THE INFLUENCE OF PSYCHOTROPIC DRUGS ON THE CEREBRAL METABOLISM OF BIOGENIC AMINES

Thesis

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Alexander T. B. Moir

Department of Pharmacology

University of Edinburgh Medical School

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"Strange things I have in head
that will to hand;
Which must be acted ere they
may be scann'd"

Macbeth, Act III, Scene IV.

Statement in Terms of Ph.D. Regulation 13 of the University of Edinburgh

Some of the results described in this thesis have been the subject of published articles.

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SECTION I

Effects of Precursor Loading on the Cerebral Metabolism of 5-Hydroxyindoles and its Use in Studying the Action of Psychotropic Drugs in Vivo.

SECTION IA - EFFECTS OF PRECURSOR ADMINISTRATION

The initial part of this section deals with the continuation of research into an aspect of cerebral metabolism which was already being investigated in the Department of Pharmacology. The results which had been obtained by other workers prior to the start of this project have been included as an appendix (Appendix I, Tables 1 - 4), as they formed a basis for the work performed in this section. Also, it is necessary to consider all the results together when discussing their probable significance.

INTRODUCTION

Since the discovery of the presence of the biologically active amine, 5-hydroxytryptamine, (5-HT) in discrete regions of brain (Amin, Crawford and Gaddum, 1954), many attempts have been made to elucidate the normal metabolic pathways of this amine and their modification by drug action and mental illness. There is no doubt that the source of brain 5-HT is tryptophan (Titus, Weissbach and Peterson 1956). Thus Udenfriend, Creveling, Posner, Redfield, Daly and Witkop (1959) showed that tryptamine is not hydroxylated to 5-HT 'in vivo', while on the other hand, Bogdanski, Weissbach

and Udenfriend (1957) showed that the administration of 5-hydroxytryptophan (5-HTP) led to an increase in the level of 5-HT in brain.

These observations, which have often been confirmed, establish that the synthesis of intracerebral 5-HT proceeds from tryptophan via the intermediate metabolite 5-HTP even though this metabolite is not normally detectable in the brain or plasma. However, the site of the hydroxylation of tryptophan remained uncertain. Following work by Cooper and Melcer(1961) on the hydroxylation of tryptophan by rat and guinea pig gut, it was suggested that tryptophan was hydroxylated extracerebrally and that the cerebral synthesis of 5-HT depended upon the rapid entry of 5-HTP into brain and its subsequent but immediate decarboxylation to 5-HT. (A possibility considered originally by Dalgliesh and Dutton 1957 after their failure to detect 5-HTP in the blood of rats under various experimental conditions).

The demonstration that cerebral tissue can hydroxylate tryptophan both <u>in vivo</u> (Gal. Poczik and Marshall 1963: Gal, Morgan and Marshall 1964) and <u>in vitro</u> (Grahame-Smith 1964) makes it probable however that brain 5-HT is derived from the hydroxylation of tryptophan

occurring mainly if not wholly in the brain. Supporting evidence was provided by Weber and Horita (1965) who showed that the rise in cerebral 5-HT in rats obtained after intraperitoneal injection of tryptophan was unaffected by evisceration and that perfusion of a cerebral hemisphere with a tryptophan solution gave rise to an elevated 5-HT concentration on the perfused side only. Despite this, Gal, Morgan, Chatterjee and Marshall (1964) concluded, from an analysis of the cerebral 'turnover' rates of 5-HT after intracerebral or intraperitoneal administration of tryptophan, that the cerebral hydroxylation of tryptophan was too slow to account for all cerebral 5-HT, and that this must be supplemented from an extracerebral supply of 5-HTP.

The investigations presented in this section support the view that the cerebral hydroxylation of tryptophan is the primary factor controlling cerebral 5-HT formation.

METHODOLOGY

ANIMAL METHODOLOGY

Rats: The same strain, sex and weight of rat and pre-experimental conditions were used as applied for the data in Appendix I, Table I, . The animals used were male Wistar rats (160-200 gm.) which were deprived of food, but not water, for 16-18 hours before

the experiment. In the tryptophan loading experiments rats were used in groups of three and the pooled brains and blood of each group were analysed. The tryptophan was injected intraperitoneally in a solution of 10 mg/ml in 0.9% saline.

In the experiments using loads of L-5-HTP, D, L-5-HTP or 5-hydroxyindol-3-ylacetic acid (5-HIAA), the analyses were done on the tissues from single animals. The dose was administered by intraperitoneal injection in approximately 1 ml. of 0.9% saline solution.

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

Dogs

ACUTE EXPERIMENTS

Technique:- The animals used were adult mongrels of both sexes. They were provided with water but food was withdrawn 12-16 hours before the experiment. The drugs were given intravenously in 0.9% saline (over a period of 2-3 minutes) in doses of L-tryptophan 50 mg/Kg, and D, L-5-HTP and L-5-HTP 10 mg/Kg, at a concentration of 10 mg/ml and α methyl dopa in doses of 50 and 100 mg/Kg

at a concentration of 20 mg/ml.

Ten minutes prior to a predetermined time after drug administration the dog was anaesthetised with intravenous sodium thiopentone and a polythene cannula was introduced into a femoral artery. Exactly at the required time the dog's head was flexed, a 21 gauge needle inserted percutaneously through the atlanto-occipital membrane into the cisterna magna and a 5 ml. sample of cerebrospinal fluid (CSF) withdrawn slowly into a disposable polythene syringe. A concurrent venous blood sample was withdrawn from a vein other than that used for drug administration and the blood collected in a heparinised polythene container. (Part of the blood collected was centrifuged immediately at 3,000 r.p.m. for 5 minutes to obtain plasma. These samples are stored at -15°C until required). 1 ml. of heparin (5,000 i u) was then given intravenously and the dog allowed to exsanguinate rapidly through the arterial cannula. During the final stage the head was raised to allow the brain to be drained of blood as far as possible. The skull was opened by means of a vibrating electric saw, the dura cut away and the brain rapidly removed in one piece.

Brain Dissection:- The cerebellum was excised in one piece then the brain was bisected longitudinally

in the median sagittal plane. Each half was then dissected as quickly as possible according to the Lim, Liu Moffit Atlas (1960) as follows:-

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

Midbrain - brain posterior to a line at right angles to the base of the brain just posterior to the mammillary bodies.

Hypothalamus - excluding the hypophysis cerebri. The anterior and lateral borders are well defined externally and the separation of the superior border of the hypothalamus from the inferior border of thalamus is easily seen in the bisected brain.

Thalamus and Caudate - have well defined borders in the bisected brain and were dissected out whole.

Hippocampus - Hippocampal gyrus through to the inferior horn of the lateral ventricle - includes the amygdala, inferior tip of the hippocampus and fornix and the surrounding cortex.

Occipital Cortex - a portion from the occipital pole, care being taken to include as little white matter as possible.

TABLE 1:1

The Weights of Regions of Dog Brain

Region	Weight in grams
Midbrain	2.02 ± 0.44 (22)
Hypothalamus	0.78 ± 0.17 (22)
Thalamus	1.77 ± 0.34 (21)
Caudate	1.75 ± 0.33 (21)
Hippocampus	3.12 ± 0.39 (22)
Hindbrain	3.93 ± 0.43 (22)
Cerebellum	6.91 ± 1.74 (22)

The mean weight of various regions of dog brain in grams ± SD (number). The weights shown include values for animals quoted in Appendix 1 as all the dissection was done by the same individual.

The animals and methods used were equivalent to those used for the experiments quoted in Appendix I, Tables 3 and 4 , except that concurrent samples of cisternal CSF were withdrawn.

The weights of the regions of brain for animals used in Section I are presented in Table 1:1.

SECOND EXPERIMENTS

The same dogs, (four mongrels - three female litter mates and their sire) and experimental techniques were used as had been used in compiling the results quoted in Appendix I, Table 2 .

Food was withdrawn 12-16 hours prior to an experiment but water was available ad libitum. L-tryptophan and α methyl dopa were administered intravenously as described above. The experiments were performed according to a 4 x 4 Latin Square design thus on each day that an experiment was performed three dogs had tryptophan administered while the fourth received none and acted as a control. [At least two weeks was left between each experiment to avoid any carry over effect between experiments.] The dogs were given a minimal anaesthetic dose of intravenous sodium thiopentone at the predetermined time so that samples of cisternal CSF could be obtained (by the technique described above (page 5)), according to the schedule - control and 1, 2 and 4 hours after

tryptophan administration.

Samples of venous blood obtained from a vein other than that used for drug administration were withdrawn from each dog at hourly intervals until after the sample of CSF had been taken.

DRUGS AND CHEMICAL REAGENTS

L-tryptophan	(Koch-Light)
L-5-HTP - Grade B	(Calbiochem)
D, L-5-HTP	(Koch-Light)
5-HT - creatinine sulphate	(Koch-Light)
5-HIAA - diethyl ammonium salt	(Roche)
5-HIAA	(Koch-Light)

α Methyl Dopa - pure substance - (Merck, Sharp and Dohme)

Sodium thiopentone - Pentothal (Abbott)

Heparin - 5,000 i u (Boots)

All chemical reagents used were Analar grade apart from hydrochloric acid which was micro analytical grade (British Drug Houses) or (Hopkin and Williams).

Water - distilled and deionised.

BIOCHEMICAL METHODOLOGY

Estimation of 5-Hydroxyindoles in Plasma and Brain

Method

Estimations of 5-HTP, 5-HT and 5-HIAA in plasma and brain samples were carried out by the method of Ashcroft, Eccleston and Crawford (1965), Eccleston (Thesis 1966) as is described briefly below.

Subsequent to the rapid removal and dissection, brain was weighed and homogenised as quickly as possible in a glass test tube homogeniser with one volume (v/w) 40% (v/v) acetic acid. Further 20% acetic acid and two drops of 2-octanol were added and homogenisation repeated to give a final brain weight to volume of 1:4 or a minimum volume of 6 ml. The homogenate was then transferred to a graduated test tube or measuring cylinder and allowed to remain at 4°C for at least 30 minutes. Brain samples which could not be homogenised immediately were stored at -15°C. (In experiments with rats homogenisation was complete within 10 minutes of death - in experiments on dogs the brain regions were homogenised in a random order and these were all completed within 60 minutes of death). Plasma was allowed to thaw at room temperature then 1 or 2 ml. samples were pipetted into 35 ml. glass stoppered centrifuge tubes and glacial acetic acid was added, 0.2 ml/ml of plasma. These tubes then remained at 4°C for at least 30 mins.

After standing for the requisite length of time the volumes of brain homogenates were recorded and a 4 ml. aliquot was pipetted into a 35 ml. glass-stoppered centrifuge tube. 24 ml. of acetone cooled to 4°C was added to precipitate protein. Similarly 18 ml. of this acetone was added to the tubes containing plasma. Both sets of tubes were shaken to allow better protein precipitation and then stored at -15°C for 45 minutes, being removed every 15 minutes for brief shaking. The precipitate was then removed by centrifuging at 2500 r.p.m. for 3 minutes in cooled centrifuge buckets and a portion of the acetone extract (24 ml. for brain extracts, 18 ml. for plasma extracts) transferred to a 35 ml. glass stoppered centrifuge tube and stored overnight at -15°C.

The following morning the acetone was evaporated by a stream of nitrogen until the volume of the brain samples was approximately 4 ml. and the plasma samples 2 ml. Most of the lipid was then removed by extracting with petroleum spirit (b.p. 40-60°C) 16 ml., and for the brain samples a second extraction with 8 ml. was carried out. Ascorbic acid 100 µg in 0.2 ml. of 80% methanol was added and the aqueous phase taken to damp dryness under reduced pressure while warmed gently by a water bath at 55°C.

The residue was dissolved in a further 0.2 ml. of 80% methanol containing 100 µg of ascorbic acid. This was applied to Whatman No. 1 chromatography paper cut in 5 cm. wide strips, along a 1 cm. band 6 cm. from the bottom, under a continuous stream of nitrogen. An ascending chromatogram was developed in an atmosphere of nitrogen with butan-1-ol: acetic acid:water (12:3:5) overnight at room temperature (approximately 15 hours - 23 cm.). The chromatogram was dried in a stream of nitrogen and cut into strips in accordance with appropriate marker chromatograms. The sections of the chromatograms were eluted into 4.2 ml of 0.1 N sulphuric acid and kept at 4°C for 15 minutes with gentle agitation at 5 minute intervals. The paper was then removed and the volume of eluate recorded and made up to 4 ml. with 0.1 N sulphuric acid if necessary. 2 ml. of concentrated hydrochloric acid containing 50 mg. of ascorbic acid per 100 ml., was added and the samples immediately read in a Farrand (1) recording spectrofluorimeter. (Types and characteristics of spectrofluorimeter used - page 21). The samples were read against a standard of 5-HT by the method of Ashcroft, Crawford, Binns and MacDougall (1964) the relative amounts of 5-HTP and 5-HIAA being quantified according to their relative fluorescence. The

5-hydroxyindoles are not stable in strong acid solution even when protected by ascorbic acid; thus they were read immediately after the addition of the concentrated hydrochloric acid .

Marker Chromatograms

These were brain and plasma extracts to which authentic 5-HTP, 5-HT and 5-HIAA were added just before applying them to the chromatography paper.

Recovery Experiments

Quantities of 0.8 µg of each 5-HTP, 5-HT and 5-HIAA were added to one of two portions of pooled brain homogenates and plasma of untreated rats before its precipitation with acetone. These samples served as internal standards for the whole procedure and the results were corrected appropriately by adjusting to 100% recovery on the average from a series of experiments. Similar recovery samples were used in the experiments on dogs by adding 0.8 µg of 5-HTP, 5-HT and 5-HIAA to dog plasma and to a portion of the cerebellum or hindbrain homogenate. It was thought necessary in discussing the results from experiments in dogs to have the data corrected to 100% recovery. However, as values for recovery samples were not available for all the previous experiments (Appendix I, Tables 3 and 4) it was considered that the best

TABLE 2:1

Recoveries of Pure Substance from Dog Cerebellum or Hindbrain

Substance	% Recovery
5HTP	52.1 ± 2.6 (18)
5HT	68.4 ± 2.4 (26)
5HIAA	70.6 ± 3.5 (26)

0.8 ug of pure substance was added to each recovery sample. % Recovery is expressed as mean ± SEM (number of observations)

No significant difference between types of brain sample used.

corrections would be to correct to 100% recovery on the basis of the values for all available recovery experiments (Table 2:1). There was no significant difference between the values for past and present recovery experiments.

Identification Procedure for 5-Hydroxyindoles

In the paper chromatographic method employed for the separation of the 5-hydroxyindoles prior to estimation, 5-hydroxytryptophol is isographic with 5-HIAA and 5-HIAA sulphate isographic with 5-HTP in the estimation of 5-HIAA and 5-HTP. To exclude the presence of the alcohol and the sulphate in the estimates of 5-HIAA and 5-HTP the following identification procedures were carried out.

Duplicate samples of rat brain and plasma from animals which had been given 40 mg/Kg of D,L-5-HTP intraperitoneally and a plasma sample from a dog 1 hour after a dose of 10 mg/Kg L-5-HTP intravenously were carried through the normal analytical procedure until after the chromatogram had been developed in n-butanol/acetic acid/water 12:3:5 (v/v). The chromatograms were dried in nitrogen and the portions of the chromatograms corresponding to the positions of the 5-hydroxyindoles in the marker chromatograms were cut out and eluted into 2 ml. of 20% (v/v) acetic acid for 15 minutes at 4°C aided

by periods of gentle agitation. Ascorbic acid, 50 µg in 0.1 ml. 80% methanol was added to each eluate and the samples taken to dryness under reduced pressure at an external temperature of 55°C. Each residue was dissolved in 0.2 ml. of 80% methanol containing 100 µg ascorbic acid and the samples were applied to Whatman No. 1 paper cut for chromatography as in the routine method.

Ascending chromatograms of the material from the 5-HTP portion of the original chromatograms were developed with aqueous sodium chloride 8% (w/v): acetic acid (100:1 by vol.) in an atmosphere of nitrogen. Ascending chromatograms of the material from the 5-HIAA portions of the original chromatograms were developed with propan-2-ol/ aqueous ammonia (S.G. 0.88/water, 85:5:15 (v/v). These chromatograms were compared with pure solution markers developed with the same systems, the portion corresponding to the expected 5-hydroxyindole was taken as one strip and the areas above and below this cut into 2 cm. portions. All these samples were then eluted into 0.1 N sulphuric acid and the 5-hydroxyindole content of the eluate estimated by their relative specific fluorescence in 3N hydrochloric acid.

Estimation of 5-Hydroxyindol-3-ylacetic acid in Cerebrospinal Fluid

0.05 ml. of concentrated hydrochloric acid was added to 4 ml . of salt (approx. 2 g.) saturated C.S.F. contained in a 35 ml. glass stoppered centrifuge tube. This was extracted by shaking for 5 minutes with 20 ml. of peroxide free, freshly distilled diethyl ether. After centrifuging for 3 minutes at 2,000 r.p.m., 18.5 ml. of the ether were transferred to another glass stoppered centrifuge tube containing 4 ml. of 0.3 M sodium phosphate buffer pH 7.0. The 5-HIAA was back extracted by shaking for 3 minutes, then the phases were separated by centrifuging for 3 minutes at 2,000 r.p.m. The ether was aspirated off and 3.5 ml. of the aqueous layer was acidified with 2 ml. of concentrated HCl, containing 50 mg of ascorbic acid per 100 ml. The 5-HIAA was estimated against a 5-HIAA standard in a Farrand (1) recording spectrofluorimeter using the specific fluorescence of 5-hydroxyindoles (activation scan with emission at 550 mμ, maximum fluorescence at 295 mμ). The samples were corrected to 100% on the basis of the recovery of an aqueous solution of 5-HIAA taken through the complete assay procedure.

Estimation of Tryptophan in Whole Blood, C.S.F. and Brain Samples:

C.S.F. -0.5ml. of cisternal C.S.F. was used - assays were only performed if the sample had no visible evidence of contamination with blood.

Whole Blood - 0.1 ml. of whole blood was used for all assays.

Brain - The acetic acid homogenate of brain (page 10) was used. The assays were performed on 1 ml. of homogenate from control animals but only on 0.5 ml. from animals which had been pre-treated with tryptophan as otherwise it was possible that the linear range of the method would be exceeded.

Assay.

The method of Hess and Udenfriend (1959) as applied by Guroff and Udenfriend (1962) was used in the form described briefly below.

Precipitation - Water was added to the samples contained in 12 ml. glass stoppered centrifuge tubes to give a total volume of 5.5 ml. (Recoveries of 2 µg of pure substance were added at this point). After inverting the tubes a few times they were allowed to stand at room temperature for approximately 10 minutes. 1 ml. of 30% trichloracetic acid was added and the sample kept well dispersed in the aqueous phase to ensure good precipitation

Table 3:1

A Comparison of the Relative Fluorescence of Precipitated and Non-Precipitated Blank Solutions for Tryptophan and Tyrosine Estimations

Estimation	Precipitated	Non-precipitated
Tryptophan	100%	98 ± 3% (10)
Tyrosine	100%	101 ± 4% (10)

Precipitated blank solutions were samples of pure water taken through the full assay procedure. Non-precipitated blanks were samples of 0.1N sulphuric acid included through the fluorophor-forming reaction only (See text, Sections I and II).

The non-precipitated blanks were expressed as a percentage of the values obtained for precipitated blanks in the same assays. The results are expressed as the mean percentage ± SEM (number of estimations).

of the protein. After exactly 10 minutes centrifugation at 3,000 r.p.m. for 7 minutes was carried out and then 3 ml. of the supernatant was transferred to 15 ml. glass stoppered tubes.

Norharman Reaction - The volume of this portion was made up to 6 ml. with 3 ml. of 0.1 N sulphuric acid. A blank containing 6 ml. of 0.1 N sulphuric acid and a standard containing 1 mg. of pure tryptophan in 6 ml. of 0.1 sulphuric acid were used for comparison. 0.2 ml. of an 18% solution formaldehyde was added, the tubes lightly stoppered and then placed in a metabolic shaker which was maintained at 100°C by lagging. After 17-20 minutes 0.2 ml. of hydrogen peroxide (approx. 17 vol. per cent) was added, the stoppers lightly replaced and the tubes incubated for a further 17-20 minutes at 100°C, following which they were cooled immediately by placing them in cold water. The samples were then read in a recording spectrofluorimeter, (Types page 21) emission 450 mμ/ excitation scan 200 - 400 mμ, maximum 315 mμ secondary peak 375 mμ.

Results - The excursion of the reagent blank is first subtracted from all sample readings. (The blank used gives similar values to a reagent blank subjected to the full precipitation method - Table 3:1). These readings

are then compared with the supplementary fluorescence produced by the added authentic tryptophan to give the quantity present in the sample. The relative efficiency of the assay is determined by comparing the fluorescence of the standard with that of the tryptophan added to samples, [redacted] No formal table of recoveries has been presented because of a marked variation from experiment to experiment due to the time-dependant nature of the fluorophor - forming reaction (Hess and Udenfriend 1957). However, a series of recoveries within an experimental run is usually fairly consistent.

Estimations carried out from plasma, red blood cells (page 18), whole blood and C.S.F. are always successful, however extreme care must be taken to produce good precipitation when analysing brain homogenate.

Spectrofluorimeters

Four different machines were used. Some of the main differences in functional characteristics are described below with a comparison of the main assays performed. The results will usually contain an indication of which spectrofluorimeter was used.

Farrand (1) (F1)

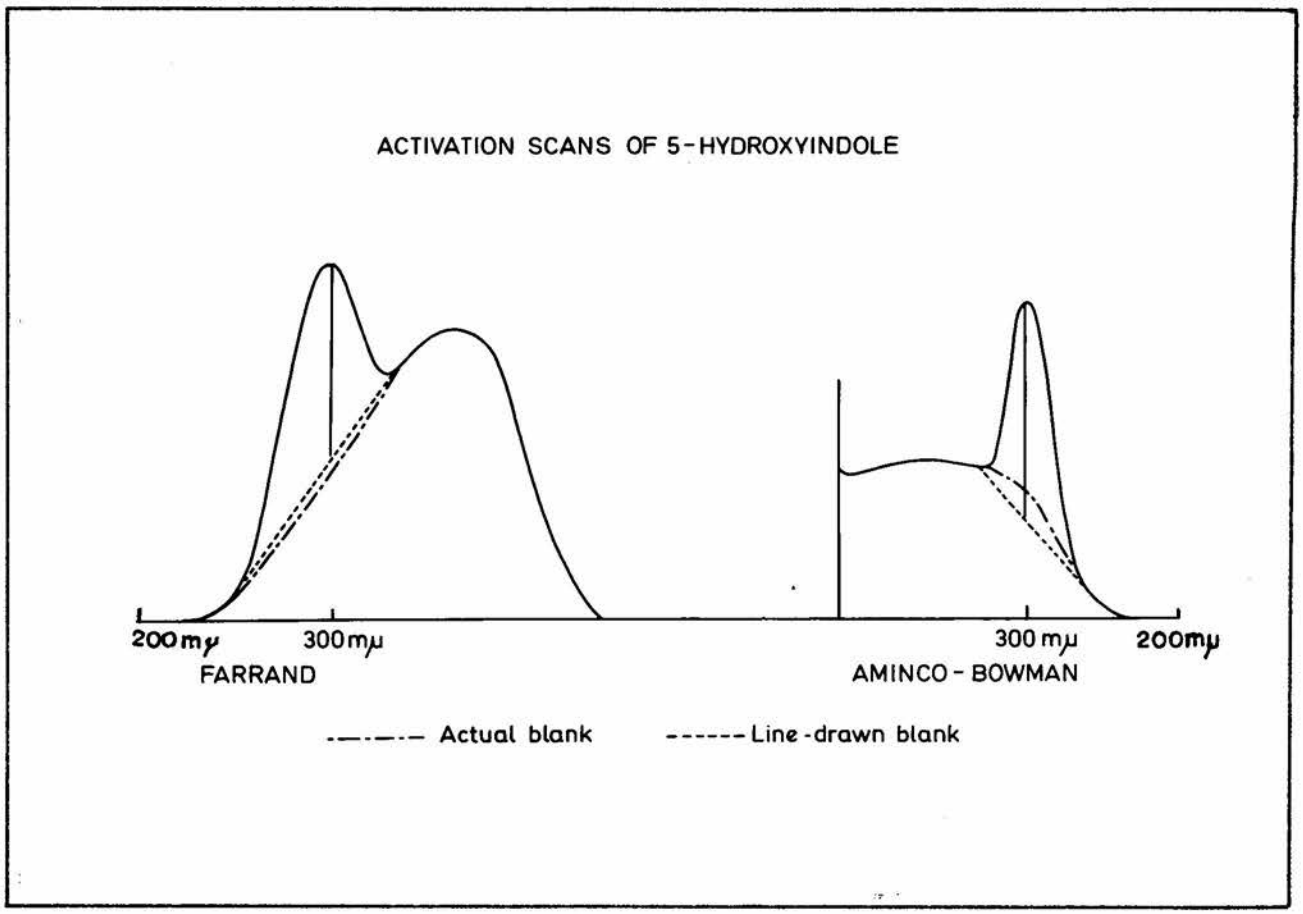
This 15 year old diffraction grating spectrofluorimeter has been fully described (Thesis, Parker-Rhodes 1966) previously. Its main features for comparison were that the power supply to the lamp was alternating current and even though a stabilising transformer was inserted in the power supply there were noticeable fluctuations in the light intensity. 5-hydroxyindole samples were each scanned twice and the average value taken as the best estimate. A standard was normally read after each three samples. The machine was generally difficult to maintain in an efficient working condition. Only a 10 ml. cuvette was available for the machine and this needed just over 5 ml. to give full window cover. All the samples from Section I of this thesis were estimated using this machine.

Farrand (2) (F2)

This machine - purchased in 1966, was basically of similar design to the above. Improved features included a stabilised direct current supply to the lamp which was fan cooled and used in conjunction with a paraboloid focusing mirror. This resulted in a stable more powerful light source. The stabilised high tension line, photomultiplier, microammeter, recording system, though no more sensitive than that of the older machine was more stable

Fig. 1:1

Spectroflurometric Activation Scans



The diagram illustrates the divergence of the line-drawn blank from the true blank value at the wavelength for maximum activation of the fluoresence of 5-hydroxyindoles. The perpendicular shown is that drawn to quantify the 5-hydroxyindole concentration.

at high sensitivity. In general, because of the increased light intensity it was possible to decrease the slit widths used thus increasing the specificity while still maintaining high sensitivity. This is of particular value when quantifying samples such as 5-HIAA obtained after routine chromatography (page 12), as this sample normally is associated with a high non-specific fluorescence due to its position near the solvent front. The machine could be used with three sizes of cuvettes, a 10 ml., 3 ml., and 0.4 ml. (See diagram, Fig. 1:1)

Aminco Bowman (A-B)

This diffraction grating spectrofluorimeter, approximately 5 years old, had a stabilised direct current supply to its fan cooled lamp resulting in a good constant light source. It used a 3 ml. cuvette which needed at least 1.3 ml. to give satisfactory readings. Estimates of hydroxyindoles tend to give high values because the blank interpolated from the excitation scan tends to be falsely low. (See diagram, Fig. 1:1)

Zeiss (Z)

This spectrofluorimeter obtained in 1967 is at present fitted with a prism monochromator to select the excitation wave-length thus resulting in non-linear input scans from which it is difficult to interpolate a satisfactory blank for 5-hydroxyindoles. However, the

input and output band width is narrow. This specificity means that fixed optimum excitation and emission settings can be used with a reagent blank as there will be little difference between samples due to non-specific fluorescence when such narrow band widths are used. The band widths on this machine may be continuously reduced and these controls may be used to obtain the best ratio of specific to non specific fluorescence depending on the sensitivity required. The 1 ml. cuvettes need at least 0.7 ml. to give satisfactory readings.

RESULTS

IDENTIFICATION OF 5-HYDROXYINDOLES

Only 5-hydroxyindoles with Rf values corresponding to 5-HTP, 5-HT and 5-HIAA were found after secondary chromatograms (page 15). Similar identification procedures had been previously carried out for samples obtained from rats which had been tryptophan loaded. (Eccleston, Ashcroft and Crawford 1965).

RATS

THE EFFECTS OF SMALL DOSES OF TRYPTOPHAN

Eccleston, Ashcroft and Crawford (1965) found the maximum brain concentration of 5-HT after intraperitoneal

TABLE 4:1

Concentrations of Tryptophan in Rat Blood and of Tryptophan, 5HT and 5HIAA in Rat Brain Tissue.

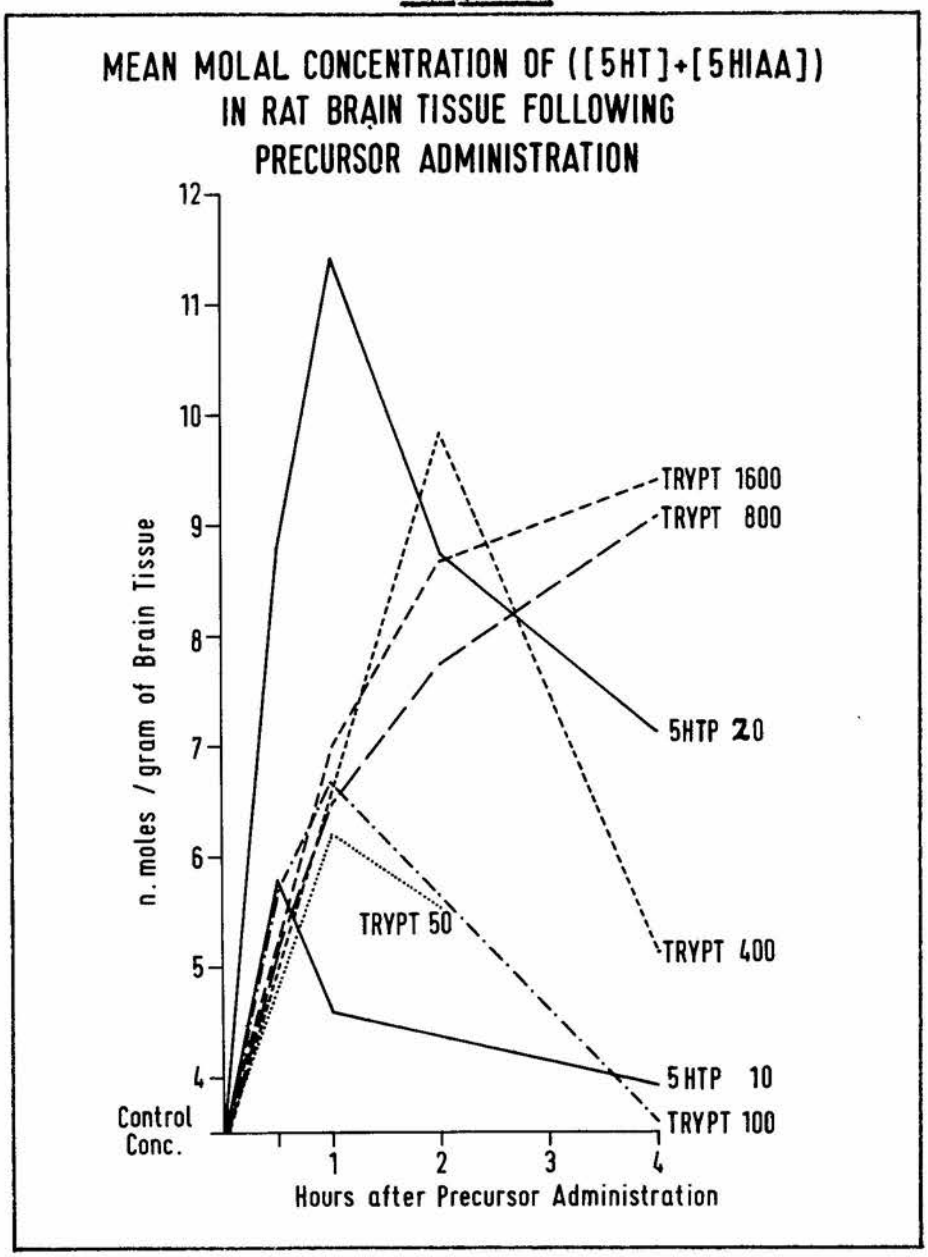
Tryptophan Dose (mg /Kg)	Time after Injection hr.	Blood Tryptophan µg/ml	Brain Tryptophan µg/g	Brain 5HT µg/g	Brain 5HIAA µg/g
	Control	18.2 ± 1.8 (6)	5.6 ± 1.7 (6)	0.38 ± 0.11 (14)	0.24 ± 0.08 (14)
50	0.5	39	43	0.53	0.36
	1	57	37	0.63	0.51
	2	26	19	0.46	0.58
100	0.5	135	50	0.64	0.39
(mean of 2 experiments)	1	74	29	0.56	0.67
	2	61	36	0.50	0.56
	4	21	7.4	0.37	0.28

5HTP could not be detected in the brains (< 0.07µg/g).

5HTP and 5HIAA could not be detected in plasma (< 0.03µg/ml).

Control values are means ± standard deviations (number of experiments).

Fig. 2:1



Values Shown

Dose Administered Intraperitoneally (mg/kg):-		Number of Estimates:-	
	TRYPT 50		Single estimate
	TRYPT 100		Mean of two estimates
	TRYPT 400		Single estimate
	TRYPT 800		Mean of three estimates
	TRYPT 1600		Single estimate
	5-HTP 10		Mean of three estimates
	5-HT 20		Mean of three estimates

The graphs show that all doses of tryptophan (TRY P.) have approximately the same initial slope signifying a similar increase in the cerebral metabolism of 5-hydroxyindoles above its normal turnover rate, but that this increased rate of metabolism can be exceeded by administration of 5-hydroxytryptophan (5-HTP).

administration of doses of up to 1600 mg/Kg to be about 0.7 ug/g (Appendix I, Table 1). Increasing the load led only to prolongation of the duration of this maximum concentration. Even with doses of tryptophan much lower than those used by Eccleston et. al. (1965) transient rises to near the maximum brain 5-HT concentration occur (Table 4:1). The maximum 5-HT concentration was reached earlier than the peak 5-HIAA concentration as was found in previous experiments (Appendix I, Table 1).

It seemed probable that, if the rate-limiting step in the metabolic pathway was the hydroxylation of tryptophan, the combined molal concentration of the total 5-hydroxyindoles might be the best index of the turnover rate of hydroxyindoles along the 5-OH-indole metabolic pathway. Consequently the gram molal concentration of 5-HT and 5-HIAA were summed for each dose and time and the results expressed graphically (Fig. 2:1). Comparable data derived from the results of Eccleston, (1965) have been included. It is clear that the initial rates of rise of the total 5-hydroxyindole concentration are almost identical within the range of tryptophan loads 50-1600 mg/Kg.

TABLE 5:1

Concentrations of 5Hydroxyindoles in Rat Plasma and Brain Following Intraperitoneal Injection of 10mg Kg of D,L-5HTP

Time after Injection hrs.	PLASMA		BRAIN		
	5HTP	5HIAA	5HTP	5HT	5HIAA
0.5	8.6±1.6	1.7±0.8	0.23±0.07	0.44±0.14	0.6 ±0.28
1	4.3±1.1*	1.9±1.6	0.11±0.11	0.34±0.12	0.51±0.21
2	0.68±0.30	0.54±0.48	0.03±0.06	0.38±0.19	0.42±0.17
4	0.04±0.03	0.11±0.15	N.D.	0.42±0.10	0.30±0.07

TABLE 5:1

Concentrations corrected * BRAIN	
5HTP	5HIAA
0.14	0.61
0.07	0.49
0.02	0.41
N.D.	0.30

Mean concentrations of 5Hydroxyindoles in brain and plasma µg/g or /ml.

± Standard deviation

Mean of 3 experiments except * when only two were performed.
N.D. Not detected in any experiment (level of detection = 0.07µg/g)

* For explanation see section on 5HIAA administration.

Conclusion

These results are in agreement with the hypothesis put forward by Eccleston et al. 1965 that the rate limiting step in the synthesis of 5-HT is most probably the hydroxylation of tryptophan. Saturation of this step can occur with much lower doses of tryptophan, than any used previously; indeed, at the lowest dose the maximum tryptophan blood level is only three times the control values.

The Effects of Small Doses of 5-Hydroxytryptophan

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

After a load of 10 mg/Kg D,L-5-HTP intraperitoneally there was no apparent rise in 5-HT concentration in the brain, but a very big increase in the concentration of 5-HIAA, so that the total 5-hydroxyindole metabolites ([5-HT] + [5-HIAA]) showed an initial rate of rise exactly comparable to that obtained after tryptophan loads. This is well illustrated in Fig. 2:1. At the same time it became possible to detect 5-HTP in the brain (Table 5:1). Bigger loads of 5-HTP (20 mg/Kg)

TABLE 6:1

Concentrations of 5Hydroxyindoles in Rat Plasma and Brain Following Intraperitoneal Injection of 20mg/Kg of D,L-5HTP

Time after Injection hrs.	PLASMA		BRAIN		
	5HTP	5HIAA	5HTP	5HT	5HIAA
0.5	17.0 ± 8.7+	3.5 ± 2.4+	0.44 ± 0.17	0.46 ± 0.14	1.16 ± 0.24
1	7.1 ± 4.4	3.0 ± 0.5	0.45 ± 0.13	0.54 ± 0.16	1.58 ± 0.03
2	4.1 ± 3.2	3.6 ± 3.4	0.25 ± 0.11	0.50 ± 0.09	1.12 ± 0.03
4	0.22 ± 0.03	0.31 ± 0.21	0.10 ± 0.11	0.48 ± 0.10	0.83 ± 0.10

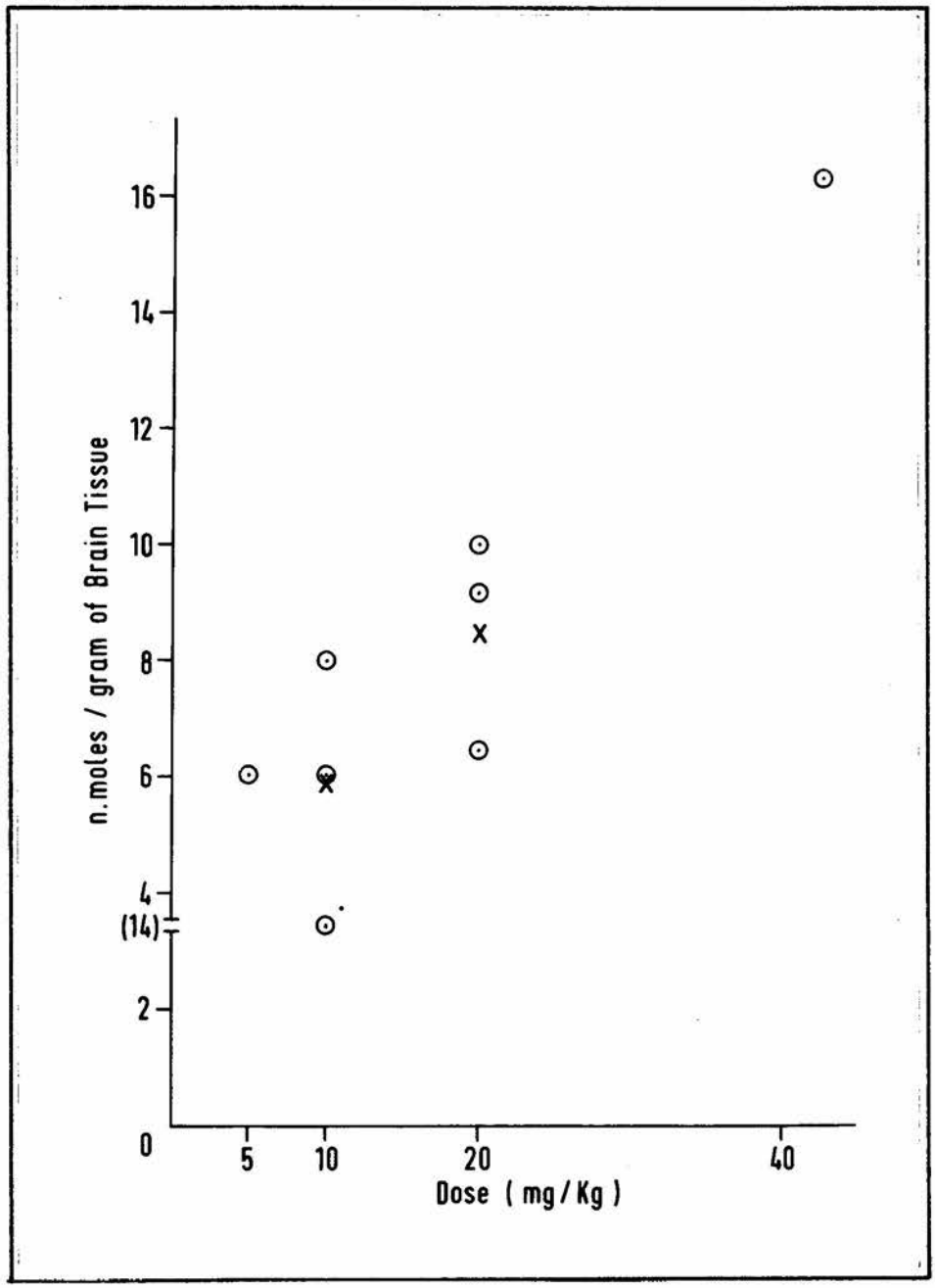
TABLE 6a:1

Concentrations corrected * BRAIN	
5HTP	5HIAA
0.26	1.12
0.38	1.55
0.21	1.09
0.10	0.82

Mean concentrations of 5hydroxyindoles in brain and plasma ug/g or /ml.
± Standard deviation
Mean of three experiments except + when only two were performed.
* For explanation see section on 5HIAA administration.

Fig. 3:1

The Total Molal Concentration of ([5HT] + [5HIAA]) in Rat Brain Tissue 30 min. After Intraperitoneal Administration of Various Doses of 5-Hydroxytryptophan.



O Single estimates
x̄ Mean value
— Limits of the standard error of the mean concentration for control animals (number of estimates)

The values of the total metabolite concentration in brain 30 min. after 5-hydroxytryptophan administration may be regarded as an index of the increase in the rate of metabolism. The above figure demonstrates that the results are compatible with either a linear or flat exponential relationship between the increased rate of metabolism and the dose of 5-hydroxytryptophan administered.

produced even more striking changes (Fig. 2:1). A dose of 20 mg/Kg i.p. (Table 6:1) produced only a moderate rise in the brain 5-HT concentration to 140% of control concentration but the concentration of 5-HIAA in brain rose rapidly to values of more than 1.5 µg/g. 650% of control concentration .

Conclusion

It would therefore appear that 5-HTP, at the above dose level is capable of passing the blood-brain barrier and of being metabolised to 5-HIAA without greatly increasing the amount of 5-HT detectable in brain. (Tables 5:1 and 6:1).

Rate Limiting Step

The initial rate of rise of total 5-hydroxyindole metabolites caused by 5-HTP loading increased with increasing loading dose. This was in direct contrast to the results obtained with tryptophan loads. Figure 3:1 shows that the initial rate of rise of total metabolites (as represented by the concentration 30 minutes after 5-HTP administration) increased with dosage and may be linearly related to the dosage of 5-HTP given intraperitoneally.

Conclusion

This finding would indicate that the rate limiting

TABLE 7:1

The Concentrations of 5HT and 5HIAA in Rat Brain Tissue and of 5HIAA in Plasma Following Intra-peritoneal Injection of 5HIAA - 20mg /Kg.

Time after Injection hrs.	Brain 5HIAA µg/g	Plasma 5HIAA µg/ml	Ratio *
Control	0.24 ± 0.08	0.06	
0.25	0.78	54.2	0.010
0.5	0.62	29.2	0.013
1	0.33	20.4	0.005
2	0.40	1.04	0.156

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

step of the conversion of 5-HTP to 5-HT and 5-HIAA is not yet saturated at these dose levels with this form of load.

5-HTP can be metabolised to 5-HT and 5-HIAA extracerebrally and these metabolites do not normally penetrate the blood brain barrier (5-HT - Axelrod and Inscoe 1963: 5-HIAA - Roos 1962). However, the very high plasma levels produced by loading or by extracerebral metabolism may contribute to the concentrations of 5-HTP and 5-HIAA apparently found in brain tissue by the contamination of the brain homogenates with plasma. The possible extent of such contamination was investigated as follows:

Systemic Administration of 5-Hydroxyindol-3-ylacetic Acid

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

If we assume that no 5-HIAA enters brain itself, then we can deduce that, since the plasma will constitute a constant fraction of the "brain" sample, the increase in "brain 5-HIAA" should be a constant fraction of the plasma 5-HIAA. The results are shown in Table 7:1. Excluding the 2 hour result (which is of very doubtful accuracy), the ratio is indeed relatively constant at

a mean value of 0.01, implying that the plasma occupied about 1% of the volume of the brain samples. This value is in good agreement with the estimate of 0.4-1.2% for the brain capillary whole blood volume made by Sokoloff (1961), based on autoradiography after I^{131} - labelled albumin injection, especially since Sokoloff took special precautions to exclude large vessels from his samples.

Using the estimated value of 1% of each sample as being occupied by plasma the results of the brain concentrations found after 5-HTP administration (Tables 5:1 and 6:1) have been corrected appropriately (Tables 5a1 and 6a1). The corrections make little difference to the estimates of cerebral 5-HIAA but reduce the estimates of 5-HTP in brain by 30-50%. Blood corpuscles and capillary endothelium may well contain relatively high concentrations of 5-HTP (Ross and Gershon 1966) due to their proximity to high concentration in plasma. Indeed marked fluorescence is found in the region of the brain capillary endothelium after 5-HTP administration to rats, (Bertler, Falck and Rosengren, 1963). Thus it is possible that even less of the 5-HTP apparently found in brain is actually present in nervous tissues.

Contamination of the brain sample by systemic 5-HT

was not corrected for, in spite of the recent observations of Bulat and Supek (1967) that a small amount of 5-HT may cross the blood brain barrier. The reason was that although the plasma 5-HT was quantified in all samples analysed, there was a vast scatter in the values found for samples from different animals under the same experimental conditions, indeed many of the highest values were for samples from control animals. The scatter of 5-HT plasma concentrations found was thought to be due mainly to a variable degree of platelet lysis prior to, and during centrifuga tion and any further changes in the free plasma 5-HT following precursor administration were obscured. Because of the doubtful value of the 5-HT plasma concentrations found they have not been included in the tables with the other data.

Having looked at the changes produced in the concentrations of whole brain homogenates with the different precursor administration, it was decided to pursue these experiments further by examining the changes produced in different regions of brain. Dog brain was chosen for this study as some of the necessary data had already been assembled.

VARIOUS BRAIN REGIONS OF DOGS

Control Dogs

Molal Ratios of 5-Hydroxytryptamine to 5-Hydroxyindol-3-ylacetic Acid.

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

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

and that, if there is no alternative route in the system, then

$$K_1 [5\text{-HT}] = K_2 [5\text{-HIAA}]$$

where K_1 and K_2 are equal to 'efflux constants' for 5-HT and 5-HIAA and $[5\text{-HT}]$ and $[5\text{-HIAA}]$ are the molal concentrations normally found. From this it was suggested that if the ratio $[5\text{-HT}] / [5\text{-HIAA}] = K_2/K_1$ was found to be constant in different parts of the brain, then it could be inferred that the turnover rates in these regions were the same and that variation in the concentrations of 5-hydroxyindoles between different brain regions reflected a variation in the concentration of serotonergic units. Tozer et al. (1966) also reported evidence favouring this in that their calculations of K_2 in rat brain, as derived from the efflux of 5-HIAA from brain after blockade of mono-amine oxidase with

TABLE 8:1

The Ratio of Molal Concentrations [5HT] / [5HIAA] in the Brains of Six Normal Dogs

Region	Mean Ratio [5HT] / [5HIAA]
Midbrain	1.12 ± 0.17
Hypothalamus	1.82 ± 0.30
Thalamus	1.06 ± 0.16
Caudate	2.17 ± 0.44
Hippocampus	2.70 ± 0.38
Hindbrain	1.06 ± 0.16

The mean ratios ± SEM of [5HT] / [5HIAA] concentrations expressed in n.moles/g of brain tissue in different regions of normal dog brain.

pargyline were similar for whole brain, brain stem and rest of brain.

While the inferences made from their data might well be correct, we have found evidence, by analysing the data of the concentrations of 5-hydroxyindoles in normal dog brain (Appendix 1, Table 3) that this ratio of molal concentrations varies in different regions of dog brain. (Table 8:1).

Conclusion

Such a finding would appear to show there are regional differences in brain in the metabolism of 5-HT and is against the concept of serotonergic units. However, it cannot pinpoint which of the individual rate constants, making up the composite "efflux constants" K_1 and K_2, are responsible or even which "efflux constant" is involved in each case. Indeed, the differences may be produced by the variable utilisation of an alternative metabolic route.

It was thought that, in view of the information obtained from the experiments with rats where the hydroxylation of tryptophan appeared limiting, the molal concentration of total 5-hydroxyindoles [5-HT] and [5-HIAA] might give the best indication of controlling mechanisms. (Table 9:1).

TABLE 9:1

Total 5Hydroxyindoles Concentration in Different Regions of the Brains of Six Control Dogs.

Regions	Dogs I	II	III	IV	V	VI	Rank of Mean	Mean of Ranks
Midbrain	8.4	11.0	8.6	8.5	12.4	8.5	1	1.17
Hypothalamus	8.8	10.5	5.8	4.8	7.2	7.3	2	1.83
Thalamus	7.6	4.6	3.1	2.7	2.5	3.9	3	4.5
Caudate	4.1	2.5	2.0	2.0	2.7	2.5	6	5.17
Hippocampus	3.4	1.7	3.4	4.3	4.1	4.2	5	5.83
Hindbrain	3.2	4.9	2.7	3.8	4.4	3.2	4	4.33
Cortex	ND	ND	ND	-	-	-	7.5	7.5
Cerebellum	ND	ND	ND	-	ND	-	7.5	7.5

Concentrations of total 5Hydroxyindoles ([5HT] + [5HIAA]) in n.m les/gram of brain tissue.

N.D. Neither 5HT nor 5HIAA detected (< C.2n.moles/gm. of each).

The amounts of 5HT and 5HIAA are presented in appendix 1 - table 3.

Table 10:1

Coefficient of Concordance for Table 9:1

Regions	Dogs 1	2	3	4	5	6	Rank of Mean	Mean of Rank
Mid brain	2	1	1	1	1	1	1	1.2
Hypothalamus	1	2	2	2	2	2	2	1.8
Thalamus	3	4	4	3	6	4	3	4.3
Caudate	6	5	6	5	5	5	6	5.3
Hippocampus	4	6	3	6	4	3	5	3.8
Hindbrain	5	3	5	4	3	6	4	4.2
Cortex	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Cerebellum	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5

The table shows the ranking of the concentration values in table 9:1.

The coefficient of concordance $= \frac{S}{\frac{1}{12}m^2(n^3-n)-m\sum_T(t^3-t)}$

Where $S = \sum$ (Average rank total of Row - actual Rank Total of Row)^2

m is the number of columns, n is the number of rows, t is the number of tied ranks in each row and $\sum_T$ is the sum of all the columns.

Thus for the example shown

$$W = \frac{1346}{1/12 \times 6^2(8-8) - 6 \times 6 \times 6/12}$$

$$= 0.899$$

$$F = \frac{(m-1)}{1-W} W = 149$$

Greater degrees of freedom $= (n-1) - \frac{2}{m} = 6.8$

Lesser degrees of freedom $= (m-1)((n-1) - \frac{2}{m}) = 54$ (Kendal, 1955)

$F(6, 30) = 5.1$ when $P = 0.001$

Thus it would appear that there is highly significant agreement as to there being an order in which the total 5-hydroxyindole metabolites of the various brain regions may be ranked. The best estimate of 'true' rank order may be given by the rank order of the total ranks for each region (Kendal, 1955) or where there is equal weight between columns, as here, by the rank order of the mean values.

Absolute Concentrations of Tryptophan, 5-Hydroxytryptamine and 5-Hydroxyindol-3-ylacetic Acid

The concentrations of tryptophan, 5-HT and 5-HIAA in the different regions of the brains of six normal dogs are presented in Appendix I, Table 3. It was shown (Parker-Rhodes 1966) that for the tryptophan concentrations there was a significant ($P < 0.05$) difference between regions but not between animals. There was also a correlation as to the order in which the regions could be ranked according to their tryptophan concentration. The degree of significance for the coefficient of concordance was however only just greater than ($P < 0.05$) and this difference between regions could not be demonstrated after tryptophan administration (Appendix I, Table 4). The best estimate of descending rank order of the concentration of tryptophan found is as follows - cerebellum, thalamus, hypothalamus hindbrain, midbrain, hippocampus and caudate. Price and West (1960) using acetone extracts also found an order in which the tryptophan concentrations of dog brain could be ranked but the order they reported was quite different from the above - the highest levels were in the hypothalamus and pons, the midbrain, cerebellum and medulla next, and the lowest concentrations in the spinal cord and cerebral hemisphere. It would

seem probable that the different orders reported in the two sets of results may well be due to differences in assay technique and may represent a variability in the extraction of tryptophan among the regions which vary with the method used. The best analysis of the hydroxyindole results is discussed below.

Total Molal Concentrations of 5-Hydroxytryptamine plus 5-Hydroxyindol-3ylacetic Acid

From the experiments with rats, it was considered that the molal concentration of the total 5-hydroxyindole metabolites ([5-HT] + [5-HIAA]) would be a highly useful parameter if the pathway was controlled by tryptophan 5-hydroxylase. Table 9:1 presents this information derived from the Table 3 in Appendix 1. From inspection of the two tables it is apparent that the form presented in Table 9:1 has lessened the variability between estimates, an unusual finding as the new information has been derived by summing independantly determined estimates.

The concentrations in the different regions of brain in each dog were assigned rank value (Table 10:1) and the coefficient of concordance (Kendal 1955) was calculated (footnote Table 10:1) and its level of significance determined from tables for the values of Snedecor's F. The value found for the coefficient of

TABLE 11:1

Total 5Hydroxyindoles Concentrations in Different Regions of Dog Brain after Intravenous Administration of L Tryptophan

Area	1 hour			2 hours			4 hours		
Midbrain	14.4	25.2	20.1	37.1	23.1	22.4	15.5	14.3	17.2
Hypothalamus	13.4	18.2	16.9	13.2	28.7	16.0	20.1	11.5	13.2
Thalamus	7.3	8.9	6.6	5.4	12.9	9.7	4.7	6.8	3.0
Caudate	4.9	4.6	4.6	4.3	7.7	6.0	4.6	3.9	3.2
Hippocampus	7.2	7.3	6.3	5.3	8.2	5.7	5.8	4.1	2.6
Hindbrain	9.7	12.9	5.4	6.6	12.8	11.8	9.4	7.3	7.9
Cortex	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cerebellum	ND	ND	ND	ND	ND	ND	ND	ND	ND

Concentrations of total 5-hydroxyindoles ([5HT] + [5HIAA]) in n.moles/gram of brain tissue following intravenous administration of 50mgm/Kg of L.Tryptophan.

N.D. Neither 5HT nor 5HIAA detected (< 0.2 n.moles/g. of each).

The amounts of 5HT and 5HIAA are presented in appendix 1 - table 4.

