THE EFFECT OF PSYCHOTHERAPEUTIC DRUGS ON THE METABOLISM AND RELEASE OF 5-HYDROXYTRYPTAMINE IN VIVO.

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SUMMARY.

This thesis describes experiments designed to investigate the actions of psychotherapeutic substances on the release of 5-hydroxytryptamine from nerve terminals in the rat forebrain.

Measurement of the brain concentrations of 5-hydroxytryptamine and of 5-hydroxyindoleacetic acid (the major identifiable metabolite of 5-hydroxytryptamine), have been made after different experimental manoeuvres.

The elevation of forebrain 5-hydroxyindoleacetic acid concentration following the electrical stimulation of the nucleus raphe medianus was significantly reduced by treatment with chlorimipramine, whereas that seen following the administration of a high dose of L-tryptophan was not. Since chlorimipramine at the dose used is known to inhibit the transport of 5-hydroxytryptamine from the synaptic cleft to the intracellular environment, these findings would suggest that the elevation of forebrain 5-hydroxyindoleacetic acid concentration, following the electrical stimulation of the nucleus raphe medianus, arises primarily as a consequence of the extra-neuronal release of 5-hydroxytryptamine followed by transportation from the synaptic cleft to the intracellular environment where it is metabolised by intraneuronal monoamine oxidase. In contrast, the extraneuronal release of 5-hydroxytryptamine appears to be involved only to a limited extent in the conversion of L-tryptophan to 5-hydroxyindoleacetic acid.

The use of the technique as a model for the investigation of the action of drugs on the extraneuronal release of 5-hydroxytryptamine is proposed.

The elevation of 5-hydroxytryptamine concentration following

L-tryptophan administration was reduced by 10 day Li^{+} pretreatment, but not by 5 day Li^{+} pretreatment, and the concentration of 5-hydroxyindoleacetic acid was simultaneously increased. This increase in the deamination of 5-hydroxytryptophan has been interpreted as a decrease in the ability of the storage compartment to accumulate synthesised 5-hydroxytryptamine.

Further studies have provided evidence to suggest that the extraneuronal release of 5-hydroxytryptamine from the nerve terminals in the rat forebrain following the electrical stimulation of the nucleus raphe medianus is inhibited by 10 day Li^{+} pretreatment.

The results of these studies also indicate that the effect of Li^{+} on the release of 5-hydroxytryptamine may not occur as a direct consequence of an effect on storage. This would suggest that the proposed effects of Li^{+} on the storage and release of 5-HT may be exerted by two independent actions.

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I N T R O D U C T I O N .

This thesis describes experiments designed to investigate the the effects of psychotherapeutic substances upon the presynaptic pharmacology of forebrain 5-hydroxytryptamine (5-HT). In particular, studies were made of the effects of psychotherapeutic agents upon the release of 5-HT from nerve terminals in the rat forebrain following the electrical stimulation of neural pathways known to contain 5-HT.

Before introducing the experiments themselves it is necessary to mention briefly why 5-HT was studied. A more detailed introduction to these aspects of the pharmacology of 5-HT which have direct relevance to the studies will be presented later.

There is now considerable evidence that 5-HT has a transmitter role in the mammalian central nervous system. The presence of 5-HT in mammalian brain was demonstrated by Twarog and Page (1953), and subsequently found to be distributed unevenly throughout various brain regions (Amin. et. al. 1954). The fluorescent histochemical technique of Falck (1962), or a modification of it (Jonsson et. al. 1969), demonstrated that the 5-HT present in the brain was located within neuronal cell bodies (Dahlstrom and Fuxe 1964), axons (Dahlstrom and Fuxe 1964, Fuxe 1965), and terminal varicosities (Fuxe 1965, Jonsson et. al. 1969). Within the nerve terminal, 5-HT has been located in synaptic vesicles (Maynert et. al. 1964), and shown to be actively accumulated by them (Maynert and Kuriyama 1964). However, in contrast to the findings in acetylcholine terminals, 5-HT does not seem to be concentrated in these organelles to the same extent as acetylcholine is by acetylcholine terminals (Zieher and De Robertis 1963, Maynert et. al. 1964, De Robertis and Rodriguez de Lores Arnaiz 1969).

L-tryptophan, the amino acid from which 5-HT is synthesised, appears to be uniformally distributed throughout the brain (Peters et.

al. 1968, Moir 1971), although active processes exist for the transportation of this amino acid into nerve cells (Grahame-Smith and Parfitt 1970, Kiely and Sourkes 1972, Belin and Pujol 1973). The synthesis of 5-HT from L-tryptophan is shown in Fig. 1. (p.3). Tryptophan is first hydroxylated to form 5-hydroxy-L-tryptophan (5-HTP) which is subsequently decarboxylated to 5-HT. The enzyme responsible for the hydroxylation is tryptophan-5-hydroxylase, and for decarboxylation, aromatic-L-amino acid decarboxylase (5-HTP decarboxylase). Two forms of tryptophan-5-hydroxylase are present in mammalian brain, a particulate found form which has a regional distribution similar to that of 5-HT which is contained in nerve terminals, and a soluble form of the enzyme which appears to be associated with brain regions which contain 5-HT cell bodies (Knapp and Mandell 1972). 5-HTP decarboxylase has been shown to be present in brain tissue (Lovenberg et. al. 1962), and to have a regional distribution similar to that of 5-HT (Kuntzman et. al. 1961, Sims et. al. 1973).

The major route of 5-HT catabolism involves the oxidative deamination of 5-HT to the aldehyde, 5-hydroxyindoleacetaldehyde, the enzyme responsible being monoamine oxidase (MAO). The unstable aldehyde is rapidly oxidised to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase. MAO seems to be uniformally distributed throughout the brain (Bogdanski et. al. 1957, Weiner 1960, La Motte et. al. 1969). Brain tissue is able to oxidise 5- hydroxyindoleacetaldehyde to 5-HIAA (Dietrich 1966), which is then probably removed from the brain by active transport processes situated at the choroid plexus (Ashcroft et. al. 1968, Czerr and Van Dyke 1972, Forn 1972, Sampath and Neff 1974) and elsewhere (Wolfson et. al. 1974).

The release of 5-HT from nerve terminals in mammalian brain has

FIG.1.

THE MAJOR ROUTE OF 5-HT SYNTHESIS AND CATABOLISM IN RAT BRAIN.

The major route of 5-HT synthesis involves the stages 1 and 2, and the major route of catabolism stages 3 and 4. The production of 5-HTOL appears to play only a minor role in 5-HT catabolism.

Abbreviations :-

5-HTP - 5-hydroxytryptophan.

5-HT - 5-hydroxytryptamine.

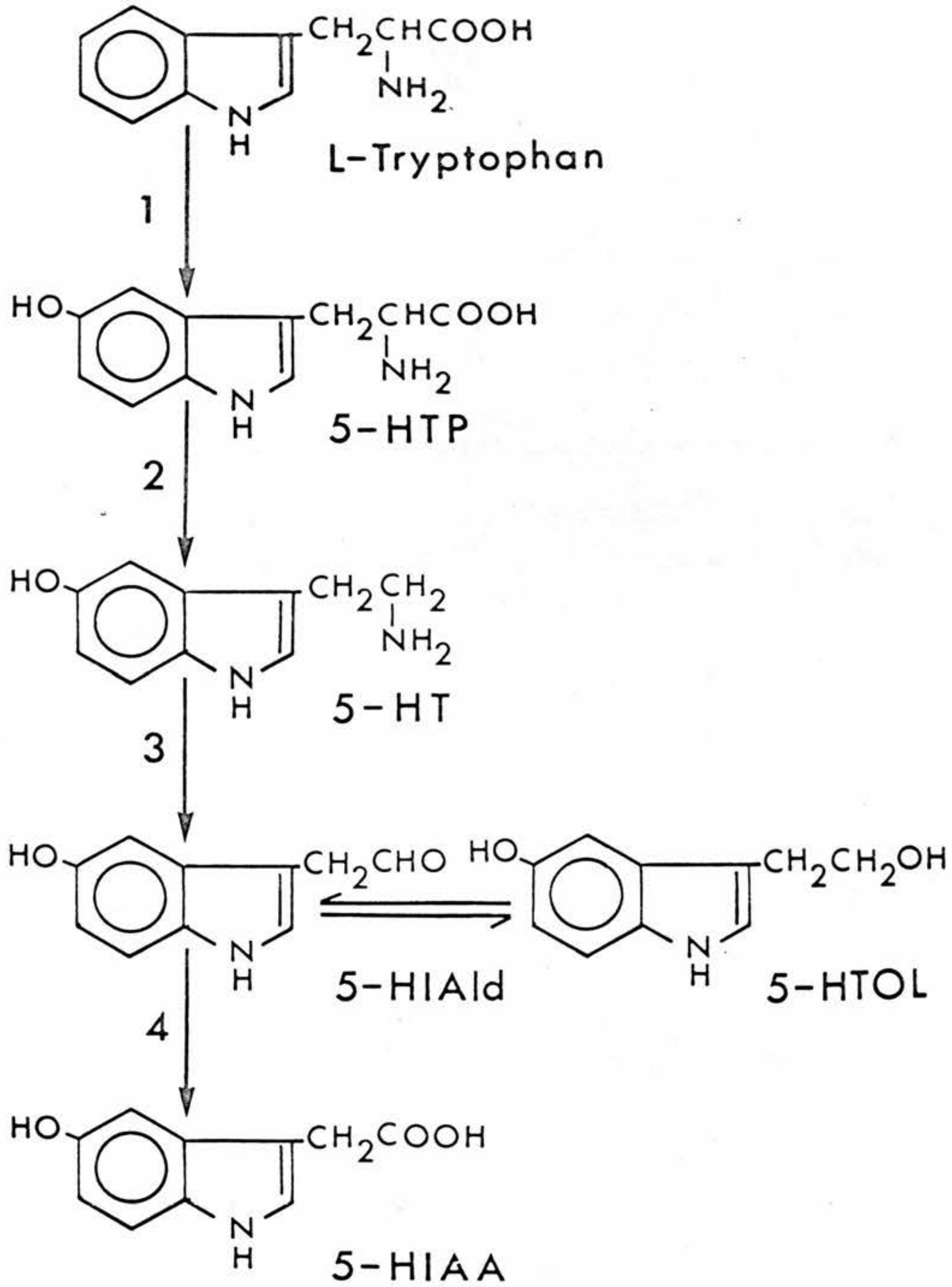
5-HIAld - 5-hydroxyindoleacetaldehyde.

5-HTOL - 5-hydroxytryptophol.

5-HIAA - 5-hydroxyindoleacetic acid.

The enzymes involved are :-

1. Tryptophan-5-hydroxylase.
2. 5-HTP decarboxylase.
3. Monoamine oxidase.
4. Aldehyde dehydrogenase.


CH2CHCOOH
NH2
N
H
L-Tryptophan
1
HO
CH2CHCOOH
NH2
N
H
5-HTP
2
HO
CH2CH2
NH2
N
H
5-HT
3
HO
CH2CHO
N
H
5-HIAld
HO
CH2CH2OH
N
H
5-HTOL
4
HO
CH2COOH
N
H
5-HIAA

been demonstrated using a variety of techniques, (Feldberg and Myers 1966, Eccleston et. al. 1969a, 1969b, Portig and Vogt 1969, Holman and Vogt 1972, Ashkenazi et. al. 1973), and a mechanizm exists for the rapid removal of released 5-HT from the synaptic cleft (see review by Iversen 1971). Finally, the microiontophoretic application of 5-HT has been found to alter the activity of single neurones in every brain region in which it has been studied (see Bloom et. al. 1972).

As a general conclusion therefore, it seems that the 5-HT of brain tissue posesses many of the properties one would expect of a substance with possible transmitter function.

If one accepts that normal brain function relies upon the transmission of correct information from neurone to neurone, it could be considered that the relaying of such information is impaired in a malfunctioning brain. Similarly, if disorders of mood arise as a consequence of the brain not functioning normally, one could postulate an impairment of one or more of the brain transmitter systems in these illnesses. The brief introduction to 5-HT outlines the basic evidence in support of the concept that 5-HT is a transmitter in mammalian brain. The first indication that disorders in brain 5-HT may play a role in mental illness was reported by Woolley and Shaw (1954). Later, Schildkraut (1965) and Bunney and Davis (1965) reviewed the current evidence and proposed the catecholamine hypothesis of affective disorders. In brief this stated "Some if not all depressions are associated with an absolute or relative deficiency of catecholamines, particularly noradrenaline, at functionally important receptor sites in the brain. Elation conversely may be associated with an excess of such amines" (Schildkraut 1965). In general, most of the evidence used to implicate noradrenaline in the aetiology of the affective dis-

orders is indirect in nature and can be applied to other biogenic amines especially 5-HT (Himwich and Alpers 1970).

Some of the more recent experimental findings supporting the contention that disorders of mood may also be related to disorders in brain 5-HT can be summarised as follows. Indirect evidence is available suggesting that the production of 5-HT from L-tryptophan may be reduced in depressed patients (Cazulla et. al. 1966, Curzon and Bridges 1970). The concentration of 5-HT, and the major metabolite 5-HIAA was found to be significantly lower in the hindbrains of depressed suicides, than in the hindbrains of non depressed subjects (Shaw et. al. 1967, Bourne et. al. 1969). Plasma monoamine oxidase activity has been reported to be higher in premenopausal depressed women than in normal subjects. Treatment of the depressed women with oestrogen resulted in a lowering of the activity of the enzyme and an improvement in the mood of the treated women (Klaiber et. al. 1972). In a similar study, Murphy and Weiss (1972) noted that the activity of platelet monoamine oxidase was slightly higher in depressed patients than in controls, and was significantly lower in manic-depressive patients. Drugs which inhibit the enzyme monoamine oxidase are effective antidepressant agents, and have been shown to elevate the concentration of 5-HT in the brain of animals (Macon et. al. 1971, Hamon et. al. 1972) and man (Maclean et. al. 1965).

Some of the most interesting findings relating mania, depression and brain 5-HT have arisen from studies of the alkaloid reserpine. This drug had been used for its sedative properties in the treatment of mania (Watt 1958). Its clinical use now is restricted to the treatment of hypertension. A small but consistant number of patients receiving reserpine developed severe depressive reactions which in some

cases required psychiatric hospitalisation and antidepressive therapy. Patients receiving other antihypertensive agents had no depressive episodes (Lemiux et. al. 1965). Results of animal studies have demonstrated that reserpine markedly reduces the concentration of both 5-HT and noradrenaline in the brain (Kuntzman et. al. 1965), and that this effect is probably exerted by inhibiting the storage of the amines within neurones (Maynert and Kuriyama 1964, Ross and Renyi 1967, Blackburn et. al. 1967, Norn and Shore 1971, Shore 1972). Further, it has been suggested that the sedation produced by reserpine is due to the loss of 5-HT in the brain rather than to a loss of noradrenaline (Kuntzman et. al. 1961).

It can be suggested from this very brief introduction that 5-HT may well be a central transmitter, and that disorders in the functioning of this transmitter may play a role in the aetiology of the affective disorders.

There are many reports describing the effects of psychotherapeutic substances on the synthesis, uptake and catabolism of 5-HT. However, there has been very little work carried out on the effects of drugs on the physiological release of 5-HT from nerve terminals (Brodie et. al. 1961, Himwich and Alpers 1970, Sulser and Sanders-Bush 1971). What little work that has been reported, has, in general been carried out on isolated brain slices in vitro (Katz et. al. 1968, Chase et. al. 1969). There seemed therefore, to be a distinct lack of information concerning the effects of these drugs on the release of 5-HT in brain tissue. The work described in this thesis was undertaken in order to try to increase our understanding of the mode of action of some psychotherapeutic substances, through an investigation into the action of these substances upon the release of 5-HT in the rat brain following

stimulation of a known neuronal pathway.

The first tasks confronting such an investigation were the identification of the neuronal pathway to be investigated, and the finding of a suitable method for the measurement of the release of 5-HT.

The cell bodies of the neurones which contain 5-HT are situated predominantly in the raphe region of the midbrain (Dahlstrom and Fuxe 1964). Nine groups of cell bodies have been identified in the medulla, pons and mesencephalon of rat brain. Those that give rise to axons ascending to the forebrain are mainly located in the nucleus raphe dorsalis and nucleus raphe medianus of the mesencephalon.

Many studies have shown that axons arising from these cell bodies ascend to the forebrain through the medial forebrain bundle (Heller et. al. 1962, Harvey et. al. 1963, Anden et. al. 1966a, 1966b, Parent 1969). More detailed mapping of the ascending 5-HT pathways demonstrated that the cell bodies of the dorsal and medial raphe nuclei gave rise to axons which ascend in the ventral part of the medial forebrain bundle (Ungerstedt 1971). The axons could be divided into a medial and a lateral component. Those axons situated medially ascend in the septum. Some turn caudally in the cingulum where a proportion lie along the dorsal surface of the corpus callosum and innervate the cortex (Fuxe et. al. 1968, Jonsson et. al. 1969). The more lateral component of the medial forebrain bundle enters the hypothalamus, the brain region most densely innervated by 5-HT terminals (Fuxe 1965), and the amygdaloid complex.

While mapping of the ascending neuronal systems is by no means complete, it is apparent from the studies outlined above that there exist neurones containing 5-HT which arise from cell bodies in the medial and dorsal raphe nuclei and ascend through the medial forebrain

bundle to innervate a number of forebrain structures. The raphe nuclei are anatomically well defined, and using the stereotaxic technique, it is relatively simple to implant electrodes into these nuclei and to activate the ascending pathways by electrical stimulation (Eccleston et. al. 1969a). It was therefore anticipated that the release of 5-HT in the rat forebrain following the stimulation of the midbrain raphe could be the model to be used in these studies. It remained now to identify a method by which the release of 5-HT following raphe stimulation could be monitored. The therapeutic response to many of the drugs used in the treatment of mania and depression often takes days or weeks to develop. Because of this any technique designed to investigate the effect of these drugs on the release of 5-HT must be applicable to the study of both acute and chronic drug treatment.

The release of 5-HT from nerve terminals in vivo has been investigated in a number of ways. Three techniques have been employed in order to collect and measure the concentration of 5-HT and its principal metabolite 5-HIAA in fluid that has been brought into contact with brain tissue. These are the cortical cup technique, the push-pull cannula, and ventricular perfusion. Following the success of the cortical cup technique in demonstrating the release of Ach from the surface of the cortex (Mitchell 1963, Collier and Murray-Brown 1968), attempts were made to employ the technique in the measurement of the release of 5-HT. Eccleston et. al. (1969a) demonstrated that 5-HT was released spontaneously into fluid contained in perspex cups applied to the sigmoid cortex of cats. The amount released was very small, but could be doubled by pretreating the cats for two days with an inhibitor of MAO. Because of the low rate of release

(0.08 ng/cm^2/min) it was necessary to pool samples for accurate estimation, and even then the release was not constant. In seven out of eight experiments conducted by the group, stimulation of the midbrain raphe for 15 minute periods, caused the appearance after a short latency (2-3 msec) of a positive evoked potential in the post sigmoid cortex and a three fold increase in the release of 5-HT into the cup fluid. The effect either coincided with or followed the period of stimulation, and recovery tended to be prolonged over the next few collection periods. The spontaneous release of 5-HIAA was unaffected by raphe stimulation. However, in a later report, Eccleston et. al. (1969b) demonstrated that stimulation of the midbrain raphe of rats produced a significant increase in the forebrain concentration of 5-HIAA which was accompanied by an increase in the release of 5-HIAA into the cortical cup fluid. The elevation of 5-HIAA both in the brain and cortical cups was believed to arise from the metabolism of released 5-HT (see later).

The push-pull cannula consists of two concentric cannulae; perfusion fluid is passed through the inner barrel and recovered by syphoning through the outer barrel. This device allowed the possible perfusion of small regions of brain tissue even at considerable depth in the brain.

Eccleston et. al. (1969a) attempted to measure the release of 5-HT in the rat caudate nucleus using this technique. The spontaneous release of 5-HT was found to be of the order of 50 pg/min, and this was found to be little changed by the stimulation of various afferent pathways. Prelabelling of the 5-HT stores with ^{14}C or ^{3}H-5-HT facilitated the measurement of 5-HT. However, the increased efflux of labelled 5-HT following stimulation of the afferent pathways was neither constant nor repeatable with an experiment. In a pre-

vious study, Chase and Kopin (1968), demonstrated an increase in the efflux of labelled noradrenaline and 5-HT in the olfactory bulb following stimulation of the olfactory tract in rats whose stores of noradrenaline and 5-HT had been previously labelled with the tritiated amine. The problems of using the push-pull cannula became apparent when the efflux of a number of metabolically inert ^{14}C-labelled compounds (inulin, urea and γ-aminoisobutyric acid) injected with the tritiated monoamines were examined. On odourous stimulation, a substantial rise in the efflux of the ^{14}C-labelled compound invariably accompanied the augmented amine efflux. Stimulus intensity studies demonstrated that there was no difference in threshold between monoamine and inulin efflux. Inulin injected into the cerebrospinal fluid remains largely in the extracellular space, thus odour induced alterations in its efflux suggest that local changes within this compartment may attend neural stimulation. Thus, while there is evidence that labelled 5-HT injected into cerebrospinal fluid is rapidly accumulated by intraneuronal stores (Aghajanian and Bloom 1967) it was impossible to determine whether the stimulus induced alterations in 5-HT efflux reflected physiological release from intracellular stores or merely extracellular shifts of residual exogenous amine.

In addition to the artificial perfusion of brain regions, a widely used and successful method for studying the release of transmitters is that of ventricular perfusion. The basis of the technique involves the perfusion of one or a number of the ventricular systems of the brain followed by the collection and estimation of substances in the perfusate. The release of transmitters in brain regions immediately adjacent to the ventricles should, theoretically, be detectable in perfusate from that region. Using this technique, the spontaneous release of 5-HT into

fluid perfusing the lateral and third ventricles has been demonstrated in the cat (Feldberg and Myers 1966), (Portig and Vogt 1969). 5-HT released into the lateral ventricles is believed to arise from the caudate nucleus, while that entering the third ventricle is believed to originate in the hypothalamus. The resting output of 5-HT was increased considerably by stimulation of the midbrain raphe nuclei (Holman and Vogt 1972, Ashkenazi et. al. 1973).

In addition to the measurement of the outflow of 5-HT into fluid which has been brought into contact with brain tissue, stimulation-induced changes in the concentration of 5-HT and its major metabolite 5-HIAA in the tissue itself has been studied.

Dahlstrom et. al. (1965) employed the Falck and Hillarp histochemical technique to investigate the fluorescence of monoamine terminals in the spinal cord in animals receiving prolonged (1-2 hours) tetanic electrical stimulation in the region of the bulbo-spinal neurones of the medulla oblongata. In the stimulated animals, there was a reduction in the number of fluorescent terminals, and many of the remaining fluorescent terminals showed a marked reduction in the fluorescent intensity of their varicosities. The stimulus-induced depletion of 5-HT fluorescence was much greater in the presence of compounds which inhibit further synthesis of the transmitter. The authors concluded that the stimulus induced depletion of nerve ending 5-HT was indicative of the release of the monoamine in the spinal cord following stimulation of 5-HT containing bulbo-spinal neurones.

Aghajanian et. al. (1967) demonstrated that stimulation of the midbrain raphe for 30 or 60 minutes induced a decrease in the concentration of 5-HT and a large increase in the concentration of 5-HIAA in the forebrain of rats. Stimulation of the lateral midbrain, an area

virtually devoid of neurones containing 5-HT, had no significant effects on the concentration of either substance. These findings were soon substantiated by a number of workers (Kostowski et. al. 1969, Gumulka et. al. 1969, Eccleston et. al. 1970, Sheard and Zolovic 1971). It was concluded that the increase in 5-HIAA and accompanying decrease in 5-HT concentration indicated a release and subsequent oxidative deamination of 5-HT. (Alternative interpretations are discussed later).

Though, with the exception of the "push-pull" cannulae, all the techniques outlined above have proved useful in demonstrating the release of 5-HT from brain tissue in vivo, few are applicable to the quantitative study of the actions of drugs on 5-HT release. The ideal technique requires that it be simple to use, accurate, consistant and easily but adequately controlled.

The quantitative determination of the loss of 5-HT following stimulation in the presence of a synthesis inhibitor requires combined histochemical and biochemical analyses to be carried out. While the technique appears adequate for the demonstration of the release of 5-HT in a particular neuronal pathway, its use in determining the effect of psychotherapeutic substances upon 5-HT release depends upon the ease in controlling for other effects of the drug. The controls that would be required to differentiate between the effects of the drugs upon synthesis, storage and release would be large. In addition, the technique has been little used, and as such, optimal conditions for experimentation would have to be determined.

The measurement of 5-HT and its principle metabolite in fluid bathing neural tissue is similarly not suitable for the proposed studies. The work of Eccleston et. al. (1969) demonstrated that the release of 5-HT into the fluid contained in cups applied to the cortex of cats and

rats was small and inconsistant. The problems of using "push-pull" cannulae were described previously.

Ventricular perfusion has been widely and successfully used to examine the stimulus induced release of a number of transmitter substances. The technique is excellent when an animal can be used as its own control in examining the effect of acute drug treatment. However, because of the very wide between animal variation that has been noted by the users of the technique (see Holman and Vogt 1972), the application of the technique to chronic drug treatments is inappropriate.

The technique of measuring the concentration of 5-HIAA in rat brain tissue following raphe stimulation appears to be accurate and consistant, providing the animals receive stimulation of similar magnitude for a set period of time (Aghajanian et. al. 1967, Kostowski et. al. 1969, Eccleston et. al. 1970). Because of this, the technique would lend itself to the examination of both acute and chronic drug treatments. It has been argued that the stimulus-induced elevation of 5-HIAA under these circumstances was produced primarily as a consequence of the extraneuronal release of 5-HT followed by catabolism to 5-HIAA (Aghajanian et. al. 1967, Kostowski et. al. 1969, Gumulka et. al. 1969). Such an interpretation is based upon the physiological processes believed to occur in the synaptic region of a tryptaminergic neurone. Fig. 2 (p.14) is a schematic representation of a 5-HT transmitting synapse. The figure shows 5-HT contained in the storage compartment within the terminal. On arrival of a nerve impulse, 5-HT is believed to be released directly from its storage site into the synaptic cleft, where it is able to diffuse across the synaptic space to activate post synaptic receptors. Following release, 5-HT in the

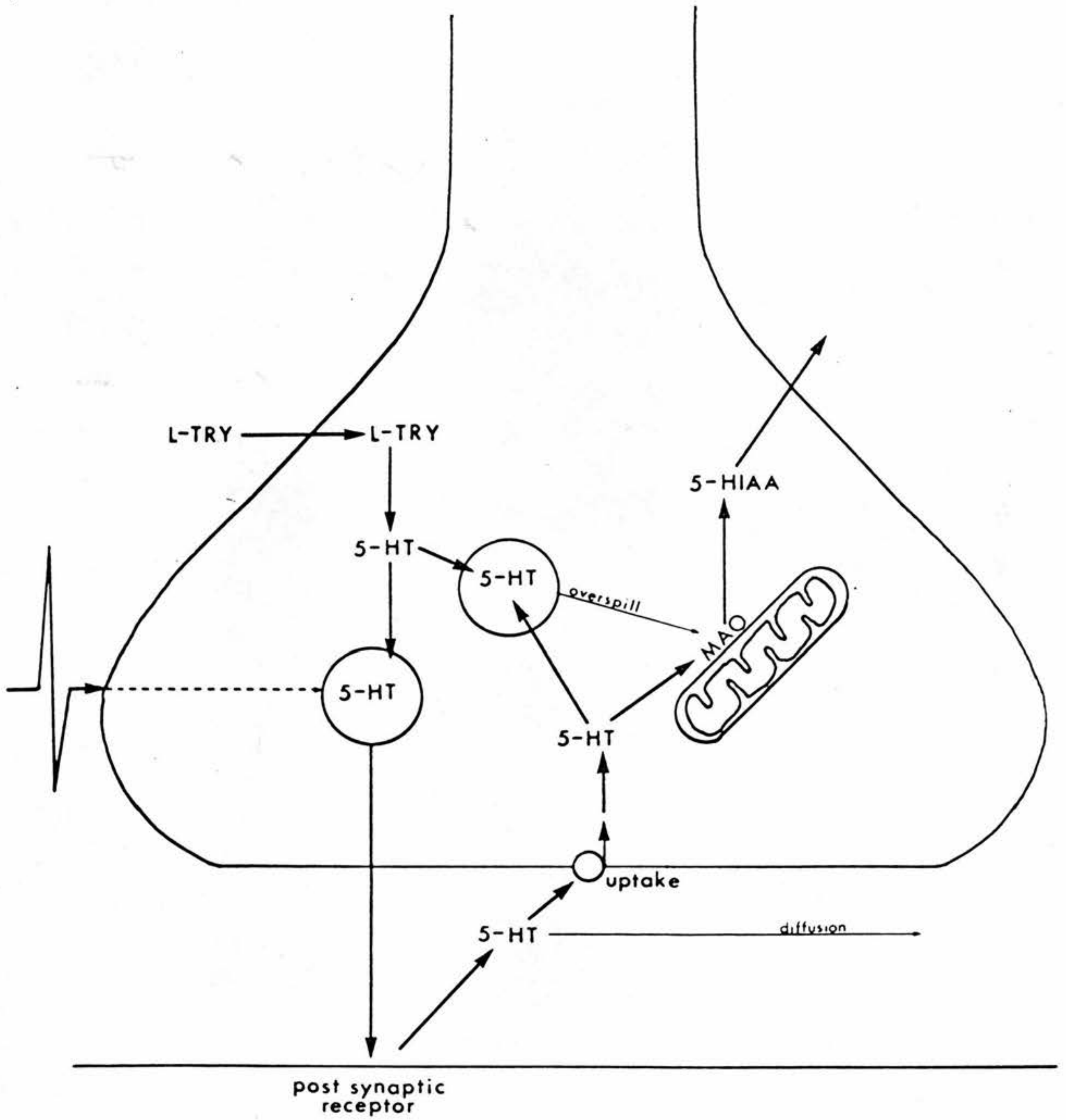
FIG. 2.

A SCHEMATIC REPRESENTATION OF A 5-HT TRANSMITTING SYNAPSE.

The present concepts of the processes occurring at the 5-HT nerve terminal are shown in the diagram.

5-HT is synthesised within the nerve terminal from L-tryptophan (L-TRY) which is transported across the terminal membrane. Free cytoplasmic 5-HT is either incorporated into the storage compartment (represented by 5-HT enclosed in circles), or metabolised by mitochondrial MAO to produce 5-HIAA which may be cleared from the neurone.

Stored 5-HT released by stimulation may be actively transported from the synaptic cleft into the intracellular environment by the membrane transport process (represented by uptake), or may diffuse away passively. 5-HT returned to the cell may then be treated as free 5-HT described above.


L-TRY
L-TRY
5-HT
5-HT
5-HIAA
overspill
MAO
5-HT
5-HT
5-HT
uptake
5-HT
diffusion
post synaptic
receptor

synaptic cleft may either diffuse away passively in the extracellular fluid or be actively transported into the presynaptic terminal by the high affinity transport process situated in the terminal membrane (Shaskan and Snyder 1970, Kuhar et. al. 1972, Wong et. al. 1973, Iversen 1971). The 5-HT which is actively transported in this way is believed to be either accumulated by the storage mechanism (Maynert and Kuriyama 1964) where it can be re-used in transmission, or it may remain free in the cytoplasm where it will be converted to 5-HIAA by MAO which is located on the outer membrane of the mitochondria (Schnaitman et. al. 1967, Tipton 1967).

According to the model of synaptic transmission outlined above, the elevation of forebrain 5-HIAA concentration following stimulation of the nucleus raphe medianus has been interpreted as follows: The passage of nerve impulses from the raphe nucleus to the axon terminals in the forebrain elicits the release of 5-HT from the terminals into the synaptic cleft. After activating post synaptic receptors, the free 5-HT in the extracellular space is actively transported into the presynaptic terminals where some will be re-stored and some converted to 5-HIAA by MAO and aldehyde dehydrogenase. The 5-HT stores are also repleted by an increase in the rate of 5-HT synthesis (Gumulka et. al. 1969, Eccleston et. al. 1970, Shields and Eccleston 1972). If this interpretation is correct, and provided the method can be easily controlled for alterations in 5-HIAA concentration by other routes, the elevation of forebrain 5-HIAA following stimulation of the midbrain raphe may well be the ideal model for these studies.

The increase in the concentration of 5-HIAA in the rat forebrain following raphe stimulation can, however, be interpreted in another way. Both the putative transmitter, and the enzymes required for its catabolism are stored in the same neurone. The elevation of forebrain

5-HIAA following raphe stimulation has been shown to be accompanied by an increase in the rate of synthesis of 5-HT (Gumulka et. al. 1969, Eccleston et. al. 1970). It follows therefore, that if the predominent effect of stimulation was an increase in the rate of 5-HT synthesis, and that a large proportion of the newly synthesised 5-HT could not be accommodated by the storage compartment, there would be an increase in the concentration of free cytoplasmic 5-HT. Since free 5-HT appears to be susceptable to deamination, there would occur a proportionate increase in the concentration of 5-HIAA. It seems possible therefore that the elevation of forebrain 5-HIAA following raphe stimulation could have arisen from a predominantly intraneuronal phenomenon of increased synthesis of 5-HT involving little or no extraneuronal release of the transmitter. In the present investigation one of the early studies was to attempt to discriminate between the two interpretations of the observed effect of raphe stimulation on the forebrain concentrations of 5-Hydroxyindoles. In order to do this, it was first necessary to identify a mechanism whereby forebrain 5-HIAA concentrations could be elevated by an essentially intraneuronal process, and secondly to select a suitable method to determine the relative contributions of intra- and extraneuronal processes in the elevation of forebrain 5-HIAA.

Moir and Eccleston (1968) demonstrated in both rat and dog brain, that the elevation of 5-HIAA concentration following a single large dose of the precursor amino acid L-tryptophan (100 mg/Kg i.p.) was far greater than the elevation of 5-HT. When plateau levels for 5-HT and 5-HIAA were produced by this procedure, the 5-HT/5-HIAA ratio was far smaller than the ratio in control brain. The authors suggested that their findings could be "due to either a progressive filling of the

5-HT stores towards saturation, with increasing 'overspill' onto mitochondrial MAO, or to 5-HTP derived from the extracerebral hydroxylation of L-tryptophan bypassing the storage mechanism for 5-HT". Moir (1971) substantiated these findings and concluded that the effect of L-tryptophan loading on the concentration of brain 5-HT and 5-HIAA was brought about by a progressive filling of the 5-HT stores so that the concentration of free cytoplasmic 5-HT increased. The catabolism of the increased concentration of free 5-HT would result in the production of large quantities of 5-HIAA. It thus appears that the administration of large single doses of L-tryptophan should provide a mechanism whereby forebrain 5-HIAA concentrations could be elevated by a predominantly intracellular process involving little extraneuronal release. Thus two methods have now been identified whereby forebrain 5-HIAA concentrations may be elevated. Electrical stimulation of the nucleus raphe medianus elevates 5-HIAA concentration by mechanisms which may or may not involve the extraneuronal release of 5-HT, whereas the elevation of 5-HIAA following L-tryptophan appears to be primarily of intraneuronal origin involving little extraneuronal release.

