The Effects of Some Neurotoxins on Tetrahydrobiopterin Metabolism.

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Doctor of Philosophy

The University of Aston in Birmingham

May 1988

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Summary:

Previous studies in man have shown that following dosing with L-ß-3,4-dihydroxyphenylalanine (L-DOPA) and cotrimoxazole, plasma biopterins were raised. By analogy with dihydropteridine reductase deficient children in whom plasma biopterins are greatly elevated and the observations that these preparations were dihydropteridine reductase inhibitors, it was assumed that these raised plasma levels were due to increased efflux from tissues which resulted in tissue depletion of biopterins. In some human disease states such as senile dementia of the Alzheimer type lowered plasma biopterins were observed; by analogy with tetrahydrobiopterin synthesis deficient children these reduced plasma biopterins were attributed to lowered tetrahydrobiopterin synthesis and concomitant low tissue biopterin levels. Because of ethical considerations it was not possible to measure directly the tissue biopterins changes in either case. The Wistar rat was used as a model for human tetrahydrobiopterin metabolism, since tissues not normally accessible for study in humans, such as the brain and liver, could be examined for the effects on tetrahydrobiopterin metabolism after administration of the various agents. Plasma total biopterins in normal conditions were found to be much higher than in healthy humans. The elevation of plasma total biopterins concentration following the administration of dihydropteridine reductase inhibitors to humans, such as L-DOPA and cotrimoxazole was not observed in the rat. However, the administration of inhibitors of *de novo* tetrahydrobiopterin biosynthesis, such as diaminohydroxypyrimidine (DAHP) and bromocriptine was shown to decrease plasma biopterins concentration. In general, hepatic biopterins were decreased after administration of both dihydropteridine reductase inhibitors and de novo biosynthesis inhibitors.

Drugs which are direct (bromocriptine) or indirect (L-DOPA and Sinemet Plus) agonists at dopamine receptors were investigated and were shown to decrease hepatic total biopterins concentration, but had no effect on brain biopterins.

Bromocriptine was demonstrated as a potent inhibitor of *de novo* tetrahydrobiopterin biosynthesis *in vivo* and *in vitro*

Cotrimoxazole decreased brain tetrahydrobiopterin concentration. DAHP was effective in causing hyperphenylalaninaemia due to tetrahydrobiopterin deficiency in the rat.

p-hydroxyphenylacetate was shown to be an effective inhibitor of dihydropteridine reductase *in vivo* Phenylacetate administration had no observable effect on tetrahydrobiopterin metabolism, but did cause tyrosinaemia. It is proposed that scopolamine reduces tetrahydrobiopterin turnover.

Lead and aluminium exposure caused deranged tetrahydrobiopterin metabolism. Aluminium, but not lead decreased brain choline acetyltransferase activity.

Phenylalanine loading in normal human subjects was followed by an elevation in plasma biopterins which was not observed after tyrosine loading. Plasma N : B ratios correlated well with VEP latencies after tyrosine loading, but not after phenylalanine loading in healthy subjects.

The use of derived pterin measurements as an indicator of tetrahydrobiopterin turnover or tetrahydrofolate status is discussed in the text.

Keywords: TETRAHYDROBIOPTERIN. BROMOCRIPTINE. DOPAMINE AGONISTS. PHENYLACETATE. DERIVED PTERIN.

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ABBREVIATIONS

GTP	Guanosine triphosphate
BH_4	L-*erythro*-5,6,7,8-tetrahydrobiopterin
NH_2TP	D-*erythro*-dihydroneopterin triphosphate
6-PTP	6-pyruvoyltetrahydropterin
PEE	Phosphate eliminating enzyme
DHPR	Dihydropteridine reductase
NADPH	Reduced nicotinamide adenine dinucleotide phosphorylated
NADH	Reduced nicotinamide adenine dinucleotide
qBH_2	Quinonoid dihydrobiopterin
$7,8\text{-}BH_2$	7,8-dihydrobiopterin
mg	milligram
p	pico
n	nano
µ	micro
mol	mole
PHS	Phenylalanine Hydroxylase Stimulating Protein
XOD	Xanthine Oxidase
FAD	Flavine adenine dinucleotide
HPLC	High performance liquid chromatography
PKU	Phenylketonuria
CNS	Central nervous system
i.p.	Intraperitoneal
C	Celsius
Tris	Tris (hydroxymethyl) amino methane
$DMPH_4$	6,7-dimethyl-5,6,7,8-tetrahydropterin

DAHP	2,4-Diaminohydroxypyrimidine
NBT	Nitro blue tetrazolium
PPO	2,5-diphenyloxazole
Dimethyl POPOP	1,4-di-2-(4-methyl-5-phenyloxazole) benzene
HCl	Hydrochloric acid
TCA	Trichloroacetic acid
l	Litre
h	hour
min	minute
KCl	Potassium chloride
UV	Utra violet
M	molar
EDTA	Ethylene diaminetetraacetic acid
CAT	Choline Acetyltransferase
NaCl	Sodium Chloride
DMSO	Dimethyl Sulphoxide
THF	Tetrahydrofolate
5-Me-THF	5-Methyltetrahydrofolate
10-CHO-THF	10-formyltetrahydrofolate
L-DOPA	L-ß-3,4-dihydroxyphenylalanine
i.g.	intra-gastric
MAO-B	Monoamine oxidase B
Ki	Inhibitor constant
Km	Michaelis constant
P:T	Phenylalanine: tyrosine ratio
p-OH	para-hydroxy
SDAT	Senile dementia of the Alzheimer type
$dFPydP_3$	Deoxyformamido-6-hydroxypyrimidine

HPA	Hyperphenylalaninaemia
IQ	Intelligence quotient
PD	Parkinsonism dementia
ALS	Amyotrophic Lateral Schlerosis
GTP-CH	Guanosine triphosphate cyclohydrolase
$Al_2(SO_4)_3$	Aluminium sulphate
GIT	Gastrointestinal Tract
Phe	L-Phenylalanine
Tyr	L-Tyrosine
ns	not significant
p	probability
cAMP	cyclic adenosine 5'monophosphate
qFH_2	Quinonoid dihydrofolate
DHFR	Dihydrofolate reductase
N:B	Neopterin: biopterin ratio
VEP	Visual evoked potential
CSF	Cerebrospinal fluid
P-HPAA	p-hydroxyphenylacetic acid
5-HT	Serotonin
Ach	Acetylcholine
P_2	Visual evoked potential response to flash stimulation
P100	Visual evoked potential response to pattern reversal stimulation
ODS	Octadecylsilane

Chapter 1. Introduction.

CHAPTER ONE.

1 INTRODUCTION.

1:1 Tetrahydrobiopterin : Background.

Tetrahydrobiopterin (BH_4) is one of a class of naturally occurring compounds called 'pterins' derived from the parent compound 2- amino-4-oxodihydro-pteridine (pterin) (1). All pteridines have a common basic nitrogen containing pyrimido (4,5-*b*)-pyrazine ring (2)(Purrman, 1940).

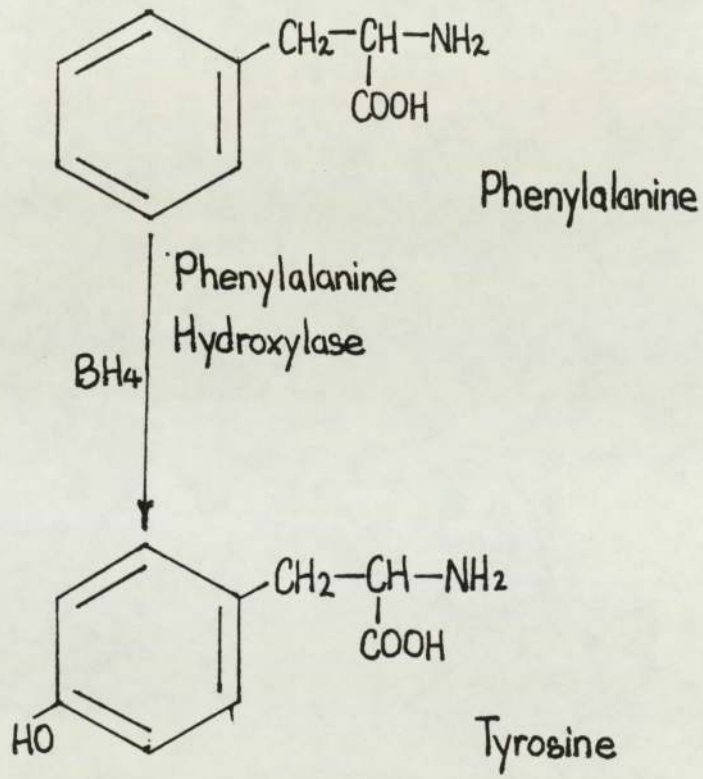
Biopterin (6-1',2'-dihydroxypropylpterin), the oxidation product of *L-erythro*-5,6,7,8- tetrahydrobiopterin (3) was isolated from human urine by Patterson *et al* in 1955, and is thought to be derived from dihydrobiopterin and tetrahydrobiopterin in mammalian fluids and tissues. Blair and Pearson (1974) showed that fully oxidized biopterin could result from free radical chain oxidation of BH_4 to 7,8-dihydrobiopterin (4) and further oxidation to biopterin (5).

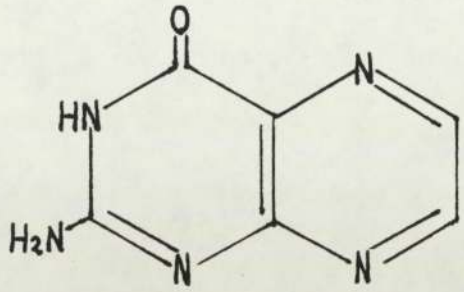
1:2 Tetrahydrobiopterin : Biosynthesis.

Guanosine triphosphate (GTP) (6) is the initial substrate for *de novo* generation of BH_4 (Fig.4). GTP is converted to D-*erythro*-dihydroneopterin triphosphate (NH_2TP)(7) in an Amadori type rearrangement by GTP cyclohydrolase (EC 3.5.4.16) ; C_8 of GTP is released as formic acid (Burg & Brown 1968) forming 2-amino-6 (triphosphoribosyl) amino -5 or 6-formamido-4-hydroxpyramidine; ring closure resulting in production of NH_2TP (Fig.5). The synthesis of BH_4 from NH_2TP is thought to proceed via tetrahydropterin intermediates.

Two separate groups have shown BH_4 is formed via 6R-(1',2'-dioxo-propyl)-tetrahydropterin or 6-pyruvoyltetrahydropterin (6-PTP)(8). This step is catalysed by a magnesium dependent enzyme designated as pyruvoyltetrahydropterin synthase by Smith & Nichol (1986) or phosphate eliminating enzyme (PEE) by Heintel

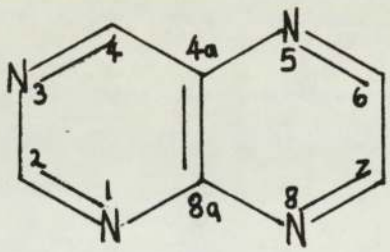
Fig1. The hepatic hydroxylation of Phenylalanine.





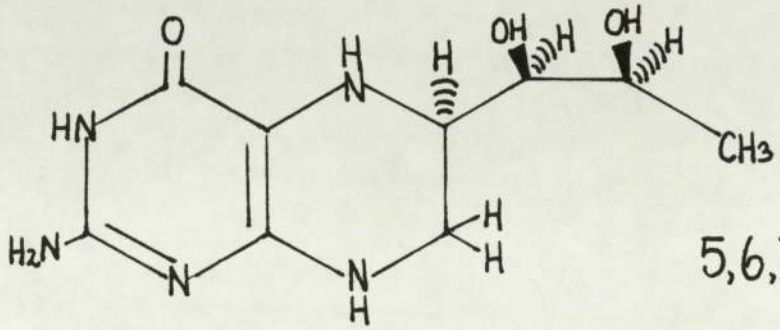
(1)

pterin



(2)

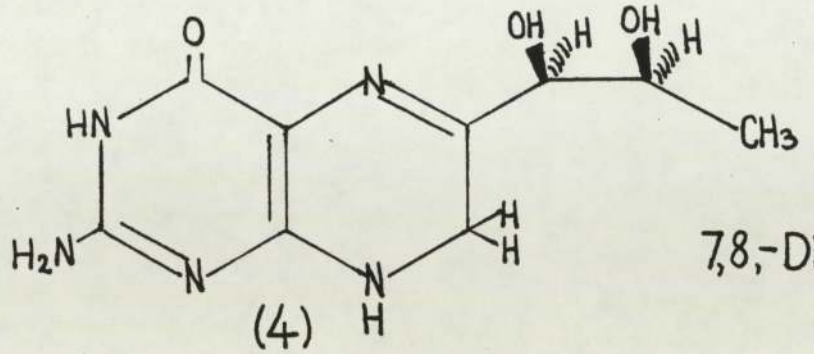
pteridine ring



(3)

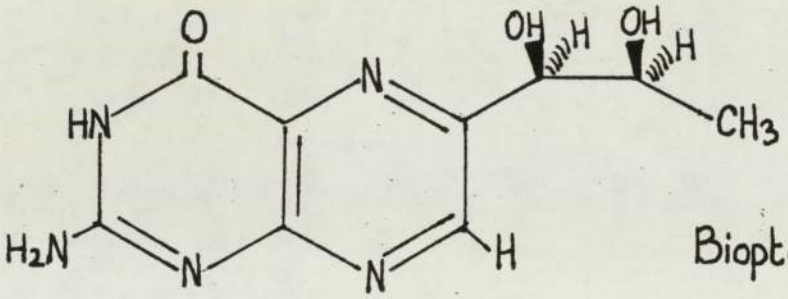
5,6,7,8-Tetrahydrobiopterin

▬ Bonds rising out of plane of the paper.
))))) Bonds going into the plane of the paper.
— Bonds in the plane of paper.



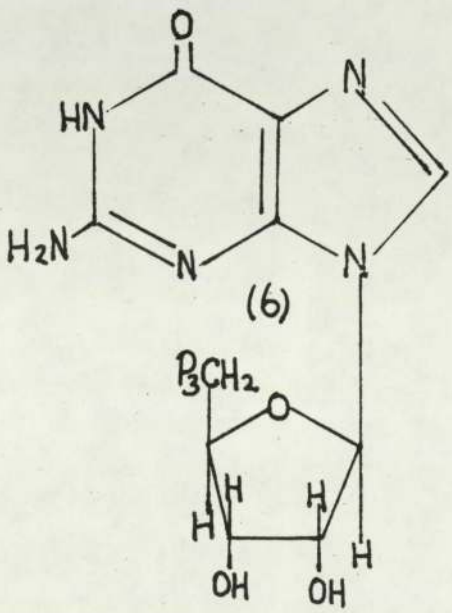
(4)

7,8,-Dihydrobiopterin



(5)

Biopterin



(6)

Guanosine triphosphate

et al (1984). 6-PTP is thought to be converted to 6R-(L-1'hydroxy -2'-propyl)tetrahydropterin or 6- lactoyltetrahydropterin (9) and this is then converted to BH_4. One enzyme catalyzes both reactions. It is similar to sepiapterin reductase in that it is inhibited by N-acetyl-serotonin and is NADPH dependent. Smith & Nichol (1986) have proposed that the enzyme be known as H_4-biopterin synthase. The rate-limiting step of BH_4 biosynthesis in humans is thought to be at the level of PEE but in the rat at the GTP-cyclohydrolase stage. Neopterin derived from NH_2TP can be detected in human but not rat tissues and fluids under normal conditions (Sawada *et al*, 1986).

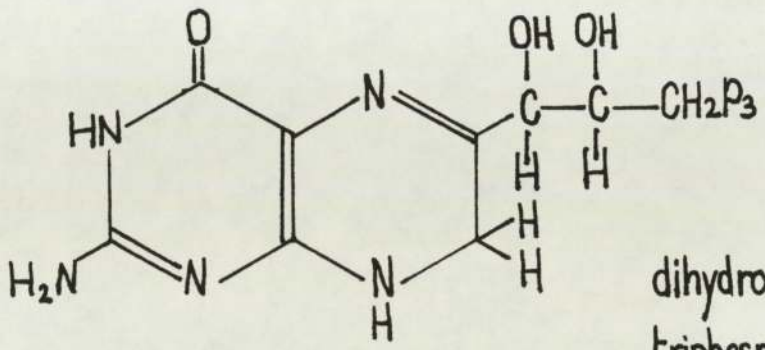
1:3 Tetrahydrobiopterin : Salvage.

Cellular BH_4 levels are maintained by a salvage pathway using the enzyme dihydropteridine reductase (DHPR) (EC 1.6.99.7) which requires NADH (Kaufman 1965, Craine *et al* 1972)(Fig.6). In the absence of this reaction, quinonoid dihydrobiopterin (qBH_2)(10) rapidly tautomerizes to 7,8-dihydrobiopterin ($7,8-BH_2$), and this is lost from the cell and excreted in the urine. $7,8-BH_2$ is not a substrate for DHPR (Kaufman 1967, Archer *et al*, 1972, Gready, 1985). qBH_2 retains the stereochemistry of the dihydroxypropyl side-chain of BH_4, whereas it is lost on formation of $7,8-BH_2$ (Pfleiderer, 1987). Dihydrofolate reductase (DHFR) (EC 1.5.1.3) is capable of reducing $7,8-BH_2$ to BH_4 (Milstien & Kaufman, 1983) although this is not thought to be of great importance under normal conditions. It may be important in DHPR deficiency (Smith *et al*, 1986).

1:4 Tetrahydrobiopterin : Functions In Neurobiochemistry.

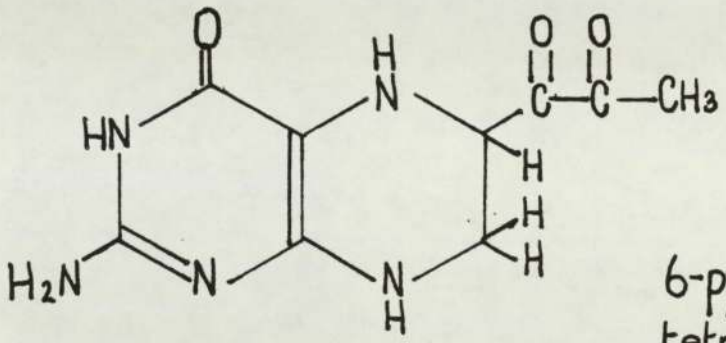
L-*erythro*-5,6,7,8-tetrahydrobiopterin is the coenzyme required for the biosynthesis of serotonin and the catecholamine neurotransmitters, dopamine and noradrenaline (Leeming, 1980). BH_4 in all of these reactions is a requirement of hydroxylase enzymes.

Kaufman (1958) showed that phenylalanine hydroxylase (EC1.14.16.1), which converts the neurotransmitter precursor phenylalanine, to tyrosine (Fig.1) requires a cofactor other than the presence of NADH and dioxygen described by Udenfriend and



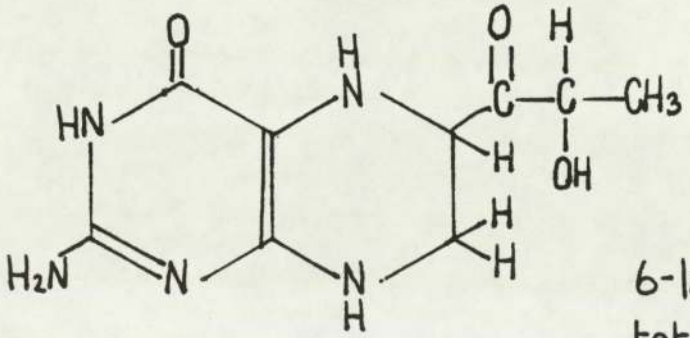
(7)

dihydroneopterin
triphosphate



(8)

6-pyruvoyl
tetrahydropterin



(9)

6-lactoyl
tetrahydropterin

Cooper (1952). Studies by Kaufman (1958), Coulson *et al* (1970) and Kaufman (1958) suggested it to be a pteridine. The natural coenzyme for phenylalanine hydroxylase is BH_4 (Kaufman 1963).

Kaufman (1964) and Brenneman and Kaufman (1964) showed that BH_4 is required by tyrosine hydroxylase (EC 1.14.16.2) to convert tyrosine to L-DOPA (Fig.2). Enzymatic production of L-DOPA from tyrosine by tyrosine hydroxylase is thought to be the rate limiting step in production of dopamine and noradrenaline neurotransmitters (Kettler, 1974). Bullard *et al*, (1978) examined the regional distribution of BH_4 in rat brain and found highest concentrations in the pineal gland (500 pmol mg^{-1}protein) and the pituitary gland (45 pmol mg^{-1}protein). Smaller amounts occur in the striatum, mesencephalon and hypothalamus. Lowest concentrations were detected in the cerebral cortex (1.5 pmol mg^{-1} protein). This regional distribution correlated with the distribution of tyrosine hydroxylase but not with that of tryptophan hydroxylase. Conversion of tryptophan to 5-hydroxytryptophan (Jequier *et al* 1969 , Friedman *et al* ,1972) by tryptophan hydroxylase (EC 1.14.16.4), the initial step in serotonin synthesis (Fig.3) might also be dependent on BH_4 , although other possible regulations exist such as partial pressure of oxygen and influence of end-product inhibition (Costa & Meek 1974).

1:5 Tetrahydrobiopterin : Oxidation.

During its activity as a coenzyme in hydroxylase reactions , BH_4 is rapidly oxidized to qBH_2 (Kaufman , 1967) ; and BH_4 is regenerated by DHPR activity (Craine 1972). The mechanism by which BH_4 is oxidized to qBH_2 is not known . *In-vitro* work suggests that the mechanism occurs by generation of a free radical at carbon 4_a ($C4_a$) ; and this radical is in turn further oxidized to qBH_2 by a one electron transfer with formation of hydrogen peroxide (Bobst 1967 , Lazarus *et al* 1982) by an autocatalytic reaction (Mager & Berends 1965 , Blair & Pearson 1974). *In-vitro*, at acidic pH the chain carrier is the hydroperoxyl radical ($HO_2\cdot$), but at neutral and alkaline pH the chain carrier is thought to be the superoxide anion radical ($O_2\cdot -$) (Blair & Pearson 1974 , Blair & Pearson 1975). The oxidation of BH_4 at neutral pH leads to quantitative amounts of pterin with little or no biopterin formation.

Fig 2. The Biosynthesis of Catecholamine Neurotransmitters.

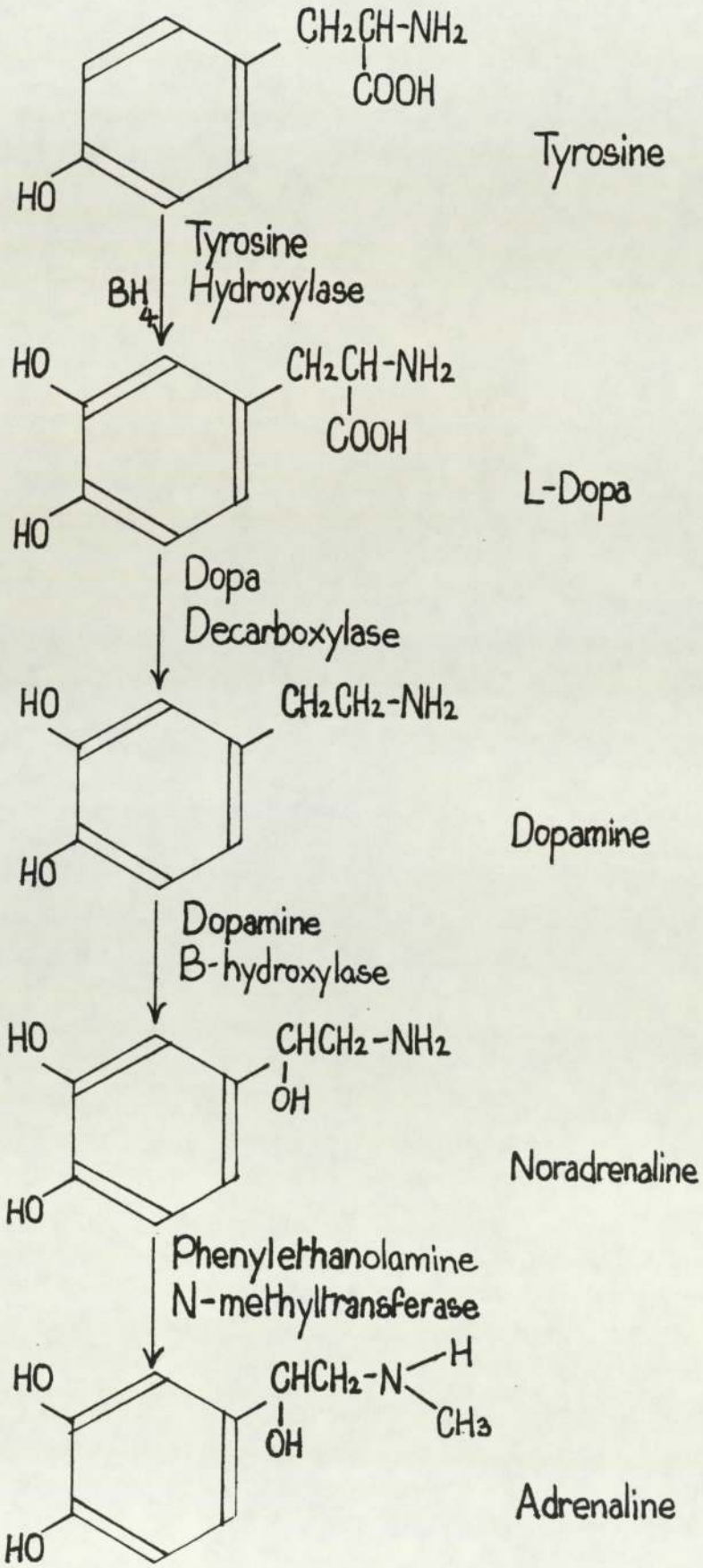
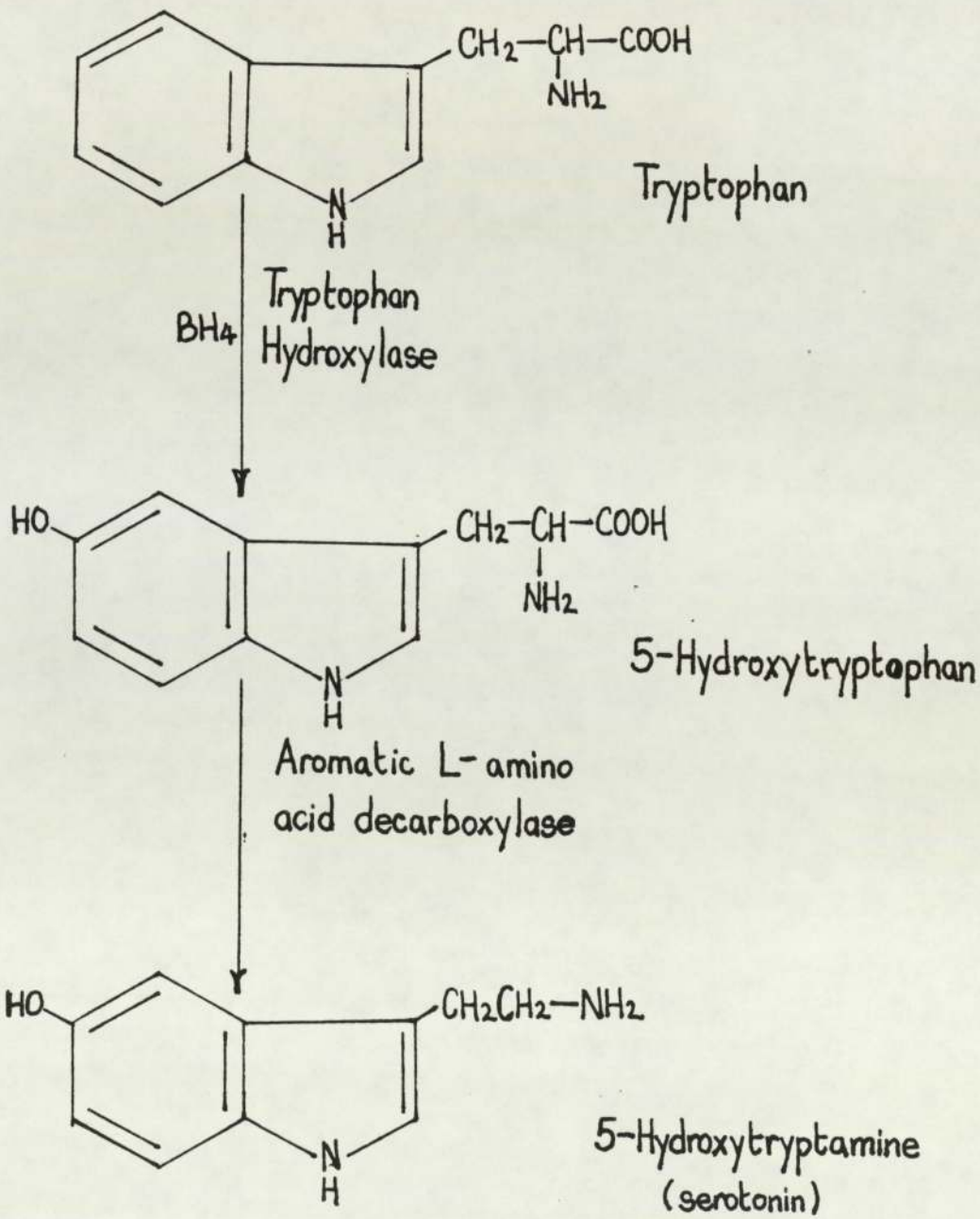


Fig3 The biosynthesis of serotonin



In-vivo, BH_4 is oxidized to quinonoid dihydrobiopterin which rearranges to 7,8-dihydrobiopterin and may be further oxidized to biopterin. 7,8-dihydropterin (11) can be produced from BH_4 by oxidation and side-chain loss. Pterin can result from oxidation of 7,8-dihydropterin (Pfleiderer, 1975; Milstien, 1983).(See Chapter 6 for the definition and discussion of 'derived pterin').

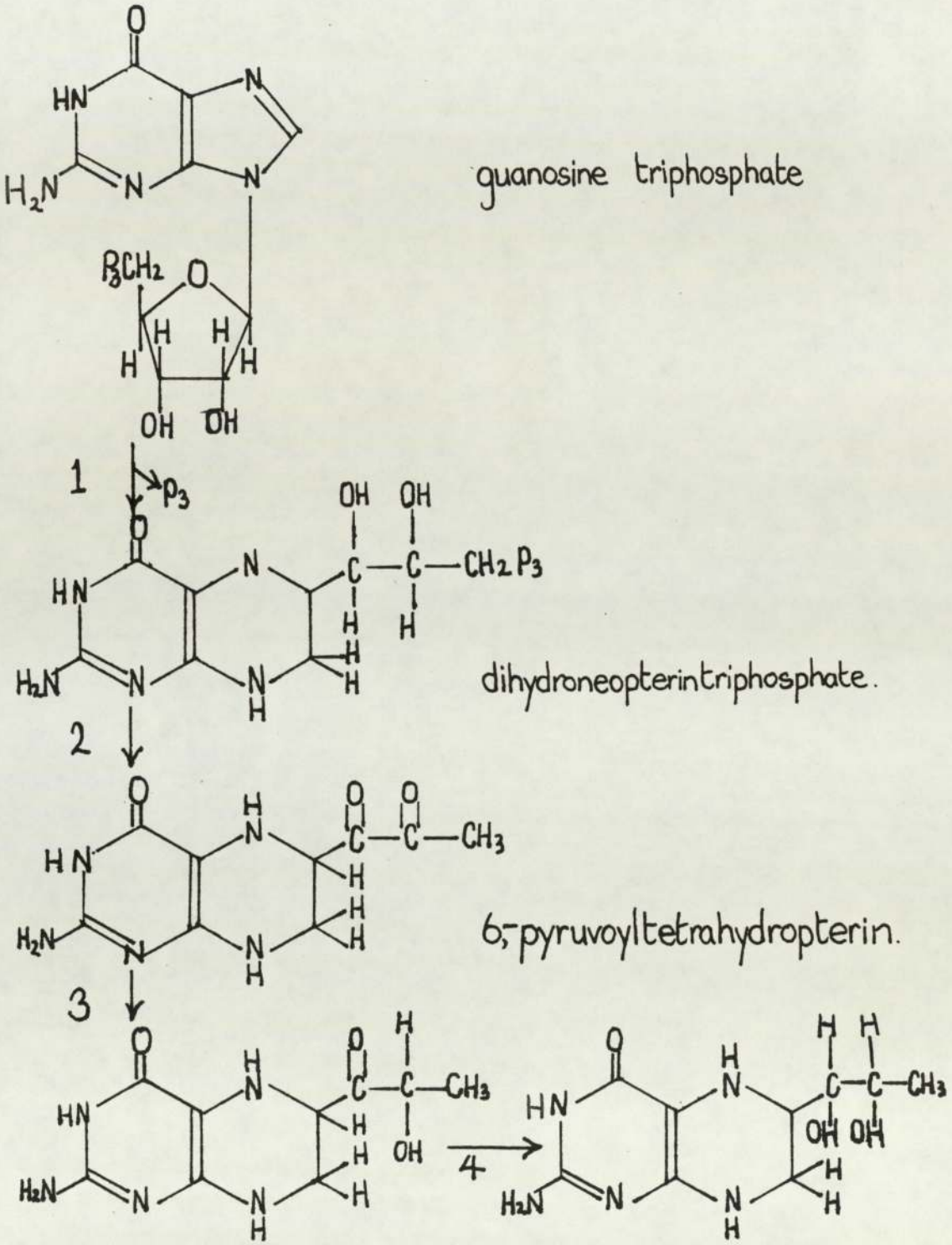
Kaufman (1986) reviewed that during enzymatic BH_4 oxidation, a protein designated the Phenylalanine Hydroxylase Stimulating Protein (PHS) catalyses the conversion of the intermediate to qBH_2 (Fig.7). This intermediate is thought to be a 4-∂-carbinolamine of BH_4, and might be formed from tetrahydropterin hydroperoxide, which may be the hydroxylating species or a precursor of it. Iron is also essential for the activity of phenylalanine hydroxylase (Fisher *et al*, 1972).

1:6 Tetrahydrobiopterin : Catabolism In The Mammal.

Besides oxidation and excretion of tetrahydrobiopterin into urine as described above, other factors are involved in the balance of cofactor pools. Rembold *et al* (1977) showed that reduced pterins are transported by high affinity binding proteins . The kidney is mainly responsible for their balance . Most of the pterins once filtered through the renal tubules are not reabsorbed but are excreted with the urine (Rembold 1983) . The second way in which pterin pools are balanced is by irreversible breakdown . This is initiated by irreversible oxidative elimination of the side-chain (Rembold 1971). Rat liver homogenate has been shown to be capable of degrading BH_4 to pterin, 7,8-dihydroxanthopterin (12) (30%), lumazine (13) and 6- (14) and 7-hydroxylumazine (15) (40%) respectively .

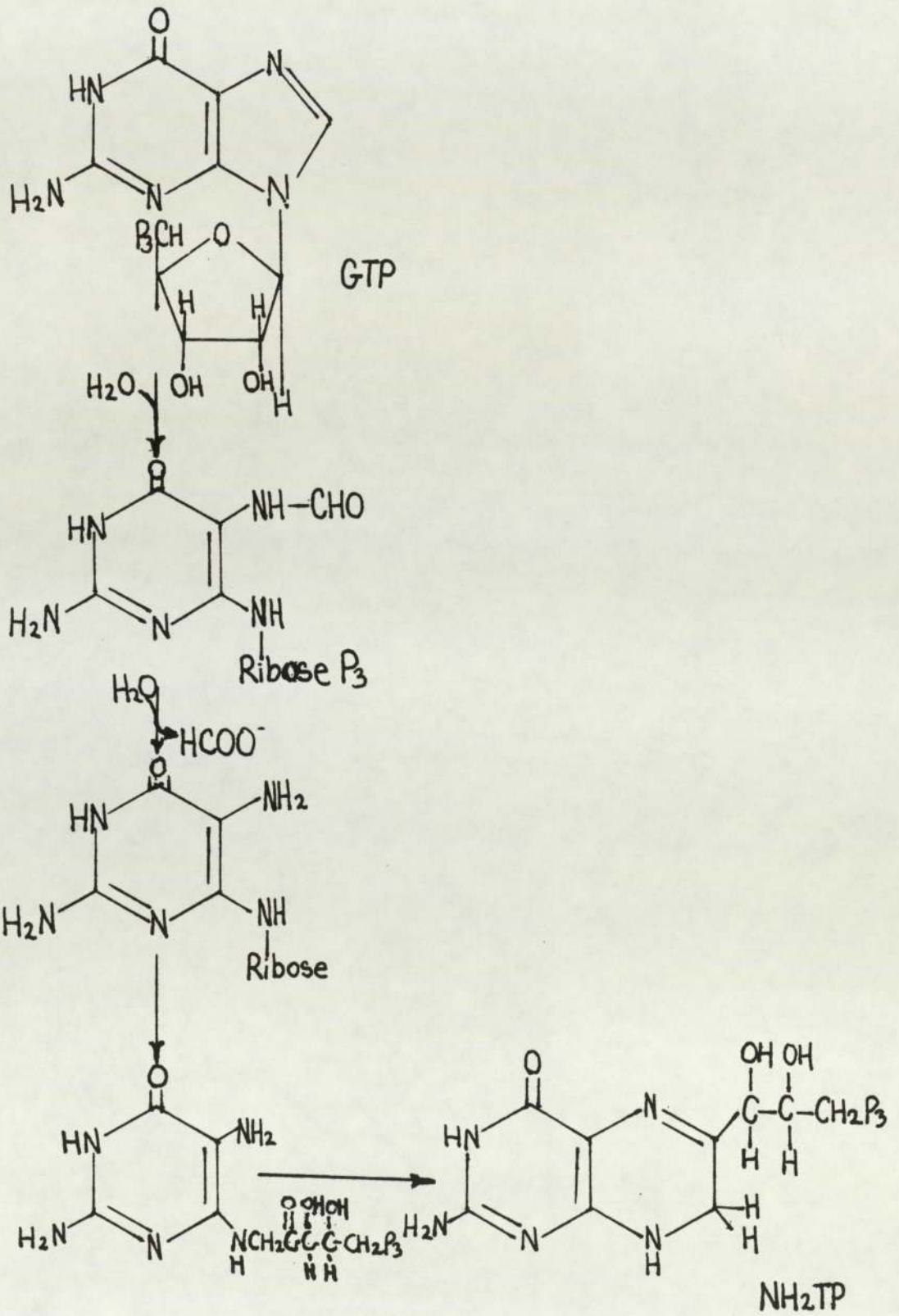
In-vivo studies using labelled BH_4 indicate a primary incorporation into liver and kidney as the main cofactor pools (Rembold 1982). Predominant metabolites in rat urine are pterin , xanthopterin (16) and its deaminated product 6-hydroxylumazine ; the deamination possibly brought about by a pterin deaminase . The same situation is present in human urine analysed for pterins. In mammals, xanthine oxidase (xanthine : oxygen oxidoreductase EC 1.2.3.2) (XOD) which contains FAD, molybdenum and iron in the ratio of 1:1:4 , catalyses oxidation of xanthine, hypoxanthine and other purines , aldehydes, NADPH and some pteridine compounds. Pterin hydroxylated in position 7 finally yields isoxanthopterin (17). Pterin is not hydroxylated in position 6 by XOD.

Fig4. The de-novo biosynthesis of tetrahydrobiopterin.



1. GTP cyclohydrolase 2.&3. 6-pyruvoyltetrahydropterin synthase, Mg^{2+} & NADPH 4. Sepiapterin reductase & NADPH

Fig 5 Formation of dihydroneopterin triphosphate from guanosine triphosphate by GTP-cyclohydrolase



Niederwieser *et al* (1986), reported that pterin deaminase is present in rat liver, but not in human liver. In man, in contrast to the rat, the side-chain of BH_4 is mainly retained and modified to 1' oxo and 2' deoxy derivatives and 2'- deoxysepialumazine (18) is the main faecal metabolite. In man, deamination occurs mainly in the gut by bacterial action.

1:7 Tetrahydrobiopterin : Analysis.

Biopterin derivatives can be measured in mammalian tissues and fluids (Baker *et al* 1974, Leeming *et al* 1976 , Leeming & Blair 1980, Frank *et al* 1963 , Pabst & Rembold 1966). The most commonly used techniques are high performance liquid chromatography (HPLC) (Fukushima & Nixon 1978, Fukushima & Nixon 1980) and microbiological assay using *Crithidia fasciculata* (Baker *et al* 1974, Leeming & Blair 1980, Blair *et al* 1983). This latter method has disadvantages in that it is unable to distinguish between different biopterin derivatives - it measures BH_4 , biopterin, 7,8-dihydrobiopterin, L-neopterin and sepiapterin and also there is a loss of BH_4 in the assay during autoclaving (Milstien 1983, Blair *et al* 1983). Another method used is an enzymatic assay using phenylalanine hydroxylase (Gurroff *et al* 1967) , however erroneously high readings are obtained due to generation of Fenton's reagent which is known to effect non-specific oxidation of phenylalanine (Blair & Pearson 1975). Other techniques are gas chromatography-mass spectrometry (GC-MS) after conversion of the pterins to their trimethylsilyl derivatives (Rothler & Karobath 1976), and by radioimmunoassay (Nagatsu *et al* 1981). Mass spectrometry of naturally occurring pteridines was first demonstrated by Blair and Foxall (1969).

1:8 Tetrahydrobiopterin Involvement In Neurological Disease.

Phenylketonuria is an inherited metabolic disorder first reported by Folling (1934), characterized by high plasma phenylalanine levels and high urinary phenylpyruvic acid. The most common form is due to a gross deficiency of phenylalanine hydroxylase (Jervis *et al* 1947). Treatment involves prolonged strict dietary restriction of phenylalanine intake.

