The effects of chronic methamphetamine treatment on amine metabolism in rat brain in vivo.

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THE EFFECTS OF CHRONIC METHAMPHETAMINE TREATMENT ON AMINE METABOLISM IN RAT BRAIN *IN VIVO*

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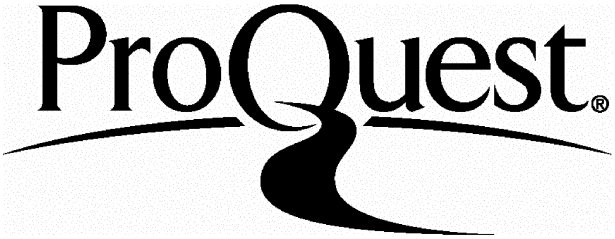

TO MY PARENTS

The Gods did not reveal, from the beginning,
All things to us, but in the course of time
Through seeking we may learn and know things better.
But as for certain truth, no man has known it,
Nor shall he know it, neither of the gods
Nor yet of all the things of which I speak.
For if by chance he were to utter
The final truth, he would himself not know it:
For all is but a woven web of guesses.

Xenophanes

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CONTENTS

I. ABSTRACT

Rats were chronically treated with methamphetamine over a seven week period during which time their weights, food consumption and drinking rates were recorded. The effects of chronic treatment with, and withdrawal from, methamphetamine on amine metabolism in selected brain regions was investigated. Three groups of experimental animals were studied: chronically treated rats, rats which had been withdrawn from the drug for 36 h and controls.

The parameters chosen to reflect changes in catecholamine metabolism in the brain were: tyrosine hydroxylase activity and the levels of noradrenaline, dopamine and their non-O-methylated metabolites and of phenylalanine and tyrosine. The metabolism of serotonin was studied by measuring the levels of "free" tryptophan in the plasma,and the levels of tryptophan and serotonin in the brain.

Initially the methamphetamine was administered in the drinking water at doses of 5, 10, 20 and 40 mg/kg/24 h increasing in a stepwise manner over a period of 20 days. This dose was found to produce toxic reactions in the rats and it was found that it was not possible to control the intake of the drug. In subsequent experiments the rats were given 5, 10 and 15 mg/kg/24 h i.p. increasing in a stepwise manner over a period of 15 days.

The first set of injected animals did not develop tolerance to the anorexic effects of methamphetamine and the chronically treated and withdrawn animals showed changes in tyrosine hydroxylase activity and in the levels of noradrenaline and dopamine.

The three other sets of injected animals did develop tolerance to the anorexic effects of methamphetamine. Tyrosine hydroxylase and the levels of noradrenaline, dopamine, their non-O-methylated metabolites and of tryptophan and serotonin did not change in these chronically-treated or withdrawn animals, but changes were seen in the levels of "free" tryptophan in the plasma and the levels of most amino acids in the brain.

II. INTRODUCTION

A. CLINICAL ASPECTS OF AMPHETAMINE USE

Historically the study of amphetamines began in 1887 when Edeleano prepared the volatile amine phenylisopropylamine (amphetamine). Barger and Dale (1910) found that it was related to adrenaline in its pharmacological actions (see Connell, 1970). In 1927 Alles synthesized amphetamine in the course of a search for a substance to replace ephedrine, which was difficult to obtain from its natural sources. This led to an exploration of the clinical uses of this group of drugs and eventually to the production of an amphetamine sulphate inhaler in 1932. Further studies led to the separation of amphetamine into dextro and laevo isomers, the former being now well known as dexamphetamine. Emde (1929) prepared the drug now known as methylamphetamine.

There are more than fifty preparations of amphetamine substances either alone or in combination with other drugs (notably barbiturates), on the market at the present time, the well known ones including amphetamine itself ('durophet'), dexamphetamine ('dexedrine'), methylamphetamine ('methedrine') and phenmetrazine ('preludin'). Some of the structures, together with the catecholamines are shown in Figure 1.

The amphetamines are a group of structurally related compounds with sympathomimetic and central stimulant properties. They were first used in medical practice in the early 1930's for the treatment of allergies (specifically those involving bronchial spasms), depression, fatigue, obesity and Parkinson's disease. Owing to their wide range of actions they rapidly came onto the general market as a general panacea to 'cure all ills'. When the addictive properties of the amphetamines were realised they were rapidly removed from the market.

Owing to the undesirable side-effects of the amphetamines which, apart from leading to dependence, have been suggested to produce behavioural and other physical pathology with prolonged use at high doses, their clinical use has been curtailed (Honigfeld and Howard, 1973; A.M.A. Drug Evaluations, 1973). Their efficacy in the treatment of depression and Parkinson's disease is also questionable. It appears that only certain forms of depression are relieved by amphetamine treatment. Amphetamine or amphetamine-barbiturate combinations have been used on a variety of depressed patients (General Practitioner Research Group, 1964; Hare *et al.*, 1962; Overall *et al.*, 1962). In all studies amphetamine was found to be no more effective than placebo, and, in some cases, patients treated with amphetamine became worse. However, Weil-Malherbe and Szara claim that amphetamine is "undoubtedly an effective antidepressant" (Weil-Malherbe and Szara, 1971), but as no reference is given to any clinical trials it is impossible to assess this statement.

Studies carried out by Finkelman and Shapiro (1937) and Davis and Stewart (1938) on patients with post-encephalitic Parkinson's disease showed that although subjective improvements in mood and energy were observed together with reversal of disordered sleep rhythms, there was no objective evidence for any improvement in the motor disturbances characteristic of Parkinson's disease. Amphetamines however did have a dramatic effect on the oculogyric crisis of post-encephalitic Parkinsonism, which was often abolished (Davis and Stewart, 1938; Matthews,1938). It is perhaps interesting to note that patients with post-encephalitic Parkinson's disease showed no signs of developing tolerance or becoming addicted to amphetamine (Solomon *et al.*, 1937).

The amphetamines are still used clinically in the treatment of narcolepsy, obesity, epilepsy, depression and various psychological disturbances which do not respond to other forms of treatment. Narcolepsy was probably the first condition for which amphetamine was used clinically (Prinzmetal & Bloomberg, 1935). It revolutionized therapy in the condition, and although not curative, in that it does nothing to eradicate the underlying disturbance, the drug may enable the patient to become symptom-free. Amphetamine was first used in epilepsy because of its action in antagonizing the sedative effects of narcotics. It was later found that amphetamine itself may have beneficial effects, particularly in the milder epileptic states. However, owing to differing responses, when given it is usually in combination with other anticonvulsant drugs (Connell, 1968).

The property of amphetamine sulphate in modifying antisocial behaviour in children was reported by Bradley & Bowen (1941). Children who were withdrawn or lethargic became more alert and sociable, and aggressive, noisy children who were hyperactive tended to calm down. Large doses were readily tolerated and as much as 60 mg of amphetamine sulphate a day was used. More recently the use of amphetamines in the treatment of hyperkinetic syndrome in children with presumed "minimal brain dysfunction" (Klein & Davis, 1969) in America has evoked controversy and the accusation that the children are being "controlled for the classroom". The possibility of side-effects from large, daily doses of amphetamine has also been raised. The apparently paradoxical effects of the amphetamines on the children had been noticed previously with psychopathic patients. The current explanation given by behavioural pharmacologists of this apparent paradox is that the hyperactive

children have a different baseline of activity from normal children, and the dose-response curve for amphetamine on locomotor activity is an inverted U-shaped function. The reason for the decrease in locomotor activity at high doses is the onset of stereotypy. Consequently the hypothesis is that the hyperkinetic children may be developing a mild form of stereotypy, which is seen as increased focussing of attention and an increased ability to perform the simple repetitive tasks demanded of children in school. Associated with this is the prediction that these children would have problems with tasks demanding a wider span of attention. This theory is, however, still purely hypothetical.

Methylamphetamine has been used in psychiatric practice as an abreactive agent in the treatment of neurotic conditions and also as an aid in the diagnosis of psychiatric disorders. However the frequency of its use in psychiatric therapy is diminishing.

The general effects of amphetamine include anorexia, although tolerance occurs rapidly to this effect, and within 2 - 3 weeks of daily treatment the drug no longer depresses food intake. Amphetamine is a strong central stimulant, decreasing sleeping, fatigue and stimulating the medullary respiratory centre. The sympathomimetic properties induce a general stimulation of the sympathetic nervous system, the effects of which include increased heart rate and dilation of the bronchioles. Amphetamine can produce repetitive motor activity (stereotyped behaviour) e.g. the assembling and disassembling of a piece of mechanical equipment. It is also reported to improve attention, especially after prolonged sleep deprivation. Subjective reports in-

dicate that amphetamine causes euphoria and an increased ability to concentrate (Goodman & Gilman, 1970; Klein & Davis, 1969).

Research into the effects of the amphetamines has undoubtedly been catalysed by the outbreaks of amphetamine taking which occurred in Sweden around 1965, and in Japan in the 1950's. While the amphetamines were still generally available, young people in America started to use benzedrine-containing inhalers for their euphoriant effects. As early as this the paradoxical effects of amphetamine were noted, as some reports suggested that a form of self-medication occurred, in the sense that the amphetamines suppressed certain forms of agitated, juvenile 'psychopathic' behaviour (Eisenberg, 1963; Pasamanick, 1951).

The Swedish outbreak of amphetamine abuse began with oral amphetamine consumption by bohemian artists, writers and actors in Stockholm in the 1940's. It spread downwards in terms of status so that anti-social and criminal groups had adopted it by the 1950's. In 1958 an increased illicit supply, brought in from Spain, allowed further non-medical use, at which time other urban centres became involved and younger people became interested. In 1965 one out of every ten people arrested in Stockholm was taking phenmetrazine intravenously. Use was largely amongst males in the twenty to thirty age-group (Bejerot, 1966).

About the same time a similar situation existed in Japan (Masaki, 1956). Here, use was attributed to the availability after the war of large military supplies of amphetamine, and to marked changes in social life following defeat in war, and democratization of government.

Young people began to use amphetamine in such numbers that a survey in one city showed that 5% of people in the 16 to 25 age-group had used amphetamines; among these non-medical users about one quarter of the group were considered dependent or suffering ill-effects. By 1954 it was estimated that there were about half a million users, with half showing dependence. Even children of 12 were giving themselves intravenous injections of methylamphetamine (WHO, 1956). A later report suggested that there were one and a half million users, almost all of whom were young people (Ministry of Health and Welfare, Japan, 1964). Again a high concentration of use was reported amongst delinquents and artists.

England also experienced an increase in non-medical and disapproved amphetamine use among young people (Bewley, 1966; Scott & Wilson, 1965), In response to public concern,generated in part by newspapers, a Drug Misuse Act was passed in 1964, making it an offence to possess amphetamine without medical authority. However, medical prescription does not guarantee limited use. A study by Kiloh & Brandon (1962) showed that five hundred people in an English town of 250,000 had become dependent on amphetamine (most of these were middle-aged women). An extrapolated estimate gives a rate of 1/1000 or 2/1000 in England for such iatrogenic dependency. As for illicit use, Bewley estimated it to be approximately the same order of magnitude.

The side effects which have been noted when therapeutic doses of amphetamine are taken include mild gastro-intestinal disturbance, anorexia, dryness of the mouth, tachycardia, cardiac arrhythmias, insomnia and restlessness (Meyler, 1966). Headache, palpitations, dizziness, vasomotor disturbances, agitation, confusion, dysphoria,

apprehension and delirium are also mentioned (Goodman & Gilman, 1966). Other side effects have been noted (Rudolph, 1955) such as flushing, pallor, sensation of swaying, excessive sweating, muscular pains. Tiredness and sleepiness, lethargy and listlessness may occur when the effect wears off, together with mild depression of mood. Side-effects on withdrawal in a patient who has taken large quantities of the drug include excessive tiredness and sleepiness, but, more important, may include severe depression with suicidal ideas and the danger of suicidal attempts.

Many amphetamine addicts have been misdiagnosed as paranoid schizophrenics; this was very common during the Japanese outbreak of amphetamine use. This psychosis, which most frequently developed in people with a long history of amphetamine abuse (Kalant, 1973), was first described by Connell (1958). Connell's study was based on a study of 42 patients who took amphetamine alone. Essentially the psychosis is a paranoid psychosis in a setting of clear consciousness; the experiences of the patient are well remembered by the patient and will include ideas of persecution and hallucinations, which may be both visual and auditory. The psychosis remits quickly in a few days and before the drug has been fully excreted.

In Sweden, where young people were injecting phenmetrazine intravenously, the symptomology included ideas of persecution by the police and was called, by the Swedish workers 'police paranoia'. It was the situation in Japan which, by virtue of the large numbers of people involved, really drew attention to the dangers of dependence, and the occurrence of the amphetamine psychosis.

Oswald & Thacore (1963) referred to the ubiquity of amphetamine addiction, and demonstrated by the use of electroencephalographic methods that the normal sleep pattern was altered on withdrawal of these drugs and that disturbance of sleep continued some days or weeks after the drug could have been assumed to have been totally excreted (but see Discussion). Many of the drug-dependent adults are first put on amphetamine by their doctor; Connell (1958) noted that 11 of the 42 patients he studied had been prescribed the drug initially by doctors. Doses the patients built up ranged from 30 - 975 mg a day. Higher doses have been reported.

In summary, the amphetamines are commonly abused drugs. Epidemics of amphetamine use have centred around the 15 - 30 age group in Japan, Sweden, England and America (four of the most technically advanced nations). Methedrine, or methamphetamine,was the most common compound taken. However there is no evidence that use in itself leads to ill-effects; some who take amphetamine in excessive doses do show toxic reactions, while others taking them on a chronic basis show dependency. Either situation may precipitate a psychosis, which has come to be known as the amphetamine psychosis.

B. BIOCHEMICAL EFFECTS OF AMPHETAMINE *IN VIVO* AND *IN VITRO*

A report by Burn and Rand (1958) suggested that amphetamine and a series of other phenylethylamine derivatives exerted their peripheral sympathomimetic actions indirectly *via* the catecholamines. Since then there has been a plethora of literature on the biochemical effects of amphetamine on adrenergic neurons. It has now become clear that amphetamine affects storage, uptake, release and metabolic degradation of the catecholamines in both the peripheral and central nervous systems. I feel that it is important to consider the effects of amphetamine on the periphery as well as the brain, as it is easy to rationalize or explain changes in the brain as though it were not connected to the rest of the body.

1. Structure-Activity Relationships

The amphetamines are a group of synthetic compounds whose basic structure is shown in Figure 1, together with those of the catecholamines; adrenaline, noradrenaline and dopamine. Amphetamine, methamphetamine and the catecholamines, noradrenaline and adrenaline, have asymmetric centres giving rise to optical activity. In the case of amphetamine, rotation occurs around the α-methyl group, and with noradrenaline and dopamine, around the β-hydroxy group. The two carbon side-chain between the phenyl and amino acids is common to the amphetamines and the catecholamines.

Daly *et al.* (1966) studied the release of [^{3}H]-noradrenaline from mouse heart induced by pharmacological agents structurally related to the catecholamines. Their experiments showed the ability of (+)- and (-)-amphetamine and methamphetamine to release noradrenaline from the mouse heart. Daly showed that progressive methylation either of the amino group or of the side-chain progressively decreases the characteristic actions of amphetamine. Shifting the α-methyl group to the β position abolishes essentially all of the central and anorexic effects of amphetamine (Biel, 1970).

Amphetamine has been shown to be a potent inhibitor of noradrenaline uptake systems (Burgen & Iversen, 1965). Iversen (1965) showed that, in rat heart, there are two separate uptake mechanisms for noradrenaline and adrenaline. Uptake$_1$ operates at low concentrations of the perfused amine and Uptake$_2$ at high concentrations of the perfused amine. The kinetic parameters of the uptake mechanisms showed that they were two distinct processes. Iversen suggested that Uptake$_2$

might be brought into action after the release of noradrenaline into the synaptic cleft. The local high concentrations of noradrenaline could be rapidly removed so as to terminate its neurotransmitter actions.

Burgen & Iversen (1965) studied the inhibition of noradrenaline uptake by sympathomimetic amines. They demonstrated the importance of the α-methyl group of amphetamine in inhibiting $Uptake_1$. The (-) form was shown to be one twentieth as potent as the (+) form , and (+) methamphetamine was 0.3 times less potent than (+) amphetamine. The ability of the various amines to inhibit $Uptake_1$ correlates well with the potency of these drugs in eliciting the characteristic behavioural responses.

$Uptake_2$ showed radically different affinities for the various inhibitors tested. Opposite effects were found with β-hydroxylation and α-methylation. β-Hydroxylation increased the affinity slightly and no difference was observed between the two optical isomers. The α-methyl group on amphetamine reduced its affinity. It was interesting that the O-methylated metabolites of adrenaline and noradrenaline (metanephrine and normetanephrine) were potent inhibitors of $Uptake_2$, but relatively poor inhibitors of $Uptake_1$.

Most of the structure-activity relationships between the amphetamines have been elucidated using the sympathetic innervation of the heart as the model system, and comparing the relative abilities of the various compounds tested to bring about the release of noradrenaline or to inhibit the uptake of noradrenaline (e.g. Daly *et al.*, 1966; Burgen & Iversen, 1965).

2. Storage of Catecholamines and other putative neurotransmitters

In this section on storage I am going to limit myself to those studies which have been carried out on rats, owing to the large number of disparate reports not only within species but between species, and in view of the fact that my studies have been carried out on rats.

(i) Catecholamines in peripheral tissues

Acute injections of amphetamine, administered i.p., s.c., or i.v. to rats, caused a decrease in noradrenaline in the heart (Axelrod & Tomchick, 1960: Axelrod *et al.*, 1962; Potter & Axelrod, 1963; Lewander, 1968 a,b; Brodie *et al.*, 1970). Noradrenaline was also found to be depleted in the stomach (Brodie *et al.*, 1970). Adrenaline and noradrenaline are depleted in the adrenal gland; the extent varies with the dose. A 3.5 mg/kg injection of (+)-amphetamine tartrate has been reported to decrease adrenaline levels by 70% and noradrenaline levels by 60% (Beauvallet *et al.*, 1962a).

The chronic administration of (±)-amphetamine sulphate caused a maximal depletion of heart noradrenaline to 40 - 50% of the control value (Lewander, 1968b, 1971a). Similar results were reported by Brodie *et al.* (1970) for the heart and stomach. Tolerance to this effect has been shown to occur in the adrenal gland. Lewander (1968a, b) showed that a single injection of 20 mg/kg of (±)-amphetamine sulphate decreased the levels of adrenaline in the adrenal gland to 80% of the control value, but after chronic administration of the drug, the levels were unchanged.

(ii) Brain catecholamines

Acute experiments in which a single dose of amphetamine is

administered (i.p., s.c. or i.v.) caused a decrease in brain noradrenaline (Moore & Lariviere, 1963, 1964; Baird & Lewis, 1963, 1964; Carlsson *et al.*, 1965; Corrodi *et al.*, 1967; Lewander, 1968 a, b; Baird, 1968; Brodie *et al.*, 1969; Groppetti & Costa, 1969b; Taylor & Snyder, 1970, 1971; Costa & Groppetti, 1970; Simon *et al.*, 1970b; Lewander, 1971 a, b; Leonard & Shallice, 1971; Leonard, 1972; Costa *et al.*, 1972; Glick *et al.*, 1974). The decrease in brain noradrenaline is dose dependent (Moore & Lariviere, 1963; Baird & Lewis, 1964; Leonard & Shallice, 1971). After sublethal doses (10 - 20 mg/kg of (+)-amphetamine sulphate) the maximal noradrenaline depletion is about 50% of the control level and doses of (+)- or (-)-amphetamine below 2 mg/kg seem to produce little, variable or no effects, (Baird & Lewis, 1964; Littleton, 1967; Corrodi *et al.*, 1967; Brodie *et al.*, 1970; Leonard and Shallice, 1971). Intracisternal injection of 40 µg of (+)-amphetamine does not change brain noradrenaline or dopamine levels (Breese *et al.*, 1970; Taylor & Sulser, 1973). Comparisons of the effect of different doses of (+)-and (-)-amphetamine show that the (+) isomer is 2 to 3 times more effective than the (-) isomer with respect to the depletion of brain noradrenaline levels. (Baird & Lewis, 1964; Taylor & Snyder, 1970, 1971; Brodie *et al.*, 1970; Lewander, 1971a; Costa *et al.*, 1972). The racemic mixture ((±)-amphetamine) gives an intermediate response (Lewander, 1971a). The decrease in noradrenaline levels seems to be variable between different brain areas (Taylor & Snyder, 1970). The time response curves show that the noradrenaline depletion persists for 1 to 4 days, depending on the dose, after single injections of (+)- or (-)-amphetamine, (Brodie *et al.*, 1969; Groppetti & Costa, 1969b; Brodie *et al.*, 1970; Costa & Groppetti, 1970; Lewander, 1970a, 1971a). The duration of

the depletion of noradrenaline levels after (-)-amphetamine is shorter (Brodie *et al.*, 1970).

Amphetamine, in combination with grid shock stress (Moore & Lariviere, 1963, 1964), forced muscular work (Beauvallet *et al.*, 1962b), cold stress (Salama & Goldberg, 1969) or increasing the room temperature to 33°C (Beauvallet *et al.*, 1967; Beauvallet, 1968), caused a more pronounced decrease in the levels of noradrenaline in the brain than amphetamine alone.

Methamphetamine also depleted the levels of noradrenaline in the brain (Cook & Schanberg, 1970; Leonard, 1972), and abolished the daily rhythm of hypothalamic noradrenaline (Asano & Moroji, 1974)

Reports on the acute effects of amphetamine on whole brain, or striatal dopamine are not consistent. Some authors have reported unchanged or slighly(less than 15%) decreased levels (Moore & Lariviere, 1963, 1964; Baird & Lewis, 1963, 1964; Carlsson *et al.*, 1965; Gunne & Lewander, 1967; Corrodi *et al.*, 1967; Baird, 1968; Breese *et al.*, 1970; Taylor & Snyder, 1970. 1971; Leonard & Shallice, 1971; Leonard, 1972; Costa *et al.*, 1972; Glick *et al.*, 1974), while others have reported an increase by up to 24%, (Littleton, 1967; Lewander, 1968a, b, 1971a, b) with doses of between 10 - 20 mg/kg about 3 h after the administration of either of the isomers or the racemate of the drug. The increase in the levels of dopamine in the brain after amphetamine administration was potentiated by keeping the animals at 33°C, which induced hyperthermia (40°C colonic temperature; Beauvallet, 1968). Lesioning the nigrostriatal dopaminergic

pathway abolished the amphetamine-induced increase in the levels of dopamine in the brain (Boulu *et al.*, 1972). Various forms of stress (grid shock, restraint, swim, sound) in combination with amphetamine had no additional effect on the levels of dopamine in the brain (Moore & Lariviere, 1963). Injection of 40 µg of amphetamine into the cerebral cisternae did not affect the concentrations of dopamine in the brain (Breese *et al.*, 1970).

Methamphetamine (15 mg/kg) caused no change in the levels of dopamine in the brain initially but the levels decreased on repeated administration of the drug. (Koda and Gibb, 1978).

(iii) Effects of chronic amphetamine administration on brain.catecholamines

Repeated injections of amphetamine of as much as 20 mg/kg of the (±)-isomer of the drug once or twice daily for as long as 1 month have been shown to induce an additional decrease in the levels of noradrenaline in the brain but never exceeding 40 to 50% of control levels (McLean & McCartney, 1961; Gunne & Lewander, 1967; Beauvallet, 1968; Javoy *et al.*, 1968; Lewander, 1968b, 1971b; Brodie *et al.*, 1970). The noradrenaline levels in the brain stem and hippocampus were reduced, but unchanged in other areas of rat brain after giving 3 mg/kg of (±)-amphetamine sulphate daily in the food for 6 months (Herman *et al.*, 1971a). No difference between controls and chronically-treated rats was found after 9 months of the same drug regime (Herman *et al.*, 1971b). Acute and chronic treatment with methamphetamine caused reduced noradrenaline levels in the brain, (Cook & Schanberg, 1970; Schanberg & Cook, 1972).

Chronic administration of (±)-amphetamine sulphate, 20 mg/kg twice daily for 1 week or 16 to 32 mg/kg twice daily for 1 month, caused a decrease to 85% and 50% respectively of the control level of dopamine in the whole brain (Gunne & Lewander, 1967; Lewander 1968b, 1971e). Daily administration of 3 mg/kg of (±)-amphetamine in the food for 6 months decreased the levels of dopamine in the hippocampus to 60% of the control level but did not change the dopamine levels in any other region including the striatum (Herman *et al.*, 1971a). According to histochemical studies, the depletion in dopamine levels after chronic treatment with amphetamine seemed to be confined to the nigrostriatal dopaminergic system and not to involve the hypothalamic dopaminergic neurons (Fuxe & Ungerstedt, 1970).

(iv) The effects of amphetamine on the levels of serotonin in the brain

Reports on the effects of amphetamine on 5-hydroxytryptamine levels are inconsistent. In rats (+) or (±)-amphetamine , in doses of between 0.6 and 30 mg/kg given 0.5 to 16 h before killing the animals did not, in most studies, affect the levels of 5-hydroxytryptamine in the whole brain, (Pletscher *et al.*, 1964; Carlsson *et al.*, 1965; Beauvallet *et al.*, 1967; Salama & Goldberg, 1969; Ganguli, 1969; Costa & Groppetti, 1970; Maickel *et al.*, 1970; Reid, 1970 a, b; Simon *et al.*, 1970b; Tagliomonte *et al.*, 1971a, b; Leonard & Shallice, 1971; Görlitz & Frey, 1972; Morgan *et al.*, 1972a; Fuller *et al.*, 1972b). Salama & Goldberg (1969) and Leonard (1972) reported a decrease in 5-hydroxytryptamine levels after amphetamine treatment. Beauvallet *et al.* (1969, 1970) found an increase in 5-hydroxytryptamine levels in the whole brain or in the striatum,

pons and mesencephalon but no change in the cortex and medulla oblongata after giving 15 mg/kg of amphetamine.

Methamphetamine in doses of 7 to 14.5 mg/kg given 1 - 6 h before death reduced the levels of 5-hydroxytryptamine in the brain by 25 - 47%; (+)- and (-)-methamphetamine were equally effective in this respect (Morgan *et al.*, 1972a; Leonard, 1972). Determination of 5-hydroxytryptamine in different brain areas (Ott *et al.*, 1971) showed that methamphetamine (5 mg/kg, 40 min before death) caused a reduction in the cortex, olfactory bulb and hippocampus, while no change occurred in six other regions. Ganguly (1969) reported an increase in the levels of 5-hydroxytryptamine in the brain after methamphetamine treatment which was abolished by chlorpromazine pretreatment.

McLean & McCartney (1961) reported a 20 - 30% increase in 5-hydroxytryptamine levels after repeated administration or chronic treatment with (+)-amphetamine (16 - 32 mg/kg). Similar results were later reported by Diaz & Huttunen (1972). The administration of 3 mg/kg of amphetamine daily did not change the levels of 5-hydroxytryptamine in the brain (Herman *et al.*, 1971b).

(v) <u>The effects of amphetamine on the levels of brain acetylcholine</u>

Acetylcholine levels in the brain have been reported to be decreased after giving 2 mg/kg of amphetamine (Domino & Olds, 1972; Domino & Wilson, 1972) but only in young or very old animals (Vasko *et al.*, 1974). The acetylcholine levels increased after amphetamine treatment (10 to 20 mg/kg) in striatal tissue from adult female rats (Consolo *et al.*, 1974; Glick *et al.*, 1974).

(vi) The effect of amphetamine on the levels of γ-amino butyric acid and substance P

Levels of brain γ-aminobutyric acid (GABA) and substance P have recently been shown to change on acute administration of amphetamine. Lynch & Leonard (1978) found that acute administration (5 mg/kg) of (+)-amphetamine caused an increase in the GABA content of the brain stem, thalamus and olfactory lobes. They found that although chlorpromazine antagonized the stereotypy induced by amphetamine, the combination of the two drugs increased the GABA content of all brain regions studied. This study is an example of the dangers of correlating biochemical observations with gross behaviour. Here it appears that GABA levels do not correlate with behaviour. Fortunately enough is known about the actions of amphetamine to say that GABA must be playing an ancillary role.

Pettibone *et al.* (1978) reported a decrease in immunoreactive substance P in the rat striatum (20 - 35% after the administration of high doses of (+)-amphetamine (10 mg/kg i.p.).

3. Uptake of Catecholamines and 5-Hydroxytryptamine

(i) Peripheral noradrenergic neurons

Axelrod and Tomchick (1960) first demonstrated (+)-amphetamine to "prevent the protective binding" of intravenously-injected radio-labelled noradrenaline and adrenaline in mice. In cats amphetamine prevented uptake and retention of [^{3}H]-noradrenaline in the heart, spleen, adrenal gland and liver but not in skeletal muscle (Axelrod *et al.*, 1961; Hertting *et al.*, 1961). Similar results were obtained with the rat heart (Axelrod *et al.*, 1962). Amphetamine also inhibited the uptake of [^{3}H]-metaraminol, a catecholamine analogue, into mouse heart (Carlsson & Waldeck, 1965). Similarly, the depletion of noradrenaline levels in the rat heart by guanethidine and bretylium (drugs which are taken up across the axonal membrane) was prevented by amphetamine pretreatment (Chang, 1965). Later, *in vitro* studies showed that noradrenaline uptake across the neuronal membrane was inhibited in the cat spleen by concentrations of amphetamine of approximately 10^{-7} M (I.D. 50) (Dengler *et al.*, 1961), in isolated rat heart (Burgen & Iversen, 1965*), or heart slices from rats and rabbits (Hendley & Snyder, 1972), slices of vas deferens from reserpine-nialamide pretreated rats (Carlsson *et al.*, 1965, 1966a, b; Häggendel & & Hamburger, 1967), isolated iris from rats and rabbits (Hamburger & Malmfors, 1967; Hendley & Snyder, 1972), and rabbit aortal strips (Ferris *et al.*, 1972; Hendley & Snyder, 1972).

Leitz (1970) showed that (+)-amphetamine inhibited metaraminol-induced release of noradrenaline from the heart by inhibiting the

*Uptake$_2$, according to Iversen (1967) was also inhibited by amphetamine at an ID50 concentration of 10^{-4}M.

uptake of metariminol. Uptake of noradrenaline into isolated bovine splenic nerve noradrenergic storage granules was inhibited by amphetamine and by methamphetamine (Euler & Lishajko, 1968).

(ii) Noradrenergic neurons in the brain

Inhibition of the uptake mechanism of noradrenergic neurons in the brain for noradrenaline *in vivo* by amphetamine has been shown by different procedures. The uptake and retention of intraventricularly-labelled noradrenaline into different brain regions was reduced in amphetamine-pretreated rats (Glowinski & Axelrod, 1964; Glowinski *et al.*, 1966a,b; Fuxe & Ungerstedt, 1968, 1970; Strada *et al.*, 1970). Methamphetamine showed the same property (Cook & Schanberg, 1970; Schanberg & Cook, 1972). In these studies an increased uptake of noradrenaline was observed 3 to 5 h after the methamphetamine injection. (+)-Amphetamine was found to be a more potent inhibitor of noradrenaline uptake in various brain areas than the (-)- isomer (Taylor & Snyder, 1970, 1971). Amphetamine also antagonised the depletion of noradrenaline in the brain caused by 4,α-dimethyl-m-tyramine (H77/77), which indicates that the uptake of this compound into rat brain noradrenergic neurons was inhibited (Carlsson *et al.*, 1969). In contrast, a potentiation of the H77/77-induced noradrenaline-depletion was reported by Leonard & Shallice (1971). This difference in results might be explained by different dosage schedules; however I feel that there is insufficient knowledge about the actions of such a drug to allow its use as a tool to determine the actions of another.

Slices from mice treated *in vivo* with amphetamine, which were then incubated *in vitro*, took up less noradrenaline than slices from control

mice (Ross & Renyi, 1966a, 1967b).

Various *in vitro* systems have been used for studies on the effects of amphetamine on noradrenaline uptake. Thus (+)-, (-)-and (±)-amphetamine have been shown to inhibit the uptake of noradrenaline into slices of cerebral cortex in mice (Ross & Renyi, 1964, 1967b; Reitz *et al.*, 1972) and rats (Carlsson *et al.*, 1965, 1966a, b; Häggendal & Hamberger, 1967; Rutledge *et al.*, 1972a; Ziance & Rutledge, 1972; Ferris *et al.*, 1972), in chopped cortex (Ziance *et al.*, 1972) in homogenates of cerebral cortex, hypothalamus, medulla oblongata and cerebellum (Coyle & Snyder, 1969b), and in synaptosomal preparations from cortex and hypothalamus (Horn *et al.*, 1971; Horn & Snyder, 1972; Ferris and Maxwell, 1972; Ferris *et al.*, 1972; Harris & Baldessarini, 1973a; Thornburg & Moore, 1973). Methamphetamine inhibited the uptake of noradrenaline into slices from the hypothalamus and brain stem but not from the telencephalon of rats (Morgan *et al.*, 1972b).

(iii) Dopaminergic neurons in brain

Amphetamine has been shown to inhibit the reserpine-resistant uptake of dopamine and noradrenaline into the tuberoinfundibular dopaminergic neurons in reserpine-nialamide treated rats *in vivo* (Fuxe *et al.*, 1967). The uptake of intraventricularly-administered dopamine into nigro-striatal dopaminergic cell bodies and terminals was inhibited by amphetamine (Fuxe & Understedt, 1968, 1970). Taylor & Snyder, (1970, 1971) showed that (+)-but not (-)-amphetamine (10 mg/kg s.c.) inhibited the uptake of intraventricularly-administered [^{3}H] -noradrenaline into the striatum. However, in a previous study by Glowinski *et al.* (1966a), (+)-amphetamine (15 mg/kg i.p.) did not

affect the uptake of intraventricularly-administered [^{3}H] -noradrenaline or [^{3}H]-dopamine into the striatum. A series of *in vitro* studies shows unequivocally that (+)- and (-)-amphetamine inhibit the uptake of dopamine into rat brain slices from striatum (Carlsson *et al.*, 1966a, b; Häggendal & Hamberger, 1967), homogenates of striatum (Coyle & Snyder, 1969 a, b) and synaptosomal preparations of striatal tissue (Horn *et al.*, 1971; Horn & Snyder, 1972; Ferris & Maxwell, 1972; Ferris *et al.*, 1972; Harris & Baldessarini, 1973a; Thornburg & Moore, 1973). Methamphetamine has similar actions (Morgan *et al.*, 1972b). Amphetamine inhibits the uptake of dopamine (and noradrenaline) into isolated synaptic vesicles from striatal tissue of pig brain (Philippu & Beyer, 1973).

Kinetic analysis of the inhibition of uptake exerted by amphetamine showed it to be competitive and reversible in both hypothalamus and striatum (Horn *et al.*, 1971). Ki values for amphetamine are in the order of 10^{-7} M in *in vitro* studies.

Controversial findings with regard to the differential potencies of (+)- and (-)-amphetamine as uptake inhibitors on noradrenergic and dopaminergic neurons have been reported. Coyle & Snyder (1969b) and Taylor & Snyder (1970, 1971) in *in vitro* and *in vivo* experiments, reported (+)-amphetamine to be up to 10 times more potent than (-)-amphetamine as uptake inhibitors for noradrenergic neurons in the brain, while the two isomers were equipotent with regard to striatal dopaminergic neurons. In contrast, Ferris *et al.* (1972), Harris & Baldessarini (1973a) and Thornburg & Moore (1973) reported (+)- and (-)-amphetamine to be about equipotent in inhibiting

noradrenaline uptake into brain noradrenergic neurons and (+)-amphetamine to be 4 - 5 times more effective than (-)-amphetamine on brain dopaminergic (striatal) neurons. A four- to six-fold difference between (+)- and (-)-amphetamine was found with regard to dopamine uptake into mesolimbic and striatal dopaminergic nerve terminals (Horn *et al.*, 1974). Differerences in experimental conditions, e.g. the use of reserpine-treated rats (Coyle & Snyder, 1969b; Hendley & Snyder, 1972), might explain the differences in these results. However, Harris & Baldessarini (1973a) point out that reserpine does not affect the "(+)/(-)-quotient" but reduces the total amounts of catecholamines taken up, which makes the results unreliable.

Other important factors affecting the outcome of stereoselective mechanisms are: thickness of slices, linearity of the kinetics and nonsaturating concentrations of amines (Hendley & Snyder, 1972). Thus, these authors found (+)-amphetamine to be more potent than (-)-amphetamine as inhibitors of uptake over the neuronal cell membrane of noradrenergic neurons in the rat heart, rabbit vas deferens, and iris; (+)- to be equal to (-)-amphetamine in rabbit heart and retinal (dopaminergic cells) and (-)-amphetamine to be more potent than (+)-amphetamine in rabbit thoracic aorta. Ferris *et al.* (1972) reported that the isomers were equipotent in the thoracic aorta of rabbits not treated with reserpine; all animals were pretreated with reserpine in the study by Hendley & Snyder (1972).

(iv) <u>The effects of amphetamine on the 5-hydroxytryptamine into neurons and platelets</u>

Total body uptake and retention of 5-hydroxytryptamine in the

mouse was not affected by amphetamine (Axelrod & Inscoe, 1963). Uptake and accumulation of 5-hydroxytryptamine into mouse cortex slices *in vitro* were inhibited by amphetamine in a concentration of 10^{-5} M (Ross & Renyi, 1967a). Similarly, Wong *et al.* (1973) found that amphetamine competitively inhibits the high affinity uptake of 5-hydroxytryptamine into isolated rat brain synaptosomes. *In vivo* models for uptake studies include histochemical evaluations of 5-hydroxytryptamine accumulation into tubero infundibular dopaminergic neurons in reserpine treated rats, which was blocked by amphetamine (Fuxe *et al.*, 1967) and accumulation of 5-hydroxytryptamine into central neurons after intraventricular administration of 5-hydroxytryptamine, which was not blocked by amphetamine (Fuxe & Ungerstedt, 1968). Depletion of serotonin in the brain by 4-methyl-α-ethyl-m-tyramine (H75/12; Carlsson *et al.*, 1969; Leonard, 1972) or (+)-p-acetyldeoxyephedrine (Dubnick *et al.*, 1973) was not blocked by (+)- or (-)-amphetamine. Methamphetamine, on the other hand, caused a decrease in the accumulation of 5-hydroxytryptamine into striatal slices, but not in slices of telencephalon, hypothalamus, or brain stem (Morgan *et al.*, 1972b).

In summary, amphetamines seem to affect the uptake mechanism for 5-hydroxytryptamine *in vitro* but a higher concentration is required ($>10^{-5}$ M) than is required for the inhibition of catecholamine uptake (greater than 10^{-6}M, Ross & Renyi, 1967a).

Blood platelets have been used as models for 5-hydroxytryptamine uptake, storage and release mechanisms. Amphetamine has been reported not to inhibit the accumulation of 5-hydroxytryptamine into rabbit platelets (Da Prada *et al.*, 1965) but to inhibit competitively the

uptake of 5-hydroxytryptamine into human platelets (Ki = 10^{-5} M) (Lemmer, 1973).

4. Release of Catecholamines, 5-hydroxytryptamine and acetyl choline

(i) Peripheral catecholamines

Amphetamine has been shown to release noradrenaline or [^{3}H] - noradrenaline previously taken up into the mouse or rat heart *in vivo* (Axelrod *et al.*, 1962; Potter & Axelrod, 1963; Benington & Morin, 1968) and [^{3}H] - noradrenaline, α-methyl-noradrenaline or metaraminol from the mouse heart (Carlsson & Waldeck, 1966a, b). The releasing action of amphetamine does not seem to be due to inhibition of the uptake mechanisms, since more potent uptake inhibitors, like desmethylimipramine (DMI) (Carlsson and Waldeck, 1968; Lundborg, 1969) or protriptyline (Carlsson & Waldeck, 1966a; Lundborg & Waldeck, 1972), do not release [^{3}H] -noradrenaline from the mouse heart. Amphetamine has to be transported into the noradrenergic neuron in order to exert its action (Lundborg & Waldeck, 1972), and it has been claimed that it releases mainly, but not exclusively, extravesicular monoamines from the mouse heart (Lundborg, 1969).

Studies *in vitro* showed that amphetamine increases the release of unchanged noradrenaline from isolated rat heart by reducing noradrenaline metabolism by deamination (Leitz & Stefano, 1971). The effect of amphetamine was inhibited by 6-hydroxydopamine pretreatment or guanethidine, α-methyl-noradrenaline or amantadine added to the slices before amphetamine, indicating that amphetamine releases noradrenaline from an extragranular storage pool. Electric field stimulation was without releasing effect in the reserpine plus monoamine oxidase inhibitor (MAOI) preparations; thus amphetamine and nerve-impulse-induced release of noradrenaline seems not be be identical (Enna & Shore, 1974). In heart slices, amphetamine caused

the release of [^{3}H]-noradrenaline newly synthesized from [^{3}H]-dopamine (Chevillard *et al.*, 1971) and this effect was potentiated by angiotensin II (Chevillard & Alexandre, 1972). Amphetamine also releases newly-synthesized noradrenaline from heart (Rapin *et al.*, 1972) and spleen slices (Rapin *et al.*, 1973b). Rather high (10^{-4} M) concentrations of amphetamine are needed for the release of noradrenaline from isolated bovine splenic nerve vesicles (Euler & Lishajko, 1968).

Catecholamines in adrenal glands from cats (Rubin & Jaanus, 1966) but not rats (Cession-Fossion, 1967) are released on perfusion with amphetamine. In this tissue amphetamine has a acetylcholine-like action, since the presence of hexamethonium or denervation does not inhibit this effect. Both in perfused cat and cow adrenals, amphetamine also causes the release of nucleotides, protein and dopamine-β-hydroxylase in addition to the catecholamines, indicating release by exocytosis at certain drug concentrations (Rubin & Jaanus, 1967; Schneider, 1972). This release is dependent on the presence of calcium ions (cf. Rubin, 1970). At high concentrations of amphetamine a direct effect on the catecholamine storage vesicles seems to be operating (Schümann & Philippü, 1962; Rubin & Jaanus, 1967; Schneider, 1972; Wagner *et al.*, 1973, 1974).

(ii) Plasma catecholamines

An amphetamine-induced increase of noradrenaline and adrenaline in plasma has been reported in dogs (Chidsey *et al.*, 1962; Harvey *et al.*, 1968). In the study by Chidsey *et al.* (1962), noradrenaline was shown by coronary sinus cannulation to be released from the heart and in the study by Harvey *et al.* (1968), increased concentrations of

adrenaline in the cannulated adrenal veins were observed after amphetamine. Dose-response relationships were established for the increase in plasma catecholamines and the effects of amphetamine on the blood pressure (Harvey *et al.*, 1968).

(iii) Urinary excretion of catecholamines

The urinary excretion of noradrenaline and adrenaline reflects primarily the release of these substances from peripheral noradrenergic neurons and the adrenal medulla brought about by nerve impulses or drugs, and also the release of catecholamines from non-innervated chromaffin cells.

An increase in the urinary excretion of noradrenaline and adrenaline in the rat after acute administration of amphetamine has been demonstrated (Biscardi *et al.*,1964; Beauvallet *et al.*, 1966 ; Gunne & Lewander, 1967; Lewander, 1968a, b; Beauvallet, 1968; Basso *et al.*, 1970). The ganglion blocking agent, mecamylamine, blocked the effect of amphetamine on the excretion of noradrenaline but not on the excretion of adrenaline; adrenal demedullation did not completely block the increase in the excretion of adrenaline after amphetamine treatment (Biscardi *et al.*, 1964). The increase in urinary adrenaline excretion was, however, absent after bilateral cutting of the splenchnic nerves (Gunne & Lewander, 1967; Lewander, 1968b). Later, Basso *et al.* (1970) could show that the adrenal demedullation could be performed more efficiently if the whole gland was removed and pieces of its cortex were grafted under the capsule of the kidney. Amphetamine administered to rats demedullated in this manner did not cause an increase in the urinary excretion of

adrenaline. It was also found (Lewander, 1968b; Basso *et al.*, 1970) that the amphetamine-induced increase in the urinary excretion of noradrenaline was more pronounced in rats with denervated or demedullated glands than in intact rats.

During chronic administration of (±)-amphetamine, 16 - 32 mg/kg twice daily for approximately 14 days per dose level, there was an initial increase in the urinary excretion of both adrenaline and noradrenaline followed by a successive decrease toward control excretion values (Lewander, 1968b). This pattern was most evident at the two higher dose levels and was considered to be a sign of tolerance to these effects of amphetamine in rats.

Amphetamine caused a decrease in the urinary excretion of dopamine in rats between 4 and 8 h after administration.

(iv) Noradrenaline and dopamine in the brain

Various methods have been used to demonstrate amphetamine-induced release of catecholamines in the brain *in vivo*. Several of these approaches utilize the hitherto accepted view that extraneuronally-liberated catecholamines are metabolized by catechol-O-methyl transferase (COMT) to the 3-O-methylated amines, which are subsequently deaminated by MAO to neutral or acidic metabolites (see reviews by Axelrod, 1959, 1965; Copin, 1964; Carlsson, 1966; Iversen, 1967; Andén *et al.*, 1969).

(v) Measurement of endogenous catecholamines and 3-O-methylated catecholamine metabolites after MAO inhibition

Amphetamine causes an increase of brain normetanephrine and 3-

methoxytyrosine, after a single dose or chronic administration in rats (Gunne & Lewander, 1967, 1968). The levels of normetanephrine and 3-methoxytyrosine are more easily detected by conventional methods in control rat or guinea pig brain after pretreatment of the animals with a monoamine oxidase inhibitor, i.e., nialamide. Administration of amphetamine after nialamide pre-treatment causes a marked increase in the normetanephrine and 3-methoxytyrosine accumulation in the brains of rats (Randrup & Jonas, 1967; Gunne & Lewander, 1968; Jonas & Scheel-Krüger, 1969; Scheel-Krüger, 1971, 1972), mice (Svensson, 1971), and guinea pigs (Lewander, 1971c) indicating extraneuronal release of both noradrenaline and dopamine in the brain.

In another series of reports, rats were pretreated with reserpine plus nialamide plus L-DOPA in order to study selectively the extravesicularly but intraneuronally located catecholamines (Carlsson *et al.*,1965, 1966a, b). In these rats very low doses of amphetamine (0.1 to 1 mg/kg) caused a depletion of the catecholamines, as shown with biochemical and histochemical methods, and an increase in the levels of normetanephrine and 3-methoxytyrosine in the brain indicated that amphetamine has a marked releasing effect on such an artificially enlarged pool of extravesicular catecholamines. Similar results have been reported by Andén and Svensson (1973) in several brain regions in reserpine-nialamide pretreated rats. Histochemical evidence for the amphetamine-induced decrease in α-methyl-noradrenaline and α-methyl-dopamine was reported in rats given tetrabenazine plus α-methyl-DOPA (Carlsson *et al.*, 1968). Potent and selective uptake inhibitors like desmethylimipramine do not cause depletion of the α-methylated amines in similar experiments (Carlsson *et al.*, 1966b).

Sectioning of dopaminergic neurons in the brain or treatment of rats with γ-hydroxybutyric acid causes an increase in the levels of dopamine in the brain. Amphetamine given before or after these treatments counteracts this increase, which indicates that amphetamine releases dopamine from the dopaminergic neurons in the brain (Andén *et al.*, 1973; Roth *et al.*, 1973). The effects of (+)-and (-)-amphetamine on the accumulation of normetanephrine and the reduction of noradrenaline, i.e., release of noradrenaline, were equal. While (+)-amphetamine was 1.7 times more potent than (-)-amphetamine on the accumulation of 3-methoxytyrosine, i.e., on the release of dopamine (Scheel-Krüger, 1972).

(vi) Levels of homovanillic acid

One to two hours after an acute dose of amphetamine (10 - 20 mg/kg i.p.) homovanillic acid levels increase in cats (Laverty & Sharman, 1965), rats and mice (Jori & Bernadi, 1969, 1972; Bizzi *et al.*, 1970; Roffler-Tarlov *et al.*, 1971; Fuentes & Del Rio , 1972; Jori & Dolfini, 1974). (+)-Amphetamine was more potent than (-)-amphetamine in this respect (Jori *et al.*, 1973; Jori & Dolfini, 1974). In some mouse strains brain homovanillic acid levels do not increase after amphetamine treatment (Jori & Garattini, 1973; Caccia *et al.*, 1973). The increase in homovanillic acid levels in the striatum in mice was antagonized by amantadine (Menon *et al.*, 1973), and γ-hydroxy-butyric acid (Menon *et al.*, 1974). However, reduced levels of homovanillic acid after amphetamine treatment have been found in dogs (Laverty & Sharman, 1965) and guinea pigs (Lewander, 1971c).

Rats, chronically treated with amphetamine (but not mice), became tolerant to the effect of amphetamine on homovanillic acid (Jori &

Bernadi, 1972); chronically treated guinea pigs had lower homovanillic acid concentrations in the brain as compared with a single dose (Lewander, 1971c).

(vii) Accumulation of [^{3}H] -normetanephrine and [^{3}H]-3-methoxytyrosine synthesized from [^{3}H] -DOPA

Amphetamine (20 mg/kg i.p.) injected before or after i.v. injection of [^{3}H]-DOPA, increases the accumulation of [^{3}H] -normetanephrine and [^{3}H]-3-methoxytyrosine in the rat brain (even without MAO inhibitors), indicating an amphetamine-induced release of catecholamines (Lewander, 1970a, 1971d).

(viii) Fate of noradrenaline and dopamine taken up into brain catecholamine neurons after intraventricular injection

[^{3}H]-noradrenaline or [^{3}H]-dopamine injected into the lateral ventricle or cisterna magna of rats is taken up primarily by central noradrenergic and dopaminergic neurons. Subsequent administration of (+)-amphetamine (10 - 15 mg/kg) accelerates the disappearance of the labelled noradrenaline and causes an increase in [^{3}H]-normetanephrine, and a decrease in deaminated catechol metabolites (Glowinski & Axelrod, 1964; Glowinski *et al.*, 1966a, b; Strada *et al.*, 1970; Tilson & Sparber, 1972). Very similar findings were reported for methamphetamine (Cook & Shanberg, 1970). Chronic methamphetamine treatment did not change this pattern of deaminated metabolites (Schanberg & Cook, 1972). Small doses (5 mg/kg) of amphetamine have no effect on the levels of [^{3}H]-normetanephrine except after repeated injections (Glowinski, 1970b). The levels of labelled 3-methoxy-4-hydroxyphenylglycol, however, were increased after 3 mg/kg

of amphetamine in such experiments (Ladisch *et al.*, 1970); progesterone mitigated this effect of amphetamine (Ladisch & Baumann, 1971).

Intraventricular administration of amphetamine also caused a decrease in labelled noradrenaline and an increase in [^{3}H]-normetanephrine (Taylor & Sulser, 1973). Intraventricularly-administered unlabelled catecholamines to reserpine-nialamide pretreated rats are also released from catecholaminergic neurons by amphetamine as demonstrated by histochemical methods (Fuxe & Ungerstedt, 1968, 1970).

(ix) Perfusion of the cerebral ventricular system

Carr & Moore (1969, 1970a, b) perfused the ventricular system in cats with [^{3}H]-noradrenaline, which was assumed to be taken up primarily by catecholaminergic neurons in the brain. Administration of amphetamine (25 - 400 µg/ml) added to the perfusion fluid, or injected i.v. (1 mg/kg), was followed by increased amounts of [^{3}H]-noradrenaline and [^{3}H]-normetanephrine in the perfusate indicating an amphetamine-induced noradrenaline release. (-)-Amphetamine was less effective than (+)-amphetamine in this respect. Release of noradrenaline has been reported in rats which have a push-pull cannula fitted on top of the skull and reaching to the ventricular system (Sparber & Tilson, 1972b). Later, ventricular perfusion experiments in cats have shown that [^{3}H]-dopamine previously taken up or newly synthesized from [^{3}H]-tyrosine or [^{3}H]-DOPA is released by amphetamine (Chiueh & Moore, 1973, 1974b). (+)-Amphetamine was 3 to 4 times more potent than (-)-amphetamine (Voigtländer & Moore, 1973a,b; Chiueh & Moore, 1974a).

Amphetamine-induced release of dopamine was reduced by sectioning the nigro-striatal dopaminergic neurons. It was also demonstrated that amphetamine potentiated the release of dopamine by electrical stimulation of the nigrostriatal bundle (Voigtländer & Moore, 1973b) Haloperidol did not antagonize amphetamine-induced dopamine release (Voigtländer & Moore, 1973a). The effect of amphetamine on catecholamine release in these experiments was not changed by α-methyl-p-tyrosine (Chiueh & Moore, 1974c) or amantadine (Gudetsky *et al.*, 1974). The lack of inhibition of amphetamine-induced release of dopamine is not in agreement with the study of Enna *et al.* (1973).

(x) Perfusion or superfusion of different brain areas *in vivo* with push-pull cannulas or cups

The output of endogenous dopamine from cat striatium was found to increase after the addition of amphetamine (0.5 mg/ml) to the perfusate through a push-pull cannula (McKenzie & Szerb, 1968). Increased amounts of newly-synthesized $[^{14}C]$-dopamine from $[^{14}C]$-DOPA or $[^{14}C]$-tyrosine was found in the perfusate after the administration of amphetamine in similar experiments (Riddell & Szerb, 1971). In rats, Stein and Wise (1967, 1969) showed that amphetamine (3 mg/kg i.p.) increased the output of $[^{3}H]$-noradrenaline in push-pull cannula perfusates placed in the amygdaloid nucleus and in the hypothalamus. Glowinski (1970b) and Besson *et al.* (1971) superfused the caudate nucleus of cats with $[^{3}H]$ -tyrosine by use of the cup technique in order to label the stores of dopamine with $[^{3}H]$ -dopamine. Addition of amphetamine (10^{-6} M) to the superfusate or i.v. injection of (-)-amphetamine (2 mg/kg) caused a release of $[^{3}H]$-dopamine into the superfusate. This result was

confirmed in a similar study in monkeys (Gauchy *et al.*, 1974).

(xi) Release of newly synthesized catecholamines in *in vitro* systems

Isolated corpus striatum (Besson *et al.*, 1969a) or slices of striatum or medulla oblongata (Besson *et al.*, 1969b; Glowinski, 1970b) from rats were superfused or incubated in the presence of [^{3}H]-tyrosine. [^{3}H]-Catecholamines synthesized during the incubation were measured in the tissues and in the media. Amphetamine, either given to the animals before killing or added to the incubations, caused a release of newly synthesized [^{3}H]-catecholamines from the tissue into the superfusion fluid or media.

(xii) Release of catecholamines previously taken up into *in vitro* tissue preparations

Ng *et al.* (1970) showed that (+)-amphetamine was more potent than (-)-amphetamine in releasing [^{3}H]-noradrenaline taken up by rat striatal slices. Farnebo (1971) demonstrated that (+)-amphetamine (10^{-6} M) released [^{3}H]-noradrenaline taken up into cortical slices and [^{3}H]-dopamine taken up into neostriatal slices. Ebstein *et al.* (1972) have reported that amphetamine caused a release of noradrenaline more efficiently in slices from the amygdaloid nucleus than from the hypothalamus. The effect was more evident when slices from reserpine plus nialamide pretreated rats were used (Farnebo, 1971). Electrical stimulation of brain slices caused an increased overflow of [^{3}H]-catecholamines in the presence of (+)-amphetamine than in its absence (Farnebo, 1971).

The release of [^{3}H]-catecholamines taken up into synaptosomal

preparations from rat cerebral cortex, hypothalamus and striatum was four times greater with (+)-amphetamine than with (-)-amphetamine (Ferris & Maxwell, 1972; Ferris *et al.*, 1972). (-)-Amphetamine (10^{-6} M) caused the release of [^{3}H] -noradrenaline taken up either by chopped tissue, homogenates or synaptosomes from rat hypothalamus (Phillipü *et al.*, 1974) and cerebral cortex (Rutledge *et al.* 1972a, b, 1973; Ziance *et al.*, 1972; Ziance & Rutledge, 1972; Azzaro & Rutledge, 1973; Azzaro *et al.*, 1974; Tseng *et al.*, 1974). (-)-Amphetamine was less active than (+)- or (±)-amphetamine. The release was temperature dependent and amphetamine did not cause unspecific release of , e.g., inulin or urea. The release process was calcium-dependent only at amphetamine concentrations below 10^{-3} M; at or above that concentration release could be demonstrated in calcium-free media. Thus, amphetamine-induced release differs from electrically-induced, potassium-induced, and tyramine-induced release, in not being completely calcium dependent (cf. Rubin, 1970).

Only a slight releasing effect of amphetamine was noticed when synaptosomes from rat brain were superfused with a solution containing amphetamine (Raiteri *et al.*, 1974). These authors concluded that the main action of amphetamines in the brain is uptake inhibition. The amphetamine metabolites, p-hydroxyamphetamine and p-hydroxynorephedrine, also released noradrenaline from chopped cortex tissue *in vitro*: p-hydroxyamphetamine was weaker in effect than amphetamine and p-hydroxyamphetamine (Wegler & Rutledge, 1974).

Enna *et al.* (1973) found that α-methyl-p-tyrosine inhibited the amphetamine-induced release of noradrenaline and some catecholamine analogues from brain slices . Cocaine and desipramine prevent

amphetamine-induced release of noradrenaline from chopped brain tissue by inhibiting the uptake of amphetamine into the neurons (Rutledge *et al.*, 1973; Azzaro *et al.*, 1974). Incorporation of α-methy-m-tyramine into dopaminergic neurons in the brain occurs after admini - stration of α-methyl-m-tyrosine (Dorris & Shore, 1974). Amphetamine caused a depletion of this false transmitter, indicating release, but by a mechanism different from release by nerve impulses (Dorris & Shore, 1974). Amantadine prevented amphetamine-induced release of α-methyl-m-tyramine (Dorris & Shore, 1974).

(xiii) The effects of amphetamine on the release of 5-hydroxytryptamine

Only a few techniques for the demonstration of amphetamine-induced release of brain 5-hydroxytryptamine *in vivo* have been applied. Fuxe and Ungerstedt (1970) reported that amphetamine caused a change toward extraneuronal accumulation of 5-hydroxytryptamine in reserpine-treated rats, as evaluated by a histochemical fluorescence microscopic technique for the visualization of monoamines. The use of intraventricularly located push-pull cannulae in rats showed that after a single dose of amphetamine there was no increase in the release of [^{14}C]-5-hydroxytryptamine, previously taken up into the serotonergic neurons, into the perfusion fluid (Sparber & Tilson, 1972a). However in rats chronically treated with amphetamine, further amphetamine caused a release of 5-hydroxytryptamine.

Superfusion of isolated corpus striata from rats *in vivo* has been used for the demonstration of release of catecholamines after amphetamine treatment. In similar experiments, 10^{-4} M concentrations of amphetamine in the perfusate caused a small release of 5-hydroxytryptamine (Glowinski, 1970a, b). (+)-Amphetamine but not (-)-amphetamine was

shown to release 5-hydroxytryptamine taken up into central cortex slices (Ng *et al.*, 1970). (+)-Amphetamine, 10^{-4} M, also induces release of 5-hydroxytryptamine from chopped pieces of tissue from the caudate nucleus, medulla oblongata and cortex of the rat brain (Rutledge *et al.*, 1972b; Azzaro & Rutledge, 1973). Amphetamine-induced liberation of 5-hydroxytryptamine from rabbit blood platelets has been reported by Da Prada *et al.* (1965) and from human blood platelets by Lemmer (1973).

(xiv) The effects of amphetamine on the release of acetylcholine

Acetylcholine release has been measured by the superfusion of an area of cat cerebral cortex *in vivo* by a cup technique. By this method amphetamine has been shown to increase the release of acetylcholine from the cat cortex (Pepeu & Bartolini, 1967; Beani *et al.*, 1968; Hemsworth & Neal, 1968a, b). This effect of amphetamine seems to be due to an indirect activation of cholinergic neurons rather than to a direct presynaptic effect, since no effect of amphetamine was obtained after undercutting of the cerebral cortex (Deffenu *et al.*, 1970; Pepeu *et al.*, 1970) or septal lesions (Nistri *et al.*, 1972). Administration of chlorpromazine (Pepeu & Bartolini, 1968; Nistri *et al.*, 1972) blocks the amphetamine-induced release of acetylcholine, while α-receptor blocking agents like phenoxybenzamine or phentolamine are without effect (Deffenu *et al.*, 1970; Pepeu *et al.*, 1970) and the β-receptor blocking agent, propranolol, has varying effects (Bartolini & Pepeu, 1970; Deffenu *et al.*, 1970; Pepeu *et al.*, 1970). These findings suggest that amphetamine-induced release of acetylcholine is a catecholamine-mediated phenomenon. The release of acetylcholine, as measured with a caudate nucleus cup in cats, was not altered by amphetamine given i.p. (5 mg/kg) or in the superfusing

medium (Jones *et al.*, 1973).

