TETRAHYDROBIOPTERIN METABOLISM, NEUROTRANSMITTERS AND BEHAVIOUR IN THE RAT.

by

JULIETTE COX

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SUMMARY

Various neurotoxins were investigated to assess their suitability for developing an animal model to study partial brain BH_4 deficiency, neurotransmitters and behavioural alterations.

Acute dosing with lead, diethylstilboestrol (DES), amphetamine and scopolamine produced no significant changes in rat brain BH_4 metabolism though total biopterins in the liver were significantly reduced by lead and DES.

Acute starvation of adult rats decreased brain biopterins. This loss of biopterins may be due to enhanced oxidative catabolism of the active cofactor caused by glutathione depletion.

Dietary administration of a BH_4 biosynthesis inhibitor, DAHP, consistently decreased brain total biopterins in weaner rats but did not alter the levels of DA, NA, 5-HT or metabolites. However the DAHP diet also induced a marked reduction in food intake. Rats subjected to an equivalent degree of food restriction without inhibitor showed significant but less severe reductions in brain biopterins and again no effect on transmitter levels.

DAHP produced a significant decrease in locomotor activity and rearing. This could not be ascribed to reduction in food intake as animals subjected to just dietary restriction showed an increase in these activities.

As gross brain levels of DA, NA and 5-HT were unaltered by DAHP the behavioural changes associated with the induced deficiency in brain total biopterins might not have been mediated through the action of these compounds. Although localised changes in neurotransmitter levels may have been obscured by gross analysis it is also possible that the behaviour changes were mediated by a role of BH_4 not yet elucidated.

Long-term administration of a high aluminium low calcium diet to mice produced no effect on gross brain total biopterins, catecholamines, serotonin or choline acetyltransferase activity though significant behavioural changes were observed.

Keywords: TETRAHYDROBIOPTERIN LEAD ALUMINIUM DAHP DIETHYLSTILBOESTROL STARVATION FOOD RESTRICTION GLUTATHIONE CATECHOLAMINES SEROTONIN BEHAVIOUR

To Susie

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ABBREVIATIONS

BBB	Blood brain barrier
BH_2	7,8-dihydrobiopterin
qBH_2	quinonoid dihydrobiopterin
BH_4	5,6,7,8-L-*erythro*-tetrahydrobiopterin
°C	Degrees centigrade
CA	Catecholamines
cAMP	Cyclic adenosine 3',5'-monophosphate
CNS	Central nervous system
CO	Catechol oestrogens
CSF	Cerebrospinal fluid
DA	Dopamine
DAHP	2,4-diamino-6-hydroxypyrimidine
Der' Pterin	Derived pterin
DES	Diethylstilboestrol
DES Dip'	Diethylstilboestrol dipropionate
DHFR	Dihydrofolate reductase
DHPR	Dihydropteridine reductase
$DMPH_4$	6,7-Dimethyl,-5,6,7,8-tetrahydropterin
L-DOPA	L-3,4-dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic acid
EDTA	Ethylenediaminetetraacetic acid
GSH	Glutathione-reduced form
GSH-PX	Glutathione peroxidase
GSSG	Glutathione-oxidised form
GTP	Guanosine triphosphate
GTP-CH	GTP cyclohydrolase
HPLC	High Performance Liquid Chromatography
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (serotonin)
HVA	Homovanillic acid
i.g	intragastric
i.p	intraperitoneal
LNAA	Large neutral amino acid
MHPA	Malignant Hyperphenylalaninaemia
n	Sample size
NA	Noradrenaline
NAD(H)	Nicotinamide adenine dinucleotide (reduced form)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced form)
NH_2	D-*erythro*-dihydroneopterin
NH_2P_3	D-*erythro*-dihydroneopterin triphophate
n.s	not significant
ODS	Octadecyl silane
PPH_4S	6-pyruvoyl tetrahydropterin synthase
P/B	Derived pterin over biopterin ratio
RT	Retention Time
SDAT	Senile dementia of the Alzheimer type
SR	Sepiapterin reductase
$\pm$ st.d	plus or minus the standard deviation
TCA	Trichloroacetic acid
Tris	Tris-(hydroxymethyl)-aminomethane
$\bar{x}$	mean

CHAPTER ONE

INTRODUCTION

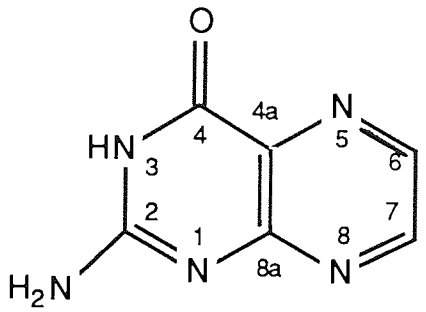
1.1 PTERINS

Pterins are a class of compounds which have in common the 2-amino-4-hydroxypteridine structure, (Pfleiderer 1964), the simplest being pterin itself (1). They can exist in 7,8-dihydro (2) and 5,6,7,8-tetrahydropterin (3) forms by reduction of the pyrazine moiety of the pterin ring system, (Pfleiderer, 1978).

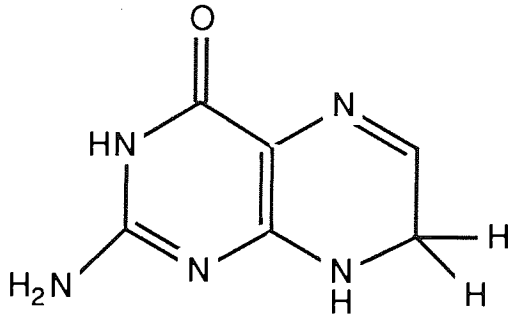
Substitution of the ring at C(6) leads to the formation of a number of biologically important pterins which are widespread in nature, (Descimon, 1975; Patterson *et al.*, 1955). They have been identified in a variety of mammalian body fluids and tissues, (Blakley, 1969; Leeming *et al.*, 1976; Fukushima and Nixon, 1980), where the predominant form is tetrahydrobiopterin, (6R-(L-*erythro*-1,2'-dihydroxy-propyl)-2-amino-4-hydroxy, 5,6,7,8-tetrahydropterin), (4) (Fukushima and Nixon, 1980; Abou-Donia and Viveros, 1981; Katoh and Sueoka, 1986). This study will consider those pterins associated primarily with tetrahydrobiopterin metabolism.

1.2 ROLE OF TETRAHYDROBIOPTERIN.

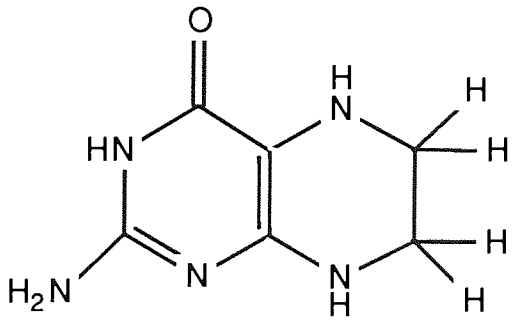
The only established physiological role for tetrahydrobiopterin (BH_4) is as the natural cofactor in the hydroxylation of the aromatic amino acids, phenylalanine, tyrosine and tryptophan (Kaufman and Fisher, 1974), catalysed by phenylalanine hydroxylase (Kaufman, 1958), tyrosine hydroxylase (Nagatsu *et al.*, 1964) and tryptophan



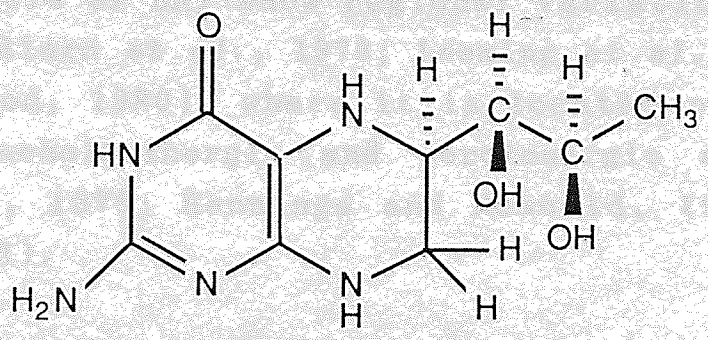
1) Pterin



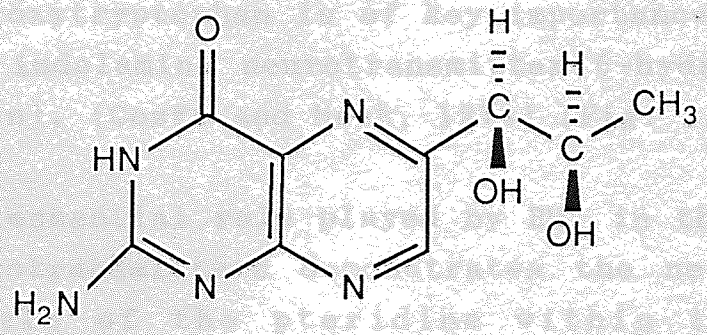
2) 7,8 -Dihydropterin



3) 5,6,7,8 - Tetrahydropterin



4) L- Erythro - Tetrahydrobiopterin



5) Biopterin

Stereochemistry of the Side Chain

out of plane of paper

into plane of paper

in plane of paper

hydroxylase (Hosoda and Glick, 1966). These reactions take place in conjunction with enzyme-bound iron and molecular oxygen (Kaufman and Fisher, 1974).

Levels of BH_4 show regional variation within the brain, (Bullard *et al.*, 1978; Leeming *et al.*, 1976; Fukushima and Nixon, 1980), where it is located primarily within the catecholaminergic and serotonergic systems, (Bullard *et al.*, 1978; Hennings and Rembold, 1982; Levine *et al.*, 1981).

The hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) is the initial and rate-limiting step in the biosynthesis of the catecholamine neurotransmitters dopamine and noradrenaline, (Levitt *et al.*, 1965), (Fig. 1.1). Similarly, the hydroxylation of tryptophan to 5-hydroxytryptophan is of key importance in the synthesis of the indolamine neurotransmitter 5-hydroxytryptamine (serotonin), (Costa and Meek, 1974) (Fig. 1.2).

The essential role played by BH_4 in the formation of these neurotransmitters demonstrates the necessity for adequate levels of the pteridine within the CNS for normal neurological functioning, (Leeming *et al.*, 1981). Where brain BH_4 deficiency has occured, a degree of concomitant neurological impairment has been observed, (see section 1.6)

1.3 MECHANISM OF ACTION OF TETRAHYDROBIOPTERIN.

The precise mechanism by which BH_4 functions as a cofactor in hydroxylation reactions has not yet been unequivocally established though it appears to be related to the ability of the pteridine to reduce molecular oxygen, (Kaufman and Fisher, 1974). Iron has been shown to be essential for the hydroxylase activity, (Fisher *et al.*, 1972), and Pearson (1974) suggested a free-chain radical process whereby BH_4,

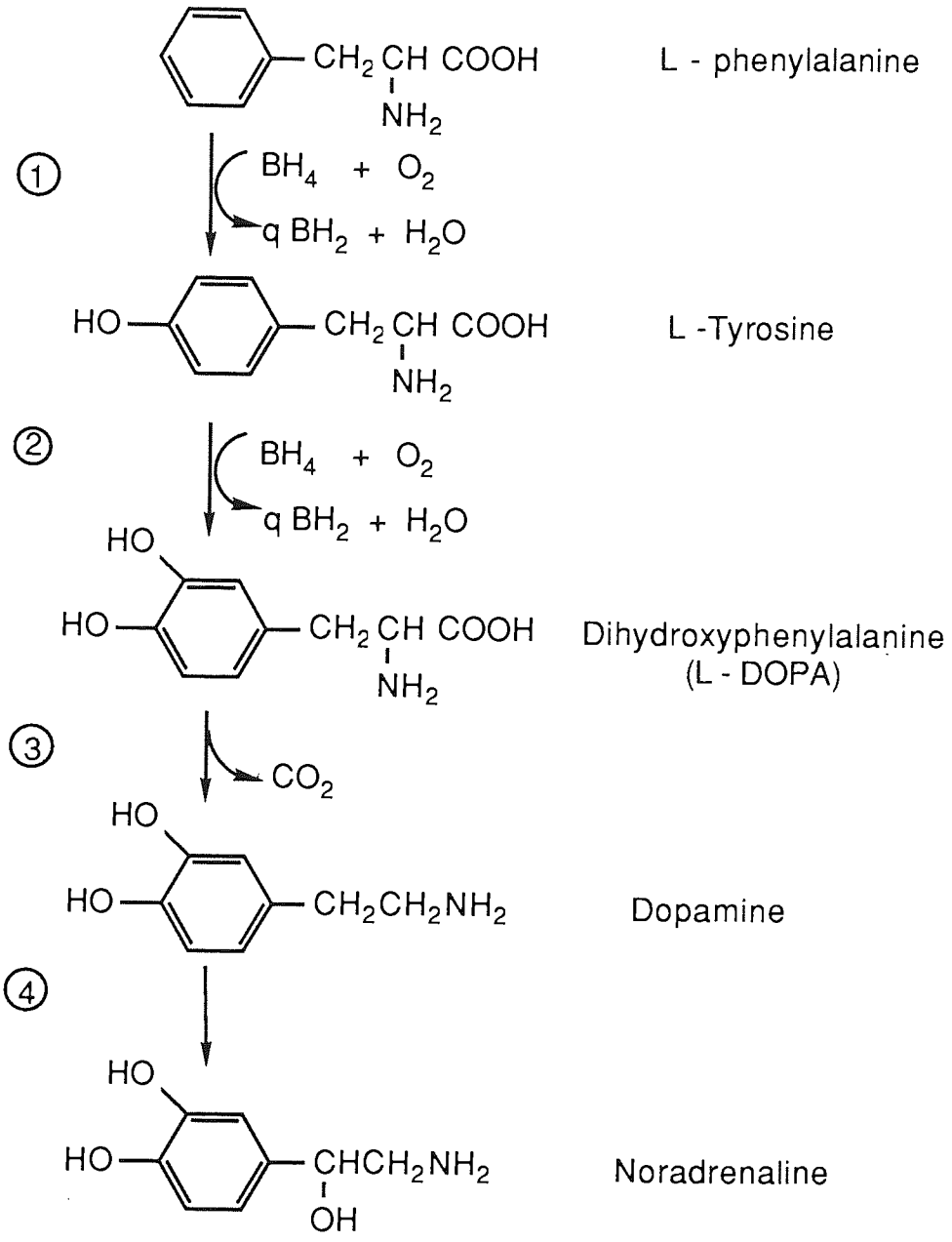


FIG. 1.1: Biosynthesis of Catecholamines

① Phenylalanine Hydroxylase
② Tyrosine Hydroxylase
③ L-aromatic amino acid decarboxylase
④ Dopamine ß-hydroxylase

qBH_2 = Quinonoid Dihydrobiopterin

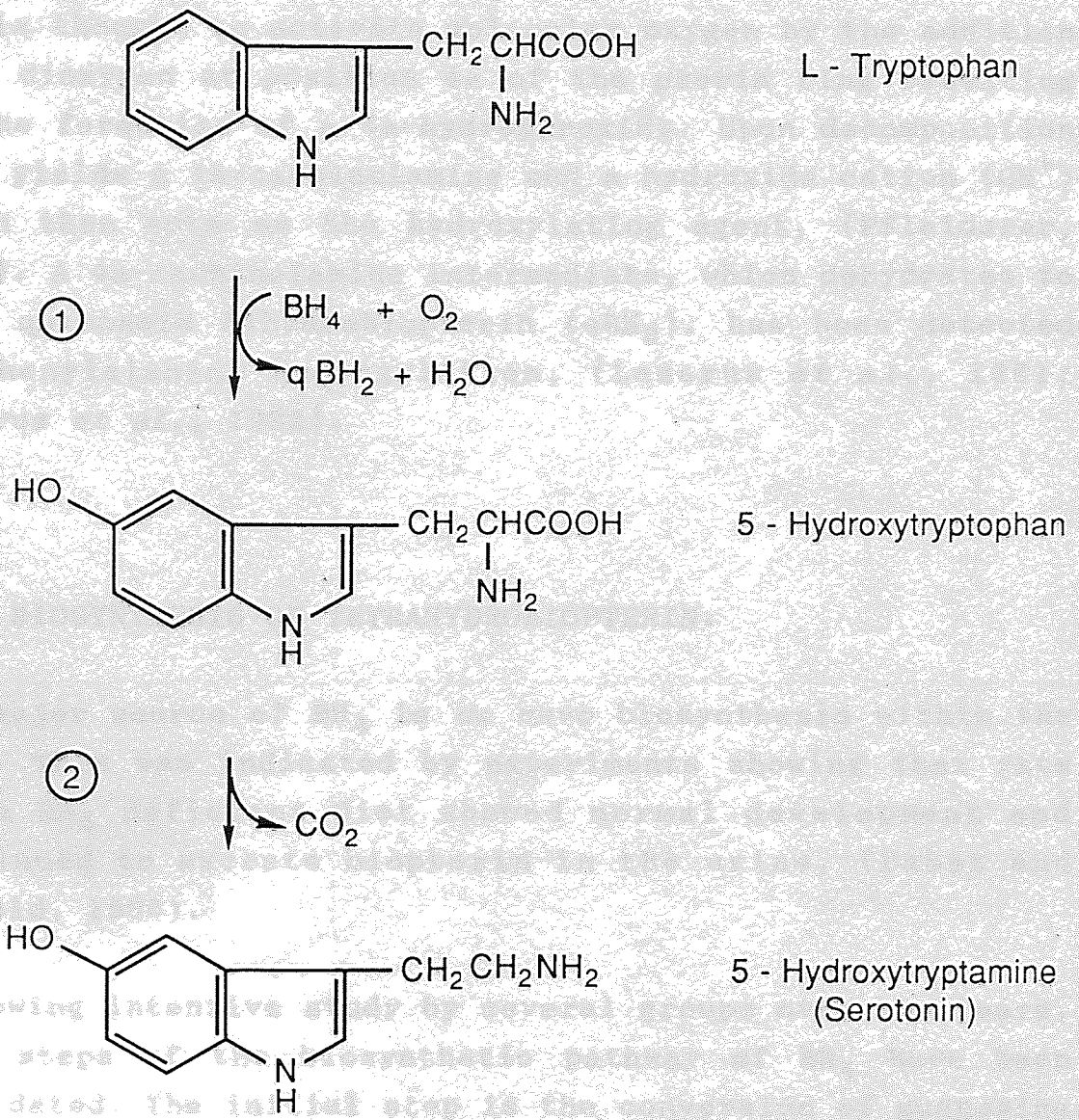


FIG. 1.2: Biosynthesis of 5 - Hydroxytryptamine

(1) Tryptophan hydroxylase

(2) L - Aromatic amino acid decarboxylase

by acting as a 2 electron reducing agent, forms an iron-peroxide complex which acts as the hydroxylating agent.

More recently, following studies on phenylalanine hydroxylase, another mechanism of action has been proposed. BH_4 is thought to activate molecular oxygen by the addition of a dioxygen at position 4a of the pterin ring resulting in the formation of a 4a-hydroperoxide. Upon decomposition this yields a 4a-carbinolamine and a hydroxide cation (OH^+) which then acts as the hydroxylating agent, (Pfleiderer, 1978). A 4a-carbinolamine intermediate, which dehydrates to give quinonoid dihydrobiopterin (qBH_2), has been detected in phenylalanine hydroxylation, (Lazarus *et al.*, 1981; Lazarus *et al.*, 1982).

1.4 BIOSYNTHESIS OF TETRAHYDROBIOPTERIN.

The major source of BH_4 is *de novo* biosynthesis within the cell. This was indicated by experiments showing that rats fed a BH_4 deficient diet showed normal development and continued to excrete biopterin in the urine, (Pabst and Rembold, 1966).

Following intensive study by several groups over the years, most steps of the biosynthetic pathway of BH_4 have been elucidated. The initial step is the conversion of guanosine triphosphate (GTP) to D-*erythro*-dihydroneopterin triphosphate (NH_2P_3) by GTP cyclohydrolase (GTP-CH), a reaction involving an Amadori rearrangement, (Burg and Brown, 1968) (Fig. 1.3). This first step is thought to be rate-limiting in the rat, (Sawada *et al.*, 1985).

Most recent data indicates that biosynthesis of BH_4 from NH_2P_3 proceeds via tetrahydropterin intermediates, formed by tautomerisation reactions involving transfer of electrons from the side chain to the pterin moiety, (Fig.

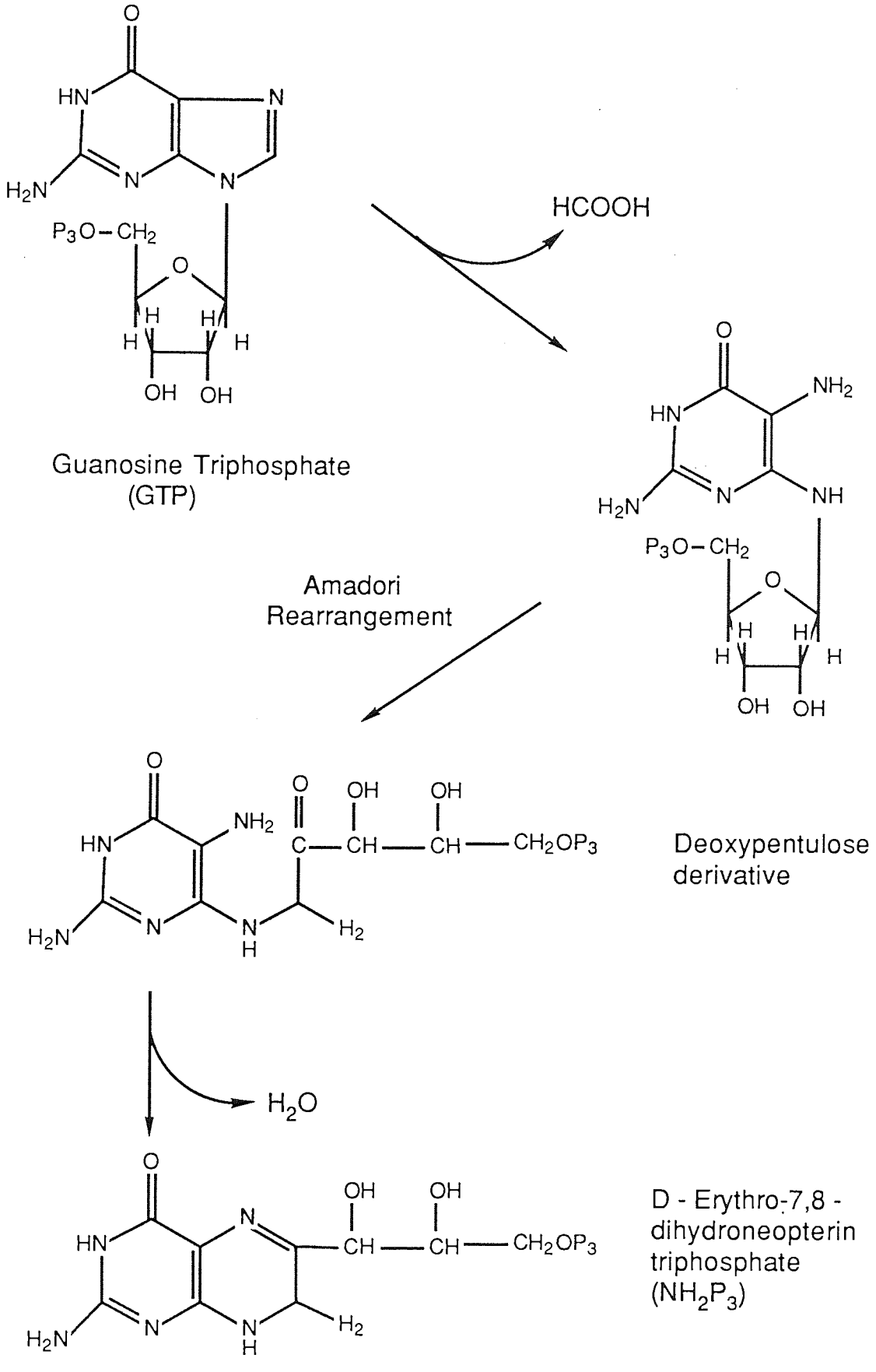


FIG. 1.3: Conversion of GTP to NH_2P_3 by GTP Cyclohydrolase

1.4) (Milstein and Kaufman, 1983; Milstein and Kaufman, 1985; Smith and Nichol, 1984; Switchenko and Brown, 1985). NH_2P_3 is converted to 6-pyruvoyl-tetrahydropterin (PPH_4) by a magnesium dependent enzyme, 6-pyruvoyl tetrahydropterin synthetase. The reaction involves the loss of the triphosphate group, hence the enzyme is also known as phosphate eliminating enzyme (Heintel *et al.*, 1985). It is a critical step in the biosynthetic pathway since it is probably rate-limiting in man, (Smith *et al.*, 1986).

The remainder of the pathway involves the two-step reduction of the keto groups of PPH_4 to produce BH_4. This is catalysed by two NADPH dependent enzymes, sepiapterin reductase (SR), that catalyses reduction of both carbonyl groups, and 2'-keto reductase which specifically reduces only the 2' carbonyl, (Milstein and Kaufman, 1985 and 1986; Smith *et al.*, 1986). It is not yet known whether the reduction occurs first at C-1' or at C-2'.

1.5 REGULATION OF TETRAHYDROBIOPTERIN LEVELS

There are three main ways in which BH_4 levels are controlled; by regulation of the biosynthetic pathway, salvage of quinonoid dihydrobiopterin and tetrahydropterin catabolism.

1.5.1 Regulation of tetrahydrobiopterin biosynthesis.

BH_4 synthesis has been shown to be controlled by a negative feedback loop *in vitro*, (Bellahsene *et al.*, 1984), and in mouse neuroblastoma cells, (Kapatos and Kaufman, 1983), in which BH_4 acts as a potent inhibitor of GTP-CH. High levels of noradrenaline and serotonin have been shown to act as competitive inhibitors of sepiapterin reductase *in vitro*, which may suggest co-regulation of BH_4 and neurotransmitter synthesis, (Katoh *et al.*, 1982).

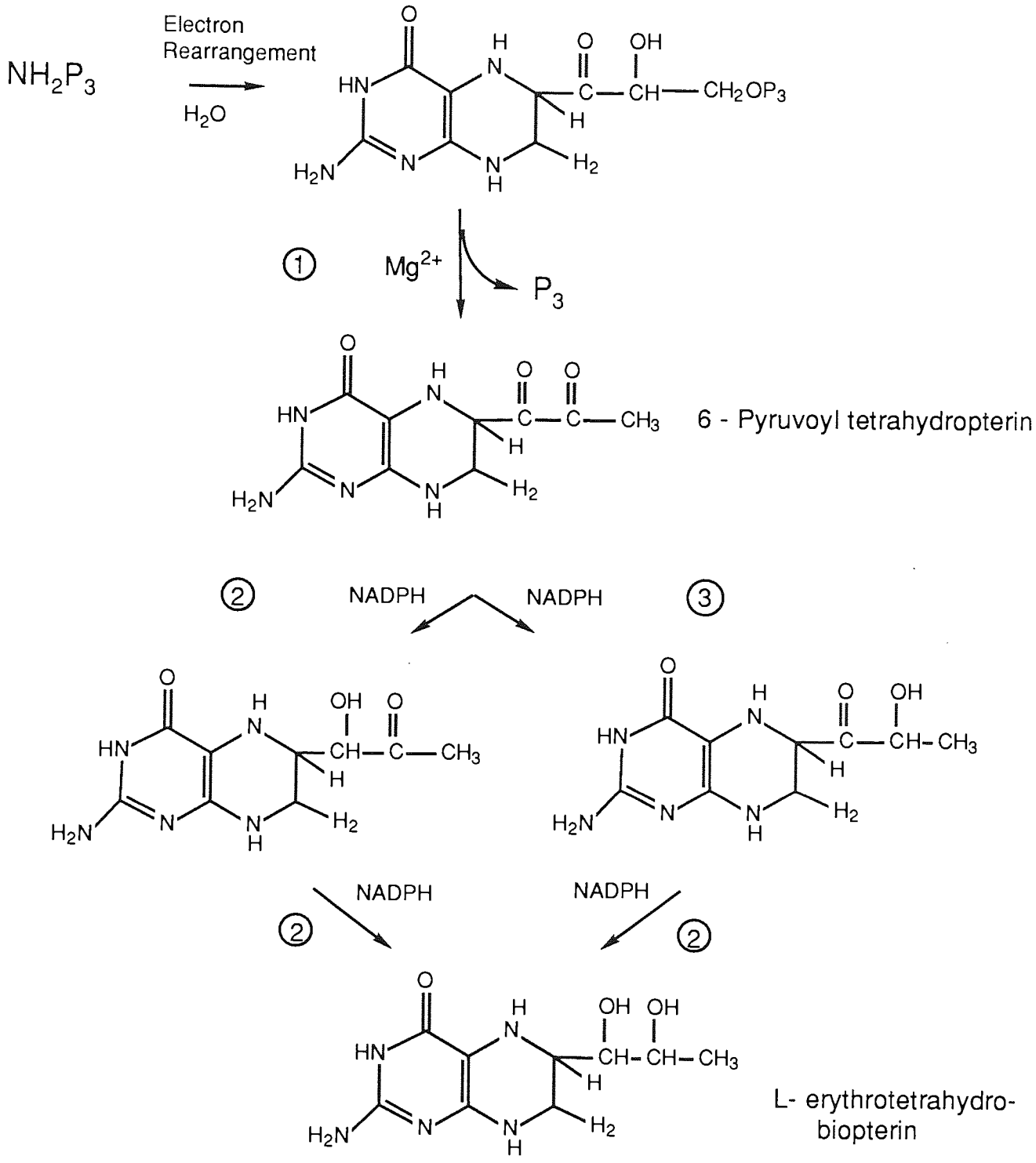


FIG. 1.4: The tetrahydropterin pathway for de novo biosynthesis of tetrahydrobiopterin.

① Pyruvoyl tetrahydropterin synthetase (phosphate eliminating enzyme)

② Sepiapterin reductase

③ 2' keto reductase

Abou-Donia *et al.*, (1981, 1986), performed experiments on adrenal medulla and cortex *in vivo* and cultures of adrenomedullary chromaffin cells, and showed that, depletion of cellular catecholamines by reserpine enhances GTP-CH activity and increases BH_4 levels. There is differential control of GTP-CH in the adrenal gland: the adrenal medulla shows enhanced GTP-CH synthesis upon increased splanchnic nerve discharge, whereas in the adrenal cortex synthesis is enhanced by ACTH release. BH_4 synthesis does not appear to be under hormonal control in the brain, (Duch *et al.*, 1986).

A cAMP-dependent mechanism by which GTP-CH activity and BH_4 levels are increased has been suggested, (Abou-Donia *et al.*, 1986). This does not occur in neuroblastoma cells in culture, (Woolf *et al.*, 1986).

1.5.2 Salvage of quinonoid dihydrobiopterin.

As a result of its cofactor activity BH_4 is oxidised to inactive quinonoid dihydrobiopterin (qBH_2). If qBH_2 is not reduced back to BH_4 it will tautomerise to give 7,8-dihydrobiopterin (BH_2) which is lost from the cell and excreted in the urine, (Archer and Scrimgeour, 1970; Leeming *et al.*, 1976). Cellular levels of BH_4 are maintained by the salvage action of dihydropteridine reductase (DHPR), an NADH dependent enzyme which regenerates BH_4 from qBH_2, (Craine *et al.*, 1972) (Fig. 1.5).

The active cofactor may also be salvaged by the activity of dihydrofolate reductase (DHFR) which can reduce BH_2 back to BH_4, (Kaufman, 1967; Nichol *et al.*, 1983) (Fig. 1.5).

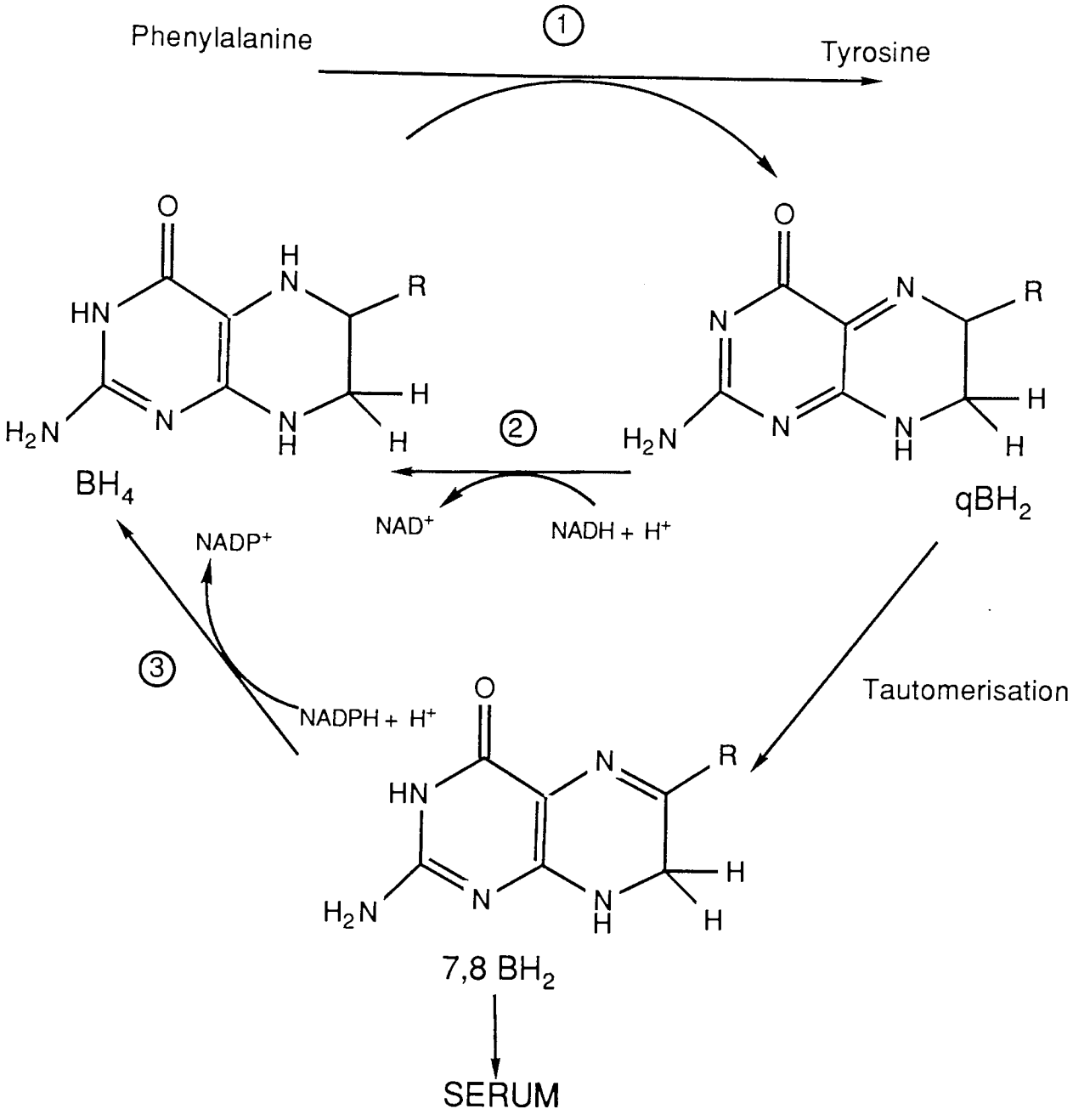


FIG. 1.5: Salvage of BH_4 from qBH_2 by DHPR in thehydroxylation of phenylalanine to tyrosine.

① Phenylalanine hydroxylase

② Dihydropteridine reductase (DHPR)

③ Dihydrofolate reductase (DHFR)

1.5.3 Catabolism of tetrahydropterins.

BH_4 catabolism in the rat occurs in the liver and starts with the oxidation of BH_2 and 7,8-dihydropterin. The resulting products include pterin, isoxanthopterin and lumazine which are excreted by the kidney, (Rembold *et al.*, 1969; Rembold, 1983). Biopterin (5) found in the urine is thought to be derived by loss from the BH_4 pool which is maintained by *de novo* biosynthesis, (Pabst and Rembold, 1966) and DHPR activity.

1.6 TETRAHYDROBIOPTERIN METABOLISM AND NEUROLOGICAL DISORDERS.

The content of BH_4 in the brain correlates well with regions containing tyrosine and tryptophan hydroxylases, (Bullard *et al.*, 1979; Levine *et al.*, 1979), that is to say the sites of catecholamine and serotonin synthesis. Evidence suggests that levels of BH_4 are subsaturating in monoaminergic tissues. Increasing the tissue concentration of BH_4, or synthetic cofactors, by exogenous administration has been shown to increase dopamine (DA) and serotonin (5-HT) synthesis in brain (Kettler *et al.*, 1974; Miwa *et al.*, 1985), synaptosomes (Patrick and Barchas, 1976; Boarder and Fillenz, 1979 and 1980), brain slices (Miwa *et al.*, 1985; Galloway and Levine, 1986; Hirata *et al.*, 1983) and cultured cells (Abou-Donia *et al.*, 1986; Levine and Kellner, 1986). These observations have contributed to the concept that certain neurological and psychiatric disorders thought to involve a biogenic amine deficiency in the brain may be, in part, due to diminished BH_4 metabolism.

1.6.1 Malignant Hyperphenylalaninaemia. (MHPA).

The study of inborn errors of BH_4 metabolism (malignant hyperphenylalaninaemias) has clearly established the relationship between a decreased availability of the cofactor, the lack of monoamine neurotransmitter synthesis and the occurrence of severe neurological deterioration (Dhondt, 1984). Reduced BH_4 levels may be due to:- diminished *de novo* biosynthesis, arising from a deficiency in either GTP-CH or phosphate eliminating enzyme (Niederwieser *et al.*, 1982 and 1984; Niederwieser *et al.*, 1985); to DHPR deficiency arising from a failure to synthesize the enzyme or from production of a mutant form which lacks catalytic activity (Kaufman *et al.*, 1975; Firgaira *et al.*, 1983).

Clinical symptoms of hyperphenylalaninaemia include progressive mental and motor regression along with convulsions and disturbances of tone and posture. Measurement of neurotransmitter metabolites in cerebrospinal fluid (CSF) and urine confirm the impaired monoamine synthesis (Brewster *et al.*, 1979) but variations in the severity of the neurotransmitter deficiency are observed. In some cases the metabolite levels are just at the lower limit of the normal range, (Dhondt, 1987).

1.6.2 Senile Dementia of the Alzheimer Type, (SDAT), and other neurological disorders.

Reduced levels of biopterin in serum, CSF and some areas of the brain have been reported in SDAT patients (Leeming *et al.*, 1979; Young *et al.*, 1982; Aziz *et al.*, 1983; Nagatsu *et al.*, 1986). Biosynthetic capacity is diminished in the temporal lobe and locus coeruleus where the biochemical lesion is suggested to occur at the level of phosphate eliminating enzyme (Barford *et al.*, 1984; Anderson *et al.*, 1986). Recent studies have demonstrated a deficit in the

noradrenergic and serotonergic systems in SDAT (Tomlinson *et al.*, 1981; Mann *et al.*, 1983).

Decreased levels of BH_4 in CSF have also been reported in Parkinson's disease (Lovenberg *et al.*, 1979), familial dystonia (Williams *et al.*, 1979; LeWitt *et al.*, 1986) and depression (Blair *et al.*, 1984; Kellner *et al.*, 1983). A key issue still unanswered is whether the cofactor abnormalities found are primary or only secondary to neuronal disease impairing all metabolic functions, including generation of the cofactor.

1.7 TETRAHYDROBIOPTERIN THERAPY.

The successful treatment of some cases of malignant hyperphenylalaninaemia indicates the potential therapeutic usefulness of BH_4 administration (Niederweiser *et al.*, 1982; Kaufman *et al.*, 1982). Benefit from BH_4 therapy has also been reported in familial dystonia (LeWitt *et al.*, 1986), Parkinson's disease (Curtius *et al.*, 1982) and depression (Curtius *et al.*, 1983).

In order to raise BH_4 concentrations in monoaminergic terminals sufficiently to increase neurotransmitter synthesis, large amounts of BH_4 must enter the brain from the periphery (Levine, 1987). This presents a major problem in BH_4 therapy since the cofactor's penetration of the blood brain barrier (BBB) is very poor (Kapatos and Kaufman, 1981). The future success of BH_4 therapy relies on the development of synthetic analogues of the cofactor which enter the brain much more readily such as 6-methyltetrahydropterin (Levine *et al*, 1987). One great drawback in the search for new pterins is that there are no good animal models of BH_4 deficiency in which to test them.

1.8 AIMS OF THE THESIS.

Tetrahydrobiopterin plays an essential role in the biosynthesis of the catecholamines and serotonin. It has been shown that a gross deficiency of the cofactor in humans leads to neurological impairment characterised by various behavioural abnormalities.

The aims of this study were:

(1) To investigate the effects of various potentially neurotoxic agents on tetrahydrobiopterin metabolism by *in vivo* analysis in the rat.

(2) Development of a suitable animal model of brain tetrahydrobiopterin deficiency in which to investigate the consequence of partial brain BH_4 depletion on brain catecholamine and serotonin content.

(3) To look for correlations between diminished brain BH_4 availability, neurotransmitter levels and alterations in general aspects of behaviour.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 MATERIALS

The following lists indicate the major chemicals and apparatus utilized during the course of this study.

2.1.1 CHEMICALS.

L-Biopterin and pterin were obtained from Dr. B. Schirks, Switzerland.

Acetyl coenzyme-A (sodium salt), albumin (bovine, fraction V), D-amphetamine sulphate, arterenol (noradrenaline NA, free base), L-ascorbic acid, choline bromide, 2,4-diamino-6-hydroxypyrimidine (DAHP), diethylstilboestrol (DES), diethylstilboestrol dipropionate (DES-dip), 3,4-dihydroxy-phenethylamine (dopamine, DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$), eserine (physostigmine), ethylenediamine tetraacetic acid (EDTA), glutathione - reduced form (GSH), guanosine-5'-triphosphate (GTP) (type II-S, sodium salt), heparin (grade II, isolated from porcine intestinal mucosa), 4-hydroxy-3-methoxy-phenylacetic acid (homo-vanillic acid HVA), horseradish peroxidase, 5-hydroxy-3-indoleacetic acid (free acid, 5-HIAA), 5-hydroxytryptamine (creatinine sulphate complex, serotonin 5-HT), nicotinamide adenine dinucleotide - reduced form (NADH, grade III, disodium salt), nicotinamide adenine dinucleotide phosphate - reduced form (NADPH, type III, tetrasodium salt), L-phenylalanine, scopolamine hydrochloride (hyoscine), tetraphenylboron (sodium salt),

and Trizma base (tris-(hydroxy-methyl)-aminomethane) were supplied by the Sigma Chemical Company Ltd., Poole, Dorset, England.

HPLC grade acetonitrile, methanol and 1-octane-sulphonic acid (sodium salt), 2,5-diphenyloxaxole (PPO), 1,4-di-2-[5-phenyloxazolyl]benzene (POPOP), lead acetate, and L-tyrosine were purchased from Fisons Laboratory Supplies, Loughborough, Leicestershire, England.

Scintran toluene and 5-sulphosalicylic acid were from BDH Chemicals Ltd., Poole, Dorset, England.

[1-^{14}C] Acetyl-coenzyme A (freeze dried solid) was supplied by Amersham International plc., Buckinghamshire, England.

Helium and liquid nitrogen were supplied by BOC Ltd., Wolverhampton, West Midlands, England.

Monobromobimane (Thiolyte) was supplied by Calbiochem Brand Biochemicals, Cambridge, England.

Metaphosphoric acid was supplied by Aldrich Chemical Company Ltd., Gillingham, Dorset, England.

All other reagents were of Analar or standard laboratory grade.

2.1.2 UV/VISIBLE SPECTROPHOTOMETERS.

Spectral data on reduced pterins and protein measurements were determined using a Schimadzu uv/visible recording double beam spectrophotometer uv-240 and graphic printer Pr-1 (Schimadzu Corporation, Japan).

Dihydropteridine reductase activity was determined using a

Pye Unicam SP 1700 double beam spectrophotometer and a Pye Unicam AR 55 linear recorder (Pye Unicam, England).

2.1.3 CENTRIFUGES.

Centrifugations over 10,000 rpm were performed using a MSE Superspeed 50 ultracentrifuge (MSE Ltd., England) and a MSE 10x10ml angle head rotor. Other centrifugations were done using a MSE swing-bucket bench centrifuge.

2.1.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY, (HPLC).

i. Fluorescence detection of pterins, amino acids and glutathione.

The individual chromatographic systems used for these analyses shared the following basic features. Separation was achieved using a 5 micron Spherisorb ODS-1 reverse phase column (25cm x 4.6mm) and a short 5 micron Whatman precolumn. Automated sample injection was carried out by a Waters Intelligent Sample Processor 710B (Waters, Millipore Ltd., USA). The solvent delivery system was an LDC Constametric III pump (Milton Roy U.K Ltd.) and detection was by an LDC Fluoromonitor III, or a Kontron Spectrofluoromonitor SFM 23 (Tegimentia, Switzerland). Peaks were recorded by a J.J. CR652A chart recorder (J.J. Lloyd Instruments Ltd., England), or a W.W. 302 chart recorder (W.W. Scientific Instruments, Switzerland).

The mobile phase for the separation of pterins consisted of 5% (v/v) methanol:glass distilled water. Flow-rate was 1ml/minute. Fluorescence detection wavelengths were excitation 360nm and emission 450nm.

Retention times were: biopterin 9.0 min
pterin 15.0 min

The mobile phase for separation of amino acids consisted of 6.8g sodium acetate and 1.05g citric acid per litre of glass distilled water. Flow-rate was 1.4ml/minute. Detection wavelengths were excitation 255nm and emission 281nm.

Retention times were: L-tyrosine 4.0 min
L-phenylalanine 9.5 min

The mobile phase for glutathione (GSH) analysis consisted of methanol:50mM ammonium dihydrogen orthophosphate (30:70) containing 10mM tetrabutylammonium hydrogen sulphate and adjusted to pH 5.9 with solid ammonium hydrogen carbonate. The flow-rate was 1ml/minute. Detection wavelengths were excitation 395nm and emission 455nm.
Retention time of glutathione (GSH) was 5.4 min

All mobile phases were degassed with helium prior to use.

ii. Electrochemical detection of neurotransmitters

Separation of neurotransmitters was achieved using an LDC Milton Roy 5 micron ODS-3 reverse phase column (25cm x 4.6mm) and a short 5 micron Whatman precolumn. The column and precolumn were maintained at 30°C by a heat block. Injection of samples was done manually. The solvent delivery system was an LDC Constametric III pump which maintained a flow rate of the mobile phase at 1ml/min. Detection was by an LDC Milton Roy e.c. monitor with a Spark Holland Amor cell. The working electrode potential was set at +0.65V and peaks were recorded by a J.J. CR652A chart recorder.