Table 12:1

Coefficients of Concordance for Table 11:1

Region	1 hour			Rank Total	2 hours			Rank Total	4 hours			Rank Total
Midbrain	1	1	1	1	1	2	1	1	2	1	1	1
Hypothalamus	2	2	2	2	2	1	2	2	1	2	2	2
Thalamus	4	4	3	4	4	3	4	4	5	4	5	4
Caudate	6	6	6	6	6	6	5	6	6	6	4	6
Hippocampus	5	5	4	5	5	5	6	5	4	5	6	5
Hindbrain	3	3	5	3	3	4	3	3	3	3	3	3
Cortex	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Cerebellum	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5

The different brain regions were assigned rank values and the coefficient of concordance (table 10:1) calculated on the total data

$W = 0.937$, $F(6.7, 54) = 119$ ($F_{(6, 40)} = 4.7$ when $P = 0.001$)

Coefficients of concordance were also calculated for the replicates at 1, 2 and 4 hours and, by ranking the total of the ranks assigned to each region at each time, between the times.

1 hour $W = 0.967$ $F(6.3, 12.6) = 60$

2 hours $W = 0.968$ $F(6.3, 12.6) = 60$

4 hours $W = 0.947$ $F(6.3, 12.6) = 36$

($F_{(6, 12)} = 8.4$ when $P = 0.001$)

Ranking the total ranks for each region at each time shows perfect correlation between the times. It can be seen from a comparison of the means of ranks of tables 12:1 and 10:1 and from the rank orders of their mean values (tables 11:1 and 9:1) that there is a high degree of correlation between the two tables.

A coefficient of concordance calculated on all the ranks assigned to the results in tables and gave $W = 0.905$ and $F(6.9, 96) = 134$ ($F_{6,60} = 4.4$ when $P = 0.001$).

concordance ω was 0.899 and its value of F very much greater than that required for a P value of 0.001. (Table 10:1). Thus it appears that there is an extremely significant agreement between dogs as to the existence of an order in which the concentration of total 5-hydroxyindoles in the different regions may be ranked. The best estimate of the 'true' rank order may be given by the rank order of the total ranks for each region (Kendal 1955) or where there is equal weight, as in this case, by the rank order of the mean values (Table 10:1).

Following Intravenous Administration of Tryptophan

Following the administration of tryptophan 50 mg/Kg intravenously, the concentration of 5-HT and 5-HIAA rose in all regions of brain where they were initially present. (Appendix I, Table 4). From this table the molal concentration of the total 5-hydroxyindole in these regions of dog brain at varying times after tryptophan administration have been derived, (Table 11:1). The statistical analysis of the data used was similar to that used for the results obtained from analysis of control dog brain. The regions of brain for each dog received rank values according to their concentrations of total metabolite (Table 12:1). A coefficient of concordance was calculated for ranks from all the dogs.

TABLE 13:1

The Concentrations of Total 5Hydroxyindoles in Different Regions of Dog Brain, Before and Following Intravenous Administration of L-Tryptophan.

Region	Control	1 hour	2 hours	4 hours
Midbrain	100	100	100	100
Hypothalamus	78 ± 20	83 ± 10	77 ± 45	95 ± 21
Thalamus	44 ± 24	39 ± 10	38 ± 21	34 ± 13
Caudate	30 ± 9	23 ± 8	24 ± 12	27 ± 2
Hippocampus	39 ± 13	37 ± 12	25 ± 11	29 ± 9
Hindbrain	39 ± 5	48 ± 21	42 ± 21	57 ± 5

The results from Tables 9:1 and 11:1 are here expressed as percentages of the concentration (n.moles/g) of the total 5Hydroxyindoles ([5HT] + [5HIAA]) in the midbrain region of each dog. Mean values ± standard deviations.

This was highly significant ($P < 0.001$). Separate concordance coefficients for data at one, two and four hours were all highly significant while the order of the rank totals at each time show perfect correlation with each other. (Table 12:1 and footnote). Thus from tables and statistical analysis it can be seen that the normal order persists throughout the load. By expressing all the concentrations of total 5-hydroxyindole as percentages of the concentration found in midbrain (Table 13:1) it can be seen that the correlation maintained throughout the load is not merely of order but of interval type.

Conclusion

These observations fit well with the idea that a precursor load of tryptophan will be controlled by the normal physiological rate determining processes.

Following Intravenous Administration of 5-Hydroxytryptophan

Following the administration of D,L-5-HTP 10 mg/Kg I.V.high concentrations of 5-HTP were achieved in the different brain areas, (plasma contamination would not give rise to a significant contribution to the levels of 5-HTP attained) and there was a rise in both 5-HT and 5-HIAA. (Table 14:1). 5-HT became detectable in the cortex and cerebellum. The caudate, the area with the highest decarboxylase activity, showed the greates t

TABLE 14:1

The Concentration of 5Hydroxyindoles in Different Regions of Dog Brain Following Intravenous 5HTP Administration.

Regions	Control (Mean)	L5HTP	D-L 5HTP			Decarb.	M.A.O.
		1 hr.	1 hr.	2 hrs.	4 hrs.	(Rank)	
Midbrain							
5HTP	ND	4.88	1.64	2.04	0.24	98	842
5HT	0.84	2.45	1.53	1.67	0.95		
5HIAA	0.92	2.70	1.75	2.67	1.79	(3)	
Rank of Total Metabolites	(1)	(3)	(2)	(3)	(1)		
Hypothalamus							
5HTP	ND	2.42	1.41	1.26	0.16	117	1624
5HT	0.81	2.60	1.45	1.80	0.79		
5HIAA	0.54	2.68	1.25	2.79	1.58	(2)	
Rank of Total Metabolites	(2)	(2)	(3)	(2)	(3)		
Thalamus							
5HTP	ND	3.38	2.19	3.10	0.57	38	940
5HT	0.34	0.56	0.66	0.56	0.52		
5HIAA	0.40	0.85	0.95	0.84	1.15	(4)	
Rank of Total Metabolites	(3)	(6)	(4)	(6)	(5)		
Caudate							
5HTP	ND	1.89	0.85	1.06	0.15	306	935
5HT	0.32	3.69	2.01	1.97	0.59		
5HIAA	0.18	2.11	1.94	4.03	1.99	(1)	
Rank of Total Metabolites	(6)	(1)	(1)	(1)	(2)		
Hippocampus							
5HTP	ND	5.71	2.54	3.23	0.36	16	1176
5HT	0.44	1.42	0.77	1.13	0.42		
5HIAA	0.18	0.92	0.65	0.79	0.54	(6)	
Rank of Total Metabolites	(5)	(4)	(5)	(4)	(6)		
Hindbrain							
5HTP	ND	4.60	2.03	1.95	0.45	30	1000
5HT	0.31	1.05	0.65	0.54	0.62		
5HIAA	0.37	1.20	0.47	1.12	1.44	(5)	
Rank of Total Metabolites	(4)	(5)	(6)	(5)	(4)		
Cortex							
5HTP	ND	5.86	3.18	3.67	0.99	7	819
5HT	ND	0.22	0.09	0.07	0.13		
5HIAA	ND	0.17	ND	ND	0.11	(8)	
Rank of Total Metabolites	(7.5)	(8)	(7.5)	(8)	(8)		
Cerebellum							
5HTP	ND	5.94	3.59	4.17	1.31	9	930
5HT	ND	0.22	0.09	0.14	0.64		
5HIAA	ND	0.14	ND	ND	0.15	(7)	
Rank of Total Metabolites	(7.5)	(7)	(7.5)	(7)	(7)		
Plasma							
5HTP	ND	2.23	4.50	-	0.45		
5HIAA	ND	0.94	0.31	-	0.13		
CSF							
5HIAA	0.028	0.127	0.080	0.102	0.118		

Concentrations of 5HTP, 5HT and 5HIAA. (µg/g of brain tissue) in different areas of dog brain at intervals after the intravenous administration of 10mg/Kg. of 5HTP (L- or D,L-form).
The corresponding mean concentrations in control dogs from Appendix 1, Table 3.

Rank of total metabolites - rank of the total molal concentration of ([5HT] + [5HIAA])

Decarboxylase activity expressed in µg of 5HT formed / gram of tissue/hour as reported by Bogdanski et al 1957.

Monoamine oxidase activity expressed in µg of 5HT destroyed / gram of tissue/hour as reported by Bogdanski et al 1957.

ND = Not detected, $<$ 0.05µg/g of 5HTP, 5HT or 5HIAA in brain

$<$ 0.03µg/ml of 5HTP or 5HIAA in plasma

increases in 5-HT and 5-HIAA concentration.

10 mg/Kg L-5-HTP was administered intravenously to one dog and the brain analysed after 1 hour. Many of the estimates of metabolites were approximately double those obtained following the same dose of D, L-5-HTP. Variation between animals and in experimental estimates precludes any attempt to establish a definite relationship between the L-isomer and its racemic mixture without many replicates of each. However, the results obtained would be compatible with the formation of the cerebral metabolites of 5-HTP from its L-isomer alone.

The ranks of 5-HTP concentrations and of the total molal concentration of [5-HT] + [5-HIAA] in the brains of dogs one and two hours after intravenous 5-HTP loading exhibit significant rank orders which correlate very well with the order of decarboxylase activity (Table 14:1) and are different from the order exhibited by the total molal concentration of ([5-HT] + [5-HIAA]) in normal dogs (Table 10:1) or in dogs during I.V. tryptophan load (Table 12:1). The rank order of ([5-HT] + [5-HIAA]) in the dog examined 4 hours after intravenous 5-HTP is different from the other time rank orders and presumably represents a change towards the physiological pattern after loading the 5-hydroxyindole pathway in an unphysiological manner.

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While the intercorrelation of the concentrations of hydroxyindoles in the brains of dogs one and two hours after loading can be seen to be of an interval type, its association with decarboxylase activity is of an order type only. However, partial rank analysis of these parameters would suggest that these are direct, significant correlations.

Statistical Analysis of Results Following 5-HTP Administration to Dogs. (Table 14:1)

The concentrations of the total 5-hydroxyindole metabolites ([5-HT] + [5-HIAA]), 1 and 2 hour dogs 'D, L-5-HTP' and 1 hour dog 'L-HTP' (Table 14:1), were ranked and their coefficient of concordance calculated. W = 0.955, $F_{(6.3,\ 12.6)} = 42.5$ ($F_{(6,\ 12)} = 8.4$ when P=0.001).

The concentrations of 5-HTP in the different regions of the brains of these dogs were ranked in inverse order of concentration and their coefficient of concordance calculated W = 0.956, $F_{(6.3,\ 12.6)} = 43$ ($F_{(6,12)} = 8.4$ when P = 0.001)

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

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

calculated by the method of Kendal (1955),

r(5-HTP, Decarb.) = 0.785, P = 0.0028

r((5-HT) + (5-HIAA), Decarb.) = 0.837, P = 0.0014

(* Gillitto 1947).

Thus it would seem that these concentrations are well correlated with decarboxylase activity and that their levels in discrete areas of brain tissue would be dependant on the decarboxylase activity of these areas. It is possible that the same findings would have been obtained if the different areas of brain had different capacities for the uptake of 5-HTP and that this had a similar rank order to decarboxylase activity. However, a Kendal partial rank correlation coefficient (Kendal 1955) was calculated excluding any direct influence of such differences in the rate of 5-HTP uptake into brain.

r((5-HT) + (5-HIAA) Decarb., 5-HTP) = 0.658.

Similarly partial rank correlation coefficients excluding the direct influence of decarboxylase activity and total ((5-HT) + (5-HIAA)) were calculated.

r((5-HT) + (5-HIAA) 5-HTP, Decarb.) = 0.294

r(5-HTP Decarb., ((5-HT) + (5-HIAA)) = 0.522

Interpretation of such correlations would suggest that the correlations of 5-HTP concentrations and of ([5-HT] + [5-HIAA]) concentrations with decarboxylase activity are the controlling correlations, and obscure any direct relationship between 5-HTP and ([5-HT] + [5-HIAA]).

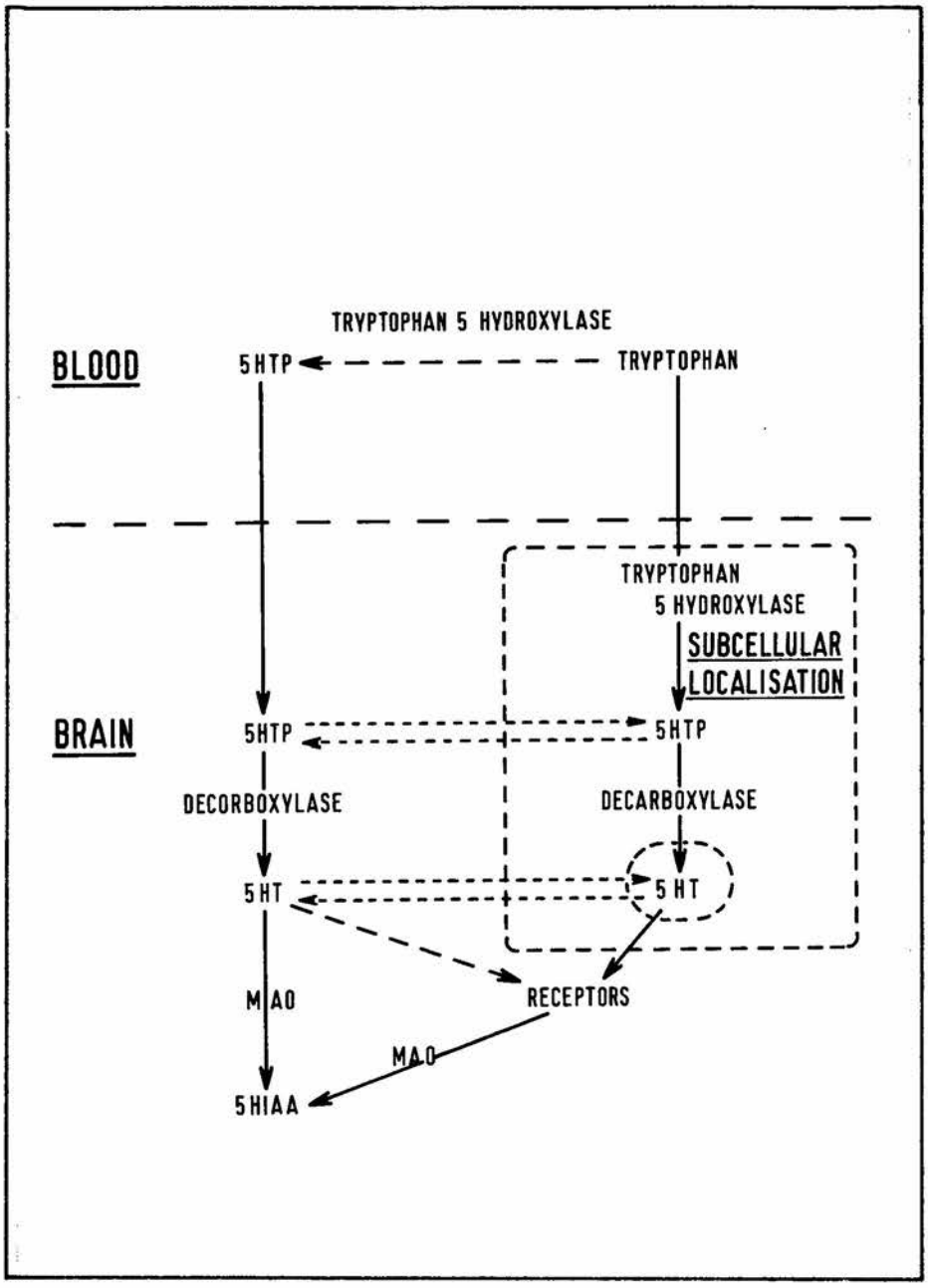
Conclusion

The ranking analysis of the concentrations of total 5-hydroxyindoles in the different areas of dog brain, in many ways an unsatisfactory method due to its ability to mask small differences in change of turnover rate, does, however, afford a means of evaluating some of the points of major importance. Thus it would appear that after tryptophan load the rate of synthesis of 5-hydroxyindoles in the different regions of brain increases in a manner which gives rise to a proportionate increase in all regions. This would happen if the metabolism during normal conditions and after tryptophan administration are being limited by the same step.

The change in the order of the increase in metabolites that occurs when loading with 5-HTP indicates that a different step is now rate limiting. Both the inverse order on ranking the 5-HTP concentration and the rank order of the concentration of total metabolites

Fig. 411

Diagram of the Cerebral Metabolism of 5-Hydroxyindoles



—— Metabolic path of major importance
--- Metabolic path of minor importance

The above diagram illustrates the author's hypothesis of the cerebral metabolic pathways concerned with 5-hydroxyindoles and emphasises the need to take account of the intracellular localisations of enzymes, their co-enzymes and substrates.

([5-HT] + [5-HIAA]) show a good correlation with the order of decarboxylase activity found in these areas by Bogdanski et al. 1957. Thus it is probable that differences in 5-HTP concentration in the different areas represent differences in the rate of utilisation controlled by decarboxylase activity and that there may be no real differences in the rate of uptake of 5-HTP into the various regions of the brain: it also follows that the step limiting the rate of production of the metabolites from 5-HTP, after 5-HTP administration is now the decarboxylase activity.

DISCUSSION OF RESULTS OBTAINED

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

(1) Entry of tryptophan into brain

(2) Hydroxylation of tryptophan, (a) extracerebrally
(b) intracerebrally

(3) Entry of 5-HTP into brain

(4) Decarboxylation of 5-HTP

(5) Intracerebral storage of 5-HT.

The tanks represent "Compartments".

The levels in the tanks represent the concentrations normally found within the compartments.

The vertical pipes represent enzymatic action.

The horizontal pipes represent diffusion.

The arrows represent direction of transfer.

The arrows with tails represent an active transport system and the direction of transport.

(1) The pipes representing diffusion have been drawn in duplicate to represent that diffusion of a substance between its compartments will depend not only on its concentration there but also on other factors such as electropotential charge and environment, and protein binding.

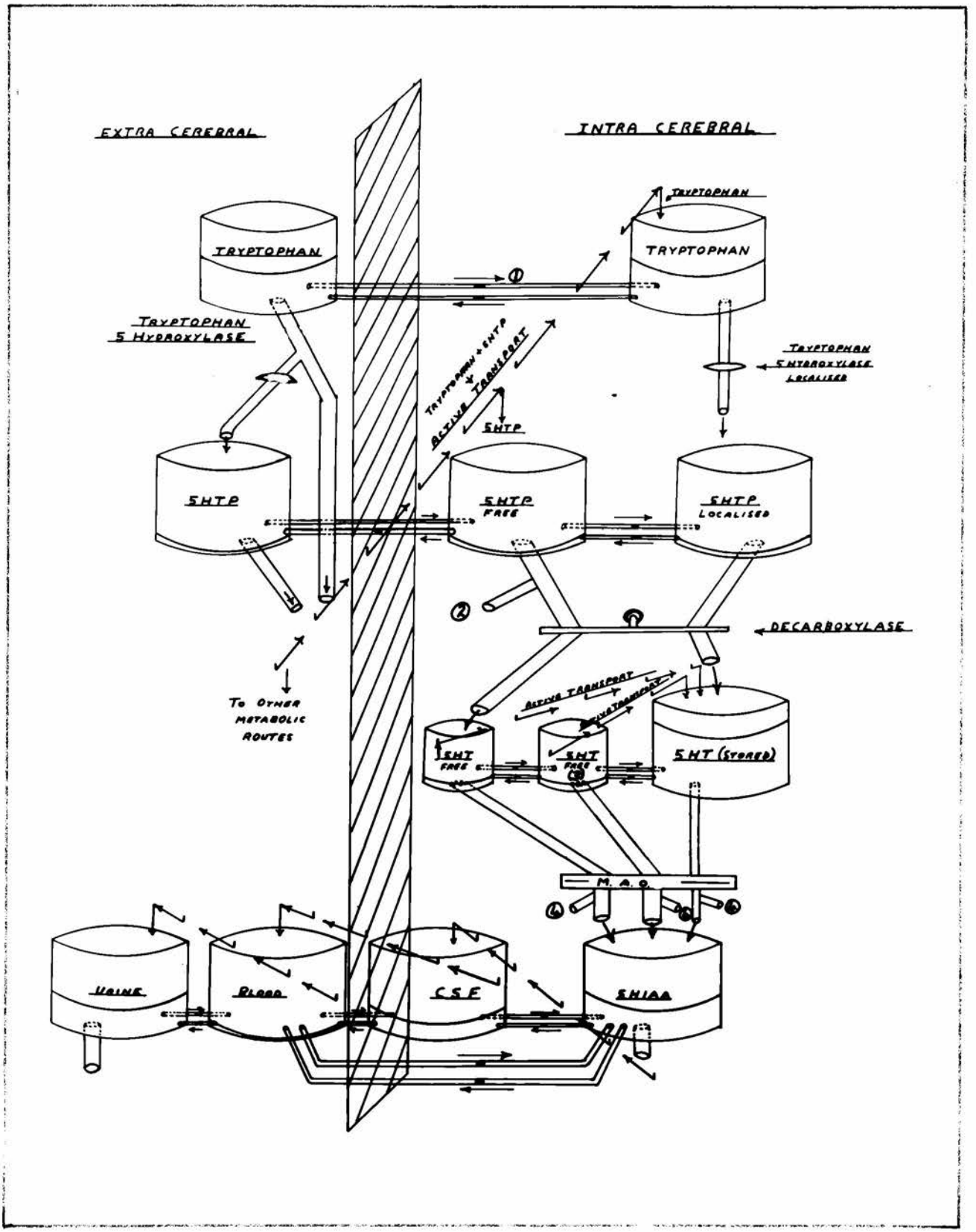
(2) It is possible that 5-hydroxytryptophan (5-HTP) is transaminated in brain to 5-hydroxyindol-3-ylpyruvic acid. (See text of discussion) N-acetylation of 5-HT might also occur, though at present there is only evidence for this in the pineal gland.

(3) "Free" 5-HT. The implication of the diagram is that functionally "free" 5-HT cannot be considered as a single compartment and that its action on "Receptors" will depend on its intracellular localisation. The hypothesis proposed is that "Free 5-HT" released from "Stored 5-HT" acts upon the "Normal receptors" while 5-HT formed "Free" by decarboxylation is rapidly destroyed, may activate "Receptors" not normally activated and may be unlikely to reach "Normal receptors". The above hypothesis explains why the two forms of precursor administration give rise to the differences in biochemical change reported here and to the differences in behavioural effects reported by many other workers.

(4) It is possible that the 5-hydroxyindol-3-ylacetaldehyde formed by monoamine oxidase (M.A.O.) will be reduced to 5-hydroxytryptophol (See text of discussion and Section IV).

Fig. 5:1

Diagrammatic Representation of Some of the Dynamic Parameters Probably Involved in the Cerebral Metabolism of 5-Hydroxyindoles.



In analysing the data obtained following loading with tryptophan or 5-HTP we have come to the conclusion that there is a degree of interplay between these steps which varies with the precursor load and animal species used, but that a general dynamic metabolic plan can be drawn. (Fig. 4:1). One can envisage two paths with multiple intercommunicating compartments which would be governed by kinetic constants in a similar manner to those governing the dynamic relationships of the mechanical system shown in Fig. 5:1.

The evidence for such a system and the limiting steps in the situations studied is considered below.

From previous (Eccleston et al. 1965) and present data on tryptophan loading in rats we have shown

(a) that concentrations of brain tryptophan increase with increasing dosage of tryptophan

(b) that 5-HTP never becomes detectable in brain even with very large loads,

(c) that the initial rate of rise in the molal concentration of total 5-hydroxyindoles ([5-HT] + [5-HIAA]) is the same over a very wide dose range (50 - 1600 mg/Kg), Fig. 2:1.

This proves that cerebral 5-hydroxyindole metabolism is limited by hydroxylation under these conditions. Two additional points were noted from previous data (Appendix I, Table 1):-

(a) that with very high doses of tryptophan, 5-HTP could be detected in small amounts in the plasma, and

(b) that when plateau levels for 5-HT and 5-HIAA could be produced as after single large doses of tryptophan the 5-HT/5-HIAA ratio was smaller than the ratio in control brain.

These findings could be due to either a progressive filling of the stores of 5-HT towards saturation with increasing overspill of 5-HT directly on to monoamine oxidase, or to 5-HTP derived from extracerebral hydroxylation of tryptophan by-passing the storage mechanism for 5-HT, thus providing a progressively increasing contribution to the brain concentration of 5-HIAA. Small doses of 5-HTP which could produce an initial rate of rise of total metabolites equivalent to the maximum that could be produced on tryptophan loading gave only an increase in 5-HIAA and none in 5-HT. Thus it appears that all 5-HT normally found in rat brain is derived via the intracerebral hydroxylation of tryptophan and that this enzyme normally limits its synthesis.

The effects seen in the whole rat brain sample could have been due to some extent to one or more regions of brain which normally have low rates of hydroxylation but have high decarboxylase activity. This type of

change is well illustrated by examining the status of the caudate nucleus in the dog brain under varying load conditions.

In dogs, regional differences in the storage, or the rate or the pathway of metabolism of 5-HT can be seen to exist from the data of Table 8:1. These results cannot merely be explained by an alternative pathway for the metabolism of 5-HT to 5-HIAA unless one assumes that the balance between such a metabolite and 5-HIAA varies in different regions as otherwise the molal ratio of [5-HT] / [5-HIAA] would still have remained constant under steady state conditions i.e.

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

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

It is interesting to speculate at this point that such a pathway as is envisaged above might involve the metabolism of 5-HT to 5-hydroxytryptophol, a pathway demonstrated for rat brain <u>in vitro</u> (Section IV). This pathway might act as a route of elimination or as a controlling shunt mechanism since both 5-hydroxytryptophol

formation (Section IV) and the formation of 5-HTP by tryptophan 5-hydroxylase localised in the mitrochondrial fraction of brain tissue, (Gal, Armstrong and Ginsborg 1966) have been shown to require the same co-enzyme $NADPH_2$.

Analysis of regions of brain at intervals following tryptophan loads in dogs shows that although there was an absolute increase of 5-HT and 5-HIAA in all regions, the pattern of metabolism remained unchanged (apart from hindbrain*).

Small doses of 5-HTP in dogs can give rise to significant rises in brain 5-HT but what is very striking is that the pattern of metabolism is not normal (Tables 10:1, 12:1 and 14:1) and that now the order of total metabolite concentration correlates very well with the order of the decarboxylase activity in dog brain reported by Bogdanski et al. 1957. From the analysis of the data (Table 14:1) it would appear that the decarboxylase activity is controlling both the concentrations of 5-HTP and total metabolites found in brain. Thus, although cerebral 5-HT can be formed from 5-HTP presented

* Footnote: The studies presented in Section III lead to the belief that the hindbrain region has a second role in metabolism concerned with the elimination of acid metabolites from the C.S.F. which would account for this divergence.

extracerebrally it can be seen (especially when one considers the status of the caudate nucleus) that again it is extremely likely that only tryptophan hydroxylated intra-cerebrally contributes to the stores of 5-HT in the brain.

Some interesting species differences are apparent from the loading experiments and show that limiting steps in metabolism depend on the species studied as well as the precursor used. In both rats and dogs only the L-isomer of 5-HTP appeared to be taken into brain, and thus it could seem likely that uptake into brain will be controlled at these dose levels mainly by an active transport process. However, there are obvious species differences in the uptake mechanism. Firstly the uptake of 5-HTP into dog brain would appear to be more efficient (Brain/plasma ratio for 5-HTP is 5-20 times greater than in rats, and the ratio of the increase of all 5-hydroxyindoles in brain to the concentration of plasma 5-HTP is also greater, Tables 5:1, 6:1 and 14:1). Secondly, the relationship of the rate of increase of metabolism to the dose of 5-HTP administered to rats shows a very flat exponential rise. It is probable, because of the doubtful significance of 5-HTP found in rat brain, that this slow rise for the rate limiting

step is dependent on the rate of entry of 5-HTP into brain. It is possible that some of the differences in uptake between the species might be due to 5-HTP and tryptophan using the same carrier mechanism into brain. Thus it is very interesting to note that our normal blood tryptophan levels for control rats were much higher than the values for control dogs.
Control dogs - Blood tryptophan 10.1±0.6 µg/ml S.E.M.(23)
Control rats - Blood tryptophan 16.2±0.7 µg/ml S.E.M. (6)
(Active transport of L-tryptophan has been demonstrated in vivo Chirigos, Greengard and Udenfriend (1960) and Eccleston et al. (1965): Schanberg (1965) has reported that "L-tryptophan is a powerful inhibitor of the 5-HTP transport system" - rat brain slices).

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

From the evidence and arguments which have been presented so far it will be obvious that to incorporate our findings into a cohesive whole it is necessary to expand the conventional concept of the 5-hydroxyindole metabolism pathway to allow for functional localisations. (Figs. 4:1 and 5:1). It is probably relevant at this point to consider how, on the basis of such concepts the metabolism of an 'unphysiological' load like 5-HTP may be envisaged.

The most likely hypothesis is that when 5-HTP is presented systemically it may be taken up into the brain and be decarboxylated generally in the cell cytoplasm. The 5-HT formed will be subsequently oxidatively de-aminated fairly readily, while the normal physiological metabolism may involve the hydroxylation of tryptophan at localised sites intraneuronally which allow 5-HT when formed in the vicinity of the 5-HT stores to have a better chance of being incorporated therein. It is one extension of the above idea that 5-HTP whether administered or derived systemically may well be transaminated (Haavaldsen 1962, Tangen, Fonnum and Haavaldsen 1965) to 5-hydroxyindol-3yl pyruvic acid in brain (which could probably not be differentiated

from 5-HIAA by our present techniques) and would thus not contribute to 5-HT stores.

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

Aghajanian and Bloom 1967 have shown that following intraventricular injection of tritiated 5-HT in rats the retention of "firmly bound label" correlates well with the areas known to have high endogenous levels of 5-HT and is predominantly associated with "nerve endings". These experiments confirm rather than contradict our findings as although their experiments use an unphysiological loading technique the analysis by autoradiography measures only that part of the total tritiated 5-HT localised with "bound" 5-HT as any "non-bound" H^3-5-HT

or H^3-5-HTP would be removed by the procedures prior to measurement. However, unless an analytical technique takes some account of the changes in the distribution of 5-HT between its intracellular compartments, (ultracentrifugation, histochemistry or autoradiography), valid interpretations based on estimates of total pool 5-HT when using loads other than tryptophan must be difficult. The same criticisms apply to the work reported by Gershon and Ross (1966a, and b) who performed similar studies following the administration of ^{14}C-D,L-5-HTP.

In the clinical sphere 5-HTP appears to have been accepted as a physiological precursor of 5-HT and has been given in the investigation of sleep, (Oswald 1965); in the investigation (Coppen, Shaw and Malleson 1965) and treatment (Kline and Sacks 1963, Kline, Sacks and Simpson 1964) of depression; in the treatment of schizophrenia (Brengelmann, Pare and Sandler 1959, Klee, Bertino, Goodman and Monson 1960) and more recently in the treatment of Down's syndrome(Bazelon, Pain, Cowie, Hunt, Houck and Mohammed, 1967). In view of the fact that low doses of 5-HTP probably do not influence intraneuronal concentrations of 5-HT in normal sites many of the conclusions drawn from these studies should

be viewed with circumspection. On the other hand it is probably worthwhile considering the administration of tryptophan as one of the best means of investigating 5-HT metabolism in man.

SUMMARY

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

Section IB

THE USE OF PRECURSOR LOADING TO STUDY THE ACTION OF α-METHYL DOPA

In view of the results obtained on determination of the concentration of 5-HIAA in samples of C.S.F. taken at intervals after precursor administration from some of the dogs used for the acute experiments, (Tables 11:1 and 14:1) it was decided that it might be possible to use this technique of C.S.F. sampling as a method of screening to find an effective dose of α-methyl dopa and to ensure that the times of acute experiments after drug administration would be planned to the best advantage.

Effects of α-Methyl Dopa on the Concentration of 5-Hydroxyindol-3-ylacetic acid in Cerebrospinal Fluid

The technique of sampling cisternal C.S.F. from dogs and the Latin square design of the experiment have been described previously (page 5). One such Latin square experiment where the C.S.F. 5-HIAA was estimated following the administration of tryptophan alone had already been performed (Appendix 1, Table 2 - Square I). Two similar Latin square experiments were performed, one an exact replica of the above - after intravenous tryptophan alone (Table 15:1 - Square II) and the other varied to include pre-treatment of the dogs with α-methyl dopa (50 mg/Kg) intravenously 30 minutes prior to administration of

Table 15:1

5-Hydroxyindol-3-ylacetic Acid Concentrations in Cisternal Cerebrospinal Fluid Following Tryptophan Administration to Dogs

Latin Square II

Experiment	Time in Hours			
	0	1	2	4
1	40	68	86	62
2	26	75	91	74
3	42	68	57	57
4	22	90	67	65

The concentrations of 5-HIAA in mμg/ml in cisternal samples of C.S.F. obtained from the cisterna magna of dogs which had been given 50 mg/Kg of tryptophan at zero time. The experiments were performed at intervals of two weeks. Each experiment involved the use of the same four dogs which were sampled at different times after tryptophan administration according to a Latin Square design.

Table 16:1

5-Hydroxyindol-3-ylacetic Acid Concentrations in Cisternal Cerebrospinal Fluid Following Tryptophan Administration to Dogs Pretreated with α-Methyl Dopa

Latin Square III

Experiment	Time in Hours			
	0	1	2	4
1	43	50	47	56
2	40	37	57	56
3	31	39	49	49
4	27	48	53	56

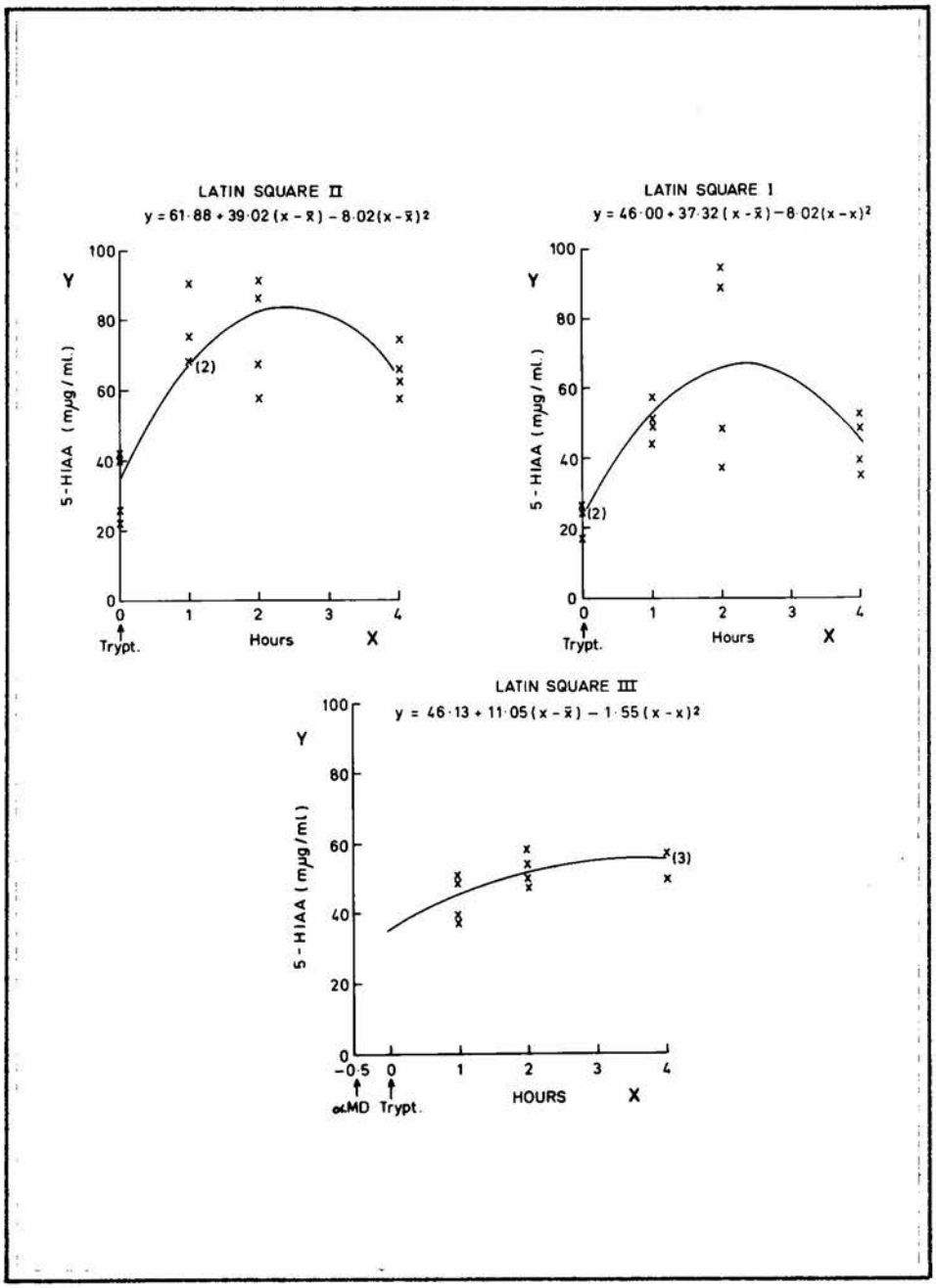
The concentrations of 5-HIAA in mμg/ml in samples of C.S.F. obtained from the cisterna magna of dogs which had been pretreated with α-methyl dopa 50 mg/Kg IV and then given 50 mg/Kg L-tryptophan intravenously at zero time. The experiments were performed at intervals of two weeks. Each experiment involved the use of the same four dogs which were sampled at different times after tryptophan administration according to a Latin Square design.

tryptophan. (Table 16:1 - Square III). It was obvious on comparison of the results that some unknown factor had caused a marked difference between the concentrations of 5-HIAA in corresponding experiments in the Square I series and the Square II series. Analysis of variance performed on the data showed that in each Latin square there was no significant variation between the dogs used nor between experimental days. However, the mean rise of 5-HIAA was found to be significantly (probability below the 0.005 level) different between the two Latin squares for tryptophan alone, Squares I and II, in spite of the fact that the design method, technique and even the animals were identical with these used to obtain the results in Square I. The reason for the difference is not known.

Originally it was intended to ignore the results in Square I, as the experiments had not been performed personally, and contrast only Squares II and III. However, it was noticed that the concentration of 5-HIAA in C.S.F. in Square I, although of lower absolute value throughout than Square II, had a similar time course of rise and subsequent fall in the concentration of 5-HIAA after precursor administration to the time course observed in Square II. It was decided that all three squares could probably be treated as regressions of 5-HIAA concentrations in C.S.F. with time after tryptophan

Fig. 6:1

Concentrations of 5-Hydroxy-3-ylacetic Acid in Cisternal Cerebrospinal Fluid



These diagrams show the best fit quadratic regression curves for 5-HIA concentration in cisternal C.S.F. against time in three experiments performed according to Latin Squere design. Latin Squares I and II were performed after the administration of 50 mg/kg of L-tryptophan intravenously. Latin Square III includes the additional variable of pretreatment with 50 mg/kg of α-methyl dopa 30 mins. prior to tryptophan administration.

administration. Since the required regressions must have contained at least two opposing exponential constants, statistical help was sought.

In view of the small amount of data available it was suggested that the best procedure was to produce 'best fit' curves of the simplest polynomial type which could reasonably fit the data. Quadratic curves were considered to fit the data fairly well and proved quite adequate to demonstrate fully the main points of interest.

The 'best fit' quadratic equations derived in the form $Y = a + b(x - \bar{x}) + c(x - \bar{x})^2$ were

Square I, $Y = 46.00 + 37.32(x - \bar{x}) - 8.02(x - \bar{x})^2$ (Fig 6.1)

Square II, $Y = 61.88 + 39.02(x - \bar{x}) - 8.02(x - \bar{x})^2$ "

Square III, $Y = 46.13 + 11.05(x - \bar{x}) - 1.55(x - \bar{x})^2$ "

Where Y is the concentration of 5-HIAA in mug/ml of cisternal C.S.F. and X is the time in hours after the administration. $\bar{X}$ the mean value will equal 1.75 hours.

Although the parameter found for this type of polynomial expression can have no direct biological meaning it reduces all the data to a form which can have statistical values calculated for the likelihood of certain hypotheses.

Firstly, where the form of the equations are similar (Squares I and II), the average value of Y is given by the first term of the right hand side, i.e. 46.00 and 61.88.

Thus for some reason as mentioned before the average response in Squares I and II is different (significant at the 0.005 probability level). Secondly however, (the main point for making this type of analysis) the 'form' of the curves for Squares I and II is remarkably similar as is shown by the similarity of the regression coefficients

$b = 37.32$, $b = 39.02$, $c = 8.02$, and $c = 8.02$

The variance of the 5-HIAA concentrations about the curve in I and II is also not significantly different. Hence, in comparing with curve III, the b's, c's and the variance about the regression curves were pooled for I and II resulting in average values

$b = 38.17$ $c = 8.02$

and the pooled variance about regression curve I and II = 186.

Since the b and c regression coefficients are statistically related the pooled results for these values from I and II were tested against b and c simultaneously, using the generalised t-test (multi-normal theory). The results were highly significant (at least at the 0.01 probability level).

Further results of interest were noted. The variance about the regression curve III was only 30.55, a value significantly smaller than that for I and II (186). Thus, not only has curve III become flattened but the variation

Table 17:1

Time Taken to Reach the Maximum Concentration of 5-Hydroxyindol-3-ylacetic Acid in Various Regions of Dog Brain Following Intravenous Tryptophan

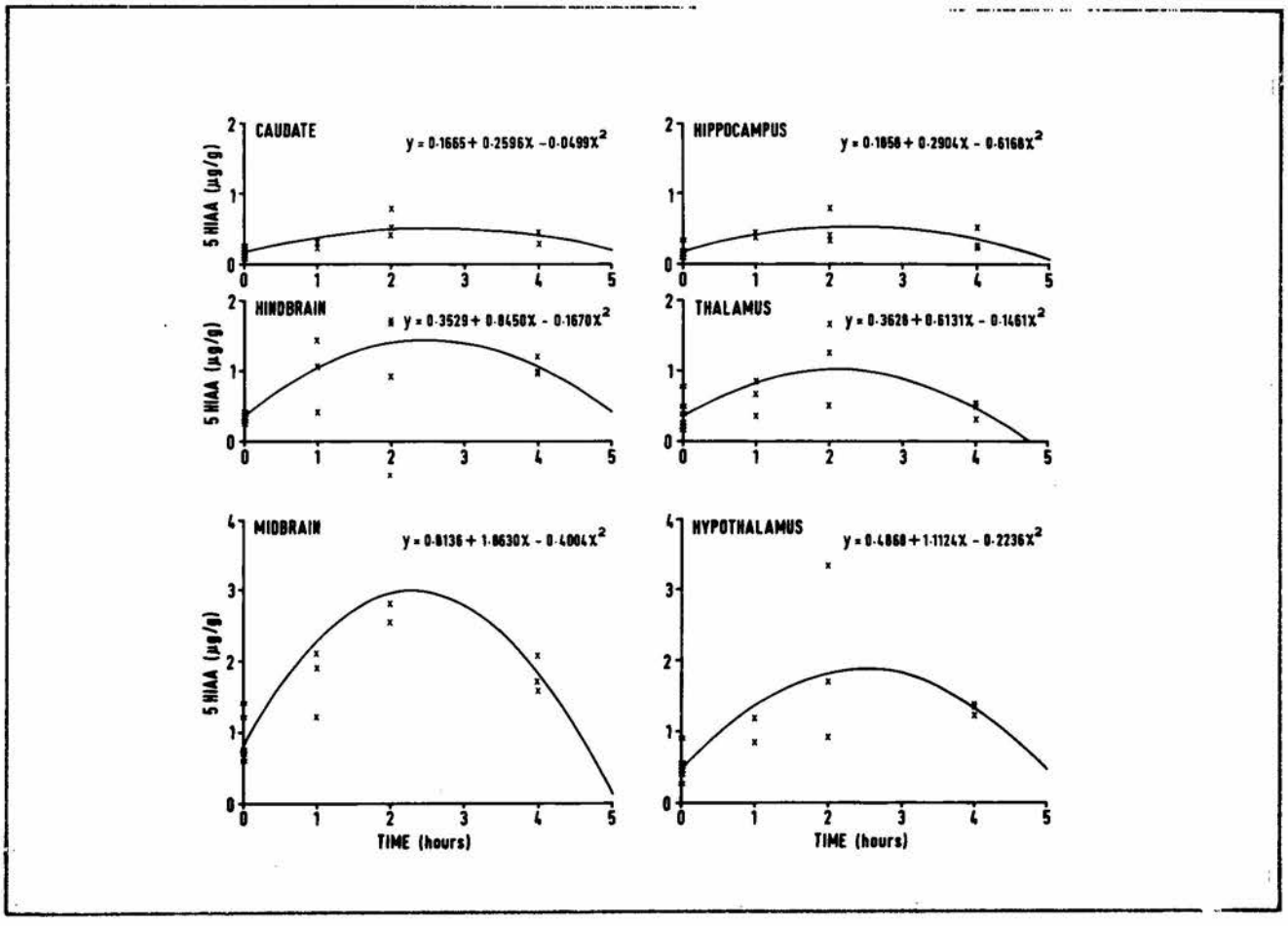
Brain Region	Estimated Time of Maximum	Standard Deviation
Hypothalamus	2.49	± .314
Midbrain	2.33	± .166
Caudate	2.60	± .397
Hippocampus	2.35	± .220
Hindbrain	2.53	± .223
Thalamus	2.09	± .197

Time to reach maximum 5-HIAA concentrations calculated from the best fit quadratic regression equations.

Dose of L-tryptophan administered 50 mg/Kg

Fig. 7:1

The Concentrations of 5-Hydroxy-3-ylacetic Acid in Various Regions of Dog Brain After Tryptophan Loading



The photograph shows the best fit quadratic regression equations and curves for the regression of 5-HIA concentration against time in various regions of dog brain.

between animals in the concentrations of 5-HIAA found in the C.S.F. has become less. The time taken to reach peak concentrations of 5-HIAA in C.S.F. were 2.33 and 2.43 hours for Squares I and II respectively, while the maximum of the curve for Square III was not reached until 3.56 hours.

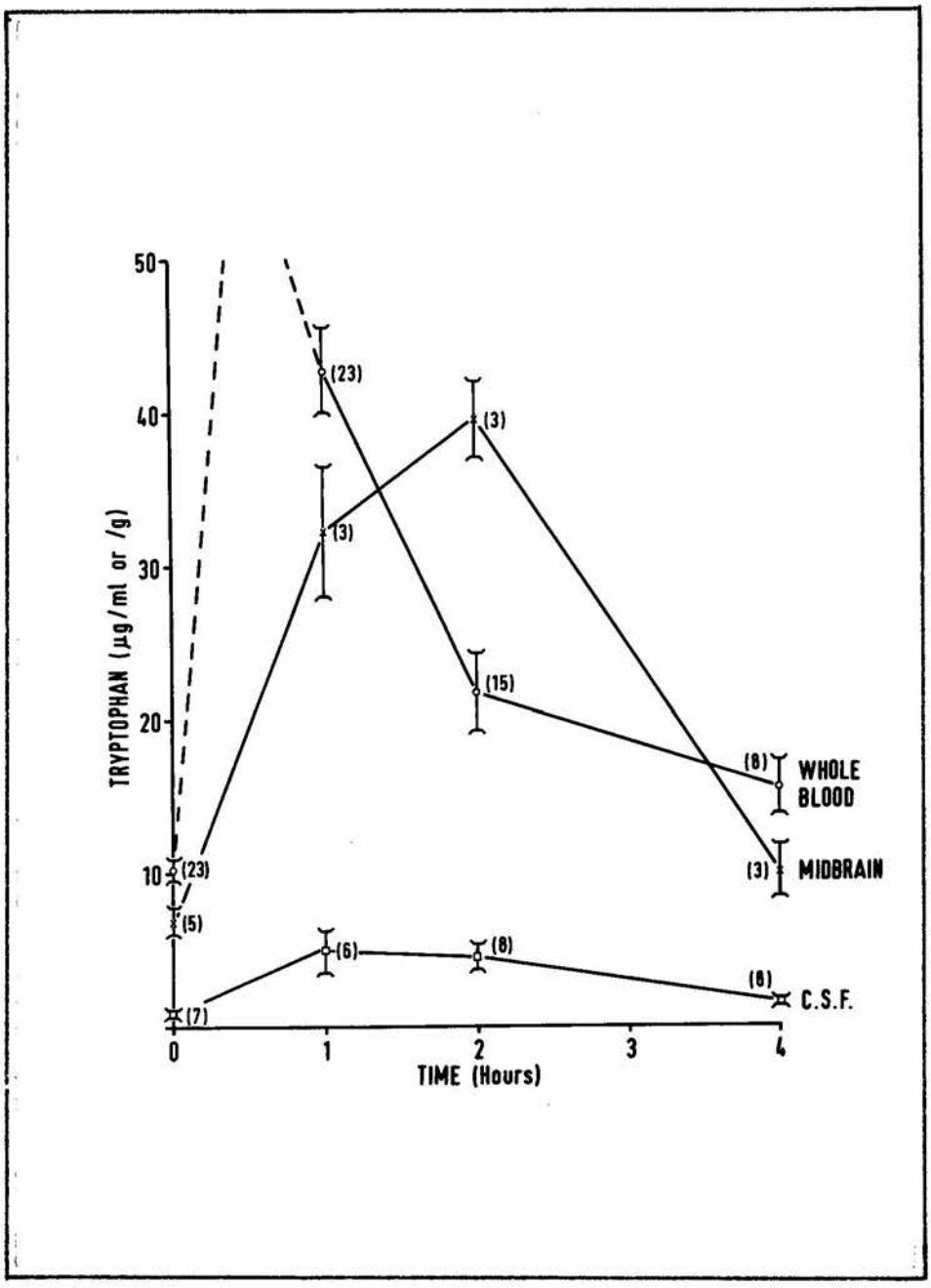
Comparison Between C.S.F. and Brain Concentration after Intravenous Tryptophan Administration.

From the data in Tables 3 and 4, Appendix I, 'best fit' quadratic curves for the changes in the concentration of 5-HIAA were fitted for the various brain regions (Fig. 7:1). Although no direct statistical comparison between the curves for the various brain regions and those for C.S.F. (Fig. 6:1, I and II) can be made due to the physical differences in the samples concerned, the time to achieve maximum concentration of 5-HIAA after tryptophan loading did not differ significantly between the various regions of brain (Table 17:1) and had a mean value of 2.34 hr. which is in good agreement with the times to maximal concentrations (2.33 and 2.43 hr.) found for C.S.F.

The concentrations of tryptophan found in different brain regions of dogs show similar regressions with time after intravenous tryptophan administration (Appendix I, Tables 3 and 4). The values of a representative area - midbrain - have been plotted together with the

Fig. 8:1

Concentrations of Tryptophan in Whole Blood, Midbrain and Cerebrospinal Fluid following Intravenous Tryptophan Administration



The figure shows concentrations of tryptophan in whole blood, midbrain and cerebrospinal fluid as their mean values ± SEM following the administration of 50 mg/kg of L-tryptophan intravenously. While the whole blood concentrations show a rapid decline, midbrain and CSF show similar time courses of rise and fall in concentration.

concentrations of tryptophan found in C.S.F. and blood during the chronic experiments reported above in Latin squares I and II (Fig. 8:1). While the concentrations of tryptophan in blood show an exponential fall, the concentrations of the amino acid in brain and C.S.F. show time courses of rise and fall which are similar to each other but different from that found for blood.

Effects of α-Methyl Dopa on the Concentrations of Tryptophan and 5-Hydroxyindoles in Dog Brain.

In view of the results obtained above it was decided to perform acute experiments according to the drug schedule used for Square III above (i.e. pretreatment with α-methyl dopa 50 mg/Kg intravenously followed 30 minutes later by intravenous tryptophan 50 mg/Kg), the acute experiments being performed at 0, 1, 2 and 4 hours with respect to tryptophan administration (Table 18:1). This experimental schedule was then repeated using a larger dose of α-methyl dopa (100 mg/Kg). Experiments were then carried out at comparable times after a dose of 100 mg/Kg of α-methyl dopa in the absence of tryptophan administration (Table 19:1).

Treatment with α-methyl dopa alone (100 mg/Kg) resulted in a slight rise in 5-HIAA with a fall in 5-HT in most regions (Table 19:1). Pretreatment with either 50 or 100 mg/Kg of α-methyl dopa prior to tryptophan (Tables 18:1 and 19:1) blocked the usual large rise in the

TABLE 18:1

Concentration of tryptophan, 5HT and 5HIAA (µg/g) in various regions of dog brain following intravenous administration of L-tryptophan (50 mg/kg) the animals being treated with α-methyl dopa intravenously (50 mg/kg) 30 min. previously

Region	Controls 0 hr	α Methyl dopa alone 0.5 hr	α Methyl Dopa + Tryptophan 1.5 hr	2.5 hr	4.5 hr
Midbrain					
Tryptophan	6.8 ± 2.1(5)	4.2	38.8	11.7	7.0
5HT	0.84 ± 0.14(6)	0.89	0.82	-	1.10
5HIAA	0.92 ± 0.19(6)	0.78	0.75	1.49	1.52
Hypothalamus					
Tryptophan	7.6 ± 2.1(5)	7.0	48.6	9.3	7.2
5HT	0.81 ± 0.21(6)	0.63	0.80	0.88	0.73
5HIAA	0.53 ± 0.21(6)	0.85	0.34	1.08	0.60
Thalamus					
Tryptophan	7.8 ± 1.9(5)	4.9	36.0	10.5	6.2
5HT	0.34 ± 0.14(6)	0.34	0.18	0.18	0.29
5HIAA	0.40 ± 0.22(6)	0.45	0.09	0.44	0.61
Caudate					
Tryptophan	5.1 ± 1.7(5)	4.5	36.9	11.2	6.9
5HT	0.32 ± 0.04(6)	0.19	0.20	0.38	0.22
5HIAA	0.19 ± 0.08(6)	0.20	0.26	0.35	0.31
Hippocampus					
Tryptophan	6.2 ± 1.7(5)	5.0	39.8	12.4	5.5
5HT	0.44 ± 0.13(6)	0.26	0.26	0.34	0.40
5HIAA	0.19 ± 0.08(5)	0.13	0.10	0.28	0.23
Hindbrain					
Tryptophan	6.9 ± 1.7(5)	5.1	40.2	13.3	7.9
5HT	0.31 ± 0.06(6)	0.19	0.34	0.35	0.15
5HIAA	0.37 ± 0.15(6)	0.28	0.40	0.89	0.48
Cortex					
Tryptophan	7.5 ± 1.3(3)	5.5	35.2	7.6	7.6
5HT	ND (3)	ND	ND	ND	ND
5HIAA	ND (3)	ND	ND	ND	ND
Cerebellum					
Tryptophan	8.5 ± 3.5(5)	6.0	79.2	9.9	8.2
5HT	ND (4)	ND	ND	ND	ND
5HIAA	ND (4)	ND	ND	ND	ND
CSF (Cisternal)					
Tryptophan	0.78 ± 0.40(7)	1.2	8.6	2.9	1.6
5HIAA	0.028 ± 0.009(8)	0.038	0.034	0.025	0.055
Blood					
Tryptophan	10.1 ± 2.8(23)	10.2	38.6	23	13.8

Mean ± SD (number of experiments) - Appendix 1 - Table 3.

5HTP not detected (<0.05 µg/g) in any region.