Amphetamine inhibits neuromuscular transmission in isolated nerve-muscle preparations (Anderson & Amman, 1963; Peterson *et al.*, 1964; Gerald & Hsu, 1975). Recently Skau & Gerald (1978) found that α- and β-receptor blockers failed to alter the blocking effects of amphetamine on the isolated rat phrenic nerve diaphragm and pretreating the rats with reserpine did not modify the response. Thus, the effects of the amphetamine isomers do not seem to be mediated by catecholaminergic interactions. The blocking of neuromuscular transmission by amphetamine resembled the non-depolarizing blockade produced by tubocurarine.

C. Biochemical Effects of Amphetamine on Metabolism

1. Degradation of the catecholamines

(i) Monoamine oxidase

Since early reports (Blaschko *et al.*, 1937; Pugh & Quastel, 1937) that amphetamine is not a substrate for monoamine oxidase (MAO) and that it inhibits the enzyme competitively and reversibly (Mann & Quastel, 1940; cf. review by Blaschko, 1952), a number of studies have confirmed this finding for a variety of species and tissues. Preparations from rat, mouse and rabbit brain (Grana & Lilla, 1959; Glowinski *et al.*, 1966a; Nielson *et al.*, 1967; Green, 1971; Möller-Nielson & Dubnik, 1970; Morgan *et al.*, 1972b), human brain and salivary gland (Blaschko & Strömblad, 1960) and other tissues including liver, kidney and heart have been studied . Concentrations of amphetamines ranging from 10^{-5} to 10^{-2} M seem to be required for inhibition of deamination of typical MAO substrates: adrenaline, noradrenaline, tyramine, oxedrine, kynuramine, benzylamine, serotonin, tryptamine, and phenethylamine. Comparative studies have shown that I.C. 50 concentrations are lowest with serotonin, tryptamine and tyramine as substrates, while phenethylamine requires 25 to 50 times higher concentrations (Everett & Yellin, 1971; Fuller, 1972).

Parmer (1966) made the observation that amphetamine protects MAO in rat liver mitochondria from other MAO inhibitors like iproniazid, tranylcypromine and nialamide. (+)-Amphetamine is generally a more potent MAO inhibitor *in vitro* than (-)-amphetamine (Pratesi & Blaschko, 1959; Grana & Lilla, 1959; Blaschko & Strömblad, 1960; Fuller & Walters, 1964; Parmer, 1966; Möller-Nielson & Dubnik, 1970) except in the rabbit liver where the two isomers are equally active (Pratesi & Blaschko, 1959).

Kinetic studies on MAO from rat liver suggest that the enzyme exists in two major forms, A and B (Johnston, 1968). Serotonin and noradrenaline have been shown to be substrates for the A form, benzylamine and phenethylamine for the B form and tyramine and dopamine as substrates for both forms. Tryptamine has been reported as a substrate for the B form and as a substrate for both forms of the enzyme, (Houslay & Tipton, 1974; Neff & Yang, 1974; Hall *et al.*, 1969; Mantle *et al.*, 1975; Johnston, 1968). The kinetic studies of Mantle *et al.* (1976) show that, in rat liver, amphetamine inhibits the A form of the enzyme. Therefore if MAO inhibition is of physiological importance, one would expect to see a differential effect between noradrenaline and dopamine metabolism. There is reason to suggest that rat liver MAO may be an adequate model for brain MAO (see Mantle *et al.*, 1976), consequently this differential effect should be seen in the brain. Glowinski *et al.* (1966) showed that, after acute injection of amphetamine there was a decrease in MAO activity; this inhibition was only mild when tryptamine was used as a substrate, but marked when noradrenaline was used as the substrate.

Salama & Goldberg (1969) found that systematically-administered amphetamine in combination with cold stress, but not other forms of stress, caused a reduced MAO activity in rat brain.

After 50 days of chronic treatment of methamphetamine to guinea-pigs there was a decrease in brain MAO activity to 75% of the controls. After withdrawal there was a compensatory increase over control levels in MAO activity (Utena *et al.*, 1959).

Rutledge (1970) and Rutledge *et al.* (1972b), have shown that amphetamine, in addition to causing release of [^{3}H]-noradrenaline and [^{3}H]-dopamine previously taken up into brain slices or chopped tissue, reduces the oxidative deamination of the catecholamines. Rutledge (1970) also found that amphetamine does not inhibit rat brain MAO from lysed synaptosomes or mitochondria from the rat brain and concluded that amphetamine causes a functional MAO inhibition by inhibiting access of the catecholamines to intraneuronal MAO by blocking the uptake mechanisms for catecholamines. In addition, the metabolic pattern of catecholamine metabolites is changed toward non-deaminated metabolites after amphetamine (Glowinski *et al.*, 1966a, b; Cook & Schanberg, 1970; Tseng *et al.*, 1974), methamphetamine (Strada *et al.*, 1970), and p-hydroxyamphetamine (Wenger & Rutledge, 1974). Similar findings were reported with the isolated perfused rat heart. It has been concluded that amphetamine protects intraneuronal catecholamines from degradation.

Studies on the endogenous concentrations of homovanillic acid, the 3-O-methylated and the deaminated product of dopamine in the brain, as an indicator of the *in vivo* importance of MAO inhibition in brain caused by amphetamine, have given conflicting results. Thus, amphetamine causes an increase in brain homovanillic acid in cats (Laverty & Sharman, 1965), rats and mice (Jori & Bernardi, 1969, 1972; Bizzi *et al.*, 1970); Fuentes & Del Rio, 1972; Menon *et al.*, 1973; Jori & Dolfini, 1974), while amphetamine causes a reduction of the levels of homovanillic acid in the dog (Laverty & Sharman, 1965) and the guinea-pig brain (Lewander, 1971c). (+)-Amphetamine was more potent than (-)-amphetamine in increasing the levels of homovanillic acid in rat brain (Jori *et al.*, 1973; Jori &

Dolfini, 1974). Roffler-Tarlov *et al.* (1971) confirmed that amphetamine increased the levels of homovanillic acid in mouse brain and showed that 3,4-dihydroxyphenylacetic acid, the deaminated metabolite of dopamine, was decreased. 3,4-Dihydroxyphenylacetic acid levels in the rat brain were also decreased after amphetamine (Roth *et al.*, 1973; Bunney *et al.*, 1973). Tolerance to the amphetamine-induced increase in homovanillic acid after repeated administration of amphetamine has been shown to occur in rats, but not in mice (Jori & Bernadi, 1972). Chronic administration of amphetamine to guinea-pigs caused a more pronounced fall in the levels of homovanillic acid in the brain than a single injection of the drug (Lewander, 1971c).

It appears that the concentration of homovanillic acid in the brain might be influenced in two directions by amphetamine: a) increased by release of dopamine from dopaminergic neuron terminals and b) decreased as a result of functional MAO inhibition and/or a negative feed-back modulation of dopamine synthesis. The resultant effective homovanillic acid concentration may thus be dependent on the concentration of amphetamine within the brain and, possibly, also on the animal species.

The early suggestion by Mann & Quastel (1940) that the central stimulant effect of amphetamine should be due to MAO inhibition is not compatible with the fact that other, more efficient MAO inhibitors do not share the central stimulant action of amphetamine (Pratesi & Blaschko, 1959; Grana & Lilla, 1959). However, recent evidence (Glowinski *et al.*, 1966a; Green, 1971; Rutledge, 1970; Leitz &

Stefano, 1971; Rutledge *et al.*, 1972b) suggest that any interpretation of the mechanism of action of amphetamine in relation to central stimulation that ignores the effect of high tissue concentrations of the drug on deamination of central and peripheral catecholamines might be incomplete. An experiment by Green *et al.* (1978) demonstrated, using a labile irreversible inhibitor of MAO (phenelzine), that MAO is inhibited *in vivo* to about 50% of its original activity.

(ii) Catechol-O-methyl transferase (COMT)

Amphetamine has been found not to change COMT activity *in vitro* (Axelrod & Tomchick, 1960). The increase in 3-O-methylated metabolites of catecholamines in various tissues is thought to be due to the increased concentration of substrate available to COMT caused by the amphetamine-induced release of catecholamines.

The interpretation of changes in O-methylated and deaminated metabolites in terms of intra- and extra-neuronal metabolism (implicit in these considerations are other considerations such as neuro-transmitter release and reuptake), rests on the assumption that COMT degrades extraneuronal catecholamines, and MAO degrades intraneuronal catecholamines. MAO is certainly intracellular (bound to mitochondrial membranes inside glial and neuronal cells), and COMT is a soluble, cytoplasmic enzyme. The situation may not be as simple as we are led to believe.

2. Synthesis of the catecholamines

(i) Tyrosine hydroxylase

Tyrosine hydroxylase, partly purified from beef or cat brain (Bagchi & McGeer, 1964; Besson *et al.*, 1973), or in a crude homogenate of rat brain (McGeer & McGeer, 1967), was not inhibited by (+)-amphetamine *in vitro* in concentrations of 10^{-4} M. Tyrosine hydroxylase activity was decreased in striatal slices *in vitro* by 10^{-7} to 10^{-4} M concentrations of amphetamine (Besson *et al.*, 1971b).

In other studies rats have been injected with amphetamine and the tyrosine hydroxylase activity has been determined in tissue homogenates or brain slices *in vitro*, either as the formation of $[^3H]$-H_2O from $[3,5-^3H]$-tyrosine or as the accumulation of $[^3H]$-catecholamine from the precursor. Thus Besson *et al.* (1969b) found that 5 mg/kg of (+)-amphetamine increased catecholamine synthesis in brain slices from the striatum and the brain stem, while in later studies (Besson *et al.*, 1971 , 1973) a reduction of the tyrosine hydroxylase activity was reported. No effect was found in the accumulation of $[^3H]$-noradrenaline from $[^3H]$-tyrosine after chronic administration of 1 mg/kg of (+)-amphetamine for 8 days (Besson *et al.*, 1973). The amphetamine-induced inhibition of tyrosine hydroxylase was non-competitive for tyrosine (Besson *et al.*,1973). Amphetamine has been reported to change the physical state of striatal but not mid-brain tyrosine hydroxylase, i.e., it becomes more membrane-bound and is thereby inactivated (Mandell *et al.*, 1972).

Harris & Baldessarini (1973b) reported that *in vivo* administration of amphetamine (5 mg/kg) to rats caused a decrease in tyrosine hydroxylase activity in striatal homogenates for 0.5 to 2 h. This

effect of amphetamine was blocked by chlorpromazine or bicuculline pretreatment, indicating that it might be secondary to indirect stimulation of dopaminergic and GABA receptors. Amphetamine added *in vitro* did not cause a decrease in the activity of the enzyme.

Administration of amphetamine in the drinking water for 3 to 7 days did not change tyrosine hydroxylase activity in the rat forebrain structures (Hulme *et al.*, 1974).

By another *in vivo* technique, i.e., interstriatal injection of labelled tyrosine and subsequent determination of $[^3H]$-H_2O and dopamine in the striatal tissue, amphetamine was found to decrease the synthesis of dopamine at the tyrosine hydroxylase step (Javoy *et al.*, 1974).

Fibiger & McGeer (1971) found that methamphetamine (10 mg/kg) had no effect on tyrosine hydroxylase in brain and adrenal glands except after repeated injection, when the activity of the caudate nucleus decreased and that of the adrenal gland increased. Similar results were reported by Koda & Gibb (1973). These changes in tyrosine hydroxylase activity in the caudate nucleus and adrenal gland could be antagonized by haloperidol (Buening & Gibb, 1974). Chlorpromazine antagonized the actions of amphetamine in the striatium but not in the adrenal gland (Buening & Gibb, 1974). Mandell & Morgan (1970) and Mandell & Knapp (1972) also reported increased tyrosine hydroxylase activity in adrenal glands of chicken after repeated administration of methamphetamine.

(ii) Aromatic amino acid decarboxylase (DOPA decarboxylase)

If amphetamine has any effect on this enzyme it has not, to my knowledge, been reported.

(iii) Dopamine-β-hydroxylase

Dopamine-β-hydroxylase has been reported to be inhibited by amphetamine, (+)-amphetamine and (±)-amphetamine (Goldstein *et al.*, 1964). Similarly dopamine-β-hydroxylase in homogenates of spleen was inhibited by amphetamine, methamphetamine and p-hydroxyamphetamine (Jacquot *et al.*, 1974).

3. Turnover of the Catecholamines

Glowinski & Axelrod (1965) injected [^{3}H]-noradrenaline intraventricularly into the rat brain and found that (+)-amphetamine (20 mg/kg) retarded its disappearance. As reported by Javoy *et al.* (1968) and later reviewed by Glowinski (1970a), smaller doses of amphetamine (5 mg/kg) accelerated the turnover of noradrenaline in the brain, while repeated or higher doses have no effect on noradrenaline turnover as measured by this method.

Determination of the rate of disappearance of catecholamines after inhibition of catecholamine synthesis at the tyrosine hydroxylase step, by giving α-methyl-p-tyrosine (Corrodi *et al.*, 1967; Andén, 1970) or 3-iodotyrosine, or at the dopamine-β-hydroxylase step, using diethyldithiocarbamate (Littleton, 1967) have also been used as methods for turnover measurements. In these studies 2 mg/kg of (+)-amphetamine accelerated noradrenaline depletion after diethyldithiocarbamate and retarded the depletion of dopamine after 3-iodotyrosine (Littleton, 1967). Large doses of (+)-amphetamine (15 - 20 mg/kg) accelerated the depletion of noradrenaline in the whole rat or mouse brain (Corrodi *et al.*, 1967; Alhava, 1973), but had no effect on noradrenaline in the rat spinal cord (Andén, 1970). In the two rat studies, dopamine depletion in the brain was not affected by amphetamine. No change in the degree of depletion of noradrenaline or dopamine was found after amphetamine in α-methyl-p-tyrosine-pretreated rats (Andén *et al.*, 1967). Methamphetamine, but not amphetamine has been reported to antagonize α-methyl-p-tyrosine-induced depletion of noradrenaline (Leonard, 1972).

A third method for the determination of brain catecholamine turnover is the accumulation of labelled catecholamines after i.v. injection of [^{3}H] - or [^{14}C] -tyrosine or of DOPA. By this technique Costa & Groppetti (1970, 1972), Persson (1970), Costa *et al.* (1972) and Groppetti *et al.* (1972) found that small doses of amphetamine caused an increase in dopamine turnover in the striatum but had no effect on noradrenaline turnover, Fulginiti & Orsingher (1971), however, reported an increased accumulation of labelled noradrenaline after giving 2 mg/kg of (±)-amphetamine. In mice implanted with amphetamine containing pellets there was an increased accumulation of both noradrenaline and dopamine from [^{3}H] -tyrosine. A large dose (10 mg/kg) of amphetamine counteracted this effect (Hitzemann *et al.*, 1973).

Acute or chronic administration of large doses of the drug (16 - 32 mg/kg i.v.) reduced the accumulation of labelled noradrenaline in rat heart and brain synthesized from tyrosine or DOPA, which was suggested to be due to release of the newly synthesised noradrenaline (Lewander, 1971d). There was no effect on dopamine levels in the brain (Lewander, 1970a, 1971d). When amphetamine was given 2 h after the catecholamine stores of the rat brain had been labelled by [^{3}H] -tyrosine administration, it was found that the disappearance of [^{3}H] -noradrenaline was accelerated and that of [^{3}H] -dopamine retarded (Lewander, 1970a). Javoy *et al.* (1970) injected [^{3}H] -tyrosine into various parts of the rat brain and measured the accumulation of [^{3}H] -catecholamines. (+)-Amphetamine (5 mg/kg) caused an increase in the accumulation of dopamine in cell bodies (substantia nigra) and a decrease in the terminals (caudate nucleus)

of dopaminergic neurons, while no effect on noradrenaline accumulation in the ventromedial nucleus of the hypothalamus was found.

Levels of tyrosine in the brain after amphetamine administration

In the aforementioned turnover studies where labelled tyrosine was used, neither plasma nor brain levels of [^{3}H]-tyrosine were affected by amphetamine; the same was found to be true of endogenous (unlabelled) tyrosine, (Rapin *et al.*, 1971). However Leonard & Shallice (1971) have reported decreased levels of tyrosine in rat brain and plasma in contrast to an increase in brain tyrosine reported by Tagliamonte *et al.* (1971a, b).

This brief summary on the reported effects of amphetamine on catecholamine turnover show that the results are contradictory. The differences may be due to the different doses of amphetamine used. It seems that low doses of amphetamine in rats cause an increase while higher doses cause a decrease or no change in brain catecholamine turnover. This is probably due to the fact that one or another of the effects of amphetamine on either uptake, release, synthesis or degradation of the catecholamines or feedback modulation of catecholamine neuron impulse flow may predominate at certain dose levels. Regional variations in turnover within the brain might be added to the list of causes for the differences in results.

Critical evaluation of methods used to measure catecholamine turnover

From the descriptions of the methods used to measure the 'turnover' of noradrenaline and dopamine it can be seen straight away that the methods which involve labelling of noradrenaline or dopamine by

injecting a labelled precursor are assuming that the label is incorporated evenly in noradrenaline/dopamine stores throughout the brain. For physical (i.e. access from the site of injection and "free" and "bound" amine interconversion) and 'compartmental' (i.e. transport mechanisms) reasons this is extremely unlikely. Therefore what is being studied is the turnover of a particular 'pool' of amine, which may vary with the precursor used and the site of injection.

Inhibition of tyrosine hydroxylase and of dopamine-β-hydroxylase have been found to be associated with different rates of noradrenaline depletion, (Persson & Waldeck, 1970). The disappearance of both [^{3}H]-noradrenaline and endogenous noradrenaline was more rapid when dopamine-β-hydroxylase was inhibited (Goldstein & Nakajima, 1967). Persson & Waldeck (1970) have presented some experimental support for the view that the activity of noradrenaline-containing neurons is affected by the activity in the dopaminergic neurons when the synthesis of both dopamine and noradrenaline are inhibited, the turnover of the latter amine appears to be slower. When only dopamine-β-hydroxylase is inhibited, the influence of the dopaminergic on the noradrenergic neurons is undisturbed (Persson & Waldeck, 1970).

The tyrosine hydroxylase inhibition as a method for the estimation of noradrenaline turnover therefore appears to have the disadvantage of slowing noradrenaline turnover. On the other hand, when isotope precursor methods are used the extent to which the isotopically-labelled amines are replaced by remaining labelled precursors is not known. Both methods therefore will show too slow

a turnover of noradrenaline. The other possible source of error, which has already been mentioned, is the unequal labelling of storage pools, since much experimental work (Hillarp, 1960: Lundborg, 1963; Häggendal & Lindqvist, 1963; Andeń & Henning, 1966; see Kopin et al, 1968) indicates the presence of a small, functionally-active pool with a high turnover and a large pool from which the amines are released slowly. During the comparatively long time intervals used for estimating the disappearance of the labelled amines in these experiments, only the turnover of the large slow pool would then be reflected in the half-lives obtained with [^{3}H]-tyrosine. Although Persson & Waldeck (1970) found no significant differences between the turnover of [^{3}H]-dopamine with or without inhibitors of its synthesis, the limitations of the methods discussed above may also apply to the estimations of dopamine turnover.

In summary, it is not surprising that there is a great disparity in the results obtained for the turnover of noradrenaline and dopamine in the brain and the changes seen on administration of amphetamine since the estimations of the catecholamine turnover in the brain with the present methods are not very accurate, and may not actually measure turnover in the usually accepted sense of the word.

4. Degradation of 5-hydroxytryptamine

Tagliamonte *et al.* (1971a, b) showed that amphetamine induces an increase in the concentrations of 5-hydroxyindoleacetic acid in the brain. In chicken, Schrold and Squires (1971) obtained similar results. Leonard (1972), however, found no change in the levels of 5-hydroxyindoleacetic acid in rat brain after a single injection or after chronic administration of amphetamine (Diaz & Huttunen, 1972). The effect of amphetamine on the levels of 5-hydroxyindoleacetic acid in the brain might be dependent on several factors including dose, time and environmental temperature. Thus, Scheel-Krüger & Hasselager (1974) reported an increase in 5-hydroxyindoleacetic acid levels in rat brain at 3 h after 5 to 15 mg/kg of amphetamine at 21°C but not at 13°C. ambient temperature. An initial (1 h) decrease and a late (4 h) increase in 5-hydroxyindole acetic acid levels in the rat brain was observed after giving 10 mg/kg of (+)-amphetamine; these fluctuations were absent in chronically-treated rats (Lewander, 1974b). A time dependency of the effect of amphetamine on the levels of 5-hydroxyindoleacetic acid in the brain might explain the inconsistent results in the earlier literature. No change in 5-hydroxyindoleacetic acid concentrations was found in the brains of dogs and cats (Laverty & Sharman, 1965). Rutledge *et al.*, (1972b) found that amphetamine decreased the deamination of 5-hydroxytryptamine *in vitro* after incubating chopped brain tissue in the presence of 10^{-3} M amphetamine. Amphetamine also inhibited the deamination of 5-hydroxytryptamine in aggregated rabbit blood platelets (Da Prada *et al.* , 1965).

5. Synthesis and turnover of 5-hydroxytryptamine

In rats, (+)-amphetamine (5 mg/kg x 2)has been shown to cause an increase in 5-hydroxytryptamine turnover as measured by the slope of the decrease in 5-hydroxyindoleacetic acid levels in the brain after inhibition of MAO by pargyline (Reid, 1970b). This effect of amphetamine was interpreted as possibly being an effect of amphetamine-induced hyperthermia. Amphetamine (1 mg/kg) decreased the rate of accumulation of 5-hydroxyindoleacetic acid after probenecid, indicating a decrease in brain 5-hydroxytryptamine turnover in rats (Görlitz & Frey, 1972).

In mice, Schuberth *et al.* (1970) found that amphetamine (15 mg/kg) reduced the accumulation of 5-hydroxytryptamine in the brain after i.v. administration of labelled tryptophan. Amphetamine also retarded the disappearance from the brain of [^{3}H]-5-hydroxyindoleacetic acid after intracerebral injection of labelled tryptophan in amphetamine-treated mice (Hitzemann *et al.*, 1970) and rats (Diaz & Huttunen, 1972). In contrast, determination of the decrease in the levels of 5-hydroxytryptamine in the brain after inhibition of its synthesis by p-chlorophenylalanine showed that amphetamine and methamphetamine accelerated the turnover as measured by this method (Leonard, 1972). Schuberth *et al.* (1970) were the first to report that amphetamine causes an increase in the brain tryptophan concentration at 3 h after its administration in mice. This finding was confirmed with dose- and time-response curves and extended to include rats as well (Schuberth & Sedvall, 1972). In addition there was an increase in plasma tryptophan concentration after amphetamine treatment, while there

was no change in the liver. Independently, Tagliamonte *et al.* (1971a, b) and Leonard & Shallice (1971) have confirmed that amphetamine-induced increased brain and plasma tryptophan concentrations, especially in rats which reacted with extreme hyperthermia. This effect on brain tryptophan is not restricted to amphetamine but is seen after a number of various drugs (Tagliamonte *et al.*, 1971b). Methamphetamine is also effective in this respect (Leonard & Shallice, 1971; Morgan *et al.*, 1972b). No definite explanation for the effect of amphetamine on brain tryptophan has been put forward. However, the amphetamine-induced changes in brain tryptophan complicate measurements of 5-hydroxytryptamine turnover.

Reports on the *in vitro* determination of tryptophan hydroxylase activity in brain tissue showed that methamphetamine or amphetamine had no effect (Morgan *et al.*, 1972b; Knapp *et al.*, 1974). Chronic administration of amphetamine in the drinking water for 3 to 7 days did not change the tryptophan hydroxylase activity in the rat forebrain tissue (Hulme *et al.*, 1971). However, striatal synaptosomes from amphetamine-treated rats showed a reduced conversion of tryptophan into 5-hydroxytryptamine when assayed *in vitro* (Knapp *et al.*, 1974).

6. Metabolism of acetylcholine

Single doses of amphetamine (5 mg/kg) to rats did not change the activities of brain choline acetylase or acetyl cholinesterase (Ho & Gershon, 1972; Mandell & Knapp, 1972). In cats an increase in acetyl cholinesterase activity in the substantia nigra has been reported (Ellinwood & Escalante, 1970a). The choline acetylase activity in the adrenal glands of chicken increased after methamphetamine administration (Mandell & Morgan, 1970; Mandell & Knapp, 1972).

D. Effects of Amphetamine on the Cyclic Nucleotides and Glycogenolysis

Since brain cAMP levels are increased by noradrenaline in brain slices, it might be expected that amphetamine should induce an increase in brain cAMP *via* the release of noradrenaline. However, several authors have been unable to show an amphetamine-induced increase in whole brain cAMP in mice or rats, in spite of sophisticated methods of rapid killing using coagulation or freezing of the brain (Paul *et al.*, 1970; Schmidt *et al.*, 1972; Ferendelli *et al.*, 1972). In fact, amphetamine and p-hydroxyamphetamine, its main metabolite in the rat, were found to inhibit noradrenaline-induced activation of adenylcyclase in brain slices *in vitro* (Palmer, 1973). Costa *et al.* (1973), however, reported that amphetamine increased cAMP in the rat caudate nucleus simultaneously with an increase in the dopamine turnover in that brain area.

cGMP was increased in mouse cerebellum after the injection of 10 mg/kg of amphetamine (Ferendelli *et al.*, 1972).

Phosphorylase b kinase and phosphorylase activity (enzymes which are thought to be important in the β-receptor mediated glycogenolysis; Sutherland & Rall, 1960) in brain have been reported to be increased by amphetamine (Breckenridge & Norman, 1965; Drummond & Bellward, 1970). (+)- And (-)-amphetamine were equipotent in increasing phosphorylase activity in the rat heart (McNeill & Muschek, 1972). High doses of the drug, however, blocked the stimulating effect of noradrenaline or isoprenaline on this cardiac enzyme (McNeill & Muschek, 1972).

Amphetamine is a potent glycogenolytic agent (Hutchins & Rogers, 1970). Hutchins & Rogers (1970) hypothesized that if the glycogenolysis is mediated by the release of endogenous catecholamines, then it should be possible to antagonize this glycogenolytic effect with drugs which impair the storage or synthesis of catecholamines. The drugs used were: p-chlorophenylalanine, diethyldithiocarbamate sodium, α-methyl-p-tyrosine and reserpine. Each of the drugs which caused depletion of the catecholamines (diethyldithiocarbamate, α-methyl-p-tyrosine and reserpine) resulted in an increase in the concentration of brain glycogen, whereas p-chlorophenylalanine, which depleted 5-hydroxytryptamine levels to less than 20% of the control values did not alter the glycogen content. These results suggest that a change in the metabolic activity of the catecholamine-containing neurons of the central nervous system can influence cerebral glycogen metabolism. After treatment with α-methyl-p-tyrosine or diethyldithiocarbamate the increase in the concentration of glycogen bore a closer temporal relationship with the loss of noradrenaline than with the change in concentration of dopamine.

None of the monoamine depleting agents, when administered individually was able to antagonize the glycogenolytic or central stimulant effect of amphetamine completely, which may be due to small amounts of catecholamines left in the neurons and able to be released by amphetamine. However, Glowinski *et al.* (1966b) found that the amount of radioactively labelled noradrenaline retained in the brains of 24 h reserpinized rats was resistant to release by amphetamine. Thus a direct stimulation of receptors by amphetamine is possible. However, Hutchins & Rogers (1973) were able to antagonize completely the effects

of amphetamine on glycogenolysis by using a combination of reserpine and α-methyl-p-tyrosine. Similar results were obtained in hypothermic mice and in mice whose body temperature had been maintained, and they are consistent with the hypothesis that amphetamine depletes brain glycogen by the release of central catecholamines rather than by a direct action at the receptors.

Although p-chlorophenylalanine did not affect the glycogenolytic effect of amphetamine, it did prevent the characteristic secondary increase in glycogen to a value greater than the control. This suggests that 5-hydroxytryptamine may play a secondary role in the replenishment of glycogen.

E. General Metabolic Effects of Amphetamine Administration.

(i) Oxygen consumption

Amphetamine increases oxygen consumption, probably *via* its peripheral actions, as isolated tissues incubated with amphetamine *in vitro* will take up oxygen, increased oxygen consumption is seen in decapitated rats, and only brain tissue seems to be insensitive to amphetamine *in vitro* (see Waterman, 1949; Andres, 1967; Weis, 1973; Utena *et al.*, 1959). Amphetamine has been found to reduce respiration (Lewis & Pollack, 1965).

The activity of the thyroid gland seems to have a profound effect on the stimulation of oxygen consumption produced by amphetamine, since this effect is enhanced in hyperthyroid rats and almost abolished in hypothyroid rats (Chu, 1969). However Chu's studies showed that amphetamine did not have a releasing action on the thyroid gland. It appears therefore that peripheral adrenergic mechanisms and the action of thyroid hormones on target cells are necessary for the effect of amphetamine on oxygen consumption.

(ii) Brain adenosine nucleotide and phosphate metabolism.

Most of the early studies can be discounted, as they do not agree with those which have been carried out recently and this is almost certainly due to *post-mortem* changes.

Nahorski & Rogers (1973) measured the concentration of glucose, glycogen, glucose-6-phosphate, lactate, ATP and phosphocreatine in the brains of mice at various intervals after the injection of

5 mg/kg of (+)-amphetamine. During the initial 30 min, amphetamine induced a fall in cerebral glycogen and phosphocreatine and an elevation of lactate. Changes in glucose and brain/blood glucose were less marked. The metabolite levels returned to control values at 60 min. The cerebral metabolic rate showed a biphasic change an initial depression of energy flux over the first 15 min following amphetamine injection was followed by an increase that appeared to be closely associated with the increase in locomotor activity over this period.

(iii) Lipid metabolism

Amphetamine in doses greater than 2 mg/kg causes an increase in free fatty acids levels in rats, the peak effect occurs after 1 h. Both isomers are active, but (+)-amphetamine is up to 10 times more potent than (-)-amphetamine (for a review see Lewander, 1977). It has been suggested that amphetamine has at least two actions on adipose tissue and lipolysis: one indirect *via* the sympathetic nervous system and a second *via* release of a lipolytic compound released from the hypothalamus, (Fassina, 1966; Opitz, 1970; Shaw *et al.*, 1972). Indirect evidence for the last proposal has been provided by Bolt *et al.* (1974), who found that the increase in free fatty acids could not be abolished by spinal transection in cats, thus excluding the possibility of a centrally elicited increase in the efferent innervation of the adipose tissue. Amphetamine does not have a direct action on fat tissue cells.

Neuronal membrane lipids

The turnover of phosphatidylcholine (a component of membranes

in nerve endings and vesicles) has been studied after acute and chronic administration of amphetamine and found to be inhibited in several brain areas (Hitzemann & Loh, 1973). *In vitro* it was found that amphetamine inhibited one specific enzymatic step in the synthesis of phosphatidylcholine (diphosphorylcholine diglyceride transferase; Hitzemann & Loh, 1973).

(iv) Protein and nucleic acid metabolism

These have been looked at to a small extent, but these results are contradictory (see Lewander, 1977).

F. Summary of the Biochemical Effects of Amphetamine

The early research into the neurochemical effects of amphetamine led to the suggestion that amphetamine might exert its action by inhibiting MAO. While this action cannot be discounted at present, there is substantial evidence that the pharmacological actions of amphetamine are mediated by the release of catecholamines from neurons in the central nervous system. The exact mechanism for the release is not known, but it seems to be different from the release induced by nerve impulses. In addition, amphetamine blocks the (re-) uptake of noradrenaline and dopamine; this together with, perhaps a similar situation existing at the intraneuronal vesicular membranes, might contribute to the increase in the concentration of catecholamines in the synaptic cleft (therefore increasing the concentration of catecoholamines at the receptor). Similarly, decreased inactivation of the catecholamines by deamination, where there is a high tissue concentration of amphetamine, leads to the same results.

Increased stimulation of pre- and/or postsynaptic catecholamine receptors caused by the increased amount of extraneuronal catecholamines might affect the rate of synthesis and turnover of noradrenaline and dopamine in the brain catecholamine neurons by negative feedback regulatory mechanisms.

The action of amphetamine is thought to be indirect, since amphetamine itself is not thought to act on receptors, but that its action is mediated by the release of catecholamines. There is evidence that amphetamine-induced release of catecholamines is

dependent on newly synthesized catecholamines(or that this pool is preferentially released).

The effects of amphetamine on 5-hydroxytryptamine storage, uptake, release and metabolism are not very clear. The effects are far less pronounced than those on the catecholamines, suggesting that 5-hydroxytryptamine may play a secondary role. The increase in brain tryptophan on treatment with amphetamine is the most pronounced effect with respect to the metabolism of 5-hydroxytryptamine, and one of the more interesting as it has yet to be fully investigated.

There is evidence that amphetamine, at least in high tissue concentrations, causes a release of 5-hydroxytryptamine,an increase in 'turnover' (perhaps due to the increased levels of tryptophan), and a change in the 5-hydroxytryptamine synthesizing capacity of striatal synaptosomes.

G. 'Catecholamines in the Brain as Mediators of the Amphetamine Psychosis'

The amphetamine psychosis has already been mentioned in section A of the Introduction. Snyder (1972) put forward a hypothesis that catecholamines mediated the amphetamine psychosis, and that research in this area could lead to a biochemical model of the paranoid state or paranoid schizophrenia. I do not feel that this model, although interesting, is very convincing. In order to introduce the catecholamine theory, Snyder analyses a study on human subjects carried out by Griffith *et al.* (1970) and Angrist & Gershon (1970) and says that they show that the psychosis is not due to sleep deprivation or to a 'latent' schizophrenic tendency in the patients. I do not think that it is possible to make the latter statement from these clinical studies, but the main point is that both sleep deprivation and personality disorder are characteristic features of chronic amphetamine users (and therefore of the psychosis); these behavioural features almost certainly involve changes in the catecholamines in the brain. He is confusing the relationships between the various disciplines involved in the study of brain function. Any discipline is potentially capable of describing and explaining an aspect of brain function but the biochemical changes occurring at the same time as the behavioural do not necessarily indicate a causal relationship. Another important aspect to bear in mind is that there is a many-to-one relationship (homomorphism) between the disciplines of biochemistry and behaviour.

Despite this criticism of the way in which Snyder presents his

case, I do feel there is good reason to believe that catecholamines may be the primary mediators of the amphetamine psychosis. I do not believe there is a good reason for extrapolating the biochemical changes which may precipitate the amphetamine psychosis to paranoid schizophrenia for the aforementioned reasons.

Crow *et al.* (1976) also proposed that the catecholamines may be involved in schizophrenia for the same reason. They point out that the psychosis occurs in most, if not all, volunteers when a sufficiently large dose is given (Griffith, 1972). This psychosis can be reversed with chlorpromazine, (Espelin & Done, 1968). I do not doubt that the catecholamines are involved in schizophrenia, but I cannot see any reason for believing at this stage that they necessarily play a primary role. It is impossible to disturb the function of one neurotransmitter in the brain without affecting others.

Animal models, by definition, are based on our preconceived ideas and therefore may not be able to provide any understanding of the cause of a particular dysfunction in man.

H. Metabolism and the Metabolites of Amphetamine

1. Uptake into and release from adrenergically innervated tissues of amphetamine itself

Amphetamine accumulates in slices of the mouse heart (Ross & Renyi, 1966b) and perfused rat heart (Thoenen *et al.*, 1968). Subcellular fractionation of the mouse heart showed that only 12% of the total amphetamine content is particle-bound 1 h after the injection of amphetamine (Obianwu *et al.*, 1968). Slices of cerebral cortex from mice (Ross & Renyi, 1966c; Ross *et al.*, 1968) and rat brain homogenate (Baldessarini & Vogt, 1971) also accumulate amphetamine *in vitro*. Subcellular fractionation of rat brains by differential or gradient centrifugation after systemic administration of amphetamine showed that amphetamine is found mainly in the high-speed supernatant (Pfeiffer *et al.*, 1969; Wong *et al.*, 1972; Fuller *et al.*, 1972b); only 7% is located in the synaptosomal fraction and less than 30% is particle bound (Wong *et al.*, 1972).

Several studies indicate that the accumulation of the drug in the tissues might be largely extraneuronal. Thus, denervation (Thoenen *et al.*, 1968; Obianwu *et al.*, 1968), incubation temperature (Baldessarini & Vogt, 1971), and metabolic inhibitors like ouabain (Ross & Renyi, 1966c; Thoenen *et al.*, 1968) do not affect amphetamine accumulation. Efficient neuronal membrane pump blockers for noradrenaline uptake like cocaine, desmethylimipramine and protriptyline have little or no effect on the uptake of amphetamine (Ross & Renyi, 1966b, c; Ross *et al.*, 1968; Thoenen *et al.*,1968; Obianwu *et al.*, 1968). Amphetamine-induced noradrenaline depletion in the rat brain and heart *in vivo* is poorly

inhibited by desmethylimipramine (Brodie *et al.*, 1968; Lewander, 1968a). Protryptyline only partially prevents amphetamine induced release of α-methyl-noradrenaline from mouse heart *in vivo* (Lundborg & Waldeck, 1972). However, more recently it has been found that cocaine and desmethylimipramine inhibit the uptake of amphetamine into central noradrenergic neurons, at low concentrations of amphetamine (Rutledge *et al.*, 1973; Azzaro *et al.*, 1974). It appears that amphetamine is, to a small extent, dependent on the noradrenaline transport mechanisms for its uptake by the adrenergic cell membrane.

Adrenal gland chromaffin vesicles take up amphetamine but to a lesser extent than tyramine and by a mechanism which is different from adrenaline (Wagner *et al.*, 1974). ^{3}H-Amphetamine taken up into peripheral tissues or cerebral cortex slices was only poorly released by electrical stimulation (Kopin & Gordon, 1963; Baldessarini, 1971).

2. Storage of amphetamine in the body.

S. Sparber (personal communication) has found that amphetamine is stored in the adipose tissue on long term treatment of rats with amphetamine. These stores of amphetamine are mobilized on subjecting the animal to cold stress. It is interesting to note that the familiar phrase 'after the drug had been eliminated from the body' may not be true. If amphetamine is stored in adipose tissue (and perhaps in other tissues) and only mobilized on stress, then measurements of blood levels of the drug may be meaningless.

3. Metabolism of amphetamine

Partridge & Conner (1973) claim there is a carrier-mediated transport mechanism for amphetamine which shows saturability, competitive inhibition with other amines and pH dependence. These authors report that a large fraction of the drug enters the brain by a non-specific free diffusion process. This free diffusion is unusual as amphetamine has a pKa of 9.9. Hence at an arterial pH of 7.4 99.7% of the plasma amphetamine will be protonated and in its lipophobic form. They postulate that the rapid onset of central nervous system stimulation could be due to this faster, carrier-mediated mechanism.

The metabolism of amphetamine is subject to large species variations. In rats amphetamine is metabolized mainly by p-hydroxylation in the liver and by β-hydroxylation in noradrenergic neurons. The p-hydroxylation step does not seem to be stereoselective, but hydroxylation in the β-position is stereospecific to the (+)-isomer of amphetamine *in vivo* (Goldstein & Anagnoste, 1965; Goldstein *et al.*, 1964; Lewander, 1971a). Direct β-hydroxylation of amphetamine can be detected *in vivo* (Goldstein *et al.*, 1964; Taylor, 1974), but is only detected in very small amounts in tissues *in vivo* and has not, so far, been found to be important for the pharmacological actions of amphetamine (Goldstein & Anagnoste, 1965; Thoenen *et al.*, 1966; Groppetti & Costa, 1969b; Brodie *et al.*, 1970; Lewander, 1971a).

The presence of p-hydroxynorephedrine was first demonstrated in the rat heart (Goldstein & Anagnoste, 1965) and cat spleen (Thoenen *et al.*, 1966). Later this metabolite was also demonstrated

in adipose tissue (Gessa *et al.*, 1969), and in the rat brain after larger doses of amphetamine (> 1 - 2 mg/kg) or repeated administration of small doses of the drug (Groppetti & Costa, 1969a, b; Costa & Groppetti, 1970; Brodie *et al.*, 1970; Lewander, 1970a, b, 1971a; Clay *et al.*, 1971; Freeman & Sulser, 1972). p-Hydroxynorephedrine is believed to be present in noradrenergic neurons and stored in noradrenergic storage vesicles. This is based on the findings that this metabolite of amphetamine is not detected in the rat liver (Goldstein & Anagnoste, 1965), that it can be liberated from the splenic nerve by sympathetic nerve stimulation in cats (Thoenen *et al.*, 1966), and that reserpine inhibits its accumulation into rat brain and heart, whether it is formed *in vivo* from amphetamine (Lewander, 1971a) or from p-hydroxyamphetamine (Lewander, 1971b). Intraventricular administration of 6-hydroxydopamine reduces the brain content of p-hydroxynorephedrine after amphetamine (Cattabini *et al.*, 1973). Methamphetamine is dealkylated to amphetamine which in turn is p-hydroxylated in the rat. No evidence of the presence of p-hydroxynorephedrine or of p-hydroxyephedrine was found in the rat brain following administration of methamphetamine (Morgan *et al.*, 1972a).

Studies on the duration of the noradrenaline depletion after (+)- or (±)-amphetamine in rats showed that control levels of noradrenaline were not reached until several hours or days after the disappearance of amphetamine from the tissues and it was demonstrated that a maintained noradrenaline depletion correlated in time with the presence of p-hydroxynorephedrine in the brain, heart

and fat tissues (Brodie *et al.*, 1969, 1970; Groppetti & Costa, 1969b; Costa & Groppetti, 1970; Lewander, 1970a, 1971a).

Lewander (1970a ,1971a) and Brodie *et al.* (1970), were the first people to suggest that p-hydroxyamphetamine and p-hydroxynorephedrine were responsible for the increase in the duration of noradrenaline and dopamine depletion after chronic amphetamine treatment. Also that p-hydroxynorephedrine acts as a false transmitter and is involved in the development of tolerance to amphetamine during chronic treatment. I will present Lewander's case, as he sees it (1977) for p-hydroxynorephedrine playing a key role in the development of tolerance to amphetamine.

Investigation of the detailed stochiometric relationship between noradrenaline depletion and the amount of p-hydroxynorephedrine present in rat brain and heart showed that these parameters were related after, but not before, the first 24 h after the amphetamine injection (Groppetti & Costa, 1969b; Costa & Groppetti, 1970; Lewander, 1971a). Thus the initial decrease in tissue noradrenaline levels seemed to be caused by mechanisms other than noradrenaline displacement due to p-hydroxynorephedrine accumulation, while the persistent noradrenaline depletion may be caused exclusively by the presence of this metabolite. The supporting evidence for this theory is:

(i) (-)-amphetamine, which is not converted to p-hydroxynorephedrine, causes only a transient ($\lesssim$ 24 h) and less pronounced noradrenaline depletion than (+)-amphetamine (Brodie *et al.*, 1970; Lewander, 1971a; Clay *et al.*, 1971).

(ii) after pretreatment of rats with drugs that inhibit p-hydroxylation of amphetamine in the rat liver, e.g. desmethylimipramine (Lewander, 1969, 1971a; Groppetti & Costa, 1969a, b; Costa & Groppetti, 1970), no p-hydroxynorephedrine was present in the tissues after (+)- or (±)-amphetamine administration and the noradrenaline depletion was of shorter duration (< 24 h), than after (+)- or (±)-amphetamine alone.

(iii) in the guinea-pig p-hydroxylation of amphetamine does not occur. Thus, p-hydroxynorephedrine is not formed in this species and the brain noradrenaline depletion after amphetamine corresponds in time with the presence of amphetamine itself (Costa & Groppetti, 1970; Lewander, 1970b, 1971c).

(iv) in peripheral noradrenergically innervated tissues, p-hydroxynorephedrine follows the noradrenaline containing vesicular fraction in density gradients, while amphetamine is found in the supernatant (Jaquot *et al.*, 1971).

Administration of p-hydroxyamphetamine to rats and mice causes depletion of heart, spleen, adipose tissue and brain noradrenaline (Maitre & Brunner, 1967; Lewander, 1969, 1971b; Groppetti & Costa, 1969b; Gessa *et al.*, 1969; Brodie *et al.*, 1969, 1970; Costa & Groppetti, 1970; McCullough *et al.*, 1970; Clay *et al.*, 1971; Rapin *et al.*, 1973b). In rats chemically sympathomimectomized by 6-hydroxydopamine, less p-hydroxynorephedrine accumulated in peripheral sympathetically innervated tissues (Jacquot *et al.*, 1973a). Inhibition of noradrenaline uptake inhibits the accumulation of p-OH-amphetamine into peripheral noradrenaline neurons (Jacquot *et al.*, 1973b).

p-Hydroxynorephedrine does not seem to accumulate in dopaminergic neurons, which may not be surprising, as they do not contain dopamine-β-hydroxylase. However, p-hydroxyamphetamine is found in the highest concentration in the caudate nucleus (Cattabeni *et al.*, 1973). This metabolite is present in rat brain for only a short (less than 24 h) period of time after administration of amphetamine (Costa & Groppetti, 1970; Lewander, 1971a). Levels of dopamine in the brain are decreased after p-hydroxyamphetamine administration in rats (Lewander, 1969, 1971b), and mice (McCullough *et al.*, 1970) and therefore p-hydroxyamphetamine may be important for the decrease in brain dopamine, particularly after chronic treatment of rats (Lewander, 1968b, 1971a). Rat brain homovanillic acid concentrations do not change after peripheral p-hydroxyamphetamine injections (Jori & Bernardi, 1972; Jori & Dolfini, 1974). After methamphetamine injections brain and heart noradrenaline is decreased, but the depletion does not last for several days as after amphetamine. Further, there is no evidence for the presence of β-hydroxylated metabolites of methamphetamine in the rat brain (Morgan, 1972a).

Intracisternally injected p-hydroxyamphetamine or p-hydroxynorephedrine causes a sustained decrease in brain noradrenaline (Breese *et al.*, 1970; McCullough *et al.*, 1970; Hitzemann & Loh, 1972; Taylor & Sulser, 1973) and, in some experiments, in the brain dopamine concentration (Hitzemann & Loh, 1972). Release of brain noradrenaline and dopamine by intracisternally administered p-hydroxynorephedrine has been shown by a decrease in labelled catecholamines and an increase in methylated

deaminated catecholamine metabolites (Hitzeman & Loh, 1972). Similar results were obtained for brain noradrenaline after intraventricular administration of both p-hydroxynorephedrine and p-hydroxyamphetamine by Taylor & Sulser (1973). In their experiments however, deaminated noradrenaline metabolites decreased. The tyrosine hydroxylase activity was reported to increase in the rat brain after p-hydroxynorephedrine (Hitzeman & Loh, 1972). Noradrenaline synthesis was, however, decreased *in vivo* after p-hydroxynorephedrine alone or in combination with amphetamine (Hitzeman & Loh, 1974). In this study, marked effects of p-hydroxynorephedrine on both synthesis, release and metabolism of brain noradrenaline were observed. The half-life of p-hydroxynorephedrine in the rat brain after intracerebral injections appears to be shorter (1.5 to 5 h; El-Guedri *et al.*, 1973; Taylor & Sulser, 1973) than after peripheral administration of the drug or its precursor (about 22 h; Costa & Groppetti, 1970; Lewander, 1971a).

It is unlikely that the formation of p-hydroxyamphetamine or p-hydroxynorephedrine are required for amphetamine to exert its acute catecholamine-mediated effects on, e.g., blood pressure, body temperature, motor activity. However, p-hydroxynorephedrine can be released from noradrenergic neurons by nerve impulses (Thoenen *et al.*, 1966) and by amphetamine itself (Brodie *et al.*, 1970; Lewander, 1971b) as a false transmitter.

Looking at the evidence critically, the hypothesis that p-hydroxynorephedrine is acting as a false neurotransmitter and is

involved in the development of tolerance to amphetamine must remain hypothetical. If p-hydroxyamphetamine is a substrate for dopamine-β-hydroxylase, it is not surprising that the product of the enzymic reaction remains in the dense-cored vesicle which contains the enzyme and noradrenaline or that it is released along with the contents of the vesicle.

Tolerance to a drug is defined behaviourally, therefore this theory that p-hydroxynorephedrine, which has weaker receptor stimulating properties than noradrenaline, may displace the transmitter from the noradrenergic storage granules and, following chronic (+)-amphetamine treatment is preferentially released resulting in diminished behavioural and physiological effects has to be subjected to behavioural tests. Lewander (1974) found that there was cross tolerance between the (+)- and (-)- isomers of amphetamine for the anorexic effect. Recently Kokkinidis and Anisman (1978) found cross-tolerance between the two isomers using a free-running Y-maze. Acute amphetamine treatment induces stimulus perseveration, i.e. animals tend to visit only two arms of the Y-maze successively (naive animals tend to enter the arm which was least recently visited). The stimulus perservation decreases after chronic treatment (10 mg/kg for 5 days resulted in tolerance to this effect). Tolerance occurred with (+)- and with (-)-amphetamine and symmetrical cross-tolerance (i.e. from (+)- to (-) and from (-)- to (+)-) was found. This suggests that p-hydroxyamphetamine is not playing a critical role in the tolerance to perserveration, even though there is evidence that suggests a noradrenergic mediation of perseverative behaviour (Anisman & Kokkinidis, 1975).

In summary, the amphetamine metabolite p-hydroxynorephedrine has been proposed to be involved in the development of tolerance to amphetamine. This has been studied extensively from a biochemical point of view. However tolerance to a drug is a behaviourally defined phenomenon, therefore investigation should be carried out, or theories confirmed in this discipline. Octopamine has been proposed as another candidate for a false transmitter by Kostopoulus and Yarbrough (1975).

J. Aim of the Research

From the above review of the literature on the actions of amphetamine it can be seen that the biochemical aspects of withdrawal have not been considered. This is because very little work has been done on this aspect of amphetamine administration.

Seiden *et al.* (1976) observed, in monkeys, that the depletion in catecholamine concentration observed in the brain after chronic treatment with methamphetamine persists for long periods of withdrawal. Herman *et al.* (1971b) treated rats chronically (3 mg/kg/24 h) with (±)-amphetamine for 9 months and found no change in 5-hydroxytryptamine, but a decrease in noradrenaline. After 12 days of withdrawal there were decreases in noradrenaline and 5-hydroxytryptamine in the pons.

A feature of the literature on catecholamine metabolism after chronic treatment with amphetamine is that no one appears to have attempted to look at the situation from a metabolic viewpoint. Thus, the aim of my research was to look at the metabolism of the catecholamines and 5-hydroxytryptamine during chronic treatment with, and withdrawal from, methamphetamine.

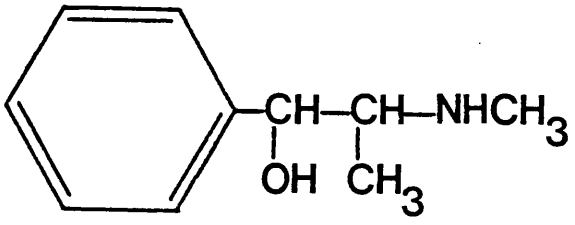
I chose to measure the levels of amines and those enzymes or transport processes which are thought to be rate-limiting steps in their synthesis (i.e. tyrosine hydroxylase and the uptake of tryptophan into the brain). The object was to see if an adaptation to the presence of the drug, *via* the regulatory mechanisms, occurred, and how any such adaptations reacted to the withdrawal of the drug.

The initial experiments were carried out using the same drug regime as Manning *et al.* (1973). In chronically-treated rats they observed that initial decreases in brain lactate and [^{14}C]-glucose incorporation had returned to control levels (suggesting tolerance). Interestingly, the changes observed in these parameters after acute treatment were reversed after withdrawal of methamphetamine.

In summary, I chose to look at the regulation of catecholamine and serotonin metabolism after chronic treatment with, and withdrawal from, methamphetamine, following the drug regime of Manning *et al.* (1973).

FIGURE 1 Chemical structures representative of the amphetamines and catecholamines

a, ephedrine; b, amphetamine; c, methamphetamine; d, phenmetrazine; e, adrenaline; f, noradrenaline; g, dopamine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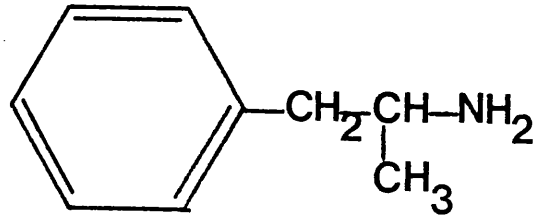
CH
NHCH3
OH
CH3

a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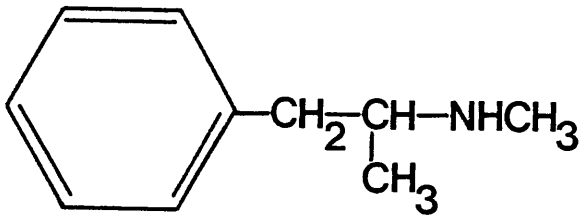
CH
NH2
CH3

b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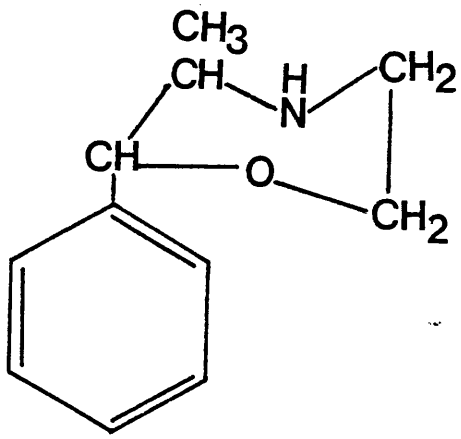
NHCH3
CH3

c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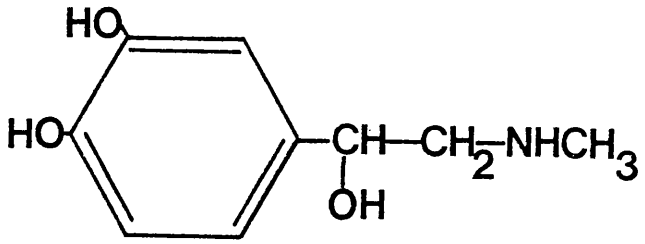
H
N
CH2
CH
O
CH2

d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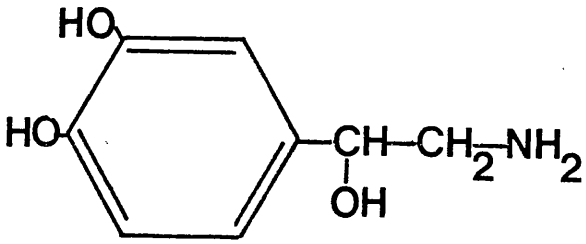
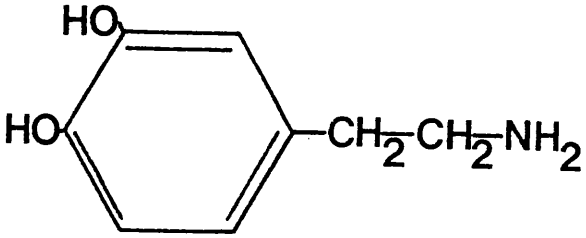
HO
CH
CH2
NHCH3
OH

e


HO
HO
CH
CH2
NH2
OH

f


HO
HO
CH2
CH2
NH2

g

SECTIONS III AND IV

MATERIALS, METHODS AND RESULTS

III. ADMINISTRATION OF METHAMPHETAMINE IN THE DRINKING WATER

A. Treatment of animals and behavioural monitoring

1. Materials and Methods

Materials

DL-Methamphetamine hydrochloride was obtained from Sigma (London) Chemical Company Ltd. The experiments were performed on remote Wistar rats (CHFB) of both sexes (Anglia Laboratories).

Methods

The rats were thirty days old on commencement of treatment. They were kept under constant conditions, i.e. light/dark cycles (12h), temperature (21°C), and diet (Oxoid rat, mouse breeding diet). Four experimental animal groups were set up as follows:

(i) 'Acute' animals were injected (i.p.) with methamphetamine hydrochloride (5 mg/kg body weight), dissolved in 0.9% (w/v) sodium chloride, one hour before death.

(ii) 'Chronic' rats received methamphetamine in their drinking water in concentrations calculated to give 5 mg/kg/24h initially. This was doubled every five days to a final intake of 40 mg/kg/24h at the end of the experimental period.

(iii) 'Withdrawn' rats were treated identically to those in the chronic group, except that the methamphetamine was removed and they were given drug-free water 24h prior to death.

(iv) Control animals. There were two groups of control rats, one of which ('injected controls') received an i.p. injection of saline 1h prior to death. The other group ('controls') received no treatment.

The rats were housed in groups of five.

2. Results

The weights and drinking rates of the rats were recorded. A graph of the weight changes is shown in Figure 2. It can be seen that the chronic male rats gained weight steadily up to 24 days at a rate slower than that of the control males, but higher than that of the control females. Between 24 and 43 days the chronic males increased in weight only very slowly, contrasting with the control males who continued to gain weight rapidly. The chronic females did not appear to gain weight at all, and at 24 days they started to lose weight. This was the only group of rats which lost weight over the experimental period.

The drinking rates are shown in Figure 3. It can be seen that they varied considerably over the experimental period not only between groups but within groups. The control males consistently drank more than the chronic males and control females. The chronic females consumed approximately the same as the last two groups until 20 - 22 days when the drinking rate fell to around 4 ml/24h/rat.

Behavioural monitoring

During the experimental period attempts were made to apply rating scales to the various stereotyped behaviours observed, and to monitor the activity of the animals. To do this a modified hole-board cage was used (File & Wardill, 1975). The size of the cage was 58 cm x 60 cm x 40 cm with 16 holes (4 x 4), diameter 2.5 cm, spaced equally apart. A representative set of figures obtained after 24 days of chronic treatment is shown in Table 1. The rats had been previously

habituated to the cage. It was observed that the chronic rats were readily disturbed by external noises; they responded with a period of immobility (about 30s), followed by a period of hyperactivity.

Table 1.

The figures represent the ratio of line-crossing (activity); climbing the sides of the cage and head-dipping (exploratory behaviour); rearing ; over a 3 min period.

Control males			Chronic males		
Rat	Wt.(g)	rating	Rat	Wt.(g)	rating
1	250	68:25:2	1	220	88:12:0
2	290	73:23:1	2	210	76:11:0
3	245	94:18:0	3	235	90:28:4
4	245	29:13:1	4	195	71:11:0
5	245	52:24:0	5	185	85:23:0
6	190	57:12:4	6	215	120:20:6
			7	200	137:34:1

It can be seen that the chronically-treated males appeared to be more active than the control males. There is no obvious difference between exploratory behaviour and rearing. The data was not analysed statistically for reasons which will become apparent later.

From subjective observations, between 0 - 3 weeks hyperactivity was marked in the chronic rats from the commencement of treatment. After 1 week an abnormal gait (recorded on film) similar to "mud-walking" was observed. This was especially prominent in the females. With-

drawal of the rats after 3 weeks of chronic treatment appeared to have a relatively depressing effect on motor activity.

Between 3 and 7 weeks of treatment the hyperactivity decreased in the chronic males and females, and the abnormal gait became less prominent, but was still observed after five weeks. At approximately 5 weeks the females showed marked wasting, and engaged in pointless, apparently stereotyped, behaviour (they clustered around the water bottle and continually rattled the ball, but the drinking rates showed that they were consuming hardly any water). By 6 weeks all the females had died; on death it could be seen that they had bitten through their skin in two, almost stereotaxically defined, positions on the upper and lower abdomen. However no bleeding was apparent.

Between 6 and 7 weeks the abnormal gait could no longer be observed in the males, neither was there any marked hyperactivity. However, they started to engage in stereotyped grooming behaviour and self-mutiliation of their claws (in some cases the claws were bitten away to the skin, which was bleeding). Withdrawal from methamphetamine at this stage appeared to have no obvious behavioural effects.

