بسم الله الرحمن الرحيم

NEUROCHEMICAL STUDIES INTO THE MODE OF ACTION OF ANTICONVULSANT DRUGS

By

ABDULLAH SALEM ELHWUEGI, B. Pharm. Sci (Cairo)

Thesis submitted to

THE UNIVERSITY OF NOTTINGHAM

for the degree of

DOCTOR OF PHILOSOPHY

1981.

PAGE NUMBERING AS ORIGINAL

Abstract

Single doses of phenobarbitone decreased the turnover rate of dopamine (DA) and noradrenaline (NA) and increased the whole brain levels of 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA). Habituation to phenobarbitone increased the levels of DA in striata and midbrain and decreased that of cerebral hemispheres, leaving the total amount unchanged. Habituation resisted the depletion otherwise caused by α-methyl-p-tyrosine (α-m.p.t.) in the striata for DA and in the cerebral hemispheres for both DA and NA. Withdrawal of phenobarbitone decreased the levels of DA in the striata and both catecholamines in the cerebral hemispheres. Withdrawal increased the depletion of DA in the striata and cerebral hemispheres and that of NA in the cerebral hemispheres and midbrain caused by α-m.p.t. Withdrawal convulsions increased the levels of DA in the striata and decreased it in the cerebral hemispheres, leaving the total amount unchanged. NA was less in this group than it was in controls. α-m.p.t. protected animals from convulsions. This group showed less DA in the striata and in the cerebral hemispheres and less NA in midbrain. Habituation to phenobarbitone increased the levels of both 5-HT and 5-HIAA. Withdrawal returned both levels to control values. While withdrawal convulsions decreased the levels of 5-HT, 5-HIAA levels were increased. Single doses of phenytoin increased the levels of DA in striata and NA in midbrain. It also increased the levels of 5-HIAA in whole brain and decreased the depletion of 5-HIAA caused by p-chlorophenylalanine (p-c.p.a.) or pargyline. Long term administration of phenytoin increased the levels of dopamine in the striata and the midbrain and decreased that of the cerebral hemispheres. It also produced an increase in the levels of NA in the cerebral hemispheres. Similar effects were observed after α-m.p.t.

Whole brain levels of 5-HT and 5-HIAA were increased after long term treatment with phenytoin. Single doses of carbamazepine increased the levels of NA in the midbrain and decreased the depletion of 5-HIAA after p-c.p.a. and pargyline. The long term treatment with carbamazepine increased the total brain levels of 5-HT and 5-HIAA and those of DA in the striata and cerebral hemispheres and for NA in the cerebral hemispheres and midbrain. The same effect was seen after α-m.p.t. While NA and GABA levels were decreased in the primary focal area one week after cobalt implantation, 5-HIAA levels were increased. The same effect was seen for NA and 5-HIAA levels two weeks after cobalt implantation.

Acknowledgments

I would like to express my gratitude for the help I have received from Professor James Crossland, who has supervised the course of my research and who originally suggested the subject of this investigation. I am grateful to him for the many discussions we have held and for his valuable criticisms and suggestions throughout the course of the work.

I also wish to thank Mrs P. Gott for her assistance with some of the experiments described in chapters 9 and 10 of this thesis.

For the help I have received from the technician staff in our department, especially that of Mr D. Reffin, I am indebted.

The patience and understanding of my wife through the whole course of my study are greatly appreciated.

I wish to acknowledge my indebtedness to Mrs J. Crossland for typing my thesis.

This thesis is dedicated to the memory of my father, SALEM ELHWUEGI.

This work was carried out during a scholarship from the University of Alfateh, Tripoli, for which I am grateful.

CONTENTS

CONTENTS - contd.

CONTENTS - contd.

PART II

CONTENTS - contd.

CONTENTS - contd.

I

INTRODUCTION

Ever since certain chemical substances in the brain were recognized to act as mediators in synaptic transmission, a great deal of interest has been centred on the possible involvement of certain neurotransmitters in the mechanisms of epilepsy. Chen, Ensor and Bohner (1954) found that reserpine (a drug which depletes brain monoamines) decreased seizure thresholds to chemically and electrically induced seizures. Using different drugs which depleted brain monoamines to a different degree, other workers reached the same conclusion as that put forward by Chen and his co-workers. that brain monoamines are involved in the manifestation of seizures.

That epilepsy might be associated with a deficiency in the monoaminergic systems was also supported by several findings that the concentrations of 5-hydroxyindole acetic acid and homovanillic acid in the cerebrospinal fluid were reduced in adults and children with epilepsy (Shaywitz, Cohen and Bowers, 1975; Papeschi, Molina-Nigro, Sourkes and Erba, 1972). 5-Hydroxyindoleacetic acid and, to a lesser degree, homovanillic acid, were reported to be increased in the cerebrospinal fluid of epileptic patients receiving therapeutic doses of phenobarbitone and phenytoin (Chadwick, Jenner and Reynolds, 1975). Moreover, 5-hydroxytryptophan in combination with a peripheral decarboxylase inhibitor was found by Chadwick, Harris, Jenner, Reynolds and Marsden (1975) to be capable of bringing about a considerable improvement or a complete cessation of myoclonus. There are also several reports confirming the original observation reported by Bonnycastle, Giarman and Paasonen (1957), that different anticonvulsant drugs increased the brain levels of 5-hydroxytryptamine.

In our effort to examine the monoamine systems in a more comprehensive way, we studied the effect of acute and chronic treatment with conventional anticonvulsant drugs on the levels and turnover of monoamines in whole and discrete regions of rat brain (Chapters 1 to 7). We made use of the original observation by Crossland and Leonard (1963) that barbiturate withdrawal after its long term administration renders nonepileptic rats acutely epileptic after subjecting them to sound stimulation, and we studied, therefore, the monoamine systems during withdrawal from phenobarbitone and during the subsequent convulsions. The levels of the monoamines were studied as well in a model of chronic epilepsy produced in rats by cobalt implantation (Chapter 9).

γ-Aminobutyric acid is another neurotransmitter which is believed to be involved in epilepsy. Drugs which decrease GABA levels will produce convulsions and some of those which increase its concentration have a protective effect against seizures (Meldrum, 1975; Wood, 1975). We studied the brain GABA levels after acute treatment with the anticonvulsant drugs we used (Chapter 8) and as well in brains of rats rendered chronically epileptic by cobalt implantation (Chapter 9). The gas chromatographic analysis of these anticonvulsant drugs in rat blood and brain and their levels after acute and chronic treatment is reported in chapter 10.

The results are discussed in chapter 11.

PART I

PART I

CHAPTER 1

DETERMINATION OF RAT BRAIN DOPAMINE AND NORADRENALINE CONTENT

The basis of the method used was first described by Anton and Sayre (1962, 1964) with the modifications suggested by Tonge (1969). It permits the determination of both dopamine and noradrenaline in the same extract. It has been successfully used in our laboratories over a number of years.

1.1 Preparation of extract

Rats were killed by cervical dislocation and decapitation, their brains were quickly removed and placed on an ice cooled glass plate. The brain tissue was freed from superficial blood clots and homogenized for five minutes in 10 ml of ice cold 0.4 M perchloric acid, using an MSE top drive homogenizer. The homogenization tubes were washed with 2.5 ml perchloric acid and the homogenates were centrifuged at 2500 rpm for ten minutes.

Ten millilitres of the supernatant solution were transferred to a 50 ml plastic beaker containing 10 mg sodium metabisulphite, 200 mg disodium ethylenediamine tetraacetic acid and 400 mg activated alumina. The preparation of the alumina is described in section 1.6. Fifteen millilitres of 0.4 M perchloric acid were added to the mixture and the pH was adjusted to 8.5 $\pm$ 0.1 with 5 M sodium hydroxide solution and maintained within these pH limits for ten minutes with 1N sodium hydroxide solution. During this time the mixture was stirred using a glass stirring rod attached to an overhead motor. The mixture was then centrifuged at 2000 rpm for ten minutes. The supernatant

solution was carefully decanted and the residue was washed with successive portions of 20 ml and 10 ml of distilled water. The catecholamines were eluted by shaking for twenty minutes with 3 ml of 0.05 M perchloric acid solution on a wrist-action shaker. The resulting mixture was transferred to a glass centrifuge tube and centrifuged at 2500 rpm for fifteen minutes.

1.2 Formation of noradrenaline fluorophore

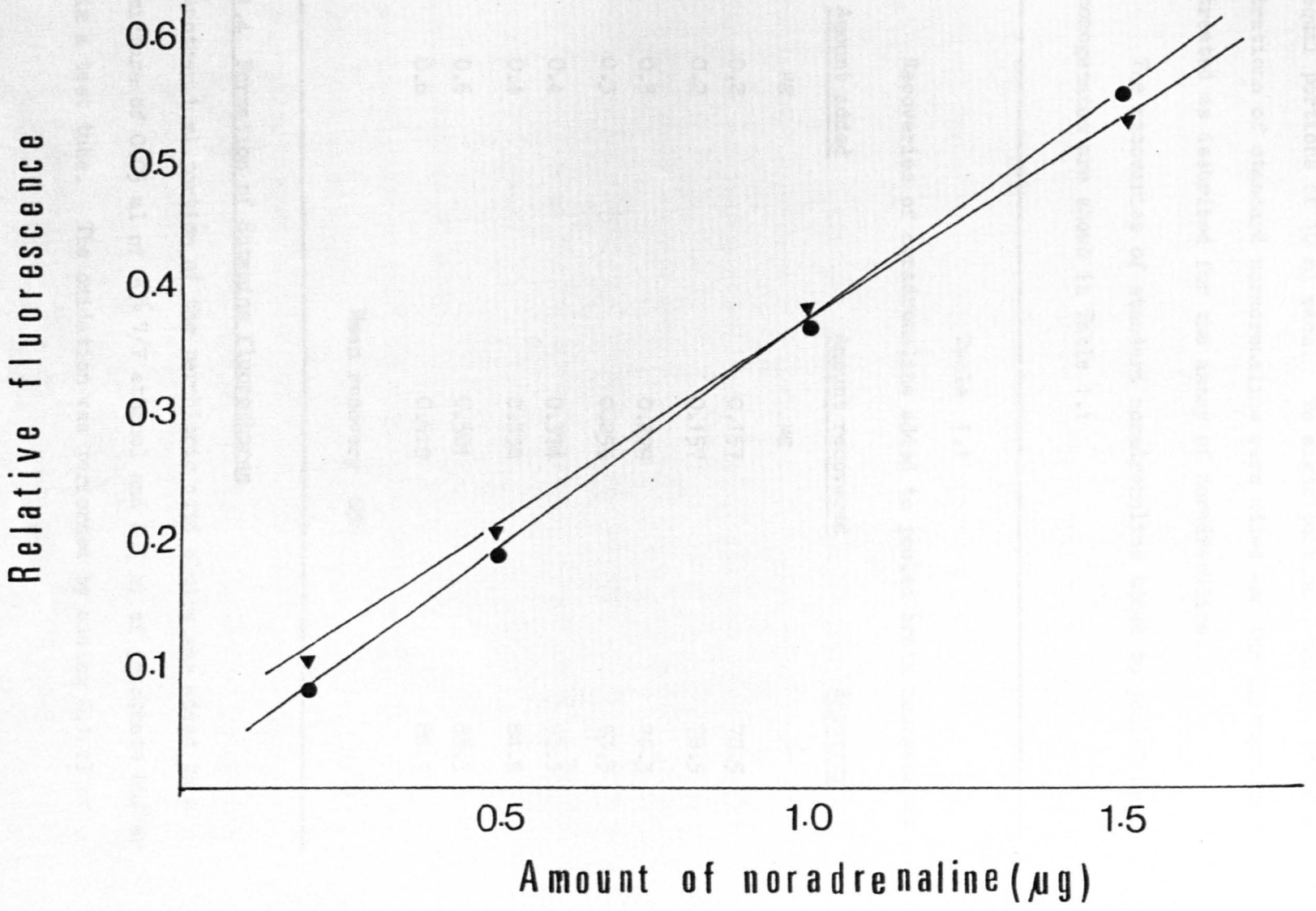
The noradrenaline fluorophores were formed before assaying the dopamine. The method used to assay the noradrenaline content incorporates the modification suggested by Gunne (1963).

A 1.0 ml aliquot of perchloric acid eluate was added to 1.0 ml of acetate buffer in a test tube. Leaving an interval of ten seconds between additions, the oxidation was performed by the addition of 1.0 ml of freshly prepared ferricyanide solution and the mixture was stirred on a vortex mixer. Exactly three minutes later, starting with the first tube to which the ferricyanide solution had been added and again leaving ten seconds between each addition, 1.0 ml of freshly prepared alkali ascorbate solution was added to each tube. The mixture was stirred on a vortex mixer and 1.9 ml of distilled water was then added. The fluorescence of this solution was read twenty minutes later at 505 nm after activation at 400 nm using a Farrand spectrophorofluorimeter.

A calibration curve was constructed by carrying solutions of standard noradrenaline in 0.4 M perchloric acid through the whole procedure side by side with the tissue extract. Examples of such curves are given in Figure 1.1

FIGURE 1.1

Typical calibration curves for the fluorimetric assay of noradrenaline obtained on two separate occasions


Relative fluorescence
0.6
0.5
0.4
0.3
0.2
0.1
0.5
1.0
1.5
Amount of noradrenaline (μg)

1.3 Recovery of standard noradrenaline added to brain homogenates

Ten rats were killed, their brains were removed and each was homogenized in 16 ml 0.4 M perchloric acid. After centrifugation the supernatant solutions were pooled, mixed and divided into fifteen equal portions of 10 ml each. To eight portions, various concentrations of standard noradrenaline were added and the mixtures were treated as described for the assay of noradrenaline.

The recoveries of standard noradrenaline added to pooled brain homogenates are shown in Table 1.1

Table 1.1

Recoveries of noradrenaline added to pooled brain homogenates

Amount added	Amount recovered	Recovery
µg	µg	%
0.2	0.157	78.5
0.2	0.157	78.5
0.3	0.229	76.3
0.3	0.250	83.3
0.4	0.374	93.5
0.4	0.338	84.5
0.6	0.501	83.5
0.6	0.519	86.5

Mean recovery 83%

1.4 Formation of dopamine fluorophores

Another 1 ml portion of the perchloric acid eluate was added to a mixture of 0.05 ml of 70% V/V ethanol and 0.5 ml of phosphate buffer in a test tube. The oxidation was performed by adding 0.1 ml of

periodate solution and the mixture was stirred on a vortex mixer. Exactly one minute after the addition of the periodate solution, 0.5 ml of alkali sulphite solution was added and the mixture was again agitated. As quickly as possible, 1.4 ml of distilled water, 0.5 ml of citrate buffer and 0.85 ml of 3 M orthophosphoric acid were incorporated into the mixture and stirred on a vortex mixer.

The fluorescence of this solution was read in the Farrand spectrophotofluorimeter at 375 nm after activation at 330 nm.

A calibration curve, using standard dopamine in 0.4 M perchloric acid, was constructed for each experiment. Three sample curves are shown in Figure 1.2. They underline the necessity of preparing a calibration curve for each experiment.

1.5 Recovery of standard dopamine added to brain homogenates

The recovery experiment was carried out in a similar fashion to that described for noradrenaline. The results are shown in Table 1.2.

The blank was obtained by carrying 25 ml of 0.4 M perchloric acid, free of dopamine and noradrenaline, through the whole procedure. The composition of the various buffers and reagents is given in the formulary. (Appendix I).

1.6 Preparation of alumina

One hundred grams of aluminium oxide for thin layer chromatography (BDH) were added to 500 ml of 2 M hydrochloric acid and heated to a temperature between 90°C and 100°C, with continuous stirring, for forty-five minutes. The alumina was allowed to settle for two and a half minutes and the yellow-tinged supernatant solution was discarded together with the fine particles of alumina floating in it.

FIGURE 1.2

Typical calibration curves for the fluorimetric assay of dopamine obtained on three separate occasions

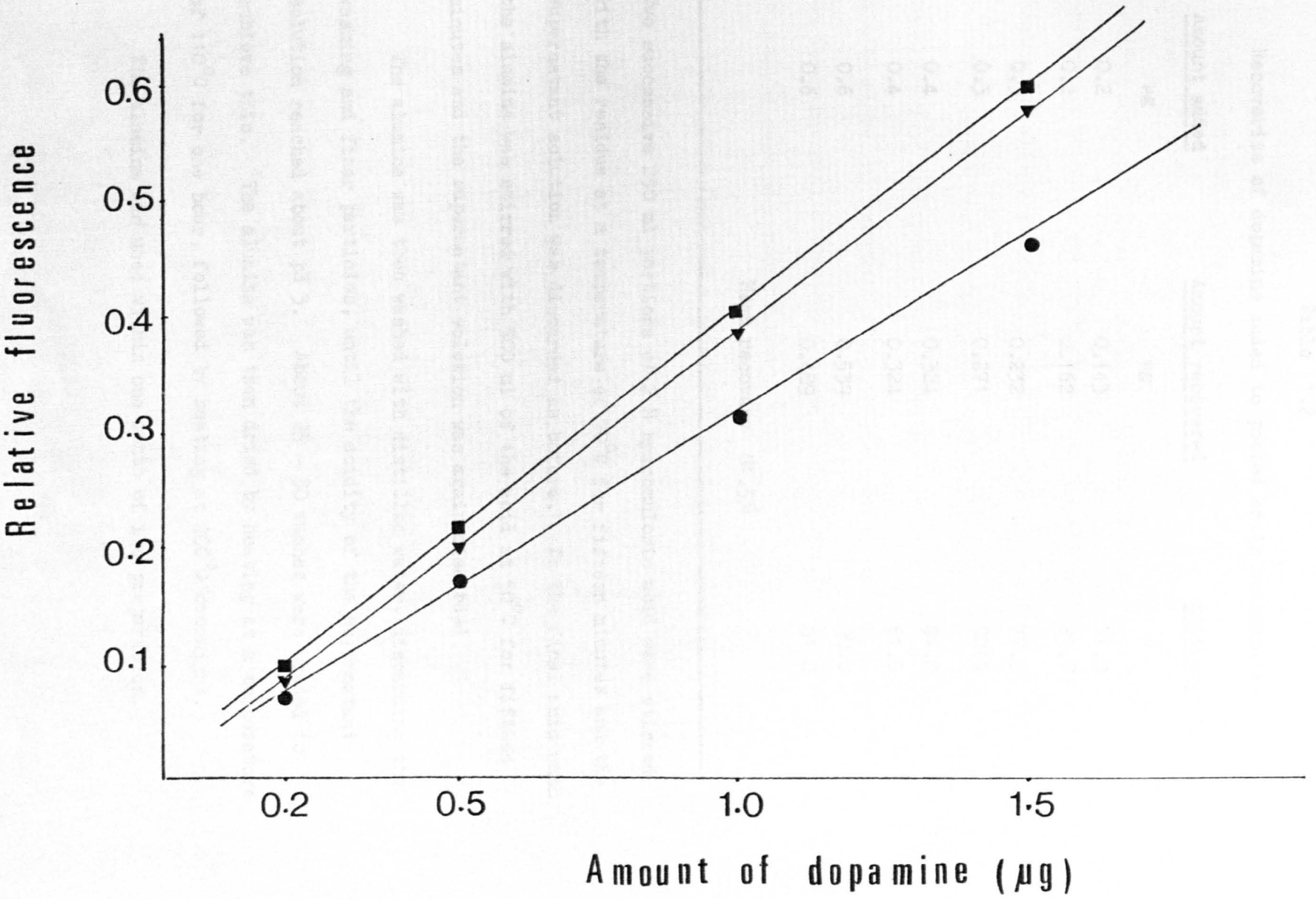

Relative fluorescence
0.6
0·5
0·4
0·3
0·2
0·1
0·2
0·5
1·0
1·5
Amount of dopamine (µg)

Table 1.2

Recoveries of dopamine added to pooled brain homogenates

Amount added	Amount recovered	Recovery
µg	µg	%
0.2	0.143	71.0
0.2	0.162	81.0
0.3	0.232	77.0
0.3	0.271	90.0
0.4	0.324	81.0
0.4	0.324	81.0
0.6	0.539	90.0
0.6	0.489	81.0

Mean recovery 81.5%

Two successive 250 ml portions of 2 M hydrochloric acid were stirred with the residue at a temperature of 70°C for fifteen minutes and the supernatant solution was discarded as before. In the final acid wash the alumina was stirred with 500 ml of the acid at 50°C for fifteen minutes and the supernatant solution was again discarded.

The alumina was then washed with distilled water, discarding the washing and finer particles, until the acidity of the supernatant solution reached about pH 3. About 25 - 30 washes were needed to achieve this. The alumina was then dried by heating at a temperature of 110°C for one hour, followed by heating at 200°C overnight.

The alumina was used within one month of its preparation.

CHAPTER 2

CATECHOLAMINE LEVELS AND TURNOVER AFTER SINGLE DOSES OF ANTICONVULSANT DRUGS

Much of the information in the literature suggests that stimulations of central dopamine and noradrenaline receptors has a predominantly inhibitory action. One might expect, therefore, that endogenous dopamine and noradrenaline in the brain might exert an inhibitory effect on seizure activity.

Kilian and Frey (1973) showed that an increase in the brain concentration of noradrenaline elevated the thresholds for maximal electroshock and pentylene tetrazole seizures in both rats and mice, on the other hand decreasing the brain concentration of the amine increased seizure susceptibility.

These data from the literature led us to investigate the effect of anticonvulsant drugs on the concentrations of dopamine and noradrenaline in rat brain.

2.1 Effect of single doses of anticonvulsant drugs on brain dopamine and noradrenaline content

Female Ash Wistar rats weighing between 140 and 160 gms were used. The animals were obtained one week before carrying out any experiment. They were caged in sixes and allowed free access to food and water. Drug doses were calculated according to the animals' body weight and were injected intraperitoneally in a solution or suspension volume of 0.5 ml per 200 gm weight.

Phenytoin was given in a dose of 75 mg/kg suspended in saline containing 1% Tween 80, phenobarbitone in a dose of 100 mg/kg in

saline, sodium bromide in a dose of 1 gm/kg also dissolved in saline and carbamazepine in a dose of 50 mg/kg in propylene glycol. Control rats received the appropriate vehicle.

Apart from sodium bromide, all the anticonvulsant drugs, in the doses employed in these experiments, produced ataxia. In the case of phenobarbitone, loss of righting reflexes occurred. The ataxia produced by carbamazepine and phenytoin started to disappear about ninety minutes after the injection, while the phenobarbitone group were affected for up to three hours, although they regained their righting reflexes about two hours after injection.

Half or one hour after receiving the doses stated, the animals were killed by cervical dislocation and their brains were quickly removed, homogenized and centrifuged.

The supernatant solutions were kept in the deep freeze until the second day when they were assayed for their dopamine and noradrenaline contents by the methods described in chapter 1. All killings were done between 14.00 and 16.00 hours.

As shown in tables 2.1(i) and 2.1(ii), none of the anticonvulsant drugs tested had any significant effect on dopamine or noradrenaline content of rat brains whether the animals were killed thirty minutes or sixty minutes after injection.

It will be seen that the estimates obtained for both the noradrenaline and the dopamine content of the brains of the untreated animals were different in the two experiments. This point is taken up again in the discussion.

Table 2.1(i)

Effect of anticonvulsant drugs on brain catecholamine content thirty minutes after injection

Drug	Dopamine ng/gm	Noradrenaline ng/gm
Vehicle (16)	725 ± 24	372 ± 5
Phenytoin (8)	729 ± 15	371 ± 15
Sodium bromide (8)	740 ± 25	343 ± 15
Carbamazepine (8)	754 ± 20	404 ± 30
Phenobarbitone (8)	725 ± 35	392 ± 10

Each value is the mean ± standard error from the number of animals, shown in parenthesis.

The figures were obtained in two different experiments.

Table 2.1(ii)

Effect of anticonvulsant drugs on brain catecholamine content sixty minutes after injection

Drug	Dopamine ng/gm	Noradrenaline ng/gm
Vehicle (16)	829 ± 30	443 ± 10
Phenytoin (8)	806 ± 10	421 ± 5
Sodium bromide (8)	785 ± 5	421 ± 10
Carbamazepine (8)	788 ± 5	429 ± 5
Phenobarbitone (8)	818 ± 10	431 ± 20

Each value is the mean ± standard error from the number of animals, shown in parenthesis.

The figures were obtained in two different experiments.

2.2 Effect of single doses of anticonvulsant drugs on the turnover rate of catecholamines in the rat brain

Costa in 1972 suggested that neuronal activity controls the rate of catecholamine turnover leaving the steady state concentration virtually unchanged. The simple measurement of the catecholamine concentration is therefore relatively uninformative in elucidating the extent to which catecholaminergic neurones participate in behavioural changes and in defining the sites of action of drugs that affect catecholamine systems. More useful information should come from measurements of turnovers. We chose to assess the turnover of catecholamines by means of α-methyl-p-tyrosine which acts by inhibition of tyrosine hydroxylase. Dolphin, Jenner and Marsden (1975) reported an interference by α-methyl-p-tyrosine in the assay procedure of noradrenaline using the method described by Maickel, Cox, Saillant and Miller (1968). We therefore performed an experiment to see whether α-methyl-p-tyrosine interferes with the assay procedure we used.

One hundred µg of α-methyl-p-tyrosine methyl ester hydrochloride were added to pooled brain homogenates and external noradrenaline and dopamine standards and the assay was performed as described in chapter 1. No alteration in dopamine, noradrenaline or blank fluorescence were observed.

The first experiment to be done was to determine the time course effect of α-methyl-p-tyrosine. A dose of 250 mg/kg of α-methyl-p-tyrosine methyl ester hydrochloride in saline was injected intraperitoneally and the animals were killed by cervical dislocation and decapitation at various times thereafter.

The results are presented in table 2.2 and figure 2.1. As can be seen from the figure, the rate of depletion of brain dopamine is greater than that of noradrenaline during the first three hours, indicating that dopamine turns over at the faster rate. By ten hours, however, the amount of dopamine in the brain is virtually indistinguishable from that of noradrenaline.

Table 2.2

Time course effect of 250 mg/kg α-methyl-p-tyrosine on brain dopamine and noradrenaline content

Time	Dopamine	Change from zero time	Noradrenaline	Change from zero time
hrs.	ng/gm	per cent	ng/gm	per cent
0	834 ± 28 (6)		422 ± 12 (6)	
1.5	496 ± 11 (3)	- 40.50	322 ± 17 (3)	- 23.60
3	308 ± 29 (3)	- 63.06	246 ± 11 (3)	- 41.70
6	180 ± 4.6 (3)	- 78.40	163 ± 11 (3)	- 61.37
10	154 ± 3.4 (3)	- 81.53	126 ± 2.8 (3)	- 70.14
24	95 ± 1.7 (3)	- 88.60	82 ± 6.0 (3)	- 80.50

Each value is the mean ± standard error from the number of animals shown in parenthesis.

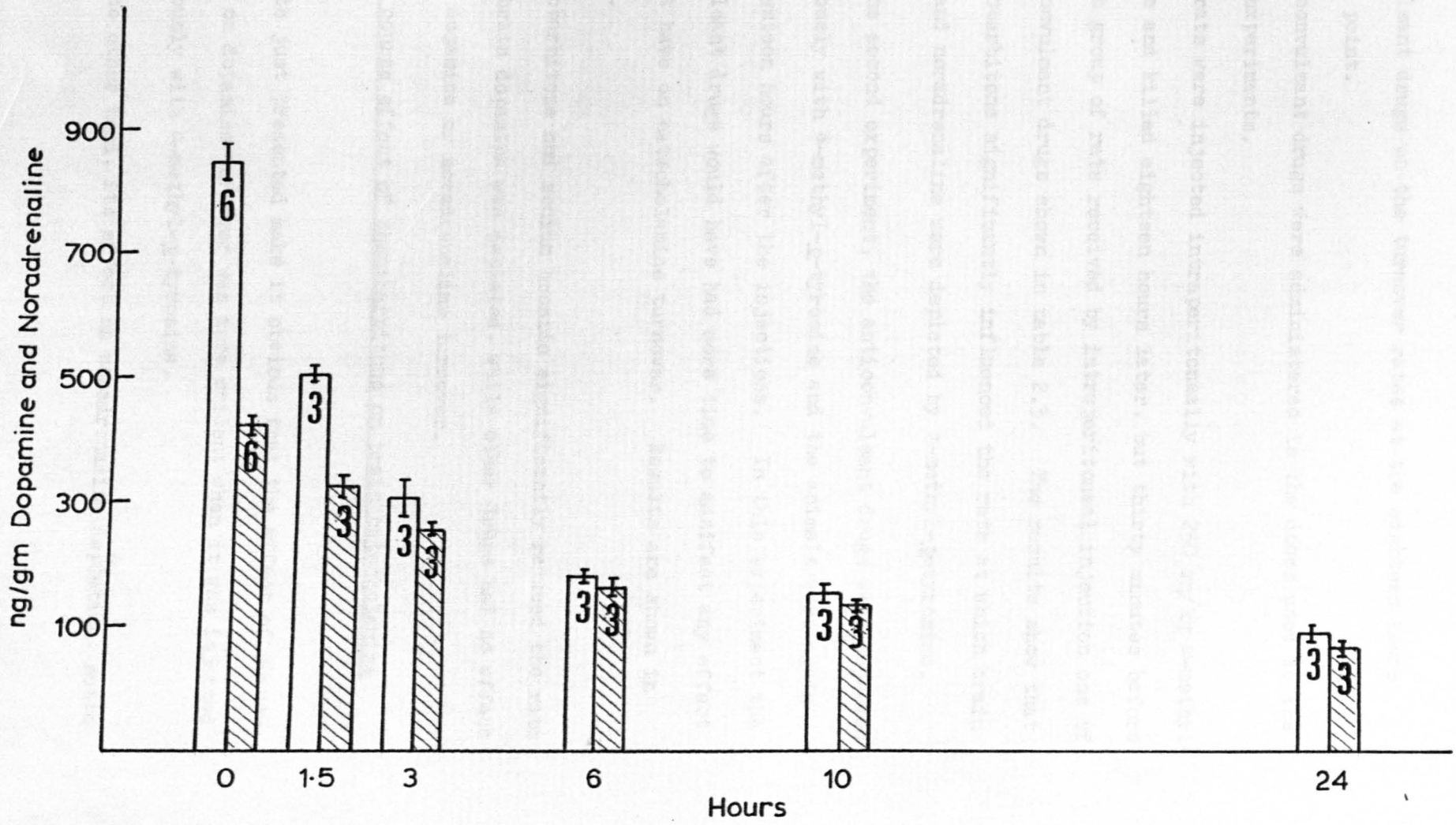
As can be seen from figure 2.1, the maximum depletion of both catecholamines occurred between ten and twenty-four hours after injection of α-methyl-p-tyrosine, so we decided to investigate the effect of

FIGURE 2.1

Time course effect of 250 mg/kg α-methyl-p-tyrosine on brain dopamine and noradrenaline content

Each bar indicates mean ± standard error for the number of animals inside the column

Open columns represent dopamine and hatched columns represent noradrenaline


ng/gm Dopamine and Noradrenaline
900
700
500
300
100
0
1·5
3
6
10
24
Hours

anticonvulsant drugs on the turnover rates at the eighteen hours depletion point.

Anticonvulsant drugs were administered in the doses used in the previous experiments.

The rats were injected intraperitoneally with 250 mg/kg α-methyl-p-tyrosine and killed eighteen hours later, but thirty minutes before death each group of rats received by intraperitoneal injection one of the anticonvulsant drugs shown in table 2.3. The results show that only phenobarbitone significantly influenced the rate at which brain dopamine and noradrenaline were depleted by α-methyl-p-tyrosine.

In the second experiment, the anticonvulsant drugs were injected simultaneously with α-methyl-p-tyrosine and the animals were again killed eighteen hours after the injections. In this experiment the anticonvulsant drugs would have had more time to manifest any effect they might have on catecholamine turnover. Results are shown in table 2.4.

Phenobarbitone and sodium bromide significantly reduced the rate at which brain dopamine was depleted, while other drugs had no effect on either dopamine or noradrenaline turnover.

2.3 Time course effect of phenobarbitone on brain catecholamine turnover

The results just presented make it obvious that the effect of phenobarbitone on dopamine turnover was more evident when it was injected simultaneously with α-methyl-p-tyrosine.

On the other hand, its effect on noradrenaline depletion. quite

Table 2.3

The effect of anticonvulsant drugs on turnover rate of brain catecholamine

Drug	Dopamine ng/gm	Noradrenaline ng/gm
Vehicle only	828 ± 30.0 (12)	416 ± 10.0 (12)
α-methyl-p-tyrosine	91 ± 5.1 (12)	97 ± 5.7 (12)
per cent change	- 89.0 %	- 76.7 %
α-methyl-p-tyrosine + phenytoin	80 ± 5.7 (4)	105 ± 3.4 (4)
per cent change	- 90.0 %	- 77.2 %
P (t test)	N.S.	N.S.
α-methyl-p-tyrosine + phenobarbitone	131 ± 5.7 (4)	138 ± 5.7 (4)
per cent change	- 84.0 %	- 66.8 %
P (t test)	< 0.02	< 0.02
α-methyl-p-tyrosine + sodium bromide	92 ± 5.7 (4)	95 ± 5.7 (4)
per cent change	- 88.9 %	- 77.1 %
P (t test)	N.S.	N.S.
α-methyl-p-tyrosine + carbamazepine	97 ± 7.7 (4)	103 ± 4.8 (4)
per cent change	- 88.2 %	- 75.2 %
P (t test)	N.S.	N.S.

The animals were killed half an hour after the anticonvulsant drugs and eighteen hours after the α-methyl-p-tyrosine.

Each value is the mean ± standard error from the number of animals shown in parentheses.

Table 2.4

The effect of anticonvulsant drugs on turnover rate of brain catecholamine

Drug	Dopamine ng/gm	Noradrenaline ng/gm
Vehicle only	828 ± 30.0 (12)	416 ± 10.0 (12)
α-methyl-p-tyrosine	93 ± 5.7 (12)	106 ± 5.1 (12)
per cent change	- 88.7 %	- 74.5 %
α-methyl-p-tyrosine + phenytoin	107 ± 5.7 (4)	106 ± 5.7 (4)
per cent change	- 87.0 %	- 74.5 %
P (t test)	< 0.1	N.S.
α-methyl-p-tyrosine + phenobarbitone	184 ± 5.7 (4)	111 ± 1.1 (4)
per cent change	- 77.7 %	- 73.3 %
P (t test)	< 0.001	N.S.
α-methyl-p-tyrosine + sodium bromide	129 ± 5.7 (4)	110 ± 4.0 (4)
per cent change	- 84.4 %	- 73.5 %
P (t test)	< 0.02	N.S.
α-methyl-p-tyrosine + carbamazepine	95 ± 6.3 (4)	105 ± 6.0 (4)
per cent change	- 88.5 %	- 74.7 %
P (t test)	N.S.	N.S.

The animals were killed eighteen hours after the simultaneous injection of the anticonvulsant drug and α-methyl-p-tyrosine.

Each value is the mean ± standard error from the number of animals shown in parentheses.

evident when it was injected thirty minutes before death, was not seen when it was injected simultaneously with α-methyl-p-tyrosine. This unexpected result led us to study the effect of phenobarbitone on the time course depletion of noradrenaline and dopamine caused by α-methyl-p-tyrosine.

Ash Wistar rats, divided into groups, received an intraperitoneal injection of either α-methyl-p-tyrosine (250 mg/kg) or of α-methyl-p-tyrosine and phenobarbitone (100 mg/kg) together. They were killed after different time intervals.

Results are shown in tables 2.5 and 2.6 and figures 2.2 and 2.3.

The results show that phenobarbitone produced a significant decrease in the rate of depletion of dopamine at all times studied, whereas it was only capable of preventing significantly the depletion of noradrenaline at the three and six hours period. The depletion was potentiated at the eighteen hours period. This point will be dealt with in detail in the discussion.

2.4 Behavioural study on α-methyl-p-tyrosine

α-Methyl-p-tyrosine is a potent inhibitor of tyrosine hydroxylase, the enzyme responsible for the rate limiting step in the synthesis of the catecholamines.

The catecholamines are thought to regulate mood and behaviour, and their reduced availability may lead to depressive disorders. In our experiment we noticed that rats which were treated with α-methyl-p-tyrosine showed behavioural symptoms reminiscent of reserpine syndrome. They looked quiet most of the time and their response to external stimuli was reduced. Moderate piloerection was also noticed and all

Table 2.5

Time course effect of phenobarbitone on brain dopamine turnover

Time	α-methyl-p-tyrosine	α-methyl-p-tyrosine + phenobarbitone	statistical significance
hrs	ng/gm	ng/gm	P
0	806 ± 10 (4)	806 ± 10 (4)	
3	348 ± 1 (3)	494 ± 10 (3)	< 0.001
6	213 ± 8 (3)	346 ± 6 (3)	< 0.001
12	170 ± 9 (3)	210 ± 8 (3)	< 0.005
18	93 ± 10 (3)	166 ± 8 (3)	< 0.005

Each value is the mean ± standard error from the number of animals shown in parentheses

Table 2.6

Time course effect of phenobarbitone on brain noradrenaline turnover

Time	α-methyl-p-tyrosine	α-methyl-p-tyrosine + phenobarbitone	statistical significance
hrs	ng/gm	ng/gm	P
0	420 ± 8.0 (4)	420 ± 8.0 (4)	
3	247 ± 4.6 (3)	289 ± 2.3 (3)	< 0.005
6	159 ± 9.8 (3)	213 ± 1.7 (3)	< 0.005
12	100 ± 7.0 (3)	110 ± 10.0 (3)	N.S.
18	127 ± 9.8 (3)	104 ± 5.7 (3)	< 0.05

Each value is the mean ± standard error from the number of animals shown in parentheses.

FIGURE 2.2

Time course effect of phenobarbitone on brain dopamine turnover

Each bar indicates mean $\pm$ standard error for the number of animals inside the columns.

Open columns represent α-methyl-p-tyrosine treatment, hatched columns represent treatment with α-methyl-p-tyrosine and phenobarbitone. Both drugs were injected simultaneously.

Significantly different by the t test (* $P < 0.005$ and ** $P < 0.001$) from those of control rats treated with α-methyl-p-tyrosine only.

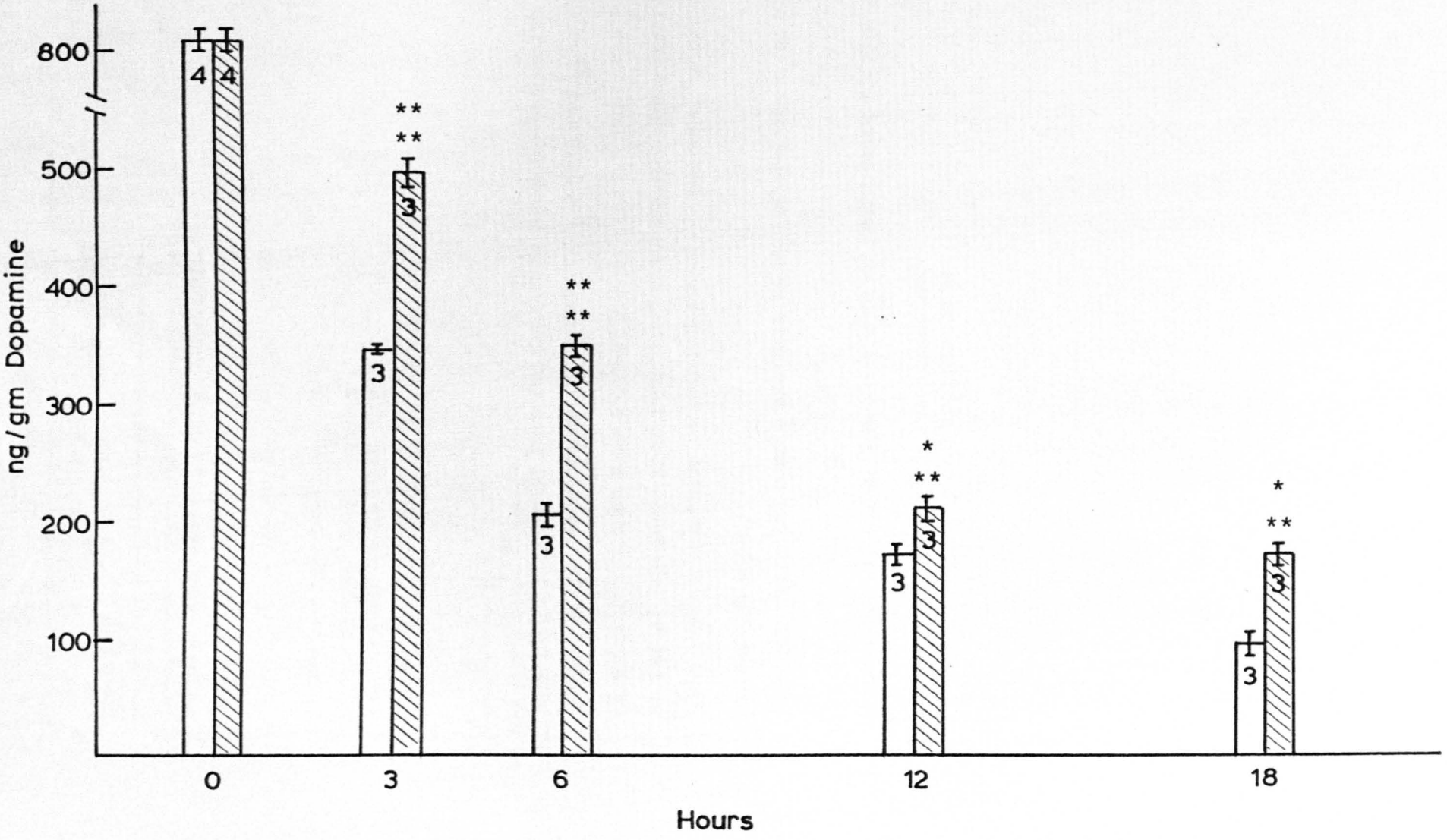

800
500
400
300
200
100
ng/gm Dopamine
4
4
3
3
3
3
3
3
**
**
**
**
*
**
*
**
0
3
6
12
18
Hours

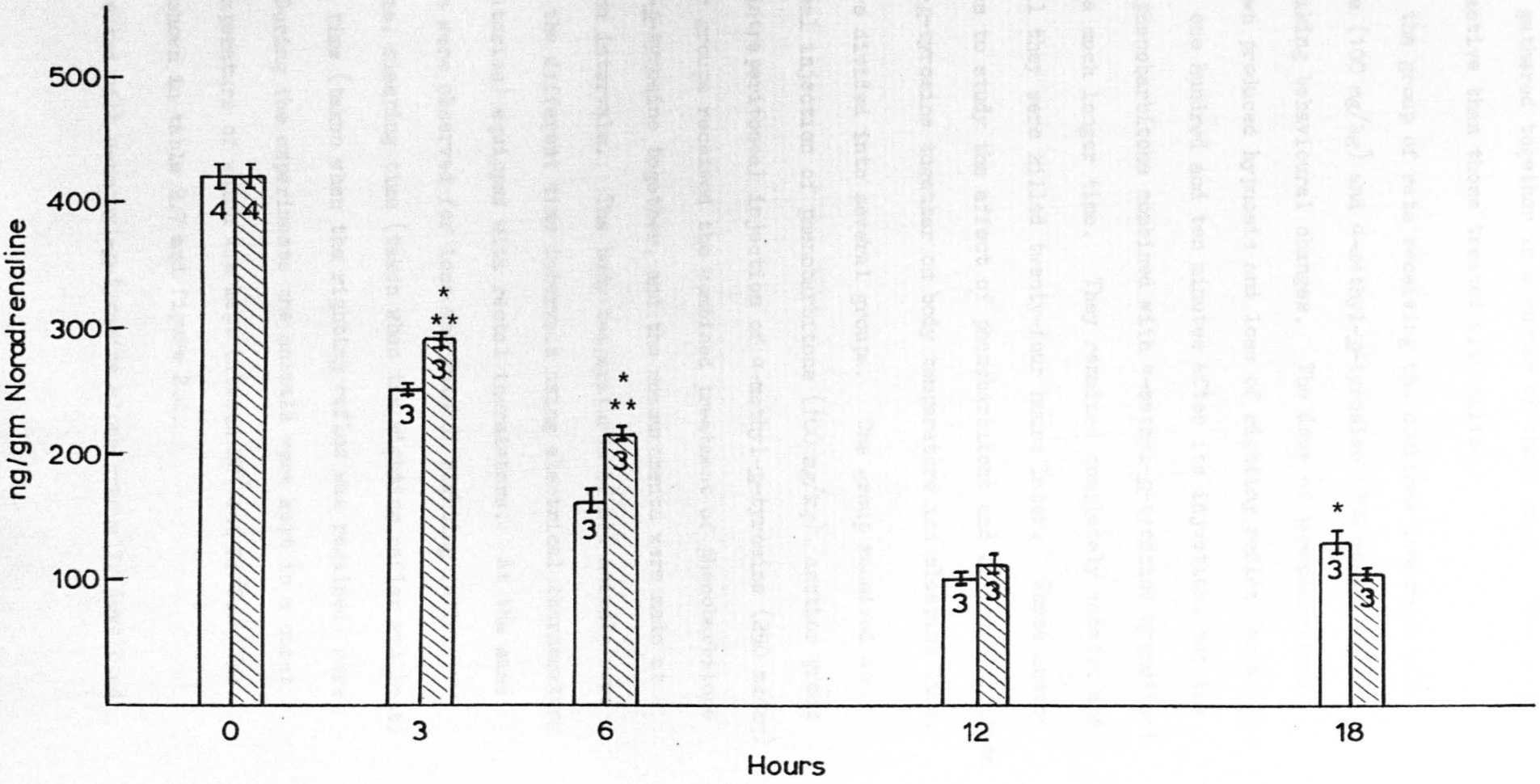
FIGURE 2.3

Time course effect of phenobarbitone on brain noradrenaline turnover

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent α-methyl-p-tyrosine treatment, hatched columns represent treatment with α-methyl-p-tyrosine and phenobarbitone. Both drugs were injected simultaneously.

Significantly different by the t test (* $P < 0.05$ and ** $P < 0.005$) from those of control rats injected with α-methyl-p-tyrosine only.


ng/gm Noradrenaline
500
400
300
200
100
4
4
3
3
3
3
3
3
*
**
*
**
*
0
3
6
12
18
Hours

the rats were gathered together in a corner of their cage. They were clearly less active than those treated with saline.

However, the group of rats receiving the combined treatment with phenobarbitone (100 mg/kg) and α-methyl-p-tyrosine (250 mg/kg) showed much more striking behavioural changes. The dose of phenobarbitone used on its own produced hypnosis and loss of righting reflex for a time of up to one hundred and ten minutes after its injection, but the same dose of phenobarbitone combined with α-methyl-p-tyrosine hypnotized the rats for a much longer time. They remained completely sedated and inactive until they were killed twenty-four hours later. These observations led us to study the effect of phenobarbitone and of phenobarbitone and α-methyl-p-tyrosine together on body temperature and sleeping time.

Rats were divided into several groups. One group received an intraperitoneal injection of phenobarbitone (100 mg/kg), another group received an intraperitoneal injection of α-methyl-p-tyrosine (250 mg/kg) and the other groups received the combined treatment of phenobarbitone and α-methyl-p-tyrosine together, and the measurements were made at different time intervals. The body temperatures of all animals were monitored at the different time intervals using electrical thermometers (Light laboratories) equipped with rectal thermistors. At the same time the rats were observed for loss and gain of righting reflex. Induction time, sleeping time (taken when the righting reflex was lost) and recovery time (taken when the righting reflex was regained) were recorded. During the experiments the animals were kept in a quiet room, the temperature of which was kept between 20° and 22°C. The results are shown in table 2.7 and figure 2.4.

Rats treated with α-methyl-p-tyrosine alone gradually developed

Table 2.7

Effect of α-methyl-p-tyrosine on sleeping time induced by injection of phenobarbitone

Drug	Induction time	Sleeping time	Recovery (induction + sleeping)
	minutes	minutes	minutes
Phenobarbitone	21.66 ± 2.9 (6)	83.3 ± 0.67 (6)	105 ± 3.6 (6)
Phenobarbitone + α-methyl-p-tyrosine	14.33 ± 2.18*(6)	220.0 ± 3.84**(6)	241.7 ± 4.4**(6)

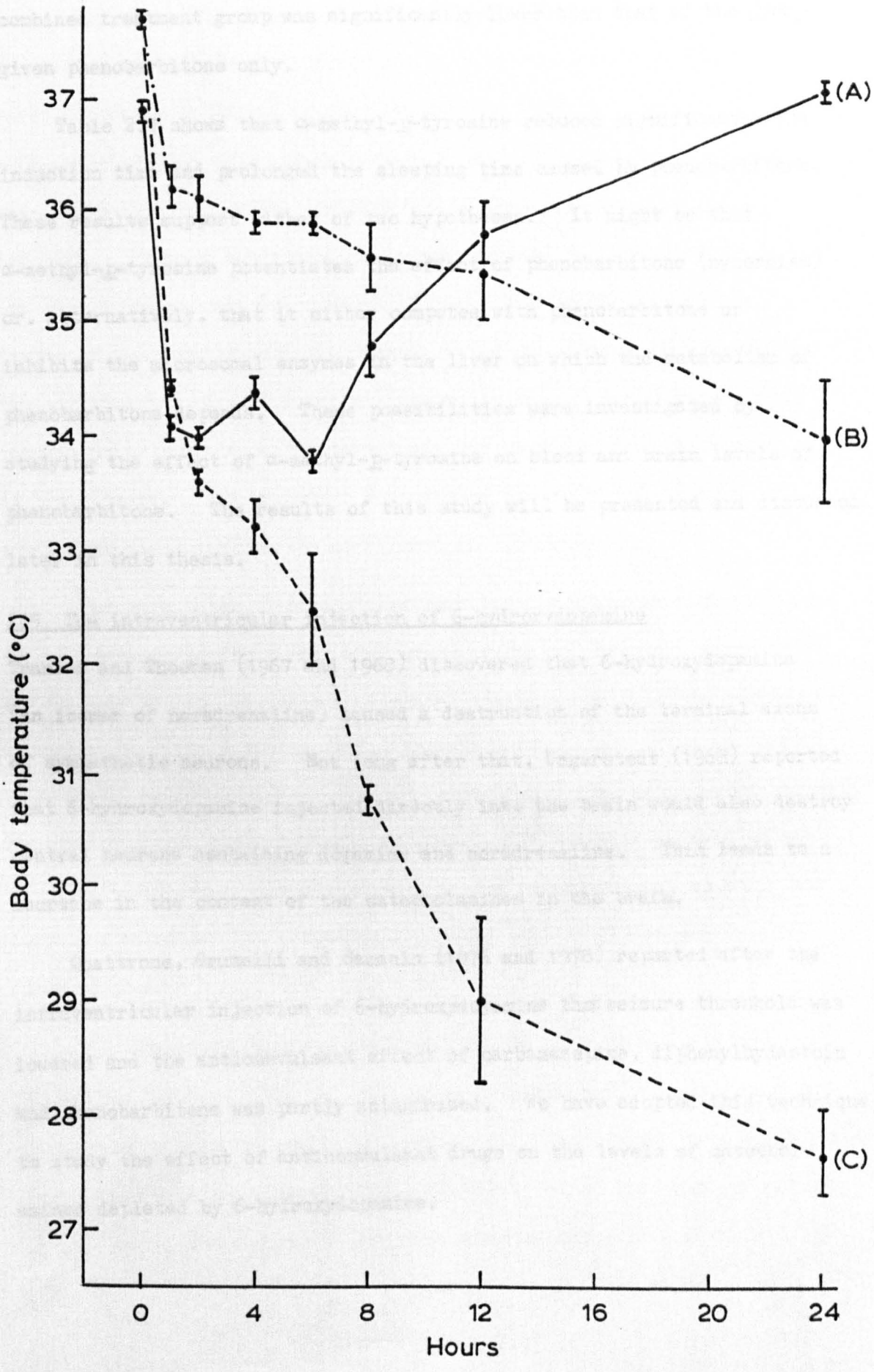
Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$ ** $P < 0.001$) from rats treated with phenobarbitone.

signs of hypothermia. The body temperature was still dropping twenty-four hours after the injection (Figure 2.4). Phenobarbitone produced a significant decrease in body temperature that reached a maximum after one hour and lasted for up to six hours after injection. It then started to recover. The combined treatment with phenobarbitone and α-methyl-p-tyrosine, however, produced a progressive and continuous fall in body temperature. For the first two hours, the fall in temperature occurred at the same rate as that following administration of phenobarbitone only. Thereafter, whereas phenobarbitone produced no further change in body temperature, the combined treatment continued to cause hypothermia, so that after four hours the temperature of the

FIGURE 2.4

Time course effect of phenobarbitone (A), α-methyl-_p_-tyrosine (B) and the combined treatment with phenobarbitone and α-methyl-_p_-tyrosine (C) on rats' body temperatures. Each point is the mean ± standard error for three rats.


37
36
35
34
33
32
31
30
29
28
27
Body temperature (°C)
0
4
8
12
16
20
24
Hours
(A)
(B)
(C)

combined treatment group was significantly lower than that of the group given phenobarbitone only.

Table 2.7 shows that α-methyl-p-tyrosine reduced significantly the induction time and prolonged the sleeping time caused by phenobarbitone. These results support either of two hypotheses. It might be that α-methyl-p-tyrosine potentiates the effect of phenobarbitone (synergism) or, alternatively, that it either competes with phenobarbitone or inhibits the microsomal enzymes in the liver on which the metabolism of phenobarbitone depends. These possibilities were investigated by studying the effect of α-methyl-p-tyrosine on blood and brain levels of phenobarbitone. The results of this study will be presented and discussed later in this thesis.

2.5 The intraventricular injection of 6-hydroxydopamine

Tranzer and Thoenen (1967 and 1968) discovered that 6-hydroxydopamine (an isomer of noradrenaline) caused a destruction of the terminal axons of sympathetic neurons. Not long after that, Ungerstedt (1968) reported that 6-hydroxydopamine injected directly into the brain would also destroy central neurons containing dopamine and noradrenaline. This leads to a decrease in the content of the catecholamines in the brain.

Quattrone, Crunelli and Samanin (1976 and 1978) reported after the intraventricular injection of 6-hydroxydopamine that seizure threshold was lowered and the anticonvulsant effect of carbamazepine, diphenylhydantoin and phenobarbitone was partly antagonized. We have adopted this technique to study the effect of anticonvulsant drugs on the levels of catecholamines depleted by 6-hydroxydopamine.

Method of injection of 6-hydroxydopamine into lateral ventricle:

The method for injection of materials into the lateral ventricle was first described by Noble, Wurtman and Axelrod (1967). Ash Wistar rats were anaesthetized with sodium pentobarbitone (50 mg/kg) and the head was placed in a horizontal position and in line with the long axis of the body. Using a sharp scalpel, a mid-sagittal incision was made. The bregma was exposed and cleaned of muscle and fascia. Using a dental drill, a small hole was made 1.5 - 2.0 mm lateral to the crossing of the sagittal and coronal sutures (Figure 2.5 (i)). The skin was then sutured using sterile cotton thread and the rats were left to recover in their cages. The procedure was carried out under semisterile conditions using instruments sterilized in 70% alcohol.

One week later, the rats were very lightly anaesthetized with ether. a 50 µl syringe equipped with a 27 gauge needle and having a stop at 3.5 mm from its tip was held in vertical position and 20 µl of 6-hydroxydopamine (containing 200 µg of the hydrochloride in normal saline with 1% ascorbic acid) was injected slowly through the hole into the ventricle. The needle was allowed to remain in place for about fifteen seconds and was then withdrawn. The second step in the experiment was to determine whether this method of administration would deliver the material into the ventricles.

Two rats were prepared as described, 20 µl of myodil iophendylate (Glaxo) was injected into the ventricles as described before and the head of the rat was exposed laterally to X-ray film. Photographs were taken immediately and again two minutes after injection. From the plate in Figure 2.5 (ii) it is clear that the solution injected was distributed

FIGURE 2.5 (i)

Drawing of rat skull to indicate the position of the injection hole

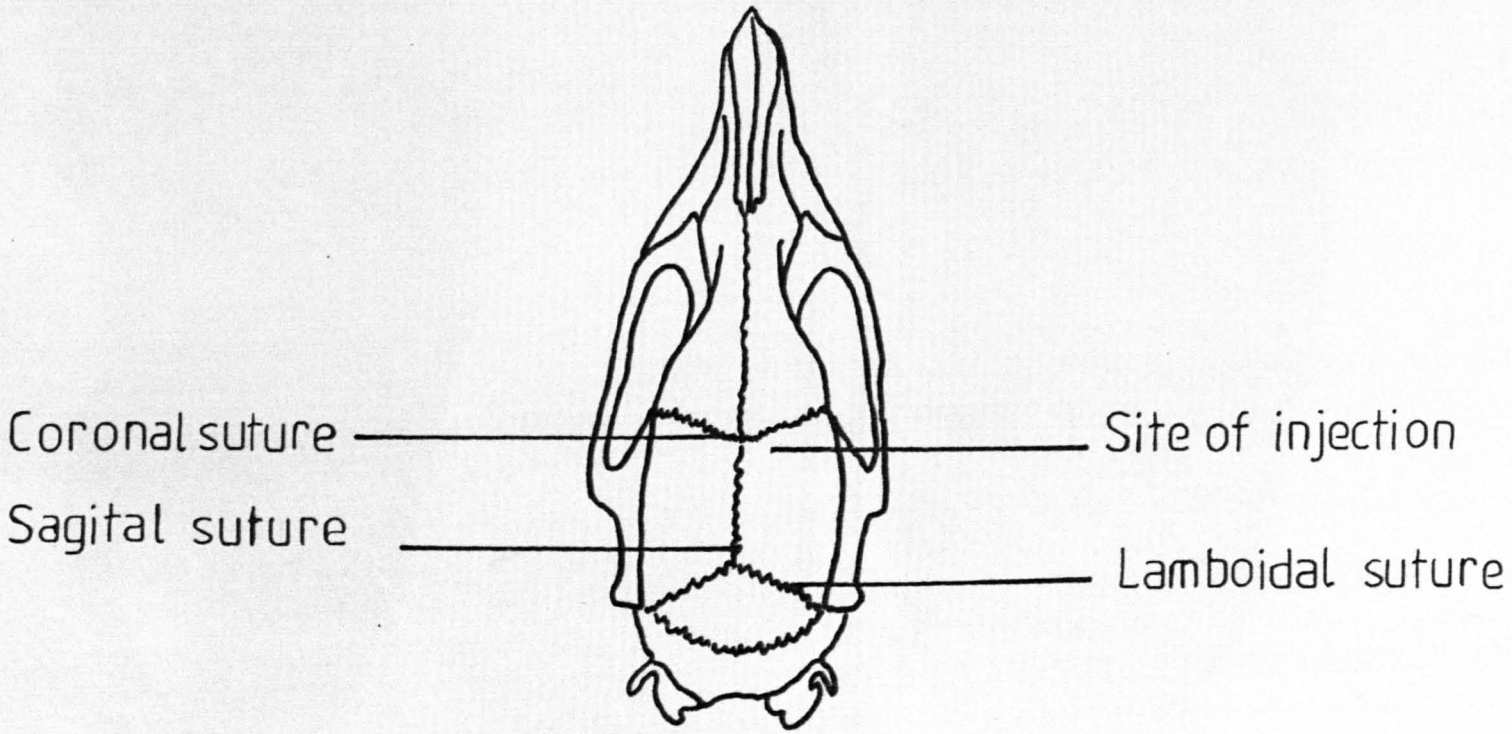
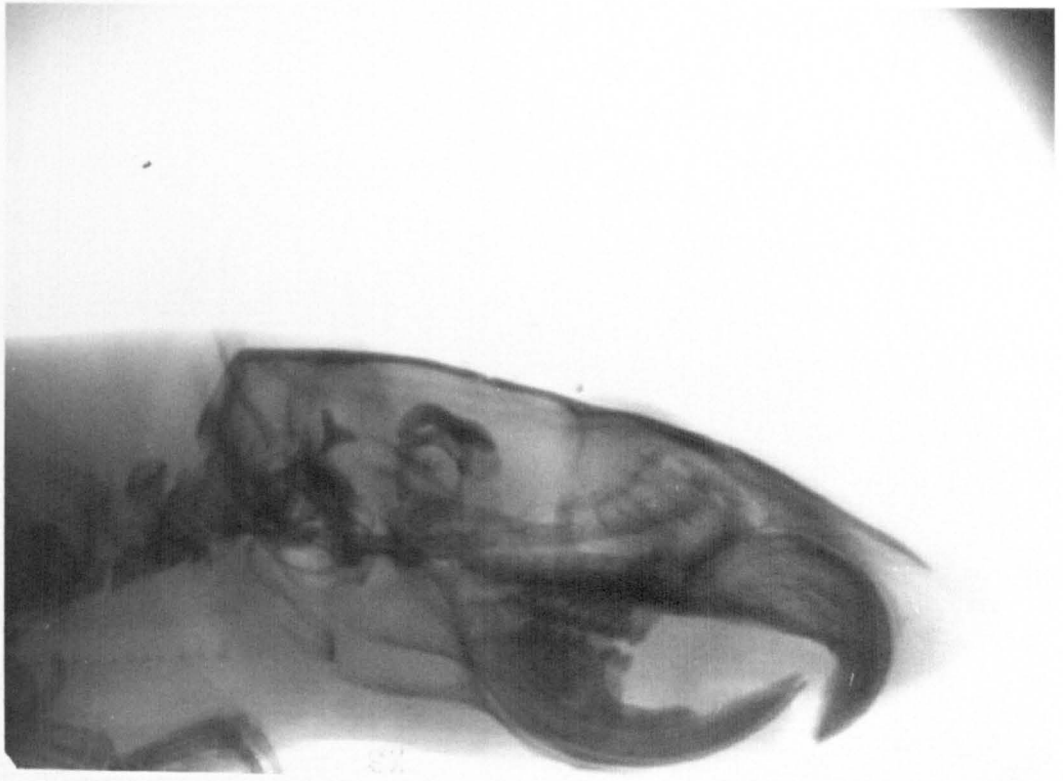
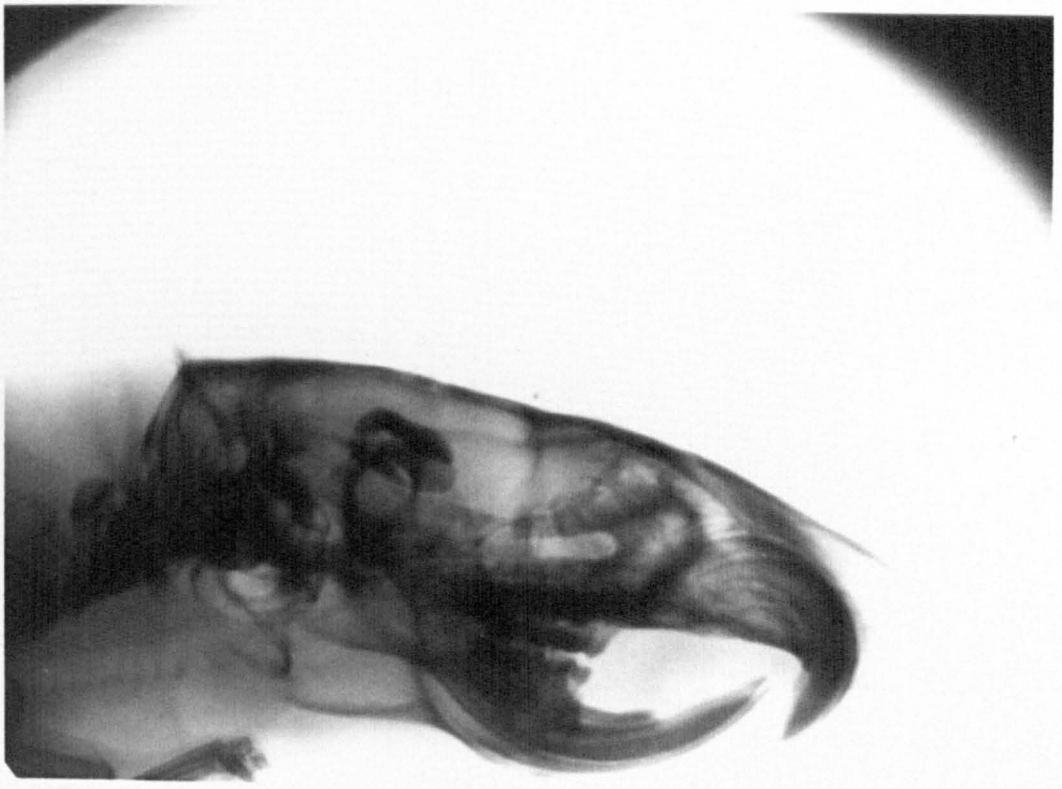

Coronal suture
Sagital suture
Site of injection
Lamboidal suture

FIGURE 2.5 (ii)

Lateral view of rat skull (a) immediately, (b) two minutes after the intraventricular injection of myodil iophendylate



(A)



(B)

to all the ventricles, but it did not fill the first ventricle completely. From this result we were satisfied that the injection was truly intraventricular.

Breese and Traylor (1970) reported that the intraperitoneal injection of pargyline (50 mg/kg) before the intraventricular injection of 6-hydroxydopamine increased the degree of depletion of dopamine. This was taken into consideration when designing the next experiment.

2.6 Time course effect of 6-hydroxydopamine on rat brain dopamine and noradrenaline

Ash Wistar rats divided into groups of four were prepared for intraventricular injection in the manner that was described before. Subsequently each group of rats received an intraperitoneal injection of either saline or pargyline (50 mg/kg) followed thirty minutes later by an intraventricular injection of 6-hydroxydopamine hydrochloride (200 µg in 20 µl saline containing 1% ascorbic acid). Each group had a control group treated in the same manner but saline containing 1% ascorbic acid was injected intraventricularly instead of 6-hydroxydopamine. The rats were killed at various times thereafter. Results are presented in tables 2.8 and 2.9 and figures 2.6 and 2.7.

The results show that 6-hydroxydopamine on its own was capable of depleting only 14% of the whole brain dopamine. On the other hand, when the administration of pargyline preceded 6-hydroxydopamine treatment by thirty minutes, the depleting action was greatly potentiated, up to 50% of the dopamine being lost. With noradrenaline, on the other hand, 6-hydroxydopamine produced about 60% depletion which was not potentiated with pagyline pretreatment. The unusually large standard error associated

Table 2.8

Time course effect of intraventricular injection of 6-hydroxydopamine on dopamine content of rat brain

Days	6-hydroxydopamine	Change	6-hydroxydopamine + pargyline	Change
	ng/gm	per cent	ng/gm	per cent
0	830 ± 10 (8)		830 ± 10 (8)	
2	791 ± 58 (4)	- 4.70	766 ± 36 (4)	- 7.71
4	780 ± 35 (4)	- 6.02	638 ± 41 (4)	- 23.13
8	784 ± 54 (4)	- 5.54	415 ± 44 (4)	- 50.00
12	713 ± 58 (4)	- 14.09	450 ± 44 (4)	- 45.78

Each value is the mean ± standard error from the number of animals shown in parentheses.

Zero time refers to control animals.

Table 2.9

Time course effect of intraventricular injection of 6-hydroxydopamine on noradrenaline content of rat brain

Days	6-hydroxydopamine	Change	6-hydroxydopamine + pargyline	Change
	ng/gm	per cent	ng/gm	per cent
0	441 ± 31 (8)		441 ± 31 (8)	
2	263 ± 16 (4)	- 40.36	301 ± 23 (4)	- 31.74
4	219 ± 12 (4)	- 50.34	187 ± 12 (4)	- 57.60
8	197 ± 22 (4)	- 55.33	161 ± 16 (4)	- 63.49
12	186 ± 12 (4)	- 57.82	179 ± 14 (4)	- 59.41

Each value is the mean ± standard error from the number of animals shown in parentheses.

Zero time refers to control animals.

FIGURE 2.6

Time course effect of 6-hydroxydopamine on brain dopamine content

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent 6-hydroxydopamine treatment, hatched columns represent treatment with 6-hydroxydopamine and pargyline.

Pargyline was injected intraperitoneally half an hour before the intraventricular injection of 6-hydroxydopamine.

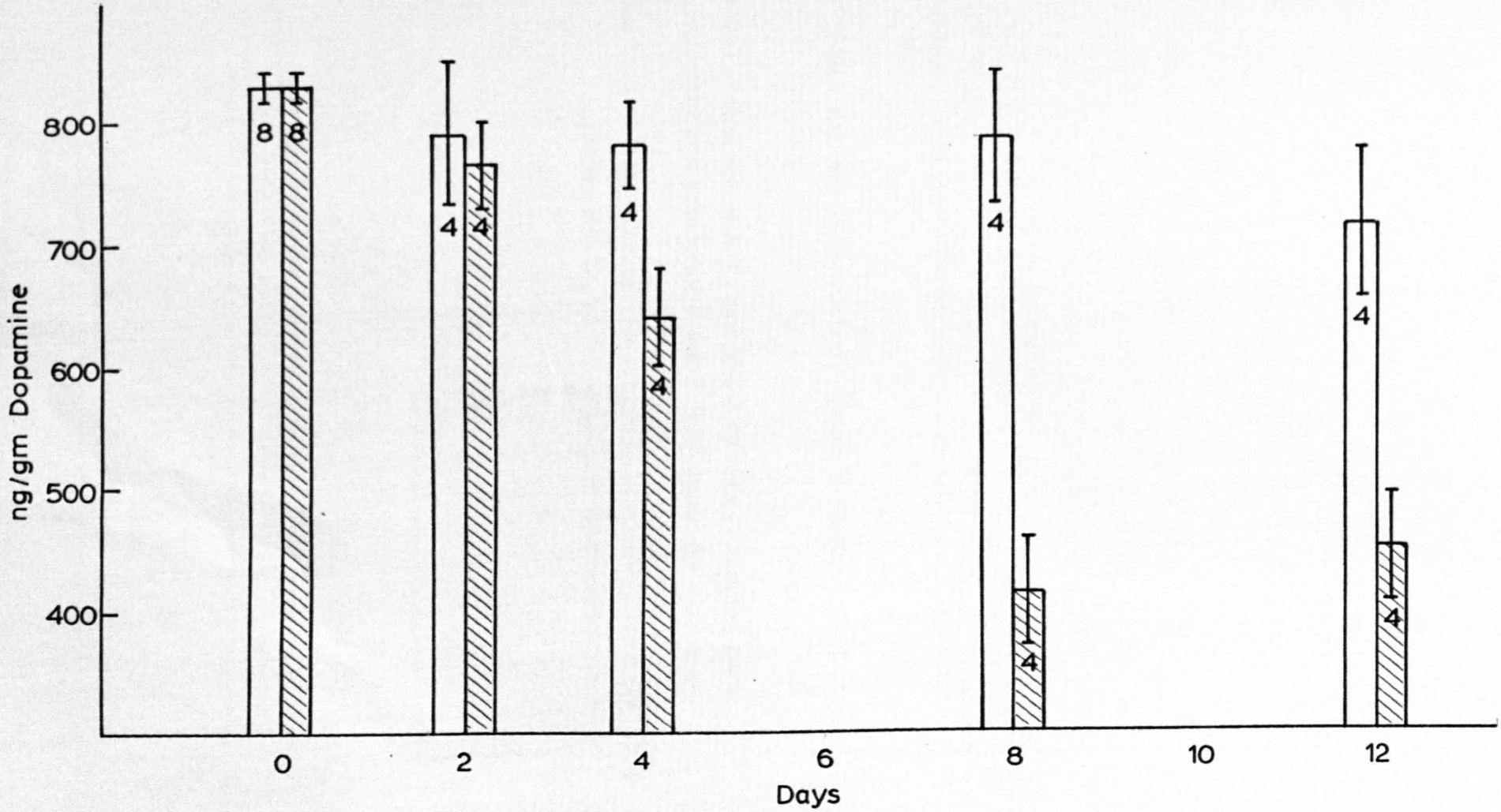

ng/gm Dopamine
800
700
600
500
400
8
8
4
4
4
4
4
4
4
4
0
2
4
6
8
10
12
Days

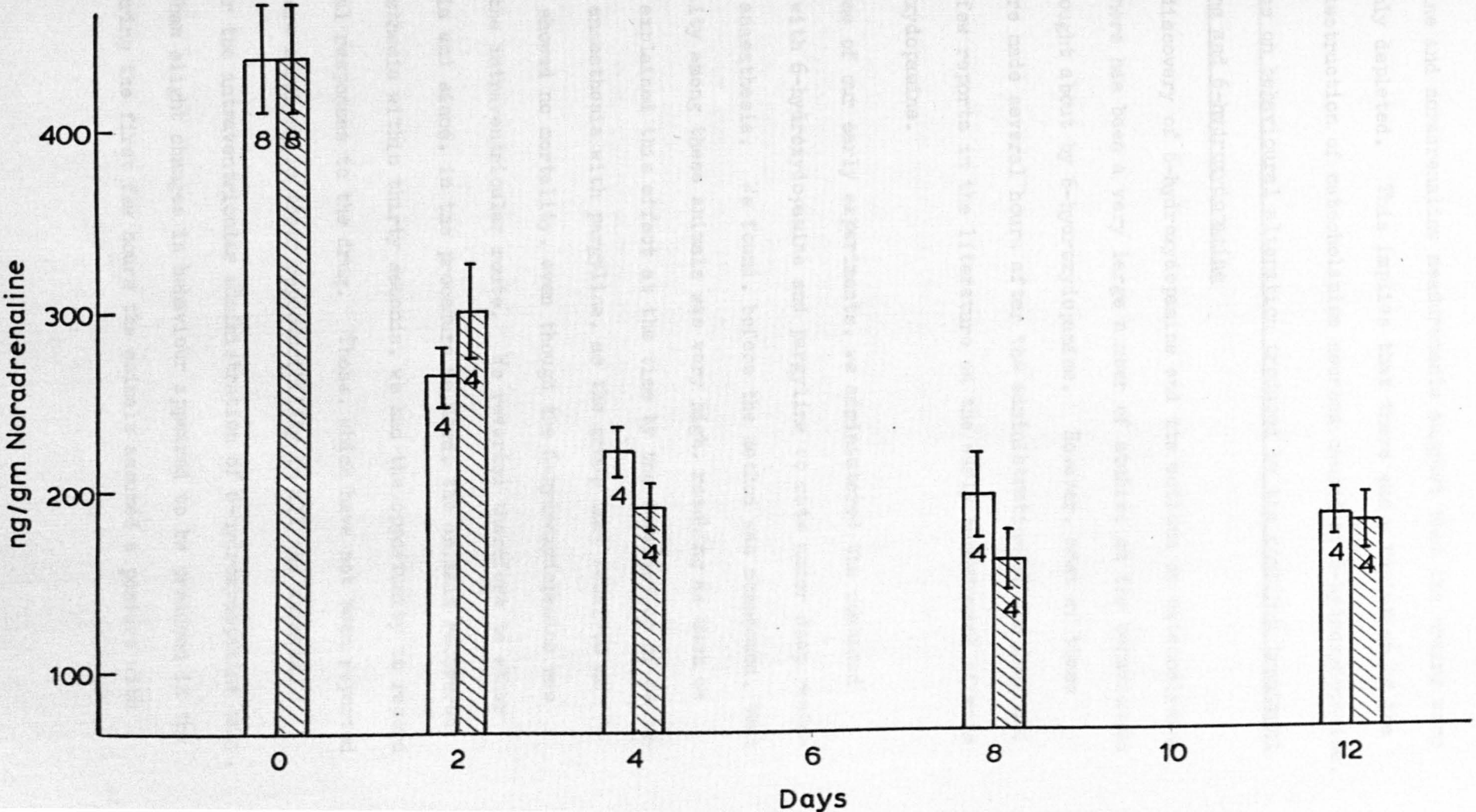
FIGURE 2.7

Time course effect of 6-hydroxydopamine on brain noradrenaline content

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent 6-hydroxydopamine treatment, hatched columns represent treatment with 6-hydroxydopamine and pargyline.

Pargyline was injected intraperitoneally half an hour before the intraventricular injection of 6-hydroxydopamine.


ng/gm Noradrenaline
400
300
200
100
8
8
4
4
4
4
4
4
4
4
0
2
4
6
8
10
12
Days

with dopamine and noradrenaline measurements suggest that the brains were not uniformly depleted. This implies that there was a variation in the degree of destruction of catecholamine neurons caused by 6-hydroxydopamine.

2.7 Studies on behavioural alteration produced by the combined treatment of pargyline and 6-hydroxydopamine

Since the discovery of 6-hydroxydopamine and its actions on catecholamine neurons, there has been a very large number of studies on the behavioural changes brought about by 6-hydroxydopamine. However, most of these studies were made several hours after the administration of the drug and there are few reports in the literature on the early behavioural effects of 6-hydroxydopamine.

In some of our early experiments, we administered the combined treatment with 6-hydroxydopamine and pargyline to rats under deep pentobarbitone anaesthesia. We found, before the method was abandoned, that the mortality among these animals was very high, reaching as much as 80%. We explained this effect at the time by the interaction of pentobarbitone anaesthesia with pargyline, as the group that received no pargyline showed no mortality, even though the 6-hydroxydopamine was given by the intraventricular route. We reverted therefore to ether anaesthesia and since, in the procedure we used, the animals recovered from anaesthesia within thirty seconds, we had the opportunity to record behavioural responses to the drug. These, which have not been reported before, were striking in their reproducibility in all the animals.

