Deep pink on standing</td>
<td>Yellow on standing</td>
<td>Unknown</td>
</tr>
<tr>
<td>0.89</td>
<td>Yellow-green fluorescence</td>
<td>Grey-blue</td>
<td>Brown</td>
<td>5-hydroxyindolacetaldehyde</td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>Yellow-green fluorescence</td>
<td>Grey-blue</td>
<td>Pale orange</td>
<td>5-hydroxytryptophol</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Yellow-green fluorescence</td>
<td>—</td>
<td>Orange</td>
<td>Unknown aldehyde?</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solvent System: iso-Propanol/Methyl Acetate/25% Ammonia, 35:45:20 (v/v)</th>
<th>RF values of components</th>
<th>Acid/acetone reagent + ultraviolet inspection</th>
<th>Cinnamaldehyde reagent</th>
<th>2:4-dinitrophenylhydrazine reagent</th>
<th>Component identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Faint yellow-green fluorescence</td>
<td>Purple</td>
<td>Yellow</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>0.925</td>
<td>Yellow-green fluorescence</td>
<td>Mauve</td>
<td>Brown</td>
<td>5-hydroxytryptophol</td>
<td></td>
</tr>
<tr>
<td>0.85</td>
<td>Yellow-green fluorescence</td>
<td>Blue-grey</td>
<td>Pale brown</td>
<td>5-hydroxyindolacetaldehyde</td>
<td></td>
</tr>
<tr>
<td>0.63</td>
<td>Faint yellow-green fluorescence</td>
<td>Pale purple</td>
<td>Yellow</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>0.47</td>
<td>Faint yellow-green fluorescence</td>
<td>Pale green</td>
<td>Yellow</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Faint</td>
<td>—</td>
<td>Yellow</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>
5-HYDROXYTRYPTOPHOL FORMATION IN BRAIN

Fig. 2. pH activity curve. 1 ml. samples of rat brain homogenate in 0.25M sucrose were incubated at 37° in the presence of 1 mg NADPH₂ and 80 μg 5HT in 1 ml. 0.5M phosphate buffer at the pH values shown. × = 5-hydroxyindolacetic acid, ○ = 5-hydroxytryptophol, ■ = 5-hydroxyindolacetaldehyde.

RESULTS

Enzymatic nature of the formation of 5-hydroxytryptophol

Heating the rat brain homogenate in 0.25M sucrose in a water bath at 100° for 30 min completely abolished its ability to produce 5-hydroxytryptophol from 5HT, even in the presence of excess NADPH₂.

Formation of 5-hydroxytryptophol, 5-hydroxyindolacetaldehyde, and 5HIAA in unfortified rat brain homogenates

The amounts of 5-hydroxytryptophol, 5-hydroxyindolacetaldehyde and 5HIAA formed from 5HT, by rat brain homogenates during one hour's incubation are given in Table 2. Without the addition of any exogenous co-factor, the formation of 5HIAA is markedly favoured and the intermediate metabolite, 5-hydroxyindolacetaldehyde, was also produced in considerable amounts.
**Table 2**

<table>
<thead>
<tr>
<th>Indole</th>
<th>5HIAA</th>
<th>5-hydroxyindolacetaldehyde</th>
<th>5-hydroxytryptophol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>60-32</td>
<td>20-86</td>
<td>1-66</td>
</tr>
</tbody>
</table>

Under such conditions, however, the rate of formation of 5-hydroxytryptophol was very low.

**Co-enzyme requirements of rat brain homogenates for the formation of 5-hydroxytryptophol**

The pyridine nucleotide requirements for the enzymatic reduction and oxidation of the intermediate 5-hydroxyindolacetaldehyde were investigated by the addition of the oxidized and reduced forms of the pyridine nucleotide co-enzymes to the incubation mixtures of rat brain homogenate and 5HT.

The results are shown in Table 3. The rat brain homogenate can utilize NADH₂ slowly for the formation of 5-hydroxytryptophol. Under these conditions, the rate of formation of the alcohol was about twice that in the absence of added co-enzyme. In contrast, 5-hydroxytryptophol formation was markedly stimulated by the addition of NADPH₂, indicating that the latter is the preferred co-enzyme.

**Table 3**

<table>
<thead>
<tr>
<th>Co-enzyme</th>
<th>5-Hydroxyindole</th>
<th>Incubation Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-Hydroxyindolacetic acid</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxytryptophol</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxyindolacetaldehyde</td>
<td>0</td>
</tr>
<tr>
<td>NADP</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxyindolacetic acid</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxytryptophol</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxyindolacetaldehyde</td>
<td>0</td>
</tr>
<tr>
<td>NADPH₂</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxyindolacetic acid</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxytryptophol</td>
<td>0</td>
</tr>
</tbody>
</table>
The addition of NADP to the brain homogenates increased the formation of 5HIAA to a greater extent than did the addition of NAD.

Formation of 5-hydroxyindole metabolites by rat brain homogenates with added NADPH₂

The formation of the three metabolites produced by rat brain homogenates from 5HT in the presence of excess NADPH₂ was followed separately for each component over a prolonged period and the results are given graphically in Fig. 3. These conditions favoured the formation of 5-hydroxytryptophol: the rates of formation of both 5HIAA and 5-hydroxytryptophol were fairly constant up to about 2 hr incubation. It appeared that 5-hydroxyindolacetaldehyde reached a steady state after about 30 min. Incubation for 2–3 hr produced maximum quantities of 5HIAA and 5-hydroxytryptophol, but periods of incubation in excess of 3 hr produced lower yields. This is thought to be due to the decomposition of these unstable compounds.

---

**Fig. 3.** Time curve for hydroxylating system. 1 ml. samples of rat brain homogenate in 0.25M sucrose were incubated at 37° C in the presence of 1 mg NADPH₂ and 80 μg 5HT in 1 ml. 0.5M pH 7.4 phosphate buffer. Samples removed at each time interval and assayed for 5-hydroxyindoles. X—X = 5-hydroxyindoleacetic acid, O—O = 5-hydroxytryptophol. •—• = 5-hydroxyindolacetaldehyde.
Use of aldehyde “trapping” agents

The addition of the aldehyde “trapping” agents, sodium metabisulphite, and hydroxylamine to the incubation mixtures resulted in an alteration in the relative yields of the 5-hydroxyindole metabolites of 5HT.

The proportion of 5-hydroxyindolacetaldehyde was increased in relation to both 5HIAA and 5-hydroxytryptophol. However, both metabisulphite and hydroxylamine, especially the latter, produced an inhibition of the overall reaction sequence (Table 4).

Table 4

<table>
<thead>
<tr>
<th>ALDEHYDE TRAPPING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation of 1 ml. rat brain homogenate in 0-25M sucrose with 80 µg 5HT in 1 ml. 0-5M phosphate buffer pH 7-4 and 1 mg. NADPH with the addition of aldehyde “trapping” agents—that is, sodium metabisulphite (0-12 mg/ml. final concn.) and hydroxylamine hydrochloride (20 µg/ml. final concn). Results expressed as µg 5-hydroxyindole formed/g (wet wt.) brain/hr.</td>
</tr>
<tr>
<td>The aldehyde oxime and sulphite compounds were decomposed by the addition of concn. hydrochloric acid (final HCl concentration, 1N) and warmed at 55°C for 10 min before extraction procedure. The 5-hydroxyindole compounds were assayed as in the Methods section.</td>
</tr>
<tr>
<td>Control (No “trapping” agent added)</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
</tr>
<tr>
<td>Hydroxylamine Hydrochloride</td>
</tr>
</tbody>
</table>

Reversibility of the oxidation-reduction reactions

The use of 5-hydroxytryptophol and 5HIAA as substrates for rat brain homogenates showed that the oxidation of 5-hydroxyindolacetaldehyde to 5HIAA was initially non-reversible, whereas the reduction of 5-hydroxyindolacetaldehyde to 5-hydroxytryptophol was reversible if excess NADP was present.

Formation of 5-hydroxytryptophol, 5-hydroxyindolacetaldehyde and 5HIAA by human brain homogenates

Table 5 gives the results of incubation of sucrose homogenates of human brain samples with 5HT and excess pyridine nucleotide co-enzyme. The samples were taken from midbrain and cortical areas, and although they were not removed from the brain until about 3 hr after death they showed a very high capacity to form 5-hydroxytryptophol. The rate of formation of 5HIAA was lower than in rat brain homogenates under similar conditions. NADPH₂ was, like rat brain, the preferred co-enzyme for the reduction of 5-hydroxyindolacetaldehyde. Cortex showed a much higher activity than midbrain for the overall system.

Test for pharmacological activity of 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol

Chromatographically purified 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol were added (final bath concentrations 87 ng/ml. and 180 ng/ml. respectively) to a rat
5-HYDROXYTRYPTOPHOL FORMATION IN BRAIN

Table 5

FORMATION OF 5HIAA, 5-HYDROXYINDOLACETALDEHYDE, AND 5-HYDROXYTRYPTOPHOL BY HOMOGENATES OF TISSUE FROM HUMAN MIDBRAIN AND CORTEX

1 ml. samples of human brain homogenates were incubated for 1 hr at 37° in the presence of 1 mg NADH₂ or NADPH₂ and 80 ng 5HT in 1 ml. 0.5M phosphate buffer pH 7.4. Results expressed as μg 5-hydroxyindole formed/g brain (wet wt.)/hr.

<table>
<thead>
<tr>
<th>Co-enzyme</th>
<th>NADH₂</th>
<th>NADPH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Hydroxy-indole</td>
<td>5-Hydroxy-indolacetic acid</td>
<td>5-Hydroxy-indolacetaldehyde</td>
</tr>
<tr>
<td>Area of brain</td>
<td>10-35</td>
<td>5-95</td>
</tr>
<tr>
<td>Midbrain</td>
<td>15-63</td>
<td>65-63</td>
</tr>
<tr>
<td>Cortex</td>
<td>82-70</td>
<td>5-51</td>
</tr>
</tbody>
</table>

Activity was not observed with either compound. Under the same conditions, 5HT (final bath concentration 2.5 ng/ml.) produced a maximal contraction.

DISCUSSION

The formation of 5HIAA from 5-HT via 5-hydroxyindolacetaldehyde in guinea-pig liver preparations was shown by Weissbach, Redfield & Udenfriend (1957) to be effected by a NAD-dependent aldehyde dehydrogenase. McIsaac & Page (1959) found a further metabolite in rat urine following the administration of 5HT, which they postulated to be 1'-N-acetyl-5-hydroxytryptamine. This was confirmed by Weissbach, Lovenberg, Redfield & Udenfriend (1961) who found a lower output of the metabolite in the urine.

Kveder et al. (1962) argued that in the metabolism of some amines, intermediate alcohols could be formed; they therefore re-examined the urine of rats injected with radioactively labelled 5HT. They were able to isolate and characterize the glucuronide of 5-hydroxytryptophol as one of the major urinary metabolites of 5HT and suggested that the similar chromatographic behaviour of 1'-N-acetyl-5-hydroxytryptamine and 5-hydroxytryptophol led to confusion of the compounds.

Feldstein & Wong (1965) showed that rat liver homogenates were capable of converting 5HT into 5-hydroxyindolacetaldehyde, 5HIAA and 5-hydroxytryptophol. They suggested liver as the major site for the conversion of 5HT to 5-hydroxytryptophol. However, urinary 5-hydroxytryptophol could also be partly derived from brain and the present experiments have confirmed this possibility and have shown that both rat and human brain homogenates possess the enzymatic mechanism to convert 5HT to 5-hydroxytryptophol, 5HIAA and 5-hydroxyindolacetaldehyde.

The synthesis of 5-hydroxytryptophol in rat and human brain is effected by an alcohol dehydrogenase which has a much higher affinity for NADP/NADPH₂ than for NAD/NADH₂. Feldstein & Wong (1965) found NADH₂ to facilitate the formation of 5-hydroxytryptophol by liver alcohol dehydrogenase, and NAD to facilitate the liver aldehyde dehydrogenase for the formation of 5HIAA. The present experiments show that the aldehyde dehydrogenase of brain has similar co-enzyme requirements for both NAD and NADP, but the specific requirements of brain alcohol dehydrogenase for
NADP/NADPH₂ distinguish this enzyme from the corresponding liver enzyme. These differences in co-enzyme specificity have a parallel in liver and retinal alcohol dehydrogenases (Zachman & Olson, 1961; Futterman, 1963).

The pharmacological significance of the complete pathway involving the formation of 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol is not understood. Neither compound exhibits the activity of 5HT on the rat's uterus but, since the equilibrium of the oxidation-reduction phases can be altered by the availability of NADP or NADPH₂, situations in which this ratio can be altered by the use of drugs or other agents may be important to brain function. In mammary gland and liver slices, ethanol affects the activity of NADP-dependent enzymes of the pentose phosphate shunt pathway (Beaconsfield & Reading, 1964). A similar situation may occur with the brain 5-hydroxytryptophol-synthesizing system.

The results of adding aldehyde "trapping agents" to the incubation system strongly support the idea that 5-hydroxyindolacetaldehyde, the primary product of monoamine oxidase action on 5HT, is the branching point for the two pathways of reduction and/or oxidation. That such a process takes place in brain is of special importance, since an inhibition of either of the branches might cause a local increase in the concentration of 5HT.

The pH optimum for the complete system appears to be in the region of pH 7 to 8, and the involvement of NADP/NADPH₂ in the formation of 5-hydroxytryptophol suggests that the reduction takes place in the soluble fraction of the cytoplasm, whereas the oxidation reaction to 5HIAA, which requires either NAD or NADP, could also take place in the particulate fractions associated with the mitochondria. Since the system has so far only been examined in homogenates, in which the normal intracellular spatial arrangements are destroyed, the extent to which the pathway functions in vivo is still an open question. However, the present experiments have demonstrated the existence of the necessary enzymes in brain tissue to produce 5-hydroxytryptophol and 5-hydroxyindolacetaldehyde, and that the activity of these pathways, at least in vitro, depends on the NADP/NADPH₂ ratio.

**SUMMARY**

1. Human and rat brain homogenates convert 5-hydroxytryptamine into 5-hydroxytryptophol and 5-hydroxyindolacetic acid via 5-hydroxyindolacetaldehyde.

2. The pH optimum for the system lies between pH 7 and pH 8.

3. The formation of 5-hydroxytryptophol is effected by an alcohol dehydrogenase which requires reduced nicotinamide adenine dinucleotide phosphate as coenzyme. Without added coenzyme, the rate of formation of 5-hydroxytryptophol is very low.

4. The coenzyme requirements for the brain alcohol dehydrogenase differ from those of the corresponding liver enzyme.

5. The rate of formation of 5-hydroxyindolacetic acid by brain homogenates is increased by both nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. However, without the addition of coenzyme the brain homogenate still forms 5-hydroxyindolacetic acid in moderate quantity.
6. Neither 5-hydroxyindolacetaldehyde nor 5-hydroxytryptophol showed pharmacological activity on the rat's uterus when compared with 5-hydroxytryptamine.

The authors are grateful to Professor W. L. M. Perry for his helpful advice and criticism and to Dr. V. O. Marquis for carrying out the pharmacological test.

REFERENCES


5-Hydroxytryptamine metabolism in brain and liver slices and the effects of ethanol

(Received 2 August 1968)

FELDSTEIN, HOAGLAND, WONG and FREEMAN (1964) reported that the ingestion of ethanol, following the administration of 5-HT caused a marked reduction in the excretion of 5-HIAA*. In a more recent series of experiments, DAVIS, BROWN, HUFF and CASHAW (1967) described alterations in the pattern of 5-HT metabolism following ingestion of ethanol in man. Ethanol ingestion, 1 hr prior to the administration of [14C]5-HT either orally or intravenously, reduced by one-half the percentage of [14C] excreted in the urine as 5-HIAA, whilst at the same time the percentage of [14C] excreted as 5-hydroxytryptophol and its conjugates rose from approximately 2 to 42 per cent. The major proportion of the 5-hydroxytryptophol was excreted in the form of a glucuronide and sulphate conjugates.

The formation of 5-hydroxytryptophol from 5-HT by rat liver homogenates was demonstrated by FELDSTEIN and WONG (1965) and this was followed by the experiments of ECCLESTON, MOIR, READING and RITCHIE (1966) who showed that a similar enzymic pathway exists in human and rat brain tissue. Since these studies were carried out on tissue homogenates, it was thought advisable to investigate the formation of 5-hydroxytryptophol from 5-HT in tissue slices, together with an appraisal of the effects of ethanol on these pathways in slices.

Experimental

Wistar strain rats of either sex, weighing 180–220 g were killed by decapitation and the brains removed as rapidly as possible. After removal of the cerebellum, the cerebral hemispheres were divided, and tissue slices made on a modified McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey). Slices of approximately 0.5 mm thickness and 55–65 mg (wet wt.) were cut and added directly to 25 ml conical flasks containing incubation medium without 5-HT substrate.

Liver slices were made from similar rats by removing the liver and after washing with water, excess water was removed by blotting. About 1 cm² portions were cut from the centre of a liver slice, and 55–65 mg (wet wt.) slices cut, using a Stadie–Riggs microtome. These slices were added directly to incubation medium as described above for brain.

Reagents used for incubation media, or for estimation purposes were of ‘Analar’ grade unless otherwise stated. 5-HT was obtained from British Drug Houses, Poole, Dorset, 5-HIAA from Roche Products, Hertfordshire and 5-hydroxytryptophol from the Regis Chemical Co., Chicago. Incubation medium consisted of Krebs–Ringer bicarbonate pH 7.4 (DAWSON, ELLIOT, ELLIOT and JONES, 1959). The final volume/tissue ratio was always 2 ml medium to 55–65 mg tissue slice. Glucose solution was added to the incubation medium to give a final concentration of 0.12%. Where necessary, ethyl alcohol was added at the commencement of incubation to give the final concentrations shown in the tables of results; all samples for incubation were done in triplicate.

After an equilibration period of 5 min in a shaking incubator, 40 μg of 5-HT (in 0.2 ml water) were added to each flask followed by a rapid gassing with 95% oxygen/5% carbon dioxide mixture before the flasks were finally stoppered. Incubations were continued for 1 hr at 37°, whereupon triplicate samples were pooled and medium and slices were homogenized in an all glass Potter-type homogenizer and the homogenate added to 2 ml 10% zinc sulphate in a glass centrifuge tube. The homogenizer was washed out with 1 ml 0.2 M-ammonium acetate pH 7.4 and the washings added to the homogenate. After thorough mixing, 0.2 ml 20% NaOH was added to the mixture and after re-mixing, each sample was centrifuged at 2000 g (4500 rev./min) for 10 min. After decantation, 3 ml portions of the supernatant from each sample were added to 1 ml 0.2 M-ammonium acetate pH 7.5, the pH of the final mixture being checked with a glass electrode and was between pH 7.4 and 7.5. Separation of the various indolalkylamine components were effected on a 7 × 70 mm column of C.G. 50, ion-exchange resin (B.D.H. Ltd) of 100–200 mesh in the ammonium form prepared as described by ECCLESTON, ASHCROFT, CRAWFORD and LOOSE (1966). This took up the 5-HT, whilst the acid and neutral metabolites passed through the column and were completely eluted by washing the column with 1 ml 0.02 M-ammonium acetate pH 7.5. This effluent was collected and assayed for 5-HIAA, 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol by a slight modification of the

* Abbreviations used: 5-HIAA = 5-hydroxyindolyacetic acid.
method previously described (ECCLESTON et al., 1966). In the present experiments, 5-HIAA was back-extracted from the ether phase into 3 ml salt-saturated 0·5 M-borate buffer pH 10. Two millilitre samples of the borate buffer extract were diluted to 4 ml with 2 ml 0·1 N-sulphuric acid and 2 ml conc. HCl containing ascorbic acid (1 mg/ml) were added prior to estimation in a Farrand spectrophotofluorimeter.

To estimate 5-HT remaining on the ion exchange resin, the column was first washed with (a) 9 ml 0·02 M-ammonium acetate pH 7·5, then (b) 4 ml 0·1 N-sulphuric acid. These washings were discarded. The 5-HT was eluted from the column in 6 ml N-sulphuric acid. Samples (1 ml) of this eluate were diluted to 4 ml with water and 2 ml conc. HCl containing 0·1 mg/ml ascorbic acid added immediately before estimation in the spectrophotofluorimeter, activation maximum set at 295 μ, with emission maximum set at 550 μ.

Recovery determinations were carried out during each experiment, by adding 40 μg 5-HT, 5 μg 5-HIAA and 5 μg 5-hydroxytryptophol to each of three non-incubated (but tissue containing) control samples immediately before homogenization etc. The pooled sample was extracted exactly as described for the incubation material and the percentage recovery of each 5-hydroxyindole determined for calculation of the final proportions.

RESULTS

Table 1 shows the rates of formation of 5-HIAA, 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol in rat brain slices. The maximum rate of 5-hydroxytryptophol production occurred in the presence of ethanol at a concentration of 10⁻³ M. The mean differences between10⁻³ M-ethanol and brain slices.

Table 1.—Rates of formation of 5-HIAA, 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol in rat brain slices

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>5-hydroxytryptophol</th>
<th>5-hydroxyindolacetaldehyde*</th>
<th>5-HIAA</th>
<th>% 5-HT used</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻³ M</td>
<td>36·5 (± 4·1)</td>
<td>30·4 (± 6·9)</td>
<td>72·9 (± 1·8)</td>
<td>44·3</td>
</tr>
<tr>
<td>10⁻² M</td>
<td>43·4 (± 3·5)</td>
<td>35·9 (± 7·6)</td>
<td>81·5 (± 7·2)</td>
<td>55·0</td>
</tr>
<tr>
<td>10⁻¹ M</td>
<td>36·9 (± 5·4)</td>
<td>31·4 (± 5·8)</td>
<td>85·9</td>
<td>56·7</td>
</tr>
<tr>
<td>10⁻² M</td>
<td>40·6</td>
<td>53·8</td>
<td>66·4</td>
<td>72·0</td>
</tr>
<tr>
<td>Control</td>
<td>33·0 (± 4·1)</td>
<td>34·0 (± 6·9)</td>
<td>78·4 (± 5·3)</td>
<td>56 (± 8·1)</td>
</tr>
<tr>
<td>Recovery</td>
<td>61·5 (± 4·2)</td>
<td>—</td>
<td>93·3 (± 7·1)</td>
<td>69·4 (± 7·7)</td>
</tr>
</tbody>
</table>

Results expressed as μg 5-hydroxyindole/g (wet wt.) brain/hr. Mean values of experiments with s.e.m. in brackets.

* Expressed as units of 5-hydroxytryptophol.

and the control showed a low degree of significance (P < 0·1). In the entire series of experiments with brain preparations it did not appear that the rate of formation of 5-HIAA was altered. The percentage of 5-HT utilized decreased with increasing concentration of ethanol.

Evidence of possible conjugation occurring in brain tissue was investigated by carrying out similar slice experiments in which the samples after incubation were assayed for 5-hydroxyindoles before and after acid hydrolysis (1 hr at pH 1 with HCl under nitrogen is scaled ampoules). No increase in the amount of 5-hydroxytryptophol was found after hydrolysis when slices were incubated with 5-HT in the presence or absence of 10⁻³ M-ethanol. Small increases amounting to about 3–7 per cent were found in the amount of 5-HIAA after hydrolysis, the lower figure being obtained in the absence of ethanol, the higher figure, in the presence of ethanol.

Table 2 shows the pattern of 5-HT metabolism in rat liver slices incubated under the same conditions as brain slices. In this case the presence of ethanol in the incubation medium induced

Table 2.—Rates of formation of 5-HIAA, 5-hydroxyindolacetaldehyde and 5-hydroxytryptophol in rat liver slices

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>5-hydroxytryptophol</th>
<th>5-hydroxyindolacetaldehyde*</th>
<th>5-HIAA</th>
<th>% 5-HT used</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻¹ M</td>
<td>236·6</td>
<td>2·2</td>
<td>42·7</td>
<td>76</td>
</tr>
<tr>
<td>10⁻² M</td>
<td>176·4</td>
<td>8·8</td>
<td>45·2</td>
<td>74</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>86·2</td>
<td>35·8</td>
<td>71·6</td>
<td>69</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>27·3</td>
<td>36·6</td>
<td>92·9</td>
<td>68</td>
</tr>
<tr>
<td>No EtOH</td>
<td>16·0</td>
<td>36·7</td>
<td>128·8</td>
<td>78</td>
</tr>
<tr>
<td>Recovery</td>
<td>83</td>
<td>—</td>
<td>110</td>
<td>55</td>
</tr>
</tbody>
</table>

Results expressed as μg 5-hydroxyindole/g (wet wt.) brain/hr. Expressions as units of 5-hydroxytryptophol.
profound changes in the rates of formation of all the 5-hydroxyindole metabolites of 5-HT. At the highest concentration of ethanol (10\(^{-1}\) m) the rate of formation of 5-hydroxytryptophol was increased about 15 times above the normal, whilst at the same time 5-HIAA formation was depressed to about one third the normal rate. These changes were reflected in the amounts of 5-hydroxyindolacetaldehyde detected; where the rate of formation of 5-hydroxytryptophol was high, the corresponding aldehyde must have had a very high rate of turnover, so that only small quantities of it were detected.

**DISCUSSION**

It would appear that ethanol in relatively high concentration has no appreciable effect on the rate of utilization of 5-HT in the liver, in contrast to the effect demonstrated in brain slices. This is possibly due to the liver being adapted to cope with much higher concentrations of alcohols without overtly affecting its overall metabolic rate. It is surprising that the rate of utilization of 5-HT was lowered by ethanol concentrations of 10\(^{-1}\) and 10\(^{-2}\)m in brain slices since McIlwain (1966) states that no inhibition in respiration of separated cerebral tissues occurs until ethanol concentrations of the order of 8 \(\times\) 10\(^{-1}\)m are reached, and Wikler (1964) has recorded that blood alcohol concentrations of 3–5 \(\times\) 10\(^{-4}\)m can be attained in normal subjects without effects on the functioning of the nervous system.

However, the effect of ethanol on the rate of utilization of 5-HT in the different tissues may have a more subtle physiological significance and may be related to the coenzyme requirements for the alcohol dehydrogenases responsible for the oxidation of ethanol and the formation of 5-hydroxytryptophol. The coenzyme requirements for the latter enzyme have previously been worked out (Eccleston et al., 1966) and it appears that the dehydrogenase catalysing the formation of 5-hydroxytryptophol in cerebral tissues has a specific requirement for reduced NADP, whilst in liver requires reduced NAD. Alcohol dehydrogenase catalysing the oxidation of ethanol in the liver requires NAD and it is possible that the alcohol dehydrogenase or dehydrogenases of cerebral tissues have a specific requirement for NADP and/or a substrate affinity for ethanol which is much lower than that for the liver enzyme. The fact that alcohol dehydrogenases of differing substrate and coenzyme specificities exist between central nervous and peripheral tissues has been established by the work of Koen and Shaw (1966) who demonstrated such differences between enzymes present in the retina and liver. Futterman (1963) earlier demonstrated similar different coenzyme specificities for retinal and liver enzymes. It is certainly well known that ethanol is not oxidized at an appreciable rate in brain tissue (McIlwain, 1966) so it is unlikely that ethanol administration will affect to any great extent the ratios of the oxidized to reduced pyridine nucleotide coenzymes in brain. Unless adequate reduced NADP is present in brain, very little enzymic formation of 5-hydroxytryptophol can take place. The marked differences in 5-HT metabolism between liver and brain slices brought about by ethanol are therefore understandable.

Evidence from the present experiments would suggest that the marked changes in the pattern of excretion of 5-HIAA and 5-hydroxytryptophol caused by the administration of ethanol to human subjects described by Davis et al. (1967) and other workers, are almost entirely due to metabolic effects occurring in the liver and possibly other peripheral organs such as gut. This raises the question of the significance of such effects on central nervous activity since it is unlikely that changes in 5-HT metabolism occur in the CNS, and there is no evidence on the passage of 5-hydroxytryptophol across the blood–brain barrier. It remains to be seen whether systemic administration of ethanol causes increases in 5-hydroxytryptophol in central structures.

**Acknowledgement—**The authors wish to thank Mr. R. Loose of the Pharmacology Department, Edinburgh University, for details of the method of estimation of 5-HT.

**REFERENCES**


SECTION IV
Release of 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid in the forebrain by stimulation of mid-brain raphé

By D. Eccleston, A. Padjen and M. Randić. M.R.C. Unit for Research in Brain Metabolism, Department of Pharmacology, University of Edinburgh, and Institute ‘Rudjer Boskovic’, Zagreb, Yugoslavia

Recently, we have shown that stimulation of the mid-brain raphé in cats consistently increased the efflux of 5-hydroxytryptamine (5-HT) but not 5-hydroxyindol-3-ylacetic acid (5-HIAA) from the cerebral cortex (Eccleston, Randić, Roberts & Straughan, 1969). The latter finding was surprising since Aghajanian, Rosecrans & Sheard (1967) had shown that the mid-brain raphé stimulation produces an increase in 5-HIAA and a decrease in 5-HT content of the rat’s forebrain. It would be reasonable to expect that the increase in the forebrain 5-HIAA level should have been reflected in the cup fluid. To clarify this discrepancy, we have repeated our experiments in rats and have attempted to correlate the efflux of 5-HIAA from the cerebral cortex with the changes in the levels of 5-HT and 5-HIAA in the forebrain produced by mid-brain stimulation. In addition, we have investigated the nature of the transfer of 5-HIAA from the brain into the cups by using probenecid.

Adult male rats were lightly anaesthetized with urethane and a bipolar stimulating electrode was placed stereotaxically into the mid-brain raphé, in both experimental and control rats, at A (ant.), 0-4 mm; L (lat.), 0-0 mm; H (horiz.), −2-6 mm. Cups containing 0-25 ml. of Krebs solution were placed on to the pial surface of the cortex and samples were removed at 30 min intervals either before or during stimulation (6 V monophasic pulses of 2 msec duration at 10/sec for 60 and/or 120 min periods). The 5-HIAA in the cup fluid was estimated by the method of Ashcroft & Sharman (1962). At the end of the experiment the rats were killed and the 5-HT and 5-HIAA content of the forebrain measured spectrophotofluorimetrically.

We found that the 5-HIAA concentration in rat brain was about 0-32 µg/g and that there was a spontaneous efflux of 5-HIAA into the cortical cup fluid (0-3 ng/cm²/min). Probenecid (200 mg/kg) produced a fourfold increase in 5-HIAA content of the rat’s forebrain, but the efflux of 5-HIAA into the cup fluid remained unchanged. This finding suggested that 5-HIAA is probably transferred from the brain into the cups by some active transport mechanism. Stimulation of the mid-brain raphé produced a significant increase in the forebrain content of 5-HIAA as in the experiments by Aghajanian et al. (1967). In these preliminary experiments this increase was accompanied by a definite rise in the 5-HIAA content of the...
cups. In contrast with the finding of Aghajanian et al. (1967) we have observed that the concentration of 5-HT either did not change or even showed some slight increase in the forebrain with stimulation. This latter finding suggests that biosynthesis of 5-HT may be increased by stimulation.

REFERENCES

Long Term Effects of Midbrain Stimulation on 5-Hydroxyindole Synthesis in Rat Brain

The association between brain monoamines and nerve terminals has been interpreted as evidence of a neurotransmitter function. It has been shown that serotonin (5-hydroxytryptamine, 5HT) is localized in fine neurones which project to the forebrain and whose cell bodies lie in the raphé nuclei of the midbrain. The transmitter hypothesis has been further substantiated by the finding that stimulation of these nuclei increases forebrain levels of 5-hydroxyindol-3-ylacetic acid (5HIAA), the metabolite of 5HT, and increases the release of 5HT from brain into cortical cups. Using the rate of formation of 5-hydroxyindoles after tryptophan administration in vivo as an index of the rate of hydroxylation of tryptophan, the limiting step in the synthesis of 5HT, stimulation has been shown not only to increase release of 5HT but also to accelerate the rate of production of the amine (unpublished observations of D. Eccleston, M. Randic and A. Padjen). This could arise from immediate electrical effects on membrane permeability with changes in transport of substrate to existing enzyme. We have examined whether the increased rate of amine production continues after stimulation has ceased, which would suggest that the stimulation caused enzyme induction.

250 g male rats were anaesthetized with 1 per cent fluothane in oxygen and a concentric bipolar stainless steel electrode was inserted into the raphé nuclei at AP +0.5; L 0.0; H −2.6. Stimulation with 3–6 V, 2 ms square wave pulses at 10 s⁻¹ was applied to the raphé for 1 h. Although an electrode was implanted in all animals, half of these served as non-stimulated controls. Fifteen minutes after the end of the stimulation period 800 mg/kg of L-tryptophan was administered intraperitoneally and the animals were killed at 0, 15, 30 or 60 min later. The forebrain was immediately removed and frozen at −20 °C, and concentrations of 5HT and 5HIAA were estimated within 24 h of obtaining the samples.

The administration of tryptophan led to an increase in the concentration of 5HT in the forebrain of both groups of animals (Fig. 1a), but this was not significantly different between the two groups of animals. The concentration of 5HIAA was significantly greater ($P < 0.01$) in the rats 15 min after the stimulation period. The increase of
Fig. 1. Concentrations of 5HT (a) and 5HIAA (b) in rat forebrain in two groups of animals, one of which has a 60 min period of stimulation by an electrode implanted in the raphé nuclei, ending 15 min before the injection of L-tryptophan (800 mg/kg). The control group had implanted electrodes without stimulation.
5HIAA proceeds linearly during the first hour (Fig. 15) at a rate of 5.6 ng/g/min in the stimulated animals. This was a significantly greater rate than the 3.0 ng/g/min in the unstimulated controls ($P < 0.05$).

Clearly, raphe stimulation leads to an increase in the rate of production of 5-hydroxyindoles which considerably outlasts the period of stimulation, and which is probably the result of an induction of the hydroxylating enzyme. This could be the result of a number of factors: increase in formation or decrease in destruction of the enzyme, increase in activation, and so on. If stimulation brings about an increase in functional enzyme protein, then activation of this monoamine system must have a regulatory role in the synthesis of 5HT. The increased formation of 5HT may, in itself, facilitate transmission. Depending on the turnover rate of the hydroxylating enzyme, this control of synthesis by stimulation should bring about changes which are observable over fairly long periods of time. The significance of this long term course would be understandable if the raphe neurones function in a tonic manner in relation to other neurophysiological systems. The identification of these long term effects may be a clue to the cause of the delay amounting to several days in the response to antidepressant drugs in man.

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EFFECTS OF ELECTRICAL STIMULATION OF RAT MIDBRAIN ON 5-HYDROXYTRYPTAMINE SYNTHESIS AS DETERMINED BY A SENSITIVE RADIOISOTOPE METHOD

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Abstract—5-Hydroxytryptamine (5-HT) synthesis has been determined in the rat brain by measuring the 5-[3H]HT formed from [3H]tryptophan in the presence of monoamine oxidase inhibitor. Electrical stimulation in the region of the midbrain raphe nucleus increased formation of 5-[3H]HT by over 100 per cent, although the level of endogenous 5-HT and the concentration and specific activity of tryptophan were unchanged; the results are interpreted in terms of a two-compartment model. The optimum stimulation parameters were determined. Three days after a single dose of the tryptophan hydroxylase inhibitor p-chlorophenylalanine, stimulation increased 5-HT synthesis by the same percentage as in untreated animals. It was also found that after the end of an hour's stimulation, synthesis returned to control values in under an hour. These results suggest that the rise in synthesis of the amine on stimulation is not due to induction of tryptophan hydroxylase, but more likely to an increase in the activity of existing enzyme.

Several methods have been used to measure 5-HT turnover in the rat brain; these include the rate of accumulation of the amine after monoamine oxidase (MAO) inhibition (NEFF and TOZER, 1968), catabolism of intracisternally-injected 5-HT (SIMMONDS, 1970), administration of loading doses of L-tryptophan to saturate tryptophan hydroxylase, the rate-limiting enzyme (ASHCROFT, ECCLESTON and CRAWFORD, 1965) and the application of steady-state kinetics to the accumulation of 5-[14C]HT from [14C]tryptophan infused intravenously (LIN, COSTA, NEFF, WANG and NGAI, 1969).

All of these methods have disadvantages. Measurement of synthesis by the rate of accumulation of the amine after MAO inhibition assumes that the drug acts immediately; the use of intracisternally-injected 5-HT is unsatisfactory as it may enter cells where it is normally absent (SIMMONDS, 1970) and in the case of tryptophan loading, a true indication of turnover is not obtained because the 5-hydroxyindole acetic acid (5-HIAA) which accumulates is constantly being transported out of the brain. The method of LIN et al. (1969) comes the closest to normal physiological conditions, but has the practical disadvantage that whenever 5-HT turnover has to be measured under different conditions, such as when a drug is given, the curve of tryptophan specific activity in plasma has to be determined as its shape may alter.

We have developed a simple method of measuring relative rates of 5-HT synthesis in rat brain which overcomes most of these problems. It involves the administration of an MAO inhibitor (pargyline) followed by [3H]tryptophan and, after a fixed length of time, the amount of 5-[3H]HT synthesized is measured. We have used this method to investigate the effects of electrical stimulation in the rat midbrain on synthesis of

¹ P.J.S. is an M.R.C. Scholar.

Abbreviations used: PCP, p-chlorophenylalanine; MAO, monoamine oxidase; 5-HIAA, 5-hydroxyindole acetic acid; PPO, 2,5-diphenyloxazole; POPOP, p-bis-[2-(5 phenyloxazolyl)]-benzene; r, coefficient of linear correlation.
5-HT. Stimulation of the raphe nuclei increases synthesis of the amine (Sheard and Aghajanian, 1968) and this increased synthesis has been reported to persist for some time after the end of stimulation (Eccleston, Ritchie and Roberts, 1970). In the latter report, maximal tryptophan hydroxylase activity was measured by giving a loading dose of tryptophan and, as under these conditions very high concentrations of tryptophan are reached in brain, the hydroxylase enzyme will be saturated. Thus, different results might be obtained from normal physiological conditions where it is not saturated (Ashcroft et al., 1965). Using the method described here, the long term effects of stimulation on 5-HT synthesis were investigated, as well as the different effects of various stimulation parameters.

**MATERIALS AND METHODS**

Male albino Wistar rats weighing 150-200 g were used. Pargyline hydrochloride (Abbott Laboratories) was injected intraperitoneally as a 30 mg/ml solution in saline. [3H]Tryptophan (generally labelled, activity 500-1000 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, and was given intraperitoneally in saline.

**Measurement of 5-HT.** Whole brains were homogenized in 5 ml of 0-4 n-perchloric acid containing 2 mg/ml ascorbic acid and centrifuged at 15,000 g for 10 min. The supernatants were adjusted to pH 7-5 with KOH, and after cooling to 4°C and removal of precipitated KClO₄ by centrifugation, were passed over 70 x 5 mm columns of Amberlite CG50 resin (100-200 mesh, NH₄⁺ form), the effluents being collected. The columns were washed with 15 ml 0.02 N-ammonium acetate buffer, pH 7-5, followed by 4 ml 0.1 N-H₂SO₄. 5-HT was eluted with 6 ml 1.0 N-H₂SO₄, with a recovery of 86 per cent. Radioactive 5-HT was measured by liquid scintillation counting of 1 ml of the eluate in 10 ml Triton X-100 scintillator (1000 ml toluene, 500 ml Triton X-100, 4g 2,4-diphenyloxazole (POPOP). 0.1 g p-bis-[2-(5 phenyloxazolyl)]-benzene (POPOP). Endogenous 5-HT was measured in a Perkin-Elmer fluorimeter at 300 nm in a recorded activation spectrum and 530 nm fluorescence, after the addition of 1 ml conc. HCl containing 2 mg/ml ascorbic acid to 2 ml of eluate.

**Identification of 5-HT.** In samples from animals which had received [3H]Tryptophan, 5-HT was identified as the only radioactive substance in the eluate by descending paper chromatography (Whatman, No. 1) in chloroform-methanol-0.88 ammonia (12:7:1, by vol.). The paper was cut into 1 cm strips, the strips eluted into 1 ml 0.1 N-H₂SO₄ and the radioactivity in each eluate determined by liquid scintillation. A sample of authentic 5-HT was run in parallel and located by spraying with Ehrlich's reagent.

**Measurement of tryptophan in brain.** The effluents from the CG50 columns were adjusted to pH 2-2 with HCl and passed over 50 x 5 mm columns of Dowex AG 50W-X4 resin (K⁺ form, 200-400 mesh). The columns were washed with 10 ml water followed by 5 ml 0.1 N-potassium phosphate buffer pH 6.5. Tryptophan was eluted with 20 ml of the same buffer, with a recovery of 89 per cent. Radioactive tryptophan was measured by liquid scintillation counting and endogenous tryptophan fluorimetrically by the method of Hess and Udenfriend (1959), as applied by Guroff and Udenfriend (1962).

**Electrical stimulation.** Rats were anaesthetised with 1-6 per cent halothane in oxygen 15 min before stimulation, and a stainless-steel concentric bipolar electrode (0.4 mm diameter, tip separation 0.6 mm) was positioned in the brain using a David Kopf No. 1530 stereotaxic frame. The position of the electrode tip was in the midline, 0.4 mm anterior to the interaural line, with the upper incisor bar set 2-4 mm below this line. The vertical position of the tip was 2.3 mm above the interaural line, except where stated otherwise. Electrode placements were controlled histologically in 13 animals by taking serial frozen sections and staining with toluidine blue. In some experiments, rats were allowed to recover after stimulation and in these cases nonrupturing ear bars (David Kopf, No. 855) were used. The scalp wound was closed with a Michel clip after withdrawal of the electrode. Animals' temperatures were maintained at 37-38°C by an electric heating blanket during stimulation and by a 200 W infra-red lamp on recovery. During stimulation, anaesthesia was maintained with 0.8 per cent halothane. Electrical stimulation was by 2 ms constant-current pulses alternating in polarity, at 0.2 mA and 10 s⁻¹, except where stated otherwise. Control animals had electrodes implanted but received no stimulation.

**RESULTS**

**Synthesis of 5-[3H]HT from [3H]tryptophan in the presence of pargyline.** Animals received pargyline (150 mg/kg) followed 15 min later by [3H]tryptophan (100 μCi/kg).
The level of 5-[3H]HT at various times after tryptophan injection is shown in Fig. 1. Figure 2 shows the amount of radioactivity in the Amberlite CG50 effluent at various times; all of this will not be [3H]tryptophan because of in vivo exchange of the tritium label: purification of the effluent on Dowex columns showed that 30 min after tryptophan injection, 48 per cent of the total radioactivity behaved as [3H]tryptophan. This figure agrees with the findings of Schubert, Nyback and Sedvall (1970). However, there was a close correlation (r = 0.98 over eight results) between the CG50 effluent counts and the [3H]tryptophan levels, so the effluent counts may be used as an index of the amount of [3H]tryptophan present in the brain. Figure 1 shows the rise of 5-[3H]HT is approximately linear during the first 30 min; thus, the method adopted to measure synthesis was to give pargyline 15 min before tryptophan and kill the animal 30 min after. There was a high correlation between the 5-[3H]HT synthesised and the CG50 effluent counts (r = 0.95 over 44 results). Therefore, to eliminate variations due to differences in the amounts of [3H]tryptophan taken up by the brain, the final results are expressed as the ratio of 5-[3H]HT d.p.m. to CG50 effluent d.p.m.

Effect of p-chlorophenylalanine (PCP). To show that the radioactivity measured as 5-HT was derived through tryptophan hydroxylase, the enzyme was inhibited with PCP (dimethyl ester), 150 mg/kg given intraperitoneally, 24 and 6 h previously to a
group of three animals. 5-HT synthesis was reduced to 14.5 per cent of the mean value of three controls.

Effects of midbrain stimulation. Stimulation in the region of the median raphe nucleus caused a large rise in 5-HT synthesis: the effects of different current strengths are shown in Table 1. The effects of varying the stimulation frequency are shown in Table 1—Effect of stimulation at different currents

<table>
<thead>
<tr>
<th>Current (mA)</th>
<th>Ratio ($\times 10^3$)</th>
<th>5-HT d.p.m./CG50 effluent d.p.m.</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32.7 ± 4.9 (9)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>49.7 ± 9.9 (3)*</td>
<td>152.0</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>70.5 ± 16.3 (9)t</td>
<td>215.7</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>82.2 ± 10.1 (3)t</td>
<td>251.6</td>
<td></td>
</tr>
</tbody>
</table>

Pargyline was given 15 min, and [3H]tryptophan 30 min after the beginning of stimulation, which was continued until the animals were killed 30 min later.

Results show mean ± s.d., with the number of animals in brackets.

* P<0.05.

† P<0.001 compared to controls (Student’s t test).

Fig. 3, the greatest rise in synthesis being at 10 s⁻¹. A greater rise in synthesis on stimulation was found with the electrode 0.9 mm above the median raphe nucleus rather than actually within it (Table 2). No effect was obtained by stimulating in the dorsal raphe nucleus. Thus, the parameters adopted for subsequent stimulation experiments were: electrode vertical co-ordinate $V = +2.3$ mm, frequency 10 s⁻¹ and current 0.2 mA. Histological examination showed that after 1 h stimulation at this current, very little tissue damage had occurred.

Effects of stimulation on tryptophan and endogenous 5-HT. Stimulation did not alter either the concentration or the specific activity of tryptophan in brain (Table 3). The level of endogenous 5-HT was also unaltered.

![Graph](image-url)

**Fig. 3.—5-HT synthesis at different stimulation frequencies. Experimental protocol is as in the footnote to Table 1. Vertical bars show s.d., with the number of observations in brackets. * P<0.025; ** P<0.001 (Student’s t test).**
Midbrain stimulation and 5-HT synthesis

Table 2.—Effect of stimulation at different electrode positions

<table>
<thead>
<tr>
<th>Vertical coordinate of electrode (V)*</th>
<th>Location</th>
<th>Ratio (× 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1·6 mm</td>
<td>Median raphe nucleus</td>
<td>45·5 ± 9·4 (7)†</td>
</tr>
<tr>
<td>2·5 mm</td>
<td></td>
<td>59·0 ± 17·7 (7)†</td>
</tr>
<tr>
<td>3·5 mm</td>
<td>Dorsal raphe nucleus</td>
<td>32·9 ± 8·0 (7)</td>
</tr>
<tr>
<td>2·5 mm (Unstimulated controls)</td>
<td></td>
<td>33·6 ± 4·2 (7)</td>
</tr>
</tbody>
</table>

Current 0·2 mA, frequency 10 s⁻¹. Protocol as in footnote to Table 1. Results show mean ± s.d., with the number of animals in brackets.

† P < 0·01 compared to controls.

Mechanism of increased 5-HT synthesis. To determine whether the increased 5-HT synthesis on stimulation was due to a rapid increase of enzyme or increased activity of existing enzyme, stimulation was given 3 days after a single dose of PCP (270 mg/kg). At this time, the drug has been virtually eliminated from the brain, but the activity of tryptophan hydroxylase is still low due to irreversible inhibition (KOE and...)

Table 3.—Effect of stimulation on brain tryptophan and endogenous 5-HT

<table>
<thead>
<tr>
<th>Group</th>
<th>Tryptophan (μg/g)</th>
<th>Tryptophan specific activity (d.p.m./ng)</th>
<th>% of CG50 effluent d.p.m. as [3H]tryptophan</th>
<th>5-HT (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated</td>
<td>5·9 ± 1·0 (4)</td>
<td>1·65 ± 0·3 (4)</td>
<td>51 ± 7 (4)</td>
<td>0·73 ± 0·04 (9)</td>
</tr>
<tr>
<td>Control</td>
<td>6·4 ± 0·7 (4)</td>
<td>1·63 ± 0·4 (4)</td>
<td>48·4 ± 4 (4)</td>
<td>0·74 ± 0·05 (9)</td>
</tr>
</tbody>
</table>

Results show mean ± s.d., with the number of animals in brackets and were measured in the 0 and 0·2 mA groups in Table 1.

WEISSMAN, 1968). After administration of PCP, the rate of 5-HT synthesis was a quarter of normal and stimulation increased it by the same percentage as in animals which had not received the drug (Table 4).

Long term effects of stimulation. 5-HT synthesis was measured in animals which had been allowed to recover from anaesthesia after an hour's stimulation. The results

Table 4.—Effect of stimulation following PCP administration

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio (× 10³)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8·0 ± 2·7 (3)</td>
<td>100</td>
</tr>
<tr>
<td>Stimulated</td>
<td>17·4 ± 5·4 (4)*</td>
<td>218</td>
</tr>
</tbody>
</table>

Animals received a single dose of PCP (270 mg/kg) 3 days before stimulation. Other protocol as in footnote to Table 1. Results show mean ± s.d., with the number of animals in brackets. * P < 0·05 compared to controls.
Stimulated

Control

0 2
0 1 2 3
Time of killing (hrs)

Fig. 4.—5-HT synthesis during and after stimulation. Animals received pargyline 45 min and [3H]tryptophan 30 min before killing. The curve shows the value of 5-HT d.p.m./effluent d.p.m. as a percentage of the initial point. Each point is the mean of four or more observations. Vertical bars show s.d.

(Fig. 4) show that synthesis returns to normal rates very soon after the end of stimulation.

To see whether continuous anaesthesia would produce a different result, a group of animals were kept anaesthetized for the whole duration of the experiment; otherwise they received the same treatment as the stimulated 2-5 h group in Fig. 4. The result was identical with that of this group.

DISCUSSION

The method presented for the measurement of 5-HT synthesis avoids most of the drawbacks of other methods. It appears to give a good indication of tryptophan hydroxylase activity when tested in situations known to produce changes in hydroxylation: synthesis is considerably reduced by inhibition of the enzyme with PCP, and increased by raphe stimulation. The relative increase in synthesis on stimulation was greater than the increase of 5-HIAA reported by SHEARD and AGHAJANIAN (1968) and thus this method could be useful as a particularly sensitive indicator of the rate of formation of the amine. MAO inhibition is apparently without effect on synthesis during the first hour: there is a linear rise of the amine for at least 60 min and the measurement of synthesis from the rate of accumulation of endogenous 5-HT gives a similar result to other methods (NEFF and TOZER, 1968; LIN et al., 1969). One report, however, shows that the accumulation of 5-HT after MAO inhibition does reduce its synthesis after 3 h (MACON, SOLOKOFF and GLOWINSKI, 1971).

Electrical stimulation increased 5-HT synthesis, though it is surprising that whereas the amount of 5-[3H]HT made rose by over 100 per cent, there was no significant change in the level of the endogenous amine. These results are comparable to those of SHEARD and AGHAJANIAN (1968), who found that in animals given an MAO inhibitor, stimulation increased 5-HT by only 10 per cent, compared to a rise of 80
Midbrain stimulation and 5-HT synthesis

per cent in 5-HIAA without the inhibitor. Also, Meek and Fuxe (1971) report that factors altering the activity of 5-HT-containing neurons in the spinal cord fail to affect the rate of accumulation of 5-HT after MAO inhibition.

Our results on the effect of varying the frequency of stimulation are in accord with those of Sheard and Aghajanian (1968), though it is surprising that no effect was obtained here on stimulating the dorsal raphe nucleus.

The results can be interpreted in terms of the following model (Fig. 5). There is a large pool of 5-HT where its synthesis is relatively insensitive to its level, where the amine accumulates after MAO inhibition. A second, small pool is available for release by the nerve impulse and its synthesis is closely controlled by its content of 5-HT. After release of the amine by the nerve impulse, the level of the small pool falls even though MAO is inhibited, as not all of the released 5-HT is taken back up into this pool. Some may be taken up into the large pool and some may be taken up by glial cells. Alternatively, this small pool could represent 5-HT whose synthesis is controlled by nervous activity through depolarization or local changes in ion concentrations activating the hydroxylase enzyme in a manner similar to that seen for adenyly cyclase in brain (Shimizu, Creveling and Daly, 1970).

The large pool could be represented by storage vesicles and the small one by membrane-bound 5-HT, which would thus be readily available for release by the nerve impulse. Alternatively, the small pool might be represented by a population of vesicles which are associated with the release of the amine: for the noradrenergic neuron it has been postulated that these vesicles may exocytose their contents, re-form and then resynthesize or take up noradrenaline for further release (Iversen and Bloom, 1970).

To explain why radioactive, but not overall, 5-HT synthesis rises on stimulation, it is also necessary to postulate that the 5-HT in the small pool is synthesized from tryptophan which has recently been taken up into the nerve, i.e. tryptophan which in these experiments, has a high specific activity. In peripheral adrenergic nerves, it has been found that noradrenaline synthesized from newly-taken up labelled tyrosine is preferentially released (Kopin, Breese, Krauss and Weise, 1968; Sedvall, 1968). It is not necessary to postulate two physically separate tryptophan pools in the nerve ending; the 5-HT in the small pool may be synthesized from tryptophan just as it is taken up, when, in this case, its specific activity would be particularly high. The

![Diagram of 5-HT synthesis and storage in rat brain](image-url)
The subcellular distribution of tryptophan hydroxylase has not yet been completely defined, but it seems that it is largely cytoplasmic, and that some may be membrane bound (Lovenberg, Jequier and Sjoerdsma, 1968).

The mechanism of increased synthesis on stimulation is probably one of increased activity of existing tryptophan hydroxylase rather than induction of new enzyme. This is indicated by the effect of stimulation after reduction of the enzyme activity by PCP. In this case, stimulation increased 5-HT synthesis in the same proportion as in animals which had not received the drug; if stimulation increased synthesis by increasing the amount of enzyme, a much larger rise would be expected.

We suggested above that 5-HT synthesis in the small pool might be subject to a sensitive feedback mechanism and that the synthesis of the amine going into this pool was increased when the pool was decreased by electrically-induced release. After PCP treatment, however, the synthesis of 5-HT to this pool would be reduced, and the fall in the level of the amine should increase the activity of the hydroxylase enzyme which is still functional. Therefore, further depletion of the pool by stimulation would be expected to result in little increase in synthesis, whereas in fact it still more than doubles. This suggests that increased synthesis on stimulation may well be mediated through an ionic or electrical mechanism.

Although stimulation did not alter overall brain tryptophan either in specific activity or absolute levels, local concentration changes of the amino acid at the enzyme site caused by its redistribution could also be important. Tryptophan levels have a large effect on 5-HT synthesis rates, the enzyme being unsaturated under normal conditions (Ashcroft et al., 1965).

We were unable to confirm the long-term effects of stimulation found by Eccleston et al. (1970). The essential difference is that in the latter report, a large dose of L-tryptophan was given 15 min after the end of stimulation and this may have sustained the increase in tryptophan hydroxylase activity by another mechanism.

REFERENCES

EVIDENCE FOR THE SYNTHESIS AND STORAGE OF 5-HYDROXYTRYPTAMINE IN TWO SEPARATE POOLS IN THE BRAIN

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Abstract—Evidence is presented to support the hypothesis that 5-hydroxytryptamine (5-HT) in the rat brain is synthesized by two separate pathways and stored in two or more compartments. Lysergic acid diethylamide in doses down to 50 μg/kg was shown to reduce the formation of 5-[3H]HT from [3H]tryptophan in the presence of a monoamine oxidase inhibitor, although the total rate of accumulation of 5-HT was unchanged. Conversely, adrenalectomy was found to increase the total synthesis of 5-HT measured in the same way, although the amount of 5-[3H]HT formed suggested that there was no increase in the synthesis of the amine. In a third experiment it was found that electrical stimulation of 5-HT-containing nerves following labelling of 5-HT stores with [3H]tryptophan led to a biphasic disappearance of 5-[3H]HT. It is suggested that the method of measuring 5-HT synthesis by measuring 5-[3H]HT formed from [3H]tryptophan in the presence of a monoamine oxidase inhibitor may be a way of selectively measuring the turnover of the functional pool of 5-HT.

There is now good evidence that the biogenic amines are stored in more than one pool in their respective neurons. In the case of noradrenaline (NA) in peripheral nerves, this is suggested by the biphasic accumulation of the amine by the rat heart (Iversen, 1963) and by several studies involving release of NA by tyramine in normal and reserpinized preparations (Potter and Axelrod, 1963; Crout, Muskus and Trendelenburg, 1962; Chidsey and Harrison, 1963). Evidence for the existence of 'functional' and 'reserve' pools of NA is also provided by the work of Kopin, Breese, Krauss and Weise (1968), who showed that [3H]NA newly synthesized from [3H]tyrosine was preferentially released. In the brain, a two-pool storage of dopamine is suggested by the results of Besson, Cheramy, Feltz and Glowinski (1969), who showed that isolated rat striata incubated in vitro with [3H]tyrosine preferentially released newly synthesized [3H]dopamine. It seems possible that 5-HT too is stored in more than one compartment in 5-HT neurons in the brain. In a previous study (Shields and Ecclestone, 1972) we showed that electrical stimulation of 5-HT-containing neurons in the rat brain increased synthesis of 5-[3H]HT from [3H]tryptophan in the presence of a monoamine oxidase inhibitor, without, however, significantly increasing the total rate of accumulation of 5-HT. These results suggested that 5-HT was stored in the brain in at least two pools, one of which was quantitatively small, but whose synthesis responded rapidly to changes in the firing rate of the neurons. It was therefore postulated that this small pool was situated at the nerve ending and represented that part of the 5-HT which was functionally active, being released by the nerve impulse. 5-HT in the second, larger pool was postulated to be synthesized by a separate

Abbreviations used: LSD, lysergic acid diethylamide; NA, noradrenaline; r, coefficient of linear correlation.
pathway which was not controlled by nervous activity. In the present report further evidence is presented to support this hypothesis.

MATERIALS AND METHODS

LSD was supplied by Sandoz Ltd. Methods were as published previously (Shields and Eccleston, 1972), with the modification that 5-[^3]H)HT was measured by dissolving 2 ml CG50 eluate in 19 ml Triton X-100 scintillator fluid with the addition of 1 ml water. Adrenalectomy (bilateral) was performed under halothane anaesthesia. On recovery, the rats were maintained on a 0.9% saline-5% glucose solution and standard rat cake. Sham-operated controls were given water to drink. The adrenalectomized animals continued to gain weight after the operation. The significance of the difference between the slopes of two linear regressions was tested by the Student t-test as described by Davies (1949). Results for 5[^3]H)HT synthesis are expressed as Ratio (x 10^3) 5[^3]H)HT d.p.m./CG50 column effluent d.p.m., as described previously (Shields and Eccleston, 1972).