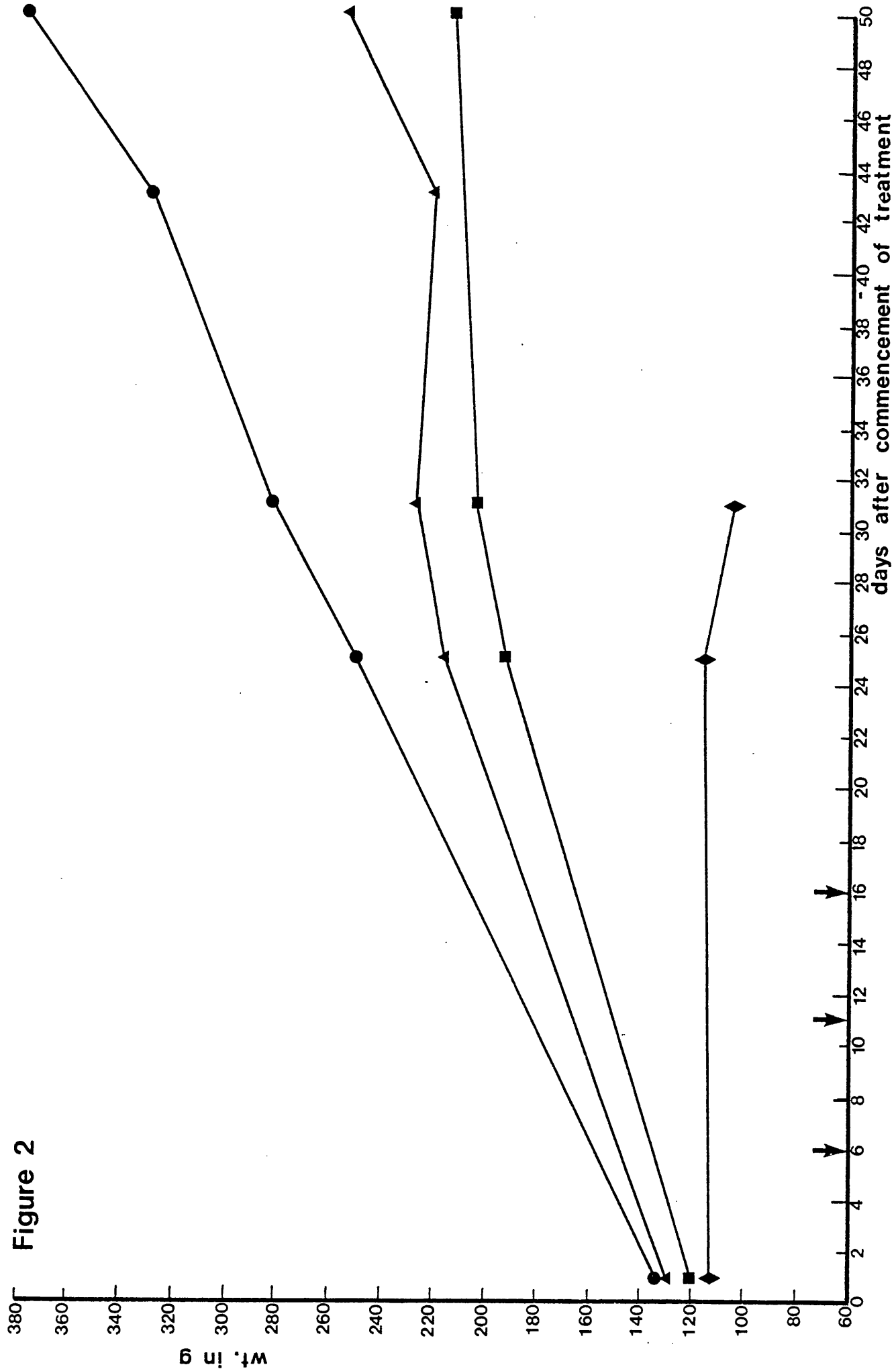
FIGURES 2 and 3 The weights and drinking rates of chronic and control rats over the experimental period

The weights of the control males (●—●), chronic males (▲—▲), control (■—■) and chronic females (◆—◆) are shown in Figure 2. Arrows on the abscissa mark the day on which the dose of methamphetamine was doubled. Figure 3 shows the drinking rates of the same animals; the key is the same as for figure 2.

The first chronically-treated female died on day 24; only one was left by day 37. All the chronically-treated females had died by day 42.

There were not enough data for a statistical analysis.

Figure 2


wt. in g
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360
340
320
300
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260
240
220
200
180
160
140
120
100
80
60
0
2
4
6
8
10
12
14
16
18
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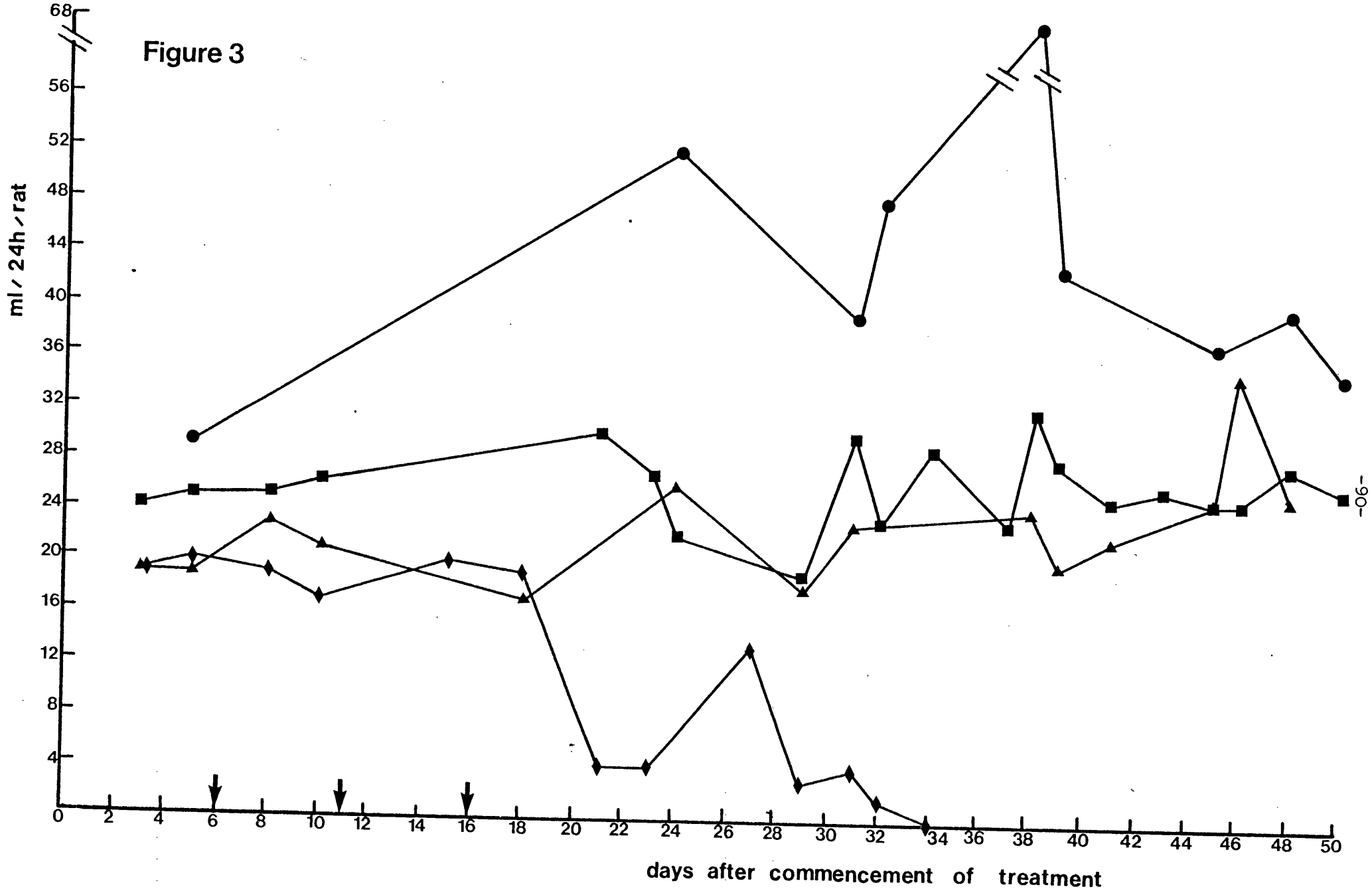
28
30
32
34
36
38
40
42
44
46
48
50
days after commencement of treatment

Figure 3


ml / 24h / rat
68
56
52
48
44
40
36
32
28
24
20
16
12
8
4
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
days after commencement of treatment

B. Choice of brain regions

This was determined by our knowledge of the distribution of the amines likely to be affected by the treatment. The regional distribution in the brain of the monoamines, noradrenaline (Vogt, 1954), dopamine (Carlsson *et al.*, 1958) and serotonin (Twarog & Page, 1953) led to the hypothesis that they were acting as neurotransmitters in the brain. Pharmacological manipulation of the monoamines has led to their implication in various neurological and psychiatric disturbances in man. Most of the dopamine in the brain was found to be localized in the neostriatum (Bertler & Ros engren, 1959a,b). Reserpine was demonstrated to deplete the stores of serotonin in the body (Shore *et al.*, 1956), and the catecholamine stores both centrally and peripherally (Holzbauer & Vogt, 1956; Carlsson & Hillarp, 1956; Bertler *et al.*, 1956). An injection of the catecholamine precursor dihydroxyphenylalanine (DOPA) resulted in a reversal of the behavioural changes brought about by reserpine administration, but the serotonin precursor 5-hydroxytryptophan lacked this effect (Carlsson *et al.*, 1957). The injection of DOPA caused a considerably greater rise in dopamine levels than in noradrenaline levels. The emergent hypothesis was that Parkinson's disease may be caused by changes in dopamine levels or its properties as a neurotransmitter (Carlsson, 1959). Other examples of drugs introduced into psychopharmacology as substances found to act by manipulating monoamine levels are chlorpromazine (Delay *et al.*, 1952), iproniazide (Loomer *et al.*, 1957; Kline, 1959) and imipramine (Kuhn, 1957). These drugs are still used as potent antipsychotic and antidepressant drugs, respectively.

The criteria for noradrenaline, dopamine and serotonin functioning as transmitters in the central nervous system have to considerable extents been experimentally established (Andén, 1964; Andén *et al.*, 1969).

The distribution of the monoamines in the central nervous system has been localized biochemically (Vogt, 1954; Bertler & Rosengren, 1959; Carlsson, 1959; Bertler, 1960) and mapped histochemically (Fuxe *et al.*, 1969).

The Falck and Hillarp histochemical technique provided the first evidence for the intraneuronal localization of noradrenaline, dopamine and serotonin in specific systems of cell bodies and nerve terminals (Carlsson *et al.*, 1962). Dahlström and Fuxe were able to make a detailed description of the exact localization of the noradrenergic, dopaminergic and serotonergic cell groups and nerve terminal areas (Dahlström & Fuxe, 1964, 1965; Fuxe, 1965 a, b). It is possible to increase the intraaxonal monoamine levels by lesioning the axons, as the interruption of axonal flow causes a pile up of fluorescent material proximal to the lesions thus making it possible to trace the axons by serial sectioning. By studying the axonal pile up and the terminal degeneration after lesions it was possible to outline the ascending monoamine pathways in the brain (Andén *et al.*, 1966). The detailed anatomy of the dopaminergic and noradrenergic pathways was elucidated by Ungerstedt (1971c) who, by superimposing his sections on the König and Klippel stereotaxic rat brain atlas (1963), made the monoamine pathways accessible to stereotaxic techniques.

Major monoamine pathways in mammalian brain

(i) Noradrenaline

Noradrenergic cell bodies, found in the brain stem, send projections via the dorsal and ventral noradrenergic bundles to innervate forebrain structures. The ventral bundle is believed to project to the hypothalamus, while the dorsal bundle innervates regions of the cerebral

cortex and mesolimbic nuclei. The locus coeruleus contains the major noradrenergic cell group. This is located in the pons in the floor of the IV th ventricle, and sends fibres mainly *via* the dorsal bundle.

(ii) Dopamine

Dopaminergic cell bodies are found predominantly in the mid-brain. Three major dopaminergic pathways have been established:

Nigro-neostriatal pathway. This originates in the cell bodies of the substantia nigra and the fibres project to the neostriatum (caudate nucleus-putamen complex).

Mesolimbic pathway. This originates in cell bodies medial to the substantia nigra and projects through the median forebrain bundle to areas of the mesolimbic system (nucleus accumbens septi, tuberculum olfactorium, amygdaloid complex).

Tubero-infundibular dopaminergic system. The cell bodies are found in the acunate nucleus of the hypothalamus and project fibres to the terminal region of the median eminence.

(iii) 5-hydroxytryptamine (serotonin)

Major serotonergic cell groups are found in the region of the Raphé nuclei (dorsal and ventral) of the upper brain stem. Other minor serotonergic cell groups are found in the pons/medulla, giving rise to various ascending and descending tracts. Fibres from the Raphé nuclei project to various forebrain structures; these fibres eventually run

with the median forebrain bundle and innervate regions of the neostriatum, cerebral cortex and mesolimbic nuclei. There are also more caudal projections to the hypothalamus and hippocampus.

A diagrammatic summary of the noradrenergic, dopaminergic (Ungerstedt, 1971**b**) and serotonergic (Dahlström & Fuxe, 1964, 1965) pathways is shown in Figure 4.

Figure 4. 4a shows the major noradrenergic pathways and the nigrostriatal dopaminergic pathway.

4b shows the major serotonergic pathways and a more detailed diagram of the dopaminergic pathways.

Fig. 4a

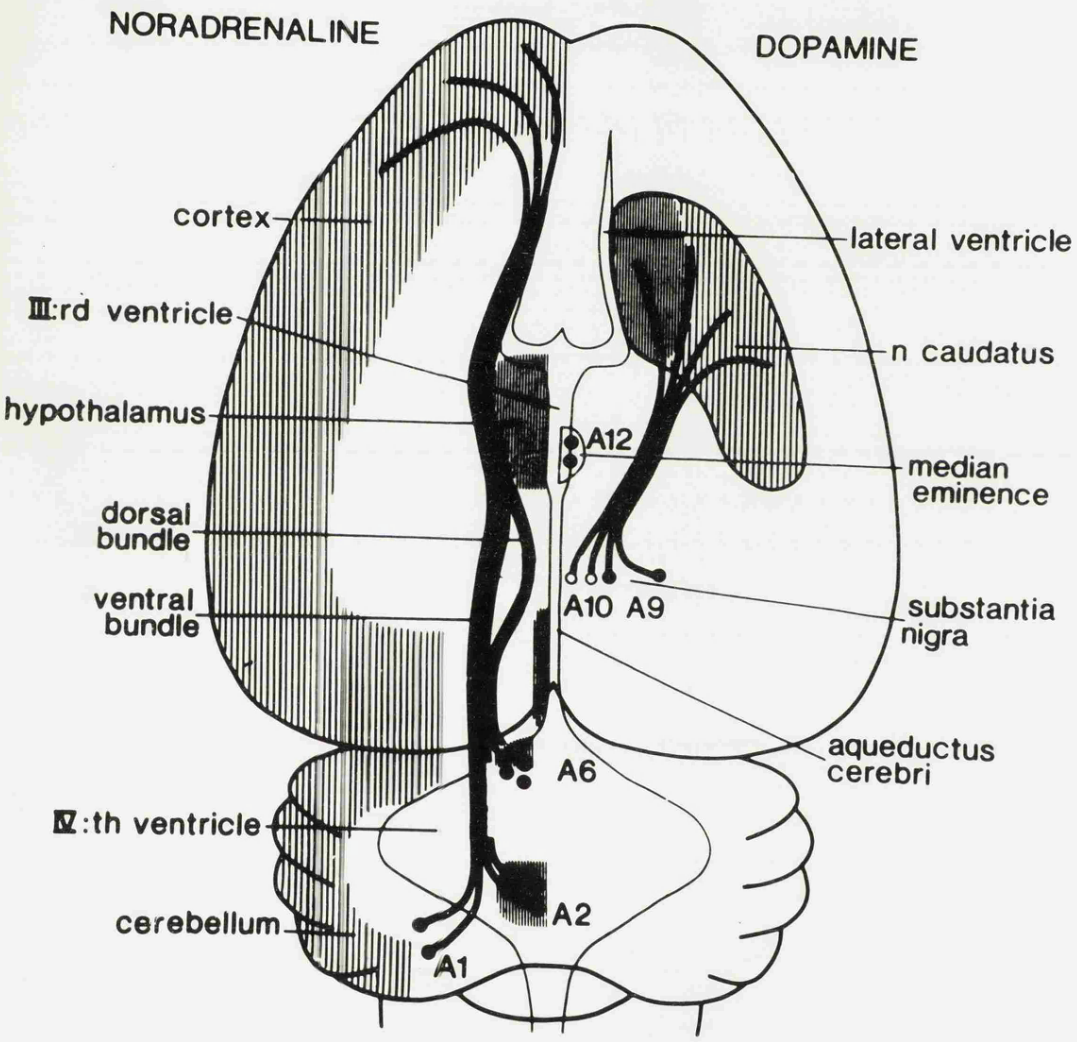
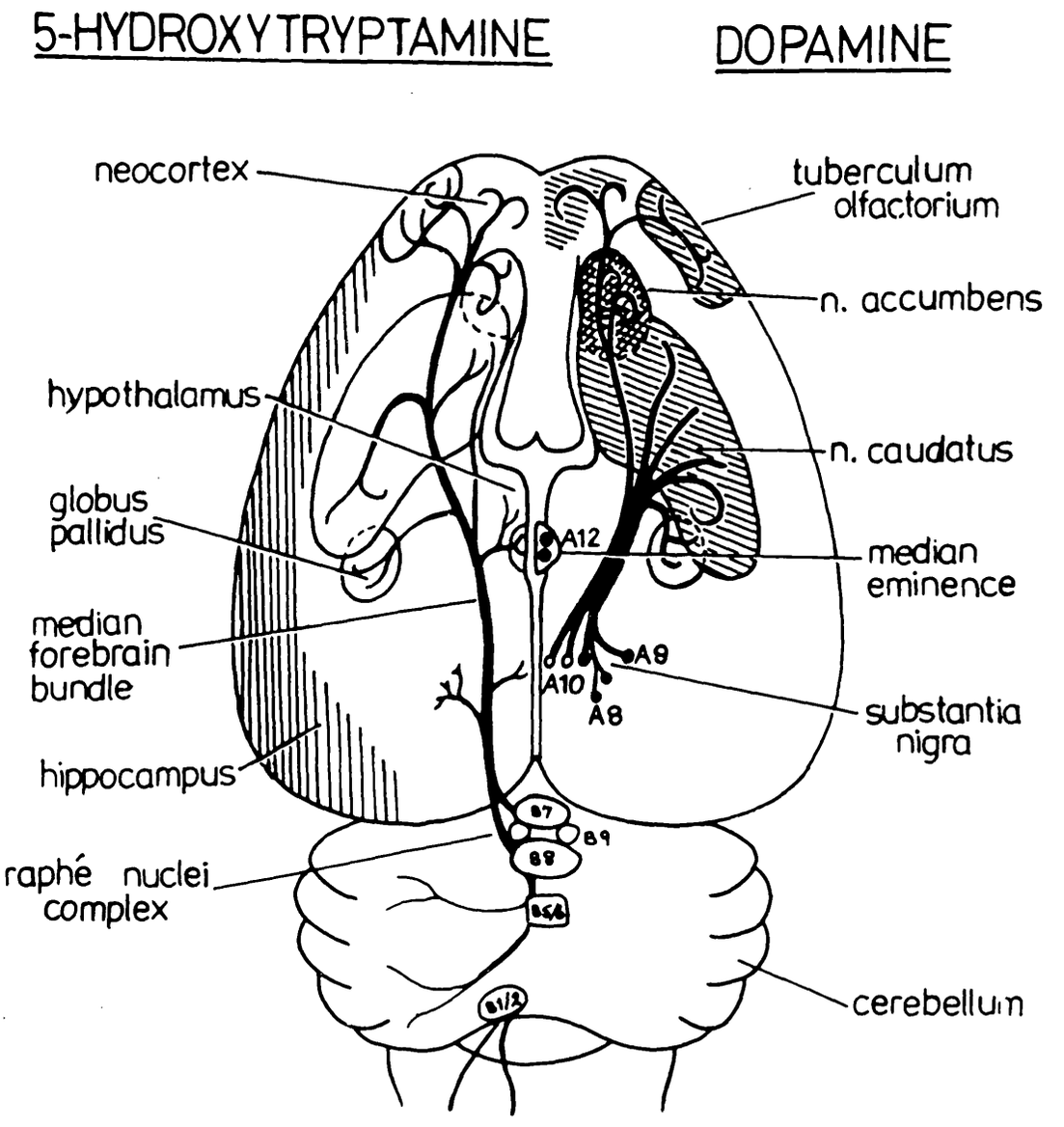

NORADRENALINE
DOPAMINE
cortex
lateral ventricle
III:rd ventricle
n caudatus
hypothalamus
A12
median eminence
dorsal bundle
ventral bundle
A10 A9
substantia nigra
aqueductus cerebri
A6
IV:th ventricle
cerebellum
A2
A1

Fig. 4b


5-HYDROXYTRYPTAMINE
DOPAMINE
neocortex
tuberculum olfactorium
n. accumbens
hypothalamus
n. caudatus
globus pallidus
A12
median eminence
median forebrain bundle
A9
A10
A8
substantia nigra
hippocampus
B7
B9
B8
raphé nuclei complex
B5&6
B1/2
cerebellum

C. Regional dissection of the brain

Post-mortem changes are obviously an important consideration at this stage. The three methods most commonly used to minimize these are:-

(i) Subjecting the rat to a short, intense burst of microwave irradiation.

(ii) Decapitation followed by dissection over ice or on a freezing block.

(iii) Decapitation followed by freezing the whole brain in liquid nitrogen (or, alternatively, killing the rat by dropping it into liquid nitrogen).

Method (i) has recently been shown to disrupt the structural integrity of the nerve fibres (Meyerhoff *et al.*, 1978). Method (iii) poses practical problems for a regional dissection of the brain, as the architecture of the brain is destroyed by freezing, and the various regions loose their colouration and texture. This means that dissection of specific regions would have to be carried out solely on their stereotaxic positions. Frozen brains are very difficult to mount on a cryostat chuck, and would have to be cut before commencing dissection. Thin blocks buckle and break more easily than the whole brain, making clean, consistent dissection hard. Palkovits (1973) has described a method employing cryostat dissection. Brain nuclei were 'punched out' of slices of frozen brain cut with a microtome. The slices were placed on a microscope slide and the required nuclei removed with a special needle, under a stereomicroscope.

I found it was not possible to raise the temperature of the brain in a cryostat to a temperature at which it could be dissected without fracturing the brain. This can only be done at $0^{\circ}C$ when the ice has melted and

the brain is no longer firm; therefore dissection is difficult.

For the above reasons Method (ii) was chosen as being the most practical. Two recent papers (McKay *et al.*, 1978 a, b) have reviewed, amongst other things, the stability of monoamine oxidase, tyrosine hydroxylase, dopamine-β-hydroxylase, dopa decarboxylase, noradrenaline, dopamine, serotonin, homovanillic acid and 5-hydroxyindoleacetic acid in the brain *post-mortem*. Tyrosine hydroxylase has been shown to be very unstable at room temperature, and only moderately stable at 4°C. The activity of this enzyme falls by approximately 50% over 3 *post-mortem* hours in rat brain (Black & Geen, 1975). The order of increasing stability is:- dopamine-β-hydroxylase, dopa decarboxylase and monoamine oxidase (which appears to be stable even at room temperature). Noradrenaline is the most unstable of the amines mentioned, although data on the metabolites are sparse, i.e. limited to those mentioned here. *Post-mortem* changes using Method (ii) are likely to be minimal with the exception, perhaps, of tyrosine hydroxylase. This will be considered in the Discussion. After dissection, the regions were weighed and dropped into liquid nitrogen. The regions were stored at -20°C.

The regions dissected were:-

(i) Cortical slice (0.33 mm slice of neocortex).

(ii) Medulla. Cuts were made in front of the superior colliculi and behind the IVth ventricle.

(iii) Striatum (including caudate nucleus - putamen and globus pallidus).

(iv) Mesolimbic regions. The tuberculum olfactorium, nucleus accumbens and amygdala were pinched out using fine forceps.

(v) Hypothalamus. This was pinched out using fine forceps.

The pinching out of regions was found to be the most rapid way of selectively obtaining these small regions, rich in nerve terminals. Precision was, to some extent, sacrificed for rapidity. The consistency of dissection was monitored by weight. The time from decapitation to the stage where all regions had been frozen in liquid nitrogen varied from 6 - 8 min. Figure 5 shows the positions of the various brain regions.

Throughout this thesis the names given to the regions do not correspond to their precise anatomical definition therefore should be prefaced by "the regions containing...", and these are defined by the dissection technique.

Fig.5 Drawing of the rat brain

The regions of the brain that were dissected out (see text) are indicated.

Key:-

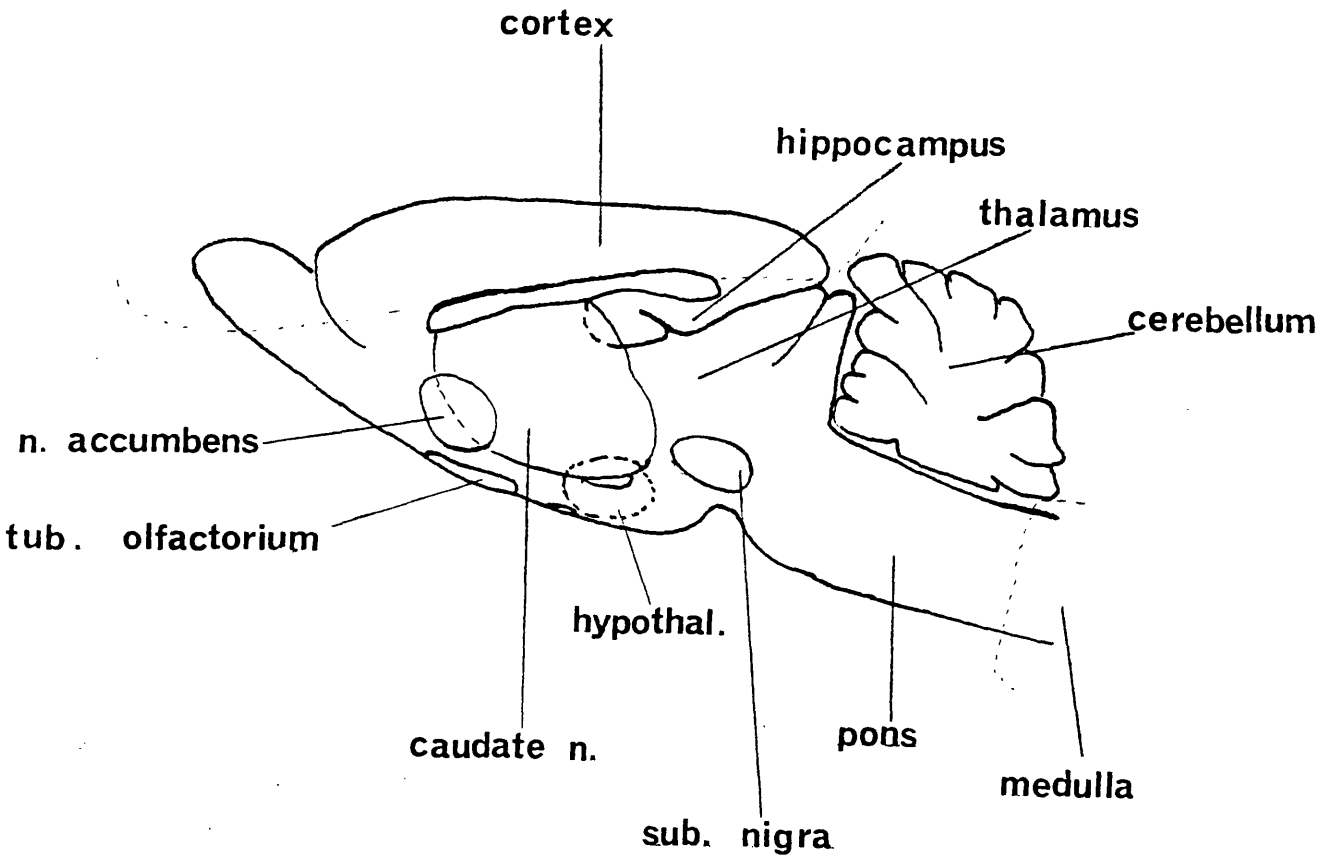
hypothal. - hypothalamus

caudate n. - caudate nucleus (striatum, globus pallidus and putamen)

tub.olfatorium - tuberculum olfatorium

n. accumbens - nucleus accumbens.

sub. nigra - substantia nigra.


cortex
hippocampus
thalamus
cerebellum
n. accumbens
tub. olfactorium
hypothal.
caudate n.
sub. nigra
pons
medulla

D. Fluorometric determination of noradrenaline, dopamine, serotonin and histamine

1. Materials and Methods

Materials

All reagents were of Analar grade. Noradrenaline bitartrate, dopamine hydrochloride, serotonin creatinine sulphate, histamine hydrochloride, orthophthalaldialdehyde and Dowex 50W (x4; H^+ form; 100 - 200 mesh) ion-exchange resin were obtained from Sigma (London) Chemical Company Ltd.

Potassium hydroxide phthalate reagent was made by adding 1.021 g of potassium hydrogen phthalate to 40 ml of water. The solution was then adjusted to pH 3.0 with 2 M hydrochloric acid. To this, 3.96 g of potassium hydroxide was added, and the final volume adjusted to 50 ml.

Narrow-range indicator paper (pHydrion paper pH 3.0 - 5.5) was obtained from Micro Essential Laboratory, Brooklyn, N.Y., U.S.A.

An Aminco Bowman spectrophotofluorometer (American Instrument Company Inc., Silver Spring, Maryland, U.S.A.) was used for all the fluorometric determinations.

Methods

The first method for the extraction and purification of the amines in brain tissue was performed according to the method of Atack & Magnusson (1970). The procedure followed was:- homogenization, neutralization and ion-exchange chromatography.

Homogenization. Each region was homogenized in 10 vol. of perchloric acid (0.4 M) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and 10 mM ascorbic acid. The EDTA and ascorbic acid were added to minimize oxidation of the amines. Homogenization was carried out using 'Uniform' glass to glass homogenizers. The procedure was standardized to ensure maximal, consistent extraction of the amines. The homogenate was kept at $0^{\circ}C$ in an ice bath, then centrifuged at 8,000 x g (16 x 15 ml head) for 15 min in an MSE 18 centrifuge. The supernatant was pipetted off, the volume measured and stored at $-20^{\circ}C$ overnight. Immediately before the amines were separated by ion-exchange chromatography, the homogenate was neutralized.

Neutralization. The extracts were adjusted to pH 4 by the addition of 1 M acetic acid (0.1 ml: 1ml extract), at $0^{\circ}C$, followed by the potassium hydroxide phthalate reagent (approximately 0.4 ml: 1 ml extract). The potassium hydroxide phthalate reagent was added with mixing to avoid high local pH changes. The precipitated potassium perchlorate was removed by centrifugation at 3,000 xg for 10 min at $0^{\circ}C$. The pH of each extract was tested using narrow-range indicator paper.

Ion-exchange chromatography. Dowex 50W (x4; H^{+} form; 100 - 200 mesh) resin was prepared by washing ten times with water, with subsequent decanting, to remove fine particles. The resin was washed in 2 M sodium hydroxide (10 vol.) containing 0.1% EDTA, followed by three washes with distilled water. Finally the resin was washed with 10 vol. of 2 M hydrochloric acid, followed by three washes in distilled water. This cycle was repeated three times, and the resin was stored in 0.1 M

sodium phosphate buffer, pH 6.5, containing 0.1% EDTA.

The columns (15 mm x 6 mm) were packed with freshly-prepared Dowex resin. The column was washed with 10 ml of 0.1% EDTA in order to remove any fluorescent material or metal ions which may have been left on the resin. A known volume of neutralized extract was then loaded onto the column and washed with 10 ml of 0.1 M sodium phosphate buffer, pH 6.5. This preliminary washing removed compounds which had not bound to the resin e.g. 5-hydroxyindoleacetic acid, 5-hydroxytryptophan and histidine. The eluate was collected and discarded. The column was then washed with 5 ml of distilled water. The amines were eluted in a stepwise manner, and the elution pattern is shown in Figure 6. This took about 6 h and the recovery was low; consequently this stage was modified.

Modified method

The method was modified as follows: noradrenaline, dopamine and histamine were eluted with 4 ml of 2.5 M hydrochloric acid, then the serotonin was eluted with 5 M hydrochloric acid in 50% methanol.

Initially it was assumed that the original order of elution corresponded to the decreasing pKa values of the amines ; therefore all should elute with 4 ml of 2.5 M hydrochloric acid. However, serotonin was found to elute very slowly even using concentrated hydrochloric acid, implying that the nature of its binding to the ion-exchange resin is not simply ionic. Serotonin was eluted with 8 ml of acid and the elution pattern was independent of the concentration of acid used between 2.5 M and 11.45 M. On using 1 M hydrochloric acid in 50%

methanol or ethanol, serotonin was eluted in 2 ml. In practice 5 M hydrochloric acid in 50% methanol was used, as the fluorometric assay requires strongly acidic conditions. Therefore the subsequent addition of hydrochloric acid in the assay procedure could be omitted, thus effectively doubling the sensitivity of the assay. It was found necessary to dry the methanol and redistill it immediately prior to use; if this was not done the blank values were very high. Methanol was chosen, rather than ethanol, as the assay for serotonin involves heating in a boiling water bath for 15 min. The evaporation of the methanol increased the sensitivity of the assay, and could be the reason why the purity of the methanol was found to be so crucial.

The flow rate of the columns using glass-wool plugs was found to be very variable. This was standardized by using polyethylene plugs. Light was partially excluded from the resin, as a precautionary measure, with opaque tape.

Each fluorometric assay was found to be specific. Methamphetamine and the major metabolites of amphetamine, norephedrine and p-hydroxyamphetamine were found not to interfere with any of the assays.

This modified procedure cut down the elution time by one third, and minimized the loss of amine due to altered elution patterns. Quenching of the fluorescence by other compounds present in the eluate, was found not to be significant (based on the use of internal standards).

Simulated assays were carried out to test the recovery of each amine at different stages in the overall procedure.

A diagrammatic summary of these, and the percentage recoveries, are shown in Figure 7 and Table 2. This was done for the pure compounds only.

Fig. 6 Elution pattern of amines from ion-exchange chromatography.

The stepwise elution of standard solutions of; noradrenaline, serotonin, dopamine and histamine from Dowex 50W (x4; H^+ form).

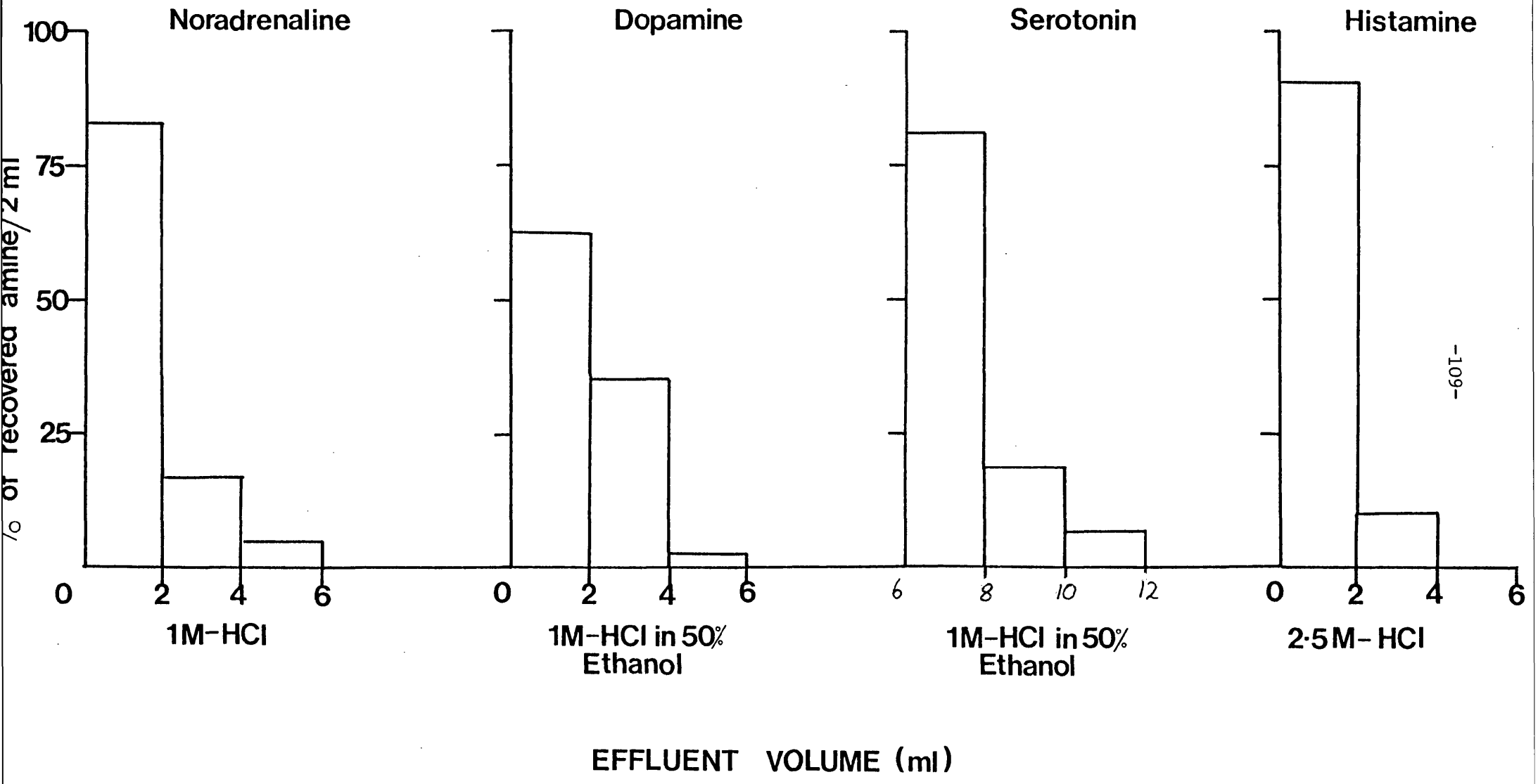

Noradrenaline
Dopamine
Serotonin
Histamine
100
75
50
25
% of recovered amine/2 ml
0
2
4
6
8
10
12
1M-HCl
1M-HCl in 50% Ethanol
1M-HCl in 50% Ethanol
2·5M-HCl
EFFLUENT VOLUME (ml)

Figure 7. Simulated Assays

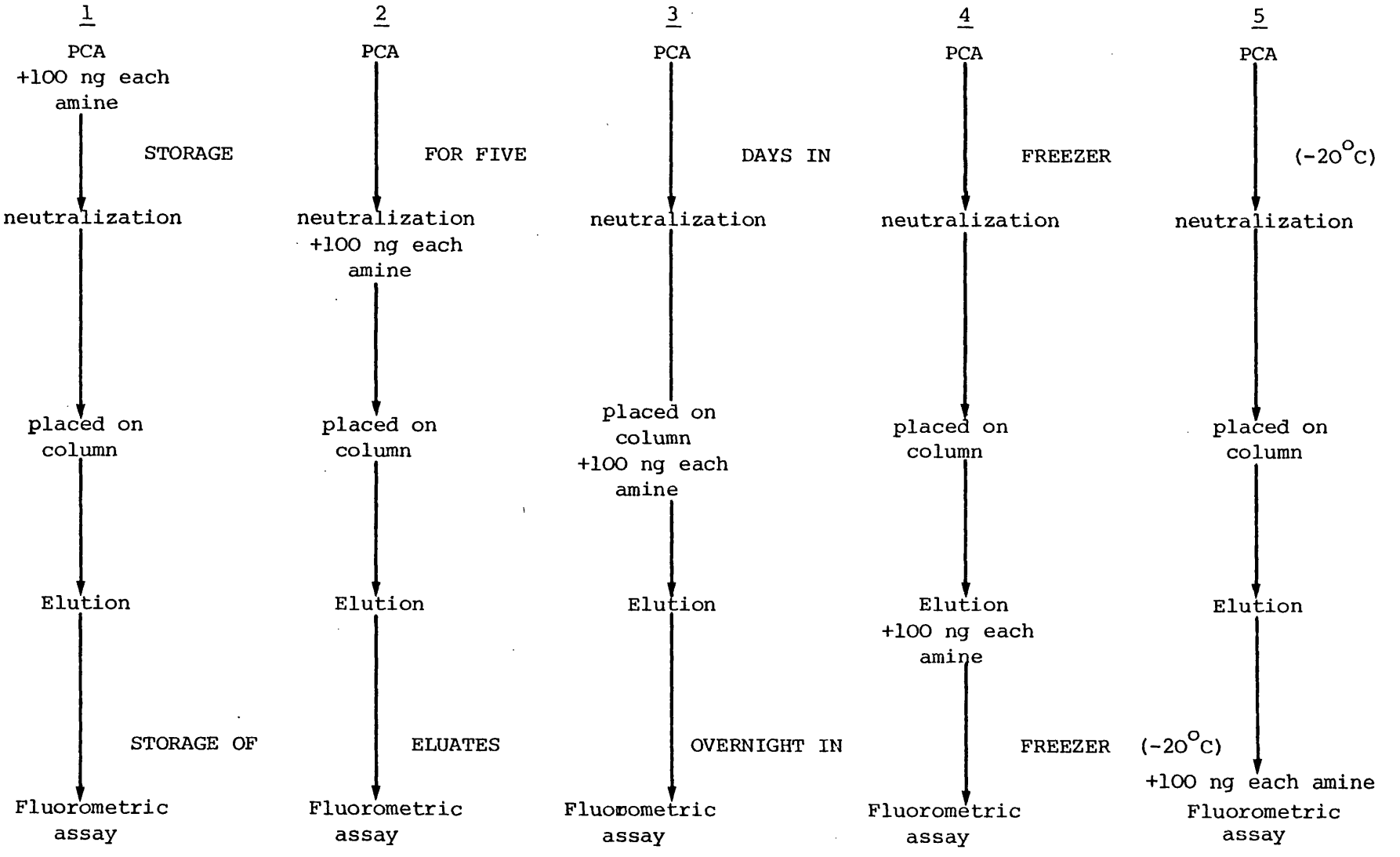


Table 2. Results of simulated assays

NORADRENALINE		DOPAMINE	
Stage	Recovery	Stage	Recovery
1	85%	1	52%
2	89%	2	52%
3	100%	3	54%
4	100%	4	88%
5	100%	5	100%

SEROTONIN		HISTAMINE	
Stage	Recovery	Stage	Recovery
1	70%	1	100%
2		2	100%
3		3	100%
4		4	100%
5		5	100%

The assays were carried out in triplicate. The assays had to be repeated for dopamine, as the first set of results showed low recovery and high interference. The second set of results are shown here.

Fluorometric determination of the levels of noradrenaline, dopamine, serotonin and histamine in brain regions

(i) Dopamine and noradrenaline

Both the noradrenaline assay (Laverty & Taylor, 1968) and the dopamine assay (Atack, 1973) are based on the formation (by oxidation) of the fluorescent trihydroxyindole derivatives. The trihydroxyindole derivative of dopamine was measured at an excitation wavelength of 330 nm, and an emission wavelength of 375 nm. The corresponding excitation and emission wavelengths for the noradrenaline derivative were 395 nm and 475 nm.

(ii) Serotonin and histamine

Both assays involve condensation with orthophthalaldialdehyde (OPT) to give a fluorescent product. In the case of serotonin the reaction takes place on heating in acid conditions, (Maickel *et al.*, 1968) to give a product which fluoresces at excitation and emission wavelengths of 295 nm and 550 nm respectively. Histamine reacts with OPT under alkaline conditions to give a fluorescent product with excitation and emission wavelengths of 350 nm and 460 nm (von Redlich & Glick, 1974). In all fluorescent studies the excitation and emission peaks are uncorrected.

Modification of fluorometric assays

The assays were modified to allow for the assay of small volumes and the higher acidity of the column eluate. In all the assays the pH has to be correct at the critical stages in the reactions to ensure maximal formation of the fluorophore.

For the assay of noradrenaline, potassium bicarbonate (2.5 M) containing potassium hydroxide (2.5 M) was added, on mixing, to 0.3 ml of eluate until the pH reached 6.5 (approximately 0.2 ml). Iodine reagent (0.1 ml of 0.02 M iodine in 5% w/v sodium iodide) was added, the solution mixed and allowed to stand for 3 min. Alkaline sulphite reagent (0.3 ml) was added on mixing and the solution left to stand for 4 min (alkaline sulphite reagent: 40% (w/v) Na_2SO_3; 1% mercaptoethanol; 5 M NaOH). Finally the solution was acidified with 0.2 ml of 10 M acetic acid.

The dopamine assay was modified as follows: EDTA (50 µl of 8%, pH 7) was added to 0.5 ml of eluate. Potassium ferricyanide (50 µl of 0.25%) was added on mixing and the solution was left to stand for 5 min. Alkaline metabisulphite (6.3 M NaOH containing 0.125 M sodium metabisulphite) was added until the solution reached a pH of 13.1 (approximately 0.2 ml). After allowing to stand for 5 min, 0.2 ml of glacial acetic acid was added.

In both these assays it was important to add the alkaline solutions on mixing on a vortex mixer to try to minimize high local pH changes which would result in oxidation of these amines. The blanks used for the noradrenaline and dopamine assays were: reagent (no amine sample) and completely reversed (acid, antioxidant and then oxidant).

Serotonin was assayed by taking 0.5 ml of eluate and adding 50 µl of 0.02% (w/v dissolved in methanol) OPT. The solution was mixed and incubated in a boiling water bath for 15 min. The tubes were left to cool before reading them in the fluorometer.

The histamine assay was carried out by adding 0.12 ml of 5 M NaOH to 0.2 ml of column eluate. The NaOH was added, on mixing, to bring the solution to a pH of 12.4 to 12.5. OPT (50 µl of 0.02% w/v dissolved in methanol) was added, the solution mixed, and allowed to stand for 4 min before adding H_3PO_4 (50 µl of 3 M) to bring the final solution to a pH of 2.4 to 2.6.

In both the serotonin and histamine assays, the OPT had to be made up immediately prior to use. It was found that the concentration of OPT used had to be increased approximately 20 fold over the published assays. Two blanks were used: a reagent blank (no amine sample) and a sample blank (methanol without OPT was added).

2. Results

One complete set of results was obtained for each of the four amines from the five experimental groups. The chronic and withdrawn animals had been on methamphetamine for seven weeks. The results are summarized in Figures 8, 9, 10, 11. The values were subjected to a statistical analysis and the test for significance which was used was the unpaired 'student's' t test.

The significant changes ($p < 0.05$) seen in the amines on chronic treatment were found mainly in serotonin. Histamine appeared to change the most on injecting the animals, although this may be due to an increased concentration of histamine in the blood. The high standard deviations,seen especially with the dopamine results,would mask small changes in the amines and relatively large changes in dopamine levels. The high standard deviations reflect the problems encountered with this assay.

It can be seen from the tables that the values for the levels of the amines are very low and that they have not been corrected for recovery. This is due to the very low, and variable recoveries obtained for the internal standards which were run at the same time as the samples. This was unexpected and casts doubt on the meaning of the results which were obtained.

FIGURES 8-11 Summary of the regional amine levels in the five experimental groups of rats after 49 days of treatment.

Figures 8 to 11 show the mean noradrenaline, dopamine, serotonin and histamine levels, respectively, in the various experimental groups. The error bars mark the standard deviation . The levels are not corrected for recovery.

Key: Control

Chronic

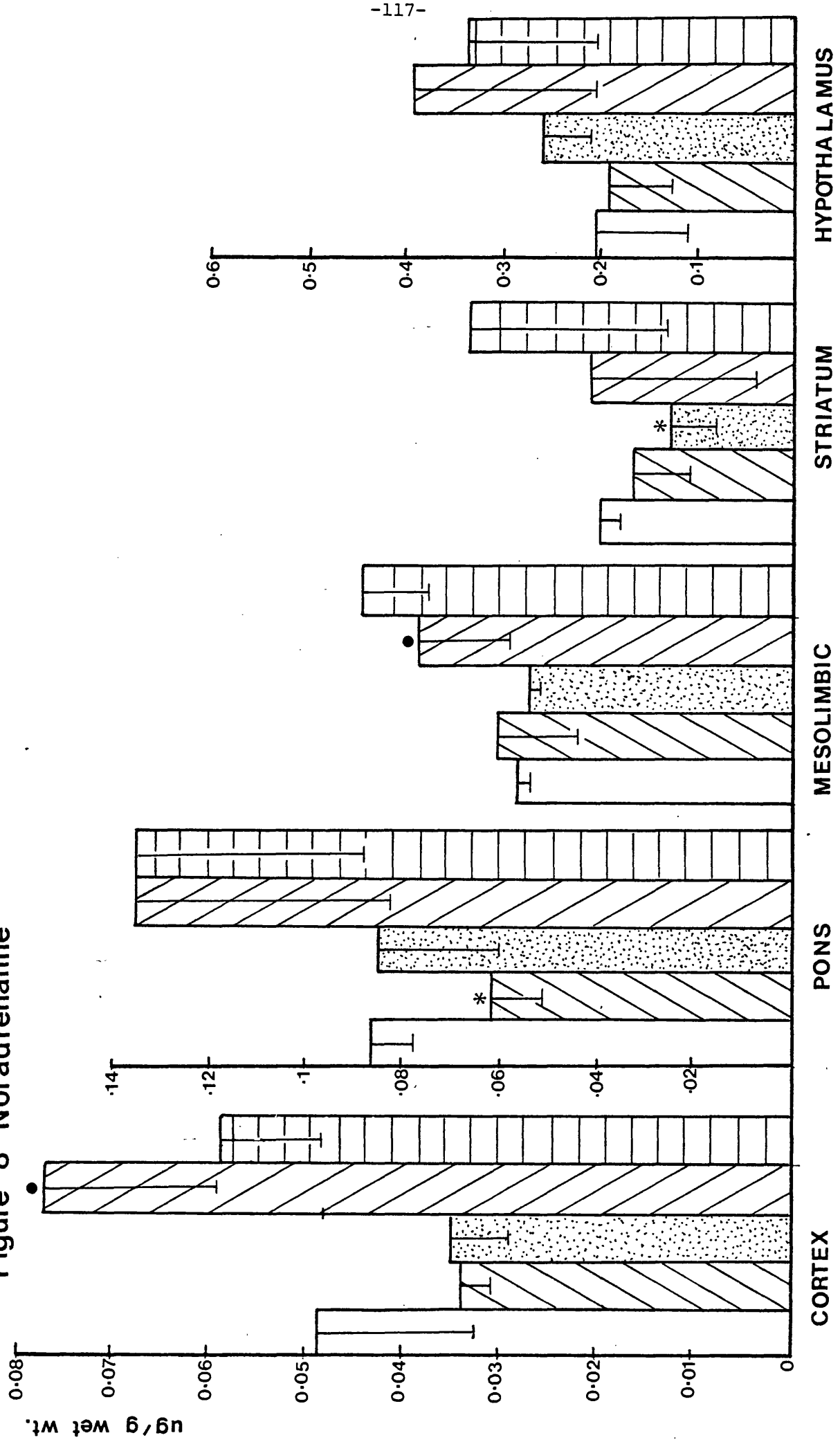
Withdrawn

Injected controls

Acute

Those values which were found to be significantly different from the relevant control levels are marked with an asterisk. • denotes a statistical (significant) difference between the controls and the injected controls.

Figure 8 Noradrenaline



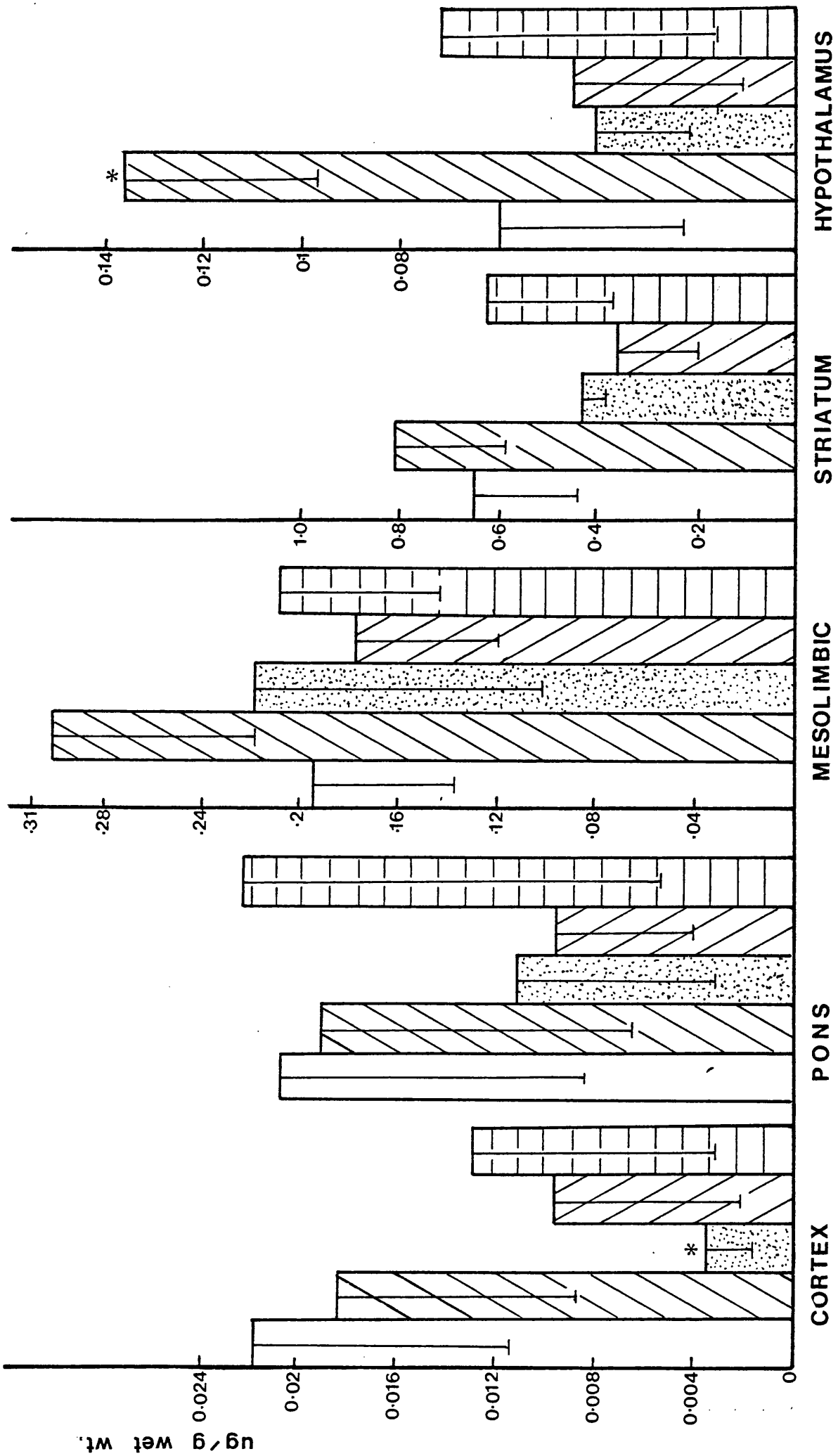


Figure 9 Dopamine
ug/g wet wt.
0
0·004
0·008
0·012
0·016
0·02
0·024
CORTEX
PONS
MESOLIMBIC
STRIATUM
HYPOTHALAMUS
*
*

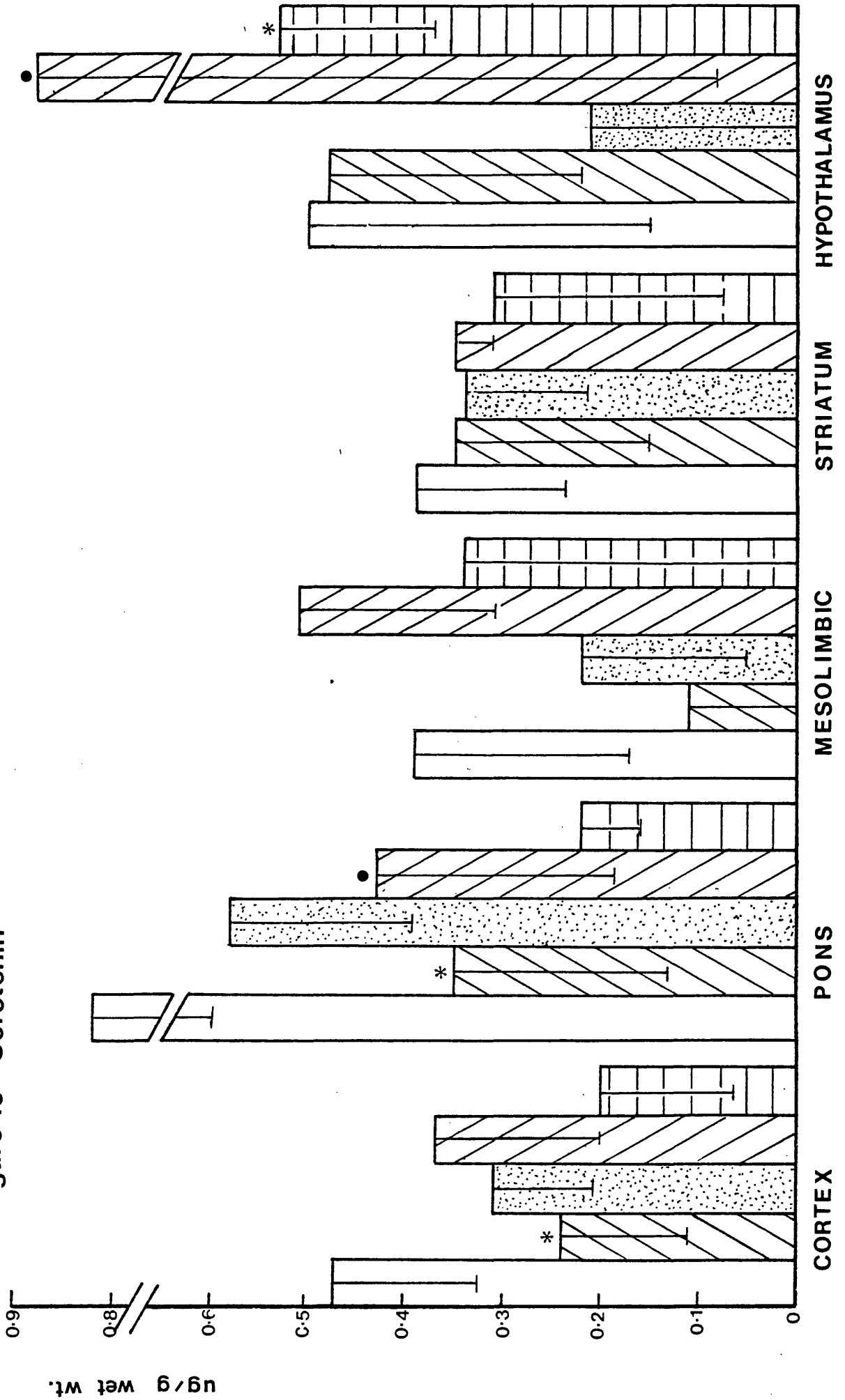


Figure 10 Serotonin
µg/g wet wt.
0·9
0·8
0·6
0·5
0·4
0·3
0·2
0·1
0
CORTEX
PONS
MESOLIMBIC
STRIATUM
HYPOTHALAMUS

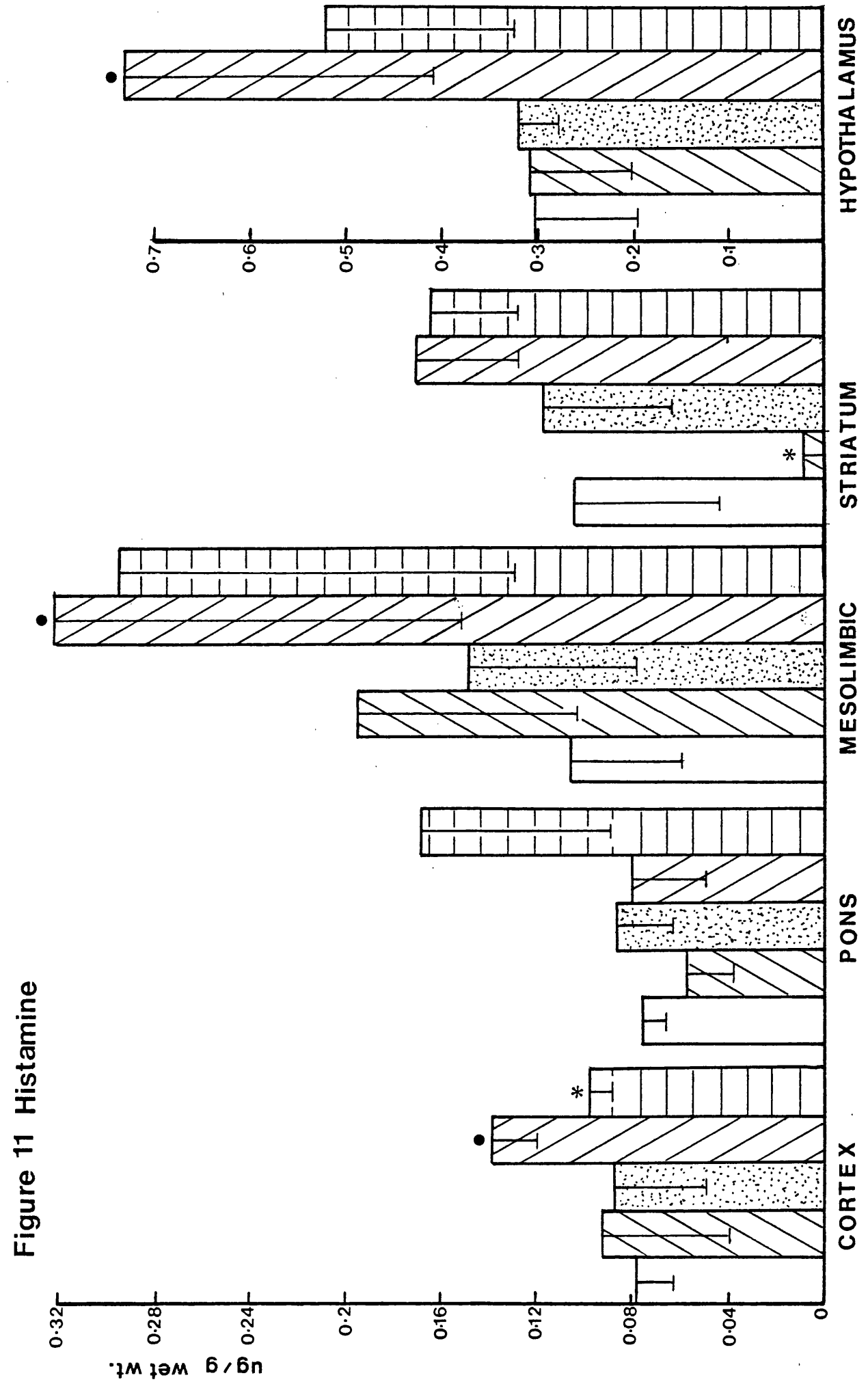


Figure 11 Histamine
ug / g wet wt.
0·32
0·28
0·24
0·2
0·16
0·12
0·08
0·04
0
CORTEX
PONS
MESOLIMBIC
STRIATUM
HYPOTHALAMUS
0·7
0·6
0·5
0·4
0·3
0·2
0·1
*
*
•
•
•

E. Comments on the route of administration of methamphetamine and the methodology used to measure amine levels

The behavioural observations showed that when methamphetamine is given in the drinking water it is not possible to know with any precision how much methamphetamine each rat has consumed. In order to control this strictly, the rats would have to be housed individually with special drinking devices which measure the total amount consumed and how much of the total was spilt. Spillage of the drinking water is an especially important consideration when some of the animals are exhibiting stereotyped behaviour. When administering drugs which have a bitter or unpleasant taste in the drinking water, it is common to sweeten the water with sucrose. However it has been shown (Young & Landsberg, 1977) that sucrose given in the water to rats already given free access to food will result in voluntary overfeeding and a stimulation of the sympathetic nervous system. For obvious reasons, the addition of sucrose to sweeten the taste of the methamphetamine would only complicate the situation further.