After the intraventricular administration of 6-hydroxydopamine only, no more than slight changes in behaviour appeared to be produced in the rat. During the first few hours the animals assumed a posture with

hunched back and raised fur, and, though they appeared to be a little irritable and excitable, they could be handled without any difficulty.

On the other hand, when the intraventricular administration of 6-hydroxydopamine was preceded by the intraperitoneal injection of pargyline, completely different responses were observed. The rats assumed a posture with hunched back, neck extensor muscles contracted, which led to a raised head (as if they were staring to the ceiling, Figures 2.8, 2.9 and 2.10). and sat in that position without showing any movement until they were disturbed, whereupon they moved to stand in another place, showing little sign of alertness. The rats were vocalizing most of the time and this increased on touch. They were irritable, appeared to be very frightened. their response to pain was greatly potentiated and they displayed increased respiration on handling. They showed some increase in muscle tone but they were not catatonic. Tail twitching or shaking resulted on touching their backs. None of these behavioural changes had been seen in rats treated with 6-hydroxydopamine only. These effects started to disappear three hours after the injections.

On the second day, most of the rats were normal although a few still showed vocalization on touch. A scoring system (Irwin primary test) was used in which the behavioural, neurological and autonomic responses were measured. The results are shown in table 2.10.

The total score for the behavioural responses is not very different in the three different groups, but as we can see from the table, some behaviours were increased and others were decreased in the group of rats treated with 6-hydroxydopamine and pargyline. These would compensate

Table 2.10

Comparison between the behavioural changes brought about by 6-hydroxydopamine, 6-hydroxydopamine and pargyline or saline and pargyline

'Irwin primary tests'

Responses	Saline + pargyline	6-hydroxydopamine	6-hydroxydopamine + pargyline
I. BEHAVIOURAL			
Alertness (4)	4.0	4.0	1.5
visual placing (4)	4.0	3.5	1.0
Passivity (0)	0.0	0.0	1.5
Vocalization (4)	0.0	0.0	4.0
Irritability (0)	0.0	0.0	2.0
Fearfulness (0)	0.0	0.0	2.0
Reactivity (4)	4.0	4.0	1.5
Spontaneous activity (4)	4.0	4.0	1.5
Touch response (4)	4.0	4.0	2.5
Pain response (4)	4.0	4.0	8.0
Total	24.0	23.5	25.5
II. NEUROLOGICAL			
Abnormal gait (0)	0.0	0.0	1.5
Body tone (4)	4.0	4.0	6.0
Abdominal tone (4)	4.0	4.0	6.0
Body posture (4)	4.0	4.0	6.0
Limb posture (4)	4.0	4.0	4.0
Total	16.0	16.0	23.5

contd.....

Table 2.10 - contd.

III. AUTONOMIC			
Piloerection (0)	0.0	1.0	2.0
Hypothermia (0)	0.0	0.0	0.0
Skin colour (4)	4.0	4.0	4.0
Heart rate (4)	4.0	4.0	6.0
Respiration (4)	4.0	4.0	6.0
Tail shaking on touch (0)	0.0	0.0	8.0
Total	12.0	13.0	26.0
Sum	52.0	52.5	75.0

The numbers are the mean of observations on two different rats.
The peak effect listed is assessed on a scale ranging from zero to eight.
Numbers in parentheses indicate the score which is given to untreated rats.

each other leaving the final score the same as for other groups. On the other hand when the neurological and autonomic scores were totalled a great difference appeared between the three different groups. Here it is noticeable that most of the neurological and autonomic responses were aggravated by the combined treatment with 6-hydroxydopamine and pargyline.

Audiogenic seizure susceptibility in rats treated with 6-hydroxydopamine

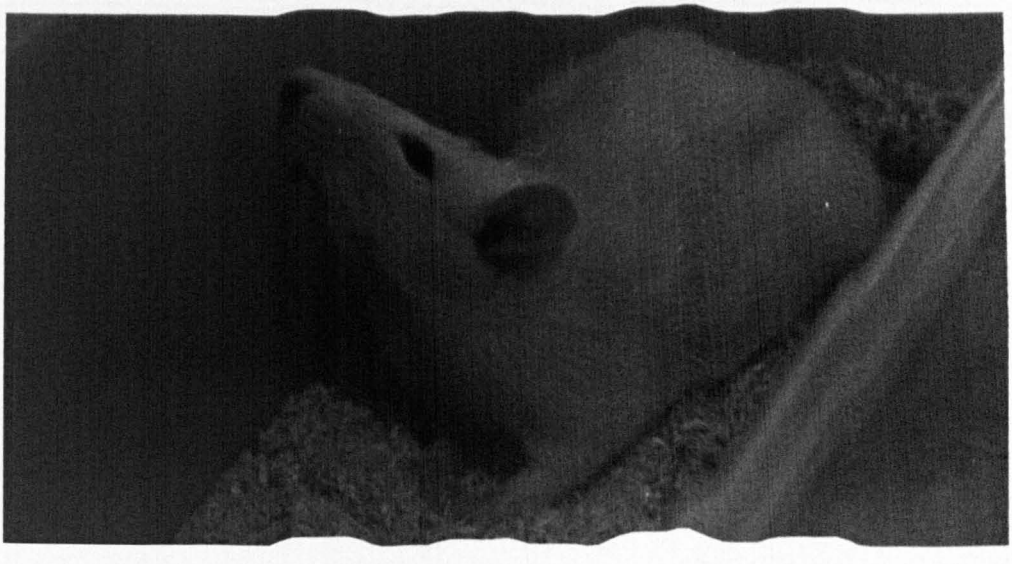
Rats which were not initially susceptible to audiogenic stimulation and which were treated with 6-hydroxydopamine showed no convulsive response to audiogenic stimulation (details of which are given in chapter 3) one, two, three and four weeks after treatment.

FIGURE 2.8

To illustrate the attitude taken up by rats that had received 6-hydroxydopamine and pargyline (A and C) in comparison with that of an untreated rat (B).

(A)

(B)



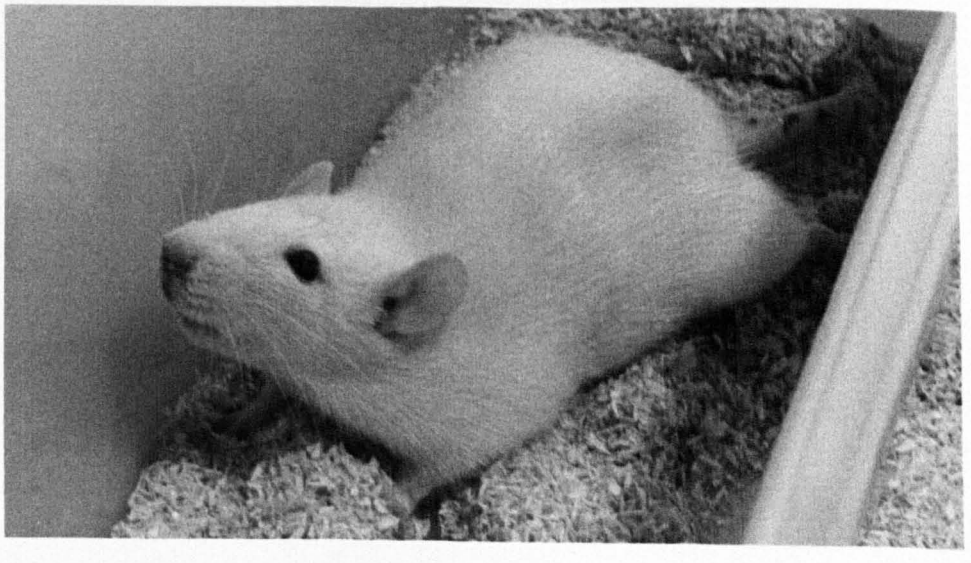
(C)

FIGURE 2.9

To illustrate the attitude taken up by rats that had received 6-hydroxydopamine and pargyline (A and C) in comparison with that of an untreated rat (B)

(A)

(B)



(C)

FIGURE 2.10

Details as in Figure 2.9: animals (A) and (C) had received 6-hydroxydopamine and pargyline; animal (B) was untreated

(A)

(B)

(C)

2.8 Effect of single doses of anticonvulsant drugs on brain dopamine and noradrenaline content depleted by 6-hydroxydopamine

Female Ash Wistar rats were prepared for the intraventricular injection as described before. One week later the rats were injected intraperitoneally with pargyline (50 mg/kg), followed half an hour later by intraventricular injection of 6-hydroxydopamine (200 µg of the hydrochloride in normal saline with 1% ascorbic acid). Twelve days later, the rats were divided into groups of four, and each group received an intraperitoneal injection of either saline or one of the anticonvulsant drugs shown in table 2.11 in the same doses as before. They were killed one hour later and their brains were quickly removed and assayed for their dopamine and noradrenaline content by the method described in chapter 1.

The results are shown in table 2.11.

The results show that while carbamazepine increased only noradrenaline, phenobarbitone and phenytoin were capable of increasing significantly the amount of both dopamine and noradrenaline in the brains of rats treated with 6-hydroxydopamine.

2.9 The regional distribution of brain dopamine and noradrenaline in rat brain

Results in section 2.1 show that the amount of dopamine and noradrenaline in whole brain did not change after the administration of single doses of anticonvulsant drugs tested. As the levels of dopamine and noradrenaline vary among the different brain regions, any change in the concentration of dopamine or noradrenaline in small areas of the brain might have been masked in an assay of the whole brain. So we decided to look at the effect of the anticonvulsant drugs on dopamine and noradrenaline content in parts of the brain.

Table 2.11

Effect of single doses of anticonvulsant drugs on brain dopamine and noradrenaline content after an intraventricular injection of 6-hydroxydopamine

The animals were killed one hour after the anticonvulsant drugs and twelve days after the 6-hydroxydopamine

Treatment	Dopamine ng/gm	Change per cent	Noradrenaline ng/gm	Change per cent
Vehicle	822 ± 13 (8)		430 ± 20 (8)	
6-hydroxydopamine	471 ± 45 (8)	- 42.7	130 ± 13 (8)	- 69.7
6-hydroxydopamine + phenobarbitone	663 ± 26 (4)**	- 19.3	189 ± 5.6 (4)**	- 56.0
6-hydroxydopamine + phenytoin	591 ± 32 (4)**	- 28.1	206 ± 26 (4)**	- 52.1
6-hydroxydopamine + carbamazepine	456 ± 25 (4)	- 44.5	162 ± 8.8 (4)*	- 62.3

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$) from those obtained after treatment with 6-hydroxydopamine.

Method of dissection

The method used for dissection was that which has previously been used in our laboratories (Jepson, 1975; Hebron, 1977). Rats were killed by cervical dislocation and decapitation, their brains were quickly removed and placed on an ice-cooled glass plate. The cerebellum was removed first by cutting the cerebellar peduncles, then the pons and medulla which lie immediately below the cerebellum were separated from the rest of the brain. The cerebral cortex was then pulled away to expose the corpus callosum. An incision was made through the midline of the corpus callosum to expose the lateral ventricles. The striata protruding from the inner surface of the cerebral hemispheres into the ventricles together with the caudate nuclei, were removed by gently scraping the inside of the ventricle with a sharp scalpel. The cerebral hemispheres were then dissected from the rest of the brain. Each part was homogenized as soon as it had been isolated and weighed. It was then assayed for its dopamine and noradrenaline content.

An example of the reproducibility of this procedure is given in table 2.12. The low coefficient of variation indicates that the procedure is confidently reproducible.

The 'rest of the brain' which will be referred to later as 'mid brain' includes the mid brain proper, the thalamus, subthalamus, hypothalamus and hippocampus.

2.10 Effect of single doses of anticonvulsant drugs on the regional distribution of dopamine and noradrenaline

These experiments were performed in the same manner as those carried out when studying the effect of the anticonvulsant drugs on whole brain catecholamines.

Table 2.12

Reproducibility of the dissection procedure

Brain region	Mean weight mg	Per cent of total brain weight	Coefficient of variation
Pons and medulla (10)	144 ± 6.5	9.26	4.5
Cerebellum (10)	242 ± 10.1	15.5	4.2
Striata (10)	38 ± 2.0	2.4	5.2
Cerebral hemispheres (10)	762 ± 43.0	49.0	5.6
Midbrain (10)	368 ± 31.0	23.7	8.4

Each value is the mean ± standard deviation from the number of animals, of body weight between 150 and 160 gms, shown in parentheses

Female Ash Wistar rats weighing approximately 150 gms were obtained one week before carrying out the experiments. Animals were killed one hour after the injection of the stated doses and their brains were quickly removed, placed on an ice cooled glass plate and dissected as described in section 2.9. Each part was homogenized as soon as it had been isolated and weighed. It was then assayed for its catecholamine content.

Results in tables 2.13 and 2.14 show that phenytoin increased the amount of dopamine in the striata where the greatest concentration of dopamine has been found and this attained a statistical significance ($P < 0.001$). However, it did not affect the concentration of dopamine

Table 2.13

Effect of single doses of anticonvulsant drugs on the regional distribution of brain dopamine content

Drug	Striata ng/gm	Cerebral ng/gm	Midbrain ng/gm
Saline	6210 ± 92 (6)	433 ± 7 (6)	260 ± 7 (6)
Phenobarbitone	6130 ± 94 (6)	453 ± 15 (6)	264 ± 17 (6)
P	N.S.	N.S.	N.S.
Saline	5710 ± 24 (6)	431 ± 28 (6)	251 ± 21 (6)
Phenytoin	6168 ± 29 (6)	406 ± 10 (6)	265 ± 21 (6)
P	< 0.001	N.S.	N.S.
Propylene glycol	6120 ± 78 (6)	443 ± 14 (6)	288 ± 10 (6)
Carbamazepine	6200 ± 23 (6)	448 ± 14 (6)	259 ± 21 (6)
P	N.S.	N.S.	N.S.

Each value is the mean ± standard error from the number of animals shown in parentheses

Table 2.14

Effect of single doses of anticonvulsant drugs on the regional distribution of brain noradrenaline content

Drug	Pons and medulla ng/gm	Cerebellum ng/gm	Cerebral ng/gm	Midbrain ng/gm
Saline	682 ± 42 (6)	406 ± 41 (6)	381 ± 14(6)	707 ± 35 (6)
Phenobarbitone	704 ± 56 (6)	393 ± 16 (6)	391 ± 14(6)	681 ± 49 (6)
P	N.S.	N.S.	N.S.	N.S.
Saline	716 ± 35 (6)	385 ± 7 (6)	392 ± 42(6)	668 ± 21 (6)
Phenytoin	801 ± 35 (6)	380 ± 21 (6)	338 ± 31(6)	749 ± 35 (6)
P	< 0.1	N.S.	N.S.	< 0.05
Propylene glycol	672 ± 21 (6)	384 ± 21 (6)	341 ± 14(6)	697 ± 35 (6)
Carbamazepine	659 ± 63 (6)	374 ± 28 (6)	391 ± 12 (6)	802 ± 35 (6)
P	N.S.	N.S.	< 0.02	< 0.05

Each value is the mean ± standard error from the number of animals shown in parentheses.

in other parts studied. Phenytoin also increased significantly the amount of noradrenaline in the midbrain and showed a tendency to lower it in the cerebral hemispheres, and to increase it slightly in the pons and medulla. Carbamazepine increased significantly noradrenaline concentration in midbrain ($P < 0.05$) and cerebral hemispheres ($P < 0.02$). Phenobarbitone had no effect on either dopamine or noradrenaline in any of the parts studied.

2.11 Effect of a single dose of phenobarbitone on dopamine turnover in selected brain regions

The results presented in section 2.2 show that phenobarbitone reduced the change produced by α-methyl-p-tyrosine on dopamine concentration in whole brain. We therefore performed experiments to study the effect of phenobarbitone on the change in dopamine concentration induced by α-methyl-p-tyrosine in discrete brain regions in order to see whether this effect was limited to one region or whether it had a more widespread effect on all the regions.

Female Ash Wistar rats were divided into groups and injected intraperitoneally either with saline and α-methyl-p-tyrosine (100 mg/kg) or with phenobarbitone (100 mg/kg) and α-methyl-p-tyrosine. The animals were killed three hours after the injection and their brains were removed, dissected as described before and the parts were assayed for their dopamine content. Results are presented in table 2.15. The depletion of dopamine in the mid brain was so great that the amount remaining could not be detected by our method. α-Methyl-p-tyrosine reduced the amount of dopamine in the striata by 56% and in the cerebral hemispheres by 61%.

Phenobarbitone reduced significantly the degree to which dopamine was depleted in the cerebral hemispheres but it did not affect the change in the striata.

Table 2.15

Effect of a single dose of phenobarbitone on dopamine turnover in parts of rat brain

Drug	Striata ng/gm	Cerebral ng/gm	Mid brain ng/gm
Saline	6320 ± 74 (6)	422 ± 16 (6)	260 ± 8 (6)
α-Methyl-p-tyrosine	2668 ± 122 (6)	163 ± 8 (6)	(see text)
% change	- 57.78	- 61.37	
Phenobarbitone + α-methyl-p-tyrosine	2799 ± 77 (6)	343 ± 8 (6)	(see text)
% change	- 55.71	- 18.72	
P (t test)	N.S.	< 0.001	

Each value is the mean ± standard error from the number of animals shown in parentheses.

The figures were obtained in two different experiments.

CHAPTER 3

BRAIN CATECHOLAMINES DURING HABITUATION AND WITHDRAWAL FROM PHENOBARBITONE

Treatment of grand mal epilepsy requires long term treatment with anticonvulsant drugs. Long acting barbiturates were found to be effective in this disorder. However, the withdrawal of barbiturates from those habituated to them can cause epileptiform convulsions even in non-epileptic individuals and the exacerbation of seizures in epileptic patients.

Crossland and Leonard (1963) developed a method by which rats became physically dependent on sodium barbitone when the drug was given in their drinking water. Animals treated in this way develop a supranormal seizure threshold during the administration of the drug and a reduced threshold during abstinence (Leonard, 1968).

We have adopted the method of Crossland and Leonard to examine the effect of habituation to and withdrawal of phenobarbitone on the levels and turnover of dopamine and noradrenaline in the whole brain, as well as on the regional distribution of the catecholamines in rat brain.

3.1 Method of habituation

Female Ash Wistar rats weighing approximately 80 gm were obtained from our usual source. All animals were first subjected to auditory stimulation by placing them singly in a glass fronted wooden box with an electric door bell attached to the roof. The wooden box was divided into two chambers, each 30 x 30 x 30 cm, and was itself enclosed in a large glass fronted case. Each rat was exposed to the sound of the bell for one minute. Any animal that exhibited convulsions after sound

stimulation was excluded from the experiment. In the course of our study, this kind of experiment was carried out several times and on each occasion the number of animals used was about forty. In each group no more than one animal usually showed convulsions, but on just one occasion we found that three rats out of the forty convulsed when subjected to auditory stimulation.

After the initial screening, the rats were housed in individual cages, where they were allowed free access to food and water. Their food and water intakes were measured daily and their body weights were taken every three days. One week later, the rats were divided into two groups. One group received phenobarbitone in their drinking water. The bitter taste of phenobarbitone was disguised by adding saccharine to the drinking water to a concentration of 0.025%. The other group of rats served as controls and received only saccharine in their drinking water.

The concentration of phenobarbitone in the drinking water was initially such as to ensure a daily intake of about 50 mg/kg for the first two weeks. The concentration was adjusted so as to increase the daily intake of phenobarbitone by about 50 mg/kg every other week, until the concentration of phenobarbitone in drinking water reached a final concentration of 150 mg/kg at which level it was continued for the last two weeks of the administration. The dose of phenobarbitone was corrected every three days in the light of changes in the fluid intake or body weight of individual animals. The drinking water was changed every day. The dose of phenobarbitone intended was calculated every three days from the following:

$$\text{mg per 100 ml} = \frac{\text{intended dose (mg/kg) x weight in grams}}{\text{fluid intake x 10}}$$

The calculated dose was obtained by taking an appropriate amount of a freshly prepared solution containing 1 gm per 100 ml of phenobarbitone and making it up to 100 ml with tap water.

Table 3.1 illustrates the mean daily intake for each week of habituation.

Table 3.1

Intake of phenobarbitone

Week	Intended intake mg/kg/day	Actual intake mg/kg/day
1	50	55.2 ± 10.6 (25)
2	50	47.6 ± 5.7 (25)
3	100	109.3 ± 10.2 (25)
4	100	97.3 ± 12.6 (25)
5	150	129.9 ± 20.9 (25)
6	150	135.3 ± 13.6 (25)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Preliminary experiments in our department revealed that there was a fluctuation between the morning and afternoon body weights, ranging between three and five grams for an individual rat, so we took all the measurements at the same time of the day (between 10.00 and 11.00 hours), during the whole period of treatment.

3.2 Behavioural studies on phenobarbitone habituation

Treatment with phenobarbitone started at a daily dose of 50 mg/kg. In spite of the slightly high pH of the phenobarbitone solution in drinking water, rats tolerated the taste and the dose very well. There was no

sign of ataxia at this dose level, although rats on phenobarbitone appeared to be less active than control rats.

After the first day of treatment, rats on phenobarbitone drank much more water and ate much more food than did the rats with saccharine only in their drinking water. These differences in food and water intake attained a high degree of significance which lasted for three or four days before both groups ate and drank the same (Figures 3.1 and 3.2).

After about ten days of treatment with daily doses of 50 mg/kg phenobarbitone, rats became slightly aggressive and exhibited a noticeable increase in biting behaviour. This disappeared after increasing the daily dose to 100 mg/kg. This latter dose produced a slight ataxia which lasted up to four days then it started to disappear. Again the food intake was increased significantly by increasing the dose of phenobarbitone, water intake remained unchanged. The aggressiveness and biting behaviour were seen again towards the end of the period of treatment with doses of 100 mg/kg, and disappeared when the daily dose was increased to 150 mg/kg. At this dose level, ataxia became apparent, as did a striking degree of passiveness, so that the rats could be positioned on the hand without showing any movement. The amount of water drunk dropped progressively till it reached a plateau which effectively prevented any further increase of barbiturate dose. The food intake did not change much at these high dose levels. Figures 3.3 and 3.4 show the changes in food and water intakes immediately after increasing the dose of phenobarbitone.

The body weight of rats treated with phenobarbitone increased progressively as did that of animals with saccharine only in their drinking water. After increasing the daily dose to 100 mg/kg, rats

FIGURE 3.1

Changes in water intakes during long term treatment with different doses of phenobarbitone

Each bar indicates mean $\pm$ standard error for 24 rats (open columns) and 8 rats (hatched columns).

Open columns represent rats receiving phenobarbitone and saccharine in their drinking water, hatched columns represent control rats receiving saccharine only in their drinking water.

Significantly different by the t test (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.001$) from those of control rats receiving saccharine only in their drinking water.

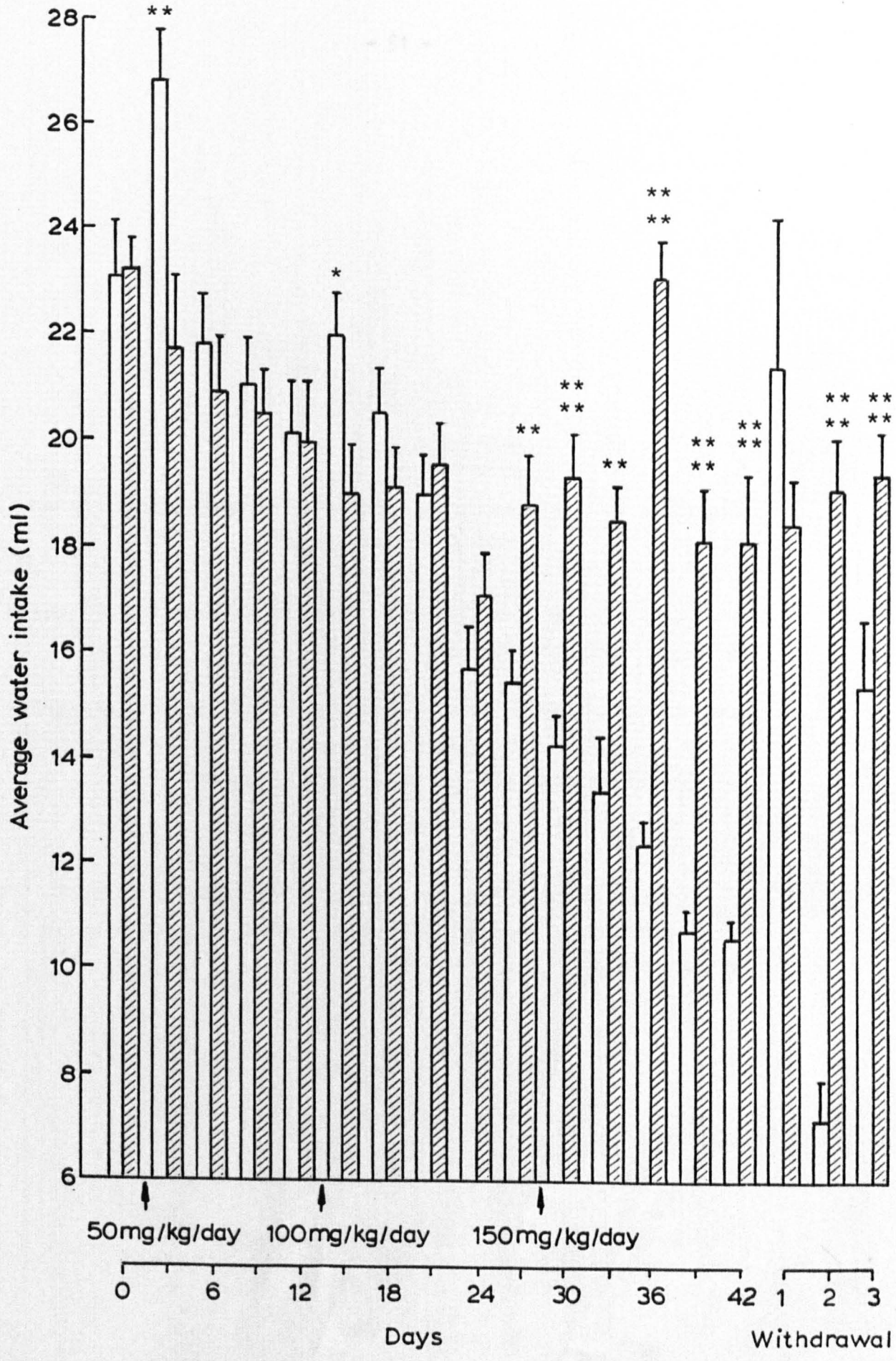

Average water intake (ml)
28
26
24
22
20
18
16
14
12
10
8
6
50mg/kg/day
100mg/kg/day
150mg/kg/day
0
6
12
18
24
30
36
42
1
2
3
Days
Withdrawal

FIGURE 3.2

Changes in food intakes during long term treatment with different doses of phenobarbitone

Each bar indicates mean $\pm$ standard error for 24 rats (open columns) and 8 rats (hatched columns).

Open columns represent rats receiving phenobarbitone and saccharine in their drinking water, hatched columns represent control rats receiving saccharine only in their drinking water.

Significantly different by the t test (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.001$) from those of control rats receiving saccharine only in their drinking water.

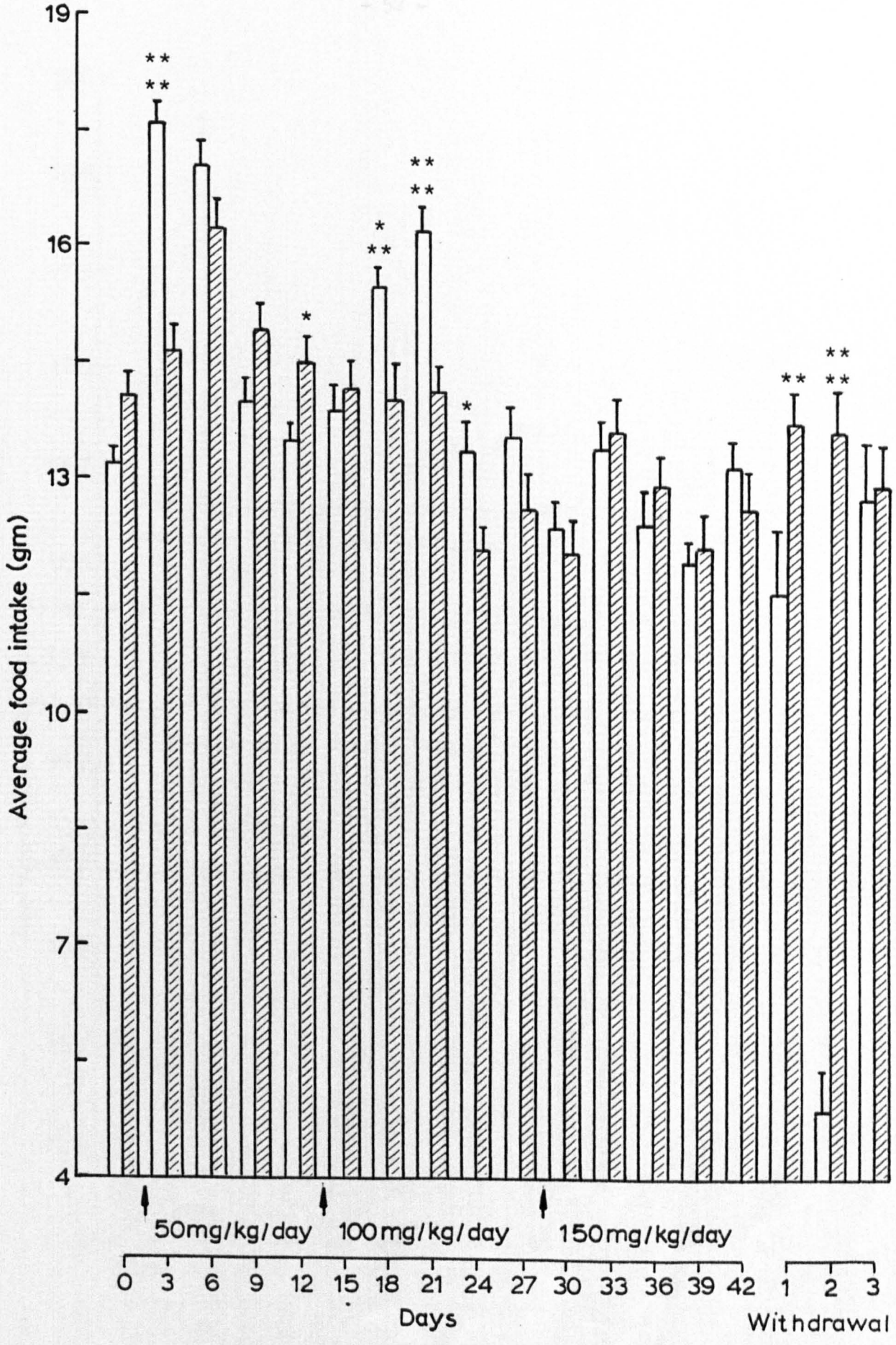

19
16
13
10
7
4
Average food intake (gm)
50mg/kg/day
100mg/kg/day
150mg/kg/day
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42
1 2 3
Days
Withdrawal

FIGURE 3.3

Daily changes in food intakes immediately after increasing the dose of phenobarbitone

Each bar indicates mean $\pm$ standard error for 24 rats (open columns) and 8 rats (hatched columns).

Open columns represent rats receiving phenobarbitone and saccharine in their drinking water, hatched columns represent control rats receiving saccharine only in their drinking water.

Significantly different by the t test (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.005$ and **** $P < 0.001$) from those of control rats receiving saccharine only in their drinking water.

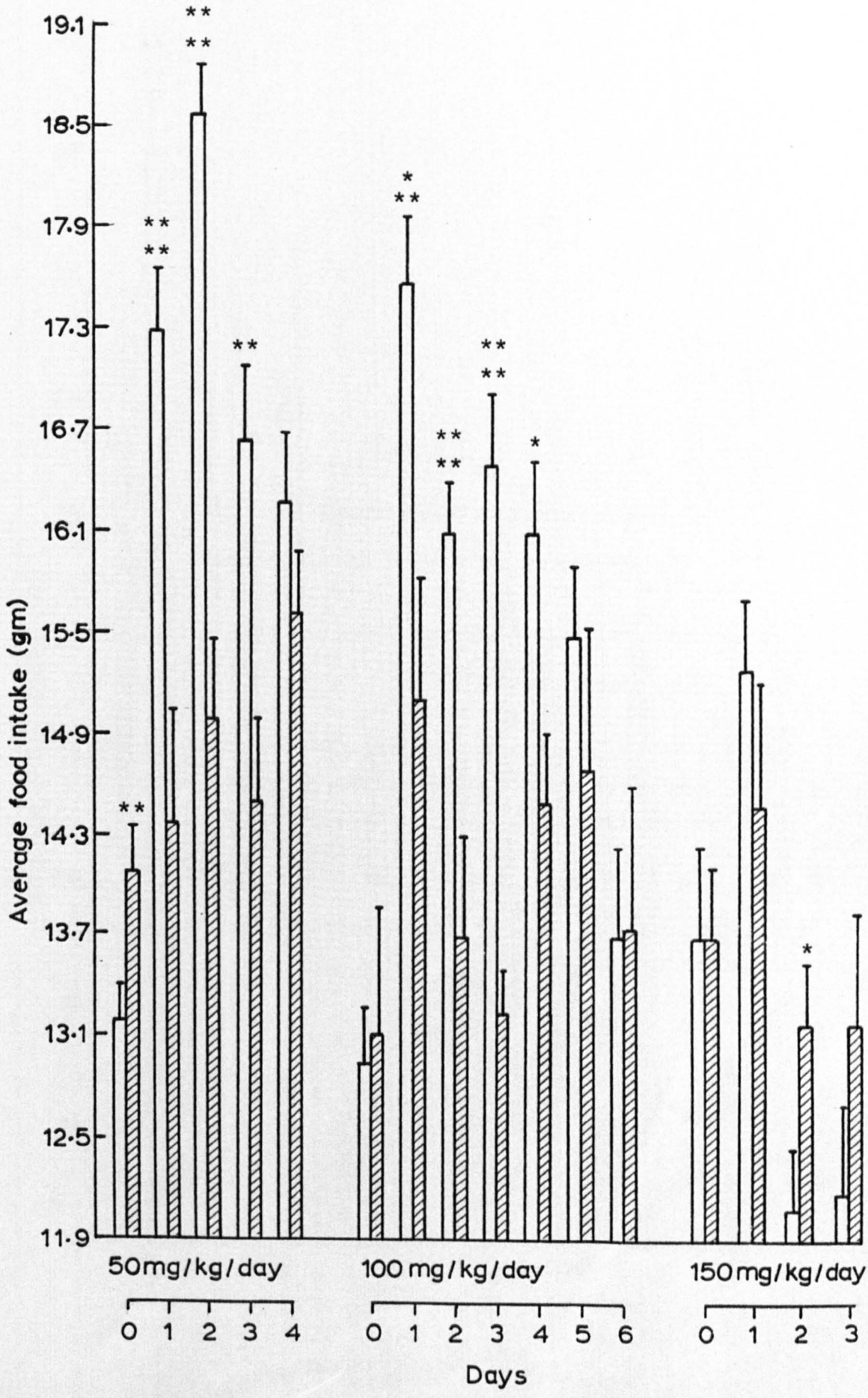

19·1
18·5
17·9
17·3
16·7
16·1
15·5
14·9
14·3
13·7
13·1
12·5
11·9
Average food intake (gm)
50mg/kg/day
100 mg/kg/day
150mg/kg/day
0 1 2 3 4
0 1 2 3 4 5 6
0 1 2 3
Days

FIGURE 3.4

Daily changes in water intakes immediately after increasing the dose of phenobarbitone

Each bar indicates mean $\pm$ standard error for 24 rats (open columns) and 8 rats (hatched columns).

Open columns represent rats receiving phenobarbitone and saccharine in their drinking water, hatched columns represent control rats receiving saccharine only in their drinking water.

Significantly different by the t test (** $P < 0.02$, *** $P < 0.005$ and **** $P < 0.001$) from those of control rats receiving saccharine only in their drinking water.

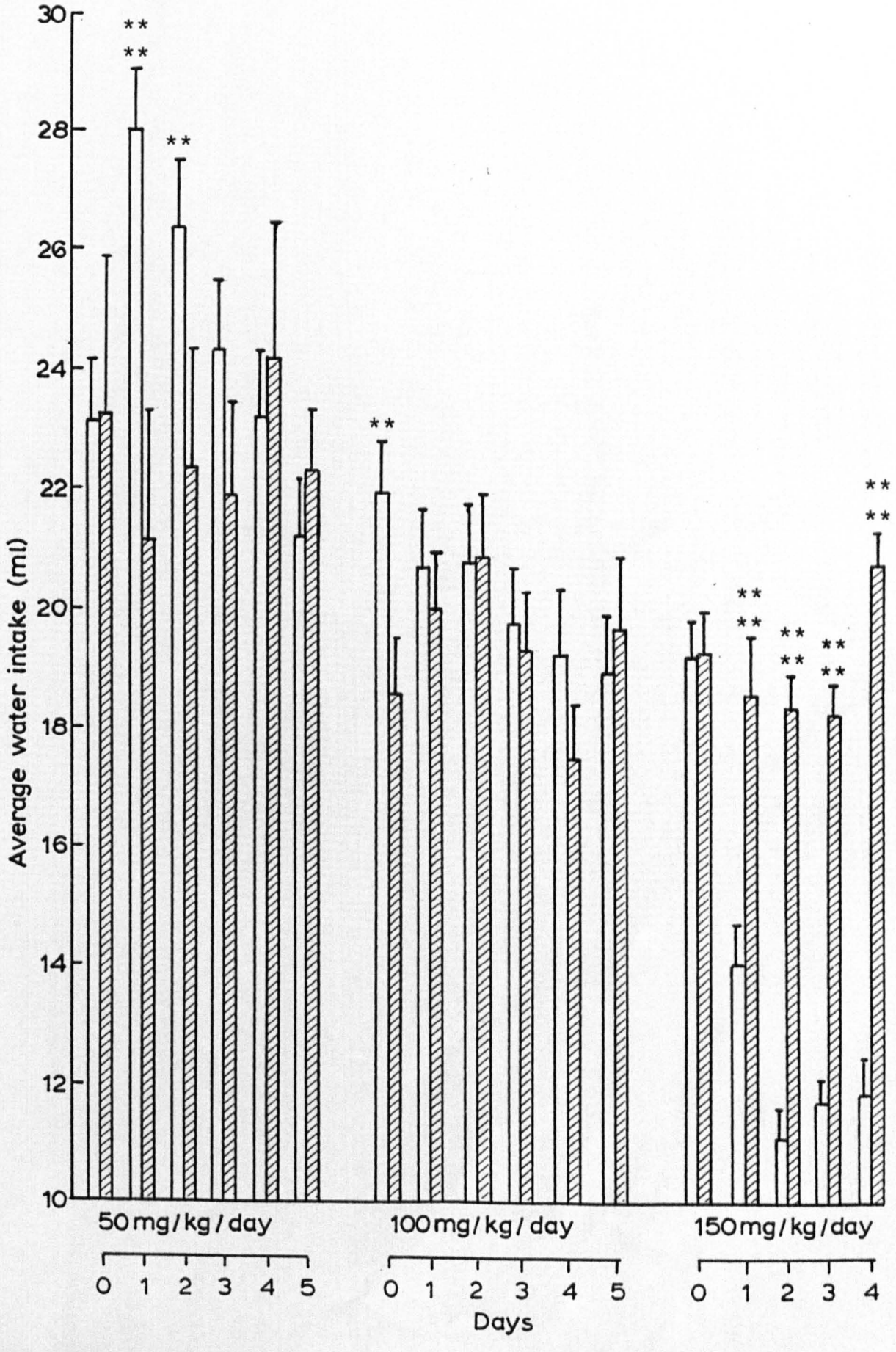

Average water intake (ml)
30
28
26
24
22
20
18
16
14
12
10
50 mg/kg/day
100 mg/kg/day
150 mg/kg/day
0 1 2 3 4 5
0 1 2 3 4 5
0 1 2 3 4
Days

FIGURE 3.5

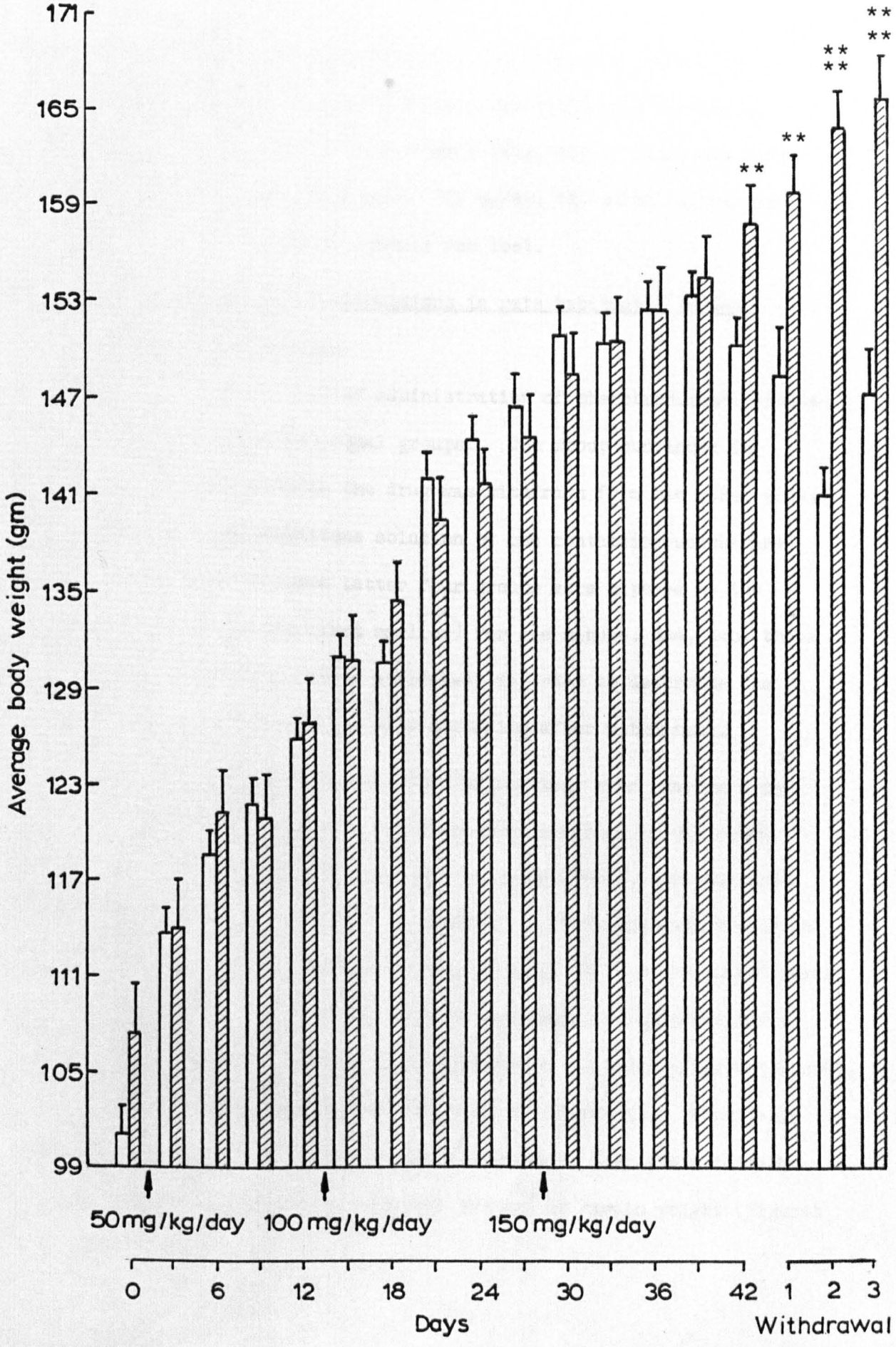
Changes in body weights during long term

treatment with different doses of phenobarbitone

Each bar indicates mean $\pm$ standard error for 24 rats (open columns) and 8 rats (hatched columns).

Open columns represent rats receiving phenobarbitone and saccharine in their drinking water, hatched columns represent control rats receiving saccharine only in their drinking water.

Significantly different by the t test (** $P < 0.02$ and *** $P < 0.001$) from those of control rats receiving saccharine only in their drinking water.


Average body weight (gm)
171
165
159
153
147
141
135
129
123
117
111
105
99
**
**
**
**
**
50 mg/kg/day
100 mg/kg/day
150 mg/kg/day
0
6
12
18
24
30
36
42
1
2
3
Days
Withdrawal

on phenobarbitone gained slightly more weight than did control rats, although the difference in weight between the two groups of animals had not attained statistical significance (Fig. 3.5). When the daily dose was increased from 100 mg/kg to 150 mg/kg, the extra weight previously gained by the treated animals was lost.

3.3 Screening for auditory convulsions in rats habituated to and withdrawn from phenobarbitone

At the end of the sixth week of administration of phenobarbitone, treated rats were divided into five equal groups. One group continued to receive phenobarbitone while the drug was withdrawn from the other groups by replacing the phenobarbitone solution by one containing saccharine only. The animals in these latter four groups were exposed to the sound of the bell (as described earlier) for one minute, one, two, three or four days respectively after withdrawal in order to determine the peak time of any convulsive activity occurring after withdrawal.

The withdrawn rats were observed for one hour every day in order to assess behavioural changes other than those revealed by the sound stimulus. Their body weight and food and water intakes were measured at different time intervals after withdrawal. Following drug withdrawal, rats which were on phenobarbitone lost an average of 10 gm in weight during the first 48 hours. Their water intakes were sharply increased during the first 24 hours and decreased significantly at 48 hours after withdrawal. Food intake was sharply reduced 48 hours after withdrawal. Ninety-six hours after withdrawal, animals began to recover. They ate and drank the same amount as did control rats and started to regain weight (Figures 3.1, 3.2 and 3.5).

These changes in food and water intakes and body weights during habituation to and withdrawal from phenobarbitone, took the same pattern in each experiment in which phenobarbitone was administered. Twenty-four hours after the removal of the drug, withdrawn rats were easily distinguished from control rats. They looked excited and restless, and were disturbed by the smallest noise. When subjected to auditory stimulation, three out of six rats showed wild running, one of the three showed convulsions and it was obvious that the other rats were upset by the sound of the bell, unlike the control rats which maintained an apparent equanimity during exposure. Two days after withdrawal, all rats subjected to auditory stimulation exhibited convulsions. The sound of the bell was stopped as soon as the convulsions started. These convulsions were characterized by a latent period in which the rat sat silent almost as if it were expecting something to happen. The animal then started to look for an escape from the sound then there followed immediately a period of wild running and jumping, in which the rat ran vigorously in close circles. After that it stood on its hind legs and started to jump violently, often reaching the roof of the box. This jumping was followed by clonic convulsions that culminated in an extensor tonic spasm. The convulsions continued in some rats for up to one minute after stopping the sound of the bell. In some cases death followed the severe convulsions. Some rats removed from the box and returned to their cages still showed sporadic jumping behaviour over a period of several minutes. Spontaneous convulsions were seen in some withdrawn rats at the forty-eight hours withdrawal period. We made no effort to measure the percentage of animals that exhibited spontaneous

convulsions as that would have required the continuous observation of the withdrawn rats over the whole twenty-four hours period, which was not possible at the time of the experiment.

Three days after withdrawal of phenobarbitone, fifty per cent of the animals still showed convulsions in response to auditory stimulation. The latent and wild running periods were longer and the duration of convulsions was shorter than at the forty-eight hours withdrawal period.

Table 3.2 and figure 3.6 show that the maximum convulsion activity occurred forty-eight hours after the drug had been withdrawn. At the peak of convulsion activity, there were strong negative correlations between the latent period and the duration of both the phases of wild running and that of the subsequent convulsions ($r = -0.880$ and -0.772 respectively). The correlation coefficient of the relationship between the period of wild running and that of the convulsions proper reached $+0.952$. These relationships are expressed graphically in figures 3.7, 3.8 and 3.9.

Seventy-two hours after withdrawal, the excitability of the animals was considerably reduced and the latent periods of the convulsions was considerably lengthened (up to 36 seconds) and the duration of convulsions was shortened (11 to 16 seconds). Interestingly enough the period of wild running remained unaltered but too much cannot be made of these observations, since so few animals exhibited convulsions at this stage.

Table 3.2

Convulsion activity of rats habituated to and withdrawn from phenobarbitone

Hours after withdrawal	Number of animals	Number of animals showing convulsions	Duration of the phase of audiogenic seizures		
			Latent period	Wild running	Convulsions
			sec.	sec.	sec.
0	6	0	0	0	0
24	6	1	30 ± 0.00	15.00 ± 0.0	10.00 ± 0.0
48	6	6	10.8 ± 0.95	12.60 ± 1.17	48.00 ± 1.46
72	6	3	28.33 ± 2.71	13.30 ± 2.18	13.50 ± 2.00
96	6	1	45.00 ± 0.00	16.00 ± 0.00	0.00

Each value is the mean ± standard error from the number of animals shown in column three

FIGURE 3.6

Duration of convulsions after withdrawal of phenobarbitone

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

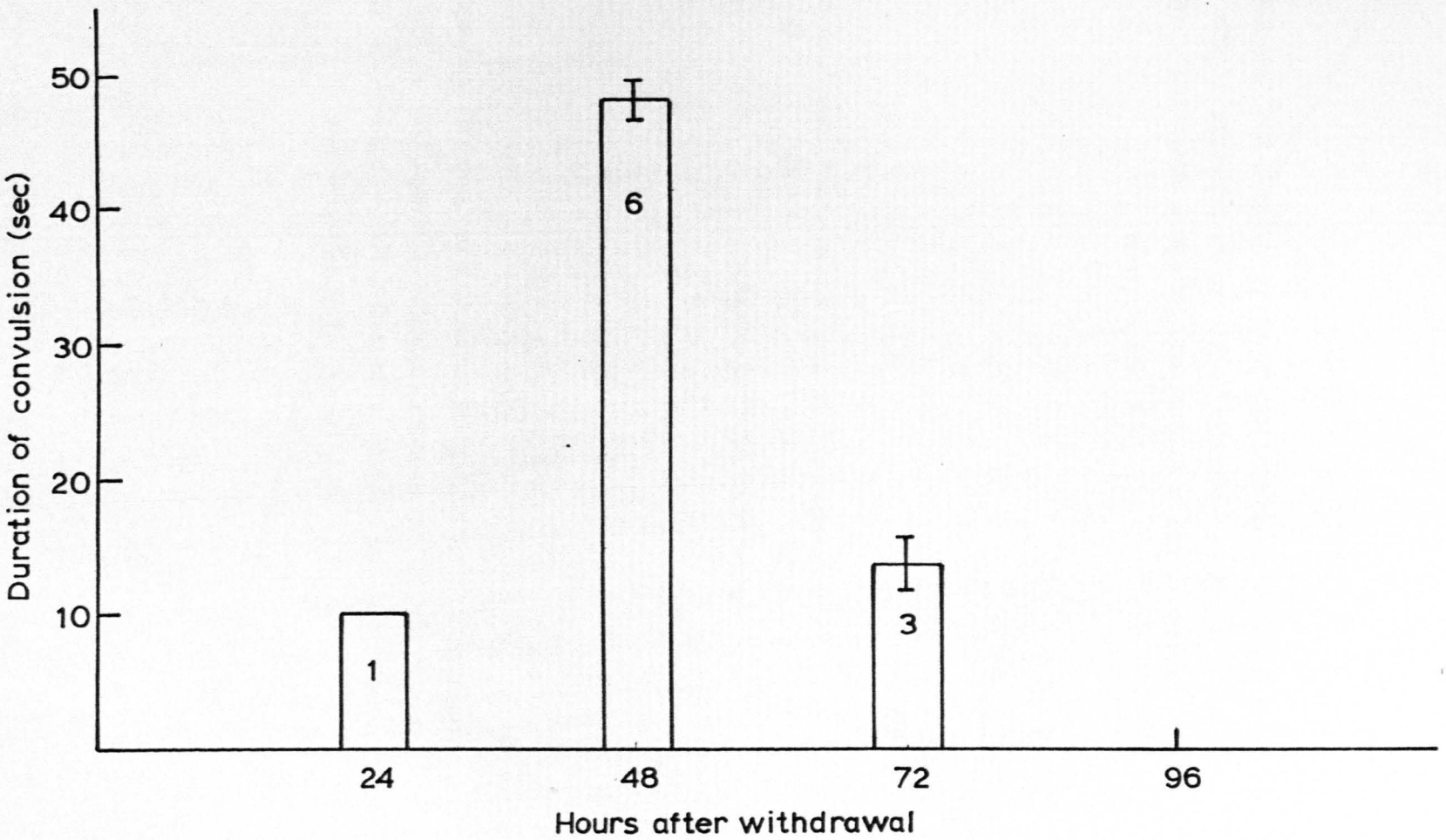
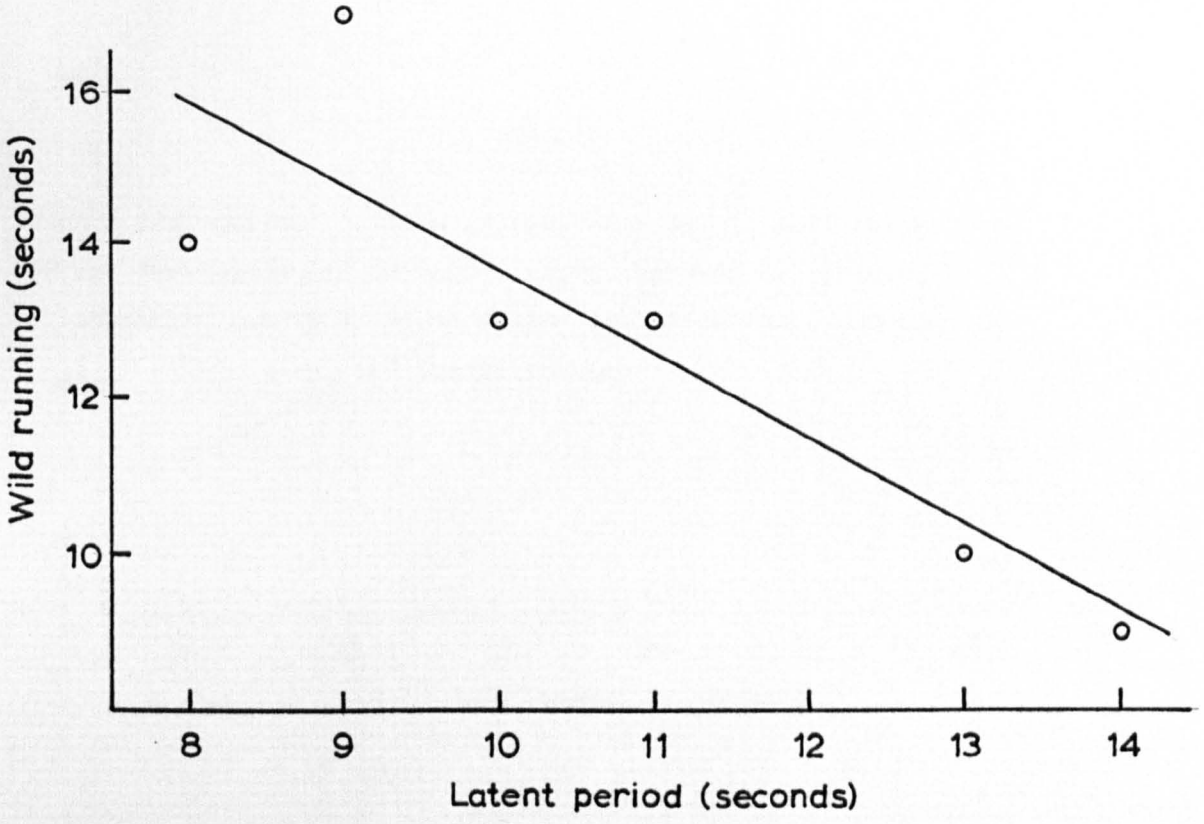

Duration of convulsion (sec)
50
40
30
20
10
1
6
3
24
48
72
96
Hours after withdrawal

FIGURE 3.7

Regression line of the relationship between the duration of the latent period (in seconds) against the duration of the wild running (in seconds), measured forty-eight hours after the withdrawal of phenobarbitone

FIGURE 3.8

Regression line of the relationship between the duration of the latent period (in seconds) against the duration of the subsequent convulsions (in seconds), measured forty-eight hours after the withdrawal of phenobarbitone


Wild running (seconds)
16
14
12
10
8
9
10
11
12
13
14
Latent period (seconds)

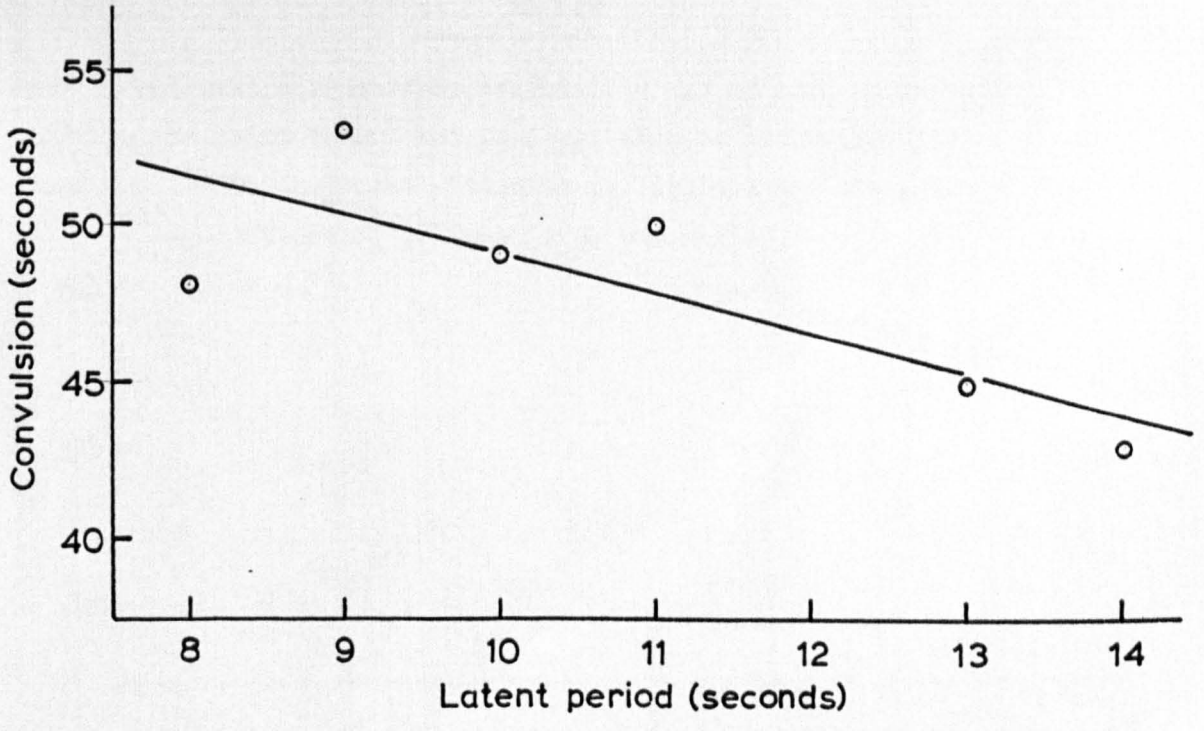
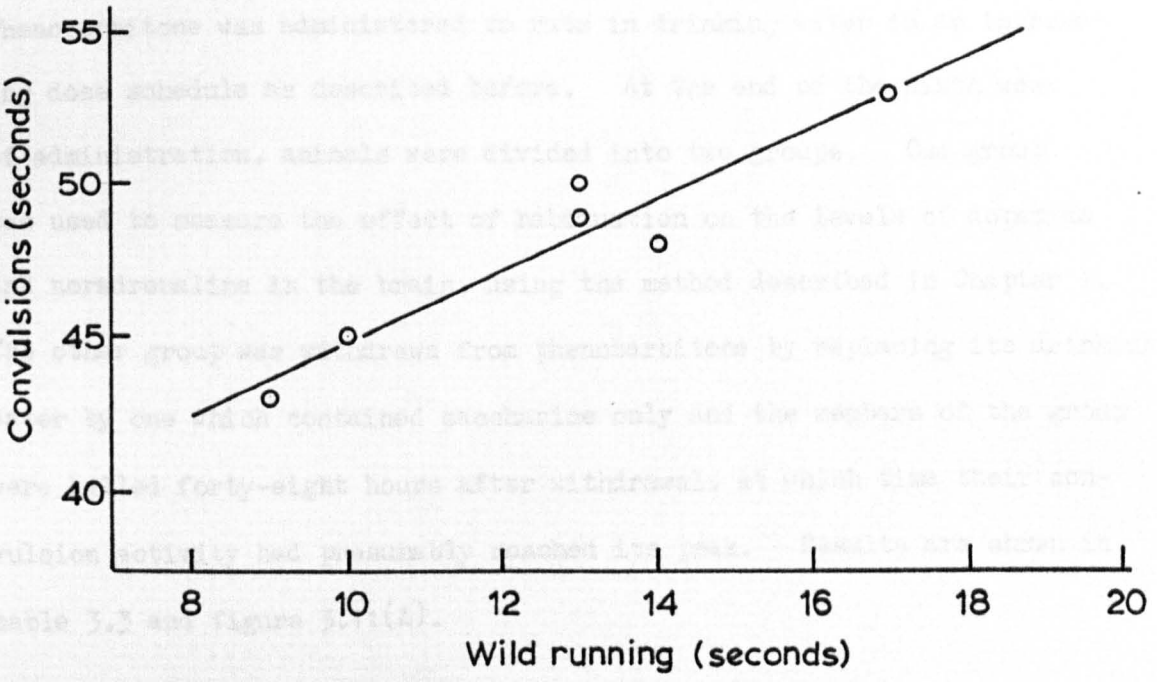

Convulsion (seconds)
55
50
45
40
8
9
10
11
12
13
14
Latent period (seconds)

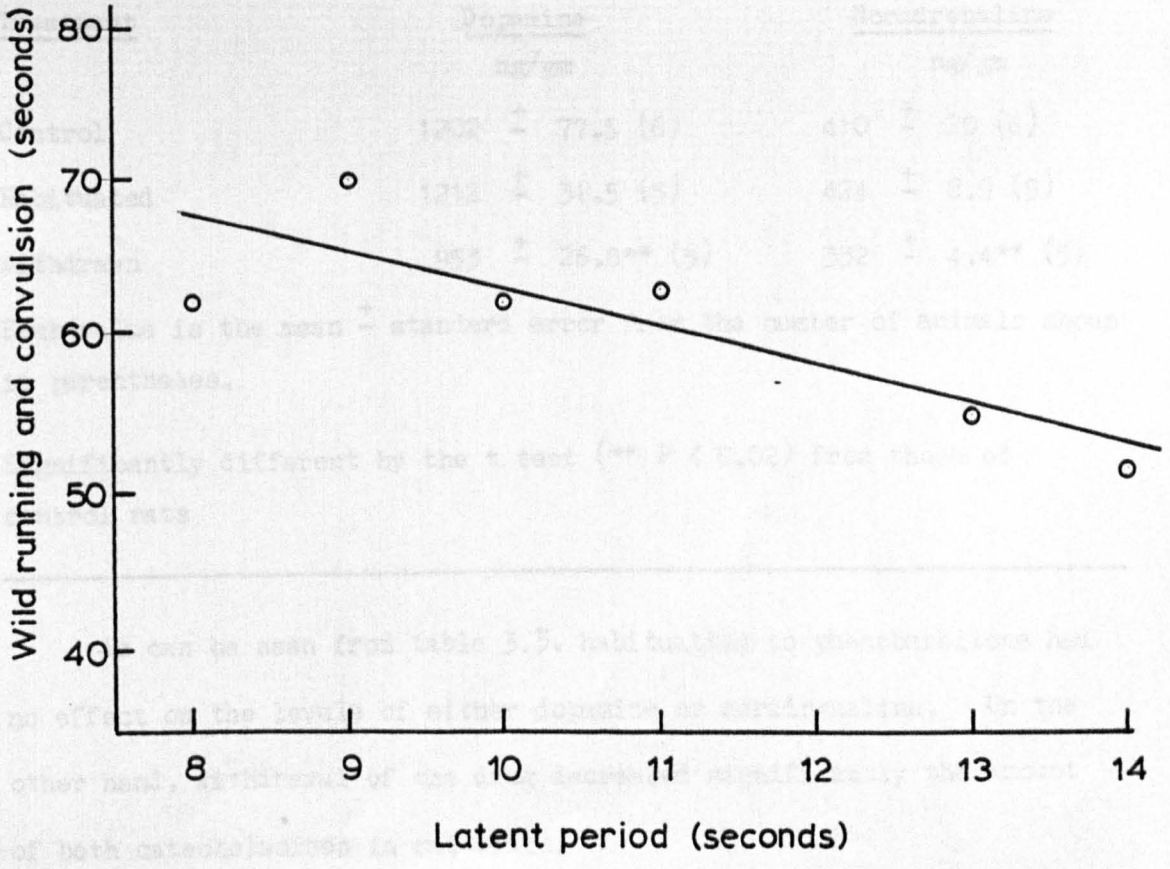
FIGURE 3.9

Regression line of the relationship between the duration of the wild running (in seconds) against that of the subsequent convulsions (in seconds), measured forty-eight hours after the withdrawal of phenobarbitone

FIGURE 3.10

Regression line of the relationship between the duration of the latent period (in seconds) against the sum of durations of wild running and convulsions (in seconds), measured forty-eight hours after the withdrawal of phenobarbitone


Convulsions (seconds)
55
50
45
40
8
10
12
14
16
18
20
Wild running (seconds)


Wild running and convulsion (seconds)
80
70
60
50
40
8
9
10
11
12
13
14
Latent period (seconds)

3.4 Brain catecholamines during habituation to and withdrawal from phenobarbitone

Phenobarbitone was administered to rats in drinking water in an increasing dose schedule as described before. At the end of the sixth week of administration, animals were divided into two groups. One group was used to measure the effect of habituation on the levels of dopamine and noradrenaline in the brain, using the method described in Chapter 1. The other group was withdrawn from phenobarbitone by replacing its drinking water by one which contained saccharine only and the members of the group were killed forty-eight hours after withdrawal, at which time their convulsion activity had presumably reached its peak. Results are shown in table 3.3 and figure 3.11(A).

Table 3.3

Effect of habituation to and withdrawal from phenobarbitone on the amount of catecholamine in rat brain

Treatment	Dopamine ng/gm	Noradrenaline ng/gm
Control	1202 ± 77.5 (6)	410 ± 20 (6)
Habituated	1212 ± 31.5 (5)	424 ± 8.9 (5)
Withdrawn	953 ± 26.8** (5)	332 ± 4.4** (5)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (** $P < 0.02$) from those of control rats

As can be seen from table 3.3, habituation to phenobarbitone had no effect on the levels of either dopamine or noradrenaline. On the other hand, withdrawal of the drug decreased significantly the amount of both catecholamines in rat brain.

FIGURE 3.11 (A)

Effect of habituation to and withdrawal from phenobarbitone on the levels of dopamine and noradrenaline in rat brain

Each bar indicates mean $\pm$ standard error for the number of animals in the text.

Open columns represent dopamine and hatched columns represent noradrenaline.

C = Control rats H = Habituated rats W= Withdrawn rats

Significantly different by the t test (** $P < 0.02$) from those of control rats.

FIGURE 3.11 (B)

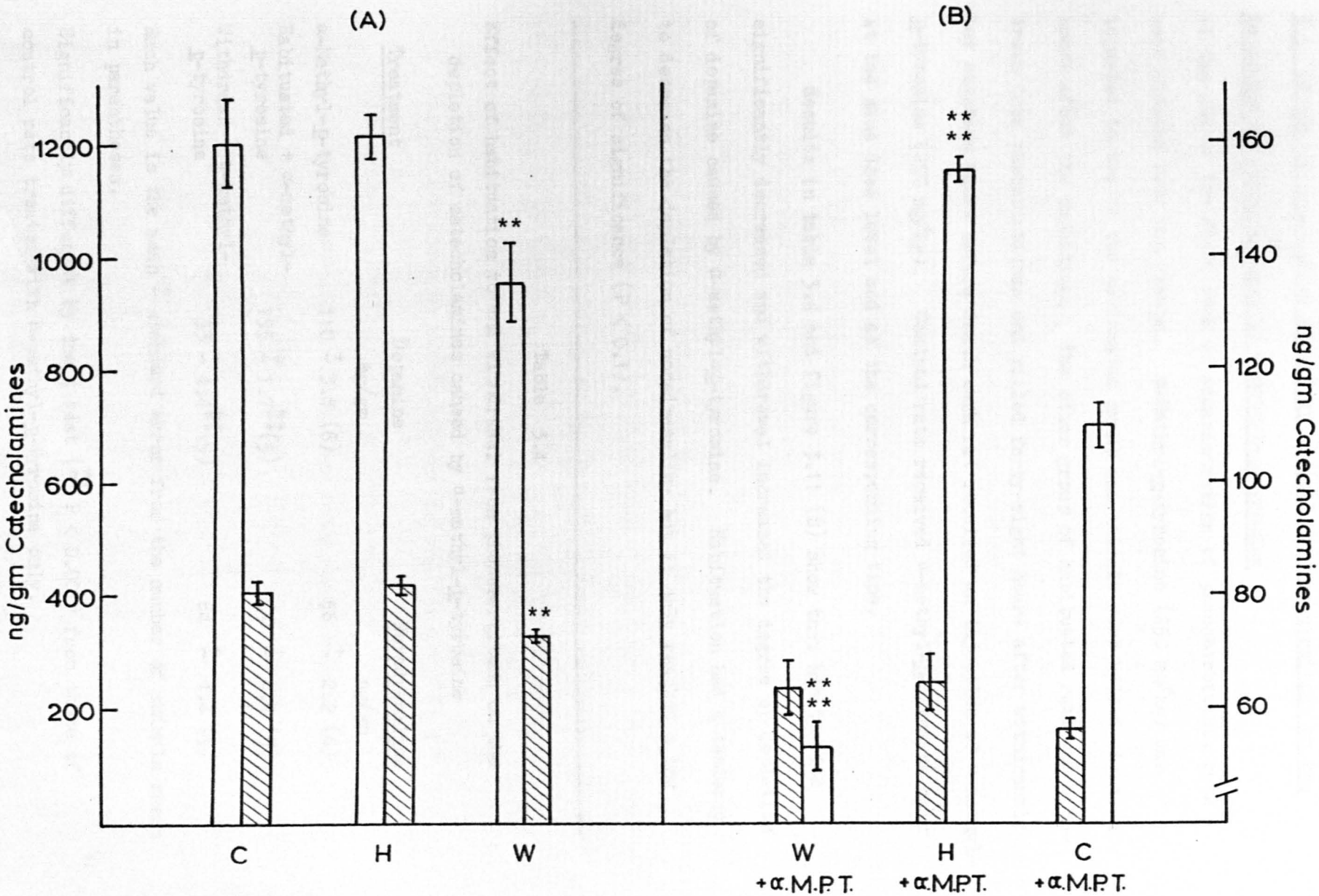
Effect of habituation to and withdrawal from phenobarbitone on the depletion of dopamine and noradrenaline caused by α-methyl-p-tyrosine

Each bar indicates mean $\pm$ standard error for the number of animals in the text.

Open columns represent dopamine, hatched columns represent noradrenaline.

C + α.m.p.t. = Control rats treated with α-methyl-p-tyrosine
H + α.m.p.t. = Habituated rats treated with α-methyl-p-tyrosine
W + α.m.p.t. = Withdrawn rats treated with α-methyl-p-tyrosine

Significantly different by the t test (** $P < 0.001$) from those of control rats treated with α-methyl-p-tyrosine only.


(A)
(B)
ng/gm Catecholamines
1200
1000
800
600
400
200
160
140
120
100
80
60
**
**
**
**
**
C
H
W
W
+α.M.P.T.
H
+α.M.P.T.
C
+α.M.P.T.

3.5 Effect of habituation to and withdrawal of phenobarbitone on the depletion of catecholamine by α-methyl-p-tyrosine

At the end of the sixth week of administration of phenobarbitone, rats were divided into two groups. α-Methyl-p-tyrosine (250 mg/kg) was injected to one of the habituated group and animals were killed eighteen hours after the injection. The other group of habituated rats was withdrawn from phenobarbitone and killed forty-eight hours after withdrawal; but eighteen hours before death each rat received an injection of α-methyl-p-tyrosine (250 mg/kg). Control rats received α-methyl-p-tyrosine only at the same dose level and at the corresponding time.

Results in table 3.4 and figure 3.11 (B) show that habituation significantly decreased and withdrawal increased the degree of depletion of dopamine caused by α-methyl-p-tyrosine. Habituation had a tendency to decrease the depletion of noradrenaline but it only reached a low degree of significance ($P < 0.1$).

Table 3.4

Effect of habituation to and withdrawal from phenobarbitone on the depletion of catecholamines caused by α-methyl-p-tyrosine

Treatment	Dopamine ng/gm	Noradrenaline ng/gm
α-Methyl-p-tyrosine	110 ± 3.8 (6)	56 ± 2.2 (6)
Habituated + α-methyl-p-tyrosine	$155 \pm 1.7^{**}$ (5)	64 ± 4.4 (5)
Withdrawn + α-methyl-p-tyrosine	$53 \pm 4.4^{**}$ (5)	64 ± 4.4 (5)

Each value is the mean $\pm$ standard error from the number of animals shown in parentheses.

Significantly different by the t test (** $P < 0.001$) from those of control rats treated with α-methyl-p-tyrosine only.

3.6 Effect of habituation to and withdrawal from phenobarbitone on the regional distribution of catecholamine in rat brain

Results presented in section 3.4 of this chapter show that, while habituation to phenobarbitone had no effect on the levels of the catecholamines, withdrawal decreased significantly the amount of both dopamine and noradrenaline in rat brain. Habituation reduced the degree of depletion of dopamine caused by α-methyl-p-tyrosine, while withdrawal potentiated it. There was no effect on the depletion of noradrenaline.

In view of these results, we performed experiments to study the effect of habituation to and withdrawal from phenobarbitone on the regional distribution of the catecholamines in order to locate the sites at which withdrawal had brought about the changes in both dopamine and noradrenaline.

Rats were habituated to phenobarbitone by the method described before. They were either killed at the end of the sixth week of administration or killed forty-eight hours after the withdrawal of the drug. Their brains were quickly removed and placed on an ice-cooled glass plate and dissected as described in section 2.9. The dissected areas of the brain were homogenized and assayed for their dopamine and noradrenaline content.

Results are shown in tables 3.5 and 3.6 and figures 3.12 and 3.13.

Habituation to phenobarbitone increased significantly the amount of dopamine in the striata and in the mid brain, and decreased it in the cerebral hemispheres. The total amount of dopamine remained unchanged.

Withdrawal of the drug decreased the concentration of dopamine in the striata and the cerebral hemispheres. Dopamine in midbrain stayed elevated. The total amount of dopamine in the brains of withdrawn rats decreased significantly below that in animals that received only saccharine in their drinking water. These results are in good

agreement with the ones reported earlier (section 3.4). Habituation had no effect on the noradrenaline levels in any part studied, but withdrawal of phenobarbitone decreased significantly the concentration of noradrenaline in the cerebral hemispheres. The total amount of noradrenaline in the brains of withdrawn rats was significantly lower than that in untreated rats.

Table 3.5

Regional distribution of brain dopamine

in rats habituated to and withdrawn from phenobarbitone

Parts of brain	Untreated (4)	Habituated (6)	Withdrawn (6)
	ng/gm	ng/gm	ng/gm
Striata	11647 ± 169	12245 ± 259*	9714 ± 49**
Cerebral hemispheres	693 ± 9.7	593 ± 21**	573 ± 19**
Mid brain	207 ± 1.96	239 ± 8**	231 ± 4**
Total dopamine	882 ± 33.4	909 ± 40	709 ± 30**

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.001$) from those of untreated rats.

Table 3.6

Regional distribution of brain noradrenaline

in rats habituated to and withdrawn from phenobarbitone

Parts of the brain	Untreated (4) ng/gm	Habituated (6) ng/gm	Withdrawn (6) ng/gm
Pons and medulla	674 ± 22.2	672 ± 20.0	651 ± 28.0
Cerebellum	300 ± 7.8	332 ± 28.0	315 ± 15.0
Cerebral hemispheres	325 ± 16.0	330 ± 17.0	205 ± 4.0**
Mid brain	620 ± 40.0	650 ± 41.6	624 ± 16.0
Total noradrenaline	415 ± 5.6	437 ± 14.5	352 ± 9.5**

Each value is the mean ± standard error from the number of animals shown in parentheses.

** Significantly different by the t test from those of untreated rats.

FIGURE 3.12

Regional distribution of brain dopamine

in rats habituated to and withdrawn from phenobarbitone

Each bar indicates mean $\pm$ standard error for the number of animals in the text.

Open columns represent untreated rats, hatched columns represent habituated rats and dotted columns represent withdrawn rats.