RESULTS

Effect of LSD. Lysergic acid diethylamide (LSD) reduces the firing rates of neurons in the raphe nuclei, where the cell bodies of most 5-HT-containing nerves are located (Foote, Sheard and Aghajanian, 1969). Therefore as a counterpart to our previous work where stimulation of these nerves was shown to increase 5-HT synthesis (Shields and Eccleston, 1972), the effect of LSD on the synthesis of the amine was tested. The method for measuring 5-HT synthesis was as described previously (Shields and Eccleston, 1972), though with altered timing. Rosecrans, Lovell and Freedman (1967) showed that after intraperitoneal injection of LSD, the level of the drug in the brain was maximal after only 10 min and then fell rapidly. Thus rats were given pargyline (a monoamine oxidase inhibitor) and LSD in the same injection, followed after 6 min by l-[^3]H)tryptophan. They were killed after a further 15 min. This schedule should have given a maximal concentration of LSD in the brain while 5[^3]H)HT was being synthesized from [^3]H)tryptophan. The results are shown in Table 1. LSD reduced the synthesis of 5[^3]H)HT, but did not significantly reduce the total rate of accumulation of 5-HT, except for a small reduction at the lowest dose of the drug. There was also no significant alteration in the rate of uptake of tryptophan by the brain, as judged by the CG50 column effluent radioactivity (which was shown previously (Shields and Eccleston, 1972) to correlate highly with the[^3]H)tryptophan

<table>
<thead>
<tr>
<th>Dose of LSD (µg/kg, i.p.)</th>
<th>Ratio (x 10^3) 5-HT d.p.m./CG50 effluent d.p.m.</th>
<th>CG50 effluent d.p.m. (x 10^-3)</th>
<th>5-HT† (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.7 ± 1.7 (14)</td>
<td>62.5 ± 4.5 (14)</td>
<td>0.50 ± 0.01 (14)</td>
</tr>
<tr>
<td>50</td>
<td>22.5 ± 2.4 (10)*</td>
<td>74.7 ± 5.5 (10)</td>
<td>0.46 ± 0.01 (10)*</td>
</tr>
<tr>
<td>100</td>
<td>17.4 ± 2.8 (11)*</td>
<td>70.5 ± 7.3 (11)</td>
<td>0.49 ± 0.01 (11)</td>
</tr>
<tr>
<td>250</td>
<td>19.9 ± 2.3 (8)†</td>
<td>72.3 ± 5.6 (8)</td>
<td>0.49 ± 0.01 (8)</td>
</tr>
</tbody>
</table>

Animals received pargyline (150 mg/kg, i.p.) and LSD followed after 6 min by l-[^3]H)tryptophan (200 µCi/kg, i.p.) and were killed after a further 15 min. Results show mean ± s.e.m., with numbers of observations in brackets.

* Significantly lower than control, P < 0.025.
† Significantly lower than control, P < 0.005 (one-tailed t-test).
‡ The 5-HT level in a group of untreated animals was 0.26 ± 0.04 µg/g (5).
Two-pool storage and synthesis of 5-HT

It is possible that the reduced synthesis of 5-[3H]HT was simply due to an increase in the level of endogenous tryptophan in the brain, which would have reduced the specific activity of the precursor [3H]tryptophan. This is unlikely, however, as the total 5-HT synthesis, which is very dependent on the level of tryptophan (Moir and Eccleston, 1968), was unchanged by the drug. A second experiment to confirm this point showed that a dose of LSD as high as 1 mg/kg had no significant effect on the level or specific activity of tryptophan in the brain (Table 2), while at the same time the synthesis of 5-[3H]HT was reduced.

Table 2.—Effect of a dose of 1 mg/kg of LSD on 5-HT synthesis and tryptophan levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio (×10³)</th>
<th>CG50 effluent d.p.m. (×10⁻³)</th>
<th>5-HT (μg/g)</th>
<th>Tryptophan (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.2 ± 2.5</td>
<td>70.6 ± 5.7</td>
<td>0.44 ± 0.01</td>
<td>3.61 ± 0.50</td>
</tr>
<tr>
<td>LSD</td>
<td>15.6 ± 2.5*</td>
<td>71.5 ± 4.4</td>
<td>0.46 ± 0.02</td>
<td>3.55 ± 0.64</td>
</tr>
</tbody>
</table>

Results show means ± s.e.m. of 10 observations.
* Significantly lower than control, P < 0.05. Other details as in footnote to Table 1.

Effect of adrenalectomy. Tryptophan hydroxylase in the brain appears to be controlled to some extent by adrenal steroid hormones (Azmitia, Algeri and Costa, 1970; Azmitia and McEwen, 1969; Green and Curzon, 1968). We therefore studied the effect of adrenalectomy on brain 5-HT synthesis, to see whether in these circumstances the synthesis of the amine would separate into two components. At either 10 or 20 days after bilateral adrenalectomy, 5-HT synthesis in the brain was measured as described by Shields and Eccleston (1972). The results are shown in Tables 3 and 4. In both cases there was a significant rise in the total rate of accumulation of

Table 3.—Synthesis of 5-HT 10 days after adrenalectomy

<table>
<thead>
<tr>
<th>Treatment ...</th>
<th>Sham-operated</th>
<th>Adrenalectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT (μg/g)</td>
<td>0.25 ± 0.01 (3)</td>
<td>0.23 ± 0.01 (3)</td>
</tr>
<tr>
<td>Rate of rise of 5-HT (μg/g/h)</td>
<td>0.27</td>
<td>0.40*</td>
</tr>
<tr>
<td>Ratio (×10³)</td>
<td>5-HT d.p.m./ CG50 effluent d.p.m.</td>
<td>3.50 ± 0.4 (5)</td>
</tr>
<tr>
<td>Tryptophan (μg/g)</td>
<td>5.94 ± 0.7 (5)</td>
<td>3.81 ± 0.39 (5)</td>
</tr>
</tbody>
</table>

Animals received pargyline (150 mg/kg, i.p.) followed after 15 min by L-[3H]tryptophan (100 μCi/kg, i.p.), and were killed after a further 30 min. Some animals were killed without treatment. Results show means ± s.e.m., with numbers of observations in brackets.
* Significantly different from control, P < 0.05. Differences between adrenalectomized and sham-operated groups were tested by a two-tailed t-test.
Table 4.—Synthesis of 5-HT 20 days after adrenalectomy

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham-operated</th>
<th>Adrenalectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment ...</td>
<td>None</td>
<td>Pargyline + [3H]tryptophan</td>
</tr>
<tr>
<td>5-HT (μg/g)</td>
<td>0.41 ± 0.01 (4)</td>
<td>0.71 ± 0.02 (5)</td>
</tr>
<tr>
<td>Rate of rise of 5-HT (μg/g/h)</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Ratio (×10³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT d.p.m./CG50 effluent d.p.m.</td>
<td>44.6 ± 9.3 (5)</td>
<td>40.1 ± 3.2 (5)</td>
</tr>
<tr>
<td>(×10⁻³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG50 effluent d.p.m. (×10⁻³)</td>
<td>33.9 ± 2.1 (5)</td>
<td>33.6 ± 2.8 (5)</td>
</tr>
<tr>
<td>Tryptophan (μg/g)</td>
<td>6.04 ± 0.17 (5)</td>
<td>5.20 ± 0.38 (5)</td>
</tr>
</tbody>
</table>

Pargyline was given 30 min before the [3H]tryptophan. Other details as in footnote to Table 3.

5-HT, whereas the results obtained using the radioactive tracer suggested that there was no significant alteration in the synthesis of the amine. Tryptophan levels were not significantly changed by the adrenalectomy; neither was the amount of [3H]tryptophan taken up changed, as judged by the CG50 effluent radioactivity.

Depletion of 5-[3H]HT by stimulation. Stimulation of the raphe nuclei increases the rate of 5-HT metabolism (Sheard and Aghajanian, 1968; Shields and Eccleston, 1972). If, as we have suggested, there is a functional pool of the amine which is

![Fig. 1](image.png)

**Fig. 1.**—Effect of stimulation on 5-[3H]HT levels following injection of [3H]tryptophan (100 μCi/kg, i.p.). Stimulation was begun 60 min after injection. There is a significant difference between the initial group and the 75 min stimulated group \((P < 0.005, t\)-test\), and between the stimulated and control groups at 120 min \((P < 0.05)\). The difference between the stimulated and control groups at 75 min approaches significance \((0.05 < P < 0.1)\). Vertical bars show S.E.M.; numbers of observations are given in brackets.
Two-pool storage and synthesis of 5-HT

Table 5.—Level and specific activity of 5-HT during stimulation

<table>
<thead>
<tr>
<th>Time from beginning of stimulation (min)</th>
<th>Controls</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT specific activity (d.p.m./μg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2965 ± 249 (12)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3123 ± 928 (14)</td>
<td>1886 ± 346 (14)*</td>
</tr>
<tr>
<td>60</td>
<td>1954 ± 326 (9)</td>
<td>1804 ± 383 (9)</td>
</tr>
</tbody>
</table>

5-HT level (μg/g)  
15 0-33 ± 0-01 (8)  0-29 ± 0-02 (8)  
60 0-36 ± 0-02 (6)  0-26 ± 0-02 (6)†

Animals received L-[3H]tryptophan (100 μCi/kg, i.p.) 60 min before the beginning of stimulation. Results show means ± s.e.m., with numbers of observations in brackets.

* Significantly lower than zero time group, $P < 0.025$.
† Significantly lower than zero time and 60 min control groups, $P < 0.005$ (one-tailed t-test).

released by the nerve impulse and is selectively labelled by [3H]tryptophan, stimulation should give a rapid release of 5-[3H]HT following labelling of the stores in this way. Thus rats were given [3H]tryptophan and the median raphe nucleus stimulated, starting 60 min after the injection. The level of 5-[3H]HT is shown in Fig. 1, and the corresponding levels of endogenous 5-HT and 5-HT specific activity are shown in Table 5. In the stimulated animals, there was a significant fall in the level and specific activity of 5-[3H]HT during the first 15 min of stimulation. The fall in endogenous 5-HT over the same period was not significant. At 60 min after the start of stimulation, the 5-HT specific activity was not significantly different in stimulated and control groups. As there was a high correlation between 5-[3H]HT and [3H]tryptophan levels in the brain ($r = 0.75$ over 58 results, $P < 0.001$ by t-test), the 5-[3H]HT values were corrected for variations in brain tryptophan levels. This was done by multiplying by the ratio: [3H]tryptophan ex/[3H]tryptophan, where [3H]tryptophan ex was the [3H]tryptophan level expected from a standard curve of its time course in brain after intraperitoneal injection (Fig. 2).

Fig. 2.—[3H]Tryptophan level in rat brain at various times after intraperitoneal injection of 100 μCi/kg L-[3H]tryptophan. Points are mean values of three or four determinations; bars show s.e.m.
DISCUSSION

The results presented here lend further support to the concept of two independent units for the synthesis and storage of 5-HT in the rat brain. In the presence of LSD, synthesis of 5-[3H]HT from [3H]tryptophan was considerably reduced, whereas the total synthesis of the amine was unaltered, as judged by its rate of accumulation in the presence of the monoamine oxidase inhibitor (Tozer, Neff and Brodie, 1966). The results show that the reduction in 5-[3H]HT formation was not due to a fall in the specific activity of precursor tryptophan. The lack of effect of the drug on the brain tryptophan level does differ, however, from the findings of Tonge and Leonard (1970), who found an increase in tryptophan after LSD. Our results suggest therefore that the pool of 5-HT which is labelled must be quantitatively insignificant compared to the other pool(s). This result parallels our previous findings, where raphe stimulation increased synthesis of 5-[3H]HT but did not affect the total amine synthesis. As the synthesis of the amine in the small pool appears to reflect the electrical activity of the neurons, this pool probably represents that fraction of the amine which is functionally active, that is, which is released by the nerve impulse. A similar situation probably exists in peripheral adrenergic nerves, as shown by the results of Crout et al. (1962): they found that exposing reserpinised guinea-pig atria to NA restored their response to tyramine to 70 per cent of normal, though the amount of NA taken up was only 2 per cent of the normal content. LSD has previously been shown to reduce 5-HT turnover (Rosecrans et al., 1967; Lin, Ngai and Costa, 1969; Schubert, Nybäck and Sedvall, 1970; Andén, Corrodi, Fuxe and Hökfelt, 1968) but in no case was any effect found with a dose of under 250 μg/kg, whereas Foote et al. (1969) found a reduction in raphe firing at doses of only 10 μg/kg. Probably no biochemical response has been detected with low doses because the concentration of the drug in the brain rises and then falls rapidly after systemic administration (Rosecrans et al., 1967); with the method used here, which had a relatively short duration, an effect was apparent at lower doses, down to 50 μg/kg.

In the case of adrenalectomy, total 5-HT synthesis was increased, but the radioactive results suggested that the synthesis of the amine was not changed. This discrepancy is unlikely to have been due to a fall in the specific activity of the precursor tryptophan in the adrenalectomized animals. If anything, the results suggest an increase in the specific activity. As the levels of tryptophan in the brain were not increased in the adrenalectomized animals, the increased 5-HT synthesis may have been due to increased levels of tryptophan hydroxylase. The lack of an increase in 5-[3H]HT synthesis may reflect the ability of the functional pool to regulate its synthesis under varying conditions, although the explanation could also be that more tryptophan hydroxylase enzyme was not present in this pool. Reports vary as to the effect of adrenalectomy on brain 5-HT metabolism. We have shown an increase in amine synthesis, and also brain 5-HIAA levels have been found to double 22 days after the operation (Ashcroft, unpublished observations). These findings are supported by those of Green and Curzon (1968), who showed that hydrocortisone injections reduced 5-HT and 5-HIAA levels in the rat brain. On the other hand, a reduced activity of tryptophan hydroxylase after adrenalectomy has been found in vivo (Azmitia et al., 1970) and in vitro (Azmitia and McEwen, 1969). Also Lovenberg (1972) reported recently that adrenalectomy had no effect on tryptophan hydroxylase activity in vitro. Stimulation of the raphe nucleus after labelling of the 5-HT stores
with \[^{3}H\]tryptophan gave a rapid fall in the level and specific activity of 5-\[^{3}H\]HT during the first 15 min of stimulation. During the next 45 min, however, the 5-HT specific activity fell only slightly. These results indicate that at the time of beginning stimulation, which was 60 min after injection of \[^{3}H\]tryptophan, the 5-\[^{3}H\]HT was present in two pools, one of which was released by the nerve impulse. The comparatively slow decline of the 5-HT specific activity over the latter 45 min of stimulation represented the turnover of the nonfunctional pool. It appears that at the start of the stimulation period, about 40 per cent of the 5-\[^{3}H\]HT was present in the functional pool and this fraction was utilized within 15 min. At the end of the stimulation period, the specific activity of 5-HT was not significantly different in stimulated and control animals, showing that in the controls, the 5-\[^{3}H\]HT in the functional pool had all been metabolized by this time.

A similar situation has been described by Kopin et al. (1968), where stimulation of the cat spleen perfused with \[^{14}C\]tyrosine led to the release of \[^{14}C\]NA with a specific activity greater than that found in the tissue. This too demonstrated the existence of a functional pool of a monoamine with a higher specific activity than the whole tissue. The biphasic disappearance of 5-\[^{3}H\]HT in the stimulated animals is comparable to the biphasic decay of 5-\[^{14}C\]HT seen after injection of this substance into the ventricles (Eccleston, unpublished observations); similar findings have also been reported after intraventricular injection of radioactively-labelled NA (Glowinski, Kopin and Axelrod, 1965). A biphasic disappearance of 5-\[^{3}H\]HT was apparent in our experiments only in the stimulated animals and not in the controls probably due to the continued synthesis of the labelled amine (Neff, Spano, Groppetti, Wang and Costa, 1971), which is not the case when labelled amine is injected intraventricularly. The evidence presented provides strong support for the hypothesis that there are not only two or more storage pools of 5-HT in the brain, but also that these pools receive amine synthesized by separate pathways. This has an important bearing on the choice of methods used to study the rate of 5-HT synthesis in the brain. Many methods used to measure this assume there is only one 5-HT compartment, and a quantitatively negligible pool could well go unnoticed. The ideal method for the assessment of functional activity of these neurons would be one selective for the functional pool—it is suggested that the method we have described might be useful in this respect.

Acknowledgements—We are grateful to Dr. G. W. Ashcroft for performing the adrenalectomies. P. J. Shields thanks the Medical Research Council for a training scholarship.

REFERENCES

THE ACCUMULATION OF C\textsuperscript{14}-SEROTONIN IN THE GUINEA-PIG VAS DEFERENS

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ABSTRACT

THOA, NGUYEN B., DONALD ECCLESTON and JULIUS AXELROD: The accumulation of C\textsuperscript{14}-serotonin in the guinea-pig vas deferens. J. Pharmacol. Exp. Ther. 169: 68–73, 1969. C\textsuperscript{14}-serotonin can be taken up by guinea-pig vas deferens tissue after an incubation in vitro. The accumulation is temperature-dependent and saturable and is blocked by cocaine, imipramine and ouabain, drugs known to interfere with the active transport of norepinephrine into the nerve cells, as well as by norepinephrine itself. The indoleamine appears to accumulate in the same intraneuronal sites which store endogenous norepinephrine. About half of the accumulated C\textsuperscript{14}-serotonin is slowly released from the vas deferens within two hours. This release is enhanced by drugs which release norepinephrine, such as reserpine, tyramine, dopamine and norepinephrine. These observations suggest that uptake and storage in the sympathetic nerve terminals of peripheral tissues may not be specific processes for norepinephrine only, but for other normally occurring amines as well.

After an injection of C\textsuperscript{14}-5-hydroxytryptophan, radioactivity presumably due to serotonin has been shown by autoradiographic techniques to accumulate in sympathetic neurons of the rat vas deferens (Taxi and Droz, 1966). The fact that the sympathetic neurons of the vas deferens could take up and store serotonin prompted us to study the in vitro uptake and release of radioactive serotonin in the isolated vas deferens of guinea pigs and the effect of norepinephrine and other drugs on these two processes. A preliminary report of this work has been published (Thoa et al., 1967).

METHODS. Male albino guinea pigs weighing 300 to 400 g were killed by a blow on the head and bled by severing both carotid arteries. Their vasa deferentia were rapidly removed, freed of mesenteric attachment and kept on crushed ice until used. In the study of the accumulation of C\textsuperscript{14}-serotonin into the vas deferens, groups of four organs each were incubated for various periods of time up to two hours with 3 ml of Krebs' solution as modified by Hukovic (1961), containing 0.4 \(\mu\)g of C\textsuperscript{14}-serotonin (specific activity = 40 mc/mmol; Nuclear-Chicago Corporation, Des Plaines, Ill.). The incubation was carried out in 20-ml beakers at 37°C and 0°C. The effect on the accumulation of C\textsuperscript{14}-serotonin at 37°C by drugs was examined by adding the drugs to the Krebs' medium 10 minutes prior to a 30-minute incubation period with C\textsuperscript{14}-indoleamine. At the end of the experiments, the vasa deferentia were washed once with Krebs' solution, blotted dry, weighed and homogenized in 3 ml of 0.4 N HClO. The homogenate was centrifuged for 10 minutes at 15,000 \(\times\) g in a refrigerated Servall centrifuge, and the supernatant was used for extraction of C\textsuperscript{14}-serotonin and its deaminated metabolite, C\textsuperscript{5}-hydroxyindole-3-acetic acid (5-HIAA), according to a previously described method (Eccleston et al., 1968). The spontaneous release of newly accumulated radioactive serotonin was examined by incubating groups of four vasa deferentia at 37°C for 30 minutes in 3 ml of Krebs' solution containing 0.25 \(\mu\)g of C\textsuperscript{14}-serotonin. At the end of this operation, the organs were washed, transferred into another series of beakers containing 3 ml of Krebs' solution and incubated at 37°C for various amounts of time in the presence or absence of drugs. The vasa deferentia were then examined for C\textsuperscript{14}-serotonin and C\textsuperscript{5}-HIAA.

The subcellular distribution of C\textsuperscript{14}-serotonin and endogenous norepinephrine was studied as follows. About 1 g of guinea-pig vas deferens was incubated at 37°C for one hour with 0.4 \(\mu\)g of C\textsuperscript{14}-serotonin. Immediately after the incubation, the
organs were homogenized in 10 volumes of cold isotonic sucrose (0.25 M), and the subcellular fractions were separated by differential centrifugation at 1,000 × g (nuclei plus unbroken cells) for 20 minutes, then at 8,000 × g (mitochondria) for 20 minutes and at 30,000 × g (microsomes) for two hours in refrigerated centrifuges. Perchloric acid (0.4 N) was added to the various subcellular fractions, which were then centrifuged. The supernatants obtained were assayed for C[14] -serotonin (Eccleston et al., 1968) and endogenous norepinephrine (Anton and Sayre, 1962). In studies on sucrose gradient, two vasa deferentia were incubated with 0.4 μc of C[14]-serotonin for one hour, then homogenized in 5 volumes of cold isotonic sucrose. Then 0.6 ml of the homogenate was layered on a density gradient made up of sucrose solutions, 0.25 to 1.75 M (Potter and Axelrod, 1962). After centrifugation at 100,000 × g for one hour in a refrigerated centrifuge, fractions of 8 drops were collected and alternately examined for C[14]-serotonin and C[14]-5-HIAA. Norepinephrine subcellular distribution was studied as for C[14]-serotonin, using another pair of vasa deferentia.

The following drugs, obtained from commercial sources, were used: cocaine HC1; dopamine HC1; desmethylimipramine HC1 (DMI); reserpine (Serpasil), 2.5 mg/ml; imipramine HC1; l-norepinephrine bitartrate; ouabain; serotonin creatinine sulfate; tryptamine HC1; and tyramine HC1. All drugs were dissolved in Krebs' solution prior to their use. The dosages used referred to their salt concentrations.

**Results.** Accumulation of C[14]-serotonin in the vas deferens. The accumulation of C[14]-serotonin in the vas deferens was examined over a period of two hours at both 37°C and 0°C (fig. 1). At 37°C there was a progressive increase in the accumulation of C[14]-serotonin with increasing duration of time. Little or no uptake occurred at 0°C. There was also a formation of the deaminated metabolite C[14]-5-HIAA at 37°C, but not at 0°C (fig. 2).

The kinetics of the accumulation of C[14]-serotonin in the vas deferens was determined by incubating longitudinal slices of vas deferens for 30 minutes at 37°C with 3 ml of Kreb's solution containing 0.25 μc of C[14]-serotonin plus various amounts of the nonlabeled amine. The results (fig. 3), expressed according to Line-weaver and Burk (1934), showed that the uptake of serotonin into the vas deferens was a saturable process with a Kₘ value about 9 × 10⁻⁶ M.

---

**Fig. 1.** Accumulation of C[14]-serotonin (5-HT) in guinea-pig vas deferens. Each point represents the mean value for at least four vasa deferentia. Vertical bars represent S.E.M. The ordinate represents C[14]-5-HT in counts per minute per milligram of wet tissue.

**Fig. 2.** Formation of C[14]-5-HIAA in guinea-pig vas deferens. Each point represents the mean value for at least four vasa deferentia. Vertical bars represent S.E.M. The ordinate indicates C[14]-5-HIAA in counts per minute per milligram of wet tissue.

**Subcellular distribution.** In the subcellular distribution studies, differential centrifugation (fig. 4) showed that C[14] -serotonin and endogenous norepinephrine accumulated mainly in the microsomal fraction of the guinea-pig vas deferens. The coarse fraction, which contained nuclei as well as unbroken cells, was found to accumulate a significant amount of both amines. Smaller quantities of both compounds were found in the mitochondrial and supernatant fractions. Continuous sucrose gradient studies (fig. 5) further showed that the distribution of C[14]-serotonin was similar to that of endogenous norepinephrine. On the other hand,
imipramine (table 1). Of all the agents used, only norepinephrine was found to inhibit 5-HIAA formation significantly (table 2). In the study of the effect of drugs on spontaneous release of C14-serotonin, reserpine, tyramine, dopamine, norepinephrine and DMI all significantly increased the release of the indoleamine at both 15 and 60 minutes. Tryptamine increased the release at 60 minutes but not at 15 minutes (table 3). Reserpine, but not the other drugs, increased the formation of released 5-HIAA (table 4).

Discussion. The guinea-pig vas deferens, an adrenergically innervated organ (Birmingham and Wilson, 1963; Burnstock and Holman, 1961; Sjöstrand, 1962), was found capable of accumulating and retaining serotonin. Subcellular studies have indicated that the sites of accumulation of serotonin are the norepinephrine (NE) storage granules of sympathetic nerves. Additional evidence that serotonin can accumulate in the same subcellular site as NE was obtained in cats in which the NE store had been depleted

C14-5-HIAA was found to be largely confined to the soluble fraction.

Spontaneous release of newly accumulated C14-serotonin. Groups of vasa deferentia were first incubated with 0.25 μg of C14-serotonin for 30 minutes at 37°C, then transferred to fresh Krebs' solution and reincubated for various amounts of time, and their content of C14-serotonin and C14-5-HIAA was determined. Figure 6 showed that the newly accumulated amine was slowly released. A proportional amount of C14-5-HIAA was also formed.

Effect of drugs on vas deferens serotonin accumulation and release. When added to the Krebs' medium containing the vasa deferentia 10 minutes prior to their incubation with C14-serotonin, cocaine, ouabain, imipramine, DMI and norepinephrine were all able to block the accumulation of C14-serotonin. The effectiveness of the compounds, in decreasing order, was: norepinephrine, DMI, ouabain, cocaine and
Subcellular distribution of C\textsuperscript{14}-serotonin (5-HT, ●), C\textsuperscript{14}-5-HIAA (□) and endogenous norepinephrine (NE, ○) in guinea-pig vas deferens by sucrose gradient technique. Two vasa deferentia were incubated with 0.4 μg of C\textsuperscript{14}-serotonin for one hour at 37°C, then quickly homogenized in 5 volumes of cold 0.25 M sucrose. Homogenate, 0.6 ml, was layered on a continuous sucrose gradient (0.25-1.75 M), then centrifuged for 60 minutes at 100,000 × g. Forty-two fractions of 8 drops each were collected into 0.5 ml of 0.4 N HClO\textsubscript{4} and extracted alternately for C\textsuperscript{14}-serotonin and C\textsuperscript{14}-5-HIAA. The same procedure was then carried out for subcellular distribution of endogenous NE. The graph shows a single experiment; similar results were obtained in a second experiment.

Spontaneous release of C\textsuperscript{14}-serotonin (5-HT) and formation of 5-HIAA in guinea-pig vas deferens. Groups of vasa deferentia were incubated in 3 ml of Krebs' solution containing 0.25 μg of C\textsuperscript{14}-serotonin for 30 minutes at 37°C. They were then transferred to another Krebs' solution and reincubated for various amounts of time, following which C\textsuperscript{14}-serotonin and C\textsuperscript{14}-5-HIAA were determined. The ordinate represents the percentage of 5-HT and 5-HIAA remaining in tissue. Each point is the mean of values obtained from at least four vasa deferentia. The vertical bars indicate S.E.M. Absolute control values at zero time: 192 ± 9.99 cpm of C\textsuperscript{14}-5-HT per mg of tissue; 218 ± 12.77 cpm of C\textsuperscript{14}-5-HIAA per mg of tissue.

with α-methyltyrosine. Incubation of the vas deferens or iris with serotonin resulted in the appearance of osmophilic dense core granules in the adrenergic terminals (Snipes et al., 1968). The accumulation of serotonin in the guinea-pig vas deferens appears to be a saturable process which is temperature-dependent and is depressed by ouabain. This would suggest that, like norepinephrine, serotonin is also taken up into the sympathetic nerves by an active process. Cocaine (Whitby et al., 1960) and imipramine derivatives (Axelrod et al., 1961) are known...
TABLE 1
Inhibition of C14-serotonin accumulation in vas deferens in vitro

Groups of four vasa deferentia were preincubated in 3 ml of Krebs’s solution containing various drugs. Ten minutes later, 0.4 μc of C14-serotonin (3.2 × 10^{-5} M) was added. Thirty minutes later, the vasa deferentia were removed, and their content of C14-serotonin was determined. Results are expressed as the percentage of inhibition of C14-serotonin accumulation as compared with control value (310 ± 35 cpm/mg of tissue).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain</td>
<td>1 × 10^{-5}</td>
<td>68 ± 2*</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1 × 10^{-4}</td>
<td>78 ± 2*</td>
</tr>
<tr>
<td>Imipramine</td>
<td>1 × 10^{-4}</td>
<td>62 ± 3*</td>
</tr>
<tr>
<td>DMI</td>
<td>1 × 10^{-6}</td>
<td>60 ± 3*</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>5 × 10^{-7}</td>
<td>80 ± 1*</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M.
* Significantly different from control (P < .05), as calculated by Student’s t test.

TABLE 2
Inhibition of formation of 5-HIAA in vas deferens in vitro

Groups of four vasa deferentia were preincubated in 3 ml of Krebs’s solution containing various drugs. Ten minutes later, 0.4 μc of C14-serotonin was added. Thirty minutes later, the vasa deferentia were removed, and their content of C14-5-HIAA was determined. Results are expressed as the percentage of inhibition of C14-5-HIAA formation, as compared with control value (365 ± 11 cpm/mg of tissue).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain</td>
<td>1 × 10^{-5}</td>
<td>27 ± 13</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1 × 10^{-4}</td>
<td>28 ± 10</td>
</tr>
<tr>
<td>Imipramine</td>
<td>1 × 10^{-4}</td>
<td>12 ± 8</td>
</tr>
<tr>
<td>DMI</td>
<td>1 × 10^{-6}</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>5 × 10^{-7}</td>
<td>45 ± 4*</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M.
* Significantly different from control (P < .05), as calculated by Student’s t test.

to block the uptake of NE into sympathetic terminals. The observation that these drugs, as well as NE, block the accumulation of C14-serotonin into the vasa deferentia suggests that norepinephrine and serotonin share the same mode of transport across the axonal membrane of the sympathetic terminals. Reserpine (Brodie et al., 1957), tyramine and dopamine (Potter and Axelrod, 1963), which release norepi-

TABLE 3
Release of C14-serotonin from vas deferens by drugs

Groups of four vasa deferentia were incubated with 0.25 μc of C14-serotonin (2 × 10^{-14} M) contained in 3 ml of Krebs’ solution for 30 minutes at 37°C. They were then transferred to fresh Krebs’ solution and incubated for 15 or 60 minutes in the presence or absence of various drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>15 min Released* after:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine</td>
<td>3 × 10^{-5}</td>
<td>47 ± 5*</td>
</tr>
<tr>
<td>Tyramine</td>
<td>1 × 10^{-4}</td>
<td>41 ± 3*</td>
</tr>
<tr>
<td>DMI</td>
<td>1 × 10^{-4}</td>
<td>76 ± 2*</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>1 × 10^{-4}</td>
<td>22 ± 12</td>
</tr>
<tr>
<td>Dopamine</td>
<td>3 × 10^{-5}</td>
<td>30 ± 5*</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>3 × 10^{-6}</td>
<td>42 ± 1*</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1 × 10^{-4}</td>
<td>71 ± 2*</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of C14-serotonin released (mean ± S.E.M.), as compared with control values.
* P < .05.

TABLE 4
Release of C14-5-HIAA from vas deferens in vitro by drugs

Groups of four vasa deferentia were incubated with 0.25 μc of C14-serotonin for 30 minutes, then transferred to fresh Krebs’ solution and incubated for 15 or 60 minutes in the presence or absence of drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>15 min Percentage Released* after:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine</td>
<td>3 × 10^{-5}</td>
<td>53 ± 6*</td>
</tr>
<tr>
<td>Tyramine</td>
<td>1 × 10^{-4}</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>DMI</td>
<td>1 × 10^{-4}</td>
<td>30 ± 22</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>1 × 10^{-4}</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>Dopamine</td>
<td>3 × 10^{-6}</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>3 × 10^{-6}</td>
<td>24 ± 10</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1 × 10^{-5}</td>
<td>37 ± 13</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of C14-serotonin released (mean ± S.E.M.), as compared with control values.
* P < .05.
serotonin from its storage site, also release the exogenous serotonin. Norepinephrine at low concentrations also effectively releases the labeled indoleamine. These observations, together with those which show that the exogenous serotonin and the endogenous NE are mainly confined to the microsomal fraction which have been shown to contain the NE storage vesicles (Potter and Axelrod, 1962), would suggest that both of these amines are stored and retained in the same storage granules.

The guinea-pig vas deferens was examined for the presence of endogenous serotonin by a sensitive method (Snyder et al., 1965), and no serotonin was detected. Thus, the uptake and storage of serotonin appeared to be exogenous phenomena. Although the brain neurons contain both serotonergic and adrenergic tracts (Dahlström and Fuxe, 1964) peripheral nerves, with the exception of the pineal gland, are innervated by adrenergic nerves (Owman, 1965). In the pineal gland, the parenchymal cells synthesize large amounts of serotonin, and a considerable portion of this is transported across the neuronal membrane of sympathetic nerves and stored within the vesicles along with NE (Owman, 1965). Our observation with the vas deferens would suggest that the uptake across the neuronal membrane and the storage in the vesicles may not be specific processes for norepinephrine only, but for other normally occurring amines such as serotonin as well.

Conclusions. Exogenous serotonin can accumulate in the guinea-pig vas deferens in vitro, presumably in sympathetic nerves, by a temperature-dependent saturable process. Drugs such as ouabain, cocaine, imipramine and its derivatives and norepinephrine block this accumulation. The serotonin accumulated is slowly released. This release can be accelerated by norepinephrine-releasing drugs, such as reserpine, tyramine and dopamine, and by norepinephrine.

REFERENCES


Inhibition by Drugs of the Accumulation in Vitro of 5-Hydroxytryptamine in Guinea-pig Vas Deferens

After an injection of $^{14}$C-5-hydroxytryptophan, autoradiographic grains were found to accumulate in the sympathetic neurones of the pineal gland and vas deferens\(^1\). These observations suggested that the sympathetic nerves of these organs might take up or retain 5-hydroxytryptamine. Subsequent work has shown that 5-hydroxytryptamine can accumulate in the guinea-pig vas deferens in vitro by an active transport process\(^2\). Subcellular distribution studies also indicated that the site of accumulation of the 5-hydroxytryptamine is the storage vesicle present in the sympathetic neurone\(^2\). Certain drugs such as imipramine\(^3\) and cocaine\(^1\) are potent inhibitors of the uptake of noradrenaline into sympathetic neurones, and those drugs also block the uptake of 5-hydroxytryptamine in platelets\(^4\) and some tissues in the rat\(^5\). Subsequent work has shown that the site of blockade of noradrenaline uptake is the neuronal membrane\(^7\). These observations prompted us to investigate the ability of these drugs to block the uptake in the vas deferens of 5-hydroxytryptamine labelled with carbon-14.

The vas deferens were obtained from guinea-pigs weighing 250-300 g. The animals were killed by a blow on the head, bled and the vas deferens rapidly removed and chilled. They were cut into segments weighing approximately 20 mg and four such segments were incubated for 30 min at 37° C in 4 ml. of Krebs–Ringer phosphate containing 0-4 mCi of $^{14}$C-5-hydroxytryptamine (specific activity 40 mCi/mnmole).

On removal from the medium they were washed, blotted and homogenized separately in 0-4 N perchloric acid and the homogenate was centrifuged at 15,000g for 15 min. A 1 ml. sample of the supernatant fluid was adjusted to pH 10 with sodium hydroxide and 1·5 ml. of 0-5 M borate buffer pH 10 and the 5-hydroxytryptamine was extracted into 6 ml. of isoamyl alcohol by shaking for 5 min. After centrifugation a 4 ml. sample of the isoamyl alcohol extract was transferred to a counting vial containing 4 ml. of ethanol and 10 ml. of phosphor. 10 mg of ascorbic acid was added to a second 1 ml. sample of the supernatant and the pH was adjusted to 1·0 by the addition of concentrated hydrochloric acid. The 5-HIAA was extracted into 5 ml. of diethyl ether by shaking for 3 min. After standing, a 4·5 ml. sample of the ether phase was shaken for 3 min with 1 ml. of 0·5 M phosphate buffer pH 7·9. An 0·4 ml. sample of the buffer extract was added to the phosphor containing 5 ml. of a mixture of ethanol : methanol (3 : 1). These methods
Fig. 1. Inhibition of uptake of $^{14}$C-5-hydroxytryptamine (5HT) in guinea-pig vas deferens by desmethylimipramine (DMI). Each group contained vas deferens from four guinea-pigs. Results are expressed as mean ± S.E.M.

had been shown to be specific by paper chromatography. Guineapig vas deferens incubated with $^{14}$C-5-hydroxytryptamine ($2.5 \times 10^{-6}$ M) for 30 min was shown to give a two-fold increase in concentration in the tissue above the medium. In addition, approximately equimolar amounts of the deaminated metabolite 5-HIAA were also present in the vas deferens. In the presence of cocaine $1 \times 10^{-4}$ M and imipramine $1 \times 10^{-4}$ M, this accumulation was reduced to 22 per cent and 38 per cent of controls, respectively. The conversion to 5-HIAA, however, was unchanged at these concentrations. Desmethylimipramine was found to inhibit uptake in concentrations as low as $3 \times 10^{-8}$ M. From $3 \times 10^{-5}$ M to $1 \times 10^{-4}$ M the inhibition was linear with the logarithm of the concentration for the drug (Fig. 1). With very high concentrations of desmethylimipramine ($10^{-3}$ M) the formation of 5-HIAA was inhibited, but at lower concentrations the formation was unaffected.

In a previous study, it was shown that the antidepressant drug imipramine and a variety of structurally related compounds blocked the uptake of tritiated noradrenaline into the intact brain. It was found that those drugs with antidepressant activity blocked the uptake into brain, but those structurally related drugs which were not antidepressant did not. A similar study was undertaken to determine the effect of tricyclic compounds with and without antidepressant activity on the blockade of
Table 1. INHIBITION OF ACCUMULATION OF 14C-5-HYDROXYTRYPTAMINE IN
GUINEA-PIG VAS DEFERENS BY ANTI DEPRESSANT DRUGS

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition of accumulation (%)</th>
<th>Clinical activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>57*</td>
<td>+</td>
</tr>
<tr>
<td>Desmethyllimipramine</td>
<td>62*</td>
<td>+</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>43*</td>
<td>+</td>
</tr>
<tr>
<td>Compound 2</td>
<td>−28*</td>
<td>−</td>
</tr>
<tr>
<td>Compound 3</td>
<td>−47*</td>
<td>+</td>
</tr>
<tr>
<td>Compound 4</td>
<td>−8*</td>
<td>−</td>
</tr>
<tr>
<td>Compound 6</td>
<td>43*</td>
<td>+</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>10</td>
<td>−</td>
</tr>
</tbody>
</table>

Groups of vas deferens obtained from five guinea-pigs were incubated with
14C-5-hydroxytryptamine. Drugs were added at a concentration of $5 \times 10^{-5}$ M.
After 1 h the uptake of 14C-hydroxytryptamine and the formation of HIAA
were measured in the tissue. Results are expressed as the percentage of
inhibition of accumulation of 5-hydroxytryptamine as compared with the
control value. Compound 2 is the isopropyl analogue of imipramine. Com¬
pound 3 is the ethyl analogue of desmethyllimipramine. Compound 4 is the
2 chloro (dimethylamino-ethoxy ethyl) derivative of phenothiazine. Com¬
pound 6 is the 2 chloro derivative of desmethyllimipramine.

* $P < 0.01$
† Clinical observations by experienced psychiatrists carried out on a
limited number of patients. Details of the tests can be obtained from Dr.
Murray Weiner, Geigy Research Laboratories, Yonkers, New York.

accumulation of 5-hydroxytryptamine by the guinea-pig
vas deferens. For this study, the concentrations were
chosen at which desmethyllimipramine produced approxi¬
mately 50 per cent inhibition ($5 \times 10^{-5}$ M). There
seemed to be a consistent relationship between the ability of
tri cyclic compounds to block the accumulation of
5-hydroxytryptamine in vas deferens and their antidepres¬
sant activity (Table 1). There was no change in the forma¬
tion of 5-HIAA.

The process of biogenic amine uptake by the vas deferens
seems to be more closely related to the brain than
other peripheral tissues. The uptake of noradrenaline
in the brain and 5-hydroxytryptamine in the vas deferens
is blocked by imipramine and its clinically active con¬
geners. Furthermore, chlorpromazine inhibits the uptake
of biogenic amines in peripheral tissues but not in brain
or vas deferens. In view of these observations the vas
deferens could serve as a useful model for brain biogenic
amine containing neurones in studying the effect of certain
drugs on amine uptake processes.

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SECTION V
Discussion of in Vivo Measurement of Brain Serotonin Turnover

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We wish to comment on two aspects of this subject.

Serotogenic Units

Neff and Tozer (p. 97) suggest that the results in their second table can be interpreted to mean that "each serotogenic unit synthesizes 5-HT at the same specific rate," and that the overall turnover rate is related to the number of serotogenic units within a brain area.

In Table 1, the results of a study are shown, in which the molar concentrations of 5-HIAA and 5-HT were examined in different areas of dog brain. If the concept proposed in the previous paper is correct, then the ratio

\[
\frac{5-HT}{5-HIAA} = \frac{k}{k_1}
\]

should be a constant. The wide variation in the ratio in the different brain areas provides evidence, in the dog, against the concept of "serotogenic units."

TABLE I

RATIO OF SEROTONIN TO 5-HYDROXYINDOLEACETIC ACID IN THE BRAINS OF SIX NORMAL DOGS

<table>
<thead>
<tr>
<th>Region</th>
<th>Mean ratio (5-HT)/(5-HIAA) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>1.12 ± 0.17</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.82 ± 0.30</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.06 ± 0.16</td>
</tr>
<tr>
<td>Caudate</td>
<td>2.17 ± 0.44</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.70 ± 0.38</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>1.06 ± 0.16</td>
</tr>
</tbody>
</table>

* The mean ratios ± SEM of (5-HT)/(5-HIAA) concentrations in nmoles per gram of brain tissue in different regions of normal dog brain.

Perfusion of the Cerebral Ventricles

We report the development of a technique which may make possible the continuous monitoring of brain amine metabolism in the conscious, unrestrained animal. The
technique is based on a recirculatory perfusion of the cerebral ventricular system of the conscious dog (Fig. 1). Guide tubes were chronically implanted into the dog's skull, using techniques previously described for the dog by Manuilov (1958), and for the goat by Pappenheimer et al. (1961). In subsequent experiments, the lateral, or third ventricle, and the cisterna magna were punctured percutaneously via the guide tubes, without anesthesia, and the ventricular space was perfused with recirculated fluid, as shown in Fig. 1. Samples of perfusate were withdrawn at half-hourly intervals for analysis.

In some experiments, 5-HIAA and inulin were injected into the perfusate at a constant rate. Clearance rates, analogous to renal clearances, were then calculated from the concentrations of 5-HIAA and inulin found when equilibration of the system had been achieved. In eight experiments, with a perfusion rate of 0.33 ml/min, the clearance of inulin was $0.06 \pm 0.005$ ml/min, and that of 5-HIAA, $0.14 \pm 0.01$ ml/min. The transport mechanism for 5-HIAA was saturated when high concentrations of 5-HIAA were injected, with a fall in the observed clearance rate. The clearance of 5-HIAA was reduced to that of inulin by pretreatment of the dog with probenecid. These data provide confirmatory evidence for the existence of an active transport mechanism from C.S.F. for 5-HIAA, as suggested by Goldberg et al. (1966) for C.S.F., and for brain by Neff et al. (1964).

The technique can be applied to the study of the release of cerebral metabolites into the ventricular perfusate. In the first part (1½ hours) of such an experiment, no exogenous substances were added, allowing the establishment of a plateau in the concentration of endogenously contributed acids in the perfusate. In the second part of the experiment, 5-HIAA and inulin were injected into the perfusate at a constant rate to obtain measures of the clearance rates. A calculation of the amount of 5-HIAA entering the perfusate per minute from brain can be made from this data, and this is in the region of 20–30 mg/min.

Preliminary experiments with reserpine indicate two effects. Perfusion 16–20 hours after a dose of 0.5 mg/kg reserpine intramuscularly shows a reduction in the clearance

![Fig. 1. Technique for the recirculatory perfusion of the cerebral ventricles in the conscious dog. At equilibrium clearance $\frac{E}{O + (E/R)}$ where $E =$ amount added per minute; $O =$ conc. per milliliter in fluid leaving cisterna; $R =$ rate of perfusion.](image-url)
rate of 5-HIAA, together with an increase in the amount of 5-HIAA entering the perfusate from the brain, per minute. These results are consistent with the effects of reserpine on turnover of 5-HIAA and clearance from brain, reported by Drs. Tozer and Neff.

We have presented a description of this technique, in the hope that it will provoke a comparison with the methods described in the previous paper, and stimulate discussion on the possible applications of both types of technique.

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Cerebral Metabolites in Cerebrospinal Fluid as a Biochemical Approach to the Brain

BY

A. T. B. MOIR, G. W. ASHCROFT, T. B. B. CRAWFORD, D. ECCLESTON AND H. C. GULDBERG


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1970
CEREBRAL METABOLITES IN CEREBROSPINAL FLUID
AS A BIOCHEMICAL APPROACH TO THE BRAIN

BY

A. T. B. MOIR, G. W. ASHCROFT, T. B. B. CRAWFORD, D. ECCLESTON AND H. C. GULDBERG

(From The Medical Research Council Unit for Research in Brain Metabolism and the Department of Pharmacology, Edinburgh University Medical School)

The study of cerebral metabolism in man presents many formidable obstacles (Mandell and Spooner, 1968) and the basic techniques which may be applied in making a direct biochemical approach to the brain are limited in number. We may use post-mortem material, biopsy samples, the arteriovenous differences in the concentration of metabolites or employ studies using cerebrospinal fluid. The last of these methods, the examination of metabolites in cerebrospinal fluid (CSF) has been used recently to study changes in cerebral amine metabolism in man in a number of pathological states.

The interpretation of the results of such investigations involves certain basic assumptions: first, that the metabolites measured in CSF are derived from cerebral and not general metabolism, and second, that levels of metabolites in CSF obtained from particular sites such as the lumbar CSF will reflect levels in specific regions of the central nervous system.

The time seems appropriate for a critical discussion of the justification for these assumptions in relation to amine metabolism including an examination of factors such as impairment of metabolite transport which may, under certain circumstances, invalidate the assumptions. Such a discussion must involve presentation of data from both animal experiments and human studies.

CEREBRAL AMINE METABOLISM

Each of the biogenic amines has its own peculiar distribution in brain and it has been suggested that they may serve as synaptic transmitters or modulators of neuronal activity in functionally distinct systems of the brain: 5-hydroxytryptamine (5-HT) in limbic structures, dopamine in the extrapyramidal system and noradrenaline in central autonomic and alerting systems.

The main factors in the metabolism of the amines are illustrated schematically in fig. 1. The precursor amino-acids cross the "blood-brain barrier," become ring hydroxylated at localized intracellular sites and then are decarboxylated to yield the amines. The amines may be taken up into storage organelles, and these storage...
organelles appear to be “coupled” (Moir and Eccleston, 1968; Udenfriend, 1968) enzymically or spatially with ring hydroxylases. The changes in the storage of amines and their release from the stored form appear to relate to many varied alterations in neuronal function.

The mechanisms available for inactivation of the amines are reuptake into nerve terminals or enzymic change by methylation or oxidative deamination. The metabolites so formed must then cross “barriers” from brain to either blood or CSF. It is with this process of removal of metabolites that we are mainly concerned; in particular, the main acid metabolites of 5-HT and dopamine, namely 5-hydroxyindol-3-ylacetic acid (5-HIAA) and homovanillic acid (HVA), respectively.

The concentration of the metabolites in brain might be expected to provide an index of turnover of the parent amine (assuming that the metabolite cannot itself enter the brain from blood). These metabolites pass into CSF and reach equilibrium with those of brain tissue; thus CSF levels of metabolites should also provide an index of turnover of the amine in brain and this body fluid would be available in living patients.

In the case of noradrenaline, vanillin mandelic acid does not appear to be the major metabolite in brain, and it is suggested that inactivation of noradrenaline in brain is by reuptake and by metabolism to the corresponding glycols and their sulphates. The factors affecting the production of these metabolites and their removal from brain and CSF are complex and require separate consideration. Histamine also has been omitted from discussion in this paper, not because we reject its possible role in cerebral function, but because the absence of techniques for the measurement of its metabolites at low concentrations have, so far, made impossible studies of the type to be described here.

**Identification of Metabolites in Brain and CSF**

In recent years it has become possible to isolate and estimate with high specificity very small quantities of the main metabolites of 5-HT and dopamine. Table I gives references to the original publications in which these metabolites were characterized and estimated quantitatively from extracts of cerebral tissue or CSF.
Table I.—Identification and Estimation of the Main Cerebral Metabolites of Dopamine and 5-Hydroxytryptamine

<table>
<thead>
<tr>
<th>Amine</th>
<th>Brain</th>
<th>Cerebrospinal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Hydroxytryptamine</td>
<td>5-HIAA (Sharman, 1960)</td>
<td>5-HIAA (Ashcroft and Sharman, 1960)</td>
</tr>
<tr>
<td></td>
<td>5-HIE (Eccleston, Moir, Reading and Ritchie, 1966)</td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>HVA (Sharman, 1963)</td>
<td>HVA (Andén, Roos and Werдинius, 1963a)</td>
</tr>
<tr>
<td></td>
<td>(Andén, Roos and Werдинius, 1963a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DOPAC (Rosengren, 1960)</td>
<td>DOPAC (Ashcroft, Crawford, Dow and Guldberg, 1968)</td>
</tr>
</tbody>
</table>

5-HIAA, 5-hydroxyindol-3-ylacetic acid; 5-HIE, 5-hydroxytryptophol; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid.

Evidence that Metabolites in Brain and CSF Are Derived from Cerebral Metabolism

5-HIAA, administered systemically, did not enter the brain of the rabbit (Roos, 1962) or rat (Moir and Eccleston, 1968) nor was intravenously administered 14C-5-HIAA detected in significant quantities in the CSF of the dog (Ashcroft, Dow and Moir, 1968). Similarly, Carlsson and Hillarp (1962) showed that systemically administered 3,4-dihydroxyphenylacetic acid (DOPAC) did not enter the brain of the rabbit. Intravenously administered HVA did not enter the brain or CSF of the cat (Bartholini et al., 1966) or the CSF of the dog (Guldberg and Yates, 1968).

These experiments show that these acid metabolites do not pass readily from blood to either brain or CSF and the most likely source of the endogenous metabolites found in these sites is cerebral metabolism.

Evidence that CSF Metabolite Concentrations Reflect the Concentrations in Adjacent Regions of Brain

Stainless steel guide tubes implanted in the dog’s skull allowed serial samples of CSF to be obtained from either the lateral ventricle (Ashcroft, Crawford, Dow and Guldberg, 1968) or the cisterna magna (Ashcroft, Dow and Moir, 1968). The use of these techniques enabled the changes produced in the metabolite concentrations of CSF to be followed during the course of action of various psychotropic drugs and permitted the relations between the CSF concentrations and various regions of brain to be determined by suitably timed acute experiments (Moir, 1967; Yates, 1967).

The caudate nucleus forms most of one wall of the lateral ventricle in the dog. Table II shows the concentrations of HVA and 5-HIAA in the caudate nucleus and lateral ventricular CSF before and following administration of a small dose of chlorpromazine. The marked rise in the acid metabolite concentration in the caudate nucleus after chlorpromazine administration was mirrored by a corresponding rise
in the acid metabolites in the CSF. The relation of a constant ratio between the acid metabolite concentrations in caudate nucleus and lateral ventricular CSF was also maintained after a larger dose of chlorpromazine which caused further alterations in the metabolite concentrations (Guldberg and Yates, 1969). This relation probably holds provided that no alteration occurs in the mechanisms responsible for the transfer of the acid metabolites from brain to CSF.

Table III shows the concentrations of 5-HIAA and HVA in the lateral ventricular CSF of "control" patients and patients with Parkinsonism and the concentration of HVA found in the caudate nucleus of similar patients. These results show a highly significant ($p < 0.001$) fall in the concentration of HVA in the lateral ventricular CSF of patients with Parkinson's disease and a corresponding percentage change in the concentration of HVA in the caudate nucleus of similar patients in the studies of Bernheimer and his co-workers. The highly significant ($p < 0.001$) fall in the concentration of 5-HIAA in lateral ventricular CSF suggests that the concentration of this metabolite in caudate nucleus will also be low in patients with Parkinson's

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**Table II.**—Relationship between Concentrations of Homovanillic Acid (HVA) and 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) in Caudate Nucleus and Lateral Ventricular CSF of Dogs Before and After Treatment with Chlorpromazine (5 mg./kg. I.V.)

<table>
<thead>
<tr>
<th></th>
<th>HVA</th>
<th>5-HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>After chlorpromazine</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
<td></td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>13.4±1.2*</td>
<td>17.0±1.1</td>
</tr>
<tr>
<td>(CN)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Lateral ventricular CSF</td>
<td>1.71±0.39</td>
<td>2.44±0.67</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Mean of the ratios (CN/CSF)</td>
<td>8.08±1.66</td>
<td>7.28±1.61</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

* Concentrations in µg./g. brain tissue or /ml. CSF. Mean ± standard deviation (No. of estimates).

---

**Table III.**—Mean Concentrations of Homovanillic Acid (HVA) in Caudate Nucleus and of HVA and 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) in Lateral Ventricular CSF in Parkinsonism and Controls

<table>
<thead>
<tr>
<th>Clinical state</th>
<th>Lateral ventricular CSF</th>
<th>Lateral ventricular CSF</th>
<th>Caudate nucleus</th>
<th>Caudate nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.111±0.050(17)</td>
<td>0.447±0.153(15)</td>
<td>3.38^a</td>
<td>7.6</td>
</tr>
<tr>
<td>Parkinsonism</td>
<td>0.059±0.027(32)</td>
<td>0.186±0.105(37)</td>
<td>1.19^a</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Mean concentration µg./ml. of CSF ± standard deviation (No. of estimate); mean concentration µg./g. brain tissue.

disease. Sourkes and Poirier (1966) have indeed shown that in monkeys with surgically induced Parkinsonian-like tremor there is a decrease in the concentration of both dopamine and 5-hydroxytryptamine in the striatum of the operated side.

Fig. 2.—Concentrations of 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the cisternal CSF and brain-stem of dogs (a) following intravenous L-tryptophan (50 mg./kg.) and (b) after intravenous α-methyl dopa (50 mg./kg.), followed by intravenous L-tryptophan (50 mg./kg.). L-tryptophan given at zero time.

Fig. 2 shows the mean concentrations of 5-HIAA in the brain-stem (mid-brain and hind-brain) of the dog and the corresponding concentrations of 5-HIAA in cisternal CSF after intravenous administration of tryptophan or α-methyl-dopa followed by tryptophan. These data (derived from the data of Eccleston, Ashcroft, Moir, Parker-Rhodes, Lutz and O'Mahoney, 1968) show that the 5-HIAA concentration in cisternal CSF followed and closely reflected the concentration of the acid in the brain-stem. The fact that the cisternal CSF concentrations of 5-HIAA reflected predominantly the brain-stem region was best demonstrated after the combination of α-methyl-dopa and tryptophan, as the metabolite changes in the other regions of the dog brain then followed a different pattern and time course from those of the brain-stem.

In general, the data presented above indicate that the metabolite concentrations found in different parts of the CSF system reflect the metabolite concentrations in the underlying brain. Nevertheless, certain other factors need to be taken into consideration.

Evidence for the Active Transport of Acid Metabolites from CSF

Guldberg et al. (1966) showed that there was a marked gradient of the concentrations of the acid metabolites, HVA and 5-HIAA, from lateral ventricle to cisterna magna of the dog, and that it was possible to reduce this gradient by administering
probencid. By the use of a recirculatory perfusion of the cerebral ventricular system in the conscious dog (Ashcroft, Dow and Moir, 1968) it was possible to investigate further the removal mechanisms of the acid metabolites from CSF.

Fig. 3 shows the result from one dog in which experiments were carried out using infusions of various concentrations of 5-HIAA and inulin. These results demonstrate that the efflux of 5-HIAA from CSF has components due to diffusion and a saturable transport mechanism, as well as a component due to bulk flow of CSF. It was possible to localize the saturable transport system to the region of the fourth ventricle and to show that it was an active transport process which removed both HVA and 5-HIAA from CSF (Ashcroft, Dow and Moir, 1968).

Table IV shows the concentrations of 5-HIAA and HVA in CSF obtained from different regions of the CSF system in dog and man. These results demonstrate that in man, as in the dog, there is a marked fall of the concentration of the acid metabolites from lateral ventricle to lumbar space.

![Graph](image-url)

Fig. 3.—The relationship of rate of efflux from CSF to the inflow concentration for 5-hydroxyindol-3-ylacetic acid (5-HIAA) and for inulin. The data were derived from the equilibrium concentrations which were achieved when various concentrations of inulin and 5-HIAA were infused into a recirculating perfusion of the cerebral ventricular system in a conscious dog. At equilibrium states the net infusion rate equals the total quantity removed from the ventricular perfusate. As the scale for inulin is one-quarter that of 5-HIAA along both axes the angular interrelationships are still valid and clearance relationships are given geometrically in the above graph by, abscissa distance/30 times ordinate distance for both inulin and 5-HIAA.
Table IV.—Mean Concentrations of Homovanillic Acid (HVA) and 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) in CSF Withdrawn from the Lateral Ventricle, Cisterna Magna or Lumbar Region of Man and Dog

<table>
<thead>
<tr>
<th></th>
<th>Ventricular CSF</th>
<th>Cisternal CSF</th>
<th>Lumbar CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man</td>
<td>Dog</td>
<td>Man</td>
</tr>
<tr>
<td>HVA</td>
<td>0.466±0.163</td>
<td>1.58±0.34</td>
<td>0.185</td>
</tr>
<tr>
<td></td>
<td>(18)</td>
<td>(6)</td>
<td>(1)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0.150±0.049</td>
<td>0.258±0.095</td>
<td>0.085</td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td>(6)</td>
<td>(1)</td>
</tr>
</tbody>
</table>

Concentration (µg./ml.) ±. Standard deviation (No. of estimates).

Species differences in the localization of active transport of substances from CSF have been observed between the goat (Pappenheimer, Heisey and Jordan, 1961), the cat (Davson, Kleeman and Levin, 1962), the rabbit (Pollay and Davson, 1963) and the dog (Ashcroft, Dow and Moir, 1968). The results in Table IV, however, seem to indicate that in man there may well be a transport system for the removal of acid metabolites from CSF located in the region of the fourth ventricle, as was shown to be the case in the dog. It is necessary to take account of the effect of this localized removal mechanism for the acid metabolites in interpreting the concentrations of the metabolites which are found in human lumbar CSF.

The Use of Quantitative Analyses of Metabolites in the Study of Drug Actions in Animals

The recently developed techniques of estimating the amine metabolite concentrations in serial samples of CSF obtained from the lateral ventricle (Ashcroft, Crawford, Dow and Guldberg, 1968) and cisterna magna have been used in association with perfusion experiments of the cerebral ventricular system which monitored the turnover rate of cerebral metabolism in the conscious animal (Ashcroft et al., 1969). Phenelzine and reserpine were shown to have inhibitory effects on the active transport of acid metabolites from CSF and it also appeared that the transport mechanisms from brain to CSF were similarly affected. These effects have to be taken into account in interpreting the drug actions.

It may be that psychiatric illnesses as well as psychotrophic drugs will be shown to affect these cerebral transport mechanisms for acid metabolites. Indeed, Glen et al. (1968) have recently shown that sodium transport into saliva is decreased in patients suffering from manic-depressive psychosis. Sodium transport is often coupled to other types of active transport; thus the probable effect of possible impairment of these transport mechanisms in brain and CSF should be taken into account in any interpretation related to the concentrations of acid metabolites found in lumbar CSF.

The experimental procedures outlined above may be used in animals to study the dynamic actions of psychotropic drugs. They also allow the planning of the best times to carry out acute experiments, since they permit a detailed study of the time course of drug action. While we have used ventricular perfusion in conscious
animals to study the removal mechanism of substances from CSF, the rate of metabolism of exogenously administered substrates and the metabolic turnover rate of endogenous cerebral metabolites, it is a technique which has extremely wide applications in neurophysiology and neuropharmacology.

**CSF Studies of Amine Metabolism in Man**

Table V shows the concentrations of 5-HIAA and HVA in lumbar CSF from patients with depressive illness or Parkinsonism from our own or similar studies. In summary our results show that, in comparison with control patients, there is a significant decrease of 5-HIAA concentration (p < 0.001) in patients with depressive symptoms and that in patients with Parkinsonism a marked fall occurs in the concentrations of HVA (p < 0.01) and a less marked one in the concentrations of 5-HIAA (p < 0.01). The results of other workers are in general agreement with these points. There are, however, further relevant factors which can be demonstrated from a comparison of the different series of investigations.