1-3% of all cases of PKU (Bartholome 1974 , Bartholome & Bird 1975 , Kaufman *et al* 1975 , Danks *et al* 1978) are termed 'malignant' or 'atypical' PKU and are not

Fig. 6 Salvage of tetrahydrobiopterin. by DHPR

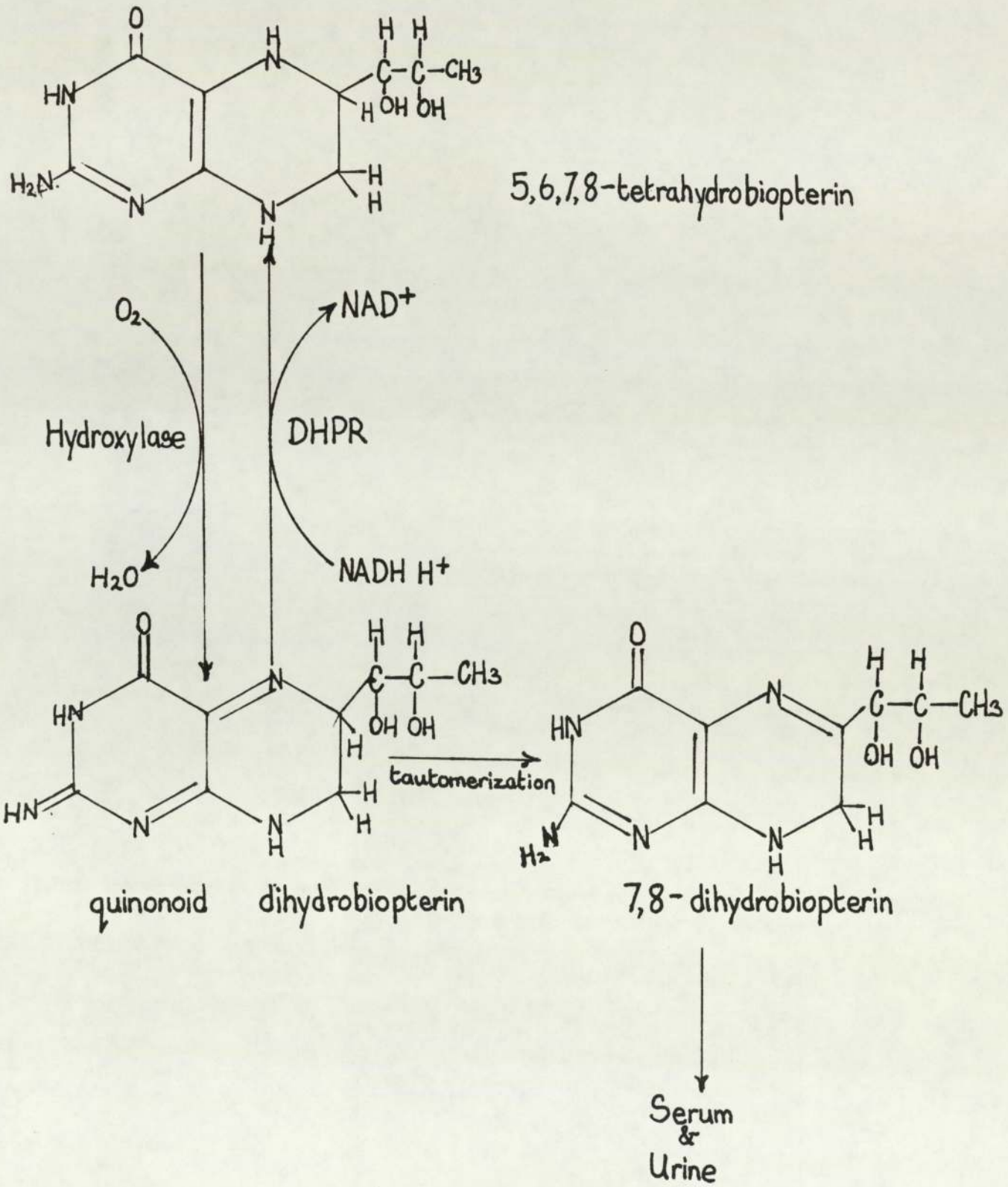
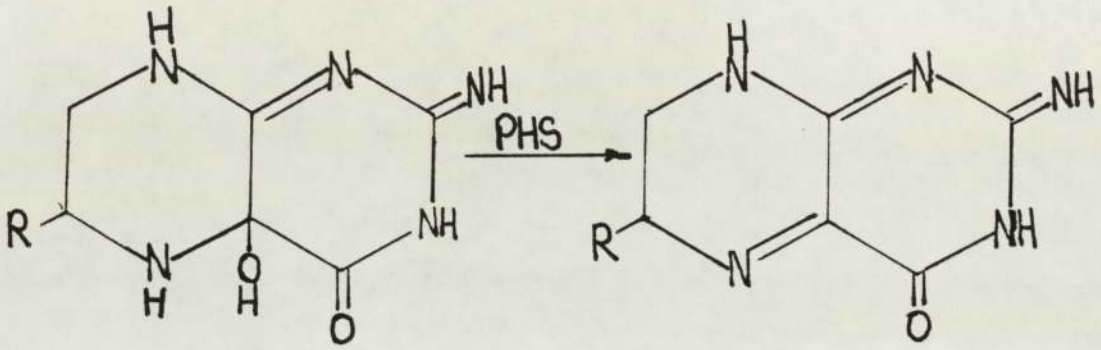
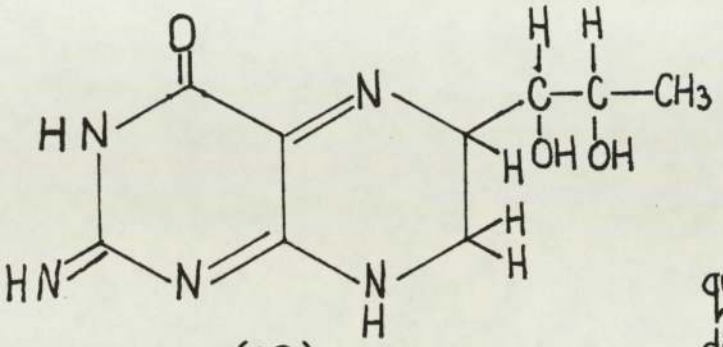


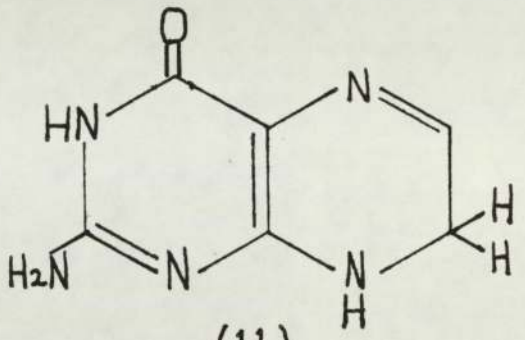
Fig7 The action of Phenylalanine Hydroxylase stimulating protein





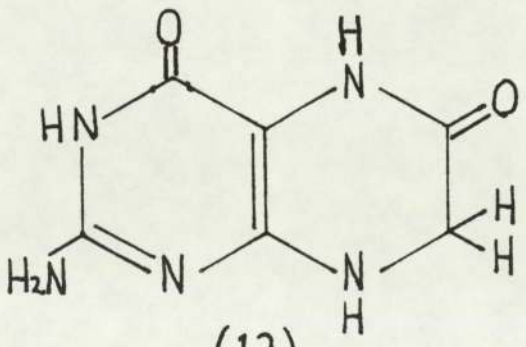
(10)

quinonoid
dihydrobiopterin



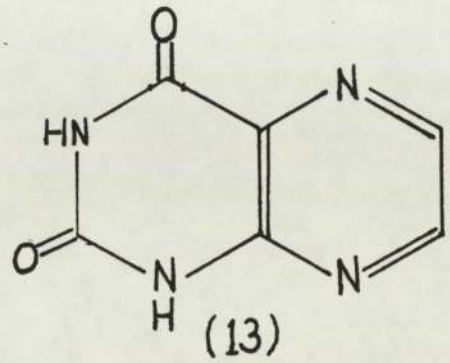
(11)

7,8-dihydropterin



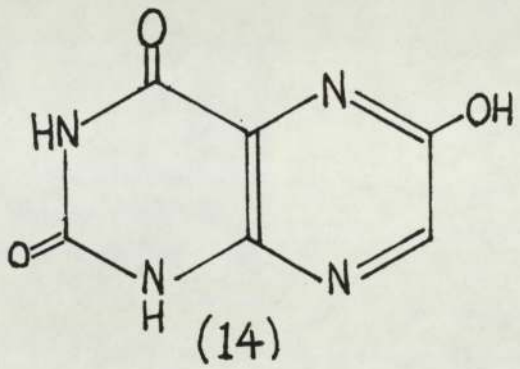
(12)

7,8-dihydroxanthopterin



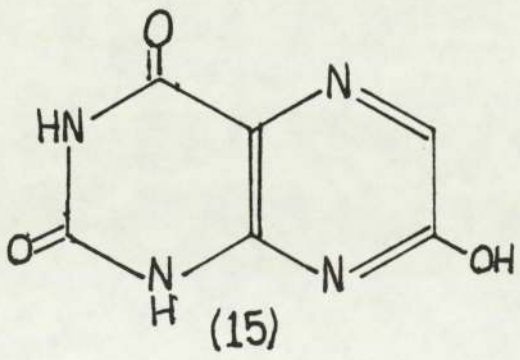
(13)

lumazine



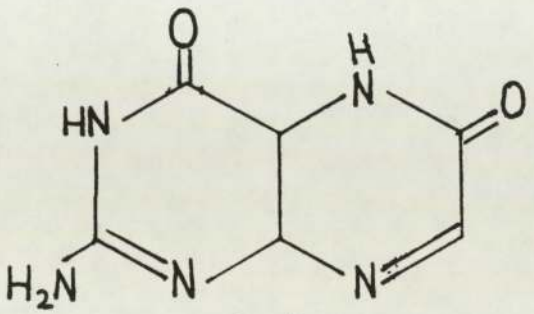
(14)

6-hydroxylumazine



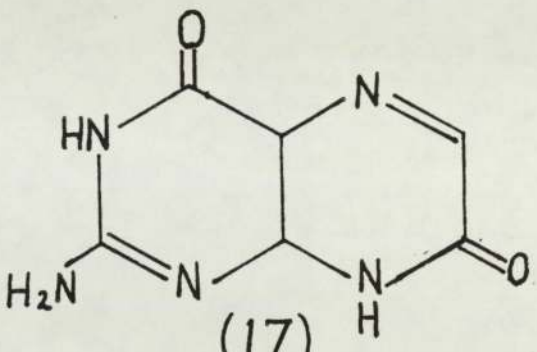
(15)

7-hydroxylumazine



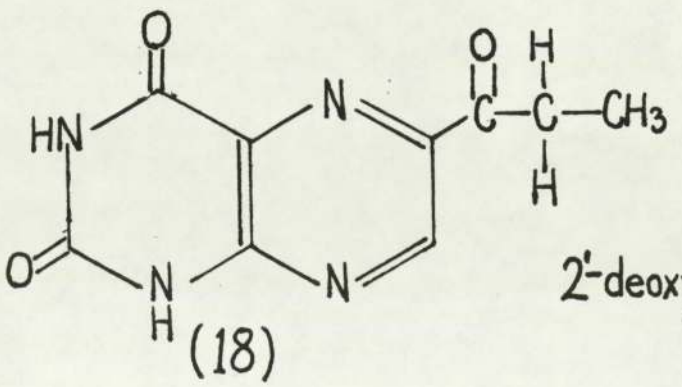
(16)

xanthopterin



(17)

isoxanthopterin.



(18)

2'-deoxysepialumazine

controllable by dietary restriction of phenylalanine . These are due to a tetrahydrobiopterin deficiency brought about by a lesion at one of a possible number of points in the *de-novo* biosynthesis of BH_4 or salvage of qBH_2 by DHPR (Kaufman *et al* 1975 , Rey *et al* 1977 , Danks *et al* 1979) . BH_4 biosynthetic deficiencies have been demonstrated in the conversion of NH_2TP to BH_4 (Rey *et al* 1977 , Bartholome *et al* 1977, Danks & Cotton 1980 , Dhondt & Farriaux 1983) ; and a block in the GTP to NH_2TP step due to a deficiency of GTP cyclohydrolase (Joller *et al* 1983 , Niederwieser *et al* 1984).

BH_4 biosynthetic deficiency is characterised by significantly lower than normal urinary and serum levels of total biopterins, and the urine level of neopterin is much elevated above normal (Leeming, Pheasant and Blair, 1981). BH_4 synthesis is absent from liver (Rey *et al*, 1977). With total DHPR deficiency, serum levels of total biopterins are much higher than normal and urinary biopterins are often increased but may be within the normal range (Leeming, Pheasant and Blair, 1981). DHPR activity is zero in erythrocytes (Barford, Blair and Leeming, 1982) and liver (Rey *et al*, 1977). The increased serum levels of total biopterins is due to increased cell efflux of biopterins caused by lack of the salvage enzyme.

In both deficiency states cell BH_4 is reduced, dopamine, noradrenaline and serotonin concentrations are substantially lowered and blood phenylalanine levels are significantly increased (Blair, 1983). The main clinical feature of all types of phenylketonuria is progressive neurological deterioration in the neonate (Smith, 1974).

Other neurological disease states in which a disturbance of BH_4 metabolism has been reported are summarized (Table 1.01).

1:9 Aims of the Thesis.

Tetrahydrobiopterin is essential for the formation of catecholamine and serotonin neurotransmitters and so is important for normal functioning of the central nervous system. It follows that a deficiency of this coenzyme can lead to neurological damage.

The aim of this thesis was to investigate the *in vivo* effects of neurotoxins either suspected, or previously shown to have *in vitro* effects on BH_4. These investigations were

further expanded with *in vitro* assays.

Brain and liver tissues and plasma were examined. BH_4 metabolism was examined in the liver so that activity could be studied in the absence of certain enzymes such as tyrosine hydroxylase and any exclusion effect of the blood-brain barrier was absent. Plasmas were used since in human studies, this fluid is one of the most convenient to obtain. By studying this fluid, changes in BH_4 metabolism in the periphery can be compared and contrasted to those in the CNS.

Table 1:01

Disease state

Decreased Blood or Serum Biopterin :	
Inherited Dystonia	LeWitt *et al* (1983) Williams *et al* (1979)
Depression	Curtius (1983)
Lead poisoning	Purdy *et al* (1981)
Senile Dementia	Leeming & Blair (1980)
Decreased Urinary Biopterin :	
Depression	Jones (1987)
Decreased Biopterin Synthesis :	
Alzheimer's Disease	Aziz *et al* (1983) Morar *et al* (1983) Jones (1987) Barford *et al* (1984)
Lead Poisoning	Purdy *et al* (1981)
Aluminium Poisoning	Cowburn (1987)

Table 1:01

Disease state.

Decreased Cerebrospinal Fluid Levels of BH_4 :	
Huntington's Chorea	Williams *et al* (1980)
Inherited Dystonia	Williams *et al* (1979)
Presenile Dementia	Williams *et al* (1980)
Parkinson's Disease	Williams *et al* (1980) Lovenberg *et al* (1980) Yamaguchi *et al* (1983) Nagatsu *et al* (1984)
Increased Serum Levels of BH_2 :	
Downs syndrome	Aziz *et al* (1982)
Decreased DHPR Activity :	
Retts Syndrome	Cowburn (1987)
Lead poisoning	Purdy *et al* (1981)
Autism	Cowburn (1987)
Aluminium Poisoning	Leeming & Blair (1979)

Table 1:01: Neurological Disease States In Which There Is Altered BH_4 Metabolism.

Chapter 2. Materials and Methods.

CHAPTER TWO.

2 MATERIALS AND METHODS.

2:1 Animals.

Animals used were male Wistar rats (supplied by Bantam and Kingman Limited, Hull, England) and maintained on a standard rat and mouse breeding diet (Pilsbury's Limited, Birmingham) with *ad libitum* access to tap water. They were kept at 26C with a 12:12 h dark : light cycle in suppliers batches. Experimental and control groups were age and sex matched.

Intraperitoneal dosing was performed using a sterile hypodermic syringe fitted with a 0.5 x 16mm gauge or 0.8 x 40mm gauge dosing needle as appropriate to the compound being administered.

Oral dosing was done using a sterile hypodermic syringe fitted with an olive bulbed oropharyngeal needle. Sacrifice was by cervical dislocation.

2:2 Design of experiments.

In all cases, experimental and control tissue was analysed on the same day with assays of both groups being run alternatively to minimize experimental error.

2:3 Statistics.

All values are expressed as arithmetic means with standard deviations and number of observations on different tissues recorded. Statistical probability was derived from unpaired two tailed Student's t tests.

2:4 Chemicals.

L-biopterin, pterin, D-neopterin and L-sepiapterin were obtained from Dr. B.

Schircks, Jona, Switzerland.

The following were purchased from the Sigma Chemical Company, Poole, Dorset: ascorbic acid, Tris (Tris(hydroxymethyl)aminomethane, reduced nicotinamide dinucleotide (NADH), 6,7 - dimethyl - 5,6,7,8 - tetrahydropterin ($DMPH_4$), sodium azide, bovine serum albumin, trichloroacetic acid, L-phenylalanine, L-tyrosine, phenylacetate, p-hydroxyphenylacetate, diaminohydroxypyrimidine (DAHP), guanosine triphosphate (GTP), reduced adenine dinucleotide phosphate (NADPH), alkaline phosphatase (Type 3 *E.Coli*), heparin, horseradish peroxidase, physostigmine salicylate (Eserine salicylate), acetyl coenzyme A, tetraphenylboron, choline bromide, scopolamine hydrochloride, dimethyl sulphoxide, nitro blue tetrazolium, Hanks solution, 2,5-diphenyloxazole (PPO), 1,4- di- 2-(4- methyl-5-phenyloxazole) benzene (dimethyl POPOP), catalase and 5-sulphosalicylic acid.

Concentrated hydrochloric acid, hydrogen peroxide, perchloric acid, HPLC grade methanol and potassium chloride were purchased from the Fison Chemical Company, Loughborough.

Sephadex G-25M columns were from Uppsala, Sweden. ^{14}C-acetyl coenzyme A was obtained from Amersham International, Amersham.

Bromocriptine mesylate (Parlodel) was a gift from Sandoz, Sinemet Plus from Merck, Sharp and Dohme, Cotrimoxazole (Septrin) from Wellcome and L-DOPA from Dr. R.J. Leeming, the General Hospital, Birmingham.

All other chemicals were of AnalaR grade or equivalent and were obtained from BDH Chemicals, Poole, Dorset.

2:5 Analysis of total biopterins and pterin.

2:5:1 Brain and liver assays.

Immediately after excision, tissue was stored at -70C. 20% homogenates (w/v) were prepared in 0.1M HCl/20% trichloroacetic acid (TCA) using 0.3g of tissue and centrifuged for 15 minutes at 100,000 g in a MSE Superspeed Ultracentifuge and a MSE 10x10 ml angle head rotor. Supernatant volume was noted and then acid/iodine oxidized by addition of 50µl of acid/iodine solution (3.0% (w/v) iodine in 1M HCl, 6.0% (w/v) KI). After 1h, ascorbate was added to reduce the iodine solution, and samples were

analysed by reverse phase HPLC with fluorescent detection (excitation wavelength = 360nm ; emission wavelength = 450nm)(Fukushima and Nixon , 1980). Liver samples were diluted 1:10 in glass distilled water. Estimation of the amount of biopterins and pterin present was achieved by determinations from calibrations prepared using standard biopterin and pterin.

The concentrations of stock standard solutions were determined by uv spectra at pH 13.0 on a Shimadzu uv 240/ uv visible recording spectrophotometer with constant temperature cell holder. Absorbance at 362nm for biopterin and 358nm for pterin was measured and concentration of standard solutions was determined using the Beer-Lambert Law where $E_{max.} = 8.3x10^{3}\ M^{-1}cm^{-1}$ for biopterin and $6.6x10^{3}M^{-1}cm^{-1}$ for pterin (Fukushima and Nixon , 1980, and Blakley, 1969 respectively). Quartz cuvettes were used.

2:5:2 Plasma total biopterins and pterin.

Blood was removed from the thorax after cardiac puncture into heparinized containers (1000 units of heparin ml^{-1} isotonic saline) and plasma was obtained after spinning down blood cells in a MSE bench centrifuge. Plasma total biopterins and pterin were measured by one of two methods:

.(1) Plasmas were deproteinated by adding 30µl of 60% perchloric acid to 300µl of sample and mixing thoroughly. 10µl of acid/iodine solution was added and mixed and left for 1h in the dark. At the end of this time excess dry ascorbate was added and supernatant was obtained after bench centrifugation. This was assayed as above by HPLC.

(2) Plasmas were assayed by *Crithidia fasciculata* at the General Hospital, Birmingham by Dr. R.J. Leeming.

2:6 DHPR activity.

2:6:1 Tissue preparation.

2:6:1:1 Brain.

The whole brain was removed and homogenized on ice. A 30% homogenate (w/v) was prepared in 0.5M Tris-maleate , pH 6.8 buffer using a Potter-Elvehjem homogenizer. Supernatant was obtained after ultracentrifugation at 100,000 g for 45 minutes at 0C

using a MSE Superspeed Ultracentrifuge and a MSE 10x10ml angle head rotor. Lipid deposits were removed by passing the supernatant through muslin cloth.

2:6:1:2 Liver.

The liver was dissected out on ice , cut up with a scissors and homogenized. Excess blood was removed by washing with buffer before homogenization and supernatant was obtained as for brain tissue. Liver supernatant was diluted 1:10 with buffer for enzyme assay.

2:6:2 Enzyme assay.

The method used was essentially that of Craine *et al* (1972). DHPR activity was determined spectrophotometrically by the disappearance of reduced nicotinamide adenine dinucleotide (NADH) at 340nm , 37C using a Unicam SP1700 uv spectrophotometer with a constant temperature cell and recorded using a Unicam AR55 linear recorder.

The standard assay contained the following in a final volume of 1ml:
0.05M Tris-maleate buffer, pH 6.8 , 250 µM sodium azide , 1 mM hydrogen peroxide, 8µg horseradish peroxidase, 0.1 mM 6,7-dimethyl-5,6,7,8 tetrahydropterin ($DMPH_4$), 100 µM NADH and 0.02ml of enzyme source. After addition of DHPR source there was an incubation period of 90s at 37C. The reaction was initiated by addition of $DMPH_4$.

Peroxidase and hydrogen peroxide were used to generate the quinonoid pterin produced by oxidation of $DMPH_4$. Sodium azide was present to inhibit catalase activity. Specific activity of DHPR was expressed as nmoles NADH oxidized $minute^{-1} mg^{-1}$ protein. Protein was determined by the biuret method (Gornall *et al*, 1949). Molar extinction coefficient of NADH was $6.22 \times 10^3 M^{-1} cm^{-1}$. Blank assays were run with distilled water in place of enzyme source.

2:7 Assay of overall tetrahydrobiopterin biosynthesis in brain and liver tissue.

BH_4 biosynthesis was measured by a method based on that of Fukushima and Nixon (1975). 25% homogenates (w/v) of brain or liver tissue as appropriate were prepared in 0.01M Tris-HCl, 0.04M KCl buffer, pH 8.0

using a Potter-Elvehjem homogenizer. Supernatants were collected after ultracentrifugation in a MSE Superspeed 50 Ultracentrifuge at 25,000g for 1 h.

The incubation medium had a final volume of 1ml and contained the following components; 6mM guanosine triphosphate, 3mM NADPH, 1mM magnesium chloride, 750µM buffer, 100µl of tissue extract and distilled water to make up to 1ml. Blank assays were prepared to measure endogenous total biopterins present in the tissue, omitting GTP and NADPH and replacing these compounds with distilled water.

Assays were incubated at 37C for 3h in the dark, after which the reaction was terminated by addition of 2ml of 0.1M HCl and acid/ iodine oxidized as described previously to measure total biopterins by the method of Fukushima and Nixon (1980).

1.0ml samples were originally freeze-dried using a Vertis freeze-drier and then reconstituted in 250µl of glass distilled water and total biopterins measured by HPLC (Fukushima and Nixon, 1980), but the assay was found to be more reproducible by running neat rather than freeze-dried samples (Heales, 1987). Biosynthetic activity of the tissue was expressed as ng of biopterin synthesized $h^{-1}mg^{-1}$ of protein. Protein was measured by the biuret method (Gornall, 1949).

2:8 Sepiapterin Reductase assay.

2:8:1 Tissue preparation.

Brain and liver tissue was prepared as for DHPR assay in 0.5M Tris-HCl buffer, pH 6.8.

2:8:2 Enzyme assay.

The method used was based on that of Matsubara *et al* (1966). Each incubation contained 50µM sepiapterin, 100µM NADPH, 0.5M Tris-HCl buffer,pH 6.8, and 50µl of enzyme extract in a total volume of 1ml. Blank assays had water in place of the enzyme. Molar extinction coefficient of sepiapterin is $10.4x10^{3}\ M^{-1}cm^{-1}$ (Sueoka 1982).

Reaction mixtures were incubated at 37C and the decrease in absorbance at 420nm was measured on a Shimadzu uv 240/ uv visible recording spectrophotometer.

Specific activities were expressed as nmoles of sepiapterin reduced $min^{-1}mg^{-1}$ protein. Protein was estimated by the biuret method (Gornall 1949).

2:9 Guanosine Triphosphate Cyclohydrolase assay.

2:9:1 Tissue preparation.

Blood was collected from rats by cardiac puncture. Whole blood was assayed after lysing by diluting a sample 1:10 in distilled water and leaving to stand for 30 minutes.

Plasma was prepared by bench centrifuging heparinized blood (1000 units of heparin ml^{-1} of isotonic saline).

Brain tissue was prepared by making up 20% homogenates (w/v) in 0.1M Tris-HCl buffer pH 7.8. Supernatants were collected after ultracentrifugation in a MSE Superspeed Ultacentrifuge at about 20,000 g for 20 minutes.

2:9:2 Enzyme assay.

The method used was that of Duch *et al* (1984). Samples were passed over a 0.5 x 6.5 cm column of Sephadex G-25 (medium) which had been equilibrated with 0.1M Tris-HCl, pH 7.8, containing 0.3M KCl, 2.5 mM EDTA and 10% (v/v) glycerol. The columns were washed with two 600µl aliquots of the same buffer; the first aliquot was discarded, and the second was retained for assay of the enzyme.

Reaction mixtures containing 200µl of the Sephadex eluate and 50µl of 60mM GTP were incubated for 90 minutes at 37C in the dark. The reaction was stopped by the addition of 25µl of acid/ iodine solution (1% I_2, 2% KI in 0.1M HCl). Excess iodine was reduced by the addition of 25 µl of 2% ascorbic acid.

The neopterin triphosphate produced was dephosphorylated by the addition of 25 µl of 1M NaOH followed by 1.5 units of bacterial alkaline phosphatase and incubated for 1h in

the dark. The reaction was terminated by the addition of 50 µl 1M acetic acid.

Following bench centrifugation, the neopterin was determined by HPLC calibrated with standard neopterin (molar extinction coefficient = 8.3×10^3 $M^{-1}cm^{-1}$, Fukushima & Nixon, 1980). Control assays (blanks) were performed by replacing water instead of GTP. Activities were expressed as ng of neopterin produced h^{-1} mg^{-1} of protein. Protein was measured by the biuret method (Gornall, 1949) on the Sephadex eluate.

2:10 Measurement of Phenylalanine and Tyrosine.

2:10:1 Tissue preparation.

2:10:1:1 Brain and liver.

The method used was based on that of Hyland *et al* (1985). Tissue was dissected and kept on ice. 20% homogenates (w/v) in 0.02M HCl were prepared and supernatant was obtained after ultracentrifugation at 100,000 g for 10 minutes using a MSE Superspeed Ultracentrifuge. The supernatant volume was noted and the sample was deproteinated with 30 mg ml^{-1} 5-sulphosalicylic acid; and ultracentrifuged at 100,000 g for 5 minutes and supernatant volume was again recorded. Samples were diluted as required for HPLC. Values were expressed as µmoles of amino acid l^{-1} tissue supernatant.

2:10:1:2 Plasma.

Whole blood was obtained by cardiac puncture in rats or by withdrawal from a vein in human subjects (by Dr. R.J. Leeming, General Hospital, Birmingham) and collected into heparinized containers (1000 units of heparin ml^{-1} of isotonic saline).

Plasma was prepared by centrifugation in a MSE bench centrifuge and volumes were noted. This was then treated with 30 mg ml^{-1} 5-sulphosalicylic acid, mixed thoroughly and ultracentrifuged as for brain and liver tissue. Plasma volume was recorded and samples were analyzed by HPLC (see below for conditions).

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

2:11 High Performance Liquid Chromatography (HPLC).

HPLC analysis of samples was performed using the components and conditions described below:

2:11:1 HPLC components.

Pump:	LDC Constametric Model 2.
Injection System :	Waters Intelligent Sample Processor (WISP), Model 710B.
Pre-column:	LichroCart ODS 5µ.
Column:	Phase Separations Spherisorb ODS 5µ 250mm x 4mm.
Detector:	Kontron SFM 23 Spectrofluorometer.
Recorder:	W&W Model 302 pen recorder.

2:11:2 HPLC conditions.

For analysis of total biopterins, neopterin and pterin:

Mobile phase: 5% methanol : glass distilled water degassed with helium.

Solvent flow rate: 1.0 ml min^{-1}.

Excitation wavelength=360nm

Emission wavelength=450nm

Retention time for biopterin = 8-9 min.

Retention time for neopterin = 4-5 min.

Retention time for pterin = 11-12min.

For analysis of aromatic amino acids:

Mobile phase: Sodium acetate (6.80g) and citric acid (1.05g) were added to 900ml of distilled water, pH was adjusted to 5.22 with NaOH and the volume made up to 1 litre with glass distilled water and degassed with helium.

Solvent flow rate: 1.4ml min^{-1}.

Excitation wavelength=255nm

Emission wavelength =281nm

Retention time for phenylalanine = 11-12min.

Retention time for tyrosine = 4- 5 min.

(conditions from Hyland *et al*, 1985).

2:11:3 Identification and quantitation.

The equipment was calibrated for quantitative determination of the compound being measured by injecting known concentrations of pure standard and calculating chromatogaphic trace peak areas by triangulation. Identification was ensured by comparison of retention times (Fukushima & Nixon, 1980), 'spiking' of tissue samples with standard compounds and comparison of chromatographic traces with blank assays as appropriate.

2:12 Ion-exchange chromatography.

Brain tissue and plasma were prepared as for HPLC and then assayed for phenylalanine and tyrosine by ion-exchange chromatography to evaluate use of the HPLC method. Assays were performed by Sheena Grant, at the Children's Hospital, Birmingham.

2:13 Choline Acetyltransferase (CAT) assay.

2:13:1 Tissue preparation.

2:13:1:1 Brain assays.

Brains were completely excised, weighed and kept cool on ice. 5% homogenates (w/v) were prepared in 10mM EDTA, pH 7.4 (Fonnum, 1975), and activated with 0.5% (w/v) Triton X-100 to ensure total release of enzyme activity (Fonnum, 1975). Supernatants were collected for assay after ultracentrifugation at 10,000g for 15 minutes.

2:13:1:2 Human blood assay.

Heparinized whole blood from a human subject was lysed by diluting 1:5 with distilled water. Protein was measured after adequate dilution by the biuret method (Gornall, 1949).

2:13:2 Enzyme assay.

Radiochemical assays on CAT must be based on reproducible, rapid and specific procedures for isolating acetylcholine from the incubation mixture. Fonnum (1975)

achieved this by isolating labelled acetylcholine by 'liquid cation exchange', using sodium tetraphenylboron.

Each incubation contained the following in a total volume of 70µl: 0.2mM acetylcoenzyme A, 300mM NaCl, 50mM sodium phosphate buffer (pH 7.4), 10^6 dpm ml^{-1} ^{14}C labelled acetyl coenzyme A, 8mM choline bromide, 20mM EDTA (pH 7.4), 0.1mM physostigmine (to inhibit acetylcholinesterase) and enzyme source.

50µl of substrate mixture and 20µl of CAT enzyme were used to give an assay volume of 70µl. Assays were incubated for 15 minutes at 37C and then washed into scintillation vials with 7ml of 10 mM sodium phosphate buffer (pH 7.4) and 3ml of acetonitrile solution containing 15mg tetraphenylboron was added, followed by 10 ml of scintillation mixture (containing scintillation grade toluene, 0.05% PPO and 0.02% dimethyl POPOP). A quench curve was prepared for the scintillation mixture.

Samples were shaken lightly for 1 minute and after standing for 10 minutes were counted in a ß-counter, together with blanks containing distilled water in place of enzyme source, and standard ^{14}C acetylcoenzyme A samples in scintillation mixture. The procedure depends on:

(1) Acetonitrile in toluene constitutes an efficient extraction solvent for liquid cation exchange extraction of acetylcholine with sodium 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 (Fonnum , 1975) whereas acetylcoenzyme A remains in the aqueous phase and is not counted since water does not function as a scintillation solvent. CAT activity was tested over a range of % (w/v) homogenates and acetyl coenzyme A concentrations to determine optimum conditions for assay.
CAT specific activity was expressed as µmoles of acetylcholine formed h^{-1} g^{-1} wet weight of tissue, for brain tissue and pmoles of acetylcholine formed h^{-1} mg^{-1} protein for blood.

2:14 Protein estimation by the biuret method

The method used was that of Gornall, 1949. Assays contained 2ml of biuret reagent (copper sulphate pentahydrate, (0.15% (w/v); sodium potassium tartrate, 0.6% (w/v); NaOH, 3.0% (w/v); KI, 0.1% (w/v); in water), 0.5ml of distilled water in blanks and

0.4 ml of distilled water in test assays, and 0.1 ml of the protein source (diluted if required, since biuret is used to measure samples containing 1-10 mg ml^{-1} of protein).

This was mixed well and left at room temperature for 30 minutes. Optical density was measured at 540 nm using a Shimadzu uv 240/ uv visible recording spectrophotometer. A protein calibration was prepared using bovine serum albumin standards made to appropriate concentrations in the required buffer system.

The biuret reaction occurs when two or more peptide bonds form a purple complex with copper salts in aqueous solution. Practically no substances other than protein normally present in biological material give the biuret reaction to an extent sufficient to cause significant interference (bile pigments absorb very weakly in the range 540-560nm) making the assay very reliable.

2:15 Nitroblue tetrazolium (NBT) assay.

This assay is used to estimate tissue oxidative stress (Bachner & Nathan, 1968). Hanks Balanced Salts Solution (Hanks BSS) was diluted 1:10 from stock with distilled water. 0.5g of brain tissue was used after dissecting the brain completely on ice and homogenizing. This was washed in Hanks and 2 ml of the Hanks solution was added to the brain tissue on ice. The sample was homogenized and incubated at 37C in 1 ml of NBT reagent for exactly 20 minutes (NBT reagent was prepared by adding 33.60 mg NBT to 400 µl DMSO and making up to 17.60 ml with Hanks).

The incubate was bench centrifuged and supernatant was discarded. 1 ml of neat pyridine was mixed with the pellet and the blue colour was extracted by boiling in a water bath (taking care not to allow the pyridine to evaporate). Again the sample was centrifuged in a MSE bench centrifuge and supernatant was decanted. 50 µl of the supernatant was immediately made up to 1 ml in distilled water (1:20 dilution). Speed was required because the supernatant frequently gels.

Absorbance of the extracted diluted blue colouration of formazan was read at 578 nm using a Shimadzu uv 240/ uv visible recording spectrophotometer. Calibrations were prepared using standard formazan. Molar extinction coefficient of formazan at 578 nm is $16.9 \times 10^{3}\ M^{-1}\ cm^{-1}$. Results were expressed as µmoles of formazan produced g^{-1} wet weight of brain tissue in a set time.

2:16 Total and differential folate assays.

2:16:1 Tissue preparation.

Brain and liver tissue was excised on ice and accurately weighed. 10 ml of 0.1 M phosphate buffer, pH 7.0 was added to 0.5 g tissue and homogenized using a Potter-Elvehjem homogenizer. 0.1 ml chick plasma conjugase (prepared by Dr. R.J. Leeming at the General Hospital, Birmingham) was added to test homogenates and incubated for 1.5 h at 37C. Blank assays were also prepared containing 0.1 ml distilled water in place of conjugase.

2:16:2 Conjugase test.

Conjugase activity was tested to validate the assays by running incubations of yeast extract in the presence and absence of the enzyme.

2:16:3 Folate measurement.

Samples were measured after appropriate dilution by the *Lactobacillus casei* assay at the General Hospital, Birmingham by Dr. R.J. Leeming.

2:16:4 Identification of folate species using *Lactobacillus casei*

2:16:4:1 Tissue preparation.

0.1M phosphate buffer, pH 7.0 homogenates (w/v) were prepared as above with and without incubating with conjugase. These samples were differentially oxidized by the method of Coppell (1984):

Neutral pH oxidation

5 mg ml^{-1} of catalase was added to 2.5 ml of homogenate, immediately followed by 0.1 ml of I_2 / KI solution (prepared as for assay of total biopterins) and 2.4 ml of phosphate buffer. After mixing, samples were incubated for 1h at room temperature. 0.05 g of ascorbate was added to terminate the oxidation.

Acid pH oxidation (pH 1.5)

0.25 ml of 2M HCl was added to 2.5 ml of homogenate followed by 0.1 ml of I_2/KI solution (as above), mixed and left for 1 h at room temperature. The pH was then brought to neutral using 0.26 ml of 1M NaOH and the volume made to 5ml using phosphate buffer. Oxidation was terminated by addition of 0.05 g of ascorbate.

No oxidation

2.5 ml of homogenate sample and 2.5 ml of phosphate buffer were mixed together and incubated for 1 h at room temperature. 0.05 g of ascorbate was then added. Samples were measured by *Lactobacillus casei* as before.

Mammalian tissue differential folate pools consists mainly of tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-Me-THF) and 10-formyltetrahydrofolate (10 CHO-THF) (Coppell, 1984).

Differential oxidation of folates:

Assay using *Lactobacillus casei*	Folate measured
(A) Total folate (no oxidizing conditions)	THF, 5 Me-THF, 10 CHO-THF.
(B) Following acid oxidation with iodine	10 CHO-THF.
(C) Following neutral pH oxidation with iodine and catalase	5 Me-THF, 10 CHO-THF.

It follows that:

THF = (A) - (C) 5 Me-THF = (C) - (B) 10 CHO-THF = (B).

2:17 Atomic absorption spectrophotometry.

Tissue samples were freeze-dried overnight in a Vertis freeze-drier. They were reconstituted with 0.4ml 0.2mM EDTA made up in AnalaR water. Tissue lead concentrations were estimated using flame atomic absorption (Perkin-Elmer 560 Atomic Absorption Spectrophotometer) after allowing time for chelation of the lead and bench centrifugation. Conditions were an air-acetylene flame, wavelength was 283.3 nm and slit width was 0.7 nm. The apparatus was calibrated with a 20 $\mu g\ ml^{-1}$ lead standard, using lead nitrate.

Chapter 3. The *In-Vivo* Effect Of Potential Dihydropteridine Reductase Inhibitors On Tetrahydrobiopterin Metabolism.

Chapter Three.

3 The *In-Vivo* Effect Of Potential Dihydropteridine Reductase Inhibitors On Tetrahydrobiopterin Metabolism.

3:1 Introduction.

The following compounds have been shown to inhibit DHPR in *in-vitro* studies.

3:1:1 L-DOPA and Sinemet Plus.

L-ß-3,4-dihydroxyphenylalanine (L-DOPA)(19) is an important precusor of dopamine (20), which is a neurotransmitter endogenously present in the central nervous system functioning in the nigro-striatal, mesolimbic-mesocortical and tuberoinfundibular pathways (Green & Costain, 1981). L-DOPA is produced by the action of tyrosine-3-hydroxylase (EC 1.14.16.2) in the brain. This enzyme requires BH_4 as cofactor (Leeming, 1981).

A deficiency of dopamine, the decarboxylated product of L-DOPA is found in Parkinson's disease in the nigro-striatal system (Ehringer & Hornykiewicz, 1960). Hornykiewicz (1966) has suggested that the release of the inhibitory control of the dopaminergic pathway from the substantia nigra to the pallidus is responsible for the rigidity associated with Parkinsonism whilst the akinesia is due to lack of dopaminergic control in the neostriatum. Following these observations, L-DOPA therapy was undertaken (Hornykiewicz review, 1973). The standard maximum dose is about 120 mg/kg body weight /day (Martindale, 1977).

Purdy *et al*(1981) showed that *in-vitro* dopamine could inhibit DHPR by 50% at concentrations of 0.11mM. L-DOPA itself had no effect in *in-vitro* studies. Aziz (unpublished) showed that low doses of L-DOPA administered to parkinsonian patients increased plasma biopterins as measured by *Crithidia fasciculata* assay, suggesting *in-vivo* DHPR inhibition. In view of these findings, the *in-vivo* effect of L-DOPA on BH_4 metabolism was investigated in rats.

When used clinically, L-DOPA has side-effects associated with its peripheral metabolism due to the wide distribution of decarboxylase enzyme in gut and blood vessels. These side-effects include nausea, vomitting and hypotension. Bartholini and Pletscher (1967) suggested the possibility of improving L-DOPA therapy and decreasing the incidence of peripheral side-effects by administration of peripheral decarboxylase inhibitors that do not cross the blood-brain barrier. Use of such inhibitors does not stop L-DOPA induced dyskinesias, which is a CNS side-effect of the drug.

One such drug, containing L-DOPA and a peripheral decarboxylase inhibitor is Sinemet Plus (Merck, Sharp & Dohme). The peripheral decarboxylase inhibitor used is carbidopa. Ratio of L-DOPA : carbidopa is 4:1. Sinemet Plus was used here to increase the dose of L-DOPA entering the CNS.

3:1:2 Cotrimoxazole.

Cotrimoxazole is a combination antibiotic containing trimethoprim (21) and sulphamethoxazole (22) in a ratio of 1: 5. England and Coles (1972) found that patients on cotrimoxazole had raised fasting serum phenylalanine levels suggesting a block in the conversion of phenylalanine to tyrosine. Andrews *et al* (1976) demonstrated that cotrimoxazole impaired the L-phenylalanine tolerance of control subjects after oral and intravenous phenylalanine loading. Leeming *et al* (1976) showed that patients on a cotrimoxazole preparation (Septrin, Wellcome) had elevated serum biopterin derivatives as measured by *Crithidia fasciculata*.