It now remains to identify a method which can be used to determine the relative contributions of the described intraneuronal and extraneuronal processes in producing the effects that have been observed following electrical stimulation. In order to do this, it would be necessary to manipulate in some way the processes that appear to be involved extracellularly in the synaptic cleft since the intraneuronal deamination of of the transmitter would be expected to be similar in both cases. It can be seen from Fig. 2 that according to the general concepts of synaptic transmission, these extracellular processes are the interaction between 5-HT and the post synaptic receptor, and the removal of the

transmitter from the synaptic cleft. Little is known about the chemical nature of the interaction between 5-HT and the post synaptic membrane, whereas there is a considerable amount of information available concerning the removal of 5-HT from the synaptic cleft, and its modification by pharmacological agents. There exists a powerful high affinity active transport process situated at the terminal membrane, responsible for the transfer of 5-HT from the extracellular fluid into the intracellular environment (Shaskan and Snyder 1970, Kuhar et. al. 1972, Wong et. al. 1973, Iversen 1971). This accumulation process is therefore responsible for bringing extracellularly located 5-HT into the environment which contains the major enzymes responsible for its catabolism. It is thus evident, that if this transport process is inhibited to some extent, there should occur a reduction in the amount of 5-HIAA formed from 5-HT which has been released into the synaptic cleft, while that which is formed from 5-HT which remains in the intracellular environment should be unaffected.

Of the substances known to interfere with the accumulation of 5-HT by brain tissue, the most widely investigated are the tricyclic antidepressive agents. Although these agents have been demonstrated to exert a number of effects upon brain 5-HT (Corrodi and Fuxe 1968, 1969, Meek and Werdinius 1970, Bruinvels 1972, Halaris et. al. 1973) the most widely documented property of these substances is their ability to inhibit the accumulation of monoamines by brain tissue (Blackburn et. al. 1967, Ross and Renyi 1967, Fuxe and Ungerstedt 1968, Carlsson et. al. 1968, 1969a, Shaskan and Snyder 1970, Meek et. al. 1970a, Lidbrink et. al. 1971).

Within the group of tricyclic drugs there is a considerable variation in the potency of their inhibitory actions with respect to the

accumulation of catecholamines and 5-HT. This difference in potency appears to be related to the chemical structure of the compounds, and to the terminal amino group in particular. Tertiary amines such as imipramine and chlorimipramine (see Fig 5 p.20), preferentially inhibit 5-HT accumulation by central neurones, whilst secondary amines preferentially inhibit the accumulation of noradrenaline. (Lidbrink et. al. 1971). The most potent of the tertiary amines studied was found to be chlorimipramine (Carlsson et. al. 1969b). This compound has been shown to inhibit the accumulation of exogenous 5-HT by brain tissue in vitro and in vivo (Shaskan and Snyder 1970, Lidbrink et. al. 1971), and there is evidence to suggest that the uptake of endogenous 5-HT from the synaptic cleft may also be inhibited (Carlsson et. al. 1969a, 1969b, Meek et. al. 1970a).

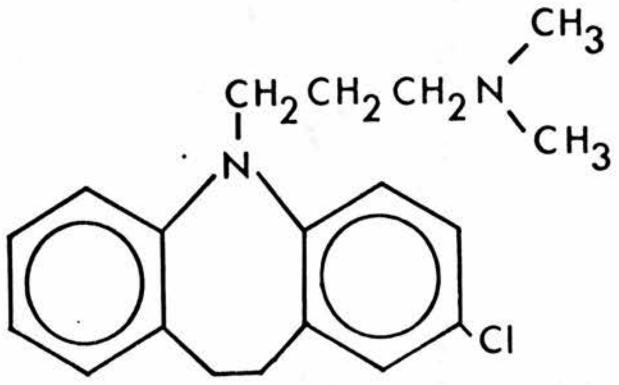
With this information, it was decided to attempt to differentiate between the intraneuronal and extraneuronal origins of the stimulus induced elevation of 5-HIAA by inhibiting the 5-HT transport mechanism with chlorimipramine. If raphe stimulation does elicit the extra-neuronal release of 5-HT, it would be expected, in the presence of chlorimipramine, that a proportion of the 5-HT released into the synaptic cleft would be denied access to intraneuronal MAO, and that the elevation of forebrain 5-HIAA concentration would be proportionately reduced. If, on the other hand, electrical stimulation of the nucleus raphe medianus elicits little extraneuronal release of 5-HT, the inhibition of the 5-HT transport process should have little effect, and the elevation of 5-HIAA produced by electrical stimulation and L-tryptophan administration would be expected to be affected similarly by pretreatment with chlorimipramine.

The changes in forebrain 5-HT and 5-HIAA elicited by L-tryptophan treatment would provide a second control system whereby any direct effect

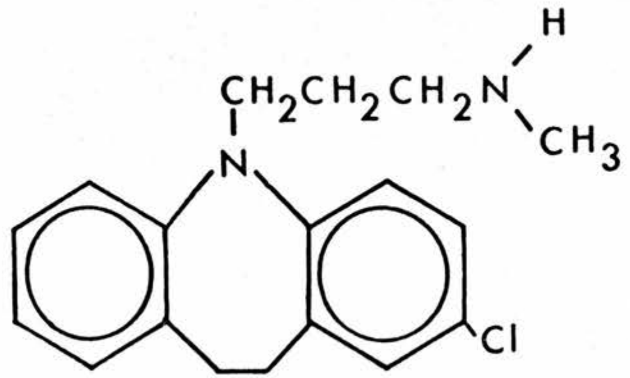
FIG. 3.

THE CHEMICAL STRUCTURE OF THE TWO MAJOR
GROUPS OF TRICYCLIC ANTIPRESSANTS.

Differences in the structure of the two groups of tricyclic antidepressants with relation to their potency as inhibitors of noradrenaline and 5-HT accumulation. A typical example of a tertiary amine is given by chlorimipramine, whilst the secondary amine is represented by the demethylated derivative chlordesmethylimipramine.



Chlorimipramine



Chlordesmethylimipramine

of chlorimipramine upon the synthesis (Bruinvels 1972), and catabolism of 5-HT (Halaris et. al. 1973) may be identified.

In summary, the first major study of the project was an attempt to examine whether the elevation of forebrain 5-HIAA concentrations induced by raphe stimulation was of predominantly intraneuronal or extraneuronal origin. By inhibiting the accumulation of extraneuronal 5-HT by pretreatment with chlorimipramine, and by comparing the effects of this treatment upon the elevation of forebrain 5-HIAA induced by raphe stimulation and by L-tryptophan administration, it should be possible to ascertain whether or not raphe stimulation elicits the extraneuronal release of 5-HT.

The second study conducted in this thesis was an investigation into the effect of Lithium (Li^+) on the release of 5-HT in the rat forebrain. Li^+ has been used extensively in the treatment of acute mania, Chronic mania, and manic depressive illnesses (Schou 1968, Davis and Fann 1971, Coppen 1973). Cade (1949) first observed the dramatic antimanic action of Li^+ in a study of ten manic patients treated with lithium carbonate or lithium citrate. This observation was soon to be supported by the findings of Noack and Trautner (1951), and the 'double-blind' clinical trials of Maggs (1963). Wharton and Fieve (1966) demonstrated that Li^+ administered to manic-depressive patients during the manic phase successfully reduced the duration of the episode in a number of patients. Subsequently it was found that in addition to the treatment of pure manic episodes, the prolonged administration of Li^+ to manic-depressive and recurrent depressive patients led to a prevention of depressions and transitional states as well (Baastrup 1964, Baastrup and Schou 1967, Schou 1971). These observations were finally substantiated by the controlled double-blind studies of Baastrup

et. al. (1970) and Melia (1971). These and other reports of the effectiveness of Li^+ as a prophylactic agent are reviewed by Coppen (1973). A recent study (Mendels et. al. 1972) demonstrated that Li^+ was as effective as desipramine in treating selected depressed patients. However, Goodwin et. al. (1972) found that depressed patients with a prior history of mania or hypomania were more likely to show an antidepressant response to Li^+ than were depressed patients without such a history. Taking this further, Kukopulos and Reginaldi (1973) observed in recurrent manic-depressive patients that a depressive episode was generally prevented or attenuated by Li^+ only when a preceeding manic episode had been prevented or attenuated by Li^+. They suggested that Li^+ acted directly on mania but only indirectly on depressions.

To summarise, Li^+ is an effective treatment for acute mania, chronic mania and manic-depressive illnesses. The efficacy of Li^+ as an antidepressive agent appears to be greater if the patient has a prior history of mania or hypomania, and there is evidence to suggest that the effectiveness of Li^+ in reducing depressive episodes in manic-depressives is dependent upon a primary effect on the previous manic phase. From this information it may be suggested that the therapuetic response to Li^+ administration in these disorders may be related to a primary antimanic action. Finally it should be noted that Li^+ therapy is the only successful treatment of manic disorders which does not sedate normal people, or interfere too much with mood between successive episodes.

The current monoamine hypothesis of affective disorders proposes a reduction in the availability of transmitters at monoamine synapses during depression, and an increase during mania. The publication of

this theory led to widespread investigations into the effect of Li^+ on the monoamine transmitters in an attempt to identify a possible mode of action. However, although a large number of investigations have taken place, the mode of action of Li^+ remains essentially unresolved. (Shaw 1973, Schou 1973, Davis and Fann 1971, Samuel and Gottesfeld 1973). The available information on the effects of Li^+ on the presynaptic physiology of brain 5-HT will be briefly reviewed here.

Schildkraut et. al. (1969) demonstrated that the turnover of 5-HT was reduced in rat brain following acute Li^+ treatment. Ho et. al. (1970) noted a 12% reduction in whole brain 5-HT turnover following prolonged Li^+ treatment. Turnover in the hypothalamus was reduced by 51%; in the brainstem by 28%; and in the cortex by 15%. Turnover in the cerebellum was increased however by 37%. Shaw et. al. (1972) using multicompartmental analysis, demonstrated a shift in the partition of tryptophan in favour of the extracellular phase at the expense of the intracellular phase in peripheral tissues of the rabbit. They suggested that if this could be extrapolated to the CNS, it could result in a decrease in 5-HT synthesis in the brain. Tagliamonte et. al. (1971) however, demonstrated an increase in total brain L-tryptophan following Li^+ treatment. According to presently held views on the control of 5-HT synthesis, (Tagliamonte et. al. 1971, 1973) this might be expected to lead to an increase in 5-HT synthesis. Sheard and Aghajanian (1970) noted an increase in 5-HT synthesis following acute administration of high doses of Li^+ and this was supported by the findings of Perez-Cruet et. al. (1971), Schubert (1973) and Poitou et. al. (1974). Bliss and Ailion (1970) were unable to detect any changes in 5-HT turnover following 14 days treatment with low doses of LiCl. Similarly Corrodi et. al. (1967) also failed to detect any changes in

5-HT turnover following acute Li^+ administration.

Knapp and Mandell (1973) in a well controlled study, compared the effects of acute and chronic Li^+ administration on brain 5-HT synthesis in rats. They found that short term Li^+ administration increased the uptake of L-tryptophan and its conversion to 5-HT by striatal synaptosomes, while at the same time cell body tryptophan-5-hydroxylase activity was reduced. By 10 days, the conversion of L-tryptophan to 5-HT in nerve endings became a joint function of a maintained increase in the uptake of L-tryptophan, and a decreased level of tryptophan-5-hydroxylase activity. The end result at 10 days was a near normal rate of 5-HT synthesis in nerve endings. The time course of these observed events was found to correlate well with the time course described for the axoplasmic flow of tryptophan-5-hydroxylase from the cell body to the terminal. As yet there are no reports of any effects of Li^+ on the storage of 5-HT in the presynaptic terminal.

The release of 5-HT from field stimulated rat brain slices in vitro was found to be inhibited by acute Li^+ pretreatment (Katz et. al. 1968), and by the addition of Li^+ to the incubation medium (Katz et. al. 1968, Chase et. al. 1969). Both acute and prolonged Li^+ treatment has been shown to slow the depletion of brain 5-HT following tryptophan-5-hydroxylase inhibition. This finding was interpreted to be a result of either a lower impulse frequency in the 5-HT neurones or an inhibition of the impulse stimulated release of 5-HT (Corrodi et. al. 1969, Schubert 1973).

The accumulation of 5-HT by synaptosomes from mouse brain was found to be unchanged following acute Li^+ pretreatment (Kuriyama and Speken 1970), and the accumulation of 5-HT by rat platelets was similarly unaffected by both acute and chronic Li^+ pretreatment. (Genefke 1972).

However, when incubated in a medium containing Li^+, 5-HT uptake into rat platelets and into platelets obtained from untreated human subjects was found to be inhibited (Genfke 1972). Murphy et. al. (1969), (1970), studied the uptake of 5-HT into platelets before, during and after Li^+ therapy in recurrent manic-depressive and depressive patients. They observed a 40 - 60% increase in 5-HT uptake during Li^+ therapy. In contrast, when Li^+ was added in vitro to platelets obtained from control subjects, there occured no change in 5-HT uptake. One report demonstrated no effect of Li^+ on the major catabolic enzyme MAO (Murphy and Weiss 1972).

The results of these studies are summarised in table 1 p. 26. It can be seen that it is difficult to relate the data obtained to any specific hypothesis. The most probable reasons for the varied and sometimes contradictory findings of previous studies are the vast differences in the doses of Li^+ administered, and the variation in the periods of time for which the animals were treated. It is always very difficult to relate the results of clinical studies with those conducted upon small laboratory animals. However, it would seem sensible to consider the therapeutic usage of the drug when designing animal experiments. The dose of Li^+ administered therapeutically is critical. Following an initial high dose of Li^+, patients suffering from recurrent affective disorders are usually maintained on a Li^+ dosage which produces a serum Li^+ concentration of between 0.8mEq/l and 1.3 mEq/l, although effective therapeutic responses are recorded with serum Li^+ concentrations maintained at 0.3 - 0.5 mEq/l (Schlagenhauf et. al. 1966). The oral dose of Li_2CO_3 required to maintain these serum levels ranges from 500 to 1800 mg/day. If the serum Li^+ concentration rises above 1.5 mEq/l, side effects invariably appear.

Table 1.

Summary of the reported effect of Li^+ on the synthesis, turnover storage, release and inactivation of 5-HT

Acute Li^+ treatment (0 - 7 days).

	Ref.	Dose (mEq/Kg/day)	Effect
Synthesis and turnover	(1)	1.2 - 4.7	Reduced
	(3)	3.3	Increased *
	(5)	4.0	Increased
	(8)	4.0	Increased
	(4)	2.5 - 7.5	Increased
	(6)	5.0 or 10.0	Increased
	(7)	Serum conc. 0.4 - 0.7 mEq/l	Increased
	(2)	In food 13.3	Reduced *
Storage		No reports.	
Release	(9)	2.5 - 7.5	Reduced
	(7)	Serum conc. 0.4 - 0.7 mEq/l	Reduced *
Uptake	(10)	3.75	Unchanged
	(11)	Serum conc.	Unchanged
MAO		No reports.	

Table 1 continued.

Chronic Li^+ treatment (10 days and over).

	Ref.	Dose. (mEq/Kg/day)	Effect.
Synthesis and turnover	(12)	2.0	Reduced
	(13)	Plasma conc.	Unchanged
	(6)	5.0 or 10.0	Unchanged
Storage		No reports	
Release	(14)	0.1	Reduced*
Uptake	(11)	Serum conc.	Unchanged
	(15)	Li_2CO_3 1.2 - 1.8g/day	Increased **
	(16)	Li_2CO_3 1.2 - 1.8g/day	Increased **
MAO	(17)	-	Unchanged **

Table 1.

Key:-

x Suppositions from indirect data.

xx Human platelets obtained during Li^+ therapy.

References:-

(1) Schildkraut et. al. (1969)

(2) Shaw et. al. (1972)

(3) Tagliamonte et. al. (1970)

(4) Sheard and Aghajanian (1970)

(5) Perez-Cruet et. al. (1971)

(6) Knapp and Mandell (1973)

(7) Schubert (1973)

(8) Poitou et. al. (1974)

(9) Katz et. al. (1968)

(10) Kuriyama and Speken (1970)

(11) Genefke (1972)

(12) Ho et. al. (1970)

(13) Bliss and Ailion (1970)

(14) Corrodi et. al. (1969)

(15) Murphy et. al. (1969)

(16) Murphy et. al. (1970)

(17) Murphy and Weiss (1972)

These include anorexia, nausea, vomiting, diarrhoea; and with high doses of Li^+ tremor, drowsiness and sometimes coma. The narrow limits between the effective and toxic doses demonstrates the extreme caution required in determining doses of Li^+ for experimental study. The normal oral maintainance dose in man is 500 - 1800 mg/day. Based on a 70 Kg. man this would be 7.1 - 25.7 mg/Kg per day of Li_2CO_3 or 0.02 - 0.7 mEq of Li^+/Kg/day. It can be seen from table 1 that the majority of studies have used doses of Li^+ vastly in excess of those used therapeutically in man. Although one can never directly relate findings in experimental animals to possible effects in man, it is more likely that experimental findings will be of some relevance if the dosage used is similar to that used in man. Consequently, it is difficult to determine whether the observed effects are relevant to the therapeutic or to the toxic effects of Li^+ seen in man. In addition, very few studies have monitored the concentration of Li^+ in either plasma or brain tissue.

The second problem in the interpretation of the results of previous studies is the relevance of the period of Li^+ dosage. In man, the therapeutic effect of Li^+ is invariably never seen until at least 5 - 7 days after treatment commenced. Therefore it is unlikely that the results of acute studies are of significant importance in identifying the mode of action of therapeutic Li^+. It would seem reasonable therefore to study the effects of chronic Li^+ treatment rather than those arising from short term administrations of the drug.

Finally, it can be seen from table 1 that very little work has been conducted upon the effect of Li^+ pretreatment on the release of 5-HT in brain tissue. The study of Katz et. al. (1968) was conducted in vitro using brain slices, and the suggestion from Corrodi et. al. (1969)

was based on indirect measurements. There do not appear to be any reports of studies conducted upon the release of 5-HT in vivo. Using the techniques developed as a result of my early investigations, and having identified the problems involved with designing the dosage regime, I entered into an investigation of the effect of Li^+ pretreatment on the release of 5-HT in the rat forebrain in vivo. The major Li^+ study investigated the effect of 10 day administration of Li^+ at a dose resembling that used therapeutically in man. In an attempt to clarify the differences between short and longer term Li^+ administration, I carried out a second study where rats received the same total amount of Li^+ administered over a 5 day period.

The final studies carried out in the course of this thesis were designed in order to attempt to clarify the interpretations of some of the findings observed during the major Li^+ study. Using synaptosomes isolated from the forebrains of rats treated and not treated with Li^+ I studied the accumulation and retention of ^{14}C-5-HT by these isolated nerve endings.

MATERIALS AND METHODS.

The experimental techniques have been developed, in the main, as a result of a series of investigations into the effect of varying the conditions of the experiment in a controlled manner. In some cases, considerable experience of particular methods were available in our research group. Where such a method appeared to be suitable for the proposed studies it was adopted.

(i) Estimation of 5-HT and 5-HIAA.

The routine estimation of the concentrations of 5-HT and 5-HIAA in a single brain sample had been carried out in this group previously using the method of Eccleston et. al. (1969c). Since the method appeared to be suitable for the purposes of measuring brain concentrations of 5-hydroxyindoles following electrical stimulation (Eccleston et. al. 1970), it was considered the method of choice in these studies. The method basically consists of the extraction of 5-HT and 5-HIAA from homogenised brain tissue, the separation of the two 5-hydroxyindoles by cation exchange chromatography, the further extraction of 5-HIAA from the effluent and the fluormetric determination of both 5-HT and 5-HIAA in a spectrofluorimeter.

In detail, the method adopted in these studies is as follows:-

Each forebrain was weighed and homogenised in four volumes of 0.4N perchloric acid (PCA) and centrifuged at 16,000 x g. for 15 minutes. 4.0 ml. of the supernatant was adjusted to pH 7.5 with KOH and the precipitated $KClO_4$ was removed by centrifugation. The extract was then passed over a 70 x 7 mm. column of Amberlite CG 50 resin (100 - 200 mesh NH_4^+ form) followed by a 2.0 ml. wash with 0.02M ammonium acetate buffer pH 7.5. The effluent containing 5-HIAA was collected. 5-HIAA was extracted from the effluent into diethyl ether, and was extracted from it into 3.0 ml. of 0.1M phosphate buffer pH 7.5.

2.0 ml. of this were removed for the fluorometric estimation of 5-HIAA. The columns were washed with 13.0 ml. of 0.02M ammonium acetate buffer pH 7.5, followed by 3.0 ml. of 0.1N H_2SO_4, and the 5-HT was eluted with 6.0 ml. of 1.0N H_2SO_4. 2.0 ml. of the elutant were removed for the fluorometric estimation of 5-HT.

1.0 ml. of concentrated HCl (Aristar) containing ascorbic acid (50 mg/100 ml.) was added to each 2.0 ml. sample of separated 5-hydroxyindole immediately before estimation in a Farrand Spectrofluorimeter. Emission at 550 nm. was recorded as a function of activation wavelength over the range 250 - 400 nm., and the emission corresponding to activation at 310 nm was read off (see fig. 4 p.31). Standard samples containing 300 ng. 5-HIAA, and 500 ng 5-HT in PCA were carried through the whole procedure with each batch of samples, and the percentage recovery determined for calculation of final proportions. The mean percentage recovery over all estimations carried out were for 5-HT - 61.9$\pm$ 2.2., and for 5-HIAA - 59.3$\pm$ 2.3.

(ii) Choice of animals.

There is some evidence that activity in hypothalamic neurones which contain 5-HT may play a role in the regulation of ovulation (Kamberi et. al. 1970, 1971).

Kamberi et. al. (1970) discovered that 5-HT injected into the third ventricle in doses of 2.5 - 5.0 μg suppressed the release of luteinising hormone (LH). When given by the stalk portal vessel or the basilar artery, at these concentrations, 5-HT had no effect on LH release. From this it was concluded that the effect may be nervously mediated in structures surrounding the third ventricle, probably the hypothalamus. Further work (Kamberi et. al. 1971) showed that 5-HT injected by the same route (intraventricularly) suppressed the release

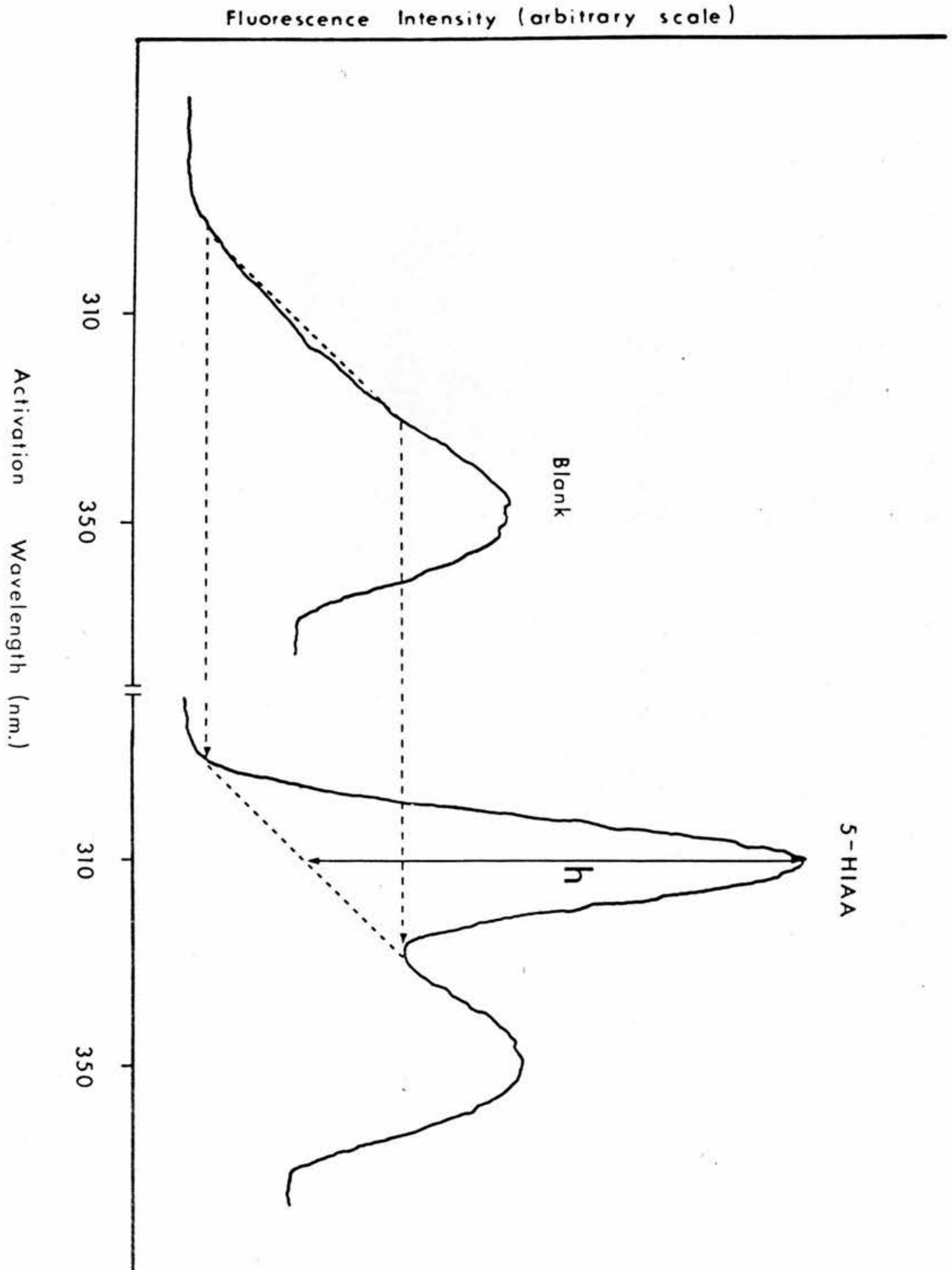
FIG. 4.

ACTIVATION SPECTRA OF A BLANK SAMPLE AND OF A SAMPLE OF 5-HIAA, TO DEMONSTRATE THE METHOD EXPLOYED IN MEASURING THE RELATIVE FLUORESCENCE OF 5-HYDROXYINDOLE SAMPLES.

The figure shows two spectra recorded over activation wave-lengths of 370 and 280 nm. The first spectrum shows the non specific fluorescence observed in a blank sample containing distilled water, HCl and ascorbic acid. The second spectrum shows the fluorescence observed in a sample containing 5-HIAA, distilled water, HCl and ascorbic acid. In this case, the non specific fluorescence, peaking at about 350 nm. is followed by the fluorescence of the 5-hydroxyindole which peaks at 310 nm.

The decline in the fluorescence of the blank sample between wave-lengths of 330 and 300 nm. can be seen to follow an approximate linear path. This decline can be fitted to the spectrum of a 5-hydroxyindole sample, as represented in the diagram by the spectrum of a sample of 5-HIAA. A perpendicular dropped from the apex of the 5-hydroxyindole fluorescence (310 nm.) to the projected decline of the blank fluorescence (h) provides the measure of the relative fluorescence arising from 5-hydroxyindole.

In extracts of brain tissue, the size of the blank has been seen to vary between samples. Because of this, the method employed in measuring the samples provides a more accurate measure of 5-hydroxyindole fluorescence since small changes in the amplitude of the blank fluorescence can be accommodated more accurately using this technique than by measuring the height of the fluorescence from a zero baseline and deducting from it the height of a single blank reading.


Fluorescence Intensity (arbitrary scale)
Blank
5-HIAA
h
310
350
310
350
Activation Wavelength (nm.)

of follicle stimulating hormone (FSH), and decreased the concentration of prolactin inhibitory factor (PIF). It was suggested from these studies that hypothalamic neurones which contain 5-HT may be involved in the control of the release of hypothalamic releasing factors which in turn control the release of the pituitary and ovarian hormones FSH and LH.

The hypothalamus contains possibly the highest concentration of nerve terminals which contain 5-HT and which originate from cell bodies in the raphe nuclei of the brainstem. Since these neurones may play a role in the control of ovulation, it was decided that the studies should be undertaken using male animals. The atlas on which the implantation of electrodes into the raphe was based, (Konig and Klippel 1963), was developed using rats weighing 150g. It was therefore decided to conduct the experiments on male Albino Wistar rats weighing between 150 - 250 g.

(iii) ANAESTHESIA.

Depression of synaptic transmission is probably the principal action of anaesthetic drugs (Wall 1967), and different anaesthetics have different effects or differing degrees of effect on nervous transmission in the CNS. It is thus important before investigating the pharmacology of central transmission to ascertain whether the proposed study can be carried out on unanaesthetised animals, and if not, to identify the anaesthetic agent which has least effect on the physiology of brain 5-HT. Electrical stimulation of the medial raphe nucleus has been carried out on unanaesthetised rats (Gumulka et. al. 1969), however, during such a procedure, it was necessary to place the animals in a "special container" to restrict movement of the head during stimulation (Kostowski et. al. 1969). These head movements and the

restraint of such movements (of the head during stimulation) are likely to be stressful to the animals, and since stress has been shown to affect 5-HT metabolism (Thierry et. al. 1968, Rosecrans and Sheard 1969, Bliss et. al. 1972), it was thought reasonable to investigate the possibility of using anaesthetised rats in experiments involving the electrical stimulation of the raphe, provided an anaesthetic with the properties designated above was available.

A good deal of work has been carried out on the effects of various anaesthetics on CNS activity both in general and with relation to specific putative transmitters. The anaesthetics in common use in animal studies are α-chloralose, urethane, the barbiturates, the volatile anaesthetics halothane, methoxyflurane and to a lesser extent ether. The general depressant effects of anaesthetics upon spontaneously active neurones in the CNS was well demonstrated by Bloom et. al. (1965). In the unanaesthetised decerebrate cat, continuous spontaneous activity was readily recordable in the caudate nucleus. Parenteral administration of anaesthetics was found to affect this activity markedly. During relatively light chloralose or barbiturate anaesthesia, the spontaneous activity of the caudate neurones was considerably less, and during deep chloralose anaesthesia, almost all spontaneous unit activity was absent.

Further investigations by Crawford (1970) demonstrated that anaesthetics administered to cerveau isole cats had essentially similar effects on both spontaneous synaptic firing of cortical neurones and on the responses to the microelectrophoretic application of acetylcholine and the excitant amino acids. Barbiturates depressed the chemical sensitivity of cortical neurones in doses well below those necessary for surgical anaesthesia. Subanaesthetic doses of urethane depressed

slightly the chemical sensitivity of the neurones, while full anaesthetic doses of -chloralose markedly reduced the chemical sensitivity of these cells for long periods.

Low to moderate concentrations of N_2O and trichloroethylene had little direct effect on the cortical neurones, while higher concentrations of N_2O induced hypoxia in the animals. Methoxyflurane at full anaesthetic doses had little effect on the sensitivity of cortical neurones. Halothane at concentrations of 0.5 - 1.0% in oxygen or air had little effect upon the spontaneous or chemically evoked firing levels despite a slow fall in systemic blood pressure. Concentrations of 1.5 - 2.0% accelerated the fall in blood pressure and reduced both the spontaneous and chemically evoked firing in these neurones.

It would thus appear from these studies that the spontaneous activity of cortical neurones is less affected by the inhalation anaesthetics than by anaesthetics administered by the intravenous route.

The use of the microiontophoretic technique made possible the investigation of the effects of anaesthetics upon the post synaptic sensitivity to 5-HT. Curtis et. al. (1961) found no effect of 5-HT when applied iontophoretically to spinal neurones of barbiturate anaesthetised cats. However, a number of spinal interneurones were found to be sensitive to 5-HT in unanaesthetised preparations (Weight and Salmoiraghi 1966, Engberg and Ryall 1966) suggesting that barbiturate anaesthesia may effect neuronal sensitivity to 5-HT. In addition, Curtis and Davis (1962) noted only depressant effects of 5-HT in the lateral geniculate nucleus of barbiturate anaesthetised cats, while Satinsky (1967) using unanaesthetised cats, observed an additional neuronal population within the lateral geniculate nucleus that is facilitated by 5-HT suggesting that barbiturate anaesthesia may alter

the proportion of neurones found sensitive to 5-HT. Roberts and Straughan (1967), investigating the responses of cortical neurones to the microiontophoretic application of 5-HT in unanaesthetised cats, found that small doses of sodium thiopentone (0.6 - 5.0 mg/Kg), administered parenterally, selectively and reversibly reduced the sensitivity of some units to excitation induced by 5-HT. In addition, they found that the responses of cortical neurones to iontophoretically applied 5-HT did not vary significantly between unanaesthetised preparations and cats anaesthetised with 40 mg/Kg α- chloralose or N_2O - halothane (0.5 - 1.0% halothane in 75% N_2O in O_2) (Johnson, Roberts and Straughan 1969). It would appear, therefore, that the neuronal sensitivity to iontophoretically applied 5-HT is susceptible to modification by anaesthetics, particularly the barbiturates.

In order to assess further the sites of action of the anaesthetics, Galíndo (1969) investigated the effects of procaine, pentobarbitone (2 - 30 mg/Kg) and halothane (1 - 2%) on pre- and post-synaptic structures in the cuneate nucleus of unanaesthetised decerebrate cats. The anaesthetics were applied parenterally (in the doses stated above), or microiontophoretically to neurones of the cuneate nucleus whilst recording simultaneously the activity of the same neurones. Synaptic transmission through the cuneate nucleus was investigated by delivering varying stimuli to peripheral nerves (movement of hair, weights applied to the skin, and movement of joints). The effect on the post-synaptic membrane was studied by observing the actions of the three anaesthetics on the firing of cuneate neurones being stimulated by the iontophoretic application of glutamate. The presynaptic element was studied by recording in the superficial radial nerve and stimulating antidromically the cuneate nucleus or the dorsal columns. The results of the study

indicated that procaine, pentobarbital and halothane all differed from one another in their site of action. Procaine was found to be equally effective in blocking antidromic, transynaptic, and direct post-synaptic stimulation of cuneate cells. Pentobarbital was more effective in blocking transynaptic and direct post-synaptic excitation, than in blocking antidromic invasion. Halothane depressed synaptic transmission through the cuneate nucleus but had no significant effects on either pre- or post-synaptic structures, suggesting that this may have been brought about by a facilitation of the inhibitory input to the system. The studies of Weakly (1969) failed to confirm the post-synaptic site of action of the barbiturates. He found that the actions of the barbiturates thiopentone and pentobarbital upon transmission in spinal motoneurones was confined to the presynaptic terminal where they reduced the amount of transmitter released by afferent impulses. It would thus appear that the barbiturate anaesthetics would be unsuitable for the study of 5-HT release.