The mobile phase was 0.15M sodium dihydrogen phosphate, 5mM sodium octane sulphonic acid, 0.1mM EDTA, and 5% methanol made up in glass distilled water and adjusted to pH 3.4 with glacial acetic acid. After filtering through a 0.45 micron millipore the solvent was continually degassed with helium during chromatographic runs.

Retention times were:

Noradrenaline (NA)	5.5 min
Dihydroxyphenylacetic acid (DOPAC)	11.4 min
Dopamine (DA)	13.5 min
5-Hydroxyindoleacetic acid (5-HIAA)	18.4 min
5-Hydroxytryptamine (5-HT)	34.5 min

Retention times of all the components were determined as the time (in minutes) from injection to the formation of the peak by the recorder.

Using known amounts of standards, calibration curves were produced by plotting peak area against concentration. Peak area was determined by hand (1/2 base x peak height).

2.1.5 LIQUID SCINTILLATION COUNTING.

Samples were counted on a Beckman LS 7500 Liquid Scintillation System.

2.1.6 ANIMALS.

Animals used in experiments reported in this thesis were; Wistar rats, grade IV, (supplied by Bantam and Kingman Ltd., England), or, C57BL/6NCrlBr black inbred female mice (Supplied by Charles River U.K/ Ltd.).

Except where special diets were administered, (indicated in individual chapters), animals were maintained on standard rat or mouse breeding diets supplied by Pilsburys Ltd., Birmingham, England. Tap water was available *ad libitum*. Rats were housed under a controlled 12:12 hour dark:light cycle. Experimental groups were in supplier's batches and, in the case of rat experiments, were compared to age and sex matched controls.

Oral dosing (intragastric, i.g) was performed using a hypodermic syringe fitted with an olive bulbed oropharyngeal needle. Intraperitoneal dosing (i.p) was performed using a sterile hypodermic syringe fitted with a 0.5mm diameter needle.

In starvation experiments animals were placed on grids to prevent coprophagy.

Animals were sacrificed by concussion and/or cervical dislocation.

2.2 METHODS

This section describes the major analytical procedures carried out during the course of this research. Details of individual experimental designs are specified in the appropriate results chapters.

2.2.1 MEASUREMENT OF BRAIN AND LIVER DERIVED PTERIN AND TOTAL BIOPTERINS.

Immediately after sacrifice brains and portions of liver were removed and stored at -70 °C until required. Brains were divided into right and left hemispheres prior to freezing. The right hemisphere was analysed for total biopterin and pterin and in some cases neurotransmitters.

Measurement of total biopterin and derived pterin was by HPLC and based on the method of Fukushima and Nixon (1980). 20% (w/v) tissue homogenates were prepared in 0.1M HCl and 20% (w/v) trichloroacetic acid (TCA), using a Potter-Elvehjem homogeniser.* Homogenates were spun at 100,000g. for ten minutes at 4 °C. To 1.0ml of supernatant, 0.25ml 3% (w/v) iodine was added and samples were left in the dark at room temperature. After 1 hour the iodine was reduced by addition of excess ascorbic acid. Following full speed bench centrifugation for two minutes to clarify them, samples were injected onto the HPLC, (for conditions see section 2.1.4). Liver samples were diluted 1/10 prior to injection onto the column. Pterin was derived by acid-iodine oxidation from THF and BH_2.

Total biopterin and derived pterin levels were calculated from calibration curves and results expressed as ng g^{-1} wet weight tissue.

* In experiments in which the same half brain was used to measure biopterin, pterin and neurotransmitters 20% (w/v) tissue homogenates were prepared in 0.1M perchloric acid instead of 0.1M HCl and TCA. This did not affect the efficiency of the pterin analysis.

2.2.2 MEASUREMENT OF PLASMA TOTAL BIOPTERINS.

Blood was removed from the thoracic region using heparinised (1000U ml^{-1} 0.9% (w/v) saline) syringes and bench centrifuged for ten minutes. Plasma was decanted off and stored at -70 °C until required.

To 0.4ml plasma, 0.02ml of 35% (v/v) perchloric acid and 0.01ml saturated iodine (36% (w/v)) were added. The samples were mixed and left in the dark for 1 hour. Excess ascorbic acid was added and samples bench centrifuged for ten minutes. Supernatants (20ul) were assayed by HPLC and

plasma biopterins calculated. Results were expressed as ng ml^{-1} plasma.

2.2.3 MEASUREMENT OF TETRAHYDROBIOPTERIN BIOSYNTHESIS IN BRAIN AND LIVER.

Brain and liver analysis of biosynthetic capacity was based on the method described by Hamon (1984), except the freeze drying step was omitted. 20% (w/v) homogenates of left brain hemispheres and liver tissue were prepared in 0.1M Tris-HCl (pH 7.6) buffer and were spun at 100,000g. for 45 minutes. Supernatants were divided into two (half was used to measure dihydropteridine reductase activity).

0.10ml of supernatant was incubated for 3 hours at 37 °C in the dark in a medium containing (final concentrations): 70mM Tris-HCl (pH 8.0), 28mM KCl, 6mM GTP, 3mM NADPH, 6mM $MgCl_2$ and distilled water in a final volume of 1.0ml. Blank assays were run in parallel and contained: 0.1ml supernatant, 70mM Tris-HCl (pH 8.0), 28mM KCl, 6mM $MgCl_2$ and distilled water in a final volume of 1.0ml.

The reaction was terminated by the addition of 2.0ml 0.1M HCl and reduced biopterins were oxidized with 0.5ml 3% iodine (w/v) in the dark. After 1 hour excess ascorbic acid was added and samples were spun briefly in a bench centrifuge to clarify them prior to injection onto the HPLC to measure biopterin levels.

The amount of biopterin produced by the supernatant was calculated by subtracting blank values from full incubations, and related to protein content of the supernatant which was determined by the biuret method. Results were expressed as ng tetrahydrobiopterin synthesised h^{-1} mg^{-1} protein.

2.2.4 ASSAY OF DIHYDROPTERIDINE REDUCTASE (DHPR) IN BRAIN AND LIVER.

Brain and liver homogenates were prepared as for the biosynthesis assay. Measurement of DHPR activity was essentially the method described by Craine *et al.*, (1972).

The assay mixture contained (final concentrations): 50mM Tris-HCl (pH 6.8), 0.25 mM sodium azide, 8ug peroxidase, 0.1mM NADH, 0.1mM hydrogen peroxide, 0.02ml tissue supernatant (enzyme source), and distilled water to give a final volume of 0.9ml. The reagents were added directly into plastic cuvettes (1cm path length), mixed by inversion and incubated at 37 °C for 90 seconds. The reaction was initiated by the addition of 0.1ml 1mM 6,7-dimethyl, 5,6,7,8-tetrahydropterin and after 30 seconds the rate of reaction was followed by measuring the rate of decrease in absorbance at 340nm. The sample mixture was run against an enzyme free blank.

Protein content of the original supernatants was measured by the Biuret method. A molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} for NADH was used to calculate DHPR activity and the results were expressed as specific activities - nmoles NADH oxidised min^{-1} mg^{-1} protein.

2.2.5 BIURET METHOD FOR PROTEIN DETERMINATION.

To 0.4ml distilled water and 0.1ml protein source, 2.0ml of Biuret reagent (0.15% (w/v) copper sulphate pentahydrate; 0.6% (w/v) sodium potassium tartrate; 3% (w/v) NaOH; 0.1% (w/v) KI in distilled water) was added, mixed well and left at room temperature for 30 min. The absorbance at 540nm was measured against a protein blank. Sample protein

concentration was calculated from a calibration curve constructed using bovine serum albumin.

2.2.6 MEASUREMENT OF PLASMA PHENYLALANINE AND TYROSINE.

The method used was based on that of Hyland *et al.*, (1985). Rat whole blood was obtained by cardiac puncture and plasma prepared by centrifugation in a bench centrifuge. To 1.0ml plasma 30mg of 5-sulphosalicylic acid was added, mixed and ultracentrifuged at 100,000g for 5 min. Supernatants were analysed by HPLC, (for conditions see section 2.1.4).

The HPLC was calibrated using L-phenylalanine and L-tyrosine standards made up in 0.02M HCl. Results were expressed as umoles of amino acid l^{-1} plasma.

2.2.7 MEASUREMENT OF BRAIN AND LIVER GLUTATHIONE, (GSH).

The method used was based on that of Burton and Aherne (1986). It involved derivatisation of the thiol using monobromobimane (mBBr) to form a fluorescent adduct which was analysed by fluorimetric HPLC.

Tissues were frozen in liquid nitrogen immediately on withdrawal from the animal. 25% homogenates (w/v) of half brains and portions of liver were prepared in 15% (w/v) metaphosphoric acid (MPA) and spun at 9950g for 5 min at 4 °C. Supernatants were decanted off and stored at -70 °C until analysed.

Supernatants were diluted before assaying to obtain a concentration within the range of the standard curve. The dilution buffer consisted of; two volumes of 10mM ammonium hydrogen carbonate, 1mM EDTA pH 8.0 and one volume of 15% (w/v) MPA having a final pH of 1.8.

20ul of diluted tissue supernatant was added to 180ul of 0.2M borate buffer and 10ul of mBBr (250ug/ml acetonitrile) were added. (The borate buffer was prepared from 0.2M KCl and 0.2M boric acid, the pH being adjusted to 10.5 with 0.2M NaOH). The mixture was incubated at room temperature in the dark for 30 min and 10ul glacial acetic acid was added to stop the reaction. Samples were then stored on ice in the dark until 50ul were analysed for glutathione (GSH) content by fluorimetric HPLC, (for conditions see section 2.1.4).

GSH standards were made up in dilution buffer and the standard curve was obtained by plotting GSH-mBBr adduct peak height (cm) against GSH concentration (pmol/50ul). Over the range of the standard curve (40-240 pmol added to column) the response of the detector was linear (correlation coefficient = 0.9987). Results were expressed as umol GSH g^{-1} wet wgt tissue.

2.2.8 MEASUREMENT OF NEUROTRANSMITTERS IN RAT BRAIN TISSUE.

Whole half rat brains were analysed for noradrenaline (NA), dopamine (DA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) content. In some experiments 3,4-dihydroxyphenylacetic acid (DOPAC) content was also measured.

20% (w/v) homogenates of right brain hemispheres, which had been frozen at -70 °C after removal from the animal, were prepared in 0.1M PCA and spun at 15,000g for 10 min at 4 °C. The supernatants were decanted and restored at -70 °C until required. Samples were defrosted and microfiltered (0.2um pore size) prior to analysis by HPLC with electrochemical detection, (for conditions see section 2.4.1). As the samples were very susceptible to oxidation in light and

air, no more than 2 were defrosted at a time and were kept on ice in the dark immediately prior to injection.

In preliminary studies it was found that brains which were frozen at -70 °C after removal from the animal and analysed within the next 72 hours showed no detectable reduction in neurotransmitter levels compared to fresh brains which were analysed immediately after excision.

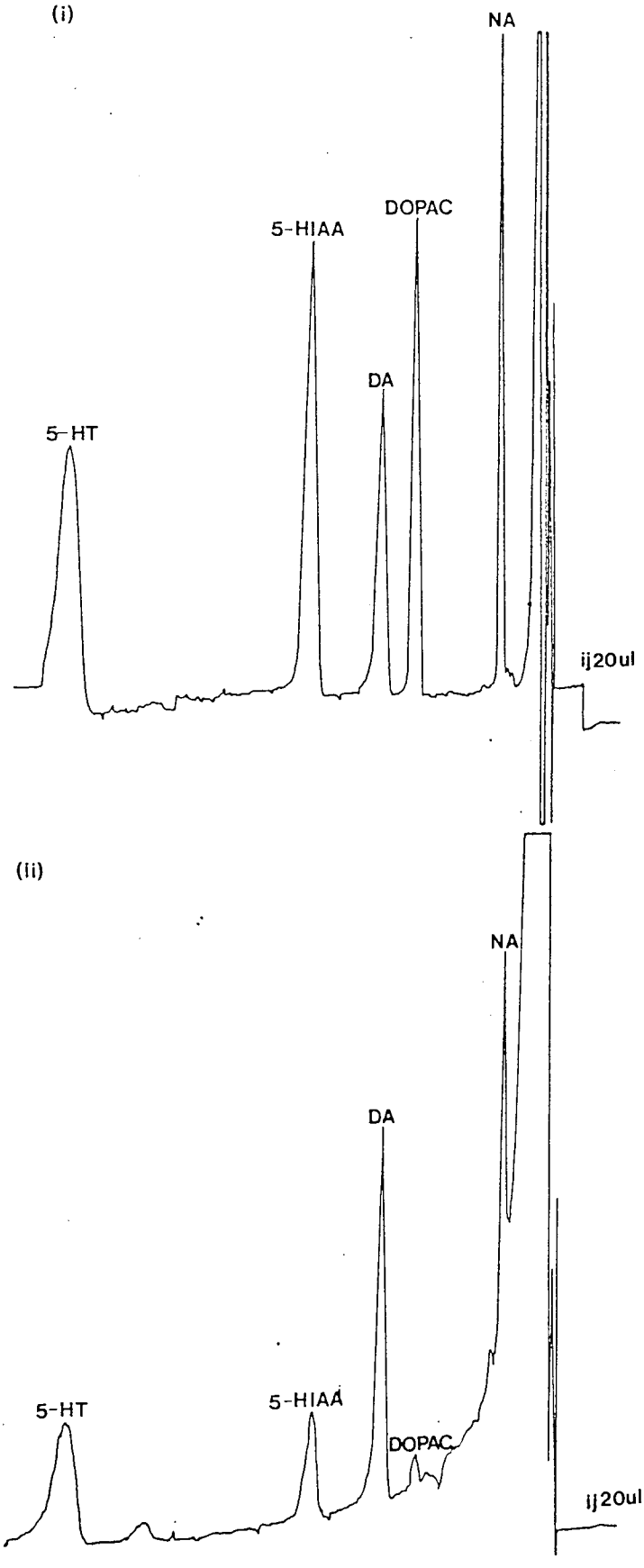
Fresh neurotransmitter standards were prepared in 0.1M PCA and standard curves constructed immediately before each experiment. Standards were run intermittantly during the analysis of samples to ensure that the conditions of the chromatographic separation had remained constant. Typical chromatograms illustrating the separation of neurotransmitter standards and tissue extracts are shown in Fig. 2.1.

2.2.9 MEASUREMENT OF CHOLINE ACETYLTRANSFERASE (CAT) ACTIVITY IN RAT BRAIN.

The method used was based on that of Fonnum (1975) who developed a radiochemical assay for CAT that involved the specific isolation of labelled acetylcholine by "liquid cation exchange" using sodium tetraphenylboron.

5% homogenates (w/v) of left hemisphere brain tissue were prepared in 10mM EDTA (pH7.4) and activated with 0.5% (w/v) Triton X-100 to ensure total release of enzyme activity. After ultracentrifugation at 10,000g for 15 min, supernatants were collected for assay which was performed in duplicate for each sample.

Fig. 2.1 Typical chromatograms illustrating the separation of neurotransmitters in (i) standard solutions and (ii) brain extracts.



RETENTION TIMES (mins)

NA	-	5.5
DOPAC	-	11.4
DA	-	13.5
5-HIAA	-	18.4
5-HT	-	34.5

ij = point of sample injection onto column.

The incubation mixture for the assay contained (final concentration): 0.2mM acetyl coenzyme A, 300mM NaCl, 50mM sodium phosphate buffer (pH 7.4), 8mM choline bromide, 20mM EDTA (pH 7.4), 0.1mM physostigmine, ^{14}C labelled acetyl coenzyme A (2.22×10^7 dpm ml^{-1} incubation mixture) and enzyme source.

4ul of enzyme source was added to 10ul of substrate mixture to give a final volume of 14ul. Assays were mixed, incubated at 37 °C for 15 min and then washed into scintillation vials with 5.0ml of 10mM sodium phosphate buffer (pH 7.4). 2.0ml of acetonitrile solution containing 15mg tetraphenylboron was added followed by 10.0ml of scintillation mixture (0.05% PPO and 0.02% dimethyl POPOP in scintillation grade toluene). Samples were shaken lightly for 1 min and after standing for 10 min were counted in a beta liquid scintillation counter, together with blanks containing water instead of enzyme source. A quench curve was prepared for the scintillation mixture.

The procedure depends on two observations: (1) acetonitrile in toluene constitutes an efficient extraction solvent for liquid cation exchange of acetylcholine with tetraphenylboron; (2) liquid scintillation counting of an organic phase can proceed undisturbed in a biphasic organic : aqueous solution mixture.

Labelled acetylcholine produced by CAT activity is extracted into the scintillation mixture and counted at high efficiency whereas acetylcoenzyme A remains in the aqueous phase and is not counted since water does not function as a scintillation solvent.

The number of counts attributable to the acetylcholine produced in the assay was determined by subtracting the counts for the assay blank from the counts measured in the sample solutions. Hence, the % conversion of the original number of counts added to the assay, in the form of acetyl

CoA, into acetylcholine can be calculated. The number of moles of acetyl-CoA added to the assay was known and thus the amount of acetylcholine produced could be determined. CAT specific activity was expressed as umoles of acetylcholine formed h^{-1} g^{-1} wet weight brain tissue.

2.2.10 BEHAVIOURAL ANALYSIS.

(a): Open-field Behaviour.

General open-field behaviour of weaner rats was investigated. This is a test widely used in pharmacological studies, (Gray, 1987). The analysis was conducted in a high walled arena the floor of which was marked off into twenty-five 11.5 x 11.5cm squares. Each animal was placed in the centre square at time zero and its activity in the arena monitored over a 5 minute period. The number of squares entered, the number of upward rears displayed and the number of times the animal groomed itself were recorded. After each animal had been in the arena the floor was wiped with a medical antiseptic and allowed to dry before the next animal was tested. This was done in order to remove traces of the path of the previous animal which may influence the movement of the animal under observation.

(b): Head-Dipping as a Measure of Curiosity in a Modified Hole-Board.

The hole-board was first introduced by Bossier and Simon (1962) and since then has been extensively used to study drug effects. The board used in this investigation was similar to that developed by File and Wardill in 1975. It consisted of a high walled arena with four holes evenly

spaced in the centre of the floor. Each hole was 4.0cm in diameter. The walls of the arena extended below the level of the floor which was thus raised to a height of 13cm. Each animal was placed singly in the centre of the board facing away from the observer and its behaviour recorded for 5 minutes. A head dip was scored if both eyes disappeared into a hole. The total amount of time spent head-dipping was also noted. As before, after each trial the floor of the arena was wiped and dried to remove traces of the previous path.

2.2.11 STATISTICAL ANALYSIS

Biochemical data is shown as the arithmetic means $\pm$ standard deviations, (ie. mean $\pm$ st.d). Comparisons between test and control data was by two-tailed Student's unpaired t tests. The results of certain experiments were also analysed by one-way or two-way analysis of variance depending on experimental design. Correlation coefficients were calculated using the least squares method. Statistical probabilities (p values) are indicated.

Behavioural analysis was by Student's unpaired t test, Chi-squared Contingency table test, two-way analysis of variance in randomised blocks and 2 factor analysis of variance split-plot.

Results giving probabilities of 0.05 (ie. $p<5\%$) and less were regarded as significant.

CHAPTER THREE

THE EFFECTS OF POTENTIALLY NEUROTOXIC AGENTS ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT.

3.1. INTRODUCTION.

Exposure to high concentrations of some environmental agents and many drugs can result in adverse effects on the human central nervous system. Often, the precise mechanism by which these compounds bring about neurotoxicity is unclear. The agents discussed below are all potential neurotoxins.

3.1.1 Lead.

Biological interest in lead was initiated as a result of its pronounced toxic properties as an industrial hazard to man (Hilburn, 1979). For many years researchers equated the lowest blood-lead level diagnostic of clinically manifest lead poisoning with the upper limits of normal (Lin-Fu, 1972). "Affected" individuals would exhibit symptoms such as anaemia, abdominal colic, and nephropathy with blood-lead levels above 60ug/dl (Kehoe, 1972). However, over the years the consensus of what is the upper limit of normal has been dropping. Concern now exists as to whether lifetime exposure to low levels of lead causes permanent neurological damage when overt signs of toxicity are absent.

Over the past decade a considerable body of evidence has been collected suggesting a relationship between childhood lead exposure and various symptoms of minimal brain dysfunction. Neonatal exposure to relatively low levels of environmental lead has been reported to cause

hyperactivity, learning difficulties and problems with motor coordination in both human and animal models (David *et al* 1972 and 1982; Faust and Brown 1987; Levin *et al* 1988; Silbergeld and Goldberg 1975; Sauerhoff and Michaelson 1973; Weinreich *et al* 1977). The developing nervous system is particularly susceptible to the adverse neurological effects of chronic lead exposure (Brown 1975; Fox *et al* 1977) as lead is absorbed and retained more readily perinatally (Forbes and Reina 1972; Mykkanen *et al* 1979). Thus, the Environmental Protection Agency (1984) now regards the maximum safe blood concentration for an individual child to be 25ug/dl. Recent epidemiological studies have reported adverse intellectual effects as a result of developmental lead exposure at lower body burdens (Bellinger *et al* 1986).

Despite extensive studies the biochemical mechanism underlying the neurotoxicity of long term lead exposure has remained unclear. Most efforts have been directed towards investigation of the effects of lead on catecholamine pathways. A detailed review of the research undertaken has been made by Winder (1982) who concluded that, despite a considerable array of hypotheses and conflicting results, the available evidence points to the existence of a catecholaminergic dysfunction at levels of lead relevant to human childhood exposure. The precise nature of the dysfunction still remains to be elucidated.

Recently the interaction between lead and tetrahydrobiopterin (BH_4) metabolism has received attention. Purdy *et al* (1981) demonstrated inhibition of rat brain BH_4 synthesis *in vitro* at lead acetate concentrations as low as 10^{-8}M, and DHPR activity in both rat brain and liver was irreversibly inhibited at 10^{-6}M lead. Positive correlations between plasma biopterin and low blood lead levels have been shown in man and the rat (Blair *et al*, 1982; McIntosh *et al*, 1982) again indicating an inhibitory effect of lead on DHPR. At higher blood lead

levels serum biopterins in man are significantly lower than normal suggesting that saturation of DHPR inhibition is accompanied by a reduction in BH_4 synthesis (Hilburn 1979; Leeming *et al*, 1981). DHPR is a thiol containing enzyme and the reaction of lead with sulfhydral groups at the active site may account in part for the neurotoxicity of the metal, (Cheema *et al*, 1973; Webber and Whitley, 1981).

Three months chronic administration of high and low levels of lead acetate to weaner rats in their drinking water resulted in significantly increased lead concentrations in brain, liver and plasma compared to controls (Edwards 1988). At the higher dose of lead brain total biopterins and pterin were significantly depressed. Both high and low doses caused increased liver and plasma total biopterins compared to controls. The increase in plasma biopterin was thought to reflect decreased DHPR activity.

A similar experiment carried out by McIntosh (1985) showed increased actual BH_4 levels in brains of rats exposed to high and low doses of lead acetate in their drinking water for 1 or 3 months. DHPR activity was also increased but only after 3 months at the lower dose of lead. These effects on BH_4 metabolism were confined to the diencephalon with the cerebellum, mid brain and telencephalon showing no changes. McIntosh concluded that the rise in DHPR activity may have been compensatory to a disruption in BH_4 metabolism at some other point, possibly *de novo* biosynthesis.

Data produced to date has therefore suggested that BH_4 metabolism appears to be particularly sensitive to the toxic effects of lead. Observations of a direct involvement of the metal with catecholaminergic function suggests that lead-induced intellectual impairment may be caused by disruption of tetrahydrobiopterin pathways in the brain.

3.1.2 Diethylstilboestrol.

Oestrogens, widely prescribed as oral contraceptives, have been shown to cause several neurologic side effects (Bickerstaff 1975). Oral contraceptive-induced chorea is a recognized, but rare example of a complication of oestrogen medication, (Fernando 1966; Pulsinelli and Hammil 1978; Nausieda *et al* 1979), and is attributed to defective central dopaminergic mechanisms, (Klawans and Weiner 1976). The influence of oestrogens on the CNS is controversial, but their main effects are on catecholaminergic function. Catechol oestrogens (CO's), important metabolites of the primary oestrogens (Ball and Knuppen 1980), can interact directly with pathways of catecholamine biosynthesis, metabolism and response (Panek and Dixon 1986).

CO's competitively inhibit tyrosine hydroxylase (Foreman and Porter 1980) and catachol-O-methyltransferase activities (Breuer and Koster 1974), and noncompetitively inhibit DHPR activity (Shen and Abell 1983) and depress monoamine oxidase activity (Ball and Knuppen 1980). These effects were observed under *in vitro* conditions at micromolar concentrations of CO's, levels two to three orders of magnitude higher than the concentration of any oestrogen in the circulation (Smith *et al* 1975). This raises doubts regarding the possible roles of these mechanisms in normal neuroendocrine function (Paden *et al* 1982).

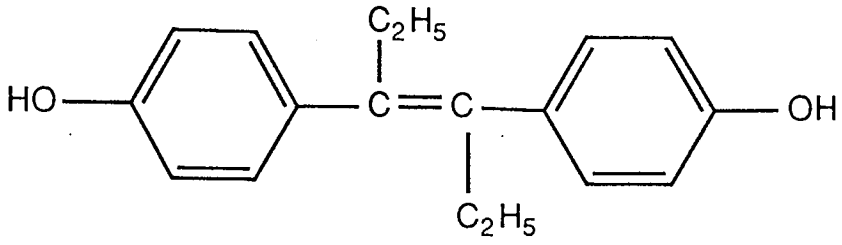
Conflicting evidence exists for the effects of oestrogens on catecholamine turnover and transmission (Parvizi and Ellendorf 1983; Bedard *et al* 1979; Panek and Dixon 1986). Di Paolo (1982) described a biphasic action of oestrogen. The effect is primarily antidopaminergic and involves decreasing dopaminergic transmission by some presynaptic effect which induces a compensatory increase in the number of postsynaptic dopamine receptors. Depletions of dopamine in the striatum, may be caused by an alteration in the

synthesis of dopamine (Di Paolo 1982).

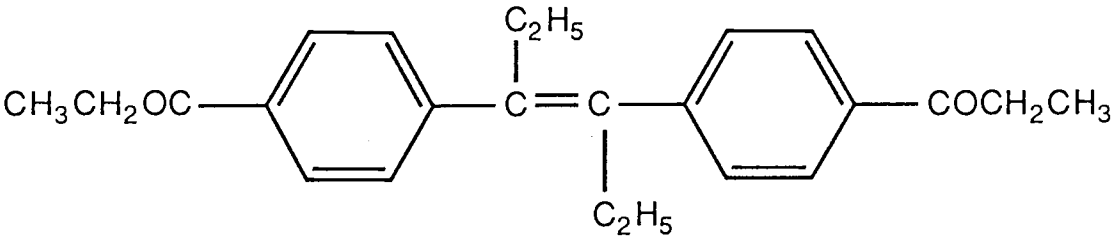
Speculation exists on the role of oestrogen-induced changes in brain monoamine concentrations in the aetiology of pre-menstrual, post-partum and menopausal depressions in women, since in all these conditions oestrogen levels fluctuate widely (Greengrass and Tonge 1971). It has been suggested that a disturbance in tetrahydrobiopterin metabolism could be involved in the antidopaminergic activity of oestrogen. Studies in women have shown that levels of serum biopterins vary with hormonal state their concentrations falling when oestrogen levels rise throughout the menstrual cycle, during pregnancy and when taking the contraceptive pill (Leeming and Blair 1980; Barford *et al* 1983). In these situations there is a compensatory rise in DHPR activity (Barford *et al* 1983).

Diethylstilboestrol, DES (1), is a synthetic non-steroidal phenolic oestrogen which was widely prescribed between 1943 and 1971 as an effective agent for minimizing complications of pregnancy, (Smith 1948), and as a post-coital contraceptive. Use of DES was terminated when an association between exposure to the compound and a rare form of reproductive tract cancer was established in female offspring (Herbst and Scully 1970).

Work was undertaken to investigate the effects of diethyl-stilboestrol, DES (1), and the derivative diethylstil-boestrol dipropionate, DES-dip (2), on BH_4 metabolism using the rat as an experimental model (Eggar *et al* 1983; Blair *et al* 1984). Oral administration of large doses of DES (500mg/kg) to weaner rats over four days was reported to have an inhibitory effect on brain BH_4 synthesis. This was reflected in a 45% decrease in total brain biopterin levels but the percentage as BH_4 remained unchanged. DHPR activity was increased. Further work using DES-dip, (Al-Salihi 1985), showed a dose-response effect of the compound on both total brain biopterin and actual BH_4 levels.



(1) DIETHYLSTILBOESTROL (DES)



(2) DIETHYLSTILBOESTROL DIPROPRIONATE (DES - dip)

Such alterations in BH_4 metabolism suggest the possible involvement of the cofactor in the psychosomatic effects of oestrogens.

3.1.3 D-amphetamine.

D-amphetamine is a phenylethylamine psychoactive drug which stimulates various changes in the central nervous system and hence animal behaviour. Considerable controversy exists as to the precise mechanism of action of the drug, as distinguishing clearly its primary effects and the relative magnitudes of each has proved problematic.

The actions of d-amphetamine on dopaminergic neurons have been studied extensively as it is believed that many of the behavioural effects induced by the drug are mediated by dopaminergic pathways (Groves and Tepper 1983). Several actions of the drug have been reported (for a review see Robinson and Becker 1986). These include; blockade of dopamine uptake (Caviness and Wightman 1982; Kuhr *et al* 1985), induction of dopamine release (Arnold *et al* 1977; Fischer and Cho 1979), increase in the synthetic rate of dopamine (Kuczenski 1977; Demarest *et al* 1983) and inhibition of MAO and thus dopamine metabolism (Miller *et al* 1980). All these mechanisms lead to increased dopamine levels in the synapse. Amphetamine-induced increases in release of noradrenaline and serotonin have also been reported (Balfour and Iyaniwura 1985; Raiteri *et al* 1975; Bradbury *et al* 1987).

However, several authors have reported that repeated large doses or chronic administration of amphetamine produces decreased dopamine levels and depressions in tyrosine and tryptophan hydroxylase activities (Besson *et al* 1971; Kuczenski 1977; Fung and Uretsky 1982; Hotchkiss *et al* 1979; Steranka and Sanders-Bush 1980), indicating reduced

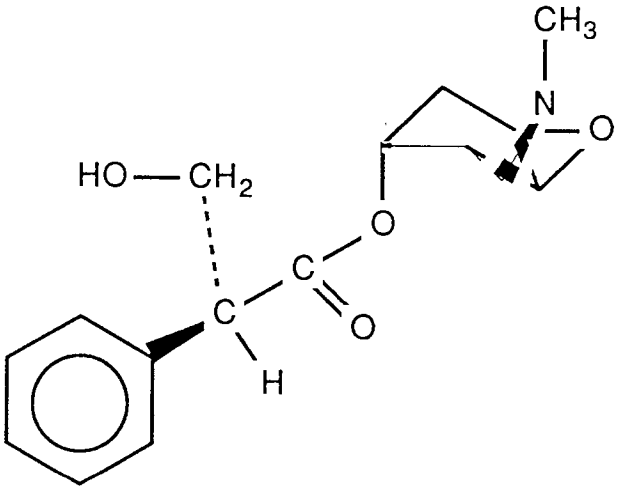
catecholamine biosynthesis. The mechanism by which amphetamine depresses these enzyme activities has not been elucidated (Egashira *et al* 1987).

Since BH_4 levels are thought to be rate-limiting in catecholamine biosynthesis, (Levitt *et al* 1965), workers have investigated the affect of amphetamine on BH_4 metabolism in rat brain (Bullard *et al* 1978, 1979: Mandell *et al* 1980; Mandell and Russo 1981; Lee and Mandell 1985a, 1985b). D-amphetamine administered i.p induced a decrease in BH_4 levels which did not exceed 25-30% with increasing doses of the drug. Recovery of control levels took several hours. Regional distribution studies showed that amphetamine decreased total biopterin levels and actual BH_4 in the major serotonin, dopamine and noradrenaline containing cell groups in a consistent way, but under no circumstances did the drug effect whole brain or striatal total biopterin levels.

Mandell *et al* (1980) proposed that utilisation and DHPR controlled regeneration of BH_4 require conformational and functional coupling to maintain normal cofactor levels and that this coupling is dynamically alterable by amphetamine such that BH_4 levels decrease. However, they concluded that the drug effect was complex since they later discovered that amphetamine-induced changes in levels of endogenous BH_4 did not correlate well with brain monoamine levels (Lee and Mandell 1985). The unique effects of amphetamine on brain BH_4 may have their functional significance and clinical implications.

3.1.4 Scopolamine.

Scopolamine (L-hyoscine) (3) is a powerful anticholinergic drug capable of crossing the blood-brain barrier (Innes and Nickerson 1970). It acts by competitive antagonism at



(3) SCOPOLAMINE

muscarinic receptors. Clinically it is known for its activity as an inhibitor of parasympathetically mediated functions, and is used in conjunction with analgesic agents in obstetrical anesthesia. Side effects of scopolamine include drowsiness, amnesia and fatigue.

There is considerable evidence that the cholinergic system plays a central role in memory and learning (Drachman and Leavitt 1974; Drachman 1977). Interference with cholinergic transmission by scopolamine results in severe cognitive impairment characterised by major disruptions in memory storage, (Drachman and Leavitt 1974), and impaired attentional vigilance, (Traub *et al* 1987). The pattern of cognitive deficits which emerges, closely resembles that seen in normal aging and dementia (Drachman and Leavitt 1974; Huff *et al* 1988). Biochemical analyses of post-mortem brain from aged human patients has demonstrated a positive correlation between the degree of change in cholinergic function and degree of senile dementia (White *et al* 1977).

Memo *et al* (1988) showed a scopolamine-induced decrease of dopamine turnover in rats. Results indicated a specific cholinergic control of the dopamine system located in those brain areas particularly involved in cognitive function, i.e. hippocampus and frontal cortex, suggesting that dopamine could be involved in the integrative functioning of memory and the learning process. However, scopolamine has been used in the therapy of Parkinson's disease (Meyers *et al* 1980) a condition characterised by dopamine deficiency.

Edwards (1988) investigated the effects of scopolamine on tetrahydrobiopterin metabolism in the rat. No change in total biopterin levels was observed but scopolamine caused a reduction in brain pterin concentrations at a dose of 0.023mg / 270g body weight.

3.2 MATERIALS AND METHODS.

The effects of lead, diethylstilboestrol, d-amphetamine and scopolamine on tetrahydrobiopterin metabolism in the rat were investigated.

Total biopterins, derived pterin, DHPR activity and biosynthetic capacity were measured as described in Chapter 2.

Some experiments were performed more than once. The results of each "trial" are recorded individually as large inter assay variability meant that no between batch comparisons could be made. Control and test samples within a particular trial were assayed together. Details of the various dosing regimes are given below.

3.2.1 LEAD.

Experiments were performed in which 150g male Wistar rats were dosed with different concentrations of lead acetate:-

Regime 1: 340mg/kg intraperitoneally (i.p.) and sacrificed after 4 hours. Brain total biopterin measured.

Regime 2: 0.3ml of 10^{-3}M lead acetate i.p. or intragastrically (i.g.) twice daily for 3 days. Sacrificed 24 hours after the last dose. Brain total biopterin, pterin and DHPR measured.

Regime 3: 0.3ml of 10^{-1}M lead acetate i.p. twice daily for 3 days. Sacrificed 24 hours after the last dose. Brain and liver total biopterin, pterin and DHPR measured.

3.2.2. DIETHYLSTILBOESTROL.

Experiments were performed in which female Wistar rats were dosed with different concentrations of diethylstilboestrol (DES) or the ester diethylstilboestrol dipropionate (DES-dip).

In experiments 1-5 animals were dosed i.g. once daily for four days with a thick suspension of DES or DES-dip in corn oil and sacrificed 24 hours after the last dose. Controls received corn oil.

Experiment 1: 100g rats given 50mg/kg DES-dip. Brain total biopterin and derived pterin measured.

Experiment 2: 50g weaner rats given 50mg/kg DES-dip. Brain total biopterin and derived pterin measured.

Experiment 3: 50g weaner rats given 500mg/kg DES-dip. Brain total biopterin and derived pterin measured.

Experiment 4: 50g weaner rats given 500mg/kg DES. Brain and liver total biopterin, derived pterin and DHPR measured.

Experiment 5: 50g weaner rats given 500mg/kg DES-dip or DES to compare the effects of the two treatments. Brain and liver total biopterin, derived pterin and DHPR measured.

DES dissolves completely in 75% ethanol. By administering a solution rather than a suspension it was thought that more of the compound might penetrate the blood-brain barrier and an effect on brain BH_4 metabolism might be more readily observed. In experiments 6 and 7 50g weaner rats were given a single dose (i.g.) of DES (500mg/kg) dissolved in 75% ethanol and sacrificed after 8 hours. Controls received 75% ethanol.

Experiment 6: The effect of giving DES in ethanol was investigated. Plasma total biopterin, brain total biopterin, derived pterin and DHPR and liver total biopterin and derived pterin were measured.

Experiment 7: An experiment was performed to test whether DES had a dose dependent effect on rat brain and liver total biopterin levels.

During the course of previous work it became evident that, with the techniques employed, a large inter assay variability in biopterin and derived pterin levels existed. This stressed the importance of analysing test and control samples together and not making between batch comparisons. This "time of analysis" effect on biopterin levels also indicated that special care should be taken in the design of experiments requiring the analysis of large numbers of samples eg. in a dose-response investigation.

Suppose thirty-six animals were taken and each assigned at random to one of six groups, (five different levels of DES and a control group), so that each group contained six animals. At the end of the dosing period all thirty-six samples would have to be analysed sequentially, over a period of ten or more hours. The "time of analysis" effect on biopterin levels would considerably increase the mean standard deviation value for each group of animals. This in turn would significantly increase the experimental error component of a one-way analysis of variance which might then mask any effect of a particular treatment.

If however the experiment was designed so that statistical analysis took account of this effect of "time" on biopterin levels and eliminated it from the overall experimental error, any effect attributable to the treatment would be more readily apparent.

Thus the dose response investigation carried out in

experiment 7 was designed in randomised blocks. On each occasion six female weaner rats were taken and five were each given a different dose of DES (50-500mg/kg body weight) dissolved in 75% ethanol. The sixth, a control, was given just ethanol. Animals were sacrificed after 8 hours and each set of six samples was analysed together. This procedure was carried out on four separate occasions each representing a block in the experimental design. Statistical analysis was by two-way analysis of variance in randomised blocks. Results are expressed as F ratios:-

F ratio (treatment) indicates the significance of any effect of the DES on biopterin or pterin levels.

F ratio (blocks) indicates the significance of any effect of doing the analyses on different occasions, (i.e. the effect of time), on biopterin or pterin levels.

3.2.3 AMPHETAMINE.

200g male rats were dosed i.p. with either 1mg/kg d-amphetamine sulphate and sacrificed after 1 hour, or 5mg/kg d-amphetamine sulphate and sacrificed after 2 hours. D-amphetamine was dissolved in isotonic saline and controls received saline alone. Brain and liver total biopterin, derived pterin and DHPR were measured.

3.2.4 SCOPOLAMINE.

270g male rats were dosed i.g. with 0.023mg or 0.23mg of scopolamine hydrochloride in distilled water and sacrificed 5 hours later. Controls were given distilled water. Brain and liver biopterin, derived pterin, DHPR activity, biosynthetic capacity and plasma biopterins were measured.

3.3 RESULTS AND DISCUSSION.

3.3.1 Lead.

There was consistently no significant effect on rat brain total biopterin levels 4 hours after the administration of an acute dose of lead acetate (340mg kg^{-1} body weight) compared to controls (Table 3.1). Similarly neither i.g. or i.p. administration of 10^{-3}M lead acetate over a 3 day period had a significant effect on rat brain total biopterin, derived pterin or DHPR levels (Tables 3.2 - 3.4). This dosing regime resulted in a total administration of 0.715mg lead acetate/rat over the three days. Cutler (1986), following a similar dosing regime (rats receiving a total of 0.911mg lead acetate i.p. over three days), did show a significant reduction in brain DHPR activity ($p<0.5\%$). However, this effect was only observed when the number of experimental animals was large (n=12). If the lead was given i.g. and n=6, DHPR activity was not significantly depressed.

Dosing with 10^{-1}M lead acetate twice daily for three days resulted in a total administration of 71.5mg lead acetate/rat. No change was detected in brain total biopterin, derived pterin or DHPR levels (Tables 3.5-3.7). Liver total biopterins were significantly decreased compared to controls ($p<0.1\%$, Table 3.8). Liver derived pterin though non-significantly reduced on one occasion was decreased significantly when the experiment was repeated ($p<0.2\%$, Table 3.9). Liver DHPR activity was unaffected (Table 3.10).

In summary, the results show that acute administration of lead acetate to adult rats does not lead to disruption of brain BH_4 metabolism as determined by measurement of whole or half brain biopterin, derived pterin and DHPR levels. However, the significant reduction in liver total biopterin

and pterin levels suggests that the absence of an effect in the brain might be due to insufficient accumulation of lead in this organ during such a short period of exposure.

It is well known that transport of lead across the blood-brain barrier is poor (DeMichelle 1984). Though the concentration of lead in all tissues rises with increased intake, most accumulation occurs in the bone and liver, with the brain receiving the smallest amounts. Chronic administration of high doses of lead to weaner rats over a 3 month period, (Edwards 1988), has resulted in significantly elevated brain lead levels compared to controls and subsequent decreases in whole brain total biopterin and derived pterin levels. Young animals, and therefore an immature nervous system, are particularly susceptible to the neurotoxic effects of lead (Rutter *et al* 1986).

Alternatively, the findings of Meredith *et al* (1988) may explain why the whole brain studies carried out in this investigation showed no apparent effect of lead on BH_4 metabolism. He carried out acute and chronic exposure studies of the effects of lead on the catecholaminergic system in 5 regions of rat brain. He concluded that both the accumulation and neurochemical effects of lead in rat brain show regional specificity. Even when a region showed significantly elevated lead levels, an affect on the catecholaminergic nervous system was not always evident. Low acute doses, similar to those administered in this investigation, failed to significantly elevate regional brain lead levels and had no effect on catecholamine levels or tyrosine hydroxylase (TOH) activity.

Thus, it seems unlikely that the short-term dosing regimes used in the current investigation produced significantly elevated whole brain lead levels. Even if levels in discrete regions had been increased and subsequent alterations in BH_4 metabolism had occured, the effects

would probably not have been detected by analysis of whole brains.

3.3.2 Diethylstilboestrol.

A low dose of DES-dip (50mg/kg body weight) given in corn oil for 4 days consistently had no effect on brain total biopterin or derived pterin levels in 100g rats (Tables 3.11 and 3.12) or 50g weaner rats (Tables 3.13 and 3.14).

A higher dose of the same compound (500mg/kg body weight) given to weaner rats also showed no effect on brain total biopterin levels (Tables 3.15 and 3.23), derived pterin levels (Tables 3.16 and 3.24) or DHPR activity (Table 3.25) compared to controls. These results do not support those of Blair *et al* (1984), or Al-Salihi (1985) who, with the same dosing regime, saw a 45% decrease in brain total biopterins, significantly reduced pterin levels and increased DHPR activity. It is not clear why these effects on brain BH_4 metabolism have not been reproducible in this investigation. It is possible that previous workers did not analyse test and control samples together in which case large inter-assay variability might have contributed to the observed effects.

DES-dip (500mg/kg) did cause significant decreases in liver total biopterin levels ($p<1\%$, Table 3.26). Liver derived pterin and DHPR were unchanged (Tables 3.27 and 3.28). An effect on biopterin levels but not DHPR supports the proposition made by Blair *et al* (1984) that oestrogens appear to inhibit BH_4 synthesis rather than the salvage pathway.

When DES was administered as the free compound (500mg/kg body weight), again in corn oil for 4 days, an increase in brain total biopterin was observed on one occasion which

was just significant at the 5% level (Table 3.17). This result was not reproducible in a second trial of the same experiment (Table 3.23). Brain derived pterin and DHPR levels were unaffected (Tables 3.18 and 3.19). DES significantly depressed liver total biopterin ($p<1\%$, Table 3.20) and derived pterin levels ($p<5\%$, Table 3.21) compared to controls but again DHPR was unaltered (Table 3.22). These results were all reproducible (Tables 3.24-3.28).

A comparison of the results for the free compound (DES) and the ester (DES-dip) revealed that they produce very similar effects. Neither altered brain BH_4 metabolism (Tables 3.23-3.25). Initial examination of the liver results indicated a lower degree of biological activity of the ester compared to the free compound with respect to reduction in biopterin and derived pterin levels (Tables 3.26 and 3.27) which may have reflected different rates or patterns of metabolism of the two agents. (DES has been shown to form highly reactive free radical intermediates during peroxidatic oxidation which may be resposible for it's genotoxic activity (Ross *et al* 1985). DES-dip, being devoid of free hydroxyl groups, does not form free radicals.).

However, when the doses were calculated on a molecular basis, it was clear that administration of equal weights of the two substances (500mg/kg), meant that animals given the free compound received approximately 30% more DES than those dosed with the ester. Hence the results shown in tables 3.26 and 3.27 may have simply reflected a dose response effect of DES.

Administration of DES dissolved in 75% ethanol failed to bring about an effect on brain biopterin, derived pterin or DHPR (Tables 3.30-3.32). Plasma biopterin levels were also unaffected (Table 3.29). Liver biopterin was significantly reduced ($p<1\%$, Table 3.33) and derived pterin was reduced but not significantly (Table 3.34). This dosing regime was used to test for a dose response effect of DES on brain and

liver BH_4 metabolism (Tables 3.35-3.38).

Two-way analysis of variance in randomised blocks showed no effect of DES on brain biopterin or derived pterin levels at any of the concentrations given, (F=0.978, Table 3.35; and F=1.607, Table 3.36 for biopterin and pterin respectively). This is in keeping with earlier results seen in this investigation. However, it is contrary to the findings of Al-Salihi (1985) who did show a dose response effect of DES on rat brain biopterin and pterin levels over a DES range of 4.3ug-500mg/kg body weight.

The importance of assaying test and control samples from the same experiment together, (or designing the experiment to allow for large inter-assay variability) was emphasised by the F ratio (blocks) values (Tables 3.35 and 3.36). In the case of biopterin and pterin there were significant differences between results obtained on different days ($p<5\%$ and $p<1\%$ respectively). Interestingly, the F ratio (blocks) for derived pterin (F=8.935) was double that for biopterin (F=4.549) indicating a greater variability in derived pterin levels between assays than biopterin levels. It is possible that the wider spread in pterin levels resulted from variation in the rate of breakdown of tetrahydrobiopterin during different analyses.

Liver, F ratio values indicated a significant effect of DES on total biopterin ($p<5\%$, Table 3.37) but not derived pterin levels (Table 3.38) supporting earlier results for DES given in ethanol. However, examination of the individual biopterin values revealed little evidence of a clear dose response effect of DES. The significant F ratio for the treatment seemed to be due primarily to the effect of the 500mg/kg dose. There was no indication of a "time of analysis" effect on biopterin or pterin levels in the liver the F ratio (blocks) values being non-significant.