** Significantly different by the t test ($P < 0.001$) from those of untreated rats.

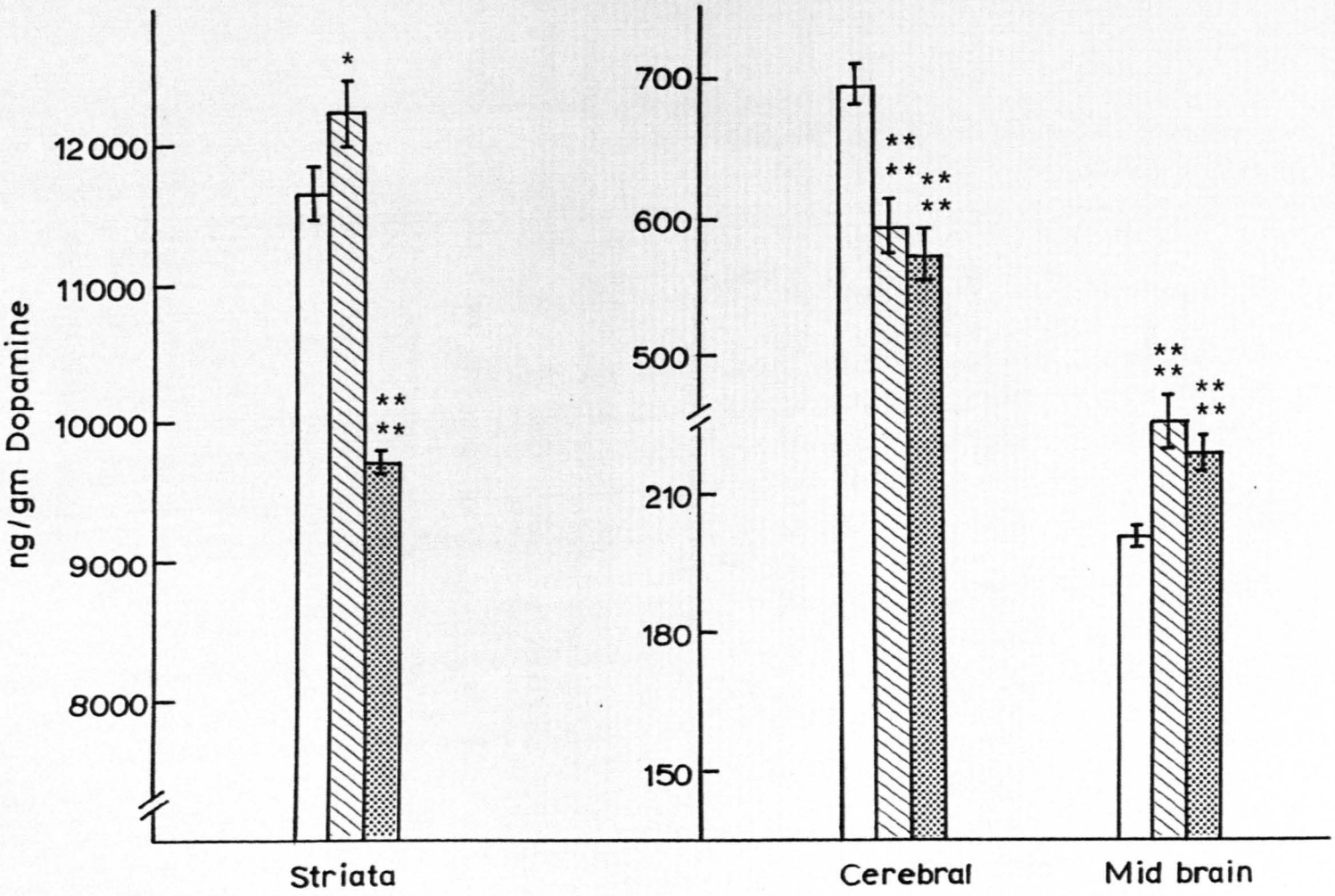

ng/gm Dopamine
12000
11000
10000
9000
8000
700
600
500
210
180
150
*
**
**
**
**
**
**
**
**
Striata
Cerebral
Mid brain

FIGURE 3.13

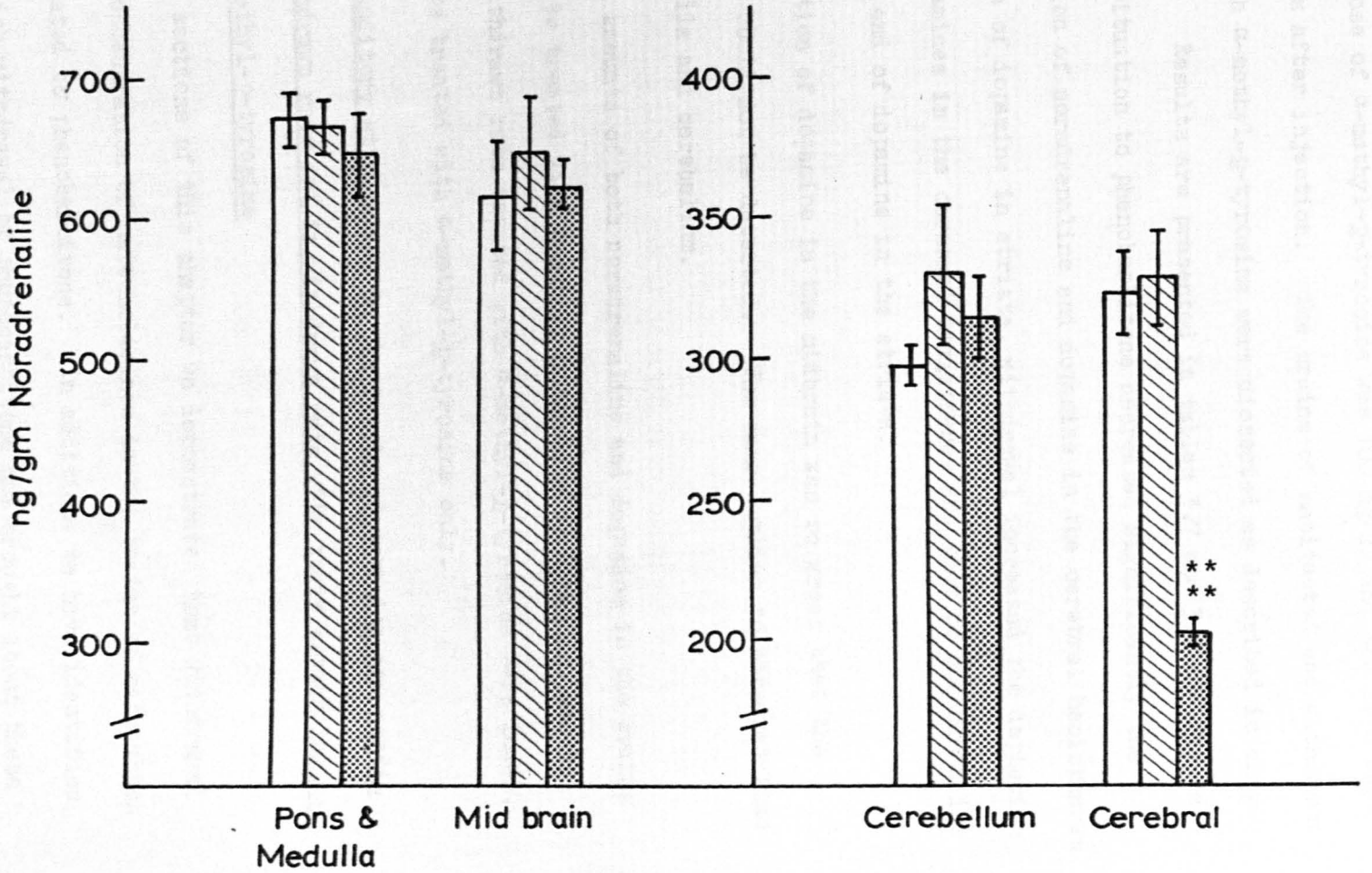
Regional distribution of brain noradrenaline

in rats habituated to and withdrawn from phenobarbitone

Each bar indicates mean $\pm$ standard error for the number of animals in the text.

Open columns represent untreated rats, hatched columns represent habituated rats and dotted columns represent withdrawn rats.

** Significantly different by the t test ($P < 0.001$) from those of untreated rats.


ng/gm Noradrenaline
700
600
500
400
300
400
350
300
250
200
**
**
Pons & Medulla
Mid brain
Cerebellum
Cerebral

3.7 Regional distribution of brain catecholamines in habituated and withdrawn rats after depletion with α-methyl-p-tyrosine

The design of these experiments is the same as that described in section 3.5, except that the dose of α-methyl-p-tyrosine was 100 mg/kg and the rats were killed three hours after injection. The brains of habituated and withdrawn rats injected with α-methyl-p-tyrosine were dissected as described in chapter 2, section 9. Results are presented in tables 3.7 and 3.8 and figure 3.14. While habituation to phenobarbitone decreased significantly the degree of depletion of noradrenaline and dopamine in the cerebral hemispheres and the depletion of dopamine in striata, withdrawal increased the depletion of both catecholamines in the cerebral hemispheres and that of noradrenaline in the mid brain and of dopamine in the striata.

The depletion of dopamine in the midbrain was so great that the amount remaining could not be detected. The same applies to noradrenaline in pons and medulla and cerebellum.

The total amounts of both noradrenaline and dopamine in the brains of habituated rats treated with α-methyl-p-tyrosine were higher, and in the brains of withdrawn rats treated with α-methyl-p-tyrosine were lower, than those in rats treated with α-methyl-p-tyrosine only.

3.8 Effect of auditory stimulation on the levels of dopamine and noradrenaline in withdrawn rats and in withdrawn rats with their catecholamines depleted by α-methyl-p-tyrosine

In the previous sections of this chapter we demonstrated that withdrawal decreased the concentration of catecholamines in the brains of rats which had been habituated to phenobarbitone. In addition, we have identified the areas in which withdrawal of phenobarbitone had brought about these changes.

Table 3.7

Effect of habituation to and withdrawal from phenobarbitone on the levels of dopamine in brains depleted by α-methyl-p-tyrosine

Part of the brain	α-methyl-p-tyrosine (4)	Habituation + α-methyl-p-tyrosine (4)	Withdrawal + α-methyl-p-tyrosine (4)
	ng/gm	ng/gm	ng/gm
Striata	5174 ± 228	5938 ± 241*	3722 ± 58**
Cerebral hemispheres	395 ± 25	541 ± 13**	254 ± 3**
Mid brain	see text	see text	see text
Total dopamine	584 ± 7	792 ± 30**	390 ± 15**

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.001$) from those of control rats treated with α-methyl-p-tyrosine.

Table 3.8

Effect of habituation to and withdrawal from phenobarbitone on the levels of noradrenaline in brains depleted by α-methyl-p-tyrosine

Part of the brain	α-methyl-p-tyrosine (4)	Habituation + α-methyl-p-tyrosine (4)	Withdrawal + α-methyl-p-tyrosine (4)
	ng/gm	ng/gm	ng/gm
Pons and medulla	see text	see text	see text
Cerebellum	see text	see text	see text
Cerebral hemispheres	164 ± 6	226 ± 10**	121 ± 5**
Mid brain	284 ± 24	304 ± 15	225 ± 6*
Total noradrenaline	207 ± 5	254 ± 15**	159 ± 10**

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (*$P < 0.05$; **$P < 0.02$; ***$P < 0.005$; ****$P < 0.001$) from those of control rats treated with α-methyl-p-tyrosine only.

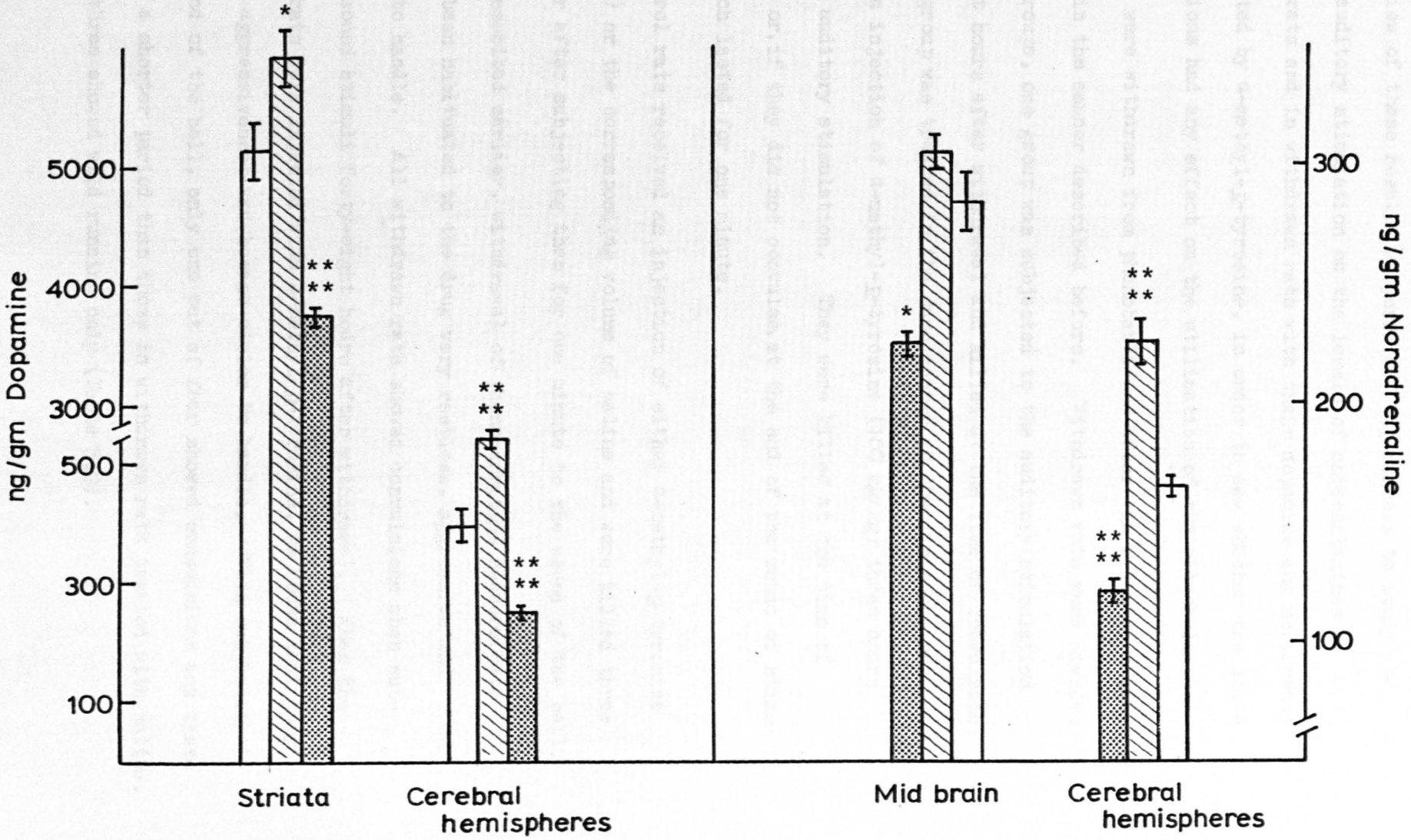
FIGURE 3.14

Regional distribution of brain catecholamines in habituated and withdrawn rats after depletion with α-methyl-p-tyrosine

Each bar indicates the mean ± standard error for the number of animals in the text.

Open columns represent control rats treated with α-methyl-p-tyrosine, hatched columns represent habituated rats treated with α-methyl-p-tyrosine and dotted columns represent withdrawn rats treated with α-methyl-p-tyrosine.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.001$) from those of rats treated with α-methyl-p-tyrosine only.


ng/gm Dopamine
5000
4000
3000
500
300
100
ng/gm Noradrenaline
300
200
100
*
**
**
Striata
Cerebral hemispheres
Mid brain
Cerebral hemispheres

In view of these results we performed experiments to study the effect of auditory stimulation on the levels of catecholamines in withdrawn rats and in withdrawn rats with their dopamine and noradrenaline depleted by α-methyl-p-tyrosine, in order to see whether the state of convulsions had any effect on the utilization of the catecholamines.

Rats were withdrawn from phenobarbitone after six weeks of administration in the manner described before. Withdrawn rats were divided into two groups, one group was subjected to the auditory stimulation forty-eight hours after withdrawal and killed at the time of convulsion. The other group was treated in the same manner as the first one but received an injection of α-methyl-p-tyrosine (100 mg/kg) three hours before the auditory stimulation. They were killed at the time of convulsion or, if they did not convulse, at the end of the sound of stimulation which lasted for one minute.

Control rats received an injection of either α-methyl-p-tyrosine (100 mg/kg) or the corresponding volume of saline and were killed three hours later after subjecting them for one minute to the sound of the bell.

As described earlier, withdrawal of phenobarbitone rendered rats which had been habituated to the drug very restless, aggressive and difficult to handle. All withdrawn rats showed convulsions when subjected to sound stimuli forty-eight hours after withdrawal. When the withdrawn rats were injected with α-methyl-p-tyrosine, we found that they lost their aggressiveness and became easier to handle. When subjected to the sound of the bell, only one out of four showed convulsions and these lasted for a shorter period than those in withdrawn rats treated with saline. The other three showed wild running only (Table 3.9).

Table 3.9

Convulsive activity of withdrawn rats treated with saline or α-methyl-p-tyrosine

Treatment	Number convulsed	Latent period (sec)	Wild running (sec)	Convulsions (sec)
Withdrawn + saline (4)	4	6.5 ± 0.64(4)	10 ± 1.3(4)	42 ± 1.9 (4)
Withdrawn + α-methyl-p-tyrosine (4)	1	20	30	22

Each value is the mean ± standard error from the number of animals that showed convulsions.

Results relating to the catecholamines concentration are shown in tables 3.10 and 3.11 and figures 3.15 and 3.16. Withdrawal convulsions had the effect of increasing the amount of dopamine in the striata and decreasing that in the cerebral hemispheres. These differences attained statistical significance of the $P < 0.025$ level of significance. Noradrenaline did not change in either the cerebral hemispheres or the mid brain. Withdrawn rats treated with α-methyl-p-tyrosine had significantly less dopamine in their striata and cerebral hemispheres than did control rats treated with α-methyl-p-tyrosine. Noradrenaline in the mid brain was significantly less in withdrawn rats treated with α-methyl-p-tyrosine, while the total amount of brain dopamine in convulsed withdrawn rats was not significantly different from that in control rats, noradrenaline was less in this group of animals. However, withdrawal still increased the degree of depletion of both catecholamines caused by α-methyl-p-tyrosine.

Table 3.10

Effect of auditory stimulation on the levels of dopamine in withdrawn rats and in withdrawn rats with their dopamine depleted by α-methyl-p-tyrosine

Treatment	Part of the brain			Total dopamine ng/gm
	Striata ng/gm	Cerebral hemispheres ng/gm	Mid brain ng/gm	
Saline + auditory stimulation (4)	11175 ± 123	771 ± 24	279 ± 2	842 ± 7.1
Withdrawal + auditory stimulation (4)	11600 ± 87**	681 ± 38*	269 ± 10	830 ± 9.2
α-methyl-p-tyrosine + auditory stimulation (4)	5538 ± 104	330 ± 7	Reading below blank values	574 ± 15
Withdrawal + auditory stimulation + α-methyl-p-tyrosine (4)	4206 ± 50**	289 ± 8**	Reading below blank values	462 ± 30**

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.025$; ** $P < 0.001$) from those of corresponding controls.

Table 3.11

Effect of auditory stimulation on the levels of noradrenaline in withdrawn rats and in withdrawn rats with their noradrenaline depleted by α-methyl-p-tyrosine

Treatment	Part of the brain		Total noradrenaline
	Cerebral hemispheres	Mid brain	
	ng/gm	ng/gm	ng/gm
Saline + auditory stimulation (4)	306 ± 14.0	603 ± 16	421 ± 2.83
Withdrawal + auditory stimulation (4)	295 ± 2.5	572 ± 15	385 ± 10.0**
α-methyl-p-tyrosine + auditory stimulation (4)	178 ± 12.0	249 ± 6	204 ± 0.7
Withdrawal + auditory stimulation + α-methyl-p-tyrosine (4)	155 ± 1.0	223 ± 10*	183 ± 4.5**

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) from those of corresponding controls.

FIGURE 3.15

Effect of auditory stimulation on the levels of dopamine in withdrawn rats and in withdrawn rats treated with α-methyl-p-tyrosine

Each bar indicates the mean $\pm$ standard error for the number of animals in the text.

(A), (C) and (E) open columns represent control rats injected with saline and subjected to audiogenic stimulation; hatched columns represent withdrawn rats injected with saline and killed at the time of convulsion during audiogenic stimulation.

(B) and (D) open columns represent control rats injected with α-methyl-p-tyrosine and subjected to audiogenic stimulation; hatched columns represent withdrawn rats injected with α-methyl-p-tyrosine and subjected to audiogenic stimulation.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$) from those of the corresponding controls.

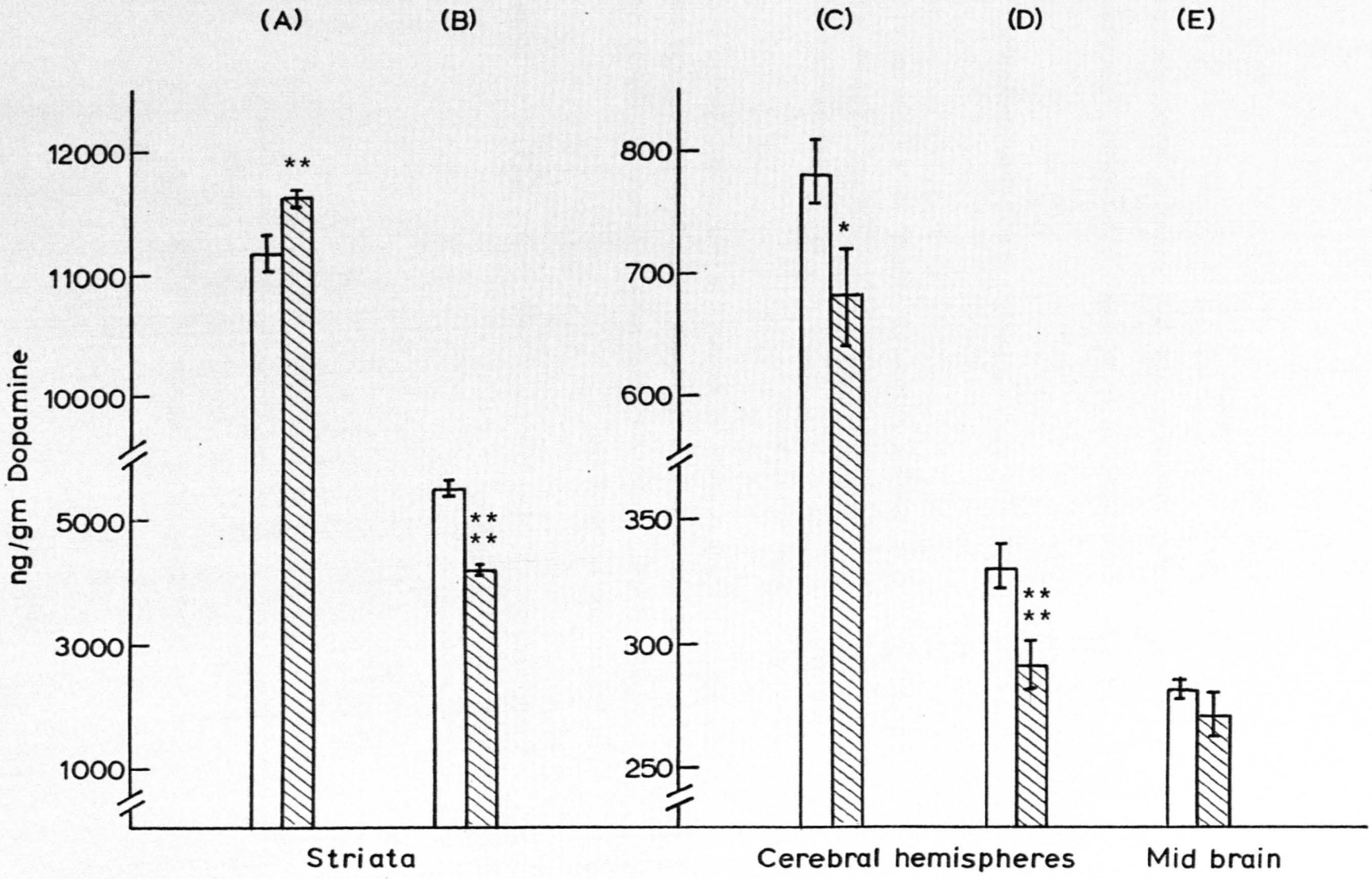

(A)
(B)
(C)
(D)
(E)
ng/gm Dopamine
12000
11000
10000
5000
3000
1000
800
700
600
350
300
250
**
**
**
*
**
**
Striata
Cerebral hemispheres
Mid brain

FIGURE 3.16

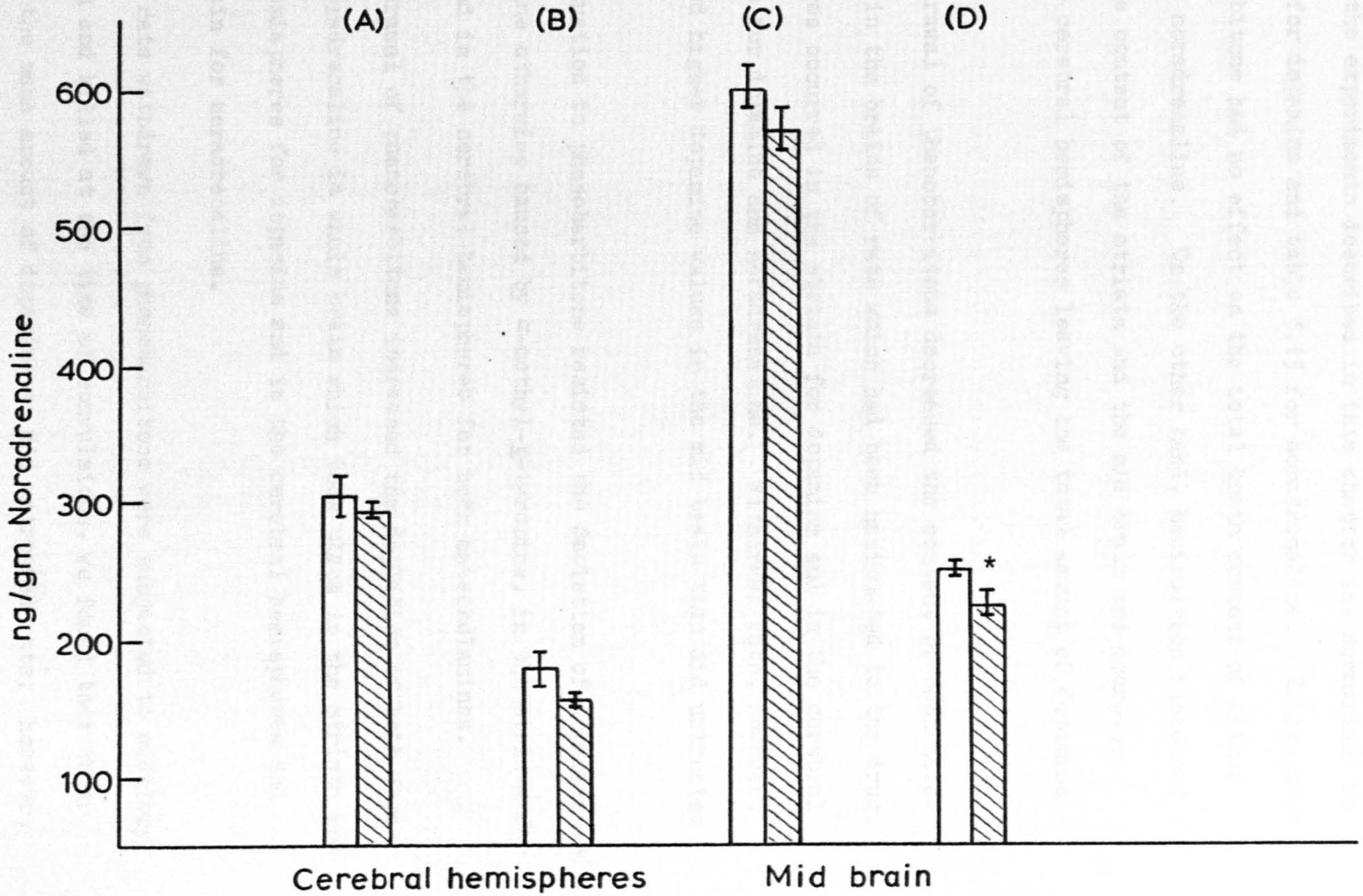
Effect of auditory stimulation on the levels of noradrenaline in withdrawn rats and in withdrawn rats treated with α-methyl-p-tyrosine

Each bar indicates the mean $\pm$ standard error from the number of animals in the text.

(A) and (C) open columns represent control rats injected with saline and subjected to audiogenic stimulation; hatched columns represent withdrawn rats injected with saline and killed at the time of convulsion during audiogenic stimulation.

(B) and (D) open columns represent control rats injected with α-methyl-p-tyrosine and subjected to audiogenic stimulation; hatched columns represent withdrawn rats injected with α-methyl-p-tyrosine and subjected to audiogenic stimulation.

Significantly different by the t test (* $P < 0.05$) from those of corresponding controls.


(A)
(B)
(C)
(D)
600
500
400
300
200
100
ng/gm Noradrenaline
*
Cerebral hemispheres
Mid brain

3.9 Summary of results presented in chapter 3

Results of the experiments described in this chapter are summarised in table 3.12 for dopamine and table 3.13 for noradrenaline. Habituation to phenobarbitone had no effect on the total brain content of either dopamine or noradrenaline. On the other hand, habituation increased the dopamine content of the striata and the mid brain and decreased that of the cerebral hemispheres leaving the total amount of dopamine unchanged.

Withdrawal of phenobarbitone decreased the content of both catecholamines in the brains of rats which had been habituated to the drug. These changes occurred in the striata for dopamine and in the cerebral hemispheres for dopamine and noradrenaline. Withdrawn rats, however, still showed higher dopamine values in the mid brain than did untreated rats.

Habituation to phenobarbitone resisted the depletion of dopamine and noradrenaline otherwise caused by α-methyl-p-tyrosine, in the striata for dopamine and in the cerebral hemispheres for both catecholamines.

Withdrawal of phenobarbitone increased the depletion of both dopamine and noradrenaline in whole brain which took place in the striata and cerebral hemispheres for dopamine and in the cerebral hemispheres and the mid brain for noradrenaline.

When rats withdrawn from phenobarbitone were subjected to auditory stimulation and killed at the time of convulsions, we found that their brains had the same amount of dopamine as did untreated rats; however, they showed significantly higher amounts of dopamine than did withdrawn rats which had not been stressed by sound stimulation. The amount of

noradrenaline was smaller in this group of rats than in the control group, but was significantly greater than in withdrawn rats which had not been stressed by sound stimulation.

Pretreatment with α-methyl-p-tyrosine prevented audiogenic convulsion in the majority of withdrawn rats. This group of animals showed less dopamine in the striata and the cerebral hemispheres and less noradrenaline in the mid brain when compared with the corresponding controls.

However, the total amount of the depleted dopamine and noradrenaline in withdrawn rats stressed by audiogenic stimulation was slightly higher than in withdrawn rats with depleted catecholamines which had not been stressed by audiogenic stimulation.

Table 3.12

Summary of dopamine results

Treatment	Striata ng/gm	Cerebral hemispheres ng/gm	Mid brain ng/gm	Total dopamine ng/gm
Control	11647 ± 169	693 ± 9.7	207 ± 1.96	882 ± 33.4
habituation	12245 ± 259*	593 ± 21.0**	239 ± 8.0**	909 ± 40.0
withdrawal	9714 ± 49**	573 ± 19.0**	231 ± 4.0**	709 ± 30**
Control + α-methyl-p-tyrosine	5174 ± 228	395 ± 25	Readings below blank	584 ± 7
habituation + α-methyl-p-tyrosine	5938 ± 241*	541 ± 13**		792 ± 30**
withdrawal + α-methyl-p-tyrosine	3722 ± 58**	254 ± 3**		390 ± 15**
Audiogenic stimulation				
+ saline	11175 ± 123	771 ± 24	279 ± 2	842 ± 7.1
+ withdrawal	11600 ± 87**	681 ± 38*	269 ± 10	830 ± 9.2
+ control + α-methyl-p-tyrosine	5538 ± 104	330 ± 7	Readings below blank	574 ± 15
+ withdrawal + α-methyl-p-tyrosine	4206 ± 50**	289 ± 8**		462 ± 30**

Each value is the mean ± standard error from the number of animals shown in the text.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; **** $P < 0.001$) from the corresponding control.

Table 3.13

Summary of noradrenaline results

Treatment	Cerebral hemispheres ng/gm	Mid brain ng/gm	Total noradrenaline ng/gm
Untreated	325 ± 16.0	620 ± 40.0	415 ± 5.6
habituated	330 ± 17.0	650 ± 41.6	437 ± 16.5
withdrawn	205 ± 4.0**	624 ± 16.0	352 ± 9.5**
Control + α-methyl-p-tyrosine	164 ± 6.0	284 ± 24.0	207 ± 5
habituation + α-methyl-p-tyrosine	226 ± 10.0**	304 ± 15.0	254 ± 15**
withdrawal + α-methyl-p-tyrosine	121 ± 5.0**	225 ± 6.0*	159 ± 10**
Audiogenic stimulation			
+ saline	306 ± 14.0	603 ± 16.0	421 ± 2.83
+ withdrawal	295 ± 2.5	572 ± 15.0	385 ± 10.0**
+ control + α-methyl-p-tyrosine	178 ± 12.0	249 ± 6.0	204 ± 7.0
+ withdrawal + α-methyl-p-tyrosine	155 ± 1.0	223 ± 10*	183 ± 4.5**

Each value is the mean ± standard error from the number of animals shown in the text.

Significantly different by the t test (*P < 0.05; ** P < 0.02; *** P < 0.005; **** P < 0.001) from corresponding controls.

CHAPTER 4

BRAIN CATECHOLAMINES AFTER LONG-TERM TREATMENT WITH PHENYTOIN, CARBAMAZEPINE AND SODIUM BROMIDE

Bromides were the first antiepileptic drugs, being introduced in 1857 by Locock. Because of their low ratio of the therapeutically effective dose to toxicity, their use was gradually abandoned when better drugs such as phenobarbitone (discovered in 1912) and phenytoin (in 1938) became available.

Carbamazepine is one of the most recent drugs for treatment of epilepsy. Blom in 1962 discovered that carbamazepine is of use in the treatment of trigeminal neuralgia and it has been applied to the treatment of diabetes insipidus.

We have seen in chapter 2 that single doses of phenytoin, sodium bromide and carbamazepine had no effect on total brain levels of either dopamine or noradrenaline. On the other hand, when we looked at the effect of these drugs on the regional distribution of the catecholamines, we found that phenytoin increased the amount of dopamine in the striata and that of noradrenaline in the mid brain. However, carbamazepine increased only noradrenaline content in the cerebral hemispheres and it had no effect on the dopamine content in any of the brain regions studied.

The fact that most antiepileptic drugs are used over a long period of time for continuous control of seizures in epileptic patients led us to investigate the effect of long term treatment with these drugs on the levels of catecholamines in rat brain.

4.1 Methods of administration

Female Ash Wistar rats weighing approximately 160 gms were obtained from our usual source. They were housed in individual cages where they were allowed free access to food and water for one week before the beginning of the experiment. During this week their food and water intakes were monitored daily and their body weights were taken every three days.

Carbamazepine is soluble in propylene glycol after moderate heating, phenytoin can be suspended in saline with the aid of 1% Tween 80, and sodium bromide is soluble in normal saline.

The animals were divided into three groups, each of which received one of the three drugs twice daily by the intraperitoneal route. The first injection was given at 10.00 hours and the other injection was given at 18.00 hours.

Phenytoin was injected in saline containing 1% Tween 80 in a volume not exceeding 2.5ml/kg body weight, at a dose level of 25 mg/kg for the first week of treatment. The dose was raised to 50 mg/kg twice daily during the second week of treatment and finally to 75 mg/kg during the third and final week of treatment. Control rats received the same volume of saline containing 1% Tween 80.

Carbamazepine was injected in propylene glycol in a volume not exceeding 1 ml/kg body weight at a dose level of 20 mg/kg twice daily for the first ten days of administration. The dose was then increased to 40 mg/kg for the next eleven days. Control rats received the corresponding volume of propylene glycol.

Sodium bromide was injected in a volume of saline not exceeding

2.5 ml/kg body weight, at a dose level of 100 mg/kg twice daily for the first week of treatment. It was then increased to 250 mg/kg during the second week of treatment and finally to 500 mg/kg during the third week. The corresponding controls received the same volume of saline twice a day.

All drug solutions for injection were prepared daily. The food and water intakes were monitored daily and body weights were taken every three days.

All the measurements for food and water intakes and body weights were taken at the same time of the day (between 10.00 and 11.00 hours), during the whole period of treatment.

4.2 Behavioural studies during the long term treatment with anticonvulsant drugs

Phenytoin

The first doses of phenytoin (25 mg/kg twice daily) produced a slight ataxia fifteen minutes after injection which disappeared completely about two hours later. Rats looked normal after the disappearance of ataxia and were as active as control rats treated with saline solution. They did not gain weight during the first week of treatment as did the control group, in fact there was a slight but nonsignificant loss in body weight. There was a fluctuation in the food intake which fell during the first two days of treatment, recovered for the next two days and then fell again. Water intakes did not change much during the first week of treatment.

When the dose was increased to 50 mg/kg twice daily at the beginning of the second week of treatment, phenytoin treated rats started to show

slight ataxia after injection which persisted till the next injection. Moreover, their gait now showed obvious differences from that of control rats with loss of muscle tone. In association with the loss of tone, the animal grip strength was weakened. They were not as active as control rats and showed hair discoloration, indicating that the rats were not cleaning themselves. They urinated a lot and we had sometimes to clean their cages daily, a process which was normally done only twice a week.

The animals' food intakes dropped immediately after increasing the dose and started to recover after the second day of treatment at this dose level. Water intakes were increased by a considerable amount.

Just before the end of treatment at this dose level, rats injected with phenytoin started to tolerate the dose; they regained a normal gait, became more active and lost most of their ataxia about three hours after injection, leaving just a little weakness of the limb muscles which could be assessed by pushing the limbs of the rat against the palm of the hand. In all other respect the rats appeared perfectly normal. In spite of the increase in food and water intakes, their body weights did not change noticeably.

On increasing the dose of phenytoin up to 75 mg/kg twice a day at the end of the second week of treatment, ataxia became more pronounced than it was with 50 mg/kg, rats remained ataxic all the time, lost their grip strength completely, looked very sedated and their muscle tone was very poor. The rats spent most of their time sleeping in a corner of their cages. They urinated a lot, their fur took on a yellow colour

and they drank much more water than previously. Their food intake dropped and started to recover four days later.

At the end of the third week of treatment, rats injected with phenytoin started to recover slightly, but they were still ataxic most of the time and were clearly less active than control rats. This persisted till the end of the treatment.

Figures 4.1, 4.2 and 4.3 show the changes in food and water intakes and body weights during the course of long term treatment with different doses of phenytoin.

Carbamazepine

The first doses of carbamazepine (20 mg/kg twice daily) produced slight ataxia which started to disappear two hours after the injection. The food intake of rats treated with carbamazepine decreased after the first day of treatment. This was accompanied by a decrease in the body weight. Water intake increased during this time. Near the end of treatment at this dose level (20 mg/kg twice daily) rats on treatment with carbamazepine started to eat more and gained more weight, and the ataxia after injection became less apparent, indicating that rats tolerated the dose very well.

When the dose of carbamazepine was increased to 40 mg/kg twice daily on the eleventh day of treatment, ataxia became pronounced and after the third dose at this dose level, it persisted till the next injection. Righting reflexes were lost about fifteen minutes after injection and were gained around three hours later. The muscle tone was low, and the rats moved with difficulty, crawling around their cages with extended hind limbs. Rats on carbamazepine continued to gain weight, there was a transient decrease in food intake which recovered after two days. The water intake was increased much more and the rats

FIGURE 4.1

Changes in food intakes during long term treatment with different doses of phenytoin

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with phenytoin and hatched columns represent rats treated with saline containing 1% Tween 80.

Significantly different by the t test ($^{*}P < 0.05$; $^{**}P < 0.02$; $^{***}P < 0.005$ and $^{****}P < 0.001$) from those of controls treated with saline only.

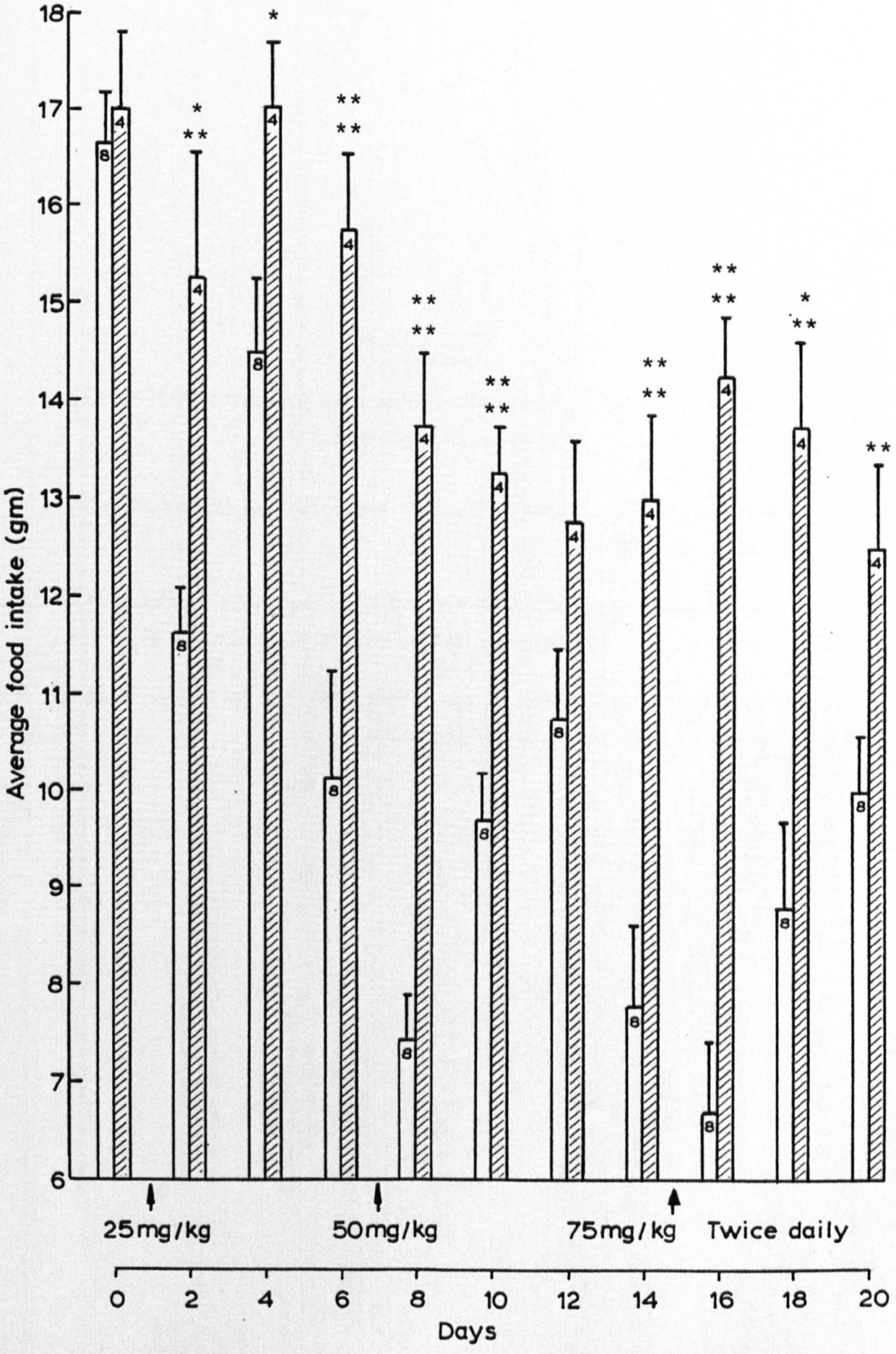

Average food intake (gm)
18
17
16
15
14
13
12
11
10
9
8
7
6
25mg/kg
50mg/kg
75mg/kg
Twice daily
0
2
4
6
8
10
12
14
16
18
20
Days

FIGURE 4.2

Changes in water intakes during long term treatment with different doses of phenytoin

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with phenytoin and hatched columns represent rats treated with saline containing 1% Tween 80.

Significantly different by the t test ($^{**}P < 0.001$) from those of control rats treated with saline only.

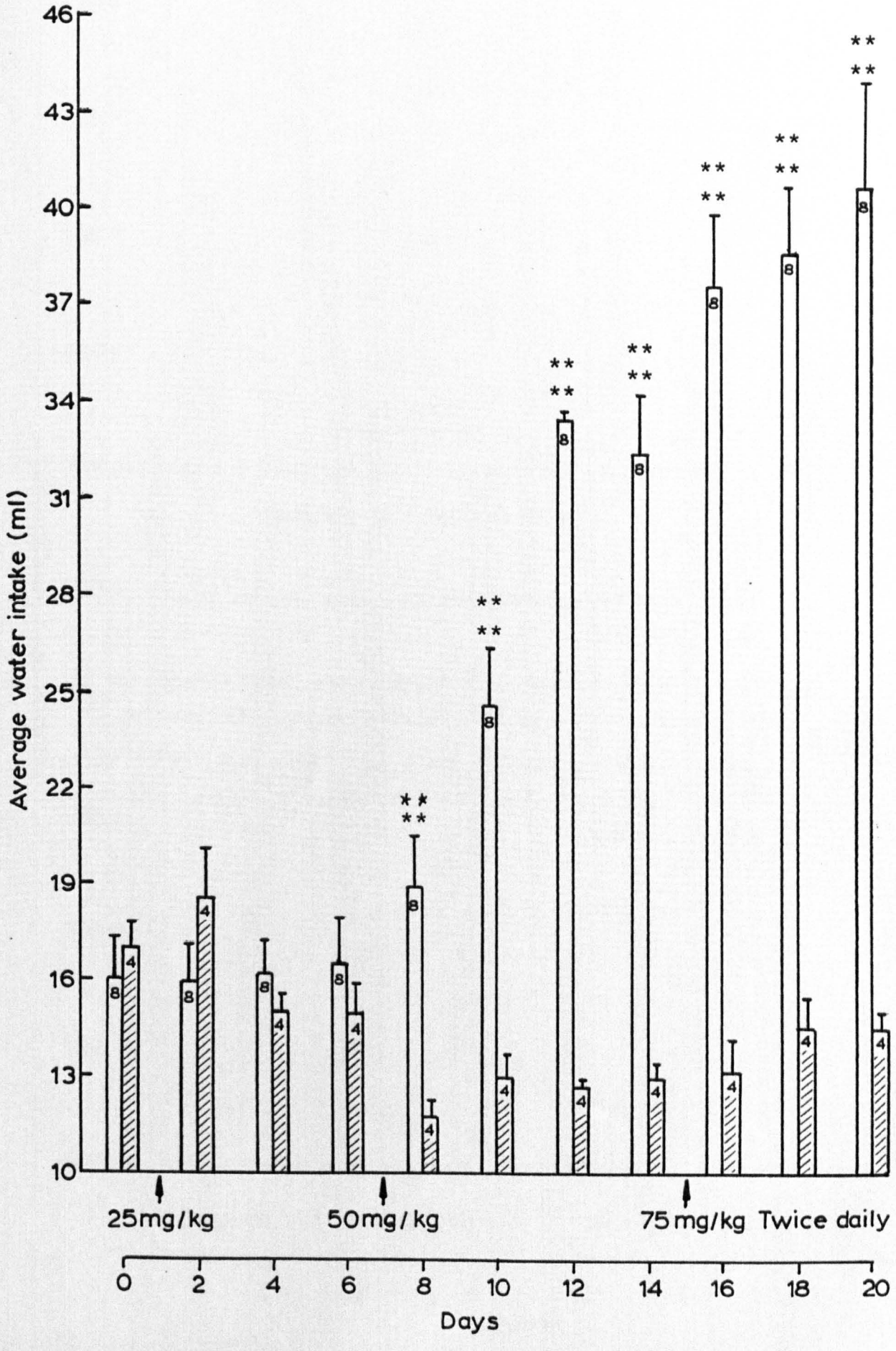

Average water intake (ml)
46
43
40
37
34
31
28
25
22
19
16
13
10
25mg/kg
50mg/kg
75mg/kg Twice daily
0
2
4
6
8
10
12
14
16
18
20
Days

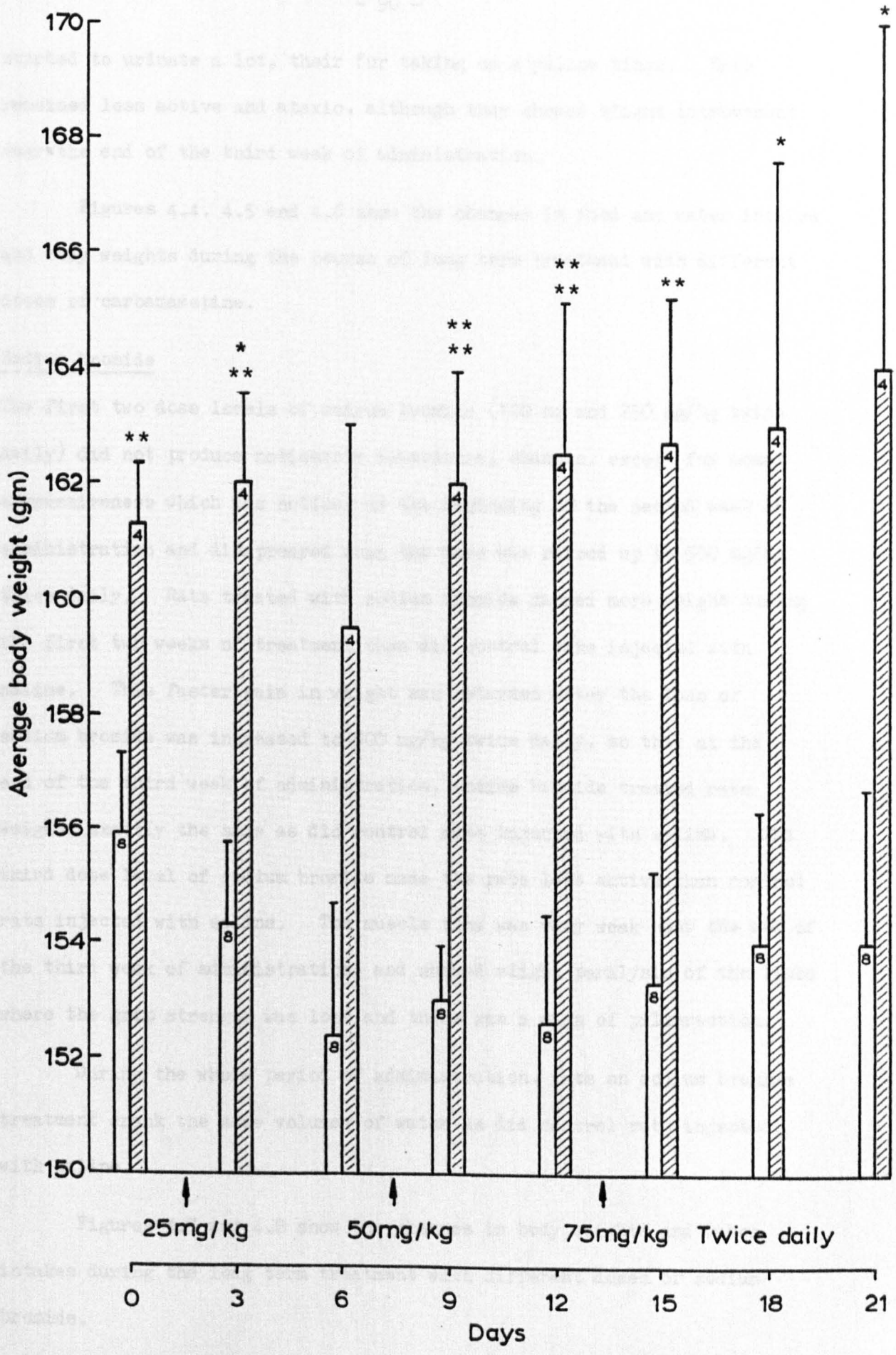
FIGURE 4.3

Changes in body weights during long term treatment with different doses of phenytoin

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with phenytoin and hatched columns represent rats treated with saline containing 1% Tween 80.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.002$; * ** $P < 0.005$ and ** ** $P < 0.001$) from those of control rats treated with saline only.


Average body weight (gm)
170
168
166
164
162
160
158
156
154
152
150
25mg/kg
50mg/kg
75mg/kg
Twice daily
0
3
6
9
12
15
18
21
Days

started to urinate a lot, their fur taking on a yellow tinge. Rats remained less active and ataxic, although they showed slight improvement near the end of the third week of administration.

Figures 4.4, 4.5 and 4.6 show the changes in food and water intakes and body weights during the course of long term treatment with different doses of carbamazepine.

Sodium bromide

The first two dose levels of sodium bromide (100 mg and 250 mg/kg twice daily) did not produce noticeable behavioural changes, except for some aggressiveness which was noticed at the beginning of the second week of administration and disappeared when the dose was raised up to 500 mg/kg twice daily. Rats treated with sodium bromide gained more weight during the first two weeks of treatment than did control rats injected with saline. This faster gain in weight was retarded after the dose of sodium bromide was increased to 500 mg/kg twice daily, so that at the end of the third week of administration, sodium bromide treated rats weighed exactly the same as did control rats injected with saline. The third dose level of sodium bromide made the rats less active than control rats injected with saline. The muscle tone was very weak near the end of the third week of administration, and showed slight paralysis of the limbs where the grip strength was lost and there was a sign of piloerection.

During the whole period of administration, rats on sodium bromide treatment drank the same volumes of water as did control rats injected with saline.

Figures 4.7 and 4.8 show the changes in body weights and water intakes during the long term treatment with different doses of sodium bromide.

FIGURE 4.4

Changes in food intakes during long term treatment

with different doses of carbamazepine

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with carbamazepine and hatched columns represent control rats treated with propylene glycol.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.005$ and ** $P < 0.001$) from those of control rats treated with propylene glycol.

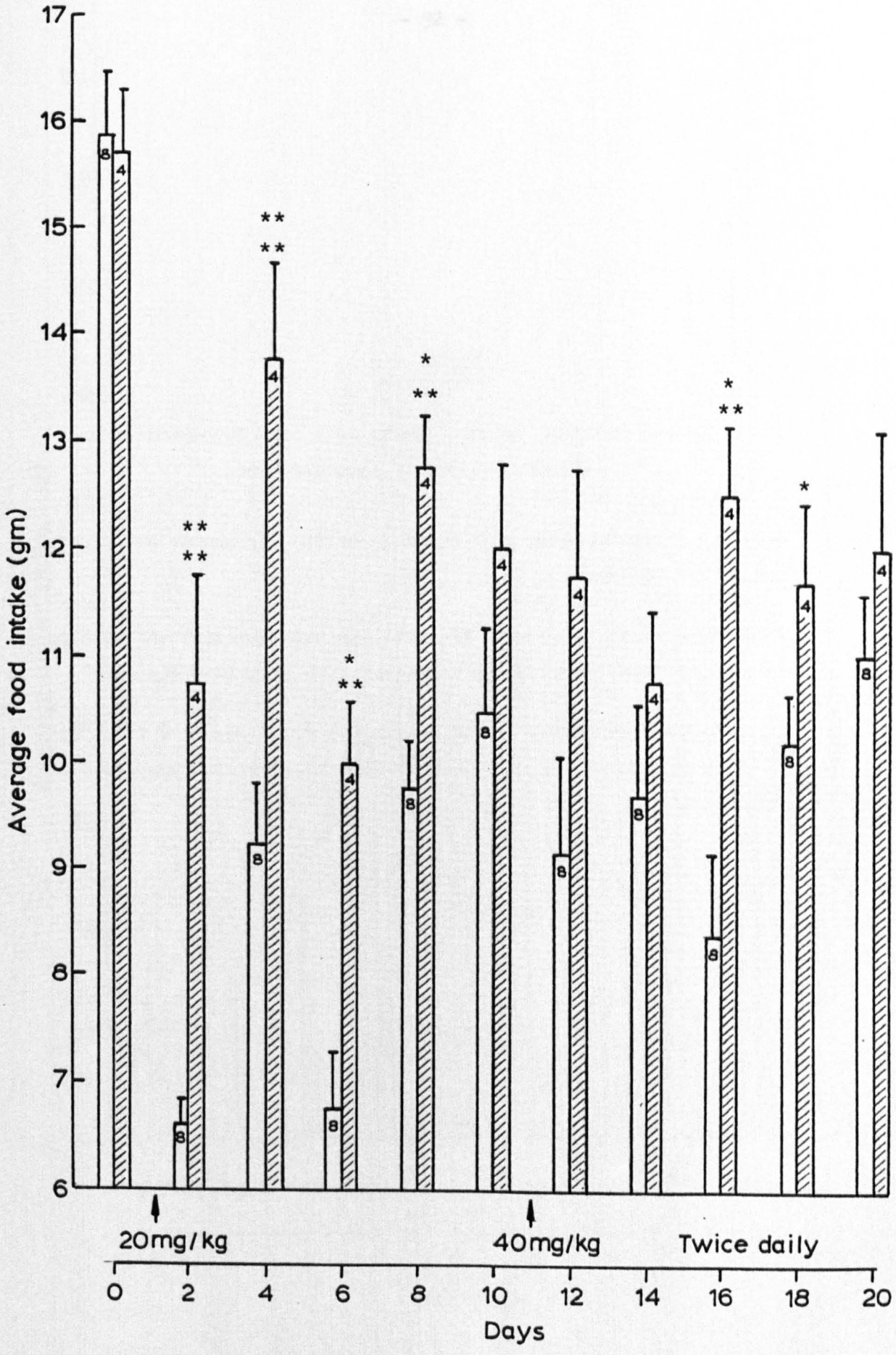

Average food intake (gm)
17
16
15
14
13
12
11
10
9
8
7
6
20mg/kg
40mg/kg
Twice daily
0
2
4
6
8
10
12
14
16
18
20
Days

FIGURE 4.5

Changes in water intakes during long term treatment with different doses of carbamazepine

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with carbamazepine and hatched columns represent control rats treated with propylene glycol.

Significantly different by the t test (** $P < 0.02$; *** $P < 0.005$; **** $P < 0.001$) from those of control rats treated with propylene glycol only.

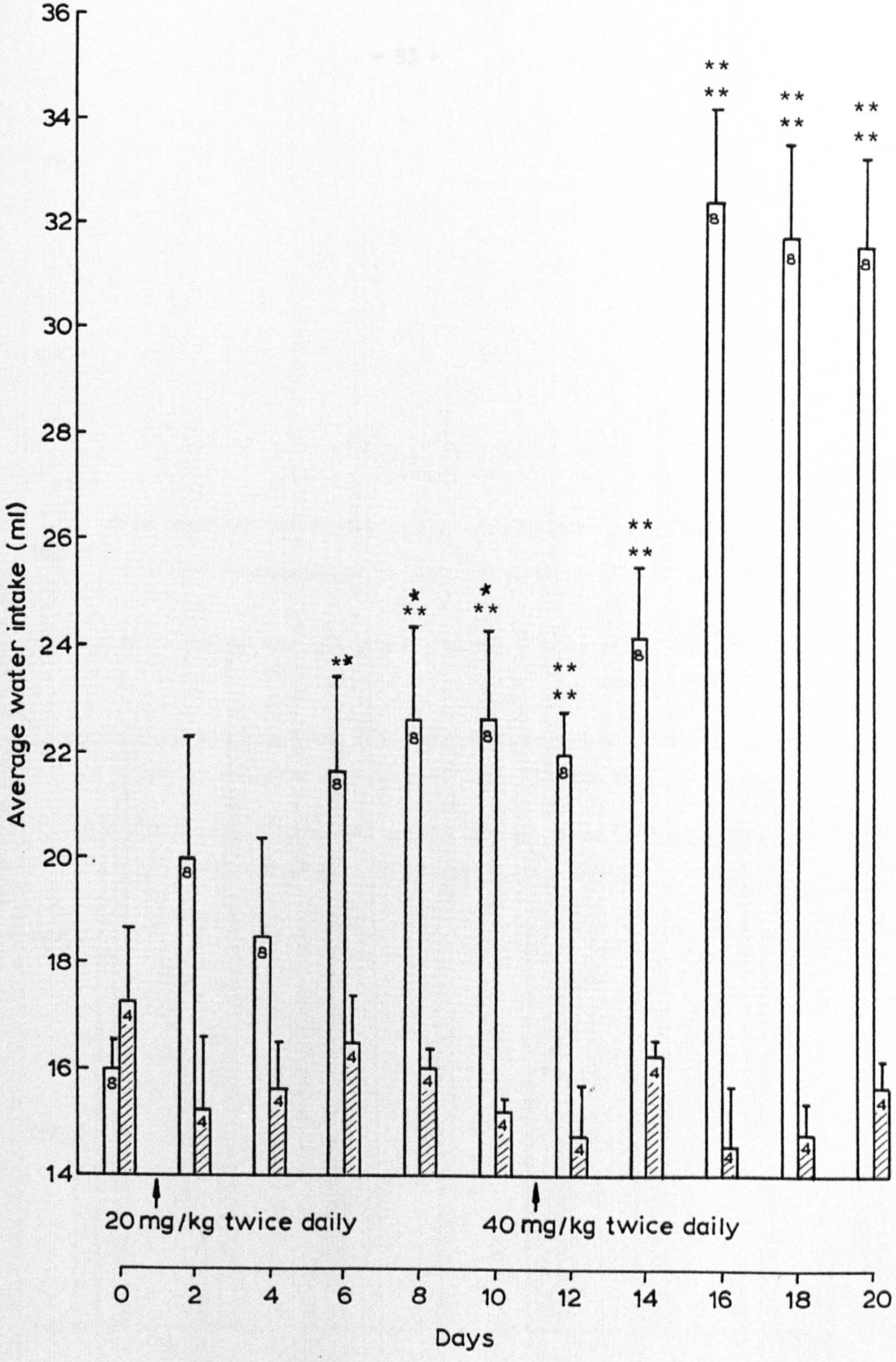

Average water intake (ml)
36
34
32
30
28
26
24
22
20
18
16
14
20 mg/kg twice daily
40 mg/kg twice daily
0
2
4
6
8
10
12
14
16
18
20
Days

FIGURE 4.6

Changes in body weights during long term treatment with different doses of carbamazepine

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with carbamazepine and hatched columns represent control rats treated with propylene glycol.

Significantly different by the t test (* $P < 0.05$ and ** $P < 0.02$) from those of control rats treated with propylene glycol.

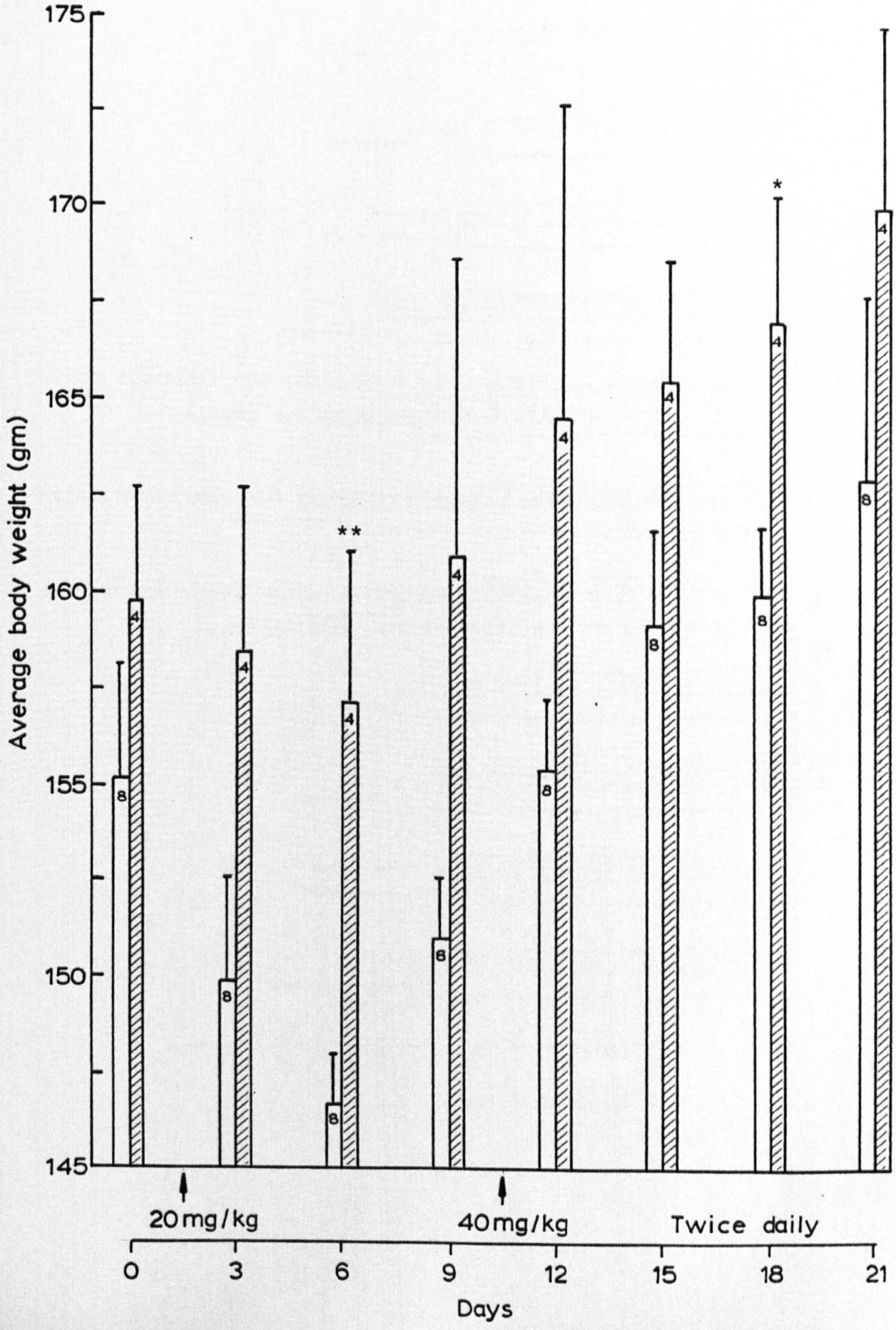

175
170
165
160
155
150
145
Average body weight (gm)
**
*
8
4
20 mg/kg
40 mg/kg
Twice daily
0
3
6
9
12
15
18
21
Days

FIGURE 4.7

Changes in water intakes during long term treatment with different doses of sodium bromide

Each bar indicates mean $\pm$ standard error for the number of animals inside the column.

Open columns represent rats treated with sodium bromide and hatched columns represent control rats treated with saline.

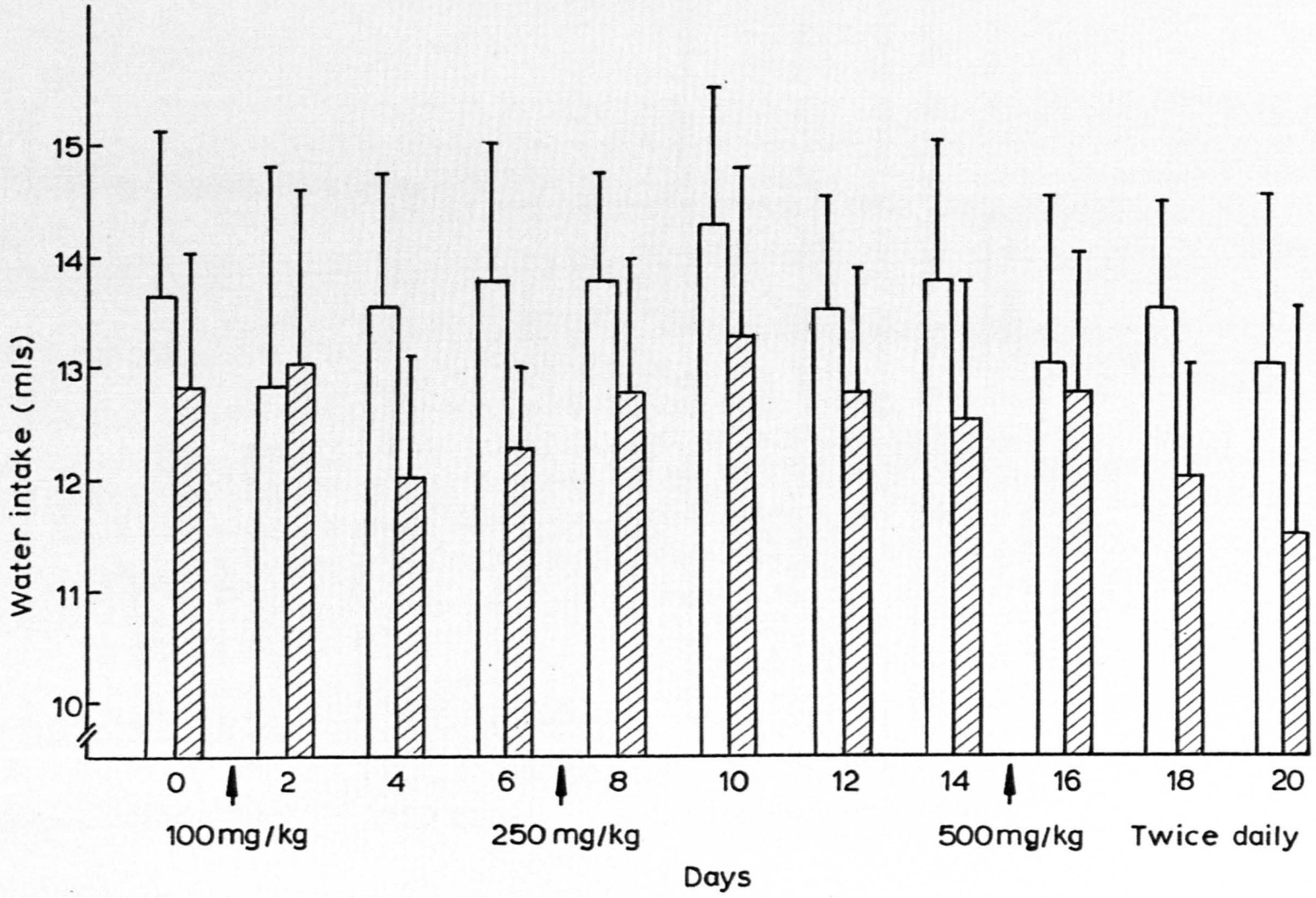

Water intake (mls)
15
14
13
12
11
10
0
2
4
6
8
10
12
14
16
18
20
100mg/kg
250 mg/kg
500mg/kg
Twice daily
Days

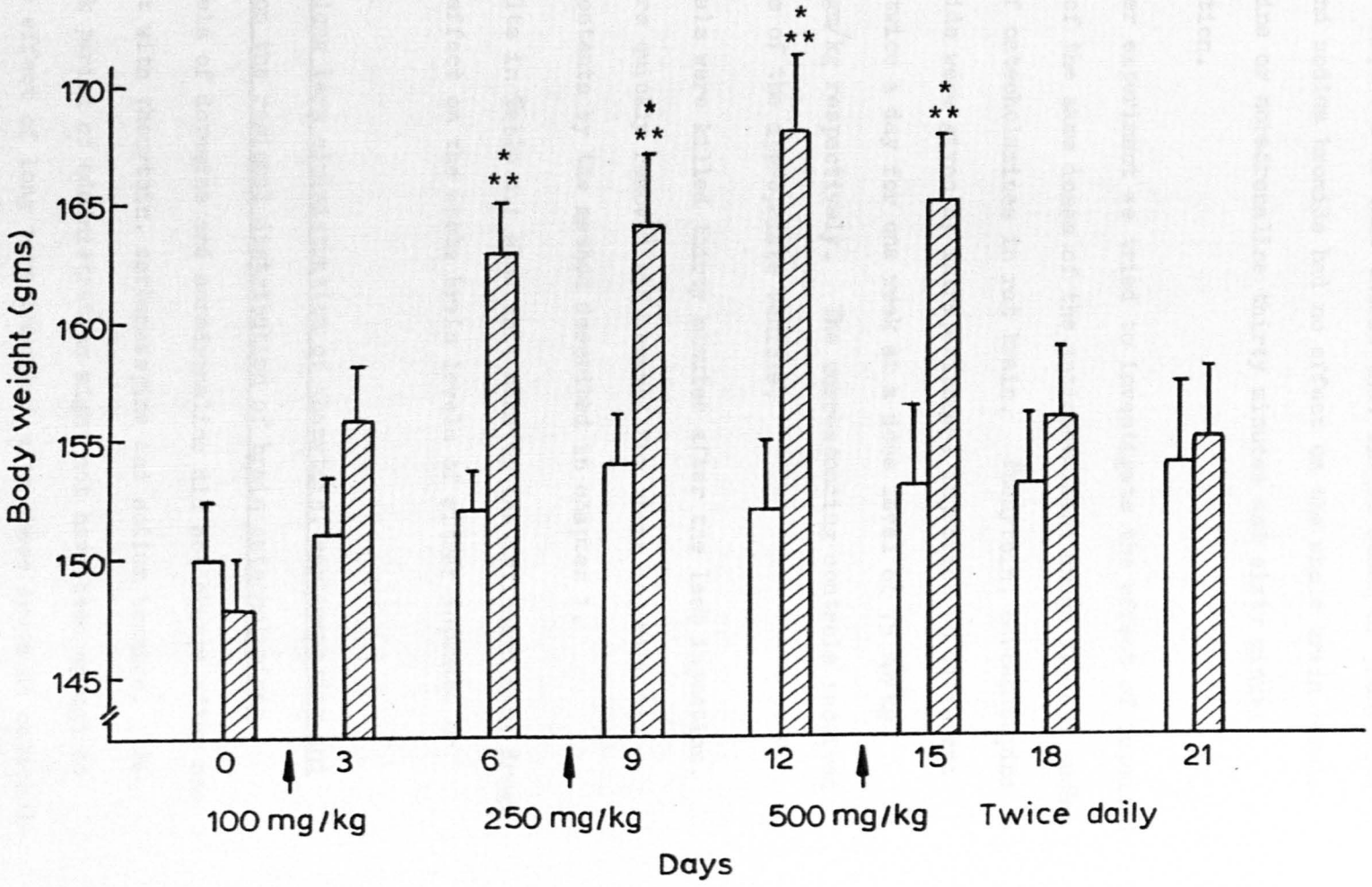
FIGURE 4.8

Changes in body weights during long term treatment with different doses of sodium bromide

Each bar indicates mean ± standard error for the number of animals inside the column.

Open columns represent rats treated with sodium bromide and hatched columns represent control rats treated with saline.

Significantly different by the t test (** $P < 0.005$) from those of control rats treated with saline.


Body weight (gms)
170
165
160
155
150
145
* **
* **
* **
* **
0
3
6
9
12
15
18
21
100 mg/kg
250 mg/kg
500 mg/kg
Twice daily
Days

4.3 Effect of repeated administration of carbamazepine, phenytoin and sodium bromide on whole brain levels of catecholamines

We have seen in chapter 2 of this thesis that single doses of phenytoin, carbamazepine and sodium bromide had no effect on the whole brain levels of either dopamine or noradrenaline thirty minutes and sixty minutes after the injection.

In another experiment we tried to investigate the effect of repeated administration of the same doses of the anticonvulsant drugs for one week on the levels of catecholamines in rat brain. Phenytoin, carbamazepine and sodium bromide were given by intraperitoneal injection to different groups of rats twice a day for one week at a dose level of 75 mg/kg, 50 mg/kg and 1 gm/kg respectively. The corresponding controls received the same volumes of the appropriate vehicle.

The animals were killed thirty minutes after the last injection, their brains were quickly removed and assayed for their dopamine and noradrenaline contents by the method described in chapter 1.

The results in Table 4.1 show that none of the anticonvulsant drugs tested had any effect on the whole brain levels of either dopamine or noradrenaline.

4.4 Effect of long term administration of phenytoin, carbamazepine and sodium bromide on the regional distribution of brain catecholamines

Whole brain levels of dopamine and noradrenaline did not change after one week's treatment with phenytoin, carbamazepine and sodium bromide. Because a one week period of administration might not have been enough to demonstrate the effect of long term treatment with these drugs on catecholamine levels, we decided to extend the period of treatment to three weeks

Table 4.1

Effect of the repeated administration of anticonvulsant drugs for one week on the levels of dopamine and noradrenaline in rat brain

Drug	Dopamine ng/gm	Noradrenaline ng/gm
Vehicle	865 ± 30 (12)	394 ± 20 (12)
Phenytoin	906 ± 20 (4)	390 ± 20 (4)
Sodium bromide	842 ± 35 (4)	353 ± 15 (4)
Carbamazepine	864 ± 50 (4)	403 ± 5 (4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

in order to allow enough time for the anticonvulsant drugs to bring about any changes they possibly effect. Female Ash Wistar rats weighing between 150 and 160 gms were obtained one week before starting the experiment. They were housed in individual cages where they were allowed free access to food and water. Seven days later, the animals were divided into several groups, and each group was treated with one of the anticonvulsant drugs or its appropriate vehicle by the same schedule described in section 1 of this chapter.

At the end of the third week of treatment with these anticonvulsant drugs, the animals were killed by cervical dislocation and decapitation half an hour after the last injection. Their brains were quickly removed, placed on ice cooled glass plate and dissected by the method desdribed in chapter 2, section 9. Each part was homogenized as soon as it had been isolated and weighed. It was then assayed for its dopamine and noradrenaline content by the method described in chapter 1.