In 1976, Stone administered high doses of trimethoprim to rats (intraperitoneal injections of 5mg and 50mg per rat-the maximum standard human dose is 40 mg/kg body weight/ day). At the lower dose range, there was no effect on liver biopterins. At the highest dose, an 18.5% drop in hepatic biopterin levels was recorded.

Brown (1981) found that both trimethoprim and sulphamethoxazole inhibited DHPR in *in-vitro* studies. Sulphamethoxazole showed mixed inhibition with respect to the pterin and competitive inhibition with respect to NADH. The Ki value for sulphamethoxazole was 8.7×10^{-5}M. Ki value for trimethoprim was 5.8×10^{-5}M and trimethoprim inhibition was competitive with respect to the pterin and mixed with respect to NADH. When used together, these drugs have a synergistic effect in the inhibition of DHPR. Since they demonstrate competitive inhibition to the different substrates, it is possible that this synergism is produced by simultaneous binding to the enzyme.

CNS side-effects have been reported during the clinical use of cotrimoxazole (American Medical Association Drug Evaluations, 1980) such as depression and psychosis.

3:1:3 Phenylacetate.

Phenylacetate (23) is a metabolite of phenylalanine produced by transamination to phenylpyruvate and subsequent decarboxylation (Edwards & Blau,1972). It is elevated when hepatic hydroxylation of phenylalanine is inhibited, such as in 'classical phenylketonuria' where there is a deficiency of hepatic phenylalanine-4- hydroxylase (EC 1.14.16.2) (Jervis, 1947 , Wen *et al*, 1980, Edwards & Blau, (1972) and 'atypical phenylketonuria' caused by BH_4 deficiency (Danks *et al* review, 1977).

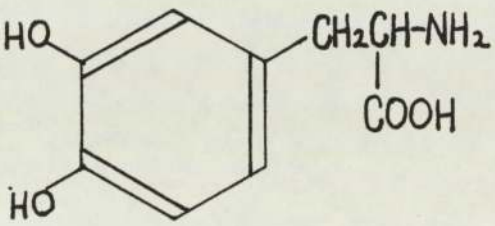
Phenylacetate poisoning in rats has been used as an experimental model of PKU where it causes neuropathological effects associated with the disease (Wen *et al*, 1980). A derivative of phenylacetate, phenylacetyl coenzyme A has been shown *in-vitro* to be a very potent inhibitor of choline acetyltransferase (Potempska *et al*, 1984).

Phenylacetate has been shown to be an *in-vitro* inhibitor of DHPR (Rong-sen Shen, 1984, Cutler, 1986). Several investigators have demonstrated that phenylacetate inhibits tyrosine-3-hydroxylase (EC 1.14.16.2) (Udenfriend *et al*, 1965) and other enzymes involved in neurotransmitter synthesis such as L-amino acid decarboxylase (EC 4.1.1.28 , Fellman, 1956).

3:1:4 p-hydroxyphenylacetate.

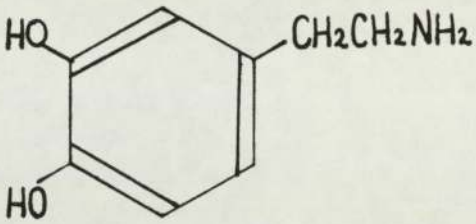
This compound is a metabolite of tyrosine produced after transamination and decarboxylation. It is found in elevated concentrations in hereditary hypertyrosinaemias (reviewed by Goldsmith, 1983). It occurs in high concentrations intracellularly in the presence of BH_4 deficiency and in 'classical' PKU due to inadequate conversion of tyrosine to L-DOPA by tyrosine hydroxylase. Acid metabolites of phenylalanine such as phenylacetate inhibit tyrosine hydroxylase (Udenfriend *et al*, 1965). Rong-sen Shen (1982) showed that p-hydroxyphenylacetate (24) is a potent non-competitive inhibitor of DHPR (Ki 74µM).

Structures of potential DHPR inhibitors.



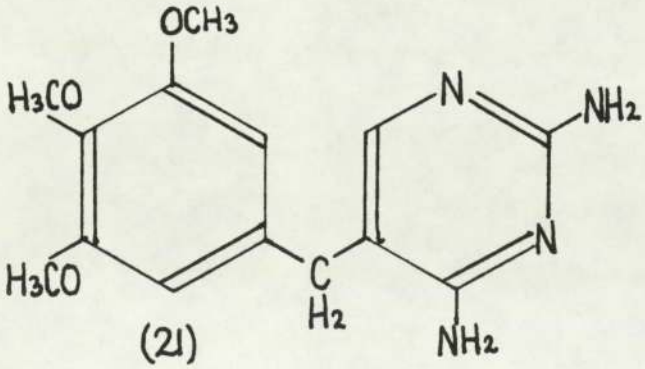
(19)

dihydroxyphenylalanine



(20)

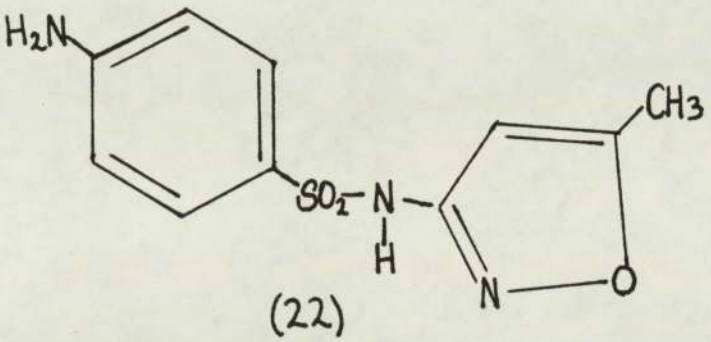
dopamine



(21)

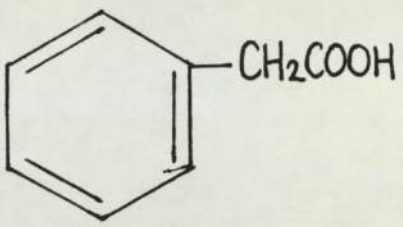
Trimethoprim

Structures of Potential DHPR inhibitors



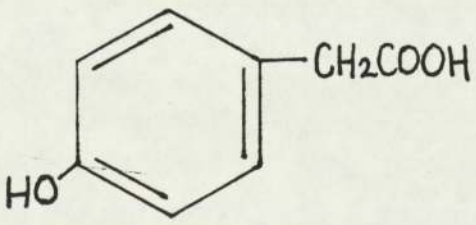
Sulphamethoxazole

(22)



phenylacetic acid

(23)



p-hydroxyphenylacetic acid

(24)

3:2 Methods.

Tissue derived pterin, total biopterins, phenylalanine, tyrosine, protein, DHPR, BH_4 biosynthesis, sepiapterin reductase, CAT and oxidative stress were assayed as described in Chapter 2.

180g male Wistar rats were dosed with 56.25 mg L-DOPA once per day for 2 days by i.p. injection and killed 24 hours later. Controls were given isotonic saline i.p. Sinemet Plus was administered in the same manner, except each dose contained 56.25 mg L-DOPA and 14.06 mg of carbidopa.

Cotrimoxazole was administered to 180g male Wistar rats as 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 hours later. Controls were given distilled water i.g.

180g male Wistar rats were administered 100mg of p-hydroxyphenylacetate i.p. over a period of 6 days. Controls received isotonic saline i.p.

40g male weanling Wistar rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Phenylacetate was omitted from the control food. Diets were given as blended food pellets *ad libitum*.

3:3 Results And Discussion.

3:3:1 L-DOPA and Sinemet Plus.

The *in-vivo* effects of L-DOPA and Sinemet Plus (L-DOPA and carbidopa) on BH_4 metabolism in the rat were examined. On administration of either drug, liver total biopterins were found to be significantly reduced (Tables 3:05, 3:06). There was no effect on either brain or plasma total biopterins (Tables 3:23, 3:25, 3:48). When L-DOPA is administered to humans, plasma biopterins are elevated (Aziz, unpublished).

Brain derived pterin was significantly reduced (Tables 3:01, 3:02), but there was no effect on liver derived pterin (Tables 3:36, 3:37). This is possibly be due to several causes. L-DOPA produces dopamine in brain tissue, which is a substrate for monoamine oxidase B (MAO-B), generating hydrogen peroxide and hydroxyl free radicals by a Fenton's type reaction (Haber & Weiss, 1934). These hydroxyl free radicals could then

oxidize reduced pterins such as tetrahydrobiopterin and tetrahydrofolate to non-pterin products (Heales, personal communication), which are non-fluorescent and not detected by HPLC.

To test this hypothesis the NBT assay was used to measure oxidative stress in cerebral tissue of dosed and control animals. Oxidative stress was found to be elevated but not significantly (Table 3:24).

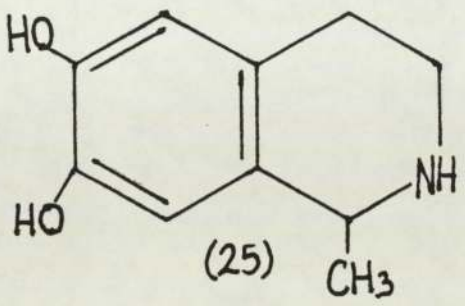
Alternatively, lowered brain derived pterin levels may reflect lowered turnover rate of biopterin. Dopamine produced by decarboxylation (Bartholini *et al*, 1967) can negatively feedback on tyrosine hydroxylase (Katoh *et al*, 1983), reducing the enzyme's requirement for BH_4 cofactor and so reducing its turnover.

Derived pterin in the brain can also reflect decreased brain tetrahydrofolate concentrations. This could arise by inhibition of DHPR and is discussed further in Chapter 6.

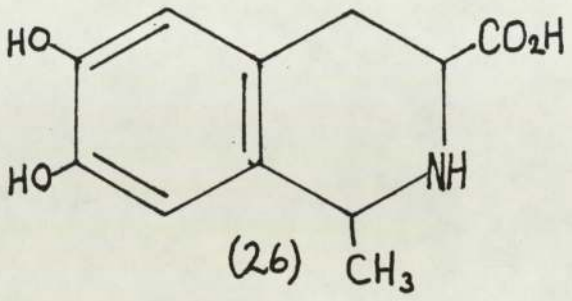
Besides the influence of dopamine and L-DOPA on *in-vivo* BH_4 metabolism, Collins (1980) reported that L-DOPA treated parkinsonian patients have significantly elevated levels of tetrahydroisoquinolones. These compounds may arise at significant rates *in-vivo* from the non-enzymatic Pictet-Spengler reaction, which is a bi-molecular condensation of a ß-arylethylamine with a carbonyl compound (Rong-sen Shen *et al*, 1982). One such dopamine derived tetrahydroisoquinolone, salsolinol is a competitive inhibitor of rat brain tyrosine hydroxylase (Ki =14µM) (Weiner & Collins, 1978). Other tetrahydroisoquinolones have been shown to be potent non-competitive inhibitors of purified human liver DHPR. Such compounds include salsolinol(25), (CIS)carboxysalsolinol(26), 4,6,7-trihydroxy tetrahydroisoquinolone (27) and tetrahydropapaveroline (28).

Since tyrosine hydroxylase occurs in the brain but not the liver (Mackay *et al*, 1978, McGeer *et al*, 1967, Fahn *et al*, 1969, Bacopoulos & Bhatnagar, 1977) and dopamine and dopamine derived tetrahydroisoquinolones can inhibit tyrosine hydroxylase as well as DHPR in the brain, but only DHPR in the liver, then the reduction in hepatic total biopterins but not of cerebral total biopterins can be explained.

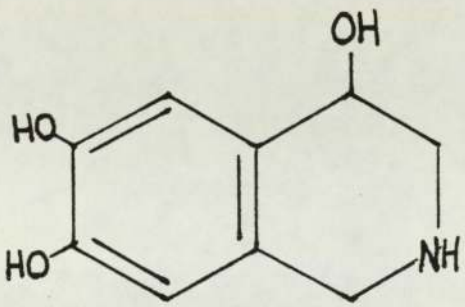
All other measured parameters did not differ significantly from control values. There was no effect on DHPR when measured (Tables 3:17, 3:33), possibly due to dilution of inhibitor out of the assay, or due to the L-DOPA/dopamine being rapidly metabolised by endogenous enzymes by the time the assay was performed. The half-life of L-DOPA in



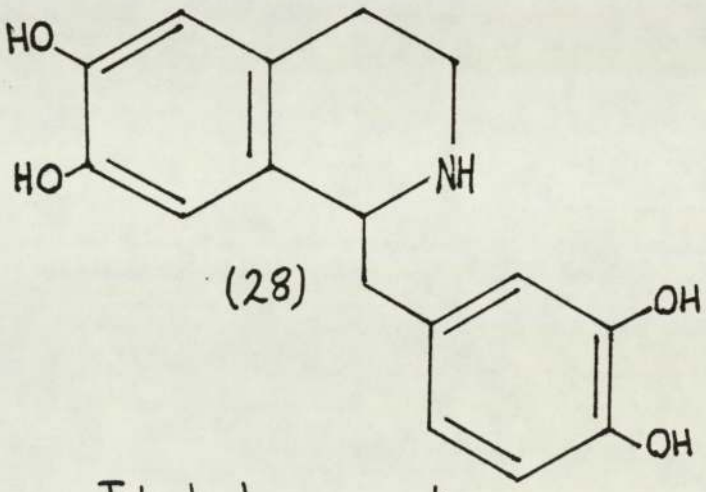
Salsolinol



(CIS) Carboxy-Salsolinol



4,6,7-Trihydroxy
Tetrahydroisoquinolone



Tetrahydropapaveroline

Tetrahydroisoquinolines related to dopamine, noradrenaline and L-DOPA (Weiner & Collins, 1978)

human plasma is about 1h (Martindale, 1977).

During the drug administration period, Sinemet Plus induced a state of hyperactivity and aggression and choreiform movements of the tail, which lasted for about three hours. L-DOPA administered on it's own caused shock, lasting several hours, and this affected appetite. Starvation has been shown to have an adverse effect on BH_4 metabolism (Edwards *et al*, 1987). Therefore, the effect of L-DOPA on BH_4 metabolism was investigated in the presence of starvation (Table 3:07). L-DOPA again caused a significant reduction in the concentration of hepatic biopterins, demonstrating a drug induced effect on BH_4 metabolism.

3:3:2 Cotrimoxazole.

Analysis of brain pteridine levels showed that both total biopterins and derived pterin were significantly reduced (Table 3:03, 3:04). Liver total biopterins were decreased, but not significantly by a t test (Table 3:39). This observation was also noted by Stone (1976) on administration of trimethoprim.

Liver derived pterin was significantly lowered. This could be due to interference with folate metabolism (Table 3:08). There was no effect on plasma biopterins (Table 3:49, 3:50). This differs from the effect on humans where there is an elevation of plasma biopterins after administration (Leeming *et al*, 1976).

No inhibition of DHPR was found (Tables 3:26, 3:40) perhaps due to dilution of reversible inhibitor out of the assay system during tissue preparation or to *in-vivo* physiological clearance of the drug by the end of the experiment. All other measured parameters were unchanged. Animals fed normally during the experiment.

3:3:3 Phenylacetate.

Phenylacetate was administered as shown in the results tables. Phenylacetate has been reported to cause a reduction in body weight and of weight of brain tissue (Wen *et al*, 1980). This was not found here (Table 3:54). Animals fed normally during the experiment.

Large amounts of phenylacetate were administered for several reasons. Phenylacetate may be greatly elevated in hyperphenylalaninaemic conditions (Loo *et al*, 1978). Also,

Potempska *et al*, (1984) found that phenylacetyl coenzyme A, which is generated *in-vivo* during detoxification of phenylacetate (Loo *et al*, 1978) is a very potent inhibitor of choline acetyltransferase, competitive for acetyl coenzyme A with Ki of 0.31μM.

Phenylacetate + ATP + Coenzyme A ⟶ Phenylacetyl Coenzyme A
- catalysed by acid-Co A ligase (EC 6.2.1.3)

Phenylacetyl Coenzyme A + glycine ⟶ Phenylacetylglycine + Coenzyme A
- catalysed by acyl-Co A glycine-N- acyl transferase (EC 2.3.1.13).

The conjugate is excreted in the urine. Glycine is used in the hepatic conjugation process in rats and glutamine is utilised in humans. The second step in the detoxification is absent in brain tissue (Loo *et al*, 1978, Woolf, 1951).

Loo *et al*, (1978) demonstrated that the detoxification process develops postnatally and that the formation of the phenylacetyl coenzyme A is the rate limiting step. The coenzyme A derivative is difficult to remove from the CNS once formed and phenylacetate readily penetrates the brain.

When measured, no inhibition of CAT was found (Table 3:30). Phenylacetate loading had no effect on brain biopterins or derived pterin (Table 3:28, 3:29). Liver biopterin levels were reduced by about 9%, which was not statistically significant (Table 3:42). No effect was found on liver derived pterin or plasma biopterins (Table 3:43, 3:52).

In SDAT, there is a deficiency in BH_4 (Perry *et al* 1981), and senile patients have been shown to have a reduced tolerance to phenylalanine loading (Leeming & Blair, 1980). CAT deficiency is a characteristic post mortem finding in SDAT and other dementias. It is possible that this may be caused by endogenous generation of aromatic amino acid coenzyme A derivatives due to intracellular BH_4 deficiency.

As the results show, phenylacetate had little effect on BH_4 metabolism. Rong-sen Shen (1984) showed that this metabolite is a poor DHPR inhibitor. Also, the detoxification mechanism may have been sufficient to prevent adequate concentrations being accumulated to have neurotoxic effects on BH_4 metabolism. A lack of *in-vivo* effect on brain CAT activity suggests that either insufficient coenzyme A derivative was generated in the duration of the experiment to inhibit CAT or, since only 5% tissue homogenates were assayed for enzyme activity, the inhibitor was diluted out during tissue preparation.

An effect was recorded on plasma tyrosine levels in dosed animals (Table 3:14). Plasma tyrosine was significantly elevated in experimental animals. There was no effect on plasma phenylalanine (Table 3:53). As a consequence, the plasma P:T ratio was lower than normal (Table 3:15). Loo (1978), found that accumumulation of phenylacetate is prevented by repeated injections of pyridoxamine. Vitamin B_6 is in general a requirement of transaminase and decarboxylase enzymes (McIlwain & Bachelard, 1985). Tyrosine is a substrate for tyrosine aminotransferase (EC 2.6.1.5 Black *et al*, 1970). Also the hydroxylated product of tyrosine, L-DOPA is decarboxylated to produce dopamine by DOPA decarboxylase (EC 4.1.1.28) (Kuntzman *et al*, 1961). It follows that vitamin B_6 is important in the control of tyrosine. The phenylacetate was given for a period of two weeks as 2.5% of a blended standard rat diet, which contains 10.10 mg of B_6 kg^{-1}(Cutler, 1986). This level of exposure may have disrupted B_6 and decreased the activity of enzymes required in the control of plasma tyrosine levels.

Wen *et al* (1980) demonstrated that phenylacetate intoxication in rats caused histological changes very similar to those found in PKU, and proposed it's use as a model for the disease state. This model could be criticised if it caused biochemical changes not found in the disease such as interference with BH_4 metabolism and B_6 metabolism. Loo & Whitaker (1967) reported that pyridoxine dependent enzymes are disturbed in PKU.

3:3:4 p-hydroxyphenylacetate.

No effect was found on brain biopterins or derived pterin (Table 3:31, 3:32), or on liver derived pterin levels (Table 3:45). However, liver total biopterins were decreased by administration of p-hydroxyphenylacetate (Table 3:09). Rong-sen Shen (1984) demonstrated that this metabolite is a better *in-vitro* inhibitor of DHPR than is phenylacetate, and this is supported here in *in-vivo* work. This suggests that *para*-phenolic structures provide a more potent inhibition of DHPR.

Except for the p-OH grouping, phenylacetate and p-hydroxyphenylacetate are identical structures. It is postulated that p-hydroxyphenylacetate may be activated with coenzyme A and metabolised in the same way as phenylacetate. If this did happen, it would be interesting to see if the suggested p-hydroxyphenylacetyl coenzyme A could inhibit CAT and contribute to the neurochemical findings in dementias such as SDAT (Perry & Perry, 1980), dementia associated with Parkinson's disease (Jellinger & Reiderer, 1984), and of Down's syndrome (Corbett, 1985). However, no effect on rat brain CAT

was observed after dosing with p-hydroxyphenylacetate (Table 3:27). Again, it cannot be ruled out that insufficient compound was present to generate enough of the potential neurotoxin in the brain, or that it was diluted out of the assay system.

No effect was noted on plasma biopterins (Table 3:51), but plasma pterin was significantly elevated (Table 3:13). As will be discussed in Chapter 6, this indicates *in-vivo* inhibition of dihydropteridine reductase. It was interesting to note that plasma phenylalanine and tyrosine levels were decreased in dosed animals (Tables 3:10, 3:11). The effect on plasma tyrosine was greater than that on plasma phenylalanine. The P: T ratio (phenylalanine : tyrosine ratio) in the plasma was significantly higher in dosed (P: Tof 1.69) compared to controls (P: T of 1.24) (Table 3:12).

Plasma P : T ratios are often used to monitor PKU patients (Tourian & Sidbury, 1983). A ratio greater than 1.00 suggests inefficient hepatic hydroxylation of phenylalanine to tyrosine. Obviously the greater the ratio, the more ineffective the hydroxylation reaction.

The reduced plasma aromatic amino acid concentrations could have been the result of increased activity of 'minor' metabolic pathways such as liver transaminases. The activity of these enzymes is enhanced in pathologic hyperphenylalaninaemic conditions.

3:4 Conclusions.

3:4:1 L-DOPA and Sinemet Plus.

Administration of these drugs caused decreased hepatic biopterins but not brain biopterins. Brain derived pterin levels, but not liver derived pterin levels were decreased. This suggests that L-DOPA dosing causes inhibition of DHPR in the liver (supported by *in-vitro* work by other workers), but L-DOPA metabolites (dopamine and dopamine derived tetrahydroisoquinolones) feedback on tyrosine hydroxylase which is present in nervous tissue rather than DHPR and decreases turnover of the biopterin cofactor in cerebral tissue. Neither Sinemet Plus nor L-DOPA caused elevated plasma biopterins, unlike human studies on the effects of these drugs where plasma biopterins are increased.

3:4:2 Cotrimoxazole.

Cotrimoxazole dosing caused decreased brain biopterins and derived pterin. This finding was not seen in liver tissue. The absence of any effect in the liver might be a

reflection of drug clearance since the half life of cotrimoxazole in humans is 10-16 h.

The effect on brain biopterins supports *in-vitro* work that this combination antibiotic may adversely affect BH_4 metabolism by synergistic inhibition of DHPR. This effect may contribute to CNS side-effects of the drug, which occur occasionally and include psychosis and depression.

Cotrimoxazole has been reported to cause an elevation of human plasma biopterins (Leeming *et al*, 1976) This was not found in rats.

3:4:3 Phenylacetate.

No effect on BH_4 metabolism was noted after administration of this compound. This may be due to detoxification of the acid metabolite or as other workers have suggested (Rong-sen Shen, 1984) that phenylacetate is a poor DHPR inhibitor.

The hypothesis that coenzyme A derivatives of phenylacetate could inhibit CAT *in-vivo* was not proved in these studies. Plasma tyrosine was elevated, possibly due to interference with vitamin B_6.

3:4:4 p-hydroxyphenylacetate.

Liver biopterins were decreased and plasma derived pterin and P: T ratios were elevated, pointing to DHPR inhibition in peripheral tissue. No effect on brain BH_4 or CAT was noted, possibly due to peripheral detoxification of the metabolite.

Rong-sen Shen (1984) demonstrated that *para*-phenolic structures provide good inhibitors of DHPR. The work here suggests the same due to the relatively lower dose of p-hydroxyphenylacetate used compared to phenylacetate (see results tables) and the effect of the p-OH metabolite on BH_4 metabolism contrasted to the lack of effect with phenylacetate.

Tables of Significant Results.

Studies on Brain Tissue.

Table 3:01

The effect of L-DOPA on brain derived pterin.

Group	Derived Pterin	
Control	86.00± 11.02 (6)	
L-DOPA	55.12± 10.13 (6)	$p < 0.010$

Values as ng of pterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:02

The effect of Sinemet Plus on brain derived pterin.

Group	Derived Pterin	
Control	82.96± 10.23 (6)	
Sinemet Plus	52.64± 10.52 (6)	$p<0.001$

Values as ng of pterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA and 14.06 mg carbidopa once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:03

The effect of cotrimoxazole on brain total biopterins.

Group	Total biopterins	
Control	95.38± 16.38 (6)	
Cotrimoxazole	68.52± 17.38 (6)	$p < 0.025$

Values as ng of biopterin/ g wet weight of tissue. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:04

The effect of cotrimoxazole on brain derived pterin.

Group	Derived Pterin	
Control	56.93± 11.65 (6)	
Cotrimoxazole	35.56± 7.12 (6)	$p < 0.020$

Values as ng of pterin /g wet weight of tissue. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Studies on Liver Tissue

Table 3:05

The effect of L-DOPA on liver total biopterins.

Group	Total biopterins	
Control	1.72± 0.40 (6)	
L-DOPA	1.10± 0.09 (6)	$p < 0.005$

Values as µg of biopterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:06

The effect of Sinemet Plus on liver total biopterins.

Group	Total biopterins	
Control	1.81± 0.41 (6)	
Sinemet Plus	1.21± 0.15 (6)	$p<0.010$

Values as µg of biopterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA and 14.06 mg carbidopa i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:07

The effect of L-DOPA on liver total biopterins on fasting.

Group	Total biopterins	
Control	1.00± 0.20 (6)	
L-DOPA	0.80± 0.10 (6)	p < 0.020

Values as µg of biopterin /g wet weight of tissue. Animals were starved overnight on grids to prevent coprophagy. 180g male Wistar rats were then dosed with 56.25 mg L-DOPA and killed 24 h. later. Controls were given isotonic saline i.p. after starving.

Table 3:08

The effect of cotrimoxazole on liver derived pterin

Group	Derived Pterin	
Control	0.31± 0.02 (6)	
Cotrimoxazole	0.25± 0.05 (6)	p< 0.025

Values as µg / g wet weight of tissue. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:09

The effect of p-hydroxyphenylacetate (P-HPAA) on rat liver total biopterins .

Group	Total Biopterins	
Control	1.80± 0.19 (6)	
P-HPAA	1.34± 0.12 (6)	p<0.001

Values as µg biopterin / g wet weight of tissue.180g male Wistar rats were dosed i.p. with 100mg P-HPAA over 6 days. Controls were given isotonic saline i.p.

Studies on Plasma

Table 3:10

The effect of p-hydroxyphenylacetate (P-HPAA) on plasma phenylalanine levels.

Group	Phenylalanine	
Control	37.32± 3.38 (6)	
P-HPAA	32.81± 2.47 (6)	p<0.050

Values as µmoles phenylalanine / litre of plasma .180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:11

The effect of p-hydroxyphenylacetate (P-HPAA) on plasma tyrosine levels.

Group	Tyrosine	
Control	30.39± 4.62 (6)	
P-HPAA	19.88± 3.52 (6)	p<0.005

Values as µmoles tyrosine / litre of plasma .180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:12

The effect of p-hydroxyphenylacetate (P-HPAA) on plasma phenylalanine : tyrosine ratios.

Group	Phe : tyr Ratio	
Control	1.24± 0.14 (6)	
P-HPAA	1.69± 0.29 (6)	p<0.010

180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:13

The effect of p-hydroxyphenylacetate (P-HPAA) on rat plasma derived pterin

Group	Derived Pterin	
Control	1.60± 2.19 (6)	
P-HPAA	5.23± 3.28 (6)	p<0.050

Values as µg pterin / litre of plasma. 180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:14

The effect of phenylacetate on rat plasma tyrosine

Group	Tyrosine	
Control	52.46± 2.12 (6)	
Phenylacetate	64.93± 6.68 (6)	p < 0.005

Values as µmoles of tyrosine / l of plasma.40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:15

The effect of phenylacetate on rat plasma phenylalanine : tyrosine ratio.

Group	Phe : Tyr ratio	
Control	0.62± 0.07 (6)	
Phenylacetate	0.50± 0.06 (6)	p < 0.010

40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Tables of Non-Significant Results

Studies on Brain Tissue

Table 3:16

The effect of L-DOPA on brain protein.

Group	Protein	
Control	5.73± 0.24 (6)	
L-DOPA	5.24± 0.57 (6)	ns

Values as mg protein / ml of a 0.5% Tris-HCl homogenate (0.5M; pH 6.8) as measured by the biuret reaction.180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:17

The effect of L-DOPA on brain DHPR activity.

Group	DHPR activity	
Control	204.77± 27.01 (6)	
L-DOPA	244.60± 45.74 (6)	ns

Values as nmoles NADH oxidised / mg protein / minute.180g male Wistar rats were dosed with 56.25 mg L-DOPA once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:18

The effect of L-DOPA on brain BH_4 biosynthesis.

Group	BH_4 biosynthesis	
Control	0.42± 0.16 (6)	
L-DOPA	0.54± 0.17 (6)	ns

Values as ng biopterin produced / mg protein / h. 180g male Wistar rats were dosed with 56.25mg L-DOPA once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:19

The effect of L-DOPA on brain sepiapterin reductase activity.

Group	Sepiapterin reductase activity	
Control	1.24± 0.37 (6)	
L-DOPA	1.38± 0.84 (6)	ns

Values as nmoles sepiapterin reduced / mg protein / minute. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:20

The effect of L-DOPA on brain tyrosine.

Group	Tyrosine	
Control	31.26± 7.78 (6)	
L-DOPA	29.38± 4.80 (6)	ns

Values as µmoles of tyrosine / litre of brain supernatant. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:21

The effect of L-DOPA on brain phenylalanine.

Group	Phenylalanine	
Control	34.38± 10.09 (6)	
L-DOPA	34.50± 8.74 (6)	ns

Values as µmoles of phenylalanine / litre of brain supernatant. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later . Controls were given isotonic saline i.p.

Table 3:22

The effect of L-DOPA on brain phenylalanine : tyrosine ratio.

Group	Phe : tyr ratio	
Control	1.12± 0.24 (6)	
L-DOPA	1.16± 0.20 (6)	ns

180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:23

The effect of L-DOPA on brain total biopterins.

Group	Total biopterins	
Control	85.26± 11.86 (6)	
L-DOPA	72.03± 8.58 (6)	ns

Values as ng of biopterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:24

The effect of L-DOPA on brain oxidative stress.

Group	Oxidative stress	
Control	0.51± 0.05 (6)	
L-DOPA	0.78± 0.42 (6)	ns

Oxidative stress in brain tissue was measured by the NBT assay. Values as µmoles of formazan / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 3 h. later. Controls were given isotonic saline i.p.

Table 3:25

The effect of Sinemet Plus on brain total biopterins.

Group	Total biopterins	
Control	89.22± 7.10 (6)	
Sinemet Plus	81.96± 11.92 (6)	ns

Values as ng of biopterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA and 14.06 mg carbidopa i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:26

The effect of cotrimoxazole on brain DHPR.

Group	DHPR	
Control	354.28± 51.31 (6)	
Cotrimoxazole	287.11± 75.07 (6)	ns

Values as nmoles NADH oxidised /min./ mg of protein. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:27

The effect of p-hydroxyphenylacetate on rat brain choline acetyltransferase (CAT) activity.

Group	CAT Activity.	
Control	15.95± 4.53 (6)	
P-HPAA	18.14± 5.52 (6)	ns

Values as µmoles of acetylcholine formed / h / g of tissue. 180g male Wistar rats were dosed with 100 mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:28

The effect of phenylacetate on rat brain total biopterins.

Group	Total Biopterins	
Control	89.48± 31.30 (6)	
Phenylacetate	79.51± 20.56 (6)	ns

Values as ng biopterin / g wet weight of tissue. 40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:29

The effect of phenylacetate on rat brain derived pterin.

Group	Derived Pterin	
Control	47.52± 5.65 (6)	
Phenylacetate	46.31± 6.26 (6)	ns

Values as ng pterin / g wet weight of tissue. 40g male weanling Wistar rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:30

The effect of phenylacetate on rat brain choline acetyltransferase.

Group	CAT Activity	
Control	8.82± 1.05 (6)	
Phenylacetate	9.23 ± 1.67 (6)	ns

Values as µmoles of acetylcholine produced/ h/ g wet weight of tissue. 40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:31

The effect of p-hydroxyphenylacetate (P-HPAA) on rat brain total biopterins.

Group	Total biopterins	
Control	79.34± 19.50 (6)	
P-HPAA	74.07± 10.07 (6)	ns

Values as ng biopterin / g wet weight of tissue. 180g male Wistar rats were dosed i.p. with 100mg P-HPAA over 6 days. Controls were given isotonic saline i.p.

Table 3:32

The effect of p-hydroxyphenylacetate (P-HPAA) on rat brain derived pterin.

Group	Derived Pterin	
Control	46.22± 5.68 (6)	
P-HPAA	45.42± 2.49 (6)	ns

Values as ng pterin / g wet weight of tissue. 180g male Wistar rats were dosed i.p. with 100mg P-HPAA over 6 days. Controls were given isotonic saline i.p.

Studies on Liver Tissue.

Table 3:33

The effect of L-DOPA on liver DHPR activity.

Group	DHPR activity	
Control	632.19± 125.80 (6)	
L-DOPA	570.99± 169.14 (6)	ns

Values as nmoles oxidised / mg protein / minute. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:34

The effect of L-DOPA on liver sepiapterin reductase activity.

Group	Sepiapterin reductase activity	
Control	10.54± 4.12 (6)	
L-DOPA	9.87± 2.27 (6)	ns

Values as nmoles of sepiapterin reduced / mg protein / minute. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:35

The effect of L-DOPA on liver biopterin biosynthesis.

Group	Biopterin biosynthesis	
Control	6.02± 3.56 (6)	
L-DOPA	4.73± 1.47 (6)	ns

Values as ng of biopterin produced / mg protein / h. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:36

The effect of L-DOPA on liver derived pterin.

Group	Derived Pterin	
Control	0.26± 0.04 (6)	
L-DOPA	0.28± 0.05 (6)	ns

Values as ng of pterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:37

The effect of Sinemet Plus on liver derived pterin.

Group	Derived Pterin	
Control	0.27± 0.10 (6)	
Sinemet Plus	0.23± 0.09 (6)	ns

Values as µg of pterin / g wet weight of tissue. 180g male Wistar rats were dosed with 56.25 mg L-DOPA and 14.06 mg carbidopa once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:38

The effect of cotrimoxazole on liver protein.

Group	Protein	
Control	67.70± 17.5 (6)	
Cotrimoxazole	53.50± 4.8 (6)	ns

Values as mg of protein /ml of 20% Tris-HCl homogenate (0.5M, pH 6.8) as measured by the biuret reaction. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:39

The effect of cotrimoxazole on liver total biopterins.

Group	Total biopterins	
Control	1.86± 0.23 (6)	
Cotrimoxazole	1.67± 0.14 (6)	ns

Values as µg / g wet weight of tissue. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:40

The effect of cotrimoxazole on liver DHPR.

Group	DHPR	
Control	546.91± 143.25 (6)	
Cotrimoxazole	519.44± 112.84 (6)	ns

Values as nmoles NADH oxidised/ min./mg of protein. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:41

The effect of cotrimoxazole on liver biopterin biosynthesis.

Group	Biopterin biosynthesis	
Control	10.23± 4.37 (6)	
Cotrimoxazole	11.64± 3.66 (6)	ns

Values as ng of biopterin synthesised / h / mg of protein. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:42

The effect of phenylacetate on rat liver total biopterins.

Group	Total Biopterins	
Control	1.67± 0.09 (6)	
Phenylacetate	1.52± 0.14 (6)	ns

Values as µg biopterin / g wet weight of tissue. 40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:43

The effect of phenylacetate on rat liver derived pterin.

Group	Derived Pterin	
Control	0.41± 0.06 (6)	
Phenylacetate	0.47± 0.06 (6)	ns

Values as µg pterin / g wet weight of tissue. 40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:44

The effect of phenylacetate on rat liver protein.

Group	Protein	
Control	77.00± 11.87 (6)	
Phenylacetate	69.20± 16.69 (6)	ns

Values as measured by the biuret reaction on diluted 20% homogenates. pH 6.8, 0.5M Tris-HCl. 40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:45

The effect of p-hydroxyphenylacetate (P-HPAA) on rat liver derived pterin.

Group	Derived Pterin	
Control	0.26± 0.05 (6)	
P-HPAA	0.21± 0.04 (6)	ns

Values as µg pterin / g wet weight of tissue. 180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:46

The effect of P-HPAA on rat liver BH_4 biosynthesis.

Group	BH_4 biosynthesis	
Control	13.62± 4.73 (6)	
P-HPAA	11.60± 1.14 (6)	ns

Values as ng of biopterin produced/h/ mg of protein. 180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:47

The effect of P-HPAA on rat liver protein.

Group	Protein	
Control	64.18± 6.62 (6)	
P-HPAA	54.23± 11.1 (6)	ns

Values as mg of protein/ ml of 20% Tris-HCl homogenates, pH 6.8, 0.5M measured by the biuret reaction after dilution. 180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Studies on Plasma

Table 3:48

The effect of L-DOPA on plasma total biopterins.

Group	Total biopterins	
Control	19.16± 6.37 (6)	
L-DOPA	18.32± 4.93 (6)	ns

Values as µg of biopterin / litre of plasma as measured by *Crithidia fasciculata* assay. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. once per day for 2 days and killed 24 h. later. Controls were given isotonic saline i.p.

Table 3:49

The effect of cotrimoxazole on plasma total biopterins.

Group	Total biopterins	
Control	17.55± 3.05 (6)	
Cotrimoxazole	18.60± 4.85 (6)	ns

Values as µg of biopterin / l of plasma. Measured by *C.fasciculata* by Dr. R.J. Leeming. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:50

The effect of cotrimoxazole on plasma total biopterins.

Group	Total biopterins	
Control	18.00± 3.32 (6)	
Cotrimoxazole	18.84± 4.10 (6)	ns

Values as µg of biopterin / l of plasma as measured by HPLC. 180g male Wistar rats were dosed with 20.53 mg sulphamethoxazole and 4.27 mg trimethoprim i.g. per day for 2 days and killed 24 h. later. Controls were given distilled water i.g.

Table 3:51

The effect of p-hydroxyphenylacetate (P-HPAA) on rat plasma total biopterins.

Group	Total Biopterins	
Control	19.51± 7.58 (6)	
P-HPAA	18.85± 4.37 (6)	ns

Values as µg biopterin / litre of plasma, as measured by HPLC. 180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

Table 3:52

The effect of phenylacetate on rat plasma total biopterins.

Group	Total biopterins	
Control	23.15± 6.35 (6)	
Phenylacetate	27.21± 6.87 (6)	ns

Values as µg biopterin / g wet weight of tissue. 40g male weanling Wistar rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:53

The effect of phenylacetate on rat plasma phenylalanine

Group	Phenylalanine	
Control	32.38± 6.43 (6)	
Phenylacetate	32.61± 4.05 (6)	ns

Values as µmoles of phenylalanine / l of plasma. 40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

Table 3:54

The effect of phenylacetate on body weight.

Group	Body weight / g	
Control	130.60± 13.2 (6)	
Phenylacetate	115.90± 10.9 (6)	ns

Animals were dosed for two weeks with phenylacetate as 2.5% of their diet. 40g male weanling Wistar rats were used.

Chapter 4. *In-Vivo Studies* On The Effect Of Neurotoxins That Inhibit BH_4 Biosynthesis.

Chapter Four.

4 *In-vivo* Studies On The Effect Of Neurotoxins That Inhibit BH_4 Biosynthesis.

4:1 Introduction.

The following agents are known neurotoxins which may have an effect on BH_4 metabolism.

4:1:1 Diaminohydroxypyrimidine.

2,4-diaminohydroxypyrimidine (DAHP)(31) was shown in 1966 to inhibit biopterin synthesis in rats as measured by the *Crithidia* assay (Pabst & Rembold). This was again shown by Gal & Whitacre (1981), who demonstrated that repeated intraventricular injections into rats caused a 60% drop in biopterins in the brain, but found no effect on serotonin levels, or on the rate of hydroxylation of L-tryptophan. This was supported by the work of Gal *et al* (1978) using another inhibitor of the *de novo* synthetic pathway, dF Pyd P_3 (deoxyformamido-6 -hydroxypyrimidine). Kettler *et al,* (1974) showed that catecholamine biosynthesis in the brain was stimulated by the intraventricular administration of BH_4 and stressed the regulatory control of reduced pterins. Gal & Whitacre (1981) demonstrated that approximately a quarter of the cerebral BH_4 pool is turned over every hour.

Cotton (1986) used DAHP to induce experimental hyperphenylalaninaemia (HPA) in mice. Serum phenylalanine concentration was directly related to dietary DAHP and liver BH_4 was inversely related to DAHP. This compound is an inhibitor of GTP cyclohydrolase.

The effects of DAHP were investigated for several reasons. The effect was tested on Wistar rats to see if the administration of the toxin to this species could also be used as a model for HPA. Since a quarter of the BH_4 is turned over every hour (Gal &

Whitacre, 1981) and pterin may be a catabolite of BH_4 (Pfleiderer, 1975) the effects of DAHP on pterin levels were followed to see if this compound may give insight into BH_4 turnover. The activity of CAT was measured to find if acute HPA can affect the enzyme *in vivo*. If disrupted BH_4 metabolism and consequential HPA could be induced by the compound, without producing overt symptoms in the animals, DAHP administration might be used to study behavioural changes caused by this biochemical lesion.

4:1:2 Lead.

The use of lead throughout the industrialized world has more than doubled during the last 30 years (Hilburn, 1979). Total body burden of lead in adults is 50 - 400mg. 10µg per day (about 4% of the lead ingested) is retained by adults. Children may retain up to 18% of their daily intake. Lead blood levels in the UK are typically 15 - 25µg/100mls. Greater than 80µg/100mls of plasma is associated with clinical signs of lead poisoning (plumbism) (Kehoe, 1972), such as anaemia, abdominal colic, nephropathy and encephalopathy.

