BIOCHEMICAL EFFECTS OF ELECTRICAL STIMULATION OF THE RAT BRAIN, WITH PARTICULAR REFERENCE TO 5-HYDROXYTRYPTAMINE

by

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SUMMARY

In Section 1 the effects of electrical stimulation of the midbrain raphe on the synthesis of 5-HT in the rat brain are described. Relative rates of synthesis were determined by measuring the amount of (^{3}H)5-HT made from intraperitoneally-injected (^{3}H)tryptophan. Pargyline, a monoamineoxidase inhibitor, was given before the (^{3}H)tryptophan to prevent catabolism of (^{3}H)5-HT. An inhibition of 5-HT synthesis by p-chlorophenylalanine was shown by this method. Electrical stimulation of the midbrain in the region of the median raphe nucleus produced an increase in (^{3}H)5-HT synthesis of over 100 per cent. Optimum stimulation parameters were found to be a current of 0.2 mA and frequency of 10/sec, with the electrode tip positioned approximately 1 mm above the centre of the median raphe nucleus. Stimulation produced however no change in the total rate of accumulation of 5-HT in the presence of the MAO inhibitor. This suggested that 5-HT might be stored in two or more separate pools in the brain, and a model was formulated to account for the observations. Further experiments were conducted to test the proposed model. LSD was shown using the same method to reduce the synthesis of (^{3}H)5-HT without reducing the total accumulation of 5-HT. Adrenalectomy was found to increase the total synthesis of 5-HT, while the formation of (^{3}H)5-HT suggested there was no increase in 5-HT synthesis. Thirdly it was shown that after labelling of 5-HT stores by giving (^{3}H)tryptophan, raphe stimulation led to a biphasic disappearance of (^{3}H)5-HT. It was concluded that 5-HT is synthesised and stored in nerves in at least two independent pools, one of which is functionally active and

is released by the nerve impulse. The synthesis of the amine in the functional pool is closely linked to the activity of the nerve.

Experiments were designed to test the hypothesis that the accelerated rate of 5-HT synthesis which occurs on raphe stimulation persists after the cessation of stimulation. 5-HT synthesis was determined both by the method described above and by the technique of tryptophan loading. In neither case was any increased synthesis found after the end of the stimulation period.

Section 2 describes the effects of raphe stimulation on RNA and protein synthesis. RNA synthesis was determined by the rate of incorporation of intraventricularly-injected (^{3}H)uridine into RNA. Stimulation at currents upto 0.4 mA was found to increase RNA synthesis by upto 60 per cent, but at currents of 0.6 and 0.8 mA, there was an apparent fall in synthesis. Measurement of levels of (^{3}H)nucleotides suggested that this reduction might have been due to depletion of precursor nucleotide pools. Protein synthesis was determined by the rate of incorporation of intraperitoneally-injected (^{14}C)leucine into protein. No changes were found following raphe stimulation.

In Section 3 the effects of electroconvulsive shock (ECS) on the metabolism of 5-HT in the rat brain are described. Various alternative methods for the measurement of 5-HT synthesis were compared. A method for the purification of (^{3}H)5-HIAA was developed. It was found however that measurement of (^{3}H)5-HT and (^{3}H)5-HIAA levels after (^{3}H)tryptophan injection did not give a good indication of 5-HT synthesis, as judged by the effects of both raphe stimulation and ECS. It was also shown that the technique of tryptophan loading is probably

not generally useful for the determination of 5-HT synthesis. It was concluded that the most satisfactory method for the present study was simply the measurement of endogenous 5-HT and 5-HIAA levels.

A single shock was found to increase the brain 5-HIAA level, measured 3 hours after the shock. A series of 8 daily shocks had no greater effect than a single shock however. When 5-HT and 5-HIAA were measured 24 hours after the last of a series of upto 16 shocks, no changes were found. It was concluded that ECS causes only a short-lasting increase in 5-HT synthesis in the brain.

ABBREVIATIONS

cpm	counts per minute
CSF	cerebrospinal fluid
DA	dopamine
dpm	disintegrations per minute
ECS	electroconvulsive shock
ECT	electroconvulsive therapy
EDTA	ethylene diamine tetra-acetic acid, disodium salt
EEG	electroencephalogram
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
HVA	homovanillic acid
i.p.	intraperitoneally
i.vtr.	intraventricularly
MAO	monoamine oxidase
NA	noradrenaline
N.S.	not significant
P	probability
PCA	perchloric acid
PCP	para-c hlorophenylalanine
r	coefficient of linear correlation
s.c.	subcutaneously
S.D.	standard deviation
S.E.M.	standard error of the mean
TCA	trichloroacetic acid

SECTION 1

THE EFFECTS OF ELECTRICAL STIMULATION OF THE RAT MIDBRAIN ON THE SYNTHESIS OF 5-HYDROXYTRYPTAMINE

INTRODUCTION

In this work the effects of electrical stimulation of the rat midbrain on the metabolism of 5-hydroxytryptamine (5-HT) were studied. There is now considerable evidence that 5-HT is a neurotransmitter substance in the mammalian central nervous system (1), and it is thought to be located in the CNS exclusively in nerve cells (2). The information obtained from the study of the effects of stimulating these neurones is mainly of two types. Firstly, it provides a knowledge of the basic biochemical mechanisms of the serotonergic (that is, 5-HT-containing) neurone, which is fundamental to an understanding of the working of the system as a whole. Secondly, these facts are necessary for the interpretation of biochemical findings in terms of the functional activity of the serotonergic neurones. A considerable number of possible functions have been ascribed to the 5-HT system in the CNS, and often an investigation involves an examination of the effects of a certain experimental situation on the metabolism of the amine. In order for measurements of its rate of metabolism to be meaningful, the relation between the activity of the 5-HT-containing neurones and the metabolism of the amine must be established.

A specific example of this is the study of amine metabolism in depressive illness. There is a good deal of evidence implicating 5-HT in the aetiology of depression: this evidence is discussed in more detail in Section 3. Obviously the means of assessing the functional activity of the 5-HT system in man are very limited; probably the most used approach is the measurement of the levels of metabolites of 5-HT in the cerebrospinal fluid (CSF) (3). With the

aid of results obtained in experimental animals, elaborate theories implicating 5-HT and other substances in the cause of mental diseases have been formulated, based on analyses of CSF (4).

In the following paragraphs a survey of the present knowledge of two relevant areas is presented. Firstly, the metabolism and storage of 5-HT in the rat brain, and the subcellular localisation of the amine and its associated enzymes; secondly, the effects of electrical stimulation of nervous tissue on the metabolism of 5-HT, and also of noradrenaline in peripheral nerves, as the noradrenaline-containing sympathetic nerve is often used as a model for monoaminergic neurones in the CNS.

Note: In the present context, 'monoamine' is used as a collective name for 5-HT, noradrenaline and dopamine.

Metabolism and storage of 5-HT in rat brain

From the fluorescence histochemical studies of Dahlström and Fuxe (2), it appears that 5-HT in the rat brain is contained only in nerve cells. All the cell bodies of 5-HT-containing neurones are located in the midbrain and brainstem, mostly in the raphe nuclei, and axons project from the cell bodies to all parts of the brain and the spinal cord (2,5). Nine groups of cells showing fluorescence characteristic of 5-HT are described; the caudal nuclei mostly send axons to the spinal cord, and the rostral nuclei send axons to the forebrain. These groups of cells correspond fairly well to the raphe nuclei in the cat as described by Taber, Brodal and Walberg (6).

The existence of pathways from the midbrain to the forebrain has been further substantiated by the measurement of forebrain 5-hydroxyindole levels after lesions in these pathways had been made

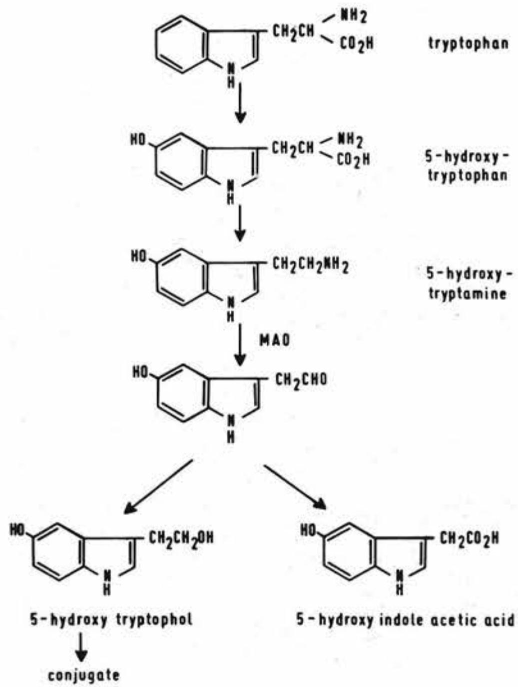

Fig. 1.1. Metabolism of 5-HT in rat brain.

(7, 8, 9), and also by following characteristic changes in the axons and cell bodies after lesioning (10, 11).

The principal metabolic pathway of 5-HT is shown in Fig. 1.1. As the blood-brain barrier is impermeable to the amine (12), it must either be synthesised from tryptophan in the brain or from 5-hydroxytryptophan (5HTP) taken up from the blood. 5-HTP however is normally undetectable in rat plasma (13); therefore the hydroxylation of tryptophan must take place in the brain itself. As the evidence described below will show, the amine is probably synthesised in the nerves where it is found.

L-tryptophan is converted to 5-HTP by the enzyme tryptophan 5-hydroxylase; its presence in brain has been shown by several groups (14, 15, 16, 17, 18). Its regional distribution in the brain was shown by Peters et al. (19) to parallel the distribution of both 5-HT and 5-HT-containing nerve terminals (as described by Dahlström and Fuxe (5)). 5-HTP is decarboxylated to 5-HT by 5-HTP decarboxylase, an enzyme widely distributed in the brain (20). The hydroxylation is the rate-limiting step in the synthesis: Ichiyama et al. (16) showed that in vitro the decarboxylase activity was far in excess of the hydroxylase, and in vivo Moir and Eccleston (21) reached the same conclusion on the results of giving 5-HTP or L-tryptophan. The latter report as well as several others (13, 22, 23, 24) also indicates that the rate of hydroxylation is highly dependent on the level of tryptophan in the brain, which in turn depends on the plasma tryptophan level.

Theoretically, 5-HT could also be synthesised by decarboxylation of tryptophan to tryptamine and then hydroxylation. However, due to

the relative affinities of the enzymes involved for their alternative substrates (16), this pathway is unimportant: tryptamine is not normally detectable in rat brain (25).

5-HT is catabolised by monoamine oxidase (MAO) to 5-hydroxyindole acetaldehyde, and thence mostly to 5-hydroxyindol-3-yl acetic acid (5-HIAA) by aldehyde dehydrogenase. Some of the aldehyde may be converted to 5-hydroxy-tryptophol by alcohol dehydrogenase, as shown in brain slices in vitro (26). Bulat et al. (27) perfused the subarachnoid space in the cat spinal cord with 5-HT and showed a conversion to 5-hydroxy-tryptophol, the amount formed being 10-15 per cent of the 5-HIAA. The concentration of 5-HT used was very high, however. Eccleston (unpublished observations) has found that the normal level of 5-hydroxy-tryptophol in the rat brain is less than one per cent of the 5-HIAA, and also that when small amounts of 5-HT are perfused through the cat ventricles, the conversion to 5-hydroxy-tryptophol is negligible.

It might be possible that some 5-HT is removed from the brain by conjugation with sulphate and thus excreted. However, 5-HT O-sulphate is undetectable in rat brain (28).

Subcellular distribution of 5-HT and associated enzymes

By fluorescence microscopy Fuxe has shown that 5-HT in brain is largely concentrated in nerve endings (5). Subcellular fractionation of brain homogenates by Whittaker (29) and Michaelson (30) showed that a substantial fraction of the 5-HT in brain is associated with pinched-off nerve endings or 'synaptosomes'. This distribution was also confirmed by Aghajanian and Bloom (31), who used autoradiography to localise intraventricularly-injected (^{3}H)5-HT: they found

that most of the radioactivity was located in nerve terminals and unmyelinated axons. In addition, they noted that most of the terminals which showed activity contained so-called dense-cored vesicles, which suggests that 5-HT may be stored in such vesicles in the nerve ending. This is supported by the findings of Maynert et al. (32), who fractionated disrupted synaptosomes, and showed that some of the 5-HT was associated with vesicles of 500 to 1,000 A° diameter.

Subcellular fractionation studies by Green and Sawyer (33) showed that 40 to 60 per cent of the tryptophan hydroxylase was present in the 'crude mitochondrial' fraction. Further fractionation into synaptosomal and mitochondrial fractions by Grahame-Smith (34) and Ichiyama (16) indicated that most of the activity of the crude mitochondrial fraction was contained in the synaptosomes rather than in the mitochondria. Very little activity was recovered in the supernatant fraction. It has, however , been possible to obtain active, partially-purified preparations of the enzyme in a soluble form (16, 34, 35): thus it remains unsettled whether this is normally a soluble enzyme.

Ichiyama et al. (16) found that when a system was set up in vitro synthesising (^{14}C)5-HT from (^{14}C)tryptophan, no (^{14}C)5-HTP mixed with exogenous 5-HTP added to the reaction mixture. This, and the fact that 5-HTP is virtually undetectable in brain even after tryptophan loading (13), suggests that the hydroxylase and decarboxylase may be bound together as a unit on a membrane surface in the nerve ending.

5-HTP decarboxylase is a soluble enzyme as far as is known (36), and its activity is distributed throughout the different

subcellular fractions (16). As it is present in great excess compared to the hydroxylase, a small membrane-bound fraction could well go unnoticed.

Monoamine oxidase is thought to be located exclusively in the mitochondria (37).

Effects of electrical stimulation of nervous tissue on monoamine metabolism

(a) Effects of stimulation of the raphe nuclei on 5-HT metabolism

The effects of midbrain stimulation on 5-HT metabolism were first investigated by Aghajanian, Sheard and co-workers (38, 39). They found that stimulation of the raphe nuclei in the rat gave an increase in 5-HIAA and a decrease in 5-HT in the forebrain, which indicates that stimulation increased 5-HT synthesis. Sites of stimulation in the midbrain which gave an increase in forebrain 5-HIAA were confined to the median and dorsal raphe nuclei, corresponding closely to the areas of 5-HT-containing nerve cell pericarya as described by Dahlström and Fuxe (2). They also showed that in animals in which the median forebrain bundle had been lesioned on one side, raphe stimulation failed to increase forebrain 5-HIAA on that side of the brain. The results lend support to the existence of a specific neural pathway of 5-HT-containing nerves from the raphe nuclei to the forebrain. Increases in 5-HIAA in the rat brain on stimulation of the raphe nuclei have also been shown by Kostowski et al. (40) and Gumulka et al. (41, 42). In addition, Sheard and Zolovick (43) stimulated the median raphe nucleus in the cat and showed a large rise in the 5-HIAA content of the cisternal CSF, and Eccleston et al. (44) found that stimulation of the median raphe in the rat gave an increased

efflux of 5-HIAA from the surface of the cerebral cortex. Holman and Vogt (45) perfused one lateral ventricle in the cat and measured the 5-HT in the perfusate, which rose by about 50 per cent when the raphe was stimulated.

A long-term effect of raphe stimulation was described by Eccleston et al. (46). The median raphe in the rat was stimulated for an hour, and 5-HT synthesis was estimated by the technique of tryptophan loading. The elevated rate of synthesis in the stimulated animals persisted for at least an hour after the end of the stimulation period.

(b) Effects of electrical stimulation of nervous tissue in vitro on 5-HT metabolism

Katz and Kopin (47) showed that radioactively labelled 5-HT previously taken up by brain slices was released by electrical stimulation. Anden et al. (48) found that in the presence of an MAO inhibitor, the amount of 5-HT released from the frog spinal cord in vitro rose by two to three times on stimulation.

(c) Effects of stimulation of catecholamine-containing nerves

An increased synthesis of noradrenaline (NA) on stimulation of peripheral adrenergic nerves has been observed in many cases (e.g. 49, 50, 51), the increased synthesis rate being accompanied by little or no depletion of the amine.

In the central nervous system, Dahlström et al. (52) found that stimulation of the medulla in the rat gave a reduction of NA in the spinal cord, the reduction being enhanced by an inhibitor of NA synthesis, thus indicating an increased catabolism of the amine. Similarly, Arbuthnott et al. (53) showed that in the presence of an

inhibitor of catecholamine synthesis, stimulation of certain noradrenaline and dopamine (DA)-containing nuclei in the rat brainstem depleted these amines in nerve terminals in the forebrain, again demonstrating an increased catabolism.

Another example of stimulation increasing monoamine catabolism, and therefore also probably increasing the synthesis and release of the amine, is described by Portig and Vogt (54). Stimulation of the substantia nigra, where the majority of DA-containing cell bodies are located (2), gave an increase in the level of homovanillic acid (the main metabolite of DA) in the ventricular CSF.

It is thus apparent that stimulation of neurones containing 5-HT, NA or DA leads to an increased synthesis, release and breakdown of the amine. In most cases the increased turnover is accompanied by little or no change in the level of the amine, indicating the existence of sensitive control mechanisms which maintain its level when the rate of catabolism is increased.

Statement of the problem

The experiments described in this section were designed to investigate the mechanism of the increase in 5-HT synthesis which occurs on raphe stimulation, with particular attention being paid to the long-term effects reported by Eccleston *et al.* (46). These authors suggested that stimulation might induce synthesis of new tryptophan hydroxylase enzyme; their results may however have been affected by the very high tryptophan concentrations which result from tryptophan loading. It was also intended to investigate the optimum stimulation parameters in more detail than was reported by Sheard and Aghajanian (38).

Before the effects of stimulation on 5-HT metabolism could be investigated however, a suitable method for measuring the rate of synthesis of the amine had to be chosen. In the following paragraphs, the various alternative methods available are described, and their relative advantages and drawbacks discussed. The actual method used in this work, and the reasons for the choice, are described in the Results section.

Methods for the measurement of 5-HT synthesis in brain

The individual advantages and drawbacks of the various available methods are described here, followed by some general considerations which apply to most of the methods.

An important assumption in any method for determining 5-HT synthesis in the brain is that the 5-HT, and 5-HIAA if measured, are made only in the brain, and do not come from the blood. This assumption does appear to be justified: Axelrod and Inscoe (12) showed that (^{14}C)5-HT scarcely penetrates to the brain when given intravenously, although Bulat and Supek (55) did find that 5-HT would pass from the blood to the brain if very large intravenous doses were given. Roos (56) showed that systemically-given 5-HIAA failed to enter the brain.

(i) Measurement of 5-HT and 5-HIAA levels

Certain experimental situations may alter 5-HT levels, and at one time reports were presented where the 5-HT level alone was used as an indication of the functional activity of the amine. As described above however (in the section on the effects of electrical stimulation of nerves), the amine level bears little relation to its rate of synthesis and breakdown. For example, reserpine lowers 5-HT

levels while actually increasing synthesis (57).

The level of 5-HIAA, the principal metabolite of 5-HT, can on the other hand be used as an indication of amine synthesis. The concentration of 5-HIAA is dependent on its rate of formation from 5-HT, and its rate of efflux from the brain. It is transported from the brain to the blood in the rat by an active transport system (58, 62), similar to the transport system also existing between the cerebrospinal fluid (CSF) and the blood in the dog (59). These transport systems resemble the renal tubular secretion of organic acids (59). The rate of efflux of 5-HIAA from the brain is directly proportional to its concentration, as shown by Tozer et al. (57) and Perez-Cruet et al. (60), who measured the decline of the acid after inhibition of MAO. Thus measurement of the 5-HIAA concentration will provide an index of 5-HT synthesis. The 5-HIAA level may also give an approximate measure of 5-HT synthesis under non-steady state conditions, although this could be misleading if, for example, 5-HT were displaced by a drug. 5-HT levels should be measured at the same time.

The advantages of this approach are that the situation is entirely physiological, involving no drug or other treatment, and it requires fewer animals than some of the other methods.

(ii) Decline of 5-HIAA after MAO inhibition

An extension of the simple measurement of 5-HIAA levels which has been used to obtain absolute, rather than relative, values for 5-HT synthesis rates, is the technique of measuring the decline of 5-HIAA after inhibition of MAO (Tozer et al. (57) and Perez-Cruet et al. (60)). As mentioned above, the elimination of 5-HIAA from the brain

was found to follow first-order kinetics, and values for the synthesis of 5-HT were obtained similar to other methods.

This method is subject to several limitations, which also apply to the interpretation of endogenous 5-HIAA levels as described in paragraph (i). Firstly, it depends on there being a first-order relationship between the 5-HIAA concentration and its efflux rate. Although the results of Perez-Cruet _et al._ (60) indicate that this holds for 5-HIAA levels upto at least 80 per cent above normal, it will not be true for all concentrations of the acid, as it is removed by an active transport system, which should follow normal enzyme kinetics and therefore be saturable. Secondly, steady-state conditions must exist, and after some drugs such as p-chlorophenylalanine (PCP), such conditions are not reached for a long time, if at all. It must also be assumed that the MAO inhibitor does not affect the acid transport system, and that alternative pathways of 5-HT catabolism such as to 5-hydroxytryptophol are negligible. This last condition may not always be true, as shown _in vitro_ in brain slices (26); however ventricular perfusion studies suggested that negligible amounts of 5-hydroxytryptophol are formed _in vivo_ (D. Eccleston, unpublished observations).

(iii) _Accumulation of 5-HIAA after probenecid administration_

The drug probenecid, which blocks the transport of certain organic acids in the kidney tubules (61), also blocks the active transport of 5-HIAA from the rat brain (62) and from the CSF in the dog. Neff and Tozer (63) reported that after administration of probenecid to rats, 5-HIAA in the brain built up linearly for as long as eight hours, reaching six times the normal level: the drug also

almost completely halted the decline in 5-HIAA after MAO inhibition (62). These results indicate that very little 5-HIAA leaves the brain other than by the active transport system.

Thus 5-HT synthesis may be measured by the rate of accumulation of 5-HIAA after probenecid. This raises the question of whether the accumulated 5-HIAA or other acid metabolites in the brain might be toxic or exert a feedback inhibition on the synthesis of 5-HT. These possibilities seem unlikely as the 5-HIAA level continues to increase linearly when it is several times normal. Satelli (64) did report an effect of intraventricularly-injected 5-HIAA on cortical evoked potentials, but the doses given were very high.

The rate of 5-HT synthesis as measured this way was found by Neff _et al._ (62) to be about the same as that calculated from the rate of fall of 5-HIAA after MAO inhibition, suggesting probenecid itself did not affect synthesis.

(iv) _Accumulation of 5-HT after MAO inhibition_

The rate of accumulation of 5-HT after inhibition of MAO has been used to measure its rate of synthesis (57, 65). This method assumes that no 5-HT is lost from the brain by diffusion, that MAO is completely inhibited, and that no other catabolic pathways are significant. A further assumption is that inhibition of MAO does not affect the rate of synthesis of the amine, either through product-inhibition as the level of the amine rises, or by a direct effect of the inhibitor.

There is some evidence for the existence of a product-inhibitory mechanism in the 5-HT pathway. After administration of a MAO inhibitor, 5-HT in the brain builds up linearly for about an hour

and eventually plateaus at around three times the normal level (57). That a plateau is reached probably indicates either a loss of the amine by diffusion, or a feedback-inhibition of its synthesis. The possibility of product-inhibition was investigated by Macon <u>et al.</u> (66), who measured the amount of (^{3}H)5-HT synthesised in a 15-minute period from intraventricular (^{3}H)tryptophan, in the presence of a MAO inhibitor. There was a reduction in (^{3}H)5-HT synthesis when measured three hours after the administration of the MAO inhibitor, compared to the initial period just after giving the drug.

Another piece of evidence suggesting that MAO inhibition is not without physiological effect is the work of Aghajanian <u>et al.</u> (67), who found that a variety of MAO inhibitors depress the firing rates of raphe neurones, the effect appearing gradually over a period of about 30 minutes. This was possibly due to the accumulation of 5-HT, as there was no depression of firing after previous depletion of 5-HT by PCP.

The fact that 5-HT initially builds up linearly when MAO is inhibited (57) does not preclude the possibility that its synthesis is reduced immediately that the drug is given. Tozer <u>et al.</u> (57) however obtained similar values for 5-HT synthesis this way as they did by other methods.

(v) <u>Catabolism of intraventricularly-injected radioactive 5-HT</u>

5-HT synthesis has been estimated by following the rate of disappearance of radioactively-labelled 5-HT injected into the ventricles or cisterna magna (68, 69). 5-HT given this way is distributed in the rat brain in 5 to 10 minutes (70), but it is very questionable whether its distribution is exactly the same as the

endogenous amine: there is evidence that it may enter catecholamine-containing or other cells (68, 71). However Simmonds (68) found that this method gave results similar to those obtained by measuring 5-HT accumulation after MAO inhibition.

(vi) Tryptophan loading

Another method which has been used as an index of the rate of 5-HT metabolism is that of 'tryptophan loading'. This consists of giving the animal a large dose of L-tryptophan so that tryptophan hydroxylase becomes saturated by its substrate, and measuring the resultant increases in 5-HT and 5-HIAA. The pattern of metabolites is the same as under normal conditions (21). This technique does not of course give an exact quantitative estimate of 5-HT synthesis, as 5-HT and 5-HIAA are turning over all the time: Ashcroft, Eccleston et al. (13, 72) showed that after rats had received a dose of 800 mg/kg of L-tryptophan i.p., 5-HT increased to a maximum after one hour, and 5-HIAA was maximal after four hours. The advantage of this approach however compared to other methods is that it gives a dynamic profile of the whole metabolic pathway. It would indicate for example whether a certain experimental situation was acting on the synthesis or breakdown of 5-HT, or the excretion of 5-HIAA.

The disadvantage of the method is that a rather unphysiological situation is produced. The level of tryptophan in the brain is many times normal (13), and the saturation of the pathway may upset its regulatory mechanisms. Aghajanian (73) has found a reduction in raphe firing rates after administration of tryptophan; as with MAO inhibition, the reduced firing is probably linked to the elevated 5-HT levels.

(vii) Infusion of (^{14}C)tryptophan

Lin et al. (74) have estimated 5-HT synthesis in rat brain by infusing tracer quantities of (^{14}C) tryptophan intravenously, and measuring the (^{14}C)5-HT formed. They deduced the rate of synthesis by a kinetic analysis, and obtained results comparable to other methods. This method is physiological in the sense that no drugs are used, but the infusion requires that the animals are restrained, which produces stress. Also, for the method to be practically useful, it must be assumed that the time course of the specific activity of plasma tryptophan is not altered in any experimental situation, which may well not be the case. Ideally, the time course should be measured in every experiment.

(viii) Single injection of radiolabelled tryptophan

A single injection of radiolabelled tryptophan has been used to overcome the difficulty of intravenous infusion. This approach was used by Azmitia et al. (75), who measured (^{3}H)5-HT in brain at two fixed times after an intravenous injection of (^{3}H)tryptophan. When using this method it is essential to measure the (^{3}H)5-HT during the rising phase of its specific activity; if measured during the falling phase, an increased turnover might give a reduced (^{3}H)5-HT level. This may be difficult to msure as brain 5-HT stores are labelled very rapidly after a systemic injection of (^{3}H)tryptophan (76, 77).

This method has been used to measure noradrenaline (NA) synthesis in a variety of tissues, including brain, by Gordon et al. (78) and Sedvall et al. (49). It is much more applicable in this case however; NA has a slower turnover in the brain than 5-HT, and the stores are labelled more slowly (49).

Schubert et al. (76) followed the disappearance of (^{3}H)5-HT in brain after labelling 5-HT stores by an intravenous injection of (^{3}H)tryptophan, in order to study the effects of LSD on 5-HT metabolism. Only qualitative results for 5-HT turnover were obtained this way. Neff et al. (77) used this method too, but applied kinetic arguments to derive a figure for the absolute rate of amine synthesis. This method is simple and efficient, and the results obtained were comparable to others.

(ix) Other methods

Anden, Corrodi, Fuxe and co-workers have used the rate of depletion of 5-HT after inhibition of its synthesis as an index of the synthesis of the amine (79). Large doses of tryptophan hydroxylase inhibitors such as α-propyldopacetamide were used, which may well have other effects: this drug for example also inhibits tyrosine hydroxylase.

Carlsson et al. (80) have measured the rate of accumulation of 5-HTP after inhibition of the decarboxylase enzyme. Again, large doses of inhibitors which may have other effects were used.

General considerations

Apart from the details described above for each method, there are also some more general considerations, each of which applies to several of these methods.

Firstly, the problem of compartmentation. There is now good evidence that NA in peripheral sympathetic nerves is stored in more than one 'pool', and evidence will be presented and discussed later that a similar situation also exists for 5-HT in brain. Some of the methods described above assume that the 5-HT is stored in a

single, open compartment, such as the isotopic methods of Lin et al. (74) and Neff et al. (77). They justified this assumption on the basis that their methods gave results almost identical to other, non-isotopic methods. It could be the case however that under the conditions tested, a small pool of the amine was not quantitatively significant, but if this pool were of functional significance, it could become quantitatively important under different conditions. These objections also apply to the use of intraventricularly-injected labelled 5-HT and the decline of 5-HT after synthesis inhibition as methods for measuring 5-HT synthesis. What these methods are in fact measuring is not the true rate of synthesis of the amine, but its turnover, i.e. the rate of replacement of the amine stores. It can no longer be assumed though that 'synthesis' and 'turnover' are synonymous. In peripheral adrenergic nerves, Sedvall et al. (49) have shown that because the possibility of compartmentation had previously been neglected, the synthesis rate of NA had been thought to be less than half its true value.

Secondly, some of the available methods require a steady-state condition, i.e. conditions where the 5-HT and 5-HIAA levels are constant at the time of doing the experiment. This applies to the measurement of 5-HIAA decline after MAO inhibition, and again to the isotopic methods of Lin et al. (74) and Neff et al. (77). Isotopic conditions may be used under non-steady state conditions, but then the indications are only qualitative as the kinetic analysis no longer applies.

A third consideration is whether the method in question will detect changes in 5-HT synthesis caused by changes in tryptophan levels.

Obviously the technique of tryptophan loading cannot do this, as it depends on the saturation of tryptophan hydroxylase by its substrate, tryptophan. In the case of isotopic methods where use is made of systemically-given labelled tryptophan, errors may arise if changes in brain or plasma tryptophan levels are not allowed for. In all the isotopic methods so far quoted however, this was taken into account.

It is concluded therefore that there is no one ideal method for measuring 5-HT synthesis in the brain. The limitations of each method must be considered, and the most appropriate method chosen for each experimental situation. In addition, the interpretation of the results must be made bearing in mind these limitations.