ND $<$ 0.05 µg/g

TABLE 19:1

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

Region	Controls	a Methyl Dopa Alone				a Methyl Dopa + Tryptophan		
	0 hr	0.5 hr	1.5 hr	2.5 hr	4.5 hr	1.5 hr	2.5 hr	4.5 hr
Midbrain								
Tryptophan	6.8 ± 2.1(5)	4.2	8.2	8.3	4.4	71.5	19.8	10.8
5HT	0.84 ± 0.14(6)	1.17	0.98	0.88	0.42	1.20	0.92	0.54
5HIAA	0.92 ± 0.19(6)	0.79	1.55	1.44	0.63	1.01	1.40	1.16
Hypothalamus								
Tryptophan	7.6 ± 2.1(5)	7.3	11	7.9	-	51.9	23.7	12.3
5HT	0.81 ± 0.21(6)	0.62	0.63	0.41	0.42	0.77	0.45	0.51
5HIAA	0.53 ± 0.21(6)	0.49	0.58	0.56	0.26	0.79	0.77	0.86
Thalamus								
Tryptophan	7.8 ± 1.9(5)	4.8	8.0	6.5	4.3	48.9	19.6	9.6
5HT	0.34 ± 0.14(6)	0.10	0.24	0.18	0.14	0.24	0.20	0.26
5HIAA	0.40 ± 0.22(6)	0.28	0.72	0.39	0.32	0.44	0.26	0.32
Caudate								
Tryptophan	5.1 ± 1.7(5)	4.9	8.0	7.7	3.7	49.4	15.6	11.6
5HT	0.32 ± 0.04(6)	0.26	0.27	0.15	0.31	0.26	0.32	0.33
5HIAA	0.43 ± 0.08(6)	0.26	0.34	0.32	0.16	0.16	0.25	0.20
Hippocampus								
Tryptophan	6.2 ± 1.7(5)	4.6	6.3	7.2	3.7	39.5	16.2	9.4
5HT	0.44 ± 0.13(6)	0.17	0.19	0.24	0.18	0.34	0.38	0.35
5HIAA	0.19 ± 0.08(5)	0.12	0.28	0.11	0.98	0.29	0.11	0.17
Hindbrain								
Tryptophan	6.9 ± 1.7(5)	4.9	8.6	7.0	4.8	49.3	20.3	10.0
5HT	0.31 ± 0.06(6)	0.10	0.14	0.18	0.14	0.19	0.36	0.26
5HIAA	0.37 ± 0.15(6)	0.23	0.34	0.34	0.27	0.51	1.11	1.23
Cortex								
Tryptophan	7.5 ± 1.3(3)	5.9	10.9	7.9	3.7	50.3	15.8	10.6
5HT	ND (3)	ND	ND	ND	ND	0.03	ND	ND
5HIAA	ND (3)	ND	ND	ND	ND	ND	ND	ND
Cerebellum								
Tryptophan	8.5 ± 3.0(5)	4.0	-	7.6	4.5	54.2	18.4	11.2
5HT	ND (4)	ND	-	ND	ND	0.02	ND	ND
5HIAA	ND (4)	ND	-	ND	ND	ND	ND	ND
CSF (Cisternal)								
Tryptophan	0.78 ± 0.40(7)	1.2	1.3	1.0	-	8.6	2.9	1.6
5HIAA	0.028 ± 0.009(8)	0.038	0.043	0.020	-	0.034	0.025	0.035
Blood								
Tryptophan	10.1 ± 2.8(23)	10.2	8.8	8.4	7.5	33.6	23.0	13.8

Mean ± SD (number of observations) - Appendix 1 - Table 3.

5HTP was detected in midbrain and hippocampus at 1.5 hr. in a concentration of 0.13 and 0.16 ug/g respectively following the administration of both tryptophan and a-methyl dopa.

ND < 0.05 ug/g

concentration of 5-HT and 5-HIAA seen with tryptophan alone (Appendix I, Table 4). The maximum rise of 5-HIAA after α-methyl dopa tended to occur later than the 2.34 hours mean for tryptophan alone (Tables 18:1, 19:1 and 17:1). This was particularly noticeable in brain stem and paralleled the changes seen in 5-HIAA in C.S.F. under the same conditions of drug administration (Fig. 6:1).

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

When α-methyl dopa (50 mg or 100 mg/Kg) was administered prior to tryptophan the 1 hour concentration for tryptophan in brain was higher (Tables 18:1 and 19:1) than in animals treated with tryptophan alone (Appendix I, Table 4) but the 2 hour values were lower.

DISCUSSION OF THE MODE OF ACTION OF α-METHYL DOPA

α-methyl dopa was used in these experiments to produce a change in the pattern of response to a tryptophan load. Previous investigators have found the drug to be a decarboxylase inhibitor *in vitro* (Sourkes, 1954) and *in vivo* in rat brain (Sharman and Smith, 1962; Eccleston, Ashcroft and Crawford, 1965). Its status as

a tryptophan hydroxylase inhibitor is disputed. Roos and Werdinius (1963) suggested that part of the fall in 5-HIAA and 5-HT in rabbit brain after α-methyl dopa was due to the inhibition of hydroxylase. Gal, Armstrong and Ginsborg (1966) showed that α-methyl dopa inhibited the tryptophan 5-hydroxylase in the mitochondrial fraction of rat brain. However, Gal, Morgan and Marshall (1965) were unable to demonstrate inhibition of tryptophan hydroxylase *in vivo* in rat brain by α-methyl dopa following an intracerebral injection of ^{14}C - labelled tryptophan. (α-methyl dopa has also been shown to inhibit the rat liver hydroxylase of tryptophan and phenylalanine *in vivo* and *in vitro* (Burkard, Gey and Pletscher, 1964) and also the *in vitro* activity of tyrosine hydroxylase (Nagatsu, Levitt and Udenfriend, 1964).)

In these present experiments when α-methyl dopa alone was administered, there was a fall in the 5-HT concentration and a rise in the 5-HIAA concentration in all regions of brain apart from the midbrain regions. This could be interpreted as the result of a release of 5-HT with or without an inhibition of synthesis of the amine. The possibility of the release of 5-HT by α-methyl dopa was first suggested by Sharman and Smith (1962) and again by Roos and Werdinius (1963). In the present experiments the rise in the concentration of both 5-HT and 5-HIAA after tryptophan was blocked in all regions by α-methyl

dopa. The inhibition was, however, incomplete. There did not seem to be a greater inhibition of the synthesis of 5-HT up to 2 hours after tryptophan with 100 mg/Kg of α-methyl dopa compared with 50 mg/Kg. However, at 4 hours the 5-HIAA and 5-HT was higher in the animals treated with the smaller doses of α-methyl dopa suggesting that the inhibition had not persisted as long as it did with 100 mg/Kg. Unlike rats under similar experimental conditions, 5-HTP did not appear in significant amounts in any brain region (Eccleston, Ashcroft and Crawford, 1965). Small quantities were however detected in midbrain and hippocampus. It was interesting to note that hippocampus had relatively low levels of decarboxylase activity and one would anticipate more complete decarboxylase inhibition by the drug in this region. Midbrain on the other hand had high levels of the hydroxyindoles and probably a high turnover and consequently relatively less decarboxylase inhibition would result in the accumulation of sufficient 5-HTP to become detectable. That 5-HTP did not accumulate in large quantities in spite of the fall in the synthesis of 5-HT and 5-HIAA would tend to indicate inhibition at the hydroxylating enzyme. The rapid fall in the concentration of tryptophan at 2 hours when compared with controls (Tables 18:1 and 19:1, and Appendix 1, Table 4) could not be explained by inhibition of active uptake of tryptophan into brain as the concentrations were

higher than with tryptophan alone at 1 hour. This finding remains unexplained.

In C.S.F. the rise of 5-HIAA after tryptophan was inhibited by α-methyl dopa and the quadratic regression curve (III) differed significantly from those obtained with tryptophan alone. The form of the curve resembled the form of change found in brain stem. The time of maximum rise of 5-HIAA in C.S.F. was delayed to 3.56 hr. The variability about the curve for 5-HIAA was also greatly diminished and might be due to a limitation of the range of response of one of the enzymes to tryptophan, presumably the hydroxylase.

Summary and Conclusions:- The overall picture presented by the data leads to the conclusion that α-methyl dopa and its metabolites have competitive actions at all points along the tryptophan-5-hydroxyindole metabolic pathway (i.e. hydroxylation, decarboxylation and amine storage). Carlsson and Lindqvist 1962 showed that the α-methylated amine derivatives of administered α-methyl dopa remain in brain for several days and are poor substrates for monoamine oxidase. This well confirmed finding means that there will also be an interaction in the 5-hydroxyindole pathway at the point of deamination.

The results also show that changes in the concentration of the metabolite 5-HIAA in C.S.F. after

tryptophan administration with and without α-methyl dopa reflect changes in the concentrations of the metabolite in brain, particularly the brain stem. In man, a study of the metabolite in C.S.F. may be useful as an indirect means of investigating cerebral 5-hydroxyindole metabolism.

SECTION II

THE INTERACTION OF 5-HYDROXYINDOLE METABOLISM AND DOPAMINE METABOLISM AND THE ACTION OF THE MONOAMINE OXIDASE INHIBITOR, PHENELZINE, ON 5-HYDROXYINDOLE METABOLISM

INTRODUCTION

This project developed because of interest in two separate aspects of the cerebral metabolism of 5-hydroxyindoles. The first was a personal observation that had been made while carrying out experiments to find a more productive way of utilising sampling of cerebrospinal fluid - (C.S.F.) as a means of monitoring changes in cerebral metabolism after the administration of intravenous tryptophan. These experiments had involved the withdrawal of samples of lateral ventricular and cisternal C.S.F. at hourly intervals from a dog maintained under light anaesthesia. While in control experiments the concentrations of 5-hydroxyindol-3-ylacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylacetic acid (HVA), an acid metabolite of dopamine had remained constant in the serial samples of C.S.F. the concentrations of both these acids were found to increase after the administration of intravenous L-tryptophan, the rise being most marked in the cisternal samples. Thus it appeared that there must be some interaction between dopamine metabolism and tryptophan metabolism.

The second point that stimulated this project was the report by Maclean, Nicholson, Pare and Stacey, (1965) that the administration of phenelzine to humans for a

period of two to four weeks prior to their death gave rise to a marked increase in the 5-HT concentration of the brain stem but the 5-HIAA concentration remained within the normal limits. Ashcroft (personal communication) has observed similar effects in rat brain following low doses of phenelzine given intraperitoneally. Maclean et al. (1965) made the interesting observation that isocarboxazide, given to another group of patients, also raised the brain 5-HT levels but this drug in contrast to phenelzine produced the expected fall in 5-HIAA concentration. These authors did not offer an explanation for the different effects of these two monoamine oxidase inhibitors on the brain 5-HIAA concentrations.

It became possible to study these two independent aspects of cerebral 5-hydroxyindole metabolism simultaneously as it became apparent during the progress of this work that the interaction between the catecholamine and 5-hydroxyindole metabolic pathways might be rendered more obvious in the presence of an inhibitor of monoamine oxidase.

METHODOLOGY

ANIMAL HUSBANDRY

The animals used were adult beagle hounds of either sex. These animals were maintained on diet (Appendix II) for at least three days prior to any experiment. Food was withdrawn from the dogs approximately 16 hours prior to an experiment but water was available at all times.

Chronic Experiments

Ventriculo-Cisternal Perfusion Experiments

The details of this type of experimental procedure are given in full in Section III of this thesis (page 156 ff. ff.).

Recirculatory perfusion from lateral ventricle to cisterna magna were carried out in conscious dogs. Phenol was added to this system at low rates of infusion and its rate of efflux from C.S.F. determined in the manner described in Section III. In one perfusion experiment a high concentration of phenylacetic acid (PAA) was infused.

Cerebrospinal Fluid and Venous Blood Samples Collected from Dogs Prior to Acute Experiments

In certain of the experiments in this section the dogs were pre-treated with the monoamine oxidase inhibitor, phenelzine (β-phenylethylhydrazine), given by subcutaneous injection in 1 ml. of 0.9% saline. The daily dose administered was 2 mg/kg of phenelzine dihydrogen sulphate.

The solution of phenelzine was prepared immediately prior to injection.

The dogs used for these experiments had had ventricular guide tubes implanted into the skull at least two weeks before any experimental procedure. During this period the animals were maintained on a diet (Appendix II) to standardise their food intake throughout the experimental period.

On certain days throughout the experimental period samples of C.S.F. and blood were obtained from the animals in the following manner:-

The dog was anaesthetised with a minimal dose of sodium thiopentone. A 21 gauge hypodermic needle of appropriate length was inserted percutaneously into the lateral ventricular guide tube and 1 ml. of ventricular C.S.F. was slowly withdrawn into a disposable polythene syringe. The needle was then removed from the ventricular guide tube. Next, the dog's head was flexed, a 21 gauge needle inserted percutaneously through the atlanto-occipital membrane into the cisterna magna and a 2 ml. sample of C.S.F. withdrawn slowly into a disposable polythene syringe. The samples of C.S.F. were transferred to glass test-tubes and were kept at -15°C until the biochemical estimations were carried out. Occasionally

a C.S.F. sample would be obtained slightly bloodstained and such a sample was immediately centrifuged at 2,000 r.p.m. for 5 min. and the clear supernatant transferred to a clean test-tube and stored at -15°C. Samples of venous blood were withdrawn into disposable polythene syringes and a 7 ml. portion of each was immediately transferred to a 12 ml. glass centrifuge tube containing 0.2 ml. of a solution of heparin (1,000 I.U./ml.). After inverting the tube a few times it was centrifuged at 3,500 r.p.m. for 8 min. Portions of plasma and the red cell layer were transferred to separate polythene containers which were kept at -15°C until the biochemical estimations were carried out.

The experimental plan used in all these experiments may be outlined as follows:-

(a) operative implantation of ventricular guide tubes, (b) two to three weeks recovery, (c) one week control period during which time samples were obtained every two to three days, (d) phenelzine administration for ten to twelve days during which time samples were obtained every two to three days (e) acute experiment.

Acute Experiments

The first acute experiment following phenelzine pre-treatment (Dog F1) was performed in an identical manner to that described in Section I (page 4 *et. seq.*) All other acute experiments in this section were carried out following an intravenous infusion of tryptophan in 0.9% saline or of an equivalent volume of saline alone.

The dog was anaesthetised with a minimal amount of intravenous sodium thiopentone and supplementary doses were administered throughout the experiment to maintain the animal in a light plane of anaesthesia. Samples of a ventricular and cisternal C.S.F. and of venous red blood cells and plasma were obtained as described on page 90 , except that only 0.5 ml. of C.S.F. was withdrawn from the lateral ventricle. The needles to the lateral ventricle and cisterna magna were maintained in place throughout the experiment.

Once the initial samples of C.S.F. had been obtained a dose of tryptophan, 50 mg/Kg, was administered intravenously into the brachiocephalic vein as a 10 mg/ml. solution in 0.9% saline. Following this, a solution of tryptophan (of the same concentration) was continuously infused intravenously at a constant rate by a pump (Watson-Marlow, Flow Inducer - type MHRE 72) at a rate

which resulted in the administration of 20 mg of tryptophan/Kg./hr. In two experiments on dogs which had been pre-treated with phenelzine an equivalent volume of 0.9% saline not containing tryptophan was administered. These animals served as controls for animals which had had tryptophan administered following phenelzine pre-treatment.

The infusions of 0.9% saline or 0.9% saline containing tryptophan were continued for four hours. At each hour samples of ventricular and cisternal C.S.F. and of venous red cells and plasma were obtained in a similar manner to those taken immediately after the administration of the anaesthetic. The samples could be obtained within a period of 2 min. and were always taken in the same order: ventricular C.S.F., cisternal C.S.F., venous blood.

At the end of the four hr. period of infusion, following collection of the final samples of C.S.F. and blood, an acute experiment was performed according to the procedure described in Section I (page 4 et. seq.).

Drugs and Solutions

L-Tryptophan		(Koch-Light)
D-L-5-HTP		(Koch-Light)
5-HT	as the creatinine sulphate	(Koch-Light)
5-HIAA	as the diethylammonium salt	(Roche)
5-HIAA		(Koch-Light)

Tryptamine as the hydrochloride (Koch-Light)

Phenylacetic Acid (PAA) (British Drug Houses)

Phenelzine as the dihydrogen sulphate (Warner)

1-Nitroso-2-naphthol Reagent - made just prior to use 0.1% (w/v) 1-nitroso-2-naphthol in 95% Ethanol.

Nitric Acid Reagent - made just prior to use. 2.5% (w/v) sodium nitrite/conc. nitric acid/water 3:25:150 (by vol)

All chemical reagents used were "Analar" grade except the 1:2 dichloro-ethane which was laboratory reagent grade (British Drug Houses) and the hydrochloric acid which was micro analytical reagent grade.

Distilled deionised water was used throughout.

BIOCHEMICAL METHODOLOGY

Estimation of the 5-Hydroxyindoles in Brain and Plasma Samples

The method of assay used was identical with that described in Section I page 10. However, each experiment contained three portions of the homogenate of cerebellum to which 200 µg of 5-HTP and 800 µg each of 5-HT and 5-HIAA were added and taken through the experimental procedure. The experiment data were corrected in each experiment separately by adjusting to 100% recovery on the basis of the average recovery in that experiment. A single similar "recovery" sample of plasma

containing 200 µg of 5-HTP and 5-HIAA was taken through the experimental procedure and was used to correct to 100% the concentrations of 5-HIAA found in plasma.

Estimation of Tryptophan and Tyrosine in Brain, Plasma and Red Blood Cells

The samples used for estimation were 0.1 ml. plasma and red blood cells, 0.5 ml. brain homogenate if the animal had had tryptophan administered and 1 ml. if only saline had been given. The dilution of the samples and their protein precipitation were performed as described in Section I, page 18 , (Estimation of Tryptophan in Blood, Brain and Cerebrospinal Fluid) except that duplicate samples containing 2 µg of tyrosine were included in each assay run to act as internal standards. The subsequent tryptophan estimations were carried out as described in Section I (page 20).

Following the precipitation of the protein in the sample, a 3 ml. portion of the supernatant was transferred to a 15 ml. glass stoppered tube for estimation of the tyrosine concentration. In addition to the samples two portions of 3 ml. of 0.1 N sulphuric acid were included as blanks and two similar portions of sulphuric acid containing 1 µg tyrosine were included as standards for the fluorophor-forming reaction. Aqueous blank solutions which had been subjected to the

precipitation procedure gave similar fluorescence intensities to the blank solutions only put through the fluorophor-forming reaction. (Table 13:1)

The reagents for the reaction were made just prior to use. To each sample was added 1.5 ml. 1-nitroso-2-naphthol reagent (page93) and, after mixing, 1.5 ml. nitric acid reagent (page93). The samples were mixed by inversion and heated at 55°C for 30 min. with shaking. After cooling, 7 ml. 1:2-dichloroethane was added to each of the samples. The mixtures were shaken vigorously for 5 min. to extract the excess reagent and then centrifuged at 2,000 r.p.m. for 3 min. A portion of the aqueous phase from each sample was removed and its fluorescence measured in a spectrofluorimeter (emission 570 mμ, activation scan 400-500 mμ, peak 450 mμ).

The concentrations of tryptophan and tyrosine were calculated by comparison with the blanks and internal standards. The recovery values quoted for the internal standards were calculated by comparison with the pure solution standards.

Estimation of Tryptophan and Tyrosine in Cerebrospinal Fluid

Protein Precipitation

To 0.75 ml. of water in a 12 ml. conical centrifuge

tube was added 0.1 ml. C.S.F. Duplicate samples containing 0.4 µg of tryptophan and tyrosine were included as internal standards for the procedure. After mixing, 0.15 ml. 30% (v/v) trichloroacetic acid was added, the sample mixed by inverting a few times and then left to stand for exactly 10 min. before centrifuging at 3,500 r.p.m. for 4 min. A 0.4 ml. portion of the supernatant was taken for estimation of the tryptophan content and a similar 0.4 ml. portion for estimation of the tyrosine content.

Tryptophan Assay

The 0.4 ml. portion of supernatant was transferred to a 15 ml. glass stoppered tube containing 0.1 ml. 0.5 N sulphuric acid. A blank containing 0.5 ml. 0.5 N sulphuric acid and a standard containing 0.2 µg tryptophan in 0.5 ml. 0.5 N sulphuric acid were used for comparison. After the addition of 0.1 ml. 4% formaldehyde solution the contents of the tubes were mixed by inversion and heated at 100°C for 20 min. with shaking. Hydrogen peroxide (0.1 ml. 4 vol. per cent) was added to each tube and the samples heated at 100°C with shaking for a further 20 min. The samples were cooled rapidly, after which they were subjected to fluorimetry - emission 450 mµ/activation scan 200-400 mµ, maximum 315 mµ, secondary peak 375 mµ. The results were calculated by comparison with the blanks and internal standards.

Tyrosine Assay

The reagents for the reaction were made just prior to use. A 0.4 ml. portion of the supernatant from the sample was transferred to a 15 ml. glass stoppered tube. A blank of 0.4 ml. water and standard containing 0.4 µg tyrosine in 0.4 ml. water were included in each assay run. To each sample was added 0.2 ml. 1-nitroso-2-naphthol reagent and, after mixing, 0.2 ml. nitric acid reagent. The samples were mixed by inversion and then heated at 55°C for 30 min. After cooling, 1 ml. 1:2-dichloroethane was added to each of the samples. These were shaken for 5 min. and then centrifuged for 3 min. at 2,000 r.p.m. The fluorescence of the aqueous phase from each sample was measured in a spectrofluorimeter: emission 570 mµ, activation scan 400-500 mµ, maximum 450 mµ. The results were calculated by comparison with the blanks and internal standards.

Estimation of 5-Hydroxyindol-3-ylacetic Acid and 3-Methoxy, 4-hydroxyphenylacetic Acid in Cerebrospinal Fluid.

The method of Guldberg (1967) was used as described below.

A measured portion of C.S.F. of the order of 0.5 ml. for ventricular or of 1.8 ml. for cisternal C.S.F. was diluted to 2 ml. with water in a 35 ml. glass stoppered centrifuge tube. This sample was acidified to pH 1-2

by the addition of 0.05 ml. conc. HCl and saturated with sodium chloride. After adding 5 ml. of ethyl acetate, the sample was shaken for 5 min. and centrifuged for 3 min. at 2,500 r.p.m. As much of the ethyl acetate layer as could be removed, without disturbing the aqueous phase was transferred to another 35 ml. glass stoppered centrifuge tube containing 2.2 ml. 0.1 M "tris" buffer pH 8.6. This tube was stored at 4°C while the aqueous phase of the original sample was extracted with a second 5 ml. of ethyl acetate in the same manner as before. After centrifuging the ethyl acetate layer of the second extraction was pooled with that of the first extraction. The weak organic acids were extracted from the ethyl acetate into the "tris" buffer by shaking for 5 min. The layers were separated by centrifuging for 3 min. at 2,500 r.p.m., the ethyl acetate layer aspirated off and 1 ml. portions of the "tris" buffer extract taken for separate assays of the 5-HIAA and HVA content.

Assay for 5-Hydroxyindol-3-ylacetic Acid.

To a 1.0 ml. portion of "tris" buffer extract 0.5 ml. conc. HCl (containing 50 mg. ascorbic acid/100 ml.) was added and the sample subjected to fluorimetry with comparable standards of pure 5-HIAA, (emission 550 mμ, activation scan 250-400 mμ, maximum 300 mμ.)

Assay for 3-Methoxy, 4-hydroxyphenylacetic Acid

A 1.0 ml. portion of the "tris" buffer extract was transferred to a tube containing 1.0 ml. 5 N ammonium hydroxide. Potassium Ferricyanide (0.2 ml. 0.1% (w/v) freshly prepared) was added to the sample, then after exactly 4 min. 0.2 ml. cysteine (0.01% (w/v) freshly prepared) was added. After at least 15 min. the fluorescence intensity of the sample was measured: activation 325 mμ/emission 430 mμ. The HVA concentrations were calculated from the readings for aqueous blank and HVA aqueous standard solutions which had been included only through the fluorophor-forming reaction. The blank used for the C.S.F. samples was taken to be 1.5 times the value of the aqueous blank (Guldberg, 1967).

Estimation of Tryptamine in Brain

The method was a modification of that described by Eccleston, Ashcroft, Crawford and Loose (1967).

In experiments on dogs PT2 and PT3 an attempt was made to estimate the brain tryptamine concentration. According to the amount of brain homogenate available after portions had been taken for estimations of tyrosine, tryptophan and 5-hydroxyindoles, measured portions of homogenate, between 1 and 2 ml. were taken for the tryptamine estimation. A duplicate sample containing

added tryptamine, 1.0 µg, served as an internal standard for the analytical procedure. Similarly 2 ml. 20% (v/v) acetic acid served as a blank.

The sample was taken to damp dryness in a 35 ml. centrifuge tube, under reduced pressure at an external temperature of 55°C, to remove most of the acetic acid. The residue was taken up in 9.0 ml. water with vigorous shaking in a mechanical shaker for 3 min. After 1 ml. 4 N perchloric acid (at 4°C) had been added, the sample was mixed by shaking and then allowed to stand at -15°C for 10 min. The sample was centrifuged at 3,500 r.p.m. for 5 min. and 9.0 ml. of the supernatant was adjusted to pH 7.4 (glass electrode) with potassium hydroxide. This solution was kept at 4°C for 10 min. to allow the precipitated potassium perchlorate to settle and then the supernatant was decanted into a 70 mm by 7. mm diam. column of Amberlite CG 50 (100-200 mesh) weak cation exchange resin which had been prepared in the ammonium form and was buffered to pH 7.4 with 0.02M ammonium acetate buffer. The sample was allowed to flow through the column under gravity at a rate of 20 ml./hr. The column was washed with 15 ml. 0.2 M ammonium acetate buffer pH 7.4 followed by 4 ml. 0.1 N sulphuric acid and then the amines such as 5-HT and tryptamine were

eluted with 6 ml. N sulphuric acid. A 1.5 ml. portion of the acid eluate was taken to estimate the 5-HT concentration, while a 4.5 ml. portion was taken for tryptamine estimation.

The 4.5 ml. portion of the column eluate was transferred to a 35 ml. glass stoppered tube containing 1 ml. 20% (w/v) sodium hydroxide (final pH of solution 10-11). This solution was saturated with sodium chloride and the tryptamine extracted by shaking for 5 min. with 22 ml. benzene which had been previously subjected to 3 washes with 0.1 N sulphuric acid. After centrifuging at 2,500 r.p.m. for 3 min. 19 ml. of the benzene phase was transferred to another 35 ml. glass stoppered tube containing 7.0 ml. 0.1 N sulphuric acid and the tryptamine extracted back into the acid by shaking for 5 min. After centrifuging at 2,500 r.p.m. for 3 min. and then aspirating off the benzene, 6.0 ml. of the 0.1 N sulphuric acid layer was transferred to a 15 ml. glass stoppered tube and the "Norharman Reaction" carried out as described on page 10. 6.0 ml. of 0.1 N sulphuric acid containing 1.0 µg tryptamine and a similar solution without tryptamine were included in the fluorophor-forming reaction as a reference standard and its blank. The recoveries of tryptamine quoted were calculated by comparison of the

fluorescent intensity of the internal standard with that of the reference standard. The relatively poor recoveries of tryptamine through the analytical procedure are probably due to the poor partition of tryptamine between benzene and water (Eccleston, 1966).

It was necessary to remove most of the acetic acid before adding the perchloric acid to ensure adequate precipitation of the protein of the sample prior to estimation of tryptamine. The other steps in this procedure have been discussed elsewhere (Eccleston, 1966).

Phenelzine Estimation in Plasma

The method used to estimate phenelzine was a modification by Dubnick (1962) of the method described by Hess, Weissbach, Redfield, and Udenfriend (1958) for the estimation of iproniazid. This method of colorimetric assay for phenelzine was based on the reaction of Folin-Ciocalteu - phosphotungstic - phosphomolybdic acid reagent with hydrazines.

A calibration curve for phenelzine added to 1.0 ml. samples of plasma showed that the method had satisfactory linearity from 2 µg to 30 µg.

Estimation of Noradrenaline, Dopamine, Methoxydopamine, 3-Methoxy, 4-hydroxyphenylacetic Acid, 3,4-Dihydroxyphenylacetic Acid and 5-Hydroxyindol-3-ylacetic Acid in Brain

The method of analyses used for these estimations

has been described and discussed in detail by Yates (1967) who undertook the supervision of these assays on portions of caudate nucleus and hypothalamus in co-operation with Guldberg. Half of the hypothalamus from one side of the bisected brain was taken for these estimations. The caudate nucleus from one side of the brain was divided after homogenisation into two portions, one being used to estimate the endogenous substances present while the other had known amounts of the various substances being estimated added to it to provide internal standards through the method. These estimates have, however, been quoted uncorrected for recovery as it was considered (Yates, 1967) that the percentage recovery of the exogenous substances did not properly reflect the recovery of the endogenous substances.

Estimation of 5-Hydroxytryptamine in Cerebrospinal Fluid

The samples of C.S.F. used were 0.5 ml. from the lateral ventricle and 1 ml. from the cisterna magna. These were diluted to 2 ml. and the method of Oates (1961) as modified by Eccleston et al. (1966), and described on page 255, was scaled to allow the elution of columns of resin 20 mm by 4 mm diam. with 1 ml. of N sulphuric acid.

Identification Procedures for 5-Hydroxyindoles

In one experiment, after the brain 5-hydroxyindoles had had their specific fluorescence in 3 N hydrochloric acid estimated, these solutions were saturated with sodium chloride, and extracted with peroxide free, freshly distilled diethyl ether as described on page 17 . The ether layer was shaken with 0.3 M sodium phosphate buffer, pH 7.0 as was also described on page 17 . The ether layer was then transferred to a 35 ml. centrifuge tube containing 100 ug ascorbic acid in 80% methanol and 0.1 ml. 20% (v/v) acetic acid and evaporated to damp dryness by a stream of nitrogen. The residue was dissolved in 1 ml. of 0.1 N sulphuric acid. To these solutions and portions of the sodium phosphate buffer half a volume of concentrated hydrochloric acid was added. The resulting solutions and portions of the original extracted samples were examined for 5-hydroxyindole fluorescence in a spectrofluorimeter (emission 550 mu/activation 200-400 mu, peak 310 mu).

RESULTS

Identification of 5-Hydroxytryptamine and 5-Hydroxyindol-3-ylacetic Acid

After estimation of their 5-hydroxyindolic content the eluates from those sections of brain extract chromatograms believed to contain 5-HT or 5-HIAA were subjected

to the identification procedure described in page 105 . Examined in this way were the '5-HT' and '5-HIAA' eluates from the chromatograms of these following brain areas from the 'Dog PT3' experiment:- midbrain, thalamus, caudate, hippocampus, hind brain, central region of "grey matter" of hind brain, cerebellum and the internal standards of pure 5-HT and 5-HIAA added to a cerebellar homogenate. In all the regions examined the endogenous "5-HT" and "5-HIAA" distributed between the different phases in the same manner as the internal standards of authentic 5-HT and 5-HIAA.

Recovery of Control Analyses of 5-Hydroxyindoles from Brain

Each experiment included three internal standards of each of 5-HTP, 5-HT and 5-HIAA added to cerebellar homogenates and the estimations for brain samples (except where stated in the tables) have been corrected to 100% based on the average recovery of each substance. It was found that the separate estimates of the recovery of any one of these substances determined in triplicate in the same experiment were satisfactorily consistent. Further evidence of the consistency of the method for the estimation of the 5-hydroxyindoles was obtained from a comparison of the average hindbrain region concentrations, which were derived from separate estimations in the central "grey matter", lateral and basal regions of

one half of the hindbrain, with the values obtained for the other half. The degree of agreement between these two estimates, having in mind that variation due to dissection will also be included, appears satisfactory.

Tyrosine Concentrations in Brain

No marked differences in the concentrations of tyrosine or its pattern of distribution in the various brain regions were observed following the different experimental procedures (Tables 1 - 10:2). The concentrations of tyrosine in the hypothalamus was on average approximately 180% and in cerebellum 140% of the concentrations found in the other brain regions. The significance of this regional distribution is not known but it is interesting to note that the hypothalamus has high concentrations of noradrenaline. It was felt that the concentration differences observed for tyrosine could not be due to differences in the extractability of this amino acid, as was postulated previously (page 43), in connection with the regional differences found for the concentrations of tryptophan in brain, because the assay for tyrosine gave consistent results for the recovery of the internal standards even when inadequate precipitation of the brain samples gave rise to a wide variation in the recovery of the internal standards used

for tryptophan.

Tryptophan and Tyrosine Concentrations in Plasma and Red Blood Cells

These data from the following experiments have not been itemised in full because only a few salient points require to be made from them and the pattern in eight of the nine dogs in which the full blood profile was studied were similar.

Normal Values in $\mu g/ml \pm$ standard deviation (no. of estimations)

Red Blood Cells; Tryptophan 8.5 ± 1.3(16)

Tyrosine 33.8 ± 8.7(16)

Plasma; Tryptophan 10.3 ± 2.9(16), Tyrosine 8.9 ± 2.5(16).

In two dogs which had saline administered after phenelzine pretreatment (Dogs P2 and P3) there was a fall of about 10% in the plasma concentrations of tryptophan and tyrosine in plasma 1 hour after the start of saline administration. This was in accord with a corresponding change in the haematocrit and presumably the amino acid concentrations reflect this dilution of the plasma with saline.

The dogs which were treated with phenelzine for 10-12 days showed no change in the concentrations of tryptophan and tyrosine, either in the plasma or in the red blood cells from those found in the same dogs during the control period.

After tryptophan had been administered intravenously the pattern of alteration of the concentrations of tryptophan and tyrosine in the plasma and red blood cells was similar whether the animals had been pretreated with phenelzine (Dogs PT1, PT2 and PT3) or not (Dogs T1, T2 and T3). In these dogs the tyrosine concentration in the plasma showed a slight fall 1 hr. after the start of the infusion similar to the slight fall produced by an infusion of saline alone. The concentrations of tyrosine in the plasma and red cells at other times were within normal limits.

The tryptophan concentrations in plasma reached peak values of 40-130 ug/ml.(range) at 1 hr., and then declined until at 4 hr., the concentrations were 25-50 ug/ml.(range). The concentrations in the red blood cells however had risen to 25-40 ug/ml.(range) at 1 hr., but in each animal the concentration reached at 1 hr., was maintained in the other samples taken at 2, 3 and 4 hours after the start of the infusion. Thus it would appear that the red blood cells have a maximum capacity for tryptophan which has been saturated throughout the experimental infusion. It would also appear that there is no inter-relationship between tryptophan and tyrosine levels in plasma or in red cells.

The high ratio for tyrosine between red blood cells and plasma of 4.1 ± 1.7 (S.D.) would make it seem probable that some active process is concerned with the uptake of tyrosine into the cells or that tyrosine is bound in some specific way within the red cells.

Dog T4, which received a tryptophan load alone, showed a high concentration of the amino acid within the red blood cells at 1 hour, 63 µg/ml. This then declined and at 2, 3 and 4 hours the tryptophan concentration was respectively 33, 33 and 28 µg/ml. The tryptophan concentrations in plasma showed a peak at 2 hr. of 120 µg. The tyrosine concentrations remained normal in both plasma and red blood cells. The blood profile of this dog has been dealt with separately as not only did it difier in its pattern of tryptophan concentrations from that found in the other dogs after tryptophan infusion but this dog also showed differences in physiological parameters during the infusion and in biochemical estimates made on the brain samples.

TRYPTOPHAN ADMINISTRATION

Tables 1 - 3:2; Dogs T1, T2 and T3

Cerebrospinal Fluid - Cisternal Samples

The tyrosine concentration remained the same in all samples. The tryptophan concentration reached a maximum

Table 1:2

Dog T1

C.S.F. PROFILE (Cisternal C.S.F. Only)

Time	5HIAA	HVA	TRYPTOPHAN	TYROSINE
0	0.030	-	0.96	3.00
1	0.048	-	10.5	2.76
2	0.080	-	9.60	2.76
3	0.093	-	8.80	3.71
4	0.088	-	7.30	3.37

Dog T1

BRAIN REGIONAL

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain	1.491	24.00	45.4	< 0.06	1.51	2.19
Hypothalamus (½)	0.372	34.84	46.6	0.25	2.55	1.84
Thalamus	1.806	24.45	50.21	< 0.06	1.10	0.78
Caudate (½)	0.581	24.67	40.56	< 0.07	0.97	0.87
Hippocampus	2.209	22.11	43.48	< 0.06	1.12	0.56
Hindbrain	3.339	20.25	70.05	< 0.06	1.03	1.48
Cortex	2.431	27.61	40.69	< 0.06	0.11	< 0.06
Cerebellum	7.527	35.60	50.24	< 0.06	0.13	< 0.06
Cord	0.525	35.60	70.49	< 0.06	0.29	0.335
Recovery 1		82%	65	64%	62%	80%
2		68%	42	59%	74%	99%
3		87		50%	56%	90%
Average Recovery		79%	54	58%	64%	90%
4 hr. Plasma				< 0.04		0.09
Plasma Recovery				37%		52%

Region	Weight	NA	DA	MDA	A	HVA	DOPAC	5HIAA
Hypothalamus (½)	0.377	0.27	-	< 0.14	< 0.03	2.0	-	1.6
Caudate (½)	0.635	< 0.10	6.39	0.65	< 0.03	14.8	1.3	0.50
Recovery		-	-	-	-	90%	80%	60%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F. given in intravenous injection of 50 mg/Kg L-tryptophan as a 10 mg/ml solution followed by an infusion at the rate of 20 mg/Kg/hr. of the same solution.

ND = Not detected.

- Unsatisfactory assay or sample not obtained.

Table 2:2

Dog T2

C.S.F. PROFILE (Cisternal C.S.F. Only)

Time	5HIAA	HVA	TRYPTOPHAN	TYROSINE
0	0.083	<0.020	0.87	1.6
1	0.137	0.260	10.10	1.4
2	0.199	0.548	9.60	1.5
3	0.336	1.180	7.90	1.7
4	0.281	1.112	5.40	1.7

Dog T2

BRAIN PROFILE

Region	Weight	Tyrosine	Tryptophan	LHTP	5HT	5HIAA
Midbrain	1.665	57.0	39.0	< 0.04	1.47	2.54
Hypothalamus (½)	0.202	118.1	68.7	< 0.06	1.23	1.19
Thalamus	1.579	41.80	44.7	< 0.04	0.72	1.12
Caudate (½)	0.587	28.9	34.4	< 0.05	0.91	0.87
Hippocampus	2.509	77.1	39.5	< 0.04	0.61	0.43
Diencephalon (½)	2.148	32.4	46.0	< 0.04	0.68	2.17
Cortex	1.273	47.1	52.9	< 0.04	0.10	< 0.02
Cerebellum	6.653	52.6	49.7	< 0.04	0.06	0.07
1 Central Grey	0.254			< 0.06	0.87	3.04
3 Basal Region	0.816			< 0.04	1.52	2.01
4 Lateral Region	0.560			< 0.05	0.70	0.80
Average Midbrain	1.630				.64	1.77
Recovery 1		61.	55.	48.	78.	75.
2		58%	54%	2[illegible]	62%	49%
3		44%	43%	35%	57%	47%
Average Recovery		48	52%	Average recovery not applicable - standards not read frequently enough. Individual readings corrected for accompanying recoveries		
4 hr. Plasma				> 0.04		0.08
Plasma Recovery				19%		44%

Region	Weight	NA	DA	MDA	HVA	DOPAC	5HIAA
Hypothalamus (½)	0.165	0.32	-	-	-	-	1.9
Caudate (½)	0.305	< 0.10	6.03	0.17	14.3	1.3	0.67
Recovery		33	-	14	85%	80%	80%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F. given in intravenous injection of 50 mg/Kg L-tryptophan as a 10 mg/ml solution followed by an infusion at the rate of 20 mg/Kg/hr. of the same solution.

ND = Not detected.

\- Unsatisfactory assay or sample not obtained.

Table 3:2

Dog T3

C.S.F. PROFILE (Cisternal C.S.F. Only)

Time	5HIAA	HVA	TRYPTOPHAN	TYROSINE
0	0.048	<0.020	0.97	3.0
1	0.101	0.105	9.16	2.8
2	0.065	0.315	13.40	2.8
3	0.113	0.618	11.30	3.0
4	0.083	0.772	8.50	3.1

Dog T3

BRAIN PROFILE

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain	2.134	21.3	47.1	<0.03	1.89	1.99
Hypothalamus (½)	0.255	46.7	44.3	<0.05	1.54	1.67
Thalamus	1.158	36.5	46.8	<0.03	1.29	1.38
Caudate (½)	0.521	21.5	32.8	<0.04	1.09	0.39
Hippocampus	2.466	30.0	39.4	<0.03	0.85	0.51
Hindbrain (½)	1.872	19.5	45.0	<0.03	0.53	1.02
Cortex	1.551	34.4	28.4	<0.03	0.09	<0.03
Cerebellum	7.845	20.3	45.4	<0.04	0.05	<0.03
HINDBRAIN Central Grey	0.523				0.97	2.81
HINDBRAIN Basal Region	0.656			<0.04	0.25	1.35
HINDBRAIN Lateral Region	0.773			<0.03	0.29	0.52
Cerebellum Hindbrain (½)	1.952				0.459	1.410
Recovery 1				43%		
2				46%	64%	49%
3				44%	76%	80%
Average Recovery		73%	73%		70%	65%
4 hr. Plasma				<0.02		0.10
Plasma Recovery				37%		51%

Region	Weight	NA	DA	MDA	HVA	DOPAC	5HIAA
Hypothalamus (½)	0.298	0.53	0.21	-	2.5	-	1.2
Caudate (½)	0.316	<0.14	6.72	ND	17.7	1.4	0.78
Recovery		41%	88%		80%	70%	95%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F. given in intravenous injection of 50 mg/Kg L-tryptophan as a 10 mg/ml solution followed by an infusion at the rate of 20 mg/Kg/hr. of the same solution.

ND = Not detected.

- Unsatisfactory assay or sample not obtained.

at 1 to 2 hours and showed a small decline thereafter. The 5-HIAA concentrations in the C.S.F. from the three dogs differed markedly [redacted] but the alterations on tryptophan loading showed the same trend in each dog. There was a rise to approximately three times the control value at 2-3 hours after the start of the infusion and then the concentrations of 5-HIAA remained at this level in subsequent samples. The HVA in cisternal C.S.F. (dogs T2 and T3) showed a similar pattern with little difference between the concentrations of the 3 hr. and 4 hr. samples. However the actual increase from the control levels was much greater, being approximately twentyfold.

These changes in the C.S.F. concentrations of 5-HIAA and HVA were known to be not due to sampling repeatedly because similar changes had been found in pilot experiments which were carried out using a serial sampling technique from both lateral ventricle and cisterna magna. If such C.S.F. samples were withdrawn after tryptophan had been given intravenously increases occurred in the concentrations of 5-HIAA and HVA in the cisternal C.S.F. However, no significant changes were seen if tryptophan was not administered.

Tryptophan Concentrations in Brain

The rank order for the tryptophan concentrations (shown previously page 17) in the different regions of brain of control dogs was no longer apparent. Similar tryptophan concentrations were present in all areas in all three dogs (dogs T1, T2, and T3).

Concentrations of 5-Hydroxytryptamine and 5-Hydroxyindol-3-ylacetic Acid in Brain

The concentrations of 5-HT found in the different regions of dog brain after this form of tryptophan load were between two and three times those found in control dogs (Appendix I, Table 3) and were greater than the concentrations arising after a single injection of 50 mg/Kg of tryptophan (Appendix I, Table 4). The concentrations of 5-HIAA were even higher, relative to their control values, than the 5-HT concentrations. This disproportionate increase in 5-HIAA was particularly marked in the hindbrain region. Thus the ratios for the molal concentrations of [5-HT] / [5-HIAA] were smaller in all regions. These results were obtained from animals with a higher 'steady state' level of the metabolism of 5-hydroxyindoles thus the decrease in the value of this ratio may indicate that an increased proportion of intracerebral 5-HIAA was being derived via extra-cerebral tryptophan 5-hydroxylase, (Section IA - Discussion) or

that some component of 5-HIAA efflux was becoming saturated. The rank order of the total molal concentrations of 5-hydroxyindoles was not in as good agreement with that of the control dogs as the rank order of this parameter which was found after the form of tryptophan load used in Section I. This was mainly due to the 5-HIAA increase which was disproportionately greater in the hindbrain region.

Concentrations of Noradrenaline, Dopamine, Methoxydopamine, 3-Methoxy-4-hydroxyphenylacetic Acid and 3-4-Dihydroxyphenylacetic Acid in Hypothalamus and Caudate Nucleus.

The estimated concentrations of these substances in the hypothalamus and caudate nucleus of control dogs are given in Appendix I - Table 5. Unfortunately, the assays performed for these estimates on dogs T1, T2 and T3 were not entirely successful. However the main point which requires to be made is that in spite of the change that occurred in the concentrations of HVA in cisternal C.S.F. after tryptophan administration there was no obvious alteration in dopamine metabolism in the caudate nucleus. Both the dopamine and 3-4-dihydroxyphenylacetic acid (DOPAC) concentrations were within normal limits as were the methoxydopamine (MDA) and 3-methoxy-4-hydroxyphenylacetic acid (HVA) concentrations, those last, however, might have been slightly higher than in control dogs.

Table 42

Dog T4

C.S.F. PROFILE

Time	5HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventri-cular	Cisternal	Ventri-cular	Cisternal	Ventri-cular	Cisternal	Ventri-cular	Cisternal
0	0.164	0.033	1.199	0.072	2.60	-	4.1	2.1
1	0.197	0.153	1.033	0.435		6.62		8.9
2	0.197	-	1.310	-	2.58	-	5.1	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-

Dog T4

BRAIN PROFILE

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain	1.598	15.2	90.5	<0.03	2.16	3.83
Hypothalamus (½)	0.276	23.2	105	<0.05	2.90	4.50
Thalamus	1.175	22.7	115	<0.03	0.85	1.35
Caudate (½)	0.593	19.5	82.7	<0.04	0.97	0.64
Hippocampus	2.169	18.5	93.8	(No separation)		
Hindbrain (½)	1.905	16.8	108	<0.03	1.38	2.52
Cortex	1.793	21.2	94.6	<0.03	0.20	<0.02
Cerebellum	7.970	30.6	115	<0.03	0.15	<0.02
HINDBRAIN Central Grey	0.350			<0.05	1.61	6.25
HINDBRAIN Basal Region	0.737			<0.03	0.51	2.02
HINDBRAIN Lateral Region	0.588			<0.04	-	1.23
Average Hindbrain	1.675				-	2.63
Recovery 1		87%	69%		59%	42%
2		77%			79%	42%
3		91%			27%	
Average Recovery		85%	69%	27%	47%	42%
4 hr. Plasma				<0.02		0.07
Plasma Recovery				37%		47%

Region	Weight	NA	DA	MDA	HVA	DOPAC	5HIAA
Hypothalamus (½)	0.274	0.44	0.10		1.6	-	1.78
Caudate (½)	0.304	<0.2	5.73	0.17	13.8	1.3	0.68
Recovery		46%	29%	14%	70%	100%	90%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F. given in intravenous injection of 50 mg/Kg L-tryptophan as a 10 mg/ml solution followed by an infusion at the rate of 20 mg/Kg/hr. of the same solution.

Dog T4 was not typical of these tryptophan loaded animals. See page for the differences found in biochemical and behavioural effects, also Tables 1-3:2.

ND = Not detected.

- Unsatisfactory assay or sample not obtained.

Physiological Responses after Tryptophan Administration.

Throughout the entire four hour period of light anaesthesia, all these dogs (T1, T2 and T3) had normal heart rate, respiration, muscle tone and there were no signs of peripheral vaso-dilatation.

Results Obtained From Dog T4

This animal was treated experimentally in a manner identical to dogs T1, T2 and T3 throughout the intravenous infusion. However the findings in this animal did not conform with those of the other three dogs.

Physiological Responses of Dog T4

This was a very excitable dog which was difficult to anaesthetize. The control C.S.F. sample was obtained without difficulty, but the C.S.F. sample obtained 1 hr. after the start of the infusion was slightly bloodstained and the 2 hr. sample looked almost like frank blood. Three and a half hours after the start of the tryptophan infusion the animal became tachypnoeic and then died from a sudden respiratory arrest 5 min. before the acute experiment was due to be performed. The animal was artificially respired, bled out immediately and the normal technical and analytical procedures were then carried out.

"Abnormalities" in Biochemical Results From Log T4

The abnormally high tryptophan concentration found in the red blood cells 1 hr. after starting the tryptophan infusion has already been mentioned (page 110). While the tryptophan concentrations found in red blood cells and plasma in the 3 and 4 hour samples were not unduly high the concentration of tryptophan found in the different regions of the dog's brain were (slightly) more than double the concentrations in the corresponding brain regions in dogs T1, T2 and T3. The concentrations of 5-HT in the different brain regions were slightly higher than were those in the other dogs but there were much greater increases in the concentration of 5-HIAA particularly in the brain stem regions. It was also noted that the tyrosine concentrations in the brain of dog T4 were slightly low.

The physiological and biochemical differences between dog T4 and dogs T1, T2 and T3 under identical experimental conditions would suggest that dog T4 itself suffered from some abnormality the nature of which is a matter of speculation. However, it would seem possible that there might have been some defect in amino acid transport. Thus not only were higher concentrations of tryptophan reached in the brain - pointing to either

Table 5:2

Dog P1

C.S.F. PROFILE

DAY	5HT		5HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal
-3	< 0.02	< 0.02	0.356	0.005	1.725	0.044	1.10	2.80	3.0	4.4
0	< 0.02	< 0.02	0.230	0.015	1.500	0.075	0.57	0.52	1.9	1.2
3	< 0.02	< 0.02	0.117	0.021	0.200	0.033	2.05	2.10	4.3	3.2
5	< 0.02	< 0.02	0.022	0.016	-	-	0.84	1.12	3.8	3.4
9	< 0.02	< 0.02	0.073	0.019	0.120	ND	1.50	0.60	3.4	1.5
10	< 0.02	< 0.02	0.145	0.019	0.320	0.014	1.05	0.65	2.4	1.5

MOIR, Alexander Thomas Boyd 33½ M.B., Ch.B., (Edin) 1963.
B.Sc. (Edin) 2nd cl. hons pharmacology 1964.
Ph.D. (Edin) 1967.

1957-1964: University of Edinburgh
1965-1972: Medical Research Council, Brain Metabolism Unit.
1965-1968: Scientific Staff
1968-1972: Clinical Scientific Staff. Engaged in research on various problems in neurochemistry. Ph.D. thesis on general neurochemical topics, involving the study of various aspects of the biochemistry of brain tissue. Since 1967 responsible for research team (1technician, 2 students).
Oct.72-date: Snr. Medical Officer, Scottish Home & Health Dept.

24 scientific papers submitted.

G. S. Boyd (F) (Prof. of Biochem., Edinburgh University)
J. H. Ottaway (A) (Snr. Lecturer, Biochem.Dept., Edinburgh Univ.)
H. W. Reading (NM) (Princ. Biochemist, MRC Brain Metabolism Unit)
T.B.B. Crawford (NM) Snr.Lect., Dept. of Pharmacology, Edinb. Univ.)

Dog P1

BRAIN PROFILE

REGION	WEIGHT	TYROSINE	TRYPTOPHAN	5HTP	5HT	5HIAA
Midbrain	1.563	32.4	8.8	< 0.06	3.53	1.21
Hypothalamus	0.821	46.0	9.3	< 0.10	4.93	0.90
Thalamus	1.452	28.8	10.1	< 0.06	1.77	0.53
Caudate	1.200	29.8	7.8	< 0.06	(No separation)	
Hippocampus	2.431	25.2	7.4	< 0.06	1.30	0.18
Hindbrain	3.522	26.6	9.3	< 0.06	1.39	0.78
Globus Pallidus	1.171	24.2	8.4	< 0.08	0.85	0.26
Cortex	2.652	26.8	8.6	< 0.06	0.07	< 0.06
Cerebellum	7.391	44.1	9.9	< 0.06	0.09	< 0.06
Recovery 1		Assay of standard failed. Corrected according to average recovery.	57%	7[illegible]	86%	63%
2			60%	7[illegible]	57%	66%
3				[illegible]9	46%	64%
Average Recovery			59	7[illegible]	63%	64%
Plasma				> 0.1		> 0.1
Plasma Recovery						57%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F.

Dog P1 was treated with 2 mg/kg phenelzine dihydrogen sulphate S.C., C.D. for 10 days. The days are numbered with respect to days of treatment with phenelzine completed.

Table 6:2

Dog P2

C.S.F. PROFILE

DAY	5HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal
-3	0.182	0.053	0.733	0.090	2.25	1.75	4.8	4.3
-1	0.320	0.110	1.060	0.104	0.88	1.53	4.0	5.9
1	0.265	0.066	0.649	0.030	1.63	1.22	3.8	4.7
4	0.132	0.024	0.164	ND	1.10	0.91	3.6	3.1
6	0.072	0.013	0.132	ND	1.48	0.86	4.7	3.1
11	0.081	0.013	0.168	ND	1.14	0.76	3.6	2.4
12	0.079	0.016	0.101	0.002	1.19	-	2.8	-
Time (hrs.)	Saline Infusion Started							
1	0.067	0.011	0.083	0.004	1.24	-	3.1	-
2	0.045	0.013	0.101	ND	1.10	1.01	2.7	2.8
3	0.056	0.021	0.097	ND	-	0.95	-	2.5
4	0.067	0.029	0.083	0.003	-	-	-	-

BRAIN PROFILE

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain	1.973	27.8	8.7	<0.05	3.42	1.67
Hypothalamus (½)	0.282	44.9	10.7	<0.05	3.82	1.51
Thalamus	0.880	31.7	11.0	<0.03	2.00	0.85
Caudate (½)	0.528	34.4	9.6	<0.04	1.30	0.38
Hippocampus	2.860	33.8	8.6	<0.05	1.78	0.22
Hindbrain	1.664	28.8	8.2	<0.03	1.08	1.15
Cortex	2.007	23.5	9.9	<0.03	0.23	<0.03
Cerebellum	7.024	35.7	10.3	<0.03	0.25	<0.03
HINDBRAIN Central Grey	0.335			<0.05	2.06	<2.33
HINDBRAIN Basal Region	0.809			<0.03	1.01	0.62
HINDBRAIN Lateral Region	0.654			<0.04	1.33	0.45
Average Hindbrain	1.798				1.32	0.87
Recovery 1		53%	72%	16%	43%	33%
2		60%	82%	44%	66%	47%
3		52%	48%	48%	48%	36%
Average Recovery				41%	52%	40%
4 hr. Plasma				<0.02		<0.04
Plasma Recovery				29%		17%

Region	Weight	NA	A	[illegible]	DA	HDA	HVA	DOPAC	5HIAA
Hypothalamus (½)	0.250	0.33	0.08	<0.07	0.14	<0.13	0.25	-	0.36
Caudate (½)	0.251	<0.28	0.10	<0.07	10.86	0.61	1.4	<.25	0.22
Recovery		58%	111%	97%	53%	68%	100%	100%	60%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F.

Dog P2 was treated with 2 mg/Kg of phenelzine dihydrogen sulphate S.C., OD. for 12 days, then an intravenous saline injection of 2 ml/Kg followed by an infusion at the rate of 2 ml/Kg/hr. for 4 hrs. The days are numbered with respect to days of treatment with phenelzine completed. The hours, with respect to the number of hours of infusion completed.

ND = Not detected.

- Unsatisfactory assay or sample not obtained.

