STUDIES OF THE CONCENTRATIONS OF

ACTIVE METABOLITES OF TRYPTOPHAN

Thesis

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by

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Some of the results described in this Thesis were presented as follows:

1. A preliminary communication before the British Pharmacological Society in July 1963. (Ashcroft G.W., Eccleston D., and Crawford T.B.B.)

2. A preliminary communication before the British Pharmacological Society in July 1965. (Eccleston D., Ashcroft G.W., Crawford T.B.B. and Loose R.E.)

3. A preliminary communication before the British Pharmacological Society in January 1966. (Ashcroft G.W., Eccleston D., and O'Mahoney D.P.)

4. As papers:

   (a) Ashcroft G.W., Eccleston D., Crawford T.B.B. (1965) J. Neurochem. 12, 483.

   (b) Eccleston D., Ashcroft G.W., Crawford T.B.B. (1965) J. Neurochem. 12, 493

   (c) Eccleston D., Ashcroft G.W., Crawford T.B.B., Loose R.E. (1966) J. Neurochem. 13, 93.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

In 1952 in a report to the British Pharmacological Society Amin, Crawford and Gaddum described the detection of 5-hydroxytryptamine (5HT) from brain. Their subsequent paper (1954) reported the detailed distribution of the amine in various areas of the brain. These findings were subsequently confirmed by other groups of workers, Twarog and Page (1953), Paasonen and Vogt (1956), Bogdanski, Weissbach and Udenfriend (1956).

The presence in brain of 5HT and amines of the catecholamine series (Vogt 1954 and Carlsson 1959) has led to speculation as to the role of these substances in normal brain function. The finding that many psychotherapeutic agents alter the metabolism of the amines (Pletscher1963) has stimulated the interest of both pharmacologists and psychiatrists, who felt it was possible that disturbance of the metabolism of biogenic amines may be of etiological significance in psychiatric (Woolleyand Shaw 1954, Gaddum and Hameed 1954, Shaw and Woolley1956, Costa 1956) and neurological disorders (Bernheimer, Birkmeyer, Hornykiewicz 1963, Poirier and Sourkes 1965).

In consequence an enormous amount of experimental work has been done on the metabolism of these substances and/

and of the influence of drugs on their metabolism. In
this laboratory the work has continued using two
particular techniques to examine the synthesis and
breakdown of 5HT. Firstly experimental techniques have
been developed to estimate in brain the known metabolites
of tryptophan along the 5-hydroxyindole pathway (Fig. 1),
5-hydroxytryptophan (5HTP), 5HT and 5-hydroxyindole-3-
ylacetic acid (5HIAA). Estimation of these metabolites
as well as tryptophan in single brain samples give a more
dynamic picture of the formation and breakdown of the
amine. Secondly it has been shown previously (Hess and
Doepfner 1961) that administration of L-tryptophan to
rats caused an elevation in the concentration of 5HT in
the whole brain. A "loading dose" of the precursor
L-tryptophan was given to rats in the hope of increasing
the turnover of the amine in brain. Under these
conditions metabolites not normally detectable by the
available techniques might reach concentrations
sufficiently high to allow their estimation.

Hess, Redfield and Udenfriend (1959) and Hess and
Doepfner (1961) also demonstrated a rise in the amine
tryptamine in brain after administration of L-tryptophan.
In that tryptamine might also have a physiological role
an attempt was made to estimate the amine along with the
5HT metabolites on single brain samples.

The/

FIGURE 1
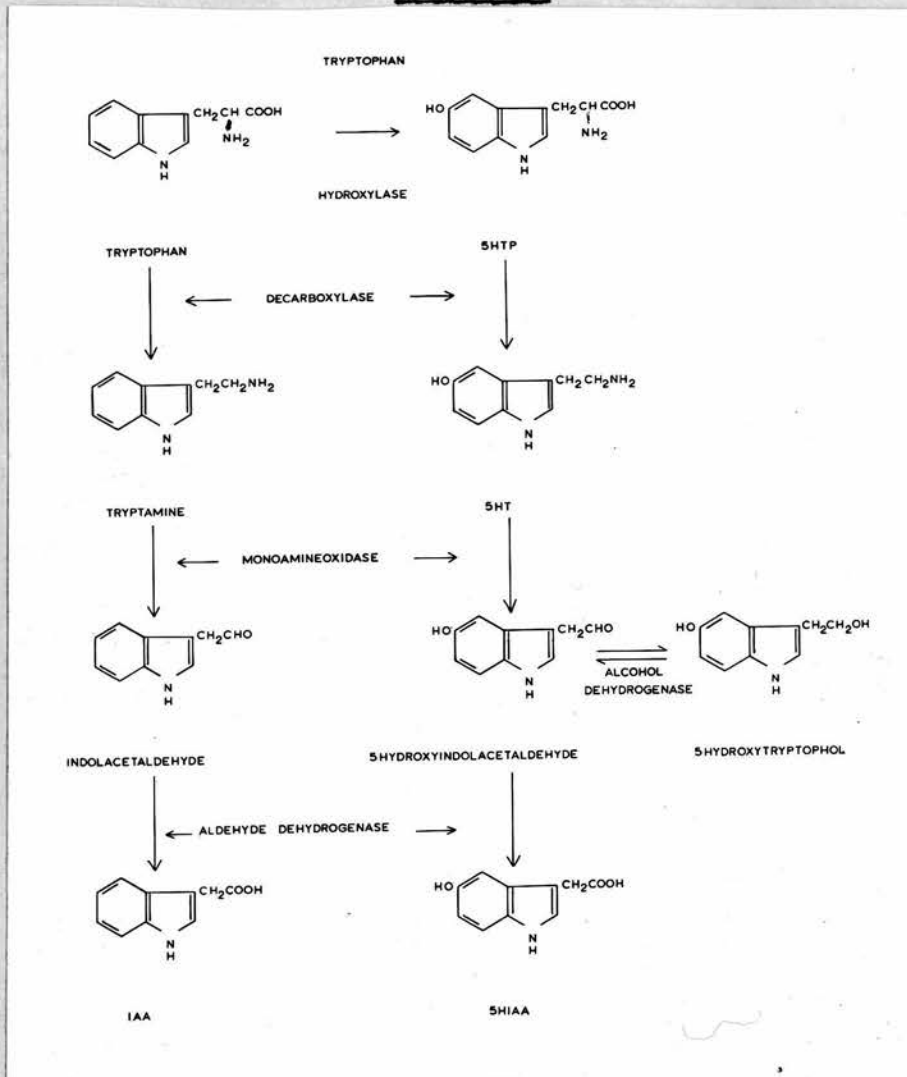
Some of the known metabolites of tryptophan along the tryptamine and 5-hydroxyindole pathway.

The thesis falls then into three sections:

The first section deals with the estimation of tryptamine in tissues in normal animals and in animals treated with L-tryptophan, with and without the amine oxidase inhibitor, "iproniazid". The results from the use of the method of Hess et al. (1959) are compared with those of paper chromatography and the discrepancies discussed. A modification of the technique of Hess et al. (1959) is described.

The second section describes a paper chromatographic technique for the estimation of 5-hydroxyindoles in brain tissue and its application to tryptophan loading in rats. The influence of drugs on the concentration of 5-hydroxyindole metabolites following loading with tryptophan is studied.

The third and final section describes experiments in which dogs are given oral tryptophan and the various areas of brain examined for their content of 5-hydroxy-indoles at varying time intervals after the administration of the amino acid.

SECTION 1

Tryptamine in tissues

INTRODUCTION

In 1922 Sullivan extracted a base which behaved like
tryptamine from the urine of patients suffering from
pellagra.   In 1956 Rodnight using ion exchange
chromatography of urine extracted$_\wedge$substance which on
subsequent paper chromatography and bioassay behaved like
authentic tryptamine.   A sensitive method for the de-
tection of tryptamine in urine was developed by Sjoerdsma,
Oates, Zaltsman and Udenfriend (1959).   The amine was
extracted into benzene from alkalinised urine and
estimated after back extraction into acid by spectro-
photofluorimetry.   The identity of the amine was con-
firmed by its fluorescent characteristics, counter current
distribution, and its behaviour in two solvent systems
on paper chromatography.   There was then, by 1959 strong
evidence to suggest that tryptamine was excreted in
urine although it was not known whether it originated
from the animals own cells or was produced by intestinal
bacteria.   Werle and Mennicken (1937) had however,
presented evidence for the production of the amine in
animal tissues.   In 1959 Weissbach, King, Sjoerdsma and
Udenfriend demonstrated the conversion of isotopically
labelled tryptophan to tryptamine by kidney homogenates.
The tryptamine was extracted from the tissues which had
been made alkaline into benzene and then back extracted
into/

into acid. The amine was identified by paper chromatography and also by its subsequent conversion by purified amine oxidase and aldehyde dehydrogenase to indol-3-ylacetic acid. Hess and Udenfriend (1959) described a solvent extraction method for the estimation of tryptamine in tissues. It was similar to that employed by Weissbach et al. (1959) consisting of an extraction into benzene from alkalinised homogenates with subsequent back extraction into acid. The amine was, however, converted into a more highly fluorescent compound and estimated by spectrophotofluorimetry. These workers were unable to detect tryptamine in tissues from normal animals. Hess, Redfield and Udenfriend (1959) found measurable quantities following the administration of either tryptophan or amine oxidase inhibitors. This remained the method employed by subsequent workers for the estimation of tryptamine (Green and Sawyer 1960, Hess and Doepfner 1961, Weber and Horita (1965).

In this laboratory during a study of the effects of tryptophan administration in rats on the concentration of 5-hydroxyindoles in brain using a paper chromatographic method we attempted to examine in parallel the levels of tryptamine in the extracts. Although for various reasons the method did not prove entirely satisfactory it was apparent that tryptamine could not be detected in the brains of normal rats, and that after administration of tryptophan/

tryptophan only trace[*] amounts (>0 <0.2 μg) of tryptamine were present.

It seemed then that there was a discrepancy between the results obtained by solvent extraction and those obtained by paper chromatography. In consequence the method of Hess and Udenfriend (1959) was modified by the addition of an ion exchange chromatographic separation of tryptophan from tryptamine prior to solvent extraction. The first section (Section A) then deals with the use of paper chromatography in the estimation of tryptamine.

The second section deals with the modified method of Hess and Udenfriend (1959) and its application to brain tissue (Section B). A comparison is made between the results obtained by the original and modified methods and a likely cause for the discrepancy between the results obtained by the two is demonstrated.

The discussion also, contains present views on the possible biogenesis of tryptamine in brain.

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[*] Trace amounts of tryptamine indicates that the activation spectrum of the sample after the norharman procedure, shows two peaks at 310 and 360 mμ, the intensity of which are not sufficiently high above the 'blank' fluorescence to make quantitative estimation meaningful.

METHODS

The Norharman Procedure

The conversion of tryptamine to norharman (Fig. 2) was originally described by Kermack, Perkin and Robinson (1921) using the Pictet-Spengler reaction (1911). Hess, Redfield and Udenfriend (1959) employed the procedure to convert tryptamine to norharman and to estimate the concentration of tryptamine indirectly by reading the fluorescence intensity of the norharman derived therefrom.

The procedure was briefly as follows. The de-proteinised tissue extract containing tryptamine was made up to 6 ml with 0.1 N H_2SO_4 in 15 ml glass tubes. To this solution was added 0.2 ml 18% (w/v) formaldehyde solution. After mixing, glass bubbles were placed on the tubes and these were heated with continuous shaking, in a boiling water bath for 20 min. Hydrogen peroxide (0.2 ml of 5 vol - 30 vol diluted 1 in 6) was added and the tubes allowed to remain for a further 20 min in the water bath. The tubes were then removed, stoppered and cooled. Vigorous shaking at this time liberated oxygen which otherwise tended to form bubbles in the cuvette and interfere with fluorimetric estimation. The fluorescence of the sample was measured in a Farrand or Aminco-Bowman recording/

FIGURE 2
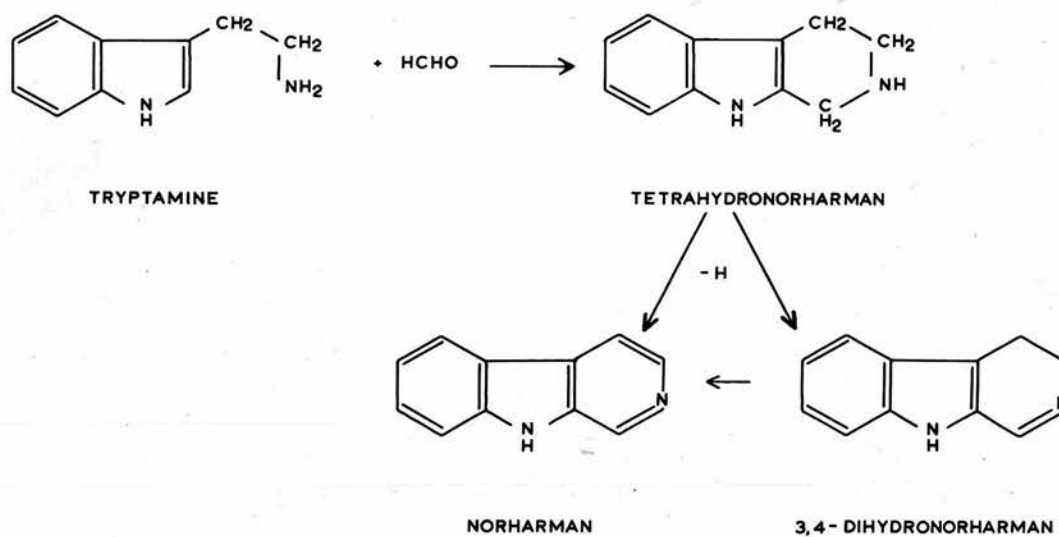
OVER - ALL REACTION FOR CONVERSION OF TRYPTAMINE TO NORHARMAN

Intermediate compounds in the conversion of tryptamine to norharman.

recording spectrophotofluorimeter with the fluorescence
monochromater set at 450 mµ (uncorrected). The activation
spectrum (Fig. 3) was recorded from 250 mµ to 400 mµ.
Activation maxima were obtained at 360 mµ and 310 mµ.
This gave some degree of specificity to the procedure.
The fluorescence intensity of the activation maximum
at 360 mµ was used to give a quantitative estimate to the
amount of tryptamine in the extract. In experiments with
tissues known amounts of tryptamine were taken through
the procedure to give estimates of recoveries. The blank
was obtained by taking the fluorescence at 360 mµ of
pure solutions of reagents taken through the procedure.

Specificity of procedure

It is to be noted that the condensation reaction can
occur with other indole-ethylamines, although highly
fluorescent compounds are not produced by the 5-
hydroxyindole derivatives. Tryptophan, when put through
the condensation reaction, yields a fluorphor with
identical fluorescence characteristics with those of
norharman. This fluorphor has also in paper chromato-
graphy, identical Rf values in two solvent systems
(n-butanol: acetic acid: water; 12:3:5; Rf 0.69 (5%;
ammonia Rf 0.15). In consequence the reaction was also
used to determine the concentration of tryptophan in
tissues./

FIGURE 3

ACTIVATION SCAN OF NORHARMAN

FLUORESCENCE AT 450 mμ.
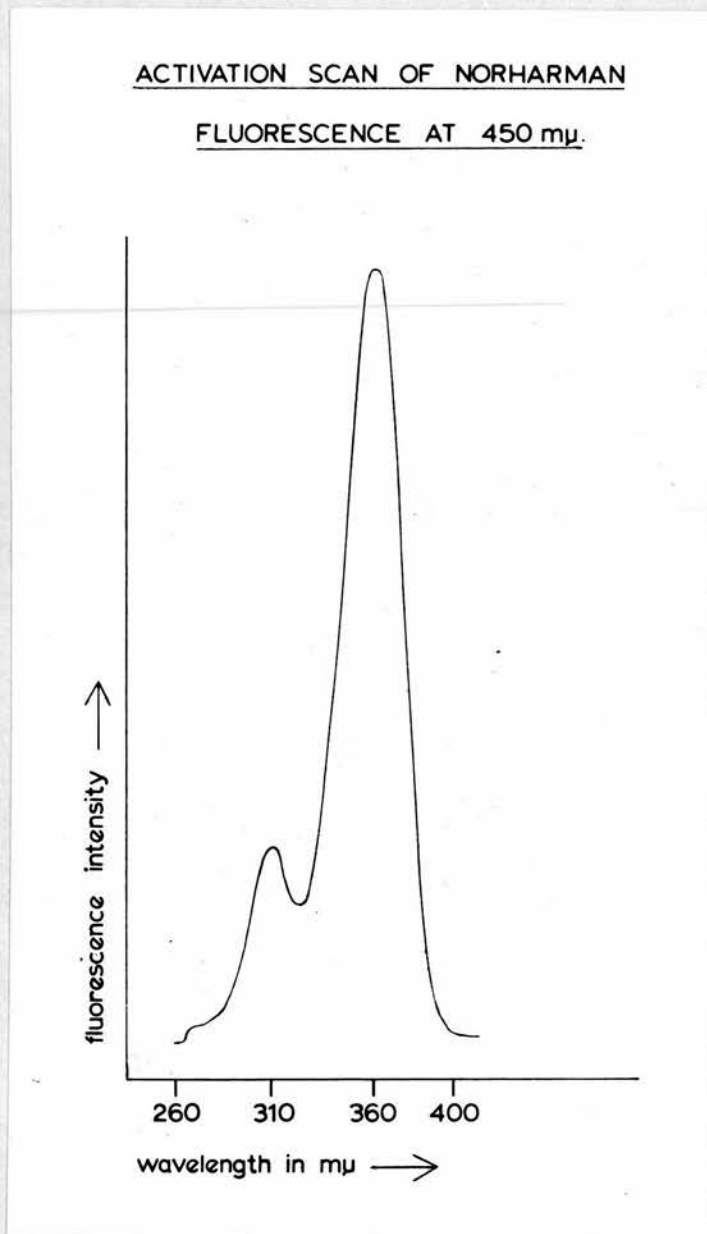
tissues.

Limits of sensitivity of norharman procedure

Quantities of tryptamine from 0.1 to 100 μg were treated by the norharman procedure as described above. The relationship between the quantity of amine and the fluorescence intensity of the norharman was found to be linear up to 10 μg. After this there was no increase in fluorescence with an increase in concentration of tryptamine (Fig. 4). Consequently when determining tryptamine or tryptophan content of tissues the final extract was diluted so that it would not contain more than 10 μg of these substances.

Application of the norharman procedure in the estimation of tryptophan in tissues.

The procedure is a modification of the method of Hess and Udenfriend (1959) as applied by Guroff and Udenfriend (1962).

(a) Brain and liver

Brain and liver homogenates were prepared as on p. 28 or on p. 57 depending on the subsequent method of estimation of tryptamine. Brain homogenate (0.5 ml) was made up to 5.5 ml with distilled deionised water in/

FIGURE 4

NORHARMAN   REACTION

CALIBRATION   CURVE

CONC. OF TRYPTAMINE IN μg. per sample
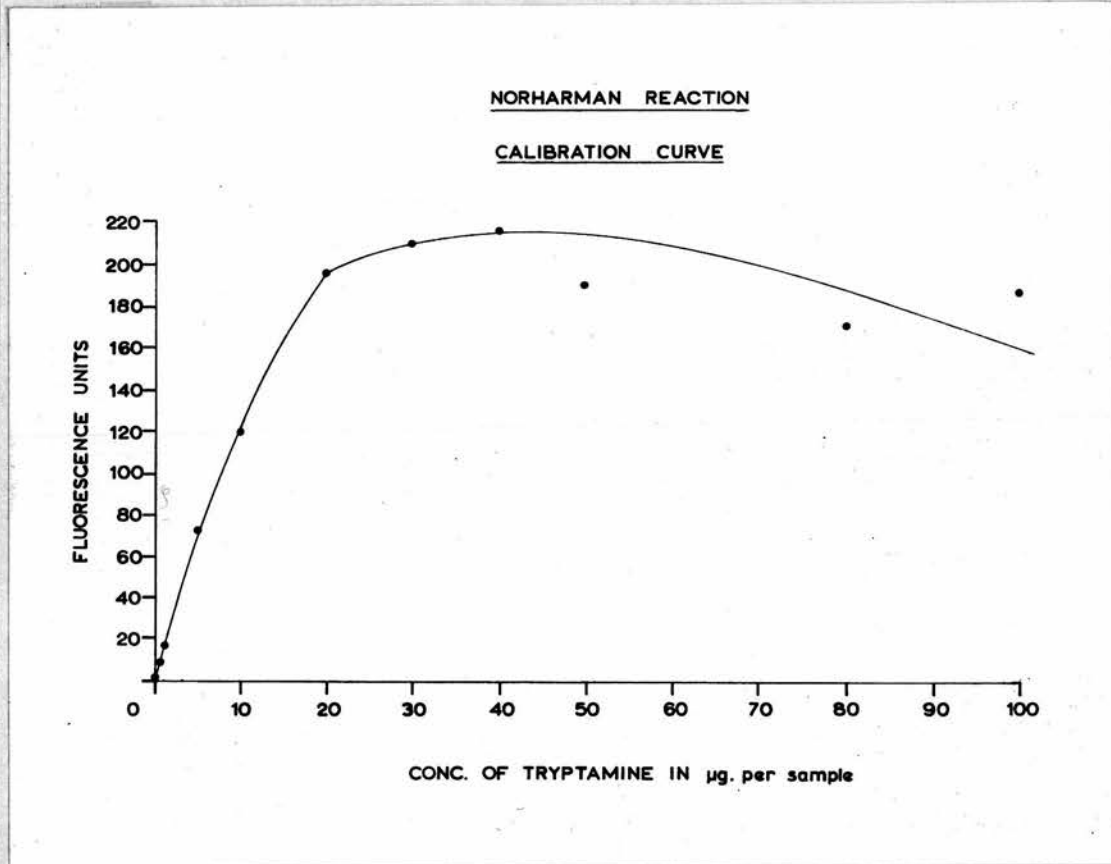
in 15 ml glass stoppered centrifuge tubes. After mixing
by gentle inversion the proteins were precipitated by
the dropwise addition of 1.0 ml 30% (w/v) trichloracetic
acid. The contents were mixed by gentle inversion and
allowed to stand for precisely 10 min. The precipitate
was centrifuged down at 2,500 r.p.m. for 10 min. Three
ml of the supernatant were pipetted into 15 ml stoppered
tubes and made up to 6 ml with 0.1 N H_2SO_4. The
quantity of supernatant from tissues expected to contain
a high tryptophan content (i.e. from tryptophan loaded
animals) was 0.5 ml. This was also made up to 6 ml with
0.1 N H_2SO_4. These extracts were subjected to the
norharman procedure as described above.

(b) Plasma and whole blood

The procedure was identical with that of brain except
that 0.1 ml of plasma or whole blood was pipetted into
5.4 ml distilled deionised water prior to precipitation
with trichloracetic acid.