METHODS

Materials

Radiochemicals were obtained from the Radiochemical Centre, Amersham. The specifications were:

L-(^{3}H)tryptophan (Generally labelled), 500-1,000 mCi/m.mole

DL-(^{3}H)5-hydroxytryptophan (Generally labelled), 100-500 mCi/m.mole

DL-(^{14}C-methylene)tryptophan, 52 mCi/m.mole

Pargyline hydrochloride was obtained from Abbott Laboratories, and Fluothane from ICI Ltd.

Lysergic acid diethylamide (LSD) tartrate was obtained from Sandoz Ltd.

Scintillator fluids were composed as follows:

Triton X-100 scintillator:

1,000 ml toluene

500 ml Triton X-100 (Rohm and Haas Co.)

4.0 g PPO (2,5 diphenyloxazole)

0.1 g POPOP (1,4 di(2-(4-methyl,5-phenyloxazolyl))benzene)

Toluene scintillator:

2,500 ml toluene

10.7 g PPO

0.28 g POPOP

All reagents were of Analar grade or better.

Water used was distilled throughout.

Glassware was cleaned in chromic acid, preceded by 'Decon' if radioactive. Rats were Wistar males, weighing 150-250g.

'Normal' saline was a 0.9 per cent solution of NaCl in water.

Estimation of 5-HT and 5-HIAA in rat brain

5-HT was estimated by the method described by Eccleston et al. (25) for tryptamine, and applied by Eccleston et al. (26) to 5-HT. Whole brains were weighed and homogenised in 5 ml ice-cold 0.4N perchloric acid (PCA) containing 2 mg/ml ascorbic acid. The homogenate was centrifuged at 15,000 g for 10 min at 4°C, and the supernatant was adjusted to pH 7.5 (glass electrode) using successively 5N, 1N and 0.1N KOH. The solution was cooled to 4°C and centrifuged at 250g for 5 min to remove precipitated $KClO_4$. The supernatant was then passed over a 70 mm high by 5 mm diameter column of Amberlite CG50 resin (NH_4^+ form, 100-200 mesh).

Before use, the resin was converted from the H^+ form to the NH_4^+ form as described by Eccleston et al. (25): it was first stirred for 30 min with 1N HCl, the acid decanted off, and this repeated twice. The resin was then washed with distilled water until chloride ions were no longer detectable in the supernatant on the addition of a few drops of 2.5% $AgNO_3$ solution. It was then stirred twice for 30 min with 3N NH_4OH, and washed repeatedly with distilled water until the pH

of the supernatant was 9. Finally the resin was washed with 0.2M ammonium acetate buffer pH 7.5 until a constant pH 7.5 was reached, and it was then stored in this buffer. Before the column of resin was used, it was washed with 10 ml 0.02M ammonium acetate buffer pH 7.5.

The effluent from the column was collected, and 1 ml 0.02M ammonium acetate buffer pH 7.5 run through and collected with the effluent. The column was washed with 14 ml of this buffer, and then with 4 ml 0.1N H_2SO_4. 5-HT was eluted with 6 ml H_2SO_4, 1N.

5-HIAA was estimated by the method of Eccleston et al. (81), slightly modified. The column effluent solution was saturated with excess NaCl (about 4 g), and about 10 mg ascorbic acid and 1 drop conc. HCl were added, before extraction into ether. Peroxide-free ether was prepared on the day of the experiment by shaking diethyl ether with saturated $FeSO_4$ solution, and washing twice with distilled water. 5-HIAA was extracted into 20 ml ether, NaCl was removed by brief centrifugation (2 min at 250g) and the 5-HIAA was extracted from the ether-phase into 6 ml 0.1M sodium phosphate buffer pH 6.5. Phosphate buffer was used instead of borate buffer as described by Eccleston et al. (81), as a lower background fluorescence was obtained. The indoles were estimated fluorimetrically in a Perkin-Elmer model MPF-2A fluorescence spectrometer. 1 ml conc. HCl containing 3 mg/ml ascorbic acid was added to 2 ml of sample immediately before reading, and the fluorescence at 530 nm measured in an activation scan from 260 to 400 nm. The slits were set at 4 to 6 (excitation) and 40 (emission), and emission filter 39 was used. The value of 3 mg/ml ascorbic acid in the conc. HCl was chosen from the results of Thompson et al. (82):

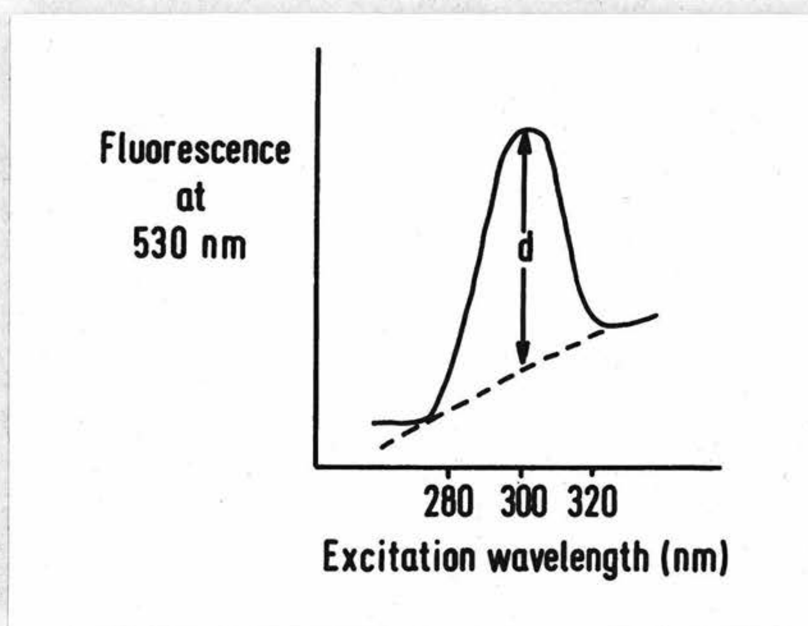

Fig. 1.2. Fluorescence spectrum of 5-HT or 5-HIAA.

their figures show that the concentration of ascorbic acid in the final mixture is fairly critical.

The appearance of the spectra of 5-HT and 5-HIAA is shown in Fig. 1.2; the peak occurs at 300 nm. The reading was corrected for blank fluorescence as shown, the distance d being taken as the true indole fluorescence. Standard solutions containing 0.1 or 0.2 µg 5-HT or 5-HIAA per sample were read at the same time.

Recovery of 5-HT and 5-HIAA

Recoveries were determined by homogenising two brains together, dividing this into two equal parts and adding 1 µg each of 5-HT and 5-HIAA to one part. These two samples were then processed in parallel with the others. The average recoveries were:

5-HT - 87 per cent ± 3.5 (25)

5-HIAA - 82 per cent ± 4.5 (15)

(The results show mean ± standard deviation with the number of measurements in brackets)

All results are corrected for recovery, and are expressed as µg 5-hydroxyindole per gram of brain weight. The mean brain weight was 1.69 ± 0.09 g.

If the recovery was not measured in an experiment, the mean value of all previous results was used. There was no noticeable trend in recovery values during the course of this work.

Estimation of radioactivity in samples

Radioactivity (^{3}H or ^{14}C) in samples was determined by liquid scintillation in a Nuclear-Chicago Mk.2 counter. Aqueous samples were dissolved in a scintillation fluid containing toluene, PPO, POPOP and the nonionic detergent, Triton X-100 (see 'Materials' section).

Usually 1 ml of sample was added to 10 ml fluid. In the case of 5-HT samples, which were usually of low activity, 2 ml of the Amberlite column eluate was added to 19 ml scintillator with 1 ml water. Without the addition of the water, a precipitate sometimes formed.

The only non-aqueous samples counted were paper chromatography strips. These were counted in a toluene-PPO-POPOP scintillation fluid.

Counting efficiency was determined using the external standard of the scintillation counter. Firstly, a calibration curve was constructed as follows. A number of samples were prepared containing 10 ml Triton X-100 scintillator, a standard amount of (^{3}H) or (^{14}C) toluene, and upto 200 microlitres of chloroform as a quenching agent. The samples were counted in the automatic external-standard mode. Values were calculated for the counting efficiency of each sample and the corresponding ratio of counts in the two channels of the external standard. The equation for a quadratic curve to fit the experimental points was then calculated by the method of least-squares (see section on 'Statistical Methods'), and thus the counting efficiency of any sample could be calculated from its external-standard channels-ratio. Counting efficiency was in the range 25-35% for tritium (^{3}H) and 65-85% for ^{14}C.

Calibration curves were constructed using 10 ml samples, but were also found to be applicable to 20 ml samples. Therefore results for both sample sizes were calculated from the same curves.

Low- activity samples were counted in polythene vials, which gave low background counts (about 20 cpm). Otherwise glass vials were used.

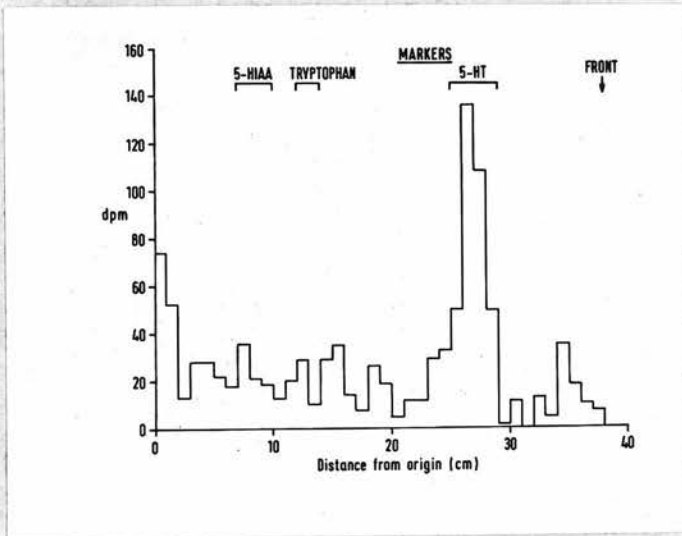

Fig. 1.3. Chromatogram of CG50 eluate.

Identification of (^{3}H)5-HT in Amberlite CG50 eluate

In several of the experiments described in this section, (^{3}H)5-HT synthesised from (^{3}H)tryptophan in the presence of pargyline is measured. The identity of the radioactive substance(s) in the CG50 eluate was determined by paper chromatography as follows.

A rat received pargyline (75 mg/kg, i.p.) followed after 15 min by (^{3}H)tryptophan (100 µCi/kg, i.p.), and was killed after a further 30 min. 5-HT was purified from the brain as described. The CG50 eluate was neutralised with 1N KOH, about 10 mg ascorbic acid added and the solution evaporated to dryness under vacuum. The residue was extracted with 0.2, and then 0.1, ml of 80% methanol - 20% water, and the solution applied under a stream of nitrogen to a 1 cm origin on a 4 cm wide strip of Whatman no.1 paper. The chromatogram was developed for 7 hr in the descending mode in an atmosphere of nitrogen in a solvent of chloroform-methanol-0.88 ammonia (12:7:1 by volume). 10 µg of 5-HT, 5-HIAA or tryptophan were each applied to separate strips as markers, these strips being developed in parallel with the test strip. After development, the chromatograms were dried and the markers located by spraying with Ehrlich's reagent (10 mg of p-N, N dimethylaminocinnamaldehyde dissolved in 50 ml acetone and acidified with 2-3 drops conc. HCl). The test chromatogram was cut into 1 cm portions, and these strips eluted by placing them in vials with 1 ml 0.1N H_2SO_4 for 15 min with occasional shaking. 10 ml Triton X-100 scintillator was added prior to counting, the strips being left in the vials.

The only significant peak of radioactivity in the chromatogram was found to correspond to the position of 5-HT (Fig. 1.3).

Estimation of tryptophan

Tryptophan was estimated in the brain at the same time as 5-hydroxyindoles by taking a fraction of the Amberlite CG50 column effluent solution. It was necessary to purify the tryptophan in this solution whether measuring endogenous or (^{3}H)tryptophan: when estimating endogenous tryptophan by conversion to norharman, perchlorate ions interfere with the reaction, and when measuring (^{3}H)tryptophan following administration of this to an animal, not all the radioactivity in the CG50 effluent is actually in tryptophan due to exchange of the tritium label, particularly with water.

Initially tryptophan was purified by the method described by Schubert et al. (76). The CG50 effluent was adjusted to pH 2.2 (glass electrode) with 1N HCl, and was passed over a 50 mm high by 5 mm diameter column of Dowex AG50W-X8 resin (K^+ form, 100-200 mesh, supplied by Bio-Rad). Before use the resin was converted from the H^+ form to the K^+ form by stirring with 1N KOH for 30 min, decanting off the solution and repeating this, and then washing with distilled water until the pH was 7. After passing the sample solution through, the column was washed with 10 ml distilled water. In the trial runs, the tryptophan was eluted with 15 ml 0.1M potassium phosphate buffer pH 6.5, followed by another 5 ml portion of the buffer, which was collected separately. However recoveries were not very high and were somewhat inconsistent. Therefore a selection of buffers was tried (Table 1.1). The most satisfactory was 0.2M ammonium chloride-ammonia pH 9.8, which gave a good recovery in 15 ml. An additional 5 ml increased the recovery by only 3 per cent; therefore 15 ml of the buffer was used in subsequent experiments.

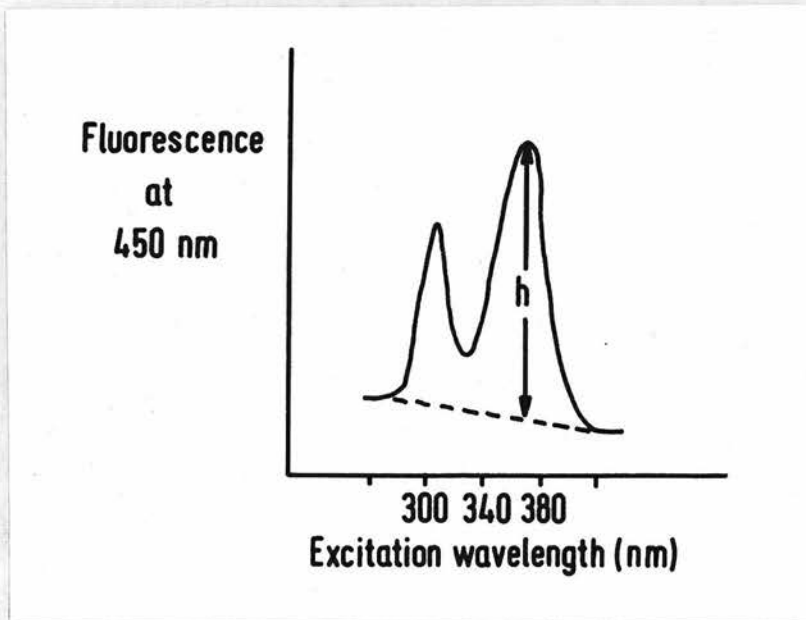

Fig. 1.4. Fluorescence spectrum of norharman.

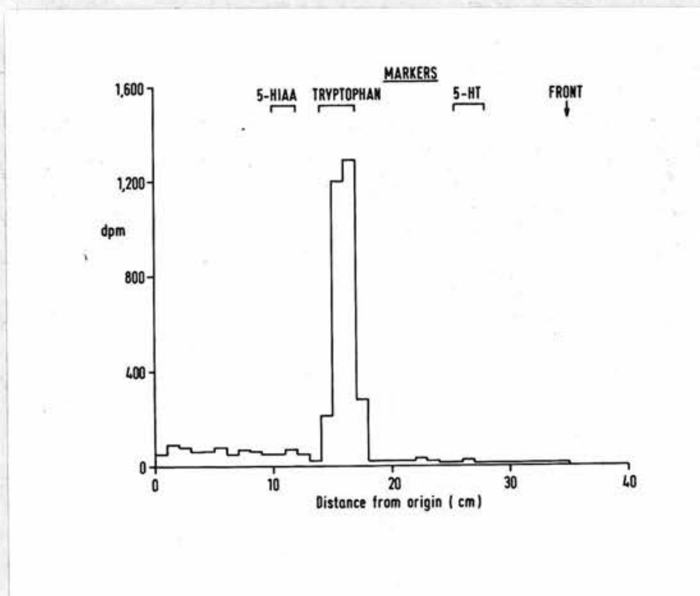

Fig. 1.5. Chromatogram of Dowex eluate.

Recoveries of tryptophan on the columns were determined by one of two methods. (a) When only radioactive tryptophan was being measured, and not endogenous, the recovery was determined by adding (^{14}C)tryptophan to a brain extract processed with the samples. The (^{14}C)tryptophan had been previously purified on a Dowex column, as described. This method of recovery was also used to compare the recoveries with different buffers. (b) When endogenous tryptophan was measured, with or without (^{3}H)tryptophan, the recovery was determined by pooling extracts from two brains, dividing into two and adding 10 μg tryptophan to one half.

The mean recovery of tryptophan was 88 per cent ± 4.2 (10).

Endogenous tryptophan was assayed by the method described by Hess and Udenfriend (83) for tryptamine, and applied by Guroff and Udenfriend to tryptophan (84), in which tryptophan is converted to norharman. 1.0 ml 2N H_2SO_4 and 0.1 ml 18% formaldehyde were added to 2.7 ml Dowex eluate, making the pH about 1. The solution was heated at 100°C in a shaking incubator for 20 min; 0.1 ml 5% H_2O_2 was then added and heating continued for 20 min. The solution was finally cooled quickly and its fluorescence measured at 450 nm with an activation scan from 250 to 420 nm. Both slits were set at 6, and no emission filter was used. The appearance of the spectrum is shown in Fig. 1.4, with peaks at 306 and 368 nm. Blank fluorescence was corrected as shown, the distance h being taken as the true norharman fluorescence.

Radioactive tryptophan was measured by dissolving 1 ml of eluate in 10 ml Triton X-100 scintillator before counting.

Table 1.1 Tryptophan recoveries on Dowex AG50W-X8

Buffer type	Percentage recovery of tryptophan		n
	In first 15 ml	In next 5 ml	
Potassium phosphate			
pH 6.5, 0.1M	59 ± 6	10 ± 1	3
pH 6.5, 0.2M	61	10	2
pH 7.5, 0.1M	50	13	1
Potassium chloride-boric acid			
pH 9.0, 0.1M	34	10	2
pH 9.8, 0.1M	33	—	2
Ammonium chloride-ammonia			
pH 9.0, 0.2M	77 ± 4	8 ± 1.6	6
pH 9.8, 0.2M	87 ± 1	3 ± 0.5	3

Results show mean recovery ± S.D.

n = number of results.

Identification of (^{3}H)tryptophan in Dowex eluate

(^{3}H)tryptophan purified as described above was identified by paper chromatography of the Dowex eluate.

The eluate was evaporated to dryness under vacuum and chromatographed as described for (^{3}H)5-HT. As more counts were expected here than with the (^{3}H)5-HT, the 1 cm strips were not eluted, but placed in 4 ml glass vials with toluene scintillator, and counted thus.

Only one peak of radioactivity was found in the chromatogram, corresponding to tryptophan (Fig. 1.5).

Measurement of acid-soluble radioactivity in plasma

The total acid-soluble radioactivity in plasma, which would include small molecules such as amino acids, was estimated as follows. On decapitation of the animal, blood was collected into a tube containing about 50 mg EDTA as an anticoagulant, and the tube was shaken vigorously. The blood was centrifuged at 1,500g for 10 min, and 1 ml of the plasma taken off and added to 4 ml 0.4N PCA to precipitate proteins. The samples were centrifuged at 2,000g for 10 min, and 1 ml supernatant added to 10 ml Triton X-100 scintillator prior to counting.

Procedure for electrical stimulation

A rat weighing 150 to 220 grams was placed in a box into which a Fluothane-oxygen mixture (approx. 5 per cent) was delivered. When anaesthetised, the rat was fixed into a David Kopf no.1530 stereotaxic frame, and anaesthetic gas was supplied to the animal by means of a plastic bag over its nose (Fig. 1.6). The concentration of anaesthetic was adjusted to give a moderately deep narcosis at this stage (about 2 per cent). Normally the sharp ear bars were

Fig. 1.6. Set-up for stimulation of raphe nucleus.

used, but in experiments where the rat was evmntually to recover, 45°-tip ear bars (David Kopf no.855) were used, which did not rupture the ear drums. After exposing the skull, the electrode, held in the electrode carrier, was aligned over the midline suture of the skull. This method was shown by histological examination to give good centring of the electrode in the brain, whereas centring initially using the ear bars was not so reliable. After marking the position of the electrode, a 2 mm diameter hole was drilled in the skull with a dental drill, and sealed with bone wax. The electrode was then lowered into the brain. After completion of surgery, the animal was kept under light anaesthesia (approx. 1 per cent Fluothane). The animal's temperature was maintained at 37-38°C by an electric heating blanket with rectal thermistor probe (Electrophysiological Instruments Ltd.). Non-stimulated controls had an electrode implanted, but no current was passed.

In recovery experiments, the hole was sealed with bone wax after withdrawal of the electrode, and the scalp wound closed with one or two Michel clips. The animals were then kept under a 200 Watt infra-red lamp in order to maintain their temperatures at 37-38°C. The intraperitoneal temperatures were checked at the time of killing using an Ellab TE3 electric thermometer with K19 needle probe. Without the lamp present, the animals' temperatures fell to around 34°.

Stimulating electrodes

The èlectrodes were concentric bipolar, made of stainless steel. They were constructed by cementing a 0.12 mm varnished stainless steel wire (Johnson Matthey Metals Ltd.) into stainless

Fig. 1.7. Stimulation electrode (Magnification x40).

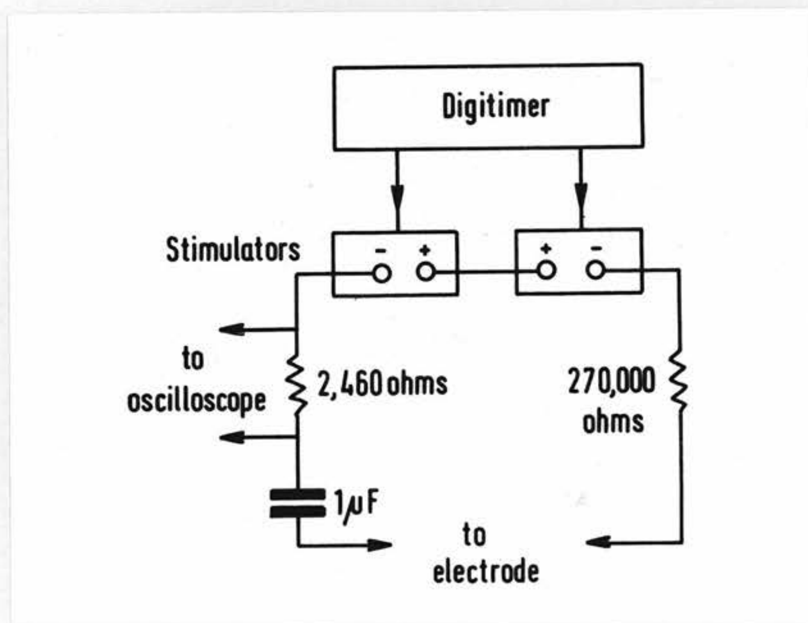

Fig. 1.8. Stimulation circuit.

steel tubing of 0.38 mm outside diameter. The cement used was Araldite varnish no. PZ985. The outside of the electrode was insulated with Bakelite lacquer no. L3128, and connecting wires were soldered on with 'Arax' acid-cored solder. The electrode is pictured in Fig. 1.7: the central wire projects 1 mm beyond the tube, and 0.4 mm of this is bared. Also the tube is bared for 0.5 mm at the end. The resistance of the electrodes was 30 to 50K ohms in 0.9 per cent saline.

Stimulation circuit

To minimise effects of polarisation of the electrode and changes in its resistance, it was decided to use constant-current pulses for stimulation. This was achieved by passing 50 to 70 volt pulses through a high resistance circuit. The current was monitored by displaying the voltage developed across a resistor on an oscilloscope (Tektronix 502A). According to Lilly (85), unidirectional stimulating pulses are injurious to nervous tissue; to avoid tissue damage, pulses should alternate in polarity, so that no net current is passed. Therefore pulses alternating in polarity were produced by two Devices Isolated Stimulators (Type 2533) connected in series and triggered alternately by a Devices Digitimer (Type 3290). The circuit is shown in Fig. 1.8. Bi-directional pulses are also necessary to avoid polarisation of the electrode: when unidirectional pulses were tried (0.2 mA, 2 mS at 10/sec) the current began to fall after only ten seconds, indicating rapid polarisation. A condenser (1 µF, 250 V) was also included in the circuit to eliminate any net current due to slight imbalance between the stimulators.

Pulses were square, with 2 mS duration. A current of

0.2 mA and frequency of 10/sec were used except where stated otherwise.

Histological examination of electrode positioning

Before any stimulation experiments were performed, the correct positioning of the electrode was ascertained by histological examination. Rats were anaesthetised and fixed into the stereotaxic frame, and an electrode was inserted into the brain at the required coordinates, and withdrawn. The animal was then decapitated and the brain removed. Anaesthetised animals were used rather than dead ones, because when the electrode is withdrawn the hole fills with blood, and the tract is easily seen when sections are made.

Production of sections

Serial frozen sections of the brain were made on a microtome as follows. After careful removal from the skull, the brain was placed with the ventral side uppermost in a cryostat at -5°C for 5-10 min, until it had a firm consistency but was not frozen. A slice about 4 mm thick was cut from the brainstem around the expected position of the electrode tract. The slice was then fixed onto the microtome chuck thus: a drop of water was placed on the end of the chuck, and the specimen placed on top with its rostral side uppermost; this assembly was then frozen rapidly in a carbon dioxide gas expansion device (South London Electrical Equipment Co.). The chuck was fixed onto the microtome, which was kept in a cryostat at -20°C (Pearse cold microtome type H, South London Electrical Equipment Co.). Serial sections 20 μ thick were taken, approximately every third one being collected on a glass coverslip. The direction of cutting was rostral to caudal, and ventral to dorsal.

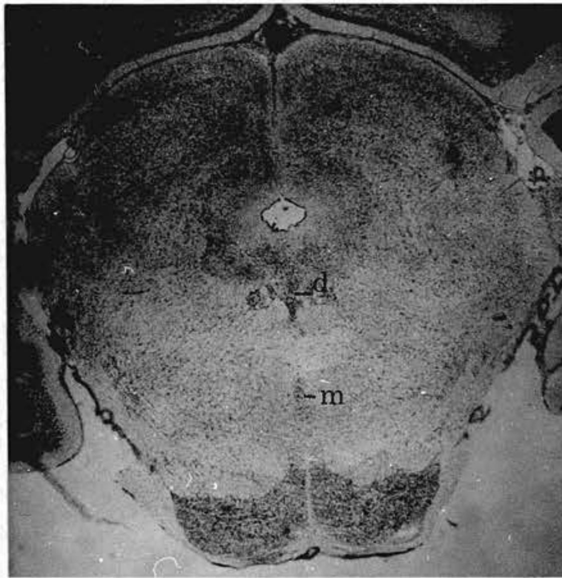

Fig. 1.9. Section of rat midbrain, at approx. A = 0.4 mm. m, median, and d, dorsal raphe nucleus. Magnification x9.

Staining of sections

Three different methods were tested for staining the sections: (a) cresyl violet; (b) luxol fast blue counterstained with cresylviolet, both as described by Carleton (86); and (c) toluidine blue (I. Laszlo, unpublished method). The toluidine blue stain was found to give the clearest separation into cell and fibre areas. The final method used was:

(1) Sections were exposed to formaldehyde gas by suspending them in a beaker containing a little formalin solution for 10 min. They were then processed in the following solutions:
(2) 100 per cent ethanol for 1 min.
(3) 50 per cent ethanol-water for 2 min.
(4) Distilled water for 2 min.
(5) 0.2 per cent aqueous solution of toluidine blue for 10 min.
(6) 50 per cent ethanol-water for 2 min.
(7) 100 per cent ethanol for 2 min.
(8) Xylene for 4 min. or more.

The sections were then mounted on slides with Canada balsam in xylene.

Appearance of sections

The sections were compared with illustrations in the stereotaxic atlas of König and Klippel (87), and were found to have a similar appearance. Fig. 1.9 shows a section of the midbrain, identified from the atlas as being about 400 μ anterior to the inter-aural line (A400 in their terminology). The median raphe nucleus is clearly visible (m). The approximate position of the dorsal raphe nucleus is indicated by d.

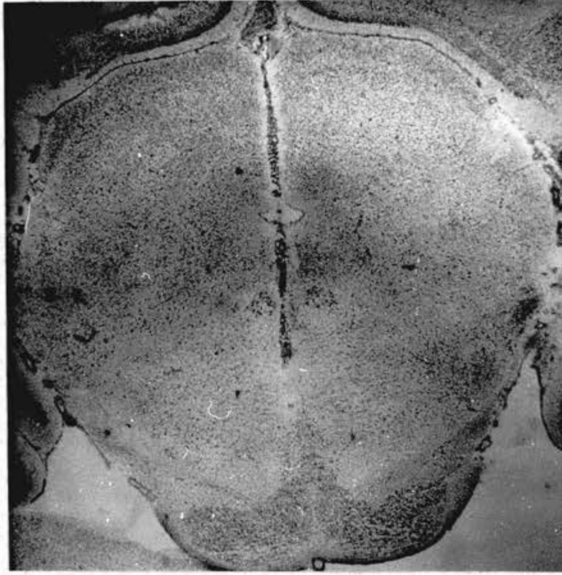

Fig. 1.10. Section of rat midbrain showing electrode tract. Magnification x9.

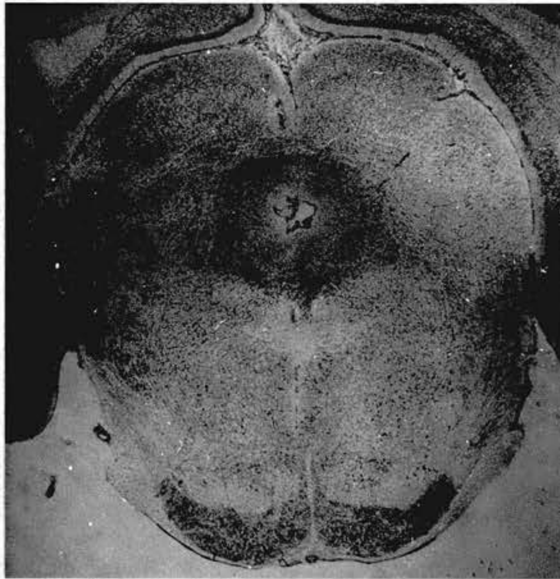

Fig. 1.11. Section of rat midbrain showing tract left by tip of electrode in the median raphe nucleus. Magnification x9.