Of the other anaesthetics that have undergone investigation, those least likely to have effects upon neural excitability are methoxyflurane, and low doses (0.5 - 1.0%) of halothane (Crawford 1970), while those having least effects upon the sensitivity of neurones to 5-HT are likely to be halothane and α-chloralose (Jonnson et. al. 1969). Since α chloralose anaesthesia has been found to have profound effects on spontaneously active neurones in the cortex (Crawford 1970), it was decided that the two inhalation anaesthetics methoxyflurane and halothane may be the most suitable for the intended studies. When used alone, induction of anaesthesia with methoxyflurane is very sluggish (Penthrane handbook, Abbot Laboratories), and for human surgery, the use of a short acting barbiturate during induction is

recommended. Experience in this department of the use of methoxyflurane as an anaesthetic for animal studies confirmed the sluggish nature of induction, and further demonstrated that changes in the depth of anaesthesia induced by changing the concentration of methoxyflurane administered are equally slow (C.M. Bradshaw personal communication). Since anaesthesia under halothane is easily controllable, and at low concentrations has little effect on spontaneous activity (Crawford 1970), or on the pre-synaptic and post-synaptic neural activity in the brain regions studied (Callindo 1969), it seemed reasonable to investigate the use of this anaesthetic for these studies.

Concentrations of halothane between 0.5 - 1.0% in oxygen or air had been demonstrated to have little effect on both the spontaneous and drug induced firing of cortical neurones, whereas concentrations of 1.5 - 2.0% depressed this activity (Crawford 1970). The depth of anaesthesia maintained by 1.0% halothane in oxygen has been found to be perfectly adequate for stimulation studies of the type intended (Eccleston et. al. 1970). It was therefore decided to investigate the effects of halothane at this concentration upon the forebrain concentrations of 5-HT and 5-HIAA. Two studies were conducted. The first study examined the effect of halothane anaesthesia (1.0% in oxygen) upon the concentration of 5-HT and 5-HIAA in the rat forebrain at various times of exposure to the anaesthetic. The second study examined the cumulative effects of anaesthesia and surgery upon forebrain 5-hydroxyindoles.

Study 1.

Single rats were placed in an induction chamber. Anaesthesia was rapidly induced by passing a high concentration (4.0% in oxygen)

of halothane through the chamber using a temperature compensated halothane vaporiser (the Fluotec 3). The rat was removed from the chamber, and anaesthesia maintained at 1.0 - 2.0% halothane in oxygen administered through a nose mask whilst the trachea was cannulated. Following tracheal intubation, the animal was transferred to a water bath at 37°C to maintain body temperature. (see section on body temperature). Anaesthesia was maintained at a halothane concentration of 1.0% in oxygen for 30, 45 or 90 minutes, after which time the animal was removed from the water bath and decapitated. The forebrain was removed by precollicular section, rapidly frozen on solid CO_2, weighed and stored at -20°C overnight for 5-hydroxyindole estimations to be carried out the next day.

At the same time that an anaesthetised animal was sacrificed, a control rat was also killed by cervical dislocation and the effect of anaesthesia was analysed by a paired comparison and t test (see statistical analyses). The forebrain concentrations of 5-HT and 5-HIAA were estimated by the method previously described.

The results of the study are shown in table 2 p.39. It can be seen that exposure to 1.0% halothane for 30minutes had no significant effect upon the forebrain concentrations of either 5-HT or 5-HIAA. The 5-HIAA concentrations were significantly elevated by 45 and 90 minutes exposure to halothane whilst the 5-HT concentration was only significantly elevated at the longer time of exposure. It thus appears that the effects of halothane anaesthesia upon forebrain concentrations of 5-HT and 5-HIAA are greater the longer the animal is exposed to the anaesthetic. It is also apparent that in experiments using halothane anaesthesia, the period of exposure must be kept constant. In these experiments it was intended that the raphe nucleus be stimulated

Table 2.

The effects of halothane anaesthesia (1% halothane in oxygen) upon the forebrain concentrations of 5-HT and 5-HIAA (ng/g wet wt.) recorded after 30, 45 or 90 minutes exposure to the anaesthetic. Results are expressed as the mean $\pm$ S.E.M. of four pairs of animals and analysed by the paired t test.

5-HT (ng/g wet weight).

Period of anaesthesia	Control.	Anaesth.	Anaesth. minus control.	P
30 min.	487$\pm$6	468$\pm$6	-19$\pm$9	N.S.
45 min.	457$\pm$29	502$\pm$19	+45$\pm$16	N.S.
90 min.	543$\pm$17	652$\pm$7	+109$\pm$18	0.01>P>0.001

5-HIAA (ng/g wet weight).

Period of anaesthesia	Control.	Anaesth.	Anaesth. minus control.	P
30 min.	276$\pm$8	293$\pm$4	+17$\pm$11	N.S.
45 min.	306$\pm$16	414$\pm$32	+108$\pm$21	0.02>P>0.01
90 min.	289$\pm$6	421$\pm$14	+132$\pm$18	0.01>P>0.001

for 30 minutes. Preliminary investigations demonstrated that the surgical procedures and the implantation of the electrode could be comfortably accomplished within 30 minutes. The total time that the animal requires to be anaesthetised is therefore 60 minutes. In order to examine the cumulative effects of surgery, electrode implantation and anaesthesia upon the forebrain concentrations of 5-HT and 5-HIAA, a second study was conducted.

Study 2.

In this study, in order to complete a reasonable number of animals per day, it was necessary to use two halothane vaporisers. This allowed animals to be maintained on 1.0% halothane whilst inducing other animals at higher halothane concentrations. Anaesthesia was maintained with 1.0% halothane in oxygen using a Fluotec 3 temperature compensated vaporiser, whilst induction was effected using an earlier model vaporiser (BOC No. 310400) without this refinement. Whilst the calibration of the instrument is reasonably accurate, changes in the temperature of the halothane would result in changes in the concentration of halothane delivered. For this reason, the temperature of the laboratory was maintained at a reasonably constant 20°C (the temperature at which the vaporiser was calibrated), and animals were transferred to the Fluotec 3 as quickly as possible.

Anaesthesia was induced as in the previous study. The trachea was cannulated and the animal transferred to the Fluotec 3 vaporiser, where anaesthesia was maintained with 1.0% fluothane in oxygen. The head of the animal was secured in a David Kopf No. 900 stereotaxic frame, and the cranium exposed. The fine tissue on the cranium was scraped off and any bleeding through the bone was arrested using plasticine. This was preferred to bone wax since the latter affected

the adhesion of the dental acrylic which was used to secure the electrode to the cranium. A hole was drilled according to the stereotaxic co-ordinates for the nucleus raphe medianus (see later), and this was plugged with plasticine. The electrode was then driven through the plasticine to enter the nucleus raphe medianus at the following coordinates. Lateral 0mm., anterio-posterior + 0.4 mm., vertical - 2.6 mm. (Konig and Klippel 1963). The electrode was secured to the skull using dental acrylic cement. When the electrode was securely held in position, the animal was removed from the stereotaxic frame and placed in a water bath at 37°C to maintain body temperature. After a total of 60 minutes anaesthesia, the animals were killed by decapitation and the forebrains removed, frozen, and stored for assay of 5-HT and 5-HIAA the following day. Control rats were treated as in the previous study and the results were analysed by a paired comparison.

It can be seen from table 3 p. 42, that the cumulative effect of 60 minutes exposure to halothane (1.0% in O_2) and surgery had no effect on the concentration of 5-HT in the forebrain, but significantly elevated the 5-HIAA concentration. The elevation of forebrain 5-HIAA may indicate either an increase in the production of 5-HIAA or a decrease in its clearance. The recent report of Bourgoin et. al. (1973) demonstrated that short term ether anaesthesia increased the turnover of 5-HT, and it is generally accepted that an increase in brain 5-HIAA with or without an increase in 5-HT may indicate an increase in turnover through the 5-HT pathway. Whether fluothane increases 5-HT turnover in a similar manner to that seen during ether anaesthesia cannot be ascertained from this study. Pentobarbital anaesthesia (35 mg/Kg) has been shown to reduce the accumulation of 5-HIAA by

Table 3.

The cumulative effect of fluothane anaesthesia (1% halothane in oxygen for 60 mins.) and surgery upon the forebrain concentration of 5-HT and 5-HIAA (ng/g wet weight). Results are expressed as the mean and S.E.M. of 10 pairs of animals and analysed by the paired t test.

Concentrations (ng/g wet weight).

	Control	Anaesthetised.	Anaesthetised minus control.	P
5-HT	488±24	527±34	+39±33	N.S.
5-HIAA	319±18	409±21	+90±26	P<0.01

isolated choroid plexuses (Cserr and Van Dyke 1971). A similar effect of fluothane anaesthesia would account for the elevation of 5-HIAA noted in these experiments. However, there is no direct evidence of this.

In an ideal experimental situation, it would be desirable that the anaesthetic should have no effect upon the system under investigation. Even though the results of the above studies demonstrated that fluothane anaesthesia affected 5-HIAA concentration, it was decided (with the knowledge of the problems associated with the use of other anaesthetics) that fluothane anaesthesia, though not ideal, would be suitable, provided the known effect was adequately controlled. All animals in the stimulation studies undergo the same surgery and are anaesthetised for the same period. They differ only in that half receive electrical stimulation, whilst the controls do not. Thus all animals will be affected to the same extent by anaesthesia and surgery, and the effects should therefore be controlled by the experimental design.

(iv) The control of body temperature.

Two factors may grossly alter the body temperature of the experimental rats. Firstly, the halothane anaesthesia will impair their ability to maintain their temperature, and secondly, experimental interference with 5-HT metabolism may affect centres in the preoptic area of the hypothalamus and possibly other forebrain sites as well (Simmonds 1970) which are involved in the control of body temperature (see Chase and Murphy 1973). It is therefore important that any studies involving measurement of forebrain 5-hydroxyindoles in fluothane anaesthetised rats should be carried out using adequate temperature control. Body temperature must be kept constant throughout the

experiment and the use of a thermostatically controlled water bath for this purpose was investigated.

The investigation was conducted upon 6 male Albino Wistar Rats. Each rat was anaesthetised as described previously and the trachea was cannulated. The rats were then placed in a water bath at 35°C and rectal temperature was recorded every ten minutes until three consecutive readings agreed. This was then recorded as the body temperature maintained by immersion in the water bath at 35°C. The procedure was repeated at water bath temperatures of 36, 37, 38 and 39°C. The results of the study are shown in table 4 p. 45. It can be seen that there is good correlation between water bath temperature and body temperature in the higher range of temperatures studied. However, body temperature never falls below 36.38°C, even when the animals are immersed in water at 35°C.

The small S.E.Ms recorded indicate that body temperature can be maintained at a reasonably constant level using this method, and at the higher temperature ranges studied, body temperature is maintained very near to the temperature of the water bath. It is therefore apparent that a thermostatically controlled water bath is very effective in maintaining body temperature at a constant level in halothane anaesthetised rats.

This method was employed in all studies in order to maintain the body temperature of anaesthetised animals at 37°C.

(v) Electrodes.

In previous studies, stimulation has been applied to the raphe nucleus through bipolar electrodes (Aghajanian et. al. 1967, Kostowski et. al. 1969, Gumulka et. al. 1969, Eccleston et. al. 1970, Sheard and

Table 4.

Body temperature of halothane anaesthetised rats attained by immersion of the animal in a water bath at temperatures of 35, 36, 37, 38 and 39°C. Results are expressed as the mean $\pm$ S.E.M. of 6 rats.

	Water bath temperature (^{o}C).				
	35.	36.	37.	38.	39.
Body temperature (^{o}C).	36.38 $\pm$0.15	36.64 $\pm$0.04	37.12 $\pm$0.04	38.06 $\pm$0.02	39.05 $\pm$0.02

Zolovic 1971). The advantage gained in the use of bipolar electrodes rather than unipolar electrodes is based on the restricted current spread between the two poles. This allows the activation of more discrete regions of brain tissue.

Two types of stainless steel bipolar stimulating electrodes were investigated, concentric bipolar electrodes, and a type of electrode referred to as the lateral bipolar electrodes. The concentric bipolar electrodes were constructed of an outer pole of stainless steel tubing with an external diameter of between 400 - 450 μ, with an inner pole of 150 μ Diamel coated stainless steel wire further insulated with a coat of Araldite PZ 985 varnish. The outside of the steel tubing was insulated with six coats of Araldite PZ 985 varnish. The tips of the electrodes were finely ground on a miniature grindstone to produce a pole separation of about 1.0 mm., with a tip diameter of about 50 μ (Fig. 5b). The lateral bipolar electrodes were constructed of two strands of Diamel coated stainless steel wire 150 μ in diameter, wound in a helical fashion using a hand drill. The electrodes were further insulated with six coats of Araldite PZ 985 varnish. The electrode tip was formed by obliquely cutting the wire to produce two poles separated by about 1.0 mm. (Fig. 5a).

The insulation of all electrodes was checked using an Avometer, and the resistance between the two poles when immersed in a solution of 0.15 M saline was measured. The resistance between the two poles was usually of the order of 30 - 50 K Ω. Electrodes with higher resistance were rejected.

A preliminary study investigated the suitability of the two types of electrode. Rats were anaesthetised and an electrode was stereotaxically positioned in the nucleus raphe medianus as described pre-

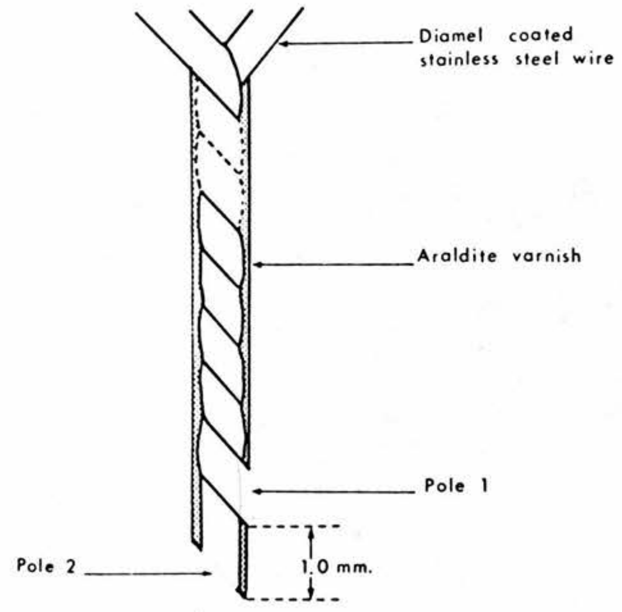
FIG. 5.

TYPES OF STIMULATING ELECTRODES EXAMINED IN THE PRELIMINARY STUDIES.

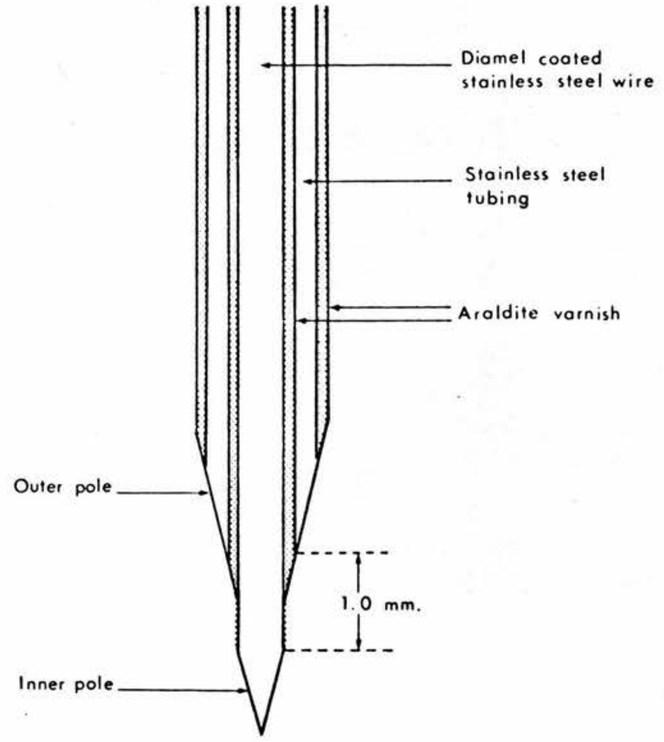
See text for detailed description of the two types of electrode.

TYPES OF ELECTRODE EXAMINED

(a) Lateral Bipolar



(b) Concentric Bipolar



viously. The animals were placed in a water bath at 37°C and stimulation was applied at a frequency of 10 Hz, a pulse width of 2.0 msec., and a voltage of 4 - 5 V (see next section) for 30 minutes. After stimulation, the animal was killed by decapitation and the forebrain was removed, rapidly frozen, weighed and stored at -20°C for the estimation of 5-HT and 5-HIAA to be carried out the following day. The midbrain was placed in 10% formol saline for histological examination of electrode placements (see histology). Control rats had electrodes implanted, but received no stimulation.

The results of the study are shown in table 5 p. 49, it can be seen that stimulation applied through the lateral bipolar electrodes had little effect upon the forebrain concentration of 5-HT and 5-HIAA, whereas stimulation at the same intensity applied through the concentric bipolar electrode produced a significant increase in the forebrain 5-HIAA concentrations without affecting the 5-HT level.

For these experiments, the stimulus current was not monitored through an oscilloscope, a technical refinement used in later studies. Stimulus intensity was therefore calculated from the known resistance of the electrode in saline and the voltage applied. Thus with an electrode of 40 KΩ resistance and a voltage of 4 V, the stimulus current should be of the order of 100 μA. This intensity of stimulation applied to the raphe was sufficient to induce considerable whisker twitching, and occasionally some movement of the eyelids and ears. It was noted during the study that in three of the six animals receiving stimulation applied through the lateral bipolar electrodes, no motor activity was visible. Examination of the electrode following removal from the brain demonstrated that some of the plasticine used to plug the hole in the skull had adhered to the tip of the electrode, and that this

Table 5.

The effect of electrical stimulation of the nucleus raphe (10 Hz, 2 msec. pulse width, 4 - 5V for 30 mins.) upon the forebrain concentration of 5-HT and 5-HIAA (ng/g wet wt.). 3a shows the effect of stimulation applied through lateral bipolar electrodes, and 3b the effect of stimulation applied through concentric bipolar electrodes. Results are expressed as the mean ± S.E.M. of six pairs and analysed by a paired t test.

3a Lateral bipolar electrodes.

	Control.	Stimulation.	Stimulation minus control.	P
5-HT (ng/g)	555±21	595±41	+40±37	N.S.
5-HIAA (ng/g)	267±12	276±39	+9±27	N.S.

3b Concentric bipolar electrodes.

	Control.	Stimulation.	Stimulation minus control.	P
5-HT (ng/g)	479±94	402±62	-77±118	N.S.
5-HIAA (ng/g)	366±15	521±18	+155±12	$P < 0.001$

had increased the resistance between the two poles considerably. Thus the current applied to the raphe in these animals was considerably less than calculated which may well have been responsible for the lack of motor activity and the small change in 5-HIAA concentration. The finer points of the concentric bipolar electrodes allowed these to pass through the plasticine with little effect on the resistance between the two poles.

In addition to the above problems, the lateral bipolar electrodes were more flexible than the quite rigid concentric electrodes. This resulted occasionally in slight bending when being manipulated in the stereotaxic frame. This, it was anticipated, could lead to unreliable implantations into the raphe region. The concentric bipolar electrodes could be effectively re-used by carefully removing the dental acrylic cement used to fix this to the skull. This procedure proved impossible with the more flexible lateral bipolar electrodes.

The preliminary study therefore demonstrated that concentric bipolar steel electrodes gave greater and more reliable elevations of 5-HIAA concentrations than the lateral bipolar electrodes, and it was decided that the former should be used in all studies involving the stimulation of the raphe nucleus.

(vi) Stimulation Parameters.

The studies of Kostowski et. al. (1969) demonstrated that electrical pulses applied to the nucleus raphe medianus at a rate of 10/sec produced greater elevations of forebrain 5-HIAA concentration than did stimulation at 2 or 60 pulses/sec. Currents of over 200 µA passed through nervous tissue can produce considerable damage to the cells surrounding the tip of the electrode. It was therefore necessary to keep the stimulus intensity below this level. In

early studies, stimulus current was not monitored through an oscilloscope, but was calculated from the known resistance of the electrode in saline, and the voltage applied. Thus by applying Ohms law, an electrode with a resistance of 40 KΩ carrying 4V would give a current of 100 μA. In these early studies therefore, the stimulus intensity was maintained at about 100 μA by calculation. In later studies, the stimulus intensity was maintained by 100 μA by measuring the voltage drop across a 40 KΩ resistor in the electrode circuit, and monitored through a Solartron CD 1400 oscilloscope.

The stimulation parameters used throughout the study were pulses of 2.0 msec duration, applied at a rate of 10/sec and at an intensity of 100 μA.

(vii) Statistical Analysis.

The concentration of 5-HT in rat brain is known to follow a circadian rhythm (Quay 1968, Okada 1971, Hery et. al. 1972). It is therefore probable that there would be considerable variation in the forebrain concentration of 5-HT measured at various times during the day. In order to assess this variation, I measured the forebrain concentration of both 5-HT and 5-HIAA at various times throughout the day. Rats used in all studies conducted in this thesis were maintained in a constant cycle of 12 hours light followed by 12 hours darkness. The period of light was from 0800 hrs. - 2000 hrs. and experiments were usually conducted between 0900 hrs. and 1700 hrs. This lighting schedule was identical to that maintained in the breeding rooms of our animal house, and by the suppliers of the rats used when our breeding programme was unable to maintain adequate supplies. The same strain of rats was used in our breeding rooms and by the suppliers.

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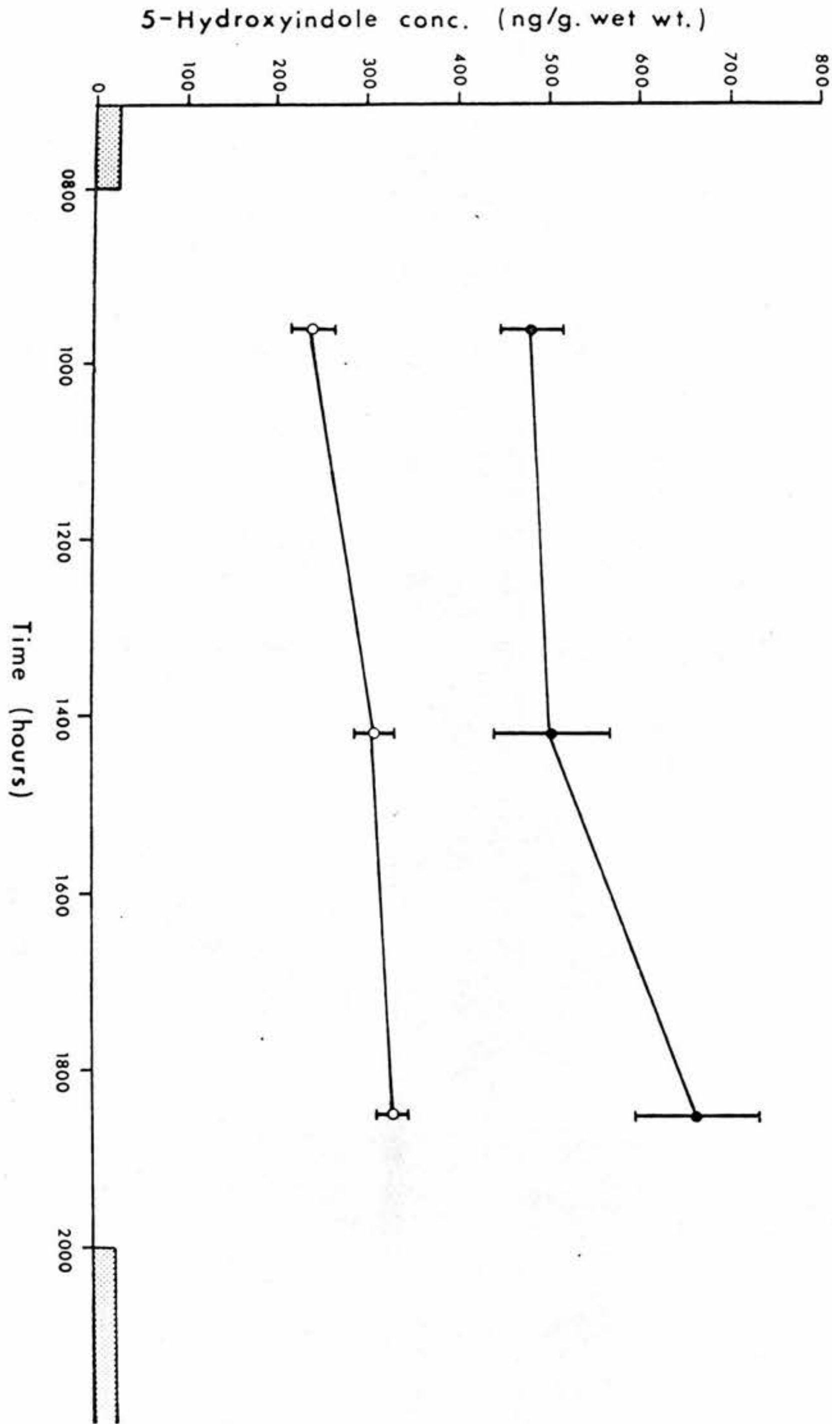
Using rats maintained on the above lighting schedule, the forebrain concentrations of 5-HT and 5-HIAA were measured in three groups of 6 rats killed at 0940, 1420 and 1830 hrs. These times were chosen to cover the time period during which it was anticipated that experiments would be conducted. The result of the study is shown in Fig. 6 p.53. It can be seen that the concentration of both 5-hydroxyindoles varied with the time of day. The 5-HT concentration appears to be higher at 1830 hrs. than at the other two times studied, and the concentration of 5-HIAA measured at 0940 hrs. is much lower than that measured later in the day. Comparison of this data with that obtained by previous workers is difficult since no measurements were made during the periods of darkness. However, previous work has shown that the concentration of 5-HT in most brain regions reaches a peak during the latter half of the period of 12 hours light, and falls to a minimum level during the early half of the dark period. The study conducted here shows that the level of both 5-hydroxyindoles was higher at 1830 hrs. than at the earlier times studied, suggesting a trend towards the circadian rhythms observed in Quay (1968) and Okada (1971).

This study, though conducted on a few animals only, confirmed that within day changes in forebrain 5-hydroxyindoles could be a considerable scource of variation in future studies. It was considered that this variation could be most effectively reduced by using a paired experimental design, and by applying the paired t test to differences between treatments. In order to ensure that all animals were anaesthetised for a constant period of time, it was impossible to run stimulated and control animals together, so there was always a short time lag between sacrifice of pairs of animals. However, control animals were prepared as soon as a stimulated animal was set up, and

FIG. 6.

THE CONCENTRATION OF 5-HT AND 5-HIAA IN THE RAT FOREBRAIN MEASURED AT THREE TIMES DURING THE 12 HOUR PERIOD OF LIGHT.

Filled circles show the 5-HT concentration (ng/g wet weight), and the open circles show the 5-HIAA concentration (ng/g wet weight). Each point represents the mean of 6 animals, and the vertical bars represent the S.E.M.'s. The shaded areas on the x axis represent the end and beginning of the 12 hour period of darkness.


5-Hydroxyindole conc. (ng/g. wet wt.)
0
100
200
300
400
500
600
700
800
Time (hours)
0800
1000
1200
1400
1600
1800
2000

the effect of stimulation was analysed by a paired comparison between treatments. The time lag between sacrifice of two animals in such an experimental design was of the order of 30 minutes. When the effect of a drug upon stimulus-induced changes in forebrain 5-HT and 5-HIAA was investigated, the experiment was designed so that pairs (stimulated and control) of animals receiving and not receiving the drug were set up alternately. The effect of the drug upon stimulus induced changes was similarly analysed by a paired comparison applied to the difference between stimulated and unstimulated animals from drug treated and saline treated groups (see Fig. 7 p.55). The maximum time between sacrifice of animals treated and not treated with the drug was 60 minutes.

Histological examination of electrode tracts was carried out in all animals receiving stimulation. Whenever an electrode was observed to be outside the raphe, that result and its pair were rejected. They were however used to ascertain the effects of stimulation of non raphe regions of the midbrain upon forebrain 5-HT and 5-HIAA concentrations.

Although no studies were conducted with the specific aim of assessing day to day variations in forebrain 5-hydroxyindole concentration, data obtained from animals studied in experiment III (c) (The effect of chlorimipramine on L-tryptophan induced changes in forebrain 5-hydroxyindoles. p.). can be utilised to demonstrate this problem. During the course of experiment III(b), one group of three animals was killed at approximately the same time on four different days. All the animals received a similar injection of saline one hour before death. The forebrain concentrations of 5-HT and 5-HIAA measured on the four days is shown in table 6a p.56. It can be seen that the concentration of

FIG.7

BASIC EXPERIMENTAL DESIGN EMPLOYED IN THE STIMULATION EXPERIMENTS

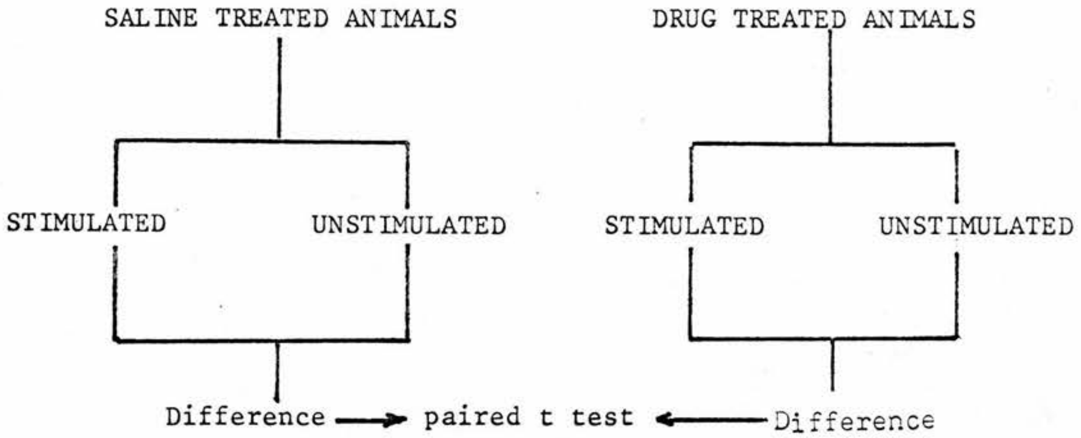


Table 6.

(a) The concentration of 5-HT and 5-HIAA in the rat forebrain measured at approximately the same time (1500 - 1530 hrs.), but on four different days. Results are expressed as the mean ± S.E.M. of three animals.

Date.	5-HT concentration (ng/g wet weight).	5-HIAA concentration (ng/g wet weight).
25/10/72	585±36	305±15
2/11/72	324±47	234±11
3/11/72	648±51	300±4
9/11/72	548±38	244±36

(b) The analysis of variance applied to the data of table 6(a). The variance ratio refers to the ratio of the mean square deviation arising between animals or days to the residual mean square deviation.

(i) 5-HT.

Origin of variance.	Sums of squares of deviation from the mean.	Degrees of freedom.	Mean square deviation.	Variance ratio.	P
Total	225170	11	-	-	-
Between animals.	12421	3	4140	0.72	N.S.
Between days.	178394	2	42815	7.47	<0.05
Residual.	34355	6	5726	-	-

(ii) 5-HIAA.

Origin of variance.	Sums of squares of deviation from the mean.	Degrees of freedom.	Mean square deviation.	Variance ratio.	P
Total	22441	11	-	-	-
Between animals.	4345	3	1448	1.53	N.S.
Between days.	12411	2	6206	6.55	<0.05
Residual.	5685	6	948	-	-

both 5-hydroxyindoles showed considerable day to day variation.

In order to assess this variation more quantitatively an analysis of variance was conducted on the data of table 6a p.56. The result of this analysis is shown in table 6b p.56. It can be seen that the between animal variation in 5-hydroxyindole concentration is not significantly different from the residual variance. However, the between day variance is significantly greater than the residual variance both for the brain concentration of 5-HT and 5-HIAA ($0.05 > P > 0.02$). It is unlikely that the day to day variation in 5-HT and 5-HIAA concentrations arose as a result of variations in the extraction and estimation of the 5-hydroxyindoles since this was always controlled by the use of both internal and external standards. What is certain however, is that the day to day variation in forebrain 5-hydroxyindole concentrations could be a considerable scource of error unless compensated for in the experimental design.

It was therefore considered that the use of a paired experimental design, as outlined above, would be the most effective way in which both the within day and day to day variations in forebrain 5-hydroxyindole concentrations could be reduced.

(viii) Histology.

After stimulation, the forebrain was separated from the midbrain by precollicular section. The former was frozen and stored for 5-hydroxyindole estimations, while the latter was fixed in a solution of 10% formol saline in preparation for the histological examination of electrode tracts.

After about 3 weeks fixation in 10% formol saline, the tissue was removed and mounted in gelatine on a freezing microtome. The gelatine and tissue were frozen by passing a stream of CO_2 through a

chamber surrounding the tissue chuck of the microtome. 40 μ transverse sections were cut and mounted on gelatine coated slides. These were then air dried and placed over formaldehyde vapour in a desiccator for 24 hours.

The sections were stained by the Thionin blue method for Nissl substance as follows :-

The sections were placed in 1.0% acid alcohol (250 ml methanol + 5 drops conc. HCl) for 5 minutes, followed by a thorough rinse in distilled water. The sections were then stained in 0.02% Thionin solution for 30 minutes at 50°C. After staining, the sections were washed in distilled water and 70% ethanol before being differentiated in a solution containing 5% aniline in absolute alcohol. The sections were then washed in a methanol/xylol mixture (66%/33% by volume) and further differentiated in a solution containing 15 parts aniline, 5 parts cajeput oil and 80 parts 70% ethanol. Sections were then washed in 95% ethanol and xylol/methanol mixture (66%/33% by volume) before being dehydrated in absolute ethanol. The sections were then cleared in xylol and mounted in Depex.