Recoveries

Known quantities of tryptophan were added to the
tissue homogenate, plasma or whole blood when these had
been diluted with distilled water. A pure solution of
tryptophan (10 μg/ml in water) was prepared, 2μg (0.2 ml)
being added to homogenates or plasma from control animals
and/

and 10μg (1 ml) to homogenates or plasma from tryptophan loaded animals. The recoveries of these amounts of tryptophan which were taken through the entire procedure give a quantitative value to the estimates of tryptophan in the tissues examined.

SECTION A

Paper chromatographic separation in the estimation of tryptamine

METHOD

In Section II (p. 57)a method for the estimation of 5-hydroxyindoles in brain and plasma involving separation by paper chromatography is described. An attempt was made to develop the method in such a way thatthe tryptamine content could be measured in parallel in the same samples. The method for the estimation of tryptamine diverges only at the stage of elution from the paper chromatogram (p. 63). The paper chromatogram was divided into 1 cm strips and these eluted into 6 ml 0.1 N H_2SO_4. After standing for 15 min with occasional gentle inversion the paper strip was removed, the volume recorded and the eluate made up to 6 ml with 0.1 N H_2SO_4 the eluate was divided into two, 3 ml being used for 5-hydroxyindole estimation. The other 3 ml of the eluate was taken and made up to 6 ml in a 15 ml glass stoppered tube. This was then put through the norharman procedure. Quantities (400 mμg to 800 mμg) of tryptamine were taken through the procedure to determine the recoveries possible by the method.

Limitations of Method

It/

It was found that recoveries of known quantities of
tryptamine added to brain and taken through the procedure
were highly variable (15-70%). It was also difficult
with the solvent system employed(n-butanol: acetic acid:
water, 12:3:5) to achieve a complete separation of
tryptamine from the large quantities of tryptophan
extracted from the tissues of animals pretreated with this
amino acid.

Tryptophan when applied as a marker in tissue
extracts was found to have a greater Rf than when applied
in pure solution. Consequently no portion of the chroma-
togram between tryptophan and tryptamine was devoid of
norharman reacting material. A final disadvantage of
paper chromatography was its failure to separate tryptamine
(Rf 0.72 in n-butanol: acetic acid: water, 12:3:5) from
two substances which appear at Rf's 0.6 and 0.75 in this
solvent system. They were detected after the norharman
reaction because of the formation of fluorophors with
an activation maximum at 310 mμ when the fluorimeter
monochromator is set at 450 mμ, the emission maximum
for norharman. Thus these fluorophores obscured the
fluorescence characteristics of tryptamine when they are
eluted together with the amine from the paper and often
rendered estimation of tryptamine impossible. These
fluorophores were present in relatively large amounts in
chromatograms of extracts of dog plasma and rat brain.
They/

They may well be the fluorophors reported by Hess, Redfield and Udenfriend (1959) to be present in the benzene extracts from dog liver. Figure 5 shows that extracts of dog plasma contained amounts of these fluorophores which increased with time after tryptophan loading with a different rate of rise in each fluorophor. They may be derivatives of tryptophan on the kynurenine metabolic pathway which are present in increased amounts following tryptophan loading. (Michael, Drummond, Anderson and Good, 1964).

Application of Method and Results

Only three experiments for the estimation of trypta-mine by paper chromatography can be considered successful from the point of view of both absolute separation of tryptamine from tryptophan and a blank sufficiently low as not to obscure the characteristic norharman spectrum.

1. Treatment of rats with tryptophan alone

Wistar strain rats 180 g. were given a dose of tryptophan 800 mg/kg by intraperitoneal injection. The animals were killed by decapitation in groups of three at varying time intervals up to 8 hr afterwards and estimates made of brain tryptamine by the method described above.

Results

No tryptamine was detected in the brains of un-treated animals. 'Trace' amounts of tryptamine (>0, <0.2 μg/g) were detected in brains of rats 1 hr after the in-jection of tryptophan. Estimates at 30 min, 2, 4 and 8 hr/

FIGURE 5
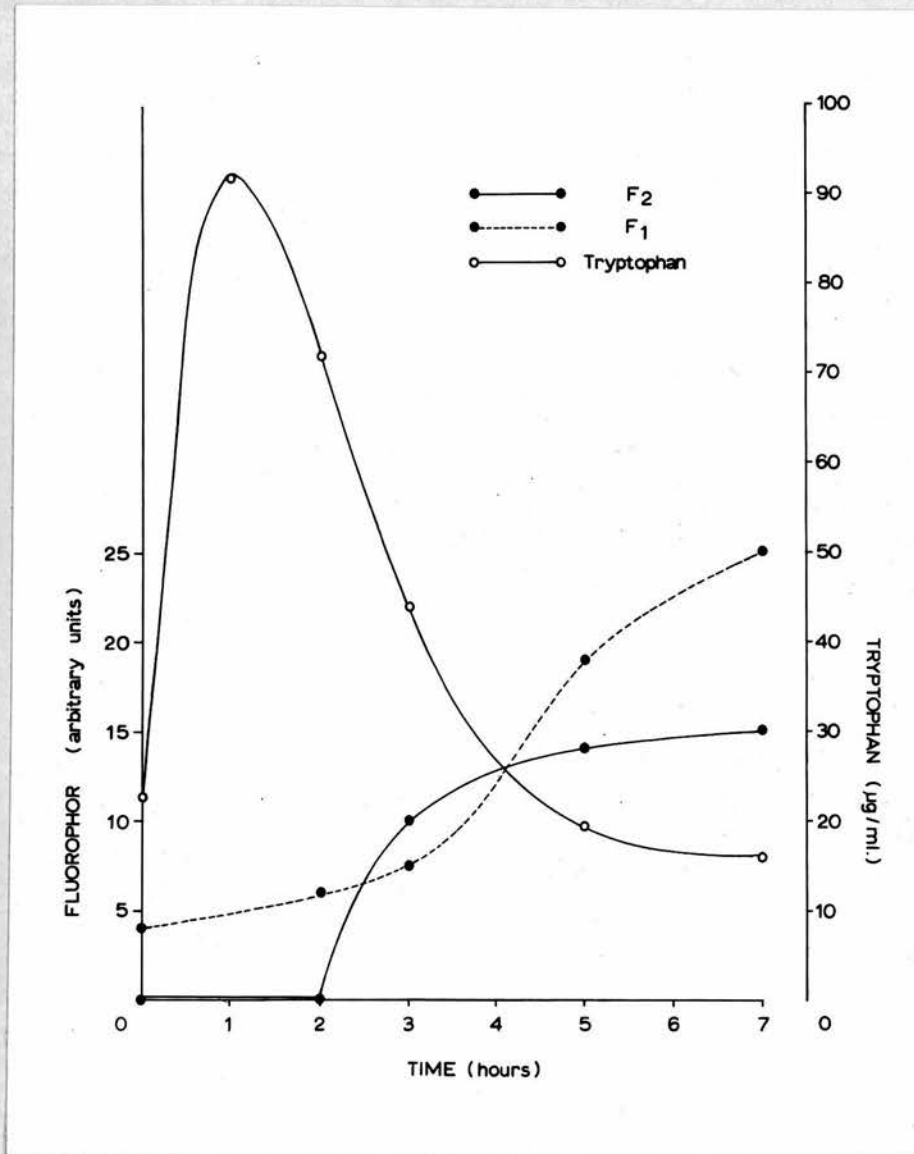
Fluorescence intensity of fluorophores appearing in
dog plasma at varying times after tryptophan loading.

hr were obscured by other fluorescent compounds running
to the Rf of tryptamine in the solvent system employed.

2.   Treatment of rats with tryptophan following
     pretreatment with a monoamine oxidase inhibitor.

Wistar strain rats, 180 g were injected intra-
muscularly with iproniazid phosphate in a dose of 5 mg/kg
for five days prior to an intraperitoneal dose of
tryptophan (800 mg/kg) as described on p. 56.   The animals
were killed by decapitation in groups of three and the pooled
brain examined for   tryptamine content.

   Results

There was no tryptamine detectable in the brains of
animals treated with iproniazid alone.   Following loading
with tryptophan, tryptamine appeared in 'trace' amounts
at 30 min (>0, <0.2 $\mu g/g$) and reached a peak con-
centration of 0.85 $\mu g/g$ at 1 hr (Table 1).   By 4 hr only
'trace' amounts of tryptamine could be detected, and at
8 hr none.   The sensitivity of the paper chromatography
procedure was low, the limit of detection being 0.2 $\mu g/g$
                      or
of tissue/per 2 ml plasma.

3.   Estimation of tryptamine in human plasma following
     tryptophan loading

A human subject was given a dose of 5 g. of tryptophan
after fasting for 12 hr.   Blood was taken off by vene-
puncture at 1, 2, 3 and 6 hr following the dose of
tryptophan/

TABLE 1

Concentration of tryptamine in rat brain as estimated by paper chromatography. The animals were pretreated with iproniazid 5 mg/kg for 5 days prior to an intraperitoneal injection of tryptophan, 800 mg/kg.

Treatment	Time after tryptophan administration	Tryptamine (μg/g)
Iproniazid	0	ND*
Iproniazid + tryptophan	30 min	$>0, <0.2$
	1 hr	0.85
	2 hr	0.48
	4 hr	$>0, <0.2$
	8 hr	ND

* ND = not detected (<0.2 μg/g)

tryptophan and placed in heparinised plastic tubes.   The
blood was centrifuged immediately and the plasma frozen
until assay by paper chromatography.

Results

The results (Table 2) show a rise in tryptamine in
plasma which is maximal at 1 hr.   Small but detectable
quantities of the amine in plasma are still present
6 hr after the ingestion of tryptophan.

TABLE 2

Concentration of tryptophan in
human plasma after an oral dose
of 5 g. of L-tryptophan.

Time after administration of tryptophan	Plasma tryptamine (μg/ml)
0	ND[*]
1 hr	0.33
2 hr	0.26
3 hr	0.26
6 hr	0.14

[*] ND = not detected. Less than 0.1 μg/ml

SECTION B

A modification of the method of Hess and
Udenfriend (1959) incorporating
ion exchange column chromatography

INTRODUCTION

Although the method involving separation by paper chromatography for the estimation of tryptamine in tissues had disadvantages the results obtained for the concentration of the amine in tissues of animals treated with L-tryptophan (800 mg/kg) with and without iproniazid (150 mg/kg) were lower than those reported by Hess et al. (1959) (Table 3 and 4). The possibility that tryptophan was a contaminant in the procedure of Hess and Udenfriend (1959) was considered and the method modified by the incorporation of an ion exchange column chromatography step for the separation of tryptophan from tryptamine prior to solvent extraction. The original method is quoted and the modified method and its application described.

METHODS

Animal treatment

The animals were killed by decapitation and the blood collected/

TABLE 3

Concentration of tryptamine in guinea pig
brain and liver after pretreatment with
iproniazid phosphate 150 mg/kg for 1 hr
with or without L-tryptophan 800 mg/kg,
the animals being killed 2 hr after the
administration of the amino acid. From
Hess, Redfield and Udenfriend (1959).

Treatment	Tryptamine	
	Liver (μg/g)	Brain (μg/g)
None	<0.1 (5)[x]	<0.1 (7)
Iproniazid	0.2 (1)	<0.1 (4)
L-tryptophan	0.2 (8)	1.4 (3)
Iproniazid and L-tryptophan	5.7 (4)	0.8 (5)

[x] The number of animals is shown in parentheses.

TABLE 4

Comparison of monoamine oxidase inhibitors on trypta-
mine formation in guinea pig brain and liver from
Hess, Redfield and Udenfriend (1959).

Drug	Duration of Pre-treatment (hours)	Tryptamine (μg/g)	
		Liver	Brain
JB-516	1	0.3	0.6
JB-516	1	0.3	0.6
JB-516	16	0.1	0.2
JB-516	16	0.3	0.5
Iproniazid	16	2.0	0.3
Iproniazid	16	3.6	0.7

Animals were pre-treated with 3 mg/kg of JB-516 or
150 mg/kg of iproniazid, intraperitoneally. After
the pre-treatment period 800 mg/kg of L-tryptophan
was injected intraperitoneally and the animals were
killed 2 hr later.

collected from the neck wound into a polythene tube
containing heparin (0.2 ml; 1000 I.U./ml). The contents
of the tube were mixed by gentle shaking.

Iproniazid phosphate (60 mg/ml in saline) was ad-
ministered 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 pretreatment
with iproniazid, and killed at comparable times following
the administration of the amino acid. A group of un-
treated 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 2,500 r.p.m.
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/

by the addition of 0.2 ml 20% (w/v) NaOH, when the tube
was immediately inverted gently three times to ensure
adequate mixing. After standing for 10 min, the tube
was centrifuged for 5 min at 2,500 r.p.m. 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 4 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 2,500 r.p.m. 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 2,500 r.p.m.
for 5 min. The supernatant fluid was separated from the
precipitated/

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.

The estimation of tryptamine in tissues by the solvent extraction technique of Hess and Udenfriend (1959)

(Method 1)

To 3 ml of tissue homogenate (as prepared above) in a glass-stoppered centrifuge tube swasadded 0.3 ml of 10 N NaOH to bring the pH to about 11. Twenty ml of benzene werethen added and the tube was shaken for 15 min on an automatic shaker. The tube was centrifuged and as much of the benzene as possible was transferred to another glass stoppered 30 ml centrifuge tube containing 4 ml of 0.1 N H_2SO_4. This tube was shaken for 5 min on an automatic shaker, centrifuged, and 3 ml of the acid layer transferred to a 15 ml stoppered tube. The volume was made up to 6 ml with 3 ml 0.1 N H_2SO_4 and the solution put through the norharman procedure. The fluorescence intensity of the norharman formed was determined using an Aminco-Bowman spectrofluorimeter.

Modification of method of Hess and Udenfriend

(a) Ionexchange column chromatography/

(a)   Ion exchange column chromatography
      (Method 2)

The method, 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 x 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 NH_4OH for 30 min.   The NH_4OH 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)   Column chromatography of extracts/

(b) Column 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 H_2SO_4 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 H_2SO_4 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 in the benzene extract was returned to aqueous solution by shaking for 5 min. with 6.0 ml 0.1 N H_2SO_4. A 5.5 ml portion of the acid extract was put through the norharman procedure. The fluorescence intensity of the norharman thus formed from tryptamine was determined, using an Aminco-Bowman spectrofluorimeter. 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/

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 50-400 mμg, were added to 5 ml portions of brain homogenates and the quantity of tryptamine estimated by the method. The results (Fig. 6) were found to be linear in this range and an amount as low as 30 mμg/g of brain was measurable.

The fluorescence from the brain sample without tryptamine added was 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% (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% (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.

FIGURE 6
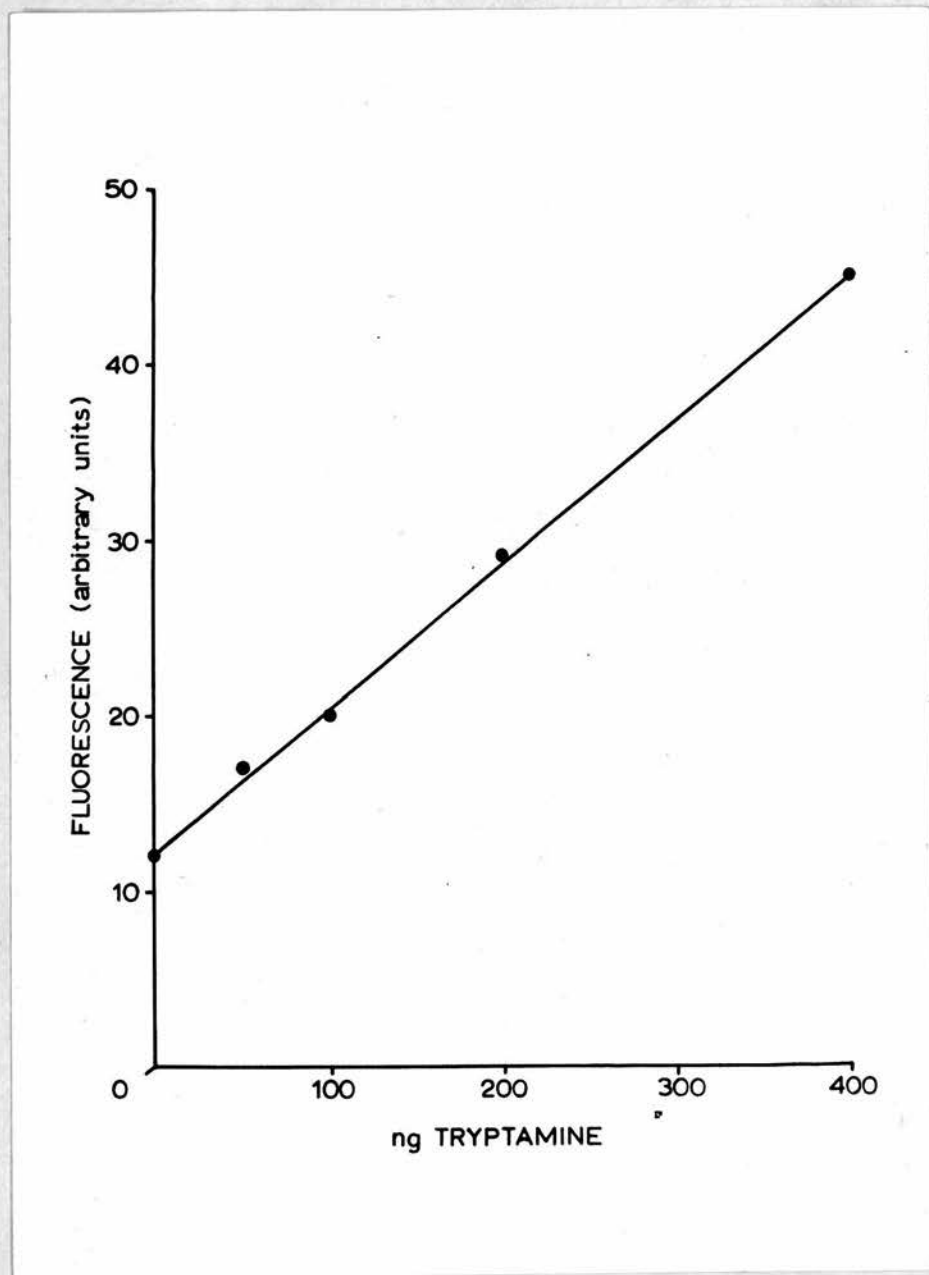
Fluorescence of norharman formed from tryptamine added
to brain homogenate from untreated guinea pigs, analysed
by modified method of Hess et al. (1959) (Method 2).
The fluorescence from the brain sample without tryptamine
added is the same as that from pure solutions of reagents
taken through the procedure.

A comparison of tryptamine estimations in guinea pig
brain and liver by the solvent extraction method of
Hess and Udenfriend (1959) (Method 1) and the modified
method as described above (Method 2).

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 the solvent extraction
procedure of Hess et al. (1959) and a 5.0 ml portion by
the modified technique. The results (Table 5) show a
discrepancy between the values for the concentration of
tryptamine in the tissues obtained by the two methods.
Table 6 shows the high concentration of tryptophan found
in these tissues and plasma at the times the animals were
killed.

Investigation of discrepancy between estimated tryptamine
concentrations in brain by the method of Hess and
Udenfriend and by the modified technique

(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 homo-
genate and this was analysed in triplicate portions by
the method of Hess and Udenfriend (1959) (Method 1).
This/

TABLE 5

Concentration of tryptamine in guinea pig brain and liver as estimated by the solvent extraction procedure of Hess et al. (Method 1) and by column chromatography (Method 2) 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). The values are given as µg/g and each estimate is from pooled tissue from three animals.

Treatment	Time after tryptophan (min)	Brain Exp. 1 Method 1	Method 2	Brain Exp. 2 Method 1	Method 2	Brain Exp. 3 Method 1	Method 2	Liver Exp. 1 Method 1	Method 2	Liver Exp. 2 Method 1	Method 2
None	0	0.1	ND[+]	ND	ND	ND	ND	0.1	ND	ND	ND
Iproniazid	0	0.1	ND	0.1	ND	0.1	ND	0.4	0.10	0.7	0.08
Tryptophan	20	0.2	ND					1.0	ND		
	40	0.2	ND					0.2	ND		
	60	0.2	ND					0.2	ND		
	80	0.5	ND					0.1	ND		
	120	0.4	ND					} 0.1*	} ND*		
	120	0.4	ND								
Tryptophan + iproniazid	20	0.1	ND	0.2	ND	0.2	ND	0.1	2.20	2.8	1.63
	40	0.1	ND	0.7	ND	1.0	ND	1.7	2.60	9.0	4.74
	50	0.2	0.05	0.4	0.10	0.3	0.04	5.3	5.50	6.6	4.20
	80	0.8	0.13	0.8	0.07	1.4	0.05	5.9	3.80	8.2	2.83
	120	0.5	0.19	1.1	0.08	1.3	0.08	} 7.0*	} 3.22*		
	120	0.9	0.27								

* Pooled tissue from six animals

+ ND = not detected   ND = not detected Method 1 <0.1 µg/g   Method 2 <0.03 µg/g

TABLE 6

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 pre-treatment for 16 hr with iproniazid phosphate (150 mg/kg). Results are given in µg/g tissue or/ml plasma.

Treatment	Time (min)	Brain Mean	Brain Range	Liver Mean	Liver Range	Plasma Mean	Plasma Range
None	0	7.5 (3)+	6.6-8.7	11.9 (2)	11.7-12.0	15.6 (2)	13.4-17.8
Iproniazid	0	8.1 (3)	5.8-10.5	12.8 (2)	11.0-14.6	17.0 (3)	15.2-18.3
Tryptophan	20	86 (1)		795 (1)		670 (1)	
	40	112 (1)		730 (1)		670 (1)	
	60	128 (1)				600 (1)	
	80	138 (1)		490 (1)		465 (1)	
	120	189 (2)	158-220	700✱ (1)		784✱ (1)	
Tryptophan + iproniazid	20	147 (3)	95-176	1015 (2)	950-1080	860 (3)	730-1000
	40	170 (3)	95-220	1188 (2)	525-1850	848 (3)	345-1280
	60	210 (3)	200-230	995 (2)	660-1330	796 (3)	600-940
	80	232 (3)	176-290	935 (2)	850-1020	823 (3)	670-1000
	120	264 (4)	210-324	800 (3)	570-950	738 (3)	560-850

✱ Pooled tissues from six animals.

+ Figures in parenthesis indicate number of experiments with groups of three animals.

This homogenate, with added tryptophan, was also analysed in triplicate by the modified procedure (Method 2). 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 Method 2.

(b) Identification of indolic substances in final extracts from two methods.

Thin layer chromatography of extract from method of Hess and Udenfriend.