The behaviours shown by individually-housed rats chronically treated with methamphetamine is radically different from those seen in group-housed rats. Groups will start to show abnormal social behaviours e.g. increased fright and flight and little social grooming. Late stages show exaggerated fright or flight, fear-like startle responses and behaviours that result in their being chased by controls (Ellison *et al.*, 1978).

The strikingly different behaviours exhibited by individually-housed and group-housed animals would suggest that their biochemistry is also different.

The rating scales which are commonly used to quantitate subjective behavioural observations (Klawans *et al.*, 1975; Ellinwood & Balster, 1974) were found to be impractical. I did not consider that this way of measuring behaviour was particularly meaningful in terms of correlating my subjective (yet quantitative, therefore'objective') ratings with other research workers in the field. My original intention had been to correlate my biochemical results with a detailed behavioural study. The alternatives to this are to carry out a properly controlled behavioural study of my own (which was clearly not feasible) or to correlate my biochemical data with results from behavioural studies by the administered dose. This has the drawback of group or individual variation to a particular dose.

It was clear that, on long-term treatment, the animals were receiving toxic doses of methamphetamine, and that the females were far more susceptible to the effects of methamphetamine (it is possible that the long-term effects were different for the two sexes).

In the light of the above observations it was decided to administer methamphetamine by intra-peritoneal injection, so ensuring that each rat received a known amount of methamphetamine. The amount of methamphetamine received by each animal was also reduced, compared with that calculated to have been received in the drinking water in the first series of experiments. The result of this was to change the

orientation of the research in the desired manner i.e. to correlate my biochemical data with published behavioural observations *via* dose rather than by behavioural assessment.

The acute group of animals was redundant as there can be no control for the acute effects of methamphetamine in chronically-treated rats even if the drug is administered by intra-peritoneal injection. As far as I am aware, the experiments which would be necessary to determine the amount of circulating and stored methamphetamine (or amphetamine) in rats which have been chronically treated with the drug, have not been done. These measurements would have to be carried out in order to set up a valid acute control.

The methodology used for the quantitation of regional amine levels was unsatisfactory for several reasons:-

(i) The separation and analysis of the four amines in five regions from twenty rat brains was tedious and impractical, as, to eliminate the possibility of inconsistent recovery at different stages in the procedure, it was preferable to run all the columns simultaneously and all the assays simultaneously.

(ii) The standard curves obtained for the fluorometric assays varied considerably even with consecutive assays, therefore a standard curve had to be carried out for every assay.

(iii) The recovery of the internal standards was variable and very low during the main experiment. This was unexpected and casts doubt on the meaning of the results. This also suggests that the published method for the purification and separation of the amines works only for the pure compounds and not for brain samples.

The original paper used rat intestinal extracts for their internal standards, and showed a good recovery. However the endogenous levels given here were from rats on an L--DOPA administration trial. In retrospect, the data presented were not convincing evidence that the method was valid. A series of experiments using internal standards was carried out subsequently, and the results confirmed that the tissue extracts do not give the same results as the pure compounds. So, short of redesigning the procedure, with no certainty of obtaining a workable method, it was decided to abandon this method in which the amines are separated by ion-exchange chromatography and assayed fluorometrically.

The attraction of the alternative radioenzymatic techniques is their sensitivity and specificity. The fluorometric methods, even when the sensitivity of the assay was increased, were not sufficiently sensitive to measure the levels of all the amines in each region (even if the recovery had been high). Another point is that in an experimental study which involves long-term drug treatment of animals it is clearly desirable to try to obtain as much biochemical information as possible, simultaneously. This not only reduces the number of animals involved, but also allows biochemical 'pictures' of individual rats to be constructed. This is more valuable when considering individual variation in response to a drug.

In summary it was decided to administer methamphetamine by intraperitoneal injection, and to use radioenzymatic techniques for the measurement of the amines, which allows other assays to be carried out on the same homogenate.

IV. ADMINISTRATION OF METHAMPHETAMINE BY INTRAPERITONEAL INJECTION

A. Treatment of Animals

1. Materials and Methods

The rats were 30 - 35 days old on commencement of treatment. They were housed in groups of five under constant conditions, i.e. light/dark cycles (12h), temperature (21 - 23°C) and diet (Oxoid 41B). Three experimental animal groups were set up as follows:

(i) 'Chronic'rats received methamphetamine by intra-peritoneal injection (one injection every 12h). The initial dose was 5 mg/kg/24h, increasing to 10 mg/kg/24h after 5 days and to 15 mg/kg/24h after 10 days. The rats were kept at this dose until the end of the experimental period.

(ii) The 'withdrawn' animals were treated identically to those in the chronic group, except that they received saline injections 36h prior to death.

(iii) The 'control' animals received saline injections over the same time period.

All three groups were killed 12h after their last injection. The weights, drinking rates and food consumption were recorded at the same time each day (12.00 - 13.00 h).

2. Results

Four sets of experimental animal groups were set up. The first set were male rats and were used to determine tyrosine hydroxylase activity and noradrenaline and dopamine levels. The second set were female rats which served as a comparison to the male rats in terms of weight gain, food consumption and drinking rates (this was felt to be important in the light of the results obtained in Section IIIA). The third set were male rats, set up for an analysis of the regional amino acid levels. The final set of rats were also male and were used for the determination of tyrosine hydroxylase activity, the determination of noradrenaline, dopamine and their non-O-methylated metabolites, tryptophan and serotonin levels in brain regions, and the levels of tryptophan, phenylalanine and tyrosine in the plasma.

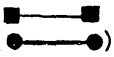
Figures 12 - 20 show the weights, food consumption and drinking rates from experimental sets 1 and 2, the weights and food consumption from experimental set 3 and the weights from experimental set 4. From the graphs it can be seen that experimental sets 2 and 3 became tolerant to the anorexic effects of methamphetamine, whereas experimental set 1 did not. A possible explanation for the first set of animals not developing tolerance to the anorexic effects of methamphetamine is stress. This set of animals showed signs of severe stress initially and also when the dose was increased (far more than any of the other experimental sets). My original intention had been to increase dose further, but because of the stress caused, I only increased it to 15 mg/kg/24h. Stress was observed in all sets of control rats (again this was more pronounced in the first set) on commencing the injections. It has been shown that amphetamine is stored in adipose tissue and released when the animal is stressed (S. Sparber, personal communication);

there are also many reports in the literature of changes in catecholamine and serotonin metabolism with stress (Curzon, 1978; Ishii *et al.*, 1975).

The chronic rats in experimental set 1 also consumed significantly less water than the controls; this was not seen with experimental set 2 or in the few measurements made for experimental sets 3 and 4. Again, the only explanation which I can suggest is the increased stress seen in the animals of set 1.

In general the stress shown by the rats receiving methamphetamine was greater than that seen in the saline injected controls. There appeared to be no obvious way of establishing a control for this.

FIGURES 12-20 Graphs of the weights, food consumption and drinking rates of the 4 experimental sets of animals over 35 days of treatment.

Significant differences between the chronic group () and the control group () are indicated by an asterisk. (* $p < 0.05$, ** $p < 0.0001$)

The error bars denote the standard error of the mean; where there are no error bars, n=1.

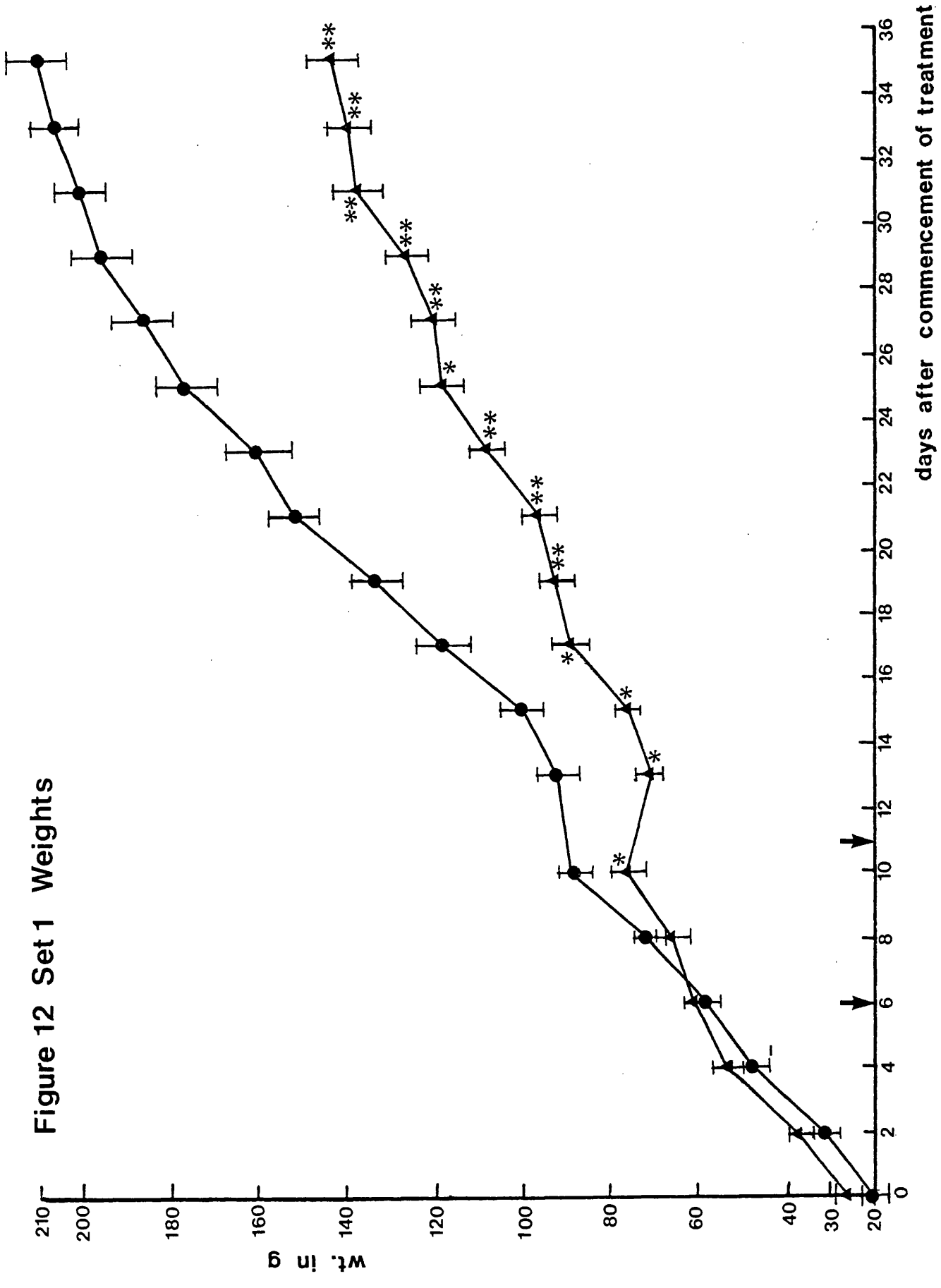
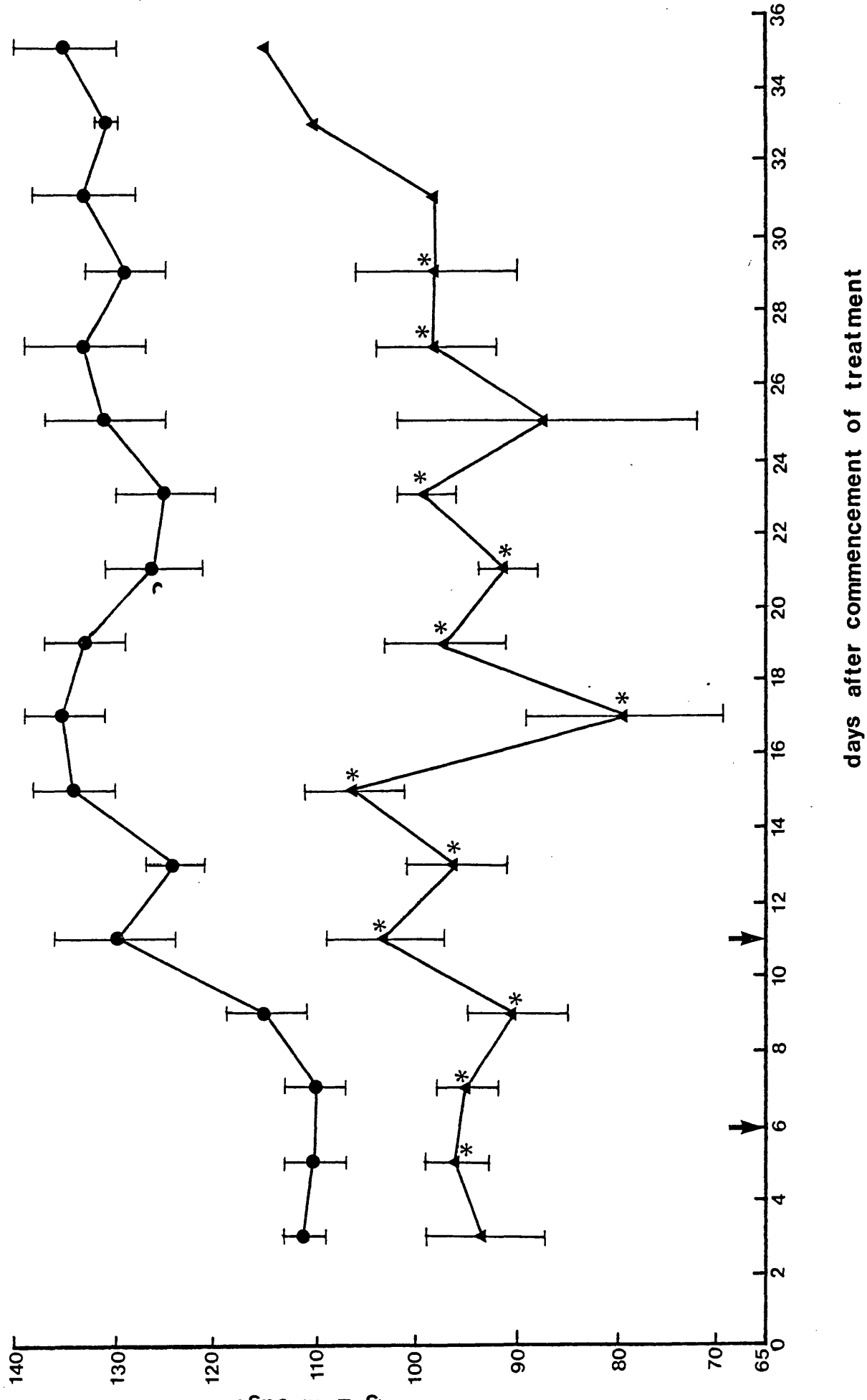


Figure 12 Set 1 Weights

Figure 13 Set 1 Food Consumption



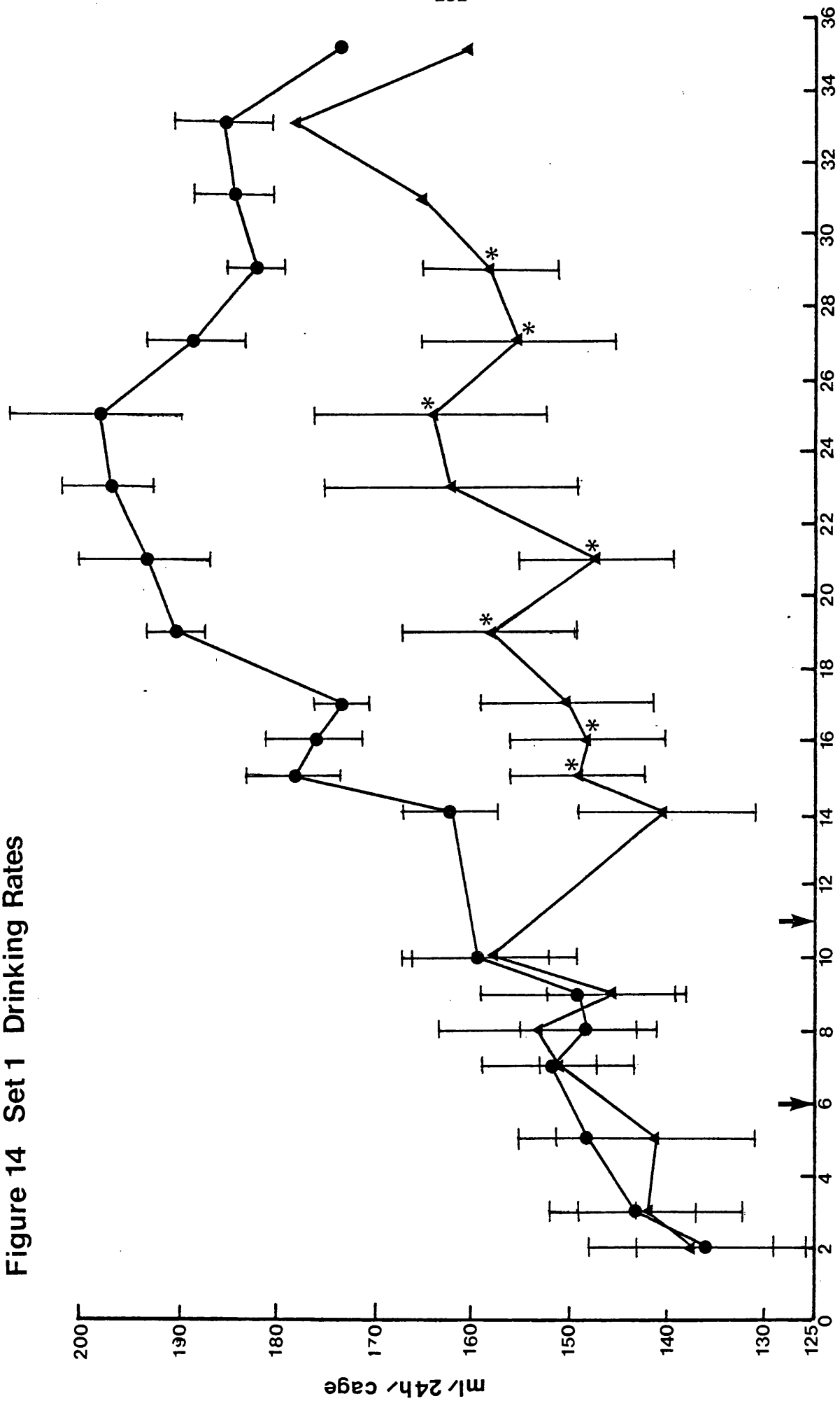


Figure 14 Set 1 Drinking Rates

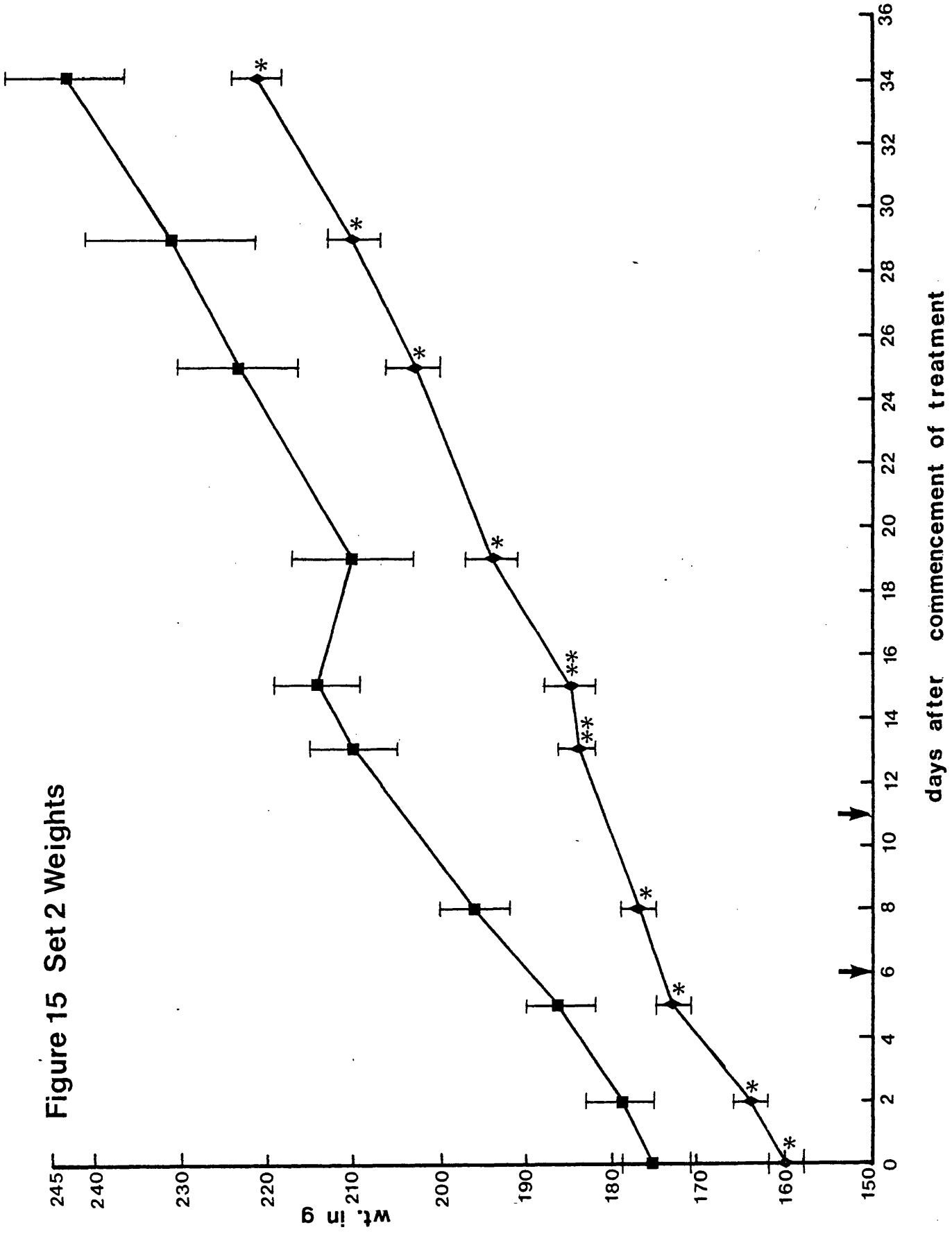


Figure 15 Set 2 Weights

Figure 16 Set 2 Food Consumption

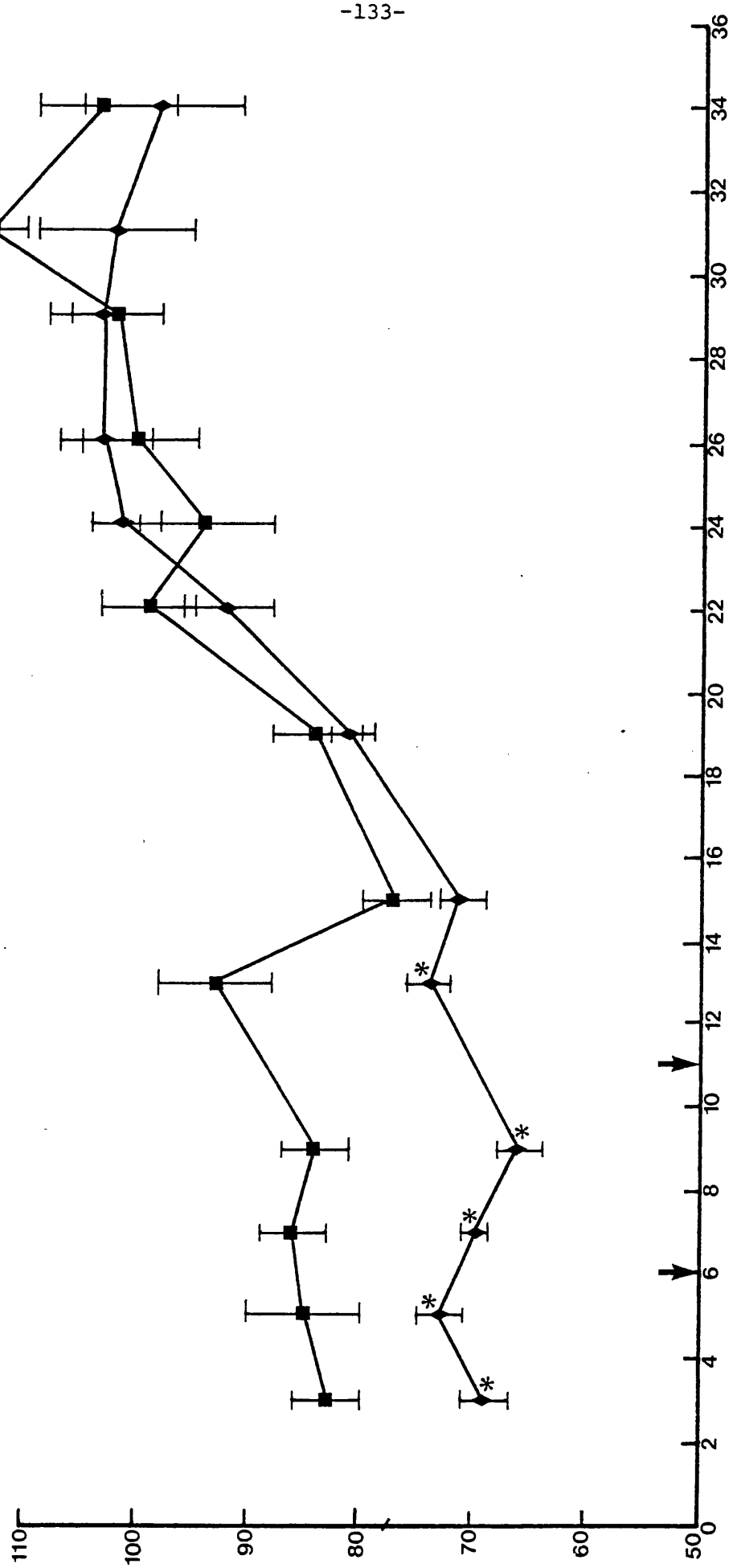


Figure 17 Set 2 Drinking Rates

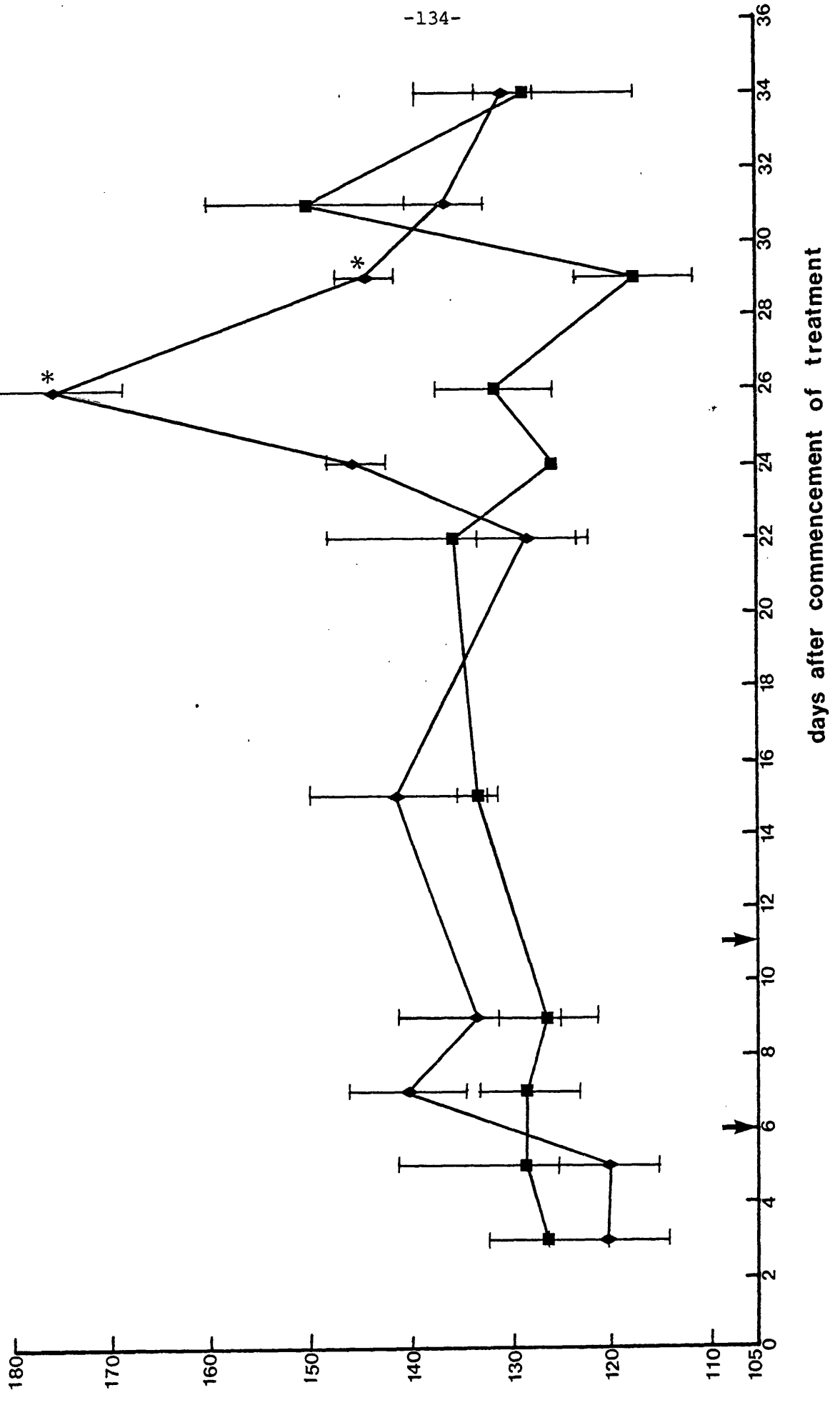
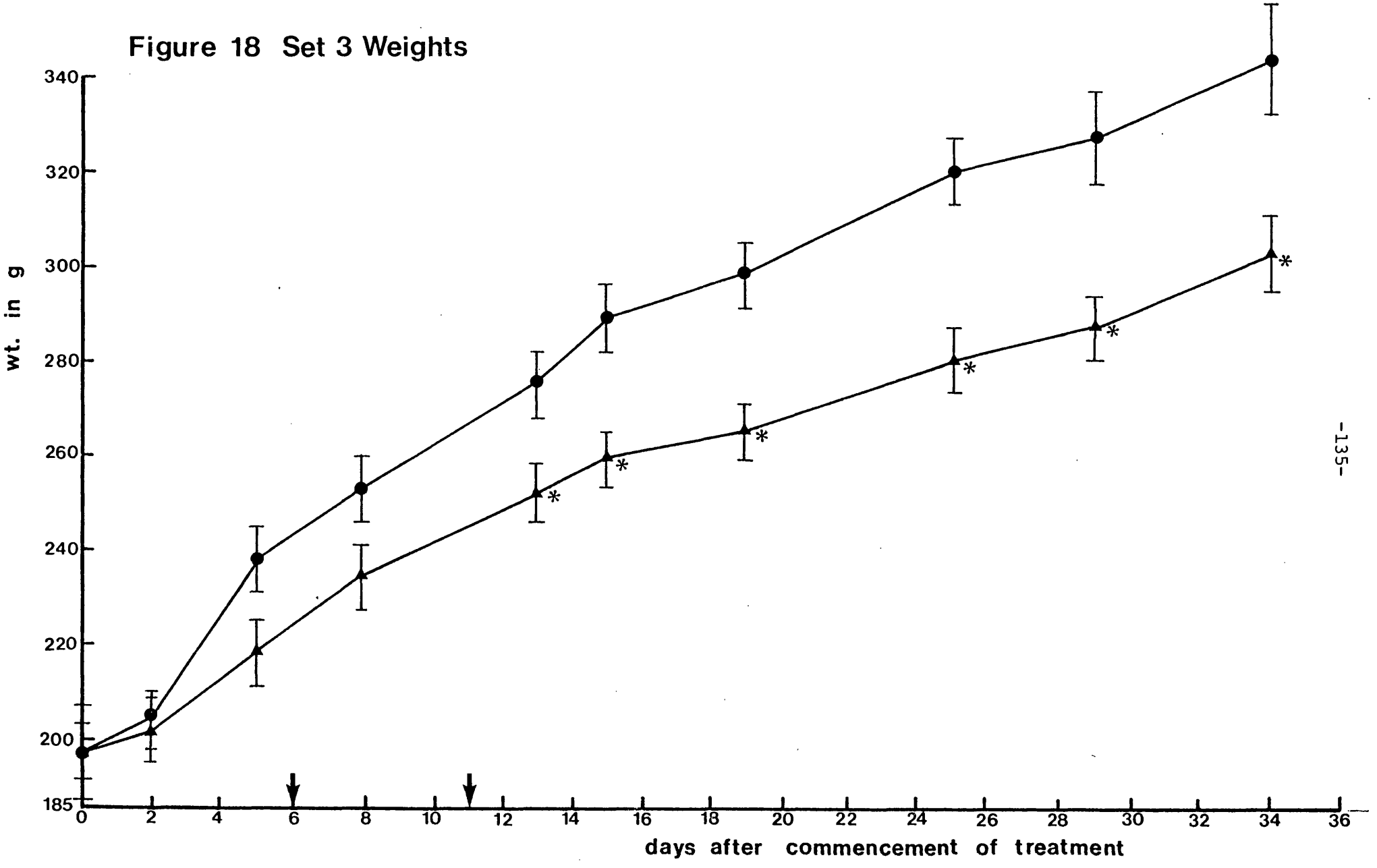


Figure 18 Set 3 Weights



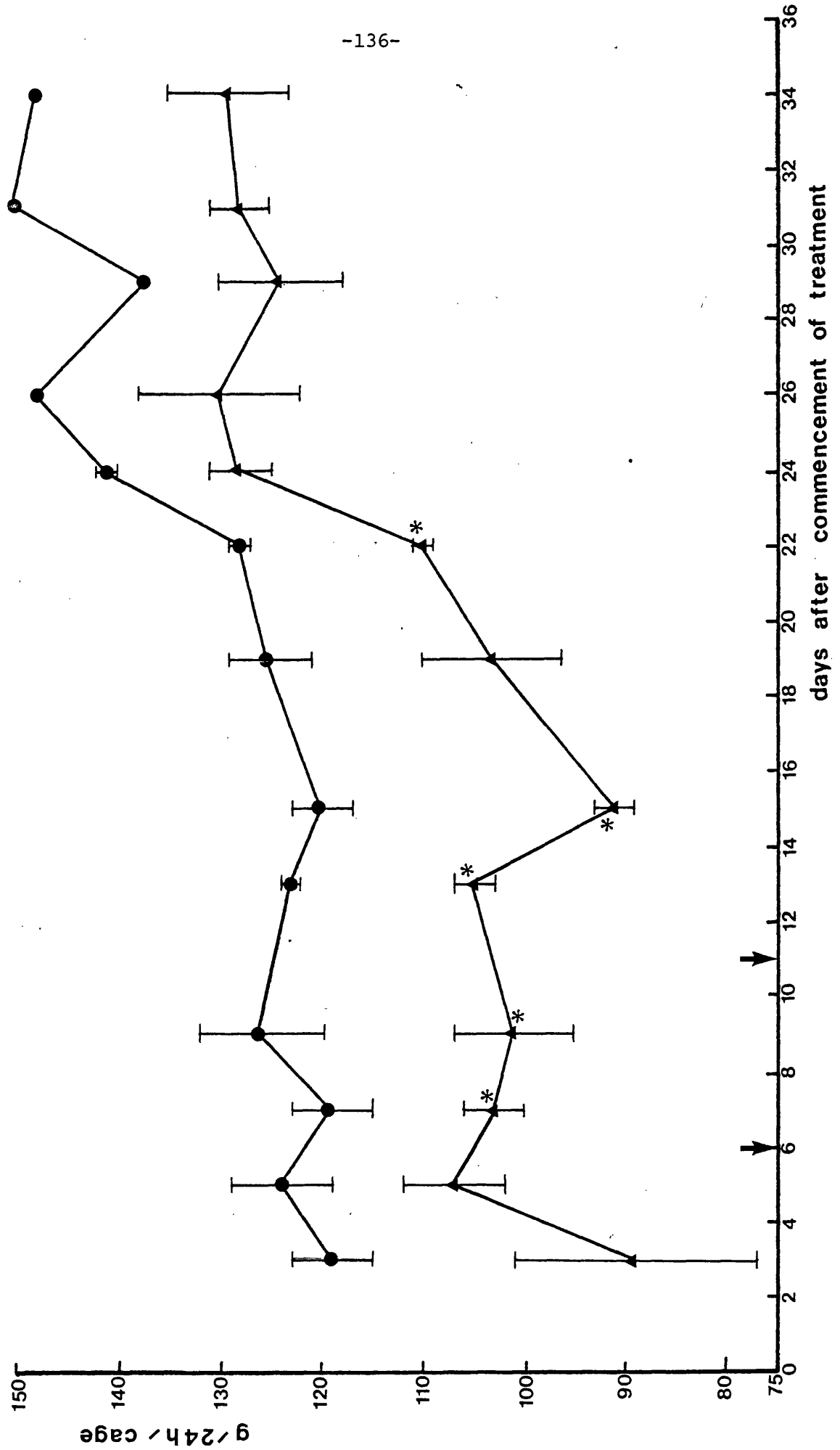


Figure 19 Set 3 Food Consumption

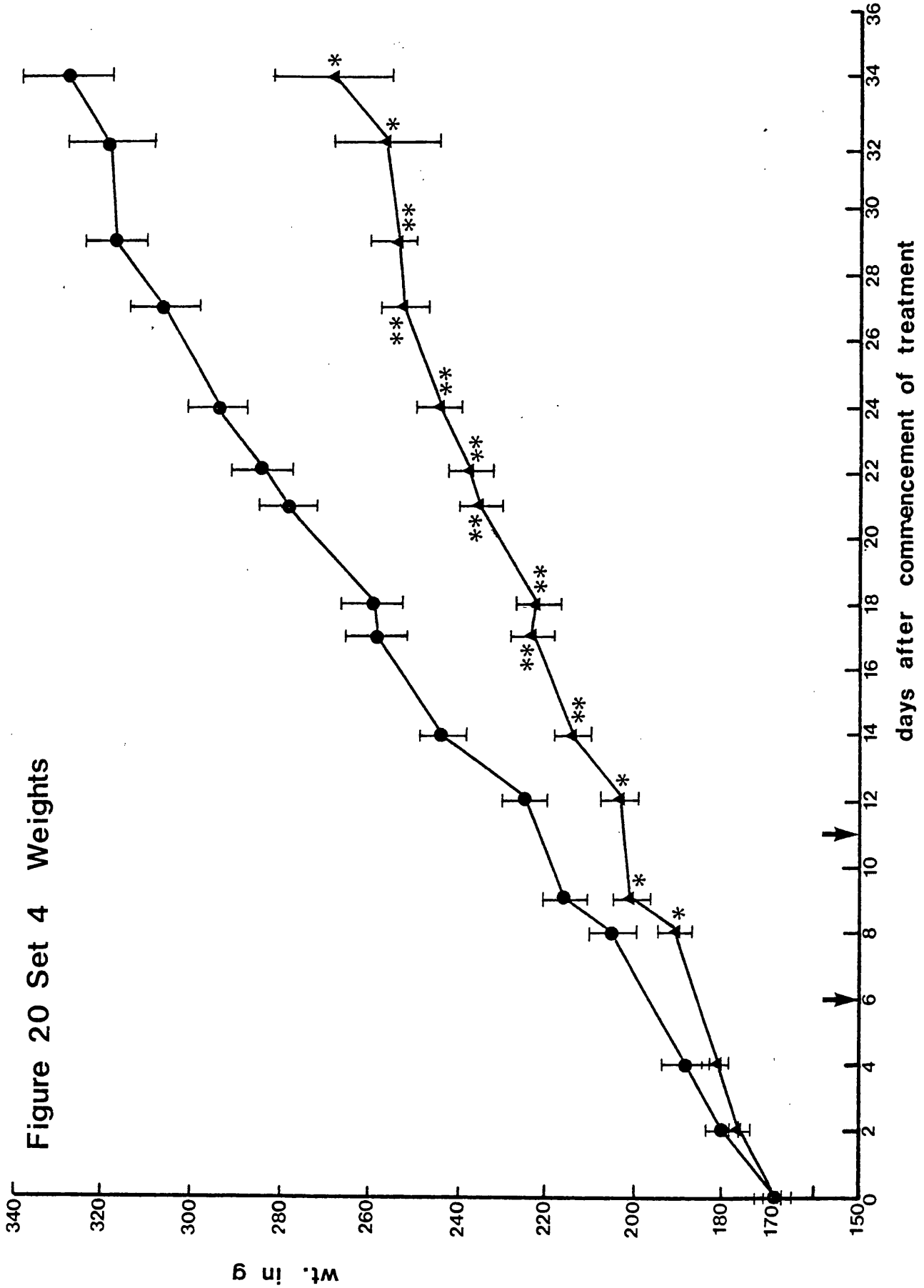


Figure 20 Set 4 Weights

B. Regional dissection of the brain

The rats were decapitated, the brains dissected over ice and the regions dropped immediately into liquid nitrogen. The regions were then weighed and stored in liquid nitrogen until analysis. The time from decapitation to the stage where all regions had been frozen in liquid nitrogen was 4 - 5 min.

The dissection technique was modified from that described in Section III to give a more reproducible and accurate dissection of the nucleus accumbens, hypothalamus and amygdala. The method used is shown in Figure 21. Otherwise the technique was the same as that described previously. The order in which the regions were dissected was: cortical slice, tuberculum olfactorium, nucleus accumbens, striatum, amygdala, hypothalamus, pons, medulla. Again the reproducibility of dissection was monitored by weight; the average weights of the regions with the standard error of the mean for one set of rats is shown in Table 3.

Fig. 21 Shows the modified dissection technique.

Fig. 21a shows a ventral view of the brain. Vertical sections were made at positions A to G.

Fig. 21b shows the sections from cuts A and B (1) and C and D (2) respectively (the relevant references to the Konig & Klippel (1963) stereotaxic rat brain atlas are given).

Cuts were made at the positions shown.

1. Shows the dissection of the accumbens and striatum (caudate nucleus)
2. Shows the dissection of the hypothalamus and amygdala. When the cuts shown were completed the section containing the hypothalamus and amygdaloid complex was turned to the ventral view when the demarcation of the hypothalamus could be seen and the hypothalamus cut out.

Key:

T	- thalamic nuclei	CN	- caudate nucleus
CC	- corpus callosum	HI	- hippocampus
H	- hypothalamus	ac	- accumbens
CAI	- capsule interna	OT	- olfactory trace
V^{111}	- IIIrd ventricle		
A	- amygdaloid nuclei		

Cuts E, F, G show the dissection of the pons and medulla (respectively).

Fig. 21a

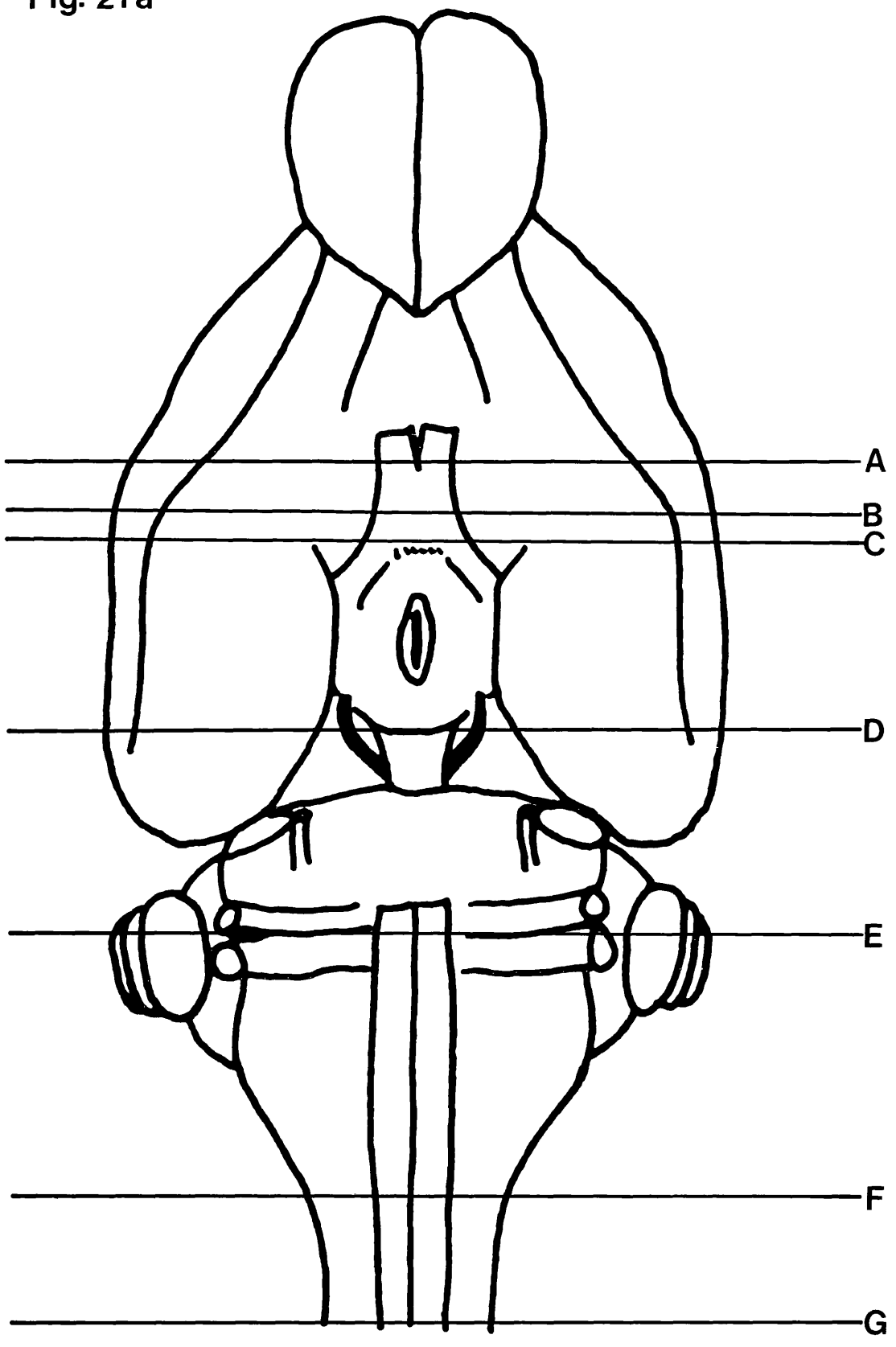
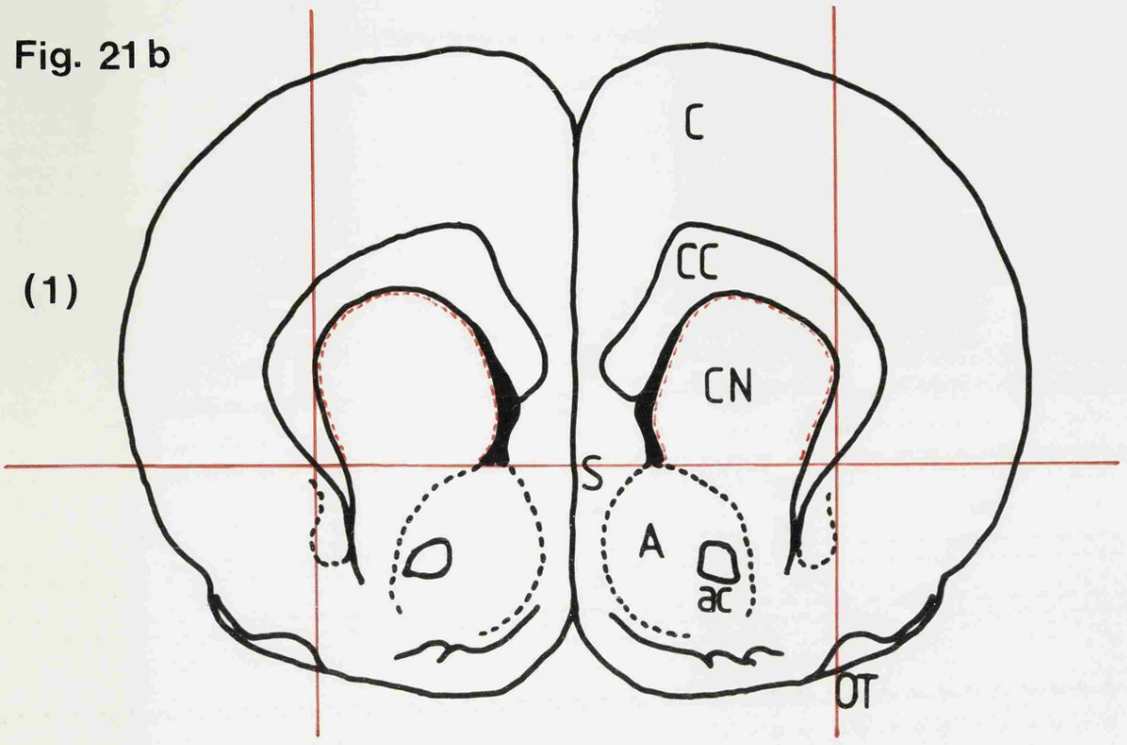
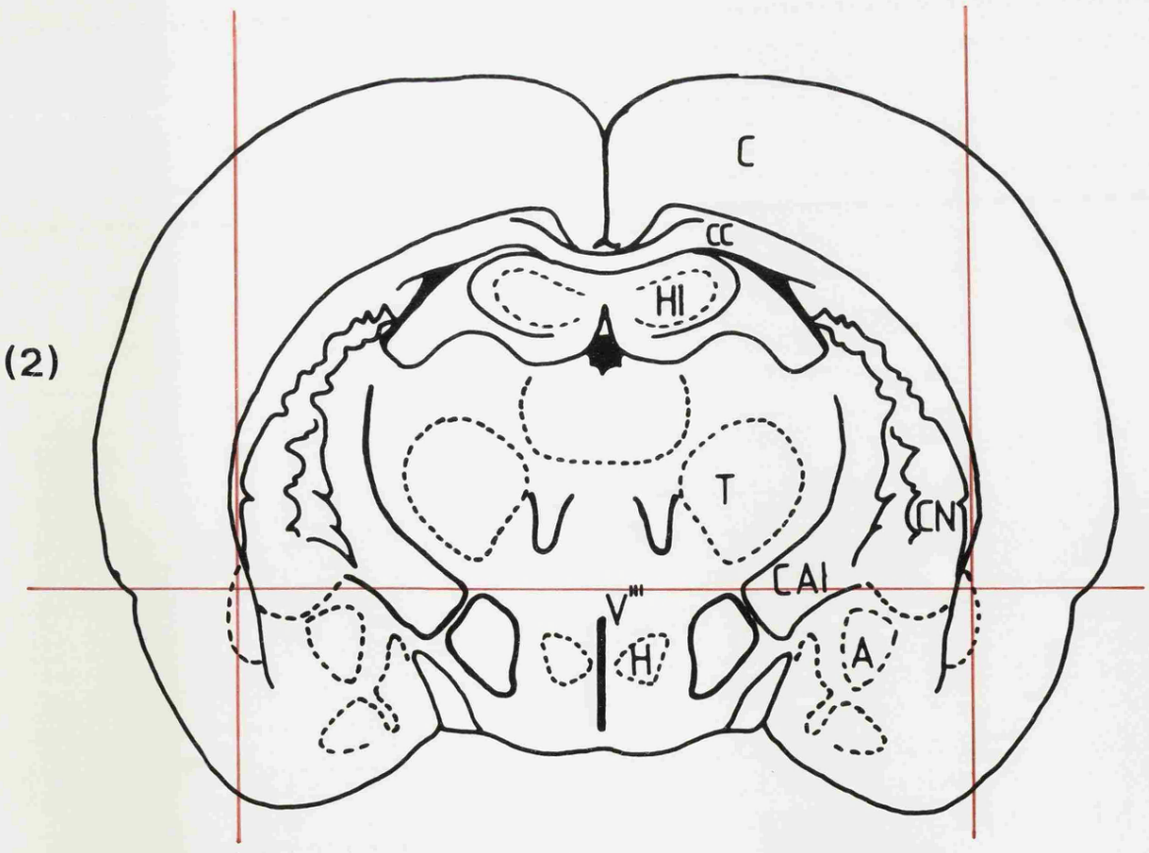


Fig. 21 b



A 9410



A 5150

Table 3. The average weights of the regions dissected from the brain ± standard error of the mean, for 45 rats

Region	Average weight ($\bar{x}$) in mg ± standard error of the mean (S.E.M.) n=45
Cortex	$\bar{x}$ = 64.5 ± 1.6
Tuberculum olfactorium	$\bar{x}$ = 9.5 ± 0.4
N. accumbens	$\bar{x}$ = 29.7 ± 0.7
Striatum	$\bar{x}$ = 46.8 ± 1
Amygdala	$\bar{x}$ = 72.8 ± 1.8
Hypothalamus	$\bar{x}$ = 63.3 ± 1.2
Pons	$\bar{x}$ = 114.8 ± 2.8
Medulla	$\bar{x}$ = 116.2 ± 2.4

C. Estimation of tyrosine hydroxylase activity

1. Materials and Methods

L- [side-chain-2,3-^{3}H]-Tyrosine (specific activity 13 Ci/mmol) and L-3,4-dihydroxy [ring-2,5,6-^{3}H]-phenyl-alanine ([^{3}H]-DOPA, specific activity 1.0 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. 6,7-Dimethyl-5,6,7,8 tetrahydropterin ($DMPH_4$), bovine serum albumin, tyrosine and Tris base (Trizma) were obtained from Sigma (London) Chemical Company Ltd. The alumina was a Merck product. NSD 1055 (m-hydroxy-p-bromobenzyloxyamine) was obtained from Sander Ltd. The tetrahydrobiopterin was a gift from Dr. R.F. Long.

Methods

Tyrosine hydroxylase activity was measured by a modification of the method described by Hendry & Iversen (1971). The assay is based on the conversion of [side-chain-^{3}H]-tyrosine to [side-chain-^{3}H]-3, 4-dihydroxyphenylalanine ([side-chain-^{3}H]-DOPA) in the presence of an aromatic amino acid decarboxylase inhibitor (NSD 1055).

(i) Homogenization

The tissue was homogenized in 5 vol. or 10 vol. (depending on the activity of the enzyme in the particular region) of 5 mM Tris-HCl buffer, pH 6.0, at 0°C.

(ii) Incubation

Tissue homogenate (10 µl) was added to tubes in an ice bath. The blank tubes contained either 10 µl of homogenizing buffer or 10 µl of homogenate. Several tubes containing 10 µl of [^{3}H] - DOPA (20 µCi/ml) served to monitor [^{3}H]- DOPA recovery. Then 10 µl of substrate-cofactor mix was added to all tubes except

the DOPA recovery tubes and the homogenate blanks. The substrate-cofactor mix was made up as follows:

A few mg of dry alumina powder was added to a small tube followed by 200 µl of 5 mM Tris-HCl buffer, pH 8.6, and 100 µl (100 µCi) of L-[side-chain-^{3}H]-tyrosine. This was mixed well and kept at 0°C. 6,7-Dimethyl-5,6,7,8 tetrahydropterin (0.5 mg) was weighed into a small tube and 50 µl of β-mercaptoethanol was added. This was mixed until the cofactor had dissolved, then 500 µl of 0.8 M KH_2PO_4 (adjusted to pH 6.0 with NaOH then diluted to 0.8 M with distilled water) containing 0.4 mg/ml of NSD 1055 and 0.4 mM tyrosine was added to the cofactor. Finally equal volumes of [^{3}H]-tyrosine and cofactor solution were mixed. The cofactor solution and the final mix were prepared immediately prior to incubation.

After the substrate-cofactor mix had been added to the tubes, they were mixed carefully and incubated in a shaking water-bath for 15 min. 2-Iodotyrosine was added to the substrate-cofactor mix to a final concentration of 0.1 mM for the homogenate blanks. After incubation, 200 µl of 0.4 M perchloric acid containing L-DOPA (2 µg/ml) was added to the tubes, and they were stored frozen at -20°C.

(iii) Isolation of product

The alumina was prepared by washing repeatedly with distilled water to remove fine particles; it was then resuspended in 0.5 M potassium phosphate buffer, pH 7.4 (approx. 0.5 M KH_2PO_4 adjusted to pH 7.4 with NaOH and diluted to 0.5 M with distilled water), and added to columns plugged with cotton wool. The columns

(i.d. 6mm) were filled with alumina to a height of 1 cm from the cotton-wool plug. The columns were then attached to glass reservoirs and washed with 5 mM Tris-HCl buffer, pH 8.6.