Table 4.2

Effect of long term administration of anticonvulsant drugs on the regional distribution of dopamine

Drug	Striata ng/gm	Cerebral hemispheres ng/gm	Mid brain ng/gm
Propylene glycol	11980 ± 285 (6)	700 ± 17 (6)	159 ± 20 (6)
Carbamazepine	14394 ± 360 (6)	937 ± 23 (6)	160 ± 12 (6)
P	< 0.001	< 0.001	N.S.
Saline + 1% Tween 80	11680 ± 396 (6)	747 ± 11 (6)	161 ± 6 (6)
Phenytoin	13690 ± 298 (6)	695 ± 16 (6)	300 ± 8 (6)
P	< 0.001	< 0.02	< 0.001
Saline	11721 ± 337 (6)	741 ± 29 (6)	166 ± 7 (6)
Sodium bromide	11933 ± 300 (6)	796 ± 13 (6)	191 ± 5 (6)
P	N.S.	N.S.	< 0.02

Each value is the mean ± standard error from the number of animals shown in parentheses.

The figures were obtained in two different experiments.

Results are presented in tables 4.2 and 4.3 and figures 4.9 and 4.10.

The long term treatment with phenytoin increased the concentration of dopamine in the striata and mid brain and decreased it in the cerebral hemispheres, while carbamazepine increased the concentration of dopamine in the striata and cerebral hemispheres, sodium bromide increased it only in the mid brain, it had no effect on the levels of dopamine in other regions studied.

The long term administration of phenytoin and carbamazepine increased

Table 4.3

Effect of long term administration of anticonvulsant drugs on the regional distribution of noradrenaline

Drug	Pons and medulla	Cerebellum	Cerebral hemispheres	Mid brain
	ng/gm	ng/gm	ng/gm	ng/gm
Propylene glycol	604 ± 28 (6)	228 ± 16(6)	309 ± 6 (6)	752 ± 20 (6)
Carbamazepine	634 ± 20 (6)	255 ± 20(6)	370 ± 8 (6)	816 ± 23 (8)
P	N.S.	N.S.	< 0.001	< 0.05
Saline + Tween 80	616 ± 14 (6)	236 ± 16(6)	313 ± 7 (6)	764 ± 18 (6)
Phenytoin	678 ± 18 (6)	249 ± 6(6)	362 ± 10(6)	1058 ± 23 (6)
P	< 0.02	N.S.	< 0.001	< 0.001
Saline	623 ± 14 (6)	231 ± 27(6)	311 ± 17(6)	760 ± 27 (6)
Sodium bromide	617 ± 17 (6)	240 ± 18(6)	322 ± 17(6)	771 ± 32 (6)
P	N.S.	N.S.	N.S.	N.S.

Each value is the mean ± standard error from the number of animals shown in parentheses.

The figures were obtained in two different experiments.

the levels of noradrenaline in the cerebral hemispheres and the mid brain. Phenytoin also increased the levels of noradrenaline in the pons and medulla. Sodium bromide had no effect on the levels of noradrenaline in any of the parts studied.

4.5 Effect of long term administration of phenytoin and carbamazepine on the turnover rates of catecholamines in discrete brain areas

Results in section 4.4 show that the long term administration of either phenytoin or carbamazepine increased the levels of noradrenaline in the

FIGURE 4.9

Effect of long term administration of anticonvulsant drugs on the regional distribution of dopamine

Each bar indicates the mean $\pm$ standard error from the number of animals shown in the text.

(A) Open columns represent control rats treated with propylene glycol, hatched columns represent rats treated with carbamazepine.

(B) Open columns represent control rats treated with saline containing 1% Tween 80, hatched columns represent rats treated with phenytoin.

(C) Open columns represent control rats treated with saline, hatched columns represent rats treated with sodium bromide.

Significantly different by the t test (** $P < 0.02$; *** $P < 0.001$) from those of corresponding controls.

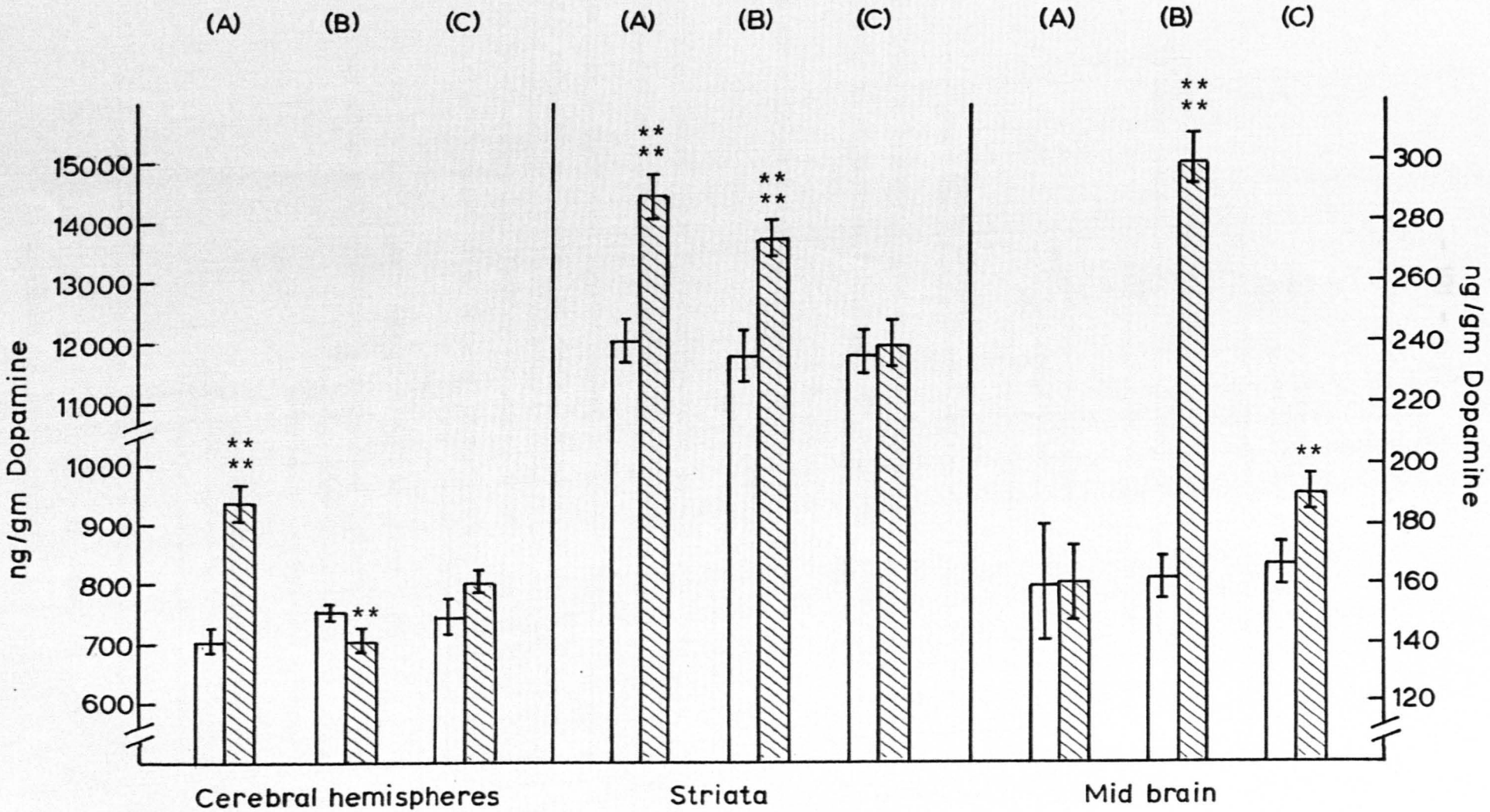

(A)
(B)
(C)
(A)
(B)
(C)
(A)
(B)
(C)
ng/gm Dopamine
15000
14000
13000
12000
11000
1000
900
800
700
600
300
280
260
240
220
200
180
160
140
120
ng/gm Dopamine
Cerebral hemispheres
Striata
Mid brain

FIGURE 4.10

Effect of long term administration of anticonvulsant drugs on the regional distribution of noradrenaline

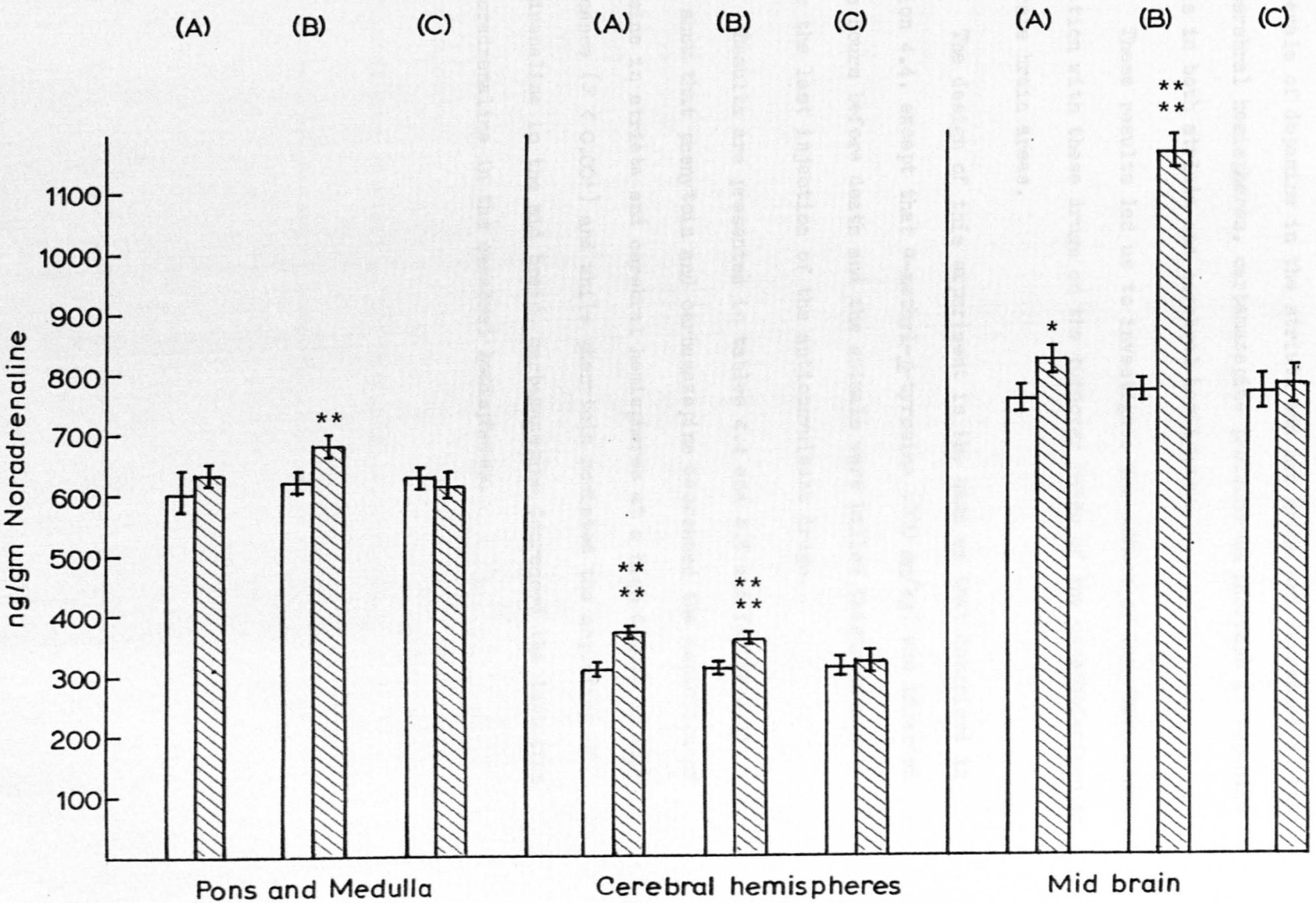
Each bar indicates the mean $\pm$ standard error from the number of animals shown in the text.

(A) Open columns represent control rats treated with propylene glycol, hatched columns represent rats treated with carbamazepine.

(B) Open columns represent control rats treated with saline containing 1% Tween 80, hatched columns represent rats treated with phenytoin.

(C) Open columns represent control rats treated with saline, hatched columns represent rats treated with sodium bromide.

Significantly different by the t test ($*$ $P < 0.05$; $**$ $P < 0.02$; $***$ $P < 0.001$) from those of corresponding controls.


(A)
(B)
(C)
(A)
(B)
(C)
(A)
(B)
(C)
ng/gm Noradrenaline
1100
1000
900
800
700
600
500
400
300
200
100
**
**
**
**
**
**
*
Pons and Medulla
Cerebral hemispheres
Mid brain

cerebral hemispheres and the mid brain, phenytoin increased as well the levels of noradrenaline in pons and medulla. While phenytoin increased the levels of dopamine in the striata and mid brain and decreased it in the cerebral hemispheres, carbamazepine produced an increase in dopamine levels in both striata and cerebral hemispheres.

These results led us to investigate the effect of long term administration with these drugs on the turnover rates of the catecholamines in discrete brain areas.

The design of this experiment is the same as that described in section 4.4, except that α-methyl-p-tyrosine (100 mg/kg) was injected three hours before death and the animals were killed thirty minutes after the last injection of the anticonvulsant drugs.

Results are presented in tables 4.4 and 4.5 and figure 4.11. They show that phenytoin and carbamazepine decreased the depletion of dopamine in striata and cerebral hemispheres at a high degree of significance ($P < 0.001$) and while phenytoin resisted the depletion of noradrenaline in the mid brain, carbamazepine decreased the depletion of noradrenaline in the cerebral hemispheres.

Table 4.4

Effect of long term administration of phenytoin and carbamazepine on the turnover rate of dopamine

Drug	Striata	Cerebral hemispheres
	ng/gm	ng/gm
Saline	11980 ± 270 (4)	700 ± 23 (4)
α-Methyl-p-tyrosine	4018 ± 177 (6)	238 ± 16 (6)
% change	- 66.46	- 66.00
α-Methyl-p-tyrosine + phenytoin	9687 ± 632 (6)	499 ± 20 (6)
% change	- 19.14	- 28.71
P (t test)	< 0.001	< 0.001
α-Methyl-p-tyrosine + carbamazepine	8060 ± 489 (6)	450 ± 20 (6)
% change	- 32.72	- 35.71
P (t test)	< 0.001	< 0.001

Each value is the mean ± standard error from the number of animals shown in parentheses.

The figures were obtained in two different experiments.

Table 4.5

Effect of long term administration of phenytoin and carbamazepine on the turnover rate of noradrenaline

Drug	Cerebral hemispheres	Mid brain
	ng/gm	ng/gm
Saline	315 ± 10 (4)	772 ± 23 (4)
α-Methyl-p-tyrosine	150 ± 8 (6)	275 ± 20 (6)
% change	- 52.38	- 64.37
α-Methyl-p-tyrosine + phenytoin	169 ± 8 (6)	522 ± 8 (6)
% change	- 46.30	- 32.38
P (t test)	< 0.1	< 0.001
α-Methyl-p-tyrosine + carbamazepine	202 ± 12 (6)	301 ± 8 (6)
% change	- 35.87	- 61.01
P (t test)	< 0.005	N.S.

Each value is the mean ± standard error from the number of animals shown in parentheses.

The figures were obtained in two different experiments.

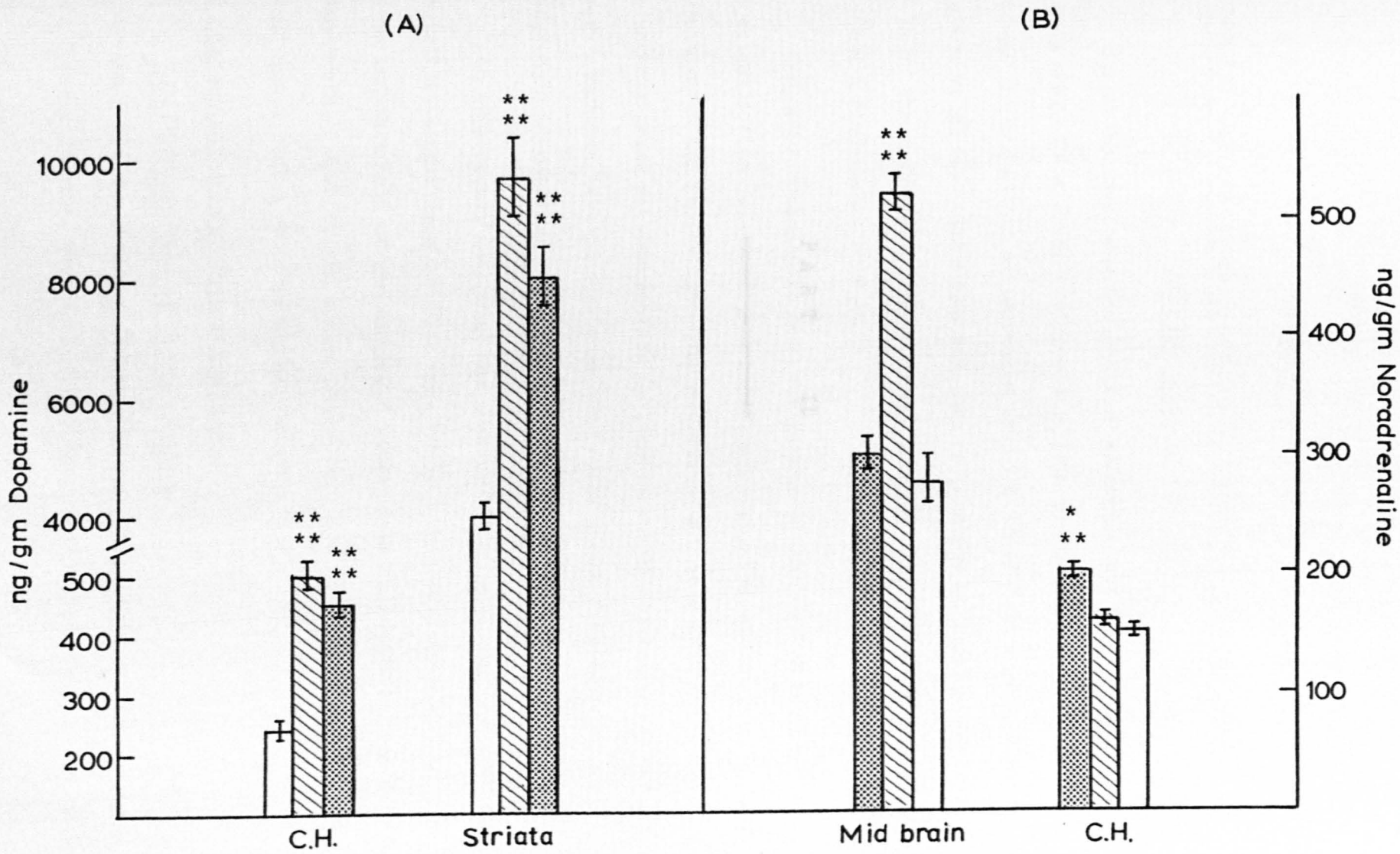
FIGURE 4.11

Effect of long term administration of phenytoin and carbamazepine on the turnover rate of dopamine (A) and noradrenaline (B)

Each bar indicates the mean $\pm$ standard error from the number of animals shown in the text.

Open columns represent control rats treated with α-methyl-p-tyrosine, hatched columns represent rats treated with phenytoin and α-methyl-p-tyrosine, dotted columns represent rats treated with carbamazepine and α-methyl-p-tyrosine.

Significantly different by the t test (* $P < 0.005$; ** $P < 0.001$) from those of control rats injected with α-methyl-p-tyrosine.


(A)
(B)
ng/gm Dopamine
ng/gm Noradrenaline
10000
8000
6000
4000
500
400
300
200
500
400
300
200
100
C.H.
Striata
Mid brain
C.H.

P A R T II

PART II

CHAPTER 5

MEASUREMENT OF RAT BRAIN 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLE ACETIC ACID

The method used for the assay of serotonin was that described by Snyder, Axelrod and Zweig (1965) with slight modifications. The method involved the extraction of serotonin into 1-butanol from a salt saturated solution at pH 10. The amine was then returned to an aqueous solution (pH 7) by the addition of n-heptane and reacted with ninhydrin by heating to yield a fluorescent product.

5-Hydroxyindoleacetic acid was assayed by the method of Giacolone and Valzelli (1966), with slight modifications. The method involved the extraction of the indole into butylacetate from a salt saturated solution. The organic phase was washed with salt saturated dilute acid, and the indole was re-extracted into phosphate buffer. The native fluorescence was measured in strong acid solution.

In experiments involving the measurement of both the amine and its metabolite, brains from two rats were used. They were divided into two halves and each half of one brain was pooled with the opposite half of the other brain. Each of the pooled halves was used for the assay of either 5-hydroxytryptamine or 5-hydroxyindoleacetic acid.

5.1 Determination of 5-hydroxytryptamine

5.1(i) Preparation of tissue extracts

Rats were killed by cervical dislocation and decapitation. Their brains were quickly removed, freed from superficial blood clots, weighed and homo-

genized in 10 ml ice-cold 0.4 N perchloric acid for five minutes using an MSE top drive homogenizer. The homogenates were then centrifuged at 2500 rpm for ten minutes. The supernatant solutions together with standard solutions were processed in identical ways.

5.1(ii) Extraction of 5-hydroxytryptamine

Seven millilitres of the supernatant solution were transferred to a plastic beaker and the pH of the solution was adjusted to 10 with 5 N and 1 N sodium hydroxide. The solution was transferred to a 50 ml glass stoppered centrifuge tube containing 0.5 ml of 0.5 M borate buffer (pH 10), 15 ml of n-butanol and approximately 1.5 g of sodium chloride. The mixture was then shaken mechanically for ten minutes, centrifuged at 2000 rpm for another ten minutes and the lower aqueous phase aspirated off and discarded.

The organic phase was then shaken for three minutes with 2 ml of salt saturated borate buffer (adjusted to pH 10 before use). After centrifugation at 2000 rpm, ten millilitres of the organic phase were transferred to another glass stoppered centrifuge tube containing 3 ml of 0.05 M phosphate buffer (pH 7) and 15 ml n-heptane. After shaking for fifteen minutes, the tube was centrifuged at 2000 rpm and the organic phase was carefully aspirated off and discarded.

5.1(iii) Fluorophore formation

Two millilitres of the buffer solution were transferred to a test tube, and 0.3 ml of 0.1 M ninhydrin solution were added. The mixture was mixed on a vortex mixer and the test tube was then sealed with a glass marble and incubated in a water bath for thirty minutes at 75°C. One hour after heating, the solution was transferred to a quartz cuvette and the fluorescence measured at 490 mμ after activation at 385 mμ, using a

Farrand spectrophotofluorimeter.

The reagent blank was obtained by carrying 7 ml of 0.4 N perchloric acid solution through the whole procedure.

A calibration curve was constructed by carrying solutions of standard 5-hydroxytryptamine in 0.4 N perchloric acid through the whole procedure side by side with the tissue extract. Examples of such curves are given in Figure 5.1 and show the high degree of reproducibility of the method.

The composition of various buffers and reagents is given in the formulary (Appendix I).

5.1(iv) Recovery of standard 5-hydroxytryptamine added to brain homogenates

The reliability of the method was examined by determining the recovery of standard 5-hydroxytryptamine added to aliquots of pooled brain extract.

The pooled supernatant solution of the brain extract from ten rats was divided into ten equal portions. To six portions, various concentrations of standard 5-hydroxytryptamine were added and the mixtures were assayed for 5-hydroxytryptamine by the method described before. The recovery of standard 5-hydroxytryptamine added to pooled brain homogenates is given in Table 5.1

5.2 Determination of 5-hydroxyindoleacetic acid

5.2(i) Preparation of tissue extracts

Rats were killed by cervical dislocation and decapitation, their brains were quickly removed, freed from superficial blood clots and homogenized in 5 ml ice-cold 0.1 N hydrochloric acid containing 0.5% ascorbic acid.

Table 5.1

Recoveries of 5-hydroxytryptamine added to pooled brain homogenates

Amount added	Amount recovered	Recovery
µg	µg	%
0.2	0.14	70
0.2	0.16	80
0.3	0.23	77
0.3	0.24	80
0.4	0.36	90
0.4	0.34	85

Mean recovery 80.3 %

Another two millilitres of the acid were used to wash the homogenization tube and the washing was added to the rest of the homogenate. The homogenate was then deproteinized with one millilitre of 10% zinc sulphate solution and 0.6 ml of N sodium hydroxide. The solutions were mixed vigorously, using a vortex mixer, and centrifuged at 16000 rpm for half an hour. After centrifugation, the clear supernatant solution was filtered through a thick filter bed made from fine glass wool fibres. The filtration was aided by using air suction.

Although the original method centrifuged the brain homogenate at 4000 rpm for only five minutes, we found that the high speed centrifugation together with the use of a thick filter bed of glass wool for filtration was necessary. If this was not done, enough soluble protein was left in the filtrate to interfere with the assay by producing a colloidal

suspension during the extraction with butylacetate or re-extraction into the phosphate buffer.

5.2(ii) Extraction of 5-hydroxyindoleacetic acid and fluorophore formation

Six millilitres of the clear filtrate were transferred to glass stoppered centrifuge tubes containing one millilitre of 2% EDTA solution, one millilitre of 6% ascorbic acid, 0.65 ml of N hydrochloric acid and approximately three grams of sodium chloride. Three millilitres of butylacetate were added to the tube and the mixture was shaken mechanically for ten minutes and centrifuged at 2000 rpm for another ten minutes. Two millilitres of the organic layer were transferred to another glass stoppered centrifuge tube. Another 2.5 ml of butylacetate were added to the first tube and the tube was shaken again and centrifuged as before.

Three millilitres of the organic layer were pooled with the first extract and shaken for ten minutes with 4 ml of salt saturated 0.1 N hydrochloric acid containing 0.5% ascorbic acid. The mixture was then centrifuged at 2000 rpm for ten minutes.

After centrifugation, a 4.5 ml portion of the organic layer was transferred to another conical glass stoppered centrifuge tube containing 2.5 ml of 0.1 M phosphate buffer (pH 7) with 0.1% ascorbic acid. After shaking for ten minutes, the tube was centrifuged at 2000 rpm for another ten minutes. The organic layer was aspirated off and discarded. Two millilitres of the aqueous phase were then combined with 0.5 ml 9 N hydrochloric acid and the fluorescence produced was measured at 540-550 mμ after activation at 295 mμ, using a Farrand spectrofluorimeter.

All solutions containing ascorbic acid had the ascorbic acid added to them just before use.

The reagent blank was obtained by carrying 6 ml of 0.1 N hydrochloric acid through the whole procedure.

A calibration curve was constructed by carrying solutions of standard 5-hydroxyindoleacetic acid in 0.1 N hydrochloric acid through the whole procedure side by side with tissue extract. Examples of such curves are given in Figure 5.2.

The composition of various buffers and reagents is given in the formulary (Appendix I).

5.2(iii) Recovery of standard 5-hydroxyindoleacetic acid added to pooled brain homogenates

A double extraction with butyl acetate was found to improve the recovery by at least 6%.

The recovery experiment for 5-hydroxyindoleacetic acid was done in the same manner as that described for 5-hydroxytryptamine and the results are given in Table 5.2.

Table 5.2

Recoveries of 5-hydroxyindoleacetic acid added to pooled brain homogenates

Amount added	Amount recovered	Recovery
µg	µg	%
0.2	0.17	85
0.2	0.17	85
0.3	0.26	86
0.3	0.28	93
0.4	0.38	95
0.4	0.37	92

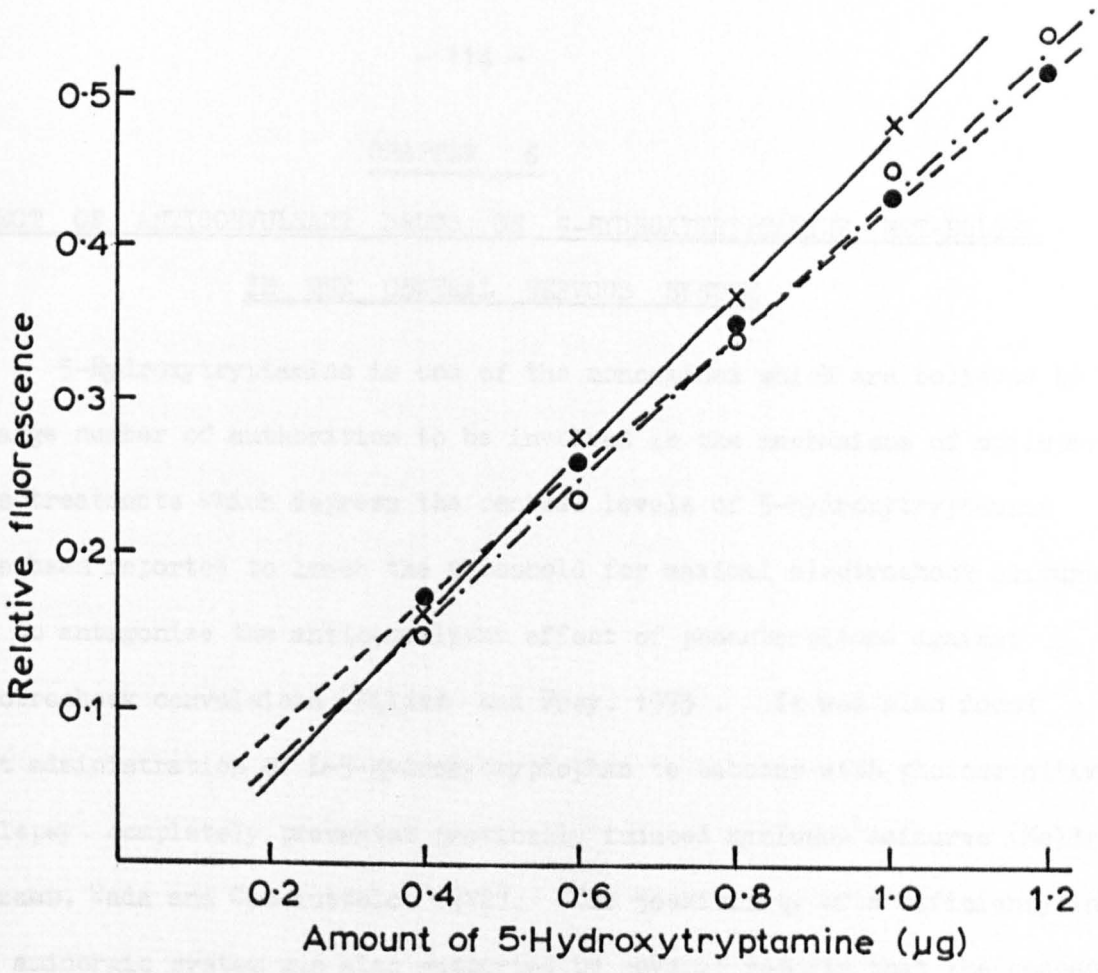
Mean recovery 89.3 %

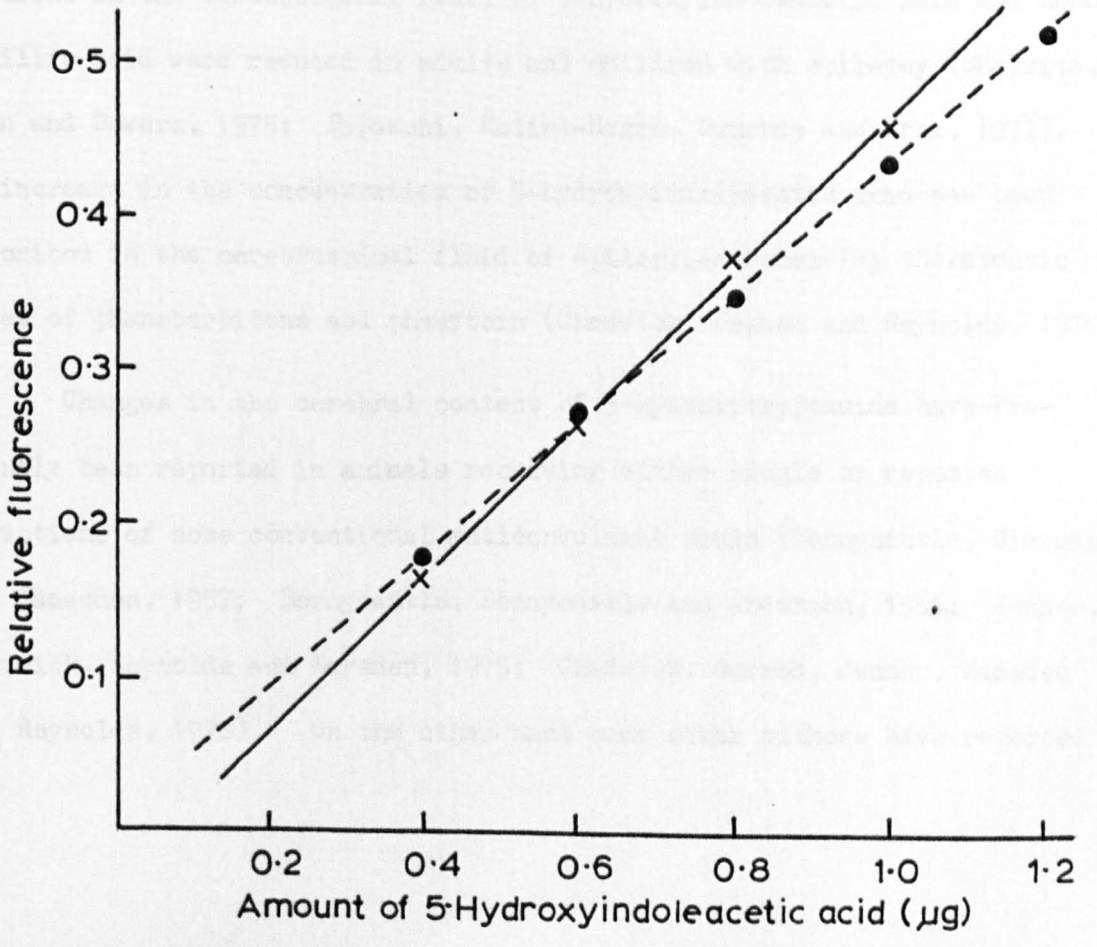
FIGURE 5.1

Typical calibration curves for the fluorimetric assay of 5-hydroxytryptamine obtained on three separate occasions

FIGURE 5.2

Typical calibration curves for the fluorimetric assay of 5-hydroxyindoleacetic acid obtained on two separate occasions


Relative fluorescence
0·5
0·4
0·3
0·2
0·1
0·2
0·4
0·6
0·8
1·0
1·2
Amount of 5-Hydroxytryptamine (µg)


Relative fluorescence
0·5
0·4
0·3
0·2
0·1
0·2
0·4
0·6
0·8
1·0
1·2
Amount of 5-Hydroxyindoleacetic acid (µg)

CHAPTER 6

EFFECT OF ANTICONVULSANT DRUGS ON 5-HYDROXYTRYPTAMINE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

5-Hydroxytryptamine is one of the monoamines which are believed by a large number of authorities to be involved in the mechanisms of epilepsy. Thus treatments which depress the central levels of 5-hydroxytryptamine have been reported to lower the threshold for maximal electroshock seizures and to antagonize the anticonvulsant effect of phenobarbitone against electroshock convulsions (Kilian and Frey, 1973). It was also found that administration of L-5-hydroxytryptophan to baboons with photosensitive epilepsy, completely prevented photically induced myclonus seizures (Meldrum, Balzano, Wada and Cacciuttolo, 1972). The possibility of a deficiency in the aminergic system was also supported by several reports that the concentrations in the cerebrospinal fluid of 5-hydroxyindoleacetic acid and homovanillic acid were reduced in adults and children with epilepsy (Shaywitz, Cohn and Bowers, 1975; Papeschi, Molina-Negro, Sourkes and Erba, 1972). An increase in the concentration of 5-hydroxyindoleacetic acid has been described in the cerebrospinal fluid of epileptics receiving therapeutic doses of phenobarbitone and phenytoin (Chadwick, Jenner and Reynolds, 1975).

Changes in the cerebral content of 5-hydroxytryptamine have frequently been reported in animals receiving either single or repeated injections of some conventional anticonvulsant drugs (Bonnycastle, Giarman and Pansonen, 1957; Bonnycastle, Bonnycastle and Anderson, 1964; Jenner, Chadwick, Reynolds and Marsden, 1975; Chadwick, Gorrod, Jenner, Marsden and Reynolds, 1978). On the other hand some other authors have reported

the lack of an ability of phenytoin to produce any change in the 5-hydroxytryptamine concentration in brain in doses sufficient to show anticonvulsant activity (Prockop, Shore and Brodie, 1959).

In the following chapters we are reporting results obtained after acute and chronic treatment with some anticonvulsant drugs on the levels and turnover of brain 5-hydroxytryptamine and its major metabolite (5-hydroxyindoleacetic acid), in an effort to study the system from a number of viewpoints.

6.1 Effect of single doses of anticonvulsant drugs on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain

Female Ash Wistar rats weighing between 140 and 160 gms were used. The animals were obtained one week before carrying out any experiment. They were caged in sixes and allowed free access to food and water. Drug doses were calculated according to the animal's body weight and were injected intraperitoneally in a solution or suspension volume of 0.5 ml per 200 g weight.

Phenytoin was given in a dose of 75 mg/kg suspended in saline containing 1% Tween 80, phenobarbitone in a dose of 100 mg/kg in saline, sodium bromide in a dose of 1 gm/kg also dissolved in saline, and carbamazepine in a dose of 50 mg/kg in propylene glycol. Control rats received the appropriate vehicle.

After receiving the doses stated, the animals were killed thirty minutes, one hour or two and a half hours after being injected and their brains were quickly removed, homogenized and centrifuged. The supernatant solutions were assayed for their 5-hydroxytryptamine and 5-hydroxyindoleacetic acid contents by the methodsdescribed in chapter 5.

Results are shown in table 6.1

Table 6.1

Effect of single doses of anticonvulsant drugs on rat brain levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Treatment	Time after injection	5-hydroxytryptamine	5-hydroxyindoleacetic acid
	hours	ng/gm	ng/gm
Saline + 1% Tween 80	-	473 ± 10 (8)	332 ± 7 (8)
Phenytoin	0.5	453 ± 11 (4)	378 ± 10**(4)
	1.0	498 ± 7 (4)	362 ± 12* (4)
	2.5	501 ± 13 (4)	366 ± 8**(4)
Saline		463 ± 15 (8)	351 ± 10 (8)
Phenobarbitone	0.5	489 ± 30 (4)	353 ± 15 (4)
	1.0	459 ± 10 (4)	349 ± 20 (4)
	2.5	533 ± 15**(4)	339 ± 15 (4)
Saline		470 ± 13 (8)	336 ± 7 (8)
Sodium bromide	0.5	477 ± 9 (4)	333 ± 10 (4)
	1.0	497 ± 11 (4)	343 ± 14 (4)
	2.5	469 ± 10 (4)	337 ± 10 (4)
Propylene glycol		561 ± 20 (8)	340 ± 10 (8)
Carbamazepine	0.5	588 ± 25 (4)	345 ± 10 (4)
	1.0	561 ± 25 (4)	343 ± 5 (4)
	2.5	572 ± 16 (4)	340 ± 7 (4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$ and ** $P < 0.005$)

Only phenobarbitone increased the levels of 5-hydroxytryptamine and this effect was only seen two and a half hours after its injection. It is interesting to note that propylene glycol produced a higher value for 5-hydroxytryptamine than did saline.

Phenytoin increased the levels of 5-hydroxyindoleacetic acid at all times studied, the maximum effect being observed half an hour after injection. This prominent effect of phenytoin on 5-hydroxyindoleacetic acid was surprising, since we had thought that any effect produced by the drug should be evident for both the amine and its metabolite. This proved not to be so. The effect of phenytoin on the metabolite suggests either an increase in the turnover of the amine (i.e. an increase in the rate of its synthesis and utilization) or that phenytoin inhibits the acid transport system in the brain thus decreasing the efflux of the metabolite from the brain.

These possibilities were examined by three different experiments. The anticonvulsant drugs were tested for their effect on the synthesis of 5-hydroxytryptamine partially inhibited by p-chlorophenylalanine; by the accumulation of 5-hydroxytryptamine and the depletion of 5-hydroxyindoleacetic acid caused by pargyline, a monoamine oxidase inhibitor; and finally by their effect on the rate of elimination of 5-hydroxyindoleacetic acid after probenecid.

6.2 Effect of p-chlorophenylalanine on rat brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

p-Chlorophenylalanine is a drug that potently inhibits the biosynthesis of 5-hydroxytryptamine (Koe and Weissman, 1966) and as well depletes brain 5-hydroxyindoleacetic acid.

The first step in the present experiment was to determine the dose of p-chlorophenylalanine that produced a satisfactory depletion of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. We chose a scheme which had been previously used in our department.

p-Chlorophenylalanine was given by oral intubation in 1 ml of mucilage of tragacanth (B.P.), two doses of the drug (320 mg/kg) being given on alternate days. Control rats received 1 ml of mucilage of tragacanth at the same time as the treated rats received p-chlorophenylalanine. Animals were killed twenty-four hours after the last dose, their brains were removed, homogenized and assayed for their 5-hydroxytryptamine and 5-hydroxyindoleacetic acid contents by the methods described in chapter 5.

Results are presented in table 6.2.

Table 6.2

Effect of p-chlorophenylalanine on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain

Treatment	5-hydroxytryptamine ng/gm	5-hydroxyindoleacetic acid ng/gm
Mucilage of tragacanth	480 ± 20 (4)	368 ± 10 (4)
p-Chlorophenylalanine	899 ± 90 (4)	130 ± 10 (4)
% change	+ 87.3%	- 64.6%

Each value is the mean ± standard error from the number of animals shown in parentheses.

Surprisingly, p-chlorophenylalanine did not deplete 5-hydroxytryptamine. Indeed, the reverse happened; there was a substantial rise in the brain 5-hydroxytryptamine content. 5-Hydroxyindoleacetic acid was depleted as we expected and only 36% of the original amount was left after p-chlorophenylalanine. We thought at the time that we might have made a mistake in the amine assay procedure which gave rise to these results.

Therefore we repeated the experiment, only to get exactly the same results as before. Our thoughts went in another direction, towards the view that the method of administration and the kind of vehicle used might have affected the assay. To test this idea, we gave the drug by intraperitoneal injection suspended in methyl cellulose (3%). Again we were faced with the same results as before. The only possibility that remained was that p-chlorophenylalanine itself might have been interfering with our assay procedure. We tested this by performing experiments in which p-chlorophenylalanine was added to brain homogenates and external 5-hydroxytryptamine standards.

p-Chlorophenylalanine in a concentration of 1 mg/10 ml was added to different concentrations of standard 5-hydroxytryptamine. Another set of standard 5-hydroxytryptamine solutions was prepared without the addition of p-chlorophenylalanine. The final volume of the solutions was 10 ml, from which seven millilitres were used for the assay.

Results are shown in table 6.3.

Brains from three rats were homogenized in 10 ml perchloric acid containing 1 mg p-chlorophenylalanine. After centrifugation, the supernatant solutions were pooled and divided into three equal portions which were then used for the assay. Another three rat brains were treated in the same manner, but no p-chlorophenylalanine was added to them.

Results are shown in table 6.4.

It is clear from these results that p-chlorophenylalanine interferes with the assay procedure we were using for 5-hydroxytryptamine. The fluorescence readings for the reagent blanks with added p-chlorophenylalanine and the standard 5-hydroxytryptamines were much greater than those given by solutions without added p-chlorophenylalanine.

Table 6.3

Effect of p-chlorophenylalanine on the relative fluorescence produced by external 5-hydroxytryptamine solutions in ninhydrin

Concentration used	Relative fluorescence without P.C.P.A.added	Relative fluorescence with added P.C.P.A.
Reagent blank	0.05	0.23
Reagent blank	0.05	0.24
0.4	0.135	0.36
0.4	0.145	0.39
0.8	0.27	0.47
0.8	0.27	0.53
1.2	0.39	0.68
1.2	0.41	0.60

Table 6.4

Effect of p-chlorophenylalanine on the relative fluorescence produced by brain 5-hydroxytryptamine extract in ninhydrin solution

Number of brain extract	Relative fluorescence without P.C.P.A.added	Relative fluorescence with added P.C.P.A.
1	0.220	0.34
2	0.210	0.37
3	0.215	0.37

Brain extract with added p-chlorophenylalanine showed more fluorescence than those of brain extract without added p-chlorophenylalanine, but the differences were not as high as those observed with the blank and standard values. The reason for this might be that some p-chlorophenylalanine

had been adsorbed by brain homogenate and had not then been extracted by the butanol.

6.3 Effect of single doses of anticonvulsant drugs on the depletion of brain 5-hydroxyindoleacetic acid caused by p-chlorophenylalanine

Female Ash Wistar rats were divided into several groups. Each animal received by oral intubation two doses of p-chlorophenylalanine (320 mg/kg), given on alternate days in one millilitre of mucilage of tragacanth. Control groups received one millilitre of mucilage of tragacanth only. The animals were killed twenty-four hours after the second dose of p-chlorophenylalanine but one hour before killing some animals received an intraperitoneal injection of phenobarbitone (100 mg/kg), phenytoin (75 mg/kg) or carbamazepine (50 mg/kg).

Results are presented in table 6.5.

Table 6.5

Effect of anticonvulsant drugs on the depletion of 5-hydroxyindoleacetic acid caused by p-chlorophenylalanine

Treatment	5-hydroxyindoleacetic acid ng/gm	Change %
Mucilage of tragacanth	340 ± 9.0 (8)	
p-chlorophenylalanine	140 ± 5.8 (8)	- 58.8
P.C.P.A. + phenobarbitone	170 ± 4.0 (4)	- 50.0**
P.C.P.A. + phenytoin	207 ± 4.0 (4)	- 39.1**
P.C.P.A. + carbamazepine	169 ± 6.0 (4)	- 50.3**

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* P < 0.005; ** P < 0.001) from the values obtained after treatment with p-chlorophenylalanine alone.

Results presented in table 6.5 show that all the anticonvulsant drugs tested reduced the degree of depletion of 5-hydroxyindoleacetic acid caused by p-chlorophenylalanine.

6.4 Effect of anticonvulsant drugs on the changes produced by pargyline on brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Udenfriend and Weissbach (1958) had suggested that the rate of increase of brain monoamine following inhibition of monoamine oxidase is indicative of the rate of biosynthesis. We have seen that phenytoin increased the levels of 5-hydroxyindoleacetic acid in the brain, and we have supposed that the increase was due to an increase in the turnover of 5-hydroxytryptamine or decreased elimination of 5-hydroxyindoleacetic acid. Hence we studied the effect of anticonvulsant drugs on the 5-hydroxytryptamine system after inhibition of monoamine oxidase.

Pargyline was used. The first step in the experiment was to determine the time at which the examination should be carried out.

Time course effect of pargyline on 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain

Female Ash Wistar rats were divided into several groups and each group received an intraperitoneal injection of pargyline hydrochloride (100mg/kg). The animals were killed by cervical dislocation and decapitation at various times after dosing. Their brains were quickly removed and assayed for their 5-hydroxytryptamine and 5-hydroxyindoleacetic acid contents by the methods described in chapter 5.

Results are shown in table 6.6 and figure 6.1.

As is clear from the table, the maximum effect of pargyline on 5-hydroxytryptamine occurs one hour after the injection. We chose this

Table 6.6

Time course effect of pargyline on brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Time hours	5-hydroxytryptamine ng/gm	Change %	5-hydroxyindoleacetic acid ng/gm	Change %
0	479 ± 13 (4)		358 ± 7 (4)	
0.5	688 ± 15 (4)	+ 43.6	250 ± 20 (4)	- 30.8
1.0	740 ± 40 (4)	+ 54.4	190 ± 9 (4)	- 46.9
1.5	751 ± 30 (4)	+ 56.8	150 ± 10 (4)	- 58.1

Each value is the mean ± standard error from the number of pairs of animals shown in parentheses

time to study the effect of anticonvulsant drugs on 5-hydroxytryptamine and 5-hydroxyindoleacetic acid contents after pargyline.

Rats were divided into groups. Each group received an intraperitoneal injection of pargyline (100 mg/kg) and the animals were killed one hour later. Thirty minutes before death, groups of rats received, by the intraperitoneal route, one of the anticonvulsant drugs shown in table 6.7 in the doses used in the previous experiments.

The results show that phenobarbitone significantly reduced the rate at which the concentration of 5-hydroxytryptamine rose in the brain of rats after pargyline treatment, while carbamazepine and phenytoin reduced the extent to which the level of 5-hydroxyindoleacetic acid was depleted by pargyline.

6.5 Effect of anticonvulsant drugs on the levels of 5-hydroxyindoleacetic acid after probenecid

We have seen that after pargyline, carbamazepine and phenytoin reduced the

FIGURE 6.1

Time course effect of pargyline on brain 5-hydroxy-tryptamine and 5-hydroxyindoleacetic acid

Each bar indicates the mean $\pm$ standard error for the number of pairs of animals indicated inside the columns.

Open columns represent 5-hydroxytryptamine and hatched columns represent 5-hydroxyindoleacetic acid.

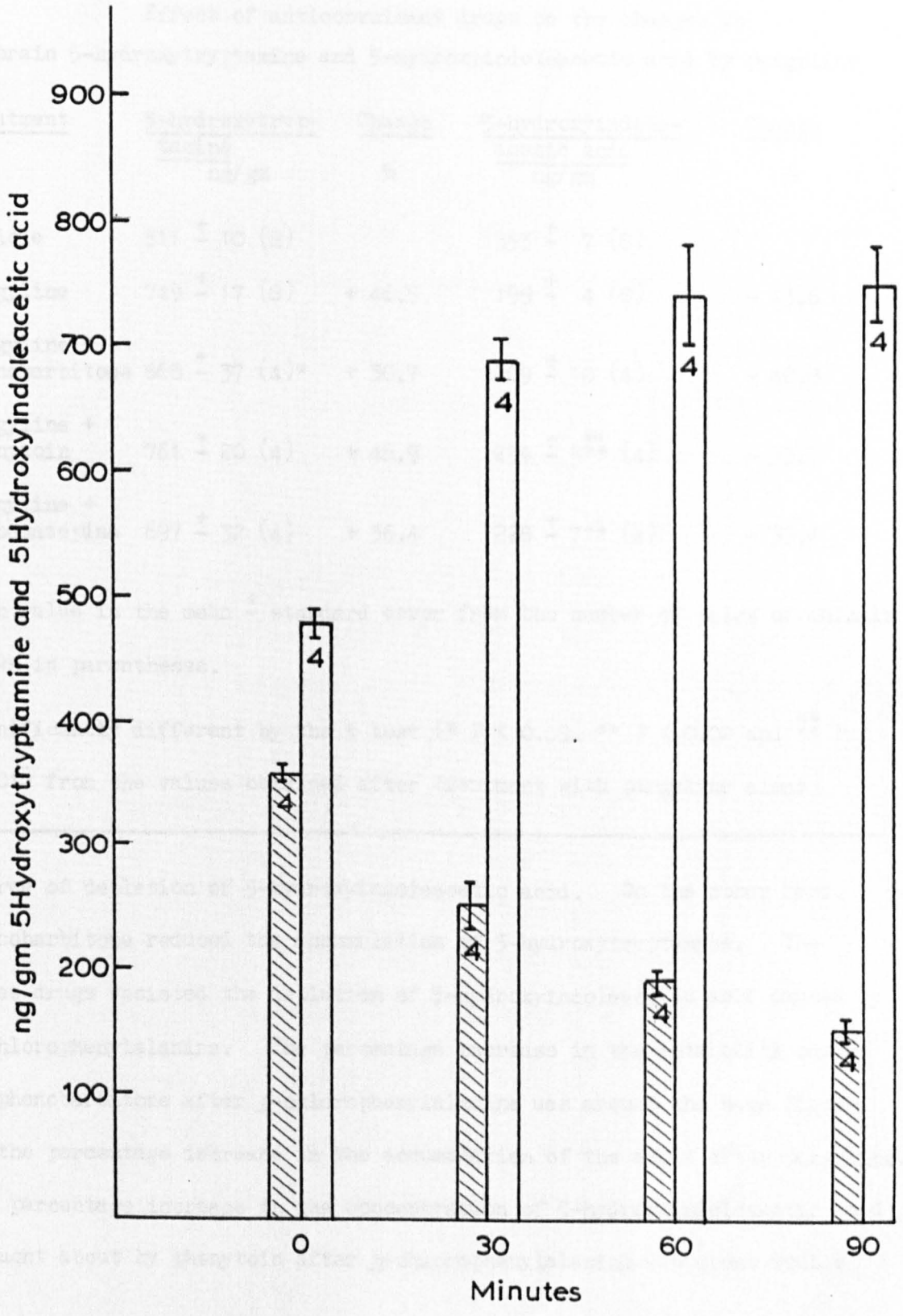

ng/gm 5Hydroxytryptamine and 5Hydroxyindoleacetic acid
900
800
700
600
500
400
300
200
100
4
4
4
4
4
4
4
4
0
30
60
90
Minutes

Table 6.7

Effect of anticonvulsant drugs on the changes in brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid by pargyline

Treatment	5-hydroxytryptamine ng/gm	Change %	5-hydroxyindoleacetic acid ng/gm	Change %
Vehicle	511 ± 10 (8)		353 ± 7 (8)	
Pargyline	749 ± 17 (8)	+ 46.5	199 ± 4 (8)	- 43.6
Pargyline + phenobarbitone	668 ± 37 (4)*	+ 30.7	209 ± 10 (4)	- 40.8
Pargyline + phenytoin	761 ± 20 (4)	+ 48.9	234 ± 5** (4)	- 33.7
Pargyline + carbamazepine	697 ± 32 (4)	+ 36.4	228 ± 7** (4)	- 35.4

Each value is the mean ± standard error from the number of pairs of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$, ** $P < 0.02$ and *** $P < 0.001$) from the values obtained after treatment with pargyline alone.

degree of depletion of 5-hydroxyindoleacetic acid. On the other hand, phenobarbitone reduced the accumulation of 5-hydroxytryptamine. The three drugs resisted the depletion of 5-hydroxyindoleacetic acid caused by p-chlorophenylalanine. The percentage increase in the metabolite caused by phenobarbitone after p-chlorophenylalanine was around the same figure as the percentage decrease in the accumulation of the amine after pargyline. The percentage increase in the concentration of 5-hydroxyindoleacetic acid brought about by phenytoin after p-chlorophenylalanine was about double

that after pargyline. This means that not all the effect was due to an increase in turnover rate of 5-hydroxytryptamine, and an inhibitory effect on the elimination of 5-hydroxyindoleacetic acid might have been taking place. This was examined by probenecid, a drug which inhibits the acid transport of 5-hydroxyindoleacetic acid out of the brain (Neff, Tozer and Brodie, 1967).

Again, the time course effect of probenecid on 5-hydroxyindoleacetic acid was to be determined first, in order to choose the time at which examination should be carried out.

Animals were injected intraperitoneally with probenecid (200 mg/kg) and were killed by cervical dislocation and decapitation at various times thereafter.

Results are presented in table 6.8 and figure 6.2.

Probenecid had a negligible effect on brain 5-hydroxytryptamine. It produced a steady increase in the concentration of 5-hydroxyindoleacetic acid content which was still rising after six hours of injection.

Anticonvulsant drugs were then tested for their effect on the accumulation of 5-hydroxyindoleacetic acid produced by probenecid. Animals were killed four hours after an intraperitoneal injection of probenecid (200 mg/kg), but one hour before death anticonvulsant drugs were injected intraperitoneally in the same doses as before.

Results are shown in table 6.9.

Phenytoin and carbamazepine increased significantly the rate of accumulation of 5-hydroxyindoleacetic acid caused by probenecid.

Table 6.8

Time course effect of probenecid on 5-hydroxytryptamine and 5-hydroxyindoleacetic acid contents of rat brain

Time hours	5-hydroxytryptamine ng/gm	Change %	5-hydroxyindoleacetic acid ng/gm	Change %
0	515 ± 15 (4)		365 ± 3 (4)	
0.5	484 ± 10 (4)	- 6.0	418 ± 3 (4)	+ 14.5
1	513 ± 6 (4)	- 0.38	490 ± 20 (4)	+ 34.2
2	517 ± 23 (4)	+ 0.38	686 ± 20 (4)	+ 87.9
3	565 ± 9.8 (4)	+ 9.7	894 ± 14 (4)	+ 144.9
6	503 ± 16 (4)	- 2.3	996 ± 10 (4)	+ 172.8

Each value is the mean ± standard error from the number of pairs of animals shown in parentheses.

Table 6.9

Effect of anticonvulsant drugs on the accumulation of 5-hydroxyindoleacetic acid after probenecid

Treatment	5-hydroxyindoleacetic acid	Change
Vehicle	350 ± 9 (8)	
Probenecid	1048 ± 14 (8)	+ 199.4
Probenecid + phenobarbitone	1075 ± 12 (4)	+ 207.1
Probenecid + phenytoin	1180 ± 17 (4)	+ 237.1**
Probenecid + carbamazepine	1095 ± 13 (4)	+ 212.8**

Each value is the mean ± standard error from the number of animals shown in parentheses.

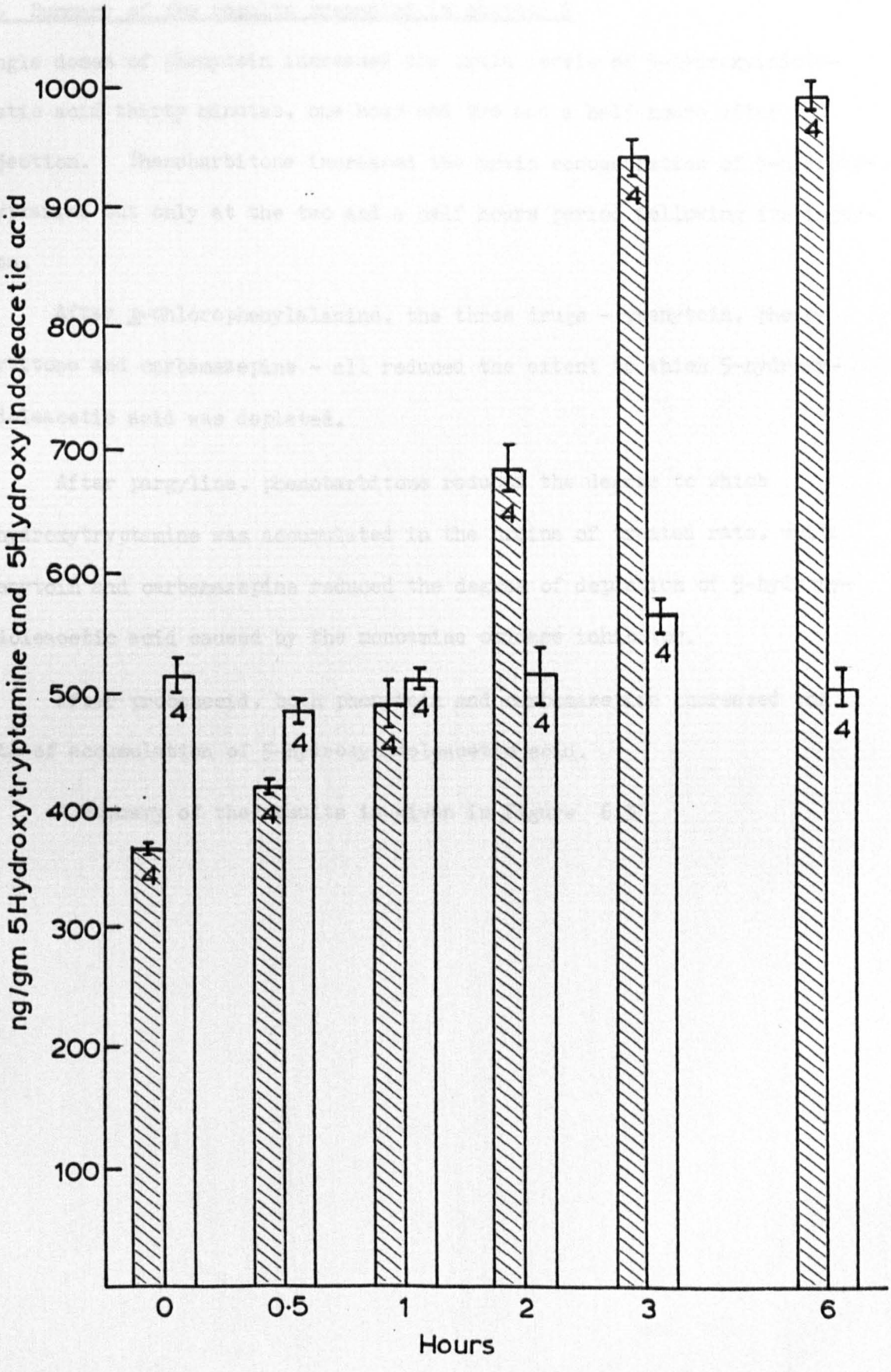
Significantly different by the t test (** $P < 0.02$ and ** $P < 0.001$) from the values obtained after treatment with probenecid alone.

FIGURE 6.2

Time course effect of probenecid on 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Each bar indicates mean $\pm$ standard error for the number of pairs of animals indicated inside the columns.

Open columns represent 5-hydroxytryptamine and hatched columns represent 5-hydroxyindoleacetic acid.


ng/gm 5Hydroxytryptamine and 5Hydroxyindoleacetic acid
1000
900
800
700
600
500
400
300
200
100
4
0
0·5
1
2
3
6
Hours

6.6 Summary of the results presented in chapter 6

Single doses of phenytoin increased the brain levels of 5-hydroxyindoleacetic acid thirty minutes, one hour and two and a half hours after its injection. Phenobarbitone increased the brain concentration of 5-hydroxytryptamine but only at the two and a half hours period following its injection.

After p-chlorophenylalanine, the three drugs - phenytoin, phenobarbitone and carbamazepine - all reduced the extent to which 5-hydroxyindoleacetic acid was depleted.

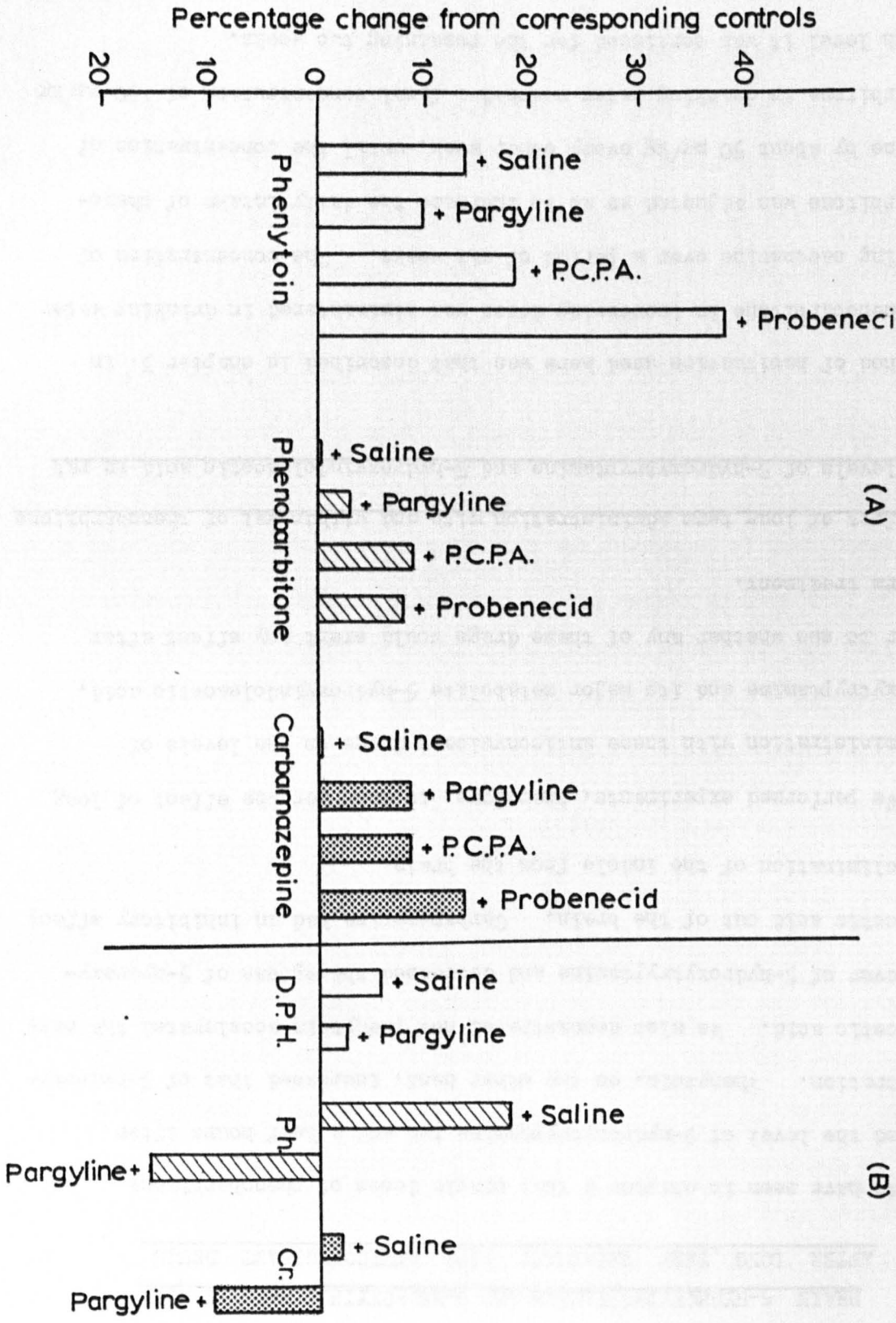
After pargyline, phenobarbitone reduced the degree to which 5-hydroxytryptamine was accumulated in the brains of treated rats, while phenytoin and carbamazepine reduced the degree of depletion of 5-hydroxyindoleacetic acid caused by the monoamine oxidase inhibitor.

After probenecid, both phenytoin and carbamazepine increased the rate of accumulation of 5-hydroxyindoleacetic acid.

A summary of the results is given in figure 6.3

FIGURE 6.3

Percentage changes in the concentrations of 5-hydroxy-indoleacetic acid (A) and 5-hydroxytryptamine (B) after different treatment


Percentage change from corresponding controls
20
10
0
10
20
30
40
Phenytoin
+ Saline
+ Pargyline
+ P.C.P.A.
+ Probenecid
Phenobarbitone
+ Saline
+ Pargyline
+ P.C.P.A.
+ Probenecid
Carbamazepine
+ Saline
+ Pargyline
+ P.C.P.A.
+ Probenecid
(A)
D.P.H.
+ Saline
+ Pargyline
Ph
+ Saline
Pargyline +
Cr
+ Saline
Pargyline +
(B)

CHAPTER 7

BRAIN 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID AFTER LONG TERM TREATMENT WITH ANTICONVULSANT DRUGS

We have seen in chapter 6 that single doses of phenobarbitone increased the level of 5-hydroxytryptamine two and a half hours after administration. Phenytoin, on the other hand, increased that of 5-hydroxyindoleacetic acid. We also demonstrated how phenytoin accelerated the rate of turnover of 5-hydroxytryptamine and decreased the egress of 5-hydroxyindoleacetic acid out of the brain. Carbamazepine had an inhibitory effect on the elimination of the indole from the brain.

We performed experiments, therefore, to look for the effect of long term administration with these anticonvulsant drugs on the levels of 5-hydroxytryptamine and its major metabolite 5-hydroxyindoleacetic acid, in order to see whether any of these drugs would exert any effect after long term treatment.

7.1 Effect of long term administration with and withdrawal of phenobarbitone on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain

The method of habituation used here was that described in chapter 3. in which phenobarbitone in increasing doses was administered in drinking water containing saccharine over a period of six weeks. The concentration of phenobarbitone was adjusted so as to increase the daily intake of phenobarbitone by about 50 mg/kg every other week, until the concentration of phenobarbitone in drinking water reached a final concentration of 150 mg/kg at which level it was continued for the remaining two weeks.

At the end of six weeks of administration, treated rats were divided into three groups. One group continued to receive phenobarbitone, while

the drug was withdrawn from the other two groups by replacing the phenobarbitone solution by one containing saccharine only. The animals in these latter groups were either killed forty-eight hours after withdrawal or exposed to the sound of the bell forty-eight hours after withdrawal (as described in chapter 3), and killed during the subsequent convulsions. The habituated group was killed at the same time. The brains of the habituated and the withdrawn rats were removed, weighed, homogenized and assayed for their 5-hydroxytryptamine and 5-hydroxyindoleacetic acid by the methods described in chapter 5.

Results are shown in table 7.1.

Habituation increased significantly the levels of both the amine and its metabolite. The percentage increase in the level of the amine was much higher than that of its metabolite which might indicate that habituation increased to a certain degree the turnover rate of 5-hydroxytryptamine, but it seems that it increased the synthesis at a higher rate than that of utilization.

Withdrawal brought down the levels of both the amine and its metabolite, which indicates either a decreased turnover or the return of the seretoninergic neurons to their steady state activity. However, withdrawal convulsions decreased the levels of the amine and increased that of its metabolite. The percentage increase in the concentration of the metabolite was very much higher than the percentage decrease in that of the amine itself, which indicates that both the rate of synthesis and that of utilization increased during the convulsions.

Table 7.1

Brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid levels after habituation to and withdrawal from phenobarbitone

Treatment	5-hydroxytryptamine ng/gm	Change %	5-hydroxyindoleacetic acid ng/gm	Change %
Control	522 ± 5 (8)		361 ± 6.7 (8)	
Habituated	641 ± 22 (8)	+ 22.8**	397 ± 17 (8)	+ 9.9*
Withdrawn	567 ± 33 (8)	+ 8.6	364 ± 14 (8)	+ 0.8
Control + sound stimulation	530 ± 6 (8)		365 ± 6 (8)	
Withdrawn + sound stimulation	487 ± 17 (8)	- 8.1**	462 ± 13.8 (8)	+ 26.5**

Each value is the mean ± standard error from the number of pairs of animals shown in parentheses.

Significantly different by t test (* $P < 0.05$, ** $P < 0.02$ and *** $P < 0.001$).

The figures were obtained in two different experiments.

7.2 Effect of long term administration of phenytoin and carbamazepine on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Both drugs were injected intraperitoneally over a period of three weeks in an increasing dosage.

Phenytoin was injected at a dose level of 25 mg/kg twice daily for the first week of treatment. The dose was raised to 50 mg/kg during the second week and finally to 75 mg/kg during the third and final week of treatment.

Carbamazepine was injected at a dose level of 20 mg/kg twice daily for the first ten days and then the dose was increased to 40 mg/kg for the last eleven days of treatment. Details of the methods are given in chapter 4.

At the end of the third week of administration, rats were killed by cervical dislocation and decapitation, their brains were quickly removed, homogenized and assayed for their 5-hydroxytryptamine and 5-hydroxyindole-acetic acid by the methods described in chapter 5.

Results are given in table 7.2.

Table 7.2

Effect of long term administration of phenytoin and carbamazepine on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid of rat brain

Treatment	5-hydroxytrypt-amine ng/gm	Change %	5-hydroxyindole-acetic acid ng/gm	Change %
Saline	511 ± 15 (8)		356 ± 4.5 (8)	
Phenytoin	598 ± 20 (8)	+ 17**	422 ± 4.0 (8)	+ 18.5**
Propylene glycol	549 ± 18 (8)		336 ± 9 (8)	
Carbamazepine	668 ± 23 (8)	+ 21.7**	495 ± 35 (8)	+ 47.5**

Each value is the mean ± standard error from the number of pairs of animals shown in parentheses.

Significantly different by the t test (** $P < 0.001$) from control values.

The figures were obtained in two different experiments.

Results show that both phenytoin and carbamazepine increased the levels of the amine and its metabolite. The percentage changes produced by phenytoin in the levels of the amine and its metabolite was the same. On the other hand, the percentage changes produced by carbamazepine on the concentration of the metabolite was double that produced in the levels of the amine itself.

CHAPTER 8

BRAIN γ-AMINOBUTYRIC ACID

γ-Aminobutyric acid is an amino acid which is present in large amounts in the mammalian central nervous system and which is believed to act as an inhibitory neurotransmitter substance there. Experimental evidence indicates that central GABA functions in epilepsy may be depressed (Meldrum, 1975).

Some of the anticonvulsant drugs (including barbiturates) have been reported to elevate brain GABA concentration (Saad, Elmasry and Scott, 1972) and to antagonize insulin convulsion in mice, by elevating the lowered GABA levels induced by insulin (Saad, 1970). These authors suggested that GABA might be a mediator in the anticonvulsant effect of the drugsthey used. In contrast, Crossland and Turnbull (1972) found no changes in the levels of GABA in the brain of rats during habituation to or withdrawal from sodium barbitone. Aminooxyacetic acid, in doses which doubled the GABA content of the brain, did not protect rats against audiogenic seizures produced as a consequence of the barbitone withdrawal. They concluded that barbitone withdrawal convulsions did not arise from deficiency in the brain GABA levels. In view of the above reported findings, we decided to see whether any of the anticonvulsant drugs we used before have any effect on GABA levels in rat brain.

8.1 Paper chromatography of GABA

The method we first used for the assay of brain GABA was the one dimensional paper chromatography method described by Maynert, Klingman and Kaji (1962), with some of our own modifications which aimed at increasing the sensitivity of the method.

8.1(i) Preparation of tissue extracts

Rats were killed by cervical dislocation and decapitation, their brains were quickly removed and homogenized in 5 ml ice cold 0.01 N hydrochloric acid, using an MSE top drive homogenizer. The brain homogenate was then mixed with 12 ml ice cold absolute ethanol and left to stand in crushed ice water bath for one hour. The mixture was then centrifuged at 11000 rpm at 0°C for ten minutes. The supernatant solution was then removed and kept in evaporation tube, and the residue was washed three times with 4 ml portions of 75% V/V ethanol, and on each occasion the mixture was centrifuged as before. The washings were pooled with the supernatant solution and the mixture was evaporated to dryness on a Buchler rotary evaporator under vacuum and at a temperature of 60°C.

The residue was then dissolved in a mixture of 1 ml of distilled water, 2 ml each of chloroform and methanol and was centrifuged at 2000 rpm for fifteen minutes.

8.1 (ii) Chromatographic separation

The solvent used for chromatography was the aqueous phase of a mixture of 50 ml of n-butanol, 12 ml of glacial acetic acid and 50 ml of water. The mixture was shaken in a separating funnel and the lower organic phase was used to saturate the atmosphere of the chromatography jar for twenty-four hours.

Fifty microlitres of the upper aqueous phase of the brain extract were applied to a sheet of Whatmann number 1 chromatography paper. The point of application was made 9 cm from either edge of the paper and the size of the spot did not exceed 0.5 cm in diameter, leaving at least 10 cm

between each point. The paper was then suspended in the chromatography jar, with the sample origin closest to the solvent trough at the top of the jar, and left for eighteen hours at room temperature.

8.1 (iii) Developing of the chromatograph

At the end of eighteen hours, the paper was removed from the tank and allowed to dry in a fume cupboard for two hours. The paper was then sprayed with freshly prepared 0.5% ninhydrin solution in 95% ethanol. It was allowed to dry for one hour in the fume cupboard and was then heated in an oven at 90°C for five minutes.

The GABA-containing spots, half way up the paper, were marked with a pencil and pieces of the paper 2 cm in diameter around the centre of the spot were cut out, chopped into small pieces and macerated in 2 ml ninhydrin solution in 95% ethanol at a temperature of 100°C for five minutes.

After the mixture had been cooled to room temperature, 5 ml of distilled water were added and it was allowed to stand for a further hour with occasional shaking. The optical density of the supernatant liquid was then measured at 570 mμ using an MSE Spectroplus spectrophotometer against a similar extract of a blank circle taken from the same paper.

A calibration curve was constructed every time by applying 50 μl of different concentrations of standard GABA in 0.01 N hydrochloric acid to the same paper. An example of the curves is given in figure 8.1

8.1 (iv) Recovery of standard GABA solution added to brain homogenates

Different concentrations of standard GABA solutions, added to pooled brain homogenates and carried through the whole procedure were recovered in excess

of 144% (table 8.1). This means that there are other substances in the brain homogenate which reacted with the added GABA to give higher reading values.

When the standard GABA solutions were added to the aqueous solution of the brain extract before application to the paper the recovery was only around 93% (table 8.2).

Table 8.1

Recoveries of GABA added to pooled brain homogenates and carried through the whole procedure

Amount added	Amount recovered	Recovery
µg	µg	%
50	62	124
50	87	174
100	166	166
100	144	144
150	193	128
150	225	150
200	280	140
200	263	131

Mean recovery 144.6%

Disadvantages of the assay of GABA by paper chromatography

Although several modifications were made to the procedure which was always conducted with great care, there were serious drawbacks in the method, which led us to look for another means of assaying GABA.