A benzene extract of 18 g. of pooled whole brain from guinea pigs treated with tryptophan and iproniazid and killed 2 hr following the administration of the amino acid, was prepared by the method of Hess and Udenfriend (1959). One ml of 40% acetic acid was added and the benzene evaporated almost to dryness under a stream of nitrogen at 55°C. This extract contained considerable amounts of lipid and was unsuitable for direct application to thin layer chromatograms. Initial paper chromatography was used to purify the extract. The residue was taken up in 10 ml of benzene and the tryptamine extracted with shaking into 3 ml 0.1 N H_2SO_4 in a stoppered centrifuge tube. The tube was centrifuged at 2,500 r.p.m. for 10 min and the acid phase pipetted off. This was adjusted to pH 4 (Bromophenol blue paper) with careful addition of/

of solid potassium carbonate and the solution evaporated
to near dryness at 55°C on a vacuum pump. The residue
was taken up in 0.2 ml 80% (v/v) methanol containing
ascorbic acid 50 mg% (w/v) and applied to a paper strip
5 cm wide under nitrogen. Quantities of tryptophan and
tryptamine were added to an identical extract to act as
markers and the chromatogram developed in isopropanol:
35% (w/v) ammonia: water; (17:1:3).

After drying in nitrogen the marker chromatogram
was divided longitudinally and half stained with
cinnamaldehyde reagent (Jepson 1963) to give the position
of tryptamine and tryptophan. Appropriate strips from
the other half of the marker and from the extract
chromatograms were eluted into 4 ml and then 3 ml 40%
acetic acid. These eluates were reduced to near dryness
in a vacuum pump at 55°C, taken up in 0.1 ml 80% (v/v)
methanol and ascorbic acid 50 mg% (w/v) and applied to a
thin layer plate of silica gel G under nitrogen and then
developed in butanol: acetic acid: water; (12:3:5).
Sufficient tryptamine and tryptophan was eluted from
half the marker paper chromatogram to again act as a
marker on the thin layer plate. The indolic substances
were visualised by spraying with cinnamaldehyde reagent.

The above procedure was adopted using butanol:
acetic acid: water; (12:3:5) as a developing agent
for the preliminary paper chromatography and methyl acetate:
isopropanol: 25% (w/v) ammonia; (9:7:4) as developing
agent/

agent for the thin layer chromatography.

Two substances were detected in the extract chromatograms which behaved like authentic tryptophan and tryptamine (Table 7) (Plate 1).

(c) Thin layer chromatography of extract produced by modified technique (Method 2).

Homogenate from 36 g. of pooled brain of guinea pigs treated with both iproniazid (150 mg/kg) and killed at 2 hr following administration of L-tryptophan (800 mg/kg) were subjected to the analytical procedure of the modified method, the benzene extracts of the amine eluate being pooled. To this was added 1 ml 40% 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% (v/v) methanol. Quantities of tryptamine were added to portions of the extract to act as a marker. The plates were developed in three solvent systems (Table 8). The plates were dried in nitrogen and visualised with cinnamaldehyde, or ultra violet fluorescence following spraying with Prochazka reagent (Stahl 1965). In the extract chromatograms a single substance corresponding to authentic tryptamine was detected. No second indolic substance corresponding to tryptophan was found.

(d) Column chromatography of extracts produced by the method of Hess and Udenfriend/

TABLE 7

Rf 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.

Preliminary paper chromatography solvent	Silica gel solvent	Rf value			
		Indole 1	Tryptophan marker in brain extract	Indole 2	Authentic tryptamine marker in brain extract
Butanol–acetic acid–water (12:3:5)	Methyl acetate–isopropanol–ammonia (45:35:20)	0.32	0.29	0.66	0.66
Isopropanol–35% ammonia–water (17:1:3)	Butanol–acetic acid–water (12:3:5)	0.38	0.38	0.47	0.47

PLATE 1
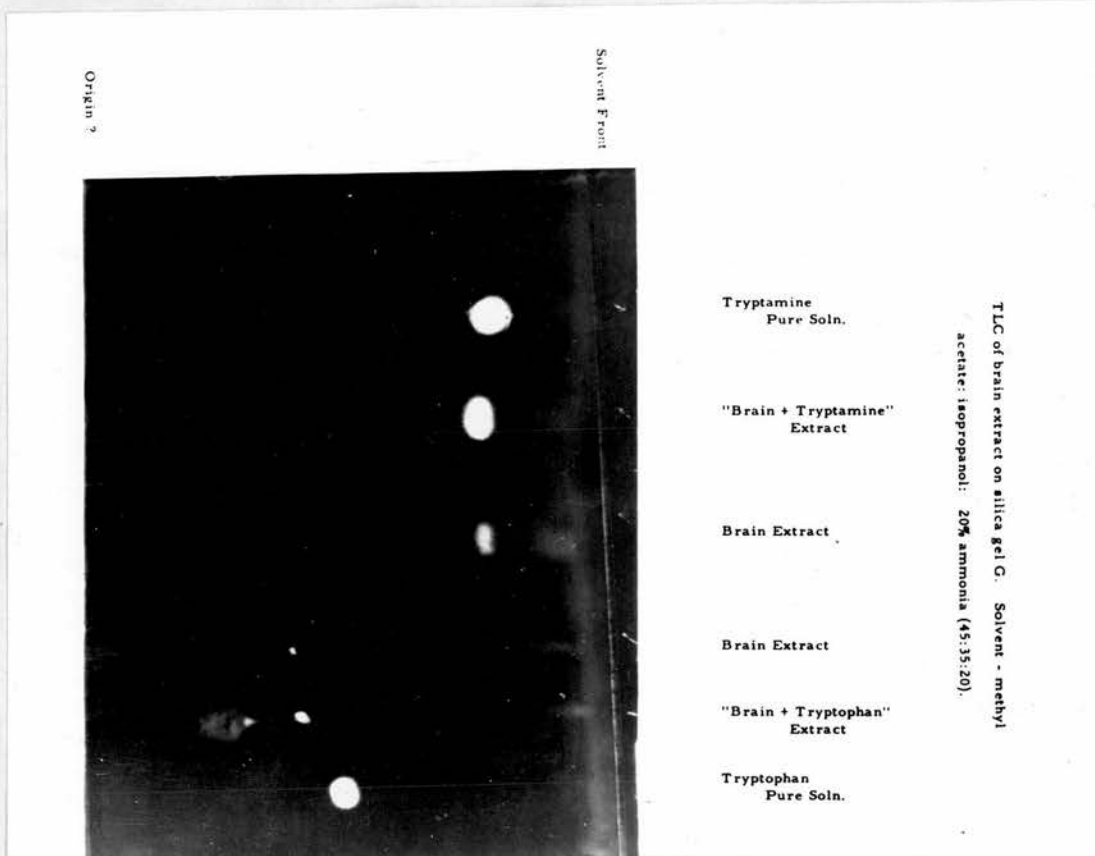
Thin layer chromatography of brain extract on silica
gel in methyl acetate:isopropanol:25% ammonia (45:35:20).

TABLE 8

Rf 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.

Solvent	Rf value	
	Indole	Tryptamine marker in brain extract
Methyl acetate–isopropanol–25% ammonia (9:7:4)	0.68	0.68
Butanol–acetic acid–water (12:3:5)	0.50	0.50
Chloroform–methanol–acetic acid (15:4:1)	0.20	0.20

(d) <u>Column chromatography of extracts produced by the method</u>
<u>of Hess and Udenfriend</u>

Homogenates of brain were prepared from guinea pigs
treated with iproniazid 150 mg/kg with and without
tryptophan 800 mg/kg. Fifteen ml of each brain homo-
genate was extracted into benzene and back-extracted
into 0.1 N H_2SO_4 by Method 1. The resulting 15 ml of
acid was then divided into two portions, one of 12 ml
and the other of 3 ml. On the 3 ml portion tryptamine
was estimated by the norharman procedure. The 12 ml
portion was adjusted to pH 7.4 (glass electrode) with
N NaOH and run through a column identical with that used
in Method 2. All the effluents and eluates were
collected in 3 ml aliquots and these put through the
norharman procedure. Five µg of tryptamine was added
as an internal standard to comparable quantities of the
brain homogenates and taken through the method, prior to
the addition of the 12 ml of buffered solution to the
column 1 µg of tryptophan was added as an internal
standard. Five ml of homogenate were examined for its
tryptamine content by the modified method of Hess et al.
(1959).

The results (Table 9) show the final acid extract as
prepared by the solvent extraction method to contain two
components which give norharman reaction. The first is
                        in the eluate
eluted from the column in which the authentic tryptophan
is/

TABLE 9

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

Treatment	Tryptamine			Tryptophan in benzene extracts from Method 1.* (μg/g)	Conc. tryptophan in brain (μg/g)
	Method 1 (μg/g)	Method 2 (μg/g)	Method 3 (μg/g)		
Tryptophan alone	0.70	0	0	0.62	294
Tryptophan plus iproniazid	0.89	0.20	0.18	0.61	300

* Tryptophan estimated after separation by column chromatography of the benzene extracts of Method 1.

is contained, and the second in the eluate containing
authentic tryptamine. Quantitatively, addition of the
two components could account for the apparent tryptamine
as found in the solvent extraction technique.

The tryptamine content of these brain homogenates
as estimated by Method 2 (0.20 μg/g) agreed well with the
amount of the amine present in the amine fraction from
the solvent extraction technique of Hess and Udenfriend
as determined after separation from the tryptophan fraction
by column chromatography, (0.18 μg/g) (Table 9).

Estimation of plasma tryptamine

The content of tryptamine in plasma was estimated
by Method 2. The results (Fig. 7, Table 10) show the
amine to be present in plasma in low concentrations after
the administration of iproniazid and tryptophan. 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.

FIGURE 7
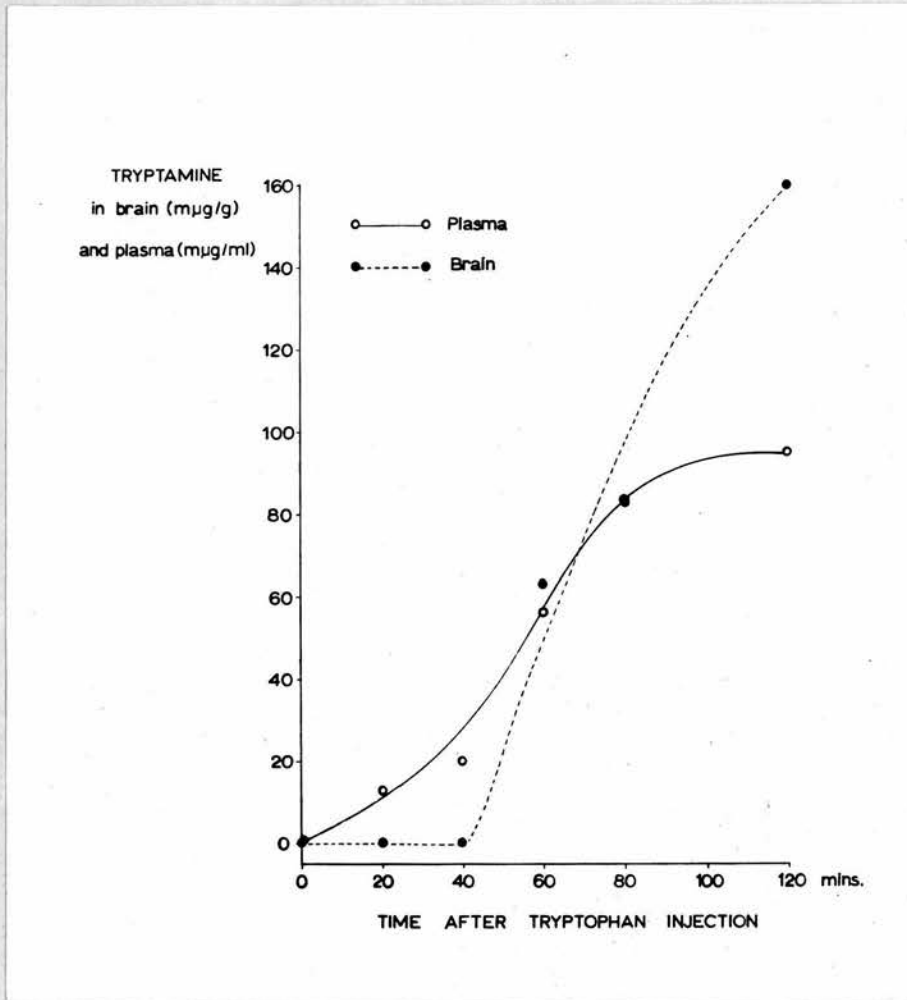
Concentration of tryptamine in guinea pig brain and plasma as estimated by the modified procedure of Hess et al. (1959) (Method 2) at varying time intervals after the intraperitoneal administration of L-tryptophan (800 mg/kg) with pre-treatment for 16 hr with iproniazid phosphate (150 mg/kg). Each point represents the mean of three experiments.

TABLE 10

Concentration of tryptamine (μg/ml) in guinea pig plasma following administration of tryptophan (800 mg/kg) to animals pre-treated with iproniazid (150 mg/kg).

Treatment	Time after tryptophan (min)	Exp. 1	Exp. 2	Exp. 3
Iproniazid	0	Trace[+]	ND[x]	ND
Iproniazid + tryptophan	20	0.04	ND	Trace
	40	Trace	Trace	0.06
	60	0.08	Trace	0.09
	80	0.15	0.04	0.06
	120	0.12	0.06	0.06
	120	0.14		

[x] ND = not detected < 0.025

[+] Trace $=> 0 < 0.025$

DISCUSSION

The method described here is basically that re-
ported by Hess and Udenfriend (1959) with the essential
additional step of ion exchange chromatography. In our
hands, the original procedure 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 to the benzene phase and finally to the acid
extract, in spite of all efforts to prevent any mechanical
carry over. Since tryptophan yields a fluorophor
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 and Udenfriend (1959)
such normal brain homogenates apparently contained 0.6
μg/g of tryptamine. The acid extract obtained by the
method of Hess and Udenfriend (1959) from the brains of
animals treated with tryptophan after prior administration
of/

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 9). Finally, it was
demonstrated by thin layer chromatography on silica gel,
using two solvent systems, that the acid extracts con-
tained indolic substances with Rf values identical to
those of authentic tryptophan and tryptamine (Table 7).

These techniques of identification were reduplicated
for the final extract of the modified method of Hess and
Udenfriend (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 8) of the final extract showed
only one indolic component with Rf values identical with
those of authentic tryptamine.

The question arises as to why the identification
procedures of the original workers did not reveal the
separate components present in the final extracts. The
answer is probably that tryptamine _per_ _se_ was not
identified from tissues, but the norharman reaction was
performed on liver extracts and the resulting fluorophor
identified by its chromatographic behaviour on paper in
two solvent systems, in comparison with authentic
norharman./

norharman. It is apparent in retrospect that any
contaminating tryptophan present would have been con-
verted to a fluorophor with identical fluorescence
characteristics to norharman and also behaving the same
way in the solvent systems used as authentic norharman.

Hess and Udenfriend state that in pure solution no
interference by tryptophan occurs even when the amino
acid is in 500-fold excess of the amine. This we also
found to be true of pure solution. It did not hold,
however, for brain homogenates, and may be due to ad-
sorption of the amino acid to some component of brain
tissue (e.g. lipids) with subsequent carry over into the
benzene phase. A similar phenomenon was observed for the
catechol amines by Von Euler$_\wedge$(1949). Consideration of
the tryptophan levels in the brain of animals pretreated
with the amino acid (Table 6) show concentrations
considerably exceeding 500 times the threshold con-
centration for the estimation of tryptamine by the
method (0.1 μg/g).

There are inconsistencies in the literature re-
garding the changes in the level of brain tryptamine
following the administration of L-tryptophan, with and
without amine oxidase inhibitors which are explicable on
the basis of methodological error. 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/

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.

Weber and Horita (1965) during the *in vivo* investigation of the conversion of tryptophan to 5HT in rat brain, estimated the concentration of tryptamine by the method of Hess and Udenfriend (1959). They treated rats with doses of tryptophan (200 mg/kg) with and without pretreatment with the amine oxidase inhibitor 2-phenyl-cyclopropyl amine 5 mg/kg subcutaneously 16 hr prior to administration of the amino acid. The animals were normal, partially (stomach intestines and spleen), or totally (stomach intestines, spleen, kidneys and liver) eviscerated. The levels of tryptamine reached in brain were 0.6 µg/g when tryptophan was administered whether or not there was pretreatment with amine oxidase inhibitor. Evisceration had no effect on these levels. These authors comment that in view of the fact that tryptamine is a better substrate/

substrate for amine oxidase than 5HT (Erspalmer et al. 1960) it is strange that the levels of tryptamine in brain are apparently uninfluenced by amine oxidase inhibitor.

In our investigations, using the modified procedure (Method 2) for the estimation of tryptamine, the amine was found in brain only after treatment with both amine oxidase inhibitor and L-tryptophan (Table 5 Fig.7). 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 ad-ministration of tryptophan produced very high con-centrations (Table 5). 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.

Biogenesis of tryptamine

Tryptamine does, then, appear in brain under certain circumstances and it is convenient at this point to dis-cuss its biogenesis.

Tryptamine/

Tryptamine may be formed in brain by the decarboxy-
lation of tryptophan. The enzyme, L-aromatic amino acid
decarboxylase, capable of effecting this conversion, is
present in brain (Lovenberg, Weissbach and Udenfriend
1962). The tryptamine may then be converted by monoamine
oxidase and aldehyde dehydrogenase to indol-3-ylacetic
acid. 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.

Tryptamine detected in brain may be synthetised at
other sites, transported in the plasma, and penetrate
into brain across the blood brain barrier. Tedeschi and
Tedeschi (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 5HT 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 the 5 mg/kg of the amine (0.173 ± 0.019 µg/g) (Mean$\pm$
S.E.M.). However, each rat received at least 1,000 µg
of/

of tryptamine by intravenous injection. The animals were killed 75 seconds 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 ionisation at body pH, and on these considerations tryptamine should penetrate the blood brain barrier.

In our investigations tryptamine was present in plasma at the time of detection in brain, rising, as in brain until the animals were killed. The question as to whether the tryptamine present in the brain of these animals is formed in brain or taken from peripheral sites is still unresolved.

Indol-3-ylacetic acid in brain might be used as an index of tryptamine turnover. In preliminary investigations the acid has been detected in brain after administration of tryptophan. It was, however, present in samples of plasma taken at the same time, and hence that present in brain may have crossed the blood brain barrier from plasma. At this stage then we cannot tell whether the brain has the potential to synthesise tryptamine from tryptophan.

SECTION 2

Tryptophan loading in rats

INTRODUCTION

In an attempt to examine all the metabolites of tryptophan along the 5-hydroxyindole pathway a paper chromatographic method was developed to estimate 5HTP, 5HT and 5HIAA from the same brain homogenate. During the preliminary experiments on the method it was found that the major source of loss of pure solution of the 5-hydroxyindoles added to brain homogenate was during evaporation of the aqueous phase obtained after acetone precipitation and subsequently blowing off the acetone with nitrogen. The optimal pH at which the solution was evaporated was found to be below 5. The acid used in the initial protein precipitation was also important. Evaporation from N/10 HCl resulted in some loss of the 5-hydroxyindole. This was minimised by the addition of ascorbic acid. The use of acetic acid in the brain homogenates gave better recoveries of 5HT taken through the procedure than any other acid which we employed in homogenisation. Initially a temperature of 37°C was used on a water pressure vacuum pump. Under these conditions it took about 45 min to evaporate 4 ml of aqueous solution. 5-Hydroxyindoles tended to be destroyed under these conditions. Using a temperature of 55°C and an efficient mechanical vacuum pump this volume of aqueous was evaporated to near dryness in 5 min, with almost 100% recovery.

The full details of the final method are given together with its application to tryptophan loading and drug studies.

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°C 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./

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 homogenised in
an all-glass homogenizer in 40% (v/v) acetic acid, 1.0
ml/g brain tissue. To the homogenate, a further 2.0
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 over-
night 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/

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 ex-
traction procedure. To the first was added 0.8 µg each
of 5HT, 5HTP and 5HIAA 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

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) 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 butan-1-ol:
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/

The developed chromatogram was dried in a glass
tank, through which was passed a stream of nitrogen. The
positions of the 5OH indoles were established from the
marker **chromatograms**. Appropriate sections of the
sample chromatogram were eluted with 4.2 ml 0.1 N H_2SO_4
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 H_2SO_4. Conc. HCl (2.0 ml containing
ascorbic acid 50 mg per 100 ml) was added and the samples
read against a standard of 5HT in a Farrand recording
spectrophotofluorimeter (Ashcroft, Crawford, Binns and
MacDougall, 1964). The relative fluorescences of standard
solutions of 5HT, 5HTP and 5HIAA were determined.