The contents of each tube were neutralized by adding 3.8 ml of a mixture containing 2 vol. 0.1 M Tris-HCl buffer, pH 8.6, 1 vol. 0.3 M NaOH and 1 vol. 0.2 M EDTA. The contents of the tube were then poured onto the column; the tube was rinsed with 5 mM Tris-HCl buffer, pH 8.6,and this was poured onto the columns. The columns were washed with 40 ml of 5 mM Tris-HCl buffer, pH 8.6. The [^{3}H]-DOPA was eluted with 1.5 ml of 1 M acetic acid into scintillation vials, and 12 ml of triton X-100/toluene scintillant (3:7 (v/v) containing 0.5% (w/v) 2,5-diphenyloxazole). It was found to be advisable to wait 45 min before counting the samples, as initially the counts could be very high. The samples were counted in a Packard Tri-Carb scintillation counter (3385).

Scintillation counting

In all experiments described in this section which involve scintillation counting, any quenching of the radioactivity was monitored by the channel ratio method (Herberg, 1965). A second channel was set to read approximately one-third of the counts obtained in the tritium channel $[^3H]$-water was used as the standard and quench curves (percentage efficiency against the ratio of the counts obtained in each channel) were constructed for each experiment, using carbon tetrachloride as the quenching agent. The maximum efficiency obtained from an unquenched $[^3H]$-water standard was 37%. However, in general, it was found that experimental error was far greater than that due to quenching, for this reason it was not considered necessary to convert the counts per minute obtained into disintegrations per minute.

A curve of counts per minute (c.p.m.) against the volume of scintillant was constructed to establish the volume of scintillant necessary to give maximum counts; this curve flattened off around 10 ml. Experiments were also carried out to determine whether a secondary scintillant was necessary. It was found that there was no difference between the counts obtained in the presence or absence of 2,2-p-phenylenebis (5-phenyloxazole) (POPOP).

Simple kinetic studies on tyrosine hydroxylase in brain homogenate

The linearity of product formation with enzyme concentration and with time was tested and is shown in Figures 22 and 23. Several experiments were carried out to look at the product formation with increasing substrate concentration and it was found that, in the substrate range employed (0.05 mM to 0.5 mM), v decreased with increasing $[S]$ indicative of substrate inhibition (Figure 24). The kinetic parameters Km, Ki and V were calculated by fitting the data to the rate equation

$$v = \frac{\underline{V}}{\left(\frac{Km}{[S]} + 1 + \frac{[S]}{Ki}\right)}$$

in the form

$$\frac{\underline{V}}{vi} - \frac{Km}{(S)} - \frac{(S)}{Ki} = 1$$

assuming simple substrate inhibition where $K_i = [ES][S] / [ES_2]$ This was done by using a computer programme which calculates all the solutions of the simultaneous equations resulting from substitution of the individual $v_i, [S]$ observations in the equation and finding the median after ranking the solutions in a manner analagous to that used to find Km and $\underline{V}$ by the direct linear plot (Eisenthal & Cornish-Bowden, 1974). Km was calculated to be approximately 0.04 mM, $\underline{V}$ = 39 mmol product formed/h/g wet wt. of tissue and Ki = 0.13 mM. These figures have to be considered as approximations as the data were not particularly good, and the values of $[S]$ were not corrected for endogenous tyrosine levels. As the values for Km and Ki are fairly low, the levels of endogenous tyrosine (65 nmol/g in striatum, the region used for these studies) would make the actual values lower than those quoted here.

Due to the very low concentrations of tyrosine at which the optimal velocity is reached, the point at which it is reached in brain homogenate will vary with endogenous tyrosine levels. The assay procedure was carried out at concentrations of tyrosine which would clearly be inhibitory, but rather than determine the mean tyrosine levels in each region together with the Vopt., it was decided to 'buffer' changes in endogenous tyrosine levels by adding cold tyrosine to a final concentration of 0.1 mM. Cicero *et al.* (1972) summarized the regional distribution of tyrosine hydroxylase activity in the brain using a tissue homogenate and a concentration of tyrosine of approximately 0.1 mM. A comparison between the values I obtained for the various brain regions are compared with the values obtained by Cicero *et al.* (1972) in Table 4.

Preliminary experiments were carried out to see if the activity of the enzyme in an homogenate was affected by added catalase, iron (Fe^{2+}), calcium, EGTA and using tetrahydrobiopterin instead of $DMPH_4$. The results indicated that adding catalase, Fe^{2+}, calcium or EGTA to the incubation mix did not significantly affect the activity of the enzyme. Tetrahydrobiopterin increased the activity of the enzyme by at least 10 fold; v against [S] and v against cofactor concentration plots suggested that the kinetic properties of the enzyme with this cofactor were also different.

My observations that neither calcium nor EGTA had any effect on the enzyme activity contradicts the findings of Morgenroth *et al.* (1975, 1976). My results do not conclusively disprove these papers, but reportshave been published recently by other researchers who are also unable to repeat the experiments of Morgenroth *et al.* (Lovenberg *et al.*, 1977).

In the preliminary experiments I found that the enzyme showed very little activity when no cofactor was added. McGeer (1967) stated that no cofactor was required in the incubation; later (1971) he proposed that the cofactor requirement depended on the brain region. His rationale was: cell body containing regions were more readily disrupted, therefore dilution accounts for the activity in these regions increasing on adding cofactor. In nerve-terminal containing regions the enzyme is tightly bound and synaptosomes are formed on homogenization, which are not easily disrupted. This accounts for the lack of effect or inhibition of enzyme activity on the addition of cofactor.

This rationale seems unlikely, especially in the light of two observations which I have made during the course of my experiments: (i) in homogenates of striatum (rich in nerve terminals), I found an absolute requirement for cofactor, (ii) in homogenate of striatum all the activity is recovered in the soluble high-speed supernatant (the homogenate was spun at 100,000 x g for 90 min in a Beckman ultra-centrifuge (model L5-657)).

Tyrosine hydroxylase is a controversial enzyme. In this section I have briefly mentioned those aspects which I have assessed. The enzyme will be considered in more detail in the Discussion.

FIGURES 22, 23 and 24

Graphs of velocity against enzyme concentration and velocity against time for tyrosine hydroxylase in a homogenate (1/5 w/v) of striatum. Fig. 24 shows enzyme activity with increasing substrate concentrations.

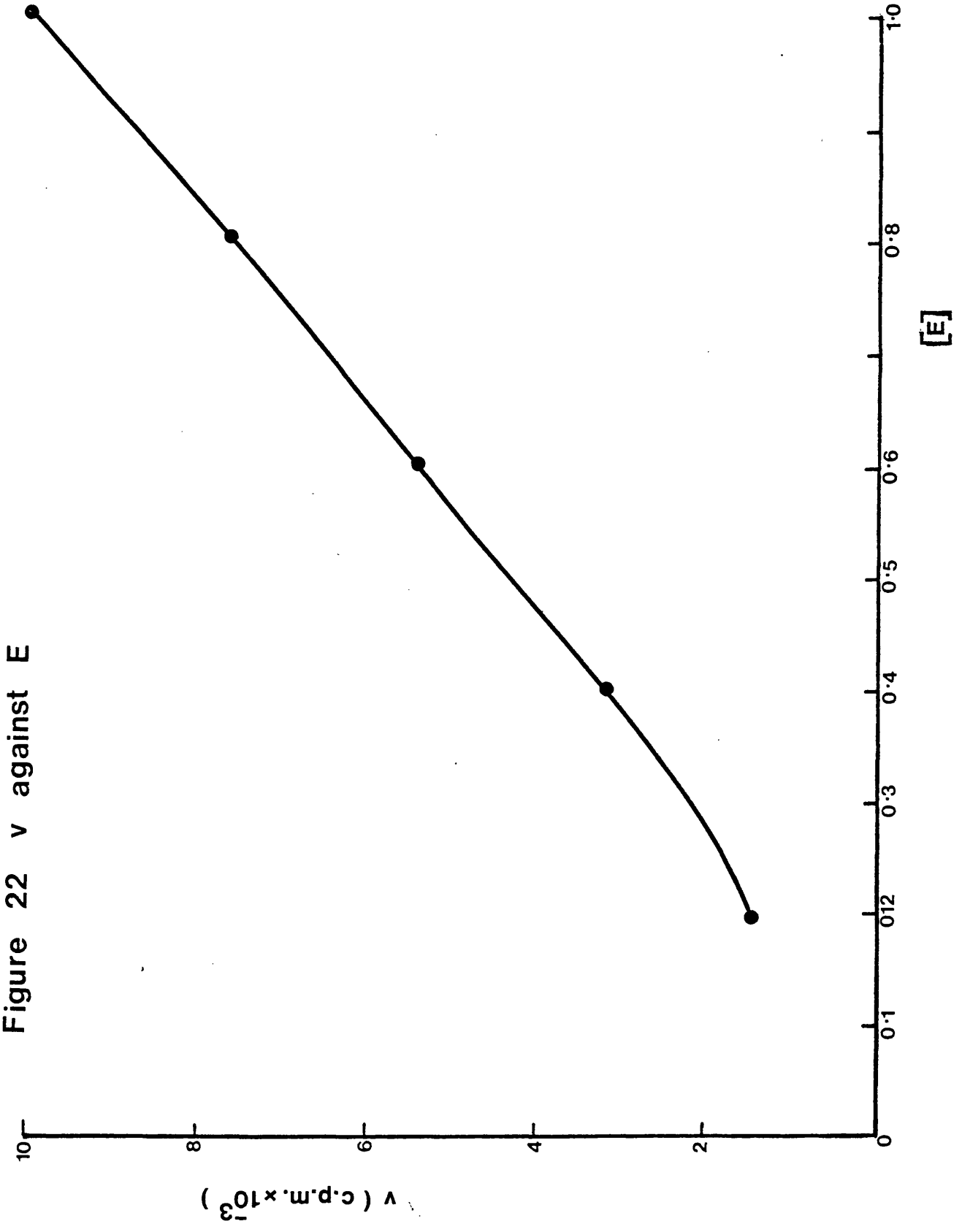


Figure 22 v against E

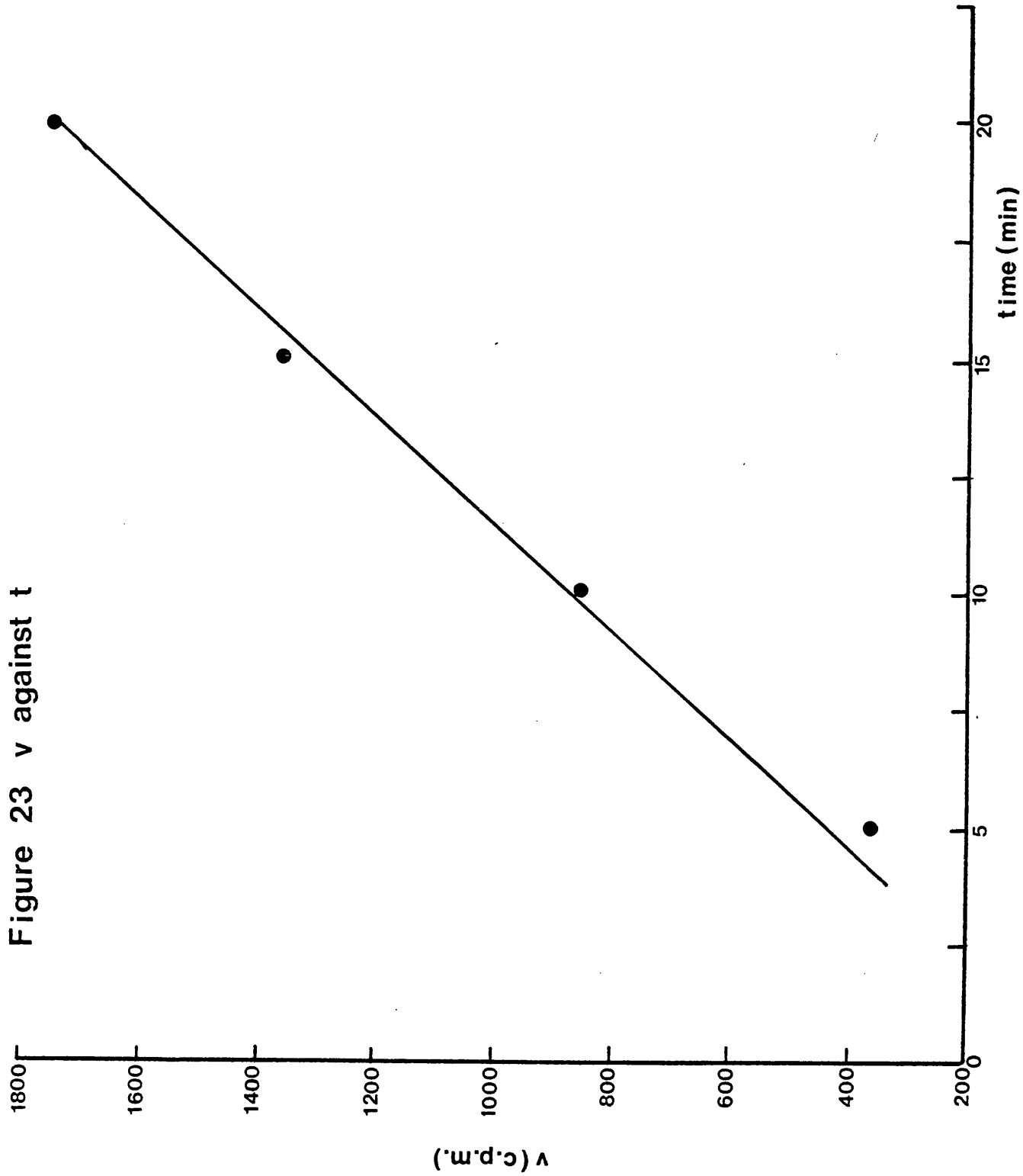


Figure 23 v against t

FIGURE 24. v against - S using the direct linear plot; this shows the characteristic pattern obtained with substrate inhibition in the range of substrate concentrations which inhibit the enzyme.

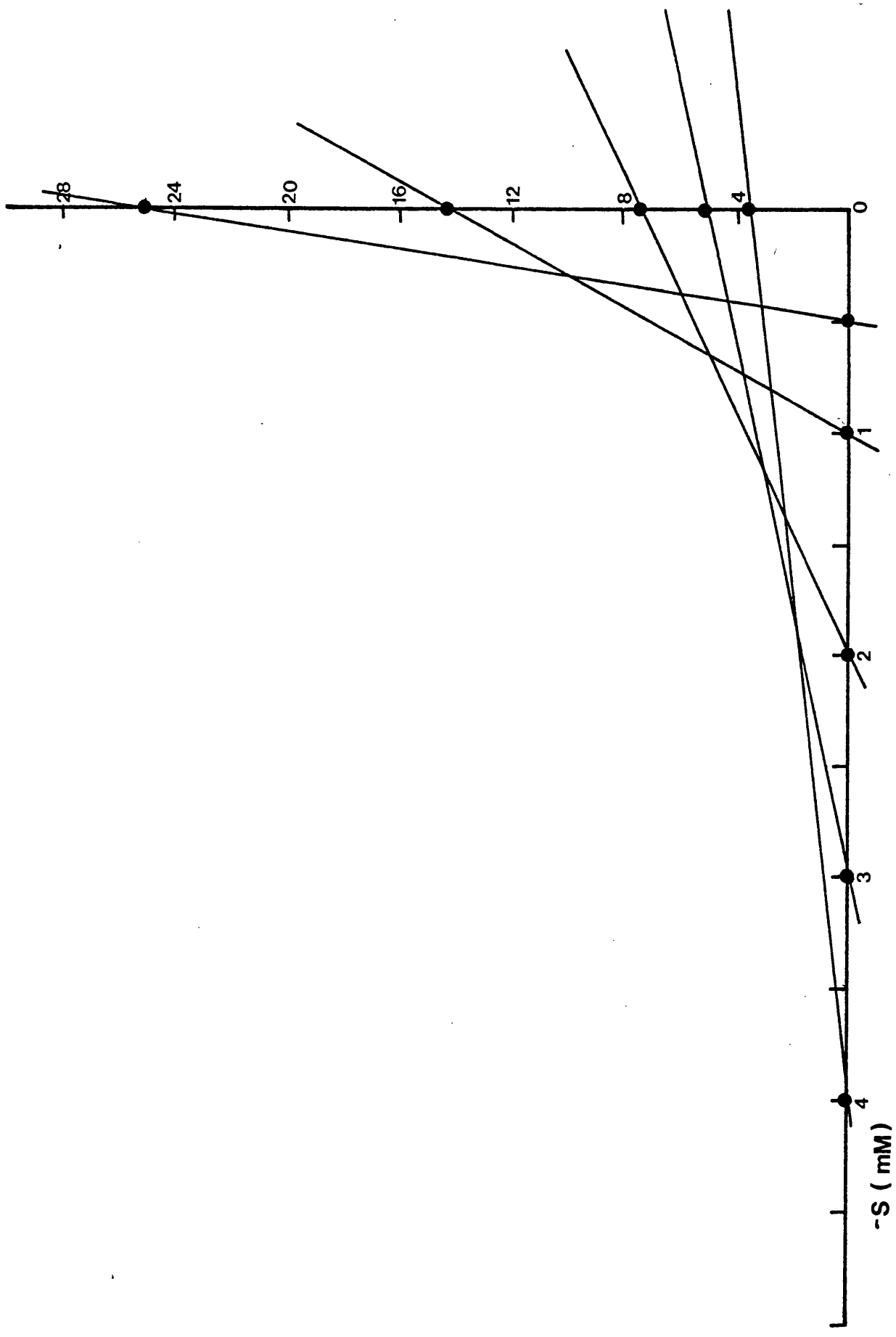


Figure 24

2. Results: Regional tyrosine hydroxylase activity in experimental animal groups

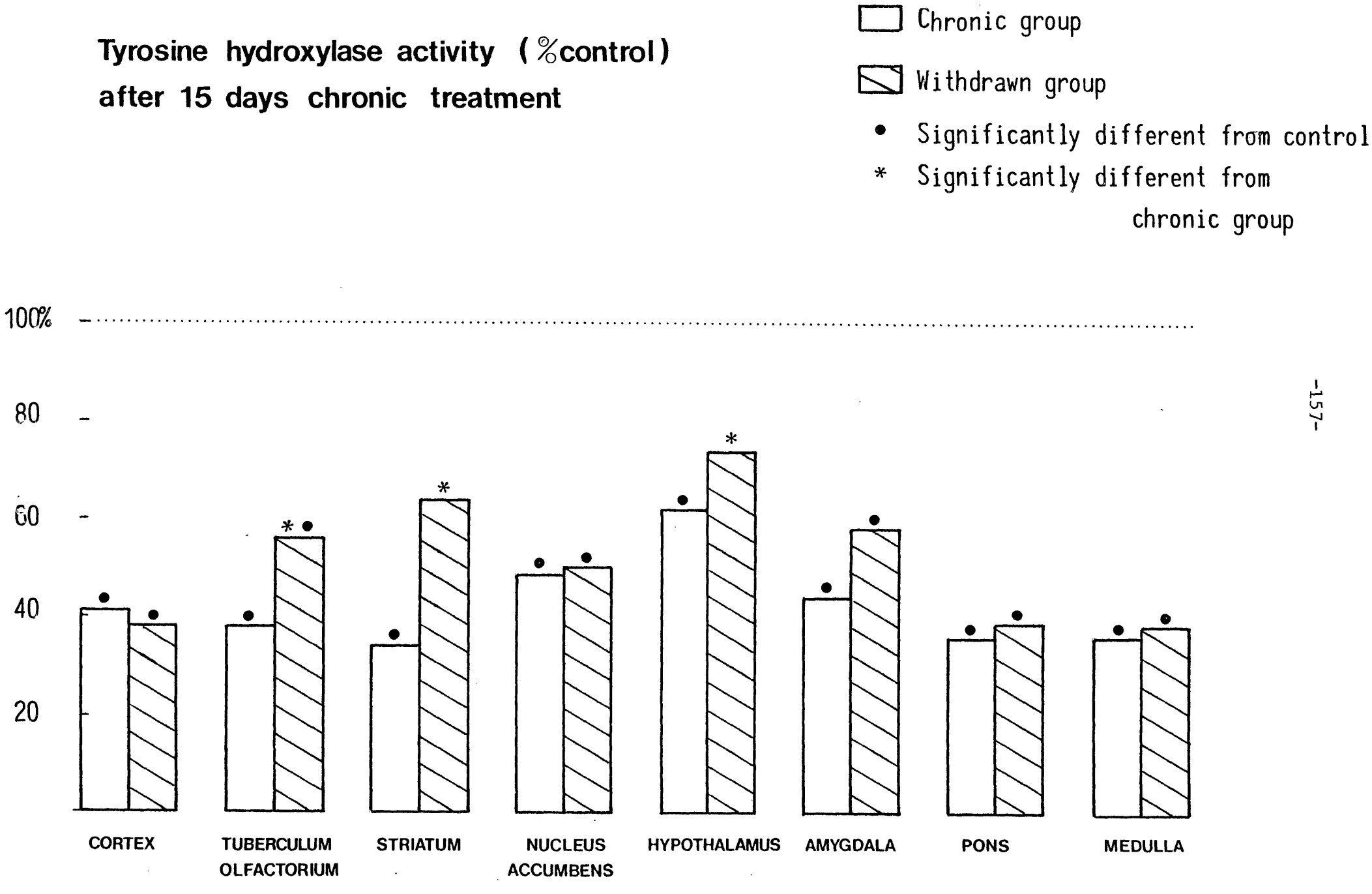
The results from the experimental groups from experimental set 1 and experimental set 4 are shown in Figures 25 and 26, and Tables 5 and 6, respectively. The groups of animals were killed after 15 days and 30 days of chronic treatment respectively. The Lowry protein assay was used to measure the amount of protein present (Lowry *et al.*, 1951). The standard protein used was bovine serum albumin.

The changes in tyrosine hydroxylase activity in the brain regions from experimental set 1 after 15 and 30 days of chronic treatment are depicted graphically,as positive changes were seen. The results from experimental set 4 after 15 and 30 days of chronic treatment show no statistically significant change between the groups in any of the regions.

Table 4 shows the control levels of activity in the various regions, comparing the levels of activity found in each set of animals analysed.

FIGURES 25 and 26. Tyrosine hydroxylase activity in the various brain regions after 15 and 30 days of chronic treatment

Tyrosine hydroxylase activity (%control)
after 15 days chronic treatment



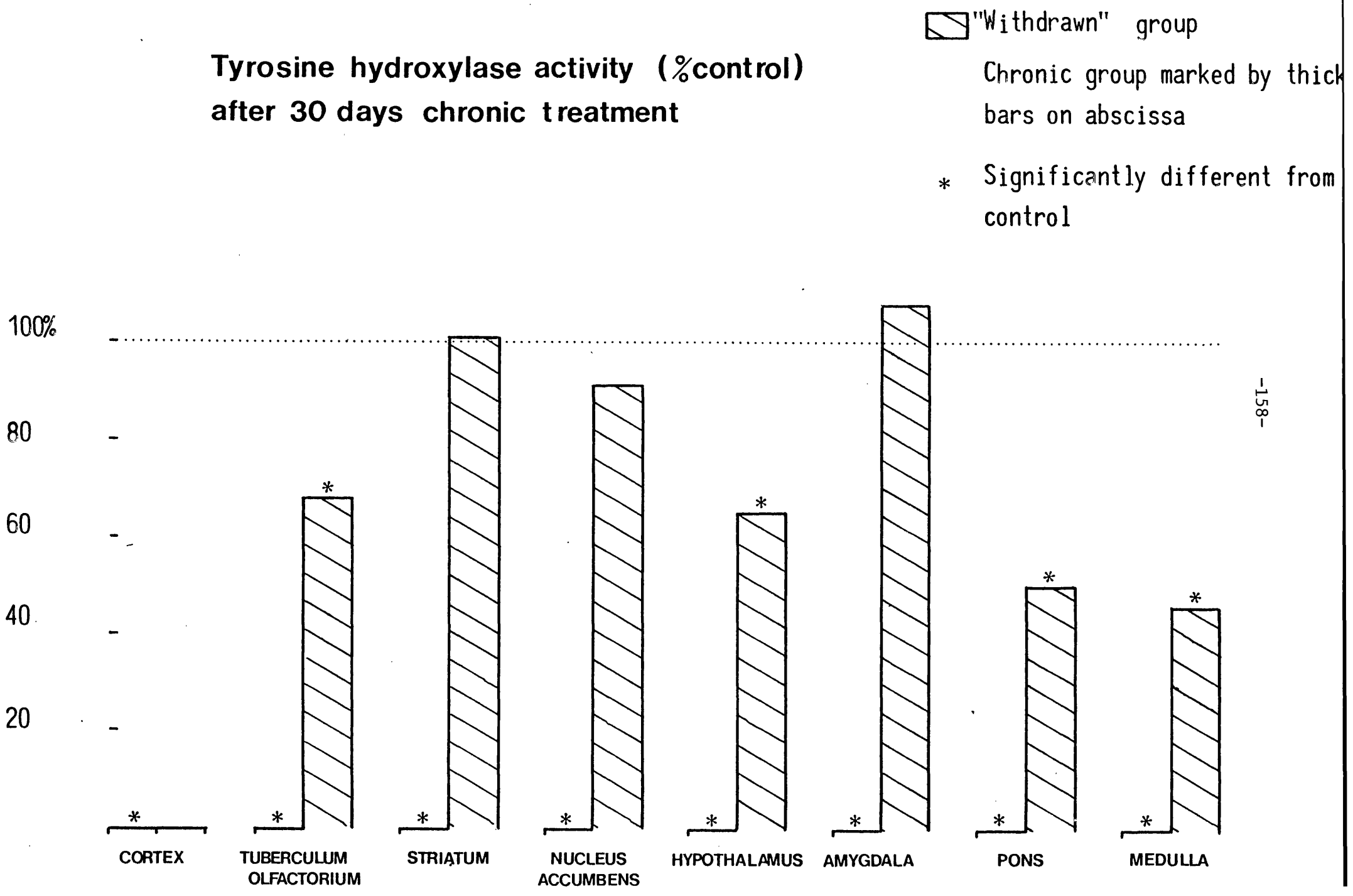

"Withdrawn" group
Chronic group marked by thick bars on abscissa
* Significantly different from control
Tyrosine hydroxylase activity (%control)
after 30 days chronic treatment
100%
80
60
40
20
CORTEX
TUBERCULUM OLFACTORIUM
STRIATUM
NUCLEUS ACCUMBENS
HYPOTHALAMUS
AMYGDALA
PONS
MEDULLA

Table 4 Comparisons between the activity of tyrosine hydroxylase obtained in the various brain regions from the control rats from each experiment and literature values for the regions using the same substrate concentrations (Cicero *et al.*, 1972).

The activity is in nmol product formed/h/g wet weight. ± S.E.M.

Region	ACTIVITY				
	Cicero *et al.*,1972	15 Day. Set 1	30 Day. Set 1	15 Day. Set 4	30 Day. Set 4
Cortex	8.87±2.29	21.13±1.36	7.56±0.01	-	2.24±0.69
Striatum	1261.22±72.90	527±81.4	268±4.8	433±79	478±45
Hypothalamus	154.16±24.67	65.6±5.2	58.9±1.1	74.4±10.7	73.6±2.4
Amygdala	28.71±3.35	-	38.9±4.8	73±9.4	53.6±6.6
Pons	76.26±3.22	32.9±2.1	26.7±1.9	-	19.9±1.9

TABLES 5 and 6 Tyrosine hydroxylase activity in the various brain regions from experimental set 4 after 15 and 30 days of treatment.

The activity is expressed in c.p.m./µg protein $\bar{x}$ is the mean, σ_{n-1} is the standard deviation of the sample and n is the number of rats in the sample.

15 days of treatment

REGION	CONTROL	CHRONIC	WITHDRAWN
ACCUMBENS	$\bar{x}$ = 127.1	$\bar{x}$ = 100.2	$\bar{x}$ = 97
	σ_{n-1} = 50	σ_{n-1} = 23.8	σ_{n-1} = 35.6
	n = 5	n = 5	n = 5
STRIATUM	$\bar{x}$ = 182.1	$\bar{x}$ = 250.4	$\bar{x}$ = 294.3
	σ_{n-1} = 80	σ_{n-1} = 46	σ_{n-1} = 99
	n = 5	n = 5	n = 5
AMYGDALA	$\bar{x}$ = 30.7	$\bar{x}$ = 44.1	$\bar{x}$ = 39.8
	σ_{n-1} = 9.5	σ_{n-1} = 25.6	σ_{n-1} = 9.5
	n = 5	n = 5	n = 5
HYPOTHALAMUS	$\bar{x}$ = 31.3	$\bar{x}$ = 30.9	$\bar{x}$ = 30.23
	σ_{n-1} = 10.8	σ_{n-1} = 6.5	σ_{n-1} = 9.3
	n = 5	n = 5	n = 5

30 days of treatment

REGION	CONTROL	CHRONIC	WITHDRAWN
CORTEX	$\bar{x}$ = 0.9	$\bar{x}$ = 1.6	$\bar{x}$ = 0.5
	σ_{n-1} = 0.6	σ_{n-1} = 1.3	σ_{n-1} = 0.3
	n = 3	n = 3	n = 4
ACCUMBENS	$\bar{x}$ = 81.6	$\bar{x}$ = 114.3	$\bar{x}$ = 93
	σ_{n-1} = 11.2	σ_{n-1} = 30.7	σ_{n-1} = 28.1
	n = 4	n = 4	n = 4
STRIATUM	$\bar{x}$ = 195.8	$\bar{x}$ = 265.2	$\bar{x}$ = 234.2
	σ_{n-1} = 46.4	σ_{n-1} = 113.9	σ_{n-1} = 27.6
	n = 4	n = 4	n = 4
AMYGDALA	$\bar{x}$ = 21.9	$\bar{x}$ = 34.7	$\bar{x}$ = 35.2
	σ_{n-1} = 6.9	σ_{n-1} = 9.6	σ_{n-1} = 0
	n = 4	n = 4	n = 2
HYPOTHALAMUS	$\bar{x}$ = 30.1	$\bar{x}$ = 31	$\bar{x}$ = 26.6
	σ_{n-1} = 2.5	σ_{n-1} = 4	σ_{n-1} = 4.5
	n = 4	n = 4	n = 4
PONS	$\bar{x}$ = 8.2	$\bar{x}$ = 8.3	$\bar{x}$ = 8.4
	σ_{n-1} = 2	σ_{n-1} = 1.9	σ_{n-1} = 2.1
	n = 4	n = 4	n = 4
MEDULLA	$\bar{x}$ = 5.9	$\bar{x}$ = 7.7	$\bar{x}$ = 5.9
	σ_{n-1} = 1.7	σ_{n-1} = 3	σ_{n-1} = 2.8
	n = 4	n = 4	n = 4

D. Radioenzymatic measurement of noradrenaline, dopamine and their metabolites

1. Materials and Methods

Materials

Dopamine hydrochloride (DA), noradrenaline bitartrate (NA), 3-methoxytyramine hydrochloride (3MT), normetanephrine hydrochloride (NM), homovanillic acid (HVA), vanillylmandelic acid (VMA), 4-hydroxy-3-methoxyphenyl glycol (MHPG), 3-methoxytyrosine, adrenaline bitartrate, metanephrine hydrochloride and S-adenosyl-L-methionine were obtained from Sigma (London) Chemical Company Ltd. Homovanillyl alcohol (HVET), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4 dihydroxyphenyl-glycol (DHPG) and 3,4-dihydroxymandelic acid (DHMA) were obtained from Calbiochem. 3,4-Dihydroxyphenethanol was obtained from Regis Chemical Company Ltd., and L-DOPA from Aldrich Chemical Company Ltd.

S-adenosyl-L-[methyl-^{3}H]-methionine was obtained from the Radio-Chemical Centre, Amersham (specific activity 8 Ci/mmol). This was diluted and stored in 100 µl aliquots at 20^{o}C). The microcrystalline cellulose thin-layer chromatography plates were a Merck product.

Methods

Determination of noradrenaline and dopamine levels

The method was based on the radioenzymatic conversion of NA and DA by catechol-O-methyl transferase, in the presence of [^{3}H]-S-adenosyl methionine,to labelled NM and 3MT respectively. The products were separated by paper chromatography, cut out and counted (Cuello *et al.*, 1973).

The procedure followed was as follows:

(i) Purification of catechol-O-methyl transferase

The enzyme was purified according to the method of Nikodijevic *et al.* (1970). The activity of the enzyme preparation was determined by the method of McCaman (1965). The linearity of product formation with enzyme concentration was tested, then the enzyme was stored at -20°C in 1 ml aliquots. The activity of the purified enzyme was found to be 4.4 - 5.4 x 10^{-2} nmol product formed/min/mg protein. The protein concentration was 40 - 45 mg/ml.

(ii) Homogenization

The brain regions were homogenized in 5 mM Tris-HCl buffer, pH 6.0, at 0°C. An aliquot was removed for a protein assay and for the assay of tyrosine hydroxylase activity. Then perchloric acid (1 M), containing 1 mM EDTA and 10 mM ascorbic acid, was added to a known volume of homogenate (which had been placed in microfuge tubes at 0°C), to give a final concentration of 0.1 M perchloric acid. The homogenates were mixed and centrifuged in a microfuge (Beckman microfuge B) at 8730 x g (11,600 rev./min) for 3 min, at 4°C. The supernatant was pipetted off and stored at -20°C overnight; the next day the supernatants were thawed to 4°C and re-centrifuged. The supernatants were then stored at -20°C until the day of assay. Internal standards were put in on homogenization in order to monitor the recovery.

A number of assays were performed on the same brain region and in order to clarifity the sequence of events a diagrammatic summary of the procedure is shown in Figure 27.

(iii) Incubation

The 'incubation mix' consisted of 50 µl of catechol-O-methyl transferase, 10 µl of 20 mM ethylene glycol-bis-(β-amino-ethyl ether) N,N-tetra acetic acid (EGTA), 30 µl of [^{3}H]-S-adenosyl methionine (250 µCi/ml), 10 µl of pargyline (16 mg/ml) in 10% β-mercaptoethanol, 63 µl of 1 M Tris-HCl buffer, pH 9, containing 3 mM magnesium chloride.

Tissue supernatants (10 µl) were placed in conical glass centrifuge tubes, in an ice bath. 'Incubation mix' (25 µl) was added to each tube, mixed and incubated at 37°C for 40 min.

(iv) Extraction

After incubation, 30 µl of a mixture of 5 vol. of saturated borate buffer, pH 10, and 1 vol. of a mixture of NM and 3MT (10 mg/ml) was added to the incubation mixture. This was mixed and 0.5 ml of toluene/isoamyl alcohol (3:2 v/v) added. This was mixed on a vortex mixer for 30 s, then centrifuged at half speed (3,000 rev./min; 8 x 15 ml head; 2065 x g) in a bench centrifuge (BTL bench centrifuge, Baird & Tatlock (London) Ltd.).

(v) Back extraction

400 µl of the toluene/isoamyl alcohol was transferred to a clean conical centrifuge tube containing 25 µl of 0.1 M HCl. This was mixed on a vortex mixer for 30 s, then centrifuged in a bench centrifuge as before. The organic phase was removed carefully.

(vi) Chromatography

Three 5 µl aliquots of acid phase were spotted onto Whatman 3 mm chromatography paper (the origins were previously spotted with 5 µl of a mixture of NM and 3MT (10 mg/ml) containing 0.1% sodium metabisulphite.

The papers were developed by descending chromatography with methylamine (25/30% w/v in water)/t-amyl alcohol (1:4 v/v) for at least 24h. The spots were visualized by spraying the paper with ammonia followed by dilute Folin's reagent (1:4 v/v).

The spots were cut out and eluted overnight in 3 ml of ethanolic ammonia (ethanol/concentrated ammonia, 100:22 v/v). This was then counted as has been described previously.

Initially internal and external standard curves were run for each assay, however, as it was found that the two were identical, only an external standard curve was run routinely (Figure 28). In practice the standard curves varied minimally between assays.

Determination of DOPA, noradrenaline, dopamine and their non-O-methylated metabolites

The method is identical to that already described, except that after incubation of the samples, 5 µl of 8 M formic acid was added to the incubation mixture, mixed and the tubes placed in an ice-bath. The compounds were then separated by the thin-layer chromatographic procedure described by Fleming & Clark (1970). Immediately prior to spotting, 5 µl of carrier methoxyamine mix was added to the mixture (NM, 3MT, HVA, VMA, MHPG, HVET and 3-methoxytyrosine; 1 µg/µl). The thin-layer plates were then spotted with 15 µl of incubation mixture and run in two dimensions. The first dimension was in butanol: methanol: 1 M formic acid (3:1:1,by vol.), and the second dimension was in chloroform:methanol:1 M ammonia (12:7:1, by vol.). The running time was approximately 3h and 2h respectively. The spots were visualized with p-nitroaniline spray, this was made up from three solutions; (i) 0.1 g of p-nitroaniline dissolved in 2 ml of concentrated HCl and made up to a final volume of 100 ml with water, (ii) 0.2% sodium nitrite (iii) 10% potassium carbonate. Immediately prior to use, 1 vol. of (i), 1 vol. of (ii) and 2 vol. of (iii) were mixed. The spray had to be used within 90 s of mixing. The spots were scraped off the plate, eluted with 0.1 ml of 0.1 M formic acid in a scintillation vial and counted. Figure 29 shows the chromatographic separation of DOPA, NA, DA and their non-O-methylated and O-methylated metabolites by the method described above.

Several problems were encountered on trying to combine the thin-layer chromatographic separation technique with the radioenzymatic method already described. Firstly the pH of the incubation mixture had

to be brought down to a pH of about 2, if the spots were to separate clearly. The only acid which could be used was formic acid (others tried were: perchloric acid, trichloroacetic acid and hydrochloric acid). The second problem was to precipitate the protein,as this could also interfere with the separation. Two other protein precipitants were tried ($ZnSO_4$ / NaOH and tungstic acid) and mercaptho-ethanol was also tried. It was found that $ZnSO_4$/NaOH and mercapto-ethanol were about equally effective. However if the activity of the catechol-O-methyl transferase is sufficiently high it can be diluted so that the amount of protein present is not great enough to interferewith the assay (2 mg/ml protein).

Metanephrine was found to chromatograph to a position which overlapped both the NM and the 3MT spots, therefore the method could not be used to measure adrenaline.

In order to test the specificity of the assay, each compound was incubated separately and chromatographed with the 'methoxyamine mix'. Each spot was scraped off and counted; Table 7 shows the results of these experiments. Interference was found to occur between NA and DA; this is almost certainly due to the NM and 3MT spots running close to each other. This is not obvious from the plate shown, but in practice, with repeated numbers of plates, sometimes these two spots are so close as to be touching each other. Therefore the NA/DA interference is probably due to overlap of spots (which can be eliminated if great care is taken on running the plates). The only other interference noted was with DHPG; most counts were, as expected, on the MHPG spot; however a significant number of counts were found on the

HVET spot. From looking at large numbers of plates, I feel it is reasonable to rule out overlap as being the cause. The only explanation I can think of is a chemical breakdown of DHPG to DHPET before, or during incubation. The experiment was repeated several days later using the same solution of DHPG, and more counts were found on the HVET spot (although the total number of counts in both spots was the same, suggesting a fairly specific breakdown).

The recovery of the radioactivity was monitored by measuring the percentage of the total counts (as measured in 5 µl of incubation mixture) recovered in the 'dry counts' (measured by spotting 5 µl of incubate on the top-right hand corner of the plate). The total counts recovered in all the spots was measured, and the counts remaining at the origin (the origin was defined by scraping a 2.25 cm^2 square of cellulose from around the origin; the individual spots were all less than 1 cm in diameter). These figures are shown in Table 8.

The standard curves for each compound are shown in Figures 30 - 36. The reproducibility of the assay (seen from the standard deviations), and the sensitivity of each amine in the assay (this is the concentration which gives 2 to 4 times blank levels of radioactivity, depending on the standard deviations)are shown in Table 8.

The levels obtained for each amine in the various regions compares favourably with literature values obtained using other techniques. Finally, the published changes observed in those amines which have been measured after chronic treatment with methamphetamine are in the same direction as my results.

Further experiments which would establish the above method as valid together with an assessment of current methods used to measure the catecholamines will be considered in the Discussion. The shape of the standard curves will also be discussed.

FIGURE 27 Diagram of the procedure by which measurements of cerebral tyrosine hydroxylase activities, amine and tryptophan levels, and plasma tryptophan, phenylalanine and tyrosine levels were performed.

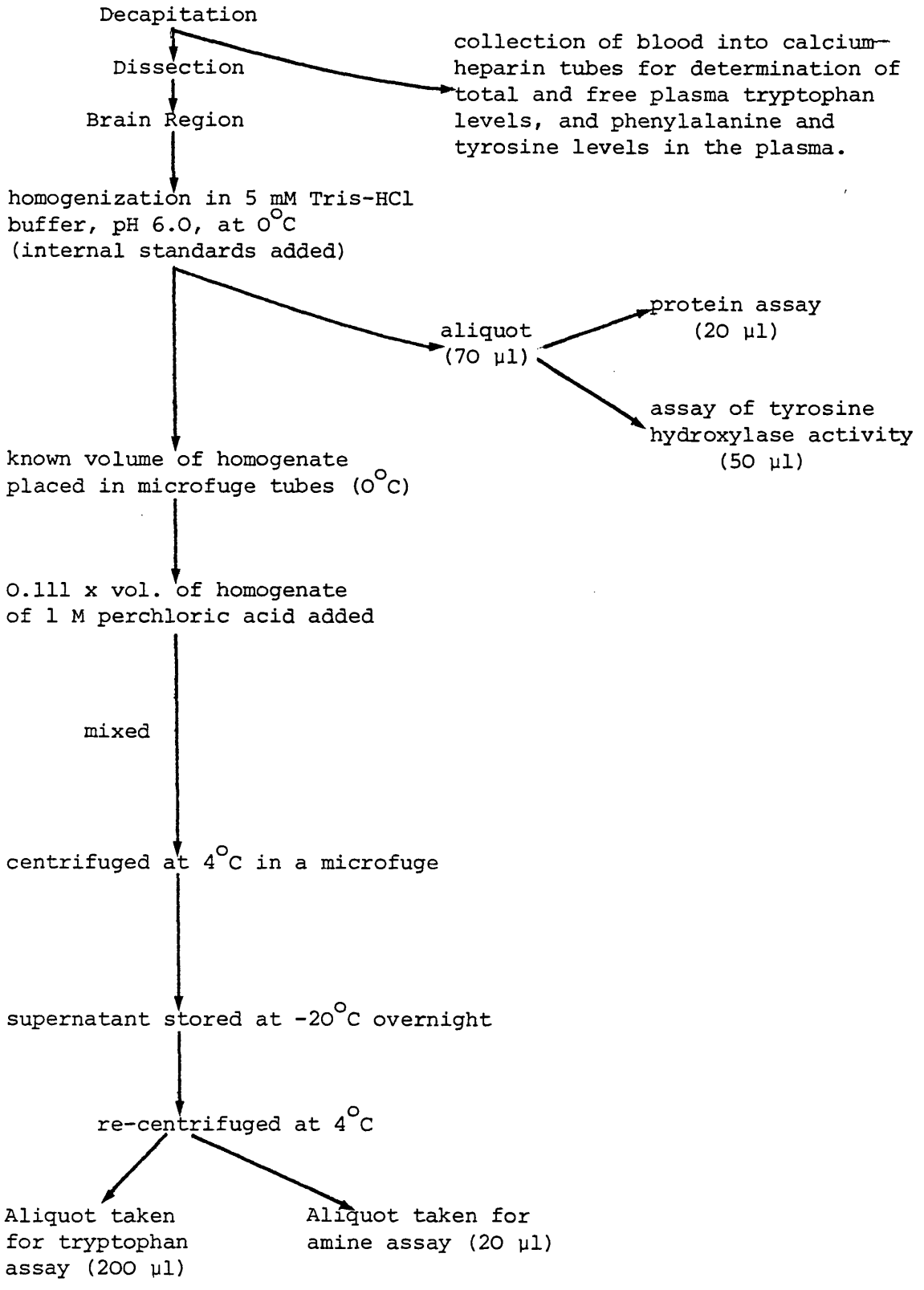

Decapitation
Dissection
Brain Region
collection of blood into calcium-heparin tubes for determination of total and free plasma tryptophan levels, and phenylalanine and tyrosine levels in the plasma.
homogenization in 5 mM Tris-HCl buffer, pH 6.0, at 0°C (internal standards added)
aliquot (70 µl)
protein assay (20 µl)
assay of tyrosine hydroxylase activity (50 µl)
known volume of homogenate placed in microfuge tubes (0°C)
0.111 x vol. of homogenate of 1 M perchloric acid added
mixed
centrifuged at 4°C in a microfuge
supernatant stored at -20°C overnight
re-centrifuged at 4°C
Aliquot taken for tryptophan assay (200 µl)
Aliquot taken for amine assay (20 µl)

FIGURE 28 Standard curves for NA (■——■) and DA (●——●). The scale on the ordinate for DA is on the right; the scale on the left relates to NA.

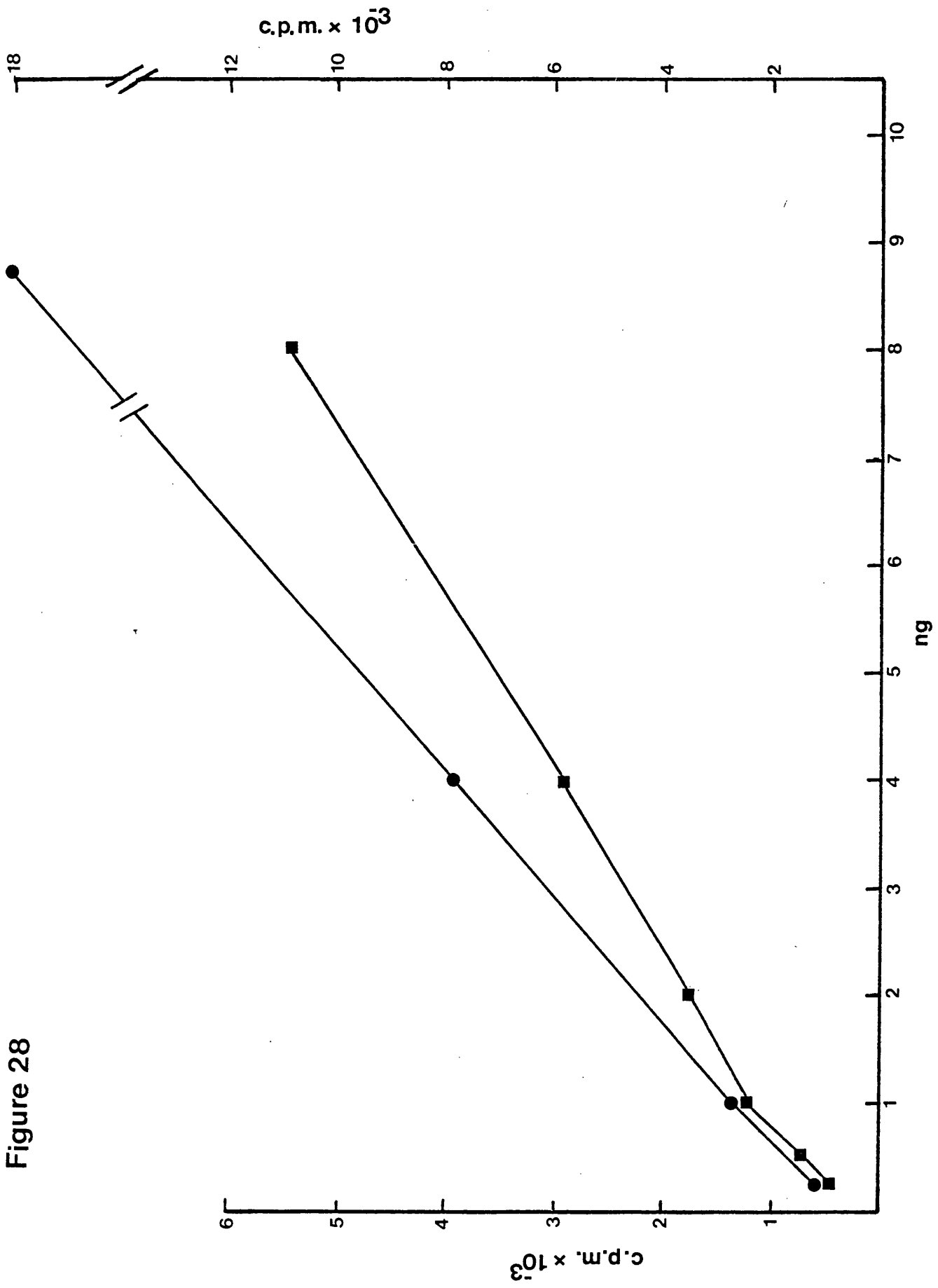


Figure 28

Figure 29. Chromatographic separation of DOPA, 3-methoxy-tyrosine (3 MeTyr), NA, DA and the O-methylated and non-O-methylated metabolites of NA and DA.

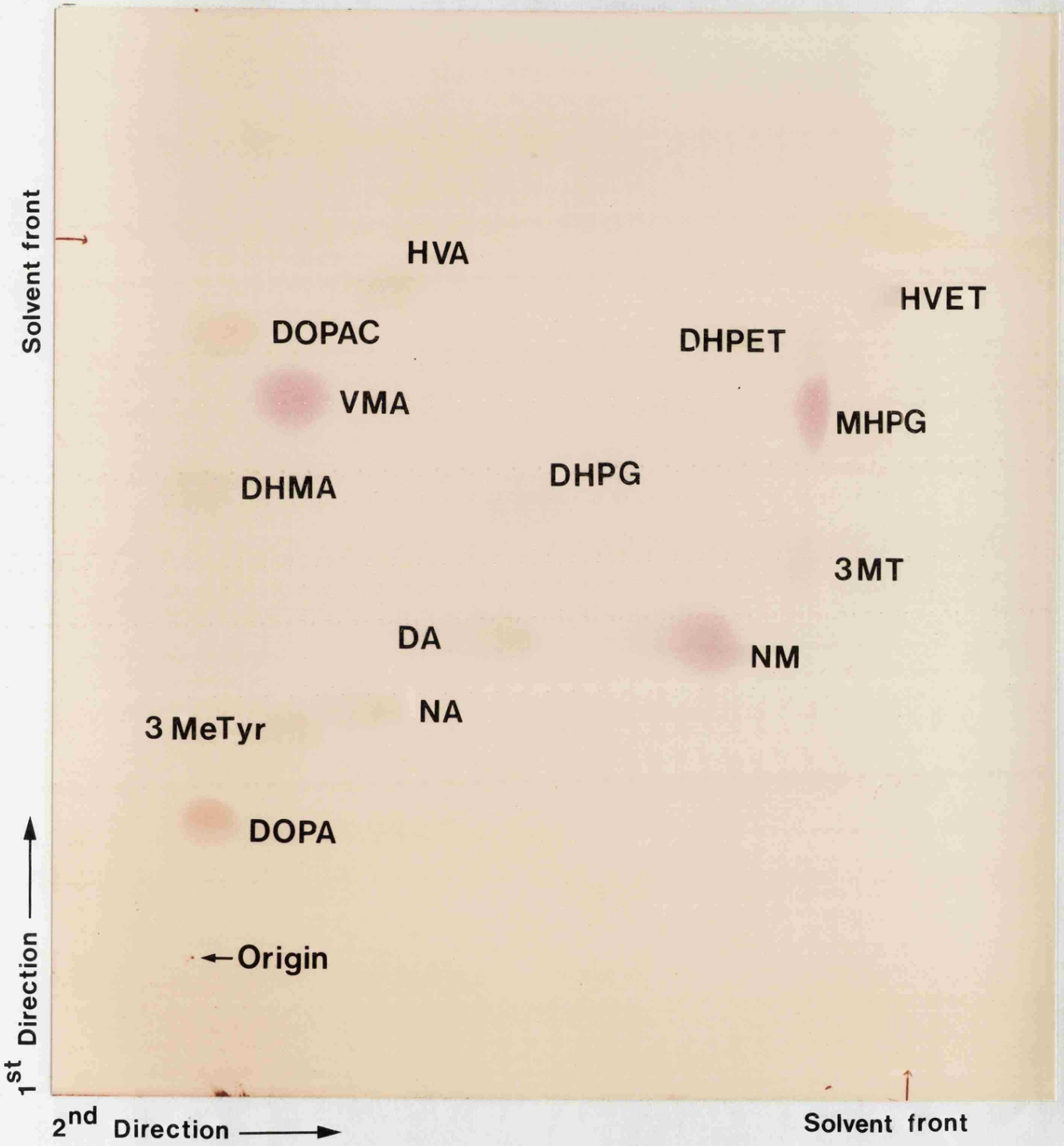

Solvent front
HVA
HVET
DOPAC
DHPET
VMA
MHPG
DHMA
DHPG
3MT
DA
NM
NA
3 MeTyr
DOPA
Origin
1st Direction
2nd Direction
Solvent front

Table 7. Percentage change in blank values on incubating 5 ng of each amine individually (n.s. indicates no significant change).

	SPOT						
Amine	3 MeTyr	NM	3MT	VMA	HVA	MHPG	HVET
DOPA	275	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NA	n.s.	188	39	n.s.	n.s.	n.s.	n.s.
DA	n.s.	n.s.	149	n.s.	n.s.	n.s.	n.s.
DHMA	n.s.	n.s.	n.s.	233	n.s.	n.s.	n.s.
DOPAC	n.s.	n.s.	n.s.	n.s.	10880	n.s.	n.s.
DHPG	n.s.	n.s.	n.s.	n.s.	n.s.	1534	47
DHPET	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	6617

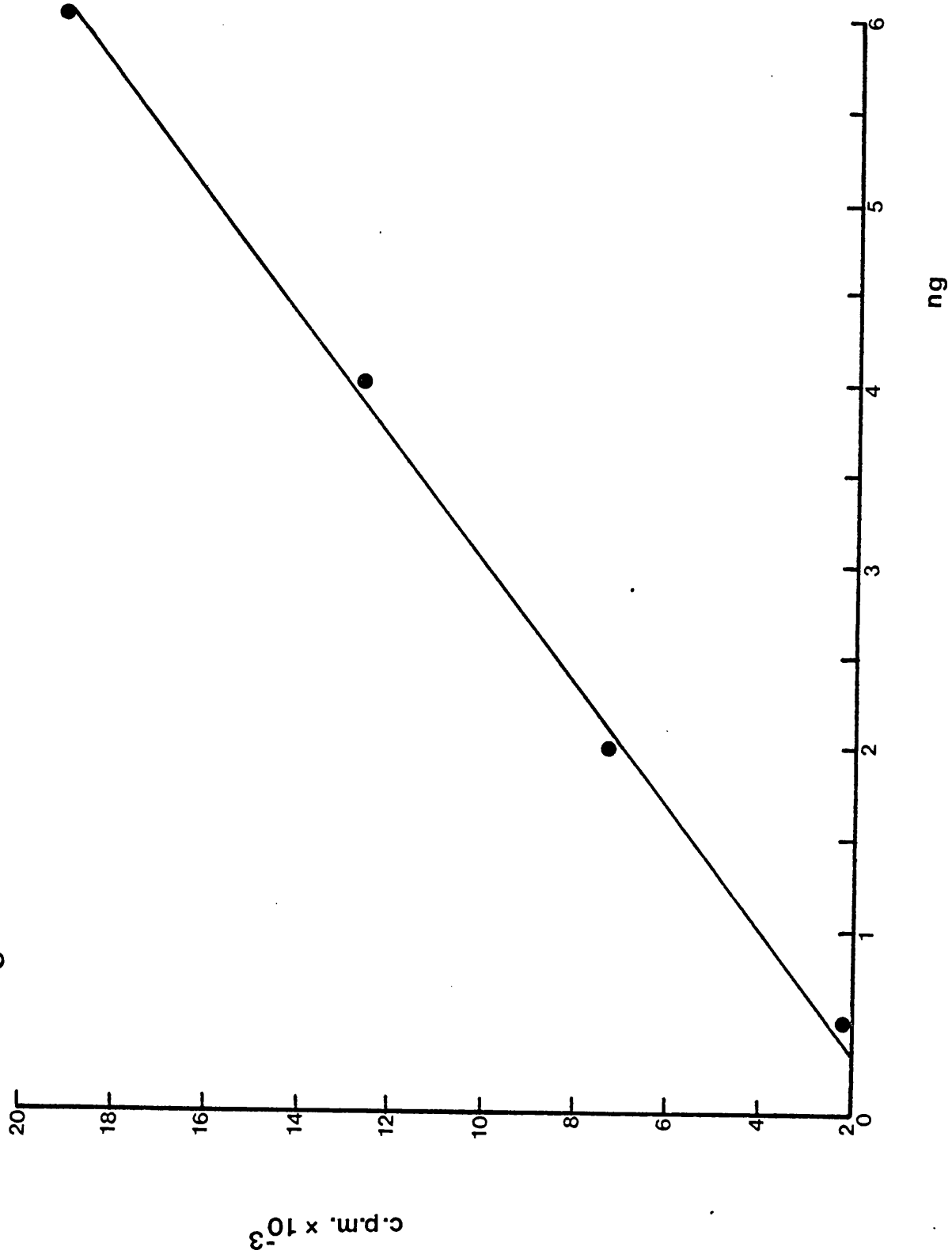
Figures 30-36 Standard curves for NA, DA, DHMA, DOPAC, DHPG and DHPET. Figure 30 shows a linear relationship between c.p.m. and concentration for NA and DA, figure 31 shows that, at lower concentrations the slope changes. Subsequent standard curves give the curves obtained at low and high substrate concentrations.