Positioning of electrode

Initially the electrode was positioned according to the atlas of König and Klippel (87). The upper incisor bar on the stereotaxic frame was set 2.4 mm below the interaural line, as they indicate. However, when an electwode was aimed at the median raphe nucleus at a vertical co-ordinate of -2.6 mm according to the atlas, it only reached -1.8 mm (Fig. 1.10) (This figure was estimated by comparing the section with the atlas). This discrepancy occurred consistently in all of 11 trials. There is also a discrepancy within the atlas: it had been taken that the atlas horizontal zero plane was 4.9 mm above the interaural line, as described in the text, yet in the diagram of the reference planes (their Fig. 1) this figure is about 3.9 mm.

The mean positioning errors are summarised in Table 1.2, which shows the difference between the actual location of the electrode tip, and what would have been expected on the basis of the atlas, taking the horizontal zero plane as 4.9 mm above the interaural line. These figures show there was no significant difference in positioning with the two types of ear bar. Also there was no significant error in the anterior-posterior direction. The error in vertical positioning however was highly significant ($P < 0.001$, t test), the overall mean being 0.75 mm. Therefore for correct electrode positioning using this atlas, the distance between the atlas zero and the interaural line should be taken as 4.15 mm.

In the following sections of this thesis, this is simplified by defining the vertical coordinate (V) as the distance above the interaural line. The centre of the median raphe nucleus is at

Table 1.2 Differences between the observed position of the electrode tip and the position expected on the basis of the stereotaxic atlas

Type of ear bar	Error in vertical direction (mm)	Error in anterior direction (mm)
Sharp	0.78 ± 0.22(4)	0.05 ± 0.18(4)
45 deg. tip	0.74 ± 0.18(7)	-0.06 ± 0.22(7)

V = +1.6 mm, and is approximately 0.4 mm anterior to the interaural line (A = + 0.4). The lateral co-ordinate (L) is zero.

Fig. 1.11 shows the tip of an electrode tract located in the median raphe nucleus; the whole of the tract is not visible in this case as the plane of cutting has not coincided exactly with the plane of the electrode.

Adrenalectomy procedure

The rat was anaesthetised fairly deeply with Fluothane and placedwith its abdomen down on the table. The hair was shaved from the back around the area to be incised, and a ½" round rod was placed under the belly, causing the back to arch slightly. A single dorsal midline incision was then made over the spine, extending from 0.5 cm above the renal angle to 2.5 cm below it (The renal angle being the angle made by the lowest rib and the dorsal muscle mass). The skin was retracted to the right or left, and the muscle exposed by cutting through the subcutaneous fascia. An incision was then made in the muscle on one side, positioned 1 to 2 mm lateral to the firm, striated muscle mass which lies on each side of spine. The incision was about 1.5 cm long, starting close to the renal angle. The kidney was seen on retracting this incision, and the adrenal gland was located usually on the upper pole of the kidney, but in a few cases more medially. The gland was distinguished from the mass of fatty tissue which surrounded it by its pea-like shape and darker colour. The tissue surrounding the gland was gently freed from the kidney by pulling with fine forceps, and then the pedicle of the adrenal was simply cut with fine scissors. No bleeding occurred at the cut. The second adrenal was removed, and

the two incisions in the muscle closed with a single gut suture in each. The skin incision was closed with 3 to 4 Michel clips, and the area sprayed with antibiotic powder.

After surgery the animals recovered in 5 to 10 minutes. They were maintained on a 0.9 per cent NaCl-5 per cent glucose solution plus normal rat cake, and were weighed either daily or on alternate days: nearly all the rats continued to gain weight after the operation. The wounds in the skin healed rapidly, and the Michel clips were removed under brief Fluothane anaesthesia after 8 days.

Non-adrenalectomised controls were taken to the stage of exposing the kidney. After the operation they were maintained on water rather than the glucose-saline solution.

Statistical methods

The significance of the difference between two independent samples was determined by Student's *t* test, as described by Davies (88). According to Siegel (89) however, parametric tests such as the *t* test are not valid when the sample sizes are very small. Therefore when there were less than six results in each sample, the randomisation test as described by Siegel (89) was used. The type of test used here is indicated by '*t* test' or 'R test'. The choice between a one-tailed test or a two-tailed test depends on the nature of the hypothesis to be tested. If the direction of the difference between the samples is stated in the hypothesis, then a one-tailed test can be used. If however the hypothesis is only that there is a difference, positive or negative, between the samples, then the less powerful two-tailed test must be used (Siegel, 89). In this thesis, one-tailed tests are used unless stated otherwise.

Best-fit straight lines for graphs were evaluated by the method of least-squares, as described by Davies (88). The significance of the coefficient of linear correlation (r) was tested by calculating Student's t. The significance of the difference between two linear regressions was tested by Student's t test as described by Davies (88).

Best-fit quadratic curves of the form $y = ax^2 + bx + c$ were evaluated by the method of least squares, as given by Davies (88).

In this thesis, results presented in the form:

$a \pm b$ (n)

represent a mean (a) plus or minus the standard deviation (b) of n observations.

The confidence limit used in assessing statistical significance was that the probability (P) was less than or equal to 0.05.

Calculations for the randomisation test and the fitting of quadratic curves were made on the Edinburgh Regional Computing Centre IBM computer using original FORTRAN programmes. Other calculations were made on an Olivetti P101 or P602 computer, also using original programmes.

RESULTS

Choice of anaesthetic for stimulation experiments

It was necessary to find an anaesthetic which did not affect 5-HT metabolism. Diaz et al. (90) for example found a large rise in 5-HIAA during ether anaesthesia, but that Fluothane did not affect the 5-HIAA level. Therefore rats were anaesthetised with different substances and the 5-HT and 5-HIAA levels compared with controls.

One group was anaesthetised with 1 per cent Fluothane in

oxygen for three hours. Another group received 2.5 g/kg urethane, injected subcutaneously (in the scruff of the neck) under brief Fluothane anaesthesia. With urethane, induction took about an hour, and the animals were killed after a further three hours. A third group of rats received pentobarbitone sodium (30 mg/kg, i.p.) three hours before killing, and the controls were given a saline injection. The temperatures of the anaesthetised animals were maintained at 37-38°C by a heating blanket.

The results are shown in Table 1.3. The only significant alteration in indole levels was a large rise in 5-HIAA with urethane. Fluothane was therefore chosen as the anaesthetic for future experiments, as it apparently did not affect 5-HT metabolism. It was preferred to pentobarbitone as the depth of anaesthesia could be varied easily, and there would be a rapid recovery to consciousness if required.

Stimulation of the raphe nucleus

As described in the introduction, several groups have shown an increase in 5-HT synthesis on raphe stimulation. As a preliminary experiment, this was confirmed as follows. A loading dose of L-tryptophan was given in order to make any change in 5-HT metabolism more readily detectable. A suspension of L-tryptophan (80 mg/ml) was made by grinding it first with a drop of Tween 80 and a few drops of 0.9% saline, and then adding the rest of the saline, as described by Ashcroft *et al.* (13). It was administered intraperitoneally, the dose being 800 mg/kg. Rats were stimulated under Fluothane anaesthesia as described. The electrode tip was positioned in the median raphe nucleus at V + 1.6 mm, A + 0.4 mm, and 0.2 mA pulses

Table 1.3 The effect of anaesthetics on 5-HT and 5-HIAA levels

Treatment	5-HT (μg/g)	5-HIAA(μg/g)
Saline	0.25 ± 0.03	0.21 ± 0.02
Fluothane	0.28 ± 0.03	0.27 ± 0.05
Urethane	0.22 ± 0.02	0.42 ± 0.04*
Pentobarbitone	0.25 ± 0.04	0.23 ± 0.04

Results show mean ± S.D. of 3 observations.

* P = 0.05 (R test) compared to controls.

were passed at 10/sec. Tryptophan was injected 15 min after the beginning of stimulation, and the animals were killed 45 min after this. The results are shown in Table 1.4, there being a highly significant rise of 41 per cent in 5-HIAA in the stimulated group. As there was no significant fall in 5-HT, there must have been an increase in 5-HT synthesis.

Development of a method for the estimation of 5-HT synthesis

To study the effects of raphe stimulation on 5-HT synthesis, a suitable method for measuring this had to be chosen: the various alternatives have been described above.

A method was needed which would be rapid and which would not depend on a steady-state being reached; on the other hand it would not be important to know the absolute rates of amine synthesis, but only the relative rates in different experimental situations. Bearing these facts in mind, it was decided to try to combine two approaches in order to produce a more efficient method. It was planned to give an MAO inhibitor, and to measure the amount of labelled 5-HT accumulated at a fixed time after a systemic injection of radioactive tryptophan. The advantage over other radioactive methods would be that an accurate indication of amine synthesis would be obtained, as none would be lost by metabolism. The advantage of using the radioactive precursor rather than simply measuring 5-HT accumulation after MAO inhibition would be that a smaller number of animals would be needed, as in the latter case the amine level would have to be measured both before _and_ after a certain time interval. Also it was hoped that the sensitivity of the

Table 1.4 Effect of stimulation on 5-hydroxyindole levels after a tryptophan load

Group	5-HT (μg/g.)	5-HIAA (μg/g.)
Control	0.71 ± 0.22	0.32 ± 0.04
Stimulated	0.54 ± 0.09	0.45 ± 0.04*

Results show mean ± S.D. of 8 observations.

* Significantly higher than controls, $P < 0.001$ (t test, one-tailed).

As the direction of change of 5-HT was not predicted, a 2-tailed t test was used. The difference was not significant.

radioactive method would allow a shorter time of measurement.

The fact that MAO inhibitors reduce the firing rates of raphe cells (67) was not considered to be important, because this work was concerned with the effects of stimulating the cells directly; in fact any changes caused by stimulation might be made clearer if firing rates were reduced below normal in the control animals.

Tests on the MAO inhibitor

Pargyline was chosen as the MAO inhibitor. It inactivates the enzyme irreversibly, and has been used before for the measurement of 5-HT turnover (57). Before proceeding with further experiments, its effectiveness was established.

(a) To show an accumulation of 5-HT after pargyline, rats received the drug (75 mg/kg i.p.) or saline, one hour before killing. The 5-HT levels were:

Control - 0.41 µg/g. (mean of 0.36, 0.46)

Pargyline - 0.80 µg/g. (mean of 0.76, 0.84)

The rate of accumulation (0.39 µg/g/hr) was similar to that reported by Tozer et al. (57).

(b) Fluothane anaesthesia reduces blood pressure: thus in rats under this anaesthetic, a poor circulation might result in slow absorption of the drug from the peritoneal space. Therefore the last experiment was repeated under Fluothane: rats were anaesthetised and 15 min later received pargyline or saline. They were killed 60 min after the injection and 5-HT assayed. The levels were:

Control - 0.55 µg/g. (mean of 0.50, 0.60)

Pargyline - 1.07 µg/g. (mean of 1.04, 1.10)

Thus the rate of accumulation of 5-HT was no smaller under anaesthesia.

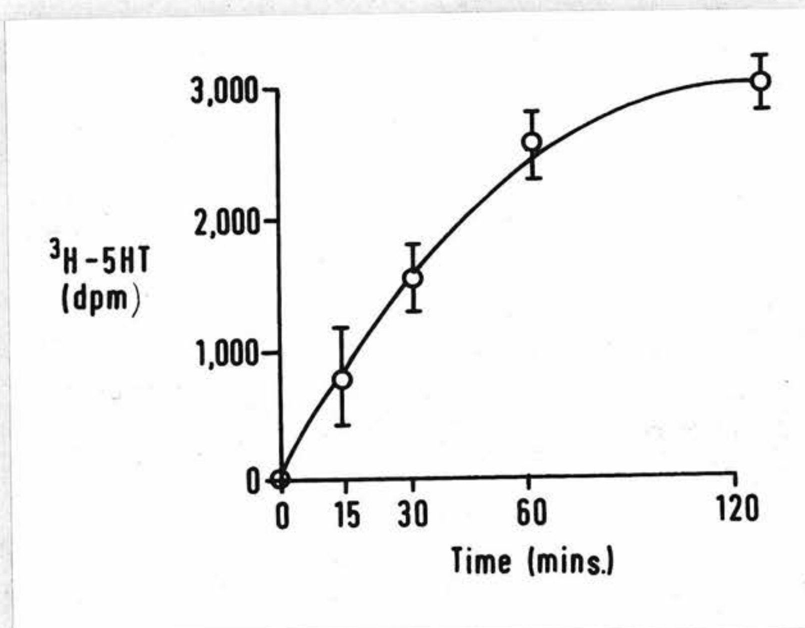

Fig. 1.12. Accumulation of (^{3}H)5-HT in brain after administration of pargyline and (^{3}H)tryptophan, i.p. Points show mean of 3 results; bars show S.D.

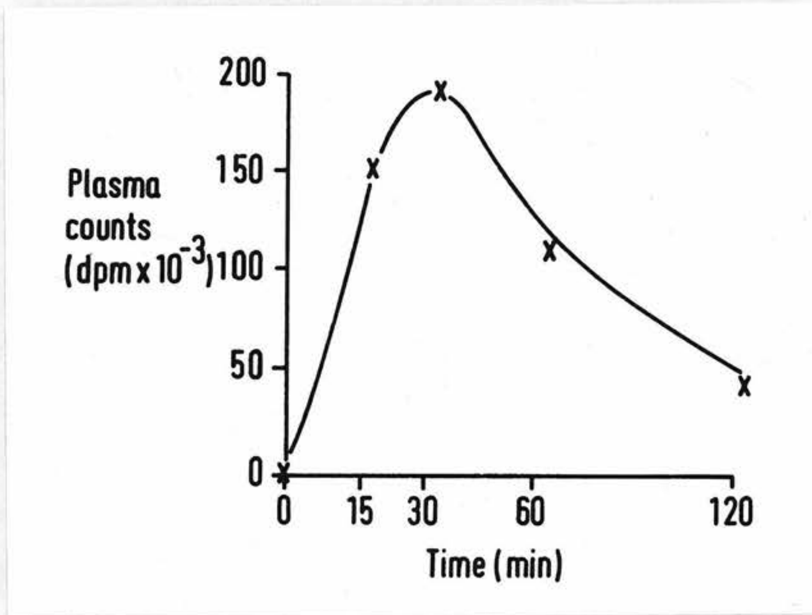

Fig. 1.13. Time course of total plasma radioactivity after i.p. injection of (^{3}H)tryptophan. Points show mean of 2 or 3 results.

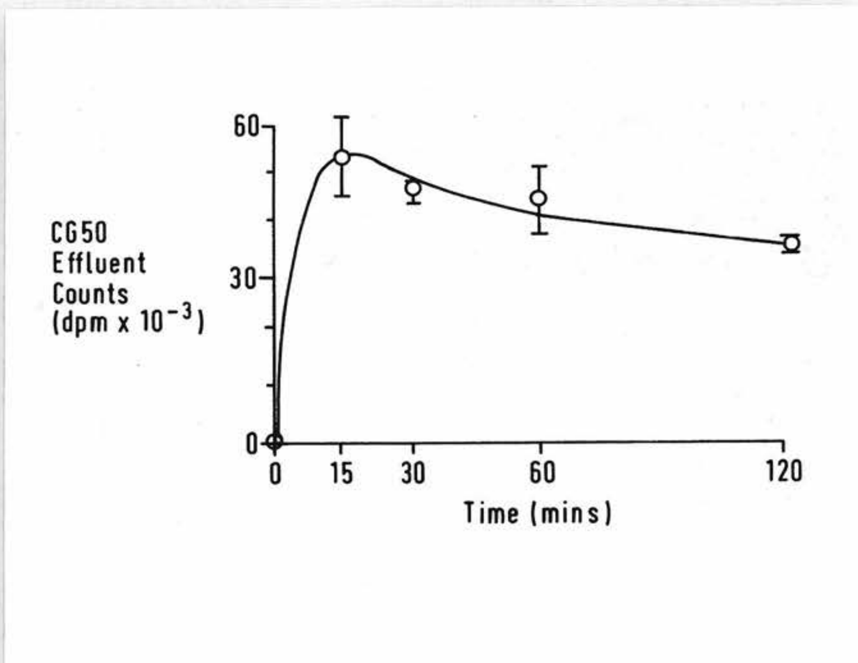

Fig. 1.14. Time course of total radioactivity in CG50 effluent after i.p. injection of (^{3}H)tryptophan. Points show mean of 3 results; bars show S.D.

Synthesis of (^{3}H)5-HT from (^{3}H)tryptophan in the presence of pargyline

The time course of (^{3}H)5-HT accumulation was determined. Rats received pargyline (75 mg/kg, i.p., 30 mg/ml in normal saline) followed 15 min later by (^{3}H)L-tryptophan (100 µCi/kg, i.p., 20 µCi/ml in normal saline). This pre-treatment time of 15 min should be sufficient according to the results of Tozer et al. (57), who obtained an immediate linear rise in brain 5-HT following intraperitoneal injection of the drug.

The dose of (^{3}H)tryptophan given represents an amount of tryptophan of 20 to 40 µg/kg. It was considered that this amount would not alter tryptophan levels in blood or brain, as it was given intraperitoneally. The amount of free tryptophan in the circulation is of the order of 500 µg/kg body weight (21), and the smallest systemically-given dose reported in the literature to raise brain 5-HT is 12.5 mg/kg (24).

The animals were killed at various times after the tryptophan injection, and (^{3}H)5-HT assayed. As described in the 'Methods' section, (^{3}H)5-HT was shown to be the only radioactive substance in the CG50 eluate. The results are shown in Fig. 1.12. The radioactivity in plasma and CG50 effluent solution was also measured, as an indication of the level of (^{3}H)tryptophan in the plasma and brain respectively (Figs. 1.13, 1.14). These curves show a rapid initial rise, followed by a slower decline.

Because the tritium label exchanges with other molecules, especially water, it would not be expected that all the radioactivity in the CG50 effluent would be (^{3}H)tryptophan. Purification of the effluent on Dowex 50 columns as described showed that about 50 per

cent of the radioactivity behaved as (^{3}H)tryptophan: this figure is in agreement with the findings of Schubert et al. (76). There was a close correlation between the CG50 effluent counts and (^{3}H)tryptophan levels in brains from animals which had all been killed at the same time (30 min) after (^{3}H)tryptophan injection ($r = 0.98$ over 8 results; $P<0.001$); in other words, the (^{3}H)tryptophan dpm were a constant fraction of the CG50 effluent dpm. Therefore it seemed justified to use the CG50 effluent dpm as an index of the amount of (^{3}H)tryptophan in the brain.

Fig. 1.12 shows that (^{3}H)5-HT in the brain rises approximately linearly for the first 30 min after the injection, and it continues to increase for some time after then. Thus a suitable time to kill the animal would be 30 min after the (^{3}H)tryptophan injection. The sequence chosen therefore for measuring 5-HT synthesis was to give pargyline followed 15 min later by (^{3}H)tryptophan, and to kill the animal after a further 30 min.

It was found in these and subsequent experiments that there was a considerable variation in the amount of (^{3}H)tryptophan present in the brain at the time of killing. This would affect the amount of (^{3}H)5-HT synthesised: in fact there was a close correlation between the (^{3}H)5-HT level and the CG50 effluent dpm ($r = 0.95$ over 44 results; $P<0.001$). Therefore to eliminate variations in the results due to differences in the amounts of (^{3}H)tryptophan taken up by the brain, the final results are expressed as the ratio of (^{3}H)5-HT dpm to CG50 effluent dpm.

The linear correlation between brain weight (which varied from 1.6 to 1.8g) and (^{3}H)5-HT dpm was tested. As it was not

significant, results are given per brain rather than per gram of brain.

Effect of increasing the dose of pargyline

The effect of increasing the dose of pargyline on the (^{3}H)5-HT measured was tested. Rats received 75 or 150 mg/kg pargyline followed by (^{3}H)tryptophan, according to the schedule described above. The results were as follows, expressed as:

Ratio(x10^3) 5-HT dpm/CG50 effluent dpm.

75 mg/kg - 33.1 (mean of 28.1, 38.1)

150 mg/kg - 36.8 (mean of 32.9, 40.7)

Thus the (^{3}H)5-HT synthesised was 11 per cent higher in the group given the higher dose of pargyline. This may not be significant, but to ensure as complete inhibition of MAO as possible, a dose of 150 mg/kg was used in subsequent experiments.

Method for measurement of 5-HT synthesis: definitive version

The definitive version of the method, as used in subsequent experiments, was: pargyline (150 mg/kg, i.p.) was given, followed after 15 min by (^{3}H)tryptophan (100 µCi/kg, i.p.), and the animals were killed after a further 30 min. The results are expressed as: Ratio(x10^3) 5-HT dpm/CG50 effluent dpm.

Effect of p-chlorophenylalanine

Tryptophan hydroxylase, the rate-limiting step in 5-HT synthesis, is inhibited by the drug p-chlorophenylalanine (PCP) (91). Therefore a test of the method would be to use it to show that 5-HT synthesis is inhibited by the drug.

Rats received PCP (500 mg/kg, i.p.) or saline 24 and 6 hours before synthesis measurement. The results were:

Ratio (x10^3)
5-HT dpm/CG50 effluent dpm.

Saline - 33.7 (mean of 29.6, 37.8)

PCP - 4.9 (mean of 4.2, 5.6)

Thus synthesis was reduced to less than 15 per cent of normal by the drug.

Level of 5-HT and (^{3}H)5-HT in blood

The rat brain contains about one per cent by volume of blood (21); therefore endogenous and (^{3}H)5-HT were measured in the blood to see if the content of these in the blood would make any significant contribution to the brain levels.

Blood was pooled from the control animals in the last experiment, and 1 ml was processed exactly as described for the brains. The concentrations were:

Endogenous 5-HT - 0.27 µg/ml.

(^{3}H)5-HT - 62 dpm/ml.

It was concluded therefore that results would not be significantly affected by the content of 5-HT or (^{3}H)5-HT in the blood trapped in brain.

Effects of stimulation on 5-HT synthesis

(a) Effect of varying the electrode position

Sheard and Aghajanian (38) reported the effects of stimulation of different midbrain areas on 5-HT metabolism. They found an elevation of brain 5-HIAA on stimulating the median and dorsal raphe nuclei, but not when other areas were stimulated. In the following experiment, the effect of stimulation at three different vertical positions was determined; the electrode was positioned in the midline and 0.4 mm anterior to the interaural line: histological

examination as described showed that the plane at AP + 0.4 mm is situated in the centre of the raphe nuclei. Stimulation parameters were 0.2 mA and 10/sec, approximately the same as used by Sheard and Aghajanian (38). 5-HT synthesis was measured as described in the previous section: pargyline was given 15 min after the beginning of stimulation, followed 15 min later by (^{3}H)tryptophan. Stimulation was continued until the animals were killed after a further 30 min. The results are shown in Table 1.5.

Stimulation of the median raphe nucleus increased synthesis of (^{3}H)5-HT, as expected from the results of Sheard and Aghajanian (38), although no rise was found on stimulating the dorsal raphe nucleus. A greater rise in synthesis was found with the electrode 0.9 mm above the centre of the median raphe rather than actually at the centre. The difference was not statistically significant, but a genuine difference may have been masked by the rather wide spread of the results in the 2.5 mm group. Therefore a vertical co-ordinate of + 2.5 mm was used in subsequent experiments.

(b) Effect of varying the stimulation current

The synthesis of 5-HT at different stimulation currents was determined. The experiment was conducted as in the last paragraph, with V = + 2.5. The results are shown in Table 1.6. 0.2 mA was chosen for future experiments, as there seemed little advantage in increasing it to 0.4 mA. Histological examination, as described in 'Methods', showed that after one hour's stimulation at 0.2 mA, negligible tissue damage had occurred.

(c) Effect of varying the frequency

In the report of Sheard and Aghajanian (38), the effects

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Table 1.5 Effect of stimulation at different electrode positions on 5-HT synthesis.

Vertical position of electrode (V)	Location	Ratio(x10^3) 5-HT dpm/ CG50 effluent dpm
1.6 mm	Median raphe nucleus	45.5 ± 9.4 (7)*
2.5 mm		59.0 ± 17.7 (7)*
3.5 mm	Dorsal raphe nucleus	32.9 ± 8.0 (7)
2.5 mm (Unstimulated controls)		33.6 ± 4.2 (7)

* Significantly higher than controls, $P < 0.01$ (t test). The 2.5 and 3.5 mm groups differ significantly ($P < 0.01$), but the 1.6 and 2.5 mm groups are not significantly different.

Table 1.6 Effect of stimulation at different currents on 5-HT synthesis

Current (mA)	Ratio($x10^3$) 5-HT dpm/ CG50 effluent dpm	Per cent Control
0	32.7 ± 4.9 (9)	100
0.1	49.7 ± 19.9 (3)*	152.0
0.2	70.5 ± 16.3 (9)**	215.7
0.4	82.2 ± 10.1 (3)**	251.6

* Significantly higher than controls, $P < 0.05$

** Significantly higher than controls, $P < 0.001$ (t test).

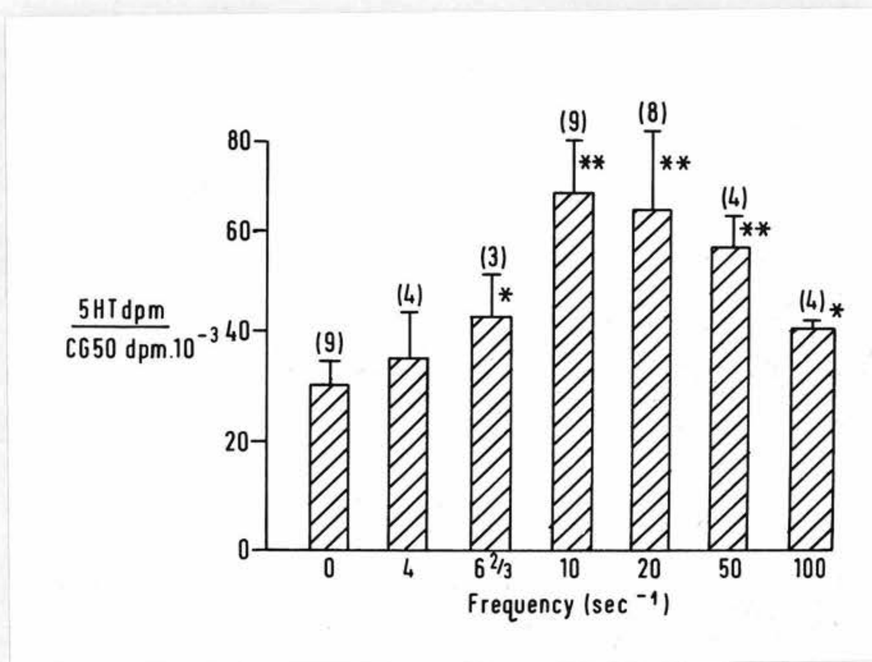

Fig. 1.15. Effect of different stimulation frequencies on 5-HT synthesis. Vertical bars show S.D., with the number of results in brackets.

*Significantly higher than control, $P < 0.025$

**Significantly higher than control, $P < 0.001$

(*t* test).

of a limited number of stimulation frequencies were described. In the following experiment, a greater number of frequencies covering a narrower range was used. Experimental details were as in paragraph (a), and a current of 0.2 mA was used. The results are shown in Fig. 1.15; the optimum frequency was 10/sec, which was used in subsequent experiments.

Effect of stimulation on tryptophan and 5-HT levels

The levels of radioactive and endogenous tryptophan and of endogenous 5-HT were measured in animals stimulated at the chosen parameters (The 0 and 0.2 mA groups in Table 1.6). The results (Table 1.7) show that stimulation did not alter the concentration or specific activity of tryptophan in the brain, and also the proportion of CG50 effluent radioactivity which behaved as (^{3}H)tryptophan was the same in both groups. There was no change in the final level of 5-HT, which shows that the total rate of accumulation of the amine in the presence of the pargyline was the same in both groups. This lack of effect on the total 5-HT level was seen in all other stimulated groups as well as this one.

The mechanism of increased 5-HT synthesis

(a) Stimulation of animals pre-treated with PCP

The following experiment was performed to determine whether the increased 5-HT synthesis on stimulation was due to a rapid increase in the amount of functional tryptophan hydroxylase, or to increased activity of the existing enzyme. PCP irreversibly inactivates brain tryptophan hydroxylase, and 3 days after a single dose of the drug, the activity of this enzyme is still low, although by this time the drug has been virtually eliminated from the brain (91).

Table 1.7 Effect of stimulation on brain tryptophan and 5-HT

Group	Tryptophan (μg/g)	Tryptophan Specific activity (dpm/ng)	Percentage of CG50 effluent dpm as (^{3}H)tryptophan	5-HT (μg/g)
Control	6.4 ± 0.7	1.63 ± 0.4	48.4 ± 4.0	0.74 ± 0.05
Stimulated	5.9 ± 1.0	1.65 ± 0.3	51.0 ± 7.0	0.73 ± 0.04

Results show mean ± S.D. of four results, except for 5-HT where the number is nine.

None of the groups differ significantly from controls (_t_ test for 5-HT; other groups: R test).

If stimulation increased 5-HT synthesis by increasing the activity of existing enzyme, the synthesis rate in PCP-treated animals should rise on stimulation by the same proportion as in untreated animals. If the amount of enzyme increased, the rise in synthesis would be proportionally greater than in untreated ones.

Rats were given PCP, 270 mg/kg, i.p. Three days later the effect of stimulation on 5-HT synthesis was determined as described above; the results are shown in Table 1.8. The increase in synthesis was in the same proportion as in untreated animals (See Table 1.6, 0 and 0.2 mA groups).

(b) Effect of stimulation on synthesis of 5-HT from 5-HTP

The experiments described above show that the activity of tryptophan hydroxylase is controlled by the nervous activity of the serotonergic neurones. It has been suggested (92) that the activity of 5-HTP decarboxylase might also partly control 5-HT synthesis, as the enzyme is inhibited *in vitro* by 5-HT. Therefore it was considered worthwhile to see whether stimulation altered the synthesis of 5-HT from 5-HTP. The experiment was conducted as described above, except that (^{3}H)5-HTP was given instead of (^{3}H)tryptophan. The result was:

Control: 1.09 ± 0.13 (4) (Ratio($\times 10^3$)5-HT dpm/CG50 effluent dpm)

Stimulated: 1.05 ± 0.16 (4)

Thus stimulation did not alter 5-HTP decarboxylase activity.

Long term effects of stimulation

Eccleston *et al.* (46) reported that after one hour's stimulation of the median raphe nucleus, 5-HT synthesis, as measured by tryptophan loading, was still higher than normal for at least an

Table 1.8 The effect of stimulation on 5-HT synthesis in PCP-treated rats

Group	Ratio($x10^3$) 5-HT dpm/CG50 effluent dpm	Per cent control
Control	8.0 ± 2.7 (3)	100
Stimulated	17.4 ± 5.4 (4)*	218

* Significantly higher than control, $P < 0.05$ (R test).