Since there is little evidence to suggest that BH_4 metabolism in the liver and brain differ greatly, the results suggest that sufficient accumulation of DES in the brain probably would reduce total biopterins. Oral administration of a DES suspension in this investigation probably resulted in poor intestinal absorption of the compound such that circulating blood levels were low. This, combined with rapid peripheral metabolism of DES to less active or non-oestrogenic products (Henry and Miller 1986) is likely to have resulted in very small quantities of the active compound actually reaching the brain.

3.3.3 Amphetamine.

Neither dose of d-amphetamine sulphate tested, (1 and 5mg/kg), showed a significant effect on rat brain total biopterin (Table 3.39) or derived pterin levels (Table 3.40). Liver biopterin, derived pterin and DHPR levels were also unchanged compared to controls (Tables 3.42, 3.43 and 3.44 respectively). The lower dose of the drug did produce an increase in brain DHPR activity which was just significant at the 5% level (Table 3.41).

Mandell *et al* (1980) showed that concentrations of D-amphetamine up to 15uM failed to alter DHPR activity. There is no clear reason why administration of the drug in the current investigation caused an increase in the activity of DHPR. The result was only just significant at the 5% level and the experiment needs to be repeated in order to confirm the effect.

Brain total biopterin results presented here are consistent with those reported by Mandell *et al* (1980) in which rat striatal total biopterins were not significantly reduced by the administration of 1 or 5mg/kg d-amphetamine sulphate. Total biopterin and actual BH_4 levels were only shown to be

decreased in discrete brain regions, these effects being masked by whole brain analyses (Lee and Mandell 1985a, 1985b).

The fact that liver biopterin levels were not affected by amphetamine in the present investigation suggests that the effects of the drug on BH_4 metabolism may be specific to the brain (changes being localised in discrete regions as indicated by Lee and Mandell). This would support the proposal that the primary effect of amphetamine is on catecholamine uptake and release in catecholaminergic neurones (Kuhr *et al* 1985; Arnold *et al* 1977). Any change in BH_4 metabolism might be a secondary effect caused by product concentration feedback mechanisms involved in catecholamine synthesis.

3.3.4 Scopolamine.

The only parameter of BH_4 metabolism affected by scopolamine was brain derived pterin which at lower doses of the drug (0.085mg/kg) was just significantly reduced at the 5% level (t=2.952) (Table 3.45). The results were consistent with those of Edwards (1988) who similarly found brain derived pterin to be depressed ($p<0.5\%$) and all other parameters of BH_4 metabolism to be unaffected. Interestingly the reduction was not seen upon administration of a ten times higher dose of scopolamine (0.85mg/kg) (Table 3.46).

The following explanation for the effect of scopolamine on brain derived pterin levels was proposed by Edwards (1988). Pterin is a catabolite of BH_4 (Pfleiderer 1975), and a reduction in derived pterin concentration might indicate decreased turnover of the cofactor. Prolonged cholinergic stimulation by a muscarinic agonist (oxotremorine) has been shown to increase tyrosine hydroxylase (TOH) activity in

certain brain areas (McIlwain and Bachelard 1985) subsequently increasing catecholamine turnover. This is brought about by activation of cAMP which lowers the Km of the enzyme for BH_4 to one sixth and increases the Ki value for inhibition by dopamine (Harris *et al* 1974). Edwards therefore suggested the possibility that a muscarinic antagonist, such as scopolamine, might cause the opposite effect, reducing TOH activity and hence catecholamine and BH_4 turnover. This could be reflected by a decrease in brain derived pterin levels as shown in the current investigation. Further work is required to test this hypothesis.

3.4 CONCLUSIONS.

Gross studies on the acute administration of lead to adult rats showed disruption of BH_4 metabolism in the liver but not the brain. Insufficient accumulation of lead during limited exposure may account for the absence of an effect in the brain. This would support the suggestion that neurotoxic effects of lead tend to be time, rather than concentration dependent (Cory-Slechta and Thompson 1979). In addition, since the effects of lead on the catecholaminergic system have been shown to be regionally specific, it is perhaps not surprising that brain BH_4 metabolism was apparently unaffected in whole brain analyses.

Like lead, DES was shown to effect BH_4 metabolism in the liver but not the brain. Again this suggests limited uptake of the compound into the brain. Liver results indicate an effect of DES on BH_4 biosynthesis rather than the salvage pathway.

High and low doses of D-amphetamine showed no effect on BH_4

metabolism in the brain or liver. These results support the regional specificity of the effects of amphetamine on BH_4 metabolism in the brain, perhaps indicating a primary effect of the drug on neuronal catecholamine uptake and release.

Scopolamine, at a low dose, caused a reduction in brain derived pterin levels which might indicate a decrease in BH_4 turnover.

The integrity of BH_4 metabolism in the brain is maintained during acute exposure to potential neurotoxins probably due to poor penetration of the blood-brain barrier by these compounds.

The results of these studies indicate the necessity for good experimental design and emphasise the importance of analysing test and control samples together.

In the following tables:-

n	=	number of animals per experimental group
p	=	probability value of a statistically significant result
n.s	=	a statistically non-significant result
st.d	=	standard deviation of the mean
trial	=	an individual occasion upon which an experiment was performed

LEAD EXPERIMENTS

DOSING REGIME ONE.

Table 3.1: The effect of lead acetate (340mg/kg$_{-1}$ body weight) on rat brain total biopterin levels (ng g^{-1} wet weight).

TRIAL	GROUP	n	Mean ± st.d.	p value
1	Control	6	46.9 ± 9.2	
	Lead	6	48.5 ± 6.2	n.s
2	Control	6	58.0 ± 8.2	
	Lead	6	56.9 ± 7.9	n.s
3	Control	6	65.6 ± 17.8	
	Lead	6	70.1 ± 20.6	n.s
4	Control	6	44.8 ± 3.4	
	Lead	6	41.5 ± 5.2	n.s

150g male rats were injected intraperitoneally with a suspension of lead acetate (340mg/kg body wgt) in isotonic sucrose (pH 7.8). Controls received sucrose alone. Animals were sacrificed 4 hours after dosing. Results were analysed by an unpaired students t-test.

DOSING REGIME TWO.

Table 3.2: The effect of intragastric (i.g) and intraperitoneal (i.p) administration of lead acetate (10^{-3}M) on rat brain total biopterin levels (ng g^{-1} wet weight).

GROUP	n	Mean ± st.d i.g	Mean ± st.d i.p
Control	6	70.9 ± 11.9	52.4 ± 5.5
Lead	6	71.5 ± 6.9	51.4 ± 12.0
(p value)		n.s	n.s

Table 3.3: The effect of i.g administration of lead acetate (10^{-3}M) on rat brain derived pterin levels (ng g^{-1} wet weight).

GROUP	n	Mean ± st.d
Control	6	64.7 ± 6.0
Lead	6	59.9 ± 5.7
(p value)		n.s

Table 3.4: The effect of i.g and i.p administration of lead acetate (10^{-3}M) on specific activity of DHPR in rat brain (nmoles NADH oxidised /min/ mg protein).

GROUP	n	Mean ± st.d i.g	Mean ± st.d i.p
Control	6	174.9 ± 33.2	91.9 ± 15.6
Lead	6	180.5 ± 51.5	86.9 ± 9.9
(p value)		n.s	n.s

TABLES 3.2 - 3.4: 150g male rats were dosed i.g or i.p with 0.3ml of 10^{-3}M lead acetate in isotonic saline twice daily for three days, (i.e 1.59mg PbAc/kg body weight/day). Controls received isotonic saline. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

DOSING REGIME THREE.

Table 3.5: The effect of lead acetate (10^{-1}M) on rat brain total biopterin levels (ng g^{-1} wet weight).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	77 ± 21	
	Lead	5	64 ± 15	n.s
2	Control	5	66 ± 10	
	Lead	6	69 ± 16	n.s

Table 3.6: The effect of lead acetate (10^{-1}M) on rat brain derived pterin levels (ng g^{-1} wet weight).

Trial	Group	n	Mean ± st.d	p value
2	Control	5	63 ± 5	
	Lead	6	67 ± 8	n.s

Table 3.7: The effect of lead acetate (10^{-1}M) on specific activity of DHPR in rat brain (nmoles NADH oxidised /min /mg protein).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	99 ± 4	
	Lead	5	93 ± 13	n.s
2	Control	5	120 ± 12	
	Lead	6	122 ± 10	n.s

TABLES 3.5 - 3.7: 150g male rats were dosed i.p with 0.3ml of 10^{-1}M lead acetate in isotonic saline once daily for three days, (i.e 158.7mg PbAc/kg body weight/day). Controls received isotonic saline. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

Table 3.8: The effect of lead acetate (10^{-1}M) on rat liver total biopterin levels (ng g^{-1} wet weight).

TRIAL	GROUP	n	Mean ± st.d	p value
1	Control	6	1058 ± 72	
	Lead	5	761 ± 94	p<0.1%
2	Control	5	1428 ± 60	
	Lead	6	1017 ± 159	p<0.1%

Table 3.9: The effect of lead acetate (10^{-1}M) on rat liver derived pterin levels (ng g^{-1} wet weight).

TRIAL	GROUP	n	Mean ± st.d	p value
1	Control	6	467 ± 61	
	Lead	5	385 ± 71	n.s
2	Control	5	634 ± 31	
	Lead	6	506 ± 53	p<0.2%

Table 3.10: The effect of lead acetate (10^{-1}M) on specific activity of DHPR in rat liver (nmoles NADH oxidised /min/mg protein).

TRIAL	GROUP	n	Mean ± st.d	p value
1	Control	6	266 ± 42	
	Lead	5	244 ± 48	n.s
2	Control	5	283 ± 28	
	Lead	6	272 ± 37	n.s

TABLES 3.8 - 3.10: 150g male rats were dosed i.p with 0.3ml of 10^{-1}M lead acetate in isotonic saline once daily for three days, (i.e 158.7mg PbAc/kg body weight/day). Controls received isotonic saline. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

DIETHYLSTILBOESTROL EXPERIMENTS.

EXPERIMENT ONE.

Table 3.11: The effect of diethylstilboestrol dipropionate (50mg/kg body weight) on brain total biopterin levels in 100g rats (ng g^{-1} wet weight tissue).

TRIAL	GROUP	n	Mean ± st.d	p value
1	Control	6	58 ± 2	
	DES Dip'	6	63 ± 11	n.s
2	Control	6	78 ± 16	
	DES Dip'	6	74 ± 67	n.s

Table 3.12: The effect of diethylstilboestrol dipropionate (50mg/kg body weight) on brain derived pterin levels in 100g rats (ng g^{-1} wet weight tissue).

TRIAL	GROUP	n	Mean ± st.d	p value
2	Control	6	120 ± 7	
	DES Dip'	6	112 ± 8	n.s

TABLES 3.11 - 3.12: 100g female rats were dosed i.g with a suspension of diethylstilboestrol dipropionate in corn oil (50mg/kg body wgt) once daily for four days. Controls were given corn oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

EXPERIMENT TWO.

Table 3.13: The effect of diethylstilboestrol dipropionate (50mg/kg body weight) on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

TRIAL	GROUP	n	Mean ± st.d	p value
1	Control	5	68 ± 16	
	DES Dip'	6	78 ± 6	n.s
2	Control	6	100 ± 17	
	DES Dip'	6	101 ± 19	n.s

Table 3.14: The effect of diethylstilboestrol dipropionate (50mg/kg body weight) on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

TRIAL	GROUP	n	Mean ± st.d	p value
1	Control	5	73 ± 6	
	DES Dip'	6	76 ± 3	n.s
2	Control	6	102 ± 7	
	DES Dip'	6	101 ± 8	n.s

TABLES 3.13 - 3.14: 50g female weaner rats were dosed i.g with a suspension of diethylstilboestrol dipropionate in corn oil (50mg/kg body wgt) once daily for four days. Controls were given corn oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

EXPERIMENT THREE.

Table 3.15: The effect of diethylstilboestrol dipropionate (500mg/kg body weight) on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	mean ± st.d	p value
Control	6	64 ± 10	
DES dip'	6	62 ± 8	n.s

Table 3.16: The effect of diethylstilboestrol dipropionate (500mg/kg body weight) on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	mean ± st.d	p value
Control	6	100 ± 6	
DES Dip'	6	99 ± 2	n.s

TABLES 3.15 AND 3.16: 50g female weaner rats were dosed i.g with a suspension of diethylstilboestrol dipropionate in corn oil (500mg/kg body wgt) once daily for four days. Controls were given corn oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

EXPERIMENT FOUR.

Table 3.17: The effect of diethylstilboestrol (500mg/kg body weight) on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	mean ± st.d	p value
Control	6	63 ± 8	
DES	5	77 ± 9	p<5%

Table 3.18: The effect of diethylstilboestrol (500mg/kg body weight) on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	mean ± st.d	p value
Control	6	174 ± 15	
DES	5	195 ± 45	n.s

Table 3.19: The effect of diethylstilboestrol (500mg/kg body weight) on specific activity of brain DHPR in 50g weaner rats (nmoles NADH oxidised /min/ mg protein).

GROUP	n	mean ± st.d	p value
Control	6	110 ± 18	
DES	5	97 ± 18	n.s

TABLES 3.17 - 3.19: 50g female weaner rats were dosed i.g with a suspension of diethylstilboestrol in corn oil (500mg/kg body wgt) once daily for four days. Controls were given corn oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

Table 3.20: The effect of diethylstilboestrol (500mg/kg body weight) on liver total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	mean ± st.d	p value
Control	6	1070 ± 154	
DES	5	714 ± 138	p<1%

Table 3.21: The effect of diethylstilboestrol (500mg/kg body weight) on liver derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	mean ± st.d	p value
Control	6	1593 ± 396	
DES	5	1028 ± 316	p<5%

Table 3.22: The effect of diethylstilboestrol (500mg/kg body weight) on specific activity of liver DHPR in 50g weaner rats (nmoles NADH oxidised /min /mg protein).

GROUP	n	mean ± st.d	p value
Controls	6	281 ± 39	
DES	5	305 ± 29	n.s

TABLES 3.20 - 3.22: 50g female weaner rats were dosed i.g with a suspension of diethylstilboestrol in corn oil (500mg/kg body wgt) once daily for four days. Controls were given corn oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

EXPERIMENT FIVE.

Table 3.23: Comparison of the effects of DES dipropionate and DES on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value
Control	6	61 ± 7	
DES Dip'	6	64 ± 6	n.s
DES	6	61 ± 5	n.s

Table 3.24: Comparison of the effects of DES dipropionate and DES on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value
Control	6	80 ± 8	
DES Dip'	6	85 ± 6	n.s
DES	6	83 ± 5	n.s

Table 3.25: Comparison of the effects of DES dipropionate and DES on specific activity of brain DHPR in weaner rats (nmoles NADH oxidised /min /mg protein).

GROUP	n	Mean ± st.d	p value
Control	6	75 ± 6	
DES Dip'	6	76 ± 3	n.s
DES	6	74 ± 4	n.s

TABLES 3.23 - 3.25: 50g female weaner rats were dosed i.g with either DES dipropionate (500mg/kg body wgt) or DES (500mg/kg body wgt) as a suspension in corn oil once daily for four days. Controls received oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

Table 3.26: Comparison of the effects of DES dipropionate and DES on liver total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value	p value
Control	6	944 ± 73		
DES Dip'	6	729 ± 118	p<1%	p<5%*
DES	6	578 ± 82	p<0.1%	

* There is a significant difference in biopterin levels (p<5%) between animals dosed with DES dipropionate and those dosed with DES.

Table 3.27: Comparison of the effects of DES dipropionate and DES on liver derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value	p value
Control	6	593 ± 94		
DES Dip'	6	524 ± 63	n.s	p<1%*
DES	6	399 ± 54	p<0.2%	

* There is a significant difference in pterin levels (p<1%) between animals dosed with DES dipropionate and those dosed with DES.

Table 3.28: Comparison of the effects of DES dipropionate and DES on specific activity of liver DHPR in weaner rats (nmoles NADH oxidised /min /mg protein).

GROUP	n	Mean ± st.d	p value
Control	6	278 ± 36	
DES Dip'	6	258 ± 45	n.s
DES	6	249 ± 40	n.s

TABLES 3.26 - 3.28: 50g female weaner rats were dosed i.g with either DES dipropionate (500mg/kg body wgt) or DES (500mg/kg body wgt) as a suspension in corn oil once daily for four days. Controls received oil alone. Animals were sacrificed 24 hours after the last dose. Results were analysed by an unpaired students t-test.

EXPERIMENT SIX.

Table 3.29: Effect of administration of diethylstilboestrol in ethanol (500mg/kg body weight) on plasma total biopterin levels in weaner rats (ng ml^{-1} plasma).

GROUP	n	Mean ± st.d	p value
Control	6	33.5 ± 8.2	
DES	6	34.4 ± 7.9	n.s

Table 3.30: Effect of administration of diethylstilboestrol in ethanol (500mg/kg body weight) on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value
Control	6	75.4 ± 6.6	
DES	6	82.4 ± 6.0	n.s

Table 3.31: Effect of administration of diethylstilboestrol in ethanol (500mg/kg body weight) on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value
Control	6	61.5 ± 4.6	
DES	5	56.5 ± 8.6	n.s

Table 3.32: Effect of administration of diethylstilboestrol in ethanol (500mg/kg body weight) on specific activity of brain DHPR in weaner rats (nmoles NADH oxidised /min /mg protein).

GROUP	n	Mean ± st.d	p value
Control	6	155 ± 16	
DES	6	165 ± 15	n.s

TABLES 3.29 - 3.32: 50g female weaner rats were dosed i.g with diethylstilboestrol (500mg/kg body wgt) dissolved in 75% ethanol. Controls received 75% ethanol. The animals were sacrificed 8 hours later. Results were analysed by an unpaired students t-test.

Table 3.33: Effect of administration of diethylstilboestrol in ethanol (500mg/kg body weight) on liver total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value
Control	6	1158 ± 181	
DES	6	866 ± 134	p<1%

Table 3.34: Effect of administration of diethylstilboestrol in ethanol (500mg/kg body weight) on liver derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d	p value
Control	6	732 ± 284	
DES	6	565 ± 257	n.s

TABLES 3.33 - 3.34: 50g female weaner rats were dosed i.g with diethylstilboestrol (500mg/kg body wgt) dissolved in 75% ethanol. Controls received 75% ethanol. The animals were sacrificed 8 hours later. Results were analysed by an unpaired students t-test.

EXPERIMENT SEVEN.

To test for a dose-response effect of DES.

Table 3.35: The effect of diethylstilboestrol concentration on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

		DES CONCENTRATION (mg/kg body weight)					
		0	50	125	250	375	500
DAY	1	81.3	79.5	87.5	83.3	80.2	73.1
	2	77.9	95.3	83.1	76.4	78.6	73.6
	3	106.3	109.1	91.6	77.0	129.2	101.0
	4	63.2	66.7	77.2	70.5	68.6	87.5

F ratio (treatment) = 0.9780 n.s
F ratio (blocks) = 4.5494 p<5%

Table 3.36: The effect of diethylstilboestrol concentration on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

		DES CONCENTRATION (mg/kg body weight)					
		0	50	125	250	375	500
DAY	1	69.7	59.5	62.4	52.1	52.4	33.8
	2	39.7	42.3	40.5	31.9	27.8	33.0
	3	45.1	39.4	39.8	28.9	39.1	29.3
	4	58.7	64.6	48.9	67.9	68.5	70.5

F ratio (treatment) = 1.6078 n.s
F ratio (blocks) = 8.9346 p<1%

TABLES 3.35 AND 3.36: Each value represents the brain biopterin or derived pterin content of a single rat. Six female 50g weaner rats were used on each day. Each received a different dose of DES dissolved in 75% ethanol except the control (0mg DES) which received ethanol alone. Animals were sacrificed 8 hours after dosing. Analysis was by two-way analysis of variance in randomised blocks. The results are indicated by the F ratios.

Table 3.37: The effect of diethylstilboestrol concentration on liver total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

		DES CONCENTRATION (mg/kg body weight)					
		0	50	125	250	375	500
DAY	1	1277	1048	1341	1343	1272	767
	2	1113	1494	1243	594	912	576
	3	1306	1409	1044	1440	504	920
	4	1446	1105	1209	1019	1039	1019

F ratio (treatment) = 3.6272 p<5%
F ratio (blocks) = 0.3552 n.s

Table 3.38: The effect of diethylstilboestrol concentration on liver derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

		DES CONCENTRATION (mg/kg body weight)					
		0	50	125	250	375	500
DAY	1	794	790	682	655	567	861
	2	832	805	594	921	1157	1114
	3	674	717	611	657	664	465
	4	998	907	629	764	650	705

F ratio (treatment) = 1.4611 n.s
F ratio (blocks) = 2.1068 n.s

TABLES 3.37 AND 3.38: Each value represents the liver biopterin or pterin content of a single rat. Six female 50g weaner rats were used on each day. Each received a different dose of DES dissolved in 75% ethanol except the control (0mg DES) which received ethanol alone. Animals were sacrificed 8 hours after dosing. Analysis was by two-way analysis of variance in randomised blocks. The results are indicated by the F ratios.

AMPHETAMINE EXPERIMENTS.

Table 3.39: The effect of D-amphetamine sulphate on rat brain total biopterin levels (ng g^{-1} wet weight tissue).

AMPHETAMINE DOSE (mg/kg body wgt)	Mean ± st.d Control	Amphetamine	p value
1mg/kg (n=6)	89 ± 8	82 ± 7	n.s
5mg/kg (n=6)	73 ± 8	68 ± 5	n.s

Table 3.40: The effect of D-amphetamine sulphate on rat brain derived pterin levels (ng g^{-1} wet weight tissue).

AMPHETAMINE DOSE (mg/kg body wgt)	Mean ± st.d Control	Amphetamine	p value
5mg/kg (n=6)	86 ± 3	85 ± 5	n.s

Table 3.41: The effect of D-amphetamine sulphate on specific activity of DHPR in rat brain (nmoles NADH oxidised /min /mg protein).

AMPHETAMINE DOSE (mg/kg body wgt)	Mean ± st.d Control	Amphetamine	p value
1mg/kg (n=6)	107 ± 16	127 ± 13	p<5%

TABLES 3.39 - 3.41: 200g male rats were dosed i.p with D-amphetamine sulphate dissolved in isotonic saline. Those given 1mg/kg body wgt were sacrificed after 1 hour; those receiving 5mg/kg were killed after 2 hours. Saline controls were used in each experiment. Results were analysed by an unpaired students t-test.

Table 3.42: The effect of D-amphetamine sulphate on rat liver total biopterin levels (ng g^{-1} wet weight tissue).

AMPHETAMINE DOSE (mg/kg body wgt)	Mean ± st.d CONTROL	AMPHETAMINE	p value
1mg/kg (n=6)	1218 ± 111	1171 ± 79	n.s
5mg/kg (n=6)	1132 ± 250	1036 ± 71	n.s

Table 3.43: The effect of D-amphetamine sulphate on rat liver derived pterin levels (ng g^{-1} wet weight tissue).

AMPHETAMINE DOSE (mg/kg body wgt)	Mean ± st.d CONTROL	AMPHETAMINE	p value
5mg/kg (n=6)	672 ± 116	566 ± 50	n.s

Table 3.44: The effect of D-amphetamine sulphate on specific activity of DHPR in rat liver (nmoles NADH oxidised /min /mg protein).

AMPHETAMINE DOSE (mg/kg body wgt)	Mean ± st.d CONTROL	AMPHETAMINE	p value
1mg/kg (n=6)	239 ± 19	249 ± 26	n.s
5mg/kg (n=6)	345 ± 39	337 ± 12	n.s

TABLES 3.42 - 3.44: 200g male rats were dosed i.p with D-amphetamine sulphate dissolved in isotonic saline. Those given 1mg/kg body wgt were sacrificed after 1 hour; those receiving 5mg/kg were killed after 2 hours. Saline controls were used in each experiment. Results were analysed by an unpaired students t-test.

SCOPOLAMINE EXPERIMENTS.

Table 3.45: The effect of scopolamine hydrochloride (0.085mg kg^{-1} body weight) on BH_4 metabolism in the rat.

	Mean ± st.d		p
	CONTROL	SCOPOLAMINE	
PLASMA BIOPTERIN (ng ml^{-1})	24.3 ± 6.3 (6)	31.9 ± 9.1 (6)	n.s
BRAIN BIOPTERIN (ng g^{-1} wbt)	65.7 ± 5.5 (6)	64.1 ± 3.6 (6)	n.s
BRAIN PTERIN (ng g^{-1} wbt)	53.9 ± 1.5 (6)	49.2 ± 3.6 (6)	p<5%
BRAIN BIOSYNTHESIS (ng biopterin hr^{-1} mg^{-1} protein)	3.799 ± 1.453 (6)	3.908 ± 1.486 (6)	n.s
BRAIN DHPR (nmol NADH oxidised min^{-1} mg^{-1} protein)	253 ± 63 (6)	252 ± 46 (6)	n.s
LIVER BIOPTERIN (ng g^{-1} wlt)	1209 ± 90 (6)	1164 ± 89 (6)	n.s
LIVER PTERIN (ng g^{-1} wlt)	931 ± 73 (6)	836 ± 104 (6)	n.s
LIVER BIOSYNTHESIS (ng biopterin hr^{-1} mg^{-1} protein)	12.16 ± 3.33 (6)	11.81 ± 3.66 (6)	n.s
LIVER DHPR (nmol NADH oxidised min^{-1} mg^{-1} protein)	376 ± 66 (6)	374 ± 46 (6)	n.s

wbt = wet brain tissue
wlt = wet liver tissue
270g male rats were dosed i.g with 0.023mg scopolamine hydrochloride per animal and sacrificed 5 hours later. Controls were given distilled water. Results were analysed by an unpaired students t test.

Table 3.46: The effect of scopolamine (0.85mg kg^{-1} body weight) on BH_4 metabolism in the rat.

	Mean ± st.d		p
	CONTROL	SCOPOLAMINE	
PLASMA BIOPTERIN ($ng\ ml^{-1}$)	51.4 ± 12.6 (6)	62.0 ± 22.2 (6)	n.s
BRAIN BIOPTERIN ($ng\ g^{-1}$ wbt)	133.7 ± 10.4 (6)	137.7 ± 9.2 (6)	n.s
BRAIN PTERIN ($ng\ g^{-1}$ wbt)	68.1 ± 6.5 (6)	72.7 ± 4.0 (6)	n.s
BRAIN BIOSYNTHESIS (ng biopterin hr^{-1} mg^{-1} protein)	10.19 ± 1.07 (6)	9.51 ± 1.48 (5)	n.s
LIVER BIOPTERIN ($ng\ g^{-1}$ wlt)	1931 ± 149 (6)	1962 ± 214 (6)	n.s
LIVER PTERIN ($ng\ g^{-1}$ wlt)	1596 ± 253 (6)	1529 ± 379 (6)	n.s
LIVER BIOSYNTHESIS (ng biopterin hr^{-1} mg^{-1} protein)	12.35 ± 1.54 (5)	11.26 ± 1.32 (6)	n.s

wbt = wet brain tissue
wlt = wet liver tissue
270g male rats were dosed i.g with 0.23mg scopolamine hydrochloride per animal and sacrificed 5 hours later. Controls were given distilled water. Results were analysed by an unpaired students t test.

CHAPTER FOUR.

INVESTIGATION OF THE EFFECTS OF STARVATION ON TETRAHYDROBIOPTERIN METABOLISM AND GLUTATHIONE LEVELS IN THE RAT.

4.1 INTRODUCTION.

"Starvation" is the term describing a severe degree of food deprivation resulting in significant loss of body weight. The biochemical alterations associated with the condition are numerous and widespread throughout the body. This chapter considers how starvation might effect brain neurobiochemistry which could subsequently lead to some form of neurological impairment.

Animal studies have shown that a major feature of neuroendocrine changes caused by starvation is low catecholamine (CA) turnover in both the periphery, (Halmi *et al* 1978; Landsberg and Young 1983), and the brain, (Schweiger *et al* 1985). The precise mechanism mediating the depression of central CA turnover in starvation remains to be elucidated.

Acute starvation results in increased formation of oxygen radicals in the brain and liver (Tateishi *et al* 1974; Isaacs and Binkley 1977; Stankiewicz 1987). This is due to decreased levels of catalase, glutathione (GSH) and glutathione peroxidase (GSH-PX) which form part of the free radical tissue defense mechanism that removes H_2O_2 produced *in vivo* (Tateishi *et al* 1974; Isaacs and Binkley 1977).

$$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2$$

$$2GSH + H_2O_2 \xrightarrow[GSH\text{-}PX]{} GSSG + H_2O$$

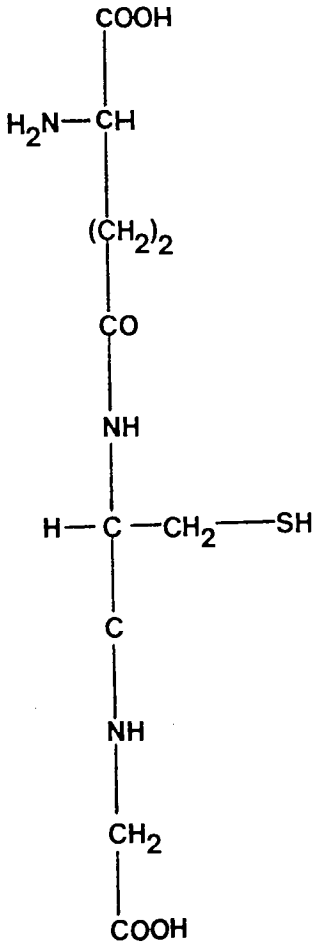
If the H_2O_2 is not removed it may generate the highly reactive and destructive hydroxyl radical ($^{\bullet}OH$) whose involvement has been inferred in the pathogenesis of a number of neurological disorders including Parkinson's disease (Cohen 1986).

The reduced form of glutathione (GSH) (i) is regenerated by the NADPH dependent glutathione reductase (Christophersen 1969; Fridovich 1976; Hothersall *et al* 1982). In starvation liver NADPH levels are diminished due to decreased activity of the enzymes glucose-6-phophate dehydrogenase and 6-phosphogluconate dehydrogenase (Martins *et al* 1985). Thus, GSH levels drop and oxygen radical formation increases.

GSH levels also decrease in starvation due to increased catabolism as it exercises its role as a cysteine reservoir during periods of inadequate cysteine or cystine intake (Cho *et al* 1981). On refeeding, GSH levels increase directly with the amount of dietary cysteine (Tateishi *et al* 1977).

The brain, having a high rate of oxygen consumption, large amounts of polyunsaturated fatty acids and comparatively lower GSH-PX and catalase levels than other tissues, is especially vulnerable to peroxidative damage and oxidative stress (DeMarchena *et al* 1974). BH_4 is particularly susceptible to free radical oxidative breakdown and autoxidation (fig 4.1) (Blair and Pearson 1974). *In vitro*, physiological concentrations of GSH have been shown to inhibit autoxidation of the cofactor induced by hydroxyl radicals (Heales 1987).

It has been proposed that the significant drop in tissue GSH, and subsequent increase in oxygen radical formation,



(i) L-Glutathione

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FIG 4.1: Autoxidation of tetrahydropterins (Blair and Pearson, 1974)

in acute starvation may disrupt BH_4 metabolism by enhancing oxidative catabolism (Heales 1987). This, in turn, would impair catecholamine and serotonin synthesis and might be the cause of neurological dysfunctions sometimes associated with starvation or inadequate nutrition, (Cravioto and DeLicardie, 1968; Young and Landsberg 1977).

Experiments designed to test this hypothesis showed that acute starvation lowered brain and transiently liver total biopterins in the rat (Heales, 1987). There was some evidence that the decreases may be due to decreased *de novo* biosynthesis and/or inhibition of the salvage pathway, but may also have been caused by enhanced oxidative breakdown of the cofactor.

The effects of L-cysteine administration to starving rats and L-buthionine sulphoximine (L-BSO) to feeding rats were investigated (Heales, 1987). L-BSO is a specific inhibitor of GSH biosynthesis (Griffith and Meister 1979). L-cysteine increased total biopterin levels in brain and liver of starved rats. L-BSO decreased total biopterin levels in the liver but not the brain. The results suggested that GSH may play a role in maintaining tissue BH_4 levels by preventing oxidative breakdown.

In the present investigation, experiments were carried out to test directly the effects of starvation on BH_4 metabolism and GSH levels. The aim was to see if the results supported the proposals put forward by Heales, which were based on indirect analysis of GSH metabolism in starvation.

4.2 MATERIALS AND METHODS.

150g male Wistar rats were allowed access to water but not

food for a specific length of time (see below). Food was removed at 17.00hrs on day one. Controls received food and water *ad libitum*. All animals were placed on grids to avoid coprophagy.

Plasma total biopterin, brain and liver total biopterin, derived pterin, tetrahydrobiopterin biosynthetic capacity, DHPR activity and reduced glutathione (GSH) levels were determined as described in Chapter 2. All samples from a single experiment, (batch), were analysed on the same day and no between batch comparisons were made.

Details of experiments:

(1). Initially, experiments were performed to see whether the starvation-induced effects on BH_4 metabolism, reported by Heales (1987), were reproducible.

The effects of 48 and 72 hours starvation on plasma, brain and liver BH_4 metabolism were investigated. The conditions of the animal experimentation licence limited the period of starvation to a maximum of 72 hours. Each experiment was carried out separately and the 72 hour investigation was repeated to ensure reproducibility of results. The results from each occasion are recorded as separate trials ie. trial 1 or 2. Results were analysed by a Students unpaired t-test.

(2). Based on the results of these preliminary experiments, a more refined experiment was set in hand to test whether starvation-induced changes in BH_4 metabolism are accompanied by alterations in reduced glutathione (GSH) levels.

Rats were starved for either 24, 48 or 72 hours, (n=5 per

group). Plasma total biopterin and brain and liver total biopterin, derived pterin and GSH levels were measured. (Half brains were analysed for their neurotransmitter contents - see Chapter 5). Results were analysed by a Students unpaired t-test and also by one-way analysis of variance (One-way ANOVA).

An experiment analysed by one-way ANOVA takes into account, and effectively eliminates, some of those factors associated with animal studies, (eg. variation between individual animals), which are likely to confound statistical analysis and partially mask any effect due to a particular treatment. The influence of such factors is included in the error term of the calculation hence any effect of a treatment is more likely to be detected.

F ratio values indicate whether or not starvation had an over-all effect on a particular parameter and least significant differences were used to compare results of individual groups.

4.3 RESULTS.

Although body weights were not measured, inspection of the animals revealed evidence of weight loss the severity of which was related to the length of starvation.

Brain total biopterin and derived pterin levels in rats starved for 24 hours were not significantly different from those in fed rats, (Tables 4.19 and 4.20). The brain pterin : biopterin ratio (P/B) was also unaffected, (Table 4.21). Twenty-four hours starvation caused lowering of liver total biopterins, ($p<5\%$, Table 4.22), while liver derived pterin and the P/B ratio were increased but not significantly, (Tables 4.23 and 4.24 respectively).

Brain total biopterin and derived pterin concentrations in rats starved for 48 hours were not significantly different from levels in fed rats (Tables 4.2, 4.3, 4.19 and 4.20). The brain P/B ratio was also unaffected (Tables 4.4 and 4.21). Brain DHPR activity was lower in animals fasted for 48 hours than non-fasted rats ($p<5\%$, Table 4.5).

48 hours starvation caused a large decrease in liver total biopterin concentrations ($p<1\%$, Table 4.6; $p<0.1\%$, Table 4.22), while liver derived pterin levels significantly increased ($p<2\%$, Table 4.7; $p<5\%$, Table 4.23). This resulted in an elevated liver P/B ratio in 48 hour starved animals compared to controls ($p<1\%$, Table 4.8; n.s increase, Table 4.24).

Plasma total biopterins showed a marked depression in rats fasted for 48 hours ($p<0.1\%$, Table 4.1), but this effect was not reproduced in a later study, (Table 4.25). Liver DHPR activity showed a non-significant decrease (Table 4.9).

Brain total biopterins were significantly decreased after 72 hours starvation compared to controls, ($p<0.2\%$, Table 4.11; n.s, Table 4.11; $p<5\%$, Table 4.19), but brain derived pterin was consistently unaffected, (Tables 4.12 and 4.20), as were brain DHPR and biosynthetic capacities (Tables 4.13 and 4.14 respectively).

Liver total biopterins were unchanged by 72 hours starvation on one occasion, (Table 4.15, trial 1), but showed large depressions compared to controls in two later studies, ($p<2\%$, Table 4.15, trial 2; and $p<1\%$, Table 4.22). Derived pterin levels in the liver were consistently increased after a 72 hour fast, (Tables 4.16 and 4.23). On all three occasions the increase was relatively large but, due to large standard deviations and small sample sizes, it only reached significance on one occasion ($p<5\%$, Table

4.16, trial 2). DHPR activity and biosynthetic capacity in the liver were both unaffected, (Tables 4.17 and 4.18 respectively).

72 hours starvation produced non-significant decreases in plasma biopterins in two separate trials (Table 4.10). The decrease was significant in a later study ($p<2\%$, Table 4.25).

Analysis of variance supported an over-all significant effect of starvation on plasma, brain and liver total biopterins, (Table 4.25, $F= 6.092$, $p<1\%$; Table 4.19, $F= 3.200$, $p<5\%$ and Table 4.22, $F=15.792$, $p<0.1\%$ respectively). This was not the case for brain or liver derived pterin levels for which the F ratio values did not reach significance, (Tables 4.20 and 4.23).

Fig. 4.2 compares how brain total biopterin, derived pterin and GSH varied with severity of starvation. Only animals starved for 72 hours had decreased brain glutathione (GSH) levels. The depression was significant compared to both controls, ($p<5\%$, Table 4.26), and animals fasted for 48 hours, ($p<5\%$. Table 4.26), and corresponded to a decline in total biopterin levels.

Brain biopterin and GSH levels did not correlate significantly, (Table 4.27), but brain pterin showed a significant negative correlation with GSH after 72 hours starvation (Table 4.29; $r= - 0.988$, $p<5\%$).

Compared to controls, liver GSH was significantly depressed in animals starved for 24 hours, (Table 4.28, $p<5\%$), and was accompanied by a decline in liver total biopterin levels, (Fig. 4.3). Values returned to normal after 48 hours and then continued to rise. After 72 hours fasting, liver GSH levels were higher than they were in controls, (not significant).

Liver biopterin showed a significant positive correlation with liver GSH at 24 hours starvation (Table 4.29; r= + 0.955, p<2%). After 48 hours the correlation was negative but non-significant (r=- 0.791). Liver derived pterin did not correlate significantly with liver GSH.

4.4 DISCUSSION.

Heales, (1987), reported lowered total biopterin levels in rat brain and liver after periods of acute starvation. Brain biopterins were consistently decreased after 24 and 48 hours but in the liver the decrease was only transient, levels being restored to normal after 48 hours.

Results from this investigation also indicated a starvation-induced decrease in brain and liver total biopterins. However, unlike Heales, the effect was only observed after 72 hours fasting in the brain, while levels were consistently lowered after 24, 48 and 72 hours in the liver.

Perhaps reflecting the lowered liver biopterin levels, plasma total biopterins showed a significant overall depression in starved animals. Although the decreases were not always statistically significant, they occurred so frequently that an overall depression was considered to be the true analysis. Heales showed no decrease in plasma biopterins after 24 or 48 hours and did not perform a 72 hour experiment.

A number of possibilities exist which may explain the observed reductions in tissue BH_4 levels. Workers have shown that starvation induces a limited availability of NADPH, (Martins *et al*, 1985), an essential component of the BH_4 biosynthetic pathway. Results from this study revealed no evidence of a reduction in biosynthetic capacity for the cofactor in either the brain or liver. Heales (1987)

similarly showed no effect on BH_4 biosynthesis in either tissue after 48 hours starvation. It should be noted however that the *in vitro* synthesis assay uses excess NADPH therefore any *in vivo* effect would probably be masked.

Diminished salvage of the active cofactor, by DHPR, might also cause a drop in tissue biopterin levels and would normally be accompanied by a rise in plasma total biopterins. In the present investigation, the most consistent finding was that both brain and liver DHPR activities were unaffected by starvation, and as mentioned above, plasma total biopterins dropped. This suggests that the decreased biopterin levels observed in starvation did not arise from a disturbance of the DHPR salvage pathway which is also consistent with the findings of Heales (1987).

Starvation is a complex phenomenon and involves complicated changes in tissue components. Glutathione, (GSH), serves as part of the body's tissue defense mechanism against oxidation by free radicals and concentrations are known to fall in starvation, (Tateishi *et al*, 1974). It is capable of reducing quinonoid dihydrobiopterin back to the active tetrahydro form;

$$GSH + qBH_2 \longrightarrow GSSG + BH_4$$

and also helps prevent oxidative breakdown of BH_4. Heales, (1987), suggested that the fall in total biopterins in starvation may be related to an increased oxidising environment within the tissues caused by decreased GSH levels. There was evidence from the results of the present investigation to support this hypothesis.

When measured in the brain, GSH levels showed no change after 24 or 48 hours and then dropped after 72 hours mirroring the fall in biopterin levels. Liver GSH levels decreased after 24 hours starvation showing a significant

positive correlation with liver total biopterins. Restoration of hepatic GSH levels after 48 hours was consistent with the findings of other workers, (Tateishi *et al*, 1974; Cho *et al*, 1981), and was followed at 72 hours by a significant increase in liver total biopterins compared to levels at 48 hours, ($p<5\%$).

An increase in liver GSH concentration after an initial decline is brought about by increased catabolism of hepatic proteins which release the amino acids required for resynthesis of the thiol, (Cho *et al*, 1981).

Pterin, measured in tissue samples by HPLC, is "derived" by acid iodine oxidation and originates, in part, from oxidation of tissue tetrahydrofolate (FH_4) (Fukushima and Nixon, 1980). Autoxidation of BH_4 results in formation of qBH_2 and subsequently 7,8-dihydropterin (Matsuura *et al*, 1986). The latter, upon acid iodine oxidation, also forms pterin (Heales 1987). Heales suggested that, formation of qBH_2, and hence 7,8-dihydropterin, from BH_4 during amino acid hydroxylation and/or oxidative catabolism *in vivo*, may therefore contribute to the derived pterin formed in tissue supernatants by acid iodine oxidation. Thus, tissue pterin levels, or the P/B ratio, may be used as indicators of BH_4 turnover or catabolism. Obviously, in the absence of information regarding tissue FH_4 levels, care must be exercised in interpreting derived pterin results.

If the fall in total biopterins during fasting was due to enhanced oxidative catabolism of BH_4, one might expect it to be reflected by a starvation-induced increase in derived pterin levels and/or the P/B ratio.

In the brain an increased P/B ratio was observed after 72 hours starvation when pterin and GSH levels showed a significant negative correlation. The ratio increased in the liver after 48 hours fasting and then dropped significantly after 72 hours following restoration of

normal liver GSH levels.

The possibility that increased derived pterin levels and P/B ratios could reflect enhanced turnover of BH_4, and not catabolism, cannot be excluded. However, given that starvation provides the potential for increased oxidative breakdown of labile species such as BH_4, by lowering levels of certain antioxidants, (eg GSH), and in the absence of any evidence for a concomitant increase in salvage or biosynthetic rate of the cofactor, this seems unlikely.

4.5 CONCLUSIONS.

Acute starvation significantly decreased total biopterins in rat brain and liver. Evidence presented here suggests this could arise from a starvation-induced decrease in tissue reduced glutathione levels (GSH) resulting in enhanced autoxidation of BH_4 and possibly decreased salvage of active BH_4 from qBH_2 by GSH. This indicates a possible role of GSH in maintaining tissue levels of BH_4 *in vivo*.

Table 4.1: Effect of 48 hours starvation on rat plasma total biopterin levels (ng ml^{-1} plasma).

	n	Mean ± st.d	p value
FED	6	22.3 ± 5.9	
STARVED	6	9.0 ± 2.1	p<0.1%

Table 4.2: Effect of 48 hours starvation on rat brain total biopterin levels (ng g^{-1} wet weight).

	n	Mean ± st.d	p value
FED	6	45.5 ± 6.0	
STARVED	6	48.8 ± 11.5	n.s

Table 4.3: Effect of 48 hours starvation on rat brain derived pterin levels (ng g^{-1} wet weight).

	n	Mean ± st.d	p value
FED	6	41.5 ± 3.6	
STARVED	6	42.0 ± 7.5	n.s

Table 4.4: Effect of 48 hours starvation on rat brain P/B ratio.

	n	Mean ± st.d	p value
FED	6	1.305 ± 0.21	
STARVED	6	1.280 ± 0.37	n.s

Table 4.5: Effect of 48 hours starvation on rat brain DHPR activty (nmoles NADH oxidised /min /mg protein).

	n	Mean ± st.d	p value
FED	5	172.0 ± 12.1	
STARVED	6	142.1 ± 24.7	p<5%

TABLES 4.1-4.5: 150g male rats were starved on grids for 48 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* Results were analysed by students unpaired t-test.

Table 4.6: Effect of 48 hours starvation on rat liver total biopterin levels (ng g^{-1} wet weight).

	n	Mean ± st.d	p value
FED	6	906 ± 84	
STARVED	6	734 ± 102	p<1%

Table 4.7: Effect of 48 hours starvation on rat liver derived pterin levels (ng g^{-1} wet weight).

	n	Mean ± st.d	p value
FED	5	299 ± 78	
STARVED	6	448 ± 85	p<2%

Table 4.8: Effect of 48 hours starvation on rat liver P/B ratio.

	n	Mean ± st.d	p value
FED	5	0.483 ± 0.177	
STARVED	6	0.858 ± 0.093	p<1%

Table 4.9: Effect of 48 hours starvation on rat liver DHPR activity (nmoles NADH oxidised /min /mg protein).

	n	Mean ± st.d	p value
FED	6	586.3 ± 88.1	
STARVED	6	542.2 ± 47.4	n.s

TABLES 4.6-4.9: 150g male rats were starved on grids for 48 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* Results were analysed by students unpaired t-test.

Table 4.6: Effect of 48 hours starvation on rat liver total biopterin levels (ng g^{-1} wet weight).

	n	Mean ± st.d	p value
FED	6	906 ± 84	
STARVED	6	734 ± 102	p<1%

Table 4.7: Effect of 48 hours starvation on rat liver derived pterin levels (ng g^{-1} wet weight).

	n	Mean ± st.d	p value
FED	5	299 ± 78	
STARVED	6	448 ± 85	p<2%

Table 4.8: Effect of 48 hours starvation on rat liver P/B ratio.

	n	Mean ± st.d	p value
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Table 4.9: Effect of 48 hours starvation on rat liver DHPR activty (nmoles NADH oxidised /min /mg protein).

	n	Mean ± st.d	p value
FED	6	586.3 ± 88.1	
STARVED	6	542.2 ± 47.4	n.s

TABLES 4.6-4.9: 150g male rats were starved on grids for 48 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* Results were analysed by students unpaired t-test.