Table 7:2

Dog P3

C.S.F. PROFILE

D/Y	5HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal
-8	0.109	0.015	1.250	0.021	0.96	0.79	5.5	2.3
-3					0.04	0.93	5.1	3.0
-1						0.71		1.8
2	0.074	0.013	0.036	ND	1.12	0.96	4.0	4.2
6	0.076	0.022	ND	0.096	0.82	0.71	5.3	4.4
8	0.076	0.055	0.090	0.016	1.73	1.17	2.9	1.3
11	0.080	0.017	0.022	ND	1.04	0.80	4.0	3.1
12	0.151	0.036	0.074	0.005	1.68	0.97	5.2	3.5
Time (hrs.)	Saline Infusion Started							
1	0.212	0.018	0.093	0.003	1.82	1.23	5.7	3.4
2	0.091	0.036	0.070	ND	1.88	1.15	5.5	3.7
3	0.204	0.045	0.096	0.005	2.12	1.35	5.5	3.4
4	0.159	0.048	0.082	0.005	3.53	-	7.4	-

Dog P3

BRAIN PROFILE

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain	2.237	19.6	8.6	<0.03	2.67	1.12
Hypothalamus	0.301	18.9	9.6	<0.05	1.95	0.81
Thalamus	1.118	19.9	8.7	<0.03	1.35	0.46
Caudate	0.599	21.7	6.9	<0.04	0.98	0.29
Hippocampus	2.889	19.1	6.6	<0.03	1.49	0.26
Hindbrain	1.847	17.0	7.0	<0.03	0.72	0.55
Cortex	1.993	20.7	8.3	<0.03	0.11	<0.03
Cerebellum	7.770	25.7	8.7	<0.03	0.08	0.06
HINDBRAIN Central Grey	0.548			<0.04	1.27	1.15
HINDBRAIN Basal Region	0.605			<0.04	0.76	0.38
HINDBRAIN Lower Region	0.705			<0.03	0.41	0.40
Average Hindbrain	1.858				0.78	0.61
Recovery 1		100%	74%	21	68%	59%
2		80%	69%	31%	70%	47%
3		91%	84%			
Average Recovery		92%	76%	26%	69%	52%
4 hr. Plasma				<0.03		<0.01
Plasma Recovery				23		60%

Region	Weight	NA	A	M	DA	MDA	HVA	DOPAC	5HIAA
Hypothalamus (½)	0.163	0.49	0.54	0.92	0.28	0	<0.1	-	0.41
Cortex	0.498	<0.15	<0.06	<0.06	<0.05	0			
Caudate (¼)	0.277	<0.27	<0.12	<0.09	0.85	0.65	0.5	<0.25	0.25
Recovery		-	68%	55%	87%	26%	80%	70%	60%

Concentration in µg/g of brain tissue or /ml of plasma or C.S.F.

Dog P3 was treated with 2 mg/Kg of phenelzine dihydrogen sulphate S.C., OD. for 3 days, then an intravenous saline injection of 5 ml/Kg followed by an infusion at the rate of 2 ml/Kg/hr. for 4 hrs. The days are numbered with respect todays of treatment with phenelzine completed. The hours, with respect to the number of hours of infusion completed.

ND = Not detected.

- Unsatisfactory assay or sample not obtained.

Table 8:2
Dog PT1

C.S.F. PROFILE

DAY	5HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventri-cular	Cisternal	Ventri-cular	Cisternal	Ventri-cular	Cisternal	Ventri-cular	Cisternal
-4					0.91	1.11	3.6	4.1
-1	0.210	0.053	1.430	0.083	1.52	0.93	6.9	5.8
1	-	-	0.623	0.034	1.00		5.3	
6	0.142	0.022	0.162	0.001	1.34	0.88	4.7	4.8
8	0.128	0.018	0.117	ND	0.91	0.57	4.2	2.3
13	0.139	0.020	0.057	0.009				
Time (hrs.)	Tryptophan Infusion Started							
1	0.113	0.025	0.213	0.077	16.00	5.94	7.4	3.5
2	0.151	0.044	0.305	0.220	11.43	5.43	6.4	2.6
3	0.093	0.048	0.201	0.272	10.82	5.35	5.7	3.0
4	0.128	0.065	0.290	0.328	11.16	6.01	5.7	3.5

Dog PT1

BRAIN PROFILE

	Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
	Midbrain	1.800	20.2	44.8	< 0.02	1.67	1.41
	Hypothalamus (½)	0.286	44.1	46.8	< 0.05	3.34	1.33
	Thalamus	1.126	27.3	37.7	< 0.03	0.53	0.29
	Caudate (½)	0.605	25.8	38.4	< 0.04	1.01	0.37
	Hippocampus	2.552	18.9	36.0	< 0.03	1.25	0.29
	Hindbrain (½)	1.632	21.9	49.5	< 0.03	1.34	0.95
	Cortex	2.704	21.7	40.8	< 0.03	0.15	< 0.02
	Cerebellum	7.080	35.2	53.5	< 0.03	0.09	0.08
HINDBRAIN	Central Grey	0.374			< 0.05	1.61	2.24
HINDBRAIN	Basal Region	0.557			< 0.04	0.51	0.75
HINDBRAIN	Lateral Region	0.717			< 0.03	0.75	0.32
	Average Hindbrain	1.648				0.86	0.90
	Recovery 1		95%	72%	31%	64%	46%
	2		99%	80%	31%	63%	50%
	3		65%	65%	29%	61%	40%
	Average Recovery		93%	72%	30%	63%	45%
	4 hr. Plasma				< 0.03		0.10
	Plasma Recovery				23%		36%

Region	Weight	NA	A	M	DA	MDA	HVA	DOPAC	5HIAA
Hypothalamus (¼)	0.308	1.36		0.07	0.73	ND	0.69	-	0.86
Caudate (¼)	0.291	0.90	-	-	5.25	3.01	1.78	1.1	0.29
Recovery		-			-		70%	80%	70%

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F.

Dog PT1 was treated with 2 mg/kg phenelzine dihydrogen sulphate for 13 days, when an intravenous injection of 50 mg/kg L-tryptophan in a 10 mg/ml solution in saline followed by an infusion at the rate of 20 mg/kg/hr. of the same solution.

The days are numbered with respect to the days of treatment with phenelzine completed. The hours with respect to the number of hours of infusion completed.

ND = Not detected

- Unsatisfactory assay or sample not obtained.

Table 9:2

Dog PT2

C.S.F. PROFILE

DAY	5HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal
-6	0.170	0.024	1.353	0.044	0.92	1.13	2.1	3.8
-2	0.164	0.015	1.260	0.039	1.15	0.86	2.2	2.5
0	0.205	0.020	1.835	ND	0.63	0.90	1.0	3.0
2	0.160	0.030	0.680	0.021	0.50	0.70	3.0	4.7
5	0.180	0.045	0.270	0.003	1.00	0.71	3.2	3.1
7	0.200	0.025	0.253	ND	1.19	0.91	4.1	4.2
12	0.133	0.020	0.164	ND	1.40	0.85	3.0	2.7
Time (hrs.)			Tryptophan Infusion Started					
1	0.126	0.022	0.390	0.063	13.00	-	2.7	-
2	0.120	0.027	0.229	0.066	-	9.85	-	2.0
3	0.185	0.037	0.433	0.236	-	11.20	-	1.8
4	0.148	0.039	0.380	0.312	12.96	10.30	4.0	4.2

Dog PT2

BRAIN PROFILE

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain	1.816		119.5	< 0.03	3.32	2.24
Hypothalamus (¼)	0.252		136.3	< 0.05	3.87	1.33
Thalamus	1.210		113.6	< 0.03	1.52	0.44
Caudate (¼)	0.575		96.1	< 0.04	1.40	0.49
Hippocampus	2.706		75.5	< 0.03	2.03	0.54
Hindbrain	1.807		98.0	< 0.03	1.18	1.14
Cortex	1.936	ASSAY	103.5	< 0.03	0.24	< 0.03
Cerebellum	7.281	FAILED	107.3	< 0.03	0.11	< 0.03
BRAIN " Central Grey	0.409			< 0.04	2.63	3.84
BRAIN Basal Region	0.786			< 0.03	1.42	1.38
BRAIN Lateral Region	0.699			< 0.03	1.26	0.58
Average Hindbrain	1.894				1.63	1.61
Recovery 1				37%	69%	62.4%
2				[illegible]	76%	45.9%
3				71%	76%	50.8%
Average Recovery			71.7 *	.0	74%	53.0
4 hr. Plasma				< 0.05		0.05
Plasma Recovery				20%		46%

Region	Weight	NA	A	M	DA	DOA	HVA	DOPAC	5HIAA
Hypothalamus (¼)	0.304	1.21	< 0.3	0.07	0.20	< 0.3	0.6	-	-
Caudate (¼)	0.275	< 0.5	-	-	8.12	1.36	1.4	-	-
Recovery		52%			168%		90%		

Concentrations in µg/g of brain tissue or /ml of plasma or C.S.F.

Dog PT2 was treated with 3 mg/Kg phenelzine dihydrogen sulphate for 14 days, then an intravenous injection of 50 mg/kg L-tryptophan as a 10 mg/ml solution in saline followed by an infusion at the rate of 20 mg/Kg/hr. of the same solution.

The days are numbered with respect to the days of treatment with phenelzine completed. The hours with respect to the number of hours of infusion completed.

ND = Not detected

\- = Unsatisfactory assay or sample not obtained.

* = Estimated from internal standards used for concurrent tryptophan assay. Values in red blood cells - Estimates not considered satisfactory.

Table 10:2

Dog PT3

C.S.F. PROBENECID

DAY	5-HIAA		HVA		TRYPTOPHAN		TYROSINE	
	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal	Ventricular	Cisternal
-3	0.045	0.011	0.081	0.020		5.00		1.0
-1	-		-	ND	-	1.07	-	2.5
0			0.128	0.080		-		2.0
	-		-	ND	-		-	
8	-	0.022	-	0.034	-	0.53	-	2.5
11	0.062	0.051	ND	0.007				
Time (hrs.)	Tryptophan Infusion started							
1	-	0.031	-	0.077	-	11.71	-	1.1
2	-	0.041	-	0.192	-	14.15	-	1.0
3	-	0.051	-	0.260	-	15.02	-	1.3
4	0.094	0.046	0.452	0.373	28.05	11.65	0.230	2.4

Dog T[illegible]

BRAIN PROFIL

Region	Weight	Tyrosine	Tryptophan	5HTP	5HT	5HIAA
Midbrain ($\frac{1}{2}$)	1.032	1 .4	40.6	<C.03	No seperation	
Hypothalamus ($\frac{1}{2}$)	0,210	27.0	40.8	< 0.05	3.75	1.05
Thalamus	1,014	10.1	[illegible]0.3	< 0.03	1.54	0.63
Caudate ($\frac{1}{2}$)	0.695	12.0	1.1	< 0.04	0.92	0.22
Hippocampus	2.76	11.6	34.4	< 0.03	1.77	0.30
Hindbrain ($\frac{1}{2}$)	2,005	13.1	32.9	< 0.03	1.06	0.77
Cortex	3,119	14.7	[illegible]1.0	< C.03	0.36	<0.03
Cerebellum	7.68	1 .8	3[illegible].3	<0.03	0.12	<0.03
H Central Grey I	0.596			< C.04	[illegible].34	2.33
D B Basal Region R	0.711			< C.03	[illegible].71	0.92
I N Lateral Region	0.751			<C.03	[illegible].26 1.24	0.32 1.11
Average Hindbrain ($\frac{1}{2}$)	2.058					
Recovery 1		64	[illegible]	22[illegible]	[illegible]	62
2		[illegible]	[illegible]	[illegible]0	69[illegible]	50[illegible]
3		81		39[illegible]	62[illegible]	55[illegible]
Average Recovery		7[illegible]	82	43[illegible]	65	55[illegible]
				<0.02		0.01
Plasma Recovery				32[illegible]		48[illegible]

Region	Weight	NA	A	M	DA	MDA	HVA	DOPAC	5HIAA
Midbrain ($\frac{1}{2}$)	1.264	0.03	0.04	0.001	0.10	0.05			
Hypothalamus ($\frac{1}{2}$)	C.313	0.25	0.25	0.07	0.16	0.13	0.8		0.4
Caudate ($\frac{1}{4}$)	0.361	0.12	< 0.08	<0.05	7.78	0.24	2.2	1.2	0.38
Recovery		20%	78%	112%	112%	126%	90[illegible]	-	60[illegible]

Concentrations in µg/g of brain tissue or /ml of plasma or C.S. .

Dog PT3 was treated with 2 mg/Kg phenelzine dihydrogen sulphate for 11 days, then an intravenous injection of 50 mg/ g L-tryptophan as a 10 mg/ml solution in saline followed by an infusion at the rate of 20 mg/Kg/hr. of the same solution.

The days are numbered with respect to the days of treatment with phenelzine completed. The hours with respect to the number of hours of infusion completed.

ND = Not detected

- Unsatisfactory assay - sample not obtained.

easier transport of this amino acid into the brain or some inhibition of its efflux from brain. The high concentrations of 5-HIAA in [illegible]in might also have been due to an impairment of the passage of this organic acid out of brain. It is interesting to reflect that such an impairment to a transport mechanism for the efflux of both these organic acids from inside cells might have applied not only to the brain cells but also the red blood cells and possibly other cells. This would explain the occurrence, in comparison with the other animals similarly treated, of both the initial high concentration found in red blood cells at 1 hour and the delay in reaching the maximum value for the concentration of tryptophan in plasma.

PHENELZINE PLUS TRYPTOPHAN TREATMENT

Dogs P1, P2 and P3 were treated with phenelzine; Tables 5 - 7:2. Dogs PT1, PT2 and PT3 were treated with phenelzine and tryptophan; Tables 8 - 10:2.

5-Hydroxytryptamine Concentration in Cerebrospinal Fluid

No 5-HT was detectable in any of the samples of C.S.F. taken from dog P1. The limit of detection was 0.02 µg/ml. for samples from both the lateral ventricle and cisterna magna.

The Concentrations of Tryptophan, Tyrosine, 5-Hydroxyindol-3-ylacetic Acid and 3-Methoxy-4-hydroxyphenylacetic Acid in Cerebrospinal Fluid Samples From the Lateral Ventricle and Cisterna Magna.

Control Period

The concentrations found in the C.S.F. from the dogs prior to treatment were:-

C.S.F. Sample	5-HIAA	HVA	Tryptophan	Tyrosine
Ventricular	0.216±0.078	1.18 ± 0.25	1.15 ± 0.52	4.3±1.6
Cisternal	0.034±0.33	0.057±0.034	1.06 ± 0.39	3.8±1.5

Results expressed as mean concentrations in µg/ml of C.S.F. ± standard deviations.

While there was an obvious gradient in the concentrations of 5-HIAA and HVA, the slight fall in the mean concentrations for tryptophan and tyrosine between lateral ventricle and cisterna could not be shown to be significant due to the variability of these estimates.

Phenelzine Administration

There was no significant alteration in the tryptophan or tyrosine concentrations in either lateral venticular or cisternal C.S.F. The 5-HIAA concentrations in the ventricular C.S.F. showed a significant ($P < 0.005$) decrease to about three-fifths of the control level; the mean value of all the samples after the start of phenelzine administration was 0.125 ± 0.051 (S.D.) µg/ml.,

of C.S.F. The 5-HIAA concentration in the cisterna, 0.025 ± 0.014 (S.D.) ug/ml. of C.S.F., also showed a fall from the control level which was not, however statistically significant ($P<0.3$). The ratio of the mean concentrations of lateral ventricular 5-HIAA to cisternal 5-HIAA was reduced from the control value of 6.3 (Guldberg, Ashcroft and Crawford, 1966 - 7.1) to one of 5.0.

The HVA concentrations in the ventricular C.S.F. showed a marked fall to about 10% of their control values. The concentrations of HVA in cisternal C.S.F. also decreased markedly but the measurement of these very low concentrations was not accurate and little weight can be placed on the actual concentrations quoted in the tables of results. Because of this it is also impossible to make any assessment of the effect of phenelzine on the ratio of the concentrations of HVA between the C.S.F. samples from the lateral ventricle and the cisterna magna.

Saline Infusion

Dogs P2 and P3 Tables 6 and 7:2. No change from the pre-infusion concentrations of HVA, tryptophan and tyrosine was found in samples of lateral ventricular and cisternal C.S.F. taken throughout the infusion period. The concentration of 5-HIAA in ventricular C.S.F. also remained unchanged but that in cisternal C.S.F. showed

a gradual increase with time. The latter was probably due to sampling of cisternal C.S.F. causing some of the ventricular C.S.F. with its higher concentration of 5-HIAA to be drawn into the cisternal space. This increase in 5-HIAA concentration with serial sampling of C.S.F. from lateral ventricle and cisterna magna was not seen in the initial pilot experiments in serial sampling from the dogs before treatment with phenelzine. The difference is presumably due to an impairment of the active transport system for the removal of 5-HIAA from C.S.F. as a result of the phenelzine treatment, (page 146 et. seq. and Section III).

Tryptophan Infusion

Dogs IT1, IT2 and IT3, Tables 8 - 10:2. During this type of infusion the tyrosine concentrations in ventricular and cisternal C.S.F. remain at their pre-infusion levels. The concentrations of 5-HIAA followed the same pattern as they did during saline infusion, the ventricular concentrations remaining at the pre-infusion level and those in the cisternal samples showing a slight increase, but no greater than occurred in the saline infused controls. The tryptophan showed a marked rise in concentration in the C.S.F. from both sites, from about 1 ug/ml. pre-infusion to about 10 ug/ml. This increase

in concentration was present 1 hour after the start of the infusion and was well maintained throughout the infusion. The tryptophan concentrations found in the C.S.F. of the dogs which had been pretreated with phenelzine (dogs PT1, PT2 and PT3) were of the same magnitude as those found in the comparable animals which had not been phenelzine pretreated (dogs T1, T2 and T3).

The concentrations in the HVA in C.S.F. showed marked increases in the samples from both lateral ventricle and cisterna magna. Ventricular C.S.F. (dogs PT1 and 2) taken 1 hour after starting the infusion, showed a rise in the HVA concentration to approximately double the pre-infusion concentration. This increase was maintained in the subsequent samples of ventricular C.S.F. The HVA concentrations in the cisterna magna showed a dramatic rise from barely detectable concentrations of approximately 0.005 µg/ml. to concentrations of between 0.3 and 0.4 µg/ml. The ability of intravenous tryptophan administration to influence the concentrations of HVA in C.S.F., as already observed in the experiments on dogs T1, T2 and T3 was again demonstrable in the phenelzine treated animals in spite of the fact that in the latter the tryptophan did not produce any increase in the C.S.F. concentration of its own metabolite, 5-HIAA.

[illegible]

Dog P1 (Table 5:2) had neither a saline infusion nor a period of prolonged anaesthesia prior to the acute experiment. However the values of the biochemical estimates on the various brain regions were in good accord with the concentrations found in the brains of dogs P2 and P3.

Tryptophan Concentrations in Various Brain Regions.

Without Tryptophan Administration

Although a significant rank order for the concentrations of tryptophan in the different brain regions from phenelzine treated dogs (P1, P2 and P3) could not be demonstrated, the concentrations found were in agreement with those of control dogs (Appendix 1, Table 5).

Following Tryptophan Administration

In the dogs which had intravenous tryptophan administered (PT1, PT2 and PT3) the concentrations of tryptophan found in the brain were of the same magnitude as those of dogs T1, T2 and T3 which had not been pre-treated with phenelzine.

Concentrations of 5-Hydroxytryptamine and 5-Hydroxyindol-3-ylacetic Acid in Various Brain Regions.

Without Tryptophan Administration

The 5-HT concentrations in the different brain regions of phenelzine treated dogs P1 and P2, showed an

increase to between 400 and 500% of the concentrations normally found (Appendix I, Table 3), the increases being most marked in the midbrain and hypothalamus. Dog P3 showed a more uniform increase in concentration to about 500% of control levels.

The 5-HIAA in all the brain regions of all three dogs (P1, P2 and P3) was increased to approximately 150% of the concentrations found in the corresponding regions in control dogs. This finding confirms the observation originally made by Kuelean et al., that phenelzine, although a monoamine oxidase inhibitor, does not cause a fall in concentration of the acid metabolite in brain.

The rank order of the total molal concentrations of the 5-hydroxyindoles in the various brain regions was the same as that found for control dogs (Section I, page 44).

Following Tryptophan Administration

The 5-HT concentrations in the various brain regions (dogs PT1, PT2 and PT3) were raised to between 300 - 400% of their control values. The most marked increase in 5-HT concentration occurred in the midbrain region. The 5-HIAA concentration was raised to between 150 and 200% of the control concentrations (Appendix I, Table 3). Thus, although tryptophan was able to enter brain as freely as it did in the animals which had not been treated

Table 11:2

The Concentrations of Dopamine, Methoxydopamine (MDA), 3-Methoxy-4-hydroxyphenylacetic Acid (HVA) and 3-4Dehydroxyphenylacetic Acid (DOPAC) in Dog Caudate Nucleus Following Various Experimental Procedures

Dog	Dopamine	MDA	HVA	DOPAC
Control	5.5 ± 1.4	0.38 ± 0.39	13.4 ± 1.14	1.3 ± 0.27
T1	6.39	0.65	14.8	1.3
T2	6.03	0.17	14.3	1.3
T3	6.72	-	17.7	1.4
T4	5.73	0.17	13.8	1.3
P2	10.86	0.61	1.4	<0.25
P3	9.85	0.65	0.5	<0.25
PT1	5.25	3.01	1.78	1.1
PT2	8.12	1.36	1.4	-
PT3	7.78	0.24	2.2	1.2

The concentrations are expressed in µg/g of brain tissue. The control values have been quoted ± standard deviation. Results taken from tables 1-4:2, 6-10:2 and Appendix 1, table 5.

Dogs T1-4 had been given tryptophan alone. Dogs P2 and P3 had been chronically treated with phenelzine. Dogs PT1-3 had been subjected to both the above treatments.

See text for the details of the experiments.

with phenelzine, it did not increase the concentrations of the 5-hydroxyindole metabolites to levels higher than would have been attained by the action of phenelzine alone.

The Concentrations of Noradrenaline, Dopamine, Methoxydopamine, 3-Methoxy-4-hydroxyphenylacetic acid and 3-4-Dihydroxyphenylacetic acid in the Caudate Nucleus Following Phenelzine Pretreatment

Following Saline Administration

Dopamine and methoxydopamine in the caudate nucleus were raised to 200% of their control concentrations while 3-4-dihydroxyphenylacetic acid became undetectable and the concentration of 3-methoxy-4-hydroxyphenylacetic acid fell to 10% of its control value (Table 11:2).

Following Tryptophan Administration

In the comparison of dogs PT, PT2 and PT3 with the dogs treated with tryptophan alone (dogs T1, T2 and T3) there were similar changes to those seen in comparing the dogs P2 and P3, treated with phenelzine alone, with control dogs. However on comparing the results of estimates from dogs PT1, PT2 and PT3 with those of dogs P2 and P3 it can be seen that the additional variable of the tryptophan load has caused a fall in dopamine and a rise in, methoxydopamine, 3-4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid in the caudate nucleus (Table 11:2). It would appear therefore that the

Table 12:2

The Effect of Phenelzine on the Clearance of 5-Hydroxyindol-3-ylacetic Acid and Inulin From a Ventricular-Cisternal Perfusion System in the Conscious Dog.

Dog	Treatment	Substance Infused	Rate of Infusion R_I (ml/min.)	Rate of Perfusion R_P (ml/min.)	Concentration of Infusion C_I (µg/ml)	Concentration of Outflow (Approx.) $C_o(\infty)$ (µg/ml)	Clearance ^1Cl (ml of inflow fluid/min.)
2	Control	Inulin	0.0128	0.33	718	123	0.049
		5-HIAA			20.5	0.951	0.146
24 lb	Phenelzine	Inulin	0.0159	0.33	800	135	0.063
		5-HIAA			21.2	2.315	0.093
3	Control	Inulin	0.0132	0.33	720	123	0.051
		5-HIAA			13.8	0.520	0.168
23 lb	Phenelzine	Inulin	0.0136	0.33	800	135	0.054
		5-HIAA			21.2	1.99	0.089

All perfusions had $C_o(\infty)$ estimated by the method of approximation (Section III).

The dogs treated with phenelzine had 2 mg/Kg S.C. daily of phenelzine dihydrogen sulphate for 10 days prior to perfusion.

The main point made from the results in this table is that phenelzine treatment has impaired the efflux of 5-HIAA from a ventricular-cisternal perfusion system in conscious dogs. The text of Section III of this thesis explains the figures given and calculations made.

tryptophan in phenelzine treated animals caused some displacement of dopamine towards its metabolites.

Tryptamine Concentration in Brain

Samples of brain homogenate from hippocampus, hindbrain, cortex and cerebellum in dogs PT2 and PT3 and from midbrain in dog PT2 were analysed for tryptamine content. No tryptamine was detected in any sample. Unfortunately, the limit of detection by this method was rather poor, being between 0.3 and 0.4 µg/gram. of brain tissue.

[illegible]

In Section III of this thesis it has been demonstrated that part of the 5-HIAA in C.S.F. is removed by an active transport system whereas inulin is only removed by bulk flow. The "Clearance" of 5-HIAA and inulin from C.S.F. were determined by means of a recirculatory perfusion from lateral ventricle to cisterna magna (Section III). Table 12:4 shows the "Clearances" of inulin and 5-HIAA in two dogs not on any drugs and in the same two dogs after ten days treatment with phenelzine dihydrogen sulphate 2 mg/Kg B.C. given daily. The results show that while the treatment with phenelzine had no effect on the "Clearance" of inulin from the C.S.F. it caused a marked impairment in the "Clearance" of 5-HIAA from C.S.F.

As it was thought that metabolites of phenelzine might be responsible for part of its actions a high concentration of the main metabolite of the drug - phenylacetic acid, was infused into the recirculatory perfusion system of a conscious dog, the concentration of HVA in the cisternal outflow showed an increase in concentration.

	Control Period	Phenylacetic acid Infusion		
Duration of Perfusion (min)	30	60	90	120
Concentration of HVA (µg/ml.)	0.34	0.31	0.44	1.13

Thus it would appear, that a high local concentration of the main acid metabolite of phenelzine was able to inhibit the active transport system for the removal of HVA and 5-HIAA from C.S.F. This is discussed in more detail in Section III.

Behavioural Effects Caused by Phenelzine

After three days treatment the dogs became progressively more easily excitable and difficult to handle. By the time of the acute experiment the animals were acutely nervous.

It was interesting that of two dogs which were perfused with phenylacetic acid each became excitable, indeed one of the experiments had to be stopped because of this fact.

DISCUSSION

The Action of Phenelzine on the Metabolism of Catecholamines

Phenelzine produced the expected result on this metabolic pathway in brain giving rise to an increase in the concentrations of the amines and a fall in the concentration of the acid metabolites of dopamine. The decrease in the caudate nucleus was particularly marked, the concentration being reduced to 10% of the control value. This decrease was parallelled by a similar dramatic fall in the concentrations of HVA in lateral ventricular and cisternal C.S.F.

The Action of Phenelzine on the Metabolism of 5-Hydroxyindoles

The various regions of dog brain all showed similar changes in response to the action of the drug, the changes being slightly more marked in the midbrain and hypothalamus. Because of this the discussion will refer to "brain" concentrations without specifying any special regions.

The metabolic pathway to be considered is that from tryptophan through 5-HTP to 5-HT and finally to 5-HIAA. Phenelzine treatment caused no change in tryptophan concentration in the red blood cells, plasma, brain or C.S.F.; no 5-HTP was detected in plasma or brain; the 5-HT concentration in brain was greatly increased and

the 5-HIAA concentration, instead of being decreased as expected was actually slightly increased. The 5-HIAA concentration in ventricular C.S.F. fell slightly while that of cisternal C.S.F. showed a similar tendency. Finally as demonstrated by the results of ventricular perfusion experiments, the efflux of 5-HIAA from C.S.F. by an active transport system was impaired. When the pathway was placed under a load by tryptophan administration, in spite of the fact that this produced the expected increases in the concentrations of tryptophan in red blood cells, plasma, brain and C.S.F. there was no change from the concentrations found on treatment with phenelzine alone, of 5-HT and 5-HIAA in brain, 5-HIAA in C.S.F., nor did 5-HTP become detectable in either plasma or brain.

These results suggested phenelzine was inhibiting the cerebral 5-hydroxyindole pathway to varying degrees at four separate points; the tryptophan 5-hydroxylase (no increase in turnover through the pathway after tryptophan administration), the monoamine oxidase (high concentrations of brain 5-HT), the efflux of 5-HIAA from brain (the rise in brain 5-HIAA concentration instead of the expected fall), and the efflux of 5-HIAA from C.S.F., (the results from ventricular perfusion).

The actions of monoamine oxidase inhibitors of the hydrazine type have been recently reviewed with particular

reference to the 5-hydroxyindole pathway (Pletscher, Gey and Burkard, 1966). These drugs have been shown to affect very many enzyme systems and as yet the exact manner in which they inhibit monoamine oxidase remains to be established.

In considering the interpretation of the biochemical effects produced by a drug it is necessary to bear in mind that metabolic derivatives of the drug may also play a part in the production of one or more of the observed changes, and this would appear to be true in the case of phenelzine.

Dubnick (1962) has shown that the major metabolite of phenelzine in the mouse is phenylacetic acid and that it is excreted in that animal as the glycine conjugate, phenaceturic acid. The pharmacological actions of the acid metabolite are less widely known than those of its parent compound, for this reason it will be dealt with more fully at this point. Phenylacetic acid (PAA) occurs endogenously in man as one of the acid metabolites of phenylalanine and it is excreted as the glutamine conjugate (Ambrose, Power and Sherwin, 1933). It was shown to have an inhibitory effect on 5-HTP decarboxylase in vitro (Davieson and Sandler, 1958) and in vivo (Dubnick, Leeson, and Phillips, 1962). Administered to

carcinoid patients PAA decreased the urinary 5-HIAA excretion, an observation interpreted by Sandler and Close (1959) and Sandler, Davis and Kimington (1959) on the basis of decreased 5-HT formation by the inhibition of 5-HTP decarboxylase. As Kilne (1959) pointed out there was insufficient evidence for this and he thought it was most likely that 5-HIAA, which had been shown by Despopoulos and Weissbach (1957) to be actively concentrated by renal cortex in vitro, would have its excretion by the kidney inhibited by PAA. Milne, Crawford Girao and Loughridge (1960) showed that 5-HIAA was actively excreted by the kidney and that this could be inhibited by p-aminohippuric acid. However, the report most relevant to the present results was that by Tozer and Neff (1967) who demonstrated that the efflux of 5-HIAA from rat brain could be inhibited by massive doses of probenecid and certain other organic acids, including PAA. Thus it would seem likely that PAA derived from phenelzine might give rise to the postulated inhibition of efflux of 5-HIAA from brain and C.S.F. This was shown to be the case for 5-HIAA if the results of the ventricular perfusion experiments reported here are considered in the light of the work described in Section III. The implication of these suggestions must be that the efflux of 5-HIAA from brain must take place to some extent by an active transport mechanism.

Inhibition of 5-HTP decarboxylase which has been shown to occur in brain after phenelzine treatment (Dubnick et al. 1962) cannot be of major importance in production of the present observed biochemical changes, as 5-HTP was never detectable even after tryptophan administration.

The inhibitory effect of phenelzine on the tryptophan 5-hydroxylase might be a direct action of the drug or one of its metabolites or might be due to an inhibitory feed back from one of the 5-hydroxyindoles - possibly 5-HT. A direct drug action would seem more likely as the extracerebral tryptophan hydroxylase must also be inhibited to some extent because otherwise systemic tryptophan administration could have given rise to an increase in brain 5-HIAA due to an increased utilisation of extracerebrally produced 5-HTP in cerebral metabolism (Discussion, Section I).

Thus in conclusion it would appear that phenelzine, a monoamine oxidase inhibitor also has inhibitory effects on extra and intra-cerebral tryptophan 5-hydroxylase and an inhibitory effect in the active transport of 5-HIAA from brain and from C.S.F., an action probably mediated by the acid metabolite of phenelzine, phenylacetic acid.

The Interaction of Dopamine and Tryptophan Metabolism in Brain

Tryptophan administration alone produced a marked rise in HVA as well as a rise in 5-HIAA concentrations in cisternal C.S.F. and yet no significant changes could be seen in the concentrations of the dopamine, HVA and DOPAC in the caudate nucleus although the HVA concentrations were marginally higher than normal. When, however, the dogs were pretreated with phenelzine the additional variable of tryptophan treatment caused a rise in HVA concentration in ventricular and cisternal C.S.F., also a definite fall in dopamine concentration and rises in the methoxydopamine HVA and DOPAC concentrations in caudate nucleus. These changes occurred even though there was no evidence of an increased turnover through the 5-hydroxyindole pathway after tryptophan administration. Thus it would appear that tryptophan itself or one of its other metabolites probably tryptamine must be responsible for the interaction between the pathways.

Tryptamine has been demonstrated in rat brain but only with phenelzine pretreatment followed by large doses of tryptophan (Eccleston et al. 1966). Failure to detect tryptamine in the present experiments was probably due to the poor sensitivity of the method used.

The most reasonable hypothesis to explain the interaction of the two pathways from the present results is to postulate that dopamine can be displaced from its "stores" and "binding sites" probably by tryptamine, but possibly also by 5-HT (Brodie, Cormer, Costa and Dlabac 1966) in the absence of phenelzine pretreatment.

The observed results could then be explained as follows. The normal turnover rate of dopamine in brain is considered to be fast and it may well be that although the amount of dopamine displaced and metabolised after tryptophan administration is sufficient to cause a noticeable increase in the H.V.A. concentration of C.S.F., the deficit produced in the "store" pool of dopamine may not be detectable due to its rapid turnover and a difference in the relative balance between the displacing agent and dopamine for occupancy of the two sites, the "binding" sites and the storage "sites". After phenelzine pretreatment it may well be that the relative proportions of the displacing agent is increased.

"Binding sites" have been shown to be of importance for the removal of circulating noradrenaline in the periphery, (Whitby, Axelrod and Weil-Malherbe, 1961) and it has also been shown that other sympathomimetic amines can block uptake into these sites (Hertting,

Axelrod and Whitby, 1961). Therefore it is possible that similar mechanisms may exist in brain for dopamine.

SUMMARY

The effects of phenelzine or tryptophan alone and the action of both together on the metabolism of dopamine and tryptophan in various regions of dog brain were studied. The results indicated that phenelzine and its metabolites inhibited the 5-hydroxyindole pathway at tryptophan 5-hydroxylase, monoamine oxidase and the active removal of 5-hydroxyindol-3-ylacetic acid from brain and cerebrospinal fluid. An interaction between dopamine metabolism and tryptophan metabolism in brain was demonstrated. It was suggested that this interaction resulted from displacement of dopamine by an amine formed from tryptophan, probably tryptamine.

SECTION III

THE ACTIVE TRANSPORT OF 5-HYDROXYINDOL-3-YLACETIC ACID AND 3 METHOXY-4-HYDROXY-PHENYLACETIC ACID FROM A RECIRCULATORY PERFUSION SYSTEM OF THE CEREBRAL VENTRICLES IN CONSCIOUS DOGS.

INTRODUCTION

In view of the recent publication by Davson, entitled "The Physiology of the Cerebrospinal Fluid (1967)", I feel that to include a general review on the value of utilising the cerebrospinal fluid (C.S.F.) as a means of studying cerebral metabolism would not only be irrelevant but presumptuous. There are, however, a few relevant general observations concerned with perfusion of the spaces containing C.S.F. which I feel might be made.

At the beginning of the century research concerned with C.S.F. dealt mainly with aspects directly related to specific clinical problems. From this time, however, many researchers have used the cerebrospinal fluid system as a means of studying cerebral metabolism, but their techniques involved either removing C.S.F. from, or adding drugs to different parts of the system.

In 1938 at this University, in the Departments of Pharmacology and Surgery the technique of perfusion of the cerebral ventricular system was developed by Adam, McNail, Obrador and Wilson. They established "perfusion" - "from lateral ventricle to subcerebellar cistern" in both cats and dogs. Using this system they perfused

with eserinised Locke solution and assayed the acetylcholine content of the perfusate following stimulation of various peripheral nerves and regions of brain. Faradic stimulation applied to the hypothalamus approximately doubled the acetylcholine concentration in the perfusate.

Leusen, in 1948, used the technique of ventriculocisternal perfusions in anaesthetised dogs. The method (as used by Leusen) had two main advantages, it provided a means firstly of reducing the concentrations of certain substances normally found in C.S.F. and secondly of maintaining a uniform environment for the region of brain under experiment. In a series of preliminary communications in 1948 Leusen described the effects of changes in the ionic concentration of potassium, calcium and magnesium in cerebrospinal fluid on the vasomotor system (Leusen 1948 a, b, c). These findings were collected together in a later paper in English (Leusen 1950).

Royer (1950) reported in detail a technique of ventriculo-cisternal perfusion in the anaesthetised rabbit, cat and dog, and he described the effects of the administration of adrenaline and acetylcholine by this route in the anaesthetised dog.

Leusen in two papers (1954a and b) described a series of experiments in which solutions with high CO_2 pressures or abnormally low bicarbonate ion concentrations caused a stimulus to respiration.

A preliminary communication was published, by Bhattacharya and Feldberg in 1957, of a ventriculo-cisternal perfusion system in the anaesthetised cat similar to that described by Adam et al. (1938) and Royer (1950). They extended this technique somewhat, however, as in some cases they cannulated the aqueduct and perfused from lateral ventricle to aqueduct. In 1958 these workers (Bhattacharya and Feldberg 1958a) studied the effects of histamine, adrenaline and tubo-curarine administration to the two perfusion systems and later (Bhattacharya and Feldberg 1958b) the recovery and release of acetylcholine into perfusing fluid containing an anticholinesterase.

Manuilov (1958) published a preliminary paper on a "Technique for Perfusion of the Ventricles in Dogs under Chronic Experimental Conditions". However, as his experiments were concerned with the addition of anaesthetics to the perfusion system, it is not clear how long his animals were perfused in a conscious state.

An important step forward was made in 1961 when Pappenheimer, Heisey and Jordan published an article in which they presented evidence for active transport of Diodrast and phenolsulphonphthalein from fluid used to perfuse the verticulo-cisternal spaces of unanaesthetised goats. They described their operative and experimental techniques in a subsequent paper (Pappenheimer, Heisey, Jordan and Downer, 1962) and in its companion paper further characterised the kinetics governing the efflux of substances from such a perfusion system (Heisey, Held and Pappenheimer, 1962).

In 1962 Davson, Kleeman and Levin reported the results of experiments concerned with the rates of removal of para-amino hippuric acid (P.A.H.), ^{24}Na and inulin from a ventricular perfusion system in the anaesthetised cat. They showed that P.A.H. had an active component of removal. In the following year (1963) Pollay and Davson published a similar paper describing a ventriculo-cisternal perfusion system in the anaesthetised rabbit and dealing with the active transport mechanisms for removal from C.S.F. of thiocyanate, iodide and P.A.H.

Use was made of a ventricular cisternal perfusion system in anaesthetised dogs by Oppelt, Maren, Owens and

Hall (1963) who, using the technique of inulin dilution, slightly modified from its description by Heisey et al. (1962), studied the effects of systemic acidosis and alkalosis on the rate of bulk (net) formation of C.S.F. They were able to demonstrate that neither respiratory nor metabolic acidosis affected its bulk formation rate, but that bulk formation could be slowed by metabolic alkalosis and, to an even more marked degree, by respiratory alkalosis. Also it was shown that the administration of acetazolamide caused even lower C.S.F. bulk formation in both types of alkalosis and even produced a fall in formation during metabolic acidosis.

The results of this work were well confirmed by Pollay and Davson (1963) and Davson and Pollay (1963) who described the similar depressive effects of acetazolamide on the bulk formation of C.S.F. and on the turnover of isotopically labelled sodium in a ventriculocisternal perfusion system in the anaesthetised rabbit.

Bering and Sato (1963) used the inulin dilution and clearance technique to calculate the bulk formation and absorption of C.S.F. as a function of pressure in localised regions of the C.S.F. pathway in both normal and hydrocephalic anaesthetised dogs.

In 1964, Carmichael, Feldberg and Fleischauer described the perfusion of localised regions of the ventricular system of the anaesthetised cat as a means of applying drugs to selected areas in the brain. Earlier research involving this type of pharmacological utilisation of the C.S.F. as a means of studying the central actions of drugs and their absorption from the ventricles has been well reviewed by Feldberg (1963) who with many co-workers was most active in this area of research from 1957-63.

Further use was made of ventricular perfusion techniques on the anaesthetised rabbit in the experiments carried out by Bradbury and Davson on the transport between C.S.F. and plasma of urea, creatinine and certain monosaccharides (1964), and also potassium (1965). A study of potassium exchange between C.S.F. and plasma which also made use of ventriculo-cisternal perfusion techniques was also reported by Csorr and Pappenheimer (1964) and described in detail by Csorr (1965). She, however, used anaesthetised rats and dogs as experimental animals.

In 1965 Pappenheimer, Fencl, Heisey and Held repeated the earlier work of Leusen (1954a and b), on the effects on respiration of carbon dioxide tension

and bicarbonate concentration in fluid perfused ventriculo-cisternally in the anaesthetised dog, along with a similar but more extensive series of experiments utilising the technique of ventriculo-cisternal perfusion in the conscious goat. The main conclusions of their elegant analysis of the experiments were in agreement with the findings of Leusen (1954a and b), except in the degree of response to the changes in osed, as they found that the unanaesthetised animals "were exquisitely sensitive to small changes in the composition of the fluid perfusing the ventricular system".

Since the work of Adam et al. (1938) and Bhattacharya and Feldberg (1958b) further perfusion experiments have also been used to study the release of acetylcholine from brain in vivo, using the anaesthetised cat and perfusing with fluid containing an anti-cholinesterase (Beleslin, Carmichael and Feldberg, 1964; Beleslin, Polak and Oproull 1965 and Vogt (1967) who also studied the release of dopamine and HVA from cat caudate in this way).

OBJECTIVES OF [illegible]

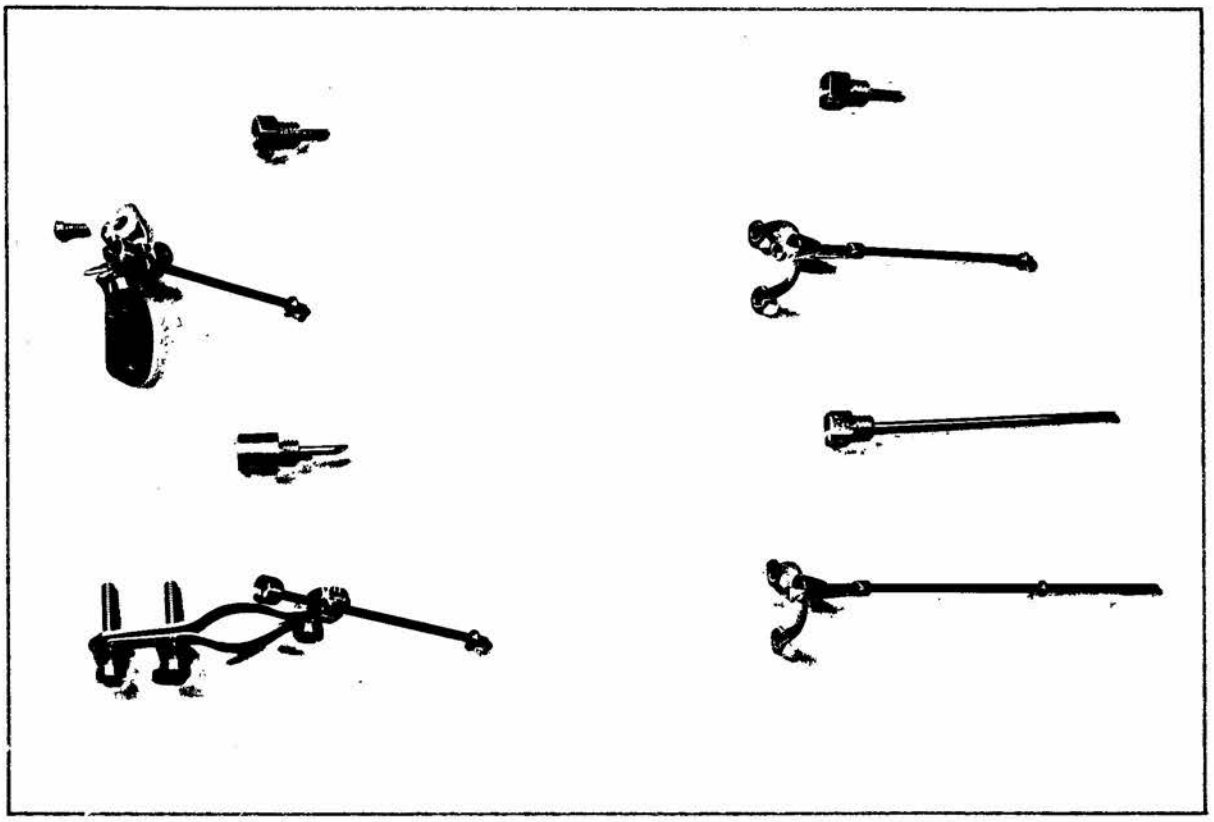
Most of these perfusion experiments have been carried out on anaesthetised animals, apart from the work of Rappenheimer and his co-workers on conscious goats (1961, 1962 and 1965) and the preliminary communication of Manuilov (1950) concerning perfusion in conscious dogs.

The present investigation had the following objectives:-

1. To establish a system of ventricular perfusion in a conscious animal, using a species of laboratory animal which had already been used extensively for research into different aspects of cerebral metabolism, so that information obtained by use of the technique could be directly correlated with existing knowledge of cerebral metabolism in that species. Also, this technique would be of more use in building up a dynamic picture of metabolism where data for the biochemical analysis of brain was already known from acute experiments. The animal chosen was the dog.
2. To study the use of a recirculatory system of ventricular perfusion and determine the characteristics of such a system. ~~minimum~~ Although it is slightly more complex to analyse than an open system of perfusion,

Fig. 1:3

Photograph of Ventricular and Cisternal Guide Tubes



Photograph shows at the left-hand side some of the early types of ventricular and cisternal guide tubes used in the development of perfusion technique. The right-hand side shows ventricular and cisternal guide tubes in present use (Bottom) as made (Top) as cut at the time of operation prior to insertion.

the use of it allows endogenous metabolites to build up in concentration, and it may have more far reaching future applications to investigations of cerebral metabolism *in vivo*.

As an initial project demonstrating the potential of the closed perfusion system, the effluxes of the acid metabolites 5-hydroxyindol-3-ylacetic acid (5-HIAA) and homovanillic acid (HVA) of the biogenic amines 5-hydroxytryptamine, (5-HT) and dopamine from C.S.F. were investigated with the idea of obtaining a means of monitoring the turnover of these amines in brain in the conscious animal.

[illegible]

Guide tubes used in earlier attempts to establish the technique of ventricular cisternal perfusion are shown to the left of Fig. 1:3. In the light of experience gained from these early attempts, and as a result of several post mortem dissections of the suboccipital region of the dog, the guide tubes were modified to the form shown on the right of Fig. 1:3 and in Fig. 2:3. They are very similar in design to those used by Mamulov (1958) in his ventriculo cisternal perfusions of dogs.

Fig. 2:3

Diagram of Ventricular and Cisternal Guide Tubes and Needles in Present Use

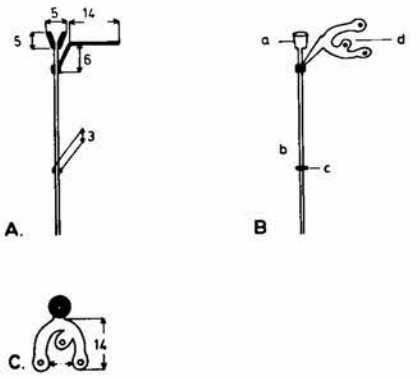


Fig.1

A. CANNULA IN SECTION

B. GENERAL VIEW OF CANNULA
- a.) Funnel of cannula
- b.) Stainless steel tube
- c.) Small collar
- d.) Horseshoe shaped plate

C. VIEW OF CANNULA FROM ABOVE (diagramatic)

(Measurements in mm)

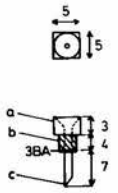


Fig.2

GUIDE TUBE FOR LATERAL VENTRICLE IN DOG BRAIN (Sectional view)
- a.) Part situated subcutaneously
- b.) Part screwed into skull.
- c.) Part inserted into brain, directed towards lateral ventricle.

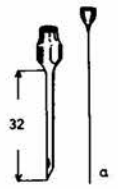


Fig.3

LUER FITTING NEEDLE WITH SIDE HOLE
- a.) Stainless steel stilette.

PRE-OPERATIVE CARE.

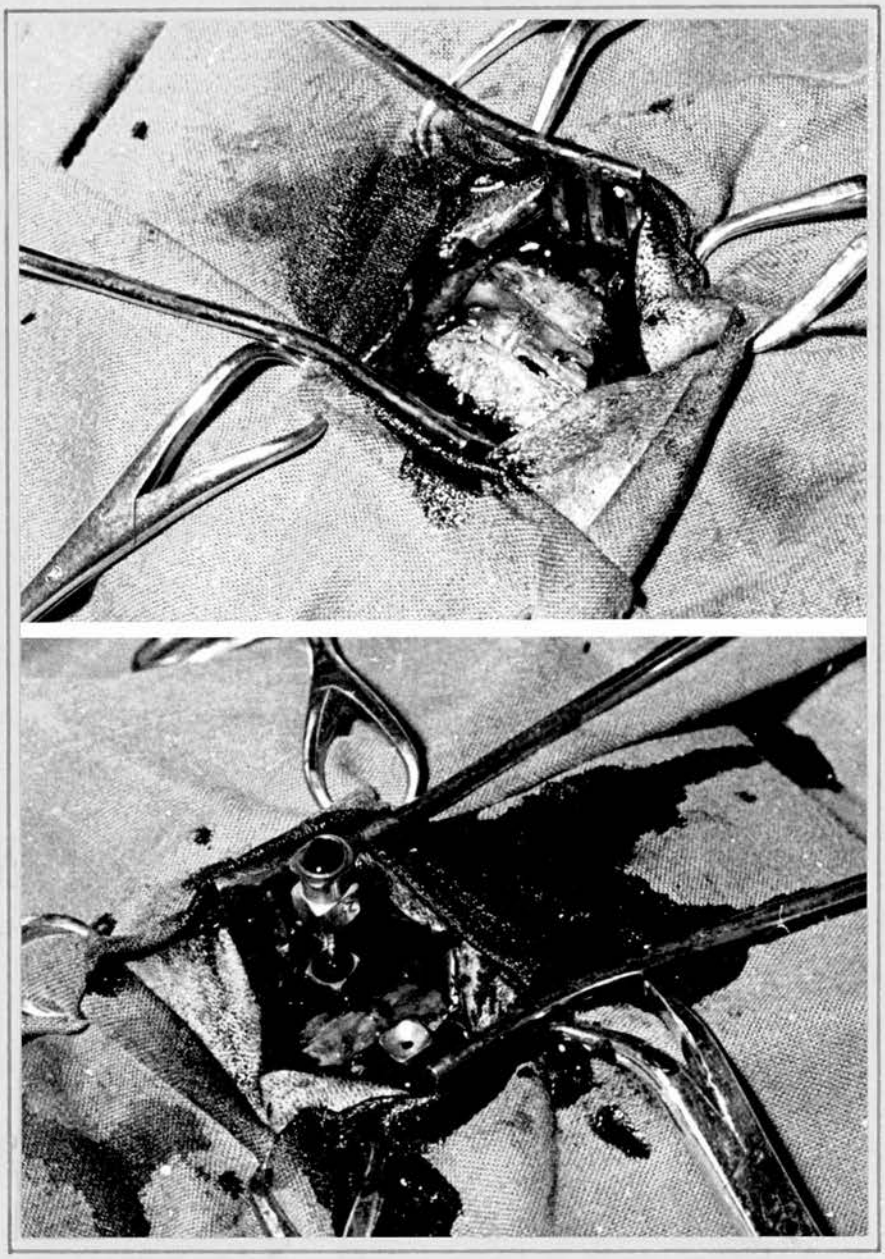
The dogs (adult Beagle hounds of either sex) were anaesthetised with intravenous sodium pentobarbitone and full surgical anaesthesia was maintained, throughout the operation by supplementary doses. The top of the dog's head and the back of the neck were first clipped and then shaved, following which an armoured cuffed endotracheal tube was inserted with the aid of an illuminated tongue depressor to maintain a free airway. The dog was placed in a prone position and for the initial part of the operation, the insertion of the ventricular cannula, the head was supported by an assistant so that the vault of the skull was in a horizontal plane. The skin was cleansed with 1% cetrimide solution (Cetavlon) followed by a dilute methanolic tincture of iodine. The entire operation was then carried out, using full aseptic surgical precautions throughout.

IMPLANTATION OF VENTRICULAR GUIDE TUBE.

A 4 cm. longitudinal incision was made through the skin. The incision was centred over a point 4 cm. anterior to the external occipital protuberance and 3 cm. from the median sagittal plane. This was necessary to avoid the scar being situated over the midline. The

Fig. 3:3

Insertion of Ventricular Guide Tubes



(Top) Operative exposure prior to insertion of the guide tube. Temporal muscles retracted to expose the temporal bone. The notch made in the sagittal crest marks the coronal plane used.

(Bottom) Guide tubes in place. The needle demonstrates that a free flow of C.S.F. can now be obtained from the lateral ventricle.

incision was then retracted into the midline and the vault of the skull exposed by incising the aponeurosis of the temporal muscle close to its origin from the sagittal crest and reflecting the temporal muscle for a distance of approximately 2 cm. A small transverse notch was made in the sagittal crest 3.7 cm. (3.5 cm. in small dogs) from the external occipital protuberance. The exposure is shown in Fig. 3:3 (Top).

A hand drill (3 mm. diam.) was centred over a point 0.8 mm. lateral to the medial sagittal plane in line with the notch in the crest, and the temporal bone trephined perpendicular to the base of the skull. The opening was then tapped with a (3Bs) tap and the cannula screwed in firmly with a specially constructed box spanner. The tip of the cannula was cut prior to its insertion so that when it was finally in position it did not reach the lumen of the ventricle, as otherwise an area of fibrosis at the tip of the cannula resulted in distortion of the ventricle and sometimes produced multiple intraventricular adhesions. Another cannula was inserted into the other lateral ventricle. Test punctures with a 21 gauge needle were always made to ensure that the guide tubes were correctly sited.

Fig. 3:3 (Bottom) shows the operation completed at this stage. The situation of the needle tract to the body of the lateral ventricule is illustrated in the brain sections (Fig. 21:3)from the post mortem performed after an early unsuccessful experiment.

The subcutaneous fascia was approximated as one layer with 3.0 chromic gut using a continuous blanket suture and the skin closed with silk using interrupted eversion matress sutures. It was found that the skin edges should be well everted as otherwise the rapid regrowth of hair tended to irritate the wound and infection might supersede.