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

It was found that the Rf values of 5HT, 5HIAA or
5HTP applied in brain extracts and applied in pure solution
were not identical, the greatest disparity occuring with
the amine./(Table 11) Routinely, therefore, the positions of these
substances in chromatograms of brain extracts were
determined from a parallel chromatogram of a similar
brain/

TABLE 11

Rf values for 5-hydroxyindole compounds

| | Indole applied to chromatogram in | |
	pure solution Rf	brain extract Rf
5HTP	0.26	0.26
5HT	0.43	0.50
5HIAA	0.70	0.68

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

TABLE 12

Recoveries of 5-hydroxyindoles from brain homogenates

	Percentage recovery[x]
5HT	87 ± 6.5 (9)
5HIAA	96 ± 10.9 (8)
5HTP	55 ± 6.9 (9)

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

[x] Mean ± standard deviation (no. of experiments)

brain extract, to which had been added 5HT, 5HIAA and
5HTP in amounts sufficient to permit easy visualisation
of the indoles (a) by the characteristic pinkish
fluorescence in ultra violet light, while the chromatogram
still retained a small amount of acetic acid from the
chromatographic solvent (Mitoma, Weissbach and Udenfriend,
1955) and (b) by the colour reaction with Ehrlich's
reagent (Rodnight, 1956), and (c) by cinnamaldehyde
(Jepson 1963)

While 5HTP and 5HT were separated by several cm,
only about 2 cm separated the upper boundary of the 5HT
area from the lower boundary of the 5HIAA 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. 8) To allow for slight
differences in the extent of solvent flow in the extract
and 'marker' chromatograms, six such templates were pre-
pared, varying in length by 0.5 cm, the shortest being
1.5 cm less than, and the longest 1.5 cm greater than
the distance from the origin to the solvent front on
the 'marker' chromatogram. The positions of the proposed
cuts were marked on each template at the appropriate
points calculated from the Rf values on the 'marker'
chromatogram and the length of the template strip. The
dried/

FIGURE 8

CHROMATOGRAM   TEMPLATE
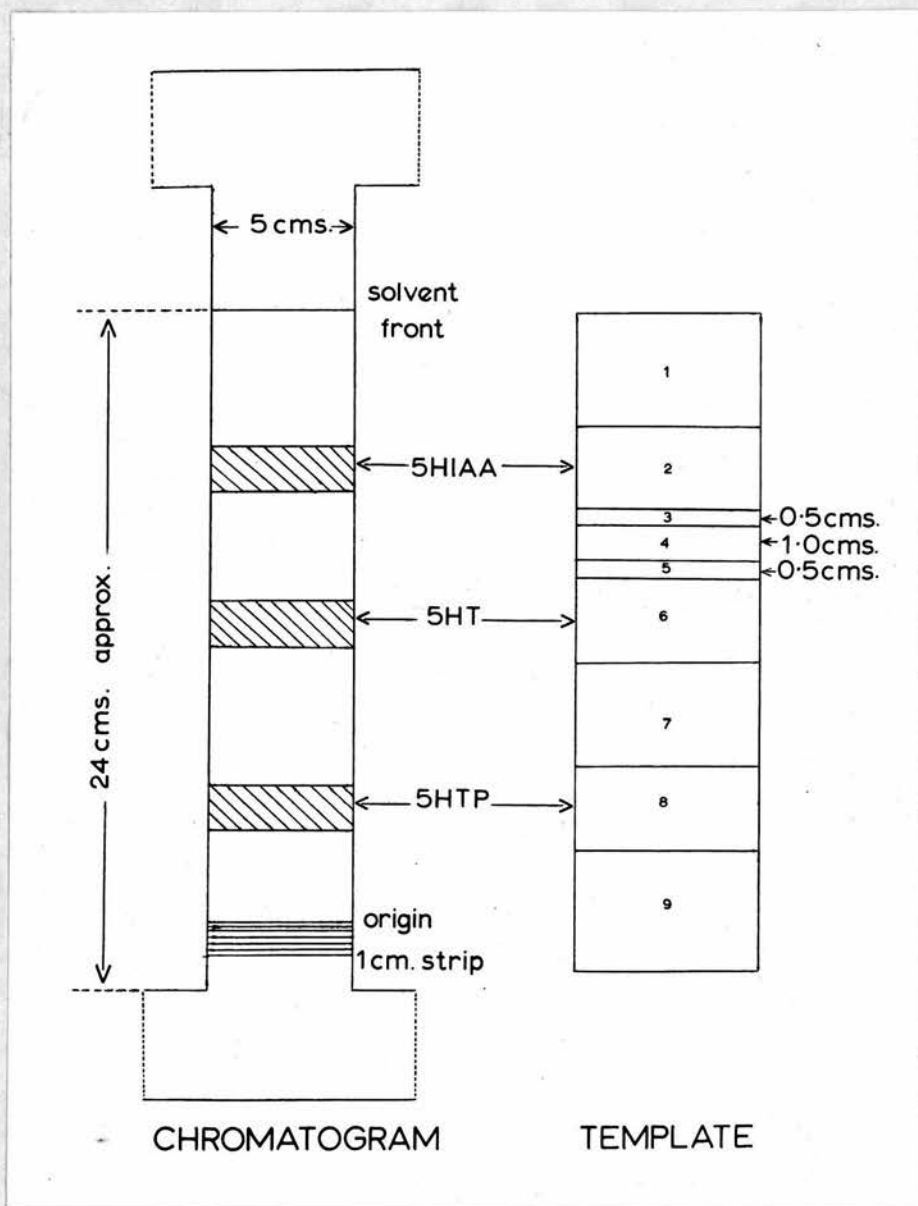
Left: Diagram of 'strip' chromatogram of 5-hydroxyindole
     compounds developed in butan-1-ol:acetic acid:water
     (12:3:5 by vol). Indoles located by the colour
     reaction with Ehrlich's reagent.

Right: Template defining positions of strips for sample
      chromatogram.

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 5OR indoles in
those sections of the chromatograms postulated to contain
none would point either to poor separation, or to the
presence of an unexpected 5OR indole, but there were no
detectable amounts of 5HT, 5HIAA or 5HTP in the eluates
of those sections.

Measurement of tryptophan in brain extracts and
whole blood as described on p. 12

Measurement of 5HTP in plasma. To 2.0 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 5HTP marker was eluted and the 5HTP assayed
spectrofluorimetrically.

Confirmation of the identity of substances in the
'5HT' and '5HIAA' 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/

and (b) comparison of biological and fluorescence assays.

(a) Solvent extraction. In one experiment, the eluates from the chromatogram strips corresponding to the marker 5HT and 5HIAA positions were subjected to the solvent extraction procedures, whilst in a second experiment only the 5HIAA 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 5HIAA, 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, Pletscher, 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. This was kindly performed by Dr. T.B.B. Crawford. Extracts were prepared from the brains of animals killed 2 hr after loading with tryptophan 800 mg/kg and also of/

of control animals.   The appropriate strips for '5HIAA'
and '5HT' from the developed chromatogram were placed
in glass tubes and dried in vacuo in a desiccator con-
taining 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 12 shows the results of the recovery ex-
periments of 0.8 μg of 5HT, 5HIAA and 5HT added to brain
homogenate and taken through the procedure.   Recoveries
of 5HTP were lower than those for 5HT or 5HIAA, the loss
occurring during protein precipitation.   The reported
estimates of the concentration of 5HTP, 5HT and 5HIAA in
brain samples have been corrected for the mean recovery of
each indole obtained during this series of experiments.
Recovery of 5HTP from plasma in a single experiment was
67%.

Identity of substances in '5HT' and '5HIAA' sections of
chromatogram

(a)   A comparison of direct estimation of 5-hydroxyindoles
in the eluates from '5HT' and '5HIAA' strips and estimates
of basic and acidic 5-hydroxyindole compounds in the
strips, obtained by the solvent extraction techniques,
are shown in Table 13.   There was good quantitative
agreement between pre- and post-extraction estimates and
                            was
no basic 5-hydroxyindole found in the '5HIAA' strip, or
acidic 5-hydroxyindole in the '5HT' strip.

(b)   A comparison of the fluorimetry and biological assay
of eluates from '5HT' and '5HIAA' strips is given in Table
14./

TABLE 13

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

| '5HT Strip' | | | '5HIAA Strip' | | |
Direct fluorescence estimate (μg 5HT per eluate)	Extractable basic 5OH indole (μg 5HT per eluate)	Extractable acidic 5OH indole (μg 5HIAA per eluate)	Direct fluorescence estimate (μg 5HIAA per eluate)	Extractable basic 5OH indole (μg 5HT per eluate)	Extractable acidic 5OH indole (μg 5HIAA per eluate)
0.94	0.92	<0.04	0.26	<0.04	0.23
0.54	0.40	<0.04	0.28	<0.04	0.28
			0.12	<0.04	0.11
			0.25	<0.04	0.23
			0.37	<0.04	0.31
			0.52	<0.04	0.53
			0.62	<0.04	0.64
			0.32	<0.04	0.27
			0.80	<0.04	0.71

TABLE 14

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

Brain sample from rats after	'5HT' section of chromatogram 5HT equivalents, µg/ml eluate			'5HIAA' section of chromatogram	
	Fluorimetry	Bioassay	Difference	5HIAA µg/ml eluate Fluorimetry	5HT activity µg/ml eluate Bioassay
No treatment	0.10	0.18	0.08	0.06	<0.025
L-tryptophan 800 mg/kg intraperitoneally 2 hr before	0.23	0.30	0.07	0.32	<0.025

14. No biological activity was detected in the '5HIAA' strip. In the '5HT' 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 5HT per ml eluate, the concentration being unaltered by tryptophan loading. No attempts have been made to investigate the discrepancy.

Application of Method

1. Variation in dose of tryptophan

Tryptophan was administered intraperitoneally as a suspension 140 mg/ml in 0.9% saline in doses of 400 mg/kg
800mg/kg
and 1600 mg/kg body weight and for each dose level a group of animals was killed 1, 2 and 4 hr after injection.

2. Drug treatments

(a) α-Methyl dopa 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 α-methyl dopa.

(b) 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 times after tryptophan administration as in (a). Animals treated for 5 days with iproniazid, but not tryptophan, were used as controls.

Confirmation of the identity of substance in 5HTP section of chromatogram level after tryptophan and α-methyl dopa Loading.

In/

In the course of the experiments, the results of the
application of the analytical procedure to extracts of
brain from animals treated with α-methyl dopa before
tryptophan administration indicated the presence of 5HTP.
Supplementary evidence of the identity of 5HTP was obtained,
as follows. The appropriate section of the paper chroma-
togram of brain extract was eluted in 2 ml 20% (v/v) acetic
acid. The eluate was evaporated to damp 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 5HTP in the solvent system composed of aqueous
NaCl8%: 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_2SO_4 (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 contain
material with the fluorescence characteristics of a 5-hy-
droxyindole (max. activation 300 mμ, max. fluorescence
550 mμ uncorrected) located solely at the same position on
the chromatogram as authentic 5HTP. The small amount of
material available prevented further characterisation
tests.

RESULTS

Uptake of amino acids by brain tissue.

The uptake of 5HTP into brain has not been studied, but Table 15 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.

Following treatment with α-methyl dopa, the blood and brain concentrations of tryptophan (Table 16) must be compared with those following administration of tryptophan alone./

TABLE 15

Tryptophan concentration in rat blood and brain at various times after intraperitoneal administration of L-tryptophan.

Time after tryptophan (hr)	Blood tryptophan (μg/ml)			Mean blood tryptophan (μg/ml)	Brain tryptophan (μg/g)			Mean Brain tryptophan (μg/g)	Blood/brain tryptophan ratio			Mean blood/brain tryptophan ratio
	Experiment				Experiment				Experiment			
	1	2	3		1	2	3		1	2	3	
0 (No tryptophan)	19.2	17.0	15.9	17.4	7.1	8.0	6.9	7.3	2.70	2.13	2.30	2.38 ± 0.29[*]
0.5	1000	890	1220	1037	177	146	150	158	5.65	6.10	8.13	6.63 ± 1.31
1	860	670	825	785	228	200	220	216	3.77	3.35	3.75	3.62 ± 0.24
2	630	650	480	587	340	300	280	307	1.85	2.17	1.71	1.91 ± 0.24
4	300	265		288	210	219		215	1.43	1.21		1.32
8	14.6	16.2		15.4	7.3	8.8		8.0	2.00	1.84		1.92

[*] Mean ± Standard deviation.   Dose of tryptophan 800 mg/kg.

TABLE 16

Tryptophan concentration in blood and brain of rats pretreated with α-methyl dopa at various times after L-tryptophan loading.

Time after tryptophan (hr)	Blood tryptophan (μg/ml)	Mean blood tryptophan (μg/ml)	Brain tryptophan (μg/g)	Mean brain tryptophan (μg/g)	Blood/brain tryptophan ratio	Mean blood/brain tryptophan ratio
0 (α-methyl dopa alone)	15.6 18.0 22.0 19.0	18.7	8.0 5.5 7.2 7.0	6.9	1.95 3.27 3.06 2.71	2.75
0.5	950 1250 970	1057	118 164 117	133	8.05 7.62 8.29	7.99
1	600 760 870	743	147 180 200	176	4.08 4.22 4.35	4.22
2	610 615 670	632	152 276 246	225	4.01 2.23 2.74	2.99
4	290 250	270	168 226	197	1.73 1.10	1.42
8	25.0	25.0	13.6	13.6	1.84	1.84

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

alone. This comparison can be made by expressing the
results as a blood/brain ratio for tryptophan (Table 17).
The ratio is increased in the α-methyl dopa group, compared
with the untreated groups, during the first 2 hr after
tryptophan administration. The difference cannot, however,
be tested for statistical significance.

In a single experiment, it was found that pretreatment
with iproniazid did not appear to alter the tryptophan
concentrations in brain or in blood at the various times
subsequent to tryptophan administration compared with
controls.

The presence of 5HTP in rat plasma following tryptophan administration

Evidence was obtained which indicated the presence
of measurable amounts of 5HTP in the plasma of animals
following tryptophan loading (Table 18). None was de-
tectable in the plasma of control animals.

Plasma levels of 5HTP were found to be lower in
animals pretreated with α-methyl dopa (Table 18).

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 5HT showed
a rise (Table 19 and Fig. 9) from a mean control
concentration/

TABLE 17

Comparison of blood/brain tryptophan ratios at various
times after administration of L-tryptophan in rats with
and without treatment previously with α-methyl dopa.

Time after tryptophan (hr)	Blood/brain tryptophan ratio[*]	
	Tryptophan alone	α-methyl dopa + tryptophan
0 (No tryptophan)	2.38 (3)	2.75 (4)
0.5	6.63 (3)	7.99 (3)
1	3.62 (3)	4.22 (3)
2	1.91 (3)	2.99 (3)

α-methyl dopa 800 mg/kg given 1 hr before tryptophan
800 mg/kg.

[*] Mean (no. of experiments)

TABLE 18

Comparison of 5HTP concentrations in rat plasma at various times after intraperitoneal injection of L-tryptophan in rats with and without pretreatment with α-methyl dopa.

Time after tryptophan (hr)	Plasma 5HTP μg/ml	
	α-methyl dopa pretreatment	No α-methyl dopa pretreatment
0 (No tryptophan)	< 0.025	< 0.025
0.5	0.06	0.13
1	0.03	0.075
2	0.04	0.075
4	-	0.045

Tryptophan 800 mg/kg, was given. Pretreatment consisted of α-methyl dopa, 800 mg/kg, 1 hr before tryptophan administration. Each result represents one estimation on pooled plasma from three rats.

TABLE 19

5-hydroxyindole concentration in brain tissue at various time intervals after intraperitoneal injection of L-tryptophan.

Time after injection (hr)	No. of experiments	Concentration ± S.D. (μg/g)		
		5HT	5HIAA	5HTP
0 (no tryptophan)	4	0.32 ± 0.06	0.24 ± 0.015	<0.05
0.5	3	0.49 ± 0.04	0.45 ± 0.03	<0.05
1	3	0.62 ± 0.04	0.57 ± 0.06	<0.05
2	4	0.59 ± 0.14	0.84 ± 0.13	<0.05
4	3	0.59 ± 0.13	1.09 ± 0.15	<0.05
6	1	0.46	1.00	<0.05
8	2	0.32 ± 0.08	0.42 ± 0.18	<0.05

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

FIGURE 9

Concentration of 5HIAA and 5HT in rat brain following intra peritoneal administration of tryptophan 800 mg./kg.
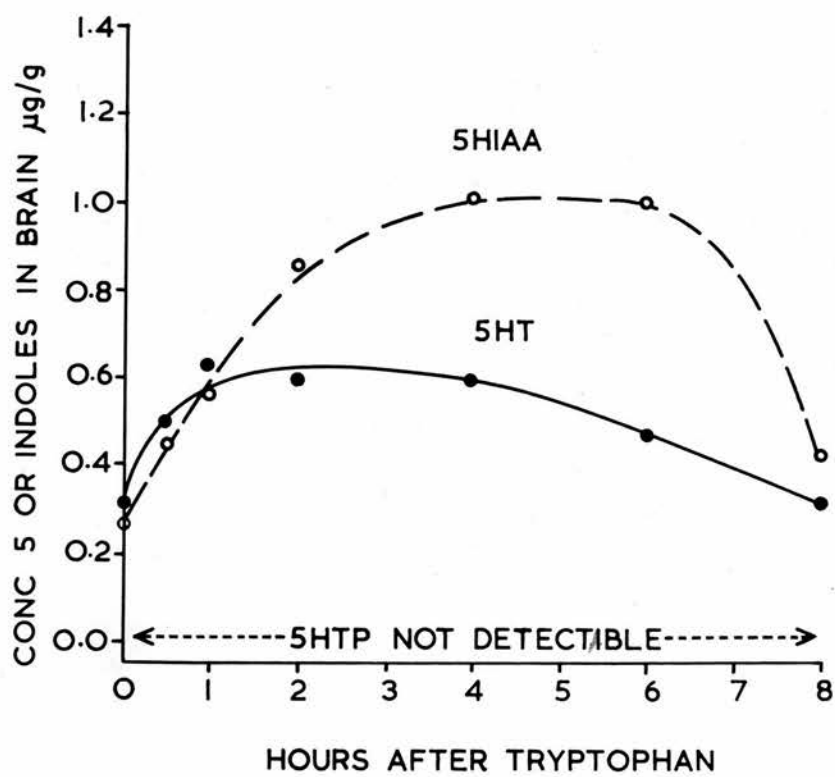
concentration of 0.3 µg/g to a maximum of 0.6 µg/g within
1 hr. This concentration was maintained during the
following 3 hr, after which it returned to the control
value within a maximum of 8 hr after the tryptophan
administration. The 5HIAA, with a control level of
0.24 µg/g also increased in concentration, but unlike
the 5HT, 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 5HIAA was maintained
at this leveloverthe 4 - 6 hr interval, or whether the
concentration did, in fact, reach a higher but relatively
unsustained maximum during this period. No 5HTP was
detectable in the brain samples at any time before or
after the tryptophan administration.

Effect of varying the dose of tryptophan

The 5HT concentration in brain rose from a control
level of 0.32 µg/g to a maximum of 0.7 µg/g (Table 20).
Alteration of the amount of tryptophan administered within
the range examined did not influence the maximum level
but only its duration.

The 5HIAA concentration in brain also increased from
a control level of 0.24 µg/g to a maximum of 1.1 µg/g,
the rate of rise apparently being similar to that of 5HT
over/

TABLE 20

Concentrations of 5HT, 5HIAA and 5HTP in rat brain tissue following intra-peritoneal injection of varying doses of L-tryptophan.

Dose tryptophan mg/kg	Time after tryptophan hr	5HT µg/g	5HIAA µg/g	5HTP µg/g
None	Control	0.32 ± 0.06 (4)*	0.24 ± 0.015 (4)*	<0.05 (4)*
400	1	0.62	0.61	<0.05
	2	0.69	1.13	<0.05
	4	0.41	0.54	<0.05
800	1	0.62 ± 0.04 (3)*	0.57 ± 0.06 (3)*	<0.05
	2	0.59 ± 0.14 (4)*	0.84 ± 0.13 (4)*	<0.05
	4	0.59 ± 0.13 (3)*	1.09 ± 0.15 (3)*	<0.05
1,600	1	0.67	0.61	<0.05
	2	0.61	1.00	<0.05
	4	0.61	1.13	<0.05

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

* Mean concentration ± standard deviation (no. of experiments) from Table 19

over the first hour. Similarly the 5HIAA appeared to
rise to a maximum concentration not correlated to the
tryptophan dose, but this was reached later than with
5HT 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.

The effect of varying the dose of tryptophan on the
concentrations of tryptophan in rat blood and brain is
shown in Table 21.

Effect of pretreatment with α-methyl dopa followed by tryptophan loading.

Table 19 and Fig. 9 shows the increase in the
concentration of 5HT and 5HIAA in brain, occurring after
treatment with tryptophan alone. This increase was not
observed in animals treated with α-methyl dopa before
the tryptophan administration (Table 22and Fig.10).
Another 5-hydroxyindole metabolite, considered to be
5HTP on the basis of its behaviour on paper chromatography,
appeared in measurable quantities in the brain extracts.

Effect of pretreatment with iproniazid on the concentrations of 5-hydroxyindole metabolites in brain following tryptophan administration.

There was an increase in the brain 5HT concentrations
to/

TABLE 21

Tryptophan concentrations in rat blood and brain at
various time intervals after intraperitoneal injection
of various doses of L-tryptophan.

Dose tryptophan (mg/kg)	Time after tryptophan (hr)	Blood tryptophan (μg/ml)	Brain tryptophan (μg/ml)
None[*]	Control	17.4 ± 1.4 (3)	7.3 ± 0.62 (3)
400	1	505	196
	2	150	152
	4	33	16.4
800[*]	1	785 ± 82.5 (3)	216 ± 11.8 (3)
	2	587 ± 75.8 (3)	307 ± 38.8 (3)
	4	288	215
1,600	1	1800	310
	2	1350	400
	4	820	545

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

[*]Mean concentration ± standard deviation (no. of experi-
ments)

TABLE 22

5-Hydroxyindole concentrations in brain tissue of α-methyl dopa pretreated rats at various times after L-tryptophan loading.

Time after L-tryptophan (hr)	5HT			5HIAA			5HTP		
	Expt. 1	Expt. 2	Mean	Expt. 1	Expt. 2	Mean	Expt. 1	Expt. 2	Mean
Untreated controls 0			0.32 ± 0.06 (4)*			0.24 ± 0.015 (4)*			< 0.05 (4)*
(α-methyl dopa alone)	0.25	0.22	0.24	0.21	0.25	0.23	0.05	0.05	0.05
0.5		0.17	0.17		0.12	0.12		0.23	0.23
1	0.25	0.22	0.24	0.27	0.19	0.23	0.11	0.41	0.26
2	0.22	0.18	0.20	0.15	0.25	0.20	0.64	0.45	0.55
4	0.12	0.22	0.17	0.19	0.22	0.21	0.50	0.27	0.39
8		0.21	0.21		0.19	0.19		0.09	0.09

Brain concentration (μg/g)

The rats were treated with 800 mg/kg α-methyl dopa 1 hr before administration of 800 mg/kg tryptophan

* Mean ± standard deviation (no. of experiments)

FIGURE 10
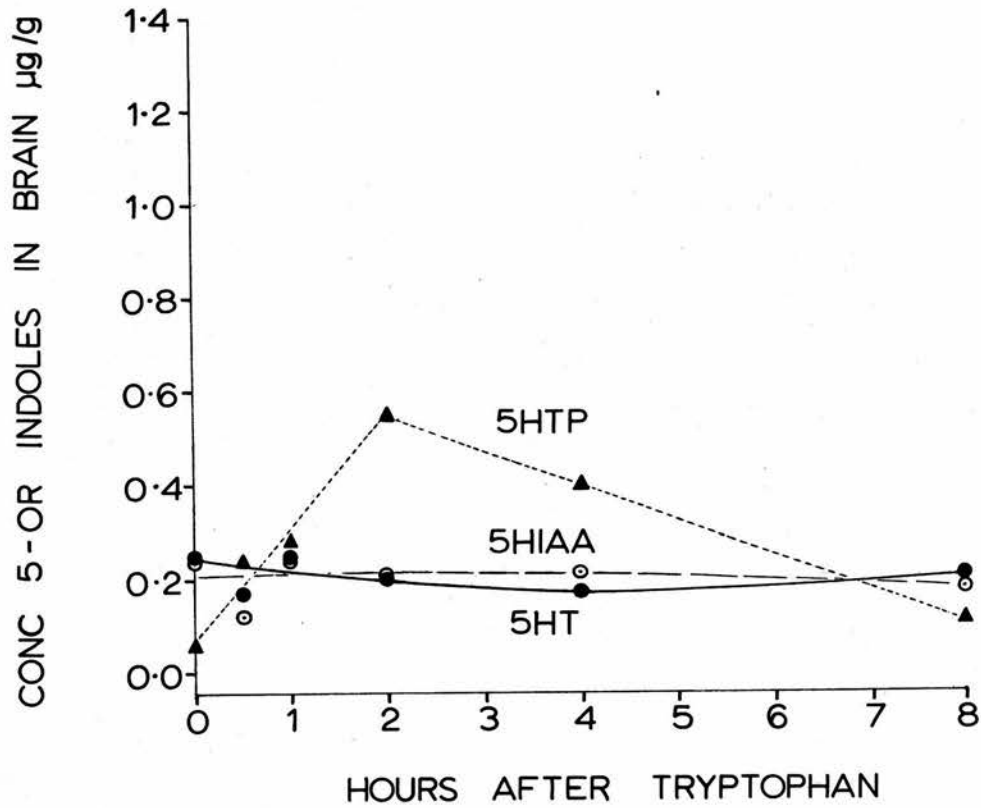
concentration of 5 hydroxyindoles in rat brain after
α-Methyl dopa 800 mg/kg, followed 1 hr later by tryptophan
800 mg/kg.

to a level higher than that reached following ad-
ministration of tryptophan alone (Table 23and Fig. 11).
The concentration of 5HT in the brains of animals
receiving only the iproniazid also showed an increase over
that of untreated animals.

