INTERACTION OF CONVULSANT AND ANTICONVULSANT DRUGS WITH ENDOGENOUS AMINES

A thesis presented by

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ABSTRACT

Evidence of an interaction of adrenergic pathways with convulsive mechanisms was sufficient to warrant an experimental study of leptazol induced convulsions.

Spectrophotofluorometric assays of noradrenaline (and adrenaline), dopamine and 5-HT, based on the methods of Bertler, Carlsson and Rosengren (1958), Carlsson and Lindqvist (1962) and Cox and Potkonjak (1967) respectively, were developed.

Catecholamine and 5-hydroxytryptamine concentrations were determined in whole brain and discrete areas of rat brain before, during and after leptazol convulsions. No significant effect was detected under these conditions. Elevation of amine concentrations with five representative MAO inhibitors (tranylcypromine, phenelzine, iproniazid, pargyline and nialamide) was without effect on leptazol threshold. Tranylcypromine, phenelzine and iproniazid possessed a transient proconvulsant action similar to that of dexamphetamine which correlated with their inherent sympathomimetic activity. COMT inhibitors, pyrogallol, catechol and β-thujaplicin, were without effect on leptazol threshold. Specific blockade of any one amine synthesis with either α-methyl-p-tyrosine, diethyldithiocarbamate or p-chlorophenylalanine, or blockade of all three syntheses simultaneously, was also without effect on leptazol threshold. Reserpine, syrosingopine or α-methyl-m-tyrosine were proconvulsant but only in doses and combinations which depleted all three amines simultaneously by at least 80-85%. Selective repletion of any one amine store to 50% of the control concentrations totally abolished this proconvulsant activity. It is concluded that the disposition of the amine stores rather than the state of the syntheses

is involved in any proconvulsant activity.

The anticonvulsants, phenobarbitone, phenytoin and chlordiazepoxide, were examined under similar conditions. Blockade of catecholamine synthesis reduced the anticonvulsant activity of chlordiazepoxide: so too did depletion of catecholamine stores with α-methyl-m-tyrosine, yet depletion with reserpine was without effect. It appears that intact reserpine-resistant catecholamine stores are necessary for the anticonvulsant activity of chlordiazepoxide and that 5-HT is in no way involved. Under these conditions the activity of phenobarbitone was unaffected.

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SECTION ONE

INTRODUCTION

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1. HISTORICAL INTRODUCTION

The term epilepsy is derived from the Greek 'epilambanein' meaning 'to seize' or 'to attack' and has been in use since 500-400 B.C. Until the Fourth century B.C. epilepsy was considered to be a sacred illness caused by demons and gods. The Greeks, however, were remarkably advanced in their concepts of epilepsy and sought a physical basis for a central nervous disorder. Despite this, it was not until the Nineteenth century that John Hughlings Jackson proposed epilepsy to be the occasional, sudden, excessive, rapid and local discharge from an abnormal focus in grey matter, the spreading of this discharge to normal brain tissue causing a generalised convulsion. Jackson's concepts have since been proved correct by electro-encephalographic and cortical excitability studies in recent years.

At the same time as Jackson proposed his concepts of epilepsy the first effective treatment was introduced in the form of Potassium Bromide by E.H. Sieveking (Sieveking, 1857), though this is ~~always~~ usually mistakenly attributed to Sir Charles Lowcock. Although the bromides are still used today, they have largely been superseded by synthetic organic drugs selected by pharmacological screening. The next effective treatment was with phenobarbitone in 1912. This was discovered by chance in the clinic and is still one of the most effective anticonvulsants for Grand mal. By the 1930's symptoms resembling convulsions could be produced in animals by electroshock techniques and used as a basis for testing anticonvulsants (Putnam & Merritt, 1937; Merritt & Putnam, 1938 a; b). These workers used this test to screen about seventy compounds and revolutionised pharmacology by establishing the value of laboratory assay techniques with their discovery of one of today's most potent anticonvulsants, 5,5-diphenylhydantoin (phenytoin) purely by

pharmacological screening. Concurrent with the discovery of electroshock testing, work was being done on antagonism of chemical convulsants by drugs (Goodman & Lih, 1941; Knoefal & Lehman, 1942) and the discovery of troxidone (Everett & Richards, 1944), established the wide use of leptazol as a convulsant in screening tests. These workers have also demonstrated that phenytoin in single doses was ineffective against leptazol convulsions. This in turn has led to the hypothesis that since tridione is effective in Petit mal and not in Grand mal, and phenytoin vice-versa, then in general the leptazol tests select Petit mal drugs and electroshock tests select Grand mal drugs. (Millichap, 1965).

2. CONVULSIVE DISORDERS IN MAN

Since the definition of epilepsy by Hughlings Jackson in the Nineteenth century, nothing significant or new has been added to his concept. Attempts have been made to correlate EEG tracings with symptoms of the most important seizure patterns, Grand mal, Petit mal, and Psychomotor convulsions. Unfortunately there is no convincing correlation. Others have attempted a pathological classification on the basis that convulsions are often associated with brain lesions, but with little more success. Since epilepsy occurs in many and varied patterns it is unlikely that one explanation will fit all symptoms. Hence we continue to use the concept of Jackson: "Epilepsies are abnormally intense, disorderly neuronal discharges originating in various regions of the brain". Signs and symptoms thus depend on the function of the affected brain areas. The cause of this abnormal discharge is as yet unknown but epilepsies are often subdivided into (a) symptomatic-in which the epilepsy is the result of traumatic, infectious, neoplastic or even emotional cause; Millichap (1960) has compiled an etiological classification of such seizures; (b) idiopathic or cryptogenic - where the cause is unknown but is suspected to be hereditary.

Despite the controversy on seizure taxonomy, the following classification appears to be most commonly accepted. Most seizures fall into one of the following patterns :- Major, minor, autonomic, psychomotor or focal.

Major seizure patterns

(a) Grand mal - This is the most common and arises from violent, prolonged neuronal discharge causing sudden loss of consciousness, followed by tonic and clonic spasms of the musculature. It is usually associated with an aura, outcry and foaming at the mouth. On regaining consciousness the patient is dazed and falls into a deep sleep for several hours. The seizure may occur as little as once in a lifetime. There is no direct correlation with EEG patterns and this together with the above symptoms suggests that the discharge is not localised but rather it is a generalised spread of the discharge.

Drug treatment consists of one of the following: Phenobarbitone, diphenylhydantoin or primidone.

(b) Status Epilepticus - This is often combined with Grand mal in classification but differs from it in that the convulsions are frequently recurring Grand mal seizures from which the patient does not completely regain consciousness. If the seizure is predominantly tonic, apnoea results and will result in death. Status epilepticus can be precipitated by rapid withdrawal of drug treatment from Grand mal patients or may arise with no apparent cause.

Drug treatment must be carefully considered in relation to the patient's state of health, e.g. hypoglycaemia, etc., but may consist of amylobarbitone, phenobarbitone or paraldehyde.

(c) Myoclonic spasms or infantile spasms - Sudden sharp jerks of the head, trunk and limbs are the common symptoms, though the whole body musculature may be involved. Each seizure occurs as four or five jerks at about 5 second intervals. Such attacks occur most frequently in the first two years of life, the majority beginning in the first year. Again there has been found no correlation between EEG and symptoms, in fact EEG reports often conflict.

Drug treatment is not totally successful, the best results being produced by primidone/acetazolamide combination, ketogenic diet and most widely used ACTH by intramuscular route.

Minor seizure patterns

(a) Petit mal - Characterised by brief lapses of consciousness or staring spells ("absence"), without aura. Though brief, this type of seizure can occur many times a day (5-100 per day). In such seizures EEG patterns are similar from patient to patient in that there is a 3 per second spike and wave pattern. The common cause is a genetically-determined predisposition to seizures.

Drug treatment is often successful with trimethadione, paramethadione and dimethadione, but these drugs can be toxic and a useful substitute has been found in meprobamate. A combination of phenobarbitone and trimethadione has also been found useful. Petit mal status is a form of Petit mal lasting for several days.

(b) Akinetic seizures - The seizure consists of loss of postural tone, falling to the floor and nodding of the head, and instantaneous recovery of posture and consciousness.

Drug treatment is not very satisfactory, the best results being obtained with primidone, diphenylhydantoin and acetazolamide.

Autonomic seizures

Can vary from abdominal pain, headaches, dizziness, emotional instability, laughing spells to vomiting.

Psychomotor seizures

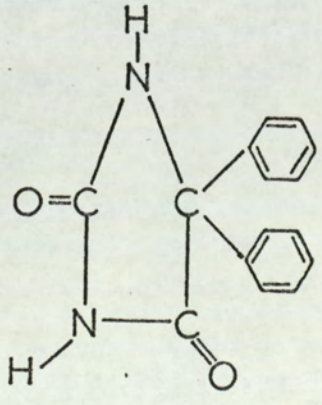
They are again varied, e.g. smacking of the lips, repeated swallowing, irrelevant speech, partial amnesia with abnormal behaviour between seizures. The seizures can often be alleviated with primidone and methsuximide, but the interseizure abnormal behaviour is not often reduced or may even become worse.

Focal seizures

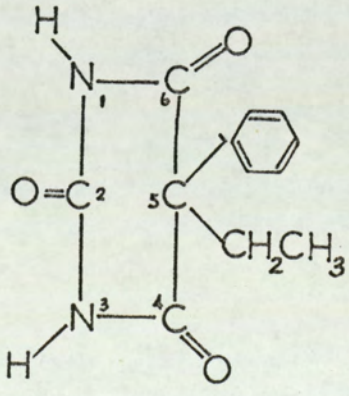
Symptoms depend on the affected brain area. The focus may arise as a result of disturbed cerebral metabolism or a lesion, etc.. Jacksonian seizures begin with clonus and numbness in one portion of the body and are then characterised by a "motor march": the seizure spreading to other muscles in conformity with the plot of these areas across the motor cortex. If the seizure spreads to both sides of the body Grand mal ensues.

3. ANTICONVULSANT DRUGS

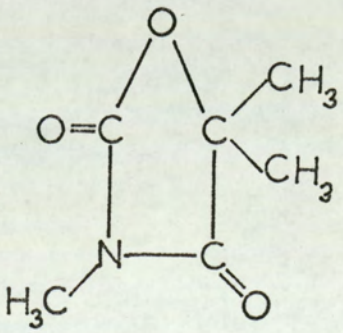
The main anticonvulsant drugs used in practice today have a similar basic nucleus as shown below:-



Diphenylhydantoin



Phenobarbitone



Trimethadione

There are four main classes of anticonvulsants. They are:

(a) Barbiturates

(b) Hydantoins

(c) Oxazolidinediones

(d) Succinimides

(a) Barbiturates

Briefly the structure-activity relationships are:-

(1) A 5 phenyl group is important but not essential.

(2) N-Methylation improves anti-leptazol potency.

(3) N-substitution of a phenyl group at N_1, increases anti-leptazol potency with absence of sedation.

(4) N-Methylation at N_1, produces anti-Grand mal potency and increases toxicity.

Phenobarbitone was introduced in 1912 and remains one of the most useful anticonvulsants today. Its major disadvantages are the sedative side effects, and the possibility of exacerbation of seizures after sudden withdrawal of the drug. The advantage of phenobarbitone is that it can be used to treat both Grand mal and Petit mal, and in experimental animals is effective against leptazol and electroshock convulsions. Phenobarbitone is effective against the clonic seizure pattern whichever way induced.

(b) Hydantoins

The hydantoins in contrast to phenobarbitones are non sedative. N-methylation improves anti-leptazol activity.

In contrast to phenobarbitone, diphenylhydantoin is particularly effective against the tonic extensor component of seizures, but previous hydration of an animal will demonstrate an anticlonic component also. Because of this selective action on the tonic component, it has been suggested that diphenylhydantoin prevents the spread of a discharge with no effect on the focus.

(c) Oxazolidinediones

These drugs have a marked anti-leptazol activity with reduced anti-electroshock activity. The heterocyclic ring may be opened with no loss of activity, e.g. phenylacetylureas. In fact this often renders the analogue more effective against both types of epilepsy. Trimethadione has specific activity against Petit mal. However, it often shows side effects when used clinically, e.g. photophobia and blood dyscrasias.

Paramethadione, though less potent, is also less toxic. It is a Petit mal drug and is often effective in cases refractory to trimethadione.

(d) Succinimides

The three drugs in use are phensuximide, ethosuximide and methsuximide. They are effective against leptazol seizures in rats and mice, phensuximide and methsuximide are also effective against the tonic extensor component of electroshock. Their side effects are disturbing in that blood dyscrasias and renal damage have been reported.

Although these are the drugs frequently in use, acetazolamide, a carbonic anhydrase inhibitor, is effective against electroshock induced tonic extension, but has little effect on leptazol clonus. However, contrary to the general rule, acetazolamide is clinically effective against Petit mal.

In recent years a class of drugs, originally introduced as tranquillizers, has become of increasing importance as anticonvulsants. These drugs are the benzodiazepines. They are also of particular interest in this project, because many drugs which affect mood, causing depression or excitation, etc., also affect leptazol convulsions. Since these drugs are thought to modify central adrenergic pathways, it is possible that leptazol also has a similar path of action.

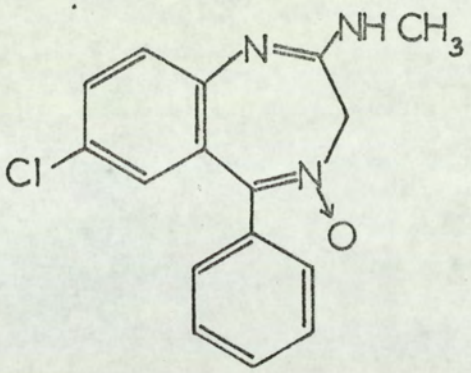
The benzodiazepines are known to be tranquillizers and antidepressives but their action on central aminergic pathways, if any, is unknown. What is known, however, is that these drugs are potent anticonvulsants. (Rosenstein, 1960; Randall, Heise & others, 1961; Hernandez-Peon, Rojas-Ramirez & others, 1964; Banziger, 1965; Eidelberg, Neer & Miller, 1965; Boyer, 1966; Swinyard & Castellion, 1966; Parsonage & Norris, 1967) From work on cats it has been postulated that diazepam (Valium-Roche) has a strong inhibitory action upon the hippocampus and amygdala (Morillo, 1962). With similar work Hernandez-Peon et al (1964) conculded that diazepam depresses after discharges from the amygdala and hippocampus, and suggested that it exerts a general depressant action upon epileptogenic structures throughout the brain. Work with rabbits has also suggested that the action of diazepam is on the hippocampus and amygdala (Arrigo, Jann & Tonali, 1965). Nitrazepam (Mogadon-Roche) has also been shown to raise the threshold of the cat amygdala during testing after discharges (Schallek & Kuhn, 1965). These same authors showed that phenobarbitone increases the threshold of the caudate nucleus to the greatest extent.

Swinyard & Castellion (1966) showed that most benzodiazepines are effective against both electroshock and leptazol convulsions, but more effective against the former. This, they suggested, was because anti-electroshock involves only preventing the spread of the discharge, whereas anti-leptazol activity requires prevention of the spread and raising the threshold. The evidence thus favours an action on the limbic system and since this class of drugs are potent anticonvulsants, e.g.

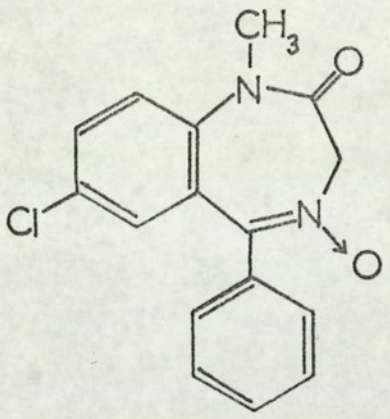
ED_{50} chlordiazepoxide = 3.6mg/kg against leptazol in mice

ED_{50} diazepam = 0.27mg/kg against leptazol in mice (Swinyard & Castellion, 1966).

their action seems to be fairly selective. Because of this potent action on the limbic system, this area suggests itself as a starting point for the investigation of catecholamine levels during convulsions.



Chlordiazepoxide



Diazepam

Mode of action of anticonvulsants

In their review Toman and Goodman (1948) proposed three categories for modes of action of anticonvulsants. They were:-

(a) Action on non-neural systems to prevent changes which might precipitate or facilitate seizure activity.

(b) Action on normal neurons to prevent their involvement in an excessive discharge originating in a focal lesion.

(c) Action on pathologically altered neurons to control an abnormal discharge.

(a) In the first category such drugs as acetazolamide, a ketogenic diet, deoxycorticosterone, corticotrophin, etc., can be included, since they prevent symptoms classified as frequent causes of seizures. (Millichap, 1960). Acetazolamide is a carbonic anhydrase inhibitor in the brain and consequently has effects on acid-base and electrolyte metabolism. Its effect on the kidney may also be important according to Millichap's classification. The corticosteroids exert their effect through electrolyte metabolism and permeability of cerebral vessels. (Aird & Gordan, 1951). A ketogenic diet has also been correlated with a systemic

balance of Na^+ and K^+.

(b) The majority of anticonvulsants are likely to fall into this class, i.e. preventing spread of discharge from the focus. Such drugs raise the excitation threshold of the neurons and this will not correct the cause of the seizure. Should there be sufficient evidence for a central sympathetic mode of action or for any other humoral transmission then anticonvulsants which block or interfere with such transmission might fall under this heading.

(c) Drugs which fall into the category do not have their action on abnormally altered neurons alone, for example phenobarbitone, which stabilises such a focus, also has an action on normal neurons which may be involved in the seizure. However, trimethadione has an anticonvulsant action on leptazol induced convulsions similar to Petit mal which may originate in a focus of abnormally altered neurons. Pyridoxine and GABA control seizures due to a dietary deficiency of pyridoxine, this deficiency may also be induced chemically.

4. EXPERIMENTAL CONVULSIVE AGENTS

The forementioned summary of convulsive disorders and their treatment serves to emphasise that no one drug will treat all forms of epilepsy and it has become necessary to use certain tests for certain epilepsies when testing for anticonvulsants. For example, electroshock tests have been found to select drugs useful against Grand mal and leptazol against Petit mal; a drug showing activity in one test may not be active in another. Therefore one of the requirements of an anticonvulsant test is that the induced convulsion should bear some resemblance to the clinical epilepsy. Consequently epileptogenic lesions would be the ideal convulsive agent; however this is both time consuming and costly. It has been found easier to induce epilepsy in animals by electroshock or chemical means or even by cross breeding mice with a genetic predisposition to audiogenic seizures. These tests do bear resemblance to epilepsy since drugs found in such tests are useful in clinical epilepsy. This interrelationship is not fully understood, but work on the state of biogenic amine concentrations, syntheses and turnovers during such convulsions might lead to an explanation of this phenomenon, and in turn elucidate the mode of action of analeptics and anticonvulsants. The main types of convulsive agents used for screening anticonvulsants are:-

(i) Electroshock

(ii) Chemical convulsants

(iii) Auditory stimulation

(iv) Epileptogenic lesions

The tests involving these agents are discussed below.

(i) Electroshock

Threshold method - the basis for this test is that a normal untreated animal when given repetitive electric shocks will convulse at a reproducible threshold. Drugs which raise the threshold are considered anticonvulsant. In the original method

(Putnam & Merrit, 1937), direct current, broken 60-80 times per second by a commutator, was passed through the brain of a cat with one electrode in the mouth and the other attached to the occiput. The convulsive threshold before and after anticonvulsant treatment was expressed as a ratio which was a measure of the anticonvulsant potency. A later modification was the use of corneal electrodes together with 60 c.p.s. alternating current (Spiegel, 1937). The method in use today was devised by Woodbury and Davenport (1952), who passed sinusoidal 60 c.p.s. shocks through either corneal or ear electrodes. The pattern of convulsions induced by this technique is essentially tonic-clonic.

There are many variations of the electroshock tests but the following are the most frequently used :-

(A) Minimal electroshock test or Normal electroshock test, (Swinyard, 1949; Swinyard, Brown & Goodman, 1952.)

A control threshold is found by shocking the animal for 0.2 seconds duration at 6-8 hour intervals and the current intensity increased by about 3% until a minimal seizure occurs. A minimal seizure is defined as ten or more seconds facial clonus without loss of righting reflex. With this procedure the individual thresholds are determined and checked three times to ascertain that they are consistent. The anticonvulsant drug is administered and the current increased by 20%. Absence of convulsions is taken as the end point and evidence of anticonvulsant activity.

Alternatively the convulsant current to 50% (CC_{50}) can be found in groups of control and test animals. Comparison of the two CC_{50} levels gives an indication of anticonvulsant activity.

(B) Maximal electroshock seizure test or Supramaximal test. (Woodbury & Davenport, 1952).

Animals are given a single supramaximal shock (150mA for rats - Swinyard, 1949; 50mA for mice - Swinyard et al, 1952), which is approximately 5 times the threshold current. Untreated animals show a tonic extension of the hind limbs. Absence of this tonic seizure is the criterion of anticonvulsant activity. N.B. all animals not exhibiting such a seizure before drug treatment are discarded.

(C) Hyponatraemic test or Hydration electroshock threshold test. (Swinyard, 1949; Swinyard et al, 1952.)

This is a variation of the minimal electroshock threshold test. (Swinyard et al, 1952). The minimal seizure threshold is determined, and four hours before the anticipated peak activity of the anticonvulsant in rats and two hours before in mice, each animal receives 10 ml/100 g body weight of 5.5% solution of glucose intraperitoneally. This was shown to reduce the minimal electroshock threshold to 44% of its initial value. (Swinyard, Toman & Goodman, 1946). To test for anticonvulsant activity a current intensity 66% of the minimal electroshock threshold is used. This represents a 50% increase in the hyponatraemic threshold. Absence of animal seizures is taken as the end point and an indication of anticonvulsant action. The advantage is that this test selects diphenylhydantoin which does not show in the Minimal electroshock threshold test.

(D) Psychomotor test. (Toman, 1951).

This was introduced with the claim that it was useful as a screen for psychomotor anticonvulsants (Toman, 1951), a claim based on

the fact that the author had found phenacemide to be positive in this test.

The method is to use 0.2m.sec. pulses at 6 per second applied with corneal electrodes for 3 second periods. This causes stunning and psychomotor symptoms. However, other workers have shown that this test is no better than Minimal electroshock threshold or Hyponatraemic tests for psychomotor drugs. (Brown, Schiffman, Swinyard & Goodman, 1953; Grewal, Swinyard, Jensen & Goodman, 1954).

(ii) Chemical convulsants

Certain drugs have been known to be convulsant in man for centuries. Strychnine and picrotoxin were used in animals in early attempts to produce experimental epilepsy, not for the purpose of testing anticonvulsants but instead to study the course of convulsions per se. Everett and Richards (1944), with their discovery of tridione, established leptazol as a useful convulsant in the screening of anticonvulsants. Today screening with leptazol is as common as with electroshock and there are probably as many variations of the leptazol procedure as there are of the electro-shock test.

Tests employing leptazol usually measure the ability of a drug to raise the leptazol threshold and not the ability to alter the seizure pattern. (cf electroshock tests).

The following tests have been used most frequently.

(a) Maximal Leptazol (Metrazol) seizures. (Orloff, Williams & Pfeiffer, 1949; Goodman, Grewal, Brown & Swinyard, 1953).

Seizures were elicited by rapid intravenous injection of 38 mg/kg leptazol as a 0.5% w/v aqueous solution. This was shown to

produce maximal convulsions in 96% of control mice. "Maximal convulsions" was defined as tonic extension of the hindlimbs. The seizure pattern was clonic-tonic but the clonic phase was so short lived that the seizure appeared to be only tonic. The mice were considered protected if the anticonvulsant drug prevented the appearance of the hindleg tonic extension.

This test is analogous to the Maximal electroshock seizure test in electroshock testing except that in the latter the pattern is tonic-clonic.

(b) Leptazol (Metrazol) seizure threshold test. (Goodman & Lih, 1941; Chen & Ensor, 1950; Swinyard, Brown & Goodman, 1952).

This test has been used to test the ability of anticonvulsant drugs to afford complete protection against seizures induced by subcutaneous injection of 85 mgm/kg (CD_{97}) leptazol in mice (Goodman & Lih, 1941) and 70mg/kg leptazol in rats. (Swinyard et al, 1952). Seizures induced by this method begin with intermittent myoclonic jerks with hind limbs apart and tail erect, followed by generalised clonus and loss of posture 3-10 minutes later. If the dose is a lethal one, tonic flexion of the forelimbs and tonic extension of the hindlimbs is seen immediately before death 5-15 minutes later. (Millichap, 1965). A modification of this test is the administration of leptazol intraperitoneally. The seizure pattern being the same but the time of onset being more rapid.

Other chemicals which have been used as convulsants include picrotoxin, strychnine, procaine, thujone, nicotine, etc., though these drugs have been used less frequently for the screening of anticonvulsants. Recently high oxygen tension has also been shown to promote convulsions (Koch & Woodbury, 1958; Haggendal, 1967; Paton, 1967).

iii) Auditory Stimuli

Recently anticonvulsants have been tested for their ability to prevent convulsions in mice genetically susceptible to sound waves, (DBA/2 mice). Continuous ringing of a 100 decibel bell causes first a running seizure, followed by a clonic-tonic-clonic seizure (Lehmann & Bushnell, 1962; Millichap, 1965; Schlesinger, Boggan & Freedman, 1965; Lehmann, 1967). This method has not been used extensively for anticonvulsant screening but has been investigated as a means of elucidating the mode of action of convulsants and anticonvulsants.

(iv) Epileptogenic lesions

Lesions produced by implanting chemically and immunologically active substances in the cerebral cortex are epiletogenic. Aluminium hydroxide has been used in monkeys (Kopeloff, Chusid & Kopeloff, 1954) and penicillin in mice and rats (Backus & Millichap, 1963). Cobalt and nickel were shown to produce epileptogenic lesions in mice (Kopeloff, 1960). Local freezing of the cortex has also been used in cats and rabbits. (Millichap, 1965).

5. MODE OF ACTION OF CONVULSIVE AGENTS

Of the many proposed modes of action of convulsants, there are several involving humoral transmission by acetylcholine, γ-amino-butyric acid and other amino acids, and noradrenaline, dopamine and 5-HT. Below, acetylcholine and γ-amino-butyric acid, etc., are discussed only briefly, whilst aminergic transmitters are discussed in greater detail.

(a) Involvement of acetylcholine (ACh) in convulsive mechanisms

Acetylcholine has been postulated to be a central transmitter as a

result of distribution studies of acetylcholine, choline acetylase and cholinesterase in the brain. (Hebb, 1957; Florey, 1961). The observed concentrations are dependent on the activity just prior to death, since increased concentrations are observed during anaesthesia and sleep, and low concentrations are observed during excitement and convulsions (Tobias, Lipton & Lepinat, 1946; Elliot, Swank & Henderson, 1950; Richter & Crossland, 1949; Crossland & Merrick, 1954; Stone, 1957). Since seizures may lead to an accumulation of acetylcholine in the cerebrospinal fluid (Stone, 1957), it has been proposed that acetylcholine is an excitatory transmitter and that inhibitors of choline acetylase and acetycholine antagonists should be anticonvulsants, ~~and vice versa~~ Epileptiform spike discharges closely resembling those seen in clinical epilepsy have been produced by the local application of acetylcholine to the cerebral cortex of the cat, (Forster & McCarter, 1945). Furthermore, convulsants cause a spread of acetylcholine-induced electrical discharges in the cerebral cortex. It was also shown that acetylcholine is produced in brain slices in response to low concentrations and is depressed by high concentrations of phenobarbitone. (McLennan & Elliot, 1951). Irreversible anticholinesterases, such as diflos, cause acetylcholine accumulation with subsequent tremor and general convulsions (Freedman, Bales, Willis & Himwich, 1949; Waelsch, 1955; Stone, 1957; Tower, 1960). This evidence taken as a whole implies that an elevation of acetylcholine concentrations is proconvulsant. ~~and vice versa.~~ However, work on human epilepsy has revealed a diminished <u>in vitro</u> formation of acetylcholine in epileptogenic cortical tissue, (Tower & Elliot, 1952), together with increased cholinesterase activity, (Pope, Morris & others, 1947) but this

may only have been the result of increased acetylcholine release. Further evidence for this hypothesis came from an observation that muscarinic and nicotinic parasympathomimetic drugs cause convulsions and a claim that antimuscarinic drugs such as atropine and scopolamine are anticonvulsant. (Spiegel, Spiegel-Adolph & others, 1947). A more recent report of the effect of parasympathetic drugs served only to emphasise that the evidence above is slender, since pilocarpine has an anticonvulsant effect, atropine an anticonvulsant effect, methacholine alone only prolongs tonic seizures, yet with anti-epileptic drugs it enhances their effects, and neostigmine and physostigmine have little effect on convulsions (Zablocka, 1963).

(b) Involvement of gamma-amino-butyric acid (GABA) and other amino acids in convulsive mechanisms

GABA (gamma-amino-butyric acid) was first detected in brain in the early 1950's, (Roberts & Frankel, 1949; Roberts, Fukuhara & Visser, 1950), and this was subsequently thought to be an inhibitory transmitter since it inhibits impulses in a stretch receptor-preparation of cray fish abdominal muscle (Florey, 1953). GABA is widely distributed throughout the brain in concentrations varying from 40-1000 μg/g, (Elliot & Florey, 1956; Elliot & Jasper, 1959). In the event of GABA being found to be an inhibitory transmitter, impairment of its synthesis would lead to hyperexcitability and seizures as a result of decreased inhibition. Doubt was thrown upon this hypothesis when dopamine was found to be 100 times more effective on the crayfish inhibitor neuron and, as such, is a far more potent inhibitory transmitter (McGeer, McGeer & McLennan, 1961). However, it is still possible that GABA is a central nervous regulator. Abnormalities in glutamic acid and GABA metabolism in human epileptogenic cortex have been reported (Tower, 1960), and

an abnormally high excretion of glutamine and other amino acids has been found in the urine of Petit mal patients (Millichap & Ulrich, 1962). Both acetazolamide and diphenylhydantoin alter brain amino acid concentrations (Vernadakis & Woodbury, 1960) and this has been suggested as the basis of the anticonvulsant action of diphenylhydantoin (Woodbury & Esplin, 1959).

Seizures can also be induced by pyridoxine deficiency, a deficiency of this sort being produced by a dietary deficiency or treatment with thiosemicarbazide or semicarbazide (Chick, El Sadir & Worden, 1940; Patton, Karn & Longenecker, 1944; Dieke, 1949, Parks, Kidder & Dewey, 1952; Preston, 1955). It was found subsequently that such convulsions are associated with a decrease in brain GABA concentrations and reduced glutamic acid decarboxylase activity (Killam & Bain, 1957), which is dependent on pyridoxal phosphate (Killam, 1957). In vivo leptazol, picrotoxin, and electroshock have each been demonstrated to have no effect on brain GABA or amino acids (Gammon, Gumnit & others, 1960).

(c) Involvement of noradrenaline, dopamine and 5-hydroxytryptamine in convulsive mechanisms

There is now substantial evidence in favour of aminergic transmission in the CNS. 5-HT was first reported to be present in mammalian brain by Twarog and Page (1953) and Amin, Crawford and Gaddum (1954). The distribution of this amine in the brain is uneven, the highest concentrations being found in the hypothalamus, low concentrations in the cerebral cortex and virtually none in the cerebellum (Bogdanski, Weissbach & Udenfriend, 1957). Noradrenaline has a similar distribution although the concentrations are higher (Amin, Crawford & Gaddum, 1954). On the basis of this evidence deficient or excess brain concentrations of 5-HT have been

considered responsible for some mental disorders such as schizophrenia (Wooley & Shaw, 1954). Later both 5-HT and noradrenaline were considered responsible (Brodie & Shore, 1957; Schildkraut, 1965; Schildkraut & Kety, 1967). Noradrenaline was first suggested as a central transmitter by Vogt (1954) and Von Euler (1956). In recent years dopamine has been found present in the brain in comparable amounts to noradrenaline (Montagu, 1957; Carlsson, Lindqvist, Magnusson & Waldeck, 1958), but with a different regional distribution, the highest concentrations being found in the corpus striatum (Bertler & Rosengren, 1959; Sano, Gamo & others, 1959; Ehringer & Hornykiewicz, 1960; Bertler, 1961). The anatomical distribution of noradrenaline and dopamine in the brain has been confirmed by the use of fluorescent histochemical techniques (Carlsson, Falk & Hillarp, 1962; Anden, Carlsson & others, 1964; Dahlstrom & Fuxe, 1964; Fuxe, 1965), by radioactive tracer technique (Glowinski & Iversen, 1966), by autoradiographic studies, (Reivich & Glowinski, 1967), and also by electron microscopic technique (Aghajanian & Bloom, 1967). In toto, this work suggests that noradrenaline and dopamine are specifically localised in a complex neuron system in the brain, and that both amines may be transmitters in the central nervous system. These are the two amines which are at present most prominent in research into mental disease.

Despite all the above suggested seizure mechanisms, the biochemical events involved in a seizure (before and after) are still obscure. It seems likely that events following a seizure are of less importance than those preceding it, and that they are only produced as a result of, rather than the cause of the seizure. Consequently, an increase in catecholamine concentrations in the brain after convulsions may only be the result of stress, and it is

the misinterpretation of such data that has given rise to the abundance of conflicting reports on the cause of convulsions. More important are the biochemical events just prior to the seizure.

Evidence accumulated during the last ten years supports an aminergic mechanism in convulsions, particularly chemically-induced convulsions. Before the era of anti-depressant drugs, many depressive patients were treated with electro-convulsive therapy or by inducing convulsions chemically with leptazol or insulin, and although dangerous, this treatment was frequently effective. Thus convulsions can alleviate depression, a factor which suggests that the two are antagonistic. The advent of anti-depressant drugs in the late 1950's produced further evidence for this hypothesis, since a number of drugs found to be anti-depressant were subsequently shown to produce an antagonistic effect on experimental convulsions. Monoamine oxidase inhibitors were claimed to be anticonvulsant (Prockop, Brodie & Shore, 1959, a,b) and so too were amphetamine (Goodman & Gilman, 1956) and imipramine (Sigg, 1959). These drugs were subsequently shown to produce their antidepressant effects by interference with the metabolism and/or synthesis of catecholamines at central ~~receptor~~ sites (Glowinski, Axelrod & Iversen, 1966). It is possible, therefore, that a similar mechanism might be involved in their anticonvulsant action.

The monoamine oxidase inhibitors inhibit the enzymic activity of monoamine oxidase (MAO), an enzyme involved in the metabolic break-down of monoamines to phenol carboxylic acids and phenol alcohols (Zeller, Barsky & others, 1952 ; see also Axelrod, 1964; Pletscher, 1964). Inhibition of this type raises brain concentrations of both noradrenaline and 5-HT. However, attempts to correlate behavioural

excitation with increased concentrations of a specific amine have given rise to considerable controversy (Kety, 1962). Spector, Hirsch and Brodie (1963) claimed to have separated these effects using various inhibitors and various animal species. Those MAO inhibitors which elevate both noradrenaline and 5-HT produce behavioural excitation which is temporarily correlated with the increased noradrenaline concentrations whereas, in species in which there is no increase in noradrenaline, no behavioural excitation is observed. These workers hypothesised that the excitation which follows administration of MAO inhibitors is associated with the spillover of free noradrenaline onto receptor sites.

Imipramine and other related tricyclic derivatives have been found to be the most clinically effective of the antidepressant drugs (Klerman & Cole, 1965). These drugs do not inhibit MAO or catechol-O-methyltransferase (COMT), but do potentiate responses to sympathetic nerve stimulation and exogenous noradrenaline (Sigg, Soffer & Gyermek, 1963; Klerman & Cole, 1965). Imipramine also potentiates the effects of 5-HT (Sigg et al, 1963). It was subsequently found that imipramine prevents re-uptake of noradrenaline at both peripheral nerve endings (Axelrod, Whitby & Hertting, 1961; Hertting, Axelrod & Whitby, 1961; Whitby, Axelrod & Weil Malherbe, 1961) and in the brain (Glowinski & Axelrod, 1964). Hertting and others (1961) suggested that imipramine may decrease cell-membrane or storage granule-membrane permeability to noradrenaline and thus impair the inactivation of free noradrenaline at the synapse by cellular re-uptake. This process would provide a mechanism for the "sensitisation of the central adrenergic synapse" which Sigg (1959) had proposed to account for the anti-depressant action of imipramine.

Amphetamine, a short acting sympathomimetic psychic stimulant, has been used for many years in the treatment of depression (Goodman & Gilman, 1965). There is evidence that amphetamine exerts its central effects by a direct action on central receptors (Vane, 1960; Rossum, Van der Schoot & Hurkmans, 1962; Smith, 1963). More recent evidence has shown that stimulant effects of amphetamine are produced through release of central endogenous amines such as noradrenaline and dopamine (Stein, 1964, Rech, 1964; Randrup & Munkvad, 1966; Weissman, Koe & Tenen, 1966; Hanson, 1966, 1967; Dingell, Owens & others, 1967; Ernst, 1969). It has been postulated that amphetamine releases bound noradrenaline in a physiologically active form from within the cell and hence by-passes metabolism by intraneuronal MAO (Glowinski et al, 1966; Hanson, 1967). In support of the latter mechanism, it has been shown that an intact noradrenaline synthesis is necessary for the stimulant effects of dexamphetamine (Weissman et al, 1966; Dingell et al, 1967; Hanson, 1967). For a 40% depletion of noradrenaline after α-methyl-p-tyrosine blocks dexamphetamine hyperactivity but a similar depletion after α-methyl-m-tyrosine or reserpine does not. Inhibition of re-uptake for both noradrenaline and dopamine has been postulated as another contributory factor to the mode of action of dexamphetamine (Iversen, 1964; Carlsson, Lindqvist & others, 1965; Glowinski & Axelrod, 1965). Carlsson, Lindqvist et al (1965) reported that only large doses of amphetamine (60mg/kg) act on storage granules to cause a release. A report by Fog, Randrup and Pakkenberg (1967) has implicated the corpus striatum as the central site of action of amphetamine.

A review of the literature indicates that all these sympathetic drugs have some effect on both clinical convulsions and experimentally-induced convulsions. However, the results are

variable and somewhat confusing. It is proposed to discuss briefly the possible involvement of aminergic pathways in audiogenic, electroshock and epileptogenic lesion induced seizures, and discuss more fully leptazol and other chemical convulsants.

(i) Audiogenic convulsions

Wada, Ikeda and McGeer (1967) have suggested neurohumoral mechanisms are involved in audiogenic seizures, and that the actions of 5-HT and DOPA are in accord with the possibility that the catecholamines are proconvulsant and that 5-HT is anticonvulsant. The fact that MAO inhibitors are anticonvulsant was said to be due to a predominance of the inhibitory effect of 5-HT. Genetic studies in mice (Schlesinger, Boggan & Freedman, 1965) showed that audiogenic susceptible mice brains contain only 44% noradrenaline concentrations and 32% 5-HT concentrations when compared with audiogenic resistant mice brains, both groups being 21 days old. When older mice are used susceptible mice become resistant and the time course is correlated with an increase in the brain amine concentrations of these mice. Thus low amine concentrations are taken to induce seizure susceptibility. The results of Lehman and Bushnel (1962) and Lehman (1967) support this conclusion. For these workers showed that MAO inhibitors, imipramine-like drugs and amphetamine are anticonvulsant to audiogenic seizures in mice, and reserpine, tetrabenazine and other catecholamine depleting agents are predictably proconvulsant. An attempt to separate the effects of 5-HT and catecholamines implied that both are equally involved.

(ii) Electroshock convulsions

MAO inhibitors have been shown to inhibit electroshock seizures (Chow & Hendley, 1959; Prockop, Brodie & Shore, 1959 a,b; Yen, Salvatore & others, 1962). Also iproniazid has been shown to potentiate the anticonvulsant activity of phenobarbitone, paramethadione, phenacemide, trimethadione and acetazolamide in the maximal electroshock test (Yen et al, 1962). The results with acetazolamide were supported by the work of Gray, Rauh and Shanahan (1963) and Gray & Rauh (1967) who showed that reserpine abolishes the anticonvulsant effect of acetazolamide and implied that an intact aminergic mechanism is necessary for this anticonvulsant action. These workers also produced evidence to show that noradrenaline is the mediator of this effect and not dopamine or 5-HT. However, De Schaepdryver, Piette and Delaunois (1962) established a correlation between dopamine concentrations and seizure susceptibility. For after a selective increase of central dopamine concentrations in rabbits there is a concomitant increase in electroshock threshold.

The converse of these results has been adequately domonstrated in that reserpine has been found to be proconvulsant in the maximal electroshock test. (Chen, Ensor & Bohner, 1954; Everett, Toman & Smith; 1955 Chen & Bohner, 1957, 1961; Prockop, Shore & Brodie, 1959b; De Schaepdryver, Piette & Delaunois, 1962).

Amphetamine has been demonstrated to be anticonvulsant in electroshock tests, (Tainter, Tainter & others, 1943; De Schaepdryver et al, 1962) but after reserpine the anticonvulsant effect of amphetamine is not evident (De Schaepdryver et al, 1962). This is indicative that intact amine stores are essential for the anticonvulsant effect.

Imipramine has also been found to be anticonvulsant against electroshock (Chow & Hendley, 1959).

Electroshock is probably the most widely investigated convulsive technique with respect to its effect on brain amines. After a single electroconvulsive shock there is no effect on brain noradrenaline concentrations but after repeated shocks there is a fall in these concentrations (Schatalova & Antonov, 1961). It has also been reported that the blood brain barrier becomes more permeable to noradrenaline after electroshock (Rosenblatt, Charley & others, 1960) but it has been pointed out that noradrenaline concentrations in the blood are neither sufficiently high nor prolonged enough to alter brain concentrations appreciably after a single electroshock (Weil-Malherbe, 1955). Kety, Javoy and others (1967) showed that there is a sustained increase in synthesis and utilisation of noradrenaline in rat brain after repeated electroshock. The effect is sustained for at least 24 hours after the electroshock. They also pointed out that the change in regulation of noradrenaline synthesis at this stage is of the same magnitude as that seen with antidepressant drugs immediately, and thus justified the empirical use of electroshock in depressive states. Haggendal (1967) quotes personal communications with Strombergson who had found that brain 5-HT concentrations increase and noradrenaline concentrations either increase or are normal after electroshock. Other reports show that significant changes in 5-HT and noradrenaline are found only after convulsions irrespective of the convulsion (Breitner, Picchioni & Chin, 1964).

(iii) Epileptogenic lesions

This method of producing an epileptic focus has been described previously. When unilateral lesions involving the medial forebrain

bundle are produced in rats there is a fall in both noradrenaline and 5-HT levels. Medial lesions involving the periventricular fibres passing to the midbrain cause a fall in 5-HT levels only (Sheard & Freedman, 1967). Moore, Bhatnagar and Heller (1966) report lateral hypothalmic lesions transecting the medial forebrain bundle in the rat decrease noradrenaline in the telencephalon (which includes hippocampus) by 52% with no effect on brain stem levels. There is also a 36% decrease in telencephalic DOPA decarboxylase activity. Medial hypothalmic lesions have no effect on telencephalic noradrenaline levels. Other reports have also shown the hippocampus to be involved in cerebellar seizure discharges and it has been suggested to play a part in epileptic seizures (Nakamura & Kurebe, 1962).

(iv) Leptazol convulsions

The results with leptazol convulsions are in most cases similar to those using electroshock. Monoamine oxidase inhibitors have been shown to be anticonvulsant (Chow & Hendley, 1959; Prockop, Shore & Brodie, 1959 a,b; Yen et al, 1962). However, there are also reports of a proconvulsant action of MAO inhibitors (Sansome & Dell'Omodarme, 1963). In this case isocarboxazid (Marplan-Roche) and two other MAO inhibitors enhanced the clonic conculsions and the mortality due to leptazol. With amphetamine the results again differ slightly. Friebel and Klatt (1959) reported that amphetamine when used in combination with phenobarbitone or the hydantoins not only inhibits the sedative effect of these drugs but also their antileptazol activity, and they concluded that amphetamine has slight proconvulsant activity per se. Amphetamine was shown to be proconvulsant when given in a mixture with phenobarbitone (Alexander & Weaver, 1955). These last two pieces of evidence might be explained by the recent work of Frey and Kampmann (1966). They showed that the simultaneous use of anticonvulsants with amphetamine results in a delay in their enteral absorption

thereby delaying their onset of action rather than a direct proconvulsant action of amphetamine.

Considerable work has been carried out with leptazol to see if one or more of the brain monoamines is involved in its convulsant action. Vogt (1954), working directly, found no correlation between leptazol convulsions and concentrations of sympathin in cat hypothalamus. On the other hand indirect results recording the effects of aminergic drugs on leptazol convulsions suggest that noradrenaline, (Pfeifer & Galambos, 1967a) 5-HT, (Lessin & Parkes, 1959) and methoxydopamines (Vanderwende & Johnson, 1966) are involved in the convulsive mechanisms. Bertaccini (1959) found a 33% increase in central 5-HT concentrations after leptazol but he considered this was not significant. Pfeifer and Galambos (1967a) showed that prenylamine decreases leptazol threshold in the presence of normal 5-HT concentrations. Whereas guanethidine, which only depletes noradrenaline, facilitates convulsions. It was also shown that reserpine lowers the convulsive threshold and that this effect is reversed by pretreatment with MAO inhibitors.

Imipramine has been reported to be anticonvulsant to leptazol (Chow & Hendley, 1959; Sigg, 1959), but other work suggested it to be inactive against high doses of leptazol and anticonvulsant to low doses (Spencer, unpublished observations).

(v) Other chemical convulsants

It has been reported that picrotoxin lowers brain noradrenaline concentrations (Vogt, 1954; Quastell & Quastell, 1962). Melville and Share (1963) and Share and Melville (1965 a,b) have suggested that catecholamine release from the brain stem after picrotoxim might explain the cardiovascular responses of this drug. The converse

result has been reported by Saito and Tokunaga (1967). They showed that elevation of central amine concentrations after intracerebrally administered adrenaline, noradrenaline and GABA is anticonvulsant but 5-HT is proconvulsant. Amphetamine has been shown to potentiate picrotoxin convulsions but in contrast to the effects on leptazol, imipramine and tranylcypromine have been demonstrated to be proconvulsant (Barron, Hall & others, 1965).

Intravenous injections of tryptamine produce characteristic convulsions which are potentiated by MAO inhibitors (Popov, Leitz & Matthies, 1967). These workers correlated tryptamine levels with convulsions but not with the time course of MAO inhibition.

High oxygen tension has recently been demonstrated to be proconvulsant (Haggendal, 1967; Paton, 1967), and has been investigated for its effects on central catecholamine concentrations (Haggendal, 1967; Neff & Costa, 1967a). Haggendal showed that high oxygen pressures produce a depletion of central dopamine and noradrenaline in rats. Neff and Costa (1967a) showed a twofold increase in noradrenaline turnover which they showed to be due to an increased synthesis rate. However, these workers did not report whether there was an elevation or depletion of catecholamine stores, for depletion can cause increased turnover or conversly either depletion or elevation can be the result of increased turnover.

Summary

Evidence has been presented for the possible involvement, in convulsive mechanisms, of acetylcholine, GABA and other amino acids and sympathetic amines. Acetylcholine was perhaps discussed the most briefly and although some results suggested it to be involved in convulsions these are few in number and conflicting. GABA has also been discussed as a convulsive mediator because it has been shown to be an inhibitory transmitter (Florey,

1953). However, dopamine was later shown to be a more potent inhibitory transmitter (McGeer et al, 1961) and hence more likely to be involved in convulsions if inhibitory transmission is a factor. There is considerable evidence found for the involvement of sympathetic amines in audiogenic, electroshock and chemically-induced convulsions.

The majority of results suggest that elevation of central amine levels is anticonvulsant, and depletion of central amine levels is proconvulsant. However, indirect evidence using aminergic drugs is both conflicting and confusing. A summary of this evidence has been tabulated (Table 1).

5. BASIS OF PROJECT

The various methods of producing convulsions have been briefly reviewed and their interactions with aminergic drugs discussed. In all cases there is evidence to indicate that central monoamines are in some way involved in the seizure mechanism. Reports are conflicting as to whether the amine involved is acetylcholine, GABA, noradrenaline, dopamine or 5-HT but there is more evidence in favour of the latter sympathetic amines. Reports are also ambiguous as to whether an elevation or depletion of such amines is the proconvulsant factor. Therefore, what seems to be more important is the disposition of these biogenic amines and the state of their synthesis, etc.

Whatever the mechanism, there is sufficient evidence to warrant an investigation of the interaction of leptazol with central aminergic pathways. Although not a part of the experimental section of this work audiogenic and electroshock convulsions have been discussed, not only to show that aminergic mechanisms might be involved in such convulsions, but also to reinforce the argument for the involvement of monoamines in leptazol convulsions.

It is proposed to reinvestigate the effects of the more common aminergic drugs on leptazol convulsions and attempt to correlate any observations with the effects on central biogenic amine concentrations. Although previous reports, (for example Vogt, 1954), show no correlation between leptazol convulsions and sympathin concentrations, it is proposed to repeat her work more extensively in the rat whole brain and in certain discrete areas of the rat brain, and investigate not only noradrenaline but also dopamine and 5-HT. Finally, the more common anticonvulsants will be used - particularly chlordiazepoxide, since it is a tranquillizer and may have some effect upon aminergic transmission. It is also a potent anticonvulsant, therefore any information about its anticonvulsant mechanism might contribute to the elucidation of its tranquillizing mechanism.