Samples of CSF in our series were obtained according to a strict routine (Ashcroft et al., 1966), which included restriction of food for sixteen hours and of movement for eight hours prior to lumbar puncture carried out in the lateral position. The estimates of the concentrations of the acid metabolites were performed on the first 5 ml. of CSF flowing from the needle and were obtained according to the method of Ashcroft and Sharman (1962) or, more

<table>
<thead>
<tr>
<th>Clinical state</th>
<th>5-HIAA (µg./ml)</th>
<th>HVA (µg./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.019 ± 0.004(21)</td>
<td>0.053 ± 0.037(11)</td>
</tr>
<tr>
<td>Depression</td>
<td>0.011 ± 0.004(24)</td>
<td></td>
</tr>
<tr>
<td>Parkinsonism</td>
<td>0.014 ± 0.003(8)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>*0.036 ± 0.011(34)</td>
<td>*0.054 ± 0.031(29)</td>
</tr>
<tr>
<td></td>
<td>0.04 ± 0.004(7)</td>
<td>0.05 ± 0.014(7)</td>
</tr>
<tr>
<td>Depression</td>
<td>Range 0.005–0.03(14)</td>
<td>Range 0.01–0.05(5)</td>
</tr>
<tr>
<td>Parkinsonism</td>
<td>*0.012 ± 0.012(24)</td>
<td>*0.018 ± 0.028(18)</td>
</tr>
<tr>
<td></td>
<td>0.02 ± 0.035(8)</td>
<td>0.02 ± 0.005(8)</td>
</tr>
<tr>
<td>Control</td>
<td>0.060(14)</td>
<td></td>
</tr>
<tr>
<td>Parkinsonism</td>
<td>&lt;0.013(17)</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from histograms of data.
† Mean concentration in µg./ml. of CSF ± standard deviation (No. of patients).
1 Ashcroft et al., 1966.
2 Dencker, Malm, Roos and Werdinius, 1966.
3 Johanssen and Roos, 1967.
4 Olsson and Roos, 1968.
5 Bernheimer, Birkmayer and Hornykiewicz, 1966.
recently, according to a modification of this method (Ashcroft, Crawford, Dow and Guldberg, 1968). The Swedish workers (Dencker et al., 1966; Johansson and Roos, 1967; Olsson and Roos, 1968) use similar biochemical methods of estimation but they use 20 ml. samples of CSF for analysis and do not impose any restrictions of food or movement on their patients prior to lumbar puncture (Roos, personal communication). It is probable that these differences in obtaining samples may account for the higher control concentration obtained by the Swedish investigators as the 20 ml. sample would be expected to include a larger portion of CSF from the brain-stem region where the 5-HIAA concentration is known to be higher (Table IV). Physical exercise influences mixing in the CSF system (Fotherby et al., 1963), and this may also help to explain the higher concentrations of 5-HIAA obtained by the Swedish workers and why their data is more variable than our own.

The concentrations of both HVA and 5-HIAA in the lateral ventricular CSF in patients with Parkinsonism show a reduction from the control values (Table III). This may well indicate that the pathological changes in the caudate nucleus are of a non-specific nature, and might be due to cell loss which has been shown to occur in surgically induced Parkinsonian-like states in animals.

Table VI shows the mean concentrations of HVA and 5-HIAA and their ratios from our series of patients suffering from depressive illness and Parkinsonism in comparison with a control group of patients.

<table>
<thead>
<tr>
<th></th>
<th>Ventricular</th>
<th>Lumbar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkinsonism</td>
<td>0.186/0.059=3.3</td>
<td>0.012/0.015=0.8</td>
</tr>
<tr>
<td>Controls</td>
<td>0.447/0.110=4.4</td>
<td>0.053/0.019=2.8</td>
</tr>
<tr>
<td>Depression</td>
<td>No results available</td>
<td>0.040/0.011=3.6</td>
</tr>
</tbody>
</table>

The ratios of HVA to 5-HIAA for control patients show that there is relatively more 5-HIAA than HVA in the lumbar CSF than in the CSF from the lateral ventricle. This may in part be because the efflux of acid metabolites from CSF that takes place in the region of the fourth ventricle is more efficient for HVA than 5-HIAA (Ashcroft, Dow and Moir, 1968). It seems likely, however, that while the major portion of HVA in the lumbar CSF will still be derived from the caudate nucleus, the 5-HIAA will be derived in greater part from regions with fast turnover of 5-hydroxyindoles nearer the site of puncture. Thus an important contribution to the 5-HIAA concentration in lumbar CSF would be expected from the brain-stem region. The results of the lumbar CSF studies (Tables V and VI) show that in Parkinsonism the most marked reduction occurs in the HVA concentration while in depression the concentration of 5-HIAA shows the major reduction.

The results discussed above indicate that in Parkinsonism there is an impairment of dopamine and 5-hydroxyindole metabolism in the caudate nucleus and that in
depression (even if cerebral transport mechanisms are shown to be inhibited) there is a selective impairment of the cerebral metabolism of the 5-hydroxyindoles. It is also clear that many factors have to be taken into consideration when trying to determine the state of cerebral metabolism from metabolite concentrations found in lumbar CSF.

**Possible Future Developments**

**(a) Probenecid.**—This drug, which blocks certain active transport mechanisms in the kidney, has been shown to inhibit the active transport of acid metabolites from brain (Neff, Tozer and Brodie, 1964) and from CSF (Guldberg, Ashcroft and Crawford, 1966; Ashcroft, Dow and Moir, 1968). Olsson and Roos (1968) gave this drug to patients suffering from Parkinsonism and were able to show that the acid metabolites in the lumbar CSF of these patients became elevated to a lesser extent than in control patients. It is possible that in this study blocking transport mechanisms allowed the metabolite concentrations found in lumbar CSF to be derived in greater part from ventricular fluid thus showing the same marked differences as were found by direct sampling of ventricular CSF (Guldberg et al., 1967). Ashcroft et al. (1966) showed that the collection of samples of CSF from the lumbar region after air had been injected during air encephalography was a useful method of obtaining CSF, which would reflect ventricular concentrations of metabolites.

Ashcroft and Sharman (1960) showed that the 5-HIAA concentration in the ventricular CSF of patients suffering from hydrocephalus was significantly raised. Recently, Andersson and Roos (1968) have shown that in experimentally induced hydrocephalus in dogs the administration of probenecid causes no further impairment of the efflux of 5-HIAA from CSF. It seems likely, therefore, that certain cerebral transport mechanisms may be impaired in some cases of hydrocephalus and that probenecid may be a useful drug to use in testing the integrity of cerebral transport mechanisms in this and other disease states.

**(b) Precursor administration.**—A common method of evaluating metabolic pathways in clinical biochemistry is to test the response to a large load of a suitable precursor. Moir and Eccleston (1968) showed that the administration of 5-hydroxytryptophan caused an abnormal pattern of cerebral metabolism of the 5-hydroxyindoles but that giving L-tryptophan allowed the turnover rate in the pathway to be increased without disturbing the normal metabolic patterns of different brain regions. In dogs the concentration of 5-HIAA in cisternal CSF after giving tryptophan reflected the concentration of 5-HIAA in the brain-stem region (Eccleston et al., 1968). Current studies indicate that comparable precursor loads in patients followed by the analysis of 5-HIAA concentrations in lumbar CSF should prove a useful means of assessing the integrity of the cerebral 5-hydroxyindole pathway in man.

**Conclusion**

The observations made in studies with experimental animals indicate that measurement of cerebral metabolites in the CSF can give information about cerebral metabolism itself. The relation between metabolite concentrations in brain
and different parts of the CSF system have been shown to be complex but appreciation of at least some of the complexities involved has, we think, enabled us to make, with greater confidence, inferences about certain aspects of cerebral metabolism in man from quantitative analyses of cerebral metabolites in lumbar CSF. We stress that interpretation of the results of such studies must be cautious because of the many complex variables involved, some probably as yet unknown. Nevertheless further recognition and unravelling of these complexities could make the analysis of samples of CSF as valuable in assessing the functional states of biochemical mechanisms in the in vivo brain as the study of urine and its modification by various manoeuvres have proved to be in assessing renal function.

**Summary**

The relationship between brain and CSF is complex and the results presented help to define this problem and to show how animal experiments aid in the interpretation of concentrations of endogenous metabolites in the lumbar CSF of patients. Parkinsonian patients show impaired metabolism of dopamine and 5-hydroxyindoles in the caudate nucleus, while patients with endogenous depression appear to have selective impairment of the cerebral metabolism of 5-hydroxyindoles. The value of analyses of lumbar CSF in the possible development of biochemical tests of brain function has been discussed.

**References**


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A Critical Evaluation of Techniques for Studying Synthesis and Turnover of 5-HT in the Brain *in vivo*

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*Introduction*

5-hydroxytryptamine (5-HT) was discovered in brain by a group of workers who were looking for substance P, a biologically active polypeptide [Amin et al., 1954]. They were aware that 5-HT could interfere with their estimations and isolated this as a contaminant of their biologically active fraction. Using bioassay techniques, 5-HT was positively identified. They found the concentration to vary between areas and for a number of years this absolute concentration was used as an index of the ‘activity’ of 5-HT in brain. A more dynamic approach was fostered by the isolation in brain of 5-HIAA, the major end product of 5-HT metabolism [Sharmann, 1960] and changes in the concentration of this metabolite in brain were used to assess the changes in 5-HT synthesis and breakdown [Ashcroft and Sharmann, 1962].

It is appropriate at this point to look at the group of methods available for estimating the synthesis and turnover of 5-HT, although they have been comprehensively reviewed in the past [Costa and Neff, 1970].

*Non-Isotopic Methods*

Under steady state conditions, these methods must rely on the use of a drug either to block the synthesis of 5-HT, or its breakdown, or block the efflux of the major metabolite, 5-HIAA, from brain. The rate of change in the various levels is then taken as an index of synthesis or turnover.
This general strategy has certain implicit criteria. Firstly, the often rapid change in pool size produced by these drugs must be unphysiological. In biochemical systems, control is often exercised either by product or end product inhibition and although there is no evidence for this factor operating in relation to 5-HIAA, there are reasonable grounds to believe that the level of amine at the terminal may govern indirectly the synthesis rate in the neuron [Aghajanian, 1972]. This will be discussed later.

Secondly, the use of drugs implies a belief in their specificity which is almost certainly unfounded. Few drugs have one action and pharmacology often deals with compounds with multiple sites of action. However, in defense of these methods, studies using various drugs have, on the whole, yielded remarkably similar results for the turnover of the amine.

Methods involving MAO Inhibition

The earliest methods [Tozer et al., 1966] depended on steady state conditions under which it was assumed that the efflux of 5-HIAA equals the synthesis of 5-HT. After MAO inhibition, the decline of 5-HIAA is exponential and by fitting the data to a log plot, a straight line is obtained which gives the turnover of 5-HT in whole brain as 0.41 µg/g/h. In the same experiments [Tozer et al., 1966], the use of the rate of rise of 5-HT after MAO inhibition was also used as an index of synthesis and a similar result is obtained (0.44 µg/g/h).

The criticisms of this method have been made before. The MAO inhibitors, particularly the hydrazine type, are highly reactive molecules and will inhibit enzymes other than MAO. Certainly in chronic administration, phenelzine has been shown to produce inhibition at several points in the cerebral metabolism of the 5-hydroxyindoles in the dog [Moir, 1971]. Secondly, it is postulated that 5-HT does not diffuse out of the brain. Thirdly, that the 5-HT system is an open, single compartment model, and finally that the rise in 5-HT does not affect synthesis by feedback inhibition. The authors are themselves aware of these possibilities. Macon et al. [1971] looked at the synthesis of $^3$H-5-HT from $^3$H-tryptophan injected intracisternally in rats treated with or without a MAO inhibitor. Their results suggest that there is a 40-percent inhibition of synthesis between 20 and 80 min after the onset of MAO inhibition. By the nature of the experiment it was not possible to determine how early the onset of this inhibition occurred. It could, in fact, have been a rapid consequence of the rise in 5-HT, possible by a neuronal feedback mechanism [Aghajanian et al., 1970].
Techniques for Studying Synthesis and Turnover of 5-HT in the Brain in vivo

**Methods involving Probenecid**

Probenecid blocks the efflux of 5-HIAA from brain. The rate of accumulation of this metabolite after administration of the drug has been used to estimate 5-HT synthesis by Tozer et al. [1967]. They showed a linear rise with time and a sustained elevation of the level in response to this drug despite MAO inhibition. Turnover by this method was similar to that obtained by the MAOI method (0.40 µg/g/h). Probenecid has, however, been found to raise tryptophan levels in brain [Tagliamonte et al., 1972] and this may, to some extent, increase the 5-HT synthesis.

**Compartmentation of the 5-HT Neuron**

One of the criticisms of the above methods is the acceptance of the assumption that the synthesis of 5-HT behaves as if it were occurring in a single, open compartment in which case, synthesis would equal turnover. If we define ‘turnover’ as the rate of replacement of stored amine, then there may be substantial differences between synthesis and turnover. Attention was focused on this problem by SEDVALL et al. [1968] in relation to the synthesis of noradrenaline (NA) in various tissues. They used a method of precursor labelling in which 14C-tyrosine was infused into animals and the formation of 14C-noradrenaline was determined after a relatively short interval. Results by this method gave synthesis rates of NA 2-to-3-fold those in the literature using other methods for the determination of turnover. This they explained by abandoning the single, open compartment model where synthesis is equal to turnover, and substituted a 2-compartment system where there was a small pool with a rapid turnover and a larger pool with a slower turnover. In this case, the apparent turnover, as determined by methods using either disappearance of endogenous noradrenaline after synthesis inhibition or by decrease in SA of 3H-NA, was 81.9 ng/g/h compared with an estimated synthesis rate of 150 ng/g/h. Similar anomalies should be considered with the 5-HT neuron.

**Isotopic Methods**

These methods should be more physiological in that drugs need not be employed and no change is produced in pool size. Secondly, they should shed some light on the possible compartmentation for the synthesis of 5-HT. LIN et al. [1969] gave an intravenous infusion of 14C-L-tryptophan in the
rat and determined the rise of labelled 5-HT. They assumed a single compartment and by elegant mathematical treatment, produced an estimate of the 5-HT synthesis. The fractional rate constants were virtually identical for 10-min periods up to 60 min indicating to them justification for the single compartment model. Their results compared favourably with other methods in quantitative terms. They point out that the specific activity of plasma tryptophan was used in calculations of synthesis rate; using specific activity of brain tryptophan the synthesis rate would have been 3 times the values accepted. The problem of plasma tryptophan is that it is largely (about 80%, varying from species to species) bound to plasma protein [McMenamy et al., 1957] and hence the specific activity of the free plasma tryptophan may be much higher than is apparent by measuring total plasma tryptophan which is what the present methods determine. Hence employing this in the calculation would give a value on 5-HT synthesis lower than the existing non-isotopic methods. In justification of this current usage, it is not known at what speed equilibration occurs between the free amino acid and that bound to plasma proteins, and if this were rapid, the assumption would be justified.

This work was extended in the method of Neff et al. [1971] who used a single, intravenous injection of $^3$H-tryptophan and estimated 5-HT synthesis. This eliminated the difficulty of restraining rats for the period of an infusion, but substituted the use of $^3$H-tryptophan rather than $^{14}$C-tryptophan. We have found, as have others [Schubert et al., 1970] that there is a rapid exchange of tritium and at 30 min 50% of the counts in brain are $^3$H-water. The adequacy of the separative procedures in relation to $^3$H-water and the metabolites become increasingly important with the duration of the experiment.

Control Mechanisms

Perhaps before embarking on further discussion, the control mechanisms for the synthesis of 5-HT should be considered and their relationship to the methods for determining synthesis and turnover.

L-Tryptophan Levels

Hydroxylation is the limiting step in the synthesis of 5-HT [Ashcroft et al., 1965]. It has also been shown that the enzyme is normally unsaturated,
Techniques for Studying Synthesis and Turnover of 5-HT in the Brain \emph{in vivo} \cite{Eccleston et al., 1965} in that increasing the concentration of the substrate increases formation of the amine. This was succinctly put by \textit{Jequier et al.} \cite{1967}: 'The relatively high Km observed for tryptophan indicates the enzyme may not be fully saturated with substrate normally and that the overall rate of serotonin synthesis may be partially dependent on available substrate.'

\textit{Tryptophan Loading}

A loading dose of tryptophan causes a rise of 5-HT synthesis and hence 5-HIAA in all areas of brain. Using this method and giving an intravenous dose of L-tryptophan (50 mg/kg) in the dog gave significant differences between areas, the highest initial rate of rise being in mid-brain and the lowest in the caudate. This method, would, however, give an indication of maximal synthesis in each area rather than the turnover under normal conditions. The tryptophan load also produces higher than normal levels of 5-HT and whether this excess 5-HT synthesised by increased tryptophan may have physiological effects is disputed. The work of \textit{Aghajanian} \cite{1972} indicates that tryptophan causes slowing of the firing rate of these neurons, possibly by receptor stimulation and neuronal feedback inhibition.

Certain drugs have been found to alter the concentration of tryptophan in brain \cite{Perez-Cruet et al., 1972}. Changes in synthesis of 5-HT in brain due to this phenomenon would be missed by a method which relied on the specific activity of plasma tryptophan. In some of our own experiments, probenecid increased not only the absolute level of tryptophan in brain, but also its specific activity. This might be by changes in binding of tryptophan to plasma proteins. Loss of binding sites may lead to increased specific activity of the free tryptophan and hence tryptophan of a higher specific activity enters the brain.

\textit{Control of Nerve Stimulation}

\textit{Aghajanian et al.} \cite{1966} have shown that stimulation of the raphe nucleus, the region of brain containing a large proportion of the cell bodies of the 5-HT neurons, results in an increase in the synthesis of 5-HT as exemplified by a rise in 5-HIAA.

We \cite{Shields and Eccleston, 1972} attempted to examine further this phenomenon by using a modification of the isotope method. $^3$H-tryptophan
Fig. 1. Accumulation of 5-(3H)HT after giving (3H) tryptophan. Results show mean ±SD of three animals.

Table I. Effect of stimulation at different currents

<table>
<thead>
<tr>
<th>Current, mA</th>
<th>Ratio (× 10³)</th>
<th>Control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT d.p.m./CG50 effluent dpm</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>32.7±4.9 (9)</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>49.7±19.9 (3)*</td>
<td>152.0</td>
</tr>
<tr>
<td>0.2</td>
<td>70.5±16.3 (9)**</td>
<td>215.7</td>
</tr>
<tr>
<td>0.4</td>
<td>82.2±10.1 (3)**</td>
<td>251.6</td>
</tr>
</tbody>
</table>

Pargyline was given 15 min and 3H-tryptophan 30 min after the beginning of stimulation, which was continued until the animals were killed 30 min later. Results show mean ±SD, with the number of animals in brackets.

* p<0.05.

** p<0.001 compared to controls (Student's t-test).

is injected after a MAO inhibitor and the rise of 3H-5-HT assessed (fig. 1). The experiments consist of stimulation of an anaesthetised rat by an electrode stereotactically placed in the raphe. Stimulation leads to a 100-percent increase in 3H-5-HT counts, but surprisingly to no detectable change in 'cold' 5-HT (table I). Sheard and Aghajanian [1968] under similar conditions, showed a 10-percent rise in the amine. This rise in 5-HT does not appear to be the result of a change of specific activity of tryptophan in brain (table II). Pretreating the animals with PCPA, a hydroxylase inhibitor, led to the finding of a similar percentage increase in activity (table III). We would interpret these findings as evidence for a small pool of hydroxylase at the nerve terminal which is 'switched on' by stimulation but which is under normal circumstances quantitatively small. Because of the small pool size, these results are not necessarily at variance with those of Carlsson presented
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**Table II.** Effect of stimulation on brain tryptophan and endogenous H-5T

<table>
<thead>
<tr>
<th>Group</th>
<th>Tryptophan, μg/g</th>
<th>Tryptophan specific activity, dpm/ng</th>
<th>Percent of CG50 5-HT, effluent dpm as 3H-tryptophan μg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated</td>
<td>5.9±1.0 (4)</td>
<td>1.65±0.3 (4)</td>
<td>51 ± 7 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>6.4±0.7 (4)</td>
<td>1.63±0.4 (4)</td>
<td>48.4±4 (4)</td>
</tr>
</tbody>
</table>

Results show mean ± SD, with the number of animals in brackets and were measured in the 0 and 0.2 mA groups in table I.

**Table III.** Effect of stimulation following PCPA administration

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio (×10^3) 5-HT dpm/CG50 effluent dpm</th>
<th>Control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.0±2.7 (3)</td>
<td>100</td>
</tr>
<tr>
<td>Stimulated</td>
<td>17.4±5.4 (4)*</td>
<td>218</td>
</tr>
</tbody>
</table>

Animals received a single dose of PCPA (270 mg/kg) 3 days before stimulation. Other protocol as in footnote to table I. Results show mean ± SD, with the number of animals in brackets.

* p<0.05 compared to controls.

at this meeting, who found a high turnover of 5-HT even after section of these neurons in brain.

**Switch On/ Switch Off Process**

Although we have not determined how rapidly hydroxylase activity increases as a consequence of stimulation, we have looked at the question of how quickly this is switched off. The experiment consists of stimulating for 1 h and then labelling the 5-HT pool by the method described above. As is seen (fig. 2) synthesis is elevated at the end of stimulation compared with controls and by 30 min is back to normal.

We also investigated whether the small pool could be preferentially released by nerve stimulation. The neuronal 5-HT is labelled using an injection of 3H-tryptophan and at 1 h stimulation is applied. It can be seen that there is a fall in 5-HT counts in the stimulated animals (fig. 3). That this is not just a release from a large pool is indicated by a significant fall in specific activity from controls, in other words, the indication of a selective release of high specific activity 5-HT.
Fig. 2. 5-HT synthesis during and after stimulation. Animals received pargyline 45 min and $^3$H-tryptophan 30 min before killing. The curve shows the value of 5-HT dpm/effluent dpm as a percentage of the initial point. Each point is the mean of four or more observations. Vertical bars show SD.

Fig. 3. Effect of stimulation on $^3$H-5-HT levels following injection of $^3$H-tryptophan (100 μCi/kg i.p.). Stimulation was begun 60 min after injection. There is a significant difference between the initial group and the 75-min stimulated group ($p<0.005$, t-test), and between the stimulated and control groups at 120 min ($p<0.05$). The difference between the stimulated and control groups at 75 min approaches significance ($0.05<p<0.1$). Vertical bars show SEM; numbers of observations are given in brackets.
Techniques for Studying Synthesis and Turnover of 5-HT in the Brain in vivo

The sensitivity of the method and the rapid switch off in synthesis is shown by the use of LSD. This drug on injection gives a rapid and almost complete inhibition of the firing of raphe cells, Foote et al. [1969]. Large doses of LSD, frequently greater than 1 mg, have been used in the past to detect the inhibition of the synthesis of 5-HT. In contrast, the doses used by Aghajanian are of the order of 10 μg/kg. In the present experiments, doses down to 50 μg/kg (fig. 4) produced significant reduction in 5-HT synthesis as judged by the labelling method. The switch off process is seen then to be rapid, both as judged by the firing rate of the cells, and also biochemically by the above experiment (21 min).

Our method does not give a quantitative but only a qualitative estimate of the synthesis of 5-HT. It may be more sensitive than the other isotope methods but has the disadvantage of using drugs, in particular MAO inhibitors, which may switch off raphe neurons, presumably by the accumulation of 5-HT and post-synaptic receptor stimulation [Aghajanian et al., 1970].

Allosteric Activation of Existing Enzyme

These results indicate a quantitatively small pool of 5-HT in which changes are rapid and in direct response to the rate at which the 5-HT neuron
is firing. The changes may be considerable on a percentage basis within this pool but because the pool is small, may not lead to a large increase in synthesis when considering total turnover. The radioisotope methods may pick up this pool, if looked at early after injection and it is postulated that this enzyme pool is closely linked with tryptophan transported in the region of the nerve ending and hence the newly transported tryptophan is preferentially converted to the amine. The results of the PCPA experiment and the speed of the switch on/switch off in relation to stimulation suggest an allosteric mechanism rather than synthesis of new enzyme [SHIELDS and ECCLESTON, 1972]. Similar suggestions have been made by MEEK and NEFF [1972] using other experimental methods.

In conclusion, the non-isotope methods are suitable for turnover studies assuming the small pool related to nervous activity is quantitatively small. The radioisotope methods give more information and can be enhanced in value by the measurement of labelled 5-HIAA [DIAZ and HUTTENON, 1972] formed. Finally, the control mechanisms operating the synthesis of 5-HT must be considered when examining methods to investigate the synthesis and turnover of 5-HT.

Summary

Both the isotopic and non-isotopic methods appear to be in good agreement on the rates of synthesis of 5-HT. The arguments mostly question whether the neuron behaves as a single or a multiple compartmental system. Experiments using electrical stimulation to increase the firing rate or LSD to decrease the firing rate of 5-HT neurons suggest the existence of a quantitatively small synthesis pool in which allosterically controlled tryptophan hydroxylase enzyme is rapidly switched on or off in response to nervous activity.

References

Techniques for Studying Synthesis and Turnover of 5-HT in the Brain in vivo


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SECTION VI
GLYCOL SULPHATE ESTER FORMATION FROM $[^{14}C]$NORADRENALINE IN BRAIN AND THE INFLUENCE OF A COMT INHIBITOR

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Abstract—A method is described allowing the identification and separation of the sulphate esters of the glycol metabolites of $[^{14}C]$noradrenaline injected into the lateral ventricle of the rat. The esters of both the 3,4-dihydroxy derivative and the 3-methoxy-4-hydroxy derivative are formed in substantial amounts. It is suggested that the quantity of the former may have been underestimated in the past. Contemporaneous administration of pyrogallol, a catechol-O-methyl transferase inhibitor, with $[^{14}C]$noradrenaline leads to a considerable fall in 3-methoxy-4-hydroxyphenylglycol sulphate without a rise in 3,4-dihydroxyphenylglycol sulphate although free 3,4 dihydroxyphenylglycol rises significantly. It is proposed that the latter may be an index of intraneuronal metabolism of noradrenaline and 3-methoxy-4-hydroxyphenylglycol that of released amine.

After injecting $[^{14}C]$noradrenaline (NA) into the lateral ventricle of the cat, MANNARINO, KIRSHNER and NASHOLD (1963) showed that it was metabolised to 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) and 3,4-dihydroxyphenylethylene glycol (DHPG). Similarly, MHPG and DHPG formation was demonstrated by Rutledge and Jonason (1967) when they incubated rabbit brain slices with either $[^{14}C]$dopamine or $[^{3}H]$NA. Schanberg, Schildkraut, Breese and Kopin (1968a) using $[^{3}H]$normetanephrine injected intraventricularly in the rat were able to show the major metabolite to be the sulphate conjugate of the glycol MHPG-SO$_4$ and postulated from this and work with labelled NA that this conjugate was also the major metabolite of NA in brain. Subsequent studies using gas chromatography have shown MHPG to be present in brain in a number of species (SCHANBERG, BRESEE, SCHILDKRAUT, GORDON and KOPIN, 1968b, SHARMAN, 1969).

Our own experiments (unpublished observations) have demonstrated that after intraventricular injection of $[^{14}C]$NA into the rat, DHPG is also produced in the conjugated form. DHPG, in common with other compounds containing the catechol group, is easily oxidised and this may lead to a low recovery through extraction methods giving a misleading impression of the amount formed. For this reason, it was thought desirable to investigate the amounts of each sulphate formed without recourse to hydrolysis. The effect of pyrogallol on the formation of these sulphates has also been examined, since as an inhibitor of catechol O-methyl transferase, it would be expected to decrease the amount of MHPG-SO$_4$ formed, and assist in the identification of the two sulphates.

Abbreviations used: NA, noradrenaline; NM, normetanephrine; MHPG, 3-methoxy-4-hydroxyphenylethylene glycol; DHPG, 3,4-dihydroxyphenylethylene glycol; DHMA, dihydroxy mandelic acid; VMA, vanillyl mandelic acid; MAO, monoamine oxidase; COMT, catechol-O-methyl transferase.

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METHODS

Male Wistar rats (120–150 g) under light ether anaesthesia were injected with $^{14}$C noradrenaline (0.25 μCi, specific activity 27.6 μCi/mmol, Radiochemical Centre, Amersham) dissolved in 0.1 ml Merlis solution according to the technique of Noble, Wurtman and Axelrod (1967). Where appropriate, pyrogallol was given in the same solution in doses of 3, 7, and 15 mg/kg. One hour after injection, the rats were killed by cervical fracture, the brains removed and homogenised separately in a glass homogeniser in 6 ml 0.4M perchloric acid with the addition of 1 mg ascorbic acid and 2 mg disodium EDTA.

After centrifugation at 10,000 g for 10 min at 5°C, the precipitate was rehomogenised in 3 ml perchloric acid and centrifuged as before. The two supernatants for each brain were pooled and the pH adjusted to 4.0 with potassium hydroxide. The precipitated potassium perchlorate was spun down at 900 g for 5 min after chilling. The supernatants were then adjusted to pH 7.5 and passed over an Amberlite CG-50 resin, 100–200 mesh (NH$_4^+$ form) in a 7 x 0.7 cm column to remove amines.

In order to extract any acids and free glycols, the effluents from the column were taken to pH 1 and extracted four times with 2 vol. ethyl acetate. A 1 ml aliquot, about one ninth, of the aqueous solution left, believed to contain only the sulphate esters of the glycols, was then chromatographed. Aliquots were taken at each stage for determination of radioactivity in different fractions.

Paper chromatography. Extracts containing the glycol sulphates were applied under a stream of nitrogen to 4 cm-wide strips of Whatman No. 1 paper, and developed by descending chromatography in tanks previously saturated in nitrogen and in the solvent to be used.

The following systems were employed:

- **System 1. Chloroform–methanol–0.88 (w/v) ammonia (12:7:1).** Developed for 12 h.
- **System 2. Isobutyl methyl ketone–4% formic acid (10:1).** The organic phase was used as the developing solvent for 6 h.
- **System 3. n-butanol–acetic acid–water (4:1:1).** Developed for 18 h.
- **System 4. methanol developed for 2 h.**

In paper chromatography for the identification of MHPG and DHPG authentic markers were added to the chromatograms and after development, these were visualised by spraying with a mixture of paranitroaniline, 0:1% in 1M HCl–10% sodium nitrite (10:1) allowed to dry and resprayed with 1M NaOH in 50% ethanol.

**Examination of the glycol conjugates.** The two conjugates were isolated as described above and separated by paper chromatography using System 1 as the developer. The appropriate strips were eluted in methanol which was then reduced in volume under a stream of nitrogen. Separate aliquots were taken through the following procedures.

(a) **Hydrolysis and extraction of glycols**

Each conjugate fraction, in a small volume of methanol, was diluted to 3 ml with water. A sulphatase–glucuronidase mixture (Industrie Biologique Française) containing 27,000 Roy units of sulphatase (1 Roy unit hydrolyses 1 μmol of nitrocatechol sulphate h at pH 5 and 37°C) in 0.3 ml molar-sodium acetate buffer, pH 5-5 was added together with two drops of chloroform to reduce bacterial decomposition and the tube incubated at 37°C for 20 h. After incubation, the solutions were saturated with sodium chloride and extracted at pH 1 three times with 2 vol. ethyl acetate. This was back extracted with 1/5 vol. of 0.5 M-phosphate buffer pH 7.6 to remove any acid impurities. The ethyl acetate containing the glycol was blown down to a small volume and chromatographed in System 2 together with authentic DHPG, MHPG, 3,4-dihydroxymandelic acid (DHMA) and vanillyl mandelic acid (VMA) (Sigma Chemical Co.).

(b) **Separation of products of hydrolysis**

An aliquot of either MHPG-SO$_4$ or DHPG-SO$_4$ was added to supernatant prepared from the brains of untreated rats as described above and incubated at 37°C with sulphatase enzyme at pH 5–5 for 20 h. The products of hydrolysis were chromatographed directly in System 2 together with authentic DHPG, MHPG, DHMA and VMA.

(c) **Re-chromatography of conjugates**

The prepared conjugates were re-chromatographed in System 1 to check their purity.

(d) **Determination of recovery**

Aliquots of either $^{14}$C MHPG-SO$_4$ or $^{14}$C DHPG-SO$_4$ prepared by the above methods, were added to the perchloric acid precipitated supernatant from the brain of untreated rats and taken through the isolation and separation procedure as described above to determine the recovery of the conjugate through the method. Similarly, recoveries were determined for free $^{14}$C MHPG and DHPG.
[*4C]DHPG prepared as described in section (a) and taken through the hydrolysis procedure and subsequent method. 

Estimation of total glycols. The effluent from the CG 50 column was incubated for 20 h with 100,000 Roy units of sulphatase in 0.1 M-acetate buffer at 37°C. The solution was salt saturated and extracted at pH 1 three times with 2 vol. ethyl acetate. This was back extracted with 1/5 vol. of 0.5 M phosphate buffer (pH 7.6) to remove the acids. The ethyl acetate fraction was reduced in volume and chromatographed in System 2.

Counting of samples. The activity on the chromatograms was established by cutting the papers into 1 cm strips which were immersed in a toluene solution containing 4.25 g/l PPO and 0.112 g/l dimethyl-POPOP. Liquid samples were dissolved in 4 ml of 3:1 ethanol-methanol mixture to which was added 10 ml scintillator solution and counted on a Nuclear-Chicago Mark II scintillation counter, the efficiency being determined with an external standard.

RESULTS

Chromatography of the conjugate fraction to System 1 gave a clear separation into two main peaks (Fig. 1). Radioactivity at the origin represents 6–10 per cent of the total on the paper. The two peaks corresponding to DHPG-SO₄ and MHPG-SO₄ are in the approximate ratio of 3:5.

Chromatography of the conjugate fraction in System 2 failed to show the presence of vanillyl mandelic acid, dihydroxy mandelic acid or either of the free glycols. In System 3, two small peaks were separated from the rest of the activity. They formed less than 6 per cent of the total. System 4 did not demonstrate any impurities.

![Fig. 1.—Descending chromatography of conjugate fraction in system 1 (chloroform-methanol-0.88 (w/v) ammonia, 12:7:1) for 12 h.](image)

When the two peaks obtained after chromatography in System 1 were eluted and hydrolysed, each on extraction with ethyl acetate gave only one major identifiable peak with the same Rₚ as either authentic DHPG (Fig. 2) or MHPG (Fig. 3) and these substances passed through the extraction procedure as do the glycols. Chromatography of the hydrolysed conjugate showed only the presence of presumed unhydrolysed conjugate and a second peak corresponding to either DHPG or MHPG (Fig. 4). Re-chromatography of the unhydrolysed conjugate showed no major breakdown product.
It is concluded that, within the limits of the chromatographic systems used, a reasonable method for isolating the two sulphates has been achieved.

*Control levels of DHPG-\(SO_4\) and MHPG-\(SO_4\).* The recovery of the two sulphate esters was found to be identical with a mean value of 69 per cent (\(n = 5\), s.d. ± 2).

The relative values for DHPG-\(SO_4\) and MHPG-\(SO_4\) are given in Table 1. Together they represent 80 per cent of the deaminated metabolites remaining with the brain 1 h after the injection of the noradrenaline, DHPG-\(SO_4\) constituting over one third of this.
FIG. 4.—After hydrolysis of the separate conjugate fractions, a small portion of each was chromatographed directly in system 2 without extraction to check whether other hydrolysis products were formed. The activity at the origin may be in part unhydrolysed conjugate which does not move on this system or may be glycol retained at the origin by protein from the enzyme preparation.

Table 1.—Levels of sulphate esters in various fractions in rats treated with pyrogallol and noradrenaline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amines retained by CG-50 column</th>
<th>Free acids and free glycols</th>
<th>DHPG-SO₄</th>
<th>MHPG-SO₄</th>
<th>Ratio DHPG-SO₄: MHPG-SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [¹⁴C]NA only</td>
<td>43·4 ± 2·3</td>
<td>15·9 ± 1·8</td>
<td>15·1 ± 2·3</td>
<td>25·5 ± 4·1</td>
<td>0·6 ± 0·06</td>
</tr>
<tr>
<td>[¹⁴C]NA + 3 mg/kg pyrogallol</td>
<td>47·0 ± 3·0</td>
<td>30·7 ± 2·1</td>
<td>19·0 ± 3·6</td>
<td>3·3 ± 1·4</td>
<td>6·2 ± 1·8</td>
</tr>
<tr>
<td>[¹⁴C]NA + 15 mg/kg pyrogallol</td>
<td>51·4 ± 5·6</td>
<td>26·2 ± 1·1</td>
<td>15·4 ± 5·1</td>
<td>0·7 ± 0·3</td>
<td>23·1 ± 4·8</td>
</tr>
</tbody>
</table>

The rats were treated intraventricularly with either 3 or 15 mg/kg of pyrogallol together with [¹⁴C] noradrenaline and killed 1 h later. Control rats received [¹⁴C] noradrenaline alone. All values given are means ± s.d. as a percentage of total activity processed. DHPG-SO₄ and MHPG-SO₄ values are corrected for recovery (69 per cent).

Effect of pyrogallol. When pyrogallol was given intraventricularly along with [¹⁴C]NA there was a marked and dose-dependent decrease in the amount of MHPG SO₄ formed (Table 1). This decrease is almost entirely responsible for the observed change in DHPG-SO₄: MHPG-SO₄ ratio, for while DHPG-SO₄ levels did rise in the group treated with 3 mg/kg pyrogallol, the change was slight and not significant (P = 0·056, one tailed Mann Whitney U-test). With 15 mg/kg the amount of DHPG-SO₄ was close to the control value. A rise in the activity of the fraction containing
free glycols and acids was noted. The size of this change is sufficient to account for the decrease in total sulphates.

**Effect of pyrogallol on total glycols.** The dose of pyrogallol used in this case was 7 mg/kg. As expected, the activity identified as MHPG is much reduced by pyrogallol (Table 2) but unlike the sulphate, total DHPG is doubled. Recoveries for $[^{14}\text{C}]$DHPG and $[^{14}\text{C}]$MHPG were found to be 11.2 per cent ± 1.3 (4) and 42.6 per cent ± 3.3 (4) respectively (mean ± s.d. and number of observations in brackets). The results (Table 2) are corrected for the recovery of free glycol, the rate of hydrolysis of the conjugate being unknown, and hence the values are approximate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity in each fraction as percentage of total activity processed.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free + conjugated</td>
</tr>
<tr>
<td>Control n = 4</td>
<td>10.7 ± 4.1</td>
</tr>
<tr>
<td>7 mg/kg pyrogallol n = 4</td>
<td>22.3 ± 2.8</td>
</tr>
</tbody>
</table>

The rats were treated intraventricularly with 7 mg/kg of pyrogallol and $[^{14}\text{C}]$noradrenaline. Control animals received $[^{14}\text{C}]$noradrenaline alone.

The values given are means ± s.d. and are corrected for the recovery of free glycol taken through the procedure.

**DISCUSSION**

In the experiments of Mannarino *et al.* (1963) in the cat, only trace amounts of DHPG were found in brain as a metabolite of $[^{14}\text{C}]$NA. Schanberg *et al.* (1968b) in similar studies in the rat also gives low values for DHPG in relation to MHPG-\(\text{SO}_4\), although it is not clear whether the concentrations are before or after enzyme hydrolysis. In contrast using rabbit brain slices Rutledge and Jonason (1967) showed that DHPG had double the rate of formation of MHPG. The sulphate conjugates were, however, not estimated and the experiment is subject to the criticism that methylation in slices may be limited by the availability of the methyl donor S-adenosyl methionine, hence likely to give high DHPG/MHPG ratios. Sharman (1969) was able to show that concentrations of endogenous free DHPG were equivalent to that of MHPG in the hypothalamus of rabbit, mouse and cat.

Our experiments were intended to identify the esters of the glycols formed from $[^{14}\text{C}]$noradrenaline and their relative proportions. After separation of amines, acid metabolites and free glycols, two radioactive metabolites remained, one of which appeared to be MHPG-\(\text{SO}_4\) and which on hydrolysis gave a substance running on paper chromatography in an identical way with authentic MHPG. The second compound behaves similarly to MHPG sulphate in the chromatographic systems used, except the alkaline system (Chloroform–methanol–ammonia) where separation occurred, indicating the presence of a weakly acidic group not present in MHPG-\(\text{SO}_4\). Hydrolysis of this compound with a sulphatase/glucuronidase mixture released a substance which behaved in a way similar to authentic DHPG both through the extraction procedures and on chromatography. Since Schanberg and his colleagues have
shown that the compound they obtained from normetanephrine was the sulphate ester rather than the glucuronide of MHPG, we would identify this second compound as DHPG-SO₄. Thus the chromatography system described was found to separate MHPG and DHPG sulphates. Separation of these two labelled esters allows a determination of their concentration prior to enzymic hydrolysis.

The administration of [¹⁴C]noradrenaline by intraventricular injection leads to reservations as to whether it is metabolised in the same way as the endogenous amine. In the doses used, Fuxe and Ungerstedt (1968) have shown by fluorescence microscopy, and Glowinski and Axelrod (1966) by radioactive methods, that the exogenously administered compounds become distributed within the same neurones as those containing endogenous noradrenaline. The pattern of metabolism, which includes the glycol sulphates has, however, not been investigated in vivo. In some of our own experiments (unpublished observations) the longer the time after administration of intraventricular noradrenaline, the higher the relative proportion of glycol metabolites compared with the acids; and one would anticipate that the later observations approach more closely to the normal metabolic pattern.

From these findings, it would appear that at one hour, the pool size of DHPG-SO₄ was large enough to suggest that deamination by monoamine oxidase without subsequent methylation formed a significant portion of the metabolism of injected noradrenaline. However the level of metabolite at a single time is insufficient evidence from which to determine turnover. Because of the close chemical similarity of the two sulphones, their clearance from brain would be expected to be of the same order, and their respective pool sizes might reflect their relative rates of formation.

![Fig. 5.—Metabolism of noradrenaline. Abbreviations—NA, noradrenaline; NM, normetanephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; DHPG, 3,4-dihydroxyphenylglycol; DHMA, dihydroxy mandelic acid; VMA, vanillyl mandelic acid; MAO, monoamine oxidase; COMT, catechol-O-methyl transferase.](image-url)
Consideration of the metabolic pathways (Fig. 5) shows two potential sources for the formation of MHPG-SO_4 either from the deamination of normetanephrine or from the methylation of DHPG. It is currently considered that monoamine oxidase is largely intraneuronal, being associated with mitochondria, although in brain it has been suggested that a portion of the activity is microsomal (de Champlain, Mueller and Axelrod, 1969). COMT on the other hand is thought to be largely extraneuronal (Glowinski, Iversen and Axelrod, 1966). However, this does not appear to be completely true, there being a fall in COMT activity after denervation of certain tissues (Jarrott and Iversen, 1971). If in brain the COMT is extraneuronal and the monoamine oxidase intraneuronal, then DHPG formed within the cell, possibly from spill over of noradrenaline from storage particles would have to pass outside the neurone to be methylated to MHPG. Alternatively, MHPG could arise from normetanephrine formed extraneuronally which is deaminated after reuptake. If this latter suggestion is true then, since normetanephrine is thought to be an index of the rate of neurone release of noradrenaline, MHPG would indicate the amount of released noradrenaline, whilst DHPG the intraneuronal turnover of the amine.

Pyrogallol was administered intraventricularly at the same time as labelled noradrenaline in an attempt to clarify the site of MHPG formation. It was found that there was a considerable fall in the MHPG-SO_4 levels without a comparable rise in DHPG-SO_4. However, when one also took into consideration free as well as conjugated DHPG, there was a rise in total DHPG. This rise could result either from an inhibition of its methylation or as a consequence of more noradrenaline being available for deamination as normetanephrine formation is prevented. A further possibility is that pyrogallol inhibits the enzymes involved in sulphate esterification, and as a result clearance rates of these compounds from the brain may change. Although pyrogallol does not appear to provide an answer to the origin of MHPG and DHPG, it does, because of the fall in presumed MHPG-SO_4, add weight to the authenticity of its identity. Further work will be necessary on this system using labelled precursors of noradrenaline to give more accurate information on the relative proportions of the two glycol conjugates.

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SULPHATE ESTER FORMATION FROM CATECHOL-AMINE METABOLITES AND PYROGALLOL IN RAT BRAIN IN VIVO

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Abstract—A sulphotransferase enzyme capable of utilizing ethanolic or glycolic catecholamine metabolites and other phenols as substrates was studied in rat brain in vivo following the intraventricular injection of sodium $[^{35}S]$ sulphate and the subsequent isolation and identification of the labelled sulphate esters. A quantitative examination was made possible by the injection of increasing amounts of substrates of the enzyme together with sodium $[^{35}S]$ sulphate and the application of Michaelis-Menten kinetics. 3-Methoxy-4-hydroxyphenylethenglycol and 3,4-dihydroxyphenylethenglycol were shown to be readily esterified as was 3-methoxy-4-hydroxyphenylethanol ('half-saturating dose' of 5, 34 and 18 nmol respectively). Three esters of pyrogallol were isolated after its administration. This compound was also shown to inhibit sulphate ester formation from both substituted glycols, probably by competitive inhibition. The amines 5-hydroxytryptamine and normetanephrine were not found to be substrates for the sulphotransferase system in brain.

Sulphate ester formation, a reaction with high energy requirements, is a major route of metabolism of compounds which contain a phenolic group (Roy, 1971). On esterification, the properties of the recipient molecule are often considerably changed, conversion being from a unionized or poorly ionized compound to one with a completely ionized sulphate residue. This may modify the molecule to transform its behaviour in relation to, for example, membrane transport. Sulphate ester formation depends on the activation of sulphate ions followed by transfer to the acceptor molecule (Fig. 1). The formation of 'active sulphate' is a two-stage process: adeny1 sulphate

\[
\begin{align*}
\text{ATP} + \text{SO}_4^{2-} & \xrightarrow{\text{ATP-sulphurylase}} \text{APS} + \text{P}_{i} \\
\text{APS} + \text{ATP} & \xrightarrow{\text{APS-kinase}} \text{PAPS} + \text{ADP} \\
\text{R.OH} + \text{PAPS} & \xrightarrow{\text{Sulphotransferase}} \text{PAP} + \text{R.OSO}_4^- \\
\end{align*}
\]

**Fig. 1.**—Scheme for formation of 'active sulphate' with subsequent sulphate esterification.

**Abbreviations used:** DHPG, 3,4-dihydroxy-phenylethenglycol; MHPG, 3-methoxy-4-hydroxy-phenylethenglycol; APS, adenosine 5'-sulphatophosphate; PAPS, 3'-phosphoadenylylsulphate; PAP, 3' phosphoadenosine-5' phosphate; P_{i} = pyrophosphate.
(adenosine 5'-sulphatophosphate, APS) is produced from ATP and sulphate ion by the enzyme ATP-sulphurylase and this is subsequently converted to 3'-phosphoadenylylsulphate (3'-phosphoadenosine, 5'-phosphosulphate, PAPS) by interaction with ATP. This reaction is catalysed by APS-kinase. The sulphate group is then transferred from PAPS to form the sulphate ester by the enzymes generally classed as sulphotransferases. Certain compounds in brain form sulphate esters, amongst which are the glycol derivatives of noradrenaline, 3-methoxy-4-hydroxyphenylethylene glycol (MHPG; Schanberg et al., 1968) and 3-4-dihydroxyphenylethylene glycol (DHPG; Sugden and Eccleston, 1971). It may be that this process transforms the glycols to compounds which can be actively transported out of brain. The present experiments investigate the formation of sulphate esters of these and other compounds in brain in vivo using intraventricular injections of sodium [35S]sulphate with the subsequent isolation of labelled sulphate materials.

METHODS

A. Administration of glycols

Male Wistar rats 150–180 g were injected intraventricularly under ether anaesthesia as described by Noble et al. (1967) with 20 µl of Merlis (1940) solution containing 50 µCi of sodium [35S]sulphate (specific radioactivity 44-6 mCi/mmol; Radiochemical Centre, Amersham) either alone or containing 40 µg MHPG or 40 µg DHPG or 20 µg each of MHPG and DHPG. For comparison, other rats were injected intraventricularly with 0-25 µCi of dl-[3H]noradrenaline (specific radioactivity 27-6 mCi/mmol). All animals recovered from anesthesia and were killed 30 min after injection. Sulphate esters were isolated by the method of Sugden and Eccleston (1971). This consists of homogenization and precipitation of brain in 0-4 M perchloric acid, neutralization of supernatant fluid to pH 7-5 and its passage over a column of Amberlite CG50 resin to remove amines. The procedure was modified so that the effluent from the Amberlite column was extracted at pH 6 with 3 × 2 ml. of ethyl acetate to remove free glycols. A 1 ml sample, about one-sixth of the aqueous solution left, was applied to Whatman No. 1 chromatography paper and developed by descending chromatography in the following systems:

- Chloroform:methanol:(0-88 w/v) ammonia, 12:7:1, developed for 22 h (system 1).
- Isobutyl methyl ketone:4% formic acid 10:1, developed for 6-5 h (system 2).
- Butanol:acetic acid:water 4:1:1, developed for 18 h (system 3).

Radioactive substances on the chromatograms were located by cutting the papers into 1 cm strips which were immersed in a toluene solution containing 4-25 g/l PPO (2,5-diphenyloxazole) and 0-112 g/l dimethyl-POPPO (1,4-di-(2-(4-methyl-5-phenyloxazolyl)) benzene) and counted in a scintillation counter, the efficiency being determined by an external standard. In the experiments in which either DHPG or MHPG had been injected together with labelled sodium sulphate, the conjugates isolated prior to chromatography were hydrolysed as described by Sugden and Eccleston (1971) and portions chromatographed in systems 1 and 2.

B. Administration of normetanephrine

Single rats were injected with 50 µCi of sodium [35S]sulphate (specific radioactivity 30-2 mCi/mmol), together with either 4 µg of DL-normetanephrine or 1-3 µCi of DL-[3H]normetanephrine (specific radioactivity 5-6 Ci/mmol, New England Nuclear). A third rat was given [3H]normetanephrine alone. The conjugates were either isolated by paper chromatography as described above and the radioactivity on the chromatograms measured by dual label counting to detect both 35S and 3H, or those solutions containing 3H-labelled conjugates were subjected to enzyme hydrolysis as described by Sugden and Eccleston (1971) and a sample subsequently chromatographed in both systems 1 and 2. [3H]MHPG was prepared in brain homogenates from DL-normetanephrine by a modification of a method described previously. (Eccleston et al., 1966). The [3H]MHPG formed was extracted into ethyl acetate which was evaporated to near dryness under nitrogen and the glycol isolated by descending paper chromatography on Whatman No. 20 developed for 6-5 h on system 2. The [3H]MHPG was eluted from the paper into methanol, concentrated under a steam of nitrogen, and subsequently used as a marker to identify the 3H-labelled compounds liberated on hydrolysis of the sulphate conjugates.
C. Quantitative aspects of sulphate formation

(a) Variation in amount of sodium [35S]sulphate injected. Rats in groups of three were injected intraventricularly with 1, 10 or 100 μCi of sodium [35S]sulphate (specific radioactivity 187 mCi/mmol; corrected for decay of sodium [35S]sulphate prior to use) and killed at 30 min. The radioactivity in the MHPG. SO4 and DHPG. SO4 fractions was determined as described above on each individual animal.

(b) Time course of glycol sulphate formation. Rats in groups of three were injected with 2-2 μCi of sodium [35S]sulphate (specific activity 82 mCi/mmol) together with 0.5 μg of MIHPG and killed at 15, 30, 45 and 60 min. MHPG.35SO4 was isolated and the radioactivity was determined on each individual animal.

(c) Variation of injected dose of MIHPG. Rats in groups of three were injected with 3-3 μCi of sodium [35S]sulphate (specific activity 123 mCi/mmol) and varying doses of MIHPG from 0.2 to 4 μg. The animals were killed at 30 min and the MHPG. 35SO4 and DHPG. 35SO4 were determined in each individual animal.

(d) Variation in dose of DHPG. Since it was apparent, using large doses of DHPG, that methylation was occurring with the formation of MHPG.SO4 groups of three rats also received smaller doses, (0.5, 1, 2 and 4 μg) of DHPG, and the MHPG and DHPG conjugates were isolated and counted in each individual animal.

D. Formation of other sulphate compounds

In order to determine the range of compounds that would undergo sulphation in this system the following substances were injected together with 4 μCi sodium [35S]sulphate (specific activity 157 mCi/mmol) and the brains assayed for conjugate as described above after 30 min. (a) The alcohol derivative of dopamine, 3-methoxy-4-hydroxyphenylethanol in amounts of 2, 4 and 8 μg. (b) The amine 5-HT in a dose of 4 μg. (c) The amine dL-nor-metanephrine in doses of 2, 4 and 8 μg. In order to prevent deamination of normetanephrine to MHPG, monoamine oxidase was inhibited with a dose of 150 mg/kg of pargyline administered intraperitoneally 15 min prior to the intraventricular injection.

E. Influence of pyrogallol on sulphate ester formation

In order to determine whether or not pyrogallol inhibited the methylation of DHPG the following experiment was performed. A group of three rats was given the combination of 4 μg DHPG and 0.6 mg pyrogallol by intraventricular injection with sodium [35S]sulphate. The sulphate esters were isolated and counted as described above and compared with those isolated from groups of three animals given either 4 μg DHPG or 0.6 mg pyrogallol both with sodium [35S]sulphate. The result of this experiment suggested that sulphate formation was being interfered with by pyrogallol and led to the following experiment. Rats in groups of three were given 1 μg MHPG and 2 mg pyrogallol with sodium [35S]sulphate or 4 μg MHPG and sodium [35S]sulphate. Because it appeared that development of chromatograms for 22 h showed a loss of labelled pyrogallol sulphate esters, a sample of these esters from animals treated with pyrogallol alone were developed for 4.5 h. The finding of a number of pyrogallol [35S]sulphate esters led to an experiment to determine the 'half saturation dose' in which 10, 20, 50 or 100 μg of this drug was injected in groups of three rats with sodium [35S]sulphate. The development time of the chromatograms was varied to give optimum separation of the pyrogallol sulphates formed.

RESULTS

A. Isolation and identification of the sulphate conjugates

Injection of radioactive sulphate alone gave rise to two small peaks on paper chromatography which, in the light of subsequent experiments, were identified as DHPG.35SO4 and MHPG.35SO4. Of the three paper chromatography systems used only system 1 proved useful in separating these two glycol conjugates and consequently this system was used in all subsequent experiments. Both system 2 and system 3 gave isographic peaks of DHPG-SO4 and MHPG-SO4 and also of conjugates formed after the injection of [14C]noradrenaline. The unchanged sodium [35S]sulphate remained at the origin. Using system 1 the injection of 40 μg of DHPG together with sodium [35S]sulphate resulted in a rise in the presumed DHPG.35SO4 peak and a small rise in the MHPG.35SO4 peak (Fig. 2) whilst injection of 40 μg MHPG resulted in a rise in presumed MHPG.35SO4 (Fig. 3) without a rise in the DHPG conjugate. Injection
of 20 µg of both DHPG and MHPG gave rise to two conjugate peaks. Hydrolysis with sulphatase enzyme resulted in a disappearance of the conjugates (Figs. 2 and 3).

B. Formation of MHPG.SO₄ from normetanephrine

When non-radioactive DL-normetanephrine was injected with sodium [³⁵S]sulphate a peak could be isolated in system 1 in the MHPG.SO₄ position. Prior hydrolysis resulted in the disappearance of this peak, suggesting it was a conjugate. The mixture of [³H]normetanephrine and labelled sulphate led to the appearance of a similar peak which on dual label counting contained both ³H and ³⁵S counts. Hydrolysis and

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**Fig. 2.**—Paper chromatography of [³⁵S]sulphates in system 1 after intraventricular injection of DHPG and sodium [³⁵S]sulphate, showing DHPG.³⁵SO₄ and MHPG.³⁵SO₄ and their disappearance on hydrolysis.

**Fig. 3.**—Paper chromatography of [³⁵S]sulphates in system 1 after intraventricular injection of MHPG and sodium [³⁵S]sulphate showing MHPG.SO₄ and DHPG.³⁵SO₄ and their disappearance on hydrolysis.
development in system 1 resulted in the loss of this peak. Development in system 2 gave two peaks, one in the MHPG position containing $^3$H and a peak at the origin containing $^{35}$S. The MHPG position in this system was confirmed by the use of $[^3]$H]MHPG, and authentic non-radioactive MHPG run separately and sprayed with diazotized $p$-nitroaniline.

C. Quantitative aspects of sulphate formation

(a) Variation in amount of sulphate injected. The amount of DHPG and MHPG sulphate formed increased with the dose of labelled sulphate injected. Using 100 $\mu$Ci of sodium $[^3]$S sulphate is equivalent to 76 $\mu$g of sodium sulphate, and this appears to be no longer a tracer dose and to contribute significantly to the sulphate pool. The ratio of DHPG.$^{35}$SO$_4^-$ to MHPG.$^{35}$SO$_4^-$ counts in these experiments was roughly unity.

(b) Time course. The time course of sulphate formation after a single dose of 2.2 $\mu$Ci of sodium $[^3]$S sulphate is shown in Fig. 4. Presumably by 60 min an equilibrium is almost reached, when sulphate formation equals removal. At 30 min after injection, the time chosen for subsequent experiments, this equilibrium had not yet been reached.

![Graph showing time course of increase in MHPG.$^{35}$SO$_4^-$ counts on intraventricular injection of sodium $[^3]$S sulphate.](image)

Fig. 4.—Time course of increase in MHPG.$^{35}$SO$_4^-$ counts on intraventricular injection of sodium $[^3]$S sulphate. Each point represents the mean of three animals unless otherwise indicated.

(c) Variation of dose injected MHPG. Increasing the dose of MHPG injected intraventricularly with 5 $\mu$Ci sodium $[^3]$S sulphate resulted in a hyperbolic increase in MHPG.$^{35}$SO$_4^-$ counts. If the $^{35}$S counts at 30 min are taken as the equivalent of initial rates of formation of sulphate ($V$), then by plotting $S$, the amount of MHPG injected against $S/V$, a straight line plot is obtained. It appears that this model system obeys classical Michaelis–Menten kinetics (Fig. 5). However, kinetic constants can only be used to describe concentrations. In this case the concentration of MHPG in brain at enzyme sites is not known because this will depend on the penetration into brain of the MHPG which may be neither complete nor homogeneous. The term 'half-saturating
dose was consequently used to avoid ambiguity and for MHPG was found to be 5.1 nmol. In this experiment the radioactivity in the DHPG.SO₄ position did not vary.

Fig. 5.—Linear plot using Michaelis–Menten equation. $S = \text{nmol of MHPG injected intraventricularly}$. $V = \text{number of counts of MHPG.}^{35}\text{SO}_4$ formed in 30 min after injection.

Fig. 6.—Linear plots using Michaelis–Menten equation. $S = \text{nmol of DHPG injected intraventricularly}$. $V_{(\text{for DHPG.}^{35}\text{SO}_4)} = \text{number of counts of DHPG.}^{35}\text{SO}_4$ and $V_{(\text{for MHPG.}^{35}\text{SO}_4)} = \text{counts of MHPG.}^{35}\text{SO}_4$, both formed 30 min after injection. Each point represents the mean of three animals.
Variation in dose of DHPG. On injection of DHPG both DHPG and MHPG sulphates appeared in the final chromatogram (Fig. 2). Treatment of the data as described above gave a 'half-saturation dose' for DHPG of 34 nmol, whilst that for MHPG plotted against DHPG injected was 5·6 nmol (Fig. 6). This value was close to that obtained by injecting MHPG (Table 1).