FIGURE 8.1

Typical calibration curves for the colorimetric paper chromatographic assay of γ-aminobutyric acid obtained on two separate occasions

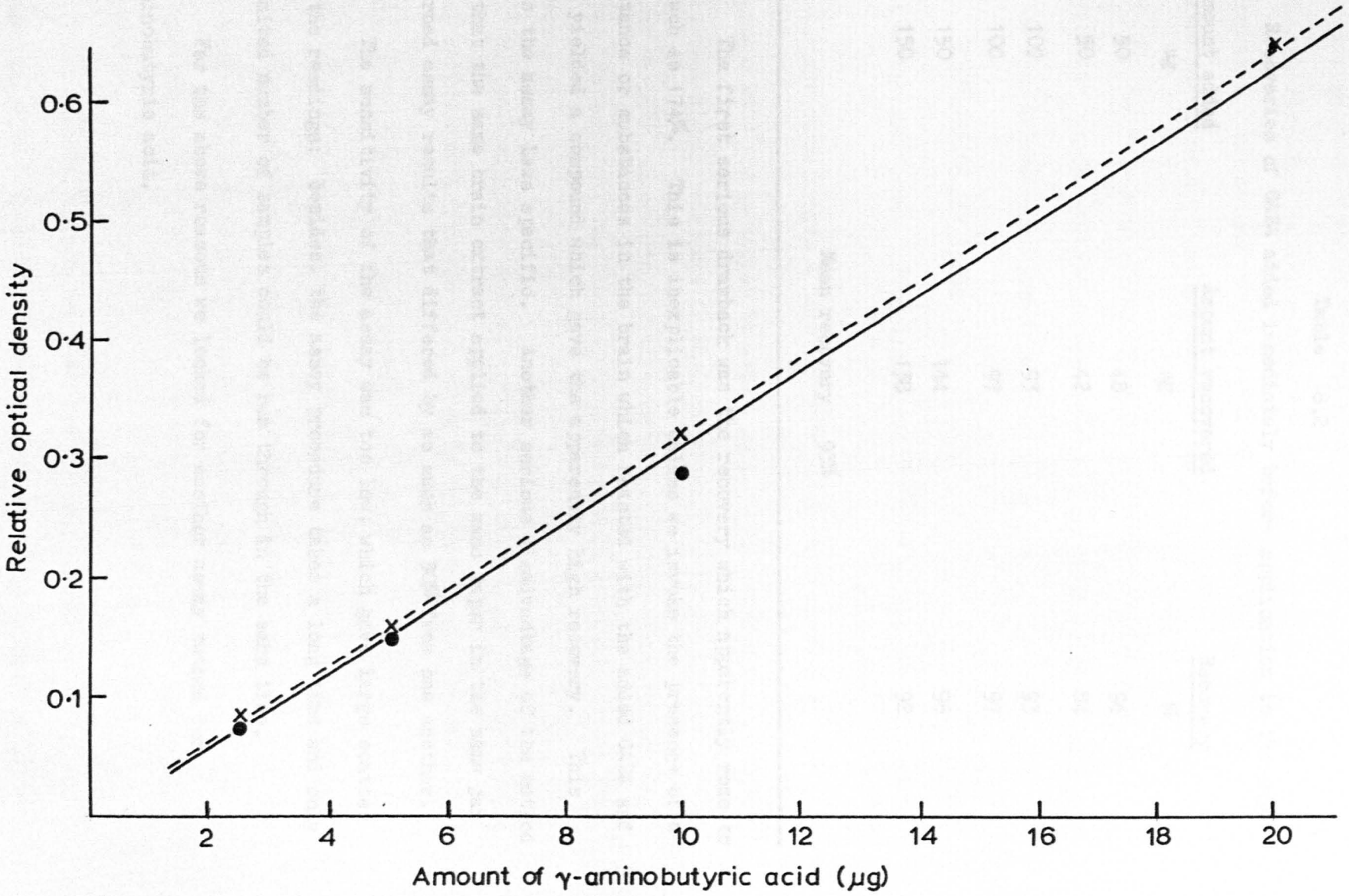

Relative optical density
0·6
0·5
0·4
0·3
0·2
0·1
2
4
6
8
10
12
14
16
18
20
Amount of γ-aminobutyric acid (μg)

Table 8.2

Recoveries of GABA added immediately before application to the paper

Amount added	Amount recovered	Recovery
µg	µg	%
50	48	96
50	42	84
100	93	93
100	97	97
150	144	96
150	138	92

Mean recovery 93%

The first serious drawback was the recovery which apparently rose to as much as 174%. This is inexplicable unless we invoke the presence of a substance or substances in the brain which reacted with the added GABA and thus yielded a compound which gave the apparently high recovery. This makes the assay less specific. Another serious disadvantage of the method was that the same brain extract applied to the same paper in the same jar returned assay results that differed by as much as 30% from one another.

The sensitivity of the assay was too low, which gave large scatter for the readings; besides, the assay procedure takes a long time and only a limited number of samples could be run through in the same time.

For the above reasons we looked for another assay method for γ-aminobutyric acid.

8.2 Enzymatic fluorometric determination of GABA in rat brain

The enzymatic measurement of GABA was first described by Jakoby and Scott (1959), who isolated the enzymes for the GABA assay from Pseudominas fluorescens. The method was then modified by Graham and Aprison (1966), to allow measurement of small amounts of GABA.

We used the assay method of Graham and Aprison but we made some modifications in the volumes of the reagents used until we found that we had the most satisfactory procedure.

Principle of the assay

1) γ-aminobutyric acid + α-ketoglutarate $\xrightarrow{\text{GABA-T}}$ succinic semialdehyde + glutamate

2) succinic semialdehyde + $NADP^+$ $\xrightarrow{\text{SSADH}}$ succinate + NADPH + H^+

The excess $NADP^+$ is destroyed by weak alkali. The NADPH which is stable in the weak alkali treatment is then converted to a highly fluorescent product of $NADP^+$ in one step by the addition of strong alkali containing hydrogen peroxide . The resultant fluorescence is a measure of the GABA present.

The composition of various buffers and reagents and the formulation of the enzyme reaction mixture are given in the formulary (Appendix I).

8.2(i) Preparation of tissue extract

Animals were killed by cervical dislocation and decapitation, the brains were quickly removed and dropped into liquid nitrogen. Five minutes later the brains were taken from the liquid nitrogen, weighed and homogenized for five minutes in 10 ml ice cold 75% ethanol, using an MSE top drive homogenizer.

After centrifugation at 14000 rpm for half an hour, 2 ml of the supernatant solution were evaporated to dryness, using a Buchler rotary

evaporator under maximum vacuum at 60°C. The residue was then dissolved in 10 ml of distilled water and the resultant solution was centrifuged for half an hour at 25000 rpm, using an MSE ultra high speed centrifuge.

8.2(ii) Enzymatic conversion of GABA and fluorophore formation

A 100 µl of the clear supernatant solution was transferred to a conical glass tube. While the tubes were standing in a rack kept in an ice water bath (0°C), 100 µl of an enzyme reaction mixture was added to each tube. The tubes were capped with parafilm, mixed on a vortex mixer and incubated at 38°C for forty-five minutes in a rotary evapomixer operated to shake at medium speed. At the end of the incubation period, the tubes were returned to the ice water bath and 250 µl of phosphate buffer were added to each tube. They were buzzed vigorously on a vortex mixer, recapped and transferred to a shaking water bath at 60°C for fifteen minutes.

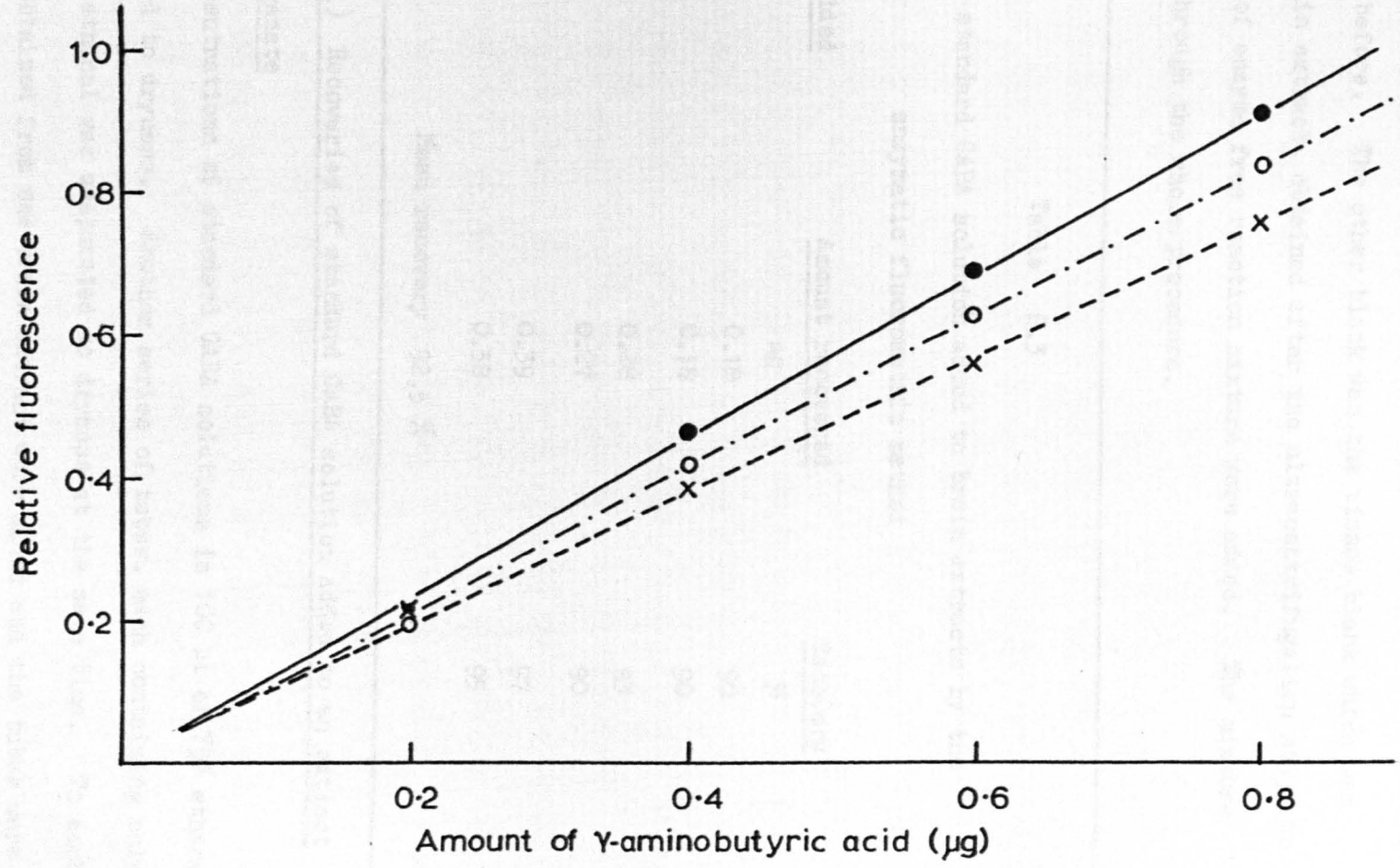
At the end of the fifteen minutes, the tubes were removed from the water bath and 500 µl of alkali peroxide was added to each one, they were again buzzed vigorously, recapped and returned to the water bath for another ten minutes. Two millilitres of distilled water was then added to each tube and they were mixed thoroughly until the very fine crystals which were produced on the addition of water had completely dissolved.

The fluorescence was then measured at 460 mµ after activation at 365 mµ, using a Farrand spectrophotofluorimeter.

A number of 100 µl portions of different concentrations of standard GABA solution in 75% ethanol were evaporated to dryness and 100 µl of the reaction mixture was added to each. The tubes were then treated in the same manner as that described for brain extracts to form a calibration curve, examples of which are given in figure 8.2.

FIGURE 8.2

Typical calibration curves for the enzymatic fluorometric assay of γ-aminobutyric acid obtained on three separate occasions


1·0
0·8
0·6
0·4
0·2
Relative fluorescence
0·2
0·4
0·6
0·8
Amount of γ-aminobutyric acid (μg)

Two blanks were carried through the whole procedure. One was an enzyme reagent blank, a 100 µl portion of ethanol evaporated to dryness to which 100 µl of the enzyme reaction mixture had been added and treated as described before. The other blank was the tissue blank which was 100 µl of brain extract, obtained after the ultracentrifugation step, to which 100 µl of enzyme free reaction mixture were added. The mixture was carried through the whole procedure.

Table 8.3

Recoveries of standard GABA solution added to brain extracts by the enzymatic fluorometric method

Amount added	Amount recovered	Recovery
µg	µg	%
0.2	0.18	90
0.2	0.18	90
0.3	0.28	93
0.3	0.27	90
0.4	0.39	97
0.4	0.38	95

Mean recovery 92.5 %

8.2(iii) Recoveries of standard GABA solution added to an extract of brain homogenate

Different concentrations of standard GABA solutions in 100 µl of 75% ethanol were evaporated to dryness. Another series of tubes, each containing only 100 µl of 75% ethanol was evaporated to dryness at the same time. To each tube, 100 µl obtained from one brain extract were added and the tubes were treated as described before for the assay of GABA.

Results are shown in table 8.3.

8.3 Post mortem changes in brain GABA levels

γ-Aminobutyric acid is one of the biologically labile compounds whose concentration in the brain was found to be increased post mortem (Alderman and Shellenberger, 1973). We therefore performed experiments to study the post mortem changes in GABA levels in rat brain, and to look as well for the best method of killing. We used liquid nitrogen in our study as it is the quickest and cheapest way of freezing.

8.3(i) Comparison between whole body immersion, decapitation, decapitation and skin removal, brain removal and dropping in liquid nitrogen

The post mortem increase in the levels of GABA in the brain is believed to arise as a result of the conversion of a labile pool of glutamic acid. The question arose in our mind whether this pool becomes active from the moment of killing or whether some time has to elapse before activation. This was tested for in four different ways. The first was the whole body immersion in liquid nitrogen; in this case complete prevention of the conversion which might be activated by death, should occur. The second experiment was to decapitate the animal and to drop the head into liquid nitrogen. Swaab (1971) has suggested that the skin of the skull is the most important temperature insulator for the cortex. This was examined by decapitating the animal, removing the skin over the skull and dropping the head into liquid nitrogen. The fourth test was done by decapitating the animal, removing the brain and dropping the brain itself into liquid nitrogen. In all the last three tests, the dropping into liquid nitrogen of either the head or the brain was done in less than thirty seconds. The brains were then treated as described in section 8.2 for the assay of GABA.

Results are given in table 8.4.

Table 8.4

Comparison between different ways of freezing on brain GABA content

Whole body immersion	Decapitation	Decapitation and removal of the skin of the head	Decapitation and removal of the brain
(See text for details)			
142	139	157	139
129	143	123	153
157	156	126	140
144	147	149	146
Mean ± S.E. 143 ± 5.7	146 ± 3.6	138 ± 8.4	144 ± 3.2

It is clear that no difference in brain concentration of GABA arises because of the four different ways of freezing. This means that neither death itself nor decapitation have an early effect on the labile glutamic acid pool. So we decided to use the easiest and quickest way of freezing, which was by removal of the brain after cervical dislocation and decapitation and then dropping the brain into liquid nitrogen.

8.3(ii) Time course of the post mortem increase in GABA of rat brain

This experiment was designed to look for the post mortem changes in GABA levels revealed by the brain freezing technique.

Ash Wistar rats were killed by cervical dislocation and decapitation, the brain was quickly removed and placed on a glass plate and left for the time required at the laboratory temperature (20°C). It was then dropped into liquid nitrogen, left for five minutes, removed and homogenized in

10 ml ice cold 75% ethanol. The brains were assayed for their GABA content by the method described in chapter 8, section 2.

Results are given in table 8.5 and figure 8.3.

Table 8.5

Time course of the post mortem changes in γ-aminobutyric acid in rat brain

Time minutes	γ-Aminobutyric acid µg/gm	Change from 30 seconds %
0.5	125.5 ± 3.8 (2)	
0.66	147.8 ± 6.4 (2)	+ 17
1	162.7 ± 4.8 (2)	+ 29.6
2	162.5 ± 2.0 (2)	+ 29.4
3	182.9 ± 4.9 (2)	+ 45.7
4	162.6 ± 1.0 (2)	+ 29.5
6	171.3 ± 5.0 (2)	+ 36
7	174.5 ± 6.0 (2)	+ 39

Each value is the mean ± standard error from the number of animals shown in parentheses.

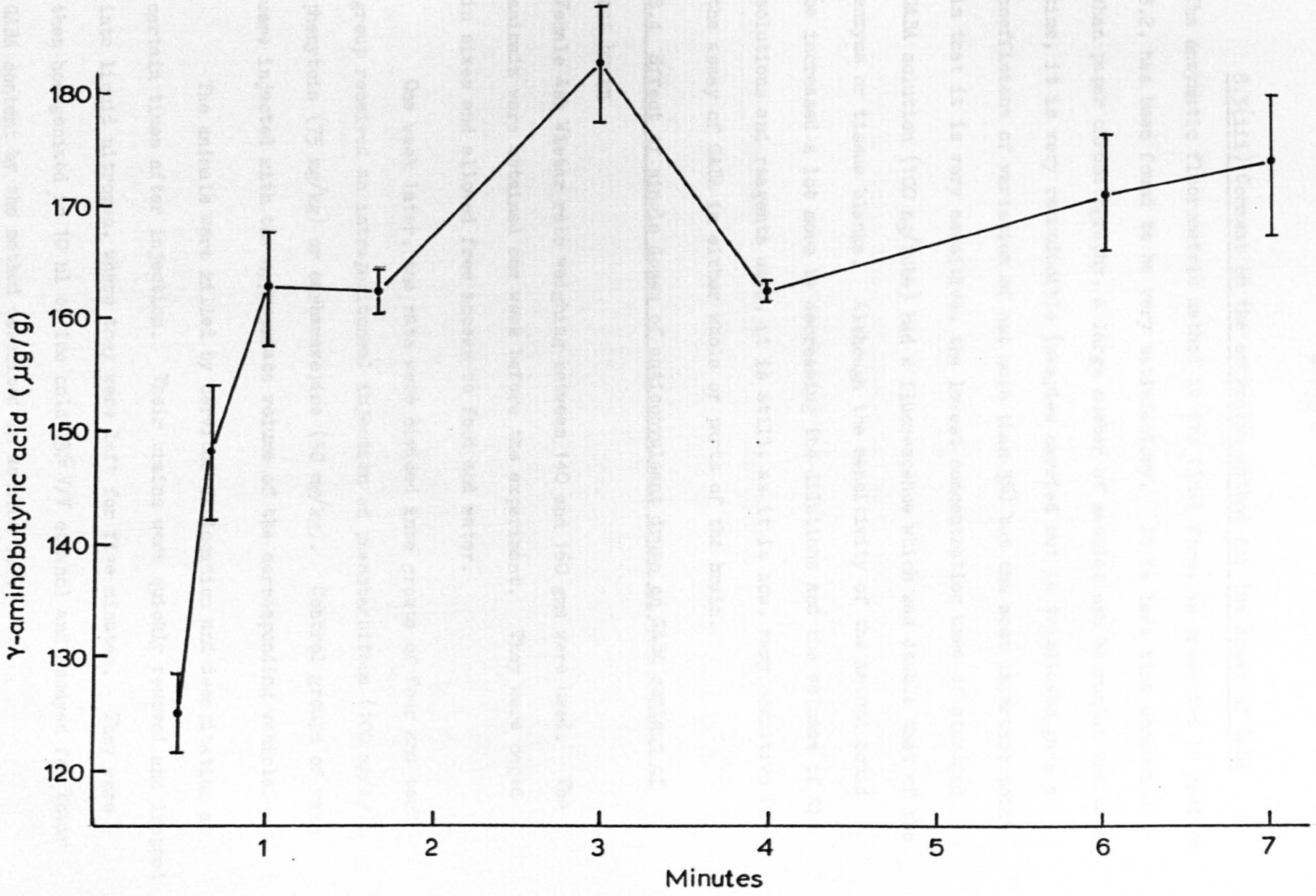
Results in the previous section show clearly that death does not by itself produce any effect on GABA content of the brain, in contrast to some other substances such as acetylcholine, the concentration of which is much affected by decapitation.

On the other hand, it is clear from the results presented in figure 8.3 that post mortem changes do begin some thirty seconds after death. These changes are apparently biphasic in nature, an early increase being followed by a decrease and then a slight increase again in the concentration of GABA.

FIGURE 8.3

Time course of the post mortem changes in

γ-aminobutyric acid concentration in rat brain


180
170
160
150
140
130
120
γ-aminobutyric acid (μg/g)
1
2
3
4
5
6
7
Minutes

8.3(iii) Comment on the enzymatic method for the assay of GABA

The enzymatic fluorometric method in its final form, as presented in section 8.2, has been found to be very satisfactory. It is less time consuming than paper chromatography, a large number of samples can be run at the same time, it is very reproducible (samples carried out in triplicate gave a coefficient of variation of not more than 3%) but the most important point is that it is very sensitive, the lowest concentration used of standard GABA solution (100 ng/tube) had a fluorescence which was double that of the enzyme or tissue blanks. Although the sensitivity of the method could be increased a lot more by decreasing the dilutions and the volumes of the solutions and reagents used, it is still, as it is now, very sensitive for the assay of GABA in either whole or parts of the brain.

8.4 Effect of single doses of anticonvulsant drugs on GABA content of rat brain

Female Ash Wistar rats weighing between 140 and 160 gms were used. The animals were obtained one week before the experiment. They were caged in sixes and allowed free access to food and water.

One week later, the rats were divided into groups of four and each group received an intraperitoneal injection of phenobarbitone (100 mg/kg), phenytoin (75 mg/kg) or carbamazepine (50 mg/kg). Control groups of rats were injected with the appropriate volume of the corresponding vehicle.

The animals were killed by cervical dislocation and decapitation at certain times after injection. Their brains were quickly removed and dropped into liquid nitrogen, where they were left for five minutes. They were then homogenized in 10 ml of ice cold 75% V/V ethanol and assayed for their GABA content by the method described in section 8.2.

Results are given in tables 8.6 and 8.7.

Phenobarbitone increased the total brain levels of GABA thirty and sixty minutes after its injection; thereafter the levels of GABA returned to their normal values. On the other hand, neither phenytoin nor carbamazepine had any effect either thirty or sixty minutes after their injection.

Table 8.6

Effect of single doses of phenobarbitone on the levels of GABA in rat brain

Time after injection minutes	GABA µg/gm	Change from zero time %	P t test
0	152.6 ± 1.9 (4)		
30	171.2 ± 1.88 (4)	+ 12.17	< 0.001
60	163.6 ± 4.8 (4)	+ 7.20	< 0.05
90	144.9 ± 7.7 (4)	- 5.07	N.S.
120	145.4 ± 10.4 (4)	- 4.73	N.S.

Each value is the mean ± standard error from the number of animals shown in parentheses

Table 8.7

Effect of single doses of phenytoin and carbamazepine on GABA levels in rat brain

Treatment	Time after injection	GABA	Change from control	P
	minutes	µg/gm	%	t test
Saline + 1% Tween 80	30 and 60	130.4 ± 7.1 (4)		
Phenytoin	30	138.3 ± 2.7 (4)	+ 6.1	N.S.
	60	136.2 ± 3.2 (4)	+ 4.5	N.S.
Propylene glycol	30 and 60	151.4 ± 3.2 (4)		
Carbamazepine	30	155.4 ± 5.2 (4)	+ 2.6	N.S.
	60	146.2 ± 5.4 (4)	- 3.4	N.S.

Each value is the mean ± standard error from the number of animals shown in parentheses

PART III

PART III

CHAPTER 9

PRODUCTION OF EPILEPTIC LESIONS IN THE RAT

After we had studied the actions of antiepileptic drugs on normal animals, the question naturally arose as to whether the neurotransmitter agents in the brains of healthy rats would behave in the same manner as those in the brains of animals producing epileptic discharges. and similarly whether an anticonvulsant drug would have the same effect on a part of the brain exhibiting focal activity as on the rest of the brain.

A search through the literature revealed that the best method of producing chronic epileptic focus in rats (which are resistant to other possible techniques such as local freezing and alumina) was that first described by Kopeloff (1960), who applied cobalt powder to the cerebral cortex. The advantages of this method were discussed by Emson and Joseph (1975), who pointed out that it gives a secondary focus in the homotopic area of the contralateral cortex free from the direct effect of the cobalt. Furthermore, the model is chronic and shows epileptiform activity for a long time.

The first step in this experiment was to find a way of measuring the brain activity in awake, unrestrained rats in order to follow the progress of epileptic activity after cobalt implantation.

9.1 Measurement of the brain activity of unrestrained, nonanaesthetized rats

De Vos and Bonta (1964) developed an electrode set which permits the electroencephalographic measurement of the unrestrained nonanaesthetized rat. We

have adopted their technique, with some modifications, to use in our study.

The electrode set consisted of five stainless steel screw electrodes, mounted on a common plastic plate the size of which was 12 x 8 mm and with a thickness of 1 mm (see figure 9.1 (A)). The electrodes were 4 mm in length but only 2 mm protruded from the lower surface of the plate. Four of the electrodes which were used for recording were mounted 6 mm apart from each other, the fifth electrode in the front point of the plate serving to provide an indifferent earth connection.

Stainless steel washers, soldered to short lengths of insulated wire, were fixed under the head of the electrodes. The short lengths of wire terminated in a common socket for connection to the recording apparatus (Figure 9.1 (B)). The total weight of the electrode set was 1 gm.

A guide plate (Figure 9.1 (C)), equipped with a small handle and made the same size and shape as the plastic plate on which the electrodes were mounted, was used for marking the holes in the skull of the rat in which the electrodes were to be implanted.

Implantation procedure

Female Ash Wistar rats weighing between 150 and 160 gms were used.

The rat was anaesthetized with an intraperitoneal injection of 50 mg/kg pentobarbitone, the skin of the head was shaved and the scalp was then cut along the midline from the level of the eyes to that of the ears. The skin was then reflected laterally as far as possible and the exposed bone was cleaned of muscle and fascia until it appeared to be dry and the bone sutures were clearly visible. The guide plate was then placed on the skull, ensuring that the front point of the plate was in

FIGURE 9.1

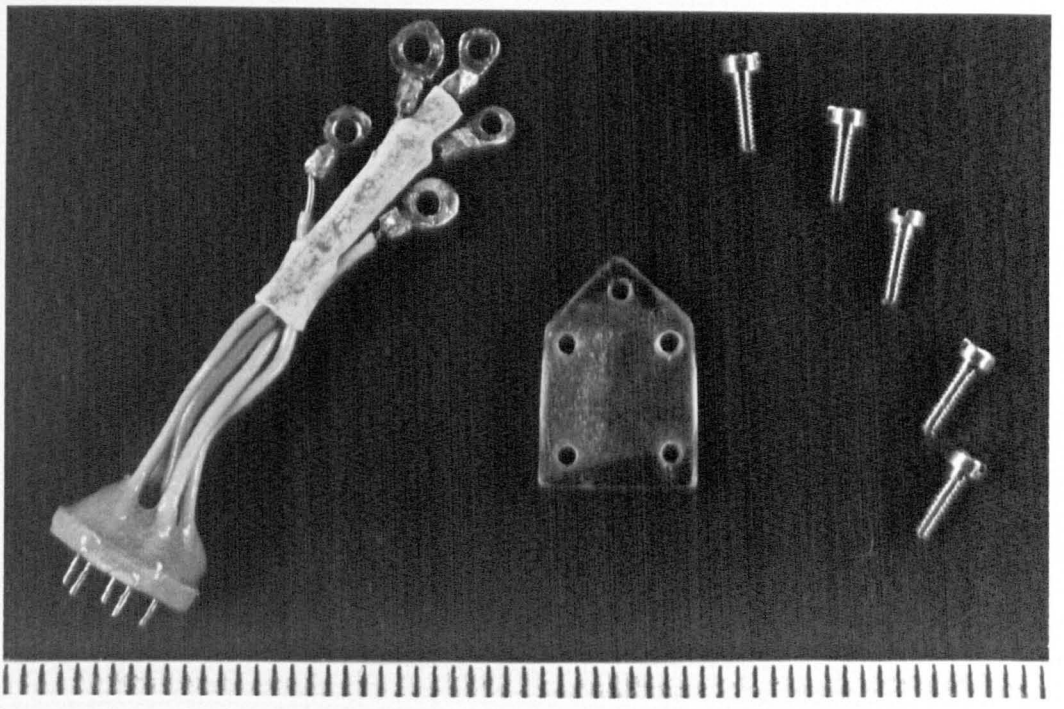
(A) Electrode set component

(B) Assembled electrode set

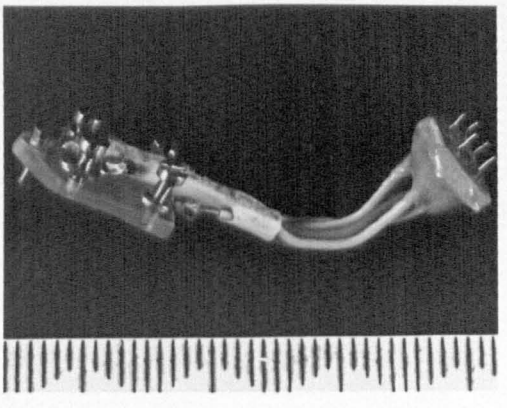
(C) Guide plate

(See text for details)

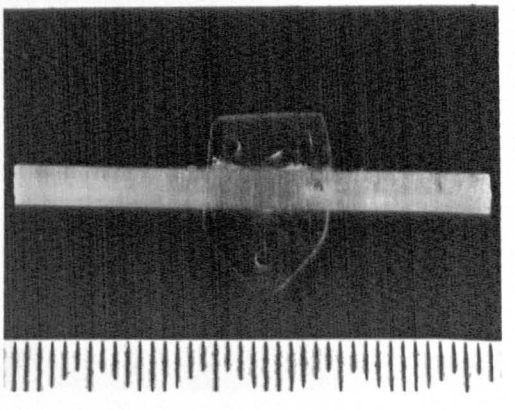
(A)



(B)



(C)



the midline of the head and that the rear end was in line with the occipital suture. The holes required for the introduction of the electrodes were drilled through the preformed holes of the guide plate with a dental drill. The diameter of the holes was slightly smaller than that of the electrodes. Thereafter the guide plate was taken off and the electrodes plate placed on the skull and the electrodes were screwed gently into the holes. The screwed electrodes fitted firmly in the skull and needed no other means of fixation.

The top of the electrodes plate was then covered with dental cement to ensure complete insulation and was left to dry. Thereafter, an antibiotic was sprayed on to the skull and the skin was sutured using a sterilized cotton thread, so that only the connecting terminal socket, which was extended to the back of the head, remained exposed.

After operation, rats were placed in individual cages.

The electrodes were located in the brain as follows: two bilaterally in the frontal cortex and the other two in the parietal cortex.

A cable coupler, which was first described by Sutton and Miller (1963), was obtained from a commercial source. This cable coupler, which was connected between the animal and a connection selector. allowed the recording cable to turn freely as the animal moved and at the same time maintained electrical continuity.

A connection selector, which was placed between the cable coupler and the recording apparatus, allowed us to select and vary the pairs of electrodes from which the recording for each channel would be taken.

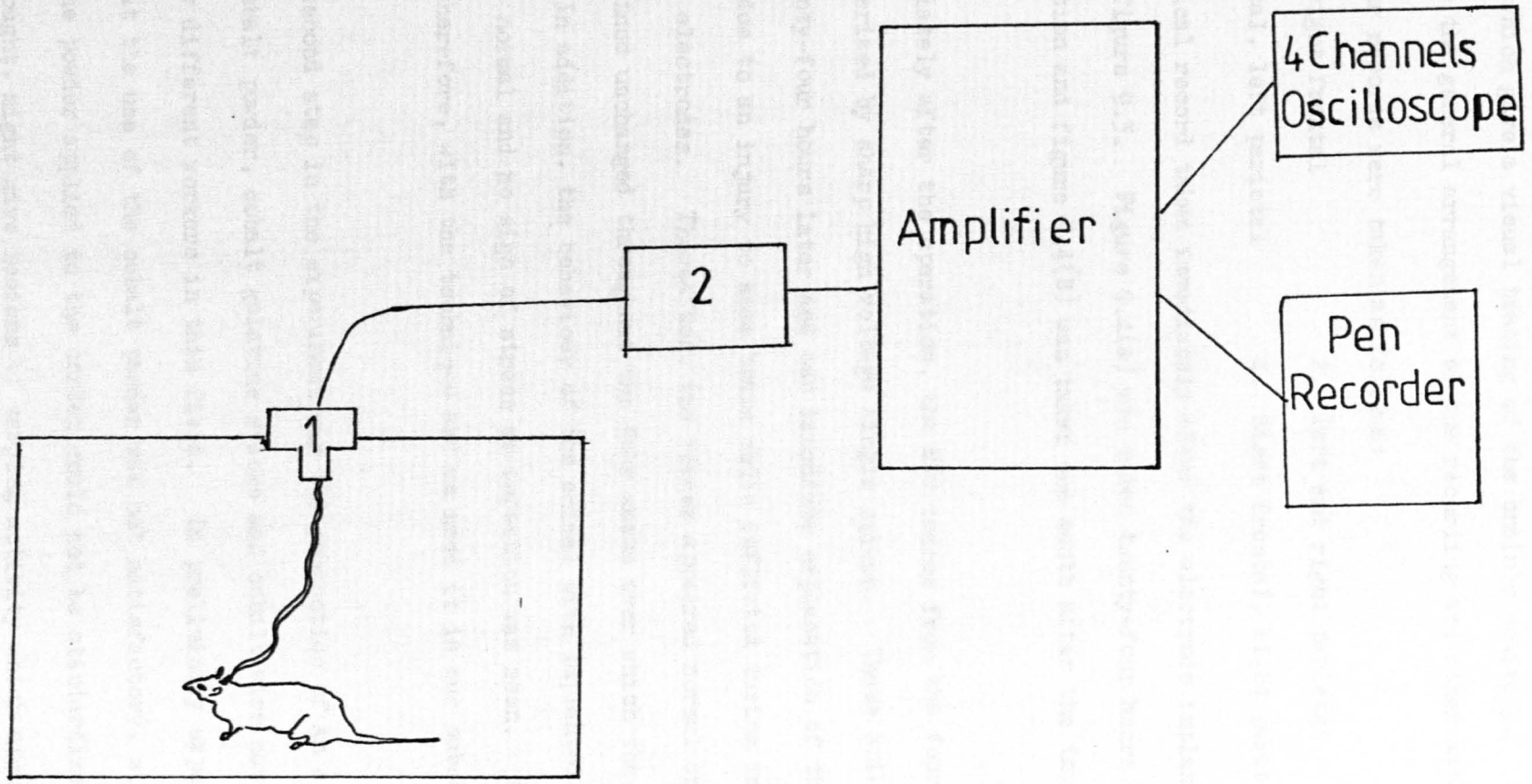
The recording apparatus was a Devices four channel physiological recorder which was connected to the connection selector and to an

FIGURE 9.2

Diagram used for the measurement of brain activity of unrestrained nonanaesthetized rats

(1) Cable coupler

(2) Connection selector


4 Channels
Oscilloscope
Amplifier
2
Pen
Recorder
1

oscilloscope which gave a visual tracing of the brain's activity. Figure 9.2 indicates the general arrangement of the recording and other apparatus.

Bipolar records were taken as follows:

1. Left and right frontal
2. Left and right parietal
3. Left frontal, left parietal
4. Right frontal, right parietal.

A typical record taken immediately after the electrode implantation is given in figure 9.3. Figure 9.4(A) was taken twenty-four hours after the implantation and figure 9.4(B) was taken one month after the implantation.

Immediately after the operation, the EEG traces from the four channels were characterized by sharp high voltage single spikes. These spikes disappeared twenty-four hours later and our immediate explanation of this was that it was due to an injury to some brain cells inflicted during implantation of the electrodes. Thereafter, the traces appeared normal and the pattern remained unchanged throughout the four weeks over which the recording was made. In addition, the behaviour of the animal with implanted electrodes was entirely normal and no sign of stress or infection was seen. We were satisfied, therefore, with the technique and we used it in our subsequent experiments.

The second step in the experiment was the production of an epileptic lesion. Cobalt powder, cobalt gelatine sticks and cobalt wire have all been used by different workers in this field. In preliminary experiments, we found that the use of the cobalt powder was not satisfactory, since the amount of the powder applied to the cortex could not be standardized, and this, we thought, might give lesions of varying activity which might, in turn, affect our results.

FIGURE 9.3

Traces of normal brain activity taken immediately after electrode implantation

1. Left and right frontal
2. Left and right parietal
3. Left frontal, left parietal
4. Right frontal, right parietal

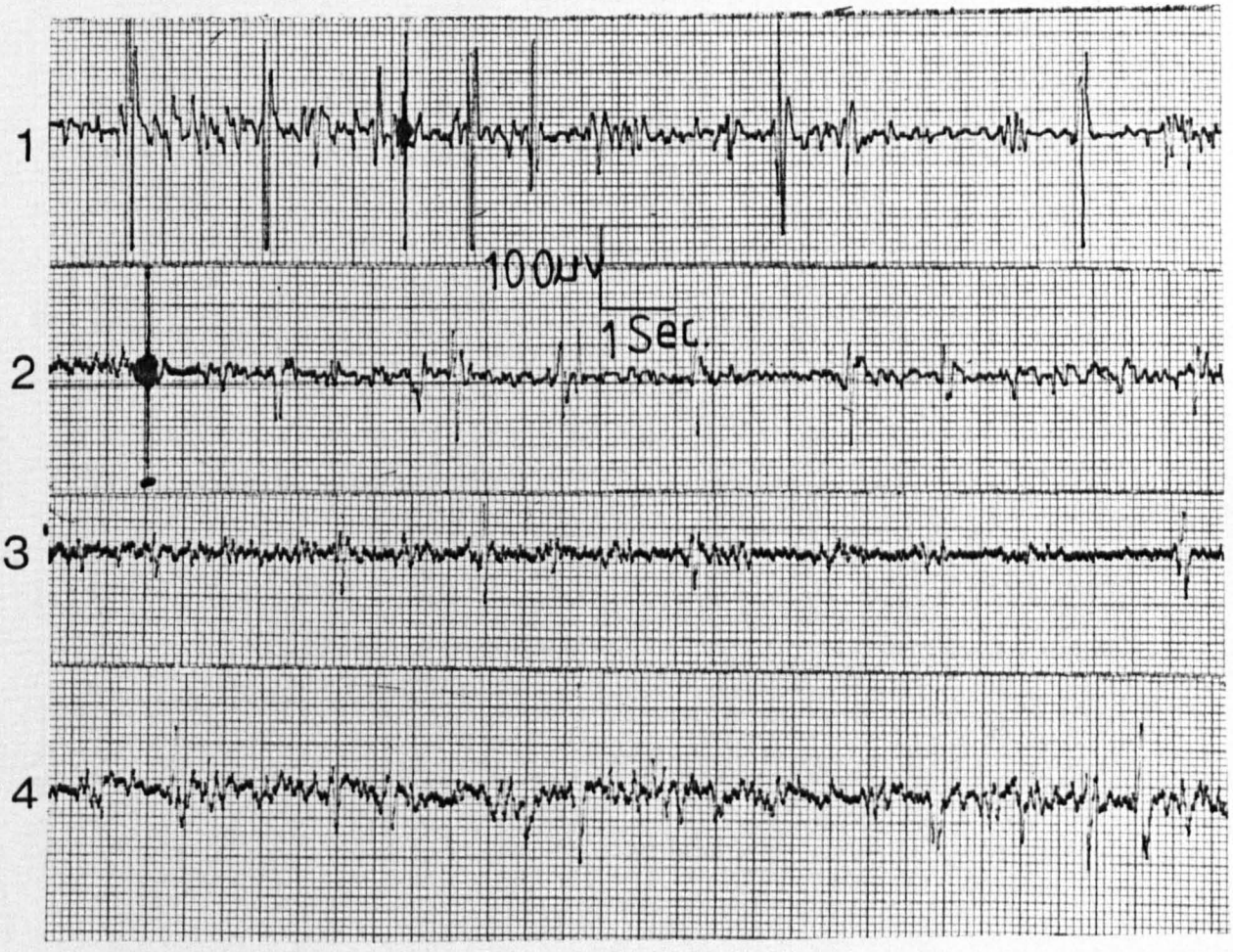
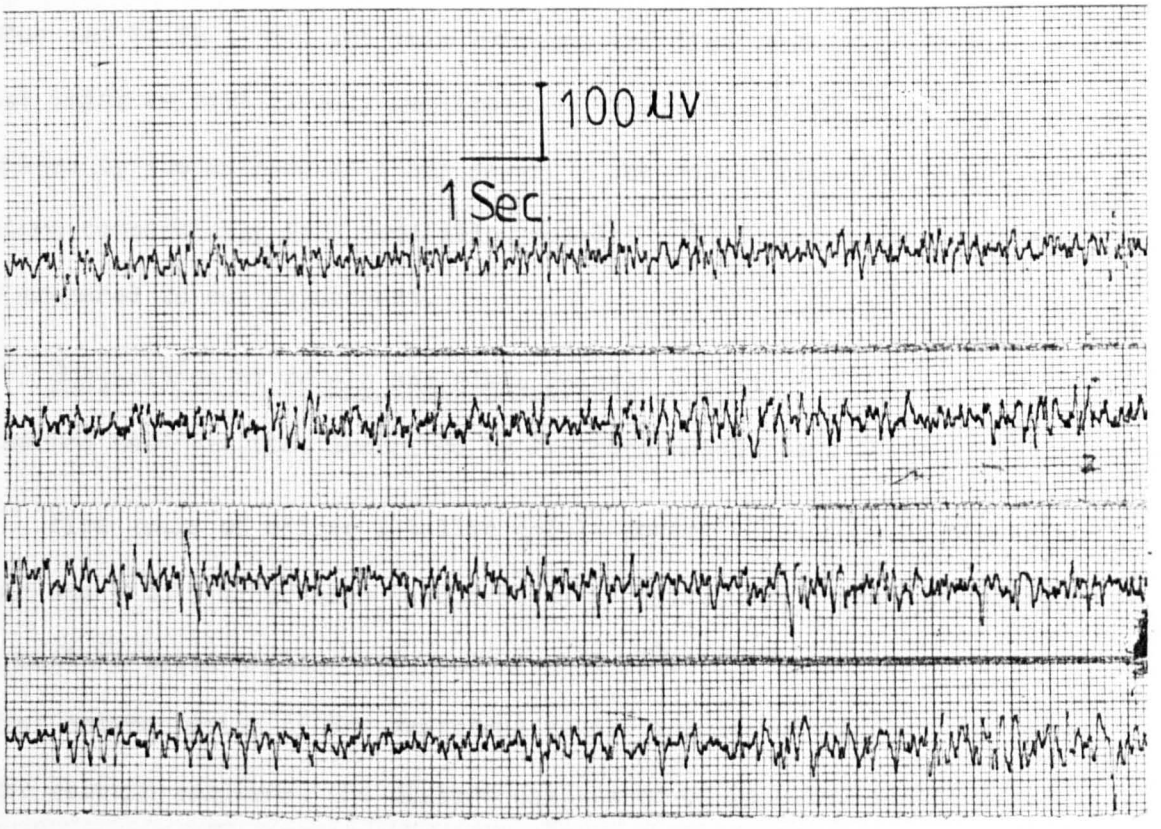

1
100μv
1 Sec.
2
3
4

FIGURE 9.4

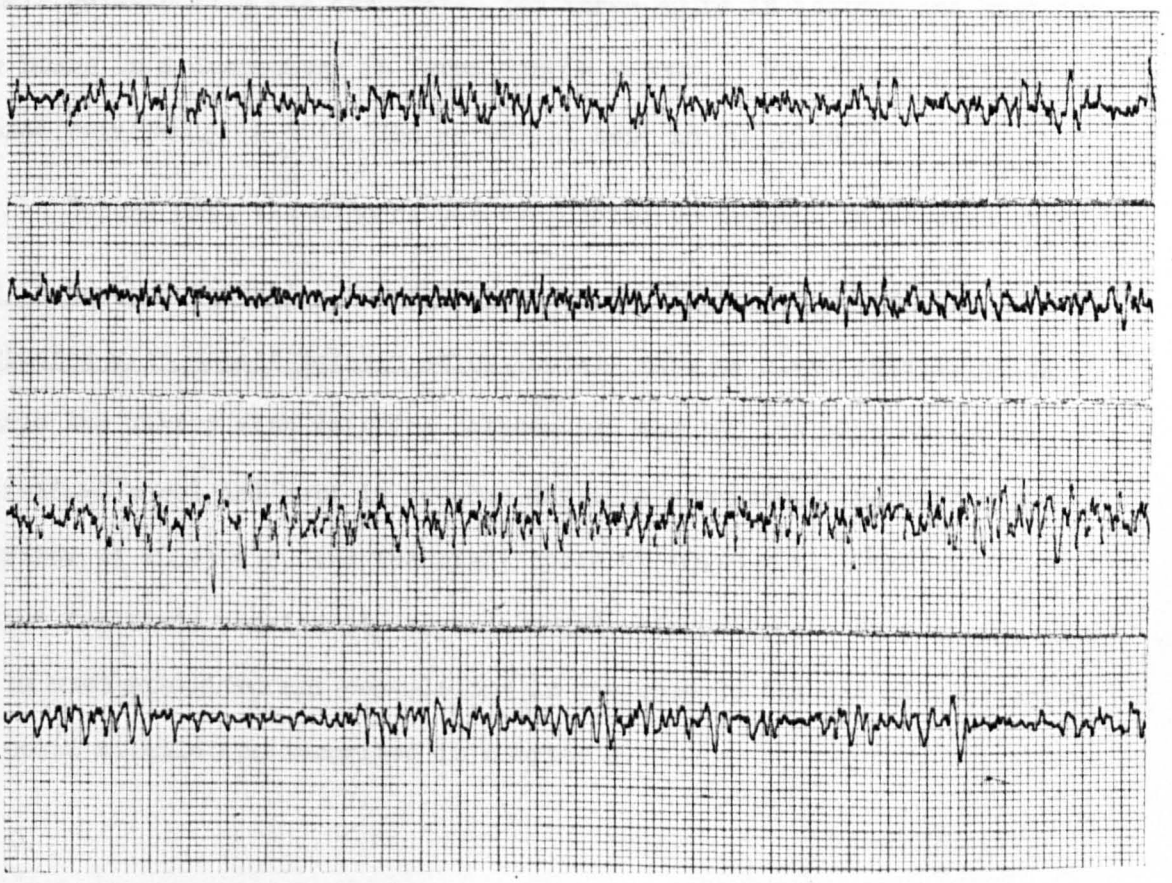
Traces of normal brain activity taken twenty-four hours (A) and one month (B) after electrode implantation

1. Left and right frontal
2. Left and right parietal
3. Left frontal, left parietal
4. Right frontal, right parietal

A



B



The use of cobalt gelatin sticks was first described by Fischer, Holubar and Malfk (1967) and this overcame the disadvantage of cobalt powder which we have already mentioned. Other advantages, too, were described by the authors in their paper. The cobalt wire and powder were obtained commercially from Goodfellow metals. The cobalt gelatin sticks were prepared in our department.

Preparation of cobalt and glass gelatin sticks

The method used was that described by Fischer, Holubar and Malfk (1967).

Five grams of either metallic cobalt powder (Mesh 200) or fine glass powder and 5 to 7 ml of warm (about 56°C) 5% gelatin solution were stirred in a mixture using a glass rod, and kept at 56°C in a test tube. After partial sedimentation of the suspended powder and decantation of the supernatant gelatin solution, the mixture was poured on to a horizontal warm slide to form a layer of about 1 mm thick. After cooling, the solidified cobalt gelatin film was dehydrated for three hours in acetone and kept for two hours in a desiccator in formaldehyde vapour. The slide was then washed repeatedly in distilled water and kept in 80% alcohol.

Before use, the alcohol was evaporated to dryness in an oven at 56°C.

9.2 Behavioural studies and the measurement of locomotor activity in rats implanted with cobalt gelatin stick, cobalt wire or glass gelatin stick

Female Ash Wistar rats weighing between 140 and 160 gms were housed in individual cages and kept in reverse daylight condition, the dark cycle starting at 10.15 a.m. and the light cycle at 10.15 p.m. The rats were tested for their sensitivity towards sound stimulation. None of the rats to be used exhibited any convulsions in response to the one minute period of sound stimulation described in chapter 3.

One week later the rats were divided into four groups of four and each was anaesthetized with 50 mg/kg pentobarbitone given by intraperitoneal injection. The skin was shaved and the scalp was cut along the midline. The skin was reflected and the exposed bone was cleaned from fascia and muscle so that the bone sutures were clearly visible.

Using a dental drill, a hole was bored in the skull at a point 2 mm to the right of the midline and 2 mm posterior to the bregma. The dura was cut by means of a fine needle and a cobalt gelatin stick, a glass gelatin stick (1 x 1 x 2 mm) or cobalt wire (1 x 2 mm) was implanted into the cortex at right angles to its surface.

The skin was then sutured using sterilized cotton thread and the animal was left to recover in its cage. The fourth group of animals was operated on as described but received no implant. This group served as a control group.

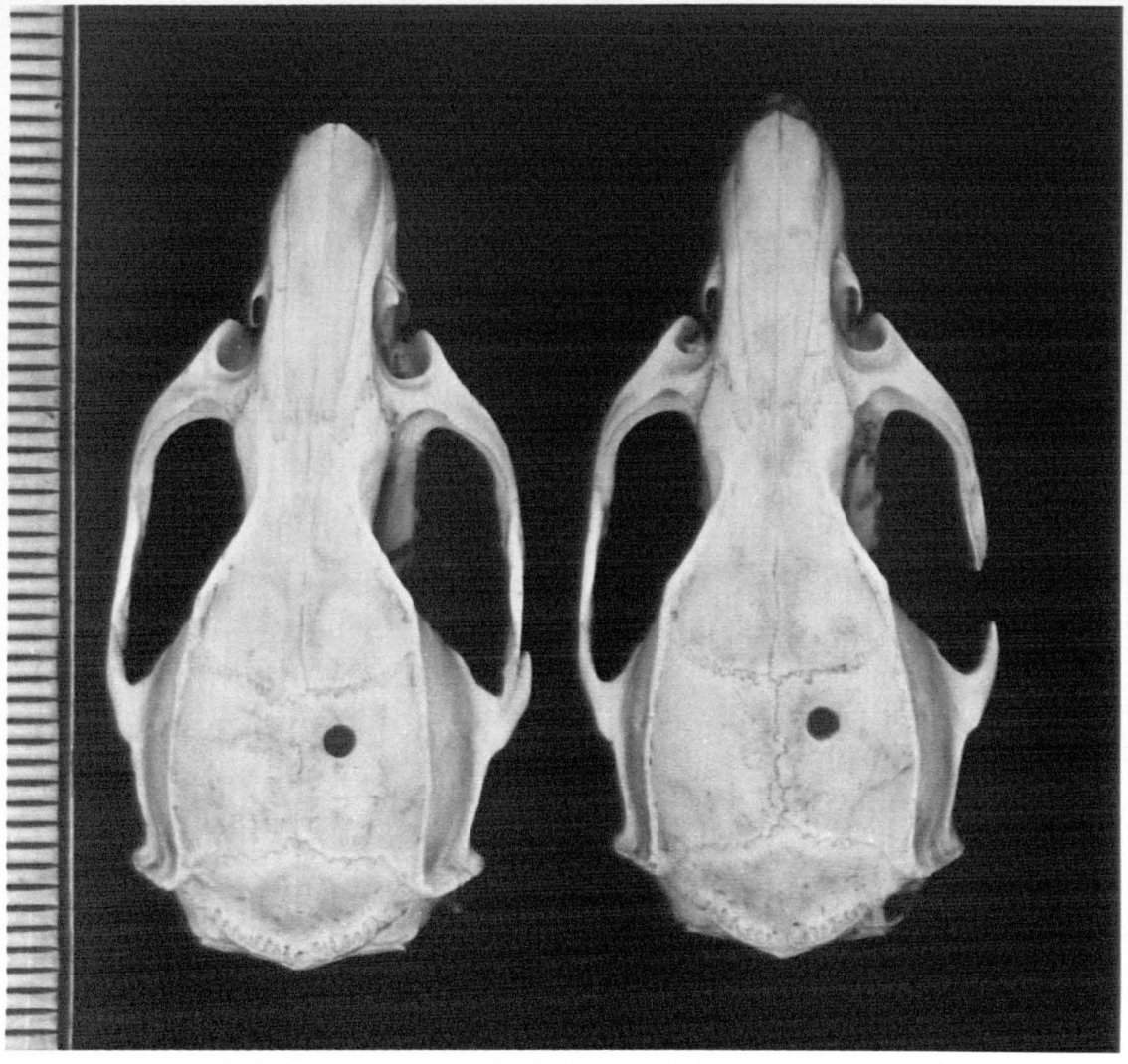
The plate in figure 9.5 shows the position of the hole in the skull.

Body weight, body temperature (measured rectally by using electrical thermometers equipped with rectal thermistors). food and water intakes were monitored daily at 9.30 a.m. One hour after starting the dark cycle (11.15 a.m.), the motor activity of three rats from each group was measured for fifteen minutes each hour for four hours using an Animex apparatus. Starting with a different group each day, the rats were left for five minutes on the apparatus before the actual counting of the motor activity started.

The Animex was set at a sensitivity of 25 microampere and it was tuned every time at 40 microampere.

FIGURE 9.5

Plate of skulls showing the position of the hole through which implantation of cobalt stick, cobalt wire or glass gelatin stick was made



After finishing measuring the motor activity (around 4.0 p.m.), each rat was assessed behaviourally for at least five minutes in order to detect and follow any behavioural changes that might be taking place.

Results

Twenty-four hours after the operation, the motor activity of rats implanted with either cobalt sticks or wire was depressed, but only the group implanted with cobalt sticks showed a significantly lower activity ($P < 0.02$) than control animals (Figure 9.6).

Rats implanted with cobalt wire ate less during the first nine days after the operation, but apart from that all groups ate and drank the same amount during the whole period of the experiment (Figure 9.7). They gained weight at the same rate, and although there were slight fluctuations in their body temperature, there was no significant difference in rectal temperature among the four groups, except on the fifteenth day, when the three implanted groups all showed slightly higher temperatures than the sham operated group (Figure 9.8).

Behaviourally, rats in the four groups were indistinguishable from each other twenty-four hours after the operation.

Three days after the operation, rats implanted with cobalt sticks started to lift their contralateral hind limb on walking and by the fifth day both cobalt groups started to show twitches of the contralateral hind-limb. These twitches were stronger when the rats were at rest. Some rats inclined towards the side of the implantation when they were displaying powerful twitches.

FIGURE 9.6

Locomotor activity of rats implanted with cobalt wire (left hatched columns), cobalt gelatin sticks (right hatched columns), glass gelatin sticks (dotted columns) or sham operated rats (open columns).

Significantly different by the t test (* $P < 0.05$; ** $P < 0.02$) from the values obtained from the sham operated group.

Each bar is the mean $\pm$ standard error of four observations, taken over a four-hour period, of three rats (see text for details).

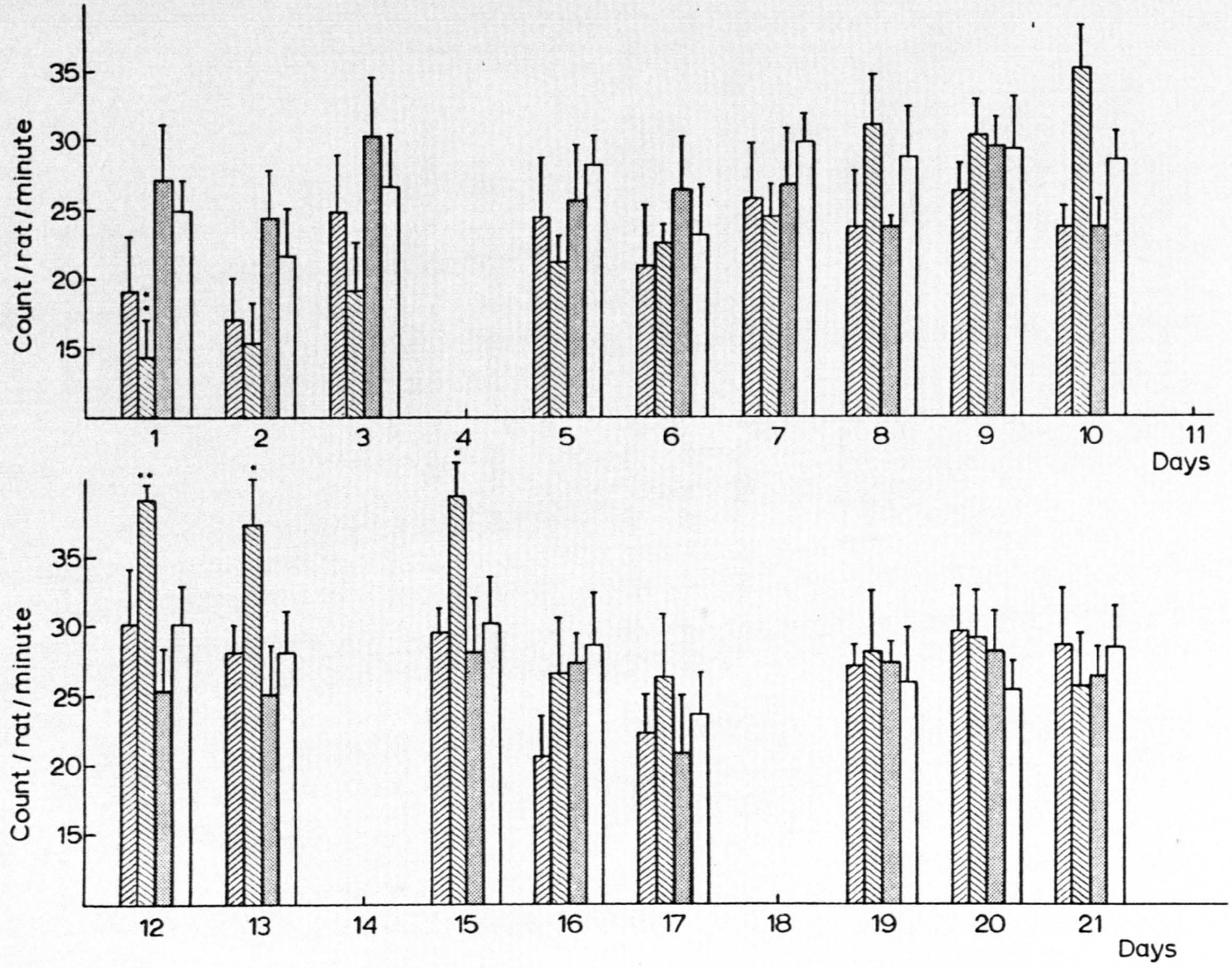

Count / rat / minute
35
30
25
20
15
1
2
3
4
5
6
7
8
9
10
11
Days
Count / rat / minute
35
30
25
20
15
12
13
14
15
16
17
18
19
20
21
Days

FIGURE 9.7

Changes in food and water intakes of rats implanted with cobalt wire (left hatched columns), cobalt gelatin sticks (right hatched columns), glass gelatin sticks (dotted columns) or sham operated rats (open columns).

Significantly different by the t test (* $P < 0.05$; ** $P < 0.001$) from the values obtained from the sham operated group.

Each bar indicates the mean $\pm$ standard error for four rats.

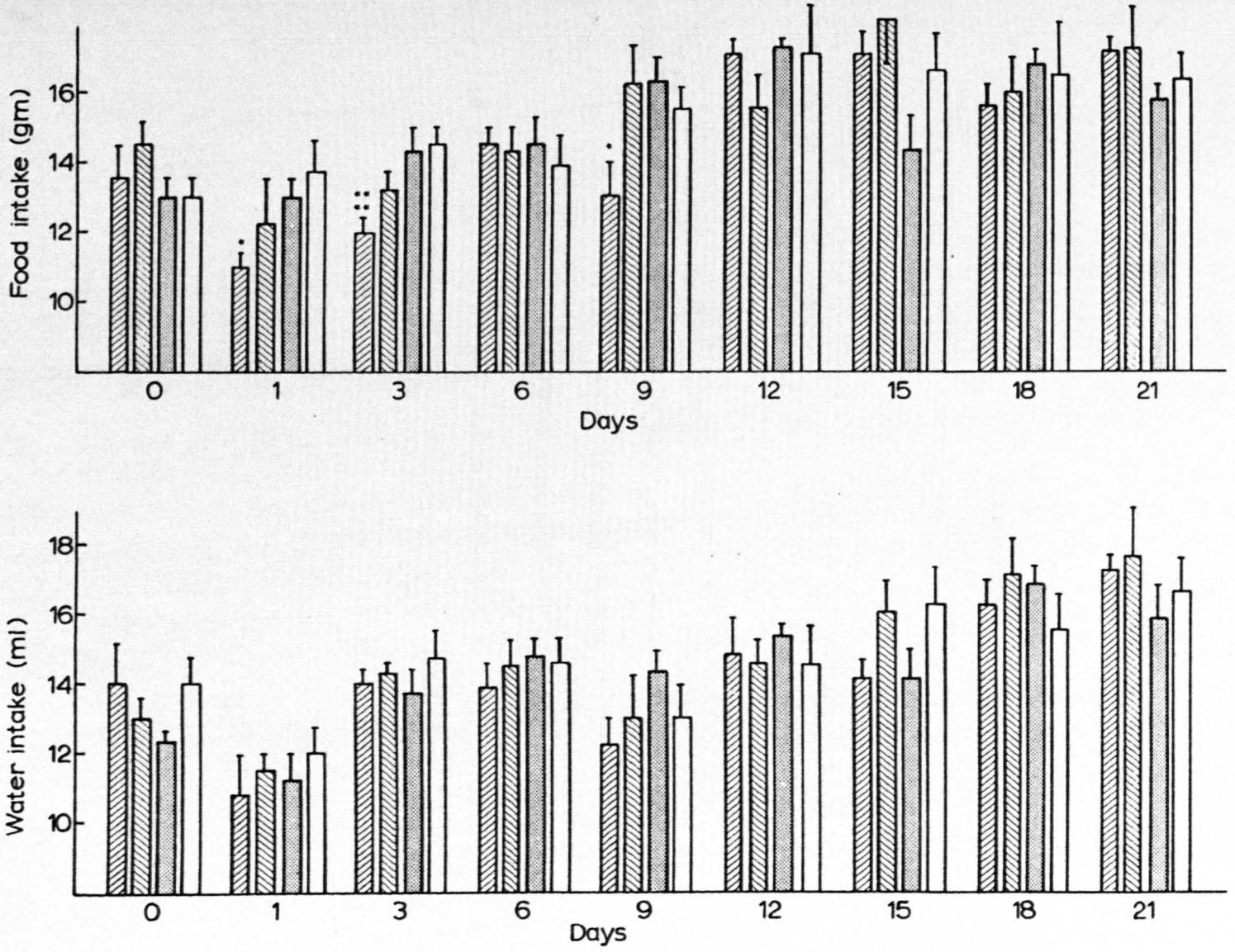
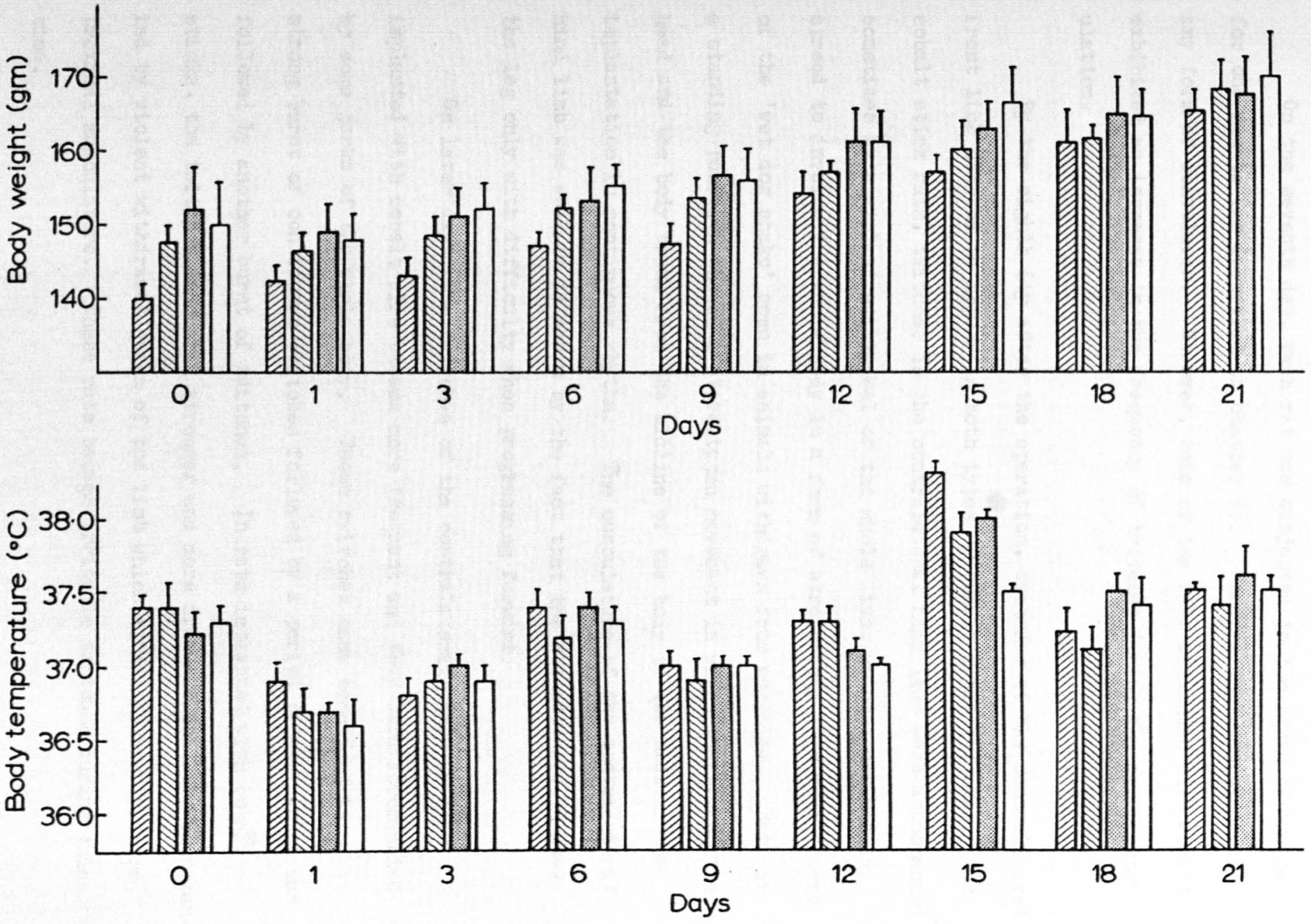

Food intake (gm)
16
14
12
10
0
1
3
6
9
12
15
18
21
Days
Water intake (ml)
18
16
14
12
10
0
1
3
6
9
12
15
18
21
Days

FIGURE 9.8

Changes of body weights and body temperatures of rats implanted with cobalt wire (left hatched columns), cobalt gelatin sticks (right hatched columns), glass gelatin sticks (dotted columns) or sham operated rats (open columns).

Each bar indicates the mean $\pm$ standard error for four rats.


Body weight (gm)
170
160
150
140
0
1
3
6
9
12
15
18
21
Days
Body temperature (°C)
38·0
37·5
37·0
36·5
36·0
0
1
3
6
9
12
15
18
21
Days

On the seventh day, each rat was subjected to the sound of the bell for one minute (as described in chapter 3). None of the rats exhibited any form of convulsion. However, some of the rats implanted with cobalt exhibited an increase in the frequency of twitches during the sound stimulation.

By the eighth day after the operation, twitches of the contralateral front limb started to appear in both types of cobalt implanted rats. In cobalt stick rats, twitches in the contralateral hind limb were accompanied sometimes by a violent withdrawal of the whole limb, which occasionally spread to involve the whole body in a form of strong body jerk reminiscent of the 'wet dog shake' seen in animals withdrawn from morphine. Rats in a standing position showed a stereotyped movement in which they moved the head and the body trunk from the midline of the body to the side of the implantation in continuous rhythm. The musculature of the contralateral hind limb was weak, as is shown by the fact that the rat appeared to move the leg only with difficulty when progressing forward.

On later days, the twitches of the contralateral limbs in rats implanted with cobalt wire became more frequent and they were accompanied by some jerks of the whole body. These twitches came sometimes as a strong burst of continuous twitches followed by a period of rest which was followed by another burst of twitches. In rats implanted with cobalt sticks, the twitches were even stronger and more frequent and were accompanied by violent withdrawal jerks of the limb which spread to involve more proximal musculature. These rats became irritable to touch during this time.

On the thirteenth day after the operation, a movie film was made (a copy of this film has been deposited in the department). It shows the peak effects described above in rats given cobalt.

The motor activity of rats with cobalt stick implants started to increase from the tenth day after the operation so that by the twelfth to the fifteenth day after operation it was significantly higher than that of control animals (figure 9.6).

After the sixteenth day, jerks became less frequent and less violent and by the end of the third week only twitches could be seen in both groups of cobalt implanted rats.

None of the rats showed any convulsions in response to sound stimulation two or three weeks after operation.

9.3 The appearance of lesions produced by cobalt implantation

One, two or three weeks after the implantation of cobalt wire, cobalt -gelatin sticks or glass gelatin sticks, rats were anaesthetized with 50 mg/kg pentobarbitone by intraperitoneal injection. The chest was opened and cardiac perfusion of about 20 ml of 15% formalin was performed into one of the ventricles after making a cut in the opposite ventricle. Ten minutes later the brain was removed and kept in formalin solution.

The plate in figure 9.9 shows the position and the extent of the lesions produced one week after implantation of cobalt wire and stick. The lesions were about 1 mm in diameter around the implantation site and they extended through the depth of the cortex. In animals given either cobalt wire or cobalt stick the contralateral cortex appeared normal. Picture no.3 of the same figure shows the response three weeks after the implantation of glass gelatin sticks; there was a very slight superficial

FIGURE 9.9

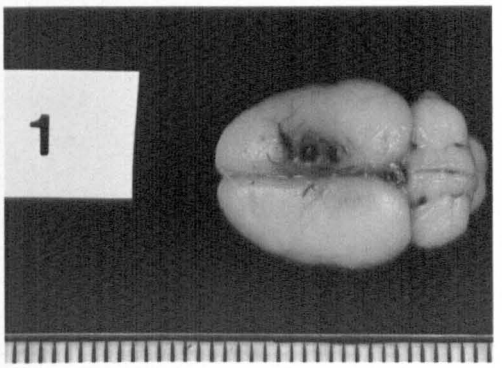
Plate showing the appearance of the brain lesions produced by implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks

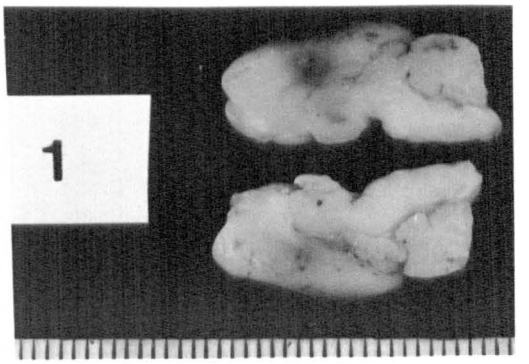
2) One week after implantation of cobalt gelatin stick

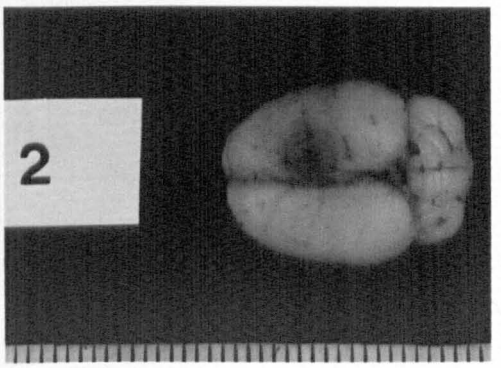
1) One week after implantation of cobalt wire

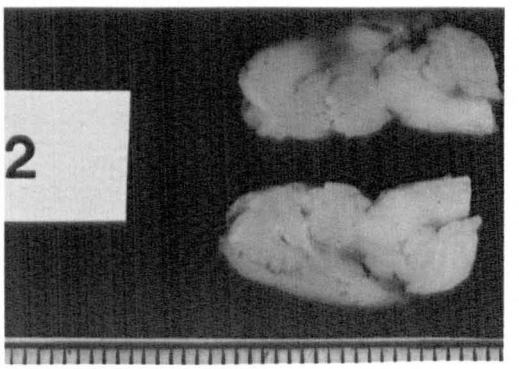
3) Three weeks after implantation of glass gelatin stick

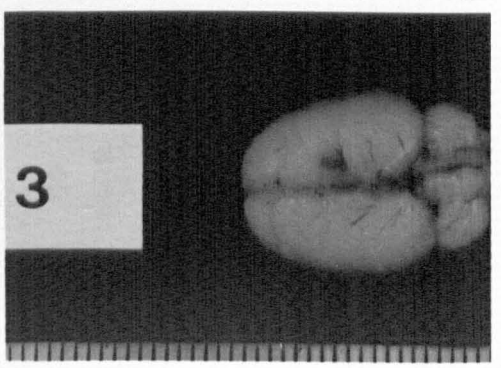
4) Brain of sham operated rat

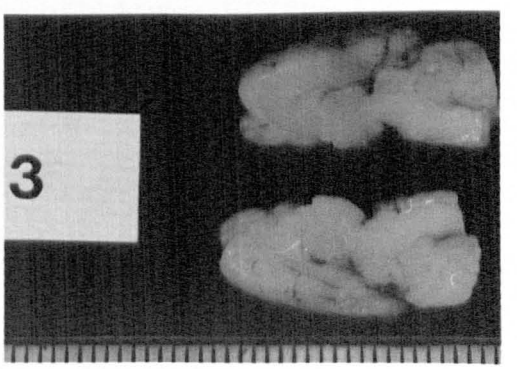

1


1


2


2


3


3

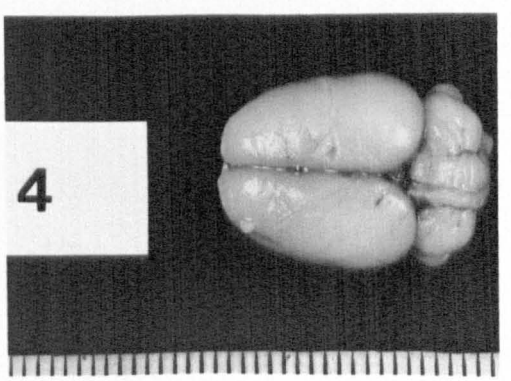

4

FIGURE 9.10

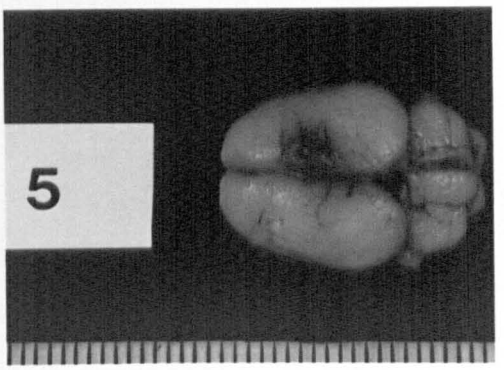
Plate showing the appearance of the brain lesions produced by the implantation of either cobalt gelatin stick or cobalt wire

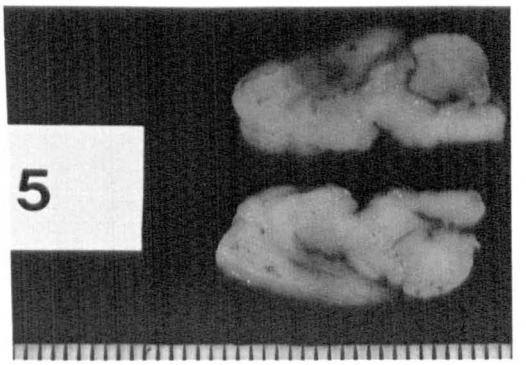
6) Two weeks after the implantation of cobalt gelatin stick

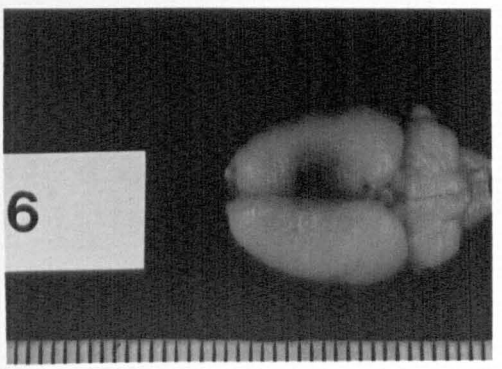
5) Two weeks after the implantation of cobalt wire

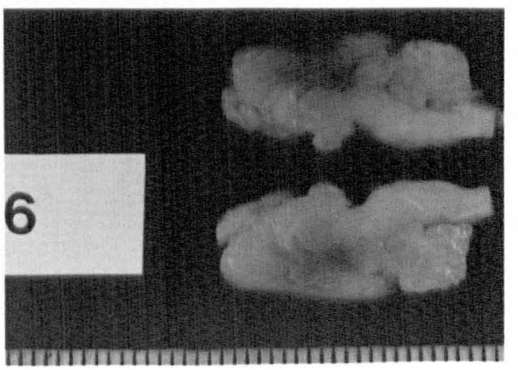
8) Three weeks after the implantation of cobalt gelatin stick

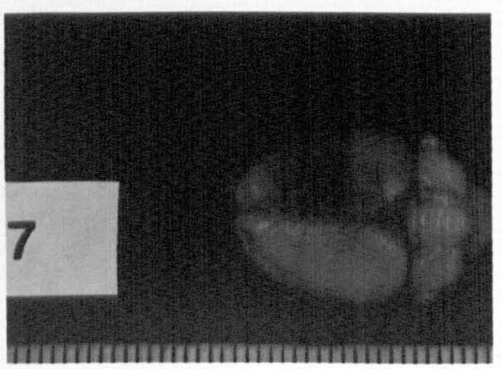
7) Three weeks after the implantation of cobalt wire

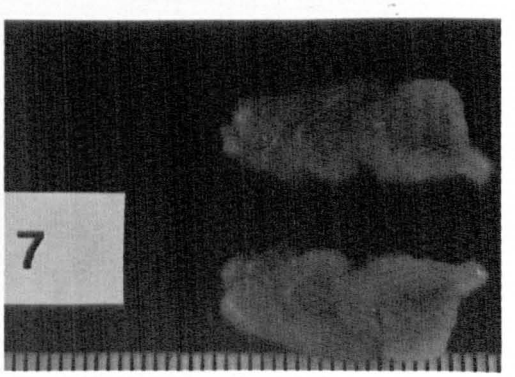

5

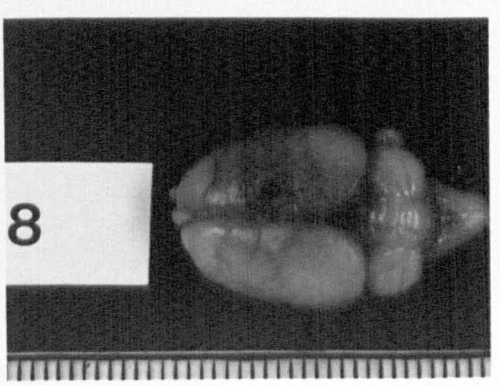

5

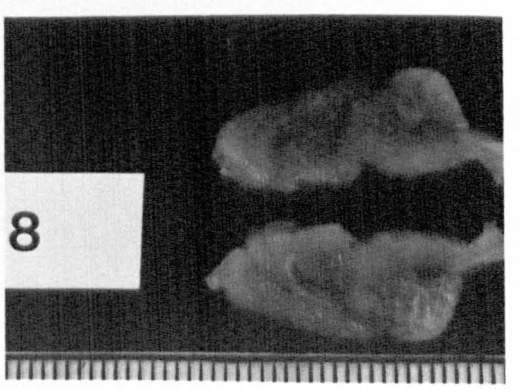

6


6


7


7


8


8

lesion which did not extend into deeper cortical areas. Picture no. 4 shows the brain of a sham operated rat three weeks after operation.