TABLE 1

SUMMARY OF EVIDENCE FOR THE INVOLVEMENT OF SYMPATHETIC AMINES IN CONVULSIVE MECHANISM

Treatment	Audiogenic Seizures	Electroshock Seizures	Leptazol Seizures	Picrotoxin Seizures
MAO inhibitor	Anticonvulsant 1, 2, 28.	Anticonvulsant 3, 4, 5, 18.	No effect 21, 23. Anticonvulsant 3, 4, 5. Convulsant 6, 7, 26.	Potentiates convulsions 7, 8.
MAO inhibitor	Higher anticonvulsant activity 1.	Higher anticonvulsant activity 1.	Anticonvulsant 24. No effect 21, 26.	
Amphetamine	Anticonvulsant 1.	Anticonvulsant 9, 10, 11. Anticonvulsant only with phenobarbitone 12.	Potentiates convulsions 7, 13. No effect 11.	Potentiates convulsions 8.
Reserpine	Potentiates convulsions 1, 2.	Potentiates convulsions 5, 9, 14, 15, 16. 17, 18, 19.	Potentiates convulsions 15, 20, 21, 22, 23, 24. No effect 27.	
Reserpine and amphetamine		No anticonvulsant activity 9.		
Imipramine	Anticonvulsant 1.	Anticonvulsant 5, 29.	Anticonvulsant 5, 29. Anticonvulsant to low leptazol doses 7.	Potentiates convulsions 8, 25.
Reserpine and Imipramine				Anticonvulsant when given after but not before reserpine 17.

KEY TO TABLE I

1. Lehmann, 1967.
2. Lehmann & Bushnel, 1962.
3. Prockop, Shore & Brodie, 1959 (a).
4. Yen et al, 1962.
5. Chow & Hendley, 1959.
6. Sansome & Dell'Omodarme, 1963.
7. Spencer, (Unpublished observations).
8. Barron et al, 1965.
9. De Schaepdryver et al, 1962.
10. Tainter et al, 1943.
11. Wolf & Stock, 1966.
12. Alexander & Weaver, 1955.
13. Friebel & Klatt, 1959.
14. Prockop, Shore & Brodie, 1959 (b)
15. Chen & Bohner, 1956.
16. Chen & Bohner, 1957.
17. Chen & Bohner, 1961.
18. Chen, Ensor & Bohner, 1954.
19. Everett, Toman & Smith, 1955.
20. Jenney, 1954.
21. Kobinger, 1958.
22. Weiss, Nelson & Tye, 1960.
23. Lessin & Parkes, 1959.
24. Pfeifer & Galambos, 1967 (a)
25. Domenjoz & Theobald, 1959.
26. Spoerlein & Ellmann, 1961.
27. Bastian, 1961.
28. Wada et al, 1967
29. Sigg, 1959.

SECTION TWO

DEVELOPMENT OF SPECTROPHOTOFLUOROMETRIC ASSAYS OF NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE

DEVELOPMENT OF SPECTROPHOTOFLUOROMETRIC ASSAYS OF NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE

page no.

DEVELOPMENT OF SPECTROPHOTOFLUOROMETRIC ASSAYS OF NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE

1. EXAMINATION OF THE SPECTROPHOTOFLUOROMETRIC ASSAY OF NORADRENALINE AND ADRENALINE

The catecholamines have no significant intrinsic fluorescence even in strongly acid or alkaline solvents. Therefore to render them suitable for such a chemical assay they must be converted to a fluorescent principle. Two procedures were available:-

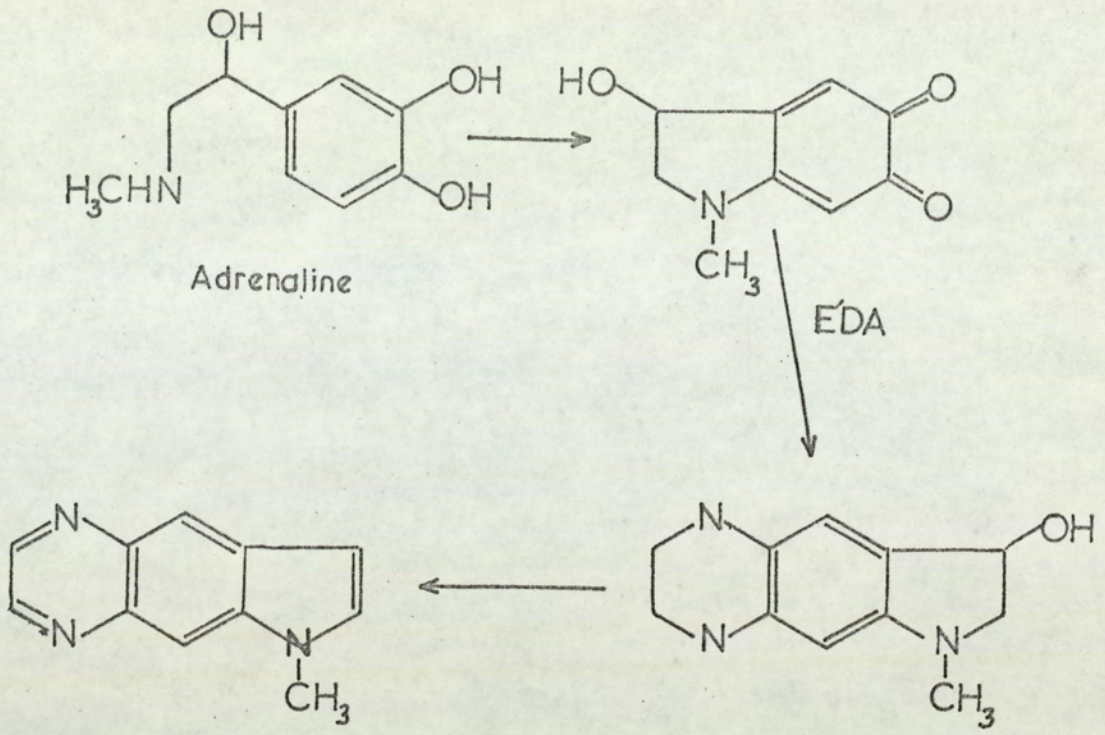
(a) Ethylenediamine condensation.

(b) Trihydroxyindole procedure.

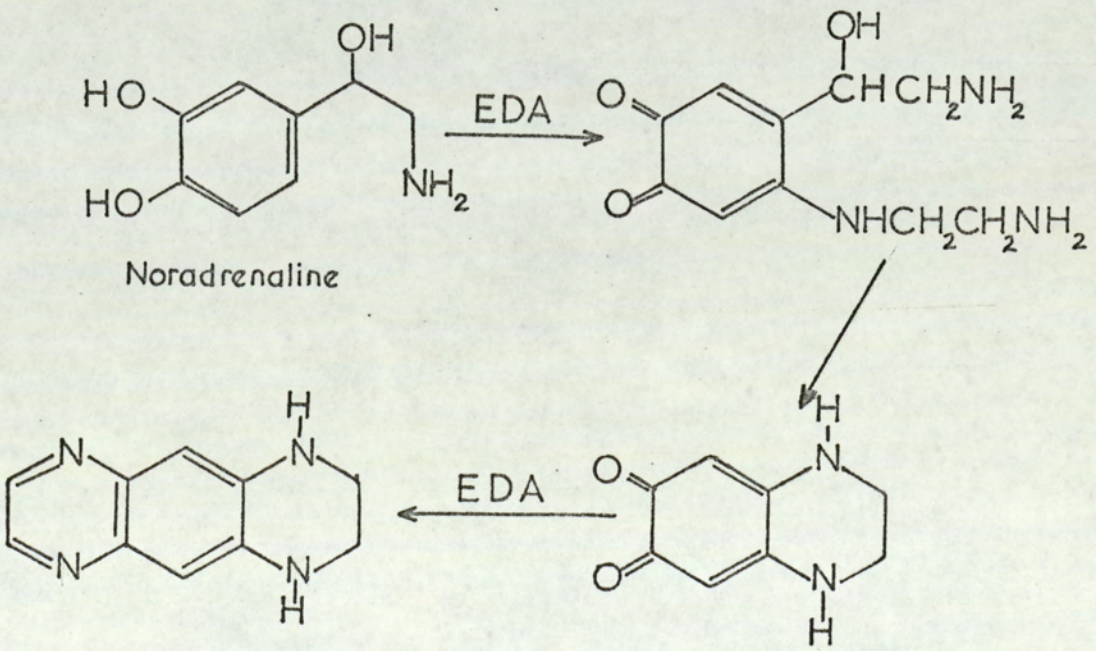
(a) Ethylenediamine condensation

This procedure was discussed at the Catecholamine Symposium, 1958 (Weil-Malherbe, 1959). It was shown to be less specific than the trihydroxyindole procedure (von Euler, 1956; Valk & Price, 1956; Haggendal, 1966). For example, dopamine was shown to interfere by adding to the noradrenaline fluorescence (Weil-Malherbe, 1959).

The formation of a fluorescent principle by this method involves the oxidation of adrenaline to adrenochrome and then condensation of the quinonoid oxygen groups with ethylene diamine (EDA).



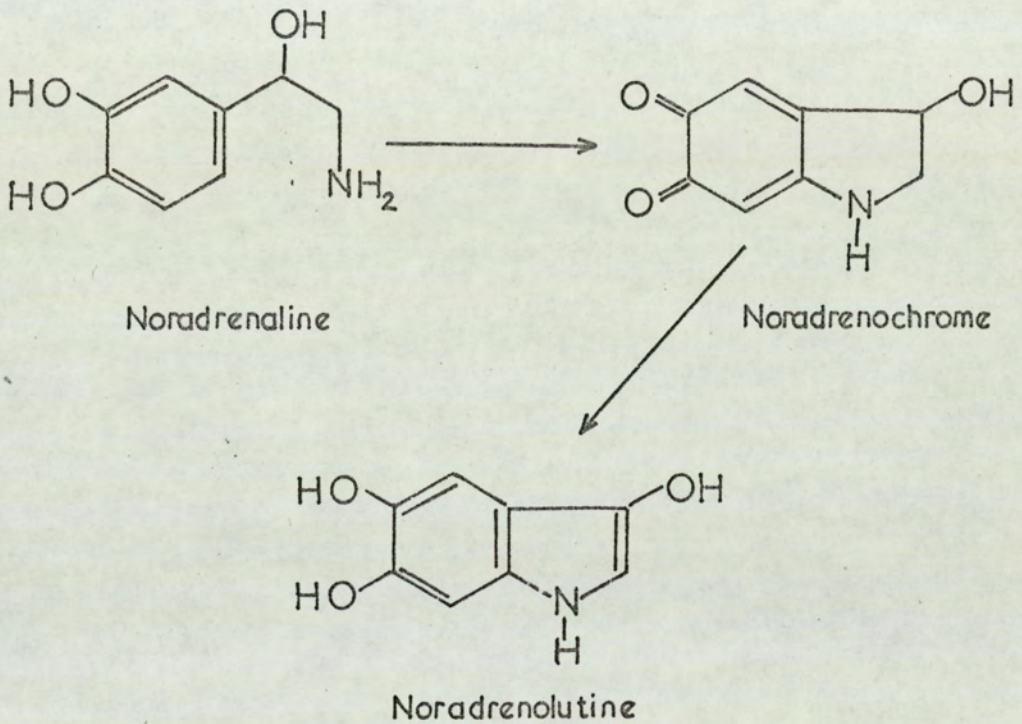
Noradrenaline forms a different condensation product because the ethylenediamine condenses on position 6 of the noradrenaline nucleus rather than on the quinonoid oxygen. Subsequently there is a ring closure which eliminates β-ethanolamine.



Because of the lack of specificity this method has fallen out of use.

(b) Trihydroxyindole procedure

This method was fully discussed in the 1958 Symposium (von Euler, 1959). Noradrenaline is converted to noradrenochrome by oxidation with iodine or potassium ferricyanide and subsequent rearrangement in alkaline conditions yields noradrenolutine the fluorescent principle.



Adrenaline and dopamine also yield fluroescent principles but nevertheless the method is specific because each displays differences in spectral characteristics.

Potassium ferricyanide was considered a better oxidant than iodine, since oxidation with the former requires only 2-3 minutes, whereas with iodine 30-60 minutes are necessary. (Anton & Sayre, 1962; Euler & Floding, 1955; Crout, 1961). Iodine also produces higher reagent blanks. Potassium ferricyanide further improves the specificity of the procedure because dopamine is not significantly oxidised (Euler, 1959; Anton & Sayre, 1962).

(i) Assay of pure solutions of adrenaline and noradrenaline

The initial procedure was essentially that used by Euler and Floding, (1955) and Bertler, Carlsson and Rosengren, (1958), except that phosphate buffer was substituted for acetate buffer and the zinc sulphate omitted, The reagents were added in the following order:-

Noradrenaline solution 1 ml.

Phosphate buffer pH 6.5 1 ml.

Water 1.9 ml.

Potassium ferricyanide 0.25% $w/_v$ 0.1 ml.

Allowed to oxidise for 3 minutes.

*Alkaline ascorbate 1 ml.

*Alkaline ascorbate - 9 parts N sodium hydroxide + 1 part 2% $w/_v$ ascorbic acid (prepared immediately before use).

Potassium ferricyanide oxidised noradrenaline to noradrenochrome, and the alkaline ascorbate served a dual purpose: (a) the ascorbic acid stopped the oxidation, (b) the sodium hydroxide rendered the solution alkaline and hence promoted the rearrangement to noradrenolutine.

A reagent blank was performed to account for background fluorescence of the reagents. In this case noradrenaline solution was replaced by 1 ml distilled water. Oxidation was allowed to proceed for 3 minutes and then only the sodium hydroxide added. After a further 5 minutes the ascorbic acid was added. This was a faded reagent blank. A similar blank was formed by allowing noradrenaline to fade for 5 minutes; this blank not only accounted for reagents but also any fluorescence contributed by the faded noradrenaline. When using pure solutions these two blanks were the same, consequently only one of the noradrenaline faded blanks was performed. Since low background fluorescence was essential for a sensitive assay the reagents were

checked initially. Water was used as a solvent for most reagents and therefore its fluorescence was examined over the noradrenaline spectrum. Three types of purification of water were tried:-

(1) deionisation

(2) distillation

(3) distillation followed by deionisation

The relative fluorescence was measured at the noradrenaline peaks. The results are shown in Table 2.

Although all three sources of water showed only an acceptable background fluorescence the distilled water was lowest and consequently was adopted for use thereafter.

(ii) Spectral characteristics of noradrenaline

Using the oxidation procedure above, the activation and emission spectra of pure solutions of noradrenaline were produced using an Aminco Bowman spectrophotofluorometer. The excitation peak was found at 395mμ and the emission peak at 500mμ (uncorrected) (Fig.1). These values were similar to those found by other workers (Bertler, Carlsson & Rosengren, 1958; Crout, 1961; Anton & Sayre, 1962). In the same determination the reagent blank did not exceed 5% fluorescence of the pure solution at any point on the spectrum. The pure noradrenaline solution contained 40ng/5ml

(iii) Stability of fluorescence

When assaying pure solutions it was noticed that the fluorescence was unstable in that it increased with time. Other workers have also found this and have tried various agents to stabilise the fluorescence e.g.5N sodium hydroxide (Euler & Floding, 1955); 10N sodium hydroxide (Anton & Sayre, 1962); B.A.L./ sodium sulphite mixture has been used to replace alkaline ascorbate (Haggendal, 1963); and sodium borohydride has been used. (Gerst, Steinsland & Walcott, 1966).

Sodium hydroxide was tried in various normalities 1N, 5N and 10N, none of which produced a significantly stable fluorescence for 30 minutes, which was the stability necessary. Since 5N had shown the best stability the assay was continued using this normality. The method of Gerst et al, (1966) was then investigated. These workers used sodium borohydride in the alkaline ascorbate in a concentration of 1% of the ascorbic acid used. There was no appreciable stabilisation of fluorescence in our hands. The procedure was repeated using 12.5% $w/_w$ of the ascorbic acid used. This proved successful and fluorescence was stable 18 minutes after oxidation and remained so for at least 30 minutes even in very dilute noradrenaline solutions. On repeating the assay but preparing the alkaline ascorbate/sodium borohydride mixture 8 minutes before oxidation instead of immediately before, the fluorescence was stabilised 10 minutes after oxidation. This indicated that the ascorbate mixture required 18 minutes to reach equilibrium and thereafter stabilised the fluorescence. Therefore in subsequent assays sodium borohydride was incorporated in a concentration 12.5% $w/_w$ of ascorbic acid in the alkaline ascorbate mixture (i.e. 2.5 mgm sodium borohydride in 10 ml alkaline ascorbate mixture) and prepared 8 minutes before oxidation. The stabilised fluorescence was then read between 10 and 40 minutes after oxidation. Subsequent work showed this fluorescence to be stable for much longer than 40 minutes but this time was sufficient in our procedure.

(iv) Spectral characteristics of adrenaline

The above oxidation procedure was used to examine the fluorescence of adrenaline at pH 6.5. From the spectra it was evident that there were two excitation peaks at 345 mμ and 410 mμ (uncorrected). Using the 410 mμ excitation peak there was an emission peak at 520 mμ (uncorrected). For assay purposes the peaks at 410/520 mμ were used but the 345 mμ peak provided a means of confirming that it was adrenaline in a given

sample. (Fig. 2).

(v) Differential assay of adrenaline (A) and noradrenaline (NA)

Since both catecholamines were extracted by the same procedure it was necessary to devise a differential assay.

The method utilising the difference in spectral characteristics required that fluorescence be measured at a constant emission peak and at two excitation wavelengths. By solving two simultaneous equations the concentrations of both amines could be calculated (Bertler et al, 1958; Vendsalu, 1960). This procedure did not prove sufficiently accurate when assaying mixtures where the NA/A ratio ranged from 1/25 to 25; all values were not within $\pm$ 5% of the correct concentration.

Another method involved using a differential antioxidant to replace alkaline ascorbate (Vochten & de Schaepdryver, 1966). Thioglycollic acid/cysteine mixture was the antioxidant which allowed adrenaline to deteriorate while noradrenaline fluorescence remained normal. Consequently, only noradrenaline fluorescence was measured and adrenaline could be calculated by difference from the standard oxidation. Using 0.01% $v/_v$ final concentration of thioglycollic (as used by the above workers) it was found that both adrenaline and noradrenaline fluorescence deteriorated to the same order as the reagent blank. (Fig. 3). When the thioglycollic acid concentration was increased to 0.1% $v/_v$, then noradrenaline fluorescence was significantly higher than the reagent blank and adrenaline, but it faded rapidly and reproducable results were unobtainable. (Fig. 4). The method was therefore discarded. A modification of the thioglycollic acid mixture was to add sodium borohydride since it had been previously found to stabilise noradrenaline. However, there was no stabilisation of fluorescence.

A third differential technique involved oxidation of catecholamines at two pH values. (Anton & Sayre, 1962). At pH 2 only adrenaline is oxidised and fluoresces, while noradrenaline fluorescence is negligible. At pH 7 both catecholamines can be oxidised to highly fluorescent principles. To achieve a solution of approximately pH 2, Anton and Sayre (1962) used 1.6 N acetic acid. Using this technique in the present investigation, it was found that there was no difference in adrenaline and noradrenaline standard fluorescence and reagent blank. Thus the acetic acid had quenched the fluorescence of both catecholamines. These authors, however, state that the oxidation was extremely sensitive to small changes in pH and therefore, various dilutions of acetic acid were investigated. The results are shown in Table 3.

The optimum mixture was the 0.2N acetic acid which allowed 98.5% oxidation of adrenaline and only 1.6% oxidation of noradrenaline. The pH of this mixture when added to standard solution was found to be 2.7; therefore this was the optimal pH for differential oxidation.

(vi) Linear relationship between fluorescence and concentration

To check that fluorescence was proportional to concentration, a series of concentrations of adrenaline and noradrenaline were assayed at pH 2.7 and pH 6.6.

A linear relationship applied to adrenaline at least up to 1 µg (total in cuvette) at both pH values. Similarly noradrenaline fluorescence was linear up to at least 1 µg at pH 6.6. At pH 2.7 fluorescence was negligible up to 100 nanograms and was only 9% of the fluorescence at pH 6.6 at higher concentrations. The fluorescence at

pH 6.6 was also linear. (See Fig. 5 and Fig. 6).

(vii) Optimum pH for oxidation of noradrenaline

The higher pH of oxidation was chosen as 6.6 since a series of tests at various pH values had shown the fluorescence to be at a peak at pH 6.7 and thereafter it fell considerably. Consequently, pH 6.6, on the plateau of the graph, was used because it gave a high fluorescence and allowed a margin of error. (Fig. 7).

(viii) Sensitivity of adrenaline and noradrenaline assays

When assaying pure solutions the reagent blank was found to show a fluorescence equivalent to approximately 2 nanograms noradrenaline or adrenaline. This was therefore the absolute limit of sensitivity of the method. The emission and excitation peaks of both amines were easily detectable when using 4 nanograms.

In tissue extraction the tissue faded blank was equivalent to no more than 12 nanograms noradrenaline and 15-20 nanograms could be accurately detected. In determinations of adrenaline in rat whole brain, the concentration of adrenaline was found to be about 20 nanograms/gramme but since this was close to the limit of sensitivity of the assay it was decided that further assays of central adrenaline would not be performed. Other workers have shown there is little adrenaline in the central nervous system (Anton & Sayre, 1962). Only in the frog brain was there a predominance of adrenaline. When tissue extracts were assayed, it was found necessary to adjust the eluate to * pH 3.3 for the adrenaline assay and then on addition of the 0.2N acetic acid the pH returned to 2.7.

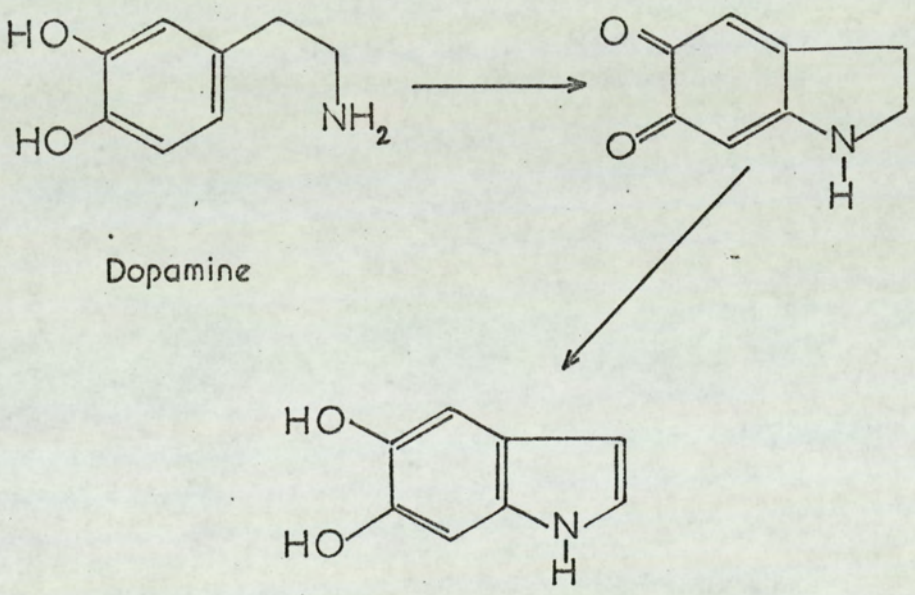
* The eluate was adjusted to pH 3.3 using 0.1N potassium carbonate at a pH meter.

2. EXAMINATION OF THE SPECTROPHOTOFLUOROMETRIC ASSAY OF DOPAMINE

(i) Assay of pure solution of dopamine

The same two chemical procedures as were used for noradrenaline can be used to produce a fluorescent derivative of dopamine. The ethylenediamine condensation is one method used to produce the fluorescent principles (Laverty & Sharman, 1965). This method was tried with little success, the reagent blanks being higher in fluorescence than the pure dopamine solutions.

The procedure using iodine as the oxidant was then used (Carlsson & Waldeck, 1958). This procedure yields a dihydroxyindole as the fluorescent principle.



Dopamine

5-6-dihydroxyindole

However, fluorescence was unstable even after U.V. irradiation as suggested by the above workers. A modification of this method was to heat the oxidised solutions in a water bath at 45°C for 30 minutes (Drujan, Sourkes & others, 1959). In our hands this procedure did not

improve the stability of the fluorescence because when placed in the fluorometer the activation wavelength of light induced a slow increase in fluorescence intensity. Attempts to stabilise the fluorescence with sodium borohydride, as used in the noradrenaline assay, only reduced fluorescence and shifted the dopamine peaks according to the concentration of sodium borohydride used. A successful modification was to heat the oxidised solutions in a boiling water bath for five minutes; the tubes were allowed to cool to room temperature (15-20 minutes) and then read in the Aminco Bowman spectrophotofluorometer. This method had previously been used in a differential assay, (Chang, 1964). Solutions treated in this way produced a fluorescence which was stable for at least 75 minutes. Having established the fluorescence, it was found that the sensitivity required improvement. The modification of this method (Carlsson & Lindqvist, 1962) using 1.6 ml of acetic acid instead of 0.6 ml and 0.1 ml of iodine solution instead of 0.05 ml was used. However, we continued to use 0.05 ml iodine and to heat the oxidised solutions at 100°C for 5 minutes instead of U.V. irradiation, and therefore only used the increased volume of acetic acid. This improved the sensitivity markedly and was the method finally adopted for the assay of dopamine.

The buffer solution was also changed. We used a mixture of 0.5M disodium hydrogen orthophosphate and 0.6M citric acid which produced a buffer of pH 5.4.

(ii) Spectral characteristics of dopamine

Dopamine when oxidised by this method showed an excitation peak at 315 mμ and maximum emission at 375 mμ (uncorrected). (Fig. 8).

(iii) Linear relationship between fluorescence and concentration

Dopamine showed a linear relationship between fluorescence and concentration, up to 800ng total in the cuvette. (Fig. 9).

(iv) Sensitivity of dopamine assay

When assaying pure solutions the faded blank displayed a fluorescent equivalent to 30 nanograms dopamine but the excitation and emission peaks were easily detected with 40 nanograms dopamine. When tissue extracts were assayed the faded blank showed a fluorescence equivalent to 50-60 nanograms dopamine. Higher values were easily detectable.

3. EXAMINATION OF THE SPECTROPHOTOFLUOROMETRIC ASSAY OF 5-HYDROXYTRYPTAMINE (5-HT)

(i) Assay of pure solutions of 5-HT

5-HT shows intrinsic fluorescence when the solvent is buffered at an acid pH (Udenfriend, 1962). In a recent paper this property has been untilised to produce a simple assay technique for 5-HT (Cox & Potkonjak, 1967). This method was examined in pure solutions. The solution used as a solvent for the 5-HT was the eluant used by Cox and Potkonjak (1967), i.e. 0.1N sodium hydroxide containing 0.2% $w/_v$ EDTA. The solutions were added in the following order:-

1.5 ml 5M sodium acetate buffer pH 4.6

14 ml 0.1N sodium hydroxide

1 ml 5-HT standard in 0.01N hydrochloric acid,

and the fluorescence spectrum followed on the Aminco Bowman spectrophotofluorometer.

(ii) Spectral characteristics of 5-HT

The spectra of 5-HT treated in this manner showed an excitation peak at 295 mμ and an emission peak at 345 mμ (uncorrected). (Fig. 10). It has been reported that in 3N hydrochloric acid the emission peak shifts to 550 mμ rendering the assay more specific. (Udenfriend, 1962). In our hands, this modification did not produce a clear peak at 500 mμ and reduced the sensitivity of the assay to 50% of the previous level.

(iii) Linear relationship between fluorescence and concentration

5-HT showed a linear relationship of fluorescence to concentration up to at least 4 µg total in cuvette. (Fig. 11).

(iv) Sensitivity of 5-HT assay

The assay method for 5-HT did not allow a faded blank to be prepared easily and therefore a simple reagent blank was used for pure solution. This blank showed a fluorescence equivalent to not more than 100 nanograms when compared with the 1 µg standard. The assay of tissue extracts involved the use of a reagent blank, in which the reagents had been passed through the Dowex column used for isolation of 5-HT. This gave a measure of fluorescent principles washed from the resin itself. Such a blank displayed a fluorescence equivalent to not more than 180 nanograms when whole brain samples showed a fluorescence equivalent to 1 µg. Samples containing 300 nanograms were easily detectable.

4. EXAMINATION OF COLUMN CHROMATOGRAPHY PROCEDURE

(i) Preparation of the ion exchange resin

AG 50 W-X 8 is a purified form of Dowex resin, (BioRad Laboratories, California) and was further purified for spectrophotofluorometric assay by washing a minimum of eight times with the following reagents as described below.

(a) 2N sodium hydroxide

(b) Distilled water until the effluent was at pH 7.0

(c) 2N hydrochloric acid

(d) Distilled water until the effluent was at pH 7.0

In the final cycle 2% EDTA was added to the sodium hydroxide solution. In the washing procedure the resin was transferred onto a Whatman filter paper in the funnel of a Buchner flask. The washing solutions were added and drawn through the resin by means of a vacuum. The pH of the effluent in the Buchner flask was checked using indicator paper or a pH meter.

The resin was stored wet in the H^+ form. A small amount of the resin was weighed, dried to constant weight, and reweighed to calculate the wet/dry ratio. This was repeated every three or four months to check that the resin was not drying out.

(ii) Preparation of mico-columns

Micro-columns were prepared by the glassblower using pyrex tubing of 7mm external diameter and 4mm internal diameter. The columns were 4cm long and gently tapered at one end. A small wad of cotton wool was pushed into the column to support the resin. 100 mg (dry weight) of the previously purified resin was washed into the column with distilled water. The dead space above the resin could be filled with glass beads but this often gave rise to air bubbles and was omitted.

(iii) Modification of the Bertler adsorption apparatus

Each column was then connected to an 'adsorption and elution assembly'. Originally the Bertler column-syringe assembly was used. (Fig. 12). (Bertler, Carlsson & Rosengren, 1958). However, in our hands this apparatus had several disadvantages:-

1. The syringe and plunger were moving parts. The plunger was expected to fall at a constant rate and force the solutions through the column at this rate. But each syringe fell at a different rate and therefore, because flow rate was critical, this meant that recovery values calculated from one column could not be applied to the next column.
2. The syringe plunger would often seize and prevent further flow.
3. The three-way tap was liable to leak and if grease was used this might release fluorescent principles.

4. Because of these disadvantages flow rate through the micro-columns could not easily be controlled.
5. The apparatus was difficult to clean and had to be reassembled for the next assay.

The major advantage of the Bertler apparatus was that once the flow had commenced the apparatus could be left and the column would not run dry.

With these properties in mind a new apparatus was designed. (Fig. 13). This was simply a 10 ml pyrex pipette opened out to a small funnel at the top. The jet was removed and the lower end fused to a pyrex glass S-bend. When the micro-column was attached, the lower tip of the column was always above the bottom of the S-bend, thus preventing syphoning and running dry. This apparatus overcame the above difficulties.

The advantages of this apparatus were:-

1. No moving parts, therefore flow could continue unimpeded.
2. The solutions passed through the column under the force of gravity. Hence the flow rate was constant and reproducible from one column to the next.
3. There were no taps in the system and thus grease was avoided.
4. The apparatus was easily dismantled and cleaned. (Only one joint - the micro-column).
5. The S-bend in the system prevented the apparatus from running dry.

5. EXAMINATION OF THE EXTRACTION PROCEDURE FOR CATECHOLAMINES AND 5-HT

The method of extraction used was essentially that of Anton and Sayre (1962). The brains were homogenised in ice cold 0.4N perchloric acid (4 ml/2g tissue) using a Jencon tissue grinder (Type MTG4 30 ml) driven at 100 rpm by an electric motor. For consistent results it was necessary to homogenise each tissue for the same length of time by the same method, four strokes of the mortar being sufficient to produce a fine homogenate. The homogenate was centrifuged at 0°C at 15,000 g for eight minutes. (30,000 g, as suggested by Anton and Sayre (1962) stressed the polycarbonate tubes with resulting leakage). It was found necessary to do a second homogenisation on the precipitated brain tissue after the supernatant had been decanted. Omission of this re-homogenisation resulted in a 20% fall in the whole brain noradrenaline levels. After re-centrifuging the supernatant was bulked with the previous extraction, made up to 16 ml with 0.4N perchloric acid, shaken and divided into two equal portions. One portion was used for catecholamine determinations and the other ~~from~~ for 5-HT determinations. Known concentrations of noradrenaline, dopamine and 5-HT were added to some extracts to produce internal standards as a check on recovery of these amines. These extracts were either used immediately or stored for no longer than 24 hours at -25°C.

6. EXAMINATION OF THE ADSORPTION AND ELUTION PROCEDURES FOR CATECHOLAMINES AND 5-HT

(i) Adsorption

The extract was titrated to pH 6.5 using 5N potassium carbonate solution and a pH meter. The precipitate of potassium perchlorate was centrifuged off at 15,000 for 6 minutes, after the mixture had been thoroughly stirred with a glass rod. (Omission of this step led to effervescence and disruption of the ion exchange columns when the supernatant was passed onto the column).

The neutralised supernatants were then passed onto the appropriate resins, 5-HT onto resin in Na^+ form, catecholamines onto resin in H^+ form (see Methods, Section 3), and allowed to run through at a flow rate not exceeding 1 ml in 2 minutes. The potassium perchlorate precipitate was washed with 10 ml distilled water and centrifuged as before. This water wash was passed through the column after the extract had run through and then the columns were ready for elution. Internal standards were treated in exactly the same way as the extracts.

(ii) Elution of catecholamines

In order to remove water from the dead space on the column 0.5 ml of 0.4N hydrochloric acid was passed onto the column but not allowed to pass through the column. Then 8 ml 0.4N hydrochloric acid was passed through the resin at a flow rate not exceeding 1 ml in 2 minutes. This was in order to elute off noradrenaline, and this 8 ml was collected for assay.

After adjusting the eluates to pH 6.5 and making up to a final volume of 10 ml, the noradrenaline was assayed by the method described earlier (in detail under Methods, Section 3).

After elution of noradrenaline, 0.5 ml 2N hydrochloric acid was passed onto the columns to displace the 0.4N acid in the dead space. The 2N hydrochloric acid was not allowed to pass to waste. To elute dopamine 8 ml 2N hydrochloric acid was passed through the column and the eluate collected for assay. Dopamine was assayed according to the method discussed earlier (described more fully under Methods, Section 3), after the eluates had been adjusted to pH 5.4 with 10N potassium carbonate and made up to a final volume of 12 ml.

Catecholamine assays were either completed on the same day as elution or within 24 hours of elution. If kept overnight eluates were stored at $-25^{\circ}C$.

(iii) Elution of 5-HT

The 5-HT was adsorbed onto the resin in the Na^+ form (see Methods, Section 3). As in the case of the catecholamine columns a small volume of the eluant was passed onto the column to displace the water from the dead space, in this case 0.5 ml 0.1N sodium hydroxide containing 0.2% $w/_v$ EDTA was used. To elute the 5-HT 15 ml of this eluant was then passed onto the column and the eluate collected in a beaker containing 1.5 ml 5M sodium acetate buffer at pH 4.6. The fluorescence of the eluate was then read directly in the Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 295 mμ and emission wavelength of 345 mμ . In all cases 5-HT was assayed immediately after elution.

After each assay the fluorescence spectrum of each biogenic amine was checked to see that it was the same as the pure standard spectrum, thus ascertaining the identity of the amine being assayed. This also served as a check on any interference produced by the drugs used.

The extraction, adsorption and elution procedure for catecholamines was essentially that used by Bertler, Carlsson and Rosengren (1958), and the method for 5-HT was essentially that of Cox and Potkonjak, (1967) with the modifications described earlier.

The ion exchange resin could have been reconstituted by washing it with 40 ml 2N hydrochloric acid, (Bertler, Carlsson & Rosengren, 1958) and rewashing the columns as before. This, however, saved little time and gave rise to a progressive increase in the fluorescence of the blanks. Therefore, each assay was performed using fresh resin and clean glassware.

7. WASHING OF GLASSWARE

All glassware used in these assays was soaked for 24 hours in a solution of Decon 75 (Medical-Pharmaceutical Developments Ltd., Ellen Street, Portslade, Brighton). The glassware was then transferred to clean tap water and rinsed free of detergent by rinsing three times. Then it was rinsed three times in fresh distilled water and finally another three times in a second container of fresh distilled water. The glassware was dried in a hot air oven for a few hours.

8. SUMMARY

The final complete assay procedures for noradrenaline, dopamine and 5-HT are described separately in the next section - (Methods).

TABLE 2

THE RELATIVE FLUORESCENCE, AT 395/500mμ , OF WATER PURIFIED BY VARIOUS METHODS

Type of purified water	Relative fluorescence
Deionised	0.031
~~Deionised~~ Distilled	0.017
Distilled-Deionised	0.024

TABLE 3

EFFECT OF ACETIC ACID, IN VARIOUS NORMALITIES, AS A BUFFER FOR OXIDATION OF CATECHOLAMINES AT LOW pH

Catecholamine Noradrenaline (NA) Adrenaline (A)	Concentration of Acetic Acid used as a 'Buffer'	RF at pH 2 approx. using Acetic Acid	RF at pH 6.6 using Phosphate buffer ONLY	% RF pH 2 / RF pH 6.6 (Ratio)
NA	1.6N	0.009	9.095	0.1%
A		0.232	13.695	1.7%
NA	0.4N	0.62	10.074	0.6%
A		5.089	12.474	40.8%
NA	0.2N	0.153	9.50	1.6%
A		12.825	13.00	98.5%
NA	0.1N	0.697	9.5	7.3%
A		10.227	13.00	79%

RF = Relative fluorescence

Relative fluorescence is the term used for Relative fluorescence intensity and is the product of the Meter multiplier reading x meter reading