In contrast, pretreatment with iproniazid reduced
the rise in concentration of 5HIAA in brain occurring
after tryptophan loading (compare Fig. 9 and 11).

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

TABLE 23

5-Hydroxyindole concentratins in rat brain at various times after administration of L-tryptophan following 5 days treatment with iproniazid phosphate.

Brain concentration in μg/g

Time after tryptophan (hr)	5HT			5HIAA			5HTP
	Expt. 1	2	Mean	Expt. 1	2	Mean	Mean
Untreated controls 0			0.32 ± 0.06 (4)*			0.24 ± 0.015 (4)*	< 0.05 (4)*
(Iproniazid alone)	0.46	0.67	0.57	0.16	0.03	0.10	<0.05
0.5	0.65	0.87	0.76	0.22	0.08	0.15	<0.05
1	0.96	1.26	1.11	0.33	0.24	0.27	<0.05
2	1.05	1.49	1.27	0.51	0.25	0.38	<0.05
4	0.85	1.61	1.23	0.52	0.25	0.39	<0.05
8	0.53	0.85	0.69	0.36	0.16	0.26	<0.05

Iproniazid 25 mg/kg daily for 5 days before administration of tryptophan, 800 mg/kg.

* Mean ± standard deviation (no. of experiments)

FIGURE 11

concentration of 5 hydroxyindoles in rat brain after
Iproniazid 25 mg/kg, daily for 5 days, followed by
tryptophan 800 mg/kg.

DISCUSSION

The major metabolic pathway of 5-hydroxyindoles in brain is tryptophan $\rightarrow$ 5-hydroxytryptophan $\rightarrow$ 5-hydroxy-tryptamine $\rightarrow$ 5-hydroxyindolacetaldehyde $\rightarrow$ 5-hydroxyindolyl-acetic acid. The analytical technique permitted the estimation of 5HTP, 5HT and 5HIAA in tissue or plasma. An alternative metabolic pathway is the formation of the neutral metabolite 5-hydroxytryptophol from the aldehyde in the reversible enzymic step mediated by alcohol dehydrogenase. This has been shown to take place in rat liver by Feldstein and Wong (1965) and in rabbit plate-lets by Bartholini, Pletscher and Bruderer (1964). It has not been demonstrated in rat brain but alcohol formation has been shown to be a minor metabolic pathway for the metabolism of the catechol amines by Goldstein and Gerber (1963). The glucuronide of 5-hydroxytryptophol has been demonstrated in rat urine by Kveder, Iskric and Keglevic (1962).

In the paper chromatography procedure described, 5-hydroxytryptophol would not separate from 5HIAA in the solvent system used (butanol: acetic acid: water; 12:3:5). However, during the identification procedure the 5-hydroxyindole on the 5HIAA strip behaved as an acid and quantitatively was recovered from an extraction procedure which would have excluded 5-hydroxytryptophol. This does not indicate, however, that in other circumstances the 5HIAA strip might not include a varying amount of 5-hydroxytryptophol./

5-hydroxytryptophol.

Limiting step in the synthesis of 5HT

The blood/brain barrier prevents uptake of 5HT from
blood into brain tissue; hence the first step in the
rise in concentrations of 5HT and 5HIAA in brain following
tryptophan loading must be either the hydroxylation of
tryptophan in the brain tissue, or the uptake into brain
of 5HTP produced elsewhere in the body. Cooper and
Melcer (1961) deduced that 5HTP 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. In
our experiments, the presence of 5HTP 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.

Gal, Morgan, Chatterjee and Marshall/investigated
(1964)
the hydroxylation of tryptophan in vivo in the pigeon
and rat. They investigated the half life of intra-
cerebrally administered 5HT and found it to be in good
agreement with the half life of 5HT formed after intra-
cerebrally injected tryptophan, (Table 24). They con-
cluded from their results that although the turnover rate
of cerebral 5HT was high the potential synthesis was low.
They/

TABLE 24

Calculated half life of 5HT ($T_{\frac{1}{2}}$) in pigeon brain from Gal et al. (1964)

Substance Administered *	Brain wt (g)	$T_{\frac{1}{2}}$ (min)	5HT	
			µg/g/hr	µg/brain/day
L—tryptophan	2	42	0.5	24.0
5HT	2	35	0.6	28.8

The values are based on average normal cerebral concentration of 5HT (0.70 µg/g)

* by intracerebral injection

They postulated that 5HT was derived from two sources
of 5HTP. The majority was derived from the hydroxylation
of tryptophan in peripheral tissues and transported to
brain and the rest from tryptophan hydroxylated in brain.
The results of Weber and Horita (1965) in the rat dis-
agreed with this finding. These workers set out to
determine the extent of extra and intra cerebral synthesis
of 5HT. They used partially (removal of stomach, in-
testines and spleen) and totally (removal of liver,
kidneys, stomach, intestines and spleen) eviscerated rats.
These animals were given an intraperitoneal injection of
L-tryptophan (200 mg/kg) and the rise in brain 5HT com-
pared with controls. Metabolism of 5HT to 5HIAA was
inhibited by the use of the amine oxidase inhibitor
(2-phenylcyclopropyl amine, PCP: 5 mg/kg). They found
that in the eviscerated animal the rise in brain 5HT
after intraperitoneal tryptophan was unimpaired. They also
demonstrated that after slow intracarotid infusion of
tryptophan in normal rats the brain 5HT concentration
rose significantly. Assay of the 5HT content of peripheral
tissues did not show a significant rise compared with
unperfused controls. It would then seem unlikely that
5HTP was synthesised from the perfused tryptophan in
peripheral tissues and transported to brain.

In our own experiments with tryptophan alone it is
not possible to determine the origin of the cerebral 5HT.
Although 5HTP does appear in plasma after tryptophan
loading,/

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

Limiting concentrations of 5HT and 5HIAA and possible
existence of metabolic pools.

The concentration of 5HT reached a maximum of
approximately 0.6 μg/g brain 1 hr after loading with
tryptophan (800 mg/kg) following which the concentration
remained at this level for the next 3 hr. The 5HIAA
levels showed no such tendency to level out at this time,
but continued to rise, reaching an apparent maximum 4 hr
after loading. Varying the dose of tryptophan by giving
either 400 or 1,600 mg/kg did not alter the maximum
concentrations which 5HT and 5HIAA reached and maintained
in brain. At the higher doses, however, these concentra-
tion maxima were maintained for longer periods.

There are at least two possible explanations.
Firstly, the presence of a 'pool' or store, of 5HT with a
limited capacity estimated at 0.6 μg/g brain. This having
filled, all the amine subsequently produced would overflow
on/

on to sites of destruction, with a continued rise of
5HIAA. An alternative explanation for the sustained
maximum concentrations of 5HT might be the existence
of a limiting supply of 5HTP, owing to either a maximum
limiting rate of formation as a result of saturation of
the hydroxylating enzyme, or to a 'feed-back' mechanism
operating on this enzyme. Given this constant supply of
5HTP, with rapid decarboxylation to 5HT, then the con-
centration of 5HT will rapidly rise to a plateau, the level
of which will depend upon the establishment of an
equilibrium between synthesis, storage and breakdown to
5HIAA. The 5HIAA will then continue to rise, reaching
a plateau at a later time. The level reach by 5HIAA will
depend on the relative rates of formation from 5HT and
removal, which, in this case, will involve passage from
brain tissue into blood and CSF.

The present results showing the same maxima for 5HT
and for 5HIAA when different tryptophan loads were
administered give evidence against a limited store for
5HT as the sole factor for, if this were the case, the
concentration of 5HIAA would be expected to rise to
higher levels when a larger dose of tryptophan was ad-
ministered. The results indicate that the hydroxylation
of tryptophan is the rate-limiting step in the bio-
synthesis of 5HT and that at all doses of tryptophan ad-
ministered the maximum rate of formation is reached, either
as a result of saturation of the hydroxylase by excess of
substrate/

substrate or as a result of a 'feed-back' mechanism
from accumulation of metabolites.

Effect of α-methyl dopa

Several points of action on the 5-hydroxyindole
metabolic pathway in brain have been suggested for
α-methyl dopa. The situation has been reviewed by
Sharman and Smith (1962), who suggested the following
possibilities:-

1.   Inhibition of the decarboxylation of 5HTP;

2.   Inhibition of the uptake of amino acid precursors
     into brain;

3.   Displacement or release of 5HT by α-methyl dopa,
     or its metabolites;

4.   Inhibition of amine oxidase by α-methyl dopamine,
     a metabolite of α-methyl dopa.

A fifth possible mechanism, inhibition of tryptophan
hydroxylase, has been suggested by Pletscher, Burkard and
Gey (1964).

Inhibition by α-methyl dopa 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 5HT by inhibition of 5HTP
decarboxylase (Westermann, Balzer and Knell, 1958).
Sharman and Smith (1962) measured total, acidic and basic
5-hydroxyindoles in rat brain following administration
of/

of α-methyl dopa and demonstrated the appearance of
neutral and/or amphoteric 5-hydroxyindolic material,
probably 5HTP, 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 5HTP
at concentrations as low as 0.025 μg/ml in plasma, or
0.05 μg/g in brain.

In animals treated with tryptophan alone 5HTP appeared
in the plasma but did not reach detectable concentrations
in brain tissue. However, following tryptophan loading
in the animals pretreated with α-methyl dopa, 5HTP
appeared in brain in concentrations increasing with time
(Table 22, Fig. 10), while 5HT and 5HIAA failed to in-
crease in concentration. These results are consistent
with the view that α-methyl dopa inhibits the decarboxy-
lation of 5HTP in vivo, resulting in the accumulation of
the substrate (5HTP) before the block and in the fall in
concentration of the metabolites (5HT and 5HIAA) beyond
it.

Despite the increase in brain concentrations of
5HTP, the plasma levels rose no higher in the α-methyl
dopa pretreated animals than in those given tryptophan
alone. The brain concentrations (μg/g) in the α-methyl
dopa 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)/

(Latha, 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 5HTP is at
least partially blocked), for uptake to proceed against
a concentration gradient and for 5HTP to accumulate in
the brain cells. This explanation assumes that 5HTP is
formed by hydroxylation of the tryptophan at extra-
cerebral sites, as suggested by Cooper and Melcer (1961)
and is transported to the brain in the plasma. A second possibility is that 5HTP
is formed in the brain by hydroxylation of tryptophan,
in the α-methyl dopa pre-treated animals, hydroxylation
would proceed in the brain with accumulation of 5HTP
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 vivo 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 α-methyl dopa than in
those treated with tryptophan alone (Table 17). These
results suggest that α-methyl dopa inhibits the uptake of
tryptophan/

tryptophan into brain. No direct evidence for an effect
of α-methyl dopa on 5HTP uptake into brain can be ad-
vanced, although the active uptake of this amino acid by
rat brain slices has been established (Schanberg and
Giarman 1960) and the inhibition of its uptake by α-methyl
dopa in rat brain slices has been demonstrated by Smith
(1963).

There was no evidence that α-methyl dopa, or its met-
abolites (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 5HIAA to show a rise in concentration
speaks against a reserpine-like action (Ashcroft and
Sharman 1962) and the lack of an increase in the 5HT levels
is contrary to expectation if amine oxidase inhibition
was occurring. Although such mechanisms are not com-
pletely excluded, in view of the reduced synthesis of
the 5HT brought about by α-methyl dopa, 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 α-methyl dopa administration (Hess,
Connamacher, Osaki and Udenfriend, 1961).

An inhibitory action on tryptophan 5-hydroxylase
has been postulated recently by Pletscher, Burkard and
Gey (1964) to account, in part, for the effect of α-methyl
dopa on cerebral 5-hydroxyindole metabolism. Two
observations/

observations in our experiments are consistent with,
but not conclusive$_\wedge$of, such an action. Firstly, the
proof
accumulation of 5HTP in the brain after pre-treatment with
α-methyl dopa and tryptophan loading did not equal or
surpass the combined increase in 5HT or 5HIAA observed
following tryptophan alone. This is not the result that
might be expected if decarboxylase inhibition was the
sole mode of action of α-methyl dopa. Secondly, the
plasma concentrations of 5HTP have been found in pre-
liminary experiments to be lower in tryptophan loaded
animals pre-treated with α-methyl dopa than in similar
animals given tryptophan alone (Table 18).

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
5HT in rat brain (Udenfriend, Weissbach and Bogdanski,
1957). In our experiments, animals loaded with tryptophan
when pre-treated with this inhibitor showed a rise in
concentration of 5HT in brain to 1.27 μg/g, considerably
higher than that seen in animals treated with tryptophan
alone (0.6 μg/g). The 5HIAA did not rise to 1.1 μg/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/

with this high dose of iproniazid, cerebral monoamine
oxidase inhibition is not complete. No effects of
iproniazid on the decarboxylation of 5HTP was 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 ex-
amination of each point of action of a drug, studying
simultaneously uptake of amino-acid precursors, synthesis
and breakdown of the amine.

SECTION 3

Tryptophan loading in dogs

INTRODUCTION

In a communication to the British Pharmacological
Society in 1952, Amin, Crawford and Gaddum reported the
presence of 5-hydroxytryptamine (5HT) in dog brain. In
their paper (1954) they described the detailed dis-
tribution of this amine in discrete areas of brain. It
was apparent that the concentration of 5HT in the various
areas was not uniform, central structures having com-
paratively high levels compared with cortex and
cerebellum. Of these central regions, hypothalamus and
midbrain had the highest concentrations. Values similar
to those reported by Amin et al. who used a bioassay
technique for the estimations, were obtained by other
workers Twarog and Page (1953), Paasonen and Vogt (1956),
Paasonen, McLean and Giarman (1957), also using a bio-
assay technique of measurement. Bogdanski, Weissbach
and Udenfriend (1957) using the fluorometric method of
assay of Bogdanski, Pletscher, Brodie and Udenfriend
(1956) found the relative distribution of the amine in
brain similar to that reported by these workers. However,
the absolute values were higher (Table 25).

In the preceeding section, it has been shown that
administration of tryptophan to rats led to an increase
in the concentration of 5HT and 5-hydroxyindol-3-ylacetic
acid (5HIAA) in whole brain. It was decided to administer
tryptophan/

TABLE 25

TABLE

Results obtained by various groups of workers for the concentration of 5-hydroxytryptamine (ng/g) in dog brain.

Area	Twang and Pape 1953		Amin, Crawford and Gaddum 1954		Garven 1955		Paasonen and Vogt 1956		Paasonen, McLean and Giarman 1957		Bogdanski, Weissbach and Udenfriend 1957		Eccleston, et al. 1965	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	± S.E. or Mean	Mean	± S.D.
Caudate			0	<4-<14 (5)*	97	45-130 (3)	262	68-480 (7)	229	213-320 (4)	720	200 (9)	210	30 (4)
Hindbrain													220	40 (5)
Midbrain			190	145-230 (3)							1000	200 (3)	560	100 (5)
Thalamus			18	14-22 (2)							570	70 (6)	240	110 (5)
Hypothalamus			280	220-330 (4)	814	415-1010 (3)	329	83-526 (7)	375	375 (1)	1700	300 (7)	550	160 (5)
Hippocampus			45	40-50 (2)					260	203-335 (3)	640	40 (3)	290	90 (5)
Cerebellum	140	60-220 (2)	0	<4-<10 (4)	0	712-730 (3)	9	7-12 (3)			<90	(3)	0	<50 (4)
Cerebral Cortex	140	90-190 (2)	0	<8-<10 (3)					36	10-75 (3)	170	80 (2)	0	<50 (4)
Extraction Method	4 vol. acetone Bioassay Venus Mercenaria		20 vol. acetone Bioassay oestrus rat uterus		20 vol. acetone Treatment with polyphenol oxidase Bioassay rat uterus		20 vol. acetone Bioassay Spisula solida		20 vol. acetone Bioassay Venus Mercenaria		2 vol. 0.1 N HCl Butanol extraction Fluorimetry		6 vol. acetone Paper chromatography Fluorimetry	

* Number of animals in parenthesis

tryptophan to dogs and observe the change in the
tryptophan and 5-hydroxyindoles in the various areas of
brain at varying time intervals after the dose of the
amino acid. Cerebrospinal fluid (CSF) was withdrawn
immediately prior to killing the animals and examined to
determine its tryptophan and 5HIAA content. The present
section gives results for oral loading of dogs and
discusses their significance.

METHODS

Analytical Procedures

    1.   5-hydroxyindoles in brain. These were estimated by the acetone extraction of homogenates of brain in acetic acid followed by paper chromatogrpahy, as described on p. 57.

    2.   5HIAA in CSF was estimated by Dr. D.P. O'Mahoney as follows:- 4 ml of CSF was acidified with 0.1 ml of conc. HCl, and saturated with sodium chloride. The 5HIAA was then extracted into peroxide free ether. The ether was washed with 0.02 N salt-saturated HCl and the 5HIAA back extracted into pH 6.5 0.3 M phosphate buffer. This was acidified with 0.5 vol. conc. HCl and estimated by spectrophotofluorimetry.

    3.   5-hydroxyindoles in plasma were estimated as described on p. 63.

    4.   Tryptophan in blood and brain. These estimations were carried out as described on p. 12.

    5.   Tryptophan in CSF. 0.5 ml CSF was diluted to 2.5 ml with distilled water in a 10 ml graduated centrifuge tube. Proteins were precipitated by the addition of 0.5 ml 30% (w/v) trichloracetic acid and the tubes allowed to stand for 10 min. They were then centrifuged at 3000 r.p.m. for 10 min. Two ml of the supernatant fluid were diluted to 5 ml with 0.1 N sulphuric acid. The tryptophan concentration in this solution was estimated by the norharman procedure (p.8).

APPLICATION OF METHODS AND RESULTS

Intraperitoneal administration of tryptophan in the
anaesthetised dog.

A dog was anaesthetised with intravenous pento-
barbitone. Anaesthesia was maintained throughout the
procedure. Tryptophan 200 mg/kg was given intra-
peritoneally in suspension in saline. Venous blood was
taken off at timed intervals up to 5 hr after the ad-
ministration of tryptophan. CSF was withdrawn at 0, 1,
from the cisterna
2 and 3 hr. Tryptophan was estimated in both sets of
samples. (Table 26).

The blood levels do not show an appreciable rise
when compared with those following intraperitoneal
tryptophan loading in the rat (400 mg/kg p. 81) (Table 20),
or those after oral loading in the conscious dog.
(Table 27).

The tryptophan in CSF showed a maximal rise of
six-fold over the control level. (Table 26).

Oral administration by capsule in the conscious animal

A venous blood sample was removed at timed intervals
after the administration of tryptophan. The blood
tryptophan (Table 27) showed a considerable rise with
the/

TABLE 26

The concentration of tryptophan in whole blood and CSF in dog following the intraperitoneal administration of tryptophan (200 mg/kg).

Time after tryptophan loading	Blood ($\mu g/ml$)	CSF ($\mu g/ml$)
0	10.4	0.6
0.5 hr	35.8	-
1 hr	37.9	3.0
2 hr	23.6	3.8
3 hr	20.0	2.6
4 hr	22.0	-
5 hr	20.8	-

TABLE 27

Concentration of tryptophan (μg/ml) in whole blood following the oral administration of L-tryptophan 50 mg/kg in the conscious dog.

Blood

Time after tryptophan loading	Tryptophan (μg/ml)
0	12.6
1 hr	92
2 hr	72
3 hr	44
5 hr	19.6
7 hr	16

the peak concentration at 1 hr, the earliest time of
sampling after the amino acid administration.

Oral administration of tryptophan in anaesthetised
animals subsequently allowed to recover.

Four dogs were used repeatedly in the same
experimental procedure over a period of several months.
For each experiment an animal was anaesthetised with
thiopentone sodium and whilst unconscious tryptophan
(200 mg/kg) in a suspension in water was given by stomach
tube. The animal was allowed to recover from this
anaesthetic and then anaesthetised once more just before
sampling of the CSF was carried out. (Table 28).

The effect of prolonged anaesthesia on the tryptophan
concentration in blood, brain and CSF following oral
administration of tryptophan to the anaesthetised animal.

Animals. Adult mongrel dogs of both sexes were
used, their weights varied from 7.7 to 21.8 kg. They
were housed separately prior to the experiment and fed
on a meat diet. Food was withdrawn 18 hr prior to the
experiment, water being available throughout the
experiment. Five dogs untreated with tryptophan acted
as controls.

Administration of tryptophan. Anaesthesia was
induced/

TABLE 28

Concentration of tryptophan in blood and CSF of
dogs following an oral load of tryptophan 200 mg/kg,
recovery being allowed after the initial anaesthetic.

Time after tryptophan loading	Blood (μg/ml)	CSF (μg/ml)
0	10.0 ± 2.6 (15)[x]	0.84 ± 0.48 (10)[x]
1 hr	46	-
2 hr	21.0 22.4	2.2
3 hr	188.0 78.0	8.1
4 hr	47.5 80.0	10.8 14.4

[x]   Mean $\pm$ Standard Deviation (number of observations)

induced with intravenous thiopentone in the dogs prior
to the administration of tryptophan (200 mg/kg) by
stomach tube, in the form of an aqueous suspension
containing 100 mg tryptophan/ml and stabilised by the
addition of a few drops of 'Tween 80'. Anaesthesia was
maintained throughout the remainder of the experiment.