Table 1.—'Half-saturation dose' for sulphate ester formation in brain using various substrates administered intraventricularly in rats

<table>
<thead>
<tr>
<th>Substrate</th>
<th>'Half-saturation dose' (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHPG</td>
<td>5·1</td>
</tr>
<tr>
<td>DHPG</td>
<td>34</td>
</tr>
<tr>
<td>MHPG (Formed from injected DHPG)</td>
<td>5·6</td>
</tr>
<tr>
<td>3-Methoxy-4-hydroxyphenylethanol</td>
<td>18</td>
</tr>
<tr>
<td>Pyrogallol conjugate A</td>
<td>20</td>
</tr>
<tr>
<td>Pyrogallol conjugate B</td>
<td>130</td>
</tr>
<tr>
<td>Pyrogallol conjugate C</td>
<td>90</td>
</tr>
</tbody>
</table>

D. Formation of other sulphate compounds

3-Methoxy-4-hydroxyphenylethanol gave a labelled conjugate which had a higher Rf in system 1 than those of the glycols, and could be isolated with only 4 h chromatographic development. Again the Michaelis–Menten plots gave a good straight line fit, with a 'half-saturation dose' of 18 nmol (Fig. 7). The amine 5-HT did not give rise to a peak in the sulphate chromatogram, nor did DL-normetanephrine when it was given to animals treated with MAO inhibitor to prevent its conversion to MHPG.

Fig. 7.—Linear plot using Michaelis–Menten equation. S = nmol of 3-methoxy-4-hydroxyphenylethanol injected intraventricularly. V = number of counts of 3-methoxy-4-hydroxyphenylethanol. $^{35}$SO₄ formed in 30 min after injection. Each point represents the mean of three animals.
Table 2.—Inhibition of sulphate conjugate formation by pyrogallol

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyrogallol (mg/kg)</th>
<th>Conjugate (%)</th>
<th>(%) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPG</td>
<td>none</td>
<td>100</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>58</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>(%) inhibition</td>
<td>42</td>
<td>74</td>
</tr>
<tr>
<td>MHPG</td>
<td>none</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>(%) inhibition</td>
<td>44</td>
<td>93</td>
</tr>
</tbody>
</table>

The results are expressed as a percentage of the major conjugate formed. DHPG\(\text{SO}_4\) taken as 100 per cent in the case of injected DHPG and MHPG\(\text{SO}_4\) as 100 per cent when MHPG was injected.

E. Pyrogallol and sulphate ester formation

The administration of the combination of pyrogallol (0-6 mg) and DHPG (4 \(\mu\)g) resulted in not only a lower concentration of MHPG\(\text{SO}_4\) than that obtained after giving DHPG alone, but the level of DHPG\(\text{SO}_4\) was also reduced (Table 2). A dose of 2 mg of pyrogallol almost completely inhibited the formation of MHPG\(\text{SO}_4\) from...

Fig. 8.—Linear plots using Michaelis–Menten equation for the formation of three conjugates of pyrogallol. \(S = \text{nmol pyrogallol injected.} \ V = \text{number of counts of pyrogallol}^{35}\text{SO}_4 \text{formed in 30 min after injection. Conjugates A, B, C, have increasing} \ R_f \text{values in that order on system 1. Each point represents the mean of three animals.}
4 μg of MHPG when given together. There was no evidence in these chromatograms of peaks representing pyrogallol [35S]sulphates. However, when the chromatograms were developed by descending chromatography for 4-5 h, three major peaks of radioactivity with high Rf values were found which must have previously run off the end of the paper. The first of these peaks may have contained two incompletely separated sulphates, since occasionally a chromatogram suggested two peaks were present. With varying doses of pyrogallol it was possible to give an estimate of the ‘half-saturating dose’ for the conjugates (Fig. 8 and Table 1).

**DISCUSSION**

GREGORY and LIPMANN (1957) when investigating the sulphotransferase system measured PAP in various tissues and found it to be present in brain. This suggested that the brain contained the PAPS, or ‘active sulphate’ synthesizing system, and this was subsequently shown to be the case (BALASUBRAMANIAN and BACHHAWAT, 1961). These authors used a supernatant fraction from rat brain and measured the incorporation of radioactive sulphate from K235SO4 into PAPS. The experiments reported here use NA235SO4 injected intraventricularly into anaesthetized rats. No attempt was made to estimate PAPS, but the sulphate esters of various compounds which were the products of the radioactive sulphate transfer from PAPS were estimated. The identification of the conjugates formed in these experiments after the intraventricular injection of labelled sodium sulphate depends on their behaviour both through the separation procedure and on paper chromatography being identical with known MHPG and DHPG sulphates formed by the intraventricular administration of [14C] noradrenaline. In experiments where sodium [35S]sulphate was injected alone, two radioactive peaks were separated which were isographic with authentic MHPG.S04 and DHPG.S04. The administration of sodium [35S]sulphate together with non-radioactive MHPG led to a rise in the conjugate identified as MHPG.S04, whilst injection of large amounts of non-radioactive DHPG and labelled sodium sulphate intraventricularly led to a large rise in the conjugate identified as DHPG.S04 and to a smaller extent MHPG.S04. In all these experiments prolonged hydrolysis with sulphatase enzyme led to the disappearance of these two peaks, as would be expected if they were true conjugates. One of the known precursors of MHPG, normetanephrine (SCHANBERG et al., 1968) when given together with sodium [35S]sulphate, led to the formation of a conjugate behaving chromatographically like MHPG.S04 and when [3H]normetanephrine was given with labelled sodium sulphate this conjugate peak was found to contain both 3H and 35S on dual label counting. Enzyme hydrolysis of the conjugate before chromatography removed it and 35S counts were then located on the paper in the free sulphate position. The 3H counts formed a peak which appeared isographic with authentic free MHPG.

It appeared, therefore, that radioactive sulphate given in this way labelled the endogenous substances, the glycol metabolites of noradrenaline in particular, and may be used to estimate indirectly their relative concentrations. The rate of formation of DHPG and MHPG has been estimated by other methods previously. SUGDEN and ECCLESTON (1971) found that after the administration of [14C]noradrenaline by intraventricular injection the molar ratio of DHPG.S04:MHPG.S04 in the rat brain was 3:5. In the present experiments this ratio was found to be unity, a ratio which has been
confirmed by measuring endogenous glycol sulphates in rat brain by gas chromatography (Walter and Eccleston, unpublished observations). Optimum parameters for investigating sulphate ester formation were found using an injection of 5 μCi of sodium [35S]sulphate of high specific activity (180 mCi/nmol) the animals being killed at 30 min. At this dose the radioactive sulphate injected did not greatly increase the sulphate pool. If the radioactivity found in the sulphate esters at this time and dose gave an index of the initial rates of formation of the esters, then the quantitative aspects of the sulphotransferase enzyme could be examined. This was achieved by increasing the dose of MHPG injected with the sulphate and determining the rates of formation of MHPG.35SO4. This is similar to a Michaelis–Menten kinetic analysis, in which the initial velocity of a reaction is determined against substrate concentration. Application of enzyme kinetics to the results appeared to be justified by the linear plots obtained if the dose of substrate (S) injected into the brain was plotted against (S/V). It could be that this particular model is an assessment of the limiting enzyme in the pathway of the formation of sulphate esters. Initially PAPS, which is a high energy compound, is formed and transfers the sulphate group to the substrate by means of the sulphotransferase enzyme. The assumption was that labelled PAPS was being formed in excess and these experiments examined the sulphotransferase enzyme in sulphate ester synthesis. Using the kinetic analysis a ‘half-saturating dose’ (equivalent to Km for in vitro enzyme studies) was estimated for MHPG as 5-1 nmol. The true molar concentration at enzyme sites is unknown because of the gradient of concentration of substrate from the ventricles into brain, but employing these calculations enables a comparison to be made between various substrates (Table 1).

When large doses, 40 μg of DHPG, were administered, there was a rise in the formation of DHPG SO4 and also the appearance of MHPG SO4. It was assumed that this was the result of methylation of the DHPG to MHPG with subsequent sulphate formation. This process was seen more clearly when smaller doses of DHPG were given (4 μg), when some 37 per cent of all sulphate counts appeared in the MHPG SO4 position. Using varying doses of DHPG, a ‘half-saturating dose’ of 34 nmol was obtained for the formation of DHPG SO4 from DHPG, which would indicate that this substance has a lower affinity for the enzyme than MHPG. However, methylation is taking place with the formation of MHPG and subsequently MHPG SO4. The ‘half-saturating dose’ for the formation of MHPG SO4 from DHPG was 5-6 nmol (Fig. 6). The amount of DHPG injected (S) was almost identical to that obtained when S was the amount of MHPG injected to investigate MHPG sulphate formation, ‘half-saturating dose’ 5-1 nmol, Fig. 5). This would suggest that when DHPG is used, sulphotransferase is the limiting enzyme step in the formation of MHPG sulphate from DHPG and that rapid methylation produces concentrations of MHPG at sulphotransferase enzyme sites as high as those obtained from direct injection of MHPG. This also means that the ‘half-saturating dose’ obtained for DHPG is probably too high.

An attempt was made to inhibit methylation of DHPG with pyrogallol so that a better estimate of the ‘half-saturating dose’ could be obtained. The results were paradoxical and showed not only inhibition of methylation which the formation of less MHPG SO4 from DHPG but also a fall in the DHPG SO4 level (Table 2). This suggested competitive inhibition of the sulphotransferase enzyme by pyrogallol, a compound with three phenolic groups, which together with the methylated derivatives
should all potentially form sulphate esters. This inhibition of the sulphotransferase enzyme was demonstrated using MHPG, pyrogallol and labelled sulphate. Sulphate ester formation from MHPG was found to be almost completely absent (Table 2). VESTERMARK and BOSTROM (1960) used a particle-free medium from liver containing the sulphate activating system and sodium $[^{35}S]$ sulphate to demonstrate the formation of labelled sulphates on incubation with pyrogallol. They concluded that both mono- and disulphates of pyrogallol were formed. As suggested by ROY (personal communication) it appears that pyrogallol in the doses used in our experiments inhibited the sulphate ester formation of the glycols by competitive inhibition, and this inhibition may account for the rise in free $[^{14}C]$MHPG and $[^{14}C]$DHPG from $[^{14}C]$NA in the presence of pyrogallol, together with a fall in DHPG.SO$_4$ and MHPG.SO$_4$, as found previously (SUGDEN and ECCLESTON, 1971). There appeared to be three or possibly four sulphate esters formed from pyrogallol. Using varying doses of pyrogallol, a 'half-saturating dose' was obtained for the formation of each of these conjugates, which could be clearly isolated from each other. The 'half-saturating dose' was of the order of 100 nmol compared with 5 nmol for MHPG and indicates a lower affinity of pyrogallol for the sulphotransferase enzyme (Table 1). This is, of course, assuming that these results reflect the activity of one sulphotransferase enzyme, which may not be the case. There may be several enzymes with differing specificities for the various substrates.

Attempts to demonstrate the formation of sulphate esters from normetanephrine, in the presence of a MAO inhibitor to prevent oxidative deamination to MHPG, were unsuccessful, as they were with 5-HT as a substrate. Even though 5-HT sulphate formation has been demonstrated in liver (HIDAKA et al., 1969), our finding is in keeping with the results of KORF and SEBENS (1970) who could not detect 5-HT sulphate in brain even after administration of a MAO inhibitor. In contrast 3-methoxy-4-hydroxyphenylethanol appeared to be a substrate for the sulphotransferase system with a 'half-saturating dose' of 18 nmol higher than that for MHPG (Table 1). KAROUN et al. (1971) have shown that 3-methoxy-4-hydroxyphenylethanol sulphate occurs in small quantities in human CSF and rat brain. These results confirm that some of the conjugates of the glycol metabolites of NA are sulphates and that brain forms esters both of these and the alcohol metabolites of dopamine as well as other 'foreign' compounds with phenolic groups such as pyrogallol. Perhaps in brain as in liver, (WILLIAMS, 1959) sulphate formation may be an important means of detoxicating potentially harmful substances, and in doing so enabling transport from the brain to be effected.

Acknowledgements—We would like to thank Dr. T. B. B. CRAWFORD and Mr. D. S. WALTER for their helpful advice.

REFERENCES


SECTION VII
INCREASE OF NORADRENALINE METABOLISM FOLLOWING ELECTRICAL STIMULATION OF THE LOCUS COERULEUS IN THE RAT

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Abstract—A sensitive gas–liquid chromatography method is described for measuring both free and conjugated 4-hydroxy-3-methoxyphenyl glycol (HMPG) in brain, which is applied to study the turnover of noradrenaline (NA) in brain when one Locus coeruleus (LC) is stimulated electrically. Stimulation was found to produce a rise in total HMPG on the ipsilateral side of brain when the current was sufficiently low to obviate current spread to the opposite LC. This suggests an increase in turnover of NA on electrical stimulation.

4-Hydroxy-3-methoxyphenylglycol (HMPG) and its sulphate conjugate have been shown by radioactive techniques to be major metabolites of noradrenaline (NA) in brain (Mannarino, Kirshner and Nashold, 1963, Schanberg, Breese, Schildkraut and Kopin, 1968a). HMPG and HMPG-SO₄ have also been demonstrated to be present in the brain of a number of species using gas–liquid chromatography (Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968b; Sharman, 1969) and HMPG–SO₄ in the rat brain by spectrophotofluorimetry (Meek and Neff, 1972). Noradrenergic nuclei in the brain stem of the rat were first demonstrated by Dahlström and Fuxe (1964) using fluorescence histochemistry. Of particular interest were two nuclei which appeared to correspond in site with the Loci coerulei, which nuclei were later shown to send NA axons to all parts of cortex (Ungerstedt, 1971). The reported experiments were designed to see whether electrical stimulation of one of these nuclei had any effect on the turnover of noradrenaline in the brain as indicated by a change in the levels of HMPG and HMPG-SO₄.

METHODS

Electrical stimulation. Male albino Wister rats 180–200 g were anaesthetised with 1-6 % halothane in oxygen, immobilized in a David Kopf No. 1530 stereotaxic frame, and anaesthesia maintained with 0-8 % halothane in oxygen. (Shields and Eccleston, 1972.) With the upper incisor bar set at 2-4 mm below the interaural line, the electrode tip was positioned at the Locus coeruleus (LC) with the following stereotaxic co-ordinates: 1-7 mm posterior to the interaural line; 1-0 mm lateral to the mid line; 6-5 mm ventral to the surface of the skull.

In initial experiments the stereotaxic coordinates of the LC in our hands was established by histology, taking frozen serial sections and staining with toluidine blue. Figure 1 shows the electrode track with the tip just dorsal to the LC. In subsequent experiments, however, no selection of animals was made on the basis of histology.

Unipolar electrodes were formed from stainless steel entymological pins 1-0 mm dia. coated with Bakelite varnish and the tip scraped clean. The indifferent electrode was clipped to the scalp wound. Stimulation was by 0-1 mA constant current pulses 2 ms in duration, alternation in polarity and at

1 Preliminary results were communicated to the 524th meeting of the Biochemical Society 27–28 March 1972 at University College London (Walter and Eccleston, 1972).

2 D. S. W. is an MRC scholar.
10/s, for 45 min. Control animals were maintained under anaesthesia for an equal time with electrodes implanted in the LC but without stimulation.

Two parameters of stimulation were investigated, the effect of the frequency of stimuli and the effect of the stimulus current strength on the concentration of total HMPG in rat cortex.

It was also of interest to see whether unilateral stimulation of the LC caused an ipsilateral rise of HMPG. Left and right cerebral cortex, therefore, was analysed separately after stimulation of the left Locus coeruleus.

**Estimation of HMPG and HMPG-SO<sub>4</sub>.** Weighed samples of brain tissue were homogenised in 5 ml of 0-4 m-perchloric acid at 5°C with an all-glass homogenizer, the homogenizer washed with a further 1 ml of 0-4 m-perchloric acid, the homogenate and wash combined and centrifuged at 10,000 g for 15 min. The clear supernatant was adjusted to between pH 4 and 5 with 5 m and 0-1 m-KOH by glass electrode. To obtain maximum precipitation of potassium perchlorate the solution was cooled to 5°C for 15 min prior to low speed centrifugation at 3000 g for 10 min at 5°C. The clear supernatant was buffered to pH 5-0 with the addition of 0-5 ml of 1-0 m sodium acetate buffer at pH 5-0.

![Graph showing hydrolysis of HMPG-SO<sub>4</sub>](image)

**Fig. 2.—Rate curves showing the hydrolysis of HMPG-SO<sub>4</sub> at different concentrations of 'Helicase' enzyme preparation. Each point represents the mean of two determinations.**

- ● 9 mg/ml
- △ 0-9 mg/ml
- × X 2 mg/ml by GLC

At this stage free HMPG may be extracted from the supernatant by shaking twice for 3 min with 15 ml and 10 ml of redistilled ethyl acetate. In each case 12 ml and 10 ml of the ethyl acetate extract was removed. Residual ethyl acetate in the aqueous phase was removed in vacuo prior to the addition of 'Helicase' enzyme. This was found to be important as ethyl acetate severely inhibits the activity of the enzyme.

A further 0-5 ml of 1-0 m-sodium acetate buffer pH 5-0 was added to the aqueous phase containing the conjugated HMPG. To each test tube was added 10 mg of 'Helicase' enzyme preparation (Industrie Biologique Francais) (0-2 ml of a solution 50 mg/ml) followed by two drops of CHCl<sub>3</sub> as an antibacterial agent. The tubes were then stoppered and placed in a water bath at 37°C.

Using [3H]HMPG-SO<sub>4</sub> the optimum conditions of hydrolysis of HMPG-SO<sub>4</sub> were determined in pilot experiments to ensure maximum hydrolysis. [3H]HMPG-SO<sub>4</sub> was prepared following an intraventricular injection of [3H]noradrenaline into rat brain (20 μCi, specific activity 5-7 Ci/mmol, New England Nuclear). After precipitation of brain protein with perchloric acid as above, amines were removed on a column of Amberlite CG50 resin, and the acid metabolites extracted from the column effluent using ethyl acetate at pH 1-0 (Sugden and Eccleston, 1971). Free glycol and amine metabolites were then extracted with 2 × 2 vol. of ethyl acetate after adjusting the pH to 5-0. Residual traces of ethyl acetate were removed from the remaining aqueous layer containing [3H]HMPG-SO<sub>4</sub> by blowing with dry nitrogen. After the addition of 0-5 ml of m-sodium acetate buffer at pH 5-0, portions of the solutions were incubated with 'Helicase' enzyme preparation at 37°C. The rate of hydrolysis of [3H]HMPG-SO<sub>4</sub> was determined from the amount of free [3H]HMPG which was extracted from the incubate at pH 5-0. The concentration of 'Helicase' generally used for urine hydrolysis (9 mg/ml) gave maximum hydrolysis after 3 h (Fig. 2) whereas a 10th of this concentration (0-9 mg/ml) gave the
Fig. 1.—Plate of frontal section of rat brain at the level of the fourth ventricle showing the electrode track ending just dorsal to the left Locus coeruleus. Toluidine blue stain. ×30. CER, cerebellum; ET, electrode track; LC, Locus coeruleus; V, Nucleus of the mesencephalic tract of the trigeminal nerve; IV Vent, Fourth ventricle. (The mid line is at the left hand border of the plate.)
same maximum after 17 h incubation. The concentration finally chosen was 1.8 mg/ml with overnight (17 h) hydrolysis. The enzyme preparation was found to be equally efficient at pH 5.0 and 6.0 but had little effect at pH 4.0.

In most experiments, free HMPG was not extracted separately but estimated along with the conjugated HMPG. Following enzymic hydrolysis, the HMPG was extracted from the solution with redistilled ethyl acetate as described above. The ethyl acetate extract was placed in a 100-ml round-bottomed flask and evaporated to dryness in vacuo at 50°C. The residue was resuspended in 0.5 ml of distilled water and applied to a 24 cm × 0.7 cm column of 'BioRad' AGI × 4 resin (200–400 mesh) in the chloride form (Antun, Pullar, Eccleston and Sharman, 1971). The flask and column were washed with 2.5 ml distilled water and the HMPG eluted with a further 4 ml of distilled water. Figure 3 shows the elution profile of HMPG.

![Figure 3](image)

**Figure 3.**—Histogram showing the elution of HMPG from 'Bio-Rad' AGI × 4 resin (200–400 mesh) in the chloride form. To the column 0.5 ml of a 2 μg/ml aqueous solution of HMPG was applied, followed by successive 0.5 ml portions of distilled water. The elution of HMPG was monitored using the fluorescence developed after the exposure to ferric ions. (Antun et al., 1971.)

**Acetylation.** HMPG was acetylated (Sharman, 1969) by the addition of 0.3 ml of redistilled acetic anhydride and 0.6 g of Analar NaHCO₃ to the 4 ml aqueous column eluate. The solution was gently shaken to promote the reaction and allowed to complete over 30 min. Acetyl-HMPG was extracted from the aqueous solution by shaking for 3 min with 10 ml of redistilled 1:2 dichloromethane. Nine ml of the lower organic layer was removed and transferred to a test tube containing 1 g Analar anhydrous sodium sulphate, stoppered and shaken to remove residual traces of water. The dry extract was filtered through a micro-Buchner funnel connected to a C24 test tube, and reduced to dryness in vacuo at 50°C. The residue was resuspended in 0.5 ml redistilled ethyl acetate, 0.4 ml of which was transferred to a 2 ml borosilicate reaction tube.

**Trifluoroacetylation.** (Bond, 1972). To the tube 0.1 ml of redistilled trifluoroacetic anhydride was added, it was stoppered and placed in an 'Eppendorf' micro-heating block at 56°C. After 15 min incubation, the solution was blown to dryness under a stream of nitrogen whilst in the heating block. This was continued for a further 15 min. The residue was made up with 0.3 ml of redistilled ethyl acetate containing 20 ng/ml of hexachlorocyclohexane.

**Gas chromatography.** A Perkin–Elmer 900 series gas chromatograph fitted with a [3H] foil electron capture detector was used to estimate the amount of HMPG present as the acetyltrifluoroacetyl derivative. The stationary phase was silicone gum rubber E301 on a chromosorb GAW-DMCS support (PE 0496-0732) and the carrier gas argon/methane 95:5. The column oven temperature was
190°C and ECD 200°C and the carrier gas flow rate 75 ml/min. Figure 4 shows a typical recorder trace of the HMPG derivative with a retention time of half that of the hexachlorocyclohexane, the latter serving as an internal standard to control injection volume. Standard HMPG taken through the method provided the means of quantitative estimation.

RESULTS

The formation of an acetyl-trifluoracetyl derivative of HMPG provides a very sensitive method for its assay in biological material. The method described for the extraction and derivative formation was found to be reproducible giving a percentage recovery of 35.1 ± 3.3 (10) (mean ± S.D. (number of observations)) for 100 ng of added HMPG. Amounts of HMPG from 50 to 150 ng were added to pooled brain tissue and taken through the method. There was found to be a linear relation between the amount added and the peak height of the acetyl-trifluoracetyl HMPG when this was corrected for volume of sample injected using the internal marker of hexachlorocyclohexane (Fig. 5). Figure 6 gives the most probable structure of the acetyl-trifluoracetyl derivative of HMPG. The mean concentrations of total HMPG found in whole rat brain, cerebral cortex and cerebellum are summarized in Table 1. In whole brain and cerebral cortex about 80 per cent of the HMPG was present as a conjugate.

The effect of electrical stimulation of the LC on the levels of free and conjugated HMPG is shown in Fig. 7. Stimulation caused no significant rise in free HMPG,
HMPG and stimulation of the *Locus coeruleus*

**Table 1.**—Concentration of free and conjugated HMPG in whole rat brain

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<tr>
<th>Area</th>
<th>Concentration of total HMPG (ng/g)</th>
<th>n</th>
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<tr>
<td>Whole brain</td>
<td>95 ± 41</td>
<td>21</td>
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<tr>
<td>Cerebral cortex</td>
<td>102 ± 26</td>
<td>65</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>89 ± 30</td>
<td>10</td>
</tr>
</tbody>
</table>

Mean (±s.d.) concentration of HMPG (free and conjugated) in whole rat brain, cerebral cortex and cerebellum, is given for the number of estimations (n).

**Fig. 5.**—Graph showing the recovery of HMPG added to pooled rat brain tissue and taken through the method. Each point is the mean of two determinations. The point of intersection with the ordinate is the endogenous level of brain HMPG.

**Fig. 6.**—The most probable chemical structure of the acetyltrifluoracetyl derivative of HMPG.
Fig. 7.—The effect of electrical stimulation of one LC on the levels of free and conjugated HMPG in whole rat brain. Comparison was made with animals in which an electrode was implanted in the LC but not stimulated. Mean ± s.d. (number of observations in brackets). Single-tailed $P$-value is calculated using the Student's $t$-test. N.S., not significant.

Fig. 8.—The effect of varying the frequency of stimuli on the rise of total HMPG concentration in rat cerebral cortex showing the mean percentage rise ($n = 4$ for each stimulated group and for controls). Single tailed $P$-values for each group are calculated using the Student's $t$-test and are related to the mean control value.
but there was a significant increase of 61 per cent in the level of conjugated HMPG ($P < 0.05$ single tailed Student's $t$-test).

Figure 8 shows the effect of varying the frequency of stimulation on the rise of the noradrenaline metabolite in cerebral cortex. The largest increase of total HMPG was noticed at a frequency of 2 stimuli/s, which is near the spontaneous firing rate of LC cells of 1.5/s reported by GRAHAM and AGHANJANIAN (1971).

The effect of varying the stimulation current strength on the total HMPG concentration in left and right cerebral cortex is shown in Fig. 9. Stimulation at 0.2 mA caused a similar increase in both left and right cortex. At 0.1 mA there was a clear distinction in the HMPG concentration between left and right, an effect which was less noticeable at 0.5 mA. In all experiments, however, where there was an increase on the ipsilateral side due to stimulation there was always some increase of total HMPG on the contralateral side.

**DISCUSSION**

The method presented here for the estimation of HMPG and HMPG-SO₄ is based on those of SHARMAN (1969) and BOND (1972) to be applicable to small samples of brain tissue. It utilizes the formation of a 4–0 acetyl derivative of HMPG, introduced by Sharman, which facilitates the shift from aqueous to organic phase, and appears to give stability and specificity during the subsequent trifluoracetylation step. Using this method it was possible to measure both free and conjugated HMPG in whole rat brain and cerebral cortex without loss of sensitivity. The disadvantages of gas chromatography; the time of analysis, and the presence of the experimenter for sample injection have been eliminated by the use of an automatic liquid sample injector (Hewlett-Packard 7670A) fitted to the gas chromatograph which enables the instrument to be preloaded with samples and used overnight.
The use of an electron capture detector in gas chromatography demands that the injected sample is highly pure. The crude enzyme preparation ‘Helicase’ is a source of contaminating electron capturing material. Most of this is removed, however, by the ion exchange resin AG1X4.

The formation of a sulphate ester would not appear to be a common metabolic route in all species since SCHANBERG et al. (1968b) could find no conjugated HMPG in the cat, and MEEK and NEFF (1972) could not detect HMPG-SO\(_4\) in mouse or guinea pig brain. The mean concentration of HMPG-SO\(_4\) in whole rat brain reported here (80 ng/g) is of the same order found by SCHANBERG et al. (1968b) 40–60 ng/g and less than that found by MEEK and NEFF (1972) (120 ng/g). Neither authors report values for free HMPG, whereas we have found values of 15–20 ng/g, about one-fifth of the total HMPG concentration in both whole rat brain and cerebral cortex. Electrical stimulation of one Locus coeruleus caused no significant rise in free HMPG but a significant (\(P < 0.05\)) (61 per cent) increase in HMPG-SO\(_4\), these results suggesting an increased turnover of NA. Also the formation of a sulphate conjugate from free HMPG appears to be rapid showing that the glycol sulphate rather than free glycol is a major metabolite in rat brain. That the conjugate of HMPG is a sulphate ester, has been confirmed in this laboratory using \([35\text{S}]\)sodium sulphate injected into the lateral ventricles of rats, with the subsequent isolation and chromatography of the conjugate fraction (Eccleston and Ritchie, 1973).

Electrical stimulation of specific 5-hydroxytryptamine (5-HT) containing areas of brain using stereotaxic location has been shown to be a useful tool in the elucidation of the metabolism of this amine. SHEARD and AGHAJANIAN (1968) showed that stimulation of the raphe nuclei increased the synthesis of 5-HT a finding endorsed and amplified by SHIELDS and ECCLESTON (1972). These experiments are essentially similar but involve stimulation of a noradrenergic nucleus. UNGERSTEIDT (1971) has described three pathways arising from the LC; a descending one, which innervates the lower brain stem nuclei thus overlapping with the termination of the ventral NA pathway, a lateral pathway which projects to cerebellar cortex, and a discrete ascending pathway, designated the dorsal pathway sending fibres to cerebral cortex and hippocampus. Although some axons were found to cross the mid-line, he describes the pathway from the LC to the cerebral cortex as uncrossed. The possibility of an ipsilateral response to electrical stimulation of the left locus coeruleus was therefore investigated by separate analysis of left and right cerebral cortex for the concentration of total HMPG. The results in Fig. 9 show that stimulation with constant current pulses at 0-1 mA gave a marked increase of noradrenaline metabolite on the stimulated side compared to the contralateral cerebral cortex, whereas stimulation at 0.2 mA caused a bilateral rise in total HMPG. The latter effect was probably due to the stimulus current having a sphere of action wide enough to simultaneously stimulate both the left and right Locus coeruleus, the separation of the two loci being only 2 mm. Stimulation at 0.1 mA would appear, therefore, to be optimum to produce a unilateral response.

Although most of the stimulation reported here was performed at a frequency of 10/s, later experiments (Fig. 8) showed that stimulation at 2/s gave a larger increase in total HMPG.

The fact that electrical stimulation of the Locus coeruleus causes a rise in the NA metabolites in cerebral cortex confirms similar conclusions of ARBURTHNOTT, CROW,
HMPG and stimulation of the Locus coeruleus

Fuxe, Olsen and Ungerstedt (1970) where NA synthesis inhibitors H44/68 and FLA 63 given prior to electrical stimulation of the dorsal pathway from the LC caused a more rapid ipsilateral depletion of noradrenaline fluorescence in rat cerebral cortex and hippocampus, and confirms our own findings that electrical ablation of the LC caused an ipsilateral decrease in total HMPG concentration in rat cerebral cortex (Arbuthnott et al., 1973).

One behavioural response connected with the nucleus has been the electrical self-stimulation behaviour in the form of repeated lever pressing produced in rats with chronically implanted electrodes with their tips at the LC (Crow, Spear and Arbuthnott, 1972) and we have reported that this self-stimulating behaviour is associated with a substantial rise of the total HMPG concentration in rat cerebral cortex (Anlezark et al., 1973).

In summary, we have described a sensitive method for the measurement of HMPG and HMPG-SO₄ in parts of rat brain. We also find that the LC with its largely ipsilateral innervation of the cortex is a useful model in studying electrical stimulation of the NA system since comparison can be made in the same animal with the opposite unstimulated cortex.

Acknowledgements—We wish to thank Dr. I. Laszlo, Dr. I. A. Pullar and Mr. P. J. Shields for advice on the development of our methods.

REFERENCES

The Effect of Unilateral and Bilateral Lesions in the Locus coeruleus on the Levels of 3-Methoxy-4-Hydroxyphenylglycol (MHPG) in Neocortex

Studies with the histochemical technique of Falck et al. indicate that fibres arising from catecholamine-containing cell bodies in the brainstem have a widespread distribution in the mammalian central nervous system. Ungerstedt has presented evidence that the noradrenergic-containing terminals in the neocortex originate from cell bodies in the ipsilateral locus coeruleus, a small nucleus in the floor of the fourth ventricle (A6 cell body group of Dahlström and Fuxe). We have recently modified the gas chromatography method of Bond to detect 3-methoxy-4-hydroxyphenylglycol (MHPG), the major metabolite of noradrenaline in rat brain, and developed a stereotaxic method for locating electrodes in the region of the locus coeruleus. In this preliminary communication, we report the effects of unilateral and bilateral lesions of the locus coeruleus on the neocortical levels of MHPG which we take to represent an index of the turnover of noradrenaline (NA) in that region.

Methods. Eighteen female albino rats were anaesthetised with halothane and immobilized in a Kopf stereotaxic apparatus. A unipolar varnished steel electrode was located in the region of the locus coeruleus in 12 rats according to the technique of Crow, et al. An electrolytic lesion was made by passing a charge of 40 mCoul through the electrode tip to an anal cathode. In 6 animals the lesion was made on the left side, and in 6 bilaterally. In 6 further animals (controls) burr holes were drilled in the skull bilaterally and the dura removed but no electrode was inserted. 3 weeks after the operation the brains were removed, the brainstems sectioned and stained by the Klüver-Barrera technique, and the neocortical portion of the prosencephalon prepared for assay of MHPG.

Results. Two rats in the group with bilateral lesions died shortly after recovery from anaesthesia. In the remaining animals, the electrolytic lesion had ablated the nucleus locus coeruleus on the appropriate side. The results of the neocortical assay for total MHPG (Table) show that on the side of the unilateral lesions, and on both sides in those rats with bilateral lesions, there was a very marked reduction in the cortical content of the noradrenaline metabolite.

Discussion. These results show that histologically confirmed lesions of the nucleus locus coeruleus cause a marked fall in the turnover of noradrenaline in the ipsilateral neocortex. This supports the suggestion arising from histochemical studies that a noradrenaline-containing system with a very wide distribution in the neocortex arises from a small nucleus in the mid-pontis. This finding would be consistent with the view that electrical self-stimulation obtained from electrodes placed using the same stereotaxic co-ordinates, and with their tips close to the locus coeruleus, is due to the activation of a noradrenergic-system, and this may provide a clue to its function. It is also of interest that in the group of rats with bilateral lesions (but not in either of the other 2 groups) we observed a striking behavioural syndrome of marked hyperkinesia and repeated jumping which appeared within 2 h of recovery from anaesthesia and lasted for 4 h. Lesions of catecholamine-containing pathways are probably followed by amine release from degenerating terminals. Anden described a form of hyperkinesia characterised by large jumps after administration of catapresan and apomorphine in rats and postulated that noradrenaline receptor stimulation was involved.

Résumé. Des lésions électrolytiques, soit unilatérales soit bilatérales, ont été pratiquées dans des cerveaux de rats, dans la région du locus coeruleus. Trois semaines après, la teneur en 3-méthoxy-4-hydroxyphénylglycol (MHPG) des deux cortices cérébraux a été analysée par chromatographie à gaz. Les lésions unilatérales ont réduit la teneur en MHPG du cortex ipsilatéral, et après les lésions bilatérales la réduction est venue dans les deux cortices.

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Electrical Ablation of locus coeruleus

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<thead>
<tr>
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<th>L. H. Cortex (ng)</th>
<th>R. H. Cortex (ng)</th>
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<td>Control</td>
<td>6 ± 16</td>
<td>42 ± 12</td>
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<tr>
<td>Unilateral (L-sided) lesions</td>
<td>6 ± 9*</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>Bilateral</td>
<td>4 ± 10*</td>
<td>17 ± 4*</td>
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*P < 0.005 vs control, *P < 0.01 vs control, *P < 0.025 vs control.
Lesions of the Locus Ceruleus and Noradrenaline Metabolism in Cerebral Cortex

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Lesions in the dorsal pons cause a reduction in the concentration of the major metabolite of noradrenaline, 4-Hydroxy-3-Methoxyphenylglycol, on the cerebral cortex of the same side. The decrease in concentration of this metabolite measured by a gas chromatographic technique parallels the cell loss in the nucleus locus ceruleus assessed histologically.

INTRODUCTION

The presence of low concentrations of noradrenaline in cerebral cortex has for long been known (2). It is likely, however, that the rate of turnover is high (12). Since the development of the Falck–Hillarp technique (7) it has been established that the noradrenaline in cortex is located within the varicosities of a diffusely distributed network of nerve terminals (1, 10, 11). Owing to the fact that the presence of catecholamines can be demonstrated by this technique only within the cell bodies and terminals (6, 10), the anatomy of the noradrenaline-containing system whose terminals are distributed to the cortex has until recently been obscure.

Using stereotaxic lesioning methods, Ungerstedt (19) recently attempted to identify the course of the catecholamine-containing axon systems arising from the numerous cell body groups situated in the brain stem described by Dahlström and Fuxe (6). By studying the accumulation of fluorescence in axons proximal to the lesion site Ungerstedt identified a “dorsal bundle” of noradrenaline-containing fibers which originate principally from the A6 area of Dahlström and Fuxe, the locus ceruleus. This small nucleus, situated in the lateral part of the floor of the fourth ventricle, has a higher density of noradrenaline-containing neurons, than is found anywhere else in the central nervous system (6, 14). Following lesions of this system the noradrena-
TABLE 1
CONCENTRATION OF TOTAL HMPG (FREE + CONJUGATED) AND LESION SIZE

<table>
<thead>
<tr>
<th>Rat</th>
<th>Size of Lesion in locus ceruleus</th>
<th>Cortical HMPG (ng/g)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>CC1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CC2</td>
<td>2</td>
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<td>CC3</td>
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<tr>
<td>CC4</td>
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<td>2</td>
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<tr>
<td>CC5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CC6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean ± sd for control rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UL1b</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>UL2b</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>UL3b</td>
<td>1</td>
<td>2</td>
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<tr>
<td>UL4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>UL5b</td>
<td>1</td>
<td>2</td>
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<tr>
<td>UL6</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Mean ± sd for unilateral lesions</td>
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<td></td>
</tr>
<tr>
<td>BL1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>BL2</td>
<td>0</td>
<td>1</td>
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<tr>
<td>BL3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BL4</td>
<td>1</td>
<td>1</td>
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<tr>
<td></td>
<td>45 ± 20</td>
<td>39 ± 24d</td>
</tr>
</tbody>
</table>

a Groups of experimental animals. UL—unilateral (left sided) lesions, BL—bilateral lesions, CC-controls. Lesions-0—locus ceruleus completely ablated; 1—partly ablated; 2—intact. Histology in rat UL6 was not available.
b A paired t test, selected on the basis of histology gives t = 9.1272 P = > 0.0025.
c P < 0.005 vs control.
d P < 0.025 vs control.

line-containing terminals in the cerebral cortex were much diminished, and on the basis of this finding Ungerstedt (19) postulated that the terminals of the fibers arising from the locus ceruleus innervate, in addition to other structures, the entire cerebral neocortex.

We have recently developed a method for locating electrode tips in the region of the locus ceruleus (5) and have modified (20) the gas chromato¬graphic method of Bond (3) to detect 4-hydroxy-3-methoxyphenylglycol (HMPG) and its sulfate conjugate in rat brain. This substance is the
Fig. 1. Regions of cell loss due to unilateral electrolytic lesions aimed at locus ceruleus (LOC. COER). Only structures adjacent to the floor of the fourth ventricle (IVthV) are shown; S.C.P.—superior cerebellar peduncle; G.F.—genu of the facial nerve; P.T.—pyramidal tract. The numbers on the left are the planes of the Fifkova and Marsala atlas (9) from which the drawings were made. The numbers above refer to individual rats.

major metabolite of noradrenaline in brain (16, 17) and can thus be used as an index of noradrenaline turnover in brain. In this paper we report the relationship between unilateral and bilateral lesions in the region of the locus ceruleus and the concentration of total HMPG in cerebral cortex in the rat.

METHODS

Using fluothane anesthesia a stainless steel electrode, varnished except for the tip, was located in the region of the locus ceruleus using the Fifkova and Marsala atlas (9) and coordinates determined according to the technique described by Crow et al. (5). Electrolytic lesions were made by passing a charge of 40 mecoulombs through the electrode tip as anode to an cathode.

Eighteen female albino rats (weight 200 ± 20 g) were divided into three groups. In six animals (group UL) lesions were made in the region of the locus ceruleus on one side only. In six animals (group BL) lesions were made on both sides, and in six further animals (group CC) bilateral burr holes were drilled in the skull, the dura was removed as in the other two groups, but no electrode was lowered into the brain.

Three weeks after operation the animals were killed by decapitation. The brains were removed, the brain stems placed in formalin, and the cortical portion of the prosencephalon dissected off and prepared for total HMPG assay as described by Walter and Eccleston (20). Serial sections of the brain stem were stained by the Kluver–Barrera technique (13).
RESULTS

Two rats in group BL died shortly after recovery from anesthesia. In three of the remaining animals in this group, but not in any of the animals in the other two groups, hyperkinesis and repeated jumping were observed this behavior coming on within 1–2 hr of recovery and lasting for approximately 4 hr.

Analysis of the cortical HMPG concentrations in the groups as a whole (Table 1) shows that there is a significant reduction ($P < 0.005$) in the HMPG content of the cortex ipsilateral to the brain stem lesion in the rats with unilateral lesions, and that there is a similar fall in the HMPG concentration in both cortices in the rats with bilateral lesions.

Examination of histological sections of the brain stem indicated that in some cases in the group with unilateral lesions (e.g., rats UL1 and UL2, Fig. 1) there had been a virtually complete ablation of the locus ceruleus. In one case (UL4) the locus ceruleus had been little affected by the lesioning procedure, and in other animals (UL3 and UL5) the nucleus had been partially damaged by the procedure. In the group with bilateral lesions (Fig. 2) the locus ceruleus had been completely ablated on one side and partly ablated on the other in some animals (rats BL1 and BL2), while in others (BL3 and BL4) there was a partial ablation on both sides. The locus ceruleus was intact on both sides in all the animals in the control (CC) group.

The range of cortical HMPG concentrations within the control group (CC) is relatively large (57–171 ng/g). Within the experimental groups however the reduction in cortical HMPG concentration on the lesioned side approximately parallels the degree of damage to the locus ceruleus (Table
1). In the group with unilateral lesions, for instance, there is a large reduction in those two rats (UL1 and UL2) with complete ablations, and in rat UL4 where there was no visible damage to the locus ceruleus the HMPG concentrations in the two cortices are approximately equal. In the group with bilateral lesions, the HMPG concentration was lower on the side in which the locus ceruleus ablation was more complete in the case of those animals where the damage to this nucleus was unequal (BL1, BL2). In the case of rat BL4 the damage to the nucleus itself was relatively small but HMPG was low in both cortices. In this particular case both lesions were placed rostroventrally to the nucleus.

DISCUSSION

The results indicate that the total HMPG concentration of the ipsilateral cerebral cortex is reduced following electrolytic ablations of the locus ceruleus, and that there is a relationship between the degree of damage to the locus ceruleus and the extent of reduction of HMPG. Since HMPG concentration reflects the turnover of noradrenaline, these results are entirely consistent with the possibility that a very diffusely distributed system of noradrenaline-containing neurons originates from the cells of the locus ceruleus. In the one case (BL4) in which the cortical HMPG was substantially reduced while the locus ceruleus ablations were of small extent, the lesions were placed rostroventrally to the nucleus on either side. There is some evidence from histochemical studies (14, 19) that the noradrenaline-containing axons travel ventrally after leaving the locus ceruleus and thus they may have been interrupted by the lesions in this rat. If this is the case it is of interest that the cells of the nucleus of origin have not degenerated as occurs with dopamine-containing cells following axonal damage (18) and this may be attributable to the fact that the ascending pathway is only one axonal branch from the cell bodies of the locus ceruleus (14, 19).

Even in those cases where the locus ceruleus ablation was complete there remained some HMPG in the ipsilateral cortex. A possible explanation is that some of the HMPG in the cortex is derived from nerves accompanying the blood vessels of the pia mater (8).

The diffuse distribution of the neuronal system arising from the locus ceruleus suggests that this system has some very general behavioral function. It has recently been suggested that some neurohumoral agents may perform a metabolic rather than an excitatory or inhibitory role (15) and this would seem a more likely possibility in the case of a system with terminals widely distributed to the whole of a large structure such as the cerebral cortex. In an earlier study (5) it was discovered that rats with electrodes implanted in the region of the locus ceruleus can be trained to self-stimulate. On the basis of this finding and other considerations (4) it
has been suggested that the system of noradrenaline-containing neurons may function as a “reinforcement” system in the sense that the term reinforcement is used in learning theories. Experiments to test this hypothesis are in progress.

REFERENCES


THE RELATIONSHIP BETWEEN NORADRENALINE TURNOVER IN CEREBRAL CORTEX AND ELECTRICAL SELF-STIMULATION THROUGH ELECTRODES IN THE REGION OF LOCUS COERULEUS

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Abstract—Rats allowed to stimulate themselves through electrodes implanted in the region of the noradrenaline-containing cells of the locus coeruleus had increased concentrations in the cerebral cortex of 4-hydroxy-3-methoxy-phenylglycol (a noradrenaline metabolite). In a group of animals stimulated under anaesthesia a rise was present if, and only if, the electrodes had previously supported self-stimulation behaviour. It is concluded that the increase in cortical 4-hydroxy-3-methoxy-phenylglycol reflects an increase in noradrenaline turnover which results from self-stimulation through electrodes in the region of the locus coeruleus. The results constitute further evidence for the involvement of a specific catecholamine-containing pathway, the coeruleo-cortical noradrenergic system, in central reward mechanisms.

The noradrenaline-containing cells of the locus coeruleus give rise to a fibre system with a diffuse terminal distribution within the prosencephalon (Ungerstedt, 1971). Lesions of the locus coeruleus decrease the content of noradrenaline (Anlezark et al., 1973b; Blanc et al., 1973; Thierry et al., 1973), and its metabolite 4-hydroxy-3-methoxy-phenylglycol (HMPG) (Arbuthnott et al., 1973; Korf et al., 1973), in the ipsilateral cerebral cortex. Stimulation through electrodes placed stereotaxically in the region of the locus coeruleus increases the concentration of HMPG in the ipsilateral cortex (Walter & Eccleston, 1972; 1973; Korf et al., 1973).

Several workers have reported a relationship between electrical self-stimulation sites and the location of catecholamine-containing nerve cell bodies (Dresse, 1966; Arbuthnott et al., 1970a,b; Crow, 1971, 1972a,b; Crow et al., 1972). Self-stimulation can be obtained with the electrode tip located either in the region of the dopamine-containing cell bodies in the ventral mesencephalon (Arbuthnott et al., 1970a; Crow, 1971, 1972a,b; Anlezark et al., 1973c) or close to the cells of the locus coeruleus in the floor of the fourth ventricle (Arbuthnott et al., 1970b; Crow et al., 1972; Anlezark et al., 1972, 1974; Ritter & Stein, 1973). Thus it has been argued that this behaviour may result from activation of either of these two systems (Anlezark et al., 1972; Crow, 1972; Crow & Arbuthnott, 1972; Crow, 1973).

Here we report that with electrodes implanted in the region of the nucleus locus coeruleus, it is possible to demonstrate a clear relationship between noradrenaline turnover as measured by HMPG concentration in the cerebral cortex and electrical self-stimulation behaviour. A preliminary report has been presented to the British Pharmacological Society (Anlezark et al., 1973a).

EXPERIMENTAL PROCEDURES

Twenty-two hooded Lister rats were studied; eighteen had had electrodes implanted in the region of the locus coeruleus according to the technique of Crow et al. (1972), and the remaining four were controls. All those with implanted electrodes were tested for self-stimulation in a standard operant conditioning apparatus using 200 ms trains of 0.5 ms unidirectional pulses at 100 Hz; self-stimulation was not obtained at any current level, up to those at which motor effects precluded further testing, in 6 animals. Self-stimulation behaviour was obtained in each of the remaining 12 rats; these animals were treated as follows: six rats were allowed to self-stimulate for 1 h; three rats were anaesthetized with fluothane and were stimulated by the experimenter for 1 h at rates and current levels similar to those at which they had previously self-stimulated (1 train every 4 s); and three rats were killed without stimulation in the previous hour.

The 6 animals which did not self-stimulate were stimulation under anaesthesia with stimulation parameters similar to those required to maintain the behaviour in the twelve rats who had self-stimulated.

At the end of the treatment period, all animals were killed by decapitation under fluothane anaesthesia.
already anaesthetized and being stimulated were killed during stimulation, while self-stimulating animals were anaesthetized with fluanthone and decapitated within 5 min of the end of stimulation. The brains were dissected out and the right and left cerebral cortices including the hippocampus were separated and rapidly frozen. Free and sulphate-conjugated HMPG were estimated by the gas chromatographic method of Walter & Eccleston (1973). Each brainstem was fixed in formalin, sectioned, and stained by the technique of Klever & Barrera (1953). Two observers assessed the electrode site independently. Subsequently they discussed their findings and agreed a location for each electrode on a particular plane in the Fifkova & Marsala (1967) atlas of the rat brain. Both biochemical assay and histological examination were conducted without knowledge of behavioural findings. Student’s t-test was applied to the comparisons of the mean HMPG values of the experimental groups.

RESULTS

The distribution of electrode sites (Fig. 1) confirms previous work (Arbuthnott et al. 1970b; Crow et al., 1972) in showing that self-stimulation can be obtained with electrodes in close proximity to the locus coeruleus. The tips of the electrodes which did not support self-stimulation were found to be located 0-5 mm or more from this nucleus.

The results of the HMPG assays are shown in Table 1 and Fig. 2. Group 1 (7 rats) includes 4 control rats and 3 rats (Fig. 1, nos. 1279, 1309, 1317) which had previously self-stimulated but which were not stimulated in the hour before being killed. The HMPG concentrations in these animals (mean 146 ng/g ± s.d. 38) were within the range of the values found in normal rats of the same age and strain (Walter & Eccleston, 1973). Group 2 (5 rats) consists of the non self-stimulating animals (Fig. 1, nos. 57, 66, 333, 356, 358). The concentration of HMPG in this group also (154 ± 23 ng/g) was not significantly different from these values ($P > 0.5$).

In group 3, the self-stimulating rats stimulated under anaesthesia, (Fig. 1, nos. 31, 234, 236) there was a marked rise in HMPG concentration (328 ± 68 ng/g) in the cortex ipsilateral to the stimulating electrode, ($P < 0.001$), but the concentration in the contralateral cortex (175 ± 37 ng/g) was not significantly different from control levels ($P = 0.25$).

In group 4, the six rats which were allowed to self-stimulate in the hour before being killed, (Fig. 1, nos. 55, 65, 78, 292, 329, 331) there was a significant increase ($P < 0.001$) in HMPG concentrations in the ipsilateral cortex (175 ± 37 ng/g) compared to controls, and a smaller increase ($P < 0.025$) in the contralateral cortex (206 ± 44 ng/g). In individual animals, the extent of the rise in HMPG concentration in the contralateral cortex showed no clear relation to current level, response rate, or electrode position.

DISCUSSION

The results show that stimulation of the locus coeruleus occurring in the course of self-stimulation behaviour is associated with increases in HMPG concentration in the ipsilateral cortex similar to those which can be demonstrated in the anaesthetised animal. The association between electrode site close to the
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat numbers</th>
<th>Stimulation parameters</th>
<th>Individual Mean ± s.d.</th>
<th>Right cortex Mean ± s.d.</th>
<th>HMPG (ng/g)</th>
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<tbody>
<tr>
<td>Group 1</td>
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<tr>
<td>Unstimulated (includes four control rats and three</td>
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<tr>
<td>self-stimulators (nos. 1279, 1309, 1317) not</td>
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<td>stimulated before being killed</td>
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<td>Group 2</td>
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<td>Non-self-stimulating rats (nos. 57, 66, 333,</td>
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<td>356, 358) stimulated under anaesthesia</td>
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<td>Group 3</td>
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<td>Self-stimulating rats stimulated under anaesthesia</td>
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<td></td>
<td></td>
<td>900 200 2</td>
<td>328 ± 68 (3)</td>
<td>175 ± 37 (3)</td>
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<tr>
<td></td>
<td>31 900 600 6</td>
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<td></td>
<td>234 900 200 2</td>
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<td>236 900 300 3</td>
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<td>55 821 150 1.5</td>
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<td>65 696 250 2.5</td>
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<td>78 1500 150 1.5</td>
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<td>292 1028 200 2.0</td>
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<td>329 750 250 2.5</td>
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<td>331 650 150 1.5</td>
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\(t\)-Test comparisons were as follows: Group 1 left vs Group 3 left: \(P < 0.001\). Group 1 left vs Group 4 left: \(P < 0.001\). Group 1 right vs Group 3 right: \(P = 0.25\). Group 1 right vs Group 4 right: \(P < 0.025\). Group 3 left vs Group 3 right: \(P < 0.05\). Group 3 left vs Group 4 left: \(P > 0.25\). Group 4 left vs Group 4 right: \(P > 0.10\).
locus coeruleus, self-stimulation behaviour, and increased cortical noradrenaline turnover, as indicated by raised HMPG concentrations, adds further support to the hypothesis that self-stimulation behaviour can result from activation of the noradrenaline-containing cells of the locus coeruleus. Although there is evidence that operant behaviour itself is associated with increased noradrenaline turnover (Lewy & Seiden, 1972; Olds & Yuwiler, 1972), the turnover changes described in the present experiments are predominantly unilateral and survive anaesthesia and therefore cannot be ascribed to the behavioural situation alone.

The rise in HMPG observed was greater in animals stimulated under anaesthesia (group 3) than in those stimulating themselves for the hour before being killed (group 4), but this difference is not significant \( (P > 0.25) \). The rise in the HMPG concentration in the cortex contralateral to the stimulating electrode is surprising in view of the evidence that the pathway is largely uncrossed (Ungerstedt, 1971). The rise was greater in conscious animals (group 4) than in the anaesthetized group (group 3) and therefore may be a consequence either of general motor activity or of lever pressing itself. Certainly we were unable to explain it on grounds of increased current spread, or in relation to electrode position. It is possible, however, that this finding indicates an interaction between the two nuclei in the conscious animal which does not take place during anaesthesia.

Our present results provide further evidence for the involvement of a specific noradrenergic system in electrical self-stimulation. This is in general agreement with the hypothesis originally proposed by Stein (1962) and Poschel & Ninteman (1963) that central noradrenaline-releasing neurones are activated in this behaviour. The coeruleo-cortical noradrenaline pathway would be further implicated if it could be demonstrated that selective removal of these neurones abolishes self-stimulation through electrodes sited close to the cell bodies. There is much evidence that drugs which inhibit the synthesis of, or otherwise deplete, catecholamines in the central nervous system reduce self-stimulation behaviour (Olds et al., 1956; Poschel & Ninteman, 1966; Dresse, 1967; Black & Cooper, 1970; Gibson et al., 1970). However, most such drugs affect both noradrenaline and its precursor dopamine, and previous anatomical evidence suggests that the dopamine-containing neurones arising from the ventral mesencephalon may also be involved (Arbuthnott et al., 1970a; Crow, 1971, 1972a,b; Anlezark et al., 1973c, 1974). The hypothesis that activation of a single noradrenaline-containing system accounts for all instances of this behaviour is also challenged by two pharmacological findings. Firstly, this behaviour is not always abolished by disulfiram, or by FLA63, dopamine-\( \beta \)-hydroxylase inhibitors which deplete noradrenaline alone (Roll, 1970; Stinus et al., 1973). Furthermore the effects of (+) and (--) amphetamine on self-stimulation responding with electrodes in lateral hypothalamic sites vary in a way which suggests that sometimes noradrenaline- and sometimes dopamine-containing neurones are involved (Phillips & Fibiger, 1973). The motor behaviours (stimulus-bound masticatory movements, presumably due to activation of the mesencephalic tract of the 5th nerve) observed in the present and in a previous study on self-stimulation with electrodes in the region of the locus coeruleus are also quite different from those which accompany self-stimulation through ventral mesencephalic electrodes (increased forward motion, sniffling, licking and gnawing behaviour)(Anlezark et al., 1972).

Thus, while the present results provide further anatomical and biochemical evidence implicating the coeruleo-cortical noradrenaline-containing system in electrical self-stimulation behaviour, we consider it probable that this is not the only catecholamine-containing system which will support this behaviour. The distribution of the terminals of the locus coeruleus system to the cerebral cortex is, however, if particular interest in relation to the hypothesis (Crow, 1968; Kety, 1970) that the noradrenergic innervation of the cortex is a necessary component of the mechanisms of learning and may mediate the 'reinforcing' as opposed to the 'motivational' aspects of rewarding stimuli (Crow & Arbuthnott, 1972; Crow, 1973) on the organism's behaviour. Anlezark et al. (1973b) have demonstrated significant learning deficits in rats with bilateral lesions of the locus coeruleus.

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SECTION VIII
Chapter 12

Adenosine 3′:5′-Cyclic Monophosphate and Affective Disorders: Animal Models

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Cyclic AMP has been shown to have a regulatory role in many intracellular biochemical events. A large range of hormones has been shown to stimulate the enzyme adenylate cyclase responsible for the conversion of ATP into cyclic AMP. This enzyme is located in cell membranes and is activated by the hormones via 'receptors' presumably on the exterior of the membrane; the activated adenylate cyclase in the inner side of the cell membrane then causes a rise in intracellular cyclic AMP. This in turn regulates intracellular biochemical events by the activation of protein kinases, which activate further enzymes by catalysing their phosphorylation by ATP (Greengard & Kuo, 1970). This has given cyclic AMP the role of 'second messenger' in many hormonally induced events (Sutherland et al., 1965), the hormone being the 'first messenger'. The system achieves specificity by the special properties of the hormone receptors and allows amplification of the hormonal message.

The brain has been found to be particularly rich in adenylate cyclase, cyclic AMP and the enzyme phosphodiesterase, which is responsible for the breakdown of cyclic AMP to 5'-AMP. This suggests that the nucleotide has an important role in the control of biochemical events in brain. Of particular interest in relation to the biogenic amines is the work of Siggins et al. (1969), who showed in physiological experiments that the iontophoresis of cyclic AMP on to Purkinje cells of the cerebellum mimicked the inhibitory action of similarly applied noradrenaline. From the biochemical standpoint a great deal of work has been done on substances that increase the rate of formation of cyclic AMP in brain. These include noradrenaline, 5-hydroxytryptamine, histamine (Kakiuchi & Rall, 1968) and, in higher doses than other catechols in vivo, dopamine (Burkard, 1972). A similar activation of adenylate cyclase is seen in response to high amounts of potassium and other depolarizing agents (Shimizu et al., 1970). A further interesting compound is adenosine, which also stimulates cyclic AMP production in brain slices (Shimizu & Daly, 1970) and which Pull & McIlwain (1972) elevate to the role of a neurohumoral agent.

In the context of this large and increasing body of facts on cyclic AMP it seemed inevitable that a place would be found for it in the biochemical aetiology of psychiatric illnesses, and in 1970 two groups working independently (Abdullah & Hamadah, 1970; Paul et al., 1970) announced their findings in relation to affective illness. Essentially, they reported that the urinary excretion of cyclic AMP
was high in hypomania and lower than normal in depression, with a return to normal on recovery. Abdullah & Hamadah (1970) suggested that the symptoms of affective illness could be attributed to changes in tissue concentrations, including that of brain, of cyclic AMP and that the therapeutic action of the tricyclic antidepressants was due to their capacity to inhibit phosphodiesterase. In our own laboratory we felt that one of the uncontrolled variables was the amount of activity in the experimental subjects. When we walked for various distances, up to 10 miles, the excretion of cyclic AMP in the urine could be correlated with the amount of exercise (Eccleston et al., 1970). The cogency of the argument of Paul and co-workers was further decreased by their report (Paul et al., 1971) that the elevation of concentrations of cyclic AMP in urine occurred only at the time of the 'switch' from depression to hypomania.