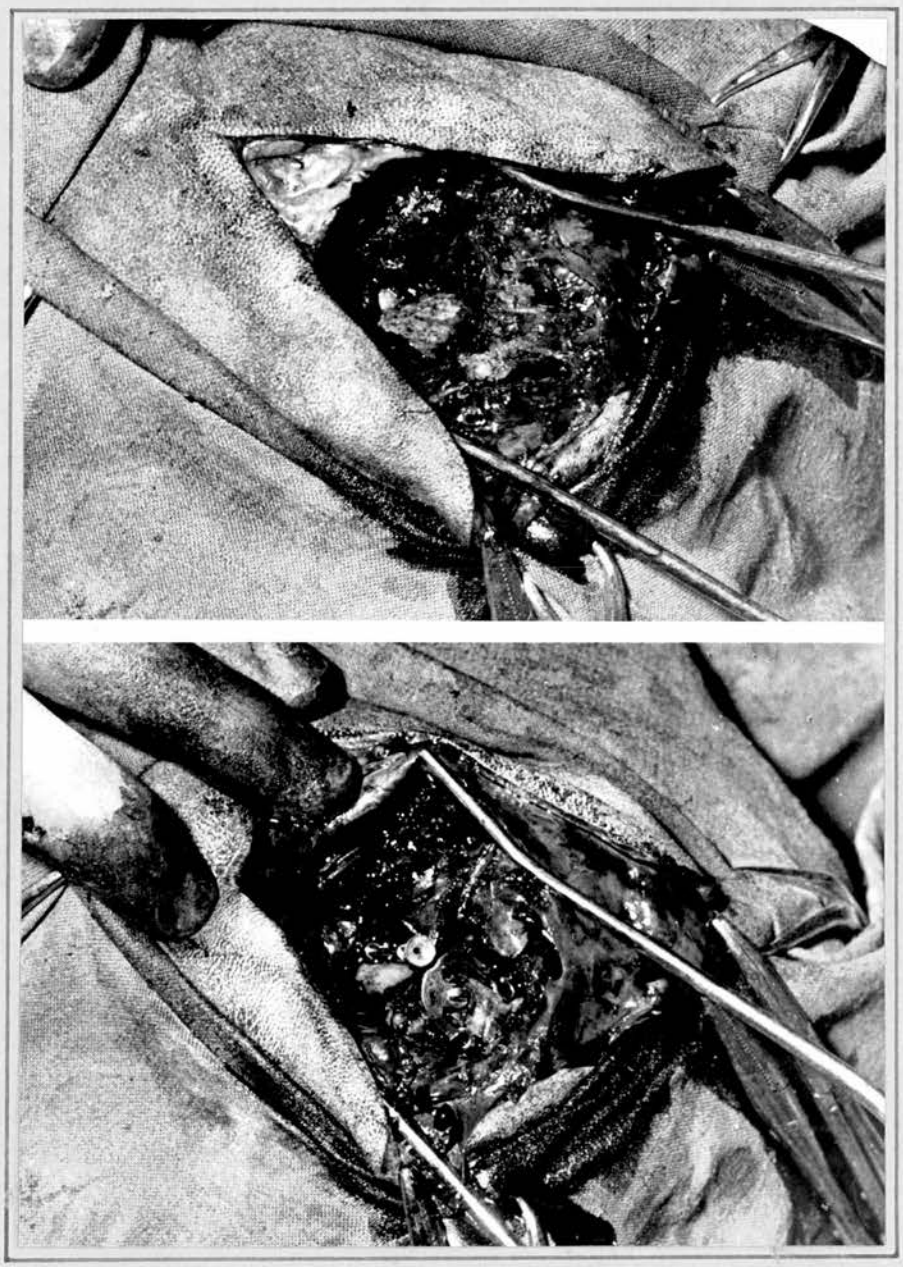
IMPLANTATION OF CISTERNAL GUIDE TUBE.

The cisternal guide tube was inserted at the same operation as the ventricular ones. First, however, it was necessary to lower a flap at one end of the operating table so that the head of the animal might be acutely flexed during the suboccipital dissection.

From a point 2 cm. lateral to the external occipital protuberance, a skin incision was made caudally for 4-5 cm. This incision was retracted into the midline and the fascia incised to expose the nuchal ligament. To minimise bleeding this was split longitudinally and the muscles of the neck retracted from the midline.

Fig. 4:3

Insertion of Cisternal Guide Tube



(Top) Operative exposure prior to the insertion of the guide tube. The nuchal muscles have been split in the midline and retracted to expose the atlanto-occipital membrane and occipital bone. The occipital protuberance and adjoining region have been cleared of muscular attachment.

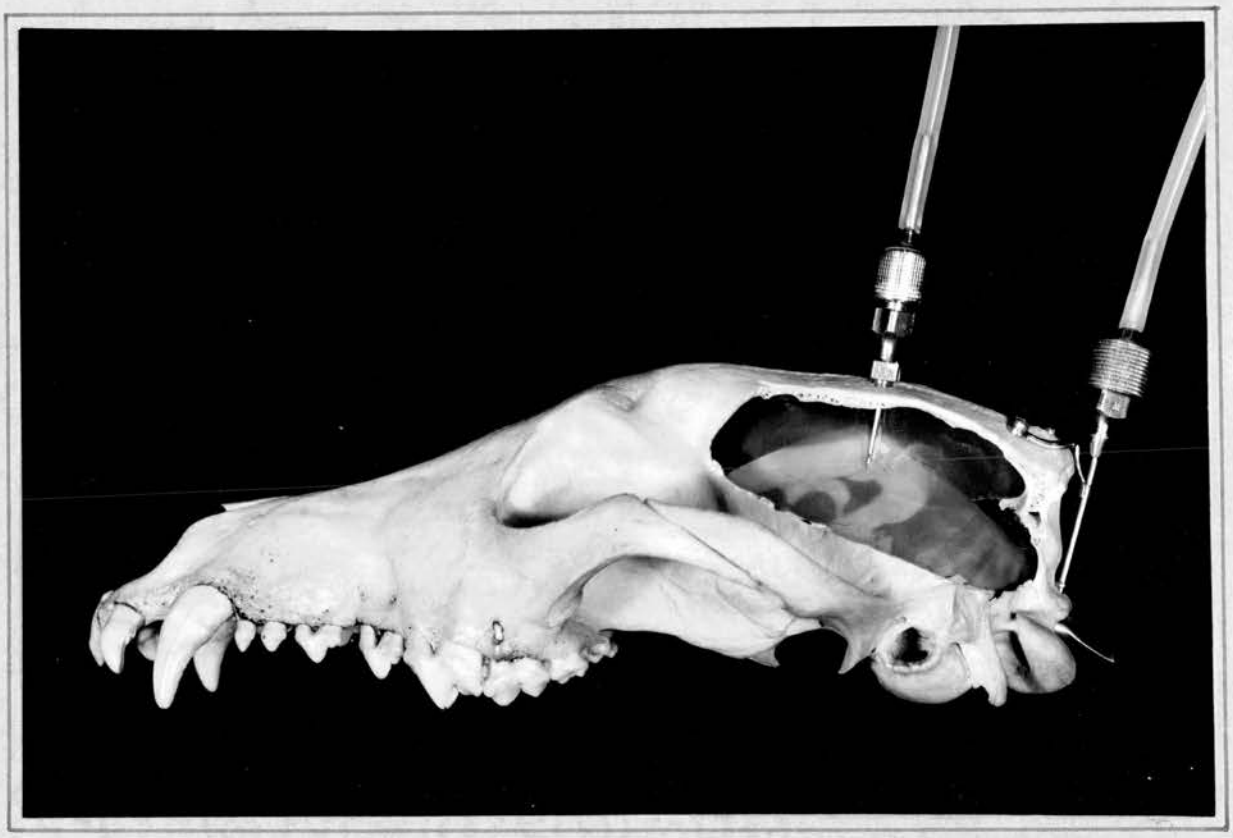
(Bottom) Guide tube in place. The tip of the tube rests in a hole drilled in the lip of the occipital bone. The top bracket is screwed firmly to the occipital protuberance by three screws.

This dissection was continued until the occipital bone was exposed from the external occipital protuberance to the atlanto occipital membrane. The ligaments and muscles overlying the external occipital protuberance were then reflected by use of an osteotome or bone scraper. If the operation was carried out in this way there was little blood loss and a major blood vessel was never encountered. Fig. 4:3 (Top), shows the full exposure made.

With a drill (1 mm. diam.) an opening was made in the midline at the edge of the occipital bone at the point of attachment of the atlanto occipital membrane. The cisternal guide tube was cut to the required length, the small stop brought to a point 2 mm. above the lower end of the tube and the horse shoe shaped plate bent so that it would grasp the external occipital protuberance firmly. While the guide tube was held in place by an assistant, holes were drilled (1 mm. diam.) in the bone of the occipital protuberance through the plate. The holes were then tapped with a (8Ba) tap and the plate fixed to the skull with three stainless steel screws. In this way the cisternal guide tube was firmly held at both ends (Fig. 4:3, Bottom). No test puncture

Fig. 5:3

Guide Tubes Inserted into a Dog Skull (Lateral View)



A X-ray showing a lateral view of the ventricles, from a dog that had an intraventricular injection of Myodil, has been inserted into the appropriate position through a window cut in the skull. The photograph demonstrates the position of the guide tubes and needles during perfusion.

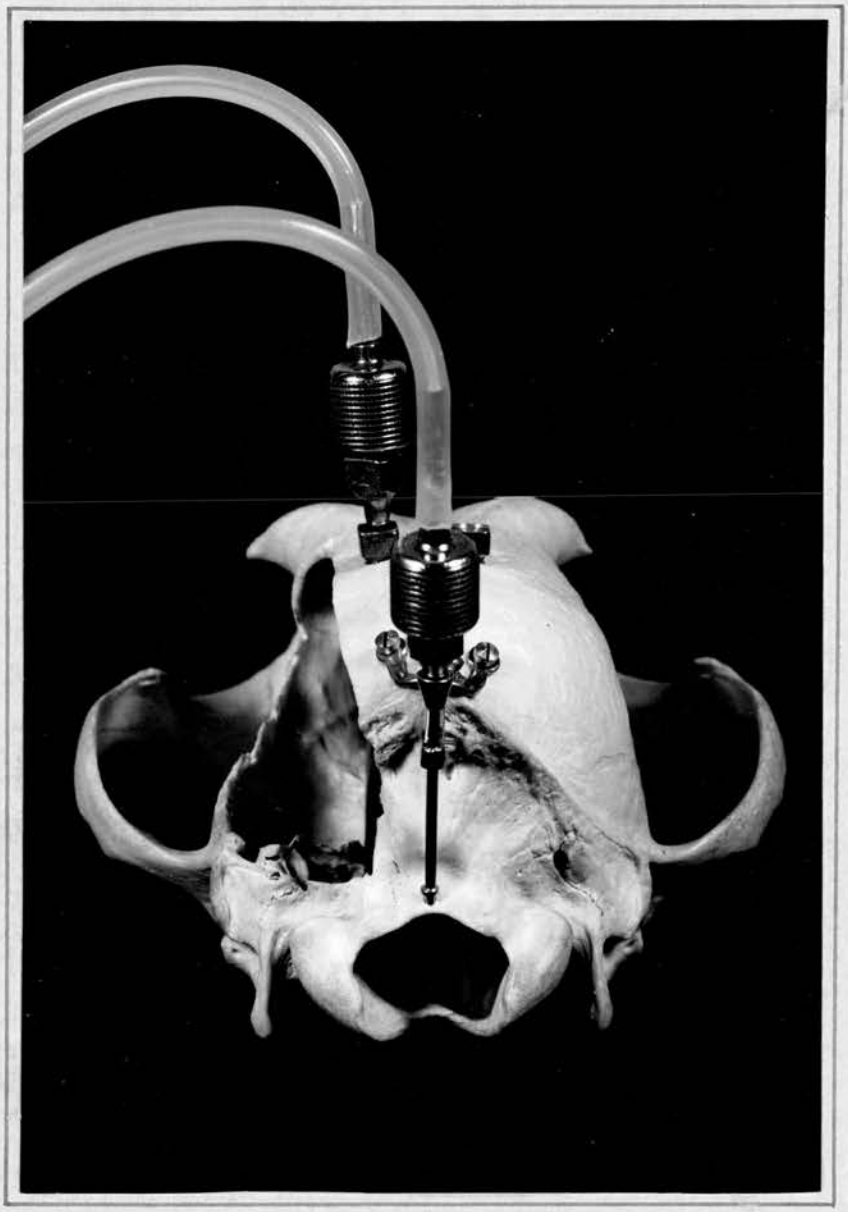
was made at the time of operation as its success was self evident. The muscles were approximated to each other and closed about the guide tube by a continuous blanket suture of 3.0 chromic gut. The fascia was then brought together as a separate layer covering the funnel of the cannula, with a similar suture, and the skin closed with interrupted everting mattress sutures. The operation is perhaps best described by Fig. 5:3, 6:3 and 7:3 which show different views of the operation performed on a dog skull.

POST OPERATIVE CARE.

Sterile dressings were bandaged in place over the wounds and the endotracheal tube removed. Care was taken to ensure that the bandage was not too tight otherwise it would cause the airway to become obstructed once the endotracheal tube was removed. A protective collar, was placed over the dog's head which prevented the animal dislodging the bandage and opening its wounds by scratching. The animal was then returned to heated kennels where the temperature was maintained at approximately 24°C. Prophylactic antibiotics were given for three days (1 amp. - Crystamycin (Glaxo) - 1,000,000 units Benzyl Penicillin + 0.5 g. Streptomycin) intramuscularly daily.

Fig. 6:3

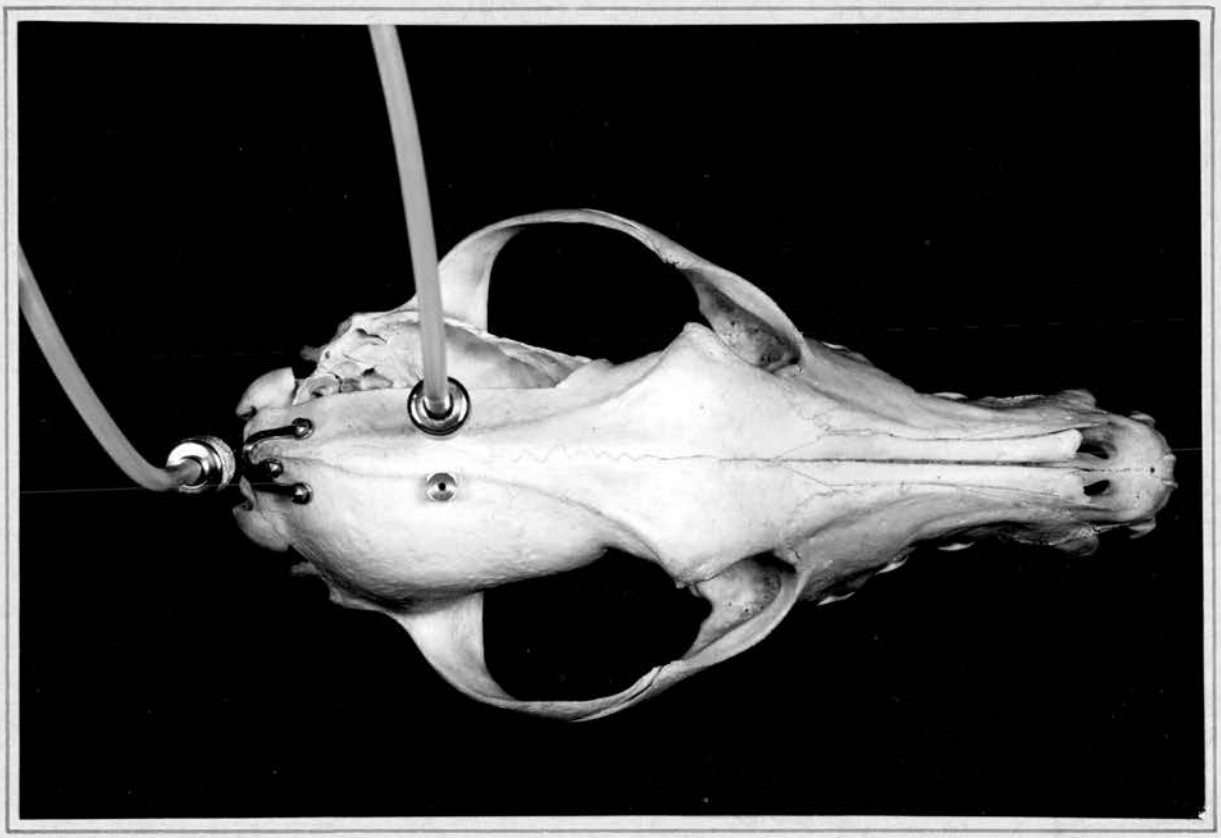
Guide Tubes Inserted into a Dog Skull (Posterior View)



The posterior view demonstrates how the tip of the cisternal guide tube is held in position, also the length of needle required for successful puncture.

Fig. 7:3

Guide Tubes Inserted into a Dog Skull (Top View)



The top view demonstrates the positioning of the ventricular guide tubes and the method of fixation of the bracket of the cisternal guide tubes.

The skin sutures were removed under light anaesthesia with sodium pentobarbitone 7 days after the operation. Between two to three weeks after the operation, once all reactionary tissue swelling had subsided, the dog was similarly anaesthetised and test punctures were made through both the cisternal and ventricular cannulae with needles of different lengths so that suitable lengths of needle might be employed in subsequent perfusion experiments. A further two weeks were allowed to elapse before the initial perfusion experiment was carried out, and subsequent experiments were also performed with an interval of at least two weeks between them.

PERFUSION TECHNIQUE

Apparatus Employed.

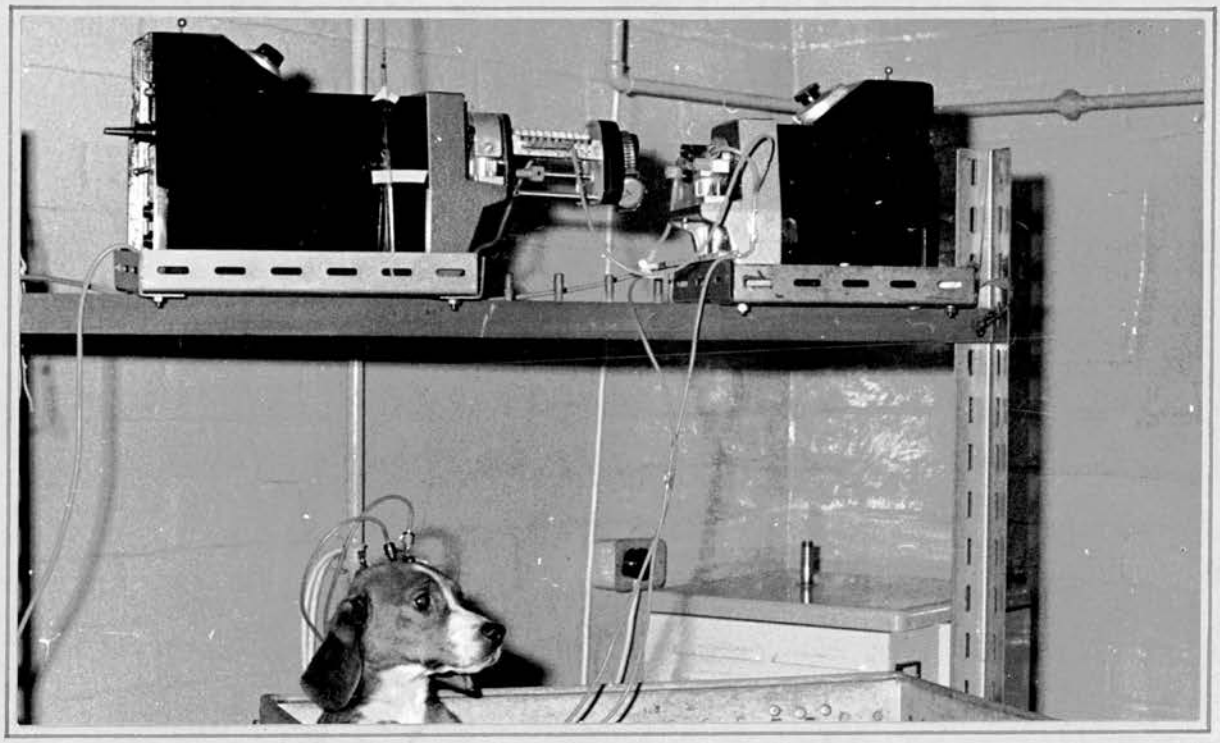
The needles used (Fig. 2:3) to make the percutaneous puncture were 21 gauge stainless steel luer lock needles which had been cut with a short bevel and with side holes near the tip. A series of such needles with lengths differing by 1 mm. were prepared so that a needle known to be of suitable size could be selected.

Perfusion Circuit

(Fig. 8:3) The tubing used for the circuit was of silicone rubber internal bore 0.8 mm. - external bore

Fig. 8:3

Dog 8 During Perfusion of the Cerebral Ventricles



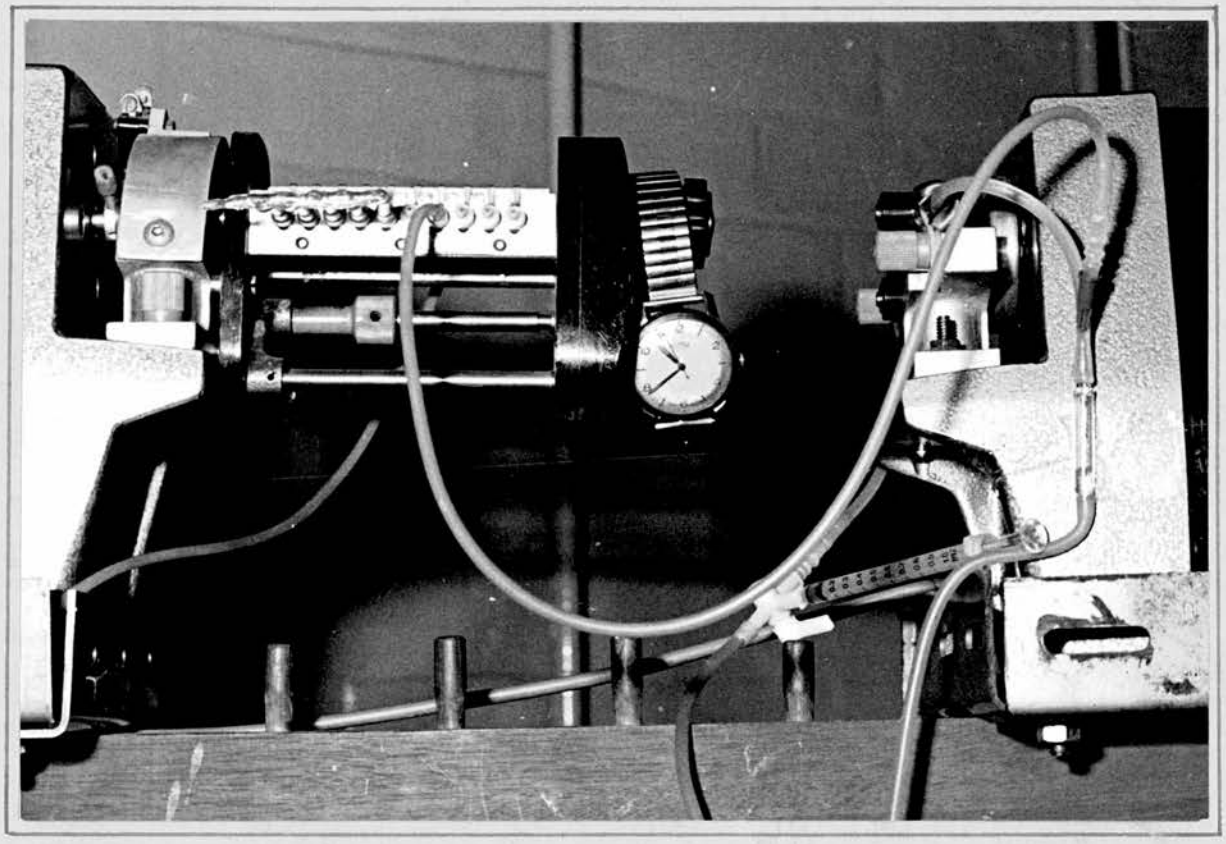
The photograph illustrates the entire apparatus used for the perfusion experiments.

It also illustrates that the dogs' behaviour during these experiments is entirely normal.

4.0 mm. (Watson-Marlow). This was connected to the needles by luer lock adaptors (Fig. 5:3). In the cisternal limb of the circuit a sterile three way tap (Pharmaseal Laboratories) was inserted so that samples might be withdrawn when required. The volume of fluid contained between this tap and the cisternal needle was 0.7 ml; thus the 0.5 ml. samples taken were truly representative of the outflow at the time of sampling. A small drip chamber was mounted in a vertical position on the pump used for perfusion, (M.H.R.E. Flow Inducer: Watson-Marlow Ltd.,) in the outflow tubing of the pump just in front of the point of entry of the infusion system, (Fig. 9:3). In this way the drip chamber not only provided a continuous monitor of the rate of perfusion but also ensured thorough mixing of the infused fluid with the perfusing fluid. The level of fluid in the drip chamber altered in the event of any blockage occurring in the system during perfusion. The pump used to maintain the flow of the recirculated perfusion fluid had a rotor carrying three rollers which, as they rotated, compressed the elastic silicone rubber tubing against a curved track. The point of contact moved round the track, pushing fluid before it, and creating an equivalent negative pressure behind. The pump, after an initial period of

Fig. 9:3

Perfusion and Infusion Pumps



A more detailed view of the infusion and perfusion pumps and their circuits. (See Fig. 8:3)

running at each time of use gave a very stable flow rate. The total volume of the perfusion circuit external to the dog was 1.85 ml.

Infusion Circuit

(Fig. 9:3) A 2 ml. graduated pipette graduated in 0.1 ml. intervals acted as a reservoir for the infusion fluid so that frequent readings could be taken of the rate of flow. Tubing of the type described above connected the reservoir to the second pump (M.H.R.A. 1000-Delta Type - Flow Inducer; Watson-Marlow Ltd.). This pump had a thin silicone rubber tube wound once round a triangular rotor giving three points of contact with the tube. It was capable of producing very slow constant flow rates. The outflow of the pump was connected by a length of silicone rubber tubing to a 26 gauge needle.

EXPERIMENTAL PREPARATION

Both the infusion and perfusion circuits were sterilised by allowing them to pump boiling water for a period of about 15 minutes and then the circuits were emptied by pumping air. The needles were also sterilised in boiling water.

C.S.F. was obtained from an anaesthetised dog by percutaneous cisternal puncture. This was pumped into

the perfusion circuit to prime it and the fluid was also used as the solvent for the infusion solution. The infusion solution was then drawn up into the reservoir, making quite sure that there was no air in the system. The point of the 26 gauge needle on the outflow side of the infusion system was then inserted through the wall of the tubing of the perfusion circuit just above the drip chambers (Fig. 9:3).

VENTRICULAR PUNCTURE

The head of the animal to be perfused was held by an assistant and the specific needle for that dog, with its stilette in position, was inserted through the skin into the ventricular cannula and thence to the ventricle. This procedure caused no distress to the animals, apart from the slight discomfort when piercing the skin. Provided that the needle was correctly selected, (when the stilette was removed) C.S.F. flowed out slowly or could be gently aspirated with a syringe. Having checked that the ventricular puncture was successful, the needle was connected to the luer lock adaptor on the inflow side of the perfusion system (Fig. 5:3).

CISTERNAL PUNCTURE

The needle required for the cisternal puncture was

Fig. 10:3

Dog 1 During Perfusion of the Cerebral Ventricles

A more detailed view of the connections between the needles and tubing. (See Fig. 8:3)

The photograph also illustrates that the behaviour of these dogs during the experiments is entirely normal.

given a slight bend in the middle so that when it was in place the spring effect of the curved needle in the straight guide tube held the needle firmly in position throughout the experiment. This simple point was important, as inadequate fixation of the cisternal needle with its dislodgement during an experiment was a common cause of failure in early experiments. With a stilette in position, the cisternal needle was inserted percutaneously into the guide tube and pushed firmly down it with a rotational action. The stilette was removed and a satisfactory flow of C.S.F. established before connecting the needle to the luer lock adaptor on the outflow side of the perfusion circuit (Fig. 5:3 and 6:3). The tubing of the perfusion system was attached to the back of the neck with adhesive tape so that there was no direct pull on the needles. Fig. 8:3 and 10:3 show experiments in progress.

EXPERIMENTAL PROCEDURE

An initial reading of the level of the infusion fluid was recorded, then both pumps were switched on after checking that they were set to the required speeds. At half hourly intervals both pumps were stopped, and samples of cisternal outflow were collected into a

disposable polythene syringe by means of the three way tap inserted in the outflow limb of the circuit. Both pumps were then switched on again, and in all it was usually only necessary to stop them for about half a minute to obtain the samples of C.S.F. Readings of the fluid level in the infusion reservoir were taken at intervals, also counts of the rate at which drops fell in the drip chamber. Thus a check was kept that there was no variation in either the rate of perfusion or the rate of infusion, but in fact they never varied unless a serious fault developed in the system.

BEHAVIOURAL EFFECTS DURING PERFUSION

Throughout the experiment the dog was placed in a large box without a top to limit its movement slightly so that it would not impose too great a strain on the length of tubing leading to the needles, or cause the needles to become dislodged. It had, however, sufficient room to walk round the box or lie down if it wished. The animal's behaviour throughout the experiment was always normal, provided there were no technical difficulties with the perfusion system. The more active dogs tended to walk round the box and expected to be petted, while the placid dogs have been known to sleep through almost the entire experiment. The dogs would take both food

and drink if presented - indeed the experimenter has had to share his lunch on more than one occasion!

The only occasions on which behaviour changes occurred were during two perfusions in which concentrated solutions of phenylacetic acid were infused into the system. In these experiments the dogs seemed tense and apprehensive and held their necks very stiff; in one of these cases the animal seemed so disturbed that the perfusion was stopped prematurely. There were, however, no long term complications in either case and subsequently both animals were perfused satisfactorily without showing similar behavioural reactions. (Section II - Venticular Perfusion, page 146).

Initial Technical Difficulties

In initial experiments the dogs were perfused at rates between 0.5 and 1 ml/min., and an artificial C.S.F. was used. The dogs tended to be extremely restless and apprehensive and perfusions had to be frequently stopped prematurely. A perfusion of about two hours always produced a subsequent spastic semi-paralysis with scissoring of the limbs. Death usually followed and marked fibrotic adhesions were usually found in the regions of the cisterna magna. Since the introduction of a stabilised cisternal needle and the use of donor C.S.F. and a slower rate of perfusion the technique has become highly successful.

BIOCHEMICAL METHODOLOGY

Drugs and Chemical reagents

Inulin	(British Drug Houses)
5-HIAA - diethylammonium salt	(Roche)
5-HIAA	(Koch-Light)
^{14}C-5-HIAA 1.86m.c./m.mole	(New England Nuclear Corp)
HVA	(Calbiochem)
Phenylacetic acid	(British Drug Houses)
Probenecid	(Merck, Sharpe and Dohme)

All chemical reagents used were Analar Grade apart from the concentrated HCl which was micro analytical grade (Hopkin and Wilkins - or British Drug Houses). Deionised distilled water was used throughout.

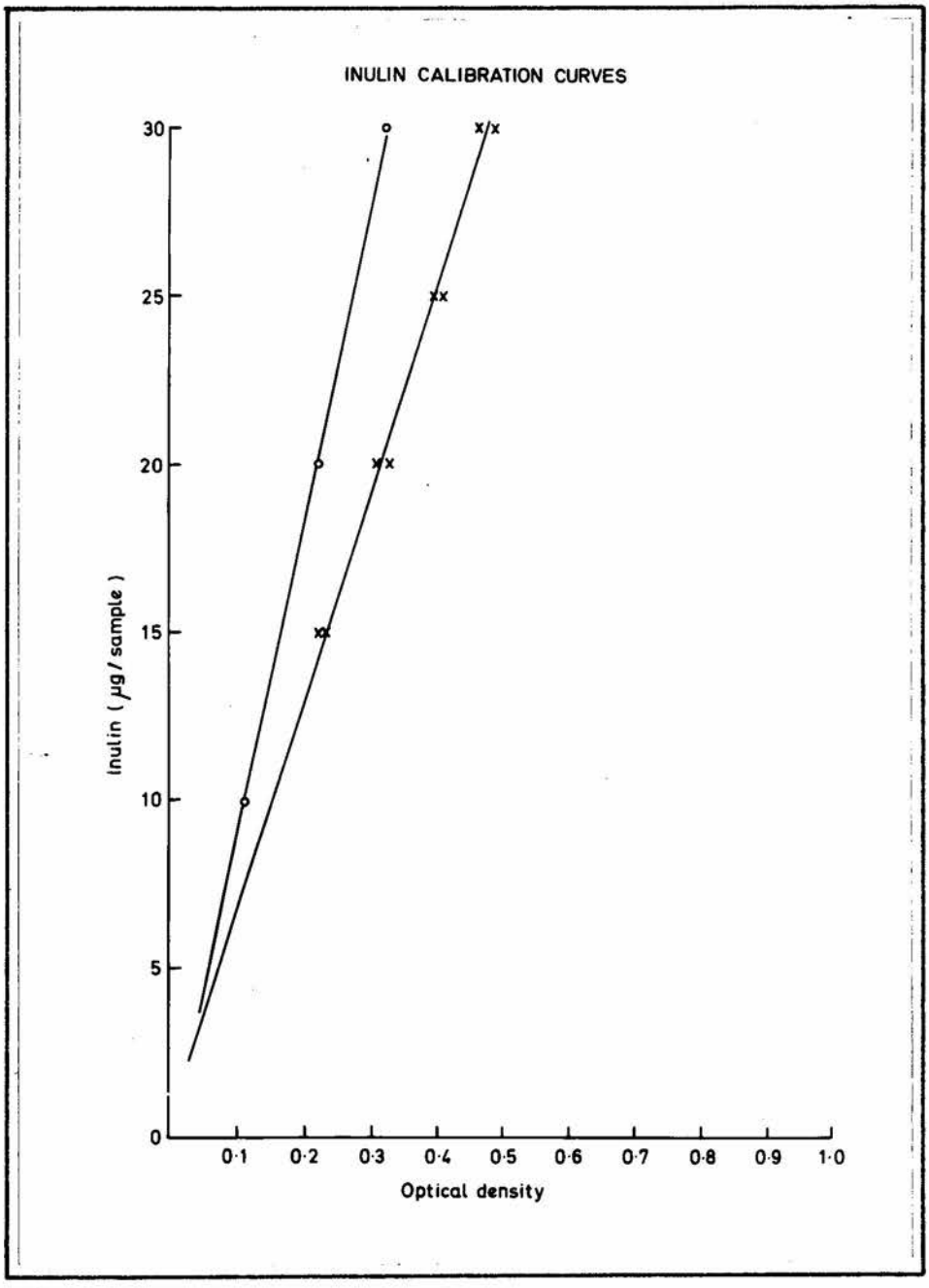
ESTIMATION OF INULIN IN C.S.F.

The method used was an adaptation of that described by Heyrovsky (1956). It was found unnecessary, however, to include the step of protein precipitation with cadmium sulphate when estimating the concentration of inulin in C.S.F.

According to the amount of inulin administered during the experiment, either 0.05 or 0.1 ml. of the C.S.F. sample was used for the estimation. The same volume of a 10-fold dilution of the infusing fluid was taken to estimate the concentration of inulin in the infusing fluid.

Fig. 11:3

Inulin Calibration Curves



The curves show good linearity but demonstrate the necessity of including standards with each assay run.

The sample size used varied to ensure that the amount of inulin for estimation remained within the linear range of the method. (Fig. 11:3).

After being transferred to a 15 ml. glass stoppered tube the sample was brought to a total volume of 0.5 ml. with water. 0.1 ml. of solution of indol-3-ylacetic acid (I.A.A.), 0.5% (w/v) in ethanol was added, followed by 4 ml. of concentrated hydrochloric acid. The samples were mixed by inversion and then heated at 37°C for 80 min. in a water bath.

An aqueous blank sample and a sample to which 20 µg of inulin had been added were also taken through the entire procedure. The samples were cooled and their optical densities at 530 mµ measured in a spectrophotometer against an aqueous blank. Either a Unicam SP800 or SP500 spectrophotometer was used.

DISCUSSION OF INULIN ASSAY

The above method involves the hydrolysis of inulin by the concentrated acid to fructose which in the presence of I.A.A. gives rise to a bluish purple colour.

There are several points to note about the method of assay. Although the method has very good linearity throughout the acceptable range of optical density (0.1 - 0.8) Fig.11:3, as a hydrolysis stage is involved it is necessary to include standards with each group

of samples assayed as the degree of hydrolysis seems to vary between runs. Secondly, the inulin used to make up the standards in the assay must come from the same batch of inulin as is used in the experiments. This is because different batches of inulin vary considerably in their degree of polymerisation and hydration, and differences in these will give rise to differences in the colour density developed for the same weight of inulin and exposure to hydrolysis. Thirdly, the colour reaction is not specific and can be produced by glucose. Thus, C.S.F. samples from perfusions in which inulin was not added gave an average reading through the method (due mainly to their glucose content) equivalent to 15 µg of inulin per ml. of C.S.F. This value was subtracted from concentrations calculated in comparison with an aqueous blank and inulin standards.

DETERMINATION OF 5-HYDROXYINDOLE ACID AND 5-HYDROXYINDOLE-3-ACETIC ACID IN C.S.F.

These were carried out by the method described in Section II page98 . Only a few procedural modifications were made. Firstly, aqueous recoveries of pure substance in amounts corresponding to the anticipated concentrations of the samples were included as internal standards and the sample concentrations were calculated by comparison

with their readings. Secondly, samples of infusion fluid were diluted 1 in 10 with water before a portion was taken for assay. Thus all the samples and the internal standards were kept within a narrow range of concentration. Thirdly, if the samples were from experiments where large concentrations of 5-HIAA or HVA had been infused, it was on occasion necessary to dilute part of the buffer into which they had been back-extracted before estimating the 5-HIAA or performing the fluorescent reaction for HVA.

Attempts were made in a few of the initial recirculating perfusion experiments to estimate 3,4-dihydroxyphenylacetic acid in the perfusate. These attempts were abandoned when it became clear that the amount of the acid present was too low to permit accurate measurement by this method (Rosengren 1960). This restriction of the assays to 5-HIAA and HVA only allowed these acids to be detected in the perfusate when they were present in even smaller concentrations than before because it was now possible to back-extract them from the ethyl acetate extract into a smaller volume of buffer since the latter required to be divided only into two and not three portions for the estimations.

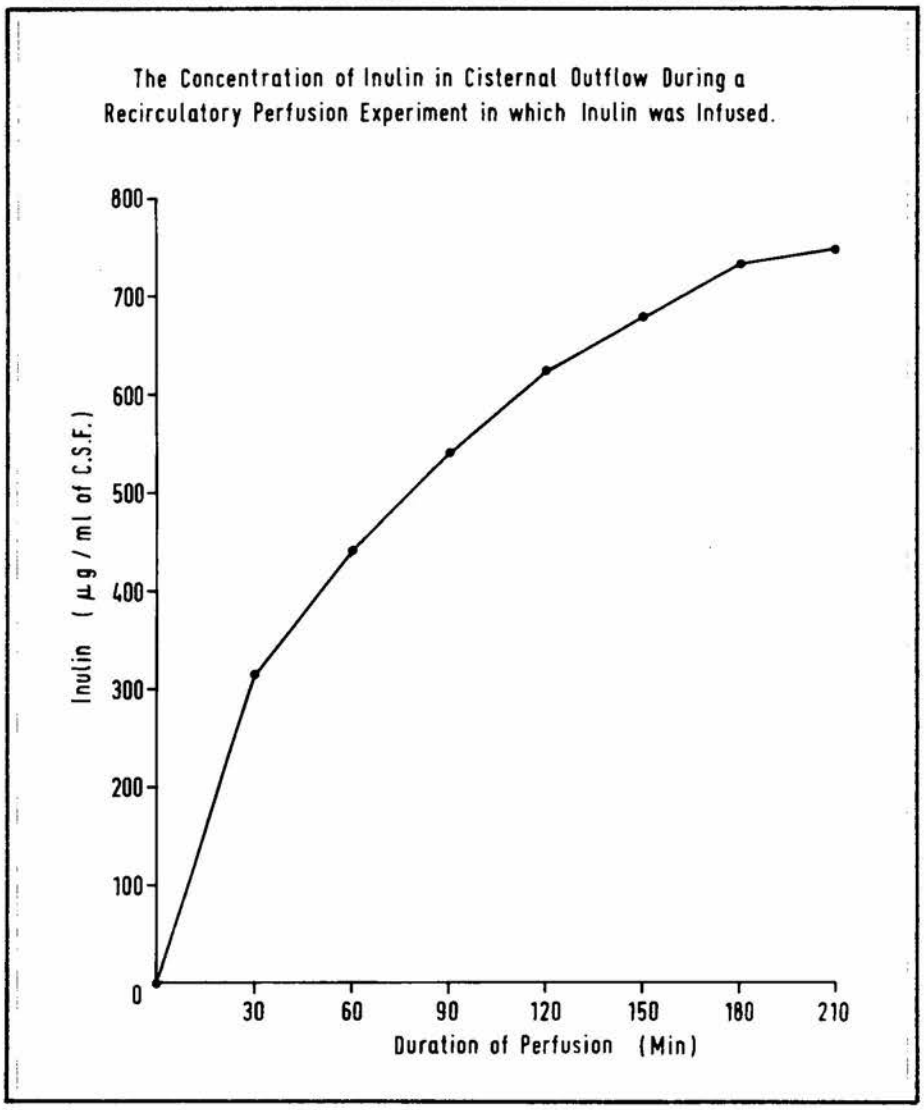
RADIO-ISOTOPE METHODOLOGY

In the experiments described in detail elsewhere (page226) ^{14}C-5-HIAA was assayed in plasma by the total radio-activity of a 0.1 ml. portion. The C.S.F. however, had its ^{14}C-5-HIAA concentration estimated by two methods so that the estimates would be comparable with those of future planned experiments.

To the C.S.F. sample water was added in a 15 ml. glass stoppered tube to give a total volume of 1.1 ml.. Of this diluted C.S.F., 0.1 ml. was taken for a direct estimate of radio-activity. The remaining 1 ml. was salt saturated and acidified with 0.05 ml. of 10 N HCl and the weak acid extracted into 7 ml. of freshly distilled peroxide-free diethyl ether by shaking the two phases together vigorously for 5 min. The phases were separated by centrifugation for 3 min. at 2000 r.p.m. in a refrigerated centrifuge and 5 ml. of the ether phase was transferred to a 15 ml. glass stoppered tube containing 1 ml. 0.3M sodium phosphate buffer pH 7.0. The tube was shaken for 5 min. and then centrifuged at 2000 r.p.m. for 3 minutes. After the supernatant ether had been separated off, 0.4 ml. of the phosphate buffer was taken for an estimate of radio-activity while a further 0.2 ml. aliquot was taken for assay of the 5-HIAA

Fig. 12:3

Inulin Concentrations in Cisternal Outflow



The figure illustrates the increase in inulin concentrations which occurs with time and how they tend towards a plateau concentration. **The concentrations of inulin obtained in the experiment illustrated are plotted logarithmically in Fig. 13:3.**

concentration by spectrofluorimetry (micro cell of Farrand (2)-spectrofluorimeter, see page 22).

Estimates of radio-activity were performed in a Packard Tri-Carb Scintillation Counter. The aqueous samples were taken up in 4 or 5 ml. of an ethanol/methanol mixture (3/1) (v/v) to which 10 ml. of scintillator (0.11 g P.O.P.O.P. + 4.26 g P.P.O./litre of toluene) was added. Quench correction was carried out by the Channels Ratio Method (Baillie 1960). The absence of severe colour quenching was checked by the use of internal standardisation on random samples.

THEORY

Fig. 12:3 shows the inulin concentration curve which was obtained during a typical experiment using a single infusion containing both inulin and 5-HIAA. It is possible from this data, obtained as described on (page 195) with a recirculatory perfusion system to arrive at values for most of the parameters which can be measured with an open ended perfusion system.

SYMBOLS EMPLOYED

C = Concentration (in µg/ml). Suffixes I, O and In indicate the concentration in the infusion fluid, the cisternal outflow and the ventricular inflow respectively.

R = Rate (in ml/min.) Suffixes I, P and S indicate rate of flow of the infusion fluid, the perfusion fluid and the average rate at which fluid is sampled. The average rate of sampling for all experiments was 0.0167 ml/min. (0.5 ml. every 30 min.)

THEORETICAL CALCULATIONS

There must be a conservation of inulin during the experiment, therefore the amount of inulin added to the system in any period of time must equal the additional amount remaining in the system plus the amount removed in that time. A substance such as inulin, which is not actively transported from C.S.F. and diffuses out only very slowly (Pappenheimer and Heisey, 1963 and Hall, Oppelt and Patlak, 1962) will be removed essentially only by bulk absorption, and, in the case of the experiment, by sampling. Thus the amount removed can be expressed in terms of a constant volume of outflow fluid. In general,

$$R_I C_I dt = K C_o (t) dt + dx \qquad (1)$$

where K is the volume continuously being removed in unit time and dx is the increment in amount of inulin in the system in time dt.

Thus, $dx/dt = R_I C_I - K C_o (t)$ (2)

But the perfusion circuit may also be regarded as a system of communicating compartments and the increase in inulin expressed as,

$$dx/dt = V_o\, dC_o/dt + V_1\, dC_1/dt + V_2\, dC_2/dt + \ldots \quad (3)$$

where $V_o + V_1 + V_2 + \ldots$ equals the total volume of the system V, and

V_o has the concentration C_o (t) at any time

V_1 has the concentration C_1 (t) at any time

V_2 has the concentration C_2 (t) at any time etc.

Where the gradient between these different areas of concentration is governed primarily by the rate of flow of fluid, the changes that occur in concentration of these compartments due to the addition of infused inulin will not alter the constant relationships of these concentrations to each other. Thus, where rate of diffusion within the system is negligible with respect to the speed of circulation

$C_1(t)$ will equal $\alpha\, C_o(t)$ at any time

and $C_2(t)$ will equal $\beta\, C_o(t)$ at any time etc.

(where α and β are constants greater than 1)

Equation (3) for the perfusion system could therefore be expressed as,

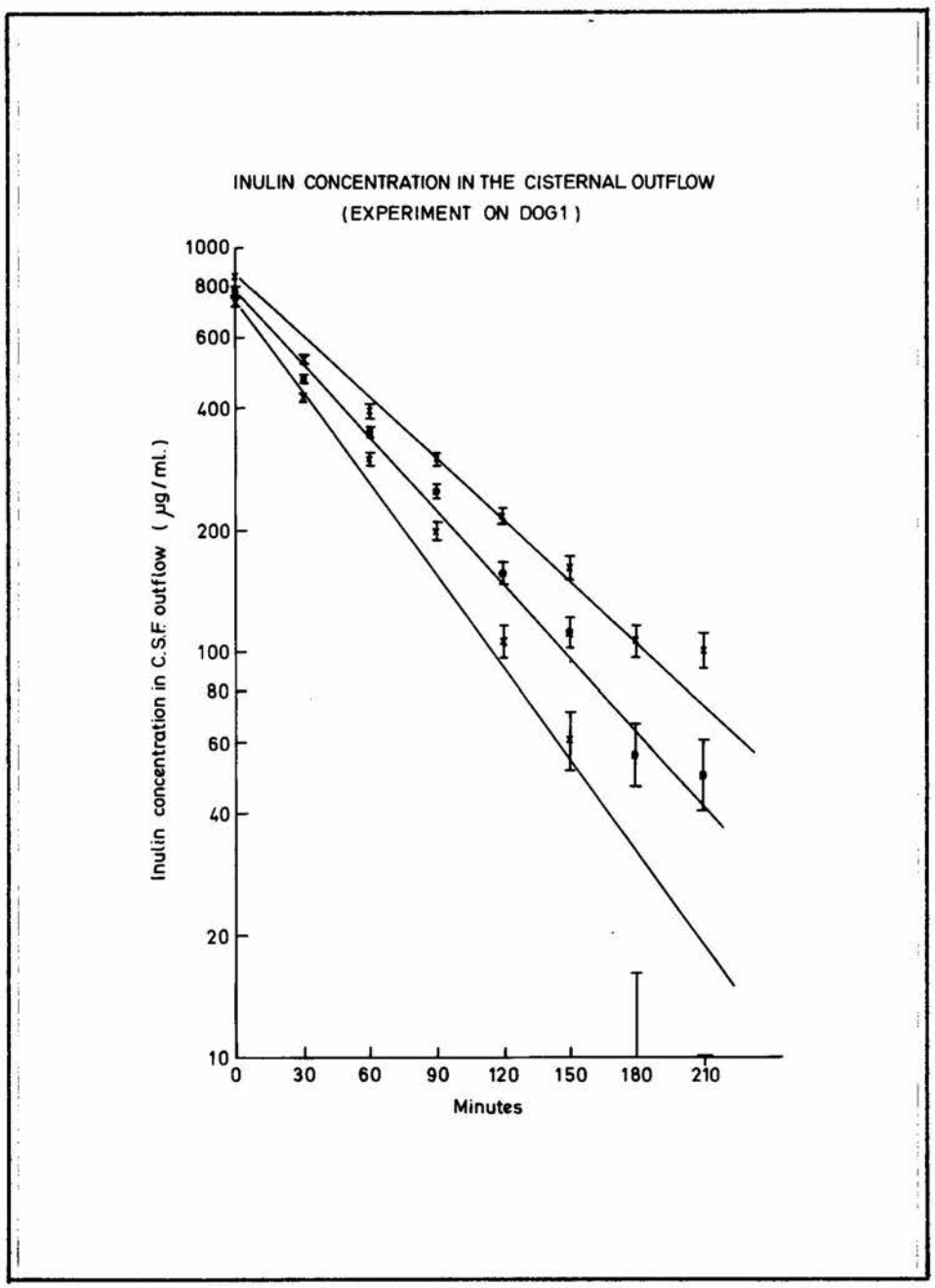
$$dx/dt = (V_o + \alpha V_1 + \beta V_2 + \ldots .)\, \frac{dC_o}{dt} \quad (4)$$

If the weighted volume

$V_o + \alpha V_1 + \beta V_2 + \ldots$ is represented by G equations

Fig. 13:3

Logarithmic Plot of $(C_o(\infty)-C_o(t))$ for the Values Illustrated in Fig. 12:3



The figure illustrates a series of plots for expected values of $C_o(\infty)$ minus the values of $C_o(t)$. I Indicates the best values for a straight line.for the data obtained in the experiment illustrated in Fig. 12:3. The value given for $C_o(\infty)$ was 800 µg of inulin/ml of C.S.F. and for γ was 63 min.

(2) and (4) may be combined

$$G \frac{dC_o}{dt} = R_I C_I - K C_o (t)$$

or $$\frac{G}{K} \frac{d C_o}{dt} = \frac{R_I C_I}{K} - C_o (t)$$

However at equilibrium when $d C_o/dt = 0$ the constant

$$\frac{R_I C_I}{K} = C_o(\infty) \qquad (5)$$

thus, $\frac{G}{K} \frac{dC_o}{dt} = C_o(\infty) - C_o (t)$ (6)

If $G/K = \tau$ (7)

then integrating gives $C_o(t) = C_o(\infty)(1 - e^{-t/\tau})$ (8)

thus

$$-t/\tau = \ln (C_o(\infty) - C_o(t)) - \ln C_o(\infty) \qquad (9)$$

and a range of $C_o(\infty)$ may be determined by trial plotting of $\ln (C_o(\infty) - C_o(t))$ against time to get a straight line (Fig. 13:3). For the best straight line a value for $C_o(\infty)$ is obtained. Also, as equation (8) shows when $t = \tau$, $(C_o(\infty) - C_o(t))$ will equal $C_o(\infty) \frac{1}{e}$, thus the best value for τ may be read from the graph.

A value for K may be obtained by inserting the best value of $C_o(\infty)$ in equation (5).

From equation (7) the volume G may be obtained.

PHYSIOLOGICAL INTERPRETATION

G represents the total volume in terms of the outflow concentration (Equation 4), and as this is the lowest concentration in the system, G gives the maximum volume possible for the perfusion system. (This value will not differ greatly from the true volume of the system as most of it is at $C_o(t)$). By subtracting the volume of the external circuit the maximum volume of the space perfused is obtained. The data shown in Fig. 13.3 gave a value of 5.55 ml. for the space perfused, a figure which is, as would be expected, compatible with but slightly larger than the mean figure of 3 (± 2.1 (SD)) ml. obtained by Bering and Sato (1963), with an open ended perfusion system, for the volume of distribution of inulin during ventriculo-cisternal perfusion of the anaesthetised dog.

The value obtained for K from equation (5) is of major physiological significance as by its definition it must represent the total volume removed in unit time. Thus, for inulin at equilibrium it is equal to the average volume removed by sampling plus the volume removed by bulk absorption. Also, because of the conservation of volume within the system, this must equal bulk C.S.F. formation F plus the volume infused.

Thus, for example in Figs. 12:3 and 13:3 K = 0.0705 ml/min and the bulk formation of C.S.F. = 0.056 ml/min (Table 2:3, Fig. 13:3).

The bulk absorption represents the clearance of inulin in the way it actually occurs, i.e. in terms of a volume of outflow. By convention, 'Clearances' in renal physiology are expressed in terms of a volume at inflow concentration. In the present system the inflow concentration can be obtained from the outflow concentration by adding the quantity of fluid and inulin known to have been infused in a fixed time,

$$C_{In} = \frac{C_o R_p + C_I R_I}{R_p + R_I} \qquad (10)$$

Thus convential clearance of inulin is expressed as

$$Cl. = \therefore \frac{C_o(\infty)}{C_{In}(\infty)} \qquad (11)$$

The derivation of 'clearance' is perhaps more simply explained when expressed in terms of the original parameters.

At equilibrium the amount of inulin infused into the system will equal the amount removed from it, thus a gross clearance may be calculated as

$$Cl.\ (gross) = \frac{C_I R_I}{C_{In}(\infty)} \qquad (12)$$

This clearance will, however, have a component due to

sampling and the actual amount cleared in terms of the inflow concentration will be given by

$$Cl = \frac{C_I R_I - C_o(\infty) R_s}{C_{In}(\infty)} \qquad (13)$$

or if expressed in terms of the observed parameters (equation 10)

$$Cl = \frac{(C_I R_I - C_o(\infty) R_s)(R_p + R_I)}{C_o(\infty) R_p + C_I R_I} \qquad (14)$$

(Equations 11 and 14 are merely alternative forms of expression).

From the practical view point it can be seen from Table (2:3) that clearances calculated in this way using the observed outflow concentration after 2½ hours are good approximations to the more accurately derived clearance values, using extrapolated values of $C_o(\infty)$.

Our present modification of this technique involves infusing at a fast rate during the first half hour of the experiment to attain near equilibrium concentrations for a subsequent slower infusion rate. Thus the method of approximation has been applied to most of the data.

RESULTS

CONCENTRATION OF ENDOGENOUS ACIDS DURING PERFUSION

Normal concentrations for the acids 5-HIAA, HVA and DOPAC in the C.S.F. of Beagle hound have been established,

Section II - corrected to 100% by average % recovery of pure substance through the analytical procedure.

Mean Values	5-HIAA	HVA	DOPAC
Ventricle	[1]0.258 ± 0.095(6)	1.577±0.336(6)	[2]0.237± 0.062(27)
Cisterna	0.039 ± 0027(6)	0.076±0.245(7)	≤0.05

[1]mean ± standard deviation, µg/ml (number of estimates).
([2]Guldberg 1967).

The concentrations of these acids which were found in the outflow of the perfusion system when no infusion was in progress are shown in Table 1:3.

Table 1:3

	5-HIAA	HVA	DOPAC
Dog 1	[1]0.118± 0.020(5)	0.278±0.032(4)	0.232± 0.068(5)
Dog 2	0.110± 0.027(5)	0.365±0.035(5)	0.150± 0.027(5)
Dog 3	0.135(2)	0.251(2)	

[1]mean ± standard deviation, µg/ml., (number of estimates).