Lead has no known biological function in mammalian systems. There is no clear threshold between 'chemical toxicity' (disruption of biochemical functions in a biological system) and 'clinical toxicity'. Betts *et al* (1973) showed that 100µg/100mls can cause lead encephalopathy in children, whereas much higher levels may be associated with only minimal cerebral dysfunction in adults. Blood lead levels much greater than 100µg/100mls in children are not always associated with detectable deficits of cerebral function.

David *et al* (1982) investigated blood lead levels of children with IQs of 55 - 84 divided according to the presence or absence of a probable aetiology for their retardations and of 40 control children. Mean blood lead of those with unknown aetiology was 25.03µg/100mls, which was significantly higher than that in the other two groups.

Purdy *et al* (1981) found *in vitro* BH_4 synthesis to be inhibited at 10^{-7}M lead acetate and the salvage enzyme, DHPR to be inhibited at 10^{-6}M. Lead may inhibit DHPR by binding with thiol groups at the active site of the enzyme. McIntosh *et al* (1985) dosed rats with 120µM and 480µM lead for 4 weeks and 12 weeks. No effect on growth was noted. Increased DHPR activity was found after 12 weeks within the 120µM group in the diencephalon, which was thought to be a compensatory rise for effects on BH_4 metabolism at some other point. In 1982, McIntosh found that humans with 0.28µM -

4.51μM blood lead had elevated serum biopterins, suggesting inhibition of DHPR. However, rats dosed with lead had low serum biopterins, demonstrating that lead has more than one effect on BH_4 metabolism. Blair *et al* (1982) showed that plasma biopterins increased as blood lead increased, and since blood phenylalanine is inversely related to IQ, disruptions in BH_4 may have effects on IQ.

Eggar *et al* (1986) discovered that DHPR is inversely related to blood lead in lead workers. Dosed rats (1.5nmol / kg and 15nmol / kg body weight) had elevated brain biopterins, but the percentage as BH_4 was decreased.

4:1:3 Aluminium.

Aluminium is the third most abundant element in the earth's crust, of which it comprises about 8%. The first reported case of adverse effects in man was by Spofforth in 1921. Toxicity of this metal has been implicated in senile dementia of the Alzheimer type (SDAT), Amyotrophic Lateral Sclerosis (ALS) and parkinsonism dementia (PD) complex (sporadic motorneurone disease), dialysis dementia and some forms of epilepsy (Wisniewski *et al*, 1985).

SDAT occurs in 5% of Americans over 65 years old. Aluminium has been suggested as a neurotoxic cause. The neurofibrillary tangle of SDAT was first considered by Kidd (1964) to be composed of twisted helices of two 100A diameter filaments. Aluminium causes similar filaments (Terry & Pena, 1965) but there are differences. Crapper *et al* (1973) reported that aluminium causes neurofibrillary degeneration in SDAT. Ward (1987) reported aluminium levels of 3.61 - 21.74 mg/g dry weight of tissue in post mortem brains.

High incidences of dementia in Guam, Kii Peninsula of Japan and southern West New Guinea may be associated with the high levels of aluminium in mineral deposits in these areas (Wisniewski *et al* review, 1985, Krishnan & Crapper McLachlan review, 1985).

Various workers have reported defects in BH_4 metabolism in SDAT (Table 1:01). Normal DHPR and GTP-CH activity were found (Barford et al, 1984), in affected brains and the lesion lay somewhere between NH_2TP and BH_4. The N:B ratios were significantly elevated in post mortem temporal lobes.

Cowburn & Blair (1987) found that the addition of aluminium acetate (1mmol/l) to human temporal and frontal cortex preparations caused a significant decrease in BH_4 synthesis. The concentration of the metal used was similar to that found in SDAT.

Alfrey *et al* (1976) first suggested that the dialysis encephalopathy syndrome may be due to aluminium intoxication. McDermott & Smith (1978) found the association of dialysis encephalopathy with high levels of aluminium in the brain and dialysis water of uraemic patients.

Dhondt *et al* (1982 and 1983) reported the increased serum N:B ratio in dialysis patients which may cause the abnormal P:T ratios and impaired neurotransmitter synthesis which occur in chronic uraemia, and that DHPR activity and biopterin levels in tissues of hamsters receiving 3% $Al_2(SO_4)_3$ in drinking water for 10 days were significantly reduced.

Altmann *et al* (1987) stressed that more than 50 - 80 µg aluminium / l of tap water in haemodialysis is dangerous. Patients on haemodialysis had raised serum biopterin levels which were not correlated to serum aluminium. DHPR is decreased, but activity increased after deferoxamine treatment (Freundlich *et al*, 1986, Malluche *et al*, 1986). Their conclusions suggested a disturbance of neurotransmitter metabolism in the brains of dialysis patients without clinical evidence of encephalopathy associated with an improvement in psychomotor function improvement after three months of aluminium chelation therapy.

4:2 Methods.

Tissue derived pterin, total biopterins, phenylalanine, tyrosine, protein, DHPR, BH_4 biosynthesis, sepiapterin reductase, lead and CAT were measured as previously described in Chapter 2.

40g male weanling Wistar rats were administered with the appropriate metal acetate as follows: Aluminium Group , 0.03% aluminium acetate for 1 month, followed by 1% for the second month, and 2% for the third month.

Lead Group 1 were administered 0.76% lead acetate for 1 month, 2% for the second month, and 4% lead acetate for the final month. Lead Group 2 were dosed with 0.39% lead

acetate for 1 month, followed by 1% for the second month, and 2% for the third month. Control animals were given ordinary tap water for the duration of the experiment.

40g male Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets. Control animals were fed blended food pellets without DAHP.

4:3 Results and Discussion.

4:3:1 Diaminohydroxypyrimidine.

DAHP caused almost 60% reduction in brain total biopterins (Table 4:07), and a 45% decrease in brain derived pterin (Table 4:08). A greater effect was seen in liver total biopterin levels which were dropped by 81% (Table 4:14). Liver derived pterin was decreased by only 44% (Table 4:15).

Pterin is a potential catabolite of both tetrahydrofolate (Fukushima & Nixon, 1980) and BH_4 (Pfleiderer, 1975, Milstien, 1983). The results indicate that pterin levels follow changes in biopterin metabolism more closely in brain than in liver, and more pterin in the brain is derived from BH_4 than in the liver.

Plasma total biopterins were significantly lower in dosed animals (Table 4:19). Plasma phenylalanine was about 3x higher than in controls (Table 4:20). No effect was observed on plasma tyrosine (Table 4:25). This resulted in elevated P:T ratios (Table 4:21). No effect was apparent on CAT activity (Table 4:04).

Liver BH_4 biosynthesis was significantly lower than that of controls (Table 4:16), and liver DHPR activity was elevated, but not significantly (Table 4:24). During the experiment, 1/6 of the animals died after 3 days of 5% w/w DAHP in the diet, so the level of administration was dropped to 3% w/w. Cotton (1986) had 25% mortality in mice with 7% w/w DAHP.

4:3:2 Lead.

Both dosed groups had lower body weights than the controls (Table 4:01). Mykkanen *et al* (1979) found that 2% lead acetate decreased growth in rats. As can be seen from the tables, Group 1 ingested twice as much lead acetate as Group 2. Animals fed normally during the experiment.

Lead Group 1 had significantly lowered brain biopterins (Table 4:03). This was not present in Group 2. Brain derived pterin was low in Group 1, but not in Group 2 (Table 4:02). Both experimental groups had very high liver total biopterins compared to controls (Table 4:13). This could be due to an analogous situation to that reported by Eggar *et al* (1986), where total biopterins were raised because of an elevation of the inactive dihydro-biopterin form because of DHPR inhibition. Liver derived pterin was not affected (Table 4:12).

There was no effect on brain protein concentration (Table 4:22) and no change was seen in CAT activity (Table 4:04). CAT activity was found to be elevated after 60 days dosing with 1% lead acetate (Modak *et al*, 1975). However, Carroll *et al* (1976) could find no change in CAT after chronic lead acetate administration.

Plasma biopterins were increased in both groups (Table 4:18), suggesting DHPR inhibition rather than the influence of inhibition of the *de novo* pathway. Measurement of lead by atomic absorption in tissues examined in this experiment showed that both groups had significantly higher levels compared to control plasmas, brains and livers.

Considering the amount of lead acetate ingested, tissue levels were unexpectedly low. Also, the only significant difference in lead levels between the two lead dosed groups was a higher level in the livers of the lower-dosed group (Table 4:17). Aungst *et al* (1981) found that blood and kidney lead concentrations in adult rats exposed for 14 days to lead *via* drinking water were not proportional to the apparent amount of lead ingested. They concluded that the relationship between oral dose and toxic effect may not be a simple one, and absorption of lead from the GIT is largely capacity limited in adult and adolescent rats.

In another experiment, the phenylalanine and tyrosine levels were examined in brains and plasmas of rats dosed for twice a day for 5 days with 0.3 ml of 10^{-3}M lead acetate. In all cases, the actual concentrations of the amino acids were unchanged (Tables 4:09, 4:10, 4:26, 4:27). The P:T ratios were normal in plasma (Table 4:28), but lower in dosed brains (Table 4:11), suggesting a derangement of their metabolism, which could be caused by interference with BH_4.

4:3:3 Aluminium.

Body weights were low compared to controls (Table 4:01), although animals fed

normally. Aluminium ingestion resulted in a decrease in brain biopterins (Table 4:03). No effect was observed on brain derived pterin (Table 4:02). Liver biopterins were elevated (Table 4:13), but derived pterin was decreased in liver (Table 4:12). Total biopterins were measured - tetrahydro-, dihydro- and biopterin after acid oxidation. The increase in liver total biopterins may have resulted from DHPR inhibition and subsequent increase in dihydrobiopterin (Eggar *et al*, 1986). No effect was found on plasma biopterins (Table 4:18).

Brain CAT activity was decreased after administration of the metal compound (Table 4:04). Yates et al (1980), reported a decrease in CAT activity in rabbits. There is a deficiency of CAT in SDAT (Bowen *et al*, 1983). this finding has been confirmed repeatedly. CAT is lowered by 20% by 550µg $AlCl_3$/g wet weight of tissue (Wisniewski *et al*, 1985), and since levels found in SDAT are much lower than this, then the reduction of enzymatic activity is not the result of direct inhibition by aluminium. This was shown here where aluminium acetate had no direct *in vitro* effect on rat brain CAT (Table 4:05, 4:06). A Tris-HCl system was also used to ensure that the EDTA present in the reaction mixture was not preventing aluminium inhibition by chelation. Boegman & Bates (1984) suggested that decreased CAT activity is the result of reduced slow transport of the enzyme from motor neurones located in the spinal cord. It follows that if aluminium is involved in the aetiology of SDAT, decreased CAT may be due to a lack of the enzyme being present rather than enzyme inhibition. No effect was found on brain protein level (Table 4:22). Blood CAT measurements were attempted (Table 4:29). Higher activity values might be achieved by measuring the enzyme in plasma. Very low activity was detected in whole blood, probably present from tissue overflow. Aluminium lowers axonal transport of CAT and this could result in reduced tissue content of the enzyme and reduced overflow into the blood. If this was the case, sensitive measurement of CAT in blood may be useful in identifying 'at risk' individuals.

As discussed previously, alterations in BH_4 metabolism have been reported in dialysis dementia. Aluminium has been implicated in this disorder, but it may not be the direct cause (Wisniewski *et al*, 1985). Therefore, BH_4 deficiency may be important. It would be interesting to know if there is a lack of CAT activity in this disorder.

Cowburn & Blair (1987) have suggested that aluminium inhibits BH_4 biosynthesis by it's effect on pyruvoyltetrahydropterin synthase by binding with the triphosphate group of intermediates in preference to magnesium (the natural cofactor) and inhibiting the breakage of the phosphate ester linkage and formation of 6-pyruvoyltetrahydropterin.

Trapp (1980) suggested a similar mechanism for mammalian hexokinase, and Lai *et a.* (1980) for inhibition of various ATPases.

4:4 Conclusions.

4:4:1 Diaminohydroxypyrimidine.

DAHP causes a decrease in tissue biopterin levels and hyperphenylalaninaemia in the rat. Derived pterin, especially in the brain is decreased after inhibiting BH_4 biosynthesis. Lower levels of DAHP administered chronically could be useful to study behavioural changes caused by BH_4 deficiency and HPA, and to see if these biochemical changes can influence the cholinergic system.

4:4:2 Lead.

Work here has confirmed that lead affects BH_4 metabolism. Whether *de novo* biosynthesis and/or DHPR inhibition occurs probably depends on many factors, such as lead exposure - dose, age and time of exposure, the amount of lead entering different tissues and organs and the relative importance of the *de novo* and salvage pathways in the maintenance of BH_4 levels in different organs.

4:4:3 Aluminium.

Aluminium intoxication can inhibit both *de novo* synthesis and salvage of BH_4 and so cause changes in the tissue concentration of total biopterins detected. Aluminium induced brain CAT deficiency was demonstrated. Both of these neurochemical findings occur in SDAT and probably in dialysis dementia and could contribute to the neurological defect. This work strengthens the hypothesis that aluminium is involved in the aetiology of these disorders.

Tables of Significant Results.

Table 4:01

The effect of chronic lead and aluminium dosing on body weight.

Group	Body Weight / g.	
Controls	483.33± 12.63 (6)	
Aluminium	454.33± 13.98 (6)	p<0.005
Lead (1)	331.50± 47.26 (6)	p<0.001
Lead (2)	387.83± 42.67 (6)	p<0.001

Initial body weight of animals three months prior to death at the start of the experiment was 40g.

Studies on brain tissue.

Table 4:02

The effect of chronic lead and aluminium dosing on rat brain derived pterin

Group	Derived Pterin	
Control	95.06± 9.98 (6)	
Aluminium	94.73± 31.02 (6)	ns
Lead (1)	70.91± 15.64 (6)	p<0.01
Lead (2)	84.41± 21.44 (6)	ns

Values as ng pterin / g wet weight of tissue.

40g male weanling rats were administered with the appropriate metal acetate as follows: Aluminium Group, 0.03% aluminium acetate for 1 month, followed by 1% for the second month, and 2% for the third month. Lead Group 1, 0.78% lead acetate for 1 month, 2% for the second month, and 4% for the third month. Lead Group 2, 0.39% lead acetate for 1 month, 1% for the second month, and 2% for the final month. Control animals were given ordinary tap water for the duration of the experiment.

Table 4:03

The effect of chronic lead and aluminium dosing on rat brain total biopterins.

Group	Total Biopterins	
Controls	75.61± 12.20 (6)	
Aluminium	53.11± 14.48 (6)	p<0.02
Lead (1)	45.36± 15.33 (5)	p<0.01
Lead (2)	60.15± 25.84 (6)	ns

Values as ng biopterin / g wet weight of tissue .

Table 4:04

The effect of chronic lead and aluminium dosing on brain choline acetyltransferase (CAT) activity .

Group	Choline acetyltransferase activity.	
Controls	11.63± 2.13 (6)	
Aluminium	7.63± 2.48 (6)	p<0.02
Lead (1)	10.09± 3.06 (6)	ns
Lead (2)	11.95± 2.57 (6)	ns

Values as µmol. acetylcholine formed / g wet weight of tissue / h.

40g male weanling rats were administered with the appropriate metal acetate as follows: Aluminium Group, 0.03% aluminium acetate for 1 month, followed by 1% for the second month, and 2% for the third month. Lead Group 1, 0.78% lead acetate for 1 month, 2% for the second month, and 4% for the third month. Lead Group 2, 0.39% lead acetate for 1 month, 1% for the second month, and 2% for the final month. Control animals were given ordinary tap water for the duration of the experiment.

Table 4:05

The *in-vitro* effect of aluminium acetate on rat brain choline acetyltransferase (CAT) in EDTA system.

Concentration of aluminium acetate	CAT Activity.
0.00	13.39± 0.57 (2)
1.43×10^{-2}	13.52± 0.21 (2)
1.43×10^{-3}	14.29± 0.86 (2)
1.43×10^{-4}	14.28± 0.83 (2)
1.43×10^{-5}	14.69± 0.50 (2)
1.43×10^{-6}	14.77± 3.04 (2)
1.43 x 10-7	12.93±3.04 (2)

Specific activity of CAT expressed as µmol. acetylcholine formed / g wet weight of tissue. Aluminium acetate is expressed as final concentration in EDTA reaction mixture pH 7.4.

Table 4:06

The *in-vitro* effect of aluminium acetate on rat brain choline acetyltransferase (CAT) in Tris-HCl system.

Concentration of aluminium acetate	CAT Activity
0.00	13.76± 0.57 (3)
$1.43x10^{-2}$	12.26± 0.86 (3)
$1.43x10^{-3}$	14.28± 0.86 (3)
$1.43x10^{-4}$	15.73± 0.67 (3)
$1.43x10^{-5}$	15.16± 0.06 (3)
$1.43x10^{-6}$	16.15± 0.16 (3)
$1.43x10^{-7}$	15.87± 0.79 (3)

Specific activity of CAT expressed as µmol acetycholine formed / g wet weight of tissue.
Aluminium acetate is expressed as final concentration in Tris-HCl reaction mixture, pH 6.8.

Table 4:07

The effect of DAHP on rat brain total biopterins.

Group	Total Biopterins	
Control	99.55± 23.66 (6)	
DAHP	42.74± 7.69 (5)	p<0.005

Values as ng biopterin / g wet weight of tissue. 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Table 4:08

The effect of DAHP on rat brain derived pterin.

Group	Derived Pterin	
Control	42.22± 15.08 (6)	
DAHP	23.45± 7.32 (5)	p<0.050

Values as ng pterin / g wet weight of tissue. 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Table 4:09

The effect of sub-chronic lead acetate dosing on brain tyrosine in rats.

Group	Tyrosine	
Control	15.94± 2.52 (6)	
Lead	18.31± 2.84 (6)	ns

Values as µmoles of tyrosine / l brain supernatant (20% w/v 0.02M HCl homogenate). 180g male Wistar rats were dosed with 0.3 ml of 10^{-3} M lead acetate i.p. twice a day for 5 days and killed 12 h later.

Table 4:10

The effect of sub-chronic lead acetate dosing on brain phenylalanine in rats.

Group	Phenylalanine	
Control	19.28± 2.83 (6)	
Lead	18.16± 1.83 (6)	ns

Values as µmoles of phenylalanine / l of brain supernatant (20% w/v 0.02M HCl homogenate). 180g male Wistar rats were dosed with 0.3 ml lead acetate i.p. twice a day for 5 days and killed 12 h later.

Table 4:11

The effect of sub-chronic lead acetate dosing on phenylalanine : tyrosine ratios in rat brains.

Group	Phe : Tyr ratio	
Control	1.22± 0.07 (6)	
Lead	1.00± 0.07 (6)	$p < 0.001$

180g male Wistar rats were dosed with 0.3 ml lead acetate i.p. twice a day for 5 days and killed 12 h later.

Studies on liver tissue.

Table 4:12

The effect of chronic lead and aluminium dosing on rat liver derived pterin

Group	Derived Pterin	
Control	0.39± 0.12 (6)	
Aluminium	0.20± 0.08 (6)	p<0.01
Lead (1)	0.49± 0.11 (6)	ns
Lead (2)	0.45± 0.14 (6)	ns

Values as µg pterin / g wet weight of tissue.

Table 4:13

The effect of chronic lead and aluminium dosing on rat liver total biopterins.

Group	Total biopterins	
Controls	1.29± 0.34 (6)	
Aluminium	1.82± 0.08 (6)	p<0.005
Lead (1)	1.95± 0.23 (6)	p<0.005
Lead (2)	1.80± 0.20 (6)	p<0.020

Values as µg biopterin / g wet weight of tissue .

40g male weanling Wistar rats were administered with the appropriate metal acetate as follows: Aluminium Group, 0.03% aluminium acetate for 1 month, followed by 1% for the second month, and 2% for the third month. Lead Group 1, 0.78% lead acetate for 1 month, 2% for the second month, and 4% for the third month. Lead Group 2, 0.39% lead acetate for 1 month, 1% for the second month and 2% for the final month. Control animals were given ordinary tap water for the duration of the experiment.

Table 4:14

The effect of DAHP on rat liver total biopterins .

Group	Total biopterins	
Controls	2.29± 0.16 (6)	
DAHP	0.43± 0.44 (5)	p<0.001

Values as µg biopterin /g wet weight of tissue . 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% DAHP for 3 days in blended food pellets.

Table 4:15

The effect of DAHP on rat liver derived pterin .

Group	Derived Pterin	
Controls	0.84± 0.05 (6)	
DAHP	0.47± 0.06 (5)	p<0.001

Values as µg pterin / g wet weight of tissue . 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Table 4:16

The effect of DAHP on rat liver biopterin biosynthesis

Group	Biopterin Biosynthesis	
Control	12.04± 4.33 (6)	
DAHP	6.04± 1.63 (5)	p<0.025

Values as ng biopterin produced / h / mg of protein . 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets. Assays performed by J . Cox.

Table 4:17

Lead levels in tissues of rats chronically dosed with lead acetate.

	Brain	Plasma	Liver
Control	0.33± 0.10 (4)	45.00± 19.15 (4)	0.63± 0.10 (4)
Lead Group 1	0.82± 0.04 (5)	95.00± 20.23 (12)	1.10± 0.32 (6)
	$p < 0.001$	$p < 0.001$	$p < 0.025$
Lead Group 2	0.75± 0.20 (6)	91.67± 11.93 (12)	1.49± 0.24 (10)
	$p < 0.005$	$p < 0.001$	$p < 0.001$

Lead concentrations were measured by atomic absorption (Chapter 2). Plasma values are expressed as µg / 100 mls of plasma. Brain and liver concentrations are as µg / g wet weight of tissue.

The only difference found in tissue content of lead between the two dosed groups was a significantly higher level in liver of the lower dosed group (Group 2) ($p < 0.020$).

Lead Group 1 was dosed with 0.78% lead acetate in the drinking water for 1 month, 2% lead acetate for the second month and 4% lead acetate for the final month.

Lead Group 2 was dosed with 0.39% lead acetate in the drinking water for 1 month, 1% lead acetate for the second month and 2% lead acetate for the final month.

Studies on plasma.

Table 4:18

The effect of chronic lead and aluminium dosing on rat plasma total biopterins.

Group	Plasma total biopterins.	
Controls	12.22± 3.25 (6)	
Aluminium	13.37± 2.32 (6)	ns
Lead (1)	23.52± 4.96 (6)	p<0.001
Lead (2)	21.91± 9.71 (6)	p<0.050

Values as µg biopterin / l plasma.
40g male weanling Wistar rats were administered with the appropriate metal acetate as follows: Aluminium Group, 0.75 mM aluminium acetate for 1 month, followed by 1% for the second month, and 2% for the third month. Lead Group 1, 0.78% lead acetate for 1 month, 2% for the second month, and 4% for the third month. Lead Group 2, 0.39% lead acetate for 1 month, 1% for the second month and 2% for the final month. Control animals were given ordinary tap water for the duration of the experiment.

Table 4:19

The effect of DAHP on rat plasma total biopterins.

Group	Total biopterins	
Controls	20.74± 3.12 (6)	
DAHP	11.84± 6.66 (5)	p<0.020

Values as µg biopterin / litre of plasma. 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Table 4:20

The effect of DAHP on rat plasma phenylalanine levels

Group	Phenylalanine	
Controls	46.54± 14.54 (5)	
DAHP	135.49± 43.01 (5)	p<0.005

Values as µmoles of phenylalanine / litre of plasma. 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Table 4:21

The effect of DAHP on plasma phenylalanine : tyrosine ratios .

Group	Phe : Tyr Ratio	
Control	0.96± 0.37 (5)	
DAHP	3.85± 1.99 (5)	p<0.020

40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Tables of non-significant results.

Studies on brain tissue.

Table 4:22

The effect of chronic lead and aluminium dosing on brain protein.

Group	Protein Concentration	
Controls	4.65± 0.87 (6)	
Aluminium	4.50± 0.35 (6)	ns
Lead (1)	4.25± 0.26 (6)	ns
Lead (2)	4.60± 0.31 (6)	ns

Values as mg protein / ml 20% w/v Tris-HCl pH 6.8 homogenate as measured by the biuret reaction .

Table 4:23

The effect of DAHP on rat brain choline acetyltransferase activity (CAT).

Group	CAT Activity	
Control	12.06± 2.04 (5)	
DAHP	14.05± 1.93 (5)	ns

Values as µmoles of acetylcholine produced / h / g wet weight of tissue . 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Studies on liver tissue

Table 4:24

The effect of DAHP on rat liver DHPR.

Group	DHPR Activity.	
Control	450.00± 75.40 (6)	
DAHP	513.29± 38.70 (5)	ns

Assays performed by J. Cox.

Studies on plasma.

Table 4:25

The effect of DAHP on rat plasma tyrosine levels

Group	Tyrosine	
Controls	56.30± 30.36 (5)	
DAHP	41.06± 16.13 (5)	ns

Values as µmoles of tyrosine / litre of plasma. 40g male weanling Wistar rats were dosed with 5% w/w for 3 days and 3% w/w DAHP for 3 days in blended food pellets.

Table 4:26

The effect of sub-chronic lead acetate dosing on plasma tyrosine in rats.

Group	Tyrosine	
Control	44.18± 8.90 (6)	
Lead	34.11± 9.49 (6)	ns

Values as µmoles of tyrosine / l of plasma. 180g male Wistar rats were dosed with 0.3 ml of lead acetate i.p. twice a day for 5 days and killed 12 h later.

Table 4:27

The effect of sub-chronic lead acetate dosing on plasma phenylalanine in rats.

Group	Phenylalanine	
Control	35.65± 8.57 (6)	
Lead	29.81± 3.94 (6)	ns

Values as µmoles of phenylalanine / l of plasma. 180g male Wistar rats were dosed with 0.3 ml of lead acetate i.p. twice a day for 5 days and killed 12 h later.

Table 4:28

The effect of sub-chronic lead acetate dosing on plasma phenylalanine : tyrosine ratios in rats.

Group	Phe : Tyr ratio	
Control	0.81± 0.12 (6)	
Lead	0.93± 0.27 (6)	ns

180 g male Wistar rats were dosed with 0.3 ml of lead acetate i.p. twice a day for 5 days and killed 12 h later.

Table 4:29

Choline acetyltransferase activity in lysed human blood.

Specific activity measured on 'day 1.'

3.31± 0.77 (12)

Specific activity re-measured on 'day 3.'

4.19± 1.41 (12) ns

Values are expressed as pmoles of acetylcholine produced / mg of protein / h. Assays were conducted on the lysed whole blood of one subject. Enzyme activity was re-measured on the same samples several days later to see if there was any quenching effect present.

Chapter 5. The Effects Of Bromocriptine And Scopolamine On BH_4 Metabolism.

Chapter Five.

5 The Effects Of Bromocriptine And Scopolamine On BH_4 Metabolism.

5:1 Introduction.

5:1:1 Bromocriptine.

Bromocriptine (29) (Parlodel-Sandoz) is an ergot compound used in the treatment of puerperal lactation (2.5-5.0 mg/ day) and parkinsonism (1.0-300.0 mg / day). It acts as a dopamine agonist. Fluorescence microscopy studies by Hokfelt and Fuxe (1972) first suggested that bromocriptine reduced neurotransmitter turnover in central dopaminergic neurones. This was proved biochemically by Corrodi *et al* (1973).

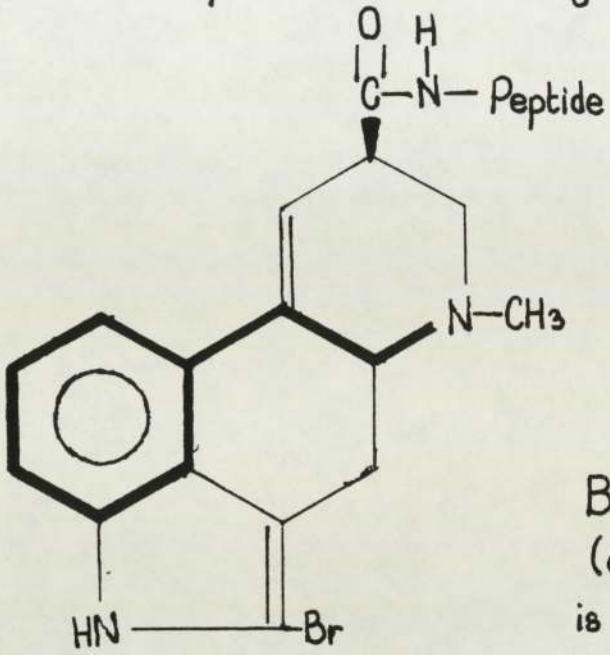
The first reported uses of bromocriptine in parkinsonism were by Calne *et al* (1974) and Lees *et al* (1975). Subsequent studies have demonstrated it's antiparkinsonism properties (Stern & Lees, 1983, Lipcsey & Peres, 1984).

Bromocriptine stimulates D_2-dopamine receptors, which are adenylate cyclase insensitive, and antagonises D_1 receptors (adenylate cyclase sensitive). Serotonin receptors which are linked to adenylate cyclase are also blocked (Rinne,1983). Dopamine agonists interact with other neurotransmitter receptors and vary in their specificity for the different subtypes of dopamine receptors. All compounds have antagonistic effects on ∂_1 (alpha-1) and ∂_2 (alpha-2) receptors.

Although believed to be a direct dopamine agonist, bromocriptine has a curious dependence on intact synthesis and storage of endogenous CNS dopamine (LeWitt *et al*, 1983). Bromocriptine treatment in parkinsonism gives improvements similar to L-DOPA (Stern & Lees, 1983).

Bromocriptine causes more nausea, sedation and mental changes but fewer dyskinesias than L-DOPA and a peripheral decarboxylase inhibitor (Rinne, 1983) and is more effective in preventing the on-off effect (Lipcsey & Peres, 1984).

Summary of structures of agents studied



(29)

Bromocriptine
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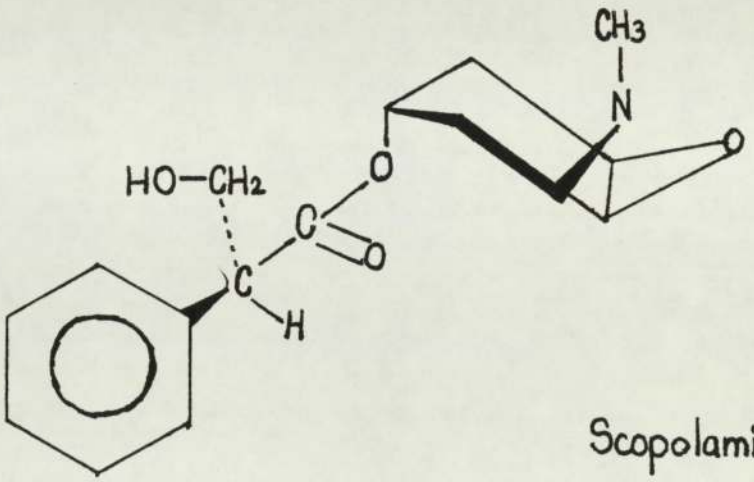
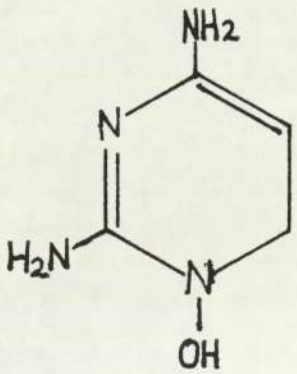


(30)

Scopolamine



(31)

DAHP

Interference with BH_4 can cause potential neurological effects. The effects of bromocriptine were investigated to compare and contrast the effects on BH_4 metabolism caused by other antiparkinsonism therapies, L-DOPA and L-DOPA plus carbidopa (Chapter 3).

5:1:2 Scopolamine.

Scopolamine (L-hyoscine) (30) is an anticholinergic drug which acts by antagonism of muscarinic receptors. Scopolamine causes the following successive changes on the central nervous system (Meyers et al, 1980):
(1) Sedation, dizziness and fatigue. (2) Excitement or delirium. Hallucinations with large doses. (3) Profound depression or coma. (4) Amnesia for the period of intoxication. (5) Convulsions. (6) Respiratory depression only with very large doses.

Learning and memory are related to the central cholinergic system. Drachman and Leavitt (1974) observed that scopolamine produced a pattern of cognitive impairment in healthy young adults which resembled that seen in 'normal aging' and in exaggerated form, in dementia. Memory for recent events was more severely impaired than memory for immediate or remote events.

In 1867, Charcot advised the use of the antimuscarinic drug, atropine (Green & Costain, 1981) in the treatment of Parkinson's disease. For the next century, atropine and related drugs formed the basis of pharmacological treatment. Scopolamine is still used infrequently in the treatment of parkinsonism to relieve the rigidity (Meyers *et al*, 1980).

The effect of scopolamine was investigated to see if an anticholinergic agent could influence BH_4 metabolism. Since scopolamine has been used in therapy of Parkinson's disease, it's effects were studied as they were for other therapeutic agents such as bromocriptine, L-DOPA and Sinemet Plus.

5:2 Methods.

Measurements of tissue derived pterin, total biopterins, BH_4 biosynthesis, DHPR, CAT, and protein were done as described in Chapter 2.

180g male Wistar rats were dosed with 5mg of bromocriptine mesylate i.p. once per day for 2 days and killed 24 hours later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2 ml of 1% lactic acid solution and 3 ml of distilled water. Controls were dosed with lactic acid solution.

270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride per animal orally, and killed 5 hours later. Controls were administered distilled water i.g.

5:3 Results and Discussion.

5:3:1 Bromocriptine.

Bromocriptine mesylate dosing caused a significant reduction in brain derived pterin concentration (Table 5:01), but had no effect on brain total biopterins (Table 5:07). Pterin is a potential catabolite of BH_4 (Pfleiderer, 1975). Enzymatic and chemical oxidation of BH_4 at pH 7.4 yields quinonoid dihydrobiopterin. This is reduced back to BH_4 by the action of DHPR. qBH_2 can rearrange to 7,8-dihydrobiopterin. 7,8-dihydropterin is also derived from the oxidation of BH_4 *via* qBH_2 and side-chain loss. Pterin is the oxidation product of dihydropterin.

The pharmacological action of bromocriptine is as a dopamine agonist (LeWitt *et al,* 1983). Dopamine agonists can decrease the activity of cerebral tyrosine hydroxylase activity (Galloway & Levine, 1986), without affecting total biopterin concentration. It follows that reduced tyrosine hydroxylase activity utilises less BH_4, and less pterin is derived from oxidation of the cofactor.

The effect of bromocriptine mesylate on peripheral BH_4 metabolism contrasted to the CNS effect. Liver total biopterins (Table 5:03) and plasma total biopterins (Table 5:06) were significantly reduced.

Liver protein concentration was not altered by administration of the drug (Table 5:10). Liver DHPR activity was not affected both in *in-vivo* (Table 5:12) and in *in-vitro* (Table 5:13) studies. Liver derived pterin was increased (Table 5:04). This finding is associated with *in-vivo* inhibition of DHPR (Edwards *et al,* 1987). One of the actions of bromocriptine is to block alpha-1 and alpha-2 adrenergic receptors. Presynaptic alpha-2 receptors act as autoreceptors (Langer, 1977) and activation causes

reduced release of neurotransmitters. Antagonism causes increased release, and it is postulated that the increase in endogenous catecholamines by bromocriptine antagonism of alpha-2 receptors allowed catecholamine inhibition of DHPR (Purdy *et al*, 1981), resulting in elevated pterin levels.

No effect was observed on *in-vivo* dosed animals liver biopterin biosynthesis (Table 5:11). However, *in-vitro* studies showed that bromocriptine mesylate is capable of inhibiting *de-novo* BH_4 synthesis (Table 5:05) by about 65%.

5:3:2 Scopolamine.

Scopolamine caused a reduction in the concentration of brain derived pterin (Table 5:02). As previously described, pterin is a catabolite of tetrahydrobiopterin (Pfleiderer, 1975) and a decrease in it's concentration can signify a decreased turnover of BH_4. It may also reflect lowered THF.

No other effect on BH_4 metabolism was found. Scopolamine had no effect on the concentration of liver, brain or plasma total biopterins (Tables 5:14, 5:08, 5:19), liver derived pterin (Table 5:18), liver protein (Table 5:15), DHPR (Table 5:16), or BH_4 biosynthetic capacity (Table 5:17).

Scopolamine did not affect the activity of brain CAT (Table 5:09). Dubois *et al* (1983) could find no change in CAT in post-mortem human brains of people who had been treated in life with anticholinergic therapies.

Prolonged cholinergic stimulation by oxotremorine (a muscarinic agonist) has been shown to increase the activity of tyrosine hydroxylase in the locus coeruleus, hypothalamus and hippocampus (reviewed by McIlwain & Bachelard, 1985). Immunoassay revealed no new protein. The increase was due to activation of existing protein, such as by changes in cyclic AMP (cAMP).

Tyrosine hydroxylase activity in the brain is directly sensitive to cyclic nucleotides. Activation by cAMP lowered the Km for BH_4 to one sixth, and the Ki value for inhibition by dopamine was increased from 0.1 to 0.6mM (Harris *et al*, 1974).

In sympathetic ganglia, acetylcholine is the transmitter at preganglionic terminals and

has been suggested to have three actions (Libet *et al*, 1975, Greengard, 1976). The first is from terminals directly on to the postganglionic cells, probably without cyclic nucleotide mediation. The second set of terminals are on dopaminergic interneurones which when activated, release dopamine at the postganglionic cells, mediated by cAMP and a dopamine mediated adenylate cyclase. The third action is suggested to be similar to the first in being an effect of acetylcholine directly on the postganglionic cell, but at different synapses, with a different muscarinic type of receptor where cyclic GMP is generated.

Within the brain and CNS, the muscarinic type of receptor outnumbers the nicotinic type by a factor between 10 and 100 (Bradford & Freeman, 1986). Preganglionic activation of muscarinic receptors (such as by oxotremorine) can cause increased activity of tyrosine hydroxylase and increased postsynaptic turnover of catecholamines. Conversely, muscarinic receptor antagonism, such as that achieved by administration of scopolamine can reduce tyrosine hydroxylase activity and catecholamine and BH_4 turnover. Decreased levels of brain derived pterin can be explained by the reduced turnover of BH_4.

5:4 Conclusions.

5:4:1 Bromocriptine.

Bromocriptine stimulates dopamine receptors in the brain which causes lowered tyrosine hydroxylase activity (Galloway & Levine, 1986). This leads to a reduced turnover of BH_4 and as a result, less derived pterin is produced from the oxidation of the cofactor. There was no effect on the concentration of total biopterins in the brain.

In-vitro studies showed that bromocriptine inhibits BH_4 biosynthesis. This was supported by the *in-vivo* findings of lowered liver and plasma total biopterin concentrations in dosed rats.

5:4:2 Scopolamine.

Scopolamine had no direct effect on BH_4 metabolism or CAT activity in the rat. Reduced brain derived pterin levels were observed probably due to preganglionic muscarinic block in sympathetic neurones preventing acetylcholine mediation in the

release of postganglionic catecholamines and reduction in tyrosine hydroxylase activity with a subsequent decrease in the turnover of BH_4.

Tables of significant results.

Studies on brain tissue.

Table 5:01

The effect of bromocriptine on rat brain derived pterin.

Group	Derived Pterin	
Controls	63.40± 7.17 (6)	
Bromocriptine	51.14± 6.93 (6)	p<0.010

Values as ng pterin / g wet weight of tissue. 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:02

The effect of scopolamine on rat brain derived pterin.

Group	Derived Pterin	
Control	69.17± 4.76 (6)	
scopolamine	49.56± 9.70 (6)	p < 0.005

Values as ng pterin / g wet weight of tissue . 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Studies on liver tissue.

Table 5:03

The effect of bromocriptine on liver total biopterins.

Group	Total biopterins	
Controls	1.57± 0.19 (6)	
Bromocriptine	1.27± 0.12 (6)	p<0.010

Values as µg biopterin / g wet weight of tissue . 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:04

The effect of bromocriptine on liver derived pterin.

Group	Derived Pterin	
Controls	0.49± 0.08 (6)	
Bromocriptine	0.66± 0.14 (6)	p<0.050

Values as µg pterin / g wet weight of tissue . 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:05

The *in-vitro* effect of bromocriptine mesylate on liver BH_4 biosynthesis.

Bromocriptine mesylate/M	BH_4 synthesis.	
0.00	43.49 ± 13.05 (3)	
1.33×10^{-7}	26.53 ± 2.42 (3)	ns
1.33×10^{-6}	20.98 ± 2.24 (3)	$p < 0.05$
1.33×10^{-5}	9.22 ± 3.81 (3)	$p < 0.02$
1.33×10^{-4}	13.18 ± 7.71 (3)	$p < 0.05$
1.33×10^{-3}	14.80 ± 0.54 (3)	$p < 0.02$

Values as ng of biopterin produced/ h / ml of supernatant.

Studies on plasma.

Table 5:06

The effect of bromocriptine on plasma total biopterins

Group	Total Biopterins	
Controls	22.23± 3.15 (6)	
Bromocriptine	14.65± 2.04 (6)	$p < 0.001$

Values as µg biopterin / litre of plasma . 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Tables of non-significant results.

Studies on brain tissue.

Table 5:07

The effect of bromocriptine on rat brain total biopterins.

Group	Total biopterins	
Controls	63.89± 8.12 (6)	
Bromocriptine	71.72± 5.06 (6)	ns

Values as ng biopterin / g wet weight of tissue. 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:08

The effect of scopolamine on rat brain total biopterins

Group	Total Biopterins	
Control	108.97± 24.38 (6)	
scopolamine	106.16± 30.32 (6)	ns

Values as ng biopterin / g wet weight of tissue. 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Table 5:09

The effect of scopolamine on rat brain choline acetyltransferase (CAT) activity.

Group	CAT Activity	
Control	9.46± 3.21 (6)	
scopolamine	9.11± 2.68 (6)	ns

Values as µmoles of acetylcholine produced / h / mg of protein. 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Studies on liver tissue.

Table 5:10

The effect of bromocriptine on liver protein concentration.