The theory was rejuvenated by a study of concentrations of cyclic AMP in cerebrospinal fluid in patients with affective illness treated with probenecid (Cramer et al., 1972). This was, at least, looking at a fluid in close contact with the nervous system. They found that the rise in the concentration of cyclic AMP in cerebrospinal fluid after probenecid treatment was higher in manic patients than in those with depression. Unfortunately, the number of cases was very small. The results did, however, suggest that the drug probenecid might be used to give an index of cyclic AMP turnover. A. I. M. Glen & D. L. W. Davidson (unpublished work) used cyclic [14C]AMP in a ventriculo-lumbar perfusion of the rabbit with and without probenecid and found a significant correlation ($P<0.001$) for the elevation of 5-hydroxyindol-3-ylacetic acid produced by the drug and the cyclic AMP concentration in cerebrospinal fluid. This suggests that cyclic AMP is removed from the cerebrospinal-fluid system by a mechanism which is similar to that involved in the removal of the acid end products of amine metabolism. Although there are many steps to examine before it can be said that concentrations of cyclic AMP in cerebrospinal fluid after probenecid

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Fig. 1. Dose-response curve for the formation of cyclic [14C]AMP from [14C]adenine in whole brain (■) and cortical (●) slices stimulated with increasing concentrations of noradrenaline

The numbers of animals are given in parentheses.
Fig. 2. Comparison of the formation of cyclic [\(^{14}\)C]AMP from [\(^{14}\)C]adenine between cortical slices from animals with a unilateral lesion of the locus coeruleus and a comparison between sides in animals in which the lesion was found histologically to be outside the locus coeruleus

(a) Lesion of one locus coeruleus; (b) lesion outside locus coeruleus. The differences are between three incubation mixtures of slices from each cortex and significance is determined by Student's t test. N.S., not significant. Means ± s.d. are indicated on the histogram. □, Lesion; ■, non-lesion.

Treatment reflect the rate of turnover of this nucleotide in brain, such measurements may provide a model for looking at changes in cyclic AMP turnover in the central nervous system in man.

In affective illness the relevance of cyclic AMP may be in relation to noradrenaline, an amine that has been postulated to be responsible for affective illness (Schildkraut, 1965). Adenylate cyclase is thought to be closely associated with both \(\alpha\)- and \(\beta\)-adrenergic receptor activity (Robinson & Sutherland, 1971). Changes in the sensitivity of adrenergic receptors in the central nervous system have been postulated to occur in affective illness (Medical Research Council Brain Metabolism Unit, 1972), and if this is correct one might anticipate changes in brain adenylate cyclase activity. Denervation of a tissue is known to produce supersensitivity to the transmitter normally released by the innervation
Lesion in locus coeruleus
P<0.0005
Lesion elsewhere
N.S.

Means±s.d. are indicated on the histograms with the numbers of animals in parentheses. The significance of differences was obtained by Student’s t test. N.S., not significant. □, Cortex of lesioned side; □, cortex of intact side.

(Trendelenberg, 1966). Such denervation supersensitivity has been described in the dopaminergic system in rat brain after lesions of the substantia nigra (Ungerstedt, 1971). The receptors on the side of the denervation were shown to be supersensitive to drugs that stimulate dopamine receptors: under these conditions the animals rotate away from the side of the lesion.

We (D. Eccleston, R. W. Loose, D. S. Walter & C. E. Anderson, unpublished work) have attempted to produce denervation supersensitivity of noradrenergic receptors in brain by making unilateral surgical lesions of the locus coeruleus in rats. This is a nucleus containing cell bodies of noradrenergic neurons that send axons ipsilaterally to the cerebral cortex. The site of the lesion in each animal was checked histologically. Slices of cerebral cortex were prepared from chronically lesioned animals, and an estimation of cyclic AMP formation was made by using the precursor-labelling method of Shimizu et al. (1969), in which the conversion of [14C]adenine into cyclic AMP is measured. Products of the incubation, including cyclic AMP, were measured after separation by paper chromatography and scintillation counting of radioactivity. Initial experiments suggested that cortical slices were superior to whole-brain slices (Fig. 1), and from the dose-
Fig. 4. Formation of cyclic $[^{14}\text{C}]\text{AMP}$ from $[^{14}\text{C}]\text{adenine}$ in animals with unilateral lesions of locus coeruleus when no noradrenaline is added to the incubation medium or when incubated with $10\mu\text{m}$ noradrenaline.

Means±S.D. are shown with the numbers of animals in parentheses. The significance of differences was obtained by a paired $t$ test.

Fig. 5. Development of increased formation of cyclic $[^{14}\text{C}]\text{AMP}$ from $[^{14}\text{C}]\text{adenine}$ on the side of lesion of locus coeruleus compared with the non-lesioned side in response to $10\mu\text{m}$-noradrenaline at various times after making the lesion.

Means±S.D. are given with the numbers of animals in parentheses.
response curve a concentration of noradrenaline of 10 μM was chosen for routine addition to the incubation medium. It was found that in animals with a lesion of the locus coeruleus there was an increased formation of cyclic AMP in response to this concentration of noradrenaline in cortical slices from the lesioned side, whereas in animals with a lesion outside the locus coeruleus no difference in cyclic AMP production between the two sides was detected (Fig. 2). To avoid bias, histochemistry was done without prior knowledge of the biochemical results. When the number of experiments was increased highly significant differences were apparent between normal and lesioned sides (Fig. 3). When the control rates of cyclic AMP synthesis (in the absence of noradrenaline in the medium) were compared in slices from the lesioned and control sides, the lesioned tissue showed a significant increase \( (P < 0.25) \) (Fig. 4). This suggests that the absolute activity of adenylate cyclase in cerebral cortex may be increased as a consequence of the lesion. It is also noteworthy that the responsiveness of cortical tissue of the lesioned side to noradrenaline increased over a 4-week period (Fig. 5), a time-course similar to that psychiatrists associate with response to antidepressant drugs. It appears then that central noradrenergic receptor supersensitivity after denervation is related to increased cyclic AMP formation. Similar results have been obtained by Palmer (1972) in animals in which cerebral noradrenergic neurons were destroyed by treatment with 6-hydroxydopamine.

In conclusion, it is postulated that changes in adrenergic receptor sensitivity may be involved in the aetiology of affective illness and that the rate of cyclic AMP production may be an index of the functional state of adrenergic receptors. The urinary output of cyclic AMP is unlikely to reflect changes in cerebral adenylate cyclase activity, but the elevation of cyclic AMP in cerebrospinal fluid in man after probenecid treatment may be useful for this purpose, provided that further animal experiments validate this method.

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Medical Research Council Brain Metabolism Unit (1972) Lancet ii, 573–577
CYCLIC AMP AND A POSSIBLE ANIMAL MODEL OF RECEPTOR SUPERSENSITIVITY

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Conventional techniques for investigating aminergic function in man: namely the estimation of amine metabolites in C.S.F. have suggested that factors other than transmitter output should be considered in affective illness. Levels of 5HIAA have been found to be low in unipolar depressed patients but normal in bipolar depression (MRC Brain Metabolism Unit, 1972). These workers also suggested that clinical observations of changes in the facial dyskinesias with the phase of manic depressive illness may be related to changes in post synaptic receptor supersensitivity. The demonstration that receptor supersensitivity in brain can be linked to adenyl cyclase activity is, then, important. In the animal model described this is indicated.

The locus coeruleus (LC) is a bilateral noradrenergic nucleus supplying cortex almost ipsilaterally (Ungerstedt, 1971). The production of supersensitivity in dopaminergic receptors has been established by lesions of the cell bodies in the substantia nigra (Ungerstedt, 1971). Similar supersensitivity in the noradrenergic system should follow lesions of one LC and this should be unilateral. In these experiments electrolytic lesions are made in one LC and the animals allowed to recover. Subsequently cortical slices are prepared and cyclic AMP production measured by the

Fig. 1.—Formation of $^{14}$C cyclic AMP from $^{14}$C adenine in animals with unilateral lesions of locus coeruleus when no NA added to incubation medium or when incubated with $10^{-5}$ M NA. (paired t-test).
method of Shimizu et al. (1969). In these experiments two points arise: firstly there is a significantly increased cyclic production on the side of the lesion both in unstimulated slices and in response to NA stimulation compared with slices prepared from the unlesioned side of the animal (Fig. 1), and secondly this increased response to NA develops over at least 4 weeks.

Perhaps the efforts now being directed to determine adenyl cyclase function in man—more specifically in brain, are important. This study which shows that the development of changes is over some weeks may give us a clue to the paradoxically slow response to the cyclic antidepressants and perhaps also to the long term phasic nature of affective illness.

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MRC brain Metabolism Unit (1972) Lancet ii, 573-577.
IS ADENYLYATE CYCLASE THE DOPAMINE RECEPTOR?

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ABSTRACT
As one test of the idea that the dopamine receptor has adenylate cyclase as one component, and that the effects of dopamine in brain are mediated via cyclic AMP, we have tried to show increases in the responses to dopamine receptor stimulation after phosphodiesterase inhibitors.

Of the drugs tested only aminophylline increased apomorphine-induced turning in 6-hydroxydopamine lesioned animals. Since it was the only central stimulant — the others (chlordiazepoxide, diazepam, ICI 63, 197) all had some central sedative properties — it seems that the increase in turning follows the known central actions of the drugs rather than their inhibitory effects on phosphodiesterase.

KEY WORDS: PHOSPHODIESTERASE INHIBITORS; CHLORDIAZEPoxide; DIAZEPAM; AMINOPHYLLINE; DOPAMINE RECEPTORS; APOMORPHINE

INTRODUCTION
Like Fuxe and Ungerstedt (4) we were interested in the idea that it may be possible to increase the efficiency of central dopamine receptor stimulants after phosphodiesterase inhibition. The suggestion stems from recent work by Greengard and his colleagues (6) and by Miller and Iversen (7), who have studied the properties of dopamine-sensitive adenylate cyclase from homogenates of various brain areas in the rat. The response of the enzyme to dopamine agonists and antagonists has many of the properties of the activation of dopamine receptors in other models. In fact, the blockade of the effect of dopamine on the adenylate cyclase follows the clinical efficacy of the neuroleptics (7) rather better than does the inhibition of turning (2). We have tried to show an effect of phosphodiesterase inhibition on the response of unilaterally 6-hydroxydopamine lesioned animals to apomorphine. The work began because in a pilot study the response of unilaterally lesioned animals to amphetamine was enhanced by caffeine. We chose a different collection of phosphodiesterase inhibitors (PDI's) and had come to the opposite conclusion from Fuxe and Ungerstedt when their paper appeared (4). We have subsequently confirmed their results with aminophylline.

MATERIAL AND METHODS
All the animals used in these experiments had been lesioned by intracerebral injection of 6-hydroxydopamine and had been subsequently tested on a dose of 0.3 mg/kg apomorphine (Evans Medical Ltd.).

Only animals making a total number of turns in excess of 200 were used in subsequent experiments. The experiments were performed in a bowl similar to that used in the rotometer (12) except the number of turns were counted by hand.
Is adenylyl cyclase the dopamine receptor?

Fig. 1. Turning rate after apomorphine. The bars represent the turning rate (mean ± 1 SD) of 23 trials with 0.3 mg/kg apomorphine injected at time 0. 0.5 ml saline was injected 30 min before. Since the initial peak occurred in animals given only saline, turns in the first 5 minutes are treated separately in Table 1.

We used a balanced experimental design using 6 rats for each drug studied. All the animals were injected (i.p.) 0.5 hour before the apomorphine (0.3 mg/kg) injection, 3 with saline, 3 with PDI. Then the test was repeated with treatments reversed. The phosphodiesterase inhibitors tested were ICI 63, 197 (10 mg/kg, 1.5 hr previously), aminophylline (25 mg/kg) Sigma Chemical Company, London, Ltd.), chlordiazepoxide (10 mg/kg) and diazepam (5 mg/kg) (Roche Products Ltd.). We used a paired t-test to compare turning in the saline tests with the drug ones. 12 normal rats were treated with ICI 63, 197 (10 mg/kg i.p.) and killed in groups of three every hour. The cyclic AMP content of their brains were analysed by the method of Brown et al. (1).

RESULTS

Fig. 1 shows a curve with the average number of turns/min against time after the apomorphine injection for all the »saline pretreatment» tests. It clearly shows an early response and then a later phase. Since the early turning can be elicited by saline injection alone, we have called this a »handling effect » and treated the total number of turns during the first 5 minutes of the test as a separate variable.

Table 1 shows the turning in this early phase after PDI pretreatment as well as

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Turns 0—5 min M ± SD</th>
<th>Turns 5 min — end M ± SD</th>
<th>P (paired t) 0—5</th>
<th>5—end</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI 63, 197</td>
<td>30 ±10</td>
<td>238 ± 62</td>
<td>p &lt; 0.0025</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>84 ± 21</td>
<td>331 ± 104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>76 ± 22</td>
<td>278 ± 108</td>
<td>p &lt; 0.0025</td>
<td>p &lt; 0.0125</td>
</tr>
<tr>
<td>Saline</td>
<td>117 ± 28</td>
<td>444 ± 177</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>52 ± 20</td>
<td>468 ± 201</td>
<td>p &lt; 0.0025</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>106 ± 25</td>
<td>518 ± 217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminophylline</td>
<td>101 ± 34</td>
<td>1060 ± 521</td>
<td>p &lt; 0.30, NS</td>
<td>p &lt; 0.025</td>
</tr>
<tr>
<td>Saline</td>
<td>93 ± 23</td>
<td>453 ± 165</td>
<td></td>
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</tbody>
</table>

TABLE 1
Effect of combined drug and apomorphine treatment on turning behaviour of experimental animals.
the total number of turns made by the animal in the succeeding time which we have called the «apomorphine-induced» turning.

Table 2 shows the results of the cyclic AMP assay after ICI 63, 197.

DISCUSSION

Although our results with aminophylline are similar to those reported by Fuxe and Ungerstedt (4) we are unwilling to assign the observed effects to the actions of the xanthines as PDI's. Biochemical work has suggested that papaverine is a very much more potent PDI than the xanthines (3, 5, 8) although it was inactive in the turning experiments (4). All the drugs we tried have been reported to inhibit phosphodiesterases (including apomorphine) (8, 10) and we have tested the effect of the ICI compound on brain cyclic AMP and confirmed Somerville's previous results in mice which showed that this compound caused an elevation of cyclic AMP in brain (Somerville, personal communication). Nevertheless this compound did not increase turning — indeed it depressed it. The depression, however, was mainly on the early »handling effect» and we were pleased to note that the minor tranquilizers, chlordiazepoxide and diazepam, also reduced the effect, suggesting that they may indeed act on «stress induced» effects although in spite of their potency as central nervous PDI's (3, 9, 11) they showed no effect on the «apomorphine-induced» turning.

The simplest explanation for our results seems to be that the central actions of the drugs determine their effect on turning rather than their PDI-like properties. However, as Fuxe and Ungerstedt also point out, the possibility of the existence of several phosphodiesterases with different actions on the brain and with differing drugs sensitivities cannot be ruled out. We hope in future experiments to explore this suggestion further.

ACKNOWLEDGEMENTS

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Is adenylate cyclase the dopamine receptor?


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SECTION IX
5-HYDROXYINDOLE COMPOUNDS IN THE CEREBROSPINAL FLUID OF PATIENTS WITH PSYCHIATRIC OR NEUROLOGICAL DISEASES

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5-HYDROXYINDOLE COMPOUNDS IN
THE CEREBROSPINAL FLUID OF PATIENTS
WITH PSYCHIATRIC OR NEUROLOGICAL
DISEASES

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An association between certain psychotic illnesses and metabolic disturbances has long been considered. There is, for example, the possibility of an abnormality in the methylation of biogenic amines in schizophrenia (Friedhoff and Van Winkle 1962) although conclusive evidence for this has not so far appeared (Lancet 1966); and a disturbance in amine metabolism in the brain has also been suggested in depressive illness.

The major obstacles to the examination of these ideas are the technical problems associated with the study of cerebral metabolism in man. The evidence so far presented in favour of a disturbance of amine metabolism in depression has been obtained either by inference from a study of the biochemical effects of drugs, which either precipitate or ameliorate depressive symptoms (Ashcroft, Eccleton, Knight, MacDougall et al. 1965) or from a

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study of the metabolites of the amines in the blood or urine (Rodnight 1961, Coppen, Shaw, Malleson, Eccleston et al. 1965). Such studies often mean that changes in peripheral metabolism must be assumed to be paralleled by changes in cerebral metabolism.

We have attempted to use the measurement of a metabolite (5-hydroxyindolylacetic acid, 5-HIAA) in the cerebrospinal fluid (C.S.F.), to reflect quantitative changes in the cerebral metabolism of the parent amine, 5-hydroxytryptamine (5-HT). Ashcroft and Sharman (1960) reported that 5-hydroxyindole compounds were present in the lumbar C.S.F. of two groups of patients, one group of depressed patients, the other of non-depressed patients with neurological disease who were undergoing lumbar air-encephalography. Significantly lower concentrations of 5-hydroxyindole compounds were found in the depressed patients, a finding which has been confirmed by Dencker et al. (1966). However, as a result of further studies, it is now appreciated that the injection of the air (10-12 c.cm.) before withdrawal of C.S.F., which is a routine procedure in air-encephalography, might itself affect the validity of the results. A further variable which might affect the concentrations could be the degree of activity of the patients just before lumbar puncture (Fotherby et al. 1963).

We present here the results of an investigation of the concentration of 5-hydroxyindole compounds in the lumbar C.S.F. of patients with neurological disease and of patients with various psychiatric illnesses, the C.S.F. being sampled under standardised conditions. We have also attempted to investigate the effect of such variables as air injection on the levels of 5-hydroxyindolyl compounds in lumbar fluid.

Materials and Methods

Selection of Patients with Depressive Illness

The depressed patients were selected from those admitted to Craig House, Royal Edinburgh Hospital over a period of nine months in 1959. The criterion for selection was simple; we included all patients in whom the main presenting symptom was depression and in whom there was no evidence of schizophrenia or of the presence of directly contributing organic disease. Twenty-four patients in this group had received no specific antidepressant therapy in the 3-week period before lumbar puncture, whilst eight were under treatment with imipramine. Routinely, night sedation was induced in all patients with quinalbarbitone ('Seconal') 200 mg. Where
lumbar puncture was not essential for diagnostic purposes, the situation was explained to the patients and their relatives and their permission was sought. Very few patients declined to cooperate. The mental state of the patient was re-evaluated on the day of lumbar puncture and all patients classified in this study as "depressed" had exhibited a sustained depression of mood from the time of admission to the time of lumbar puncture, in most cases a period of 3 to 4 days.

The biochemist was unaware of the clinical diagnosis and the results of clinical and biochemical evaluations were not compared until the end of the study. In an attempt to correlate severity of depression with biochemical findings, a simple ranking procedure was used, one of the clinicians (E. M.) being asked to place the patients in rank order of severity of illness.

In ten patients, the lumbar punctures and estimates were repeated after remission of the depressive symptoms.

Patients who had been receiving monoamine-oxidase inhibitors within 3 weeks of hospital admission were excluded from the main study, but a small group of such patients was studied separately.

Patients with Neurological Disease

Patients with organic disease of the central nervous system were selected from those undergoing diagnostic lumbar puncture in the same hospital as the depressed patients and also from the neurological unit of a general hospital. C.S.F. samples were obtained from a consecutive series of patients from each unit, irrespective of diagnosis. Lumbar C.S.F. samples were also obtained from the neurological unit at diagnostic lumbar air-encephalography.

Samples of C.S.F. obtained during ventricular drainage from six patients in a neurosurgical unit were also examined.

Patients with Psychiatric Conditions other than Depression

Night sedation was standardised as for the depressed patients. Lumbar puncture was carried out, with the patient's agreement, in the following conditions: (a) acute and chronic schizophrenia; (b) chronic alcoholism; and (c) hypomania. Repeat studies after remission were carried out in some of these patients. All the schizophrenic patients were receiving phenothiazines at the time of C.S.F. sampling.

Collection of C.S.F.

Lumbar fluid.—Each specimen was collected by allowing approximately 5 ml. of fluid to drop from the needle into a chemically clean 10 ml. glass graduated test-tube containing 5 mg. ascorbic acid to prevent oxidation of the 5-hydroxyindolyl compounds. Specimens were transported in an ice-packed container to the laboratory and were kept on ice until the estimation was carried out later the same day.

The lumbar puncture, performed with the patient in the lateral position, was done between 8 and 10 A.M., no food having
been taken since 5.30 p.m. the previous day. To reduce the possible increase, by exercise, in the mixing of the C.S.F. from the various compartments, the patients were not allowed up from bed on the morning of the lumbar puncture until after the procedure was completed.

**Lumbar Fluid Sampling at Air-encephalography**

The patient was given six paracetamol ("Panadol") tablets, which was the routine premedication for this diagnostic examination. The procedure was carried out with the patient in the sitting position, 10-12 c.cm. of air being injected slowly over a 10-minute period before the first sample of C.S.F. was taken.

**Estimation of 5-hydroxyindolyl Compounds in C.S.F.**

The estimations were carried out on 5.0 ml. samples of C.S.F., using the modification of the method of Weissbach et al. (1958), as described by Ashcroft and Sharman (1962). Protein precipitation by zinc sulphate and sodium hydroxide was followed by direct fluorimetric assay of the 5-hydroxyindolic material in the supernatant fluid made 3 N with respect to hydrochloric acid, using standard solutions of 5-H.I.A.A. as reference. This method will detect 5-hydroxyindolyl compounds in C.S.F., including 5-H.T., 5-H.I.A.A. and, 5-hydroxytryptophan (5-H.T.P.) should these be present. Recoveries of 5-H.T. and 5-H.I.A.A. were of the order of 85% but, as a result of losses during the protein-precipitation stage, recoveries of 5-H.T.P. through the method varied from 10% to 40%.

Sharman (1960) and Roos (1963) have demonstrated that the only 5-hydroxyindolyl compound present in measurable concentrations in human C.S.F. under normal conditions is 5-H.I.A.A. Hence, the estimates of the concentrations of "5-hydroxyindoles" reported in this study can almost certainly be equated with concentrations of 5-H.I.A.A., with the possible exception of those referable to patients receiving monoamine-oxidase inhibitors. The estimates of the concentrations of 5-hydroxyindoles cited in the text and in the table have therefore been expressed in terms of the equivalent amount of 5-H.I.A.A.

**Results**

The mean (± S.D.) of 5-hydroxyindoles in the different groups of patients are shown in the table.

**Lumbar Puncture**

*Patients with Neurological Disease or Alcoholism*

There was no significant difference (p > 0.1) between the mean concentrations in the C.S.F. from the groups of patients from the two hospitals. There were no obvious correlations with sites of the lesion. Levels in the C.S.F. from alcoholic patients were not significantly different from
**CONCENTRATION OF 5-HYDROXYINDOLE COMPOUNDS IN CEREBROSPINAL FLUID**

<table>
<thead>
<tr>
<th>Type of patient</th>
<th>No.</th>
<th>Site and method of C.S.F. sampling</th>
<th>Concentration of 5-hydroxyindoles † (ng. per ml. ±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurosurgical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurological (organic disease of the central nervous system):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurological unit</td>
<td>28</td>
<td>Lumbar air-encephalography</td>
<td>33.3(±11.2)</td>
</tr>
<tr>
<td>Psychiatric unit</td>
<td>8</td>
<td>Lumbar puncture</td>
<td>20.0(±4.7)</td>
</tr>
<tr>
<td><strong>Alcoholic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed:</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>On imipramine</td>
<td>8</td>
<td>Lumbar puncture</td>
<td>8.8(±2.4)</td>
</tr>
<tr>
<td>On no specific antidepressant</td>
<td>24</td>
<td>Lumbar puncture</td>
<td>11.1(±3.9)</td>
</tr>
<tr>
<td><strong>Hypomanic</strong></td>
<td></td>
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</tr>
<tr>
<td>Schizophrenic:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>7</td>
<td>Lumbar puncture</td>
<td>10.9(±2.2)</td>
</tr>
<tr>
<td>Chronic</td>
<td>7</td>
<td>Lumbar puncture</td>
<td>16.4(±2.9)</td>
</tr>
</tbody>
</table>

† Concentration of 5-hydroxyindoles expressed as 5-H.I.A.A.

those of the neurological patients, and these estimates have therefore been included along with those from the neurological group to give a “control” group of 21 estimates of the concentration of 5-hydroxyindolyl compounds in the lumbar fluid of non-depressed patients.

**Depressed Patients**

There seemed to be no correlation between drug (imipramine) treatment and the concentration of 5-hydroxyindolic substances. The mean level in the “depressed patients” (10.3 ng. per ml.) was significantly lower than that of the “controls” (19.1 ng. per ml.) (p < 0.001).

We attempted a rough correlation between biochemical findings and severity of depression by comparing the mean levels of 5-hydroxyindolyl compounds in the C.S.F. of successive groups of eight patients with decreasing levels of severity of depression (increasing rank order). Such a comparison gave the following results (mean ± s.d., ng. per ml.): ranks 1–8, 8.0 (±2.0); ranks 9–16, 9.5 (±2.2); ranks 17–24, 12.0 (±2.9); and ranks 25–32, 11.8 (±4.1).

Another method of investigation was provided by a comparison of levels of 5-hydroxyindolic substances in the lumbar C.S.F. of ten patients before and after remission.
of depressive symptoms. The mean (±S.D.) concentration before remission was 11·9 (±5·0) ng. per ml. and after remission it was 16·5 (±4·2) ng. per ml. Analysis of paired differences showed a significant (p < 0·02) increase in levels after remission, with no apparent relationship to the type of therapy (electroconvulsive therapy [E.C.T.] alone in three patients, imipramine alone in two, and imipramine plus E.C.T. in five).

A subsidiary investigation was carried out in six patients who had been receiving monoamine-oxidase inhibitors (phenelzine in five, tranylcypromine in one) before admission and up to the time of the lumbar puncture. In these patients, all still depressed at the time of lumbar puncture, the mean concentration of 5-hydroxyindolyl compounds in the lumbar C.S.F. was 21·7 (±3·5) ng. per ml. This concentration was much higher than that in depressed patients not on these drugs, and was in fact higher, although clearly not significantly so, than that in the non-depressed group.

Other Psychiatric Conditions

Schizophrenia.—The results showed a difference between the concentrations in the C.S.F. of patients with acute (recent onset) and of those with chronic schizophrenia. The mean concentration in the patients with acute schizophrenia was of the same order as that seen in depressive illness, and was significantly lower (p < 0·01) than that of the control group. The chronic patients, however, showed a mean concentration not significantly different from the control group (p > 0·1).

Hypomania.—Four hypomanic patients were examined before and after remission. The concentration during the hypomanic phase was not significantly different from that of the control group. There was no consistent change in levels following remission, mean (±S.D.) 17·5 (±8·6) ng. per ml.

Air-Encephalogram Studies

We measured levels of 5-hydroxyindolic compounds in the lumbar fluid, withdrawn after the injection of the air into patients undergoing air-encephalography. No general correlation was evident between C.S.F. level and the diagnostic category, but it is of interest that four out of the five patients with C.S.F. concentrations greater than 40 ng. per ml. had abnormalities affecting the brain stem or central structures, areas of brain which in dogs have been shown to have high concentrations of 5-H.T.A.A.
(Eccleston 1966). Two of these patients had petit mal (C.S.F. 5-hydroxyindole concentration, 43 and 63 ng. per ml.) and two were diagnosed as having had cerebrovascular accidents affecting brain-stem structures (C.S.F. 5-hydroxyindole concentration, 41 and 59 ng. per ml.).

VENTRICULAR-DRAINAGE SAMPLES

The concentration of 5-hydroxyindolic compounds in ventricular fluid in six patients undergoing ventricular drainage after operation for cerebral tumours or head injury was high (881 \( \pm 22.6 \) ng. per ml.).

Discussion

The work was complete in 1960 and at the time posed considerable problems in the interpretation of the results, since we were unable to explain the gradient between ventricular and lumbar C.S.F. levels of 5-H.I.A.A., or the results after air-encephalography. Since this time, we have paid considerable attention to the mechanisms of addition and removal of 5-H.I.A.A. to C.S.F. in animals. In addition, sufficient background information regarding the relationship of C.S.F. and cerebral levels of 5-H.I.A.A. is now available to us to attempt to explain some of our findings. One advantage of carrying out this work in 1959 was that it preceded widespread use of antidepressant drugs in general practice, hence there was a large number of depressed patients available for study who were not taking specific antidepressant drugs.

We had assumed at the start of this work that the concentration of a metabolite 5-H.I.A.A. in the C.S.F. would reflect and give information regarding the rate of turnover in nervous tissue of the biogenic amine, 5-H.T. This concept is supported by Eccleston's (1966) studies on the cisternal-fluid levels of 5-H.I.A.A. in animals, which showed a tendency for the C.S.F. concentrations to follow the 5-H.I.A.A. concentrations in brain-stem structures.

However, our results illustrate that the C.S.F. does not represent a homogeneous system, at least in so far as the concentration of 5-hydroxyindolic substances is concerned. There is a gradient in levels, the concentration in ventricular fluid being considerably higher than in the lumbar fluid. The fluid obtained at lumbar air-encephalography gives levels between the two extremes; the most obvious explanation of this finding is that it results from the mixing of the displaced ventricular fluid containing a high concentration with the rest of the C.S.F. Other
explanations are possible. For example, the injected air may release 5-hydroxyindolic substances from neural tissue or interfere with a mechanism for the removal of these substances from C.S.F. Evidence for the existence of an active transport mechanism for the removal of 5-H.I.A.A. from the C.S.F. has been presented (Guldberg et al. 1966).

The source of the 5-H.I.A.A. in C.S.F. has not yet been defined precisely. In animals, it would seem that C.S.F. levels follow brain, rather than blood, concentrations (Eccleston 1966), and hence a central-nervous-system source seems likely. The different concentrations at different levels of the C.S.F. system may be accounted for by a process involving an addition of the 5-H.I.A.A. in the ventricles, followed by a progressive reduction in concentration by mechanisms such as active transport, diffusion, and dilution. A contribution from the spinal cord to the lumbar C.S.F. must also be considered.

The conclusions drawn from our results must, therefore, be tentative. Depressed patients show lower concentrations of 5-hydroxyindolic compounds (considered to be mainly 5-H.I.A.A.) in C.S.F., when compared with a non-depressed group with neurological diseases. The levels in the depressed patients seem to correlate with the severity of the depression, and also to increase after remission. That lowered levels are not diagnostic solely of depressive illness is illustrated in the concentration found in the C.S.F. of acute schizophrenic patients, which are of a similar order to those seen in depression. It should be noted that all schizophrenic patients were receiving drugs of the phenothiazine type and it is possible that the results may have been influenced by this therapy.

The results in the four hypomanic patients are interesting. They show "normal" levels in the hypomanic phase and there is no significant change during remission. Unfortunately, none of these patients was studied in a depressed phase. Unexpected results were obtained in the depressed patients on monoamine-oxidase inhibitors, the levels being higher than those in the other depressed patients. Unfortunately, the 5-hydroxyindolic compounds in the C.S.F. were not fractionated and it might be that the amine, 5-H.T., rather than the acid, 5-H.I.A.A., was present. Roos (1963) studied the effect of monoamine-oxidase inhibitors in schizophrenic patients and found no reduction in 5-H.I.A.A. levels in the lumbar
C.S.F. Maclean et al. (1965) also found that the levels of brain 5-H.I.A.A. in patients who had been receiving nialamide, a monoamine-oxidase inhibitor, for several weeks before death, were not reduced from the normal level. These findings cast further doubt on the hypothesis that the therapeutic effect of these drugs is in fact related to their property of cerebral monoamine-oxidase inhibition.

The results of the estimations in lumbar C.S.F. of depressed patients are explicable in terms of a defect in the synthesis or in the release of the amine, 5-H.T., in neural tissue, this being reflected by a fall in the levels of its metabolite, 5-H.I.A.A., in lumbar C.S.F. However, the study of the effect of air-encephalography on lumbar levels of 5-H.I.A.A. casts some doubt on this interpretation, and points to the fact that other variables, such as decreased mixing of C.S.F. from various levels, which might result from decreased physical activity could produce such changes in lumbar fluid 5-H.I.A.A. levels. Despite careful attempts to control such variables in this study, these possibilities have not been completely ruled out.

If it is subsequently proved beyond doubt that the 5-H.I.A.A. in C.S.F. is derived solely from cerebral metabolism, and if the difficulties of representative sampling can be overcome, then the measurement, in C.S.F., of this and other central-nervous-system metabolites may prove a valuable tool in the investigation of the metabolism of biogenic amines in the human brain and spinal cord.

Summary

Levels of 5-hydroxyindole compounds (almost exclusively 5-hydroxyindolylacetic acid [5-H.I.A.A.]) in cerebrospinal fluid (C.S.F.) may reflect the brain metabolism of the parent biogenic amine 5-hydroxytryptamine (5-H.T.). This approach has been used in the analysis of C.S.F. samples from patients with neurological or psychiatric diseases. Levels vary with the site of sampling, indicating that C.S.F. is not a homogeneous system; and since the precise source of 5-H.I.A.A. in the C.S.F. is not known, any conclusions must be tentative. Levels in C.S.F. obtained by lumbar puncture are lower (10-3 ng. per ml.) in depressed patients than in patients with other neurological disease (19-1 ng. per ml.). This finding is consistent with a defect in synthesis or release of 5-H.T. in the depressed patient.
We thank the medical and nursing staff at Craig House and Dr. Elizabeth Robertson, Prof. W. K. M. Perry, and Prof. F. J. Gillingham for their co-operation in this study.

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Changes on recovery in the concentrations of tryptophan and the biogenic amine metabolites in the cerebrospinal fluid of patients with affective illness

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Changes on recovery in the concentrations of tryptophan and the biogenic amine metabolites in the cerebrospinal fluid of patients with affective illness

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SYNOPSIS The concentration of the acid metabolites of dopamine, and 5-hydroxytryptamine (5-HT), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5-HIAA) respectively, were estimated in the cerebrospinal fluid of patients suffering from either unipolar or bipolar affective illness, both before and after recovery. Significantly low concentrations of HVA and 5-HIAA (P<0.01 and 0.05 respectively) were found in the unipolar depressed group and these did not return to normal on recovery. Depressed bipolar patients had levels within normal limits. In bipolar manic patients the HVA concentration fell on recovery to a level significantly lower (P<0.05) than controls. There was no difference in the levels of tryptophan in the CSF of any of the groups of patients nor was there any alteration on recovery. There was a high correlation between 5-HIAA and HVA in the same CSF. These findings are against the amine hypothesis which postulated in depression a lowered concentration of transmitter amine at synaptic junction.

Circumstantial evidence for the involvement in affective illness of the neuronal systems which have the biogenic amines as their transmitters has been derived from studies of drugs which either precipitate or alleviate these disorders. Previous studies in depressed patients (Ashcroft and Sharman, 1960; Ashcroft, Crawford, Eccleston, Sharman, MacDougall, Stanton, and Binns, 1966; Dencker, Malm, Roos, and Werдинius, 1966; Van Praag, Korf, and Puire, 1970) have shown a reduced concentration of 5-hydroxyindolacetic acid (5-HIAA), the major metabolite of 5-hydroxytryptamine (5-HT) in the cerebrospinal fluid. Low levels of homovanillic acid (HVA), the major metabolite of dopamine, have also been reported (Roos and Sjöström, 1969; Papeschi and McClure, 1971).

In this paper we report a series of patients suffering from affective illness, who have been classified into unipolar or bipolar groups and whose CSF levels of HVA and 5-HIAA have been measured both before and after treatment.

METHODS The study involved 31 psychiatric and 31 neurological patients. The psychiatric patients had either a unipolar or bipolar affective disorder (either depression or hypomania) of sufficient severity to warrant hospital admission and the neurological 'control' group had been investigated for neurological disorder by lumbar puncture, but had neither gross brain damage nor psychiatric symptoms. Diagnosis of the patients with affective illness was established by two psychiatrists as well as by consulting the case notes for previous episodes of hypomania or depression. Such previous episodes were diagnosed only if they required treatment by drugs or ECT. To enable this study to be compared with others in the future further diagnostic criteria were established by the use of the Wing Present State Examination (Wing, 1970). During the patients' stay in the ward, symptoms were rated by trained nursing staff using the Hargreaves Rating Scale (Hargreaves, 1968). Treatment was either by ECT or psychotropic drugs of the phenothiazine or butyrophenone groups. Drugs were withdrawn five days before the first and the second lumbar puncture. The psychiatric patients were also maintained on a diet standardized to 0.1 g of each

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concentrations of HVA and 5-HIAA were estimated in the CSF essentially according to the methods described by Guldberg and Yates (1968). 0.15 M borate buffer pH 8.6 was used instead of 0.1 M tris buffer pH 8.6 in order to reduce the blank. Tryptophan in the CSF samples was estimated by the method of Hess and Udenfriend (1959), as modified by Guroff and Udenfriend (1962).

Fig. 1 Concentration (mg/ml.) of homovanillic acid (HVA) in the lumbar CSF of unipolar depressed (UPD), bipolar depressed (BPD), and bipolar manic (BPM) patients before treatment as compared with a control neurological group. Each value is the mean ± SEM. The number of patients is in parentheses. NS: not significant.
RESULTS

In the unipolar depressed patients the concentrations of both HVA and 5-HIAA in the lumbar CSF before treatment were significantly lower (\( P < 0.01 \) and 0.05 respectively) than those in the control neurological group (Figs. 1 and 2). On recovery these levels did not alter significantly regardless of whether the statistical analysis was by paired difference or by a \( t \) test between the depressed and recovered groups (Figs. 3 and 4). This differs from the findings in bipolar depressed patients while depressed, when the concentrations of both HVA and 5-HIAA are within the normal range (Figs. 1 and 2). On recovery a paired difference analysis showed a significant fall in the HVA levels (\( P < 0.005 \)) in the bipolar depressed group, even though there was no significant difference between the levels before treatment and after recovery on a simple Student's \( t \) test (Fig. 3). There was no significant alteration in the concentration of 5-HIAA in the lumbar CSF of this group of patients on recovery (Fig. 4).

The bipolar manic patients, while still in a hypomanic episode, had, like the bipolar depressed group, HVA and 5-HIAA levels which did not differ significantly from the neurological patients (Figs. 1 and 2). On recovery the concentrations of HVA fell to a level significantly lower (\( P < 0.05 \)) than the control group (Fig. 3). There was no significant alteration in the concentrations of 5-HIAA on recovery (Fig. 4).

In view of the reports of increasing HVA and 5-HIAA levels in the lumbar CSF with increasing age (Gottfries, Gottfries, Johansson, Olsson, Persson, Roos, and Sjöström, 1971), we looked for a similar correlation in our own data. We were unable to show any significant correlation between age and the concentrations of HVA and 5-HIAA in the lumbar CSF of any of the psychiatric groups of patients or in the neurological controls. There was, however, a highly significant correlation between the concentrations of HVA and 5-HIAA in the control group (\( r = 0.81, P < 0.001 \)), the pre-treatment psychiatric group (\( r = 0.63, P < 0.001 \)), and the psychiatric group after recovery (\( r = 0.70, P < 0.001 \)).
Tryptophan levels in CSF did not differ significantly between the groups of patients and when compared with controls (Table). There was no correlation between 5-HIAA and tryptophan levels in the CSF of individuals.

DISCUSSION

The concentration of acid end-products of both 5-HT and dopamine metabolism have been extensively studied in brain and CSF in animals (Guldberg, Ashcroft, and Crawford, 1966; Eccleston, Ashcroft, Moir, Parker-Rhodes, Lutz, and O'Mahoney, 1968) and certainly ventricular and cisternal CSF metabolite levels reflect the levels in brain and their variation with time. In particular, elevating the turnover of 5-HT in the brain of dogs by tryptophan administration produces a rise of 5-HIAA in brain with a contemporaneous rise of this metabolite in cisternal CSF. Studies in man (Eccleston, Ashcroft, Crawford, Stanton, Wood, and McTurk, 1970) suggest that the rise in lumbar fluid depends upon a rise in metabolite in CSF in the ventriculo-cisternal space with subsequent diffusion down to the lumbar region. Concentration of these metabolites both with and without probenecid, a drug which inhibits transport of acids out of CSF, have been used in many psychiatric studies, although the validity of the findings has been questioned by Bulat and Zivkovic (1971).

By dividing the patients into groups with unipolar or bipolar affective illness we have demonstrated that the lowered levels of 5-HIAA previously reported in depressive illness (Ashcroft and Sharman, 1960; Ashcroft et al., 1966; Dencker et al., 1966; Van Praag et al., 1970) are confined to patients with unipolar depression. The concentrations in the bipolar depressed and bipolar manic patients do not differ from those in neurological controls (Fig. 2). Up to the present time the finding of lowered 5-HIAA levels in CSF in depression has indicated a reduction in some aspect of the function of the 5-HT system in brain, and the argument in some of the amine theories has implied a causal relation-

![Fig. 3](image-url)  
**Fig. 3** Change in the concentrations (μg/ml) of homovanillic acid (HVA) in the lumbar CSF of unipolar depressed (UPD), bipolar depressed (BPD), and bipolar manic (BPM) patients on treatment resulting in recovery, as compared with a neurological control group expressed as the mean ± SEM of 31 patients. NS: not significant.
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ship between depression and reduced 5-HT synthesis. This clearly cannot be the case in all depressed patients, since in the two subgroups, unipolar and bipolar, the affect, depression, cannot be distinguished, and yet the bipolar group do not appear to have a reduction in central 5-HT turnover if lumbar concentration of 5-HIAA is taken as an index of this. Lowered HVA concentrations have also been demonstrated only in the unipolar depressed group (Fig. 1). This then tends to isolate the unipolar group biochemically and it is also of interest that they have been found to have different genetic and clinical backgrounds (Perris, 1966; Blackburn, 1972).

The rate of formation of 5-HT in brain depends to some extent on the concentration of the amino acid precursor, the enzyme tryptophan 5-hydroxylase being unsaturated with tryptophan under normal conditions (Jequier, Lovenberg, and Sjoerdsma, 1967). The concentration of tryptophan in CSF in our group of patients did not differ significantly from that of controls. This is contrary to the finding of Coppen, Brooksbank, and Peet (1972), who reported lowered levels in depression compared with neurological patients. No comment was, however, made as to whether the patients were fasting up to the time of sampling. In our own series standard diets had been given for at least five days before the first, and continued to the second lumbar puncture. The patients were fasted for 12 hours before the lumbar puncture. This made the psychiatric patients comparable within groups in relation to diet. There is, however, no information on the dietary status of the neurological group. This situation is complicated by the fact that tryptophan is bound to plasma protein (McMenamy, Lund, and Oncley, 1957) and the concentration in CSF probably relates to the free amino acid in plasma rather than the total concentration, as has been shown in brain (Knott and Curzon, 1972). The concentration of amino acid in plasma is influenced by such factors as fasting and stress (Knott and Curzon, 1972). Certainly, the total plasma tryptophan can show a threefold rise in total tryptophan on the eating of a 700 g steak (Lockie and Eccleston, unpublished observations) with,
The used in the 'amine apparent aminergic two be related above results demonstrate the if of the affective illness. This might show in the of 5-HIAA (Ungerstedt, 1963). In our patients these drugs were discontinued at least five days before the lumbar puncture. Any residual effect on the second lumbar puncture would have been predicted to show a rise in HVA. This acid did, however, fall significantly in the group in which the drug was used and hence an effect of this treatment may be discounted. The concentrations of 5-HIAA in the lumbar CSF of these patients show the same trends on recovery as those of HVA, but the changes for this acid are not significant (Fig. 4). There is a highly significant correlation (P<0.001) between the concentrations of HVA and 5-HIAA in the lumbar CSF of the affective group and also in the control patients. This might suggest either a functional relation between the 5-HT and dopaminergic systems or the effect of a common mechanism of elimination of these metabolites from the CSF. If the results are not due to the latter then the above results demonstrate that mood state cannot be related directly to the functioning of these two aminergic systems as demonstrated by their apparent turnover, and, therefore, do not support the ‘amine hypothesis’ as it is at present stated. The circumstantial evidence in relation to drugs used in affective illness still indicates that the biogenic amine systems are in some way involved. However, because of the above results we feel it necessary to formulate a new hypothesis. Neural transmission is influenced by not only transmitter availability but also the sensitivity of the post-synaptic receptors to the transmitter. Changes in central dopaminergic receptor sensitivity have been demonstrated in animals (Ungerstedt, 1971) and there is also evidence for an inhibitory neuronal feed back mechanism in the 5-HT system. Hence, amine output receptor sensitivity and feedback mechanisms should be considered in any modified amine hypothesis (M.R.C. Brain Metabolism Unit, 1972).

In this paper we have not studied the concentration of the metabolite of noradrenaline in the lumbar CSF of the patients with affective illness. It is thus possible that the amine hypothesis could be sustained solely on the influence of this amine. The pharmacological evidence, however, is against the sole involvement of noradrenaline in mood change. Depression is treated with drugs which potentiate noradrenergic (Iversen, 1967) and possibly 5-HT (Carlsson, Fuxe, and Ungerstedt, 1968) transmission, but probably not that of dopaminergic neurons (Fuxe and Ungerstedt, 1968), while the treatment of hypomania uses drugs which, although not specifically, are potent dopaminergic receptor blocking drugs (Ungerstedt, 1971). Further research is needed to examine the factors which influence receptor sensitivity as well as the functioning of the aminergic systems in patients with affective illness.

We wish to acknowledge the help of our nursing staff and the clinicians in the Edinburgh area who referred patients for this study.

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Effect of tryptophan administration on 5HIAA in cerebrospinal fluid in man

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Effect of tryptophan administration on 5HIAA in cerebrospinal fluid in man

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Because of the relative inaccessibility of the brain, attempts to quantify in man the rate of cerebral metabolism of any particular compound are fraught with difficulties. Measurements of urinary end products of brain metabolism are often invalidated by the presence of the same metabolites which originate in other tissues. Cerebral arteriovenous differences in the concentration of metabolites may be technically difficult to estimate and rely on the demonstration of a concentration gradient across the brain to evaluate the rate of production. The concentration of metabolites in the cerebrospinal fluid (CSF) may, however, provide an index of the turnover of substances in the brain.

5-Hydroxytryptamine (5HT) is found in high concentration in central structures of brain of animals and man (Garattini and Valzelli, 1965). It appears to be localized in fine neurones whose cell bodies arise in the mid-brain raphe nuclei and ramify both upwards to other structures, including the cortex, and downwards to the spinal cord (Andén, Dahlström, Fuxe, Larsson, Olson, and Ungerstedt, 1966). The function of these 5-hydroxytryptaminergic neurones is not known, but the system has been implicated in the control of sleep (Koella and Czicman, 1966; Oswald, Ashcroft, Berger, Eccleston, Evans, and Thacore, 1966), temperature (Feldberg and Myers, 1964), and central autonomic processes (Brodie and Shore, 1957). Consequently, any technique which determines the turnover rate of 5HT in brain may shed some light on its role in the regulation of these functions.

The metabolite of 5HT, 5-hydroxyindol-3-yiacetic (5HIAA), is found both in the brain and in CSF. The administration of tryptophan to animals increases the concentration of both 5HT and 5HIAA in brain (Ashcroft, Eccleston, and Crawford, 1965) and of 5HIAA in CSF. The change in the concentration of 5HIAA in cisternal CSF in the dog shows a very precise time relation to the changes in brain after an intravenous dose of amino acid, and it seems that the 5HIAA in CSF may be used to make an indirect determination of the concentration in brain (Eccleston, Ashcroft, Moir, Park-Rhodes, Lutz, and O'Mahoney, 1968). In this study, tryptophan was given in man in order to determine the resultant changes in the concentration of 5HIAA in lumbar CSF and to relate this to blood and CSF tryptophan levels.

METHODS

LUMBAR PUNCTURE Patients who were investigated for neurological disease by lumbar puncture, but had neither gross brain damage nor psychiatric symptoms, were given an oral dose of L-tryptophan (50 mg/kg) in milk at various times up to 18 hours before sampling CSF. To obviate variable mixing of the CSF patients were rested in bed before lumbar puncture. The first 5 ml of CSF withdrawn was used for the estimation of tryptophan and 5HIAA. Blood was taken for estimation of tryptophan at various times before lumbar puncture.

AIR ENCEPHALOGRAMS Patients who were to be investigated for neurological disorders by air encephalography were given L-tryptophan (50 mg/kg) four hours before the procedure. Five millilitres of fluid were withdrawn, air injected, and a further sample of CSF taken. Patients who were not given tryptophan served as controls.

CHEMICAL ESTIMATION 5HIAA in CSF was estimated by a modification of the procedure of Ashcroft and Sharman (1962). Tryptophan was estimated by the method of Hess and Udenfriend (1939), as modified by Guroff and Udenfriend (1962).

RESULTS

AIR ENCEPHALOGRAPHY PROCEDURE After the injection of air in control subjects there is a significant
(P < 0.01, paired t test) rise in the concentration of 5HIAA in the CSF withdrawn from the lumbar space (Table). In the control subjects there is no difference between concentrations of tryptophan in the fluid samples withdrawn before or after air. Four hours after the dose of tryptophan there is a small, but significant (P < 0.05, paired t test) rise in the concentration of the amino acid in the CSF sample obtained after the injection of air. The administration of tryptophan four hours before the procedure does not alter either the absolute value of 5HIAA or the ratio of the concentration in the fluid after the injection of air to that before.

**TABLE**

<table>
<thead>
<tr>
<th>Group</th>
<th>5HIAA in CSF (µg/ml)</th>
<th>Before air injection</th>
<th>After air injection</th>
<th>Mean ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.7 ± 1.9 (3)</td>
<td>27.7 ± 5.2 (3)</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>19.8 ± 19 (3)</td>
<td>33.3 ± 20 (3)</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

Tryptophan in CSF (µg/ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Tryptophan</th>
<th>0.49 ± 0.04 (3)</th>
<th>0.45 ± 0.03 (3)</th>
<th>0.45 ± 0.03 (3)</th>
<th>0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.3</td>
<td>2.4 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

1 mean ± SEM (no. of observations).

LUMBAR PUNCTURE After tryptophan administration the concentration of 5HIAA in lumbar CSF takes some four hours to show an initial rise (Fig. 1). The maximum level is reached between six and 12 hours and is normal again at 18 hours. Tryptophan on the other hand, shows a significant rise at two hours (Fig. 2). High levels occur during the six to 12 hour period as with the 5HIAA, both substances then showing similar rates of disappearance to return to control levels at about 18 hours after tryptophan administration. As expected, blood levels of tryptophan show an early rise, concentrations returning to normal at 12 hours.

**DISCUSSION**

The acid metabolite of 5HT, 5HIAA, is not normally detectable in plasma. Active transport mechanisms prevent its accumulation in brain from the blood stream (Moir and Eccleston, 1968), and hence the 5HIAA found in CSF probably has its origin in neural tissue. The gradient in the concentration of the 5HIAA which exists from the ventricles to lumbar space (Ashcroft et al., 1968) is maintained by active transport of the 5HIAA out of CSF by a mechanism resembling renal tubular secretion of organic acids (Pappenheimer, Heisey, and Jordan, 1961; Ashcroft et al., 1968). Entry of air into the subarachnoid space during encephalo¬graphy leads to mixing and displacement downwards of CSF resulting in a two-fold increase in the concentration of 5HIAA in fluid removed from the lumbar space (Table). The corresponding con-
centration ratio for tryptophan is unity, indicating a more uniform entry of tryptophan along the CSF space. However, after administration of tryptophan, a small but significant gradient for the amino acid develops, suggesting that the influx under these conditions is not entirely uniform, there being a greater entry at higher levels.

A rise in the concentration of tryptophan in lumbar CSF is already apparent two hours after an oral dose of the amino acid. This is in contrast with the concentration of 5HIAA which only begins to rise some four hours after tryptophan administration and is maximal at about eight hours. This time scale is in good agreement with the work of Pletscher, Bartholini, and Tissot (1967), who found that after intravenous administration, in man, of labelled 3,4-dihydroxyphenylalanine, the precursor of dopamine, there was a slow rise in the concentration of the labelled metabolite, homovanillic acid in lumbar CSF reaching a maximum at eight hours. Entry of 5HIAA into the CSF space does not appear to be uniform, but that in the lumbar region seems to depend on the diffusion of the acid down from higher levels. The delayed increase of this metabolite in lumbar CSF would also suggest that the spinal cord does not contribute to any great extent to the 5HIAA content of lumbar fluid.

The level of 5HIAA in brain and ultimately in CSF is probably a measure of the turnover of 5HT in the 5-hydroxytryptaminergic neurones. It may not, however, give an index of their functional activity. The acid can come from at least two sources. 5HT is released on stimulation of the nerve cell bodies of those neurones situated in the mid-brain raphe nuclei (Aghajanian, Rosecrans, and Sheard, 1967; Eccleston, Padjen, and Randic, 1969) and rapidly converted to 5HIAA. Tryptophan administration also leads to the formation of 5HT and 5HIAA in these neurones. The 5HT formed is not necessarily functionally released and some may merely overspill onto monoamine oxidase and give rise to 5HIAA within the neurone. The total output of 5HIAA from the neurone is ultimately limited by the enzyme tryptophan 5-hydroxylase, which catalyses the formation of 5-hydroxytryptophan, the immediate precursor of 5HT. Nerve stimulation appears to increase the rate of tryptophan hydroxylation probably by stimulation of the tryptophan 5-hydroxylase, thus the administration of tryptophan after stimulation leads to the production of more 5HIAA than in the absence of stimulation (Eccleston, Ritchie, and Roberts, 1970).

In man, then, the lowered concentration of 5HIAA found in the CSF of certain cases of depression (Ashcroft and Sharman, 1960; Dencker, Malm, Roos, and Werdinius, 1966; Ashcroft, Crawford, Eccleston, Sharman, MacDougal, Stanton, and Binns, 1966) could be the result either of a lowered release of 5HT from 5-hydroxytryptaminergic neurones, or of lowered tryptophan 5-hydroxylase activity setting a lower than normal limit to the synthesis of 5HT. The results of animal experiments (Eccleston, Padjen, and Randic, unpublished observations) suggest that there is a greater production of 5HIAA as a result of tryptophan administration (the amount being limited by the quantity of tryptophan 5-hydroxylase) than by nerve stimulation. Hence a failure of tryptophan administration in patients with depression to produce rises in 5HIAA in CSF comparable with normal subjects (Ashcroft, unpublished observations) would suggest reduced activity of tryptophan 5-hydroxylase in this illness. This could, however, be the late result of reduced functional activity of 5-hydroxytryptaminergic neurones, assuming normal activity has a regulatory role in the formation of this particular enzyme.

**SUMMARY**

Oral administration of tryptophan produces a rise in the concentration of the amino acid in lumbar CSF. It also leads to the formation of 5HT in brain with an increase in the metabolite 5HIAA which appears in lumbar CSF after a delay that is presumably due to the time taken to diffuse down from the brain to the lumbar space.

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5-Hydroxtryptamine metabolism in affective illness: the effect of tryptophan administration

From the Medical Research Council Brain Metabolism Unit, University Department of Pharmacology, Edinburgh

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5-Hydroxytryptamine metabolism in affective illness: the effect of tryptophan administration


From the Medical Research Council Brain Metabolism Unit, University Department of Pharmacology, Edinburgh

SYNOPSIS 1-tryptophan 50 mg/kg was administered orally to patients suffering from either unipolar or bipolar affective illness, and the concentration of 5-hydroxyindol-acetic acid estimated in their cerebrospinal fluid eight hours later. There was no significant difference between the patient groups or between these and patients with neurological disease. These findings suggest a reduced neuronal activity in the 5-hydroxytryptaminergic system in some depressed patients rather than an absolute deficiency of tryptophan-5-hydroxylase. The synthesis of 5-HIAA in response to tryptophan varied with age.

The biogenic amines are a group of compounds which probably act as neurotransmitters and are located within the central nervous system in neuronal tracts which reach most areas of the brain. 5-hydroxytryptamine (5-HT) is predominantly located within nerves whose cell bodies lie in the brain stem raphe nucleus and whose axons spread both downwards to the spinal cord and upwards to areas including the cortex. In particular, they supply the limbic system. The end product of 5-HT metabolism is 5-hydroxyindol acetic acid (5-HIAA) and is found both in brain and cerebrospinal fluid (CSF). Measurement of the concentration of 5-HIAA in the brain and/or CSF is used as an index of 5-HT metabolism.

In depressive illness it has been shown that the concentration of 5-HIAA is lower than in a control group (Ashcroft and Sharman, 1960; Ashcroft, Crawford, Eccleston, Sharman, MacDougall, Stanton, and Binns, 1966; Dencker, Malm, Roos, and Werdinius, 1966; Van Praag, Korf, and Puite, 1970) and it has been put forward that this is due to a reduced turnover of 5-HT.

The possibility that the reduced concentration of 5-HIAA in the lumbar CSF of depressed patients is due to a deficiency of the enzyme tryptophan-5-hydroxylase has been explored in the present paper. Tryptophan was administered to patients with affective illness and, for comparative purposes, to neurological 'controls', in order to give increased synthesis of 5-HT by exposing the enzyme to an elevated concentration of the amino acid.

METHODS

The study involved 19 psychiatric patients whose illness required admission to hospital, and 13 neurological patients. The psychiatric patients had either a unipolar or bipolar-affective disorder. These patients had episodes in the past which required treatment with drugs or ECT. The neurological 'control' patients had been investigated for neurological disease by lumbar puncture, but had neither gross brain damage nor psychiatric symptoms. Diagnosis of the psychiatric patients was established by two psychiatrists as well as by consultation of the case notes for previous episodes of hypomania or depression. The average age of the psychiatric patients was 52 ± 10 (mean ± SD) and that of the neurological controls 46 ± 13.

Before administration of 1-tryptophan the patients including the controls were placed on a diet for at least three days, and usually five, designed to stabilize the intake of tryptophan and indol-like substances which might interfere with the estimations. 1-
Tryptophan (50 mg/kg) was given orally in milk at 2 a.m. on the fifth day after the commencement of the diet and the patients were rested in bed until a lumbar puncture was performed on them eight hours later at 10 a.m. A time of eight hours between the administration of L-tryptophan and the removal of CSF was chosen, as a previous study (Eccleston, Ashcroft, Crawford, Stanton, Wood, and McTurk, 1970) had shown that after this time interval the concentration of the 5-hydroxytryptamine metabolite, 5-HIAA, in the lumbar CSF had reached a maximum.

Samples of lumbar CSF were obtained from the patients who had given their permission for the lumbar puncture to be performed, after it had been explained to them that the procedure was for research and not for any diagnostic purpose. The patients were placed in a lateral position and approximately 5 ml CSF were dripped from a needle inserted into the lumbar region into a clean, glass-stoppered test tube. The cerebrospinal fluid was placed at -20°C until analyses were carried out within not more than seven days after the samples had been withdrawn.

Samples of venous blood were obtained at one hour before, at the time of, and one hour after, lumbar puncture. The blood samples were placed in heparinized containers and were placed at 4°C until used for the estimation of tryptophan in plasma and whole blood within one week of removal.

5-HIAA in the lumbar CSF was estimated by a modification of the procedure described by Guldberg and Yates (1968). Essentially, 0.15 M borate buffer pH 8.6 was used instead of 0.1 M tris buffer pH 8.6 in order to reduce the blank. Tryptophan in whole blood, plasma, and CSF was estimated by the method of Hess and Udenfriend (1959) as modified by Guroff and Udenfriend (1962).