ELIMINATION OF 5-HYDROXYINDOLE-3-ACETIC ACID

The clearance of 5-HIAA was calculated similarly to the approximate values derived for inulin clearance. First it is necessary to subtract from the observed outflow concentration of 5-HIAA the concentration which is due to the addition of endogenous 5-HIAA (Table 1:3). Even this approximate correction is only of significance when very small amounts of 5-HIAA are infused. From the corrected outflow 'equilibrium' concentration a clearance value may be derived by equation 14.

TABLE 2:3

Perfusion Experiments with [5]Dog 1 Using Various Rates of Infusion of Inulin and 5-HIAA

Substance Infused	Rate of Infusion R_I (ml/min.)	Rate of Perfusion R_P (ml/min.)	Concentration of Infusion C_I (ug/ml)	Concentration of Outflow (Approx.) $Co(\infty)$ (ug/ml)	Clearance Cl (ml/min. of Inflow Fluid)	Concentration of Outflow (Extrapolated) $Co(\infty)$ (ug/ml)	Clearance Cl using $Co(\infty)'$	Bulk CSF Absorption [4] A (ml/min.)	Bulk CSF Formation [4] F (ml/min.)
Inulin	0.0118	0.33	479	68	0.055	78	0.047	0.056	0.061
5-HIAA			11.0	0.515	0.138				
Inulin [2]	0.0152	0.33	737	112	0.067			0.083	0.085
5-HIAA			18.2	1.36	0.121				
Inulin	0.0163	0.33	812	151	0.059	151	0.059	0.071	0.072
5-HIAA			37.1	2.45	0.139				
Inulin	0.0075	0.33	960	60[1]	0.077 [1]			0.103 [1]	0.112 [1]
5-HIAA			229	7.17	0.132				
Inulin	0.0156	0.33	1000	221	0.047	221	0.047	0.054	0.055
5-HIAA			223	26.71	0.086				
Inulin	0.0146	0.33	3850	749[3]	0.051	800	0.046	0.054	0.056
5-HIAA			894	108	0.080				
Inulin [2]	0.0152	0.33	620	104	0.061			0.074	0.076
Inulin	0.0124	0.33	780	103	0.062	133	0.048	0.056	0.060

[1] Initial experiment in which the concentrations of inulin were really too low for the assay method employed.

[2] Experiments in which a loaded circuit was employed in an attempt to reach equilibrium values with a short perfusion.

[3] The figure quoted is at 3½ hours. At 2½ hours - the time used for experiments other than[2] - the concentration in the outflow was 678 µg/ml which if used as $Co(\infty)$ gives a clearance of 0.055 ml of inflow fluid/min.

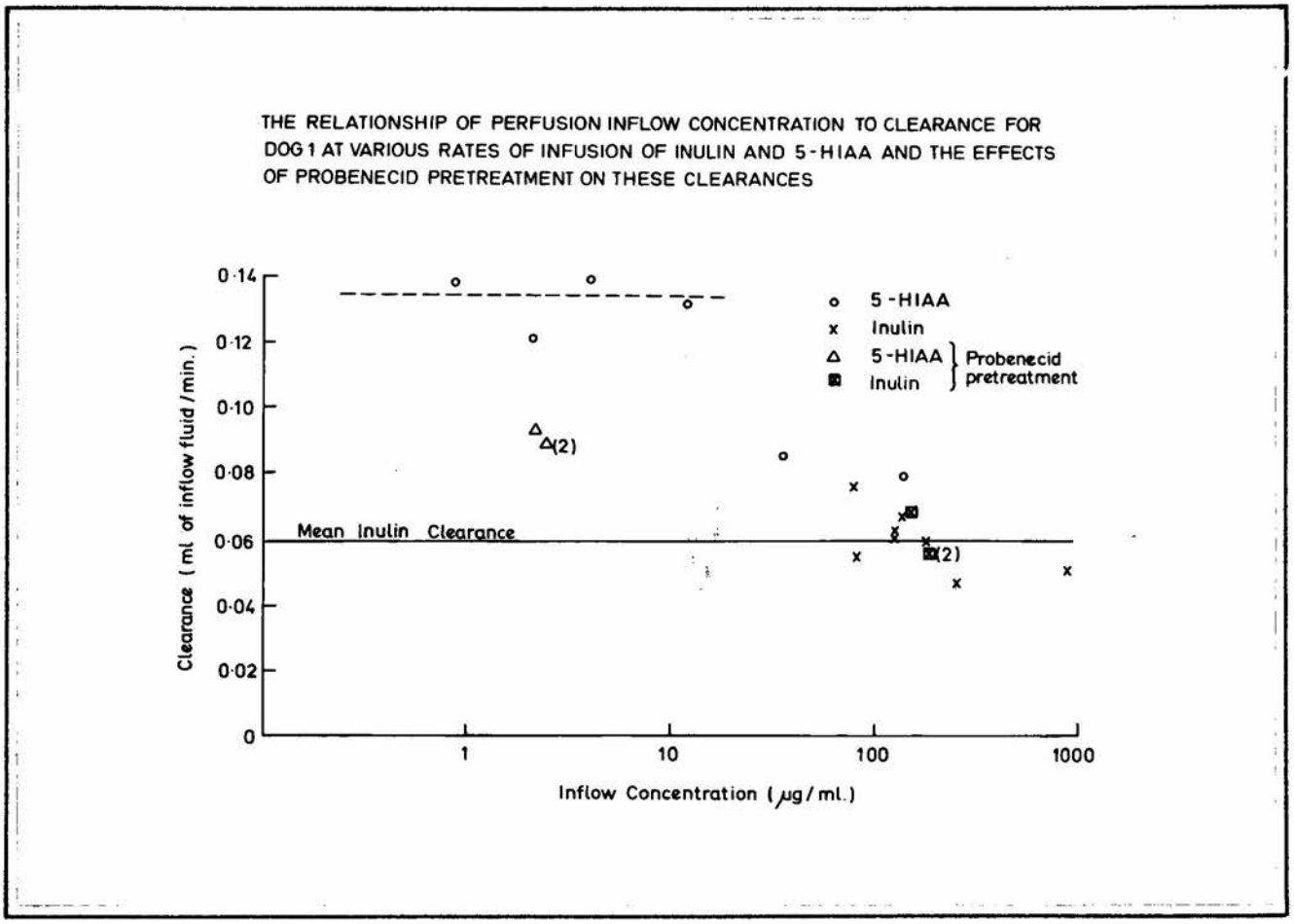
[4] Bulk Absorption and Formation have been calculated using $Co(\infty)'$ (extrapolated value) where these are available.

[5] Dog 1 was a male Beagle - 23 lb. weight.

CLEARANCES OF INULIN AND 5-HYDROXYINDOL -3-YLACETIC ACID FROM C.S.F.

A series of experiments was performed on Dog 1 infusing different concentrations of inulin and 5-HIAA (Table 2:3). These results show that when inulin was infused it was cleared at a rate of approximately 0.06 ml. of inflow fluid/min. regardless of the rate of infusion (C_I R_I). Table 2:3 also shows that the equilibrium outflow concentrations $C_o(\infty)$ tend to agree fairly closely, whether the value estimated after 2.5 hours is used as an approximation to $C_o(\infty)$ or the theoretically more satisfactory method of extrapolating the value of $C_o(\infty)$, by fitting all the data obtained during the experiment to the best straight line log plot, is used. The method of extrapolation gives slightly higher values for $C_o(\infty)$ and correspondingly lower values for clearance. The values for C.S.F. bulk absorption and formation have been derived from the values of the extrapolated $C_o(\infty)$ by the method described on page 98. Where extrapolated values are not calculable, because of the use of a "load infusion method", approximated values have been used for these calculations. The values derived from extrapolated $C_o(\infty)$ tend to be slightly lower and are probably more accurate. However, as

Fig. 1413



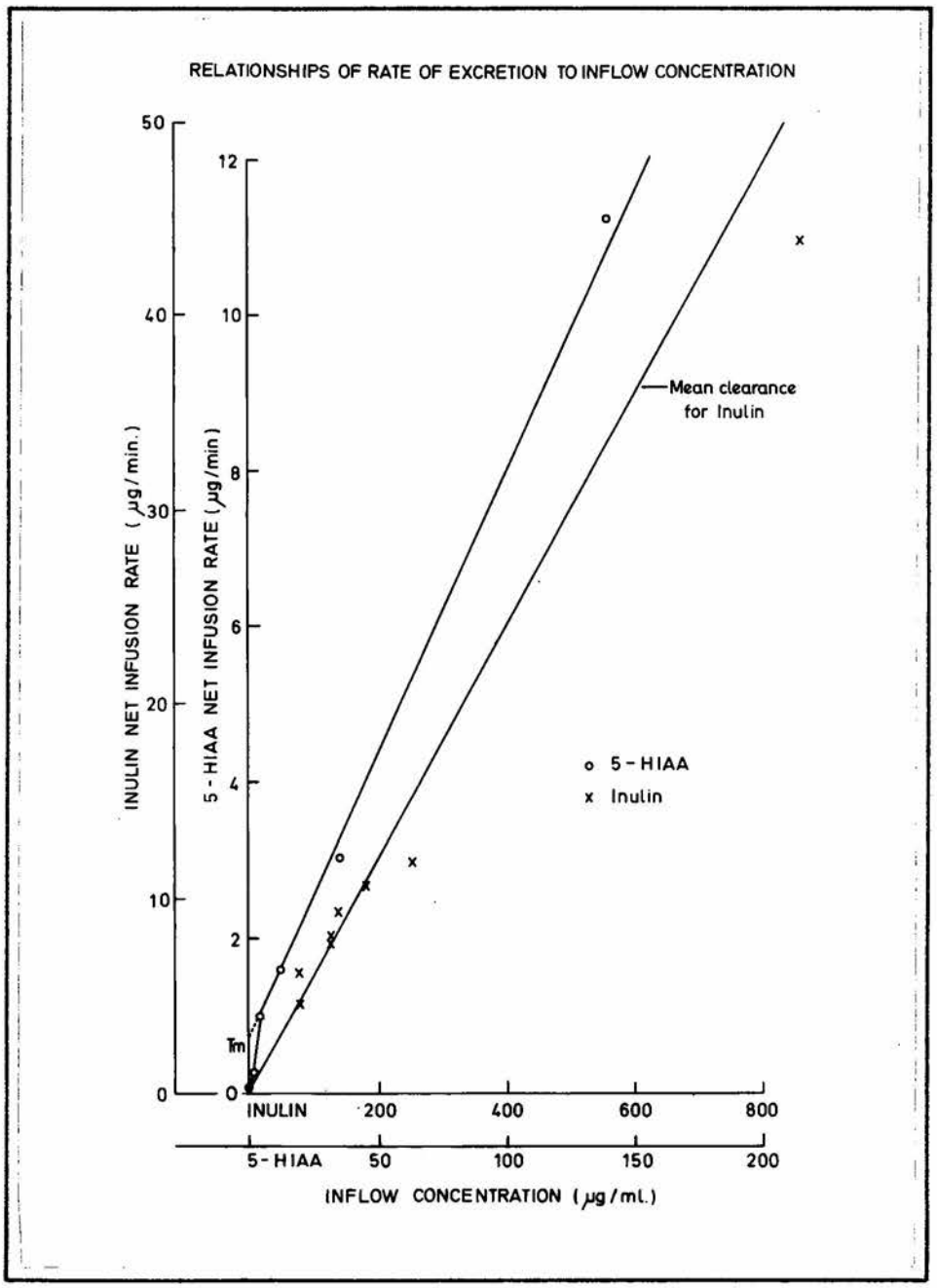
Footnote:- The figure demonstrates the relationship between clearance and inflow concentration which for inulin is fixed independent of inflow concentration while the values for 5HIAA indicate that one form of its clearance must be saturated at high inflow concentrations and inhibited by pretreatment with probenecid.

the discrepancy between approximation and extrapolation is slight, and not all the experiments performed are suitable for deriving $C_o(\infty)$ by extrapolation, approximated values of $C_o(\infty)$ have been used in all the graphical explanations and text of these experiments and in all the data reported subsequently. This consistency allows valid intercomparison to be made between most experiments.

The method of approximation for $C_o(\infty)$ when applied to 5-HIAA has even greater validity than for inulin, as experimentally the outflow levels usually plateau within the 2.5 hours of the perfusion and the best estimate of $C_o(\infty)$ may be given by the average of the concentrations of the last two or three samples obtained. The rapid achievement of plateau levels is probably due to the mechanisms other than bulk flow which exist for the removal of 5-HIAA (see following discussion).

Table 2:3 also shows that 5-HIAA is cleared from C.S.F. at slightly more than double the inulin clearance when infused at low rates, but at values approximating to the inulin clearance when infused at high rates. Such self-inhibition by the administration of large amounts of a substance is characteristic of the saturation

Fig. 15:3



The figures graphed are all derived from experiments on dog (table 3:1). Net infusion rate (µg/min.) given by ($C_I R_I - C_o(\infty) R_s$) is for equilibrium states equal to the total efflux of substance removed by the dog. Inflow concentration at "equilibrium" (ug/ml) calculated by equation (10) (page 100). (Approximated value of $C_o(\infty)$ used for all plotted values.)

Footnote:- As the scale for inulin is one quarter that of 5-HIAA along both axes the angular inter-relationships are still valid and clearance relationships are given geometrically in the above graph by, abcissa distance/30 x ordinate distance for both Inulin and 5-HIAA.

of an active transport system for the substance, thus the net clearance rates fall towards those due to the passive modes of transport namely diffusion and bulk flow. The conventional renal graphical plot of the data in Table 2:3 (Clearance against Inflow Concentration) well illustrates the self inhibition of transport by high rates of 5-HIAA infusion (Fig. 14:3).

If the data in Table 2:3 is graphically plotted as the Net Rate of Infusion ($C_I R_I - C_o(\infty) R_s$) against the Inflow Concentration (C_{In}) (Fig. 15:3), this gives a graph in which the clearance of any value is given as the tangent of the angle between the abscissa and the line joining the value to the origin allowing for scale differences per µg. The 5-HIAA values plotted in this manner show that even after saturation of the active transport component lines drawn for the efflux coefficients of inulin and 5-HIAA do not become parallel but continue to diverge. This indicates that a significant part of 5-HIAA efflux occurs by diffusion.

The quantitative measurements for the efflux of 5-HIAA by active transport and diffusion are perhaps better illustrated if these components themselves are graphed against inflow concentration for each experiment in Table 2:3 (Fig. 15:3). A graphical estimate made

The values plotted are all derived from the results of experiments on dog 1 (table 2:3). (Approximated values of $C_o(\infty)$ were used in calculating all the values plotted) 5HIAA inflow concentration was calculated by equation (10) (page 100). 5HIAA efflux due to diffusion and active transport is derived for each experiment in table 2:3 as the rate of removal of 5HIAA minus the rate of removal of 5HIA by bulk flow and sampling, i.e. $CI\ RI - C_o(\infty)\ (A + R_B)$

Footnote:- The graph indicates two clearance mechanisms for 5HIAA apart from bulk flow. One mechanism can be saturated - transport maximum (T_m) = 0.55 ug/min. - while the other mechanism shows no saturation and has a clearance coefficient of 0.0177 ml/min. (given by the slope of the line D) greater than the approximated inulin clearance.

Fig. 16:3

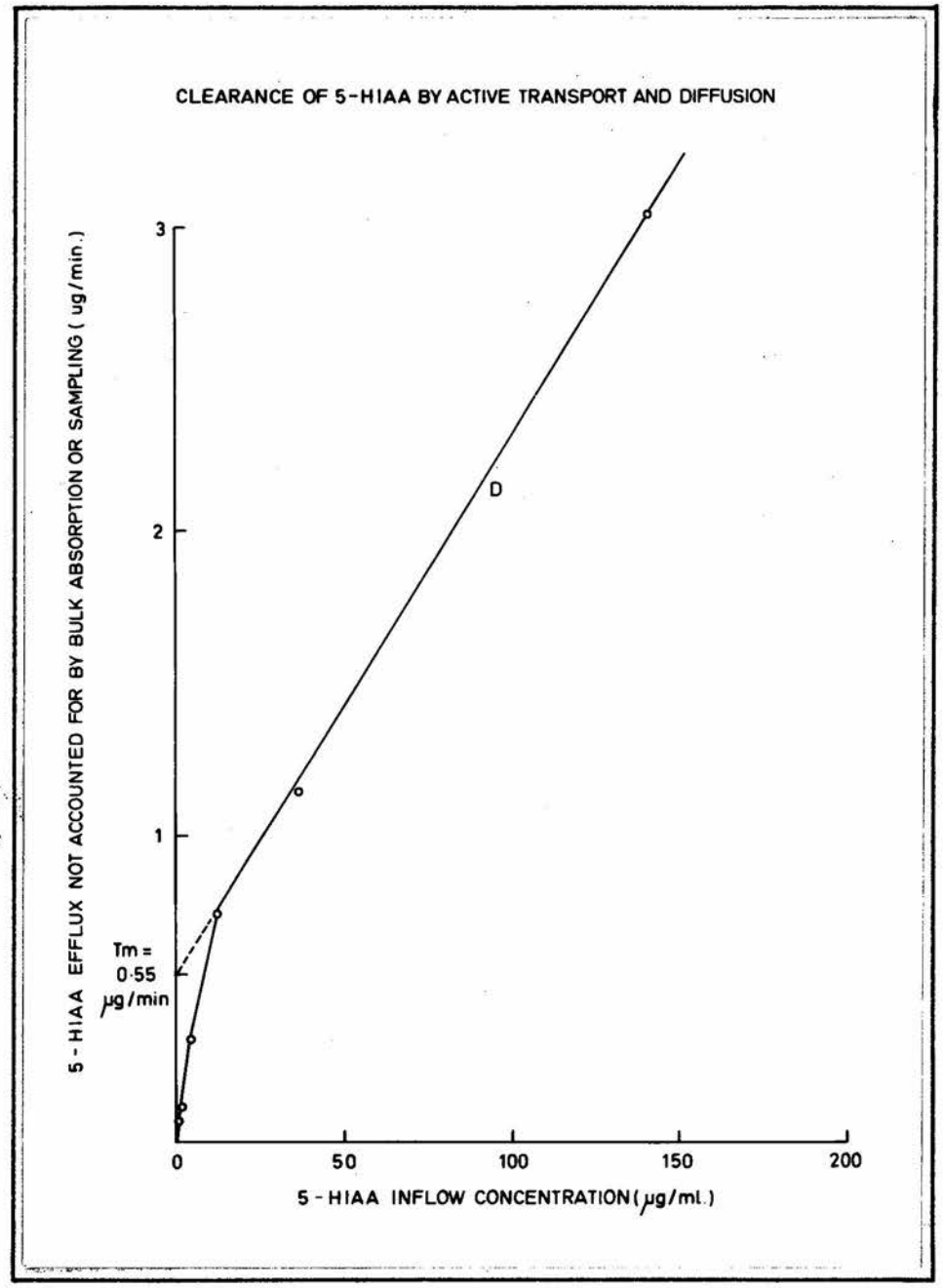

CLEARANCE OF 5-HIAA BY ACTIVE TRANSPORT AND DIFFUSION
5 - HIAA EFFLUX NOT ACCOUNTED FOR BY BULK ABSORPTION OR SAMPLING (ug/min.)
3
2
1
D
Tm = 0·55 µg/min
0
50
100
150
200
5 - HIAA INFLOW CONCENTRATION (µg/ml.)

TABLE 3:3

Clearance of 5-HIAA at Low and High Rates of Infusion in Comparison with Simultaneously Determined Inulin Clearances

Dog	Substance Infused	Rate of Infusion R_I (ml/min)	Rate of Perfusion R_p (ml/min)	Concentration of Infusion C_I (ug/ml)	Concentration of Outflow (Approx.) $C_o(\infty)$ (ug/ml)	Clearance Cl (ml of inflow fluid/min)
2	Inulin	0.0128	0.33	718	123	0.049
	5-HIAA			20.5	0.951	0.146
24 lb	Inulin	0.0165	0.33	1100	242	0.050
	5-HIAA			794	91.4	0.093
3	Inulin	0.0132	0.33	720	123	0.051
	5-HIAA			13.8	0.520	0.168
23 lb	Inulin	0.0133	0.33	916	162	0.050
	5-HIAA			651	79.1	0.073

All perfusions carried on for 2.5 hours and $C_o(\infty)$ (Approx.) taken as the value of the concentration in the last sample.

in this way (Fig. 16:3) indicates that the transport maximum for the active transport of 5-HIAA from C.S.F. in Dog 1 is of the order of 0.55 µg/min. The diffusion component for the efflux of 5-HIAA from C.S.F. in this dog was estimated as having an efflux coefficient of 0.0177 ml. in excess of the inulin clearance. While the estimate of the total component of clearance of 5-HIAA by passive means should be reasonably accurate, as mentioned before (page 20θ, the approximated inulin clearances used here are slightly too large, thus the proportion of efflux occurring by diffusion will tend to be under-estimated in the results presented here.

Table 3:3 shows the results from two dogs which had 5-HIAA administered at both high and low rates of infusion. These results are in agreement with the conclusions arrived at from the results of the larger series of experiments performed on Dog 1.

CLEARANCE OF HOMOVANILLIC ACID FROM CEREBROSPINAL FLUID

In a series of experiments performed on different dogs (Dogs 4-7), HVA was infused at both high and low rates. Concurrent inulin studies were also performed. These results (Table 4:3) show that while at low rates of infusion HVA was cleared at slightly more than three times the inulin clearance, when administered at a high

TABLE 4:3

Clearance of HVA at Low and High Rates of Infusion in Comparison with Simultaneously Determined Inulin Clearances

Dog	Substance Infused	Rate of Infusion R_I (ml/min.)	Rate of Perfusion R_P (ml/min.)	Concentration of Infusion C_I (ug/ml)	Concentration of Outflow (Approx.) $C_o(\infty)$ (ug/ml)	Clearance Cl (ml of inflow fluid/min.)	Bulk CSF Absorption A (ml/min.)	Bulk CSF Formation (ml/min.)
4 [1] 31 lb.	Inulin	0.00562	0.325	4740	425	0.039	0.046	0.057
	HVA			215	5.6	0.121		
5 [2] 22 lb.	Inulin	0.0110	0.33	852	121	0.051	0.061	0.066
	HVA			351	9.31	0.183		
6 [1] 31 lb.	Inulin	0.0091	0.33	4160	494	0.050	0.060	0.068
	HVA			1043	56	0.104		
7 [1] 25 lb.	Inulin	0.0073	0.30	5460	465	0.055	0.069	0.078
	HVA			1660	65.5	0.108		

[1] Initial fast rate of infusion to load circuit - total perfusion time 2 hours

[2] Constant rate of infusion - perfusion time 2.5 hours

rate the clearance fell to approximately double that of inulin. Thus, from the self inhibition exhibited by HVA in these experiments, it would seem that the efflux of HVA from C.S.F. also has important components due to active transport and probably - as the clearance still remains high at high rates of infusion - diffusion.

INHIBITION OF THE ACTIVE TRANSPORT OF ONE ACID BY ANOTHER ACID

The samples from the experiment on Dog 7 (Table 4:3) when HVA was infused at a high rate, showed an increase in 5-HIAA content 0.142 µg/ml., a value in excess of those normally expected (Table 1:3). Also, in an experiment where the first period had involved recirculation perfusion alone and the second period had included an infusion of a 9 mg/ml. solution of HVA at the rate of 0.015 ml/min., the mean 5-HIAA concentration for the first period was 0.108 µg/ml., and for the second period 0.126 µg/ml. The specifity of the assay for 5-HIAA is such that even the presence of very large amounts of HVA does not alter the estimates.

During experiments in which high concentrations of 5-HIAA were infused the estimates of HVA in the C.S.F. outflow were raised above the levels found in experiments where low concentrations of 5-HIAA were infused. However,

it was found that very high concentrations of 5-HIAA interfere with the estimation of HVA by giving a non-specific peak at the assay wavelength.

As part of the series of experiments to elucidate the action of phenelzine in dogs - Section II - perfusions were performed on dogs pretreated with phenelzine and with the infusion of the main acid metabolite of phenelzine, phenylacetic acid (PAA). In both cases the active transport mechanism concerned with the removal of 5-HIAA and HVA appeared to have been inhibited. These results are discussed in more detail in Section II, Ventricular Perfusion Experiments, page 146.

The general conclusion from this series of experiments is that 5-HIAA, HVA and PAA may all be transported from C.S.F. by the same active carrier mechanism and that if any one of those acids is present in sufficient quantity then the active transport of the other acids using the carrier system will be inhibited.

Clearance of Inulin, 5-hydroxyindole-3-acetic acid and homovanillic acid from cerebrospinal fluid

Inhibition of Active Transport with Probenecid

Pretreatment - The two dogs used weighed 23 and 24 lbs. and were both given 0.5 g of probenecid P.O. at 1 p.m., 5 p.m. and 9 a.m. for three days and perfusion

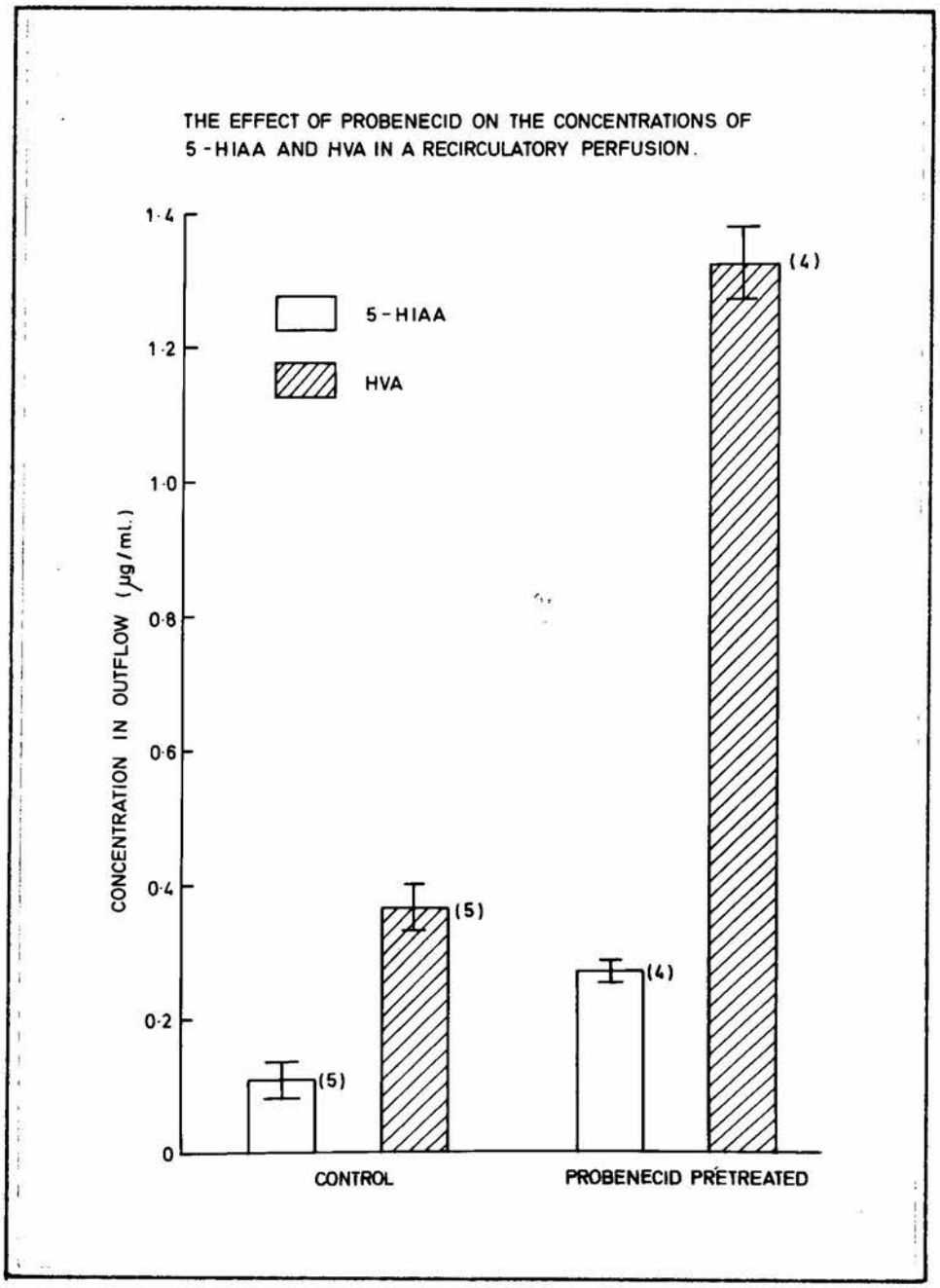
TABLE 5:3

The Effect of Probenecid Pretreatment on the Clearance at Low Rates of Infusion of 5-HIAA

Dog	Pretreatment	Substance Infused	Rate of Infusion R_I (ml/min.)	Rate of Perfusion R_P (ml/min.)	Concentration of Infusion C_I (ug/ml)	[1]Concentration of Outflow (Approx.) $C_o(\infty)$ ug/ml)	Clearance (ml of inflow fluid/min.)
1	Control	Inulin	0.0152	0.33	737	112	0.067
		5-HIAA			18.2	1.36	0.121
23 lb.	Probenecid	Inulin	0.0114	0.33	1064	118	0.068
		5-HIAA			20.3	1.57	0.093
2	Control	Inulin	0.0128	0.33	718	123	0.049
		5-HIAA			20.5	0.951	0.146
24 lb.	Probenecid	Inulin	0.0124	0.33	1064	155	0.056
		5-HIAA			20.3	1.82	0.089

[1] Constant rate of infusion - perfusion time 2.5 hours

Fig. 17:3



Concentrations of 5-HIAA and HVA in C.S.F. from the outflow of recirculatory perfusions performed on dog 2 with and without probenecid pretreatment (page215).

was started two hours after the last tablet.

Experiments Performed - Dog 2 was perfused without infusion and the equilibrium levels found are shown in Fig. 17:3. The concentrations found for 5-HIAA are 2.4 times its control level and for HVA 3.6 times control. This finding could be due to either an increased addition of these acids to C.S.F. or a decreased removal from it.

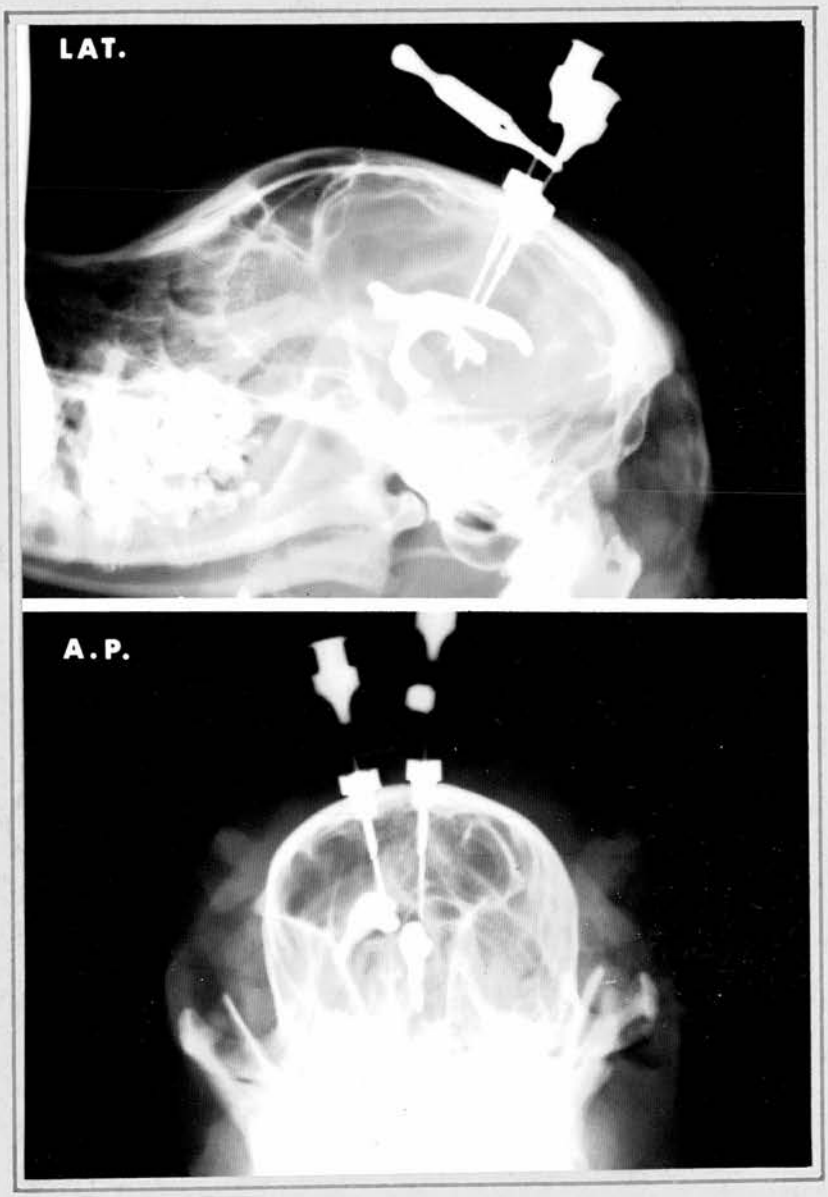
On a subsequent occasion dogs 1 and 2 pretreated as above were perfused while infusing small amounts of 5-HIAA, (Table 5:3). The results graphed on Fig. 14:3 show that while there was no change in inulin clearance the clearance of 5-HIAA fell markedly, thus indicating a block in the active transport component. This is the result one would anticipate, as probenecid has long been known to block the active transport of organic acids by kidney.

LOCALIZATION OF SITE OF ACTIVE TRANSPORT

By a modification of the method used to insert needles into the lateral ventricles, a cannula was inserted into the skull so that the third ventricle could be punctured. The success of this technically difficult operation was checked by X-ray (Fig. 18:3). Three weeks later concurrent 0.5 ml. samples were withdrawn from the lateral and from the third ventricle and then 2 ml. from

Fig. 18:3

X-Rays Showing the Position of a Needle in the Third Ventricle



X-rays showing the outline of the ventricular system in dog 8 after the injection of Myodil into the right lateral ventricle and illustrating that a needle (marked with a clip) can be inserted into the third ventricle.

TABLE 6:3

Clearances of Inulin and 5-HIAA at Low Rates of Infusion of 5-HIAA from Localised Perfusion of the Ventricular System

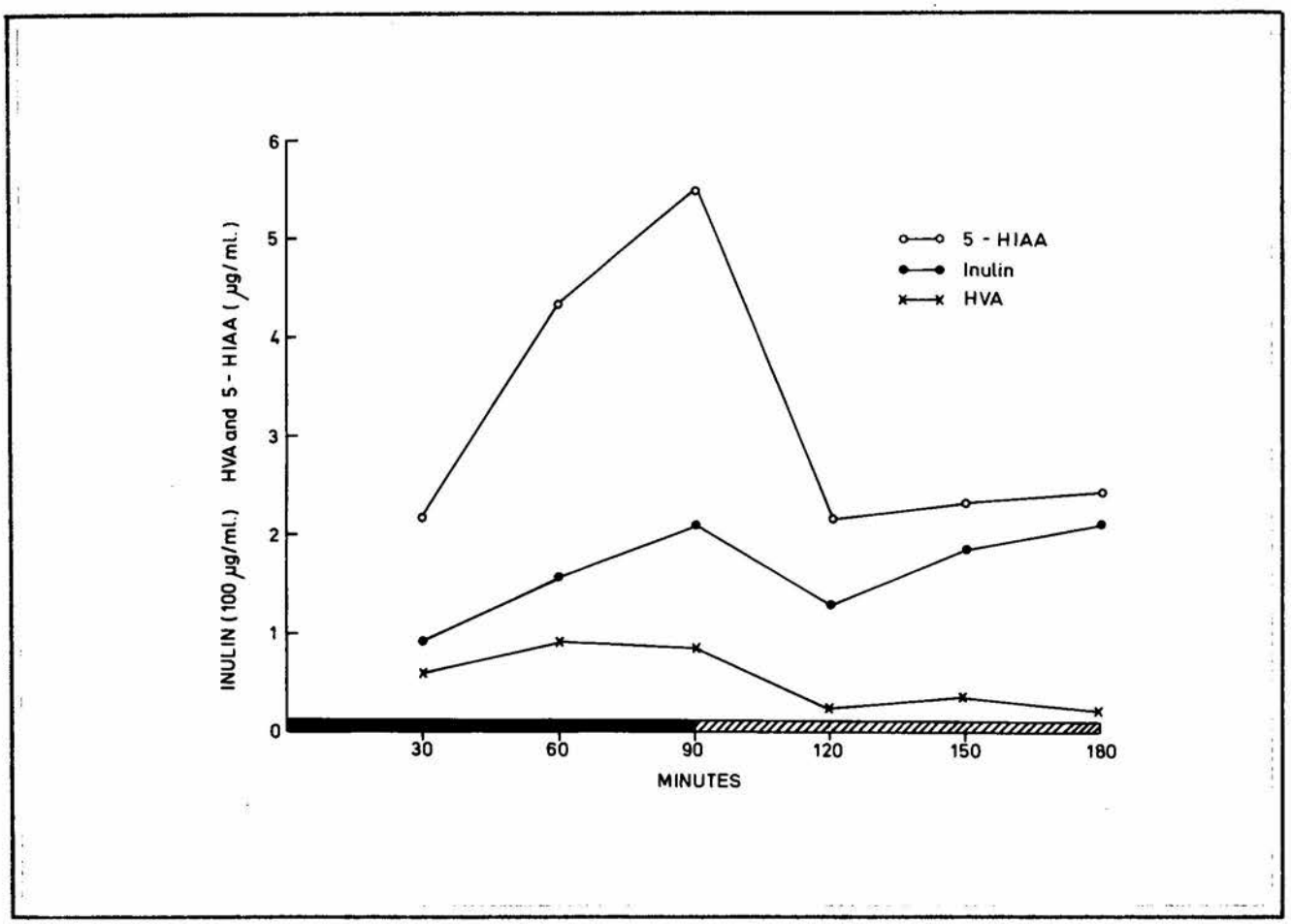
[1] Experiment	Dog	Rate of Infusion R_I (ml/min.)	Rate of Perfusion R_P (ml/min.)	Concentration of Infusion C_I (ug/ml)	Concentration of Outflow (Approx.) $C_o(\infty)$ (ug/ml)	Clearance Cl[2] (ml of inflow fluid/min.)	Bulk CSF[2] Absorption A (ml/min.)	Bulk CSF[2] Formation F (ml/min.)	Substance Infused
A	8, 0+ 12 lb.	0.0167	0.36	740	210	0.038	0.042	0.042	Inulin
				13.32		0.036			5-HIAA
B		0.0167	0.3[illegible]	740	210	0.038	0.042	0.042	Inulin
				.32		0.899			5-HIAA
C	5, 0+ 22 lb.	0.0117	0.33	384	66	0.044	0.051	0.056	Inulin
				[illegible].3	1.69	0.101			5-HIAA
D		0.0117	0.33	[illegible]	116	0.021	0.022	0.027	Inulin
				[illegible]	4.86	0.035			5-HIAA

[1] Experiments - A Perfusion for 90 mins. from lateral ventricle to third ventricle
B Perfusion for 90 mins. from third ventricle to cisterna magna
C Perfusion for 90 mins. from lateral ventricle to cisterna magna
D Perfusion for 90 mins. from lateral ventricle to opposite lateral ventricle

[2] Clearances, Bulk Formation and Absorption of CSF have been calculated from the 90 min. outflow concentration and as Fig. 193 shows they will only be rough approximations of the true values.

Fig. 19:3

Localised Ventricular Perfusion



Initial 90 min. of perfusion was carried out from lateral ventricle to third ventricle, the following 90 min. was carried out from third ventricle to cisterna magna.

The Figure shows the concentrations of 5-hydroxyindol-3-ylacetic acid (5-HIAA), 3-methoxy-4-hydroxyphenylacetic acid and inulin in the cisternal outflow during the infusion of a solution of inulin and a low rate of infusion of 5-HIAA.

the cisterna. An assay for 5-HIAA gave the following concentrations (µg/ml),

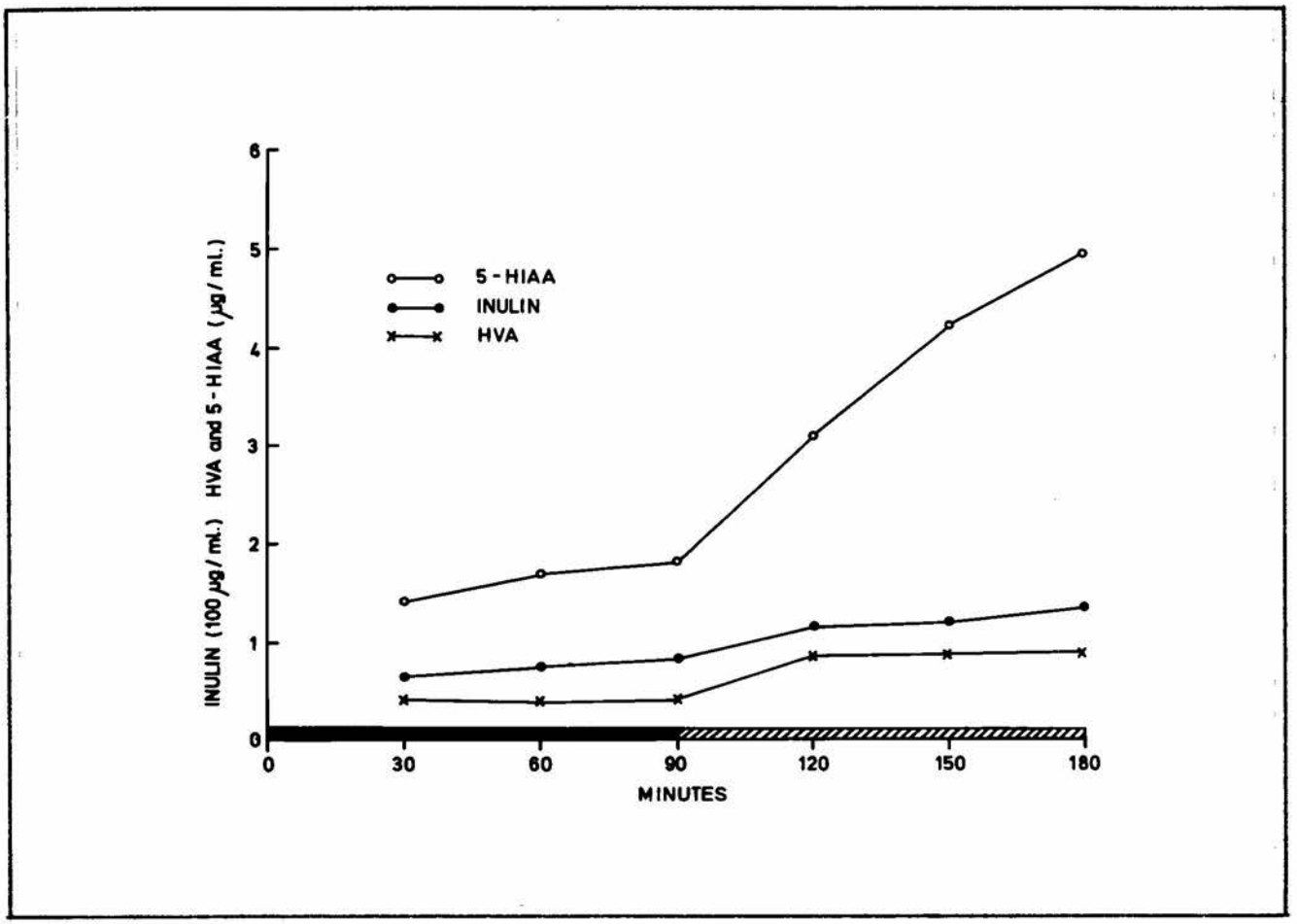
Lateral Ventricular C.S.F.	0.231
3rd Ventricular C.S.F.	0.313
Cisternal C.S.F.	0.040

The high value in the sample from the third ventricle may relate to its proximity to the hypothalamus which has high concentrations of 5-HIAA and 5-HT.

While maintaining continuous infusion of a low concentration of 5-HIAA dog 8 was perfused for ninety minutes from right ventricle to the hind part of the third ventricle and then for a further ninety minutes from the third ventricle to cisterna (Fig. 19:3). Taking the ninety minute values as approximate equilibrium values, clearances may be calculated for these localised perfusions (Table 6:3). Although approximate, these values clearly show that the portion of the C.S.F. system from third ventricle to cisterna is concerned with removal of 5-HIAA and HVA. The clearance of inulin, however, appears fairly uniform, indicating that the lateral and third ventricles may be responsible for the formation of almost the entire bulk of the C.S.F. produced within the ventriculo-cisternal system, as inulin clearance will be caused by the displacement of perfusing fluid from the system by newly formed C.S.F.

Fig. 20:3

Localised Ventricular Perfusion



Initial 90 min. of perfusion was carried out from lateral ventricle to cisterna magna. The following 90 min. was carried out from lateral ventricle to the opposite lateral ventricle.

The Figure shows the concentrations of 5-hydroxyindol-3-ylacetic acid (5-HIAA), 3-methoxy-4-hydroxyphenylacetic acid and inulin in the cisternal outflow during the infusion of a solution of inulin and a low rate of infusion of 5-HIAA.

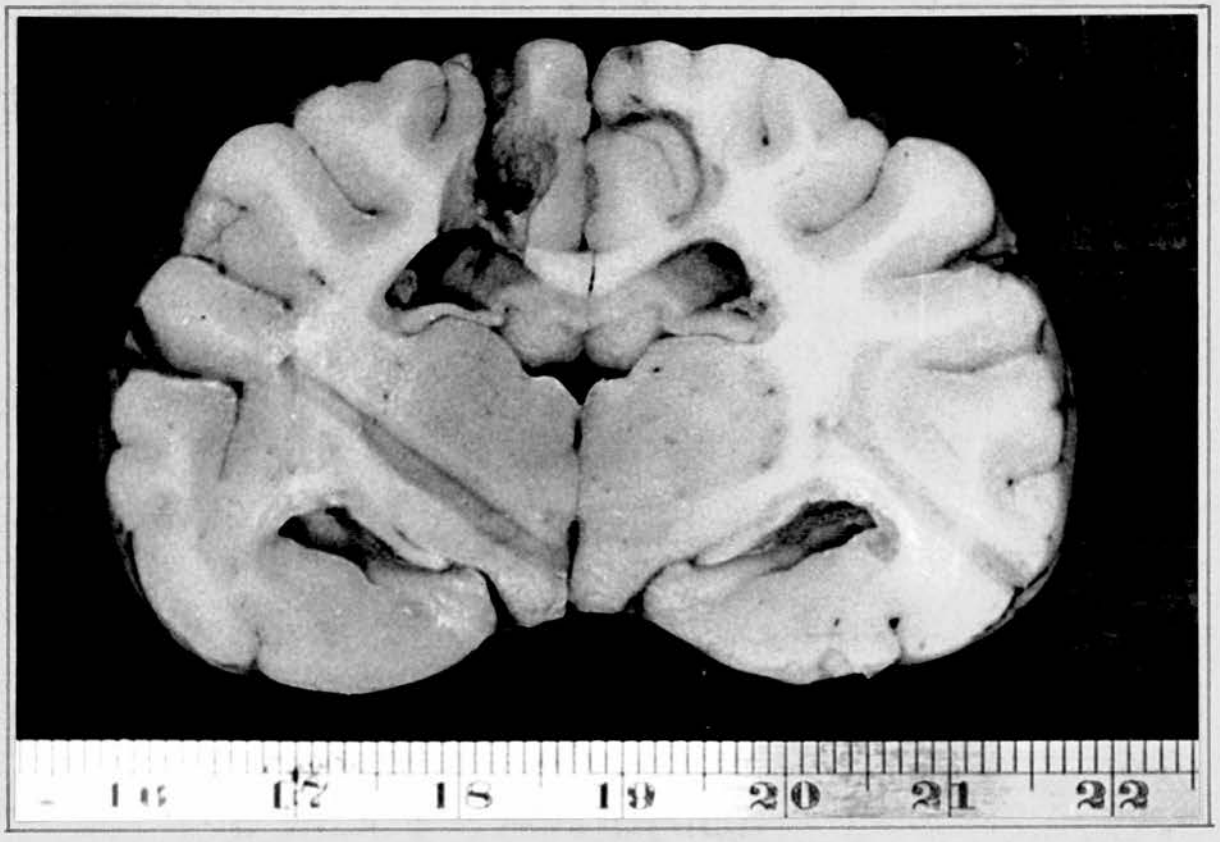
While maintaining an infusion of a similar low concentration of 5-HIAA dog 5 was perfused for the first ninety minutes in the normal way from right ventricle to cisterna and then for a further ninety minutes from right lateral ventricle to left lateral ventricle (Table 6:3). It is obvious from the marked rise in the concentrations of both HVA and 5-HIAA (Fig. 20:3) that their efflux is now impaired. This is also demonstrated by the finding that the divergence between 5-HIAA clearance and inulin clearance has become less (Table 6:3), and from Fig. 20:3 it can be seen that the 5-HIAA concentrations are still rising steeply and it is probable that the clearance calculated for 5-HIAA from the true outflow concentration at infinity would be in even closer agreement with the inulin clearance. What is rather surprising is the marked rise in inulin concentration (Fig. 20:3). This rather implies, in view of the result of the last experiment, that a comparatively large amount of the bulk of C.S.F. is formed from the choroid plexus in the third ventricle.

PRELIMINARY SUMMARY AND

From the extrapolation of the graph of the relationship of inflow concentration to clearance of 5-HIAA (Fig. 14:3) it is apparent that, in the absence of any

Fig. 21:3

Coronal Section of Dog Brain



This photograph illustrates the dissected tract made by the guide tube to the body of the lateral ventricle.

infused 5-HIAA, the endogenous 5-HIAA in dog 1 will be cleared at approximately 0.135 ml/min. The results derived from recirculation without infusion can now be inserted into a modified form of equation (13) as inflow and outflow concentrations are now equal.

$$X = (Cl_{(5\text{-HIAA})} + Is)\, C_o(\infty) \qquad (15)$$

where X is the quantity of 5-HIAA received from brain in unit time.

The values from Table 1:3 show that dog 1 will have 0.018 µg/min. added to the ventriculo-cisternal space perfused and similarly from Table 1:3 and 3:3 it can be seen that similar values will apply to the other dogs studied.

Preliminary Studies of Ventricular Perfusion System Using Radio Isotopes

This study of the recirculatory perfusion system in the dog has been directed to defining the system sufficiently to enable it to be used in monitoring cerebral metabolism in vivo.

There were several outstanding points about the relationship of cerebral 5-hydroxyindole metabolism to the perfusing system which were more satisfactorily resolved using radio-isotope techniques.

TABLE 7:3

Transfer of 5HIAA From Plasma to CSF in Dog 1

Time	Cisternal CSF		Ventricular CSF		Plasma
(min.)	cpm of Sample	muc/ml	cpm of Sample	muc/ml	muc/ml
0	48	ND	50	ND	ND
30	47	ND	51	ND	2.02
60	59	0.031	50	ND	3.04
90	62	0.042	52	0.007	2.30

ND indicates that the number of counts per minute was not significantly different from the average background count of 50 cpm

[illegible] OF ^{14}C-5-HYDROXYINDOLE-3-YLACETIC ACID FROM PLASMA AND ITS TRANSFER FROM PLASMA TO CEREBROSPINAL FLUID.

Dog 1 was infused intravenously while conscious at the rate of 0.0274 ml/min. with a solution of ^{14}C-5-HIAA 1.02 mg/ml., 10 µc/ml. Samples of C.S.F., each 0.2 ml., were withdrawn from a lateral ventricle and the cisterna magna via needles inserted percutaneously into the guide tubes. Concurrent samples of venous blood were also withdrawn. The results are shown in Table 7:3. The actual observed count rates have been shown, because, for the C.S.F. samples, they were hardly above background.

An index of the maximum transfer of 5-HIAA from blood to C.S.F. will be of the order of 100 x 0.042/2.45 = 1.7% (the maximum C.S.F. concentration expressed as a percentage of the average plasma concentration). The actual plasma concentrations in this experiment would be of the order of 0.25 µg/ml. 5-HIAA is not normally detectable in dog plasma and concentrations of 0.25 µg/ml are not even achieved with the systemic administration of large amounts of tryptophan (Sections I and II). Thus the transfer of 5-HIAA from blood to C.S.F. may be neglected in analysing data in subsequent experiments.

As the plasma concentrations of 5-HIAA have reached a plateau it was also possible to calculate the clearance

of 5-HIAA from plasma. Thus the rate of excretion by the kidneys must equal the rate of infusion and clearance will be given by 0.0274 x 10,000/2.45 = 112 ml/min. Kelly and McDonald (1948) quoted mean glomerular filtration rates (G.F.R.) for three conscious female mongrel dogs with weights of 19, 23 and 19 lbs., of 55, 46 and 42 ml/min. Dog 1 was a male beagle hound weighing 24 lbs and would probably have a similar G.F.R. If dog 1 had a G.F.R. of 52 ml/min then the amount of 5-HIAA cleared by active renal transport would be 60 x 2.45 x 102 mμg/min. i.e. 15 μg/min if the carrier mechanism was fully saturated at this rate of administration or at a greater rate if the carrier system was still unsaturated.

CLEARANCE OF ^{14}C-5-HYDROXY INDOL-3-YL ACETIC ACID FROM CEREBROSPINAL FLUID

It was thought that it was just possible that the "active transport" process which apparently existed for the efflux of HVA and 5-HIAA from brain and which could be inhibited by probenecid and phenylacetic acid could also be explained by a conjugating enzymic reaction. Such an enzymic reaction would give rise to the same results as were observed in the preceding experiments purporting to show clearance from C.S.F. either if the organic acid conjugates could not be extracted from salt

TABLE 8:3

Clearance of ^{14}C 5-HIAA from Cerebrospinal Fluid

Method of Assay	Concentration of Infusion C_I	Concentration of Perfusion Outflow (Approx.) C_o(∞)	Clearance Cl (ml of inflow fluid/min.)
Total Radioactivity (aliquot of C.S.F.) (mμc/ml)	536	35.1	0.099
Ether extractable Radioactivity (mμc/ml)	574	40.7	0.093
5-HIAA Assay by Spectrofluorimeter (μg/ml)	50.16	3.8	0.088
Inulin (μg/ml)	3620	451	0.055

Dog 9 - rate of perfusion (R_P) = 0.33 ml/min.
rate of infusion (R_I) = 0.0104 ml/min.

saturated acidified aqueous solution into ethyl acetate, or if the conjugates did not give the final assay reaction. If such conjugates were formed both possibilities would be likely.

To exclude this possibility a perfusion experiment was carried out in which ^{14}C-5-HIAA was infused (Table 8:3) The results show that the clearance of 5-HIAA from C.S.F. is of the same order whether this is calculated as the clearance of "Total counts per min", of the "Ether extractable counts per min", or of the "5-HIAA fluorescence", thus confirming the opinion that "Clearance" represents physical removal from C.S.F.

DISCUSSION

Parameters Measured by Inulin Estimation

Bulk formation and absorption of C.S.F. have been measured by many workers using the technique of inulin dilution and clearance in an 'open ended' perfusion system and assuming that little of the inulin will be lost by diffusion through normal ventricular ependyma, (Pappenheimer et al. 1961, Heisey et al. 1962, Davson with various co-workers 1962, 1963, 1963, and 1965, Oppelt et al. 1963, Bering and Sato 1963, and Cserr 1965). That such an assumption was valid was demonstrated by Rall,

Oppelt and Patlak (1962) and by Pappenheimer and Heisey (1963). Pollay and Davson 1963 showed the inulin dilution measurement of C.S.F. production in the rabbit was in good agreement with direct measurement.