In sections cut and stained in this manner, the nucleus raphe medianus is clearly visible as a well defined group of cells occupying a position in the midline of the section. The electrode track is visible as a small lesion (Fig. 8 p.59). The position of the electrode in relation to the nucleus raphe medianus can be clearly ascertained by examination of the midbrain sections under a low power microscope.

(ix) Summary of the techniques used in the stimulation study.

As a result of the controlled investigations outlined in sections i - viii, the following technique was adopted in all studies involving

FIG 8.

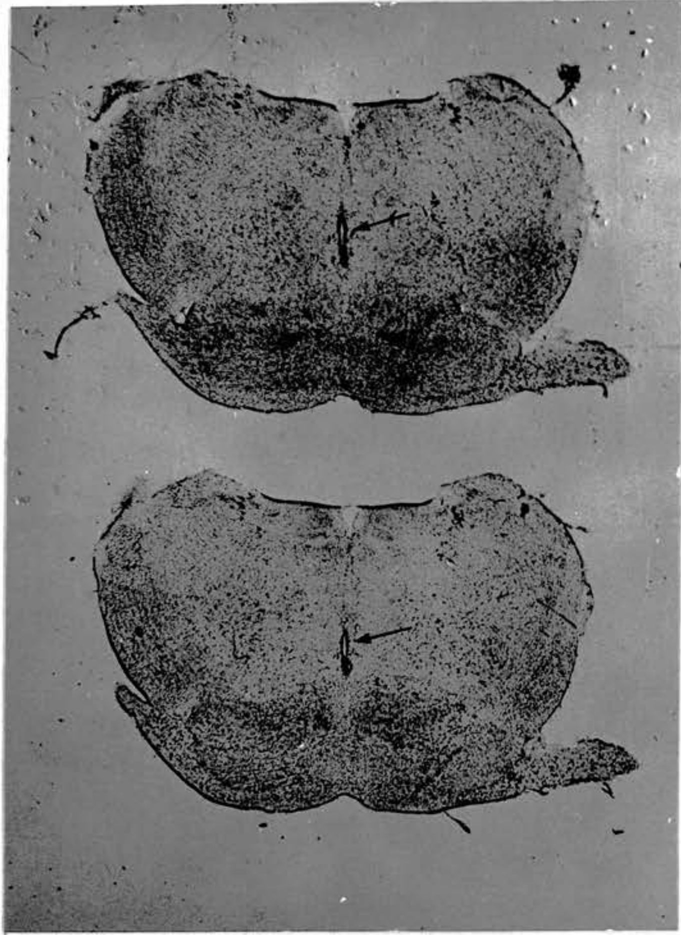
HISTOLOGICAL LOCATION OF ELECTRODE PLACEMENT.

The figure shows two photographs of sections through the midbrain of rats. The position of the tips of the stimulating electrode is visible as a small lesion (arrowed).

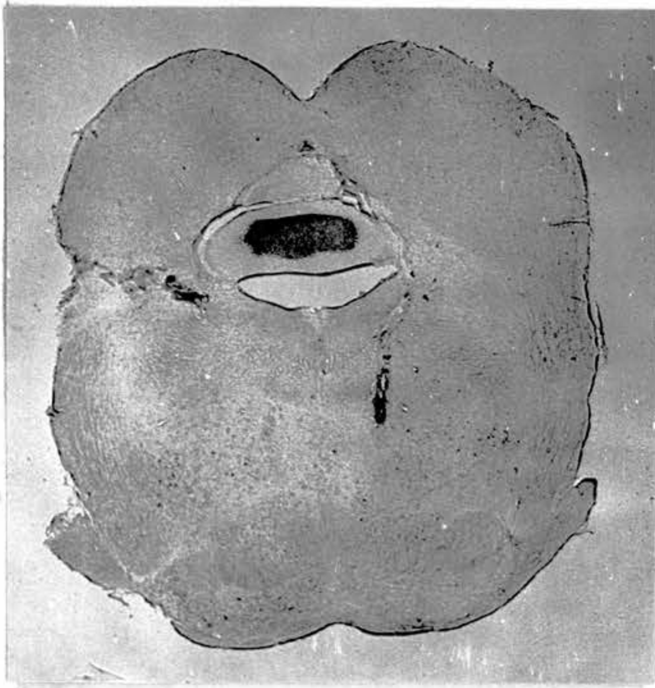
Photograph (a) shows two sections from the same animal in which the tip of the stimulating electrode is correctly positioned in the midline raphe nucleus.

Photograph (b) shows a section from an animal in which the electrode tip was misplaced dorsolaterally.

(a)



(b)



stimulation of the nucleus raphe medianus.

Animals were anaesthetised with 1.0% halothane in oxygen and a cannula was implanted into the trachea. A stainless steel bipolar electrode (500 μ diameter, tip separation 1 mm.) was stereotaxically positioned in the nucleus raphe medianus according to the following co-ordinates, lateral 0, anterio-posterior + 0.4 mm., vertical -2.6 mm. (Konig and Klippel 1963) using a David Kopf No 900 stereotaxic frame. Following electrode implantation, body temperature was maintained at 37°C by immersing the animal in a water bath. Half of the animals in each group were stimulated by monopolar pulses of 2 msec duration, 10 Hz, 4 - 5 V, producing a stimulation current of about 100 μA delivered from a Grass SD 5 stimulator. The other half of the group remained as unstimulated controls. Stimulation was applied for 30 minutes. At the end of this period, all animals were killed by decapitation and each forebrain was removed by precollicular section, rapidly frozen, then weighed. Samples were stored overnight at -20°C and 5-HT and 5-HIAA estimations carried out the following day.

The histological location of electrode placements was identified in all animals receiving stimulation. Whenever an electrode was observed to be outside the nucleus raphe, that result and its pair were rejected.

(x) Tryptophan.

L-tryptophan was obtained from British Drug Houses Ltd.

(a) Method of administration.

Being a very insoluble amino acid, L-tryptophan was administered as a 10 mg/ml suspension in saline containing 0.1 ml/20 ml polyoxyethylene sorbitan mono oleate (Tween 80) as an emulsifying agent. Control animals were given saline containing the same con-

centration of Tween 80.

(b) Basic experimental design.

In these studies the animals were not anaesthetised. Following administration of L-tryptophan by intraperitoneal injection, the animals were maintained for the relevant period of time in a quiet environment before being sacrificed by cervical dislocation. The forebrain was removed by precollicular section, frozen on solid CO_2, weighed and stored at -20°C for estimation of 5-HT and 5-HIAA as described previously. Pairs of animals receiving and not receiving L-tryptophan were sacrificed at the same time and differences in brain concentrations of 5-HT and 5-HIAA analysed by a paired comparison of the differences between treatments.

(c) Measurement of tryptophan in brain and plasma.

(i) Extraction of tryptophan from brain tissue :-

Rats were sacrificed by cervical dislocation and decapitation. Blood was collected from the neck into glass tubes containing 500 I.U. sodium heparin. The brain was rapidly excised and the forebrain was removed by precollicular section and weighed.

Tryptophan was extracted from the forebrain by a modified method of Ashcroft et. al. (1965). The tissue was homogenised in a 1.0 volume of 40% v/v acetic acid using an all glass homogeniser. A further 1.0 volume of 20% v/v acetic acid was added to the homogenate, and the tissue was re-homogenised. The homogenate was then diluted 1:10 with distilled water. 1.0 ml of 30% w/v trichloracetic acid (TCA) was added to 5.0 ml samples of diluted homogenate, and the precipitated proteins removed by centrifugation at 2,500 x g. 5.0 ml of supernatant were removed for the fluorometric determination of tryptophan (see later).

(ii) Extraction of tryptophan from plasma :-

McMenamy and co-workers investigated the distribution of tryptophan in human plasma and found that normally about 80% of the L- tryptophan is not dialysable but is bound to serum albumen in a specific manner (McMenamy and Oncley 1958). The proportion of dialysable tryptophan in plasma can be reliably estimated in the plasma ultrafiltrate (McMenamy et. al. 1961). Since that proportion of plasma tryptophan available for 5-HT synthesis in the brain must be in the free form, the concentration of tryptophan in both whole plasma and ultrafiltrate was measured.

Blood samples were treated as described by Moir (1971). Blood was centrifuged at 2,500 x g. for 8 minutes. 0.1 ml. of plasma was diluted 1:50 with distilled water and the proteins were precipitated by the addition of 1.0 ml of 30% w/v TCA. Precipitated proteins were removed by centrifugation at 2,500 x g. for 7 minutes, and 3.0 ml. of supernatant was removed for tryptophan determination.

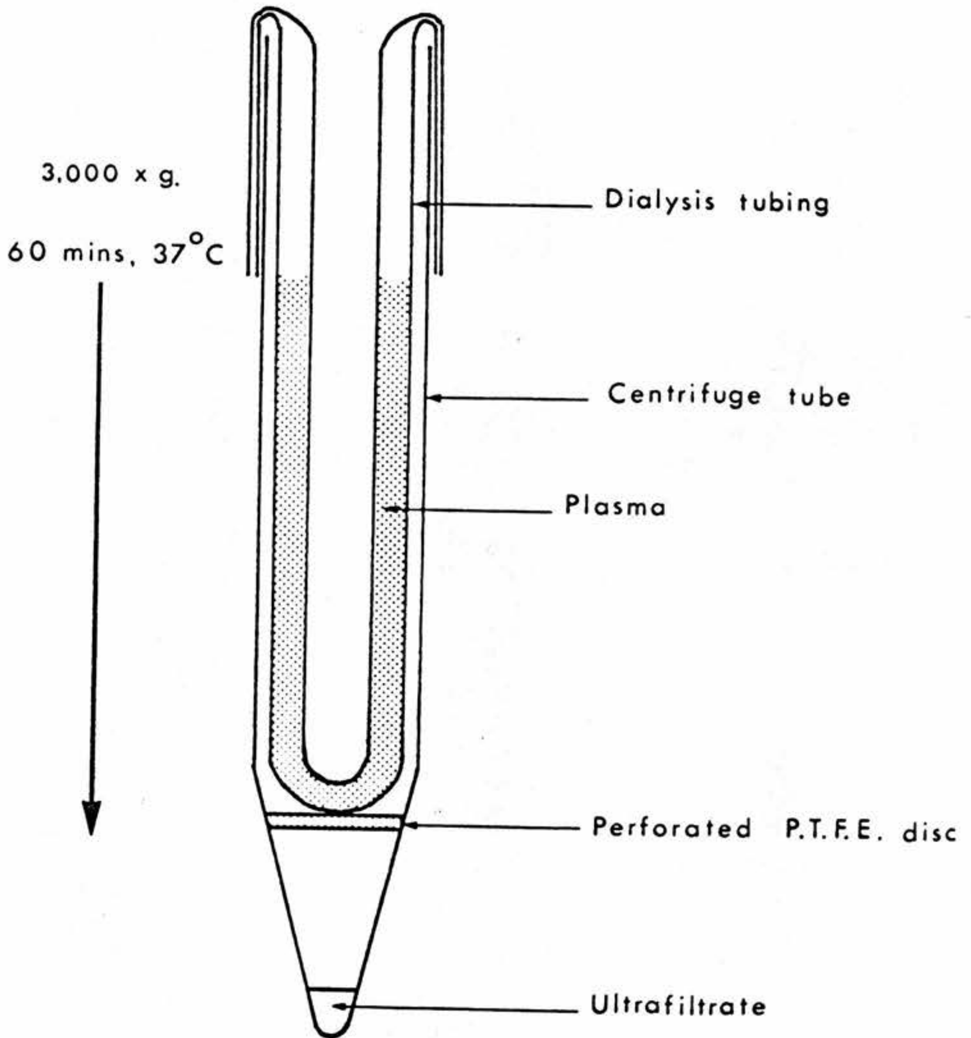
The plasma ultrafiltrate was prepared according to the method of McMenamy et. al. (1961) as modified by Moir (1971). Nine inch lengths of Visking dialysis tubing 8/32 (Scientific Instrument Centre Ltd.) were soaked in distilled water. A glass rod inserted along the whole length to ensure patency and the tubing was dried. A perforated PTFE disc was placed in a centrifuge tube to fit just above the taper (See Fig. 9 p.63). The dry dialysis tubing was bent into a U shape and placed in the tube with the bend of the U resting on the PTFE disc. 1.0 ml of plasma was placed in the tubing, and the ends of which were then secured to the outside of the tube with adhesive tape. The top of the tubes were covered with sealing tissue. The tubes were centrifuged at 3,000 g. for 60 minutes at 37°C, after which

FIG. 9.

DIAGRAM TO SHOW THE BASIC METHOD EMPLOYED IN THE PREPARATION OF THE PLASMA ULTRAFILTRATE.

See text for details.

METHOD USED IN THE PREPARATION OF THE PLASMA ULTRAFILTRATE



time the clear ultrafiltrate was visible in the tip of the centrifuge tube. 0.1 ml. of ultrafiltrate was diluted 1:40 with distilled water and 3.0 mls were removed for tryptophan determination.

(iii) Fluorometric estimation of tryptophan in brain and plasma extracts:-

The concentration of L-tryptophan in brain, and plasma extracts was measured by the fluorometric method of Hess and Udenfriend (1959).

Standard solutions against which the L-tryptophan concentrations were estimated were prepared by adding known concentrations of L-tryptophan to brain homogenates, plasma and plasma ultrafiltrates. These were then carried through the assay procedure with each batch of samples. The fluorescence due to tryptophan was calculated by deducting the fluorescence of the blanks (no tryptophan added) from that obtained in the paired samples containing added L-tryptophan. The tryptophan concentrations of the standards used were for brain 1.0 n.mol/ml. diluted homogenate, for plasma 60 n.mol/ml. undiluted plasma, and for the ultrafiltrate 500 p.mol/ml. undiluted ultrafiltrate. The percentage recovery from the extraction procedures was calculated by comparing the fluorescence of L-tryptophan added to the tissue samples with that obtained from external standards containing equivalent concentrations of L-tryptophan in distilled water. The tryptophan concentration of the tissues was then corrected according to the % recovery.

The mean percentage recoveries over all estimations carried out were for brain - 75.6 ± 2.3, plasma - 76.3 ± 6.4, and for ultra filtrate - 84.9 ± 8.1.

The 3.0 ml. samples of the final extracts and standards were added to 0.1 ml. of 20% v/v formaldehyde solution and placed in a boiling water bath for 20 minutes. The tubes were then removed from

the water bath and 0.1 ml. of 5% aqueous H_2O_2 added. The samples were replaced in the boiling water bath for a further 20 minutes after which time they were removed and allowed to cool to room temperature. The fluurescence intensity of the samples was measured at 365 nm. (excitation) and 440 nm. (emission).

(xi) Drugs.

(a) Chlorimipramine:-

Chlorimipramine (Geigy) was prepared as a 5 mg/ml solution in saline. The dosage used throughout the study was 5 mg/Kg.

(b) Lithium:-

The lithium salt used in all studies was LiCl. Isotonic (0.15M) LiCl was administered by the intraperitoneal route in doses of 1.5 mEq/Kg/day for 5 days, or 0.75 mEq/Kg/day for 10 days.

(xii) Estimation of Lithium concentrations in brain and plasma.

The concentration of Li^+ in brain and plasma following the i.p. administration of 0.15M LiCl in doses of 1.5 mEq/Kg/day for 5 days or 0.75 mEq/Kg/day for 10 days was estimated according to the method of Schou (1958).

Animals pretreated with LiCl as described above were killed by cervical dislocation and decapitation. Blood samples were collected from the neck as described previously and the brain was removed. Forebrains were removed by precollicular section, weighed and placed in centrifuge tubes. An equal volume of concentrated HNO_3 was added, and the tubes were placed in a boiling water bath for 60 minutes. The tubes were then removed from the water bath and allowed to cool to room temperature, whereupon the contents of the tubes were thoroughly mixed with 3 volumes of 10% v/v isopropanol in distilled water. The tubes were centrifuged at 1,500 x g. and the supernatant removed for estimation.

Blood samples were centrifuged at 1,500 x g. for 15 minutes. The plasma was removed, diluted 1:2 with 10% v/v isopropanol, and recentrifuged at 1,500 g. for 30 minutes. The supernatant was removed for estimation.

Lithium concentrations were measured by flame emission spectrophotometry using an EEL 240 Mark II Atomic Absorption Spectrophotometer. Emission was recorded at a wavelength of 670.5 nm using a 10.0 flame and slit width no. 4. Standard Li^+ samples were made up in mock plasma or brain extracts. The ionic composition of the solutions was for brain, Na^+50 mEq/l, K^+100 mEq/l, Ca^{2+}5 mEq/l, and Mg^{2+}8 mEq/l; and for plasma Na^+152 mEq/l, K^+5 mEq/l, Ca^{2+}5 mEq/l, and Mg^{2+}3 mEq/l. The mock solutions were diluted with distilled water according to the dilutions of the brain and plasma extracts, and standard Li^+ added to produce final concentrations of 0.02 - 0.10 mEq/l. The Li^+ standard was prepared by dissolving dried Li_2CO_3 in 1N HCl and diluted to volume with distilled water (Little et. al. 1968). The Li^+ concentration in each sample of brain and plasma was estimated by comparing the percentage luminance of the samples with the prepared standard curves.

In order to evaluate the effect of other cations on the emission of Li^+ at 670.5 nm, two studies were undertaken. The first study investigated the effect of cations in general on the luminance recorded from standard Li^+ solutions at the parameters outlined previously. Standard Li^+ solutions containing 0.2 - 0.8 mEq/l were made up in distilled water, mock plasma extract, and mock brain extract. The luminance recorded for each concentration of Li^+ in the three fluids is presented in table 7 p.67. It can be seen from the table that the major physiologic cations Na^+, K^+, Ca^{2+}, and Mg^{2+} in the concentrations likely to be present in the brain and plasma extracts had little effect upon luminance recorded from Li^+ standards made up in glass-distilled water.

Amdisen (1971) pointed out that the background emission from Na^+

Table 7.

The luminance recorded for standard Li^{+} solutions made up in glass distilled water, mock brain extract (Na^{+} 50 mEq/l, K^{+} 100 mEq/l, Ca^{2} 5 mEq/l, Mg^{2+} 8 mEq/l, and mock plasma extract (Na^{+} 152 mEq/l, K^{+} 5 mEq/l, Ca^{2+} 5 mEq/l, Mg^{2+} 3 mEq/l). The results are expressed as the mean $\pm$ S.E.M. of 3 estimations.

Li^{+} concentration (mEq/l)	Luminance (arbitrary units)		
	Dist H_2O	Mock brain extract	Mock plasma extract
0.02	11.3$\pm$0.3	12.1$\pm$0.1	11.7$\pm$0.2
0.04	22.4$\pm$0.5	22.1$\pm$0.5	22.7$\pm$0.3
0.06	32.7$\pm$0.8	33.1$\pm$0.6	33.6$\pm$0.6
0.08	43.8$\pm$1.0	44.1$\pm$0.8	44.1$\pm$0.7

and K^+ are of very minor importance when recording Li^+ emission at 671 nm. However, it was pointed out that unless very small slit widths are used, the background emission for Ca^{2+} can be considerable, and could lead to erroneously high Li^+ estimations. In order to evaluate the interference from Ca^{2+} more directly, the effect of adding high concentrations of Ca^{2+} to standard Li^+ solutions made up in distilled water was investigated. The emission recorded from standard Li^+ solutions (0.1 mEq/l) at the parameters outlined previously was compared with that recorded for Li^+ solutions containing 118.5 or 237 mEq Ca^{2+}/l. The result of the study is shown in table 8 p.69. It can be seen that the addition of Ca^{2+} to Li^+ standards had no significant effect on the emission recorded at the Li^+ wavelength (670.5 nm). Only at the higher Ca^{2+} concentration (237 mEq/l) was the emission elevated, and that not significantly.

It was concluded from the two studies, that the interference from the major physiologic cations Ca^{2+}, Mg^{2+}, K^+, and Na^+ in the estimation of Li^+ by flame emission spectrophotometry using the techniques outlined previously was insignificant. The effect of the major interfering cation Ca^{2+} was only just evident at concentrations vastly in excess of that expected in brain or plasma. Thus it can be assumed that the technique used in these studies for the estimation of Li^+ was perfectly valid and produced accurate measurements of the cation in the tissues studied.

(xiii) Studies using radioactive 5-HT.

The last part of the results section describes some preliminary studies into the effect of Li^+ on the uptake and retention of ^{14}C-5HT by a crude preparation of isolated nerve endings (synaptosomes). The techniques employed in the study are discussed below.

Table 8.

The effect of Ca^{2+} on Li^{+} emission recorded at 670.5 nm., using a 10 cm. flame and no. 4 slit width. Emission is expressed as the percent luminance recorded with or without added Ca^{2+}. Results are expressed as the mean ± S.E.M. of four pairs and analysed by the paired t test.

Concentration of Ca^{2+} added	Luminance (arbitrary units)				
	Li^{+} (0.1 m/Eq/1) alone.	Li^{+} plus added Ca^{2+}	Difference	t	P
118.5 mEq/1	60.08±1.96	60.15±1.33	0.07±0.15	0.49	N.S.
237.0 mEq/1	59.90±0.94	57.23±1.03	2.67±1.11	2.41	N.S.

(1) Preparation of the crude synaptosomal fraction.

Crude synaptosomal fractions were prepared according to the method of Gray and Whittaker (1962). Whole forebrains were homogenised in 10 volumes of ice cold 0.32M sucrose using a teflon pestle and glass tube. The clearance between pestle and tube was 0.30 mm. The homogenate was centrifuged at 1,000 x g. for 10 minutes at 4°C. The pellet containing nuclei and large cell debris was discarded, and the supernatant was further centrifuged at 17,000 x g. for 30 minutes. The resulting supernatant containing mostly microsomes was discarded, and the pellet containing synaptosomes, myelin fragments and mitochondria was resuspended in 26 ml. of ice cold Krebs solution containing 2g/1 glucose. This suspension constituted the crude synaptosomal suspension used in all incubation studies.

(2) Incubation procedures.

Two types of experiment were conducted. The first investigated the accumulation of 5-HT by the crude synaptosomal fraction, and the second examined the retention of the accumulated 5-HT at 37°C.

(i) Accumulation study.

5.0 ml samples of the synaptosomal suspension were placed in incubation flasks containing the necessary volume of Krebs solution to bring the final volume to 6.0 mls. on addition of ^{14}C-5HT. This procedure brought the final volume of synaptosomes to the order of 1 mg protein/ml of suspension. The flasks were placed in a water bath and preincubated for 10 minutes at 37°C. The ^{14}C-5HT was then added to the suspension, and incubated for varying periods of time from 0 - 10 minutes. Following incubation, the flasks were removed from the water bath and cooled rapidly in an ice bath. The incubation mixtures were then centrifuged at 9,000 x g. for 5 minutes at 4°C. The supernatant was discarded and the pellet washed by resuspending in 5.0 mls of ice cold

Krebs solution. The resuspended pellet was then further centrifuged at 9,000 x g. for 5 minutes at 4°C. The resulting supernatant was discarded, and the pellet resuspended in 1.0 ml. of 0.4 N PCA. The suspension in PCA was then centrifuged at 10,000 x g. for 5 minutes at 4°C and the supernatant removed for counting (see later). The pellet of precipitated protein was stored for estimation of protein concentration by a modified method of Lowry et. al. (1952) (see later).

(ii) Retention study.

The initial procedures of the retention study were identical to those used in the accumulation study outlined above. Following incubation with ^{14}C-5HT for 10 minutes at 37°C, the flasks were removed from the water bath and rapidly cooled. The incubation mixture was centrifuged at 9,000 x g. for 5 minutes at 4°C, and the synaptosomal pellet washed in 5.0 ml of ice cold Krebs as described above. The washed pellet was resuspended in 6.0 ml of ice cold Krebs solution and re-incubated for 0 - 30 minutes. The flasks were then removed from the water bath and rapidly cooled in an ice bath. The incubation mixture was then treated as in the first study. The PCA extract was removed for counting while the precipitated protein was stored for estimation by the modified Lowry method.

The specific activity of the 5-HT used in the studies involving incubation with low concentrations of 5-HT (0.1 µMØ was 19.3 m Ci/m.mole, and for those involving incubation at the high concentration of 5-HT (1.0 µM) it was 9.65 m.Ci/m.mole. ^{14}C-5HT creatinine sulphate was obtained from the Radiochemical Centre Amersham.

(3) Measurement of 5-HT and 5-HT metabolites.

The major aim of these studies was to determine that proportion of the accumulated 5-HT that can be retained by the synaptosome in its

original form, and how much is metabolised. In the absence of the availability of ^{14}C-5HIAA from commercial scources, the concentration of labelled metabolites of 5-HT was calculated by measuring the total radioactivity of the PCA extract and deducting from it the radioactivity of authentic 5-HT separated by ion exchange chromatography.

(i) Total radioactivity:-

0.1 ml of the PCA extract was added to 10 ml of scintillation fluid (see later) and counted in a Packard Tricarb 526 Liquid Scintillation Counter. Blank samples containing 0.1 ml of PCA in 10 ml of scintillation fluid were used for background counting.

(ii) Authentic 5-HT:-

0.8 ml of the PCA extract was added to 1.0 ml of 0.2 M ammonium acetate buffer pH 7.5. The pH of the sample was adjusted to 7.5 with KOH, and the precipitated $KClO_4$ was removed by centrifugation. The sample was then passed over a 5.x 50 mm. column of Amberlite CG 50 (100 - 200 mesh NH_4^+ form), and the effluent discarded. The columns were washed with 5.0 ml 0.02 M ammonium acetate buffer, followed by 3.0 ml of 0.1 N H_2SO_4. The washings were discarded and the 5-HT eluted in 4.0 ml of 1.0 N H_2SO_4. 1.0 ml samples of the elutant were added to 10.0 mls of scintillation fluid (see below) and counted in the Packard Tricarb 526 Liquid Scintillation Counter. 1.0 ml samples of 60 nM ^{14}C-5HT in PCA were carried through the separation procedure with each batch of samples, and the percentage recovery determined for calculation of final proportions. Blank samples containing 1.0 ml of blank column elutant in 10.0 ml of scintillation fluid were used for counting background activity.

(4) Quench Correction.

Sample quenching was monitored by the channels ratio method.

Standard samples of ^{14}C-Toluene were quenched by the addition of varying amounts of carbon tetrachloride. The resulting plots of percentage efficiency versus channel ratio were used to calculate the efficiency of the counting of each sample. Sample counts were then corrected according to the recorded efficiency.

(5) Scintillation Fluid.

The scintillation fluid used in the studies was composed of 3.2 g. 2,5 - Diphenyloxazole (PPO) and 80 mg. 1,4 Bis - (5-phenyloxazol - 2 - yl) benzene (POPOP) dissolved in 400 ml Triton X-100 and 800 ml toluene.

(6) Protein Estimation.

The precipitated protein was redissolved in 5.0 ml of 2 N NaOH. This was then diluted 1:4 and 0.4 ml samples taken for estimation.according to the method of Lowry et. al. (1952). Standard samples containing 50, 100 and 200 µg bovine serum albumin in 0.4 ml of distilled water were prepared. 1.0 ml of 0.4 N PCA was added to each tube and the denatured protein was precipitated by centrifugation. The supernatant was removed and the precipitated protein was redissolved in 0.2 ml of 1N NaOH. 0.2 ml of distilled water was then added to each tube to raise the volume to 0.4 ml and reduce the NaOH concentration to 0.5 N. Blank samples contained 0.2 ml of 1.0 N NaOH and 0.2 ml of distilled water.

Lowrys' solution D was prepared by adding 1.0 ml of a solution of 0.5% $CuSO_4 5H_2O$ in 1.0% sodium citrate to 50 ml of 2.0% Na_2CO_3. 2.0 ml of solution D were then added to each of the blanks, standards and samples. Ten minutes later, 0.2 ml of Folins reagent were added to each tube and the colour was allowed to develop for 30 minutes. The absorbance at 750 nm. was recorded in a Pye Unicam SP 800 spectrophoto-

meter, and the protein concentration was determined from the standard curves by linear regression.

RESULTS

Experiment 1

The effect of electrical stimulation of the nucleus raphe medianus on the forebrain concentration of 5-HT and 5-HIAA.

(i) Aim.

The aim of this experiment was to examine the changes in the concentration of 5-HT and 5-HIAA following electrical stimulation of the nucleus raphe medianus, and to see how the results obtained in this laboratory compared with those of previous workers.

(ii) Plan of experiment.

The techniques employed in this study have been fully described in the previous section. The experiment was conducted upon 28 male Albino Wistar rats weighing between 150 and 200 g. All animals were anaesthetised, and all had electrodes implanted into the nucleus raphe medianus. Body temperature was maintained around 37°C using the water bath technique.

Fourteen of the animals received raphe stimulation, while the others remained as unstimulated controls. Following stimulation, the animals were killed by decapitation and the forebrains were removed, rapidly frozen and stored at -20°C overnight. The estimation of 5-HT and 5-HIAA was carried out the next day. Histological examination of the midbrain revealed misplaced electrodes (see legend Fig. 8) in 3 animals receiving stimulation. These were rejected and the paired t test was applied to data derived from 11 pairs of animals only.

(iii) Results.

The results of the study are shown in table 9 p. 76. It can be seen that electrical stimulation of the nucleus raphe medianus significantly elevated the forebrain concentration of 5-HIAA ($P<0.001$), and did not affect significantly the 5-HT concentration.

Table 9.

The effect of electrical stimulation of nucleus raphe medianus (2 msec. duration, 10Hz, 4-5V. for 30 minutes) upon the forebrain concentration of 5-HT and 5-HIAA (ng/g. wet weight). Results are expressed as the mean ± S.E.M. of 11 matched pairs, and analysed by the paired t test.

	Control	Stimulated	Stimulated minus Control.	P
5-HT (ng/g)	479±58	426±50	-53±74	N.S.
5-HIAA (ng/g)	328±16	447±25	+119±17	$P<0.001$

Experiment II

The effect of electrical stimulation of midbrain regions outside the nucleus raphe medianus on the forebrain concentration of 5-HT and 5-HIAA.

(i) Aim.

It was mentioned in the previous section that whenever a stimulating electrode was identified to be outside the nucleus raphe medianus, that result and its pair were not included in the analysis of the data from that particular experiment. The results of such misplacements were however retained, together with the next pair of animals which had electrodes correctly positioned in the nucleus raphe medianus. The data derived from the groups of animals which had received stimulation of non-raphe regions, and those which had received raphe stimulation was then used to evaluate the selectivity of raphe stimulation in eliciting changes in forebrain concentrations of 5-HT and 5-HIAA.

(ii) Plan of experiment.

Only animals which had received no other treatment than electrical stimulation were used in this evaluation. Because of the diversity of accidental misplacements, only those animals in which the electrode was seen to be of the order of 0.5 mm. outside the anatomical borders of the nucleus raphe medianus were included. Data derived from 9 pairs of animals in each group were analysed. Of the 9 misplaced electrodes, 5 were misplaced laterally, 3 dorsally, and 1 dorso-laterally. The effect of stimulation on forebrain 5-hydroxyindole concentrations in each group was analysed by a paired t test applied to the difference between stimulated and non-stimulated pairs of animals. The difference between stimulation of raphe and non-raphe regions of the midbrain was analysed by a paired comparison of the differences between the two

treatments. (See Appendix I Section (a).

(iii) Results.

The results of this study are shown in table 10 p.79. It can be seen that stimulation applied to either the raphe or the non-raphe regions of the midbrain produced no significant effects on the forebrain concentration of 5-HT. On the other hand, stimulation applied to both sites significantly elevated the forebrain concentration of 5-HIAA. The elevation of 5-HIAA induced by stimulation of non-raphe regions was, however, significantly ($0.01 > P > 0.001$) lower than that induced by stimulation of the nucleus raphe medianus.

Experiment III

The effect of chlorimipramine on changes in forebrain 5-hydroxyindoles induced by electrical stimulation of the nucleus raphe medianus or by the administration of L-tryptophan.

This experiment was undertaken to attempt to ascertain what proportion of the 5-HIAA produced by raphe stimulation arose as a consequence of the deamination of extraneuronally released 5-HT, and how much may be derived from the direct intraneuronal deamination of 5-HT.

The experiment consisted of two studies. The first examined the effect of the uptake inhibitor chlorimipramine on the changes in forebrain 5-hydroxyindoles induced by raphe stimulation; and the second examined the effect of chlorimipramine on the changes in forebrain 5-hydroxyindoles induced by a high dose of L-tryptophan. The effect of chlorimipramine on the resting levels of 5-hydroxyindoles was also determined from data obtained in the course of the above studies.

(a) The effect of chlorimipramine on the resting levels of 5-HT and 5-HIAA in the rat forebrain.

Table 10.

A comparison of the effects of electrical stimulation (2 msec. duration, 10 Hz, 4-5V. for 30 minutes) of raphe and non-raphe regions of the rat midbrain upon the forebrain concentrations of 5-HT and 5-HIAA (ng/g. wet weight). Results are expressed as the mean S.E.M. of 9 pairs of animals. Analysis is by the paired t test.

5-HT concentration (ng/g wet weight).

Site of stimulation	Control.	Stimulated.	Stimulated minus control.	P
Raphe	451±62	456±38	+5±72	N.S.
Non-raphe	435±58	457±46	+22±32	N.S.
Difference between sites.	-16±74	+1±56	-17±79	
P	N.S.	N.S.	N.S.	

5-HIAA concentration (ng/g wet weight).

Site of stimulation	Control.	Stimulated.	Stimulated minus control.	P
Raphe	326±22	488±27	+162±25	$P < 0.001$
Non-raphe	349±28	428±28	+79±27	$0.05 > P > 0.02$
Difference between sites.	23±27	60±31	-83±24	
P	N.S.	N.S.	$0.01 > P > 0.001$	

(i) Plan of experiment.

The effect of chlorimipramine on the resting levels of 5-HT and 5-HIAA was determined from animals used in studies (b) and (c) (outlined below) which received either an injection of chlorimipramine, or an injection of saline, but which did not receive tryptophan or electrical stimulation. The effect of chlorimipramine was analysed by a paired comparison of the differences between the two treatments (see Appendix I section b and c).

It should be noted that study (b) was conducted on anaesthetised animals whereas study (c) used unanaesthetised animals. The effect of chlorimipramine on the resting levels of 5-HT and 5-HIAA have therefore been assessed separately in both anaesthetised and unanaesthetised animals.

(ii) Results.

The results of the study are shown in table 11 p.81. It can be seen that chlorimipramine had no significant effects on the forebrain concentration of 5-HT in either the anaesthetised or unanaesthetised animals. The forebrain concentration of 5-HIAA was, however, significantly reduced by chlorimipramine treatment in the unanaesthetised animals ($0.05 > P > 0.02$), but not in those anaesthetised with halothane.