Key:

●—● high substrate concentration

■—■ low substrate concentration

Figure30 NA



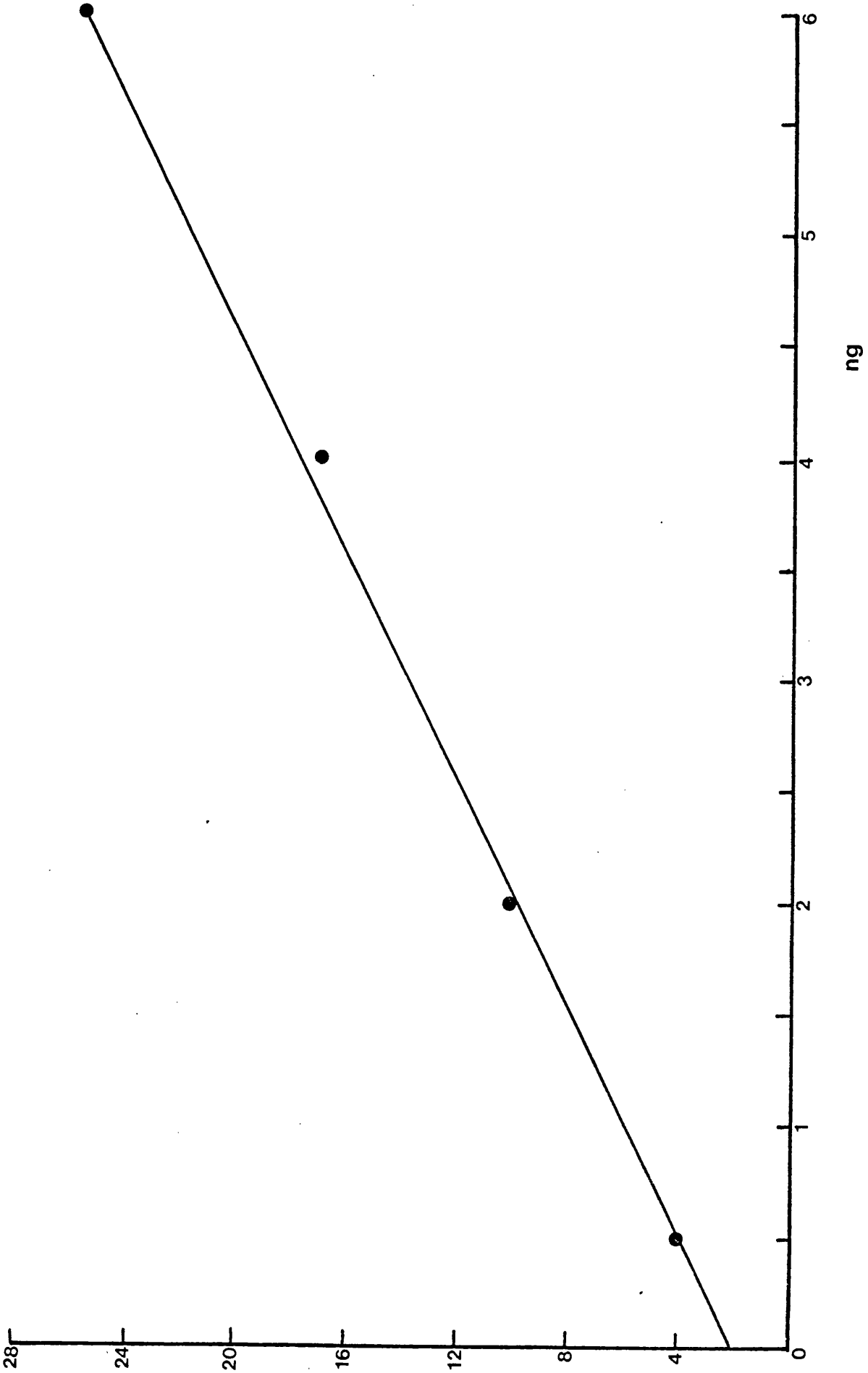


Figure 30a DA

Figure 31 NA

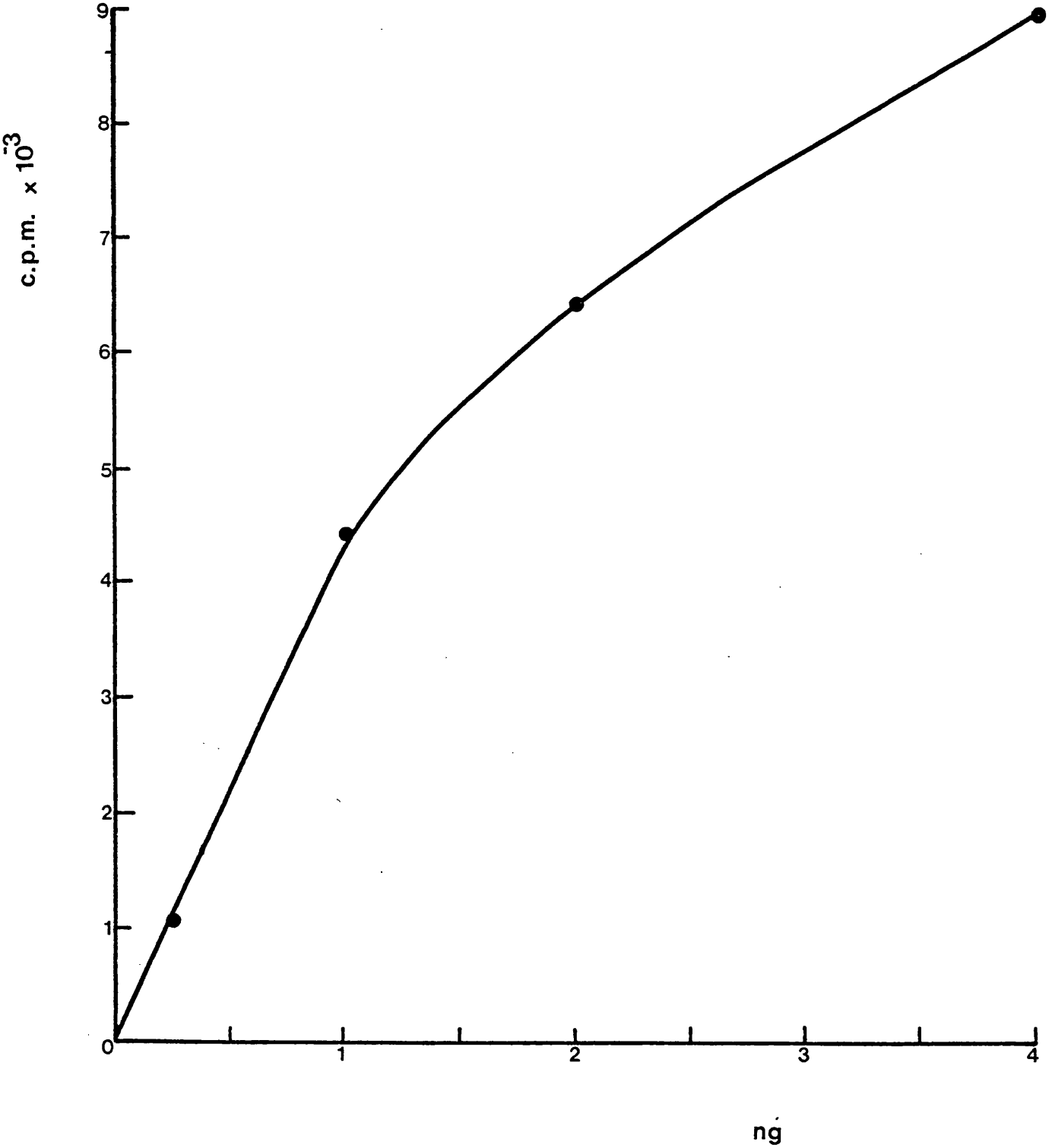
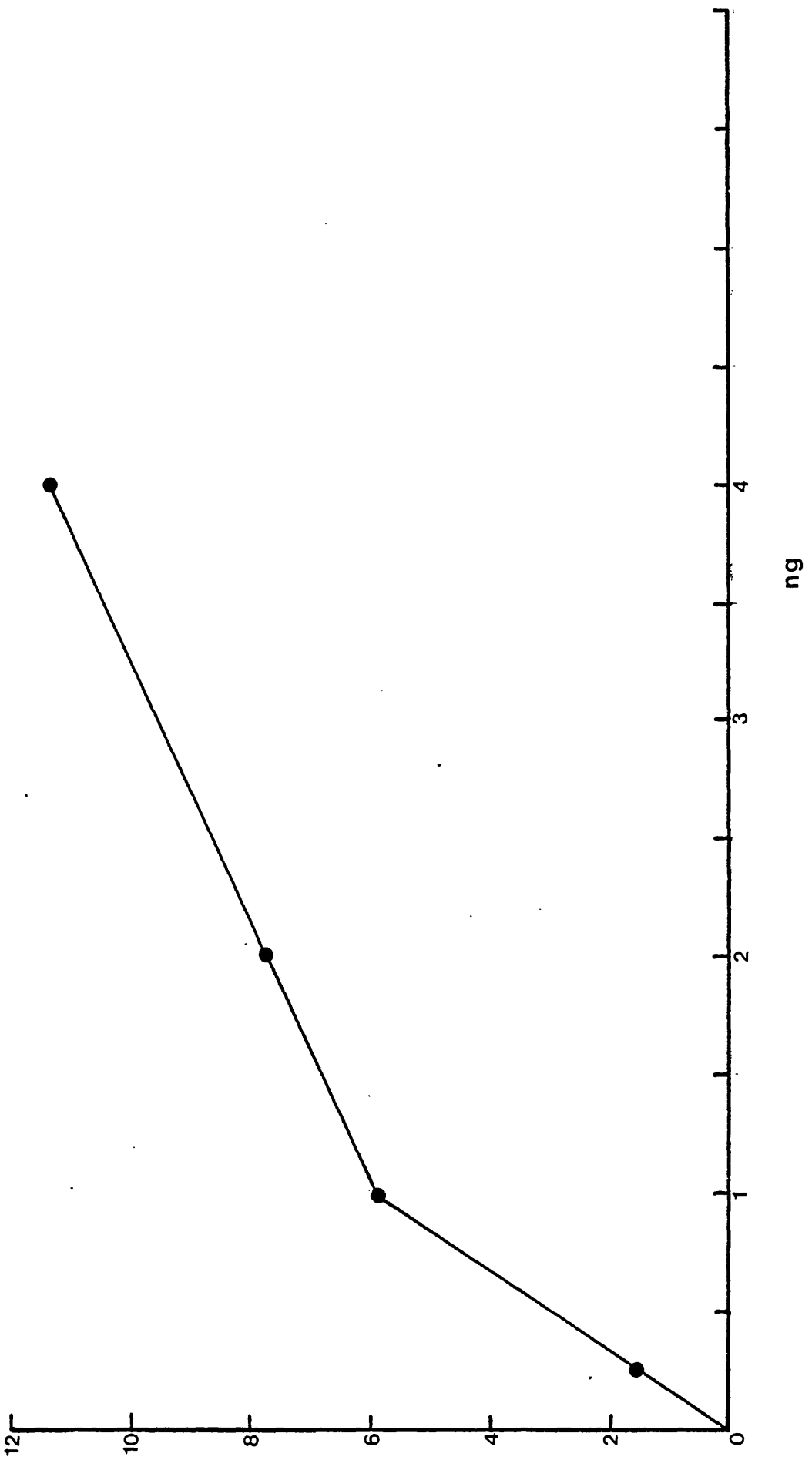


Figure 32 DA



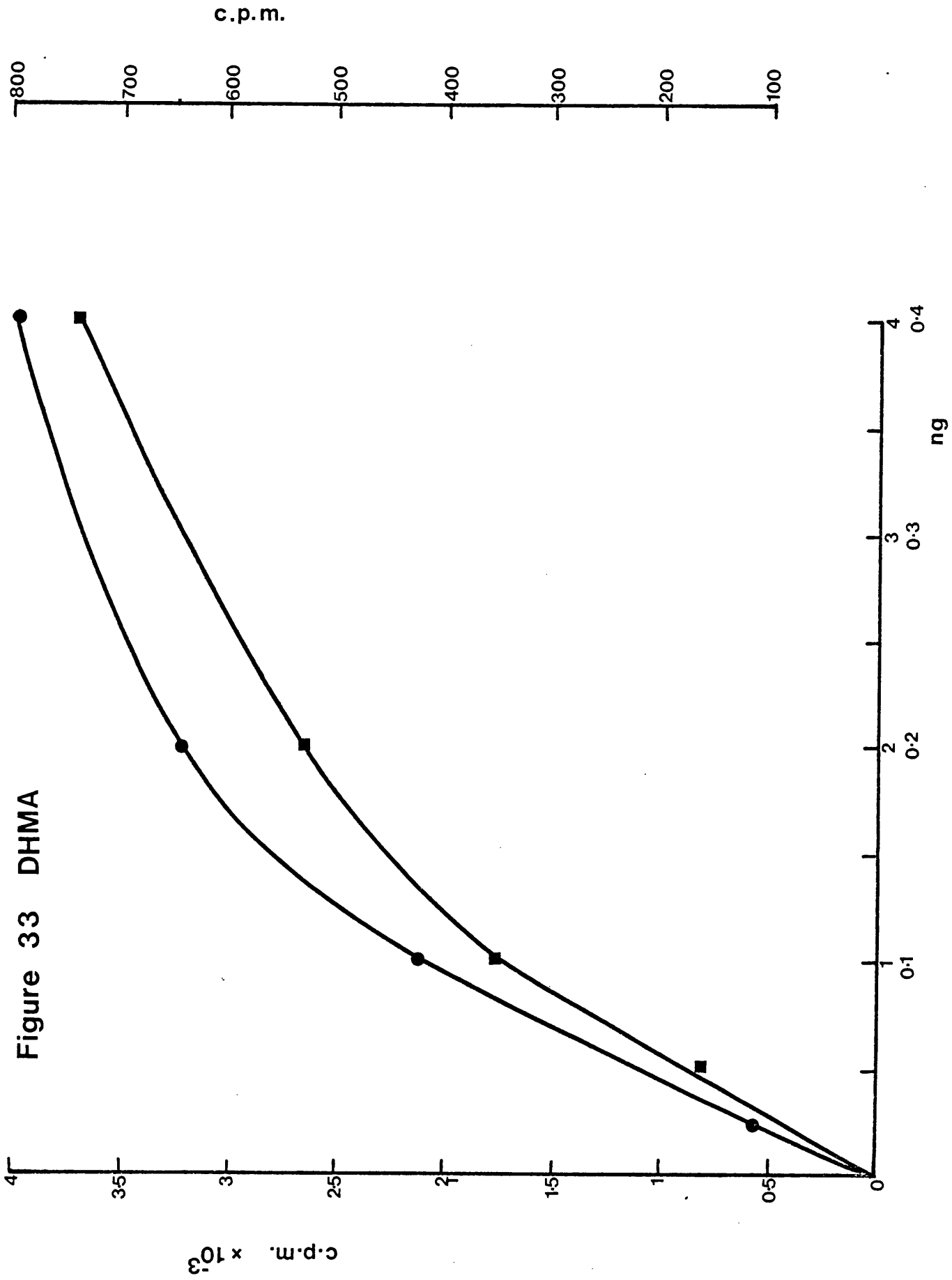


Figure 33 DHMA

Figure 34 DOPAC

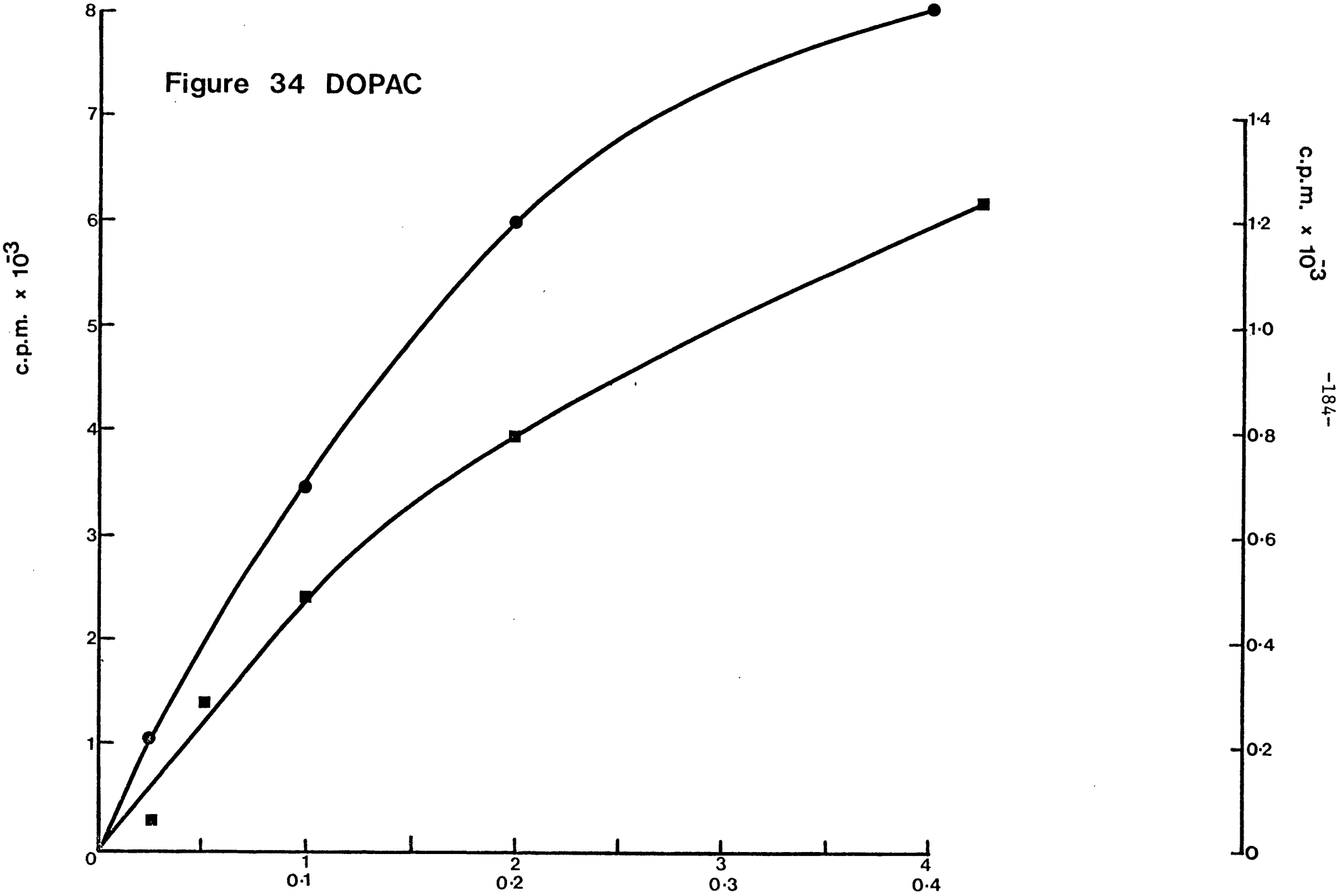
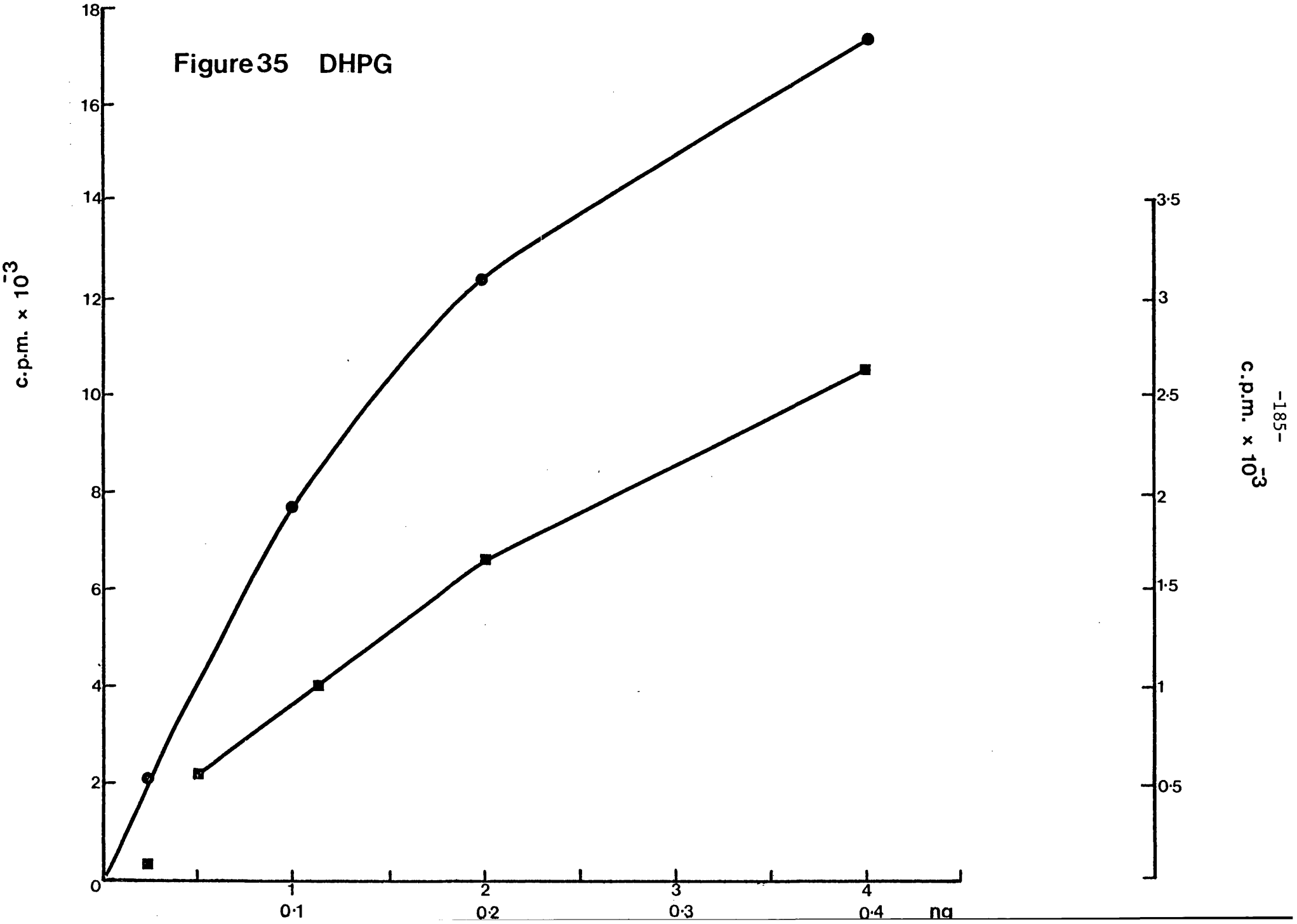


Figure 35 DHPG



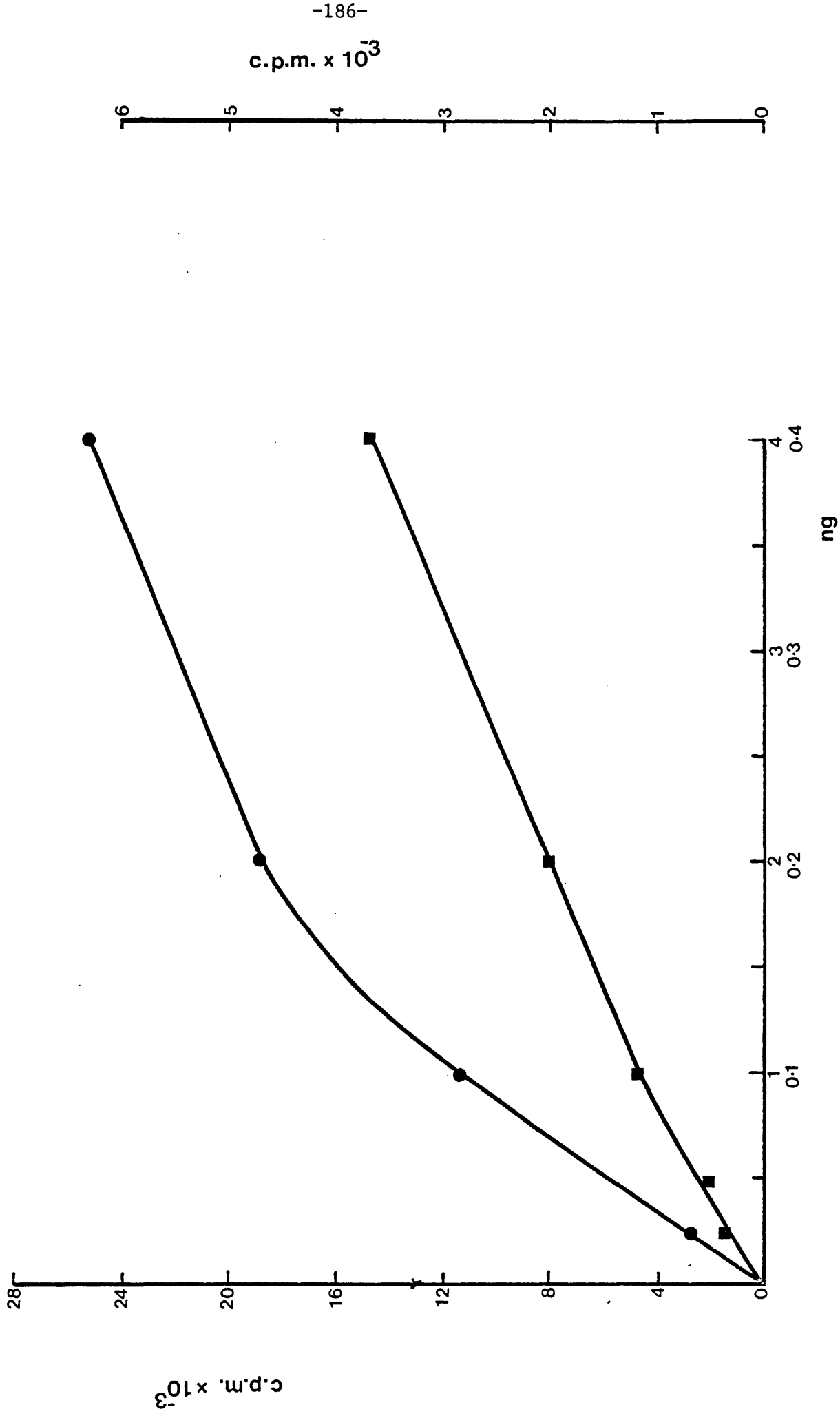


Figure 36 DHPET

Table 8. Distribution of radioactivity on thin-layer chromatography plates.

Blank plate

"dry counts" = 67% of "wet counts"

origin = 79% of "dry counts"

counts recovered on spots = 4% of "dry counts"

radioactivity unaccounted for = 96 % of "dry counts"

= 44,000 c.p.m.

Approximate sensitivity of the assays:

NA: 20 pg	DOPAC: 10 pg
DA: 200 pg	DHPG: 20pg
DHMA: 20 pg	DHPET: 10 pg

2. Results: Regional amine levels in experimental animal groups

The levels of noradrenaline and dopamine were determined in the three groups of experimental set 1 after 30 days of chronic treatment. The results are shown in Figure 37. In all the regions analysed there were decreased average levels of noradrenaline and dopamine; significant decreases were found in NA and DA all regions in either the chronic or withdrawn animal groups except for NA in the nucleus accumbens.

Using the modified radioenzymatic method, results were obtained for two regions (striatum and hypothalamus) in the three groups from experimental set 4 after 30 days of treatment. These two regions were selected as amphetamine is thought to act primarily on the nigroneostriatal dopaminergic pathway and the hypothalamus is rich in noradrenaline and dopamine, therefore any differential effect of amphetamine on the two transmitters in terms of the levels of NA and DA and their metabolites would be seen. The results are shown in Table 9.

Figure 37. Noradrenaline and dopamine levels in selected brain regions after 30 days of chronic treatment.

Key:

NA noradrenaline

DA dopamine

* significantly different from control

● significantly different from chronic

Control

Chronic

Withdrawn

Figure 37

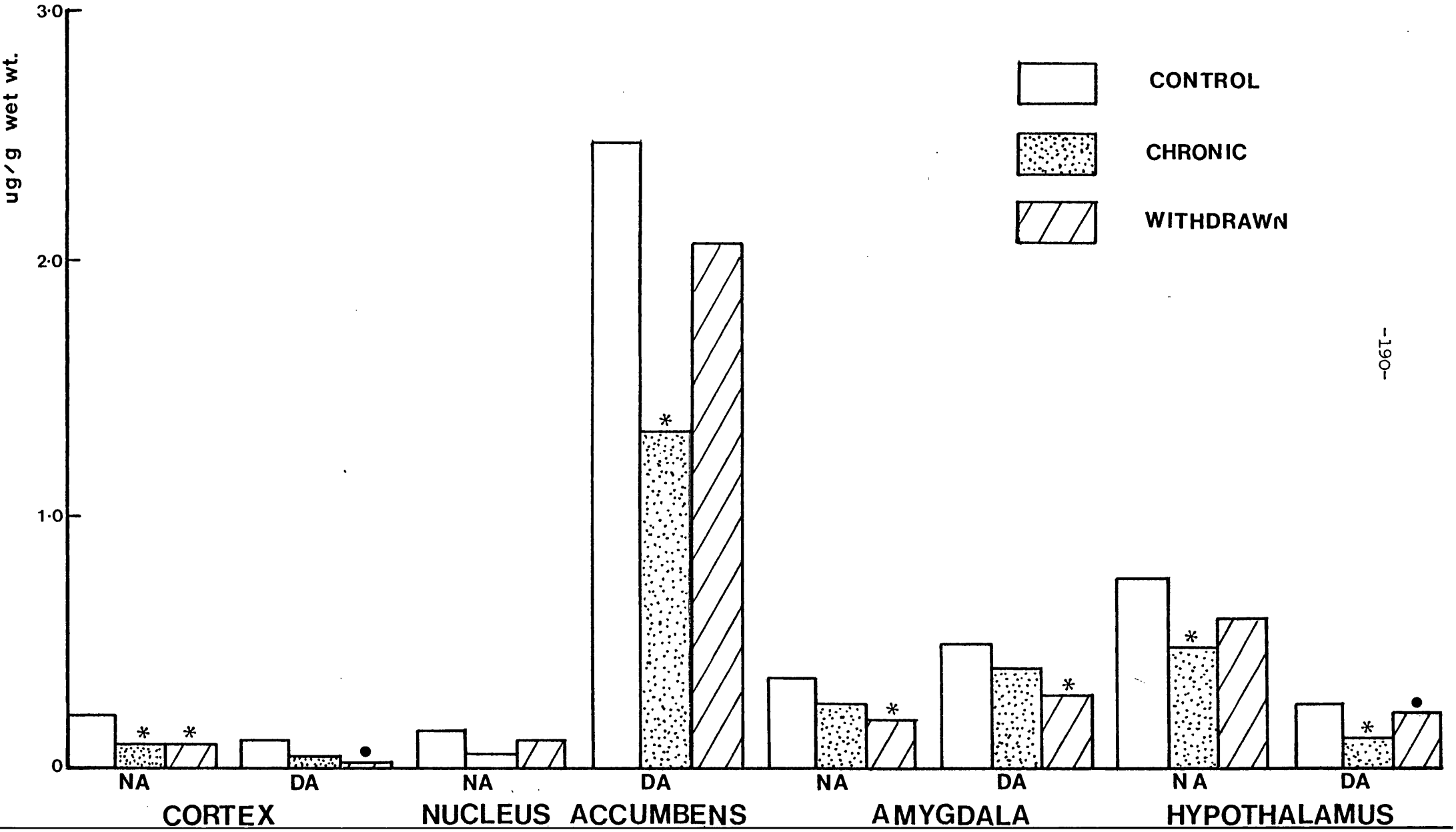

3·0
2·0
1·0
0
ug/g wet wt.
CONTROL
CHRONIC
WITHDRAWN
NA
DA
NA
DA
NA
DA
NA
DA
CORTEX
NUCLEUS ACCUMBENS
AMYGDALA
HYPOTHALAMUS

Table 9. Levels of noradrenaline, dopamine and their non-O-methylated metabolites in the striatum and hypothalamus from experiment Set 4, after 30 days of chronic treatment. Levels are in mg/g wet wt. of tissue ± standard deviation.

Key:

NA - noradrenaline

DA - dopamine

DHMA - 3,4-dihydroxymandelic acid

DOPAC - 3,4-dihydroxyphenylacetic acid

DHPG - 3,4-dihydroxyphenylglycol

DHPET - 3,4-dihydroxyphenethanol.

	NA	DA	DHMA	DOPAC	DHPG	DHPET
Control						
30 day Striatum	0.3±0.1	4.3±1.3	0.05±0.02	1.45±0.14	-	0.19±0.04
30 day Hypothalamus	1.5±0.4	0.3±0.2	0.05±0.03	0.08±0.01	-	0.04±0.03
Chronic						
30 day Striatum	0.15±0.03	3.7±0.6	0.05±0.02	0.95±0.27	-	0.23±0.11
30 day Hypothalamus	1.3±0.05	0.5	0.05±0.03	0.09±0.01	-	0.05±0.005
Withdrawn						
30 day Striatum	0.32±0.14	5.4±1.3	0.045±0.01	2.05±0.5	-	0.13±0.05
30 day Hypothalamus	2.2±1.0	0.4±0.5	0.03±0.001	0.11±0.02		0.03±0.002

E. Determination of brain tryptophan levels

1. Materials and Methods

Tryptophan was measured by virtue of its native fluorescence under strongly alkaline conditions (Udenfriend, 1962); the fluorescence intensity is greatest at pH 10.9. The brain regions were homogenized in Tris-HCl buffer and the protein precipitated with 0.1 M perchloric acid. The tryptophan assay was carried out on the resulting supernatant; consequently the fluorescence measured corresponds to the free tryptophan and the tryptophan present in proteins. As no extraction step was employed there is likely to be non-specific interference.

n-Butylamine buffer, pH 11.0 (24.56 ml of 0.2 M n-butylamine, 18.46 ml of 0.1 M HCl, made up to 200 ml with distilled water) (900 µl) was added, on mixing,to 100 µl aliquots of samples or standards, and allowed to stand at room temperature for 30 min. The samples or standards were read at an excitation wavelength of 287 nm and an emission wavelength of 376 nm (uncorrected values). All glassware used in this assay was soaked in 15% HCl overnight, then rinsed 4 times with distilled water, and dried.

The characteristic spectrum of tryptophan is shown in Figure 38. The external standard curve is shown in Figure 39; the sensitivity of the assay was about 10 ng (twice blank value). The internal standard curves (Figure 40) were less sensitive and more variable, as can be seen from the two internal standard curves shown. An internal standard curve was run for every assay.

The reproducibility of the assay was assessed by measuring tryptophan levels in 23 cerebellar samples from 2 rats. The standard deviation was found to be 2 to 4 ng.

2. Results: Regional tryptophan levels in the experimental animal groups

The results obtained for the various brain regions from the experimental animal groups are shown in Table 10. The values are not corrected for recovery; the average recovery was 83%. No significant differences can be seen between the three experimental groups after 15 and 30 days of treatment in experimental set 4. In the 15 day animals the control group showed significantly lower levels of tryptophan in the cortex compared to the striatum ($p < 0.005$) and hypothalamus ($p < 0.001$) and significantly lower levels in the medulla as compared to the striatum ($p < 0.002$). These regional variations were not seen in the 30 day rats.

The 35 day rats from experimental set 1 do show significant differences between the experimental groups. There is a depletion of tryptophan in the striatum and mesolimbic regions (nucleus accumbens and amygdala) in the chronic group of animals. On withdrawal the levels are between those of the control and chronic groups, but not significantly different from either. There is a trend towards a depletion in the hypothalamus in the chronic group and an apparent partial recovery on withdrawal but this did not reach significance owing to the high standard deviation. The pons and medulla appear to show opposite trends i.e. an increase in tryptophan levels in the chronic group, but again this did not reach significance.

As with the 15 day rats from set 4 the levels of tryptophan were lower in the cortex and medulla compared with the other regions. The cortical levels of tryptophan in the controls were significantly lower

than the levels in the striatum ($p < 0.05$), nucleus accumbens ($p < 0.05$) and amygdala ($p < 0.01$). The control levels in the medulla were significantly lower than the levels in the nucleus accumbens ($p < 0.05$), striatum ($p < 0.05$) and the amygdala ($p < 0.02$).

The large standard deviations seen in the table probably reflect a large variation between individual rats. In most methods for measuring tryptophan levels in brain tissue there is an extraction step (usually using a buffered cation exchange resin or by partitioning between two phases of solvent mixtures), before the measurement of tryptophan fluorometrically. Some of the methods have been reviewed by Gaitonde (1974), and he presents the levels of tryptophan obtained in whole brain and specific regions of the brain, found by various research workers. These values varied from 12.5 to 65.1 nmol/g of tissue. The levels of tryptophan which I have reported here vary between 28 and 38 nmol/g of tissue for experimental set 4, which had much lower standard deviations than the first experiment on the 35 day rats from experimental set 1 (these values varied from 22 to 61nmol/g). Therefore this method gives values similar to those obtained when an extraction step is included and has the advantage of being simple and rapid. The method described here is, however, not very sensitive and would not detect any small regional changes in tryptophan. By comparing the published methods which include an extraction step with mine it is not possible to tell whether these methods are more sensitive.

The changes observed between experimental groups in experimental set 1 after 35 days of chronic treatment are compatible with the results of the tyrosine hydroxylase activity and amine levels in that

this experimental set appeared to react more, in biochemical terms, than did experimental set 4.

FIGURE 38 Excitation and emission spectrum of 50 ng of L-tryptophan, pH 11.0, 25°C
F is the intensity (uncorrected)

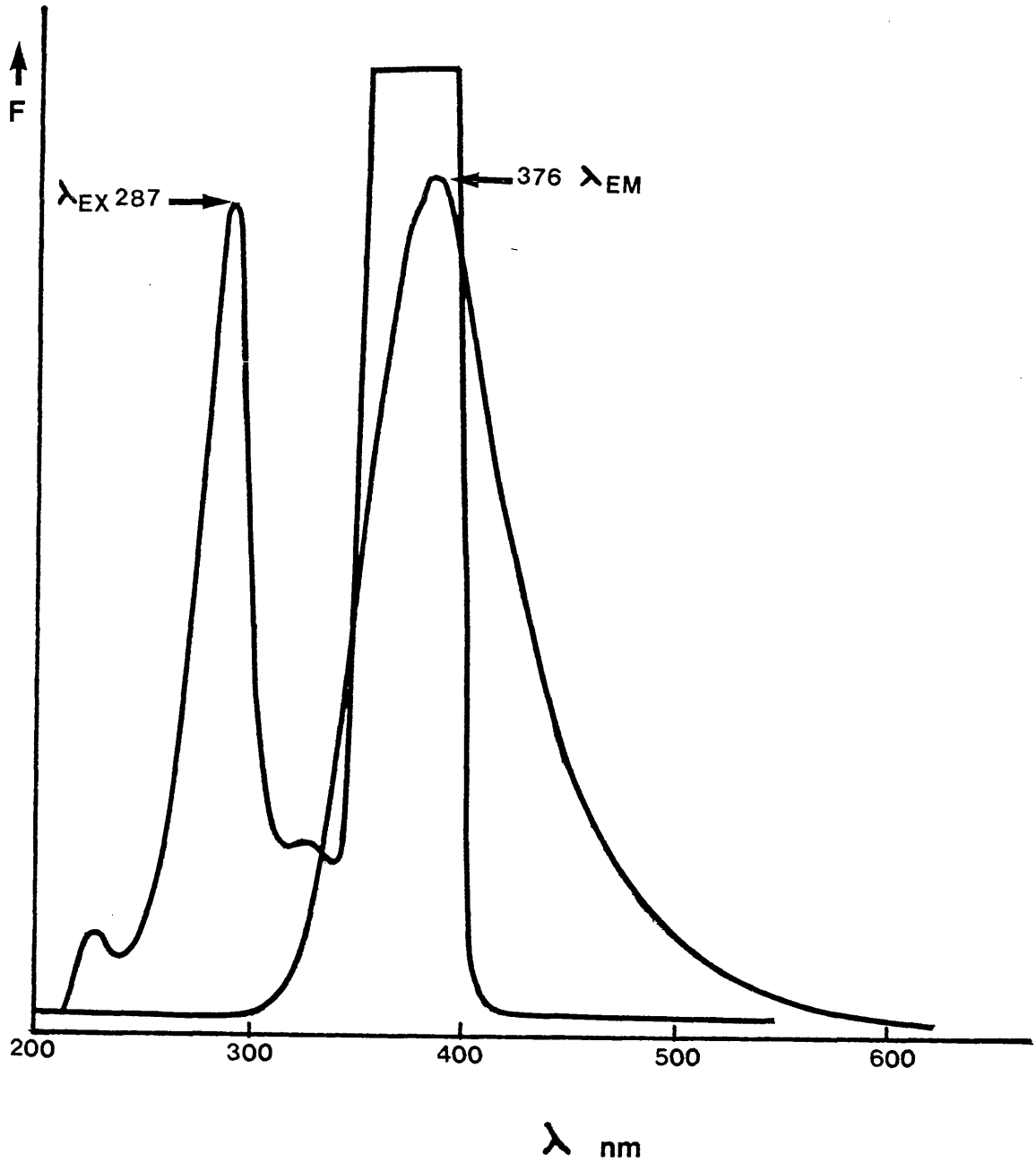

F
λEX 287
376 λEM
200
300
400
500
600
λ nm

FIGURE 39 External standard curve for tryptophan assay, pH 11, 25°C

F is the uncorrected intensity in arbitrary units.

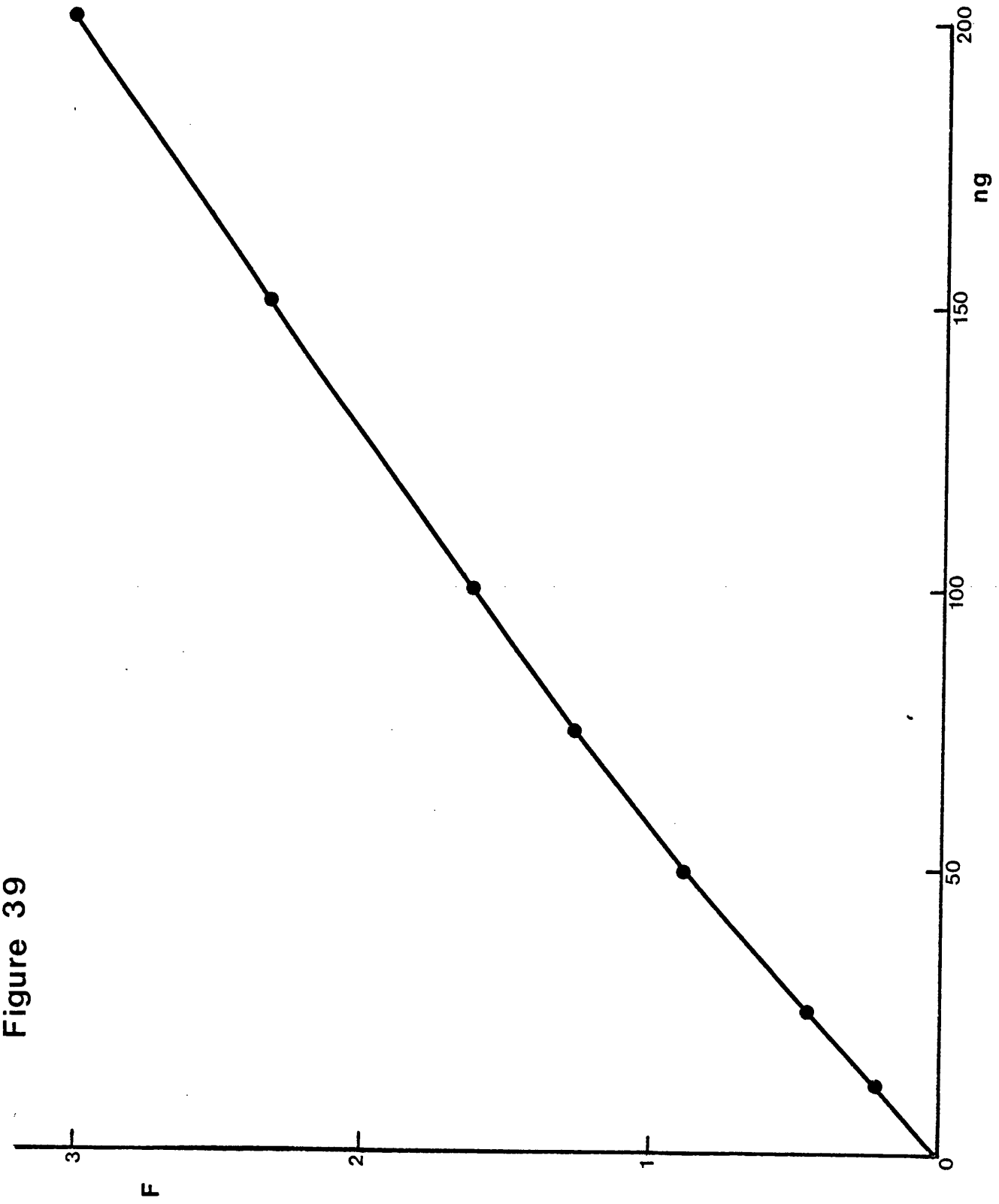


Figure 39

FIGURE 40 Internal standard curves for tryptophan assay. Blank values were 0.42 (curve a) and 0.51 (curve b). F - intensity (uncorrected) in arbitrary units.

Figure 40

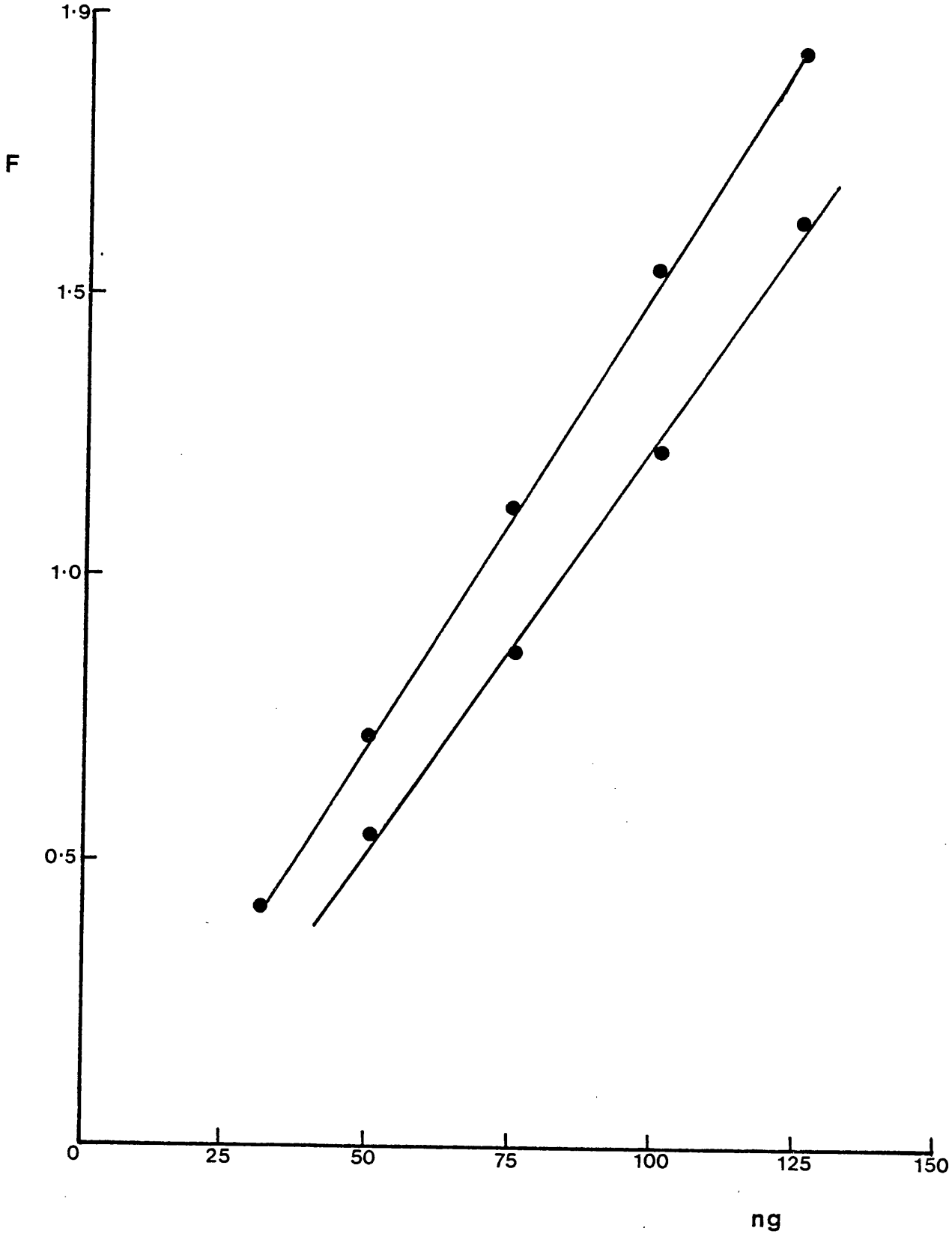


Table 10 Levels of tryptophan in the various brain regions after 35 days of chronic treatment in experimental set 1, and after 15 and 30 days in experimental set 4. The table gives the mean ($\bar{x}$), the number of observations (n) and the standard deviation (σ_{n-1}); * denotes that the results in the experimental group were significantly different from the control values.

Experimental set 1 35 day rats (levels are in ng/mg protein

	Cortex	N. Accumbens	Striatum	Amygdala	Hypothalamus	Pons	Medulla
Control							
$\bar{x}$	45	125	116	110	107	73	48
n	2	3	3	3	3	3	3
n-1	16	26	19	6	40	27	18
Chronic							
$\bar{x}$	63	47*	47*	63*	58	121	118
n	3	3	3	2	3	3	3
n-1	9	9	6	16	12	20	46
		$p < 0.02$	$p < 0.01$	$p < 0.02$			
Withdrawn							
$\bar{x}$	41	83	47*	76	79	51	55
n	3	3	3	3	3	3	3
n-1	15	19	31	41	32	26	20
			$p < 0.05$				

Experimental set 4 (levels are in ng/mg protein)

15 days

	Cortex	N. accumbens	Striatum	Amygdala	Hypothalamus	Pons	Medulla
Control							
$\bar{x}$	58	78	85	64	77	75	68
n	4	4	4	4	4	4	4
σ_{n-1}	5	13	6	9	3	7	2
Chronic							
$\bar{x}$	73	103	85	80	84	77	87
n	4	4	4	3	4	4	4
σ_{n-1}	20	15	18	8	19	4	27
Withdrawn							
$\bar{x}$	68	90	85	71	79	72	64
n	4	4	4	4	4	4	4
σ_{n-1}	6	16	10	11	17	17	14
30 days							
Control							
$\bar{x}$	68	75	66	69	78	63	64
n	4	4	4	4	4	3	4
σ_{n-1}	11	5	11	8	13	9	5
Chronic							
$\bar{x}$	63	79	64	61	70	61	68
n	4	4	4	4	4	4	4
σ_{n-1}	11	17	5	3	8	7	14
Withdrawn							
$\bar{x}$	54	76	66	76	73	50	55
n	4	3	4	4	4	4	4
σ_{n-1}	9	15	9	6	7	9	5

F. Measurement of brain serotonin levels

1. Materials and Methods

Materials

Acetyl coenzyme A (acetyl CoA) and serotonin creatinine sulphate (5-HT) were obtained from Sigma (London) Chemical Company Ltd. A stock solution of serotonin creatinine sulphate was stored in 0.1 M HCl in the deep freeze. Under these conditions it was stable for 2 to 3 weeks.

S-adenosyl-L-[methyl-^{3}H]-methionine ([^{3}H]-SAM ; specific activity 8 Ci/mmol) and [1-^{14}C]-acetyl coenzyme A (specific activity 50 mCi/mmol) were obtained from the Radiochemical Centre, Amersham.

Methods

The method used was identical to that described by Saavedra *et al.* (1973). This radioenzymatic assay is based on the following reactions:

$$\text{5-HT + Acetyl CoA} \xrightarrow[\text{(rat liver)}]{\text{N-acetyl transferase}} \text{N-acetyl-5-HT + CoA}$$

$$\text{N-acetyl-5-HT + [}^{3}\text{H]-SAM} \xrightarrow[\text{(bovine pineal)}]{\text{HIOMT}} \text{[}^{3}\text{H]-Melatonin}$$

(N-acetyl-5-methoxytryptamine)

The melatonin is extracted, and the amount of [^{3}H] melatonin formed should be directly proportional to the amount of 5-HT in the original sample.

(i) Purification of N-acetyl transferase

N-acetyl transferase was purified from rat liver by a modification of the method described by Weissbach *et al.* (1961). All purification procedures were carried out at 4°C. Rat livers (50 g) were homogenized in 100 ml of 0.1 M sodium phosphate buffer, pH 7.2, and centrifuged at 100,000 x g for 1 h in a Beckman Ultracentrifuge (model L5-65). The supernatant was saturated to 40% with solid ammonium sulphate, allowed to stand for at least 15 min, then centrifuged for 20 min at 18,000 rev./min (36,000 x g) in an M.S.E. 18 centrifuge. In all steps which involve the addition of solid ammonium sulphate, it was added slowly with constant stirring. The precipitate was dissolved in 10 ml of 20 mM sodium phosphate buffer, pH 7.2. Ammonium sulphate was added to give 65% saturation and this was centrifued for 20 min at 36,000 x g. The precipitate was dissolved in 10 ml of 20 mM sodium phosphate buffer, pH 7.2,and dialysed three times (3h each) against 2,000 ml of this buffer. After dialysis a precipitate formed in the dialysis residue which was centrifuged (in a bench centrifuge at 3,000 rev./min (2065 x g)) and discarded. The enzyme was stored with 10% ethylene glycol, neutral EDTA (0.1 mM) and 1 mM mercaptoethanol (Riddle and Jenks, 1971). The enzyme was stored in 200 µl aliquots at -20°C. Under these conditions it was found to be stable for 6 months.

The activity of the enzyme was assayed by the method of Deguchi & Axelrod (1972). The activity of the enzyme was found to be 700 - 800 nM product formed/h/ml. The velocity was linear with enzyme concentration.

Purification of hydroxyindole-O-methyl transferase

Hydroxyindole-O-methyl transferase was partially purified from bovine pineal glands by a modification of the method described by Axelrod & Weissbach (1961). All purification procedures were carried out at 4°C. Frozen bovine pineals (10 g) were homogenised in 1.1% KCl. The homogenate was centrifuged at 100,000 x g for 1 h. To the soluble supernatant fraction, solid ammonium sulphate was added to 35% saturation; the supernatant was allowed to equilibrate for 1 h and was then centrifuged at 18,000 rev./min (36,000 x g) for 20 min. Solid ammonium sulphate was added to the soluble supernatant fraction to reach 65% saturation; it was added as before and left to stand for 1 h. This was centrifuged at 18,000 rev./min (36,000 x g) for 20 min. The pellet was dissolved in 5 ml of 50 mM sodium phosphate buffer, pH 7.9, and dialysed three times (each for 3h) against 1 litre of this buffer. After dialysis the enzyme was stored in 200 µl aliquots at -20°C. It was found to be stable for about 6 months.

The activity of the enzyme was assayed by the method described by Saavedra et al (1973). The activity of the enzyme was 250 nmol product formed/h/ml. (this is below the values reported by Saavedra et al (1973). The velocity was linear with enzyme concentration.

Assay for serotonin

The frozen brain regions were homogenised in 0.1 M perchloric acid (containing ascorbic acid and EDTA as before), or 0.1 M HCl (1:10 w/v). The homogenate was frozen and thawed before centrifuging

in a microfuge and assaying for serotonin. The assay was performed in 3 stages:

(i) N-acetylation. 25 µl of samples (standards or tissue samples) were placed in stoppered glass tubes; 45 µl of 'incubation mix' was added and the tubes incubated at 37°C for 30 min. The reaction was stopped by transferring the tubes to an ice bath. The 'incubation mix' consisted of 25 µl of a solution made with 10 ml of 0.2 M sodium phosphate buffer, pH 7.9, and 1.1 ml of 1 M sodium hydroxide, 10 µl of N-acetyl transferase and 10 µl of acetyl CoA (this has to be made up freshly, immediately prior to use.)

(ii) O-methylation. After stage (i), 22 µl of a mixture containing 10 µl of hydroxyindole-O-methyl transferase, 2.0 µl of [^{3}H]-S-adenosyl-L-methionine (8.0 Ci/mmol) and 10 µl of 0.2 M sodium phosphate buffer, pH 7.9, was added.

The reaction was stopped with 0.5 ml of 0.5 M borate buffer, pH 10.0 (10 vol) plus melatonin (1 vol. of 1 mg/ml in 25% ethanol.)

(iii) Extraction. 6 ml of toluene was added to the incubation mixture, which was then mixed on a vortex mixer for 30 s, and centrifuged at half-speed on a bench centrifuge (2065 x g) for 2 min. The toluene extract (5 ml) was transferred to clean glass scintillation vials containing 3ml of fresh toluene. These were incubated overnight at 80°C in an oven.

It was noted that, in this stage of the procedure, the temperature

of the oven was crucial. It was important for the temperature to be homogeneous, and that the temperature should not exceed 80°C otherwise the melatonin will break down.

The product was identified by thin-layer chromatography. The [^{3}H]-melatonin was dissolved in 75 µl of ethanol, and 30 µl applied to activated (heated at 100°C for 1 h) silica gel plates (home-made). The melatonin was separated by ascending chromatography in toluene: acetic acid:ethyl acetate: water (80:40:20:4, by vol.). The melatonin was seen as a bright orange spot under ultra-violet light.

The thin-layer chromatography step can be included in the assay in order to obtain maximal sensitivity. In these experiments it was only used to identify the product of the reaction.

2. Results

The procedure described by Saavedra *et al* (1973) employs HCl only in the homogenization step. This is unusual, as most methods for the measurement of brain amine levels use a protein precipitating agent such as perchloric acid or trichloroacetic acid. Firstly a standard curve was obtained for the procedure exactly as outlined (Figure 41), then the tissue was homogenized in perchloric acid. For the assay to work in perchloric acid the buffer for the N-acetylation stage had to be altered. (10 ml sodium phosphate buffer, pH 7.9 plus 0.6 ml 1M NaOH). Using HCl the pH at this stage in the reaction was found to be 7.6; using perchloric acid and using the modified buffer it was found to be pH 7.0. The standard curve was found to be linear (Figure 42), however the modified method could not be used on tissue samples as the buffer for the N-acetylation stage did not give a constant pH. As the same kind of variation in pH occurred if the samples were homogenized in water before adding the hydrochloric acid to 0.1 M, it seems that the buffer must be inadequate. The reason for wishing to modify the assay was so that all the assays described in previous sections could be carried out on the same brain sample. In order to take this line of investigation further, the first parameter which ought to be tested is the buffer for reaction (i). HEPES would be a more efficient buffer at the pH opt. of N-acetyl transferase.

As it proved to be difficult to combine this assay with the others, more rats from experimental set 4 were killed after 30 days of chronic treatment. After dissection of the brain and freezing in liquid nitrogen, the assay was carried out exactly as that described by Saavedra *et al.* (1973).

The results are shown in Table 11. It can be seen that after 30 days of chronic treatment there is no significant change in the serotonin levels in any of the regions from the three experimental animal groups, except in the amygdala.

Figures 41 and 42 Internal standard curves for serotonin using 0.1 M HCl and 0.1 M Perchloric acid respectively, as the homogenizing medium. The graphs show c.p.m. against the concentration of serotonin in ng.