RESULTS

The concentration of 5-HIAA in the lumbar CSF of the psychiatric patients eight hours after the administration of L-tryptophan (50 mg/kg) did not differ significantly from that of a neurological 'control' group treated in the same way. There was also no significant difference in the 5-HIAA concentration between patients with unipolar or bipolar affective disorders or between bipolar manic and bipolar depressed patients (Fig. 1).

There appeared to be a parabolic relationship between the age of the patient at lumbar puncture and the concentration of 5-HIAA in their CSF. Constructing the best fit quadratic curve by the method of least squares gave a minimum value for the concentration of 5-HIAA at 42 years.

![Graph](image-url)

**Fig. 1** The concentration (μg/ml) of 5-hydroxyindole acetic acid (5-HIAA) in the lumbar CSF of unipolar depressed (UPD), bipolar depressed (BPD), and bipolar manic (BPM) patients eight hours after the administration of L-tryptophan (50 mg/kg) as compared with a control group (continuous horizontal line with SEM broken lines) treated in the same way. Each value represents the mean ± SEM. NS: not significant.
When these data are examined by the Eta correlation there was found to be a significant variation with age ($y = 0.67$, $P < 0.05$). Even when this age distribution of 5-HIAA concentrations is taken into consideration, there is no difference between the four groups of patients (Fig. 2).

The concentration of tryptophan in the lumbar CSF of the three groups of psychiatric patients did not differ significantly from that of the 'control' neurological group (Table). There was, however, found to be a significant correlation ($r = 0.58$, $P < 0.005$) between the concentration of tryptophan and 5-HIAA in the lumbar CSF of all the groups of patients eight hours after the administration of L-tryptophan (Fig. 3). There was a significant correlation ($r = 0.52$, $P < 0.02$) between tryptophan in the CSF and the age of the patient in the group above 45. Unfortunately, there are only a few estimates available in patients below 45 years of age (Fig. 4), and it is not clear whether for tryptophan a 'U'-shaped correlation with age exists as with 5-HIAA.

From an hour before the lumbar puncture until an hour after, the concentration of tryptophan in whole blood and in plasma was dropping gradually. There was no correlation between the concentration of tryptophan in blood at the time of lumbar puncture and that in the lumbar CSF. It should be pointed out that tryptophan is found in plasma both bound to protein and free (McMenamy, Lund, and Oncley, 1957) and one would anticipate that the CSF concentration would be more directly related to the free amino acid. Knott and Curzon (1972) have found that stress in rats causes an increase in both free plasma tryptophan and total brain tryptophan without a significant change in total plasma level.
Tryptophan loading in affective illness

TABLE

CONCENTRATION OF TRYPTOPHAN IN LUMBAR CSF OF UNIPOLAR DEPRESSED (UPD), BIPOLAR DEPRESSED (BPD), BIPOLAR MANIC (BPM), AND NEUROLOGICAL CONTROL PATIENTS, EIGHT HOURS AFTER ADMINISTRATION OF TRYPTOPHAN (50 MG/KG)

<table>
<thead>
<tr>
<th></th>
<th>UPD</th>
<th>BPD</th>
<th>BPM</th>
<th>Neurological controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan (µg/ml)</td>
<td>2.4 ± 1.0 (10)</td>
<td>3.3 ± 1.0 (5)</td>
<td>2.1 ± 0.5 (3)</td>
<td>2.8 ± 1.4 (12)</td>
</tr>
</tbody>
</table>

Mean concentration ± standard deviation (no. of patients.)

DISCUSSION

Since the discovery of low 5-HIAA levels in the CSF of depressed patients (Ashcroft and Sharman, 1960), much work and speculation has been centred around the possibility that, in these states, there exists a deficiency in transmission in neuronal systems in which the biogenic amines act as neurotransmitters.

It has been suggested that the low levels of 5-HIAA in depression are due to a defect in the synthesis of the amine 5-HT. The rate limiting step in the formation of the amine is probably the enzyme tryptophan-5-hydroxylase (Moir and Eccleston, 1968) and it is change at this step which produces alteration of 5-HT synthesis. The substrate for this enzyme in brain is the amino acid L-tryptophan, and normally the enzyme is unsaturated with this substrate. Hence, when the brain levels of tryptophan are raised, the synthesis of 5-HT increases. 5-HT synthesis is also increased when the neurons are stimulated electrically (Sheard and Aghajanian, 1968), and this increase does not appear to require a change in the level of tryptophan in the brain (Shields

FIG. 3 Correlation between the concentration (µg/ml) of 5-hydroxyindol acetic acid (5-HIAA) and the concentration (µg/ml) of tryptophan in the lumbar CSF of patients with affective illness and also control patients eight hours after the administration of L-tryptophan (50 mg/kg).
and Eccleston, 1972). These authors suggested that electrical stimulation results in an allosteric change in the enzyme tryptophan 5-hydroxylase resulting in an accelerated synthesis of 5-HT. Reduced synthesis in this system could be due to an abnormally low concentration of tryptophan in brain, to a reduced amount of the enzyme tryptophan-5-hydroxylase, or to a reduced firing rate in the system. In the above experiments these possibilities have been explored by giving large doses of L-tryptophan in order to test the maximum potential synthesis of 5-HT in the brains of patients with various affective illnesses and in neurological controls.

It has been established in experimental animals (Eccleston, Ashcroft, Moir, Parker-Rhodes, Lutz, and O'Mahoney, 1968) that changes in cisternal CSF 5-HIAA reflect changes in brain 5-HIAA and that high levels indicate a high turnover of 5-HT and vice versa. That 5-HIAA in CSF bears a relationship to brain 5-HT turnover has been questioned by Bulat and Zivković (1971). However, their experiments were performed in the cat, an animal with extremely high levels of the acid in brain and spinal cord (Eccleston and Roberts, unpublished observations). In previous experiments (Eccleston et al., 1970) in man, the rise in 5-HIAA in lumbar CSF after a loading dose of tryptophan became apparent only after four to six hours, reaching a maximum at eight hours. The response in terms of increased synthesis of 5-HT and hence 5-HIAA production is an immediate consequence of the rise in tryptophan in neural tissue (Moir and Eccleston, 1968). Hence, if spinal cord in man had contributed significantly to the rise in 5-HIAA one would have anticipated an almost immediate rise in 5-HIAA in lumbar CSF, certainly occurring earlier than four hours. The delay in the rise of 5-HIAA at the lumbar space is compatible with the view that this is due to the time taken for diffusion of the acid from ventricular fluid down the CSF axis of the lumbar region. In that there is no difference in the concentration of 5-HIAA in the CSF after loading between the groups, then it is possible to infer that the low level of 5-HIAA in depression is not due to a reduction in the enzyme tryptophan-5-hydroxylase (assum-

![FIG. 4 Correlation between the concentration of tryptophan in the CSF of unipolar depressed (●), bipolar depressed (■), bipolar manic (○), and control neurological patients (×) eight hours after the administration of 50 mg/kg tryptophan and their age.](image-url)
ing it is not a genetically different enzyme in this illness). Since tryptophan levels in CSF in depression are the same as control subjects (Ashcroft, Blackburn, Eccleston, Glen, Hartley, Kinloch, Lonergan, Murray, and Pullar, 1973), it would suggest that there is not an abnormally low level of the amino acid in brain, and hence the low 5-HIAA is due to diminished neuronal activity in this system in depression.

The results also show that the accelerated synthesis of 5-HIAA in response to a tryptophan load varies with age in a complex fashion. That this accelerated synthesis is likely to be near maximum is demonstrated by the finding that this dose of L-tryptophan (50 mg/kg) gives maximal synthesis of 5-HT in the brain of the rat (Moir and Eccleston, 1968). There appears to be a parabolic curve in the 5-HIAA response with age which gives a minimum level at about the age of 42 and which is similar to that described, without loading, by other workers (Bowers and Gerbode, 1968). When these data are examined by the Eta Correlation there was found to be a significant variation with age (yx = 0.67, P < 0.05).

It is also apparent that the tryptophan in the CSF correlates significantly with the concentration of 5-HIAA.

This correlation is seen more clearly in considering the concentration of tryptophan in CSF after the age of 42, too few points being available before this age to decide whether there is a correlation between the amine and the amino acid. As with 5-HIAA there is a significant (P < 0.02) rise in tryptophan in CSF with age at eight hours after loading. It could be considered that the high 5-HIAA concentrations are a direct result of high levels of tryptophan in brain and vice versa. One would then be observing the result of a combination of transport of tryptophan and hydroxylation under these conditions. A further possibility is that the mechanisms responsible for the efflux of these compounds from CSF were less efficient with age after 42 years. The important finding is, however, that even when this age factor is considered, there is no difference in the response to tryptophan in terms of 5-HT synthesis between the affective groups studied and the neurological controls. This, with the presence of normal tryptophan levels in these illnesses, implies that the low levels of 5-HIAA in depressive illness are due neither to a reduced availability of trypto-

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SECTION X
The question of the relationship between biogenic amines and depression has been adequately reviewed in the past (Schildkraut, 1965; Coppen, 1967).

Perhaps I could take the opportunity to cover the recent literature and to mention some of our own observations.

It seems that we have to answer three questions:

1. Is there sufficient evidence to implicate the biogenic amines in the aetiology of depression?
2. If they are concerned, then is 5-hydroxytryptamine or noradrenaline, or even tryptamine the amine mediating the mood change?
3. If one of the amines is primarily involved, would precursor loading be likely to increase its physiological effects?

The evidence for the role of biogenic amines is largely circumstantial, and stems from the fact that drugs which are used in the treatment of other conditions have been found to produce changes in mood. One of these illnesses was hypertension, and the drugs which interfered with noradrenergic transmission were also found to cause significant incidence of depression amongst the patients.

Reserpine, for example, is known to deplete biogenic amines in brain. The incidence of depression amongst patients treated with this drug was as high as 15% (Bunney and Davis, 1965). On the other hand, drugs used in the treatment of depression were found to have an action which was likely to potentiate any amines which were released onto receptor sites (fig. 1). Noradrenaline is released on nerve stimulation. Its major mode of inactivation is re-uptake into the neurone (Whitby et al., 1961). The intraneuronal noradrenaline is metabolised by monoamine oxidase. It has been found that drugs
of the tricyclic antidepressant group, of which imipramine was the first, are capable of inhibiting this re-uptake mechanism into the nerve terminal and, in consequence, of potentiating noradrenaline released on nerve stimulation (Sigg, 1959).

Both histochemical and biochemical techniques (Carlsson et al., 1966; Glowinski and Axelrod, 1966) have shown that there appears to be such a re-uptake mechanism into noradrenergic neurones in brain, and that this process itself is susceptible to inhibition by drugs

![Fig. 1. Model of noradrenergic terminal.](image)

of the tricyclic group. Glowinski and Axelrod (1964) working on this model, injected labelled noradrenaline intraventricularly and determined whether the re-uptake was inhibited by a number of drugs which were antidepressants and some of their structural analogues which were not antidepressants. They found a direct relationship between inhibition of uptake of noradrenaline into brain and the clinical activity of the drug. As we have seen in the model, the intraneuronal metabolism of noradrenaline appears to be by monoamine oxidase. Iproniazid used in the treatment of tuberculosis was found to cause elevation of mood and was subsequently shown to be a monoamine oxidase inhibitor. The difficulty was that there appeared to be a very poor correlation between clinical activity and monoamine oxidase inhibition. It has been proposed by (Collins et al., 1970) that this is related more to the inhibition by a particular drug of one of the isoenzymes of monoamine oxidase which is found in brain, and that the therapeutically more potent drugs significantly inhibit those isoenzymes which are responsible for terminating the action of the amine mediating mood change. On the other hand, Hendly and Snyder (1968) found that the monoamine oxidase inhibitors, such as phenelzine, are themselves capable of inhibiting
the re-uptake mechanism. This gives a unitary action to both monoamine oxidase inhibitors and tricyclic antidepressants. There does, then, appear to be a reasonable body of evidence to implicate the biogenic amines in the aetiology of depressive illness.

The question of which amine is primarily involved in the aetiology of depression is important in the reasoning behind precursor loading. If it is the 5HT system, then tryptophan or 5HTP is the precursor of choice, and if noradrenaline, then dihydroxyphenylalanine (DOPA). Perhaps it is also relevant at this point to put a final nail in the tryptamine coffin. The finding of tryptamine in tissues and its elevation on tryptophan loading was proved to be an artefact of the method involved in its detection. The “tryptamine” was merely tryptophan carried through into the final fluorescence assay by its absorption onto lipid (ECCLESTON et al., 1966). BJORKLUND and FALCK (1969) have attempted to find tryptamine in brain by fluorescence microscopy but has evinced evidence for its presence only in the pars intermedia of the pituitary gland. Most of the pharmacological evidence for the biogenic amine hypothesis involves the use of drugs which have similar actions on both 5-hydroxytryptaminergic and noradrenergic neurones. This appears also to be true for the tricyclic antidepressants. Electron microscopic studies combined with autoradiography (AGHAJANIAN et al., 1966) and fluorescence studies (FUXE and UNGERSTEDT, 1967) have shown that when 5HT was given intraventricularly in brain, there was an accumulation of the amines in fine varicose fibres, cell bodies and terminals lying close to the ventricles. Fluorescence of products were also seen in the cell bodies of, for example, the dorsal raphe nuclei. This uptake process was inhibited by imipramine and chlorimipramine (CARLSSON et al., 1968). We have then the situation in which the drugs which were postulated to either cause or cure depressive illness can be shown to interfere in a similar way with the function and metabolism of both noradrenaline and 5-hydroxytryptamine. There are differences in activity on the other two systems amongst the tricyclic antidepressants. Imipramine was not a potent uptake blocker of noradrenaline but was of 5HT whilst the reverse seems to be true for desipramine. However, these experiments were conducted in the rat, and because of species differences, it may not be possible to generalise the finding to man.

In these experiments in animals, the effects of drugs are seen im-
mediately, and this does not fit in with the clinical experience which shows the improvement on the tricyclic antidepressants may take from ten days to several weeks. This was initially explained by postulating that time was required for concentrations of active drug or metabolite to build up in brain (Brodie et al., 1961; Moody et al., 1967). More recently Schildkraut et al. (1970) have shown that in acute experiments, not only is the uptake of noradrenaline inhibited into the central noradrenergic neurones by imipramine but the release from brain is slowed. However, when dosage of imipramine is continued for three weeks, the uptake of noradrenaline is still blocked, but there is now an accelerated loss of noradrenaline from the neurone. In other words, during this period of three weeks, there has been an acceleration in the rate of metabolism in these neurones. The implication is that changes in receptor activity have a regulatory effect on the biosynthesis of the amine.

Our own work has attempted to show the relationship between the function of the 5HT system and the synthesis of 5HT. This amine is contained in fine neurones, the largest collection of whose cell bodies lie in the raphe nuclei of the brain stem (Dahlstrom and Fuxe, 1965) and whose axons ramify to all regions of brain including the cortex. By implanting an electrode in these nuclei, electrical stimulation of this system (Aghajanian et al., 1967) has been shown to accelerate the synthesis of 5HT. We wondered whether this increase in synthesis outlasted the stimulation and consequently, we stimulated an anaesthetised rat for one hour (Eccleston et al., 1970) stopped stimulation, and waited 15 minutes. The rate of synthesis of 5HT was then determined by the administration of a loading dose of L-tryptophan, a procedure which gives an index of maximum hydroxylation rate. The results (fig. 2) show that the increased synthesis outlasts stimulation. It seems feasible that this mechanism functions by way of increase in functional hydroxylase enzyme which may be by the formation of additional hydroxylase enzyme. In other words, these changes may reflect an increased protein synthesis which should produce changes on a much longer time base than that indicated by the half life of 5-hydroxytryptamine itself.

We must now answer the third question: is precursor loading likely to increase the physiological effect of the amine?

I would like to focus attention on tryptophan loading. I would
not like to comment on the clinical effects from previous studies in depression. In the remaining literature, I can find only the effects of tryptophan alone on sleep where it appears to bring forward the onset of rapid eye movement sleep in susceptible individuals (Oswald et al., 1966) and to increase its duration in normal individuals (Hartmann, 1967) and narcoleptics (Evans and Oswald, 1966).

Fig. 2. Concentration of 5HIAA in rat forebrain in two groups of animals one of which had a 60 min. period of stimulation by an electrode implanted in the raphe nuclei, ending 15 min. before the injection of L-tryptophan (800 mg/kg). The control group had implanted electrodes without stimulation.

In the experimental animals, tryptophan has been found to potentiate the spinal reflex of the rat (Meek et al., 1970). This reflex has a 5-hydroxytryptaminergic component. The time of onset of the potentiation after tryptophan was found to be two hours, which agrees well with maximum levels of 5HT achieved after tryptophan loading (Ashcroft et al., 1966). The effect of tryptophan was found to be further potentiated by pre-treatment with chlorimipramine. Attention should, however, be drawn to the fact that these experiments culminated in the examination of tissue by fluorescence
microscopy, and required that the animals were treated with both reserpine and a monoamine oxidase inhibitor.

There are, of course, in the literature a number of references to the physiological effects of 5-hydroxytryptophan. We feel, however, that tryptophan should be considered the more physiological precursor. We have undertaken some quantitative fluorescence studies

![Graph](image)

Fig. 3. Concentration of forebrain 5HT and 5HIAA in control rats, and rats treated with either L-5HTP (50 mg/kg) or L-tryptophan (800 mg/kg) with or without pretreatment with nialamide (200 mg/kg) 3 hours before the amino-acid. The animals were killed 1 hour after treatment with the amino-acid.

on brain after either tryptophan loading or 5HTP loading. Our previous work (MOIR and ECCLESTON, 1968) had indicated that even with a relatively small dose of tryptophan both 5HT and its metabolite 5HIAA rose in brain. When 5HTP was administered there was a marked increase in the concentration of 5HIAA and this always assumed a proportionately higher level than 5HT when compared with tryptophan. The question arises as to where this 5HT is formed. Consequently we (Dowson et al. — unpublished observations) conducted histochemical studies using the formaldehyde
fluorescence method, and quantitative studies were made by microspectrophotometry. This was compared with a chemical assay for 5HT and 5HIAA in forebrain, the other half of the brains of rats treated with either 5HTP or tryptophan. The chemical assays are shown on fig. 3. The administration of L-tryptophan gave a rise in both 5HT and 5HIAA. When combined with a MAOI this gave, as anticipated, higher levels of 5HT and lower of 5HIAA. L-5HTP (50 mg/kg) gave a much larger rise in 5HIAA than 5HT. When combined with an MAOI the 5HT levels were extremely high.

Sections of the cortex from these animals show that L-tryptophan leads to an increase in the parenchymal fluorescence compared with the sections of cortex from untreated animals. This is not significantly increased by the combination with an MAOI. The emission maximum from this diffuse green fluorescence is at approximately 490 m, and corresponds to that obtained with tryptophan in model experiments. Hence it is probably due to the high concentrations of this amino-acid following the large dose administered. It is important to point out that alone and in the presence of an MAOI tryptophan gives no increase in capillary wall fluorescence. L-5HTP (50 mg/kg) on the other hand gives an increase in capillary wall fluorescence. This could, of course, be due to the accumulation of 5HT, of 5HTP, or of a mixture of the two substances. In combination with an MAOI this fluorescence becomes extremely marked and suggests that 5HT forms a significant part of this fluorescence. These findings would indicate that a large proportion of the 5HT formed and estimated chemically after 5HTP, is not localised intraneuronally, whilst after tryptophan it occupies normal physiological sites.

Even if 5HT concentration does increase intraneuronally on administration of tryptophan without a monoamine oxidase inhibitor, then this does not necessarily mean that there is an increased release of the amine onto receptors (fig. 4). One may indeed merely have increased synthesis of 5HT filling stores, followed by an increased overspill from these stores onto monoamine oxidase with subsequent pumping out of the 5HIAA from the neurone. These findings may go some way to explaining the lack of efficiency of 5HTP precursor loading in man, and may also indicate that therapeutic results achieved with tryptophan would indeed only be anticipated in combination with a monoamine oxidase inhibitor.

A number of studies have shown that 5HIAA is lowered in the
cerebrospinal fluid of patients suffering from depression (Ashcroft et al., 1966; Dencker et al., 1966). This could be interpreted either as a lowering function of the 5-hydroxytryptaminergic function or else a failure of function in this system because of limits on the synthesis of 5HT placed by a deficiency of tryptophan 5-hydroxylase. To test this hypothesis, we established what we hoped would be the normal 5HIAA response to a tryptophan load (50 mg/kg) in the cerebrospinal fluid in patients suffering from neurological disorders who were about to have a lumbar puncture (fig. 5). As you can see, after a large dose of tryptophan the levels of 5HIAA peak only after some 8 to 10 hours (Eccleston et al., 1970). A group

Fig. 4. Model of 5HT terminal.

Fig. 5. Concentration (mean ± s.e.m.) of 5HIAA (µg/ml) in lumbar CSF at times up to 18 hours after oral administration of L-tryptophan (50 mg/kg). Number of subjects indicated in parenthesis.
Amines and Affective Disorders

of patients suffering from various forms of depressive illness were tryptophan loaded (50 mg/kg) the CSF and 5HIAA being assayed after 8 hours. These were then compared with a group of neurological patients similarly loaded. What we found was a very wide range of concentrations of 5HIAA after the procedure (fig. 6). There appears to be no difference between controls and depressed patient. The 5HIAA response to tryptophan increases significantly \((p < 0.005)\) with age, and may merely reflect a less effective mechanism for removing 5HIAA from cerebrospinal fluid with increased age. This age difference in 5HIAA in CSF has been reported previously in unloaded subjects (Bowers and Gerbode, 1968).

Summarising our questions then:

![Fig. 6. Concentration of 5HIAA in lumbar CSF of patients suffering from either manic depressive psychosis (depressed or manic) reactive depression or under investigation for neurological disease. The CSF was taken 8 hours after a loading dose of L-tryptophan (50 mg/kg).]
1. There appears to be sufficient evidence to implicate the biogenic amines in the aetiology of depression.

2. Whether 5-hydroxytryptamine or noradrenaline is aetiological remains an open question.

3. There is not a great deal of evidence that the administration of tryptophan alone gives detectable physiological effects in animals or man.

   Perhaps then, we are asking the wrong questions. Is it reasonable to implicate a single system in brain? It seems probable that we are dealing with inter-related systems. A very exciting paper has recently been published by Couch (1970) which gives evidence that the firing rate of single cells in the region of the raphe nuclei may be either stimulated or depressed by noradrenaline or 5-hydroxytryptamine. On a small majority of these cells, 5-hydroxytryptamine and noradrenaline produce opposite effects. The suggestion is then that certain cells have terminals of both 5-hydroxytryptaminergic neurones and noradrenergic neurones on them, and that whether their firing rate increases or not may depend on the relative rate of discharge of these two amines onto them.

   Originally, the function of these various systems was judged by the level of the amine in brain, later the concept of turnover being related to function was accepted, but now because certain examples are arising where turnover and firing rate are not related (Sheard and Aghajanian, 1970) we must look at the elegant experiments of Aghajanian et al. (1970) on determining the firing rates of the systems. Our own workers tended to show that the rates of firing in themselves lead to changes in the enzyme activity in the systems, and that these effects may, in fact, be quite long term.

   As psychiatrists, we seldom see pure endogenous depression or manic depressive psychosis. Psychogenic factors are almost invariably present. Perhaps because of the relationship between the function of these systems and the long term biochemical changes it is now the biological psychiatrist who can find some common ground with the analysts.

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MODIFIED AMINE HYPOTHESIS FOR
THE AETIOLOGY OF AFFECTIVE ILLNESS

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Reports of low levels of 5-hydroxyindolacetic acid (5-H.I.A.A.) in cerebrospinal fluid (C.S.F.) in depressive illness have been confirmed, and low levels of homovanillic acid (H.V.A.) have also been reported. However, there is no consistent relation between these abnormal levels and mood state—e.g., low levels of 5-H.I.A.A. found in depression and mania did not rise when the patient recovered. If we accept that these low metabolite levels reflect a fall in the activity and metabolism of serotoninergic neurons, then these experimental clinical studies are not consistent with the amine hypothesis as it was simply stated—namely "that depression can occur when the levels of biological amines at reactive sites within the brain are reduced and that anti-depressant drugs will be those which increase the levels of the amines at the reactive sites". The object of this communication is, therefore, to develop a modification of the amine hypothesis which will more closely fit the facts and which may prove susceptible to further testing. In constructing the new hypothesis, we will summarise the results of two of our studies on amine metabolism in the C.N.S. In making comparisons between affective disorders in man and the behavioural changes which accompany the experimental manipulation of amine-mediated systems in animals, we have found it necessary to emphasise behavioural aspects of mania and depression: and in this paper more weight is given to a

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discussion of these than to the description of the "feeling state" with which no ready parallel can be drawn from animals.

C.S.F. STUDIES IN MAN

*Amine Metabolites in Unipolar and Bipolar Affective Illness*

In most of the studies carried out on depression, including our own, there has been no attempt to designate specific diagnostic subgroups. We have recently separated patients into unipolar (recurrent depressive) and bipolar (manic depressive) subgroups. In the bipolar group all patients have a history of both manic and depressive attacks whereas the unipolar group have a history only of previous depressive episodes.

The results (table 1) show that the concentration of 5-H.I.A.A. and H.V.A. in lumbar C.S.F. in unipolar depression is significantly lower than in the control group, but in the bipolar depressive group the level of both these metabolites are within the normal range and significantly higher than in the unipolar group:

| TABLE I—MEAN ± S.D. CONCENTRATIONS (ng./ml.) OF 5-H.I.A.A. AND H.V.A. IN LUMBAR C.S.F. OF PSYCHIATRIC PATIENTS BOTH BEFORE AND AFTER TREATMENT AND OF CONTROLS |
| --- | --- | --- | --- | --- |
| | Unipolar depression | Bipolar depression | Bipolar mania | Controls subjects |
| 5-H.I.A.A. before treatment | 10 ± 4 (11)* | 18 ± 8 (9) | 15 ± 6 (11) | 16 ± 8 (30) |
| 5-H.I.A.A. after treatment | 12 ± 3 (10) | 17 ± 11 (6) | 17 ± 6 (4) | 15 ± 8 (30) |
| Change on treatment (paired difference) | None | None | None | None |
| H.V.A. before treatment | 20 ± 9 (11) | 34 ± 16 (9) | 35 ± 15 (11) | 41 ± 23 (30) |
| H.V.A. after treatment | 23 ± 10 (9)* | 28 ± 14 (7) | 17 ± 4 (6)* | 21 ± 20 (30) |
| Change on treatment (paired difference) | None | Fall* | None | None |

Numbers of observations are shown in parentheses.
* p < 0.05. *Comparison with controls; Student's t test.
TABLE II—MEAN±S.D. CONCENTRATIONS OF 5-H.I.A.A. AND TRYPtopHAN IN THE LUMBAR C.S.F. OF PSYCHIATRIC PATIENTS 8 HR. AFTER THE ORAL ADMINISTRATION OF L-TRYPTOPHAN (50 mg./kg.) AS COMPARED WITH CONTROLS TREATED SIMILARLY

<table>
<thead>
<tr>
<th></th>
<th>Unipolar depression</th>
<th>Bipolar depression</th>
<th>Bipolar mania</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-H.I.A.A. (ng./ml.)</td>
<td>32±13 (10)</td>
<td>26±14 (5)</td>
<td>27±7 (4)</td>
<td>29±14 (13)</td>
</tr>
<tr>
<td>Tryptophan (μg./ml.)</td>
<td>2·4±1·0 (10)</td>
<td>3·3±1·0 (5)</td>
<td>2·1±0·5 (3)</td>
<td>2·8±1·4 (12)</td>
</tr>
</tbody>
</table>

The concentrations of 5-H.I.A.A. and tryptophan in any of the psychiatric groups of patients do not differ significantly (p>0·05) from those of the control group.

c.s.f. levels are thus clearly not related to mood—e.g., in the unipolar depressed group, a change in mood state is not related to a change in level of the metabolites.

Synthesis of 5-H.T. in Depression

One suggested explanation for low levels of 5-H.I.A.A. in unipolar depression would be a defect in synthesis of the amine. Such a deficiency might be due to a defect in the activity of the rate-limiting enzyme, tryptophan-5-hydroxylase.10

The synthetic capacity for the formation of 5-H.T. can be evaluated by administering a loading dose of tryptophan and later measuring the increased level of 5-H.I.A.A. in c.s.f. The results (table II) seem to suggest that there is no reduced potential synthesis of 5-H.T. in affective illness.

The findings of these two investigations suggest that in unipolar depressive illness there is a change in functional release of amine transmitters (at least for 5-H.T.) without a change in the capacity for amine synthesis. However, if we wish to formulate a general hypothesis to acknowledge a central role of the amine-containing system in the different types of affective illness, then we must search for a modification of the simple amine hypothesis normally put forward.

ANIMAL STUDIES

Structure and Function of Amine-containing Systems in the C.N.S.

The presence of high concentrations of 5-H.T., noradrenaline, and dopamine in brainstem, limbic structures, and basal ganglia has been recognised for some time.12–14 With the introduction of the techniques of fluorescence microscopy by Swedish workers,15 specific amine-containing neuronal systems have been clearly delineated.16 In
general, cell bodies of the aminergic neurons are in brainstem with projections to corpus striatum, to amygdala and hippocampus, to cortex, and also, for some systems, downwards to the spinal cord.

**Amine-mediated Synaptic Transmission**

A schematic representation of transmission at a synapse mediated by an amine transmitter is shown in fig. 1.

The enzymatic machinery for the synthesis of amines is transported down the axon, and at the nerve terminal vesicles of transmitter release their contents into the synaptic cleft. Inactivation appears to be mainly by reuptake into the presynaptic nerve terminal, although enzymatic catabolism by monoamine oxidase and catechol-O-methyl transferase is also important. The active re-uptake mechanisms are ion dependent and inhibited by the tricyclic group of antidepressant drugs. In this respect, dopamine may be the exception. Little is known of the receptor, although there is evidence that the synthesis of the biogenic amines in the presynaptic neuron is influenced by receptor activity, possibly through an inhibitory neuronal feedback mechanism.

**Changes in Receptor Sensitivity and Feedback Control of Transmitter Synthesis at Synapse**

In animals the administration of haloperidol, a drug which blocks cerebral dopamine receptors, results in an increase in the turnover of cerebral dopamine. The administration of lysergide (L.S.D.), a compound which in low dosage stimulates cerebral 5-H.T. receptors, is accompanied by a decreased turnover of 5-H.T. Recording from the presynaptic 5-H.T.-containing neurons shows that the administration of L.S.D. produces a fall in firing-rate, whilst the firing-rate in postsynaptic cells is increased. The results are explained by the presence of a neuronal inhibitory feedback mechanism (fig. 2). Increased receptor activity in the postsynaptic neuron in some

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**Fig. 1—Model of noradrenergic synapse.**

NA = Noradrenaline.
MAO = Monoamine oxidase.
COMT = Catechol-O-methyl transferase.
way feeds back to inhibit the firing of the presynaptic aminergic neuron and in addition results in a simultaneous reduction in amine synthesis and turnover. Receptor blockade is thought to release the inhibition and result in an increased firing-rate in the presynaptic neuron and an increase in amine synthesis. Amine turnover may thus show an inverse relation to the activity of the postsynaptic neuron. Activity in the postsynaptic neuron will be dependent on the events occurring in the synaptic cleft and in the postsynaptic receptor membrane. Thus the factors involved will include the level of presynaptic transmitter release, the efficiency of inactivation of released transmitter by re-uptake and/or enzymatic degradation, and the "sensitivity of the postsynaptic receptors". That receptor sensitivity is capable of variation is shown in the peripheral autonomic nervous system, where denervation (e.g., sympathectomy) increases the sensitivity of the postsynaptic neurons to available transmitter. In the central nervous system there is now evidence suggesting the presence of a similar mechanism—e.g., lesions in the substantia nigra which destroy dopaminergic neurons have been shown to lead to an increase in the sensitivity of dopaminergic receptors in postsynaptic neurons.

**BEHAVIOUR AND THE AMINE-CONTAINING SYSTEMS**

**In Animals**

Many attempts have been made to draw inferences regarding the functions of amine-containing systems from a study of the behavioural effects of psychotropic drugs known to be acting on these neurons (e.g., reserpine). Lack of specificity of these drugs has, however, made it difficult to elucidate the role of individual systems.
More recently, however, the use of stereotaxic lesions and electrical stimulation via implanted electrodes has been used to investigate function. From these investigations certain general conclusions can be drawn regarding the role of the systems in the control of animal behaviour. The noradrenergic system has cell bodies lying in the brainstem projecting to many other areas, in particular, the locus coeruleus projects ipsilaterally to the cortex in the rat. The dopaminergic neurons arise primarily in the substantia nigra and project to the corpus striatum. Stimulation of dopaminergic receptors by apomorphine leads to stereotyped behaviour consisting of sniffing, licking, and biting. Dopaminergic activity also seems to control gross motor activity. Lesions of the substantia nigra result in profound hypokinesia in rats, presumably analogous to the symptoms of parkinsonism: “all animals showed pronounced hypokinesia, loss of exploratory behaviour and curiosity and an inability to initiate activity”. On the other hand, ablation of the striatum and cortex in rats and monkeys leads to pronounced hyperactivity with a stereotyped component. It would seem from these animal observations that “it is more probable that the dopaminergic system and striatum control a general arousal or drive level that is necessary for performing a number of activities”.

The noradrenergic system, on the other hand, has been implicated in excitation and in exploratory behaviour. A further facet of behaviour has been studied in self-stimulation experiments, which have shown that animals will self-stimulate when electrodes are placed either in the substantia nigra (the dopaminergic system) or in the locus coeruleus (a noradrenergic system). It therefore becomes apparent that both systems may be necessary for this particular pattern of behaviour. Similarly, we have also found this to be true by studying the effect of amphetamine in rats. This drug will give overactivity and stereotyped behaviour. Lesions of the substantia nigra destroying dopaminergic neurons modify this effect. In a preliminary study we have found that bilateral lesions of the locus coeruleus also almost completely abolishes this response to amphetamine. Again it appears that both noradrenergic and dopaminergic function are necessary for this particular behavioural manifestation.

5-H.T. has been implicated in many forms of
behaviour. The strongest evidence, however, is its role in the mediation of sleep. Cats with lesions in the raphe nuclei—an area which contains many of the cell bodies of the 5-h.t. neurons—show prolonged insomnia.\textsuperscript{38} 5-h.t. neurons have also been postulated to control sensory input into the brain; stimulation of the raphe nuclei reduces the normal habituation to repeated stimuli,\textsuperscript{39} which is a phenomenon also found in animals treated with L.S.D.,\textsuperscript{40} a drug which in small doses stimulates 5-h.t. receptors.\textsuperscript{20} It has been suggested \textsuperscript{36} that the 5-h.t. system antagonises the behavioural effects of dopaminergic and noradrenergic systems, reminding one of the earlier theories \textsuperscript{41} of trophotrophic and ergotrophic systems in brain with opposing functions. The results of these experimental lesions or stimulation of particular systems lead to the exhibition of fragments of behaviour and not to complex behaviour patterns which might be considered to be examples of adaptive behaviour expected in the normal environment. However, these fragments may be the building blocks of more complex adaptive reactions. McLean \textsuperscript{42} has reviewed the neuronal systems whose activity is associated in animals with certain types of behaviour—namely, social behaviour, aggressive/submissive behaviour, sexual behaviour, and oral behaviour. These neurons form, in particular, part of the limbic system and seem to be identical to the amine-mediated pathways described above.

In Man
We can now attempt to examine the problems of relating the activity of neuronal systems to what we understand as “mood” in man. In general, mood is regulated so that it is syntonic with environmental events and pressures, as they are assessed in terms of previous experience. In man, it seems that the label “mood change” is used to cover complex subjective and objectively observed changes in the organism. The components of mood response include a subjective feeling state, cognitive changes with appropriate colouring of thought content, behavioural changes, and changes in the state of arousal. The feeling state and cognitive aspects can only be examined in man because he is the only species to communicate feelings by language. It seems likely, however, that certain of the behavioural and autonomic changes may have much in common in man and other species. In
animals, as we have seen above, there is evidence linking behavioural components of the mood response with activity in specific subcortical and limbic neuronal systems. If it is possible to make valid behavioural comparisons between the behaviour of animals subjected to stimulation or lesions in specific systems and humans with affective illness, then we may be advancing to a position where we can draw conclusions about the basic mechanisms by which behavioural components of the affective disorders are mediated. This is not to claim that we would be any nearer the cause of depression and mania—we would merely be examining the mechanisms by which some aspects of mood are expressed.

The development of complete mood response must involve many stages and many neuronal systems. We suggest that the results of animal experiments relating transmission and metabolism in amine-containing neuronal systems may indicate the mechanisms by which function may be disrupted in similar systems in man. Whilst experiments comparable to those done in animals are impossible in man, we believe that circumstantial evidence permits certain tentative conclusions regarding the state of the aminergic systems in affective illness.

EVIDENCE IN MAN FOR ROLE OF AMINE-CONTAINING SYSTEMS IN AFFECTIVE ILLNESS

Exploratory and Stereotyped Behaviour in Depression and Mania

We would postulate that changes in exploratory and stereotyped behaviour patterns are seen in both depression and hypomania. Changes in exploratory behaviour are seen as a change in the overall activity level of these patients. Changes in the stream of mental activity can also be seen in these terms, thus in mania there is often a constantly changing stream of mental activity. In depression, there is a lack of exploration both in motor and mental activity. Stereotyped motor activity and stereotyped thinking may, however, be accentuated in depression, particularly in so-called agitated depression.

In mania, stereotyped activity may also be an important component of the clinical picture—most psychiatrists will recall a manic patient rummaging through drawers for hours or days at a time, apparently without purpose. Whilst in most manic patients a combination of exploratory and stereotyped activities make up the clinical state, in others, one or other can predominate. We are struck by the similarity between this situation and the effects noted on
amphetamine administration to both animals and man. Here both increase in exploratory and stereotyped behaviour patterns are reported. In animal studies it has been postulated that the increase in exploration results from stimulation of noradrenergic systems while increase in stereotyped behaviour patterns results from stimulation of dopaminergic systems. If this difference were to operate also in man, the varying proportions of exploratory and stereotyped activity in mania might be related to varying activities of these two systems.

In addition to complex stereotypes, other simpler stereotypes can also be observed in patients with affective illness. Chewing, mouthing, tongue-protruding movements are seen in many patients with bipolar affective illness. They resemble the faciobuccal dyskinesias reported in other conditions (e.g., some parkinsonian patients treated with levodopa). The movements come and go at particular times in the cycle of affective illness, recurring at the same phase in subsequent cycles, and they seem unrelated to drug treatment. The movements are identical with those seen in amphetamine addicts and similar to those seen in animals treated with large doses of this drug, where they are attributed to stimulation of dopaminergic systems. By analogy we might argue that stimulation of dopaminergic systems occurs at certain stages in a bipolar affective illness.

**Paradoxical Response to Drug Therapy**

In the treatment of mania with haloperidol or phenothiazines, we find that as long as the underlying mania persists then motor side-effects of drug therapy do not develop and large doses of the drugs can be used because tolerance is high. Suddenly during treatment patients may develop symptoms of parkinsonism and akathisia, and when they do so withdrawal of the drug or reduction of dose invariably reveals a remission or alleviation of the mania. This suggests that a fundamental change in sensitivity of these motor systems is present in mania which disappears coincidentally with remission of the mania. A change in postsynaptic receptor sensitivity might be consistent with these findings.

A basic change in receptor sensitivity might be invoked to explain variations in response to other drugs both in animals and man. Ungerstedt notes that the administration of 6-hydroxydopamine destroys the dopaminergic systems in rat brain and this is followed by the development of supersensitivity of postsynaptic receptors to apomorphine and levodopa. He suggests that some parkinsonian patients show dyskinetic side-effects on levodopa because of a similar type of receptor supersensitivity.

Some hyperkinetic children show a reduction in hyperkinesia and an improvement in behaviour when treated with small doses of amphetamine. Paradoxically, in some patients single doses of amphetamine provoke an
increase in hyperkinesia and the development of severe faciobuccal dyskinesias usually seen only with much higher doses of the drug. These findings would be consistent with the hypothesis that hyperkinesia in some children is due to a specific overactivity of dopaminergic systems resulting from a supersensitivity of postsynaptic receptors. This might lead to a sensitivity to the administration of amphetamine in a single dose, but continued administration of small doses might lead to a suppression of receptor sensitivity as a result of constant low-level stimulation.

If this type of therapy is considered logical in the hyperkinetic child, then a similar reasoning might also be applied to the management of rare cases of recurrent mania. In these patients we might postulate recurrent episodes of increase in receptor sensitivity in one or more amine-containing systems. If our hypothesis regarding the mode of action of amphetamine in hyperkinetic children is correct, then the administration of small doses of this drug to patients with recurrent mania may suppress receptor sensitivity, at least in dopaminergic systems and control manic episodes. Suitable cases resistant to other treatments are rare. There is, however, a report of treatment of mania in this way, and we can confirm the effect in one case.

**Lithium Treatment**

One might also re-examine the role of lithium in bipolar affective illness from the standpoint of its influencing, perhaps stabilising, the sensitivity of amine-containing systems. U'Prichard and Steinberg have reported that lithium will block certain types of behaviour induced by a mixture of chlordiazepoxide and amphetamines in rats which they have advanced as an animal model for mania. This action is interesting in view of our suggestions about the relation between certain aspects of animal behaviour, induced behaviour, and components of the manic response. Lithium might reduce the availability of amine at the receptor by promoting ion-mediated re-uptake into nerve terminals.

**CONCLUSIONS**

We suggest that animal studies may give a lead to the identification of neuronal systems which are involved in the mediation of patterns of behaviour seen during "mood responses" and in affective illness in man. These behaviour patterns include changes in motor behaviour, aggressive/submissive patterns of behaviour, and changes in social and sexual behaviour patterns. The motor patterns seen in animal experiments during stimulation of amine-containing systems may represent non-adaptive "components" or "fragments" of these more complex behaviours. We have attempted to equate the neuronal systems involved
with the limbic and brainstem systems described by McLean and others, in some of which transmission, in at least one synapse, may be mediated by one or other of the biogenic amines.

We have argued that in depression and mania there will be changes in one or more of these systems and that the resulting behaviour modifications can be observed. The change in function and activity in the neuronal systems may result from a changed appraisal of the environmental situation dependent on higher cortical systems, in which case the changes are to be found in input and output into the aminergic systems without any primary change in their sensitivity. In the case of depressive illness, this may be reflected in low output of amine transmitters and in low levels of one or more amine metabolites in C.S.F.

Change in function in aminergic systems may, however, result from a change in sensitivity or threshold setting, possibly as a result of a change in postsynaptic receptor sensitivity. In depression, this would involve a fall of sensitivity in one or more groups of amine-mediated synapses with, at least initially, normal transmitter output and normal C.S.F. amine metabolite levels.

The conclusion we would draw in relation to depression is that both low output of amine transmitters (low-output depression) and low sensitivity of amine receptors (low-sensitivity depression) may give rise to a similar clinical picture. Our preliminary results suggest that the clinical division into unipolar and bipolar depression might parallel such a classification.

A similar subdivision into high output and high sensitivity mania does not yet emerge from the clinical data, but certainly this is susceptible to further testing.

A consideration of the interactions between transmitter output and receptor sensitivity lead us to certain conclusions regarding recovery from depression and mania. Recovery from depression may involve a rise in receptor sensitivity or a rise in transmitter output, or both. The possibility for an "overswing" in functional activity is present both in low output and low sensitivity depression, but we might postulate that a normal functioning of control mechanisms for receptor sensitivity would rapidly restore this in unipolar cases. If the homoeostatic mechanisms for the setting of receptor sensitivity are at fault in bipolar cases, then overswing to mania may occur.

Secondary changes in transmitter output may
follow the primary changes in receptor sensitivity in bipolar affective illness, making the interpretation of C.S.F. metabolite levels difficult.

We would state our modified amine hypothesis as follows:

The activities of amine-mediated synapses in brain are modified in affective illness either as a result of altered input into the neuronal systems from other centres or as a result of altered sensitivity of the postsynaptic receptors.

It will be the balance between transmitter availability at the receptor and receptor sensitivity which will determine the functional state of the systems. We would also postulate that one or more of the amine-mediated systems may be implicated and that the pattern of involvement of the systems will be important in determining the characteristic pattern of behaviour and autonomic components comprising the clinical picture.

Functional recovery of these systems in depression will follow either a rise in receptor sensitivity or a rise in transmitter output or both. True functional restoration of the systems will, however, occur only when both transmitter output and receptor sensitivity and the reactivity of the system to incoming stimuli are all restored to normal.

To test this hypothesis further we must develop tests of neuronal sensitivity in man. In addition, we must re-examine our therapies to consider effects on postsynaptic as well as presynaptic events. A search for methods of influencing receptor sensitivity may be of value, in particular, methods for stabilising these mechanisms.

None of these techniques will provide a treatment for depression—a complex human reaction. All they may be expected to do is to restore the integrity of the behavioural systems to respond to the environment as assessed by the individual.

Requests for reprints should be addressed to G. W. A.

REFERENCES

The biochemistry of human moods

The notion that moods can be determined by biochemical defects in the brain may be difficult to accept. Now, 20 years after the first indications, evidence is accumulating in support of primary biochemical lesions as a cause of a number of psychiatric disorders.

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Affective illness has always fascinated psychiatrists, presenting as it does a striking exaggeration of normal mood: patients may suffer profound depression with feelings of hopelessness and ideas of guilt accompanied in some circumstances by a slowing both of thought processes and movements; the converse, an elevated mood, is to the patient often an exhilarating experience, a feeling of boundless energy, of infinite potential. Unfortunately, such patients may embark on inappropriate financial ventures or disastrous sexual exploits. The two states may occur within the same patient (bipolar illness) or with depressive episodes recurring at intervals of months or years (unipolar depression). Most people experience depressed and elevated moods, but in affected illness, the control of mood is lost so that the patient’s mood becomes inappropriate to his life situation.

The brain amine trio

The first real indication that these illnesses could be the result of biochemical disturbances in the brain was raised in the early 1950s when drugs became available which could inhibit the breakdown of certain substances in brain known collectively as the biogenic amines. Headed the list of these amines was 5-hydroxytryptamine (5HT) which was discovered by accident in the pharmacology department of Edinburgh University by a team of scientists, one an eccentric genius, another a brilliant methodologist and the third, a man capable of doing long and careful experiments. Noradrenaline (NA) was found some few years later in the same laboratories. Dopamine, the third important brain amine was discovered by Swedish workers (see Figure 1).

Using fluorescence microscopy the Swedes located the amines in specific neuronal systems which tend to have their cell bodies in the central regions of the brain with ramifications of the cell processes to all areas including the cortex. Biogenic amines are now recognised as important neurotransmitters. They were implicated in mood regulation by the finding that the group of drugs useful in depression, the monoamine inhibitors, prevent their breakdown by the enzyme monoamine oxidase. A second series of compounds (the tricyclic antidepressants) is also effective in depression, probably by potentiating biogenic amine transmitters after their release.

A second indication of the implication of 5HT in affective illness came in 1960 when George Ashcroft and Denis Sharman estimated the concentration of the end product of its metabolism—5-hydroxy-indole acetic acid (5HIAA)—in cerebrospinal fluid (CSF) and found it to be low in patients suffering from depression; they assumed that the turnover of 5HT was low in this illness. In spite of problems of accuracy in measuring CSF content, subsequent research authenticated the original finding of low levels of 5HIAA in depression. But the picture became more completed when one study divided the depressed group clinically into unipolar and bipolar subgroups. Here the low levels of 5HIAA are found only in the unipolar group. In other words the lowered turnover seems to be limited to people who suffer from depression only and have not in the past had an attack of mania. Unipolar depressives also have low levels of homovanillic acid (HVA), the metabolite of dopamine; together with 5HIAA, the levels of HVA do not change on recovery.

Evidence from CSF studies therefore point to a decline in the function of the 5HT system as being responsible for some depressions. Whether there are changes in the noradrenergic neurones, however, is less clear. One problem is the difficulty in obtaining data from CSF on the concentration of the end product of NA metabolism. To make matters worse, little additional help is obtainable by considering drugs used in affective illness which tend to have rather non-specific effects on the biogenic amine systems. For example, certain drugs used in the treatment of depression—the tricyclic anti-depressants—relate for their therapeutic effect on the capacity to block the re-uptake of amine released on stimulation at the nerve terminal. This re-uptake is a potent mechanism for the rapid inactivation of transmitter released into the synaptic cleft (the gap between the nerve terminal and the surface of the next nerve). These drugs probably allow a greater accumulation of amine at the receptor with a consequent increase in activity. The drugs are active against both 5HT and NA but not dopamine.

Possible defects in 5HT metabolism

If we can’t at this time look at noradrenergic activity in patients, is it possible to examine more closely the 5HT hypothesis? Low levels of 5HIAA in CSF suggest a lowered turnover of 5HT. Like many biochemical systems the formation of 5HT has a limiting step; this is at the first enzymic reaction, the conversion of tryptophan to 5-hydroxytryptophan by the enzyme tryptophan-5-hydroxylase; the other step, the decarboxylation of this amino acid, is not limiting, the enzyme being present in considerable excess. As in other biochemical systems control on the rate of formation of the product is exerted at the limiting enzyme. Tryptophan-5-hydroxylase appears to have two such mechanisms. Under normal circumstances the enzyme is unsaturated with its substrate tryptophan. Hence, when the concentration of
this amino acid is raised in the brain the synthesis of 5HT will increase. (Some researchers suggest that lowered levels of tryptophan are responsible for the low levels of 5HIAA in depression.) The second control of 5HT synthesis is the rate of electrical activity or firing of the system. George Aghajanian in America shows that electrical stimulation using an electrode implanted in the raphe nuclei—a midline group of cells bodies of the 5HT neurones—causes an increase in synthesis of 5HT.

If there is a decrease in the formation of 5HT in depression there could be three possible mechanisms. First, an absolute decrease in the amounts of the enzyme tryptophan-5-hydroxylase present in the neurones; secondly, a fall in the concentration of tryptophan substrate for this enzyme; and thirdly, a reduction in the firing rate of the system resulting in a fall in 5HT synthesis.

That there was a fall in the absolute amount of enzyme could be tested by saturating it with tryptophan and determining the 5HT synthesis which should be maximal. In 1970 Ashcroft and I did this in man by giving a dose of tryptophan by mouth which, on considering parallel animal experiments, should have been sufficient to saturate the enzyme. CSF sampled some eight hours after the dose shows no difference in either manic or depressed patients when compared with controls; this suggested that all had potentially the same amount of hydroxylase present. The second possibility—a defect in the transport of tryptophan—was also eliminated; this left the third alternative, a reduction in the firing of the system.

Sensitivity of nerve receptors

The discovery that the lowered turnover of the 5HT system, and possibly the dopaminergic system, was confined to a specific group of depressions made it evident that turnover was not the whole story and that other factors should be considered. One of these factors is the sensitivity of the receptor to the released transmitter. If, for example, the sensitivity of the receptor fails there would be a decrease in the firing rate of the post synaptic cell. Conversely, an abnormal rise in the sensitivity of the receptor would result in an increased firing of the post synaptic cell in response to a normal release of transmitter. Hence the hypothesis has been advanced that affective illness is the result of changes in amine transmission either as a result of altered synthesis of the transmitter or changes in post synaptic receptors. Is there evidence of receptor change? We have tackled this question and so far we’ve accumulated some fascinating results on the role of that ubiquitous molecule, cyclic AMP, in receptor activity, but we still do not have the full answer.

Recently, the not unexpected suggestion has been made that the symptoms of affective illness are attributable to a failure in production of cAMP. Work on this interesting compound has mushroomed since it turned out to be an important intermediate in many biochemical events, particularly those initiated by hormones. The enzyme adenyl cyclase, which produces cAMP from adenosine triphosphate (ADP) in the first instance, is the rate limiting step in the process. The hormone impinging on the outside of the cell activates adenyl cyclase in the membrane and results in an increase in cAMP within the cell; this flood of cAMP then initiates further biochemical events within the cell: hence the concept of its activity as a second messenger of the hormone. The system also gives an amplification of the message since stimulation of the adenyl cyclase enzyme by a small number of hormone molecules results in the conversion of many molecules of ATP to cAMP and their subsequent alteration of intracellular biochemical systems. It is conceivable, therefore, that a defect in the cAMP system in the postsynaptic membrane could be important in affective diseases.

Making use of rats

To try to establish the role of adenyl cyclase in mania and depression we performed some experiments in rats. It is known that when the nerve supply to a tissue is severed the tissue becomes more sensitive—supersensitive—to the transmitter which is normally released at the nerve endings. We attempted to denervate one side of the brain of noradrenaline neurones by making an electrical lesion in the locus coeruleus. This is a collection of NA cell bodies lying in the brain stem; whole processes go forward to the cortex, very largely to the same side of the brain. Hence we had an animal which had an intact NA system on one side and one which had degenerated on the other. Noradrenaline is known to stimulate the activity of adenyl cyclase in brain slices and so we prepared slices from both the intact side and the denervated side. After incubating the slices with a standard amount of NA we measured the amount of cAMP found in them. It turned out that the denervated, supersensitive side of the brain produced more cyclic AMP in response to NA than the intact side. This experiment indicates that cyclic AMP may be an index of receptor sensitivity in the NA and perhaps other amine systems.

Considered together with clinical data these results on rats become important. It seems that the amount of cAMP excreted in the urine of manic patients is substantially raised above normal; in depressed patients cAMP output is reduced. Presumably the increased cAMP turnover in mania reflects a greater receptor activity in this condition, and vice versa for depression. The problem still to be solved, though, is whether the alteration in receptor activity is caused by changes in NA stimulation or is the primary defect.

Embracing as it does both complicated neurochemistry in a very difficult experimental animal (man), and the observation of subtle behavioural moods, it is perhaps not surprising that research on biogenic amines will continue. A great deal of fundamental knowledge has been accumulated so far in animals and, by the very nature of the research, it must give the lead to the inevitably slow progress in human studies.
MODERN VIEWS ON DIAGNOSIS AND CLASSIFICATION OF DEPRESSIONS

BY

II. UNITED KINGDOM

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Modern Views on Diagnosis and Classification of Depressions
II. United Kingdom

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For almost a decade the catecholamine hypothesis and its variants for the cause of affective disorders has held sway. Murmurs of discontent and rival theories are emerging. The biological evidence in man for the catecholamine hypothesis is negligible. Yet for all that, the currently accepted mode of action of drugs used in this illness still attractively grace the theory. At least they did until it was realized that perhaps drugs which were useful in mania, a supposed polar opposite of depression, were if anything more potent in the blockade of central dopaminergic receptors rather than noradrenergic, and that recent reports had revealed the possibility that increased central cholinergic activity also produced a transient defervescence of manic symptoms. Animal experiments are beginning to suggest that the dopaminergic system provides the master drive to which others such as hunger, thirst and possibly sex and exploration are secondary. The possibility will be explored that the dopamine system in man is the background driving force important to him not only with his basic biological drives but to propel him forwards into his social contracts and integration. Mood may be a partially dependent variable which can and does become clinically dissociated from the psychomotor phenomena. A closer examination of the clinical states of depression, mania and Parkinsonism is warranted to explore this aspect.

Introduction

Ten years ago the catecholamine hypothesis for the cause of depression was stated by Schildkraut (1965) as follows:

'The catecholamine hypothesis of affective disorders proposes that some, if not all, depressions are associated with an absolute or relative deficiency of catecholamines, particularly norepinephrine, at functionally important adrenergic receptor sites in the brain. Elation conversely may be associated with an excess of such amines.'

Since then variants of this theory have been proposed, some postulating that 5-hydroxytryptamine (5-HT) and not noradrenaline is aetiological and others that the cholinergic system bears an important functional role in the control of affect. Abullah & Hamadah (1970) proposed that changes in cyclic AMP in the body may be the sole cause of this disorder. Unfortunately, biochemical evidence in man for any of these theories is not available. Hope was originally put on studies of cerebrospinal fluid with some initial measure of success, for example 5 hydroxy indole acetic acid (5-HIAA) the metabolite of 5-HT, was found to be low in depression. Subsequent studies, however, have shown that this lowering cannot be correlated with mood and in some cases persists when the patient has recovered. In fact the whole situation is that how closely levels of metabolites in lumbar cerebrospinal fluid reflect what is going on in brain has been called into question and it has certainly been found that for 5-HIAA part of this metabolite has its origin in spinal cord rather than in brain.

'Vivalan' is a trade mark
However, the original hypothesis still stands on the evidence of drugs used in the treatment of depression. In particular the tricyclic anti-depressants have been shown quite clearly to inhibit the re-uptake of noradrenaline into sympathetic nerve terminals and hence potentiate its action (Iversen 1967). There is no evidence in animals that these drugs potentiate the effect of dopamine at their synapse. In animal experiments the tricyclic anti-depressants do not inhibit the re-uptake of dopamine into striatal neurones. When we consider hypomania then the drugs used here are catecholaminergic receptor blockers. In particular the drugs most effective in mania are those which block the dopaminergic receptors. There have been recent reports (1972) that physostigmine which gets into brain and potentiates acetylcholine can produce a transient defervescence of symptoms in hypomania. We ourselves have only tried this in a single case of hypomania, the experiment was not blind and therefore cannot be regarded as scientific. Twenty minutes after treatment with physostigmine, for a very short period, that is to say minutes, all the patient's symptoms seemed to disappear and he had apparently full insight into his condition. This is not incompatible with the idea that dopamine has a role in hypomania in that it has recently been postulated that one of the interneurones within the striatum activated by dopamine is cholinergic and that dopamine is inhibitory on this system (Fibiger & Darsham, 1974). The summary of the drug evidence then is that drugs which potentiate noradrenaline will treat depression whereas drugs which prevent hypomania will block in particular the effects of dopamine.

Animal Evidence

Can this drug evidence be translated into what is known about the neuronal systems concerned? Of the dopamine system which has been most extensively studied, that which arises from the substantia nigra to terminate in the striatum has been most widely studied, whilst the system for noradrenaline which arises in the locus coeruleus and terminates in cortex has received probably more attention than other noradrenergic systems. Interestingly enough these two systems, as has been demonstrated by Crow and co-workers (1972), support self-stimulation, that is, if electrodes are placed in either substantia nigra or the locus coeruleus then the animals will deliver, by bar pressing, an electrical stimulus to themselves which they will continue doing at very high bar-pressing rates to the exclusion of all other drives. The central effects are felt to be in some way rewarding or lead the animal to expect a reward. These experiments had as their forerunner electrical self-stimulation which occurred in lateral hypothalamus, the region where it is known that both dopaminergic and noradrenergic fibres traverse in a bundle of fibres, the medial forebrain bundle.

Perhaps one of the crucial experiments which was done was that by Valenstein (1968). He implanted an electrode in the lateral hypothalamus and placed the rat in a cage where three specific objects were available. These were either eating, drinking or gnawing on a block of wood. He found that on electrical stimulation the animal either drank or ate or gnawed. Whenever stimulation was repeated the animal would return to that particular form of activity which was his particular pattern. Interestingly enough if in animals which always drank, water was taken away, then the animal would switch to eating or gnawing each time electrical stimulation was applied. Occasionally he noted a spontaneous switch from one form of activity to the other on electrical stimulation and he postulated that the drive mechanisms were being operated and that the plasticity existed beyond this point of stimulation. Crow (1973) has subsequently taken up this theme and has postulated that the dopaminergic system is concerned with the animal's overall drive (incentive motivation) and that the noradrenergic system is concerned with reinforcement. Again we have the picture emerging of two systems which, although related, have different functions.