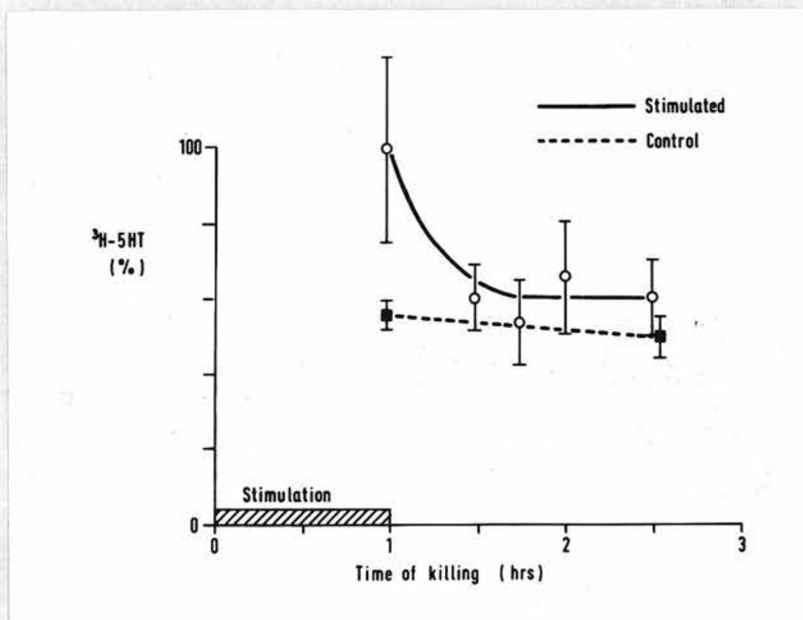

Fig. 1.16. 5-HT synthesis during and after stimulation. Animals received pargyline 45 min and (^{3}H)tryptophan 30 min before killing. The curve shows the value of the Ratio 5-HT dpm/CG50 effluent dpm, as a percentage of the initial stimulated group.
Each point is the mean value of 4 or more results; bars show S.D.
Only the initial point is significantly higher than control, $P < 0.005$ (t test).

hour after the cessation of stimulation. The continuation of the increased synthesis could have been related to the presence of the very high levels of tryptophan reached in the brain after tryptophan loading (13): perhaps tryptophan hydroxylase was induced by the tryptophan, as is tryptophan pyrrolase (93). Therefore 5-HT synthesis was measured after stimulation using the present method.

Rats were stimulated for one hour and then allowed to recover. A good recovery was made in 10-20 minutes: the animals showed normal feeding and grooming behaviour. The rate of 5-HT synthesis is shown in Fig. 1.16. The synthesis rate was only increased above normal when measured during stimulation and not afterwards, indicating a rapid reduction immediately the stimulation stopped.

The experiment of Eccleston _et al._ (46) was performed under continuous anaesthesia, which might account for the difference between it and the present results. Therefore some animals were kept under continuous anaesthesia for the whole duration of the experiment, and were killed 2½ hours after the beginning of stimulation, thus corresponding to the final point of Fig. 1.16. There was no significant difference between the stimulated group and controls:

Control - 31.7 ± 4.6 (7) (Ratio($x10^3$)5-HT dpm/CG50 effluent dpm)

Stimulated - 28.5 ± 6.8 (7)

Synthesis of 5-HT after stimulation and tryptophan loading

The previous experiment showed there was no long-term effect of stimulation on 5-HT synthesis as measured by the radioactive method. Possibly then, the long-term effect found by Eccleston _et al._ (46) is only seen in the presence of a tryptophan load. The

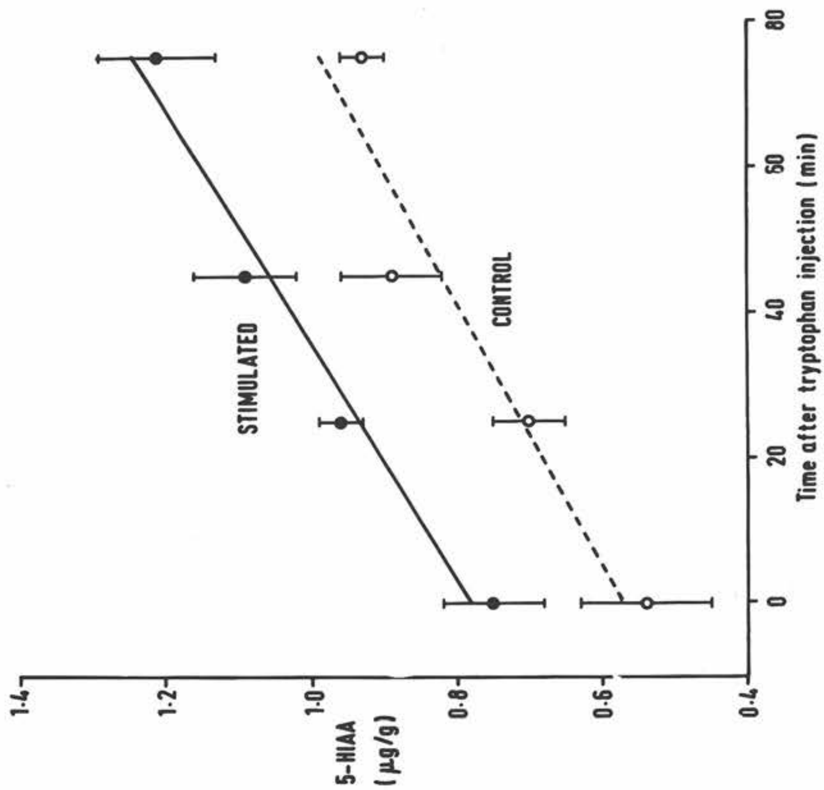

Fig. 1.17. Effect of tryptophan loading immediately after stimulation.

Points show the mean of 3 or 4 results; bars show S.D. The slopes of the two lines are not significantly different (t test).

following experiment tested whether an increased synthesis was apparent on giving a tryptophan load one hour after the end of the stimulation period.

Rats were stimulated for one hour and allowed to recover. One hour after the end of stimulation they were given L-tryptophan (800 mg/kg, i.p.) or saline, and were killed after a further 30 min. The results are shown in Table 1.9. Although there are insufficient observations to apply conventional statistics, the results strongly suggest that there was no greater rate of synthesis in the stimulated animals.

These results show either that tryptophan must be given much earlier in order to maintain the increased synthesis (Eccleston _et al._ gave it 15 min after the end of stimulation), or that their results were not a genuine finding. The following experiment is a repeat of that of Eccleston _et al._, except that tryptophan was given immediately after the end of stimulation: if it were the case that the tryptophan stabilised tryptophan hydroxylase in a more active form, giving it earlier should enhance its effect.

Rats were stimulated for one hour, and L-tryptophan (800 mg/kg, i.p.) was given at the end of this period. Anaesthesia was maintained until the animals were killed at various times later. The rates of accumulation of 5-HIAA are shown in Fig. 1.17. The rates were virtually identical in stimulated and control groups: Stimulated, 6.2 ng/g/min; control, 5.6 ng/g/min. The rate of accumulation of 5-HT was also no higher in the stimulated group (Stimulated, 2.7 ng/g/min; control, 3.9 ng/g/min), and thus the accumulation of total 5-hydroxyindoles was not altered by stimulation

Table 1.9 Effect of stimulation on 5-HT metabolism, determined by tryptophan loading one hour after stimulation

Group	5-HT	5-HIAA	Total 5-hydroxyindoles	Tryptophan
		(All in units of µg/g)		
Control, saline	0.36(2)	0.28(2)	0.64	5.2(2)
Control, tryptophan	0.59(2)	0.49(3)	1.08	62.4(3)
Stimulated, saline	0.43(2)	0.38(2)	0.81	4.9(2)
Stimulated, tryptophan	0.60(3)	0.50(3)	1.10	46.2(3)
Increase due to tryptophan load				
Control	0.23	0.21	0.44	
Stimulated	0.17	0.12	0.29	

Results are the mean values of the number of observations in brackets.

(Stimulated, 8.9 ng/g/min; control, 9.5 ng/g/min). 5-HIAA levels were consistently higher in the stimulated animals, indicating a rise in synthesis during stimulation.

Thus no long-term effects of stimulation on 5-HT synthesis were found in any of these experiments.

Effect of L.S.D. on 5-HT synthesis

In the experiments above it was described how raphe stimulation increased the synthesis of (^{3}H)5-HT from (^{3}H)tryptophan in the presence of pargyline, without significantly increasing the total rate of accumulation of 5-HT. This suggests that 5-HT in the brain might be stored in two or more separate pools, and in the Discussion, a model for the synthesis and storage of the amine is presented. In the following paragraphs, three separate experiments are described which were performed to test this hypothesis.

Lysergic acid diethylamide (LSD) reduces the firing rates of neurones in the raphe nuclei (94). Therefore as a counterpart to the previous experiments where stimulation of these nerves was shown to increase 5-HT synthesis, the effect of LSD on the synthesis of the amine was tested.

The method for measuring 5-HT synthesis was as described previously though with altered timing. Rosecrans et al. (95) showed that after intraperitoneal injection of LSD, the level of the drug in the brain was maximal after only ten minutes, and then fell rapidly. Thus rats were given pargyline and LSD in the same injection, followed after 6 minutes by (^{3}H)tryptophan. They were killed after a further 15 min. This schedule should have given a maximal concentration of LSD in the brain while (^{3}H)5-HT was being

synthesised from (^{3}H)tryptophan.

The results are shown in Table 1.10. LSD reduced the synthesis of (^{3}H)5-HT, but did not significantly reduce the total rate of accumulation of 5-HT, except for a small reduction at the lowest dose of the drug. There was also no significant alteration in the rate of uptake of tryptophan by the brain, as judged by the CG50 column effluent radioactivity (which was shown previously to correlate highly with the (^{3}H)tryptophan level). It is possible that the reduced synthesis of (^{3}H)5-HT was simply due to an increase in the level of endogenous tryptophan in the brain, which would have reduced the specific activity of the precursor (^{3}H)tryptophan. This is unlikely however as the total 5-HT synthesis, which is very dependent on the level of tryptophan (Moir and Eccleston, 21), was unchanged by the drug. A second experiment to confirm this point showed that a dose of LSD as high as 1 mg/kg had no significant effect on the level or specific activity of tryptophan in the brain (Table 1.11), while at the same time the synthesis of (^{3}H)5-HT was reduced.

Effect of adrenalectomy

Tryptophan hydroxylase in the brain appears to be controlled to some extent by adrenal steroid hormones (Azmitia et al. 96; Azmitia and McEwan, 97; Green and Curzon, 98). We therefore studied the effect of adrenalectomy on brain 5-HT synthesis, to see whether in these circumstances the synthesis of the amine would separate into two components.

At either 10 or 20 days after bilateral adrenalectomy, 5-HT synthesis in the brain was measured as described earlier. The

Table 1.10 Effect of LSD on 5-HT synthesis

Dose of LSD (μg/kg, i.p.)	Ratio (x10^3) 5-HT dpm/CG50 effluent dpm	CG50 effluent dpm (x10^{-3})	5-HT ‡ (μg/g)
0	28.7 ± 6.5(14)	62.5 ± 16.9(14)	0.50 ± 0.05(14)
50	22.5 ± 7.5(10)*	74.7 ± 17.3(10)	0.46 ± 0.04(10)*
100	17.4 ± 9.2(11)†	70.5 ± 24.3(11)	0.49 ± 0.03(11)
250	19.9 ± 6.4(8)†	72.3 ± 15.8(8)	0.49 ± 0.04(8)

Animals received pargyline (150 mg/kg, i.p.) and LSD followed after 6 min by L-(^{3}H)tryptophan (200 μCi/kg, i.p.), and were killed after a further 15 min.

* Significantly lower than control, $P < 0.025$

† Significantly lower than control, $P < 0.005$ (t test)

‡ The 5-HT level in a group of untreated animals was 0.26 ± 0.04 μg/g (5)

Table 1.11 Effect of a dose of 1 mg/kg of LSD on 5-HT synthesis and tryptophan levels

Group	Ratio($\times 10^3$) 5-HT dpm/CG50 effluent dpm	CG50 effluent dpm ($\times 10^{-3}$)	5-HT (μg/g)	Tryptophan	
				Sp. Ac. (dpm/ng)	Level (μg/g)
Control	23.2 ± 8.0	70.6 ± 17.9	0.44 ± 0.04	3.61 ± 1.57	6.64 ± 0.64
LSD	15.6 ± 7.8*	71.5 ± 14.0	0.46 ± 0.04	3.55 ± 2.03	6.78 ± 0.97

Results show mean ± S.D. of 10 observations.

* Significantly lower than control, $P < 0.05$ (t test)

Other details as in footnote to Table 1.10.

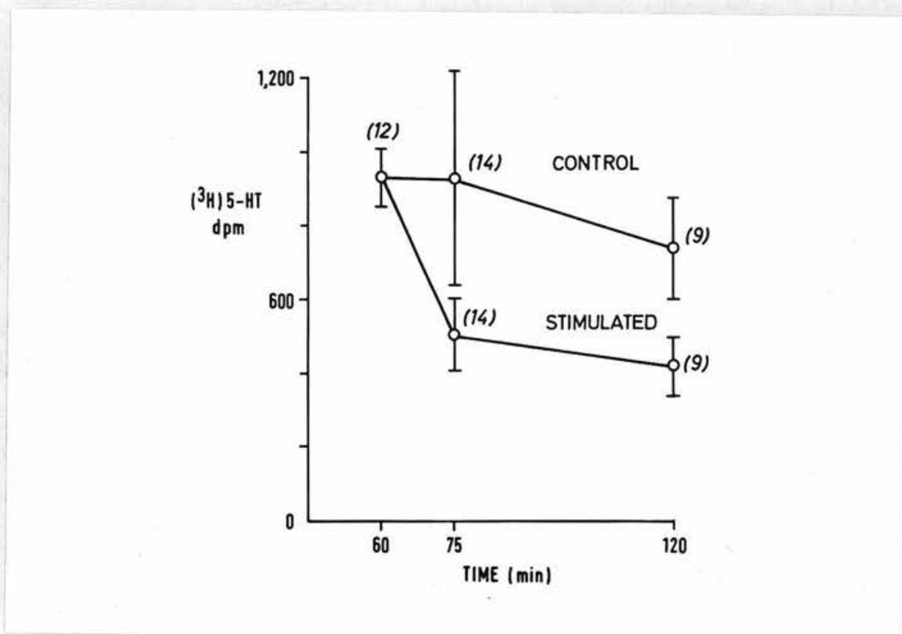

Fig. 1.18. Effect of stimulation on (^{3}H)5-HT levels following injection of (^{3}H)tryptophan (100 µCi/kg, i.p.). Stimulation was begun 60 min after injection. There is a significant difference between the initial group and the 75 min stimulated group ($P < 0.005$, _t_ test), and between the stimulated and control groups at 120 min ($P < 0.05$). The difference between the stimulated and control groups and 75 min approaches significance ($0.05 < P < 0.1$). Vertical bars show S.E.M.; numbers of results are given in brackets.

results are shown in Tables 1.12 and 1.13. In both cases there was a significant rise in the total rate of accumulation of 5-HT, whereas the results obtained using the radioactive tracer suggested that there was no significant alteration in the synthesis of the amine. Tryptophan levels were not significantly changed by the adrenalectomy; neither was the amount of (^{3}H)tryptophan taken up changed, as judged by the CG50 effluent radioactivity.

Depletion of (^{3}H)5-HT by stimulation

Stimulation of the raphe nuclei increases the rate of 5-HT metabolism, as described above. If there is a functional pool of the amine which is released by the nerve impulse, and is selectively labelled by (^{3}H)tryptophan, stimulation should give a rapid release of (^{3}H)5-HT following labelling of the stores in this way. Thus rats were given (^{3}H)tryptophan and the median raphe nucleus stimulated, starting 60 min after the injection. The level of (^{3}H)5-HT is shown in Fig. 1.18, and the corresponding levels of endogenous 5-HT and 5-HT specific activity are shown in Table 1.14. In the stimulated animals, there was a significant fall in the level and specific activity of (^{3}H)5-HT during the first 15 min of stimulation. The fall in endogenous 5-HT over the same period was not significant. At 60 min after the start of stimulation, the 5-HT specific activity was not significantly different in stimulated and control groups.

As there was a high correlation between (^{3}H)5-HT and (^{3}H)tryptophan levels in the brain ($r = 0.75$ over 58 results, $P < 0.001$ by *t* test), the (^{3}H)5-HT values were corrected for variations in brain (^{3}H)tryptophan levels. This was done by multiplying by the ratio: (^{3}H)tryptophan *ex*/(^{3}H)tryptophan, where (^{3}H)tryptophan *ex*

Table 1.12 Synthesis of 5-HT 10 days after adrenalectomy

Group:	Sham-operated		Adrenalectomised	
Treatment:	None	Pargyline+ (^{3}H)tryptophan	None	Pargyline+ (^{3}H)tryptophan
5-HT (μg/g)	0.25±0.01(3)	0.45±0.05(5)	0.23±0.01(3)	0.53± 0.04(4)
Rate of rise of 5-HT (μg/g/hr)	0.27		0.40*	
Ratio (x10^3) 5-HT dpm/CG50 effluent dpm		34.0±7.5(5)		40.9±7.5(3)
CG50 effluent dpm (x10^{-3})		35.3±9.2(5)		49.8±5.4(3)
Tryptophan (μg/g)	5.94±1.7(5)		3.81±0.87(5)	

Animals received pargyline (150 mg/kg, i.p.) followed after 15 min by (^{3}H)tryptophan (100 μCi/kg, i.p.), and were killed after a further 30 min.

Some animals were killed without treatment.

Results show mean ± S.D., with numbers of observations in brackets.

* Significantly different from control, $P < 0.05$. Differences between adrenalectomised and sham-operated groups were tested by a 2-tailed R test, except for the rate of rise of 5-HT, which was tested by a 2-tailed special form of the t test, as described in Methods.

Table 1.13 Synthesis of 5-HT 20 days after adrenalectomy

Group:	Sham-operated		Adrenalectomised	
Treatment:	None	Pargyline+ (^{3}H)tryptophan	None	Pargyline+ (^{3}H)tryptophan
5-HT (µg/g)	0.41±0.02(4)	0.71±0.05(5)	0.36±0.02(4)	0.73±0.04(5)
Rate of rise of 5-HT (µg/g/hr)	0.30		0.37*	
Ratio (x10^3) 5-HT dpm/CG50 effluent dpm		44.6±20.9(5)		40.1±7.1(5)
CG50 effluent dpm (x10^{-3})		33.9±4.6(5)		33.6±6.2(5)
Tryptophan (µg/g)	6.04±0.37(5)		5.20±0.84(5)	

Pargyline was given 30 min before the (^{3}H)tryptophan. Other details as in footnote to Table 1.12.

Table 1.14 Level and specific activity of 5-HT during stimulation

Time from beginning of stimulation (min)	Controls		Stimulated
		5-HT specific activity (dpm/µg)	
0		2,965±861(12)	
15	3,123±3,473(14)		1,886±1,293(14)*
60	1,954 ±978(9)		1,804±1,149(9)
		5-HT level (µg/g)	
0		0.33±0.02(8)	
15	0.33±0.02(8)		0.29±0.07(8)
60	0.36±0.05(6)		0.26±0.05(6) †

Animals received (^{3}H)tryptophan (100 µCi/kg, i.p.) 60 min before the beginning of stimulation.

* Significantly lower than zero time group, $P < 0.025$.

† Significantly lower than zero time and 60 min control groups, $P < 0.005$ (t test).

was the (^{3}H)tryptophan level expected from a standard curve of its time course in brain after intraperitoneal injection (Fig. 3.5).

DISCUSSION

Method for measurement of 5-HT synthesis

The method described avoids many of the drawbacks of other methods. Probably the main objection to it is the fact that raphe cell firing is gradually inhibited in the presence of MAO inhibitors. As mentioned earlier however, this would not be important in the present experiments as the cells are being stimulated directly. The method appears to give a good indication of tryptophan Hydroxylase activity when tested in situations known to produce changes in the rate of hydroxylation: thus 5-HT synthesis was shown to be increased by raphe stimulation, and reduced by inhibition of the enzyme with PCP. In addition, a reduction in synthesis was seen following LSD administration, a drug which rapidly reduces raphe cell firing rates (94). This finding suggests that the gradual reduction in raphe firing which is seen after inhibition of MAO may not invalidate the method as one which is generally useful.

Determination of optimum stimulation parameters

In the experiments where the midbrain was stimulated at various vertical co-ordinates, an increase in 5-HT synthesis was found on stimulating the median, but not the dorsal, raphe nucleus. An increase in synthesis on stimulating the dorsal, as well as median, raphe nucleus was found however by Sheard and Aghajanian (38) and Gumulka *et al.* (42), the positive sites of stimulation thus corresponding to the areas of 5-HT-containing cells described by Dahlström and Fuxe (2). The origin of this discrepancy may lie in

the difficulty of visualising the dorsal nucleus in stained sections. Unlike the median raphe nucleus, which is easily recognisable (Fig. 1.9), the dorsal nucleus lies within the periaqueductal grey area, and without the use of fluorescence microscopy it is difficult to tell exactly where the cell bodies of the 5-HT-containing neurones are located. In this work the position of the dorsal raphe was estimated with the help of the photographs of Konig and Klippel (87). It is possible therefore that the site of stimulation did not coincide exactly with the position of the 5-HT-containing cells.

The results suggested that a greater effect of stimulation might be obtained with the electrode tip placed between these two raphe nuclei, rather than with the tip actually within the median raphe. If this were the case, it could be due to the inclusion of cell bodies and axons from both raphe nuclei in the stimulated area.

A stimulation current of 0.2 mA was chosen as the optimum, as little increased effect was seen on doubling this to 0.4 mA, which might have caused tissue damage. The increase in amine synthesis as the current was increased from 0.1 mA probably simply reflected the extension of the stimulated area to include more of the serotonergic neurones.

The rate of 5-HT synthesis at different frequencies followed the pattern of results of Sheard and Aghajanian (38), with an optimum frequency of 10/sec, and a diminished response at higher or lower frequencies. Also Kostowski et al. (40) found a greater rise in forebrain 5-HIAA when the median raphe was stimulated at 10/sec, rather than 2/sec or 60/sec. It is interesting that a maximal effect is obtained at a stimulation frequency of 10/sec, as this is about an

order of magnitude above the natural firing rate of the raphe neurones (20-40/min: ref. 94).

Development of a model for synthesis and storage of 5-HT in the brain

It was found that raphe stimulation increased synthesis of (^{3}H)5-HT by over 100 per cent, and yet there was no change in the level of endogenous 5-HT at the time of killing: thus there was no change in the total rate of accumulation of the amine in the presence of the MAO inhibitor. Comparable results were obtained by Meek and Fuxe (99), who showed that the rate of accumulation of 5-HT in the spinal cord after MAO inhibition was not changed by factors altering the activity of the 5-HT neurones, such as LSD or spinal section. Also Sheard and Aghajanian (38) found that raphe stimulation in animals given a MAO inhibitor increased the 5-HT level by only 10 per cent compared to unstimulated controls, whereas there was a rise of 5-HIAA of 80 per cent without the inhibitor. These findings suggested that 5-HT in the brain might be present in more than one compartment, and thus a model was developed here for the synthesis and storage of the amine.

A useful parallel is the storage of NA in sympathetic nerves. There is now strong evidence that NA is stored in more than one compartment in these nerves, and as the sympathetic nerve is often used as a model for central monoaminergic neurones, some of the more important evidence is described as follows.

Iversen (100) found that when the isolated rat heart was perfused with a solution containing radioactively-labelled NA, the amine accumulated biphasically in the tissue. The first compartment filled very rapidly, and appeared to contain only a small amount of

NA. The second compartment contained 25 to 30 per cent of the endogenous amine. The second compartment could be depleted of NA by reserpine, but the first appeared to be resistant to the drug. Altogether, only 25 to 30 per cent of the NA in the heart exchanged with the perfused labelled NA. This was not due to a lack of exchange of NA across the storage granule membrane, as upto 80 per cent of the NA in isolated adrenergic storage granules exchanges rapidly (von Euler et al., 101). Potter and Axelrod (102) showed that labelled NA recently taken up by the heart was largely released by tyramine, but several hours after the NA had been taken up, much less of it was released by tyramine.

The existence of a small, functionally-active pool of NA in sympthetic nerves is supported by the work of Crout, Muskus and Trendelenburg (103). They showed that the contractile response of guinea-pig atria to tyramine was not reduced by reserpine until NA stores had been depleted to a large extent. The response to tyramine was largely restored by exposure of the tissue to NA, although the amount of NA taken up was only 2 per cent of the normal content. It was also shown that the recovery of the response to tyramine after reserpine treatment occurred much quicker than the recovery of the NA level.

After administration of reserpine to an animal, the content of NA in sympathetically-innervated tissues declines biphasically. The first phase is rapid, and depletes the tissues of about 85 per cent of the NA; the remaining 15 per cent however declines only slowly, but can be rapidly depleted by nervous stimulation (Sedvall and Thorson, 104).

Further evidence for the existence of 'functional' and 'non-functional' pools of NA comes from the work of Sedvall, Kopin and others, who used (^{14}C)tyrosine as a tracer. Sedvall et al. (49) infused (^{14}C)tyrosine intravenously into rats, and measured the amount of (^{14}C)NA which accumulated. It was found that for several different tissues, the minimal estimate of the rate of NA synthesis was 2 to 3 times greater than estimates of NA turnover obtained by other methods. The authors showed that this discrepancy could be explained in terms of a two-compartment system for NA storage. Kopin et al. (105) perfused the isolated cat spleen with (^{14}C) tyrosine and measured the (^{14}C)NA released by nerve stimulation. They found that stimulation released NA with a higher specific activity than was found in the whole tissue, indicating a selective release of newly-synthesised NA.

There is also a good deal of evidence suggesting that the monoamines in the brain are stored multicompartmentally. Glowinski et al. (106) found that intraventricularly-injected (^{3}H)NA disappeared from the brain in a multiphasic manner, and Javoy and Glowinski (107) found that after inhibition of tyrosine hydroxylase, DA in the striatum declined biphasically. They interpreted these results in terms of a two-compartment model, consisting of a 'storage' pool and a 'functional' pool. The storage pool was three times the size of the functional pool, but the turnover rate of the latter was four times that of the former. Besson et al. (108) found a preferential release of newly-synthesised (^{3}H)DA from striatal slices incubated with (^{3}H)tyrosine, thus obtaining results comparable to those of Kopin et al. (105) in sympathetic nerves.

The presence of more than one storage form of NA in the brain is indicated by the results of Glowinski and Axelrod (109), comparing the effects of amphetamine and reserpine on NA metabolism. They found that amphetamine released (^{3}H)NA giving an increase in (^{3}H)normetanephrine in the brain (this metabolite is the O-methylated derivative of NA, formed extraneuronally by catechol-O-methyl transferase (COMT)), whereas reserpine led to an increase in (^{3}H)deaminated metabolites (which are formed mostly intraneuronally by MAO). Similar results have been obtained in sympathetic nerves by Kopin (110), comparing the releasing actions of tyramine and reserpine: this supports the use of the peripheral adrenergic nerve as a model for central adrenergic nerves. Schildkraut et al. (111) observed that when rats were given a single electroconvulsive shock soon after an intraventricular injection of (^{3}H)NA, the (^{3}H)NA released was largely converted to (^{3}H)normetanephrine. If the shock was given 5 hours after the injection however, the (^{3}H)normetanephrine formed was only a few per cent of the (^{3}H)NA released. These results show that NA is stored in pools in the brain which differ in their turnover and relative pathways of metabolism.

There have been numerous studies on the effects of reserpine on behaviour and brain amines, and it has been a general finding that animals recover from the effects of the drug much more quickly than do the levels of NA and 5-HT in the brain. It has been suggested that the behavioural recovery parallels the appearance of a small store of brain amines (112), though according to Brodie et al. (113), the sedative action of the drug correlates with the rate of release of the amines rather than the extent of depletion.

Chan and Webster (114) found that some of the drugs which depleted brain catecholamines reduced locomotor activity in rats, but that some of these drugs did not affect the activity. They suggested from these observations that there might be functional and nonfunctional pools of NA in the brain.

Weissman <u>et al.</u> (115) showed that the behavioural effects of amphetamine in rats were antagonised by α-methyl tyrosine, an inhibitor of tyrosine hydroxylase, before there had been a significant depletion of NA from the brain. This shows that the functional activity of NA is dependent on an intact synthesis rather than intact stores, and again provides evidence for the presence of a small, highly active pool of the amine. Kopin <u>et al.</u> (105) studied the effect of α-methyl tyrosine in the cat spleen, and similarly concluded that the maintenance of transmitter release was more dependent on new synthesis than on mobilisation of existing amine stores.

NA appears to play a role in temperature regulation, and Brodie and Reid (116) have described how rats treated with α-methyl tyrosine are unable to withstand exposure to a temperature of 4°C. Reserpinised rats depleted of NA are able however to maintain their temperatures as efficiently as normal animals. Again, the synthesis of the amine appears to be of greater functional importance than the presence of full stores.

Much less information is available on the storage of 5-HT in the brain than is the case with NA. A biphasic disappearance of intraventricularly-injected (^{14}C)5-HT was observed by Eccleston (unpublished observations), as was found by Glowinski <u>et al.</u> (106) for (^{3}H)NA.

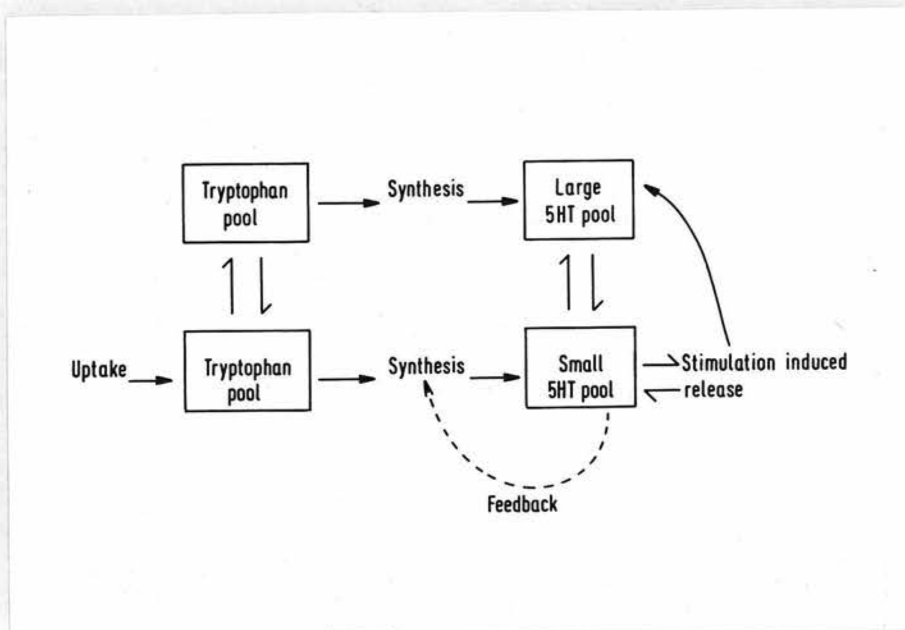

Fig. 1.19. A model for the synthesis and storage of 5-HT in the rat brain.