By the second week after the implantation of either cobalt wire or sticks (plate shown in figure 9.10), the lesions were large and in the case of the cobalt gelatin sticks, dark necrotic tissue in the central area around the implantation site was seen.

By the third week after implantation, an increased vascularity was obvious in the brains of animals given cobalt wire or sticks (figure 9.10).

Although the contralateral cortex seemed to be unaffected superficially, two or three weeks after the implantation of either cobalt wire or cobalt stick, inspection of the deeper layers (revealed after hemisection of the brain) showed that the lesions spread to the contralateral cortex by the second week after implantation (figure 9.10 (5), (6), (7) and (8)).

9.4 Brain activity of rats implanted with cobalt gelatin stick, cobalt wire or glass gelatin stick

The following experiment was carried out in order to follow the changes in the brain activity of rats implanted with cobalt gelatin sticks, cobalt wire or glass gelatin sticks.

Female Ash Wistar rats weighing between 140 and 160 gms were used. The rats were housed in individual cages on reverse daylight. One week after housing, each rat was anaesthetized with 50 mg/kg pentobarbitone by intraperitoneal injection and a cobalt gelatin stick, cobalt wire or glass gelatin stick was implanted as described in chapter 9, section 2. Each rat received a set of electrodes as described in chapter 9, section 1. The rats were then left to recover in their cages where their brain activity was monitored twenty-four hours later, as described in section 9.1.

In order to follow the progressive change in brain activity, a scoring system which involved counting the number of bursts of spike discharge for a certain period of time was devised. The brain activity recorded by each of the four channels for each rat was followed visually using the oscilloscope for five minutes, and the total number of discharges for each rat over a period of twenty minutes was taken. Only continuous bursts of spikes containing no fewer than five high voltage spikes per burst were counted, so that isolated single spikes were not counted. The total number of the continuous bursts of spikes in the four channels was then plotted against the time in days that had elapsed since the operation.

Results are shown in figure 9.11.

The results show that the total number of spikes was maximum between day eight and day fifteen after implantation of either cobalt stick or cobalt wire. Glass gelatin sticks gave spikes as well but they were very few. They, too, reached a maximum eight days after implantation and thereafter declined.

Figures 9.12, 9.13 and 9.14 show traces of brain activity taken five days after implantation of glass gelatin sticks, cobalt gelatin sticks and cobalt wire respectively. The brain activity of the rats implanted with cobalt gelatin sticks showed a higher voltage background activity than those implanted with cobalt wire or glass gelatin sticks. The burst of spikes recorded in channels one and four from rats implanted with cobalt wire were of a higher frequency and voltage than those from rats that had received cobalt gelatin sticks.

Fourteen days after the implantation, cobalt gelatin sticks produced a higher background activity than did cobalt wire in channels 1, 3 and 4; beside the burst of high voltage spikes, the background activity was characterised by a large number of high voltage single spikes (figure 9.16).

FIGURE 9.11

Development of abnormal spike activity in rats implanted with glass gelatin stick (A), cobalt gelatin stick (B) and cobalt wire (C)

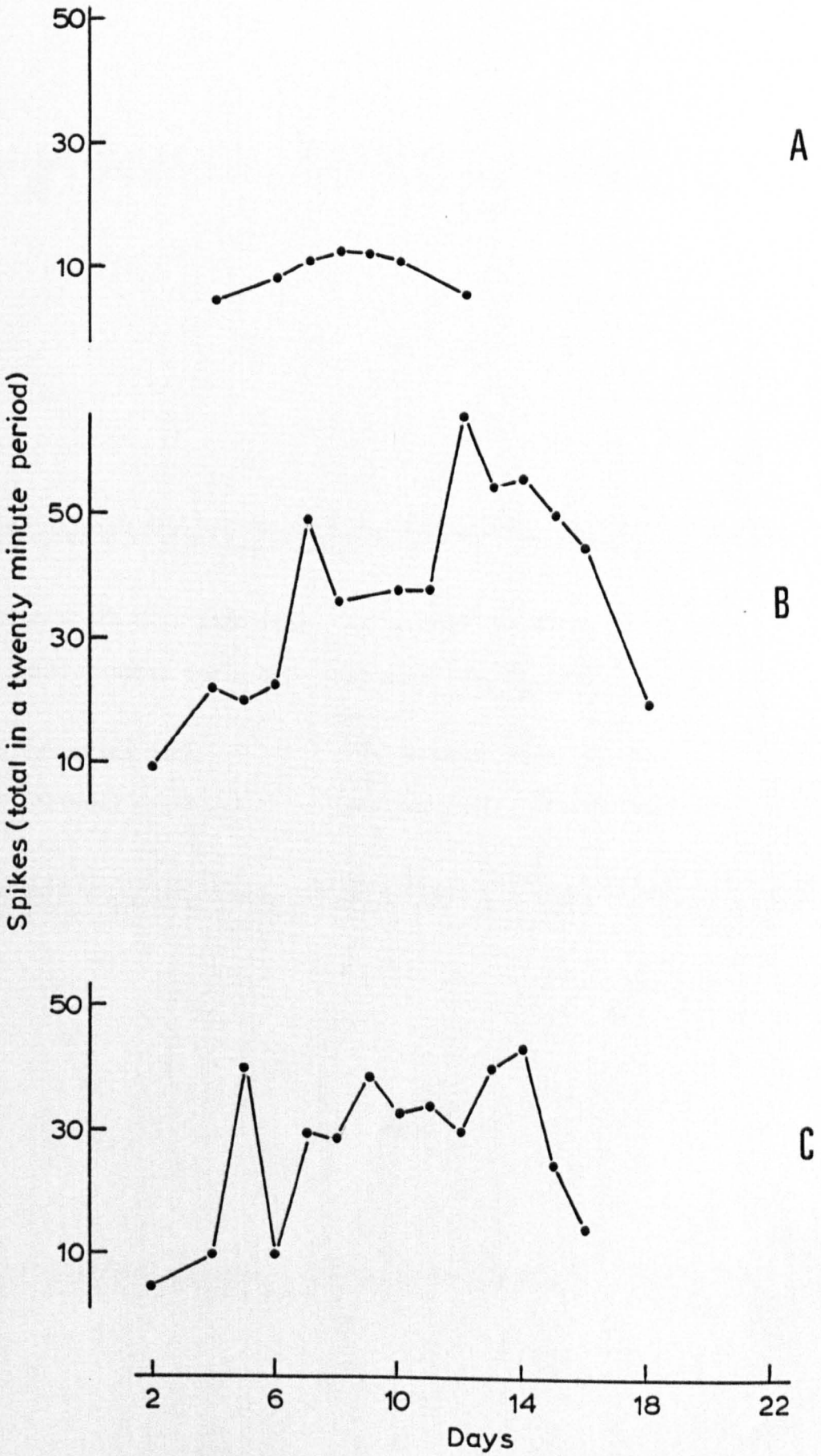

50
30
10
A
Spikes (total in a twenty minute period)
50
30
10
B
50
30
10
C
2
6
10
14
18
22
Days

FIGURE 9.12

Traces of brain activity of rat implanted with glass gelatin stick into the right cortex taken five days after implantation

1. left and right frontal
2. left and right parietal
3. left frontal, left parietal
4. right frontal, right parietal

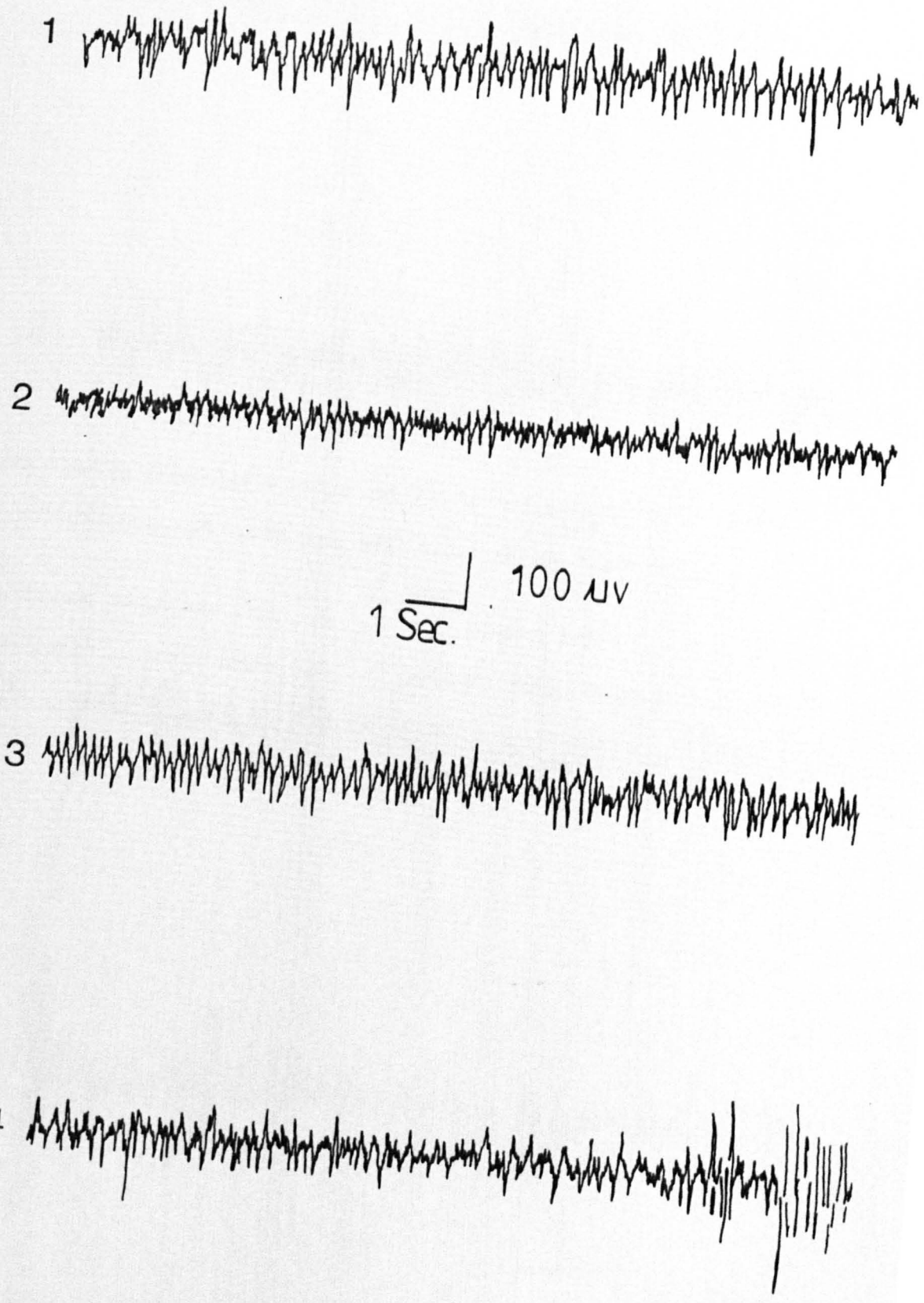

1
2
100 uv
1 Sec.
3
4

FIGURE 9.13

Traces of brain activity of rat implanted with cobalt gelatin stick into the right cortex taken five days after the implantation

1. left and right frontal
2. left and right parietal
3. left frontal, left parietal
4. right frontal, right parietal

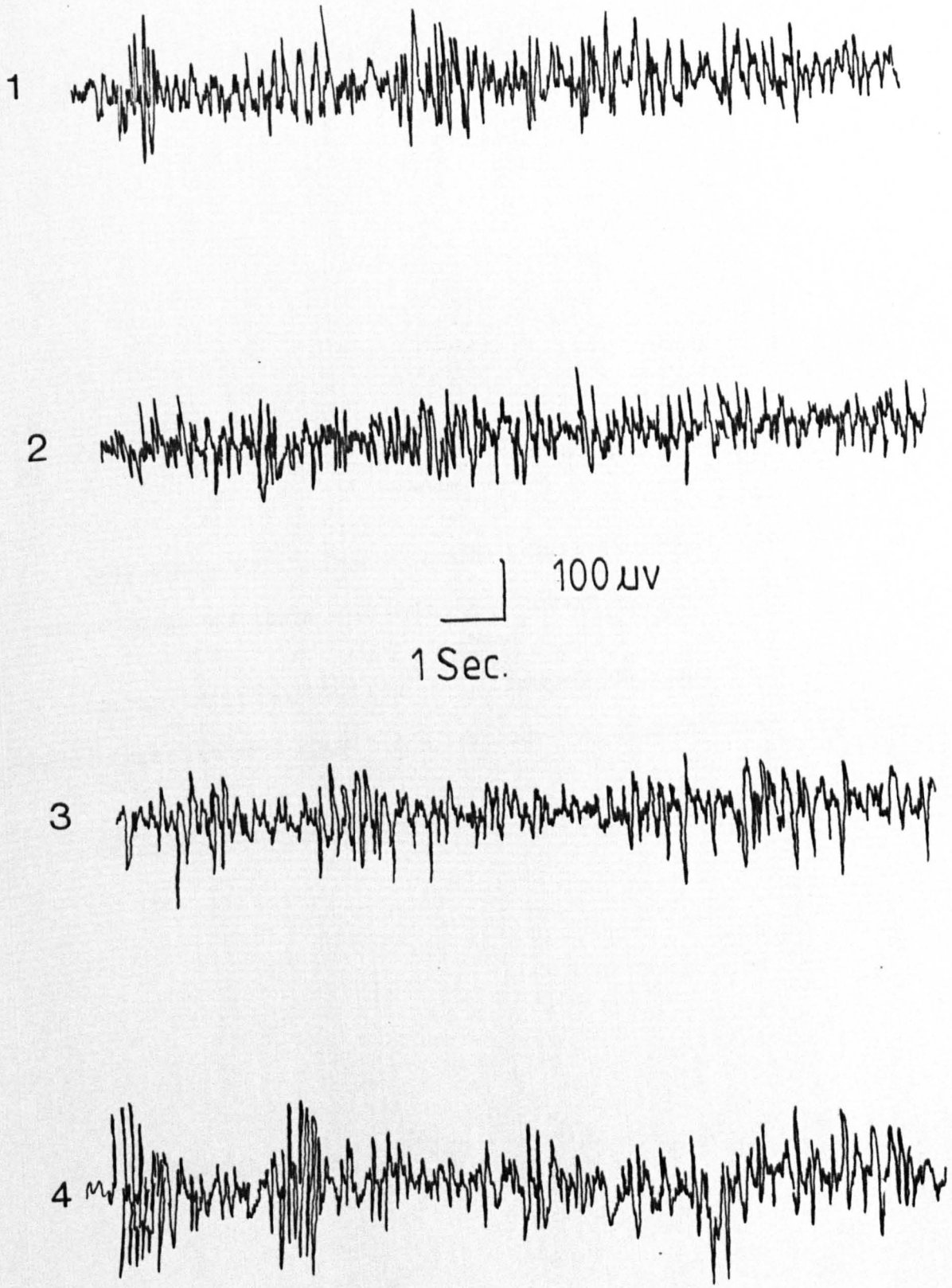

1
2
100 µv
1 Sec.
3
4

FIGURE 9.14

Traces of brain activity of rat implanted with cobalt wire into the right cortex taken five days after the implantation

1. left and right frontal
2. left and right parietal
3. left frontal, left parietal
4. right frontal, right parietal

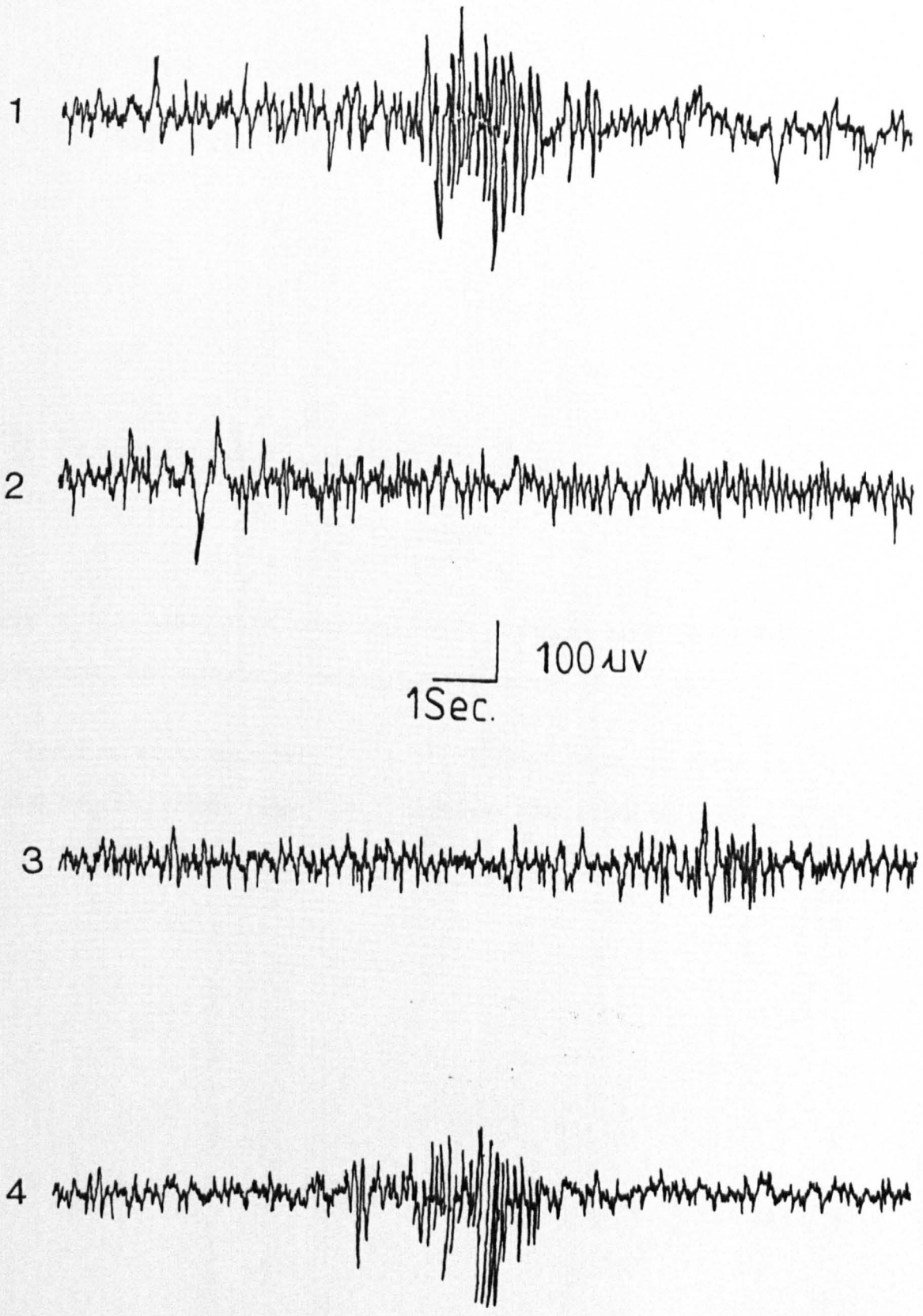

1
2
100 µv
1Sec.
3
4

FIGURE 9.15

Traces of brain activity of rat implanted with glass gelatin stick into the right cortex taken fourteen days after the implantation

1. left and right frontal
2. left and right parietal
3. left frontal, left parietal
4. right frontal, right parietal

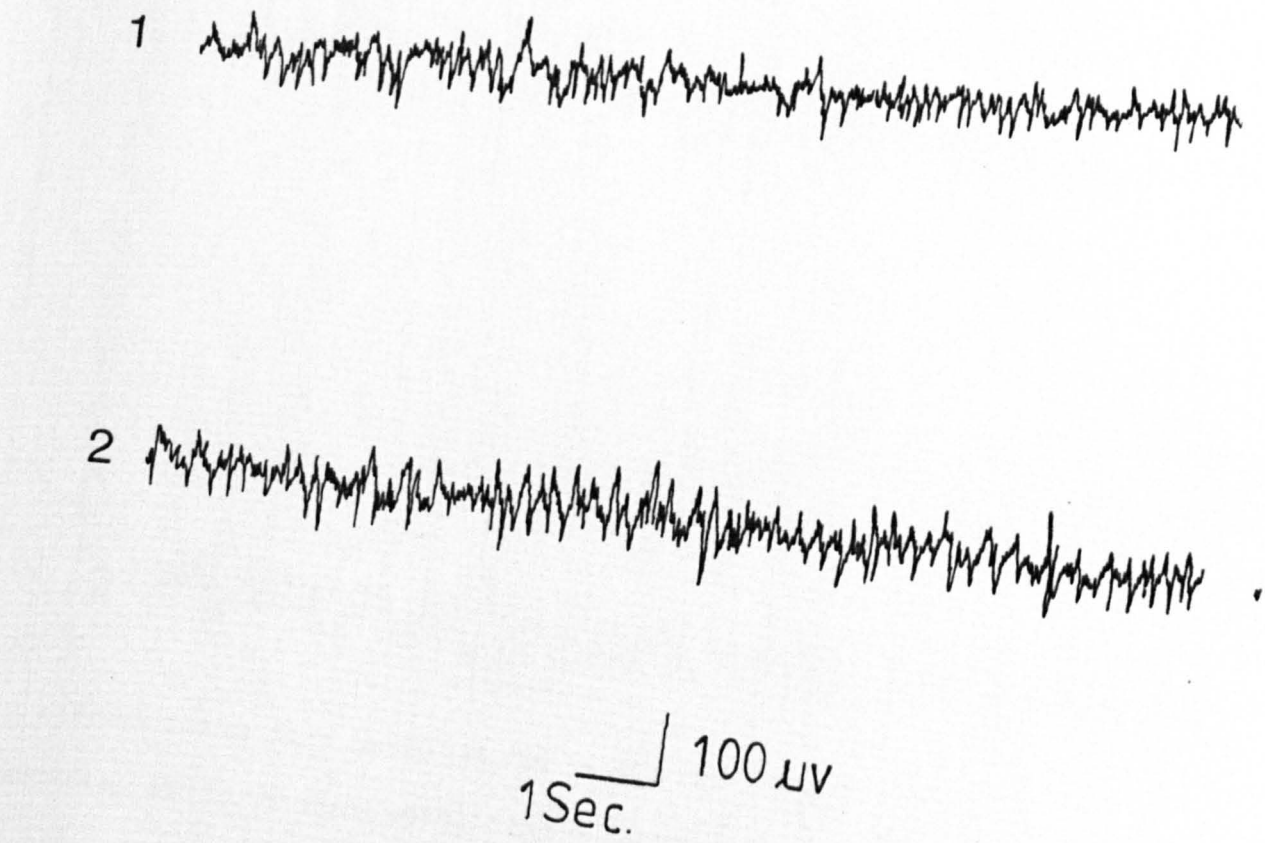

1
2
100 µv
1 Sec.
3
4

FIGURE 9.16

Traces of brain activity of rat implanted with cobalt gelatin stick into the right cortex taken fourteen days after the implantation

1. left and right frontal
2. left and right parietal
3. left frontal,left parietal
4. right frontal, right parietal

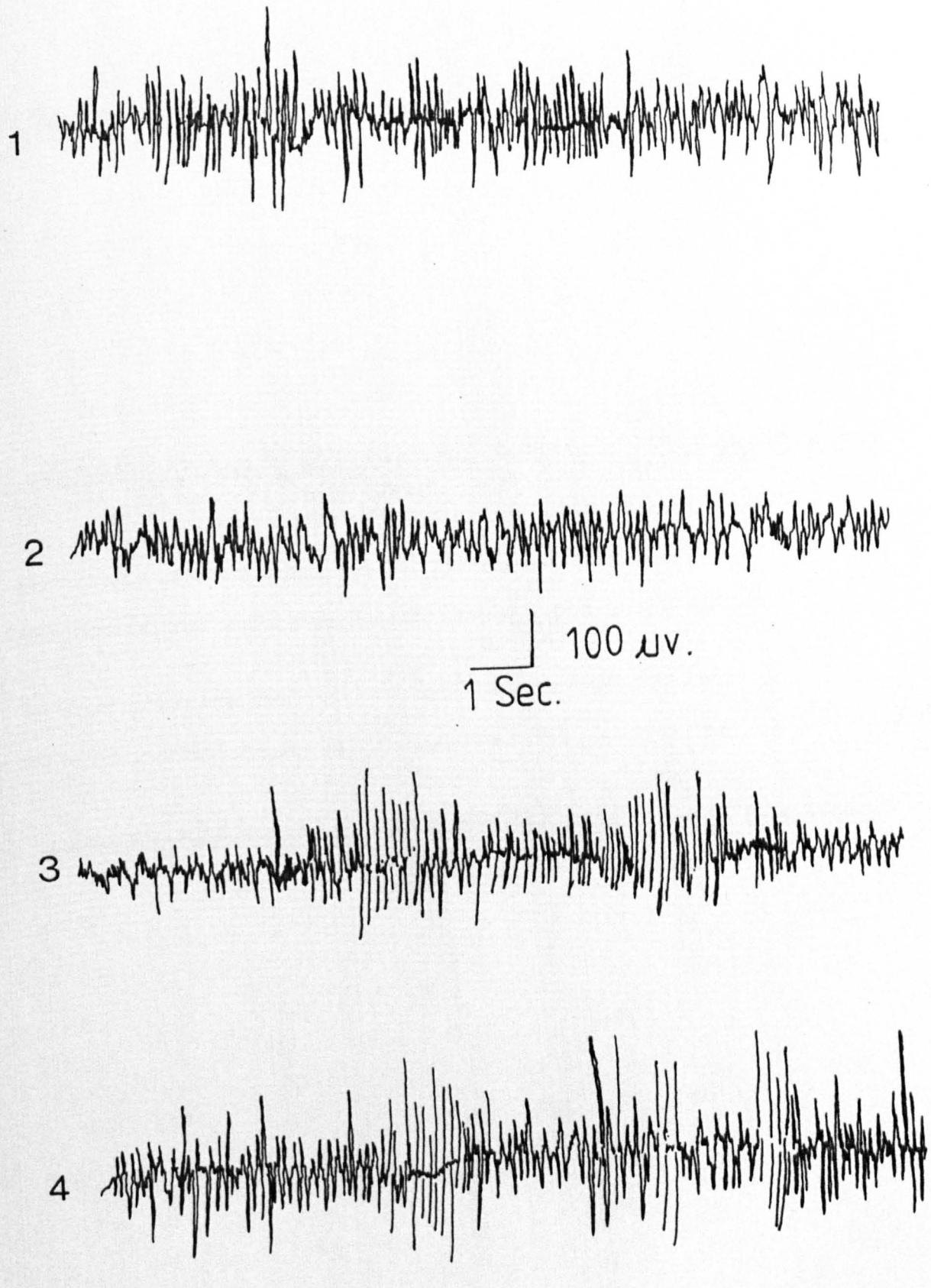
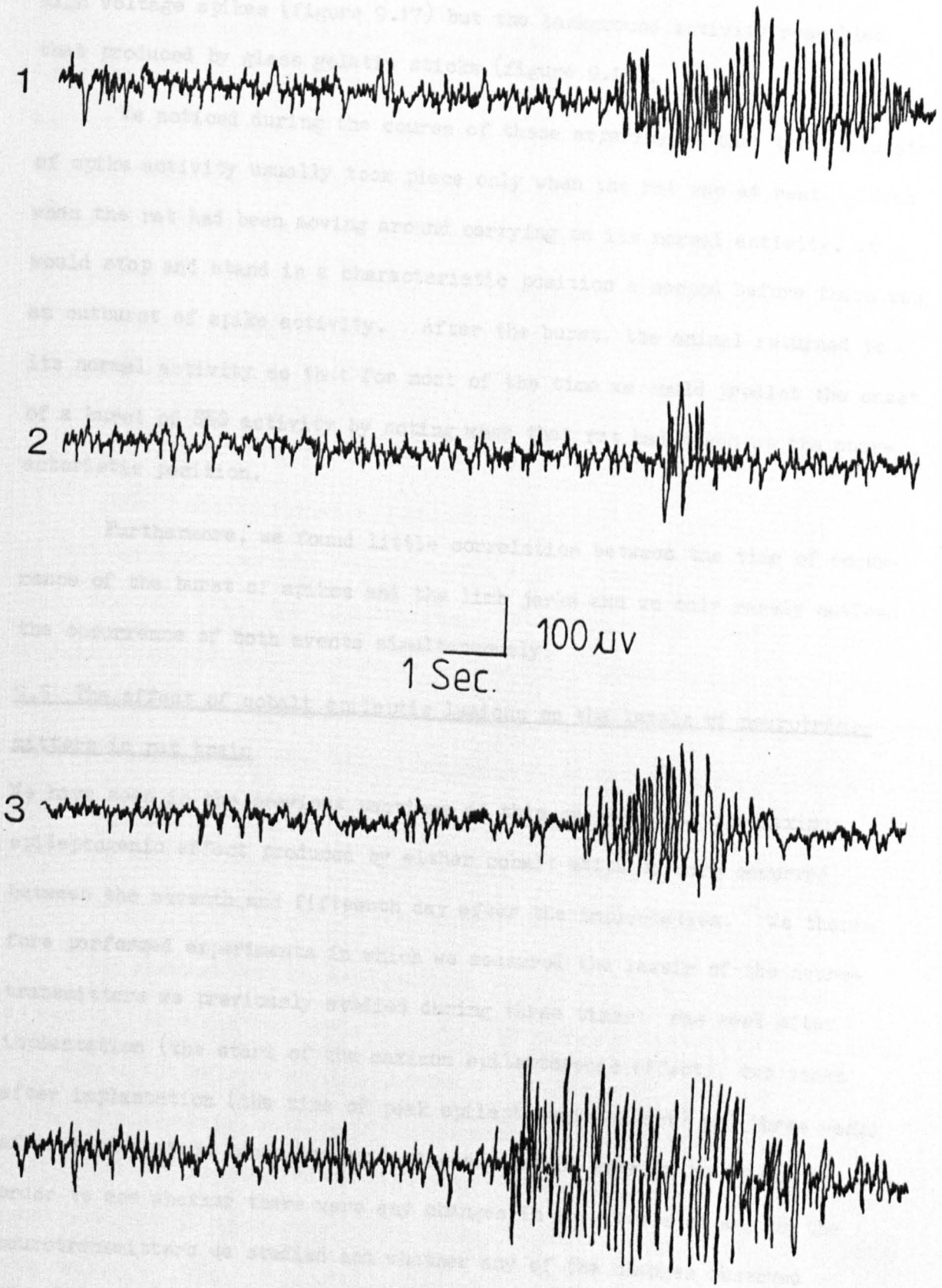

1
2
100 µv.
1 Sec.
3
4

FIGURE 9.17

Traces of brain activity of rat implanted with cobalt wire into the right cortex taken fourteen days after the implantation

1. left and right frontal
2. left and right parietal
3. left frontal, left parietal
4. right frontal, right parietal


1
2
100 µv
1 Sec.
3
4

On the other hand, cobalt wire produced characteristic bursts of very fast high voltage spikes (figure 9.17) but the background activity resembled that produced by glass gelatin sticks (figure 9.15).

We noticed during the course of these experiments that the outbursts of spike activity usually took place only when the rat was at rest. Even when the rat had been moving around carrying on its normal activity, it would stop and stand in a characteristic position a second before there was an outburst of spike activity. After the burst, the animal returned to its normal activity so that for most of the time we could predict the onset of a burst of EEG activity by noting when that rat had taken up the characteristic position.

Furthermore, we found little correlation between the time of occurrence of the burst of spikes and the limb jerks and we only rarely noticed the occurrence of both events simultaneously.

9.5 The effect of cobalt epileptic lesions on the levels of neurotransmitters in rat brain

We have seen in the previous sections of this chapter that the maximum epileptogenic effect produced by either cobalt stick or wire occurred between the seventh and fifteenth day after the implantation. We therefore performed experiments in which we measured the levels of the neurotransmitters we previously studied during three times: one week after implantation (the start of the maximum epileptogenic effect), two weeks after implantation (the time of peak epileptogenic effect) and three weeks after implantation (when the epileptogenic effect started to fade), in order to see whether there were any changes in the levels of any of the neurotransmitters we studied and whether any of the changes observed correlated in any way with changes observed in the motor and brain activity.

Female Ash Wistar rats weighing between 140 and 160 gms were used. Cobalt gelatin sticks, cobalt wire or glass gelatin sticks were implanted into the right cortex by the method described in section 9.2. The rats were killed at various times thereafter and their brains were removed and cut longitudinally along the midline into two halves. Each half was then assayed for its dopamine and noradrenaline content by the method described in chapter 1 or for its 5-hydroxytryptamine and 5-hydroxyindoleacetic acid by the methods described in chapter 5 or for its GABA content by the method described in section 8.2.

Results are given in tables 9.1 to 9.7.

Cobalt sticks and wire produced a significant decrease in the levels of both noradrenaline and γ-aminobutyric acid in the implanted half of the brain one week after the implantation. At the same time, cobalt sticks produced a significant decrease in the levels of noradrenaline in the contralateral half of the brain as well. Dopamine and 5-hydroxytryptamine content were not changed by either cobalt sticks or wire but 5-hydroxyindoleacetic acid was significantly increased by both cobalt wire and sticks in the implanted half of the brain. Cobalt wire produced a significant increase in the levels of the indole in the contralateral half as well.

Two weeks after the implantation, the effect on GABA levels disappeared. On the other hand, the levels of noradrenaline in rats implanted with cobalt sticks were still depressed. At this time, too, the levels of 5-hydroxyindoleacetic acid were significantly increased in both halves of brains that had been implanted with cobalt stick, while cobalt wire produced a significant increase in the levels of this indole in the implanted side only.

Table 9.1

Effect of one week implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks on the levels of dopamine and noradrenaline in rat brain

Treatment	Right half	Left half	Right half	Left half
	Dopamine ng/gm		Noradrenaline ng/gm	
Cobalt gelatin sticks	893 ± 29.3(4)	912 ± 17.7(4)	348 ± 6.2**(4)	366 ± 13.7**(4)
Cobalt wire	922 ± 16.4(4)	921 ± 23.1(4)	352 ± 3.5**(4)	432 ± 16.3 (4)
Glass gelatin sticks	933 ± 19.0(4)	897 ± 32.1(4)	467 ± 12.0 (4)	482 ± 22.0 (4)
Sham operated	929 ± 17.8(4)	899 ± 19.3(4)	493 ± 12.3 (4)	453 ± 27.0 (4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (** $P < 0.02$; ** $P < 0.005$) from the values obtained from the sham operated group.

Table 9.2

Effect of two weeks implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks on the levels of dopamine and noradrenaline in rat brain

Treatment	Right half	Left half	Right half	Left half
	Dopamine ng/gm		Noradrenaline ng/gm	
Cobalt gelatin sticks	913 ± 68(4)	909 ± 41(4)	401 ± 14.9**(4)	493 ± 4.5(4)
Cobalt wire	933 ± 24(4)	890 ± 57(4)	482 ± 24.0 (4)	514 ± 4.3(4)
Glass gelatin sticks	925 ± 32(4)	899 ± 26(4)	473 ± 27.0 (4)	479 ± 11.6(4)
Sham operated	933 ± 28(4)	885 ± 19(4)	486 ± 12.0(4)	491 ± 8.4(4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (** $P < 0.001$) from the values obtained from the sham operated group.

Table 9.3

Effect of three weeks implantation of cobalt gelatin sticks. cobalt wire or glass gelatin sticks on the levels of dopamine and noradrenaline in rat brain

Treatment	Right half	Left half	Right half	Left half
	Dopamine ng/gm		Noradrenaline ng/gm	
Cobalt gelatin sticks	949 ± 4(4)	937 ± 3(4)	398 ± 10(4)	478 ± 12(4)
Cobalt wire	898 ± 28(4)	963 ± 17(4)	483 ± 20(4)	488 ± 18(4)
Glass gelatin sticks	936 ± 56(4)	946 ± 35(4)	485 ± 5(4)	473 ± 19(4)
Sham operated	961 ± 18(4)	897 ± 34(4)	458 ± 37(4)	474 ± 17(4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Table 9.4

Effect of one week implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks on the levels of 5-hydroxytryptamine and 5-hydroxy-indoleacetic acid in rat brain

Treatment	Right half	Left half	Right half	Left half
	5-Hydroxytryptamine ng/gm		5-Hydroxyindoleacetic acid ng/gm	
Cobalt gelatin sticks	512 ± 38.4(4)	511 ± 19.7(4)	372 ± 13**(4)	318 ± 13 (4)
Cobalt wire	548 ± 27.7(4)	498 ± 9.1(4)	371 ± 13**(4)	391 ± 5**(4)
Glass gelatin sticks	503 ± 20.8(4)	500 ± 19.3(4)	341 ± 10 (4)	333 ± 15 (4)
Sham operated	499 ± 17.3(4)	492 ± 13.8(4)	323 ± 11 (4)	321 ± 12 (4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (** $P < 0.02$; *** $P < 0.001$) from the values obtained from the sham operated group.

Table 9.5

Effect of two weeks implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain

Treatment	Right half	Left half	Right half	Left half
	5-Hydroxytryptamine ng/gm		5-Hydroxyindoleacetic acid ng/gm	
Cobalt gelatin sticks	549 ± 21.6(4)	500 ± 20.5(4)	365 ± 17.1**(4)	361 ± 14.9**(4)
Cobalt wire	516 ± 4.8(4)	519 ± 17.0(4)	346 ± 13.6* (4)	339 ± 12.4(4)
Glass gelatin sticks	521 ± 6.38(4)	528 ± 10.9(4)	330 ± 7.0 (4)	330 ± 4.2(4)
Sham operated	531 ± 18.5(4)	503 ± 14.1(4)	313 ± 3.5(4)	311 ± 8.1(4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.02$) from the values obtained from the sham operated group.

Table 9.6

Effect of three weeks implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks on the levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat brain

Treatment	Right half	Left half	Right half	Left half
	5-Hydroxytryptamine ng/gm		5-Hydroxyindoleacetic acid ng/gm	
Cobalt gelatin sticks	533 ± 18.1(4)	521 ± 16.0(4)	316 ± 15.1(4)	303 ± 11.7(4)
Cobalt wire	503 ± 19.0(4)	499 ± 11.9(4)	317 ± 18.0(4)	293 ± 11.0(4)
Glass gelatin sticks	511 ± 7.8 (4)	512 ± 18.1(4)	313 ± 18.2(4)	315 ± 15.9(4)
Sham operated	513 ± 16.2(4)	506 ± 14.1(4)	319 ± 18.7(4)	311 ± 5.6(4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Table 9.7

Effect of one week, two weeks and three weeks implantation of cobalt gelatin sticks, cobalt wire or glass gelatin sticks on the levels of GABA in rat brain

Treatment	Right half	Left half
	γ-Aminobutyric acid μg/gm	
ONE WEEK		
Cobalt gelatin sticks	128.8 ± 3.08**(4)	139 ± 6 (4)
Cobalt wire	139.3 ± 2.79* (4)	159 ± 8.1 (4)
Glass gelatin sticks	152.6 ± 6.3 (4)	156.6 ± 7.7 (4)
Sham operated	156.9 ± 7.3 (4)	155.2 ± 6.06(4)
TWO WEEKS		
Cobalt gelatin sticks	149.1 ± 2.6 (4)	161.6 ± 3.7 (4)
Cobalt wire	162.8 ± 8.1 (4)	165.6 ± 11 (4)
Glass gelatin sticks	165.8 ± 6.3 (4)	163.9 ± 2.4 (4)
Sham operated	161.9 ± 7.08(4)	167.78 ± 11.4(4)
THREE WEEKS		
Cobalt gelatin sticks	155.16 ± 4.2(4)	166.0 ± 2.9 (4)
Cobalt wire	164.2 ± 6.6 (4)	166.2 ± 13.3(4)
Glass gelatin stick	165.0 ± 16.3(4)	159.7 ± 12.8(4)
Sham operated	168.3 ± 5.7(4)	164.1 ± 2.5(4)

Each value is the mean ± standard error from the number of animals shown in parentheses.

Significantly different by the t test (* $P < 0.05$; ** $P < 0.02$) from the values obtained from the sham operated group.

Neither cobalt sticks nor cobalt wire had any effect on the levels of any of the transmitters and of 5-hydroxyindoleacetic acid three weeks after implantation.

Glass gelatin sticks did not produce any change at any of the times studied.

9.6 Review of the results presented in chapter 9

The production of model epilepsies in animals which resemble those found in human subjects would be a very useful tool for penetration into the unknown mysterious world of epilepsy.

Cobalt implantations into rat brain produced some central changes which resembled those occurring in grand mal epilepsy in the human being and at the same time it did not produce any major neurological defects in the rat which might have complicated the results obtained by this model. Rats implanted with either cobalt sticks or cobalt wire looked healthy at all times and although at the peak of the response they showed very strong jerks and twitches, these did not affect in any way their normal pattern of behaviour and the rats still ate, drank and gained weight as well, and looked as healthy as those with glass implantation or simply a hole only in their skull. Cobalt sticks proved to be a better agent than cobalt wire, since the jerks they produced were stronger and more frequent. The changes produced by cobalt sticks in the motor activity were noticeable at the peak of their effect and they produced the most profound changes in the brain activity.

Neurochemically, only a few changes could be seen: GABA content was decreased by both cobalt sticks and wire, but this occurred only in the implanted half of the brain and only for one week after implantation.

Noradrenaline was decreased in both brain halves by cobalt sticks and only in the implanted half by cobalt wire, one week after implantation. However, only cobalt sticks produced changes in noradrenaline two weeks after the implantation (when the maximum epileptogenic effect was observed) and this only in the implanted half of the brain. This might indicate an increased utilization of the amine in response to the increased activity of the brain.

A surprising result was obtained with 5-hydroxyindoleacetic acid, which was increased in the implanted half by both cobalt sticks and wire and this persisted for two weeks after implantation. The wire increased the indole in the contralateral brain half one week after implantation and the stick did so for two weeks after implantation.

5-Hydroxytryptamine itself did not change at any time during the experiment and the increased concentration of metabolite might indicate an increase in the rate of synthesis and at the same time a correspondingly increased utilization of 5-hydroxytryptamine.

CHAPTER 10

LEVELS OF ANTICONVULSANT DRUGS AFTER ACUTE AND CHRONIC ADMINISTRATION

Adequate drug therapy of epilepsy in human beings requires that a constant serum concentration of the antiepileptic drug be maintained, high enough to prevent seizures but not so high as to give toxic effects. This can only be achieved by continuous determination of the drug serum concentration. The importance of ensuring a steady concentration of the drug has only recently been fully appreciated.

In our field of research, the idea of developing a technique for the measurement of serum levels of anticonvulsant drugs in animals came from our wish to see what serum levels were being developed in response to our treatment regimen and whether the times at which we measured the effect of the anticonvulsant drugs on the various neurotransmitter agents coincided with the drug's peak serum and brain levels.

Several methods of analysis are available for the estimation of anticonvulsant drugs. Fluorometric assays for phenytoin were described by Dill and Glazko (1972) and by Dill, Leuny, Kinkel and Glazko (1976). A colorimetric method of assay for phenobarbitone was described by Bush (1961) and for carbamazepine by Morselli, Gerna and Garattini (1971). In addition a considerable number of gas chromatographic analyses for anticonvulsant drugs, using either flame ionization or nitrogen detectors, have been developed.

Gas liquid chromatography is considered to be one of the best available methods for the determination of anticonvulsant drugs in biological fluids. The method is more sensitive, gives much more accurate

results and requires smaller sample volumes than does any other. The most important advantage is that several anticonvulsant drugs can be assayed simultaneously in the same specimen.

10.1 Gas chromatographic analysis of anticonvulsant drugs

Of the several methods we tried for the gas chromatographic analysis, we found that the method described by Dorrity and Linnoila (1976) gave the best results in terms of sensitivity and recovery, without any need for solvent evaporation.

10.1(i) Method of extraction

Half a millilitre of serum was placed in a 15 ml round bottomed glass stoppered centrifuge tube, to which 1 ml of 3M sodium dihydrogen phosphate, dissolved in methanol water mixture (1/9 V/V) was added. Ten millilitres of a mixture of ether-toluene (3/7 V/V) containing 25 µg of 5(4-methyl phenyl)-5 phenyl hydantoin as internal standard were added and the mixture was then shaken in a rest hand shaker for ten minutes and centrifuged at 2000 rpm for another ten minutes. Nine millilitres of the upper organic layer were transferred to another glass stoppered conical centrifuge tube containing 75 µl of 0.2 M tetramethylammonium hydroxide in methanol. The tube was then shaken and centrifuged as before.

After centrifugation, the upper ether-toluene layer was aspirated off by suction and the last traces of the solvent were carefully aspirated from the interface. The remainder of the tetramethylammonium hydroxide (in the conical bottom of the tube by then it developed a slight brownish colour) was transferred to a microsample vial. The tube was then washed with 50 µl methanol and the washing was incorporated with the tetramethylammonium hydroxide in the microsample vial.

One microlitre of the mixture was then injected into the chromatograph.

10.1(ii) Conditions for gas chromatography

A Pye 104 with dual flame-ionization detector was used. The chromatograph was connected to an Infotronics CRS 309 automatic integrator set at low noise and to measure peak areas resolved within 8 seconds.

The columns used were made of 'Chromatoglass' and were 180 cm long and 2 mm internal diameter. They were packed with 3% OV 17 on 'Chromosorb' WHP of mesh size 80-100.

The columns were conditioned before the first use for forty-eight hours at 280°C with nitrogen flow of 40 ml per minute. The columns were conditioned every time the packing material was partly or completely changed.

We found during the course of the experiments that an overnight conditioning of the columns was necessary before use. This conditioning gave a steady low base line and better resolution. Bleeding of the columns occurred only rarely under these conditions. The temperature dial of the injection port was set at maximum. which at oven temperature of 250°C was 280°C. Calibration of the injection temperature is given in table 10.1.

Phenobarbitone and carbamazepine was chromatographed by using a temperature programming. The column temperature was held at 160°C for one minute after injection and programmed at 16°C per minute up to 280°C (7.5 minutes) and the temperature was held at 280°C for another five minutes.

Phenytoin was chromatographed at constant temperature (280°C). Gas flow rates were nitrogen 40 ml per minute, air and hydrogen were 10 pounds per square inch.

Table 10.1

Calibration of injection temperature of the Pye Unicam 104 chromatograph

(A) Constant temperature

Scale	Oven temperature (°C)	Injection temperature (°C)
0	150	138
5	150	168
Maximum	150	210
Maximum	250	286

(B) Programmed temperature (from 150°C to 260°C at maximum dial)

Oven temperature (°C)	Time after the start of the programme (minutes)	Injection temperature (°C)
150	0	208
250	6*	232
250	10	250
250	16	280

* End of programme

10.1(iii) Calibrations and recoveries of anticonvulsant drugs

(A) Phenobarbitone

The working standards used for the construction of a calibration curve for phenobarbitone were 10, 20, 30 and 40 mg/litre.

To 0.5 ml of drug-free serum, 100 µl of different concentrations of phenobarbitone standard and 0.9 ml of 3.3 M sodium dihydrogen phosphate were added and extraction was done as described earlier. Each concentration was carried out in duplicate.

We found that the use of temperature programming gave better

resolution of the peaks and low base line than using constant temperature. Examples of the calibration curve are given in figure 10.1. Chromatograms obtained after the column injection of 1 µl reagents blank extract (A) and reagents blank plus internal standard extract (B) are shown in figure 10.2.

Chromatograms obtained after the column injection of 1 µl of different extract of different concentrations of phenobarbitone plus the internal standard are given in figure 10.3.

The retention time for phenobarbitone was six and a half minutes and of the internal standard eleven minutes.

The reliability of the method was tested by studying the recovery of phenobarbitone.

The recovery was carried out by adding different concentrations of phenobarbitone solution in methanol to a half millilitre drug-free serum to which 0.9 ml of 3.3 M sodium dihydrogenphosphate was added. The tubes were then treated in the manner described before. Results are given in table 10.2.

(B) Phenytoin

The working standards and the construction of the calibration curve for phenytoin was carried out in the same manner as that described for phenobarbitone.

Examples of the calibration curve are given in figure 10.4.

Chromatograms obtained after the injection of 1 µl reagent blank (A), reagent blank plus internal standard (B), 2.5 mg/litre phenytoin (C and E) and 5 mg/litre phenytoin (D and F) are given in figure 10.5.

Phenytoin added to blood serum was recovered in the range of 90%. Results are given in table 10.3

Table 10.2

Recoveries of phenobarbitone added to rat serum

Amount added mg/litre	Amount recovered mg/litre	Recovery %
10	8.8	88
10	8.2	82
20	17.8	89
20	18.6	93
30	29	96.6
30	28.5	95

Mean recovery: 90.6%

Table 10.3

Recoveries of phenytoin added to the serum of rat blood

Amount added mg/litre	Amount recovered mg/litre	Recovery %
5	4.2	84
5	4.6	92
10	8.7	87
10	9.0	90
20	19	95
20	18.7	93.5

Mean recovery: 90%

FIGURE 10.1

Typical calibration curves for the gas chromatographic analysis of phenobarbitone obtained on three separate occasions

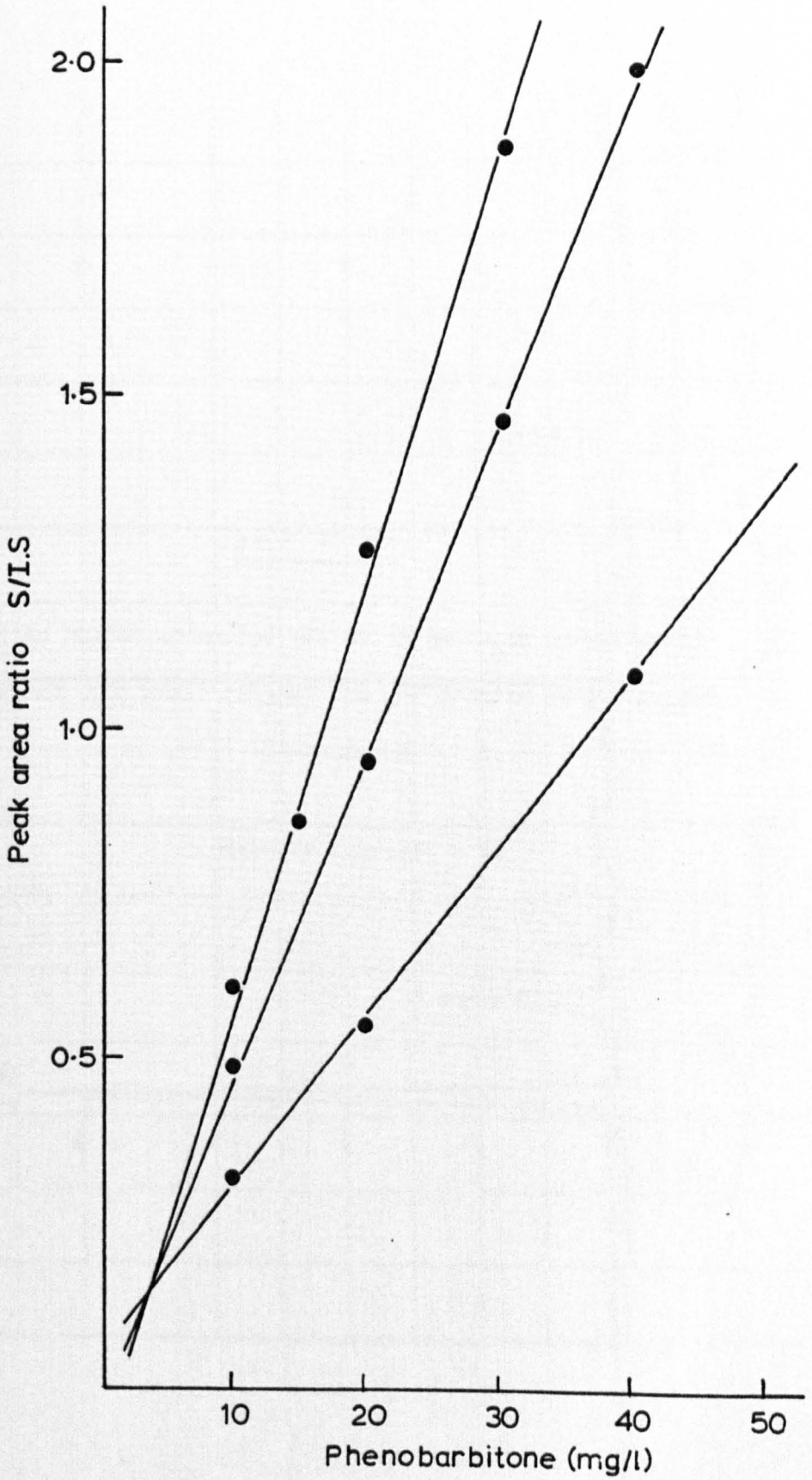

2·0
1·5
1·0
0·5
Peak area ratio S/I.S
10
20
30
40
50
Phenobarbitone (mg/l)

FIGURE 10.2

Chromatograms obtained after the column injection of 1µl of reagent blank extract (A) and reagent blank plus internal standard (B)

IS = internal standard

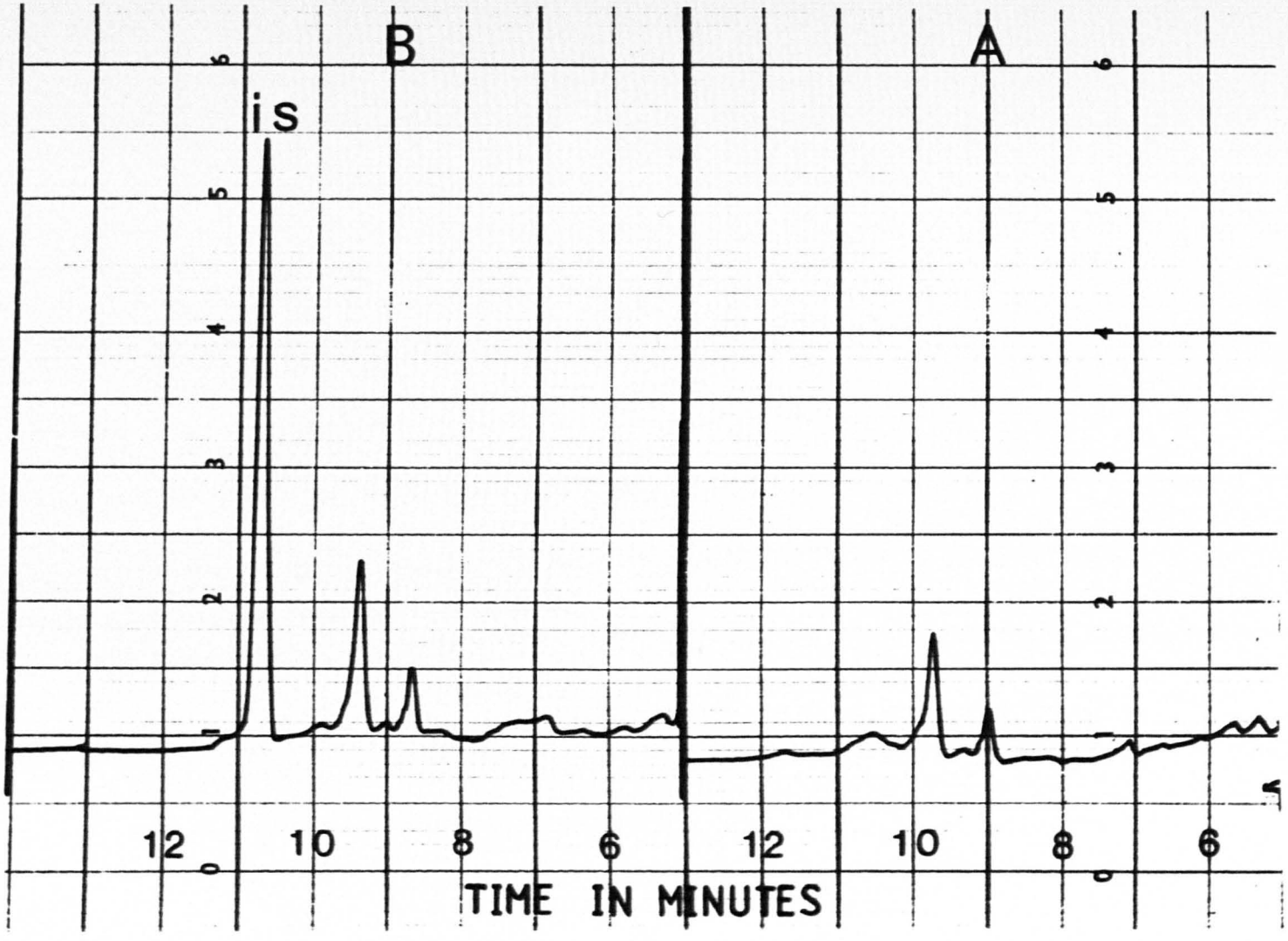

B
A
is
12
10
8
6
12
10
8
6
TIME IN MINUTES

FIGURE 10.3

Chromatograms obtained after the column injection of 1 µl of different concentrations of phenobarbitone extracts

(A) 10 mg/litre phenobarbitone (B) and (C) 20 mg/litre phenobarbitone

Ph = phenobarbitone IS = internal standard

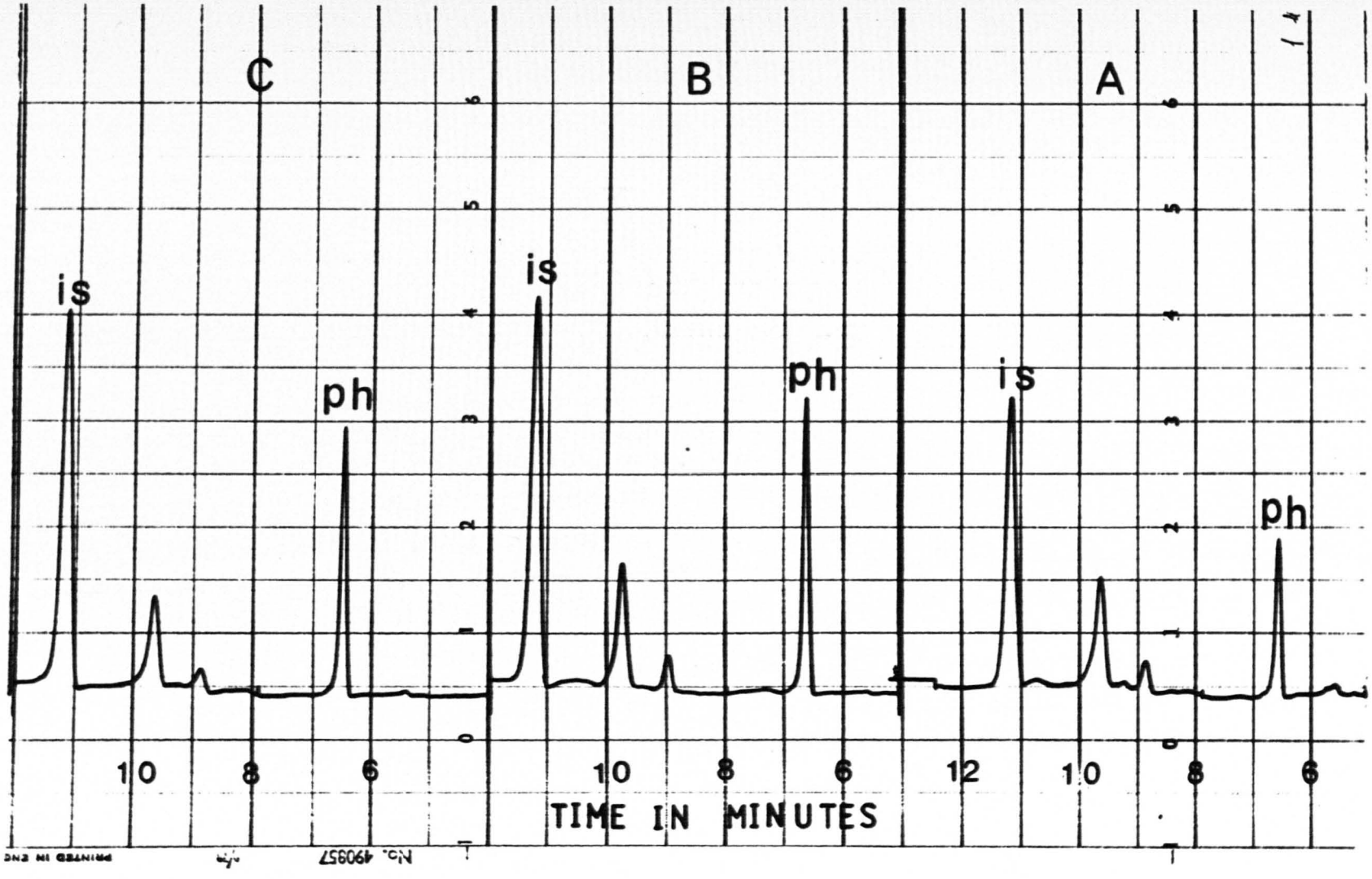

C
B
A
is
ph
is
ph
is
ph
10
8
6
10
8
6
12
10
8
6
TIME IN MINUTES

FIGURE 10.4

Typical calibration curves for the gas-chromatographic analysis of phenytoin, obtained on two separate occasions

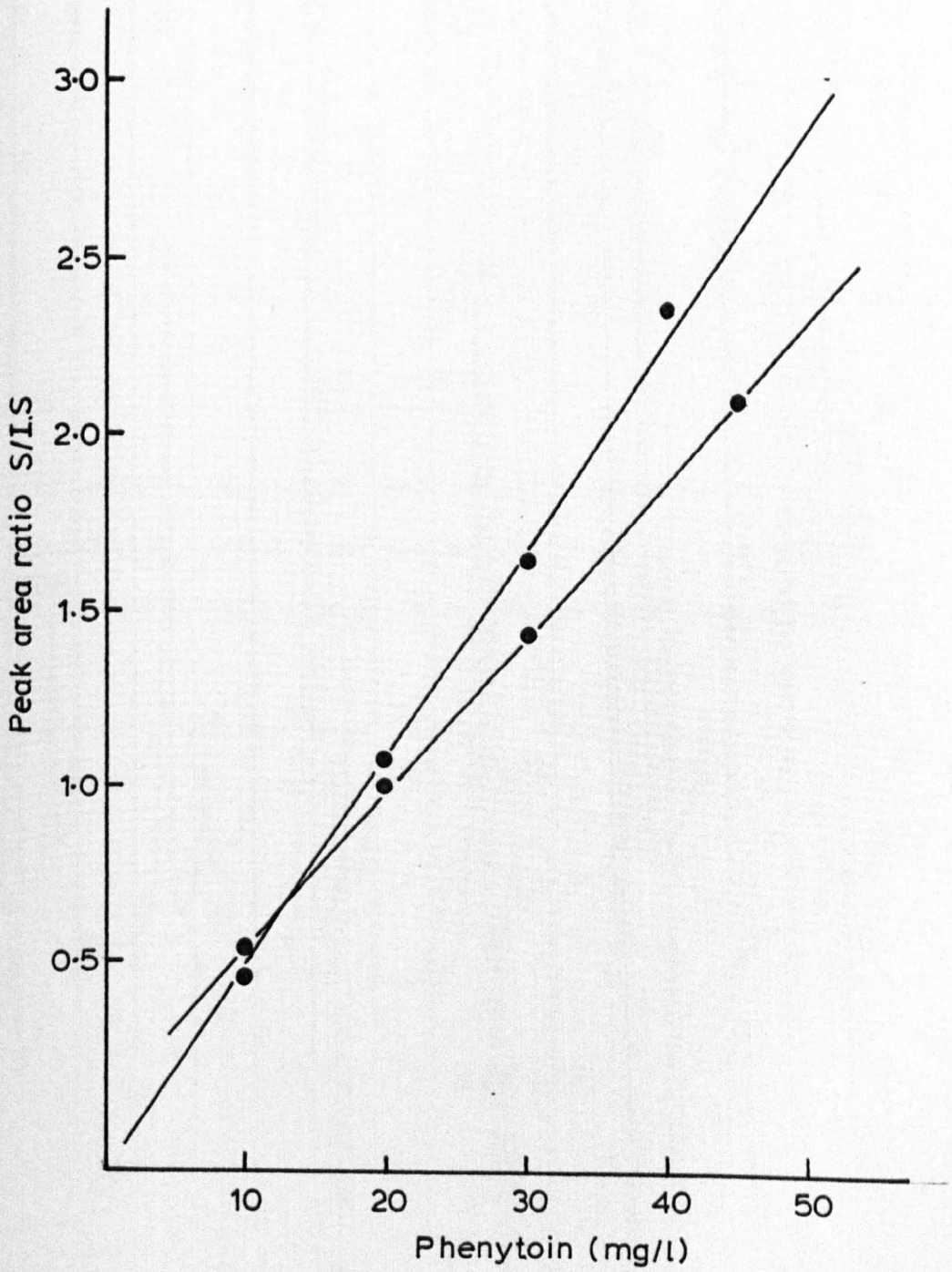
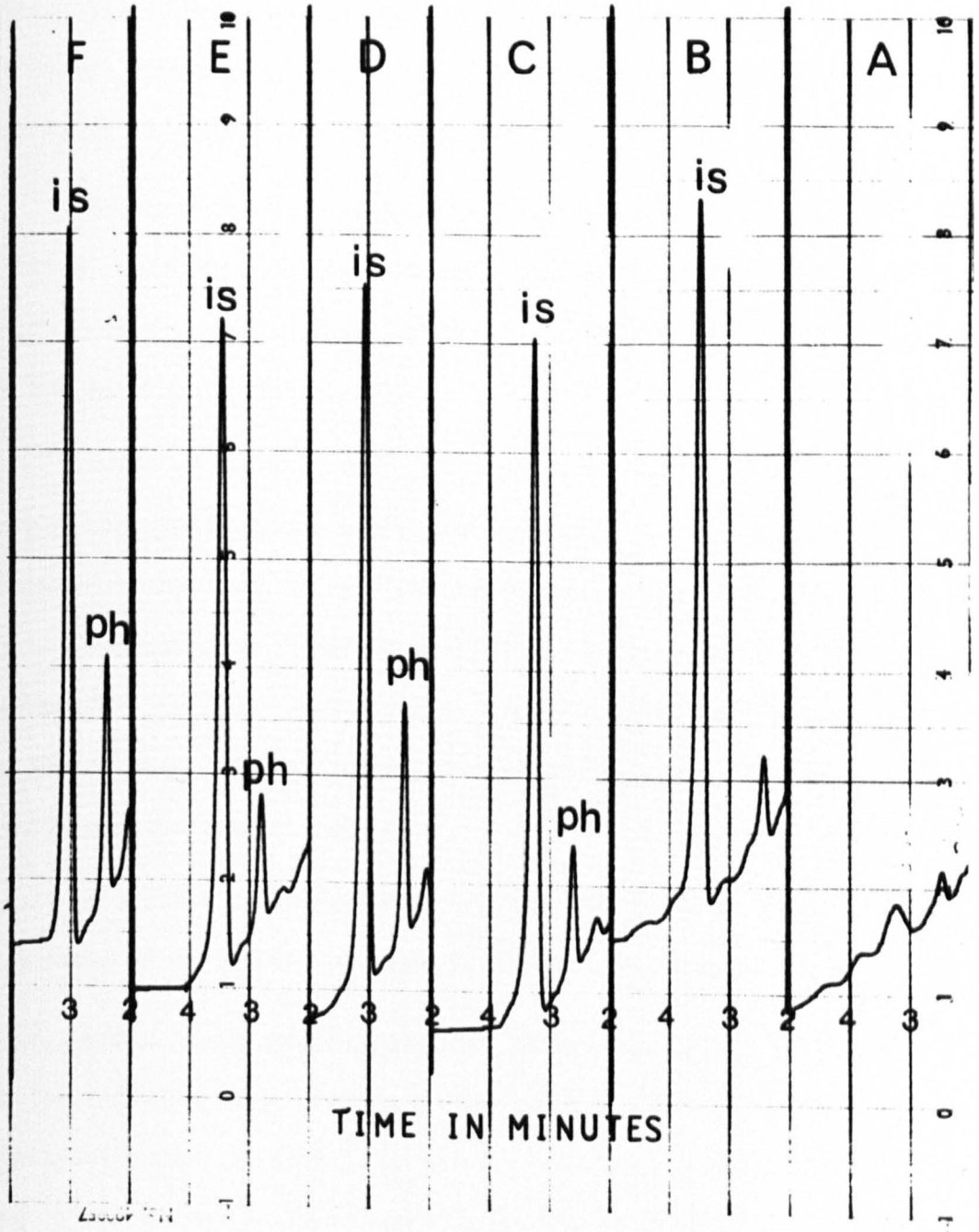

3·0
2·5
2·0
1·5
1·0
0·5
Peak area ratio S/I.S
10
20
30
40
50
Phenytoin (mg/l)

FIGURE 10.5

Chromatograms obtained after the column injection of reagent blank extract (A), reagent blank extract plus internal standard (B), 2.5 mg/l phenytoin (C and E), 5 mg/l phenytoin (D and F).


F
E
D
C
B
A
is
ph
TIME IN MINUTES

(C) Carbamazepine

When carbamazepine was chromatographed under the same conditions as those described for phenobarbitone, we found that it yielded two adjacent peaks which were not reagent blanks. The first peak was resolved half a minute before the second one and the height of each peak was not the same after different injections, although we found that the sum of the heights of the two peaks was always the same (Figure 10.6).

The only possible explanation that could be given for this phenomenon at that time was that carbamazepine underwent an erratic decomposition which yielded two different compounds, the sum of which corresponds to that of the original carbamazepine.

Later we found a report that confirmed our conclusion. It transpires that carbamazepine decomposes partially to iminostilbene at the elevated temperature of the gas chromatography and that this decomposition is not reproducible (Kupferberg, 1972). Kupferberg developed a different method for the gas chromatographic analysis of carbamazepine which depends on the formation of the trimethylsilyl derivative of the drug, using cyheptamide as internal standard.

A calibration curve obtained from the ratio of the heights of the two peaks of carbamazepine against internal standard is given in figure 10.7.

10.2 Levels of phenobarbitone after acute and chronic treatment

In the following experiments, we are reporting results obtained by using different routes of administration in order to see what levels we are achieving by our method of injection and to compare it with the levels obtained after using a different route of administration.

FIGURE 10.6

Chromatograms obtained after the column injection of 1 µl of 7.5 mg/litre carbamazepine (A) and (B) and 10 mg/litre carbamazepine (C) and (D)

car. = carbamazepine IS = internal standard

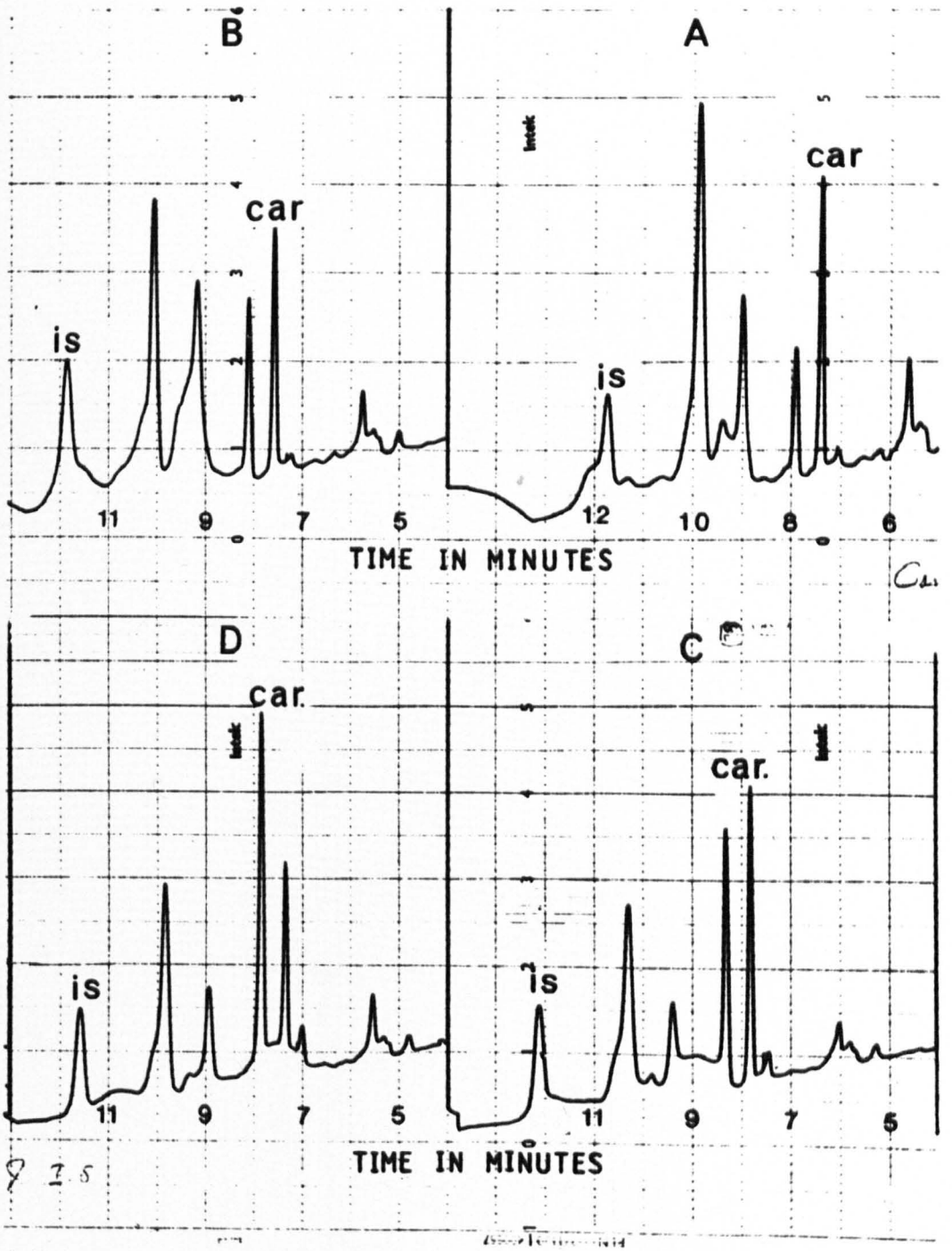
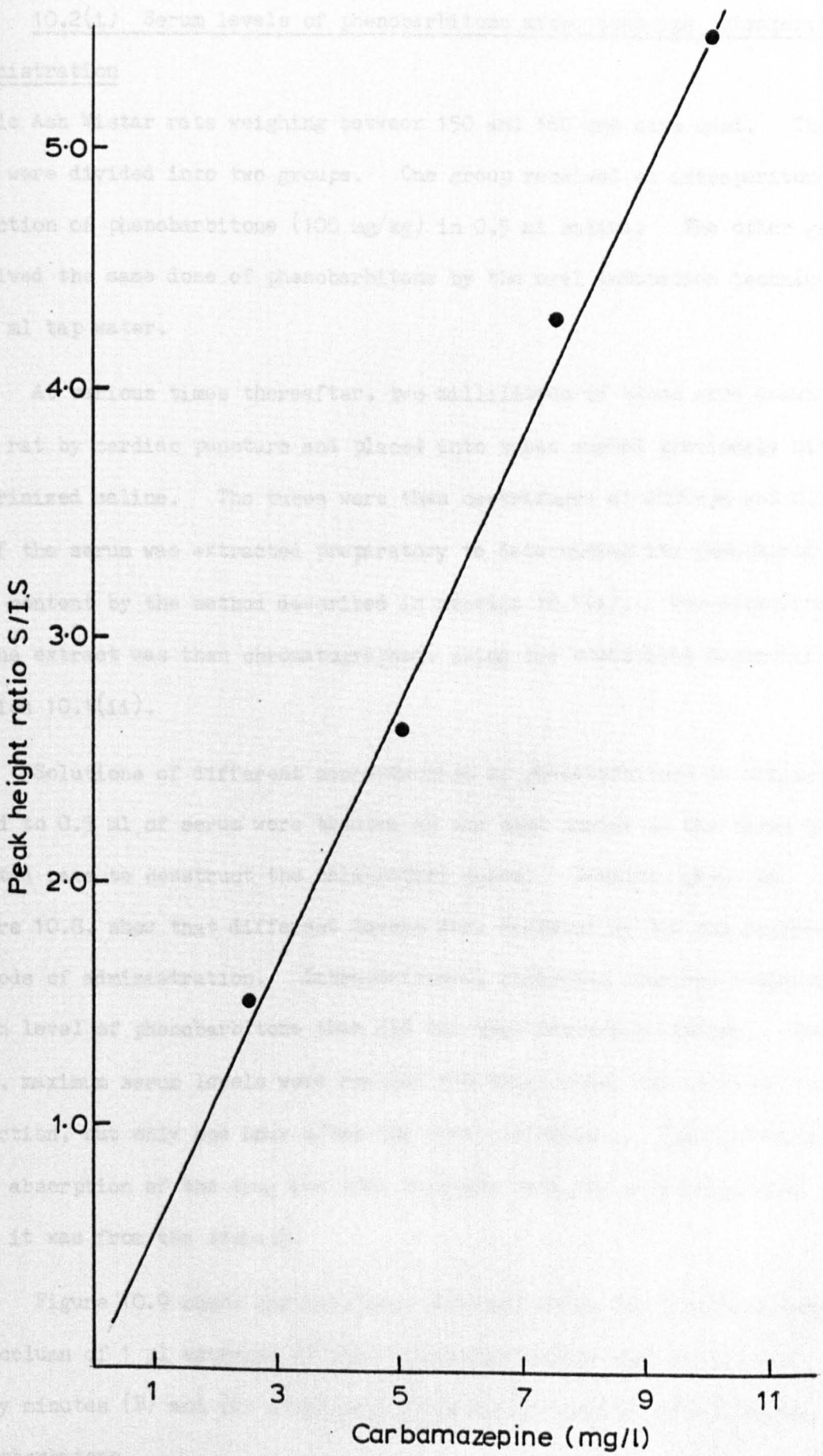

B
A
car
car
is
is
11
9
7
5
12
10
8
6
TIME IN MINUTES
D
C
car.
car.
is
is
11
9
7
5
11
9
7
5
TIME IN MINUTES

FIGURE 10.7

A calibration curve for the gas chromatographic analysis of carbamazepine


Peak height ratio S/IS
5·0
4·0
3·0
2·0
1·0
1
3
5
7
9
11
Carbamazepine (mg/l)

10.2(i) Serum levels of phenobarbitone after oral and intraperitoneal administration

Female Ash Wistar rats weighing between 150 and 160 gms were used. The rats were divided into two groups. One group received an intraperitoneal injection of phenobarbitone (100 mg/kg) in 0.5 ml saline. The other group received the same dose of phenobarbitone by the oral intubation technique in 1 ml tap water.