Group	Protein	
Control	72.48± 9.41 (6)	
Bromocriptine	63.32± 5.22 (6)	ns

Values as mg of protein/ ml of 20% Tris-HCl homogenates, pH 6.8 after appropriate dilution, as measured by the biuret reaction. 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:11

The *in-vivo* effect of bromocriptine on liver biopterin biosynthesis.

Group	Biopterin biosynthesis	
Control	8.97± 1.97 (6)	
Bromocriptine	9.90± 3.32 (6)	ns

Values are expressed as nmoles of ng of biopterin produced/ h/ mg of protein. 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:12

The *in-vivo* effect of bromocriptine on liver DHPR activity.

Group	DHPR activity	
Control	534.38± 56.75 (6)	
Bromocriptine	534.09± 56.75 (6)	ns

Values are expressed as nmoles of NADH oxidized / minute/ mg of protein. 180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate i.p. once/ day for 2 days and killed 24h later. Stock solution of bromocriptine mesylate was prepared fresh each day by dissolving 50 mg of bromocriptine mesylate in 2ml of 1% lactic acid and 3 ml distilled water. Controls were dosed with lactic acid solution.

Table 5:13

The *in-vitro* effect of bromocriptine on liver DHPR activity.

Bromocriptine mesylate concentration	DHPR activity	
0.00 µM	23.69± 3.40 (3)	
0.13 µM	22.40± 0.77 (3)	ns
1.25 µM	24.79± 1.58 (3)	ns
12.50 µM	25.40± 1.81 (3)	ns
25.00 µM	21.50± 3.60 (3)	ns
50.00 µM	16.03± 3.90 (3)	ns

Values are expressed as µmoles of NADH oxidized/ minute/ ml of supernatant.

Table 5:14

The effect of scopolamine on rat liver total biopterins

Group	Total Biopterins	
Control	1.15± 0.24 (6)	
scopolamine	1.12± 0.32 (6)	ns

Values as µg biopterin / g wet weight of tissue . 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Table 5:15

The effect of scopolamine on liver protein concentration.

Group	Protein	
Control	51.70± 8.30 (6)	
scopolamine	60.00±17.10 (6)	ns

Values as mg of protein / ml of 20% Tris-HCl homogenate, pH 6.8 after appropriate dilution as measured by the biuret reaction. Measured by S. Jones. 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Table 5:16

The effect of scopolamine on liver DHPR activity.

Group	DHPR activity	
Control	169.40± 64.00 (6)	
scopolamine	218.30± 172.70 (6)	ns

Values as nmoles of NADH oxidized / minute/ mg of protein. Measured by S. Jones. 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Table 5:17

The effect of scopolamine on liver biopterin biosynthesis.

Group	Biopterin synthesis	
Control	12.16± 3.33 (6)	
scopolamine	11.81± 3.66 (6)	ns

Values expressed as ng of biopterin produced/ h /mg of protein. Measured by J. Cox. 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Table 5:18

The effect of scopolamine on rat liver derived pterin .

Group	Derived Pterin	
Control	0.45± 0.10 (6)	
scopolamine	0.42± 0.05 (6)	ns

Values as µg pterin / g wet weight of tissue . 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Studies on plasma.

Table 5:19

The effect of scopolamine on rat plasma total biopterins.

Group	Total biopterins	
Control	28.69± 7.50 (6)	
scopolamine	32.30± 7.74 (6)	ns

Values as µg biopterin / litre of plasma. 270g male Wistar rats were dosed with 0.023 mg scopolamine hydrochloride / animal orally and killed after 5h. Controls were given distilled water i.g.

Chapter 6. Use Of Derived Pterin As A Non-specific Indicator Of Reduced Pteridine Metabolism.

Chapter Six.

6. Use of Derived Pterin as a Non-specific Indicator of Reduced Pteridine Metabolism.

6:1 Introduction.

The term 'derived pterin' is used to describe pterin measured by HPLC after acid -iodine oxidation, which consists of that present endogenously mainly as a catabolite of BH_4 as dihydropterin , and that formed from the acid-iodine oxidation of THF.

Pterin (2-amino-4-oxodihydro-pteridine) (1) is the basic molecule of most naturally occurring pteridine derivatives (Pfleiderer, 1978). Pfleiderer (1975) found that 5,6,7,8-tetrahydrobiopterin is oxidized by air at pH 7.5 to three main products identified as biopterin , 7,8-dihydroxanthopterin and pterin. The same reaction at pH 4.0 also led to biopterin and pterin , but in addition pterin-6-carboxylic acid, sepiapterin and isosepiapterin were found. Heales (1987) found that air oxidation of BH_4 at pH 7.6 yielded 31% biopterin, 15% xanthopterin and 60% pterin.

Fukushima and Nixon (1980) concluded that pterin is also derived from tetrahydrofolic acid or it's derivatives. Neutral oxidation of THF gives quinonoid dihydrofolate (qFH_2) and dihydropterin. Acid oxidation of THF yields approximately 100% pterin as product.

The aim of this study was to investigate the origins of tissue derived pterin and to see if it has a potential use as an indicator of BH_4 and THF metabolism.

6:2 Materials and Methods.

Total and differential folate measurements, total biopterins and derived pterin were all assayed as described in Chapter 2.

6:3 Results and Discussion.

DAHP administration to rats (as described in Chapter 4) caused a significant decrease in both brain and liver derived pterin (Table 6:06). DAHP depletes total biopterins by inhibition of GTP cyclohydrolase. Lowered derived pterin levels would result from the decreased biopterin precursor concentration. GTP cyclohydrolase also catalyses the first step in folate biosynthesis (Blakley, 1969) but it is unlikely that THF levels were affected by DAHP since folates are vitamins in the rat, and are not endogenously produced.

A decreased derived pterin concentration was found in the brains of bromocriptine dosed rats (Chapter 5 & Table 6:06). Bromocriptine stimulates dopamine receptors and can decrease tyrosine hydroxylase. Galloway and Levine (1986) demonstrated this effect of dopamine agonists. Decreased tyrosine hydroxylase activity lowers the requirement for BH_4 cofactor and reduces it's turnover. Low brain derived pterin levels can reflect lowered turnover of BH_4.

No biochemical or clinical effect of bromocriptine therapy on folate metabolism has been reported in the literature. Another drug which caused lowered brain derived pterin levels, probably by decreased tyrosine hydroxylase activity, and reduced turnover of BH_4 was scopolamine (Table 6:06 & Chapter 5).

The increase in plasma derived pterin levels (Table 6:06) by p-hydroxyphenylacetate (as described in Chapter 3) is thought to be due to inhibition of tissue DHPR (Shen, 1984) causing an efflux of BH_2 and dihydropterin into plasma, which is detected after acid-iodine oxidation.

Some agents may have an effect on both BH_4 and THF, especially those that inhibit DHPR. In the brain, DHPR may have a role in it's maintenance of THF levels (Pollock & Kaufman, 1978). DHPR can reduce qFH_2 to THF (Lind,1972). Very little DHFR is present in the brain (Makulu *et al*, 1973). Secondary THF deficiency has been reported in patients with DHPR deficiency (reviewed by Smith *et al*, 1986), and folinic acid is required. The following drugs may have this dual effect.

Cotrimoxazole is a combination antibiotic consisting of trimethoprim and sulphamethoxazole. The trimethoprim component acts by inhibition of DHFR. DHPR is also inhibited by cotrimoxazole (Brown,1981). Rats treated with the drug (Chapter 3) were found to have lowered derived pterin levels in both the brain and the liver (Table 6:06). Since this antibiotic can potentially affect both BH_4 and THF metabolism, the decreased derived pterin levels might be the reult of inhibition of one or both enzymes.

The same situation is present in rats dosed with L-DOPA or Sinemet Plus (as in Chapter 3). L-DOPA is metabolised to dopamine and other metabolites which inhibit DHPR (Purdy *et al*, 1981, Shen *et al*, 1980), and could decrease both BH_4 and THF concentrations.

Tables 6:01 and 6:02 compare total biopterins concentrations against derived pterin, and total folates against derived pterin. There was no significant difference in the brain, but both total folates and total biopterins in the liver were significantly higher than derived pterin concentration.

The THF portion of the folate pool is the immediate precursor of derived pterin (Fukushima & Nixon, 1980). If derived pterin can arise from BH_4 metabolism, then derived pterin levels should be higher than THF.

Differential oxidation of rat tissues were performed (Tables 6:04 & 6:05) to detect various folate species. The results show that approximately 19% of total folates are as tetrahydrofolate in the brain. Liver concentration of THF was negligable, and this could be due to air oxidation of the assay system, where 'no oxidizing conditions' are used to measure total folates by the method of Coppell, (1984) (Chapter 2).

It follows that brain derived pterin concentration is much higher than THF. However, the percentage of total folates reported as THF varies (Wilson & Horne, 1986). Table 6:03 summarizes percentage of total folates as THF found by other workers. Wilson & Horne (1986) found that percentage of folates as THF was the same in the brain and the liver. Since there is such a wide discrepancy in literature reports of percentage THF, THF concentration was calculated as a percentage found of total folates measured in the brain and the liver (Tables 6:01 and 6:02) by the method stated of each worker in Table 6:03.

In all cases, brain derived pterin levels are higher than THF. The situation is not so clear in the liver, but it seems likely that liver derived pterin is higher than liver THF concentration.

Tables 6:07-6:10 demonstrate that detected changes in derived pterin did not arise from air oxidation of intact tissue during assay preparation and dissection procedures.

6:3 Conclusions.

Pterin is easily detected by HPLC after acid-iodine oxidation. Derived pterin concentration in the brain and probably in the liver is higher than THF, demonstrating that a portion of the derived pterin pool arises from BH_4 metabolism. Analysis of derived pterin is complicated because of it's dual origin. Certain agents, such as bromocriptine probably cause changes in levels of tissue derived pterin by only affecting BH_4 metabolism. Others may cause changes in tissue derived pterin levels by only affecting THF status. Nitrous oxide has been shown to have this effect (Heales, 1987). Drugs such as cotrimoxazole and L-DOPA may affect both THF and BH_4 metabolism. It is very important too know folate status when examining BH_4 metabolism to use derived pterin measurements accurately. Derived pterin measurements are useful as a non-specific indicator of reduced pteridine metabolism.

Table 6:01

A comparison of total biopterins and derived pterin, and total folates and derived pterin in the rat brain.

Total folates	Free folates	Derived pterin	Total biopterins
390.98± 51.28 (4)	318.30± 59.94 (4)	336.51± 94.61 (4)	333.78± 67.92 (4)

Derived pterin v. Total biopterins ns.

Derived pterin v. Total folates ns.

Values as pmoles / g wet weight

Table 6:02

A comparison of total biopterins and derived pterin, and total folates and derived pterin in the rat liver.

Total folates	Free folates	Derived pterin	Total biopterins
5.84± 1.47 (4)	4.94± 1.46 (4)	3.11± 0.22 (4)	5.04± 0.99 (4)

Derived pterin v. Total biopterins $p < 0.010$.

Derived pterin v. Total folates $p < 0.020$.

Values as nmoles / g wet weight.

Total folates are the polyglutamate and monoglutamate forms. The samples were treated with conjugase. Free folates are those in the monoglutamate form. Samples were not treated with conjugase. The *L. casei* assay detects folates in the monoglutamate form.
Total biopterins and derived pterin were measured by HPLC. Folates were measured by *L. casei* assay.

Test for conjugase activity: Yeast extract + conjugase, Total Folates = 14.6ng/ ml
Yeast extract - conjugase, Total Folates = 1.6ng/ ml

Table 6:03

A comparison of tissue derived pterin with tissue tetrahydrofolate levels.

Reference	Calculated brain THF	Calculated liver THF
McMartin *et al*, (1981) found 42% as THF.	**164.21± 21.54 (4)** p< 0.020	**2.45± 0.62 (4)** ns
Eto & Krumdieck, (1982) found 19% as THF.	**74.28± 9.74 (4)** p< 0.005	**1.11± 0.28 (4)** p< 0.001
Duch *et al* (1983) found 22% as THF.	**86.02± 11.28 (4)** p< 0.005	**1.28± 0.32 (4)** p< 0.001
Gregory *et al* (1984) found 44% as THF.	**172.03± 22.56 (4)** p< 0.020	**2.57± 0.65 (4)** ns
Wilson & Horne, (1984) found 33% as THF.	**129.02± 16.92 (4)** p< 0.005	**1.93± 0.49 (4)** p< 0.005
Wilson & Horne, (1986) found 43% as THF.	**168.12± 22.05 (4)** p< 0.020	**2.51± 0.63 (4)** ns
Present study, 19% as THF in the brain.	**74.28± 9.74 (4)** p< 0.005	–
Derived pterin	**336.51± 94.61 (4)**	**3.11± 0.22 (4)**

Liver values as nmoles/ g wet weight. Brain values as pmoles/ g wet weight. Derived pterin was measured by HPLC.THF was measured by differential oxidation and *L. casei* assay (Dr. R.J. Leeming) and also estimated as a percentage of measured total folates by the methods of other workers and compared to tissue derived pterin concentrations by t tests.

Table 6:04

Differential oxidation of rat brain to detect various folate species.

Total folate	5-Me.THF	10-CHO.THF	THF
392.43± 35.25 (4)	55.30± 31.26 (4)	263.91± 34.18 (4)	72.89± 36.13 (4)

Table 6:05

Differential oxidation of rat liver to detect various folate species.

Total folate	5-Me.THF	10-CHO.THF	THF
3.94± 0.92	1.24± 0.42	2.52± 0.51	0.00

5-Me.THF = 5-methyltetrahydrofolate, 10-CHO.THF = 10-formyltetrahydrofolate, THF = tetrahydrofolate.
Brain values as pmoles/ g wet weight. Liver values as nmoles/ g wet weight.

Table 6:06

Summary Table of Discussed Derived Pterin Results.

Agent	Tissue	Controls	Dosed
DAHP	Brain	42.22± 15.08 (6)	23.45± 7.35 (5) p< 0.050
DAHP	Liver	0.84± 0.05 (6)	0.47± 0.06 (5) p< 0.001
L-DOPA	Brain	86.00± 11.02 (6)	55.12± 10.13 (6) p< 0.010
Sinemet Plus	Brain	82.96± 10.52 (6)	52.64± 10.52 (6) p< 0.001
Cotrimoxazole	Brain	56.93± 11.65 (6)	35.56± 7.12 (6) p< 0.020
Cotrimoxazole	Liver	0.31± 0.02 (6)	0.25± 0.05 (6) p< 0.025
p-hydroxyphenyl-acetate	Plasma	1.60± 2.19 (6)	5.23± 3.28 (6) p< 0.050
Bromocriptine	Brain	63.40± 7.17 (6)	51.14± 6.93 (6) p< 0.010
Scopolamine	Brain	69.17± 4.76 (6)	49.56± 9.70 (6) p< 0.005

Brain values are as ng/ g wet weight, liver values are as µg/ g wet weight and plasma values are µg/ l of plasma.

Table 6:07

The effect of 2h air oxidation on brain biopterin levels.

Group	Total Biopterins	
T=0h	86.61± 15.43 (6)	
T=2h	69.00± 17.66 (6)	ns

Values as ng biopterin / g wet weight of brain tissue.
Animals used were 180g male Wistar rats. Brains were dissected and stored at -70 C until assay. Tissues were defrosted at room temperature and immediately assayed for total biopterins. The same tissues were allowed to oxidize under room temperature for 2 h and then reassayed for total biopterins.

Table 6:08

The effect of 2h air oxidation on brain derived pterin levels.

Group	Derived Pterin	
T=0h	55.01± 7.86 (6)	
T=2h	52.94± 12.66 (6)	ns

Values as ng of pterin / g wet weight of brain tissue.
Animals used were 180g male Wistar rats. Brains were dissected and stored at -70 C until assay. Tissues were defrosted at room temperature and immediately assayed for pterin. The same tissues were allowed to oxidize under room temperature for 2h and then reassayed for pterin.

Table 6:09

The effect of 6h air oxidation on brain total biopterin levels.

Group	Total Biopterins	
T=0h	78.77± 16.03 (4)	
T=6h	92.36± 16.57 (4)	ns

Values as ng of biopterin / g wet weight of tissue.
Animals used were 180g male Wistar rats. Brains were dissected and stored at -70 C until assay. Tissues were defrosted at room temperature and immediately assayed for total biopterins. The same tissues were allowed to oxidize under room temperature for 6h and then reassayed for total biopterins.

Table 6:10

The effect of 6h air oxidation on brain derived pterin levels.

Group	Derived Pterin	
T=0h	54.18± 15.23 (4)	
T=6h	63.93± 10.64 (4)	ns

Values as ng of pterin / g wet weight of brain tissue. Animals used were 180g male Wistar rats. Brains were dissected and stored at -70 C until assay. Tissues were defrosted at room temperature and immediately assayed for pterin. The same tissues were allowed to oxidize under room temperature for 6h and then reassayed for pterin.

Chapter 7. The Effects Of Phenylalanine and Tyrosine Loading On Pteridine Metabolism and Visual Evoked Potentials in Normal Human Subjects.

Chapter Seven.

7 The Effects of Phenylalanine and Tyrosine Loading on Pteridine Metabolism and Visual Evoked Potentials in Normal Human Subjects.

7:1 Introduction.

Many workers have shown low biopterins in serum (e.g. Aziz *et al*, 1983, Young *et al*, 1982), CSF (e.g. Morar *et al*, 1983) and post mortem brain samples of brain tissue (e.g. Barford *et al*, 1984) in patients with Alzheimer's disease. There is thought to be a lesion in the *de novo* biosynthetic pathway between dihydroneopterin triphosphate and BH_4. Neopterin levels are normal (Barford *et al*, 1984, Young *et al*, 1982), resulting in abnormal N:B ratios.

Harding *et al* (1985) investigated the use of the flash and pattern reversal visual evoked potentials (VEPs) in the diagnosis of presenile dementia. The results from 20 patients with primary senile dementia were compared with those from a control group of normals of equivalent ages and from a control group of 20 patients with cortical atrophy but no dementia. Presenile dementia caused a slowing of the major positive (P2) component of the VEP to flash stimulation. However, the VEP to pattern reversal stimulation (P100) was of normal latency. The difference between these two latencies characterises the combination of results and is a specific diagnostic indicator of primary senile dementia.

Hamon *et al* (1987) suggested that it may be possible to relate the N:B ratio to the degree of dementia as measued by VEP. The high correlation between N:B ratios and VEP scores show that N:B ratios reflect pathology in the brain, and the higher ratio corresponds to the amount of organic deterioration as shown by the VEP. From a VEP point of view, they indicate that the delay of the flash VEP is closely associated with a deficiency of BH_4 and hence dopamine and noradrenaline neurotransmitters.

Leeming *et al* (1976) studied plasma biopterin derivatives in 10 normal and 21 phenylketonuric children and in adults given phenylalanine (10 subjects) and tyrosine

(1 subject) loads. It was found that biopterin derivatives correlated with plasma phenylalanine concentrations, but in normal adults given an oral load of phenylalanine the rate of increase of plasma biopterin derivatives differed from that in the PKU patients. An oral tyrosine load did not produce any significant alteration in plasma biopterins in one subject examined. However, Nagatsu *et al* (1984) found that oral tyrosine in mice increased plasma biopterins in the striatum, adrenal and serum but not the liver. Yamaguchi *et al* (1983) showed that after administration of tyrosine to rats, total biopterins were increased in the striatum, adrenal glands and serum and also demonstrated an increase in serum biopterins in humans after tyrosine ingestion.

In view of these findings, the response of phenylalanine and tyrosine loading in human subjects was studied to see if the aromatic amino acids influenced the VEP changes associated with Alzheimer's disease. The effect on plasma biopterin and neopterin levels were investigated.

7:2 Methods.

7:2:1 Experimental.

After an overnight fast, subjects were given 7g oral loads of L-phenylalanine (3 subjects) or L-tyrosine (5 subjects) on a marmalade sandwich. A Placebo was administered at another time, which consisted of a marmalade sandwich. Blood samples were taken before and 2 hourly for 6 hours after the load.

7:2:2 VEP Technique.

VEPs were recorded (Dr. U. Danesha, Dept. of Vision Sciences, Aston University) from occipital electrodes O2 and O1 referred to central electrodes C4 and C3 respectively (international 10-20 system of nomenclature). 50 sweeps were averaged by a Cadwell 5200 A averager. The pattern stimulus consisted of red and black checks of 50 minutes of arc reversing twice per second by a Gross P522 stroboscope at intensity 2. The flash stimuli were produced twice per second by a Gross PS22 stroboscope at intensity 2.

The latencies in milliseconds for the flash P2 and pattern reversal P100 were measured and the latencies for the right and left brain hemispheres were averaged.

7:2:3 Plasma Analyses.

7:2:3:1 Pteridines.

Biopterin and neopterin were measured by HPLC as described in Chapter 2 by Dr. R.J. Leeming (General Hospital, Birmingham) and Dr. C.G.B. Hamon (Aston University)(Tables 7:07, 7:09). Biopterins were also measured by *C. fasciculata* assay.

7:2:3:2 Amino Acids.

Phenylalanine and tyrosine were measured by HPLC as described in Chapter 2.

7:3 Results and Discussion.

7:3:1 Phenylalanine Loading.

Figure 7:1 shows that after ingesting a 7.0g load of phenylalanine, plasma phenylalanine increases and this is followed by a significant increase in plasma total biopterins ($R=+0.89$, $p< 0.01$). This could arise from an increased activity of hepatic phenylalanine hydroxylase, hydroxylating phenylalanine to tyrosine and utilising BH_4 as cofactor (Kaufman, 1964). The increased enzyme activity uses more of the cofactor, causing a subsequent loss of oxidised forms of biopterin (BH_2 and biopterin) into the plasma. Consequently, after phenylalanine loading, there was a rise in plasma tyrosine, and this correlates significantly with plasma biopterins ($R=+0.68$, $p<0.02$) (Figure 7:2).

From Figures 7:1 and 7:2, at plasma phenylalanine and tyrosine concentrations of 0µM, plasma biopterins concentration would be approximately 2-3 µg/L. This might represent non-specific loss of cellular coenzyme.

Plasma phenylalanine levels correlate with plasma tyrosine after ingestion of phenylalanine ($R=+0.80$, $p<0.01$) (Figure 7:3). Plasma tyrosine rises due to it's production from phenylalanine. At a plasma phenylalanine concentration of 0µM, plasma tyrosine levels would be about 18µM and this is probably tyrosine from dietary sources.

Peak plasma phenylalanine and tyrosine were typically achieved 2h. after the load. The fall in plasma tyrosine is slower than that of phenylalanine (Figures 7:4 and 7:5).

No significant positive correlation was present between neopterins and biopterins ($R=+0.52$, ns, $n=12$) (Figure 7:6). A plasma biopterins concentration of 0µM corresponds to plasma neopterins of 3.8µg/L, which might arise due to stimulation of the immune system (Huber *et al*, 1984).

No correlation was found after phenylalanine loading between, plasma biopterins and flash VEPs (Figure 7:7), plasma biopterins and pattern VEPs (Figure 7:8), plasma tyrosine and flash VEPs (Figure 7:9), plasma tyrosine and pattern VEPs (Figure 7:10) or plasma phenylalanine and flash VEPs (Figure 7:11). However, as plasma phenylalanine levels increased, pattern VEPs decreased significantly ($R=-0.65$, $p<0.05$) (Figure 7:12). No correlation was found between VEP latencies (Flash-Pattern) and plasma N : B ratios ($R=-0.21$, $n=12$) (Fig. 7:27). The results differ to those of Hamon *et al* (1987), who found N:B ratios to correlate with flash VEPs and the latency difference between flash and pattern VEPs in the absence of phenylalanine or tyrosine loading. . The anomaly can be explained by the influence of elevated aromatic amino acids levels after phenylalanine loading which have been shown to influence plasma biopterin metabolism (Figure 7:1 and 7:2).

7:3:2 Tyrosine Loading.

No significant correlation was found between plasma phenylalanine and biopterins after tyrosine loading (Figure 7:13) or between plasma tyrosine and biopterins (Figure 7:14). This supports the work of Leeming *et al* (1976) but contrasts to the finding of Nagatsu *et al* (1984) and Yamaguchi *et al* (1983).

Nagatsu *et al* (1984) found that a 7.0g oral load of tyrosine administered to six control subjects and six parkinsonian patients increased serum biopterin levels by 3-7 fold in controls and <3 fold in the parkinsonian patients.

Yamaguchi *et al* (1983) demonstrated an increase in the total concentration of biopterin in the striatum, adrenal glands and serum of rats after administration of 1g of tyrosine/kg body weight in male Sprague-Dawley rats. This very high level administration of tyrosine could potentially generate large amounts of metabolites such as p-hydroxyphenylacetate (Chapter 3) and tyramine (Edwards *et al*, 1987) which can inhibit DHPR and cause elevated serum biopterins by this mechanism. Biopterins in the

other tissues could be elevated for a time because of the high BH_2 concentration present due to DHPR inhibition before loss into serum.

The sensitivity and specificity of the *Crithidia fasciculata* method of measuring biopterins has been well established (Nagatsu *et al*, 1981, Blair *et al*, 1983). Figure 7:25 demonstrates that the HPLC method agrees very well with the *Crithidia fasciculata* method of measuring biopterins ($R=+0.88$, $p< 0.01$, $n=31$)(Figure 7:25). Nagatsu and associates used a radioimmunoassay technique which they claimed was specific for L-*erythro*-biopterin. It is possible that the differences found on plasma biopterin levels after tyrosine loading may be due to the methodology used.

No correlation was apparent between plasma phenylalanine and tyrosine after tyrosine loading (Figure 7:15). Plasma tyrosine levels peaked about 4h. after ingestion of the tyrosine load (Figure 7:16). Yamaguchi (1983) found plasma tyrosine to peak 3h. in his subjects after a 7.0g load. Plasma phenylalanine levels remained unaltered after tyrosine loading (Figure 7:17), demonstrating no reverse conversion of tyrosine to phenylalanine.

No significant correlation after ingestion of 7.0g of tyrosine was found between plasma biopterins and pattern VEPs (Figure 7:18), plasma biopterins and flash VEPs (Figure 7:19), plasma tyrosine and pattern VEPs (Figure 7:20), plasma tyrosine and flash VEPs (Figure 7:21), plasma phenylalanine and pattern VEPs (Figure 7:22), plasma phenylalanine and flash VEPs (Figure 7: 23) or plasma biopterins and neopterins (Figure 7:24). However, a good correlation was obtained between plasma N : B ratios and VEP latencies ($p<0.01$, $R=+0.69$, $n=18$) (Figure 7:26) after tyrosine loading.

7:4 Conclusions.

Phenylalanine loading produced an increase in plasma biopterins which correlated positively with plasma phenylalanine. Tyrosine loading did not cause an increase in plasma biopterins, when measured by HPLC or *Crithidia fasciculata* methods.

It has been suggested that high correlations between N:B ratios and the VEP measures indicate that the delay of the flash VEP in SDAT is closely associated with a deficiency of BH_4 and hence catecholaminergic neurotransmitter systems (Hamon *et al*, 1987). The loss of correlation between plasma N : B ratios and VEP latencies after loading with phenylalanine could be due to the observed increase in plasma biopterins. Plasma N : B ratios correlated well with VEP latencies after tyrosine loading.

Table 7:01.

The Effects of a 7.0g Phenylalanine Load on Subject A.H.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	102.0	95.0	3.49
2	100.7	92.7	9.40
4	100.2	93.7	7.55
6	102.5	97.2	6.12

Tyrosine/µM	Neopterin/µg/L	Phenylalanine/µM	Crithidia/µg/L
7.51	4.76	10.82	1.70
51.11	5.50	216.10	7.50
55.80	5.86	149.70	7.80
47.97	6.86	112.80	5.10

Table 7:02

The Effects of a 7.0g Phenylalanine Load on Subject P.E.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	139.5	102.5	2.90
2	139.5	90.5	6.10
4	130.0	96.0	5.90
6	131.5	101.5	5.20

Tyrosine/µM	Neopterin/µg/L.	Phenylalanine/µM	Crithidia/µg/L.
25.23	3.30	17.01	0.70
57.08	3.80	216.30	3.00
51.07	4.30	98.43	2.90
55.08	4.30	76.56	2.30

See text for details.

Table 7:03.

The Effects of a 7.0g Phenylalanine Load on Subject C.H.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	106.3	94.8	1.12
2	98.2	91.8	9.50
4	98.3	94.5	7.14
6	102.1	94.8	3.43

Tyrosine/µM	Neopterin/µg/L.	Phenylalanine/µM	Crithidia/µg/L.
35.5	4.32	14.7	1.50
93.7	6.05	334.1	7.00
85.7	6.41	166.0	6.50
56.1	5.78	69.7	5.00

Table 7:04

The Effects of a 7.0g Tyrosine Load on Subject C.H.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	105.7	97.5	2.70
2	106.7	97.3	3.00
4	102.7	97.3	2.40
6	104.3	98.8	2.30

Tyrosine/µM	Neopterin/µg/L.	Phenylalanine/µM	Crithidia/µg/L.
18.02	2.70	14.58	2.90
124.90	3.00	16.71	2.80
150.20	2.40	16.71	1.90
81.11	2.30	10.94	2.00

See text for details.

Table 7:05.

The Effects of a 7.0g Tyrosine Load on Subject P.E.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	140.0	102.0	2.07
2	139.0	98.0	1.00
4	130.0	96.0	3.60
6	132.0	101.0	0.88

Tyrosine/µM	Neopterin/µg/L.	Phenylalanine/µM	Crithidia/µg/L.
14.15	7.40	16.71	1.50
89.20	7.30	17.92	1.80
104.50	7.00	16.27	1.50
62.00	6.90	14.50	1.80

Table 7:06

The Effects of a 7.0g Tyrosine Load on Subject R.J.C.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	116.1	101.8	1.50
2	122.5	97.8	1.40
4	119.4	101.3	1.30
6	114.5	103.2	1.30

Tyrosine/µM	Neopterin/µg/L.	Phenylalanine/µM	Crithidia/µg/L.
15.02	3.60	13.67	1.00
76.61	2.90	15.19	1.10
94.33	2.70	16.71	0.80
52.47	2.30	16.71	0.90

See text for details.

Table 7:07

The Effects of a 7.0g Tyrosine Load on Subject J.C.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	122.0	98.5	2.50
2	118.0	97.5	-
4	118.5	97.3	2.30
6	124.0	99.5	2.10

Tyrosine/µM	Neopterin/µg/L.	Phenylalanine/µM	Crithidia/µg/L.
16.22	1.60	18.23	1.60
128.50	-	18.23	1.80
139.90	2.30	13.37	1.40
78.11	2.10	19.75	1.40

Table 7:08

The Effects of a 7.0g Tyrosine Load on Subject A.H.

Time/h.	Flash/ms	Pattern/ms	Biopterin/µg/L.
0	106.3	101.9	2.30
2	110.2	104.4	1.70
4	108.2	105.1	2.40
6	111.8	102.4	2.70

Tyrosine/µM	Neopterin/µg/L	Phenylalanine/µM	Crithidia/µg/L
14.42	2.60	16.71	1.30
159.82	1.50	14.58	1.30
153.51	1.70	15.19	1.10
83.12	1.90	12.76	1.00

See text for details.

Table 7:09.

The Effects of a Placebo on Subject C.H.

Time/h.	Flash/ms	Pattern/ms	Biopterin/ug/L.
0	97.2	96.0	1.96
2	94.7	95.3	2.55
4	98.5	96.0	2.35
6	96.7	97.0	2.60

Tyrosine/uM	Neopterin/ug/L.	Phenylalanine/uM	Crithidia/ug/L.
9.26	7.50	11.41	1.50
11.35	6.16	14.14	1.80
10.88	6.09	9.82	1.50
10.28	8.31	10.59	1.80

See text for details.

Fig. 7:01. Correlation Between Plasma Biopterins And Phe. After Phe. Loading.

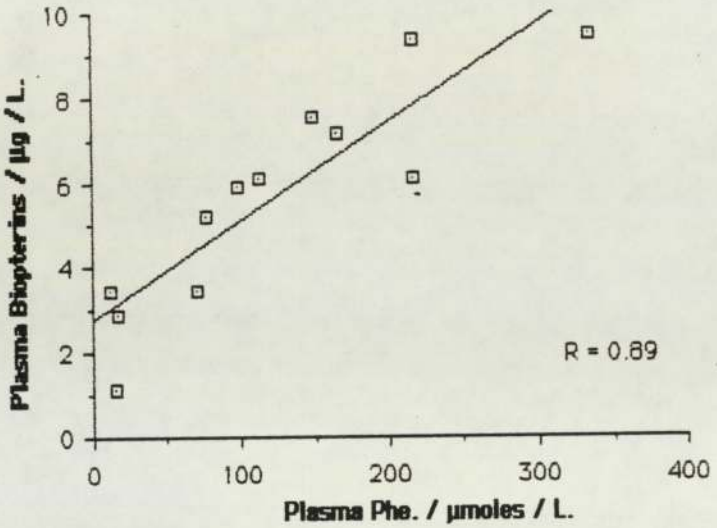
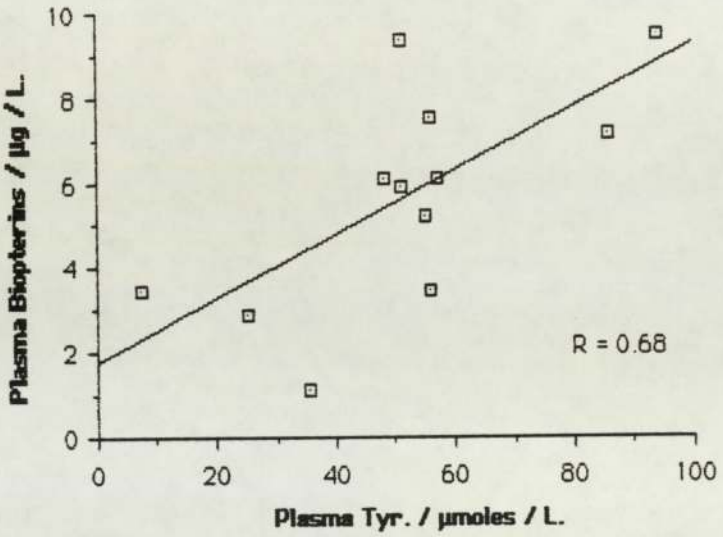


Fig. 7:02. Correlation Between Plasma Biopterins And Tyr. After Phe. Loading.



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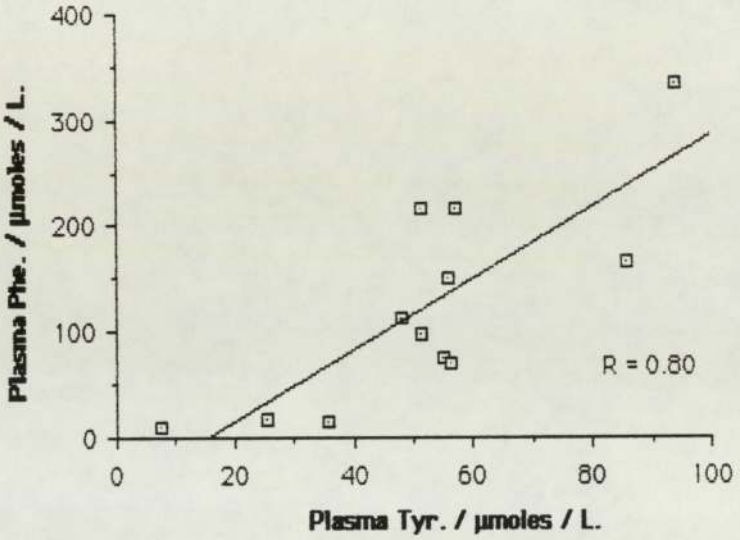

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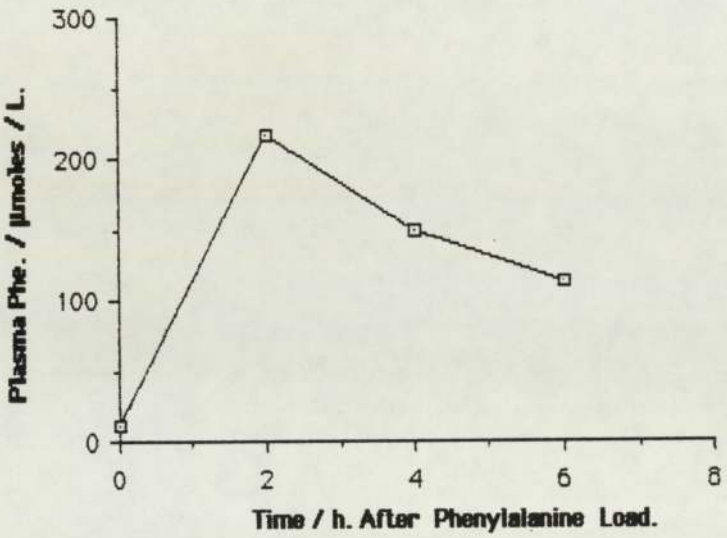

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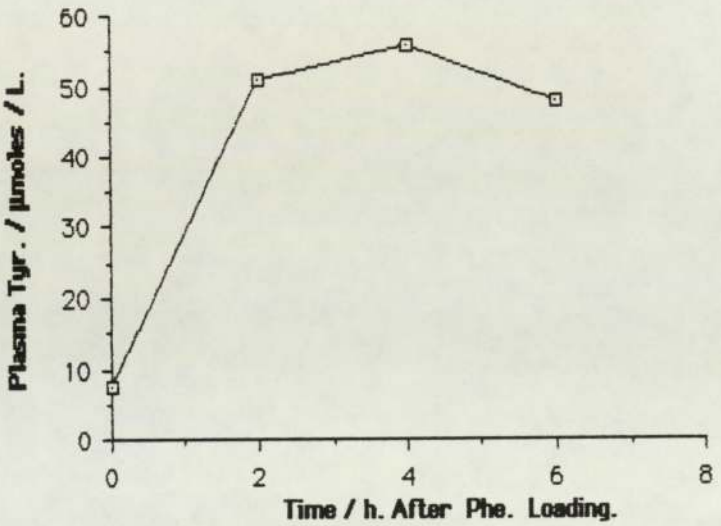

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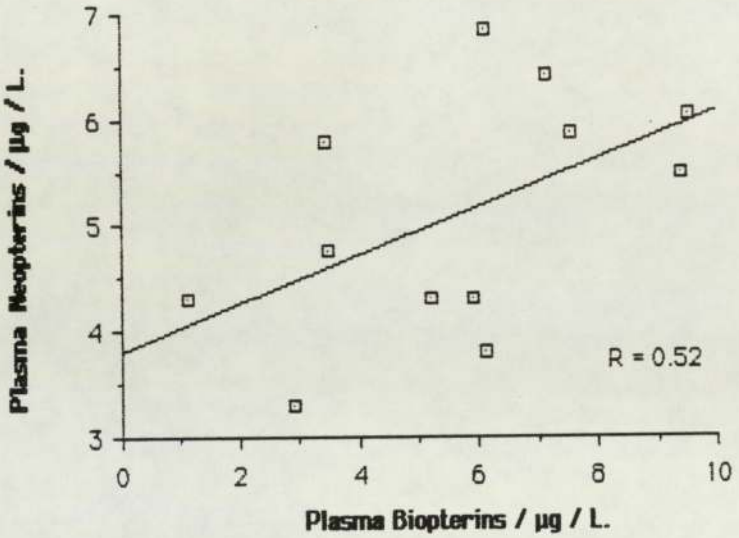
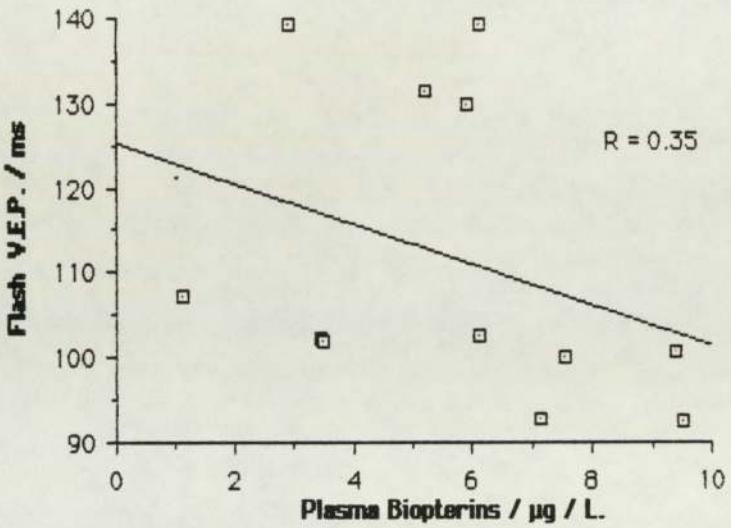


Fig. 7:07. Correlation Between Plasma Biopterins And Flash V.E.P.s After Phe. Loading.