A 3-compartment system for 5-HT storage in the nerve ending was suggested by Aprison and Hingtgen (117), consisting of a storage pool, a functional pool released by the nerve impulse, and a free pool in the cytoplasm. The model was based on various observations concerning the temporal relationships between types of learned behaviour and 5-HT levels after giving different drugs.

Jouvet (118) found that lesions in the raphe nuclei or inhibition of tryptophan hydroxylase caused insomnia in cats. Sleep could be made normal however by administration of 5-HTP in doses which gave only a small restoration of the brain 5-HT content.

There is, therefore, a wealth of data suggesting that the monoamines may be located in several compartments in their neurones. In the present work this concept is extended and a new model for the synthesis and storage of 5-HT in the brain is proposed.

The model is shown in Fig. 1.19. It was formulated principally to explain the finding that raphe stimulation doubled the synthesis of (^{3}H)5-HT from (^{3}H)tryptophan in the presence of pargyline, though the total rate of accumulation of the amine was unchanged. There is a large pool of 5-HT where the synthesis of the amine is relatively insensitive to its level, and where 5-HT accumulates after inhibition of MAO. A second, smaller pool is available for release by the nerve impulse, and its synthesis is closely controlled by its content of 5-HT. This second pool is selectively labelled by newly taken-up (^{3}H)tryptophan. When 5-HT is released from the functional pool by the nerve impulse, its level falls even though MAO is inhibited, as not all of the released amine is taken up back into this pool: some may be taken up into the large pool, and some may enter glial

cells. The possibility of feedback inhibition was mentioned in the Introduction. The large accumulation of 5-HT in the brain after inhibition of MAO (57) is against this concept, though evidence for feedback inhibition has been presented by Macon _et al._ (66) and Hamon _et al._ (119) using isotopic tracer methods. Perhaps a positive effect was found when tracers were used due to selective labelling of the functional pool by the radioactive tryptophan. Regulation of NA synthesis in adrenergic nerves by the level of NA in a small, functional pool has already been suggested by Weiner and Rabadjija (120).

An alternative explanation of why (^{3}H)5-HT synthesis rises even though MAO is inhibited is that the activity of the tryptophan hydroxylase is controlled by nervous activity directly through depolarisation, or via local changes in ion concentrations.

As the total accumulation of 5-HT was not significantly increased by stimulation, the rate of synthesis in the small pool must be relatively slow compared to that in the large pool. To take some hypothetical figures, if the synthesis rate into the large pool was 10 units, and that to the small pool 1 unit, a doubling of the synthesis to the small pool would mean a total increase in synthesis of about 10 per cent. This would be within the range of experimental error, and would probably go unnoticed. The relatively small contribution of the small pool may be due to the lowered firing rates of the raphe cells which follows MAO inhibition (67); under normal circumstances this pool is probably quantitatively significant, as shown by the large rise of forebrain 5-HIAA on raphe stimulation (38).

The findings presented here also show that the 5-HT in the

two pools is synthesised from two separate pools of tryptophan. The amine in the functional pool is synthesised from newly taken-up tryptophan: thus in these experiments the small pool would be selectively labelled by tryptophan which had a high specific activity. A similar situation has been shown to exist in sympathetic nerves by Kopin et al. (105), who found that newly taken-up labelled tyrosine was preferentially released by nerve stimulation. Also Spector et al. (121) found that in the guinea-pig heart, (^{14}C)tyrosine in the perfusate did not equilibrate with tyrosine in the heart, and that the tyrosine in the perfusate was the relatively undiluted precursor of NA in the tissue.

An attempt to calculate the absolute rate of 5-HT synthesis shows that compartmentation of tryptophan must occur in the brain. Sedvall et al. (49) estimated NA synthesis according to the formula:

Synthesis rate = NA dpm/mean tyrosine specific activity

This expression requires two correction factors: as applied here, these are for the change in molecular weight when tryptophan is converted to 5-HT, and also for an assumed loss of two tritium atoms per molecule in the conversion. These two factors cancel each other out however almost exactly. The mean brain (^{3}H)tryptophan level was estimated from Fig. 3.5, and using this, a figure for brain 5-HT synthesis of 0.49 µg/g/hr was obtained, which is greater than the total rate of accumulation of the amine (0.40 µg/g/hr)! Sedvall et al. (49) used the plasma tyrosine specific activity in their calculations however. In the case of tryptophan though there is a problem in that only about 10 per cent of the tryptophan in rat plasma

is in free solution (A.T.B. Moir, unpublished observations; a similar finding was reported by McMenamy et al. (121a) for human plasma), and it is not known how fast this exchanges with the bound tryptophan. In the following calculation it was assumed that no exchange occurred in the 30 minutes of the experiment, and using an estimate of mean plasma (^{3}H)tryptophan from Table 3.3, a figure of 0.03 µg/g/hr for 5-HT synthesis was obtained. If the (^{3}H)5-HT synthesised during the first 30 min after (^{3}H)tryptophan injection is exclusively in the small pool, this figure gives the rate of amine synthesis in this pool. Thus by making certain assumptions it is possible to obtain a figure for 5-HT synthesis in the small pool which is less than 10 per cent of the total. This condition would be necessary for there to be no significant alteration in total amine synthesis on stimulation, given that there is an increase in (^{3}H)5-HT synthesis of over 100 per cent.

Although Fig. 1.19 shows tryptophan uptake into only one tryptophan pool, an alternative arrangement is possible where the tryptophan is taken up into a third pool, with which the other two pools equilibrate. The rate of equilibration would be much faster with the pool from which the 'functional' 5-HT is synthesised than with the other tryptophan pool.

Further evidence supporting the proposed model

Additional support for the concept of two independent pathways for the synthesis and storage of 5-HT in the rat brain is provided by the effects of LSD, adrenalectomy and raphe stimulation.

In the presence of LSD, synthesis of (^{3}H)5-HT from (^{3}H)tryptophan was considerably reduced, whereas the total synthesis

of the amine was unaltered, as judged by its rate of accumulation in the presence of pargyline. The results show that the reduction in (^{3}H)5-HT formation was not due to a fall in the specific activity of precursor tryptophan. The lack of effect of the drug on the brain tryptophan level does differ however from the findings of Tonge and Leonard (122), who found an increase in tryptophan after LSD. The results suggest therefore that the pool of 5-HT which is labelled must be quantitatively insignificant compared to the other pool(s). This result parallels the previous findings, where raphe stimulation increased synthesis of (^{3}H)5-HT but did not affect the total amine synthesis.

LSD has previously been shown to reduce 5-HT turnover (Rosecrans et al., 95; Lin et al., 123; Schubert et al., 76; Anden et al., 124), but in no case was any effect found with a dose of under 250 μg/kg, whereas Aghajanian et al. (94) found a reduction in raphe firing at doses of only 10 μg/kg. Probably no biochemical response has been detected with low doses because the concentration of the drug in the brain rises and then falls rapidly after systemic administration (95); with the method used here, which had a relatively short duration, an effect was apparent at lower doses, down to 50 μg/kg.

In the case of adrenalectomy, total 5-HT synthesis was increased, but the radioactive results suggested that the synthesis of the amine was not changed. This discrepancy is unlikely to have been due to a fall in the specific activity of the precursor tryptophan in the adrenalectomised animals. If anything, the results suggest an increase in the specific activity. As the levels

of tryptophan in the brain were not increased in the adrenalectomised animals, the increased 5-HT synthesis may have been due to increased levels of tryptophan hydroxylase. The lack of an increase in (^{3}H)5-HT may reflect the ability of the functional pool to regulate its synthesis under varying conditions, although the explanation could also be that more tryptophan hydroxylase enzyme was not present in this pool.

Reports vary as to the effect of adrenalectomy on brain 5-HT metabolism. We have shown an increase in amine synthesis, and also brain 5-HIAA levels have been found to double 22 days after the operation (G.W. Ashcroft, unpublished observations). These findings are supported by those of Green and Curzon (98), who showed that hydrocortisone injections reduced 5-HT and 5-HIAA levels in the rat brain. On the other hand, a reduced activity of tryptophan hydroxylase after adrenalectomy has been found <u>in vivo</u> (Azmitia <u>et al.</u>, 75) and <u>in vitro</u> (Azmitia and McEwen, 97). Also Lovenberg (125) reported recently that adrenalectomy had no effect on tryptophan hydroxylase activity as measured <u>in vitro</u>.

Stimulation of the raphe nucleus after labelling of the 5-HT stores with (^{3}H)tryptophan gave a rapid fall in the level and specific activity of (^{3}H)5-HT during the first 15 min of stimulation. During the next 45 min however, the 5-HT specific activity fell only slightly. These results indicate that at the time of beginning stimulation, which was 60 min after injection of (^{3}H)tryptophan, the (^{3}H)5-HT was present in two pools, one of which was released by the nerve impulse. The comparatively slow decline of the 5-HT specific activity over the latter 45 min of stimulation represented the turnover

of the nonfunctional pool. It appears that at the start of the stimulation period, about 40 per cent of the (^{3}H)5-HT was present in the functional pool, and this fraction was utilised within 15 min. At the end of the stimulation period, the specific activity of 5-HT was not significantly different in stimulated and control animals, showing that in the controls, the (^{3}H)5-HT in the functional pool had all been metabolised by this time.

A similar situation has been described by Kopin _et al._ (105), where stimulation of the cat spleen perfused with (^{14}C) tyrosine led to the release of (^{14}C)NA with a specific activity greater than that found in the tissue. This too demonstrated the existence of a functional pool of a monoamine with a higher specific activity than the whole tissue.

The biphasic disappearance of (^{3}H)5-HT in the stimulated animals is comparable to the biphasic decay of (^{14}C)5-HT seen after injection of this substance into the ventricles (D. Eccleston, unpublished observations). A biphasic disappearance of (^{3}H)5-HT was apparent in our experiments only in the stimulated animals and not in the controls probably due to the continued synthesis of the labelled amine (Neff _et al._, 77), which is not the case when labelled amine is injected intraventricularly.

The evidence presented thus provides strong support for the hypothesis that there are not only two or more storage pools of 5-HT in the brain, but also that these pools receive amine synthesised by separate pathways.

Anatomical concepts of the model

The existence of more than one compartment where 5-HT is

stored in neurones in the brain does not necessarily imply that these pools are separated by a membrane barrier. It is possible that they could be represented by different parts of the neurone, that is the cell body, the axon or the terminal; also the compartments could represent different types of nerve cell. Presumably however the functional pool is located at the nerve ending, where it is available for release into the synaptic cleft. It is also unlikely that the nonfunctional pool of the amine is only in the cell body, as most of the 5-HT in brain seems to be located in the nerve endings (2).

As is the case with NA, some of the 5-HT in the nerve ending appears to be stored in small membranous vesicles, usually termed 'storage granules'. On the basis of many types of cytological and histochemical observation, Bloom (126) concluded that the 'small granular vesicles' seen in electron micrographs were of major importance in the storage of 5-HT (and also NA) in the brain. Another type of vesicle, the 'large granular vesicle', which was found in nerve endings along with the small vesicles but in smaller numbers, appeared to play only a minor role in amine storage. Furthermore, there seemed to be more than one type of small granular vesicle, one of them apparently being resistant to reserpine.

The subcellular fractionation studies of Maynert (32) have provided some direct evidence for the storage of 5-HT in vesicles in the nerve ending. It is difficult to tell however how much of the amine is stored in cellular organelles and how much is in the cytoplasm, as some is released into the surrounding solution during the fractionation procedure. In the case of NA in the heart, there

have been reports of between 25 and 70 per cent of the amine being in the particulate fraction (127).

Several mechanisms have been suggested for the release of NA from adrenergic nerves, which may serve as useful models for 5-HT in the brain. At the moment the two mechanisms thought to be most likely (Iversen and Bloom, 128) are exocytosis of the amine from the storage granules, and release of the amine from a small membrane-bound pool. There is a good deal of evidence available in support of both theories, and both of them are compatible with the model postulated above. The functional pool could be membrane-bound, adjacent to the synapse, with the nonfunctional pool in the storage granules and cytoplasm; alternatively the granules could represent the functional pool and the cytoplasm the nonfunctional one. Another possibility is that the two pools represent two populations of granules (as described by Bloom, 126), or there could be different types of binding sites within one granule. It is not likely however that the functional pool simply represents the population of granules which is close to the nerve-ending membrane, as the two pools seem to have different properties; the synthesis of 5-HT appears to be controlled by its level in one pool, but not in the other. It could be the case though that tryptophan hydroxylase is only sensitive to a <u>fall</u> in the level of 5-HT, and not to an increase above normal levels.

One problem hindering the interpretation of results is that the subcellular distribution of tryptophan hydroxylase is not known. According to Lovenberg (35), the enzyme is present mostly in the cytoplasm, but some may be membrane bound. An analogous situation exists for tyrosine hydroxylase, which is also thought to be a

soluble enzyme in the cytoplasm, though a small proportion has been found in the storage granules by Fahn <u>et al.</u> (129).

On balance, the evidence presented here probably favours the concept of a small pool of 5-HT bound to the nerve-ending membrane, and which is released by the nerve impulse. This model also allows for control of 5-HT synthesis in the functional pool by an electrical or other biophysical mechanism, as an alternative to product-inhibition. This idea is expanded in the next paragraph, headed 'Mechanism of 5-HT synthesis regulation'.

An attractive concept is that of a functional complex of tryptophan hydroxylase and 5-HTP decarboxylase, loosely bound to the nerve-ending membrane, and intimately coupled in some way to the release of 5-HT. Such an arrangement seems likely in view of the very low levels of 5-HTP found, and the lack of exchange of endogenous with exogenous 5-HTP, as described in the Introduction. Several examples of functional complexes of 'soluble' enzymes loosely bound together on a membrane are already known. Membrane fragments from broken erythrocytes and yeast cells have been shown to catalyse the complete sequence of glycolysis (130), the various enzymes being bound to the membrane to varying extents. Other examples of such complexes are the enzymes which collectively constitute the α-keto acid dehydrogenases, fatty acid synthetase, and tryptophan synthetase (131).

The model in Fig. 1.19 shows two hypothetical pools of tryptophan. These however need not be physically distinct; 5-HT in the functional pool may be synthesised from tryptophan just as it is taken up into the nerve ending, when, in this case, its specific

activity would be particularly high.

Mechanism of 5-HT synthesis regulation

The increased 5-HT synthesis which occurs on raphe stimulation probably involves an increased activity of existing tryptophan hydroxylase, rather than induction of new enzyme. This is indicated by the effect of stimulation after reduction of the enzyme activity by PCP. In this case, stimulation increased 5-HT synthesis in the same proportion as in animals which had not received the drug: if stimulation increased synthesis by increasing the amount of enzyme, a much larger rise would have been expected. Induction of tryptophan hydroxylase is also made unlikely by the rapidity of the changes in 5-HT synthesis in response to stimulation.

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

Such a mechanism would act through local changes in ion concentrations near the nerve-ending membrane. Depolarising agents such as ouabain and potassium ions have been found to increase the activities of tyrosine hydroxylase in the vas deferens (132) and

adenyl cyclase in the brain (133). Alternatively, there might be a direct allosteric interaction between tryptophan hydroxylase and molecules in the nerve-ending membrane at the site of transmitter release.

Another possible mechanism by which 5-HT synthesis might be controlled is through alterations in the rate of tryptophan transport to the site of 5-HT synthesis. No changes in the total tryptophan level in the brain were detected on stimulation, but local changes could be important. Possibly measurement of the tryptophan concentration in isolated synaptosomes could resolve this.

As stimulation did not alter the synthesis of (^{3}H)5-HT from (^{3}H)5-HTP, it is concluded that the activity of 5-HTP decarboxylase is probably not affected by nervous activity. It could be the case however that there is a small fraction of this enzyme which is intimately associated with tryptophan hydroxylase, and which may in fact be subject to a regulatory mechanism. The common supposition that tryptophan hydroxylase, rather than the decarboxylase, is the rate-limiting step in 5-HT synthesis might thus be treated with caution.

One more way in which 5-HT synthesis at the nerve ending might be controlled is through changes in the rate of transport of tryptophan hydroxylase from the cell bodies to the nerve terminals. Nerve cells are able to transport protein along their axons at rates upto 20 mm/hour (Lasek, 134; Sjostrand, 135; Ochs and Ranish, 136), although the rate of transport of tryptophan hydroxylase in the spinal cord is only 5 to 7 mm/day (Meek and Neff, 137). Also stimulation of nerves has been reported to have no effect on the rate of axonal

transport of protein (Lux et al., 138). These results make it unlikely therefore that changes in transport of tryptophan hydroxylase are important in acute changes in 5-HT synthesis in response to changes in nervous activity.

Long-term effects of stimulation.

The interest in the 'long-term' effects of stimulation stemmed primarily from the work of Eccleston et al. (46), who showed that an increased rate of 5-HT synthesis outlasted the stimulation period by at least an hour. As they had used tryptophan loading however, which may have affected the result, the experiment was repeated using the method for 5-HT synthesis measurement developed in this section. It was found in this case that the rate of 5-HT synthesis returned to normal almost immediately after the cessation of stimulation. The next experiment was designed to see whether a prolonged increase in synthesis was only seen in the presence of a tryptophan load. Tryptophan was given one hour after the end of the stimulation period, but again no difference was found between stimulated and control animals. The remaining possibility was that tryptophan would maintain the hydroxylase enzyme in a more active form if given during or immediately after the stimulation period. However, even when the tryptophan load was administered at the time of stopping stimulation (15 min earlier than Eccleston et al.), no difference was found between the two groups.

Thus the results of Eccleston et al. could not be reproduced here. It is interesting to note that these authors found a rate of accumulation of 5-HIAA of 3.0 ng/g/min in the controls and 5.6 ng/g/min in the stimulated animals, and that the latter figure is about the

same as was found in both groups in the results described here. It could have been the case therefore that the rate of 5-HT synthesis in the animals used by Eccleston et al. was abnormally low, and was 'switched on' by the stimulation.

It might be mentioned here that there is evidence for induction of tyrosine hydroxylase in response to stimulation of adrenergic nerves. Thoenen et al. (139) found that a variety of drugs which gave an increase in sympathetic nerve activity increased the in vitro activity of adrenal tyrosine hydroxylase, and Weiner and Rabadjija (140) found an increase in NA synthesis in the vas deferens which outlasted stimulation. In both cases the increases were sensitive to inhibitors of protein synthesis.

The results presented here indicate that if tryptophan hydroxylase synthesis is affected at all by the rate of discharge of the serotonergic neurones, this must be a relatively long-term adaptation mechanism. The existence of such a mechanism at all is questioned by findings of the effects of tryptophan loading in depressed patients. The response to a tryptophan load in these patients has shown that the activity of tryptophan hydroxylase is normal (141), despite the finding that their lumbar CSF 5-HIAA levels are much below normal (142, 143, 144). This suggests that the reduced 5-HIAA levels are a consequence of reduced firing of the serotonergic nerves, and in turn that the synthesis of tryptophan hydroxylase is not related to these firing rates.

SECTION 2

THE EFFECTS OF ELECTRICAL STIMULATION OF THE RAT MIDBRAIN ON THE SYNTHESIS OF RIBONUCLEIC ACID AND PROTEIN

INTRODUCTION

A knowledge of the relationship between the electrical activity of a neurone and its synthesis of ribonucleic acid (RNA) and protein is fundamental to the understanding of basic neurochemical processes, and to brain function as a whole. Considerable attentionn has been focussed recently for example on the role of RNA and protein synthesis in the storage of memory. Barondes (145) has investigated the effects of inhibiting protein synthesis with the drug cycloheximide on memory storage in rats and mice, and has shown that immediately after training, the memory is susceptible to electroconvulsive shock (ECS) but not to cycloheximide, but after several hours, the memory becomes obliterated by the drug but not by ECS. This suggests that the memory trace is stored initially in some way electrically, but is gradually changed into a more permanent form dependent on the synthesis of new protein. Work on amnesia induced by another inhibitor of protein synthesis, puromycin, has been published by Flexner (146) and Agranoff (147), and work by Hyden and Lange (148) suggests that synthesis of the protein S-100 may be specifically involved in the memory storage process. Bennett et al. (149) found that rats reared in a 'complex' environment where they were exposed to constant behavioural stimulation had an increased protein content and acetylcholinesterase activity in the cerebral cortex compared to controls subjected to minimal behavioural stimulation.

Various types of 'physiological' stimulation have been found to alter the RNA and protein content of nerve cells. Exhaustive muscular effort was found by Hyden (150) to decrease the RNA and protein content of spinal cells, whereas after a complex vestibular training

where the rats were trained to climb up an inclined wire, there was an increase in RNA in the brain. The newly-synthesised RNA had a different base-ratio from the original, and similar changes also took place in glial cells (151, 152). These changes were shown to depend upon the process of learning, and were not simply due to increased motor activity. Dewar and Reading (153) found that photic stimulation of rats increased RNA synthesis in the visual cortex.

Many of the different methodological approaches to the study of the effect of increased functional activity of nerve cells on RNA and protein metabolism introduce complicating factors which may distort the results. Such changes include alterations in hormonal balance and hypoxia, both of which affect macromolecular synthesis (154, 155). Stimulation of nervous tissue directly may help to reduce factors such as these resulting from changes in the activity of other parts of the body. There have been many studies concerning the effects of electrical stimulation of nervous tissue on RNA and protein synthesis, but not many of these have involved stimulation of intact brain _in situ_: mostly work has involved study of _in vitro_ systems or peripheral nerves. Several reports have been published on the effects of ECS, but such stimulation can hardly be considered to be a normal physiological state. Some of the published work dealing with electrical stimulation of neurones and synthesis of RNA and protein is described as follows.

Effects of stimulation on RNA synthesis

Stimulation of brain slices was found by Orrego (156) to reduce the incorporation of (^{14}C)uridine into RNA by 40 per cent, partly due to a decreased formation of UDP and UTP. Similar results were obtained by Prives and Quastel (157).

ECS has been found to reduce RNA levels in brain, as shown by Mihailovich et al. (158), who found a decrease of about 25 per cent, depending on the brain area. Einarson and Krogl (159) stimulated the giant sea-snail Aplysia sufficiently to cause general tetanic seizures, and found a considerable reduction in cytoplasmic RNA in the abdominal ganglia of the animal. This might suggest an increased activity of the enzyme ribonuclease, though Vesco and Giuditta (160) failed to show any increased activity of the enzyme in the rat brain after ECS.

In the peripheral nervous system, Stoller and Wayner (161) found that stimulation had no effect on RNA synthesis in rat dorsal root gänglion cells and motoneurones from the cat and guinea pig. Hyden (150) however found that stimulation of spinal ganglion cells increased their RNA and protein content.

Some interesting results have been obtained in invertebrate systems. Grampp and Edstrom (163) found that generation of action potentials for several hours by the lobster stretch receptor did not alter the total amount of RNA in the cell body, but the RNA base-ratio was significantly changed. D'Yakonova et al. (164) stimulated the isolated abdominal chain of the earthworm Lumbricus and found an increase in RNA; they concluded that the total synthesis of RNA was proportional to the rate of discharge of the cells.

Particularly informative results were obtained by Peterson and Kernell (165, 166), working on identified giant neurones in the abdominal ganglion of Aplysia. The cells were stimulated either trans-synaptically via afferent nerves, or by intracellular micro-electrodes. They found that synaptic activation led to a large

increase in the incorporation of labelled nucleotides into RNA of a wide range of types, but there was no increase in incorporation when the cells were stimulated via the intracellular electrode. Furthermore, weak stimulation of the afferent nerves, which produced few discharge spikes in the ganglion cells, but mostly only slight depolarisation, gave a small but significant increase in RNA labelling. These results strongly suggest that the increased labelling was due to an effect of synaptic transmission other than the production of an action potential in the post-synaptic cell. Berry (167) also found an increase (of 80 per cent) in the incorporation of labelled uridine into RNA in the same preparation following trans-synaptic stimulation. Comparable results were obtained by Gisinger (168) working on the isolated rat sympathetic ganglion. An increase in the incorporation of labelled uridine into RNA was found on either preganglionic stimulation or application of acetylcholine. The increased labelling of RNA was not reduced by preventing depolarisation of the ganglion cells with tetrodotoxin; however depolarisation of the cells with KCl failed to increase the RNA synthesis. Thus the critical event appeared to be the interaction of the acetylcholine with its receptor, rather than just the depolarisation of the ganglion cell.

A corollary of these findings is provided by the work of Kupfer and Downer (169), who showed that 5 days after section of the optic nerve in the monkey, RNA in the lateral geniculate body (to which the optic nerve leads) fell to 30 per cent of the normal level. Thus in this case there seemed to be a reduction in RNA synthesis following reduced synaptic input to a group of neurones.

The findings described so far represent all the possible types of response of RNA synthesis to electrical stimulation, that is, an increase, a decrease, or no change. The work of Peterson and Kernell (165, 166) and Gisinger (168) suggests that trans-synaptic excitation of the nerve cell is necessary for increased RNA synthesis to be seen; thus the negative results obtained by some workers might be ascribed to their use of direct, rather than synaptic, stimulation of the nervous tissue. On the other hand, the different results may reflect the different properties of the various preparations studied.

In the cases where a decrease in RNA level or synthesis was observed, the results seem to be correlated with a very intense stimulation, such as ECS. What may be happening here is that intense depolarisation of the neurones gives an imbalance of Na^+ and K^+ ions, activation of ATPase-linked ion transport systems, and a consequent diversion of nucleotide stores and synthesis from other pathways, including RNA synthesis: reduced levels of uridine nucleotides were found by Orrego (156) and Prives and Quastel (157) after stimulation of brain slices. In hardly any of the experiments where a controlled stimulus of moderate intensity was used was there a reduction in RNA synthesis or concentration. In his own experiments, Hyden (170) has found that in general, situations where there is moderate stimulation of nerve cells increase RNA synthesis, and more intense stimulation reduces the synthesis.

Effects of stimulation on protein synthesis

Ungar et al. (171) found that stimulation of the sciatic nerve, which contains cortical afferents, eventually caused proteolytic

activity and protein breakdown in both the nerve and the cerebral cortex. Hyden (150) however found that electrical stimulation of spinal ganglion cells increased their protein content.

There have been many studies of the effects of ECS on protein synthesis in brain. Dingman et al. (172) found no change in the incorporation of (^{14}C)proline into protein, but Geiger et al. (173) found an increased incorporation of radioactivity from (^{14}C)glucose. Dawson and Richter (174) showed a 50 per cent reduction in incorporation of ^{32}P into phosphoprotein, Gaitonde and Richter (175) reported a small reduction in the incorporation of (^{35}S)methionine into protein, and Dunn (176) found a 50 per cent reduction in incorporation of (^{14}C)leucine. A possible explanation for the differences between the reports of the various investigators is suggested by Hemmer (177), who found that ECS only caused changes in brain metabolite levels when there were greater changes in the liver: the liver apparently compensated largely for the increased metabolism of the brain. Thus the effect of ECS on brain metabolism may depend on the metabolic state of the whole animal. A reduction in protein synthesis after ECS might be anticipated in view of the findings of Vesco and Giuditta (160), who showed that ECS caused extensive disaggregation of polysomes into free ribosomes in the cerebral cortex. They suggested that this might be due to alterations in ion levels, particularly in Na^+, K^+, and Mg^{+2} ions, to which polysomes are especially sensitive.

Some positive results of stimulation on protein synthesis have been obtained by measuring individual enzymes rather than total protein. Thoenen et al. (139) treated rats with a variety of drugs

which led to an increase in the electrical activity of the sympathetic nerves, and found an increase in the _in vitro_ activity of adrenal tyrosine hydroxylase, indicating a probable _de novo_ synthesis of the enzyme. Weiner and Rabadjija (140) showed that stimulation of the isolated guinea-pig vas deferens increased NA synthesis, and that this elevated synthesis persisted after stimulation had stopped. The increased synthesis did not persist after cessation of stimulation however if puromycin was given to inhibit protein synthesis, thus suggesting that stimulation had induced synthesis of tyrosine hydroxylase or an associated protein. Musacchio _et al._ (178) showed that chronic ECS given to rats increased the _in vitro_ activity of brain tyrosine hydroxylase.

There is, therefore, a wealth of data suggesting that the metabolism of RNA and protein in nerve cells is linked in some way to their electrical activity. So far however, hardly any reports have been published dealing with the effects of moderate stimulation on the synthesis of these macromolecules in the brain _in situ_. The metabolism of RNA in the rat brain has been investigated in this laboratory in some detail (153), and the opportunity arose for the study of the effects of stimulation on RNA synthesis. The need to stimulate a well-defined system in the brain rather than at an arbitrary position is obvious: in this respect, the median raphe nucleus was a very satisfactory choice for the present purposes, as it projects to all parts of the brain, and may be a general modulator of brain activity. This is suggested for example by the work of Jouvet (179) connecting the raphe system with sleep function. Also some information is available suggesting a direct connection between

the transmitter substance in the raphe system, i.e. 5-HT, and RNA. Siegel and Salinas (180) showed that an elevation of brain 5-HT levels reduced RNA polymerase activity, and in a study of electroshock-induced amnesia and 5-HT metabolism, Essman (181, 182) found that the decrease in RNA levels after electroshock correlated with the elevation of 5-HT.

In the present study, the effects of raphe stimulation on synthesis of RNA and protein were investigated. The rate of incorporation of (^{3}H)uridine into RNA was used as the index of RNA synthesis, and in the case of protein, the incorporation of (^{14}C)leucine.

METHODS

Materials

Radiochemicals, obtained from the Radiochemical Centre, Amersham, were:

(5-^{3}H)uridine, 25 Ci/m.mole

L-(1-^{14}C)leucine, 63 mCi/m.mole

Cycloheximide was obtained from Sigma Ltd, and Hyamine 10-X hydroxide, as a 1M solution in methanol, from Packard Ltd.