At various times thereafter, two millilitres of blood were drawn from each rat by cardiac puncture and placed into tubes washed previously with heparinized saline. The tubes were then centrifuged at 2000rpm and 0.5 ml of the serum was extracted preparatory to determining its phenobarbitone content by the method described in section 10.1(i). One microlitre of the extract was then chromatographed, using the conditions described in section 10.1(ii).

Solutions of different concentration of phenobarbitone in methanol added to 0.5 ml of serum were treated in the same manner as the serum of treated rats to construct the calibration curve. Results, given in figure 10.8, show that different levels were achieved by the two different methods of administration. Intraperitoneal injection produced a higher serum level of phenobarbitone than did the oral intubation method. Moreover, maximum serum levels were reached two hours after the intraperitoneal injection, but only one hour after the oral intubation. This indicates that absorption of the drug was more complete from the peritoneal site than it was from the stomach.

Figure 10.9 shows chromatograms obtained after the injection into the column of 1 µl extracts of serum collected thirty minutes (A) and sixty minutes (B) and (C) after intraperitoneal injection of 100 mg/kg phenobarbitone.

FIGURE 10.8

Serum levels of phenobarbitone obtained after the administration of 100 mg/kg of the drug by intraperitoneal injection (A) and oral intubation (B)

Each point indicates the mean $\pm$ standard error for 4 rats

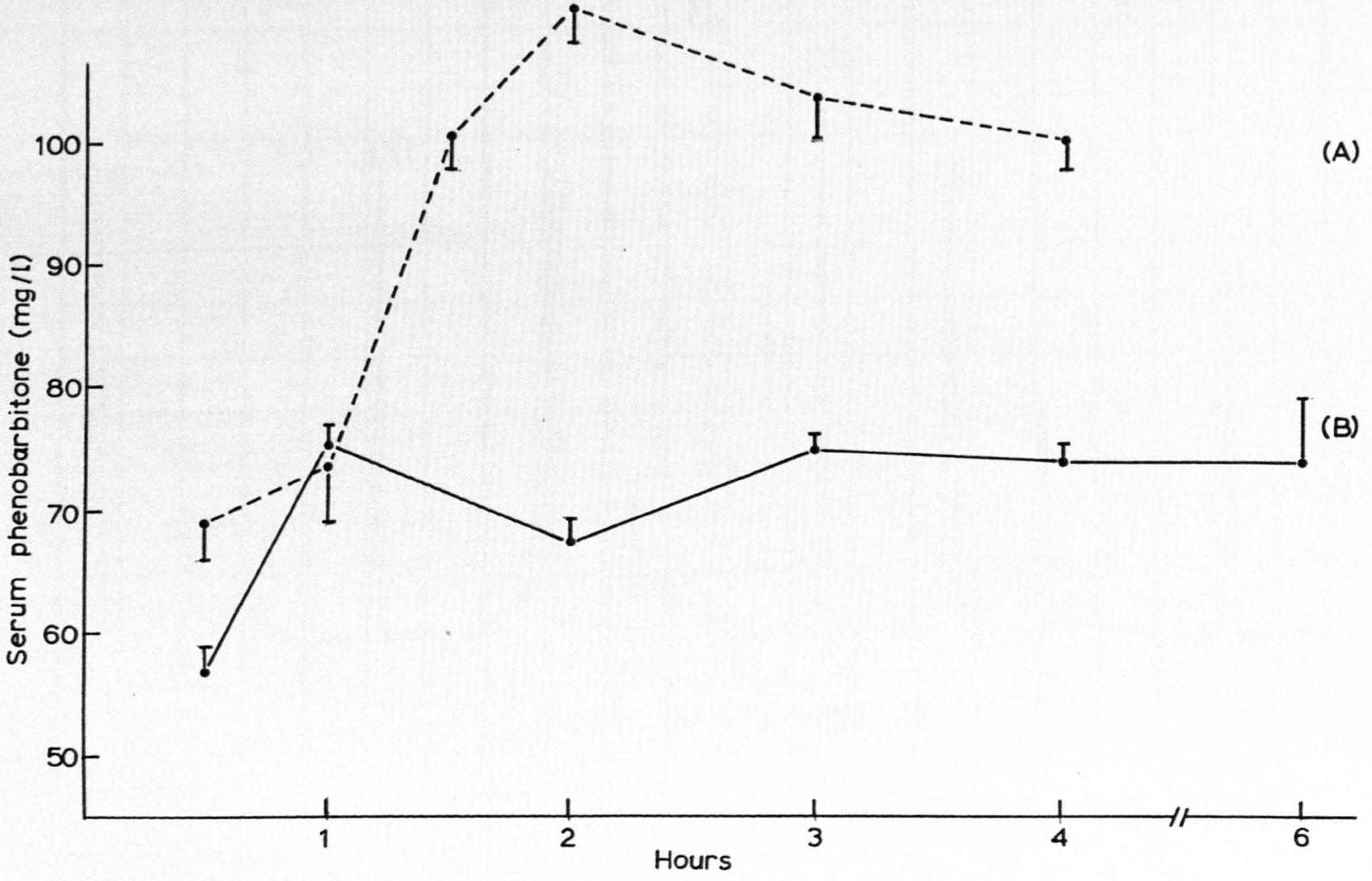
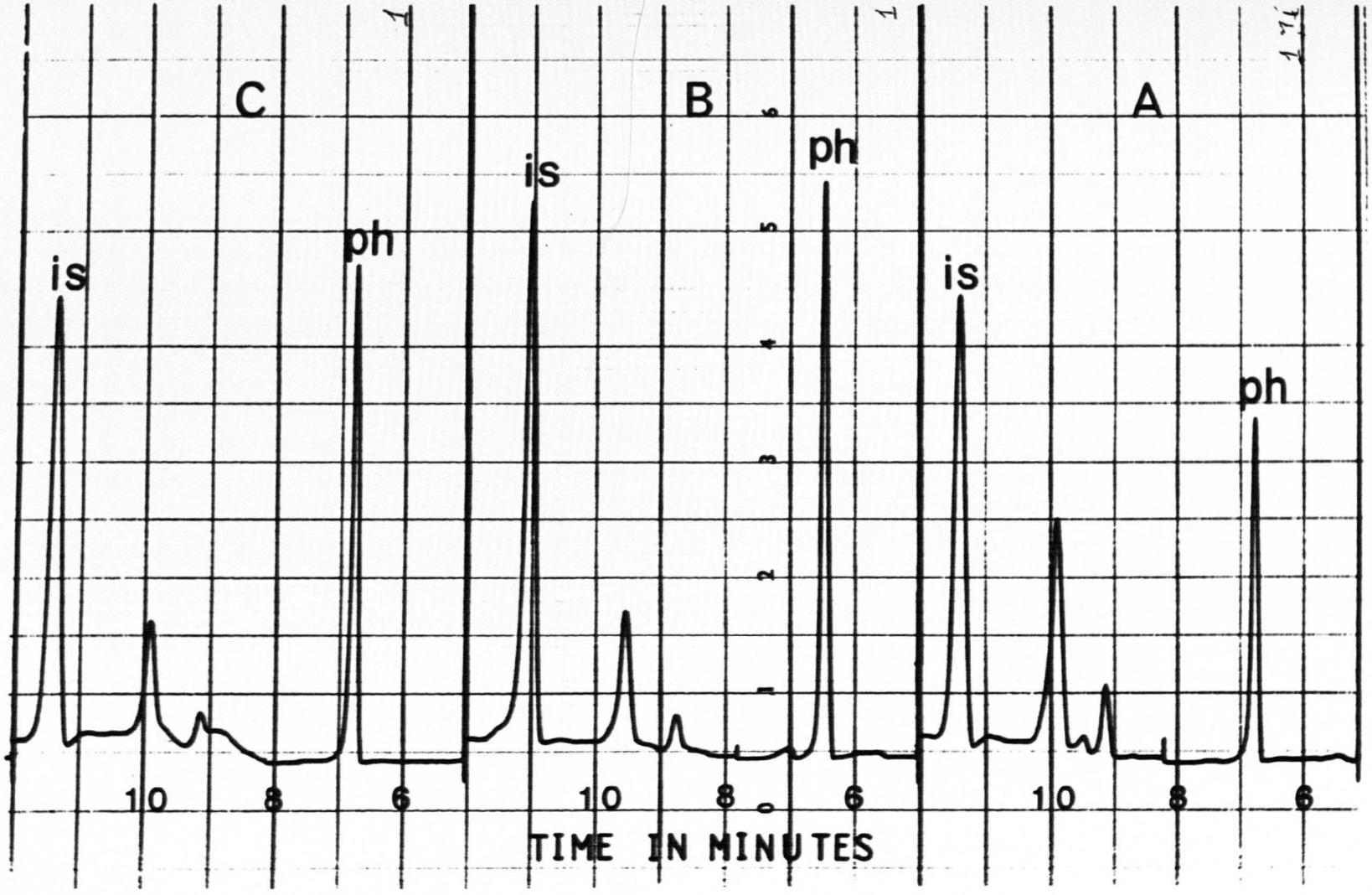

Serum phenobarbitone (mg/l)
100
90
80
70
60
50
1
2
3
4
6
Hours
(A)
(B)

FIGURE 10.9

Chromatograms obtained after the column injection of 1 µl extracts of serum collected from rats thirty minutes (A) and sixty minutes (B) and (C) after intraperitoneal injection of 100 mg/kg phenobarbitone


C
B
A
is
ph
is
ph
is
ph
10
8
6
10
8
6
10
8
6
TIME IN MINUTES

10.2(ii) The effect of α-methyl-p-tyrosine on serum and brain levels of phenobarbitone

In chapter 2, section 4 of this thesis we saw that α-methyl-p-tyrosine reduced significantly the induction time and prolonged the sleeping time caused by phenobarbitone. We saw as well that the combined treatment with the two drugs produced a progressive and continuous fall in body temperature which neither of the two drugs produced when given on its own. Two possibilities arose from these results. Either α-methyl-p-tyrosine potentiates the effect of phenobarbitone at a central level (synergism) or alternatively α-methyl-p-tyrosine competes with phenobarbitone or inhibits the microsomal enzymes in the liver on which the metabolism of phenobarbitone depends.

These possibilities were tested by measuring serum and brain levels of phenobarbitone after α-methyl-p-tyrosine.

Female Ash Wistar rats weighing between 150 and 160 grams were used. The rats were divided into two major groups. One group was divided into several subgroups and each received an intraperitoneal injection of phenobarbitone (100 mg/kg). The other group was also divided into several subgroups and each received the combined treatment by α-methyl-p-tyrosine (250 mg/kg) and phenobarbitone (100 mg/kg).

Each subgroup in the two major groups was killed at corresponding and predetermined times thereafter by decapitation. Blood was collected directly from the decapitated body into tubes previously washed with heparinized saline. The brain was removed and homogenized with 5 ml distilled water. The blood and brain homogenate were then centrifuged at 2000 rpm for ten minutes and 0.5 ml of the serum and 1 ml of the supernatant solution from the brain homogenate had their phenobarbitone extracted by the method described in section 10.1(i). One microlitre of the extract was then

injected into the gas chromatography column under the conditions described in section 10.1(ii).

Results are presented in figures 10.10 and 10.11.

The results show that α-methyl-p-tyrosine retarded the elimination of phenobarbitone from both serum and brain.

Table 10.4 shows the percentage changes in the levels and elimination rates of phenobarbitone. Although the table shows that phenobarbitone concentration in the brain dropped at a faster rate than that in the serum, the elimination rate per hour, calculated from the amount of phenobarbitone left after certain times, was slower in the brain than it was for the serum. For the purposes of these calculations, zero time was taken as three hours. In both serum and brain the elimination rates decreased with time. α-Methyl-p-tyrosine, on the other hand, seems to reverse the pattern completely. In the presence of this drug, the phenobarbitone concentration in the brain fell off much more slowly than it did in the serum. While elimination rates were very low in the brain, they seemed to increase with time. The opposite state of affairs obtained in the serum.

Although we could not find any statistical correlation among these numbers, the results indicate that α-methyl-p-tyrosine changed the way by which phenobarbitone was metabolized in the brain or serum. Although it is only speculative, it is tempting to wonder whether the increased elimination rate of phenobarbitone by the brain might be an indication that the brain was reacting in an adaptive way to the persisting high concentration of the drug. However, our main concern was to find whether α-methyl-p-tyrosine potentiated the effect of phenobarbitone through a synergistic effect or through effecting a delay of its metabolism. Our results indicate

clearly that it does decrease the metabolism of the drug, probably by inhibition of the microsomal enzyme. On the other hand, some synergistic effect could not be ruled out completely. However, α-methyl-p-tyrosine has been reported to be toxic for the kidney (Moore, Wright and Bert, 1967) and when we remember that 27% of phenobarbitone is excreted unchanged in the urine (Glasson and Benakis, 1973), along with its other metabolites, it is tempting to suggest that a decreased elimination of phenobarbitone by the kidneys may be in part a supplementary factor in elevating the levels of the drug we saw in serum and brain after α-methyl-p-tyrosine.

Correlation of brain and serum levels of phenobarbitone at different times after injection are given in figure 10.12; these show a high correlation ($r = 0.927$) between brain and serum levels.

Table 10.4

Percentage change and elimination rates of phenobarbitone with or without α-methyl-p-tyrosine treatment

Hours after administration (3 hrs as zero time)		Phenobarbitone change %	Elimination rate mg/hr	Phenobarbitone + αmpt. %	Elimination rate mg/hr
SIX HOURS	Serum	- 12.9	3.98	- 7.63	2.32
	Brain	- 36.10	3.16	- 1.09	0.09
TWELVE HOURS	Serum	- 30.30	3.11	- 11.78	1.19
	Brain	- 42.29	1.23	- 5.87	0.16
TWENTY-FOUR HOURS	Serum	- 52.89	2.32	- 25.85	1.12
	Brain	- 69.20	0.865	- 18.8	0.22

FIGURE 10.10

Serum levels of phenobarbitone, for rats treated with phenobarbitone only (100 mg/kg), continuous line; and rats treated with phenobarbitone plus α-methyl-p-tyrosine (250 mg/kg), dotted line

Each point indicates the mean ± standard error for three rats.

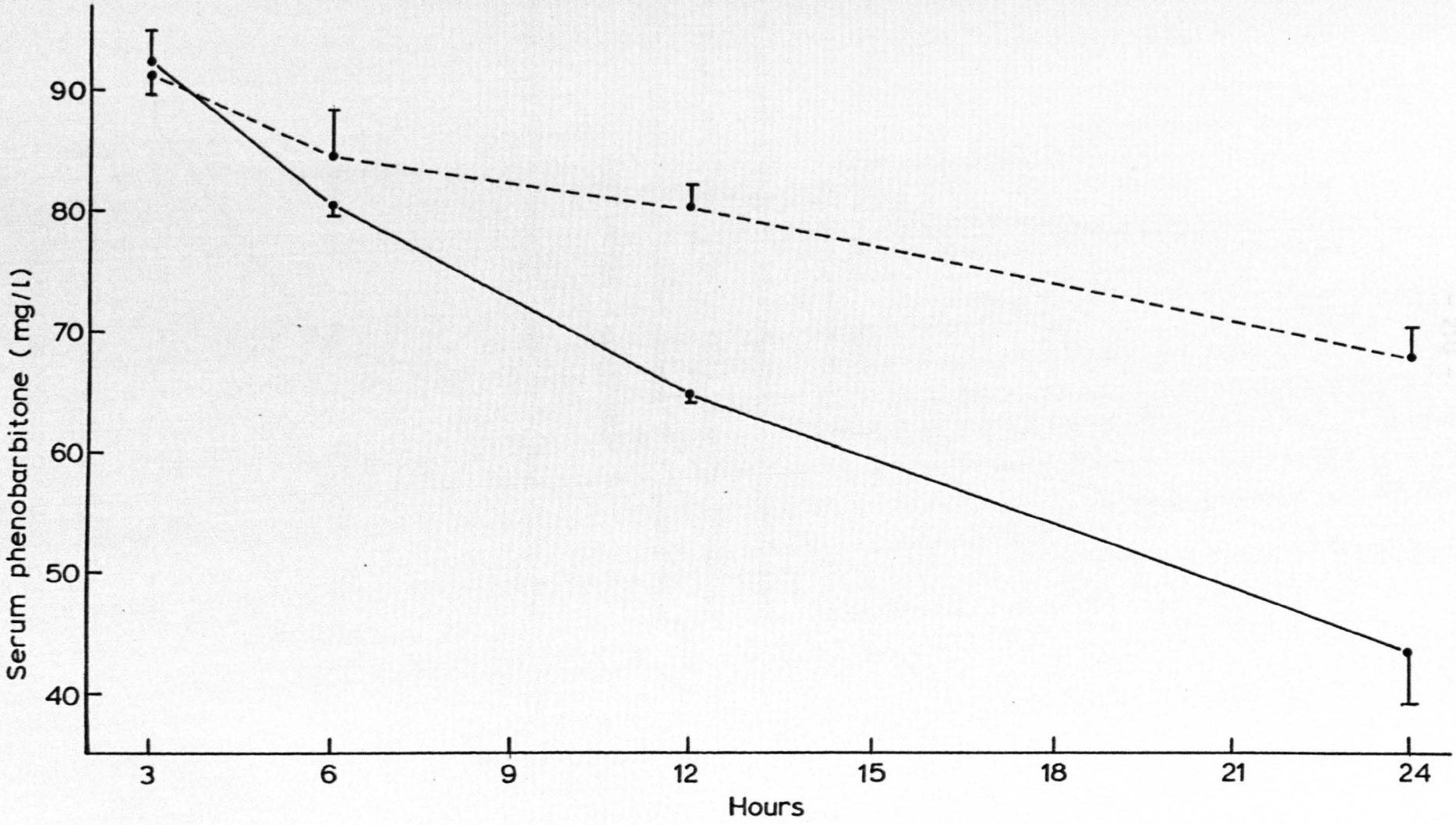

Serum phenobarbitone (mg/l)
90
80
70
60
50
40
3
6
9
12
15
18
21
24
Hours

FIGURE 10.11

Brain levels of phenobarbitone, for rats treated with phenobarbitone only (100 mg/kg), continuous line; and rats treated with phenobarbitone plus α-methyl-p-tyrosine (250 mg/kg), dotted line

Each point indicates mean $\pm$ standard error for three rats

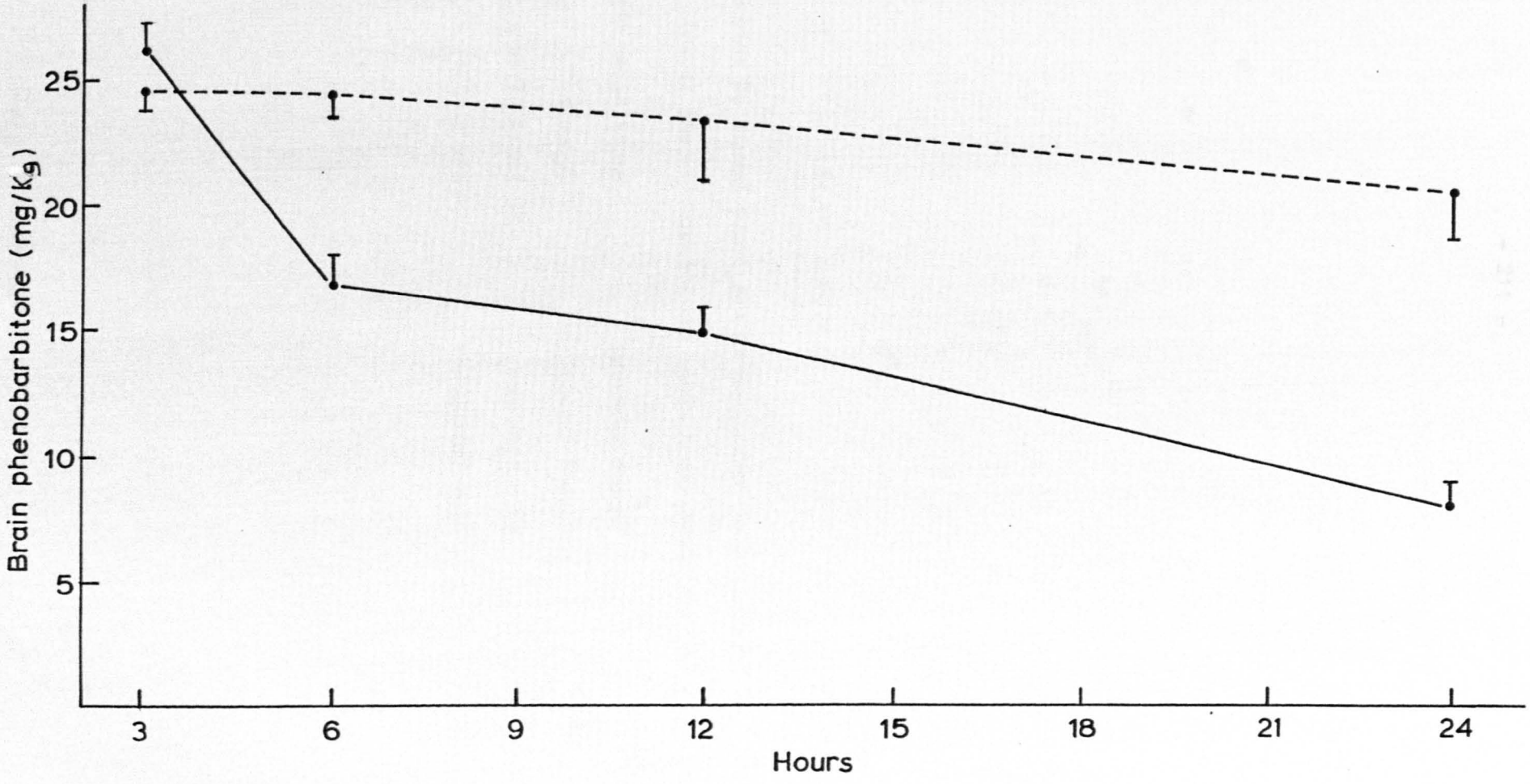
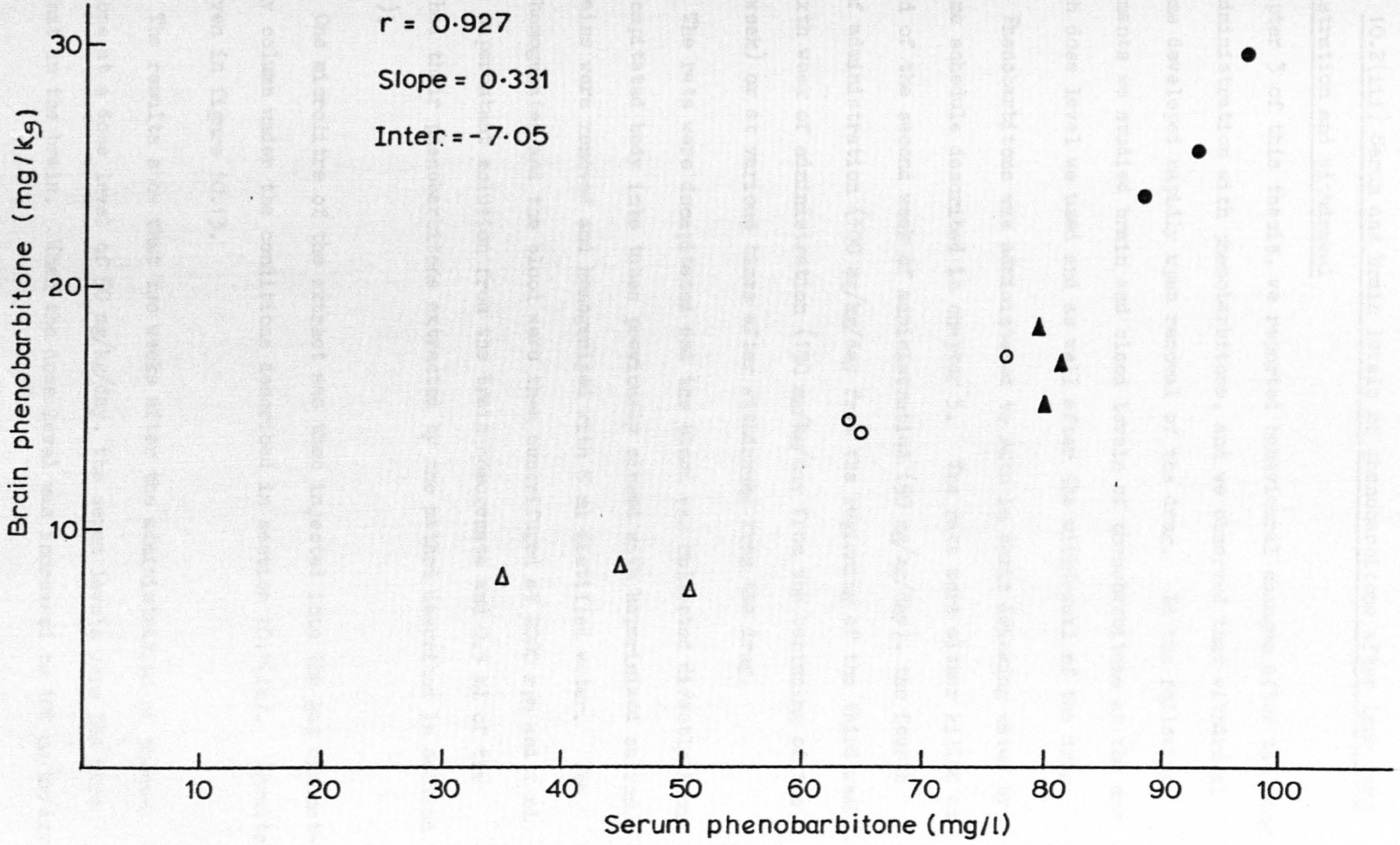

Brain phenobarbitone (mg/Kg)
25
20
15
10
5
3
6
9
12
15
18
21
24
Hours

FIGURE 10.12

Correlations between serum and brain levels of phenobarbitone obtained at different times after the intraperitoneal injection of 100 mg/kg.

Closed circles:	3 hours after injection
Closed triangles:	6 hours after injection
Open circles:	12 hours after injection
Open triangles:	24 hours after injection


r = 0·927
Slope = 0·331
Inter = -7·05
Brain phenobarbitone (mg/kg)
30
20
10
10
20
30
40
50
60
70
80
90
100
Serum phenobarbitone (mg/l)

10.2(iii) Serum and brain levels of phenobarbitone after long term administration and withdrawal

In chapter 3 of this thesis, we reported behavioural changes after the long term administration with phenobarbitone, and we observed that withdrawal symptoms developed rapidly upon removal of the drug. In the following experiments we studied brain and blood levels of phenobarbitone at the end of each dose level we used and as well after the withdrawal of the drug.

Phenobarbitone was administered to rats in their drinking water by the same schedule described in chapter 3. The rats were either killed at the end of the second week of administration (50 mg/kg/day), the fourth week of administration (100 mg/kg/day from the beginning of the third week). the sixth week of administration (150 mg/kg/day from the beginning of the fifth week) or at various times after withdrawal from the drug.

The rats were decapitated and the blood was collected directly from the decapitated body into tubes previously rinsed with heparinized saline. The brains were removed and homogenized with 5 ml distilled water. The brain homogenates and the blood were then centrifuged at 2000 rpm and 1 ml of the supernatant solution from the brain homogenate and 0.5 ml of the serum had their phenobarbitone extracted by the method described in section 10.1(i).

One microlitre of the extract was then injected into the gas chromatography column under the conditions described in section 10.1(ii). Results are given in figure 10.13.

The results show that two weeks after the administration of phenobarbitone at a dose level of 50 mg/kg/day, the serum levels were 38% more than that in the brain. When the dose level was increased to 100 mg/kg/day,

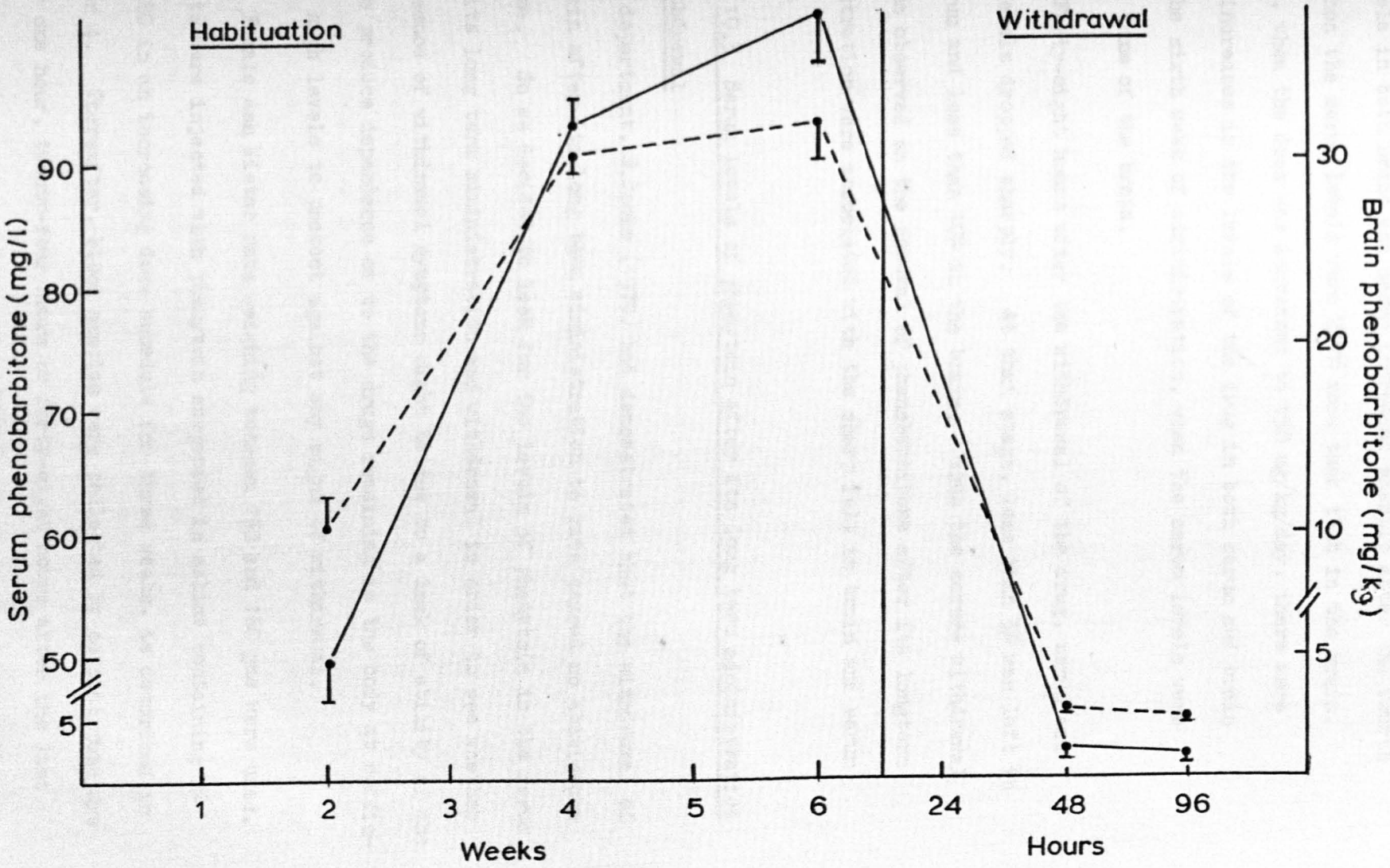
FIGURE 10.13

Serum and brain concentration of phenobarbitone during its habituation and withdrawal

Continuous line - serum; broken line - brain

See text for dosage

Each point indicates mean $\pm$ standard error for three rats.


Habituation
Withdrawal
Serum phenobarbitone (mg/l)
90
80
70
60
50
5
Brain phenobarbitone (mg/kg)
30
20
10
5
1
2
3
4
5
6
24
48
96
Weeks
Hours

the levels in both brain and serum increased sharply after the fourth week, when the serum levels were 207% more than that in the brain. However, when the dose was increased to 150 mg/kg/day, there were slight increases in the levels of the drug in both serum and brain after the sixth week of administration, when the serum levels were 225% of those of the brain.

Forty-eight hours after the withdrawal of the drug, serum and brain levels dropped sharply. At that stage, less than 3% was left in the serum and less than 10% in the brain. Thus the severe withdrawal symptoms observed on the removal of phenobarbitone after its longterm administration were associated with the sharp fall in brain and serum levels.

10.3 Serum levels of phenytoin after its long term administration and withdrawal

In our department, S.Cooke (1979) had demonstrated that the withdrawal of phenytoin after its long term administration to rats caused no abstinence syndrome. So we decided to look for the levels of phenytoin in the serum after its long term administration and withdrawal in order to see whether the absence of withdrawal symptoms might be due to a lack of ability of the drug to produce dependence or to the drugs remaining in the body at sufficiently high levels to protect against any signs of withdrawal.

Female Ash Wistar rats weighing between 150 and 160 gms were used. The rats were injected with phenytoin suspended in saline containing 1% Tween 80 in an increasing dose schedule for three weeks, as described in chapter 4. Thereafter, blood samples were collected by cardiac puncture either one hour, twenty-four hours or forty-eight hours after the last injection into tubes previously rinsed with heparinized saline. The tubes were centrifuged at 2000 rpm and 0.5 ml of the serum had its phenytoin

Table 10.5

Serum levels of phenytoin after its long term administration and withdrawal

Time after last injection hours	Number of animals	Phenytoin mg/litre
1	5	29.65 ± 2.01
24	4	3.28 ± 0.1
48	4	3.069 ± 0.09

Each value is the mean ± standard error

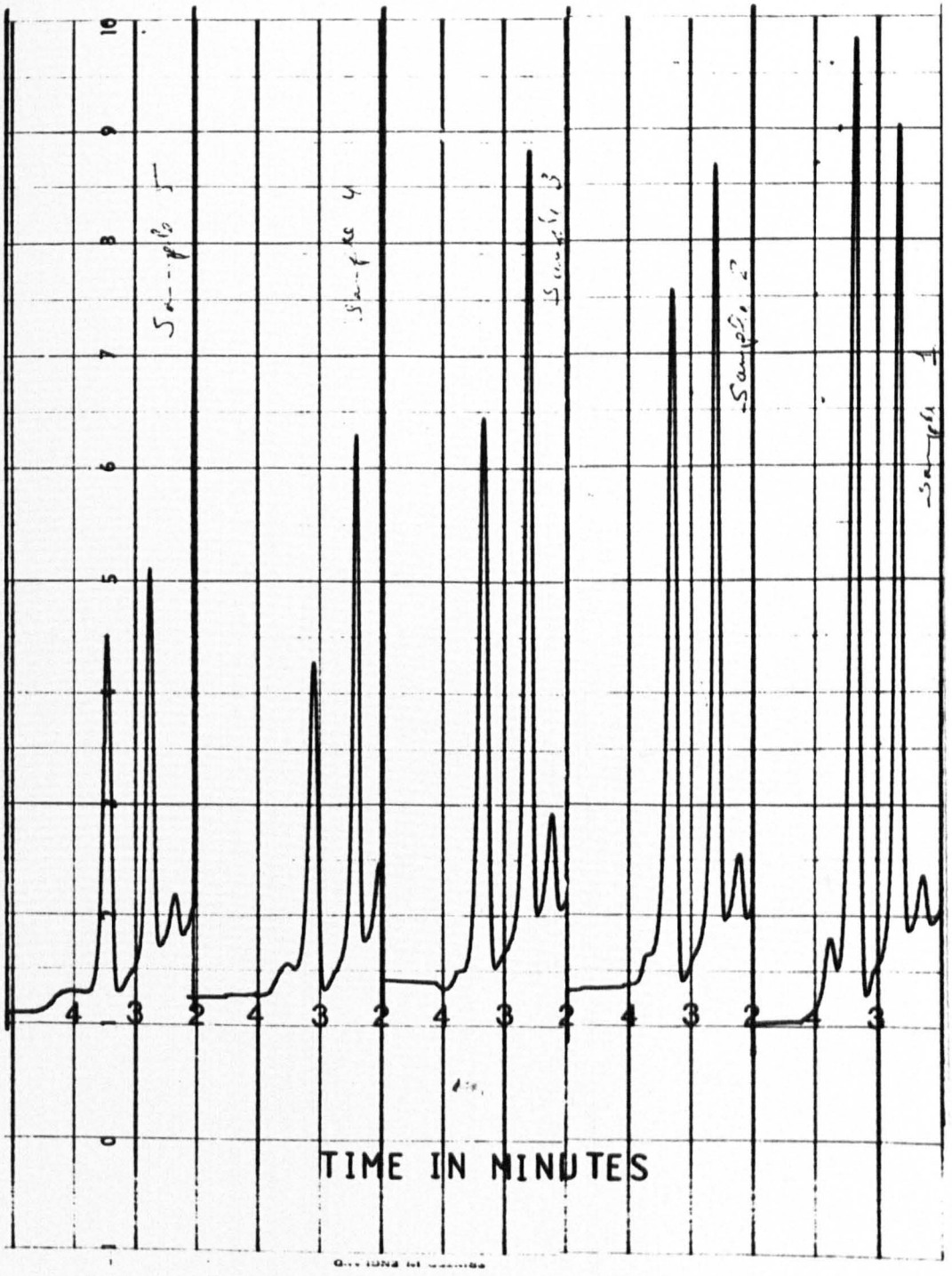
extracted by the method described in section 10.1(i). One microlitre of the extract was then injected into the gas chromatography column under the conditions desdribed in section 10.1 (ii). Results are given in table 10.5.

The results show that phenytoin levels dropped sharply twenty-four hours after the last injection and only 11% of the original concentration remained. From these results, we can conclude that phenytoin is a poor drug to produce dependence and the sharp fall in the serum levels did not produce any withdrawal symptoms.

Figure 10.14 shows chromatograms obtained after the column injection of 1 µl of different serum extracts of rats treated with phenytoin.

FIGURE 10.14

Chromatograms obtained after column injection of different serum extracts from rats that had been injected for three weeks with phenytoin


TIME IN MINUTES

PART IV

PART IV

CHAPTER 11

GENERAL DISCUSSION

Epilepsy was defined by Hughlings Jackson as 'a state produced by an abnormal excessive neuronal discharge within the central nervous system'.

It is now an accepted fact that the transmission of impulses across synapses in the vertebrate central nervous system is mediated for the most part by the intervention of specific chemical transmitter substances, some inhibitory and others excitatory. Thus it is tempting to speculate that epilepsy might be accompanied or preceded by a disturbance of the pre- or post-synaptic mechanisms involved in neurotransmission, resulting in disorganized neuronal activity in the form of an epileptic discharge. The spread of this epileptic activity to normal neighbouring neurons also involves synaptic transmission. thus further underlining the importance of the study of central synaptic transmission in the epileptic state.

It is reasonable to believe that the concentration of the neurotransmitter in the synaptic cleft should be the parameter most directly related to the level of functioning of the transmitter, but since the determination of this concentration is beyond the scope of current technological methods, only indirect estimation of the parameter can be obtained. For example, changes in whole brain concentration of a neurotransmitter or in its turnover rates may reflect changes in the neurotransmitter concentration in the synaptic cleft. On the other hand, they may not.

There are, of course, factors other than the neurotransmitters themselves which may affect neurotransmission in epilepsy. Known influences include electrolyte concentration, the ionic pumps and hormones. A study of these factors is outside the scope of our study.

In our effort to study the mode by which an anticonvulsant drug exerts an inhibitory effect on epilepsy, we examined the effect of some anti-epileptic drugs of known efficacy on some chemical substances (and their metabolites) present in the brain and generally accepted as transmitters.

Because of the large amount of data reported in our experimental studies, we shall first discuss the results in the sequence in which they are presented in the experimental chapter. Later we attempt to relate the results with each other.

I. Catecholamines and anticonvulsant drugs

The possible role of brain biogenic amines in experimental seizures and the mode of action of anticonvulsant drugs has been a subject of considerable interest ever since Chen, Ensor and Bohner (1954) demonstrated in mice that reserpine lowered maximum electroshock seizure and chemoshock seizure thresholds and antagonized the anticonvulsant effect of phenytoin. Rudzik and Mennear (1965) suggested that the antagonism of reserpine to the anticonvulsant effect of phenytoin was not the result of amine depletion, since other amine depletors (α-methyl dopa, α-methyl-m-tyrosine and α-methyl-p-tyrosine) were ineffective in this respect. Although they found no correlation between the brain amine concentrations and the incidence of minimal electroshock seizures, Azzaro, Wenger, Craig and Stitzel (1971) came to a conclusion which supported the original one of Chen and his

colleagues that brain amines (noradrenaline, dopamine and 5-hydroxytryptamine) are all intimately involved in the effect of reserpine on electrically induced seizures. They based their conclusion on the finding that the administration of amine precursors to animals treated with reserpine resulted in both an increase in brain amine concentration and an antagonism of the effect of reserpine on seizure incidence. Similarly, they found that the administration of inhibitors of catecholamine and/or 5-hydroxytryptamine synthesis to reserpine-treated mice prevented brain amines from returning towards their control levels and prolonged the effect of reserpine on the minimum electroshock seizure threshold. Moreover, Azzaro et al. showed that the action of reserpine can be approximated in control mice by the concomitant inhibition of catecholamine and 5-hydroxytryptamine. In a later study, this same group of research workers (Wenger, Stitzel and Craig, 1973) provided data which suggested that noradrenaline and 5-hydroxytryptamine, but not dopamine, are important in regulating minimal electroshock seizure susceptibility. They suggested that the total amine concentrations are less important than the restoration of the ability of the tissue to synthesize and store the transmitters, since inhibitors of noradrenaline and 5-hydroxytryptamine synthesis in reserpine-treated animals prevented the minimal electroshock seizures from returning to their control intensity. Kilian and Frey (1973) and Meyer and Frey (1973) demonstrated that inhibition of noradrenaline synthesis in the brain would decrease the convulsive threshold and antagonize the anticonvulsant effect of phenytoin and phenobarbitone against electrically and chemically induced convulsions in mice and rats. This suggested a role for noradrenaline in these effects.

Destruction of catecholamine neurons in the brain by the intraventricular injection of 6-hydroxydopamine increased the severity of audiogenic seizures in susceptible rats (Bourne, Chin and Picchioni, 1972), increased the susceptibility to both electroshock and chemically induced seizures (Browning and Maynert, 1970; Quattrone, Crunelli and Samanin, 1978) and antagonized the anticonvulsant effect of phenytoin, phenobarbitone, carbamazepine and acetazolamine against electrically induced convulsions (Browning and Simonton, 1978; Quattrone and Samanin, 1977; Quattrone, Crunelli and Samanin, 1978).

Studies in experimental animals of a genetic strain highly susceptible to audiogenic seizures have likewise provided data indicating a relationship of brain moncamines to seizure phenomena. Schlesinger, Boggan and Freedman (1965) and Kellogg (1976) found that the DBA/2 strain of mice (three weeks old) exhibited a high degree of sensitivity to audiogenic seizures together with low levels of brain noradrenaline and 5-hydroxytryptamine, indicating a possible role for these amines in seizure mechanisms. However, in a more recent report, Lints, Willott, Sze and Nenja (1980) reported no change in the concentration of either of the two amines measured in the same strain of mice at the same time as that reported by Schlesinger and his co-workers. It must be added, however, that Anlezark, Horton and Meldrum (1978) had found that dopamine agonists reduced the susceptibility of the DBA strain of mice to audiogenic seizures. This effect was blocked by haloperidol, a dopamine antagonist, thus suggesting a role for dopamine in audiogenic seizure susceptibility.

In another strain of mice (EL) susceptible to convulsions produced by postural stimulation, Kohsaka, Hiramatsu and Mori (1978) demonstrated

that postural stimulation produced 50% depletion of brain dopamine. A further decrease was observed in the levels of dopamine which was accompanied by a fall in noradrenaline levels at the preconvulsive stage. The concentration returned during the convulsions to the levels seen at the resting position.

We began our investigation into the relationship between anti-convulsant drugs and catecholamines (noradrenaline and dopamine) by simply looking at the effects of single doses of some of these drugs on the whole brain levels of the catecholamines.

Before discussing these results, however, we have to refer to the fact, mentioned briefly in an earlier section of this thesis that resting brain levels of dopamine in untreated animals varied from one experiment to another.

During the course of our studies, different batches of alumina were used. We noticed that some batches are more effective in adsorbing dopamine than others. Even in the same batch, alumina prepared on different days gave different degrees of adsorption. This should not give rise to the large differences we observed in our animals, since a calibration curve was constructed for each experiment and any effects on the degree of adsorbtion should happen equally to both the brain extract and to the external dopamine. It can only be concluded (and this somewhat lamely) that some components of the brain extract affected the adsorption or elution of brain dopamine and that the extent of this effect was dependent on subtle differences among different batches of the alumina.

Single doses of phenytoin, phenobarbitone, carbamazepine and sodium bromide had no effect on the whole brain levels of either catecholamine, thirty minutes and one hour after injection. Twice daily doses of the same drugs, given for one week, also failed to influence the amount of dopamine or noradrenaline in the rat brain.

The fact that the levels of dopamine and noradrenaline vary among the different brain regions might result in changes in the concentration of either catecholamine in a small area of the brain's being masked in assays of the whole brains. With this in mind, we turned our attention to the effect of these drugs on the regional distribution of the catecholamines, and some interesting results emerged. Single doses of phenytoin increased the concentration of dopamine in the striata and of noradrenaline in the midbrain, the areas of the brain which contain the greatest concentrations of dopamine and noradrenaline respectively. Carbamazepine increased significantly the amount of noradrenaline in the midbrain and the cerebral hemispheres, but it did not affect the dopamine levels in any part studied. Phenobarbitone did not change the concentration of either catecholamine in any part of the brain. Phenytoin would seem to decrease the utilization, increase the synthesis or inhibit the breakdown of dopamine in the striata and to have a similar effect on the noradrenaline in the midbrain.

Azzaro, Gutrecht and Smith (1973) reported that low concentrations of phenytoin (10^{-5} to 10^{-4}) facilitated, and higher concentrations (10^{-3}) had no effect on the twenty minute accumulation of radio-active noradrenaline. The higher concentration (10^{-3}) produced a severe reduction in the five minute accumulation of [^{3}H] noradrenaline. Furthermore, phenytoin produced an inhibition of the oxidative catabolism of the radio labelled

amine in cerebral cortex slices and in whole and lysed synaptosomal fractions of the cerebral cortex. The authors concluded that the facilitating action of phenytoin on the twenty minute accumulation of [^{3}H] noradrenaline was due to an inhibitory effect of phenytoin on the oxidative catabolism of the radio labelled amine and not to an effect on the uptake which was inhibited, at least by the higher concentrations of phenytoin, at the five minute stage. In another report, Azzaro and Gutrecht (1975) have identified the characteristics of the inhibitory action of phenytoin on monoamine oxidase to be of a competitive nature. They also found that phenytoin reduced the activity of type A monoamine oxidase to 20 per cent of its control values, while type B was inhibited to the extent of 50 per cent.

Thus phenytoin seems to increase the levels of noradrenaline in the midbrain and of dopamine in the striata by inhibiting their catabolism. Chang, Okerholm and Glazko (1972) provided data that showed that phenytoin is metabolized to a corresponding catechol which is extensively methylated to the 3-O-methyl catechol. Thus the possibility exists that the free catechol metabolite of phenytoin may compete with catecholamines for the 3-O-methylation, thereby exerting a sparing effect and prolonging the life time of the catecholamines at their receptor sites. Whether the ability of carbamazepine to increase the noradrenaline content in the midbrain and the cerebral hemispheres can be explained by a similar mechanism must remain a speculation, but it may not be irrelevant to recall that carbamazepine has antidepressant properties so that it is not unreasonable to predict that it might intervene in some way in the noradrenaline system. This point is taken up again later.

Many writers suggest that the absolute concentration of a transmitter in the brain may bear little relationship to the actual activity

of the system under study. It may be that the estimation of turnover rates of the catecholamines in central neurons can be used as a better index of the functional state of these neurons. Many techniques, isotopic and nonisotopic, are being utilized for the measurement of catecholamine turnover in the central nervous system. One of the simplest ways involves following the disappearance rate of the endogenous catecholamine after inhibition of its biosynthesis by α-methyl-p-tyrosine.

In our study on the effect of anticonvulsant drugs on the turnover rates of catecholamines, we found that only phenobarbitone influenced the rate at which the catecholamines were depleted by α-methyl-p-tyrosine. The results, presented in chapter 2.3, show that phenobarbitone reduced the rate at which dopamine was depleted at all times studied. On the other hand, the effect on noradrenaline depletion was seen only during the first six hours of α-methyl-p-tyrosine treatment. Thereafter, phenobarbitone no longer inhibited noradrenaline turnover. Indeed, eighteen hours after α-methyl-p-tyrosine was given there was a slight but significant stimulation of turnover. Our results support those of Corrodi, Fuxe and Hokfelt (1966), who reported results similar to ours in regard to dopamine. On the other hand, these workers found that phenobarbitone produced no change in noradrenaline depletion by α-methyl-p-tyrosine.

It is reasonable to suppose that following the administration of α-methyl-p-tyrosine, the rate of depletion of catecholamines will be proportional to their rate of release and subsequent removal from the receptor sites, and this will depend on the activity of the neurons in question. Thus, the higher the impulse flow along neuronal fibres, the faster will be the rate of depletion of catecholamines from those fibres and vice versa (Cooper, Bloom and Roth, 1978; Hebron, 1977).

In our experiments, phenobarbitone decreased the rate of depletion of dopamine at all times studied, and those of noradrenaline at the early hours after administration of α-methyl-p-tyrosine. If the above supposition is valid in our case, we can suggest that phenobarbitone inhibited the neuronal activity of dopaminergic neurons at all times studied and that of noradrenergic neurons only for the first few hours after its administration.

When we looked for the region in which phenobarbitone resisted the depletion, we found it to be in the cerebral hemispheres.

Our behavioural studies on the interaction between phenobarbitone and α-methyl-p-tyrosine proved to be very interesting. Both α-methyl-p-tyrosine and phenobarbitone given alone produced sedation and hypothermia. The hypothermia produced by phenobarbitone was rapid in onset and showed recovery after five hours, whereas that produced by α-methyl-p-tyrosine was slow in onset, but continued to the end of the time of measurement (twenty-four hours - see Figure 2.4, page 23). Sedation went in the same way as hypothermia. When both drugs were injected simultaneously the hypothermic and sedative effects were greatly potentiated. α-Methyl-p-tyrosine reduced significantly the induction time and prolonged the sleeping time caused by phenobarbitone. We supposed at the time that α-methyl-p-tyrosine either potentiated the effect of phenobarbitone by central synergistic mechanisms or by inhibiting the microsomal enzymes in the liver. The results presented in chapter 10.2 show that α-methyl-p-tyrosine delayed the metabolism of phenobarbitone in both the serum and the brain. We could not rule out completely the existence of some synergistic effect between the two drugs, and we discussed in addition the possibility that α-methyl-p-tyrosine might affect the elimination of phenobarbitone.

Papeschi and Randrup (1973) suggested that the hypothermia produced by α-methyl-p-tyrosine was the result of blockade of the synthesis of dopamine and noradrenaline in peripheral organs and of dopamine centrally, since loading of catecholamine stores by L-DOPA antagonized the hypothermia induced by α-methyl-p-tyrosine with a lesser degree of effectiveness after peripheral decarboxylase inhibition. Whether phenobarbitone potentiated the hypothermic effect of α-methyl-p-tyrosine through an effect on the catecholamine system remains an open question for further research in this field.

One of the major means of studying neuronal function in a particular organ or system has been the observation of functional impairment after removal of the neuronal input. 6-Hydroxydopamine, a neurotoxin which causes destruction of neurons containing catecholamines, is one of the drugs which are useful for this purpose. We therefore used 6-hydroxydopamine as a further means of studying the effect of anticonvulsant drugs on the catecholamine system.

In our experiments, a single dose of 250 µg of 6-hydroxydopamine injected intraventricularly effectively depleted only noradrenaline stores, dopamine being depleted by only 14 per cent twelve days after the injection. When pargyline preceded 6-hydroxydopamine treatment, the depleting action on dopamine was greatly potentiated while noradrenaline depletion stayed the same. Pargyline inhibits monoamine oxidase, thereby increasing the amount and the duration of the effect of 6-hydroxydopamine in the brain, so producing a greater degree of destruction of dopaminergic neurons (Breese and Traylor, 1970).

When we looked at the effect of single doses of anticonvulsant drugs on the levels of catecholamines in the brains of rats treated with

6-hydroxydopamine twelve days earlier, we found that phenytoin and phenobarbitone increased significantly the amount of both amines, while carbamazepine increased only that of noradrenaline. These results recall those in animals that had not received 6-hydroxydopamine.

It is known that 6-hydroxydopamine treatment lowers seizure thresholes and antagonises the anticonvulsant effect of phenytoin, phenobarbitone and carbamazepine against electroshock seizures (Quattrone, Crunelli and Samanin, 1978). In our experiments, these drugs increased the amount of the amines present in the stores, which might have been expected to exert an effect against the electrically-induced seizures. However, this was not so.

After 6-hydroxydopamine treatment, there is a reduction in the number of catecholamine-containing neurons, which is accompanied by a decrease in tyrosine hydroxylast activity and a loss of noradrenaline uptake sites (Bell, Iverson and Uretsky, 1970). Because of this reduction in the number of neurons and the activity of tyrosine hydroxylase, the total turnover rates of catecholamines in the brain would be expected to decrease, and this was found to be so (for references see Kostrzewa and Jacobowitz, 1974). Thus, although in our experiments the anticonvulsant agents produced an increase in the levels of the amines in the surviving neurons, the total size of the catecholamine stores was reduced and smaller amounts of the amine could be released. This might not be sufficient to antagonize the electrically induced seizures, unlike, perhaps, the state of affairs in the experiments described by Quattrone et al. If this assumption is true, we would expect that the reloading of the catecholamine stores would reverse the enhanced effect of 6-hydroxydopamine towards the electrically induced seizure. The work of Stull,

Jobe and Geiger (1977) showed that this is indeed so. These authors found that the enhanced electroshock seizure intensity produced by the depletion of noradrenaline and dopamine in and near the ventricular area by the intracerebroventricular administration of benzoquinolizine was antagonized by the systemic administration of L-DOPA.

The behavioural changes (described in chapter 2.7) produced by combined treatment with an intraventricular injection of 6-hydroxydopamine and an intraperitoneal injection of pargyline were unique in their features. Vetulani, Reichenberg and Wiszniowska (1972) reported some early behavioural changes in rats treated with 6-hydroxydopamine given by unilateral intraventricular injection and a monoamine oxidase inhibitor (nialamide) given intraperitoneally. They described the behavioural changes as strong stimulation, postural changes and circling behaviour. They claimed that these changes were completely absent when treatment was effected under ether anaesthesia and if the rats were not separated. Fighting behaviour developed if the rats were put together in one cage two hours after treatment, but failed to do so if they were put together immediately after the injections. Our experimental results are entirely different from those described by Vetulani et al., although we adhered to the same protocol they used. Giving 6-hydroxydopamine unilaterally before the monoamine oxidase inhibitor, we observed some behavioural changes in rats after the recovery from the short ether anaesthesia, but this occurred whether the animals were caged singly or together (see Figures 2.8, 2.9 and 2.10). We did not observe any circling behaviour. No fighting behaviour developed any time after the injection, either in the singly caged or the grouped rats. Vetulani and his co-workers detected an increase in dopamine concentration in the injected half of the brain in their animals and the behavioural changes were prevented completely with spiroperidole, a dopamine receptor blocker. In view of the circling behaviour displayed

by the treated animals and the increase in dopamine concentration in the injected half of the brain, these authors suggested that the damage produced by 6-hydroxydopamine was not symmetrical.

Fibiger, Lonsbury and Cooper (1972) described early behavioural changes similar to some of the ones we noticed. They reported that animals treated with pargyline and 6-hydroxydopamine became aphagic and adipsic, that they did not groom, assumed a hunched back posture and showed increased irritability. However, they also reported that their animals exhibited a pronounced state of hyperactivity throughout the first twelve hours after administration. Although we did not measure the spontaneous activity, it was obvious, even by casual observation, that rats exposed to the combined treatment were less active than rats treated with either pargyline or 6-hydroxydopamine alone.

In cats, displayed rage and occasional convulsions have been reported after combined treatment with pargyline and 6-hydroxydopamine (Howard and Breese, 1974). Rats injected intraventricularly with 6-hydroxydopamine on the right but not the left side of the brain, exhibited two and a half hours later short periods of violent activity to the extent of convulsions (Laverty and Taylor, 1970). Similar results were reported in rats twenty-four hours after intracisternal injection of 6-hydroxydopamine. Scotti De Carolis, Ziegler, Dell Bass and Longo (1971) have examined the electroencephalographic and behavioural alterations and reported motor depression intermingled with spells of hyperirritability and seizures. In our experiments we observed the motor depression together with continuous hyperirritability, but we saw no convulsions during the period of our observation, or when the treated rats were subjected to auditory stimulation at various times thereafter.

The behavioural changes observed in our experiments may possibly be subserved by a dopaminergic mechanism, since neither pargyline nor 6-hydroxydopamine alone can bring about all of the observed changes we noticed. Jonsson and Sacks (1971), demonstrated that monoamine oxidase inhibition increases the accumulation of 6-hydroxydopamine in sympathetically innervated tissue. Thus pargyline, by increasing both the duration of the effect of and the uptake of, 6-hydroxydopamine by the dopaminergic neurons might be expected to increase the release of dopamine by displacement mechanisms and so make more dopamine available at the receptor site.

The question arises as to whether the results presented and discussed so far enable us to say that the activity of an anticonvulsant drug depends in part on the catecholamine systems in the brain. This is difficult to answer as yet, but we can say that some promising results emerged from our preliminary experiments, in which we saw that single doses of phenytoin increased the levels of dopamine in the striata and of noradrenaline in the midbrain and increased the levels of both amines in the brains of rats treated with 6-hydroxydopamine. We discussed the possibility that these effects of phenytoin might have been the result of inhibition of the oxidative catabolism of the catecholamines. Phenobarbitone reduced the depletion of both dopamine and noradrenaline in the early hours after α-methyl-p-tyrosine and did so also after 6-hydroxydopamine and we discussed the possibility that phenobarbitone produced these changes by inhibiting neuronal activity. Carbamazepine, on the other hand, seems to have an effect on noradrenaline, where single doses increased the amount of amine in the midbrain and cerebral hemispheres and did so for the whole brain after 6-hydroxydopamine.

So far the results were promising, and because most of the drugs

used for the treatment of epilepsy are given over a long period of time, we extended our study to inspect the effect of anticonvulsant drugs on the levels and turnover rates of catecholamines after long term administration.

Chapter 3 desdribes results obtained after long term administration and withdrawal of phenobarbitone. The method of habituation we used was very successful in producing dependence on phenobarbitone, since withdrawal of the drug produced severe abstinence symptoms, characterized by irritability, aggressiveness, a decrease in food intake, loss of body weight and convulsions.

A tolerance to the lower doses of phenobarbitone developed about ten days after treatment at each dose level of the drug, where a slight aggressiveness and biting behaviour were noticed and rats conducted themselves in a completely different manner from that seen during the first days of treatment at each dose level. We called this phenomenon self withdrawal, since phenobarbitone stimulates its own metabolism as well as that of other barbiturates (Conney, Davidson, Gastel and Burns, 1960; Kato and Chiesara, 1962; Frey and Kampmann, 1965). It is well known as well that tolerance to the sedative and hypnotic action of phenobarbitone develops after a few days of treatment and then disappears (Frey and Kampmann, 1965). So phenobarbitone acting by stimulating its own metabolism might decrease the levels of phenobarbitone in serum giving rise to the mild withdrawal symptoms described earlier.

The first dose of phenobarbitone we used (50 mg/kg daily) produced an increase in food and water intakes. These persisted for two days, but then fell so that on the fourth day and thereafter both treated and control groups ate and drank the same amount. The same thing happened again with food intake when the dose was increased up to

100 mg/kg daily. Crossland and Turnbull (1971) related the increase in food intake on the long term treatment with sodium barbitone to the stimulation of the growth rate of young rapidly growing rats, as indicated by the increase in the body weight and skeletal length. In our experiments, there was evidence that rats on phenobarbitone grew at a faster rate than did control rats, but this was seen only at the low dose levels and it was reversed when the daily dose was increased to 150 mg per kilo. It is possible that the observed increase in food intake was caused by an action of phenobarbitone on the hypothalamus.. Feldberg (1960) noticed that injection of barbiturate into the lateral ventricles often resulted in hyperphagia before the induction and during recovery from anaesthesia.

When we studied the effect of long term administration of phenobarbitone on the catecholamine content of rat brain, we found that habituation to phenobarbitone had no effect on the total content of either dopamine or noradrenaline. However, it was associated with an increase in the dopamine content of the striata and midbrain and an equivalent decrease in that of the cerebral hemispheres, so that the total amount of dopamine remained unchanged.

The dopaminergic nigrostriatal system is believed to be concerned with the integration of incoming sensory stimuli and the fine control of movement. It was clear that at the end of the period of habituation to phenobarbitone, the rats were ataxic, their response to external stimuli was depressed and movement was impaired. Whether we can attribute the changes in dopamine content of the striata and the midbrain to a decreased activity of the nigrostriatal system (among others), and whether, if so, these changes are also related to the behavioural changes, we will leave as an open question.

The decreased dopamine concentration in the cerebral hemispheres could conceivably be the result of an increased release and utilization of this amine in habituated rats. It might be relevant to note this point since the motor centres are located in the cortex.

Before considering any explanation for the above reported results, let us go a little further in order to try and see the whole picture.

The catecholamine system in the brain of rats which had been habituated to phenobarbitone was studied after the withdrawal of the drug. Two days after the withdrawal of phenobarbitone, the amounts of dopamine and noradrenaline were decreased in the brains of the previously habituated rats. When we looked at the regional distribution of the catecholamines in the brains of this group of rats, we found that the changes took place in the striata for dopamine and in the cerebral hemispheres for both amines. The loss of dopamine in the cerebral hemispheres of withdrawn rats was greater than that in habituated rats. However, withdrawal of phenobarbitone was not associated with any decrease in dopamine levels in the midbrain which had been elevated by the previous long term administration of the drug. This might indicate a slow turnover rate of dopamine in the midbrain.

The experiments of Morgan, Pfeil and Gonzales (1977) to some extent pre-dated ours. These workers reported an increase in dopamine concentration in the striata of rats dependent on sodium barbitone. Twenty-four hours after withdrawal of the drug, dopamine concentration was lowered to that of control value and forty-eight hours after withdrawal there was a further depletion. Withdrawal of sodium barbitone decreased the concentration of noradrenaline in the cerebral cortex and the thalamus twenty-four hours after the removal of the drug. Habituation in Morgan's experiments

had no effect on the levels of noradrenaline. The results reported by Morgan and his co-workers are in good agreement with the results we reported with respect to the elevation of dopamine in the striata after habituation to phenobarbitone and the decrease of dopamine in the striata and of noradrenaline in the cerebral hemispheres after withdrawal of the drug. Unfortunately, Morgan and his co-workers did not report what had happened to dopamine in the cerebral hemispheres during habituation to and withdrawal from sodium barbitone.

To summarize the results so far, habituation decreased the levels of dopamine in the cerebral hemispheres and increased it in the striata and midbrain, while withdrawal brought about a decrease in dopamine level in the striata and a further decrease in the cerebral hemispheres. Noradrenaline content decreased only in the cerebral hemispheres.

In order to investigate our results further, we examined the effect of habituation to and withdrawal of phenobarbitone on the turnover rate of the catecholamines after α-methyl-p-tyrosine.

We found that habituation to phenobarbitone decreased the depletion of both catecholamines, normally brought about in the striata for dopamine and in the cerebral hemispheres for both amines.

We should draw attention at this point to an apparent discrepancy between the results we obtained for whole brain and those relating to the regional distribution when we were considering the noradrenaline content in habituated rats treated with α-methyl-p-tyrosine. Whereas habituation to phenobarbitone had no apparent effect on the depletion of whole brain noradrenaline brought about by α-methyl-p-tyrosine, when we conducted our regional studies we found that habituation reduced the depleting effect of α-methyl-p-tyrosine. This difference, of course, arose from the difference

in the time of killing after α-methyl-p-tyrosine. In the whole brain assays the killings were made eighteen hours after α-methyl-p-tyrosine, while in the regional studies the animals were killed only four hours after α-methyl-p-tyrosine. This is in good agreement with the results obtained on the effect of single doses of phenobarbitone on the disappearance rate of noradrenaline after α-methyl-p-tyrosine, where a delaying effect was seen only during the early hours after α-methyl-p-tyrosine.

Our results after α-methyl-p-tyrosine are consistent with those obtained for the effect of habituation on the unimpaired catecholamine system except for the results for dopamine in the cerebral hemispheres. For this catecholamine in the unimpaired system, there was an increased turnover which changed to a decreased turnover when the system was impaired by the administration of α-methyl-p-tyrosine. On the other hand, withdrawal of phenobarbitone increased the depletion of both dopamine and noradrenaline in whole brain, an effect that was found to be localized in the striata and cerebral hemispheres for dopamine and in the cerebral hemispheres and midbrain for noradrenaline. These observations, too, are consistent with those reported after withdrawal of the drug in an unimpaired system.

Morgan, Pfeil, Huffman and Gonzales (1978) reported that withdrawal increased the rate of depletion of both dopamine and noradrenaline in the brains of barbitone dependent rats, suggesting a role for catecholamines in the manifestation of barbiturate withdrawal symptoms. These results again are consistent with the results we reported after withdrawal of phenobarbitone.

It is now clear that habituation decreased and withdrawal increased the turnover rate of dopamine and noradrenaline. The effect of habituation was more pronounced on the dopamine system. We identified the areas at

which these changes took place and as well confirmed the effects in animals given α-methyl-p-tyrosine.

Let us consider now the possible explanations of the results we reported above.

The reduced turnover of dopamine during habituation may be theoretically due to inhibition of monoamine oxidase, to a direct stimulation of dopamine receptors, to a reduced dopamine release from its nerve terminals or finally a decreased flow of nerve impulses in dopamine neurons (Corrodi, Fuxe, Lidbrink and Olson, 1971).

Lidbrink, Corrodi, Fuxe and Olson (1972) also reported that single doses of phenobarbitone (100 mg/kg) had no effect on the total amount of noradrenaline and dopamine in rat brain, a result which was confirmed by our preliminary work. On the other hand, the same dose of phenobarbitone decreased the depletion of dopamine brought about by α-methyl-p-tyrosine in the striatum and of noradrenaline in the cerebral hemispheres. These results, too, are in good agreement with our findings. In this same paper, Lidbrink and his co-workers found that phenobarbitone was no different in its effect on monoamine oxidase than was α-methyl-p-tyrosine. This finding applied to the cerebral cortex, the neostriatum and the hypothalamus and it excludes the idea that inhibition of monoamine oxidase might be responsible for the reduced dopamine or noradrenaline turnover. The second possibility was tested by the same authors, by the injection of phenobarbitone into rats with a unilateral lesion of the nigrostriatal dopamine pathways. They found no asymmetry or rotation in those rats, thus excluding the possibility that a direct stimulation of dopamine receptors was responsible for the observed reduction in dopamine turnover.

Cooper, Bloom and Roth (1978) proposed that an alteration in the turnover rate of a neurotransmitter is not necessarily a clear indication that there has been a change in impulse flow in a given neuronal pathway. Although these authors were speaking about the catecholamine system, their statement seems to be contradictory to the work of Anden, Corrodi, Fuxe and Ungerstedt (1971) and of Nyback (1972), who reported that the turnover of dopamine and noradrenaline was mediated by changes in nervous activity and we conclude tentatively that the reduced dopamine turnover seen after phenobarbitone habituation was caused by a decreased impulse flow in dopaminergic neurons.

For the observed decrease in the degree of depletion of noradrenaline in the cerebral hemispheres of habituated rats treated with α-methyl-p-tyrosine, similar mechanisms to that described for dopamine can be suggested. Lidbrink and Farnebo (1973) found that phenobarbitone, in a wide range of concentrations, did not interfere with the uptake, retention and the spontaneous or evoked release of [3H] noradrenaline in rat cerebral cortex. This again supports the concept that a decrease in neuronal activity is responsible for the reduced turnover of noradrenaline observed in the cerebral hemispheres of the habituated rats seen during the early hours after giving α-methyl-p-tyrosine.

It may be relevant to point to the reports that drugs which block impulse flow in nigrostriatal dopaminergic neurons increase instead of decreasing dopamine biosynthesis (for references see Roth, Nowycky, Walters and Morgenroth, 1977). Thus in conclusion we can assume that phenobarbitone produced a decrease in the turnover of catecholamines by

decreasing the impulse flow in neurons.

After withdrawal the picture was reversed and there was an increase in the turnover of both catecholamines.

Crossland (1971) has put forward a hypothesis that adaptation of the central nervous system to a depressant drug might stem from an increase in the liberation of an excitatory transmitter or a decrease in the liberation of inhibitory transmitter sufficient to overcome the depressed condition of the nerve cell. Barbiturates are known for their depressant effect on neuronal firing and the catecholamines for their inhibitory role in neurotransmission. The theory of Crossland fits in well with our conclusion that phenobarbitone decreased the impulse flow in inhibitory neurons.

After withdrawal, the inhibitory effect of phenobarbitone on catecholamine neurons would be removed and the neurons would presumably fire at a faster rate, resulting in an increased turnover of the amine. This is what we have found to happen. It is interesting to note that the concentration of phenobarbitone in the brain was very low forty-eight hours after withdrawal. It was at this time that our measurements of the catecholamines were made (Chapter 10.2(iii).)

We extended our investigation much further by studying the catecholamine systems during the onset of convulsions produced in the withdrawn rats by audiogenic stimulation, in order to see to what extent the catecholamine systems would react in convulsions.

When rats withdrawn from phenobarbitone forty-eight hours earlier were subjected to auditory stimulation and killed at the time of convulsions, we found that their total brain levels of dopamine were unchanged

from those of untreated animals also subjected to stimulation. However, they showed significantly higher amounts of dopamine in the striata and lower amounts in the cerebral hemispheres, which is exactly the same situation we found in the brains of habituated rats. The brains of convulsed rats showed higher levels of dopamine than the brains of withdrawn rats which had not been stressed by audiogenic stimulation. Noradrenaline levels were lower in this group of rats than in the corresponding untreated rats subjected to stimulation, but contained significantly higher amounts of noradrenaline than did the brains of withdrawn rats which had not been stressed by audiogenic stimulation.

It is obvious that withdrawal convulsions changed the activity of the catecholaminergic system. The mechanisms by which the brain reacted during convulsions are difficult to explain on the basis of a single effect of a neurohumoral agent, although an explanation is a most desirable goal. The convulsion state itself could bring about a lot of changes in the activity of other putative neurotransmitters, especially since it has been demonstrated already that other neurohumours, like acetylcholine and γ-aminobutyric acid, control the activity of dopaminergic and noradrenergic neurons. However, we will try to explain our findings on the basis of results obtained by other researchers in this field.

Calderini, Carlsson and Nordström (1978) reported that rats with sustained epileptic seizures produced by intravenous injection of bicuculline showed a reduced level of noradrenaline after thirty minutes and that the catecholamine remained low during the two hours of sustained epileptic activity. This is in agreement with the lower values of noradrenaline we observed in the brains of withdrawn rats during convulsions.

The same authors reported that dopamine was increased one hour after the sustained seizure activity and remained so a further hour later. This again supports the results we obtained for dopamine in the striata, but does not confirm those observed in the cerebral hemispheres in which we found a decrease during convulsions. Tyrosine was reported by the same researchers to be elevated by bicuculline and the accumulation of DOPA following inhibition of L-aromatic acid decarboxylase was found as well to increase in the striatum, limbic forebrain and the hemispheres during the period of seizures. This increase was found to be most pronounced in the striatum and the hemispheres, which indicates an increased hydroxylation of tyrosine during epileptic seizures.

Therefore it seems reasonable to suppose that the low levels of dopamine in the cerebral hemispheres seen in withdrawn rats during audiogenic convulsions are best explained by an extraordinarily high functional activity in the dopaminergic neurons exceeding the rate of transmitter synthesis. The same can be applied to noradrenaline. The increased levels of dopamine in the striata can be simply explained on the basis of decreased functional activity.

Catalepsy always follows convulsions. Catalepsy is thought to be caused by a depression of dopaminergic mechanisms in the striatum (Papeschi, 1977). Therefore the decreased dopaminergic functions in the striatum during convulsions would decrease the amount of dopamine at the receptor site, which in turn might result in catalepsy. This might give further support to our findings. Furthermore, Farjo and Mcqueen (1979) have suggested that a reduction in the activity of striatal dopamine neurons might release the cortical neurons from striatal inhibition and perhaps potentiate epileptic charges in the

cortex. Thus, if this mechanism takes place in audiogenic convulsions, we would expect that the decreased striatal activity during sound stimulation would release the cerebral cortex from its inhibition and may contribute in part to the convulsions.

In conclusion, withdrawal related convulsions seem to increase the activity of noradrenergic neurons. The tone in dopaminergic neurons, on the other hand, was increased in the cerebral hemispheres and decreased in the striatum. When compared to withdrawn rats without sound stimulation, withdrawn convulsed rats had higher levels of dopamine and noradrenaline which might reflect an increase in the rate of synthesis of these amines during convulsions. A support for this suggestion comes from the findings of Calderini and his co-workers (1978) that sustained seizures increased the rate of uptake and hydroxylation of tyrosine.

When we extended our study on the mechanisms of convulsions by studying the turnover of catecholamines after α-methyl-p-tyrosine, we found that α-methyl-p-tyrosine protected most withdrawn rats from sound convulsions, although the rats which did not convulse showed wild running. This group of rats still showed less dopamine in their striata and cerebral hemispheres than the corresponding control group treated with α-methyl-p-tyrosine and subjected to audiogenic stimulation, but these values were higher than those of withdrawn rats with depleted catecholamine stores that had not been stressed by audiogenic stimulation. Moreover, noradrenaline was also reduced in amount in the midbrain of this group of rats, when compared with those of control group treated with α-methyl-p-tyrosine and subjected to audiogenic stimulation. The total amounts of depleted dopamine and noradrenaline in the brains of withdrawn rats which had been stressed by audiogenic stimulation were slightly higher than

in the brain of withdrawn rats with depleted catecholamines and which had not been stressed by audiogenic stimulation.

This indicates that catecholamine systems after α-methyl-p-tyrosine in sound stimulated withdrawn rats reacted in a manner between that seen during withdrawal and that in convulsions and may indicate that stimulation in protected rats activated in part the mechanisms we described as taking place during convulsions.