Table 4.10: Effect of 72 hours starvation on plasma total biopterin levels (ng ml^{-1} plasma).

Trial	Group	n	Mean ± st.d.	p value
1	Fed	6	31.2 ± 15.8	
	Fasted	6	21.0 ± 5.9	n.s
2	Fed	6	27.6 ± 9.0	
	Fasted	5	19.6 ± 11.2	n.s

Table 4.11: Effect of 72 hours starvation on brain total biopterin levels (ng g^{-1} wet weight).

Trial	Group	n	Mean ± st.d.	p value
1	Fed	6	95.7 ± 16.3	
	Fasted	6	65.2 ± 7.1	p<0.2%
2	Fed	6	88.3 ± 12.1	
	Fasted	6	75.0 ± 11.4	n.s

Table 4.12: Effect of 72 hours starvation on brain derived pterin levels (ng g^{-1} wet weight).

Trial	Group	n	Mean ± st.d.	p value
2	Fed	6	49.0 ± 9.2	
	Fasted	6	53.3 ± 8.0	n.s

TABLES 4.10-4.12: 150g male rats were starved on grids for 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* The experiment was carried out on two occasions, (Trials 1 and 2). Results were analysed by a students unpaired t-test.

Table 4.13: Effect of 72 hours starvation on rat brain DHPR activity (nmoles NADH oxidised / min /mg protein).

Trial	Group	n	Mean ± st.d.	p value
1	Fed	6	216.2 ± 31.1	
	Fasted	6	202.2 ± 21.8	n.s
2	Fed	6	159.6 ± 42.9	
	Fasted	6	159.3 ± 21.8	n.s

Table 4.14: Effect of 72 hours starvation on rat brain biosynthetic capacity (ng biopterin /hr/mg protein).

Trial	Group	n	Mean ± st.d.	p value
2	Fed	6	3.65 ± 3.01	
	Fasted	6	3.30 ± 1.83	n.s

Table 4.15: Effect of 72 hours starvation on liver total biopterin levels (ng g^{-1} wet weight).

Trial	Group	n	Mean ± st.d.	p value
1	Fed	6	1310 ± 70	
	Fasted	6	1310 ± 123	n.s
2	Fed	6	1535 ± 84	
	Fasted	6	1407 ± 66	p<2%

TABLES 4.13-4.15: 150g male rats were starved on grids for 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* The experiment was carried out on two occasions, (Trials 1 and 2). Results were analysed by a students unpaired t-test.

Table 4.16: Effect of 72 hours starvation on liver derived pterin levels (ng g^{-1} wet weight).

Trial	Group	n	Mean ± st.d.	p value
1	Fed	6	624 ± 105	
	Fasted	6	670 ± 80	n.s
2	Fed	6	326 ± 44	
	Fasted	6	417 ± 87	p<5%

Table 4.17: Effect of 72 hours starvation on liver DHPR activity (nmoles NADH oxidised /min /mg protein).

Trial	Group	n	Mean ± st.d.	p value
2	Fed	6	439 ± 81	
	Fasted	6	403 ± 56	n.s

Table 4.18: Effect of 72 hours starvation on rat liver biosynthetic capacity (ng biopterin /hr/mg protein).

Trial	Group	n	Mean ± st.d.	p value
2	Fed	6	3.65 ± 3.01	
	Fasted	6	3.30 ± 1.83	n.s

TABLES 4.16-4.18: 150g male rats were starved on grids for 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* The experiment was carried out on two occasions, (Trials 1 and 2). Results were analysed by a students unpaired t-test.

Table 4.19: Effect of various lengths of starvation on brain total biopterin levels (ng g^{-1} wet weight).

Group	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	65.2 ± 5.2		3.200	p<5%	5.42
24 hr	5	65.2 ± 5.7	n.s			n.s
48 hr	5	61.3 ± 1.7	n.s			n.s
72 hr	5	58.7 ± 1.7	p<5%			*

* at 72 hours biopterin levels are significantly decreased compared to the other 3 groups, (p<5%).

Table 4.20: Effect of various lengths of starvation on brain derived pterin levels (ng g^{-1} wet weight).

Group	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	52.3 ± 5.4		1.135	n.s	5.84
24 hours	5	50.1 ± 2.9	n.s			n.s
48 hours	5	56.4 ± 4.2	n.s *			**
72 hours	5	54.4 ± 3.2	n.s			n.s

* and ** : brain derived pterin level at 48 hours is significantly increased compared to that at 24 hours (p<5%).

TABLES 4.19-4.20: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 4.21: Effect of various lengths of starvation on brain P/B ratio.

Group	n	Mean ± st.d.	p value
Control	5	0.809 ± 0.126	
24 hours	5	0.818 ± 0.071	n.s
48 hours	5	0.921 ± 0.074	n.s
72 hours	5	0.959 ± 0.058	p<5% *

* P/B ratio at 72 hours is significantly increased compared to that at 24 hours (p<1%).

Table 4.22: Effect of various lengths of starvation on liver total biopterin levels (ng g^{-1} wet weight).

Group	n	Mean ± st.d.	T-TEST p		ONE-WAY ANOVA F ratio	p	LSD
Control	5	1404 ± 150			15.792	p<0.1%	145.1
24 hours	5	1175 ± 96	p<5%				*
48 hours	5	946 ± 95	p<0.1%				*
72 hours	5	1083 ± 80	p<1%	#			*

* biopterin levels were significantly decreased at 24, 48 and 72 hours compared to controls.
# biopterin levels at 72 hours are significantly greater than at 48 hours (p<5%).

TABLES 4.21-4.22: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 4.23: Effect of various lengths of starvation on liver derived pterin levels (ng g^{-1} wet weight).

Group	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	508 ± 114		2.089	n.s	168.7
24 hours	5	568 ± 141	n.s			n.s
48 hours	5	691 ± 110	p<5%			*
72 hours	5	645 ± 136	n.s			n.s

* derived pterin levels were significantly increased at 48 hours compared to controls, (p<5%).

Table 4.24: Effect of various lengths of starvation on liver P/B ratio.

Group	n	Mean ± st.d.	p value
Control	5	0.368 ± 0.108	
24 hours	5	0.486 ± 0.121	n.s
48 hours	5	0.729 ± 0.067	p<0.1% *
72 hours	5	0.591 ± 0.083	p<1.0% #

* P/B ratio at 48 hours is significantly greater than at 24 hours (p<1%).
# P/B ratio at 48 hours is significantly greater than at 72 hours (p<5%).

TABLES 4.23-4.24: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 4.25: Effect of various lengths of starvation on plasma total biopterin levels (ng ml^{-1}).

Group	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	35.2 ± 11.2		6.092	p<1%	10.73
24 hours	5	39.9 ± 8.3	n.s			n.s
48 hours	5	35.7 ± 7.0	n.s			n.s
72 hours	5	19.8 ± 3.6	p<2%			*

* biopterin levels were significantly decreased at 72 hours compared to the other 3 groups.

TABLE 4.25: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 4.26: Effect of various lengths of starvation on brain glutathione levels (umol g^{-1} wet weight tissue).

Group	n	Mean ± st.d	p value
Control	5	0.894 ± 0.106	
24 hours	5	0.924 ± 0.193	n.s
48 hours	5	0.918 ± 0.063	n.s
72 hours	5	0.606 ± 0.255	p<5% *

* glutathione level at 72 hours is significantly lower than that at 48 hours (p<5%).

Table 4.27: Correlations of brain glutathione with (i) brain total biopterins, and (ii) brain derived pterin after various lengths of starvation.

(i). Brain biopterin vs. Glutathione.

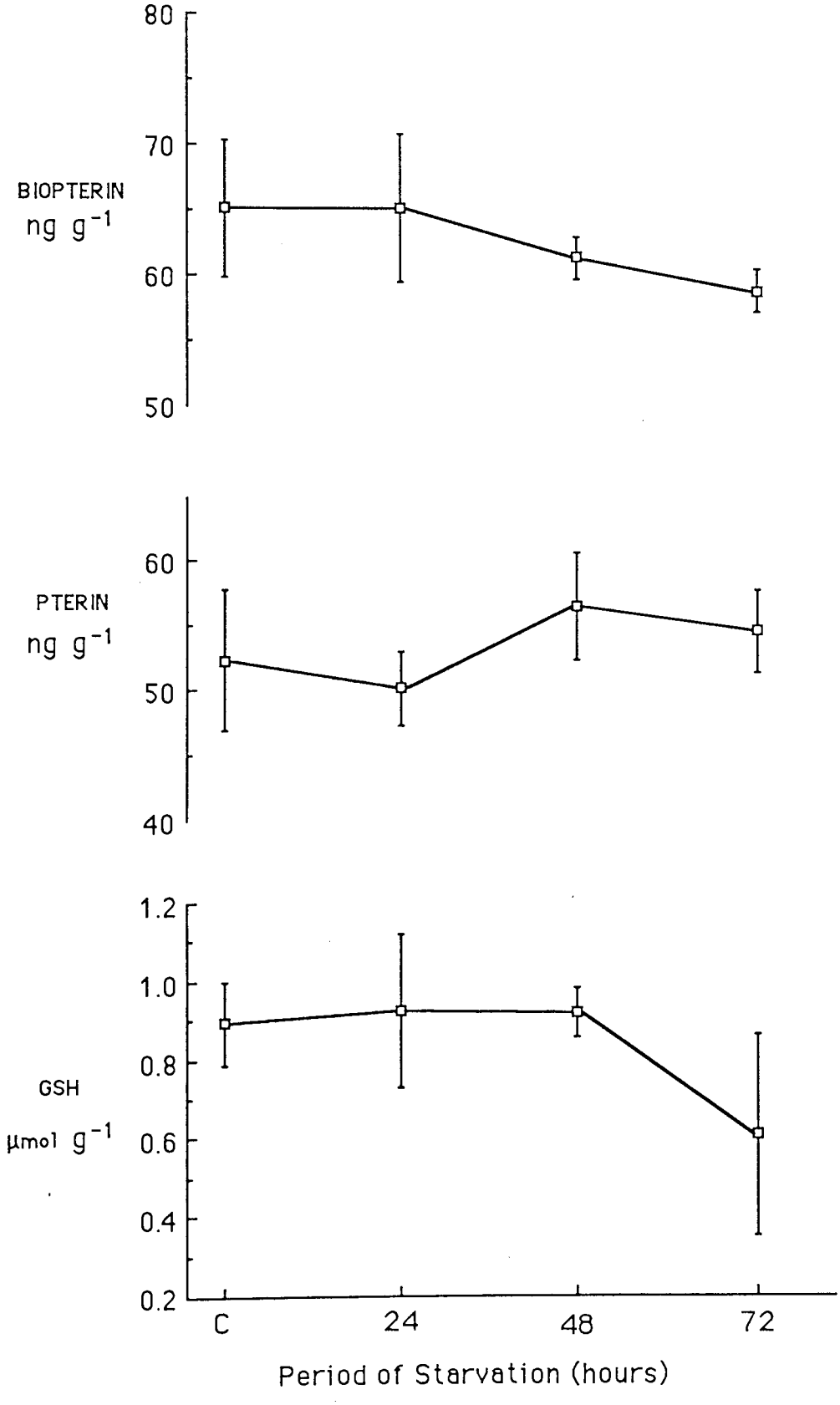
Group	r value	p value
Control	+ 0.016	n.s
24 hours	- 0.371	n.s
48 hours	- 0.113	n.s
72 hours	+ 0.548	n.s

(ii). Brain derived pterin vs. Glutathione.

Group	r value	p value
Control	- 0.268	n.s
24 hours	+ 0.307	n.s
48 hours	+ 0.025	n.s
72 hours	- 0.988	p<0.5%

TABLES 4.26-4.27: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. P values refer to comparisons made between test groups and the control group.

FIG 4.2: Variation in brain total biopterins, derived pterin and glutathione over 24, 48, and 72 hours starvation.



150g Male rats starved for 24, 48 or 72 hours. Controls received food ad lib. Each point is the mean of five observations ± st.d

Table 4.28: Effect of various lengths of starvation on liver glutathione levels (umol g^{-1} wet weight tissue).

Group	n	Mean ± st.d.	p value
Control	5	3.643 ± 0.845	
24 hours	5	2.103 ± 1.173	p<5%
48 hours	5	3.063 ± 1.863	n.s
72 hours	5	5.660 ± 2.875	n.s *

* glutathione level at 72 hours is significantly higher than that at 24 hours (p<5%).

Table 4.29: Correlations of liver glutathione with (i) liver total biopterin, and (ii) liver derived pterin after various lengths of starvation.

(i). Liver biopterin vs. Glutathione.

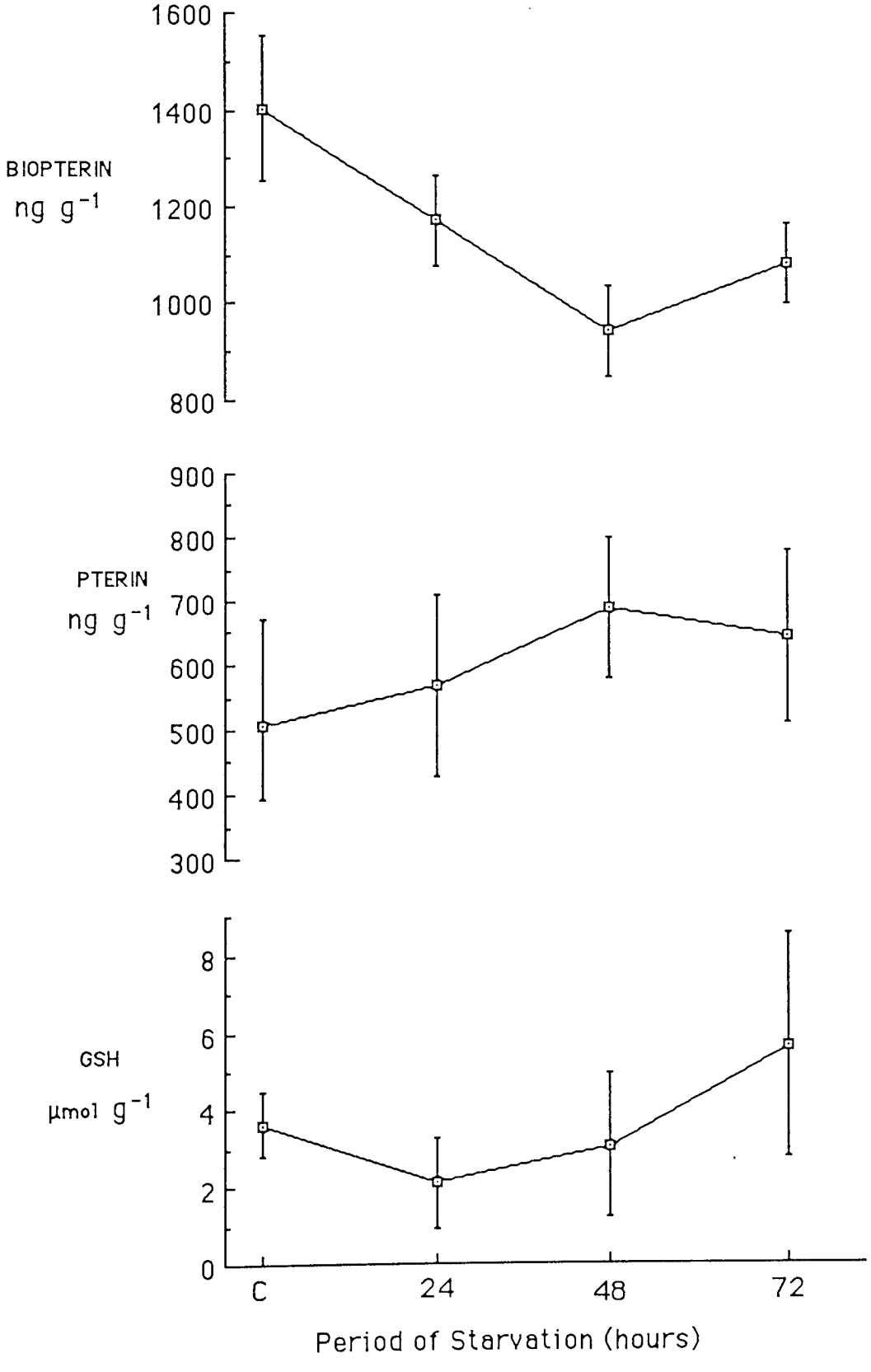
Group	r value	p value
Control	+ 0.237	n.s
24 hours	+ 0.955	p<2%
48 hours	- 0.791	n.s
72 hours	+ 0.304	n.s

(ii). Liver derived pterin vs. Glutathione.

Group	r value	p value
Control	+ 0.445	n.s
24 hours	+ 0.235	n.s
48 hours	- 0.626	n.s
72 hours	+ 0.010	n.s

TABLES 4.28-4.29: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. P values refer to comparisons made between test groups and the control group.

FIG 4.3: Variation in liver total biopterins, derived pterin and glutathione over 24, 48, and 72 hours starvation.



150g Male rats starved for 24, 48 or 72 hours. Controls received food *ad lib*. Each point is the mean of five observations ± st.d

CHAPTER 5.

THE EFFECT OF A STARVATION-INDUCED REDUCTION IN BRAIN TOTAL BIOPTERINS ON NEUROTRANSMITTER LEVELS IN THE RAT.

5.1 INTRODUCTION.

Undernutrition in early life can inhibit maturation of the brain and thus modify its physical and biochemical composition (Eichenwald 1969). Studies in animals (Levitsky and Barnes 1970) and children (Cravioto and DeLicardie 1968) suggest that this can lead to impaired intellectual and emotional development. Severe weight loss, even in adult life, may be associated with changes in brain biochemistry and hence neurological function as shown by human (Ebert *et al* 1984) and animal studies (Curzon *et al* 1972).

A major feature of neuroendocrine changes caused by starvation is low catecholamine (CA) turnover in both the periphery (Gross *et al* 1979; Halmi *et al* 1978; Landsberg and Young 1978; Landsberg and Young 1983) and the brain (Pirke and Spyra 1982; Schweiger *et al* 1985). Decreased steady state levels of noradrenaline (NA) and dopamine (DA) have been reported in certain areas of rat brain after 48 hours starvation (Glick *et al* 1973; Stachowiak *et al* 1978) and after undernutrition of rats from mid-gestation to weaning (Shoemaker and Wurtman 1971).

Evidence suggests that low peripheral CA turnover is under central nervous system control (Young and Landsberg 1977). The precise mechanism mediating the depression of central CA turnover in starvation remains to be elucidated.

The availability of the neurotransmitter precursors phenylalanine, tyrosine and tryptophan within the central nervous system is one of the major factors influencing the synthesis and turnover of dopamine, noradrenaline and serotonin (5-HT) (Wurtman *et al*, 1974; Wurtman 1982). At normal brain physiological concentrations of the amino acids tyrosine hydroxylase (TOH) and tryptophan hydroxylase (TryOH) are unsaturated, thus alterations in precursor amino acid levels will alter the rates of hydroxylation to DA, NA and 5-HT (Wurtman *et al*, 1974; Schweiger 1985).

These precursors, and other large neutral amino acids (LNAAs), share the same competitive uptake system across the blood-brain barrier (BBB). Each amino acid has a unique affinity (Km) for the transport carrier (Fernstrom 1979), and consequently brain levels of each depend on the concentration ratio of the individual amino acid to the other LNAAs in the plasma (Fernstrom and Wurtman 1972).

Food intake, by influencing directly the plasma levels of the LNAAs, will modify brain neurotransmitter synthesis. 24 hour starvation has been shown to lower plasma tyrosine and tryptophan concentrations in rats (Arola 1984). Schweiger *et al* (1985) reported that semistarvation significantly decreased the plasma tyrosine:LNAA ratio, brain tyrosine levels and NA turnover in rats. He also stated that amino acid availability was unlikely to be the only factor governing reduced NA turnover in starvation.

Other factors affecting synthesis, turnover and hence steady state levels of the neurotransmitters include, the degree of neuronal activity, catabolism and the availability of the hydroxylase enzymes and cofactor (BH_4) required for synthesis.

It has been established that acute starvation reduces total biopterin levels in rat brain and liver, (see Chapter 4).

The effect of fasting on steady state levels of brain catecholamines and serotonin was investigated to test whether starvation-induced depressions in cofactor availability may be partly responsible for the diminished neurotransmitter turnover reported in food deprivation.

5.2 MATERIALS AND METHODS.

Neurotransmitter analysis was carried out on the brains of rats used in the large starvation experiment outlined in Chapter 4, (Tables 4.19-4.29). 150g male rats were subjected to 24, 48 or 72 hours starvation. Animals were allowed free access to water. Controls received food and water *ad libitum*. Food was removed at 17.00 hours on day one. All animals were placed on grids to minimise coprophagy.

Analyses of whole half brain levels for total biopterins, derived pterin, noradrenaline (NA), dopamine (DA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and dihydroxyphenylacetic acid (DOPAC) were carried out as decribed in Chapter 2. Results were analysed by students unpaired t-test and by one-way analysis of variance.

Brain derived pterin and total biopterins were correlated with brain neurotransmitter and transmitter metabolite levels to look for a relationship between BH_4 and neurotransmitter concentrations within the brain using whole half brain analysis.

5.3 RESULTS.

The effects of starvation on rat brain biopterin and derived pterin levels have been detailed in Chapter 4. To

summarise, brain total biopterins decreased after 72 hours starvation ($p<5\%$, Table 5.1), while pterin levels were non-significantly increased after 48 and 72 hours, (Table 5.2). As the length of the starvation period increased there was a gradual rise in the P/B ratio which reached significance after 72 hours compared to controls, ($p< 5\%$, Table 5.3).

There was no evidence of a significant effect of starvation on brain NA, DA or DOPAC levels or the DA:DOPAC ratio, (Tables 5.4-5.7 respectively).

There was a significant over-all effect of starvation on brain 5-HT ($F= 4.208$, $p<5\%$) and 5-HIAA levels ($F= 8.129$, $p<1\%$), (Tables 5.8 and 5.9). 5-HT was elevated in animals starved for 48 and 72 hours compared to controls, ($p<2\%$ and $p<0.1\%$ respectively). 5-HIAA was significantly increased after 24 and 48 hours fasting, ($p<1\%$, table 5.9), but levels declined after 72 hours causing a corresponding fall in the 5-HIAA:5-HT ratio (n.s, Table 5.10).

Animals starved for 24 hours showed a significant positive correlation between brain total biopterin and NA levels, (Table 5.11, Fig. 5.1; $r= + 0.8833$, $p<5\%$).

A relationship between brain biopterin and DA levels in rats starved for 24 and 48 hours may have been indicated, (Fig. 5.2; $r = + 0.8696$, $p<10\%$ and Fig. 5.3; $r = - 0.8274$, $p<10\%$ respectively). When the results for the control and 24 hour group were pooled, the correlation between brain biopterin and DA was statistically significant (Fig. 5.4; $r= + 0.7847$, $p<1\%$).

Brain derived pterin correlated significantly with the 5-HIAA:5-HT ratio in rats starved for 48 hours, (Table 5.12, Fig 5.7; $r= + 0.8938$, $p<5\%$). The P/B ratio and 5-HIAA:5-HT ratio in animals fasted for 24 hours also showed a significant correlation, (Table 5.12, Fig 5.8; $r= + 0.8939$, $p<5\%$).

Those correlations only reaching the 10% level of significance are illustrated since it is possible that with larger groups of animals they might achieve statistical significance at the 5% level. These include, derived pterin with DA after 72 hours starvation, (Fig. 5.5), and pterin with 5-HT after 48 hours, (Fig. 5.6).

5.4 DISCUSSION.

A starvation-induced decrease in brain total biopterins did not alter steady-state levels of brain catecholamines. Neurotransmitter analysis, in this investigation, used whole half brains. This, together with the use of tissue weight for normalising measurements of compounds in specialised cell populations, could have obscured any significant alterations in transmitter levels that might have occured in specific regions.

Since BH_4 levels can be rate-limiting in the synthesis of DA some dependency between brain biopterin and DA levels was anticipated. A significant positive correlation was achieved by combining biopterin and DA data from control and 24 hour starved rats. This pooling of data was considered justifiable since there was no difference in the mean levels of either parameter between the two groups.

It should be noted however that when data for control, 24 and 48 hour rats was pooled again a positive correlation between biopterin and DA levels with $r = + 0.6018$ was achieved which was significant at the 2% level. The correlation is clearly invalid since biopterin and DA in 48 hour animals were negatively correlated, ($p<10\%$)as biopterin levels started to fall. Hence, care should be exercised in the interpretation of statistical correlations

and examination of the scatter of points carried out to establish the real significance and validity of any given r value.

Further evidence for a link between brain BH_4 status and DA content was observed. As derived pterin levels increased after 48 hours fasting, the biopterin/DA correlation became negative, (only significant at the 10% level). As mentioned in chapter 4, a rise in pterin levels, or the P/B ratio, may be indicative of increased BH_4 turnover. The negative correlation may therefore have reflected enhanced utilisation of BH_4 resulting in increased DA production.

However, as there was no significant indication of increased DA synthesis in starved rats these suggestions remain purely speculative. No data was obtained in the present investigation regarding brain tyrosine content in starvation. A reduction in plasma and brain levels of the amino acid has been reported in semistarvation due to a lowered tyrosine:other LNAA ratio and enhanced competition (especially from tryptophan) for entry into the brain, (Schweiger *et al*, 1985; Wurtman *et al*, 1974). Shoemaker and Wurtman, (1971), showed no alteration in whole brain tyrosine levels in malnourished weaner rats compared to well fed controls however tyrosine status in just those brain cells that convert the amino acid to catecholamines could not be measured.

There was little evidence for a relationship between brain biopterin and NA levels. The validity of the correlation shown in 24 hour starved animals is questionable since controls, who had virtually identical mean concentrations of biopterin and NA to animals starved for 24 hours, failed to display a similar correlation. As BH_4 levels are not directly rate-limiting for the hydroxylation of DA to NA by dopamine beta-hydroxylase these findings are probably not surprising.

A gradual rise in 5-HT and 5-HIAA levels over the first 48 hours starvation indicated enhanced 5-HT synthesis and turnover. Levels of tryptophan, the precursor of 5-HT synthesis, are more readily influenced by diet than catecholamine precursor amino acid levels (Fernstrom and Wurtman 1971). Food deprivation though causing a general reduction in plasma LNAA levels has been reported to cause an elevated plasma tryptophan:other LNAA ratio (Fernstrom and Wurtman 1971 and 1972; Fernstrom 1979). This was probably responsible for an enhanced influx of tryptophan into the brain causing a corresponding rise in 5-HT synthesis and turnover.

The rise in 5-HT concentration over the three day starvation period indicated enhanced tryptophan hydroxylase activity. This suggests that the BH_4 concentration did not become rate-limiting for the hydroxylation reaction, even when levels dropped after 72 hours.

The P/B ratio showed a significant positive correlation with the 5-HIAA/5-HT ratio in 24 hour fasted rats, and derived pterin correlated significantly with 5-HIAA levels after 48 hours. This may indicate a concomitant increase in utilisation of the cofactor and 5-HT at least over the first 48 hours starvation.

As there has been no evidence to-date of a starvation-induced increase in BH_4 biosynthesis, an elevated utilisation of the cofactor would eventually cause levels to become exhausted and this may explain the decline in total biopterins after 72 hours. As 5-HT levels continue to rise one might question the extent to which BH_4 is a rate-limiting factor in serotonin biosynthesis.

5.5 CONCLUSIONS.

There was little evidence of a concomitant starvation-induced decrease in brain total biopterins and catecholamines. This may highlight the limited usefulness of using whole half brain analysis in measuring compounds which are present in specific cell populations.

Acute starvation produced a significant increase in brain 5-HT levels. This was probably due to an increased influx of tryptophan into the brain resulting in elevated synthesis of the transmitter. The increase in brain 5-HT was probably associated, at least initially, with increased neuronal firing indicated by the significant rise in 5-HIAA levels.

There was no evidence that a reduction in total biopterins after 72 hours significantly lowered 5-HT levels. This may question the extent to which BH_4 is a rate-limiting factor in serotonin synthesis.

Table 5.1: Effect of various lengths of starvation on brain total biopterin levels (ng g^{-1} wet weight).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	65.2 ± 5.2		3.200	p<5%	5.42
24 hr	5	65.2 ± 5.7	n.s			n.s
48 hr	5	61.3 ± 1.7	n.s			n.s
72 hr	5	58.7 ± 1.7	p<5%			*

* at 72 hours biopterin levels are significantly decreased compared to the other 3 groups.

Table 5.2: Effect of various lengths of starvation on brain derived pterin levels (ng g^{-1} wet weight).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	52.3 ± 5.4		1.135	n.s	5.84
24 hours	5	50.1 ± 2.9	n.s			n.s
48 hours	5	56.4 ± 4.2	n.s *			**
72 hours	5	54.4 ± 3.2	n.s			n.s

* and ** : brain derived pterin level at 48 hours is significantly increased compared to that at 24 hours (p<5%).

TABLES 5.1-5.2: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 5.3: Effect of various lengths of starvation on brain P/B ratio.

GROUP	n	Mean ± st.d.	p value
Control	5	0.809 ± 0.126	
24 hours	5	0.818 ± 0.071	n.s
48 hours	5	0.921 ± 0.074	n.s
72 hours	5	0.959 ± 0.058	p<5% *

* P/B ratio at 72 hours is significantly increased compared to that at 24 hours (p<1%).

There is a significant positive correlation between the length of starvation and the P/B ratio (p<5%).

TABLES 5.3: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 5.4: Effect of various lengths of starvation on brain noradrenaline, (NA), levels (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	283.3 ± 20.2		0.385	n.s	43.98
24 hours	5	284.0 ± 50.8	n.s			n.s
48 hours	5	300.1 ± 34.0	n.s			n.s
72 hours	5	298.6 ± 11.6	n.s			n.s

Table 5.5: Effect of various lengths of starvation on brain dopamine, (DA), levels (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	746.6 ± 58.9		1.454	n.s	155.6
24 hours	5	719.3 ± 84.3	n.s			n.s
48 hours	5	715.8 ± 53.1	n.s			n.s
72 hours	5	799.7 ± 102.2	n.s			n.s

Table 5.6: Effect of various lengths of starvation on brain DOPAC levels (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	63.8 ± 9.5		0.137	n.s	14.3
24 hours	5	61.6 ± 4.8	n.s			n.s
48 hours	5	61.0 ± 16.8	n.s			n.s
72 hours	5	59.7 ± 7.7	n.s			n.s

TABLES 5.4-5.6: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 5.7: Effect of various lengths of starvation on brain DA:DOPAC ratios.

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	11.80 ± 0.93		0.783	n.s	2.73
24 hours	5	11.75 ± 1.81	n.s			n.s
48 hours	5	12.40 ± 3.09	n.s			n.s
72 hours	5	13.49 ± 1.72	n.s			n.s

Table 5.8: Effect of various lengths of starvation on brain serotonin, (5-HT), levels (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	501.3 ± 27.6		4.208	p<5%	104.5
24 hours	5	603.3 ± 112.9	n.s			n.s
48 hours	5	643.2 ± 95.8	p<2%			*
72 hours	5	661.0 ± 39.8	p<0.1%			*

* 5-HT levels were significantly increased at 48 and 72 hours compared to controls.

TABLES 5.7-5.8: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 5.9: Effect of various lengths of starvation on brain 5-hydroxyindoleacetic acid, (5-HIAA), levels (ng g^{-1} wet weight tissue).

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	185.0 ± 24.8		8.129	p<1%	24.2
24 hours	5	233.8 ± 19.3	p<1%			*
48 hours	5	233.2 ± 10.4	p<1%			*
72 hours	5	213.3 ± 14.2	n.s **			*

** 5-HIAA level at 72 hours was significantly lower than that at 48 hours (p<5%).
* 5-HIAA levels at 24, 48 and 72 hours were significantly increased compared to controls.

Table 5.10: Effect of various lengths of starvation on brain 5-HIAA:5-HT ratios.

GROUP	n	Mean ± st.d.	T-TEST p	ONE-WAY ANOVA F ratio	p	LSD
Control	5	0.371 ± 0.060		1.57	n.s	0.072
24 hours	5	0.396 ± 0.057	n.s			n.s
48 hours	5	0.369 ± 0.060	n.s			n.s
72 hours	5	0.324 ± 0.031	n.s *			*

* the 5-HIAA:5-HT ratio at 72 hours is significantly lower than that at 24 hours (p<5%).

TABLES 5.9-5.10: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.* All samples were analysed on the same day. Results were analysed by a students unpaired t-test, (p values refer to a comparison made with the control group); and by one-way analysis of variance (one-way anova), with the F ratio indicating any over-all effect of the treatment, and using the least significant difference (LSD) to compare the results for individual groups.

Table 5.11: Correlations of brain total biopterin with neurotransmitter levels in fed rats and those subjected to various lengths of starvation.

CORRELATION PERFORMED	EXPERIMENTAL GROUP(s)	n	r value	p value
Biopterin vs. Noradrenaline	controls (c)	5	- 0.0482	n.s
	24 hr	5	+ 0.8833	p<5%
	48 hr	5	- 0.4528	n.s
	72 hr	5	+ 0.6854	n.s
Biopterin vs. Dopamine	c	5	+ 0.7178	n.s
	24 hr	5	+ 0.8696	p<10%
	48 hr	5	- 0.8274	p<10%
	72 hr	5	+ 0.3557	n.s
	c+24	10	+ 0.7847	p<1%
	c+24+48	15	+ 0.6018	p<2%
Biopterin vs. 5-HT	c	5	- 0.6439	n.s
	24 hr	5	+ 0.7840	n.s
	48 hr	5	- 0.0900	n.s
	72 hr	5	+ 0.4708	n.s
Biopterin vs. 5-HIAA	c	5	- 0.1470	n.s
	24 hr	5	+ 0.8001	n.s
	48 hr	5	- 0.5817	n.s
	72 hr	5	- 0.2029	n.s
Biopterin vs. 5-HIAA : 5-HT	c	5	+ 0.0672	n.s
	24 hr	5	+ 0.0397	n.s
	48 hr	5	+ 0.2212	n.s
	72 hr	5	- 0.4586	n.s

TABLE 5.11: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.*

Various correlations were performed to test for;
a) a relationship between biopterins and neurotransmitter levels in the brain, and
b) any indication that a starvation-induced decrease in brain biopterin levels causes associated decreases in brain neurotransmitter levels.
Significant correlations and those approaching significance, (p<5% and 10% respectively), are illustrated in the following graphs.

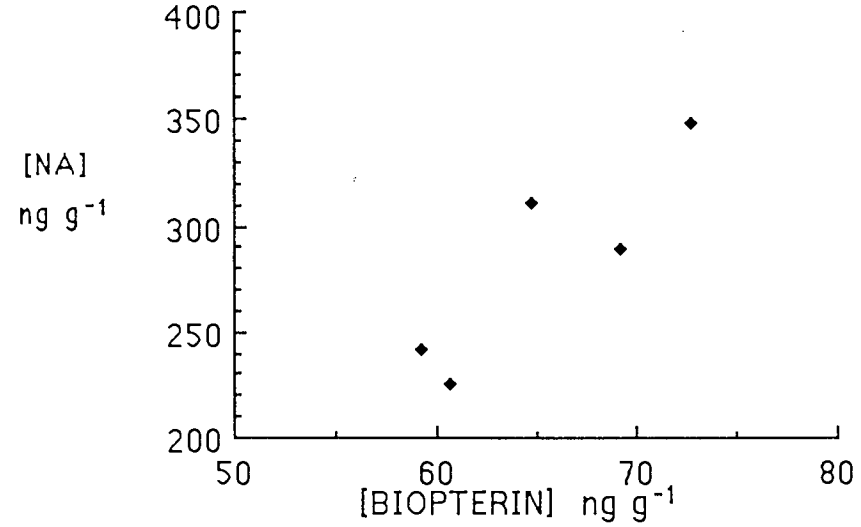
Table 5.12: Correlations of brain derived pterin and derived pterin : biopterin ratios with neurotransmitter levels in fed rats and those subjected to various lengths of starvation.

CORRELATION PERFORMED	EXPERIMENTAL GROUP	n	r value	p value
Der' Pterin vs. Noradrenaline	controls (c)	5	- 0.3649	n.s
	24 hr	5	+ 0.4919	n.s
	48 hr	5	- 0.2144	n.s
	72 hr	5	+ 0.1183	n.s
Der' Pterin vs. Dopamine	c	5	- 0.2496	n.s
	24 hr	5	+ 0.0981	n.s
	48 hr	5	- 0.2144	n.s
	72 hr	5	- 0.8164	p<10%
Der' Pterin vs. 5-HT	c	5	+ 0.2761	n.s
	24 hr	5	+ 0.0218	n.s
	48 hr	5	- 0.8347	p<10%
	72 hr	5	+ 0.2495	n.s
Der' Pterin vs. 5-HIAA	c	5	+ 0.4714	n.s
	24 hr	4	- 0.8289	n.s
	48 hr	5	+ 0.3903	n.s
	72 hr	5	+ 0.6963	n.s
Der' Pterin vs. 5-HIAA : 5-HT	c	5	+ 0.3358	n.s
	24 hr	5	+ 0.3540	n.s
	48 hr	5	+ 0.8938	p<5%
	72 hr	5	+ 0.3650	n.s
Pterin:Biopterin vs. 5-HIAA:5-HT	c	5	+ 0.1265	n.s
	24 hr	5	+ 0.8939	p<5%
	48 hr	5	+ 0.7436	n.s
	72 hr	5	+ 0.4850	n.s

TABLE 5.12: 150g male rats were starved on grids for 24, 48 or 72 hours. Food was removed on day one at 4.00pm. Water was available *ad libitum*. Controls received food and water *ad. lib.*

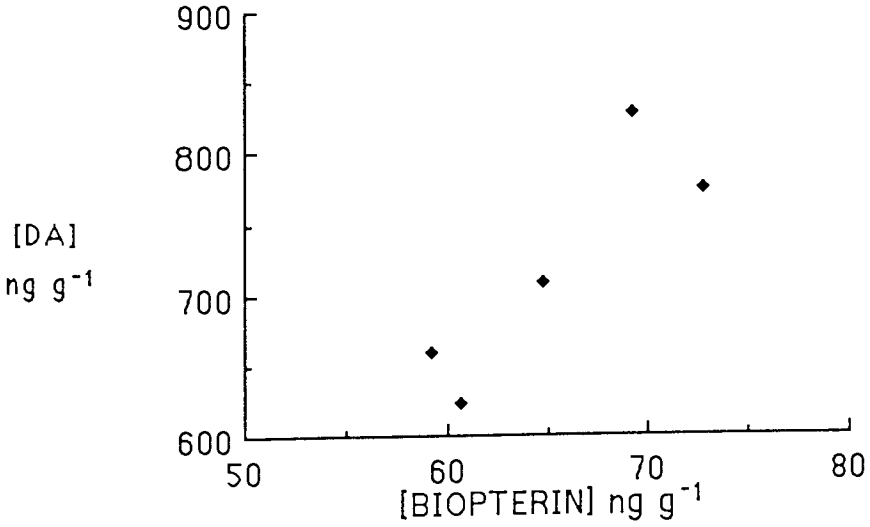
Various correlations were performed to test for;
a) a relationship between pterin and neurotransmitter levels in the brain, and
b) any indication that a starvation-induced effect on brain pterin causes associated effects in brain neurotransmitter levels.
Significant correlations and those approaching significance, (p<5% and 10% respectively), are illustrated in the following graphs.

FIG 5.1: Correlation of Brain Biopterin with noradrenaline in 24hr starved rats.



r = +0.8833
p < 5%

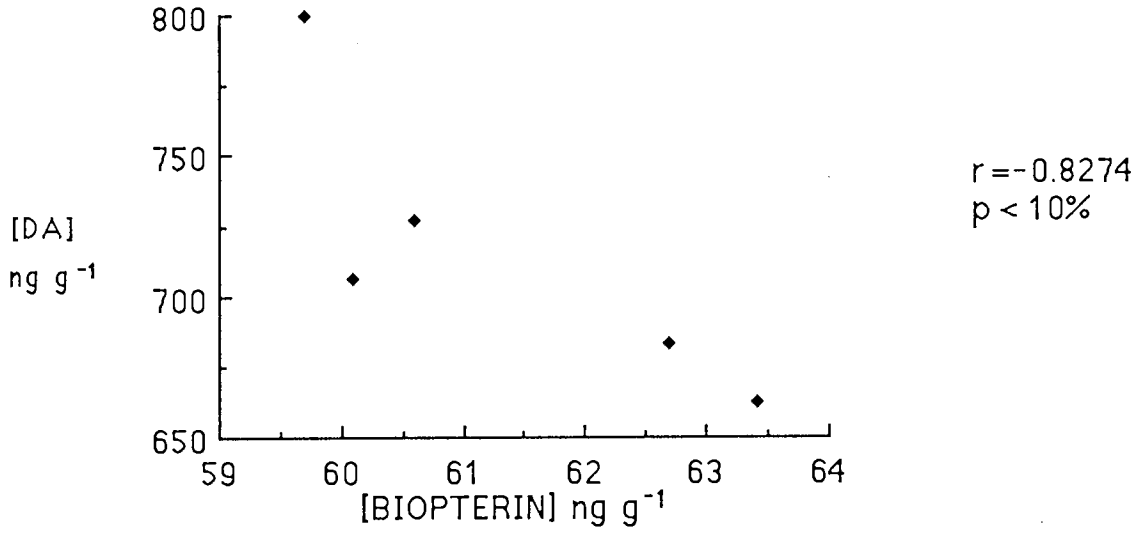
FIG 5. 2: Correlation of Brain Biopterin with Dopamine in 24hr starved rats.



r = +0.8696
p < 10%

150g Male rats were starved for 24, 48 and 72 hours. Food was removed on day one at 4 pm. Water was available *ad lib.* Controls received food and water *ad lib.* Units are ng g^{-1} wet weight tissue.

FIG 5.3: Correlation of Brain Biopterin with Dopamine in 48 hr starved rats.



150g Male rats were starved for 24, 48 and 72 hours. Food was removed on day one at 4 pm. Water was available *ad lib*. Controls received food and water *ad lib*. Units are ng g^{-1} wet weight tissue.

FIG 5.4: Correlation of Brain Biopterin with Dopamine in Control (◇) and 24hr (◆) Starved Rats.

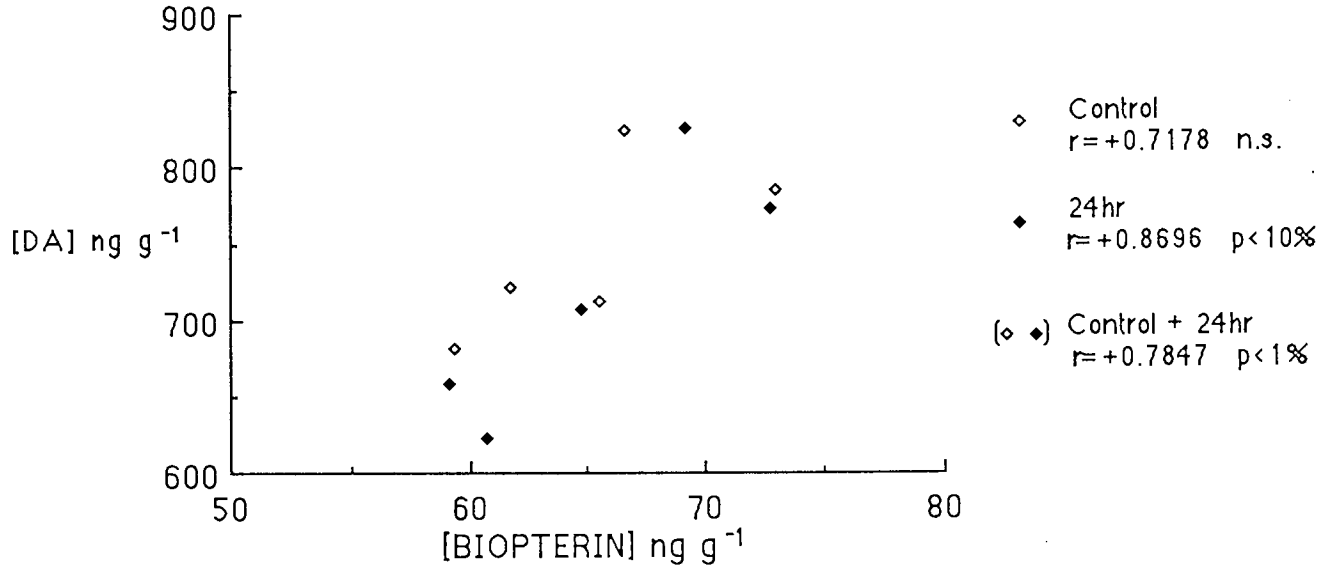
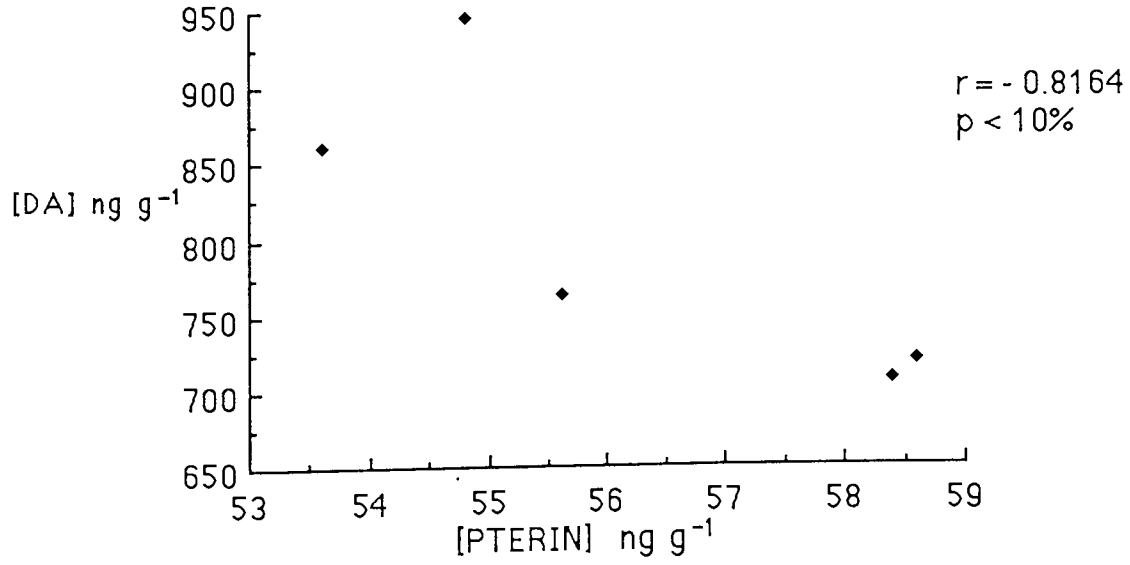


FIG 5.5: Correlation of Brain Pterin with Dopamine in 72 hr starved Rats.



150g Male rats were starved for 24, 48 and 72 hours. Food was removed on day one at 4 pm. Water was available *ad lib*. Controls received food and water *ad lib*. Units are ng g^{-1} wet weight tissue.