Concentration of tryptophan in blood. Two ml of
blood was taken from a foreleg vein prior to tryptophan
administration and at the time of sacrifice (Dogs 2A,
2B, 2C and 4A). Blood was taken off prior to tryptophan
administration and at times $\frac{1}{2}$, 1, 2 and 3 hr (Dogs 3A
and 3B) and also a further sample at 4 hr in Dog 4B.
Table 29 shows the blood levels of tryptophan in five
dogs at intervals following the administration of the
amino acid. The figures demonstrate in the dogs where
the whole absorption curve for tryptophan was known
that the extent and time of rise of tryptophan in blood
showed considerable variation from animal to animal.
For Dog 3A there was evidence of a rise in blood
tryptophan only 2 hr following its administration. In
Dog 3B the rise in blood tryptophan, although occurring
in the first hour, was never particularly increased above
resting levels.

Concentration of tryptophan in brain.
Brain removal and dissection. A polythene cannula
was/

TABLE 29

Concentration of tryptophan (µg/ml) in whole blood of anaesthetised dogs following oral administration of tryptophan 200 mg/kg.

Time	Dog			Dog		Dog	
	2A [x]	2B	2C	3A	3B	4A	4B
0	14.8	8.6	10.2	8.3	8.0	10	6.4
0.5 hr	-	-	-	7.7	8.8	-	8.0
1 "	-	-	-	8.2	14.0	-	11.7
2 "	30.0	26.3	22.0	29.8	13.3	-	34.0
3 "	-	-	-	25.5	18.8	-	40.0
4 "	-	-	-	-	-	110	55.0

[x] Nomenclature adopted: number indicates time of sacrifice (hr after tryptophan load). The number together with a letter identifies the dog killed at that time.

was introduced into the femoral artery and after
administration intravenously of 1 ml (5000 units)
heparin the animal was bled out. As soon as the heart
stopped beating, the skull was opened by means of a
vibrating electric saw and the brain rapidly removed.
The brain was dissected rapidly into the various regions
to be analysed. Each part of brain was placed in a
plastic vessel, weighed and stored at -18°C for not more
than 30 min prior to homogenisation in acetic acid, in
the way described for rat brain (p. 57).

The following areas of brain, as defined, were
analysed:

1.   Cerebellum.

2.   Hindbrain. The brain posterior to the junction
between the pons and midbrain extending posteriorly
as far as the lower border of the medulla.

3.   Hypothalamus included the mamillary body but
not the hypophysis.

4.   Midbrain. That region from the upper border
of the pons to a point posterior to the mamillary
bodies.

5.   Caudate nuclei.) The boundaries of these areas

6.   Thalamus.   ) were visible to the naked eye.

7.   Hippocampus including the amygdala.

8.   Occipital cortex. This was a portion of the
occipital pole of the cortex, care being taken to
include as little white matter as possible.

Tryptophan levels in brain/

Tryptophan levels in brain.   The tryptophan levels
in the brain of normal animals was found to vary between
individuals (Table 30) and also between regions.[*] After
the administration of tryptophan, the brain concentration
of tryptophan rose (Table 31).   The levels attained at
the time of death did not however relate to the time
interval elapsing between tryptophan administration and
sacrifice, nor did they consistently relate to the
concentrations in whole blood at the time of death.
This is probably due to the inconsistent rate and degree
of absorption of the amino acid from the intestine.

Tryptophan concentration in CSF.   Prior to bleeding
out, the neck of the animal was flexed and 5 ml CSF was
taken from the cisterna into a polythene syringe.   The
fluid was stored in an all-glass 10 ml stoppered tube
at -18°C until estimation of tryptophan was performed
(not more than 4 days).

The results (Table 32) demonstrate a rise in the
concentration of tryptophan in CSF following the
tryptophan load.   The rise is, however, variable and
not directly related to the concentration of the amino
acid in brain at the time of death.

[*]   For evaluation see Appendix 2 (p.166).   (Table 51).

TABLE 30

Concentration of tryptophan in various areas of the brains of 5 normal dogs.

Areas	Tryptophan (μg/g)					Mean	$\pm$S.D.
	1	2	3	4	5		
Caudate	6.1	5.8	3.4	3.3	7.0	5.1	1.7
Hindbrain	6.6	9.8	5.3	6.5	6.4	6.9	1.7
Midbrain	6.1	10.0	4.3	6.4	7.4	6.8	2.1
Thalamus	9.5	9.7	8.0	6.3	5.6	7.8	1.8
Hypothalamus	7.0	9.5	4.5	9.5	✻7.3	7.6	2.1
Hippocampus	8.1	7.8	4.1	5.7	5.4	6.2	1.7
Cerebellum	8.4	14.3	5.0	6.8	8.2	8.5	3.5
Cortex	8.7	-	-	6.1	7.8	7.5	1.3

✻ Figure regenerated by statistical methods.

TABLE 31

Concentration of tryptophan (μg/g) in various areas of the brains of dogs at varying times up to 4 hours following the oral administration of L-tryptophan 200 mg/kg.

Tryptophan μg/g

Areas	Control Mean	2A	2B	2C	3A	3B	4A	4B
Caudate	5.10	31.0	19.8	-	13.4	15.0	79.0	70.0
Hindbrain	6.9	17.5	27.0	15.8	16.0	32.0	25.2	79.0
Midbrain	6.8	32.5	30.0	15.0	13.2	20.3	78.0	88.0
Thalamus	7.8	38.0	36.0	15.3	16.0	16.8	64.0	87.0
Hypothalamus	7.6	25.4	27.0	16.5	16.7	40.0	94.0	79.0
Hippocampus	6.2	24.5	34.0	15.1	13.6	29.6	85.0	84.0
Cerebellum	8.5	30.0	36.0	17.9	17.7	18.8	35.5	94.0
Cortex	8.7	35.0	-	21.7	-	-	29.0	-

TABLE 32

Concentration of tryptophan (µg/ml)
in CSF obtained immediately prior to
sacrifice.

Time after tryptophan load	Dog	Tryptophan (µg/ml)
0	OC	1.1
	OD	1.1
2 hr	2A	12.1
	2B	4.7
	2C	4.2
3 hr	3A	3.6
	3B	1.2

The concentration of 5-hydroxyindole substances in the
brain, plasma and CSF of the anaesthetised dog.

The homogenates obtained as described, from the
above dogs (pp. 57,112) were analysed for 5-hydroxyindoles.

The concentration of 5-hydroxyindoles in the brains
of control animals.

The estimates of the concentration of these
substances are show in Tables 33, 34 . These figures
have not been corrected for losses inherent in the
analytical procedure.

They show the uneven distribution of the 5-
hydroxyindolic compounds throughout the brain and also
their variation from one animal to another in any one
brain region. Midbrain and hypothalamus showed the
highest levels of both 5HIAA and 5HT. Neither 5HIAA nor
5HT was found in cerebellum and cortex. The ratio of
5HIAA to 5HT appeared to be a constant for each area.

Concentration of 5-hydroxyindoles in brain after oral
tryptophan loading.

Oral administration of tryptophan caused a rise in
both/

TABLE 33

Concentration of 5-hydroxytryptamine (5HT) in the brains of 5 normal dogs.

Areas	5HT µg/g					Mean	+S.D.
	1	2	3	4	5		
Caudate	-	0.19	0.19	0.19	0.25	0.21	0.03
Hindbrain	0.23	0.20	0.19	0.19	0.28	0.22	0.04
Midbrain	0.54	0.44	0.58	0.54	0.72	0.56	0.10
Thalamus	0.42	0.24	0.21	0.19	0.13	0.24	0.11
Hypothalamus	0.70	0.69	0.53	0.31	0.53	0.55	0.16
Hippocampus	0.31	0.14	0.30	0.30	0.39	0.29	0.09
Cerebellum	0	0	0	0			
Cortex	0	0	0	0			

TABLE 34

Concentration of 5-hydroxyindol-3-ylacetic acid (5HIAA) in the brains of 5 normal dogs.

Areas	5HIAA µg/g					Mean	±S.D.
	1	2	3	4	5		
Caudate	-	0.11	0.06	0.19	0.09	0.11	0.06
Hindbrain	0.17	0.44	0.15	0.30	0.26	0.26	0.12
Midbrain	0.53	1.00	0.51	0.54	0.86	0.69	0.23
Thalamus	0.56	0.35	0.18	0.13	0.19	0.28	0.18
Hypothalamus	0.40	0.64	0.19	0.30	0.37	0.38	0.17
Hippocampus	0.10	0.08	0.12	0.25	0.12	0.13	0.07
Cerebellum	0	0	0	0			
Cortex	0	0	0	0			

Corrected for 5HIAA fluorescence but not recovery.

both 5HT and 5HIAA in all areas at all times up to 4 hr
with two isolated exceptions (Tables 35 and 36)
The rise was greatest in those areas which begin with high
levels, the hypothalamus and midbrain.   Trace amounts
(< 0.05 µg/g) of 5HT were found in the cerebellum and
cortex of Dogs 2C and 4A.   These trace amounts may have
been derived from residual platelets in the tissue, the
amount present depending on how well the animal had been
bled out.   No 5HIAA appeared in these areas following
tryptophan loading.   5HTP was not detected (< 0.05 µg/g)
in any area at any time after tryptophan loading.

The concentration of 5-hydroxyindoles in dog plasma after
oral tryptophan loading.

Plasma obtained from Dogs 2A, 2B and 4A when the
animal was finally bled out was examined for 5-hydroxy-
indoles.   No 5HIAA or 5HTP was detected (< 0.05 µg/ml).
Very low levels (< 0.05 µg/ml) of 5HT were found to be
present, but as no particular precautions were taken to
avoid platelet destruction during centrifugation, it is
uncertain whether this was 'free' 5HT in plasma or that
released from platelets.

Concentration of 5HIAA in CSF following oral loading with
tryptophan.

There was a steady rise in the concentration of
5HIAA/

TABLE 35

Concentration of 5-hydroxytryptamine (μg/g) in various areas of the brains of dogs at varying times up to 4 hours following oral administration of L-tryptophan 200 mg/kg.

5HT μg/g

Areas	Control Mean	2A	2B	2C	3A	3B	4A	4B
Caudate	0.21	0.44	0.39	-	0.32	0.30	0.45	0.36
Hindbrain	0.22	0.43	0.36	0.33	0.35	0.38	0.74	0.42
Midbrain	0.56	1.04	1.00	0.93	0.79	1.02	1.40	0.65
Thalamus	0.24	0.30	0.29	0.55	0.36	0.36	1.04	0.65
Hypothalamus	0.55	0.59	0.88	0.58	0.79	0.90	1.88	1.25
Hippocampus	0.29	0.29	0.31	0.19	0.55	0.52	0.80	0.28
Cerebellum	-	0	-	0.05	-	-	<0.05	-
Cortex	-	0	-	0.05	-	-	<0.05	-

TABLE 36

Concentration of 5-hydroxyindol-3-ylacetic acid ($\mu g/g$) in various areas of the brains of dogs at varying times up to 4 hours following oral administration of l-tryptophan 200 mg/kg

5HIAA $\mu g/g$

Areas	Control Mean	2A	2B	2C	3A	3B	4A	4B
Caudate	0.11	0.21	0.21	-	0.17	0.10	0.26	0.19
Hindbrain	0.26	0.69	0.63	0.71	0.54	0.36	1.46	0.57
Midbrain	0.69	1.26	1.1	1.05	0.98	0.72	2.08	1.10
Thalamus	0.28	0.67	0.42	0.58	0.71	0.29	0.96	0.91
Hypothalamus	0.38	0.76	0.81	0.29	0.95	0.50	0.93	0.95
Hippocampus	0.13	0.23	0.20	-	0.35	0.22	0.31	0.14
Cerebellum	-	0	-	0	-	-	0	-
Cortex	-	0	-	0	-	0	0	-

5HIAA in CSF following oral loading with tryptophan
(Table 37). The /level of 5HIAA corresponded quite well
mean
in its rate of rise with the 5-hydroxyindoles in brain,
especially in relation to hypothalamus (Fig. 12). There
was, however, quite a wide variation between animals in
these levels at the various time intervals after tryptophan
loading.

Recoveries of 5HT, 5HIAA and 5HTP added to brain homogenate

The three 5-hydroxyindoles in 0.2 ml aqueous solution
containing 800 mµg of each were added to 4 ml of cere-
bellum or cerebral cortex homogenate. The mixture was
processed in parallel with homogenates from these and
other brain regions. The recoveries from several such
experiments are shown in Table 38. The variability of the
recoveries was found to be greater than that found using
rat whole brain.

Cerebellum and cerebral cortex were used for the
recovery experiments in that the 5-hydroxyindoles were not
normally found in these areas. Consequently any 5-
hydroxyindoles present in the recovery sample were
entirely due to added compounds and the variation in the
recovery was not influenced by the variation due to
duplicates as would have been the case should an area
containing 5-hydroxyindoles have been used. The figures
given for the concentrations of 5-hydroxyindoles in brain
were not corrected for these recoveries for two reasons.
Firstly/

TABLE 37

Concentration of 5HIAA (ng/ml) in CSF following oral
load of tryptophan 200 mg/kg. in the unconscious dog.

Dog	5HIAA Concentration
OA	25
OB	20
OC	56
Mean	33.7
2A	25
2B	63
2C	71
Mean	53
3A	127
3B	52
Mean	89.5
4A	126
4B	250
Mean	188

FIGURE 12

Concentration of 5 HT and 5HIAA in hypothalamus and 5HIAA in CSF (line) after oral loading of tryptophan (200 mg./kg.) in dog.
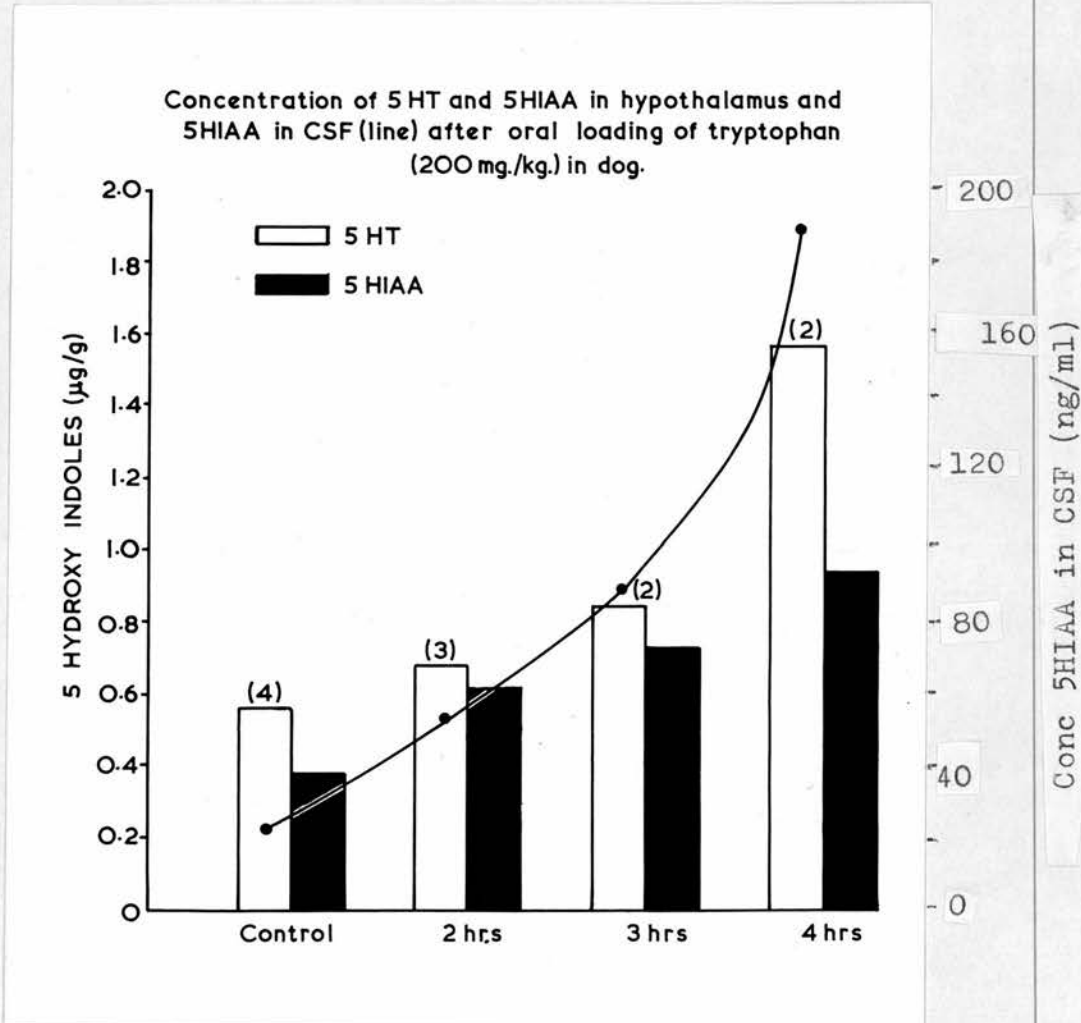
TABLE 38

Recoveries (%) of 800 mμg of pure solution of 5-hydroxyindoles added to brain homogenate and taken through the method.

5-Hydroxy indole	OA	OD	OD	OE	2A	2B	2C	3A	3B	4A	Mean±S.D.
5HTP	50	51	39	47	-	58	40	45	70	55	50.6±9.6
5HT	71	70	49	64	87	72	69	73	105	105	76.5±17.7
5HIAA	73	75	70	44	67	71	88	57	90	63	69.8±13.6

Firstly the variation in the recovery of 800 mμg of the 5-hydroxyindoles was large, and correction by the use of a mean figure for these recoveries would not have had a great deal of meaning. Secondly there was no evidence that the recovery of these indoles through the method was linear with the amount of indole added.

DISCUSSION

The Concentration of Tryptophan in various areas of brain

In 1960 Price and West, using acetone extracts identified tryptophan in various areas of brain in a number of animal species. They found a wide variation in the concentration in the different areas and, in the dog, a large range in the concentration of this amino acid between any one area in different animals (Table 39). The hypothalamus and pons had the highest levels; the midbrain, the cerebellum and the medulla were next in order, with spinal cord and cerebral hemispheres having the lowest concentrations. Their estimates of the concentration of 5HT in these areas correlated fairly well with the concentration of tryptophan in the same areas. However, the method of estimation employed by these workers (paper chromatography followed by colour reaction with a spray reagent) was insufficiently precise to give this ranking very much significance, and was not reasonable evidence for their hypothesis that 5HT was formed from tryptophan in brain. It is incorrect to assume that the concentration of substrate in a particular area is an index of the turnover of that substrate through a particular pathway without additional evidence.

In our own investigations, tryptophan was estimated in the various areas of five control dogs (Table 30). There appeared to be a difference in the concentration of the/

TABLE 39

Concentration of tryptophan and 5HT (μg/g) in various areas of dog brain with approximate rank order from Price and West, 1960.

Area	Tryptophan		5HT
	Concentration	Rank Order	
Hypothalamus	5 - 40	1	0.40
Pons	1 - 20	1	0.05
Midbrain	2 - 8	2	0.35
Cerebellum	1 - 4	2	0.02
Medulla	2 - 5	2	0.25
Cerebral hemispheres	0.1	3	0.01
Spinal cord	0.2	3	0.02

the amino acid between the areas. An analysis of
variance on the figures showed a significant difference
($P < 0.05$) in the concentration of the amino acid between
areas. When these concentrations were analysed by a
method which gave each area for each dog a rank order,
it was found that there was again a significant difference
in ranking for the areas ($P < 0.05 > 0.01$). The best
estimate of rank order is shown on Table 40, Appendix 2.

Whether these absolute levels have any physiological
significance is uncertain. It is however, interesting
to note that they are very roughly inversely correlated
with the concentration of dopa decarboxylase in the
various areas as given by Bogdanski et al. (1957)
(Table 40). This enzyme is thought to be identical with
5HTP decarboxylase (Holt z and Westerman 1957). This
variation in tryptophan concentration may be correlated
with the density of a particular cell type in the areas
examined. The amino acid pool or the rate of utilisation
of tryptophan may vary from cell to cell. It could,
however, be merely an artifact of the method of estimation
of tryptophan. This amino acid becomes strongly adsorbed
onto protein and cleavage of the adsorbed tryptophan
from the protein under the acid conditions brought about
by the addition of trichloracetic acid. The degree of
adsorption may vary from area to area. Multiple recovery
experiments would be necessary to prove this point.

A/

TABLE 40

Best estimate of rank order of brain areas with respect
to the concentration of tryptophan. Comparison of rank
order with dopa decarboxylase concentration.

Brain Area	Best Estimate of Rank Order	Dopa [x] Decarboxylase Activity $\mu g/5HT/g/hr$
Cerebellum	1	9
Thalamus	2	38
Hypothalamus	2	117
Hindbrain	4	28 (Pons) 32 (Medulla)
Midbrain	5	98
Hippocampus	6	16 (Ammon's Horn)
Caudate nucleus	7	306

[x] Bogdanski et al. (1957)

A two-way analysis of variance (Table 52) on these
figures shows a highly significant (P< 0.01) variation
in the concentration of tryptophan in the brains of
different dogs. This may be due to normal biological
variation or be methodological in origin.

Administration of tryptophan

Tryptophan was administered to the dogs orally
following a preliminary experiment in which a dog was
anaesthetised and an intraperitoneal injection made of
tryptophan (200 mg/kg) in saline suspension. Blood levels
of tryptophan showed no appreciable rise (Table 26) in
contrast to the markedly raised levels observed following
intraperitoneal injection of tryptophan (400 mg/kg) to
rats (p. 80). In view of the fact that oral dosage of 5 g.
(70 mg/kg approx.) tryptophan to man (Oswald, Ashcroft,
Berger, Eccleston, Evans and Thacore (1966) (Table 41)
resulted consistently in high levels of tryptophan within
1 hour of administration it was decided to use oral
loading tryptophan in dogs. Initially the tryptophan
(50 mg/kg) was given in capsule form in the conscious
animal. A good rise in the concentration of the amino
acid in blood resulted (Table 27). A dose of 200 mg/kg
required a large number of capsules (14-16) and it became
difficult to administer this number even to trained dogs.
Consequently the tryptophan was administered in sus-
pension in water by stomach tube after anaesthetising the
dog/

TABLE 41

Concentration of tryptophan in whole blood following
a loading dose of 5 g. tryptophan in man.

	Tryptophan μg/ml			
Subject	H	M	W	L
0 hours	7.3	6.9	9.2	7.5
½ "	8.8	17.8	29.2	42.0
1 "	38.0	32.2	75.0	76.5
2 "	67.5	30.8	108.0	67.3
6 "	67.5	35.4	45.3	52.9

dog with thiopentone sodium. Initially it was planned
to allow the animals to recover from the anaesthetic and
to readminister the anaesthetic at the time of killing
the animal. Four dogs, treated this way but not killed
at the second anaesthetic showed increases in tryptophan
in blood and CSF (Table 28). In another group of
animals in which it was intended to examine the brain
as well as the CSF, the first dog used was allowed to
recover from the first anaesthetic. It was not a dog
which had become used to these procedures and thereafter
showed such marked excitement that intravenous ad-
ministration of thiopentone was impossible. It was
consequently killed under nembutal anaesthesia given
intraperitoneally. After this experience the anaesthetic
for all other animals was continued from the time of
administration of tryptophan to bleeding out and death.
Initially tryptophan in blood was estimated in this group
of animals only at the beginning of the experiment and at
the time of death. Later, when it became apparent that
the tryptophan levels were not reaching the anticipated
concentrations throughout the experiment, estimates of
tryptophan concentration in blood were carried out at
timed intervals over the full duration of the experiment.