FIGURE 1

EXCITATION AND EMISSION SPECTRA OF NORADRENALINE

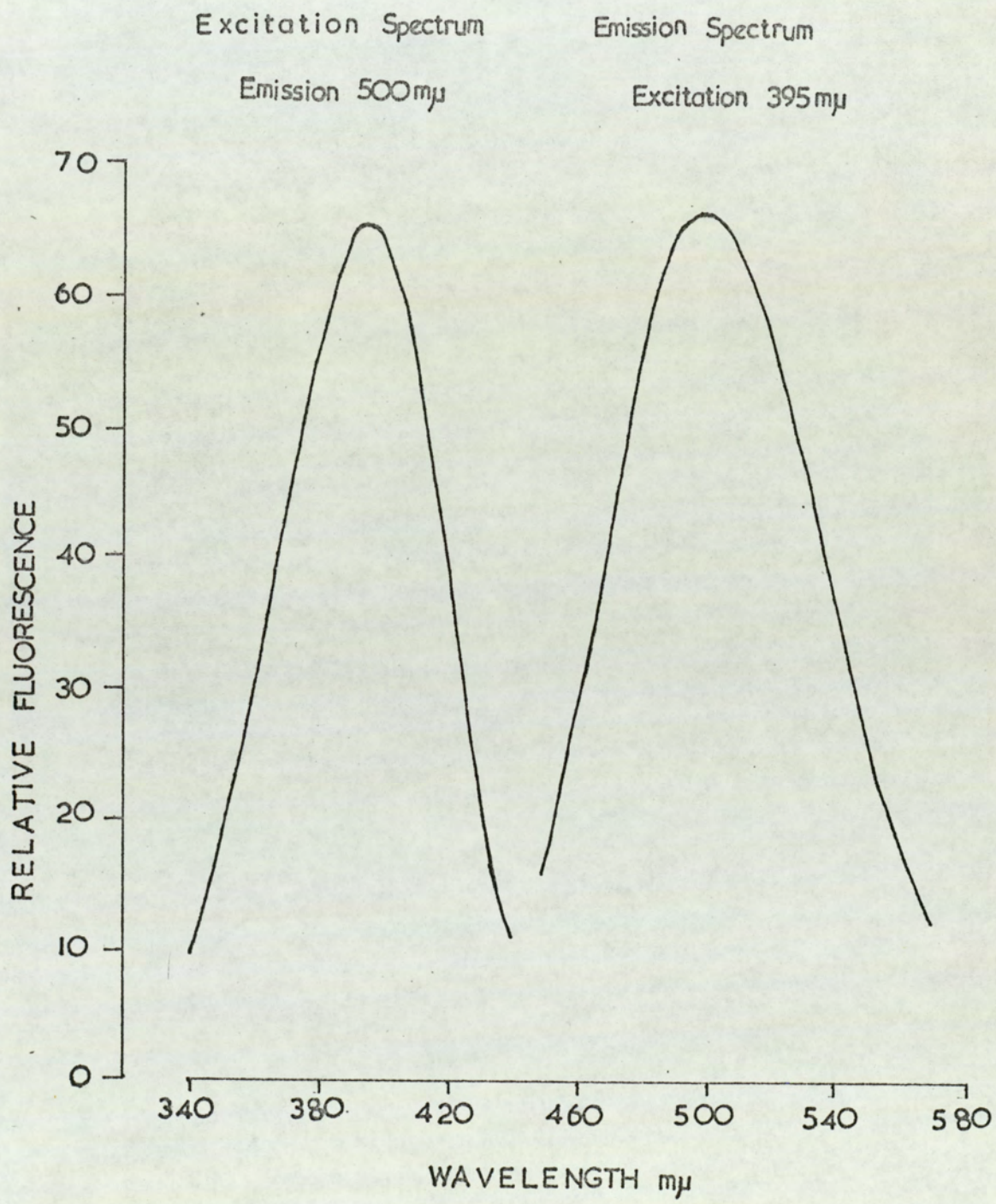


FIGURE 2

EXCITATION AND EMISSION SPECTRA OF ADRENALINE

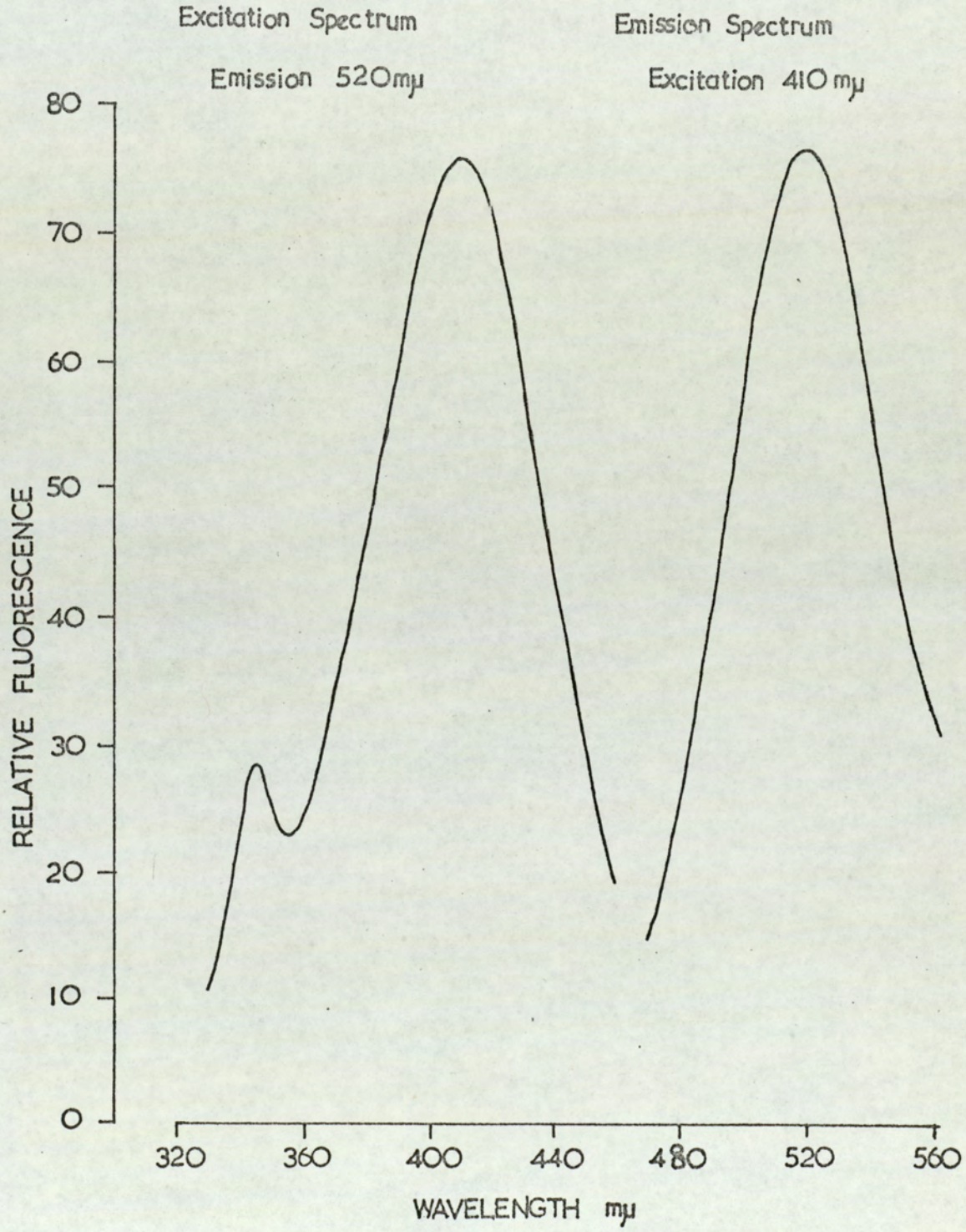


FIGURE 3

STABILITY OF CATECHOLAMINE FLUOPHORES IN 0.01% v/v TRIOGLYCOLLIC ACID

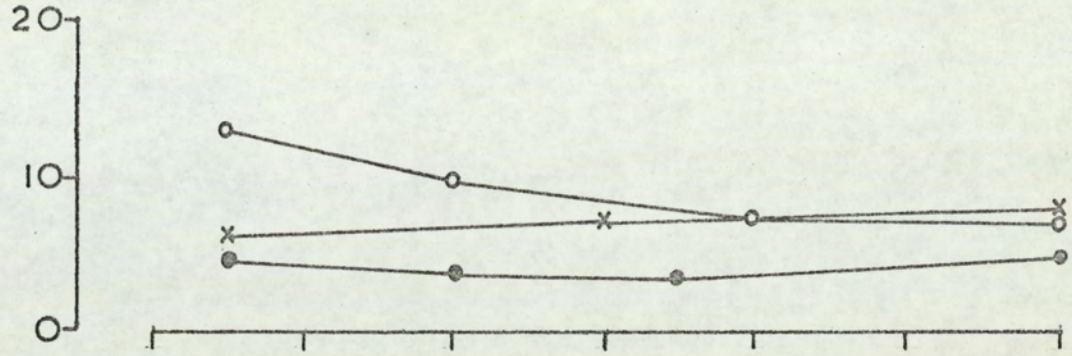


FIGURE 4

STABILITY OF CATECHOLAMINE FLUOPHORES IN 0.01% v/v TRIOGLYCOLLIC ACID

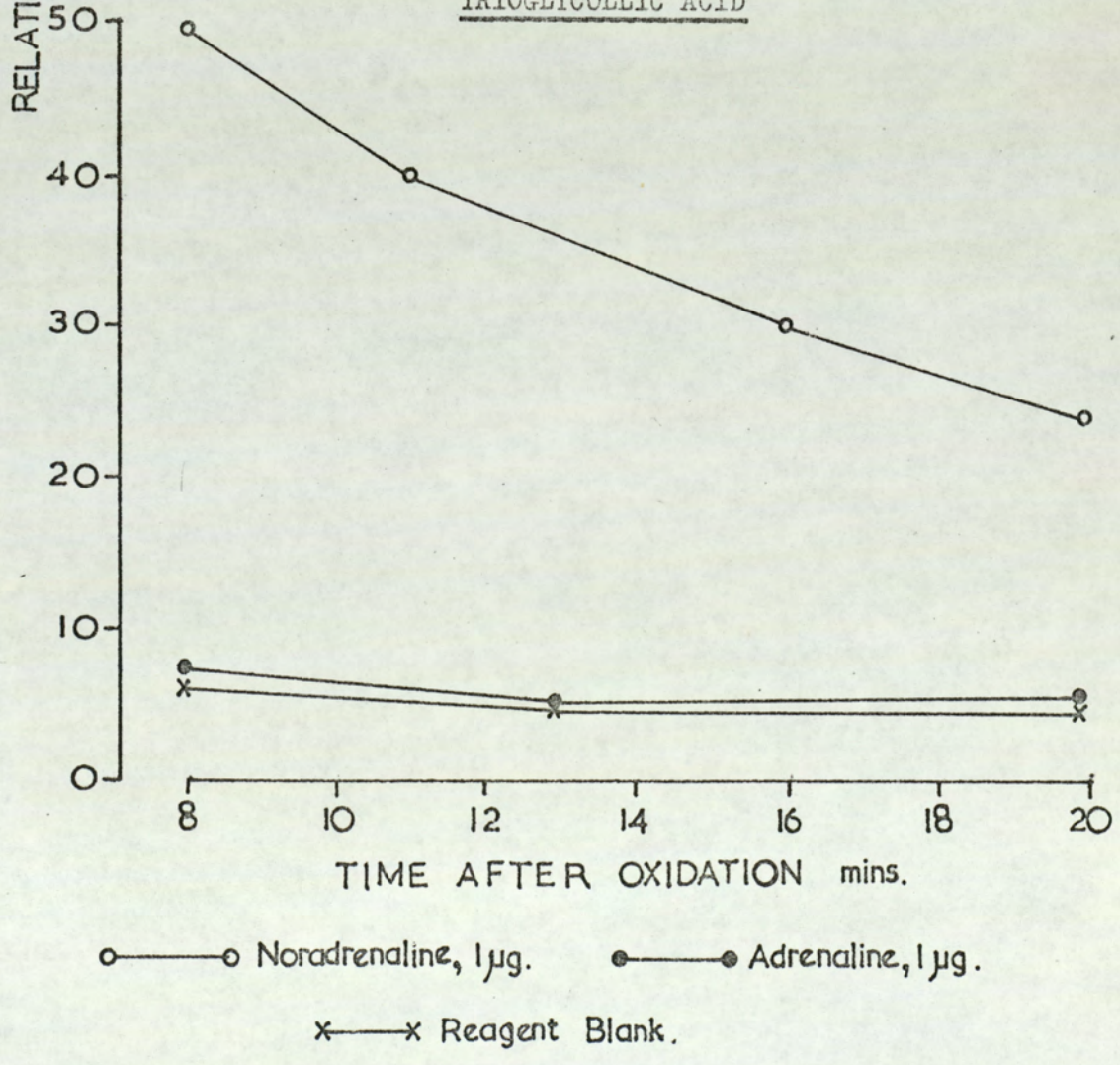


FIGURE 5

FLUORESCENCE OF NORADRENALINE OXIDISED AT pH 2.7 AND pH 6.6

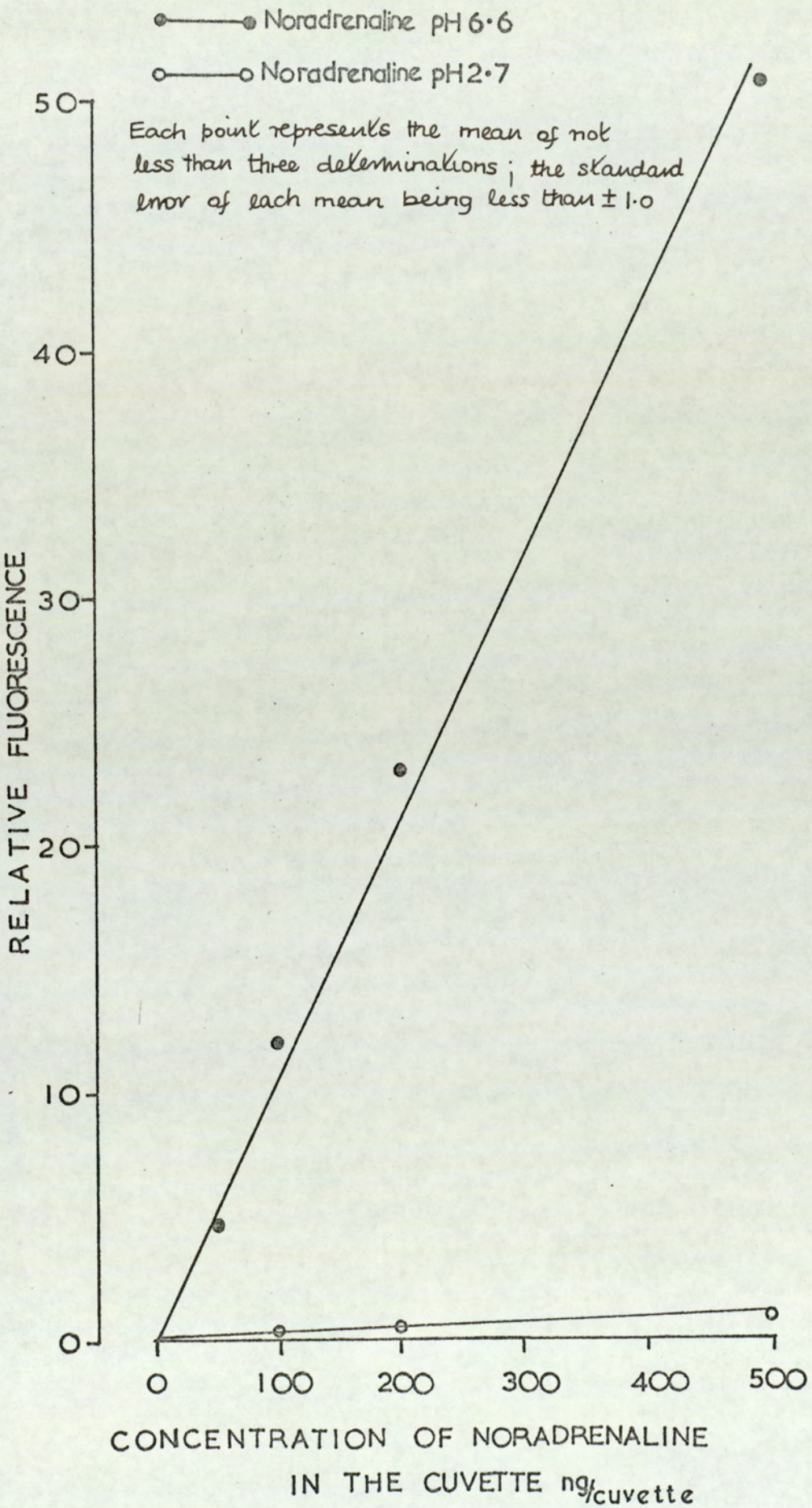


FIGURE 6

FLUORESCENCE OF ADRENALINE OXIDISED AT pH 2.7 AND pH 6.6

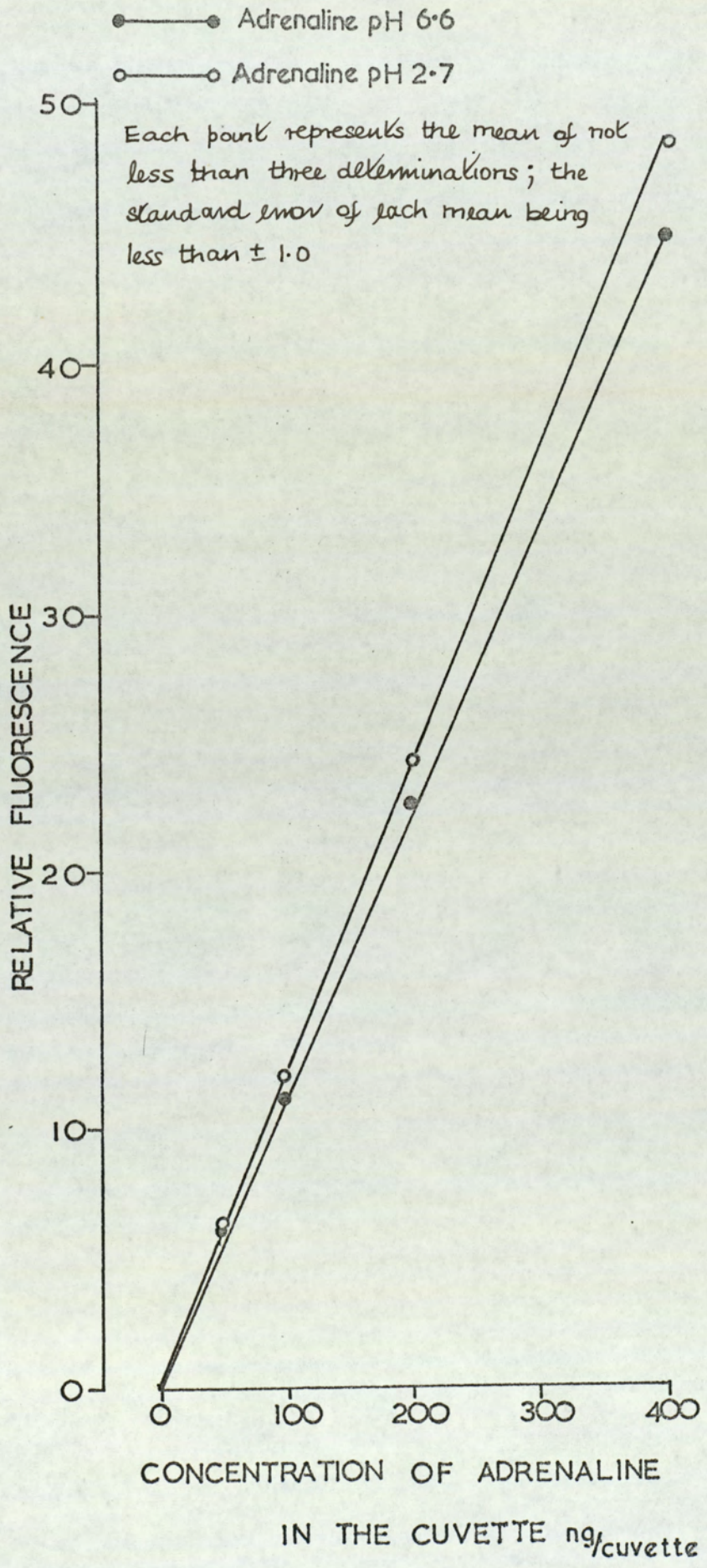


FIGURE 7

EFFECT OF pH ON THE OXIDATION OF CATECHOLAMINES

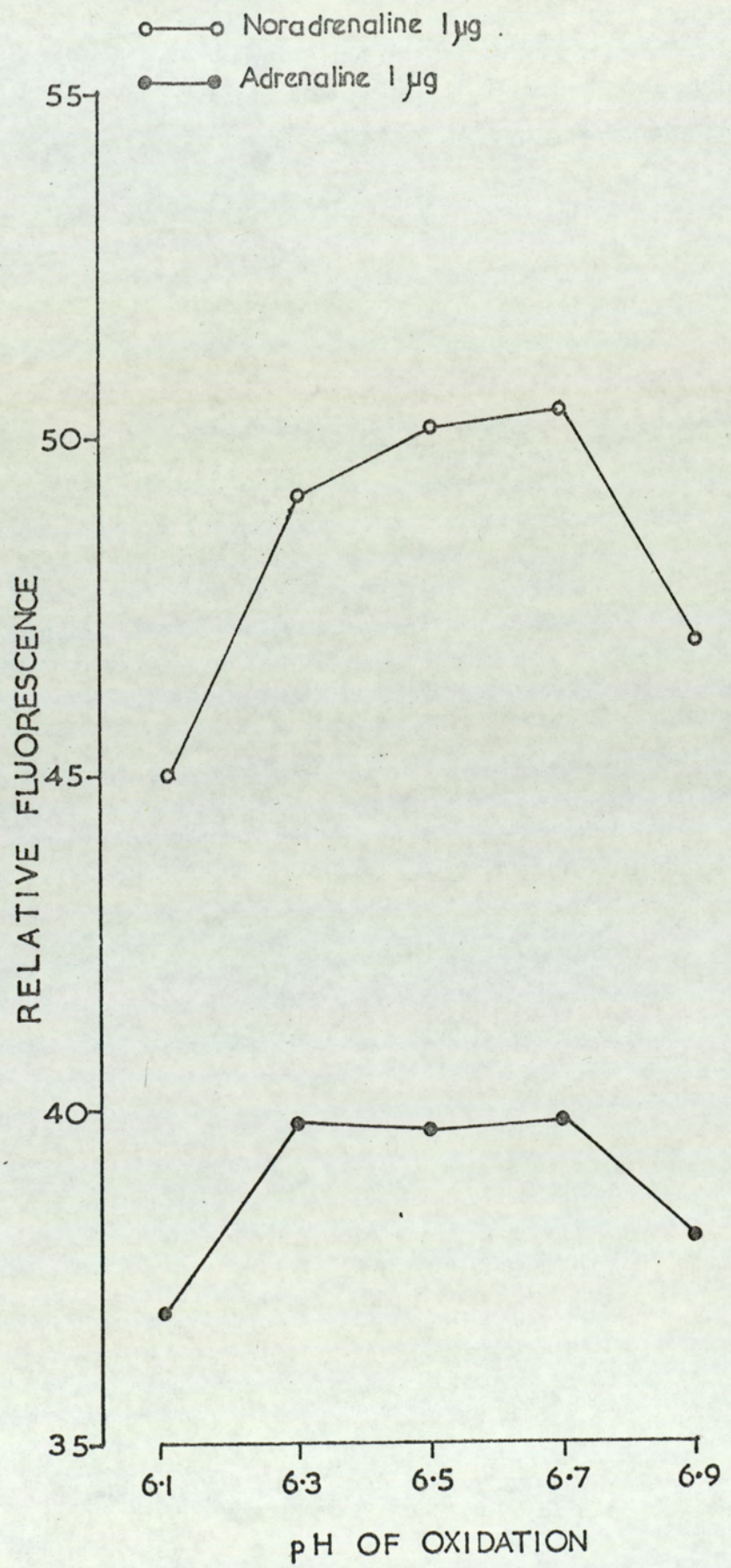


FIGURE 8

EXCITATION AND EMISSION SPECTRA OF DOPAMINE

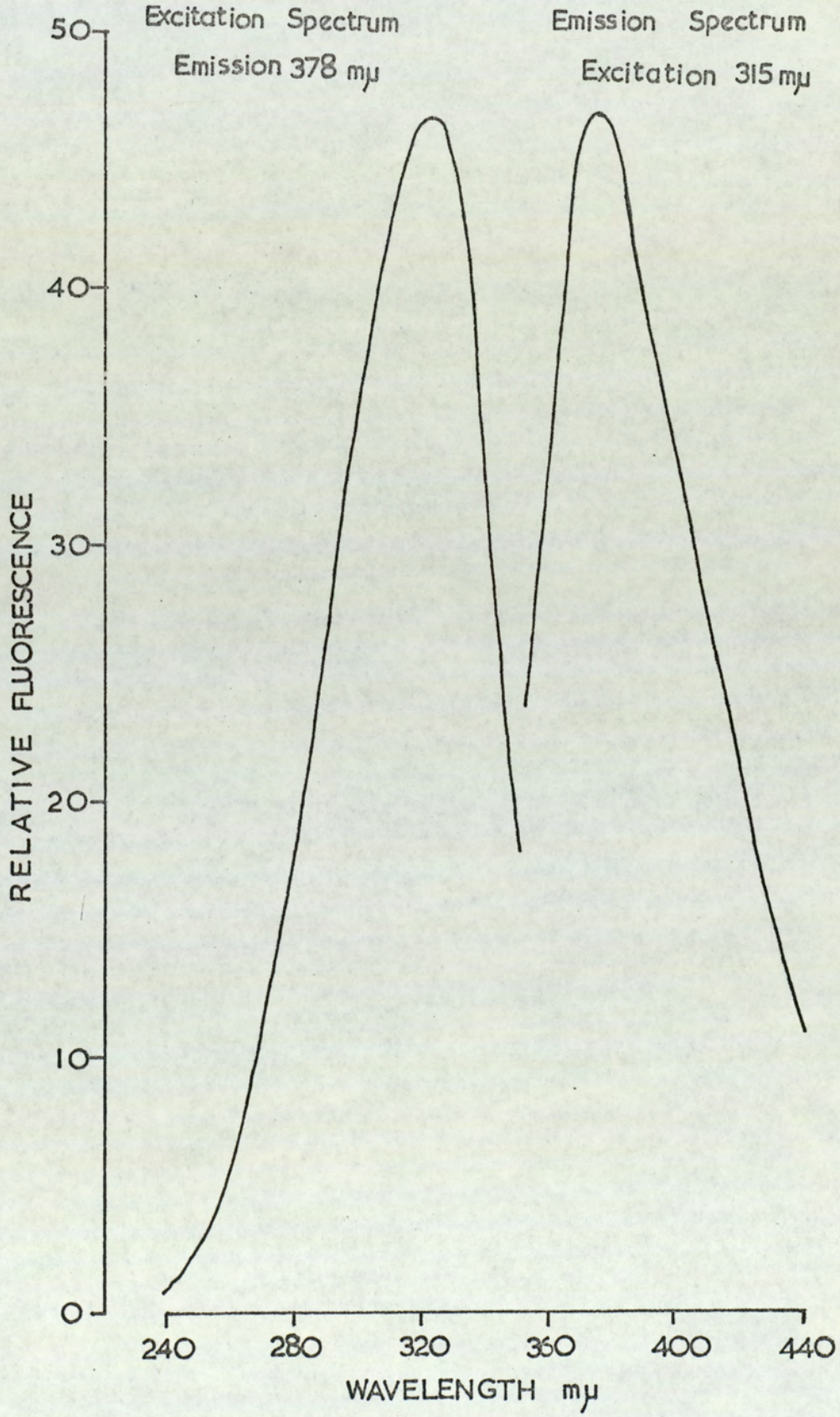


FIGURE 9

THE LINEAR RELATIONSHIP BETWEEN DOPAMINE FLUORESCENCE AND CONCENTRATION

Each point represents the mean of not less than three determinations; the standard error of each mean being less than ± 2·0

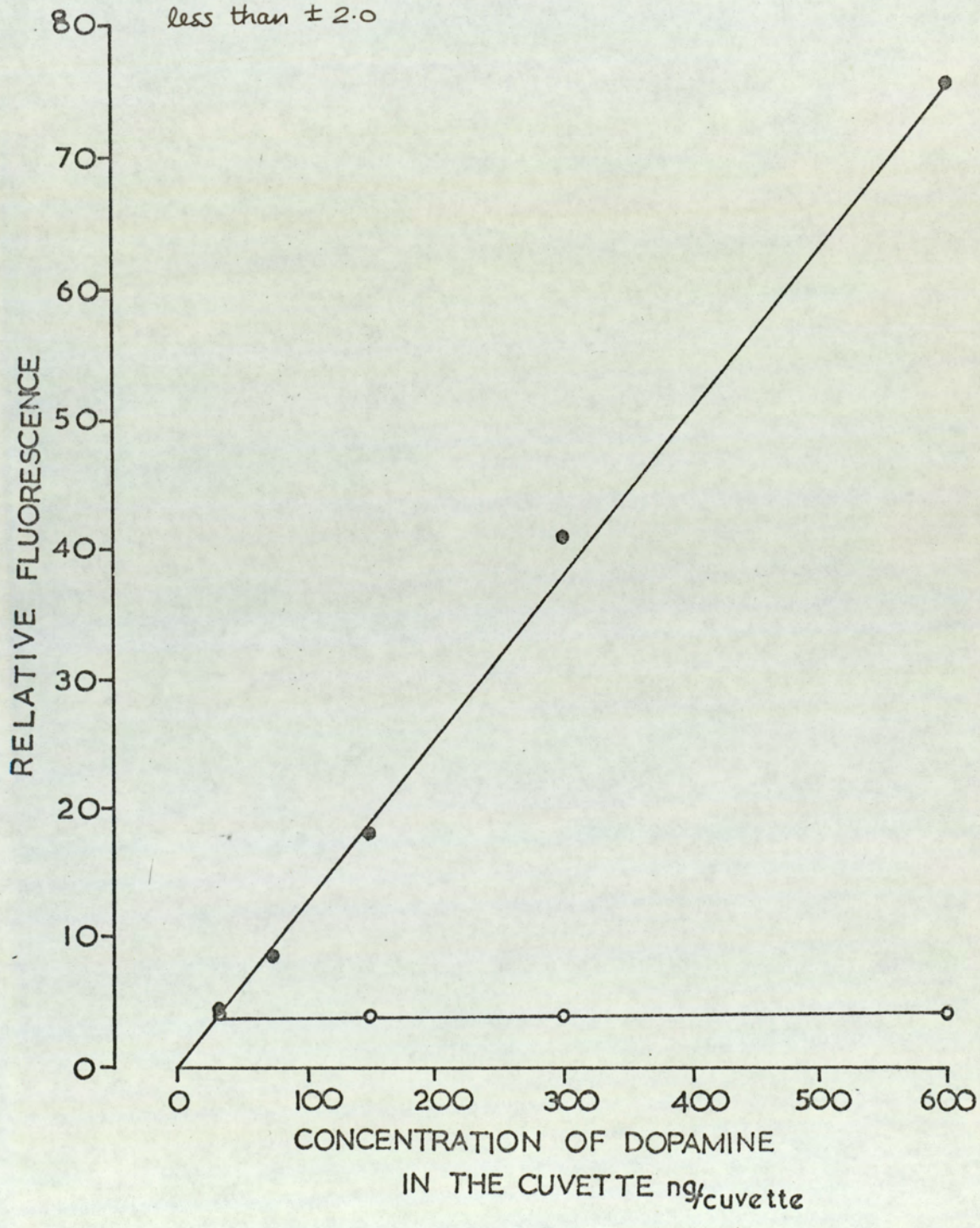


FIGURE 10

EXCITATION AND EMISSION SPECTRA OF
5-HYDROXYTRYPTAMINE (5-HT)

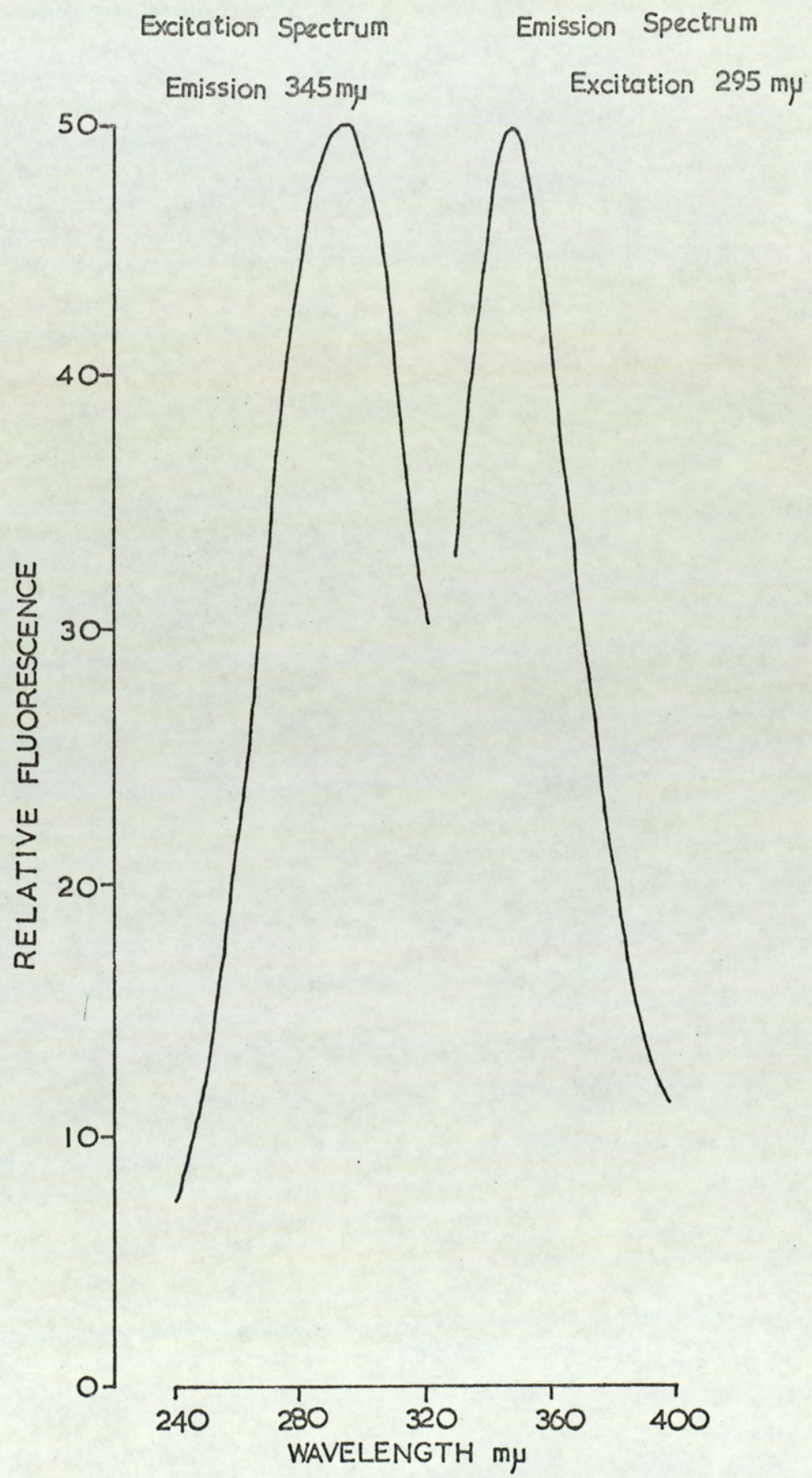


FIGURE 11

THE LINEAR RELATIONSHIP BETWEEN 5-HYDROXYTRYPTAMINE (5-HT) FLUORESCENCE AND CONCENTRATION

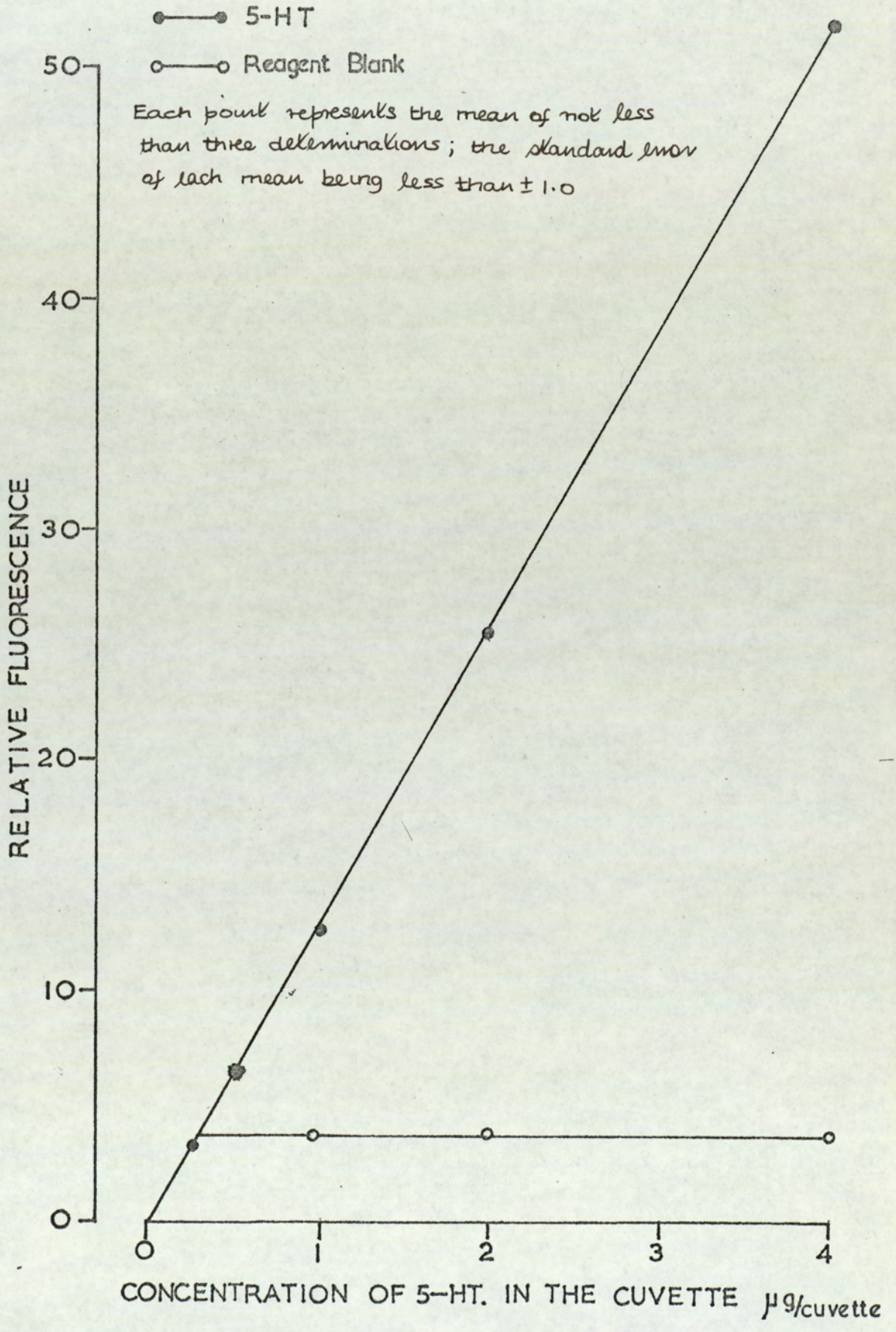


FIGURE 12

THE BERTLER ADSORPTION APPARATUS

(Bertler et al 1958)

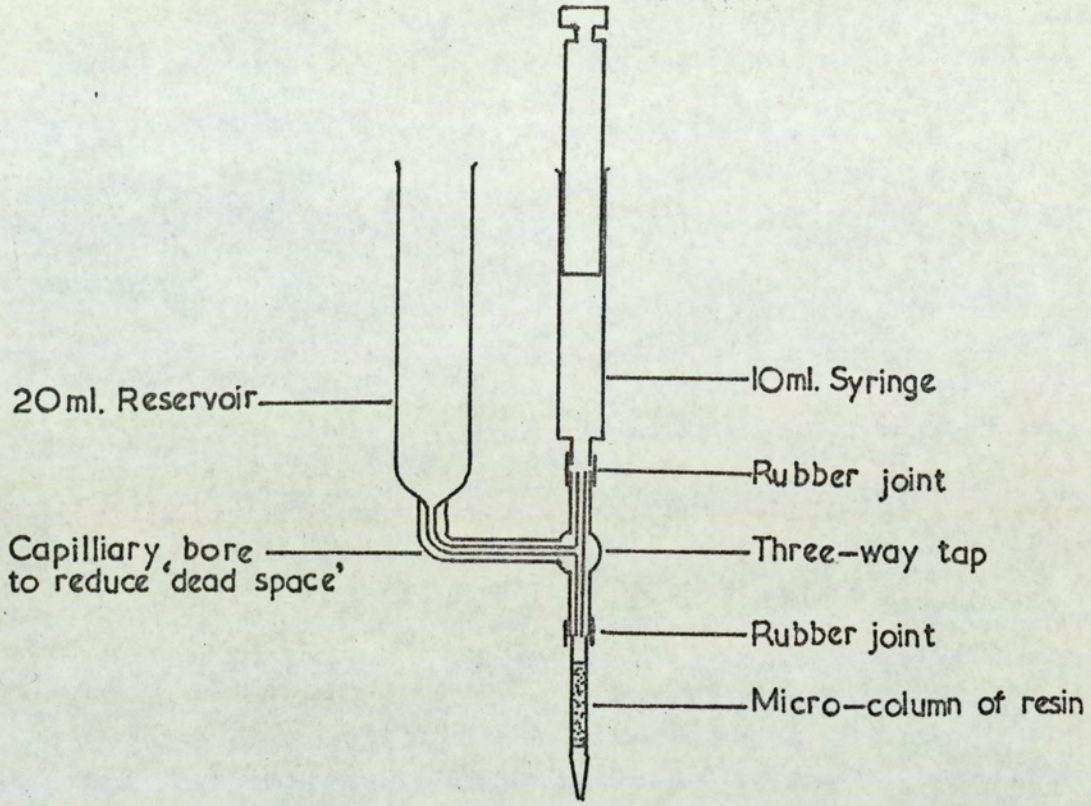
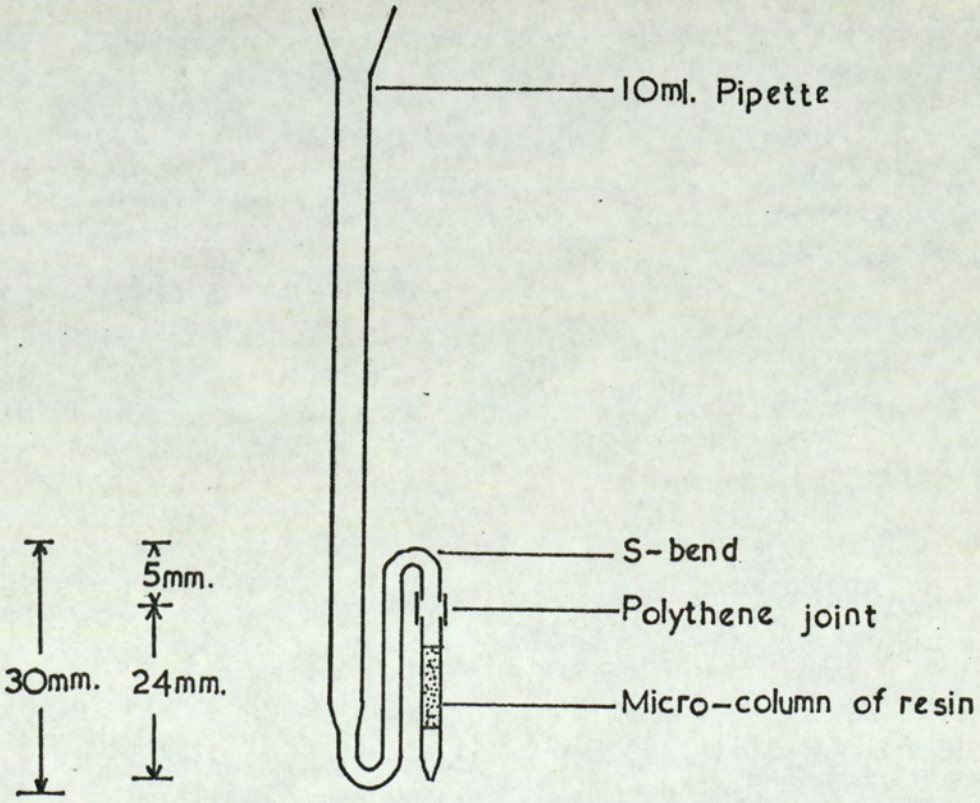


FIGURE 13

ADSORPTION APPARATUS USED IN THIS INVESTIGATION



SECTION THREE

METHODS

METHODS

1. SPECTROPHOTOFLUOROMETRIC DETERMINATION OF BIOGENIC AMINES

Animals were killed by cervical dislocation, the brains were dissected out, weighed, and homogenised in 4 ml of 0.4N perchloric acid at 0°C. The homogenate was centrifuged at 15,000 g for 8 min. at 0°C and the supernatant stored at 0°C. A second homogenization using a further 2 ml 0.4N perchloric acid was performed on the original sample and recentrifuged as before, the second supernatant being bulked with the first. The total clear supernatants from two brains were combined, made up to a volume of 16ml with 0·4N perchloric acid shaken and divided into two equal portions, one for dopamine and noradrenaline determination and the other for 5-HT determination. Known amounts of the three amines were added to some extracts as a check on the recovery of these amines.

(i) Noradrenaline and dopamine estimation

The 8ml aliquot of clear supernatant was titrated to pH 6.5 using 5N potassium carbonate at a pH meter. The precipitate of potassium perchlorate thus produced was removed by centrifugation at 15,000 g for 6 min. at 0°C, and the clear supernatant passed onto a Dowex 50 W.X.8 resin (100 mg dry weight) column which had been washed previously with:

(1) 8ml 2N hydrochloric acid;

(2) 10ml distilled water;

(3) 5ml 0.5 M-phosphate buffer pH 6.5;

(4) 10ml distilled water;

(5) Two further 10ml volumes of water.

The dimensions of the washed resin column were 4mm diameter and 12-15mm in length.

The supernatant was passed through the resin at a flow rate not exceeding 1ml in 2 min. After adsorption of the amines, the

columns were washed with 10ml distilled water. Then, after passing 0.5ml 0.4N hydrochloric acid onto the column to displace the water, the noradrenaline was eluted with 8ml 0.4N hydrochloric acid at a flow rate not exceeding 1ml every 2 min. The dopamine was then eluted with 2N hydrochloric acid at the same flow rate (having first displaced any 0.4N hydrochloric acid with 0.5ml 2N hydrochloric acid). This procedure was a modification of that used by Bertler, Carlsson and Rosengren, (1958).

The noradrenaline was assayed by a trihydroxyindole method evolved from those of Euler and Floding, (1955) and Bertler et al, (1958). Phosphate buffer was used instead of acetate buffer, and zinc sulphate was omitted from the method. In the alkaline ascorbate solution, sodium borohydride was found to stabilize fluorescence (Gerst, Odd, Steinsland & Walcott, (1966), although it was necessary to use a concentration of sodium borohydride ten times higher than that suggested by these workers. This stabilized the fluorescence of noradrenaline for at least 60 min. The fluorescence of noradrenaline was read at the activation and emission wavelengths 395/500 mμ (uncorrected) respectively in an Aminco Bowman spectrophotofluorometer. Table 4 shows the volumes of reagents used in a series of tubes in a noradrenaline determination.

Dopamine was assayed by the method of Carlsson and Waldeck (1958), with the modification of Carlsson and Lindqvist (1962). However, only 0.05ml iodine solution was used instead of 0.1ml in the oxidation and maximum fluorescence developed without the use of ultraviolet irradiation. The fluorescent principle produced by this procedure was unstable in that it faded rapidly when subjected

to the activation light in the fluorometer, but if the tubes were immersed in a boiling water bath for 5 min. immediately after the oxidation and then allowed to cool to room temperature the dopamine fluorescence was stabilized at its maximum for at least 60 min. The fluorescence was then read at the activation and emission wavelengths 325/378 mμ (uncorrected) respectively. Table 5 shows the volumes of reagents used in a series of tubes in a dopamine determination.

(ii) 5-Hydroxytryptamine (5-HT) estimation

The 5-HT aliquot was neutralised with 5N potassium carbonate and centrifuged as for catecholamine determination and the clear supernatant passed onto a column of Dowex 50 W.X.8 resin (100mg, dry weight) previously prepared in the sodium form with:

(1) 8ml 1N sodium hydroxide;

(2) 15ml distilled water;

(3) 15ml 0.1N sodium hydroxide containing 0.2% $w/_v$ EDTA;

(4) 10ml distilled water;

(5) Two further 10ml volumes of distilled water.

The clear supernatant was passed through this column at a flow rate not exceeding 1ml every 2 min. The 5-HT was then eluted from the column with 15ml 0.1N sodium hydroxide (containing 0.2% $w/_v$ EDTA) into 1.5ml sodium acetate buffer, pH 4.6, and read directly in the spectrophotofluorometer at the activation and emission wavelengths 295/345 mμ respectively. This method is similar to that used by Cox and Potkonjak (1967), but these authors eluted the catecholamines from this column with 1M potassium chloride before elution of 5-HT. In our hands the presence of potassium chloride gave rise to higher blanks and significantly reduced the sensitivity of the 5-HT assay.

The excitation of emission spectra of the extracted amines were always checked to ascertain that they were the same as those of the authentic standards. This also served as a check on any possible interference by the drugs used.

The recoveries of the three amines by the methods outlined above were:

Noradrenaline - 72% - 95%

Dopamine - 65% - 85%

5-HT - 70% to 100%

2. DISSECTION OF DISCRETE AREAS OF RAT BRAIN

After removal of the rat brain it was dropped into ice-cold 0.4N perchloric acid for 5 minutes, blotted dry and placed on a glass slab on ice and the dissection completed as quickly as possible (5 minutes). The areas used for noradrenaline determinations were, (a) midbrain, (b) hypothalmus, (c) midbrain, (d) striatum and (e) hippocampus. For dopamine only striatum and whole brain concentrations were determined. These areas have been described in detail by Glowinski and Iversen (1966) and the dissection procedure of these workers was followed precisely. The weights dissected were reproducible; the variation in weight for a given area being no more than $\pm$ 15%. However, in no determination did the hypothalmus weigh 110 mgm. as quoted by these workers. In our hands the mean weight of the hypothalmus was 70 mgm.

Six areas were pooled for each determination and extracted and assayed in the same way as whole brain.

3. ANIMALS

The majority of experiments reported in this thesis were carried out on adult male Wistar albino rats, although some preliminary experiments were also carried out on adult male T.O. albino mice.

(a) Rats

Male SPF. Wistar rats (Scientific Products Farms Limited, Ash, Kent) were used initially but because this supply was not always available another source was used. Therefore male Porton Wistar rats (Animal Unit, Fisons Pharmaceuticals, Holmes Chapel, Crewe) were used exclusively during the latter two years of this project. One notable difference between the rats from these sources, was seen in the response to leptazol: the SPF Wistar rats consistently showed all the phases of convulsions after a large dose of leptazol, (see Section 4 - Chapter 1.) but the Porton Wistar rats never displayed a hindlimb extensor tonic phase even when treated with supramaximal doses of leptazol. However, pre-treatment with some adrenergic drugs, dexamphetamine, reserpine and guanethidine, lowered the leptazol threshold and an extensor tonic phase was seen. This difference did not affect the results since the clonic convulsive phase was used as the criterion by which the severity of convulsions was judged.

(b) Mice

In all experiments using mice, male TO albino mice (Scientific Products Farms Limited, Ash, Kent) were used exclusively.

4. ANIMAL HUSBANDRY

After receipt, all animals were maintained in the animal house for a period of not less than 14 days prior to the experiment. Mice were received as weaners and were used for experiment when they weighed 18-20g. Rats were received at 100-150g. and were used for experiment when they weighed 200-250g. All animals were maintained on a conventional 41B cube diet (supplied by Pilsburys Limited) and allowed tap water ad libitum to drink. (Mice were kept in groups of 50 in polypropylene cages (40cm x 30cm) until 18 hours prior to experiment, when they were randomly divided into groups of ten and kept in smaller cages

(10 cm x 27 cm). At this stage they were transferred to a temperature controlled room at 20°C ± 0.5°C in preparation for experiment. Food and water were withdrawn 2 hours before the experiment.

Rats were kept in groups of 5 in polypropylene cages (40 cm x 30 cm) until 8 hours prior to the experiment when they were transferred to a temperature controlled room at 20°C ± 0.5°C. Food and water were withdrawn 2 hours before the experiment.

All experiments were made in the temperature controlled room and performed as far as possible between the hours 1.00 pm and 6.00 p.m. to minimise the effect of any circadian variation in leptazol threshold (Webb & Russell, 1966).

The animal house conditions were - relative humidity 50-55% and temperature 21-23°C. There was a normal light/dark cycle determined predominantly by natural lighting.

5. LEPTAZOL CONVULSIONS

All animals were housed individually during investigation of leptazol convulsions.

(i) Intraperitoneal administration

Convulsions were produced in groups of 10 mice by intraperitoneal injection of leptazol (80mg/kg) in 0.9% $w/_v$ sodium chloride solution (0.1 ml/20g), and in groups of 5 rats by intraperitoneal injections of leptazol (55mg/kg) dissolved in 0.9% $w/_v$ sodium chloride solution (0.5ml/100g). 0.9% $w/_v$ sodium chloride alone produced no convulsions. The results with mice were expressed as a mortality ratio (MR): the mortality ratio was the proportion of mice dead in the test group divided by the proportion dead in the control group, 15 minutes after the leptazol challenge.

$$MR = \frac{(\%\text{ dead in test group})}{(\%\text{ dead in control group})} \text{ @ 15 mins.}$$

Each % is expressed as a % of the maximum.

In rats the seizure pattern after high doses of leptazol, always showed two clonic convulsive phases and this was chosen as the most consistent parameter by which to measure the extent of convulsions. (See Section 4, Chapter 1). The results were expressed either as a clonic convulsive ratio or as a percentage of maximum clonic convulsions.

The clonic convulsive ratio (CCR) was the number of clonic convulsive episodes in a group of test rats divided by the number of episodes in a control group of equal size, during the 15 minute period after the leptazol challenge.

$$CCR = \frac{(\%\text{ clonic convulsions in test group})}{(\%\text{ clonic convulsions in control group})} \text{ @ 15 mins.}$$

Each % is expressed as a % of the maximum.

The percentage of maximum clonic convulsions was the number of clonic phases in a group of 5 rats expressed as a percentage of the maximum, 10 - (2 per rat). This percentage was calculated for both test and control groups.

Mortality, or convulsive ratios greater than one indicated a proconvulsant action and ratios less than one indicated an anti-convulsant effect.

(ii) Subcutaneous administration

Convulsions were produced in groups of 10 mice by subcutaneous injection of leptazol (80mg/kg) in 0.9% $w/_v$ sodium chloride solution (0.1ml/20g) in neck region. In groups of 5 rats, convulsions were produced by subcutaneous injection of leptazol (65mg/kg) in 0.9% $w/_v$ sodium chloride solution (0.5ml/100g). 0.9% $w/_v$ sodium chloride solution alone produced no convulsions.

Results were expressed either as mortality or convulsive ratios or percentage of maximum clonic convulsions, but the animals were observed for a period of 30 min. after the leptazol challenge.

(iii) Intravenous administration

Convulsions were induced in mice by the intravenous administration of leptazol by a method similar to that described by Orloff, Williams and Pfeiffer, (1949). A 0.05% $w/_v$ solution of leptazol in 0.9% $w/_v$ sodium chloride solution was injected into the lateral tail vein of the mouse using a 2ml Jencon Repette which delivered 0.05ml per injection. The leptazol was injected at a rate of 0.05ml/10 seconds and the time to various stages of the convulsion recorded.

6. DRUG ADMINISTRATION BY THE INTRAVENOUS ROUTE

Rats were lightly anaesthetised with a mixture of nitrous oxide 500ml/min and oxygen 100ml/min., containing 3.5% $w/_v$ halothane, and the drug injected into the penile vein in a dose volume of 0.1ml/100g. Control animals were anaesthetised in the same manner and the vehicle injected into the penile vein in the same volume. Anaesthesia was so light as to allow the rats to recover within one to two minutes of the injection.

Mice were injected intravenously in the lateral tail vein (0.1ml/20g).

7. MEASUREMENT OF BODY TEMPERATURE

Oesophageal temperature was measured as an estimate of deep body temperature since this has been reported to provide a more accurate assessment of true body temperature than does rectal temperature. The method of Brittain and Spencer (1964) was employed, using groups of 10 mice. A thermocouple attached to an electric thermometer (Light Laboratories) was inserted into the oesophagus to a depth of approximately

2cm. The temperature was noted when the reading became constant (after 4-6 sec.).

8. MEASUREMENT OF SPONTANEOUS LOCOMOTOR ACTIVITY

Groups of 4 mice received injections of the drug under investigation and controls received the vehicle. Locomotor activity was recorded for successive 30 minute intervals using a Faraday Animal Activity Recorder (Hawkelsy & Sons, Lancing, Sussex). The apparatus consisted of two polypropylene cages (10cm x 27cm) at least 25cms apart. Each was surrounded by two aerial plates at right angles to each other which detected movement within the cage. To cancel out effects of feeding and drinking, on this count, leads from the top and bottom grilles of each cage were connected and this information fed into the integrating system where it was subtracted from the total count. Movement within each cage activated an integrating amplifier and digital counter. Prior to experiment the cages were balanced electronically against each other so that the digital records were the same for similar degrees of movement. Control mice were housed in one cage and treated mice in the other.

The mice were previously acclimatized to the procedure of injection and their home cages in the apparatus, so reducing the contributions of fear and exploration to a minimum.

9. ASSESSMENT OF INHIBITION OF MAO ACTIVITY

The MAO inhibitors were initially assessed by a method based on the report by Corne, Pickering and Warner, (1963). Groups of 10 mice were injected intraperitoneally with 5-hydroxytryptophan (50mg/kg) and observed for 2 minutes commencing 24 minutes after the 5-hydroxytryptophan.

During this 2 minute period the number of mice out of 10 showing at least one 'head twitch' was recorded for both MAO inhibitor-pretreated and control mice. Potentiation of this 'head twitch' response in test animals as compared with the controls was indicative of effective MAO inhibition.

TABLE 4

VOLUMES OF REAGENTS (ml) USED IN THE NORADRENALINE SPECTROPHOTOFLUOROMETRIC ASSAY

Tube No.	Code	Extract	Noradrenaline Standard 40ng/ml	0.5M Phosphate Buffer	Potassium Ferricyanide 0.25% w/v	Alkaline Ascorbate/ Sodium Borohydride	Distilled Water
1	E	2	-	1	0.1	1	0.9
2	E	2	-	1	0.1	1	0.9
3	E.IS	2	-	1	0.1	1	0.9
4	E.IS	2	-	1	0.1	1	0.9
5	NASt	-	2	1	0.1	1	0.9
6	NASt	-	2	1	0.1	1	0.9
7	NAFB	-	2	1	0.1	0.9 NaOH 0.1 Vit.C	0.9
8	Oxid IS	2	0.5	1	0.1	1	0.4
9	EFB	2	-	1	0.1	0.9 NaOH 0.1 Vit.C	0.9

E = Eluate

IS = Internal standard containing 100ng/2ml of added noradrenaline

NASt = Noradrenaline standard

NAFB = Noradrenaline faded blank

EFB = Eluate faded blank

Oxid IS = Oxidation internal standard

TABLE 5

VOLUMES OF REAGENTS (ml) USED IN THE DOPAMINE SPECTROPHOTOFLUOROMETRIC ASSAY

Tube No.	Code	Extract	Dopamine Standard 100ng/ml	0.5M Phosphate/ Citrate Buffer pH 5.4	0.02N Iodine	Alkaline Sulphite Solution	5N Acetic Acid
1	E	3	-	1	0.05	0.5	1.6
2	E	3	-	1	0.05	0.5	1.6
3	EIS	3	-	1	0.05	0.5	1.6
4	EIS	3	-	1	0.05	0.5	1.6
5	DMSt	-	3	1	0.05	0.5	1.6
6	DMSt	-	3	1	0.05	0.5	1.6
7	DMFB	3	-	1	0.05	0.45NaOH 0.05Na_2SO_3	1.6
8	Oxid IS	2	1	1	0.05	0.5	1.6
9	EFB	3	-	1	0.05	0.45NaOH 0.05Na_2SO_3	1.6

E = Eluate

IS = Internal standard containing 250ng/3ml of added dopamine

DMSt = Dopamine pure standard

DMFB = Dopamine faded blank

EFB = Eluate faded blank

Oxid IS = Oxidation internal standard

SECTION FOUR

EXPERIMENTAL RESULTS:

STUDIES OF THE EFFECTS OF AMINERGIC DRUGS ON THE CONVULSANT ACTIONS OF LEPTAZOL IN MICE AND RATS.

CHAPTER ONE

EXAMINATION OF THE CONVULSANT EFFECTS OF LEPTAZOL IN MICE AND RATS

EXAMINATION OF THE CONVULSANT EFFECTS OF LEPTAZOL IN MICE AND RATS

EXAMINATION OF THE CONVULSANT EFFECTS OF LEPTAZOL IN MICE AND RATS

The discovery of tridione as an anticonvulsant established leptazol as a useful experimental convulsant in screening for anticonvulsant activity. (Everett & Richards, 1944). Many variations of the original test have been used, each claimed to have certain advantages. The two commonly used tests have been discussed earlier (see Experimental Convulsive Agents). These two tests were investigated to determine the pattern and duration of convulsions after various doses of leptazol and to determine the method most suitable to the needs of this project.

1. LEPTAZOL CONVULSIONS IN MICE

(i) Maximal Leptazol Seizure Test

Leptazol was administered intravenously by a modification of the Orloff, Williams and Pfeiffer (1949) technique (see Methods). Each mouse was observed individually for the various stages of the convulsions. Onset of the convulsion was rapid (within 2 minutes) and was almost immediately maximal in that the clonic phase was followed immediately by the tonic phase. Consequently it was difficult to calculate a clonic convulsive dose and differentiate it from tonic convulsive dose. A disadvantage of this 'all or none' response was that only the accepted potent anticonvulsants could be detected by it and the less potent adrenergic drugs showed no effect. Dexamphetamine was subsequently shown by other techniques to be proconvulsant but in this test its effects could not be distinguished from those of controls. Therefore the maximal leptazol seizure test was discarded as unsuitable to the needs of this project involving adrenergic drugs.

(ii) Leptazol Seizure Threshold Test

These tests were similar to those used by Goodman and Lih (1941) and Swinyard, Brown and Goodman (1952). Both intraperitoneally and subcutaneously-administered leptazol were investigated.