FIG 5.6: Correlation between brain Pterin and 5HT in 48 hour starved animals.

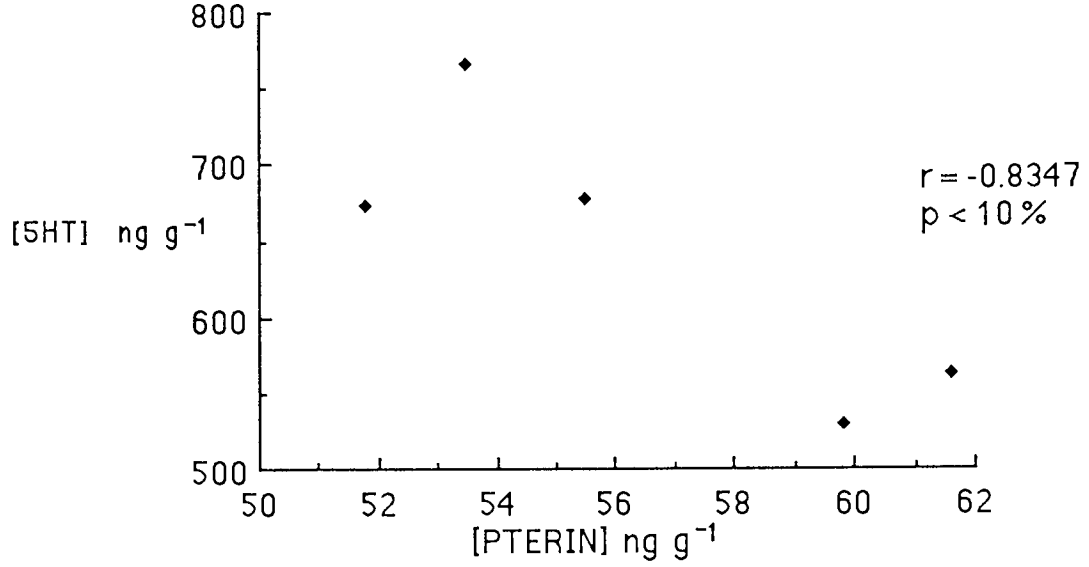
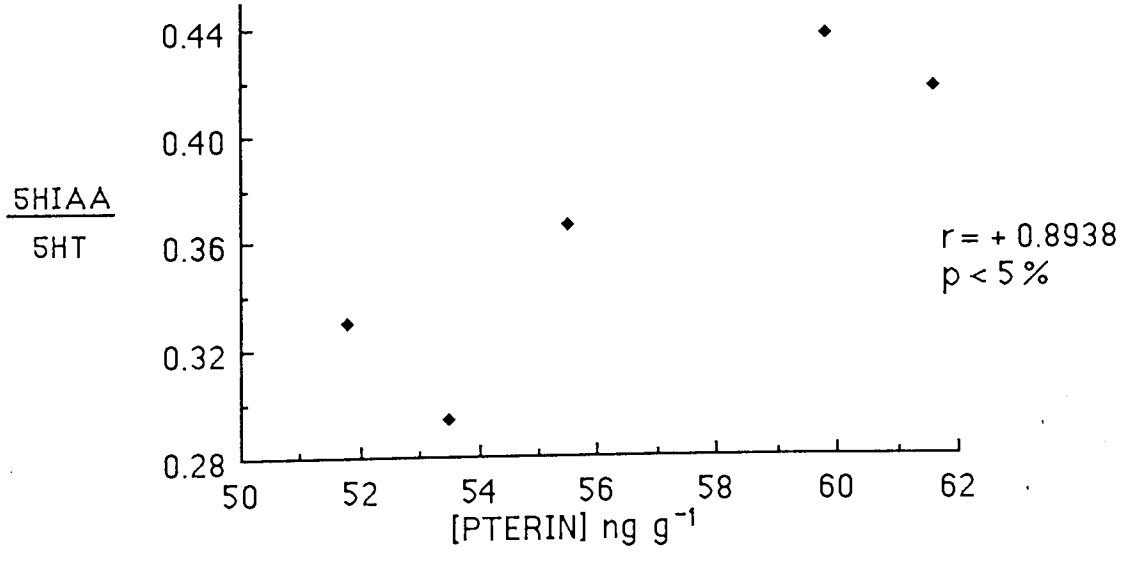
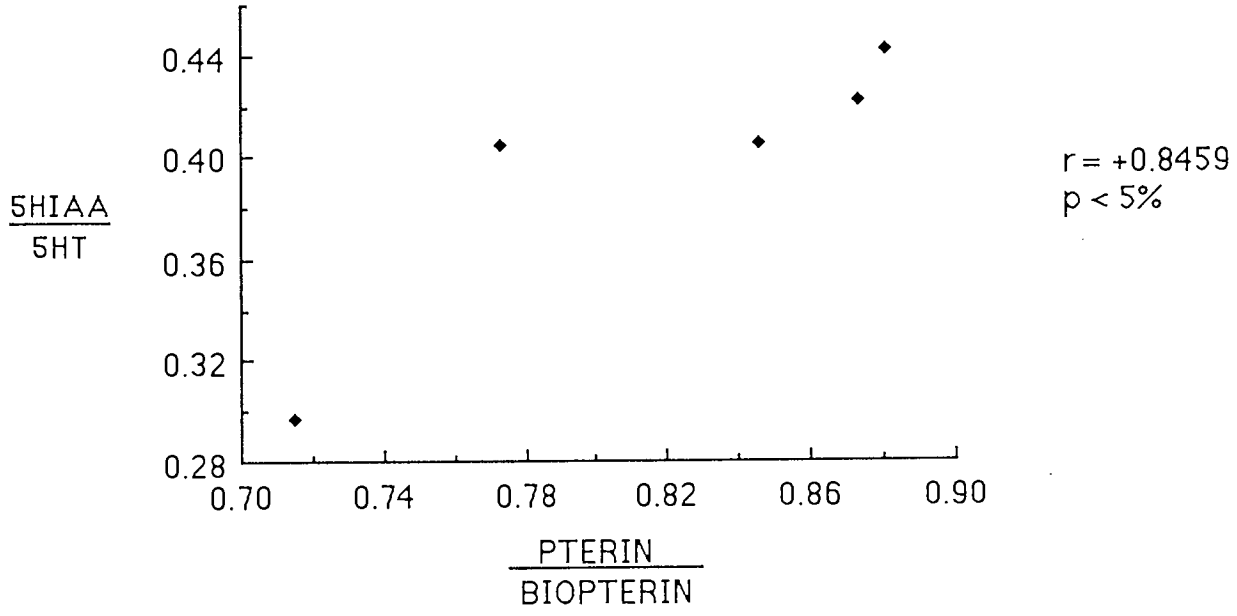


FIG 5.7: Correlation between brain pterin and 5HIAA : 5HT in 48 hr starved rats.



150g Male rats were starved for 24, 48 and 72 hours. Food was removed on day one at 4 pm. Water was available *ad lib.* Controls received food and water *ad lib.* Units are ng g^{-1} wet weight tissue.

FIG 5.8: Correlation between brain Pterin : Biopterin and 5HIAA: 5HT in 24 hour starved rats.



150g Male rats were starved for 24, 48 and 72 hours. Food was removed on day one at 4 pm. Water was available *ad lib.* Controls received food and water *ad lib.* Units are ng g^{-1} wet weight tissue.

CHAPTER 6.

DIETARY ADMINISTRATION OF 2,4-DIAMINO-6-HYDROXYPYRIMIDINE AS A MODEL FOR BRAIN TETRAHYDROBIOPTERIN DEFICIENCY IN THE RAT.

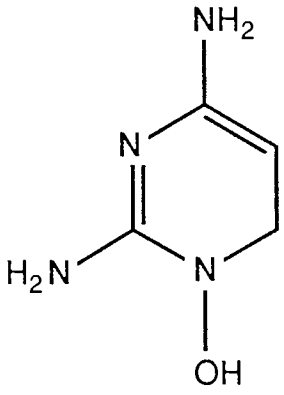
6.1 INTRODUCTION.

In 1966 administration of a diet containing 1% (w/w) 2,4-diamino-6-hydroxypyrimidine, (DAHP) (i), was shown to inhibit tetrahydrobiopterin biosynthesis and reduce urinary biopterins by about 80% as measured by Crithidia assay, (Pabst and Rembold, 1966).

Much later, Gal and coworkers, (Gal and Sherman 1976; Gal *et al* 1978; Gal and Whitacre 1981), confirmed the inhibitory effect of DAHP on BH_4 synthesis and the subsequent loss of the biopterin pool in bacteria and in rat brain. *In vitro* analyses showed that the pyrimidine blocked the conversion of GTP to NH_2P_3 by inhibiting GTP cyclohydrolase, the rate limiting enzyme in BH_4 biosynthesis in the rat.

These inhibitory properties of DAHP suggested its potential usefulness in creating an animal model of the type of BH_4 deficiency which has been reported in humans, (Joller *et al* 1983; Niederwieser *et al* 1984). Clearly, such a model would provide the facility in which to test the therapeutic efficacy of new pterins for treatment of BH_4 deficiency.

Recently Cotton, using DAHP, described a model of BH_4 deficiency which successfully induced a hyperphenylalaninaemic state in mice (Cotton 1986). Adult and weaner animals were fed a diet containing between 1 and 7% DAHP for five



(1). 2,4 - DIAMINO - 6 - HYDROXYPYRIMIDINE (DAHP)

days. At higher concentrations of the compound biopterin levels were reduced, the effect being restricted to the periphery in adult animals although weaners did show BH_4 deficiency in the brain.

Work was undertaken to see whether dietary administration of DAHP might be used to create an animal model of brain BH_4 deficiency in weaner rats similar to that described by Cotton in weaner mice. If successful the model would be used to investigate behavioural and neurobiochemical consequences of partial brain BH_4 deficiency.

6.2 MATERIALS AND METHODS

6.2.1 Administration of DAHP.

(i) In the diet. 2,4-diamino-6-hydroxypyrimidine (DAHP) was blended (2% or 5% w/w) with standard rat breeder diet, to produce a homogeneous powder. Controls received standard food pellets also ground into powder form. The diet was given for 6 days.

(ii) Oral dosing. Animals were dosed by stomach intubation with a thick suspension of DAHP in corn oil for 5 days, (0.1g DAHP / 0.4ml corn oil / rat / day). This was the maximum concentration of DAHP that could be given orally due to the insoluble nature of the compound. (No other suitable vehicle could be found in which the drug was more soluble). Controls received 0.4ml corn oil / rat / day. All animals were allowed access to normal food *ad lib*.

6.2.2 Details of Experiments Performed.

(1). The effect of 6 days dietary administration of 5% DAHP on BH_4 metabolism in weaner, (3 week old), 180 and 500g male rats was investigated. Where experiments were performed more than once, each occasion is referred to as a separate trial, (eg. trial 1, 2 etc). In some experiments animal weights and approximate food intakes were recorded over the 6 day administration period.

(2). DAHP was administered orally to male weaner rats, for 5 days, (0.1g DAHP / 0.4ml corn oil / rat / day). Body weight changes were monitored.

(3). A single large experiment was conducted in which male weaner rats were fed either a standard rat diet or a 2% or 5% DAHP diet for 6 days. Body weights and daily food intake were monitored. In addition to biochemical analyses, behavioural studies were carried out on these animals over the 6 day experimental period, (see Chapter 7).

(4). Animals receiving the DAHP diet ate significantly less food than controls. As starvation has been shown to affect biopterin metabolism, (chapter 4), an experiment was designed to act as a "control" to animals receiving DAHP. Male weaner rats were given standard rat breeder diet but in restricted amounts for 6 days. The quantities of food given were equivalent (in % terms compared to controls) to the daily food intake of animals on the 5% DAHP diet compared to their controls. To try and ensure that food restricted animals ate the appropriate amounts of food, control and test animals were housed individually. In section 6.3, experiments in which animals received DAHP are referred to as Expt. A; associated food restriction experiments as Expt. B.

This type of analysis, comparing 5% DAHP administration and

food restriction was performed twice, (Trials 1 and 2). In trial 1 behavioural analysis was performed; in trial 2 brain neurotransmitter levels were assessed, see Chapter 7.

6.2.3 Biochemical Analyses.

After sacrifice, some or all of the following parameters were measured as described in Chapter 2. Brain and liver total biopterins, derived pterin, biosynthetic capacity and DHPR activity, plasma total biopterins, phenylalanine and tyrosine levels. Some behavioural and neurotransmitter analyses were performed, (see Chapter 7).

6.3 RESULTS AND DISCUSSION

Effect of a 5% DAHP Diet in Weaner Rats.

Administration of a 5% DAHP diet to male weaner rats always produced a significant decrease in brain total biopterin levels, (Table 6.2 trials 1 and 2, $p<0.1\%$; Table 6.42 trials 1 and 2 Expt's A, $p<1\%$). Biopterins were reduced to between 43 and 80% of controls. The results support those of Cotton, (1986), who reported brain BH_4 deficiency in weaner mice fed a DAHP diet.

As DAHP is an inhibitor of GTP cyclohydrolase, a fall in brain total biopterins would presumably arise from depressed BH_4 biosynthesis in the brain. However, results consistently failed to indicate any significant effect of DAHP on brain BH_4 biosynthetic capacity, (Table 6.5 trials 1 and 2, and, 6.44 trial 1).

It is possible that inhibition of synthesis may have occured *in vivo* but was not revealed by the *in vitro* assay. If a competitive inhibitor, such as DAHP, was diluted out sufficiently in the assay, a false negative result might be observed. Levels of the inhibitor in the tissue were likely to be small as transport of the compound across the blood-brain barrier is poor, (Hasegawa *et al*, 1988).

Liver total biopterin concentrations in rats fed DAHP were significantly depressed in 4 separate trials, (Table 6.6 trials 1 and 2, and, Table 6.45 trials 1 and 2; $p<0.1\%$). Levels fell to between 19 and 62% of control values. Cotton observed the same effect of DAHP in weaner mice, (1986).

Results indicate that the fall in liver biopterins was due to inhibition of BH_4 biosynthesis. Liver biosynthetic capacity fell by approximately 50% in two out of three trials, (Tables 6.9 trial 1, $p<2\%$; Table 6.47 trial 1, $p<0.1\%$). Reduced liver total biopterins were not caused by disruption of the salvage pathway as DHPR activity was consistently unaffected in rats fed DAHP, (Table 6.8).

Liver BH_4 was clearly functionally inadequate in DAHP treated rats as reflected by the induced state of hyperphenylalaninaemia. Plasma phenylalanine levels increased approximately three fold in animals fed 5% DAHP compared to controls, (Table 6.12, $p<1\%$), while plasma tyrosine was not significantly affected, (Table 6.11). Cotton reported inconsistent increases in serum phenylalanine levels in mice fed a 5% DAHP diet, (1986).

Workers from this laboratory have shown that phenylalanine loading in rats by oral dosing, (600-900 mg /kg body weight), causes a reduction in brain total biopterin levels and actual BH_4 content, (Blair *et al* 1984). Lower doses, (300mg /kg body weight), did not reduce total biopterins but decreased the % as BH_4. This is consistent with inhibition of rat brain DHPR by phenylalanine or one of its

metabolites, probably phenylpyruvate, (Cutler 1986).

This suggests the possibility that the reduction in brain biopterins by 5% DAHP, with no detectable concomitant decrease in biosynthetic capacity, could have been caused by inhibition of DHPR activity brought about by elevated plasma and brain levels of phenylalanine or its metabolites. However, inhibition of DHPR would lead to increased loss of 7,8 BH_2 into the blood and high plasma biopterins, (Leeming *et al* 1976). Brain DHPR levels were unaffected by DAHP administration (Table 6.4) and plasma biopterins were decreased, (Table 6.37).

Perhaps reflecting the decline in liver biopterins, plasma total biopterin levels were significantly reduced in all trials to between 35 and 57% of control levels, (Table 6.10 trial 1, $p<5\%$; Table 6.10 trial 2 and Table 6.48 trials 1 and 2, $P<0.1\%$).

The effect of DAHP on derived pterin was less consistent. Brain levels were only depressed in one trial, (Table 6.3 trial 1, $p<5\%$). Liver derived pterin dropped dramatically on two occasions, (Table 6.7 trial 1, and Table 6.46 trial 1; $p<0.1\%$), but showed no significant decline in two other trials, (Table 6.7 and 6.46).

Pterin is a potential catabolite of both tetrahydrofolate (THF), (Fukushima and Nixon, 1980), and BH_4, (Pfleiderer, 1975; Milstein, 1983). In 1988 Hasegawa *et al* found that DAHP administration to mice caused digestive dysfunction. This might result in decreased folate absorption and subsequent reduction in tissue THF levels possibly accounting for the lowered levels of derived pterin observed in rats fed DAHP. The inconsistency in the results could be a reflection of variation in the severity of the digestive disorder in individual rats.

Over the six day experimental period visual inspection of

the animals revealed that those fed DAHP declined in health indicated in part by the poor quality of their coats. One animal died after 3 days administration of the diet. Cotton, (1986), also observed a decline in the health of mice fed DAHP and reported a 25% death rate and weight loss.

In the present investigation control animals gained weight normally over the 6 days but those fed DAHP were not only significantly lighter than controls at the end of the experiment, (Table 6.1, trial 1 $p<0.1\%$, trial 2 $p<0.2\%$), but actually weighed less than their original weights on day one, (Table 6.41 trials 1 and 2).

Effect of a 5% DAHP Diet in 180 and 500g Rats.

As in weaner rats, six days dietary administration of 5% DAHP produced loss of weight in both 180 and 500g rats while controls gained weight normally, (Table 6.13). Table 6.23 shows the daily food intake of 500g rats fed DAHP to have been 50% or less of that eaten by controls.

The effects of DAHP on BH_4 metabolism in 180 and 500g rats were similar to those observed in weaners. Liver and plasma total biopterin levels and liver biosynthesis were all significantly depressed, (Tables 6.17, 6.22 and 6.19 respectively), while brain biosynthesis was not, (Table 6.16). Tables 6.21 and 6.22 show that dietary administration of 5% DAHP to 180g rats produced elevated plasma phenylalanine levels, ($p<2\%$), tyrosine levels remaining unchanged confirming results found in weaner rats.

As in weaners derived pterin levels continued to show a degree of variation. Brain concentrations were unaffected in both groups, (Table 6.15), and in the liver, levels were

not significantly affected in 180g rats but were significantly depressed in 500g animals, (Table 6.18, $p<3.5\%$).

The only discrepancy between the results for weaner and adult rats was that 500g animals fed DAHP showed no significant depression in brain total biopterins though levels were reduced to 80% of control values, (Table 6.14). 180g rats showed a significant 37% reduction in brain biopterins, (Table 6.14). Cotton, (1986), observed no effect of the inhibitor on brain BH_4 in adult mice and suggested that this was due to the inability of DAHP to cross the mature blood-brain barrier.

Comparison of the Effects of a 2% and 5% DAHP Diet on BH_4 Metabolism, Food Intake and Body Weight in Weaner Rats.

Most of the parameters depressed in weaner rats by a 5% DAHP diet were also significantly decreased by a 2% diet. These included liver total biopterins, (Table 6.34, $p<0.1\%$), liver derived pterin levels, (Table 6.35, $p<1\%$), liver biosynthesis, (Table 6.36, $p<2\%$), and plasma total biopterins, (Table 6.37, $p<1\%$).

The noticeable exception was brain total biopterin levels which, though reduced in both 2% and 5% groups, only reached significance in the 5% group, (Table 6.32). Brain biosynthesis was not significantly affected in either group, (Table 6.33), which was in keeping with previous findings.

Close inspection of the data showed that the decreases produced by the 5% DAHP diet were, in all cases, more pronounced than those seen with the 2% diet. In some instances the difference between the two groups was significant, ie. total biopterins in brain (Table 6.32. $p<1\%$), liver (Table 6.34, $p<0.2\%$), and plasma (Table 6.37,

p<5%). This initially suggested the possibility of a dose-response effect of DAHP as reported by Cotton, (1986).

Table 6.38, shows the daily food intake of animals fed 2% or 5% DAHP, (calculated as a % of the quantity eaten by the controls). Food intake was inversely proportional to the DAHP content of the diet and this was reflected by the weight changes in the three groups after 6 days, (Table 6.31). It was therefore calculated that animals on the 2% and 5% diets actually ate similar amounts of the inhibitor, (Table 6.39).

This suggested that the differences in amplitude of the effects of the 2% and 5% DAHP diets on BH_4 metabolism were unlikely to be associated with a dose-response effect of the inhibitor and were probably attributable to the variation in food intake between the groups.

In an attempt to overcome the confounding effect of variation in food intake between animals fed DAHP and controls, a different DAHP diet was prepared, (described by Pabst and Rembold, 1966), which was designed to disguise the bitter taste of the pyrimidine. The diet was based primarily on starch, sugar and cocoa fat and is detailed in appendix 1. Though the diet was used successfully in 1966, it proved to be ineffective in the current investigation. Animals receiving the DAHP treated diet still consumed smaller amounts of food than controls.

The Effect of Oral Administration of DAHP to Weaner Rats.

Weaner rats receiving standard rat breeder diet *ad libitum* and dosed daily by stomach intubation with a suspension of DAHP in corn oil showed weight gains comparable to controls who were orally dosed with untreated corn oil, (Table 6.24).
Of the parameters measured, (Tables 6.25-6.30), only liver

total biopterin levels were significantly decreased compared to controls, (Table 6.27, $p<5\%$).

The amount of inhibitor given by this dosing regime (0.1g / day) was less than that received by animals fed 2% or 5% DAHP in the diet, (between 0.1-0.3g / day, Table 6.39). This may have accounted for the lack of any effect on parameters other than liver biopterins.

However, the results may suggest that administration of DAHP to rats, without an accompanying weight loss due to reduced food intake, limits the inhibitory effect of the drug on BH_4 metabolism restricting it to the periphery. If this was so, it would seriously question the validity of using DAHP to create an animal model of BH_4 deficiency.

Comparison of the Effects of a 5% DAHP Diet and Food Restriction on BH_4 Metabolism in Weaner Rats.

Animals subjected to enforced dietary restriction (group B), and those fed DAHP (group A) showed loss in body weight over the 6 day experimental period while controls showed a 45% gain, (Table 6.41).

Since, in a particular trial, rats in groups A and B ate approximately the same amounts of food the relative weight losses should have been similar in both groups as was seen in trial 2. In trial 1 however, animals fed DAHP showed a much smaller mean weight loss than their food restricted controls. The group of 8 animals receiving DAHP were caged together and while 6 showed weight losses comparable to their food restricted controls, 2 consumed more food, indicated by a slight gain in weight, and thus caused the group mean weight change to be higher than was typical for most members of the group. This situation was avoided in the food restriction experiment as animals were caged individually.

Food restriction was shown to have a similar effect on some parameters of BH_4 metabolism as administration of a 5% DAHP diet. Brain, liver, and plasma total biopterin levels were all significantly reduced in both food restricted rats and those fed DAHP, (Tables 6.42, 6.45 and 6.48 respectively). The results were reproducible in plasma and liver in trial 2 but not in the brain.

In trial 2 animals ate greater quantities of the DAHP diet than in trial 1 hence the enforced food restriction was also less severe than in trial 1, (see Table 6.40). This may explain the lack of effect on brain total biopterins in food restricted rats in the second trial.

As with administration of the DAHP diet there was some inconsistency in the effect of food restriction on tissue derived pterin levels. Concentrations in the brain were reduced in trial 1 but not trial 2, (Table 6.43), an effect shown on occasion upon administration of DAHP, (Table 6.3). Liver derived pterin levels were reduced but not significantly in food restricted rats, (Table 6.46), while DAHP had been shown to produce a significant decrease in 2 out of 4 trials.

Food restriction had no significant effect on BH_4 biosynthesis in either the brain, (Table 6.44), or liver, (Table 6.47). DAHP, while apparently having no effect on BH_4 production in the brain, consistently reduced it in the liver.

The results show that the effects of DAHP on BH_4 metabolism will be confounded to some degree by the effects of reduced dietary intake. Acute starvation has been shown to significantly reduce BH_4 metabolism in the rat, (Chapter 5), and results presented here suggest that non-absolute food deprivation may produce similar but less severe effects.

6.4. CONCLUSIONS.

Dietary administration of DAHP to rats caused deficiencies in BH_4 metabolism in the brain and liver. Decreased total biopterins in the liver were caused by a reduction in biosynthesis of the cofactor resulting in a hyperphenylalaninaemic state in the periphery.

Rats fed a DAHP diet showed diminished food intake compared to controls fed standard rat breeder diet. There is strong evidence to suggest that reduced dietary intake is at least a contributing factor to observed changes in BH_4 metabolism in animals receiving DAHP in the diet.

This questions the validity of using dietary administration of DAHP as a specific model of BH_4 deficiency as the effects of the food restriction will confound any results obtained. It also highlights the necessity for careful monitoring of dietary intake in experiments designed to investigate BH_4 metabolism in the rat.

Table 6.1: Effect of dietary administration of 5% DAHP for six days on body weight in weaner rats.

Trial	% weight of controls	p value
1	68.2 %	p<0.1%
2	80.6 %	p<0.2%

Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed access to normal food *ad lib.* The body weight of animals given DAHP after 6 days is expressed as a % of the weight of controls. All animals (six per group) were approximately the same weight at the start of the experiment.

Table 6.2: Effect of a 5% DAHP diet on brain total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	99.6 ± 23.7	
	DAHP	5	42.7 ± 7.7	p<0.1%
2	Control	6	85.2 ± 8.0	
	DAHP	6	67.5 ± 1.5	p<0.1%

TABLES 6.1-6.2: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.3: Effect of a 5% DAHP diet on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	42.2 ± 15.1	
	DAHP	5	23.5 ± 7.3	p<5%
2	Control	6	34.4 ± 2.2	
	DAHP	6	34.1 ± 3.2	n.s

Table 6.4: Effect of a 5% DAHP diet on brain DHPR activity in weaner rats (nmol NADH oxidised /min/ mg protein).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	93.1 ± 24.9	
	DAHP	5	109.2 ± 22.6	n.s
2	Control	6	138.2 ± 11.7	
	DAHP	6	154.4 ± 16.5	n.s

TABLES 6.3-6.4: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.5: Effect of a 5% DAHP diet on brain biosynthetic capacity in weaner rats (ng biopterin /hr/mg protein).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	1.275 ± 0.459	
	DAHP	5	1.814 ± 1.100	n.s
2	Control	6	2.059 ± 0.510	
	DAHP	6	1.968 ± 0.516	n.s

Table 6.6: Effect of a 5% DAHP diet on liver total biopterin levels in weaner rats (ng g^{-1} wet weight tissue).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	2290 ± 160	
	DAHP	5	430 ± 44	p<0.1%
2	Control	6	1689 ± 115	
	DAHP	6	1050 ± 235	p<0.1%

TABLES 6.5-6.6: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.7: Effect of a 5% DAHP diet on liver derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	840 ± 50	
	DAHP	5	470 ± 60	p<0.1%
2	Control	6	275 ± 107	
	DAHP	6	363 ± 59	n.s

Table 6.8: Effect of a 5% DAHP diet on liver DHPR activity in weaner rats (nmol NADH oxidised /min/ mg protein).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	450 ± 75	
	DAHP	5	513 ± 39	n.s
2	Control	6	321 ± 52	
	DAHP	6	307 ± 31	n.s

TABLES 6.7-6.8: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.9: Effect of a 5% DAHP diet on liver biosynthetic capacity in weaner rats (ng biopterin /min/ mg protein).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	12.04 ± 4.33	
	DAHP	5	6.42 ± 1.63	p<2%
2	Control	6	13.16 ± 4.04	
	DAHP	6	12.19 ± 2.38	n.s

Table 6.10: Effect of a 5% DAHP diet on plasma total biopterin levels in weaner rats (ng ml^{-1} plasma).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	20.7 ± 3.1	
	DAHP	5	11.8 ± 6.7	p<5%
2	Control	6	46.7 ± 8.8	
	DAHP	6	23.5 ± 6.3	p<0.1%

TABLES 6.9-6.10: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.11: Effect of a 5% DAHP diet on plasma tyrosine levels in weaner rats (ug ml^{-1} plasma).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	10.20 ± 5.50	
	DAHP	5	7.44 ± 2.92	n.s

Table 6.12: Effect of a 5% DAHP diet on plasma phenylalanine levels in weaner rats (ug ml^{-1} plasma).

Trial	Group	n	Mean ± st.d	p value
1	Control	6	7.69 ± 2.40	
	DAHP	5	22.38 ± 7.11	p<1%

TABLES 6.11-6.12: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.13: Effect of dietary administration of 5% DAHP for six days on body weights of 180g and 500g rats.

Weight	Group	% wgt loss or gain after 6 days
180g	Control	+22.5%
	Test	-20.7%
500g	Control	+ 1.6%
	Test	-11.1%

TABLE 6.13: Values are expressed as a percentage loss, (-), or gain, (+), of the body weight at the start of the experiment after a 6 day period and are means of a group of six animals.

Table 6.14: Effect of a 5% DAHP diet on brain total biopterin levels in 180g and 500g rats (ng g^{-1} wet weight tissue).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	209.7 ± 37.2	
	DAHP	6	131.5 ± 37.8	p<1%
500g	Control	6	78.1 ± 12.5	
	DAHP	6	62.7 ± 15.3	n.s

TABLE 6.14: Male rats (180 or 500g) were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.15: Effect of a 5% DAHP diet on brain derived pterin levels in 180g and 500g rats (ng g^{-1} wet weight tissue).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	120.9 ± 4.2	
	DAHP	6	119.5 ± 9.7	n.s
500g	Control	6	61.0 ± 9.1	
	DAHP	6	54.4 ± 8.0	n.s

Table 6.16: Effect of a 5% DAHP diet on brain biosynthetic capacity in 180g and 500g rats (ng biopterin /hr/mg protein).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	9.85 ± 2.95	
	DAHP	5	7.10 ± 1.95	n.s
500g	Control	6	3.74 ± 2.04	
	DAHP	6	2.488 ± 1.73	n.s

TABLES 6.15-6.16: Male rats (180 or 500g) were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.17: Effect of a 5% DAHP diet on liver total biopterin levels in 180g and 500g rats (ng g^{-1} wet weight tissue).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	4431 ± 458	
	DAHP	5	988 ± 435	p<0.1%
500g	Control	6	1044 ± 136	
	DAHP	6	431 ± 352	p<1%

Table 6.18: Effect of a 5% DAHP diet on liver derived biopterin levels in 180g and 500g rats (ng g^{-1} wet weight tissue).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	1300 ± 154	
	DAHP	5	1212 ± 105	n.s
500g	Control	6	820 ± 187	
	DAHP	6	587 ± 95	p<3.5%

TABLES 6.17-6.18: Male rats (180 or 500g) were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.19: Effect of a 5% DAHP diet on liver biosynthetic capacity in 180g and 500g rats (ng biopterin /hr/mg protein).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	6.95 ± 1.29	
	DAHP	5	3.93 ± 1.25	p<0.2%
500g	Control	6	8.92 ± 1.05	
	DAHP	6	6.64 ± 1.05	p<1%

Table 6.20: Effect of a 5% DAHP diet on plasma total biopterin levels in 180g and 500g rats (ng ml^{-1} plasma).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	22.3 ± 3.9	
	DAHP	5	8.9 ± 7.6	p<1%
500g	Control	6	22.1 ± 4.7	
	DAHP	6	12.8 ± 2.8	p<0.2%

TABLES 6.19-6.20: Male rats (180 or 500g) were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.21: Effect of a 5% DAHP diet on plasma phenylalanine levels in 180g rats (ug ml^{-1} plasma).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	8.0 ± 2.4	
	DAHP	5	34.2 ± 20.5	p<2%

Table 6.22: Effect of a 5% DAHP diet on plasma tyrosine levels in 180g rats (ug ml^{-1} plasma).

Weight	Group	n	Mean ± st.d	p value
180g	Control	6	23.8 ± 7.1	
	DAHP	5	26.9 ± 6.9	n.s

TABLES 6.21-6.22: 180g male rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.23: Approximate food intake over six days of 500g rats fed a 5% DAHP diet compared to controls.

	DAY					
	1	2	3	4	5	6
Approximate food intake / rat (% of controls)	9.7	10.1	47.5	32.4	50.5	47.4

TABLE 6.23: 500g male rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* The food intake of rats fed DAHP was calculated and expressed as a % of the food eaten by the controls.

Table 6.24: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on body weight in weaner rats.

% weight of controls after 5 days	p value
97.7 %	n.s

Male weaner rats were dosed orally with a suspension of DAHP in corn oil for 5 days, (0.1g DAHP / 0.4ml corn oil / rat / day). Controls received 0.4ml corn oil / rat / day for 5 days. All animals were allowed access to normal food *ad lib.* The body weight of animals given DAHP after 5 days is expressed as a % of the weight of controls. All animals (six per group) were approximately the same weight at the start of the experiment.

Table 6.25: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on total brain biopterins in weaner rats (ng g^{-1} wet weight tissue).

Group	n	Mean ± st.d	p value
Control	6	109.8 ± 8.5	
DAHP	6	118.6 ± 15.4	n.s

Table 6.26: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on brain derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

Group	n	Mean ± st.d	p value
Control	6	48.9 ± 4.1	
DAHP	6	47.7 ± 2.9	n.s

TABLES 6.25-6.26: Male weaner rats were dosed orally with a suspension of DAHP in corn oil for 5 days, (0.1g DAHP / 0.4ml corn oil / rat / day). Controls received 0.4ml corn oil / rat / day for 5 days. All animals were allowed access to normal food and water *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.27: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on total liver biopterins in weaner rats (ng g^{-1} wet weight tissue).

Group	n	Mean ± st.d	p value
Control	6	1449 ± 144	
DAHP	6	1268 ± 133	p<5%

Table 6.28: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on liver derived pterin levels in weaner rats (ng g^{-1} wet weight tissue).

Group	n	Mean ± st.d	p value
Control	6	550 ± 77	
DAHP	6	469 ± 74	n.s

TABLES 6.27-6.28. Male weaner rats were dosed orally with a suspension of DAHP in corn oil for 5 days, (0.1g DAHP / 0.4ml corn oil / rat / day). Controls received 0.4ml corn oil / rat / day for 5 days. All animals were allowed access to normal food and water *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.29: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on liver biosynthetic capacity in weaner rats (ng biopterin /hr/mg protein).

Group	n	Mean ± st.d	p value
Control	5	16.04 ± 2.15	
DAHP	4	13.58 ± 1.64	n.s

Table 6.30: Effect of five days oral administration of DAHP (0.1g DAHP/rat/day) on plasma total biopterin levels in weaner rats (ng ml^{-1} plasma).

Group	n	Mean ± st.d	p value
Control	6	32.5 ± 10.4	
DAHP	6	37.4 ± 10.0	n.s

TABLES 6.29-6.30: Male weaner rats were dosed orally with a suspension of DAHP in corn oil for 5 days, (0.1g DAHP / 0.4ml corn oil / rat / day). Controls received 0.4ml corn oil / rat / day for 5 days. All animals were allowed access to normal food and water *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.31: Effect of dietary administration of 2% or 5% DAHP on body weight in weaner rats.

Group	n	Mean % change in body weight after six days
Controls	8	+ 44.4%
2% DAHP	8	+ 26.5%
5% DAHP	8	- 3.2%

Values are expressed as a % loss or gain of the group weights prior to the onset of the experiment.

Table 6.32: Effect of dietary administration of 2% or 5% DAHP on brain total biopterin levels in weaner rats, (ng g^{-1} wet wgt).

Group	n	Mean ± st.d	p value	
Controls	8	147.3 ± 23.0		
2% DAHP	8	136.4 ± 12.1	n.s	
5% DAHP	8	118.2 ± 11.3	p<1%	p<1%

Table 6.33: Effect of dietary administration of 2% or 5% DAHP on brain biosynthetic capacity in weaner rats, (ng biopterin /hr/mg protein).

Group	n	Mean ± st.d	p value
Controls	8	3.97 ± 2.40	
2% DAHP	8	3.63 ± 1.97	n.s
5% DAHP	8	3.22 ± 1.56	n.s

TABLES 6.31-6.33: Male weaner rats were fed either a 2% or 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.34: Effect of dietary administration of 2% or 5% DAHP on liver total biopterin levels in weaner rats, (ng g^{-1} wet wgt).

Group	n	Mean ± st.d	p value	
Controls	8	1701 ± 152		
2% DAHP	8	1087 ± 117	p<0.1%	
5% DAHP	8	727 ± 237	p<0.1%	p<0.2%

Table 6.35: Effect of dietary administration of 2% or 5% DAHP on liver derived pterin levels in weaner rats, (ng g^{-1} wet wgt).

Group	n	Mean ± st.d	p value
Controls	8	835 ± 82	
2% DAHP	8	619 ± 146	p<1%
5% DAHP	8	567 ± 120	p<0.1%

Table 6.36: Effect of dietary administration of 2% or 5% DAHP on liver biosynthetic capacity in weaner rats, (ng biopterin /hr/mg protein).

Group	n	Mean ± st.d	p value
Controls	8	12.46 ± 1.78	
2% DAHP	8	9.63 ± 2.26	p<2%
5% DAHP	8	6.89 ± 2.88	p<0.1%

TABLES 6.34-6.36: Male weaner rats were fed either a 2% or 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.37: Effect of dietary administration of 2% or 5% DAHP on plasma total biopterin levels in weaner rats, (ng ml^{-1}).

Group	n	Mean ± st.d	p value	
Controls	8	17.1 ± 3.6		
2% DAHP	8	10.7 ± 4.5	p<1%	
5% DAHP	8	6.0 ± 4.2	p<0.1	p<5%

TABLE 6.37: Male weaner rats were fed either a 2% or 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t-test.

Table 6.38: Approximate food intake over six days of weaner rats fed a 2% or 5% DAHP diet compared to controls.

Group	Food intake / rat / day (% of controls)						
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	$\bar{x}$
2% DAHP	33.8	44.5	52.8	56.4	94.5	77.1	59.9
5% DAHP	10.3	39.9	25.6	22.8	33.3	31.8	27.3

TABLE 6.38: Male weaner rats were fed either a 2% or a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* The food intake of rats fed DAHP was calculated and expressed as a % of the food eaten by the controls. Each value is a mean for six animals.

Table 6.39: Approximate DAHP intake / day of rats fed a 2% or 5% DAHP diet determined from the quantity of food eaten.

Group	g DAHP / rat / day						
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	TOTAL
2% DAHP	0.10	0.15	0.21	0.24	0.38	0.30	1.38
5% DAHP	0.08	0.35	0.25	0.24	0.34	0.30	1.56

TABLE 6.39: Male weaner rats were fed either a 2% or a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* The food intake / day of rats fed DAHP was recorded and thus the approximate amount of DAHP eaten / day by each of the experimental groups was calculated. Each value is a mean for six animals.

Table 6.40: Food intake over six days of weaner rats fed a 5% DAHP diet compared to controls. Results of two separate investigations.

	Food intake / rat / day (% of controls)					
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
Trial 1	10.3	39.9	25.6	22.8	33.3	31.8
Trial 2	16.9	46.9	66.4	50.2	71.1	58.5

TABLE 6.40: Male weaner rats were fed a 5% DAHP diet for six days. Controls were allowed normal food *ad lib.* The food intake of rats fed DAHP was calculated and expressed as a % of the food eaten by the controls. The investigation was performed on two occasions, (trials 1 and 2). In trial 1 each value is the mean for 8 animals, in trial 2 for 6 animals. The amounts of food eaten by the DAHP animals determined the severity of food restriction in subsequent experiments, (see over page).

Table 6.41: The effect of a 5% DAHP diet and food restriction on body weight in weaner rats. Results of two separate investigations.

Trial	Expt	Group	n	Mean % change in body weight after six days
1	A	Controls	8	+ 44.4%
		5% DAHP	8	- 3.2%
	B	Controls	8	+ 52.1%
		F.R	7	- 19.9%
2	A	Controls	6	+ 35.8%
		5% DAHP	6	- 7.2%
	B	Controls	6	+ 43.3%
		F.R	5	- 13.4%

Values are expressed as a % loss or gain of the group weights prior to the onset of the experiments.

TABLE 6.41: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). This type of investigation was performed on two separate occasions, (Trials 1 and 2).

Table 6.42: Comparison of the effects of a 5% DAHP diet and food restriction on brain total biopterin levels in weaner rats, (ng g^{-1} wet wgt). Results of two separate investigations.

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control	8	147.3 ± 23.1		
		DAHP	8	118.2 ± 11.3	p<1%	80.2%
	B	Control	8	108.6 ± 10.8		
		F.R	6	92.8 ± 11.68	p<5%	85.5%
2	A	Control	5	76.5 ± 8.7		
		DAHP	5	49.7 ± 11.5	p<1%	65.0%
	B	Control	6	112.8 ± 11.5		
		F.R	5	112.3 ± 15.2	n.s	99.6%

TABLE 6.42: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). This type of investigation was performed on two separate occasions, (Trials 1 and 2). Results were analysed by an unpaired students t-test.

Table 6.43: Comparison of the effects of a 5% DAHP diet and food restriction on brain derived pterin levels in weaner rats, (ng g^{-1} wet wgt). Results of two separate investigations.

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control		NO RESULTS OBTAINED		
		DAHP				
	B	Control	8	108.6 ± 10.8		
		F.R	6	92.8 ± 11.68	p<5%	85.5%
2	A	Control	5	75.0 ± 23.5		
		DAHP	5	88.6 ± 19.8	n.s	117.0%
	B	Control	6	34.0 ± 4.5		
		F.R	5	33.7 ± 3.6	n.s	99.1%

TABLE 6.43: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). This type of investigation was performed on two separate occasions, (Trials 1 and 2). Results were analysed by an unpaired students t-test.

Table 6.44: Comparison of the effects of a 5% DAHP diet and food restriction on brain biosynthetic capacity in weaner rats, (ng biopterin /hr/mg protein).

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control	8	3.97 ± 2.40		
		DAHP	8	3.22 ± 1.56	n.s	81.1%
	B	Control	8	3.99 ± 1.77		
		F.R	7	3.89 ± 1.87	n.s	97.5%
2	NOT PERFORMED					

TABLE 6.44: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). The investigation was only performed on one occasion. Results were analysed by an unpaired students t-test.

Table 6.45: Comparison of the effects of a 5% DAHP diet and food restriction on liver total biopterin levels in weaner rats, (ng g^{-1} wet wgt). Results of two separate investigations.

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control	8	1701 ± 152		
		DAHP	8	727 ± 237	p<0.1%	42.7%
	B	Control	8	1574 ± 149		
		F.R	7	1284 ± 128	p<1%	81.6%
2	A	Control	6	3356 ± 533		
		DAHP	6	1139 ± 646	p<0.1%	33.9%
	B	Control	6	594 ± 61		
		F.R	5	418 ± 134	p<5%	70.4%

TABLE 6.45: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). This type of investigation was performed on two separate occasions, (Trials 1 and 2). Results were analysed by an unpaired students t-test.

Table 6.46: Comparison of the effects of a 5% DAHP diet and food restriction on liver derived pterin levels in weaner rats, (ng g^{-1} wet wgt). Results of two separate investigations.

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control	8	835 ± 82		
		DAHP	8	567 ± 120	p<0.1%	67.9%
	B	Control	8	700 ± 148		
		F.R	7	613 ± 138	n.s	87.6%
2	A	Control	6	1393 ± 437		
		DAHP	6	1118 ± 46	n.s	80.3%
	B	Control	6	387 ± 61		
		F.R	5	354 ± 95	n.s	91.5%

TABLE 6.46: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). This type of investigation was performed on two separate occasions, (Trials 1 and 2). Results were analysed by an unpaired students t-test.

Table 6.47: Comparison of the effects of a 5% DAHP diet and food restriction on liver biosynthetic capacity in weaner rats, (ng biopterin /hr/mg protein).

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control	8	12.46 ± 1.78		
		DAHP	8	6.89 ± 2.88	p<0.1%	55.3%
	B	Control	8	12.83 ± 2.27		
		F.R	6	10.91 ± 2.47	n.s	85.0%
2	NOT PERFORMED					

TABLE 6.47: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). The investigation was only performed on one occasion. Results were analysed by an unpaired students t-test.

Table 6.48: Comparison of the effects of a 5% DAHP diet and food restriction on plasma total biopterin levels in weaner rats, (ng ml^{-1}). Results of two separate investigations.

Trial	Expt	Group	n	Mean ± st.d	p value	% of control
1	A	Control	8	17.1 ± 3.5		
		DAHP	8	6.0 ± 4.2	p<0.1%	35.1%
	B	Control	8	38.3 ± 10.6		
		F.R	7	18.3 ± 5.4	p<0.1%	47.8%
2	A	Control	6	78.5 ± 8.3		
		DAHP	6	36.7 ± 9.7	p<0.1%	46.8%
	B	Control	6	11.3 ± 2.9		
		F.R	5	8.3 ± 1.0	p<5%	73.5%

TABLE 6.48: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. Controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A, (i.e both groups ate the same relative quantities of food determined as a % of the amounts eaten by their respective control groups). This type of investigation was performed on two separate occasions, (Trials 1 and 2). Results were analysed by an unpaired students t-test.

CHAPTER 7

DOES A REDUCTION IN BRAIN BH_4 METABOLISM BY DIETARY ADMINISTRATION OF DAHP OR FOOD RESTRICTION EFFECT BRAIN LEVELS OF CATECHOLAMINES, SEROTONIN OR BEHAVIOURAL PATTERNS IN THE RAT?

7.1 INTRODUCTION.

Severe loss of brain BH_4 affects the functioning of three of the major neurotransmitter systems in the brain, namely the dopaminergic, noradrenergic and serotonergic systems, (illustrated in figs. 7.1, 7.2 and 7.3 respectively), (Dhondt 1984). This has been clearly established by studies of inborn errors of metabolism and accounts for the profound and extensive array of behavioural defects seen in patients with malignant hyperphenylalaninaemia (MHPA).

The search for improved treatment of MHPA would benefit greatly from an animal model in which to test the efficacy of new compounds in overcoming the clinical symptoms of the disorder. A suitable model would exhibit not only deficiencies in BH_4, catecholamines and serotonin but would also display changes in those aspects of behaviour thought to be controlled by DA, NA and 5HT.

Roles of Various Neurotransmitter Systems in Behaviour.