Figure 41

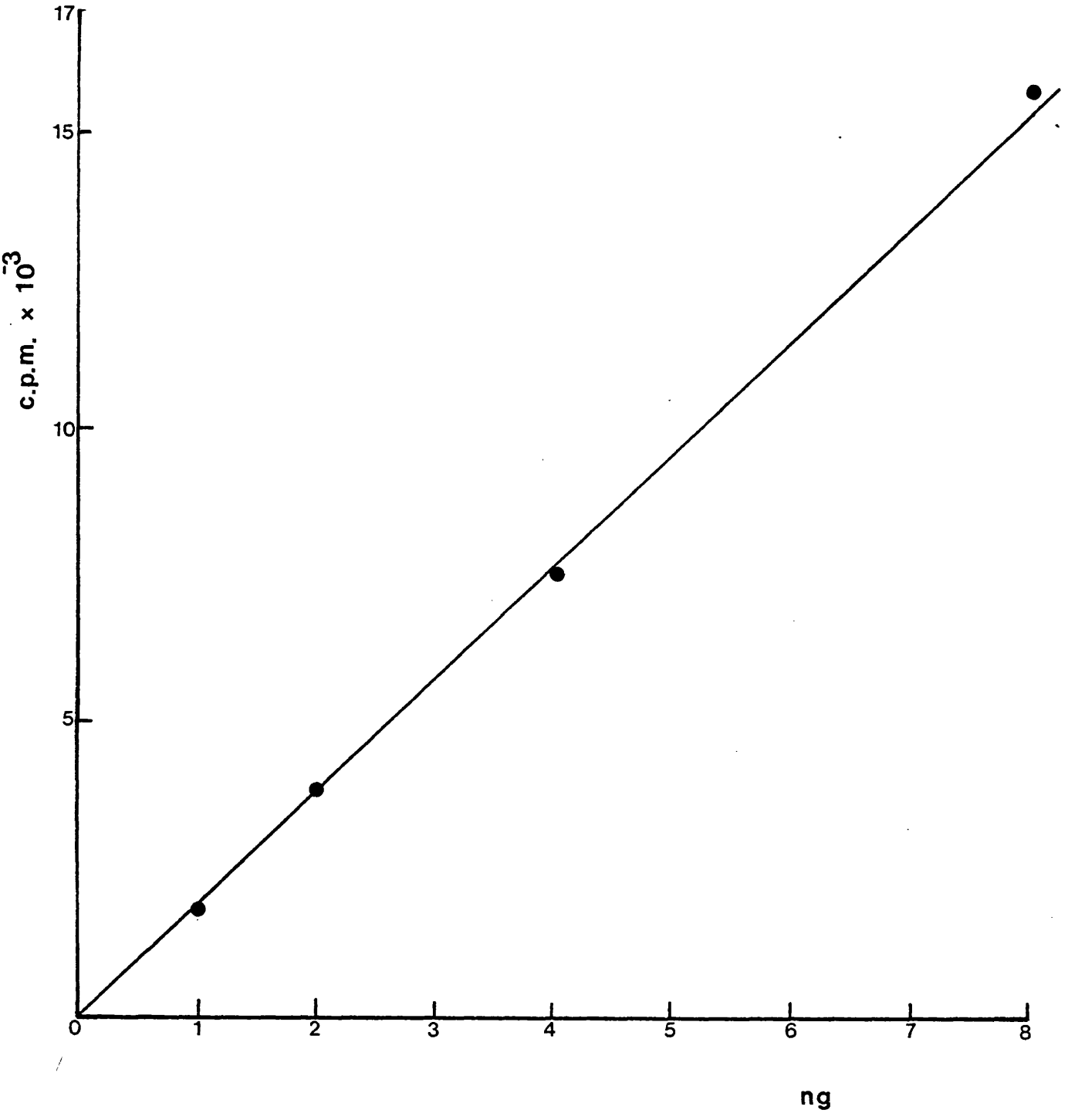


Figure 42

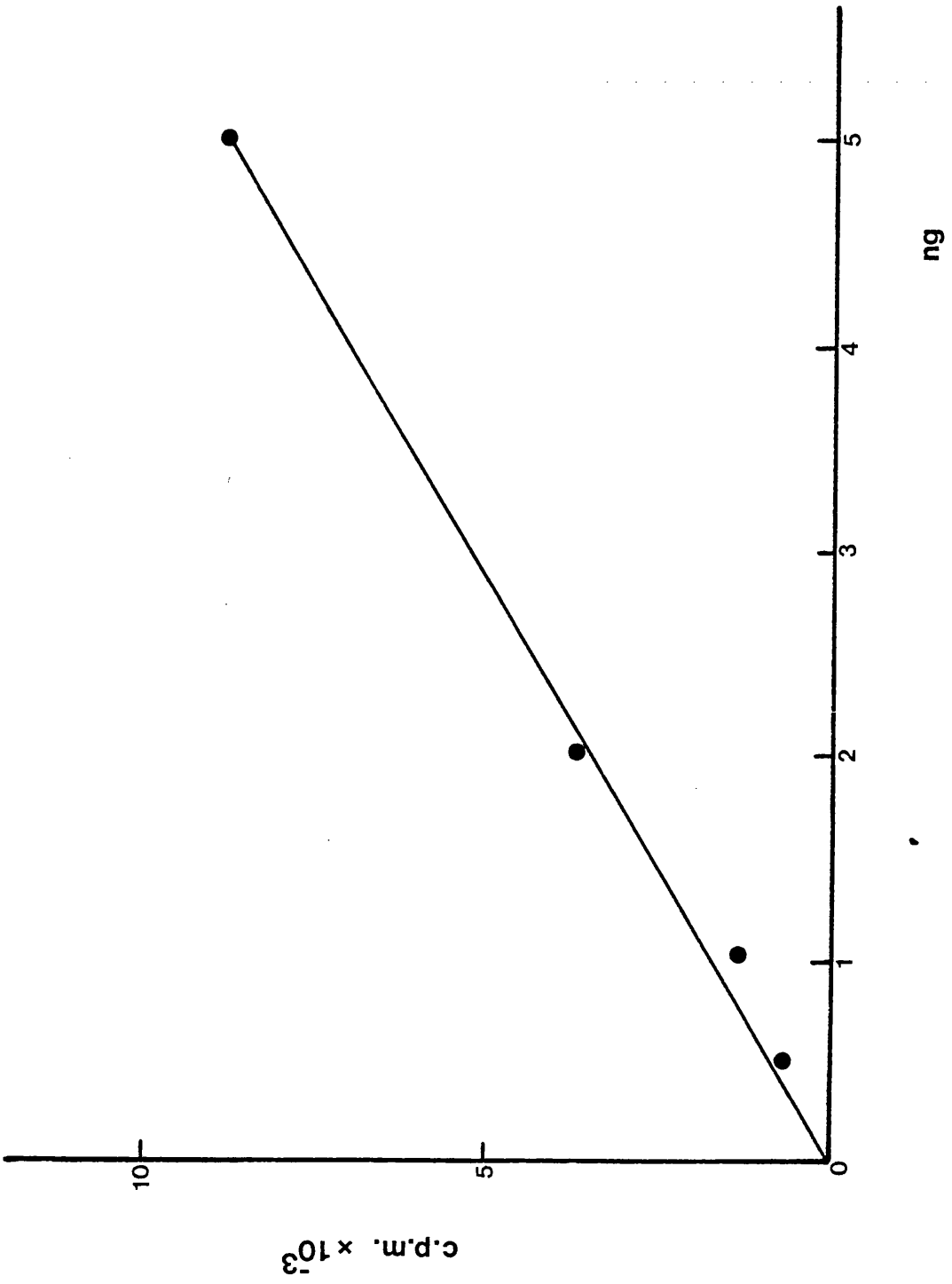


Table 11. Levels of serotonin in the three experimental groups of animals from Set 4. in ng/g wet wt. of tissue

Key:

* indicates the value is significantly different from control.

Region	Control	Chronic	Withdrawn
Cortex	$\bar{x}$ = 268	$\bar{x}$ = 332.5	$\bar{x}$ = 316
	σ_{n-1} = 43	σ_{n-1} = 50.2	σ_{n-1} = 61
	n = 3	n = 2	n = 2
Accumbens	$\bar{x}$ = 316	$\bar{x}$ = 439	$\bar{x}$ = 665
	σ_{n-1} = 99	σ_{n-1} = 134	σ_{n-1} = 238
	n = 3	n = 2	n = 3
Striatum	$\bar{x}$ = 230	$\bar{x}$ = 301	$\bar{x}$ = 408
	σ_{n-1} = 85	σ_{n-1} = 151	σ_{n-1} = 257
	n = 3	n = 2	n = 3
Amygdala	$\bar{x}$ = 411	$\bar{x}$ = 854.5*	$\bar{x}$ = 650
	σ_{n-1} = 112	σ_{n-1} = 139	σ_{n-1} = 228.5
	n = 3	n = 2	n = 3
Hypothalamus	$\bar{x}$ = 504	$\bar{x}$ = 549	$\bar{x}$ = 609
	σ_{n-1} = 93	σ_{n-1} = 12.7	σ_{n-1} = 165
	n = 2	n = 2	n = 2
Pons	$\bar{x}$ = 237	$\bar{x}$ = 273	$\bar{x}$ = 300
	σ_{n-1} = 38	σ_{n-1} = 68	σ_{n-1} = 127
	n = 2	n = 2	n = 2
Medulla	$\bar{x}$ = 238.5	$\bar{x}$ = 436.5	$\bar{x}$ = 338.5
	σ_{n-1} = 19	σ_{n-1} = 78.5	σ_{n-1} = 165
	n = 2	n = 2	n = 2

G. Determination of amino acid levels in brain regions

1. Materials and Methods

The frozen brain regions were weighed and homogenised in 3% sulphosalicylic acid (10 ml/g). The sulphosalicylic acid contained 55.6 nM of norleucine/ml, to give a final concentration of 50 nM/ml. Norleucine is used as an internal standard for the amino acid analyzer. The homogenates were left in ice for about 30 min, then spun at 6,000 rev/min (4335 x g) for 30 min in a Christ microfuge (V.A. Howe Ltd.). The supernatant was filtered through an 8 μ millipore filter , to remove fats, and stored at -20°C.

The animals used in this experiment were killed after 15 and 30 days of treatment. It was necessary to pool the samples; consequently the two time intervals have only two samples of each region per experimental group.

The samples were run on a Chromaspek auto amino acid analyser (Rank-Hilger Ltd., Margate, Kent). This analyser separates the amino acids by ion-exchange chromatography. The pH gradient for elution is continuous and provided by means of an electronic gradient programmer. The composition of the acidic and basic buffers are as follows: the acidic buffer contains anhydrous lithium chloride (0.84% w/v), citric acid (1.05% v/v) and isopropanol (1% v/v), pH 2.2; the basic buffer contains lithium hydroxide (0.84% w/v), boric acid (0.31% w/v) and citric acid (1.05% w/v), pH 12.0. The amino acids are reacted with ninhydrin as they come off the column, heated in an oil bath maintained at 90°C, cooled and a photocell measures the intensity of colour formed in the reaction with each amino acid; this is then recorded.

The Chromaspek which I used was connected to a computer which was set to integrate the areas under the peaks.

The supernatants were run at two dilutions; 1 in 10 and 1 in 100. The first dilution resolved the neutral and basic amino acids, and the second dilution resolved the acid amino acids.

2. Results

The levels of amino acids were calculated using the norleucine internal standard. This was used as the measure of recovery of the amino acids. A standard mixture of amino acids was run every 3 or 4 samples; this provided a standard value for the particular amino acid from which, knowing the recovery, the concentration of each amino acid present could be calculated.

Table 12 shows the levels of amino acids in the pooled regions from the 3 groups of rats after 15 and 30 days of treatment.

Table 12a Regional levels of tyrosine, phenylalanine and tryptophan in various brain regions from experimental set 3 after 15 and 30 days of chronic treatment, as measured by a Chromaspek auto amino acid analyser

Table 12b This shows the changes in the other amino acids which were measured (only a representative selection of all the results is shown here). Asparagine does not appear to be present in brain tissues, or is it present in such low amounts in all brain regions that it is below the sensitivity of the auto amino acid analyzer.

For both tables the levels are in μM, or μmoles/0.1 g wet wt. of tissue. The mean ± the standard deviation is shown where no standard deviation is given, n = 1.

Key: ǂ significantly different from control

O significantly different from chronic

Table 12a

Region	Group	Phenylalanine	Tyrosine	Tryptophan
Hypothalamus	Control 15 days	4.64 ± 1.94	6.8 ± 1.74	trace
	Chronic 15 days	4.32 ± 0.375	5.75 ± 0.32	trace
	Withdrawn 15 days	87.12 ± 14.72*	47.3 ± 5.61*	5.74 ± 0.74
	Control 30 days	5.13	7.94	trace
	Chronic 30 days	4.93	4.65	trace
	Withdrawn 30 days	9.2 ± 0.19	8.87 ± 0.14	trace
Amygdala	Control 15 days	6.8 ± 1.32	10.7 ± 3.54	trace
	Chronic 15 days	-	-	-
	Withdrawn 15 days	64.3 ± 10.75*	42.35 ± 3.9*	5.58 ± 2.93
	Control 30 days	6.18	10.5	-
	Chronic 30 days	4.31 ± 2.42	5.86 ± 2.47	-
	Withdrawn 30 days	21.05 ± 8.13	15.1 ± 1.27	trace

Region	Group	Phenylalanine	Tyrosine	Tryptophan
N. accumbens	Control 15 days	5.94	7.45 ± 3.75	trace
	Chronic 15 days	-	-	-
	Withdrawn 15 days	25.9	16.6	trace
	Control 30 days	6.97	8.99	trace
	Chronic 30 days	4.37	5.22	trace
	Withdrawn 30 days	8.42	7.36	trace
Cortex	Control 15 days	4.82	9.93	trace
	Chronic 15 days	-	-	-
	Withdrawn 15 days	29.9 ± 2.97	27.2 ± 1.98	trace
	Control 30 days	7.62	7.42	trace
	Chronic 30 days	5.12 ± 0.83	8.31 ± 2.58	trace
	Withdrawn 30 days	12.01 ± 2.06	1.35 ± 1.68	trace

Region	Group	Phenylalanine	Tyrosine	Tryptophan
Striatum	Control 15 days	4.4	6.28 ± 0.42	trace
	Chronic 15 days	-	-	-
	Withdrawn 15 days	49.8 ± 2.4	27.6 ± 2.12*	-
Pons	Control 15 days	6.03	7.54 ± 2.11	trace
	Chronic 15 days	-	-	-
	Withdrawn 15 days	12.9 ± 16.97	60 ± 8.49	13.5 ± 6.22
	Control 30 days	5.21	7.18	trace
	Chronic 30 days	6.14 ± 1.43	5.62 ± 0.79	trace
	Withdrawn 30 days	33.7	19.0	trace
Medulla	Control 15 days	-	4.81 ± 0.59	trace
	Chronic 15 days	-	-	-
	Withdrawn 15 days	29.15 ± 26.09	15.99 ± 11.76	4.95

Region	Group	Phenylalanine	Tyrosine	Tryptophan
Medulla	Control 30 days	-	4.05	trace
	Chronic 30 days	3.48	3.9 ± 0.33	trace
	Withdrawn 30 days	11.8 ± 6.22	58.25 ± 3.61	13.85 ± 2.05

Table 12b

	HYPOTHALAMUS			AMYGDALA		
	Control	15 DAYS Chronic	Withdrawn	Control	15 DAYS Chronic	Withdrawn
Asp	387±50.9	308±26	768±432	722±40.3		1326±163*
Thr	60.9±0.95	32.4±13.3	153±11.3*	78.1±6.6		164±9.19*
Ser	73.9±4.65	83.75±15.1	269±16.3*	137±7.78		330±707
Glu	852±5.73	763±70	608±209	1606±139		1225±345
Gln	311	240±21.2	310±0.01	496±7.57	NO	497±2.83
Pro	11.8	trace	72.9±25.6	-		112.5±14.8
Gly	131±0.2	111±8.9	365±24*	124±14.95		388±4.95*
Ala	94.5±0.15	60.8±1.03*	201±10.7*	153±42.4	RESULTS	253±2.83
Cit	-	7.19	34.3 1.44	-		30.±8.4
Val	8.07±3.8	6.42±0.97	70.91±9.45*	14.8±1.13		78.2±13.6*
Cys	-	-	-	-		-
Met	202.8	3.82	53 2.97	9.15±8.98		32.3±2.9*
Ileu	trace	trace	51.2±2.03	3.63±1.02		41.7±5.52*
Leu	6.86±1.29	6.42±0.3	127±6.28	8.88±1.06		99.2±15.3
Gaba	98.6±4.51	673±71.5	528±595*	1018±180		1877±603
Hist	25.4±4.55	19.9±5.18	8.76±0.23*	21.75±1.77		95.8±0.14*
Orn	6.18±2.18	trace	16.8±0.54*	4.29±1.77		18.85±5.66*
Lys	34±3.62	26.4±1.67	108±5.55*	38.65±7		116±5.66*
Arg	15.6±9.14	11±1.79	118±7.48*	25.15±2.19		140±27.58*

	STRIATUM 15 DAYS			PONS 15 DAYS		
	Control	Chronic	Withdrawn	Control	Chronic	Withdrawn
Asp	204±4.24		no result	572±14.85		973±38.89*
Thr	53.6±5.23		no result	70.5±0.71		192±30.55*
Ser	88.9±0.99		200	62.2±3.25		315±27.58*
Glu	687±16.97		657±192	789±141		529±15.56
Gln	309±7.78	NO RESULTS	207±41	309±24.75	NO RESULTS	305±19.8
Pro	-		76.4	trace		85.8±5.37
Gly	80.38±13.54		209±495*	432±9.9		563±86
Ala	104±2.83		132±4.24	157±27.58		223±6.36
Cit	9.98		17±3.82	-		56.15±3.04
Val	6.17±0.81		34.35±1.77*	6.8±2.14		67.9±9.05*
Cys	-		-	-		-
Met	6.84±1.1		28.15±1.91*	16.62±1.45		76,45±8*
Ileu	trace		30±3.82	trace		64.7±6.36
Leu	5.56±0.73		70.5±3.54*	6.89±1.22		158±20.51*
Gaba	524±16.97		570±20.78	399±77.8		373±14.85
Hist				12.85±2.05		131±30.4*
Orn	-		5.43	trace		15.69±10.5
Lys	18±0.85		55.6±6.08*	46.75±10.25		142±33.59
Arg	87.45±61.59		-	30.6±11.31		257±58.69*

	PONS 30 DAYS			MEDULLA 15 DAYS		
	Control	Chronic	Withdrawn	Control	Chronic	Withdrawn
Asp	651	556±49.7	849	576±26.87		495±62.23
Thr	104	68.6±7.78	87.3	54.45±0.78		80.55±27.51
Ser	87	61±3.27	116	49.85±1.63		99.15±55.23
Glu	955	769±124	647	1005±108		554±23.3*
Gln	317	277±28.68	266	217±15.56		184±7.78
Pro	trace		-		NO	
Gly	472	426±25.72	434	376±9.19		441±24.4
Ala	187	145±20.55	172	114±11.31	RESULTS	172±10.61*
Cit	-			5.53±0.35		15.66±8.4
Val	16.5	6.37±1.48		5.72±0.04		20.44±12.3
Cys	-			trace		trace
Met	21.2	15.6±5.1	30.2			16.66±15.75
Ileu	trace	trace	18.1			15.81±13.63
Leu	7.4	6.53±1.08	42.1	5.08±0.6		37.85±20.51
Gaba	525	436±51	397	548±15.56		619±20.51
Hist	15	11.27±1.26	45	9.49±1.15		31.1±23.69
Orn	trace	49.5±6.78	12.3			
Lys	75.9		77.1	28.4±0.56		52.65±19.17
Arg	23.7	18.13±3.69	69.6	8.15±1.25		46.85±36

	MEDULLA		
		30 DAYS	
	Control	Chronic	Withdrawn
Asp	420	321±27.58	791±24.75
Thr	79.7	49.15±6.15	184±8.49
Ser	59.9	45.75±6.58	298±24
Glu	593	548±23.3	602±9.19
Gln	214	152±9.19	229±17.68
Pro	trace	trace	78.5±3.96
Gly	402	355±14.14	583±19.09
Ala	174	119±20.51	231±14.85
Cit			56.25±1.91
Val	7.24	5.01±1.03	78.25±4.88
Cys	trace	trace	
Met			67.1±4.81
Ileu			61.3±3.96
Leu	4.42	5.07±0.93	147±10.61
Gaba	474	511±36.77	594±36.77
Hist	8.75	8.67±0.46	98.2±2.62
Orn	trace	trace	13.8±0.85
Lys	36.9	32.02±4.07	123±8.49
Arg	27.1	9.49±5.61	213±53.74

H. Determination of total and free tryptophan levels, phenylalanine and tyrosine levels in plasma

1. Materials and Methods

Blood was collected, on decapitating the rats, in calcium-heparin (2 I.U./ml) tubes. This was centrifuged immediately (3,000 x g for 10 min at 4°C) and the plasma transferred immediately into plain plastic tubes. The plasma was then frozen in liquid nitrogen.

(i) Total and free plasma tryptophan

Equilibrium dialysis of plasma samples was carried out as described by Wood *et al.* (1977) with the dialysis carried out at room temperature; equilibrium was attained after 4 h, when the samples were extracted. The plasma total and free tryptophan was measured fluorometrically by a modification of the method described by Eccleston (1973). The method was modified in the following ways: 4 ml of 10% (w/v) trichloroacetic acid, 0.5 ml of 1.8% (w/v) formaldehyde and a final strength of 1.5×10^{-4} M $FeCl_3$ were used. The samples were centrifuged at 3,000 x g for 10 min at 20°C and the ferric chloride solution was added to the samples immediately prior to incubation in a boiling water bath. The samples were put in to the incubation bath within 15 min of being centrifuged (Wood *et al.*, 1977).

(i) Plasma phenylalanine

Plasma phenylalanine levels were measured using a method based on that of McCaman & Robbins (1962) in which the phenylalanine is measured by virtue of the fluorescence obtained when phenylalanine is reacted with ninhydrin in the presence of a sensitizing peptide

(L-leucyl-L-alanine). The success of the method depends on the strict control of pH (5.8 ± 0.1) by means of a succinate buffer; this optimizes fluorescence and maximises specificity. The method is described in Sigma Tech. Bulletin No. 60F/70F (7-74): the reagents were obtained from Sigma (London) Chemical Company Ltd. in the form of a 'kit'.

(iii) Plasma tyrosine

Plasma tyrosine was measured by the method of Wong *et al.* (1964). The basis of the assay is the formation of a fluorescent derivative formed by the reaction of tyrosine with 1-nitroso-2-napthol after the mixture is heated in the presence of nitric acid. The reagents were provided in a kit form by Sigma (London) Chemical Company Ltd.

Plasma 'free' phenylalanine and tyrosine were also determined by equilibrium dialysis.

2. Results

It has been shown that the freezing of plasma samples does not affect the total and free tryptophan levels in plasma (K. Wood, personal communication). The measurements were carried out on animals which had been chronically treated with methamphetamine for 30 days and the results are shown in Tables 13 and 14. It can be seen that there is no change in total tryptophan between the groups. The % free tryptophan is significantly lower in the chronic group compared with the controls. The free tryptophan levels are significantly lower in the chronic group compared with the controls and the withdrawn animals. There is no significant difference between the groups in bound tryptophan levels.

The phenylalanine and tyrosine results show no significant differences between the groups.

Tables 13 and 14 Table 13 shows the total, free and bound levels of tryptophan in the plasma (in µg/ml) from rats from experimental set 4 after 30 days of chronic treatment.

Table 14 shows the levels of phenylalanine and tyrosine (in µg/ml) in plasma from the same rats. The % of free amino acid is also given.

Key: $\bar{x}$ is the mean
σ_{n-1} is the standard deviation
n is the number

The figures were analysed statistically using Satterthwaite's approximation and the "student's" t test.

* indicates a significant difference from the control group

o indicates a significant difference from the chronic group

Table 13

Group	Total Tryptophan	% free Tryptophan	free Tryptophan	bound Tryptophan
Control				
mean ($\bar{x}$)	22.3	20.6	4.53	17.7
σ_{n-1}	2.38	9.50	1.9	3.16
n	7	7	7	7
Chronic				
mean ($\bar{x}$)	19.8	11.4*	2.14	17.7
σ_{n-1}	4.82	3.18	0.29	4.76
n	6	6	6	6
Withdrawn				
mean ($\bar{x}$)	21.5	13.9	2.99°	18.5
σ_{n-1}	1.30	1.20	0.24	1.23
n	6	6	6	6

Table 14

Group	Free Phenylalanine	% free Phenylalanine	free Tyrosine
Control			
mean ($\bar{x}$)	15.2	98.3	20.24
σ_{n-1}	1.13	0.82	1.39
n	7	5	7
Chronic			
mean ($\bar{x}$)	15.7	95.4	19.86
σ_{n-1}	1.11	-	2.00
n	4	1	4
Withdrawn			
mean ($\bar{x}$)	15.0	98.7	19.11
σ_{n-1}	1.62	1.29	1.14
n	6	4	6

In all samples the tyrosine was completely free

DISCUSSION

A. Catecholamine Metabolism

1. Levels of phenylalanine and tyrosine in the plasma and in the brain

The levels of phenylalanine and tyrosine in the plasma were unchanged in all three experimental groups. The binding of other amino acids, apart from tryptophan, to serum proteins is not usually considered as playing any regulatory role: it was interesting that about 10% of the phenylalanine is bound to serum proteins although this did not change in any of the experimental groups. That the levels of these amino acids are unchanged on chronic treatment with, or withdrawal from, methamphetamine is a good indication that their nutritional status is the same in all the groups. Consequently any change observed in the levels of amino acids in the brain is unlikely to be due to a nutritional deficit caused by the anorexic effects of methamphetamine which is subsequently compensated for on withdrawal.

The levels of phenylalanine and tyrosine in the various regions of the brain did not appear to change on chronic treatment with methamphetamine but increased dramatically on withdrawal in the regions examined.

2. Tyrosine hydroxylase activity

The results obtained for the activity of tyrosine hydroxylase show, in the case of experimental set 1, a decrease in both the 15 and 30 day animals after chronic treatment followed by a complete or partial recovery on withdrawal. After 30 days the decrease in activity on chronic treatment was so great that it was below the sensitivity of the assay. However, experimental set 4 did not show any significant change in tyrosine hydroxylase activity in any of the experimental groups. P. Vaughn (personal communication) has found, with studies on synaptosomes, that the effect of amphetamine on tyrosine hydroxylase activity *in vitro* depends on the control levels. If these are high then amphetamine tends to decrease the activity and *vice versa*. The table of control levels of tyrosine hydroxylase in the various experimental groups (Table 4) does not support this fact for my *in vivo* studies.

The graphs of the drinking rates, weights and food consumption (Figures 12 - 20) for the four experimental sets show that the chronically-treated animals from experimental set 1 differed from the other three experimental sets in not developing tolerance to the anorexic effects of methamphetamine and they drank significantly less water. Subjective observations indicated that they were under considerably greater stress than the other sets. Stress had already been shown to correlate with alterations in catecholamine metabolism (Ishii, 1975; Curzon, 1978) and to increase the effect of methamphetamine on amine depletion (Beauvallet *et al.*, 1962b, 1967; Moore & Lariviere, 1963, 1964; Salama & Goldberg, 1969). Stress also releases amphetamine which was stored in rats chronically treated with amphetamine (S. Sparber, personal communication). Consequently the measurements from the first experimental set will be considered separately.

Ellison *et al.* (1978), using pellet implantation to treat rats chronically with (+)-amphetamine found, after sectioning the brains and visualizing the amines with formaldehyde vapour, that there was a reduced fluorescence in the forebrain after 2 days and "diffusion around cortical axons". After 5 days, they found that the axons in the caudate nucleus had become swollen, which is very similar in appearance to the caudate after lesions have been made by partial aspiration (Andén *et al.*, 1965). The implication is that continous amphetamine treatment damages the dopaminergic terminals in the caudate. The swollen axons are thought to reflect an accumulation of amines in the remaining axons. On withdrawing the rats from amphetamine for 5 to 33 days, a gradual and partial reappearance of the background fluoresence and finer axons was usually seen, but bright and extremely swollen axons were still present in some regions. Ellison *et al.* (1978) measured tyrosine hydroxylase activity in several brain regions and found that in the caudate the activity had dropped to 50% of control levels and that it had not returned to control (74%) 110 days after withdrawal, although the activity was elevated in some regions. This supports the results from the fluorescence studies in that amphetamine, administered by pellet implantation, causes selective alterations in nigrostriatal dopaminergic fibres. In order to show that chronic amphetamine administration by pellet implantation is not the same as i.p. injections and is a better model for amphetamine use in man, where chronic users develop patterns where, for several days they are in a permanent state of intoxication, Ellison *et al.* (1978) carried out a similar study to that already described except that they administered the same dose (3.2 mg/kg/24h) i.p.

once a day for 30 days and found that the activity of tyrosine hydroxylase did not decrease.

Thus, one possible theory which would explain the disparate results obtained for tyrosine hydroxylase in my experiments is that the first experimental set were in a permanent state of intoxication with the drug. I administered the drug by 2 i.p. injections per day in order to make my model resemble the human state more closely. The animals in experimental set 1 were stressed, therefore it seems possible that the amount of circulating drug which would otherwise be stored in adipose tissue was continually being mobilized. It is also possible that these animals may have been more sensitive to the effects of amphetamine. It has been observed, using operant conditioning, that individual rats can behave completely differently to the same dose of amphetamine (L.H. Fossum, personal communication).

3. Levels of catecholamines and their metabolites in various brain regions

In experimental set 1 only noradrenaline and dopamine levels were measured in the cortex, nucleus accumbens, amygdala and hypothalamus. In all regions the levels of noradrenaline and dopamine tended to decrease on chronic treatment; this decrease reached significance inthe noradrenaline levels in the cortex, dopamine levels in the nucleus accumbens and noradrenaline and dopamine levels in the hypothalamus. On withdrawal the levels of noradrenaline and dopamine in the cortex in the amygdala were significantly decreased. The levels of noradrenaline and dopamine tended to recover in the nucleus accumbens and the hypothalamus but only the dopamine levels in the hypothalamus were significantly above the chronic levels.

The modified radioenzymatic assay method which allows noradrenaline, dopamine and their non-O-methylated metabolites to be measured simultaneously was used for experiment set 4. No significant change in any of the amines or their metabolites was found. In order to get an idea of the changes which had occurred in the metabolites in the samples from experimental set 1, I examined a chronic and a control sample from the hypothalamus from this set of animals after 30 days of chronic treatment. The decrease in noradrenaline, dopamine and their non-O-methylated metabolites in the chronic animals is shown below, as a % of control.

Noradrenaline:	23%
3,4-dihydroxymandelic acid:	no change
3,4-dihydroxyphenylglycol:	78%
Dopamine:	no change
3,4-dihydroxyphenylacetic acid:	47%
3,4-dihydroxyphenethanol:	63%

Tentatively it would appear that, in the hypothalamus, noradrenaline was affected more than dopamine but the metabolites of dopamine were affected more than the noradrenaline metabolites. This is the reverse of what would be predicted from the MAO inhibition studies (Mantle *et al.*, 1976). However if MAO inhibition is physiologically significant, an explanation for this apparent discrepancy could be that the concentration of dopamine in the hypothalamus is not significantly different from the control values whereas noradrenaline is only 23% of control values. The hypothalamus contains a higher endogenous level of noradrenaline, but if we are only dealing with intraneuronal deamination this is not an important consideration. Consequently any intraneuronal inhibition of MAO will affect dopamine more than noradrenaline.

The results of the amine levels in the experiment set 1 and experimental set 4 agree with the theory put forward in section 2 above to explain the different results obtained for tyrosine hydroxylase. Ellison *et al.* (1978) found decreased levels of dopamine in the striatum on chronic treatment by pellet implantation and decreased levels of noradrenaline in the cortex, hypothalamus and brain stem. There were increased levels of dopamine in the

brainstem on chronic treatment. On withdrawal, the levels returned to control.

It is worth mentioning here that although I do not consider the results from Section III to be reliable due to the problem of the internal standards noted previously, rats with electrolytic lesions in the medial Raphé nuclei have been found to engage in the same "mud-walking" (C. Pycock, personal communication) which I saw in the animals described in Section III. The Raphé nuclei are serotonergic therefore these lesions would be expected to decrease the levels of serotonin in the brain, and this was the main change which I found in my animals.

4. Conclusions

Experimental set 1, owing to a high level of stress, resembled the model of chronic amphetamine treatment which is produced by pellet implantation, i.e. the animals may be in a permanent state of intoxication induced by the drug. The extent of the decrease of tyrosine hydroxylase increases with the duration of treatment which is in keeping with the hypothesis that structural damage may be occurring. In my studies the enzyme activity returned to control values in the cortex, striatum, nucleus accumbens and amygdala, but was still significantly lower than control in the tuberculum olfactorium, hypothalamus, pons and medulla after 30 days of treatment, and only returned to control levels in the striatum and hypothalamus after 15 days of treatment.

The levels of noradrenaline tended to return to control values in only two of the regions studied (nucleus accumbens and hypothalamus). The noradrenaline and dopamine levels in the cortex and amygdala were significantly decreased on withdrawal.

These findings can be explained in two ways: (1) assuming structural damage to dopaminergic neurons (ii) assuming that the only changes are metabolic.

Damage to the neurons by lesioning causes a loss in tyrosine hydroxylase activity due to damage of cell bodies (Creese & Iversen, 1975) and a supersensitivity of the receptors (Ungerstedt, 1971a) to that amine; therefore as the levels of the amine return to control levels on withdrawal, this supersensitivity may persist, resulting in a feedback

inhibition of tyrosine hydroxylase. On withdrawal the situation starts to return to normal.

The actual situation is probably a combination of (i) and (ii). To shed more light on the situation I would have to study withdrawal over a longer period of time.

In experimental set 4 amine metabolism appears to be unaffected by the treatment, in keeping with the hypothesis that in experimental sets 2 and 3 the stress was low and the animals did not seem to be permanently intoxicated with the drug. This agrees with my subjective observations. Ellison *et al.* (1978) has shown that permanent and intermittent intoxication with amphetamine during a period of chronic treatment are qualitatively(and quantitatively)different. However in experimental sets 2 to 4 biochemical changes are occurring in the amino acid levels in the brain. Phenylalanine and tyrosine increased up to 18 fold in some brain areas on withdrawal and this effect is not due to nutritional factors as shown by the constant levels of these amino acids in the blood. Tolerance to the drug appears to have developed and, whatever adaptive metabolic process has occurred, perhaps at the transport stage is disturbed by the withdrawal of the drug, resulting in the increase of brain phenylalanine and tyrosine. Other possible explanations for this increase will be discussed in the section on amino acids.

B. Serotonin Metabolism

1. Levels of tryptophan in the plasma and in the brain

The total level of tryptophan in the plasma was unchanged in all three experimental groups. The percentage of free tryptophan and the concentration of free tryptophan is significantly lower in the chronic animals (Satterthwaite's approximation for % free tryptophan $f = 7$, $d = 2.408$, $p < 0.05$. For the concentration of free tryptophan $f = 6$, $d = 3.271$, $p < 0.02$). The concentration of free tryptophan in the withdrawn group is significantly higher than the chronic group ($p < 0.001$). As free tryptophan decreases with stress (Curzon, 1978) it is difficult to know whether this finding is a concomitant of stress or an effect of chronic treatment with methamphetamine. As I have mentioned earlier, using injected controls does not control for the stress induced in rats chronically treated with methamphetamine by i.p. injection.

The levels of tryptophan found in the brain did not change significantly in any of the animals from experimental set 4. Experimental set 1 did show significant changes; in this set of animals a decrease of tryptophan was found in the striatum, nucleus accumbens and amygdala in the chronic group. The overall tendency appeared to be a decrease of tryptophan in the nerve-terminal containing regions (striatum, nucleus accumbens and amygdala), and an increase in the cell body containing regions.

Measurement of tryptophan on the Chromaspek autoamino acid analyser is unreliable (M. Grant, personal communication). In a few cases (15 day hypothalamus, 15 day amygdala, 15 day pons and 15 and

30 day medulla) the levels of tryptophan appeared to increase on withdrawal. In summary it is probably better to consider only those results obtained using fluorometry and bear in mind that changes in the levels of tryptophan probably are occurring, but the assay is not sufficiently sensitive to detect them.

It is important to question the assumption that the free tryptophan in the plasma regulates the concentrations of tryptophan in the brain and that the transport of tryptophan into the brain is the rate limiting step in the synthesis of serotonin Brain serotonin concentration and metabolism have been shown to be affected by the uptake of tryptophan into the brain (Green *et al.*, 1962; Moir & Eccleston, 1968; Grahame-Smith, 1971; Fernstrom & Wurtman, 1971; Yuwiler, 1973; Gal & Drews, 1962). This is explained as reflecting the relatively high Km of tryptophan hydroxylase which approximates to the normal brain tryptophan concentration. This is thought to be the rate-limiting enzyme for the synthesis of serotonin in the brain (Fernstrom & Wurtman, 1971; McKeen *et al.*, 1968; Eccleston *et al.*, 1970). Thus basal serotonin synthesis is thought to be both substrate and enzyme dependent with a basal rate approximating $\frac{1}{2}\underline{V}$. Since the tryptophan in the brain is taken up from the blood, research has tended to concentrate on the "free" tryptophan in the blood as being an indicator of brain levels and hence the rate of serotonin synthesis. This is based on the assumption that the Kd for tryptophan bound to the serum proteins is sufficiently low that most of the bound tryptophan is not going to dissociate as the tryptophan is being transported into the brain.

The existence of a saturable aromatic amino acid carrier system has been well documented (Lajtha & Toth, 1961; Neame, 1964; Tsukada *et al.*, 1963). Oldendorf (1971) has shown that most neutral amines can interfere with tryptophan transport to the brain. Perez-Cruet *et al.* (1972) speculated that this was of physiological importance in regulating brain tryptophan concentration, which was confirmed by Fernstrom & Wurtman (1972).

McMenemy & Oncley (1958) reported that tryptophan exists in the free form and bound to albumin in the blood. The binding is pH-sensitive and tryptophan can be displaced from albumin binding sites by agents such as free fatty acids (Curzon *et al.*, 1973). Both total blood tryptophan and free tryptophan have been correlated with brain serotonin changes following various treatments (Tagliamonte *et al.*, 1973; Curzon & Knott, 1973; Fernstrom & Wurtman, 1971).

As tryptophan binding to albumin is a pH-dependent equilibrium, it seems likely to be influenced by the pH difference between venous and arterial blood. Also the microcapillary bed has a small diffusion pathway, therefore the rate of uptake in the microcapillary bed of the brain might not be reflected in the bulk equilibrium conditions and between free and bound tryptophan in the blood.

Yuwiler *et al.* (1977) showed by experiments which manipulated the levels of free and bound tryptophan that the bound tryptophan contributes to the tryptophan taken up by the brain. They also found that other amino acids may strongly influence the uptake of tryptophan into the brain. They argue that a considerable portion

of the bound tryptophan is dissociated from the albumin during its passage through the capillary bed in the brain. They calculated the "effective" tryptophan concentration in albumin-tryptophan mixtures from the measured uptake into the brain and found that the "effective" tryptophan concentration greatly exceeds the measured free tryptophan.

Yuwiler *et al.*, (1977) also calculated that, at normal blood concentrations, other neutral amino acids inhibit tryptophan uptake in the order leucine > tyrosine > threonine > phenylalanine = histidine > isoleucine = methionine > valine. This is of importance when considering the large increases in phenylalanine and tyrosine in the brain on withdrawal, and with reference to the other amino acid levels in the brain.

The assumption that the sole role of tryptophan in increasing serotonin formation is as a substrate is questionable. Firstly the effective half-saturation constant for tryptophan transport into neurons is likely to be considerably greater than 60 μM since it has been shown that several amino acids interfere with tryptophan transport at such concentrations (Grahame-Smith & Parfitt, 1970). This would tend to linearize the relationship between blood tryptophan and the rate of tryptophan hydroxylation. Secondly, the concentration of tryptophan at the neuronal uptake site and the hydroxylation site may not reflect the mean concentration of this amino acid in brain. If these concentrations were higher than the mean, the effectiveness of changes in available blood tryptophan would be minimized whereas, if lower, the effectiveness would be

magnified. Finally tryptophan may do more in influencing the rate of 5-hydroxytryptophan synthesis than merely act as a substrate. By analogy with the tryptophan oxygenase system, tryptophan may help stabilize the hydroxylase, may influence co-factor binding, or may help maintain the integrity and function of the pteridine reductase system (Etienne *et al.*, 1976; Yuwiler *et al.*, 1977).

Peripheral considerations

Peripheral administration of tryptophan is a 'stressor', as evidenced by elevated adrenal corticoids and some 'stressors' lead to elevations in brain serotonin (Barchas & Freedman, 1963; Thierry *et al.*, 1963), but not all (Yuwiler, 1971) 'stressors' result in an elevation of brain serotonin, presumably as a complex function of hormonally-induced enzyme changes, alterations in central nervous system activity and even such factors as stress-induced changes in feeding behaviour. Thus adrenal corticoids are reported to elevate enzymic activity of mid brain tryptophan hydroxylase in adrenalectomized animals (Azmitia & McEwen, 1969) and of aromatic amino acid decarboxylase (Davis, 1963), hepatic tryptophan pyrrolase (E.C. 1.11.1.4) and tryptophan amino transferase (Knox, 1963). in intact animals. The first two could facilitate conversion of tryptophan to serotonin and the latter could redirect tryptophan to alternative metabolic pathways.

The amount of free tryptophan in the blood is frequently correlated with the levels of brain tryptophan and brain serotonin. The explanations put forward however have to be made with all the afore-

mentioned factors in mind, especially when considering, as I am, the chronic administration of a drug which has central and peripheral actions by intra-peritoneal injection.

2. Brain serotonin

There were no significant changes in the levels of serotonin in any of the regions from experimental set 4, except in the amygdala where the levels were significantly increased.

In summary the significantly lower levels of free tryptophan in the chronic and withdrawn animals do not correlate with the levels of brain tryptophan or serotonin. Brain tryptophan and serotonin levels either do not change, or decrease in the nerve-terminal containing regions and increase in the cell body containing regions. The low free tryptophan is not related to a nutritional deficit as the total plasma levels of tryptophan remain unchanged.

C. Amino Acid Uptake

There was no clear pattern in the groups of amino acids which were increased in the brain on withdrawal. Most of the amino acid concentrations increased with the exception of GABA which was not considered as the levels of this amino acid are known to increase dramatically *post-mortem* owing to the high activity of glutamic acid decarboxylase. Consequently it was not possible to interpret the data in terms of known carriers for groups of amino acids (e.g. neutral, basic, acidic, aromatics; see Oldendorf & Partridge, 1977).

The generalized changes in amino acid levels suggest that there may be a change in some metabolic pathway which is common and essential to the transport of all amino acids e.g., the γ-glutamyl cycle (Meister, 1973) which has been suggested to be involved. Another possibility is a general effect on the subsequent metabolism of these amino acids e.g. the availability of pyridoxal phosphate, a cofactor which is essential for many of the enzyme catalysed reactions in amino acid metabolism, or changes in the activity of the transaminases in the brain.

To investigate these metabolic parameters was not the aim of this work therefore the possible explanations for the increased concentrations of amino acids in the brain seen on withdrawal will not be considered in any more detail.

D. Radioenzymatic Assays

The catecholamines and indoleamines are usually measured fluorometrically, radioenzymatically, by gas-liquid chromatography or using high-pressure-liquid chromatography. Of these methods only the last three were sufficiently sensitive for my needs and only the radioenzymatic assays were practical under my circumstances.

There are a number of radioenzymatic assays which have been published for the measurement of the catecholamines and indoleamines, all of which are based on the conversion of the amine to its O-methylated derivative in the presence of catechol-O-methyl transferase (COMT; E.C. 2.1.1.6) with $[^{14}C]$- or $[^{3}H]$-methyl-S-adenosyl methionine as the methyl donor.

For the measurement of noradrenaline and dopamine, I used the method of Cuello *et al.* (1973). More sensitive assays for noradrenaline and dopamine have been reported by Palkovits *et al.*, (1974) and Gauchy *et al.* (1976) and modifications by Chiueh & Kopin (1978) and Martin *et al.* (1978).

Assays which measure noradrenaline, dopamine and adrenaline simultaneously have been reported (Da Prada & Zürcher, 1976; Ben-Jonathan & Porter, 1976; Peuler & Johnson, 1977). Of the improvements and modifications reported, only two stand out. These are: the use of sodium tetraphenylborate as a complexing agent for the extraction of the O-methylated amines and the use of thin-layer chromatography instead of paper chromatography.

Hefti & Lichtensteiger (1976) reported a method for the measure-

ment of DOPA, and Argiolas *et al.* (1977) and Kebabien *et al.* have published methods for the measurement of 3,4-dihydroxyphenylacetic acid.

It has been reported (Gauchy *et al.*, 1976) that Ca^{2+} salts inhibit the reaction and refuted by Chiueh & Kopin (1978), therefore the alumina step employed by Gauchy *et al.* (1976) is probably unnecessary. In all these publications on the radioenzymatic measurement of catecholamines no one has looked for a thin-layer chromatographic procedure which is capable of measuring DOPA, noradrenaline, dopamine and their non-O-methylated metabolites simultaneously. The method which I have described in Section IV is potentially capable of measuring all these compounds simultaneously.

However I believe that there is still more work to be done on this assay. The use of sodium tetraphenylborate to increase the sensitivity could be examined; the pH opt. of COMT isolated from rat brain is 7.2 - 7.4 (P. Gulliver & K. F. Tipton, personal communication) but in all the methods reported a Tris buffer was used and the pH varied from 8.6 to 10.4. I used a Tris buffer, pH 9.0, but I did find that the pH of the incubation mixture was not constant (it varied between 7.8 and 8.2). I feel that it would be advisable to try using a phosphate buffer, pH 7.2 to 7.4.

The standard curves which I obtained for noradrenaline and dopamine using the method of Cuello *et al.* (1973) and my thin-layer chromatographic method were not linear. In general I found it to be linear below 1 ng and above 1 ng (see figures 28 and 30 to 36).

The gradient of the line was lower above 1 ng suggestive of inhibition of the enzyme or an insufficient enzyme concentration or S-adenosyl methionine. The most likely of these possibilities is inhibition of the enzyme by S-adenosyl homocysteine formed by the action of COMT. This can be removed by the use of adenosine deaminase, purified from Takadiastase (Kaplan, 1955), which converts S-adenosyl homocysteine to S-inosyl methionine. Adenosine deaminase from Takadiastase has the advantages of having a broad specificity, broad pH optimum and being stable (Kaplan, 1955).

The sensitivity of my method for measuring noradrenaline, dopamine and their non-O-methylated metabolites is in the pg range. The reproducibility of the assay determined from the standard deviations of the mean c.p.m. obtained for each concentration of amine was not very reproducible. The amine levels were calculated from the standard curve which was run with every assay (Figures 30 - 36 show the standard curves obtained from one assay).

The radioenzymatic assay which I used to determine the levels of serotonin (Saavedra, 1974) was inadequately buffered at the first reaction step i.e. the N-acetylation of serotonin. Again I think alternative buffers ought to be tried (the pHopt for serotonin quoted by Weissbach (1961) is 7.5 - 8.5) e.g. N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES) or triethanolamine hydrochloride-NaOH.

It has been proposed that a sulphomucopolysaccharide may be involved in the binding of catecholamines inside vesicles (Unväs,

1973). It is reasonable to assume that the amines are in a bound form in the nerve terminal. Some work has been done on the hypothesis that a sulphomucopolysaccharde is involved in the binding of catecholamines in the adrenal medulla. However little work has been done on the brain and that which has been done has been on whole brain. Pycock *et al.* (1975) reported a study in which adult rats which had treated at birth with 6-hydroxydopamine were injected with ^{35}S-labelled sodium sulphate. Synaptosomes were prepared from various regions and it was found that the ^{35}S content of each synaptosomal preparation correlated with the decreases in catecholamine content brought about by 6-hydroxydopamine treatment. Sucrose gradient centrifugation studies showed that ^{3}H-noradrenaline labelled synaptosomes showed similar equilibrium characteristics as the distribution of ^{35}S on the gradient. Their results showed that the main sulphomucopolysaccharides occurring in the synaptosome pellet were chondroitin sulphate and heparan sulphate.

The results of the study by Pycock *et al.* (1975) suggest to me the possibility of binding of the amines to chondroitin sulphate or heparan sulphate in synaptic vesicles. Although this is purely hypothetical the question which would follow this is what fraction or 'pool' of amines is being measured by the various assay techniques available? This is, I believe an important question which ought to be investigated.

E. Behavioural Correlates

As the concepts of drug addiction, tolerance and withdrawal are all behaviourally defined, I am going to describe a behavioural study on rats which were on a similar drug regime for a similar period of time and in which withdrawal was studied. This will be prefaced by a summary of the background to behavioural studies of this sort. For reviews on the behavioural analysis of amphetamine intoxication and behavioural theories on the effects of amphetamine see Lyon & Robbins (1975) and Ellinwood *et al.* (1972).

Stereotypy and the approaches used to determine a biochemical mechanism

Behavioural studies have indicated that treatment with amphetamine produces a sensitization to its central stimulant effects, i.e. the dose-response curve for stereotypy moves to the left (Hitzemann *et al.*, 1973). Stereotypy has been shown to be mediated by dopaminergic mechanisms. Stereotyped behaviour can be seen in most species of animals if they are given a sufficient dose of amphetamine. The topography of the stereotyped behaviour patterns differs between species and it should be emphasized that the responses characterised by stereotyped behaviour are not outside the animals' general repertoire, but are observed in caged animals under a variety of conditions, although they ordinarily occur at a much lower rate. They are classified as stereotyped when their rate increases and therefore the rate of other types of behaviour decreases (e.g. eating and drinking). In rats stereotyped behaviour is characterized by repetitive licking and sniffing, periodically interrupted by gnawing on the cage floor or the rats own forelegs (Randrup & Munkvad, 1968; Scheel-Krüger, 1971). The rat often assumes a characteristic crouched posture against the side of the cage. The frequencies of another behaviours such as eating, drinking, grooming

and forward locomotion are greatly diminished.

Psychomotor stimulants which elicit stereotypic behaviour fall into two groups (Randrup & MunkVad, 1970; Scheel-Krüger, 1972). In the first group the occurrence of stereotypic behaviour can be blocked by α-methyl-p-tyrosine but not by reserpine. This group includes amphetamine, methamphetamine and phenmetrazine. In the second group the occurrence of stereotypic behaviour can be blocked by reserpine but not α-methyl-p-tyrosine; this group includes pipradol and methyl phenidate. It was therefore suggested that the former group released catecholamines from the newly synthesized pool, while the latter group releases catecholamines from storage sites depleted by reserpine.

The conclusion that the activation of dopamine receptors in the corpus striatum appears to be responsible for amphetamine induced stereotyped behaviour is based on three experimental approaches: (i) Drug-drug interactions; (ii) neurotoxin-drug interactions; (iii) lesion-drug interactions. (These approaches epitomize the way in which biochemical theories of types of behaviour are constructed. The global nature of this kind of investigation leads to 'simplistic' biochemical explanations, i.e. the words used are the same but the concepts are not).

(i) Drug-drug interactions:

Many of these types of study were cited in the Introduction, here they are being looked at from a different viewpoint.

It was noted that α-methyl-p-tyrosine blocks amphetamine-induced sterotypy, while reserpine does not. From this finding it was suggested that amphetamine-induced stereotypy is dependent on the newly synthesized pool of catecholamines. L-DOPA can antagonize the blockade of amphetamine-induced sterotypy produced by α-methyl-p-tyrosine, suggesting that dopamine plays an important role in stereotypic behaviour. Administration of a dopamine-β-hydroxylase inhibitor does not antagonize the effects of L-DOPA, suggesting that noradrenaline is not required for amphetamine-induced stereotyped behaviour. That noradrenaline does not play a role in stereotypic behaviour is further supported by experimental data showing that neither α- nor β-adrenergic blocking agents have an effect on stereotypy. On the other hand, either i.p. administration of large doses of L-DOPA or direct injection of dopamine into the caudate nucleus elicits stereotypy. The importance of dopamine in stereotyped behaviour is also supported by evidence which indicates that dopamine acts at post-synaptic sites to produce stereotypic behaviour. The methylated metabolite of dopamine has been shown to increase after an effective dose of amphetamine in both reserpine-pretreated and normal animals (Randrup & Munkvad, 1970).

(ii) Neurotoxin-drug interactions:

Creese & Iversen (1973) used the neurotoxin, 6-hydroxydopamine, in a series of experiments designed to examine the importance of the nigrostriatal dopaminergic system in the mediation of amphetamine-induced stereotypy in rats. 6-

Hydroxydopamine is presumed to destroy presynaptic catecholaminergic terminals. Rats were treated with 6-hydroxydopamine intraventricularly at 5, 7 and 9 days of age. Control rats were injected in the same manner, but with the 6-hydroxydopamine vehicle. Although special feeding procedures were necessary after weaning, the 6-hydroxydopamine rats were at normal body weight with a normal diet when tested. When control rats were given 5 mg/kg of (+)-amphetamine, stereotypic behaviour occurred throughout the 2 h experimental session, whereas when the 6-hydroxydopamine treated rats were given the same dose of (+)-amphetamine, stereotypy did not occur. Similarly, a 10 mg/kg dose of (+)-amphetamine elicited stereotypy in controls, but very little, if any, in 6-hydroxydopamine treated animals. On the other hand, apomorphine, a putative dopaminergic agonist, elicited stereotypic behaviour in 6-hydroxydopamine treated rats which was 2 to 5 times greater than control rats. Biochemical data showed that, following 6-hydroxydopamine treatment, tyrosine hydroxylase was only 2% of control levels in the striatum and dopamine levels were so low they could not be measured. Tyrosine hydroxylase activity and noradrenaline levels in the hypothalamus were 58% and 35% of control respectively. These data indicate that dopamine plays a major role in the expression of stereotypy. The increased stereotypy seen in 6-hydroxydopamine-treated rats following apomorphine suggests that the dopamine receptors were intact and functional, following 6-hydroxydopamine, and that they may be supersensitive owing to the destruction of presynaptic dopaminergic terminals. The results also indicate that (+)-amphet-

amine-induced stereotypy depends on intact presynaptic dopaminergic neurons and is mediated by dopamine release.

(iii) Lesion-drug interactions:

Evidence from selective brain lesions also indicates that dopamine plays an important role in stereotyped behaviour in adult rats. Creese & Iversen (1975) made lesions in the substantia nigra that caused a 99% decrease in striatal tyrosine hydroxylase activity and completely blocked amphetamine-induced stereotypy, whereas partial nigra lesions that caused only an 80% decrease in tyrosine hydroxylase activity enhance amphetamine-induced stereotypy. Lesions in the dorsal and ventral noradrenergic tracts caused no modification of drug-induced stereotypy. The markedly different effect of amphetamine depending on whether the lesion produced 80% or 99% depletion of tyrosine hydroxylase suggests that the small remaining pool of catecholamines may be of functional importance (Fibiger *et al.*, 1973.)

On the basis of discrete lesions in the dopaminergic system, Asher & Aghajanian (1974) concluded that only lesions which reduced levels of dopamine in the caudate (i.e. nigrostriatal lesions) blocked stereotypy. Other evidence questions whether the nigrostriatal dopaminergic system is necessary for stereotypy. Costall *et al.* (1972) lesioned the substantia nigra electrolytically and found no change in amphetamine-induced stereotypic behaviour; moreover the response to apomorphine was decreased.

It is argued by many authors (e.g. Seiden, 1978) that as Costall *et al.* (1972) did not report dopamine levels, it is impossible to draw conclusions concerning the functional state of the dopaminergic system. Costall *et al.* (1972) provide morphological evidence concerning the precision of the lesion anatomically; Creese & Iversen (1975) do not. The argument is in terms of pathways, therefore surely the anatomical evidence is crucial.

This global and rather destructive set of approaches towards a biochemical mechanism for the behavioural action of a drug, although highly regarded is, in my opinion, crude and of little value to the neurochemist. More important I believe that many neurochemical concepts have been arrived at in this fashion. This is especially true with the biochemical actions of amphetamine. One further comment is that, throughout, the interpretations of these studies attribute a causal relationship to the biochemical explanation. Temporally the cause has to precede the event.

The behavioural study where the treatment of the rats was very similar to mine was one by Hitzemann *et al.* (1977). They injected rats s.c. with 3, 6 and 12 mg/kg of (+)-amphetamine twice daily on a weekly increasing "staircase" schedule. On days 1, 7, 14 and 28 after the last injection of amphetamine the animals were challenged with 1 and 3 mg/kg of (+)-amphetamine and their behaviour observed.

Stereotypy, measured by a rating scale, and exploratory activity by square-crossing, showed that for at least 28 days after withdrawal from chronic amphetamine treatment, the dose of amphetamine

necessary to induce stereotyped behaviours in the rat is considerably reduced. Square-crossing and rearing activities were depressed from control levels in the withdrawn animals administered 3 mg/kg of (+)-amphetamine. However there was an increase in these behaviours if the withdrawn animals were administered 1 mg/kg of (+)-amphetamine. This inverted U shaped dose-response curve is also seen in control animals (Hitzemann & Loh (1974), although higher doses are required to depress the square-crossing activity. Thus, in the withdrawn rats the dose-response curve moves to the left. While reserpine pretreatment markedly potentiates both amphetamine-induced square-crossing and stereotyped behaviours in normal animals, reserpine pretreatment had little or no effect on these behaviours in the withdrawn animals. The authors suggest that the storage of dopamine may have been altered by the chronic drug treatment causing dopamine to be more easily displaced by (+)-amphetamine.

Hitzemann *et al.* (1977) found that α-methyl-p-tyrosine blocked the (+)-amphetamine-induced increase in square crossing and stereotypy, but only 20 - 30 min after administration of the amphetamine; between 0 - 10 min α-methyl-p-tyrosine potentiated these behaviours. α-Methyl-p-tyrosine blocked the (+)-amphetamine-induced stereotypy at both time intervals in the withdrawn rats, i.e. the effect of the drug, at this dose, depends on the uninterrupted synthesis of catecholamines. α-Methyl-p-tyrosine was not effective in blocking the effects of 3 mg/kg of (+)-amphetamine in the withdrawn animals; in fact the square crossing activity was potentiated during the 20 - 30 min interval. This suggests that the effects of amphetamine at this dose are no longer dependent on catecholamine synthesis.

Thus, behavioural studies have shown that there are long-term behavioural changes after chronic amphetamine administration in the rat (Hitzemann *et al.*, 1977) mouse and cat (Short & Shuster, 1976; Ellinwood, 1974).

F. General Comments

1. Controls

As has been mentioned before it was not possible to set up adequate controls for this experiment. Firstly the injected control group, after the first week, were not apparently upset by the injections. The chronic group reacted to every increase in dose with increased stress for several days. At best they were still more stressed than the control group. Secondly it was not possible to set up a control for the acute effects of methamphetamine as methamphetamine is stored in the body and mobilized by stress. Thus, one would have to determine the dose equivalent to the circulating and easily mobilized pools of amphetamine stored by the chronic animals.

2. Enzymic Adaptation

The term 'enzymic adaptation' was first introduced by Karström in 1930 - 38 in order to describe the development of an enzyme activity by micro-organisms only when the relevant substrate was provided (see Richmond, 1968, for a general account). It has been shown that tolerance occurs at least to the anorexic effects of methamphetamine in my experiments, and that in those animals there is a change in amino acid levels in the brain on withdrawal, suggesting that metabolic adaptation had taken place. Logically I would expect any metabolic adaptation in the central nervous system to occur at the tyrosine hydroxylase step, however my results seem to indicate that it may be occurring at the level of transport of amino acids into the brain. However it is impossible to tell whether the *in vitro* activity of tyrosine hydroxylase is representative of its *in vivo* activity. It is possible that the results may have been affected by *post mortem* changes in the activity of the enzyme as the dissection time of set 1 was slower than that for the other sets. (6 to 7 min. as compared to 4 min.)

3. Tyrosine Hydroxylase

The literature on the kinetic properties of tyrosine hydroxylase is diffuse and contradictory (see Mandell, 1978 for a review). Four problems which make generalizations about the enzyme difficult are:

(i) The purification of the enzyme using either proteolytic digestion, detergents (Kuczenski, 1973a; Kuczenski, 1973b) or affinity chromatography (Poillon, 1971), provide contradictory results on the kinetic properties of the enzyme.

(ii) The kinetic properties of the enzyme appear to differ depending on the source e.g. adrenal medulla (Petrack *et al.*, 1968; Musacchio *et al.*, 1971); rat striatum (Kuczenski, 1973) and dopaminergic and noradrenergic neurons and sympathetic ganglia (Joh & Reis, 1975).

(iii) The cofactor used to assay the enzyme. Usually 6,7-dimethyl-pterin or 6-monomethyl pterin is used. These are not, however the natural cofactors for the enzyme. The use of what is considered to be the natural cofactor (biopterin) gives completely different kinetic parameters and suggests alternative regulatory roles for tyrosine hydroxylase (Black, 1975; Mandell, 1978).

(iv) Purification of the enzyme from different sources show that the enzyme aggregates, therefore exists in more than one form *in vitro* (Poillon, 1971; Petrack *et al.*, 1968).

4. Alterations in the "blood-brain barrier" on amphetamine treatment

Carlsson & Johansson (1978) have suggested that amphetamine causes blood brain barrier "dysfunction" in rats. The theory is based on the altered permeability of Evans blue dye into the brain. This type of experimental design which is used to demonstrate alterations in the blood-brain barrier is adequately criticized by Dobbing (1968). However, that there may be alterations in the "blood-brain barrier" after amphetamine treatment cannot be totally dismissed.

G. Conclusions

In this thesis I believe that I have studied the metabolic changes in what are, essentially two animal models of chronic methamphetamine administration.

Experimental set 1 closely resembled a model in which the animal is in a constant state of intoxication with methamphetamine. In this group the progressive decrease in the levels of activity of tyrosine hydroxylase with the duration of treatment together with the decrease in the levels of noradrenaline indicates either an enzymic adaptation to methamphetamine, a feedback inhibition due to the increased activity of the dopaminergic and noradrenergic neurons, or damage to the catecholaminergic neurons.

The results from experimental sets 3 and 4 were completely different behaviourally and biochemically. The only changes observed were in amino acid levels. The levels of free tryptophan in the plasma which were decreased on chronic treatment are difficult to interpret as there was no 'stressed' control. The increase in amino acid levels in the brain on withdrawal from methamphetamine strongly indicate a metabolic adaptation at the level of transport of essential substrates into the brain or on their subsequent metabolism. If the blood-brain barrier is damaged by amphetamine, as has been suggested (Carlsson & Johansson, 1978), the metabolic control in the brain may be completely altered. However the changes in amino acid levels in the brain did not appear to be altered on chronic treatment, only on withdrawal from the drug.

Tolerance to the anorexic effects of methamphetamine only occurred in experimental sets 2 to 4. Therefore I am proposing that metabolic adaptation to the effects of methamphetamine occurs primarily at the level of transport of essential substrates into the brain.

H. Future Work

This thesis has covered a fairly wide range of biochemical variables rather than investigating one in detail. I feel that this overview was a necessary prelude to more detailed work. A few suggestions which emerge as a direct consequence from this work are:

1. An investigation into the changes in amine levels or tyrosine hydroxylase activity using pellet implantation as the means of administering methamphetamine, and comparing the results with the same dose administered intraperitoneally.

2. A more detailed investigation into the inhibition of tyrosine hydroxylase which it is possible to induce on chronic amphetamine treatment. Mixing the homogenates from the chronic and control rats would provide some information on the nature of the inhibition. Further kinetic studies could elucidate whether the change in activity is a metabolic adaptation or due to feedback inhibition.