Purification and assay of RNA

RNA was extracted by the method of Guroff et al. (183) slightly modified. Procedures were carried out at 0-4°C where possible. The brains were homogenised in 10 ml 6 per cent TCA and allowed to stand for 30 min. The homogenate was centrifuged at 3,000 g for 5 min, and 1 ml of the supernatant removed for determination of acid-soluble radioactivity. The precipitate was washed twice with 5 ml 6 per cent TCA and defatted with 5 ml ethanol-diethyl ether (3:1),

followed by 3x5 ml diethyl ether. The TCA solution contained 2 μg/ml of the ribonuclease inhibitor polyvinyl sulphate. The defatted precipitate was suspended in 5 ml 0.3N NaOH and incubated at 37°C for 18 hr. 0.5 ml 50 per cent TCA was then added and the mixture allowed to stand at 0°C for 30 min. The resulting precipitate was removed by centrifugation, and 0.1 ml of supernatant taken for assay of the hydrolysed RNA. The radioactivity of the RNA was determined by adding 1 ml supernatant to 10 ml dioxane-based scintillator fluid before counting. The acid-soluble counts were determined similarly.

RNA was assayed by the Ceriotti orcinol method (184), as modified by Guroff _et al._ (183). 0.1 ml of the supernatant was made up to 1.5 ml with distilled water. 1.5 ml 0.05 per cent $FeCl_3$ in conc. HCl was added, and after mixing, 0.2 ml 6 per cent orcinol in ethanol was added. The samples were heated at 100°C for 20 min. The tubes were cooled and the green reaction product read at 665 nm against the yellow reagent blank in a Perkin-Elmer SP500 ultraviolet spectrophotometer. Yeast RNA was used as a standard.

Estimation of (^{3}H)uridine nucleotides

The amounts of radioactivity in uridine and uridine nucleotides in the acid-soluble fraction were estimated by paper chromatography. The fraction was washed with 5x10 ml water-saturated ether to remove TCA. The solution was freeze-dried and the residue dissolved in 0.2 ml ethanol. This was applied to a 1 cm origin on a 4 cm-wide strip of Whatman no.3 paper, and the chromatogram was developed for 6 hr in the descending mode in a solvent of ethanol-ammonium acetate solution (1M, pH 7.4) (2:1 by volume). After drying,

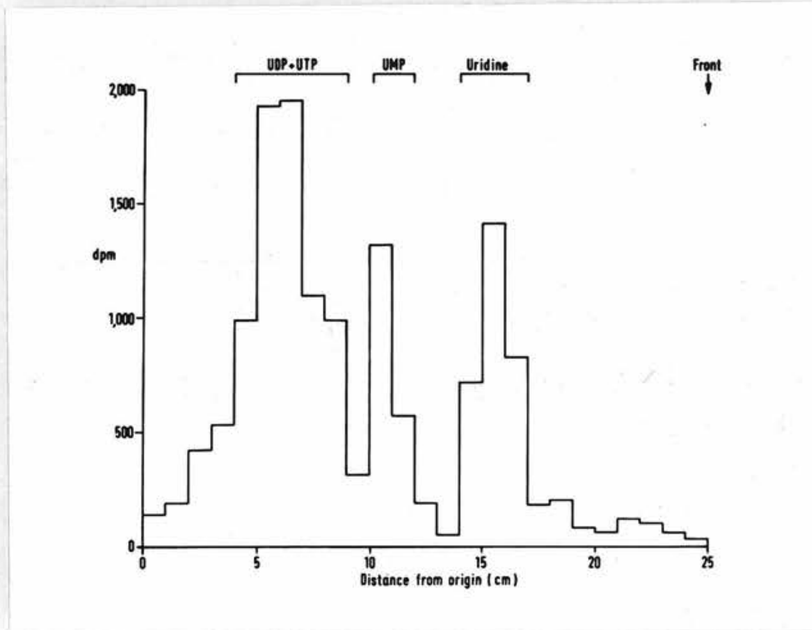

Fig. 2.1. Paper chromatogram of acid-soluble fraction after i.vtr. injection of (^{3}H)uridine.

the chromatogram was cut into 1 cm portions, which were eluted into 1 ml 0.1N HCl before addition of scintillator and counting.

Chromatograms prepared from rats treated with (^{3}H)uridine showed three distinct peaks of radioactivity. Analogy with markers run in parallel showed they were uridine, UMP and UDP+UTP (Fig. 2.1). Intraventricular injection was performed as described by Noble et al. (185). A small hole was drilled in the skull with a sharp ice-pick, 1.5 mm lateral to the sagittal suture, and 1.5 mm posterior to the coronal suture. The solution was injected from a Hamilton syringe fitted with a needle guarded to within 3.5 mm of the tip, thus keeping the depth of insertion of the needle constant. The dose of (^{3}H)uridine given was in each case 5 µCi, dissolved in 20 µL of Merle's solution.

Measurement of radioactively-labelled protein

Protein labelled by systemic injection of (^{14}C)leucine was measured as follows.

Brains were homogenised in 5 ml 0.4N PCA and centrifuged at 15,000 g for 10 min. The supernatant was kept for determination of acid-soluble counts. The pellet was resuspended in 10 ml 0.4N PCA by homogenisation and centrifuged at 1,500 g for 10 min; this wash was then repeated once. After the second wash, the supernatant contained negligible radioactivity. The pellet was dissolved in 15 ml 98-100 per cent formic acid by incubating for 12-16 hr at 50°C. 1 ml of the solution and 2 ml water were added to 18 ml Triton X-100 scintillator: this formed a clear solution. The counting efficiency was much lower than normal, being about 40 per cent. In view of this, the efficiency of each sample was determined by an internal standard

rather than using the external standard as described in Section 1. This involved counting the sample, adding a standard amount of (^{14}C)toluene, and re-counting.

An alternative method for solublising the protein was tried, using a solution of Hyamine 10-X hydroxide (Packard). However this substance gave very high background counts, probably due to chemiluminescence.

The acid-soluble counts in the original supernatant were determined by neutralisation with 5N KOH, centrifuging off precipitated $KClO_4$, and counting 1 ml in 10 ml Triton X-100 scintillator.

RESULTS

Effects of raphe stimulation on RNA synthesis

(a) Effects of stimulation on incorporation of (^{3}H)uridine into RNA

In the following experiments the median raphe nucleus was stimulated at a frequency of 10/sec, as described in Section 1, with the electrode tip positioned in the centre of the median raphe nucleus at A + 0.4 mm, V + 1.6 mm. The degree of incorporation of intraventricularly-injected (^{3}H)uridine into RNA was measured. Reports in the literature on the effects of stimulation on RNA synthesis indicate that the effect observed is probably dependent on the intensity of stimulation, and therefore a range of currents was used; the range chosen was of the same order as that used in Section 1 for the study of the effects of stimulation on 5-HT synthesis.

In the first experiment, rats were stimulated for 30 min before being given an intraventricular injection of 5 µCi of (^{3}H)uridine. As Fluothane anaesthesia has possibly been found to affect RNA metabolism (186), stimulation was always begun exactly

15 min after the time of anaesthetising, thus keeping the duration of anaesthesia constant in each experiment. The animals were killed 30 min after the uridine injection, stimulation being continued during this period. The brains were then assayed for endogenous and radioactive RNA: the results are shown in Table 2.1.

The actual amount of uridine injected was less than one per cent of the endogenous uridine content of the rat brain (according to the value for the latter given by Minard and Grant (187)), and so would be unlikely to interfere quantitatively with the normal uridine pool. It was found that there was a fairly wide variation in the acid-soluble counts in identically-treated animals, but there was a close correlation between the acid-soluble and RNA counts ($r = 0.98$ over 10 results (the controls), $P < 0.001$). Thus in order to reduce variations in the results due to differences in the amounts of (^{3}H)uridine injected or the rate of transport of this from the CSF to the brain, the results are expressed as the ratio of the RNA specific activity to the acid-soluble counts.

Table 2.1 shows that there were no significant alterations in the RNA labelling in any of the stimulated groups compared to controls, although there is a suggestion of an increase at a current of 0.2 mA.

In the next experiment a longer time of stimulation was allowed before injection. Rats were stimulated at a single current, 0.2 mA, for 60 min, (^{3}H)uridine injected, and the animals killed after a further 30 min. In this case there was a large increase in RNA labelling (Table 2.2). A single current was used at this stage in order to provide more duplicate observations; the results in Table 2.1

Table 2.1 Effect of raphe stimulation on incorporation of (^{3}H)uridine into RNA; 30 min stimulation before injection.

Current (mA)	RNA relative specific activity (dpm x10^{3}/mg/acid-soluble dpm)
0	105 ± 20
0.1	82 ± 18
0.2	134 ± 56
0.4	107 ± 25

Animals were stimulated for 30 min before receiving (^{3}H)uridine. Stimulation was continued until killing after a further 30 min. Results show mean ± S.D. of 5 observations.

No group is significantly different from control (R test).

Table 2.2 Effect of stimulation on incorporation of (^{3}H)uridine into RNA, with 60 min stimulation before injection.

Group	RNA relative specific activity (dpm x10^3/mg/acid-soluble dpm)	Per cent Control
Control	70 ± 16	100
Stimulated (0.2 mA)	113 ± 28*	161

*Significantly higher than control, $P < 0.001$ (t test)

Results show mean ± S.D. of 10 observations.

may represent real changes which are masked by the rather wide scatter of the results.

In a separate experiment, a range of currents was used from 0.1 to 0.8 mA. The results (Table 2.3) show an increase in RNA labelling at currents of 0.1 to 0.4 mA, but a decrease at 0.6 and 0.8 mA. As in the last experiment, the initial stimulation period was 60 min and the labelling period 30 min.

The work of Balazs and Cocks (188) showed that the degree of labelling of RNA continues to rise for about 60 hours after injection of labelled precursor, before starting to decline. To see whether the above findings represented a prolonged effect, the labelling period was increased to 24 hours. Rats were stimulated for one hour, injected with (^{3}H)uridine, and stimulation continued for 30 min. At the end of this period, they were allowed to recover and were killed 24 hours after the injection. The results (Table 2.4) showed no differences in the degree of RNA labelling at stimulation currents which had either given an increase or a decrease in labelling at 30 min after injection.

(b) Effect of stimulation on total RNA level

The total amount of RNA in the brain at the end of a 90 min stimulation period was found to be unaltered from control levels in all cases (Table 2.5).

(c) Effect of stimulation on uridine phosphorylation

Orrego (156) suggested that the reduction in RNA labelling he found in stimulated brain slices might be due, at least in part, to depletion of nucleotide pools. Therefore the levels of (^{3}H)uridine nucleotides were measured in the brains of some of the

Table 2.3 Effect of various stimulation currents on RNA labelling

Current (mA)	RNA relative specific activity (dpm x10^3/mg/acid-soluble dpm)	Per cent Control
0	89.9 ± 13.3 (3)	100
0.1	134.6 (mean of 68.2, 201.0)	150
0.2	147.5 (" " 156.4, 138.7)	164
0.4	130.0 (" " 127.8, 132.2)	147
0.6	48.8 (" " 48.3, 49.2)	54
0.8	48.9 (" " 47.7, 50.2)	54

The 0.6 and 0.8 mA groups taken together show a significant reduction compared to controls, $P < 0.05$ (R test).

Table 2.4 Effect of stimulation on RNA labelling in a 24 hour period.

Current (mA)	RNA relative specific activity (dpm x10^3/mg/acid-soluble dpm)
0	1,142 ± 102
0.2	1,297 ± 138
0.8	1,231 ± 150

Results show mean ± S.D. of 4 observations.

Neither stimulated group differs significantly from control (R test).

Table 2.5 Effect of stimulation on brain RNA content

Current (mA)	RNA (mg/g. wet weight)
0	2.32 ± 0.07 (11)
0.2	2.33 ± 0.18 (11)
0.4	2.38 ± 0.14 (3)
0.6	2.31 ± 0.04 (3)
0.8	2.31 ± 0.05 (3)

Rats received 90 min. stimulation.

stimulated animals. These animals were those stimulated for 60 min before injection, whose results are shown in Table 2.3. The levels of (^{3}H)uridine and uridine nucleotides found are shown in Table 2.6: there appears to have been little change at a current of 0.2 mA, but at 0.8 mA there was a reduction in the (^{3}H) UDP + UTP level, although the total acid-soluble counts were unchanged. Thus the reduction in RNA labelling found at higher currents could well have been due to the depletion of uridine nucleotide pools.

<u>Effect of raphe stimulation on protein synthesis</u>

(a) <u>Effect of cycloheximide on incorporation of (^{14}C)leucine into protein</u>

It was proposed to measure the rate of protein synthesis by giving (^{14}C)leucine intraperitoneally, and measuring the amount of radioactivity incorporated into protein. A simple method was used to separate the protein, as described in the Methods section, where the whole acid-insoluble fraction was dissolved in formic acid and counted, without any separation into protein, nucleic acids etc. Because only a short time was to be used for labelling the brain protein (30 min), it was expected that little radioactivity would be incorporated into other acid-insoluble material. This assumption was verified by using the inhibitor of protein synthesis <u>cycloheximide</u> to prevent incorporation of label from (^{14}C)leucine into protein. Cycloheximide causes a rapid and specific block of protein synthesis in all eucaryotic cells, and has been shown to have little or no effect on energy metabolism in various <u>in vitro</u> systems (189), or on RNA synthesis <u>in vitro</u> (189) and in rat brain (190).

Rats received various doses of cycloheximide, injected

Table 2.6 Effect of stimulation on levels of (^{3}H)uridine and uridine nucleotides

	Current (mA)		
	0	0.2	0.8
Uridine	269 (219, 320)	326 (252, 399)	492 (468, 516)
UMP	135 (102, 167)	93 (86, 100)	163 (157, 180)
UDP + UTP	538 (473, 604)	605 (524, 686)	322 (244, 400)
Acid-soluble counts	1410 (1170, 1650)	1415 (1100, 1730)	1405 (1200, 1610)

Results (dpm x10^{-3}) show mean values of two measurements, with the individual results in brackets.

intraperitoneally in saline. Two hours later they were given 25 µCi/kg (^{14}C)L-leucine i.p., and were killed after a further 30 min. The degree of incorporation of the label into the acid-insoluble fraction is shown in Table 2.7.

Possible contamination of the protein by free (^{14}C)leucine was tested by homogenising a brain with 0.02 µCi (^{14}C)leucine, and isolating the insoluble material as described. Less than 0.25 per cent was retained in this fraction.

(b) Effect of raphe stimulation on protein synthesis

As described above, raphe stimulation was shown to increase RNA synthesis. It was possible that there would be a consequent increase in protein synthesis; thus the effect of raphe stimulation on incorporation of (^{14}C)leucine into protein was investigated.

Initially the timing of the experiment was the same as in the RNA experiment which showed an increase of labelling on stimulation. Rats were stimulated for 60 min at various currents; otherwise the parameters were the same as were used in the RNA experiments. They then received (^{14}C)leucine (25 µCi/kg, i.p.), and stimulation was continued until the animals were killed 30 min later.

There was a high correlation between the protein dpm and the acid-soluble dpm ($r = 0.855$ over 24 results, $P < 0.001$); thus to correct for variations in the amount of (^{14}C)leucine taken up into the brain, the results are expressed as the ratio of the protein dpm to the acid-soluble dpm.

The results are shown in Table 2.8, and suggest that stimulation had little effect.

It is possible however that a certain time would be needed

Table 2.7 Effect of cycloheximide on incorporation of (^{14}C)leucine into acid-insoluble material

Dose of cycloheximide (mg/kg)	Radioactivity in acid-insoluble material (dpm/g. fresh brain)	Per cent inhibition
0	9,239 ± 359 (4)	0
1.7	5,580 (mean of 2)	40.0
2.5	4,184 (")	54.8
11.5	1,101 (")	88.1
52.0	185 (")	98.0
250.0	32 (1 result)	99.7

Table 2.8 Effect of stimulation on incorporation of (^{14}C)leucine into protein

Current (mA)	Ratio Protein dpm/ acid-soluble dpm.	Acid-soluble dpm
0	1.94 (1.57; 2.31)	1,155 (1,381; 929)
0.1	2.20 (1.71; 2.58)	754 (669; 839)
0.2	1.62 (1.02; 2.22)	902 (1,082; 723)
0.6	3.39 (2.28; 4.49)	767 (747; 787)

Results show mean of two values, with the individual figures in brackets.

between an increase in RNA synthesis, which occurs in the nucleus, and an increase in protein synthesis, which occurs in the cytoplasm. Therefore rats were stimulated for 90 min, the electrode withdrawn and the animals allowed to recover. 30 min after the end of stimulation, they were given the injection of (^{14}C)leucine, and were killed after a further 30 min. The result is shown in Table 2.9, the stimulation current being 0.2 mA: there was no significant rise in protein labelling. In a third experiment, the stimulation period was again 90 min, but the recovery time was increased to 90 min. The leucine was injected and the animals killed after a further 30 min as before. The result (Table 2.10) showed that there was again no significant increase in protein labelling.

DISCUSSION

The results show that stimulation of the median raphe nucleus at a certain current strength can cause an increase in labelling of RNA by (^{3}H)uridine in a 30 minute period, and that stimulation at higher currents can cause a decrease in the labelling.

The actual amount of uridine incorporated into RNA during the labelling period will be given by the ratio of the RNA radioactivity to the mean specific activity of its immediate precursor, in this case (^{3}H) UTP. In these experiments, the total acid-soluble counts at the time of killing were used as an index of the mean UTP specific activity. This approximation allowed for variations in the amount of uridine injected or taken up into the brain: it is assumed that the acid-soluble counts are due mostly to uridine and uridine nucleotides. The use of the acid-soluble counts as an index of precursor specific activity was justified by the very close correlation

Table 2.9 Incorporation of (^{14}C)leucine into protein, injected 30 min after a 90 min stimulation period

Group	Ratio Protein dpm/acid-soluble dpm
Control	2.47 ± 0.39 (4)
Stimulated	2.57 ± 0.82 (4)

Stimulation current 0.2 mA.

The result of the stimulated group is not significantly higher than the control (R test).

Table 2.10 Incorporation of (^{14}C)leucine into protein, injected 90 min after a 90 min stimulation period

Group	Ratio Protein dpm/acid-soluble dpm
Control	2.00 ± 0.54 (4)
Stimulated	2.35 ± 0.75 (4)

Stimulation current 0.2 mA.

The result of the stimulated group is not significantly higher than the control (R test).

of RNA specific activity with the acid-soluble counts in the control animals, despite a rather wide scatter in the values of both of these; by taking the ratio, the scatter was very much reduced. What was not allowed for however was an alteration in the rate of phosphorylation of uridine. Table 2.6 shows that the amount of (^{3}H)UDP + UTP in the brains of animals stimulated at 0.8 mA (the highest current used) was much reduced, though the total acid-soluble counts were unchanged. Changes in the concentration of uridine in the brain were also not allowed for, which would have affected the specific activity of the uridine pool.

As there appeared to be no change in the level of (^{3}H)UDP + UTP in animals stimulated at 0.2 mA, the results suggest a genuine increase in the rate of RNA synthesis at this current. There may have been however a decrease in the rate of breakdown of the labelled RNA, which would have given the same effect.

The results suggest that there may be a time lag between the beginning of stimulation and the increase in RNA labelling, as an increased labelling was found when the uridine was injected 60 minutes after starting stimulat ion, but not when this time was 30 min.

How the increase in RNA labelling was mediated is unknown. Substantial ion fluxes occur when nerves are depolarised, and RNA polymerase is sensitive to ion concentrations (191, 192). The work of Peterson and Kernell (165) and Gisinger (168) suggests however that the increase in RNA labelling on nerve stimulation depends on something other than just depolarisation: the essential event seems to be the binding of the neurotransmitter to its receptor in the postsynaptic membrane. Gisinger (168) suggested that the binding of

the transmitter to the receptor might release regulatory proteins or other molecules from the inside of the cell membrane by a conformational change or ion-exchange effect. It is not possible from this work to tell whether the effect was a post-synaptic one or not; perhaps an analysis of different brain areas after stimulation and RNA labelling would throw light on this. Stimulation of the raphe nucleus does of course increase the release of 5-HT from serotonergic nerve-endings, which could affect RNA synthesis in the post-synaptic cells. Siegel and Salinas (180) found a reduction in RNA polymerase activity when brain 5-HT levels were raised (though the method of raising the 5-HT levels was not stated), and an inhibition of the enzyme by 5-HT *in vitro*. These results do not contradict the present findings though, as it would be very unlikely that 5-HT released from the nerve ending would have a direct effect on nuclear RNA polymerase in the post-synaptic cell.

With higher stimulation currents - 0.6 and 0.8 mA - there was a significant fall in RNA labelling. This pattern of an apparent increase in RNA synthesis at a certain level of stimulation, and an apparent decrease with more intense stimulation, has been noted on several occasions by Hyden (170). A reduction in RNA labelling was seen on stimulation of brain slices *in vitro* by Orrego (156) and Prives and Quastel (157), which they claimed was attributable, at least in part, to depletion of nucleotide pools. Depolarisation of nerve cells leads to an influx of sodium into the cell and an efflux of potassium. ATP-linked ion transport mechanisms are then activated, with a consequent diversion of ATP and energy metabolism from other pathways, including UTP synthesis, which requires ATP (154). A fall

in the level of (^{3}H)UDP + UTP was in fact observed here at a current of 0.8 mA, though there was no change in the total acid-soluble counts; this suggests a reduction in the rate of uridine phosphorylation, rather than a reduction in the amount of (^{3}H)uridine taken up. At the higher currents, there would presumably be greater disturbances of ionic equilibria. It seems likely that the reduction in the (^{3}H)UDP + UTP level reflects a reduction in the endogenous pool of UDP and UTP, and thus the fall in RNA labelling represents a true reduction in RNA synthesis, as a consequence of the reduced precursor levels. The fall in RNA labelling was of approximately the same magnitude (40 per cent) as the fall in the (^{3}H)UDP + UTP level.

An alternative explanation for the fall in synthesis at higher currents is cellular damage. Histological examination showed however that lesioning was slight, and certainly not large enough to account for the reduction in labelling found.

No alteration in the total RNA content of the brain was seen after 90 minutes' stimulation. This could have been because the rate of synthesis of RNA was small in relation to the total pool of RNA, and hence a rise in its synthesis over a relatively short period would have had little effect on the total brain RNA. Stimulation might also have increased the breakdown of RNA, thus increasing its turnover without altering its concentration. Another possibility is an increase in the RNA content of the neurones, with a concomitant reduction in glial RNA, as has been observed by Hyden (170): many reports suggest a close functional connection between neurones and glia (154). It could also be argued that the majority of the cells in the brain are not affected by the stimulation, and thus any changes

in the RNA content of the affected cells would be diluted to a large extent by the rest of the brain. A change in apparent RNA synthesis was seen however in the whole brain, which seems to invalidate this argument. Probably cytochemical techniques would be useful in establishing whether any changes in the RNA content of the stimulated neurones took place.

There was no effect of stimulation on RNA labelling measured 24 hours after the uridine injection. This suggests that the effects of stimulation noted with a 30-minute labelling period were only of short duration, and also that the extra RNA synthesised following stimulation turned over in less than 24 hours, unless of course the increased synthesis was followed by a compensatory decrease.

Thus this work has confirmed the results of other workers who studied RNA synthesis in peripheral nerves or *in vitro* systems. For this reason it is unlikely that these findings are a special property of serotonergic nerves; it is more likely that the magnitude of the observed increase is attributable to the wide projection of the raphe system.

In the work on protein synthesis, the use of a relatively simple method for the measurement of labelled protein was justified by the virtually complete inhibition of incorporation of (^{14}C)leucine by cycloheximide, a specific inhibitor of protein synthesis.

The results are expressed as the ratio of the acid-insoluble to the acid-soluble counts, as there was a high correlation between them: this allowed for variations in the amount of (^{14}C)leucine taken up into the brain. As was noted above in the case of RNA, ideally the ratio of the protein counts to the mean leucine specific

activity should be taken as the index of protein synthesis. In these experiments, the acid-soluble counts are used as an approximation to the mean specific activity of leucine. Stimulation appeared to have little effect on the uptake of leucine by the brain (Table 2.8), although there still could have been changes in the concentration of the amino acid in the brain caused by altered protein breakdown, energy metabolism etc.

No changes in protein synthesis were found on stimulation, either using the same timing as with the RNA experiments which gave a rise in RNA labelling, or when allowing upto 90 minutes after stimulation before injection of (^{14}C)leucine. Egyhazi and Hyden (193) showed that RNA appears in the cytoplasm of nerve cells only 15 minutes after being synthesised in the nucleus, although according to Balazs and Cocks (188), this time is somewhat longer. The time allowed in the present experiments should however have been long enough for RNA made in the nucleus to reach the site of protein synthesis in the cytoplasm. It is therefore uncertain whether any of the increase in RNA synthesis was of messenger RNA; in any case the increase would have been unlikely to be <u>all</u> messenger RNA, which constitutes only 1-2 per cent of the total RNA content of the cell.

Jakoubek and Semiginovsky (154) pointed out that even if a certain experimental situation failed to change the specific activity of both the protein and acid-soluble fractions, this would not necessarily mean there had been no change in protein synthesis, as the time course of the specific activity of the amino acid pool could have been altered. To be absolutely sure there had been no change in protein synthesis, the time course of the amino acid specific

activity should be determined in both control and experimental groups.

Another possibility to be considered is that of a reciprocal change in protein synthesis in neurones and glia, giving a net change of zero.

A further factor to be considered is the great variation in the half-lives of proteins in the CNS, ranging from seconds to years (154). The duration of labelling of protein used will determine which part of the spectrum of half-lives is seen: with a certain labelling time, the synthesis rate of only a certain fraction of the proteins will be measured. Thus stimulation might affect the synthesis of proteins whose half-lives are much shorter than the labelling period, without any change in the incorporation of label into the protein fraction.

There are therefore many limitations in the interpretation of the present findings. All that can be said is that stimulation did not produce a striking alteration in the apparent rate of protein synthesis, even though the rate of RNA synthesis seemed to be considerably increased.

SECTION 3

THE EFFECTS OF ELECTROCONVULSIVE SHOCK ON THE METABOLISM OF 5-HYDROXYTRYPTAMINE IN THE RAT BRAIN

INTRODUCTION

There is now considerable evidence which suggests that changes in amine metabolism may be related to depressive illness. The evidence falls mainly into three categories. Firstly, some of the drugs used in the treatment of hypertension cause depression in a proportion of patients, and these drugs, such as reserpine and tetrabenazine, have a common property of depleting the brain of monoamines. Lemieux (194) found that fifteen per cent of hypertensive patients treated with reserpine became depressed, but none of those treated with other drugs did so. Secondly, many of the drugs used to treat depression are known to interact with monoaminergic systems. These drugs include the imipramine group, which block the re-uptake of the amines at the nerve ending, and the MAO inhibitors (195). So far this evidence may implicate either 5-HT or the catecholamines, but there is also evidence specific for 5-HT. The therapeutic value in depression of L-tryptophan, as shown by Coppen et al. (196), could be included in the last category. The third type of evidence, which provides a specific indication that 5-HT may be involved in depressive illness, consists of measurements of levels of the amines and their metabolites in brain and CSF. The level of 5-HIAA has been found to be significantly below normal in the lumbar CSF of depressed patients (142, 143, 144, 197), and in the brains of depressive suicides (198).

Electroconvulsive shock therapy (ECT) is still probably the most effective treatment for depression: according to Lehmann (199) and Greenblatt et al. (200), about 80 per cent of cases show some improvement. There is now some evidence that ECT affects the amine-containing systems in the brain. Ashcroft et al. (144) reported

that 5-HIAA levels in the lumbar CSF rose significantly after remission of depressive symptoms in ten patients, eight of whom were treated with ECT. The 5-HIAA levels after treatment were within the normal range. (Although in a recent study it was reported that in a majority of patients the 5-HIAA levels did not return to normal upon remission of symptoms (4)).

Varying reports have appeared on the effects of electro-convulsive shock (ECS) on brain amines in rats and other experimental animals. A significant increase in the 5-HT level in the rat brain after a single shock was found by Garattini et al. (201, 202), Hinesley et al. (203) and Bertaccini (204), but no change was found by Breitner et al. (205). Kety et al. (206) and Kato et al. (207) reported significant rises in the 5-HT level, measured 24 hours after the last of seven (ref. 206) or eleven (ref. 207) daily shocks, but according to Feighner et al. (208) the 5-HT level was unchanged after a series of twelve shocks given in 14 days. Thus these various reports conflict somewhat, but in any case, as described in Section 1, the 5-HT level alone gives little indication of the functional activity of the serotonergic nerves.

Some other workers have attempted to obtain more meaningful results by measuring parameters other than just 5-HT levels. Cooper et al. (209) found that a series of shocks raised 5-HIAA in dog ventricular CSF, but there was no change after a single shock. Essman (210) reported that a single shock raised rat brain 5-HT and lowered 5-HIAA, and the rate of accumulation of 5-HT after inhibiting MAO was reduced. Engel et al. (211) used the rate of depletion of 5-HT after inhibition of tryptophan hydroxylase as an index of the

turnover of the amine, and found that the rate of depletion was increased after a series of five shocks given in a three-day period. Paradoxically however, the 5-HIAA levels were unaltered. The work of Feighner _et al._ (208) showed that ECS speeded the recovery of the 5-HT level in the brainstem following depletion by reserpine, though no change was seen in the midbrain-hypothalamic area.

Changes in noradrenaline (NA) and dopamine (DA) metabolism have also been reported after ECS. Feighner _et al._ (208) found a significant fall in NA in the brainstem after twelve shocks, and as with 5-HT, there was a more rapid recovery of NA stores after reserpine depletion. Musacchio _et al._ (178) and Kety _et al._ (206) found that ECS given twice daily for seven days increased NA turnover in the brainstem and forebrain: this was measured 24 hours after the last shock by the rate of disappearance of intraventricularly-injected (^{3}H)NA. They also showed that there was an increase in the _in vitro_ activity of tyrosine hydroxylase. An increased release and breakdown of NA was shown after a single shock by Schildkraut _et al._ (111), and a fall in the NA level was found by Breitner _et al._ (205) after a series of six shocks given in ten minutes, though not after a single shock. An increase in DA turnover was shown by Engel _et al._ (212), who found a large increase in the striatal HVA content after a single shock; at the same time there was a small rise in the level of DA itself.

It is apparent therefore that the numerous studies of the effects of ECS on brain amines have produced a variety of conflicting results. These studies have used different schedules of administration of shocks, different periods between giving the last shock and killing

the animal, and different methods for measuring the turnover rate or level of the amine. In the case of 5-HT, virtually none of the available reports represents a systematic attempt to find out whether ECS increases 5-HT turnover in the brain after a single shock or at some time during a series of shocks given at regular intervals. The closest approach to this is the work of Cooper et al. (209), who measured 5-HIAA levels in the CSF of two dogs during chronic ECS treatment.