The goal of defining mammalian behaviour in terms of specific neurotransmitter systems is still long from being achieved. A particular neurotransmitter pathway may play an initiating or dominant role in triggering a specific behaviour but it is never the only system involved. Table

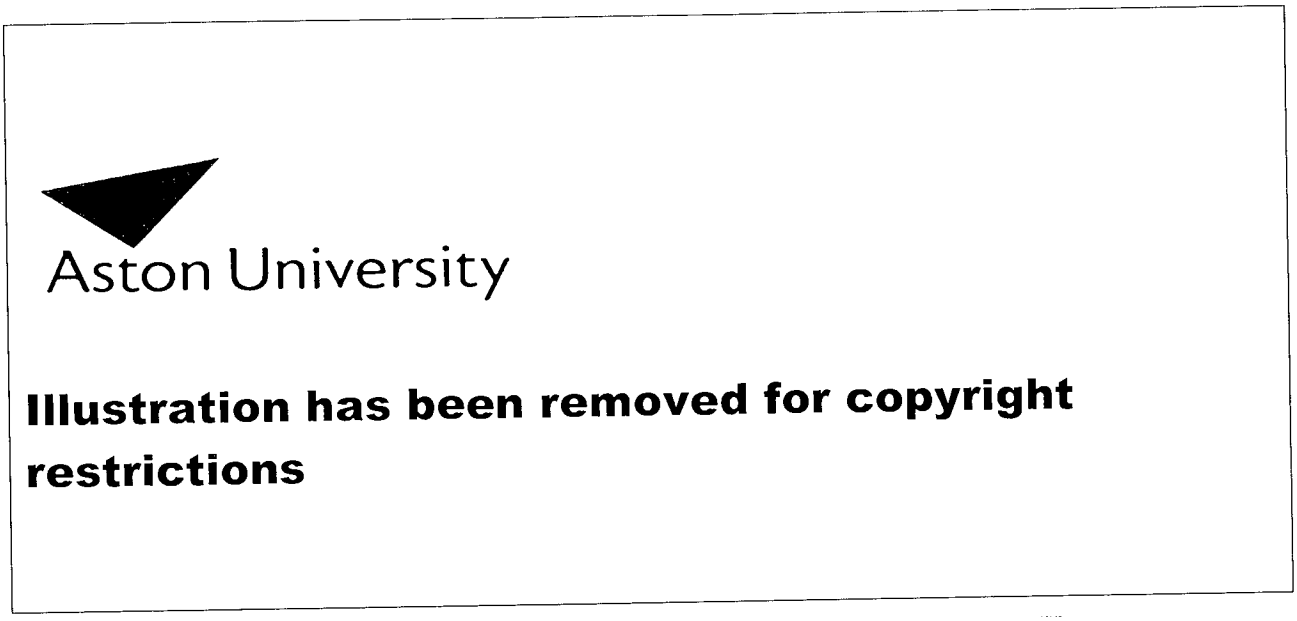


FIGURE 7.1: (A) Dopamine pathways in rat brain shown in saggital section. (B) Dopamine and noradrenaline pathways shown in horizontal section. Hatched areas represent nerve terminal fields and the numbers refer to defined groups of neurones.
(from Ungerstedt, 1971).

FIGURE 7.2: Noradrenaline pathways in rat brain shown in saggital section. Hatched areas represent nerve terminal fields. Pathways descending to the cerebellum and brain stem are not shown. (from Ungerstedt 1971).

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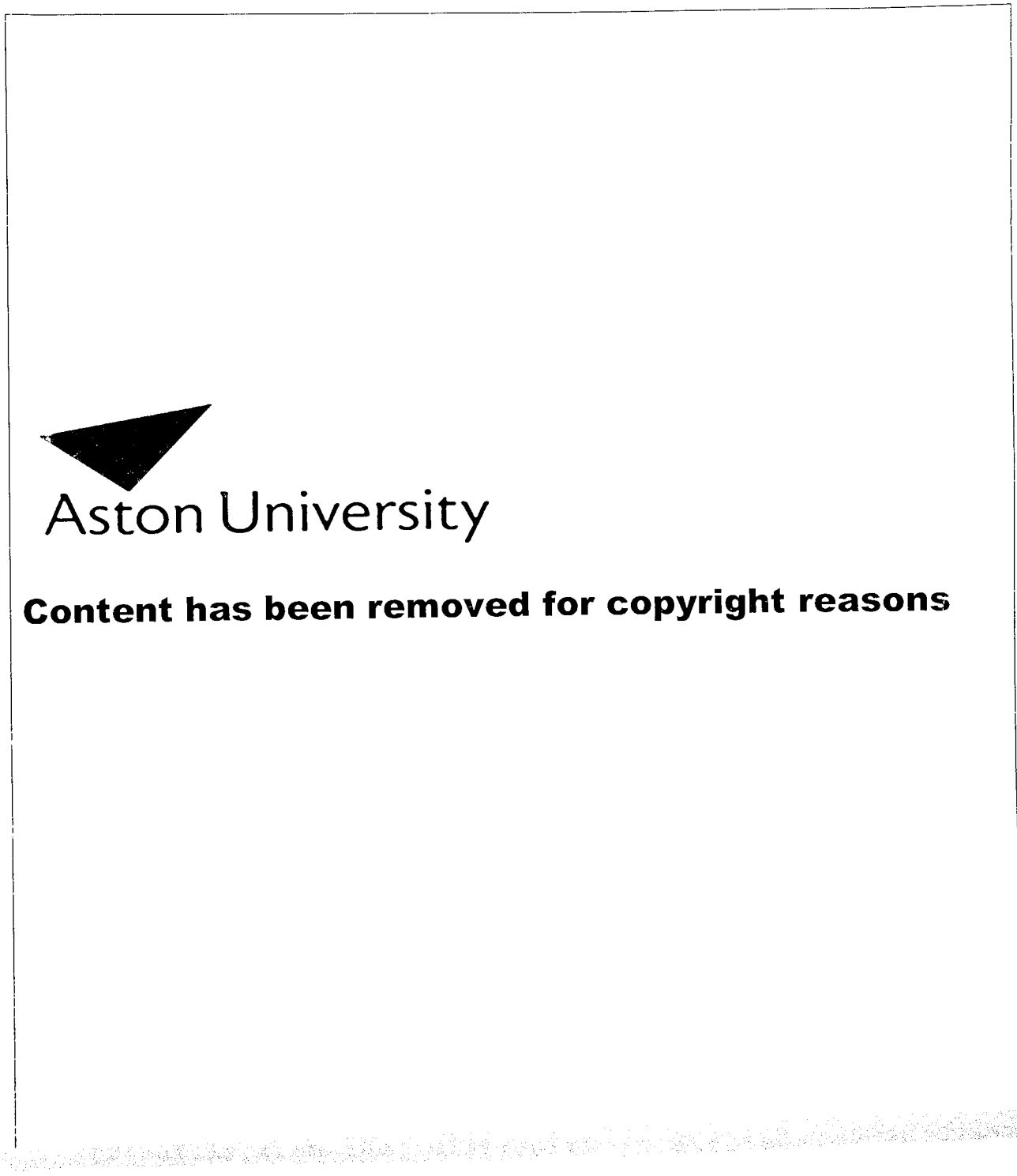
FIGURE 7.3: Serotonin pathways in rat brain shown in saggital (A) and horizontal (B) projection. The numbers refer to defined groups of neurones.
(from Fuxe and Jonsson, 1974).

Table 7.0: Monoamine and Cholinergic Pathways in Behaviour.



(From H. F. Bradford 1986).

7.2 MATERIALS AND METHODS.

Full details of all DAHP experiments performed have been outlined in section 6.2.2 of Chapter 6. Below is a summary of those experiments in which behavioural or neurotransmitter analyses were carried out.

7.2.1 The effect of administration of a 2% or 5% DAHP diet or food restriction on brain total biopterins and behaviour in weaner rats.

(a). Male weaner rats were fed either a standard rat breeder diet or a 2% or 5% DAHP diet for six days during which time various behavioural analyses were carried out, (see below). After sacrifice rat brains were analysed for total biopterin levels as described in Chapter 2.

(b). A subsequent experiment was performed which was designed to act as a control to the DAHP study outlined above. Male weaner rats were subjected to a degree of food restriction over a 6 day period during which time the same behavioural analyses were performed. The degree of enforced dietary restriction was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet. Total biopterins were analysed after sacrifice.

Behavioural analyses to test for catecholaminergic or serotonergic impairment.

Deciding which aspects of behaviour to assess in order to identify impairment in specific neurotransmitter pathways is clearly not straightforward. The problems arise primarily from the difficulty in attributing specific behaviours to the activity of a single neurotransmitter

system.

In the present study the problem was reduced by the fact that we simply wanted to detect any visual impairment of the catecholaminergic or seratonergic systems. Hence, simple behaviours that are easy to study and known to primarily involve at least one or more of the neurotransmitters DA, NA or 5HT were performed. Any changes in behaviour would encompass at least one of these transmitters. The behavioural tests chosen are described below:

(a). The Open-Field Test.

This test is frequently used in behavioural pharmacology being generally sensitive to the effects of toxicants, (Tilson, 1987). Basically, an animal is placed in a large arena and its exploratory behaviour, (ambulation and rearing), grooming and sometimes level of defaecation and/or urination are measured, (Gray, 1987).

Behaviour in the open-field is complex and reflects motor and exploratory activity, sensorimotor functions and fearfulness, (Tilson 1987; Gray 1987). As such it probably involves the activity of a number of neurotransmitter systems.

In this study animals were placed in an arena, (described in Chapter 2, section 2.2.10), and their activity monitored over a 5 minute period. The number of squares entered, (with all four paws), upward rears and the total number of grooming bouts performed by each animal were recorded.

Open field behaviour was assessed two days before the experimental diets were administered, (B1), and on days 1 and 4 after the diet had been given, (T1 and T4 respectively).

(b). Head-Dipping as a Measure of Exploration / Curiosity in a Modified Hole-Board.

This analysis was based on the method of File and Wardill, (1975), who proposed two criteria for considering head-dipping as a measure of exploration: firstly that it should refect novel aspects of the environment; secondly that exposure to the hole-board should result in information storage and hence habituation on re-exposure to the apparatus.

The test basically involves placing an animal into an arena with four holes evenly spaced in the centre of the floor and recording both the number of times it dips its head into any of the four holes and also the total amount of time it spends head-dipping, (see Chapter 2 section 2.2.10 for further details). On subsequent exposures to the hole-board objects were either introduced or removed from beneath the holes in order to present a novel situation to the animals.

Exploration was assessed on the day before the experimental diets were administered, (B2), and on days 2 and 5 after the diet had been given, (T2 and T5 respectively). It was on the third exposure to the hole-board that objects were introduced beneath the holes to present a novel situation to the animals.

File and Wardill (1975) claimed that the hole-board apparatus allows an animals exploration to be assessed independently of its locomotor activity. However for the purpose of the present investigation this distinction was not of paramount importance as catecholaminergic and/or serotonergic systems are likely to be involved to some extent in both aspects of behaviour. The mass of experimental work in this area does not suggest that any one brain neurotransmitter pathway is specifically involved in the control of exploratory behaviour.

7.2.2 The effect of administration of a 5% DAHP diet or food restriction on brain total biopterins and neurotransmitter levels in weaner rats.

This study employed different animals to those used in the behavioural experiments outlined in section 7.2.1. Due to an insufficient amount of time to complete the thesis no single experiment was performed which combined BH_4, neurotransmitter and behavioural analyses.

Male weaner rats were fed a 5% DAHP diet or subjected to food restriction over a 6 day period. Again, the degree of enforced dietary restriction was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet. After sacrifice rat brains were assayed for total biopterin, NA, DA, DOPAC, 5HT and 5HIAA concentrations as described in Chapter 2.

7.2.3 Statistical Analysis.

Analysis of biochemical data, (neurotransmitter and biopterin measurements), was by a students unpaired t-test.

Behavioural data was collated and analysed in three ways each analysis having specific advantages and disadvantages over the others:-

i. A X^2 contingency table test was carried out on the data for total group performance. This detects any change in the difference between control and test animals after treatment compared with initial performances before treatment. Analysis of total group performances is an important way of looking at data but does not account for inherent variation between

individual animals making it less specific than other tests. However in instances where this variation is particularly large, (as in behavioural studies), the X^2 test can reveal effects of a treatment which might otherwise be obscured due to the extent of the variation.

ii. A two-factor analysis of variance split-plot was performed on the data with treatment as the major factor and time as the minor factor. The advantage of this analysis is that it looks at the data as a whole and provides a lot of information revealing major trends in the data and differences in the effects of treatments compared to controls, time trends in each behaviour and whether the effects of time on each behaviour are consistent among the different treatments, (ie. the interaction between treatment and time). Again individual animal variation is taken into account in the analysis compared with the analysis of total group performance.

The disadvantage is that when the inherent inter-animal variation is very significant this type of analysis can obscure any effects of the treatments. Also, this test makes the assumption that the variation is consistent throughout the data ie. that the variation in control and treated animal groups is the same. In pharmacological studies this is quite often not seen to be the case as animals often respond very differently to the same drug bringing a degree of heterogeneity to test groups which is not observed in controls.

iii. A two-way analysis of variance in randomised blocks was performed on the data to detect any time trend in a particular behaviour in each of the different experimental groups. This analysis takes into account

inherent variation between individual animals by treating the animals as randomised blocks. It has an advantage over the 2 factor split-plot analysis in that it does not assume consistent variation throughout the data and looks at each treatment group as a separate entity and not at the data as a whole.

The disadvantage is that because the variation is not assumed to be consistent between the groups the analysis does not make direct statistical comparisons between the different treatments.

7.3 RESULTS.

7.3.1 The effect of a DAHP diet on brain total biopterin levels, open-field behaviour and curiosity in weaner rats.

(i) Total biopterins.

Administration of a 5% DAHP diet to weaner rats caused a significant decrease in brain total biopterins compared to controls, (Table 7.1, $p<1\%$), while a 2% diet produced no such reduction.

(ii) Open-field Behaviour.

X^2 analysis of open-field behaviour revealed a significant difference in activity and rearing between controls and 5% DAHP fed animals after administration of the treated diet compared to initial performances, (Table 7.2, $p<0.1\%$ for activity; $p<2.5\%$ for rearing). All experimental groups showed a decrease in activity and rearing on T1 compared to initial levels on B1. However, while the levels measured on

B1 were regained in control and 2% DAHP rats on T4, they remained low in 5% DAHP animals, (Figure 7.4).

X^2 analysis revealed no significant effect of DAHP on grooming, (Table 7.2), however figure 7.4 shows that the pattern of variation in grooming with time was different in DAHP fed animals and contols. Two-way analysis of variance of the data, (Table 7.3), revealed that in the 5% DAHP group this time-trend was significant, ($p<5\%$).

Two-way ANOVA showed no significant time-trends in other aspects of open-field behaviour in any of the experimental groups, (Table 7.3). However, with the exception of rearing in 5% DAHP rats, the F ratios for blocks were highly significant for each behaviour in all groups indicating marked variation in performance between animals within a particular experimental group.

When the data was analysed by two-factor ANOVA split-plot, there was no evidence of a significant over-all effect of DAHP administration on open-field behaviour, (Table 7.4). The F ratio (time) values indicated that activity, rearing and grooming all showed a significant variation with time, ($p<0.1\%$ activity; $p<1\%$ rearing and grooming), which was consistent among the different treatments (shown by the non-significant F ratios for interaction). This is illustrated in figure 7.4.

(iii) Curiosity.

X^2 analysis showed a significant difference in curiosity, (assessed as the number of head-dips), between control and 5% DAHP animals after treatment compared to the initial performances, (Table 7.5, $p<5\%$). Animals on a 5% DAHP diet showed a slight but progressive increase in curiosity on successive assessments as opposed to an initial marked decline and subsequent rise in curiosity observed in

controls and rats on the 2% diet, (Fig. 7.5).

Only rats given the 2% DAHP diet showed a significant variation in curiosity with time, (Table 7.5, 2-way ANOVA), and this only occured if curiosity was assessed as the number of head-dips not as the amount of time spent head-dipping. The controls showed a similar pattern with time, (Fig. 7.5), but this was not significant probably due to the less severe decline in curiosity at T2 compared to the 2% group.

The F ratio block values, (Table 7.5, 2-way ANOVA), were very significant, once again illustrating the marked variation between the behaviour of individual animals within the three experimental groups. This was particularly evident in the 2% DAHP rats.

Curiosity data analysed by two-factor ANOVA split-plot, (Table 7.6), showed no over-all significant effect of DAHP administration on curiosity once animal variation had been accounted for. A significant variation in curiosity with time was observed, ($p<5\%$), which was consistent among the different treatments, (F ratio (for interaction) = n.s).

7.3.2 Comparison of the effects of 5% DAHP and food restriction on total brain biopterins, open-field behaviour and curiosity in weaner rats.

(i) Total Brain Biopterins.

Total brain biopterin levels in weaner rats were significantly decreased by both a 5% DAHP diet, ($p<1\%$), and enforced food restriction, ($p<5\%$), (Table 7.7). In a later study, the effect was reproducible for DAHP but not for food restriction, (Table 7.13).

(ii) Open-field Behaviour.

X^2 analysis of total group performances revealed that both 5% DAHP and food restriction had significant effects on activity and rearing in the open-field, (Table 7.8). However the effects of the treatments were quite different. While 5% DAHP caused a decrease in activity, ($p<0.1\%$), and rearing, ($p<2.5\%$), food restriction appeared to increase them, ($p<0.1\%$, activity and rearing), resulting in quite different time-trend patterns (Figure 7.6).

Regarding variation in activity and rearing with time, significant effects were only observed for activity in food restricted animals, ($p<1\%$), as shown by two-way ANOVA, (Table 7.9). F ratio block values again showed a significant degree of variation in each behaviour between animals within each group. However, a notable exception to this was in activity in food restricted animals.

Neither treatment significantly affected grooming as assessed by X^2 analysis (Table 7.8). Grooming did show a significant variation with time in 5% DAHP rats, ($p<5\%$), but not food restricted animals (Table 7.9).

Analysis of open-field behaviour by two-factor ANOVA split-plot, (Table 7.10), showed that food restriction had an over-all significant effect on both activity ($p<0.1\%$), and rearing ($p<5\%$), causing both of them to increase compared to controls, (Fig. 7.6). The F ratio treatment values for DAHP animals were non-significant in all behaviours.

Further evidence for an effect of food restriction on activity was provided by the significant F ratios for time, ($p<0.1\%$), and interaction, ($p<0.1\%$), which indicated a significant variation in activity with time in the food restricted group but not the controls. Similarly, the variation in rearing with time was not significant in controls or food restricted animals but the patterns of

variation were different in the two groups, (F ratio (x), $p<1\%$). Open-field behaviour in DAHP animals showed a significant trend with time, (Table 7.10), but the effect was consistent with that seen in the control group.

(iii) Curiosity.

X^2 analysis revealed significant differences in curiosity, (assessed as number of head-dips), between animals fed DAHP or subjected to food restriction and their respective control groups after treatment compared to the initial performances on B2, (Table 7.11).

As mentioned earlier, with DAHP the effect was due to a slight but progressive increase in curiosity after administration of DAHP, and an initial marked decline in curiosity in the controls followed by a subsequent increase, (see fig. 7.8). In the case of food restriction the difference was attributable to a large decline in curiosity in controls on days T2 and T5 compared to B2 and very little change in the food restricted group, levels remaining quite high. The effects of food restriction and DAHP on curiosity were therefore similar.

Two-way ANOVA revealed no significant effect of time on curiosity in DAHP rats or their controls. Food restricted rats also showed no time trend in curiosity but, as expected, their control group did, ($p<0.1\%$). F ratio block values were again very significant with the exception of those for food restricted rats which were very low indicating little variation in curiosity between the animals in this group.

A two-factor ANOVA split-plot, (Table 7.12), showed no significant over-all effect of either DAHP or food restriction on curiosity; a significant time-trend in the level of curiosity in the DAHP experiment, ($p<5\%$), that was

consistent between DAHP rats and controls; and significant time-trends in curiosity in both food restricted rats and their controls which were not the same. Curiosity decreased dramatically in controls on day 2 in food restricted animals it remained high.

7.3.3 The effect of a 5% DAHP diet and food restriction on brain total biopterins, NA, DA, 5HT, 5HIAA and DOPAC levels in weaner rats.

A 5% DAHP diet produced a significant decline in brain total biopterins, (Table 7.13, $p<1\%$), while food restriction did not decrease this parameter compared with previous experiments.

Neither food restriction or DAHP had a significant effect on brain levels of NA, DA, 5HT, 5HIAA or DOPAC, (Tables 7.14 and 7.15).

7.4 DISCUSSION.

Great care needs to be taken in the interpretation of the results of animal behaviour experiments.

7.4.1 Limitations of sample size in behavioural analysis.

In the present investigation clearly the size of the experimental groups may have been too small to counteract for the large contribution that individual animal variation made to the error term in statistical analyses.

It was therefore not surprising that X^2 analysis, which compares total group performances and does not take account of animal variation within a group, revealed significant effects of DAHP and food restriction on behaviours which were not, in most cases, supported by a significant analysis of variance result.

7.4.2 Effect of a 5% DAHP diet and food restriction on open field behaviour.

There was evidence from X^2 analysis of open-field behaviour that after treatment with a 5% DAHP diet, rats not only had decreased total brain biopterins but also displayed lowered levels of activity and rearing compared to controls.

As these animals also underwent a degree of volountary food deprivation, as a result of the treatment, a second study in which animals were subjected to an equivalent amount of enforced food restriction was carried out. Food restricted, (F.R), rats also had significantly lowered brain biopterins but demonstrated elevated levels of activity and rearing compared to controls. This was shown by both X^2 analysis and two-factor analysis of variance split-plot.

The increased activity observed in F.R rats, not receiving DAHP, may have been due to the partial induction of the "5HT Hyperactivity Syndrome" which can result from increased synaptic 5HT availability.

Starvation is known to enhance tryptophan entry into the brain and can thereby increase the activity of central 5HT systems. It may be that non-absolute food deprivation has the same effect and was the cause of the increased activity seen in F.R animals. This is plausible since an animal increasing its activity also improves its chances of finding food. However, as no other characteristics of the

syndrome were noted and neurochemical analysis of whole brains in food restricted rats showed no elevation of 5HT or 5HIAA levels, the evidence in support of this hypothesis is not strong.

As both DAHP administration and food restriction caused a degree of food deprivation, the opposing effects of the two treatments on open-field behaviour may indicate an inhibitory effect of DAHP on the hyperactivity associated with lowered food intake. This may be connected with its property as an inhibitor of BH_4 synthesis.

7.4.3 Effect of a 5% DAHP diet and food restriction on exploratory behaviour/curiosity.

File and Wardill, (1975), anticipated that if the hole board apparatus was a valid test of exploratory behaviour, then enhanced curiosity should follow the presentation of novel stimuli beneath the holes, while habituation should follow re-exposure to unchanged conditions.

DAHP controls did show habituation on the second exposure to the hole board when the environment was unchanged, and increased curiosity on the third exposure when objects were placed beneath the holes. However this was not so in the case of the F.R controls which, after showing habituation on the second exposure, proceeded to demonstrate even less curiosity on the third exposure. There was no apparent reason for the discrepancy in these results, but it may question the validity of the apparatus for use in weaner rats.

Animals fed a 2% DAHP diet showed similar trends in curiosity to their controls but the 5% DAHP group revealed a significant difference, (assessed by X^2 analysis), in that on the second exposure they failed to show habituation

and maintained the initial levels of curiosity observed on B2. F.R animals also showed a persistently high degree of head-dipping, (unlike their controls), though on subsequent exposures the dips were shorter as indicated by a progressive decline in the mean time spent head-dipping. The results may have been caused by increased activity in the F.R rats, but it seems reasonable that the enhanced curiosity in both DAHP and F.R groups may have been caused by hunger.

Many of the behaviours studied showed significant variation with time. This was expected as even if assessments are made at the same time on each day there are many uncontrolled factors, besides administration of a drug, that may influence the way in which an animal may respond to a given situation on a particular day.

However, if behavioural measurements are performed both before and after the administration of a test compound, then a significant variation in the behaviour with time may be indicative of an effect of the treatment if the observed time-trends are different in control and test animals. This was observed in two-factor ANOVA split-plot analysis of F.R results for curiosity and activity providing further evidence for a significant effect of F.R on these behaviours. However, care should be taken in interpreting these results as one can only indirectly infer a difference between treatments with this type of analysis.

7.4.4 The effect of a 5% DAHP diet and food restriction on brain catecholamine and serotonin levels.

Whole brain neurotransmitter analysis in DAHP fed and food restricted rats revealed no significant alterations in catecholamine, serotonin or metabolite levels even when DAHP rats showed a 35% decrease in total brain biopterins.

Gal and Whitacre, (1981), by direct intracerebral injection of DAHP causing a 60% drop in the reduced biopterin pool also showed no significant change in the synthesis or level of 5HT in whole brain. Similarly, Sherman and Gal, (1978), found no decrease in cerebral catecholamine levels after lowering the cofactor pool by 50%.

The data probably highlights the limitations of whole brain neurochemical analyses which may mask alterations in neurotransmitter levels occurring in specific cell populations. Levels of NA and DA have been significantly reduced in the brain stem by acute i.p administration of DAHP to weaner rats who showed a 66% decrease in whole brain total biopterins, (Suzuki *et al*, 1988). These workers found no effect on 5HT or 5HIAA contents in any brain region.

There are obvious limitations associated with performing biochemical and neurotransmitter analyses on *post mortem* brain samples and trying to correlate the results with behaviour in the living animal. Use of *in vivo* voltametry to measure actual neurotransmitter release in the living animal, or neurochemical analysis of blood and/or CSF levels while the animal is still alive are possible solutions to this problem but were beyond the scope of the work undertaken here.

7.5 CONCLUSIONS.

Animals fed a 5% DAHP diet always produced a decline in brain total biopterins and showed consistent decreases in activity and rearing in the open-field. Rats subjected to food restriction also on occasion exhibited reduced biopterin levels but activity and rearing in these animals was enhanced. These observations may indicate different mechanisms of action of the two treatments on neurochemical pathways in the brain.

There was no evidence from this investigation that significant reductions in brain total biopterins by either DAHP administration or food restriction were accompanied by decreased steady-state levels of gross brain catecholamines or serotonin.

Table 7.1: Effect of dietary administration of 2% or 5% DAHP on brain total biopterin levels in weaner rats, (ng g^{-1} wet wgt).

Group	n	Mean ± st.d	p value
Controls	8	147.3 ± 23.0	
2% DAHP	8	136.4 ± 12.1	n.s
5% DAHP	8	118.2 ± 11.3	p<1%

Table 7.2: The effect of a 2% or 5% DAHP diet on open field behaviour in weaner rats, assessed by X^2 analysis.

GROUP (n)		BEHAVIOUR		
		Activity	Rearing	Grooming
2% DAHP (n=8)	X^2 value p value effect	0.595 (n.s) -	3.742 (n.s) -	3.638 (n.s) -
5% DAHP (n=8)	X^2 value p value effect	41.25 (p<0.1%) decrease	7.54 (p<2.5%) decrease	5.01 (n.s) -

TABLES 7.1-7.2: Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days, controls were allowed normal food *ad lib.* Total biopterins were analysed by a students unpaired t-test. Open-field behaviour was assessed two days before the diet was administered, (B1), and on days 1 and 4 after the diet had been given, (T1 and T4 respectively). Data was collated and then analysed by a X^2 contingency table test for total group performance.

Table 7.3: The effect of a 2% or 5% DAHP diet on open field behaviour in weaner rats, assessed by two-way analysis of variance.

GROUP (n)		BEHAVIOUR		
		Activity	Rearing	Grooming
Control (n=8)	F ratio (t)	1.168	0.485	0.301
	p value	(n.s)	(n.s)	(n.s)
	F ratio (b)	11.70	6.11	4.40
	p value	p<0.1%	p<1%	p<1%
2% DAHP (n=8)	F ratio (t)	0.943	1.039	1.441
	p value	(n.s)	(n.s)	(n.s)
	F ratio (b)	13.90	14.39	6.15
	p value	p<0.1%	p<0.1%	p<1%
5% DAHP (n=8)	F ratio (t)	3.202	1.347	3.888
	p value	(n.s)	(n.s)	(p<5%)
	F ratio (b)	6.98	3.05	17.21
	p value	p<1%	(n.s)	p<0.1%

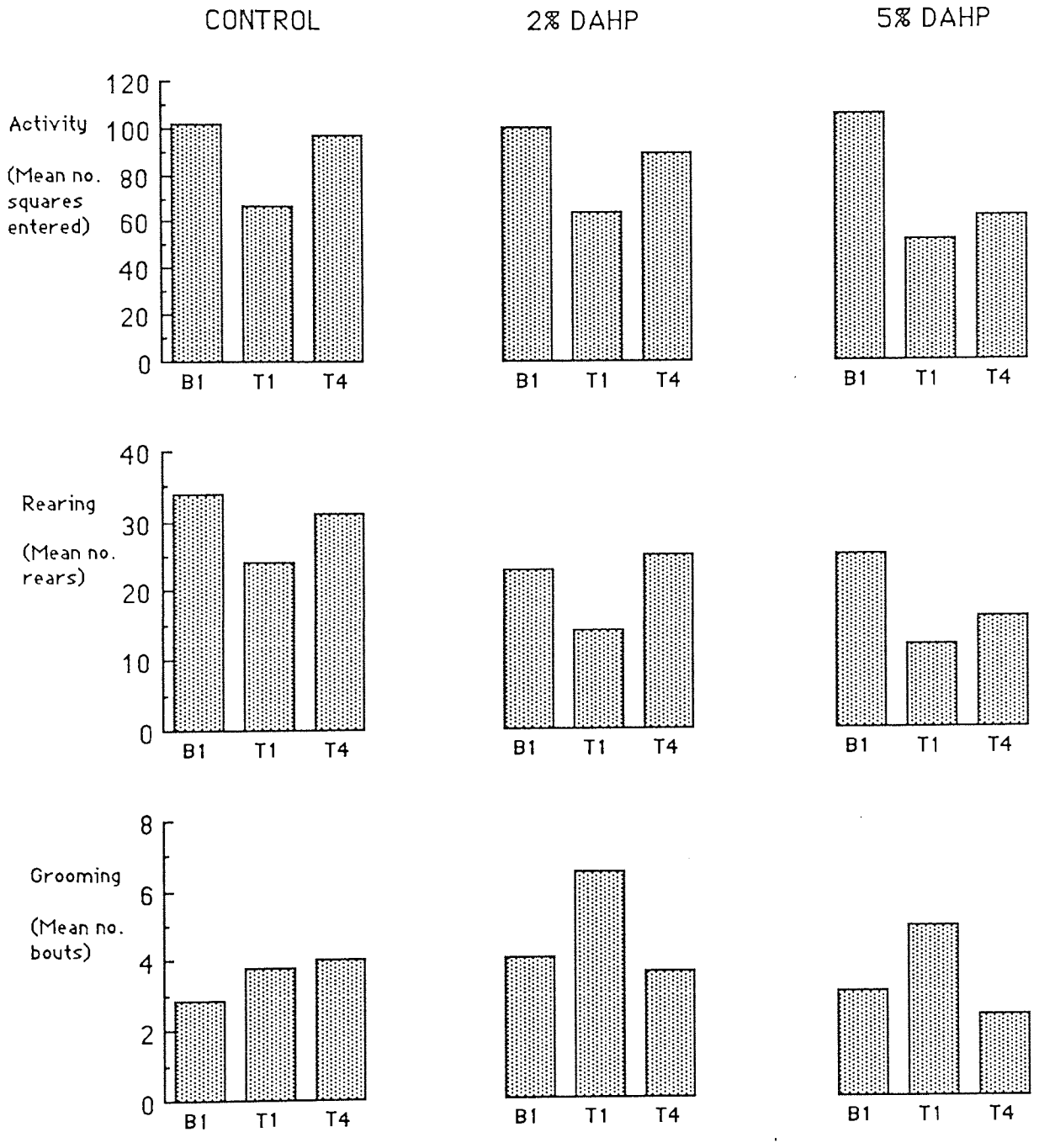
TABLE 7.3: Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days, controls were allowed normal food *ad lib.* Open field behaviour was assessed two days before the diet was administered, (B1), and on days 1 and 4 after the diet had been given, (T1 and T4 respectively). A two-way analysis of variance was carried out on the data. F ratio (t) indicates the effect of time; F ratio (b) indicates the degree of variation between the animals.

Table 7.4: The effect of a DAHP diet on open field behaviour in weaner rats, assessed by two-factor analysis of variance split-plot.

F ratio	BEHAVIOUR		
	Activity	Rearing	Grooming
F ratio (trt)	0.675 (n.s)	2.773 (n.s)	1.711 (n.s)
F ratio (time)	14.711 (p<0.1%)	6.046 (p<1%)	6.385 (p<1%)
F ratio (x)	1.218 (n.s)	0.257 (n.s)	1.800 (n.s)

TABLE 7.4: Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days, controls were allowed normal food *ad lib*. Open field behaviour was assessed two days before the diet was administered, (B1), and on days 1 and 4 after the diet had been given, (T1 and T4 respectively). A two-factor analysis of variance split-plot was carried out, using all of the data, to test for an over-all treatment effect, (F ratio trt), time trend, (F ratio time), or any interaction between the two, (F ratio x). The level of significance attained is indicated in parenthesis.

FIG 7.4: The effect of a 2% and 5% DAHP diet on open-field behaviour in weaner rats.



Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days. Controls were allowed normal food and water. Open field behaviour was assessed two days before the diet was administered, (B1) and on days 1 and 4 after the diet had been given, (T1 and T4, respectively).

Table 7.5: The effect of a 2% or 5% DAHP diet on curiosity in weaner rats, assessed by X^2 and two-way analysis of variance.

CURIOSITY	STATISTICAL TEST	GROUP		
		Control	2% DAHP	5% DAHP
NUMBER OF HEAD DIPS	X^2			
	X^2 value	N.A	0.933	6.34
	p value		n.s	p<5%
	2-way anova			
	F ratio (t)	0.456	5.404	0.075
	p value	n.s	p<5%	n.s
	F ratio (b)	12.86	29.91	6.58
	p value	p<0.1%	p<0.1%	p<1%
TIME SPENT HEAD DIPPING	X^2			
	X^2 value	N.A	N.A	N.A
	p value			
	2-way anova			
	F ratio (t)	0.327	1.569	1.181
	p value	n.s	n.s	n.s
	F ratio (b)	7.37	13.24	7.07
	p value	p<1%	p<0.1%	p<1%

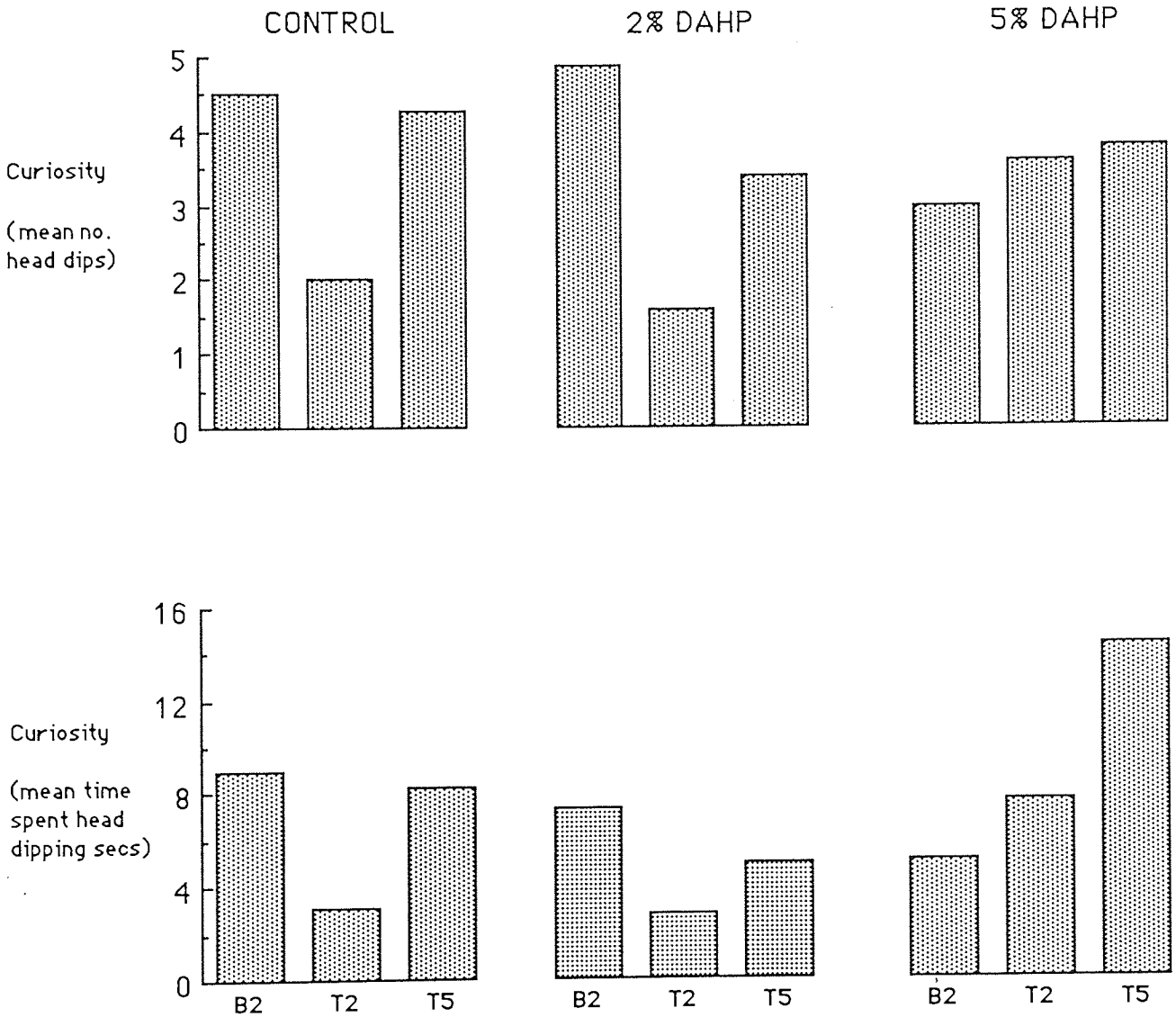
TABLE 7.5: Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days, controls were allowed normal food *ad lib*. Curiosity was assessed on the day before the diet was administered, (B2), and on days 2 and 5 after the diet had been given, (T2 and T5 respectively). The data were analysed by a X^2 contingency table test for total group performance and by a two-way analysis of variance. F ratio (t) indicates the effect of time; F ratio (b) indicates the degree of variation between the animals.

Table 7.6: The effect of a DAHP diet on curiosity in weaner rats, assessed by two-factor analysis of variance split-plot.

F ratio	CURIOSITY	
	No. head dips	Time spent head dipping
F ratio (trt)	2.869 (n.s)	1.063 n.s
F ratio (time)	3.801 (p<5%)	3.230 (p<5%)
F ratio (x)	1.733 (n.s)	2.098 (n.s)

TABLE 7.6: Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days, controls were allowed normal food *ad lib*. Curiosity was assessed on the day before the diet was administered, (B2), and on days 2 and 5 after the diet had been given, (T2 and T5 respectively). A two-factor analysis of variance split-plot was carried out, using all of the data, to test for an over-all treatment effect, (F ratio trt), time trend, (F ratio time), or any interaction between the two, (F ratio x). The level of significance attained is indicated in parenthesis.

FIG 7.5: The effect of a 2% and 5% DAHP diet on curiosity in weaner rats.



Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days. Controls were allowed normal food ad lib. Curiosity was assessed on the day before the diet was administered, (B2) and on days 2 and 5 after the diet had been given, (T2 and T5, respectively).

Table 7.7: Comparison of the effects of a 5% DAHP diet and food restriction on brain total biopterin levels in weaner rats, (ng g^{-1} wet wgt).

EXPT	Group	n	Mean ± st.d	p value
A	Controls	8	147.3 ± 23.0	
	5% DAHP	8	118.2 ± 11.3	p<1%
B	Controls	8	108.6 ± 10.8	
	F.R	6	92.8 ± 11.68	p<5%

Table 7.8: Comparison of the effects of a 5% DAHP diet and food restriction on open field behaviour in weaner rats, assessed by X^2 analysis.

GROUP (n)		BEHAVIOUR		
		Activity	Rearing	Grooming
5% DAHP (n=8)	X^2 value	41.25	7.54	5.01
	p value	(p<0.1%)	(p<2.5%)	(n.s)
	effect	decrease	decrease	-
F.R (n=7)	X^2 value	315.8	37.8	3.43
	p value	(p<0.1%)	(p<0.1%)	(n.s)
	effect	increase	increase	-

TABLES 7.7-7.8: Male weaner rats were fed a 5% DAHP diet, (Expt. A), or subjected to food restriction, (F.R), (Expt. B), for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A. Total biopterins were analysed by an unpaired students t-test. Open-field behaviour was assessed two days before any dietary manipulation (B1), and then on days 1 and 4 after the diets had been changed, (T1 and T4 respectively). A X^2 contingency table test was carried out for total group performance.

Table 7.9: Comparison of the effects of a 5% DAHP diet and food restriction on open field behaviour assessed by two-way analysis of variance.

EXPT	GROUP (n)		BEHAVIOUR		
			Activity	Rearing	Grooming
A	Control (n=8)	F ratio (t)	1.168	0.485	0.301
		p value	(n.s)	(n.s)	(n.s)
		F ratio (b)	11.70	6.11	4.40
		p value	p<0.1%	p<1%	p<1%
	5% DAHP (n=8)	F ratio (t)	3.202	1.347	3.888
		p value	(n.s)	(n.s)	(p<5%)
		F ratio (b)	6.98	3.05	17.21
		p value	p<1%	(n.s)	p<0.1%
B	Control (n=8)	F ratio (t)	0.216	0.503	0.091
		p value	(n.s)	(n.s)	(n.s)
		F ratio (b)	5.24	9.17	1.73
		p value	p<1%	p<0.1%	(n.s)
	F.R (n=8)	F ratio (t)	6.909	2.045	1.500
		p value	(p<1%)	(n.s)	(n.s)
		F ratio (b)	2.53	3.70	6.65
		p value	(n.s)	p<5%	p<1%

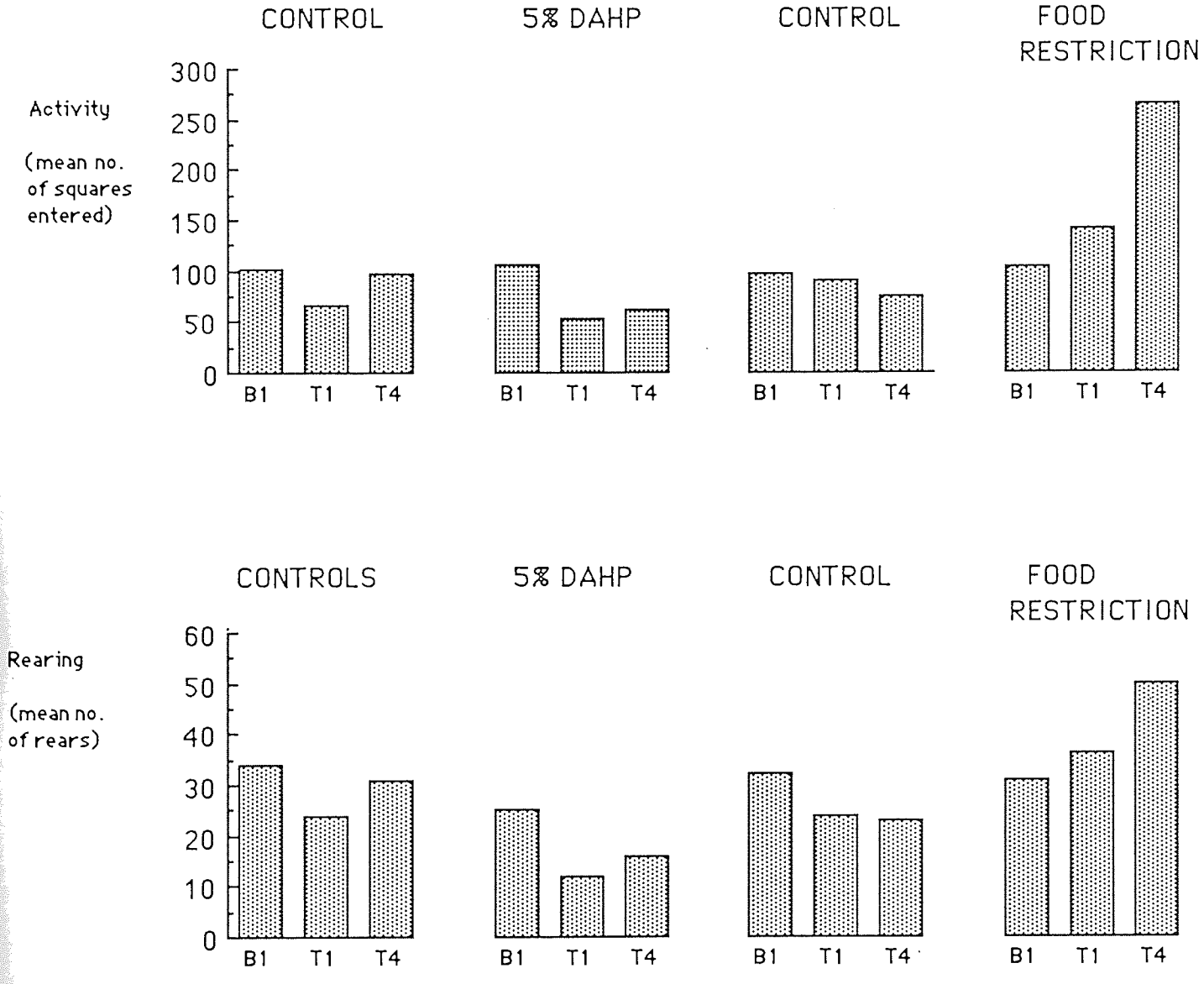
TABLE 7.9: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (Expt B), for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A. Open field behaviour was assessed two days before any dietary manipulation (B1), and then on days 1 and 4 after the diets had been changed, (T1 and T4 respectively). A two-way analysis of variance was carried out on the data.

Table 7.10: The effect of a DAHP diet and food restriction on open field behaviour in weaner rats, assessed by two factor analysis of variance split-plot.