Human Evidence

Oliver Sacks in his book 'Awakenings' describes a series of patients who suffered from encephalitis lethargica and who were chronic patients in a New York hospital where they had often remained for up to 30 years, some almost mute or with minimal activity. These
patients were treated with l-dopa and almost without exception the results were startling although often not maintained. Characteristically the patient literally awakened from the incarceration of his disease and could vocalize feelings of his past illness together with feelings of his present as one of these cases illustrates.

Leonard L was a man of 46;

‘completely speechless and completely without voluntary motion except for minute movements of the right hand’.

He had only been able to communicate by tapping messages on a small letter board for the past 16 years. For all that, he was ‘a man of most unusual intelligence, cultivation and sophistication’.

One of his descriptions of his state of illness is as follows;

‘a sort of total calmness, a nothingness, which is by no means unpleasant. It’s a let-up from the torture. On the other hand, its something like death. At these times I feel I’ve been castrated by my illness, and relieved from all the longings other people have’.

He was given l-dopa and the following transpired,

‘he was like a man who had awoken from a nightmare or a serious illness, or a man released from entombment or prison, who is suddenly intoxicated with the sense and beauty of everything around him. During these two weeks, Mr L was drunk on reality —on sensations and feelings and relations which had been cut off from him, or distorted, for many decades. He loved going out into the hospital garden: he would touch the flowers and leaves with astonished delight, and sometimes kiss them or press them to his lips’.

However, a month later;

‘In April, intimations of trouble appeared. Mr L’s abundance of health and energy—of ‘grace’, as he called it—became too abun-
dant and started to assume an extravagant, maniacal and grandiose form; at the same time a variety of odd movements and other phenomena made their initial appearance. His sense of harmony and ease and effortless control was replaced by a sense of too-muchness, of force and pressure, and a pulling apart—a pathological driving and fragmentation which increased obviously and visibly, with each passing day. Mr L passed from his sense of delight with existing reality to a peremptory sense of mission and fate: he started to feel himself a Messiah, or the Son of God. After two months he was thoroughly ‘charged up’, in his own words, ‘charged and super-charged’ with a great surplus, a great pressure, of libidinous and aggressive feelings, with an avidity and voracity which could take many forms’.

Clearly one can see that this patient became clinically hypomanic and this case was not unique. Calne et al (1969) describes hypomania and overactivity in his series of post-encephalitic patients. In a series of depressed patients described by Goodwin et al (1970) it was found that on giving large doses of l-dopa a high proportion of patients became hypomanic. Analysis of the data shows that five of the six patients who became hypomanic suffered from a bipolar illness. The other interesting point about this was that they showed increased drive and activity although virtually no change in affect, ‘minimal or transient changes in depression’. Perhaps it is not without some importance that one considers that the post-encephalitic group of patients because of the destruction of the presynaptic dopaminergic neurones will almost certainly have receptor super sensitivity and this same phenomenon has been postulated as being aetiological in bipolar affective illness.

In animal brain, dopa leads to the formation of greatly increased amounts of dopamine with little, if any, increase in noradrenaline. It could then be postulated that the dopaminergic system mediates the primary drive mechanisms which was a consequence of the other drives to follow and in man, the social interaction is perhaps an important sequel. In the rat there are prominent olfactory inputs into the substantia nigra
region and in man inputs from a higher level could, of course, be important.
We would like to look at the hypothesis that drive and mood are mediated by separate neuronal systems, that for drive being the dopaminergic system and possibly the noradrenergic for mood. These systems are in the normal functionally dependent on each other. In affective illness they become dissociated, changes in drive being evident as well as mood. This would mean that mania was not the polar opposite of depression as suggested by Goodwin et al (1970). It would explain the common failure to have normality intervening between mania and depression. It could also mean that one should treat not only mood but drive in these illnesses.

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SECTION XI
A FLUORIMETRIC METHOD FOR THE DETERMINATION OF 4-HYDROXY-3-METHOXYPHENYLGLYCOL IN URINE

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(Received March 16, 1971)

SUMMARY

A sensitive method is described for the estimation of 4-hydroxy-3-methoxyphenylglycol in urine. Separation of the glycol is effected, after preliminary enzymatic hydrolysis, by solvent extraction and ion exchange chromatography. The glycol is estimated spectrophotofluorimetrically after oxidation by ferric chloride.

INTRODUCTION

Recent reports have indicated that the major metabolite of noradrenaline in brain is 4-hydroxy-3-methoxyphenylglycol (HMPG)\(^1\)-\(^4\) and that this in some species is metabolised to the sulphate conjugated prior to excretion\(^3\),\(^4\). In contrast, the metabolism of noradrenaline in the periphery leads mainly to the formation of vanillyl mandelic acid (VMA)\(^1\). Hence an estimation of HMPG in urine might give a good index of the turnover of noradrenaline in brain. Changes in the function of the noradrenergic neurones in brain have been implicated in the aetiology of depressive illness\(^6\). Recent studies in animals have shown that long term treatment with tricyclic antidepressants leads to an increase in turnover of noradrenaline in the central nervous system\(^8\). At present, the assessment of turnover of noradrenaline in brain in man must remain indirect, and a simple method for the estimation of HMPG in urine may lead to advances in the investigation of depressive illness\(^7\). The present paper describes such a spectrophotofluorimetric method and its application in human subjects. The method uses the ion exchange separative procedure of Wilk et al.\(^9\) followed by a fluorimetric assay based on that described by Sharman\(^9\) for the estimation of 4-hydroxy-3-methoxyphenylacetic acid.

MATERIALS

HMPG was obtained from Sigma London Chemical Company. All chemicals were of analytical reagent grade or purer. The resin was Bio-Rad AG-1-X4, 200–400
mesh in the chloride form (Calbiochem). "Helicase" enzyme (Industrie Biologique Française) was used which contained 100 000 units (Fishman) of β-glucuronidase and 15 000 000 units (Roy) of sulphatase per g. Glass distilled water was used throughout.

METHODS

Isolation of HMPG

Urine was collected over a 24 h period into bottles containing 10 ml 6 N HCl as a preservative, and stored at -20° until estimation, which was within 24 h of collection. A 5 ml portion of urine was adjusted to pH 1 with conc. HCl and the organic acids were extracted by shaking the sample with 10 ml of ethyl acetate. After centrifugation at 2000 g for 5 min the organic phase was removed and the extraction repeated twice with two further 10 ml quantities of ethyl acetate. These ethyl acetate extracts were discarded. To 3 ml of the aqueous phase was added 0.3 ml of 1 M sodium acetate buffer pH 5 and the pH adjusted to 5 with 1 N NaOH (glass electrode). The sample was then incubated in a water bath at 35° for 24 h with 27 mg "Helicase" (9 mg/ml urine). In order to prevent any bacterial decomposition during hydrolysis, 2 drops of chloroform were added to each tube prior to incubation.

The hydrolysed sample was centrifuged for 5 min at 3500 g and the supernatant retained. The residue was washed with 0.5 ml distilled water and centrifuged. The combined supernatants were adjusted to pH 6 with 1 N NaOH and passed over a column (11 × 0.7 cm) of Bio-Rad AG50-X4 resin. The resin was washed with 6 ml of water and the HMPG eluted with 12 ml water. The eluate was saturated with sodium chloride and extracted twice with 12 ml and a third time with 6 ml of ethyl acetate. The combined ethyl acetate extracts of the column eluates were evaporated nearly to dryness under a stream of nitrogen at room temperature. The sides of the tube were washed with 3 ml water, 2 ml of which were taken for the determination of HMPG by fluorimetry.

Fluorimetric method

The reaction to produce the fluorophor from HMPG was carried out in 15 ml glass stoppered centrifuge tubes. Because of the light sensitivity of the reaction it was carried out in a dimly lit room. The FeCl₃ reagent was freshly prepared prior to each batch of estimations by adding 0.1 ml 2 N NaOH to 5 ml 1% FeCl₃ solution freshly diluted from a 60% solution. The mixture was shaken thoroughly. The precipitate of ferric hydroxide formed a dark brown colloidal solution. To the 2 ml sample containing HMPG was added 0.2 ml of the ferric chloride reagent, the mixture shaken thoroughly and the reaction allowed to proceed for exactly 2 min. At the end of that period the reaction was stopped by the addition of 0.2 ml 5 N NaOH and the precipitate of ferric hydroxide removed by centrifugation at 5000 g for 1 min. The supernatant was transferred to a 3 ml cuvette and the fluorescence estimated by activation scans on a Farrand spectrophotofluorimeter. Fluorescence maxima occurred at 325 nm activation wavelength and 430 nm emission wavelength (uncorrected values).

A blank was prepared by taking 3 ml distilled water through the extraction and fluorimetric procedure. Recoveries were assessed by processing duplicate samples of urine, to one of which was added 5 μg HMPG after the first ethyl acetate extraction, but prior to the treatment of the sample with "Helicase".

ESTIMATION OF URINARY HMPG

RESULTS

Specificity of isolation procedure

The substances found in urine giving a positive reaction with the ferric chloride oxidation include o-methylated catechol derivatives. Of these, 90% of the acids, 3-methoxy-4-hydroxymandelic (VMA) and 3-methoxy-4-hydroxyphenylacetic (HVA) are removed at pH 1 in the initial ethyl acetate extraction. The remainder adsorb onto the resin and are not eluted in the fraction containing HMPG. Of the basic compounds, metanephrine and 3-methoxytyramine, 85% pass through the column in the effluent and first wash. The other 15% remain in the aqueous phase of the second ethyl acetate extraction (pH 5–6) which extracts HMPG.

The alcohol metabolite of dopamine, 4-hydroxy-3-methoxyphenylethanol (HMPT) could still be a contaminant. However, chromatography, as described below, failed to show the presence of this compound.

The method is essentially a determination of the conjugated HMPG in urine. The "free" HMPG was found to be 70% removed by the ethyl acetate extraction at pH 1 for the removal of acids.

Chromatography of presumed HMPG

In order to validate the identity of the compound in the final extract as HMPG, qualitative thin layer chromatography was performed. The final ethyl acetate extract of the column eluate was evaporated to dryness in a stream of nitrogen, the residue dissolved in 0.2 ml methanol and applied under a stream of nitrogen to a thin layer plate of cellulose (Merck precoated 20 x 20 cm 0.10 mm thick). The plates were developed in one of the following solvent systems:

1. Isobutyl methyl ketone: 4% formic acid (10:1 v/v)

The following pure substance markers were spotted on the plates—HMPG, HMPT, VMA, HVA, normetanephrine and metanephrine. After development, the plates were sprayed with freshly prepared diazotised p-nitroaniline (mixture 10:1 v/v of saturated solution of paranitroaniline in 0.5 N HCl and 10% sodium nitrite) and the phenolic compound visualized by a final spraying of the dry plate with 1 N

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rp values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent system</td>
</tr>
<tr>
<td>HMPG</td>
<td>0.29</td>
</tr>
<tr>
<td>Vanillyl mandelic acid</td>
<td>0.48</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>0.84</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxyphenylethanol</td>
<td>0.81</td>
</tr>
<tr>
<td>Metanephrine</td>
<td>0.00</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>0.00</td>
</tr>
<tr>
<td>Supposed HMPG in urine</td>
<td>0.28</td>
</tr>
</tbody>
</table>


NaOH in 5% ethanol. In the sample extract a single spot was visible with an \( R_f \) identical with that for authentic HMPG as indicated in Table I.

**Enzyme hydrolysis**

The efficiency of the enzyme hydrolysis was assessed by means of radioactively labelled HMPG sulphate. This was prepared from whole brain of rats treated with an intraventricular injection of normetanephrine-\( \text{\textsuperscript{73}} \text{H} \) (10 μC SA 35 C/mM in 20 μl Merles solution) and killed one hour later. The HMPG sulphate was purified from the supernatant after protein precipitation, by the removal of amine metabolites on a column of Amberlite CG50 resin, mesh 100-200 in the ammonium form at pH 7.5. The acids were extracted with ethyl acetate at pH 1 and the final purification was effected by paper chromatography (Sugden, Yates and Eccleston, unpublished observations). About 10 000 d.p.m. of labelled conjugate found to give single peaks on high voltage electrophoresis and thin layer chromatography was added to each of three urine samples. Two of these were enzymatically hydrolysed as described in the method, and the third sample was incubated without enzyme and acted as a control. The products (mainly free HMPG and residual unhydrolysed conjugate) were determined by high voltage electrophoresis, thin layer chromatography and column chromatography. The estimate of the percentage hydrolysis was 80 ± 2% (ref. 12) mean ± S.D. (number of observations).

**Recoveries through the method**

Recoveries of 5 μg HMPG were assessed at various points through the procedure.

(a) **Column recoveries.** The resin only adsorbs the HMPG loosely\(^8\) retarding its flow through the column. The volume of sample:wash:eluate was maintained at a constant ratio of 1:2:4 and by altering the size of the column in relation to these washes, it was possible to reduce the quenching due to urinary pigments to an 8% decrease in fluorescence intensity.

The mean recovery through the column was 84 ± 6% (ref. 9) mean value ± S.D. (number of observations).

(b) **Ethyl acetate extraction of column eluate.** Under the conditions of the method, 80% of the HMPG in the column eluate was extracted into the ethyl acetate.

**TABLE II**

**Recovery through the method of 5 μg of HMPG in eight different samples of urine**

<table>
<thead>
<tr>
<th>Urine sample (number)</th>
<th>Recovery of HMPG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55 ± 2 (3)*</td>
</tr>
<tr>
<td>2</td>
<td>57 ± 3 (3)</td>
</tr>
<tr>
<td>3</td>
<td>59 ± 3 (3)</td>
</tr>
<tr>
<td>4</td>
<td>61 ± 3 (3)</td>
</tr>
<tr>
<td>5</td>
<td>60 ± 3 (3)</td>
</tr>
<tr>
<td>6</td>
<td>56 ± 4 (3)</td>
</tr>
<tr>
<td>7</td>
<td>63 ± 2 (3)</td>
</tr>
<tr>
<td>8</td>
<td>60 ± 2 (3)</td>
</tr>
</tbody>
</table>

Total mean recovery 59 ± 4 (24).

* Mean value ± S.D. (number of estimates).

Total recovery. This was assessed by processing, in triplicate, eight different samples of urine to each of which had been added 5 μg HMPG prior to the enzyme hydrolysis step. The recoveries from each sample are given in Table II.

Linearity of fluorescence reaction

The fluorescence reaction gave a linear relation between the fluorescence produced and the concentration of HMPG within the range 100 ng to 10 μg in 2 ml deionized water.

Concentration of HMPG in normal human urine

Urine was collected over a 24 h period from 5 male and 7 female subjects (age range 20-40 years) who were on a diet which reduced the intake of sympathomimetic compounds and their precursors. The results for the concentration of HMPG corrected for recoveries in these samples are given in Table III.

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>CONCENTRATION (μg/24 h) OF HMPG IN THE URINE OF NORMAL HUMAN SUBJECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>519</td>
<td>1510</td>
</tr>
<tr>
<td>1220</td>
<td>1170</td>
</tr>
<tr>
<td>939</td>
<td>670</td>
</tr>
<tr>
<td>1240</td>
<td>314</td>
</tr>
<tr>
<td>697</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>531</td>
</tr>
<tr>
<td>Mean 923 ± 317 (5)*</td>
<td>637 ± 529 (7)*</td>
</tr>
</tbody>
</table>

Overall mean 756 ± 459 (12)*.  
* Mean ± S.D. (number of estimates).

DISCUSSION

The methods described in the literature for the estimation of HMPG either involve the use of spectrophotometry or gas chromatography. The sensitivity of the spectrophotometric methods is low and the specificity is reduced by interference from p-hydroxyphenolic acids. Gas chromatography, however, combines a high sensitivity with a good specificity, but is time consuming for routine studies. The method described has a high degree of specificity, a reasonable sensitivity and is relatively easy to carry out.

In pure solution, the limit of sensitivity of the fluorimetric procedure, taken as being the amount of HMPG giving a fluorescence intensity equal to twice the blank, was of the order of 50 ng. In urine samples the limit of detection was found to be 100 ng per ml.

Using this method, the values for the concentration of HMPG in the urine of normal subjects (756 μg/24 h) fell within the range published by Wilk et al. of 0.86 μg/mg creatinine, but were lower than the values of Ruthven and Sandler of 3000 μg/24 h.

We were unable to detect any differences between males and females in the urinary concentration of HMPG, but the range of concentrations was found to be very large.
REFERENCES


SECTION XII
Some Experiments in the Chemistry of Normal Sleep

By IAN OSWALD, G. W. ASHCROFT, R. J. BERGER, D. ECCLESTON, J. I. EVANS and V. R. THACORE

Sleep is essential for physical and mental health. In the last 15 years there has grown up the concept of the brain stem reticular activating system. Electroencephalographic studies have shown two qualitatively different and alternating kinds of sleep, the orthodox ("slow wave", or "fore-brain") and the paradoxical ("hind-brain", "rapid eye movement", "activated", or "dreaming") phases (Akert et al., 1965). It may be predicted that in the next decade attention will turn increasingly to the chemical basis of sleep. If a man is deprived of sleep for 100 hours, it is extremely difficult to keep him awake and one may suppose that an abnormal biochemical state exists within his central nervous system.

It has been shown that selective deprivation of either the paradoxical phase (Dement, 1960; Kales et al., 1964; Jouvet et al., 1964) or the more profound stages of the orthodox phase (Agnew et al., 1964) is followed when subsequent sleep is undisturbed by an increased proportion of the kind of sleep that was lost. This may be compensatory, being a need for each kind of sleep, with a different neurochemical substratum for each. There is generally an abrupt switch from one to the other. What controls the switching? At the beginning of the night, orthodox sleep predominates and, as if with high priority, will often momentarily obstruct into the first period of paradoxical sleep. The converse occurs when there is an increased "pressure" towards paradoxical sleep, created by, for example, amphetamine withdrawal. Can one therefore conceive of a delicate balance between two competing systems? Some substances having sedative properties, such as barbiturates (Oswald et al., 1963) and alcohol (Gresham et al., 1963), suppress paradoxical sleep, as also, curiously enough, do amphetamine (Rechtschaffen and Maron, 1964) and tranylcypromine (Le Gassicke et al., 1965).

Shift of the balance in favour of paradoxical sleep by the administration of 4-butyrolactone and sodium 4-hydroxybutyrate to certain cat brain stem preparations was reported by Jouvet et al. (1961). A means of producing such a shift in intact man is described below.

Our study has involved the oral administration of L-tryptophan (Oswald, 1963; Oswald et al., 1964) and of intravenous DL-5-hydroxytryptophan (5HTP). These are precursors of 5-hydroxytryptamine (5HT or serotonin), a substance increasingly considered to play a major role in brain function. 5-HT, given peripherally is unsuitable for investigation of its central action because it passes the blood brain barrier with difficulty. Tryptophan, however, is rapidly absorbed from gut (see, for example, Table I) and, like 5-HTP, quickly reaches the brain, bringing about a rapid rise in the 5-HT content of the parts where it is normally found (Costa and Rinaldi, 1958; Bogdanski et al., 1957; Hess and Doepfner, 1961). Oral tryptophan has been administered experimentally to schizophrenics by Pollin et al. (1961) and to depressed patients by Coppen et al. (1963) to determine whether changes in central 5-HT result in changes in observable symptoms. Drowsiness was reported as one side-effect by these authors, and also by Smith and Prockop (1962) and was noted by ourselves in experiments with normal people. A subjective feeling of being slightly drunk and some euphoria was apparent within 20 minutes after ingestion upon an empty stomach.

Physiological Index Studied

It is usual for indices of physiological functions to show a scatter about a mean. One can attempt to demonstrate that a function is altered by some externally applied variable in
either of two ways. Most commonly, with the use of mathematical techniques and using a large number of individuals, one attempts to show that the applied variable causes a shift in the value of the mean so large that chance is an improbable explanation. Or one can arbitrarily select for that function a value which occurs so rarely in the normal population that it can be used as a limit of normal, and then make attempts deliberately to cause the function concerned to be abnormal (outside the chosen limit). The second method has here been used as the first would have been uneconomic of labour.

The normal human who falls asleep at night passes first into orthodox sleep. After about an hour the first period of paradoxical sleep with rapid eye movements begins. The delay between falling asleep (onset of sleep spindles) and the first rapid eye movement is not less than 45 minutes in normal persons (Dement and Kleitman, 1957; Oswald and Thacore, 1963; Rechtschaffen and Verdone, 1964). This delay period has been the criterion used in this study. Apart from its use in an arbitrary manner, the subjects served as their own controls in experiments which have been essentially exploratory in nature. Fig. 1 indicates how the delay period is observed and measured, techniques similar to those previously reported (Oswald and Thacore, 1963) being used.

The Experiments

It was first observed that rats looked sleepy after intraperitoneal L-tryptophan. A pilot study over 12 nights of sleep, using 2 human volunteers given 5 grams of L-tryptophan orally upon retiring, revealed nothing to suggest an effect upon the amounts of the different phases of sleep over the night as a whole. In all, 16 normal young adult males received 5 to 10 grams of oral tryptophan before sleep on 1–3 pilot nights each. Five gave delay periods of under 45 minutes at least once. Two, Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time</th>
<th>5-HIAA</th>
<th>IAA</th>
<th>5-HT</th>
<th>Tryptamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>9 a.m.–12 noon</td>
<td>600</td>
<td>1,000</td>
<td>8.7</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>12 – 6 p.m.</td>
<td>4,150</td>
<td>5,400</td>
<td>353</td>
<td>256</td>
</tr>
<tr>
<td>M</td>
<td>9 a.m.–12 noon</td>
<td>—</td>
<td>—</td>
<td>11.2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>12 – 6 p.m.</td>
<td>—</td>
<td>—</td>
<td>186</td>
<td>242</td>
</tr>
<tr>
<td>W</td>
<td>9 a.m.–12 noon</td>
<td>700</td>
<td>415</td>
<td>9.5</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>12 – 6 p.m.</td>
<td>2,960</td>
<td>2,600</td>
<td>382</td>
<td>449</td>
</tr>
<tr>
<td>L</td>
<td>9 a.m.–12 noon</td>
<td>440</td>
<td>495</td>
<td>17.2</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>12 – 6 p.m.</td>
<td>3,200</td>
<td>5,100</td>
<td>310</td>
<td>215</td>
</tr>
</tbody>
</table>

Reactors H and M  Non-reactors W and L

Table II

Concentration of 5-Hydroxyindol-3-acetic Acid (5-HIAA), Indole Acetic Acid (IAA), Indole Lactic Acid (ILA), 5-Hydroxytryptamine (5-HT) and Tryptamine in Urine During Control Period (3 hours) and Following the Oral Administration of 5 g. Tryptophan (6 hours)
Fig. 1—A four channel recording from Subject M. The upper excerpt is that of drowsiness with irregular low voltage EEG activity and rolling eye movements. Arrows point to what looks like beginnings of spindle activity on the original record. At 12.15 well-developed spindles begin to appear, showing themselves strongly in the anterior derivations, principally intended to show eye movement potentials.

The lower excerpt shows the high voltage slow wave pattern of profound orthodox sleep at 12.40. Seven minutes later the EEG is of lower voltage, and then, after a series of slow wave complexes, rapid eye movements (REMs) begin at 12.49.
SOME EXPERIMENTS IN THE CHEMISTRY OF NORMAL SLEEP

Figs. 2 and 3.—The scale across the top indicates the number of minutes between falling asleep (EEG spindles onset) and first rapid eye movements (REMs) of the first period of paradoxical sleep. The horizontal bars indicate the duration of the delay period on the various dates in 1963-5 shown on the left. Where no tryptophan was given the horizontal bars show that the delay period was always normal, in that it exceeded 45 minutes. Where tryptophan alone was given prior to sleep the delay period was always abnormal, under 45 minutes.

Methysergide in particular, given for three prior days, prevented the tryptophan effect.
M and H, did so consistently and have been studied over a two year period. The experiments with these subjects are portrayed in Figs. numbered 2 and 3. Lengthy intervals between experimental nights were used deliberately to minimize carry over effects between treatments.

In these two subjects, it will be observed that on the combined total of 20 nights, when substances other than tryptophan were given (lactose, amino acids, saline), the delay period was always normal (over 45 minutes). It was always under 45 minutes on each of the 11 nights when tryptophan alone was given. Lactose capsules, similar to the tryptophan capsules, were used as a control to determine whether the results obtained were not specific to tryptophan. Other amino-acids, L-tryptophan 10 grams, and DL-methionine 16 grams, each as a suspension in a quantity of water similar to that used for swallowing the tryptophan capsules, were administered.

Prior to October 1963, Subject M received tryptophan in 5 gram quantities, thereafter in 7.5 quantities. Subject H received 5 grams of tryptophan on his first night, 10 grams on the next occasion, 28th June 1963 (when he took about 20 minutes to fall asleep and had the very short delay period before paradoxical sleep of 5 minutes); thereafter he received 7.5 gram doses. Another Subject (S), studied over 18 months, produced delay periods of under 45 minutes on 7 out of 11 occasions after tryptophan alone; on none of 6 occasions when lactose was administered; on none of 3 occasions after both methysergide and tryptophan; and on 1 of 2 occasions after both alpha methyl dopa and tryptophan. The results showing the effect of tryptophan on the onset of REM sleep are combined for the 3 subjects in Table III. \( \chi^2 \) (with Yates' correction) = 21.7 (p < 0.001). The other two tryptophan reactors amongst the original total of 16 volunteers were not subjected to further study.

Alpha methyl dopa, 500 mg., 150 minutes before the tryptophan appeared only irregularly to prevent the action of tryptophan; indeed, Subject H revealed his shortest delay period under these circumstances when, on March 7, 1965, only 19 minutes after swallowing the tryptophan, he passed into paradoxical sleep, after less than a minute of orthodox sleep with EEG spindles, and continued therein for 15 minutes. Analysis of the combined results of these experiments (Table IV) showed no significant difference between the effect of administration of tryptophan alone and pretreatment with alpha methyl dopa, \( \chi^2 = 0.33 \).

Methysergide ("Deseril", Sandoz), a specific 5-HT blocking agent (Doepfner and Cerletti, 1968), 3 mg. daily for three days prior to tryptophan, prevented the action of tryptophan on 10 out of 10 occasions. These results are shown in Table V and the difference between treatment with tryptophan alone and pre-treatment with Deseril is significant, \( \chi^2 = 15.52 \), p < 0.001. It was noted that the onset of REM sleep, following pretreatment with Deseril, appeared to be occurring even later than on control nights. Taking an arbitrary division of 60 minutes, the results of these two groups are shown in Table VI. The difference is not significant, \( \chi^2 = 2.32 \). The possibility that the effect is mediated by Deseril alone and not by its combination with tryptophan was not investigated further in this series of studies.

Intravenous saline injections prior to sleep were without effect, but 40 mg. of 5-HTP intravenously in 10 ml. of saline caused Subject M to have his shortest delay period of 16 minutes. The injection procedure may have been more anxiety-provoking than capsules and each subject took over 30 minutes to fall asleep on these nights, which may have provided a long enough interval for most of the 5-HTP to be metabolized prior to sleep, the extreme response of 16 minutes by Subject M being the only presumed response among the 6 observations.

**Waking Observations**

An investigation carried out with six subjects having lactose on two occasions and tryptophan, 5 grams, on two occasions (through the kind co-operation of Dr. J. Laidlaw and Miss J. Catlin) failed to reveal any clear effect of tryptophan upon either waking EEG rhythms (an automatic frequency analyser being used) or upon performance in various simple tasks requiring attention.
### Table III

**Effect of Tryptophan on onset of paradoxical (REM) sleep in 3 subjects**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(a) With Onset REM under 45 min.</th>
<th>(b) With Onset REM at 45 min. or later</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls—No treatment: (Saline or Lactose Capsules)</td>
<td>... ...</td>
<td>...</td>
<td>16</td>
</tr>
<tr>
<td>Tryptophan (5–10 grams)</td>
<td>... ...</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>... ...</td>
<td>18</td>
<td>38</td>
</tr>
</tbody>
</table>

### Table IV

**Effect of administration of Alpha Methyl Dopa, 500 mg., 150 min. prior to Tryptophan on-set REM sleep in 3 subjects**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(a) With Onset REM under 45 min.</th>
<th>(b) With Onset REM at 45 min. or later</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan (5–10 grams.)</td>
<td>... ...</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>Alpha Methyl Dopa (500 mg.) 150 min. prior to Tryptophan (5–10 gr.)</td>
<td>... ...</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>... ...</td>
<td>26</td>
<td>34</td>
</tr>
</tbody>
</table>

### Table V

**Effect of “Deseril” on REM sleep in 3 subjects**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(a) With Onset REM under 45 min.</th>
<th>(b) With Onset REM at 45 min. or later</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan (5–10 gram)</td>
<td>... ...</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>“Deseril” (3 mg. daily for 3 days)</td>
<td>... ...</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Tryptophan (5–10 gram)</td>
<td>... ...</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>... ...</td>
<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>

### Table VI

**Effect of “Deseril” on REM sleep in 3 subjects**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(a) With Onset REM under 60 min.</th>
<th>(b) With Onset REM at 60 min. or later</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls—No Treatment: (Saline or Lactose Capsules)</td>
<td>... ...</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>“Deseril” (3 mg. daily for 3 days)</td>
<td>... ...</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Tryptophan (5–10 gram)</td>
<td>... ...</td>
<td>14</td>
<td>26</td>
</tr>
</tbody>
</table>
Some normal volunteers had responded to the tryptophan by having delay periods of under 45 minutes, while others did not do so. It was thought probable that this merely reflected differences in degree of responsiveness rather than a sharp division between two categories of person, but in order to determine whether there was a difference in the whole body metabolism of tryptophan along the tryptamine and 5-hydroxytryptamine pathways between the "reactors" and "non-reactors", Subjects H. and M. were investigated in a metabolic unit, and compared with Subjects W. and L. who had not shown response to tryptophan. Having fasted in the previous 12 hours, urine was collected over a 3 hour control period. A 5 g. dose of L-tryptophan was administered in gelatin capsules and urine collected over a subsequent 6 hour period. Blood samples were withdrawn at \( \frac{1}{2}, 1, 2, 3 \) and 6 hours. Subjects H. and W. experienced some nausea.

Blood tryptophan was estimated by the method of Hess and Udenfriend (1959) as applied by Guroff and Udenfriend (1962). 5-Hydroxyindol-3-ylacetic acid (5-HIAA), indol-3-ylacetic acid (IAA), tryptamine and 5-hydroxytryptamine (5-HT) were determined as enumerated in the paper of Le Gassicke et al. (1965).

The results obtained (Tables I and II) show no noteworthy difference between "reactors" and "non-reactors" in the excretion of 5-HIAA, IAA, 5-HT and tryptamine following this loading dose of tryptophan. The concentration of these metabolites was rather high in all these young subjects.

When these waking subjects had received tryptophan upon an empty stomach they noticed a slight euphoria, a mild feeling of drunkenness and drowsiness after about 20 minutes, persisting about one hour. It was noticeable on these occasions (and also when another normal subject received intravenous 5-HTP) that their conversation tended to become lewd. Only subsequently did we discover Smith and Prockop's description (1952) of similar behaviour after tryptophan leading to complaints from nursing staff. Tryptophan given to dogs can provoke spontaneous orgasms (Himwich and Costa, 1960). These observations are consistent with the penile erections that regularly accompany human paradoxical sleep (Fisher et al., 1965) and the other links between that phase of sleep and sexual behaviour (Faure and Bensch, 1962).

**Discussion**

Jouvet (1962) located a pontine centre controlling the release of paradoxical sleep. The biogenic amine, 5-HT, is distributed throughout the central regions of brain (Amin et al., 1954; Costa and Rinaldi, 1958; Bogdanski et al., 1957; Price and West, 1960). The administration of L-tryptophan has been shown to increase the absolute amount (Hess and Doepfner, 1961) and turnover (Ashcroft et al., 1965) of the amine in the brain. We may reasonably postulate that the action of tryptophan in the present experiments has been an action in the brain, as opposed to, for example, some unknown reflex arising from an action on the gut.

A non-specific effect of amino acid administration has been ruled out by the failure of tyrosine or methionine to produce the effect.

Although the injected 5-HTP reproduced the tryptophan effect only once out of six times, it caused the shortest delay period in Subject M, of 16 minutes. Delay-periods of that order do not normally occur. One may suppose the 5-HTP to have been responsible, suggesting that the observed action of tryptophan depended upon its metabolism to 5-HTP and thence to 5-HT. This would seem a reasonable inference, in view of the blocking of the tryptophan effect by a specific 5-HT antagonist, methysergide. It is known from animal experiments that methysergide reaches the brain (Doepfner, 1962) and that it can inhibit the central action of 5-HT (Karfa et al., 1961).

Alpha methyl dopa has been shown to block the synthesis of 5-HT from tryptophan in brain (Eccleston et al., 1965) and was therefore tried out in the present experiments. The inconsistent results therefrom may have arisen because alpha methyl dopa also reduces the cerebral concentration of other biogenic amines, which may play a role in cerebral function (Sourks, 1965).

Recording only the first hour of sleep, as was the case on most of the nights, had obvious
practical advantages. It meant, however, that a record of the duration of each first period of paradoxical sleep was not regularly kept, though durations of a few seconds to as long as 17 minutes were seen—a normal range. It has now been shown by Rechtschaffen and Verdone (1964) that in a normal night’s sleep a shorter delay period is correlated with a shorter duration of the first period of paradoxical sleep. Dement (personal communication) points out that if the early, tryptophan-induced initial period of paradoxical sleep is as long, on average, as the normal, then a factor making for increase of duration as well as early onset should be postulated.

The effect of L-tryptophan upon normal sleep prompted a study of its effect in idiopathic narcolepsy, where it is found to double the duration of the period of paradoxical sleep into which such patients, unlike normal persons, will at once pass upon falling asleep (Evans and Oswald, 1965). Faure (1965) has reported that tryptophan enhances paradoxical sleep in the rabbit.

**Summary**

Upon falling asleep there is normally at least 45 minutes of orthodox (“slow wave”) sleep before the onset of paradoxical sleep (rapid eye movement phase). Laevro-tryptophan, 5 to 10 grams orally upon retiring to bed resulted in delays of less than 45 minutes in 5 of 16 normal young male adults, of whom three were studied in detail and among whom no evidence of unusual tryptophan absorption or metabolism was found.

Control amino-acids, tyrosine and methionine did not produce the response. It is postulated that the effect on sleep results from the metabolism of tryptophan to 5-hydroxytryptamine (serotonin). Further results described support this hypothesis, particularly the fact that the specific anti-serotonin agent, methysergide, prevented the effect of tryptophan.

**Acknowledgments**

We are much indebted to Messrs. Sandoz Ltd. for generous supplies of tryptophan, 5HTP and methysergide (Deseril). Messrs. I.C.I. (Pharmaceuticals) Ltd. also kindly gave some tryptophan. The Medical Research Council gave financial aid.

**References**


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THE CLINICAL STATE, SLEEP AND AMINE METABOLISM OF A TRANYLCYPROMINE ('PARNATE') ADDICT

BY

J. LE GASSICKE, G. W. ASHCROFT, D. ECCLESTON,
J. I. EVANS, I. OSWALD and E. B. RITSON

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The Clinical State, Sleep and Amine Metabolism of a Tranylcypromine (‘Parnate’) Addict

By J. LE GASSICKE, G. W. ASHCROFT, D. ECCLESTON, J. I. EVANS, I. OSWALD and E. B. RITSON

A number of drugs capable of mono-amine oxidase inhibition have been marketed with the claim that they alleviate depressive illness. One of these is tranylcypromine (‘Parnate’), which has won notoriety on account of the severe headache experienced by those receiving the drug when they also eat ripe cheese (Lancet, 1964). In chemical structure, it is related to amphetamine. Amphetamine is a drug of addiction, and addiction to tranylcypromine was therefore to be expected. A preliminary notice of the present patient has already been published (Le Gassicke, 1962).

Amphetamine and phenmetrazine addicts previously studied (Oswald and Thacore, 1963) were found to show striking and easily measurable neurophysiological abnormalities upon withdrawal. These affected the proportion of their nocturnal sleep occupied by the so-called “hind-brain”, “paradoxical”, “dreaming”, or “rapid eye movement” phase of sleep. This phase of sleep normally appears abruptly for some 20 minutes or so about 5 times per night, and is characterized, inter alia, by jerky rapid eye movements and a low voltage electroencephalogram with characteristic 2–3 c/second “saw-toothed” waves most marked frontally (Oswald, 1964).

Upon falling asleep, normal people always pass first into the “orthodox” or “fore-brain” phase of sleep (with spindles and high voltage slow waves in the EEG, and ocular reposé). After about an hour, the first rapid eye movement (REM) period of sleep begins. Findings published by various authors (Dement and Kleitman, 1957; Dement, 1960; Berger and Oswald, 1962; Oswald and Thacore, 1963; Rechtschaffen et al., 1963; Rechtschaffen and Maron, 1964; Feinberg et al., 1964) and other unpublished work indicate that in normal adults the delay between first falling asleep (onset of EEG sleep spindles) and the onset of rapid eye movements in the first episode of paradoxical sleep is normally not less than 45 minutes. The total proportion of the night’s sleep, measured by the “REM-time”, spent in paradoxical sleep varies between about 12 per cent. and 32 per cent. with a mean around 22 per cent. More extreme values do not appear to occur more than once or twice per hundred instances.

Since it had been found that amphetamine and phenmetrazine withdrawal caused (a) an abnormally short delay between first falling asleep and the first onset of paradoxical sleep with rapid eye movements (as short as 4 minutes), and (b) a huge rise in the proportion of the whole night spent in the latter kind of sleep (up to 48 per cent.), it was decided to embark on similar studies with the present patient. It should, however, be noted that, whereas the amphetamine addicts had maintained a steady dosage for such a long period that while on the drug their sleep approximated to normal, the present patient’s dosage before withdrawal was highly erratic.

Since tranylcypromine was introduced because of its effect upon amine metabolism, an attempt was made to relate the neurophysiological findings to the patient’s amine metabolism. The practical difficulties of working with unco-operative drug addicts are considerable. We realize that our studies lack the elegant design of a deliberate experiment. Any attempt at the latter, however, could not involve comparable drug dosage.
THE CLINICAL STATE OF A TRANYLCYPROMINE ADDICT

CLINICAL SUMMARY

The patient is an unmarried man, born in 1939. His mother separated from his father, a publican, in the patient's infancy, and he was brought up in a household consisting of mother, elder half-sister, grandmother, and maternal aunt. The grandmother was the dominant person and owned the house. When she died in 1960, the family had to move, and the patient's difficulties dated from then.

He had always been a shy and solitary individual. At puberty, he developed facial acne. On leaving school at 15, he did a number of jobs and left each on the pretext that he could not face people because of his acne. From 1958 to the summer of 1962, he worked as a bus conductor, but his attendance at work was intermittent for the same reason. He has not worked since the summer of 1962.

He was referred in November, 1961. It was thought that his preoccupation with his acne had the quality of an over-valued idea and he was diagnosed as being a "sensitive personality".

Early in 1962, he was given phenelzine ("Nardil") for his depressive symptoms, with no effect. He went off work completely in April, 1962, and was put on etryptamine maleate ("Monase"). This had a dramatic effect on him and he was able to get out to work by himself and took a confident interest in the outside world. This drug was withdrawn from the market in the same month, and he was then given nialamide ("Niamid"). This was ineffective and he returned to his former solitary state.

In May, 1962, he was given tranylcypromine ("Parnate") which had an effect on him similar to that of etryptamine maleate. However, he became dependent on the drug and started to increase the dose. Since then, he has been taking large doses intermittently, up to 700 mg. in one day, though more usually about 200 mg. daily. He has used illicit methods to get the drug and has changed his general practitioner seven times. His activity was related to supplies of the drug. When the latter was available, he got out and about. When it was in short supply, he would restrict his drug to the day he had to visit the Labour Exchange, general practitioner, or out-patient clinic. When without Parnate, he was morose, virtually house-bound and terrified to go out because he was convinced that people stared at his acne.

He was eventually persuaded to come into hospital and was admitted informally on 10 December, 1962. He was given 120 mg. of tranylcypromine daily until 16 December. Later, he admitted that he had been consuming his own supplies as well. On 17 December, he was given 12 dummy tablets instead. On that day, he was kept in bed in a different ward and hourly blood pressure readings were taken. The procedure deprived him of access to his secret supplies of Parnate (hidden in a chimney). He was sleepy, and complained of dreaming a lot during the day. On 18 December, he was permitted the drug again, but became restless, excited and paranoid, and was found dialling the police. He left hospital against advice.

He was constantly warned of the dangers associated with this drug. He would only agree to admission if we could give him the drug, and this we agreed to do for the first part of his re-admission. He was re-admitted on 10 February, 1964. On 12 February, dummy tablets were given and he was, as before, confined strictly to bed in another ward. On 13 February, tranylcypromine was re-introduced, but an increasing proportion of the dose was replaced each day with dummy tablets, and the drug was totally replaced in nine days. He had left hospital against advice on the morning of 13 February, vowing that he would get Parnate for himself, but returned later that day. He finally left against advice on 28 February.

His clinical state from 13-28 February suggested that he was consuming supplies of his own. He afterwards admitted that he took at least 400 tablets of his own over the 18-day admission period. We are certain he did not have any drug on 12 February.

The following are the main clinical features associated with tranylcypromine medication in this patient.

(a) On Preferred Dose (about 300 mg. daily)


Mental. Alert, cheerful, makes conversation readily. Seeks attention and makes jokes. States he hears and sees things vividly.

(b) Immediately After Withdrawal


Mental. Depressed and apprehensive. Complains of recurrent dreams by day and of vivid nightmares. States everything about him looks grey and colourless. Now notices the scratches and dirty marks on the wall. Feelings of intense antipathy towards doctors looking after him.

After 28 February, he found himself unable to get further Parnate. He was seen again after lacking Parnate for ten days. He stated that he had been agitated, restless, and terrified. He had slept intermittently day and night, with frequent nightmares. Visual hallucinations (dreams?) of doctors in his house. Convinced he had been assaulted in hospital and that his leg had been kicked until bruised. These symptoms had lessened as the ten days progressed, and he returned to his former, virtually housebound, state.
Silver cup electrodes containing saline jelly were fixed to the scalp with collodion and to the face with adhesive plaster in order to record the EEG and eye movements respectively throughout the whole night. The patient slept alone in a room at a short distance from the EEG room.

The principal findings are shown in Table I. When the patient was getting Parnate in quantity, paradoxical (or REM) sleep was wholly suppressed on 5 nights. On two other nights, it occupied 2.3 per cent. and 6.4 per cent. of the total sleep time, both values being abnormally low. An outstanding feature was the retention on all these nights of a high level of muscle tone visible as continuous muscle artifact in all channels. The level would occasionally decline sharply for a period and then return. Normally, muscle tone declines so much upon falling asleep that muscle artifact is not seen from such scalp derivations except transiently. In this respect, the patient, while on Parnate, was unique in our experience. Despite the muscle tension, the underlying EEG waves could (at times only just) be discerned sufficiently to enable the stages of sleep to be distinguished.

On the two individual nights following drug withdrawal, he was fearful of being left alone, wanted the light left burning, had difficulty in

<table>
<thead>
<tr>
<th>Date</th>
<th>Parnate 10 mg. Tablets</th>
<th>Total Sleep Time (minutes)</th>
<th>REM Sleep (minutes)</th>
<th>% REM Sleep</th>
<th>Whole Blood 5-Hydroxytryptamine (milli-micrograms per ml.)</th>
<th>Urine, 12 Hours (micrograms)</th>
<th>Urine, 12 Hours (milligrams)</th>
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<td>Own supplies</td>
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</tr>
</tbody>
</table>

The Patient's Sleep

Silver cup electrodes containing saline jelly were fixed to the scalp with collodion and to the face with adhesive plaster in order to record the EEG and eye movements respectively throughout the whole night. The patient slept alone in a room at a short distance from the EEG room.

The principal findings are shown in Table I. When the patient was getting Parnate in quantity, paradoxical (or REM) sleep was wholly suppressed on 5 nights. On two other nights, it occupied 2.3 per cent. and 6.4 per cent. of the total sleep time, both values being abnormally low. An outstanding feature was the retention on all these nights of a high level of muscle tone visible as continuous muscle artifact in all channels. The level would occasionally decline sharply for a period and then return. Normally, muscle tone declines so much upon falling asleep that muscle artifact is not seen from such scalp derivations except transiently. In this respect, the patient, while on Parnate, was unique in our experience. Despite the muscle tension, the underlying EEG waves could (at times only just) be discerned sufficiently to enable the stages of sleep to be distinguished.

On the two individual nights following drug withdrawal, he was fearful of being left alone, wanted the light left burning, had difficulty in
falling asleep, and repeatedly awakened in the earlier part of the night. The most remarkable feature was his frequent passage from wakefulness and normal drowsiness (in which the EEG alpha rhythm becomes slowed or lost while low voltage irregular slow activity at about 4-7 c./seconds appears, and during which the eyeballs make slow rolling movements) directly into paradoxical or REM sleep, with not only the characteristic clusters of jerky eye movements but also the “saw-toothed” frontal waves at 3 c./seconds. This direct transition into REM sleep occurred 12 times from 17 periods of wakefulness on the first drug withdrawal night, and twice from 5 periods of wakefulness on the second occasion of drug withdrawal. In both cases, the first sleep onset of the night was directly into REM sleep (Fig. 1).

On both withdrawal nights, a huge proportion of the night’s sleep (75.7 per cent. and 59.4 per cent.) was spent in REM sleep. Once on each of these nights he awoke from REM sleep emitting repeated blood-curdling shrieks. On each occasion, he described a nightmare. As such an incident has not hitherto been reported, we show Figure 2 in which the presumed course of the nightmare dream, as reflected by the EEG pens, can be seen. Spasmodic contraction of facial muscles preceded awakening by half a minute. He complained of feeling terrified after this and did not fall asleep again for two and a half

![Diagram](image-url)

**Fig. 1.**—Illustrates the transition from wakefulness, with EEG alpha rhythm, through drowsiness with rolling eye movements and loss of alpha rhythm, directly into paradoxical sleep with frontal 3 c/sec. EEG waves and rapid eye movements. The second and third excerpts are continuous with one another. Bottom excerpt—33 minutes later the paradoxical sleep period still continues. Time constants 0.3 second.
hours. He described having had a dream in which an electric machine removed half of his face, and said that doctors then threw him into a lime-pit in which were people in nurses' uniforms who were preventing him from escaping.

The night of 14 December, 1963, was different. He had been supplementing his Parnate ration since admission on 10 December, and for certainly the two (and probably the four) nights prior to the 14th had had no REM sleep. We believe he exhausted his own supplies
that day, because on going to bed he tried to persuade us to give him more Parnate, claiming (untruthfully) that the nurse had not given him his ration at 6 p.m. The next day, while out of hospital for a few hours (we were unable to prevent him doing this), he probably obtained further supplies. On falling asleep on the 14th, he passed into orthodox sleep in the normal way. The first REM period began after 63 minutes and occupied 3 minutes, an hour later a REM period began which lasted 48 minutes, then 43 minutes later, at 2.43 a.m., he entered a REM period which continued for 227 minutes till he finally awoke. A REM period of over 90 minutes has never before been seen in our laboratory.

AMINE METABOLISM

Methods

Whole blood 5-hydroxytryptamine (5-HT) concentrations were estimated by the method described by Ashcroft et al. (1964). Urinary 5-HT was measured by the method of Oates (1961) involving extraction of the urine using ion exchange chromatography, followed by spectrofluorimetric assay in 3-N-hydrochloric acid (Udenfriend, Bogdanski and Weissbach, 1955).

Urinary tryptamine was extracted, using the same ion exchange chromatography as for 5-HT. Elution from the column was with N sulphuric acid. Since it was found that this purification technique alone was not sufficient to remove all interfering substances, the eluate was subjected to the solvent extraction procedure and norharman fluorescence technique described for the estimation of tryptamine by Hess and Udenfriend (1959).

5-Hydroxyindol-3-ylacetic-acid in urine was measured by the technique described by Udenfriend, Titus and Weissbach (1953).

Indol-3-ylacetic acid was measured by the method of Weissbach et al. (1959).

Results

The results are shown in Table I. Blood 5-HT concentrations are seen to fall when the patient was receiving sustained high doses of tranylcypromine.

The patient often left hospital briefly, so urine collections were for periods of twelve hours, when the patient had been under close supervision. The tryptamine excretion increased to extraordinarily high levels (Table I), and followed closely the dose of tranylcypromine.

The 5-HT excretion never rose above a normal level of 60 micrograms/12 hours. The excretion of 5-HIAA decreased to well below the normal level (1-4 mg./12 hours) but despite the increase in tryptamine excretion the levels of IAA were never reduced below normal (1-5-4 mg./12 hours).

Discussion

The patient's sleep showed unprecedented abnormalities—successive nights without paradoxical sleep but with retention of considerable muscle tone, a single period of paradoxical sleep lasting nearly six hours, and 75-7 per cent. of paradoxical sleep on one night. Most striking was the direct onset of paradoxical sleep from initial drowsiness. Hitherto, this has only been described (Rechtschaffen et al., 1963) in the case of patients suffering from idiopathic narcolepsy, an observation confirmed in our own laboratory. Abnormally short delays between falling asleep and the onset of paradoxical sleep have also been observed in three other categories of “sleepy” persons, namely, amphetamine addicts after withdrawal (Oswald and Thacore, 1963), some normal persons after ingesting l-tryptophan (Oswald et al., 1964), and some brain-damaged persons with “légers troubles de la conscience” (Schwartz and Gilbaud, 1964).

The patient's subjective experience of recurrent daytime dreaming on the days when he received dummy tablets was presumably related to recurrent brief episodes of either actual or incipient states of paradoxical sleep. This tremendous “pressure” towards paradoxical sleep, in which it not only took precedence over orthodox sleep at night but obtruded into the daytime life of the patient, can be interpreted as a compensation for the total loss of that kind of sleep while he was taking the large doses of Parnate. The “need” for paradoxical sleep and compensatory increase following experimental
deprivation has been reported (Dement, 1960; Berger and Oswald, 1962; Rechtschaffen and Maron, 1964; Williams et al., 1964), even to the extent of its immediate onset upon falling asleep (Dement, 1963). It might be supposed that on 14 December, a small dose of Parnate by day was sufficient at first during the night to suppress paradoxical sleep, after which it "broke through" in a prolonged and compensating period when the Parnate had been largely metabolized. Experiments in Chicago by Rechtschaffen (personal communication) have shown that therapeutic doses when first given to normal persons will also markedly suppress paradoxical sleep.

The improvement in the patient's clinical state when he received tranylcypromine confirms the potential of drugs of this kind, particularly as he had at times in the past received a few amphetamine preparations without subjective benefit. The extent to which the observed neurophysiological abnormalities can be directly related to the patient's amine metabolism is uncertain. The observations provide hypotheses for further work, some of which we hope to publish later.

The effect of amine oxidase inhibitors on blood 5-HT levels has previously been described by Pletscher and Bernstein (1958), and a typical result with therapeutic dose of tranylcypromine (15 mg. t.i.d.) was described by Ashcroft et al. (1964). There is a steady rise in blood (really platelet) 5-HT concentrations during such therapy.

Urinary tryptamine levels have been used as an index of the degree of amine oxidase inhibition in man by Sjoerdsma et al. (1959).

The pharmacological effects of tranylcypromine have been described and include at least two distinct components.

1. Amine oxidase inhibition.
2. Sympathomimetic effects.

The present case was of interest as the dose of tranylcypromine was much in excess of normal therapeutic dose levels and several unexpected effects on indolalkylamine metabolism were noted.

The anticipated rise in blood 5-HT levels was not found; instead, on the higher doses of tranylcypromine, a progressive fall in blood 5-HT to zero was noted. It is suggested that this may represent an effect on platelet uptake of 5-HT similar to that described for imipramine by Marshall et al. (1960). In vitro experiments (Long and Lessin, 1962) on platelet uptake of 5-HT have shown such an effect for amphetamine, and tranylcypromine is structurally similar. At high dose levels tranylcypromine may be inhibiting two methods of amine inactivation, the enzyme amine oxidase and the process of tissue uptake.

The rise in tryptamine in urine is consistent with the inhibition of oxidative deamination, but no significant rise of 5-HT excretion was found despite a fall in 5-HIAA excretion. This suggests either that another route of excretion exists for 5-hydroxyindole compounds, or that a feedback mechanism is operating to limit 5-HT production.

Summary

A twenty-four-year-old male addicted to tranylcypromine is described. He consumed up to 700 mg. daily. While taking the drug he was restless, cheerful and confident. Without it he was apprehensive and housebound.

Large tranylcypromine intake was associated with high muscle tension during sleep and sometimes abolition of the paradoxical or "rapid eye movement" phase of sleep. Drug withdrawal was associated with complaints of dreaming by day, and, by night, an immediate onset and unprecedented duration of "paradoxical" sleep, with nightmares.

The biochemical effects of tranylcypromine at the high dose levels differed significantly from those associated with therapeutic doses. Tryptamine excretion was much increased but 5-HT excretion was not. Blood levels of 5-HT showed a progressive fall, and it is suggested that this may be a result of interference by tranylcypromine at high concentrations with uptake of 5-HT by platelets.

Acknowledgments

Our thanks are owed to Professor F. J. Fish, Dr. T. B. B. Crawford, Professor G. M. Carstairs, and Professor W. L. M. Perry. Messrs. Smith, Kline and French kindly provided dummy Parnate tablets. Financial aid was received from the Medical Research Council.
THE CLINICAL STATE OF A TRANYLCYPROMINE ADDICT

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References


CHANGES IN AMINE METABOLISM PRODUCED BY ANTIDEPRESSANT DRUGS

G. W. ASHCROFT, D. ECCLESTON, F. KNIGHT, ELIZABETH J. McDOUGALL and J. L. WADDELL

The idea that changes in amine metabolism may be related to the development of depressive illness has gained prominence in the last few years and we make no claim that the views put forward in this paper are new. Rather, we wish to present a review of the evidence recently accumulated and suggest how it might be applied in the clinical field.

To form a basis for discussion, we shall present a simplified, challenging hypothesis, regarding the role of changes in amine metabolism in depression.

Hypothesis. That depression can occur when the levels of biological amines at reactive sites within the brain are reduced, and that anti-depressant drugs will be those which increase the levels of the amines at the reactive sites.

Evidence

Precipitation of depression by drugs. Drugs have been introduced into medicine for the treatment of hypertension which will precipitate a depressive illness in susceptible individuals, viz. reserpine, guanethidine and α-methyldopa. They all have in common the property of depleting the brain of the catecholamines and 5-hydroxytryptamine (Hess et al., 1956; Carlsson and Lindqvist 1962).

A study of these drugs may throw light on the basis of naturally occurring depressive illness and provide support for the first part of the hypothesis.

Treatment of depression by drugs. This topic is our main concern and evidence will be presented to suggest that these drugs have in common the property of potentiating the effect of amines centrally, by increasing the effective concentrations at reactive sites. This evidence can be presented under two main headings:

(i) The evidence obtained by the physiologists and pharmacologists in animal experimental studies.

(ii) The evidence presented by the clinician, as a result of clinical observation (including toxic reactions) and biochemical studies.

We can, then, with this information, attempt an integration of the evidence presented by the two disciplines, which, if successful, should explain the mode of action of the drugs, basis for toxic reactions, means by which these might be avoided, or treated, and also reasons for failures in drug therapy. Such an approach should also enable us to predict the properties to be sought in a new anti-depressant drug.

1. EVIDENCE FROM PHYSIOLOGY AND PHARMACOLOGY

We cannot hope to understand the basic mechanisms of the actions of the drugs we are using, without a knowledge of the recent work carried out in relationship to the peripheral actions and metabolism of the biogenic amines, in particular the catecholamines, and an understanding of the basis for toxic reactions, e.g. hypertensive crises.
The study of the cerebral metabolism of the amines in man is complicated by its inaccessibility and by its relative isolation from the rest of the body by the blood/brain barrier. In animals, much has been learned of the cerebral metabolism of the amines, but recent rapid advances in knowledge have been mainly concerned with peripheral mechanisms. These studies have revealed the complex nature of the mechanisms involved in amine synthesis, storage, release and inactivation. Excellent reviews of different aspects of the problem have been provided by Axelrod (1963), Kopin (1964) and Shore (1962).

The catecholamines are stored in peripheral sympathetic post-ganglionic neurones, released by nerve stimulation and inactivated rapidly after release. A schematic representation of the mechanisms of storage, release and inactivation is presented in Fig. 1.

**Fig. 1. Schematic representation of mechanisms of storage, release and inactivation of noradrenaline**

**Storage.** At least three different stores exist, in relationship to the sympathetic nerves:

(i) Most of the catecholamines are stored in intraneural vesicles.
(ii) A second "store", or pool, acts as the source of noradrenaline, released by nerve stimulation. It may be in the nerve membrane and be replenished by noradrenaline from the intraneural vesicle.
(iii) An extraneural pool, resistant to release by reserpine, but released by tyramine and other sympathomimetic amines.

**Inactivation.** Noradrenaline, released by nerve stimulation, is inactivated rapidly by the following mechanisms:

(i) The enzyme catechol-O-methyl transferase.
(ii) Re-uptake into the nerve terminal, this being carried out by an active transport mechanism. The amine is then either returned to the stores, or de-aminated by the enzyme monoamine oxidase. The suggested role of mono-amine oxidase, in relation to noradrenaline metabolism, is the control of the level of free amine within the cell.
(iii) Uptake on to tissue sites, or "silent receptors", where the amine is held in inactive form, to be liberated slowly thereafter.

The presence of three mechanisms ensures rapidity and efficiency of inactivation and termination of effect, and accounts for failure of drugs affecting only one mechanism to produce significant potentiation of the endogenous catecholamine effects at peripheral sites.

Central mechanisms. The existence of similar mechanisms in the central nervous system is still largely conjecture, but it is on the assumption that such a parallel can be drawn that the subsequent discussion of the mode of action of anti-depressant drugs is based.

Anti-depressant drugs

The following effects have been considered for each type of drug:

(a) Inhibition of mono-amine oxidase in vivo.
(b) Blockade of uptake on to non-specific uptake sites, or "silent receptors".
(c) Release of catecholamines at peripheral sites, with production of sympathomimetic effects.
(d) Direct effects on central catecholamine receptors.
(e) Blockade of peripheral catecholamine receptors.
(f) Action on peripheral and central tryptamine receptors is not considered here, but is considered in detail elsewhere in this symposium (Dewhurst, 1964; Marley, 1964).

A summary of the effects produced by each group of drugs is shown in Table 1. The actions of chlorpromazine are shown, for comparison.

The feature shown in common by all the anti-depressant drugs is the possibility of potentiation of cerebral amine effects (Sigg, 1959) either as a result of the liberation of amines, or interference with their inactivation. It should be noted at this point, however, that blockade of one route of amine inactivation may cause no potentiation of peripheral catecholamine effects, unless it is accompanied by release of the amines (Kopin, 1964).

Table 1.—Effects on catecholamine metabolism

<table>
<thead>
<tr>
<th>Type of drug</th>
<th>Inhibits mono-amine oxidase in vivo</th>
<th>Block of uptake into non-specific uptake sites</th>
<th>Releases catecholamines with sympathomimetic effects at peripheral sites</th>
<th>Possible direct effect on central catecholamine receptors</th>
<th>Blocks catecholamine effect at peripheral receptors</th>
<th>Action on tryptamine receptors</th>
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It must be emphasised, too, that we have deliberately ignored the possibility that the defect in amine metabolism in depression might not be a simple deficiency of catecholamines, but might involve an upset in balance between systems represented on the one hand by the catecholamines and on the other by the indolalkylamines.