In the present work, the hypothesis was tested that ECS causes a prolonged increase in the turnover of 5-HT in the brain. ECT used in psychiatric treatment can give a long-lasting cure of depression; thus for any biochemical effect of ECS to be meaningful in terms of its value in the treatment of depression, it would have to be more than just a transient change.

The problem remained of deciding on a suitable method for measuring the rate of 5-HT synthesis. As described in Section 1, not all of the available methods may give a meaningful indication of the functional activity of the serotonergic nerves, which is the object of measuring the synthesis of the amine. The method used in Section 1, where (^{3}H)5-HT synthesised from (^{3}H)tryptophan in the presence of pargyline was measured, was developed specifically for studying the effect of raphe stimulation on 5-HT synthesis. This method may not be generally useful however, as the firing of the serotonergic neurones is depressed in the presence of MAO inhibitors, perhaps as a result of 5-HT accumulation (67). Therefore before work was started on the effects of ECS on 5-HT metabolism, some alternative approaches to the measurement of the synthesis of the amine were investigated.

As described in Section 1, several groups have measured the (^{3}H)5-HT synthesised from (^{3}H)tryptophan under steady-state conditions, particularly Lin et al. (74), who applied kinetic arguments to derive a quantitative figure for the rate of synthesis of the amine. This approach has the disadvantages that a steady-state condition must exist, and also it is questionable whether the kinetic analysis, which assumes an open, single compartment of 5-HT, is always valid. Few workers have extended the approach of using tracer amounts of (^{3}H)tryptophan by also measuring the (^{3}H)5-HIAA formed. By determining this product as well as (^{3}H)5-HT it should be possible to obtain more information, in a way somewhat analogous to the measurement of 5-HT and 5-HIAA levels after tryptophan loading. A further possibility would be to block the efflux of (^{3}H)5-HIAA with the drug probenecid, and thus obtain a more quantitative index of 5-HT synthesis. The possible advantage compared to simply measuring 5-HIAA accumulation in the presence of probenecid is that the radioactive tracer may selectively label the functional pool of 5-HT, as was suggested by the results of Section 1. Measurement of the synthesis of the amine in the functional pool should give a more accurate indication of the functional activity of the serotonergic nerves than measurement of the total 5-HT synthesis.

In this section the investigation of these possibilities is described.

METHODS

Materials

Probenecid ('Benemid' pure substance) was obtained from Merck, Sharpe and Dohme Ltd, and sulphinpyrazone ('Anturan' pure substance) from Geigy Ltd.

Measurement of (^{3}H)5-HIAA

5-HIAA was extracted from the brains of rats treated with (^{3}H)tryptophan, as described in Section 1. It was found that the level of radioactivity in the phosphate buffer extract was four to five times that of the 5-HT fraction, which suggested that there might be some contamination, possibly by (^{3}H)tryptophan or ^3H_2O. The carry-over of these substances was estimated by adding samples of (^{14}C)tryptophan or ^3H_2O to acidified salt-saturated buffer, extracting into ether and back into buffer as usual. There was a penetration of 1-2 per cent of both tryptophan and water, which would probably mean a significant contamination of the (^{3}H)5-HIAA. Therefore a method was sought which would separate (^{3}H)5-HIAA from (^{3}H)tryptophan and ^3H_2O.

Trial of an anion exchange column. At neutral pH, 5-HIAA should be retained on an anion exchange resin, while tryptophan and water pass through. This was tested by passing a solution of 5-HIAA and (^{14}C)tryptophan at pH 7.5 over a 60x5 mm column of Dowex AG1-X4 resin, 100-200 mesh (Bio-rad). The column was washed with 15 ml water and eluted first with 6 ml 1N NH_3, and then 6 ml 1N KOH. Tryptophan was retained on the column and eluted mostly by the KOH; 5-HIAA was retained and not eluted. Thus this procedure was not suitable for purifying 5-HIAA. This difficulty in removing the acid from anion exchange resins has been found previously in this laboratory.

<u>Trial of Sephadex G10</u>. A method for 5-HIAA purification on columns of Sephadex G10 was described by Korf <u>et al.</u> (213), where the solutions were applied at pH 2 to 3, and 5-HIAA eluted in 0.01N NH_3. This was tested by passing a solution of 1 ug 5-HIAA, a measured tracer amount of (^{14}C)tryptophan and 10 μg tryptophan (as a carrier) at pH 2.5 over a 30x5 mm column of Sephadex G10, which was then washed with 5 ml 0.01N acetic acid. No 5-HIAA was eluted in 6 ml of 0.01N NH_3, but was eluted with 2 ml 1N NH_3, with a recovery of 72 per cent. Most of the tryptophan passed through the column, less than 4 per cent being eluted with the 5-HIAA. The whole procedure was tested as follows. Samples were prepared containing 6 ml water, 4 g NaCl, 10 mg ascorbic acid, 1 drop conc. HCl, and 1 μg 5-HIAA. To each was added either a (measured) tracer amount of (^{14}C)tryptophan plus 10 μg tryptophan as carrier, or a tracer amount of ^3H_2O. The samples were extracted into 20 ml ether and back into 6 ml phosphate buffer as usual. 5 ml of this buffer was adjusted to pH 2.5 with 1N HCl (glass electrode) and passed over a 30x5 mm column of Sephadex G10. The columns were washed with 5 ml 0.01N acetic acid, and eluted with 2 ml 1N NH_3. The recoveries were:

5-HIAA:	44.4 ± 2.0 per cent	(5)
(^{14}C)tryptophan:	0.09 ± 0.1 " "	(4)
^3H_2O:	1.2 " "	(2)

These results show that the contamination by tryptophan would be negligible but the ^3H_2O level was still too high. Increasing the acetic acid wash of the Sephadex to 20 ml reduced this figure to about 0.5 per cent, but this was still considered to be too high. Therefore to remove more ^3H_2O, the ether extract was shaken with 10 ml

NaCl-saturated 0.1N HCl before extracting it into the phosphate buffer. With this additional precaution, ^3H_2O was undetectable in the final eluate.

The levels of the substances in question were also measured in the phosphate buffer, following the addition of the extra ether-washing step. The recoveries were:

5-HIAA	:	70 ± 10 per cent (3)
(^{14}C)tryptophan	:	0.24 ± 0.00 per cent (3)
^3H_2O	:	0.05 ± 0.00 per cent (3)

These figures suggested that the Sephadex column might in fact not now be necessary. In some further tests on the purification method, the results before and after the Sephadex column were compared.

<u>Tests on (^{3}H)5-HIAA purification.</u> Two rats were given (^{3}H)tryptophan (100 μCi/kg, i.p.), 45 minutes before killing. The brains were processed as described previously, the extracts being passed over CG50 columns, extracted into ether, the ether back-washed before being extracted into buffer, and the solutions passed over Sephadex columns. Samples were taken for determination of radioactivity and 5-HIAA fluorescence before and after the Sephadex columns, and also samples were taken for paper chromatography. The paper chromatography was carried out as descfibed for (^{3}H)5-HT in Section 1, with the exceptions that the system was butanol-acetic acid-water (12:3:5, by volume), and the chromatograms were developed for 18 hours. A comparison of the recoveries of endogenous and radioactive 5-HIAA over the Sephadex column showed that 90 per cent of the radioactivity in the phosphate buffer behaved as 5-HIAA:

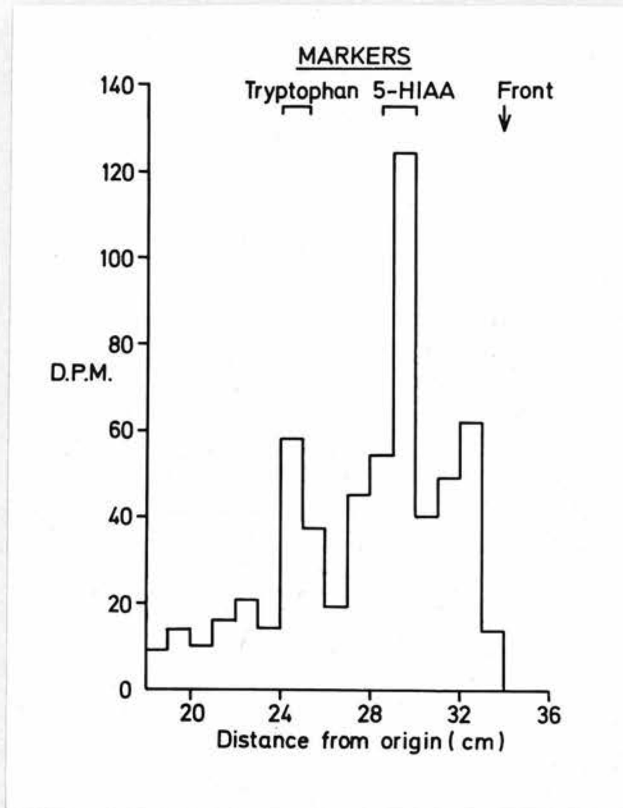

Fig. 3.1. Paper chromatogram of phosphate buffer during purification of (^{3}H)5-HIAA in brain, following injection of (^{3}H)tryptophan i.p.

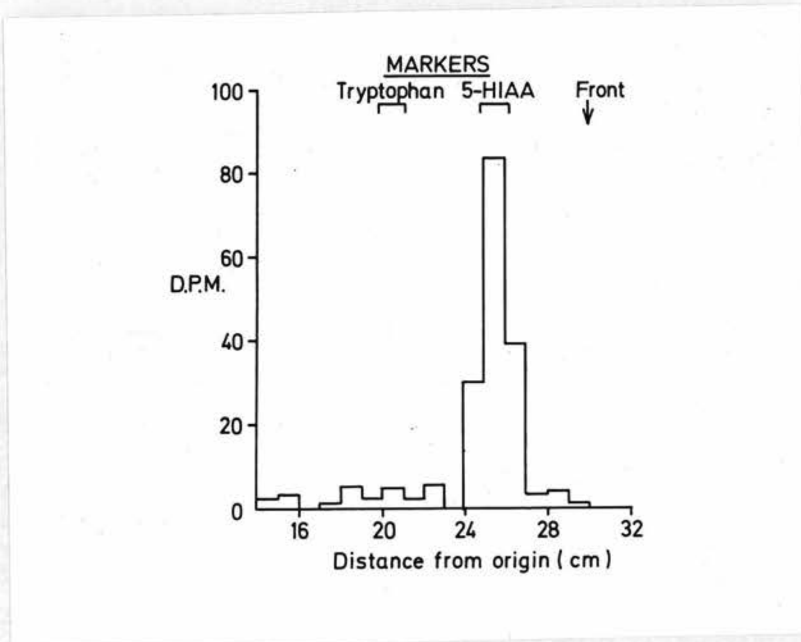

Fig. 3.2. Paper chromatogram of Sephadex eluate during purification of (^{3}H)5-HIAA.

	Endogenous 5-HIAA	Total radioactivity
Column 1:	60	55 (per cent
Column 2:	75	68 recovery)

In each case the recovery of total radioactivity was about 90 per cent of the recovery of endogenous 5-HIAA.

The paper chromatograms of the phosphate buffer and the Sephadex eluate are shown in Figs. 3.1 and 3.2. The Sephadex eluate shows a single clear peak of radioactivity at the position of 5-HIAA. In the chromatogram of the phosphate buffer however, there is also a peak at the position of tryptophan, which represents approximately 13 per cent of the total counts. It is likely however that a substantial proportion of the 5-HIAA decomposed, and therefore that the tryptophan really represents a much smaller fraction of the total radioactivity: in the other chromatogram, the amount of radioactivity measured was less than 40 per cent of what was originally applied to the paper.

Effect of MAO inhibition. If a MAO inhibitor were given before the administration of (^{3}H)tryptophan, in theory no (^{3}H)5-HIAA should be detected. Therefore three rats received 150 mg/kg pargyline 30 min before (^{3}H)tryptophan, were killed after a further 45 min, and the counts in the phosphate buffer compared with those in the previous experiment. The results were:

Controls: 600 $\pm$ 51 dpm (3)

Pargyline-treated: 164 $\pm$ 44 dpm (3)

Not all the counts in the buffer in the pargyline-treated group are

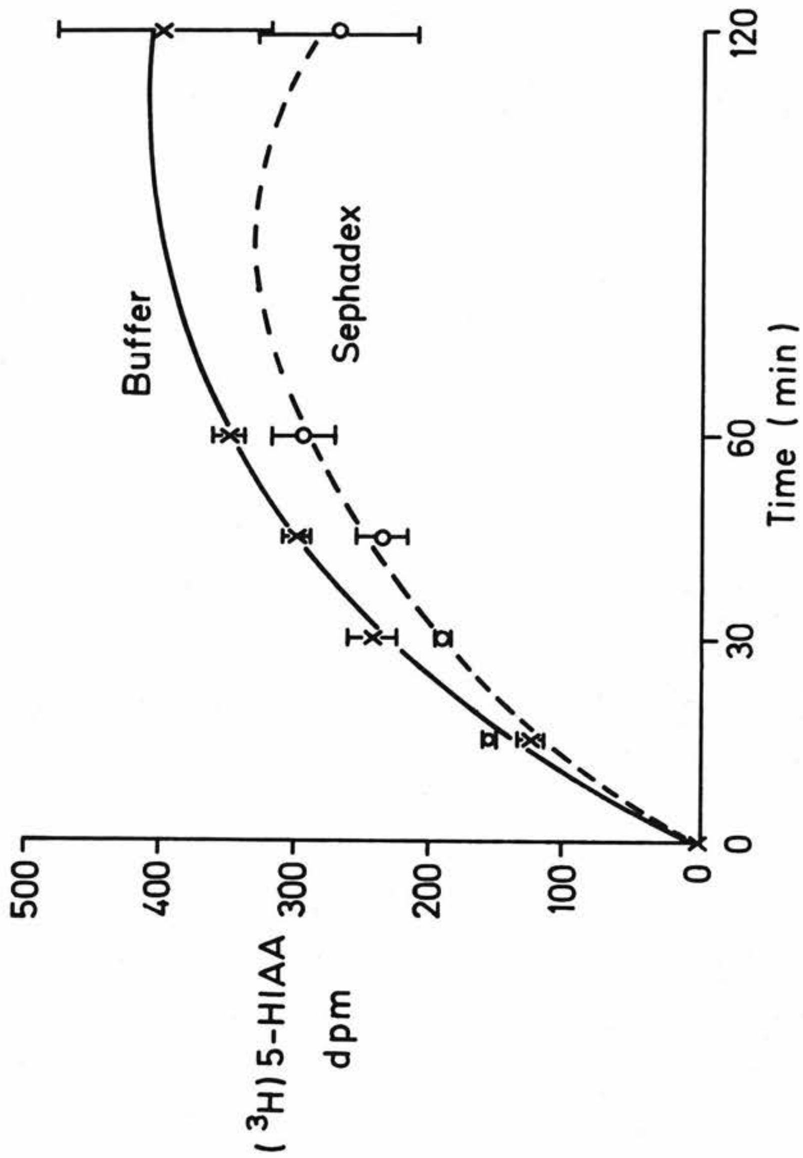

Fig. 3.3. Level of (^3H)5-HIAA in brain, as measured in the phosphate buffer and Sephadex eluate, at various times after injection of (^3H)tryptophan (100 µCi/kg, i.p.). Points show mean of 3 or 4 values; bars show S.D.

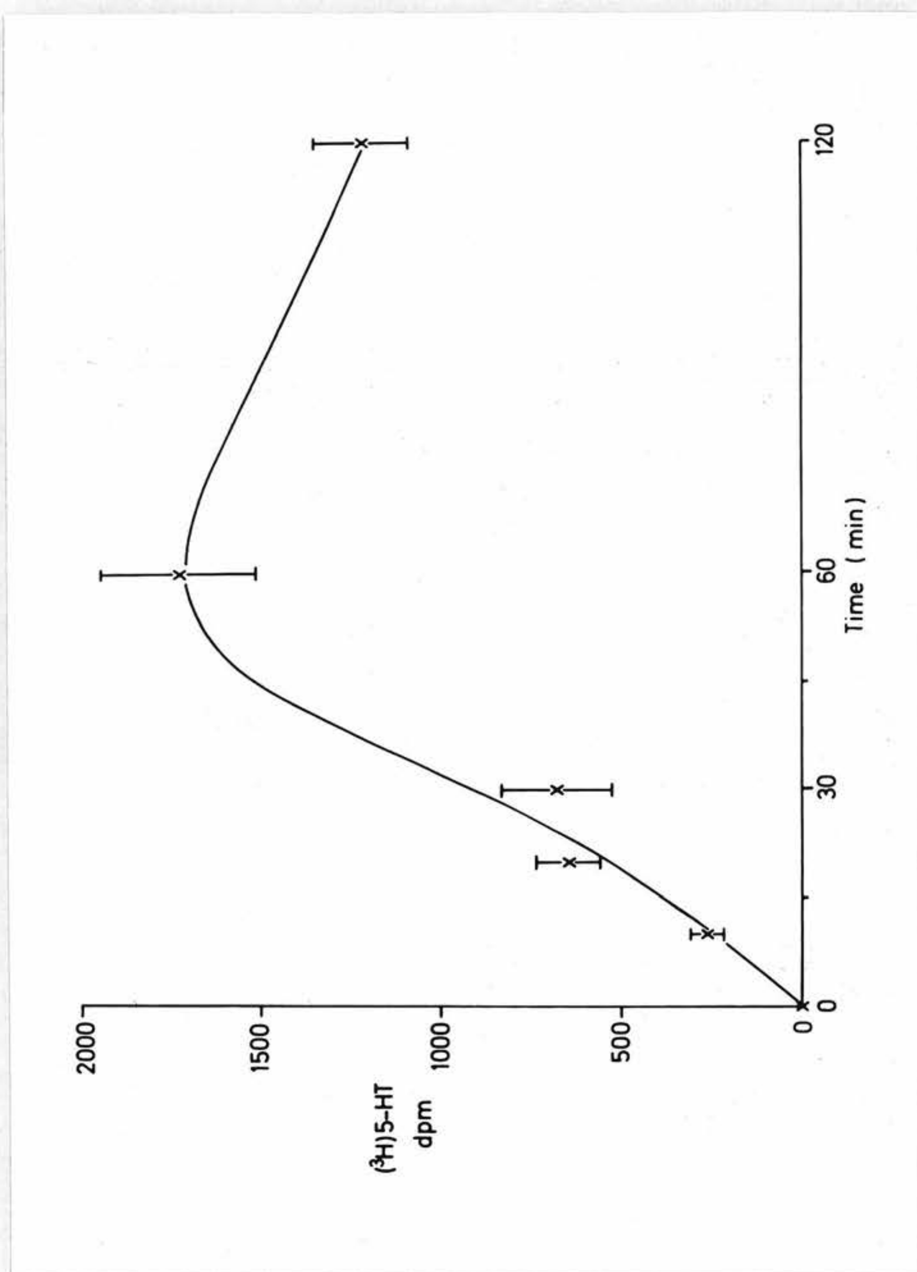

Fig. 3.4. Time course of (^{3}H)5-HT in brain, after injection of (^{3}H)tryptophan.

Points show mean of 3 or 4 values; bars show S.D.

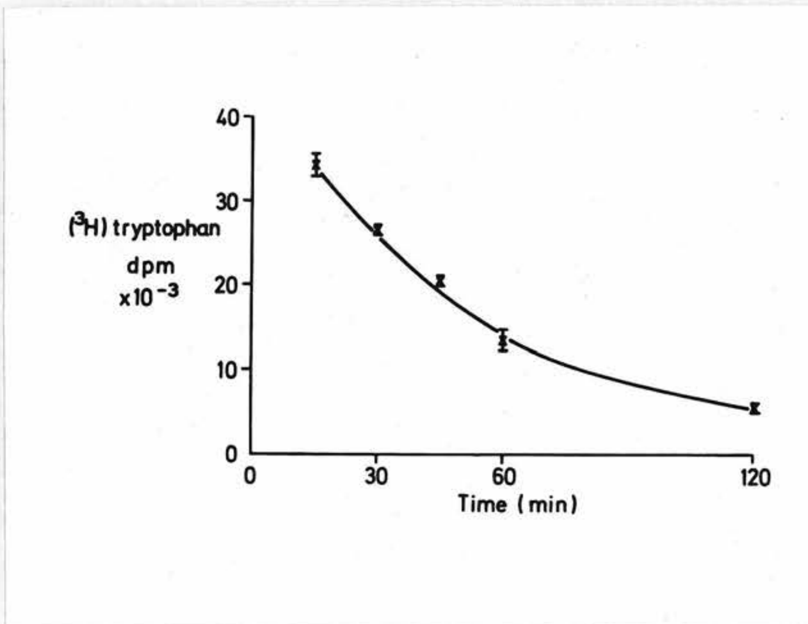

Fig. 3.5. Time course of (^{3}H)tryptophan in brain after intraperitoneal injection of 100 μCi/kg of (^{3}H)tryptophan. Points show mean of 3 or 4 values; bars show S.D.

necessarily contamination; MAO inhibition might not have been 100 per cent, and also there could have been an exchange of the tritium label with endogenous 5-HIAA.

Time course of (^{3}H)5-HIAA in brain. The time course of (^{3}H)5-HIAA in brain following systemic administration of (^{3}H)tryptophan was determined in both the phosphate buffer and Sephadex eluate fractions. The results (Fig. 3.3) show a trend towards slightly elevated counts in the buffer fraction, suggesting some contamination. The discrepancy is especially noticeable at 120 min, and may be related to the appearance of other metabolites, or to increasing exchange of the tritium label. The time courses of brain (^{3}H)5-HT and (^{3}H)tryptophan were also determined (Figs. 3.4, 3.5).

As the degree of contamination of the (^{3}H)5-HIAA appears to increase with time, it seems unlikely that a major contaminant is (^{3}H)tryptophan, which has a quite different time course (Fig. 3.5).

Conclusions. The paper chromatography indicates that virtually all of the radioactivity in the Sephadex eluate is (^{3}H)5-HIAA. Probably ^3H_2O would not give a peak on the chromatogram, but the tests with samples of pure ^3H_2O indicate that a negligible amount is carried right through the whole procedure.

At the phosphate buffer stage, it appears that about 10-15 per cent of the radioactivity is not (^{3}H)5-HIAA, the amount of contamination increasing with time after (^{3}H)tryptophan injection.

It is probably sufficient therefore to use the phosphate buffer counts as a measure of (^{3}H)5-HIAA at times upto an hour, provided that the levels of it are not unusually low, e.g. after MAO inhibition.

Administration of electroconvulsive shock

In most of the reports published on ECS, shocks were given to rats via ear-clips or corneal electrodes. The parameters usually used were a voltage of 100-170 or a current of 60-150 mA, at 50 or 60 c/s, applied for 0.2-1.0 sec. In some cases (214, 215), 1 mS square-wave pulses at 150 c/s were used. In the following experiments, shocks of 50 c/s sinusoidal current were delivered for 1.0 sec from an electroconvulsive apparatus (Multitone Electric Co., type 103). It was found necessary to use the maximum voltage available on the machine (150v) in order to produce convulsions consistently. As was described in Section 1, it would be preferable to use constant-current stimulation in order to avoid changes in the level of stimulation due to changes in the resistance of the circuit. However as the apparatus was not available to do this, the actual current passed was monitored by measuring the voltage across a 33 ohm resistor in series with the circuit using an oscilloscope. This was so that the current could be compared with results in the literature, where the current and not the voltage was specified. The mean current passed was 104 ± 40 mA (n = 168).

The rats were kept on a constant light-dark cycle of 8 am on, 8 pm off, and ECS was administered always at 10 am. Each animal was lightly anaesthetised with Fluothane, and clips applied to the ears. While current was being passed, the animal exhibited a tonic convulsion. Firm pressure was applied to its back to avoid

damage to the spine. The tonic convulsion was followed by clonic convulsions, particularly of the limbs, and also in some cases there was vocalisation. Occasionally the shock failed to produce clonic convulsions. This appeared to be due to the anaesthesia being too deep, and in these cases a second shock was given which always gave convulsions. The reason for doing this was that King et al. (245) found that biochemical changes (in energy metabolism) normally seen after ECS were not apparent if convulsions were prevented by barbiturate narcosis.

After treatment, the animals made a normal recovery in 5 to 15 min. Control animals were anaesthetised only.

RESULTS

Tests on probenecid

As was described in Section 1, the drug probenecid has been used to block the efflux of 5-HIAA from the rat brain. In these experiments it was desired to block the efflux of (^{3}H)5-HIAA in order to measure the synthesis rate of (^{3}H)5-HT from (^{3}H)tryptophan. Therefore the efficacy of the drug was tested by measuring 5-HIAA levels after its administration.

Probenecid was dissolved as recommended by the manufacturers. The solid was suspended in water and 5N KOH added to bring the final KOH concentration to 0.2N. A clear solution was formed. Sodium dihydrogen phosphate was added to a final concentration of 0.01M. The pH was adjusted to 7.5-8 (glass electrode) with 1N HCl.

(i) Rats were given probenecid at a dose of 200 mg/kg, i.p., the dose used by Neff and Tozer (63). They were killed at various times after the injection. The 5-HIAA levels were:

Time (hr)	5-HIAA (μg/g)
0	0.20 (mean of 2 values)
1	0.25 (do.)
2	0.34 (do.)
3	0.36 (do.)

The mean rate of rise was 0.055 μg/g/hr, which was statistically significant ($P < 0.001$). This compares with a rate of accumulation of 5-HT after MAO inhibition of 0.39 μg/g/hr (see Section1), which would be expected to be about the same.

(ii) Neff et al. (62) found that the accumulation of 5-HIAA was maximal with a dose of probenecid of 200 mg/kg. Higher doses were also tested here however in case this dose was not sufficient. Rats received different doses of probenecid, or saline, and were killed after 2 hr. The results were:

Dose (mg/kg)	5-HIAA (μg/g)
0	0.25 (mean of 2 values)
200	0.25 (do.)
400	0.26 (do.)
600	0.21 (do.)

Thus in this experiment, no accumulation at all of 5-HIAA was seen.

(iii) Neff et al. (62) reported that probenecid stopped the fall in brain 5-HIAA which is normally found after inhibition of MAO. In the following experiment an attempt was made to repeat this. Probenecid (200 mg/kg) was given 30 min before pargyline (150 mg/kg). The animals were killed at various times, and 5-HIAA assayed:

Time after giving pargyline (min)	5-HIAA (μg/g)
0	0.24 (mean of 2)
30	0.22 (do.)
60	0.17 (do.)
90	0.17 (do.)

The rate of fall of 5-HIAA was 0.048 μg/g/hr, which was significantly greater than zero ($P < 0.005$). Thus the efflux of the acid was not prevented.

(iv) It was possible that the lack of effect of probenecid compared with the results of other laboratories was due to differences in the strain of rat used. Therefore the drug was tested on another strain: PVG (female). A dose of 200 mg/kg was given 2 hr before killing. The 5-HIAA levels were:

Group	5-HIAA (μg/g)
Control (received saline)	0.34 ± 0.02 (4)
Probenecid	0.39 ± 0.01 (4)

The rise in 5-HIAA was statistically significant ($P < 0.025$, R test), but was only of 14 per cent.

Tests on Anturan

Anturan is a drug with similar action to probenecid, and is said to be more effective than the latter in reducing plasma uric acid levels (216). Thus this drug was tested for blocking of 5-HIAA efflux from the rat brain. It was dissolved as described for probenecid and administered in various doses to Wistar (male) and PVG (female) rats. The animals were killed after 2 hours and brain 5-HIAA measured. The results (Table 3.1) show significant increases in 5-HIAA in both strains of rat, but the rates of increase were less than a quarter of either the rate reported by Neff et al. (62), or the rate of rise of 5-HT after MAO inhibition. It is concluded therefore that neither Anturan nor probenecid provide an effective block of the efflux of 5-HIAA from the brain in either of the two strains of rat tested.

Effect of stimulation on the time course of (^{3}H)5-HT and (^{3}H)5-HIAA

It was hoped that it would be possible to use (^{3}H)5-HT and (^{3}H)5-HIAA levels after (^{3}H)tryptophan injection as an index of 5-HT synthesis, despite the lack of a means of blocking 5-HIAA efflux. In the following experiment the time courses of (^{3}H)5-HT and (^{3}H)5-HIAA were determined in animals stimulated in the raphe nucleus, and in non-stimulated controls. As stimulation increases 5-HT synthesis, this should show whether the measurement of (^{3}H)5-HT and (^{3}H)5-HIAA levels is a useful index of the synthesis of the amine.

Rats were stimulated as described in Section 1, and 10 min after the start of stimulation were given (^{3}H)tryptophan (100 μCi/kg, i.p.). They were killed at various times after the injection, stimulation being continued until killing. (^{3}H)5-HIAA was measured

Table 3.1 Effect of Anturan on brain 5-HIAA levels

Dose of Anturan (mg/kg, i.p.)	5-HIAA (μg/g)	
	Wistar	PVG
0	0.24 ± 0.01 (4)	0.34 ± 0.02 (4)
100	0.32 ± 0.06 (4)*	0.49 ± 0.02 (4)**
150	0.37 ± 0.01 (4)**	-
200	Convulsions and death (both strains)	

* Significantly higher than controls, $P < 0.05$ (R test)

** Significantly higher than controls, $P < 0.025$ (R test)

in the phosphate buffer, the Sephadex column not being considered necessary at this stage.

The (^{3}H)5-HT and (^{3}H)5-HIAA levels are shown in Table 3.2. These figures were corrected to allow for variations in the level of (^{3}H)tryptophan at the site of 5-HT synthesis. It is not possible to measure this quantity directly, but it should be possible to obtain a reasonable index of it from other measurements. In Section 1, the level of (^{3}H)tryptophan in the brain was used as an index of the specific activity of the tryptophan from which 5-HT was synthesised. The results however indicated that 5-HT was not derived from a single, open pool of tryptophan in the brain. Also Spector <u>et al.</u> (121) showed that in the perfused guinea-pig heart, (^{14}C)tyrosine in the perfusate did not equilibrate with tyrosine in the heart, and that the tyrosine in the perfusate was the relatively undiluted precursor of NA in the tissue. Hence it seemed that a better estimate of the precursor specific activity might be obtained here by measuring the (^{3}H)tryptophan in plasma. A significant correlation was found between plasma (^{3}H)tryptophan levels and brain (^{3}H)5-HT and (^{3}H)5-HIAA ($P < 0.025$ in each case). As measurements were made at different times after injection of (^{3}H)tryptophan, it would obviously not be correct simply to divide each result by the plasma (^{3}H)tryptophan level. Instead, each result was corrected according to the formula:

$$\text{Corrected result} = \text{original result} \times \frac{\text{Expected plasma } (^3\text{H})\text{tryptophan}}{\text{Measured plasma } (^3\text{H})\text{tryptophan}}$$

The 'expected' plasma (^{3}H)tryptophan levels were taken from a curve of the time course of (^{3}H)tryptophan in plasma, constructed from the

Table 3.2 Effect of stimulation on (^{3}H)5-HT and (^{3}H)HIAA levels

Time after injection (min)	Group	(^{3}H)5-HT (dpm)	(^{3}H)5-HIAA (dpm)
10	Stimulated	355 ± 128 (5)	226 ± 72 (4)
	Control	262 ± 115 (6)	233 ± 42 (5)
20	Stimulated	603 ± 208 (6)	501 ± 300 (6)
	Control	649 ± 223 (6)	490 ± 195 (6)
30	Stimulated	846 ± 360 (6)	703 ± 492 (6)
	Control	679 ± 383 (6)	840 ± 350 (6)
60	Stimulated	1186 ± 645 (6)	1237 ± 818 (6)
	Control	1726 ± 534 (6)	907 ± 174 (6)
120	Stimulated	995 ± 359 (5)	1396 ± 364 (5)*
	Control	1222 ± 296 (5)	991 ± 164 (5)

Rats received 100 µCi/kg (^{3}H)tryptophan 10 min after beginning of stimulation.