EXPT.	F RATIO	BEHAVIOUR		
		Activity	Rearing	Grooming
DAHP	F ratio (trt)	0.675 (n.s)	2.773 (n.s)	1.711 (n.s)
	F ratio (time)	14.711 (p<0.1%)	6.046 (p<1%)	6.385 (p<1%)
	F ratio (x)	1.218 (n.s)	0.257 (n.s)	1.800 (n.s)
FOOD RESTRICTION	F ratio (trt)	35.41 (p<0.1%)	6.493 (p<5%)	-
	F ratio (time)	9.643 (p<0.1%)	1.073 (n.s)	-
	F ratio (x)	16.42 (p<0.1%)	6.036 (p<1%)	-

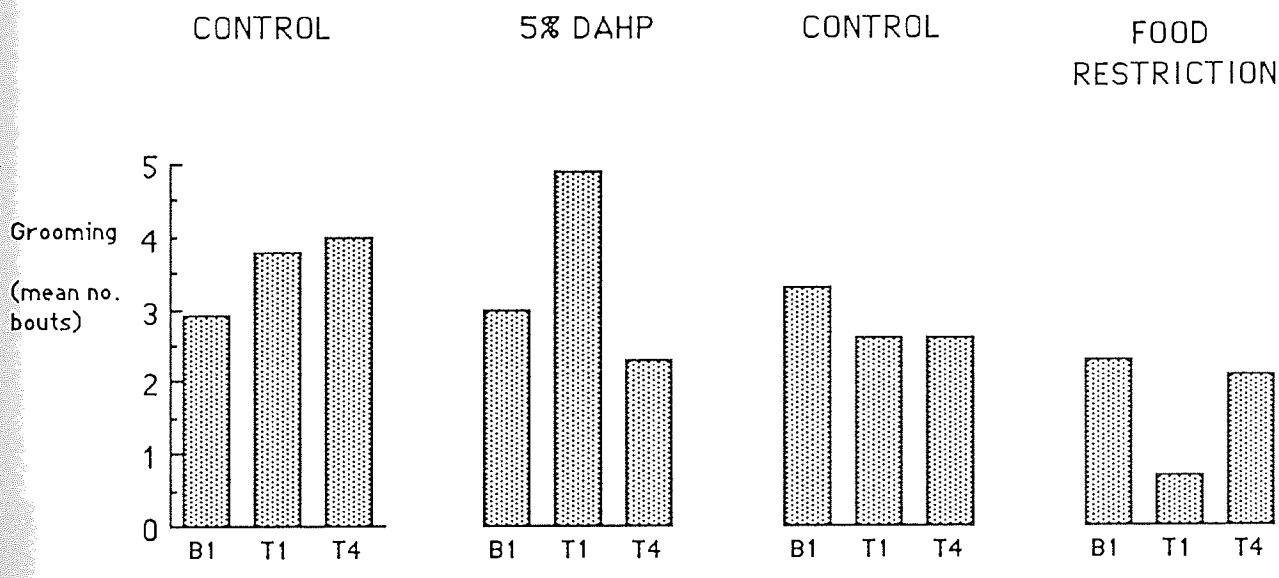
TABLE 7.10: Male weaner rats were fed either a 2% or 5% DAHP diet or subjected to food restriction, for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet. Open field behaviour was assessed two days before any dietary manipulation (B1), and then on days 1 and 4 after the diets had been changed, (T1 and T4 respectively). A two-factor analysis of variance split-plot was carried out.

FIG 7.6: The effect of a 5% DAHP and food restriction on open-field behaviou in weaner rats.



Male weaner rats were fed a 5% DAHP diet or subjected to food restriction for 6 days, controls were allowed normal food ad lib. Open field behaviour was assessed two days before the diet was administered, (B1), and on days 1 and 4 after the diet had been given, (T1 and T4, respectively).

FIG 7.7: The effect of a 5% DAHP diet and food restriction on open-field behaviour in weaner rats.



Male weaner rats were fed a 5% DAHP diet or subjected to food restriction for 6 days, controls were allowed normal food *ad lib*. Open field behaviour was assessed two days before the diet was administered, (B1) and on days 1 and 4 after the diet had been given, (T1 and T4, respectively).

Table 7.11: Comparison of the effect of a 5% DAHP diet and food restriction on curiosity in weaner rats, assessed by X^2 and two-way analysis of variance.

CURIOSITY	STATISTICAL TEST	EXPT. 1.		EXPT. 2	
		Control	5% DAHP	Control	F.R.
NUMBER OF HEAD DIPS	X^2				
	X^2 value	N.A	6.34	N.A	30.38
	p value		(p<5%)		(p<0.1%)
	2-way anova				
	F ratio (t)	0.456	0.075	8.540	0.075
	p value	(n.s)	(n.s)	(p<1%)	(n.s)
	F ratio (b)	12.87	6.58	19.65	0.883
	p value	p<0.1%	p<1%	p<0.1%	(n.s)
TIME SPENT HEAD DIPPING	X^2				
	X^2 value	N.A	N.A	N.A	N.A
	p value				
	2-way anova				
	F ratio (t)	0.327	1.181	4.920	0.904
	p value	(n.s)	(n.s)	(p<5%)	(n.s)
	F ratio (b)	7.37	7.07	8.93	1.16
	p value	p<1%	p<1%	p<1%	(n.s)

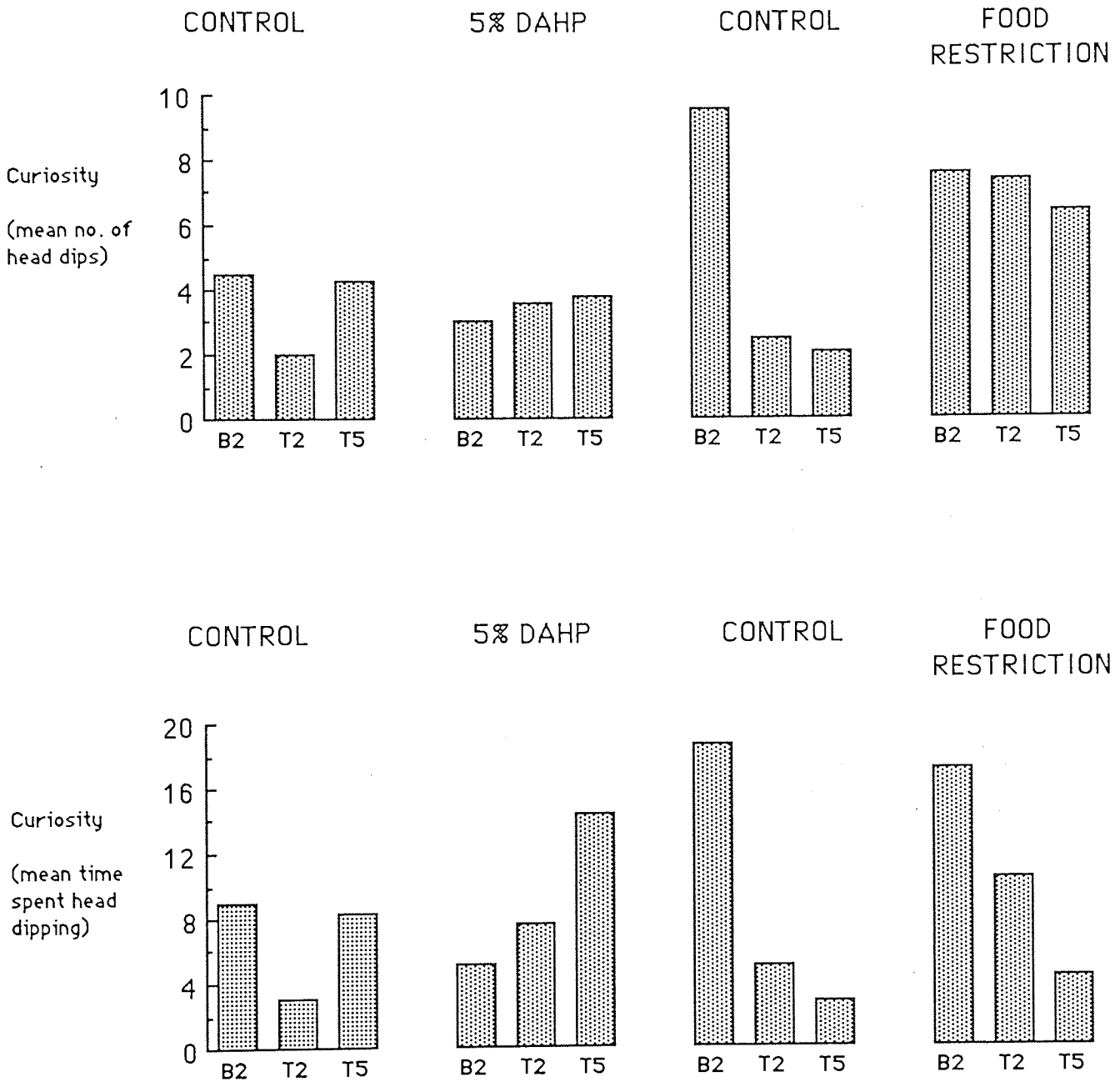
TABLE 7.11: Male weaner rats were fed a 5% DAHP diet, (Expt 1), or subjected to food restriction, (F.R), (Expt 2), for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt 2 was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt 1. Curiosity was assessed on the day before the diets were administered, (B2), and on days 2 and 5 after the diets had been given, (T2 and T5 respectively). A X^2 analysis and two-way analysis of variance was carried out on the data.

Table 7.12: Comparison of the effect of a 5% DAHP diet and food restriction on curiosity in weaner rats, assessed by two-factor analysis of variance split-plot.

CURIOSITY	F RATIO	DAHP EXPT.	F.R. EXPT.
NUMBER OF HEAD DIPS	F ratio (trt)	2.869	2.754
	p value	(n.s)	(n.s)
	F ratio (time)	3.801	6.392
	p value	(p<5%)	(p<1%)
	F ratio (x)	1.733	6.123
	p value	(n.s)	(p<1%)
TIME SPENT HEAD DIPPING	F ratio (trt)	1.063	-
	p value	(n.s)	
	F ratio (time)	3.230	-
	p value	(p<5%)	
	F ratio (x)	2.098	-
	p value	(n.s)	

TABLE 7.12: Male weaner rats were fed either a 2% or 5% DAHP diet or subjected to food restriction, (F.R.), for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet. The level of curiosity shown by the animals was assessed on the day before any dietary manipulation (B2), and then on days 2 and 5 after the diets had been changed, (T2 and T5 respectively). A two-factor analysis of variance split-plot was carried out. The level of significance attained is indicated in parenthesis.

FIG 7.8: The effect of a 5% DAHP diet and food restriction on curiosity in weaner rats.



Male weaner rats were fed a 5% DAHP diet or subjected to food restriction for 6 days, controls were allowed normal food *ad lib*. Open field behaviour was assessed on the day before the diet was administered, (B2) and on days 2 and 5 after the diet had been given, (T2 and T5, respectively).

Table 7.13: Comparison of the effects of a 5% DAHP diet and food restriction on brain total biopterin levels in weaner rats, (ng g^{-1} wet wgt).

Expt	Group	n	Mean ± st.d	p value	% of control
A	Control	5	76.5 ± 8.7		
	DAHP	5	49.7 ± 11.5	p<1%	65.0%
B	Control	6	112.8 ± 11.5		
	F.R	5	112.3 ± 15.2	n.s	99.6%

Table 7.14: The effect of a 5% DAHP diet and food restriction on brain neurotransmitter levels in weaner rats, (ng g^{-1} wet weight).

EXPT	GROUP	n	NEUROTRANSMITTER LEVEL		
			Noradrenaline	Dopamine	Serotonin
A	Control	6	2119 ± 262	355 ± 31	302 ± 52
	5% DAHP	6	1946 ± 464 (n.s)	289 ± 48 (n.s)	326 ± 61 (n.s)
B	Control	5	311 ± 25	689 ± 91	451 ± 62
	F.R.	5	288 ± 36 (n.s)	676 ± 32 (n.s)	482 ± 60 (n.s)

TABLES 7.13-7.14: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A. Results were analysed by an unpaired students t-test.

Table 7.15: The effect of a 5% DAHP diet and food restriction on brain neurotransmitter metabolite levels and metabolite : neurotransmitter ratios in weaner rats, (ng g^{-1} wet weight).

EXPT	GROUP	n	METABOLITE OR RATIO		
			5-HIAA	DOPAC	5-HIAA : 5-HT
A	Control	6	136 ± 31	-	0.450 ± 0.085
	5% DAHP	6	138 ± 34 (n.s)	-	0.433 ± 0.127 (n.s)
B	Control	5	246 ± 41	79 ± 8	0.545 ± 0.041
	F.R.	5	260 ± 43 (n.s)	84 ± 12 (n.s)	0.547 ± 0.117 (n.s)

TABLE 7.17: Male weaner rats were fed a 5% DAHP diet, (Expt A), or subjected to food restriction, (F.R), (expt B), for six days. In each experiment controls were allowed access to normal food *ad lib.* The degree of enforced dietary restriction in expt B was equivalent to the amount of self-imposed restriction observed by animals on the 5% DAHP diet in expt A. Results were analysed by an unpaired students t-test.

CHAPTER 8.

THE EFFECT OF AGING AND LONG-TERM ADMINISTRATION OF A HIGH ALUMINIUM DIET ON BRAIN TOTAL BIOPTERINS AND NEUROTRANSMITTER LEVELS IN THE MOUSE. A MODEL FOR ALZHEIMER'S DISEASE?

8.1 INTRODUCTION

Though mild neurological degradation is part of the normal aging process, at least 5% of people over 65 suffer from an age related disorder causing severly impaired cognitive and intellectual function called dementia. Sixty percent of these are believed to have Alzheimers Disease (AD), (Roth, 1980; Gottfries, 1985).

In the early stages it is hard to differentiate between AD and normal aging as both are often characterised by memory deficits. However, the neuropathological and neurochemical changes occuring in AD are far more severe than in the normal aging brain and as AD progresses victims can become mentally and physically totally incapacitated, (Reisberg *et al*, 1982).

Diagnosis of AD is usually confirmed at *post mortem* by the presence of numerous senile plaques and tangles in the neocortex, hippocampus and certain subcortical neurones, (Alzheimer, 1907). The aetiology of the disease is unknown but toxic substances including aluminium, (Al), have been implicated.

Aluminium as a neurotoxin.

The neurotoxic effects of Al have been demonstrated by experimentally induced encephalopathy in animals, (Crapper

and De Boni, 1980), and in dialysis dementia syndrome in man, (Sideman and Manor, 1982; Arieff *et al* 1979). Both are associated with an accumulation of Al in body tissues, including the brain, (Arieff *et al*, 1979; McDermott and Smith, 1978), and have been shown to cause neurological degeneration, (Brun and Dictor, 1981; Wisniewski *et al*, 1982). The neuronal damage observed is similar but not identical to that found in AD. Animals treated with Al have shown impaired learning, memory and motor responses, (Crapper and Dalton, 1973; Commissaris *et al*, 1982).

Aluminium and Alzheimer's Disease.

Direct evidence that Al is the causative agent in AD is lacking, (Perl, 1983; Crapper *et al*, 1980), but since a feature of Al neuroencephalopathy is neurofibrillary degeneration, (Wisniewski *et al*, 1982), a link between Al and AD has been proposed, (Kolata, 1981; Crapper and De Boni, 1978).

There is considerable evidence for the presence of increased levels of Al in brains of AD patients and furthermore that the Al is localised only in those neurones that have undergone neurofibrillary degeneration, (Crapper *et al*, 1976; McDermott *et al*, 1979; Candy *et al*, 1986). Whether Al accumulation represents non-specific absorption by the degenerating system with no pathological consequences, or is a causative agent in neurofibrillary degeneration is unclear.

Mann, (1983), proposed that the degeneration occuring in the locus coeruleus with age would lead to inadequate control of brain homeostasis such that toxic substances such as Al could gain entry to and accumulate within brain tissue. The accumulation may then precipitate or predispose the individual to AD upon further metabolic insult. There are no acute neurological effects after ingestion of Al,

(Bjorksten, 1982), however a lifetime exposure to the element may lead, by way of progressive alterations, to neuronal damage.

Whatever the role of Al in AD, it is associated with the pathology of the syndrome. Whether or not it is a cause or an effect is very much open to debate.

Most experiments looking at the neurotoxicity of Al have been conducted in young animals and often the fact that AD is age-related has been neglected in the search for a model of the neuropathology of the disease. Neurones undergo age-related changes and it is likely that their response to Al would also be altered with age.

As part of his postgraduate research Mr. D Myers, (Aston University), carried out an experiment to examine the effects of normal aging and long-term administration, (over 2 years), of a high aluminium, low calcium/magnesium or high lecithin diet on behaviour and neuropathology in the mouse.

Brain tissue from some of these animals was made available for biochemical analysis. Neurochemical and total biopterin measurements were carried out to try and resolve the effects of aging and long-term Al administration on neurotransmitter metabolism and look for a possible role of BH_4 in Al neurotoxicity.

8.2 MATERIALS AND METHODS.

The experimental design is briefly described below. Full details can be found in Myers Ph.D Thesis (1989).

120 black inbred female mice (strain C57BL/6NCr/Br) were taken at 3 months of age and divided at random into three

groups of 40 animals. All groups were housed in cages in a 12:12 hour light:dark cycle at 23 C and had access to food and deionised water *ad libitum*.

Towards the end of the longitudinal study an additional 40 untreated young animals (3 months old) were taken to be compared to aged controls.

The various groups received the following diets;

Group 1 (aged controls): standard mouse breeder diet.

Group 2 (aluminium treated): an aluminium enriched, low calcium and magnesium diet, (prepared by Pilsbury's Birmingham). The aluminium was in the form of bentonite and aluminosilicate clay used naturally in the production of pelleted rodent diets. The diet contained 900% more Al than the control diet (1035mg / kg food), 64% less calcium and 50% less magnesium.

Group 3 (lecithin treated): a lecithin enriched diet, (prepared by Pilsbury's Birmingham).

All diets were in solid pellet forms and were visually identical.

Animals were maintained under these conditions for 23 months. During this period various behavioural tests were performed on animals from each of the experimental groups. Some of analyses carried out are listed in Table 8.9 of the results section.

After 23 months all animals were sacrificed. Brains were removed and while most were used for histological examination (see Myers, 1989), six from each of the aged, lecithin and young groups and 5 from the aluminium group were randomly selected for biochemical analysis.

Whole half brains were analysed for; total biopterin, derived pterin, NA, DA, 5-HT and 5-HIAA levels and choline acetyltransferase, (CAT), activity as described in Chapter 2.

Biochemical results were analysed by one-way analysis of variance and students unpaired t-test. Comparisons were made between appropriate control and test groups ie;

Young and aged animals, Y vs. A.
Aged and aluminium treated animals, A vs. Al.
Aged and lecithin treated animals, A vs. L.
Lecithin treated and aluminium treated animals, L vs. Al.

A general summary of the results of the behavioural tests is shown in Table 8.9.

8.3 RESULTS

Tables 8.1 and 8.2 show the mean levels of whole half brain total biopterin and derived pterin levels in young, aged, aluminium fed and lecithin treated mice. There was no significant difference in the levels of either parameter between the four experimental groups as indicated by one-way analysis of variance and t-tests.

One-way ANOVA of half brain NA levels, (Table 8.3), gave an F ratio of 3.0504 which was not quite significant at the 5% level, (F_{tables} = 3.10), indicating some variation in NA levels between the different groups. Young and aged mice had similar brain concentrations of NA, while levels in the Al and lecithin groups were lower but not significantly so.

Brain dopamine levels, (Table 8.4), were highest in young mice and lowest in aged controls, however statistical analysis showed no significant difference between DA levels in any of the four groups.

The results of serotonin analysis are shown in tables 8.5-8.7. The various groups showed no statistically significant differences in whole half brain 5-HT levels, (Table 8.5), though there was a non-significant decrease in Al and lecithin treated mice compared to the young group. The serotonin metabolite 5-HIAA also showed no over-all difference between the groups, (Table 8.6). Levels in the Al treated mice were raised compared to young and aged animals but the differences were not statistically significant.

Analysis of variance indicated some variation in the ratio of 5-HIAA to 5-HT between the different experimental groups which again was not quite significant at the 5% level, (F ratio (calc) = 2.979; F ratio (tables) = 3.10). This was due to an increase 5-HIAA:5-HT ratio in aluminium treated animals compared to young which was just outside the 5% level of significance.

There was no significant difference in the levels of CAT activity between the four experimental groups, (Table 8.8).

Table 8.9 summarises the results of behavioural tests carried out on the various experimental groups over the 2 year period. Age-related behavioural changes were observed which included impairments in motor activity, sensorimotor coordination, spatial reference memory and olfaction.

There was evidence from some of the behavioural tests, (but not all), that administration of a high Al diet increased the degree of general motor and sensorimotor impairment seen in normal aging. Swim performance and spatial reference memory were both impaired at a much earlier age in Al treated mice compared to age-matched controls. There was also evidence that long-term administration of a high lecithin diet may produce a slight lowering in the degree of impairment in spatial reference memory observed in normal aging.

8.4 DISCUSSION.

(i). The effect of aging on brain BH_4 metabolism and neurotransmitter levels.

No age-related effects on whole half brain catecholamine (CA) levels were observed in the mouse. Other workers have also been unable to demonstrate changes in whole brain CA levels in mice, (Finch, 1973), and humans (McGeer *et al*, 1971). However, regional age-related decreases in DA and NA have been consistently reported, (Roubein 1986; Winblad *et al*, 1985; Robinson *et al*, 1977). It is likely that whole, or half brain neurotransmitter analysis masks any alterations which might occur in specific regions of the brain and accounts for the apparent absence of any significant effect of age on CA's in this investigation.

Several investigators, (McGeer and McGeer, 1975; Winblad *et al*, 1985), have reported substantial age-related decreases in the activity of the rate-limiting enzyme for CA synthesis, tyrosine hydroxylase (TOH), in specific areas of the brain.

As BH_4 is the essential cofactor required for the hydroxylation reactions carried out by TOH, a disturbance in BH_4 metabolism could be partly responsible for a fall in TOH activity and subsequent decrease in CA synthesis. BH_4 metabolism in the aging brain has not been examined in detail but some evidence for an age-related reduction in the availability of the cofactor has been shown.

Leeming and Blair, (1980), reported an increase in human serum biopterin levels with age suggesting a loss of brain DHPR. Anderson, (1987), showed a significant negative correlation between DHPR activity and aging in the temporal and frontal cortices in human brain preparations. Lewitt *et al*, (1982), noted an increase in the CSF neopterin : biopterin ratio with age suggesting a possible deficit in

the BH_4 biosynthetic pathway.

Results from the present investigation indicate that brain BH_4 metabolism in the aged mouse is unaffected. This might explain the concommitant lack of any effect on brain CA levels. However, the limited specificity of the analysis performed, ie. total biopterin and derived pterin levels in whole half brains, reveals no information as to the BH_4 or CA status in specific areas of the brain. A much more detailed assessment of the effects of aging on brain BH_4 metabolism is required looking at possible changes in levels of the various forms of the cofactor in specific brain areas and also at the enzymes involved in BH_4 biosynthesis.

Some workers have reported a decline in brain 5-HT levels in aging but again these were noted in certain areas not in whole brain, (Mackay *et al*, 1978; Carlsson *et al*, 1985). No effect of aging on serotonin metabolism was noted in the present investigation using whole brain analysis.

Similarly large decreases in choline acetyltransferase, (CAT), activity have been reported in certain regions of human brain in aging. McGeer and McGeer, (1975), reported a 40-66% decrease in the level of cortical CAT between the ages of 20 and 50. Gross brain CAT activity was found to be unaffected in aged mice compared to young controls in the current investigation.

(ii). The effect of chronic administration of a high aluminium diet on brain BH_4 metabolism and neurotransmitter levels.

There is not a great deal of available data on the effect of aluminium intoxication on brain neurotransmitter status,

and existing results are contradictory. Some workers have shown Al to influence the properties of enzymes involved in choline and DA uptake in synaptosomal membranes, (Lai *et al*, 1981), others that increased concentrations of the metal in the brain affects DA and NA levels and DBH activity in some regions. However, the precise affect of the metal on these parameters is not always consistent and has been shown to depend on the dietary intake of other metals, (Wenk and Stemmer, 1981 and 1982).

Neurochemical analysis of dialysis dementia brains, (Perry *et al*, 1985), showed that MHPG, HVA and 5-HIAA levels were normal in the frontal cortex in which Al levels were significantly raised, suggesting that CA and serotonin activity is not impaired in the disorder.

In the present investigation there was no evidence of a significant effect of long-term administration of a high Al, low Ca/Mg diet on gross brain CA content in the mouse. Activity of CAT was also unaffected in Al treated mice compared to age-matched controls.

There may have been some evidence for an Al-related disturbance in serotonin metabolism. Aluminium treated mice showed lowered brain 5-HT and raised 5-HIAA levels compared to age-matched controls which gave rise to an increased 5HIAA:5HT ratio in the Al group. None of these changes were statistically significant however this could have been due to the small number of samples analysed from each experimental group. Clearly further work is required using more animals to confirm an effect of the metal on 5-HT metabolism.

Al has been found to inhibit DHPR *in vitro* and *in vivo*, (Leeming and Blair, 1979; Dhondt and Bellahsene, 1983), and Dhondt *et al*, (1982), showed an increase in the neopterin : biopterin ratio in serum of ureamic patients on maintenence dialysis indicating impaired BH_4 metabolism.

However, results presented here revealed no evidence for an effect of long-term dietary Al administration on brain BH_4 metabolism in the mouse as shown by total biopterin and derived pterin analysis of half brains. These findings are in keeping with those of Anderson, (1987), who showed no effect of either acute oral dosing, (50mg Al/kg body wgt / day for 7 days), or chronic dietary administration of Al, (30mg Al / kg body wgt / day for 6 weeks), on whole brain total biopterins or BH_4 synthesis in the rat.

Impaired BH_4 metabolism has been observed *in vivo* by 3 months administration of aluminium acetate to weaner rats in their drinking water, (Edwards, 1988; Cowburn, 1989). It would appear that the occurrence of brain alterations in BH_4 and neurotransmitter levels *in vivo* depends to some extent on the aluminium species presented to the animals and the way in which the species is administered. Dosing regimens involving addition of aluminium salts, (eg. the acetate), to drinking water would probably be more likely to increase tissue levels of the metal than dietary administration of more insoluble species such as the aluminosilicates which will probably be less well absorbed by the gut and show poorer penetration of the blood brain barrier.

In the present investigation no analysis of brain levels of aluminium was carried out, hence it is impossible to say how much of the metal, if any at all, crossed the blood brain barrier and accumulated in the brain. It is argued that the low Ca and Mg in the diet enhances the intestinal and tissue absorption of the Al species present in the diet, (Candy *et al*, 1986).

(iii). The effect of lecithin supplementation on BH_4 metabolism and neurotransmitter levels.

The most marked and consistent change in AD in terms of neurotransmitters is a severe cholinergic lesion in the cerebral cortex and hippocampus, and at *post mortem* this is seen as a pronounced fall in brain levels of CAT and acetylcholine esterase, (AChE), (Perry *et al*, 1978). Other neurotransmitter systems show more inconsistent impairments between patients.

The significance of the ACh system in AD has resulted in recent interest in lecithin, (a substrate for ACh synthesis), as a dietary supplement to AD patients. Orally administered lecithin has yielded variable results in terms of treatment of senile dementia with reports of alleviation of motor, memory and cognitive disturbances in some cases, (Barbeau, 1978; Perry and Perry, 1980), but as yet no long-term or consistent benefits have been observed in these patients.

In the present investigation there was no difference in levels of brain CAT activity between lecithin treated mice and age-matched controls indicating firstly no effect on ACh function in aging mice and secondly no apparent lecithin-related increase in ACh synthesis.

Interestingly, lecithin treated mice showed slightly decreased NA levels compared to young mice and age-matched controls and also lowered brain 5HT compared to age-matched controls. These results did not however reach statistical significance and further work is required to establish the reproducibility of these effects.

(iv). Life-long exposure to Al as a model for Alzheimers disease in the mouse.

Alzheimer's disease has been shown to produce significant neurochemical changes in certain regions of human brain which are quite distinct from those observed in normal aging, (Arai *et al*, 1984; Carlsson *et al*, 1985). Several groups have reported deficiences in BH_4 metabolism in AD patients, (Leeming and Blair 1980; Lewitt *et al* 1985; Young *et al* 1982; Barford *et al* 1984; Nagatsu *et al* 1986; Anderson 1987).

In the present investigation there were no statistically significant differences in neurotransmitter or BH_4 levels between aluminium treated mice and normal age-matched controls. This may suggest a resistance in mouse brain to the neurotoxic effects of Al indicating the unsuitability of either the species or the metal as a model for AD.

However, the behavioural differences shown between aged and Al treated mice together with the possible indication of an Al-related disturbance in NA and 5HT metabolism, (even by gross brain analysis), suggests that a degree of neurological dysfunction has occured in Al treated mice compared to age-matched controls.

There was no evidence for a deficit in DA levels in Al treated mice. Similarly the dopaminergic system seems to be virtually intact in AD. Areas showing the greatest neuropathological changes in AD patients, ie. the neocortex and hippocampus, show normal DA levels, (Arai *et al*, 1984).

In summary, given that the neurochemical changes observed in aging and AD occur in specific areas of the brain it is perhaps not surprising that the rather limited scope of gross brain analysis provided no stronger evidence for differences in neurotransmitter and BH_4 levels between the various groups.

Clearly further work using a greater number of animals and examination of parameters in specific areas of the brain is necessary to resolve the neurotoxic effects of Al in the mouse and to assess the efficacy of chronic Al administration as a model for AD.

Table 8.1: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain total biopterins (ng/g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	135.6 ± 10.7	F= 0.1374 (n.s)	Y vs A	n.s
(A)	6	139.1 ± 16.3		A vs L	n.s
(L)	6	138.3 ± 12.6		A vs Al	n.s
(Al)	5	139.9 ± 9.9		L vs Al	n.s

Table 8.2: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain derived pterin levels (ng/g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	55.3 ± 3.6	F= 2.0132 (n.s)	Y vs A	n.s
(A)	6	53.5 ± 5.5		A vs L	n.s
(L)	6	52.9 ± 3.4		A vs Al	n.s
(Al)	5	49.3 ± 5.0		L vs Al	n.s
				Y vs Al	n.s

Tables 8.1-8.2: Total biopterins and derived pterin were measured in the brains of four groups of female mice. Y = 3 month old mice; A = 23 month old mice fed standard mouse breeder diet since weaning; L = 23 month old mice fed a high lecithin diet since months; Al = 23 month old mice fed a high aluminium, low calcium and magnesium diet since 3 months. Animals were housed in groups in a 12:12 hour light:dark cycle and allowed food and water *ad libitum*. Results were analysed by one-way ANOVA and by students unpaired t-tests to look for differences between specific groups.

Table 8.3: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain noradrenaline levels (ng/g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	405.8 ± 45.3	F= 3.0504 (n.s)	Y vs A	n.s
(A)	6	392.6 ± 36.4		A vs L	n.s
(L)	6	359.8 ± 23.0		A vs Al	n.s
(Al)	5	369.9 ± 20.4		L vs Al	n.s
				Y vs L	n.s
				Y vs Al	n.s

Table 8.4: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain dopamine levels (ng/g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	1323.8 ± 139.7	F= 0.8813 (n.s)	Y vs A	n.s
(A)	6	1239.8 ± 63.9		A vs L	n.s
(L)	6	1286.5 ± 52.5		A vs Al	n.s
(Al)	5	1278.1 ± 77.3		L vs Al	n.s

Tables 8.3-8.4: Noradrenaline and dopamine levels were measured in the brains of four groups of female mice. Y = 3 month old mice; A = 23 month old mice fed standard mouse breeder diet since weaning; L = 23 month old mice fed a high lecithin diet since months; Al = 23 month old mice fed a high aluminium, low calcium diet since 3 months. Animals were housed in groups in a 12:12 hour light:dark cycle and allowed food and water *ad libitum*. Results were analysed by one-way ANOVA and by students unpaired t-tests to look for differences between specific groups.

Table 8.5: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain serotonin levels (ng/g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	1083.8 ± 64.0	F= 0.5245 (n.s)	Y vs A	n.s
(A)	6	1059.2 ± 84.2		A vs L	n.s
(L)	6	1046.8 ± 67.9		A vs Al	n.s
(Al)	5	1041.5 ± 130.6		L vs Al	n.s
				Y vs AL	n.s

Table 8.6: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain 5-HIAA levels (ng/g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	280.9 ± 17.0	F= 2.1319 (n.s)	Y vs A	n.s
(A)	6	283.6 ± 28.6		A vs L	n.s
(L)	6	294.0 ± 29.5		A vs Al	n.s
(Al)	5	327.7 ± 54.5		L vs Al	n.s
				Y vs Al	n.s

Tables 8.5-8.6: Serotonin and its metabolite, 5HIAA were measured in the brains of four groups of female mice. Y = 3 month old mice; A = 23 month old mice fed standard mouse breeder diet since weaning; L = 23 month old mice fed a high lecithin diet since months; Al = 23 month old mice fed a high aluminium, low calcium diet since 3 months. Animals were housed in groups in a 12:12 hour light:dark cycle and allowed food and water *ad libitum*. Results were analysed by one-way ANOVA and by students unpaired t-tests to look for differences between specific groups.

Table 8.7: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on the brain 5HIAA:5HT ratio (ng/g wet wgt) in the mouse.

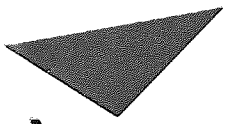
Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	0.260 ± 0.017	F= 2.9790 (n.s)	Y vs A	n.s
(A)	6	0.269 ± 0.033		A vs L	n.s
(L)	6	0.282 ± 0.032		A vs Al	n.s
(Al)	5	0.318 ± 0.059		L vs Al	n.s
				Y vs Al	n.s

Table 8.7: The brain 5HIAA:5HT ratio was calculated for four groups of female mice. Y = 3 month old mice; A = 23 month old mice fed standard mouse breeder diet since weaning; L = 23 month old mice fed a high lecithin diet since 3 months; Al = 23 month old mice fed a high aluminium, low calcium diet since months. Animals were housed in groups in a 12:12 hour light:dark cycle and allowed food and water *ad libitum*. Results were analysed by one-way ANOVA and by students unpaired t-tests to look for differences between specific groups.

Table 8.8: The effect of aging, a high aluminium (low calcium) diet or high lecithin diet on brain choline acetyltransferase (CAT) activity (umoles acetylcholine / hr /g wet wgt) in the mouse.

Group	(n)	Mean ± st.d	One-way ANOVA	Comparison	t-test p
(Y)	6	20.40 ± 6.46	F= 1.4531 (n.s)	Y vs A	n.s
(A)	6	18.36 ± 6.09		A vs L	n.s
(L)	6	17.86 ± 7.37		A vs Al	n.s
(Al)	5	21.83 ± 3.47		L vs Al	n.s

Table 8.8: Levels of brain CAT activity were measured in four groups of female mice. Y = 3 month old mice; A = 23 month old mice fed standard mouse breeder diet since weaning; L = 23 month old mice fed a high lecithin diet since months; Al = 23 month old mice fed a high aluminium, low calcium diet since 3 months. Animals were housed in groups in a 12:12 hour light:dark cycle and allowed food and water *ad libitum*. Results were analysed by one-way ANOVA and by students unpaired t-tests to look for differences between specific groups.



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CHAPTER 9.

GENERAL DISCUSSION.

As the natural cofactor required for the hydroxylation of phenylalanine to tyrosine, tyrosine to dopa and tryptophan to 5-hydroxytryptophan, BH_4 is crucial for the synthesis of the neurotransmitters noradrenaline, (NA), dopamine, (DA), and serotonin, (5-HT). It is therefore logical to expect a significant depletion of the cofactor to result in severe neurological impairment.

This anticipation has been borne out by the discovery of certain inborn errors of BH_4 metabolism, namely the malignant hyperphenylalaninaemias, or atypical phenylketonuria, in which gross BH_4 deficiency is observed to have significant clinical impact, characterised primarily by high blood phenylalanine levels and severe neurological dysfunction, (Danks *et al*, 1978; Dhondt, 1984).

The defect in BH_4 may arise from a deficiency in one of the enzymes involved in BH_4 synthesis namely GTP cyclohydrolase, (GTP-CH), (Niederwieser *et al*, 1982 and 1984), or 6-pyruvoyl-tetrahydropterin synthase, (PPH_4S) (Niederwieser *et al* 1985) or from a deficiency in dihydropteridine reductase, (DHPR) (Kaufman *et al* 1975), the enzyme responsible for salvaging the fully reduced active form of the cofactor after amino acid hydroxylation.

Though the results of gross BH_4 deficiency in humans have been well established what has not yet been fully resolved are the precise clinical effects of a partial brain BH_4 deficiency. ie. to what extent is a partial decline in cofactor levels a causative agent of neurological dysfunction? Some speculation on this matter has been presented from studies of BH_4 metabolism in human

neurological disorders.

PPH_4S deficiency is heterogeneous and both partial and peripheral forms of the defect have been recognised in humans, (Niederwieser *et al*, 1987; Dhondt, 1987). In these cases, though measurement of biochemical parameters in blood, urine and CSF has clearly indicated impaired BH_4 metabolism, patients may not present with any observable neurological dysfunction and normal levels of CSF neurotransmitter metabolites have been measured.

On the other hand, patients suffering from marked neurotransmitter deficiencies in disorders such as Alzheimer's disease, dystonia, Parkinson's disease and depression have shown partial reductions in levels of the cofactor, (Lovenberg *et al* 1979; LeWitt *et al* 1986; Leeming *et al*, 1979), which have been alleged to possibly contribute to the neurological symptoms associated with these disease states.

Clearly there is still some uncertainty as to the extent to which BH_4 metabolism can be impaired before concomitant disruption of neurotransmitter synthesis and subsequent behavioural abnormalities occur.

The objectives of this study were firstly to identify situations in the rat in which brain total biopterin levels are consistently depressed and then to examine in these situations brain levels of biogenic amines and their metabolites and look for any signs of alterations in animal behaviour. It is the first time that a detailed study of this type looking at BH_4 metabolism, neurotransmitters and behaviour simultaneously has been reported.

A collaborative study is also reported which examined the effects of long-term administration of a high aluminium, low calcium/magnesium diet on behaviour, histopathology and neurobiochemistry in the mouse. I performed the biochemical

analyses.

There are obvious limitations associated with the use of animal models for analysis of biochemical pathways *in vivo*. Care should be exercised in the interpretation of results with respect to man. Extrapolation of circumstances observed in animals to "parallel" situations in humans is not always appropriate. This is not simply because of specific differences in metabolism which exist between different species.

Behavioural experiments serve as a clear example of where particular care should be taken. Human behaviours incorporate much more subtle components than those seen in animals.

Also many experiments carried out in this thesis involved acute exposure of rats to neurotoxins over very short periods of time, whereas in man the impact of a potentially harmful compound or situation is often likely to be spread over a number of months or even years. The results of the experiments performed in this investigation are discussed in the light of these limitations.

9.1: THE EFFECTS OF LEAD AND DIETHYLSTILBOESTROL ON BH_4 METABOLISM IN THE RAT.

A number of potentially neurotoxic agents have been previously reported to decrease rat brain total biopterin levels. The effects of some of these substances were re-examined in order to assess their suitabilities as vehicles with which to produce an animal model of brain BH_4 deficiency.

The integrity of brain BH_4 metabolism was maintained

during the acute exposure of rats to lead and diethylstilboestrol (DES), (Table 9.1). Dosing regimes for these compounds which have been previously reported to decrease rat brain total biopterin levels (Cutler, 1986: Al-Salihi, 1985; Edwards, 1988) failed to do so in the course of the present investigation.

Table 9.1: The effect of acute exposure to lead and DES on brain total biopterin and derived pterin levels in the rat, (ng g^{-1} wet weight tissue).

TREATMENT	PARAMETER	n	Mean ± St.d CONTROL	Mean ± St.d TEST	p value
LEAD	biopterin	5	66 ± 10	69 ± 16	n.s
	pterin	6	63 ± 5	67 ± 8	n.s
DES	biopterin	6	61 ± 7	61 ± 5	n.s
	pterin	6	80 ± 8	83 ± 5	n.s

Lead:- 150g male Wistar rats given 75.9mg PbAc / kg body weight i.p in isotonic saline once daily for 3 days.

DES:- 50g female weaner Wistar rats given 500mg DES /kg body wgt i.g as a suspension in corn oil once daily for 4 days.

Food and water were available *ad lib.* Results expressed as mean ± st.d and were analysed by a students unpaired t test.

Significant reductions in liver total biopterin and derived pterin levels in lead and DES treated rats, (Table 9.2), suggested that insufficient passage of the compounds across the blood-brain barrier, (BBB), may have explained the lack of effects observed in the brain. Passage of lead into the brain is known to be low, (DeMichelle, 1984), and poor intestinal absorption of orally administered DES in a

suspension of corn oil or in ethanol together with rapid peripheral metabolism, (Henry and Miller, 1986), would result in very little accumulation of the active compounds in the brain.

Table 9.2: The effect of lead and DES on liver total biopterin and derived pterin levels in the rat, (ng g^{-1} wet weight tissue).

TREATMENT	PARAMETER	n	Mean ± St.d CONTROL	Mean ± St.d TEST	p value
LEAD	biopterin	5	1428 ± 60	1017 ± 159	p<0.1%
	pterin	6	634 ± 31	506 ± 53	p<0.2%
DES	biopterin	6	944 ± 73	578 ± 82	p<0.1%
	pterin	6	593 ± 94	399 ± 54	p<0.2%

Lead:- 150g male Wistar rats given 75.9mg PbAc / kg body weight i.p in isotonic saline once daily for 3 days.

DES:- 50g female weaner Wistar rats given 500mg DES /kg body wgt i.g as a suspension in corn oil once daily for 4 days.

Food and water were available *ad lib.* Results expressed as mean ± st.d and analysed by students unpaired t test.

In addition use of whole half brain analyses would mask small alterations in brain biopterin levels occuring in specific brain regions. Meredith *et al*, (1988), showed the accumulation and neurochemical effects of lead in the brain to be regionally specific. The results may therefore highlight the limited use of gross brain analysis of biochemical parameters which are known to show regional specificity in their distributions.

So from a preliminary survey, neither of the agents were found to be suitable for producing a model of brain BH_4 deficiency in the rat though they showed signs of being neurotoxic with respect to BH_4 as indicated by the significant reduction in total biopterin and derived pterin levels in the liver.

9.2: TETRAHYDROBIOPTERIN METABOLISM IN STARVATION.

Starvation is known to produce complex biochemical effects. Low catecholamine, (CA), turnover in the periphery and the brain has been reported in starving animals, (Landsberg and Young, 1983; Schweiger *et al*, 1985), and abnormal catecholamine metabolism noted in primary anorexia nervosa in humans, (Young and Landsberg, 1977). The mechanism of this reduction in catecholamine turnover is not fully understood, but limited precursor amino acid availability (ie. tyrosine), has been implicated as a factor, (Schweiger *et al*, 1985).

Earlier recordings by Heales, (1987), that starvation produces a decrease in brain and liver total biopterin levels were re-examined to assess the reproducibility and nature of the reduction and also investigate the possibility that brain BH_4 levels, in addition to amino acid availability, may regulate neurotransmitter synthesis during starvation.

Animals were starved for 24, 48 or 72 hours and various parameters of BH_4 metabolism in the brain and liver measured. Results confirmed Heales' findings of a starvation-induced decline in both brain and liver total biopterin levels in the rat, (Table 9.3). However whereas Heales showed a drop in brain biopterins after 24 and 48 hours but only a transient fall in the liver after 24 hours, in the present investigation brain biopterins

decreased only after a more extensive starvation period, (ie. 72 hours), and liver levels consistently decreased after 24, 48 and 72 hours.

Table 9.3: The effect of starvation on brain and liver total biopterin levels, (ng g^{-1} wet wgt).

TISSUE	LENGTH OF STARVATION PERIOD			
	0 hr	24 hr	48 hr	72 hr
BRAIN BIOPTERIN	65.2 ± 5.2	65.2 ± 5.7	61.3 ± 1.7	58.7 ± 1.7
p value		n.s	n.s	p<5%
LIVER BIOPTERIN	1404 ± 150	1175 ± 96	946 ± 95	1083 ± 80
p value		p<5%	p<0.1%	p<1%

150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib*. Controls received food and water *ad lib*. Number of animals per group = 5. Results expressed as mean ± st.d and analysed by a students unpaired t test.

Workers have shown starvation to induce a limited availability of NADPH, an essential component of the BH_4 biosynthetic pathway, (Martins *et al*, 1985). However, results presented here show no evidence that the decrease in total biopterins in brain or liver after 72 hours starvation was due to a reduction in biosynthesis of the cofactor, (Table 9.4). Heales, (1987), similarly showed no reduction in brain or liver BH_4 synthesis after 48 hours starvation.

There was some evidence of possible disruption of the salvage pathway in brains of rats starved for 48 hours, (Table 9.5), but as DHPR activities were unaffected when brain and liver biopterins were depressed, and since plasma biopterins were significantly decreased not elevated it seems unlikely that impaired salvage of BH_4 was responsible

for the starvation-induced decreases in total biopterins observed. These findings were also consistent with those of Heales, (1987).

Table 9.4: Effect of 72 hour starvation on brain and liver biosynthetic capacity in the rat, (ng biopterin /hr /mg protein).

TISSUE	PERIOD OF STARVATION	Mean ± st.d FED	Mean ± st.d STARVED	p value
BRAIN	72 hours	3.65 ± 3.01	3.30 ± 1.83	n.s
LIVER	72 hours	3.65 ± 3.01	3.30 ± 1.83	n.s

150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib.* Controls received food and water *ad lib.* Number of animals per group = 5. Results expressed as mean ± st.d and analysed by a students unpaired t test.

Table 9.5: Effect of 48 and 72 hour starvation on brain and liver DHPR activity in the rat, (nmoles NADH oxidised /min /mg protein).

TISSUE	PERIOD OF STARVATION	Mean ± st.d FED	Mean ± st.d STARVED	p value
BRAIN	48 hours	172.0 ± 12.1	142.1 ± 24.7	p<5%
	72 hours	159.6 ± 42.9	159.3 ± 21.8	n.s
LIVER	48 hours	586.3 ± 88.1	542.2 ± 47.4	n.s
	72 hours	439.0 ± 81.0	403.0 ± 56.0	n.s

150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib.* Controls received food and water *ad lib.* Number of animals per group = 5. Results expressed as mean ± st.d and analysed by a students unpaired t test.

9.2.1: REGULATION OF TETRAHYDROBIOPTERIN IN STARVATION.

Acute starvation results in an increased tissue oxidising environment due to a reduction in catalase and glutathione, (GSH), levels, (Tateishi *et al*, 1974). This together with findings that physiological concentrations of GSH inhibit autoxidation of labile BH_4 *in vitro* , led Heales, (1987), to propose that depressions in total biopterins observed in starvation may arise from increased oxidative catabolism of the cofactor due to a reduction in GSH levels and an enhanced oxidising environment in the tissues.