The mean values for C.S.F. production in the conscious dog presented in this thesis when calculated on the best estimate of the steady state outflow concentration (that derived by extrapolation) give values in good accord with these in the literature for anaesthetised dogs (0.047 ± 0.025, (S.D.) ml/min - Bering and Sato, 1963 and 0.057 ± 0.012 (S.D.) ml/min - Oppelt et al. 1963). The figures given when the steady state outflow concentration is considered to be reached after 2.5 hr. tend, as would be expected, to be slightly larger but these values are of sufficient accuracy to be used routinely.

It is also possible to calculate a value for the maximum space perfused with the system used if this is desired and as is considered in the theoretical section (page 199). This will be a good approximation to the actual space perfused.

The experiments with localised perfusion would suggest that most of the bulk C.S.F. formation takes place in the ventricles but a rather large proportion of this, 30-40%, is formed in the third ventricle. This finding

is completely in accord with the results of Bering and Sato (1963) on the localization of bulk C.S.F. production and is only really surprising (Davson 1967) if one neglects to consider that bulk C.S.F. production is only the net volume produced after local reabsorption, and that production in the lateral ventricles may well be offset by a large surface at which reabsorption can take place.

Active Transport From Cerebrospinal Fluid

In 1961 Pappenheimer et al., demonstrated the active reabsorption of Diodrast and phenolsulphonphthalein (P.S.P.) from a lateral ventricle-cisterna magna perfusion system in the unanaesthetised goat. The criteria used by them in defining active transport were (1) self-saturation of the mechanism, (2) inhibition of Diodrast transport by large amounts of P.S.P. and P.A.H. and (3) transport against a concentration gradient. By stop flow analysis, as was originally applied by Wilde and Malvin 1958 to the localisation of renal secretory activity, Pappenheimer and his co-workers calculated that active transport occurred from the caudal 1.5-2.5 ml. of the ventriculo-cisternal system. They suggested that the choroid plexus of the 4th ventricle or possibly the area postrema might be implicated in this active transport.

Davson, Kleeman and Levin (1962) demonstrated the active transport of P.A.H. from C.S.F. of anaesthetised cats by use of a perfusion system. They showed that the extraction ratio of P.A.H. decreased when large concentrations were perfused and that when high concentrations of P.A.H. were maintained in plasma over a 2 hr. period the maximum concentration of P.A.H. reached in the C.S.F. in that time was only 0.2% of the average plasma concentration. In perfusions from the third ventricle to cisterna the extraction ratios for P.A.H. and inulin were similar; it was concluded therefore that active transport of P.A.H. occurred in the lateral ventricles and was probably carried out by their choroid plexuses.

In 1963 Dolley and Davson demonstrated that iodide, thiocyanate and P.A.H. were all actively removed from a ventriculo-cisternal perfusion system in the anaesthetised rabbit. The same carrier mechanism was postualted for these substances as cross inhibition could be demonstrated. They claimed that active reabsorption of these substances in the rabbit involved all the choroid plexuses. However, they presented no real evidence to prove this point.

These species differences in the localisation of active transport systems for reabsorption from C.S.F. may be important and require further definition. Already

there is evidence to suggest that the efflux of certain metabolites from brain takes place by active transport and that species differences may exist. Perhaps the most relevant references to this discussion are those of Tozer and Neff (1967) who observed that continuous dosage of probenecid blocked the efflux of 5-HIAA from rat brain, and of Ashcroft (personal communication) who noted that low dosage of probenecid would allow systemically administered 5-HIAA to enter rat brain, in contrast to the finding of Berdinius (personal communication) who has apparently been unable to produce any rise in the 5-HIAA concentration of rabbit brain even with toxic doses of probenecid.

Carrier mechanisms have also been implicated in the transport of certain monosaccharides (Bradbury and Davson 1964) and potassium (Bradbury and Davson 1965, Oberr 1965). However the relationships of such mechanisms in the transport between plasma, brain and C.S.F. are of a more complex type due to their inter-relationships with cerebral metabolism and cannot be summarised or discussed briefly.

The present results clearly show that 5-HIAA and HVA are actively removed from C.S.F. Firstly, a full clearance profile of 5-HIAA was constructed in one dog

allowing the maximum capacity of the active transport system and the diffusion constant to be determined for the efflux of 5-HIAA from the perfusion system in this dog. Experiments in other dogs showed that these constants would probably be of the same order in different dogs. The results of experiments with infusions of HVA into the perfusion system demonstrated that HVA, like 5-HIAA, had a component of its efflux which could be saturated.

The efflux of 5-HIAA from C.S.F. was also shown to be inhibited by large amounts of HVA or phenylacetic acid (Section II, page 146).

Probenecid, a drug known to block renal tubular secretion of organic acids and demonstrated to block the active uptake of 5-HIAA by renal cortical slices (Despopoulous and Weisbach, 1957) and its transport out of rat brain in vivo (Tozer and Hefft, 1967) caused the inhibition of a component of 5-HIAA efflux from C.S.F. The raised HVA levels obtained with recirculatory perfusion after probenecid pretreatment (Fig. 17:3) and the decrease in the concentration gradient from lateral ventricle to cisterna (Guldberg, Ashcroft and Crawford 1966) also point to the efflux of HVA from C.S.F. being similarly inhibited by probenecid.

A clearance experiment for 5-HIAA was performed with ^{14}C-5-HIAA to confirm that 'clearance' of 5-HIAA from the C.S.F. was in fact due to removal from C.S.F. and not due to its enzymatic conversion.

With the knowledge of the rate of clearance of 5-HIAA from the perfusion system it became possible to calculate the rate at which endogenous 5-HIAA was being added and thus actually arrive at a figure which represented the turnover of 5-HIAA in part of brain in a conscious animal. The location of the active transport system for 5-HIAA was shown to be in the region between the third ventricle and the cisterna magna and is thus similarly positioned to the site of active transport for Diodrast and P.S.P. in the goat. The most probable site for this active reabsorption is the choroid plexus of the fourth ventricle. The function of the active transport system for these endogenous acids is not known but it may well serve as a protection to the brain in the same way as the 'blood-brain' barrier.

The ultra-structure of the choroid plexus has many features in common with the renal capillary endothelium (Pisloeki and Ladman 1958, Tennyson and Pappas 1961). In vitro the choroid plexus has been shown to be capable of concentrating within its stroma certain organic acid

dyes, (Cameron 1953, Lumsden 1958, Kall and Sheldon 1961), I^{131} (Welch 1962a), thiocyanate (Welch 1962b) and probably certain proteins (Smith, Streicher, Milkovic and Klatzo 1964). It is intended in the near future to study the active uptake of 5-HIAA by the choroid plexus *in vitro*

General Discussion of the Uses of Ventricular-Cisternal Perfusion

The value of such a technique is well demonstrated by the literature summarised in the introduction to this section. It shows that many sophisticated questions about the transport of substances between the blood, brain and C.S.F. and about the production of C.S.F. can be answered by such a technique.

Apart from the work of Pappenheimer and his co-workers 1961, 1962 and 1965, all perfusions have been carried out in anaesthetised animals. This is obviously unsatisfactory as the brain is undoubtably the target organ of anaesthetics and although the actual mechanism of anaesthesia is not understood alterations must occur in at least some, if not all, aspects of cerebral metabolism. Thus it is not really normal cerebral metabolism that is being studied in such experiments.

Indications of the added interaction of anaesthesia on this type of study have already been shown. Bradbury and Davson 1965 while studying plasma C.S.F. potassium interchange noted that the plasma potassium fell steadily after the induction of anaesthesia, this result occurred with several forms of anaesthesia. Pappenheimer et al. (1965), using conscious goats, repeated many of the experiments of Lowsen (1954a and b) who varied the CO_2 tension and bicarbonate concentration in the perfusion of the ventriculo-cisternal system of anaesthetised dogs. While the nature of the responses were the same in both series of experiments the conscious animals were much more sensitive to changes in the perfusate.

The main disadvantage of Pappenheimer and his co-workers' series of experiments lies in their choice of experimental animal - the goat - an animal in which very few physiological studies have been carried out. It is also an animal which few research workers can have facilities to study.

To enable the technique to have wider application and the results obtained from cerebral ventricular perfusions to be viewed in perspective with an existing accumulation of physiological and experimental data,

we chose to develop the technique in dogs. Its use in this context is well demonstrated in Section II of this thesis where its application causes much of the other data to be readily interpretable.

The use of an "open ended" ventriculo-cisternal perfusion system has some advantages over a recirculatory system in that it is simpler to analyse kinetically and steady states are established more quickly.

We chose to study a closed circuit perfusion system because we believe it is, in general, closer to the physiological state, firstly, because donor C.S.F. from another dog may be used to prime the circuit of the system (less than 2 ml.) and secondly, because as it is a closed system and as long as the rate of perfusion is not too fast, the dog itself will regulate the pressure within its ventricular system.

Several other advantages of this technique are perhaps most easily illustrated by a brief description of work currently in progress.

Firstly, it allows the build up of metabolites from cerebral metabolism to approach their normal concentrations in C.S.F. while the efflux of these metabolites from C.S.F. may be monitored by the efflux of infused tracer

amounts of isotopically labelled metabolite. Thus, the observed concentrations of endogenous metabolite serve as a continuous monitor of the metabolic turnover of a region of brain in the conscious animal.

This "metabolite efflux labelling technique" is of value particularly where it is difficult to label specific endogenous pools physiologically without causing the production of a great variety of radio-active metabolites which would then require rigorous separation before individual estimation. Thus this technique is pre-eminently suitable for the study of cerebral 5-HT metabolism under varying experimental conditions since Section I of this thesis makes very apparent the difficulties of labelling the endogenous 5-HT pools physiologically. In conjunction with studies of the above type, however, it is proposed to carry out studies of a more pharmacological type for the purpose of comparison. These involve the infusion of a solution containing radio-isotopes of 5-HT and 5-HIAA, one of these carrying a ^{14}C label and the other ^{3}H. It is then possible to follow the efflux of the 5-HT, its subsequent conversion to 5-HIAA, the entry of 5-HIAA into C.S.F. and its efflux from C.S.F. and to arrive at transfer constants for this two compartment system of brain and C.S.F. Initial experiments of both types

have already been carried out.

An adaptation of the recirculatory ventriculo-perfusion system described above is at present being used in the cat (Eccleston, Roberts and Straughan, personal communication) to load the intraneuronal stores with radioactively labelled 5-HT, to study firstly its metabolism and secondly its release by nerve stimulation. Use is at present being made of the recirculatory perfusion in the dog to study factors affecting the cerebral metabolism of glucose.

Another development of the technique in the dog is the miniaturisation of the external circuit and to this end we have designed a small pump which can be easily carried on the dog's back and will power the perfusion system. Thus it should be possible to study various aspects of cerebral metabolism of an unrestrained animal under different environmental conditions, e.g. awake or asleep. Also perfusion techniques are being developed for conscious perfusion of small laboratory animals.

The use of dogs with chronically implanted guide tubes directed to the ventricles and cisterna is not limited however, to perfusion studies. Such prepared dogs are also useful in experiments which involve repeated sampling of C.S.F. and this can be done not

only in the anaesthetised dog (Guldberg et al. 1966, Guldberg 1967), but also in the conscious animal. In this connection a different degree of response in the pH changes after metabolic and respiratory acidosis and alkalosis has been noted between ventricular and cisternal C.S.F. and is at present being further investigated.

Such dogs may also be used to administer precursors, metabolites or drugs via the C.S.F. into the brain either by injection or perfusion. The active carrier system or systems concerned with transport from C.S.F. such as have been described in this section, may easily be termed "cerebral kidneys" and they await further investigation by more refined renal physiology techniques utilising chronically prepared experimental animals like those used in this study.

SUMMARY

An operation on dogs for the implantation of guide tubes to the lateral ventricle and cisterna magna has been described, also how these animals can be repeatedly perfused ventriculo-cisternally while conscious and unrestrained. The characteristics of a recirculatory

perfusion system have been examined, and calculations for the bulk formation and absorption of C.S.F. and the space perfused have been derived from equilibrium inulin concentrations. It has been found that 5-HIAA and HVA are both removed from C.S.F. by an active transport system localised in the area of the fourth ventricle.

The potential of experimental animals such as those described has been discussed, also some work projected and in progress.

SECTION IV

THE FORMATION OF 5-HYDROXYTRYPTOPHOL IN BRAIN IN VITRO

INTRODUCTION

Udenfer, Iskric and Keglevic (1962) demonstrated '*in vivo*' that rats had the capacity to form 5-hydroxytryptophol (5-HTE) by showing that part of the radioactivity of a dose of tritiated 5-HT could be trapped by a simultaneously administered dose of authentic 5-HTE. They also showed that the glucuronide of 5-HTE was a normal metabolite in rat urine. Bartholini, Pletscher and Bruderer (1964) described the formation of 5-HTE from 5-HT by isolated platelets from rabbits pre-treated with reserpine. Subsequently, the isolation of the alcohol (5-HTE) from bovine pineal gland by McIsaac, Farrell, Taborsky and Taylor (1965) led to the present attempt to synthesise 5-HTE with brain homogenates using 5-HT as a substrate.

Soon after the start of this project, Feldstein and Long (1965) reported the detection of the formation of both 5-hydroxyindol-3-ylacetaldehyde (5-HIAAL) and 5-HTE following the incubation of rat liver homogenates with ^{14}C-5-HT. This confirmed the impression that the metabolism of 5-HT to 5-HTE might be of importance. Their paper also described briefly the method used by them to assay the neutral 5-hydroxyindoles. Much of their method was incorporated with the method of assay for 5-HTE in use at that time to allow a more effective estimation of 5-HTE

and 5-HIAAL.

Because of the changes in methodology which it was necessary to make during this project it has been considered that it would perhaps be more logical to describe the methodological development in its chronological order together with the results, rather than to write a separate section dealing with the methodology in isolation.

5-HIE and 5-HIAAL in 3N HCl give the specific 5-hydroxyindole fluorescence with activation/emission maxima at 295-300/550 mu. As no reliable estimate could be made for the molar fluorescence of 5-HIA L in 3N HCl all the 5-hydroxyindoles have been expressed in terms of 5-HT, in moles, equivalent to the relative fluorescence intensity of the samples containing the 5-hydroxyindolic substance. The molar specific fluorescence relative to 5-HT is approximately 74% for 5-HIAA and 38% for 5-HIE, the precise figure varying slightly with concentration.

DRUGS, CHEMICALS AND REAGENTS

Disulfiram - Tetraethylthiuram disulphide
(pure substance - Gideon Richter)

Nicotinamide adenine dinucleotide (NAD)
(purity 98%) - Koch-Light

Nicotinamide adenine dinucleotide reduced ($NADH_2$)
(purity 98%) - Koch-Light

Nicotinamide adenine dinucleotide phosphate, mono-sodium salt (NADP)
(purity 80%) - Koch-Light

Nicotinamide adenine dinucleotide phosphate reduced, tetra-sodium salt ($NADPH_2$) (purity 90%) - Koch-Light

5-HT - as the creatinine sulphate - Koch-Light

5-HIAA - as the diethylammonium salt - Roche

5-Hydroxytryptophol (5-HIE) - The National Institutes of Health, Bethesda.

Amberlite resin CG-50, chromatographic grade type 1, 100-200 mesh. - British Drug Houses. Prepared in the ammonium form and buffered to pH 7.4 with ammonium acetate buffer.

All reagents and chemicals "Analar" grade except hydrochloric acid which was "Micro Analytical Reagent" grade (British Drug Houses or Hopkin and Williams).

The diethyl ether was "Analar" grade but prepared peroxide-free and freshly distilled just prior to use.

Spray Reagents Used:-

Acid/Acetone - HCl:Acetone, (5:95), (v/v)

Cinnamaldehyde Reagent - p-3-4dimethylaminocinnamaldehyde: HCl:Acetone, (0.1:5:95), (w/v/v)

2-4-Dinitrophenylhydrazine Reagent - 2-4dinitrophenyl-hydrazine 0.1% in 2N HCl

Distilled, deionised water was used throughout.

The Farrand (1) spectrofluorimeter was used throughout this Section to assay all 5-hydroxyindoles.

RESULTS

Initial Successful Formation of 5-Hydroxytryptophol

The first successful attempt at causing brain to produce 5-HIE was an experiment in which the hypothalami of freshly decapitated rats were pooled and homogenised in an all glass homogeniser with 0.3M sodium phosphate buffer pH 6.9 to give final homogenate containing 20 mg. of brain tissue/ml. 1 ml. samples of homogenate contained in a 30 ml. glass centrifuge tube were preincubated at 37°C in a metabolic incubator with shaking for 15 min. Then 0.1 ml 4×10^{-4}M solution of disulfiram (tetraethylthiuram disulphide) in 20% ethanol was added to one sample while 0.1 ml 20% ethanol was added to two other samples which acted as a control and a marker. 40 µg of 5-HT was added to the sample containing disulfiram and its control sample in 0.2 ml of water and all the samples were then incubated with shaking for a further 60 min. After this time, 0.2 ml glacial acetic acid and 8 ml chilled acetone were added to each sample and the mixtures left to extract at - 15°C, with occasional shaking to disperse the precipitate, for a period of 45 mins. The samples were centrifuged at 2,500 r.p.m. for 3 min. and an 8.5 ml portion of each supernatant was transferred to a 35 ml glass stoppered centrifuge tube and stored overnight at - 15°C. The following morning

Table 1:4

Identification of the Formation of 5-Hydroxytryptophol (5-HTL) by Homogenates of Rat Brain Hypothalami

Thin-layer Chromatography on Silica Gel H (250 μ) (Merck) - Solvent System: Propan-2-ol:aqueous ammonia (S.G. 0.880):water (8:1:2)

Compound	Rf values of Samples			
	Control	Disulfiram	Marker	Pure Soln.
5-	-	0.90	0.84	0.90
5-	0.52	0.54	0.52	0.44
5-	0.50	0.53	0.47	0.30

The 5-HTL gave the characteristic fluorescence after spraying with acid/acetone reagent and examining under ultra-violet light. In another similar chromatographic plate the 5-HTL was identified by its characteristic colour with cinnamaldehyde reagent.

50 µg ascorbic acid in 0.1 ml 80% methanol was added to each extract and the acetone evaporated off by a stream of nitrogen with no external heating until the volume was approximately 1 ml. Then the samples were taken to damp dryness under reduced pressure at an external temperature of 55°C. Each residue was dissolved in 0.1 ml 80% methanol containing 50 µg ascorbic acid and these extracts were stored overnight at - 15°C. Next morning, after authentic 5-HT, 5-HIAA and 5-HIE had been added to one of the extracts from a control sample, each extract and pure solution markers were spotted onto two thin layer chromatography plates (Silica Gel H (Merck) - depth 0.25 mm) which were developed with propan - 2-ol/aqueous ammonia (S.G. 0.88)/water, 20:1:2 (by vol.). (Time 3 hr. - Solvent front 10.5 cm). One plate was sprayed with acid acetone and examined under ultra violet light while the other plate was sprayed with cinnamaldehyde reagent. The results given in table 1:4 show 5-HIE had apparently been formed in the presence of disulfiram but not in the control.

This initial sighting experiment strengthened the belief in the hypothesis that the metabolism of the amine (5-HT) to the corresponding alcohol (5-HIE) might be increased by this drug. It has been well demonstrated that disulfiram inhibits the oxidation of acetaldehyde to

acetic acid both *in vivo* and *in vitro* as a result of its competition with NAD for the active centres of the aldehyde dehydrogenase. It was thought that disulfiram might also inhibit conversion of 5-HIAAL to 5-HIAA because this had been shown to be NAD-dependant *in vitro* (Weisbach, Redfield and Udenfriend 1957) and in this way might reveal the capacity of brain tissue to form 5-HIE from 5-HIAAL, the latter arising from the action of monoamine oxidase on 5-HT.

Methodological Development

From the initial successful experiment, methodological development progressed over a series of experiments.

Procedure for Homogenisation and Extraction

Several initial experiments attempting to cause homogenates of whole rat brain to form 5-HIE had failed, (this may have been due to factors other than the homogenate source), but it was found that its production could be detected if homogenates of the central structures of rat brain were used. This dissection involved the removal of the cerebellum and the cerebral hemispheres and the samples of brain thus produced were the source of rat brain homogenate for subsequent experiments.

It was also found that more satisfactory homogenates could be produced if the brain was homogenised in 0.25 M

Table 2:4

Separation of 5-Hydroxytryptophol (5-HIE), 5-Hydroxytryptamine (5-HT) and 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) by Paper Chromatography

Compound	Solvent			
	1	2	3	4
5-HIE	0.71	0.66	0.74	0.73
5-HT	0.44	0.43	0.23, 0.55	0.48
5-HIAA	0.70	0.33	0.49	0.54

The compounds were added to the residues of diethyl ether extracts of rat brain homogenates and developed overnight (15-16 hr.) in an atmosphere of nitrogen on Whatman No. 1 paper by ascending solvent systems.

Solvent 1 Butan-1-ol:acetic acid:water (12.5:5) (v/v)

Solvent 2 Propan-2-ol:aqueous ammonia (s.g. 0.88):water (85:5:15) (v/v)

Solvent 3 Methanol:butan-1-ol:benzene:water (4:2:2:2) (v/v)

Solvent 4 Propan-2-ol:0.2 M sodium borate buffer pH 10 (2:1) (v/v)

sucrose chilled to 4°C to give a final homogenate concentration of 40 mg brain/ml. The substrate was then added to the 1 ml portions of homogenate at the start of the reaction in 1 ml 0.5 M phosphate buffer pH 7.5 which had been preincubated at 37°C for 15 min. This change caused acetone extraction to be unsatisfactory because of the formation of gums. As a result of this the enzymic reaction was stopped by the addition of 0.2 ml of 10 N HCl, duplicate samples pooled, centrifuged at 3,000 r.p.m. for 7 min., the supernatant decanted into a 35 ml centrifuge tube containing 2 g of sodium chloride and extracted by shaking for 5 min. with 20 ml of diethyl ether.

Chromatographic Techniques

Attention was also given to the development of a method by which the different 5-hydroxyindoles could be separated and assayed quantitatively. Of the numerous solvent systems examined, for the separation of the 5-hydroxyindoles on thin layers of silica gel, during the qualitative phase of this project, the solvent systems shown in table 7:4 proved to be the most satisfactory for the final characterisation of the neutral 5-hydroxyindole metabolites. It was thought, however, that a paper chromatographic method would be more suitable in the separation of the 5-hydroxyindoles prior to quantitative assay. Table 2:4 shows the results obtained with four

Table 3:4

The Initial Detection and Quantitation of the Formation of 5-Hydroxytryptophol (5-HTE) by Homogenates of Rat Brain

5-Hydroxyindole	Time of Incubation (min.)				
	0	15	30	45	60
5-HIAAL + 5-HT	29	41	52	51	66
5-HIAA	10	46	53	59	62
5-HIE	0	7	10	16	30

Results in n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of rat brain homogenate incubated at 37 C for times shown, in the presence of 450 n.moles of 5-HT in 1 ml of 0.5M phosphate buffer pH 7.4, 1 mg of $NADH_2$ and 40 n.moles of disulfiram. The results were determined by the method on page 254 and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL).

solvent systems on Whatman No. 1 paper in which the separation of authentic 5-HT, 5-HIAA nd 5-HIE, added to the residue of extracts of control incubations, was attempted. The separation achieved with propan-2-ol/ aqueous ammonia (SG 0.88)/water (85:5:15) seemed the most satisfactory and this was incorporated into the methodology.

Initial Detection of 5-Hydroxyindole-3-ylacetaldehyde

At this point it was thought that the method would be adequate for quantitative experiments. The first experiment undertaken used homogenates to which both disulfiram and $NADH_2$ were added, as it was anticipated that these might give optimal conditions for the formation of 5-HIE. The results (Table 3:4) showed time of incubation increased the yield of 5-HIE. 5-HIAA formation also showed an increasing yield with time. A surprising feature of the experiment, however, was that the small amount of 5-HT, known to be carried into the ether extracts also apparently showed an increase with time. 5-HIE was well separated from the other 5-hydroxyindoles but the 5-HT appeared to be poorly separated from the 5-HIAA. A further experiment using the same methodology was carried out omitting the addition of disulfiram and using either NAD or $NADH_2$ as co-enzyme. 5-HIE was formed with both co-enzymes in detectable quantities and again it was noted that poor separation occurred between 5-HT and

5-HIAA and that the 5-HT, like the metabolites, appeared to increase with the duration of incubation.

The probable explanation of the increase in '5-HT' with time of incubation was thought to be that a metabolite other than 5-HIAA or 5-HIE was being formed by the brain homogenates and had an Rf. value in the chromatographic system used similar to that of 5-HT. It was considered that 5-hydroxyindol-3-ylacetaldehyde (5-HIAAL) was the most likely metabolite to be formed in these experiments.

Extraction of 5-Hydroxytryptamine

In view of the above finding it was decided to introduce an additional step into the method to remove the bulk of 5-HT from the homogenate supernatant, prior to extraction with diethyl ether. Columns (10 x 0.7 cm) of a weak cation exchange resin, (Amberlite, C.G.50-mesh 100-200) were prepared in the ammonium form buffered to pH 7.4 with 0.02 M ammonium acetate buffer according to the method of Oates (1961) as modified by Eccleston, Ashcroft, Crawford and Loose (1966). These columns had been proved previously to be an excellent means of extracting 5-HT from urine and tissue extracts. Two ml 0.02 M ammonium acetate buffer pH 7.4 containing 1 µg of 5-HIAA or 5-HIE was allowed to pass through a column of

the resin under gravity followed by 1 ml of the ammonium acetate buffer. The total effluent was collected and assayed for 5-hydroxyindoles. Recoveries of 100% of 5-HIAA and 96% of 5-HIE were achieved. Thus this step was included in the method.

Assay Method 1 - For the Formation of 5-Hydroxyindole Metabolites After Incubation with 5-Hydroxytryptamine

Preparation of Brain Homogenates and Incubation Mixtures

The rats were killed by decapitation and the brain quickly removed. After excising the cerebral hemispheres and cerebellum the remainder of the brain was homogenized in 25 vol. 0.25M sucrose at 4°; 1 ml portions of the homogenate were preincubated in 35 ml centrifuge tubes in a metabolic shaking incubator for 15 min. at 37°C. In experiments in which disulfiram was present during the incubation, 0.1 ml of a 10^{-4}M solution in 20% ethanol was added at the start of the preincubation period. The reaction was started in each tube by the addition of 450 n.moles 5-HT in 1 ml 0.5M sodium phosphate buffer, pH 7.4, containing 1 mg of one of the pyridine nucleotide co-enzymes, NAD, $NADH_2$, NADP and $NADPH_2$. The sodium phosphate buffer was preincubated for 15 min. at 37°C before it was added to the homogenates. The reaction was stopped by the addition of 0.1 ml conc. HCl.

Estimation of 5-Hydroxytryptophol, 5-Hydroxyindol-3-ylacetaldehyde and 5-Hydroxyindol-3-ylacetic Acid

Pairs of duplicate incubation mixtures were pooled and centrifuged at 3,000 r.p.m. for 7 min. After decantation the supernatant was adjusted to pH 7.4 (glass electrode) using 5N NaOH, and passed at the rate of 20 ml/hour through a 10 x 0.7 cm column of Amberlite C.G. 50 ion-exchange resin in the ammonium form at pH 7.4 prepared as described by Eccleston et al (1966). The resin extracted most of the residual 5-HT. The acid and neutral metabolites were washed through the column with 1 ml 0.02M pH 7.4 ammonium acetate buffer. The effluent was collected in a 35 ml glass stoppered centrifuge tube, saturated with sodium chloride and acidified by the addition of 0.1 ml conc. HCl. The neutral and acidic components were extracted into 20 ml freshly distilled peroxide-free diethyl ether by shaking for 5 min., then the two phases were separated by centrifuging at 2,000 r.p.m. for 3 min. From each of a pair of duplicate samples, 18.5 ml of the ether layer was transferred to a tube containing 100 µg of ascorbic acid in 0.1 ml 80% methanol and 0.1 ml 20% (v/v) acetic acid. This was then evaporated to damp dryness by a stream of nitrogen. The residue in 0.1 ml 80% methanol containing 100 µg was applied to chromatography paper cut as described

Table 4:4

The Detection and Quantitation of the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HTL) by Homogenates of Rat Brain

5-Hydroxyindole	Time of Incubation (min.)			
	0	15	30	60
5-HIAAL	1	6	23	25
5-HIAA	6	23	39	76
5-HTL	0	4	9	14

Results, n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of rat brain homogenate incubated at 37°C for the times shown, in the presence of 450 n.moles of 5-HT in 1 ml of 0.5M phosphate buffer pH 7.4, 1 mg of $NADH_2$ and 40 n.moles of disulfiram. The results were determined by "Assay Method 1" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIAL, 74% for 5-HTL and undetermined for 5-HIAAL.

previously (page 12) and an ascending chromatogram developed overnight with propan-2-ol/aqueous ammonia (S.G. 0.88)/water (85:5:15) (by vol.). The chromatogram was dried with nitrogen and the appropriate strips as determined from a concurrent marker chromatogram, eluted with 0.1N sulphuric acid and assayed for 5-hydroxyindoles by the specific fluoresence in 3N HCl. Emission set at 550 mμ (Ashcroft et al 1964). Activation maximum was attained at 295 mμ.

Results of Experiments with Assay Method 1

Confirmation of the Detection of 5-Hydroxyindol-3-ylacetaldehyde.

Incubations were carried out in the presence of disulfiram and $NADH_2$ as described on page 256 but the method of assay used was that described above (Assay Method 1). The results, (table 4:4) show that amounts of 5-HIAA and 5-HIE comparable to the amounts shown in table 3:4 were produced. On comparing the two tables (table 3:4 and 4:4) it is obvious that the removal of the 5-HT in the second experiment had clearly revealed the presence of a metabolite increasing with the duration of the incubation. This metabolite was thought to be 5-hydroxyindol-3-ylacetaldehyde (5-HIAAL).

Table 5:4

The Effects of Oxidised and Reduced Nicotinamide Adenine Dinucleotide on the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Rat Brain Homogenates in the Presence of Disulfiram

Co-enzyme	5-Hydroxyindole	Time of Incubation (min.)				
		0	15	30	60	120
NAD	5-HIAAL	2	11	12	22	19
	5-HIAA	5	31	50	79	80
	5-HIE	0	3	3	5	3
$NADH_2$	5-HIAAL	2	12	18	61	45
	5-HIAA	11	49	60	140	117
	5-HIE	0	4	5	16	25

Results, n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of rat brain homogenate incubated at 37 C for the times shown in the presence of 450 n.moles of 5-HT in 1 ml of phosphate buffer, 1 mg of co-enzyme and 40 n.moles of disulfiram. The results were determined by "Assay Method 1" and are uncorrected for recovery. The approximate molar fluoresence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

Determination of Optimal Conditions for the Formation of 5-Hydroxytryptophol

Because the homogenates of rat brain were formed from fresh tissue in each experiment it was felt that while comparison within experiments would be valid, comparisons between experiments should be made with caution. A short series of experiments was carried out to try to determine the optimum cofactors for the production of 5-HIE by brain homogenates.

In the first experiment disulfiram and either NAD or $NADH_2$ were added to the homogenates. The results (Table 5:4) show that 5-HIAAL, 5-HIAA and 5-HIE were all formed and showed increases with the duration of incubation in the presence of either coenzyme but that $NADH_2$ was more effective than NAD, producing approximately three times the amount of 5-HIAAL, one and a half times the amount of 5-HIAA and five times the amount of 5-HIE. This difference in effectiveness was also more apparent with time. Thus the reduced form of coenzyme caused a stimulus to monoamine oxidase and increased the proportion of 5-HIAAL reduced to the 5-HIE by the alcohol dehydrogenase.

As $NADP/NADPH_2$ has been found to be more specific than $NAD/NADH_2$ as a coenzyme for retinene reductase (Zachman and Olson (1961)) it was felt that it might also

Table 6:4

The Effects of Oxidised and Reduced Nicotinamide Adenine Dinucleotide Phosphate on the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic acid (5-HIAA) and 5-Hydroxytryptophol (5-HTE) by Rat Brain Homogenates in the Presence and Absence of Disulfiram

Disulfiram	Co-enzyme	5-Hydroxyindole	Time of Incubation (min.)				
			0	15	30	60	120
Present	NADP	5-HIAAL	6	47	74	151	118
		5-HIAA	1	29	50	88	89
		5-HTE	1	7	10	20	16
Present	$NADPH_2$	5-HIAAL	14	57	104	140	159
		5-HIAA	6	27	68	80	114
		5-HTE	2	7	20	59	67
Absent	$NADPH_2$	5-HIAAL	5	29	33	36	48
		5-HIAA	6	51	83	130	162
		5-HTE	0	15	27	58	157

Results, n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of rat brain homogenate incubated at 37 C for the times shown in the presence of 40 n.moles of 5-HT in 1 ml of phosphate buffer and 1 mg of co-enzyme, and in the presence or absence of 40 n.moles of disulfiram. The results were determined by "Assay Method 1" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38 for 5-HIAAL, 74 for 5-HIAA and undetermined for 5-HIAAL.

be more specific for the cerebral alcohol dehydrogenase being studied. An experiment was performed in which the time course of metabolite production was followed with $NADPH_2$ as coenzyme, and also with NADP or $NADPH_2$ as coenzymes in the presence of disulfiram (table 6:4).

The results of this experiment demonstrate some interesting points. On comparing the amounts of metabolites formed in the presence of disulfiram with $NAD/NADH_2$ as coenzymes (table 5:4) and with $NADP/NADPH_2$ as coenzymes (table 6:4), it is apparent that $NADP/NADPH_2$ have produced greater amounts of 5-HIAAL and 5-HIE while the amount of 5-HIAA is the same. Thus $NAD/NADH_2$ have been able to cause more of the 5-HIAAL available to be oxidised to 5-HIAA. While interpretation between experiments should be made with caution because of possible differences in the enzymic activity of the homogenates, the results suggest $NADP/NADPH_2$ are more specific for the monoamine oxidase and alcohol dehydrogenase, but that $NAD/NADH_2$ are more specific for the aldehyde dehydrogenase. The other main point illustrated by the results in table 6:4 is the contrast in the pattern of metabolites formed with $NADPH_2$ as coenzyme, in the presence or absence of disulfiram. While disulfiram obviously causes an increased yield of 5-HIAAL, it is apparent that this is due not only to an

inhibition of the oxidation of 5-HIAAL to 5-HIAA but also to an inhibition of the reduction of 5-HIAAL to 5-HIE.

Initial Characterisation of 5-Hydroxyindol-3-ylacetaldehyde, 5-Hydroxytryptophol and 5-Hydroxyindol-3-ylacetic Acid

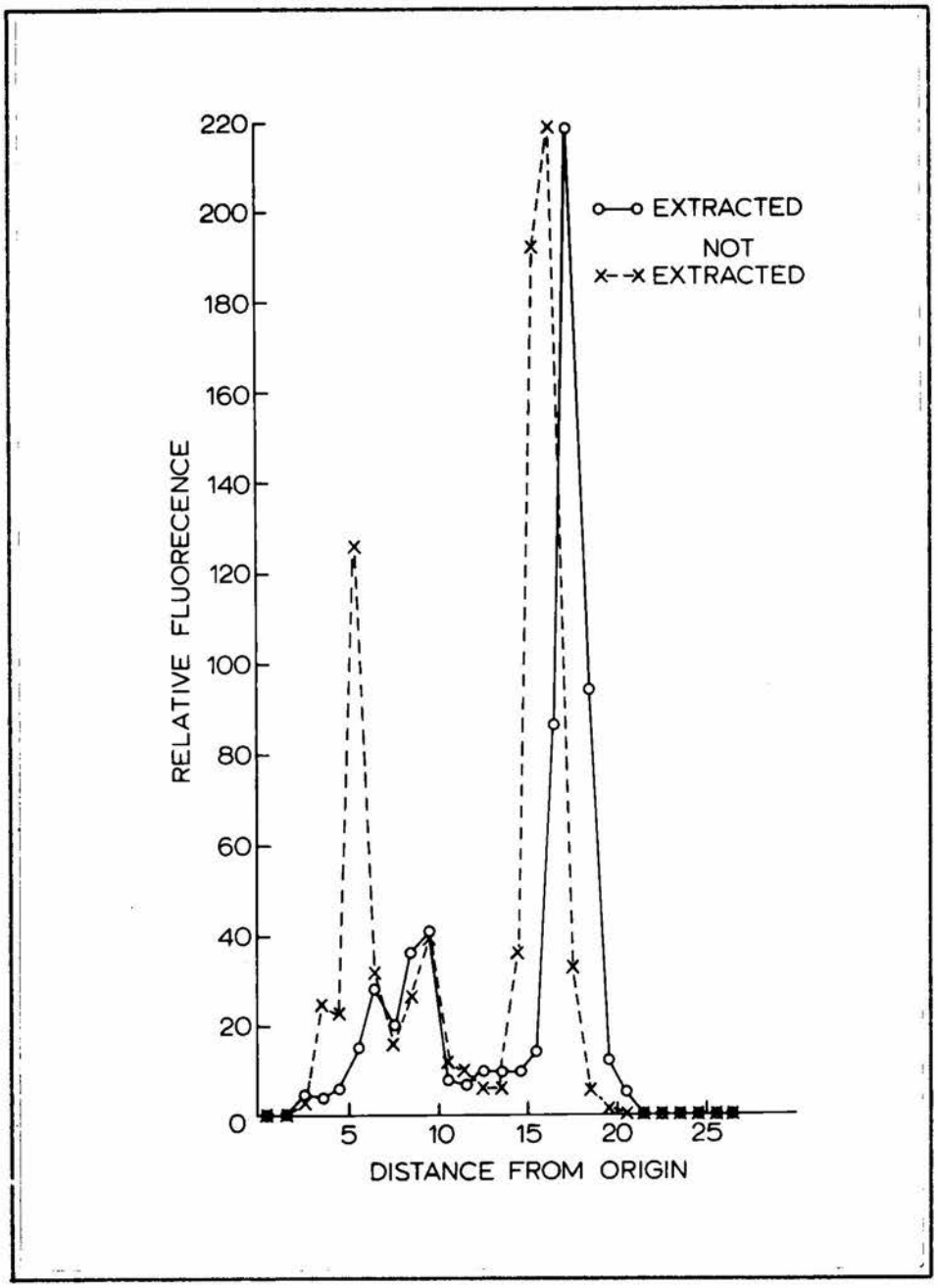
On several occasions after samples containing maximal quantities of 5-HIAAL and 5-HIE had been estimated spectrofluorometrically, the 3N acid solutions were salt-saturated and extracted with four vol. freshly distilled, peroxide-free diethyl ether by shaking for 5 min. After centrifuging for 3 min. at 2,000 r.p.m., the ether layer was transferred to a tube containing 0.1 ml of an 80% methanol solution of ascorbic acid (1 mg/ml) and 0.1 ml of 20% acetic acid (v/v), and then evaporated to damp dryness by a stream of nitrogen. When the residue was diluted with water and assayed for 5-hydroxyindole fluorescence the average percentage recovery was 15% for 5-HIE and only 1% for 5-HIAAL, thus neither the 5-HIAAL nor the 5-HIE had survived the ether extraction from strong acid solution.

Re-extraction of the eluates of chromatogram containing 5-HIAAL and 5-HIE was also unsuccessful, presumably due to similar poor recoveries.

It was decided that the best way to characterise the neutral 5-hydroxyindoles was to follow the procedure of Feldstein and Wong (1965), and separate the acid from the

Fig. 1:4

Paper Chromatographic Separation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE)



The Figure shows the relative specific 5-hydroxyindole fluorescence of 1 cm strips of chromatograms of the residues of ether extracts of rat brain homogenates which had been incubated at 37°C for 1 hour in the presence of 450 n.moles of 5-HT, 1 mg of $NADH_2$ and 40 n.moles of disulfiram. The "extracted" sample had the ether extract shaken with salt saturated 0.2M borate buffer pH 10 for 3 mins before the ether layer was evaporated to provide the residue for chromatography. Ascending chromatogram - Whatman No. 1 Paper Solvent system. Propan-2-ol:aqueous ammonia (SG 0.88):water (85:5:15). Duration of chromatographic run - 16 hours.

neutrals. Accordingly assay method 1 was followed until the 5-HIAA and neutral 5-hydroxyindoles substances had been extracted into the diethyl ether. 18.5 ml of ether extract from each sample was then transferred to a 35 ml glass stoppered centrifuge tube containing 5 ml 0.2 M sodium borate buffer, pH 10 saturated with sodium chloride. The 5-HIAA was extracted back into the borate buffer by shaking for 5 min. After centrifuging for 3 min. at 2,000 r.p.m., a 17.5 ml portion of the ether layer from a sample was transferred to an another 35 ml centrifuge tube along with an equal volume of ether from a duplicate sample. The ether extract was evaporated to damp dryness in the presence of acetic acid and ascorbic acid as described previously (page 259).

Fig. 1:4 shows the relative 5-hydroxyindole fluorescence, found in strips of chromatograms developed on Whatman No. 1 paper with an overnight ascending run of propan-2-ol/aqueous ammonia (SG 0.88)/water (85:5:15), for samples before and after back extraction of 5-HIAA with salt saturated borate buffer. This demonstrates that better separation of the 5-hydroxyindoles can be achieved if only the neutral fraction is subjected to chromatography.

Further chromatograms of the neutral components of extracts of incubations of rat brain homogenates with 5-HT

TABLE 7:4

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

Solvent System: Ethyl Acetate/Chloroform, 3:1 (v/v)

R_f values of components	Acid/acetone reagent + ultraviolet inspection	Cinnamaldehyde reagent	2:4-dinitrophenyl-hydrazine reagent	Component identified
0.92	-	Deep pink on standing	Yellow on standing	Unknown
0.83	Faint yellow-green fluorescence	Light brown--blue-grey on standing	Reddish-brown	5-hydroxy-indolacetaldehyde
0.5	Yellow-green fluorescence	Green--blue on standing	Light brown	5-hydroxy-tryptophol
0.42	-	Pink	Pink	Urea
0.292	-	Light blue	Faint brown	Unknown
0.15	Yellow-green fluorescence	Very pale purple	Pale yellow	5HIAA as oxidation product? (5HIAA is held at the origin in this solvent)

Solvent System: Ethyl Acetate

1	-	-	Yellow	Unknown
0.94	-	Deep pink on standing	Yellow on standing	Unknown
0.89	Faint yellow-green fluorescence	Grey-blue	Brown	5-hydroxy-indolacetaldehyde
0.7	Yellow-green fluorescence	Grey-blue	Pale orange	5-hydroxy-tryptophol
0	Faint yellow-green fluorescence	-	Orange	Unknown aldehyde?

Solvent System: iso-Propanol/Methyl Acetate/25% Ammonia, 35:45:20(v/v)

1	Faint yellow-green fluorescence	Purple	Yellow	Unknown
0.925	Yellow-green fluorescence	Mauve	Brown	5-hydroxy-tryptophol
0.85	Yellow-green fluorescence	Blue-grey	Pale brown	5-hydroxyindol-acetaldehyde
0.63	Faint yellow-green fluorescence	Pale purple	Yellow	Unknown
0.47	Faint yellow-green fluorescence	Pale green	Yellow	Unknown
0	Faint	-	Yellow	Unknown

and $NADPH_2$ were run this time on silica gel H (Merck) (300 μ). The 5-hydroxyindoles were visualized by spraying first with acid acetone and observing under ultraviolet light, followed by spraying with cinnamaldehyde reagent. Comparisons of R_F were made against extracts containing additional authentic 5-hydroxytryptophol. Two major 5-hydroxyindolic components were found in the neutral 5-hydroxyindole fractions by thin layer chromatography. The first behaved like authentic 5-hydroxytryptophol. The second gave a brown colour reaction with the aldehyde spray reagent, 2:4 dinitrophenylhydrazine (0.1g in 100 ml 2N HCl) and was presumed to be 5-hydroxyindolacetaldehyde. Chromatographic properties of the components are shown in table 7:4.

The borate buffer containing the acidic 5-hydroxyindoles was acidified to pH 1 with HCl, saturated with sodium chloride and the acids were extracted into diethyl ether. This ether extract was taken to damp dryness after the addition of acetic acid and ascorbic acid, as described above. Chromatography on thin layers of Silica gel H showed that the single 5-hydroxyindolic component of the acidic fraction had an R_F value identical with that of authentic 5-HIAA.

FURTHER METHODOLOGICAL DEVELOPMENT

In view of the rather poor separation of 5-HIAA and 5-HIAAL by ascending paper chromatography with propan-2-ol: aqueous ammonia (S.G. 0.88): water (85:5:15), it was thought that a more satisfactory method of separation prior to assay might be achieved by removing both the 5-HT and 5-HIAA and then subjecting the remaining neutral 5-hydroxyindoles to chromatographic separation.

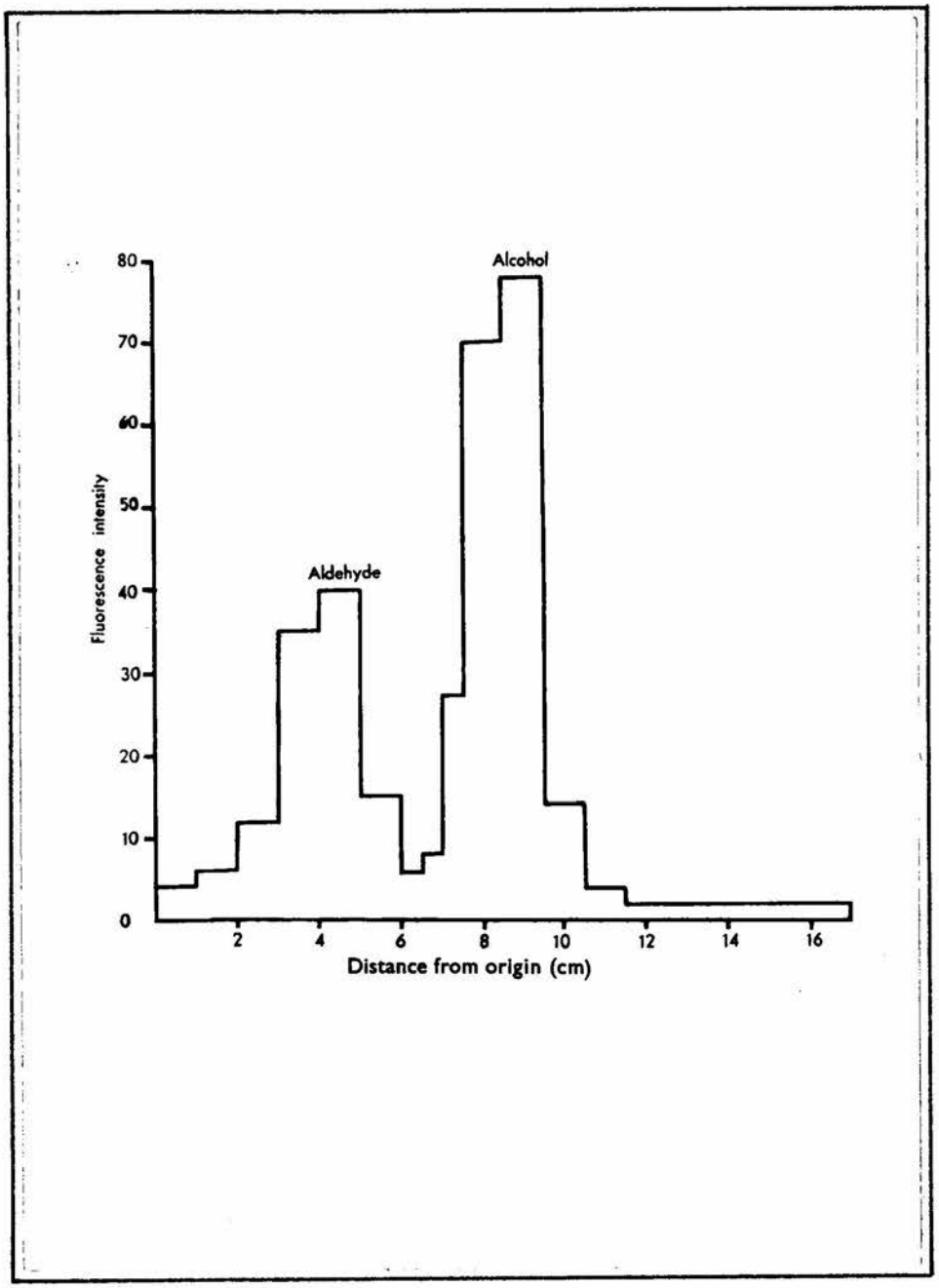
Assay Method 2

Incubations, the extraction of 5-HT, and the extraction of 5-HIAA 5-HIAAL, and 5-HIE into diethyl ether were carried out as in assay method 1 (page 256). A volume of 18.5 ml. of the ether layer from a sample was transferred to a 35 ml. glass stoppered centrifuge tube containing 5 ml. 0.2M sodium borate buffer, pH 10 saturated with sodium chloride and the 5-HIAA was extracted back from the ether into the sodium borate buffer by shaking for 5 min. After centrifuging at 2,000 r.p.m. for 3 min. the two separated phases were treated as follows.

To a 1 ml. portion of the sodium borate phase was added 3 ml. of 0.1N sulphuric acid and 2 ml. of 10 N. hydrochloric acid containing 1 mg/ml of ascorbic acid.

Fig. 2:4

Paper Chromatographic Separation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL) and 5-Hydroxytryptophol (5-HIE)



The figure shows the relative specific 5-hydroxyindole fluorescence in 1 cm strips of a chromatogram of the residue of an ether extract containing neutral 5-hydroxyindoles formed by "Assay Method 2". The chromatogram was developed on Whatman No. 1 paper in an ascending solvent system of 8% aqueous sodium chloride (W/V):glacial acetic acid (100:1 by vol) for three hours in an atmosphere of nitrogen.

The 5-HIAA concentrations in the samples were quantified by comparing the specific fluorescence with comparable standard solutions of 5-HIAA as described previously (page 259).

A 17.5 ml. portion of the ether phase which contained the 5-HIAAL and 5-HIE was transferred to a 35 ml. tube along with a like amount from a duplicate sample. 100 ug ascorbic acid as 0.1 ml. of a solution in 80% methanol and 0.1 ml. 20% (v/v) acetic acid were both added to the ether extract.

The diethyl ether was evaporated to damp dryness by a stream of nitrogen and the residue was dissolved in 0.2 ml. 80% methanol containing 200 µg. of ascorbic acid. This solution was applied under nitrogen to Whatman No.1 paper strips as described previously (page 12). Ascending chromatograms were developed with aqueous sodium chloride 8% (w/v): acetic acid (100 to 1 by vol.) in an atmosphere of nitrogen. (Feldstein and Long, 1965) to separate 5-HIE from 5-HIAAL (Fig. 2:4). Initially, authentic 5-hydroxytryptophol was added to one sample to act as a marker and its position in the developed chromatogram made visible with the cinnamaldehyde reagent. Subsequently the 5-hydroxyindoles were localised by cutting a 0.5 cm. longitudinal strip from one of the

paper chromatograms, spraying it with acid acetone and then viewing it under ultra violet light.

5-HIE and 5-HIAA were eluted from the appropriate sections of paper of the sample chromatograms into 4.2 ml. of 0.1 N sulphuric acid. The amount of 5-hydroxyindole present in each eluate was determined by spectrofluorimetry as described above. The separation of the compounds was checked by dividing the paper segment between the aldehyde and the alcohol positions into four 0.5 cm. strips, eluting each with 4.2 ml. 0.1 N sulphuric acid and demonstrating the absence of detectable 5-hydroxyindolic substance by fluorimetry in at least one of these eluates.

RESULTS IN ASSAY METHOD 2

Recovery Experiments

The recovery 5-HIE and 5-HIAA through the analytical procedure tended to be low. Authentic 5-HIE added to control samples in amounts of 2.5, 5 and 10 µg. and taken through assay method 2, gave recoveries of 57, 43 and 45% respectively. 5-HIE was not detected in the pH 10 borate buffer used to extract the acid. 5-HIAA, 10 µg. taken through the procedure gave a recovery of 51%. 5-HIAA was not detected in the final paper chromatogram. 5-HT did not interfere with the estimations at any stage.

Further Characterisation of 5-Hydroxytryptophol and 5-Hydroxyindol-3-ylacetaldehyde

The "5-HIE" formed by brain homogenates had already been shown to be isographic with authentic 5-HIE in three solvent systems on thin layers of silica gel (Table 7:4) and on Whatman No. 1 paper chromatograms with ascending systems of aqueous sodium chloride 8% (w/v): acetic acid (100 to 1 by vol.) and propan-2-ol: aqueous ammonia (S.G. 0.88): water (85:5:15 by vol.). Its formation in the presence of the pyridine nucleotide co-enzymes and disulfiram was also in accord with what would be anticipated.

The 5-hydroxyindolic substance suspected to be 5-HIAL gave a brown colour with the aldehyde spray reagent 2:4 dinitrophenylhydrazine thus suggesting the presence of a carbonyl group. The formation of this substance in the presence of the pyridine nucleotide co-enzymes and disulfiram was also in accord with its identity as 5-HIAL.

Some further confirmation of the identity of these 5-hydroxyindolic substances as 5-HIE and 5-HIAL was sought.

Ultra-violet Absorption Characteristics of 5-Hydroxytryptophol.

The substance, suspected to be 5-hydroxytryptophol was purified and isolated by paper partition chromatography

Table 8:4

The Effect of Aldehyde "Trapping" Agents on the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Rat Brain Homogenates in the Presence of Reduced Nicotinamide Adenine Dinucleotide Phosphate.

Trapping Agent	5-Hydroxyindole	Time of Incubation (hrs.)	
		½	1
NONE (Control)	5-HIAAL	36	69
	5-HIAA	156	456
	5-HIE	40	275
Sodium Metabisulphite	5-HIAAL	99	139
	5-HIAA	133	365
	5-HIE	12	94
Hydroxylamine Hydrochloride	5-HIAAL	101	79
	5-HIAA	191	329
	5-HIE	53	195

Results n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of brain homogenates incubated at 37°C for the times shown in the presence of 450 n.moles of 5-HT and 1 mg of $NADPH_2$.

Aldehyde "trapping" agents added per sample - sodium metabisulphite 105 n.moles - hydroxylamine hydrochloride 575 n.moles.

The results were determined by "Assay Method 2" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

from the neutral fraction of an extract made from brain homogenate incubated with 5-HT. It was eluted from the paper chromatogram with spectroscopically pure ethyl alcohol. The ultraviolet spectrum of this solution was examined in a Unicam SP 800 recording spectrophotometer. The E_{max} of this solution at 278 mμ was 5,950 and at 302 mμ, 4,140. These were in agreement with the E_{max} for authentic 5-hydroxytryptophol (278 mμ - 5,553 and 303 mμ - 4032).

The Use of Aldehyde "Trapping Agents"

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

The proportion of 5-HIAAL was increased in relation to both 5-HIAA and 5-HIE particularly in the presence of sodium metabisulphite (Table 8:4). Hydroxylamine tended to give rise to pigmented samples.

Infra-red Spectroscopy of the Carbonyl Group of 5-Hydroxyindol-3-ylacetaldehyde

An attempt was made to obtain the characteristic spectrum of the carbonyl group of 5-HIAAL (Diffraction Grating Infrared Spectrophotometer 237 - Perkin Elmer). However, diethyl ether proved to be a poor solvent for this technique and 100-200 μg/ml. of aldehyde compounds

were necessary to give a characteristic spectrum even using a balanced cell technique. This was a much larger quantity of 5-HIAAL than could be made conveniently.