(b) <u>The effect of chlorimipramine on changes in forebrain 5-hydroxyindoles induced by electrical stimulation of the nucleus raphe medianus.</u>

(i) Plan of experiment.

The experiment was conducted upon 76 male Albino Wistar rats weighing between 150 and 250 g. The animals were divided into two groups of 38 animals. All animals in one group received an intraperitoneal (i.p.) injection of chlorimipramine (5mg/Kg), while those in the control group received an equivalent volume of saline. Three hours after re-

Table 11.

The effect of chlorimipramine alone on the concentration of 5-HT and 5-HIAA (ng/g wet weight) in the rat forebrain measured 4 hours after the injection of chlorimipramine in anaesthetised and unanaesthetised animals. Results are expressed as the mean ± S.E.M. of 14 and 13 matched pairs respectively. Analysis is by the paired t test.

5-HT concentration (ng/g wet weight).

	Control.	Chlorimipramine.	Chlorimipramine minus control.	P
Unanaesthetised (n = 13 pairs).	536±39	557±49	+21±44	N.S.
Anaesthetised (n = 14 pairs).	571±31	511±36	-60±51	N.S.

5-HIAA concentration (ng/g wet weight).

	Control.	Chlorimipramine.	Chlorimipramine minus control.	P
Unanaesthetised (n = 13 pairs).	266±14	227±12	-39±17	0.05>P>0.02
Anaesthetised (n = 14 pairs).	322±19	303±18	-19±19	N.S.

ceiving the injection of chlorimipramine or saline, each animal was anaesthetised and electrodes were implanted as described previously. Half of the animals in each group received raphe stimulation while the other half remained as paired unstimulated controls. Animals were killed following the thirty minute period of stimulation or sham treatment (4 hours after the injection of chlorimipramine). The forebrains of all animals were assayed for 5-HT and 5-HIAA as described previously. The effect of electrical stimulation was analysed by a paired t test, and the effect of chlorimipramine on stimulus induced changes in forebrain 5-hydroxyindoles was analysed by a paired t test between adjacent pairs (stimulated and non-stimulated) of animals receiving and not receiving chlorimipramine (see Appendix I section B).

Histological examination of the midbrain revealed misplaced electrodes in 5 animals receiving stimulation. These were rejected and the t test was applied to data derived from 14 pairs of animals.

(ii) Results.

The effect of raphe stimulation on the forebrain concentration of 5-HT and 5-HIAA in saline control animals and animals treated with chlorimipramine (5mg/Kg) is shown in table 12 p. 83. It can be seen that raphe stimulation in saline treated animals induced a small fall in 5-HT concentration and a significant increase in 5-HIAA concentration ($P<0.001$). In chlorimipramine treated animals stimulation again significantly increased the 5-HIAA concentration ($0.01>P>0.001$) and increased slightly the 5-HT concentration. The differences between the effects of stimulation on groups treated and not treated with chlorimipramine are shown in table 13 p. 84. It can be seen that the elevation of 5-HT by stimulation in the chlorimipramine treated animals was significantly different from the reduction of 5-HT by stimulation in the saline treated

Table 12.

The effect of raphe stimulation on the 5-HT and 5-HIAA concentration (ng/g. wet weight) in rat forebrain in control animals and in animals treated with chlorimipramine (5 mg/Kg). Results are expressed as the mean S.E.M. of 14 matched pairs. Analysis is by the paired t test.

	Unstimulated.	Stimulated.	Stimulated minus unstimulated.	P
5-HT (ng/g)				
Control	571 ± 31	524 ± 39	-47 ± 35	N.S.
Chlorimipramine	511 ± 36	552 ± 34	$+41 \pm 25$	N.S.
5-HIAA (ng/g)				
Control	322 ± 19	469 ± 33	$+147 \pm 21$	$P<0.001$
Chlorimipramine	303 ± 18	341 ± 21	$+39 \pm 12$	$0.01>P>0.001$

Table 13.

The effect of chlorimipramine (5 mg/Kg i.p.) on stimulus-induced changes in forebrain 5-HT and 5-HIAA concentrations (ng/g. wet weight). Results are expressed as the mean ± S.E.M. of 14 matched pairs and analysed by the paired t test.

	Control.	Chlorimipramine	Chlorimipramine minus control.	P
Change in 5-HT concentration (ng/g)	-47±35	+41±25	+88±26	$0.01 > P > 0.001$
Change in 5-HIAA concentration (ng/g)	+147±21	+39±12	-108±26	$0.01 > P > 0.001$

animals ($0.01>P>0.001$). The elevation of 5-HIAA by stimulation was significantly less in the chlorimipramine treated animals ($0.01>P>0.001$). It can also be seen that chlorimipramine reduced the concentration of 5-HIAA in the stimulated brain and correspondingly increased the concentration of 5-HT.

(c) <u>The effect of chlorimipramine in L-tryptophan induced changes in forebrain 5-hydroxyindoles.</u>

(i) Plan of experiment.

The experiment was conducted upon 52 male Albino Wistar rats weighing between 150 and 250 g. The animals were divided into two groups of 26 animals. All animals in one group received an i.p. injection of chlorimipramine (5 mg/Kg) while those in the control group received an equivalent volume of saline. Three hours later, half of the animals in each group received an i.p. injection of L-tryptophan (100 mg/Kg), while the others received an equivalent volume of saline. One hour later (4 hours after the initial injection of chlorimipramine), all animals were sacrificed and the forebrain removed for 5-hydroxyindole estimations. The effect of L-tryptophan on forebrain concentrations of 5-hydroxyindoles was analysed by a paired t test between animals receiving and not receiving L-tryptophan. The effect of chlorimipramine on L-tryptophan-induced changes in forebrain 5-hydroxyindoles was analysed by a paired t test applied between adjacent pairs (tryptophan and control) of animals receiving and not receiving chlorimipramine (see Appendix I section c).

(ii) Results.

Table 14 p.86 shows the change in forebrain levels of 5-HT and 5-HIAA one hour after the i.p. injection of L-tryptophan. L-tryptophan induced significant elevations of forebrain 5-HT and 5-HIAA in both the

Table 14.

The effect of L-tryptophan on the 5-HT and 5-HIAA concentrations (ng/g wet weight) in rat forebrain in controls and in animals treated with chlorimipramine (5mg/Kg i.p.). Results are expressed as the mean S.E.M. of 15 matched pairs and analysed by the paired t test.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
5-HT (ng/g)				
Control	536±39	821±74	285±72	0.01>P>0.001
Chlorimipramine	557±47	855±65	298±68	P<0.001
5-HIAA (ng/g)				
Control	266±14	475±31	209±26	P<0.001
Chlorimipramine	227±12	531±17	304±16	P<0.001

chlorimipramine and saline pretreated animals. A comparison of the effects of tryptophan between animals treated and not treated with chlorimipramine (table 15 p.88) demonstrated that chlorimipramine did not have significant effects upon the elevation of 5-HT by L-tryptophan, but enhanced the elevation of 5-HIAA by L-tryptophan ($0.01>P>0.001$).

Experiment IV

The effect of 10 day lithium pretreatment (0.75 mEq/Kg/day) on changes in forebrain 5-hydroxyindoles induced by raphe stimulation or L-tryptophan administration.

This experiment applied the techniques developed in experiment III to a study of the effect of chronic Li^+ pretreatment on the release of 5-HT in vivo. As in experiment III, two studies were conducted. The first examined the effect of Li^+ pretreatment on changes in forebrain 5-hydroxyindoles induced by the administration of L-tryptophan, and the second examined the effect of Li^+ on changes in forebrain 5-hydroxyindoles induced by raphe stimulation. The effect of Li^+ on resting levels of 5-hydroxyindoles was also determined from data obtained in the course of the above studies.

(a) The effect of 10 day Li^+ pretreatment on the resting levels of 5-HT and 5-HIAA in the rat forebrain.

(i) Plan of experiment.

The effect of Li^+ on the resting levels of 5-HT and 5-HIAA was determined from animals used in studies (b) and (c) (outlined below) which received either 10 days treatment with Li^+ (0.75 mEq/Kg/day) or 10 days treatment with saline but which did not receive tryptophan or raphe stimulation. The effect of Li^+ was analysed by a paired comparison of the differences between the two treatments (see Appendix I

Table 15.

The effect of chlorimipramine (5 mg/Kg i.p.) on tryptophan-induced changes in forebrain 5-HT and 5-HIAA concentrations (ng/g wet weight). Results are expressed as the mean ± S.E.M. of 13 matched pairs and analysed by the paired t test.

Change in 5-HT concentration (ng/g)

Control.	Chlorimipramine.	Chlorimipramine minus control.	P
285±72	298±68	+13±89	N.S.

Change in 5-HIAA concentration (ng/g)

Control.	Chlorimipramine.	Chlorimipramine minus control.	P
209±26	304±16	+94±27	$0.01>P>0.001$

sections b and c). It should be noted that as in experiment III, study (b) was conducted on unanaesthetised animals whereas study (c) used anaesthetised animals. The effect of Li^+ on the resting levels of 5-HT and 5-HIAA have therefore been assessed separately in both anaesthetised and unanaesthetised animals.

(ii) Results.

The results of the study are shown in tables 16 and 17 p. 90. It can be seen that Li^+ pretreatment had no significant effects on the resting 5-HT concentration in either anaesthetised or unanaesthetised animals, but significantly elevated the 5-HIAA concentration in both groups.

(b) <u>The effect of 10 day Li^+ pretreatment on L-tryptophan induced changes in forebrain 5-hydroxyindoles.</u>

(i) Plan of experiment.

The experiment was conducted upon 124 male Albino Wistar rats weighing between 150 and 250 g. Animals were divided into two groups of 62 animals. All animals in one group received an i.p. injection of isotonic (0.15M) LiCl 0.75 mEq/Kg day for 10 days, while the control group received an equivalent volume of isotonic (0.15M) NaCl. On the 11th day of treatment (24 hours after the last dose of Li^+ or saline), half of the animals in each group received an i.p. injection of L-tryptophan (100 mg/Kg), while the others were given an equivalent volume of saline. Pairs of animals (consisting of one animal which had received tryptophan and one which had received saline) from both groups of animals were then sacrificed 30, 60 or 90 minutes after the L-tryptophan or saline injection. The forebrains were removed for 5-hydroxyindole estimation as described previously.

The effect of L-tryptophan on forebrain 5-hydroxyindole concentra-

Table 16.

The effect of 10 day Li^+ pretreatment on the resting concentration of 5-HT and 5-HIAA (ng/g wet weight) in the rat forebrain of unanaesthetised animals. Results are expressed as the mean $\pm$ S.E.M. of 31 matched pairs and analysed by the paired t test.

	Control.	Lithium.	Lithium minus control.	P
5-HT (ng/g).	499$\pm$24	546$\pm$29	+47$\pm$29	N.S.
5-HIAA (ng/g).	285$\pm$11	317$\pm$13	+32$\pm$8	$P<0.001$

Table 17.

The effect of 10 day Li^+ pretreatment on the resting concentration of 5-HT and 5-HIAA in the forebrain of anaesthetised animals. Results are expressed as the mean $\pm$ S.E.M. of 9 pairs of animals and analysed by the paired t test.

	Control.	Lithium.	Lithium minus control.	P
5-HT (ng/g)	614$\pm$67	562$\pm$42	-51$\pm$78	N.S.
5-HIAA (ng/g)	334$\pm$17	374$\pm$15	+40$\pm$10	$0.01>P>0.001$

tions, and the effect of Li^+ upon the tryptophan-induced changes in forebrain 5-hydroxyindole concentrations were analysed as described in experiment III (c) and shown diagrammatically in Appendix I section (c).

(ii) Results.

The effect of L-tryptophan administration (100 mg/Kg) on the forebrain concentrations of 5-HT and 5-HIAA measured 30, 60, and 90 minutes after the injection of tryptophan is shown in tables 18 (a), (b) and (c) p.92, 93 and 94. It can be seen that the forebrain concentrations of both 5-HT and 5-HIAA were significantly elevated by L-tryptophan administration when measured 30 minutes after its injection in both the saline and lithium pretreated groups (table 18a p.92). However, when measured 60 or 90 minutes after the injection of L-tryptophan, the 5-HT concentration in the Li^+ pretreated animals was no longer significantly elevated, whereas the concentration measured in the control animals was still significantly higher than that in animals which had received no tryptophan (table 18b p. 93) and (table 18c p. 94). The forebrain concentration of 5-HIAA was significantly elevated by L-tryptophan treatment in both the saline and Li^+ pretreated animals (tables (a), (b) and (c) pages 92, 93 and 94). From this data, a comparison of the effects of L-tryptophan administration between animals treated and not treated with Li^+ is shown in table 19 p. 95. It can be seen that the elevation of 5-HT and 5-HIAA concentrations seen 30 and 90 minutes after the injection of the amino acid are not significantly different in the saline and Li^+ pretreated groups. However, when measured 60 minutes after the injection, the elevation of 5-HT concentration in the Li^+ pretreated group is significant- less than that in the saline pretreated group ($0.05>P>0.02$). Accompany-

Footnote:- The term Li^+ when used in this section of the thesis will refer to isotonic (0.15M) LiCl.

Table 18(a).

The forebrain concentrations of 5-HT and 5-HIAA (ng/g wet weight) measured 30 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 10 day Li^+ pre-treatment. Results are expressed as the mean $\pm$ S.E.M. of 8 matched pairs and analysed by the paired comparison and t test.

5-HT (ng/g)

	Saline.	Tryptophan.	Tryptophan minus saline.	P.
Control.	492$\pm$31	637$\pm$48	+145$\pm$50	$0.05>P>0.02$
Lithium.	506$\pm$57	647$\pm$74	+142$\pm$52	$0.05>P>0.02$

5-HIAA (ng/g)

	Saline.	Tryptophan.	Tryptophan minus saline.	P.
Control.	280$\pm$13	355$\pm$26	+75$\pm$19	$0.01>P>0.001$
Lithium.	286$\pm$14	402$\pm$17	+117$\pm$15	$P<0.001$

Table 18(b).

The forebrain concentration of 5-HT and 5-HIAA (ng/g wet weight) measured 60 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 10 day Li^+ pretreatment. Results are expressed as the mean ± S.E.M. of 13 matched pairs and analysed by the paired t test.

5-HT (ng/g)

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Control.	495±43	778±84	+283±59	P<0.001
Lithium.	568±46	700±62	+132±71	N.S.

5-HIAA (ng/g)

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Control.	287±25	488±51	+201±39	P<0.001
Lithium.	336±29	616±41	+280±23	P<0.001

Table 18(c).

The forebrain concentration of 5-HT and 5-HIAA measured 90 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 10 day Li^+ pretreatment. Results are expressed as the mean $\pm$ S.E.M. of 10 matched pairs and analysed by the paired t test.

5-HT (ng/g)

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Control.	508±48	711±53	+202±53	$0.01>P>0.001$
Lithium.	550±51	622±76	+72±80	N.S.

5-HIAA (ng/g)

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Control.	286±10	540±28	+254±30	$P<0.001$
Lithium.	316±8	551±47	+235±47	$P<0.001$

Table 19.

The effect of 10 day Li^+ pretreatment on L-tryptophan-induced changes in forebrain 5-hydroxyindoles measured 30, 60 and 90 minutes after the injection of L-tryptophan. Results are expressed as the mean $\pm$ S.E.M. of 8, 13 and 10 matched pairs respectively. Statistical analysis is by the paired t test.

Change in 5-HT concentration (ng/g)

Time after L-tryptophan.	Control.	Lithium.	Lithium minus control.	P
30 min.	$+145\pm50$	$+142\pm52$	-3 ± 77	N.S.
60 min.	$+283\pm59$	$+132\pm71$	-151 ± 64	$0.05>P>0.02$
90 min.	$+202\pm53$	$+72\pm80$	-130 ± 96	N.S.

Change in 5-HIAA concentration (ng/g)

Time after L-tryptophan.	Control.	Lithium.	Lithium minus control.	P
30 min.	$+75\pm19$	$+117\pm15$	$+42\pm24$	N.S.
60 min.	$+201\pm39$	$+280\pm23$	$+79\pm35$	$0.05>P>0.02$
90 min.	$+254\pm30$	$+235\pm47$	-29 ± 39	N.S.

ing this change is a significant increase in the elevation of 5-HIAA in the Li^+ pretreated animals ($0.05 > P > 0.02$). Thus it has been demonstrated that Li^+ reduces the maximum elevation in forebrain 5-HT concentration that can be obtained following a single i.p. injection of L-tryptophan (100 mg/Kg), and increases the elevation of 5-HIAA measured 60 minutes after the injection.

(c) <u>The effect of 10 day Li^+ pretreatment on stimulation-induced changes in forebrain 5-hydroxyindoles.</u>

(i) Plan of experiment.

The experiment was conducted upon 104 male Albino Wistar rats weighing between 150 and 250 g. The animals were divided into two groups of 52 animals. One group received 10 day administration of LiCl (as described in experiment IV b), while the others received an equivalent volume of saline. On the 11th day, each group was further divided into two sub-groups. Animals in one sub-group were given chlorimipramine (5 mg/Kg i.p.), while the others were given an equivalent volume of saline. On each day of the experiment, 8 animals were used, one pair from each sub-group. Three and a half hours after the injection of chlorimipramine or saline, one animal in each pair received raphe stimulation for 30 minutes, while the other animal received a similar but sham operation. Animals were killed immediately following the 30 minute period of stimulation or sham treatment (4 hours after chlorimipramine). The forebrain of each animal was removed, frozen, and stored at -20°C for estimation of 5-HT and 5-HIAA carried out the next day.

The benefits of using a paired design for reducing the within day variation in forebrain 5-hydroxyindole concentration are lessened somewhat when conducting an experiment of complex design such as this.

However, because the day to day variation in 5-hydroxyindole concentrations are significant (table 6 p. 56), the paired analysis was applied to this study. The effect of electrical stimulation was analysed by a paired t test applied between animals of the same sub-group receiving and not receiving electrical stimulation. The effect of Li^+ on stimulus-induced changes in forebrain 5-hydroxyindoles was analysed by applying the paired t test to changes induced in pairs (stimulated and control) of animals treated and not treated with Li^+. Similarly, the effect of chlorimipramine on stimulus-induced changes in forebrain 5-hydroxyindoles was analysed by applying the paired t test to changes observed in pairs (stimulated and control) of animals treated and not treated with chlorimipramine (see Appendix I section d). Histological examination revealed 4 misplaced electrodes, consequently data derived from only 9 pairs of animals in each sub-group was used in the final analysis.

(ii) Results.

The effect of raphe stimulation on the forebrain concentration of 5-HT and 5-HIAA in control animals and in animals which had received 10 day Li^+ pretreatment is shown in table 20 p. 98. Raphe stimulation had no significant effects on the forebrain concentration of 5-HT in either group of animals. However, the concentration of 5-HIAA was elevated by raphe stimulation in both the saline pretreated ($P<0.001$), and Li^+ pretreated animals ($0.01>P>0.001$). A comparison of the effects of stimulation between animals treated and not treated with Li^+ is shown in table 21 p. 99. It can be seen that the changes in forebrain 5-hydroxyindoles produced by raphe stimulation are not significantly affected by Li^+ pretreatment.

Table 22 p. 100, shows the effect of chlorimipramine (5 mg/Kg i.p.)

<u>Table 20.</u>

The effect of raphe stimulation on the 5-HT and 5-HIAA concentration (ng/g wet weight) in rat forebrain in control animals and in animals which had received 10 day Li^+ pretreatment. Results are expressed as the mean $\pm$ S.E.M. of 9 matched pairs and analysed by the paired t test.

		Unstimulated.	Stimulated.	Stimulated minus unstimulated.	P
5-HT (ng/g)	Control.	614 ± 67	658 ± 53	$+44 \pm 53$	N.S.
	Lithium.	562 ± 42	634 ± 53	$+71 \pm 50$	N.S.
5-HIAA (ng/g)	Control.	334 ± 17	427 ± 16	$+93 \pm 12$	$P<0.001$
	Lithium.	374 ± 15	472 ± 14	$+98 \pm 21$	$0.01>P>0.001$

Table 21.

The effect of 10 day Li^+ pretreatment on stimulus-induced changes in forebrain 5-HT and 5-HIAA concentration (ng/g wet weight). Results are expressed as the mean $\pm$ S.E.M. of 9 matched pairs and analysed by the paired t test.

	Control.	Lithium.	Lithium minus control.	P
Change in 5-HT concentration (ng/g)	$+44\pm53$	$+71\pm50$	$+27\pm93$	N.S.
Change in 5-HIAA concentration (ng/g)	$+93\pm12$	$+98\pm21$	$+5\pm17$	N.S.

Table 22.

The effect of chlorimipramine (5 mg/Kg i.p.) on the stimulus-induced changes in forebrain 5-hydroxyindoles in control animals and in animals which had received 10 day Li^+ pretreatment. Results are expressed as the mean $\pm$ S.E.M. of 9 pairs of animals, and analysed by the paired t test.

Change in 5-HT concentration (ng/g)

	Saline.	Chlorimipramine.	Chlorimipramine minus saline.	P
Control.	+44$\pm$53	+116$\pm$59	+72$\pm$99	N.S.
Lithium.	+71$\pm$50	-42$\pm$51	-113$\pm$48	0.05>P>0.02

Change in 5-HIAA concentration (ng/g)

	Saline.	Chlorimipramine.	Chlorimipramine minus saline.	P
Control.	+93$\pm$12	-16$\pm$15	-109$\pm$20	P<0.001
Lithium.	+98$\pm$21	+78$\pm$14	-20$\pm$22	N.S.

on the changes in forebrain 5-HT and 5-HIAA induced by raphe stimulation in saline and Li^+ pretreated animals. The change in 5-HT induced by raphe stimulation in the saline pretreated animals is unaffected by chlorimipramine treatment; however, chlorimipramine significantly reduced the concentration of 5-HT in the stimulated animals which had received Li^+ pretreatment ($0.05 > P > 0.02$). The elevation of 5-HIAA induced by raphe stimulation was significantly reduced by chlorimipramine in the saline treated animals ($P < 0.001$), but was unchanged in the Li^+ pretreated animals.

In summary, raphe stimulation had no significant effect on the forebrain 5-HT concentration in either the saline or Li^+ pretreated groups, however, the forebrain 5-HIAA concentration was significantly elevated by this procedure in both groups of animals. The elevation of 5-HIAA produced by raphe stimulation was significantly reduced by chlorimipramine treatment in the saline pretreated animals, but not in those receiving Li^+. Chlorimipramine significantly reduced the forebrain 5-HT concentration following raphe stimulation in the Li^+ pretreated animals but had no effect in the saline pretreated group.

Experiment V

The effect of 5-day Li^+ pretreatment (1.5mEq/Kg/day) on changes in forebrain 5-hydroxyindole concentrations induced by raphe stimulation or L-tryptophan administration.

This experiment was conducted to determine whether treatment with Li^+ (1.5 mEq/Kg/day) for 5 days exerted similar effects to those observed following treatment with Li^+ (0.75 mEq/Kg/day) for 10 days (experiment IV). As in the previous experiments, two studies were undertaken. The first examined the effect of Li^+ on changes in forebrain 5-hydroxyindoles induced by a dose of L-tryptophan, while the second examined

the effect of Li^+ on changes in forebrain 5-hydroxyindoles induced by raphe stimulation. The effect of Li^+ on the resting levels of 5-hydroxyindoles was also determined from data obtained in the course of the above two studies.

(a) <u>The effect of 5 day Li^+ pretreatment on the resting levels of 5-HT and 5-HIAA in the rat forebrain.</u>

(i) Plan of experiment.

The effect of Li^+ on the resting levels of 5-HT and 5-HIAA was determined from animals used in studies (b) and (c) (outlined below) which received either 5 days treatment with Li^+ or 5 days treatment with saline, but which did not receive tryptophan or raphe stimulation.

The effect of Li^+ was analysed by a paired comparison of the differences between the two treatments (see Appendix I sections b and c). It should be noted that as in the previous two experiments, study (b) was conducted on unanaesthetised animals, whereas study (c) used anaesthetised animals. The effect of Li^+ on the resting levels of 5-HT and 5-HIAA have therefore been assessed separately in both anaesthetised and unanaesthetised animals.

(ii) Results.

The results of this study are shown in table 23 p. 103. It can be seen that Li^+ pretreatment had no significant effect on the 5-HT concentration in either the anaesthetised or unanaesthetised groups. On the other hand, the 5-HIAA concentration was significantly increased by Li^+ pretreatment in the unanaesthetised group ($0.01 > P > 0.001$), but not in the anaesthetised group.

(b) <u>The effect of 5 day Li^+ pretreatment on L-tryptophan-induced changes in forebrain 5-hydroxyindoles.</u>

(i) Plan of experiment.

Table 23.

The effect of 5 day Li^+ pretreatment alone on the forebrain concentrations of 5-HT and 5-HIAA (ng/g wet weight) in anaesthetised and unanaesthetised animals. Results are expressed as the mean $\pm$ S.E.M. of 19 (unanaesthetised) and 11 (anaesthetised) pairs of animals. Statistical analysis is by the paired t test.

5-HT concentration (ng/g wet weight).

	Control.	Lithium.	Lithium minus control.	P
Unanaesthetised (n= 19 pairs).	545$\pm$33	547$\pm$39	+2$\pm$33	N.S.
Anaesthetised (n= 11 pairs).	432$\pm$36	403$\pm$35	-27$\pm$45	N.S.

5-HIAA concentration (ng/g wet weight).

	Control.	Lithium.	Lithium minus control.	P
Unanaesthetised (n= 19 pairs).	286$\pm$12	327$\pm$22	+41$\pm$11	$0.01 > P > 0.001$
Anaesthetised (n= 11 pairs).	298$\pm$17	324$\pm$ 19	+26$\pm$14	N.S.

The experiment was conducted upon 76 male Albino Wistar rats weighing between 150 and 250 g. Animals were divided into two groups of 38 animals. All animals in one group received an i.p. injection of isotonic (0.15M) LiCl (1.5 mEq/Kg/day) for 5 days, while the control group received an equivalent volume of isotonic (0.15M) NaCl. On the 6th day of treatment (24 hours after the last dose of Li^+), half of the animals in each group received an injection of L-tryptophan (100 mg/Kg i.p.) while the others received an equivalent volume of saline. Pairs of animals (tryptophan treated and control) from both groups were then sacrificed 30 or 60 minutes after the L-tryptophan or saline injection. The forebrain was removed from each animal and the 5-hydroxyindole concentrations measured as described previously. The effect of L-tryptophan on forebrain 5-hydroxyindole concentrations, and the effect of Li^+ on the tryptophan-induced changes in forebrain 5-hydroxyindole concentrations was analysed as described in experiment III(c) and shown in Appendix I section c.

(ii) Results

The effect of L-tryptophan administration (100 mg/Kg i.p.) on the forebrain concentrations of 5-HT and 5-HIAA measured 30 and 60 minutes after the injection of tryptophan is shown in table 24 p. 105. Table 24(a) p. 105, shows that the forebrain concentration of 5-HT measured 30 minutes after the injection of tryptophan was significantly increased in the Li^+ pretreated group only. However, L-tryptophan treatment significantly elevated the 5-HIAA concentration in both saline and Li^+ pretreated animals. Table 24(b) p. 106 shows that the forebrain concentrations of 5-HT and 5-HIAA were significantly elevated by L-tryptophan treatment in both the saline and Li^+ pretreated groups when measured 60 minutes after the injection. The comparison of the

Table 24(a).

The forebrain concentration of 5-HT and 5-HIAA (ng/g wet weight) measured 30 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 5 day Li^{+} pretreatment. Results are expressed as the mean ± S.E.M. of 8 matched pairs and analysed by the paired t test.

		Saline.	Tryptophan.	Tryptophan minus saline.	P
5-HT (ng/g)	Control.	563±64	670±95	+108±84	N.S.
	Lithium.	584±60	734±81	+150±52	0.05>P>0.02
5-HIAA (ng/g)	Control.	253±7	348±15	+95±13	P<0.001
	Lithium.	287±6	431±21	+144±19	P<0.001

Table 24(b).

The forebrain concentration of 5-HT and 5-HIAA measured 60 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 5 day Li^+ pretreatment. Results are expressed as the mean ± S.E.M. of 11 matched pairs and analysed by the paired t test.

		Saline.	Tryptophan.	Tryptophan minus saline.	P
5-HT (ng/g)	Control.	533±35	717±74	+184±69	$0.05>P>0.02$
	Lithium.	520±53	762±26	+242±39	$P<0.001$
5-HIAA (ng/g)	Control.	309±16	541±17	+232±40	$P<0.001$
	Lithium.	356±17	655±33	+299±26	$P<0.001$

effects of L-tryptophan between animals treated and not treated with Li^+ (table 25 p.108) demonstrated that the changes in forebrain 5-HT and 5-HIAA concentrations induced by L-tryptophan administration were not significantly affected by 5 day Li^+ pretreatment.

(c) <u>The effect of 5 day Li^+ pretreatment on stimulation-induced changes in forebrain 5-hydroxyindoles.</u>

(i) Plan of experiment.

The experiment was conducted on 56 male Albino Wistar rats weighing between 150 and 250 g. Animals were divided into two groups of 28 animals. One group received Li^+ pretreatment (administered as described in experiment Vb), while the other received an equivalent volume of saline. On the 6th day of treatment (24 hours after the last dose of Li^+) half of the animals in each group received electrical stimulation (at the parameters described previously), while the other half remained as paired unstimulated controls. Following the period of stimulation or sham treatment, all animals were sacrificed and each forebrain was removed and stored for the 5-hydroxyindole estimations which were carried out the next day.

The effect of Li^+ on stimulus-induced changes in forebrain 5-hydroxyindole concentrations was analysed by the paired t test applied to changes observed in adjacent pairs (stimulated and control) of animals receiving and not receiving Li^+ pretreatment. (see Appendix I section b).

(ii) Results.

The effect of raphe stimulation on the forebrain concentrations of 5-HT and 5-HIAA in control animals and in animals which had received 5 day Li^+ pretreatment is shown in table 26 p. 109. Raphe stimulation had no significant effect on the 5-HT concentration in either the saline

Table 25.

The effect of 5 day Li^+ pretreatment on L-tryptophan-induced changes in forebrain 5-hydroxyindoles measured 30 and 60 minutes after the injection of L-tryptophan. Results are expressed as the mean ± S.E.M. of 8 and 11 matched pairs respectively. Statistical analysis is by the paired t test.

Change in 5-HT concentration (ng/g)

Time after L-tryptophan.	Control.	Lithium.	Lithium minus control.	P
30 min.	+108±84	+150±52	+42±80	N.S.
60 min.	+184±69	+242±39	+57±105	N.S.

Change in 5-HIAA concentration (ng/g)

Time after L-tryptophan.	Control.	Lithium.	Lithium minus control.	P
30 min.	+95±13	+144±19	+49±24	N.S.
60 min.	+232±40	+299±26	+68±45	N.S.

Table 26.

The effect of raphe stimulation on the 5-HT and 5-HIAA concentration (ng/g wet weight) in rat forebrain in control animals which had received 5 day Li^+ pretreatment. Results are expressed as the mean $\pm$ S.E.M. of 11 matched pairs and analysed by the paired t test.

		Unstimulated.	Stimulated.	Stimulated minus unstimulated.	P
5-HT (ng/g)	Control.	432 ± 36	444 ± 28	$+12 \pm 44$	N.S.
	Lithium.	403 ± 35	406 ± 36	$+3 \pm 32$	N.S.
5-HIAA (ng/g)	Control.	298 ± 17	417 ± 29	$+119 \pm 28$	$0.01 > P > 0.001$
	Lithium.	324 ± 19	461 ± 41	$+138 \pm 34$	$0.01 > P > 0.001$

or Li^+ pretreated group, but significantly elevated the 5-HIAA concentration in both groups of animals. A comparison of the effects of raphe stimulation between animals treated and not treated with Li^+ is shown in table 27 p. 111. It can be seen that the effects of raphe stimulation on the forebrain concentrations of 5-HT and 5-HIAA did not differ significantly between animals which had received 5 day saline or 5 day Li^+ pretreatments.

Experiment VI

The measurement of Li^+ concentrations in brain and plasma following 5 or 10 day Li^+ pretreatment.

(i) Aim.

This experiment was conducted for two reasons. Firstly, to determine whether the brain and plasma concentration of Li^+ differed following treatment with Li^+ for 5 or 10 days, and secondly to examine whether the differences between 5 and 10 day treatment observed in experiments IV and V may be related to differences in the brain concentration of Li^+.

(ii) Plan of experiment.

The experiment was conducted upon 16 male Albino Wistar rats weighing between 150 and 250 g. 8 animals were given an i.p. injection of isotonic LiCl 0.75 mEq/Kg/day for 10 days, and the other 8 received 1.5 mEq/Kg/day for 5 days. The dosage schedules were arranged so that the animals received their 10th and 5th injection on the same day. On the next day, 24 hours after the last dose of Li^+, all animals were sacrificed by cervical dislocation and decapitation. The Li^+ concentration in the plasma and forebrain was then measured as described previously.

Table 27.

The effect of 5 day Li^+ pretreatment on stimulus-induced changes in forebrain 5-hydroxyindole concentration (ng/g wet weight). Results are expressed as the mean ± S.E.M. of 11 pairs of animals and analysed by the paired t test.

Change in 5-HT concentration (ng/g)

Control.	Lithium.	Lithium minus control.	P
+12±44	+3±32	-9±61	N.S.

Change in 5-HIAA concentration (ng/g)

Control.	Lithium.	Lithium minus control.	P
+119±28	+138±34	+19±34	N.S.

(iii) Results.

Table 28 p. 113 shows the concentrations of Li^+ measured in brain, and plasma following 5 day (1.5 mEq/Kg/day) or 10 day (0.75 mEq/Kg/day) Li^+ pretreatment. It can be seen that the concentration of the cation measured in both tissues after 5 days treatment with 1.5 mEq/Kg/day was approximately double that measured after 10 days treatment with 0.75 mEq/Kg/day.

Experiment VII

The effect of 10 day Li^+ pretreatment on changes in the concentration of tryptophan in plasma, plasma ultrafiltrate and brain tissue following the injection of L-tryptophan.

(i) Aim.

This experiment was conducted to determine whether treatment with Li^+ for 10 days exerted significant effects on the gross distribution of tryptophan between plasma and brain tissue.

(ii) Plan of experiment.