It was difficult to observe groups of ten mice for various stages of convulsions and so the number of dead mice was recorded in each group of ten mice 15 minutes after leptazol intraperitoneally. This was construed as a measure of the extent of tonic convulsions, because tonic convulsions were consistently lethal to mice. Several doses of leptazol were used and log. dose plotted against probability response: the LD_{50} was calculated according to the method of Litchfield and Wilcoxon (1949). The LD_{50} of intraperitoneally administered leptazol was 79.8 mg/kg (Fig. 14). The log. dose-probability line was very steep, a small change in dose producing a large difference in response. Consequently small changes in environment made mice more or less susceptible to leptazol and produced variation in response from day to day. One such source of error was change in ambient temperature. There was no direct relationship between temperature and leptazol threshold since both increased and decreased ambient temperature led to higher lethality after leptazol. This was consistent with reports that leptazol itself will either produce hyperthermia or hypothermia depending on the ambient temperature. (Shemano & Nickerson, 1959; Oberdorf & Meyer, 1960). However Stormont and Hook (1941) showed that low body temperature did not affect leptazol threshold. Subsequent experiments were carried out in a temperature controlled room to minimise this variation. The route of administration itself also was a source of variable results, for rate of absorption of leptazol from the peritoneal cavity and hence severity of the convulsion would depend to some

extent on when the animal last fed. Subsequent experiments were performed two hours after food had been withdrawn, and resulted in more consistent results.

After intraperitoneally administered leptazol, the onset of the convulsions was extremely rapid and it was difficult to distinguish between clonic and tonic phases. For this reason leptazol administered subcutaneously was investigated. The LD_{50} was found to be 90 mg/kg (Fig. 15) but the convulsion was spread out over a period of 30 minutes facilitating observation of both clonic and tonic phases. Because of this distinct advantage the majority of work was carried out using leptazol subcutaneously. This route also produced more consistent lethality in mice.

2. LEPTAZOL CONVULSIONS IN RATS

(i) Leptazol Seizure Threshold Test

Similar problems were encountered with rats as for mice, in that intraperitoneally administered leptazol produced convulsions with rapid onset, short duration and little separation of clonic and tonic phases. A further problem was that a consistent tonic phase was unobtainable, and after high doses of leptazol those rats which showed a tonic phases lived in respiratory distress and had to be killed. Therefore calculation of an LD_{50} was not possible and the tonic phase was unsuitable as a parameter by which to judge proconvulsant or anticonvulsant drugs. However there was a linear relationship between log. dose of leptazol and probability of clonic convulsions. For reasons given later (see Pattern of Convulsive Phases) each rat was capable of having two clonic phases. The clonic convulsive score in groups of 5 rats was used to determine the clonic convulsive dose to 50% (CCD_{50}) by the method of Litchfield and Wilcoxon (1949). The CCD_{50} of intraperitoneally administered leptazol was found to be 55mg/kg (Fig. 16).

Subcutaneous leptazol showed the same advantages with rats as with mice, the most useful advantage being the separation of clonic and tonic phases. The CCD_{50} by this route was 65mg/kg (Fig. 17) and was very consistent. The fact that rats are less susceptible to changes in ambient temperature may have contributed to the reproducibility of this result. This dose induced one clonic phase in each rat and any potentiation or block of this effect was easily detected.

3. PATTERN OF CONVULSIVE PHASES

The pattern of convulsions for mice and rats was very similar and since rats were more frequently used in this project, they have been discussed below and where necessary any differences from mice have been considered.

In low doses (60mg/kg intraperitoneally), leptazol induced myclonic jerks about 3 minutes after injection. This lasted about 30 seconds and was followed by facial clonus and generalised body clonus which lasted for about 10 seconds. At this dose the convulsion went no further and the rat recovered completely in 20 minutes. Higher doses (80mg/kg intraperitoneally), produced the above stages followed by a second clonus 1 - 2 minutes after the first. When the dose was increased to 90mg/kg a convulsion followed which rapidly passed through the above phases and terminated in the tonic phase which consisted of a forelimb flexor tonus followed immediately by the hindlimb extensor tonus. Very high doses (120mg/kg) threw the rat into an immediate tonic convulsion within 40 seconds of administration.

When completely separated the convulsive pattern was as follows:-

(i) Myoclonic jerks.

(ii) Facial clonus and general body clonus.
(First clonic phase).

(iii) Sedation. (lasting 1 or 2 minutes)

(iv) Facial and general clonus. (Second clonic Phase).

(v) Forelimb flexor tonus.

(vi) Hindlimb extensor tonus.

(vii) Death or respiratory distress.

Depending on the dose used, one or all of these phases were seen. The dose also determined the time between each phase and the duration of the whole convulsion. Rats consistently showed two clonic phases if the dose was high enough. Therefore, for the purpose of screening, each rat was considered capable of two clonic phases and the number of clonic phases exhibited in a group of 5 rats was expressed as a percentage of the maximum (10), percentage maximum convulsions. Increase or decrease in this percentage after drug treatment was used to determine whether the drug was anticonvulsant or proconvulsant.

Mice showed these same stages of convulsions except that the forelimb flexor tonus and hindlimb extensor tonus occurred simultaneously.

Because of the advantages already mentioned, rats were used more frequently than mice in this project. However one variation in rats was discovered when a different source of rats had to be used. Scientific Products Farm Wistar rats showed the full convulsion as described above, but Fisons Porton Wistar rats only exhibited a forelimb flexor tonus as the tonic phase. No matter how high the dose of leptazol these rats did not exhibit the hindlimb extensor tonus. But, then treated with some adrenergic drugs e.g. reserpine, dexamphetamine, and guanethidine, these rats exhibited the full tonic phase. This phenomenon was not investigated fully but was taken as a positive sign of proconvulsant activity.

4. DISCUSSION

Mice showed all the typical phases of leptazol convulsions but results were not very consistent. The results could be rendered more consistent by careful control of ambient temperature, withdrawal of food for two hours before experiment and administration of leptazol by the subcutaneous route. The LD_{50} of leptazol in mice was 90mg/kg subcutaneously.

Rats, having a smaller surface area/body weight ratio, were less susceptible to changes in ambient temperature but, as in the case of mice, withdrawal of food two hours before experiment and administration of leptazol subcutaneously produced the most consistent results. Results with rats were so reproducible that it was decided to use rats in preference to mice in this project whenever possible. The clonic convulsive phase of the convulsion was selected as the most reliable parameter by which to judge whether pretreatment was pro- or anti-convulsant. The CCD_{50} of leptazol in control rats was 65mg/kg subcutaneously.

FIGURE 14

PROBIT % MORTALITY AGAINST LOG. DOSE
INTRAPERITONEAL LEPTAZOL IN MICE

LD_{50} = Lethal dose to 50% of mice tested

i. p. LD_{50} = 79·8 mg/kg (mice)

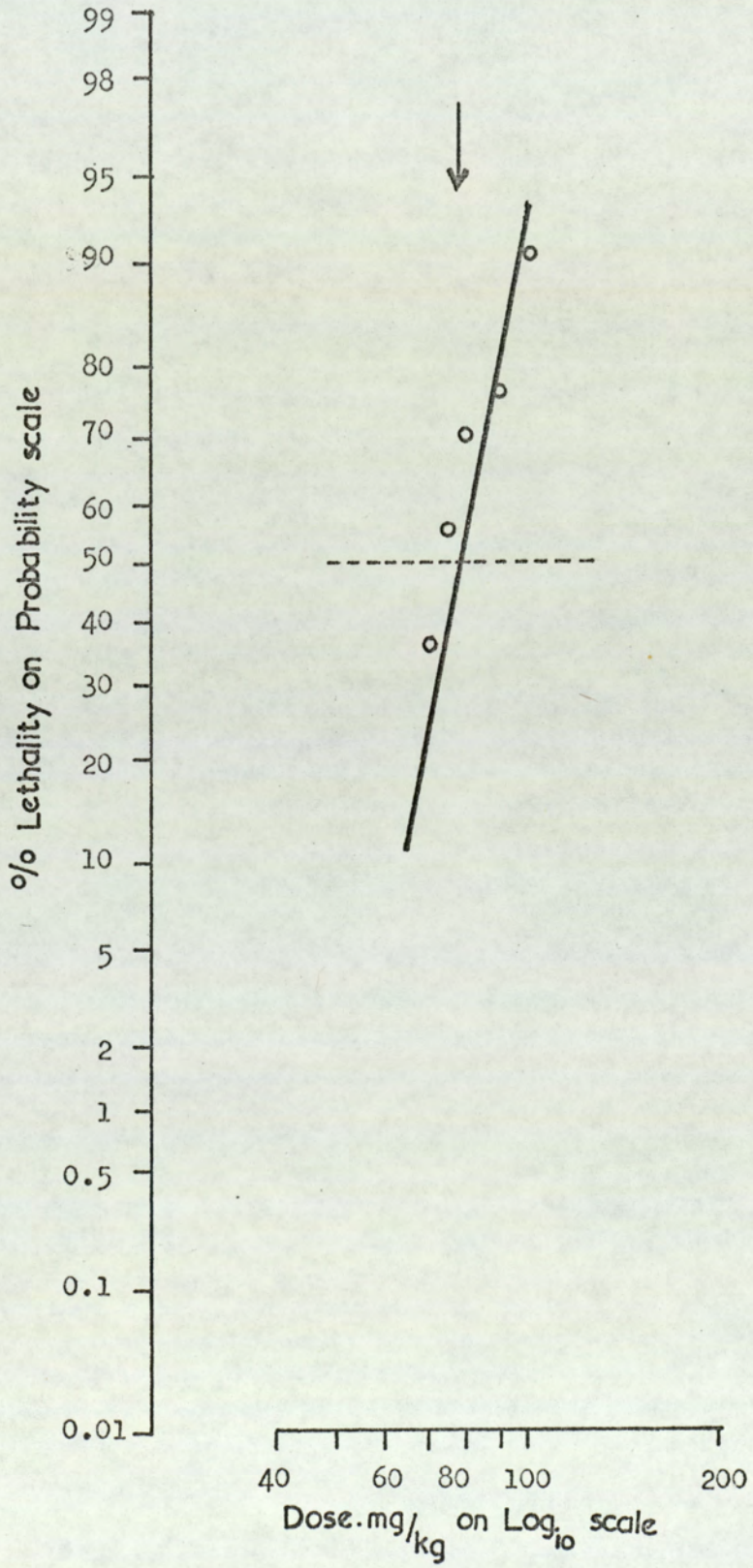


FIGURE 15

PROBIT % MORTALITY AGAINST LOG. DOSE
SUBCUTANEOUS LEPTAZOL IN MICE

LD_{50} = Lethal dose to 50% of mice tested

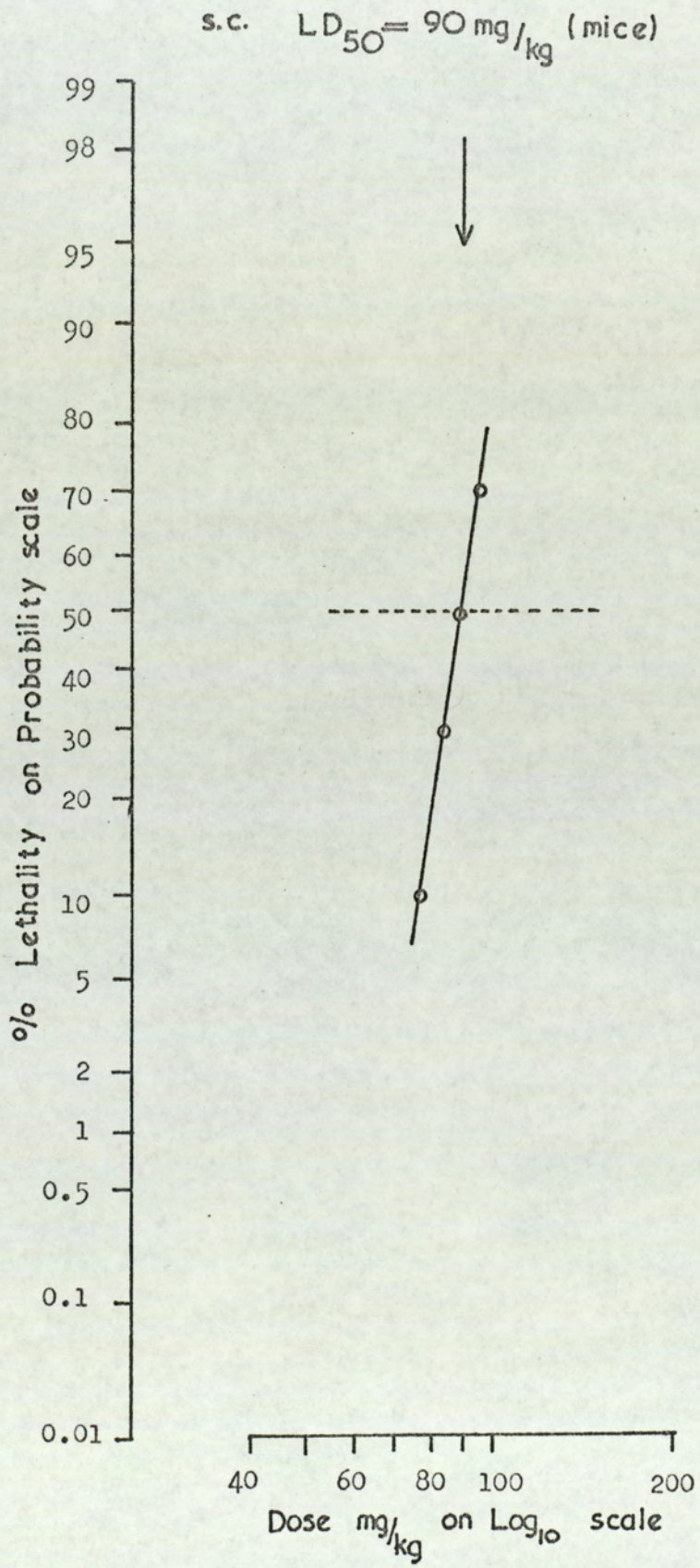


FIGURE 16

PROBIT % CLONIC CONVULSIONS AGAINST LOG. DOSE INTRAPERITONEAL LEPTAZOL IN RATS

CCD_{50} = Clonic convulsive dose to 50% of rats tested

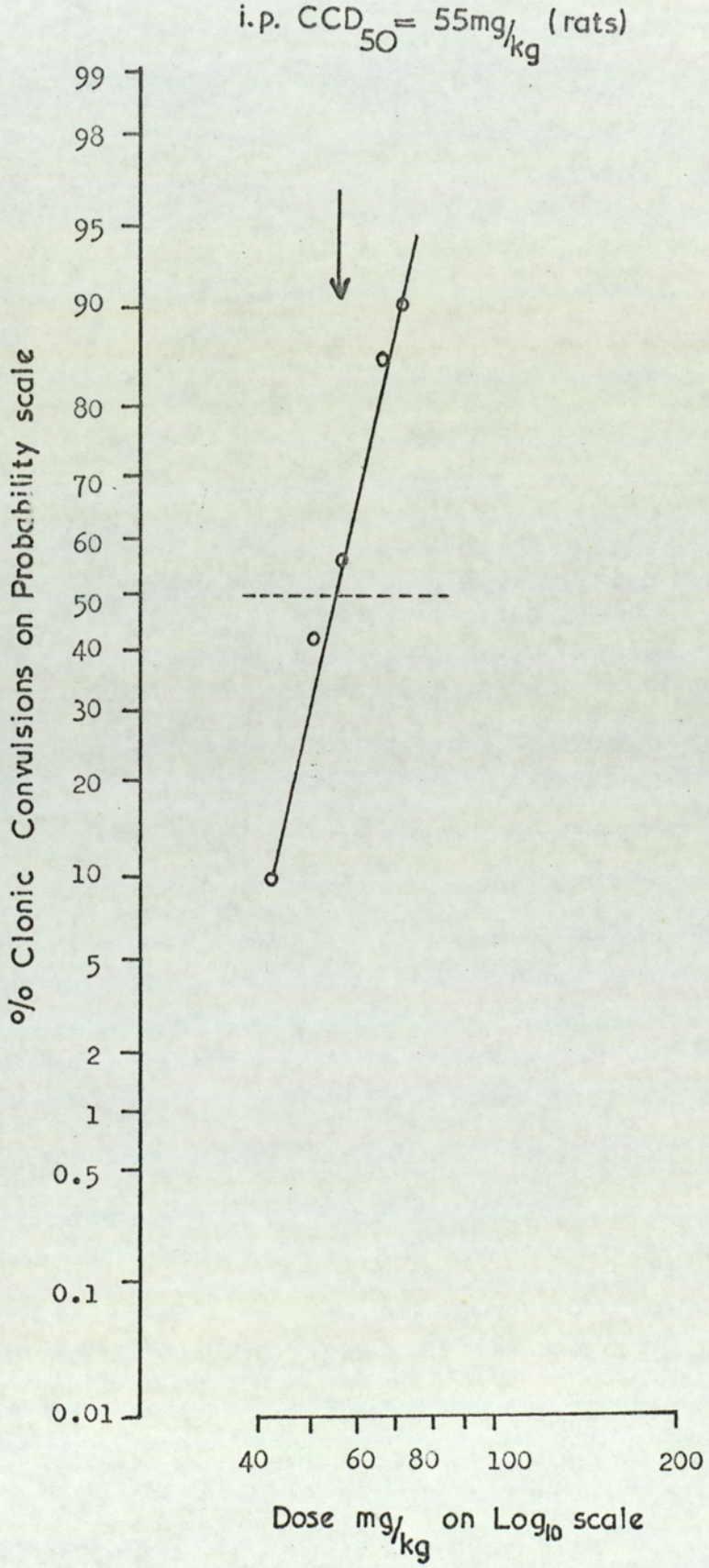
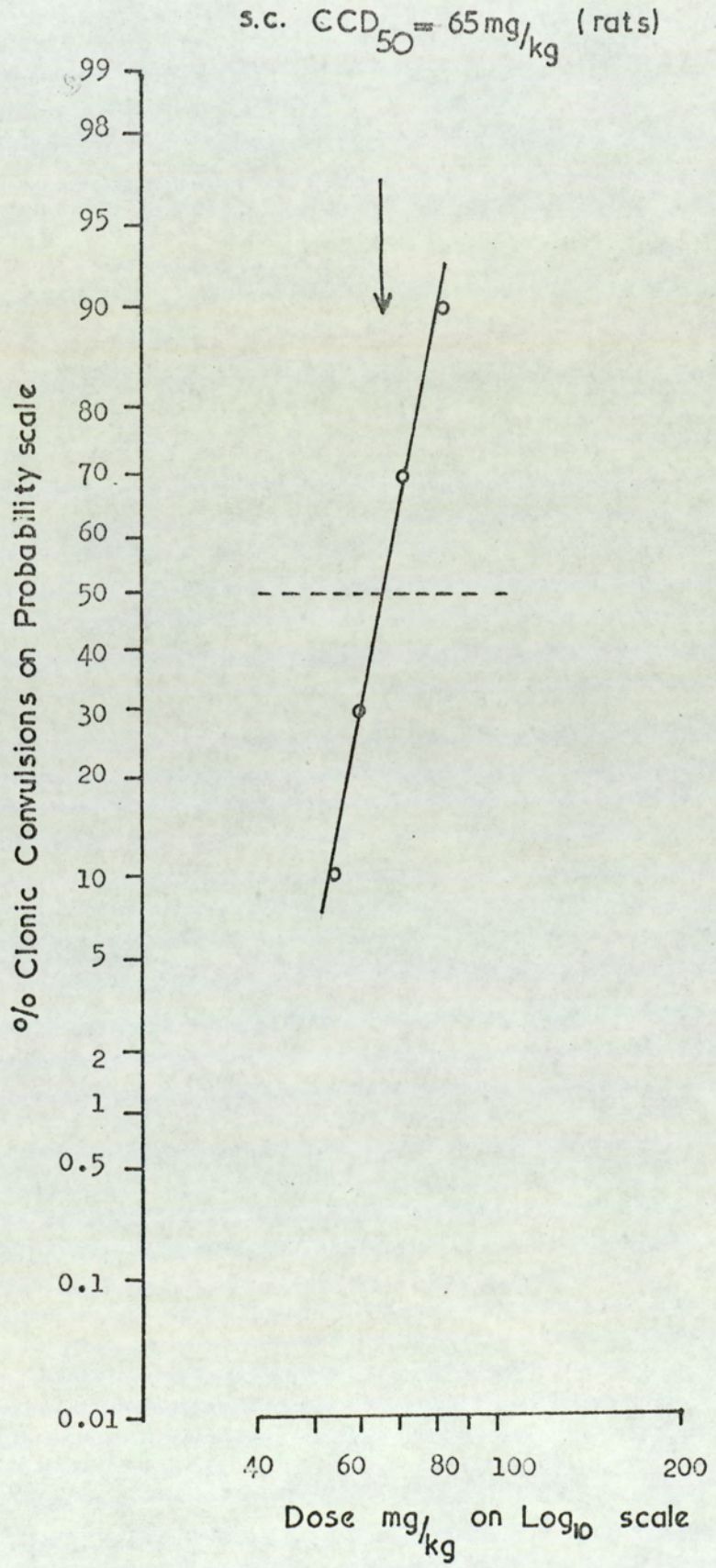


FIGURE 17

PROBIT % CLONIC CONVULSIONS AGAINST LOG. DOSE SUBCUTANEOUS LEPTAZOL IN RATS.

CCD_{50} = Clonic convulsive dose to 50% of rats tested



CHAPTER TWO

EFFECT OF LEPTAZOL ON BIOGENIC AMINE CONCENTRATIONS IN RAT BRAIN

EFFECT OF LEPTAZOL ON BIOGENIC AMINE CONCENTRATIONS IN RAT BRAIN

EFFECT OF LEPTAZOL ON BIOGENIC AMINE CONCENTRATIONS IN RAT BRAIN

The possible involvement of biogenic amines in leptazol convulsions was reviewed in the introduction (Experimental Convulsive Agents). Various workers have produced evidence that noradrenaline (Pfeifer & Galambos, 1967 a), dopamine (De Schaepdryver et al, 1962) and 5-HT (Lessin & Parkes, 1959) were involved. This evidence was based on the results of work with various aminergic drugs and as such was indirect. Direct evidence, i.e. measurement of amines during or after leptazol, was difficult to find; Vogt (1954) had measured sympathin concentrations in the cat hypothalamus after leptazol but established no correlation with convulsive activity. Bertaccini, (1959) had demonstrated an increase of 33% in central 5-HT concentrations after electroshock and leptazol convulsions but did not consider this significant. Breitner and others (1964) had demonstrated significant changes in central 5-HT and noradrenaline concentrations after convulsions irrespective of how the convulsions were produced and said these changes were the result of and not the cause of the convulsions. Since no reference could be found to any determination of amine levels before and during convulsions, this was undertaken as the preliminary investigation in this project.

1. WHOLE BRAIN BIOGENIC AMINE LEVELS DURING AND AFTER THE CONVULSION

Rats were injected intraperitoneally with leptazol (90mg/kg) and killed at the various stages of the convulsions. For the post-ictal phase, rats were killed 2 minutes after the tonic phase. Whole brains were assayed for noradrenaline, dopamine and 5-HT. The results are shown in Table 6.

No significant change in noradrenaline, dopamine or 5-HT concentrations was detected after the convulsion and no change in catecholamine concentrations was observed during the convulsion. This result did not rule out the possibility of involvement of aminergic mechanisms, for mechanisms such as increased turnover of the amines or inhibition of uptake would not necessarily be detected by simply measuring amine concentrations. Also, the fact that the whole convulsion lasted only 15 minutes might not have been long enough for any small change to be reflected in whole brain amine concentrations. Therefore a second determination of whole brain amine concentrations was made 30 minutes after leptazol (65mg/kg) subcutaneously. This dose produced only one clonic convulsive phase in each rat. The results are shown in Table 7.

This method of inducing the convulsion over a longer period of time produced no significant change in the central concentrations of the three biogenic amines studied.

2. WHOLE BRAIN BIOGENIC AMINE LEVELS AFTER SUBCONVULSIVE DOSES OF LEPTAZOL

Convulsive doses of leptazol produced no effect on central biogenic amine levels. However this was still not indicative that leptazol produced no effect on aminergic processes. It was possible that leptazol exerted an effect, discreetly, upon synthesis or turnover, etc. Neither radioactive tracer techniques nor amine metabolite assays were available to determine this. However it was considered that determination of amine concentrations after long term treatment with subconvulsive doses might unmask any such action.

Rats were treated twice daily for 3 days with a subconvulsant dose of leptazol (40mg/kg intraperitoneally) and killed on the fourth day, one hour after the seventh dose of leptazol. Whole brain amine concentrations were determined. The results are shown in Table 8.

No significant difference was detected in any of the amine concentrations of the treated and control rats.

3. CATECHOLAMINE CONCENTRATIONS IN DISCRETE AREAS OF THE RAT BRAIN AFTER A CONVULSIVE DOSE OF LEPTAZOL

Whole brain determinations of amine concentrations after convulsive doses of leptazol showed no difference from control levels. However small changes, such as 10 - 15% in a discrete area, may not have been reflected in whole brain determinations. Consequently the investigation was repeated in discrete areas of the brain.

Rats received leptazol 90mg/kg intraperitoneally and the convulsion was allowed to proceed to the post-ictal phase. The rats were then sacrificed and the discrete brain areas dissected out as described under 'Methods'. The areas used were:- midbrain, hippocampus, hypothalamus, striatum and whole brain. The results are shown in Table 9.

No significant effect on catecholamine concentrations was detected in any of the areas examined. Therefore it was concluded that a change in catecholamine concentrations did not occur as a result of the convulsions and no evidence was found for such a change being the cause of the convulsions.

4. DISCUSSION

The effect of leptazol on central catecholamine and 5-HT concentrations has been investigated because no reports could be found in the literature which cited the effects before and/or during a convulsion, but many conclusions had been drawn from the concentrations found after the convulsion. (see Introduction). Induction of severe convulsions with leptazol (90mg/kg intraperitoneally) produced no change in catecholamine or 5-HT concentrations in rat whole brain or in any of the discrete areas examined. Chlordiazepoxide has been shown to be a potent

depressant at the amygdalo-hippocampal level in cats (Morillo, Revzin & Knauss, 1962) and a potent anticonvulsant against leptazol (Swinyard & Castellion, 1966) and therefore the hippocampus was considered a likely site for changes to occur. Since no effect was detected it is suggested that chlordiazepoxide does not exert its anticonvulsant effect by a simple reversal or prevention of an elevation or depletion of catecholamines at the hippocampal level. However, it remains possible that chlordiazepoxide might exert its effect on synthesis and turnover of amines and this will be investigated later.

Contrary to the report of Breitner and others (1964), no post-ictal effect on amine levels was detected.

Since La Brecque and Goldberg (1967) had successfully treated geriatric patients for depression with subconvulsive leptazol and niacin for several days we reasoned that this might be due to an effect on central amine levels. No such effect could be detected in whole brain concentrations after similar treatment (omitting niacin) in rats. Niacin was omitted because we wished to determine if leptazol alone exerted any effect under these conditions. This negative result was an indicator that leptazol did not significantly effect the synthesis of any of these three amines, for if it had, some effect on amine concentrations would have been expected after 3 days' treatment.

Having completed this initial investigation it was only possible to say that leptazol produced no effect on central catecholamine and 5-HT concentrations. Nevertheless an effect on synthesis and turnover could not be dismissed. These possibilities must be investigated indirectly using aminergic drugs to alter the disposition of central amines and noting the effect on leptazol convulsions.

TABLE 6

CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RAT WHOLE BRAIN AT VARIOUS STAGES OF A CONVULSION INDUCED BY LEPTAZOL (90mg/kg INTRAPERITONEALLY)

Convulsive phase	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Controls	360 ± 14 (15)	707 ± 27 (5)	627 ± 14 (5)
Myoclonus	308 ± 22 (5)	696 ± 33 (6)	
Clonus	327 ± 14 (5)	702 ± 33 (6)	
Forelimb flexor tonus	356 ± 27 (4)	732 ± 22 (3)	
Post-ictal	356 ± 13 (16)	722 ± 35 (3)	636 ± 21 (6)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

ng = nanogram = 10^{-9} gramme

TABLE 7

CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RAT WHOLE BRAIN 30 MINUTES AFTER LEPTAZOL (65mg/kg SUBCUTANEOUSLY)

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Controls	311 ± 23 (5)	679 ± 38 (3)	587 ± 22 (4)
Leptazol 65mg/kg (subcutaneously) 30 minutes	308 ± 17 (5)	741 ± 18 (4)	638 ± 25 (5)

TABLE 8

EFFECT OF LEPTAZOL, (40mg/kg INTRAPERITONEALLY) TWICE DAILY FOR THREE DAYS, ON RAT WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS

Treatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Controls	333 ± 17 (5)	656 ± 18 (5)	594 ± 11 (5)
Leptazol 40mg/kg intraperitoneally twice daily for three days	322 ± 16 (5)	623 ± 26 (5)	624 ± 22 (5)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

ng = nanogram = 10^{-9} gramme

TABLE 9

EFFECT OF A CONVULSIVE DOSE OF LEPTAZOL (90mg/kg INTRAPERITONEALLY) ON CATECHOLAMINE CONCENTRATIONS IN DISCRETE AREAS OF THE RAT BRAIN

Brain area	Noradrenaline ng/g	Dopamine ng/g
Whole brain		
Controls	328 ± 23	593 ± 35
Leptazol treated	320 ± 18	592 ± 26
Hypothalamus		
Controls	1667 ± 156	
Leptazol treated	1467 ± 116	
Striatum		
Controls	163 ± 14	3526 ± 501
Leptazol treated	135 ± 7	4240 ± 840
Hippocampus		
Controls	286 ± 11	
Leptazol treated	246 ± 16	
Midbrain		
Controls	375 ± 20	
Leptazol treated	319 ± 25	

Six determinations were made on each area.

Results are expressed as group mean ± standard error.

ng = nanogram = 10^{-9} gramme.

CHAPTER THREE

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DRUGS PRODUCING INCREASED BIOGENIC AMINE CONCENTRATIONS AT THE AMINERGIC RECEPTORS

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DRUGS PRODUCING INCREASED BIOGENIC AMINE CONCENTRATIONS AT THE AMINERGIC RECEPTORS

EFFECT ON LEPTAZOL CONVULSIONS OF DRUGS PRODUCING INCREASED BIOGENIC AMINE CONCENTRATIONS AT THE AMINERGIC RECEPTORS

1. MONOAMINE OXIDASE INHIBITORS

There are several reports that monoamine oxidase (MAO) inhibitors are anticonvulsant to leptazol (Chow & Hendley, 1959; Prockop, Shore & Brodie, 1959 a, b.). There are also reports that MAO inhibitors produce no effect under these conditions (Kobinger, 1958; Lessin & Parkes, 1959) and yet others postulating a proconvulsant action (Spoerlain & Ellman, 1961; Sansome & Dell'Omodarme, 1963). A reappraisal of the effects of MAO inhibitors on leptazol was undertaken using five representative MAO inhibitors.

If MAO inhibitors were anticonvulsant then an increasing anticonvulsant activity would follow the time course of MAO inhibition with each of the inhibitors used. Tranylcypromine, a potent MAO inhibitor, has been found to produce maximal inhibition one hour after administration and this inhibition lasted for four days (Green & Erikson, 1960). These workers also showed that noradrenaline levels in rat brain were maximal 4 to 6 hours after administration, and were significantly elevated after 4 days.

Tranylcypromine (2mg/kg) was administered intraperitoneally to mice and the effect of a leptazol challenge (80mg/kg intraperitoneally) observed after various pretreatment times up to 6 hours. Other MAO inhibitors used intraperitoneally were phenelzine (6mg/kg), pargyline (50mg/kg), iproniazid (65mg/kg) and nialamide (10mg/kg). These drugs were investigated for their effect on leptazol convulsions after $\frac{1}{2}$, $1\frac{1}{2}$, 4 and 6 hours pretreatment. The results are shown in Table 10.

A transient but significant proconvulsant effect was observed after pretreatment with iproniazid, phenelzine and tranylcypromine, but nialamide and pargyline produced no effect (Fig. 18). These doses were shown to inhibit effectively MAO, using the 5-HTP head-twitch test (Corne, Pickering & Warner, 1963) - (see Methods). The proportion of control mice eliciting the characteristic head-twitch response varied between 0 and 10% when examined 25 minutes after 5-HTP (50mg/kg). The proportion of mice giving this response after pretreatment with an MAO inhibitor was consistently 60% or greater. This potentiation of the head-twitch response was indicative of effective inhibition of MAO activity.

These results showed that the proconvulsant action of an MAO inhibitor was not related to MAO inhibition per se, nor could it be attributed to similarity in chemical structure. A feature common to the proconvulsant MAO inhibitors was their inherent sympathomimetic activity (Furgiule, Kinnard & Buckley, 1962; Goldberg, 1964; Pirch & Norton, 1965). To test this hypothesis dexamphetamine (5mg/kg intraperitoneally) was investigated in the same way as the MAO inhibitors (Fig. 18). Dexamphetamine produced a marked and slightly more prolonged potentiation of leptazol convulsions and lethality than the MAO inhibitors. Yet after 6 hours pretreatment, there was a definite anticonvulsant effect. This may have been a post-ictal (rebound) effect following the earlier intense adrenergic stimulation produced by dexamphetamine (Goodman & Gilman, 1965). The time course of this potentiation followed the same time course as that of increased locomotor activity after dexamphetamine (Table 11, Fig. 19). Since increased locomotor activity could not be detected after the proconvulsant MAO inhibitors, increased locomotor activity itself was not the proconvulsant factor. No significant effect on body temperature (Table 12, Fig. 20) could be detected after this dose of dexamphetamine, therefore neither

hypothermia nor hyperthermia contributed to the proconvulsant activity.

The effect of tranylcypromine, phenelzine, pargyline and dexamphetamine on clonic convulsions due to leptazol (55mg/kg intraperitoneally) was also investigated in rats. The results are shown in Table 13. Phenelzine, tranylcypromine and dexamphetamine produced a transient potentiation of leptazol, whilst pargyline produced no effect. With tranylcypromine there was an anticonvulsant effect but this was also transient and followed the proconvulsant effect (Fig. 21). The results were similar to those in mice.

Dexamphetamine is believed to exert its central effects via catecholamines (Glowinski, Axelrod & Iversen,1966; Hanson, 1967). Therefore central amine concentrations, after treatment with dexamphetamine and the MAO inhibitors, were investigated in an attempt to correlate any amine effect due to dexamphetamine with the proconvulsant effects of the MAO inhibitors and to dissociate this effect from MAO inhibition per se. Amine determinations were made at the time of peak activity of dexamphetamine (30 min.) and the time course of tranylcypromine and pargyline followed for 4 hours. The results are shown in Table 14 and Figure 22. At a time when it produced a maximum potentiation of leptazol convulsions, dexamphetamine produced no significant change in the concentrations of any of the three amines investigated. Tranylcypromine produced a 30% elevation of noradrenaline concentrations ($p < 0.001$) after 30 minutes and a 130% elevation after 4 hours: dopamine was elevated by 100% ($p < 0.001$) within 30 minutes and reached a maximum after 90 minutes. 5-HT was elevated by 12% ($p < 0.001$) after 30 minutes and by 50%, 90 minutes after tranylcypromine. Pargyline did not produce such a marked effect upon noradrenaline concentrations. Although a 30% elevation ($p < 0.01$) was produced after 90 minutes, this was transient and levels decreased

after 4 hours. However, dopamine was elevated by 92% ($p < 0.001$) after 30 minutes and had not reached a maximum after 4 hours. 5-HT showed a similar elevation to that produced after tranylcypromine. Thus we were unable to establish any correlation between central amine concentrations and proconvulsant activity after MAO inhibitors.

2. AMINE PRECURSORS

The effects of the monoamines in the brain can be investigated through studies of the functional changes induced by injection of the noradrenaline and dopamine precursor, dihydroxyphenylalanine (DOPA) and the 5-HT precursor, 5-hydroxytryptophan (5-HTP). However, because of the widespread distribution and non-specificity of aromatic-L amino acid decarboxylase, the actual cellular localization of the catecholamines and 5-HT formed by decarboxylation of these precursors, may be artificial (Iversen, 1967). Therefore, the results from such studies must be interpreted cautiously.

The work with MAO inhibitors shows that after tranylcypromine, pargyline, etc., there is an elevation of the central concentrations of all 3 amines investigated. To investigate the effects after elevation of only one, 5-HT or catecholamines, it is necessary to load the appropriate stores by pretreatment with one of the precursors. It is also possible to pretreat with a MAO inhibitor followed by the precursor. The latter method not only induces a rapid increase of the amine formed from the exogenous precursor, but also promotes an increase in the other amines (from their endogenous precursors), and therefore nullifies, to some extent, the purpose of selective loading of an amine's stores. The method using the amine precursor *per se* is more selective but nevertheless subject to the limitations discussed by Iversen (1967).

Lessin and Parkes (1959) used both of these methods to look for an effect on leptazol convulsions in mice. After a MAO inhibitor, 5-HTP lengthened the survival time but DOPA had no effect. Neither 5-HTP nor DOPA alone produced any effect on leptazol convulsions. Bonnycastle, Giarman and Paasonen (1957) also showed that 5-HTP per se was without effect on leptazol convulsions in rats. Schmidt (1964) demonstrated that DOPA and 5-HTP were anticonvulsant after MAO inhibitors but had no effect per se. On the other hand, Wada (1961) has shown 5-HTP to produce a marked activation of the epileptogenic cerebral electrical activity in chronically epileptic cats and monkeys.

Having investigated the MAO inhibitors and found that they possess no anticonvulsant activity but that some possess proconvulsant activity, it was decided to investigate the amine precursors to check if they produce similar effects. Rats were pretreated for one hour with DL-5-HTP 50mg/kg or DL-DOPA 100mg/kg by intraperitoneally injection and then given a leptazol challenge (65mg/kg subcutaneously) and observed for clonic convulsions. The results are shown in Table 15. Neither of the precursors produced any significant effect on leptazol convulsions. Biogenic amine concentrations were determined under the same conditions to verify that an elevation of amine concentrations did occur. The results are shown in Table 16 and Figure 23. DL-DOPA treated rats showed no difference from controls with respect to their sensitivity to leptazol convulsions, yet this pretreatment produced a 50% elevation of central noradrenaline ($p < 0.05$) and a 33% elevation of dopamine concentrations ($p < 0.001$) and simultaneously a 25% decrease in central 5-HT concentrations ($p < 0.01$). 5-HTP pretreatment produced no significant effect on the sensitivity of rats to leptazol convulsions despite a 20% elevation of central 5-HT concentrations ($p < 0.05$).

These results are similar to the results of work with MAO inhibitors in that an elevation of central concentrations is without effect on the leptazol convulsive threshold. The results with the amine precursors also show that a selective increase of either catecholamine or 5-HT concentrations is without effect on this convulsive threshold.

3. CATECHOL-O-METHYL TRANSFERASE INHIBITORS

Catechol-O-methyl transferase (COMT) is considered to be responsible for the metabolism of circulating catecholamines (Axelrod, Inscue & others, 1958), though Brodie and Costa (1962) disagree that COMT is essential in this breakdown because there are many alternative pathways. However, the major metabolite of dopamine in the central nervous system appears to be homovanillic acid and hence COMT is of importance in this metabolic pathway (Murphy, Robinson & Sharman, 1969). The effect of blocking this metabolic pathway was investigated for its effect on leptazol convulsions.

Many drugs are known to inhibit COMT but generally these drugs are non-specific (Carlsson, 1964). The most commonly used inhibitors are the polyhydroxyphenols, pyrogallol and catechol (Axelrod & Larocke, 1959; Carlsson, Lindqvist & others, 1962; Carlsson, 1964; Ross & Haljasmaa, 1964, a;b; Iversen, 1967; Rogers, Angel & Butterfield, 1968). A more specific but less frequently used group of inhibitors is the tropolone series (Belleau & Burba, 1961; Carlsson, Lindqvist & others, 1962; Carlsson, 1964; Ross & Haljasmaa, 1964, a;b; Burba & Murnaghan, 1965; Murphy & others, 1969). One such tropolone is β-thujaplicin which was used in rats and shown to be an effective COMT inhibitor over a 24 hour period (Mussachio & Goldstein, 1962).

Recent work has shown that pyrogallol, injected intraperitoneally to mice, reaches peak levels in the brain after only 10 minutes

(Rogers et al, 1968). Therefore, rats were injected intraperitoneally with pyrogallol and 10 minutes later they were given a leptazol challenge (65mg/kg subcutaneously). The results are shown in Table 17. The lower dose of pyrogallol (120mg/kg) had no effect on leptazol convulsions, but higher doses blocked the convulsive effect of leptazol. At larger doses pyrogallol (500mg/kg) was itself convulsant, but was anticonvulsant against leptazol - (Fig. 24). This indicated that pyrogallol and leptazol might compete for some part of the convulsive mechanism or that one was inactivating the other in some way. If, however, this effect was due to COMT inhibition, then the smaller non-convulsant dose (120mg/kg) should be anticonvulsant after a longer pretreatment period. However, pyrogallol (120mg/kg) administered 1 hour prior to the leptazol challenge was without significant effect on leptazol convulsions. (Fig. 24).

A further check on the hypothesis, that COMT inhibition might be anticonvulsant, was performed using a second COMT inhibitor, catechol. Catechol was tested in the same way as pyrogallol but using a pretreatment time of 5 minutes, after which maximum levels have been found in the CNS (Rogers & others, 1968), and after 1 hour when COMT inhibition should be significantly effective. Catechol itself was a convulsant at 30mg/kg and 60mg/kg intraperitoneally. Therefore, the effects of two subconvulsant doses and one mildly convulsive dose were investigated for the effect on leptazol. The results are shown in Table 17 and Figure 24. Catechol produced no significant effect on leptazol convulsions at doses of 10, 20 and 30mg/kg, though 20mg/kg reduced the % maximum convulsions to 33.3%, this was statistically not significantly different from the controls. In contrast to pyrogallol, a convulsive dose of catechol (30mg/kg) was not significantly anti-convulsant against leptazol. A dose of 20mg/kg showed some evidence of anticonvulsant activity though this was statistically not significant.

This dose was investigated for its effect on leptazol convulsions after a 1 hour pretreatment period when COMT inhibition should have been significant. This pretreatment was without effect on leptazol convulsions (Fig. 24): in fact, if catechol has any anticonvulsant activity, it was less evident after 1 hour's pretreatment (40% convulsions) compared with the 5 minute pretreatment, when such activity was maximal (33% convulsions).

Further evidence that COMT inhibition is without effect on leptazol convulsions, was produced with the results of β-thujaplicin pretreatment. β-Thujaplicin is a tropolone. (2, 4, 6 -cycloheptatrien-1-one-2-hydroxy-4-isopropyl). At a dose level of 40mg/kg intraperitoneally it has been shown to reduce the methoxy-catechol fraction and increase the catechol fraction in the urine of rats, collected over a 24 hour period (Mussachio & Goldstein, 1962). This was attributed to COMT inhibition.

Rats were pretreated with β-thujaplicin (40mg/kg intraperitoneally) 24 hours prior to the leptazol challenge. (Table 17 and Fig. 24). This pretreatment did not alter the leptazol threshold. Therefore, β-thujaplicin (40mg/kg intraperitoneally) was administered 1 and 2 hours before the leptazol challenge: no significant effect on leptazol threshold was evident (Table 17 and Fig. 24). Amine determinations were not performed after pretreatment with these drugs because it has been demonstrated previously that COMT inhibition produces no effect on central catecholamine concentrations in the rat (Crout, Creveling & Udenfriend, 1961; Weil Malherbe, Posner & Bowles, 1961; Glowinski & Axelrod, 1965).

4. IMIPRAMINE-LIKE DRUGS

Imipramine has been reported to block the reuptake of noradrenaline both peripherally (Axelrod, Whitby & Hertting, 1961,

for other references see Introduction), and centrally (Glowinski & Axelrod, 1964). The reports of its effect on leptazol convulsions are consistent in that it is always reported to be anticonvulsant (Sigg, 1959; Chow & Hendley, 1959; Spencer, unpublished observations). Imipramine has also been reported to be anticonvulsant to audiogenic seizures (Lehmann & Bushnel, 1962) and to electroshock (Chow & Hendley, 1959; Sigg, 1959). The anticonvulsant activity of other imipramine-like drugs is not well documented. Therefore, the effects of imipramine, desipramine and protriptyline on leptazol convulsions have been investigated in rats in this project. Desipramine and protriptyline prevent reuptake of noradrenaline at nerve endings both centrally (Carlsson, Fuxe, Hamberger & Lindqvist, 1966) and peripherally (Carlsson & Waldeck, 1965; Iversen, 1967). Centrally these two drugs block only the reuptake of noradrenaline and not dopamine (Carlsson, Fuxe & others, 1966). Imipramine is a weaker uptake inhibitor of noradrenaline (Carlsson, Fuxe & others, 1966) but in addition it blocks reuptake by 5-HT neurons, whereas desipramine and protriptyline do not (Carlsson, Fuxe & Ungerstedt, 1968). Further it has been reported that desipramine and protriptyline increase the turnover of central noradrenaline but not of dopamine (Neff & Costa, 1967 b).

With these properties in mind the effects of desipramine, protriptyline and imipramine were investigated: Imipramine is rapidly metabolised in rats to desipramine, its active metabolite (Gillette, Dingell & others, 1961). After 1 hour over 60% of the imipramine is metabolised to desipramine (Bickel, 1967). Therefore, in order to see the effects of imipramine and not those of desipramine a 15 minute pretreatment period was used and a dose of 20mg/kg intraperitoneally. Desipramine and protriptyline are the most potent imipramine-like drugs (Carlsson, Fuxe & others, 1966), desipramine being ten times more potent than imipramine as an uptake inhibitor (Iversen, 1967),

therefore, a dose of 10mg/kg and a pretreatment period of 1 hour was used.

Rats were pretreated with one of the imipramine-like drugs as described above and then subjected to a leptazol challenge (65mg/kg subcutaneously) and observed for the frequency of clonic convulsions. The results are shown in Table 18. Imipramine produced no significant effect on the clonic convulsive phase and although desipramine and protriptyline produced an increase in the % maximum clonic convulsions, this effect was statistically not significant.

Amine determinations were not made after these three drugs because no significant proconvulsant or anticonvulsant effect was produced. However, previous reports indicate that the imipramine-like drugs do not alter brain concentrations of 5-HT, noradrenaline or dopamine (Sulser & Brodie, 1961; Sulser, Watts & Brodie, 1962). Although earlier reports claim that imipramine is anticonvulsant to leptazol, we were unable to reproduce this anticonvulsant activity. Consequently, the results of this work with differential uptake-inhibitors did not implicate any one amine as being involved in convulsive mechanisms.

5. DISCUSSION

The proconvulsant action of some MAO inhibitors, demonstrated both in rats and mice, was not due to MAO inhibition *per se*. All five drugs were effective MAO inhibitors but only three (tranylcypromine, phenelzine and iproniazid) were proconvulsant. Nor could this effect be attributed to similarity in chemical structure; of the three hydrazines studied, iproniazid and phenelzine were proconvulsant whilst nialamide had no effect. In the non-hydrazine group tranylcypromine was proconvulsant yet pargyline was not. However, the proconvulsant

MAO inhibitors have been shown to possess inherent sympathomimetic activity (Furgiuele et al, 1962; Goldberg, 1964; Pirch & Norton, 1965). Results with dexamphetamine confirmed that sympathomimetic activity was a proconvulsant factor. Though the potentiation of leptazol after dexamphetamine followed the same time course as the increased locomotor activity, no increased locomotor activity was detected after the proconvulsant MAO inhibitors. The anticonvulsant activity of MAO inhibitors described by other workers (Chow & Hendley, 1959; Prockop et al, 1959 a,b; Yen et al, 1962) could not be confirmed. A transient anticonvulsant activity followed the proconvulsant effect of tranylcypromine in rats and dexamphetamine in mice. This activity was not reproducible in all the proconvulsant inhibitors and was assumed to be a 'rebound post ictal depression' following the earlier intense adrenergic stimulation (Goodman & Gilman, 1965). An attempt was made to correlate brain amine concentrations with the behavioural effects of these drugs, yet no correlation was obvious. A host of workers have shown that dexamphetamine depletes central noradrenaline concentrations (McLean & McCartney, 1961; Sanan & Vogt, 1962; Moore & Lariviere, 1963; Carlsson, Lindqvist, Dahlstrom, Fuxe & Masuoka, 1965; Smith, 1965), and it was considered possible that the proconvulsant MAO inhibitors also might produce a small initial amine depletion before being followed by the predictable elevation. In our hands dexamphetamine (5mg/kg) produced no depletion of central amines. This was not surprising since in all reports of depletion by dexamphetamine very high doses had been used. The lowest dose reported by others to produce a depletion was 10mg/kg (Moore & Lariviere, 1963) such a dose was far greater than that necessary to potentiate leptazol convulsions in the present experiments. Therefore, since no depletion

was produced with 5mg/kg dexamphetamine, this factor could not be correlated with the proconvulsant effect.

Another possibility was that the proconvulsant MAO inhibitors produced a selective elevation of one amine, as has been reported by Spector, Hirsch and Brodie, (1963). Although pargyline did not produce a large elevation of noradrenaline whilst tranylcypromine did, this does not account for the difference in their behavioural effects. Tranylcypromine was only proconvulsant after 30 minutes, when the noradrenaline concentration was 307ng/g. Pargyline produced this noradrenaline concentration after $1\frac{1}{2}$ hours but without effect on leptazol convulsions. The extremely rapid rise in dopamine concentrations was not the proconvulsant factor: both MAO inhibitors produced this same effect. The elevation of central 5-HT concentrations was also similar after each MAO inhibitor. This evidence, therefore, implies that central amine concentrations are not involved in the proconvulsant effect of these drugs.

Since the proconvulsant MAO inhibitors have been shown to possess amphetamine-like activity (Goldberg, 1964) and the above proconvulsant action was not associated with the relase of amines, it appears that these inhibitors exert a transient stimulant effect by a direct (amphetamine-like) sympathomimetic stimulation of receptors rather than by an indirect action involving the release of central amines.