The protective effect of α-methyl-p-tyrosine on audiogenic seizures in rats withdrawn from phenobarbitone is surprising, since it is known that disruption of catecholamine systems either by inhibition of storage (Chen, Ensor and Bohner, 1954; Azzaro, Wenger, Craig and Stitzel, 1972; Wenger, Stitzel and Craig, 1973, and many others) or the destruction of catecholaminergic neurons by 6-hydroxydopamine (Browning and Maynert, 1970; Bourn, Chin and Picchioni, 1972; Browning and Simonton, 1978, and many others) usually results in an increased susceptibility of animals to chemically, electrically and sound induced convulsions. However, the results reported with the effect of α-methyl-p-tyrosine have been inconsistent. For whereas Chin, Ensor and Bohner (1968) reported that α-methyl-p-tyrosine lowered the extensor electroshock seizure threshold in mice, Jobe, Picchioni and Chen (1973) observed no change in susceptibility to audiogenic seizures in rats treated with this drug. Furthermore, the anticonvulsant effect of phenytoin against supramaximal electroshock was not modified by pretreatment with α-methyl-p-tyrosine (Bhattacharya, Reddy and Das, 1976). On the other hand, Wilkison and Halpern (1979) reported that α-methyl-p-tyrosine administered to rats twelve hours before grouped trial sessions significantly depressed the amygdalar after-discharge. Reserpine significantly augmented the amygdalar after-discharge, which is in line with other findings that disruption of catecholamine storage system

increases seizure activity. Our results were supported by those reported by Morgan, Pfeil and Gonzales (1978), who reported a protective effect of α-methyl-p-tyrosine against withdrawal related convulsions in barbitone dependent rats.

These conflicting results make the protective effect of α-methyl-p-tyrosine against audiogenic induced withdrawal convulsions more difficult to explain. However, there are several possibilities which might contribute to the observed effect. α-Methyl-p-tyrosine produces hypothermia and hypothermia below 28°C provides complete protection against chemically, electrically and sound induced seizures (Essman and Sudak, 1964). Hypothermia as well was reported to produce a marked decrease in the concentration of glutamate (which is believed to act as an excitatory neurotransmitter) in rat cerebral cortex (Tower, 1959).

The other possibility is that α-methyl-p-tyrosine itself might be metabolized to the corresponding catecholes (α-methyl noradrenaline and α-methyl dopamine) and that these might be released and act as false neurotransmitters at the catecholamine receptors and so provide a protection effect against audiogenic seizure. This view is supported by the finding of Maitre (1965) who reported that after treatment with α-methyl-p-tyrosine, α-methyl noradrenaline and α-methyl dopamine could be detected in the brain tissue of guinea pigs in amounts averaging 240 and 355 per cent respectively of that of the remaining noradrenaline. However, all these suppositions demand further investigation.

With the above arguments in mind, we should try to elucidate the functions of catecholaminergic systems during habituation to and withdrawal from phenobarbitone and during withdrawal convulsions. The simplest

hypothesis that can be given at this stage is that habituation to phenobarbitone decreases the turnover of catecholamines, most probably by decreasing flow in nerve impulses. When the drug is withdrawn, the depressant effect is removed and the neurons return to firing at a faster rate, resulting in increased turnover of transmitters. Withdrawal related convulsions would bring about an increase in the rate of synthesis and an increase in the activity of dopaminergic neurons in the cerebral hemispheres and noradrenergic ones in the whole brain. At the same time they evidently also produce a decrease in the activity of dopaminergic neurons in the striata.

Experiments in chapter 4 describe results obtained after long term administration of phenytoin, carbamazepine and sodium bromide on the content and turnover of catecholamines in rat brain. The most striking changes observed in rats treated with phenytoin and carbamazepine were the high increase in water intake and the increased urination produced by both drugs. Phenytoin is known to inhibit the central release of antidiuretic hormone in the human being (Fitchman and Kleeman, 1970) and probably acts by the same mechanism in rats leading to an increase in water intake to balance the water lost by the increased urination. The effect of carbamazepine, on the other hand, perhaps cannot be so easily explained. It seems probable that the effect of carbamazepine arises from its toxic effect on the kidneys of the treated rats. However, this is only a matter of speculation and a proper explanation is as yet impossible.

Neurochemically, long term administration of phenytoin and carbamazepine resulted in increased levels of noradrenaline in the cerebral

hemispheres and midbrain, while phenytoin also slightly increased the levels of the amine in the pons and medulla. While phenytoin increased the levels of dopamine in striata and midbrain and decreased it in the cerebral hemispheres, carbamazepine increased the levels of the amine in the striata and the cerebral hemispheres. The effect of phenytoin on the dopamine concentration in different parts of the brain is exactly the same as that obtained after long term administration with phenobarbitone. It is tempting to speculate, therefore, that phenytoin might have exerted its effect on dopaminergic systems by mechanisms similar to those described for phenobarbitone. The effect of phenytoin on noradrenaline levels, on the other hand, is different from that of phenobarbitone, which had no effect on noradrenaline levels but did delay the disappearance of noradrenaline from the cerebral hemispheres after α-methyl-p-tyrosine.

Phenytoin was reported by Azzaro, Gutrecht and Smith (1973) to inhibit monoamine oxidase. It produced a greater inhibition of monoamine oxidase type A, which prefers noradrenaline and serotonin as substrates (Yang and Neff, 1974) than it did with monoamine oxidase type B (Azzaro and Gutrecht, 1975). Phenytoin was reported as well to inhibit the calcium dependent potassium stimulated release of [3H] noradrenaline from rat brain slices (Pincus and Lee, 1973) and to block the uptake of radioactively labelled noradrenaline by rat brain synaptosomes under physiological conditions (Hadfield, 1972; Azzaro and Gutrecht, 1973). Phenytoin, therefore, seems to have different effects on noradrenaline neurones. It inhibits the intraneuronal degradation of noradrenaline, decreases its release and blocks the uptake of the released amine. The other possibility is that the catechol metabolite of phenytoin may compete with noradrenaline for 3-O-methylation thereby exerting a sparing

effect and prolonging the life of noradrenaline in the synaptic cleft.

The mechanisms we described above might explain our findings with regard to noradrenaline after long term administration of phenytoin.

Carbamazepine, on the other hand, had slightly different effects on the levels of dopamine in the cerebral hemispheres. This indicates that carbamazepine might have different mechanisms of action on dopamine activity in the cerebral hemispheres, especially when we remember that carbamazepine is a different class of anticonvulsant drug from those of phenytoin and phenobarbitone and resembles the tricyclic antidepressant agents. Furthermore, carbamazepine is more usually used in the treatment of grand mal epilepsy associated with depression. The difference in its effect on dopamine from those observed with phenytoin and phenobarbitone might be associated with this difference in therapeutic use.

The effect of carbamazepine on noradrenaline levels, on the other hand, resembles that of phenytoin. In fact there is a report which shows that carbamazepine shares some of the properties described for phenytoin. Purdy, Julien, Fairhurst and Terry (1977) found that a concentration of 10^{-4} of carbamazepine significantly inhibited both the release and the uptake of tritiated noradrenaline from rabbit brain synaptosomes, an effect which was described earlier for phenytoin (see above). Since the effect occurred at a concentration that is higher than the therapeutic dosage, the authors concluded that this effect was insufficient to account for an anticonvulsant effect in this drug. Nevertheless, these results may at least suggest a common effect of phenytoin and carbamazepine on the noradrenaline system.

In support of the observed effect of carbamazepine on the

noradrenaline system, imipramine, which is structurally related to carbamazepine, was reported to decrease the intensity of audiogenic seizures in genetically susceptible rats and strongly to antagonize the seizure enhancing effect of a benzoquinolizine derivative. It also restored the fluorescence in the hypothalamus (Jobe, Picchioni and Chin, 1973). This effect of imipramine was suggested by Jobe et al. to be caused by an accumulation of noradrenaline at the synaptic cleft, itself caused by blockade of uptake. This, it was proposed, would produce an anticonvulsant effect in response to central noradrenergic discharge. Therefore it is possible that carbamazepine produces its anticonvulsant effect by mechanisms similar to those described by Jobe et al. for imipramine.

Sodium bromide was tested for its effect on catecholamine systems just to compare the effect of modern antiepileptic drugs with an old fashioned one. Sodium bromide had virtually no effect on the catecholamine systems. It only produced a slight increase in the concentration of dopamine in the midbrain after its long term administration, and delayed the disappearance of dopamine after α-methyl-p-tyrosine. It had no effect on noradrenaline levels or turnover. Little can be made from these results.

In order still further to investigate our results, we examined the effect of long term administration of phenytoin and carbamazepine on the turnover rate of catecholamines after α-methyl-p-tyrosine. Phenytoin decreased the depletion of noradrenaline in the midbrain and (but only slightly and with a low degree of significance) in the cerebral hemispheres. This is in good agreement with the results obtained without α-methyl-p-tyrosine treatment where the predominant effect of phenytoin on noradrenaline

levels was observed in the midbrain. Carbamazepine decreased only the degree of depletion of noradrenaline in the cerebral hemispheres. Both drugs resisted the depletion of dopamine in the striata and cerebral hemispheres. The effect of phenytoin is similar to that obtained after long term administration of phenobarbitone, where the decreasing effect on dopamine concentration in the cerebral hemispheres was not seen after inhibition of synthesis by the administration of α-methyl-p-tyrosine. In fact there was a reversal of the effect, the drugs resisting the depletion of dopamine caused by the synthesis inhibitor. These observations further underline the similarity between the effects of phenytoin and those of phenobarbitone on the dopamine system. This is perhaps not surprising in view of the similarity in structure between the two drugs.

Elliott, Jenner, Chadwick, Reynolds and Marsden (1977) found that phenytoin produced a dose dependent inhibition of both apomorphine and amphetamine induced circling behaviour in rats with unilateral nigrostriatal lesions, an effect which was seen as well after L-DOPA treatment (Mendez, Cotzias, Mena and Papavasilion, 1975), suggesting that phenytoin produced a functional blockade of striatal dopamine activity. Such an effect would be expected to cause an increase in the turnover rates of dopamine and as well an increase in the concentration of dopamine metabolite due to the feedback activation of dopaminergic neurons. Neither could be detected by these authors. In fact, they reported a decrease in dopamine turnover and this occurred only with one dose and at one time interval (40 mg/kg after 2.5 hours). In our experiments single doses of phenytoin produced no change in the whole brain turnover of dopamine. There is a possibility that the catechol metabolite of phenytoin reported by Chang,

Okerholm and Glazko (1972) might have acted as a partial antagonist at dopamine receptors in the striatum and that this might have accounted for the blockade effect observed by Elliott and his colleagues.

In conclusion, we can say that phenytoin acted on dopaminergic system by mechanisms similar to those described for phenobarbitone. Carbamazepine, on the other hand, acted a little differently, but its major effects on dopamine resembled those of phenobarbitone.

Phenytoin acted on noradrenergic neurons by inhibiting monoamine oxidase, inhibiting release and as well inhibiting the uptake of the released amine. Its catechol metabolite would compete with catecholamines for 3-O-methylation, thus prolonging the life of the catecholamines at their receptor sites. Carbamazepine shares some of the properties of phenytoin by inhibiting the release and as well the uptake of noradrenaline and we suggested that they might have a common mechanism of action on the noradrenergic neurons.

II. Indoleamines and anticonvulsant drugs

The finding by Bonnycastle, Giarman and Pàasonen (1957) that a series of anticonvulsant drugs significantly elevated the brain 5-hydroxytryptamine content, opened a new era in the field of research into the mechanisms of action of anticonvulsant drugs. Although the original work by Bonnycastle et al. showed that elevation of the brain amine content by a monoamine oxidase inhibitor or an amine precursor provided no protection against leptazol convulsions, work carried out later by other workers provided the opposite results. Thus treatments which lower central levels of 5-hydroxytryptamine (by inhibiting its synthesis) have been

reported to lower the threshold for maximal electroshock seizures (Kilian and Frey, 1973) to intensify audiogenic seizures in genetically susceptible rats (Jerliez, Kostowski, Bidzinski and Hauptmann, 1978) and to potentiate leptazol convulsions in rats (Bhattacharya, Ghosh and Bose, 1978). Moreover, the anticonvulsant effect of phenobarbitone against electroshock seizures was inhibited by treatments that lowered the central 5-hydroxytryptamine content and potentiated by pre-treatment with both 5-hydroxytryptophan and L-DOPA (Meyer and Frey, 1973). It was also found that 5-hydroxytryptophan administered to baboons with photosensitive epilepsy, completely prevented photically induced myoclonus seizures (Meldrum, Balzamo, Wada and Cacciuttolo, 1972). In human beings, 5-hydroxytryptophan in combination with a peripheral decarboxylase inhibitor was found by Chadwick, Harris, Jenner, Reynolds and Marsden (1975) to be capable of bringing about a considerable improvement or a complete cessation of myoclonus. These patients had low levels of 5-hydroxyindoleacetic acid in their cerebrospinal fluid. That epilepsy might be associated with a deficiency in the aminergic system was also supported by several findings that the concentrationsof 5-hydroxyindoleacetic acid and homovanillic acid in the cerebrospinal fluid were reduced in adults and children with epilepsy (Papeschi, Molina-Negro, Sourkes and Erba, 1972; Shaywitz, Cohen and Bowers, 1975). Chadwick, Jenner and Reynolds (1975) reported an increase in the concentration of 5-hydroxyindoleacetic acid in the cerebrospinal fluid of epileptics receiving therapeutic doses of phenytoin and phenobarbitone. They also found a slight increase in the levels of homovanillic acid, but this did not reach statistical significance. On the other hand, Ito, Okuno, Mikawa and Osumi (1980) reported an increase in the levels of homovanillic acid in the cerebrospinal fluid of children with infantile spasm, status epilepticus or febrile convulsions. The first two groups were receiving anticonvulsant drugs in various combinations,

but Ito et al., strangely enough, excluded the possibility that the increased levels of homovanillic acid were a consequence of the anti-convulsant drug therapy, because the patients with febrile convulsions showed increased levels of homovanillic acid, although they had not been treated with any of the anticonvulsant drugs. Another factor which complicated this study is the fact that the cerebrospinal fluid samples were taken within a short time of a convulsive attack. We can conclude that these results in humans, supported by those observed in animals, provide some but not conclusive evidence that an anticonvulsant drug may exert its effect, in part at least, by modifying the serotoninergic mechanisms in the brain.

In our experiments on the effect of single doses of anticonvulsant drugs on the 5-hydroxytryptamine system, we found that only phenobarbitone increased significantly the levels of 5-hydroxytryptamine (5-HT) in the brain and this was only seen two and a half hours after its injection. Phenytoin, on the other hand, increased the amount of 5-hydroxyindoleacetic acid (5-HIAA) at all times studied, the maximum effect being observed half an hour after injection. Our results concerning the level of 5-HT after phenytoin are contradictory to those reported by Bonnycastle, Bonnycastle and Anderson (1962) and supported by other workers (Jenner, Chadwick, Reynolds and Marsden, 1975; Chadwick, Gorrod, Jenner, Marsden and Reynolds, 1978) who found an increase in cerebral 5-HT and 5-HIAA levels in mice treated with single doses of phenytoin. However, our results confirm those reported by Prokop, Shore and Brodie (1959) and Green and Grahame-Smith (1975), who found in rats that phenytoin in a single dose had no effect on the steady state levels of 5-HT.

The effect of phenytoin on the metabolite suggests either that it

causes an increase in the turnover of the amine or that it inhibits the acid transport system in the brain thus decreasing the efflux of the metabolite from the brain. These possibilities were examined in different experiments.

Phenytoin, phenobarbitone and carbamazepine, all reduced the depletion of 5-HIAA brought about by p-chlorophenylalanine (PCPA), phenytoin being the most effective in this respect. The proportional change produced by phenytoin in the level of the amine metabolite after PCPA was greater than that produced by phenytoin alone. This could suggest the possibility that phenytoin might have increased both the rate of synthesis and that of the breakdown of 5-HT (an effect which was suggested by Green and Grahame-Smith, 1975), thus resulting in higher values for 5-HIAA seen after the synthesis inhibitor. If this assumption is true, we would expect that phenytoin would elevate the levels of 5-HT if its degradation were blocked by a monoamine oxidase inhibitor. However, after pargyline, phenytoin did not produce any change on 5-HT levels, but still produced a decrease in the degree of depletion of 5-HIAA. It is difficult to explain this lack of effect of phenytoin after pargyline. There is a possibility that pargyline did not inhibit the monoamine oxidase completely and that the enzyme was still capable of metabolizing the amine, or alternatively that 5-HT might have been metabolized along other pathways. However, these are unlikely possibilities, particularly since phenytoin itself was reported to have some ability to inhibit monoamine oxidase (Azzaro, Gutrecht and Smith, 1973). On the other hand, the elevating effect of phenytoin on 5-HIAA after pargyline indicates that the drug does inhibit the acid transport of the metabolite out of the brain. This is supported by the fact that the same effect was also demonstrated after probenecid

which is known to block the acid transport system of the brain. These findings give support to the conclusion reached by Chase, Katz and Kopin (1969) that phenytoin produces a restraining effect on the efflux of 5-HIAA from the brain.

The results for carbamazepine are all consistent with each other, for the drug reduced the depletion of 5-HIAA after PCPA and pargyline and increased the accumulation of the metabolite after probenecit. These results all indicate that carbamazepine decreased the efflux of 5-HIAA from the brain.

The results obtained with phenobarbitone are not as consistent as those for phenytoin and carbamazepine. A single dose of phenobarbitone increased the 5-HT content of the brain 2.5 hours after its injection, but produced no effect on 5-HIAA. After pargyline, phenobarbitone decreased the rate of accumulation of 5-HT, which was in contrast with the results obtained by the drug only, but we should point out here that the measurement was made at a time when phenobarbitone on its own had no effect on the steady state levels of 5-HT. Phenobarbitone had no effect on the acid transport of the metabolite but decreased its depletion by PCPA.

Phenobarbitone was reported by Lidbrink, Corrodi and Fuxe (1974) not to change the steady state levels of 5-HT in either the cerebral cortex or the rest of the brain but to reduce significantly the degree of depletion of 5-HT caused by α-propyldopacetamide (a tryptophan hydroxylase inhibitor) in both areas. These workers claimed that this effect of phenobarbitone was due to reduced nervous activity in the 5-HT neurons.

The long term treatment with phenobarbitone increased significantly

the brain levels of both 5-HT and 5-HIAA. The percentage increase in the level of the amine was much higher than that of its metabolite. Several possibilities might be responsible for these effects. If we consider the effect on 5-HT first we can suggest that the long term administration of phenobarbitone might increase the rate of synthesis, decrease the rate of release or inhibit the intraneuronal degradation of the amine.

Among other neurotransmitters, serotonin is believed to be dependent for its release on intracellular calcium ions and inhibition of the efflux of calcium ions to the synaptic terminals would be expected to reduce transmitter release. A drug with this property would be expected to produce membrane stabilization.

Sohn and Ferrendelli (1976) provided data which shows that phenobarbitone and phenytoin, in concentrations similar to those used clinically for the treatment of epilepsy, inhibited the uptake of $^{45}Ca^{++}$ into potassium depolarized synaptosomes from rabbit neocortex. Richter and Jackson (1980) found that potassium stimulated release of [^{3}H]-5HT from rat midbrain slices was calcium dependent and that the release was inhibited by adding phenobarbitone to the medium. Thus phenobarbitone by decreasing the calcium influx into neurons would stabilize its membranes and reduce the release of 5-HT. This is a very likely mechanism, since phenobarbitone was demonstrated by Lidbrink, Corrodi, Fuxe and Olson (1972) to have no effect on monoamine oxidase, making it less likely that enzyme inhibition is responsible for the elevation in the levels of serotonin (5-HT) observed after habituation. The increased concentration of the metabolite might have been caused by an inhibitory effect of phenobarbitone on its elimination. Phenobarbitone was found by Chase, Katz and Kopin (1969) to retard the transfer of ^{14}C-labelled

5-HIAA out of the brain, a finding that supports our proposal.

We found that withdrawal of phenobarbitone brought down the levels of both the amine and its metabolite, a finding that can be explained on the same basis as those described for the effect on catecholamines, that is that withdrawal of the drug would relieve the serotoninergic neurons from the drug depressant effect, resulting in the neurons firing at a faster rate, thus reducing the levels of the amine in the stores. However, the levels of serotonin did not sink below control values, unlike the catecholamines after phenobarbitone withdrawal.

Withdrawal convulsions decreased the brain levels of serotonin and increased that of its metabolite. The percentage increase in the concentration of the metabolite was very much higher than the percentage decrease in that of the amine itself, which indicates that both the rate of synthesis and the rate of utilization of serotonin increased considerably during convulsions.

Calderini, Carlsson and Nordström (1978) showed that sustained seizure activity induced in rats by the intravenous injection of bicuculline produced an increase in the levels and hydroxylation of tryptophan, decreased the levels of serotonin during the first thirty minutes of sustained seizure activity and substantially increased the levels of 5-HIAA up to three hours after injection. These authors concluded that seizures induced by bicuculline lead to an increased functional activity in serotonin neurons, which resembles that observed in our experiments after withdrawal-related audiogenic seizures.

After the long term administration of phenytoin and carbamazepine, the levels of serotonin and of 5-HIAA were both increased.

Phenytoin produced identical percentage changes in the levels of both the amine and its metabolite. Phenytoin produced the same effect on the calcium efflux as did phenobarbitone (Sohn and Ferrendelli, 1976), thus reducing the release of 5-HT as a consequence of its stabilizing effect on the membrane. Phenytoin was also reported to have a monoamine oxidase inhibitor activity (Azzaro, Gutrecht and Smith, 1973), which might in part contribute to the effect on 5-HT observed after its long term administration. The elevating effect on 5-HIAA comes from the inhibitory effect of phenytoin on its elimination by the brain (Chase et al., 1969). The percentage changes produced by the long term administration of carbamazepine on the concentration of 5-HIAA was about double that produced in the levels of the parent amine, which might indicate that the inhibitory effect of carbamazepine on the acid transport of the metabolite is greater than that produced by phenytoin. The effect of carbamazepine on serotonin is similar to that of phenytoin and could have been brought about by similar mechanisms.

It seems probable from the foregoing discussion that serotoninergic mechanisms may be involved in part in the mechanisms by which anticonvulsant drugs act in the brain. Our results after acute doses are not as impressive as those seen after long term treatment with the anticonvulsant drugs. These results indicate that they altered the 5-hydroxytryptamine system and support the hypothesis that the effect of anticonvulsant drugs on 5-hydroxytryptamine may be in part responsible for their antiepileptic effect.

III. γ-Aminobutyric acid and anticonvulsant drugs

The biochemical factors that determine the amount of γ-aminobutyric acid (GABA) in the brain are the activity of the enzyme responsible for its synthesis (glutamic acid decarboxylase, GAD) and that responsible for its metabolism (GABA-aminotransferase, GABA-T).

In spite of many recent studies, the role of GABA in epilepsy is still a matter of controversy. Although agents that elevate brain GABA levels protect animals from convulsions, no clear cut relationship exists between the degree of elevation of the GABA levels in the brain and the extent of the anticonvulsant effect. Indeed, some drugs which elevate central GABA levels have convulsant activity (for review see Wood, 1975). This lack of relationship between GABA levels and the anticonvulsant effect was first demonstrated by Kuriyama, Roberts and Rubinstein (1966), who showed that the anticonvulsant action attributable to amino-oxyacetic acid was observed one and a half hours after its administration, at which time GABA levels were elevated, but no anti-convulsant effect was observed six hours after administration although the central GABA levels were still elevated at that time. Similar findings were reported by Crossland and Turnbull (1972), who found that the high dose of amino-oxyacetic acid (50 mg/kg) protected rats withdrawn from sodium barbitone from audiogenic seizures and increased the levels of GABA by twofold ninety minutes after injection. Smaller doses of amino-oxyacetic acid (20 mg/kg), which produced the same rise in GABA levels, produced no protection effect against audiogenic seizures. Crossland and Turnbull concluded that the amino-oxyacetic acid effect was associated with its generally depressant effect on motor functions.

Substances which depress central GABA levels, by inhibiting the enzyme required for its synthesis (GAD) lead to seizures (Meldrum, 1975) but again no clear relationship exists between the degree of inhibition of GAD and the onset of seizures (Wood, 1975).

It is an old observation that isoniazid causes convulsions and also that it depletes brain GABA levels and this is the starting point

of many later studies. Vernadakis and Woodbury (1960) showed that phenytoin increased the levels of GABA in the cerebral hemispheres of rat brain and decreased that of glutamate, and several later reports on the effect of anticonvulsant drugs on the levels of GABA in animal brain have appeared. The most significant report was probably that of Saad, Elmasry and Scott (1972), who found that single doses of phenytoin, phenobarbitone, methylphenobarbitone and mesontoin increased significantly the levels of GABA in the cerebral hemispheres of mice. The most interesting effect observed by these authors was that the anticonvulsant drugs produced a significant elevation in the amount of GABA in the cerebral hemispheres of animals treated with isoniazid and that this elevation was accompanied by a disappearance of the characteristic isoniazid convulsions. This finding was later supported by Löscher and Frey (1977), who found that phenytoin and phenobarbitone prevented the decrease of GABA levels in the brains of mice treated with isoniazid but did not effect the inhibition in GAD and GABA-T produced by the convulsant drug. However, Löscher and Frey (1977) excluded the possibility that the increase in GABA levels seen after phenytoin and phenobarbitone was responsible for their anticonvulsant effect, since phenytoin did not protect the animals against convulsions induced by picrotoxin, leptazol and bicuculline. On the other hand, these authors concluded that the anticonvulsant effect of diazepam, ethosuximide, trimethadone and sodium valproate may well be related to their effect on the metabolism of GABA, since they reversed the inhibition of GAD activity and elevated above the control values the depressed GABA levels in mice pretreated with isoniazid.

Insulin was also reported to lower cerebral GABA levels and of course it produces convulsions (Maynert and Kaji, 1962). Saad (1970) found that phenobarbitone, methylphenobarbitone and primidone protected

mice from insulin convulsions and raised the lowered GABA content of the cerebral hemispheres. Tunnicliff (1976) found that single doses of phenytoin did not change whole brain levels of GABA in rats.

In a more recent report, Patsalos and Lascelles (1981) found that long term treatment with phenytoin (10 days) decreased brain levels of GABA in the cerebellum, hypothalamus and striatum. By contrast, phenobarbitone produced an elevation in GABA in all brain regions and sodium valproate had a similar effect everywhere except in the striatum. When phenytoin was administered with phenobarbitone, elevated GABA levels were found. Patsalos and Lascelles (1981) concluded that these anticonvulsant drugs had different actions on the GABA system.

Abdul-Ghani, Coutinho-Netto, Druce and Bradford (1981) have recently put forward the suggestion that anticonvulsant drugs might produce their effects partially or entirely by raising GABA content or by causing a rise in the concentration of this inhibitory agent in the extracellular fluid of the brain by inhibiting the uptake of GABA following its release. They tested the second possibility by following the *in vivo* and *in vitro* release of GABA and found that acute doses of phenobarbitone, phenytoin, carbamazepine and sodium valproate caused no detectable release of endogenous GABA *in vivo* or of exogenous GABA *in vitro*. These authors suggested that there is no correlation between anticonvulsant activity of these drugs and changes in GABA accumulation extracellularly. We should point out here that both phenytoin and pentobarbitone were reported to increase the high affinity uptake of GABA in synaptosomes of the central nervous system of rats (Weinberger and Nichlas, 1976 and Weinberger, Nichlas and Berl, 1976). These observations are not entirely consistent with the hypothesis put forward by Abdul-Ghani and his colleagues, for they imply that the drugs decrease, rather than increase, GABA levels in the extracellular fluid of the brain.

In our experiments, single doses of phenobarbitone increased the total brain levels of GABA thirty and sixty minutes after its injection. Thereafter the levels of GABA returned to their normal values. On the other hand, neither phenytoin nor carbamazepine had any effect either thirty or sixty minutes after their injection.

Our results with respect to phenobarbitone are in agreement with those of Saad et al. (1972) and of Patsalos and Lascelles (1981) for we could not observe any effect after phenytoin or carbamazepine. It is worth noticing, however, that Saad et al. (1972) found an effect of phenytoin on GABA levels three hours after its administration, a time at which we did not measure the effect of the drug.

Unfortunately, we did not have time to extend our experiments any further, and little can be concluded from our observation on the effect of phenobarbitone on GABA levels, but it may be relevant to point out that pentobarbitone was reported by Weinberger and Nichlas (1976) to increase twofold the active uptake of GABA into rat brain synaptosomes. Whether phenobarbitone produced its elevation effect on GABA by a similar mechanism remains only a speculation and is offered as a suggestion for further research in this topic.

Before we end our discussion on the role of GABA in the anticonvulsant effect of antiepileptic drugs, we would like to point to the work reported by Wood, Russel, Kurylo and Newstead (1979), who suggested that the synaptosomal-enriched fraction of rat brain could be used as a model to evaluate drug induced changes in GABA levels in nerve endings. They concluded from in vivo experiments that hydrazine, isonicotinic acid hydrazide and amino-oxyacetic acid brought about similar decrease in the GABA content of the synaptosomal-enriched fractions prepared from the

brains of rats taken at the onset of seizure, despite the fact that no correlation was observed between seizure activity and whole brain GABA levels. Although the hypothesis that the levels of GABA at nerve endings, and not whole brain GABA levels, determine seizure susceptibility was suggested a long time ago, no experimental evidence supporting this hypothesis was available until the work of Wood and his co-workers. It would be interesting to note what this model will provide in the future as a key to a new field of research into the mechanism of action of anticonvulsant drugs.

IV. Cobalt induced epilepsy and neurotransmitters

We saw in Chapter 9 that the insertion of cobalt stick or cobalt wire into the motor cortex led to a reproducible seizure state characterized by the slow onset and prolonged duration of an epileptic-like encephalograph pattern in the side of the implantation as well as in the contralateral cerebral hemisphere. In addition, myoclonic jerks were obvious on the side contralateral to the implantation. We did not see any generalized convulsions even after strong sound stimulation. Cobalt induced seizures in rats might seem therefore to be a useful experimental model for determining the aetiology of the seizure state. The idea behind our experiments with cobalt was to see whether the prolonged seizure activity produced by cobalt implantation would bring about any change in the neurotransmitter levels we studied and to compare any that were found with those obtained after the brief seizures provoked by audiogenic stimulation after ceasing the long term administration of phenobarbitone.

Several reports in the literature indicate that cobalt implantation in the motor cortex produces changes in some neurotransmitters and

in the enzymes related to their synthesis and degradation. Craig and Hartman (1973) reported that cobalt wire implantation in rats' parietal cortex decreased the levels of glutamic acid, aspartatic acid and taurine and increased that of lysine and alanine at the site of the lesion. Emson and Joseph (1975) demonstrated that cobalt gelatine sticks implanted in the frontal cortex of rats produced a decrease in the levels of GABA, glutamate and aspartate in the lesion and primary focal areas five to ten days after implantation. These authors excluded the hypothesis that spike activity in the cortex is associated with selective elevation of excitatory amino acids or selective reduction of inhibitory amino acids since glutamate and aspartate (known as excitatory amino acids) as well as GABA (an inhibitory amino acid) were all reduced in the primary focus. Furthermore, no change was observed in these amino acid levels in the secondary focus. On the other hand, it must be noted that Dodd, Bradford, Abdul-Ghani, Cox and Continho-Netto (1980) demonstrated that superfusion with an in vivo cannula system in the brain of rats implanted with perforated discs of cobalt produced an increased release of glutamate, valine and glycine in the lesioned side when compared with unlesioned tissue. Unfortunately, they could not detect GABA in their samples. Bradford and his colleagues excluded the possibility that the increased release of glutamate was caused by tissue damage, since another metal (nickel) produced an equivalent amount of tissue damage but little epileptic activity and little release of glutamate. Thus the glutamate released at the cobalt focus could well be responsible for the hyperactivity.

Balcar, Pumain, Mark, Borg and Mandel (1978) reported that the high affinity uptake of glutamate in the epileptogenic focus of rats implanted with cobalt powder eight to ten days earlier was unchanged, while that of GABA was significantly lower than that in the non-implanted controls or in

those that had received an implant on the contralateral side. Cobalt itself had no effect on GABA or glutamate uptake. These observations are not, of course, entirely consistent with those of Bradford and his co-workers. This effect of cobalt implantation on GABA high affinity uptake in the primary focal area was confirmed in a recent report by Ross and Craig (1981).

Whole brain levels of noradrenaline, dopamine and 5-hydroxytryptamine of rats at various times after implantation of cobalt powder were reported to remain unchanged (Colasanti and Craig, 1973). The turnover rates of noradrenaline and dopamine remained unaltered in the epileptic rats, while 5-hydroxytryptamine metabolism showed complicated changes. These authors concluded that the involvement of brain biogenic amines in the development and maintenance of chronic epileptic states differs markedly from that which would be expected in view of their apparently well established roles as neurotransmitters.

The enzymes associated with the synthesis and metabolism of neurotransmitters were also reported to be changed by cobalt implantation. Thus, the activity of the enzyme responsible for acetylcholine synthesis (cholinacetyltransferase) and for its hydrolysis (cholinesterase) were both reported to be depressed in the primary focus area in the brain of rats implanted one week earlier with cobalt gelatin sticks (Goldberg, Pollock, Hartman and Craig, 1972). The same results were reported by Emson and Joseph (1975), who found as well a decrease in the levels of glutamate decarboxylase and aromatic amino acid decarboxylase in the lesion area and primary and secondary foci produced four to eight days after implantation of cobalt gelatin stick. The levels of these enzymes returned to normal by twenty-four days after operation. The same was reported for γ-aminobutyrate aminotransferase (Clayton and

Emson, 1975). In this same paper, Clayton and Emson reported that the activity of the enzyme responsible for the synthesis of catecholamines (tyrosine hydroxylase) was inhibited, reaching its maximum by the sixth day after cobalt implantation in both the primary and secondary foci and remaining so up to seventy-five days after implantation. The same was seen for the catecholamine degrading enzymes (monoamine oxidase and catechol-O-methyl transferase) which were inhibited to the same degree as tyrosine hydroxylase. Clayton and Emson suggested that the long term inhibition of the enzymes may reflect a slow rate of repair of noradrenergic neurons. Emson (1976) has reported that the acute treatment with amino-oxyacetic acid of rats with cobalt implantation produced a reduction in the spiking activity in both the primary and secondary focus five to ten minutes after injection and persisting for thirty to ninety minutes. These doses of amino-oxyacetic acid (20 - 60 mg/kg), which produced marked sedation and inhibition of motor activity, also produced an elevation of brain GABA, but the author found no correlation between GABA content and spike activity. Sodium valproate had no effect on spike frequency. Chronic treatment with low doses of amino-oxyacetic acid (25 - 5 mg/kg) reduced the epileptiform spike frequency which was most striking in the secondary focus and it produced an inverted dose effect relationship, the lower doses having the largest effect. On the other hand, the compound produced a dose dependent increase in the levels of GABA and a decrease in the activity of GAD and GABA-T. Sodium valproate had no effect on the development or the numbers of spikes in the cobalt focus despite the elevation in GABA levels. The author concluded that the anticonvulsant action of sodium valproate and probably of amino-oxyacetic acid is not likely to be mediated through mechanisms involving elevation of brain GABA.

It has been suggested that dopamine might play an antiepileptic role in cobalt epilepsy. Farjo and Mcqueen (1979) found that dopamine agonists

(apomorphine, lisuride and bromocriptine) inhibited spike activity in established primary and secondary foci in a dose dependent manner, while a dopamine antagonist (pimozide) had the opposite effect and blocked the antiepileptic effect induced by dopamine agonists. Because the intra-striatal injection of dopamine or apomorphine suppressed epileptiform spikes in the cortex and the destruction of striatal catecholaminergic terminals by 6-hydroxydopamine increased the spike activity, Farjo and Mcqueen concluded that the striatum may play an essential part in mediating the antiepileptic effect of dopamine and its agonists in the cobalt model of epilepsy.

In our experiments with cobalt, GABA levels were decreased only in the implanted side of the brain and only one week after the implantation of either cobalt sticks or cobalt wire. These findings are in agreement with those reported by Emson and Joseph (1975), who reported a decrease in GABA levels in the lesion and primary focal area five to ten days after cobalt stick implantation. This decrease in GABA levels could be the result of neuronal damage caused by cobalt or to a decreased synthesis or increased utilization of the aminoacid. The lack of any effect on GABA in the implanted side of the brain two or three weeks after implantation argues against the possibility that the neuronal damage was the cause of the decreased GABA levels, since at that time the tissue damage was most marked. The possibility of decreased synthesis is supported by the fact that the activity of GAD was reported to be inhibited at the same time as we found that the GABA had reached its minimum level. The GAD activity recovered to the control values by the third week (Emson and Joseph, 1975). On the other hand, the decreased GABA levels in the implanted side might possibly be due to an inability of the tissue surrounding the lesion to take up the released GABA as reported by Baclar and his co-workers (1978). However, this too depends on the notion that the decrease was attributable to a loss of neuronal tissues.

While dopamine levels remained unchanged in the brains of rats one, two or three weeks after cobalt implantation, noradrenaline levels were decreased in the implantation side and in the contralateral half of the brain one week after the implantation of cobalt sticks. Cobalt wire decreased the levels in the implanted side only. In the second week the decreased noradrenaline content was seen only in rats implanted with cobalt sticks and only in the implanted half of the brain. No change was seen in the noradrenaline levels three weeks after the implantation of either cobalt wire or sticks.

The change in noradrenaline levels in the secondary focus seen one week after cobalt stick implantation is of particular interest, as this area is free of the major necrotic changes caused by cobalt. The mechanisms by which this decrease took place are difficult to assess, since the enzymes involved in the biosynthesis and in the intra- and extracellular degradation of noradrenaline are all reported to be inhibited in the primary and secondary focus (Clayton and Emson, 1975). There is a possibility that increased release with a decreased synthesis due to the inhibition of tyrosine hydroxylase may be a contributing factor.

While 5-hydroxytryptamine levels did not change one, two or three weeks after implantation of either cobalt wire or stick, its major metabolite (5-hydroxyindoleacetic acid) was increased by both cobalt wire and cobalt sticks in the implanted half of the brain one week after implantation. Cobalt wire produced a significant increase in the level of the indole in the contralateral half of the brain as well.

Two weeks after implantation, cobalt sticks produced a significant increase in the concentration of the indole in both halves of the brain, while cobalt wire did so in the implanted side only.

As monoamine oxidase was reported to be inhibited by the cobalt implantation (Clayton and Emson, 1975), this increase in 5-HIAA could not be explained simply by an increase in the turnover rate of 5-hydroxy-tryptamine. On the other hand it may be so. However, there is a possibility that cobalt produced damage to the blood brain barrier which in turn affected the acid transport of the indole and contributed to the observed rise in 5-HIAA.

If we compare the results obtained with the cobalt model just discussed with those obtained after withdrawal from phenobarbitone, we can conclude that the mechanisms involved in the production of the chronic epileptic state differ markedly from those operating in the production of the acute state. However, although the cobalt model produced peripheral changes characterized by myoclonic jerks on the contralateral side to the implantation and electroencephalograph manifestation resembling those obtained from patients with grand mal epilepsy, we never saw the generalized convulsions of the clonic-tonic type (even after strong sound stimulation) that were seen in rats withdrawn from phenobarbitone. The cobalt model, of course, carries the advantage of giving a chronic effect which is not the case with convulsions resulting from phenobarbitone withdrawal but we believe that withdrawal convulsions the more closely resemble those seen in grand mal epilepsy. Moreover, no interference with brain structure took place,as is the case with the cobalt model.

V. Conclusion

Neurotransmitters represent the chemical substances that mediate communication at synapses. Therefore the elucidation of the effect of anticonvulsant drugs on these neurotransmitters would probably provide meaningful insights into the biochemical mechanisms by which anticonvulsant

drugs exert their effects on the central nervous system. The most significant results in our research are those obtained for the effect of anticonvulsant drugs on monoamine systems. We saw that the long term administration of phenobarbitone, phenytoin and carbamazepine produced an increase in the levels of monoamines in certain areas of the brain. We have proposed that phenobarbitone produced these effects by inhibiting neuronal activity of the monoamine systems. Phenytoin, on the other hand, produced its effect probably by inhibiting the intra-neuronal degradation of the monoamines and may also share some of the properties described for phenobarbitone. Carbamazepine was reported to share some of the effects exerted by phenytoin - inhibiting the release of noradrenaline and inhibiting the re-uptake of the released amine - and we have suggested a common mechanism of action between phenytoin and carbamazepine.

Thus it is evident that anticonvulsant drugs cause the accumulation of noradrenaline, dopamine and 5-hydroxytryptamine in certain areas of the brain. These probably take place at the nerve endings and since the monoamines are believed to act as inhibitory transmitters it is possible that part of the effect of these anticonvulsant drugs may be mediated through an effect on these monoamines, first by accumulating the amine at the nerve endings of certain areas of the brain, which upon stimulation would release this amine in sufficient quantity to exhibit inhibition on excitated neurons and secondly by inhibiting the uptake of the released amine thus prolonging its effect at the synapse, as suggested for phenytoin and carbamazepine. Now, the question that might be asked is whether these effects are genuinely an anticonvulsant effect and whether it is possible to replicate these effects in genuinely epileptic animals. This is our proposal for further work in this field. A step

in this direction was taken when we began to study a model of chronic epilepsy in rats using cobalt as the epileptogenic agent. The study of the effect of anticonvulsant drugs on the monoamine system in these animals (especially in the secondary focus which is free from the direct effect of cobalt) should provide at least a part answer to the questions we have posed.

It certainly seems to be the fact that these monoamines are involved in the manifestation of the epileptiform withdrawal convulsions from phenobarbitone. We have suggested that there is an increase in the rate of synthesis and an increased activity of dopaminergic neurons in the cortex and of noradrenergic neurons in whole brain and a decreased activity of dopaminergic neurons in the striatum during convulsions, though these may represent the effect rather than the cause of convulsions. Further work on this point is necessary to study the extent to which the rate of synthesis and utilization of monoamines have been affected in phenobarbitone withdrawal convulsions.

γ-Aminobutyric acid is another neurotransmitter of interest. We have used a very sensitive method for its assay and the next step for studying the GABA system is to look for the effect of anticonvulsant drugs on GABA at the synaptic levels, using the model suggested by Wood and his co-workers (1979). Interestingly, Wood used the same principle for assaying GABA as that we adopted.

APPENDIX I

FORMULARY

Chapter 1

Reagents and buffer used for the formation of noradrenaline fluorophore

Acetate buffer at pH7

Sodium acetate	20.5 gms
1.0 M hydrochloric acid	Q.S.
Distilled water	to 250 ml

The sodium acetate was dissolved in about 220 ml of the water and the acidity of the solution was adjusted to pH 7.0 with the hydrochloric acid. The solution was diluted to 250 ml with more water.

Ferricyanide solution

Potassium ferricyanide	125 mg
Distilled water	to 50 ml

Alkali ascorbate solution

2% Ascorbic acid solution	5.0 ml
Ethylenediamine	1.0 ml
5.0 M Sodium hydroxide solution	to 50 ml

Reagents and buffers used for the formation of dopamine fluorophore

0.5 M Phosphate buffer at pH7

Sodium dihydrogen phosphate 19.50 gm. dissolved in 250 ml distilled water

Disodium hydrogen phosphate 17.74 gm. dissolved in 250 ml distilled water

Sodium dihydrogen phosphate solution was added gradually to the disodium hydrogen phosphate solution until pH 7 was obtained.

Periodate solution	Always freshly prepared before use
Sodium periodate	50 mg
Distilled water	to 10 ml

Alkali sulphite solution	Always freshly prepared before use
Anhydrous sodium sulphite	500 mg
Distilled water	4 ml
5.0 M sodium hydroxide solution	to 20 ml

0.5 M citrate buffer at pH4

Citric acid	26.27 gms.
5.0 M Sodium hydroxide solution	Q.S.
Distilled water	to 250 ml

The citric acid was dissolved in about 230 ml of the distilled water and the acidity of this solution was adjusted to pH 4.0 with the sodium hydroxide solution. The solution was then diluted to 250 ml with more distilled water.

Chapter 5

Buffers used for the assay of 5-hydroxytryptamine and 5-hydroxyindole-acetic acid

0.5 M Borate buffer at pH 10

Boric acid	6.18 gms
5 M Sodium hydroxide solution	Q.S.
Distilled water	to 200 ml

The boric acid was dissolved in about 190 ml of the distilled water and the solution was adjusted to pH 10 with the sodium hydroxide solution. The solution was then diluted to 200 ml with distilled water.

0.1 M Salt saturated Borate buffer at pH 10

Boric acid	2.47 gms
5 M Sodium hydroxide solution	Q.S.
Distilled water	to 400 ml

Prepared as 0.5 M borate buffer but sodium chloride was added with shaking till saturation. The buffer was adjusted to pH 10 before use.

0.05 M Phosphate buffer at pH 7

Sodium dihydrogen phosphate	1.95 gm dissolved in 250 ml distilled water
Disodium hydrogen phosphate	1.77 gm dissolved in 250 ml distilled water

Prepared as described before.

0.1 M Phosphate buffer at pH 7

Sodium dihydrogen phosphate	3.90 gm dissolved in 250 ml distilled water
Disodium hydrogen phosphate	3.55 gm dissolved in 250 ml distilled water

Prepared as described before.

Chapter 8.2

Reagents and buffers used for the enzymatic assay of GABA

0.1 M Sodium pyrophosphate buffer at pH 8.4

Sodium pyrophosphate	4.46 gm
1.0 N Hydrochloric acid	Q.S.
Distilled water	to 100 ml

The sodium pyrophosphate was dissolved in about 90 ml of the distilled water. The solution was adjusted to pH 8.4 with the hydrochloric acid solution and brought up to a final volume of 100 ml with distilled water. The solution was then treated with Norit-A and filtered to remove fluorescent background.

Reconstitution of GABAse

GABAse is a partially purified cell-free preparation from Pseudomonas fluorescens containing GABA-α-ketoglutarate transaminase and succinic semi-aldehyde dehydrogenase, purchased from Sigma Chemical Company.

Ten units of the enzyme were dissolved in its container with 5 ml of 0.075 M phosphate buffer at pH 7.2 containing 25% (V/V) glycerol. While the container was in ice bath, the enzyme solution was divided into 50 portions of 100 µl each in 2 ml volume test tubes. The test tubes were capped with Parafilm and kept in deep freeze at a temperature below $-20^{\circ}C$. One tube was used for the construction of enzyme reaction mixture each time the assay of GABA was performed. The phosphate buffer used for the reconstitution of the enzyme was 0.585/50ml sodium dihydrogen phosphate, to which a solution of 0.532/50 ml disodium hydrogen phosphate was added to pH 7.2, followed by the addition of 25% (V/V) glycerol.

Solutions used for the construction of enzyme reaction mixture

All freshly prepared

NADP 1 mM

1 mg was dissolved in 1.2 ml distilled water

2-Mercaptoethanol

50 µl was dissolved in 7 ml distilled water

0.06 M α-ketoglutarate

88 mg was dissolved in 9 ml of pyrophosphate buffer, adjusted to pH 7.9 and brought up to ten millilitres with distilled water.

The construction of enzyme reaction mixture

To each tube containing 0.1 ml of GABAse solution, one millilitre of pyrophosphate buffer was added, followed by 0.2 ml of NADP solution, 0.2 ml of α-ketoglutarate solution and 0.2 ml mercaptoethanol solution.

The mixture was mixed thoroughly and kept in ice bath until it was used. For enzyme FREE reaction mixture used for the tissue blanks, 0.1 ml of the pyrophosphate buffer was used to replace the 0.1 ml GABAse solution.

Phosphate solution was prepared by dissolving 15.2 gms sodium phosphate ($NA_3PO_4 12H_2O$) and 5.3 gm disodium hydrogen phosphate in 100 ml water ($0.4\ M\ PO_4 \equiv 0.2\ M\ HPO_4$)

Alkali peroxide was prepared immediately before use by diluting 100 µl of 3% hydrogen peroxide to 10 ml with 10 N sodium hydroxide solution.

APPENDIX II

REFERENCES

ABDUL-GHANI, A.S., COUTINHO-NETTO, J., DRUCE, D. & BRADFORD, H.F. (1981) Effects of anticonvulsants on the *in vivo* and *in vitro* release of GABA. Biochem.Pharmacol., 30, 363-368.

ALDERMAN, J.L. & SHELLENBERGER, M.K. (1973) γ-Aminobutyric acid (GABA) in the rat brain: re-evaluation of sampling procedures and the post-mortem increase. J.Neurochem., 22, 937-940.

ANDEN, N.E., CORRODI, H., FUXE, K. & UNGERSTEDT, V. (1971) Importance of nervous impulse flow for the neuroleptic induced increase in the amine turnover in central dopamine receptors. Eur.J.Pharmacol., 15, 193-199.

ANLEZARK, G.M., HORTON, R.W. & MELDRUM, B.S. (1978) Dopamine agonists and reflex epilepsy. Advances in biochemical psychopharmacology, 19, 383-388.

ANTON, A.H. & SAYRE, D.F. (1962) A study of the factors affecting the aluminium oxide trihydroxyindole procedure for the assay of catecholamines. J.Pharmacol.Exp.Ther., 138, 360-375.

ANTON, A.H. & SAYRE, D.F. (1964) The distribution of dopamine and DOPA in various animals and a method for their determination in diverse biological material. J.Pharmacol.Exp.Ther., 145, 326-336.

AZZARO, A.J. & GUTRECHT, J.A. (1975) Characteristics of the inhibitory action of diphenylhydantoin on brain monoamine oxidase. Neurology, 25, 378.

AZZARO, A.J., GUTRECHT, J.A. & SMITH, D.J. (1973) Effect of diphenylhydantoin on the uptake and catabolism of L[^{3}H]norepinephrine *in vitro* in rat cerebral cortex tissue. Biochem.Pharmacol., 22, 2719-2729.

BELL, L.J., IVERSEN, L.L. & URETSKY, N.I. (1970) Time course of the effects of 6-hydroxydopamine on catecholamine-containing neurons in rat hypothalamus and striatum. Brit.J.Pharmacol., 40, 490-499.

AZZARO, A.J., WENGER, G.R., CRAIG, C.R. & STITZEL, R.E. (1972) Reserpine-induced alterations in brain amines and their relationship to changes in the incidence of minimal electroshock seizures in mice. J.Pharmacol.Exp.Ther., 180, 558-568.

BACLAR, V.J., PUMAIN, R., MARK, J., BORG, J. & MANDEL, P. (1978) GABA-mediated inhibition in the epileptogenic focus, a process which may be involved in the mechanisms of the cobalt induced epilepsy. Brain Research, 154, 182-185.

BHATTACHARYA, S.K., GHOSH, P. & BOSE, R., (1978) Pentylenetetrazole induced clonic convulsions in rat. Role of brain monoamines. Materia Medica Polona, 10(3), 184-187.

BHATTACHARYA, S.K., REDDY, P.K. & DAS, P.K. (1976) Studies on the role of brain monoamines in the anticonvulsant action of diphenyl-hydantoin and nialamide-induced potentiation of diphenyhydantoin in albino rats. In: Drugs and central synaptic transmission (Bradley, P.B. & Dhawan, B.W., Eds.) pp.155-164. The Macmillan Press, Ltd.

BLOM, S. (1962) Trigeminal neuralgia: its treatment with new anti-convulsant drug (G.32883). Lancet 1, 839-840.

BONNYCASTLE, D.D., BONNYCASTLE, M.F. & ANDERSON, E.G. (1962) The effect of a number of central depressant drugs upon brain 5-hydroxy-tryptamine levels in the rat. J.Pharmacol.Exp.Ther., 135, 17-22.

BONNYCASTLE, D.D., GIARMAN, N.J. & PAASONEN, M.K. (1957) Anticonvulsant compounds and 5-hydroxytryptamine in rat brain. Br.J.Pharmacol., 12, 228-231.

BOURN, W.M., CHIN, L. & PICCHIONI, A.L. (1972) Enhancement of audiogenic seizure by 6-hydroxydopamine. J.Pharm.Pharmac., 24, 913-914.

BREESE, G.R. & TRAYLOR, T. (1970) Effect of 6-hydroxydopamine on brain norepinephrine and dopamine: evidence for selective degeneration of catecholamine neurons. J.Pharmacol.Exp.Ther., 174, 413-420.

BROWNING, R.A. & MAYNERT, E.W. (1970) Increased seizure susceptibility in 6-hydroxydopamine treated rats. Fed.Proc., 29, 699.

BROWNING, R.A. & SIMONTON, R.L. (1978) Antagonism of the anticonvulsant action of phenytoin, phenobarbital and acetazolamide by 6-hydroxydopamine. Life Sci., 22, 1921-1930.

BUSH, M.T. (1961) Extraction and identification of barbiturates. Microchem.J., 5, 73-90.

CALDERINI, G., CARLSSON, A. & NORDSTRÖM, C.H. (1978) Monoamine metabolism during bicuculline induced epileptic seizure in the rat. Brain Research, 157, 295-302.

CHADWICK, D., GORROD, J.W., JENNER, P., MARSDEN, C.D. & REYNOLDS, E.H. (1978) Functional changes in cerebral 5-hydroxytryptamine metabolism in the mouse induced by anticonvulsant drugs. Br.J.Pharmacol., 62, 115-124.

CHADWICK, D., HARRIS, R., JENNER, P., REYNOLDS, E.H. & MARSDEN, C.D. (1975) Manipulation of brain serotonin in the treatment of myoclonus. The Lancet, 6, 434-435.

CHADWICK, D., JENNER, P., & REYNOLDS, E.H. (1975) Amines, anticonvulsants and epilepsy. Lancet, I, 473-476.

CHANG, T., OKERHOLM, R.A. & GLAZKO, A.J. (1972) A 3-O-methylated catechol metabolite of diphenylhydantoin (Dilantin) in rat urine. Res. Commun.Chem.Path and Pharmacol. 4(1), 13-23.

CHASE, T.N., KATZ, R.I. & KOPIN, I.J. (1969) Effect of anticonvulsants on brain serotonin. Trans.Am.Neurol.Assoc., 94, 236-238.

CHEN, G., ENSOR, C.R. & BOHNER, B. (1954) A facilitation action of reserpine on the central nervous system. Proc.Soc.exp.Biol.Med., 86, 507-510.

CHEN, G., ENSOR, C. & BOHNER, B. (1968) Drug effects on the disposition of active biogenic amines in the central nervous system. Life Sci., 7, 1063-1074.

CLAYTON, P.R. & EMSON, P.C. (1975) Changes in monoamine related enzymes in cobalt induced epilepsy. Biochem.Soc.Trans., 3, 261-263.

COLASANTI, B.K. & CRAIG, C.R. (1973) Brain concentrations and synthesis rates of biogenic amines during chronic cobalt experimental epilepsy in rat. Neuropharmacology, 12, 221-231.

CONNEY, A.H., DAVISON, C., GASTEL, R. & BURNS, J.J. (1960) Adaptive increases in drug-metabolizing enzymes induced by phenobarbitone and other drugs. J.Pharmacol.Exp.Ther., 130, 1-8.

COOKE, S.S. (1978) Brain folate and anticonvulsive activity. PhD thesis, University of Nottingham.

COOPER, J.R., BLOOM, F.E. & ROTH, R.H. (1978) The biochemical basis of neuropharmacology. pp. 179-181. Oxford University Press.

CORRODI, H., FUXE, K. & HÖKFELT, T. (1966) The effects of barbiturates on the activity of the catecholamine neurons in the rat brain. J.Pharm.Pharmacol., 18, 556-558.

CORRODI, H., FUXE, K., LIDBRINK, P. & OLSON, L. (1971) Minor tranquilizers, stress and central catecholamine neurons. Brain Research, 29, 1-16.

COSTA, E. (1972) Studies of neurotransmitters at the synaptic level. Advances in behavioural psychopharmacology, 6, 182. Raven Press, New York.

CRAIG, C.R. & HARTMAN, E.R. (1973) Concentrations of amino acids in the brain of cobalt epileptic rat. Epilepsia, 14, 409-414.

CROSSLAND, J. (1971) Neurohumoral substances and drug abstinence syndrome. In: Advances in neuropharmacology (Vinar O. et al., Eds) pp.497-507 N.Holland, Amsterdam.

CROSSLAND, J. & LEONARD, B.E. (1963) Barbiturate withdrawal convulsions in the rat. Biochem.Pharmacol., 12 (Supp.), 103.

CROSSLAND, J. & TURNBULL, M.J. (1972) The effect of the chronic administration of barbitone on the growth rate of the rat. Neuropharmacology, II, 163-176.

CROSSLAND, J. & TURNBULL, M.J. (1972) γ-Aminobutyric acid and barbiturate abstinence syndrome in rats. Neuropharmacology, II, 733-738.

DE VOS, C.J. & BONTA, I.L. (1964) A simple permanent electrode set for tracing cortical electrograms in the rat. Arch.Int.Pharmacodyn., 147, 280-284.

DILL, W.A. & GLAZKO, A.J. (1972) Fluorometric assay of diphenylhydantoin in plasma or whole blood. Clin.Chem., 18(7), 675-676.

DILL, W.A., LEUNG, A., KINKEL, A.W. & GLAZKO, A.J. (1976) Simplified fluorometric assay for diphenylhydantoin in plasma. Clin.Chem., 22(6), 908-911.

DODD, P.R., BRADFORD, H.F., ABDUL-GHANI, A.S., COX, D.W.G. & CONTINHO-NETTO, J. (1980) Release of amino acids from chronic epileptic and subepileptic foci in vivo. Brain Research, 193, 505-517.

DOLPHIN, A., JENNER, P. & MARSDEN, C.D. (1975) Interference by α-methyl-p-tyrosine with three procedures for the assay of cerebral catecholamines. J.Neurochem., 25, 897-898.

DORRITY, F. & LINNOILA, M. (1976) Rapid gas-chromatographic measurement of anticonvulsant drugs in serum. Clin.Chem., 22(6), 860-862.

ELLIOTT, P.N.C., JENNER, P., CHADWICK, D., REYNOLDS, E. & MARSDEN, C.D. (1977) The effect of diphenylhydantoin on central catecholamine containing neuronal systems. J.Pharm.Pharmacol., 29, 41-43.

EMSON, P.C. (1976) Effect of chronic treatment with aminooxyacetic acid or sodium n-dipropylacetate on brain GABA levels and the development and regression of cobalt epileptic foci in rats. J.Neurochem., 27, 1489-1494.

EMSON, P.C. & JOSEPH, M.H. (1975) Neurochemical and morphological changes during the development of cobalt induced epilepsy in the rat. Brain Research, 93, 91-110.

ESSMAN, W.B. & SUDAK, F.N. (1964) Audiogenic seizure in genetically induced mice, relation of hypothermia to onset and susceptibility. Exp.Neurol., 9, 228-235.

FARJO, I.B. & McQUEEN, J.K. (1979) Dopamine agonists and cobalt-induced epilepsy in the rat. Br.J.Pharmacol., 67, 353-360.

FELDBERG, W.S. (1960) Catatonia, anaesthesia and sleep-like conditions. In: Chemical Concepts of Psychosis (Rinkel, M., Ed.) pp.277-291. P. Owen, London.

FIBIGER, H.C., LONSBURY, B. & COOPER, H.P. (1972). Early behavioural effects of intraventricular administration of 6-hydroxydopamine in rats. Nature (New biol.) 236, 209-211.

FISCHER, J., HOLUBAR, J. & MALÍK, V. (1967) A new method of producing chronic epileptogenic cortical foci in rats. Physiol.Bohemoslov., 16, 272-277.

FITCHMAN, M.B., KLEEMAN, C.R. & BETHUNE, J.E. (1970) Inhibition of anti-diuretic hormone secretion by diphenylhydantoin. Arch.Neurol. 22, 45-53.

FREY, H.H. & KAMPMANN, E. (1965) Tolerance to anticonvulsant drugs. Acta pharmacol. et toxicol. 22, 159-171.

GIACOLONE, E. & VALZELLI, L. (1966) A method for determination of 5-hydroxyindoleacetic acid in rat brain. J.Neurochem., 13, 1265-1266.

GLASSON, B. & BENAKIS, A. (1973) Absorption, distribution, excretion and metabolism of anticonvulsant drugs in animals. In: Anticonvulsant drugs, Vol.I (Mercier, J., Ed.) pp.242-252, Pergamon Press.

HOWARD, J.L. & BREESE, G.R. (1974) Physiological and behavioural effects of centrally administered 6-hydroxydopamine in rats. Pharmacol. Biochem. Behav., 2, 651-661.

GOLDBERG. A.M., POLLOCK, J.J., HARTMAN, E.R. & CRAIG, C.R. (1972) Alterations in cholinergic enzymes during the development of cobalt induced epilepsy in the rat. Neuropharmacology, II, 253-259.

GRAHAM, L.T. & APRISON, M.H. (1966) Fluorometric determination of aspartate, glutamate and γ-aminobutyrate in nerve tissue using enzymic methods. Anal.Biochem., 15, 487-497.

GREEN, A.R. & GRAHAME-SMITH, D.G. (1975) The effect of diphenylhydantoin on brain 5-hydroxytryptamine metabolism and function. Neuropharmacology, 14, 107-113.

GUNNE, L.M. (1963) Catecholamine and 5-hydroxytryptamine in morphine tolerance and withdrawal. Acta physiol.Scand., 58 Suppl., 204, 5-91

HADFIELD, M.G. (1972) Uptake and binding of catecholamines. Arch.Neurol., 26, 78-84.

HEBRON, B.S. (1977) Some aspects of the neurochemistry of tremor. PhD Thesis, University of Nottingham.

ITO, M., OKUNO, T., MIKAWA, H. & OSUMI, Y. (1980) Elevated homovanillic acid in cerebrospinal fluid of children with infantile spasms. Epilepsia, 21, 387-392.

JENNER, P., CHADWICK, D., REYNOLDS, E.H. & MARSDEN, C.D. (1975) Altered 5-hydroxytryptamine metabolism with clonazepam, diazepam and diphenyhydantoin. J.Pharm.Pharmacol., 27, 707-710.

JEPSON, E.P. (1975) Neurochemical aspects of narcotic analgesia and dependence. PhD Thesis, University of Nottingham.

JERLICZ, M., KOSTOWSKI, W., BIDZINSKI, A. & HAUPTMANN, M. (1978) Audiogenic seizures susceptibility in rats with lesioned raphe nuclei and treated with p-chlorophenylalanine. Pol.J.Pharmacol.Pharm., 30, 63-68.

KOSTRZEWA, R.M. & JACOBOWITZ, D.M. (1974) Pharmacological actions of 6-hydroxydopamine. Pharmacological Reviews, 26, 199-286.

JOBE, P.C., PICCHIONI, A.L. & CHIN, L. (1973) Role of brain norepinephrine in audiogenic seizures in the rat. J.Pharmacol.Exp.Ther., 184, 1-9.

JONNSON, G. & SACHS, C.H. (1971) Uptake and accumulation of [3H] 6-hydroxydopamine in adrenergic nerves. Eur.J.Pharmacol., 16, 55-62.

KATO, R. & CHIESARA, E. (1962) Increase of phenobarbitone metabolism induced in rats pre-treated with some centrally acting compounds. Br.J.Pharmacol., 18, 29-38.

KELLOGG, C. (1976) Audiogenic seizures: relation to age and the mechanisms of monoamine neurotransmission. Brain Research, 106, 87-103.

KILIAN, M. & FREY, H.H. (1973) Central monoamines and convulsive thresholds in mice and rats. Neuropharmacology, 12, 681-692.

KOE, B.K. & WEISSMAN, A. (1966) p-Chlorophenylalanine: a specific depletor of brain serotonin. J.Pharmacol.Exp.Ther., 154, 499-516.

KOHASAKA, M., HIRAMATSU, M. & MORI, A. (1978) Brain catecholamine concentration and convulsions in EL mice. Advances in biochemical psychopharmacology, 19, 389-391.

KOPELOFF, L.M. (1960) Experimental epilepsy in the mouse. Proc.Soc. Exp.Biol., 104, 500-504.

KUPFERBERG, H.J. (1972) GLC determination of carbamazepine in plasma. J.Pharmaceutical sci. 61(2), 284-286.

KURIYAMA, K., ROBERTS, E. & RUBINSTEIN, M.K. (1966) Elevation of γ-aminobutyric acid in brain with amino-oxyacetic acid and susceptibility to convulsive seizures in mice: a quantitative evaluation. Biochem.Pharmacol., 15, 221-236.

LAVERTY, R. & TAYLOR, K.M. (1970) Effects of intraventricular 2,4,5-trihydroxyphenylethylamine (6-hydroxydopamine) on the rat behaviour and brain catecholamine metabolism. Br.J.Pharmacol., 40, 836-846.

LIDBRINK, P. & FARNEBO, L.O. (1973) Uptake and release of noradrenaline in rat cerebral cortex in vitro: no effect of benzodiazepines and barbiturates. Neuropharmacology, 12, 1087-1095.

LEONARD, B.E. (1968) The effect of chronic administration of sodium barbitone on chemically and electrically induced convulsions in the rat. Inst.J.Neuropharmacol. 7, 463-468.

LIDBRINK, P., CORRODI, H., FUXE, K. & OLSON, L. (1972) Barbiturate and meprobamate; decreases in catecholamine turnover of central dopamine and noradrenaline neuronal systems and the influence of immobilization stress. Brain Research, 45, 507-524.

LIDBRINK, P., CORRODI, H. & FUXE, K. (1974) Benzodiazepines and barbiturates: turnover changes in central 5-hydroxytryptamine pathways. Eur.J.Pharmacol., 26, 35-40.

LINTS, C.E., WILLOTT, J.F., SZE, P.Y. & NENJA, L.H. (1980) Inverse relationship between whole brain monoamine levels and audiogenic seizure susceptibility in mice: failure to replicate. Pharmacol., Biochem. and Behav., 21, 385-388.

LÖSCHER, W. & FREY, H.H. (1977) Effect of convulsant and anticonvulsant agents on level and metabolism of γ-aminobutyric acid in mouse brain. Naunyu-Schmiedeberg's Arch.Pharmacol., 296, 263-269.

MAITRE, L. (1965) Presence of α-methyl DOPA metabolites in heart and brain of guinea pigs treated with α-methyl-tyrosine. Life Sci., 4, 2249-2256

MAYNERT, E.W., & KAJI, H.K. (1962) On the relationship of brain γ-aminobutyric acid to convulsions. J.Pharmacol.Exp.Ther., 137, 114-121.

MAYNERT, E.W., KLINGMAN, G.I. & KAJI, H.K. (1962) Tolerance to morphine II: Lack of effect on brain 5-hydroxytryptamine and γ-aminobutyric acid. J.Pharmacol.Exp.Ther., 135, 296-299.

MELDRUM, B.S. (1975) Epilepsy and γ-aminobutyric acid-mediated inhibition. Int.Rev.Neurobiol., 17, 1-36.

MELDRUM, B.S., BALZAMO, E., WADA, J.A. & VUILLON-CACCIUTTOLO, G. (1972) Effect of L-tryptophan, L-3,4-dihydroxyphenylalanine and tranylcypromine on the electroencephalogram and on photically induced

epilepsy in the baboon papio papio. Physiol.Behav., 9, 615-621.

MENDEZ, J.G., COTZIAS, G.C., MENA, I. & PAPAVASILIOU, P.S. (1975) Diphenylhydantoin. Blocking of levodopa effects. Arch.Neurol, 32, 44-46.

MEYER, H. & FREY, H.H. (1973) Dependence of anticonvulsant drug action on central monoamines. Neuropharmacology 12, 939-947.

MOORE, K.E., WRIGHT, P.F. & BERT, K.K. (1967) Toxicologic studies with α-methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase. J.Pharmacol.Exp.Ther., 155, 506.

MORGAN, W.W., HUFFMAN, R.D., PFEIL, K.A. & GONZALES, E.G. (1978) Effect of synthesis inhibition on the levels of brain catecholamines in barbital-dependent rats. Psychopharmacology, 56, 41-44.

MORGAN, W.W., PFEIL, K.A. & GONZALES, E.G. (1977) Catecholamine concentration in discrete brain areas following the withdrawal of barbital dependent rats. Life Sci., 20, 493-500.

MORGAN, W.W., PFEIL, K.A. & GONZALES, E.G. (1978) Effect of α-methyl-p-tyrosine on the incidence of spontaneous convulsions observed following barbital withdrawal. Neuropharmacology, 17, 115-119.

MORSELLI, P.L., GERNA, M. & GARATTINI, S. (1971) Carbamazepine plasma and tissue levels in the rat. Biochem.Pharmacol., 20, 2043-2047.

NEFF, N.H., TOZER, T.N. & BRODIE, B.B. (1967) Application of steady state kinetics to studies of the transfer of 5-hydroxyindoleacetic acid from brain to plasma. J.Pharmacol.Exp.Ther., 158, 214-218.

NOBLE, E.P., WURTMAN, R.J. & AXELROD, J. (1967) A simple and rapid method for injecting [3H] norepinephrine into the lateral ventricle of the rat brain. Life Sci., 6, 281-291.

NYBACK, H. (1972) Effect of brain lesions and chloropromazine on accumulation and disappearance of catecholamines formed *in vivo* from (^{14}C) tyrosine. Acta Physiol.Scand. 84, 54-64.

PAPESCHI, R. (1977) The functional pool of brain catecholamines: its size and turnover rate. Psychopharmacology, 55, 1-7.

PAPESCHI, R., MOLINA-NEGRO, P., SOURKES, T.L. & ERBA, G. (1972) The concentration of homovanillic and 5-hydroxyindoleacetic acids in ventricular and lumbar CSF. Neurology, 22, 1151-1159.

PAPESCHI, R. & RANDRUP, S. (1973) Catalepsy, sedation and hypothermia induced by alpha-methyl-p-tyrosine in the rat. An ideal tool for screening of drugs active on central catecholaminergic receptors. Pharmakopsychiatr. Neuropsychopharmakol., 6, 137-157.

PATSALOS, P.N. & LASCELLES, P.T. (1981) Changes in regional brain levels of amino acid putative neurotransmitters after prolonged treatment with the anticonvulsant drugs diphenylhydantoin, phenobarbitone sodium valproate, ethosuximide and sulthiane in the rat. J.Biochem., 36(2), 688-695.

PINCUS, J.H. & LEE, H. (1973) Diphenylhydantoin and calcium. Arch. Neurol., 29, 239-244.

PROCKOP, D.J., SHORE, P.A. & BRODIE, B.B. (1959) Anticonvulsant properties of monoamine oxidase inhibitors. Ann.N.Y.Acad.Sci., 80, 643-651.

PURDY, R.E., JULIEN, R.M., FAIRHURST, A.S. & TERRY, M.D., (1977) Effect of carbamazepine on the in vivo uptake and release of norepinephrine in adrenergic nerves of rabbit aorta and in whole brain synaptosomes. Epilepsia, 18, 251-257.

QUATTRONE, A., CRUNELLI, V. & SAMANIN, R. (1978) Seizure susceptibility and anticonvulsant activity of carbamazepine, diphenylhydantoin and phenobarbitone in rats with selective depletions of brain monoamines. Neuropharmacology, 17, 643-647.

QUATTRONE, A. & SAMANIN, E. (1977) Decreased anticonvulsant activity of carbamazepine in 6-hydroxydopamine-treated rats. Eur.J.Pharmacol., 41, 333-336.

SCOTTI DECAROLIS, A., ZIEGLER, H., DEL BASSO, P. & LONGO, V.G. (1971) Central effects of 6-hydroxydopamine. Physiology and Behaviour, 7, 205-708.

RICHTER, J.A. & JACKSON, S.K. (1980) [3H] acetylcholine and [3H] 5-hydroxytryptamine release from rat midbrain slices and the effects of calcium and phenobarbitone. Neurochemical Research, 5(7), 719-730.

ROSS, S.M. & CRAIG, C.R. (1981) Studies on γ-aminobutyric acid transport in cobalt experimental epilepsy in the rat. J.Neurochem., 36, 1006-1011.

ROTH, R.H., NOWYCKY, M.C., WALTERS, J.R. & MORGENROTH, V.H. (1977) γ-Hydroxybutyrate: effect on nonstriatal dopaminergic neurons. In: Advances in Biochemical Psychopharmacology, Vol.16 (Costa E. & Gessa, G.L., Eds.) pp. 483-488 Raven Press, New York.

RUDZIK, A.D. & MENNEAR, J.H. (1965) The mechanism of action of anticonvulsants I. Diphenylhydantoin. Life Sci., 4, 2373-2382.

SAAD, S.F. (1970) Effect of isoniazid and some anticonvulsant drugs on the γ-aminobutyric acid content of mouse brain in insulin hypoglycaemia. J.Pharm.Pharmacol., 22, 372-374.

SAAD, S.F., ELMASRY, A.M. & SCOTT, P.M. (1972) Influence of certain anticonvulsants on the concentration of γ-aminobutyric acid in the cerebral hemispheres of mice. Eur.J.Pharmacol., 17, 386-392.

SCHLESINGER, K., BOGGAN, W. & FREEDMAN, D.X. (1965) Genetics of audiogenic seizures I. Relation to brain serotonin and norepinephrice in mice. Life Sci., 4, 2345-2351.

SCOTT, E.M. & JAKOBY, W.B. (1959) Soluble γ-aminobutyric-glutamic transaminase from Pseudomonas fluorescens. J.Biol.Chem., 234 (4), 932-936.

SHAYWITZ, B.A., COHEN, D.J. & BOWERS, M.B. (1975) Reduced cerebrospinal fluid 5-hydroxyindoleacetic acid and homovanillic acid in children with epilepsy. Neurology, 25, 72-79.

SOHN, R.S. & FERRENDELLI, J.A. (1976) Anticonvulsant drug mechanisms. Arch.Neurol., 33, 626-629.

STULL, R.E., JOBE, P.C. & GEIGER, P.F. (1977) Brain areas involved in the catecholamine mediated regulation of electroshock seizure intensity. J.Pharm.Pharmacol., 29, 8-11.

SUTTON, O. & MILLER, J.M. (1963) Implanted electrodes: cable coupler for elimination of movement artifact. Science, 149, 988-989.

SWAAB, D.F. (1971) Pitfalls in the use of rapid freezing for stopping brain and spinal cord metabolism in rats and mice. J.Neurochem., 18, 2085-2092.

SYNDER, S.H., AXELROD, J. & ZWEIG, H. (1965) A sensitive and specific fluorescence assay for tissue serotonin. Biochem.Pharmacol., 14, 831-835.

TONGE, S.R. (1969) Some pharmacological and biochemical properties of hallucinogenic drugs. PhD thesis, University of Nottingham.

TOWER, D.P. (1959) Glutamic acid metabolism in the mammalian central nervouc system. In: Biochemistry of central nervous system. Proc.IV Int.Biochem.Congr. Vol.3, pp. 213 Vienna.

TRANZER, J.P. & THOENEN, H. (1967) Ultra-morphologische Veranderungen der sympatischen Nervendigunden der katze nach Vorbehandlung mit 5- und 6-hydroxy-dopamine. Naunyn-Schmiedebergs Arch.Pharmakol. Exp.Pathol., 257, 343-344.

TRANZER, J.P. & THOENEN, H. (1968) An electron microscopic study of selective acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. Experientia (Basel), 24, 155-156.

TUNNICLIFF, G. (1976) Centrally acting drugs and formation of γ-amino-butyric acid. Gen.Pharmacol., 7, 259-262.

UDENFRIEND, S. & WEISSBACH, H. (1958) Turnover of 5-hydroxytryptamine (serotonin) in tissue. Proc.Soc.Exp.Biol.Med., 97, 748-751.

UNGERSTEDT, U. (1968) 6-Hydroxydopamine induced degeneration of central monoamine neurons. Eur.J.Pharmacol., 5, 107-110.

VERNADAKIS, A. & WOODBURY, D.M. (1960) Effects of diphenylhydantoin and adrenocortical steroids on free glutamic acid, glutamine and γ-aminobutyric acid concentration of rat cerebral cortex. In: Inhibition in the Nervous System and Gamma Aminobutyric Acid (Roberts, E., Ed.) pp.242-248. Pergamon, Oxford.

VETULANI, J., REICHENBERG, K., & WISZNIOWSKA, G. (1972) Asymmetric behavioural and biochemical effects of unilateral injections of 6-hydroxydopamine into the lateral brain ventricle of the rat. Eur.J.Pharmacol., 19, 231-238.

WEINBERGER, J., NICKLAS, W.J. & BERL, S. (1976) Mechanism of action of anticonvulsant: role of the differential effects on the active uptake of putative neurotransmitters. Neurology, 26, 162-166.

WENGER, G.R., STITZEL, R.E. & CRAIG, C.R. (1973) The role of biogenic amines in the reserpine-induced alteration of minimal electroshock seizure threshold in the mouse. Neuropharmacology, 12, 693-703.

WILKISON, D.M. & HALPERN, L.M. (1979) The role of biogenic amines in amygdalar kindling I. Local amygdalar after-discharge. J.Pharmacol.Exp.Ther., 211, 151-158.

WOOD, J.D. (1975) The role of γ-aminobutyric acid in the mechanism of seizure. Prog.Neurobiol., 5, 79-95.

WOOD, J.D., RUSSELL, M.P., KURYLO, E. & NEWSTEAD, J.D. (1979) Stability of synaptosomal GABA levels and their use in determining the in vivo effects of drugs: convulsant agents. J.Neurochem., 33, 61-68.

YANG, N.H. & NEFF, N.H. (1974) The monoamine oxidases of brain: selective inhibition with drugs and consequences for the metabolism of the biogenic amines. J.Pharmacol.Exp.Ther., 189, 733-740.