The experiment was conducted upon 72 male Albino Wistar rats weighing between 150 and 250 g. The animals were divided into two groups of 36 animals. One group received 10 day Li^+ pretreatment (0.75 mEq/Kg/day) while the others received control injections of saline. On the 11th day (24 hours after the last dose of Li^+), half of the animals in each group were given an i.p. injection of L-tryptophan (100 mg/Kg), while the others received a control injection of saline. Animals were killed 30, 60 and 90 minutes after the injection of L-tryptophan, and the concentration of the amino acid was measured in the forebrain, whole plasma and plasma ultrafiltrate using the methods previously described.

The effect of Li^+ on changes in tryptophan concentration was

Table 28.

The concentration of Li^+ in brain (nEq/Kg) and plasma (nEq/1) following 5 day (1.0 mEq/Kg/day) and 10 day (0.75 mEq/Kg/day) Li^+ pretreatment. Results are expressed as the mean ± S.E.M. of 8 animals.

Period of treatment.	Plasma Li^+ concentration (µEq/1)	Brain Li^+ concentration (µEq/Kg brain tissue)
5 days.	59±0.7	144±5.2
10 days.	32±2.7	70±0.5

analysed by a paired comparison and t test of the differences between treatments. (See Appendix I section c).

(iii) Results.

The effect of 10 day Li^+ pretreatment alone on the concentration of tryptophan in brain, plasma and plasma ultrafiltrate are shown in table 29 p. 115. It can be seen that Li^+ pretreatment had no significant effect on the tryptophan concentration in any of the three tissues.

Table 30 p. 116 - 118, shows the effect of L-tryptophan administration (100 mg/Kg i.p.) on the concentration of tryptophan in forebrain, plasma and plasma ultrafiltrate measured 30, 60 and 90 minutes after the injection. It can be seen that the concentration of tryptophan in all three tissues is significantly elevated at 30 (table 30a p. 116), 60 (table 30b p. 117), and 90 (table 30c p. 118), minutes after the injection of L-tryptophan both in the control group of animals, and in those which had received 10 day Li^+ pretreatment. A comparison of the effects of L-tryptophan administration between animals treated and not treated with Li^+ is shown in table 31 p. 119 - 121. It can be seen that 10 day Li^+ pretreatment had no significant effects on the elevation of tryptophan concentrations observed in any of the three tissues following the i.p. administration of the amino acid.

Experiment VIII

The effect of 10 day Li^+ pretreatment on the accumulation and retention of ^{14}C-5-HT by isolated synaptosomes.

This study was conducted in order to examine more directly some possible actions of Li^+ which were suggested by the results of experiment IV(b).

Two major studies were undertaken. The first examined the effect of 10 day Li^+ pretreatment on the accumulation of ^{14}C-5-HT by isolated

Table 29.

The effect of 10 day Li^+ pretreatment alone on the concentration of tryptophan in brain, plasma, and plasma ultrafiltrate. Results are expressed as the mean ± S.E.M. of 18, 18 and 16 matched pairs respectively. Analysis is by the paired t test.

Tryptophan concentration.

	Control.	Lithium.	Lithium minus control.	P
Brain (ug/g)	4.77±0.26	4.91±0.25	+0.14±0.12	N.S.
Plasma (ug/ml)	34.34±2.44	32.98±1.96	-1.36±1.56	N.S.
Ultrafiltrate (ug/ml)	3.53±0.45	3.81±0.44	+0.27±0.18	N.S.

Table 30(a).

The concentration of tryptophan in rat forebrain (ug/g wet weight) plasma (ug/ml) and plasma ultrafiltrate (ug/ml) measured 30 minutes after an i.p. injection of L-tryptophan (100 mg/Kg), in control animals and in animals which had received 10 day Li^{+} pretreatment. Results are expressed as the mean $\pm$ S.E.M. of 6, 6, and 4 matched pairs respectively. Statistical analysis is by the paired t test.

Control animals.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Brain (ug/g).	5.42 $\pm$0.63	28.77 $\pm$4.34	+23.35 $\pm$3.99	0.01>P>0.001
Plasma (ug/ml).	40.24 $\pm$2.15	174.30 $\pm$16.21	+134.06 $\pm$15.29	P<0.001
Ultrafiltrate (ug/ml).	4.21 $\pm$1.64	30.09 $\pm$7.51	+25.88 $\pm$7.28	0.05>P>0.02

Lithium pretreated animals.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Brain (ug/g).	5.17 $\pm$0.60	28.92 $\pm$3.12	+23.75 $\pm$2.80	P<0.001
Plasma (ug/ml).	35.97 $\pm$2.45	178.56 $\pm$12.07	+142.59 $\pm$13.34	P<0.001
Ultrafiltrate (ug/ml).	4.37 $\pm$1.57	29.49 $\pm$8.8	+25.12 $\pm$6.05	0.05>P>0.02

Table 30(b).

The concentration of tryptophan in rat forebrain (ug/g wet weight) plasma and plasma ultrafiltrate (ug/g) measured 60 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 10 day Li^+ pretreatment. Results are expressed as the mean ± S.E.M. of 8 matched pairs and analysed by the paired t test.

Control animals.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Brain (ug/g).	4.59 ±0.24	32.74 ±3.24	+28.15 ±3.18	$P<0.001$
Plasma (ug/ml).	31.43 ±3.85	128.96 ±15.97	+97.54 ±13.26	$P<0.001$
Ultrafiltrate (ug/ml).	3.68 ±0.39	28.52 ±4.82	+24.84 ±4.74	$0.01>P>0.001$

Lithium pretreated animals.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Brain (ug/g).	4.92 ±0.24	28.43 ±3.31	+23.51 ±3.20	$P<0.001$
Plasma (ug/ml).	31.74 ±3.64	134.66 ±18.94	+102.92 ±17.10	$P<0.001$
Ultrafiltrate (ug/ml).	3.95 ±0.48	29.19 ±5.98	+25.24 ±5.57	$0.01>P>0.001$

Table 30(c).

The concentration of tryptophan in rat forebrain (ug/g wet weight) plasma and plasma ultrafiltrate (ug/ml) measured 90 minutes after an i.p. injection of L-tryptophan (100 mg/Kg) in control animals and in animals which had received 10 day Li^+ pretreatment. Results are expressed as the mean ± S.E.M. of 4 matched pairs and analysed by the paired t test.

Control animals.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Brain (ug/g).	4.68 ±0.77	21.76 ±5.36	+17.08 ±4.75	0.05>P>0.02
Plasma (ug/ml).	31.30 ±5.59	95.23 ±10.74	+63.93 ±10.40	0.01>P>0.001
Ultrafiltrate (ug/ml).	2.60 ±0.39	16.00 ±3.96	+13.40 ±3.82	0.05>P>0.02

Lithium pretreated animals.

	Saline.	Tryptophan.	Tryptophan minus saline.	P
Brain (ug/g).	4.48 ±0.61	23.16 ±3.49	+18.68 ±3.05	0.01>P>0.001
Plasma (ug/ml).	30.95 ±3.78	97.25 ±5.34	+66.30 ±4.69	P<0.001
Ultrafiltrate (ug/ml).	3.02 ±0.26	15.62 ±1.66	+12.60 ±1.45	0.01>P>0.001

Table 31(a).

The effect of 10 day Li^+ pretreatment on changes in the concentration of brain tryptophan measured 30, 60 and 90 minutes after the i.p. injection of L-tryptophan (100 mg/Kg). Results are expressed as the mean ± S.E.M. of matched pairs and analysed by the paired t test.

Change in brain tryptophan concentration (ug/g)

Time after L-tryptophan (mins)	Control.	Lithium.	Lithium minus control.	P
30 mins. (n = 6 pairs)	+23.35±3.99	+23.75±2.80	+0.40±3.81	N.S.
60 mins (n = 8 pairs)	+28.15±3.18	+23.51±3.20	-4.64±3.03	N.S.
90 mins. (n = 4 pairs)	+17.08±4.75	+18.68±3.05	+1.60±4.93	N.S.

Table 31(b).

The effect of 10 day Li^{+} pretreatment on changes in the concentration of plasma tryptophan measured 30, 60 and 90 minutes after the i.p. injection of L-tryptophan (100 mg/Kg). Results are expressed as the mean ± S.E.M. of matched pairs and analysed by the paired t test.

Change in plasma tryptophan concentration (ug/ml)

Time after L-tryptophan (mins)	Control.	Lithium.	Lithium minus control.	P
30 mins. (n = 6 pairs)	+134.06±15.29	+142.59±13.34	+8.53±17.53	N.S.
60 mins. (n = 8 pairs)	+97.54±13.26	+102.92±17.10	+5.38±17.43	N.S.
90 mins. (n = 4 pairs)	+63.93±10.40	+66.30±4.69	+2.37±7.50	N.S.

Table 31(c).

The effect of 10 day Li^+ pretreatment on changes in tryptophan concentration of the plasma ultrafiltrate measured 30, 60 and 90 minutes after the i.p. injection of L-tryptophan (100 mg/Kg). Results are expressed as the mean ± S.E.M. of matched pairs and analysed by the paired t test.

Change in ultrafiltrate tryptophan concentration (ug/ml)

Time after L-tryptophan (mins)	Control.	Lithium.	Lithium minus control.	P
30 mins. (n = 4 pairs)	+25.88±7.28	+25.12±6.05	-0.76±1.98	N.S.
60 mins. (n = 8 pairs)	+24.84±4.75	+25.24±5.57	+0.40±3.45	N.S.
90 mins. (n = 4 pairs)	+13.40±3.82	+12.60±1.45	+0.08±3.03	N.S.

synaptosomes, and the second examined the effect of Li^+ on the retention of the accumulated 5-HT. In addition, a preliminary study was conducted to test the functional integrity of the prepared synaptosomal suspensions.

(a) The effect of temperature on the accumulation of ^{14}C-5-HT by isolated synaptosomes.

(i) Plan of experiment.

This preliminary study to check the functional integrity of the synaptosomal suspension was carried out on 2 rats only.

Crude synaptosomal suspensions were prepared as described previously. Eight 5.0 ml samples of the suspension were placed in incubation flasks containing 0.9 ml. Krebs solution. Four of the flasks were pre-incubated at 37°C for 10 minutes while the other four were pre-incubated at 2°C for 10 minutes. 0.1 ml of ^{14}C-5-HT (specific activity 19.5 mCi/mmole) was added to each flask (final 5-HT concentration 0.1 μM). Flasks were then incubated for 1.0, 2.0, 5.0 and 10.0 minutes at 37°C or 2°C. Following incubation, the flasks were rapidly cooled in an ice bath, and the accumulated 5-HT extracted and measured as described in section (xiii).

(ii) Results.

Table 32 p.123 shows the effect of temperature on the accumulation of ^{14}C-5-HT by isolated synaptosomes incubated in 0.1 μM ^{14}C-5-HT. It can be seen that 5-HT is rapidly accumulated by synaptosomes when incubated at 37°C, but not when incubated at 2°C.

(b) The effect of 10 day Li^+ pretreatment on the accumulation and metabolism of 5-HT by isolated synaptosomes.

(i) Plan of experiment.

The accumulation of ^{14}C-5-HT against time was studied at an

Table 32.

The concentration of 5-HT (pmoles/mg protein) accumulated by isolated synaptosomes incubated in 0.1 uM ^{14}C-5-HT (S.A. 19.3 mCi/mmole) for 1.0 - 10 minutes at 37°C or 2°C. Results are expressed as the mean of two animals.

5-HT accumulated (pmoles/mg protein).

Incubation time (mins)	2°C	37°C
1.0 min.	0.46	2.59
2.0 min.	0.43	3.38
5.0 min.	0.59	5.50
10.0 min.	0.55	6.11

incubation concentration of 0.1 µM. At this concentration, the 5-HT should be transported primarily by the high affinity uptake process (Shaskan and Snyder 1970, Wong et. al. 1973). The study was conducted upon 18 male Albino Wistar rats weighing between 150 and 250 g. The animals were divided into two groups of 9 animals. One group received 10 day Li^+ pretreatment (0.75 mEq/Kg/day) while the others received a control injection of saline. On the 11th day, all animals were killed and synaptosomal suspensions prepared from each forebrain. Synaptosomes were incubated in 0.1 µM ^{14}C-5-HT for 0.5, 1.0, 2.0, 5.0 and 10.0 minutes. Following incubation, labelled 5-hydroxyindoles were extracted, separated and measured as described in section (xiii). Synaptosomes prepared from Li^+ and saline pretreated animals were incubated at the same time and the effect of Li^+ on the accumulation and metabolism of 5-HT was analysed by the paired t test.

(ii) Results.

The accumulation of 5-HT by rat forebrain synaptosomes prepared from control animals which had received 10 day Li^+ pretreatment is shown in table 33(a) p. 125. It can be seen that ^{14}C-5-HT is continually accumulated by the synaptosomal fraction and that this accumulation is unaffected by Li^+ pretreatment.

Table 33(b) p. 126 shows the concentration of 5-HT metabolites measured in synaptosomes following incubation for 0.5 - 10.0 minutes. There appears to be a reasonably constant concentration of 5-HT metabolites present in the synaptosomes over all periods of incubation both in the control and Li^+ pretreated animals. Li^+ pretreatment had no significant effects on the synaptosomal concentration of 5-HT metabolites.

Table 33(a).

The effect of 10 day Li^+ pretreatment on the accumulation of 5-HT by rat forebrain synaptosomes incubated in 0.1 uM ^{14}C-5-HT (Specific activity 19.3 mCi/mmole) for 0.5 - 10.0 minutes at 37°C. Paired control animals received 10 day saline treatment. Results are expressed as the mean ± S.E.M. of 9 matched pairs and analysed by the paired t test.

Synaptosomal 5-HT concentration (pmoles/mg protein).

Incubation time (minutes).	Control.	Lithium.	Lithium minus control.	P
0.5	2.59±0.15	2.15±0.13	-0.44±0.21	N.S.
1.0	2.84±0.24	2.98±0.17	+0.14±0.23	N.S.
2.0	3.80±0.31	3.61±0.08	-0.19±0.31	N.S.
5.0	4.66±0.34	5.13±0.46	+0.53±0.61	N.S.
10.0	6.40±0.62	6.22±0.38	-0.18±0.68	N.S.

Table 33(b).

The effect of 10 day Li^+ pretreatment on the synaptosomal metabolism of 5-HT following incubation in 0.1 uM ^{14}C-5-HT (S.A. 19.3 mCi/mmole) for 0.5 - 10 minutes at 37°C. Paired control animals received 10 day saline pretreatment. Results are expressed as the mean ± S.E.M. of 9 matched pairs and analysed by the paired t test.

Synaptosomal concentration of 5-HT metabolites (pmoles/mg protein).

Incubation time (minutes).	Control.	Lithium.	Lithium minus control.	P
0.5	0.87±0.23	0.88±0.12	+0.01±0.21	N.S.
1.0	0.87±0.28	1.07±0.41	+0.20±0.46	N.S.
2.0	0.72±0.32	1.26±0.29	+0.53±0.37	N.S.
5.0	1.06±0.22	0.73±0.50	-0.33±0.39	N.S.
10.0	1.00±0.57	1.18±0.42	+0.18±0.73	N.S.

(c) The effect of 10 day Li^+ pretreatment on the retention of ^{14}C-5-HT by isolated synaptosomes.

(i) Plan of experiment.

This study was conducted on 36 male Albino Wistar rats weighing between 150 - 250 g. Rats were divided into two groups of 18 animals. One group received 10 day Li^+ pretreatment (0.75 mEq/Kg/day) while the others received an equivalent volume of saline. On the 11th day, all animals were killed, and synaptosomal suspensions prepared as before.

Synaptosomes prepared from 8 animals in each group were incubated in 0.1 μM ^{14}C-5-HT for 10 minutes. Following the incubation, the synaptosomes were washed, resuspended in fresh Krebs solution and re-incubated for 0, 5.0, 10.0, 20.0, or 30.0 minutes. Following incubation the 5-hydroxyindoles were extracted and measured as described previously.

Synaptosomes prepared from the remaining 10 animals in each group were incubated in 1.0 μM ^{14}C-5-HT for 10 minutes before being washed and re-incubated in fresh Krebs as described above. Following incubation, the 5-hydroxyindoles were extracted and measured as before. The experimental design and the statistical evaluation was carried out as described in experiment VIII (b).

(ii) Results.

The effect of 10 day Li^+ pretreatment on the retention of accumulated 5-HT at 37°C is shown in table 34(a) p. 128. The concentration of 5-HT retained by the synaptosome gradually declined over the 30 minute period of investigation in synaptosomes isolated from both the saline and Li^+ pretreated animals. However, the initial fall in 5-HT concentration measured after 5 minutes incubation was significantly

Table 34(a).

The effect of 10 day Li^{+} pretreatment on the retention of 5-HT by synaptosomes at 37°C following pre-incubation for 10 minutes in 0.1 uM ^{14}C-5-HT (S.A. 19.3 mCi/mmole). Control pairs of animals received 10 day saline pretreatment. Results are expressed as the mean ± S.E.M. of 8 matched pairs and analysed by the paired t test.

Synaptosomal 5-HT concentration (pmoles/mg protein).

Incubation time (minutes)	Control.	Lithium.	Lithium minus control.	P
0	5.83±0.41	5.66±0.62	-0.17±0.32	N.S.
5.0	5.42±0.48	4.82±0.48	-0.60±0.25	$0.05 > P > 0.02$
10.0	4.61±0.26	4.50±0.39	-0.09±0.20	N.S.
20.0	4.04±0.46	3.86±0.30	-0.18±0.25	N.S.
30.0	3.36±0.30	3.72±0.46	+0.37±0.21	N.S.

greater in synaptosomes isolated from Li^+ pretreated animals ($0.05 > P > 0.02$).

Table 34(b) p. 130 shows the concentration of 5-HT metabolites present in the synaptosomes during the same 30 minute period of investigation. It can be seen that the synaptosomal concentration of 5-HT metabolites gradually falls throughout the study. Li^+ pretreatment had no significant effects on the concentration of 5-HT metabolites over the first 20 minutes of the study, however, when measured after 30 minutes incubation there was a significantly higher concentration of metabolites in synaptosomes prepared from Li^+ pretreated animals.

Table 35 p. 131 and 132 shows the results of a similar study to that reported in table 34. However, in this study, synaptosomes were preincubated in 1.0 μM ^{14}C-5-HT and not 0.1 μM ^{14}C-5-HT as in the previous study. Again the concentration of 5-HT retained can be seen to decline over the 30 minute period of incubation. 10 day Li^+ pretreatment appeared to have no significant effect on this decline (table 35(a) p.131. However, the initial fall in 5-HT concentration (as measured by the difference between 0 and 5 minutes incubation) is significantly greater in synaptosomes isolated from Li^+ pretreated animals (table 35(a)(i)). p. 131.

Table 35(b) p. 132 shows the concentration of 5-HT metabolites present in the synaptosomes during the 30 minute incubation period. The concentration of 5-HT metabolites in synaptosomes from control animals can be seen to decline gradually after an initial large fall in concentration. Conversely, in the synaptosomes prepared from Li^+ pretreated animals, the concentration of 5-HT metabolites first increases over the first 5 minute period of incubation then gradually falls towards a

Table 34(b).

The effect of 10 day Li^+ pretreatment on the metabolism of 5-HT by synaptosomes at 37°C following preincubation for 10 minutes in 0.1 uM ^{14}C-5-HT (S.A. 19.3 mCi/mmole). Control pairs of animals received 10 day saline treatment. Results are expressed as the mean ± S.E.M. of 8 pairs of animals and analysed by the paired t test.

Synaptosomal concentration of 5-HT metabolites (pmoles/mg protein).

Incubation time (minutes)	Control.	Lithium.	Lithium minus control.	P
0	3.99±0.44	3.37±0.57	-0.62±0.27	N.S.
5.0	2.77±0.40	2.78±0.38	-0.04±0.10	N.S.
10.0	2.28±0.33	2.18±0.22	-0.10±0.22	N.S.
20.0	1.98±0.21	2.25±0.19	+0.27±0.21	N.S.
30.0	1.50±0.15	1.93±0.13	+0.43±0.15	0.05>P>0.02

Table 35(a).

The effect of 10 day Li^+ pretreatment on the retention of 5-HT by synaptosomes at 37°C following preincubation for 10 minutes in 1.0 uM ^{14}C-5-HT (S.A. 9.67 mCi/mmole). Control animals received 10 day saline treatment. Results are expressed as the mean ± S.E.M. of 10 pairs of animals and analysed by the paired t test.

Synaptosomal 5-HT concentration (pmoles/mg protein)

Incubation time (minutes)	Control.	Lithium.	Lithium minus control.	P
0	16.25±0.99	17.35±0.66	+1.10±0.87	N.S.
5.0	14.37±0.69	14.15±0.69	-0.22±0.65	N.S.
10.0	12.85±0.64	12.65±0.64	-0.20±0.48	N.S.
20.0	10.55±0.54	10.25±0.38	-0.30±0.44	N.S.
30.0	8.63±0.36	9.81±0.56	+1.18±0.71	N.S.

Table 35(a)(i).

Initial fall in synaptosomal 5-HT concentration (pmoles/mg protein) measured as the difference between 0 and 5.0 minutes incubation.

Control.	Lithium.	Lithium minus control.	P
1.88±0.57	3.19±0.39	+1.31±0.56	$0.05 > P > 0.02$

Table 33(b).

The effect of 10 day Li^{+} pretreatment on the metabolism of 5-HT by synaptosomes at 37°C following preincubation for 10 minutes in 1.0 uM ^{14}C-5-HT (S.A. 9.67 mCi/mmole). Control animals received 10 day saline pretreatment. Results are expressed as the mean ± S.E.M. of 10 matched pairs and analysed by the paired t test.

Synaptosomal concentration of 5-HT metabolites (pmoles/mg protein)

Incubation time (minutes)	Control.	Lithium.	Lithium minus control.	P
0	7.89±2.45	8.03±2.60	+0.14±0.71	N.S.
5.0	4.74±1.52	11.23±2.63	+6.49±1.46	0.01>P>0.001
10.0	4.29±1.19	4.03±1.20	-0.27±0.45	N.S.
20.0	3.24±0.69	3.46±0.84	+0.22±0.52	N.S.
30.0	2.55±0.57	3.49±0.61	+1.44±0.35	0.01>P>0.001

plateau level recorded after 20 and 30 minute periods of incubation. The concentration of metabolite measured after 5 and 30 minutes incubation are significantly higher in synaptosomes prepared from Li^+ pretreated animals ($0.01 > P > 0.001$). The increase in metabolite concentration measured in the synaptosomes from Li^+ pretreated animals after 5 minutes incubation coincides with a significantly larger initial fall in synaptosomal 5-HT concentration recorded in these synaptosomes ($0.05 > P > 0.02$) (table 35(a)(i)) p. 131.

D I S C U S S I O N

(1) The effects of electrical stimulation on forebrain 5-HT and 5-HIAA.

Experiment I was conducted to determine whether electrical stimulation of the nucleus raphe medianus using the techniques developed in our laboratories produced changes in forebrain 5-hydroxyindole concentrations which were comparable with those previously reported.

The results of the experiment (table 9 p. 76) demonstrated that stimulation of the nucleus raphe medianus produced a significant elevation of forebrain 5-HIAA concentration without significantly affecting the 5-HT concentration. These observations agreed generally with the observations of Aghajanian et. al. (1967) and Kostowski et. al. (1969), but the amplitude of the changes observed in this study were smaller. There was no significant reduction in 5-HT concentration following stimulation whereas a fall had been previously reported and the elevation of 5-HIAA was slightly less than that observed by the previous workers. Apart from the different anaesthetic used in this study, the major difference in the experimental design was that both Aghajanian et. al. (1967) and Kostowski et. al. (1969) repetitively stimulated the raphe for 60 minutes whilst in this study the period of stimulation was only 30 minutes. This may be sufficient to account for the observed differences.

The shorter period of stimulation has been retained in this study because the longer period used by Aghajanian et. al. (1967) would have involved a longer total time under the anaesthetic which could have increased the effect of anaesthesia on the forebrain concentrations of 5-HT and 5-HIAA (table 2 p. 39).

(2) The effects of variation in electrode placement.

Experiment II examined the selectivity of raphe stimulation in eliciting the observed changes in forebrain 5-hydroxyindoles.

Electrical stimulation applied to regions of the midbrain situated approximately 0.5 mm from the dorsal and lateral borders of the nucleus raphe medianus induced significant increases in the forebrain concentration of 5-HIAA (table 10 p. 79). However, the increase in 5-HIAA concentration observed following such treatment was significantly less than that observed following stimulation of the nucleus raphe medianus. (table 10 p. 79).

It has been demonstrated that the region of the midbrain most densely populated with cell bodies which contain 5-HT are the raphe nuclei (Dahlstrom and Fuxe 1964). In the region of the nucleus raphe medianus, although the majority of cell bodies are located within the nucleus, some have been located laterally and dorsally to the main population of cells (Dahlstrom and Fuxe 1964). The smaller elevation of forebrain 5-HIAA observed following stimulation of regions outside the nucleus raphe medianus could therefore have arisen as a consequence of :-

(1) The activation of a smaller and less dense population of cell bodies or their axons which contain 5-HT and which project to the forebrain.

(2) The activation of a group of afferent fibres coursing to the nucleus raphe medianus where they excite a small population of cells.

(3) The spread of current flowing from the stimulating electrode and exciting a small group of cells situated in the peripheral regions of the nucleus raphe medianus.

From the results of this study, it is impossible to ascertain which of these interpretations is the most accurate. Stimulation applied through electrodes situated laterally to the raphe could be activating the nucleus reticularis paragigantocellularis. This

nucleus appears to send afferent fibres to the raphe nuclei (Couch 1970). Electrical stimulation of this structure has been shown to increase the rate of firing of raphe neurones (Couch 1970). Thus the elevation of forebrain 5-HIAA concentration seen following the stimulation of non raphe regions in the lateral aspect of the midbrain may be related to the excitation of this afferent input to the raphe nuclei. It is probable however, that a combination of all three effects may give rise to the small but significant elevation in 5-HIAA following stimulation of non raphe sites. Previous workers have demonstrated that stimulation of a region of the rat midbrain 1.0 mm from the lateral border of the nucleus raphe medianus failed to elicit any changes in the forebrain concentration of either 5-HT or 5-HIAA (Aghajanian et. al. 1967, Kostowski et. al. 1969). Similarly, Sheard and Aghajanian et. al. (1968) observed close correlation between the site of stimulation and changes in the forebrain concentration of 5-hydroxy-indoles. Only when applied to those regions of the midbrain which contain 5-HT neurones did stimulation produce an increase in the fore-brain 5-HIAA concentration.

It would appear that the findings of this study are in agreement with those of previous workers and support the general concept that the amplitude of the elevation of 5-HIAA concentration produced following stimulation of the rat midbrain decreases as the point of stimulation moves away from the region of the nucleus raphe medianus. Since the vast majority of neurones which contain 5-HT, and ascend to terminate in the forebrain, arise from cell bodies situated in the nucleus raphe medianus, it can be proposed that changes in the forebrain concentration of 5-HIAA resulting from stimulation of this nucleus are related to the activation of the ascending neuronal pathways which emanate from it.

(3) The effect of chlorimipramine on forebrain 5-HT and 5-HIAA.

Background to the study.

The elevation of forebrain 5-HIAA concentration following stimulation of the nucleus raphe medianus has been widely interpreted as a consequence of the release of 5-HT from nerve terminals followed by the uptake into cells and conversion to 5-HIAA by monoamine oxidase and aldehyde dehydrogenase (Aghajanian et. al. 1967, Kostowski et. al. 1969) but there is an alternative interpretation of these findings. Both 5-HT and the enzymes required for its catabolism are stored within nerve terminals. The elevation of forebrain 5-HIAA concentration following raphe stimulation has been shown to be accompanied by an increase in the rate of synthesis of 5-HT (Gumulka et. al. 1969, Eccleston et. al. 1970, Shields and Eccleston 1972). If the predominant effect of stimulation was to increase the rate of 5-HT synthesis, and if a large proportion of the newly synthesised 5-HT could not be accommodated in the storage compartment of the presynaptic terminal, there would be an increase in the concentration of free cytoplasmic 5-HT. Since free 5-HT appears to be susceptible to deamination (Ross and Renyi 1967, Blackburn et. al. 1967), this might cause an increase in the concentration of 5-HIAA. The elevation of forebrain 5-HIAA following raphe stimulation might therefore be a consequence of an intraneuronal increase in the synthesis of 5-HT independent of extraneuronal release of the transmitter.

Experiment III was designed to determine what proportion of the changes in forebrain 5-hydroxyindole concentrations induced by raphe stimulation may arise as a consequence of the extraneuronal release of 5-HT, and how much may be due to intracellular effects. One mechanism requires that 5-HT released into the synaptic cleft should be

transported back into the cytoplasm of the nerve endings before it becomes accessible to the monoamine oxidase. The other mechanism requires that the changes in forebrain 5-HIAA are produced by purely intracellular process.

The biochemical processes involved in the catabolism of 5-HT may be assumed to be similar in both instances. It was therefore necessary to modify a process which is suggested to be a principal factor in one of the two mechanisms but not in the other. The effect of this modification on the change in forebrain 5-HT and 5-HIAA evoked by raphe stimulation may then provide some information as to the mechanism of action of raphe stimulation in eliciting the effects observed in the previous experiments. The process which is the special feature of the first interpretation is the transport of 5-HT back into the nerve terminal. There are many reports that the tricyclic antidepressants are powerful inhibitors of this transport process (Carlsson et. al. 1969a, 1969b, Shaskan and Snyder 1970, Meek et. al. 1970, Lidbrink et. al. 1971), and that among this group of compounds, chlorimipramine appears to have the most potent inhibitory action on the 5-HT accumulation process (Carlsson et. al. 1969b, Lidbrink et. al. 1971).

The increase in brain concentration of 5-HIAA following the administration of L-tryptophan has been interpreted as a predominantly intracellular process, due to progressive saturation of 5-HT stores, which could lead to a sharp increase in cytoplasmic 5-HT concentration. This could result in an increase in the rate of conversion of 5-HT to 5-HIAA. (Moir and Eccleston 1968, Moir 1971). Contrasting the effects of chlorimipramine upon the elevation of 5-HIAA by stimulation, and by L-tryptophan administration, should differentiate between the intracellular and extracellular origins of the changes in 5-HIAA concentra-

tion induced by these two procedures.

The dose of chlorimipramine used in these studies has been shown to inhibit the transport of 5-HT into nerve terminals by about 60% (Carlsson et. al. 1969). Under these circumstances, a proportion of the 5-HT which passes into the extracellular compartment will not be removed from that compartment by the transport process. If the major scource of free cytoplasmic 5-HT, accessible to monoamine oxidase, is the 5-HT transported back into the nerve terminal from the extracellular fluid, then chlorimipramine should increase the 5-HT content and decrease the 5-HIAA content of brain. In the absence of deliberate stimulation of the 5-HT neurones these effects might not be detectable. After prolonged stimulation they ought to become more marked.

(4) The effect of chlorimipramine on the resting levels of 5-HT and 5-HIAA.

Table 11 p. 81, shows that in unanaesthetised animals chlorimipramine produced a non-significant increase in 5-HT and a significant decrease in 5-HIAA, though the effects on anaesthetised animals were not significant. The reduction in 5-HIAA concentration seen following the injection of chlorimipremine in the unanaesthetised animals may be interpreted in three ways. (i) As a direct reduction in the intracellular catabolism of 5-HT. (ii) As an increase in the clearance of 5-HIAA from brain tissue. (iii) As an indirect reduction in the catabolism of 5-HT brought about by the inhibition of the transport of spontaneously released 5-HT back into the nerve terminal.

(i) Possible direct effects of chlorimipramine on the catabolism of 5-HT.

The depression of brain 5-HIAA concentration observed following

chlorimipramine administration has also been reported by previous workers. Bruinvels (1972) observed that the reduction in 5-HIAA seen following imipramine administration was accompanied by a reduction in 5-HT turnover which could be causally related to the impairment of the transport of tryptophan into brain cells. Halaris et. al. (1973) also suggested that the reduction in brain 5-HIAA concentration seen some time after the injection of chlorimipramine, but not immediately after the injection, could be related to a decrease in the synthesis and catabolism of 5-HT. If the major effect of low doses of chlorimipramine was to reduce directly the rate of synthesis and catabolism of 5-HT, it would be expected that chlorimipramine would affect changes in forebrain 5-HT and 5-HIAA following L-tryptophan administration in a similar manner to that seen in the control animals. However, rather than reducing the elevation of 5-HIAA seen after L-tryptophan administration, chlorimipramine actually increased the forebrain concentration of 5-HIAA in tryptophan treated animals (table 15 p. 88). It seems unlikely therefore that the effect of chlorimipramine (5 mg/Kg) on the forebrain concentration of 5-HT and 5-HIAA could be related to a direct inhibition of the rate of metabolism of the monoamine. The finding that chlorimipramine failed to reduce the production of 5-HT and 5-HIAA following the injection of L-tryptophan would also tend to suggest that at this dose, chlorimipramine is not affecting the availability of tryptophan for the production of 5-hydroxyindoles (Bruinvels 1972).

(ii) Possible effects of chlorimipramine on the transport of 5-HIAA.

The reduction in 5-HIAA concentration seen in the chlorimipramine treated animals may reflect a facilitation of the transport of 5-HIAA out of brain tissue. However, the finding that chlorimipramine

actually increased the elevation of 5-HIAA induced by L-tryptophan is difficult to reconcile with such an interpretation.

(iii) The effect of chlorimipramine on the uptake of 5-HT.

There are many reports of the powerful inhibitory action of the tricyclic antidepressants on the 5-HT uptake process. (Carlsson et. al. 1969a, 1969b, Shaskan and Snyder 1970, Meek et. al. 1970, Lidbrink et. al. 1971). In addition, it has been shown that in comparison with other members of this group of compounds, chlorimipramine has the most potent action on the 5-HT transport process (Carlsson et. al. 1969b, Lidbrink et. al. 1971). Because of this known action of chlorimipramine, and the difficulty in fitting the findings of this study with the alternative interpretations discussed above, the observed reduction in the forebrain concentration of 5-HIAA following the administration of chlorimipramine may best be interpreted as a consequence of the slowing of the transport into nerve terminals of spontaneously released 5-HT, thus reducing its rate of access to intracellular MAO. If this is the predominent effect of small doses of chlorimipramine, then increasing the rate of extracellular release of 5-HT should markedly increase the effect of chlorimipramine on the brain levels of these two indoles.

(5) The modification of the effect of stimulation by chlorimipramine.