3. Full use was not made of the method which evolved for the measurement of DOPA, noradrenaline, dopamine and their non-O-methylated metabolites. Only a partial study was possible. The method should be improved as suggested in Section 4 of the Discussion by using adenosine deaminase to remove the inhibitor S-adenosylhomocysteine.

4. The amino acid results were interesting as they were completely unexpected. Further work ought to be done in order to repeat

this and obtain more statistically significant results. As well as providing fresh data on the biochemical actions of amphetamine it may well provide insights into the amino acid transport mechanisms from the blood into the brain.

5. An investigation into the changes in amino acid uptake into the brain over a longer period of withdrawal.

6. A determination of the circulating and tissue levels of methamphetamine and its metabolites over a course of chronic treatment. A suitable experiment could be designed where the animal is subjected to mild and moderate stress and the circulating and tissue levels redetermined. From this one can attempt to set up a suitable acute control.

REFERENCES

Alhava, E. (1973). Acta Pharmacol. Toxicol. 32, 119-128.

Alles, G.A. (1927). J. Pharmacol. 32, 121.

A.M.A. Drug Evaluations, 2nd edn. (1973).

Andén, N.-E. (1964). Studier över monoaminer ochderas roll som transmittorsubstanser. Thesis, Göteborg, 1-29. Cited by Persson, T. (1970).

Anden, N.-E. (1970). in Amphetamines and related compounds (Costa, E. & Garattini, S., eds.), pp. 447-462. Raven Press, New York.

Anden, N.-E., Carlsson, A. & Häggendal, J. (1969). Ann. Rev. Pharmacol. 9, 119-134.

Andén, N.-E., Dahlström, A., Fuxe, K. & Larsson, K. (1965). Amer. J. Anat. 116, 329. Cited by Ellison et al. (1978).

Andén, N.-E., Dahlstrom, A., Fuxe, K., Larsson, L., Olson, L. & Ungerstedt, U. (1966). Acta Physiol. Scand. 67, 313-326.

Anden, N.-E. & Henning, M. (1966). Acta physiol. Scand. 67, 498-504.

Andén, N.-E., Magnusson , T. & Stock, G. (1973). Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 178, 363-372.

Andén, N.-E., Rubenson, A., Fuxe, K. & Hökfelt, T. (1967). J. Pharm, Pharmacol. 19, 627-629.

Andén, N.-E. & Svensson, T.H. (1973). J. Neural. Transm. 34, 23-30.

Anderson, E.G. & Ammann, A. (1963). J. Pharmacol. Exp. Ther. 140, 179-182.

Angrist, B.M. & Gershon, S. (1970). Biol. Psychiat. 2, 95-107.

Anisman, H. & Kokkinidis, L. (1975). Psychopharmacologia 45, 55-64.

Argiolas, A., Fadda, F., Stefanini, E. & Gessa, G.L. (1977). J. Neurochem. 29, 599-601.

Asano, Y. & Moroji, T. (1974). Life Sci. 14, 1463-1472.

Asher, I.M. & Aghajanian, G.K. (1974). Brain Res. 82, 1-12.

Atack, C.V. (1973). Brit. J. Pharmacol. 48, 699-714

Atack, C.V. & Magnusson , T. (1970). J. Pharm. Pharmacol. 22, 625-627.

Axelrod, J. (1959). Physiol Rev. 39, 751-776.

Axelrod, J. (1965). in Recent Progress in Hormone Research pp·597-622. Academic Press, New York.

Axelrod, J., Hertting, G.& Potter, L. (1962). Nature (London) 194, 297.

Axelrod, J. & Inscoe, J.K. (1963). J. Pharmacol. Exp. Ther. 141, 161-165.

Axelrod, J. & Tomchick, R. (1960). J. Pharmacol. Exp. Ther. 130, 367-369.

Axelrod, J., Whitby, L.G. & Hertting, G. (1961). Science 133, 383-385.

Azmitia, E.E. & McEwen, B.S. (1969). Science 166, 1274.

Azzaro, A.J. & Rutledege, C.O. (1973). Biochem. Pharmacol. 22, 2801-2813.

Azzaro, A.J., Ziance, R.J. & Rutledge, C.O. (1974). J. Pharmacol. Exp. Ther. 189, 110-118.

Baird, J.R.C. (1968). J. Pharm. Pharmacol. 20, 234-235.

Baird, J.R.C. & Lewis, J.J. (1964), Biochem. Pharmacol. 13, 1475-1482.

Baird, J.R.C. & Lewis, J.J. (1963). Biochem. Pharmacol. 12, 577-602.

Bagchi, S.P. & McGeer, P.L. (1964). Life Sci. 3, 1195-1200.

Baldessarini, R.J. & Vogt, M. (1971). J. Neurochem. 18, 2519-2533.

Barchas, J.D. & Freedman, D.X. (1963). Biochem. Pharmacol. 12, 1232.

Bartolini, A. & Pepeu, G. (1970). Pharmacol. Res. Commun. 2, 23-29.

Basso, P. del, Rusca, G. & Carpi, A. (1970). Eur. J. Pharmacol. 13, 83-89.

Beani, L., Bianchi, C., Santinoceto, L. & Marchetti, P. (1968). Int. J. Neuropharmacol. 7, 469-481.

Beauvallet, M. (1968). Actualités pharmacol. 21, 15-39.

Beauvallet, M., Fugazza, J. & Legrand, M. (1966). C.R.Soc. Biol. 160, 546-550.

Beauvallet, M., Fugazza, J. & Legrand, M. (1967). Thérapie 22, 1273-1276.

Beauvallet, M., Fugazza, J. & Solier, M. (1962a). C.R. Soc. Biol. 156, 1258-1260.

Beauvallet, M., Halpern, B.N., Fugazza, J. & Drudi-Baracco, C. (1962b). Biochem. Pharmacol. 12, suppl. 8.

Beauvallet, M., Legrand, M., Bernard, J. & Solier, M. (1969). C.R. Soc. Biol. 163, 855-856.

Beauvallet, M., Legrand, M., Solier, M. (1970). C.R. Soc. Biol. 164, 1462-1467.

Bejerot, N. (1966). Paper given at the Symposium on the Pharmacological and Epidemiological aspects of AdolescentDrug Dependence, London Hospital Medical College.

Ben-Jonathan, N. & Porter, J.C. (1976). Endocrinology 98, 1497-1507.

Benington, F. & Morin, R.D. (1968). J. Med. Chem. 11, 896-897.

Bertler, Å. (1960). Acta Physiol. Scand. 51, 97-107.

Bertler, Å., Carlsson, A. & Rosengren, E. (1956). Naturwissenschaften 22, 521.

Bertler, Å. & Rosengren, E. (1959a). Acta Physiol. Scand. 47, 350-361.

Bertler, Å. & Rosengren, E. (1959b). Experientia 25, 10.

Bertler, Å. & Rosengren, E. (1959). Experientia 25, 382.

Besson, M.J., Cheramy, A., Feltz, P. & Glowinski, J. (1969a). Proc. Nat. Acad. Sci. U.S.A. 62, 741-748.

Besson, M.J., Cheramy, A., Gauchy, C. & Musacchio, J. (1973). Eur. J. Pharmacol. 22, 181-186.

Besson, M.J., Cheramy, A. & Glowinski, J. (1969b). Eur. J. Pharmacol. 7, 111-114.

Besson, M.J.,Cheramy, A. & Glowinksi, J. (1971). J. Pharmacol. Exp. Ther. 177, 196-205.

Bewley, (1966). Bulletin on Narcotics 18, 1-13.

Biel, J.H. (1970). in Amphetamines and related compounds, (Costa, E. & Garattini, S., eds.), pp. 3-19. Raven Press, New York.

Biscardi, A.M., Carpi, A. & Orsingher, O.A. (1964). Brit. J. Pharmacol. 23, 529-539.

Bizzi, A., Bonaccorsi, A., Jespersen, S., Jori, A. & Garattini, S. (1970). in Amphetamines and Related Compounds. (Costa, E. & Garattini, S., eds.), pp. 577-595. Raven Press, New York.

Black, I.B. (1975). Brain Res. 95, 170-176.

Black, I.B. & Geen, S.C. (1975). Archives of Neurology and Psychiatry 32, 47-49.

Blaschko, H. (1952). Pharmacol. Rev. 4, 415-458.

Blaschko, H., Richter, D. & Schlossman, H. (1937). Biochem. J. 31, 2187-2196.

Blaschko, H., Strömblad, B.C.R. (1960). Arzneim. Forsch. 10, 327.

Bolt, A.G., Mulligan, B.M. & Graham, C. (1974). Res. Commun. Chem. Pathol. Pharmacol. 9, 182-192.

Boulu, R., Rapin, J.R., Lebas, M., Jacquot, C. (1972). Psychopharmacologia 26, 54-61.

Breckenridge, B.M. & Norman, J.H. (1965). J. Neurochem. 12, 51-57.

Breese, G.R., Kopin, I.J. & Weise, V.K. (1970). Brit. J. Pharmacol. 38, 537-545.

Brodie, B.B., Costa, E., Groppetti, A. & Matsumoto, C. (1968). Brit. J. Pharmacol. 34, 648-658.

Brodie, B.B., Cho, A.K. Stefano, F.J.E. & Gessa, G.L. (1969). Advan. Biochem. Psychopharmacol. 1, 219-238.

Brodie, B.B., Cho, A.K. & Gessa, G.L. (1970). in Amphetamines and Related Compounds. (Costa, E. & Garattini, S., eds.), pp. 217-230. Raven Press, New York.

Buening, M.K. & Gibb, J.W. (1974). Eur. J. Pharmacol. 26, 30-34.

Bunney, B.S., Walters, J.R., Roth, R.H. & Aghajanian, G.K.A. (1973). J. Pharmacol. Exp. Ther. 185, 560-571.

Burgen, A.S.V. & Iversen, L.L. (1965). Brit. J. Pharmacol. 25, 34-49.

Burn, J.P. & Rand, M.J. (1958). J. Physiol.(London) 144, 314-336.

Caccia, S., Ceccheti, G., Garattini, S. & Jori, A. (1973). Brit. J. Pharmacol. 49, 400-406.

Carlsson, A. (1959). Pharmacol. Rev. 11, 490-493.

Carlsson, A. (1966). in Mechanisms of Release of Biogenic Amines (Euler, U.S. von, Rosell, S. & Uvnäs, B., eds.), pp. 331-346. Pergamon Press, Oxford.

Carlsson, A., Corrodi, H., Fuxe, K. & Hökfelt, T. (1969). Eur. J. Pharmacol. 5, 367-373.

Carlsson, A., Fuxe, K., Hamberger, B. & Lindqvist, M. (1966a). Acta Physiol.Scand. 67, 481-497.

Carlsson, A., Falck, B. & Hillarp, N.-A. (1962). Acta Physiol. Scand. 56, suppl. 196, 1-28.

Carlsson, A., Fuxe, K. & Hökfelt, T. (1968). Eur. J. Pharmacol. 2, 196-201.

Carlsson, A. & Hillarp, N-A. (1956). Kgl. Fysiogr. Sällsk. Lund Förh. 26, no. 8. Cited by Persson, T. (1970).

Carlsson, C. & Johanson, B.B. (1978). Acta Neuropathologica 41, 125-129.

Carlsson, A., Lindqvist, M., Dahlström, A., Fuxe, K. & Masuoka, D. (1965). J. Pharm. Pharmacol. 17, 521-523.

Carlsson, A., Lindqvist, M., Fuxe, K. & Hamberger, B. (1966b). J. Pharm. Pharmacol. 18, 128-130.

Carlsson, A., Lindqvist, M., Magnusson , T. & Waldeck, B. (1958). Science 127, 471.

Carlsson, A., Lindqvist, M. & Magnusson T. (1957). Nature (London) 180, 1200.

Carlsson, A. & Waldeck, B. (1965). J. Pharm. Pharmacol. 17, 243-244.

Carlsson, A. & Waldeck, B. (1966a). J. Pharm. Pharmacol. 18, 252-253.

Carlsson, A. & Waldeck, B. (1966b). Acta Pharmacol. Toxicol. 24, 255-262.

Carlsson, A, & Waldeck, B. (1968). Eur. J. Pharmacol. 4, 165-168.

Carr, L.A. & Moore, K.E. (1969). Science 164, 322-323.

Carr, L.A. & Moore, K.E. (1970a). Biochem. Pharmacol. 19, 2361-2374.

Carr, L.A. & Moore, K.E. (1970b). Biochem. Pharmacol. 19, 2671-2675.

Cattabeni, F., Racagni, G. & Groppetti, A. (1973). in Frontiers of Catecholamine Research (Usdin, E. & Snyder, S., eds.), pp. 1035-1037, Pergamon Press, New York.

Cession-Fossion, A. (1967). Arch. Int. Physiol. Biochim. 75, 303-309.

Chang, C.C. (1965). J. Pharm. Pharmacol. 17, 818-820.

Chevillard, C. & Alexandre, J.-M. (1972). Eur. J. Pharmacol. 19, 223-230.

Chidsey, C.A., Harrison, D.C. & Braunwald, E. (1962). Proc. Soc. Exp. Biol. (N.Y.) 109, 488-490.

Chiueh, C.C. & Kopin, I.J. (1978). J. Neurochem. 31, 561-564.

Chiueh, C.C. & Moore, K.E. (1973). Brain Res. 50, 221-225.

Chiueh, C.C. & Moore, K.E. (1974a). Res. Commun. Chem. Pathol. Pharmacol. 7, 189-199.

Chiueh, C.C. & Moore, K.E. (1974b). J. Neurochem. 23, 159-168.

Chiueh, C.C. & Moore, K.E. (1974c). J. Pharmacol. Exp. Ther. 190, 100-108.

Chu, H., Opitz, K. & Intemann, E. (1969). Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 263, 358-362.

Cicero, T.L., Sharpe, L.G., Robins, E. & Grote, S.S. (1972). J. Neurochem. 19, 2241-2243.

Clay, G.A., Cho, A.K. & Roberfroid, M. (1971). Biochem. Pharmacol. 20, 1821-1831.

Connell, P.H. (1958). 'Amphetamine Psychosis' Maudsley monograph No. 5, Oxford University Press, London.

Connell, P.H. (1970). Paper given at the 60th Anniversary of the Jellinek Clinic, Amsterdam 7-9th April, pp. 1-7.

Consolo, S., Ladinsky, H., Garattini, S. (1974). J. Pharm. Pharmacol. 26, 275-277.

Cook, J.D., Schanberg, S.M. (1970). Biochem. Pharmacol. 19, 1165-1179.

Corrodi, H., Fuxe, K. & Hökfelt, T. (1967). Eur. J. Pharmacol. 1, 363-368.

Costa, E., Carenzi, A., Guidotti, A. & Revuelta, A. (1973). in Frontiers of Catecholamine Research (Usdin, E. & Snyder, S., eds.), pp. 1003-1010, Pergamon Press, New York.

Costa, E. & Groppetti, A. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 231-355, Raven Press, New York.

Costa, E. & Groppetti, A. (1972). in Current Concepts on Amphetamine Abuse (Ellinwood, E.H. & Cohen, S., eds.), pp. 117-124, DHEW Publ. No. (HSM) 72-9085, U.S. Government Printing Office, Washington D.C.

Costa, E., Groppetti, A. & Naimzada, M.K. (1972). Brit. J. Pharmacol. 44, 742-751.

Costall, B., Naylor, R.J. & Olley, J.E. (1972). Eur. J. Pharmacol. 18, 95-106.

Coyle, J.T. & Snyder, S.H. (1969a). Science 166, 899-901.

Coyle, J.T. & Snyder, S.H. (1969b). J. Pharmacol. Exp. Ther. 170, 221-231.

Creese, I. & Iversen, S.D. (1973). Brain Res. 55, 369-382.

Creese, I. & Iversen, S.D. (1975). Brain Res. 83, 419-436.

Crow, T.J., Johnstone, E.C., Deakin, J.F.W. & Longden, A. (1976). Lancet 2, 563-566.

Cuello, A.C., Hiley, R. & Iversen, L.L. (1973). J. Neurochem. 21, 1337-1340.

Curzon, G. (1978). in Chemical Communication Within the Nervous System and its Disturbance in Disease (Taylor, A. & Jones, M.T., eds.), pp. 45-47. Pergamon Press, Oxford.

Curzon, G., Friedel, J. & Knott, P.J. (1973). Nature (London) 242, 198-200.

Curzon, G. & Knott, P.J. (1975). Brit. J. Pharmacol. 54, 389-396.

Dahlström, A. & Fuxe, K. (1965). Acta Physiol. Scand. 64, Suppl. 247, 1-36.

Daly, J.W., Creveling, C.R. & Witkop, B. (1966). J. Med. Chem. 9, 273-284.

Davis, V.E. (1963). Endocrinology 72, 33.

Davis, P.L. & Stewart, W.B. (1938). J. Amer. Med. Assoc. 110, 1890-1892.

Deffenu, G., Bartolini, A. & Pepeu, G. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 357-368, Raven Press, New York.

Deguchi, T. & Axelrod, J. (1972). Anal. Biochem. 50, 174-179.

Delay, J., Deniker, P. & Harl, J.M. (1952). Annls. Méd. Psychol. 110, 112-117. Cited by Persson, T. (1970).

Dengler, H.J., Spiegel, H.E. & Titus, E.O. (1961). Nature (London) 191 , 816-817.

Diaz, J.-L. & Huttunen, M.O. (1972). Psychopharmacologia 23, 365-372.

Dobbing, J. (1961). Physiol. Rev. 41, 130-188.

Domino, E.F. & Olds, M.E. (1972). Psychopharmacologia 23, 1-16.

Domino, E.F. & Wilson, A. (1972). Psychopharmacologia 25, 291-298.

Dorris, R.L. & Shore, P.A. (1974). Biochem. Pharmacol. 23, 867-872

Drummond, G.I. & Bellward, G. (1970). J. Neurochem. 17, 475-482.

Dubnick, B., Ruchi, E.W. & Salama, A.I. (1973). Eur. J. Pharmacol. 22, 121-128.

Ebstein, R.P., Ebstein, B.S., Samuel, D. & Berger, B.D. (1972). J. Neurochem. 19, 2703-2705.

Eccleston, D., Ritchie, I.M. & Roberts, M.H.T. (1970). Nature (London) 226, 84-85.

Eisenberg, L. (1963). Amer. J. Orthopsychiatry 33, 431-437.

Eisenthal, R. & Cornish-Bowden, A. (1974). Biochem. J. 139, 715-720.

El Guedri, H., Jacquot, C., Rapin, J. & Cohen, Y. (1973). J. Pharmacol. (Paris) 4, 453-463.

Ellinwood, E.H. Jr. & Balster, R.L. (1974). Eur. J.Pharmacol. 28, 35-41.

Ellinwood, E.H.Jr. & Escalante, O. (1970a). Biol. Psychiat. 2, 27-39.

Ellinwood, E.H., Sudilovsky, A. & Nelson, L. (1972). Biol. Psychiat. 4, 215-230.

Ellison, G., Eison, M.S. & Huberman, H.S. (1978). Psychopharmacology 56, 293-299.

Ellison, G., Eison, M.S., Huberman, H.S. & Daniel, F. (1978). Science 201, 276-278.

Emde, H. (1929). Helv. Chim. Acta 12, 265.

Enna, S.J., Dorris, R.L. & Shore, P.A. (1973). J. Pharmacol. Exp. Ther. 184, 576-582.

Enna, S.J.& Shore, P.A. (1974). J. Neural. Transm. 35, 125-135.

Espelin, D.E. & Done, A.K.N. (1968). Engl. J. Med. 278, 1361. Cited by Crow et al. (1976).

Etienne, P., Young, S.N. & Sourkes, T.L. (1976). Nature (London) 262, 144-145.

Euler, U.S. von & Lishajko, F. (1968). Acta Physiol. Scand. 73, 78-88.

Everett, G.M. & Yellin, T.O. (1971). Res. Commun. Chem. Pathol. Pharmacol. 2, 407-414.

Farnebo, L.-O. (1971). On transmitter release evoked by field stimulation of monoamine neurons, pp. 1-44, Department of Histology, Karolinska Institutet, Stockholm. Cited by Lewander (1977).

Fassina, G. (1966). Arch. Int. Pharmacodyn. Ther. 161, 410-422.

Fernstrom, D. & Wurtman, R.J. (1971). Science 173, 149-152.

Fernstrom, J.D. & Wurtman, R.J. (1972). Science 178, 414-416.

Ferrendelli, J.A., Kinscherf, D.A. & Kipnis, D.M. (1972). Biochem. Biophys. Res. Commun. 46, 2114-2120.

Ferris, R.M. & Maxwell, R.A. (1972). Fed. Proc. Fed. Amer. Soc. Exp. Biol. 31, abs. 2175.'

Ferris, R.M., Tang, F.L.M. & Maxwell, R.A. (1972). J. Pharmacol. Exp. Ther. 181, 407-416.

Fibiger, H.C., Fibiger, H.P. & Zis, A.P. (1973). Brit. J. Pharmacol. 47, 683-692.

Fibiger, H.C. & McGeer, E.G. (1971). Eur. J. Pharmacol. 16, 176-180.

File, S.E. & Wardill, A.G. (1965). Psychopharmacologia 44, 47-51.

Finkelman, I. & Shapiro, L.B. (1937). J. Amer. Med. Assoc. 109, 344-346.

Fleming, R.M. & Clark, W.G. (1970). J. Chromatogr. 52, 305-312.

Freeman, J.J. & Sulser, F. (1972). J. Pharmacol. Exp.Ther. 183, 307-315.

Fuentes, J.A. & Del Rio, J. (1972). Eur. J. Pharmacol. 17, 297-300.

Fulginiti, S. & Orsingher, O.A. (1971). Arch. Int. Pharmacodyn. Ther. 190, 291-298.

Fuller, R.W. (1972). Advan. Biochem. Psychopharmacol. 5, 339-354.

Fuller, R.W., Schaffer, R.J., Roush, B.W. & Molloy, B.B. (1972b). Biochem. Pharmacol. 21, 1413-1417.

Fuller, R.W. & Walters, C.P. (1964). Biochem. Pharmacol. 14, 159-163.

Fuxe, K. (1965a). Acta Physiol. Scand. 64 Suppl. 247, 36-85.

Fuxe, K. (1965b). Z. Zellforsch. 65, 573-596. Cited by Ungerstedt, U. (1971).

Fuxe, K., Hamberger, B. & Malmfors, T. (1967). Eur. J. Pharmacol. 1, 334-341.

Fuxe, K., Hökfelt, T. & Ungerstedt, U. (1968) in Metabolism of Amines in the Brain. Symposium of the British and Scandinavian Pharmacological Societies, Edinburgh (Hooper, G., ed.), pp. 10-22. MacMillan, London.

Fuxe, K. & Ungerstedt, U. (1968). Eur. J. Pharmacol. 4, 135-144.

Fuxe, K. & Ungerstedt, U. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 257-288, Raven Press, New York.

Gaitonde, M.K. (1974). Biochem J. 139, 625-631.

Gal, E.M. & Drews, P.A. (1962). Proc. Soc. Exp. Biol. Med. 110, 368-371. Cited by Muwiler et al. (1977).

Ganguly, D.K. (1969). Experientia 25, 1153-1154.

Gauchy, C., Bioulac, B., Cheramy, A., Besson, M.J., Glowinski, J. & Vincent, J.D. (1974). Brain Res. 77, 257-268.

Gauchy, C., Tassin, J.P., Glowinski, J. & Cheramy, A. (1976). J. Neurochem. 26, 471-480.

General Practitioner Research Group (1964). Practitioner Report No. 51, 192, 151-154.

Gerald, M.C. & Hsu, S.Y. (1975). Neuropharmacology 14, 115-123.

Gessa, G.L., Clay, G.A. & Brodie, B.B. (1969). Life Sci. 8, 135-141.

Glick, S.D., Jerussi, T.P., Waters, D.H., & Green, J.P. (1974). Biochem. Pharmacol. 23, 3223-3225.

Glowinski, J. (1970a). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 301-316. Raven Press, New York.

Glowinksi, J. (1970b). in New Aspects of Storage and Release Mechanism of Catecholamines (Schüman, H.J. & Kroneberg, G., eds.), pp. 237-247, Springer, Berlin-Heidelberg-New York.

Glowinski, J. & Axelrod, J. (1964). Nature (London) 204, 1318-1319.

Glowinski, J. & Axelrod, J. (1965). J. Pharmacol. Exp. Ther. 149, 43-49.

Glowinski, J., Axelrod, J. & Iversen, L.L. (1966a). J. Pharmacol. Exp. Ther. 153, 30-41.

Glowinski, J., Iversen, L.L. & Axelrod, J. (1966b). J. Pharmacol. Exp. Ther. 151, 385-399.

Goldstein, M. & Anagnoste, B. (1965). Biochim. Biophys. Acta 107, 166-168.

Goldstein, M., McKereghan, M.R. & Lauber, E. (1964). Biochim. Biophys. Acta 89, 191-193.

Goldstein, M. & Nakajima, K. (1967). J. Pharmacol. Exp. Ther. 157, 96-102.

Goodman, L.S. & Gilman, A. (1970). The Pharmacological basis of Therapeutics McMillan Co., N.Y.

Görlitz, B.-D. & Frey, H.-H. (1972). Eur. J. Pharmacol. 20, 171-180.

Grahame-Smith, D.G. (1971). J. Neurochem. 18, 1053-1066.

Grahame-Smith, D.G. & Parfitt, A.G. (1970). J. Neurochem. 17, 1339-1353.

Grana, E. & Lilla, L. (1959). Brit. J. Pharmacol. 14, 501-504.

Green, A.L. (1971). Biochem. J. 121, 37.

Green, H., Greenberg, S.M., Erickson, R.W., Sawyer, J.L. & Ellison, T. (1962). J. Pharmacol. Exp. Ther. 136, 174-178.

Green, A.L., Mayyada, A.S. el Hait (1978). J. Pharm. Pharmacol. 30, 262-263.

Griffith, J.D., Cavanaugh, J.M., Held, J. & Oates, J. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 897-904. Raven Press, New York.

Griffith, J.D., Cavanaugh, J., Held, J. & Oates, J.A. (1972). Arch. Gen. Psychiat. 26, 97. Citec by Crow et al. (1976).

Groppetti, A. & Costa, E. (1969a). Fed. Proc. Fed. Amer. Soc. Exp. Biol. 28, 795.

Groppetti, A. & Costa, E. (1969b). Life Sci. 8, 653-665.

Groppetti, A., Misher, A., Naimzada, M., Revuelta, A. & Costa, E. (1972). J. Pharmacol. Exp. Ther. 182, 464-473.

Gudetsky, G.A., McCall, R.B., Chiueh, C.C. & Moore, K.E. (1974). Res. Commun. Chem. Pathol. Pharmacol. 9, 653-660.

Gunne, L.-M. & Lewander, T. (1967). in Molecular Basis of Some Aspects of Mental Activity,Vol. 2, pp. 75-81. Academic Press, New York.

Gunne, L.-M. & Lewander, T. (1968). in The Addictive States, Vol. XLVI, pp. 106-116, Williams and Wilkins Company, Baltimore.

Häggendal, J. & Hamberger, B. (1967). Acta Physiol. Scand. 70, 277-280.

Häggendal, J. & Lindqvist, M. (1963). Acta Physiol. Scand. 57, 431-436.

Hall, D.W.R., Logan, B.W. & Parsons, G.H. (1969). Biochem. Pharmacol. 18, 1447.

Hamberger, B. & Malmfors, T. (1967). Acta Physiol. Scand. 70, 412-418.

Hare, E.H., Dominian, J. & Sharpe, L. (1962). Brit. Med. J. 1, 9-12.

Harris, J.E. & Baldessarini, R.J. (1973a). Neuropharmacology 12, 669-679.

Harris, J.E. & Baldessarini, R.J. (1973b). J. Pharm. Pharmacol. 25, 755-757.

Harvey, S.C., Sulkowski, T.S. & Weenig, D.J. (1968). Arch. Int. Pharmacodyn. Ther. 172, 301-322.

Hefti, F. & Lichtensteiger, W. (1976). J. Neurochem. 27, 647-649.

Hemsworth, B.A. & Neal, M.J. (1968a). Brit. J. Pharmacol. 32, 416-417.

Hemsworth, B.A. & Neal, M.J. (1968b). Brit. J. Pharmacol. 34, 543-550.

Hendley, E.D. & Snyder, S.H. (1972). Eur. J. Pharmacol. 19, 56-66.

Hendry, I.A. & Iversen, L.L. (1971). Brain Res. 29, 159-162.

Herberg, R.J. (1965). Packard Tech. Bulletin no. 15.

Herman, Z.S., Kmieciak-Kolada, K., Drybański, A., Sokola, A., Trzeciak, H. & Cruściel, T.L. (1971a). Psychopharmacologia 21, 66-75.

Herman, Z.S., Trzeciak, H., Cruściel, T.L., Kmieciak-Kolada, K., Drybański, A. & Sokola, A. (1971b). Psychopharmacologia 21, 74-81.

Hertting, G., Axelrod, J. & Whitby, G.L. (1961). J. Pharmacol. Exp. Ther. 134, 146-153.

Hillarp, N-Å. (1960). Acta Physiol. Scand. 50, 8-22.

Hitzemann, R.J. & Loh, H.H. (1972). Pharmacology 8, 361-367.

Hitzemann, R.J. & Loh, H.H. (1973). Biochem. Pharmacol. 22, 2731-2741.

Hitzemann, R.J. & Loh, H.H. (1974). Eur. J. Pharmacol. 27, 89-98.

Hitzemann, R.J., Loh, H.H., Carves, F.B. & Domino, E.F. (1973). Psychopharmacologia 30, 227-240.

Hitzemann, R.J., Loh, H.H. & Domino, E.F. (1970). Pharmacologist 12, 197.

Hitzemann, R.J., Tseng, L.F., Hitzemann, B.A., Sampath-Khanna, S. & Loh, H.H. (1977). Psychopharmacology 54, 295-302.

Ho, A.K.S. & Gershon, S. (1972). Eur. J. Pharmacol. 18, 195-200.

Holzbauer, M. & Vogt, M. (1956). J. Neurochem. 1, 8-11.

Honigfelt, G. & Howard, A. (1973). Psychiatric drugs, A desk reference Academic Press, N.Y.

Horn, A.S., Coyle, J.T. & Snyder, S.H. (1971). Mol. Pharmacol. 7, 66-80.

Horn, A.S., Cuello, A.C. & Miller, R.J. (1974). J. Neurochem. 22, 265-270.

Horn, A.S. & Snyder, S.H. (1972). J. Pharmacol. Exp. Ther. 180, 523-530.

Houslay, M.D. & Tipton, K.F. (1974). Biochem. J. 139, 645.

Hulme, E.C., Hill, R., North, M. & Kibby, M.R. (1974). Biochem. Pharmacol. 23, 1393-1404.

Hutchins, D.A. & Rogers, K.J. (1970). Brit. J. Pharmacol. 39, 9-25.

Hutchins, D.A. & Rogers, K.J. (1973). Brit. J. Pharmacol. 48, 19-29.

Ishii, Y., Homma, M. & Yoshikawa, Y. (1975). Neuropharmacology 14, 155-157.

Iversen, L.L. (1965). Brit. J. Pharmacol. 25, 18-33.

Iversen, L.L. (1967). The Uptake and Storage of Noradrenaline in Sympathetic Nerves Cambridge University Press, London.

Jacquot, C., L'Hermite, P., Cohen, Y. & Valette, G. (1971). C.R. Acad. Sci. Ser. D. 273, 1640-1642.

Jacquot, C., L'Hermite, P., Rapin, J.R. & Cohen, Y. (1973a). C.R. Soc. Biol. 167, 221-225.

Jacquot, C., Rapin, J., El Guedri, H. & Cohen, Y. (1973b). C.R. Acad. Sci. Ser. D. 276, 2851-2853.

Jacquot, C., Rapin, J. Renault, H. & Cohen, Y. (1974). C.R. Soc. Biol. 168, 479-484.

Javoy, F., Agid, Y., Bouvet, D. & Glowinski, J. (1974). J. Pharm. Pharmacol. 26, 179-185.

Javoy, F., Hamon, M. & Glowinski, J. (1970). Eur. J. Pharmacol. 10, 178-188.

Javoy, F., Thierry, A.M., Kety, S.S. & Glowinski, J. (1968). Commun. Behav. Biol. 1, 43-48.

Joh, T.H. & Reis, D.J. (1975). Brain Res. 85, 146-151.

Johnston, J.P. (1968). Biochem. Pharmacol. 17, 1285.

Jonas, W. & Scheel-Krüger, J. (1969). Arch. Int. Pharmacodyn. Ther. 177, 379-389.

Jones, B.E., Guynet, P., Chéramy, A., Gauchy, C. & Glowinski, J. (1973). Brain Res. 64, 355-369.

Jori, A. & Bernardi, D. (1969). J. Pharm. Pharmacol. 21, 694-697.

Jori, A. & Bernardi, D. (1972). Eur. J. Pharmacol. 19, 276-280.

Jori, A. & Dolfini, E. (1974). Pharmacol. Res. Commun. 6, 175-178.

Jori, A., Dolfini, E., Tognoni, G. & Garattini, S. (1973). J. Pharm. Pharmacol. 25, 315-318.

Jori, A. & Garattini, S. (1973). in Frontiers in Catecholamine Research (Usdin, E. & Snyder, S., eds.), pp. 939-941, Pergamon Press, New York.

Kalant, O.J. (1966). 'The Amphetamines', University of Toronto. Cited by Connell, P.H. (1970).

Kaplan, N.O. (1955). Methods in Enzymol. II, 475-478.

Kebabian, J.W., Saavedra, J.M. & Axelrod, J. (1977). J. Neurochem. 28, 795-801.

Klawans, H.L., Margolin, D.I., Dana, N. & Crosset, P. (1975). J. Neurological Sciences 25, 283-289.

Klein, D.F. & Davis, J.M. (1969). Diagnosis and Drug Treatment of Psychiatric Disorders. Williams and Wilkins Co., Baltimore.

Kline, N.S. (1959). Res. Publ. Ass. Nerv. Ment. Dis. 37, 218-244. Cited by Persson, T. (1970).

Knapp, S., Mandell, A.J. & Geyer, M.A. (1974). J. Pharmacol. Exp. Ther. 189, 676-689.

Knox, W.E. (1963). Trans. N.Y. Acad. Sci. 25, 503.

Koda, L.Y. & Gibb, J.W. (1973). J. Pharmacol. Exp. Ther. 185, 42-48.

Kokkinidis, L. & Anisman, H. (1978). Neuropharmacology 17, 95-102.

König, J.F.R. & Klippel, R.R. (1963). The rat brain: a stereotaxic atlas of the forebrain and lower parts of the brain stem. Williams & Wilkins Co., Baltimore, pp. 1-162.

Kopin, I.J. (1964). Pharmacol. Rev.16, 179-191.

Kopin, I.J., Breese, G.R., Krauss, K.R. & Weise, W.K. (1968). J. Pharmacol. Exp. Ther. 161, 271-278.

Kopin, I.J. & Gordon, E.K. (1963). J. Pharmacol. Exp. Ther. 140, 207-216.

Kostopoulos, G.K. & Yarbrough, G.G. (1975). J. Pharm. Pharmacol. 27, 408-412.

Kuczenski, R. (1973a). J. Biol. Chem. 248, 5074-5080.

Kuczenski, R. (1973b). J. Biol. Chem. 248, 2261-2265.

Kuhn, R. (1957). Schweiz. Med. Wschr. 87, 1135. Cited by Persson, T. (1970).

Ladisch, W., Volbehr, H. & Matussek, N. (1970). Neuropharmacology 9, 303-310.

Ladisch, W. & Baumann, P. (1971). Neuroendocrinology 7, 16-24.

Lajtha, A. & Toth, J. (1961). J. Neurochem. 8, 216-225.

Laverty, R. & Sharman, D.F. (1965). Brit. J. Pharmacol. 24, 759-772.

Laverty, R. & Taylor, K.M. (1968). Anal. Biochem. 22, 269-279.

Leitz, F.H. (1970). J. Pharmacol. Exp.Ther. 173, 152-157.

Leitz, F.H. & Stefano, F.J.E. (1971). J. Pharmacol. Exp. Ther. 173, 152-157.

Lemmer, B. (1973). Eur. J. Pharmacol. 21, 183-188.

Leonard, B.E. (1972). Biochem. Pharmacol. 21, 1289-1297.

Leonard, B.E. & Shallice, S.A. (1971). Brit. J. Pharmacol. 41, 198-212.

Lewander, T. (1968a). Eur. J. Pharmacol. 5, 1-9.

Lewander, T. (1968b). Psychopharmacologia 13, 394-407.

Lewander, T. (1969). Eur. J. Pharmacol. 6, 38-44.

Lewander, T. (1970a). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 317-329, Raven Press, New York.

Lewander, T. (1970b). Acta Pharmacol. Toxicol. 44, 62.

Lewander, T. (1971a). Acta Pharmacol. Toxicol. 29, 33-48.

Lewander, T. (1971b). Acta Pharmacol. Toxiol. 29, 20-32.

Lewander, T. (1971c). Acta Pharmacol. Toxicol. 29, 209-225.

Lewander, T. (1971d). Naunyn-Schmiedebergs Arch. Pharmakolo. 271, 211-233.

Lewander, T. (1974b). in Neuropharmacology of Monoamines and their Regulatory Enzymes (Usdin, E., ed.), pp. 221-239. Raven Press, New York.

Lewander, T. (1977). in Handbook of Experimental Pharmacology (Born, G.V.R., Eichler, O., Farah, A., Herken, H. & Welch, A.D., eds.), pp. 160-166, Springer-Verlag, Berlin-Heidelberg-New York.

Lewis, J.J. & Pollack, D. (1965). Life Sci. 4, 21-26.

Littleton, J.M. (1967). J. Pharm. Pharmacol. 19, 414-415.

Loomer, H.P., Saunders, J.S.C. & Kline, N.S. (1957). Amer Psychiat. Ass. Psychiatric Res. Reports 8, 129. Cited by Persson, T. (1970).

Lovenberg, W., Ames, M.N. & Lerner, P. (1978). Adv. Biochem. Psychopharmacol. 16, 461-464.

Lowry, O.H., Rosenbrough, N.J., Parr, A.L. &Randall, R. (1951). J. Biol. Chem. 193, 265.

Lundborg, P. (1963). Experientia 19, 479-480.

Lundborg, P. (1969). J. Pharm. Pharmacol.21, 266-268.

Lundborg, P. & Waldeck, B. (1972). Acta Pharmacol. Toxicol. 30, 339-347.

Lynch, M.A. & Leonard, B.E. (1978). Biochem. Pharmacol. 27, 1853-1855.

Lyon, M. &Robbins, T. (1975). in Current Developments in Psychopharmacology Vol. 2, pp. 81-161. Spectrum Publications Inc.

Maickel, R.P., Cox, R.H.Jr., Ksir, C.J., Snodgrass, W.R. & Miller, F.P. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 747-759. Raven Press, New York.

Maickel, R.P., Cox, R.H., Saillant, J. & Miller, F.P. (1968). Int. J. Neuropharmacol. 7, 275-281.

Maitre, L. von, Brunner, H. (1967). Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 257, 40.

Mandell, A.J. (1978). Ann. Rev. Pharmacol. Toxicol. 18, 461-493.

Mandell, A.J. & Knapp, S. (1972). in Current Concepts on Amphetamine Abuse (Ellinwood, E.H. & Cohen, S., eds.), pp. 77-86. DHEW Publ. No. (HSM) 72-9085. U.S. Government Printing Office, Washington D.C.

Mandell, A.J., Knapp, S., Kuczenski, R.T. & Segal, D.S. (1972). Biochem. Pharmacol. 21, 2737-2750.

Mandell, A.J. & Morgan, M. (1970). Nature (London) 227, 75-76.

Mann, P.J.G. & Quastel, J.H. (1940). Biochem. J. 34, 414-431.

Manning, D.H., Strang, R.H.C. & Bachelard, H.S. (1974). Biochem. Pharmacol. 23, 1205-1209.

Mantle, T.J., Tipton, K.F. & Garrett, N.J. (1976). Biochem. Pharmacol. 25, 2073-2077.

Mantle, T.J., Wilson, K. & Long, R.F. (1975). Biochem. Pharmacol. 24, 2039.

Martin, I.L., Baker, G.B. & Fleetwood-Walker, S.M. (1978). Biochem. Pharmacol. 27, 1519-1520.

Masaki, T. (1956). Sixth report of the expert committee on drugs liable to produce addiction. W.H.O. technical report Series 102, 14-21.

Matthews, R.A. (1938). J. Med. Sci. 195, 448-452.

McCaman, R.E. (1965). Life Sci. 4, 2353-2359.

McCaman, M.W. & Robbins, E. (1962). J. Lab. Clin. Med. 59, 885.

McCullough, D.O., Milberg, J.N. & Robison, S.M. (1970). Brit. J. Pharmacol. 40, 219-226.

McGeer, E.G., Gibson, S. & McGeer, P.L. (1967). Can. J. Biochem. 45, 1557-1563.

McGeer, E.G. & McGeer, P.L. (1967). Can. J. Biochem. 45, 115-131.

McGeer, E.G., McGeer, P.L. & Wada, J.A. (1971). J. Neurochem. 18, 1647-1658.

McKay, A.V.P., Davis, P.,Dewar, A.J. & Yates, C.M. (1978a). J. Neurochem. 30, 827-839.

McKay, A.V.P., Yates, C.M., Wright, A., Hamilton, P. & Davies, P. (1978b). J. Neurochem. 30, 841-848.

McKenzie, G.M. & Szerb, J.C. (1968). J. Pharmacol. Exp. Ther. 162, 302-308.

McKean, C.M., Boggs, D.E. & Peterson, N.A. (1968). J. Neurochem. 15, 235-241.

McLean, J.R. & McCartney, M. (1961). Proc. Soc. Exp. Biol. Med. (N.Y.) 107, 77-79.

McMenamy, R.H. & Oncley, J.L. (1958). J. Biol. Chem. 233, 1436-1447.

McNeill, J.H. & Muschek, L.D. (1972). Arch. Int. Pharmacodyn. Ther. 197, 236-244.

Meister, A. (1973). Science 180, 33-39.

Menon, M.K., Clark, W.G. & Fleming, R.M. (1973). Eur. J. Pharmacol. 21, 311-317.

Menon, M.K., Fleming, R.M. & Clark, W.G. (1974). Biochem. Pharmacol. 23, 879-885.

Meyerhoff, J.L., Kant, G.J. & Lenox, R.H. (1978). Brain Res. 152, 161-169.

Ministry of Health and Welfare Japan (1964). Pamphlet, December 1964.

Moir, A.B.T. & Eccleston, D.J. (1968). J. Neurochem. 15, 1093-1108.

Møller-Nielson, I. & Dubnick, B. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 63-74. Raven Press, New York.

Moore, K.E. & Lariviere, E.W. (1963). Biochem. Pharmacol. 12, 1283-1288.

Moore, K.E. & Lariviere, E.W. (1964). Biochem. Pharmacol. 13, 1098-1100.

Morgan, C.D., Cattabeni, F. & Costa, E. (1972a). J. Pharmacol. Exp. Ther. 180, 127-135.

Morgan, C.D., Löfstrandh, S. & Costa, E. (1972b). Life Sci. 11, 83-96.

Morgenroth, V.H. III, Boadle-Biber, M.C. & Roth, R.H. (1975). Mol. Pharmacol. 11, 427-435.

Morgenroth, V.H.III, Boadle-Biber, M.C. & Roth,R.H. (1976). Mol. Pharmacol. 12, 41-48.

Musacchio, J.M., Wurzburger, R.J. & D'Angelo, G.L. (1971). Mol. Pharmacol. 7, 136-146.

Nahorski, S.R. & Rogers, K.J. (1973). J. Neurochem. 21, 679-686.

Neame, K.D. (1964). J. Neurochem. 11, 67-76.

Neff, N.H. & Yang, H.-Y.T. (1974). Life Sci. 14, 2061.

Ng, K.Y., Chase, T.N. & Kopin, I.J. (1970). Nature (London) 228, 468-469.

Nielsen, C.K., Magnusson , M.P., Kampmann, E. & Frey, H.-H. (1967). Arch. Int. Pharmacodyn. Ther. 170, 428-442.

Nikodijevic, B., Daly, J. & Creveling, C.R. (1969). Biochem. Pharmacol. 18, 1577-1584.

Nistri, A., Bartolini, A., Deffenu, G. & Pepeu, G. (1972). Neuropharmacology 11, 665-674.

Obianwu, H.O., Stitzel, R. & Lundborg, P. (1968). J. Pharm. Pharmacol. 20, 585-594.

Oldendorf, W.H. (1971). Amer. J. Physiol. 221, 1629-1639.

Opitz, K. (1970). in Amphetamines and Related Compounds (Costa, E. & Garattini, S., eds.), pp. 627-639. Raven Press, New York.

Oswald, I. & Thacore, V.R. (1963). Brit. Med. J. 11, 427.

Ott, T., Schmitt, M., Pohle, W. & Matthies, H. (1971). Brain Res. 25, 171-178.

Overall, J.E., Hollister, L.E., Shelton, J., Porkorny, A.D., Casey, J.F. & Katz, G. (1962). Clin. Pharmacol. Ther. 3, 16-22.

Palkovits, M. (1973). Brain Res. 59, 449-450.

Palkovits, M., Brownstein, M., Saavedra, J.M. & Axelrod, J. (1974). Brain Res. 77, 137-149.

Palmer, G.C. (1973). Life Sci. 12, 345-355.

Parmar, S.S. (1966). Biochem. Pharmacol. 15, 1497-1505.

Partridge, W.M. & Connor, J.D. (1973). Experientia 29, 302-4

Pardridge, W.M. & Oldendorf, W.H. (1977). J. Neurochem. 28, 5-12.

Pasamanick, B. (1951). Arch. Neurology and Psychiatry 65, 752.

Paul, M.I., Pauk, G.L.& Ditzion, B.R. (1970). Pharmacology 3, 148-154.

Pepeu, G. & Bartolini, A. (1967). Boll. Soc. Ital. Biol. Sper. 43, 1409-1413. Cited by Lewander (1977).

Pepeu, G. & Bartolini, A. (1968). Eur. J. Pharmacol. 4, 254-263.

Pepeu, G.C., Bartolini, A. & Deffenu, G. (1970). in Drugs and Cholinergic Mechanisms (Heilbronn, E. & Winters, S., eds.), pp. 387-396. Res. Inst. Natl. Def., Stockholm.

Perez-Cruet, J., Tagliamonte, P. & Gessa, G.L. (1972). Life Sci. 11, 31-39.

Persson, T. (1970). Thesis from the Department of Pharmacology, Psychiatric Research Centre, St. Jörgen's Hospital, University of Göteborg, Sweden 4.

Persson, T. (1970). Acta pharmacol. Toxicol. 28, 378-390.

Persson, T.& Waldeck, B. (1970). J. Pharm. Pharmacol. 22, 473-478.

Peterson, D.I., Hardinge, M.G. & Tilton, B.E. (1964). J. Pharmacol. Exp. Ther. 146, 175-179.

Petrack, B., Sheppy, F.& Feltzer, V. (1968). J. Biol. Chem. 243, 743-748.

Pettibone, D.J., Wurtman, R.J. & Leeman, S.E. (1978). Biochem. Pharmacol. 27, 839-842.

Peuler, J.D. & Johnson, G.A. (1977). Life Sci. 21, 625-636.

Pfeiffer, A.K., Csáki, L., Fodor, M., György, L. & Ökrös, I. (1969). J. Pharm. Pharmacol. 21, 687-689.

Philippu, A. & Beyer, J. (1973). Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 278, 387-402.

Philippu, A., Glowinski, J. & Besson, M.J. (1974). Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 258, 238-250.

Pletscher, A., Bartholini, G., Bruderer, H., Burkhard, W.P. & Gey, K.F. (1964). J. Pharmacol. Exp. Ther. 145, 344-350.

Poillon, W.N. (1971). Biochem. Biophys. Res. Commun. 44, No. 1.

Potter, L.T. & Axelrod, J. (1963). J. Pharmacol. Exp. Ther. 140, 199-206.

Prada, M. Da, Bartholini, G. & Pletscher, A. (1965). Biochem. Pharmacol. 14, 1721-1726.

Prada, M.Da & Zürcher, G. (1976). Life Sci. 19, 1161-1174.

Pratesi, P. & Blaschko, H. (1959). Brit. J. Pharmacol. 14, 256-260.

Prinzmetal, M. & Bloomberg, W. (1935). J. Amer. Med. Assoc. 105, 2051.

Pycock, C., Blaschke, E., Bergqvist, U. & Unväs, B. (1975). Acta Physiol. Scand. 95, 373-382.

Raiteri, M., Levi, G. & Federico, R. (1974). Eur. J. Pharmacol. 28, 237-240.

Randrup, A. & Jonas, W. (1967). J. Pharm. Pharmacol. 19, 483-484.

Randrup, A. & Munkvad, I. (1968). Pharmakopsychiatrie Neuro-Psychopharmakologie 1, 18-26. Cited by Seiden, L.S. & Dykstra, L.A. (1977).

Randrup, A. & MunkVad, I. (1970). Cited by Seiden, L.S. & Dykstra, L.A. in Psychopharmacology: A Biochemical and Behavioural Approach, Van Nostrand, Reinhold Co., New York.

Rapin, J., Hamar, C. & Cohen, Y. (1972). J. Pharmacol. (Paris) 3, 537-538.

Rapin, J.R, Jacquet, C., Cohen, Y. & Valette, G. (1971). C.R. Soc. Biol. 165, 1576-1581.

Redlick, D.V. & Glick, D. (1965). Anal. Biochem. 10, 459-467.

Reid, W.D. (1970a). Fed. Proc. Fed. Am. Soc. Exp. Biol. 29,747 Abs.

Reid, W.D. (1970b). Brit. J. Pharmacol. 40, 483-491.

Reitz, R.H., Bocknik, S.E. & Kulkarni, A.S. (1972). J. Pharm. Pharmacol. 24, 830-832.

Richmond, M.H. (1968). Essays in Biochemistry 4, 105-154.

Riddell, D. & Szerb, J.C. (1971). J. Neurochem. 18, 989-1006.

Roffler-Tarlov, S., Sharman, D.F. & Tegerdine, P. (1971). Brit. J. Pharmacol. 42, 343-351.

Ross, S.B. & Renyi, A.L. (1964). Acta Pharmacol. Toxicol. 21, 226-239.

Ross, S.B. & Renyi, A.L. (1966a). J. Pharm. Pharmacol. 18, 322-323.

Ross, S.B. & Renyi,A.L. (1966b). J. Pharm. Pharmacol. 18, 756-757.

Ross, S.B. & Renyi, A.L. (1966c). Acta Pharmacol. Toxicol.24, 297-309.

Ross, S.B. & Renyi, A.L. (1967a). Life Sci. 6, 1407-1415.

Ross, S.B. & Renyi, A.L. (1967b). Eur. J. Pharmacol. 2, 181-186.

Ross, S.B., Renyi, A.L. & Brunfelter, B. (1968). J. Pharm. Pharmacol. 20, 283-288.

Roth, R.H., Walters, J.R. & Aghajanian, G.K. (1973). in Frontiers in Catecholamine Research (Usdin, E. & Snyder, S., eds.), pp. 567-574. Pergamon Press, New York.

Rubin, R.P. (1970). Pharmacol. Rev. 22, 389-428.

Rubin, R.P. & Jaanus, S.D. (1966). Naunyn-Schmiedebergs Arch. Pharmak. Exp. Pathol. 264, 125-137.

Rubin, R.P. & Jaanus, S.D. (1967). Biochem. Pharmacol. 16, 1007-1012.

Rutledge, C.D. (1970). J. Pharmacol. Exp. Ther. 171, 188-195.

Rutledge, C.D., Azzaro, A.J. & Ziance, R.J. (1972a). Fed. Proc. Fed. Am. Soc. Exp. Biol. 31, 601.

Rutledge, C.D., Azzaro, A.J. & Ziance, R.J. (1972b). Biochem. Psychopharmacol. 5, 379-392.

Rutledge, C.D., Azzaro, A.J. & Ziance, R.J. (1973). In Frontiers in Catecholamine Research (Usdin, E. & Snyder, S., eds.), pp. 973-975. Pergamon Press, New York.

Saavedra, J.M., Brownstein, M. & Axelrod, J. (1973). J. Pharmacol. Exp. Ther. 186, 508-515.

Salama, A.I. & Goldberg,M.E. (1969). Arch. Int. Pharmacodyn. Ther. 181,474-483.

Schanberg, S.M. & Cook, J.D. (1972). in Current Concepts on Amphetamine Abuse (Ellinwood, E.H. & Cohen, S., eds.), pp. 87-95. DHEW Publ. No. (HSM) 72-9085. U.S. Government Printing Office, Washington D.C.

Scheel-Krüger, J. (1971). Eur. J. Pharmacol. 14, 47-59.

Scheel-Krüger, J. (1972). Eur. J. Pharmacol. 18, 63-73.

Scheel-Krüger, J. (1972). Psychiat. Neurol. Neurochir. 75, 179-192. Cited by Seiden, L.S. & Dykstra, L.A. (1977).

Scheel-Krüger, J. & Hasselager, E. (1974). Psychopharmacologia 36, 189-202.

Schmidt, M.J., Hopkins, J.M., Schmidt, D.E. & Robinson, G.A. (1972). Brain Res. 42, 465-477.

Schneider, F.H. (1972). J. Pharmacol. Exp. Ther. 183, 80-89.

Schrold, J. & Squires, R.F. (1971). Psychopharmacologia 20, 85-90.

Schuberth, J., Fyrö, B., Nybäck, H. & Sedvall, C. (1970). J. Pharm. Pharmacol. 22, 860-862.

Schuberth, J. & Sedvall, G. (1972). J. Pharm. Pharmacol. 24, 53-62.

Schumann, H.J. & Philippü, A. (1962). Int. J. Neuropharmacol. 1, 179-182.

Scott, & Wilcox, (1965). Brit. J. Psychiat. 3, 865-875.

Seiden, L.S. & Dykstra, L.A. (1977). Psychopharmacology: A Biochemical and Behavioural Approach Van Nostrand Reinhold Co., New York.

Seiden, L.S., Fischman, M.W. & Schuster, C.R. (1976). in Cocaine and other Stimulants (Ellinwood, E.H. & Kilbey, M.H., eds.), pp. 179-186. Plenum Press, New York.

Shaw, W.N., Fuller, R.W. & Matsumoto, C. (1972). Eur. J. Pharmacol. 19, 98-103.

Shore, P.A., Silver, S.L. & Brodie, B.B. (1956). Science 122, 284-285.

Simon, P., Tillement, J.-P., Larousse, C., Breteau, M., Guernet, M. & Boissier, J.-R. (1970b). J. Pharmacol. (Paris) 1, 95-108.

Skau, K.A.& Gerald, M.C. (1978) Neuropharmacology 17, 271-276.

Snyder, S.H. (1972). Arch. Gen. Psychiat. 27, 169-178.

Solomon, P., Mitchell, R.S. & Prinzmetal, M. (1937). J. Amer. Med. Assoc. 108, 1765-1770.

Sparber, S.B. & Tilson, H.A. (1972a). Psychopharmacologia 23, 220-230.

Sparber, S.B. & Tilson, H.A. (1972b). Life Sci 11, 1059-1067.

Stein, L. & Wise, C.D. (1967). Fed. Proc. Fed. Am. Soc. Exp. Biol. 26, 651.

Stein, L. & Wise, C.D. (1969). J. Comp. Physiol. Psychol. 67, 189-198.

Strada, S.J., Sanders-Bush, E. & Sulser, F. (1970). Biochem. Pharmacol. 19, 2621-2629.

Sutherland, E.W. & Rall, T.W. (1960).Pharmacol. Rev. 12, 265-299.

Svensson, T.H. (1971). Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 271, 170-180.

Tagliamonte, A., Biggio, G., Vargiu, L. & Gessa, G.L. (1973). Life Sci. 12, 277-287.

Tagliamonte, A., Tagliamonte, P., Perez-Cruet, J. & Gessa, G. (1971a). Nature (London) 229, 125-126.

Tagliamonte, A., Tagliamonte, P., Perez-Cruet, J., Stern, S. & Gessa, G. (1971b). J. Pharmacol. Exp. Ther. 177, 475-480.

Taylor, B. (1974). J. Biol. Chem. 249, 454-458.

Taylor, K.M. & Snyder, S.H. (1970). Science 168, 1487-1489.

Taylor, K.M. & Snyder, S.H. (1971). Brain Res. 28, 295-310.

Taylor, W.A. & Sulser, F. (1973). J. Pharmacol. Exp. Ther. 185, 620-632.

Thierry, A.M., Fekete, M. & Glowinski, J. (1968). Eur. J. Pharmacol. 4, 384.

Thoenen, H., Hürlimann, A., Gey, K.F. & Haefely, W. (1966). Life Sci. 5, 1715-1722.

Thoenen, H., Hürlimann, A. & Haefely (1968). J. Pharm. Pharmacol. 20, 1-11.

Thornburg, J.E. & Moore, K.E. (1973). Res. Commun. Chem. Pathol.Pharmacol. 5, 81-89.

Tilson, H.A. & Sparber, S.B. (1972). J. Pharmacol. Exp. Ther. 181, 387-398.

Tseng, L.F., Hitzemann, R.J. & Loh, H.H. (1974). J. Pharmacol. Exp. Ther. 189, 708-716.

Tsukada, Y., Nagata, Y., Hirano, S. & Matsutame, T. (1963). J. Neurochem. 10, 241-256.

Twarog, B.M. & Page, I.H. (1953). Amer. J. Physiol. 175, 157-161.

Udenfriend, S. (1962). Fluorescence Assay in Biology and Medicine pp. 162-164. Academic Press, London.

Ungerstedt, U. (1971a). Acta Physiol. Scand. suppl. 367, 49-68.

Ungerstedt, U. (1971b). Acta Physiol. Scand. Suppl. 367, 1-48.

Ungerstedt, U. (1971c). Thesis from the Department of Histology, Karolinska Institutet, Stockholm, Sweden.

Unväs, B. (1973). Acta Physiol. Scand. 87, 168-173.

Utena, H., Ezoe, T., Kato, N. & Hada, H. (1959). J. Neurochem. 4, 161-169.

Vasko, M.R., Domino, L.E. & Domino, E.F. (1974). Eur. J. Pharmacol. 27, 145-147.

Vogt, M. (1954). J. Physiol. (London) 123, 451-481.

Voigtländer, P.F. von, Moore, K.E. (1973a). Neuropharmacology 12, 451-462.

Voigtländer, P.F. von, Moore, K.E. (1973b). J. Pharmacol. Exp. Ther. 184, 542-552.

Wagner, L.A., Koerker, R.L. & Schneider, F.H. (1973). Biochem. Pharmacol. 22, 430.

Wagner, L.A., Koerker, R.L. & Schneider, F.H. (1974). J. Pharmacol. 26, 464-467.

Waterman, F.A. (1949). Proc. Soc. Exp. Biol. Med. 71, 473-475.

Weil-Malherbe, H. & Szara, S.I. (1971). The Biochemistry of Functional and Experimental Psychoses.

Weis, J. (1973). Life Sci. 13, 475-484.

Weissbach, H., Redfield, B.G. & Axelrod, J. (1961). Biochim. Biophys. Acta 54, 190-192.

Wenger, G.R. & Rutledge, C.O. (1974). J. Pharmacol. Exp. Ther. 189, 725-732.

Wong, D.T., Horng, J.S. & Fuller, R.W. (1973). Biochem. Pharmacol. 22, 311.

Wong, P.W.K., O'Flynn, M.E. & Inuoye, T. (1964). Clin. Chem. 10, 1098.

Wong, D.T., Van Frank, R.M., Horng, J.S. & Fuller, R.W. (1972). J. Pharm. Pharmacol. 24, 171-173.

Wood, K., Swade, C., Harwood, J., Eccleston, E., Bishop, M. & Coppen, A. (1977). Clin. Chim. Acta 80, 299-303.

Yuwiler, A. (1973). J. Neurochem. 20, 1099-1109.

Yuwiler, A., Oldendorf, W.H., Geller, E. & Braun, L. (1977). J. Neurochem. 28, 1015-1023.

Ziance, R.J. & Rutledge, C.O. (1972). J. Pharmacol. Exp. Ther. 180, 118-126.

Ziance, R.J., Azzaro, A.J. & Rutledge, C.O. (1972). J. Pharmacol. Exp. Ther. 182, 284-294.