It was found (Table 29) that of these dogs examined
in this way, one showed a 2 hr delay in the initial rise
in the blood concentration (Dog 3A) while another showed
a peak blood tryptophan concentration which was consis-
tently lower than that of other animals (Dog 3B). It
would/

would appear then that oral loading in the unconscious animal does not give a predictable rise in either blood or brain tryptophan levels (Table 29) presumably due to delayed gastric emptying or impaired absorption. This may be a major factor in causing the highly variable results for the concentration of 5-hydroxyindoles in brain between dogs killed at the same time after the administration of tryptophan. Subsequent experiments have shown that intravenous administration of tryptophan (10 mg/ml in saline) in a dose of 50 mg/kg give predictable rises in the concentration of blood and brain tryptophan.

The concentration of 5-hydroxyindoles in the brains of control animals.

The control group in this series consisted of five dogs. There was a wide variation in the concentrations of 5HT and 5HIAA between the areas examined (Tables 33 and 34) The highest concentration of 5HT was found in midbrain and hypothalamus. The concentration of the acid metabolite paralleled that of the amine and was also highest in midbrain and hypothalamus. The concentration of these metabolites in specific areas, however, varied from dog to dog. This has been observed for 5HT by other workers (Paasonen and Vogt, 1956; Laverty and Sharman (1965) and may not be methodological in origin.

Changes in the concentrations of 5-hydroxyindoles in brain following oral tryptophan loading.

From/

From these results (Tables 35 and 36, Fig. 13)
it can be seen that following tryptophan administration
there was a rise in the concentration of both 5HIAA and
5HT in all the areas examined apart from cerebellum and
cortex. In these areas neither of the indoles were de-
tectable in control animals. However, very small amounts
of 5HT ($<$ 0.05 µg/g) were detected in the cortex and
cerebellum of two animals after tryptophan loading. These
small quantities may have been derived from blood still
present in the tissues at death in spite of the exs-
anguination. The rise in 5HT and 5HIAA in other areas
was very variable between animals at particular times
after administration of tryptophan. This may be due to
a combination of biological variation and variable
tryptophan absorption. If the full absorption curve
for each dog had been known prior to death then the
animals might have fallen into a different order with
respect to the time the tryptophan had been elevated in
blood prior to killing. The present order assumes,
incorrectly, that immediately the tryptophan is administered
absorption begins.

The significance of changes in the concentration of 5HIAA
in brain.

Ashcroft and Sharman (1962) investigated the effects
of reserpine on the concentrations of the acidic (probably
5HIAA) and basic (probably 5HT) 5-hydroxyindoles in the
caudate/

FIGURE 13
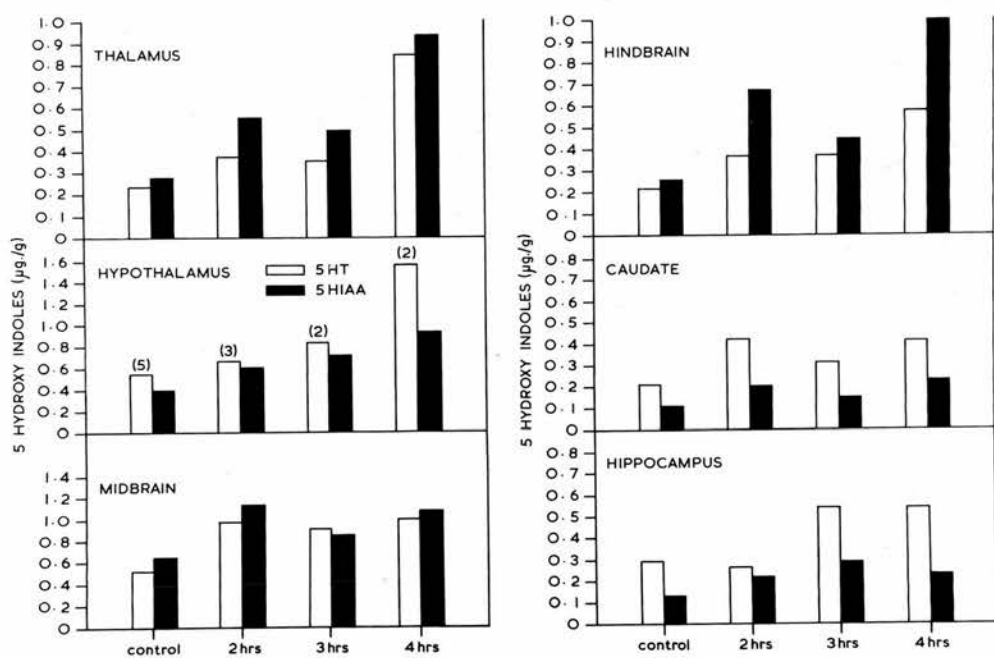
CONCENTRATION OF 5 HIAA AND 5HT IN VARIOUS AREAS OF DOG
BRAIN AT VARIOUS TIMES AFTER ORAL ADMINISTRATION OF TRYP-
TOPHAN 200mg./kg.

caudate nucleus of the dog. They found (Table 42) that
during the first two hours after the administration
of the drug (2 mg/kg intravenously) the concentration of
the basic 5-hydroxyindoles fell, that of acidic 5-
hydroxyindoles rose, but the concentration of the total
5-hydroxyindoles remained virtually unchanged. They
concluded that the increase in concentration of acidic
5-hydroxyindoles was probably a measure of the basic
5-hydroxyindoles released from stores by the action of
the reserpine, and converted to the acid by amine oxidase
and aldehyde dehydrogenase. The possibility thus arose
that the concentration of acid 5-hydroxyindoles might be used
as an index of 5HT turnover.

For the concentration of 5HIAA in brain to be useful
as an index of 5HT turnover certain criteria should be
fulfilled. Firstly the catabolism of 5HT by amine oxidase
and aldehyde dehydrogenase to 5HIAA should be the major
pathway in the metabolism of the amine. Secondly the
5HIAA present in brain must be derived almost solely
from the amine. The amount produced via other metabolic
pathways would require to be almost negligible in
proportion, particularly in situations in which a
decreased formation from the amine was occurring. In
connection with the foregoing suggestion of alternative
pathways for 5HIAA production it seems theoretically
possible, although there is no experimental evidence
for it that the acid might arise from 5-hydroxyindolyl-
pyruvic acid formed as a result of deamination of 5HTP.
Thirdly/

TABLE 42

Concentration of 5OR indolyl compounds in the caudate nucleus of the dog.

Concentrations expressed as mµg 5HIAA/g tissue from Ashcroft and Sharman (1962)

	Total 5OR indolyl compounds	Acidic 5OR indolyl compounds	Basic 5OR indolyl compounds
Control dogs	610	184	485
	845	285	631
2 hr after reserpine	650	478	172
(2 mg/kg intravenously)	670	557	189

Thirdly for an acid metabolite to reflect changes in the rate of synthesis of the amine it should accumulate in brain when an increase in synthesis occurs. On the other hand the acid should leave the brain cell and the brain as a whole sufficiently rapidly so that any decrease in the turnover of the amine is quickly reflected by a fall in the concentration of the acid metabolite. Finally the acid metabolite should not readily penetrate the brain from plasma.

The concentration of 5HIAA present in brain tissue at any point in time will be determined by the difference between its rate of synthesis and rate of removal, assuming that no further metabolism of the acid takes place. A rise in the concentration of the acid metabolite with time could result either from an increase in turnover of the amine or from a decrease in the transport of the metabolite out of brain. The converse of these would hold for a fall in the concentration of the metabolite. Its egress may be by way of the blood stream or the CSF or both. It is not known what is the relative importance of these processes. The changes in the levels of 5HIAA in CSF follow quite closely changes in the levels of the acid in brain after tryptophan loading (Fig12). It seems likely that this acid, like other organic acids might be transported out of the CSF by some mechanism located in the region of the fourth ventricle (Pappenheimer 1961). 5HIAA was/

was not detectable in the plasmas of dogs we have examined after tryptophan loading. There is evidence (Milne, Crawford, Girao and Loughridge 1960) that this acid is actively transported out of the plasma by the kidney and hence the plasma is rapidly cleared of this metabolite.

In our observations in rat and dog brain after tryptophan loading the rise and fall in the concentration of 5HIAA followed closely that of 5HT. It will be seen from Fig. 9   that, following tryptophan loading, there was a fall in the concentration of 5HIAA in whole rat brain of 0.6 µg/g over the two hour period covered by the seventh and eighth hours after the tryptophan administration. The potential rate of loss of 5HIAA from brain would be higher than this in that during this period normal or supranormal synthesis of 5HIAA would still be proceeding. The level of 5HT in the stores was continuing to decrease and was presumably being metabolised to 5HIAA. On this evidence it would seem that 5HIAA can leave brain fairly rapidly.

There is, however, experimental evidence which apparently conflicts with this conclusion. Ashcroft and Crawford (unpublished observations) treated rats with reserpine (5 mg/kg intraperitoneally) and observed the changes in the concentration of the 5HIAA and 5HT in whole brain at times from 10 min to 48 hr after the administration of the drug. As can be seen (Table 43) there was/

TABLE 43

Concentration of 5HT and 5HIAA in whole rat brain
up to 48 hr following the intraperitoneal injection
of reserpine 5 mg/kg (Ashcroft and Crawford, Un-
published observations).

Treatment	Time	5HT $\mu g/g$	5HIAA $\mu g/g$	5HT + 5HIAA $\mu g/g$
None	0	0.62	0.18	0.80
Reserpine	10 min	0.49	0.30	0.79
	45 min	0.43	0.49	0.92
	90 min	0.23	0.48	0.71
	21.5 hr	0.07	0.46	0.53
	48 hr	0.13	0.30	0.43

was a significant fall in 5HT and rise in 5HIAA within
10 min of injection of the drug. The concentration of
the acid metabolite remained elevated long after the 5HT
had been depleted. In similar experiments performed on
rabbits, Roos (1962) (Table 44) also found that the 5HT
and Werdinius
concentration in brain rapidly decreased but the
concentration of 5HIAA remained elevated for some 24 hr
after the maximal depletion of 5HT. The observations
of Roos and Ashcroft and Crawford would appear to be
against the hypothesis that the brain levels of 5HIAA
would be an index of 5HT turnover. However, this
apparently contrary evidence is not conclusive. Other
explanations are possible. Reserpine might increase the
turnover of 5HT in the absence of a storage mechanism for
the amine. Reserpine might interfere with a mechanism of
active transfer transporting 5HIAA from the cell in the
brain.   In consequence a "pool" of 5HIAA would
be formed the rate of removal of which was determined
purely by the physico chemical properties of 5HIAA in
relation to its permeability of lipid membranes. Our
own observations have shown that the drug probenecid used
to influence the transport of organic acids in kidney, is
effective in preventing the egress of 5HIAA from CSF. In
high doses Sharman (personal communication) has demon-
strated that it causes a rise in homovanillic acid in rat
brain, and in our own laboratory under similar conditions
5HIAA/

TABLE 44

Concentration of 5-hydroxytryptamine and 5-hydroxy-3-ylacetic acid in rabbit brain at varying intervals following intravenous injection of reserpine (2-5 mg/kg body weight) after Roos (1962).

No. of animals	Time after injection	Brain stem	
		5HT	5HIAA
8	0 hr	0.7	0.94 ± 0.041
2	2 hr	0.02	1.3
2	6 hr	0.01	1.7
5	12 hr	0.02	1.5
2	24 hr	0.01	1.0
2	48 hr	0.07	0.5

5HIAA has been shown to rise after administration of the drug. This drug may well prove useful in the investigation of this transport process.

The significance of the ratios of 5HIAA to 5HT in the various regions of dog brain.

Examination of the data (Tables 33/ and 34 for 5HT and 5HIAA in the various regions of dog brain showed that the ratio of the concentration of the acid metabolite to that of the amine was not constant throughout the brain (Table 45). Although it is not possible to test by statistical analysis whether the ratio is, in fact, significantly different between one area and another, it is of interest to consider the implications following the assumption that the differences are indeed real. If the level of 5HIAA can be equated with the turnover of 5HT then areas with similar 5HT concentrations but different 5HIAA/5HT ratios would have different rates of turnover of the amine and consequently the turnover of 5HT is not related to the storage capacity for 5HT.

On this basis, the turnover of 5HT in the hindbrain and thalamus would appear to be similar but greater than that in either the caudate nucleus or the hippocampus. Also the midbrain with 5HT levels similar to that in the hypothalamus shows a greater turnover of the amine than the hypothalamus.

Such/

TABLE 45

Ratio of concentration of 5HT to 5HIAA in the
various areas of 5 normal dogs.

Area	Dog					Mean
	1	2	3	4	5	
Caudate	-	1.7	3.2	1.0	2.8	2.2
Hindbrain	1.4	0.45	1.3	0.63	2.3	1.2
Midbrain	1.0	0.44	1.1	1.0	0.83	0.87
Thalamus	0.75	0.68	1.2	1.5	0.68	0.96
Hypothalamus	1.8	1.1	2.8	1.0	1.4	1.6
Hippocampus	3.1	1.8	2.5	1.2	3.3	2.4

Such conclusions are dependent on the following additional assumptions; firstly that the facility for removal of 5HIAA does not vary from region to region; secondly that the differences in the relative concentrations of 5HIAA and 5HT do not merely reflect differences in the post-mortem activity of amine oxidase in the various regions.

GENERAL CONSIDERATIONS

The main advantages of the technique of tryptophan loading combined with the simultaneous examination of several metabolites in the synthesis and breakdown of 5HT is that a more dynamic picture of the metabolism of the amine can be gained and that the action of drugs like α-methyl dopa and iproniazid are more easily demonstrated.

In both dog and rat there was a rise in the concentrations of the 5-hydroxyindolyl compounds in brain following oral administration of tryptophan. That these increases took place at all was rather surprising, the tendency being for biochemical systems to possess feedback mechanisms which prevent over-synthesis of a particular metabolite. Although these concentrations rose, there were no apparent behavioural effects in the conscious dog. It could well be that the release of 5HT on to (what are still hypothetical) receptor sites was unchanged, and the additional 5HT formed was rechannelled into stores, or sites of loss such as monoamine oxidase. There is indeed no reason to assume that the 5HIAA is formed exclusively by oxidative deamination of 5HT or indeed that at all times after the administration of tryptophan the proportion metabolised through any particular pathway is constant.

From a consideration of experimental design the use of/

of oral loading in the unconscious dog is unsatisfactory because absorption from the gut is unpredictable and often delayed, hence the rise in the 5-hydroxyindoles in brain cannot be related on a time basis from the time of administration of the amino acid. This difficulty could be overcome by more frequent sampling of blood with estimation of its tryptophan content. Even so, the absolute rise in the concentration of the amino acid varies from animal to animal, and even in the same animal varies between experiments. Subsequent work has shown that reproducible changes in the concentration of 5-hydroxyindolic compounds in brain can be produced by the intravenous administration of L-tryptophan in the dog.

APPENDIX 1

Further investigation of acetone extraction procedure
as applied to dog brain.

Comparison of results of acetone extraction procedure with
a perchloric acid precipitation of the acetic acid
homogenate followed by ion exchange column chromatography.

An aliquot of brain homogenate from various areas of
the brain of dog 4B which was being examined by paper
chromatography was analysed in parallel for its content
of 5HT by a method involving perchloric acid pre-
cipitation and column chromatography. The actual volumes
of homogenate precipitated by this method depended on the
residual volume after 4 ml homogenate had been removed from
the total for estimation by paper chromatography. Con-
sequently, the volume of midbrain used was 1.5 ml, caudate
2 ml. Five ml of hippocampus cerebellum, midbrain and
thalamus of which there is a considerable initial volume
of homogenate, was processed by the ion exchange method.
The method is that described for guinea pig brain in
Section 1 (p. 30) except that to the amine containing
fraction from the ion exchange column is added conc. HCl
(50% by vol) and the concentration of 5HT estimated by
spectrophotofluorimetry on an Aminco-Bowman fluorimeter.
The areas examined are shown in Table 46.

Similar parallel estimates were made on homogenates
of/

of brain from various areas of two dogs given an
intravenous load of tryptophan (50 mg/kg) in a 10 mg/ml
solution in saline, the animals being killed 2 and 4 hr
after injection (Dogs IV 2B and IV 4C respectively. Table
46).

The results show that analyses by the column method,
with few exceptions give consistently higher values for
the concentration of 5HT in the various areas of brain
than the estimate reached by acetone precipitation and
paper chromatography.

Examination of the petrol ether phase used to extract
lipids from the aqueous phase after acetone extraction

The two methods differ in the additional step of the
use of petrol ether to remove lipids after acetone pre-
cipitation. As it is possible that 5HT is lost in this
phase complexed with lipid the petrol ether phase used
in an extraction of the extract of midbrain, thalamus and
hypothalamus was retained and analysed for its content
of 5-hydroxyindoles (this would also contain any 5HIAA
transferred from the extract). The petrol ether phase
was blown down to dryness with nitrogen. The lipid was
taken up in 4 ml of butanol and the 5-hydroxyindoles
extracted by shaking with 2 ml 0.1 N H_2SO_4 after addition
of 4 vol (16 ml) of heptane to the butanol. The 2 ml
H_2SO_4 was made up to 4 ml with 2 ml 0.1 N H_2SO_4 and 2 ml
conc./

TABLE 46

Comparison of concentration of 5-hydroxytryptamine in brain homogenates as estimated by acetone precipitation and paper chromatography, perchloric acid precipitation and column chromatography.

Dog	Area	PCA and column	Acetone and paper
4B	Midbrain	2.3[+]	1.307[*]
	Caudate	0.66	0.85
	Thalamus	1.02	0.47
	Hippocampus	0.57	0.37
	Hind brain	0.45	0.55
IV. 2B	Mid brain	1.74	1.54
	Caudate	1.05	0.58
	Thalamus	0.73	0.65
	Hippocampus	0.74	0.64
	Hind brain	0.44	0.64
	Hypothalamus	2.14	1.75
	Cortex	$>0 <0.05$	0.05
	Cerebellum	$>0 <0.05$	0.05
IV. 4C	Mid brain	0.94	0.71
	Caudate	0.34	0.25
	Thalamus	0.37	0.25
	Hypothalamus	1.00	0.41

[+]Results corrected for 76.5% recovery of 5-HT

[*]Corrected for recovery of individual experiment

conc. HCl added. The solution was examined by Farrand spectrophotofluorimeter for the presence of compounds with 5-hydroxyindole fluorescence.

Only traces (> 0 < 0.05 µg) of 5-hydroxyindole could be detected in the petrol ether.

Extraction of precipitate remaining after acetone precipitation with perchloric acid.

In that the difference between the two methods could be accounted for by a difference in the percentage extraction of 5HT present in the brain (as opposed to that quantity of pure solution added to the homogenate to act as an internal standard) the precipitate remaining after acetone precipitation was extracted with perchloric acid and examined by ion exchange column chromatography. The precipitate remaining after removal of the aqueous acetone supernatant from midbrain and hippocampus of dog was blown to dryness with nitrogen. This was then homogenised with 10 ml 0.4 N perchloric acid, allowed to extract overnight, and then centrifuged. The supernatant was adjusted to pH 7.4 and run over the columns as described above. The amine fraction was examined for the presence of 5-hydroxyindoles. Only traces (> 0 < 0.05 µg/g) of 5-hydroxyindole was detected by this method.

Examination of supernatants by ion exchange chromatography after P.C.A. precipitation compared with acetone precipitation.

It/

It was possible that the ion exchange column chromatography procedure itself for some unknown reason gave high values for 5HT and hence the supernatants after acetone precipitation and perchloric acid precipitation were examined by ion exchange column chromatography. The brain of a dog which had been killed by a large dose of intravenous Nembutal was dissected to remove midbrain and thalamus. These parts were homogenised in 1 vol 40% (v/v) and 2 vol 20% (v/v) acetic acid. Each homogenate was divided into two portions. One portion was analysed by perchloric acid precipitation of the homogenate followed by ion exchange column chromatography and spectrophotofluorimetry of the amine fraction using an Aminco-Bowman fluorimeter. The proteins of the other portion were precipitated by addition of 6 vol acetone and the aqueous phase prepared as described above. The lipids were removed by shaking with petrol ether. The aqueous phase was made up to 6 ml with distilled water and the pH adjusted to 7.4 (glass electrode). The 5HT was estimated after separation by column chromatography and spectrophotofluorimetry on an Aminco-Bowman spectrophotofluorimeter. Amounts of 500 mµg of pure solution of 5HT were added to equal volumes of homogenate and taken through the procedure to act as an internal standard.

After calculating for recovery the results (Table 47) show no great discrepancy between the two methods.

TABLE 47

Concentration of 5HT (μg/g) in two areas of brain
as estimated by (a) perchloric acid precipitation
and column chromatography and (b) acetone precipi-
tation and column chromatography.

Method

Area	P.C.A. and Column	Acetone and Column
Hindbrain	1.04	1.15
Thalamus	0.34	0.21

A comparison of the estimates of the concentration of
5HT by the Aminco-Bowman and Farrand spectrophoto-
fluorimeter in the same samples.

The various areas of the brains of two dogs were
examined by the procedure of acetone precipitation and
paper chromatography. The eluates from the 5HT and 5HIAA
containing strips were acidified with concentrated HCl and
for each sample the fluorescence due to the 5-hydroxyindole
present was determined using a Farrand spectrophoto-
fluorimeter and immediately therafter the determination
was repeated using an Aminco-Bowman spectrophotofluorimeter.
The estimates corresponding to the two determinations are
shown in Table 48. It is evident that the estimate of
the 5-hydroxyindole content on any one sample was higher
when the Aminco-Bowman fluorimeter was used for determining
the fluorescence intensity.