Loading specific amine stores by treatment with the amine precursors, DL-DOPA and DL-5-HTP, produced no significant effect on the convulsive threshold of leptazol and confirmed the results of Bonnycastle and others (1957), Lessin and Parkes (1959) and Schmidt (1964). An interesting observation during the course of this work was that pretreatment with DL-DOPA depleted whole brain 5-HT concentrations by 25% ($p < 0.01$) and conversely pretreatment with 5-HTP elevated central

dopamine concentrations by 15% ($p < 0.05$) suggesting that there is an interaction between these two amines.

No attempt was made to investigate the effect of pretreatment with MAO inhibitor/amine precursor combinations on the leptazol threshold. Elevation of amine concentrations by this method has been demonstrated to produce pharmacological properties distinctly different from those seen after elevation of endogenous amines (Green & Sawyer, 1964). However, the effects of these amine precursors after reserpine pretreatment have been examined (Chapter 4 - Reserpine and amine precursors).

The inactivity of COMT inhibitors, in non-toxic doses, against leptazol convulsions can perhaps be explained by our observation that leptazol fails to release catecholamine into the extraneuronal space, the site of action of COMT. This inactivity also demonstrated that the metabolism of dopamine to homovanillic acid, which utilises COMT, is in no way involved. Since "submaximal" doses of the polyhydroxyphenols produced no effect on leptazol but maximal doses were anticonvulsant, this suggests that the polyhydroxyphenols may either compete with leptazol for the convulsive "receptor site" (for both are convulsants) or that they inactivate leptazol or that their presence in the brain in high concentrations prevents leptazol from crossing the blood-brain barrier. That both polyhydroxyphenols are convulsant might be explained by the observations of Roberts and Broadley (1965) that decreased COMT activity might lead to formation of noradnamine, and Cowell (1969) that intracerebral injections of noradnamine are convulsant. These two COMT inhibitors might produce sufficient noradnamine in the brain to produce convulsions. However, results with β-thujaplicin, a COMT inhibitor with a potency thirty

times that of pyrogallol (Belleau & Burba, 1961), do not lend support to this hypothesis, for β-thujaplicin does not produce convulsions,

Our results with imipramine-like drugs were disappointing. Imipramine has been reported to be anticonvulsant against leptazol by Sigg (1959) who showed that 10mg/kg imipramine was without effect but that 15 minutes pretreatment with 30mg/kg blocked 90% of convulsions and only 45% when the pretreatment time was extended to 1 hour. This suggested to us that as metabolism to desipramine occurred, so the anticonvulsant activity decreased. Another report by Giurgea, Dauby and others (1967) shows that the ED_{50} of desipramine against leptazol in mice is 58mg/kg and that of imipramine is 35mg/kg, which supports the hypothesis that imipramine, the weaker uptake inhibitor, is a more potent anticonvulsant than desipramine. It would be possible to explain this from the results of Carlsson, Fuxe and Ungerstedt (1968), who reported that imipramine blocks 5-HT reuptake but desipramine does not. However, in our hands neither imipramine, desipramine nor protriptyline were anticonvulsant. Therefore, our results allow the following conclusions:-

(a) Inhibition of reuptake of 5-HT by imipramine (Carlsson, Fuxe & Ungerstedt, 1968) was without effect on leptazol convulsions.

(b) Inhibition of the reuptake of noradrenaline by desipramine or protriptyline (Carlsson, Fuxe & others, 1966) was without effect on leptazol convulsions.

(c) Any increased turnover of central noradrenaline due to desipramine or protriptyline (Neff & Costa, 1967 b) produced no effect on leptazol convulsions.

In conclusion, the results with drugs increasing the concentrations of the catecholamines and 5-HT at aminergic receptors imply that no matter how the increase is produced whether it be by blocking metabolism or reuptake, whether it be an increase of one specific amine or of all three amines studied, there is no significant effect on leptazol threshold in rats.

TABLE 10

EFFECT OF MAO INHIBITORS AND DEXAMPHETAMINE ON MORTALITY IN MICE AFTER LEPTAZOL (80mg/kg INTRAPERITONEALLY)

Results are expressed as a Mortality Ratio (see Methods). Each result is the mean of at least 3 determinations. For control mice the Mortality Ratio $\pm$ standard deviation = 1.0 ± 0.215 ($45.6 \pm 9.7\%$). Mortality Ratios outside the value $\bar{x} \pm 2$ SD are significant at $p < 0.05$ (Documenta Geigy Scientific Tables). Limits of significance are 1.00 ± 0.43 (1.43 - 0.57). Ratios greater than 1.43 show significant proconvulsant activity (**). Ratios less than 0.57 show significant anticonvulsant activity (*).

Pretreatment	Leptazol-induced Mortality ratios in mice following pretreatment at various times			
	30 mins.	90 mins.	240 mins.	360 mins.
Saline controls	$\bar{x} \pm 2$ SD = 1.00 ± 0.43			
Tranylcypromine 2mg/kg	1.54 **	0.82	1.20	1.10
Phenelzine 6mg/kg	1.2	1.48 **	0.82	0.98
Pargyline 50mg/kg	0.98	1.10	0.88	1.31
Nialamid 10mg/kg	0.98	0.77	1. 0	0.88
Iproniazid 65mg/kg	1.64 **	1.31	1.20	1.10
Dexamphetamine 5mg/kg	1.97 **	1.54 **	0.88	0.44 *

FIGURE 18

EFFECT OF MAO INHIBITORS AND DEXAMPHETAMINE ON MORTALITY IN MICE AFTER LEPTAZOL (80mg/kg. INTRAPERITONEALLY)

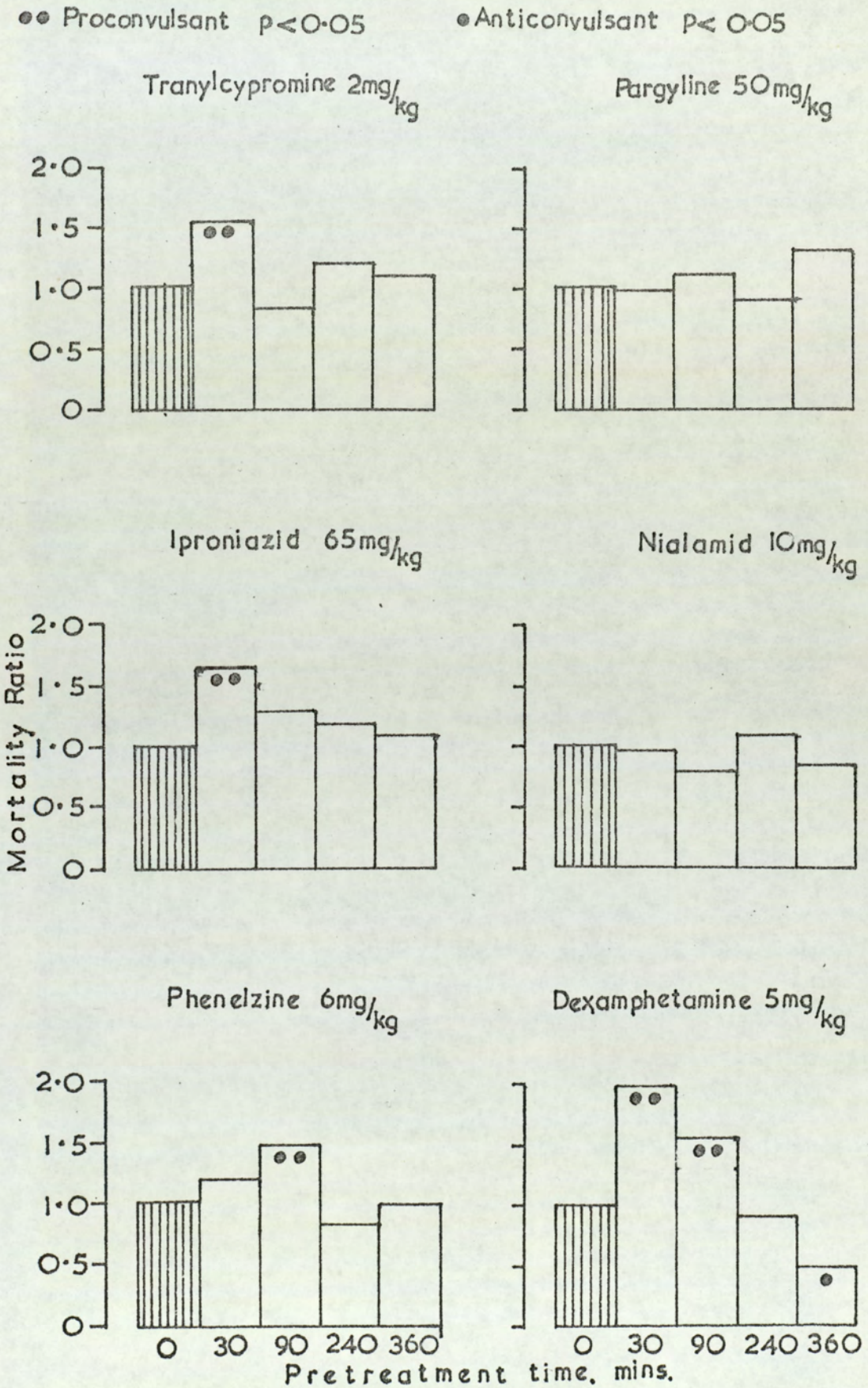


TABLE 11

LOCOMOTOR ACTIVITY COUNTS IN MICE AFTER PRETREATMENT WITH DEXAMPHETAMINE (5mg/kg INTRAPERITONEALLY) OR N.SALINE

Time in hours	Dexamphetamine-Treated		Saline-Treated Controls	
	Cumulative count	30 minute increment	Cumulative count	30 minute increment
0	0	-	0	-
$\frac{1}{2}$	2,832	2,832	227	227
1	5,906	3,074	270	43
$1\frac{1}{2}$	8,840	2,934	356	86
2	12,000	3,160	388	32
$2\frac{1}{2}$	14,979	2,979	496	108
3	18,009	3,030	523	27
$3\frac{1}{2}$	19,966	1,957	582	59
4	20,675	709	652	70
$4\frac{1}{2}$	20,971	296	687	35
5	21,258	283	767	80

FIGURE 19

SPONTANEOUS LOCOMOTOR ACTIVITY IN MICE AFTER PRETREATMENT WITH DEXAMPHETAMINE (5mg/kg, INTRAPERITONEALLY) OR N. SALINE (CONTROLS)

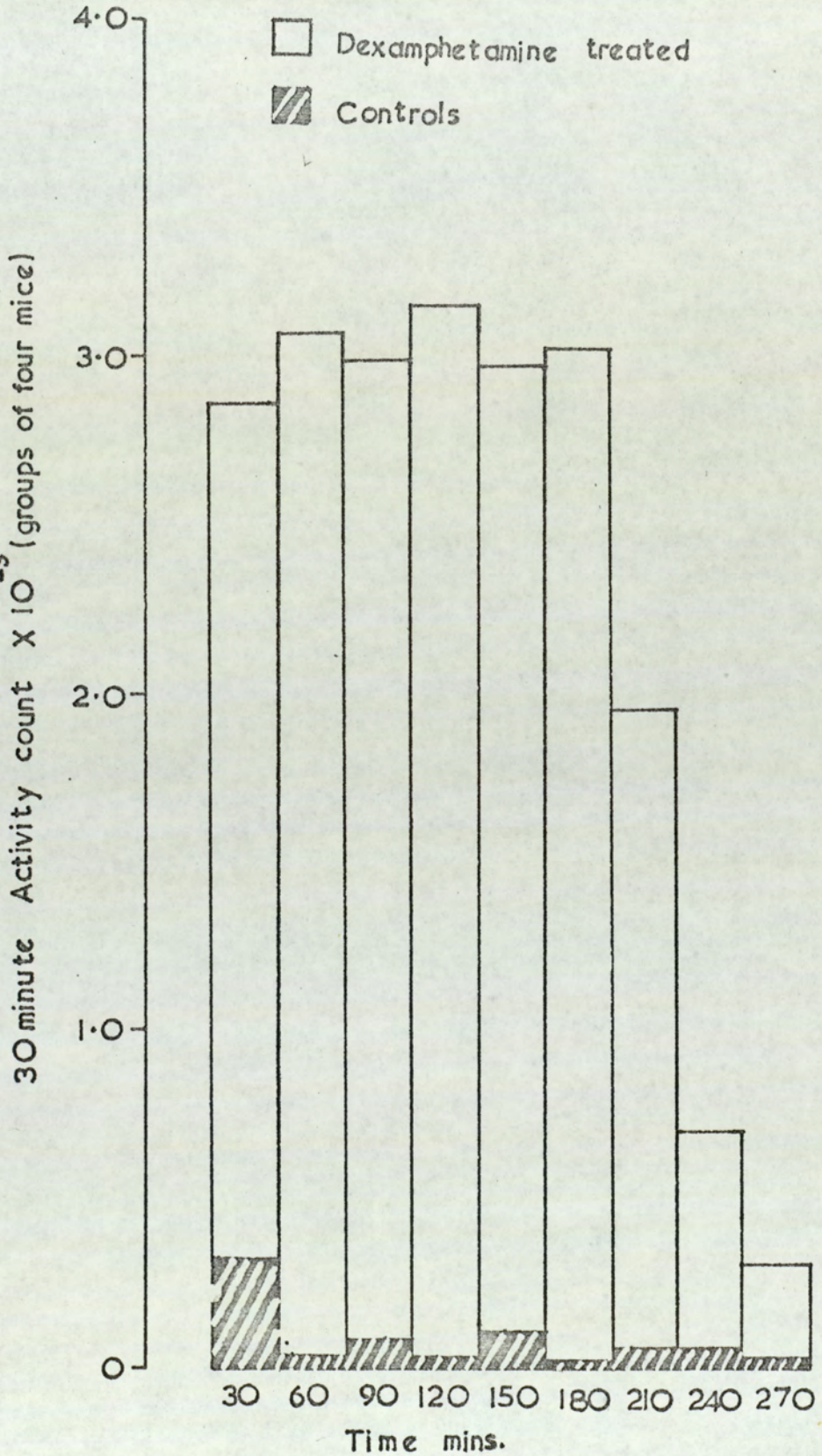


TABLE 12

EFFECT OF DEXAMPHETAMINE (5mg/kg INTRAPERITONEALLY) ON THE OESOPHAGEAL TEMPERATURE (°C) OF MICE.

Pretreatment time in hours	Dexamphetamine treated mice	Saline treated controls
0	36.4 ± 0.21	36.3 ± 0.28
½	36.5 ± 0.20	37.4 ± 0.17
1	36.8 ± 0.22	37.3 ± 0.21
1½	37.2 ± 0.30	37.0 ± 0.21
2	37.3 ± 0.28	36.8 ± 0.30
2½	36.9 ± 0.31	36.8 ± 0.24
3	36.7 ± 0.18	36.7 ± 0.18
3½	36.6 ± 0.15	36.4 ± 0.27
4	36.2 ± 0.17	36.4 ± 0.27
5½	36.3 ± 0.24	36.1 ± 0.19
6	36.3 ± 0.24	36.2 ± 0.21

Ambient temperature 21°C

Results are expressed as the group mean ± standard error of ten results

FIGURE 20

EFFECT OF DEXAMPHETAMINE (5mg/kg, INTRAPERITONEALLY) ON THE OESOPHAGEAL TEMPERATURE OF MICE

Ambient temperature 21°C

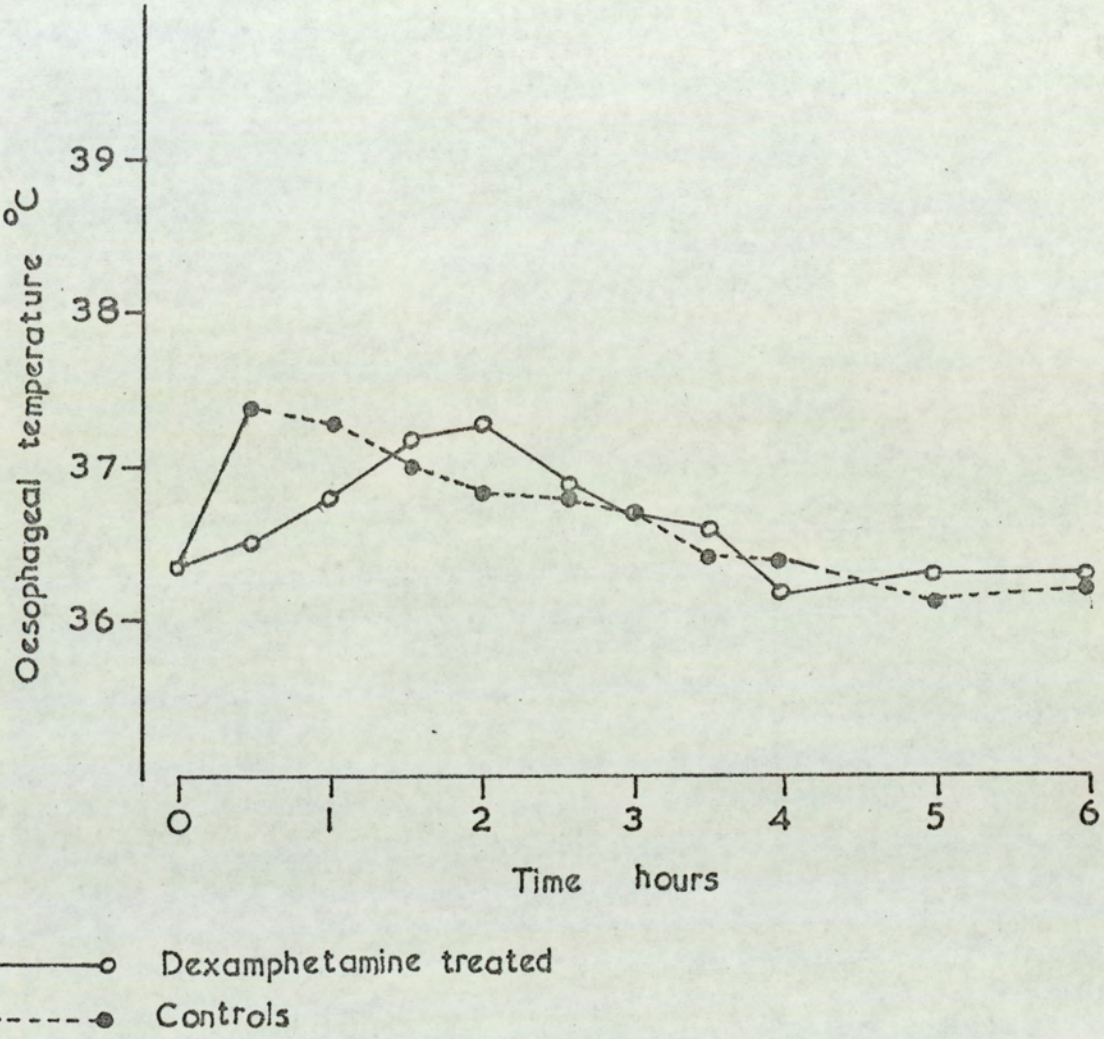


TABLE 13

EFFECT OF MAO INHIBITORS AND DEXAMPHETAMINE ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (55mg/kg, INTRAPERITONEALLY)

Results are expressed as a Clonic Convulsive Ratio (see Methods). Each result is the mean of at least 3 determinations. For control rats the Clonic Convulsive Ratio $\pm$ standard deviation = 1.00 ± 0.115 ($40.6 \pm 9.3\%$). Clonic Convulsive Ratios outside the value $x \pm 2SD$ are significant at $p < 0.05$ (Documenta Geigy Scientific Tables). Limits of significance are 1.00 ± 0.23 (1.23 - 0.77). Ratios greater than 1.23 show significant proconvulsant activity (**). Ratios less than 0.77 show significant anticonvulsant activity (*).

Pretreatment	Leptazol-induced Clonic Convulsive Ratios in rats following pretreatment at various times		
	30 mins.	90 mins.	240 mins.
Saline controls	$\bar{x} \pm 2SD = 1.00 \pm 0.23$		
Tranylcypromine 2mg/kg	1.36 **	0.62 *	0.99
Pargyline 50mg/kg	0.99	0.99	0.99
Phenelzine 6mg/kg	1.11	1.11	1.36 **
Dexamphetamine 5mg/kg	1.6 **	1.6 **	1.11

FIGURE 21

EFFECT OF MAO INHIBITORS AND DEXAMPHETAMINE ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (55mg/kg, INTRAPERITONEALLY)

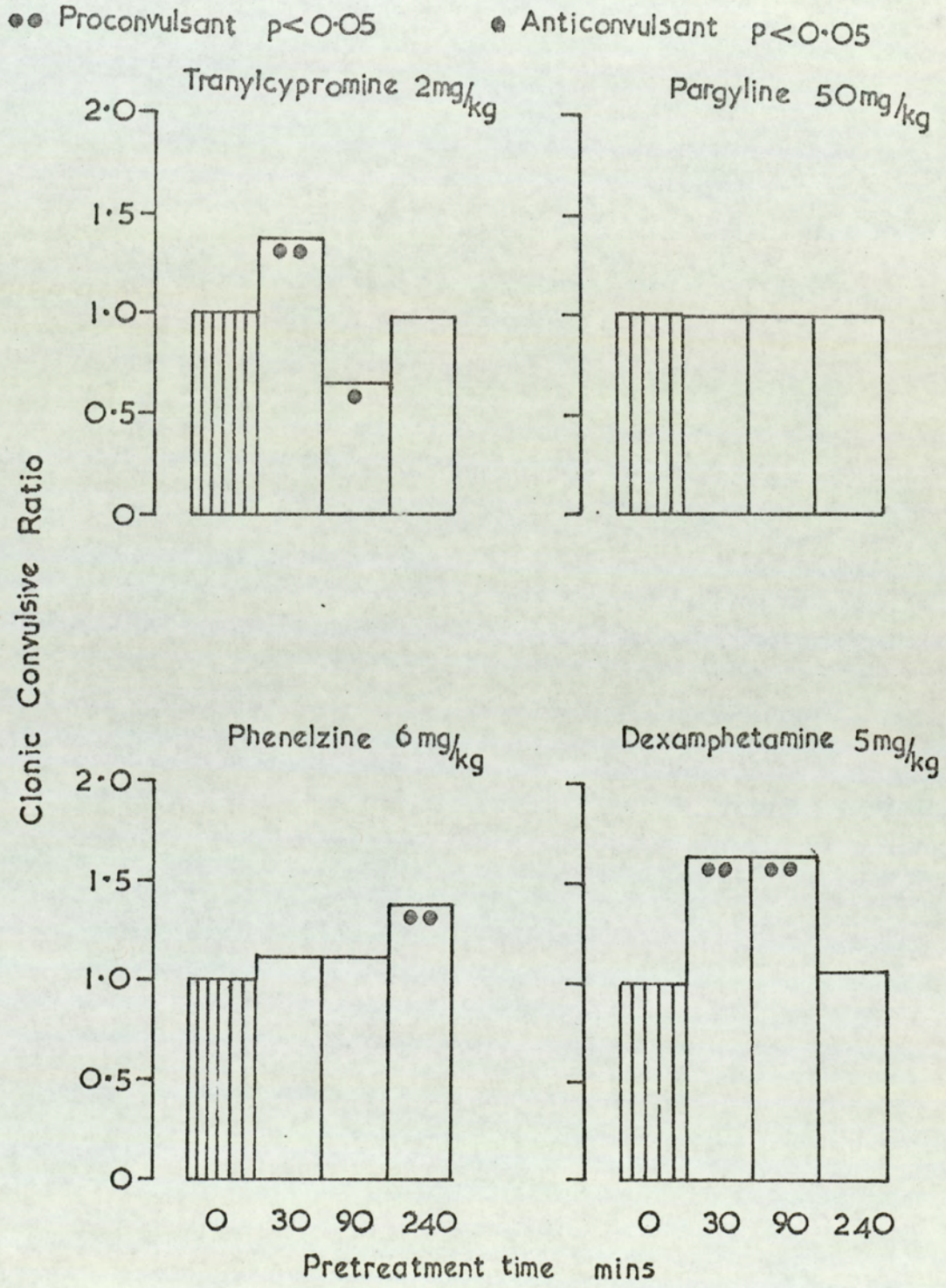


TABLE 14

EFFECT OF DEXAMPHETAMINE AND MAO INHIBITORS ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g			Dopamine ng/g			5-HT ng/g		
	Pretreatment time in minutes								
	30	90	240	30	90	240	30	90	240
Controls	233 ± 12 (8)			643 ± 29 (7)			540 ± 8 (8)		
Dexamphetamine 5mg/kg intraperitoneally	201 ±9 (4)			637 ±12 (4)			560 ±10 (4)		
Tranylcypromine 2mg/kg intraperitoneally	307 ±8 (3) ***	377 ±15 (3) ***	541 ±35 (3) ***	1261 ±38 (3) ***	1423 ±34 (3) ***	1467 ±99 (3) ***	609 ±12 (3) ***	804 ±36 (3) ***	929 ±26 (3) ***
Pargyline 50mg/kg intraperitoneally	242 ±10 (3)	307 ±22 (3) **	278 ±23 (3)	1235 ±36 (3) ***	1397 ±57 (3) ***	1756 ±113 (3) ***	607 ±8 (3) ***	699 ±39 (3) ***	855 ±27 (3) ***

Results are expressed as group mean ± standard error.

ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

** = $p < 0.01$

*** = $p < 0.001$

FIGURE 22

EFFECT OF DEXAMPHETAMINE AND MAO INHIBITORS ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

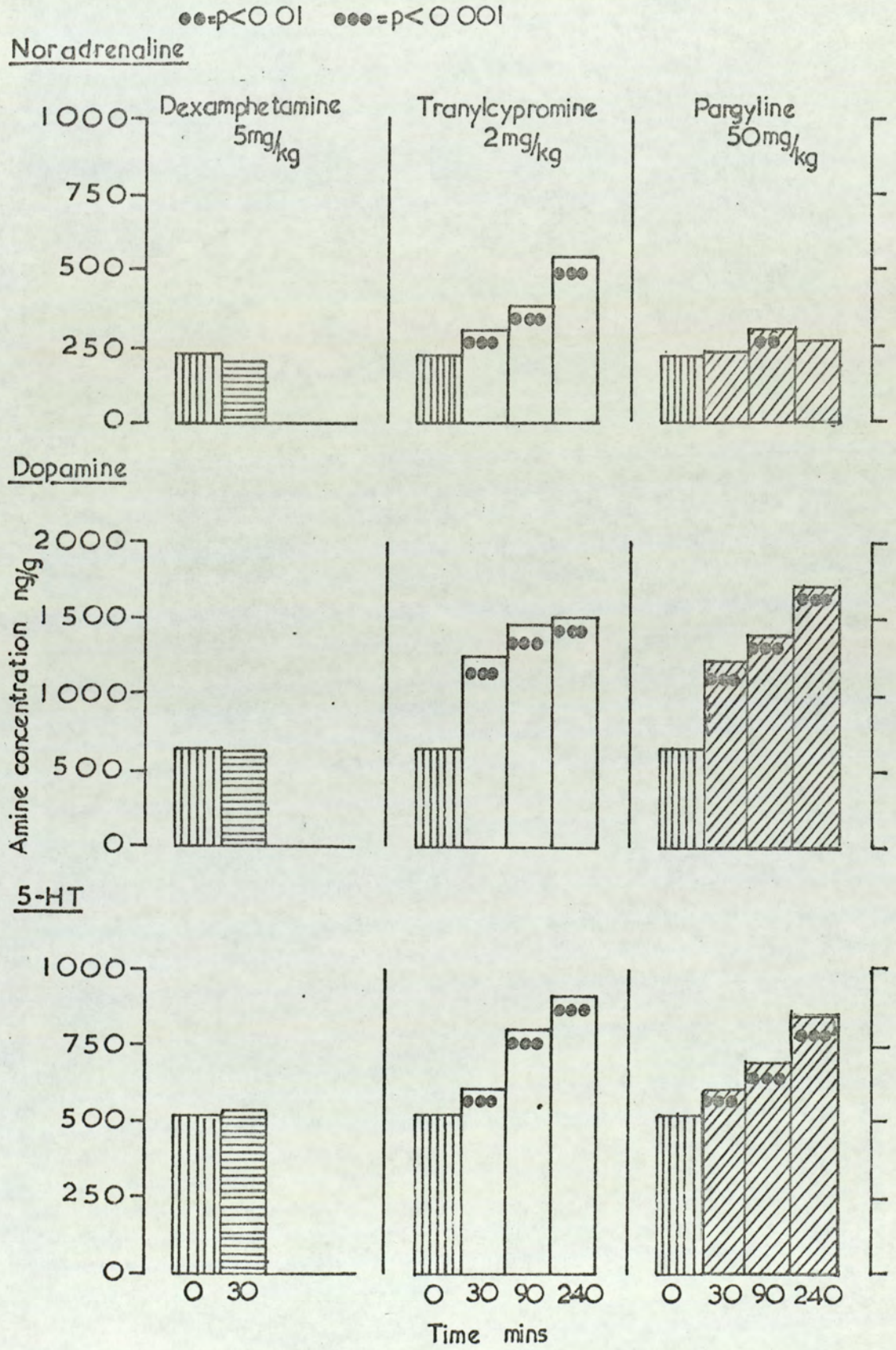


TABLE 15

EFFECT OF PRETREATMENT WITH THE AMINE PRECURSORS, DL-DIHYDROXYPHENYLALANINE (DL-DOPA) AND 5-HYDROXY-DL-TRYPTOPHAN (5-HTP), ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Pretreatment	% Maximum Clonic Convulsions
Saline (intraperitoneally) 1 hour Leptazol 65mg/kg (subcutaneously)	43 ± 3 (16)
DL-DOPA 100mg/kg (intraperitoneally) 1 hour Leptazol 65mg/kg (subcutaneously)	50 ± 4 (4)
5-HTP 50mg/kg (intraperitoneally) 1 hour Leptazol 65mg/kg (subcutaneously)	36 ± 6.5 (4)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

TABLE 16

EFFECT OF PRETREATMENT WITH DL-DOPA (100mg/kg, INTRAPERITONEALLY) AND 5-HTP (50mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Amine concentrations in rat whole brain ng/g		
	Noradrenaline	Dopamine	5-HT
Controls	230 ± 13 (4)	736 ± 12 (4)	597 ± 27 (4)
5-HTP, 50mg/kg intraperitoneally (1 hour)	300 ± 35 (4)	840 ± 39 (4) *	727 ± 39 (4) *
DL-DOPA, 100mg/kg intraperitoneally (1 hour)	352 ± 35 (3) *	970 ± 13 (4) ***	448 ± 4 (4) **

Results are expressed as group mean ± standard error.

ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

FIGURE 23

EFFECT OF PRETREATMENT, FOR 1 HOUR, WITH DL-DOPA (100mg/kg, INTRAPERITONEALLY) OR DL-5HTP (50mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS AND ON CLONIC CONVULSIONS AFTER A LEPTAZOL CHALLENGE (65mg/kg, SUBCUTANEOUSLY IN RATS

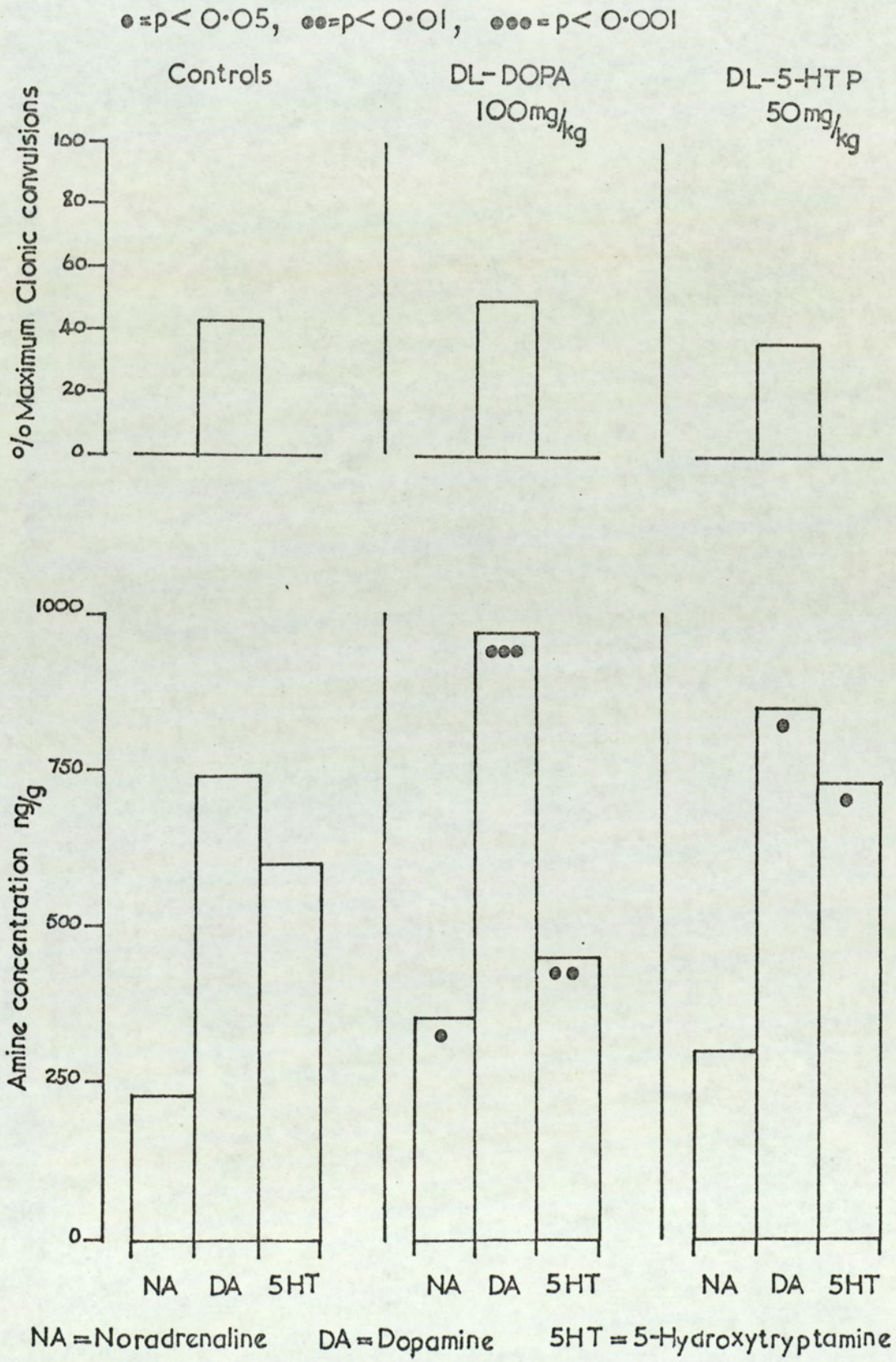


TABLE 17

EFFECT OF COMT INHIBITORS ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg SUBCUTANEOUSLY)

COMT Inhibitor	Dose mg/kg intraperitoneally	Pretreatment time prior to leptazol challenge	Percentage Maximum Clonic Convulsions
-	-	Saline controls	47.5 ± 4.8 (5)
Pyrogallol	120	10 mins.	55 ± 3 (2)
Pyrogallol	240	10 mins.	10 ± 3 (2)
Pyrogallol	500	10 mins.	10 ± 3 (2)
Pyrogallol	120	1 hour	50 ± 4 (3)
Catechol	10	5 mins.	50 ± 3 (2)
Catechol	20	5 mins.	33 ± 3 (3)
Catechol	30	5 mins.	55 ± 3 (2)
Catechol	20	1 hour	40 ± 7 (2)
β -Thujaplicin	40	1 hour	50 (1)
β -Thujaplicin	40	2 hours	50 (1)
β -Thujaplicin	40	24 hours	50 (1)

Results are expressed as group mean ± standard Error.

Figures in parentheses indicate the number of determinations made.

FIGURE 24

EFFECT OF COMT INHIBITORS ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

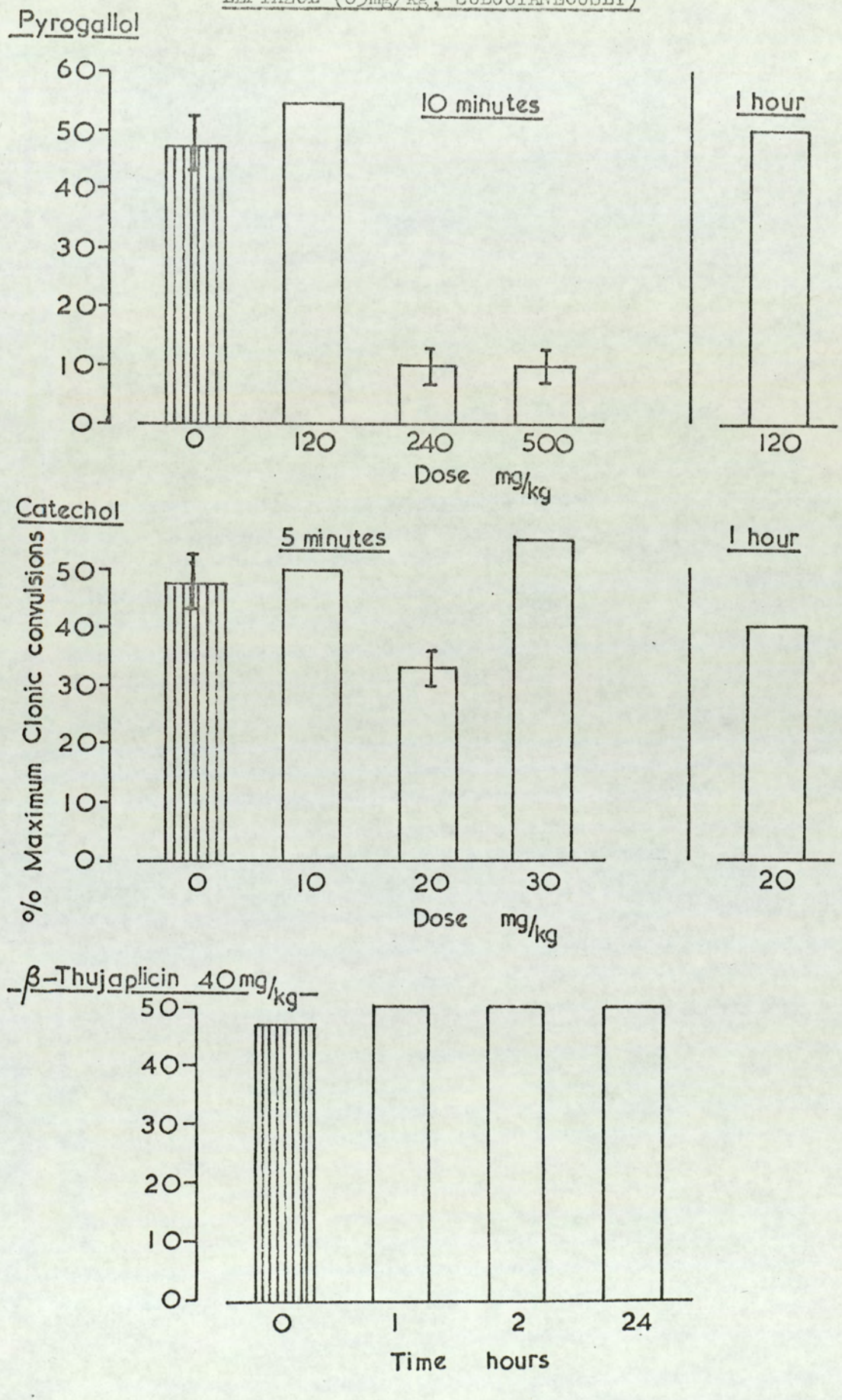


TABLE 18

EFFECT OF 2 HOURS PRETREATMENT WITH DESIPRAMINE (DMI) (10mg/kg, INTRAPERITONEALLY) AND PROTRIPTYLENE (10mg/kg, INTRAPERITONEALLY), AND 15 MINUTES PRETREATMENT WITH IMIPRAMINE (20mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Pretreatment prior to the leptazol challenge	% Maximum Clonic Convulsions
Saline intraperitoneally (2hours) Leptazol	45 ± 3 (4)
D.M.I. 10mg/kg intraperitoneally (2 hours) Leptazol	58 ± 5 (4)
Protriptyline 10mg/kg intraperitoneally (2 hours) Leptazol	58 ± 5 (4)
Imipramine 20mg/kg intraperitoneally (15 mins.) Leptazol	45 ± 3 (2)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

CHAPTER FOUR

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DRUGS PRODUCING DEPLETION OF BIOGENIC AMINES

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DRUGS PRODUCING DEPLETION OF BIOGENIC AMINES

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DRUGS PRODUCING DEPLETION OF BIOGENIC AMINES

1. DRUGS PRODUCING DEPLETION BY SPECIFIC INHIBITION OF AMINE SYNTHESIS

During the last ten years various inhibitors of monoamine biosynthesis have been described. Spector, Sjoerdsma and Udenfriend (1965) reported that α-methyl-p-tyrosine reduced tissue catecholamine concentrations by specific inhibition of tyrosine hydroxylase, the rate limiting enzyme in the biosynthesis (Nagatsu, Levitt & Udenfriend, 1964). The enzymology of this system has recently been reviewed (Udenfriend, 1966), and so too has the pharmacology of α-methyl-p-tyrosine (αMPT), (Spector, 1966).

Disulfiram, (tetraethylthiuram disulphide), is an inhibitor of dopamine-β-oxidase and as such reduces tissue noradrenaline concentrations (Goldstein, Anagnoste, Lauber & McKereghan, 1964); Hashimoto, Ohi & Imaizumi, 1965; Maj & Przegalinski, 1967 a;b;). The active metabolite of disulfiram, diethyldithiocarbamate, (Goldstein, 1966) is about equipotent with disulfiram as a dopamine-β-oxidase inhibitor (Maj & Przegalinski, 1967 b) and has been shown to deplete central noradrenaline concentrations with no significant effect on dopamine (Carlsson, Fuxe & Hokfelt, 1967).

More recently p-chlorophenylalanine (p-ClPhA) has been reported to be a specific depletor of central and peripheral 5-HT (Koe & Weissman, 1966) and is proposed to produce this effect by inhibition of tryptophan hydroxylase.

(a) α-methyl-p-tyrosine (αMPT)

The effect of αMPT on convulsions is not extensively reported. Weissman, Koe and Tenen (1966) showed αMPT to exert no anticonvulsive effect against leptazol, strychnine or fluorotyrosine, the latter a chemically related convulsant.

Since other synthesis blocking agents were to be investigated, αMPT was reinvestigated for its effects on leptazol convulsions in rats and for its effects on central amine concentrations. Rats were pretreated with αMPT 100mg/kg intraperitoneally and subjected to a leptazol challenge (65mg/kg subcutaneously) 4 hours later. The results are shown in Table 19. Catecholamine and 5-HT concentrations were determined in rat whole brain after 4 hours pretreatment with this same dose of αMPT. The results are shown in Table 20.

αMPT produced a significant decrease in both central noradrenaline and dopamine concentrations with no effect on 5-HT concentrations. Noradrenaline was depleted to 50% of control values ($p < 0.001$) and dopamine to 30% of control values ($p < 0.001$). This depletion was without any significant effect on leptazol induced convulsions (Fig. 25) and is therefore in agreement with the result of Weissman, Koe and Tenen (1966). These results do however show that an intact noradrenaline synthesis is not necessary for leptazol to exert its convulsive effect.

(b) Sodium diethyldithiocarbamate (DDC)

Reports from one group of workers on the effect of disulfiram on leptazol convulsions are confusing. Maj and Przegalinski (1967, b) reported that disulfiram is without effect on leptazol convulsions in mice, but then later (Maj, Przegalinski & Wielosz, 1968) claimed that disulfiram potentiates subconvulsive doses of leptazol.

The active metabolite, DDC, was investigated for its effects on leptazol in rats. Rats were treated with DDC 400mg/kg intraperitoneally for 4 hours and then subjected to a leptazol challenge (65mg/kg subcutaneously). The results are shown in Table 19. Central biogenic amine concentrations were determined in rat brain after this pretreatment with DDC (Table 21). DDC reduced noradrenaline concentrations to 43% of control concentrations ($p < 0.001$) and elevated dopamine to 124% of control concentrations ($p < 0.005$) (Fig. 26). These changes in biogenic amine concentrations produced no effect on susceptibility to leptazol seizures.

This result indicates that an intact noradrenaline synthesis is not necessary for leptazol to exert its convulsive effect. It is, however, possible that the excess dopamine available as a consequence of dopamine- β -oxidase inhibition, could mask the effects of noradrenaline depletion by replacing noradrenaline at the receptor site. Since noradrenaline was depleted by 57% and dopamine elevated by only 24% such a replacement is unlikely.

(c) p-Chlorophenylalanine (p-ClPhA)

p-ClPhA has been shown to specifically deplete 5-HT concentrations (Koe & Weissman, 1966), but no report of the activity against leptazol was made. Another 5-HT depleting agent, p-chloroamphetamine (Fuller, Hines & Mills, 1965), has been shown to be anticonvulsant against leptazol in rats and mice (Pfeifer & Galambos, 1967,b), but these workers concluded that this anticonvulsive effect is not due to changes in brain 5-HT concentrations but to direct stimulation of noradrenergic receptors.

We have investigated the effects of p-chlorophenylalanine on leptazol convulsions. Rats were pretreated with p-ClPhA (320mg/kg, intraperitoneally) for 3 days (Koe & Weissman, 1966) and then subjected to a leptazol challenge (65mg/kg subcutaneously). The effect on clonic convulsions is shown in Table 19. Central biogenic amine concentrations were determined after this same pretreatment with p-ClPhA; the results are shown in Table 22. p-ClPhA produced no effect on leptazol convulsions but reduced central 5-HT concentrations to 30% of control concentrations ($p < 0.001$) and dopamine to 86% of control concentrations ($p < 0.01$). These results confirm those of Pfeifer and Galambos (1967 b) that depletion of central 5-HT is without effect on leptazol convulsions. Our results also show that an intact 5-HT synthesis is not necessary for leptazol to exert its convulsant effect.

(d) p-ClPhA and αMPT

No significant effect on leptazol convulsions was produced after specifically blocking 5-HT or catecholamine syntheses, consequently the effect of blocking the synthesis of 5-HT and catecholamines was investigated.

Rats were pretreated with p-ClPhA (320mg/kg, intraperitoneally) for 3 days and then with αMPT (100mg/kg, intraperitoneally) 4 hours before a leptazol challenge (65mg/kg, subcutaneously). The effect on leptazol convulsions is shown in Table 19. No significant effect was produced. Catecholamine and 5-HT concentrations after this pretreatment were assumed to be the resultant of administering the two drugs individually, in which case whole brain amine concentrations as compared to controls were:- 5-HT - 30%, noradrenaline - 50% and dopamine - 30% (Table 20.) Such an extensive depletion was without effect on leptazol convulsions and the result demonstrates that decreased amine synthesis has no effect on the convulsive mechanism.

2. DRUGS PRODUCING DEPLETION BY INTERFERENCE WITH AMINE STORAGE MECHANISMS

The uptake of catecholamines in tissues has been the subject of numerous studies. Two levels of uptake are now thought to exist in adrenergic nerves: one at the cell membrane which is insensitive to reserpine (Hamberger, Malmfors, Norberg & Sachs, 1964; Carlsson, 1966) and a second in the amine granules, by means of a Mg^{++}-ATP dependent and reserpine-sensitive uptake-storage mechanisms (Carlsson, Hillarp & Waldeck, 1963; Dahlstrom, Fuxe & Hillarp, 1965). A reserpine-

resistant uptake mechanism has also been demonstrated at the amine granule level (Malmfors, 1965; Hamberger & Malmfors, 1967). Therefore, reserpine acts at the amine granule level to prevent storage of amines. As a consequence the synthesis of noradrenaline is also blocked, for reserpine prevents the storage of dopamine in the amine granules which contain dopamine-β-oxidase (Weiner & Rutledge, 1966; Roth & Stone, 1968). The overall effect of reserpine is therefore complex but essentially it prevents storage of amines in the amine granules.

α-Methyldopa and α-methyl-m-tyrosine (αMMT) lower tissue noradrenaline concentrations by a similar mechanism. These amino acids are metabolised to amine products which are active noradrenaline releasers (Porter, Totaro & Leiby, 1961; Carlsson & Lindqvist, 1962; Udenfriend & Zaltzman-Nirenberg, 1962). It has been shown that metaraminol, the metabolite of αMMT, is taken up into the amine granules first by the reserpine-resistant mechanism and thereafter transferred to the reserpine-sensitive pool of the granules (Lundborg & Stitzel, 1967) where it replaces noradrenaline stoichiometrically (Shore, Busfield & Alpers, 1964).

(a) Reserpine

Reserpine prevents storage of amines in the intracellular amine granules and as a consequence there is a long lasting depletion of catecholamines and 5-HT (Holzbauer & Vogt, 1956; Carlsson, Rosengren, Bertler & Nilsson, 1957), which occurs both peripherally and centrally (Carlsson, 1964). Depletion is complete within 24 hours and tissue amine levels return to normal only after 7-14 days (Carlsson, Rosengren & others, 1957).

The effect of reserpine on leptazol convulsions has been investigated previously and in nearly all cases it was shown to potentiate leptazol convulsions in mice (Jenney, 1954; Chen & Bohner, 1956; Kobinger, 1958; Lessin & Parkes, 1959; Weiss, Nelson & Tye, 1960; Pfeifer & Galambos, 1967 a;b; Jones & Roberts, 1968), and in rabbits (Reuse, 1960). In one report reserpine was said to have no effect in mice (Bastian, 1961). Where reserpine was proconvulsant it was assumed that depletion of biogenic amines was the proconvulsant factor since pretreatment with MAO inhibitors abolished reserpine potentiation (Chen & Bohner, 1956; Lessin & Parkes, 1959) or the combination was anticonvulsant (Pfeifer & Galambos, 1967a; Weiss et al, 1960). Lessin and Parkes (1959) correlated the proconvulsant action with the time course of 5-HT depletion but Chen, Ensor and Bohner (1954) showed the proconvulsant time course to be the same as that of noradrenaline depletion.