Stimulation of the nucleus raphe medianus increased the forebrain concentration of 5-HIAA without affecting that of 5-HT (table 12 p. 83). Chlorimipramine profoundly diminished the effect of stimulation on 5-HIAA concentration and correspondingly increased the concentration of 5-HT (table 13 p. 84). The finding that chlorimipramine reduces the stimulation-induced elevation of 5-HIAA is in agreement with Samanin et. al. (1972) who reported that the related antidepressant imipramine, at a dose of 5.0 mg/Kg i.p. completely blocked the elevation of 5-HIAA

following raphe stimulation. In addition, they noted that the secondary amine desipramine administered in similar doses had no effect on the change in forebrain 5-HIAA induced by raphe stimulation. Since desipramine is a relatively weaker inhibitor of 5-HT transport than is imipramine, the authors concluded that the reduction in 5-HIAA seen in the stimulated animals given imipramine, could have arisen as a consequence of the inhibition of 5-HT uptake denying 5-HT access to intraneuronal MAO. Although the conclusion of Samanin et. al. (1972) may well be correct, these authors failed to control for possible direct effects of imipramine on the catabolism of 5-HT. Thus in the absence of control studies, both the findings of Samanin et. al. and the results of the study reported here would fail to determine whether the reduction in the stimulation-induced elevation of 5-HIAA concentration observed in the chlorimipramine treated animals arose as a consequence of the inhibition of 5-HT uptake (as described above), or as a consequence of a direct reduction in the intracellular catabolism of 5-HT. In the experiment reported here however, a study was conducted in order to determine to what extent a direct effect of chlorimipramine on the catabolism of 5-HT may be responsible for the effects observed following raphe stimulation. The results of this study are discussed below.

(6) The modification of the effects of L-tryptophan by chlorimipramine.

Table 14 p. 86 shows that the forebrain concentration of both 5-HT and 5-HIAA was significantly increased by the administration of L-tryptophan. As discussed previously, there is evidence that the increased concentration of 5-HIAA seen in brain tissue following the administration of a high dose of L-tryptophan is produced by the catabolism of the large amount of free cytoplasmic 5-HT which is made available by this procedure (Moir and Eccleston 1968, Moir 1971). If chlorimipramine is having a

direct effect on the catabolism of 5-HT, it might be expected that chlorimipramine would alter the levels of 5-hydroxyindoles following tryptophan administration in a manner similar to that observed following stimulation. The elevation of 5-HT by tryptophan was unaffected by chlorimipramine, whereas the elevation of 5-HIAA by tryptophan was increased by chlorimipramine (table 15 p. 88). Chlorimipramine therefore had opposite effects on the change in 5-HIAA levels induced by stimulation and L-tryptophan administration. The results of this study would therefore suggest that chlorimipramine has little or no direct inhibitory effects on the catabolism of 5-HT in brain tissue. This being so, it can be suggested that the reduction in the stimulation-induced elevation of 5-HIAA observed in the chlorimipramine treated animals may best be interpreted as a consequence of the inhibition of the 5-HT uptake process which would deny extracellularly released 5-HT access in intraneuronal monoamine oxidase.

These findings would support the concept that a large proportion of the 5-HIAA produced following raphe stimulation arises as a consequence of the extracellular release of 5-HT, followed by uptake into cells and conversion to 5-HIAA. In contrast, it would appear that the extraneuronal release of 5-HT is only involved to a small extent in the conversion of L-tryptophan to 5-HIAA. That chlorimipramine actually increases the elevation of 5-HIAA following L-tryptophan suggests either an acceleration of the metabolism of L-tryptophan through the 5-HT pathway or the inhibition of 5-HIAA transport from the brain. From these studies I am unable to determine whether chlorimipramine has any direct action on either of these two processes. While the results of the above studies have demonstrated that chlorimipramine is unlikely to be exerting any direct effects on the synthesis and catabol-

ism of 5-HT, one possible indirect effect of chlorimipramine on 5-HT turnover which may be influencing the response to stimulation has yet to be examined. This is discussed in the following section.

(7) Possible effects of a neuronal feedback system on the synthesis of 5-HT.

It has been reported that chlorimipramine reduces the synthesis and metabolism of 5-HT (Corrodi and Fuxe 1969, Meek and Werdinius 1970, Halaris et. al. 1975). Such a reduction in turnover would account for the lower levels of 5-HIAA in the forebrain following administration of chlorimipramine in both stimulated and unstimulated animals. However, as mentioned previously the increase in the elevation of 5-HIAA seen following administration of chlorimipramine animals given L-tryptophan suggests that a direct inhibition of 5-HT synthesis is unlikely.

In order to account for the observed reduction in 5-HT synthesis, Corrodi and Fuxe (1968, 1969) suggested that a reflex inhibition of the rate of discharge of the 5-HT-containing presynaptic neurone could be brought about by an increase in the rate of discharge of the post synaptic cell. Tricyclic antidepressants, by inhibiting the transport of 5-HT away from the synaptic cleft, would further increase the rate of discharge of the postsynaptic cell (Meek et. al. 1970, Clineschmidt et. al. 1971) and thus produce a stronger reflex inhibition of the rate of firing of the presynaptic neurone.

Corrodi and Fuxe further suggested that the rate of synthesis of 5-HT was dependent upon the rate of discharge in the neurones which contain 5-HT. On this basis they suggested that the reduction in 5-HT synthesis seen following the administration of tricyclic antidepressants was directly related to the build up of 5-HT in the synaptic cleft which activated the inhibitory feedback system described above.

Bramwell (1972, 1974) has shown that imipramine inhibits the spontaneous discharge of neurones in the nucleus raphe dorsalis in the rat, and that there was a strong correlation between the potency of a tricyclic antidepressant in inhibiting 5-HT transport into cells, and its potency in diminishing the frequency of discharge of these neurones. Corrodi et. al. (1967) and Corrodi and Fuxe (1968) similarly demonstrated good correlation between inhibition of 5-HT uptake and reduction of 5-HT synthesis.

Although it is possible that these correlations could exist without such a feedback system being operated, it was necessary to ensure that such a system, if present, was not influencing to any great extent the results obtained in these studies. The major difference between my experiments and those of Bramwell (1972, 1974), and Corrodi and coworkers (1967, 1968, 1968), is that the latter were investigating the responses of spontaneously active raphe neurones, whereas in my experiments, the activity in the 5-HT pathways was evoked by repetitive stimulation of the nucleus raphe medianus. In this situation, it would be necessary for the feedback mechanism to reduce the effectiveness of stimulation in order to have profound effects on the synthesis and metabolism of 5-HT.

At this point I would like to examine some possible electrical changes that may be occurring in neural tissue in response to applied current, and how these changes may be influenced by an inhibitory afferent input. With peripheral nerves, the nerve is usually placed on two electrodes. Current flows from the anodic electrode along the nerve fibre to the cathodic electrode where it leaves. Depolarisation occurs where the current leaves the nerve, that is at the catho-ic electrode. In the situation employed in my experiments, the anode

and cathode are separated by about 1.0 mm. and current flows from the anode to the cathode through the cell mass of the raphe cell bodies. In this situation it may be assumed that cells are depolarised at the point where current leaves the cell on route to the cathode. In my experiments, the direction of current flow is changed so that for a 15 minute period of stimulation the inner pole is cathodic. It is then made anodic for a second period of 15 minutes. (The order in which these changes were made was random.) With stimulation applied in this manner, cells lying close to the cathode will receive a higher intensity of stimulation than those lying close to the anode, and changing the direction of current flow will ensure that regions of high stimulus intensity are notlocalised to one particular group of cells. However, it is apparent that when applying current in this way some cells will receive a higher intensity of stimulation than others.

When applying pulses to a mass of cells, the concept of threshold, suprathreshold, maximal and supramaximal stimulus intensities becomes entirely nebulous. Even if current was being applied to a homogeneous collection of cells, it is very unlikely that the thresholds of individual cells will be similar. Consequently, a current of supramaximal intensity for any one cell may well be subthreshold for another. Thus we have no real indication of what is happening when electrical current is being applied in this manner. Cells of probably widely differing thresholds are being subjected to an electrical field in which the current density applied to any one cell varies with the position of that cell within the field.

The stimulus current employed in my experiments (100 μA) is about half that required to produce electrolytic lesions in this area of the brain. It might be valid to assume in this situation that some of

the cells receiving a high current density may be influenced by a supramaximal stimulus, whereas those situated on the edge of the electric field may be barely excited, if at all. Conversely, the threshold of these cells may be so low that all cells are excited supramaximally. Let us assume for a moment that these cells are receiving an intact inhibitory input which is being driven by the build up of 5-HT at the post synaptic receptors. Such an inhibitory input would be expected to initiate an inhibitory post synaptic potential (IPSP) on the raphe cell membrane. The result of this procedure would be an increase in the potential difference across the cell membrane and thus an increase in the threshold of these cells. Most of the IPSP's recorded in the CNS are only a few millivolts in amplitude, and in situations where the cells are being excited by pulses of supramaximal intensity, there is an excellent chance that the membrane will be depolarised sufficiently to overcome the IPSP and still initiate firing in that neurone. Conversely, in cells which have either an inherently high threshold, or which are being excited by just suprathreshold stimulation, the IPSP may be sufficient to inhibit firing in that cell. Thus in situations where raphe cells are being excited by electrical pulses of moderate intensity, there is a chance that inhibitory inputs to some cells will be overcome by the intensity of electrical stimulation. Thus the inhibitory influences arising as a result of the proposed negative feedback mechanism could well be overcome by the electrical pulses in some of the raphe neurones.

In support of the theoretical considerations outlined above, there is some evidence that electrical stimulation of the raphe is indeed able to activate ascending fibres from these nuclei in the presence of drugs believed to activate the proposed inhibitory feedback system. In

addition to the tricyclic antidepressants, other drugs are also believed to reduce the activity of raphe neurones by activating an inhibitory neural input to them (Aghajanian 1972). One such drug is Lysergic acid diethylamide (LSD). This drug has been shown to reduce the synthesis and turnover of 5-HT in the brain (Lind et. al. 1969, Rosecrans et. al. 1967), and to decrease the rate of firing of raphe neurones in rats (Aghajanian et. al. 1968, 1970). The inhibition observed at very low doses of LSD (10 μg/Kg) applied parenterally was found to be highly selective for raphe neurones. In addition to a direct inhibitory action on the raphe cells (Aghajanian et. al. 1972), it has been proposed that LSD may mimic the action of 5-HT on post-synaptic receptors, and in doing so activate the negative feedback loop (Aghajanian 1972). The interaction between LSD and electrically stimulated raphe neurones has been studied by Bloom et. al. (1972). These workers investigated the activity of single neurones in the suprachiasmatic nucleus of the cat. The suprachiasmatic nucleus is one of the regions of the brain which receives a dense innervation of efferent raphe terminals. The activity of these neurones was found to be markedly inhibited by electrical stimulation of the nucleus raphe medianus. Although low (10 μg/Kg) doses of LSD administered parenterally have been found to markedly inhibit the firing of spontaneously active raphe neurones, electrical stimulation of the nucleus raphe medianus continued to inhibit activity in the suprachiasmatic nucleus even after the parenteral administration of large doses (up to 200 μg/Kg) of LSD. Thus, in this situation, electrical stimulation of the nucleus raphe medianus was sufficient to overcome the inhibitory effects of LSD on raphe neurones. If LSD and tricyclic antidepressants do affect raphe neurones by indirect activation of the same

inhibitory input, it seems likely that electrical stimulation applied to the raphe could overcome the inhibitory action of both groups of drugs. Thus any indirect inhibition of raphe cells exerted by chlorimipramine in my experiments may be overcome by the manner in which the cells of the raphe nucleus were activated. It may be suggested therefore that the feedback system proposed by Corrodi and Fuxe (1968, 1969) is unlikely to provide the basis of our observations in these experiments.

In summary, the results of experiment III provide evidence supporting the view that the elevation of forebrain 5-HIAA concentration induced by electrical stimulation of the nucleus raphe medianus originates mainly as a result of the extraneuronal release of 5-HT followed by uptake and catabolism. In addition the results of the studies support the use of the technique as a model for the investigation of the actions of psychotherapeutic substances on the release of 5-HT in the rat forebrain.

Using the model developed in experiment III, the next study investigated the action of Li^+ treatment on changes in forebrain 5-hydroxyindoles induced by electrical stimulation or L-tryptophan administration.

(8) The effect of 10 day lithium pretreatment on the concentration of 5-HT and 5-HIAA in the rat forebrain.

Pretreatment with Li^+ for 10 days (0.75 mEq/Kg/day) had no significant effects on the forebrain concentration of 5-HT in either unanaesthetised or anaesthetised animals. However, the concentration of 5-HIAA was elevated in both groups of animals (tables 16 p.90 and 19 p.95). An increase in 5-HIAA without a change in 5-HT concentration

may indicate either an increase in the turnover of 5-HT or an inhibition of the transport of 5-HIAA out of the brain. Previous workers who have examined the effect of Li^+ treatment for periods of 10 days or more have noted either no change in the synthesis and turnover of 5-HT (Bliss and Ailion 1970, Knapp and Mandell 1973), or a reduction (Ho et. al. 1970). The possibility that Li^+ is increasing the turnover of 5-HT therefore, does not agree with previous data. The differences may be due to the much lower dose of Li^+ used in my experiments. (see table 1 p. 26). It should also be noted that at this lower dose, Li^+ had no effect on brain or plasma tryptophan concentrations (table 29 p. 115). This would suggest that any effect that Li^+ is exerting on the synthesis and turnover of 5-HT is unlikely to be related to a change in brain and free circulating tryptophan concentrations. (Tagliamonte et. al. 1971, 1973, see later).

Secondly, there is a possibility that Li^+ may be inhibiting the transport of 5-HIAA out of the brain. 5-HIAA appears to be transported from the brain by a probenecid-sensitive active transport process (Neff et. al. 1967, Ashcroft et. al. 1968, Cserr and Van Dyke 1972, Forn 1972, Sampath and Neff 1974, Wolfson et. al. 1974). There are no direct reports of the effect of Li^+ on the transport of 5-HIAA from brain tissue. However, Sheard and Aghajanian (1970) reported that acute Li^+ treatment appeared to have no effects on this transport process. Obviously direct measurement of the transport of 5-HIAA from c.s.f. to blood are required before one can ascertain whether or not the treatment received in these experiments is affecting 5-HIAA transport.

In conclusion, it can be stated that at this stage, it is difficult to determine the relative contributions of these two possible actions of Li^+ in producing the effects observed in this experiment.

(9) The modification of the effect of L-tryptophan by pretreatment with lithium for 10 days.

The administration of L-tryptophan (100 mg/Kg i.p.) significantly elevated the forebrain concentration of 5-HIAA measured 30, 60 and 90 minutes after the injection in both the saline controls and in animals which had received 10 day Li^+ pretreatment. Similarly, the forebrain concentration of 5-HT was also significantly elevated by this treatment in the control group of animals. However, in the Li^+ pretreated animals, the 5-HT concentration was only significantly increased by L-tryptophan when measured 30 minutes after the injection (table 19, p. 92). The comparison of the effects of L-tryptophan administration between animals treated and not treated with Li^+ (table 18 pages 92, 93 and 94) shows that the changes in forebrain 5-hydroxyindole concentrations measured 30 and 90 minutes after the administration of tryptophan are not significantly different between the two groups of animals. However, the elevation in forebrain 5-HT measured 60 minutes after L-tryptophan administration was significantly less in the Li^+ pretreated animals. At the same time, the elevation in 5-HIAA induced by L-tryptophan administration was significantly greater in the Li^+ pretreated animals. Thus 10 day Li^+ pretreatment significantly reduces the maximum increase in 5-HT concentration induced by L-tryptophan administration and enhances the increase in 5-HIAA.

The result of this study may be interpreted in four ways. (i) Li^+ may increase the rate of deamination of 5-HT, either by increasing the activity of the deaminating enzyme MAO, or by inhibiting the storage of 5-HT within the neurone. (ii) Li^+ may divert the metabolism of L-tryptophan away from the production of 5-HT and towards the production of 5-hydroxyindolepyruvic acid through the 5-HTP aminotransferase pathway.

(iii) Li^+ may reduce the clearance of 5-HIAA. (iv) Li^+ may affect the rate at which tryptophan is made available for the synthesis of 5-hydroxyindoles.

(ia) Possible effects of Li^+ on the deamination of 5-HT - MAO activity.

The most straightforward interpretation of this data is that Li^+ pretreatment increases the deamination of 5-HT. As stated above, this could be brought about by an increase in the activity of the deaminating enzyme MAO. It is now generally believed that 5-HT is first synthesised in the cytoplasm and then transported into the storage compartment. In this situation, MAO and the storage process may be considered to compete for the newly synthesised 5-HT while it is still free in the cytoplasm. An increase in MAO activity may therefore increase the proportion of free 5-HT which can undergo deamination and correspondingly reduce the amount available for storage. Such an effect could account for the smaller amount of 5-HT and the larger amount of 5-HIAA produced from the loading dose of tryptophan in the Li^+ pretreated animals.

A number of factors indicate however, that the effects observed in this study may not readily be interpreted as an increase in the activity of MAO. The enzyme is present in great excess in the brain (Gey and Pletscher 1961), therefore in order to have profound effects on the deamination of 5-HT, the activity of the enzyme would have to be increased to a considerable degree. In addition, because MAO is present in great excess, it is likely that the major factor which limits the rate of 5-HT deamination is the ease with which the substrate and enzyme are able to interact (see next section), rather than the activity of the enzyme itself. Finally, there is little information available in the literature concerning the action of Li^+ on MAO activity. No

animal studies comparable to those described here appear to have been reported in the literature. Although not strictly comparable with our study, it is interesting to note that Murphy and Weiss (1972) reported no change in human platelet MAO activity following treatment with Li^+.

In conclusion, I should like to suggest that the apparent increase in the deamination of 5-HT observed in this experiment is unlikely to be due to a direct effect of Li^+ on MAO activity.

(ib) Possible effect of Li^+ on the deamination of 5-HT - storage.

It is now universally accepted that 5-HT is stored in the presynaptic terminal in a form which protects it from deamination by MAO (Ross and Renyi 1967). The results of the previous experiment (experiment III) confirmed that the elevation of 5-HIAA produced by a loading dose of L-tryptophan most probably arose as a consequence of the saturation of the 5-HT storage compartment with a resultant increase in the concentration of 5-HT free in the cytoplam which was available for metabolism by intraneuronal MAO (Moir and Eccleston 1968, Moir 1971). In this situation, the ease with which newly synthesised 5-HT can be stored determines the concentration of free cytoplasmic 5-HT. The reduction in the tryptophan-induced elevation of 5-HT concentration, accompanied by the increased elevation of 5-HIAA seen in the Li^+ pretreated animals, could have arisen as a result of a reduction in the ability of the storage compartment to retain all the newly synthesised 5-HT. In this situation, a greater proportion of the synthesised 5-HT would remain free in the cytoplasm and be susceptible to deamination by MAO. This would lead to the observed increase in the tryptophan-induced elevation of 5-HIAA and the concomitant decrease in 5-HT.

An interesting finding of this study was that the changes in 5-HT

and 5-HIAA seen following the loading dose of L-tryptophan were not apparent in animals which had received Li^+ pretreatment only (see section 8, tables 16, 17 page 90, c.f. tables 18, 19 pages 92 - 95). The concentration of 5-HIAA was, in fact, significantly increased by treatment with Li^+, but the concentration of 5-HT remained unchanged (tables 16 and 17 page 90). With respect to the proposed effect of Li^+ on the storage of 5-HT, these findings could be accounted for by proposing that Li^+ affects the rate at which 5-HT can be transported from the cytoplasm to the storage compartment. As a consequence of this it could be proposed that the rate of 5-HT synthesis in animals given Li^+ alone was sufficiently slow to allow the storage compartment to accumulate most of the newly synthesised 5-HT. However, following a loading dose of L-tryptophan, the rate at which 5-HT is being produced would be greatly in excess of the rate at which the 5-HT could be transported into the storage compartment. Alternatively, it could be that the maximum capacity of the store is reduced by Li^+ pretreatment. In this situation, the storage compartment would be able to accomodate the synthesised 5-HT during near normal rates of synthesis. However, in situations where the rate of 5-HT production is high, such as that following a loading dose of L-tryptophan, the storage compartment would be saturated more quickly and excessive "overspill" into the cytoplasm would occur. The possibility that Li^+ may be affecting the storage of 5-HT in a manner similar to that outlined above, prompted the investigation of the effect of Li^+ on the accumulation and retention of 5-HT by isolated synaptosomes. The results of this investigation and a more detailed discussion of the possible effects of Li^+ on the storage of 5-HT are presented later.

(ii) Possible effect of Li^+ on the route of tryptophan metabolism.

It has been demonstrated that a number of aromatic amino acids can undergo transamination in brain tissue (Albers et. al. 1962, Tangen et. al. 1965). The transamination of 5-HTP in rat brain opens a route of tryptophan metabolism which can functionally "bypass" 5-HT and MAO through the production of 5-hydroxyindolepyruvic acid (Fig 9) (Millard and Gal 1971). A change in the direction of tryptophan metabolism through this pathway would result in a change in the proportions of 5-HT and 5-HIAA produced from a loading dose of L-tryptophan. An increase in the transamination of 5-HTP at the expense of decarboxylation would be expected to increase the production of 5-HIAA through 5-hydroxyindolepyruvic acid, and to decrease the production of 5-HT from 5-HTP. The results of this study may therefore indicate that Li^+ could be influencing tryptophan metabolism by increasing the transamination of 5-HTP. At the present time, little information is available concerning this metabolic pathway. Until the direct examination of the effect of Li^+ on this pathway is conducted it must be considered a possible site of action of Li^+ in exerting the effects observed in this study.

<u>(iii) Possible effect of Li^+ on the transport of 5-HIAA.</u>

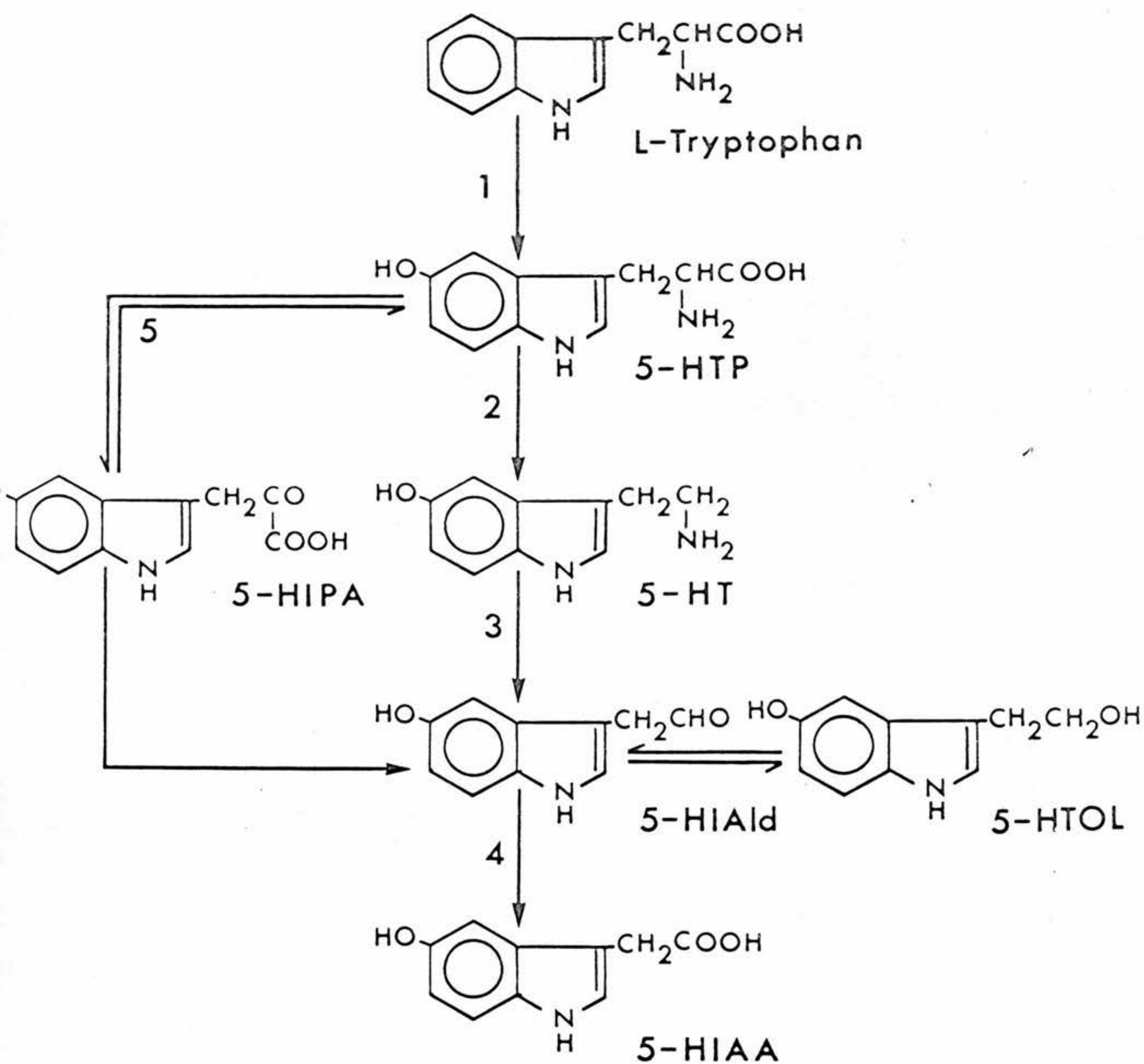
The increased concentration of 5-HIAA seen both in animals given Li^+ alone, and 60 minutes after the i.p. administration of L-tryptophan, may indicate that Li^+ is inhibiting the clearance of 5-HIAA from the brain. This could not, however, account for the reduction in 5-HT concentration which accompanied the latter increase in 5-HIAA, unless one postulated a negative feedback system. Such a feedback system would require an increase in the concentration of 5-HIAA to feed back and inhibit further synthesis of 5-HT. This could account both for the smaller changes in 5-HT measured 60 and 90 minutes after the trypto-

FIG. 9.

THE MAJOR ROUTES OF 5-HYDROXYINDOLE SYNTHESIS AND METABOLISM.

The abbreviations used are the same as those used in Fig 1. The diagram shows how the transamination of 5-HTP (reaction 5), can effectively route L-tryptophan metabolism away from the production of 5-HT, with the subsequent production of larger amounts of 5-hydroxyindoleacetaldehyde.

5-HIPA - 5-hydroxyindolepyruvic acid. The enzyme catalysing reaction 5 is 5-HTP aminotransferase.


CH2CHCOOH
NH2
N
H
L-Tryptophan
1
HO
CH2CHCOOH
NH2
5
N
H
5-HTP
2
CH2 CO
COOH
N
H
5-HIPA
HO
CH2CH2
NH2
N
H
5-HT
3
HO
CH2CHO
N
H
5-HIAld
HO
CH2CH2OH
N
H
5-HTOL
4
HO
CH2COOH
N
H
5-HIAA

phan injection, and for the decline in 5-HIAA concentration seen 90 minutes after tryptophan. As yet there is no evidence that such a feedback system exists; on the contrary there is evidence to suggest that inhibition of 5-HIAA clearance does not inhibit the rate of 5-HT synthesis. The estimation of 5-HT turnover rates by measuring the rate of accumulation of 5-HIAA after completely inhibiting 5-HIAA clearance by probenecid has been used extensively in the past. Studies comparing different methods of estimating 5-HT turnover have tended to provide comparable results (Neff et. al. 1969). The most direct method of estimating the rate of 5-HT synthesis is to measure the conversion of ^{14}C-tryptophan to ^{14}C-5-HT. A comparison of the rate of 5-HT synthesis as measured by this technique, and by the accumulation of 5-HIAA after probenecid showed that in the same strain of rats, the rate of 5-HT synthesis was almost identical (Neff et. al. 1969). It would thus appear that the build up of 5-HIAA in brain tissue does not inhibit the further synthesis of 5-HT. It is unlikely therefore that changes in the transport of 5-HIAA are responsible for the effects of Li^+ observed in this experiment.

(iv) Possible effects of 10 day lithium pretreatment on the availability of tryptophan for 5-HT synthesis.

It can also be seen from table 18 pages 92 - 94 that there are some basic differences in the pattern of changes in forebrain 5-hydroxyindoles following tryptophan treatment in the saline and Li^+ treated groups. In the saline group, the maximum elevation of 5-HT concentration occurred 60 minutes after the injection of tryptophan. Thereafter, the 5-HT concentration declined. In the Li^+ treated animals however, the maximum elevation of 5-HT occurred 30 minutes after the injection and declined slowly over the next 60 minutes. Similarly, the elevation

of 5-HIAA concentration in the saline treated animals was still increasing when measured 90 minutes after the injection whereas in the Li^+ treated animals the elevation of 5-HIAA had reached a maximum 60 minutes after the injection. It looks therefore, as though Li^+ pretreatment may be increasing the rate at which the administered tryptophan is made available for conversion to 5-HT. Although the elevation of 5-HT seen in the Li^+ pretreated animals was considerably lower than that seen in the saline group, it is impossible to say whether or not the maximum elevation in 5-HT occurred between 0 and 30 minutes after the injection of tryptophan. In this situation, measurement of the 5-HT concentration 30, 60 and 90 minutes after tryptophan may only give an indication of the decline in 5-HT concentration from a maximum. The changes in 5-HIAA concentration would also support the suggestion that Li^+ is increasing the availability of tryptophan. The maximum increase in 5-HIAA concentration in the control group of animals appeared to lag the maximum increase in 5-HT by at least 30 minutes. Thus the peak increase in 5-HIAA concentration seen 60 minutes after the injection of L-tryptophan in the Li^+ treated animals may indicate that the maximum elevation in 5-HT could have occurred between 0 and 30 minutes.

The examination of the distribution of tryptophan between plasma and brain tissue following a loading dose of L-tryptophan may provide some indication of what is happening here. L-tryptophan has been shown to be present in human, dog and rat plasma in two forms. A large proportion is bound to plasma albumen, and a smaller proportion remains free in the tissue fluid (McMenamy and Oncley 1958, McMenamy et. al. 1961, Moir 1971a, Tagliamonte et. al. 1973). That portion of plasma tryptophan which remains unbound will be available for transpor-

tation from the plasma to the tissues. There is some indication that the concentration of tryptophan in the brain is directly related to the free plasma concentration of the amino acid (Knott and Curzon 1972), Tagliamonte et. al. 1973), and that this in turn is similarly related to the rate at which 5-HT is produced (Tagliamonte et. al. 1971, 1973, Moir 1971, Knott and Curzon 1972). Thus there is some suggestion that the brain concentration of tryptophan and the synthesis of 5-HT can be influenced by the level of circulating free tryptophan. Any changes in the utilisation of tryptophan between these two compartments may therefore be detected by the direct measurement of the amino acid in these tissues. McMenamy et. al. (1961) demonstrated that the small proportion of human plasma tryptophan which was dialysable could be reliably estimated by the concentration found in an ultrafiltrate of the plasma. In this experiment, an indication of the concentration of free plasma tryptophan was therefore obtained by preparing a plasma ultrafiltrate.

The concentration of tryptophan in brain, plasma, and plasma ultrafiltrate was measured 30, 60, and 90 minutes after the injection of the amino acid. Administration of L-tryptophan (100 mg/Kg i.p.) significantly increased the concentration of the amino acid in all three tissues at all times measured in both groups of animals (table 30 pages 116 - 118). A comparison of the changes in tryptophan concentration observed between animals treated and not treated with Li^+ (table 31 pages 119 - 121) demonstrated that Li^+ had no significant effects on the increase in tryptophan concentrations elicited by the injection of the amino acid. If, as has been suggested, Li^+ increases the rate of utilisation of tryptophan, one might have expected the concentration of tryptophan in the brain to follow a pattern similar to that observed for the changes in brain 5-HT in these animals. Although there were no significant differences in the changes

in tryptophan concentration observed following saline or Li^+ pretreatment, it can be seen from table 31a p. 119 (c.f. table 18), that the pattern of change in brain tryptophan concentration was indeed similar to the change in 5-HT concentration both in the saline and Li^+ pretreated groups. Thus the maximum change in 5-HT occurred 60 minutes after the injection in the saline pretreated group, while in the Li^+ pretreated group these changes were greater 30 minutes after the injection.

Since only a portion of the brain tryptophan will be entering the 5-HT pathway, it is possible that a change in the metabolism of tryptophan through the 5-HT pathway could have occurred without significantly affecting the brain concentration of the amino acid. However, if Li^+ is increasing the utilisation of tryptophan through the 5-HT pathway such that the maximum change in 5-hydroxyindole production occurs more rapidly, there must be some form of limiting factor, since the brain concentration of tryptophan remains significantly elevated at 90 minutes both in the saline and Li^+ pretreated groups. Thus, at this time, the brain still contains sufficient tryptophan to allow the production of 5-HT and 5-HIAA to continue to a similar degree in both groups of animals. It is unlikely, therefore, that an increase in the rate at which the administered tryptophan is made available for the production of 5-hydroxyindoles , forms the basis for the effects of Li^+ observed in this study.

(10) The effect of 10 day lithium pretreatment on the uptake and retention of ^{14}C-5-HT by isolated synaptosomes.

The two interpretations which best fit the data derived from the previous experiments are that 10 day Li^+ pretreatment may be either affecting the ability of the 5-HT nerve terminals to store 5-HT, or influencing the route through which tryptophan is metabolised.

In order to determine which of the two interpretations is the more

likely, experiments were designed to study the accumulation and retention of ^{14}C-5-HT by isolated synaptosomes. In this way it should be possible to examine in a more direct manner any effects on the storage of 5-HT.

Two experiments were conducted. The first examined the accumulation of ^{14}C-5-HT at 37°C, and the second examined the ability of the synaptosomes to retain accumulated ^{14}C-5-HT at 37°C.

Wong et. al. (1973) demonstrated that 5-HT was accumulated by rat brain synaptosomes by means of a high affinity process, and a low affinity process. There is some evidence that the high affinity process represents transport into authentic 5-HT terminals, whereas the low affinity process may be responsible for the transportation of 5-HT into catecholamine terminals (Shaskan and Snyder 1970, Wong et. al. 1973). In the initial studies conducted in this investigation, synaptosomes were incubated in 0.1 μM 5-HT. This concentration of 5-HT is sufficiently low to enable the amine to be transported primarily by the high affinity process. (Shaskan and Snyder 1970, Wong et. al. 1973).

<u>(11) The effect of temperature on the accumulation of 5-HT by synaptosomes.</u>

Table 32 p. 123 shows that the synaptosomes accumulated 5-HT much more rapidly at 37°C than at 2°C suggesting that the transport process in the synaptosomes prepared in these experiments was behaving normally with respect to temperature dependence.