It is of interest that many of the drugs which block the uptake of the catecholamines (Hertting et al., 1961) on to non-specific sites have also been shown by Axelrod and Inscoe (1963) to block the uptake of 5-hydroxytryptamine at peripheral sites in the mouse. It may be that the blockade of platelet uptake of 5-hydroxytryptamine by drugs in man will provide an index of this effect (Marshall et al., 1960).

2. ATTEMPTS TO PROVIDE EVIDENCE IN MAN TO SUPPORT THE HYPOTHESIS REGARDING THE MODE OF ACTION OF ANTI-DEPRESSANT DRUGS

We present here preliminary investigations on these topics:

(a) Evidence for the multiple actions of tranylcypromine on 5-hydroxytryptamine metabolism in man

From Table 1, it can be seen that tranylcypromine (Parnate) exerts multiple actions on catecholamine metabolism. The effects on two other amine pathways, those of 5-hydroxytryptamine and tryptamine, have been examined in a patient addicted to the drug and the results are to be published in detail elsewhere (le Gassicke et al., 1965). Whole blood levels of 5-hydroxytryptamine were measured by the method described by Ashcroft et al., (1964), and the change in these levels in two patients, one receiving a therapeutic dose of tranylcypromine and the other an addict taking the drug in excess, are shown in Fig. 2. The expected response to a drug which is a mono-amine oxidase inhibitor is seen in the patient on therapeutic dosage, i.e. a progressive rise in blood 5-hydroxytryptamine. In the case of the addict, however, a paradoxical response to the drug is seen, where there is a progressive fall in levels with continued high dosage of tranylcypromine. This result may be explained as an effect of tranylcypromine at high concentrations on the uptake of the amine by platelets. Long and Lessin (1962) have shown that in vitro amphetamine, a closely related compound, is one of the basic substances which prevent uptake of 5-hydroxytryptamine by platelets.

(b) Investigation of the effect of Amitriptyline on the excretion of indol-3-ylacetic acid following intravenous infusion of tryptamine in man

In animals, drugs of this type have been shown to inhibit tissue uptake of catecholamines (Hertting et al., 1961) and of 5-hydroxytryptamine, (Axelrod and Inscoe, 1963). Amitriptyline has been shown to prevent platelet uptake of 5-hydroxytryptamine (Yates et al., 1964).

We postulate that, if the drug in man blocks the uptake of amines on to sites in the tissues, then its administration in patients should increase the percentage of an infused dose of the amine excreted during the first few hours after the infusion.

The amine chosen for infusion was tryptamine, shown by Axelrod and Inscoe (1963) to compete for uptake sites with 5-hydroxytryptamine in the mouse, but not concentrated in 5-hydroxytryptamine stores, such as platelets (Stacey, 1961). Tryptamine 6 mg was infused intravenously, slowly and steadily in 30 ml 5% dextrose, over a
Changes in amine metabolism produced by antidepressant drugs

period of 30 min in a fasting patient, diuresis being maintained by the administration of 100 ml water at hourly intervals, from 2 hr before the infusion until the end of the investigation.

Urine collections were made hourly, over 4 hr. The first hour was a control period before infusion, and the second hour included the infusion and the subsequent 30 min period. Tryptamine was estimated in the urine by a combination of ion exchange chromatography (Oates, 1961) with benzene extraction of the eluted amine fraction and fluorescence assay, as described by Hess and Udenfriend (1959). Total urinary indol-3-ylacetic acid was determined after preliminary acid hydrolysis, by the method of Weissbach et al. (1959).

It was found that, in normal controls and in depressed patients receiving no drugs, no significant increase in urinary tryptamine occurred following the infusion. In these studies, there was a transient rise in urinary indol-3-ylacetic acid, but this had returned to the control level by the fourth hour of the investigation in all cases. The results are expressed as the increase in total excretion of indol-3-ylacetic acid following infusion, i.e. total IAA excretion, hours 2 + 3 minus total IAA excretion, hours 1 + 4. The results in four patients are shown in Fig. 3, together with the results of a second infusion in three patients following treatment with amitriptyline. A third infusion was carried out in one patient.

The percentage of the injected tryptamine excreted as the IAA over the period of test is seen never to rise above 10 per cent in the untreated patient, suggesting that either the compound is retained in the body for a longer period, or metabolised along an alternative route. The results are consistent with the observations made by Jepson (1963).

These investigations must be considered as a preliminary study, in which inadequate account was taken of factors such as urinary pH and volume, which can alter indol-3-ylacetic acid rates, Milne et al. (1960). However, as can be seen in Fig. 3, three out of four patients showed an increase in the amount of indol-3-ylacetic acid excreted.

---

**Fig. 2.** Changes in whole blood levels of 5-hydroxytryptamine produced by therapeutic ▲ and excess ● doses of tranylcypromine.

---
following the injection of 6 mg tryptamine when under amitriptyline therapy, an
effect that would be consistent with the action of the drug being a reduction in tissue
uptake of the amine. The fourth patient showed an anomalous result in three separate
infusions; he excreted less indol-3-ylacetic acid following tryptamine infusion than in

![](image)

**Fig. 3.** Increase in “total” indol-3-ylacetic acid excretion, following intravenous
injection of 6 mg. tryptamine in depressed patients.

a control period. No explanation can be offered to account for this response, but it
should be noted that this patient was suffering from the depressive phase of a manic-
depressive psychosis, and that he had a strong family history of manic-depressive
illness, father and three siblings, one sister and two brothers, having suffered from the
disease.

**DISCUSSION**

The hypothesis presented here is not new, but we believe that it may now prove
possible to devise methods to test its validity in man.

The first part of the hypothesis, that levels of amines at active sites in brain are
reduced, may perhaps be investigated further by a study of the relationship of drug-
induced depression to that form of the illness which develops spontaneously. A direct
attack on the problem may be possible by a study of the levels of amine metabolites
in cerebrospinal fluid (Ashcroft and Sharman, 1960).

The second part of the hypothesis, that anti-depressant drugs will be those which
potentiate amines at active sites, already has much support from animal studies
(Sigg 1959; Hertting et al., 1961). It appears that many such drugs may have multiple
effects on amine metabolism, and consideration must be given to each possible action,
e.g. amine oxidase inhibition, release of catecholamines, or blockade of uptake sites,
in the evaluation of the mode of action.
We would postulate that, in the normal, there are fluctuations in response (mood) in relation to environmental situations and that the biochemical background to these fluctuations may be represented by changes in amine levels at reactive sites in brain. In the depressed patient, these changes in amines may be seen as still operating in response to environmental stimuli, but now, at reduced levels, insufficient to produce behavioural effects. Drugs possessing the property of potentiating the action of the amines might amplify the subthreshold changes, in response to environmental stimuli, to a level at which behavioural, or mood, changes would again occur. Substances of this type, e.g. imipramine and some amine oxidase inhibitors, could be considered as approaching the ideal in the treatment of depression, as they would still allow fluctuations in mood in response to environmental situations, whilst drugs of the amphetamine type, with a component of direct stimulation, might prove less satisfactory by eliminating the possibility of such mood changes.

Combinations of drugs with different effects on release, or inhibition of inactivation of catecholamines, e.g. drugs blocking non-specific uptake sites, combined with mono-amine oxidase inhibitors, would be expected to produce severe toxic effects. This is borne out in practice; Howarth (1961) showed that the combination of imipramine and phenelzine resulted in toxic reactions. Serious hypertensive crises may result from the effects of tranylcypromine being potentiated by cheese, caused by the release of catecholamines at peripheral sites by the tyramine in the cheese (Lancet, 1964).

SUMMARY

The hypothesis is advanced that depressive illness occurs when the level of amines is reduced at reactive sites in the brain, and that anti-depressant drugs will be those which increase the levels of amines at these sites.

The evidence for the mechanisms of potentiation of peripheral effects of amines by mono-amine oxidase inhibitors, imipramine and amphetamine, is reviewed and the presence of multiple effects of single drugs on amine metabolism acknowledged.

The mechanisms by which toxic effects are produced by single drugs and drug combinations is examined.

Preliminary evidence for an effect of amitriptyline on the pattern of excretion of injected tryptamine in man is presented.

Acknowledgements—We gratefully acknowledge the help given during these studies by our clinical colleagues, in particular to Dr. Elizabeth E. Robertson, Consultant Psychiatrist, Royal Edinburgh Hospital, and also Professor W. L. M. Perry and Dr. T. B. B. Crawford in the Pharmacology Department, Edinburgh University.

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AMNIOTIC FLUID COMPOSITION IN MALFORMATIONS OF THE CENTRAL NERVOUS SYSTEM

BY
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AMNIOTIC FLUID COMPOSITION IN MALFORMATIONS OF THE CENTRAL NERVOUS SYSTEM

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Summary

In pregnancies in which the fetus had a malformation of the central nervous system (anencephaly, spina bifida, or anencephaly with spina bifida) amniotic fluid levels of 5-hydroxyindole acetic acid (5HIAA), expressed on a protein basis, were less than in normal pregnancies of comparable gestation. This was not observed with a number of other substances present in amniotic fluid nor in 5HIAA levels in cases of polyhydramnios where the fetus was normal. The urinary excretion of 5HIAA in children with spina bifida was normal. A number of possible interpretations are discussed, including the idea that the reduced levels of 5HIAA may reflect the reduction in functioning neural tissue in more severe CNS malformations.

The antenatal diagnosis of a number of genetic disorders is now possible from the cytogenetic and biochemical study of cultured amniotic fluid cells (Emery, 1970a). With the selective abortion of abnormal fetuses this is becoming an established procedure in the management of families at high risk of having a child with a serious genetic disorder. However, these techniques are not applicable to congenital malformations not associated with any recognized biochemical or chromosomal abnormality. In the case of congenital malformations of the central nervous system (CNS) there are three possible approaches to the problem of antenatal diagnosis by examination of amniotic fluid. Firstly, a fortuitous biochemical abnormality may be found in the amniotic fluid, an example being the non-specific increase in optical density at 450 nm observed in anencephaly (Cassady and Cailliateau, 1967; Lee and Wei, 1970) with or without spina bifida (Emery, 1970b). Secondly, an increased concentration may be found of metabolites known to be excreted in excess amounts in the urine of affected children since fetal urine contributes to the formation of amniotic fluid from at least the 12th week of gestation. It has been reported for example, that the urinary excretion of various catecholamine metabolites is increased in children with spina bifida (McKibbin, O’Gorman and Duckworth, 1969). Unfortunately we have been unable to detect any abnormality in the catecholamine metabolite content of amniotic fluid in CNS malformations (Emery and McKibbin, 1971). Thirdly, there might be an increased concentration in amniotic

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fluid of substances which could possibly have leaked (directly, or perhaps as a transudate) from the CNS. Substances most likely to be informative in this regard are those which have a higher concentration in cerebrospinal fluid compared with normal amniotic fluid (at least in mid-pregnancy) and include magnesium, glucose, total cholinesterase, and 5-hydroxyindole acetic acid (5HIAA). However, apart from 5HIAA we have found normal levels of these various substances (as well as sodium, potassium, chlorine, total protein and urea) in amniotic fluid in CNS malformations. The present communication concerns 5HIAA levels in amniotic fluid which, contrary to expectations, were found to be lower than normal in CNS malformations.

MATERIALS AND METHODS

Normal specimens of amniotic fluid were obtained at delivery in normal pregnancies, or by transabdominal amniocentesis during the management of rhesus-incompatible pregnancies in which the mother was subsequently delivered of a perfectly healthy child, or from pregnancies being terminated for social or psychiatric reasons in which the fetus, after careful examination, appeared normal. Specimens from pregnancies with CNS malformations were obtained at therapeutic abortion (one specimen) or at delivery (spontaneous or induced). The duration of pregnancy was estimated from the first day of the last menstrual period. CNS malformations have been classified as anencephaly, spina bifida, or anencephaly with spina bifida when anencephaly was associated with a variable defect of the spinal vertebrae. All these fetuses were believed to be alive at the time when amniotic fluid was obtained.

Specimens in which there was any suggestion of contamination with maternal blood were discarded. After centrifuging, the cell-free supernatant was usually analyzed immediately, but when this was not possible the sample was stored at -25 °C. but never for more than two weeks. 5HIAA was estimated by a spectrophotofluorimetric method (Loose and Paterson, 1966) whereby fluorescence is measured in the presence of hydrochloric acid and ascorbic acid after deproteinization and ether extraction. A modification of a colorimetric method was used for determining 5HIAA levels in urine samples (Macfarlane et al., 1956). All 5HIAA estimations were carried out without knowledge of the source of the specimens.

Protein was estimated according to the method of Papadopoulos et al. (1959).

RESULTS

The amount of 5HIAA in normal amniotic fluid was found to be related to the duration of pregnancy, there being little in the first half of pregnancy but a rapid increase throughout the latter half of pregnancy (Fig. 1), and this confirms previous findings (Loose and Paterson, 1966). However, in the majority of specimens from cases in which the fetus had a CNS malformation 5HIAA levels, whether expressed as a concentration (ng. per ml.) or on a protein basis (ng. per mg. protein), were less than normal. All the mothers of fetuses with a CNS malformation were healthy apart from one mother who had mild pre-eclampsia, and in this case a comparatively high level of 5HIAA (24·4 ng. per mg. protein) in amniotic fluid was observed at 38 weeks. This might find explanation in the recent report that 5HIAA levels in fetal urine are raised in pre-eclampsia (Loose, Paterson and Scrimgeour, 1970). However, another specimen of amniotic fluid with a comparatively high level of 5HIAA (24·0 ng. per mg. protein) at 41 weeks was not associated with pre-eclampsia. Polyhydramnios was present in the first of these cases but not in the second.

Since most of the cases of CNS malformations (17 out of 21) were associated with varying degrees of polyhydramnios, 5HIAA levels were determined in a number of pregnancies in which there was polyhydramnios but the fetus was normal, and the mother was healthy and did not have pre-eclampsia. These latter values fell within the range for control values of comparable gestation (Fig. 1).

5HIAA in amniotic fluid, at least in later pregnancy, probably originates from fetal urine (Loose, Paterson and Scrimgeour, 1970). We therefore investigated the 24-hour urinary excretion of 5HIAA in a number of children with spina bifida and controls of comparable age. Both patients and controls were in hospital, the
latter for minor surgical procedures, and were on the same diet. The results have been expressed as μg. of 5HIAA excreted per 24 hours. It seemed unwise to express the results in terms of creatinine as is customary, since the urinary excretion of this substance is reduced in spina bifida probably as a result of the reduction in functioning muscle tissue. The results (Table 1) indicate that in childhood there is a gradual increase in the urinary excretion of 5HIAA with increasing age, but there was no consistent difference between the patients with spina bifida and their controls. The excretion of 5HIAA in patients...
with spina bifida appeared to be un influenced by the presence or absence of a shunt.

**Table I**

<table>
<thead>
<tr>
<th>Age</th>
<th>Spina bifida</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>528</td>
<td>120</td>
</tr>
<tr>
<td>4-6 weeks</td>
<td>687</td>
<td>109</td>
</tr>
<tr>
<td>16-21 months</td>
<td>1769</td>
<td>1208</td>
</tr>
<tr>
<td>50-60 months</td>
<td>1763</td>
<td>1948</td>
</tr>
<tr>
<td>84-90 months</td>
<td>1716</td>
<td>1040</td>
</tr>
<tr>
<td>102-126 months</td>
<td>2489</td>
<td>2842</td>
</tr>
<tr>
<td>130-132 months</td>
<td>2189</td>
<td>2690</td>
</tr>
</tbody>
</table>

**Discussion**

5-hydroxyindole acetic acid (5HIAA) is the major end product of 5-hydroxytryptamine (5-HT) metabolism, the first step in the synthesis of which is the hydroxylation of tryptophan which takes place in both brain and gut (Loose and Paterson, 1966). 5HIAA has been detected in human cerebrospinal fluid (Ashcroft and Sharman, 1960), but not in plasma. The formation of 5HIAA in brain depends on the amount of tryptophan ingested, there being a large rise when this amino acid is administered (Ashcroft, Eccleston and Crawford, 1965) and on the functional activity of the 5-hydroxytryptaminergic neurones (Sheard and Aghajanian, 1968). It seems likely that in the human fetus 5HIAA is probably excreted by two routes; directly by the placental circulation and indirectly via fetal urine into the amniotic fluid. The proportion of 5HIAA excreted into the amniotic fluid is unknown as is the relative contribution of brain and intestine to the total 5HIAA excretion.

The hypothesis was considered that in congenital malformations of the CNS a leak (directly, or perhaps as a transudate) of 5HIAA from the CNS into the amniotic fluid might occur, and so lead to an increase in the amniotic fluid concentration. Contrary to our expectations, the levels obtained at least in later pregnancy were less than in controls, for which values were similar to those reported by other investigators (Loose and Paterson, 1966). There are several possible explanations for this observation. There is a tendency for polyhydramnios to be associated with such malformations and it may be that all that is observed is a "dilution" effect. However, the values were less than normal when expressed on a protein basis, the phenomenon was not observed with other substances (e.g. cholinesterase, electrolytes, various catecholamine metabolites and total protein) and was not observed in cases of polyhydramnios when the fetus was normal. Alternatively, the reduced levels of 5HIAA in amniotic fluid may result from a relative failure of renal function in these malformations so that a larger proportion of the metabolite is directed into the maternal circulation. Since the urinary excretion of 5HIAA appears to be normal in children with spina bifida if there is a defect in renal function this may be present only in the more severe CNS malformations or be manifest only in utero. Finally, the reduced levels may be a consequence of reduction in the formation of 5HIAA either as a result of a relative deficiency of precursors or to reduced synthesis. The latter might result from a reduction in the extra-neural synthesis of 5HIAA, probably from the intestine, or merely reflect the reduction of functioning neural tissue in these malformations. The congenital defect in liveborn children with spina bifida is considerably less than in the CNS malformations investigated in this study and if the latter explanations were true then a significant reduction in the synthesis, and therefore urinary excretion, of 5HIAA in these children would not be expected. These various possibilities are being further investigated.

**Acknowledgements**

We wish to acknowledge the excellent technical assistance of Mrs. I. M. Ritchie and Mr. D. Burt. We also thank Dr. J. G. Robertson for his help in obtaining specimens of amniotic fluid in mid-pregnancy, Dr. D. B. Horn and his staff for electrolyte and urea determinations, Dr. G. W. Ashcroft and Dr. D. J. H. Brock for helpful discussions and the nursing staff for their help in collecting 24-hour urinary samples.

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Summary

The results are presented of measurements of optical density at 450nm (OD$_{450}$), 5-hydroxyindole acetic acid (5-HIAA), a-fetoprotein (AFP) and amino acids in amniotic fluid at various stages of gestation in over 60 pregnancies in which the fetus had a neural tube defect namely anencephaly and/or spina bifida. Before 20 weeks’ gestation neither OD$_{450}$ nor 5-HIAA levels were abnormal. AFP levels were more reliable than amino acid concentrations for the antenatal diagnosis of neural tube defects, particularly in early pregnancy. Various interpretations for these findings are discussed including the possibility that OD$_{450}$ and AFP levels in amniotic fluid might reflect altered maternal metabolism.

The study of the composition of amniotic fluid in anencephaly and/or spina bifida could prove helpful in their antenatal diagnosis, and could throw some light on the pathogenesis and aetiology of these malformations.

Cassady and Cailliteau (1967) and Lee and Wei (1970) found an increase in amniotic fluid optical density at 450 nm (OD$_{450}$) in late pregnancy in anencephaly. We have confirmed this finding and have also observed a decrease in 5-hydroxyindole acetic acid (5-HIAA) (Emery et al., 1972), an increase in alpha-fetoprotein (AFP) (Brock and Sutcliffe, 1972) and an increase in certain amino acids (Emery et al., 1973) in amniotic fluid in patients with a fetus who had a neural tube defect, namely, anencephaly and/or spina bifida. The object of this report is to present the results of OD$_{450}$, 5-HIAA, AFP and amino acid studies in amniotic fluid at various stages of gestation in over 60 pregnancies complicated by a fetus with a neural tube defect and to compare the relative merits of these four determinations in antenatal diagnosis. Possible interpretations of these findings are also considered.

MATERIALS AND METHODS

Specimens of amniotic fluid were obtained at therapeutic abortion or at delivery (after spontaneous or induced labour) and in the case of AFP determinations also from the rhesus isoimmunization clinic and in the relief of hydramnios for various reasons. The duration of pregnancy was estimated from the first day of the last menstrual period and, where possible, correlated with the crown-rump or heel-toe lengths of the fetus.

The OD$_{450}$ was only determined if the mother was rhesus positive and the amniotic fluid contained no meconium. Specimens in
which there was any obvious contamination with blood were discarded. After centrifugation the cell-free supernatant was either analyzed immediately or after storage at −70 °C.

The $\Delta OD_{450}$ was determined according to the method described by Robertson (1969). Values were considered abnormal if they were above the normal curve given by Carlton and Sinha (1970). The 5-HIAA level was estimated by a spectrophotofluorimetric method (Loose and Paterson, 1966) and values were considered abnormal if below the normal range for any given gestational age (Emery et al., 1972). AFP levels were determined by counter-immunoelectrophoresis (Brock and Sutcliffe, 1972) and were considered abnormal if above the normal range for any given period of gestation. Amino acid concentrations were determined by ion-exchange chromatography, normal values at various stages of gestation having been previously reported (Emery et al., 1970). A specimen of amniotic fluid was considered abnormal if the concentration of more than one amino acid was greater than two standard deviations above the normal mean value for any given period of gestation.

It was not possible to carry out all the investigations on every specimen of fluid because the amount of fluid available was sometimes small, and because AFP determinations were only made in the later stages of the investigation.

**RESULTS**

The results of $\Delta OD_{450}$, 5-HIAA and AFP determinations are shown in Figures 1, 2 and 3. These and the amino acid results are summarised in Table 1. In early pregnancy (before 20 weeks) neither $\Delta OD_{450}$ measurements nor 5-HIAA levels were abnormal. AFP determinations gave a more reliable indication of a neural tube defect than did amniotic fluid amino acid measurement, particularly in early pregnancy.
Anencephaly can be diagnosed at 17 weeks' gestation by ultrasonography but this technique requires specialized equipment and the results can be difficult to interpret (Campbell et al., 1972). The value of fetoscopy is its theoretical ability to detect spina bifida as well as anencephaly but the technique is difficult and dangerous to the fetus (Scrimgeour, 1973). At present the most rewarding approach to the problem of diagnosing neural tube defects antenatally appears to be the study of amniotic fluid. Our results indicate that estimation of AFP in amniotic fluid, and probably maternal serum (Brock et al., 1973), is the best means currently available for detecting neural tube defects in utero in early pregnancy. However, spina bifida may be missed if the lesion is small or closed (Laurence et al., 1973).

Abnormalities in the biochemical composition of amniotic fluid in the presence of neural tube defects have usually been attributed to leakage of fetal C.S.F. or blood into the amniotic fluid. However, at least some of the observed changes in amniotic fluid composition might be due to transfer of metabolites from the mother into the amniotic fluid, reflecting changes in maternal metabolism which may be of aetiological significance.

It has been shown previously that the optical density of amniotic fluid at 450nm may be increased in late pregnancy complicated by an

### Table I

Proportions of specimens of amniotic fluid with neural tube defects in which significant biochemical abnormalities were detected

<table>
<thead>
<tr>
<th>Gestation (weeks)</th>
<th>△OD&lt;sub&gt;450&lt;/sub&gt;</th>
<th>5-HIAA</th>
<th>AFP</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 or less</td>
<td>More than 20</td>
<td>20 or less</td>
<td>More than 20</td>
</tr>
<tr>
<td>Anencephaly</td>
<td>0 / 1</td>
<td>20 / 27</td>
<td>0 / 1</td>
<td>20 / 26</td>
</tr>
<tr>
<td>Spina bifida</td>
<td>0 / 3</td>
<td>2 / 7</td>
<td>0 / 3</td>
<td>6 / 9</td>
</tr>
<tr>
<td>Anencephaly + spina bifida</td>
<td>0 / 4</td>
<td>13 / 20</td>
<td>0 / 4</td>
<td>12 / 19</td>
</tr>
<tr>
<td>Total</td>
<td>0 / 8</td>
<td>35 / 54</td>
<td>0 / 8</td>
<td>38 / 54</td>
</tr>
<tr>
<td>per cent</td>
<td>0 / 65</td>
<td>65 / 100</td>
<td>0 / 70</td>
<td>70 / 100</td>
</tr>
</tbody>
</table>

**Fig. 3**

AFP levels in amniotic fluid. The curve represents the upper limit of the normal range.
anencephalic fetus (Cassady and Cailliteau, 1967; Lee and Wei, 1970). Our results indicate that an increase in $\triangle OD_{450}$ may also occur in spina bifida. The $\triangle OD_{450}$ is believed to represent unconjugated (indirect) bilirubin in rhesus sensitization (Brazie et al., 1966: Mandelbaum and Robinson, 1966). This is probably also true in neural tube defects for amniotic fluid bilirubin levels measured by chemical methods have been high in anencephaly (Stewart and Taylor, 1964). This could be due to leakage of fetal blood or to regurgitation of bile as a consequence of defective fetal swallowing. The latter explanation might also account for the observed increase in $\triangle OD_{450}$ in congenital atresia of the duodenum (Liley, 1963; Grimes and Cassady, 1970) and ileum (Grimes and Cassady, 1970). However, it could not account for the increase in $\triangle OD_{450}$ which has been reported in a fetus with both oesophageal and anal atresia (Ojala, 1971) and in congenital pyloric stenosis (Willoughby et al., 1969). In the latter case the mother had subclinical jaundice with a total serum bilirubin of 2·9 mg./100 ml. and it is known that an increase in $\triangle OD_{450}$ can be associated with maternal hyperbilirubinaemia (Emery, 1973; Willoughby et al., 1973). Whether or not the increase in $\triangle OD_{450}$ in amniotic fluid in pregnancy with a fetal neural tube defect is associated with a defect in the maternal metabolism of bilirubin is not known.

In a case of pregnancy complicated by fetal oesophageal atresia, Stewart and Taylor (1964) showed increased amniotic fluid bilirubin measured by chemical methods. In congenital oesophageal atresia there is also an increase in $\triangle OD_{450}$ in amniotic fluid and in amniotic fluid AFP (Seppälä, 1973) and both these changes may have a common origin. In neural tube defects raised levels of AFP in amniotic fluid have generally been considered to be due to leakage of fetal CSF or blood. However, the elevations of maternal serum AFP which have been observed (Brock et al., 1973; Leek et al., 1973; Brock et al., 1974) are not so easy to explain, for fetal serum AFP concentrations are normal in anencephaly (Brock, 1974). It is possible that amniotic fluid AFP diffuses into the maternal circulation. Alternatively, the mother may respond to the abnormal fetus by an increased synthesis of AFP. The peak of serum AFP reached at 32 to 35 weeks of normal pregnancy when fetal AFP production is decreasing, suggests that AFP may be of maternal origin (Seppälä and Ruoslahti, 1973; Ishiguro and Nishimura, 1973).

The observed changes in amniotic fluid composition in neural tube defects may thus be due to alternations in maternal metabolism rather than to leakage of fetal CSF or blood.

ACKNOWLEDGEMENTS

We thank all the obstetricians, particularly Dr. J. G. Robertson and Dr. J. B. Scrimgeour, who have provided us with specimens of amniotic fluid and Mrs. I. M. Richie and Miss S. Brown for excellent technical assistance. This work was supported by research grants from the Children’s Research fund, the Distillers’ Company and the Scottish Hospital Endowments Research Trust.

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Tryptamine in the Blood and Urine of a Patient with a Carcinoid Tumour

Patients with the carcinoid syndrome typically show high blood levels of 5-hydroxytryptamine and increased urinary excretion of 5-hydroxyindolyl-3-acetic acid. Variants of the tumour have, however, been described in which the major secretion is 5-hydroxytryptophan.

Recently, during studies of blood of a male patient with a carcinoid tumour with extensive metastases, an indole compound was detected which failed to give the fluorescence or colour reactions typical of 5-hydroxyindole compounds.

This substance was not detected in similar examinations of blood samples from normal individuals or from another carcinoid patient. The identity of this compound with tryptamine was indicated by the following evidence:

(a) Rf values identical with authentic tryptamine on paper chromatograms in three solvent systems: (1) 20 per cent potassium chloride (w/v); (2) butanol-acetic acid/water (12:3:5 by vol.); (3) propan-2-ol/aqueous ammonia solution (sp. gr. 0.88)/water (20:1:2 by vol.).

(b) Norharman formation, detected by fluorescence characteristics following condensation of tryptamine with formaldehyde and subsequent dehydrogenation of the tetrahydronorharman with hydrogen peroxide.

This patient was also found to be excreting large amounts of tryptamine in the urine as estimated by the method of Sjoerdsma, Oates, Zaltzman and Undenfriend. In a 24-h urine sample the excretion was 84 mg, which is about 1,000 times the normal level of 0.006–0.12 mg/day (ref. 3). It is of interest that the urinary indolyl-3-acetic acid and its acid hydrolysable conjugates showed little or no rise above the normal concentrations, estimated by the method of Weissbach, King, Sjoerdsma and Undenfriend. The urinary indolyl-3-acetic acid was found to be 12 mg/24 h (normal, 3–8 mg/24 h). After preliminary acid hydrolysis of the urine, the estimates increased to 16 mg/24 h (normal, 3–13.8 mg/24 h).

So far as we are aware, this is the first report of the finding of tryptamine in human blood either in normal human beings or patients with carcinoid syndrome.

This work was done with the help of Mr. Adam Smith, Western General Hospital, Edinburgh, whose patient this is.

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G. W. Ashcroft

University of Edinburgh Medical School, Edinburgh, S.
SOME OBSERVATIONS ON THE METABOLISM OF INDOLES IN TWO PATIENTS WITH THE CARCINOID SYNDROME

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The relationship of the venous blood levels of 5-hydroxytryptamine to the symptoms of the carcinoid syndrome, e.g., the flush and endocardial lesions, is still far from clear. Studies by many workers have shown that other substances, such as histamine and bradykinin, may be involved. It would appear that many biologically active substances may be released into the blood stream of such patients. They may not all originate from the tumor. Some may be substances normally present in the body which have been activated or caused to accumulate because of the influence on their metabolism of the high levels of the 5-hydroxyindoles. The nature of the circulating substances may vary greatly from patient to patient and possibly from hour to hour in a given patient, hence the difficulty in implicating directly one or more in the production of different aspects of the syndrome.

The present studies were carried out solely in relationship to indolalkylamine metabolism and illustrate further the diversity of the biochemical picture in two patients, and the changes seen during repeated sampling of body fluids in each case. Within the limits of investigation imposed by such clinical studies, they appear to show a possible interaction between the 5-hydroxytryptamine and tryptamine metabolic pathways, and add two further substances, tryptamine and indole-3-ylacetic acid to the list of compounds which may fluctuate in concentration in body fluids in patients with this syndrome.

The clinical details of these patients are described in the previous paper. Patient 1 had an argentaffin tumor of the intestinal tract and patient 2 an "adenoma of the bronchus" of argentaffin cell origin, both with extensive liver metastases.

Biochemical Investigations

Methods

All chemicals were of "Analytical Reagent" grade and "Microanalytical Reagent" grade (for concentrated hydrochloric acid). Deionized distilled water was used throughout. Evaporations were carried out under reduced pressure at an external temperature of less than 30 C. Fluorimetric estimations were made using a Farrand spectrophotofluorimeter, measurements being taken from the activation scan recordings at the appropriate maxima for activation and fluorescence, in comparison with those of standard solutions of authentic compounds, using internal standards when appropriate.

Collection of Blood Samples

Venous samples. Each venous blood sample, withdrawn into a disposable sterile plastic syringe, was immediately transferred to the bottom of an all polythene stoppered tube containing heparin (approximately 40 iu/ml blood). The tubes containing blood samples were carried from ward to laboratory on a layer of ice in a vacuum flask. The blood was stored at -15 C until the estimations were carried out.

Arterial samples. These were collected, using siliconized syringes, by percutaneous puncture of the femoral artery.
Cardiac catheter samples. These were collected by plastic syringes from the cardiac catheter.

**Estimations**

(a) Estimations of indolic compounds in whole blood using solvent extraction techniques. All solvent extraction procedures were carried out on blood extract obtained by protein precipitation by zinc sulphate and sodium hydroxide of samples of whole blood previously hemolyzed by freezing and thawing. 5-Hydroxytryptamine was estimated in a portion of deproteinized blood by butanol extraction, followed by fluorimetric assay; tryptamine by the method for tissue extracts described by Hess and Udenfriend; and 5-hydroxyindol-3-ylacetic acid by the method of Udenfriend et al.

(b) Estimation of 5-hydroxyindolyl compounds in cerebrospinal fluid. 5-Hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid were estimated in the manner outlined for their determination in blood samples.

(c) Bioassay of 5-hydroxytryptamine in cerebrospinal fluid. Assays were performed on the isolated rat uterus using suitable dilutions of the cerebrospinal fluid in 0.9% (w/v) sodium chloride. Inhibition by 2-bromolysergic acid diethylamide of the uterine contraction induced by the cerebrospinal fluid was used as a criterion of identity of the active substance with 5-hydroxytryptamine.

(d) Estimation of indole substances in urine. 5-Hydroxyindol-3-ylacetic acid was estimated by the method of Udenfriend et al., indol-3-ylacetic acid by the method of Weissbach et al., and tryptamine by the method of Hess and Udenfriend. 5-Hydroxytryptamine was estimated by the method of Oates.

**Qualitative Chromatography of Extracts of Carcinoid Blood**

Preparation of blood extract for chromatography. 5 ml of blood were hemolyzed by freezing and thawing and gassed with carbon monoxide to diminish the destruction of indolic substances during the subsequent protein precipitation by the addition of 45 ml of acetone. After intermittent shaking for 1 hr at 4°C, the mixture was centrifuged and the protein precipitate reextracted in a similar manner with 25 ml 90% (v/v) acetone. The combined supernatant fluids were evaporated to 3 to 4 ml and shaken with 25 ml light petroleum (bp 40 to 60°C) to remove lipid material. The aqueous phase was taken to damp dryness under reduced pressure. The residue was extracted three times with 0.5 ml 80% (v/v) acetone and, after centrifugation to remove insoluble material, these combined acetone extracts were evaporated to dryness. The residue was dissolved in 0.5 ml 75% (v/v) ethanol and, for chromatography, portions of the solution were applied to paper, the solvent being evaporated in a continuous stream of nitrogen to minimize oxidation.

**Paper chromatography.** The blood extracts were examined by one-dimensional ascending (Whatman No. 1) chromatography in a nitrogen atmosphere, using as solvents: (1) butan-1-ol:acetic acid:water (12:3:5 by vol); (2) propan-2-ol:aqueous ammonia (sp gr 0.88): water (20:1:2 by vol); (3) 20% (w/v) potassium chloride.

The identification of the indole substances in the extracts was found to be facilitated by the following technique. Partial separation of the constituents of an extract was obtained on a preliminary ascending chromatogram developed with the butanol-acetic acid solvent, or with distilled water. After drying in nitrogen, the developed chromatogram was divided horizontally into strips, the position of the cuts being determined by the location of the regions in which indole compounds were visualized on a narrow vertical strip of the chromatogram by treatment with Ehrlich's reagent (see below). The paper segments were eluted with 0.01 N HCl and each eluate was evaporated to dryness under reduced pressure after pH adjustment to about 4 (bromphenol blue indicator paper) with solid sodium bicarbonate. Each residue was leached with 0.5 ml 75% (v/v) ethanol and portions of the solution were chromatographed in each of the three solvent systems referred to above. Since each eluate contained, at the most, only two indole substances, these were more readily identifiable by their chromatographic behavior in the three solvent systems than in one-dimensional chromatograms of the more complex original extracts.

Cerebrospinal fluid extracts were examined by two-dimensional chromatography, using solvent 1, followed by solvent 2.

**Identification of Indolic Compounds in Blood and Cerebrospinal Fluid Extracts**

The identification of a particular indole substance in a blood and C.S.F. extract was deduced from its position on the chromatograms developed in the various solvent systems and from its behavior in the tests referred to below, comparison being made with the position and
behavior of a specimen of the pure indole chromatographed in parallel.

The principal test used for the detection and identification of the indoles on the paper chromatograms was Ehrlich's p-dimethy lamino-aldehyd reagent. Indole substances carrying a 5-OR (R = H, alkyl or aryl) group were further differentiated by their greenish-yellow, or pinkish-yellow fluorescence in ultraviolet light, after spraying a chromatogram with 3% HCl. The a-nitroso-β-naphthol reaction was used on occasion as a test for indoles bearing a 5-hydroxy group. The ninhydrin reaction provided an additional confirmatory test for 5-hydroxytryptophan when other indications of its presence were obtained.

Identification of Tryptamine in Urine

A 24-hr specimen of urine from patient 1 was collected directly into a polythene bottle containing 4 ml of 2N sulphuric acid. Benzene extracts from a 70-ml portion of the urine from patient 1 and from a like volume of urine from a control subject were prepared by the method used for tryptamine estimations described by Sjoerdsma et al. Benzene soluble basic substances were returned to aqueous medium by extraction with 0.1N sulphuric acid. This acid extract was divided into three portions.

The first portion was assayed for tryptamine by fluorimetry in pH 10 buffer. The second was assayed for tryptamine by fluorimetry of the norharman prepared from tryptamine by reaction with formaldehyde and hydrogen peroxide. The third portion of the urine extract was adjusted to pH 11 with NaOH (20 w/v) and re-extracted with benzene. Acetic acid (0.5 ml) was added to the benzene phase and the extract evaporated under reduced pressure to damp dryness. The residue was dissolved in methanol (80% v/v) and portions of the solution subjected to one-dimensional paper chromatography in each of the solvent systems 1, 2, and 3, described above, and in a fourth solvent system—aqueous sodium chloride 8%:acetic acid (100:1 by vol). Comparison was made with the extract from the control urine with authentic tryptamine and with authentic tryptamine added to the urine extracts.

Arteriovenous Difference in the Concentration of Indolic Compounds

In patient 2, blood was obtained from the pulmonary artery, brachial artery, and antecubital vein during a cardiac catheterization study, and repeat samples were obtained from the first two sites, after epinephrine infusion. Samples were also obtained on another occasion from femoral artery and femoral vein of both patients and examined by the qualitative and quantitative methods described above.

Results

(1) Qualitative Paper Chromatography

Patient 1. The examination of blood samples from patient 1 indicated the presence of 5-hydroxytryptophan, 5-hydroxytryptamine, tryptophan, tryptamine, and indol-3-ylacetic acid. No 5-hydroxyindol-3-ylacetic acid was detected by chromatography in any of the blood samples.

Patient 2. In another patient, indollic substances, apart from 5-hydroxytryptamine and tryptophan, were detected in a blood sample from the femoral artery, while blood from the femoral vein contained, in addition, indol-3-ylacetic acid. A sample of venous blood obtained 9 months later also contained 5-hydroxytryptophan.

(2) Arteriovenous Studies

In patient 2, blood was obtained from the pulmonary artery, brachial artery, and antecubital vein during a cardiac catheterization study, and repeat samples were obtained from the first two sites after epinephrine infusion. In these studies, no gradient in 5-hydroxytryptamine levels was noted from pulmonary to brachial artery (12 μg/ml in both blood samples), but after the infusion of 10 μg epinephrine given over a period of 10 min there was an increased concentration of 5-hydroxytryptamine in samples from both of these sites (pulmonary, 21 μg/ml; bronchial, 19 μg/ml). In the single antecubital vein sample taken before the epinephrine infusion, the 5-hydroxytryptamine level was 8 μg/ml. There was thus apparently a fall in 5-hydroxytryptamine concentration from peripheral artery to vein and this finding was studied further in both patients.

To this end, samples were obtained from femoral artery and femoral vein and examined by the qualitative and quantitative methods described above. In both patients, there was a marked fall in the concentration of 5-hydroxytryptamine from artery to vein.
to vein. The 5-hydroxytryptamine concentration in the arterial samples were 100 μg/ml (patient 1) and 29 μg/ml (patient 2) and in the venous samples, 15 μg/ml (patient 1) and 5 μg/ml (patient 2). Estimation of 5-hydroxyindol-3-ylacetic acid in the blood samples showed no change in the concentration of the acid from artery to vein; 0.4 μg/ml in both samples from patient 1 and 0.06 μg/ml in both samples from patient 2. In contrast, however, visual inspection of chromatograms sprayed with α-nitroso-β-naphthol reagent showed there was a marked increase in the concentration of indol-3-ylacetic acid on passage from artery to vein in both patients.

(3) Venous Blood 5-Hydroxyindole Concentrations

The venous blood levels of 5-hydroxyindoles in these patients are considerably higher than any previously reported. The concentrations of total 5-hydroxyindoles in patient 1 varied between 2 and 130 μg/ml—nine estimates over a period of 5 years; and in patient 2 between 5 and 11 μg/ml—four estimates over a period of 4 months.

(4) Tryptamine Metabolism

Patient 1. This patient was found to be excreting large amounts of tryptamine (84 mg in one 24-hr sample of urine), as previously reported by Eccleston et al. This is about 1.000 times the normal level of 0.036 to 0.12 μg/day. The concentration in a venous blood sample at this time was found to 0.12 μg/day. The concentration in a tamine in the blood being confirmed by paper chromatography. No tryptamine was found in the blood of two control subjects and Rodnight states that under normal conditions it is not detectable.

The urinary excretion of indol-3-ylacetic acid was found to be 12 mg/24 hr and, after hydrolysis in N HCl, the estimate increased to 15 mg/24 hr. Weissbach et al. give the normal excretion as free, 3.1 to 8.1 mg/24 hr; total, after acid hydrolysis, 5.2 to 13.8 mg/24 hr. They also quote figures for the excretion of indol-3-ylacetic acid in two patients with carcinoid tumor. Both showed slightly raised levels: free, 7.0 and 16.4 mg/24 hr, and total, 14.4 and 21.9 mg/24 hr after acid hydrolysis.

One year later, patient 1 was found to be still excreting large amounts of tryptamine, 57 mg/24 hr. The urinary excretion of other indoles during this 24-hr period was indol-3-ylacetic acid, 0.5 mg increasing to 4.2 mg following acid hydrolysis; 5-hydroxytryptamine, 16 mg; 5-hydroxyindol-3-ylacetic acid, 127 mg.

This persistently high tryptamine excretion should be contrasted with the levels seen in eight patients with carcinoid tumor examined by Levine and Sjoerdema. In three the excretion of tryptamine fell within the normal range and in five it was below the normal range.

The identity of the apparent tryptamine in extracts from the urine of patient 1 was deduced from the following observations. The fluorescence and activation spectra of the apparent tryptamine at pH 10 and of the product from the norharman reaction were identical with those of authentic tryptamine. The estimates of the apparent tryptamine, using these two fluorescent methods, were in good agreement, 61 μg/ml urine by fluorimetry at pH 10 and 57 μg/ml urine by fluorimetry of the norharman product.

The behavior of the apparent tryptamine in the extract from the carcinoid urine was the same as authentic tryptamine on paper chromatography in four solvent systems. The chromatograms are reproduced in figure 1.

It seemed possible that venous blood levels of tryptamine might be showing considerable variation during the course of a day, and four further venous blood samples, taken at hourly intervals in the same day, were obtained to investigate this question. The results (fig. 2) show the variability in the concentration of both tryptamine and 5-hydroxyindoles in the different samples. There was no obvious relationship between the levels found and the presence or absence of carcinoid flush.

Patient 2. In a single 24-hr sample of urine from patient 2, we found the tryptamine excretion to be within normal limits.
Fig. 1. Paper chromatograms of benzene extracts of urine from patient 1 and normal control. Comparison is made with authentic tryptamine and 5-hydroxytryptamine in four solvent systems. Fig. 1a. Butan-1-ol:acetic acid:water (12:3:5 by vol). Fig. 1b. Propan-2-ol: aqueous ammonia (S.G. 0.88):water (20:1:2 by vol). Fig. 1c. 20% (w/v) potassium chloride. Fig. 1d. 8% (w/v) sodium chloride:acetic acid (100:1 by vol).
(5) Estimation of 5-Hydroxyindolyl Compounds in Cerebrospinal Fluid

Samples of cerebrospinal fluid were obtained from the lumbar region and were free from visible blood contamination.

There was good agreement between the estimates for 5-hydroxytryptamine obtained by fluorimetric and biological assay. In a sample of cerebrospinal fluid from patient 1 the 5-hydroxytryptamine concentration was estimated by fluorimetry to be 20 µg/ml and by bioassay, 17 µg/ml. In a sample from patient 2 the estimates were 16 µg/ml and 15 µg/ml, respectively. 5-Hydroxytryptophan was detected in the cerebrospinal fluid from patient 1, and the concentration, estimated semiquantitatively from the chromatograms, was approximately 50 µg/ml. 5-Hydroxyindol-3-ylacetic acid was present in considerably smaller amount, 0.3 µg/ml (patient 1) and 0.04 µg/ml (patient 2).

Discussion

5-Hydroxytryptophan was detected in the blood of patient 1 on the three occasions it was examined. In patient 2, although not present initially, this amino acid was found in a sample taken 9 months later. The blood levels of 5-hydroxytryptamine show a marked fall from peripheral artery to vein in both cases without a concomitant rise in the acid metabolite, 5-hydroxyindol-3-ylacetic acid. Removal of 5-hydroxytryptamine from blood by peripheral tissues could not in this case be by oxidative deamination. An alternative route of metabolism for 5-hydroxytryptamine could account for this finding, although no other indolic substance which could be a metabolite was identified in the blood on qualitative chromatography.

Tryptamine was detected in the venous blood and in high concentration in the urine of one of the patients (case 1). It is not known whether it was secreted by the tumor, or was tryptamine formed elsewhere, the metabolism of which had been altered by the high levels of 5-hydroxyindoles.

A possible explanation of the changes observed in both cases in the concentrations of indolic compounds during the passage of the blood through a limb might be the existence of tissue binding sites for 5-hydroxytryptamine and tryptamine. The sudden release of large quantities of 5-hydroxytryptamine from a tumor into the blood stream might be followed by rapid uptake onto tissue binding sites. If tryptamine was already present at some of these sites, it might be displaced and the events reflected in venous blood by an increase in tryptamine or indol-3-ylactic acid concentration, or both. This would presumably be an intermittent phenomenon, with release of 5-hydroxytryptamine from the sites during periods of low secretion from the tumor. An exchange mechanism in the periphery, if producing the release of biologically active compounds, might explain the observation that 5-hydroxytryptamine levels in venous blood so often do not relate to the severity of the symptoms. Some support for this hypothesis is provided by the work of Axelrod and Inscoe, who have obtained evidence for the presence of tissue binding sites for 5-hydroxytryptamine in the mouse. These may resemble the uptake sites for 5-hydroxytryptamine on platelets investigated by Stacey who showed that competition for uptake onto them may take place between tryptamine and 5-
hydroxytryptamine. Evidence for the peripheral uptake of the two amines in man has been demonstrated by Ashcroft et al. (Changes in amine metabolism produced by antidepressant drugs. Unpublished) following their intravenous infusion.

5-Hydroxyindol-3-ylacetic acid showed a high renal clearance. In contrast, the urinary excretion of indol-3-ylacetic acid was not unduly high in spite of the raised blood levels. This could be accounted for either by further metabolism of indol-3-ylacetic acid prior to excretion or to a low renal clearance which, in the case of this acid, is known to be influenced by urinary pH.¹⁰

Unlike the cases reported by Sjoerdsma et al.³¹ both our patients had a high concentration of 5-hydroxytryptamine in the cerebrospinal fluid; 5-hydroxytryptophan was present in the blood and may have given rise to intracerebral 5-hydroxytryptamine after passage through the blood/brain barrier. 5-Hydroxytryptamine is thought³² to pass through this barrier with difficulty.

Summary

In the peripheral blood of two patients with the carcinoid syndrome, numerous indoles in abnormally high concentration were detected. These were 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindol-3-ylacetic acid, and indol-3-ylacetic acid. In one of the cases, tryptamine also was detected in the blood. This patient excreted unusually large amounts of this amine in the urine, a finding which we believe has not been reported hitherto in the carcinoid syndrome.

In both patients, there was a marked fall in the blood 5-hydroxytryptamine concentration on passage from peripheral artery to vein, while the indol-3-ylacetic acid concentration showed a rise. It is suggested that these arteriovenous differences may be a result of an uptake of 5-hydroxytryptamine onto binding sites in the tissues, possibly with competitive displacement of tryptamine already present, which is then partly or wholly metabolized to indol-3-ylacetic acid.

5-Hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid were present in relatively high concentration in samples of cerebrospinal fluid from both patients. 5-Hydroxytryptophan was detected in the cerebrospinal fluid of one patient.

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REFERENCES

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The remarkable potential of carcinomas of the bronchus to produce endocrine disorders, such as Cushing's syndrome (Brown, 1928), hyponatraemia (Winkler and Crankshaw, 1938), gynaecomastia (Hardy, 1960), and hypercalcaemia (Connor et al., 1956), is now well recognized. The most recent addition to this group of disorders is the carcinoid syndrome, and this is known to be associated with 5-hydroxytryptamine (5-H.T.) secretion (Williams and Azzopardi, 1960) and also with 5-hydroxytryptophan (5-H.T.P.) secretion (Gowenlock et al., 1964). However, 5-H.T. and its metabolites are probably not the direct mediators of carcinoid flushes, and the release of a kinin peptide from the tumour has recently been suggested as responsible (Oates et al., 1964).

We recently had the opportunity to study 5-H.T. metabolism and kinins in a patient with many features of the carcinoid syndrome who was ultimately shown to have an oat-cell carcinoma of the bronchus. As this association has only rarely been recognized and our ignorance of the mechanism responsible for the flushing in the carcinoid syndrome continues, it was thought that our investigations of a single patient would be of sufficient interest to justify a case report.

Case Report

A bus-conductor aged 43 was admitted to hospital with a history of upper abdominal discomfort, nausea, anorexia, and loss of weight for the previous three months; during the month preceding admission he had had intermittent aching pain in the right side of his chest spreading into his shoulder and neck. He had smoked 30
cigarettes a day for many years and had a mild “smoker’s cough.” For a week or two prior to admission his face had been puffy and rather highly coloured; during this period he had collapsed on one occasion on the floor after defaecation, and had then vomited.

Examination showed redness of his face and neck of variable intensity, becoming paler at times, but never clearing completely or extending to the trunk or limbs; there was puffiness around the eyes and fullness of the face, not unlike Cushing’s syndrome; and moderate sacral and ankle oedema was present. His liver was palpable 2 in. (5 cm.) below the subcostal margin and was hard and irregular. Marked hyperperistalsis was audible on direct auscultation; but there was no diarrhoea until shortly before death; barium enema and barium-meal examination revealed no abnormality. No abnormal signs were detected on clinical examination of the chest, but radiography showed an opacity in the right hilum with elevation of the diaphragm.

Blood count, erythrocyte sedimentation rate, and electrolytes were normal, although hypokalaemia (lowest serum potassium 2.4 mEq/l.) developed a month after admission, necessitating potassium supplements. Urinary 17-ketosteroids were 5–6 mg./day and 17-hydroxycorticosteroids were 17–18 mg./day on two estimations. Liver-function studies showed that the serum alkaline phosphatase was raised to 22 King-Armstrong units and the serum glutamic pyruvic transaminase to 123 units. Liver biopsy showed tumour cells as described below.

The patient’s condition steadily deteriorated, and he died two months after admission.

Necropsy Findings

A small tumour nodule, 2.5 cm. in diameter, of pale grey colour with a dark pink mottling, was ulcerating the wall of the right upper lobe apical bronchus and was infiltrating the surrounding lung, but encroaching little on the bronchial lumen. Metastases were seen in the hilar lymph nodes, in the paratracheal region, and in the anterior mediastinum. Both lungs were congested. There was no evidence of tumour in the intestinal tract, but scarring in the lesser curve of the stomach suggested a healed ulcer. The colon showed marked oedema of the mucosa. Two small tumour nodules were also present in the body and the tail of the pancreas. The liver was grossly enlarged (3,400 g.) and distorted by nodules of tumour. Tumour nodules were also found in the third lumbar vertebral body, in both kidneys, and in the thyroid gland. Histological examination showed the tumour in the lung to be an oat-cell carcinoma arising from a small bronchus, with sheets of small round, oval, or spindle-shaped cells with hyperchromatic nuclei; in some areas, particularly secondary deposits, there was better differentiation, and in others the cells were all of spindle form. Mitotic figures were frequent and a number of giant cells were present. There was considerable haemorrhage and necrosis. The histological features corresponded closely with those described by Azzopardi (1959).

The heart valves and endocardium were normal. The adrenal glands were enlarged (15 g. each) and a small haemorrhagic area was present in the right adrenal cortex. Both adrenal cortices were
hyperplastic and consisted almost entirely of compact zona fasciculata cells with only a few atrophied glomerulosa layer cells; microscopic deposits of tumour were present. The renal tubules showed no changes of hypokalaemic nephropathy.

Argentaffin and diazo staining of tumour tissue removed from the liver within half an hour of death was negative. Argyrophil staining, by a modified Bodian’s method (Azzopardi and Pollock, 1963), of bronchial, liver, and pancreatic tumour tissue was negative. The sensitivity of the staining method was checked by positive and negative controls. Unstained sections of the same specimens mounted in Univert failed to show auto-fluorescence in ultra-violet light.

Biochemical Investigations

Methods.—Ten 24-hour urine collections were made before and during treatment and the indolic compounds were determined in aliquots. 5-Hydroxyindole acetic acid (5-H.I.A.A.) was determined by the method of Udenfriend et al. (1955), indole acetic acid (free I.A.A.) by the method of Weissbach et al. (1955), 5-H.T. by the method of Oates (1961), and tryptamine by the method of Hess and Udenfriend (1959). Total 5-hydroxyindoles were measured in whole blood by the method of Ashcroft et al. (1964), and 5-H.T.P. and 5-H.I.A.A. in blood and tumour by the method of Ashcroft et al. (1965).

The kinin content of heparinized venous whole blood was estimated by a modification (J. Z.) of the methods used by Brocklehurst and Lahiri (1962) and by Gaddum and Horton (1959).

Results

The results of the estimation of indolic compounds in the urine and the response to treatment are summarized in the Table.

The 5-H.I. level in whole venous blood was raised to 415 mμg./ml. (normal range 60–120 mμg./ml.) when the flushing was mild, and to 820 mμg./ml. when intense. The 5-H.I.A.A. was 65 mμg./ml. (normal value nil). No 5-H.T.P. was detected. These estimations were made prior to treatment. 5-H.T. was found in a concentration of 1.4 μg./g. in the liver metastasis, but no 5-H.T.P. or 5-H.I.A.A. was detected. The lung and pancreas tumour tissue examined contained no 5-H.T., 5-H.T.P., or 5-H.I.A.A.

The only biochemical alteration demonstrated with treatment was a fall in the urinary 5-H.T. excretion on the administration of chlorpromazine. The apparent fall in the 5-H.I.A.A. is an artifact due to interference with the colour reaction by chlorpromazine.

The kinin content of venous whole blood before drug therapy was 25 mμg./ml. equivalent of standard bradykinin (normal less than 0.4 mμg./ml.). The P.C.V. was 42%, making the kinin content of the plasma 43.1 mμg./ml. equivalent of bradykinin (normal less than 0.7 mμg./ml.). Control experiments showed that the activity measured was bradykinin-like and could not have been due to 5-H.T. or histamine or substance P as has been suggested (Lancet, 1964).
### Response of Urinary 5-H.T., Tryptamine, 5-H.I.A.A., and I.A.A. to Treatment. (Normal Range in Parentheses)

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<th>Therapy</th>
<th>24-hour Urine</th>
<th>Clinical Features</th>
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<td>5-H.T.</td>
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</table>

### Effects of Therapy

An attempt was made to alleviate the patient's symptoms by short courses of potentially effective drugs, and the response of the clinical signs and changes in the excretion of the urinary metabolites of tryptophan were observed.

Although its exact mode of action is unknown, chlorpromazine is said to reduce 5-H.T. turnover in the brain (Gey and Pletscher, 1962), but this may be due to the hypothermia induced (Bartlet, 1965). An anti-bradykinin effect is also described (Spector and Willoughby, 1963a), and in addition its well-known antiemetic and tranquilizing effect might have been expected to provide some relief. In this patient the nausea and abdominal discomfort were lessened, but there was no change in the facial appearance and the flush. The fall in 5-H.T. excretion was probably a genuine response to the drug, but needs further confirmation in other cases. As mentioned above, the fall in 5-H.I.A.A. is an artifact.

Methysergide (Deseril), a potent inhibitor of 5-H.T. (Doepfner and Cerletti, 1958), showed no effect clinically or chemically.

Oates et al. (1964) have shown that bradykinin, a kinin peptide formed by the proteolytic action of kallikrein on plasma α2-globulin,
could induce the characteristic flush in carcinoid patients. Trasylol, an inhibitor of kallikrein and other proteolytic enzymes, was discovered by Frey et al. (1950). It was used by Frey (1953–4) in the treatment of acute pancreatitis on account of its trypsin-inactivating effect, and has been used extensively since then with promising results (Maurer, 1961). It seemed reasonable to see if inhibition of kallikrein and hence bradykinin formation would produce any beneficial effect. No clinical improvement occurred, however.

Homochlorcyclizine, which has been shown to have an antihistamine and antiserotonin action, and inhibits slow release substance in animals (Kimura et al., 1960), had no clinical effect; nor had aspirin, which also has an inhibitory effect on kinins and slow release substance in animals (Collier, 1963; Spector and Willoughby, 1963b).

Discussion

The facial flush, oedema, and hyperperistalsis together suggested the diagnosis of the carcinoid syndrome, and this was confirmed by the raised blood levels of 5-H.T. and kinins, and the increased urinary excretion of 5-H.T. and 5-H.I.A.A. Radiological examination of the chest suggested that the primary tumour might be in the lung, and at necropsy an oat-cell carcinoma was found in the right upper lobe apical bronchus, and this was thought to be the primary lesion; there were multiple secondary deposits in the liver. 5-H.T. was found in small amounts in the liver metastases only.

Despite the presence of tumour tissue in the liver containing 5-H.T. and the relatively high blood levels of 5-H.T., there were no fibrotic lesions in the heart or other organs, as have been described in the carcinoid syndrome, and which have recently been reviewed by Hallén (1964). It may be that the rapid downhill course of this patient did not permit time for the deposition of the characteristic fibrous plaques on the heart and great vessels.

The hypokalaemia noted, together with the hyperplasia of the adrenal cortex, suggested the possibility that the tumour was producing a substance with corticotrophin-like activity, as described by Holub and Katz (1961) and by Meador et al. (1962). Apart from the facies and hypokalaemia, however, there are no grounds for postulating persistent adrenocortical overactivity.

Treatment with a series of drugs had no clinical effect and only slight biochemical changes were noted with one drug, chlorpromazine; no attempt was made to detect alterations in blood kinin levels. It must be concluded that 5-H.T. and kinin antagonists in the dosage given were of no value in this patient, and their administration did not throw further light on the cause of the carcinoid flush, as might have been hoped.
Summary

The case of a patient with the carcinoid syndrome is described in which there was an associated oat-cell carcinoma of the bronchus.

Antagonists to 5-H.T. and kinin peptides were administered with no demonstrable effect, except some subjective improvement and a slight fall in the 5-H.T. urinary excretion during chlorpromazine administration.

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References


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