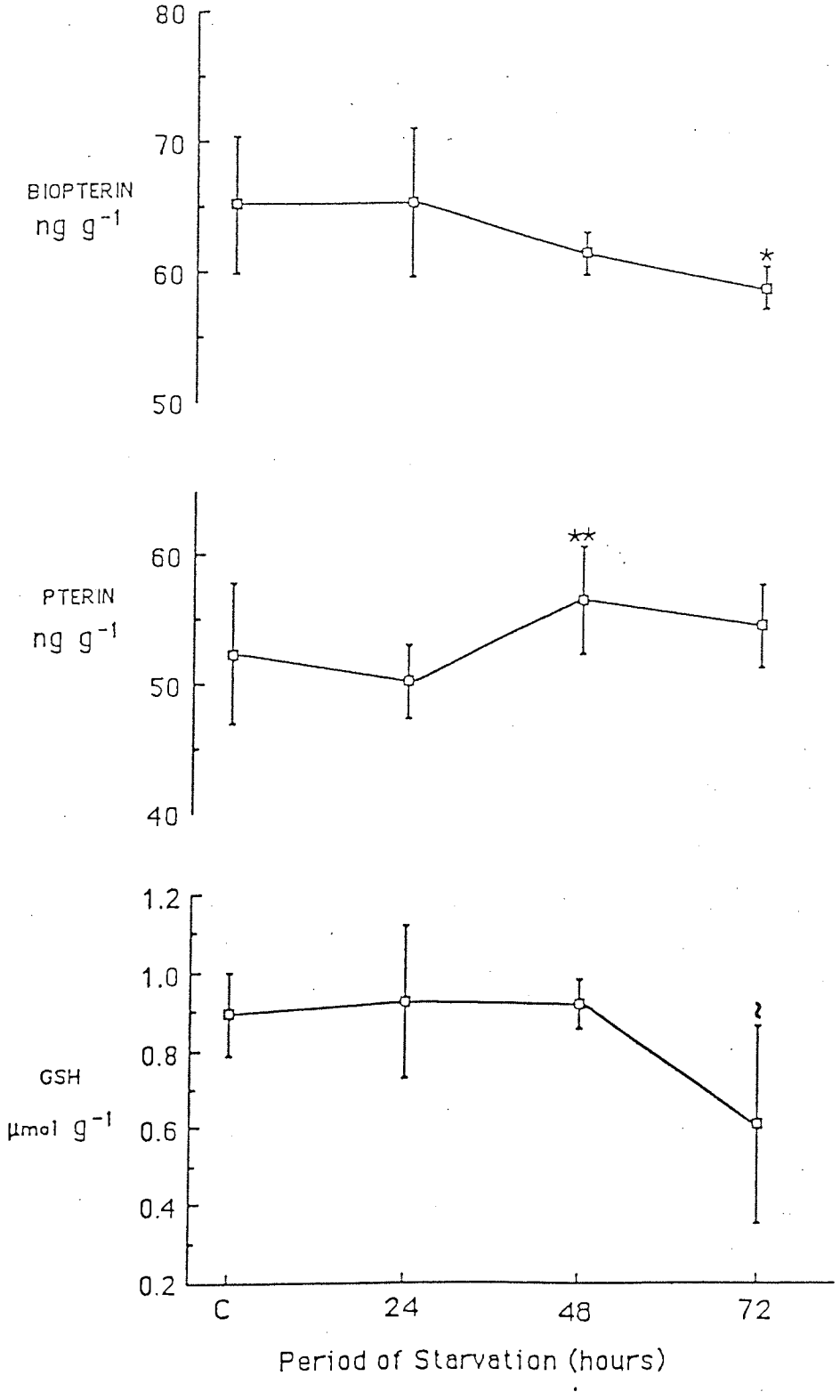
Experiments conducted by Heales, (1987), involving manipulation of the oxidising environment in rat tissue by administration of L-buthionine sulphoximine and L-cysteine produced indirect evidence supporting a link between reduced biopterin and GSH levels in starvation.

In order to confirm and investigate further Heales' work experiments were conducted to directly assess actual GSH levels in rat brain and liver in starvation and look for a possible correlation between tissue levels of the thiol and total biopterins.

There was evidence to indicate that a fall in tissue GSH levels in starvation might cause a subsequent decrease in tissue BH_4 status, (Figs. 9.1 and 9.2).

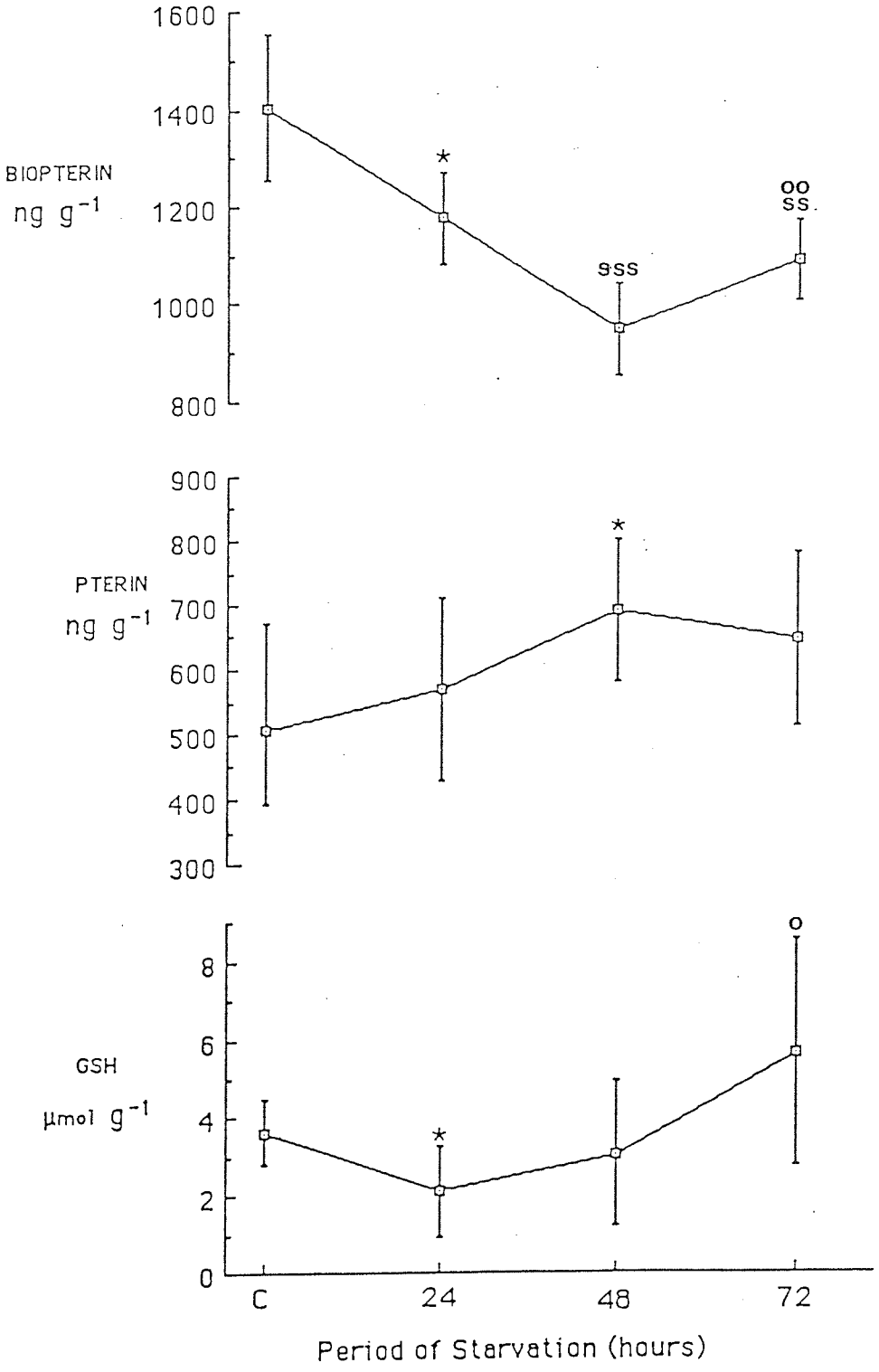
Brain GSH was unaffected until 72 hours starvation when levels dropped mirroring the decline in brain total biopterins. Liver GSH levels decreased after 24 hours showing a significant positive correlation with liver total biopterin, ($p<2\%$). A rise in liver GSH at 48 hours is consistent with the findings of others, (Tateishi *et al*, 1974; Cho *et al*, 1981), and when levels had surpassed those in controls at 72 hours, liver biopterins began to rise.

Fig. 9.1: Variation in brain total biopterin, derived pterin and reduced glutathione levels over 24, 48 and 72 hours starvation in the rat.



150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib.* Controls received food and water *ad lib.* Each point is the mean of 5 observations ± st.d. Results were analysed by a students unpaired t test.
GSH = reduced glutathione.
* = decreased compared to other 3 groups, ($p<5\%$).
** = increased compared to level at 24 hours, ($p<5\%$).
ɀ = decreased compared to level at 48 hours, ($p<0.5\%$).

Fig. 9.2: Variation in liver total biopterin, derived pterin and reduced glutathione (GSH) levels over 24, 48 and 72 hours starvation in the rat.



150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib.* Controls received food and water *ad lib.* Each point is the mean of 5 observations ± st.d. Results were analysed by a students unpaired t test.

GSH = reduced glutathione.
* = p<5% compared to controls.
ss = p<1% compared to controls.
sss = p<0.1% compared to controls.
o = increased compared to level at 24 hours, (p<5%).
oo = increased compared to levels at 48 hours, (p<5%).

Evidence for increased oxidative breakdown of BH_4 in starvation brought about by a decline in tissue GSH levels may also come from examination of brain and liver derived pterin levels and P/B ratios, (Table 9.6).

Table 9.6: The effect of starvation on brain and liver derived pterin levels, (ng g^{-1} wet wgt), and the liver P/B ratio.

TISSUE	LENGTH OF STARVATION PERIOD			
	0 hr	24 hr	48 hr	72 hr
BRAIN PTERIN p value	52.3 ± 5.4	50.1 ± 2.9 n.s	56.4 ± 4.2 n.s	54.4 ± 3.2 n.s
BRAIN P/B p value	.809 ± .126	.818 ± .071 n.s	.921 ± .074 n.s	.959 ± .058 p<5%*
LIVER PTERIN p value	508 ± 114	568 ± 141 n.s	691 ± 110 p<5%	645 ± 136 n.s
LIVER P/B p value	.368 ± .108	.486 ± .121 n.s	.729 ± .067 p<0.1%	.591 ± .083 p<1%

150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib.* Controls received food and water *ad lib.* Number of animals per group = 5. Results expressed as mean ± st.d and analysed by a students unpaired t test.
* = significant increase compared to 24 hrs (p<1%).

An increase in the tissues of either of these parameters may, with caution, be used as an indicator of enhanced BH_4 catabolism, (Heales, 1987).

In the brain an increased P/B ratio was observed after 72 hours starvation and pterin and GSH levels showed a significant negative correlation, (p<0.5%). Derived pterin

levels in the liver, (Table 9.6), were significantly elevated as was the P/B ratio and though no correlation between liver derived pterin and GSH levels was observed both pterin levels and the P/B ratio dropped as normal GSH levels were surpassed.

Data presented here therefore confirms Heales' findings of a starvation-induced disruption of BH_4 metabolism in the rat and supports the proposal that it could arise from a drop in GSH levels in the fasted state and prevention of oxidative breakdown of the active cofactor.

9.2.2: STARVATION AND BRAIN NEUROTRANSMITTER STATUS.

Having established that starvation produces a decrease in rat brain total biopterins the fasted rat was used to test whether these depressions in cofactor availability may be in part responsible for the diminished neurotransmitter turnover that has been reported in various states of food deprivation, (Halmi *et al*,1978; Landsberg and Young, 1983; Pirke and Spyra, 1982; Schweiger *et al*, 1985).

A fall in whole half brain total biopterins after 72 hours starvation was accompanied by no change in half brain levels of the catecholamines noradrenaline, (NA), or dopamine (DA), and an elevation in both serotonin (5-HT) and the metabolite 5-hydroxyindoleacetic acid (5-HIAA), (Table 9.7).

Many investigators report that brain monoamine metabolite content provides a good estimate for an increase or decrease in the turnover rate of the parent monoamine, (Eccleston *et al*, 1970; Roth *et al*, 1976). The rise in brain 5-HT and 5-HIAA levels with starvation observed in the present investigation indicated increased serotonin synthesis and turnover. This was almost certainly due to a

starvation-induced increase in the ratio of tryptophan to other large neutral amino acids in the plasma and a subsequent enhanced entry of the amino acid into the brain, (Fernstrom, 1979). The reduced levels of BH_4 in the brain did not affect tryptophan hydroxylation or subsequent 5HT synthesis.

Table 9.7: Effect of starvation on brain total biopterin, NA, DA, 5HT and 5HIAA levels in the rat.

Parameter	Length of starvation period			
	0 hr	24 hr	48 hr	72 hr
Biopterin	65.2 ± 5.2	65.2 ± 5.7	61.3 ± 1.7	58.7 ± 1.7
p value		n.s	n.s	p<5%
NA	283.3 ± 20.2	284.0 ± 50.8	300.1 ± 34.0	298.6 ± 11.6
p value		n.s	n.s	n.s
DA	746.6 ± 58.9	719.3 ± 84.3	715.8 ± 53.1	799.7 ± 102
p value		n.s	n.s	n.s
5HT	501.3 ± 27.6	603.3 ± 113	643.2 ± 95.8	661.0 ± 39.8
p value		n.s	p<2%	p<0.1%
5HIAA	185.0 ± 24.8	233.8 ± 19.3	233.2 ± 10.4	213.3 ± 14.2
p value		p<1%	p<1%	n.s **
5HIAA/5HT	0.37 ± 0.06	0.40 ± 0.06	0.37 ± 0.06	0.32 ± 0.03
p value		n.s	n.s	n.s *

150g male Wistar rats were starved on grids for either 24, 48 or 72 hours. Water available *ad lib.* Controls received food and water *ad lib.* Each value is the mean of 5 observations ± st.d. Results were analysed by a students unpaired t test.
p values in the table refer to comparisons made with the control group only. Any other significant comparisons have been assigned a symbol which are listed and explained below.
* decreased compared to that at 24 hours, (p<5%).
** decreased compared to that at 48 hours, (p<5%).

So in so far as gross changes in the brain are concerned, there was no evidence that BH_4 levels are rate-limiting for catecholamine and particularly serotonin synthesis as shown in starvation in the rat.

The obvious limitation of using whole half brain analysis to measure small quantities of compounds which are known to be located in specialised cell populations must be considered in the interpretation of the results. However the apparent lack of regulation of catecholamine and particularly serotonin levels by brain BH_4 content presented here has also been reported by other workers, (Suzuki *et al*, 1988), who have suggested that BH_4 concentrations *in situ* are not so subsaturating for tyrosine and tryptophan hydroxylase activities as proposed in previous studies, (Kettler *et al*, 1974; Miwa *et al*, 1985; Sawada *et al*, 1986).

9.3: DIETARY ADMINISTRATION OF 2,4-DIAMINO-6-HYDROXYPYRIMIDINE AS A MODEL FOR BRAIN BH_4 DEFICIENCY IN THE RAT.

In 1966 Pabst and Rembold reported that rats fed a diet containing 1% 2,4-diamino-6-hydroxypyrimidine, (DAHP), had significantly reduced urinary biopterin levels. It has since been confirmed that DAHP is an inhibitor of GTP cyclohydrolase the enzyme involved in the first step of BH_4 biosynthesis, (Gal and Sherman,1976; Gal and Whitacre, 1981). As such, the potential for using DAHP administration to create an animal model of BH_4 deficiency was recognised.

Cotton, (1986), described a model of hyperphenylalanineamia produced by dietary administration of DAHP, in which weaner mice showed both significantly increased serum phenylalanine levels and BH_4 deficiency in the brain.

Work was undertaken to test the validity of using dietary administration of DAHP to create a model of brain BH_4 deficiency in the rat which might be used for neurotransmitter and behavioural analysis. The inhibitor which was in powder form was blended with standard rat chow which had also been ground into a powder. Animals were fed either a control diet, (standard none-treated powdered rat chow), or DAHP treated diet for 6 days.

A 5% DAHP diet, consistently decreased total biopterin levels in both the brain and liver, (Table 9.8). In the liver, but not brain, there was also evidence for a reduction in synthesis of the cofactor, (Table 9.9).

Table 9.8: Brain and liver total biopterin levels in weaner rats after administration of a 5% DAHP diet expressed as a % of control values.

TRIAL	BRAIN		LIVER	
	% biopterins	p value	% biopterins	p value
1	43%	$p<0.1\%$	19%	$p<0.1\%$
2	79%	$p<0.1\%$	62%	$p<0.1\%$
3	80%	$p<1.0\%$	43%	$p<0.1\%$
4	62%	$p<1.0\%$	34%	$p<0.1\%$

Male weaner rats fed a 5% DAHP diet for 6 days. Results analysed by a students unpaired t test and expressed as a % of the total biopterin levels observed in control animals which were fed normal food *ad lib.* p values relate to differences between means ($\pm$ standard deviations), in the control and test groups.

As DAHP competitively inhibits GTP cyclohydrolase the absence of impaired BH_4 biosynthesis in the brain was a little surprising. It is possible that such a small amount of the compound accumulated in the brain tissue that it was diluted out sufficiently in the *in vitro* biosynthesis assay so as to produce a "false-negative" result.

Table 9.9: Brain and liver BH_4 biosynthetic capacities in weaner rats after administration of a 5% DAHP diet expressed as a % of control values.

Trial	Brain		Liver	
	% biosynthesis	p value	% biosynthesis	p value
1	142%	n.s	53%	p<2.0%
2	76%	n.s	93%	n.s

Male weaner rats were fed a 5% DAHP diet for 6 days. Results were analysed by a students unpaired t test and are expressed as a % of the levels observed in control animals which were fed normal food *ad lib.* p values relate to differences between means (± standard deviations), in the control and test groups.

That BH_4 was functionally deficient in the liver was confirmed by a considerable rise in plasma phenylalanine levels, (Table 9.10), supporting claims by Cotton of a DAHP-induced hyperphenylalanineamic state in mice, (Cotton, 1986).

Table 9.10: Plasma phenylalanine, tyrosine and total biopterin levels in weaner and 180g rats after administration of a 5% DAHP diet expressed as a % of control values.

Trial	Phenylalanine	Tyrosine	Total biopterins
1 (weaners)	291% (p<1%)	73% n.s	57% (p<5%)
2 (180g rats)	428% (p<2%)	113% n.s	40% (p<1%)

Male weaner rats were fed a 5% DAHP diet for 6 days. Results were analysed by a students unpaired t test and are expressed as a % of the total levels observed in control animals which were fed normal food *ad lib.* Values in parenthesis indicate levels of significance.
p values relate to differences between means (± standard deviations), in the control and test groups.

The rise in plasma phenylalanine levels may explain the observed decrease in brain total biopterins in the absence of any observable fall in brain BH_4 biosynthetic capacity. Blair *et al*, (1984), showed that rats dosed orally with high levels of phenylalanine, (600-900mg /kg body wgt), had reduced brain total biopterin and actual BH_4 levels. Increased plasma levels of phenylalanine will cause enhanced entry of the amino acid into the brain where it or one of its metabolites, probably phenylpyruvate, inhibits DHPR activity, (Cutler, 1986). However, brain DHPR activity was unaffected by DAHP and plasma total biopterins showed a decrease not the elevation expected in the event of DHPR inhibition.

Having discovered a substance with which brain total biopterin levels could be consistently depressed it seemed that a potential model for investigating neurotransmitter status and behaviour in the BH_4 deficient rat had been found.

However, it was observed that animals fed DAHP diets showed significant weight loss, due to reduced food intake, compared to controls which gained weight gradually over the 6 day experimental period. It was also noted that animals fed 2% or 5% DAHP diets for 6 days actually consumed similar amounts of DAHP, the major variable between the two groups being the total quantities of food consumed rather than the amounts of inhibitor they were exposed to, (Table 9.11). Despite similar intakes of DAHP the two groups showed variable degrees of BH_4 deficiency, (Fig. 9.3).

In the light of experiments conducted on starvation it was of some concern that part of the decrease in total biopterins may have been attributable to a starvation effect and not exclusively an inhibitor effect.

Table 9.11: Daily food intake, DAHP intake and change in body weights in animals fed 2% or 5% DAHP diets over 6 days.

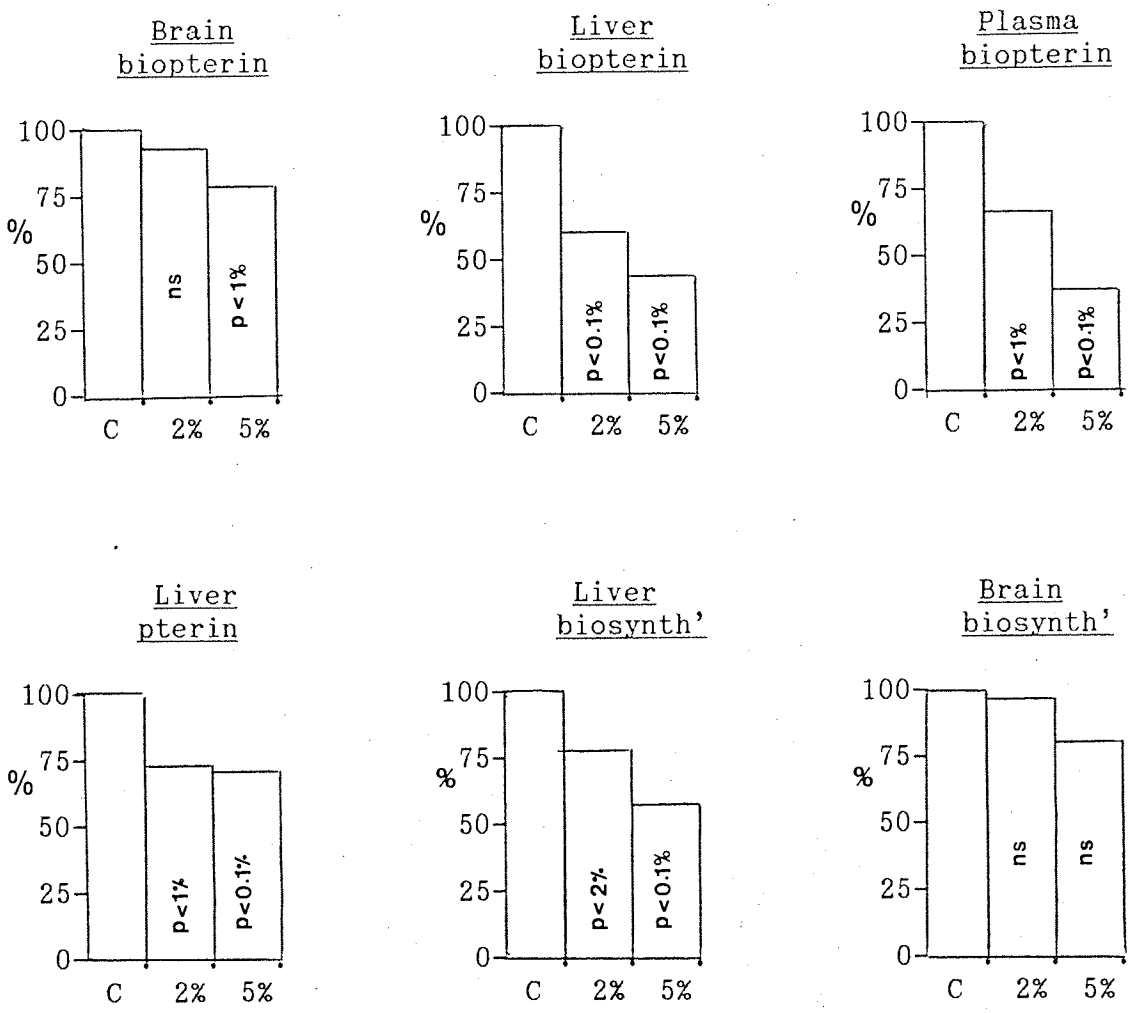
Group	Mean % change body wgt after 6 days	Mean daily food intake / rat (% of controls)	Mean daily DAHP intake / rat (g)
2% DAHP (n=6)	+26.5	59.9	0.23
5% DAHP (n=6)	- 3.2	27.3	0.26

Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days. Controls were allowed normal food *ad lib*. Body wgt changes are expressed as a % loss or gain of the group wgts prior to the onset of the experiment

Experiments were set up to compare the effects of administration of a 5% DAHP diet, (incorporating a degree of voluntary food restriction), and food restriction alone on BH_4 metabolism in weaner rats. The degree of imposed food restriction experienced by the latter group was equivalent to that seen in animals on the 5% DAHP diet. This type of experiment was performed on two separate occasions denoted by trials 1 and 2 in table 9.12.

Both treatments produced depressions in total biopterins in brain, liver and plasma compared to their respective control groups. The decreases observed in rats subjected to enforced food restriction were significant but not as severe as those seen in DAHP treated animals who underwent additional voluntary food restriction.

Fig. 9.3: Comparison of the effects of a 2% or 5% DAHP diet on BH_4 metabolism in weaner rats.



Male weaner rats were fed either a 2% or 5% DAHP diet for 6 days. Controls were allowed normal food *ad lib.* Results were analysed by a students unpaired t test.

There was some suggestion of a relationship between the severity of food restriction and intensity of the affect on BH_4 metabolism, (as was seen in the case of acute starvation),(Table 9.12). When food restriction was less intense, as in trial 2 where DAHP and hence food restricted animals ate more food, there was no decrease in brain total biopterins and the depression seen in the plasma was smaller.

Table 9.12: Comparison of the effects of a 5% DAHP diet and food restriction on BH_4 metabolism in weaner rats.

Trial	Group	% change in b.w	Brain biopterin	Plasma biopterin	Liver biopterin	Liver biosynth
1	Control	+44.4				
	5% DAHP	- 3.2	80.2%*	35.1%**	42.7%**	55.0%**
	Control	+52.1				
	F.R	-19.9	85.5%#	47.8%**	81.6%*	85.0%
2	Control	+35.8				
	5% DAHP	- 7.2	65.0%*	46.8%**	33.9%**	-
	Control	+43.3				
	F.R	-13.4	99.6%	73.5%#	70.4%#	-

Male weaner rats were fed a 5% DAHP diet or subjected to food restriction for six days. Controls were allowed access to normal food *ad lib*. The investigation was performed twice, (ie. trials 1 and 2). Results were analysed by a students unpaired t test and are expressed as %'s of control values.
b.w = body weight; values are expressed as a % loss or gain of the group wgts prior to the onset of the experiment.
F.R = imposed food restriction.
N.B. Levels of significance, (# = $p<5\%$; * = $p<1\%$; ** = $p<0.1\%$), relate to differences between means ($\pm$ standard deviations), in the respective control and test groups.
Trial 1:- Controls and DAHP n=8; F.R n=7.
Trial 2:- Controls and DAHP n=6; F.R n=5.

These observations indicate that while DAHP itself clearly reduces brain total biopterin levels in the rat, when it is administered in the diet there is an additional and confounding effect of food restriction. Given that the spectrum of biochemical alterations associated with starvation is so vast, the results may question the validity of using dietary administration of DAHP to create a specific model of brain BH_4 deficiency in which to assess neurochemical and behavioural status.

The reason why non-absolute food deprivation caused a decrease in brain total biopterins when absolute starvation for 24 and 48 hours did not is probably related to the ages of the animals used in the respective experiments. Weight losses incurred by food restriction in weaner rats are likely to have been at least equivalent in terms of relative severity to those weight losses seen in adult rats subjected to 24 and 48 starvation.

Alternative ways of giving the DAHP to rats were examined to try and overcome the problem of associated food restriction. Administration of a DAHP diet specifically designed to disguise the bitter taste of the pyrimidine, (described by Pabst and Rembold, 1966) (appendix 1), still produced significantly lower levels of food consumption in test animals than controls.

Oral dosing by stomach intubation was confounded by the extreme insolubility of DAHP in suitable vehicles for animal use. Insufficient quantities could be delivered in corn oil to bring about a decrease in brain biopterins. The insoluble nature of the compound similarly made intraperitoneal injection impossible.

9.4: NEUROTRANSMITTER LEVELS AND BEHAVIOURAL STATUS IN THE BRAIN BH_4 DEFICIENT RAT.

As dietary administration of DAHP seemed to be the only situation in which a drop in brain total biopterins was consistently observed, this protocol of DAHP treatment was ultimately used to investigate neurochemical and behavioural status in the biopterin deficient rat.

9.4.1: Behaviour.

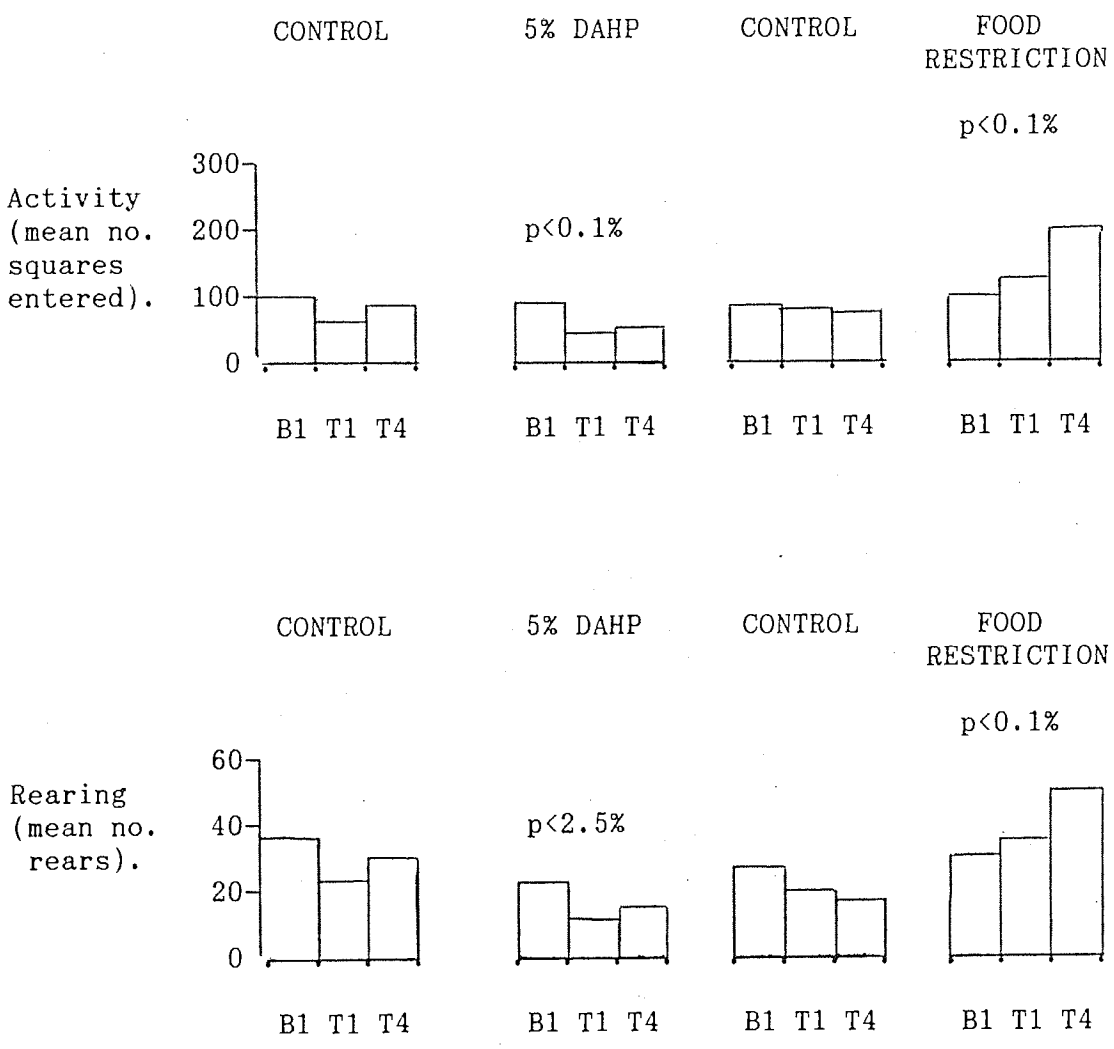
We are unable to attribute specific behaviours to the activity of a single neurotransmitter system, (Gray, 1987). Owing to the role of BH_4 in NA, DA and 5-HT synthesis general behaviours, components of which are known to be primarily controlled by one or more of these transmitters, were measured.

Behavioural analysis was restricted by small sample size and the inherent large degree of variation in behaviour observed between individual animals. Hence any significant differences between the behaviours of the various treatment groups were obscurred by statistical analyses which took into account this variation. However when total group performances were analysed by X^2 analysis differences in behaviour between the groups were apparent.

When brain total biopterins were significantly decreased to 80% of control values by administration of a 5% DAHP diet a decrease in general activity and rearing was observed (Fig. 9.4), a result similar to those also observed in preliminary experiments conducted by Suzuki *et al*, (1988) who showed decreases in locomotor activity in DAHP fed rats. This suggested the possibility of a causative relationship between a decrease in these behaviours and reduced biopterin levels. However, when a corresponding food restriction experiment was performed a decrease in brain total biopterins of 14.5% was accompanied by a rise in activity and rearing, (Fig.9.4).

The opposing effects of DAHP and food restriction suggested that though both treatments produced independent declines in brain biopterin levels they subsequently influenced different neurochemical systems in the brain and thus different behavioural responses.

Fig. 9.4: The effect of a 5% DAHP diet and food restriction on activity and rearing in weaner rats.



Male weaner rats were fed a 5% DAHP diet or subjected to food restriction for 6 days. Controls were allowed normal food *ad lib.* Open field behaviour was assessed two days before the diets were administered, (B1), and on days 1 and 4 after the diets had been given, (T1 and T4 respectively). Total group perfomances were analysed by a X^2 contingency table test and significance levels are indicated on the histograms.

Starvation has been associated with the induction of the "5-HT Hyperactivity Syndrome" (Green *et al*, 1981), resulting from increased synaptic 5-HT avaiability. Acute starvation has been shown to enhance 5-HT synthesis and turnover in the present investigation, (Table 9.7). Partial starvation, ie food restriction, may have resulted in enhanced activity and rearing through similar mechanisms.

Unfortunately, neurotransmitter analysis was not carried out on the food restricted rats described above who showed decreases in brain biopterins and increased levels of activity. A separate food restriction experiment did reveal a slight increase in brain 5-HT and 5-HIAA levels in deprived rats compared to controls, (Table 9.13). However the degree of food deprivation in this experiment was not so severe as before, and the elevations in 5-HT and 5-HIAA were not significant. Brain total biopterins were also unaffected in this experiment and behavioural analysis was not performed.

The ultimate decreases in activity and rearing seen in rats fed DAHP, may arise as a result of the combined effects of the inhibitor and associated food restriction. Both treatments produce a decrease in brain total biopterins and the cumulative effect may override the behavioural effect of food restriction alone causing a decline in activity and rearing which could be directly associated with the intensified reduction in cofactor levels.

9.4.2: Neurotransmitters.

The hypothesis outlined above assumes that brain levels of some or all of the neurotransmitters that depend on adequate BH_4 levels for their synthesis, ie NA, DA and 5-HT levels, are decreased upon DAHP administration thus causing disruption of neurological pethways associated with activity and rearing.

Results of neurotransmitter analyses in rats showing a 35% DAHP-induced drop in brain total biopterins revealed no significant changes in brain NA, DA, 5-HT or 5-HIAA levels, (Table 9.13). Again, it must be considered that use of gross half brain analysis may have masked significant alterations in regional transmitter levels.

Table 9.13: The effect of a 5% DAHP diet and food restriction on brain BH_4 and neurotransmitter status in weaner rats, (ng g^{-1} wet wgt).

GROUP (n)	Values expressed as a % of control values					
	TOTAL BIOPTERINS	NA	DA	5HT	5HIAA	5HIAA:5HT
DAHP n=6	65% (p<1%)	92% (n.s)	81% (n.s)	108% (n.s)	101% (n.s)	96% (n.s)
F.R n=5	99.6% (n.s)	93% (n.s)	98% (n.s)	107% (n.s)	106% (n.s)	100% (n.s)

Male weaner rats were fed a 5% DAHP diet or subjected to food restriction, (F.R), for six days. Each experiment had its own separate control group which was allowed access to normal food *ad lib*. The degree of enforced dietary restriction was equivalent to the amount of self-imposed restriction observed by animals fed DAHP. Results were analysed by an unpaired students t-test and are expressed as a % of the respective control values.
N.B. Levels of significance, (in parenthesis), relate to differences between means (± standard deviations), in the respective control and test groups.

However, other workers while showing decreases in rat brain biopterin pools in excess of 35% with DAHP administration similarly reported no significant alterations in steady-state levels or synthesis of catecholamines or serotonin even in specific regions of the brain, (Gal and Whitacre 1981; Sherman and Gal 1978; Suzuki *et al*, 1988). The results of these DAHP experiments and others are summarised in Table 9.14. Only after severe i.p dosing regimes in weaner rats, producing a 72% reduction in total biopterins were depressions in NA and DA levels observed, and these were only noted in the brain stem an area rich in tyrosine and tryptophan hydroxylase activities, (Suzuki *et al* 1988). Levels of 5-HT remained unaffected.

Table 9.14: Summary of previous experiments conducted with DAHP showing the effects of the inhibitor on BH_4 and neurotransmitter levels.

STUDY	BH_4 STATUS	CATECHOLAMINES + METABOLITES	SEROTONIN + 5HIAA
In vitro study of PC-12 cell culture incubated with $4x10^{-4}$M DAHP. *(Brautigam et al 1984)*	40% ↓ in BH_4 pool	10% ↓ in DOPA production	-
Intracerebral injection of DAHP (3 doses at 90 min intervals of 300ug/ 20ul saline) 250g rats. *(Gal and Whitacre 1981)*	mean 60% ↓ in whole brain reduced biopterin pool	-	no change in whole brain synthesis or level of 5HT
Repeated intraventricular injection of DAHP in rats. *(Sherman and Gal 1978)*	cerebral cofactor pool ↓ by 50%	steady-state synthesis unaffected	steady-state synthesis unaffected
7% DAHP diet to adult mice for 5 days (n=3) *(Cotton 1986)*	whole brain biopterins increased	In whole brains: DOPAC v by 35% NA & DA n.s ↓	-
	Total Biopterins		
4 i.p injections of 1g/kg DAHP in 20ul PBS to weaner rats at 4hr intervals. *(Suzuki et al)*	whole brain ↓ by 66%	-	-
	neocortex + striatum ↓ by 50%	n.s affected	UNAFFECTED IN ALL REGIONS
	diencephalon ↓ by 67%	NA & DA n.s affected DOPAC & HVA ↓ by 45%	
	brain stem ↓ by 72%	NA v by 22% DA v by 35% DOPAC & HVA ↓ by 24%	

In the table above: ↓ = decrease (a value "decreased by x%" is compared to controls values).

In the light of previous work, the biochemical and behavioural results presented in this thesis suggest a number of considerations regarding the role of BH_4 in neurotransmitter synthesis and neurological function.

Firstly, partial reductions in brain total biopterins are not consistently accompanied by a depression in brain neurotransmitter levels in the rat. In fact a large body of evidence shows that brain cofactor pools have to show an extremely marked decline before neurochemical pathways are clearly affected. In humans, even when biochemical measurements clearly indicate impaired BH_4 synthesis, (eg. in PPH_4S deficiency), patients may present with normal levels of neurotransmitter metabolites in the CSF and no observable neurological dysfunction.

This raises questions as to the extent to which partial declines in brain BH_4 levels are rate-limiting for brain monoamine and especially serotonin synthesis and possibly indicates a higher degree of stability in the overall integrity of the BH_4 / monoamine system than has previously been suggested. In 1988 McDonald *et al* identified a hyphenylalaninaemic mouse mutant, (*hph-1*), showing greatly diminished GTP-cyclohydrolase activity but still sufficient BH_4 biosynthetic capacity to allow normal development and neurological function.

A second interesting observation has emerged from this study. Dietary administration of DAHP, an inhibitor of BH_4 biosynthesis, to weaner rats significantly and consistently depressed brain total biopterin levels there being evidence for an associated concomitant decrease in locomotor activity and rearing. However, administration of the same inhibitor produced no alterations in brain neurotransmitter levels in weaner rats indicating a lack of correlation between cofactor levels, neurotransmitter status and behavioural dysfunction.

These observations allow us to speculate as to the possible existence of another role of BH_4 as yet unidentified which accounts for the behavioural changes seen in DAHP administration in the absence of monoamine deficiency. Such a role could be linked to the reducing properties of BH_4 or the compound itself may behave directly as a "neurotransmitter-like" substance thus promoting changes in behaviour which cannot by DA, NA and 5-HT analysis be attributed to monoamine deficiency.

9.5: THE EFFECT OF AGING AND CHRONIC LONG-TERM ADMINISTRATION OF A HIGH ALUMINIUM, LOW CALCIUM DIET ON BRAIN TOTAL BIOPTERIN AND NEUROTRANSMITTER LEVELS IN MICE.

Though direct evidence that aluminium is a causative agent in the development of Alzheimers Disease (AD) is lacking, (Crapper *et al*, 1980), it has been well established that the metal is associated with the pathology of the disease, (Crapper *et al*, 1976; Mann 1983 Candy *et al*, 1986).

As part of his postgraduate research programme Mr. D. Myers, (Aston University), set up an experiment to investigate the effects of normal aging and life-long administration of a high aluminium, low calcium/magnesium diet on brain neuropathology and behaviour in the mouse, with the possibility that such a dosing regime may provide an animal model of an Alzheimers like syndrome, (Ph.D thesis, 1989),

As aluminium is implicated in the development of AD, which is afterall age-related, it seems likely that the neurotoxic effects would be most readily observed in aged animals following a life-time exposure to the compound. Until now, most neurotoxicity studies with aluminium have

involved short-term exposure in young animals.

Brain tissue of aged (23 months), long-term aluminium treated (23 months) and young control mice (3 months), from the Myers study was made available to myself for biochemical analysis.

Whole half brain analyses revealed no significant effect of either aging or aluminium on brain total biopterin, catecholamine or serotonin levels compared to controls. Choline acetyl transferase activity was similarly unaffected.

Anderson, (1987), failed to show a change in whole brain total biopterins or BH_4 biosynthetic capacity upon chronic dietary administration of Al in the form of $Al(OH)_3$ to rats for 6 weeks and other workers have been unable to demonstrate changes in whole mouse brain catecholamine levels with aging, (Finch, 1973).

The possibility that the analyses were restricted by the use of gross brain measurements cannot be ruled out especially since decreases in both NA and DA levels in aging have been consistently reported, (Robinson *et al* 1977; Winblad *et al* 1985;), and workers have shown Al to affect transmitter levels only in certain areas of rat brain.

In the present investigation Al was administered to mice in the diet in the form of aluminosilicates which show low solubility properties and no effect on BH_4 metabolism was observed. It is argued that the low calcium and magnesium content of the diet enhances the absorption of the aluminium species, (Candy *et al* 1986). Unfortunately, no assessment of the amounts of Al that actually accumulated in the brains was made. Impaired BH_4 metabolism has been induced on two separate occasions *in vivo* by 3 months administration of Al to rats in their drinking water,

(Edwards 1988; Cowburn 1989). The Al was given in the more soluble salt form as the acetate.

It is possible that the subsequent neurotoxicity of aluminium is related to the species, or more specifically the solubility of the species, in which it is presented to the living organism.

A series of sophisticated behavioural experiments conducted by Myers indicated significant disturbances in certain aspects of behaviour in the aluminium treated mice compared to age-matched controls. This therefore provides yet another situation in which clearly the presence of neurological dysfunction is not accompanied by central neurotransmitter deficiencies although in this situation brain total biopterin levels were also unaffected as measured by gross half brain analysis.

In order to determine the implications of these observations in terms of the clinical significance of the rate-limiting role of BH_4 in neurotransmitter synthesis, further analyses should be carried out to assess actual BH_4, catecholamine and serotonin status in specific brain areas which have, in so far as is possible, been connected with specific behaviours.

9.6 GENERAL CONCLUSIONS AND FURTHER WORK.

1. While BH_4 metabolism in the liver has been shown to be significantly depressed by acute administration of potential neurotoxins, eg. lead and DES, the integrity of the system in the brain was apparently maintained.

   This may have indicated either:-a greater stability of BH_4 in the brain than the liver due to efficient protective mechanisms in salvage and biosynthesis pathways; failure of the neurotoxins to penetrate the blood brain barrier and accumulate in the brain in sufficient quantities; or the limited efficiency of whole brain analyses which could mask important alterations in biopterin levels in specific brain regions.

   Further work should involve the measurement of actual quantites of compounds entering the brain and also total biopterin levels in specific regions of the brain not just gross analysis of the whole brain. Such studies should help determine the presence , absence and nature of effects of neurotoxins on BH_4 metabolism in the brain.

   Studies should also be carried out to determine long-term rather than acute effects of these neurotoxins on BH_4 metabolism and thus match more closely the situations which might prevail in humans.

2. Long-term (23 month) dietary administration of aluminium to mice in the form of aluminosilicates failed to alter brain total biopterin levels. In the light of previous results showing significant disruption of BH_4 metabolism upon relatively short-term (3 weeks) administration of aluminium salts to rats in

their drinking water further work should be done to determine the relative importance of,

a) the species of aluminium which is administered

b) the vehicle by which it is given,

in aluminium neurotoxicity. These experiments should be performed as life-time exposure studies in the rat and/or mouse and should include determination of final body burdens of aluminium especially in the brain.

3. Acute starvation and non-absolute food restriction in rats resulted in depressions in brain and liver total biopterins. In acute starvation there was evidence that lowered levels of reduced glutathione (GSH) could have induced oxidative breakdown of BH_4 by enhancing the oxidising environment within the tissues. This suggests the possibilty for a role of GSH in maintaining tissue BH_4 levels *in vivo*.

Further work should involve measurement and correlation of GSH levels with biopterins in partially food restricted animals in order to further investigate a possible role of GSH in maintaining tissue BH_4 levels.

Reduction of tissue biopterins by starvation indicates the necessity for careful monitoring of dietary intake when attempting to establish the *in vivo* effects of an experimental agent on BH_4 metabolism. Experiments conducted with DAHP highlighted the importance of this as food restriction associated with administering the compound in the diet was seen to confound measurements of BH_4 metabolism. This certainly limits the usefulness of using DAHP to create an animal model of brain BH_4 deficiency and further work should be done to find an agent which will produce brain BH_4 deficiency without incorporating additional dietary problems.

4. An inhibitor of BH_4 synthesis, DAHP, was shown to decrease total biopterin levels in rat whole brain and also cause inhibition of activity and rearing behaviour. There were however no observable effects of the inhibitor on brain catecholamine or serotonin levels. This may suggest another role of BH_4 which has not yet been shown by which the cofactor regulates neurological function by mechanisms not directly involving NA, DA or 5-HT.

However, further work needs to be done to analyse neurotransmitter levels in specific brain regions in order to eliminate the possibility that alterations in brain neurotransmitters did occur but that these were masked by gross brain analysis.

The difficulties in trying to correlate post-mortem measurements of neurochemical indices with behavioural trends observed in the living animal have been highlighted. Use of *in vivo* voltametry would be one way of overcoming these problems. Further work should also look at more specific behaviours which so far as is possible can be related to the predominant involvement of one neurotransmitter.

APPENDIX 1

The ingredients contained in the DAHP diet prepared to try and disguise the bitter taste of the inhibitor is described below;-

24% (w/w)	Casein
40% (w/w)	Rice Starch
14% (w/W)	Raw Sugar
10% (w/w)	Cocoa fat
7% (w/w)	Cellulose powder
4% (w/w)	Sodium chloride
1% (w/w)	2,4-diamino-6-hydroxypyrimidine (DAHP)

(DAHP was replaced by cellulose in control animals).

Vitamin supplementation was achieved by adding appropriate amounts of crushed commercial vitamin tablets to the diet such that vitamin levels were approximately equivalent to those present in standard rat chow.

The ingredients were blended together and then bound into dough form with a little water. The dough was then minced into small pieces and left to air dry before it was administered to the rats as dry pellets.

The diet was originally described by Pabst and Rembold (1966).

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