<u>(12) The effect of 10 day lithium pretreatment on the accumulation of 5-HT by synaptosomes.</u>

Table 33a p. 125 shows that synaptosomes incubated in 0.1 μM

^{14}C-5-HT rapidly accumulate the amine over the 10 minute incubation period studied. It can also be seen that the accumulation of 5-HT by synaptosomes prepared from rats which had received 10 day Li^+ pre-treatment was not significantly different from that observed in the control group of animals. Table 33b p. 126 shows that the concentration of 5-HT metabolites present in the synaptosomes is reasonably constant over the 10 minute incubation period. It can also be seen that Li^+ pretreatment had no significant effects on the synaptosomal concentration of metabolites derived from accumulated 5-HT. If Li^+ pre-treatment is affecting the rate at which 5-HT is transported from the cytoplasm to the storage compartment, one might have expected to see an increase in the synaptosomal concentration of metabolites accompanied by a reduction in 5-HT concentration. This was not so. However, in order for this assumption to be valid, it must be assumed that the concentration of 5-HT measured in the synaptosome represents wholly that which is present in the storage compartment. For this to be so it would also be necessary for cytoplasmically located 5-HT to be immediately catabolised by MAO. If a significant proportion of the 5-HT measured is free in the cytoplasm, an effect of Li^+ on the storage compartment may be masked by a high cytoplasmic 5-HT content. Studies of the accumulation of 5-HT by rat brain slices prepared from untreated animals and animals which had received an injection of the MAO inhibitor pheniprazine, demonstrated that the amount of 5-HT accumulated was markedly greater in slices from the pheniprazine treated animals (Ross and Renyi 1967). However, the degree to which MAO inhibition increased the amount of 5-HT accumulated differed considerably depending on the time for which the slices were incubated. At incubation times of up to 5 minutes there appeared to be no difference in the amount of

5-HT accumulated by the brain slices from treated and untreated animals. It was only after 10 minutes incubation that an increase in the amount of 5-HT accumulated was detectable in the pheniprazine treated animals, and the effect was not maximal until the slices had been incubated for 30 or 60 minutes. Blackburn et. al. (1967) reported similar effects following the addition of the MAO inhibitor iproniazid directly to the medium in which rat brain slices were incubated.

The lack of effect of the MAO inhibitors on the accumulation of 5-HT following the shorter periods of incubation may indicate that 5-HT is able to enter the nerve cell more rapidly than MAO is able to deaminate it. In my experiments, the maximum incubation time used was 10 minutes. It is possible therefore that a proportion of the 5-HT measured in the synaptosomal extract may have been located in the cytoplasm so that an effect on the storage compartment may have remained undetected.

A further point to consider is that if Li^+ acts to reduce the maximum capacity of the storage compartment, it may be expected that 5-HT would be accumulated at an identical rate in both saline and Li^+ pretreated animals until the maximum capacity is reached. Thereafter, a difference between Li^+ and saline pretreatment would be expected to become detectable. If the maximum capacity of the 5-HT store is greater than 7.0 pmoles/mg protein, an effect of Li^+ on this compartment would not be apparent from the results of this experiment.

In conclusion, the results of this experiment while failing to confirm an action of Li^+ on the storage of 5-HT were also unable to deny that Li^+ may be affecting 5-HT storage. In an attempt to overcome some of the problems outlined above, further experiments were conducted and these are discussed below.

(13) The effect of 10 day lithium pretreatment on the retention of 5-HT by isolated synaptosomes.

Two points needed clarifying from the previous experiment. The first point refered to the location of the 5-HT measured in the synaptosomes, and the second to the concentration of 5-HT required to be stored by the synaptosome. In an attempt to clarify these points, the ability of synaptosomes to retain accumulated 5-HT at 37°C was investigated. Synaptosomes were incubated in 0.1 µM or 1.0 µM ^{14}C-5-HT for 10 minutes at 37°C. These were then washed and reincubated in fresh Krebs at 37°C for periods of 0 - 30 minutes. In this situation, any 5-HT which is unable to be retained by the storage compartment should be metabolised by MAO. Thus any effect of Li^{+} on the storage compartment that may have been masked by cytoplasmic 5-HT should then become visible. In addition, the incubation of the synaptosomes in a higher concentration of 5-HT may expose a limiting factor on the size of the storage compartment.

It can be seen from table 34a p. 128 that the concentration of 5-HT retained by the synaptosome declined gradually over the 30 minute period of incubation, both in synaptosomes prepared from saline controls, and Li^{+} pretreated animals. It can also be seen that the concentration of 5-HT retained by the synaptosomes was significantly smaller in the Li^{+} pretreated group following incubation for 5 minutes. However, there were no significant differences observed at any other period of incubation. The reasons for the slow decline in 5-HT concentration cannot be ascertained from this data. Because of the relatively active nature of rat brain MAO (Huszti 1972), it is unlikely to be the slowness of the removal of cytoplasmic 5-HT. On the other hand it has been previously demonstrated that vesicles isolated from the caudate

nucleus are relatively thermolabile and release dopamine spontaneously when incubated in mock intracellular fluid at 37°C (Philippu and Beyer 1973). Though in this case, the vesicles are enclosed within the synaptosomal membrane, and will be in contact with true intracellular fluid, it is possible that the decline in 5-HT concentration observed in both groups of synaptosomes may be related to the spontaneous leakage of 5-HT from the storage compartment.

The rapid fall in 5-HT concentration in the synaptosomes isolated from Li^+ pretreated animals may indicate that the ability to retain 5-HT is hampered in these animals. Though there were no significant changes in 5-HT concentration measured at the other incubation times, the fact that it was lower in the Li^+ pretreated group over the first 20 minutes of the incubation period may support this supposition.

The synaptosomal concentration of 5-HT metabolites (table 34 pages 128 and 130) was also found to decline gradually over the 30 minute period of incubation. This would suggest that the clearance of metabolites from the synaptosome is occurring more rapidly than is the production of metabolites from the 5-HT which has leaked from the storage compartment. The significant rise in the concentration of metabolites seen after 30 minutes in the synaptosomes prepared from the Li^+ pretreated group indicates a retardation in the decline in metabolite concentration. Why this would be is difficult to ascertain. It is unlikely to be due to a sudden increase in the release of 5-HT from the storage compartment since the concentration of 5-HT in these synaptosomes did not decline rapidly between the 20 and 30 minute period of incubation. The most likely explanation of this finding is that the transport of 5-HT metabolites out of the synaptosome gradually slows. The slow onset of this effect would suggest that it may be related to the gradual depletion of

a substrate or factor required for the transport of 5-HT metabolites out of the synaptosome. The nature of this transport process is as yet unknown. Until the transport process is identified it is impossible to suggest what this factor may be.

The retention of 5-HT by synaptosomes after incubation in 1.0 μM ^{14}C-5-HT follows similar pattern to that seen following incubation at the lower concentration. The synaptosomal concentration of 5-HT gradually declined over the 30 minute period of incubation. Pretreatment with Li^+ had no significant effects on the synaptosomal concentration of 5-HT, but the initial fall was significantly greater in these animals. (table 35(a)(i) p. 151). The initial fall in 5-HT was accompanied by a significant increase in the concentration of 5-HT metabolites (table 35(b) p. 152) suggesting that this may be related to the rapid metabolism of unstored 5-HT. Following the rapid initial fall, the 5-HT concentration declined at a slower rate which was probably related to the spontaneous leakage of 5-HT from the storage compartment. The rapid removal of the 5-HT metabolites between 5 and 10 minutes incubation would suggest that the process responsible for the removal of such metabolites is reasonably efficient. Though the initial fall in 5-HT was significantly greater in synaptosomes prepared from Li^+ pretreated animals, the final concentration reached was only slightly lower than that in synaptosomes from control animals. Thus it would appear that synaptosomes prepared from Li^+ pretreated animals, when incubated in 1.0 μM 5-HT are able to accumulate slightly more 5-HT than those prepared from control animals, however, a larger proportion of accumulated 5-HT remains unstored. The study failed however, to provide any information that may help to identify whether Li^+ affects the rate at which 5-HT may be transported from the cytoplasm to the

storage compartment. In addition, it is still impossible to determine whether or not Li^+ reduces the maximum capacity of the storage compartment.

More information will be obtained by future investigations of the effect of Li^+ on the accumulation and retention of 5-HT by sub-synaptosomal fractions. My laboratory was not previously equipped to do this. At the moment however, in the absence of such studies, it can be said that the results of the investigations reported here, provide some evidence to support the contention that 10 day Li^+ pretreatment (0.75 mEq/Kg/day) may reduce the ability of 5-HT terminals to store transmitter.

(14) Support from previous findings for an effect of Li^+ on the storage of 5-HT.

There is some support in the literature of an effect of Li^+ on the storage of brain momoamines. Schildkraut et. al. (1966, 1969) demonstrated that both acute and chronic Li^+ treatment produced a shift in noradrenaline metabolism from O-methylation to deamination. Catechol-O-methyltransferase is primarily an extracellular enzyme, whereas MAO is located intraneuronally. The authors suggested that noradrenaline released from the neurones is either accumulated by nerve endings, or O-methylated extraneuronally. Conversely, noradrenaline released from storage granules within the neurone would be expected to be deaminated. From this they suggested that an increase in the intraneuronal deamination of noradrenaline may reduce the amount of noradrenaline available for release (Schildkraut et. al. 1966). As in our investigations (table 16 p. 90) these workers were unable to detect a significant reduction in the brain concentration of the parent monoamine.

More direct evidence of an effect of Li^+ on storage of monoamines

has been provided by the work of Komisky and Buckner (1974). These workers examined the uptake and retention of metaraminol and octopamine by synaptosomes isolated from animals which had been treated with Li^+ or saline for 10 days. It was found that the uptake and retention of metaraminol was markedly increased in the Li^+ treated animals whereas the uptake of octopamine was little affected.

According to Komisky and Buckner (1974), octopamine and metaraminol occupy storage sites within adrenergic neurones and both are transported across the cell membrane by the same process. The two amines differ in that only octopamine appears to be a substrate for monoamine oxidase. With this information, Komisky and Buckner (1974) concluded that the different effects of Li^+ on the uptake and retention of octopamine and metaraminol may best be interpreted by proposing that Li^+ increases the rate of transport of both amines across the cell membrane, but reduces the ability of the storage compartment to retain the transported amine. As octopamine is a substrate for monoamine oxidase, that fraction of transported octopamine which is not sequestered in the storage compartment would be deaminated. Conversely, as metaraminol is not a substrate for monoamine oxidase, the transported amine which is not stored could remain free in the cytoplasm in an intact form.

As far as can be ascertained, the physiocochemical basis of noradrenaline, octopamine and 5-HT storage appears to be similar. All appear to require the formation of nucleotide-divalent cation aggregates to which the monoamine becomes bound (Berneis et. al. 1971, Rajan et. al. 1972). Though the dependence upon nucleotides and divalent cations has been confirmed for noradrenaline in the CNS (Philippu et. al. 1968), it has yet to be shown that this is so for 5-HT. Both noradrenaline and 5-HT have, however, been located in synaptic vesicles (see Shore

1972). Thus, although the mechanism of storage of 5-HT in vivo has yet to be elucidated, there is good evidence that the physiocochemical basis of the binding of monoamines <u>in vitro</u> is similar. Thus the findings of Schildkraut et. al. (1966, 1969) Komisky and Buckner (1974) and the results of this study may be related to a common effect of Li^+ on the intraneuronal storage of monoamines.

<u>(15) The effect of 5 day lithium pretreatment on the concentration of 5-HT and 5-HIAA in the rat forebrain.</u>

The administration of Li^+ (1.5 mEq/Kg/day) for 5 days had no significant effects on the concentration of 5-HT in the rat forebrain, but elevated the concentration of 5-HIAA (table 23 p. 103). A comparison of table 16 p. 90 with table 23 p.103 demonstrates that both acute (5 day) and prolonged (10 day) lithium administration had similar effects on the forebrain concentration of 5-HT and 5-HIAA.

An increase in 5-HIAA without a change in 5-HT concentration may indicate either an increase in the turnover of 5-HT or an inhibition of the transport of 5-HIAA out of the brain. The finding that 5 day Li^+ pretreatment did not greatly increase the production of 5-HIAA following L-tryptophan administration (table 24 pages 105, 106 and table 25 p. 108) would tend to deny that Li^+ may be reducing the clearance of 5-HIAA from the brain. Thus, the increase in the forebrain 5-HIAA concentration may reflect an increase in the synthesis and metabolism of 5-HT. Such an interpretation is supported by a number of previous reports which have demonstrated that short term Li^+ administration to rats increases the synthesis and turnover of 5-HT in the brain (Tagliamonte et. al. 1971, Sheard and Aghajanian 1970, Perez-Cruet et. al. 1971, Knapp and Mandell 1973, Schubert 1973, Poitou et. al. 1974). On some occasions, the increase in 5-HT turnover was accompanied by an increase in brain

5-HIAA concentration without a change in the concentration of 5-HT (Sheard and Aghajanian 1970, Poitou et. al. 1974).

(16) The modification of the effect of L-tryptophan by 5 day lithium pretreatment.

Tables 24 and 25 pages 105 - 108, show that 5 day Li^+ pretreatment had no significant effects on the changes in forebrain 5-HT and 5-HIAA concentration induced by L-tryptophan.

If, as has been suggested, 5 day Li^+ pretreatment enhances the synthesis and metabolism of 5-HT, one might have expected the elevation of 5-HIAA by L-tryptophan to be increased by Li^+ treatment. One of the suggested mechanisms by which acute Li^+ administration may increase the synthesis and turnover of 5-HT is by increasing the transport of the precursor amino acid L-tryptophan into brain (Tagliamonte et. al. 1971, Schubert 1973), and nerve terminals (Knapp and Mandell 1973). Under normal circumstances, an increase in the influx of tryptophan into the neurone would be expected to increase the synthesis of 5-HT, since tryptophan-5-hydroxylase is relatively unsaturated (Friedman et. al. 1972). However, under situations where the influx of tryptophan into the brain is markedly enhanced, such as that following a loading dose of the amino acid, the enzyme or the process transporting tryptophan into the neurone (Belin and Pujol 1973) may become saturated. Thus any change in the availability of substrate exerted by Li^+ pretreatment could be masked by the saturation of the hydroxylating enzyme or the tryptophan transport process. This may account for the lack of effect of Li^+ on the forebrain concentration of 5-HT and 5-HIAA following L-tryptophan administration.

(17) A comparison of the effects of 5 and 10 day lithium pretreatment

<u>on the response to L-tryptophan administration.</u>

In contrast to the effects of 10 day Li^+ pretreatment on the response to the administration of L-tryptophan, 5 day Li^+ treatment had no significant effects on changes in forebrain 5-HT and 5-HIAA induced by this procedure. This may indicate that 5 day Li^+ pretreatment is not influencing the storage of 5-HT to any great extent.

The different effects of 5 and 10 day Li^+ administration may be related to the time course required for a discrete change to occur in the brain, or to the time needed to build up a sufficient concentration of Li^+ in the brain compartment in which the effect is exerted.

The first thing to identify was whether or not the differences in brain Li^+ concentration following 5 or 10 day Li^+ treatments could provide some information that could be related to the differences in effect on 5-HT storage. Previous work had demonstrated that Li^+ passes slowly from plasma to brain tissue (Davenport 1950, Corrodi et. al. 1967), thus it is possible that brain levels may not have equilibrated after 5 day treatment. Measurements of the concentration of Li^+ in plasma and brain however, demonstrated that in these experiments, the concentration of Li^+ in both tissues obtained from animals receiving 5 days Li^+ pretreatment (1.5 mEq/Kg/day), was approximately double that measured in rats receiving 10 days Li^+ pretreatment at the lower dose (0.75 mEq/Kg/day) (table 28 p. 113). Thus it would appear that the brain concentration of Li^+ may have equilibrated after both 5 and 10 day treatments. Thus, if the difference observed between the two treatments is related to the brain concentration of Li^+, and not to the time for which it was maintained, it would appear that a lower brain concentration of Li^+ is required to exert a noticeable effect on storage. At higher concentrations, the Li^+ must be expected to either antagonise the effect

of low doses of Li^+ or to exert a greater effect on another process which would appear to mask an effect on storage.

Although total forebrain Li^+ concentration has probably equilibrated well within the 10 day period of treatment, it is possible that the build up of Li^+ in the subcellular organelles involved in the synthesis and storage of 5-HT could take a longer period. A recent report however, demonstrated that Li^+ tends to accumulate in the particulate fraction of brain tissue following long term administration (Christensen 1974).

Although the differences observed between long and short term Li^+ administration may be related to the brain concentration of the cation, it is probably more likely to be related to the time course required for a discrete change to occur in the 5-HT neuronal system. In the peripheral adrenergic neurone, there is evidence to suggest that the storage vesicles are pre-formed in the cell body of the neurone and carried by axoplasmic flow to the nerve terminals (Dahlstrom and Haggendal 1967). The latency in the recovery of adrenergic storage function following reserpine treatment has been related to the time taken for new granules to travel from the cell body to the terminal. This takes many days. (Dahlstrom and Haggendal 1966, Haggendal and Dahlstrom 1971). Support for this proposal was given by the finding that the action of reserpine on adrenergic storage is a two phase process. The first phase of reserpine action is believed to be a reversible inhibition of the transport of amines across the granular membrane. This phase has a half life of 12 to 18 hours. After this initial effect, a proportion of reserpine appears to remain permanently bound to the storage granule where it is believed to reduce the binding of the amine within the granule (Norn and Shore 1971). The slow recovery of amine storage is be-

lieved to be due to the flow of new, unaffected granules from the cell body to the terminal.

If the storage vesicles in central 5-HT neurones are similarly manufactured in the cell body, and are transported by axoplasmic flow to nerve terminals in the forebrain, one could postulate that those effects of long term Li^+ pretreatment on the storage of 5-HT may be related to this phenomenon. Thus the principal effect of Li^+ could be exerted on the formation of the storage granules within the cell body. The time lag observed for an effect on 5-HT storage to be detected, may then be related to the gradual replacement of functional vesicles by Li^+ affected ones.

(18) The modification of the effect of stimulation by lithium pretreatment.

If the postulates outlined above concerning an action of Li^+ on amine storage are correct, it might be expected that a reduction in the storage of 5-HT may be reflected as a reduction in the stimulation-induced release of the monoamine. The results of experiment III provided evidence that the elevation of forebrain 5-HIAA concentration following stimulation of the nucleus raphe medianus was derived primarily as a consequence of the release of 5-HT followed by uptake and intraneuronal metabolism. Thus the last study conducted in the course of this thesis investigated the effects of Li^+ pretreatment on the changes in forebrain 5-hydroxyindole concentration induced by electrical stimulation of the nucleus raphe.

Raphe stimulation was observed to significantly elevate the forebrain concentration of 5-HIAA without affecting the 5-HT concentration in both control animals, and in animals which had received 5 or 10 day Li^+ administration (tables 20, 26 pages 98 and 109). A comparison of

the effect of stimulation between animals treated and not treated with Li^+ demonstrated that Li^+ pretreatment had no significant effect on the stimulation-induced changes in forebrain 5-HT and 5-HIAA (tables 21 and 27 pages 99 and 111). Time did not permit a more detailed investigation into the effect of 5 day Li^+ pretreatment on these changes. However, in an attempt to identify the site of origin of the changes in 5-HIAA concentration observed in animals which had received 10 day Li^+ pretreatment, the second part of the experiment investigated the effect of chlorimipramine on these changes.

(19) The modification of the effect of stimulation by lithium plus chlorimipramine.

Chlorimipramine (5 mg/Kg i.p.) administered 3.5 hours before stimulation had no effect on the change in 5-HT concentration induced by raphe stimulation in the control group of animals, but significantly reduced the concentration of 5-HT in the Li^+ pretreated animals (table 22 p. 100). In addition, chlorimipramine completely abolished the stimulation-induced increase in 5-HIAA concentration in the control group without significantly affecting that in the Li^+ pretreated group.

The reduction in the increase in forebrain 5-HIAA concentration seen in the control group confirms the findings of experiment III.

The fact that chlorimipramine was unable to significantly reduce the stimulation-induced increase in 5-HIAA concentration in the Li^+ pretreated group may indicate that most of the 5-HIAA produced by stimulation in these animals was derived intracellularly, and not primarily from the uptake of extraneuronally released 5-HT. It is possible however, that the effects observed may have resulted from interactions between Li^+ and chlorimipramine.

(20) Possible interactions between chlorimipramine and lithium.

The major important common site of action for Li^+ and chlorimipramine, at least with respect to this experiment, is the transport of 5-HT across the terminal membrane. Although the acute effects of Li^+ pretreatment on the accumulation of 5-HT by synaptosomes have been reported in the literature (Kuriyama and Speken 1970), there are no comparable reports as to the long term effects of Li^+ on this process. Because of the physiological similarities between synaptosomes and blood platelets (Paasonen et. al. 1971), it has become increasingly common to use these structures as models of 5-HT terminals. The accumulation of 5-HT by platelets obtained from patients before, during and after Li^+ therapy has been shown to be increased during the periods in which Li^+ was administered (Murphy et. al. 1969, 1970). Genefke (1972) however noted no change in the accumulation of 5-HT by rat platelets following prolonged Li^+ treatment. The results of my studies would suggest that 10 day Li^+ pretreatment tended to enhance slightly, but not significantly the transport of 5-HT across the terminal membrane. In contrast, chlorimipramine is well known to inhibit this process (Blackburn et. al. 1967, Ross and Renyi 1967, Fuxe and Ungerstedt 1968, Carlsson et. al. 1968, 1969a, Shaskan and Snyder 1970, Meek et. al. 1970, Lidbrink et. al. 1971). Thus it is possible for drug interactions to take place at this level. If Li^+ was able to overcome a chlorimipramine induced inhibition of 5-HT transport from the extracellular to the intracellular environment, a greater proportion of the extraneuronally released 5-HT would be able to gain access to intraneuronal MAO. The apparent lack of effect of chlorimipramine on the elevation of 5-HIAA induced by raphe stimulation in the Li^+ pretreated animals, may therefore be due to the deamination of 5-HT which has in fact been released, and

which was able to return to the intracellular environment. This possible drug interaction could easily have been investigated by examining the accumulation of 5-HT by isolated synaptosomes from animals treated with these drugs. Although time did not permit such a study it is one of a number of investigations which will be conducted in order to follow up the studies reported in this thesis. In the absence of this, such an interaction must be considered as a possible contributing factor to the effects observed in this study.

(21) Possible effects of lithium on the release of 5-HT.

If Li^+ pretreatment is not antagonising to any great extent the chlorimipramine-induced inhibition of 5-HT transport, one must consider that the lack of effect of chlorimipramine on the stimulation-induced changes in forebrain 5-HIAA could indicate that a greater proportion of the 5-HIAA is derived from 5-HT which has not been released extracellularly. As a consequence, it can be proposed that Li^+ inhibits in some way the impulse stimulated release of 5-HT. It remains to be determined whether this possible effect of Li^+ on the extraneuronal release of 5-HT is related to an effect on the storage of the monoamine within the nerve terminal. The result of the previous study suggested that Li^+ may affect the ability of the storage compartment to bind 5-HT. Such an effect could be expected to directly reduce the impulse-stimulated release of 5-HT from nerve terminals in the forebrain. The results of the stimulation study however, do not entirely fit such a hypothesis. If Li^+ is affecting the rate at which 5-HT is transported from the cytoplasm to the storage compartment, one would expect a lower proportion of the 5-HIAA produced by raphe stimulation to be derived from 5-HT which has been released extracellularly. The findings of this experiment would suggest that this may be so. However, it might also

be expected that the repletion of 5-HT stores following release by stimulation would also be slowed. Accordingly, one would expect the forebrain 5-HT concentration to be reduced by raphe stimulation. Although this was so in the group of animals which had received both Li^+ and chlorimipramine, it was not so in those receiving Li^+ alone. One would have expected the forebrain concentration of 5-HT to be similar in both cases. Why chlorimipramine should reduce the concentration of 5-HT in the stimulated brain is difficult to determine. It is unlikely to be due to a reduction in synthesis, since the 5-HIAA level was still significantly elevated. If 5-HT present in the synaptic cleft was able to diffuse away from brain tissue, it could be suggested that the reduction in 5-HT concentration in the presence of chlorimipramine may indicate that under the influence of Li^+, 5-HT which has been transported across the terminal membrane is stored more readily than is newly synthesised amine. Such an effect could also account for the changes in forebrain 5-HT and 5-HIAA seen in Li^+ pretreated animals given L-tryptophan. However, the trend towards an increase in brain 5-HT concentration seen in stimulated control animals given chlorimipramine, would suggest that a large proportion of released 5-HT is unable to diffuse away from the synaptic cleft (experiment IIIb and this study).

Since the concentration of 5-HT in the forebrain of control animals is not significantly changed by raphe stimulation, it would appear that the storage compartment in this situation, is repleted at a rate which is proportional to the rate at which 5-HT is released from it. In this situation, the storage compartment will not be required to store large amounts of amine and an effect of Li^+ on the maximum capacity of the storage compartment may not be detectable in this experiment.

The results of this study are therefore unable to determine whether

the possible effects of Li^+ on the release of 5-HT are directly related to an effect on the storage compartment. The findings of the previous experiments (experiment IVb and Vb) suggested that the effect of Li^+ on the storage of 5-HT may take many days to become apparent. Time did not permit the investigation of the effect of chlorimipramine upon changes in forebrain 5-hydroxyindole concentrations induced by raphe stimulation in animals which had received 5 day Li^+ pretreatment. Thus the time period required for the reduction in the impulse-stimulated release of 5-HT to occur cannot be ascertained. It is interesting to note however, that Katz et. al. (1968) and Chase et. al. (1969) observed significant reductions in the impulse-stimulated release of 5-HT from rat brain slices following the addition of Li^+ directly to the incubation medium. It is therefore possible that a direct effect of Li^+ on the extraneuronal release of 5-HT from nerve terminals may occur as soon as the Li^+ concentration at the terminal reaches a critical level, whereas a longer period may be required to exert a noticeable effect on storage.

(22) Possible mechanisms by which lithium may affect 5-HT release.

Stimulation of the midbrain raphe nuclei increases the rate of 5-HT synthesis (Shields and Eccleston 1972). It is normally considered that this increase in 5-HT synthesis is responsible for the repletion of 5-HT which has been released by electrical stimulation. The stimulus for this effect could be a feedback system triggered by the loss of 5-HT from the storage compartment. Such a loss of 5-HT may be brought about either by the extracellular release of 5-HT into the synaptic cleft, or by the intracellular release of 5-HT from storage sites into the cytoplasm. The effect of chlorimipramine on the elevation of 5-HIAA by raphe stimulation in control animals (experiment

IIIb and IVc) would suggest that under normal circumstances, 5-HT is released from the storage sites into the synaptic cleft. However, under the influence of Li^+, 5-HT may be released from the storage sites into the cytoplasm where it may be metabolised by MAO. In this situation, the elevation of forebrain 5-HIAA concentration could occur with little or no extraneuronal release of the amine.

The mechanism of 5-HT release from nerve terminals in the CNS has yet to be elucidated. In simplest terms it must involve a process whereby the storage compartment, or a proportion of the storage compartment, is suitably positioned for the release of 5-HT into the synaptic cleft; and secondly 5-HT must be released from the storage sites. Thus Li^+ may be influencing the ability of the storage compartment to adopt a suitable position for the extraneuronal release of 5-HT, without affecting the cleavage of 5-HT from its binding sites. In response to stimulation, 5-HT would then be released into the cytoplasm instead of into the synaptic cleft. The final result of such a process would be a reduction in the impulse-stimulated extraneuronal release of 5-HT.

(25) General conclusions.

The results of these studies have provided some evidence that 10 day Li^+ pretreatment may inhibit the storage and extraneuronal release of 5-HT in the rat forebrain. Evidence was presented which suggested that the effect of Li^+ on the release of 5-HT may not occur as a direct consequence of an effect on storage, but may be an independent effect of Li^+.

These studies were conducted in a clinical department, with the aim of increasing our understanding of the mode of action of psychotherapeutic substances. The experiments described in this thesis were, how-

ever, conducted using rats, and it is therefore difficult to relate the findings directly to the possible effects of Li^+ in man. If, however, the interpretation of my findings is later shown to be valid, a study of the effect of Li^+ on the storage of 5-HT in human platelets (see Paasonen 1971) may provide a link between this work and the possible therapeutic effects of the drug. However, it can be said that with respect to the anti-manic effect of Li^+, the results of these studies would fit currently-held views on the relationship between brain monoamines and the affective disorders.

APPENDIX I.

DIAGRAMMATIC EXPLANATION OF STATISTICAL ANALYSIS

(a) Experiment II.

	Stimulation.	No stimulation.
Raphe.	A	B
Non-raphe.	C	D

A,B,C and D refer to the concentration of 5-HT or 5-HIAA in the forebrain of animals receiving the experimental manoeuvers outlined above and to the side of the table.

The effect of raphe stimulation was determined by a paired t test applied to A and B, and the effect of stimulation of non-raphe sites by a paired t test applied to C and D.

The difference between stimulation at the two sites was analysed by a paired t test applied to (A-B) and (C-D).

(b) All experiments involving raphe stimulation in saline controls and drug treated animals (experiments IIIb and Vc).

	Stimulation.	No stimulation.
Saline treated.	A	B
Drug treated.	C	D

A,B,C and D again refer to the concentration of 5-HT and 5-HIAA in animals receiving the experimental manoeuvers outlined above and to the side of the table.

The effect of drug treatment on the resting levels of 5-hydroxyindoles was determined by a paired t test applied to B and D.

The effect of stimulation in saline treated animals was determined by a paired t test applied to A and B and the effect of stimulation in drug treated animals by a paired t test applied to C and D.

The effect of drug treatment on stimulation-induced changes in forebrain 5-hydroxyindoles was analysed by a paired t test applied to (A-B) and (C-D).

(c) All experiments involving L-tryptophan administration in saline controls and drug treated animals. (Experiments IIIc, IVb, Vb and VIII).

	Tryptophan.	Saline.
Saline treated.	A	B
Drug treated.	C	D

A,B,C and D refer to the concentration of 5-HT, 5-HIAA or tryptophan in animals receiving the experimental manoeuvers outlined above and to the side of the table.

The effect of drug treatment on the resting levels of 5-hydroxyindoles or tryptophan was determined by a paired t test applied to B and D.

The effect of L-tryptophan administration in saline treated animals was determined by a paired t test applied to A and B and the effect of tryptophan in drug treated animals by a paired t test applied to C and D.

The effect of drug treatment on tryptophan-induced changes in 5-HT, 5-HIAA or tryptophan was analysed by a paired t test applied to (A-B) and (C-D).

(d) Experiment IVc.

	Saline plus stim.	Saline plus no stim.	Chlorimipramine plus stimulation.	Chlorimipramine plus no stimulation.
Control animals.	A	B	C	D
Li^+ treated animals.	E	F	G	H

A - H refer to the concentration of 5-HT and 5-HIAA in animals receiving the experimental manoeuvers outlined above and to the side of the table.

The effect of stimulation in control animals was determined by a paired t test applied to A and B, and the effect of stimulation in Li^+ treated animals by a paired t test applied to E and F.

The effect of Li^+ treatment on stimulation-induced changes in 5-hydroxyindole concentration was analysed by a paired t test applied to (A-B) and (E-F).

The effect of chlorimipramine on stimulation-induced changes in forebrain 5-hydroxyindoles in the control group was analysed by a paired t test applied to (A-B) and (C-D), and in the Li^+ treated group by a paired t test applied to (E-F) and (G-H).

APPENDIX II.

A SUMMARY OF THE MAJOR FINDINGS OF THE STUDIES.

This table summarises the major findings of the studies. The table is divided into two sections. Section (A) describes the responses observed in control animals following stimulation of the nucleus raphe medianus or the administration of L-tryptophan. An upward pointing arrow (↑) indicates an increase in the forebrain concentration of the substances, a downward pointing arrow (↓) indicates a decrease in the forebrain concentration of the substance, and a zero (0) indicates no change in concentration.

Section (B) describes the responses observed in drug treated animals, and how these responses differ from those observed in their paired controls. Each column in section (B) is divided into two sub-columns. The left hand sub-column indicates how the forebrain concentration of 5-hydroxyindoles or tryptophan is affected by electrical stimulation or L-tryptophan administration. The symbols used in this column are the same as those used in section (A). Thus an upward pointing arrow (↑) indicates an increase in the forebrain concentration of the substance, a downward pointing arrow (↓) indicates a decrease in the forebrain concentration of the substance, and a zero (0) indicates no change in concentration. The right hand sub-column indicates how the responses observed in drug treated animals differ from those observed in their paired controls. The symbols used indicate significant differences between paired control and drug treated groups. An upward pointing arrow (↑) indicates that the response to stimulation or L-tryptophan treatment was greater in animals receiving drugs. A downward pointing arrow (↓) indicates that the response was decreased by drug treatment, and a zero (0) indicates that the response was unaffected by drug treatment. A dash (-) indicates that no study was conducted.

The numbers in parenthesis refer to the particular tables from which this summary chart was constructed.

SUMMARY CHART OF THE EFFECTS OF DRUGS ON THE RESPONSES TO STIMULATION AND L-TRYPTOPHAN ADMINISTRATION.

Table 36.

	Change in concentration of 5-HT and 5-HIAA induced by stimulation.		Changes in concentration of 5-HT, 5-HIAA and tryptophan, 30 minutes after the administration of L-tryptophan.			Changes in concentration of 5-HT. 5-HIAA and tryptophan, 60 minutes after the administration of L-tryptophan.		
	5-HT	5-HIAA	5-HT	5-HIAA	Tryptophan	5-HT	5-HIAA	Tryptophan
(A) CONTROL RESPONSES.	0 (10)	↑(10)	↑(17)	↑(17)	↑(30)	↑(17)	↑(17)	↑(30)
(B) MODIFICATION BY DRUG TREATMENT.								
(1) MODIFICATION BY CHLORIMIPRAMINE.	0 (12) ↑(13)	↑(12) ↓(13)	-	-	-	↑(14) 0 (15)	↑(14) ↑(15)	-
(2) MODIFICATION BY Li^+ 10 DAYS.	0 (20) 0 (21)	↑(20) 0 (21)	↑(17) 0 (18)	↑(17) 0 (18)	↑(30) 0 (31)	↑(17) ↓(18)	↑(17) ↑(18)	↑(30) 0 (31)
(3) MODIFICATION BY Li^+ 10 DAYS PLUS CHLORIMIPRAMINE.	↓(22) ↓(22)	↑(22) 0 (22)	-	-	-	-	-	-
(4) MODIFICATION BY Li^+ 5 DAYS.	0 (26) 0 (27)	↑(26) 0 (27)	↑(24) 0 (25)	↑(24) 0 (25)	-	↑(24) 0 (25)	↑(24) 0 (25)	-

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