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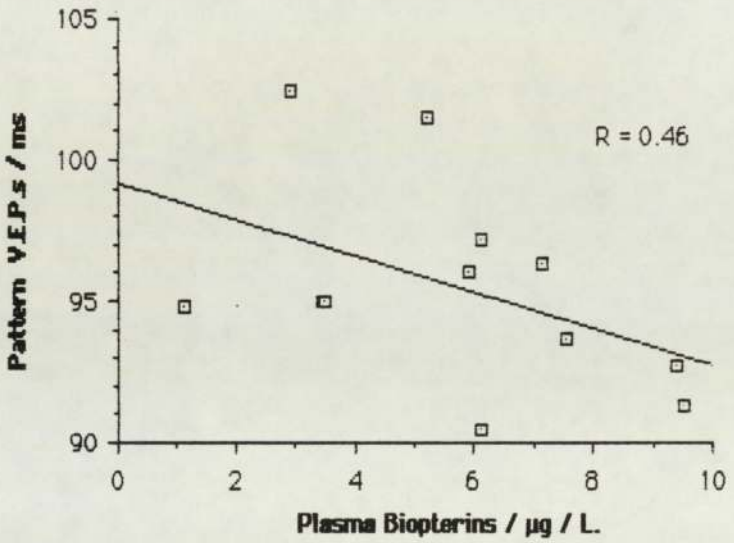

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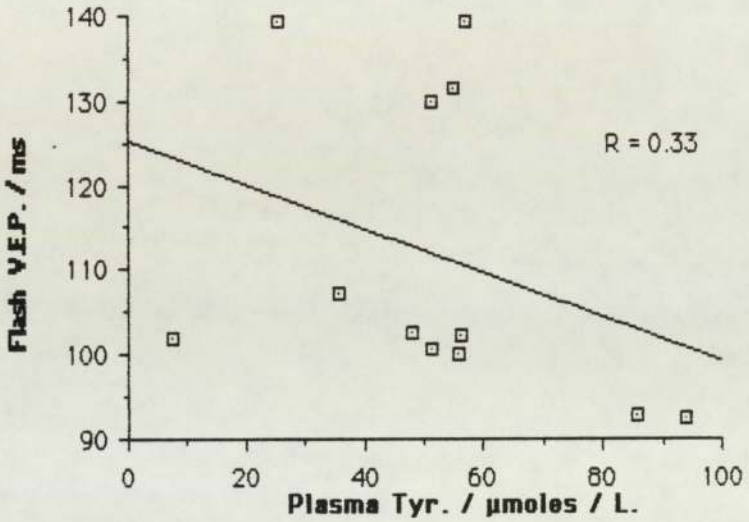

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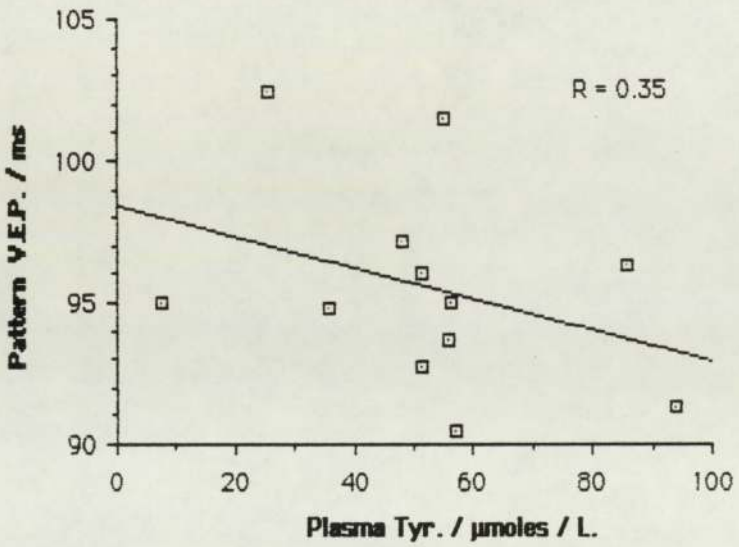

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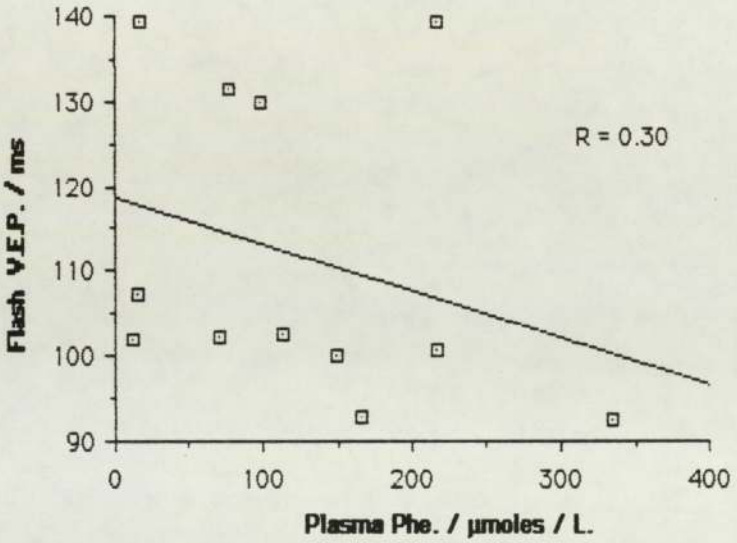

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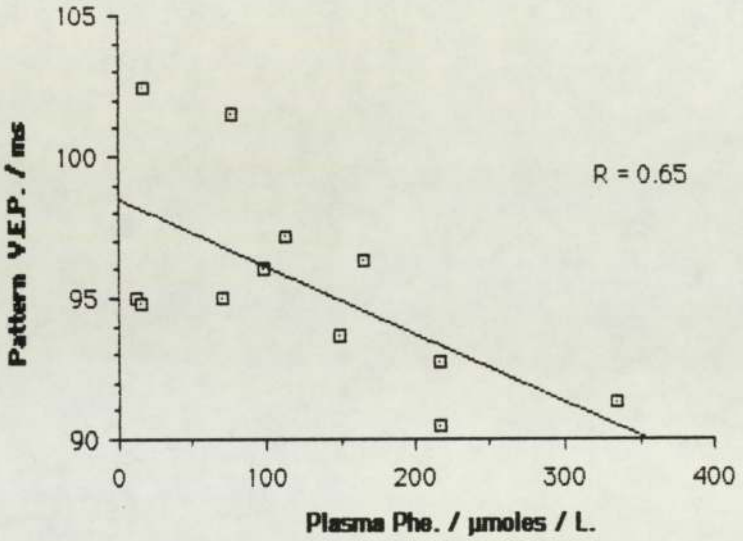
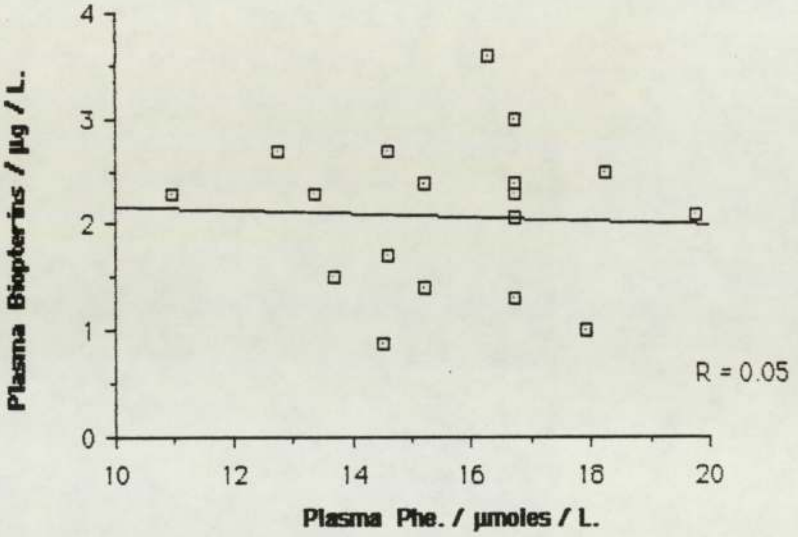


Fig. 7:13. Correlation Between Plasma Phe. And Biopterins After Tyr. Loading.



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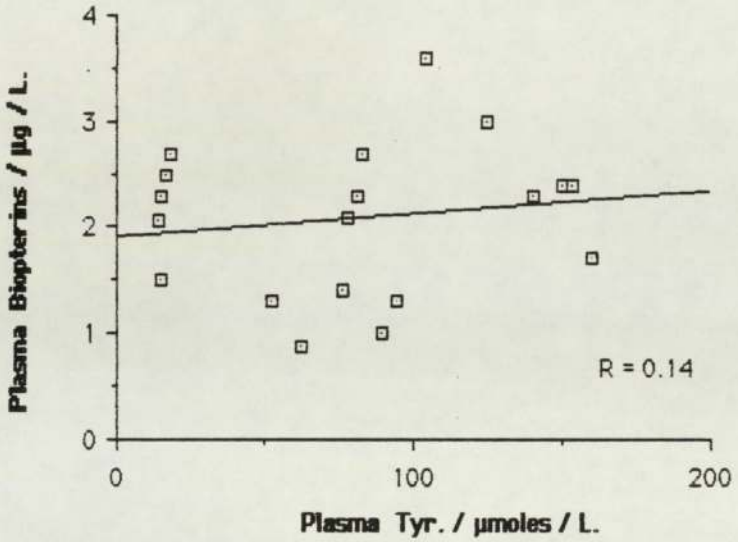

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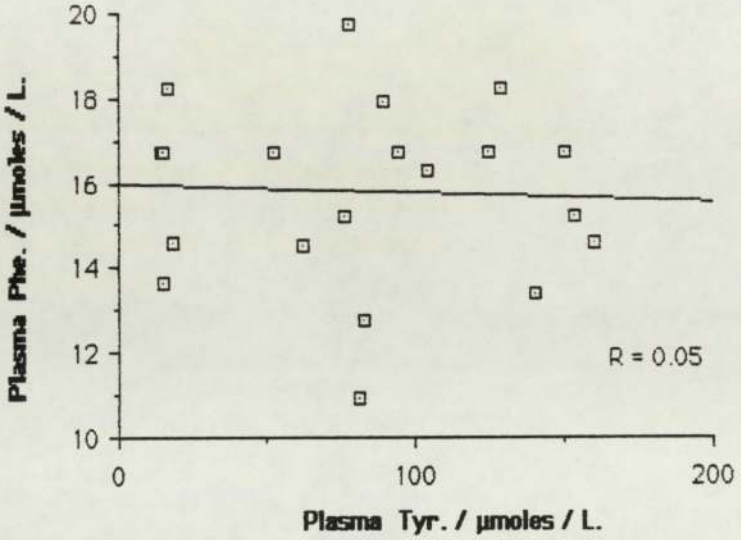

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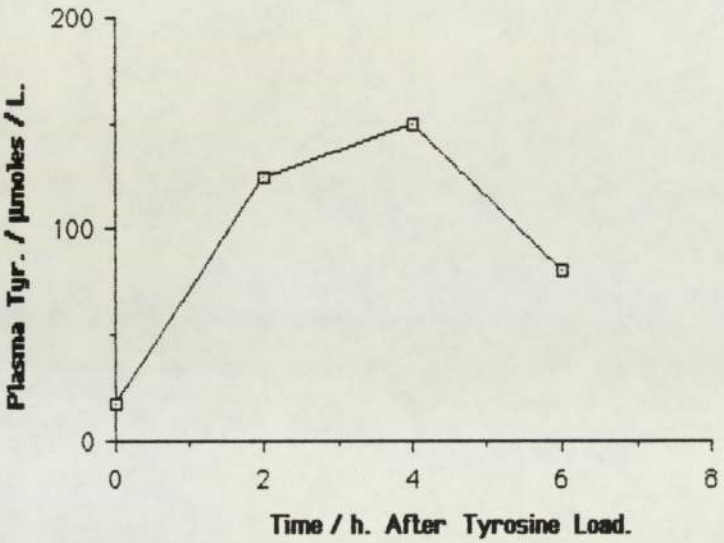

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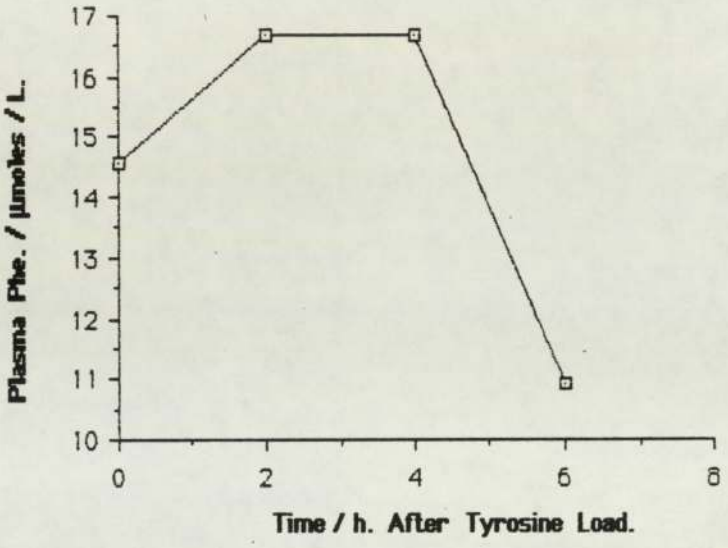

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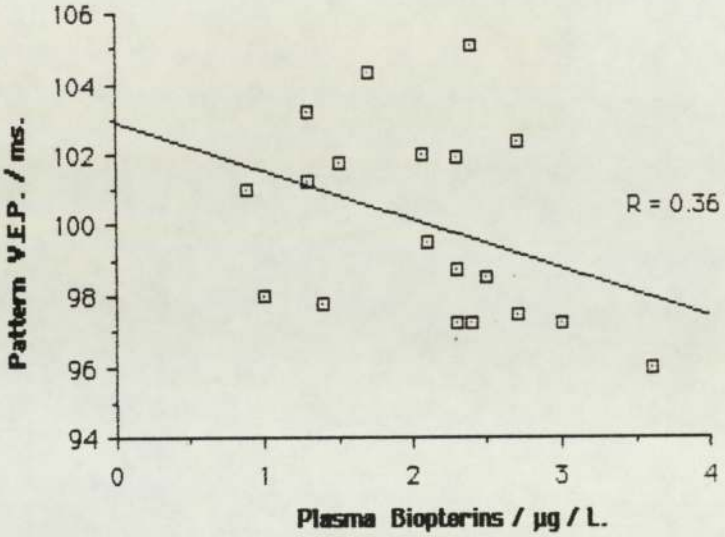

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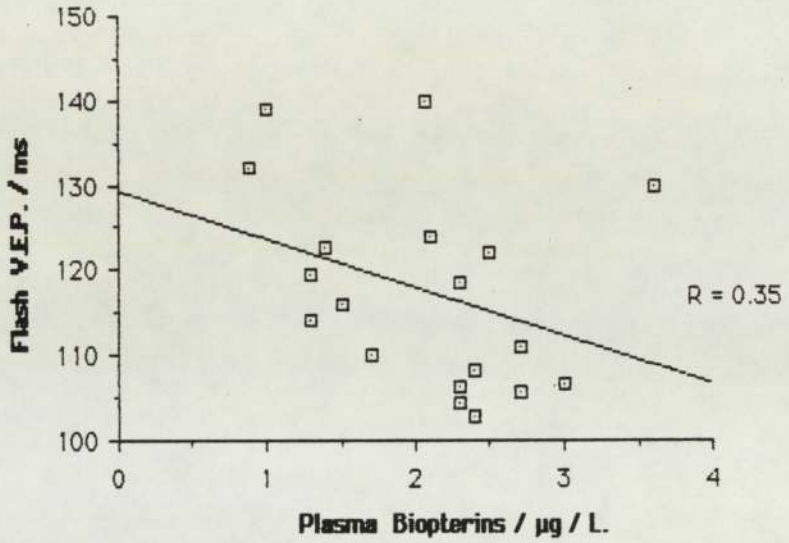

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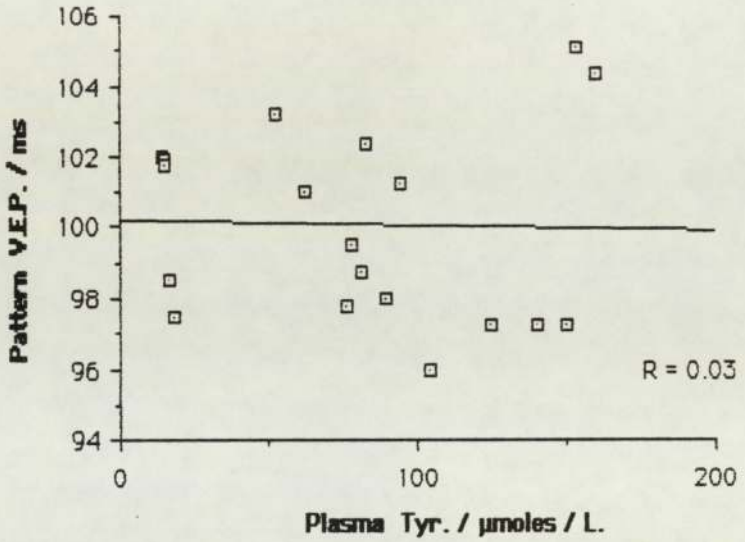

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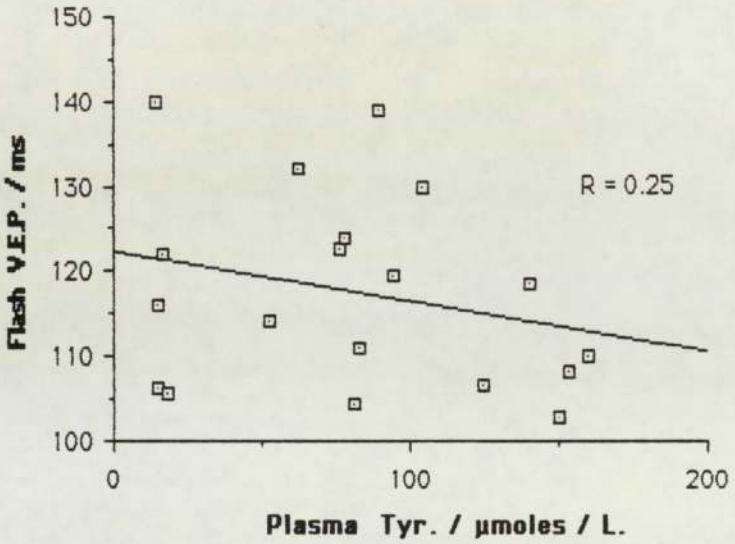

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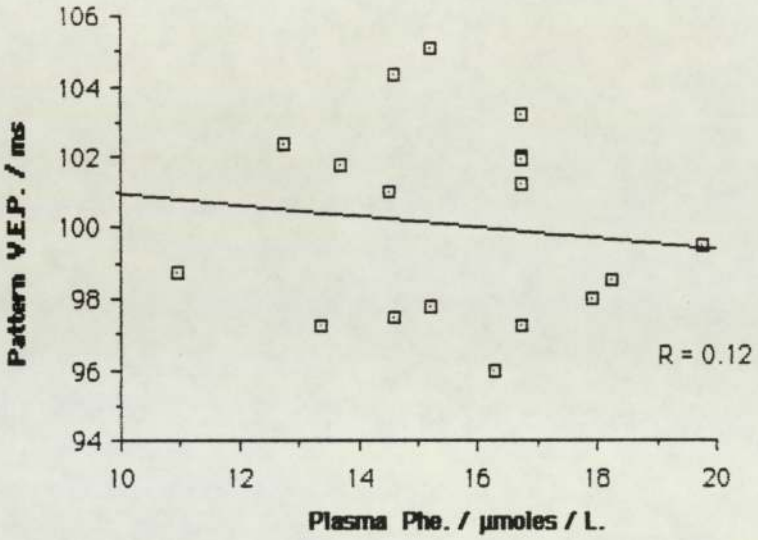

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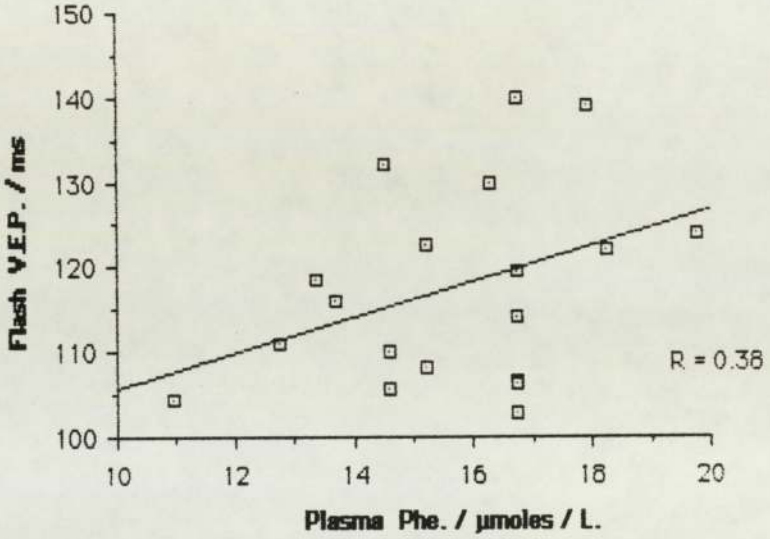


Fig. 7.24. Correlation Between Plasma Biopterins And Neopterins After Tyr. Loading.

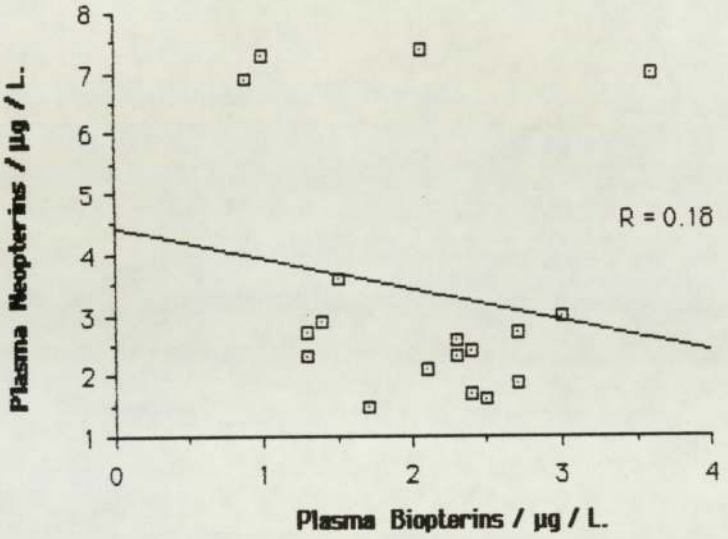


Fig. 7:25. Correlation Between Biopterins As Measured By C. fasciculata And H.P.L.C.

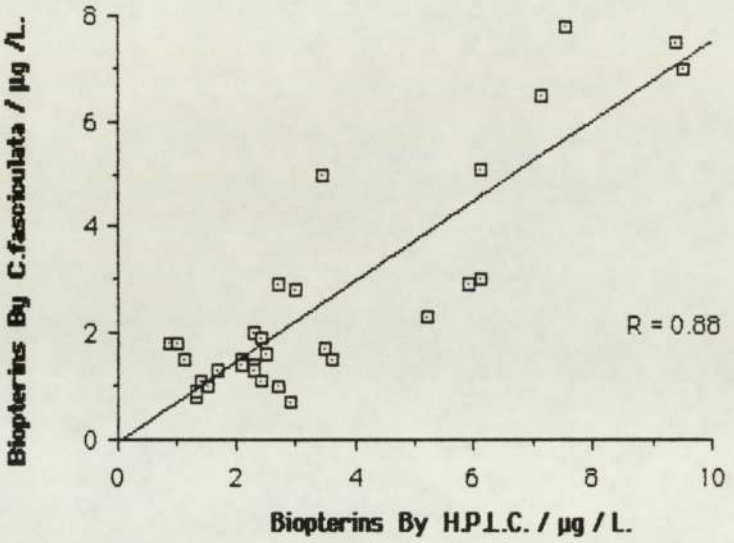


Fig. 7:26 Correlation between VEP latencies and plasma N : B ratios after tyrosine loading.

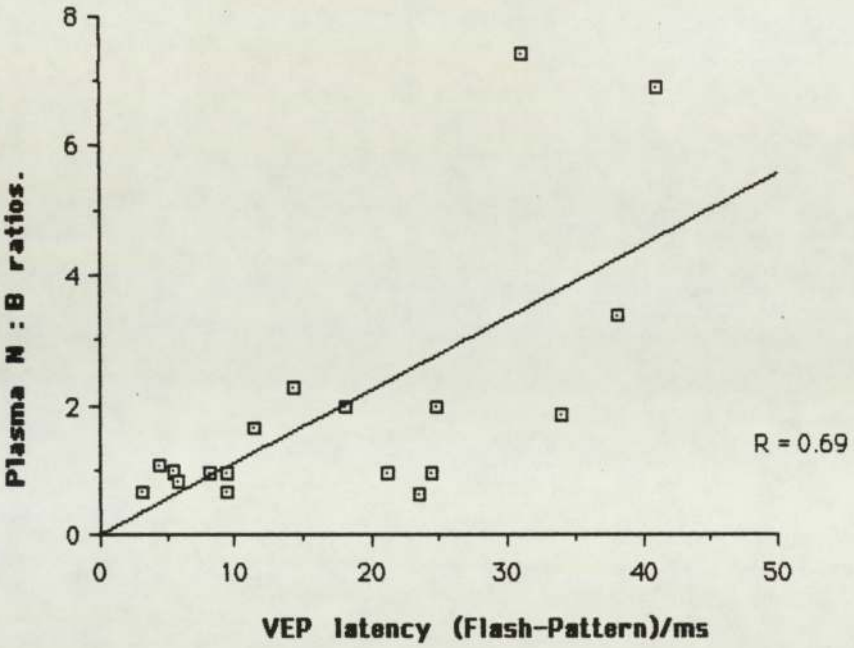
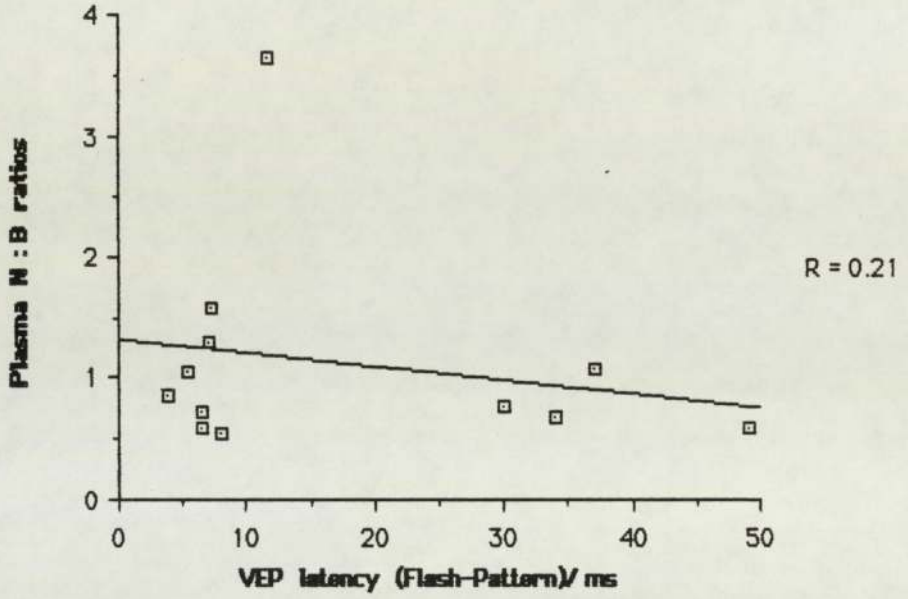


Fig. 7:27 Correlation between VEP latencies and plasma N : B ratios after phenylalanine loading.



Chapter 8. General Discussion.

Chapter Eight.

8. General Discussion.

8:1 Introduction.

L-*erythro*-5,6,7,8-tetrahydrobiopterin is the hydroxylase coenzyme required for the biosynthesis of dopamine, noradrenaline and serotonin (Kaufman, 1987). Because of its essential role in the production of these neurotransmitters, disturbances in BH_4 metabolism can have severe neurological consequences. BH_4 levels are maintained by two pathways. A *de novo* biosynthetic pathway starting from guanosine triphosphate (Fig. 4) and a salvage pathway utilizing DHPR (Fig. 6). Rare inherited metabolic disorders exist of the synthesis of BH_4 involving a deficiency in the conversion of NH_2TP to BH_4 (Rey *et al*, 1977, Bartholome *et al*, 1977, Danks and Cotton, 1980, Dhondt and Farriaux, 1983), a block in the GTP to NH_2TP step due to a deficiency of GTP cyclohydrolase (Joller *et al*, 1983, Niederwieser *et al*, 1984) and of the salvage of $_qBH_2$ by DHPR (Kaufman *et al*, 1975, Rey *et al*, 1977, Danks *et al*, 1979). Deficiency causes gross neurological failure (Danks *et al*, 1978).

The rate limiting enzyme in the *de novo* biosynthetic pathway is phosphate eliminating enzyme (PEE) in man, and guanosine triphosphate cyclohydrolase in the rat. This is demonstrated by the fact that neopterin is not detected in the tissues of rats, but is detected in man (Sawada *et al*, 1986). Other differences exist in the catabolism of BH_4. Niederweiser *et al* (1986) reported that pterin deaminase is present in rat liver but not in human liver. In man, in contrast to the rat the side-chain of BH_4 is mainly retained and modified to 1'-oxo and 2'-deoxy derivatives and 2'-deoxysepialumazine is the main faecal metabolite. In humans, deamination occurs mainly in the gut by bacterial action.

A compilation of reference biochemical parameters in the adult male Wistar rat is reported in Table 8:01 obtained from control samples in the thesis. (See Chapter 2 for the methodology).

Table 8:01.

Compilation of biochemical parameters in normal fed adult male (180g) rats.

Liver biopterins	1.72± 0.39 (30) /µg/g wet weight
Brain biopterins	82.94± 21.69 (30) /ng/g wet weight
Plasma biopterins	19.29± 6.68 (30) /ng / ml
Liver derived pterin	0.48± 0.21 (30) / µg/g wet weight
Brain derived pterin	57.41± 16.38 (30)/ ng/g wet weight
*Brain BH_4 biosynthesis (freeze-dried method)	0.35± 0.19 (12)/ng biopterin/h/mg protein
*Liver BH_4 biosynthesis (freeze-dried method)	8.72± 4.85 (12)/ ng biopterin/h/mg protein
*Liver BH_4 biosynthesis	12.04± 4.33 (6)/ ng biopterin/h/mg protein
Brain sepiapterin reductase	1.51± 0.33 (25)/nmol sepiapterin reduced/ min./mg protein
Liver sepiapterin reductase	17.75± 3.97 (7)/nmol sepiapterin reduced/ min./mg protein
Brain GTP-CH	1.01± 0.50 (7)/ng neopterin/h/mg protein
Plasma GTP CH	48.57± 10.01 (6)/ng neopterin/h/mg protein
Whole blood GTP-CH	61.76± 13.04 (6)/ng neopterin/h/mg protein

Table 8:01 (continued).

Brain DHPR	263.77± 68.94(30)/ nmol NADH oxidized/ min./mg protein
Liver DHPR	397.03± 200.71 (30)/ nmol NADH oxidized/ min./mg protein
Plasma Tyrosine	55.52± 22.71 (30)/ µmol/L
Plasma Phenylalanine	37.36± 18.17 (30)/ µmol/L
Brain CAT	11.59± 3.66 (30)/µmol Ach/h/mg protein

Values are given as mean± SD. Number of samples in brackets. *See Chapter 2, 2:7 for the differences in the BH_4 biosynthesis assay.

Another important difference between the two species is illustrated in Table 8:02, which shows that control rat plasma biopterins are much higher than control human plasma biopterin levels.

Table 8:02

Comparison of control human and rat plasma biopterins concentration.

Humans	Rats
4.61± 3.21 (12)	15.24± 7.33 (11)

Values as ng/ml plasma. $p< 0.001$. Measured by HPLC as described in Chapter 2.

Comparison of control group measurements showed it was essential to use matched controls and tests when investigating the effects of various neurotoxins on BH_4 metabolism in the rat, and to perform assays on both control and experimental tissue on the same day because of the variation in tissue biopterins and derived pterin concentration between days (Table 8:03).

Table 8:03.

Variation in brain biopterins and derived pterin concentration between days.

Brain biopterins concentration.

Batch 1	Batch 2
85.26± 11.86 (6)	63.89± 8.12 (6)

Values as ng of biopterin /g wet weight of tissue. $p< 0.001$.

Brain derived pterin concentration.

Batch 1	Batch 2
63.40± 7.17 (6)	42.22± 15.08 (6)

Values as ng of pterin/g wet weight of tissue. $p< 0.001$.

The theme of the thesis was to study the effects of various agents on brain BH_4 metabolism, but assays were performed on the liver and plasma as well as the brain for the following reasons. BH_4 metabolism was investigated in the liver so that activity could be studied in the absence of tyrosine hydroxylase, and any exclusion effect of the blood-brain barrier was absent. Plasmas were studied, since this fluid is one of the most convenient to obtain from humans so that changes in BH_4 metabolism in the periphery can be compared and contrasted to those in the CNS, and between the two species. The use of the rat model was useful because it allowed examination of BH_4 metabolism in tissues such as brain and liver after administration of the potential neurotoxins, which is not possible in human studies.

8:2 Derived pterin and reduced pteridine metabolism

Acid-iodine oxidation of rat brain, liver or plasma followed by HPLC assay for total biopterins measurement also showed the presence of pterin which could be quantitatively estimated. This "derived pterin" is formed from endogenous dihydropterin from BH_4 turnover and from the rearrangement of any quinonoid BH_2 not salvaged by DHPR; from acid-iodine oxidation of THF and dihydropterin and as an oxidation product of BH_4 or THF. Table 6:03 (Chapter 6) shows that brain derived pterin is much higher than THF (THF concentration was 22-51% of derived pterin concentration), demonstrating that a

large proportion of derived pterin arises from BH_4 turnover. This is supported by the work presented later, where L-DOPA and bromocriptine caused decreased derived pterin in brain tissue. Both of these agents can decrease tyrosine hydroxylase activity and reduce catecholamine turnover.

The situation present in the liver is less clear. THF concentration in the liver was estimated to be 36-83% of the derived pterin concentration (Table 6:03). Cook and Blair (1979) found that about 50% of liver folates in rats was as THF. This would suggest that most of the hepatic derived pterin would result from THF rather than BH_4 metabolism.

Measurement of derived pterin may be useful as an indicator of reduced pteridine metabolism turnover but changes associated with derived pterin can be complicated and ideally should be attributed to changes in BH_4 or THF metabolism only after measurement of both parameters.

8:3 Dopamine Agonists.

8:3:1 L-DOPA and Sinemet Plus.

L-DOPA can be considered to be an indirect dopamine agonist because it is metabolized *in vivo* to dopamine. A deficiency of dopamine, the decarboxylated product of L-ß-3,4-dihydroxyphenylalanine (L-DOPA) is found in the nigro-striatal pathway of parkinsonian patients (Ehringer and Hornykiewicz,1960).

L-DOPA therapy is effective in the control of the clinical symptoms of Parkinson's disease. The standard maximum dose is 120 mg/kg body weight (Martindale, 1977), although is no longer used alone, but in combination with peripheral decarboxylase inhibitors (e.g. carbidopa), monoamine oxidase inhibitors (e.g. deprenyl) or anti-emetics (e.g. cyclizine).

Leeming *et al* (1976) did not observe any change in the plasma concentration of biopterins in L-DOPA treated parkinsonian patients, although there have been reports of decreased BH_4 levels in this disease (Table 1:01). This could have been due to L-DOPA therapy raising plasma biopterins into the normal range. Purdy *et al* (1981) showed that *in vitro* dopamine could inhibit DHPR by 50% at concentrations of 0.11mM. Shen

(1985) reported Ki values for L-DOPA on DHPR in rat striatal synaptosomes as 8.1×10^{-5}M and 9.0×10^{-5}M in human liver, and Ki values for dopamine on DHPR in rat striatal synaptosomes as 1.3×10^{-5}M and 1.4×10^{-5}M for human liver.

Aziz, Blair and Leeming (unpublished) showed that single oral 250mg doses of L-DOPA caused a rise in plasma biopterins in humans, suggesting *in vivo* DHPR inhibition. The rise in plasma biopterins observed in humans after L-DOPA ingestion was not found in rats following administration of L-DOPA (Table 8:06), although there was a decrease in hepatic total biopterins (Tables 8:04 and 8:06).

Amarago and Waring (1983) suggested that *in vitro* inhibition of DHPR by dopamine was due to oxidative products, the dopaminochromes, which would be produced by the action of hydrogen peroxide on dopamine in the reaction system. Purdy (1981) reported that L-DOPA itself had no effect in *in vitro* studies. Shen (1985) showed that dopaminochromes were not responsible for the inhibition of DHPR, and dopamine was an inhibitor. Furthermore, the quinone structure of dopamine was required for nucleophilic attack and subsequent inhibition of DHPR.

It is probable that other products of L-DOPA metabolism besides dopamine would cause *in vivo* inhibition of DHPR such as catecholamine derived tetrahydroisoquinolines (e.g. salsolinol, (CIS) carboxysalsolinol, 4,6,7-trihydroxytetrahydroisoquinoline and tetrahydropapoveroline). Collins (1980) reported that L-DOPA treated parkinsonian patients have significantly elevated levels of tetrahydroisoquinolines and that these are capable of inhibiting DHPR.

No direct effect was found on DHPR here, possibly due to dilution of reversible inhibitor out of the assay system during preparation of the tissue homogenate (Edwards *et al*, 1987) (Tables 3:12 and 3:33) and rapid clearance of the drug. The half life of L-DOPA in human plasma is about 1 hour (Martindale, 1977).

Table 8:04

The effects of Sinemet Plus on tissue biopterins.

Tissue	Control	Dosed	
Brain	89.22± 7.10 (6)	81.96± 11.92 (6)	ns
Liver	1.81± 0.41 (6)	1.21± 0.15 (6)	$p < 0.010$

Brain values/ng/g wet weight. Liver values/ µg/g wet weight.

Table 8:05

The effects of Sinemet Plus on tissue derived pterin.

Tissue	Control	Dosed	
Brain	82.96± 10.25 (6)	52.64± 10.52 (6)	$p < 0.001$
Liver	0.23± 0.09 (6)	0.27± 0.10 (6)	ns

Brain values as ng/g wet weight. Liver values as µg/g wet weight.

180g male Wistar rats were dosed with 56.25 mg L-DOPA and 14.06 mg Carbidopa /day for 2 days and killed 24h later. Controls were given isotonic saline i.p.

Table 8:06

The effects of L-DOPA on tissue biopterins.

Tissue	Controls	Dosed	
Brain	85.26± 11.86 (6)	72.03± 8.58 (6)	ns
Liver	1.72± 0.40 (6)	1.10± 0.09 (6)	$p<0.005$
Plasma	19.16± 6.37 (6)	18.32± 4.93 (6)	ns

Brain values as ng/g wet weight. Liver values as µg/g wet weight. Plasma values as ng/ml.

Table 8:07

The effects of L-DOPA on tissue derived pterin.

Tissue	Controls	Dosed	
Brain	86.00± 11.02 (6)	55.12± 10.13 (6)	$p< 0.010$
Liver	0.26± 0.04 (6)	0.28± 0.05 (6)	ns

Brain values as ng/ g wet weight. Liver values as µg/ g wet weight. 180g male Wistar rats were dosed with 56.25 mg L-DOPA i.p. /day for 2 days and killed 24h later. Controls were given isotonic saline i.p.

Another *in vivo* biochemical marker of DHPR inhibition is an elevation of tissue derived pterin levels. Pterin can arise from both tetrahydrobiopterin and tetrahydrofolate. Neutral oxidation of BH_4 and THF yield $_qBH_2$ and $_qFH_2$ respectively, and dihydropterin (Fukushima and Nixon, 1980, Heales, 1987). Acid oxidation of THF yields 100% pterin as product.

Derived pterin was decreased in the brains of L-DOPA and Sinemet Plus dosed rats (Tables 8:06 and 8:07). This is surprising since DHPR inhibitors should cause greater loss of $_qBH_2$ to BH_2 and PH_2 and an increase in derived pterin would be expected. The lowered derived pterin is thought to result from a reduced turnover of BH_4 in the brain brought about by negative feedback of dopamine directly on to tyrosine hydroxylase (Katoh *et al*, 1983) and indirectly *via* activation of dopamine receptors (Galloway and Levine, 1986). This decrease probably involves cAMP. Tyrosine hydroxylase activity in the brain is directly sensitive to cyclic nucleotides. Harris *et al* (1974) found that activation by cAMP lowered the Km for the pteridine cofactor to one-sixth and the Ki value for inhibition by dopamine was increased from 0.1 to 0.6mM.

An alternative hypothesis tested to explain the decreased cerebral derived pterin was increased oxidative stress generated during the catabolism of the L-DOPA and dopamine. Dopamine is a substrate for monoamine oxidase B (MAO B). During enzymatic activity, hydrogen peroxide and hydroxyl free radicals are generated by a Fenton's type reaction (Haber and Weiss, 1934). These hydroxyl free radicals could then oxidize BH_4, THF and other reduced pterins to non-pterin products (Heales,1987). On acid-iodine oxidation, the pterin produced would be lower. This was investigated by measurement of oxidative stress in the brain after L-DOPA administration by the non-specific NBT assay (Chapter 2). An increase in oxidative stress was found, but it was not statistically significant by t-test (Table 8:08).

The reason for the use of Sinemet Plus was to ensure that high concentrations of L-DOPA penetrated the CNS. This combination drug contains L-DOPA and a peripheral decarboxylase inhibitor, carbidopa.Sinemet Plus induced a state of hyperactivity and choreiform movements of the tail which lasted about 3 hours. L-DOPA caused a state of shock lasting 4-6 hours and a probable loss in appetite. Starvation has been shown to adversely affect BH_4 metabolism (Cutler, 1986, Edwards *et al*, 1987). The decrease in hepatic biopterins after drug administration was confirmed in the presence of starvation state (Table 8:09) proving that the observation was attributed to drug action and not nutritional status.

Table 8:08

The effect of L-DOPA on brain oxidative stress

Controls	Dosed	
0.51± 0.05 (6)	0.78± 0.42 (6)	ns

Oxidative stress was measured by the NBT assay. Values as nmoles of formazan/ g wet weight. 180g male Wistar rats were dosed with 56.25mg L-DOPA i.p./day for 2 days and killed 3h later. Controls were given isotonic saline i.p.

Table 8:09

The effect of L-DOPA on liver total biopterins on fasting.

Controls	Dosed	
1.00± 0.20 (6)	0.80± 0.10 (6)	p< 0.020

180g male Wistar rats were fasted overnight on grids, dosed i.p. with 56.25mg L-DOPA and killed 24h later. Controls were given isotonic saline i.p. in place of L-DOPA. Values as µg of biopterin/ g wet weight.

8:3:2 Bromocriptine.

Bromocriptine is an effective therapy in the control of the clinical symptoms of Parkinson's disease (Calne *et al*, 1974, Lees *et al*, 1975, Stern and Lees, 1983, Lipcsey and Peres, 1984). Bromocriptine is believed to be a direct dopamine agonist but it is unusual in that it has a dependence on intact synthesis and storage of endogenous dopamine (LeWitt *et al*, 1983). Use in the treatment of parkinsonism gives improvements similar to L-DOPA (Stern and Lees, 1983).