* Significantly higher than control, $P < 0.05$ (R test)

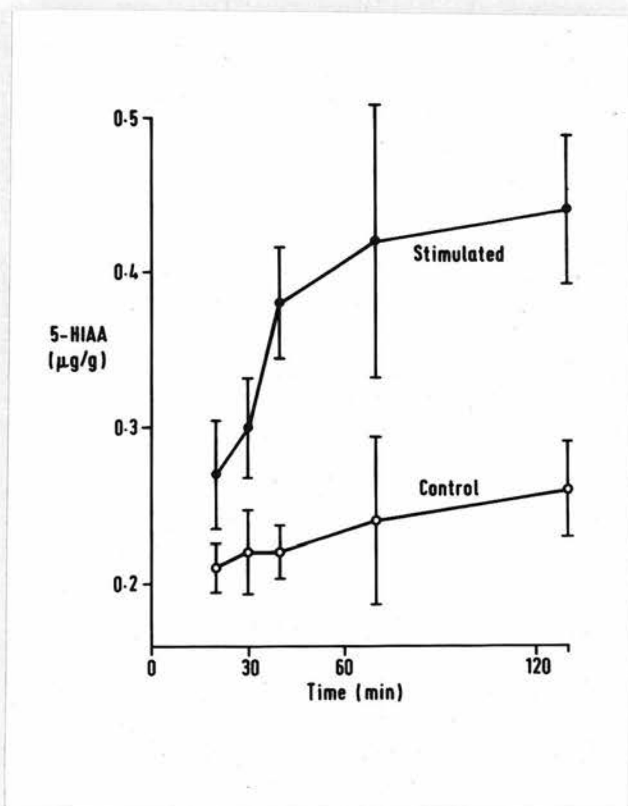

Fig. 3.6. Effect of stimulation on 5-HIAA levels. Each point is the mean of 5 or 6 results; vertical bars show S.D.

values measured in this experiment. As the levels were not significantly different in stimulated and control groups, all the values at each time were combined (Table 3.3).

The levels of 5-HIAA measured at the same time are shown in Fig. 3.6. The level of the acid was significantly higher in stimulated than in control animals at each time ($P < 0.025$, or better). The 5-HT levels remained constant with time in both stimulated and control groups. The mean level in the stimulated animals (0.30 ± 0.05 µg/g (27)) was significantly lower than the mean level in the controls (0.35 ± 0.05 µg/g (18)), $P < 0.005$, $\underline{t}$ test.

The results show that although stimulation produced a clear rise in endogenous 5-HIAA, the changes in (^{3}H)5-HIAA were not very marked: the level was higher in a stimulated group only at the latest time (120 min). In the case of 5-HT, there appeared to be a tendency to a more rapid elimination of (^{3}H)5-HT in the stimulated animals, though none of the groups were significantly different from controls. One problem seemed to be the wide scatter of results of the (^{3}H)5-hydroxyindoles, which might have masked genuine differences.

It is concluded therefore that the measurement of (^{3}H)5-HT and (^{3}H)5-HIAA is unlikely to provide a more sensitive index of 5-HT synthesis than simply measurement of endogenous 5-hydroxyindole levels.

Effects of ECS on 5-HT metabolism as determined by tryptophan loading

In the following experiments tryptophan loading was used to determine the effects of ECS on 5-HT metabolism. This technique appears to provide a good indication of the functional activity of

Table 3.3 Level of (^{3}H)tryptophan in plasma after intraperitoneal injection

Time after injection (min)	Plasma (^{3}H)tryptophan (dpm x10^{-3}/ml)
10	83.4 ± 21.2 (11)
20	79.8 ± 14.2 (12)
30	109.0 ± 33.6 (12)
60	83.7 ± 26.0 (12)
120	51.9 ± 7.7 (10)

Rats received 100 µCi/kg (^{3}H)tryptophan i.p.

the 5-HT-containing neurones, as shown by the stimulation experiments in Section 1.

<u>Effect of a single shock on 5-HT metabolism</u>

Rats were given a single shock, and L-tryptophan (800 mg/kg, i.p.) or saline given immediately afterwards. The animals were killed after 60 min; the results are shown in Table 3.4. There were no significant differences between any of the control and ECS groups, and also no alteration in the rates of accumulation of 5-HT and 5-HIAA after tryptophan loading. Probably the most useful index of 5-HT synthesis in the case of tryptophan loading is the rate of rise of total 5-hydroxyindoles, i.e. 5-HT + 5-HIAA: thus these figures are also presented in these tables.

<u>Effects of a series of shocks on 5-HT metabolism</u>

In these experiments the effects of chronic ECS treatment on 5-HT metabolism were investigated. In order to determine the duration of any effect found, the tryptophan load was given at 0, 3 or 24 hr after the last shock.

(i) Rats were given ECS once daily for 8 days, and tryptophan was given immediately after the last shock. The animals were killed after 60 min, as in the last experiment. The results (Table 3.5) again show no significant effect of ECS, although there is a suggestion of a rise in the endogenous 5-HIAA level in the experimental group. Owing to the loss of two samples however, this rise is not statistically significant.

Table 3.4 Effect of a single shock on 5-hydroxyindole levels

Group	5-HT	5-HIAA (µg/g)	Total 5-hydroxyindoles
Control, saline	0.40 ± 0.03	0.26 ± 0.02	0.66 ± 0.03
ECS, saline	0.34 ± 0.03	0.29 ± 0.02	0.63 ± 0.05
Control, tryptophan	0.67 ± 0.02(3)	0.60 ± 0.06	1.25 ± 0.08(3)
ECS, tryptophan	0.62 ± 0.11	0.62 ± 0.05	1.24 ± 0.14
<u>Increase due to tryptophan load</u>			
Control	0.27	0.34	0.61
ECS	0.28	0.33	0.61

Results show mean ± S.D. of 4 observations, except where marked (3)

Table 3.5 Effect of 8 daily shocks on 5-hydroxyindole levels: tryptophan given immediately after the last shock

Group	5-HT	5-HIAA	Total 5-hydroxyindoles
		(μg/g)	
Control, saline	0.40 ± 0.07	0.26(2)	0.63(2)
ECS, saline	0.48 ± 0.11	0.32 ± 0.02	0.80 ± 0.12
Control, tryptophan	0.71 ± 0.05	0.52 ± 0.04	1.23 ± 0.08
ECS, tryptophan	0.79 ± 0.13(3)	0.54 ± 0.03(3)	1.32 ± 0.12(3)
Increase due to tryptophan load			
Control	0.29	0.26	0.55
ECS	0.31	0.22	0.52

Results show mean of 4 observations, except where marked otherwise

(ii) Rats were given ECS daily for 8 days, and this time the tryptophan was given 3 hours after the last shock. They were killed after 60 min. The results are shown in Table 3.6. In this case there was a significant rise in the endogenous 5-HIAA level in the ECS group; the 5-HIAA level was also significantly higher after tryptophan loading, but the rate of increase of both 5-HT and 5-HIAA was almost identical in ECS and control groups.

(iii) In the third experiment of this series, the tryptophan was given 24 hr after the last shock. The results (Table 3.7) show a similar pattern to those of the preceding experiment (Table 3.6), in that ECS raised the endogenous 5-HIAA level, but failed to increase the rate of accumulation of the 5-hydroxyindoles after tryptophan loading.

<u>General effects of chronic ECS treatment</u>

There was a marked reduction in the rate of increase of body weight in rats given chronic ECS. The average rate of increase over the 8 days was 2.3 g/day in the treated rats, and 4.1 g/day in the controls. The only other noticeable effect of the treatment was an increased excitability.

<u>Effect of tryptophan loading in the presence of chlorimipramine</u>

The results presented above are paradoxical in that chronic

Table 3.6 Effect of 8 daily shocks on 5-hydroxyindole levels: tryptophan given 3 hr after the last shock

Group	5-HT	5-HIAA	Total 5-hydroxyindoles
		(μg/g)	
Control, saline	0.38 ± 0.04	0.31 ± 0.02	0.69 ± 0.05
ECS, saline	0.36 ± 0.02(3)	0.40 ± 0.03(3)*	0.76 ± 0.03(3)
Control, tryptophan	0.55 ± 0.06	0.59 ± 0.02	1.13 ± 0.08
ECS, tryptophan	0.54 ± 0.07	0.67 ± 0.06*	1.26 ± 0.14
<u>Increase due to tryptophan load</u>			
Control	0.17	0.28	0.45
ECS	0.18	0.27	0.45

Results show mean of 4 observations, except where marked otherwise.

* Significantly higher than control, $P < 0.05$ (R test)

Table 3.7 Effect of 8 daily shocks on 5-hydroxyindole levels: tryptophan given 24 hr after the last shock

Group	5-HT	5-HIAA	Total 5-hydroxyindoles
		(μg/g)	
Control, saline	0.41 ± 0.04(9)	0.26 ± 0.02(9)	0.67 ± 0.06(9)
ECS, saline	0.38 ± 0.06(6)	0.33 ± 0.08(5)*	0.71 ± 0.13(5)
Control, tryptophan	0.58 ± 0.09(7)	0.58 ± 0.04(7)	1.16 ± 0.12(7)
ECS, tryptophan	0.54 ± 0.08(6)	0.56 ± 0.02(7)	1.11 ± 0.09(6)
<u>Increase due to tryptophan load</u>			
Control	0.17	0.32	0.49
ECS	0.16	0.23	0.39

* Significantly higher than control, $P < 0.025$ (*t* test)

ECS appeared to increase 5-HT synthesis, as there was an increase in the 5-HIAA level with no change in 5-HT, yet no difference was shown by tryptophan loading. The increased level of endogenous 5-HIAA was unlikely to be due to a reduced efflux of the acid from the brain, as a change would also have been seen after tryptophan loading. Thus perhaps ECS produces an increase in 5-HT synthesis, possibly due to increased nervous activity, but this increased synthesis is suppressed in the presence of high concentrations of tryptophan. This possibility is supported by the recent findings of Aghajanian (73), who showed that systemically-given tryptophan reduces the firing rates of raphe neurones.

It is therefore questionable whether tryptophan loading would show changes in the rate of 5-HT synthesis due to alterations in the firing rates of the serotonergic nerves. In the following experiment the usefulness of tryptophan loading was investigated by determining the effect of tryptophan on 5-hydroxyindole levels in the presence of the drug chlorimipramine, which reduces 5-HT synthesis. This drug inhibits the uptake of 5-HT by rat brain slices, and in this situation is the most potent of the imipramine group (217). It has been found to reduce 5-HT turnover in the rat brain, measured by either the depletion of 5-HT after inhibition of its synthesis (218, 219), or by the accumulation of 5-HIAA after probenecid (220). Corrodi and Fuxe (218) suggested that 5-HT turnover is reduced following a reduction in the activity of the serotonergic neurones: the drug acts by blocking the re-uptake of 5-HT at the nerve ending, thus increasing the activity of the 5-HT receptor, which leads to an inhibitory nervous feedback onto the serotonergic nerves. An

inhibitory effect on raphe firing of the related drug imipramine has been found by Bramwell (221).

Rats received 10 mg/kg chlorimipramine or saline i.p., followed 2 hr later by L-tryptophan (800 mg/kg, i.p.) or saline. The animals were killed after a further 60 min. The results are shown in Table 3.8. In animals which had not received tryptophan, there was a significant fall in the 5-HIAA level, suggesting a reduction in 5-HT synthesis. The increases in 5-HT, 5-HIAA and total 5-hydroxyindoles after tryptophan loading were not significantly reduced by the drug however (*t* test). The indications obtained from this experiment are not very marked, but tend to confirm the observations noted above, i.e. that it is possible to alter the rate of 5-HT synthesis by some experimental manipulation, but that this change may not be seen using tryptophan loading.

<u>Use of radioisotope techniques in the investigation of the effects of ECS on 5-HT synthesis</u>

The results presented so far suggest that a series of 8 daily ECS treatments increases 5-HT synthesis. The technique of tryptophan loading however appears to be unsuitable for this investigation. At this stage, the possibility of using an isotopic method for measuring 5-HT synthesis was tested. The value of using (^{3}H)5-HT and (^{3}H)5-HIAA levels after (^{3}H)tryptophan injection was rather doubtful, as shown by the effect of raphe stimulation on these quantities. Another possibility was the (^{3}H)tryptophan-MAO inhibitor method which was used in Section 1.

Table 3.8 Effect of tryptophan loading in the presence of chlorimipramine

Group	5-HT	5-HIAA	Total 5-hydroxyindoles
		(μg/g)	
Control	0.33 ± 0.06(6)	0.19 ± 0.03(6)	0.52 ± 0.08(6)
Chlorimipramine	0.38 ± 0.06(6)	0.16 ± 0.01(6)*	0.54 ± 0.06(6)
Tryptophan	0.46 ± 0.06(4)	0.66 ± 0.06(4)	1.12 ± 0.09(4)
Tryptophan + chlorimipramine	0.54 ± 0.09(4)	0.57 ± 0.10(4)	1.12 ± 0.19(4)
<u>Increase due to tryptophan load</u>			
Control	0.13	0.47	0.60
Chlorimipramine	0.16	0.41	0.57

* Significantly lower than control, $P < 0.05$ (*t* test)

Effect of a series of shocks on (^{3}H)5-hydroxyindole levels

Rats were given 8 daily shocks, and (^{3}H)tryptophan (100 µCi/kg, i.p.) was given 3 hours after the last shock. The animals were killed after a further 30 min. The results (Table 3.9) show that although there was a marked rise in the endogenous 5-HIAA level, the levels of (^{3}H)5-HT and (^{3}H)5-HIAA were unaltered. These results confirm those obtained on raphe stimulation, showing that measurement of the (^{3}H)5-hydroxyindole levels is not useful as a sensitive index of 5-HT synthesis.

Effect of a series of shocks on synthesis of (^{3}H)5-HT in the presence of pargyline

The effect of a series of shocks on 5-HT synthesis was tested using the method described in Section 1. Rats were given 8 daily shocks, and pargyline (150 mg/kg) was given 2 hr 45 min after the last shock. (^{3}H)tryptophan (100 µCi/kg) was given 15 min later, and the animals were killed after a further 30 min. The results (Table 3.10) show a significant rise in synthesis of (^{3}H)5-HT. There was no rise in the total level of 5-HT however, and as previous results showed no change in normal 5-HT levels after 8 days' ECS treatment, it appears that the total rate of accumulation of 5-HT in the presence of the MAO inhibitor was also unchanged. Despite the fairly large number of animals used, the difference in (^{3}H)5-HT between the groups was only just significant however, due to the wide scatter of the results. It was concluded that this method too was unlikely to provide a sensitive index of 5-HT synthesis, particularly

Table 3.9 Effect of 8 daily shocks on (^{3}H)5-HT and (^{3}H)5-HIAA levels after administration of (^{3}H)tryptophan i.p.

Group	5-HIAA (μg/g)	(^{3}H)5-HT	(^{3}H)5-HIAA
		(Ratio x10^3 dpm/CG50 effluent dpm)	
Control	0.37 ± 0.06 (8)	41.6 ± 8.2 (8)	30.6 ± 4.9 (7)
ECS	0.56 ± 0.07 (7)*	44.3 ± 8.7 (4)	28.3 ± 9.2 (7)

* Significantly higher than control, $P < 0.001$ (*t* test).

Table 3.10 Effect of 8 daily shocks on synthesis of (^{3}H)5-HT in the presence of pargyline

Group	5-HT (μg/g)	Ratio (x10^{3}) 5-HT dpm/CG50 effluent dpm
Control	0.75 ± 0.08 (7)	26.1 ± 16.5 (15)
ECS	0.72 ± 0.06 (5)	37.5 ± 14.0 (14)*

* Significantly higher than control, $P < 0.05$ (*t* test)

in view of the fact that the firing of the serotonergic nerves is suppressed in the presence of MAO inhibitors (67). This suppression of firing might mask small changes in 5-HT synthesis which result from alterations in neuronal activity.

The experiments so far have suggested that ECS may increase 5-HT synthesis, but of the methods tested, only the endogenous 5-HIAA level seemed to provide a sensitive index of the synthesis of the amine. In the following experiments, the effects of ECS on 5-HT metabolism were examined in greater detail, using the 5-HT and 5-HIAA levels as an index of amine synthesis. As mentioned in Section 1, a particular virtue of this approach is that the situation is entirely physiological, as it does not require administration of drugs or any other treatment, which might distort the results.

Effect of a single ECS treatment on 5-HT synthesis

The results presented so far show clearly that 5-HT synthesis is accelerated when measured 3 hours after the last of 8 daily shocks. In the following experiment, the effect of a single shock was determined. The effect of a single shock on 5-HT synthesis was described above (results in Table 3.4), but in this case, tryptophan loading was used which might have suppressed any changes. Also the measurements of endogenous 5-HT and 5-HIAA levels were made at only one hour after ECS. There was a suggestion of an increase in 5-HIAA, but the numbers of results were too small.

Thus rats were given a single shock and killed after 3 hr. The results (Table 3.11) show that 5-HIAA was raised by over 60 per

Table 3.11 Effects of a single ECS on brain 5-HT, 5-HIAA and tryptophan levels

Group	5-HT	5-HIAA (µg/g)	Tryptophan
Control	0.22 ± 0.04 (8)	0.12 ± 0.03 (8)	6.06 ± 0.71 (8)
ECS	0.24 ± 0.05 (8)	0.20 ± 0.04 (7)*	5.17 ± 0.30 (8)

Animals were killed 3 hr after ECS.

* Significantly higher than control, $P < 0.001$ (t test).

cent compared to the controls; 5-HT levels were not significantly altered. The apparent increase in 5-HT synthesis was at least as great as occurred after 8 daily shocks. The brain tryptophan level was not significantly altered.

For these results to be meaningful in terms of the clinical use of ECT however, it would be necessary to show an effect lasting for longer than 3 hours. In the following series of experiments, the effect of chronic ECS on 5-HT and 5-HIAA levels measured 24 hr after the last shock was determined.

Effects of chronic ECS treatment on 5-HT synthesis

Animals were given ECS once daily for upto 16 days, and were killed 24 hr after the last shock. The 5-HT and 5-HIAA levels are shown in Table 3.12. In no case was any significant change found. It appears therefore that ECS is capable of producing only short-lasting increases in 5-HT synthesis, and that chronic ECS treatment has no greater effect than a single shock.

DISCUSSION

The discussion of the results of this section falls into two sections. These are (a) the investigation of methods for the measurement of 5-HT synthesis, and (b) the effects of ECS on 5-HT synthesis.

Investigation of methods for determination of the rate of 5-HT synthesis

The purification of (^{3}H)5-HIAA on columns of Sephadex G10 was found to be highly satisfactory. This method has also been used recently by Diaz and Huffunen (222) and Hamon *et al.* (223). However,

Table 3.12 Effect of chronic ECS on brain 5-HT and 5-HIAA levels

Number of days' treatment	Group	5-HT (μg/g)	5-HIAA
6	Control	0.24 ± 0.03 (8)	0.15 ± 0.02 (8)
	ECS	0.25 ± 0.04 (8)	0.16 ± 0.03 (8)
8	Control	0.32 ± 0.04 (7)	0.20 ± 0.03 (7)
	ECS	0.33 ± 0.03 (8)	0.20 ± 0.02 (8)
12	Control	0.26 ± 0.03 (8)	0.18 ± 0.04 (8)
	ECS	0.27 ± 0.02 (7)	0.19 ± 0.03 (7)
16	Control	0.28 ± 0.04 (8)	0.17 ± 0.02 (8)
	ECS	0.27 ± 0.03 (6)	0.16 ± 0.10 (6)

Animals were killed 24 hr after the last shock.

with the addition of the extra 'backwash' stage to the normal 5-HIAA extraction procedure, a good degree of purity was obtained without the Sephadex column: about 85-90 per cent of the radioactivity was (^{3}H)5-HIAA. This was considered to be a sufficient degree of purity, at least for the preliminary experiments. Thus in the results presented in this section, the Sephadex column was not used.

It was shown that probenecid had little effect in blocking the efflux of 5-HIAA from the brain. This was in spite of the fact that the drug has been shown in the past to be effective in blocking 5-HIAA efflux from the brain in the rat (62, 224), cat (225), dog (226) and man (227). It is possible that the lack of effect found here was a property of the two strains of rat tested. This is suggested by the results of Andersson and Roos (228), who found that the effectiveness of probenecid in raising the 5-HIAA level in the rabbit brain was highly dependent on the strain: in one type of rabbit, the drug had no significant effect, and in two other types, no effect was apparent until 4 hours after giving the drug. Thus there may also be a prolonged time-lag in some cases before the drug is effective. A further limitation with the use of probenecid is that its site of action is unknown. Padjen and Randic (229) and Eccleston <u>et al.</u> (44) found that although the drug increased the 5-HIAA level in the rat brain, there was no change in the rate of efflux of the acid into cups placed on the cerebral cortex. Furthermore, Padjen and Randic (229) showed that although raphe stimulation normally produced a large rise in 5-HIAA in the forebrain and in the rate of efflux of 5-HIAA into the cortical cups, no changes were seen on stimulation after giving probenecid. Similar results were

reported by Sheard and Aghajanian (38), who found that raphe stimulation in the presence of probenecid increased forebrain 5-HIAA by only 25 per cent, compared to a rise of 80 per cent without the drug. These findings are somewhat paradoxical, and suggest perhaps that probenecid blocks the transport of 5-HIAA out of the nerve ending, but not of 5-HIAA formed from 5-HT which is released and metabolised elsewhere. Thus the interpretation of 5-HIAA accumulation in the presence of probenecid as an index of neuronal activity is open to question.

In the case of Anturan, a more positive effect on 5-HIAA levels was found, but again the blocking action seemed to be relatively small.

The principal objective of the initial experiments described in this section was to develop a method for measurement of 5-HT synthesis, based on the measurement of (^{3}H)5-HT and (^{3}H)5-HIAA levels after injection of (^{3}H)tryptophan. The usefulness of this approach was assessed by determining the effect of raphe stimulation on the time courses of (^{3}H)5-HT and (^{3}H)5-HIAA, as stimulation produces a large rise in 5-HT synthesis. Stimulation was seen to have a marked effect on the synthesis of 5-HT by the rapid rise in the level of 5-HIAA in the brain. The effect of stimulation on the time courses of (^{3}H)5-HT and (^{3}H)5-HIAA however was not very pronounced. The level of (^{3}H)5-HT showed a tendency to fall more quickly in the stimulated animals, but showed little tendency to rise any faster during the first 30 minutes after (^{3}H)tryptophan injection. In no case was there a statistically significant difference between stimulated and control groups. In the case of (^{3}H)5-HIAA the level

in the stimulated animals was significantly higher than in the controls only at the latest time measured (120 min). One problem was the wide dispersion of results, which probably masked some genuine changes. It was apparent however that the level of endogenous 5-HIAA gave a more sensitive index of 5-HT synthesis than did (^{3}H)5-HIAA; this was surprising in view of the results of Section 1 which appeared to show a selective labelling of the functional pool of 5-HT by (^{3}H)tryptophan. At the moment it is difficult to find an explanation for this, unless the pargyline which was used in Section 1 affects the distribution of (^{3}H)tryptophan in the brain. Some evidence suggesting that pargyline does affect the distribution of (^{3}H)tryptophan has been presented by Millard _et al._ (230).

The results obtained with tryptophan loading seriously question the general usefulness of this technique for the measurement of 5-HT synthesis. ECS was shown to increase 5-HT synthesis, and chlorimipramine to reduce it, yet in neither case was there a change in the rate of accumulation of 5-hydroxyindoles after tryptophan loading. Aghajanian (73) found that systemically-given tryptophan suppressed the firing of raphe neurones. Thus a likely explanation of these observations is that the firing rate of the serotonergic neurones was altered by ECS or chorimipramine, but that in the presence of tryptophan, the firing of the neurones was suppressed in both experimental and control groups. It is concluded that the technique of tryptophan loading is likely to be useful only for detecting changes in the _amount_ of tryptophan hydroxylase enzyme present, and not changes in the activity of the enzyme caused by alterations in the firing rates of the serotonergic neurones.

In the preliminary experiments on the effects of ECS, an increased 5-HT synthesis after a series of 8 daily shocks was shown by the increased level of endogenous 5-HIAA. Two possible radio-isotope methods for measuring 5-HT synthesis were then tested. Measurement of (^{3}H)5-HT and (^{3}H)5-HIAA levels after (^{3}H)tryptophan injection confirmed the previous findings of the effects of raphe stimulation on these quantities: no change was seen, in spite of a significant rise in the endogenous 5-HIAA level. In the case of the pargyline-(^{3}H)tryptophan method, as described in Section 1, a significant rise in synthesis was found however. This indicates that the serotonergic nerves are not completely 'switched off' in the presence of the MAO inhibitor, assuming that ECS increases 5-HT synthesis by increasing the cell firing rate (an increased synthesis due to higher tryptophan levels is unlikely, as 5-HT accumulated at the same rate in both groups). The results of Aghajanian *et al.* (67) do in fact show that after giving a MAO inhibitor the raphe cells' firing rates are reduced gradually over a period of about 30 minutes. In spite of this however, this method was not thought to be entirely suitable for the present study. Due to the wide scatter of the results, the difference in 5-HT synthesis in the experiment described only just reached statistical significance, although fairly large numbers of animals were used. Also the fact that the activity of the raphe cells is reduced in the presence of the MAO inhibitor might mean that small changes in firing rates and hence in amine synthesis might be suppressed.

It seemed therefore that the most useful measure of 5-HT synthesis for the present study was simply the determination of

endogenous 5-HT and 5-HIAA levels. This leads to the question of to what extent the 5-HIAA level in the rat brain is determined by the activity of the serotonergic nerves. We know that electrical stimulation of these nerves produces large increases in the 5-HIAA level, but it is not known what the level of this metabolite would be if the nerves were not firing at all. Some information is available however from the effects of drugs and lesions. LSD causes a rapid and complete inhibition of raphe firing (94), and several investigators have measured 5-HIAA levels after giving this drug. Rosecrans et al. (95) found a transient fall of 5-HIAA of upto 20 per cent, and likewise Diaz et al. (90) also found a 20 per cent fall. The difficulty with LSD, as discussed in Section 1, is that it is cleared from the brain very quickly (95), which makes its instanteneous effects on metabolism hard to assess. In the case of lesions in brain, Weiss and Aghajanian (231) found no fall in 5-HIAA in the forebrain $3\frac{3}{4}$ hours after destruction of the dorsal and median raphe nuclei. Anden et al. (232) on the other hand found that after transection of the rabbit spinal cord, 5-HIAA in the caudal part of the cord fell by 60 per cent after 2 days. There was no fall in the 5-HT level, which shows that the metabolism of the nerve endings was still intact at this time.

It appears from these observations that the 5-HIAA level in the brain probably does fall when the firing rates of the serotonergic nerves are reduced. Thus measurement of the level of the acid can be used as an indication of a decrease, as well as an increase, of the activity of these nerves.

Effects of ECS on 5-HT synthesis

It was apparent from the preliminary experiments that 8 daily ECS treatments increased the synthesis of 5-HT, when synthesis was measured 3 hours after the last shock. A subsequent experiment showed however that an increase as least as large was apparent 3 hours after a single shock: thus the chronic treatment appeared to have no greater effect than a single shock. The treatment caused no change in the brain tryptophan level, which suggests that the most likely cause of the increased 5-HT synthesis was an increased firing of the serotonergic nerves. In support of this is the fact that ECT causes marked changes in the EEG, often for long periods, especially as regards the sleep EEG (233, 234, 235). This is particularly relevent as the raphe system is thought to be active in producing the sleep state (179).

An increased level of brain tryptophan 3 hours after a single shock has been found by Tagliamonte et al. (236), though Cooper et al. (209) found no changes in the concentrations of tryptophan or tyrosine in dog ventricular CSF during a series of ECS treatments.

When 5-HIAA was measured 24 hours after the last of a series of daily ECS treatments however, no changes were apparent with treatment periods ranging from 6 to 16 days. It thus appears that ECS causes only short-term increases in 5-HT synthesis, as the rate of metabolism returned to normal in under 24 hours. Tagliamonte et al. (236) found that a single shock elevated the 5-HIAA level in the rat brain, but the level returned to normal after about 6 hours.

It might be questioned therefore whether the efficacy of ECT in the treatment of depression is due to an effect on the 5-HT

system in the brain, and indeed whether a dysfunction in this system is a causative factor in this illness. Given the facts that (a) the level of 5-HIAA in the CSF is reduced in depression and returns to normal when the patient recovers after ECT (144), and (b) that ECS has no prolonged effect on 5-HT metabolism in experimental animals, it seems that the apparently-reduced activity of the 5-HT system in depressed patients is only secondary to the true cause of the disease. It could also be argued of course that ECS only affects the serotonergic nerves when their activity is reduced below normal, and not in normal experimental animals.

The picture may be clarified somewhat by recent studies by the M.R.C. Brain Metabolism Unit (4). In a carefully controlled study of selected unipolar depressed patients over a considerable period of time, it was found that the 5-HIAA level in the lumbar CSF was still below normal when the patients had recovered. In the light of these results, the present findings of a lack of effect of ECS on 5-HT metabolism in animals are still compatible with an amine hypothesis of depression. This could be now that depression is due to a dysfunction of the 5-HT system, and that on recovery this dysfunction is counterbalanced by some change in another system due to the shock treatment. Thus patients make a normal recovery even though the original defect is still present.

These theories are still very embryonic and it is hoped that other clinical and animal data will throw light on this obscure problem.

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