Reserpine was first briefly investigated in a conventional manner. Rats were pretreated with reserpine (5mg/kg, intraperitoneally) and then challenged with leptazol (65mg/kg, subcutaneously) at various times. The result was predictable in that reserpine was found to be proconvulsant. Only one group of five rats was used at each pretreatment time: the results are shown in Table 23 and Figure 28. After 6 hours there was a marked potentiation of leptazol which reached a maximum after 18 hours and thereafter decreased until control levels were reached after 4 days. Amine concentrations were not determined at this dose level but it would appear that susceptibility to leptazol-induced seizures after reserpine follows a similar time course to that of biogenic amine depletion. This result correlates with the time course of amine depletion described by Carlsson, Rosengren and

others (1957) and has been demonstrated previously by other workers (Chen, Ensor & Bohner, 1954; Lessin & Parkes, 1959). During the course of these experiments it became obvious that the rats were severely reserpinised, for they were totally sedated, and suffered severe diarrhoea and maximal ptosis. This was considered to be a supramaximal effect, and, as such, of no use for testing the effect of other drugs on this reserpine potentiation. Such severe reserpinisation also made it impossible to establish any definite correlates with aminergic processes, because at this dose reserpine is probably non-specific. A more suitable dose of reserpine was sought by administering smaller doses of reserpine intravenously. Rats were pretreated for 4 hours with various doses of reserpine intravenously and challenged with leptazol (65mg/kg, subcutaneously). The effects on leptazol convulsions are shown in Table 24. It is evident that doses of reserpine up to and including 200 μg/kg were without a significant effect on leptazol convulsions, but 400 μg/kg produced a significant potentiation of leptazol. This "all or none" response suggested that a threshold level of some kind had to be reached for reserpine to potentiate leptazol. It was conceived that either a certain level of amine depletion was necessary to potentiate leptazol or that reserpine might exert a differential effect centrally and peripherally. In the latter possibility 200 μg/kg might only deplete peripherally and 400 μg/kg might deplete centrally as well and therefore be the proconvulsant factor. Consequently the effects of these two doses on central and peripheral (cardiac) amine concentrations were investigated (Table 25 and Figure 29). Although 200 μg/kg did not potentiate leptazol, central noradrenaline was depleted to 27% of control concentrations ($p < 0.001$), dopamine to 55% of

control concentrations ($p < 0.001$), 5-HT to 62% of control concentrations ($p < 0.001$) and cardiac noradrenaline to 64% of control concentrations ($p < 0.001$). The result with central noradrenaline is particularly interesting for it implies that such an extensive depletion of central noradrenaline does not produce a potentiation of leptazol convulsions. After 400 μg/kg reserpine, the concentrations of central noradrenaline, dopamine and cardiac noradrenaline were depleted to levels beyond the limits of sensitivity of assay. Central 5-HT was depleted to 29% of control concentrations ($p < 0.001$). This dose produced a significant potentiation of leptazol. There are numerous possible conclusions:-

For reserpine to potentiate leptazol one of the following conditions must be complied with:

(i) Total depletion of central noradrenaline. (73% depletion was not proconvulsant).

or (ii) A depletion of central dopamine greater than 45% is necessary.

or (iii) Total depletion of central dopamine.

or (iv) A depletion of central 5-HT greater than 38%.

or (v) A depletion of cardiac noradrenaline greater than 36%.

or (vi) All these conditions must be satisfied.

It is unlikely that (v) by itself can be the proconvulsant factor because leptazol is a centrally acting convulsant (for references see Hahn (1960): it could nevertheless be a contributory factor. The extent of peripheral involvement has been investigated further (see Syrosingopine).

(b) Reserpine and MAO inhibitors

Reserpine has been shown to potentiate leptazol. This potentiation followed by a similar time course to that of biogenic amine depletion described by Carlsson, Rosengren and others (1957). We have also shown MAO inhibitors are without any anticonvulsant effect on leptazol. It was therefore pertinent to investigate the combination of MAO inhibitor and reserpine for its effect on leptazol convulsions. Rats were pretreated with tranylcypromine (2.5mg/kg, intraperitoneally) and 2 hours later with reserpine (400 µg/kg, intravenously). Four hours later the rats received leptazol (65mg/kg, subcutaneously) and were observed for the effects on clonic convulsions (Table 26.) Tranylcypromine pretreatment prevented the reserpine induced potentiation of leptazol but was not anticonvulsant when compared with the leptazol controls. Similar results have been reported by Lessin and Parkes (1959), Reuse (1960) and Chen and Bohner (1961), but Pfeifer and Galambos (1967 a) report a similar combination to be anticonvulsant. Our results show that depletion of central biogenic amines was a proconvulsant factor and by preventing this depletion with a MAO inhibitor the potentiation of leptazol was blocked. It is also evident that this proconvulsant mechanism does not have an opposite mechanism producing an anticonvulsant action for MAO inhibitors increase amine concentrations but have no anticonvulsant effect.

(c) Reserpine and amine precursors

Reserpine (400 μg/kg, intravenously) has been shown to deplete central noradrenaline, dopamine and 5-HT to very low concentrations (Fig. 29) and to potentiate leptazol, but pretreatment with a MAO inhibitor, which elevates all three amines, prevents this potentiation (Table 26). An attempt was made to replace these three amines selectively to determine if one or all three amines were involved in this proconvulsant action, or conversely for the prevention of this potentiation.

Rats were pretreated with reserpine (400 μg/kg, intravenously) and 3 hours later with either DL-5-HTP (50mg/kg, intraperitoneally) or DL-DOPA (100mg/kg, intraperitoneally) followed 1 hour later by a leptazol challenge (65mg/kg, subcutaneously. The effects on clonic convulsions are shown in Table 27 and Figure 30. DL-5-HTP and DL-DOPA have been shown to produce no effect on leptazol convulsions in intact animals (Chapter 3 - Amine precursors) even though central amines were selectively elevated. In the present section of the work DL-5-HTP was shown to prevent reserpine induced potentiation of leptazol ($p < 0.001$) and so was DL-DOPA ($p < 0.005$). However DL-5-HTP was far more effective for it maintained the level of clonic convulsions at control levels, whereas although DL-DOPA reduced the proconvulsant effect, there remained a significantly proconvulsant effect, ($p < 0.01$). Thus it appeared that depletion of 5-HT stores was the proconvulsant factor. The effect of reserpine and amine precursor combination on central amine concentrations was investigated (Table 28 and Figure 30). As

expected DL-5-HTP selectively repleted 5-HT stores to control level after reserpine pretreatment. This correlated with reversal of reserpine-induced potentiation of leptazol. DL-DOPA only produced a small selective repletion of dopamine stores with no effect on noradrenaline stores, but nevertheless produced a 50% reduction in the reserpine-induced potentiation of leptazol. This indicates that if dopamine stores were to be fully repleted after reserpine it, too, might totally prevent the potentiation of leptazol. Thus repletion of only one amine store is necessary to prevent the proconvulsant effect of reserpine. In this work DL-5-HTP has been found to be the most effective treatment for it causes an accumulation of central 5-HT, whereas DL-DOPA does not produce such an effective accumulation of dopamine or noradrenaline.

(d) Reserpine and dexamphetamine

Having shown that repletion of amines prevents the proconvulsant activity of reserpine it was considered pertinent to investigate the effects of a sympathomimetic amine on this potentiation by reserpine. Dexamphetamine has already been demonstrated to be proconvulsant (Chapter 3). It is interesting that two drugs (reserpine and dexamphetamine) with opposite mechanisms should both be proconvulsant, and it was thought that together they might have no effect. Rats were pretreated with reserpine (400 μg/kg, intravenously) and $3\frac{1}{2}$ hours later given dexamphetamine (5mg/kg, intraperitoneally) and 30 minutes later a leptazol challenge (65mg/kg, subcutaneously). The effects on clonic convulsions are shown in Table 29. Reserpine or dexamphetamine alone were proconvulsant, so too was the reserpine/dexamphetamine combination. However, this combination was not synergistic for the proconvulsant activity was only equivalent to

that of either reserpine or dexamphetamine and not the sum of both. Dexamphetamine was not an effective anticonvulsant after reserpine, as was endogenous 5-HT or dopamine. It is possible that reserpine prevented the uptake of dexamphetamine which would block any direct or indirect action on adrenergic receptors and therefore any possible anticonvulsant action. This must be considered as a real possibility since the reserpine and dexamphetamine were not synergistic and it could therefore be argued that reserpine prevented dexamphetamine from exerting its proconvulsant action, and that only the proconvulsant effect of reserpine was displayed.

Consequently, although this combination has been shown to be anticonvulsant to electroshock (Jurna & Regelhy, 1968), it was not possible from our brief investigation with leptazol to support or to refute this previous result with certainty.

(e) Syrosingopine

During the investigation of reserpine it was found that a peripheral depletion of (cardiac) noradrenaline could be correlated with the proconvulsant activity. It was considered extremely unlikely that this was the sole proconvulsant factor but that it might contribute to the proconvulsant mechanism. For this reason the effects of syrosingopine on leptazol convulsions were investigated.

Syrosingopine, a synthetic analogue of reserpine, has been demonstrated to deplete only peripheral aminergic stores (Plummer, Barrell & others, 1959; Brodie, 1960; Orlans, Finger & Brodie, (1960). Maximum depletion occurs after 4 hours pretreatment (Orlans, Finger & Brodie, (1960). In this investigation rats were

pretreated for 4 hours with doses of syrosingopine from 100 μg/kg to 2 mg/kg intraveously, and then subjected to a leptazol challenge (65mg/kg, subcutaneously). The results on clonic convulsions are shown in Table 30. Doses up to and including 800 μg/kg intravenously were without effect on leptazol convulsions and were assumed to be peripheral depleting doses. A dose of 2mg/kg intravenously produced a marked potentiation of leptazol from 52% to 100% and this was thought to be a dose which would deplete centrally. This hypothesis was tested by measuring central and peripheral (cardiac) amine levels after syrosingopine (800 μg/kg and 2mg/kg, intravenously). The results are shown in Table 31 and Figure 31. In a dose of 800 μg/kg syrosingopine produced complete depletion of cardiac noradrenaline and central noradrenaline and dopamine with no effect on leptazol convulsions. Central 5-HT was only depleted to 70% ($p < 0.001$) of control concentrations. At a dose level of 2mg/kg there was significant proconvulsant activity ($p < 0.001$) and a depletion of central 5-HT concentrations to 50% of control levels ($p < 0.001$). The only difference between the ineffective dose and the proconvulsant dose of syrosingopine was that the latter was a more effective 5-HT depleting dose.

These results allow us to rule out the possibility of a peripheral adrenergic contribution to the leptazol convulsive mechanism, because 800 μg/kg was without effect on leptazol but produced total depletion of cardiac noradrenaline. It is also evident that at these doses syrosingopine is not a specific peripheral depleting agent. It does, however, have possibilities

as a specific noradrenaline and dopamine depleting agent for it is relatively ineffective against central 5-HT. A smaller dose (400 μg/kg) might totally deplete noradrenaline and dopamine whilst leaving 5-HT intact: it would be worthwhile investigating this as a possible pharmacological tool. This work also serves to confirm our earlier results with reserpine and reduce the number of possible convulsive mechanisms discussed (see Reserpine, (a)) to one: that a total depletion of central noradrenaline and dopamine is not sufficient to potentiate leptazol unless a depletion of 5-HT greater than 38% is achieved.

(f) α-Methyl-m-tyrosine (αMMT)

αMMT depletes aminergic stores both centrally and peripherally (Carlsson, 1964; Carlsson & Lindqvist, 1967) but is selective in that 5-HT stores are unaffected (Carlsson & Lindqvist, 1967). It is metabolised to metaraminol which replaces noradrenaline stoichiometrically (Shore, Busfield & Alpers, 1964). Carlsson and Lindqvist (1962) postulated that lack of behavioural effects after depletion with αMMT can be explained by assuming that the metabolite, metaraminol, is a "false transmitter" and as such can act on noradrenergic receptors and mask the effects of depletion.

The effect of this type of depletion was investigated on leptazol convulsions. Rats were pretreated with αMMT (100mg/kg, intraperitoneally) for 4 hours and then subjected to a leptazol challenge (65mg/kg, subcutaneously). The effect on leptazol convulsions is shown in Table 32 and Figure 32. No significant effect was observed. Central amine concentrations were determined after 4 hours pretreatment with this dose of

αMMT (Table 33 and Figure 32). Both noradrenaline and dopamine were depleted significantly: noradrenaline was depleted to 22% of control concentrations ($p < 0.001$) and dopamine to 20% of control concentrations ($p < 0.001$). 5-HT was only depleted to 82% of control concentrations ($p < 0.01$). Therefore, even after this depletion no proconvulsant activity was observed. It can be argued that metaraminol, the "false transmitter" and metabolite of α MMT, is masking the effects of depletion of noradrenaline and dopamine (Carlsson & Lindqvist, 1962). However, in later work with αMMT (Chapter 7 - Chlordiazepoxide), we have shown this is not the case. We have also shown earlier that a similar effect on control amines by syrosingopine is without proconvulsant activity until a significant depletion of 5-HT is produced. Therefore, this result with αMMT, is taken as confirmation of our earlier results that as long as one amine store, whether it be noradrenaline, dopamine or 5-HT, is intact no proconvulsant activity will be produced.

(g) p- ClPhA and αMMT

A method of depleting all three amines, 5-HT, noradrenaline and dopamine, significantly was investigated. Rats were pretreated with p-ClPhA (320mg/kg, intraperitoneally) for 3 days and then 4 hours before the leptazol challenge (65mg/kg, subcutaneously) with αMMT (100mg/kg, intraperitoneally). The effect on leptazol convulsions is shown in Table 34. Although individually p-ClPhA (Table 19) and αMMT (Table 32) produce no significant effect on leptazol convulsions, when both are administered to the same rats (Table 34), there is a marked potentiation ($p < 0.001$) of

leptazol convulsions. Amine determinations, after this combination, were not made but it was assumed that the net effect on whole brain amines would be the same as administration of the drugs individually. If so, whole brain amines, as compared to controls, were 5-HT 30% (Table 22), noradrenaline 22% and dopamine 20% (Table 33). This confirms our earlier conclusions that extensive depletion of all three amines is necessary before a significant potentiation of leptazol occurs after such treatment.

3. DISCUSSION

Specific depletion of both noradrenaline and dopamine, or noradrenaline alone or 5-HT alone by specific inhibition of synthesis with αMPT, DDC or pClPhA respectively was without effect on the convulsive threshold of leptazol in rats. Each of the synthesis inhibitors reduced the respective whole brain amine concentration by at least 50%. When αMPT and pClPhA were administered to the same group to rats so as to block the syntheses of noradrenaline, dopamine and 5-HT simultaneously, this was also without effect on leptazol threshold. This evidence implies that neither the synthesis of noradrenaline and dopamine, nor the synthesis of 5-HT, is involved in the convulsive mechanism of leptazol.

Depletion of these amines by interference with storage mechanisms produced more positive effects on leptazol convulsions in rats. Reserpine is consistently reported to potentiate leptazol convulsions; only one report, claiming reserpine to be without effect on convulsions, (Bastian, 1961) was found in a search of the literature. In our initial investigation a dose of 5mgm/kg reserpine was used. This produced a long lasting potentiation of leptazol convulsions which

correlated with the time course of catecholamine depletion reported by Carlsson, Rosengren and others (1957). At this dose level the rats were severely "reserpinised, suffering extreme diarrhoea, ptosis and loss of weight overnight: these conditions were considered unfavourable for investigating leptazol convulsions for it was possible that this stress alone might render the animal more susceptible to leptazol and that non-specific effects were likely to be produced by reserpine at this high dose. Using smaller doses of reserpine, administered intravenously, we were able to show that a dose of 200 μg/kg, (which produced at least 40% depletion of noradrenaline, dopamine and 5-HT simultaneously), was without effect on leptazol threshold. A dose of 400 μg/kg produced a marked potentiation of leptazol convulsions, accompanied by a depletion of central noradrenaline and dopamine, and cardiac noradrenaline to levels below the sensitivity of the assay, and central 5-HT to 29% of control concentrations. A similar investigation with syrosingopine demonstrated that a dose of 800 μg/kg, intravenously, produced complete depletion of central noradrenaline and dopamine, and cardiac noradrenaline and 30% depletion of central 5-HT, without effect on leptazol convulsions, but that 2mg/kg syrosingopine markedly potentiated leptazol convulsions. The only difference in brain amine concentrations was that central 5-H T was further depleted to 50% of control levels. When these two results are compared, it appears that in order to potentiate leptazol either central 5-HT must be depleted to at least 50% of control concentrations or that all three amines must be simultaneously depleted to below 50% of control concentrations. The latter is more likely because specific depletion of 5-HT with pClPhA was without effect on the convulsive threshold. The results with amine precursors

after reserpine, support this conculsion: for selective repletion of 5-HT to control concentrations completely abolished the proconvulsant activity of reserpine. Selective repletion of dopamine, to 24% of control concentrations, was sufficient to reduce the proconvulsant effect of reserpine by 50%. It is concluded that if two of these three amine stores are severely depleted then any proconvulsant activity is controlled by the last 50-60% of the remaining amine store.

An attempt to load the catecholamine stores with dexamphetamine, to mimick repletion of catecholamines and perhaps reveal an anti-convulsant effect by dexamphetamine instead of the normal proconvulsant effect, was unsuccessful. Dexamphetamine was not anticonvulsant under these conditions nor was it proconvulsant. It is possible that reserpine prevented the uptake of dexamphetamine: no measurement of central dexamphetamine concentrations were made.

Depletion of catecholamine stores, to 20% of control concentrations, after αMMT produced no anticonvulsant activity. This might have been explained by the theory of Carlsson and Lindqvist (1962), that metaraminol, the metabolite of αMMT, is an effective false transmitter and masks the effects of depletion. However, αMMT after pClPhA, a combination producing severe depletion of catecholamines and 5-HT simulatenously, was markedly proconvulsant. This negates the above false transmitter theory and also supports the premise that depletion of all three amines is necessary to reveal a proconvulsant activity.

TABLE 19

EFFECT OF α-METHYL-p-TYROSINE (αMPT), DIETHYLDITHIOCARBAMATE (DDC) AND p-CHLOROPHENYLALANINE (p-ClPhA) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Pretreatment	% Maximum Clonic Convulsions
Leptazol 65mg/kg (subcutaneously)	43 ± 3 (8)
αMPT 100mg/kg (intraperitoneally) 4 hours Leptazol 65mg/kg (subcutaneously)	52 ± 5 (4)
DDC 400mg/kg (intraperitoneally) 4 hours Leptazol 65mg/kg (subcutaneously)	45 ± 8 (3)
p-ClPhA 320mg/kg (intraperitoneally) 3 days Leptazol 65mg/kg (subcutaneously)	50 ± 5 (2)
p-ClPhA 320mg/kg (intraperitoneally) 3 days αMPT 100mg/kg (intraperitoneally) 4 hours Leptazol 65mg/kg (subcutaneously)	50 ± 7 (2)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

TABLE 20

EFFECT OF 4 HOURS PRETREATMENT WITH α-METHYL-p-TYROSINE (α MPT 100mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINES AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Saline controls (4 hours)	217 ± 13 (9)	657 ± 65 (4)	509 ± 19 (5)
α MPT 100mg/kg intraperitoneally (4 hours)	109 ± 5 (9) ***	198 ± 14 (5) ***	516 ± 22 (5)

Results are expressed as group mean ± standard error.

ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

TABLE 21

EFFECT OF 4 HOURS PRETREATMENT WITH DIETHYLDITHIOCARBAMATE (DDC, 400mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Saline controls (4 hours)	239 ± 10 (13)	649 ± 6 (5)	520 ± 12 (20)
DDC 400mg/kg intraperitoneally (4 hours)	101 ± 8 (17) ***	800 ± 32 (5) **	524 ± 9 (30)

Results are expressed as group mean ± standard error.

ng/g = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

TABLE 22

EFFECT OF 3 DAYS PRETREATMENT WITH p-CHLOROPHENYLALANINE (p-ClPhA, 320mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Saline controls (3 days)	268 ± 30 (5)	642 ± 16 (5)	550 ± 24 (5)
p-ClPhA 320mg/kg intraperitoneally (3 days)	311 ± 18 (5)	550 ± 25 (5) **	184 ± 18 (5) ***

Results are expressed as group mean ± standard error.

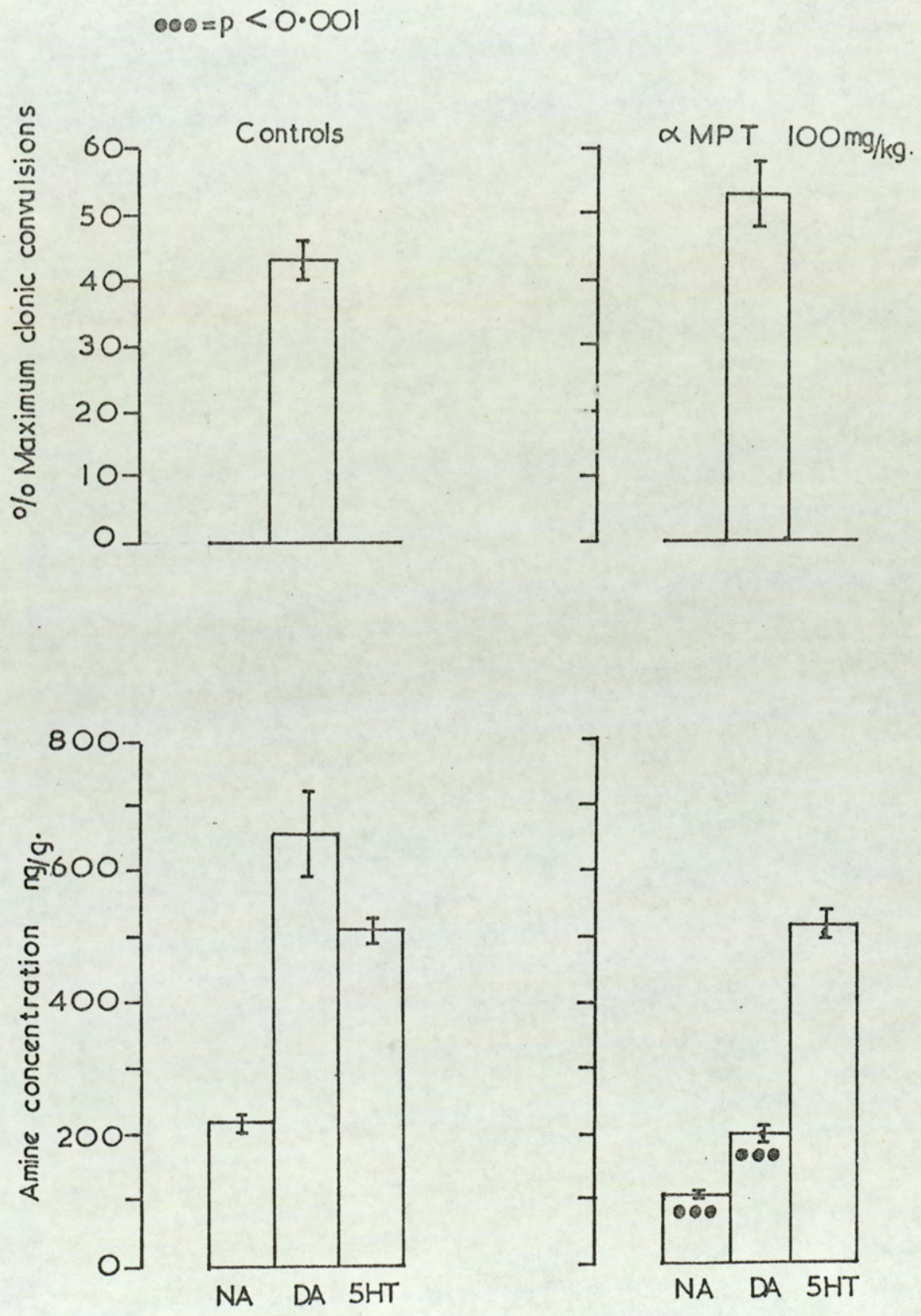
ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 25

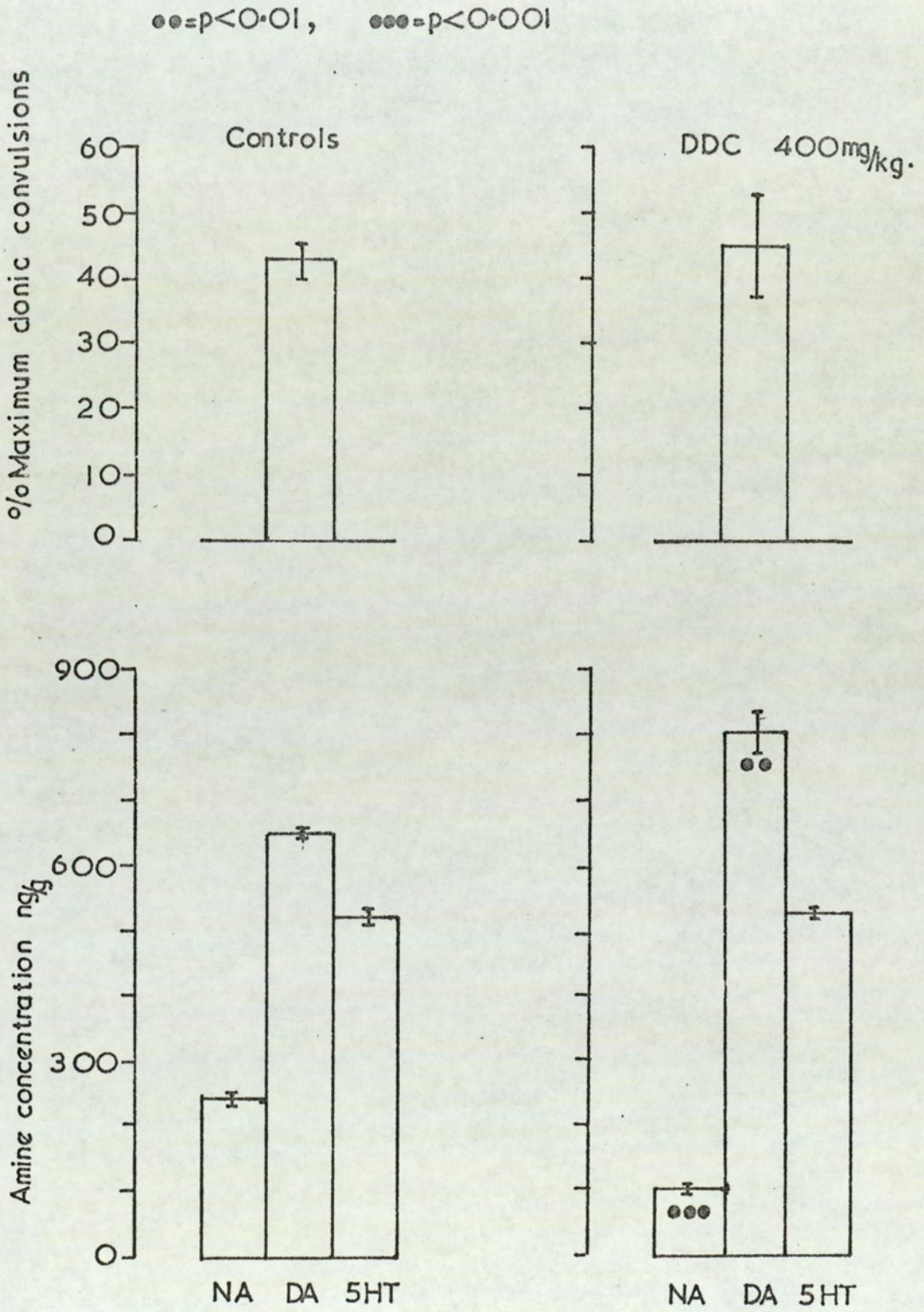
EFFECT OF α-METHYL-p-TYROSINE (α MPT, 100mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS



Vertical bars indicate standard errors of the mean

FIGURE 26

EFFECT OF DIETHYLDITHIOCARBAMATE (DDC, 400mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL 65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

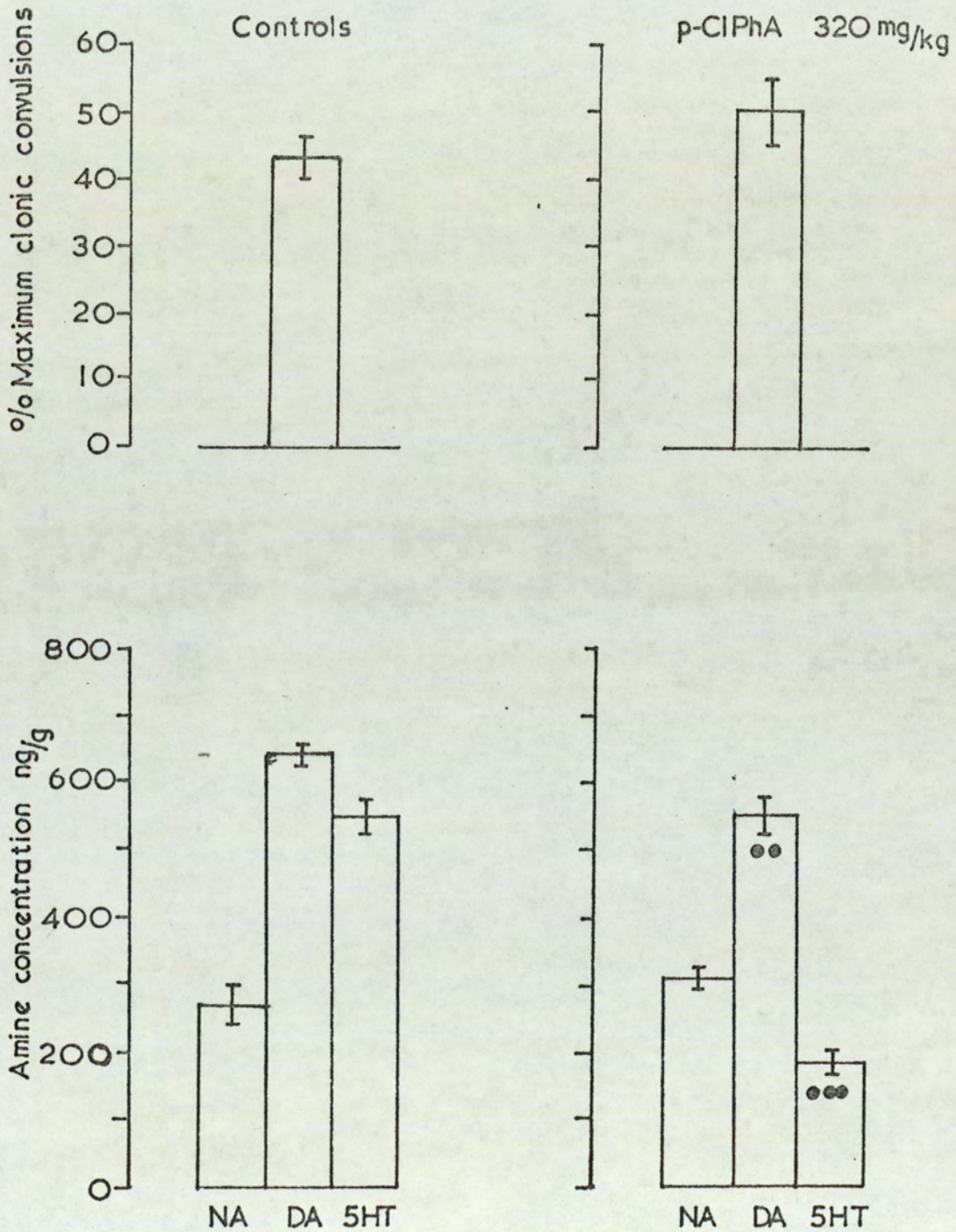


Vertical bars indicate standard errors of the mean

FIGURE 27

EFFECT OF p-CHLOROPHENYLALANINE (pClPhA, 320mg/kg, INTRAPERITONEALLY ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL 65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

●● = $p < 0.01$, ●●● = $p < 0.001$



Vertical bars indicate standard errors of the mean

TABLE 23

EFFECT OF RESERPINE (5mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Reserpine pretreatment time	% Maximum Clonic Convulsions
0 (Vehicle controls)	37.5 ± 2.2 (5)
6 hours	90 (1)
18 hours	100 (1)
24 hours	80 (1)
48 hours	50 (1)
96 hours	30 (1)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

FIGURE 28

EFFECT OF RESERPINE (5mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

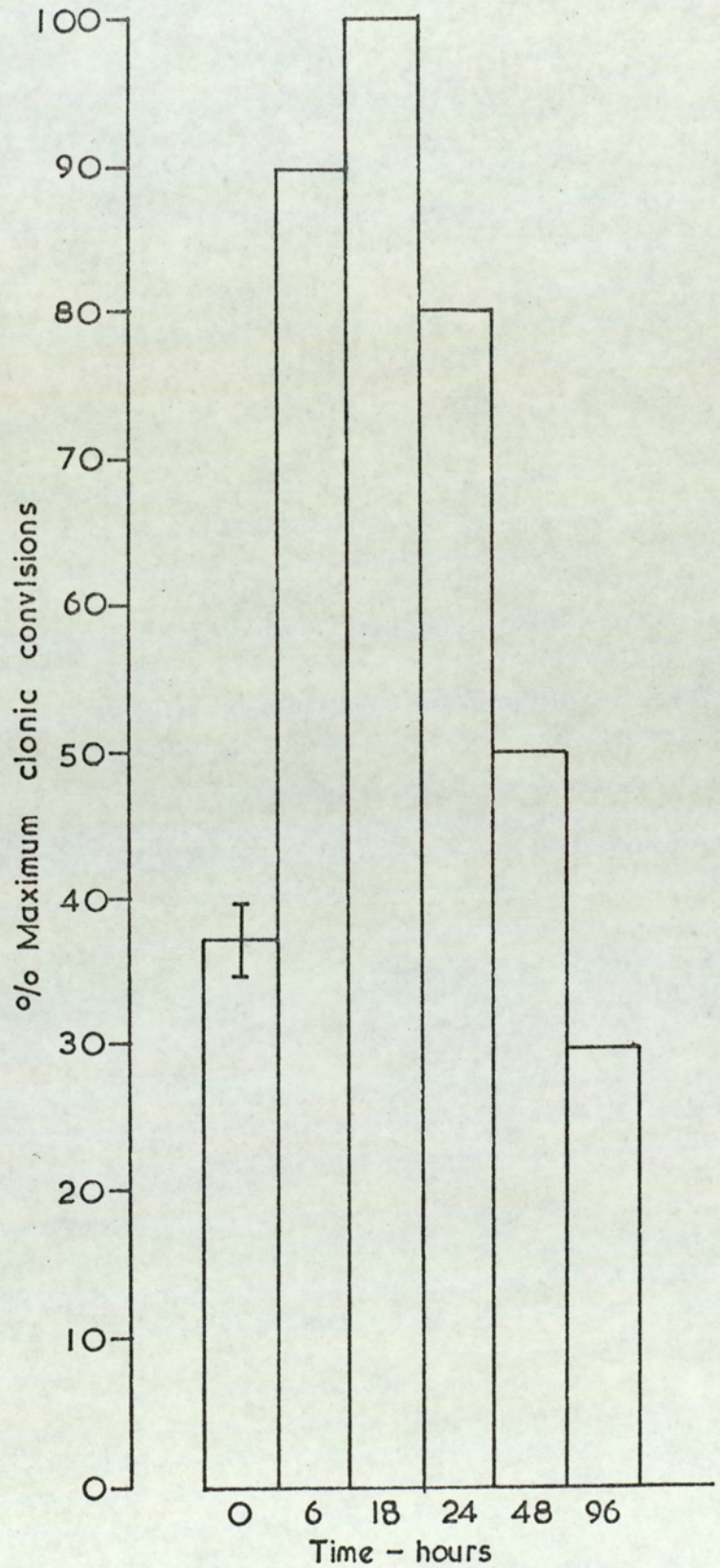


TABLE 24

EFFECT OF 4 HOURS PRETREATMENT WITH RESERPINE (INTRAVENOUSLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Dose of reserpine µg/kg (intravenously)	% Maximum Clonic Convulsions
0 (Vehicle controls)	47 ± 2 (6)
100	55 ± 3 (2)
200	40 ± 7 (5)
400	79 ± 2 (9)

Results are expressed as group mean ± standard error.

Figures in parenthese indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

TABLE 25

EFFECT OF 4 HOURS PRETREATMENT WITH RESERPINE (INTRAVENOUSLY) ON WHOLE BRAIN AND HEART AMINE CONCENTRATIONS IN RATS

Pretreatment	Brain			Heart
	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g	Noradrenaline ng/g
Vehicle controls	304 ± 17	611 ± 23	571 ± 14	377 ± 17
Reserpine 200 µg/kg (intravenously)	82 ± 8 (6) ***	335 ± 30 (6) ***	354 ± 18 (5) ***	239 ± 30 (5) ***
Reserpine 400 µg/kg (intravenously)	< 40 (5)	< 60 (5)	184 ± 9 (3) ***	< 40 (5)

Results are expressed as group mean ± standard error.

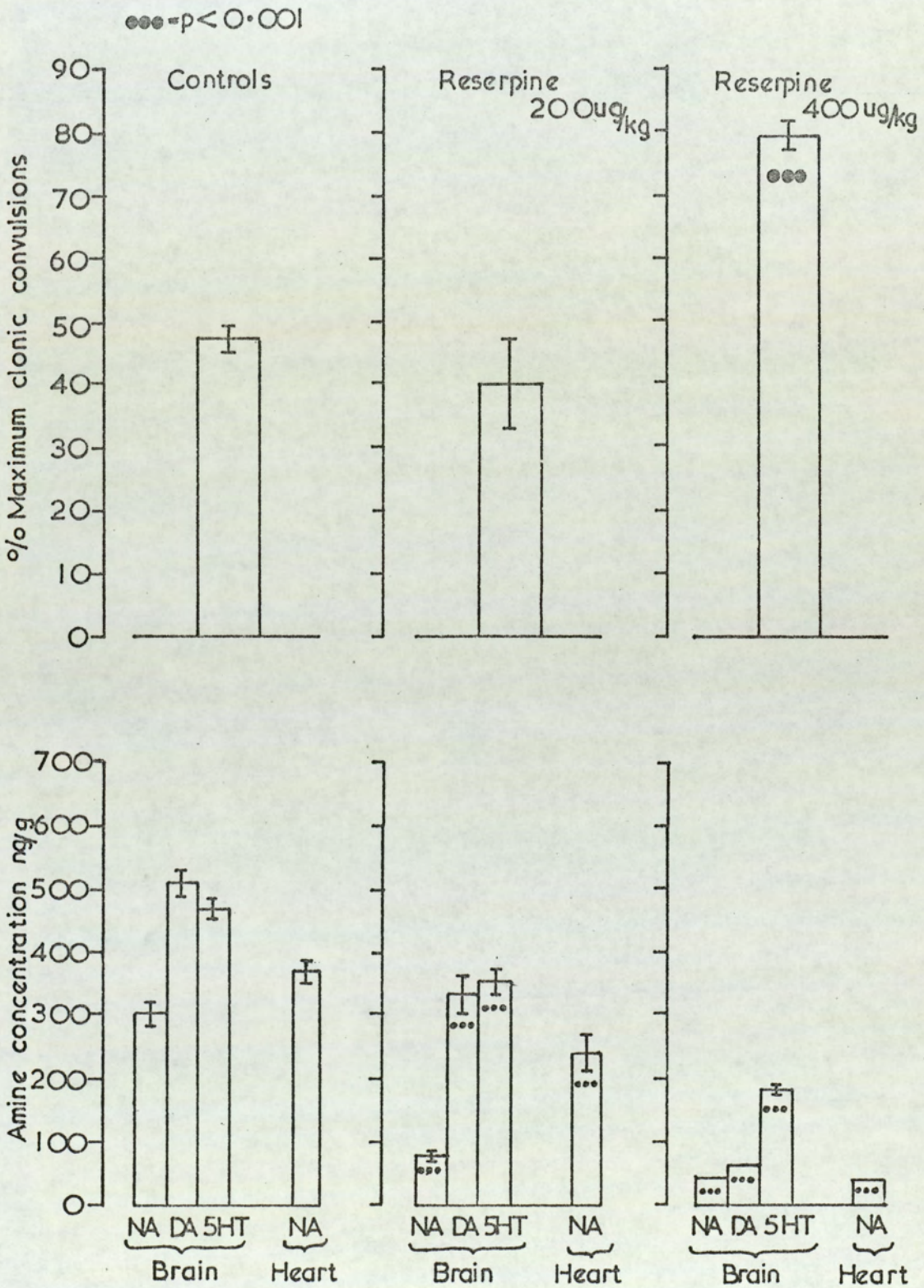
ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 29

EFFECT OF 4 HOURS PRETREATMENT WITH RESERPINE (INTRAVENOUSLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN AND HEART AMINE CONCENTRATIONS IN RATS



Vertical bars indicate standard errors of the mean

TABLE 26

EFFECT OF TRANYLCYPROMINE (2.5mg/kg, INTRAPERITONEALLY ON THE RESERPINE-INDUCED POTENTIATION OF LEPTAZOL CONVULSIONS IN RATS

Pretreatment	% Maximum Clonic Convulsions
Leptazol 65mg/kg (subcutaneously)	52.5 ± 5 (4)
Reserpine 400 µg/kg (intravenously) 4 hours Leptazol 65mg/kg (subcutaneously)	82.5 ± 3 (4) ***
Tranylcypromine 2.5mg/kg (intraperitoneally) 6 hours Leptazol 65mg/kg (subcutaneously)	55 ± 5 (2)
Tranylcypromine 2.5mg/kg (intraperitoneally) 6 hours Reserpine 400 µg/kg (intravenously) 4 hours Leptazol 65mg/kg (subcutaneously)	45 ± 5 (2)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$

*** = $p < 0.001$

TABLE 27

EFFECT OF DL-5HTP (50mg/kg, INTRAPERITONEALLY) AND DL-DOPA (100mg/kg, INTRAPERITONEALLY) ON THE RESERPINE-INDUCED POTENTIATION OF LEPTAZOL CONVULSIONS IN RATS

Pretreatment	% Maximal Clonic Convulsions
Leptazol 65mg/kg (subcutaneously)	43 ± 3 (6)
Reserpine 400 µg/kg (intravenously) 4 hours Leptazol 65mg/kg (subcutaneously)	79 ± 2 (9)
DL-5HTP 50mg/kg (intraperitoneally) 1 hour Leptazol 65mg/kg (subcutaneously	36 ± 7 (4)
DL-DOPA 100mg/kg (intraperitoneally) 1 hour Leptazol 65mg/kg (subcutanesouly)	50 ± 4 (4)
Reserpine 400 µg/kg (intravenously) 4 hours DL-5HTP 50mg/kg (intraperitoneally) 1 hour Leptazol 65mg/kg (subcutaneously)	46 ± 4 (5) ***
Reserpine 400 µg/kg (intravenously) 4 hours DL-DOPA 100mg/kg (intraperitoneally) Leptazol 65mg/kg (subcutaneously)	62 ± 4 (5) **

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

TABLE 28

EFFECT OF RESERPINE AND AMINE PRECURSOR COMBINATIONS ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Controls	304 ± 17 (11)	611 ± 23 (10)	623 ± 20 (12)
Reserpine 400 µg/kg (intravenously) 4 hours	40 (5)	60 (5)	204 ± 9 (3)
Reserpine 400 µg/kg (intravenously) 4 hours DL-5-HTP 50mg/kg (intraperitoneally) 1 hour	40 (4)	88 ± 7 (4)	593 ± 55 (4) ***
Reserpine 400 µg/kg (intravenously) 4 hours DL-DOPA 100mg/kg (intraperitoneally) 1 hour	40 (4)	158 ± 16 (4) ***	Reserpine/DOPA combination interfered with 5-HT assay

Results are expressed as group mean ± standard error.

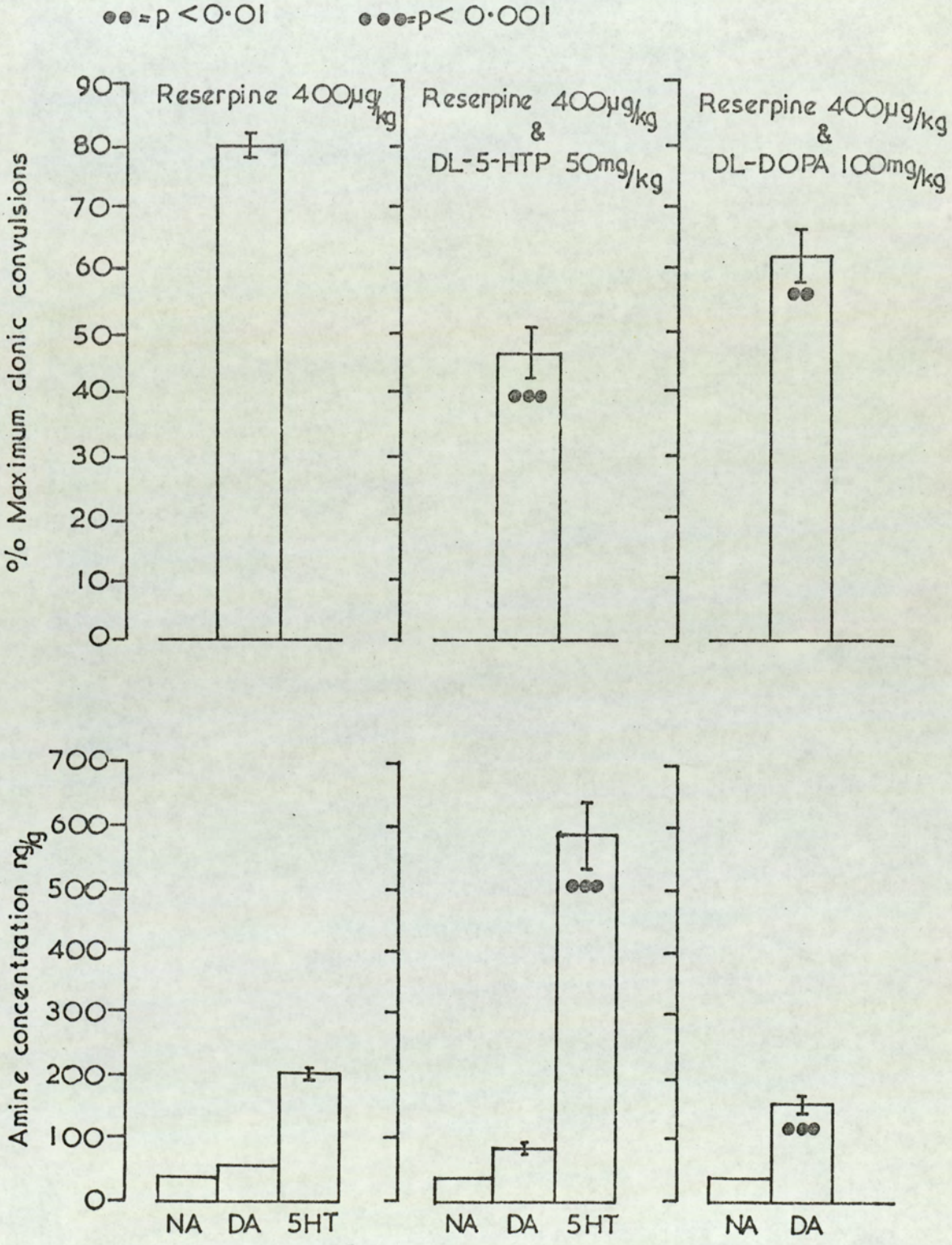
ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 30

EFFECT OF RESERPINE AND AMINE PRECURSOR COMBINATIONS ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS



Vertical bars indicate standard errors of the mean

TABLE 29

EFFECT OF DEXAMPHETAMINE (5mg/kg, INTRAPERITONEALLY) ON RESERPINE-INDUCE POTENTIATION OF LEPTAZOL CONVULSIONS IN RATS

Pretreatment	% Maximum Clonic Convulsions
Leptazol 65mg/kg (subcutaneously)	47 ± 2 (16)
Reserpine 400 µg/kg (subcutaneously) 4 hours Leptazol 65mg/kg (subcutaneously)	79 ± 2 (9)
Dexamphetamine 5mg/kg (intraperitoneally) 30 minutes Leptazol 65mg/kg (subcutaneously	92 ± 3 (9)
Reserpine 400 µg/kg (intravenously) 4 hours Dexamphetamine 5mg/kg (intraperitoneally) 30 minutes Leptazol 65mg/kg (subcutaneously)	85 ± 3 (2)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

TABLE 30

EFFECT OF 4 HOURS PRETREATMENT WITH SYROSINGOPINE (INTRAVENOUSLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Dose of Syrosingopine µg/kg (intravenously)	% Maximum Clonic Convulsions	
Vehicle treated controls	52 ± 4	(5)
100	40	(1)
200	50	(1)
400	40	(1)
800	55 ± 5	(2)
2000	100 ± 0 ***	(2)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ *** = $p < 0.001$

** = $p < 0.01$

TABLE 31

EFFECT OF 4 HOURS PRETREATMENT WITH SYROSINGOPINE (INTRAVENOUSLY) ON WHOLE BRAIN AND HEART AMINE CONCENTRATIONS IN RATS

Pretreatment	Brain			Heart
	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g	Noradrenaline ng/g
Vehicle controls	310 ± 21 (5)	653 ± 20 (5)	618 ± 17 (10)	377 ± 17 (13)
Syrosingopine 800 µg/kg (intravenously) 4 hours	40 (5)	60 (5)	425 ± 10 (5) ***	40 (5)
Syrosingopine 2mg/kg (intravenously) 4 hours	40 (5)	60 (5)	316 ± 21 (5) ***	40 (5)

Results are expressed as group mean ± standard error.