Attempts to Quantify the Molar Fluorescence of 5-Hydroxyindol-3-ylacetaldehyde

As mentioned previously, no standard of 5-HIAAL existed and its relative molar fluorescence is not recorded in the literature. Two attempts to derive the molar fluorescence of 5-HIAAL relative to that of 5-HT were made but neither was considered successful. Therefore all results in this section have been quoted in terms of n. moles of 5-HT giving rise to fluorescence of an intensity equivalent to that from the 5-hydroxyindolic compound.

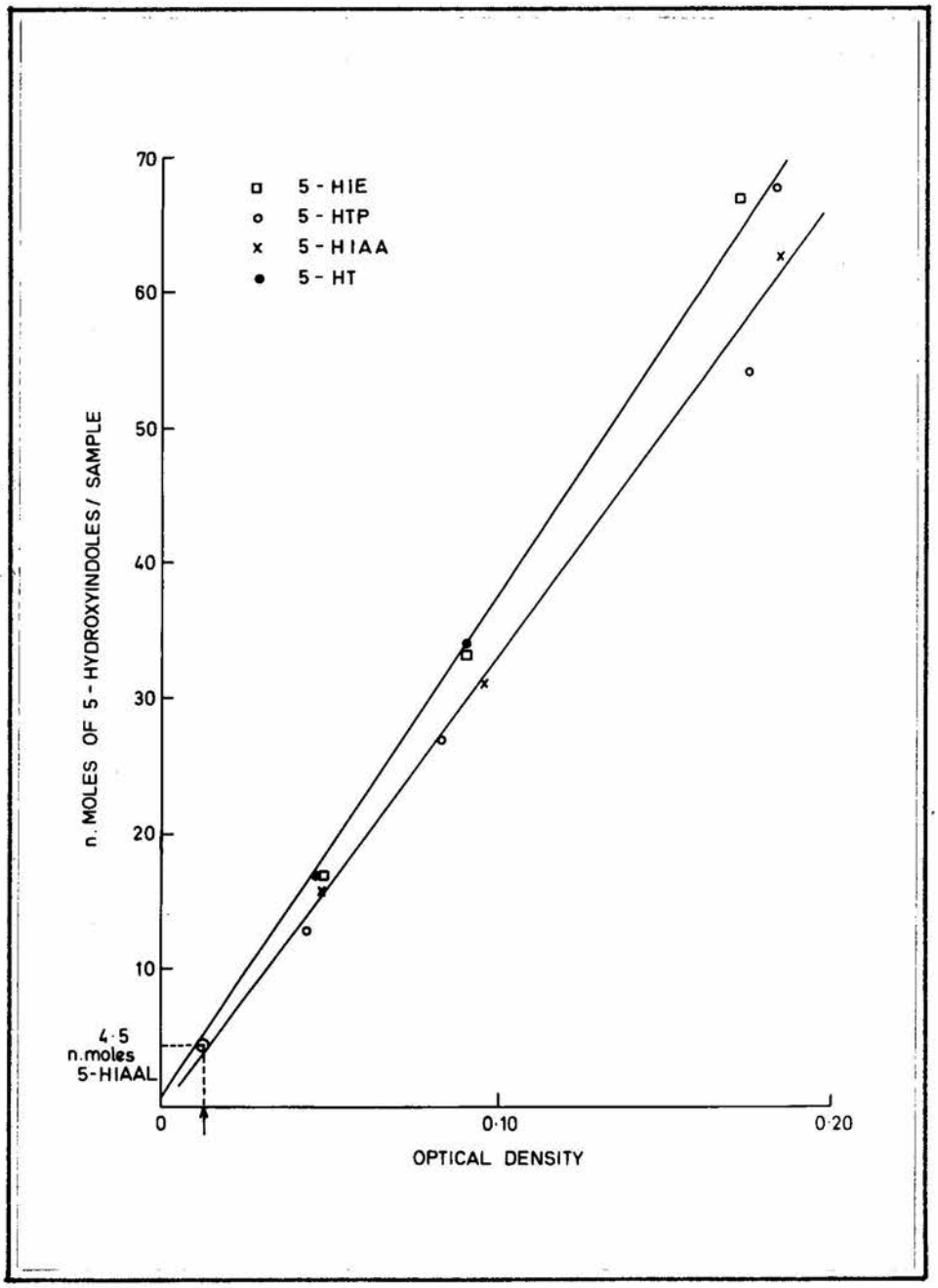
Use of the 1-Nitroso-2-naphthol Reaction

The first attempt to determine the molar fluorescence of 5-HIAAL involved assaying two portions of a chromatographically pure solution of 5-HIAAL in 0.1 N sulphuric acid, one according to its specific 5-hydroxy indole/fluorescence and the other portion according to its 5-hydroxyindolic content as determined by the 1-nitroso-2-naphthol reaction.

This reaction was originally described for 5-hydroxyindoles by Udenfriend and Clark (1955) and although the precise nature of the compounds formed is not known, the

Fig. 3:4

Calibration Curves for 5-Hydroxytryptophol (5-HIE), 5-Hydroxytryptophan (5-HTP), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptamine (5-HT) Using the 1-Nitroso-2-Naphthol Reaction



The figure demonstrates that the 1-nitroso-2-naphthol reaction, which is said to be specific for 5-hydroxyindoles (see text), gives very similar calibration curves for 5-HIE, 5-HTP, 5-HIAA and 5-HT. The assumption that a similar calibration curve applies to 5-hydroxyindol-3-acetaldehyde allows its quantitation.

reaction is considered specific for 5-hydroxyindoles.

Duplicate samples of 5-HIAAL were prepared fresh from incubations of brain homogenates with 5-HT by the use of assay method 2 and the relative 5-hydroxyindole fluorescence determined. The samples of 5-HIAAL were contained in 1.4 ml. 0.1N sulphuric acid. Duplicate standards 3, 6 and 12 µg. of 5-HT, 5-HIAA, 5-HTP and 5-HIL, were contained in similar solutions. Two samples of 0.1 N sulphuric acid were included as blanks. To each sample 0.7 ml. of 0.1% (w/v) 1-nitroso-2-naphthol reagent in 95% ethanol was added. After mixing, 0.7 ml. of nitrous acid reagent (a freshly prepared mixture of 25 ml. 2N sulphuric acid and 1 ml. 2.5% sodium nitrite), was added to each of the samples. The tubes were stoppered shaken and incubated at 55°C for 5 min. The samples were shaken for 5 min. with 7 ml. of 1:2 dichloro-ethane to remove the excess reagent . After centrifuging at 2000 r.p.m. for 3 min. a portion of the aqueous layer was transferred to a clean tube and the optical density of the purple chromophore was determined in a Unicam 600 spectrophotometer at 540 mµ against the blank solutions.

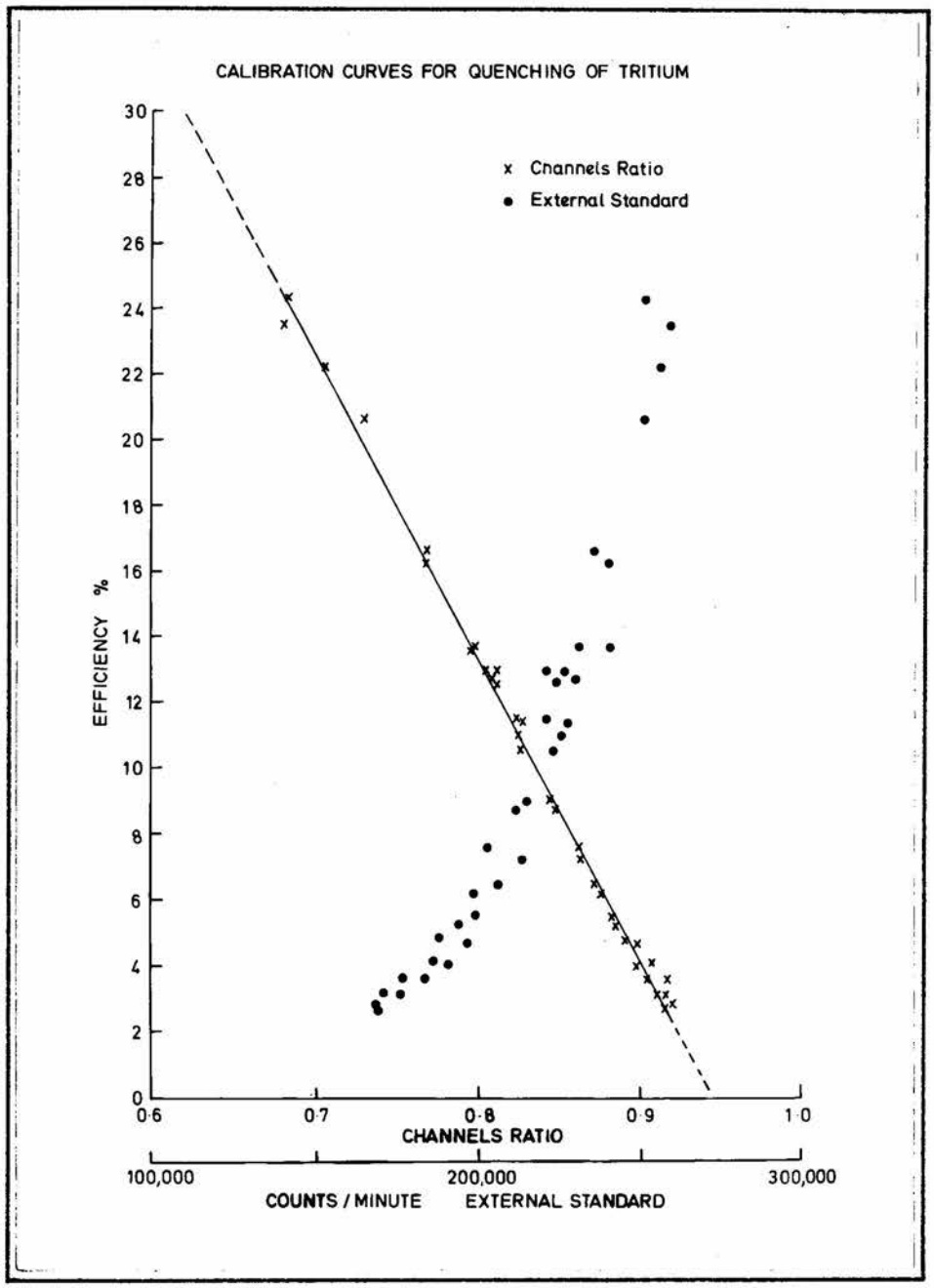
Fig. 3:4 shows a plot of optical density against the total moles of 5-hydroxyindole in the sample. Although the optical density range used is below that

normally considered adequate the duplicate samples agreed well and linearity was maintained. As the calibration curves of the molar concentration against the optical density given by the 1-nitroso-2-naphthol reaction were approximately the same for 5-HTP, 5-HT, 5-HIAA and 5-HIAL, a value for the molar concentration of the samples of 5-HIAL could be calculated by assuming that a similar calibration curve applied for 5-HIAL. In comparison with the relative 5-hydroxyindole fluorescence obtained for the 5-HIAL samples the molar 5-hydroxyindole fluorescence of 5-HIAL was calculated to be 130% that of 5-HT.

Use of the ratio of Specific Activity to 5-Hydroxyindole Fluorescence.

A small quantity of ^{3}H-5-HT was added to the 5-HT to be used as substrate for the incubations with brain homogenate. Incubations were carried out for 1 hr. in the absence of any co-enzyme or disulfiram. A number of homogenates had 0.1 ml. conc. hydrochloric acid added before the substrate and thus acted as controls. The standard method of assay (Assay Method 2) was carried out for 5-HIAA and 5-HIAL. In addition 5-HT was eluted from the columns of C.G.50 ion exchange resin with 6 ml. N sulphuric acid after washing the columns with 14 ml. 0.02 M ammonium acetate buffer pH 7.4 and 4 ml. 0.1 N

Fig. 4:4



Channels Ratio - $\frac{50\text{-}300}{50\text{-}1000}$ at 59% Gain in both Channels.

External Standard - Radium Source; window setting - 50-1000 at 4% Gain

The figure shows quench curves for ^{3}H-toluene using chloroform as a quenching agent. It can be seen that the quench curve for the method of external standardisation became non-linear with high quenching of a low energy βemitter such as tritium.

sulphuric acid (Eccleston et al. 1966). The 5-hydroxy-indoles were quantified by the specific fluorescence in 3 N hydrochloric acid in comparison with standard solutions of 5-HT. No 5-HIAA or 5-HIAAL was detectable in the control samples.

After spectrofluorometric assay, 0.2 ml. portions of the samples were taken for determination of radio-activity. Each 0.2 ml. portion was diluted in 4 ml. of ethanol/methanol (3:1, v/v) to which was added 10 ml. of scintillator (0.11 g P.O.POP + 4.26 g P.P.O/litre of toluene) and the sample was counted in a Tri Carb Scintillation Counter (Packard). Quenching was allowed for by the "Channels Ratio Method" (Baillie, 1960) as the counting efficiency was in the region of 12% and method of "External Standardisation" with a radium source has poor linearity at this level of efficiency for tritium (Fig. 4:4). Colour quenching was found to be absent, because the addition of tritium standards to the control samples and samples from homogenates which were allowed to incubate for 1 hour but had no ^{3}H-5-HT added to the substrate agreed with the values found by the "Channels Ratios Method". A slight amount of radio-activity was detected in the "5-HIAA" samples from control homogenates but none in their "5-HIAAL" samples.

The specific activities of the 5-HT, 5-HIAA and 5-HIAL should all have been identical, and therefore the ratios of the quantity of specific 5-hydroxyindole fluorescence for any fixed number of disintegrations per second should have been equal to the ratios of the molar fluorescence. Taking the fluorescence of 5-HT to be 100, the results gave values for the molar fluorescence of 5-HIAA and 5-HIAL of 63 and 2.5 respectively. The value by direct comparison for the molar fluorescence of 5-HIAA with respect to 5-HT is 74 but it varies slightly with concentration.

The molar fluorescent value for 5-HIAL given by this method seemed extremely low and was widely discrepant from the molar fluorescence estimated using the 1-nitroso-2-naphthol reaction. It is probable that the estimate given by the latter method is the more accurate in spite of the low values of the optical density range at which the measurements were made.

The reasons for the discrepancy when using the specific activity as a means of quantifying the molar fluorescence are twofold. Firstly, as the isotope used was generally labelled with tritium it is possible that, due to exchange of tritium and the difference in the structure of the metabolites, 5-HIAA and 5-HIAL would

become more heavily labelled than 5-HT. (The experiment should have been performed using ^{14}C-5-HT). However, such a mechanism alone could not account for the very low molar fluorescence derived for the aldehyde. The other reason, which is more likely, is that some of the 5-HIAL had undergone polymerisation after chromatography and this would give rise to radio-activity in the absence of specific 5-hydroxyindole fluorescence. Such a mechanism is very probable because aldehydes as a class of compounds tend to polymerise easily. Also Weissbach, Redfield and Udenfriend (1957) were frustrated in their attempts to isolate 5-HIAL as the compound presented as a brown pigment which they considered to be a polymer of 5-HIAL. Certain of the other results presented here suggest that 5-HIAL polymerises rather easily. Firstly, 5-HIAL failed to run in more than a single chromatogram without loss to some form other than a 5-hydroxyindole: secondly, it was similarly lost on extraction from strong acid: finally the initial attempts at aldehyde trapping were occasionally completely unsuccessful and no 5-HIAL could be obtained. It was noted, however, that the supernatant of these samples was always a brownish colour.

While the 1-nitroso-2-naphthol reaction offers a

possible means of quantifying the molar fluorescence of 5-HTAL, the technique was not pursued further because of the relatively large amounts of 5-HTAL which would have been necessary to give adequate optical density. Also it was felt that little dependence could have been placed on a value that would be arrived at by subjecting aliquots of solutions of such an unstable substance as 5-HTAL to two different procedures and having to assume that the same proportion of the 5-HTAL remained in the 5-hydroxyindole form in both.

[illegible] ACTIVITY OF 5-HT [illegible] SOLUTIONS AND 5-HYDROXYINDOLE-3-ACETALDEHYDE

5-HTAL and 5-HIE were both prepared from incubates of brain homogenates by assay method 2 and eluted from the chromatogram in 0.9% saline. A portion of each of the solutions was quantified for 5-hydroxyindoles by the specific fluorescence in 3N hydrochloric acid relative to standards of 5-HT. The solutions of 5-HTAL and 5-HIE were tested on a rat uterus preparation (in oestrus) in de Jalon's solution. This preparation gave a maximal contraction to 5-HT at 0.014 n. moles/ml. (final bath concentration) but failed to show any activity to 0.5 n. moles/ml. of 5-HTAL or 1.0 n. moles/ml. of 5-HIE (final bath concentrations).

TABLE 9:4

The Formation of 5-Hydroxyindol-3-ylactaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Rat Brain Homogenates in the Presence of Excess Pyridine Nucleotide Co-enzyme

Co-enzyme	5-Hydroxyindole	Incubation Time (hrs.) 0	½	1
None	5-HIAAL	0	60	-
	5-HIAA	0	158	-
	5-HIE	0	5	-
NADP	5-HIAAL	0	103	98
	5-HIAA	0	324	550
	5-HIE	0	17	25
$NADPH_2$	5-HIAAL	0	69	118
	5-HIAA	0	323	602
	5-HIE	0	120	282
NAD	5-HIAAL	0	47	75
	5-HIAA	0	265	398
	5-HIE	0	9	12
$NADH_2$	5-HIAAL	0	49	72
	5-HIAA	0	236	401
	5-HIE	0	13	19

Results n. moles of 5-hydroxyindole/g (wet wt.) brain 1 ml of rat brain homogenate incubated at 37°C for the times shown in the presence of 450 n. moles of 5-HT in 1 ml 0.5 M phosphate buffer pH 7.4 and 1 mg co-enzyme. The results were determined by "Assay Method 2" and the results were uncorrected for recovery. The approximate molar fluorescence, relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

CO-[illegible] FOR THE [illegible] OF 5-HYDROXY-[illegible]

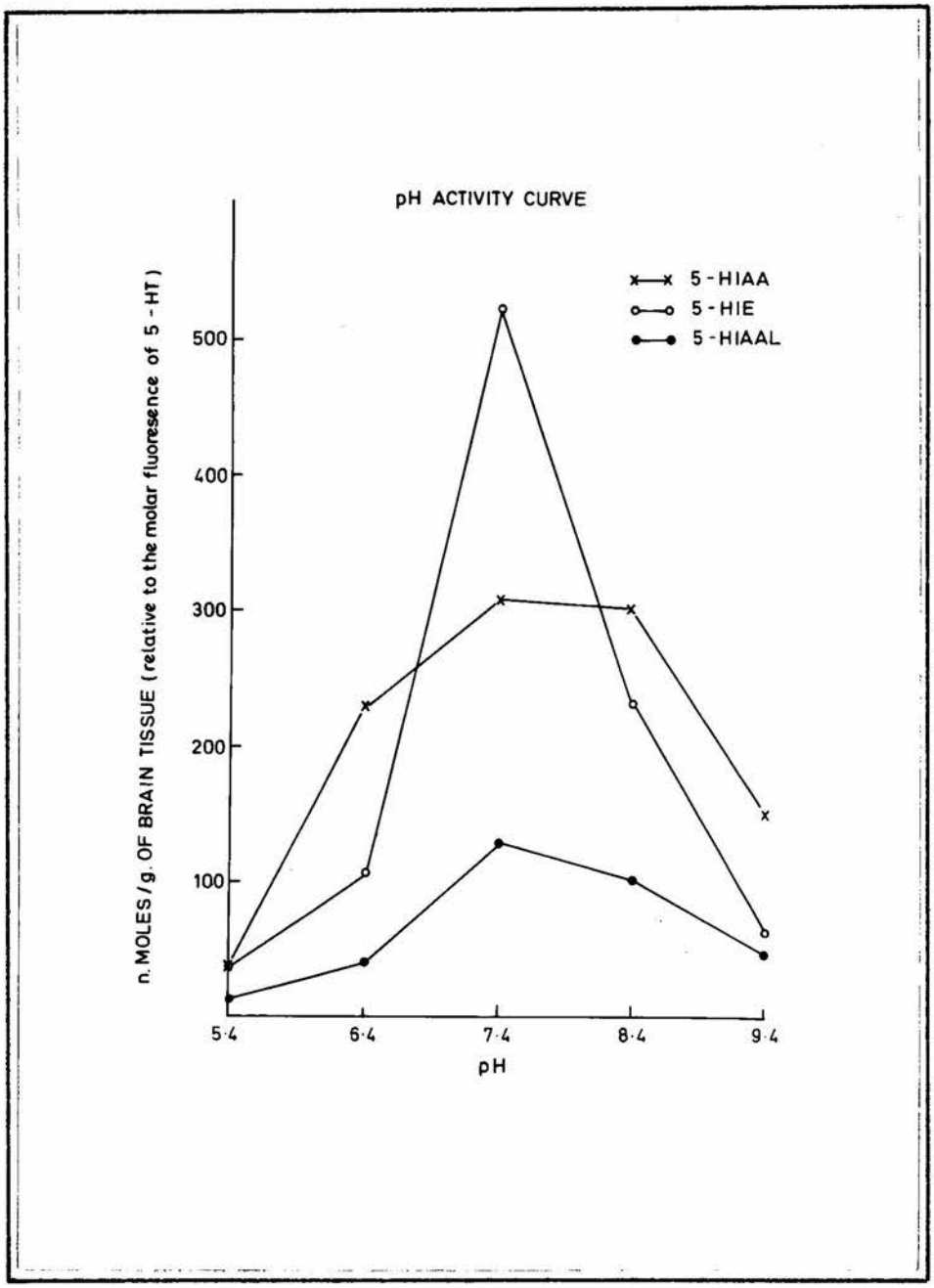
In comparing the results of separate experiments with the pyridine nucleotide co-enzymes in the presence of disulfiram (Tables 5:4 and 6:4) it seemed that the 5-HIE alcohol dehydrogenase had a preference for the NADH/NADH$_2$ co-enzymes. Table 9:4 shows the results of an experiment in which the effects of the different co-enzymes on the metabolites of 5-HT formed by rat brain homogenates were compared within a single experiment. These results show that the NADH$_2$/NADH co-enzymes are preferred by both monoamine oxidase and alcohol dehydrogenase and that, as expected, the reduced form of co-enzyme is more efficient in causing the reduction of 5-HIAL to 5-HIE. The oxidation of 5-HIAL to 5-HIAA is aided by the oxidised form of the co-enzymes and it would appear from the ratios of 5-HIAL to 5-HIAA that there is little difference in specificity with NAD possibly being marginally more efficient than NADP.

[illegible] THE [illegible] OF 5-HYDROXYTRYPTOPHOL

Homogenates of rat brain which had been subjected to heat at 60°C for 10 min. were incubated at 37°C with 450 n. moles of 5-HT and 1 mg. NADH$_2$ in pH 7.4 phosphate buffer for a period of 1 hr. No 5-HIAL or 5-HIE and

Fig.5:4

The Effect of pH on the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid and 5-Hydroxytryptophol (5-HIE) by Rat Brain Homogenates in the Presence of Reduced Nicotinamide Adenine Dinucleotide Phosphate



1 ml samples of rat brain homogenate in 0.25 M sucrose were incubated at 37° C for 1 hour in the presence of 1 mg $NADPH_2$ and 450 n.moles of 5-HT in 1 ml of sodium phosphate buffer at the pH values shown. The results were determined by "Assay Method 2" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

only a trace of 5-HLAA occurred in other extracts of these incubations.

REVERSIBILITY OF THE OXIDATION - [illegible] REACTIONS.

The use of 5-HLE and 5-HLAA as substrates for rat brain homogenates in the presence of excess pyridine nucleotide co-enzymes showed that the oxidation of 5-HLAAL to 5-HLAA was non-reversible, whereas the reduction of 5-HLAAL to 5-HLE was reversible when 5-HLE was used as a substrate in the presence of either excess NADP or $NADPH_2$.

pH ACTIVITY CURVE

Incubations of 5-HT with rat brain were continued for 1 hr. in sodium phosphate buffer at varying pH values from 5.4 to 9.4. The 5-hydroxyindoles were determined by assay method 2. Fig. 5:4 shows duplicate estimations of the metabolites formed at the different pH values chosen. The optimum pH for 5-HLE formation proved to be in the region of 7.4.

THE TIME COURSE OF FORMATION OF 5-HYDROXYINDOLE METABOLITES

Using optimal pH (7.4) and co-enzyme conditions (excess $NADPH_2$) incubations were continued over a 4 hr. period to determine the curves for the time course of the formation of the 5-hydroxyindole metabolites. The mean results of duplicate estimates are shown graphically

Table 10:4

The Effect of Various Concentrations of Ethanol on the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Rat Brain Homogenates in the Presence of Reduced Nicotinamide Adenine Dinucleotide Phosphate.

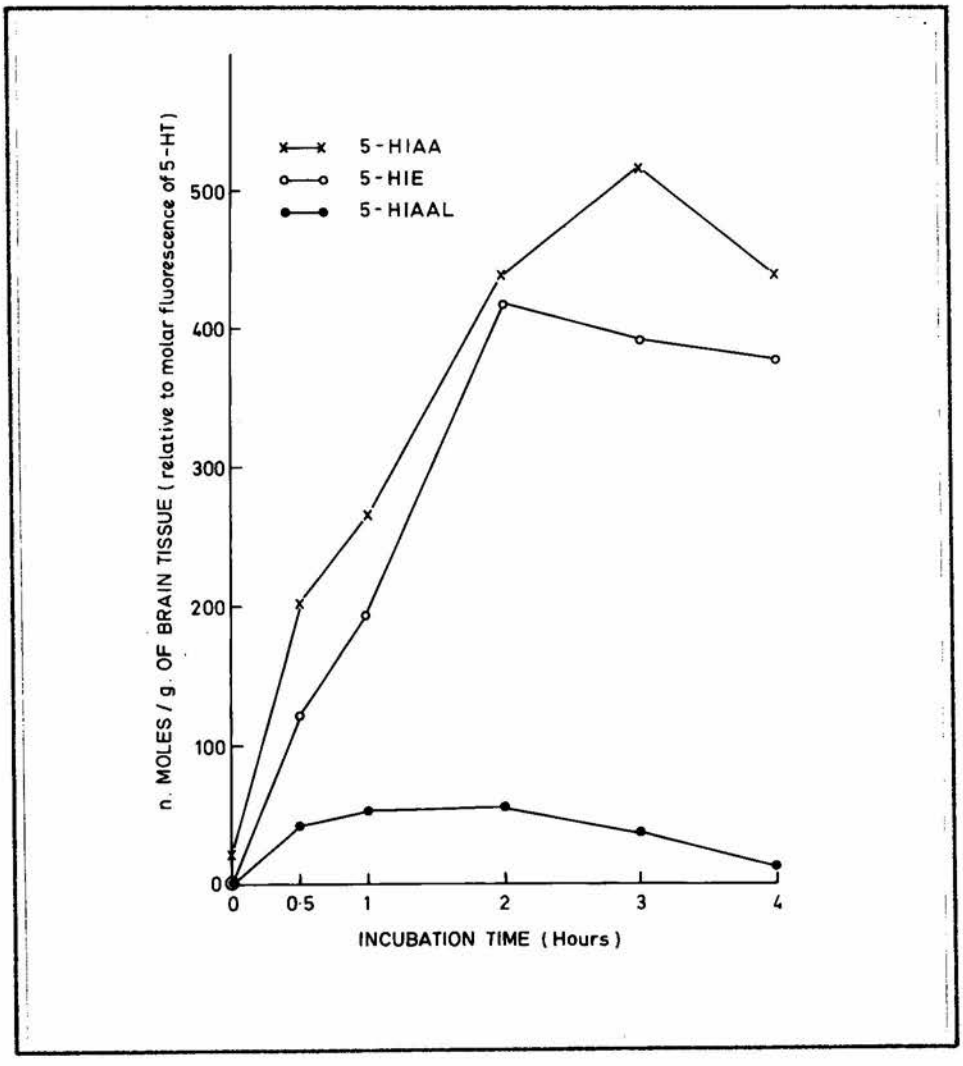
Ethanol (Molar Concentration)	5-Hydroxyindole	Time of Incubation (min.) 0	30	60
None (Control)	5-HIAAL	12	166	376
	5-HIAA	0	99	235
	5-HIE	3	72	122
10^{-2} M	5-HIAAL	5	276	286
	5-HIAA	19	99	217
	5-HIE	3	107	159
10^{-3} M	5-HIAAL	11	206	254
	5-HIAA	15	91	227
	5-HIE	3	76	141
10^{-4} M	5-HIAAL	4	241	244
	5-HIAA	15	89	220
	5-HIE	2	97	123

Results n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of brain homogenates incubated at 37°C for the times shown in the presence of 450 n.moles of 5-HT and 1 mg of $NADPH_2$.

The results were determined by "Assay Method 2" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

Fig. 6:4

The Time Course of the Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Rat Brain Homogenates in the Presence of Reduced Nicotinamide Adenine Dinucleotide Phosphate



1 ml samples of rat brain homogenate in 0.25 M sucrose were incubated at 37°C for the times shown in the presence of 1 mg of $NADPH_2$ and 450 n.moles of 5-HT in 1 ml of sodium phosphate buffer at pH 7.4. The results were determined by "Assay Method 2" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

in Fig 6:4. The rates of formation of both 5-HIAA and 5-HIE were fairly constant up to about 2 hrs. of incubation. It appeared that 5-HIAAL reached a steady state after about 30 min. Incubation for 2-3 hr. produced maximum quantities of 5-HIAA and 5-HIE but periods in excess of 3 hours produced lower yields, probably the result of the decomposition of these unstable compounds.

THE EFFECTS OF ETHANOL ON THE FORMATION OF 5-HT METABOLITES FROM 5-HTP IN VITRO

It was thought that the action of ethanol in the *in vitro* system under study might be of great interest as it was possible that its presence might act as a "pool" in which 5-HIAL might become "trapped", thus the action of the alcohol dehydrogenase on the 5-hydroxyindoles 5-HIAAL and 5-HIAL would have taken place essentially only in the direction of reduction and the yield of 5-HIE should have increased. The effects of varying amounts of ethanol in the incubation mixtures of rat brain homogenates in the presence of excess $NADH_2$ on the production of the 5-hydroxytryptamine metabolites a re shown in Table 10:4. With the higher concentrations of ethanol there was an increase in the amount of 5-HIE formed. The amount of 5-HIAAL produced after 30 min. incubation was also greater when ethanol was present. Whether this was due to some transient "pooling" effect

Table 11:4

The Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Homogenates of Human Midbrain and Cortex in the Presence of Reduced Pyridine Co-enzymes.

Co-enzyme	5-Hydroxyindole	Region of Brain	
		Midbrain	Cortex
$NADH_2$	5-HIAAL	59	90
	5-HIAA	135	347
	5-HIE	34	31
$NADPH_2$	5-HIAAL	34	40
	5-HIAA	82	196
	5-HIE	467	603

Results n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of brain homogenates incubated at 37°C for 1 hour in the presence of 450 n.moles of 5-HT and 1 mg of either reduced nicotinamide adenine dinucleotide ($NADH_2$) or reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$). The results were determined by "Assay Method 2" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 58% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

due to acetaldehyde possibly protecting initially against the loss of 5-HIAL by polymerisation must remain a matter for speculation.

[illegible]

Two studies were carried out with homogenates of human brain. These samples were obtained 3-4 hr. after death and kept at -15°C until required. The subsequent experiment was performed within 24 hr. The homogenates were made in 0.5 M sucrose, as for rat brain, and the incubations and assay procedure were carried out according to assay method 2.

In the initial experiment homogenates from the midbrain region and from the grey matter of the cerebral cortex were incubated for 1 hr. with an excess of either $NADH_2$ or $NADPH_2$ at pH 7.4. The results (Table 11:4) showed that these homogenates had a high capacity to form 5-HIE under these conditions, that $NADPH_2$ is the more effective co-enzyme for the formation of 5-HIE and that the homogenate of cortical grey matter appeared to be the more efficient at 5-HIE formation.

The other experiment carried out with human brain homogenates compared the amounts of the metabolites formed from 5-HT by homogenates from different regions

Table 12:4

The Formation of 5-Hydroxyindol-3-ylacetaldehyde (5-HIAAL), 5-Hydroxyindol-3-ylacetic Acid (5-HIAA) and 5-Hydroxytryptophol (5-HIE) by Homogenates of Various Regions of Human Brain in the Presence of Reduced Nicotinamide Adenine Dinucleotide Phosphate

Region of Brain	5-Hydroxyindole		
	5-HIAAL	5-HIAA	5-HIE
Corpus Callosum	16	0	68
Putamen	24	51	125
Thalamus	41	15	146
Ammon's Horn	64	41	154
Amygdala	50	44	158
Globus Pallidus	33	5	151
Caudate	32	115	151

Results n.moles of 5-hydroxyindole/g (wet wt.) brain. 1 ml of brain homogenates incubated at 37°C for the times shown in the presence of 450 n.moles of 5-HT and 1 mg of $NADPH_2$.

The results were determined by "Assay Method 2" and are uncorrected for recovery. The approximate molar fluorescence relative to 5-HT is 38% for 5-HIE, 74% for 5-HIAA and undetermined for 5-HIAAL.

of human brain after 1 hr. incubations at pH 7.4 in the presence of excess NAD^+. Table 12:4 shows the results which again demonstrate that the human brain homogenates under these conditions had a very marked ability to form 5-HIE. It was also interesting to find that all the regions of "grey matter" from the central nuclei showed similar capacities for 5-HIE formation. The amounts of 5-HIAA produced varied markedly between the duplicates and so little importance can be attached to the average figures quoted except to note that much greater amounts of 5-HIE were formed. The region of "white matter" from which homogenates were made was from the rostral portion of the corpus callosum, an area known to be devoid of neurones. The homogenates from this region formed only half the amount of 5-HIE produced by the homogenates of "grey matter" and the formation of 5-HIAA was not detected. The ability of human brain homogenates to form a larger ratio of 5-HIE to 5-HIAA than rat brain homogenates may be due to the differences between the regions of brain studied, or to a relative destruction of aldehyde dehydrogenase caused by freezing the samples of human brain, or it may represent a true species difference.

DISCUSSION

Identification of 5-Hydroxyindole Metabolites Formed after Incubation of 5-Hydroxytryptamine with Brain Homogenates

"5-HIAL" gave a brown colour with 2:4 dinitrophenylhydrazine thus suggesting the presence of a carbonyl group. It was possible to "trap" the substance with sodium metabisulphite in the incubation media. There is no other 5-hydroxyindolic substance which could be formed from 5-HT which would have the above properties, apart from 5-HIAL. Also suggestive of this was the finding that occasionally 5-HIAL would be lost during an assay and a brown pigment formed. Weissbach, Redfield and Udenfriend, (1957) found brown pigments formed during their attempts to isolate 5-HIAL and concluded that these pigments were polymers of the aldehyde.

"5-HIE" formed by the brain homogenate was found to be isographic with authentic 5-HIE in the five different solvent systems reported here, and in several other systems not reported as they failed to give good separation of 5-HIE from other 5-hydroxyindoles. The ultraviolet absorption maxima and λ_{max} of "5-HIE" when formed by brain homogenates was identical with that of authentic 5-HIE.

The "5-HIAA" formed during incubation had the

properties of a 5-hydroxyindolic acid and could only be 5-HIAA from the *in vitro* conditions used. The "5-HIAA" formed was isographic with authentic 5-HIAA in several solvent systems.

Factors Influencing the In Vitro Metabolism of 5-Hydroxytryptamine

The initial experiments performed with assay method 1 are difficult to analyse precisely due to the poor chromatographic separations of the 5-hydroxyindoles and also due to the possible interaction of disulfiram and the ethanol, which acted as its solvent, which were both present in the incubation media. However, these experiments allowed us to detect the formation of 5-HIE and 5-HIAAL *in vitro* and suggested that their formation involved specific pyridine nucleotide co-enzymes.

The formation of 5-HIE by brain homogenates occurred most efficiently at physiological pH (7.4) and this pH was used in carrying out experiments to find what other factors had effects on the metabolism of 5-HT by brain homogenates.

Feldstein and Wong (1965) showed that rat liver homogenates were capable of converting 5-HT into 5-HIAAL, 5-HIAA and 5-HIE. They suggested the liver as the major site for the conversion of 5-HT to 5-HIE. However,

urinary 5-HIE could be derived also partly from brain and the present experiments provide evidence for this possibility and have shown that both rat and human brain homogenates possess the enzymatic mechanisms to convert 5-HT to 5-HIAAL, 5-HIAA and 5-HIE.

The synthesis of 5-HIE in rat and human brain is effected by an alcohol dehydrogenase which has a much higher affinity for NADP/$NADPH_2$ than for NAD/$NADH_2$. Feldstein and Wong (1965) found $NADH_2$ to facilitate the formation of 5-HIE by liver alcohol dehydrogenase, and NAD to facilitate the formation of 5-HIAA by liver aldehyde dehydrogenase (for the formation of 5-HIAA.) The present experiments have shown that the aldehyde dehydrogenase of brain has similar co-enzyme requirements for both NAD and NADP, but the specific requirements of brain alcohol dehydrogenase for NADP/$NADPH_2$ distinguish this enzyme from the corresponding liver enzyme. These differences in co-enzyme specificity have a parallel in liver ethanol dehydrogenase and the retinal alcohol dehydrogenase retinene reductase (Zachman and Olson, 1961: Futterman, 1963).

The pharmacological significance of the complete pathway involving the formation of 5-HIAAL and 5-HIE is not understood. Neither compound exhibits the activity

of 5-HT on the rat's uterus but, since the equilibrium of the oxidation-reduction phases can be altered by the availability of NADP or $NADPH_2$, situations in which this ratio of metabolism to the alcohol or aldehyde metabolite can be altered in brain by the use of drugs or other agents may be important to cerebral function.

In brain homogenates the addition of ethanol resulted in a slightly increased yield of 5-HIAL and 5-HIE above that occurring in the presence of excess $NADPH_2$ alone. In mammary gland and liver slices, ethanol affects the activity of NADP-dependent enzymes of the pentose phosphate shunt pathway (Beaconsfield and Reading 1964). Ethanol similarly affects the utilisation of the pentose shunt pathway in rat brain homogenates similar to these studied and in slices of rat brain cortex (Reading, personal communication). Thus, in vivo, ethanol would be expected not only to act as a metabolic "pool", "trapping" the 5-HIE but also to increase the amount of $NADPH_2$ available by its own metabolism to acetaldehyde and the subsequent oxidation of acetaldehyde. In 1963 Ridge demonstrated that following the ingestion of ethanol by rats, fluctuations occurred in brain acetaldehyde concentrations and that these fluctuations were accompanied by fluctuations with a similar time

course in the brain concentrations of pyruvate, lactate, α-oxoglutarate and NAD. These findings are consistent with the view that acetaldehyde metabolism is linked to carbohydrate metabolism through the NAD/$NADH_2$ co-enzyme system and a similar linkage might be expected to occur for ethanol and the 5-hydroxyindole pathway through the NADP/$NADPH_2$ co-enzyme system. It is also possible that the increased concentrations of acetaldehyde occurring after ethanol ingestion may inhibit competitively the oxidation of 5-HTAL to 5-HIAA because of the marked potential of the acetaldehyde itself to be oxidised by aldehyde dehydrogenase.

Kveder et al. (1962) showed that the glucuronide of 5-HTE was a normal constituent of rat urine. Davis, Cashaw, Huff and Brown (1966) demonstrated that 5-HTE, its sulphate and glucuronide all occurred in human urine as minor metabolites after the oral administration of ^{14}C-5-HT and that these compounds could be isolated in the urine of patients with carcinoid tumours. In a later short communication (Davis, Brown, Huff and Cashaw 1966), these workers reported that normally only 2% of the urinary metabolites of orally administered ^{14}C-5-HT was present as 5-HTE and its conjugates. If however, ethanol was ingested one hour before the ^{14}C-5-HT, 46%

of the urinary metabolites of the isotopically labelled 5-HT was present as 5-HIE and its conjugates.

These studies raise the possibility that the metabolism of 5-HT to 5-HIE might be responsible to some extent for the psychotropic effects of ethanol. There are several other important possible points of interaction to consider. It may be that the reduction of 5-HIAAL to 5-HIE causes some degree of inhibition of tryptophan-5-hydroxylase an enzymic reaction shown to require the reduced form of a co-enzyme such as $NADPH_2$ (Gal, Armstrong and Ginsoorg, 1960). In this way the synthesis of 5-HT might be inhibited. It is also evident from the present studies that mono-amine oxidase may utilise the NAD+/$NADH_2$ co-enzyme system thus, in regions where 5-HIAAL was preferentially reduced to 5-HIE it is possible that the action of mono-amine oxidase would be facilitated and that lower concentrations of 5-HT would be present in such areas.

Conversion of 5-HIAAL to 5-HIE may be considered to be a possible route of metabolism of 5-HT in brain but there are several ways in which this metabolic pathway may be utilised. A first possibility is that if pulses of 5-HT are released from neurons high localised concentrations will result which would give rise to similar

quantities of 5-HIAAL, part of which may be "shunted" to 5-HIE. This 5-HIE could then be oxidised to 5-HIAAL and 5-HIAA during a quiescent period. A second possibility is that both 5-HIAAL and 5-HIE, as neutral compounds, will easily pass the "brain-blood barrier". Bartholini, Pletscher, and Bruderer (1964) have shown that blood platelets contain mono-amine oxidase and alcohol dehydrogenase, the former of which can utilise 5-HT and the latter, indol-3-ylacetaldehyde or 5-HIE as substrates. They also found that red blood cells contain an aldehyde dehydrogenase which will convert 5-HIAAL to 5-HIAA. The balance of these enzymes in normal rabbit blood in vitro was found to be such that both 5-HT and 5-HIE were promptly metabolised to 5-HIAA. Thus, it is probable that even if cerebral 5-HT was metabolised to 5-HIE in vivo most of this compound would not be isolatable as such. A third possibility is that 5-HIE might become conjugated in vivo, probably as the glucuronide or sulphate. While conjugation is normally associated with liver and kidney metabolism there is evidence that some conjugation of alcohols such as 5-HIE might occur in the brain, at least in rats (Goldstein and Gerber, 1963, Schanberg, personal communication).

While 5-HIE has not yet been demonstrated to be a cerebral metabolite of 5-HT in the rat or man *in vivo*, it has been shown to be in the cat (Eccleston, personal communication). The difficulty in detecting 5-HIE formation *in vivo* will be appreciated if it is considered that although the metabolism of noradrenaline in brain *in vitro* has been shown to go predominantly to the alcohol metabolites, 3,4-dihydroxyphenylglycol and 3, methoxy-4,hydroxyphenylglycol (Rutledge and Jonason, 1967) there is so far no report of their detection from the brain *in vivo*. However, Kopin has recently claimed to have detected 3, methoxy-4-hydroxyphenylglycol in human C.S.F. (Kopin personal communication) and it may be possible to initiate *in vivo* studies of these alcohol metabolites in the near future. The results of such studies of cerebral metabolism involving the formation of alcohols from the biogenic amines will however always be difficult to interpret because of the reversible nature of the reaction governed by the alcohol dehydrogenase, the lipid solubility of the alcohols and the probable ability of these alcohols to form conjugates both intra- and extra-cerebrally.

SUMMARY

A method is described for demonstrating the formation of 5-hydroxyindol-3-ylacetaldehyde (5-HIAAL) and 5-hydroxytryptophol (5-HIE) from 5-HT by homogenates of rat and human brain and for their subsequent quantitative assay. The optimum pH for the incubation system was found to be in the region of pH 7.4. The reduction of 5-HIAAL to 5-HIE by brain homogenates required $NADPH_2$ and in this respect the brain alcohol dehydrogenase differed from the co-enzyme requirements which have been reported elsewhere for the corresponding liver enzyme. The oxidation of 5-HIAAL to 5-hydroxyindol-3-ylacetic acid in brain homogenates was facilitated by the addition of NAD or NADP but occurred in the absence of both. The effects of ethanol in the metabolism of 5-HT were demonstrated *in vitro*. The potential importance of the formation of 5-HIE in brain metabolism is discussed.

GENERAL SUMMARY

L-tryptophan and D,L-5-hydroxytryptophan were administered to rats. The increased metabolism along the 5-hydroxyindole pathway appeared to be governed by tryptophan 5-hydroxylase. The maximum concentrations of 5-hydroxyindolic metabolites in whole brain which could be achieved by tryptophan administration could be exceeded by the administration of 5-hydroxytryptophan. However, 5-hydroxytryptophan produced only slight increases in the cerebral concentrations of 5-HT.

Examination of the concentrations of 5-HT and 5-hydroxyindol-3-ylacetic acid in various regions of dog brain revealed that there were regional differences in the metabolism of 5-hydroxyindoles and it was possible to demonstrate the existence of a pattern of distribution in normal dogs for the concentrations of total 5-hydroxyindolic metabolites between the various brain areas. The administration of tryptophan or 5-hydroxytryptophan to dogs produced increases in the concentrations of 5-hydroxyindolic metabolites in brain. However, while the normal pattern of the various regions for the total 5-hydroxyindolic metabolites was maintained following tryptophan administration a new pattern agreeing with that for the distribution of the concentrations of 5-hydroxy-

tryptophan decarboxylase was established after the administration of 5-hydroxytryptophan.

The above findings were interpreted as demonstrating that the rate of metabolism of cerebral 5-hydroxyindoles is normally governed by an intracerebral tryptophan 5-hydroxylase and also that it is necessary to take account of intracellular localisations of enzymes, their co-enzymes and substrates in explaining the responses of cerebral metabolism to precursor administration.

The administration of tryptophan by a single intravenous injection to dogs was used to allow the inhibitory actions of α-methyl dopa on cerebral metabolism of 5-hydroxyindoles to be studied. The conclusions reached were that, although the biphasic action of the precursor load made interpretation difficult it appeared likely that α-methyl dopa and its metabolites acted competitively as substrates at all enzymic steps in the 5-hydroxyindole metabolic pathway.

The technique of intravenous infusion of tryptophan in dogs was employed in an attempt to produce a "steady state" of increased cerebral metabolism of 5-hydroxy-indoles. This technique was employed to study the action of the monoamine oxidase inhibitor, phenelzine on the cerebral metabolism of 5-hydroxyindoles. In addition to inhibition of monoamine oxidase, the results showed that

inhibition of tryptophan 5-hydroxylase and efflux of 5-hydroxyindol-3-ylacetic acid from brain and cerebrospinal fluid also occurred.

The series of experiments involving phenelzine and tryptophan infusions showed evidence for an interrelationship between the metabolism of tryptophan and dopamine in the caudate nucleus of dogs.

A technique was described for the recirculatory perfusion with donor cerebrospinal fluid of the cerebral ventricular system of conscious unrestrained dogs. The characteristics and potential use of this technique were discussed. The efflux of 5-hydroxyindol-3-ylacetic acid, 3methoxy-4-hydroxyphenylacetic acid and inulin from the system were studied. It was demonstrated that inulin was removed only by displacement of cerebrospinal fluid from the system, while 5-hydroxyindol-3-ylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid were removed from the cerebrospinal fluid by diffusion and an active transport mechanism as well.

A technique was described demonstrating the formation of 5-hydroxytryptophol and 5-hydroxyindol-3-ylacetaldehyde from 5-hydroxytryptamine by the action of homogenates of rat and human brain. Methods for the estimation of 5-hydroxytryptophol, 5-hydroxyindol-3-ylacetic acid and 5-hydroxyindol-3-ylacetaldehyde in such homogenate

preparations were developed.

The effects of various substances in the formation of these metabolites by rat brain homogenates were examined. The substances studied included pH, pyridine nucleotide co-enzymes, sodium metabisulphite, hydroxylamine hydrochloride, disulfiram and ethanol.

APPENDIX I

TABLES

TABLE 1

Concentrations of 5-HT, 5-HIAA and 5-HTP in Rat Brain Tissue following Intraperitoneal Injection of Varying Doses of L-Tryptophan

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

Mean ± SD (number of experiments)

Except where stated, results are from a single experiment. Each result represents an estimation on the pooled brains of three rats. 5-HTP <0.05 µg/g in all samples.

Table quoted from Eccleston, Ashcroft and Crawford 1965.

Table 2

5-Hydroxyindol-3-ylacetic Acid Concentrations in Cisternal Cerebrospinal Fluid Following Tryptophan Administration To Dogs

Latin Square I

Experiment	Time in Hours			
	0	1	2	4
1	26	44	94	39
2	17	57	37	48
3	26	49	88	35
4	25	51	48	52

The concentrations of 5-HIAA in mμg/ml in cisternal samples of C.S.F. obtained from the cisterna magna of dogs which had been given 50 mg/Kg of tryptophan at zero time. The experiments were performed at intervals of two weeks. Each experiment involved the use of the same four dogs which were sampled at different times after tryptophan administration according to a Latin Square design.

TABLE 3

Concentration of 5-Hydroxyindoles and Tryptophan in Different Regions of the Brains of Six Control Dogs

Region	Dogs 1	2	3	4	5	6	Mean ± Standard Deviation
Midbrain							
Tryptophan	6.1	10.0	4.3	6.4	3.4	-	6.84 ± 2.09(5)
5HT	0.79	0.64	0.85	0.79	1.05	0.94	0.84 ± 0.14(6)
5HIAA	0.75	1.42	0.72	0.76	1.22	0.60	0.92 ± 0.19(6)
Hypothalamus							
Tryptophan	7.0	9.5	4.5	9.5	7.3	-	7.56 ± 2.08(5)
5HT	1.02	1.01	0.77	0.45	0.77	0.85+	0.81 ± 0.21(5)
5HIAA	0.57	0.91	0.27	0.42	0.53	0.48+	0.53 ± 0.21(5)
Thalamus							
Tryptophan	9.5	9.7	8.0	6.3	5.6	-	7.82 ± 1.85(5)
5HT	0.61	0.35	0.31	0.28	0.20	0.31	0.34 ± 0.14(6)
5HIAA	0.79	0.50	0.25	0.18	0.27	0.40	0.40 ± 0.22(6)
Caudate							
Tryptophan	6.1	5.8	3.4	3.3	7.0	-	5.12 ± 1.68(5)
5HT	0.35+	0.28	0.28	0.28	0.36	0.36	0.32 ± 0.04(5)
5HIAA	0.21	0.16	0.08	0.27	0.13	0.28	0.19 ± 0.08(6)
Hippocampus							
Tryptophan	8.1	7.8	4.1	5.7	5.4	-	6.22 ± 1.69(5)
5HT	0.45	0.20	0.44	0.44	0.56	0.56	0.44 ± 0.13(6)
5HIAA	0.14	0.11	0.17	0.35	0.17	0.18	0.19 ± 0.08(5)
Hindbrain							
Tryptophan	6.6	9.8	5.7	6.5	6.1	-	6.92 ± 1.09(5)
5HT	0.34	0.29	0.28	0.28	0.41	0.26	0.31 ± 0.06(6)
5HIAA	0.24	0.62	0.21	0.42	0.37	0.33	0.37 ± 0.15(6)

All concentrations in ug/g of brain tissue

No 5HT and 5HIAA was detected in the cerebellum or cortex of any dog (limit of detection 0.06 for each)

+ Best estimates made of missing samples to minimise the error of the sum of squares of the whole table (Finney 1964)

TABLE 4

The Effect of Tryptophan Administration on the Concentrations of Tryptophan and 5-Hydroxyindoles in Various Regions of Dog Brain.

Region	Time								
	1 hour			2 hours			4 hours		
Midbrain									
Tryptophan	31.5	25.4	40	34.9	43.5	40	6.8	12.2	11
5HT	1.40	2.31	1.77	2.28	1.72	1.34	1.15	1.04	1.10
5HIAA	1.23	2.12	1.91	4.62	2.55	2.82	1.71	1.59	2.08
Hypothalamus									
Tryptophan	38	28.8	41.3	29.8	40.5	37.6	4.8	4.4	11.5
5HT	1.56	1.95+	1.86	1.46	1.96	1.43	2.25	0.89	1.05
5HIAA	0.86	1.36+	1.20	0.93	3.34	1.70	1.39	1.23	1.37
Thalamus									
Tryptophan	39	28.1	33.4	31.7	39.5	38.8	5.8	13.3	9.8
5HT	0.94	0.77	0.50	0.47	0.73	0.54	0.35	0.69	0.25
5HIAA	0.37	0.86	0.69	0.52	1.66	1.26	0.51	0.54	0.31
Caudate									
Tryptophan	32.4	23.2	40	18.7	43.3	31	6.2	11	7.4
5HT	0.64	0.58	0.54	0.37	0.64	0.57	0.34	0.25	0.28
5HIAA	0.24	0.24	0.30	0.42	0.78	0.52	0.50+	0.47	0.30
Hippocampus									
Tryptophan	36	24.8	40	31.4	40.7	34.8	6.2	11.1	7.7
5HT	0.88	0.88	0.76	0.60	0.72	0.63	0.51	0.47	0.22
5HIAA	0.41	0.44	0.38	0.37	0.79	0.41	0.55	0.28	0.25
Hindbrain									
Tryptophan	32.8	27	40	38.6	44.5	45.8	7	13.1	11.3
5HT	0.73	0.95	0.57	0.32	0.72	0.51	0.53	0.38	0.51
5HIAA	1.06	1.43	0.41	0.91	1.66	1.70	1.22	0.98	0.96

Concentrations of 5HT and 5HIAA (ug/g of brain tissue) in various regions of dog brain following administration of intravenous tryptophan 50 mg/Kg. No 5HT and 5HIAA was detected in the cerebellum or cortex of any dog. (Limit of detection 0.06 for each)

+ Best estimate made of missing sample to minimise the error of the sum of squares in the time block concerned (Finney 1964)

Table 5

Concentrations of Noradrenaline, Adrenaline, Ketanepherine, Dopamine, Methoxy-dopamine, 3-Methoxy-4-Hydroxyphenylacetic Acid and 3-4-Dihydroxyphenylacetic Acid in Hypothalamus and Caudate Nucleus of Normal Dogs.

Substance Assayed	Caudate	Hypothalamus
NA	0.2	0.40 ± 0.12
A	0.03	0.08 ± 0.03
M	0.05	0.05
DA	5.5 ± 1.4	0.15 ± 0.05
MDA	0.38 ± 0.39	0.06
HVA	13.4 ± 1.14	2.5 ± 0.10
DOPAC	13 ± .27	0.09 ± 0.03
5-HIAA	0.35± 0.06	0.67 ± 0.11

Results in ug/g. of brain tissue. Mean ± Standard Deviation,

APPENDIX II

Diet for Dogs Used for Chronic Experiments

There have been numerous reports that the dietary concentration of phenylalanine is capable of influencing the concentrations of brain 5-hydroxytryptamine in rats. Because of this the dogs used for the experiments reported in Sections II and III of this thesis were maintained on a 'diet'. The purpose of this was to minimise any alterations in the cerebral metabolism of 5-hydroxyindoles caused by dietary factors throughout the duration of the experimental period.

Each dog was given daily 13½ oz. of "Chum", 4 oz. "Lassie" (Petfoods Ltd.) and 4 oz. of "Ful-O-PEP" (Quaker Oats Ltd.) at approximately 3 p.m. On the day prior to those on which experiments were performed food was withdrawn at 5 p.m., water was available overnight, and the experiments were started in the morning.

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