Bromocriptine mesylate administration to rats caused a significant decrease in brain derived pterin but there was no effect on the concentration of brain total biopterins (Table 8:10). Liver total biopterins and plasma total biopterins were significantly lowered (Table 8:10). This would suggest that bromocriptine mesylate inhibits the biosynthesis of BH_4. This was confirmed (Fig. 8:1) where addition of bromocriptine mesylate to liver homogenate caused inhibition of BH_4 biosynthetic capacity at concentrations higher than 10^{-7}M. Price *et al* (1978) determined plasma bromocriptine levels following oral doses in parkinsonian patients. Mean peak levels were attained after 102± 9.6 minutes.Peak clinical response occurred shortly afterwards. Improvement in parkinsonism lasts 6-8 hours compared with 1-2 hours for L-DOPA. A single 100mg dose caused a peak plasma concentration of 24.60 ng/ml (0.32×10^{-7}M). The maximum dose used in Parkinson's disease is 300mg/day. It would seem likely that concentration of bromocriptine required to inhibit BH_4 biosynthesis could be achieved during therapy.

The most likely enzyme to be inhibited by bromocriptine would be sepiapterin

reductase, which is inhibited by catecholamines (Katoh *et al*, 1982). Bromocriptine contains a catecholamine "skeleton" (29)

As noted previously, the CNS effect differed from the peripheral effect of bromocriptine on BH_4 metabolism. Galloway and Levine (1986) found that the regulation of tyrosine hydroxylase activity can be accomplished by stimulation of dopamine receptors and the biopterin pool is relatively resistant to acute fluctuations under these conditions. Reduced tyrosine hydroxlase activity would decrease catecholamine and BH_4 turnover, reflected by lowered brain derived pterin. No effect was found on the DHPR salvage pathway, although liver derived pterin was increased.

Table 8:10

The effect of bromocriptine on BH_4 metabolism in the rat.

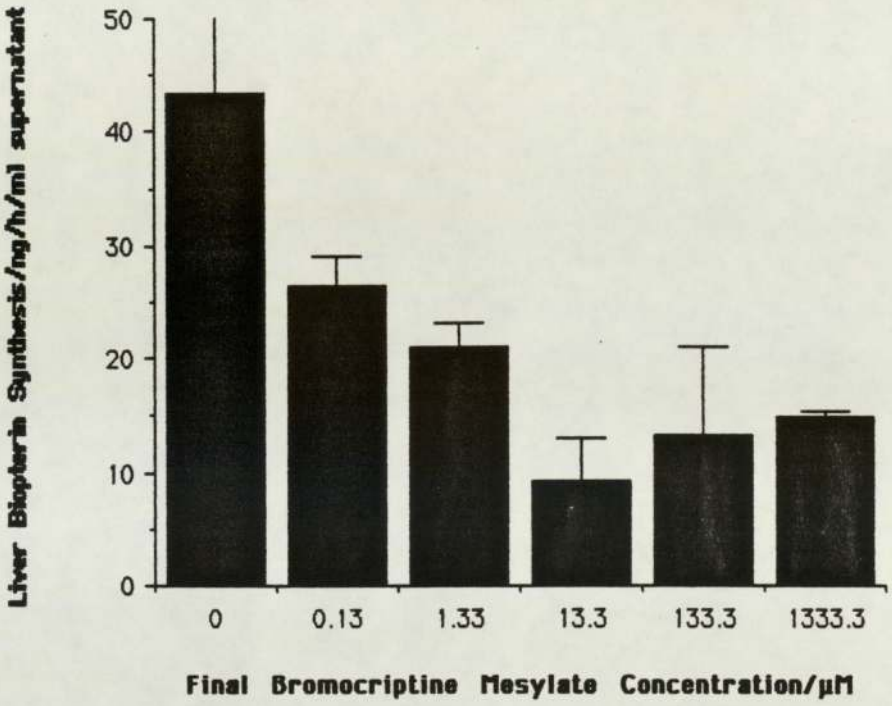
Parameter	Tissue	Control	Dosed	
Derived pterin	Brain	63.40± 7.17(6)	51.14±6.93(6)	p<0.010
Biopterins	Brain	63.89± 8.12(6)	71.72± 5.00(6)	ns
Biopterins	Liver	1.57± 0.19 (6)	1.27±0.12(6)	p<0.010
Derived pterin	Liver	0.49±0.08 (6)	0.66± 0.14(6)	p<0.050
Biopterins	Plasma	22.23± 3.15 (6)	14.65± 2.04(6)	p<0.001

Liver values as µg/g wet weight, brains as ng/g wet weight and plasmas as ng/ml.
180g male Wistar rats were dosed with 5 mg of bromocriptine mesylate /day for 2 days and killed 24h later.
Controls were dosed with lactic acid solution.

8:4 Investigation of the effects of psychotropic drugs on BH_4 metabolism.

In sub-chronic experiments, the administration of direct and indirect dopamine agonists to rats (bromocriptine and L-DOPA/ Sinemet Plus respectively) were found to have no effect on the concentration of the brain biopterin pool, but decreased derived pterin concentration was evident which probably reflects reduced turnover of BH_4 because of decreased tyrosine hydroxylase activity. Peripheral biopterin levels in the liver were decreased. This suggests that a primary control of catecholamine production would be by negative feedback onto tyrosine hydroxylase and not by decreased biopterin concentrations.

Fig 8:1 Effect of bromocriptine on liver Biopterin Synthesis.



Bromocriptine is thought to decrease hepatic biopterins by inhibition of *de novo* synthesis, and L-DOPA by dopamine-inhibition of DHPR.

Mandell *et al* (1980) could find no effect on rat striatal concentrations in a survey of the effects of over thirty psychotropic agents. Only reserpine and physostigmine induced increases, and these changes were both small and variable. As shown above, this does not prove conclusively that BH_4 metabolism is unaffected by any of the drugs they tested. The importance of studying biopterin levels in tissues after the administration of compounds which do not contain substantial tyrosine hydroxylase (e.g. the liver) is paramount for detecting adverse effects on BH_4 metabolism from chronic exposure to compounds.

8:5 Scopolamine

Scopolamine is an anticholinergic drug which acts by antagonism of muscarinic receptors. Antimuscarinic drugs are occasionally used in the treatment of parkinsonism to relieve the rigidity (Meyers *et al*, 1980) The effect of scopolamine was investigated to see if an anticholinergic agent could influence BH_4 metabolism. Scopolamine caused a reduction in the concentration of brain derived pterin (Table 8:11), suggesting a decreased turnover of BH_4 or a decrease in THF concentration.

Table 8:11

The effect of scopolamine on rat brain derived pterin

Control	Dosed	
69.17± 4.76 (6)	49.56± 9.70 (6)	$p<0.005$

Value as ng pterin/ g wet weight of tissue. 270g male Wistar rats were dosed with 0.023mg scopolamine hydrochloride/ animal orally and killed 5h later. Controls were given distilled water i.g.

8:6 Cotrimoxazole

Cotrimoxazole is a combination antibiotic containing trimethoprim and sulphamethoxazole in a ratio of 1:5. England and Coles (1972) found that patients on cotrimoxazole had raised fasting serum phenylalanine levels suggesting a block in the formation of tyrosine from phenylalanine. Andrews *et al* (1976) reported that cotrimoxazole impaired the

L-phenylalanine tolerance of control subjects after oral and intravenous phenylalanine loading. Serum biopterins were elevated in patients taking cotrimoxazole (Leeming *et al*, 1976). Brown (1981) found that both trimethoprim and sulphamethoxazole inhibited DHPR in *in vitro* studies and that they act synergistically. Following these observations, the effects of cotrimoxazole administration on BH_4 metabolism in the rat was investigated.

After administration of cotrimoxazole to 180g male Wistar rats (Table 8:12) brain total biopterins and brain and liver derived pterin concentrations were significantly lower than control values. DHPR was normal when measured, probably because of dilution effects as described for L-DOPA. Trimethoprim is a DHFR inhibitor and the decreased derived pterin might be from loss of THF.

Adverse reactions involving the CNS have occasionally been reported resulting in psychosis or depression (American Medical Association Drug Evaluations, 1980). Reduced biopterin levels have been associated with these clinical conditions (Table 1:01). Decreased BH_4 and/or THF could result in inefficient neurotransmitter synthesis thereby contributing to the neuropathology. No elevation of plasma biopterins was found in rats (Table 8:12) which differs to the observed effect of cotrimoxazole in humans (Leeming *et al*, 1976). This situation is analogous in some ways to the effects of L-DOPA administration on BH_4 metabolism in the rat, where there was found to be no elevation in plasma biopterins after administration, which contrasts to the rise in plasma biopterins found in humans.

Table 8:12

The effect of cotrimoxazole on pteridine metabolism in the rat.

Parameter	Tissue	Control	Dosed
Biopterins	Brain	95.38± 16.38(6)	68.52± 17.38(6) $p<0.025$
Derived pterin	Brain	56.93± 11.65(6)	35.56± 7.12 (6) $p<0.020$
Derived pterin	Liver	0.31± 0.02 (6)	0.25± 0.05 (6) $p<0.025$
Biopterins	Liver	1.86± 0.23 (6)	1.67± 0.14 (6) ns
Biopterins	Plasma	17.55± 3.05 (6)	18.60± 4.85 (6) ns

Brain values as ng/g wet weight, livers as µg/g wet weight and plasmas as ng/ml. 180g male Wistar rats were dosed with 20.53mg sulphamethoxazole and 4.27mg trimethoprim i.g. /day for 2 days and killed 24h later. Controls were given distilled water i.g.

8:7 DHPR and cerebral THF maintenance

DHPR is the salvage enzyme responsible for maintenance of BH_4 (Craine *et al*, 1972). In the brain, DHPR may have a role in it's maintenance of THF levels (Pollock and Kaufman, 1978). DHPR can reduce $_qFH_2$ to FH_4 (Lind,1972). Very little DHFR is present in the brain (Makulu *et al*, 1973). Secondary THF deficiency has been reported in patients with DHPR deficiency (Smith *et al*, 1986), and folinic acid therapy is required. If indeed DHPR is important in control of THF metabolism in the brain, agents that can inhibit DHPR could potentially decrease both BH_4 and THF levels if they are capable of crossing the blood-brain barrier. Examples would include L-DOPA, cotrimoxazole and lead. This is another possible explanation for decreased brain derived pterin.

Alternatively, Hyland *et al*, (1985) suggested that the folate deficiency arising from DHPR deficiency may be due to competitive inhibition by the elevated concentration of dihydrobiopterins of both 5,10-methylenetetrahydrofolate reductase and DHFR. This would lead to decreased concentrations of methyl and other tetrahydrofolates in the brain and to neurological deterioration. This hypothesis is consistent with the response to folinic acid therapy in DHPR deficent patients since folinic acid is converted to 5,10-methylene THF *in vivo* and could then provide an increase of natural substrate for 5,10-methylene THF reductase to overcome the competitive inhibition caused by dihydropterins. Administration of DHPR inhibitors may act similarly in generating dihydrobiopterins and dihydropterin which may inhibit folate enzymes and derived pterin would be decreased after acid-iodine oxidation. Heales (1987) reported that acid-oxidation of THF yields 100% pterin, and so derived pterin may also reflect THF status.

8:8 Phenylacetate

Phenylacetate is a metabolite of phenylalanine (Edwards and Blau, 1972) (23) which is found in increased concentrations when hepatic hydroxylation of phenylalanine is inhibited, such as in "classical phenylketonuria" or "atypical phenylketonuria" (Loo *et al*, 1978).

Phenylacetate has been shown to be capable of inhibiting enzymes involved in

neurotransmitter biosynthesis, such as DHPR (Shen, 1984, Cutler, 1986), tyrosine hydroxylase (Udenfriend *et al*, 1965), and L-amino acid decarboxylase (Davison and Sandler, 1956). This metabolite has been used as an experimental model for phenylketonuria (Wen *et al*, 1980) where it causes neuropathological effects associated with the disorder.

Since phenylacetate has been shown *in vitro* to be capable of inhibiting DHPR (Shen, 1984), it's *in vivo* effects on the biochemistry of BH_4 were investigated. No effect was found on any of the BH_4 parameters measured (Chapter 3). This supports Shen's views (1984) that phenylacetate is too weak an inhibitor of DHPR to have physiological or pathological significance ($Ki = 8.5x10^{-4}M$, Cutler, 1986). However, phenylacetate would be raised in tissues in states of BH_4 deficiency. ($Ki = 8.5 \times 10^{-4}M$).

8:8:1 Phenylacetate and CAT activity

Potempska *et al* (1984) found that phenylacetylcoenzyme A, which is produced *in vivo* during detoxification of phenylacetate (Loo *et al*, 1978) is a very potent inhibitor of choline acetyltransferase (CAT) in *in vitro* studies, competitive for acetyl coenzyme A with Ki of 0.31μM.

There have been many reports of BH_4 deficiency in Alzheimer's disease (Table 1:01) and senile patients have been shown to have a reduced tolerance to phenylalanine loading (Leeming and Blair, 1980). Choline acetyltransferase deficiency is a post mortem finding in SDAT and other dementias (Bowen *et al*, 1983). It is postulated here that impaired hydroxylation of phenylalanine due to BH_4 deficiency would lead to chronic accumulation of endogenous phenylacetylcoenzyme A and this might result in lowered CAT activity. Potempska *et al* (1984) have suggested that this occurs in phenylketonuria.

CAT activity was measured in the brains of rats after administration of phenylacetate, but no difference was found when compared with controls (Table 8:13). However, the experiment was conducted over a relatively short period of time, and 5% tissue homogenates (w/v) were used for enzyme assay, which could have diluted any competitive enzyme inhibitor out of the tissue, as is thought to occur when measuring DHPR activity after administration of inhibitors.

Table 8:13

The effect of phenylacetate on rat brain CAT activity

Controls	Dosed	
8.82± 1.05 (6)	9.23± 1.67 (6)	ns

Values as µmoles Ach produced/ h/ gwet weight of tissue. 40g male weanling rats were dosed for 2 weeks with phenylacetate (2.5% w/w) in blended food pellets *ad libitum*.

8:8:2 Phenylacetate and vitamin B6 metabolism

Plasma tyrosine levels were significantly elevated in experimental animals (Table 8:14). There was no effect on plasma phenylalanine. As a consequence, the plasma P:T ratio was lower than normal (Table 8:15).

Loo and Whitaker (1967) investigated the effects of phenylalanine metabolites on B6 metabolism. They found that phenylethylamine forms an aldimine with pyridoxal, pyridoxylidene-ß-phenylethylamine which inhibits pyridoxal kinase in the liver and prevents formation of the active (phosphorylated) vitamers of B6.

Loo *et al* (1967) reported that in suckling rats treated with parachlorophenylalanine, the predominant aromatic amino acid metabolite in the brains of these animals was phenylacetic acid with decreasing amounts of phenylpyruvic acid, phenyllactic acid and mandelic acid. Pyridoxamine, when injected repeatedly was effective in significantly reducing the amount of phenylacetate that accumulated in the brain over a period of 6 hours. In view of these findings they speculated that pyridoxamine phosphate is involved in the removal of aldehydes formed from amines, thus preventing the accumulation of the corresponding acids in the brain. Wen *et al* (1980) found that phenylacetate in rats caused histological changes in the brain similar to B6 deficiency.

Tyrosine is a substrate for tyrosine aminotransferase (Black *et al*, 1970) , which is a B6 dependent enzyme. Also, the hydroxylated product of tyrosine, L-DOPA is decarboxylated to produce dopamine by DOPA decarboxylase (Kuntzman *et al*, 1961) which also requires pyridoxal phosphate, as does the decarboxylation of tyramine to p-hydroxyphenylacetate (Easton *et al*, 1972).It follows that vitamin B6 is essential for the metabolic control of tyrosine.

Phenylacetate was administered for 2 weeks as 2.5% of a blended standard rat diet,

which contains 10.10 mg B_6 / kg (Cutler, 1986). This level of exposure could have disrupted B6 and decreased the activity of enzymes required in the control of plasma tyrosine. Easton *et al* (1972) showed that tyrosinaemia was produced in Wistar rats deficient in vitamin B6.

It would seem important in the light of the above discussion that B6 status is investigated in hyperphenylalaninaemic conditions such as DHPR deficiency, BH_4 synthesis deficiency, "classical" PKU or after exposure to agents which can decrease hepatic BH_4 levels, to determine if pyridoxine supplementation is required in these individuals.

Table 8:14

The effect of phenylacetate on rat plasma tyrosine

Controls	Dosed	
52.46± 2.12 (6)	64.93± 6.68 (6)	p< 0.005

Value as µmol/L of plasma.

Table 8:15

The effect of phenylacetate on rat plasma phenylalanine: tyrosine ratios

Controls	Dosed	
0.62± 0.07 (6)	0.50± 0.07 (6)	p< 0.010

40g male weanling rats were dosed for 2 weeks with phenylacetate as 2.5% of their diet. Diets were given as blended food pellets *ad libitum*.

8:9 P-Hydroxyphenylacetate

P-hydroxyphenylacetate (P-HPAA) (24) is a catabolite of tyrosine. Levels are elevated in Tyrosinaemia and related disorders, namely Richner-Hanhart syndrome, neonatal tyrosinaemia and tyrosinosis. Plasma levels of 0.36 mg/100ml have been reported (Goldsmith, 1983).

P-HPAA has been reported as a potent *in vitro* inhibitor of DHPR (Shen, 1984 $Ki=7.4x10^{-5}M$). As displayed in Table 8:16 administration of P-HPAA to rats caused typical biochemical findings in BH_4 metabolism associated with DHPR inhibition. Liver total biopterins were significantly decreased, and plasma tyrosine and phenylalanine were

significantly lowered, with an overall increase in the phenylalanine: tyrosine ratio, signifying inefficient phenylalanine hydroxylation. Plasma derived pterin was increased in dosed animals. Changes in BH_4 metabolism were not found in the CNS. This may represent exclusion of the toxic metabolite by the blood-brain barrier. No effect was found on plasma biopterins, liver BH_4 synthesis or derived pterin, brain total biopterins, derived pterin concentration or CAT (Chapter 3).

There have been cases of Richner-Hanhart syndrome and neonatal tyrosinaemia where sufferers have been mentally retarded. The finding that *in vivo* administration of a p-hydroxyphenolic acid metabolite of tyrosine can cause alteration in BH_4 metabolism, presumably by DHPR inhibition, and inefficient phenylalanine hydroxylation could be partially responsible for the neurological symptoms of the diseases.

Shen (1984) suggested that efficient inhibitors of DHPR require a p-hydroxyphenol structure. Since administration of phenylacetate in greater concentrations than P-HPAA and for a longer period of time had less of an effect on BH_4 metabolism (Chapter 3) in *in vivo* studies, work here supports this hypothesis.

Table 8:16

The effect of P-HPAA on BH_4 metabolism and aromatic amino acids in the rat

Parameter	Tissue	Controls	Dosed
Biopterins	Liver	1.80± 0.19 (6)	1.34± 0.12 (6)p<0.001
Phenylalanine	Plasma	37.32± 3.38 (6)	32.81± 2.47 (6) p<0.050
Tyrosine	Plasma	30.39± 3.38 (6)	19.88± 3.52 (6)p<0.005
Derived pterin	Plasma	1.60± 2.19 (6)	5.23± 3.28 (6)p<0.050
P : T ratio	Plasma	1.24± 0.14 (6)	1.69± 0.29 (6)p<0.010

Liver biopterins as µg/ g wet weight. Plasma amino acid as µmol/ L and plasma derived pterin as ng/ ml. 180g male Wistar rats were dosed with 100mg P-HPAA i.p. over 6 days. Controls were given isotonic saline i.p.

8:10 DAHP

Cotton (1986) used DAHP to induce experimental hyperphenylalaninaemia in mice. DAHP is an inhibitor of GTP cyclohydrolase, and it's administration to rats has been used as an experimental model for BH_4 deficiency in humans due to a block in the GTP to NH_2TP step of biosynthesis because of GTP cyclohydrolase deficiency (Joller *et al*, 1983, Niederwieser *et al*, 1984). DAHP was used to investigate the effects of decreased BH_4 levels on tissue derived pterin.

DAHP caused a 60% decrease in brain total biopterins (Table 8:17) and a 45% decrease in brain derived pterin. A greater effect was seen in the liver total biopterin levels which were dropped by 81%. Liver derived pterin was decreased by 44%.

Derived pterin is a potential catabolite of both tetrahydrofolate (Fukushima and Nixon, 1980) and BH_4 (Pfleiderer, 1975, Milstien, 1983). The results indicate that derived pterin levels follow changes in biopterin metabolism in the brain more closely than in the liver. However, Hasegawa *et al*(1988) found that DAHP administration to mice caused disorder of digestive functions, which would decrease folate absorption and derived pterins would be lowered because of decreased THF content in tissues.

Plasma total biopterins were significantly lower in dosed animals (Table 8:17) indicating decreased BH_4 biosynthesis. Plasma phenylalanine was higher in experimental animals compared with controls. No effect was observed on plasma tyrosine and this resulted in elevated P : T ratios, showing inefficient phenylalanine hydroxylation. No effect was apparent on brain CAT activity (Chapter 4).

The work presented here supports that of Cotton (1986). DAHP administration may be a useful model of BH_4 synthesis deficiency, and behavioural and biochemical changes associated with the induced lesion could be studied under varying severity of imposed BH_4 deficiency in the Wistar rat.

Table 8:17

The effect of DAHP on rat pteridine metabolism and aromatic amino acids

Parameter	Tissue	Controls	Dosed	
Biopterins	Brain	99.55± 23.66 (6)	42.74± 15.08 (5)	p<0.005
Derived pterin	Brain	42.22± 15.08 (6)	23.45± 7.32 (5)	p<0.050
Biopterins	Liver	2.29± 0.16 (6)	0.43± 0.44 (5)	p<0.001
Derived pterin	Liver	0.84± 0.05 (6)	0.47± 0.06 (5)	p<0.001
BH_4 synthesis	Liver	12.04± 4.33 (6)	6.04± 1.63 (5)	p<0.025
Biopterins	Plasma	20.74± 3.12 (6)	11.84± 6.66 (5)	p<0.020
Phenylalanine	Plasma	46.54± 14.54 (5)	135.49± 43.01(5)	p<0.005
P: T ratio	Plasma	0.96± 0.37 (5)	3.85± 1.99 (5)	p<0.020

Brain biopterin and derived pterin values as ng/ g wet weight, livers as µg/ g wet weight and plasmas as ng/ ml. Phenylalanine as µmol/L. Biosynthetic capacity as ng biopterin / h/ mg protein.40g male weanling Wistar rats were dosed with 5% DAHP for 3 days and 3% w/w for 3 days in blended food pellets.

8:11 Neurotoxic Metals

8:11:1 Lead

Lead is a known neurotoxin. Hrdina *et al* (1980) in their review summarized the neurochemical effects of lead. Both dopamine and 5-HT are decreased in the brains of lead exposed animals. Lead inhibits DHPR (Purdy *et al*, 1981, McIntosh *et al*, 1982, Cutler,1986, Eggar *et al*, 1987) as well as the *de novo* biosynthetic pathway for BH_4 (Purdy *et al*, 1981, McIntosh *et al*, 1985).

Chronic dosing of lead was found to increase plasma biopterins significantly in the two experimental groups compared with the control group, indicating DHPR inhibition (Table 8:18).

Liver total biopterins were found to be elevated compared with controls (Table 8:18). Eggar *et al* (1987) found elevated levels of total biopterins after administration of lead to rats because of an increase in the inactive BH_2 form after DHPR inhibition. Brain total biopterins were decreased in Group 1 but not in the lower dosed Group 2 (Table 8:18).

Measurement of lead by atomic absorption in tissues examined in this experiment showed that both dosed groups had significantly higher levels (Table4:17) compared with control brains, plasmas and livers. Differences in lead levels between the two leaded groups were unexpectedly low, and the only significant difference in lead levels between the two experimental groups was a higher level in the livers of the lower dosed group.

Aungst *et al* (1981) found that tissue lead levels were not proportional to the apparent amount of lead ingested and concluded that the relationship between oral ingestion of lead and toxic effect may not be a simple one and absorption of lead from the GI tract is largely capacity limited in adult and adolescent rats. It follows that the absorption process of lead in the experimental groups could have been saturated.

In the chronic exposure experiment, no effect was seen on brain CAT in either group (Chapter 4). Modak *et al* (1975) found CAT to be elevated, but Carroll *et al* (1976) could find no change in CAT after chronic lead acetate dosing.

Table 8:18

The effect of chronic lead ingestion on BH_4 metabolism in the rat.

Parameter	Tissue	Controls	Group 1	Group2
Biopterins	Brain	75.61±12.20 (6)	45.36±15.33 (5) **p< 0.010**	60.14±25.84 (6) **ns**
Derived pterin	Brain	95.06±9.98 (6)	70.91±15.64 (6) **p< 0.010**	84.41±21.44 (6) **ns**
Biopterins	Liver	1.29±0.34 (6)	1.95±0.23 (6) **p< 0.005**	1.80±0.20 (6) **p< 0.020**
Derived pterin	Liver	0.39±0.12 (6)	0.49±0.11 (6) **ns**	0.45±0.14 (6) **ns**
Biopterins	Plasma	12.22±3.25 (6)	23.52±4.96 (6) **p< 0.001**	21.91±9.71 (6) **p< 0.050**

Brain values as ng/ g wet weight of tissue, livers as µg/ g wet weight and plasmas as ng/ ml. 40g male weanling rats were administered lead acetate as follows: Lead Group 1, 0.78% lead acetate for the first month, 2% for the second month and 4% for the third month. Lead Group 2, 0.39% lead acetate for the first month, 1% for the second month and 2% for the third month.

In another experiment, phenylalanine and tyrosine levels were examined in the brains and plasmas of rats dosed sub-chronically with lead acetate (Table 8:19). In all cases,

actual amino acid concentrations were in the normal range, but P:T ratios were lower in dosed brains, but normal in plasmas, which may have been the result of interference with BH_4.

Table 8:19

The effect of sub-chronic lead administration on P : T ratios in rat brains

Controls	Dosed	
1.22± 0.07 (6)	1.00± 0.07 (6)	$p< 0.001$

180g male Wistar rats were dosed with 0.3ml lead acetate (10^{-3}M) twice a day for 5 days and killed 12h later. Controls were given i.p. injections of isotonic saline.

Defective BH_4 metabolism because of interference by lead could be responsible for the neurochemical findings in experimental animals of decreased catecholamine and serotonin neurotransmitters (Hrdina, 1980) and associated with the mental retardation in children with blood lead levels greater than matched controls (David *et al*, 1982).

8:11:2 Aluminium

Brain total biopterins were decreased in rats chronically ingesting aluminium acetate (Table 8:21). No effect was found on plasma biopterins. Liver biopterins were elevated as was found for the lead dosed animals, and this is thought to be due to the production of excessive BH_2 by DHPR inhibition (Eggar *et al*, 1987).

Dhondt *et al* (1982, 1983) reported the increased N:B ratios in dialysis patients may be responsible for abnormal P:T ratios and impaired neurotransmitter synthesis which can occur in chronic uraemia, and that DHPR activity and biopterin levels in hamsters receiving aluminium sulphate in their drinking water (3% for 10 days) were significantly reduced.

Cowburn and Blair (1987) reported that the addition of aluminium acetate to temporal and frontal cortex preparations caused a significant decrease in BH_4 synthesis. The concentration of the metal used was similar to that found in SDAT. They suggested an effect on phosphate eliminating enzyme by binding with the triphosphate group of intermediates in preference to magnesium and inhibiting the breakage of the phosphate ester linkage and formation of 6-pyruvoyltetrahydropterin.

Altmann *et al* (1987) showed that haemodialysis patients had increased serum biopterins and lowered DHPR activity. DHPR activity increased after deferoxamine treatment.

Brain CAT activity was decreased after chronic administration of aluminium to rats (Table 8:20). Yates *et al* (1980) reported a decrease in CAT activity in rabbits caused by aluminium. There is a deficiency of CAT in SDAT (Bowen *et al*, 1983).

Wisniewski *et al* (1985) found that CAT activity is lowered by 20% by 550µg of aluminium chloride/ g wet weight of tissue. Since levels found in SDAT are much lower than this, then the reduction of enzymatic activity is not the result of direct inhibition by aluminium. This can also be seen in Chapter 4 (Tables4:05, 4:06) where aluminium acetate had no direct effect *in vitro* on CAT activity either in a Tris-HCl or EDTA reaction system. Boegman and Bates (1984) suggested that decreased CAT activity is the result of reduced slow transport of the enzyme from slow neurones located in the spinal cord.

Table 8:20

The effect of chronic aluminium ingestion on rat brain CAT

Controls	Dosed	
11.63± 2.83 (6)	7.63± 2.48 (6)	p< 0.020

Values as µmol Ach/ h/ mg protein. See Table 8:21 for dosing procedure.

Table 8:21

The effect of chronic aluminium ingestion on BH_4 metabolism in the rat.

Parameter	Tissue	Controls	Dosed	
Biopterins	Brain	75.61± 12.20 (6)	53.11±14.48 (6)	p<0.020
Derived pterin	Brain	95.06± 9.98 (6)	94.73± 31.02(6)	ns
Biopterins	Liver	1.29± 0.34 (6)	1.82± 0.08 (6)	p<0.005
Derived pterin	Liver	0.39± 0.12 (6)	0.20± 0.08 (6)	p<0.010
Biopterins	Plasma	12.22± 3.25 (6)	13.32± 2.32 (6)	ns

40g male weanling Wistar rats were administered 0.03% aluminium acetate for 1 month, followed by 1% for the second month and 2% for the third month. Control animals were given ordinary tap water for the duration of the experiment.

In conclusion, aluminium intoxication can inhibit both the *de novo* synthesis and salvage of BH_4 and cause changes in concentrations of total biopterins. This would affect catecholamine and serotonin neurotransmitter production.

Aluminium induced CAT deficiency was demonstrated. Neurochemical deficits associated with SDAT and probably dialysis dementia include altered BH_4, catecholamine, serotonin and CAT metabolism, and this work supports the hypothesis that aluminium is involved in the biochemical aetiology of these disorders.

8:12 Tissue distribution of *de novo* and salvage BH_4 pathways.

Table 8:23 compares liver and brain BH_4 metabolism in the rat. Liver BH_4 biosynthetic capacity is 10-12x higher than in the brain, DHPR is 1-2x higher in the liver, and the concentration of total biopterins is 20x higher than in the brain. It can be seen that in these tissues, *de novo* and salvage pathways make different contributions to the tissue maintenance of the BH_4 pool.

In tissues where maintenance of BH_4 levels is relatively more important by the salvage pathway compared with other tissues, inhibitors of DHPR would have a greater effect on BH_4 levels. The same argument applies for tissues where the contribution of the *de novo* pathway is relatively more important than in other tissues.

Table 8:22

Comparison of BH_4 metabolism in the brain and liver of the rat.

Parameter	Brain	Liver	Liver:Brain
DHPR	263.77±68.94 (30)	397.00±200.71 (30)	1.51
BH_4 Synthesis	1.00±0.36 (6)	12.04±4.33 (6)	12.00
Sepiapterin Reductase	1.51±0.33 (30)	17.75±3.97 (7)	11.75
Total biopterins	82.94±21.60 (30)	1720.00±390.00(30)	20.74

DHPR/nmol NADH oxidized/min/mg protein. BH_4 Synthesis/ng biopterin/h/ mg protein. Sepiapterin Reductase/ nmol sepiapterin reduced/ min./ mg protein.Total biopterins/ ng/ g wet weight.

8:13 The effects of phenylalanine and tyrosine loading on pteridine metabolism in normal human subjects

A 7.0g load of phenylalanine administered orally to healthy volunteers caused an increase in plasma total biopterins (Chapter 7). There was a significant positive correlation between plasma phenylalanine and total biopterins ($R = +0.89$, $p < 0.01$, $n = 12$, Fig. 7:01).

Plasma tyrosine increased following phenylalanine loading (Fig. 7:02, $R = +0.68$, $p < 0.02$, $n = 12$). No significant correlation was found between plasma phenylalanine and biopterins after tyrosine loading (Fig. 7:13) or between plasma tyrosine and biopterins (Fig. 7:14). These findings support Leeming, (1976) but not the work of Yamaguchi *et al* (1983) and Nagatsu *et al* (1984), who claimed to find elevations in serum biopterins on administration of tyrosine to control subjects and parkinsonian patients. The different methods used may offer an explanation. Biopterin was measured by HPLC and *Crithidia fasciculata* here, whereas the Japanese groups used radioimmunoassay. Correlation of biopterin measurement by HPLC compared to *Crithidia fasciculata* correlated very well ($R = +0.89$, $p < 0.01$, $n = 31$) (Fig. 7:25).

8:14 The effect of phenylalanine and tyrosine on visual evoked potentials in normal human subjects.

N:B ratios are abnormal in senile dementia, and Hamon *et al* (1987) suggested the possibility of relating the N:B ratio to the degree of dementia as measured by VEP. Harding *et al* (1985) found that presenile dementia caused a slowing of the major positive (P2) component of the VEP to flash stimulation but the VEP to pattern reversal stimulation (P100) was of normal latency. The difference between these two latencies is a specific diagnostic indicator of primary senile dementia. Hamon *et al* (1987) showed that the high correlation between N:B ratio and latency differences in the VEPs show that N:B ratios reflect pathology, and the higher ratio corresponds to the amount of organic deterioration as shown by the VEP.

A good correlation was found between plasma N:B ratios and VEP latencies after tyrosine loading ($p<0.01$, $R=+0.69$, $n=18$, Fig. 7:26), which was not found when normal subjects ingested phenylalanine loads ($R=-0.21$, $n=12$, ns, Fig. 7:27).

8:15 Further work

Following the decrease in brain derived pterin after the administration of DHPR inhibitors such as L-DOPA and cotrimoxazole, it would be important to determine the definitive cause of this finding. As previously discussed, DHPR may be involved in the maintenance of cerebral THF, and THF measurements should be done in the brains of animals dosed with DHPR inhibitors. In other studies on the effects of neurotoxins on BH_4 metabolism, changes in tissue derived pterin should be further investigated by THF measurements. The potential for using derived pterin as an indicator of BH_4 turnover should be studied further.

The observation that phenylacetate caused an increase in plasma tyrosine, and previous reports that phenylalanine and phenylalanine metabolites affect vitamin B6 metabolism, lead to the suggestion that vitamin B6 status of hyperphenylalaninaemic patients should be routinely investigated.

Since BH_4 metabolism is disturbed in SDAT, and this may increase phenylacetate, and consequently phenylacetylcoenzyme A in the brain, which is a potential inhibitor of CAT, an interesting study would be a comparison of the levels of these metabolites in the post mortem brains in SDAT and control subjects.

Aluminium was found to decrease the CAT content in rat brains after chronic ingestion of the acetate salt. This decrease is thought to result from inhibited cellular transport of the enzyme, not direct inhibition by the metal. This is postulated to lead to decreased tissue overflow of CAT, and development of CAT measurement in plasma may be important in identifying "at risk" populations in aluminium associated diseases.

The use of the Wistar rat as a model for human BH_4 metabolism should be further investigated by a comparison of the relative activities of the *de novo* biosynthetic and salvage pathways and their contribution to maintenance of BH_4 levels in the same tissues of the two species.

DAHP administration to rats caused a hyperphenylalaninaemic condition biochemically similar to GTP cyclohydrolase deficiency in humans. The use of this compound should be undertaken to study biochemical (including CAT activity) and behavioural effects of hyperphenylalaninaemic disorders under varying severity of the biochemical lesion.

The finding that a commonly used therapy of Parkinson's disease, bromocriptine, inhibits *de novo* biosynthesis of BH_4 and the greatly diminished BH_4 content of parkinsonian patients would suggest the prophylactic co-administration of BH_4 with the bromocriptine therapy. This might also be beneficial with L-DOPA administration, which inhibits DHPR.

The point of inhibition of *de novo* BH_4 biosynthesis by bromocriptine should be investigated. A likely lesion would be at the sepiapterin reductase step. The effect of bromocriptine should be determined on sepiapterin reductase, GTP cyclohydrolase and phosphate eliminating enzyme.

It would be interesting to determine the mechanism by which ingestion of phenylalanine causes an elevation of plasma biopterins, which is not observed after ingestion of tyrosine.

A study should be done on the correlation of N : B ratios with VEP latencies in normal subjects under normal dietary conditions, and after tryptophan loading, since it may be possible to influence brain serotonin levels in this way.

Appendix.

Comparison of the Measurement of Phenylalanine and Tyrosine by Ion Exchange Chromatography and reverse phase HPLC.

Plasma Phenylalanine Concentration / µmol./L

Ion-exchange method	HPLC	
62.50± 22.97 (6)	43.00± 5.00 (6)	ns

Plasma Tyrosine Concentration / µmol. /L

Ion-exchange method	HPLC	
73.50± 22.89 (6)	57.00± 8.50 (6)	ns

Brain Phenylalanine Concentration / µmol. /L

Ion-exchange method	HPLC	
17.17± 1.60 (6)	20.00± 3.50 (6)	ns

Brain Tyrosine Concentration / µmol. /L

Ion-exchange method	HPLC
Not Resolved	25.00± 4.50 (6)

Discussion.

Plasma values are expressed as µmol./L of plasma. Brain values are expressed as µmol./L of brain supernatant from a 20% homogenate w/v 0.02M HCl. HPLC method used was based on that of Hyland *et al*, (1985) as described in Chapter 2. Ion-exchange chromatography was performed by Sheena Grant, Birmingham Childrens' Hospital in the routine clinical chemistry laboratory. The HPLC method was found to be faster (single sample can be analysed in 15 minutes, compared with overnight runs for ion-exchange chromatographs). Also, brain tyrosine chromatographs were not resolved by the ion-exchange method but were easily quantitated by HPLC.

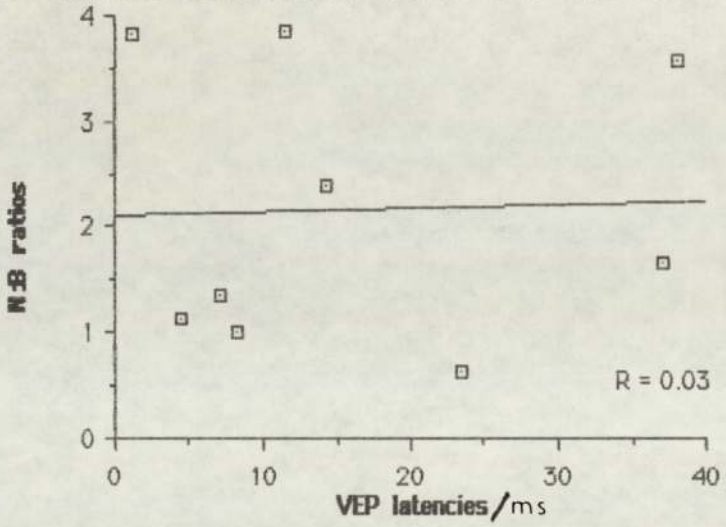
Plasma phenylalanine and tyrosine concentrations, and brain phenylalanine in the Wistar rat were not significantly different when measured by reverse phase HPLC or ion-exchange chromatography.

Appendix 2.

Correlation Between N:B Ratios and VEP Response Latencies in Normal, Unloaded Subjects.

The table overleaf (Page 217) shows the poor correlation obtained ($R=0.03$, $n=9$, ns) between neopterin : biopterin ratios and VEP response latencies in normal subjects used in the phenylalanine and tyrosine loading experiments at Time=0 (i.e. before ingestion of the amino acid). The subjects were not examined for any possible causes of elevated neopterins concentration, such as infections. The experiment should be repeated using subjects who are known to have no elevated activity of the immune system.

Correlation between N:B ratios and VEP latencies in normal, unloaded subjects.



References.

REFERENCES

Alfrey A.C., Le Gendre G. R., Kaehny W.D. (1976).
The Dialysis Encephalopathy syndrome.
New. Eng. J. Med., 294, 4, 184-188.

Altmann P., Al-Salihi F., Cutler P., Cunningham J., Marsh J.P., Blair J.A. (1987).
Aluminium and Tetrahydrobiopterin metabolism.
Clinical and Biochemical Aspects of Pteridines. Vol. 5., de Gruyter (Berlin) 129-146.

Amarego W.L.J., Waring P. (1983).
Inhibition of Human Brain Dihydropteridine Reductase (E.C.1.6.99.10) by the oxidation Products of Catecholamines, the Aminochromes.
Biochem. Biophys. Res. Comm. 113, 895-899.

American Medical Association Drug Evaluations. 4th Ed. (1980) John Wiley & Sons Inc. New York.

Andrews J.M., Purkiss P., Chalmers R.A., Watts R.W.E. (1976).
Effect of Cotrimonazole on the Response to Phenylalanine Loading in Man.
Clinica Chimica Acta, 68, 17-30.

Archer M.C., Vanderschmitt P.J., Scrimgeour K.G. (1972).
Mechanism of Oxidation of Tetrahydrobiopterin.
Can. J. Biochem., 50, 1174-1182.

Aungst B.J., Doke J.A., Fung H.L. (1981).
The Effect of Dose on the Disposition of Lead in Rats after Intravenous and Oral Administration.
J. App. Pharmac., 61, 48-57.

Aziz A.A., Leeming R.J., Blair J.A. (1983).
Tetrahydrobiopterin Metabolism in Senile Dementia of Alzheimer Type.
J. Neurol. Neurosurg. Psychiatry 46, 410-413.

Aziz A.A. (1985).
Effect of L-DOPA on Human Plasma Biopterins.
(Unpublished).

Aziz A.A., Blair J.A., Leeming R.J., Sylvester P.E. (1982).
Tetrahydrobiopterin Metabolism in Down's Syndrome and in Non-Down's Syndrome Mental Retardation.
J. Ment. Defic. Res., 26, 67-71.

Bachner R.I., Nathan D.G. (1968).
Quantitative nitroblue Tetrazolium Test in Chronic Granulomatosis Disease.
New. Eng. J. Med. 278, 18, 971-976.

Bacopoulos N.G., Bhatnagar R.K. (1977).
Correlation Between Tyrosine Hydroxylase Activity and Catecholamine Concentration on Turnover in Brain Regions.
J. Neurochem. 29, (4), 639-643.

Baker H., Frank O., Bacchi C.J., Hutner S.H. (1974).
Biopterin Content of Human and Rat Fluids and Tissues Determined by Protozoology.
Am. J. Clin. Nutr., 27, 1247-1253.