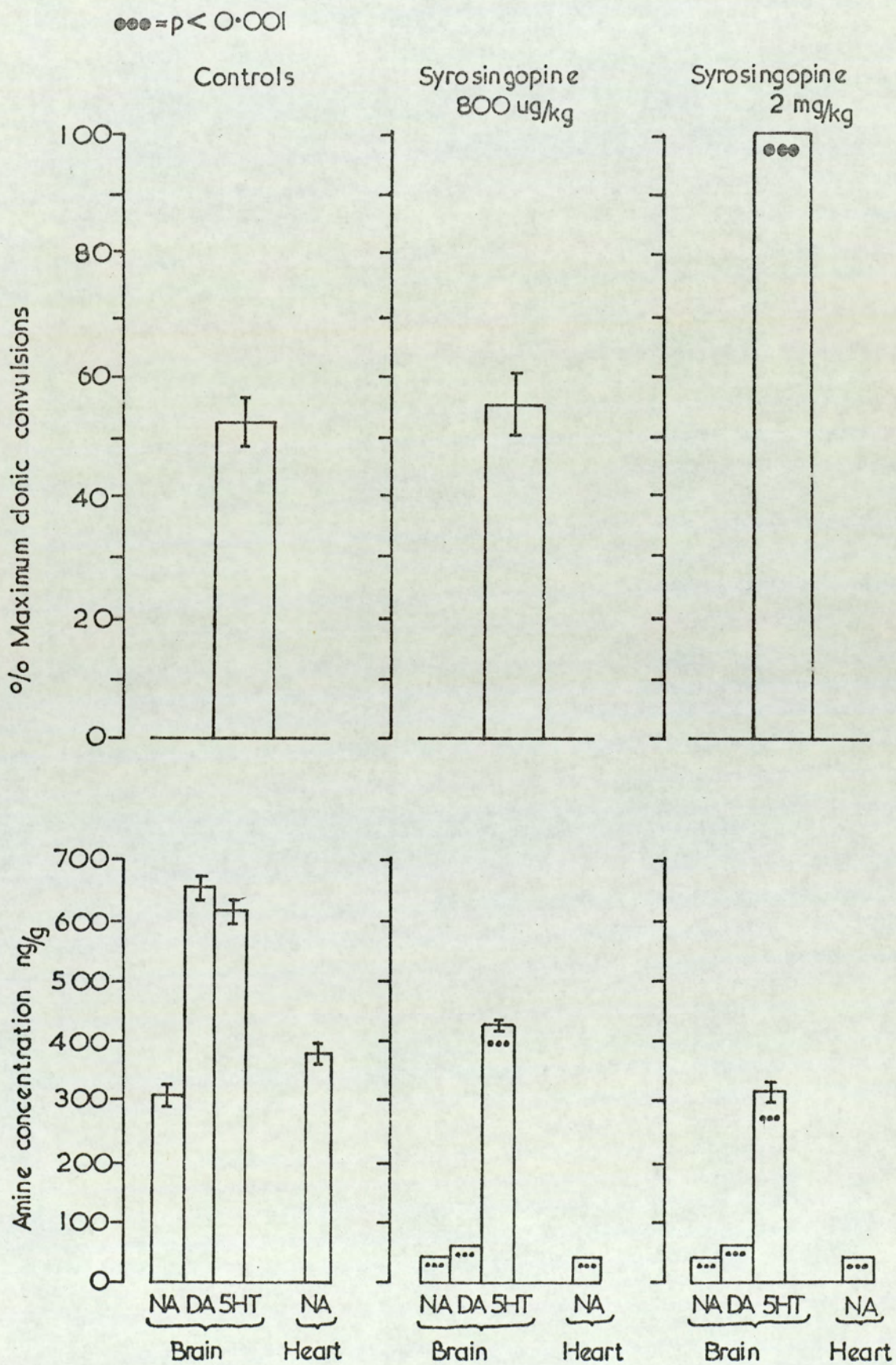
ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 31

EFFECTS OF 4 HOURS PRETREATMENT WITH SYROSINGOPINE (INTRAVENOUSLY) CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS



Vertical bars indicate standard errors of the mean

TABLE 32

EFFECT OF 4 HOURS PRETREATMENT WITH α-METHYL-m-TYROSINE (αMMT, 100mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY

Pretreatment	% Maximum Clonic Convulsions
Leptazol 65mg/kg (subcutaneously)	47 ± 2 (16)
αMMT 100mg/kg (intraperitoneally) 4 hours Leptazol 65mg/kg (subcutaneously)	45 ± 3 (2)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

TABLE 33

EFFECT OF 4 HOURS PRETREATMENT WITH α-METHYL-m-TYROSINE (αMMT, 100mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Controls	283 ± 30 (5)	665 ± 47 (4)	546 ± 22 (5)
αMMT 100mg/kg (intraperitoneally)	62 ± 6 (6) ***	133 ± 12 (6) ***	449 ± 13 (5) **

Results are expressed as group mean ± standard error.

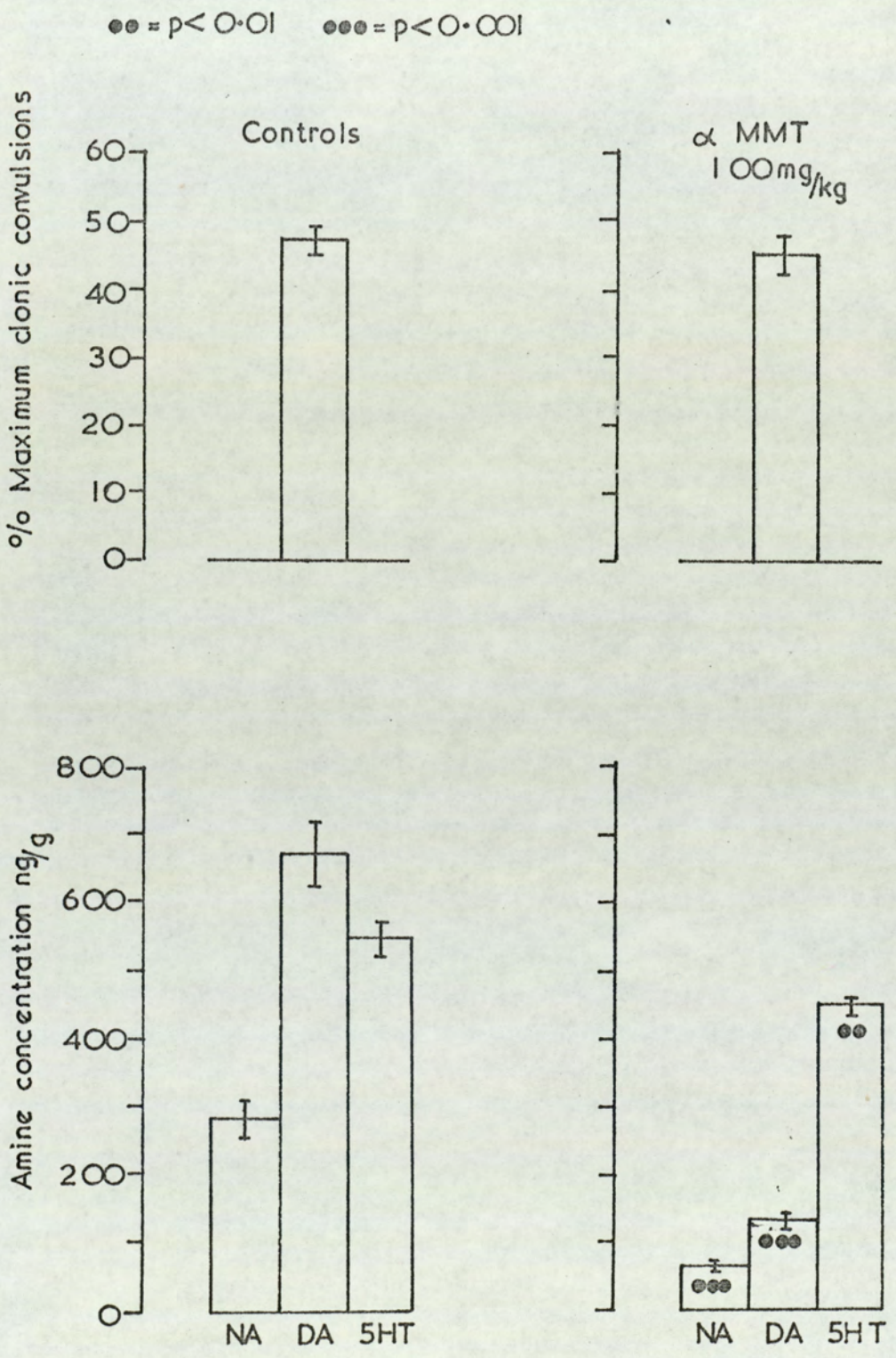
ng = nanogram = 10^{-9} gramme.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 32

EFFECT OF α-METHYL-m-TYROSINE (αMMT, 100mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY) AND ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS



Vertical bars indicate standard errors of the mean

TABLE 34

EFFECT OF p-CHLOROPHENYLALANINE AND α-METHYL-m-TYROSINE (α MMT) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Pretreatment	% Maximum Clonic Convulsions
Leptazol 65mg/kg (subcutaneously)	43 ± 3 (8)
pClPhA 320mg/kg (intraperitoneally) 3 days. α MMT 100mg/kg (intraperitoneally) 4 hours. Leptazol 65mg/kg (subcutaneously).	85 ± 3 (2) ***

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$

*** = $p < 0.001$

CHAPTER FIVE

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DEXAMPHETAMINE AND FENCAMFAMIN

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DEXAMPHETAMINE AND FENCAMFAMIN

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH DEXAMPHETAMINE AND FENCAMFAMIN

Amphetamine is frequently classified as an analeptic with leptazol, picrotoxin, etc., (Hahn, 1960; Bowman, Rand & West, 1968). The mode of action of amphetamine has largely been elucidated in recent years (for references see Introduction) but the mode of action of leptazol remains obscure. Since amphetamine is a sympathomimetic and leptazol has been demonstrated to possess sympathomimetic activity (see Introduction and review by Hahn (1960)), leptazol has now been investigated and compared with amphetamine under similar conditions to those which elucidated the mode of action of amphetamine. This was an attempt to implicate biogenic amines in the convulsant mechanism of leptazol. Fencamfamin also has been investigated under these conditions because, unlike dexamphetamine, it possesses only central stimulant properties with no peripheral activity (Hotovy, Enenkel & others, 1961).

1. DEXAMPHETAMINE

Dexamphetamine, the most active isomer of amphetamine, is a central stimulant in most species. Two mechanisms have been postulated to account for its effects. The first is that dexamphetamine causes a direct stimulation of central receptors (Vane, 1960; Rossum & others, 1962; Smith, 1963). The second and more recent hypothesis is that dexamphetamine releases central catecholamines and thus stimulates central receptors indirectly (Stein, 1964; Rech, 1964; Randrup & Munkvad, 1966). This mechanism has been demonstrated to be dependent on an intact catecholamine synthesis (Weissman & others, 1966; Hanson, 1966; 1967; Dingell & others, 1967). Attempts to determine which catecholamine is involved have implicated noradrenaline (McLean &

McCartney, 1961; Sanan & Vogt, 1962; Moore & Lariviere, 1963; Baird & Lewis, 1964; Carr & Moore, 1969), dopamine (Fog, Randrup & Pakenberg, 1967; McKenzie & Szerb, 1968; Ernst, 1969) or the three amines, noradrenaline, dopamine and 5-HT (Smith, 1965). In all cases a significant release of central amines was not evident in doses of dexamphetamine less than 10mg/kg, but increased locomotor activity and other stimulant effects could be elicited by as little as 3mg/kg (Smith, 1965).

The effect of dexamphetamine on leptazol convulsions has been reported to be proconvulsant (Friebel & Klatt, 1959), without effect (Wolf & Stock, 1966) and anticonvulsant in combination with phenobarbitone (Weaver & Alexander, 1955). Dexamphetamine has also been reported to abolish clinical seizures and abnormal EEG discharges (Goodman & Gilman, 1965). In this project dexamphetamine has already been demonstrated to be markedly proconvulsant (Chapter 3). In this section of the project, this proconvulsant activity was re-investigated after specific inhibition of the syntheses of the three amines studied, and compared with the activity of leptazol under similar conditions.

(a) Dose-response relationship of dexamphetamine-induced proconvulsant activity

Rats were pretreated with dexamphetamine, at various dose levels, 30 minutes before a leptazol challenge (65mg/kg, subcutaneously). The effect of clonic convulsions is shown in Table 35 and Figure 33. At the lowest dose level (1.25mg/kg) dexamphetamine exerted a small and statistically not significant proconvulsant activity. As the dose was increased the proconvulsant activity increased and was significant ($p < 0.001$) at 2.5mg/kg, 5mg/kg and 10mg/kg. In subsequent work we have used the submaximal dose - 5mg/kg.

(b) Dexamphetamine-induced proconvulsant activity after specific inhibition of amine syntheses

Recent investigations into the mode of action of dexamphetamine have utilised α-methyl-p-tyrosine, a specific inhibitor of noradrenaline and dopamine syntheses (Weissman, Koe & Tenen, 1966; Dingell, Owens & others, 1967; Weissman & Koe, 1967). In this project these investigations have been extended to include diethyldithiocarbamate (DDC), an inhibitor of noradrenaline synthesis only (Goldstein, Anagnoste & others, 1964) and p-chlorophenylalanine (pClPhA), a specific inhibitor of 5-HT synthesis (Koe & Weissman, 1966). These drugs have been demonstrated to be effective synthesis-inhibitors in the doses used below (Chapter 4).

(i) α-Methyl-p-tyrosine (α MPT)

Rats were pretreated with α MPT (100mg/kg, intraperitoneally) $3\frac{1}{2}$ hours prior to dexamphetamine (in various doses). 30 minutes after dexamphetamine, the rats were given a leptazol challenge (65mg/kg, subcutaneously) and observed for the effect on clonic convulsions. (Table 35 and Figure 33). Pretreatment with αMPT inhibited noradrenaline and dopamine syntheses (Figure 25) and reduced the proconvulsant activity of dexamphetamine 2.5mg/kg ($p < 0.02$) and 5mg/kg ($p < 0.001$), yet was without effect on 10mg/kg dexamphetamine. Thus αMPT effectively inhibited the proconvulsant activity of submaximal doses of dexamphetamine but was ineffective against maximal doses.

(ii) Diethyldithiocarbamate (DDC)

Inhibition of synthesis of both noradrenaline and dopamine has been demonstrated to block the proconvulsant activity of dexamphetamine. Therefore to determine whether it is an intact noradrenaline or dopamine synthesis which is necessary for this proconvulsive action of dexamphetamine, DDC, an inhibitor of noradrenaline synthesis at the dopamine-β-oxidase stage, was used under similar conditions to αMPT. Rats were pretreated with DDC (400mg/kg, intraperitoneally) $3\frac{1}{2}$ hours prior to dexamphetamine (5mg/kg, intraperitoneally). 30 minutes after the dexamphetamine the rats were given a leptazol challenge (65mg/kg, subcutaneously) and observed for the effect on clonic convulsions, (Table 36). The proconvulsant activity of dexamphetamine was in no way affected. This dose of DDC has been shown to effectively inhibit noradrenaline synthesis with no effect on leptazol convulsions (Figure 26). In contrast to αMPT, DDC was without effect on dexamphetamine induced proconvulsant activity which suggests that it is the dopamine synthesis that is involved in this action of dexamphetamine and not the noradrenaline synthesis.

(iii) p-Chlorophenylalanine (pClPhA)

To ascertain that 5-HT is not involved in the proconvulsant activity of dexamphetamine, the effect of pClPhA was investigated under similar conditions to αMPT and DDC.

Rats were pretreated with pClPhA (320mg/kg, intraperitoneally) for 3 days. On the third day the rats received

dexamphetamine (5mg/kg, intraperitoneally) and 30 minutes later a leptazol challenge (65mg/kg, subcutaneously) and the effect on clonic convulsions observed (Table 36). This dose of pClPhA has previously been shown to effectively inhibit 5-HT synthesis with no effect on leptazol convulsions (Figure 27). In this experiment inhibition of 5-HT synthesis produced no effect on the proconvulsant activity of dexamphetamine which implies that 5-HT is not necessary for this activity.

2. FENCAMFAMIN

Fencamfamin (2-ethylamino-3 phenyl-norcamphane) is a drug possessing marked central stimulating properties with practically no peripheral effect (Hotovy, Enenkel & others, 1961). It has been investigated in this project because dexamphetamine, shown here to be proconvulsant, is both a central and peripheral stimulant: though the proconvulsant activity is more likely to be a central effect, this is not certain. Hotovy, Enenkel and others (1961) reported that fencamfamin, in small doses (0.5mg/kg, orally) to mice is a weak proconvulsant and in larger doses (2 - 5mg/kg, orally) is a weak anticonvulsant. These effects were quite small and no limits of significance were given. However, the relative inactivity against leptazol convulsions compared with dexamphetamine suggests that the marked proconvulsant activity of the latter might be mediated peripherally rather than centrally. Therefore this possibility has been investigated using fencamfamin.

(a) Investigation of a dose-response relationship for the effects of fencamfamin on leptazol convulsions

Rats were pretreated with fencamfamin (5 and 10mg/kg, intraperitoneally) for 30 minutes and then subjected to a leptazol

challenge (65mg/kg, subcutaneously). The effect on clonic convulsions is shown in Table 37 and Figure 34. At the low dose level (5mg/kg) fencamfamin induced a small and statistically not significant proconvulsant effect. However, at 10mg/kg fencamfamin induced a marked proconvulsant activity ($p < 0.001$) which was maximal. This appeared to be an "all or none" effect with fencamfamin, in contrast to dexamphetamine where there was a dose-related effect. In contrast to Hotovy, Enenkel and others (1961) we found a marked proconvulsant activity after fencamfamin.

(b) Fencamfamin-induced proconvulsant activity after specific inhibition of amine synthesis

The proconvulsant activity of dexamphetamine was blocked by inhibition of dopamine synthesis but not by inhibition of noradrenaline or 5-HT synthesis. The effects of fencamfamin have been investigated under similar conditions to ascertain whether or not dopamine is the mediator of this proconvulsant effect.

(i) α-Methyl-p-tyrosine (α MPT)

Rats were pretreated with α MPT (100mg/kg, intraperitoneally) $3\frac{1}{2}$ hours prior to fencamfamin (10mg/kg, intraperitoneally). 30 minutes after fencamfamin the rats were given a leptazol challenge (65mg/kg, subcutaneously) and observed for the effect on clonic convulsions (Table 38 and Figure 35). Although α MPT inhibited noradrenaline and dopamine syntheses (Figure 25) it did not produce any significant effect on the proconvulsant activity of fencamfamin. This indicates that in contrast to dexamphetamine, fencamfamin displays a proconvulsant activity in the absence of an intact

noradrenaline synthesis, an effect observed with the largest doses of dexamphetamine.

(ii) Diethyldithiocarbamate (DDC)

Rats were pretreated with DDC (400mg/kg, intraperitoneally) $3\frac{1}{2}$ hours prior to fencamfamin (10mg/kg, intraperitoneally). 30 minutes after fencamfamin the rats were given a leptazol challenge (65mg/kg, subcutaneously) and observed for effects on clonic convulsions (Table 38 and Figure 35). Inhibition of noradrenaline synthesis after DDC (Figure 26) produced no significant effect on fencamfamin-induced potentiation of leptazol and thus supported the similar result after α MPT.

(iii) p-Chlorophenylalanine (pClPhA)

Rats were pretreated with pClPhA (320mg/kg, intraperitoneally) for 3 days. On this third day the rats received fencamfamin (10mg/kg, intraperitoneally) and 30 minutes later a leptazol challenge (65mg/kg, subcutaneously). The effect on clonic convulsions is shown in Table 38 and Figure 35. Inhibition of 5-HT synthesis after pClPhA (Figure 27) was without effect on the potentiation of leptazol by fencamfamin showing that fencamfamin does not require an intact 5-HT synthesis to exert this effect.

3. EFFECT OF DEXAMPHETAMINE AND FENCAMFAMIN ON WHOLE BRAIN AMINE CONCENTRATIONS IN RATS

Whole brain amine concentrations were determined in rats pretreated with dexamphetamine or fencamfamin in an attempt to correlate their

proconvulsant activities with the disposition of central amines and to explain the results with the synthesis inhibitors.

Rats were pretreated with either dexamphetamine (5mg/kg, intraperitoneally) or fencamfamin (10mg/kg, intraperitoneally) and sacrificed 30 minutes later. Whole brain amines were then determined (Table 39 and Figure 36). The effect of dexamphetamine on central amines has already been reported in this project (Table 14 and Figure 22). This result is repeated in Table 39 and Figure 36 only for the purpose of comparison with fencamfamin. Dexamphetamine produced no significant effect on whole brain amine concentrations at this dose level, consequently the proconvulsant activity could not be correlated with changes in whole brain biogenic amine concentrations. Fencamfamin was also markedly proconvulsant but unlike amphetamine it produced a significant elevation ($p < 0.02$) of whole brain dopamine concentrations, with no effect on noradrenaline and 5-HT concentrations.

4. DISCUSSION

Both fencamfamin and dexamphetamine have been demonstrated to be proconvulsant with leptazol. However the mechanisms of proconvulsant activity appear to differ, for several interesting differences between these drugs have been shown. The proconvulsant activity of dexamphetamine has been demonstrated to be dose-dependent. In contrast the proconvulsant activity of fencamfamin was an "all or none" effect: 5mg/kg being without effect and 10mg/kg producing 100% (maximum) convulsions. This suggests that the mechanism of fencamfamin involves a threshold limit which must be reached to initiate the proconvulsant activity.

The proconvulsant activity of dexamphetamine (2.5 and 5mg/kg) was blocked by α MPT which depleted whole brain noradrenaline to 50% and dopamine to 30% of control levels (Figure 25). This supports the hypothesis that an intact noradrenaline synthesis is necessary for dexamphetamine to exert its effects (Weissman & others, 1966; Dingell & others, 1967; Hanson, 1967; Weissman & Koe, 1967). However, α MPT depleted both noradrenaline and dopamine and therefore only dopamine synthesis may be necessary for this effect of amphetamine. DDC, a dopamine-β-oxidase inhibitor, which effectively blocked noradrenaline synthesis and allowed a small accumulation of dopamine, was without effect on the proconvulsant activity of dexamphetamine. This implies that an intact dopamine synthesis is necessary for the proconvulsant activity of dexamphetamine and supports recent reports by McKenzie and Szerb (1968) and Ernst (1969) who demonstrated that dexamphetamine releases dopamine from the caudate nucleus in cats (McKenzie & Szerb, 1968) and in rats (Ernst, 1969). However, other reports claim that dopamine is only responsible for stereotypy and noradrenaline for hyperactivity after dexamphetamine (Randrup & Munkvad, 1967; Scheel-Kruger & Randrup, 1967). It is unlikely that the proconvulsant activity is associated with stereotypy but rather it is associated with hyperactivity in which case our results are contrary to those of the above workers. The proconvulsant activity of dexamphetamine was not affected by inhibition of 5-HT synthesis with pClPhA, confirming reports that 5-HT is not involved in the mechanism of action of dexamphetamine (Frey & Magnussen, 1968).

It is difficult to explain why the proconvulsant activity of dexamphetamine is dependent upon an intact dopamine synthesis when dexamphetamine itself produces no effect on central noradrenaline or

dopamine concentrations. The depletion that occurs after inhibition of the synthesis is not responsible for the anti-amphetamine properties of α MPT, for a similar depletion after reserpine does not produce anti-amphetamine effects (Chapter 4). It is possible that dexamphetamine exerts its effects only via newly synthesised dopamine. If the mechanism were to be inhibition of reuptake of noradrenaline and dopamine at the neuronal membrane (as suggested by Carlsson, Lindqvist, Dahlstrom, Fuxe and Masuoka (1965)) and only involved newly synthesised dopamine, this would explain why inhibition of synthesis and not depletion produces anti-amphetamine effects and why there is no noticeable effect on central amine concentrations. It would also explain reports of enhanced amphetamine activity after reserpine (Stolk & Rech, 1967): depletion of catecholamine stores by reserpine increases the synthesis rate of catecholamines by a negative feedback mechanism (Neff & Costa, 1967 a) which increases the amount of newly synthesised dopamine available for amphetamine to utilise. This hypothesis is summarised in the form of a diagram (Figure 37).

In this work it has been shown that the proconvulsant activity of high doses (10mg/kg) of dexamphetamine was not blocked by inhibition of dopamine synthesis with α MPT. This indicates that although the synthesis of dopamine was blocked, dexamphetamine was able to exert a proconvulsant effect without the aid of newly synthesised dopamine. It is possible that these conditions revealed a second and more direct action of dexamphetamine. Therefore, dexamphetamine may possess an indirect action utilising newly synthesised dopamine and a direct action on adrenergic receptors; the latter is only evident after high doses.

Fencamfamin exerted a proconvulsant activity even after inhibition of catecholamine synthesis with α MPT or DDC or inhibition of 5-HT synthesis with pClPhA. Thus in contrast to dexamphetamine, the mode of action of fencamfamin does not require an intact amine synthesis. Fencamfamin does not release brain amines as shown by the lack of depletion. These two observations suggest that fencamfamin does not exert an indirect effect via catecholamines, but rather that it may exert its proconvulsant effect by a direct stimulation of adrenergic receptors, and as such is similar to high doses of dexamphetamine. It is of course possible that the increased dopamine concentration after fencamfamin might be the proconvulsant factor and that a similar mechanism might be involved in the proconvulsant activity of dexamphetamine at 10mg/kg. This is extremely unlikely because a similar selective elevation of dopamine after DL-DOPA exerted no proconvulsant activity (Chapter 3 - Amine precursors) and neither did a similar selective elevation after pargyline (Chapter 3 - Monoamine oxidase inhibitors). It is also unlikely that this is the mechanism of action of dexamphetamine at 10mg/kg because dexamphetamine is only reported to deplete catecholamines at high doses and never to elevate dopamine concentrations. McKenzie and Szerb (1968) and Ernst (1969) reported that dexamphetamine releases dopamine; Smith (1965) reported that 10 - 30mg/kg of dexamphetamine decreased both noradrenaline and dopamine concentrations. Moore and Lariviere (1963) reported that these same doses produced a significant depletion of noradrenaline with no effect on dopamine concentrations. Many other works have demonstrated only depletion of whole brain noradrenaline after dexamphetamine in rats (Baird & Lewis, 1964; Baird, 1968), in rabbits Sanan & Vogt, 1962) and in cats (Carr & Moore, 1969). It is therefore considered that the small elevation of dopamine after fencamfamin is

incidental and not responsible for the proconvulsant activity. A similar elevation of dopamine by dexamphetamine at a dose of 10mg/kg has been shown to be extremely improbable. What appears more likely is that fencamfamin and high doses of dexamphetamine (10mg/kg) possess a common mode of proconvulsant action; that of direct stimulation of adrenergic receptors.

In conclusion, it has been demonstrated that dexamphetamine and fencamfamin possess marked proconvulsant activity. Dexamphetamine at low doses exerts an indirect effect, utilising freshly-synthesised dopamine, but large doses (10mg/kg) exert a direct stimulation of adrenergic receptors. Fencamfamin appears to possess no indirect mechanism and exerts its proconvulsant effect only by direct stimulation of adrenergic receptors. The "all or none" effect of fencamfamin indicates that a threshold concentration must be achieved at the receptors before any proconvulsant effect is produced.

TABLE 35

EFFECT OF 30 MINUTES PRETREATMENT WITH DEXAMPHETAMINE ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY) AND THIS SAME EFFECT AFTER α-METHYL-p-TYROSINE, (αMPT, 100mg/kg, INTRAPERITONEALLY)

Dose of dexamphetamine mg/kg (intraperitoneally)	% Maximum Clonic Convulsions after leptazol	% Maximum Clonic Convulsions after αMPT
0 (controls)	43 ± 3 (6)	52 ± 3 (4)
1.25	53 ± 3 (3)	-
2.5	77 ± 3 (3)***	53 ± 3 (3)**
5.0	89 ± 2 (10)***	57 ± 5 (6)***
10.0	100 ± 0.0 (3)***	93 ± 7 (3)

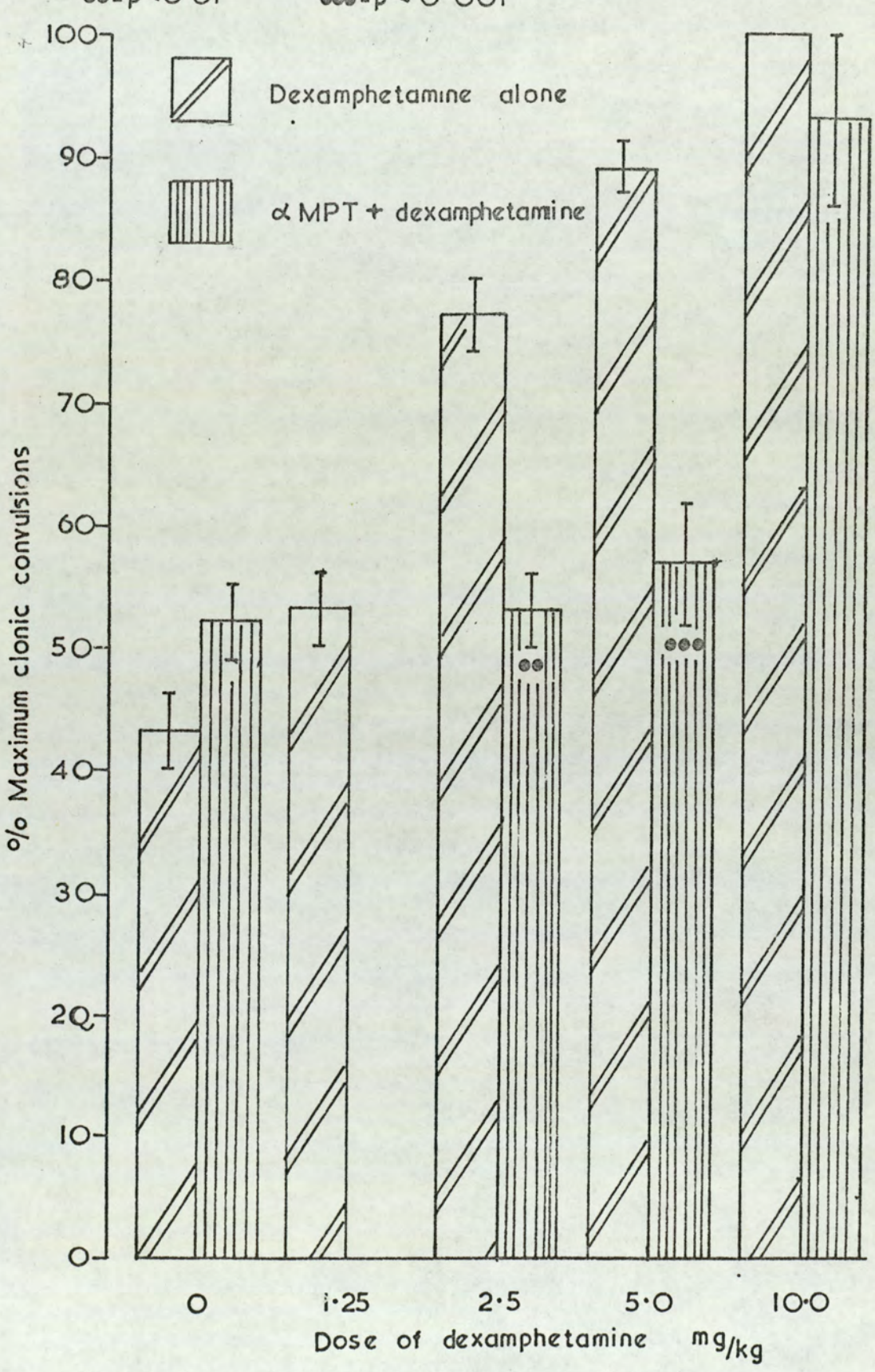
Results are expressed as group mean $\pm$ standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 33

EFFECT OF DEXAMPHETAMINE ALONE (5mg/kg, INTRAPERITONEALLY) AND THE EFFECT OF THIS SAME DOSE OF DEXAMPHETAMINE AFTER PRETREATMENT WITH α-METHYL-p-TYROSINE (100mg/kg, INTRAPERITONEALLY), ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)



Vertical bars indicate standard errors of the mean.

TABLE 36

EFFECT OF DIETHYLDITHIOCARBAMATE (DDC 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE (pClPhA 320mg/kg, INTRAPERITONEALLY) ON THE DEXAMPHETAMINE-INDUCED POTENTIATION OF LEPTAZOL CONVULSIONS IN RATS

Pretreatment	% Maximum Clonic Convulsions
Controls	43 ± 2 (16)
Dexamphetamine 5mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, (subcutaneously).	89 ± 2 (10) ***
DDC 400mg/kg, (intraperitoneally) 4 hours. Dexamphetamine 5mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, (subcutaneously)	87 ± 3 (3) ***
pClPhA 320mg/kg, (intraperitoneally) 3 days. Dexamphetamine 5mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, (subcutaneously)	90 ± 6 (3) ***

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

TABLE 37

EFFECT OF 30 MINUTES PRETREATMENT WITH FENCAMFAMIN ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Dose of fencamfamin mg/kg intraperitoneally	% Maximum Clonic Convulsions	
0 (controls)	53 ± 5	(4)
5	53 ± 3	(3)
10	100 ± 0 ***	(4)

Results are expressed as group mean $\pm$ standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$

*** = $p < 0.001$

TABLE 38

EFFECT OF α-METHYL-p-TYROSINE (αMPT 100mg/kg, INTRAPERITONEALLY) DIETHYLDITHIOCARBAMATE (DDC 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE (pClPhA 320mg/kg, INTRAPERITONEALLY) ON FENCAMFAMIN-INDUCED (10mg/kg, INTRAPERITONEALLY) POTENTIATION OF LEPTAZOL CONVULSIONS IN RATS

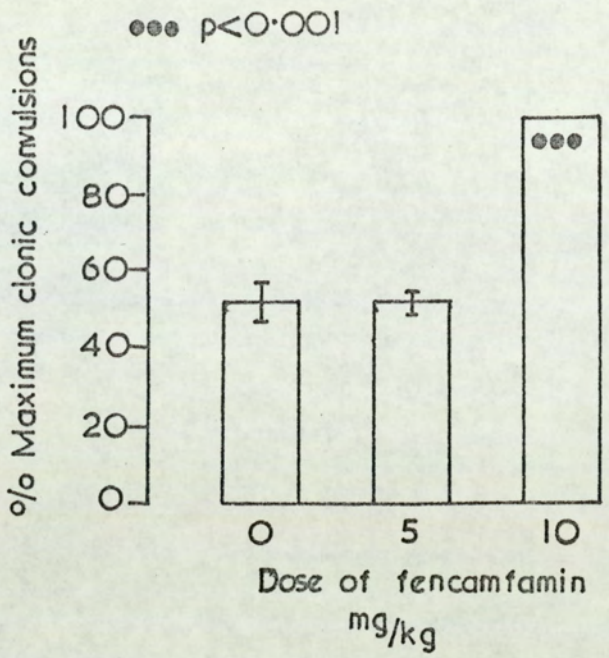
Pretreatment	% Maximum Clonic Convulsions
Fencamfamin 10mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, subcutaneously)	100 ± 0 (4)
αMPT 100mg/kg, (intraperitoneally) 4 hours. Fencamfamin 10mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, (subcutaneously)	100 ± 0 (3)
DDC 400mg/kg, (intraperitoneally) 4 hours. Fencamfamin 10mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, (subcutaneously)	95 ± 2 (4)
pClPhA 320mg/kg, (intraperitoneally) 3 days. Fencamfamin 10mg/kg, (intraperitoneally) 30 mins. Leptazol 65mg/kg, (subcutaneously)	100 ± 0 (3)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

FIGURE 34

EFFECT OF 30 MINUTES PRETREATMENT WITH FENCAMFAMIN ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)



Vertical bars indicate standard errors of the mean

FIGURE 35

EFFECT OF α-METHYL-p-TYROSINE (αMPT, 100mg/kg, INTRAPERITONEALLY), DIETHYLDITHIOCARBAMATE (DDC, 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE (p-ClPhA, 320mg/kg, INTRAPERITONEALLY) ON THE FENCAMFAMIN INDUCED (10mg/kg) POTENTIATION OF CLONIC CONVULSIONS IN RATS

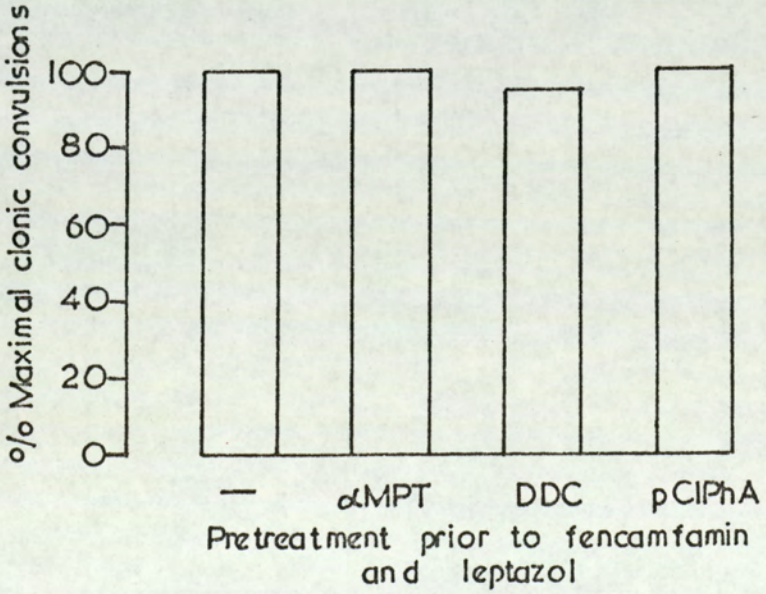


TABLE 39

EFFECT OF 30 MINUTES PRETREATMENT WITH DEXAMPHETAMINE (5mg/kg, INTRAPERITONEALLY) OR FENCAMFAMIN (10mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINE AND 5-HT CONCENTRATIONS IN RATS

Pretreatment	Noradrenaline ng/g	Dopamine ng/g	5-HT ng/g
Controls	230 ± 13 (4)	628 ± 9 (5)	537 ± 22 (4)
Dexamphetamine 5mg/kg intraperitoneally	201 ± 9 (4)	637 ± 12 (3)	560 ± 10 (4)
Fencamfamin 10mg/kg intraperitoneally	208 ± 10 (4)	708 ± 22 (3) *	529 ± 8 (4)

Results are expressed as group mean ± standard error.

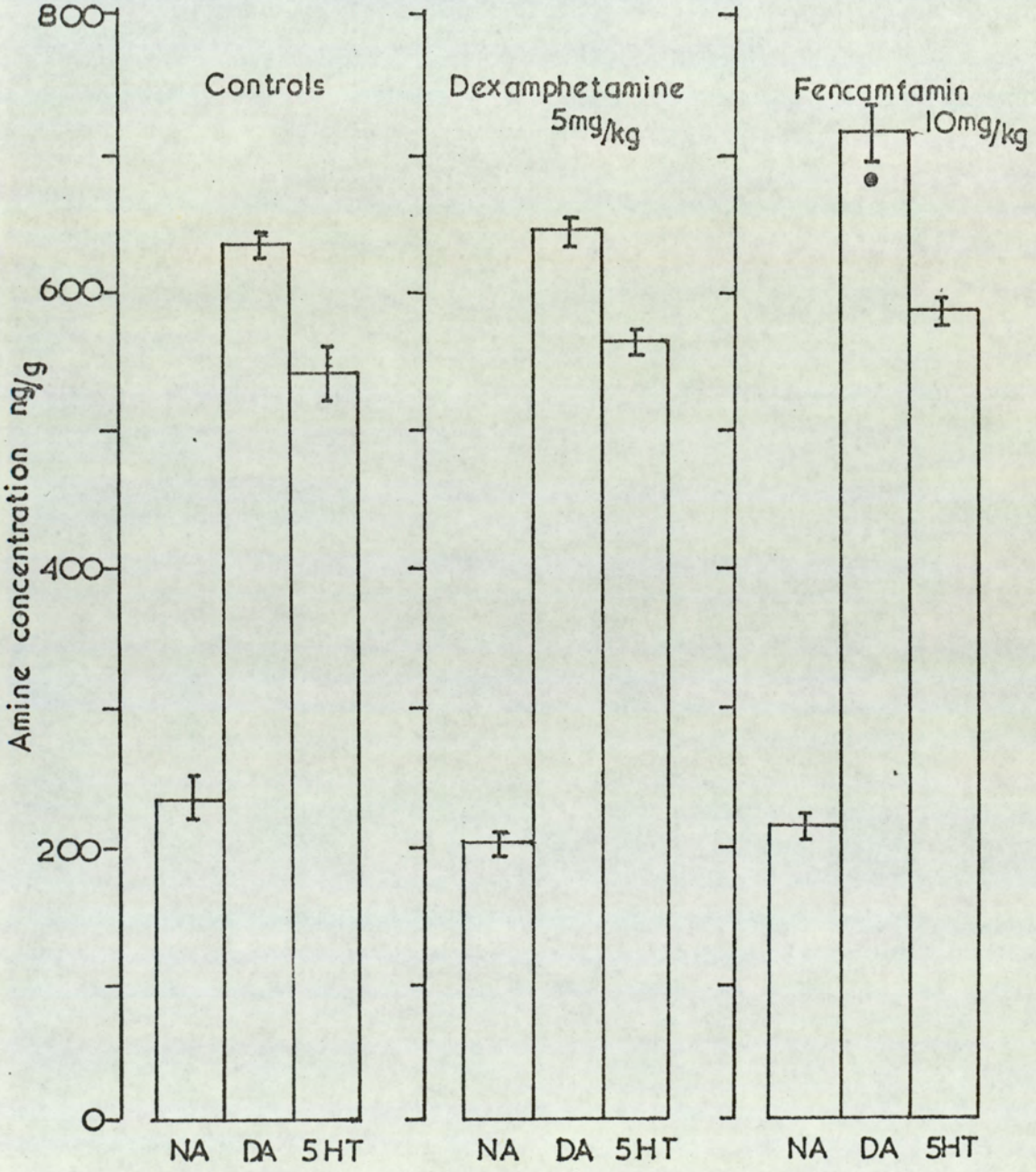
Figures in parentheses indicate the number of determinations made.

ng = nanogram = 10^{-9} gramme.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 36

EFFECT OF 30 MINUTES PRETREATMENT WITH DEXAMPHETAMINE (5mg/kg, INTRAPERITONEALLY) OR FENCAMFAMIN (10mg/kg, INTRAPERITONEALLY) ON WHOLE BRAIN CATECHOLAMINES AND 5-HT CONCENTRATIONS IN RATS

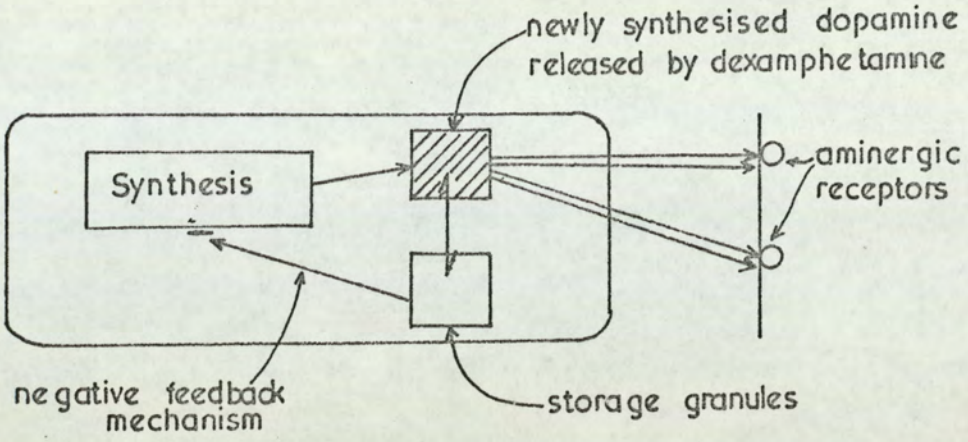


Vertical bars indicate standard errors of the mean

FIGURE 37

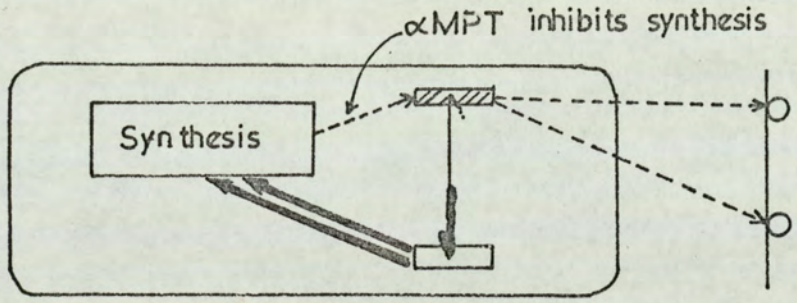
DIAGRAMMATIC REPRESENTATION OF AN ADRENERGIC TERMINAL TO SHOW THE PROPOSED MODE OF PROCONVULSANT ACTION OF DEXAMPHETAMINE AFTER (a) NO OTHER PRETREATMENT (b) α MPT PRETREATMENT AND (c) AFTER RESERPINE PRETREATMENT

(a)



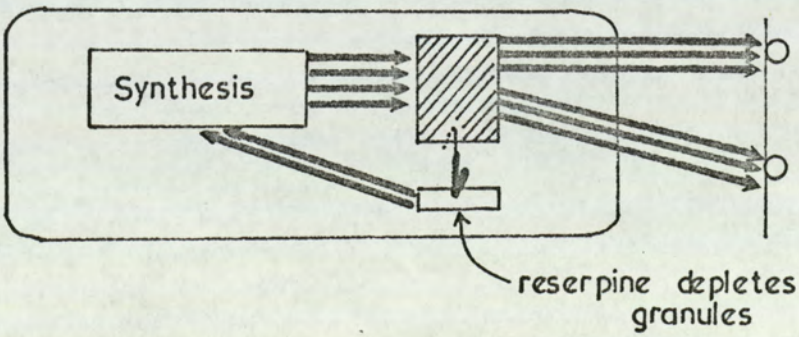
Synthesis provides newly synthesised dopamine which dexamphetamine releases onto the receptors. Excess dopamine is stored in the granules which control further synthesis by a negative feedback mechanism.

(b)



α MPT blocks dopamine synthesis and this prevents dexamphetamine from exerting any effect. Although the feedback mechanism demands faster synthesis, as a result of reduced stores, this is blocked by αMPT.

(c)



Reserpine depletes the amine stores which in turn increases synthesis rate via the negative feedback mechanism. As a result there is an increased concentration of freshly synthesised dopamine available which dexamphetamine can release to produce a greater stimulation.

CHAPTER SIX

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH ADRENERGIC BLOCKING DRUGS

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH ADRENERGIC BLOCKING DRUGS

LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH ADRENERGIC BLOCKING DRUGS

Several classes of drugs which modify adrenergic mechanisms have already been investigated for effects on leptazol-induced convulsions. Although α and β receptors known to exist in peripheral tissues have not been demonstrated to be present in the CNS, for the sake of completeness a brief investigation of phentolamine, pronethalol and the adrenergic neurone blocking agent guanethidine has been performed.

1. PHENTOLAMINE

Phentolamine is an adrenergic blocking agent which acts at α receptors. It is more potent and has less non-specific effects than tolazoline (Goodman & Gilman, 1965) or phenoxybenzamine (M.D. Day - personal communication), two other widely used adrenergic blocking agents. No reports on the effects of phentolamine on leptazol convulsions could be found, whilst a report on the activity of phenoxybenzamine in the psychomotor test (low-frequency electroshock) claimed that it is without effect on this type of seizure (Yeoh & Wolf, 1968).

Rats were pretreated with phentolamine, 20mg/kg, intraperitoneally, 30 minutes before a leptazol challenge (65mg/kg, subcutaneously) and observed for the effect on clonic convulsions (Table 40). This dose of phentolamine produced a degree of sedation and was significantly anticonvulsant ($p < 0.01$), reducing the incidence of convulsions from 46.7% to 26.7%. However it was noticed that phentolamine had produced a considerable delay in the onset of convulsions and therefore the rats

were observed for a further 30 minute period. In this second observation period the rats, which had been protected previously, all convulsed and the overall incidence of convulsions was not significantly different from controls; the control rats showed no further incidence of convulsions in this second observation period. Hence phentolamine was anticonvulsant only in so far as it delayed the onset of clonic convulsions.

2. PRONETHALOL

Pronethalol is a β-adrenergic blocking agent (Goodman & Gilman, 1965) related to propranolol which has the same mode of action. Propranolol has been reported to be anticonvulsant to electroshock and convulsions induced by nicotine and strychnine but without effect on leptazol convulsions (Leszkovszky & Tardos, 1965). In another report both propranolol and pronethalol were demonstrated to be anticonvulsant to leptazol, electroshock and nicotine-induced convulsions, but pronethalol was relatively ineffective against strychnine convulsions (Murmann, Almirante & Saccani-Guelfi, 1966). In this investigation pronethalol was used because it has been reported to cross the blood-brain barrier more readily than propranolol (Bowman, Rand & West, 1968).

Rats were pretreated with pronethalol, 10mg/kg, intraperitoneally, 30 minutes before a leptazol challenge (65mg/kg, subcutaneously) and observed for the effect on clonic convulsions (Table 40). No significant effect was observed. The anticonvulsant effect reported by Murmann and others (1966) was against the tonic component of leptazol convulsions and thus may be inactive in the test using the clonic component, in the same way as diphenylhydantoin is.

3. GUANETHIDINE

Guanethidine should not strictly be classed as an adrenergic blocking agent; it is an adrenergic neuron blocking agent in that it is believed to act by preventing the release of transmitter from the adrenergic nerve ending (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960). Pfeifer and Galambos (1967 a) have demonstrated that guanethidine produces a 40% depletion of central noradrenaline in mice and a 20% decrease in the leptazol threshold using the Maximum Leptazol Seizure test. In this project guanethidine has been investigated against the clonic component to leptazol convulsions in rats. Rats were pretreated with guanethidine, 4mg/kg intraperitoneally, and two hours later received a leptazol challenge (65mg/kg, subcutaneously). The effect in clonic convulsions is shown in Table 40. There was no significant change in the incidence of leptazol-induced clonus.