TABLE 48

Area	5HT (µg/g)		5HIAA (µg/g)	
	Farrand	Aminco-Bowman	Farrand	Aminco Bowman
Dog 1				
Midbrain	0.64	0.70	0.43	0.66
Hindbrain	0.18	0.25	0.23	0.33
Hippocampus	0.38	0.49	0.13	0.18
Caudate	0.25	0.74	0.20	0.35
Thalamus	0.21	0.30	0.28	0.39
Cerebral Cortex	0.19	0.49	< 0.05	0.30
Dog 2				
Midbrain	0.752	0.89	0.70	1.06
Hypothalamus	0.82	1.03	0.65	0.99
Hindbrain	0.22	0.27	0.22	0.32
Hippocampus	0.33	0.41	0.20	0.35
Caudate	0.25	0.35	0.20	0.55
Thalamus	0.30	0.40	0.39	0.66
Cerebral Cortex	< 0.05	0.21	< 0.05	0.08

DISCUSSION

Several workers have considered whether the figures
they obtained for the concentration of 5HT in brain were,
for any reason, low. Amin, Crawford and Gaddum (1954)
used a bioassay technique to determine the concentration
of the amine. They made comparisons between various
biological preparations and, hence, established to some
measure the specificity of their assays. This also gave
a quantitative verification of the amount of the amine
present in their final extracts. They did, however,
consider the possibility of interfering substances
reducing the sensitivity of their preparations. During
their investigations, they considered that the two
possible interfering substances were adrenaline and
noradrenaline but the concentration of the latter as
indicated in the work of Vogt (1954) would be insufficient
to interfere with the estimation of 5HT. Adrenaline could,
however, have been present in certain areas in sufficient
concentration to produce inhibition of the biological
preparation and, hence, apparently low values for 5HT.

Garven (1955), investigating the problem of the
interference of adrenaline and noradrenaline in the
bioassay of 5HT extracted from dog brain, used mushroom
extracts containing polyphenol oxidase to break down any
catechol amines extracted by the procedure of Amin et al.
(1954). The remaining 5HT was assayed using the isolated
oestrus/

oestrus rat uterus preparation. She detected the amine
in caudate nucleus and hypothalamus obtaining higher
figures for the concentration of the amine than reported
by Amin et al. (1954) (Table 49). However, the bio-
logical response of the preparation was not completely
abolished by lysergic acid diethylamide, and hence the
possibility arose that some of the biologically active
material was other than 5HT.

Another source of error is the extraction procedure
itself. Amin et al. (1954) extracted brain tissue with
20 ml of acetone per g of tissue followed by re-extraction
of the insoluble residue with 95% acetone. Their
acceptance of the efficiency of the extraction procedure
was a 98% recovery of relatively large amounts of 5HT
(5 μg) added to 10 g of minced horse intestine. Twarog
and Page (1953) found that, using 20 vol of acetone
recoveries were 60% from brain. Correale (1956) in-
vestigating the concentration of acetone to water which
produced the best recoveries, found 80% (4 vol) to be
optimal, whereas 95% (20 vol) gave fairly low recoveries.
Bogdanski et al. (1956) also found the recoveries using
95% acetone to be low and variable. Sharman (1960)
re-examined the extraction method of Amin, et al (1954)
and in some of his initial experiments found a variable
proportion of 5HT to remain in the precipitate following
acetone extraction. He found, however, that careful
mincing of the tissues eliminates this loss.

Another/

TABLE 49

Acetone extracts of dog hypothalamus and caudate nucleus (mushroom treated) from Garven (1955).

Method of Assay	5HT equivalent ng/g	
	Hypothalamus	Caudate nucleus
Rat uterus	770	130

Another source of loss he found to be in the
extraction of some of the 5HT into light petroleum ether
in the course of lipid removal from the aqueous residues
left after removal of the acetone extracts. He found
quite large quantities (50% of total) of 5HT entered this
phase (Table 50). In our own experiments, recoveries
although somewhat variable (Table 38), were quite high.
Even so, in the figures we obtained for 5HT in dog brain,
when corrected up for these recoveries are still low,
compared with those obtained by Bogdanski et al. (1956)
(Table 25). It would, therefore, be necessary to postulate
either that there is considerable biological variation and
in the group of animals which we have used the levels en-
countered by Bogdanski were not reached or, secondly, that
a certain portion of the 5HT in brain did not behave
identically with the 5HT added in pure solution to
give an estimate of the recovery through the procedure.
A possibility was that the tissue 5HT might be complexed
with lipid or other material and in this form was not
completely extractable under the conditions we employ.
It might be that to break such complexes, fairly acid
conditions would be necessary, those for instance
obtaining in the procedures of Bogdanski et al. (1956)
The pH of their HCl homogenates diluted with water to
9 vol is found to be about 2.0. Secondly, we may have
been losing 5HT complexed with lipid into the light
petroleum phase (Gal et al. 1964). We investigated this
on/

TABLE 50

Biological estimation of 5HT and fluorescence
estimation of 5-hydroxyindolyl compounds in the
fractions of an acetone extract (Amin et al.) of
rat brain (estimated as 5HT) from Sharman, 1960.

	Estimation on rat fundus strip (5HT)	Fluorescence (as 5HT)
Final aqueous extract	2.0 µg	2.8 µg
Light petroleum washings	1.0 µg	1.7 µg

on three samples from different brain areas by the method
described by Sharman, but could only detect insignificant
amounts of 5-hydroxyindole. The difference between our
own extraction method and that employed by Sharman was
that the acetone extracts were prepared in the presence
of acetic acid and this acid in the aqueous phase might
prevent the formation of a 5HT lipid complex and, hence
reduce the extractability of the 5HT into the light
petroleum.

The effect on the 5HT estimates resulting from the
use of different methods of protein precipitation
was examined by comparing the estimates of the con-
centration of 5HT in portions of the same brain homogenate
treated by acetone precipitation and by perchloric acid
precipitation and column chromatography. Higher levels
were found by the latter technique. This would tend to
support the view that we were failing to extract some
'bound' 5HT at the pH (about 3) of the precipitations when
compared with that obtained during perchloric acid
precipitation (about 1).

To investigate this question of extractability, the
concentration of 5HT obtained by precipitating a portion
of the same brain homogenate with acid acetone estimated
by column chromatography procedure and fluorimetry (by
Aminco-Bowman fluorimeter) was compared with the con-
centration found by perchloric acid precipitation,
column/

column chromatography and fluorimetry (Aminco-Bowman
fluorimeter). The results under these circumstances
were in good agreement. Only two areas were, however,
examined in this way.

Precipitates incompletely extracted by the acid
acetone should contain 5HT extractable by perchloric acid.
An attempt to extract this residual 5HT by perchloric
acid followed by separation by column chromatography and
estimation (Aminco-Bowman fluorimeter) showed negligible
amounts (< 0.1 μg) to be extracted by this procedure.

During these investigations one point had been
overlooked. The final fluorescence measurement was
carried out using either a Farrand or an Aminco-Bowman
spectrophotofluorimeter. With our instruments, the latter
was more sensitive than the former. The Aminco-Bowman
spectrophotofluorimeter was generally used after column
chromatographic procedures when the volume of homogenate
used was smaller than for the paper chromatographic
procedure and hence greater sensitivity of the measuring
instrument was necessary. An experiment was performed
in which the eluate from paper strips containing either
5HIAA or 5HT in varying concentrations from various
areas of dog brain were measured on both instruments.
Table 48 shows that there was a discrepancy between the
estimates as obtained by measuring the fluorescence on the
two instruments, the figures from the Aminco-Bowman being
higher.

It/

It would thus appear that we have introduced an
artifact in to our methods of investigating the dis-
crepancies in the reported concentration of 5HT in brain.
The estimates of the concentration of 5HT following
perchloric acid precipitation and column chromatography
were based without exception on readings obtained from
the Aminco-Bowman spectrophotofluorimeter, those from
paper chromatography on readings obtained from the
Farrand spectrophotofluorimeter. In the absence of any
real difference between the efficacies of the extraction
procedures, those results from procedures involving column
chromatography would give higher figures because of the
fluorimeter variation. This discrepancy does not account
for the large differences in our own results to those in
the literature.

APPENDIX 2

Ranking analysis of tryptophan.

TABLE 51

Areas	Dogs					Rank Totals	Best estimate of rank order
	1	2	3	4	5		
Caudate	6.5	7	7	7	4	31.5	7
Hindbrain	5.0	3	2	3	5	18.0	4
Midbrain	6.5	2	5	4	2	19.5	5
Thalamus	1.0	4	1	5	6	17.0	2=
Hypothalamus	4.0	5	4	1	3	17.0	2=
Hippocampus	3.0	6	6	6	7	28.0	6
Cerebellum	2.0	1	3	2	1	9.0	1
	28.0	28	28	28	28	140.0	

Expected rank total if no difference between samples

$$= \frac{m(n + 1)}{2} = \frac{5 \times 8}{2} = 20$$

where m = number of columns = 5

n = number of rows = 7

If no true agreement between dogs the sum of squares of differences between observed and expected rank totals are maximum and given by:-

Smax $= \frac{m^2 (n^3 - n)}{12}$

In that there may be only partial agreement between dogs the sum of squares will be S.

The/

The coefficient of concordance, $W = \dfrac{S}{S_{max}}$

$$W = \frac{339.5^{\times12}}{25(336)}$$

$$= \underline{0.485}$$

Test of significance using Snedecors distribution of F

Continuity correction of W:

$$W' = \frac{(S-1)}{\dfrac{m^2(n^3-n)}{12} + 2}$$

$$W' = 0.48219$$

Snedecor's $F = \dfrac{(m-1)W'}{1-W'}$

$$= 3.7248$$

Degrees of Freedom $= (n-1) - \dfrac{2}{m}, \left[(m-1)(n-1) - \dfrac{2}{m}\right]$

$$= \underline{5.6, \quad 22.4}$$

There is thus a significant ranking of the results ($P < 0.05, > 0.01$).

Regeneration of 'lost' hypothalamus result in dog (p.117).

TABLE 52

Areas	Dogs					$\sum xj$
	1	2	3	4	5	
Caudate	6.1	5.8	3.4	3.3	7.0	25.6
Hindbrain	6.6	9.8	5.3	6.5	6.4	34.6
Midbrain	6.1	10.0	4.3	6.4	7.4	34.2
Thalamus	9.5	9.7	8.0	6.3	5.6	39.1
Hypothalamus	7.0	9.5	4.5	9.5	y'	30.5 R
Hippocampus	8.1	7.8	4.1	5.7	5.4	31.1
Cerebellum	8.4	14.3	5.0	6.8	8.2	42.7
$\sum xi$	51.8	66.9	34.6	44.5	40.0	237.8 G

Finney $y' = \dfrac{rR + cC - G}{(r-1)(c-1)}$

When y' = term to be regenerated

r = number of rows (Areas)

R = total of row (Hypothalamus) without missing number

c = number of columns (Dogs)

C = total of column (Dog 5) without missing number

G = grand total without missing number.

$y' = \dfrac{7 \times 30.5 + 5 \times 40.0 - 237.8}{6 \times 4}$

$= \underline{7.32 \ \mu g/g}$

TABLE 53

Two-way analysis of variance of tryptophan concentration in various

areas of the brains of 5 dogs

Areas	Dogs						$\sum x^2 j$	$T^2 j$
	1	2	3	4	5	Total		
Caudate	6.1	5.8	3.4	3.3	7.0	25.6	142.3	655.36
Hindbrain	6.6	9.8	5.3	6.5	6.4	34.6	250.9	1197.16
Midbrain	6.1	10.0	4.3	6.4	7.4	34.2	251.42	1169.64
Thalamus	9.5	9.7	8.0	6.3	5.6	39.1	319.39	1528.81
Hypothalamus	7.0	9.5	4.5	9.5	7.3+	37.8	303.04	1428.84
Hippocampus	8.1	7.8	4.1	5.7	5.4	31.1	204.91	967.21
Cerebellum	8.4	14.3	5.0	6.8	8.2	42.7	413.53	1823.29
Total	51.8	66.9	34.6	44.5	47.3	245.1	1885.49	8770.31
$T^2 i$	2683.24	4475.61	1197.16	1980.25	2237.29	12573.55		

+ Figure regenerated statistically

Two-way analysis of variance of tryptophan concentration in various areas of the brains of 5 dogs cont'd.

Variation (Total) $= \sum \sum x_{ij}^2 - \dfrac{\bar{I}^2}{N}$

$= 1885.49 - \dfrac{(245.1)^2}{35}$

$= 1885.49 - 1716.40$

$= \underline{169.0897}$

Variation (Areas) $= \sum n_j (\bar{x}_j - \bar{x})^2$

$= j \left\{ \dfrac{T_j^2}{n_j} \right\} - \dfrac{T^2}{N}$

$= \dfrac{8770.31}{5} - 1716.40$

$= \underline{37.662}$

Variation (Dogs) $= \sum n_1 (\bar{x}_1 - \bar{x})^2$

$= 1 \left\{ \dfrac{T_1^2}{n_1} \right\} - \dfrac{T^2}{N}$

$= \dfrac{12573.55}{7} - 1716.40$

$= \underline{79.8214}$

Error $=$ Total $-$ Areas $-$ Dogs

$= 169.0897 - 37.662 - 79.8214$

$= \underline{51.606}$

Two-way analysis of variance of tryptophan concentration in various areas of the brains of 5 dogs.   cont'd

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Areas	6	37.662	6.277	$\frac{6.277}{2.150} = 2.9195$
Dogs	4	79.8214	19.955	$\frac{19.955}{2.150} = 9.281$
Error	23	51.606	2.150	
Total	33	169.0897		

Between Areas $P \left\{ F = 2.51, \ d.f. \ (6.23)* \right\}$ <0.05 significant

Between Dogs $P \left\{ F = 4.22, \ d.f. \ (4.23) \right\}$ <0.01 very significant

* Regenerated result not included in degrees of freedom.

SUMMARY

SUMMARY

SECTION 1 - Tryptamine

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, in-
corporating 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 ad-
ministered following pretreatment with an amine oxidase
inhibitor.

SUMMARY

SECTION 2 - Tryptophan loading in rats

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 5HTP, 5HT and 5HIAA were determined
in the brain at times up to 8 hr after loading with
tryptophan.

No 5HTP was detected in the brain after tryptophan
loading, although it appeared in measurable amounts in
the plasma. The 5HT in brain rose rapidly to a level
which remained constant over a period of hours. The
5HIAA in brain also increased rapidly, reaching a maximum
at a time later than 5HT. These concentrations were
maintained for a longer period with larger doses of
tryptophan.

a-Methyl dopa treatment before tryptophan loading
prevented the increases in 5HT and 5HIAA which occur
following tryptophan administration alone, and also
resulted in the appearance of the amine precursor, 5HTP;
it may also interfere with the uptake of tryptophan from
plasma/

plasma into the brain.   Iproniazid treatment before

tryptophan loading increased the concentration of 5HT

and diminished the rise of 5HIAA, with no apparent effect

on the uptake of tryptophan or the decarboxylation of

5HTP in the brain.

SUMMARY

Section 3 - Tryptophan loading in dogs.

The technique of estimating the concentration of
the 5-hydroxyindoles and tryptophan is applied to various
areas of the brains of five normal dogs. The concentration
of the 5HT and 5HIAA varies greatly from area to area,
midbrain and hypothalamus having the highest levels,
cortex and cerebellum having amounts below the limits
of detection of the method. Tryptophan also shows a
definite order in its concentration from area to area.

Following the oral administration of tryptophan
the concentration of 5HIAA and 5HT rises in all areas and
at all times at which estimations are made (up to 4 hr).
Oral loading in this case was in the unconscious animal
and, possibly because of a delay in gastric emptying,
the absorption of tryptophan was very variable in its rate
and time of onset. As a result the rise in the 5-
hydroxyindoles varied very greatly between dogs killed at
the same time after tryptophan loading.

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REFERENCES

Amin A.H., Crawford T.B.B., and Gaddum J.H. (1952)
Communication to British Pharmacological Society,
July 1952.

Amin A.H., Crawford T.B.B., and Gaddum J.H. (1954) J.
Physiol. (Lond.). 126, 596.

Ashcroft G.W., and Sharman D.F. (1962) Brit. J. Pharmacol
and Chemotherap. 19, 153.

Ashcroft G.W., Crawford T.B.B., Binns J.K. and MacDougall
E.J. (1964) Clin. Chim. Acta. 9, 364.

Bartholini G., Pletscher A., Bruderer H. (1964) Nature
(Lond.) 203, 1281.

Bernheimer H., Birkmayer W., Hornykiewicz.O (1963) Klin.
Wschr. 41, 465.

Bogdanski D.F., Pletscher A., Brodie B.B. and Udenfriend
S., (1956) J. Pharmacol. 117, 82.

Bogdanski D.F., Weissbach H., and Udenfriend S. (1957)
J. Neurochem. 1, 272.

Carlsson A., (1959) Pharmacol. Rev. 11, 490.

Carlsson A. and Lindqvist M. (1962) Acta. Physiol.
Scand. 54, 83.

Chirigos M.A., Greengard P. and Udenfriend S. (1960)
J. biol. Chem. 235, 2075.

Cooper J.R. and Melcer I. (1961) J. Pharmacol. Exp.
     Ther. 132, 265.

Correale P. (1956) J. Neurochem. 1, 22.

Costa E. (1956) Proc. Soc. Exptl. Biol. Med. 91, 39

Eccleston, D., O'Mahoney D.P. and Ashcroft G.W. (unpublished
                                        observations)(1966)

Erspamer V. and Testini A. (1959) J. Pharm. and
     Pharmacol. 11, 618.

Euler, U.S. von and Hamberg U. (1949) Science 110, 561.

Feldstein A. and Wong K.K. (1965) Life Sciences 4, 183.

Gaddum J.H and Hameed K.A.(1954) Brit. J. Pharmacol.
     9, 240.

Gal E.M., Poczik M. and Marshall F.D. (1963) J.
     Biochem. Biophys. Res. Commun. 12, 39.

Gal E.M., Morgan M., Chatterjee S.K. and Marshall F.D.
     (1964) Biochem. Pharmacol. 13, 1639

Garven J.D. (1955) Ph.D. Thesis for University of
     Edinburgh.

Goldstein M., and Gerber H. (1963) Life Sciences
     2, 97.

Green H. and Sawyer J.L. (1960) Proc. Soc. exp. Biol.
     (N.Y.) 104, 153.

Guroff G. and Udenfriend S. (1962) J. Biol. Chem.
     237, 803.

Grahame-Smith, D.G. (1964) Biochem. J. 92, 52.

Hess S.M., Redfield B.G. and Udenfriend S. (1959)
J. Pharmacol. exp. Ther. 127, 178.

Hess S.M. and Udenfriend S. (1959) J. Pharmacol. exp.
Ther. 127, 175.

Hess S.M., Connamacher R.H., Ozaki M. and Udenfriend S.
(1961) J. Pharmacol. exp. Ther. 134, 129.

Hess S.M. and Doepfner W. (1961) Arch. Int. Pharmacodyn.
134, 89.

Hornykiewicz O. (1963) Wien Klin. Wsch. 75, 309

Holtz, P. and Westermann E. (1956) Arch. exp. Path. Pharmak. 227 538

Jepson J.B. (1963) In: Chromatographic and Electo-
phoretic Techniques (Edited by Smith) Vol. 1
p. 194 Heinemann, London.

Kermack W.O., Perkin W.H. Jr. and Robinson R. (1921)
J. Chem. Soc. 119, 1602.

Kveder S., Iskric S. and Keglevic D. (1962) Biochem. J.
85, 447.

Laverty R. and Sharman D.F. (1965) Brit. J. Pharmacol.
and Chemotherap. 24, 759.

Lajtha A. (1964) In: Comparative Neurochemistry
(Edited by Richter D.) p. 193. Pergamon Press
Oxford.

Lovenberg, W., Weissbach H. and Udenfriend S. (1962)
J. Biol. Chem. 237, 89.

McMenamy R.H., Lund C.C. and Oncley J.L. (1957) J.

Clin. Inv. 36, 1672

Doeden D.
Michael A.F., Drummond K.N., Anderson J.A. and Good R.A.

(1964) J. Clin. Inv. 43, 1730

Milne M.D., Crawford M.A., Girao C.B. and Loughridge L.

(1960) Clin. Sci. 19, 165.

Mitoma C., Weissbach H. and Udenfriend S. (1955) Nature(Lond) 175, 994.

Oates J.A. (1961) Meth. Med. Res. 9, 169.

Oswald I., Ashcroft, G.W., Berger J., Eccleston D., Evans

J.I., Thacore V.R. (1966) Brit. J. Psychiatry 112 391

Pappenheimer J.R., Heisey S.R., and Jordan E.F. (1961)

Am. J. Physiol. 200, 1.

Paasonen M.K., MacLean P.D. and Giarman N.J. (1957) J.

Neurochem 1, 326

Pictet A. and Spengler T. (1911). Ber. dtsch. chem.

Ges. 44, 2030.

Pletscher A. (1963). In: Clinical Chemistry of

Monoamines (Edited by Varley H. and Gowenlock A.H.)

p. 191 Elsevier, Amsterdam.

Pletscher A., Burkard W.P. and Gey K.F. (1964) Biochem.

Pharmacol. 13, 385.

Poirier L.J. and Sourkes T.L. (1965) Brain 88, 181.

Price S.A.P, West G.B., (1960) Nature 185, 470

Paasonen M.K. and Vogt M. (1956) J. Physiol (Lond.)

131, 617.

Rodnight R. (1956) Biochem. J. 64, 621.

Roos B and Werdinius R. (1962) Life Sciences 1, 105

Schanberg S., and Giarman N.J. (1960). Biochem.
    Biophys. Acta (Amst.) 41, 556

Sharman D. F. (1960) Ph.D. Thesis. University of
    Edinburgh.

Sharman D.F. and Smith S.E. (1962) J. Neurochem. 9, 403.

Shaw E.N., and Woolley D.W. (1956) Science 124, 121.

Sjoerdsma, A., Oates J.A., Zaltsman J.N., and Udenfriend S.
    (1959) J. Pharmacol exp. Ther. 126, 217.

Smith S.E., (1963) Brit. J. Pharmacol. 20, 178.

Sourkes T.L. (1954) Arch. Biochem. 51, 444

Stahl E. (1965) In: Thin Layer Chromatography p. 298
    Academic Press. New York.

Sullivan M. (1922) J. Biochem. 50, XXXIX

Tedeschi D.H., Tedeschi R.E. and Fellows E.J. (1959)
    J. Pharmacol exp. Ther. 126, 223.

Twarog B. and Page I.H. (1953) Amer. J. Physiol.
    175, 157

Udenfriend S., Titus E. and Weissbach H. (1955) J.
    Biol. Chem. 216, 499.

Udenfriend S., Weissbach H. and Bogdanski D.F. (1957)
    J. Pharmacol. exp. Ther. 120, 255.

Vogt M. (1954) J. Physiol (Lond.) 123, 451

Weber L.J., and Horita A. (1965) Biochem Pharmacol. 14, 1141.

Weissbach H., King W. Sjoerdsma A. and Udenfriend S. (1959) J. Biol. Chem. 234, 81.

Werle, E., and Mennicken G. (1937) Biochem. Z. 291 325

Westermann E., Balzer H. and Knell J. (1958) Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 234, 194

Woolley D.W. and Shaw E. (1954) Science 119, 587.

Zeller E.A. and Barsky J. (1952) Proc. Soc. exp. Biol. (N.Y.) 81, 459.