4. DISCUSSION

Although the α-adrenergic blocking agent, phentolamine, produced a significant anticonvulsant effect, this was shown to be due only to a delay in onset of action of leptazol. The β-blocking agent, pronethalol, was without effect on leptazol clonus and so was the neuronal blocking agent, guanethidine. Previous work on the effects of adrenergic blocking drugs on low frequency electroshock seizures by Yeoh and Wolf (1968) has demonstrated that pronethalol and propranolol elevated the electroshock threshold and phenoxybenzamine reduced this threshold. These workers suggested that a central adrenergic system, involving antagonistic types of receptors, was involved. The work in this project did not support their hypothesis

but tended to support the work of Murmann and others (1966), namely that these drugs exert a CNS depressant effect which explains any anticonvulsant activity they might possess. This general depressant action might be responsible for the anticonvulsant activity of phentolamine, but it is also possible that phentolamine prevents leptazol from readily crossing the blood-brain barrier.

The conclusion to be drawn from this investigation is that although pronethalol (Murmann & others, 1966) and guanethidine (Pfeifer & Galambos, 1967 a) have previously been demonstrated to be anticonvulsant against the tonic component of leptazol convulsions, they are not anticonvulsant against the clonic component.

TABLE 40

EFFECT OF PHENTOLAMINE (20mg/kg, INTRAPERITONEALLY) PRONETHALOL (10mg/kg, INTRAPERITONEALLY) AND GUANETHIDINE (4mg/kg, INTRAPERITONEALLY) ON CLONIC CONVULSIONS INDUCED IN RATS BY LEPTAZOL (65mg/kg, SUBCUTANEOUSLY)

Pretreatment	% Maximum Clonic Convulsions
Controls (untreated) Leptazol 65mg/kg, (subcutaneously)	46.7 ± 3.3 (3)
Phentolamine 20mg/kg intraperitoneally 30 mins; then, Leptazol 65mg/kg, (subcutaneously)	26.7 ± 3.3 (3) **
Pronethalol 10mg/kg intraperitoneally 30 mins; then, Leptazol 65mg/kg, (subcutaneously)	40 ± 6 (3)
Guanethidine 4mg/kg intraperitoneally 2 hours; then, Leptazol 65mg/kg, (subcutaneously)	43.3 ± 3.3 (3)

Results are expressed as group mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$

*** = $p < 0.001$

CHAPTER SEVEN

THE ACTIVITY OF ANTICONVULSANT DRUGS AGAINST LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH ADRENERGIC DRUGS

THE ACTIVITY OF ANTICONVULSANT DRUGS AGAINST LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH ADRENERGIC DRUGS

THE ACTIVITY OF ANTICONVULSANT DRUGS AGAINST LEPTAZOL CONVULSIONS AFTER PRETREATMENT WITH ADRENERGIC DRUGS

In the preceding chapters various adrenergic drugs have been examined for effects on leptazol-induced convulsions. It has been demonstrated that interference with adrenergic mechanisms can produce changes in the leptazol threshold. It was therefore possible that similar interference with adrenergic mechanisms might affect the anti-convulsant activity of phenobarbitone, diphenylhydantoin and chlordiazepoxide. Phenobarbitone sodium is a potent anti-leptazol drug (Swinyard, Brown & Goodman, 1952) and so is chlordiazepoxide (Swinyard & Castellion, 1966). In contrast diphenylhydantoin is ineffective against leptazol (Goodman, Toman & Swinyard, 1948; Swinyard, Brown & Goodman, 1952). Nevertheless diphenylhydantoin was included in these tests for, although it is not active in the Minimal Electroshock Threshold test, it can be rendered active in a similar test, the Hyponatraemic Electroshock Threshold test, by pretreating the animals with isoosmolar glucose solution which lowers extracellular sodium and electroshock threshold (Swinyard, Toman & Goodman, 1946). Therefore, diphenylhydantoin was included to see if altering the balance of aminergic mechanisms would reveal diphenylhydantoin to be anticonvulsant against leptazol. It was necessary to use adrenergic drugs which themselves had previously been found to be neither anticonvulsant nor proconvulsant, otherwise this would have led to erroneous conclusions. For example reserpine is proconvulsant and although phenobarbitone would block leptazol convulsions it may not block the proconvulsant activity of reserpine. Such results could unreasonably be interpreted as evidence that intact amine stores are necessary for the anticonvulsant action of phenobarbitone. This is not necessarily so; it may be that the previously effective dose of

phenobarbitone was sufficient to block the normal level of convulsive activity after leptazol, but that a higher dose of phenobarbitone would have been necessary to inhibit the superimposed proconvulsant activity of reserpine.

With these limitations in mind the three anticonvulsants were investigated against the synthesis blocking agents α-methyl-p-tyrosine, diethyldithiocarbamate and p-chlorophenylalanine, and against the depleting agent α-methyl-m-tyrosine, all of which have been found inactive against leptazol alone.

The dose of anticonvulsants to be used were extracted from the literature. Swinyard, Brown and Goodman (1952) demonstrated that sodium phenobarbitone was an effective anticonvulsant against leptazol in a dose of 30mg/kg, the time of peak activity being 2 hours. They also showed that diphenylhydantoin was ineffective against leptazol in doses as high as 100mg/kg, whereas a dose of 10mg/kg was markedly effective against electroshock convulsions, with a peak activity at 30 minutes. We therefore used diphenylhydantoin at 40mg/kg with a 30 minute pretreatment period. Phenobarbitone sodium was used at a dose level of 30mg/kg and pretreatment period of 90 minutes. Waite (1969) has demonstrated that the ED_{90} of chlordiazepoxide in mice is 2.5mg/kg with a pretreatment period of 30 minutes. Such a submaximal dose suits the purpose of this experiment, for our intention was to see if adrenergic drugs reduced the efficacy of the anticonvulsants; supramaximal doses may mask any such effect.

These doses were examined for their efficacy in a small initial experiment. The anticonvulsants were administered intraperitoneally and leptazol subcutaneously. Only one group of 5 rats was used for

each drug. Chlordiazepoxide and phenobarbitone produced 100% inhibition of leptazol but these doses were not supramaximal because in each group there were one or two rats seen to be on the threshold of a convulsion. Diphenylhydantoin was ineffective against leptazol as described by Swinyard and others (1952).

1. PHENOBARBITONE SODIUM

The mode of anticonvulsant activity of phenobarbitone is known only in vague terms. Goodman, Grewal, Brown and Swinyard (1953) report that phenobarbitone elevates seizure threshold and inhibits seizure spread. However the mechanism which achieves this ~~end~~ remains obscure. In a recent report, phenobarbitone sodium was shown to decrease impulse flow in dopamine neurones of the rat brain with no effect on noradrenaline neurones (Corrodi, Fuxe & Hokfelt, 1966). This decrease was revealed after pretreatment with α-methyl-p-tyrosine. The decreased impulse flow in dopamine neurones might contribute to the anticonvulsant activity of phenobarbitone. Therefore, the disposition of central amines, with particular reference to dopamine, was investigated for any effects on the anticonvulsant activity of phenobarbitone sodium.

Rats were pretreated with one of the following: αMPT, 100mg/kg intraperitoneally, for $2\frac{1}{2}$ hours; DDC, 400mgm/kg intraperitoneally, for $2\frac{1}{2}$ hours; or pClPhA, 320mg/kg intraperitoneally, for 3 days. In each case, phenobarbitone sodium, 30mg/kg intraperitoneally, was given $1\frac{1}{2}$ hours before the leptazol challenge (65mg/kg, subcutaneously). The effect on clonic convulsions is shown in Table 41. The anticonvulsant activity of phenobarbitone sodium was not reduced significantly by inhibition of the syntheses of noradrenaline, dopamine or 5-HT, and this implies that an intact synthesis of any one of these three amines is not necessary for the anticonvulsant effect of

phenobarbitone. These results also show that depletion of amine stores does not reduce the anticonvulsant activity of phenobarbitone sodium.

2. DIPHENYLHYDANTOIN

Although diphenylhydantoin is ineffective against leptazol-induced clonic convulsions (Swinyard & others, 1952), like phenobarbitone its mechanism of action is vague. Goodman, Grewal and others (1953) reported that diphenylhydantoin only prevents the spread of the seizure discharge and does not elevate the threshold. This being the case, it was considered pertinent to investigate whether interference with adrenergic mechanisms might potentiate such a mechanism and reveal an anti-clonic component similar to that produced by hyponatraemia (Swinyard, Toman & Goodman, 1946).

Rats were pretreated with one of the following: αMPT, 100mg/kg intraperitoneally, for $3\frac{1}{2}$ hours; DDC, 400mg/kg intraperitoneally, for $3\frac{1}{2}$ hours; or pClPhA, 320mg/kg intraperitoneally for 3 days. In each case diphenylhydantoin, 40mg/kg intraperitoneally, was given 30 minutes prior to a leptazol challenge 65mg/kg, subcutaneously) and observed for the effects on clonic convulsions. (Table 42). Diphenylhydantoin produced a small but significant potentiation ($p < 0.01$) of the clonic phase of leptazol induced convulsions. This is confirmation of a similar report by Mitchell and Keasling (1960). However pretreatment with the selective synthesis inhibitors did not reduce or increase this potentiation and it is concluded that biogenic amines are not involved in the mechanism of action of diphenylhydantoin. Rudzik and Mennear (1965) arrived at this same conclusion using the Maximal Electroshock seizure test, a test in which diphenylhydantoin is markedly anticonvulsant.

3. CHLORDIAZEPOXIDE

Chlordiazepoxide has been demonstrated to be both a potent tranquillizer and anticonvulsant (Randall, Schallek & others, 1960; Randall, Heise & others, 1961). Since the majority of antidepressants and tranquillizers have been demonstrated to exert effects on adrenergic mechanisms it was considered possible that chlordiazepoxide might exert its anticonvulsant effects via the adrenergic system. A brief discussion of the evidence on the mode of action of chlordiazepoxide was included in the Introduction. (Section 1 - Anticonvulsant Drugs.

The effects of various adrenergic drugs on the anticonvulsant potency of chlordiazepoxide have been briefly investigated in rats. Rats were pretreated with either αMPT (100mg/kg, intraperitoneally) for 3½ hours, DDC (400mg/kg, intraperitoneally) for 3½ hours or pClPhA (320mg/kg, intraperitoneally) for 3 days. In each case chlordiazepoxide 2.5mg/kg, intraperitoneally) was given 30 minutes before a leptazol challenge (65mg/kg, subcutaneously). The effects on clonic convulsions are shown in Table 43 and Figure 38. Chlordiazepoxide effectively reduced the incidence of leptazol-induced convulsions from 45% to 3.3%. However, after αMPT this anticonvulsant activity of chlordiazepoxide was significantly reduced ($p < 0.005$), for the incidence of clonic convulsions increased to 30%. Neither DDC nor pClPhA produced any significant effect on the anticonvulsant activity of chlordiazepoxide. Thus neither noradrenaline nor 5-HT syntheses are involved in this mechanism. Instead, an intact dopamine synthesis and/or intact dopamine stores is necessary for the anticonvulsant activity of chlordiazepoxide. To determine which of these might be involved it was necessary to deplete central amine stores with drugs which were not themselves

proconvulsant. The drugs used were α-methyl-m-tyrosine (α MMT) which has been shown to deplete only noradrenaline and dopamine stores (Chapter 4) and reserpine at a dose level of 200 μg/mg, intravenously, which has been shown to deplete central amine stores with no effect on leptazol convulsions (Chapter 4).

Rats were pretreated with either αMMT (100mg/kg, intraperitoneally) for 3½ hours or reserpine (200 μg/kg, intravenously) for 3½ hours. In each case, chlordiazepoxide (2.5mg/kg, intraperitoneally) was given 30 minutes before a leptazol challenge. The effects on clonic convulsions are shown in Table 44 and Figure 39. Depletion with αMMT reduced the anticonvulsant activity of chlordiazepoxide; the incidence of clonic convulsions increased from 3.3% to 26.7% ($p < 0.02$). Reserpine was without effect on the activity of chlordiazepoxide. Thus these two methods of depleting central catecholamines produced different effects on the activity of chlordiazepoxide.

4. DISCUSSION

The anticonvulsant site and mode of action of barbiturates remains obscure, Goodman, Grewal, Brown and Swinyard (1953) postulated that phenobarbitone elevates seizure threshold and prevents seizure spread. The mechanism by which it produces this effect was not discussed and has not been elucidated. Exley (1954) has reported that barbiturates depress transmission in sympathetic ganglia in concentrations that are without effect on nerve conduction, neuroeffector junctions or smooth muscle. However the potencies of barbiturates as ganglionic depressants do not correlate with their potencies as central nervous depressants. Nevertheless, a similar mechanism at central synapses might explain the anticonvulsant action. The central depressant action of barbiturates is believed to occur at the level of the reticular activating system

(Goodman & Gilman, 1965). However, phenobarbitone increases the threshold for electrical stimulation of the motor cortex (the area which must ultimately be involved in a convulsion) in doses that minimally affect the threshold of the reticular system (Aston & Domino, 1961). The report by Corrodi, Fuxe and Hokfelt (1966) that barbiturates decrease neuronal flow in dopamine neurones of the caudate nucleus and putamen might explain these threshold elevating effects cited by Aston and Domino (1961). This evidence prompted an investigation of the effects of interference with adrenergic mechanisms on the anticonvulsant efficacy of phenobarbitone. It was considered that if catecholamines or 5-HT were involved in the anticonvulsant mechanism then inhibition of amine synthesis or depletion of stores might reduce this effect. No such effect was observed. It must therefore be assumed that the disposition of these amines is not involved in the anticonvulsant effect of phenobarbitone.

Diphenylhydantoin, in contrast to phenobarbitone, is considered to only prevent seizure spread with little effect on seizure threshold (Goodman, Grewal & others, 1953). The only evidence for an effect of diphenylhydantoin on synaptic transmission is that it reduces postetanic potentiation of synaptic transmission in the spinal cord of the cat, (Esplin, 1957). Although diphenylhydantoin has been shown to elevate central 5-HT concentrations in rats (Bonnycastle & others, 1957), the dose used was a toxic dose (100mg/kg and was administered sixteen times. Such a result cannot be extrapolated to explain the anticonvulsant activity. These two reports were the only evidence that central transmission might be involved in the anticonvulsant activity of diphenylhydantoin. Consequently it was not surprising to find that interference with adrenergic mechanisms did not uncover an anticonvulsant

effect for diphenylhydantoin against leptazol clonus. A more suitable experiment would have been to examine diphenylhydantoin, under these conditions, against the tonic component of leptazol convulsions. This was not possible in this project since the rats used did not exhibit a consistent tonic component. Rukzik and Mennear (1965) have demonstrated that adrenergic mechanisms were not involved in the anti-convulsant activity of diphenylhydantoin against maximal eletroshock. Although our evidence is negative we must arrive at a similar conclusion using leptazol. What is a more likely mechanism is the decrease in intracellular concentrations of sodium in brain cells produced by diphenylhydantoin (Woodbury, 1955). It also prevents the intracellular increase of sodium in the Hyponatraemic Electroshock Threshold test, a test in which diphenylhydantoin lowers the electroshock threshold. Diphenylhydantoin also prevents the increase of intracellular sodium after maximal electroshock induced seizures.

The results which chlordiazepoxide were more positive: αMPT significantly reduced the anticonvulsant activity of chlordiazepoxide, whereas DDC and pClPhA did not. This suggested that chlordiazepoxide requires an intact dopamine synthesis for its anticonvulsant activity. However, a similar depletion of dopamine and noradrenaline produced by αMMT also reduced this anticonvulsant activity. Thus amine stores rather than synthesis appeared to be involved. As a further check, the effect of reserpine was investigated. Although reserpine produced a depletion of noradrenaline, dopamine and 5-HT, it was without effect on the anticonvulsant activity of chlordiazepoxide. It followed that the difference in depletion of amines would explain the lack of effect of reserpine. αMMT, the depleting agent which inhibited the anticonvulsant action, is metabolised to metaraminol which in turn displaces noradrenaline from the stores (for references see Chapter 4 - α-Methyl-m-tyrosine). This displacement is achieved by uptake of

metaraminol into the reserpine-resistant stores which is then transferred to the reserpine-sensitive stores (Lundborg & Stitzel, 1967). It follows that metaraminol not only depletes reserpine-sensitive but also reserpine-resistant noradrenaline stores, and herein lies the difference between αMMT-induced depletion and reserpine-induced depletion. αMMT depletes reserpine-resistant catecholamine stores and inhibits the anticonvulsant activity of chlordiazepoxide. Reserpine cannot deplete the resistant stores and is without effect on the anticonvulsant activity. Therefore, chlordiazepoxide requires intact reserpine-resistant catecholamine stores to achieve its anticonvulsant activity. The blocking effect of αMPT can be explained by assuming that inhibition of catecholamine synthesis first depletes the reserpine-resistant stores. This can be envisaged if newly synthesised catecholamines are first taken up into the reserpine-resistant stores and thereafter transferred to reserpine-sensitive stores. This being the case, inhibition of synthesis would allow transference of catecholamines from resistant to sensitive stores but would not allow repletion of the resistant stores. Inhibition of noradrenaline synthesis with DDC did not reduce the anticonvulsant activity of chlordiazepoxide. It is therefore assumed that dopamine rather than noradrenaline is the catecholamine involved. Corrodi, Fuxe and Hokfelt (1967) have demonstrated that chlordiazepoxide in extremely large doses (300mg/kg to rats) does not influence the activity of central catecholamine neurones, however this high dose did elevate central dopamine concentrations by 30%. Sharman (1966) has demonstrated that chlordiazepoxide in non-toxic doses (50mg/kg to mice) does not alter the metabolism of central dopamine. Thus the elevation of dopamine reported by Corrodi and others (1967) is only produced as a result of using a very high dose. Taken together these two reports

show that chlordiazepoxide in doses less than 50mg/kg is without effect on catecholamine neurones or stores. Our results suggest that reserpine-resistant dopamine stores are utilised by chlordiazepoxide and are in conflict with the above reports. However, an elevation or depletion of such a small pool would not be detected by the method used by Sharman (1966) and Corrodi and others (1967).

In conclusion it has been demonstrated that, despite a report that barbiturates decrease impulse flow in dopamine neurones (Corrodi, Fuxe & Hokfelt, 1966), adrenergic mechanisms are unlikely to be involved in the anticonvulsant activity of phenobarbitone. Diphenylhydantoin remained inactive against leptazol-induced clonus after inhibition of synthesis of either 5-HT, noradrenaline or dopamine. The anticonvulsant activity of chlordiazepoxide against leptazol has been demonstrated to be dependent on the disposition of central catecholamines, and reserpine-resistant catecholamine stores are involved. Possibly intact reserpine-resistant dopamine stores rather than noradrenaline stores are the more necessary for this anticonvulsant activity.

TABLE 41

EFFECT OF THE AMINE SYNTHESIS INHIBITORS, α-METHYL-p-TYROSINE (αMPT, 100mg/kg, INTRAPERITONEALLY), DIETHYLDITHIOCARBAMATE (DDC, 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE (pClPhA 320mg/kg, INTRAPERITONEALLY) ON THE ANTICONVULSANT ACTIVITY OF PHENOBARBITONE SODIUM (30mg/kg, INTRAPERITONEALLY) AGAINST LEPTAZOL IN RATS

Pretreatment	% Maximum Clonic Convulsions
Controls (untreated) Leptazol 65mg/kg, subcutaneously	45 ± 3 (4)
Phenobarbitone sodium 30mg/kg intraperitoneally 1½ hours. Leptazol 65mg/kg, subcutaneously.	0.0 ± 0 (3)
αMPT 100mg/kg intraperitoneally 4 hours Phenobarbitone sodium 30mg/kg intraperitoneally 1½ hours. Leptazol 65mg/kg, subcutaneously.	3.3 ± 3.3 (3)
DDC 400mg/kg intraperitoneally 4 hours. Phenobarbitone sodium 30mg/kg intraperitoneally 1½ hours. Leptazol 65mg/kg subcutaneously.	3.3 ± 3.3 (3)
pClPhA 320mg/kg intraperitoneally 3 days. Phenobarbitone sodium 30mg/kg intraperitoneally 1½ hours. Leptazol 65mg/kg subcutaneously.	0.0 ± 0 (2)

Results are expressed as mean ± standard error.

Figures in parentheses indicate the number of determinations made.

TABLE 42

EFFECT OF AMINE SYNTHESIS INHIBITORS, α-METHYL-p-TYROSINE (αMPT 100mg/kg, INTRAPERITONEALLY), DIETHYLDITHIOCARBAMATE (DDC 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE (pClPhA 320mg/kg, INTRAPERITONEALLY) ON THE ACTIVITY OF DIPHENYLHYDANTOIN IN THE LEPTAZOL SEIZURE THRESHOLD TEST IN RATS

Pretreatment	% Maximum Clonic Convulsions
Controls (untreated) Leptazol 65mg/kg, subcutaneously.	45 ± 3 (4)
Diphenylhydantoin 40mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg, subcutaneously.	58 ± 2 (5)
αMPT 100mg/kg intraperitoneally 4 hours. Diphenylhydantoin 40mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg, subcutaneously.	53.3 ± 3.3 (3)
DDC 400mg/kg intraperitoneally 4 hours. Diphenylhydantoin 40mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg, subcutaneously.	55 ± 5 (2)
pClPhA 320mg/kg intraperitoneally 3 days. Diphenylhydantoin 40mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg, subcutaneously.	55 ± 5 (2)

Results are expressed as mean ± standard error.

Figures in parentheses indicate the number of determinations made.

TABLE 43

EFFECT OF AMINE SYNTHESIS INHIBITORS, α-METHYL-p-TYROSINE (αMPT 100mg/kg, INTRAPERITONEALLY), DIETHYLDITHIOCARBAMATE (DDC 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE (pClPhA 320mg/kg, INTRAPERITONEALLY) ON THE ANTICONVULSANT ACTIVITY OF CHLORDIAZEPOXIDE (CDZP 2.5mg/kg, INTRAPERITONEALLY) IN RATS

Pretreatment	% Maximum Clonic Convulsions
Controls (untreated) Leptazol 65mg/kg subcutaneously.	45 ± 3 (4)
CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	3.3 ± 3.3 (3)
αMPT 100mg/kg intraperitoneally 4 hours. CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	30 ± 2 (3) **
DDC 400mg/kg intraperitoneally 4 hours. CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	10 ± 6 (3)
pClPhA 320mg/kg intraperitoneally 3 days. CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	6.7 ± 3.3

Results are expressed as mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 38

EFFECT OF AMINE SYNTHESIS INHIBITORS, α-METHYL-p-TYROSINE (αMPT, 100mg/kg, INTRAPERITONEALLY), DIETHYLDITHIOCARBAMATE (DDC, 400mg/kg, INTRAPERITONEALLY) AND p-CHLOROPHENYLALANINE pClPhA 320mg/kg, INTRAPERITONEALLY) ON THE ANTICONVULSANT ACTIVITY OF CHLORDIAZEPOXIDE (CDZP) 2.5mg/kg, INTRAPERITONEALLY) AGAINST LEPTAZOL IN RATS

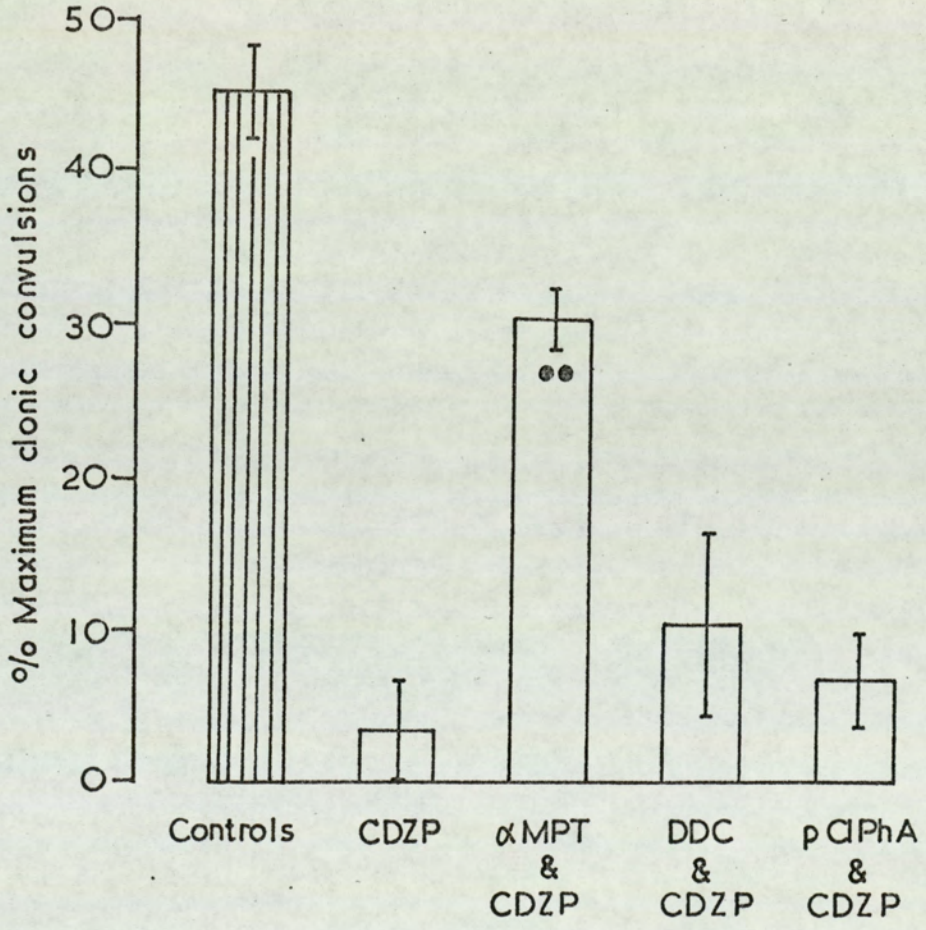


TABLE 44

EFFECT OF α-METHYL-m-TYROSINE (α MMT 100mg/kg, INTRAPERITONEALLY) AND RESERPINE (200 µg/kg, INTRAVENOUSLY) ON THE ANTICONVULSANT ACTIVITY OF CHLORDIAZEPOXIDE (CDZP 2.5mg/kg, INTRAPERITONEALLY) AGAINST LEPTAZOL IN RATS

Pretreatment	% Maximum Clonic Convulsions
Controls (untreated) Leptazol 65mg/kg subcutaneously.	45 ± 3 (4)
CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	3.3 ± 3.3 (3)
α MMT 100mg/kg intraperitoneally 4 hours. CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	26.7 ± 3.3 (3) **
Reserpine 200 µg/kg intravenously 4 hours. CDZP 2.5mg/kg intraperitoneally 30 mins. Leptazol 65mg/kg subcutaneously.	0.0 ± 0 (2)

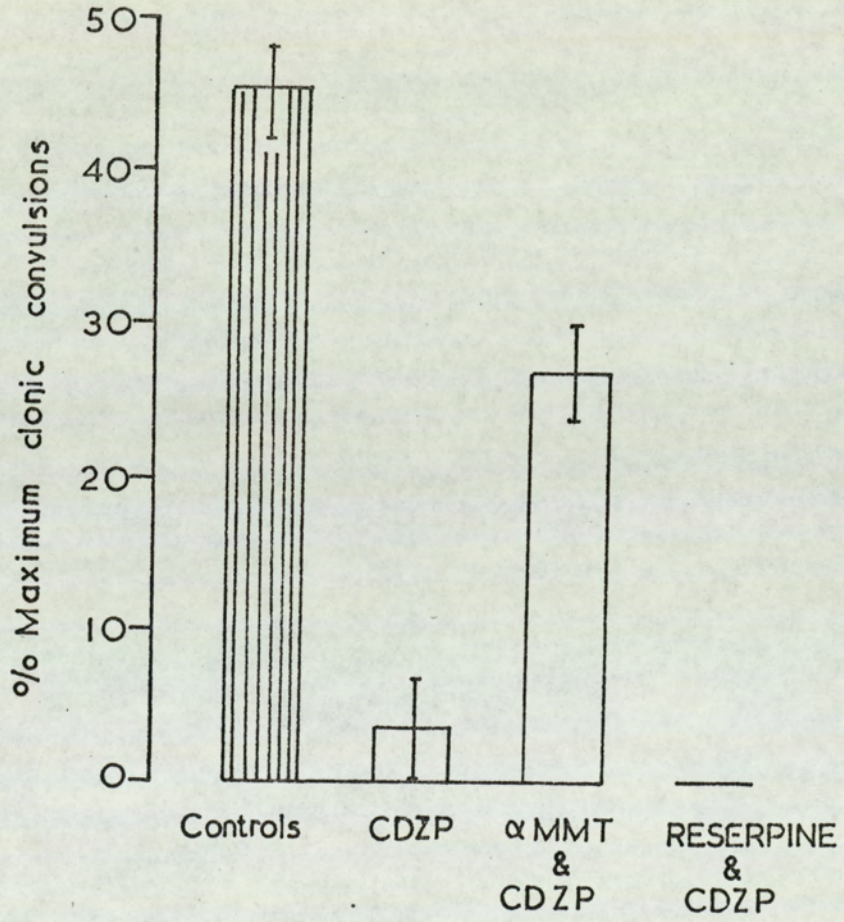
Results are expressed as mean ± standard error.

Figures in parentheses indicate the number of determinations made.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

FIGURE 39

EFFECT OF α-METHYL-m-TYROSINE (α MMT, 100mg/kg, INTRAPERITONEALLY) AND RESERPINE (200 µg/kg, INTRAVENOUSLY) ON THE ANTICONVULSANT ACTIVITY OF CHLORDIAZEPOXIDE (2.5mg/kg, INTRAPERITONEALLY) AGAINST LEPTAZOL IN RATS



CHAPTER EIGHT

GENERAL DISCUSSION

GENERAL DISCUSSION

The literature contains considerable evidence that interference with central aminergic mechanisms (using such drugs as MAO inhibitors, amphetamine, reserpine, etc.), can alter the convulsive threshold of animals to leptazol. So too can interference with cholinergic mechanisms or central GABA concentrations. The majority of previous evidence favoured the involvement of aminergic mechanisms in leptazol convulsions. There was also similar evidence for aminergic involvement in audiogenic and electroshock convulsions, two areas of anticonvulsant testing where evidence of other neurohumoral mechanisms was not so convincing.

Chlordiazepoxide, an effective tranquillizer, is also known to be a potent anticonvulsant (Rosenstein, 1960; Randall, Heise & others, 1961). Since many other psychoactive drugs are thought to exert their action via the central adrenergic system and are also reported, by other workers, to possess anticonvulsant activity, it was reasoned that chlordiazepoxide too might exert its anticonvulsant effect via an adrenergic mechanism. This was the rationale behind the decision to investigate the possible involvement of aminergic (adrenergic & tryptaminergic) mechanisms in leptazol-induced convulsions. The mode of action of leptazol is obscure, but leptazol has been used successfully clinically, albeit empirically, as an antidepressant therapy. It has also been demonstrated to activate the sympathetic division of the autonomic nervous system in subconvulsant and convulsant doses (see review by Hahn, (1960)). Therefore leptazol might utilise the central aminergic system in some way.

Our aims were two-fold: firstly to investigate, via its convulsive action, the possible interaction of leptazol with central aminergic mechanisms, and secondly to investigate the possible interaction of chlordiazepoxide with similar or the same mechanisms, via its anticonvulsive action. It was hoped that this might contribute to the elucidation of the modes of action of both leptazol and chlordiazepoxide.

The means of investigating aminergic mechanisms was by simple measurement of amine concentrations in rat brain after various types of pretreatment. A method of assay in which noradrenaline, dopamine and 5-HT could be determined from the same extract was required. A large number of assay procedures were available but in most cases the extraction procedure recovered either the catecholamines or 5-HT, but not both. The solvent extraction of 5-HT (Udenfriend, Weissbach & Clark, 1955) was similar to that for catecholamines (Shore & Olin, 1958), but the former was carried out at pH 10 and at this pH the catecholamines are rapidly destroyed. However, it has been shown recently that the extraction procedure for catecholamines, using 0.4N perchloric acid (Bertler & others, 1958), also extracts 5-HT (Cox & Potkonjak, 1967). In our hands the catecholamine assay of Cox and Potkonjak (1967) was less sensitive than that of Bertler and others (1958) and so we combined the two, using the catecholamine assay of Bertler and others (1958), and the 5-HT assay of Cox and Potkonjak (1967). Therefore, these amines were separated on different ion exchange resins using equal portions of the same extract. Because of the time taken to develop this, it has not been possible to investigate all possible aminergic mechanisms, and this project should be considered as an initial investigation providing pointers to further work.

The investigation of the convulsive effects of leptazol in Chapter 1 clearly indicated that the method of inducing leptazol convulsions, best suited to this project, was the subcutaneous leptazol test. Because our strain of rats (although ideal in every other way) did not exhibit a consistent tonic component, we were restricted to using the clonic component as the criterion by which drugs were assessed as pro- or anti- convulsant. Nevertheless, to elicit clonic convulsions, a smaller dose of leptazol was required, and it was considered that this might detect weakly pro- or anti-convulsant drugs better than the higher doses of leptazol that would have been required to elicit a tonic component. It was appreciated that certain types of drug, which are only anticonvulsant against the tonic component, e.g. diphenylhydantoin, might be overlooked. However, since the pattern of leptazol convulsions is clonic-tonic, any anti-leptazol drug should show an effect on the clonic component if it is to be considered anticonvulsant. Rats were used not only because they showed more consistent control levels of convulsive activity but also because they were more convenient for the subsequent assay work.

Determinations of biogenic amine concentrations in whole brain and discrete areas of the rat brain revealed no changes either before, during or after leptazol induced clonic or tonic convulsions. It follows that any possible aminergic mechanism of leptazol is not revealed by simple determination of the levels of these amines. Therefore, indirect methods using aminergic drugs to alter the disposition of biogenic amines were next investigated. The MAO inhibitors have been reported to be anticonvulsants (Chow & Hendley, 1959; Prockop, Shore & Brodie, 1959 a,b), yet we were unable to

reproduce this anticonvulsant activity using tranylcypromine, pargyline, phenelzine, nialamide and iproniazid. In this investigation the anticonvulsant activity was tested against the clonic component in rats and the tonic component in mice, both results being essentially the same. There was however some proconvulsant activity with tranylcypromine, phenelzine and iproniazid, and this was correlated with their inherent sympathomimetic activity; subsequently dexamphetamine proved to be a potent proconvulsant. Tranylcypromine displayed a transient anticonvulsant activity but this appeared to be a post-ictal 'rebound' effect as a result of earlier intense adrenergic stimulation. It was only possible to conclude that elevation of central amine levels, as a consequence of MAO inhibition, was without anticonvulsant activity. A similar elevation of central amines with the amine precursors, DL-DOPA and DL-5-HTP, and the subsequent lack of effect on the leptazol threshold supported this conclusion. Inhibition of the metabolism of extracellular catecholamines by catecol-O-methyl-transferase, using pyrogallol, catechol and β-thujaplicin, did not protect rats from the convulsive action leptazol.

Depletion of central amines was proconvulsant with leptazol, but only if all three amines studied were simultaneously and almost totally depleted. Thus depletion with reserpine was not proconvulsant if only noradrenaline and dopamine were totally depleted. It required a higher dose of reserpine (400 μg/kg, intravenously) to further deplete the 5-HT and initiate the proconvulsant mechanism. Depletion with syrosingopine had the same charateristics, and specific depletion of the catecholamines, noradrenaline and dopamine, with αMMT was without effect. It appeared that the concentration of 5-HT was the factor which triggered the proconvulsant activity especially when it was demonstrated that depletion with αMMT was proconvulsant if the

5-HT had been previously depleted with pClPhA. Nevertheless, depletion of 5-HT *per se* was without effect on the leptazol threshold and it was concluded that depletion of all three amines was essential to demonstrate any proconvulsant activity. Repletion of one amine, for example 5-HT with 5-HTP, totally abolished the proconvulsant activity of reserpine; a similar repletion of catecholamines with DL-DOPA reduced the proconvulsant activity by about 50%. It appeared that 5-HTP was a more potent anticonvulsant than DL-DOPA, although the determination of central amines after this pretreatment showed that the catecholamine stores were not repleted after DL-DOPA as effectively as were those of 5-HT after 5-HTP. This suggested, that reserpine depletes the catecholamines more easily than it depletes 5-HT, for both these precursors would be synthesised to their respective amines. However, the catecholamines were not taken up into the storage granules as easily as 5-HT. Therefore, the only partial anticonvulsant activity of DL-DOPA, compared with that of 5-HTP, may be attributed to this reduced uptake of catecholamines. It follows that if catecholamine stores could be selectively and totally repleted, then this should totally abolish the proconvulsant activity of reserpine. The results of depletion with reserpine and syrosingopine, and the results of repletion with amine precursors after reserpine, all suggest that reserpine and syrosingopine act mainly on catecholamine stores and less effectively on 5-HT stores. It is suggested that a more extensive investigation of amine levels after smaller doses of syrosingopine would reveal a definite selective action on catecholamine stores whilst leaving 5-HT stores intact, and a similar effect might be seen with the smaller doses of reserpine. Initially it was considered that the rapid release of catecholamines

might be the proconvulsant mechanism rather than the subsequent depletion, but this proconvulsant activity was evident 3 or 4 days after a single dose of reserpine. The lack of biogenic amines is therefore the proconvulsant factor and a direct effect of the biogenic amines themselves does not appear to be involved in this proconvulsant activity. The fact that such an extensive depletion is necessary to produce a proconvulsant effect indicates that the depletion of the large proportion of the amines stores has no effect on the convulsive mechanism but that depletion of the remaining, (more resistant) store is responsible for the proconvulsant activity. In this connection, depletion of the reserpine-resistant stores with αMMT reduced the anticonvulsant activity of chlordiazepoxide. It follows that reserpine-resistant stores might contribute an anticonvulsant influence under normal conditions and that reserpine in sufficient doses might deplete these normally resistant stores. When the anticonvulsant influence is removed, proconvulsant activity ensues. Yet, the fact that depletion of reserpine-resistant stores with αMMT does not potentiate leptazol, tends to contradict this hypothesis. Alternatively it would seem that severe depletion of all stores, which must include the reserpine-resistant catecholamine stores, is necessary to reveal the proconvulsant activity. The fact that αMMT is proconvulsant after 5-HT has been depleted with pClPhA is support for this latter explanation. This concept of an overall depletion producing a proconvulsant activity is discussed later in connection with a possible effect on the extrapyramidal system.

The uptake mechanism at the level of the cell membrane, another reserpine-resistant site, does not appear to control convulsive activity; inhibition of this mechanism with imipramine-like drugs,

(imipramine, desipramine and protriptyline), was without effect on the convulsive threshold to leptazol.

A second group of potent proconvulsant agents was the amphetamine-like drugs, dexamphetamine and fencamfamin. It is interesting to note that dexamphetamine exerts its proconvulsant activity by stimulation of adrenergic receptors, whereas reserpine mediated its proconvulsant effect via depletion of biogenic amines. In low doses (5mg/kg), dexamphetamine was shown to exert its proconvulsant action via newly synthesised dopamine. Inhibition of catecholamine synthesis with αMPT blocked this proconvulsant effect of dexamphetamine, but selective inhibition of either 5-HT or noradrenaline syntheses did not. However, at higher doses of dexamphetamine (10mg/kg), the proconvulsant effect appeared now to be due to a direct stimulation of adrenergic receptors, because this action was no longer blocked by αMPT. Thus dexamphetamine, at low doses, exerts a proconvulsant effect by an indirect mechanism involving newly synthesised dopamine, and by a direct effect at higher doses. The mechanism by which dexamphetamine exerts an indirect proconvulsant effect is not simply the same mechanism by which leptazol exerts its convulsive effect for, although this proconvulsant activity of dexamphetamine can be blocked by αMPT, the convulsive effect of leptazol cannot. It is possible that leptazol might exert a direct stimulation of central synapses similar to that produced by higher doses of dexamphetamine (10mg/kg) at adrenergic receptors. Fencamfamin, a central stimulant with little peripheral activity (Hotovy, Enenkel & others, 1961), was shown to possess a marked proconvulsant activity which was not blocked by inhibition of any of the syntheses studied. It therefore resembled the higher doses of dexamphetamine in that it appeared to exert a direct stimulation of central receptors. Fencamfamin was not unlike

leptazol since neither agent was blocked by αMPT, and both agents have been accused, in this project, of producing a direct stimulation of central receptors. This possible direct effect is later discussed in relation to the extrapyramidal system.

The anti-adrenergic drugs pronethalol, phentolamine and guanethidine, were not effective anticonvulsants. Because guanethidine, an adrenergic neurone blocking agent, was ineffective against the clonic component of leptazol convulsions, and that a similar dose (5mg/kg) has been shown previously to deplete central noradrenaline (Pfeifer & Galambos, 1967 a), it must be assumed that central release of noradrenaline at nerve endings is not involved in these clonic convulsions. The inactivity of phentolamine and pronethalol might be evidence that leptazol does not act directly on central adrenergic receptors for blockade of either α or β receptors would be expected to block this effect. However it has not yet been demonstrated conclusively that α and β receptors exist in the CNS, and the work of Murmann and others (1966) suggests that any central activity that these drugs might have is a result of CNS depressant effects. We did demonstrate that phentolamine exerted an anticonvulsant effect by delaying the onset of convulsions, but that this was due to α receptor blockade is by no means certain.

The brief investigation of the anticonvulsants revealed no adrenergic component in the anticonvulsant mechanism of phenobarbitone; the activity of phenobarbitone was unaffected by depletion of catecholamine stores with αMMT, or by inhibition of amine synthesis and depletion of stores with specific synthesis inhibitors. It is of course possible that the decreased neuronal flow in dopamine neurons cited by Corrodi, Fuxe and Hokfelt (1966) is the anticonvulsant mechanism, for the adrenergic drugs used in the present investigation do not enhance or block this decrease in neuronal flow, they only

reveal it. A decreased neuronal flow in catecholamine neurone is not produced by chlordiazepoxide (Corrodi, Fuxe & Hokfelt, 1967), which is also an anticonvulsant: however, it is unlikely that all anticonvulsants have a common mode of action. The anticonvulsant activity of chlordiazepoxide was clearly demonstrated to be reduced by interference with adrenergic mechanisms: both inhibition of synthesis with αMPT and depletion of stores with αMMT reduced its anticonvulsant effect. Neither inhibition of only noradrenaline synthesis, (with DDC), nor depletion of amine stores, (with reserpine), affected this anticonvulsant activity. It was concluded that depletion of dopamine was responsible for the reduced activity of chlordiazepoxide, and that only the reserpine-resistant dopamine stores appeared to be involved.

These results taken as a whole allow several tentative conclusions. It is not possible to say that any one of the biogenic amines studied is directly responsible for leptazol-induced convulsions. However the adrenergic proconvulsant drugs indicate that adrenergic pathways are involved in the convulsive mechanism. The proconvulsant activity of reserpine was shown to correlate with an extensive depletion of biogenic amines which probably included the reserpine-resistant dopamine stores. The same mechanism was not responsible for the convulsions after leptazol per se because leptazol did not produce a depletion of any of the amines studied. Dexamphetamine was shown to exert an indirect effect via newly synthesised dopamine and possibly a direct effect on receptors, both of which potentiated leptazol convulsions. It has been suggested that leptazol resembles more closely the direct effect of dexamphetamine for neither are blocked by αMPT. Fencamfamin also exerts a direct effect which is not blocked by αMPT and leptazol resembles more closely fencamfamin than it does dexamphetamine; neither

fencamfamin (Hotovy, Enenkel & others, 1961) nor leptazol exert significant peripheral activity (the peripheral effects of leptazol are considered to be mediated centrally, see Hahn (1960)) whereas dexamphetamine possesses considerable peripheral activity. In this connection it is interesting to note that many of our results imply that dopamine might be involved (e.g. - chlordiazepoxide, reserpine and dexamphetamine results). If fencamfamin and leptazol exert a common mode of action i.e. direct stimulation of central dopamine receptors, this would explain their lack of peripheral effects (there is so far, no direct evidence of true dopaminergic neurons in the periphery and no transmitter function for peripheral dopamine can be postulated (Hornykiewicz, 1966). The possibility of such a direct action of leptazol and the possible involvement of dopamine suggests the extrapyramidal centres as the site of such actions. This system is responsible for motor coordination and dopamine has been implicated as the main transmitter at this site (Hornykiewicz, 1966). It is tentatively suggested that interference with the disposition of dopamine in the extrapyramidal centres might remove the inhibitory influence of the descending reticular formation and allow a predominance of the facilitatory effects of such pathways as the vestibulo-spinal tract: the result being potentiation of convulsive mechanisms. For example, it is possible that dexamphetamine, which exerts its indirect mechanism via newly synthesised dopamine, might ~~block~~ potentiate the ~~inhibitory~~ facilitatory influence of the ~~descending~~ ascending reticular formation and potentiate convulsions; ~~and that~~ Reserpine removes dopamine, the inhibitory influence in the descending reticular system and thereby facilitates convulsions; and that chlordiazepoxide exerts its anticonvulsant effects via reserpine-resistant dopamine in the ~~extrapyramidal~~ descending system, so that removal of this dopamine (with αMMT) removes the inhibitory influence and blocks the anticonvulsant effect of

chlordiazepoxide. The earlier postulate, that leptazol exerts a direct effect in this system, is best explained by assuming that leptazol acts by direct stimulation of dopaminergic receptors in the ascending reticular activating system, a system in apposition to the descending reticular formation. It is suggested that such a stimulation of the reticular activating system leads to subsequent stimulation of the cortex and then convulsions. It is possible that the proposed direct effect of dexamphetamine and fencamfamin is exerted on the same ascending reticular pathway as leptazol, but since neither dexamphetamine nor fencamfamin produce convulsions, it is ~~more~~ also likely that they interact with the descending inhibitory pathways and produce a predominance of facilitatory effects. The possible involvement of these two pathways in apposition also explains why drugs which potentiate convulsions can be blocked by drugs which do not block leptazol itself. In support of this proposed mechanism of convulsions, there is already evidence in the literature that leptazol interacts with a neurotransmitter in the reticular activating system (Ingvar, 1954; Purpura, 1956), and that leptazol acts chiefly by stimulation of excitatory synapses at this site (Purpura & Grundfest, 1957).

In conclusion, aminergic drugs have been investigated for their effects on leptazol convulsions. The results at this stage only allow a tentative hypothesis that leptazol might exert its convulsive activity by stimulation of excitatory synapses in the reticular activating system and that certain aminergic drugs potentiate convulsions by reducing the inhibitory influence of the descending reticular formation. It is also suggested that dopaminergic receptors are involved. Furthermore, the use of certain adrenergic drugs has produced additional information on the actions of dexamphetamine and

chlordiazepoxide, and has suggested that reserpine, and certainly syrosingopine, in accurately controlled doses might be used as selective depleting agents of catecholamines.

Clinical implications

The original project had three possible clinical implications; firstly a possible pharmacological basis of antidepressant action of leptazol, secondly a possible explanation of its convulsant action, and finally an explanation of the anticonvulsant activity of chlordiazepoxide.

The first we have failed to do, since it seems hardly likely that depression originates from the extrapyramidal centres, which normally control motor coordination rather than emotion.

The second we have achieved in part, for a mechanism by which leptazol exerts its convulsant effects has been suggested. A mechanism by which these convulsive effects of leptazol are potentiated by aminergic drugs has also been proposed. The mechanisms involve dopamine receptors in the extrapyramidal system and as such might suggest a link between leptazol convulsions and Parkinsonism.

Finally, we have shown that the anticonvulsant activity of chlordiazepoxide can be affected by the disposition of catecholamines. This does not contradict the conclusions of other workers, who have shown chlordiazepoxide to alter the electrical activity of the brain. Electrical activity of the brain and biogenic amines may be interrelated (e.g. decreased neuronal impulse flow might reduce electrical activity and amine release). The result with chlordiazepoxide has a further

implication in that it was affected by aminergic drugs whereas phenobarbitone was not. If further similar work with other benzodiazepines produces the same results it might be possible to use this procedure for the separation of chlordiazepoxide-like drugs from compounds simply found to be anticonvulsant. This in turn would mean that such anticonvulsants might be useful minor tranquillizers.

S E C T I O N F I V E

APPENDICES

DRUGS AND VEHICLES USED

All drugs were dissolved in N.saline except where otherwise stated. Doses quoted are in terms of free base.

Leptazol (Pentylenetetrazol)	-	Sigma
Dexamphetamine sulphate	-	Ward Blenkinsop
Tranylcypromine sulphate	-	Smith, Kline and French (Parnate)
Pargyline hydrochloride	-	Abbotts Laboratories (Eutonyl)
Phenelzine hydrogen sulphate		
Iproniazid phosphate	-	Roche Ltd. (Marsilid)
Nialamide	-	Pfizer Ltd. (Nialamid)
DL-DOPA DL-5-HTP	-	Sigma suspended in N.saline, stirred before withdrawal of each dose.
Pyrogallol	-	BDH
Catechol	-	BDH
β-Thujaplicin	-	Koch-Light Labs. Ltd. dissolved in 1 ml dimethyl sulphoxide and diluted slowly to 10 ml with distilled water.
Imipramine hydrochloride	-	Geigy (Tofranil)
Desipramine hydrochloride	-	Geigy (Pertofran)
Protriptyline hydrochloride	-	Merck, Sharpe and Dohme.
α-Methyl-p-tyrosine	-	Regis Chemical Co. - (RM 2700) suspended in N.saline, stirred before withdrawal of each dose.
Diethyldithiocarbamate (disodium salt)	-	BDH

p-Chlorophenylalanine methyl ester hydrochloride	-	Pfizer Ltd.
Reserpine	-	BDH 20 mg dissolved in 0.4 ml propylene glycol, 0.4 ml 95% ethyl alcohol, 4 drops lactic acid, warmed on a water bath to drive off alcohol, made up to 10 ml with distilled water. Further dilutions with distilled water.
Syrosingopine	-	Nicholas Research Institute same vehicle as reserpine.
α-Methyl-m-tyrosine	-	Regis Chemical Co. (RM 2600)
Fencamfamin hydrochloride	-	Allenbury's
Phentolamine methane sulphonate	-	Ciba Ltd. (Rogitine)
Pronethalol hydrochloride	-	I.C.I. Ltd. (Alderlin)
Guanethidine monosulphate	-	Ciba Ltd. (Ismelin)
Diphenylhydantoin	-	Parke-Davis (Epanutin)
Phenobarbitone sodium	-	BDH
Chlordiazepoxide hydrochloride	-	Roche Ltd. (Librium)

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