Barford P.A., Blair J.A., Eggar C., Hamon C., Leeming R.J. (1983).
Tetrahydrobiopterin Metabolism and Mental Disease.
Biochemical and Clinical Aspects of Pteridines, Vol 2, (Curtius H-Ch., Pfleiderer W., Wachter H), de Gruyter (Berlin) 303-315.

Barford P.A., Blair J.A., Eggar C., Hamon C., Morar C., Whitburn S. (1984).
Tetrahydrobiopterin Metabolism in the Temperol Lobe of Patients Dying with Senile Dementia of the Alzheimer type.
J. Neurol. Neurosurg. Psychiatry, 47, 736-738.

Bartholini G., Pletscher A. (1987).
Cerebral Accumulation and Metabolism of ^{14}C - DOPA after Selective Inhibition of Peripheral Decarboxylase.
J. Pharmac. Exp. Ther. 161, 14-20.

Bartholome K., Byrd P.J. (1975).
L-DOPA and 5-hydroxytryptophan Therapy in Phenylketonuria with Normal Phenylalanine Hydroxylase Activity.
Lancet ii, 1042-1043.

Bartholome K. (1974)
A New Molecular Defect in Phenylketonuria
Lancet ii, 1580.

Bartholome K., Byrd D.J., Kaufman S., Milstein S. (1977)
Atypical Phenylketonuria with Normal Phenylalanine Hydroxylase and Dihydropteridine Reductase Activity *In-Vitro*
Pediatrics 59, 757-761.

Bellahsene Z., Dhondt J.L., Farriaux J.P. (1984)
Guanosine Triphosphate Cyclohydrolase Activity in Rat Tissues.
Biochem. J. 217, 59-65.

Betts P.R., Astley R., Raine D.N. (1973)
Lead Intoxication in Children in Birmingham
Br. Med. J., 1, 402-406.

Black J. B. (1970)
Regulation of Hepatic Transaminase *In-Vivo* through Interaction of Norepinephrine and the Pyridoxal Phosphate Cofactor.
J. Pharmacol. Exp. Ther. 174, 2283-289.

Blair J.A., Whitburn S.B., Pheasant A.E., Leeming R.J., Morar C., Al-Beir A. (1983)
A Critical Appraisal of Methods for the Quantitative Analysis of BH_4, BH_2 and B in Human Urine, Serum and C.S.F.
Chemistry and Biology of Pteridines, de Gruyter (Berlin) 165-162.

Blair J.A. (1983)
Tetrahydrobiopterin Metabolism, Neurological Disease and Mental Retardation
(Personal Communication).

Blair J.A., Pearson A.J. (1974).
Kinetics and Mechanism of the Autoxidation of
2-amino-4-hydroxy-5,6,7,8-tetrahydrobiopterin.
J. Chem. Soc. Perkin. Trans. II 80-88.

Blair J.A., Hilburn M.E., Leeming R.J., McIntosh M.J., Moore M.R. (1982)
Lead and Tetrahydrobiopterin Metabolism: Possible Effects on I.Q.
Lancet 1, 964.

Blair J.A., Foxall C.D. (1969)
Mass Spectrometry of Naturally Occurring Pteridines.
Organic Mass Spectrometry Vol 2. 923-928.

Blair J.A., Pearson A.J. (1975)
Non-Enzymatic Tetrahydrobiopterin Hydroxylation of Phenylalanine.
J. Chem. Soc. Perkin Trans. II 245-249.

Blair J.A., Parveen H., Barford P.A., Leeming R.J. (1984)
Aetiology of Parkinson's Disease
Lancet i, 167.

Blakley R.L. (1969)
The Biochemistry of Folic Acid and Related Pteridines.
North Holland, Amsterdam.

Bobst A. (1967)
Radikalbilding Wahrendden Oxydation von Tetrahydrofolsaure und 5,6,7,8-tetrahydropterin.
Helv. Chim. Acta. 50, 2222-2225.

Boegman R.J., Bates L.A. (1984)
Neurotoxicity of Aluminium.
Can. J. Physiol. Pharmacol. Vol. 62, 1010-1014.

Bowen D.M., Allen S.J., Benton J.S., Goodhardt M.L., Haan E.A., Palmer A.M., Sims N.R., Smith C.C.J., Spillane J.A., Esini M.M., Neary D., Snowden J., Wilcock G.K., Davison S.N. (1983).
Biochemical Measurement of Serotonergic and Cholinergic Dysfunction and Cerebral Atrophy in Alzheimer's Disease.
J. Neurochem. 41 266-272

Bradford H.J. (1986).
Chemical Neurobiology. An Introduction to Neurochemistry.
W. H. Freeman & Company. New York

Brenneman A.R., Kaufman S. (1964).
The Role of Tetrahydropteridines in the Enzymatic Conversion of Tyrosine to 3,4-dihydroxyphenylalanine.
Biochem. Biophys. Res. Commun. 17, 177-183.

Brown S.E. (1981).
The Biosynthesis of Tetrahydrobiopterin in the Rat.
Ph.D Thesis. University of Aston in Birmingham.

Bullard W.P., Guthrie P.B., Russo P.U., Mandell A.J. (1978)
Regional and Subcellular Distribution and some Factors in the Regulation of Reduced Pterins in Rat Brain.
J. Pharm. Exp. Ther. 206, 4-20.

Burg A.W., Brown G.M. (1968)
The Biosynthesis of Folic Acid. VIII Purification and Properties of the Enzyme that Catalyses the Production of Formate from Carbon Atom 8 of GTP.

J. Biol. Chem. 243 (9) 2349-2358.

Calne D.B., Teychenne P.J., Claveria L.E., Eastman R., Greenacre J.K. (1974).
Bromocriptine in Parkinsonism.
Br. Med J. 4, 442-444.

Carroll P.J., Silbergeld E.K., Goldberg A.M. (1976).
Alteration of Central Cholinergic Function by Chronic Lead Acetate Exposure.
Biochem. Pharmacol. 26, 397-402.

Collins M.A. (1980).
'Neuroamine Condensation, in Human Subjects'.
Adv. Exp. Med. Biol. 126, 87-102.

Cook R.J., Blair J.A. (1979).
The Distribution and Chemical Nature of Radioactive Folates in Rat Liver Cells and Rat Liver Mitochondria.
Biochem. J. 178, 651-659.

Coppell A.D. (1984).
Identification of Folate Species using *L. casei*
Personal Communication.

Corbett J. (1985).
Is Down's Syndrome a Progressive Condition?
J. Roy. Soc. Med. 78 (6) 499-502.

Corrodi H., Fuxe K., Hokfelt J., Lidbunk P., Ungerstedt U. (1973).
Effect of Ergot Drugs on Central Catechalamine Neurons: Evidence for a Stimulation of Central Dopamine Receptors.
J. Pharm. Pharmac. 25, 409-411.

Costa E., Meek S.L. (1974).
Regulation of Biosynthesis of Catecholamine and Serotonin in the Central Nervous System.
Ann. Review Pharmacol. 14, 491-511.

Cotton R.G.H. (1986).
A Model for Hyperphenylalaninaemia due to Tetrahydrobiopterin Deficiency.
J. Inher. Metab. Dis. 9, 4-14.

Coulson W.J., Powers M.J., Jepson J.B. (1970).
Hydroxylation of Aromatic Amino Acids Mediated by a Tetrahydropterin.
Biochim. Biophys. Acta 222, 606-610.

Cowburn J.D., (1987).
Personal Communication

Cowburn J.D., Blair J.A. (1987).
Effect of Aluminium on *in vitro* Tetrahydrobiopterin Synthesis in Brain Preparation.
The Lancet i, 105.

Craine J.E., Hall E.S., Kaufman S. (1972).
The Isolation and Characterisation by Dihydropteridine Reductase from Sheep Liver.
J. Biol. Chem. 247, 6082-6091.

Crapper D.R., Krishnan S.S., Dalton A.J. (1973).

Brain Aluminium Distribution in Alzheimer's Diseas and Experimental Neurofibrillary Degeneration.
Science 180, 511-513.

Curtius H-Ch (1983).
Successful Treatment of Depression with Tetrahydrobiopterin.
Lancet i, 657-658.

Cutler P. (1986).
Dihydropteridine Reductase
Ph.D. Thesis. University of Aston in Birmingham.

Danks D.M., Bartholome K., Clayton B.E., Curtius H., Grobe H., Kaufman S., Leeming R.J., Pfleiderer W., Rembold H., Rey J. (1978).
Malignant Hyperphenylalaninaemia - Current Status (June 1977).
J. Inherited Metab. Dis. 1, 49-53.

Danks D.M., Cotton R.G.H. (1980).
Early diagnosis of Hyperphenylalaninaemia Due to Tetrahydrobiopterin deficiency (Malignant Hyperphenylalaninaemia).
J. Pediatr. 96, 854-856.

Danks D.M., Schlesinger P., Firgaira F., Cotton R.G.H., Watson B., Rembold H., Hennings G. (1979).
Malignant Hyperphenylalaninaemia: Clinical Features, Biochemical Findings and Experience with Administration of Biopterin.
Pediatr. Res. 13, 1150-1155.

David O.J., Grad G., McGann B., Kolt A. (1982).
Mental Retardation and 'Non-Toxic' Lead Levels.
Am. J. Psychiatry, 139, 6, 806-809.

Dhondt J.L. (1984).
Tetrahydrobiopterin Deficiencies: Preliminary Analysis from an International Survey.
J. Pediatrics 104, 4, 501-508.

Dhondt J.L., Bellahsene Z. (1983).
Inhibition of DHPR by Pteridines, Monoamines and Metallic Compounds.
Biochemical and Clinical Aspects of Pteridines Vol.2 (Curtius H-Ch., Pfleiderer W., Wachter H.) de Gruyter (Berlin) 139-146.

Dhondt J.L., Farriaux J.P. (1983).
Dihydroneopterin Synthetase Deficiency.
Biochemical and Clinical Aspects of Pteridines Vol 2. (Curtius H-Ch., Pfleiderer W., Wachter H.) de Gruyter (Berlin) 317-324.

Dhondt J.L. Bellahsene Z., Vanhille P., Noel C. (1982).
Tetrahydrobiopterin Metabolism in Chronic Uraemia. Possible Explanation of Dialysis Encephalopathy.
Lancet ii, 491.

Drachmann D.A. (1977).
Memory and Cognitive Function in Man: does the Cholinergic System have a Specific Role?
Neurology 27, 783-790.

Drachmann D.A., Leavitt J. (1974).

Human Memory and the Cholinergic System. A Relationship to Ageing?
Arch. Neurol. 30, 113-121.

Dubois B., Ruberg M., Javoy-Agid F., Ploska A., Agid Y. (1983).
A Subcortico - Cortical Cholinergic System is Affected in Parkinson's Disease.
Brain Research 288, 213-218.

Duch D.S., Bowers S.W., Woolf J.H., Nichol C.A. (1984).
Biopterin Cofactor Biosynthesis: GTP Cyclohydrolase, Neopterin and Biopterin in Tissues and Body Fluids of Mammalian Species.
Life Sciences Vol 35, 1850-1901.

Duch D.S., Bowers S.W., Nichol C.A. (1983).
Analysis of Folate Cofactor Levels in Tissues using High Performance Liquid Chromatography. *Anal. Biochem. 130, 385-392.*

Easton E.J., Simpson I., Martin J.K., Campbell D.J.(1972). Tyrosinaemia induced by a pyridoxine antagonist, desoxypyridoxine. *Clin. Chem. 2 18 161-163.*

Edwards P., Heales S.J.R., Cutler P., Blair J.A., Leeming R.J. (1987).
The *in-vivo* Effect of L-DOPA, Phenelzine and Starvation on Tetrahydrobiopterin Metabolism in the Rat.
Biochemical and Clinical Aspects of Pteridines Vol.5. de Gruyter (Berlin) 119-129.

Edwards D.J., Blau K. (1972).
Aromatic Acids Derived from Phenylalanine in the Tissues of Rats with Experimentally Induced PKU-like Characteristics.
Biochem. J. 130-495-503.

Eggar C., Hamon C.G.B., Morar C., Al-Salihi F., Blair J.A., Barford P.A. (1986).
The Effect of Lead on Tetrahydrobiopterin Metabolism. A Possible Mechanism for Neurotoxicity.
Clinica Chimica Acta 161, 103-109.

Ehringer H., Hornykiewicz O. (1960).Verteilung von Noradrenalin und Dopamin (3-Hydroxytyramin) im Gehirn des Extrapyramidalen Systems.
Kline Wochenschr. 38, 1236-1239.

England J.M., Coles M. (1972).
Effect of Cotrimoxazole on Phenylalanine Metabolism in Man.
The Lancet 1341-1343.

Eto J., Krumdieck C.L. (1982).
Determination of Three Different Pools of Reduced One-Carbon-Substituted Folates III, Reverse-Phase High Performance Liquid Chromatography of the Azo Dye Derivatives of p-aminobenzoylpoly-γ-glutamates and its Application to the Study of Unlabelled Endogenous Pteroylglutamates of Rat Liver.
Anal. Biochem. 120, 323-329.

Fahn S., Rodman J.S., Cote L.J. (1969).
Assocation of Tyrosine Hydroxylase with Synaptic Vesicles in Bovine Caudate Nucleus.
J. Neurochem. 16, 1293-1300.

Fellman J.H. (1956). Inhibition of DOPA Decarboxylase by Aromatic Acids Associated with Phenylpyruvic Oligophrenia.
Proc. Soc. Exp. Biol. Med. 93, 413-414.

Flood J.F. (1986).
Scopolamine Effects on Memory Retention in Mice: A Model of Dementia?
Behav. Neurol. Biol. 45 (2) 169-184.

Folling A. (1934).
Uber Ausscheidung von Phenylbenztraulensauze in der Harm als Staffwechselanomalie in Verbindung mit Imbezilliatat.
Z. Physiol. Chem. 227-169-176.

Fonnum F. (1975).
A Rapid Radiochemical Method for the Determination of Choline Acetyltransferase.
J. Neurochem. 24, 407-409.

Frank O., Baker H., Sobtka H. (1963).
Blood and Serum Levels of Soluble Vitamins in Man and Animals.
Nature 197, 490-491.

Freundlich M., Zilleruelo G., Faugere M.C., Abitbol C., Strauss J., Malluche H.H. (1986).
Treatment of Aluminium Toxicity in Infantile Uraemia with Deferoxamine.
J. Ped. Vol. 109. (1) 140-143.

Friedman P.A., Kappelman A.H., Kaufman S. (1972).
Partial Purification and Characterisation of Tryptophan Hydroxylase from Rabbit Hindbrain.
J. Biol. Chem. 247, 4165-4173.

Fukushima J., Nixon J.C. (1978).
Reverse Phase Separation of Unconjugated Pterins.
Chemistry and Biology of Pteridines. de Gruyter (Berlin) 35-36.

Fukushima J., Nixon J.C. (1980).
Analysis of Reduced Forms of Biopterin in Biological Tissues and Fluids.
Anal. Biochem. 102, 176-188.

Gal E.M., Nelson J.M., Sherman A.D. (1978).
Biopterin III Purification and Characterisation of Enzymes Involved in the Cerebral Synthesis of 7,8-Dihydrobiopterin.
Neurochem. Res. 3, 69-88.

Gal E.M., Whitacre D.H. (1981).
Biopterin VII Inhibition of Synthesis of Reduced Biopterins and its Bearing on the Function of Cerebral Tryptophan-5-Hydroxylase *in-viva*
Neurochem. Res. 233-240.

Galloway M.P., Levine R.A. (1986).
Modulation of Dopamine Synthesis by Nerve Terminal Autoreceptors: A Role for Tetrahydrobiopterin?
Chemistry and Biopterin of Pteridines. de Gruyter (Berlin) 347-350.

Goldsmith A.L. (1983).
Tyrosinaemia and Related Disorders.
Ch. 13, p287-299, 5th Ed. The Metabolic Basis of Inherited Disease. Stanbury J.B., Wyngaarden J.B., Fredrickson D.S., Goldstein J.L., Brown M.S., (McGrow Hill).

Gornall A.G., Bardawill C.J., David M.M. (1949).
Determination of Serum Proteins by Means of the Biuret Reaction.
J. Biol. Chem. 177, 751-766.
Guroff G., Rhoades C.A., Abramowitz A. (1967)*Anal. Biochem. 21 273*

Haber J., Weiss J. (1934).
The Catalytic Decomposition of H_2O_2 by Iron Salts.
Proc. Roy. Soc. A. 147, 332-351.

Hamon C.G.B., Edwards P., Jones S.A., Anderson J.M., Cattell R.J., Wright C.E., Blair J.A. (1987).
Tetrahydrobiopterin Metabolism in Senile Dementia.
Biochemical and Clinical Aspects of Pteridines Vol. 5, (Eds Pfleiderer W., Wachter H., Blair J.A.) de Gruyter (Berlin).

Harding G.F.A., Wright C.E., Orwin A. (1985).
Primary Senile Dementia; the Use of the Visual Evoked Potential as a Diagnostic Indicator.
Br. J. Psychiatr. 147, 532-539.

Harris J.E., Morgenroth V.H., Roth R.H., Baldessanini R.J. (1974).
Regulation of Catecholamine Synthesis in the Rat Brain *in-vitro* by cAMP.
Nature 252, 156-158.

Hasegawa H., Kobayashi T., Ichiyama A. (1988)
DAHP Induces Intestinal Disorder in Mice: An Animal Model of Serotonin Deficiency by Inhibition of Tetrahydrobiopterin Synthesis.
Seventh Winter Workshop on Pteridines 369, 532.

Heales S.J.R. (1987).
Personal Communication

Heintel D., Ghisla S., Curtius C.H., Niederwieser A., Levine R.A. (1984).
Biosynthesis of Tetrahydrobiopterin. Possible Involvement of Tetrahydropterin Intermediates.
Neurochem. Int.. 6, 141-155.

Hetnarski B., Wisniewski H.M., Iqbal K., Dzilzie J.D., Lajtha A. (1980).
Central Cholinergic Activity in Aluminium Induced Neurofibrillary Degeneration.
Annals. of Neurology Vol. 7, No. 5, 489-490.

Hilburn M.E. (1979).
Environmental Lead in Persepctive.
Chem. Soc. Reviews Vol. 8, No 1, 63-84.

Hokflet J., Fuxe K. (1972).
Effects of Prolactin and Ergot Alkaloids on the Tuberoinfundibular Dopamine (DA) Neurons.
Neuroendocrinology 9, 100-122.

Hornykiewicz O. (1966).
Dopamine (3-hydroxytyramine) and Brain Function.
Pharmacol. Rev. 18, 925-964.

Hornykiewicz O. (1973).
Dopamine in the Basal Ganglia: its Role and Therapeutic Implications (Including the Clinical Use of L-DOPA).

Br. Med. Bull. Vol. 29, No,2, 172-178.

Hosoda S., Glick D. (1966).
Studies in Histochemistry. LXXIX Properties of Tryptophan Hydroxylase from Neoplastic Murine Most Cells.
J. Biol. Chem. 241, 192-196.

Hrdina P.D., Hanin J., Dubos J.C. (1980).
Neurochemical Correlates of Lead Toxicity.
Lead Toxicity. Ed. Singhal R.L., Thanas J.A. 273-297 (Urban and Schwarzenberg).

Huber C., Batchelor J.R., Fuchs D., Hausen A., Long A., Niederwieser A., Reibnegger G., Swetly P., Troppmair J., Wachter H. (1984).
Immune Response Associated Production of Neopterin. J. Exp. Med. 160, 310-316.

Hyland K., Smith J., Howells D.W., Clayton P.J., Leonard J.V. (1985).
The Determination of Pterins, Biogenic Amines, Metabolites and Aromatic Amino Acids in C.S.F. using Isocratic Reverse Phase H.P.L.C. with In Series Dual Cell Coulometric Electrochemical and Fluorescence Detection. Use in the Study of Inborn Errors of Dihydropteridine Reductase and 5,10-Methylenetetrahydrofolate Reductase.
Biochemical and Clinical Aspects of Pteridines Vol. 4, de Gruyter (Berlin) 85-89.

Jellinger K., Reiderer P. (1984).
Dementia in Parkinson's Disease and (Pre) Senile Dementia of Alzheimer's Type: Morphological Aspects and Changes in the Intracerebral M.A.O. Activity.
Adv. in Neurology 40, 199-209.

Jequier E., Lovenberg W., Sjoerdoma A. (1967).
Tryptophan Hydroxylase Inhibition: The Mechanism by which p-Chlorophenylalanine Depletes Rat Brain Serotonin.
Molecular Pharmacol. 3, 274-278.

Jequier E., Robinson P.S., Lovenberg W., Sjoerdoma A. (1969).
Further Studies on Tryptophan Hydroxylase in Rat Brain Stem and Beef Pineal.
Biochem. Pharmacol. 18, 1071-1081.

Jervis G.A. (1947).
Studies on Phenylpyruvic Oligophrenia: the Position of the Metabolic Error.
J. Biol. Chem. 169, 651-656.

Joller P.W., Blau N., Atares M., Niederwieser A., Cardesa-Garcia J. (1983).
Guanosine Triphosphate Cyclohydrolase Deficiency: Analysis of the Influence on the Immune Parameters in a Girl.
Biochemical and Clinical Aspects of Pteridines Vol. 2, de Gruyter (Berlin) 167-176.

Jones S.A. (1987).
Personal Communication.

Katoh S., Sueoka, T., Yamada S. (1983).
Inhibition of Brain Sepiapterin Reductase by a Catecholamine and an Indoleamine.
Chemistry and Biology of Pteridines. de Gruyter (Berlin) Ed. Blair J.A. 789-793

Kaufman S. (1967).
Metabolism of the Phenylalanine Hydroxylase Cofactor.
J. Biol. Chem. 242, 3934-3943.

Kaufman S. (1963).

The Structure of the Phenylalanine Hydroxylase Cofactor.
Proc. Natl. Acad. Sci. 50, 1085-1092.

Kaufman S. (1964).
The Role of Pteridines in the Enzymatic Conversion of Phenylalanine to Tyrosine.
Trans. N.Y. Acad. Sci. Ser. II 26, 977-983.

Kaufman S. (1986).
The Metabolic Role of Tetrahydrobiopterin.
Chemistry and Biology of Pteridines de Gruyter (Berlin) 185-199.

Kaufman S. (1964).
Studies on the Structure of the Primary Oxidation Product Formed from Tetrahydropteridines During Phenylalanine Hydroxylation.
J. Biol. Chem. 239, 332-338.

Kaufman S. (1958).
A New Cofactor Required for the Enzymatic Conversion of Phenylalanine to Tyrosine.
J. Biol. Chem. 230, 931-939.

Kaufman S., Holtzman N., Milstien S., Butler J.J., Krumholz A. (1975).
Phenylketonuria Due to a Deficiency of Dihydropteridine Reductase.
New. Eng. J. Med. 293, 785-789.

Kaufman S. (1987) in:
Unconjugated Pterins in Neurobiology. Eds. Lovenberg W., Levine R.A.

Kehoe R.A. (1972).
Occupational Lead Poisoning.
J. Occup. Med. 14, 2298-300.

Kettler R., Bartholini G., Pletscher A. (1974).
In-vivo Enhancement of Tyrosine Hydroxylase in Rat Striatum by Tetrahydrobiopterin.
Nature 249, 476-477.

Kidd M. (1964).
Alzheimer's Disease - an Electron Microscopical Study.
Brain 87, 307-319.

Krishnan S.S., Crapper-McLachlan D.R. (1985).
Aluminium: Is it Toxic to the Human Brain?
Sci. Total Environ. 41, 203-205.

Kuntzman R., Shore P.A., Bogdanski D., Brodie B.B. (1961).
Microanalytical Procedures for Fluorometric Assay of Brain DOPA - 5HTP Decarboxylase, Norepinephrine and a Detailed Mapping of Decarboxylase Activity in Brain.
J. Neurochem. 6, 226-232.

Lai J.C.K., Guest J.F., Leung J.K.C., Lim L., Davison N. (1978).
The Effect of Cadmium, Manganese and Aluminium in Sodium - Potassium Activated and Magnesium Activated Adenosine Triphosphatase Activity and Choline Uptake in Rat Brain Synaptosomes.
Biochem. Pharmacol. 29, 141-146.

Langer S.Z. (1977). Presynaptic Receptors and Their Role in the Regulation of Transmitter Release. *Brit. J.Pharmacol. 60, 481-493.*

Lazarus R.A., De Brosse C.W., Berkovic S.J. (1982).
Structural Determination of Quinonoid Dihydropterins.
J. Am. Chem. Soc. 104, 6871-6872.

Lee E.H.Y., Mandell A.J. (1985).
Relationships between Drug Induced Changes in Tetrahydrobiopterin and Biogenic Amine Concentrations in Rat Brain.
J. Pharmac. Exp. Ther. Vol. 234, No. 141-146.

Leeming R.J., Blair J.A., Green A., Raine D.N. (1976).
Biopterin Derivatives in Normal and Phenylketonuric Patients after Oral Loads of L-Phenylalanine, L-Tyrosine and L-Tryptophan.
Arch. Dis. Child. 51, 771-777.

Leeming R.J., Blair J.A. (1974).
Crithidia factors in Human Urine.
Biochem. Med. 11. 122-128.

Leeming R.J., Blair J.A., Melikian V., O'Gorman D.J. (1976).
Biopterin Derivatives in Human Body Fluids and Tissues.
J. Clin. Path. 29, 444-451.

Leeming R.J., Blair J.A. (1980).
The Effect of Pathological and Physiological Processes on Biopterin Derivative Levels in Man.
Clin. Chim. Acta. 108, 103-111.

Leeming R.J., Blair J.A. (1980).
Serum Crithidia Levels in Disease.
Biochem. Med. 23, 122-125.

Leeming R.J., Blair J.A. (1979).
Dialysis Dementia, Aluminium and Tetrahydrobiopterin Metabolism.
Lancet i, 556.

Leeming R.J., Pheasant A.E., Blair J.A. (1981).
The Role of Tetrahydrobiopterin in Neurological Disease: A Review.
J. Ment. Defic. Res. 25, 231-241.

Lees A.J., Stern G.M. (1983).
Sustained Low Dose Levodopa Therapy in Parkinson's Disease: A three year follow-up.
Adv. Neurology, Vol.37, 9-15.

Lees A.J., Shaw K.M., Stern. G.M. (1975).
Bromocriptine in Parkinsonism.
The Lancet 709-710.

Levitt M., Spector S. (1964).
Elucidation of the Rate Limiting Step in Norepinephrine Biosynthesis in the Perfused Guinea Pig Heart.
J. Pharm. Exp. Ther. 148, 1-8.

Lewitt P.A., Neumann R.P., Miller I.P., Lovenberg W., Eldridge R. (1983).
Treatment of Dystonia with Tetrahydrobiopterin.
New. Eng. J. Med. 308, 157-158.

Lewitt P.A., Miller L.P., Levine R.A., Lovenberg W., Newman R.P., Papavasilou A., Rayes A., Eldridge R., Burns R.S. (1986).
Tetrahydrobiopterin in Dystonia: Identification of Abnormal Metabolism and Therapeutic Trials.
Neurology, 36, 760-764.

Lewitt P.A., Miller L.P., Newman R.P., BUrns R.S., Insel J., Levine R.A., Lovenberg W., Calne D.B. (1982).
Tyrosine Hydroxylase Cofactor (Tetrahydrobiopterin)in Parkinsonism.
Adv. Neurol. 40, 459-462.

Lewitt P.A., Burns R.S., Calne D.B. (1983).
Lisuride Treatment in Parkinson's Disease: Clinical and Pharmacokinetic Studies.
Adv. Neurol. 37, 131-140.

Libet B., Kobayashi H., Janaka J. (1975).
Synaptic Coupling into the Production and Storage of a Neuronal Memory Trace.
Nature 258, (5531) 155-157.

Lind K.E. (1972).
Dihydropteridine Reductase: Investigation of the Specificity for Quinonoid Dihydropteridine and the Inhibition by 2,4-Diaminopteridines.
Eur. J. Biochem. 25, 3, 560-562.

Lipcsey A., Peres A. (1984).
Clinical Administration of Parlodel in Low Doses.
Adv. Neurol. 40, 557-561.

Loo J.H., Whitaker V.P. (1967).
Pyridoxal Kinase in Brain and its Inhibition by Pyridoxylidene- ß-Phenylethylamine.
J. Neurochem. 14, 997-1011.

Loo J.H., Fulton J., Wisniewski H.M. (1978).
Vulnerability of the Immature Brain to Phenylacetate Intoxication: Tissue Permeability to Phenylacetate.
J. Neurochem. 32, 1697-1698.

Loo J.H., Fulton J., Wisniewski H.M. (1978).
Vulnerability of the Immature Brain to Phenylacetate Intoxication: Postnatal Development of the Detoxification Mechanism.
J. Neurochem. 32, 1699-1705.

Loo J.H., Scotto L., Horning M.G. (1972).
Aromatic Acid Metabilites of Phenylalanine in the Brain of the Hyperphenylalaninaemic Rat: Effect of Pyridoxamine.
J. Neurochem. 29, 411-415.

Lovenberg W., Levine R.A., Robinson D.S., Ebert M., William A.C., Calne D.B. (1979).
Hydroxylase Cofactor Activity in Cerebrospinal Fluid of Normal Subjects and Patients with Parkinson's Disease.
Science 204, 624-626.

Mackay A.V.P., Davies P., Dewar A.J., Yates C.M. (1978).
Regional Distribution of Enzymes Associated with Neurotransmission by Monoamines, Acetylcholine and GABA in the Human Brain.
J. Neurochem. (30) 4, 827-839.

Mager H.I.X., Berends W. (1965).
Hydroperoxides of Partially Reduced Quinonalines, Pteridines and (Iso) Alloxazines: Intermediates in Oxidation Processes.
Rel. Trans. Chim. Pays Bas. 84, 1329-1343.

Makulu D.R., Smith E.J., Bertino J.R. (1973).
Lack of Dihydrofolate Activity in Brain Tissue of Mammalian Species: Possible Implications.
J. Neurochem. 21, 241-245.

Malluche H.H., Smith A.J., Abreo K., Faugere M.C. (1984).
The Use of Deferoxamine in the Management of Aluminium Accumulation in Bone in Patients with Renal Failure.
New Eng. J. Med. 31, 140-144.

Mandell A.J., Russo P.V. (1981).
Short Term Regulation of Hydroxylase Cofactor Activity in Rat Brain.
J. Neurochem. 37, 1573-1578.

Martindale W. (1977).
The Extra Pharmacopoeia. 27th Edition.
Ed. Wade A.
London Pharmaceutical Press.

Matsubara M., Katoh S., Akino M., Kaufman S. (1966).
Sepiapterin Reductase.
Biochim. Biophys. Acta. 122, 202-212.

McDermott J.R., Smith A.Z., Iqbal K., Wisniewski H.M. (1979).
Brain Aluminium in Aging and Alzheimer's Disease.
Neurol. 29, 809-824.

McIlwain H., Bachelard S.H. (1985).
Biochemistry and the Central Nervous System.
5th Edition. Churchill Livingstone.
(Edinburgh, London, Melbourne, New York).

McGeer P.L., McGeer E. G. (1976).
Enzymes Associated with the Metabolism of Catecholamines, Acetylcholine and GABA in Human Controls and Patients with Parkinson's disease and Huntington's Chorea.
J. Neurochem. 6, 65-76.

McIntosh M.J., Meredith P.A., Goldberg A.A., Moore M.R. (1985).
Alteration of Dihydropteridine Reductase Activity by Lead.
Biochim. Soc. Trans. 610th Meeting Sterlin. 13, 375-376.

McIntosh M.J., Moore M.R., Blair J.A., Hilburn M.E., Leeming R.J. (1982).
Lead and Tetrahydrobiopterin Metabolism in Man and Animals.
Clin. Sci. Mol. Med. 63, 44.

McMartin K.E., Virayotha V., Tephly J.R. (1981).
High Pressure Liquid Chromatography Separation and Determination of Rat Liver Folates.
Arch. Biochem. Biophys. 209, 127-136.

Meyers J.H., Jawetz E., Goldfien A.
Review of Medical Pharmacology 7th Ed.

Lange 1980.

Milstien S., Kaufman S. (1985).
Biosynthesis of Tetrahydrobiopterin: Conversion of Dihydroneopterin Triphosphate to Tetrahydropterin Intermediates.
Biochem. Biophys. Res. Comm. 128, 3, 1099-1107.

Milstien S. (1983).
Tetrahydrobiopterin is Destroyed by Autoclaving at pH 4.5. A Comparison of Crithidia and HPLC Assays.
Biochemical and Clinical Aspects of Pteridines. Vol. 2, Ed H-Ch Curtius, Pfleiderer W., Wachter H. de Gruyter (Berlin) 65-70.

Milstien S., Kaufman S. (1983).
Tetrahydrosepiapterin is an Intermediate in Tetrahydrobiopterin Biosynthesis.
Biochem. Biophys. Res. Commun. 115, 888-893.

Milstien S., Kaufman S. (1983).
Dihydrofolate Reductase Catalyses the Reduction of 7,8-Dihydrobiopterin in Liver and Brain.
Biochemical and Clinical Aspects of Pteridines. Vol. 2. Ed H-Ch. Curtius, Pfleiderer W., Wachter H. de Gruyter (Berlin) 133-137.

Modak A.T., Weintraub S.T., Stavinoha W.B. (1975).
Effect of Chronic Ingestion of Lead on the Central Cholinergic System in Rat Brain Regions.
Toxicol. App. Pharmacol. 34, 340-347.

Morar C., Whitburn S.B., Blair J.A., Leeming R.J., Wilcock G.K. (1983).
Tetrahydrobiopterin Metabolism in Senile Dementia of the Alzheimer Type.
J. Neurol. Neurosurg. Psychiatry. 46, 582.

Musacchio J.M., D'Angelo G.L., McQueen C.A. (1971).
Dihydropteridine Reductase: Implication of the Regulation of Catecholamine Biosynthesis.
Proc. Nat. Acad. Sci. U.SA., 68, 9,, 2087-2091.

Mykkanen H.M., Dickerson J.W.J., Lancaster M.C. (1979).
Effect of Age on the Tissue Distribution of Lead in the Rat.
Toxicol. Appl. Pharmacol. 51, 447-454.

Nagatsu J., Mizutami K., Nagatsu J., Matsuura S., Sugimoto J. (1972).
Pteridines as cofactor or Inhibitors of Tyrosine Hydroxylase.
Biochem. Pharm. 21, 1945-1953.

Nagatsu J., Yamaguchi J., Rahman K., Troiewicz J., Olza K., Hirata Y., Nagatsu J., Narabayoshi H., Kondo J., Jzuka R. (1984).
Catecholamine Related Enzymes and the Biopterin Cofactor in Parkinson's Disease and Related Extrapyramidal Disorders.
Adv. Neurol. 40, 467-473.

Nagatsu R., Levitt M., Udenfriend S. (1964).
Tyrosine Hydroxylase.
J. Biol. Chem. 239, 2910-2917.

Nagatsu J., Yamagucchi J., Kato J., Sugimoto J., Matsuura S., Akino M., Isushimo S., Nakazaqwa N., Ogawa H. (1981).

Radioimmunoassay for Biopterin in Body Fluids and Tissues.
Anal. Biochem. 110, 182-189.

Niederwieser A., Blau N., Wong M., Joller P., Atares M., Condesa-Garcia J. (1984).
GTP -Cyclohydrolase Defiency: A New Enzyme Defect causing Hyperphenylalaninaemia with Neopterin, Biopterin, Dopamine and Serotonin Deficiencies and Muscular Hypotonia.
Eur. J. Pediatr. 141, 208-214.

Niederwieser A., Matasovic A., Kuster J.H., Staudenmann W., Pfleiderer W., Scheibenreiters S. (1986).
Catabolism of Tetrahydrobiopterin in Man.
Chemistry and Biology of Pteridines. de Gruyter (Berlin) 305-308.

Niederwieser A., Blau N., Standermann W., Wong M., Curtius H-Ch., Atares M., Condesa-Garcia J. (1983).
Neopterin Deficiency (GTP-Cyclohydrolase I Deficiency). A new variant of Hyperphenylalaninaemia.
Chemistry and Biology of Pteridines (Ed. Blair J.A.) de Gruyter (Berlin) 183-187.

Pabst W., Rembold H. (1966).
The Behaviour of Biopterins in the Mammalian Body: The Effect of Avitaminosis and of an Antagonist on the Excretion of Biopterin and the Growth of the Rat.
Hoppe-Seylers Z., Physiol. Chem. 344 (1/3) 107-112.

Patterson E.L., Broquist H.P., Albrecht A.M., Von Saltza M.H., Stockstad E.L.R. (1955).
A New Pteridine in Urine Required for the Growth of the Protozoon *Crithidia fasciculata*
J. Amer. Chem. Soc. 77, 3167-3168.

Perry E., Tomlinson G., Blessed G., Perry R., Cross A.,Crow J. (1981).
Noradrenergic and Cholinergic Sytstems in Senile Dementia of the Alzheimer Type.
Lancet i, 149.

Pfleiderer W. (1978).
Chemistry of Dihydropterins and Tetrahydropterins.
J. Inher. Metab. Dis. 1. 54-60.

Pfleiderer W. (1975).
Synthesis and Absolute Configuration of Sepiapterin.
Chemistry and Biology of Pteridines (Ed. Pfleiderer W.) de Gruyter (Berlin) 941-949.

Pollock R.J., Kaufman S. (1978).
Dihydropteridine Reductase may Function in Tetrahydrofolate Metabolism.
J. Neurochem. 42, 1499-1501.

Potempska A., Loo Y.H., Wisniewski H.M. (1984).
On the Possible Mechanism of Phenylacetate Neurotoxicity: Inhibition of Choline Acetyltransferase by Phenylacetyl-CoA. J. Neurochem. 42, 1499-1507.

Price P., Debono A., Parkes J.D., Massder C.D. (1978).
Plasma Bromocriptine Levels, Clinical and Growth Hormone Responses in Parkinsonism.
Br. J. Clin. Pharmac. 6, 303-309.

Purdy S.E., Blair J.A. (1980).
Rat Liver Dihydropteridine Reductase Inhibition.

Biochem. Soc. Trans. 8, 565-566.

Purdy S.E., Blair J.A., Leeming R.J. (1981).
Effect of Lead on Tetrahydrobiopterin Synthesis and Salvage: A cause of Neurological Dysfunction.
Intern. J. Environ. Studies. 17, 141-145.

Purrman R. (1940).
Die Synthese des Xanthopterins.
Liebigs. Am. Chem. 546, 98-102.

Rembold H., Buff K., Hennings G. (1977).
Specificity and Binding Capacity of Human Blood Serum for Tetrahydropterins.
Clinica Chimica Acta 76, 329-338.

Rembold H., Metzger H., Gutensohn W. (1971).
Catabolism of Pteridine Cofactors.
Biochem. Biophys. Acta. 230, 117-126.

Rembold H. (1983).
Catabolism of Pterin Pools.
Biochemical and Clinical Aspects of Pteridines Vol. 2, de Gruyter (Berlin, New York) 107-122.

Rey J., Harpey J.P., Leeming R.J., Blair J.A., Acardi J., Rey J. (1977).
Les Hyperphenylalaniemies avec Activitie Normale de la Phenylalanine Hydroxylase: Le Deficity en Tetrahydrobiopterin et la en Dihydropteridine Reductase.
Arch. Franc. Pediatrics. 34, CIX-CXX.

Rinne U.K. (1983).
Dopamine Agonists in the Treatment of Parkinson's Disease.
Adv. Neurol. 37, 141-149.

Rothler J., Karobath M. (1976).
Quantitative Determination of Unconjugated Pterins in Urine by Gas Chromatography/Mass Fragmentography.
Clin. Chim. Acta. 69, 457-462.

Sawada M., Horikoshi J., Masada M., Akino M., Sugimoto J., Matsuura S., Nagatsu J. (1986).
A Sensitive Assay of GTP-Cyclohydrolase I Activity in Rat and Human Tissues using Radioimmunoassay of Neopterin.
Anal. Biochem. 154, 361-366.

Segal D.S., Mandell A.J. (1974).
Long Term Administration of d-Amphetamine: Progressive Augmentation of Motor Activity and Stereotypy.
Pharmacol. Biochem. Behav. 2 249-255

Shen R.S., Smith R.V., Davis P.J., Brubaker A., Abell C.W. (1982).
Dopamine Derived Tetrahydroisoguinolines.
J. Biol. Chem. 254, 13, 7294-7297.

Shen R.S. (1984).

Potent Inhibitory Effects of Tyrosine Metabolites on Dihydropteridine Reductase from Human and Sheep Liver.
Biochim. Biophys. Acta. 785, 181-185.

Shen R.S. (1985).
Inhibition of Dihydropteridine Reductase in Rat Striatal Synaptosomes and from Human Liver by Metabolites of Biogenic Amines.
J. Enzyme Inhibition, 1, 61-66.

Spofforth J. (1921).
Case of Aluminium Poisoning.
Lancet i, 1301.

Stern G.M., Lees A.J. (1983).
Sustained Bromocriptine Therapy in 50 Previously Untreated Patients with Parkinson's Disease.
Adv. Neurol. 37, 17-21.

Stone K.J. (1976).
The Role of Tetrahydrofolate Dehydrogenase in the Hepatic Supply of Tetrahydrobiopterin in Rats.
Biochem. J. 157, 105-109.

Sueoka J., Katoh S. (1982).
Purification and Characterisation of Sepiapterin Reductase from Rat Erythroyctes.
Biochim. Biophys. Acta. 717, 265-271.

Switchenko A.C., Brown G.M. (1985).
The Enzymatic Conversion of Dihydroneopterin Triphosphate to Triphosphate and 6-pyruvoyl-Tetrahydropterin, an Intermediate in the Biosynthesis of other Pteridines in *Drosophila Melanogaster.*
J. Biol. Chem. 260, 5, 2945-2951.

Switchenko A.C., Primus J.P., Brown G.M. (1980).
Intermediates in the Enzymatic Synthesis of Tetrahydrobiopterin in *Drosophila Melanogaster.*
Biochem. Biophys. Res. Comm. 120, 3, 754-760.

Terry R.D., Pena C. (1965).
Experimental Production of Neurofibrillary Degeneration II Electron Microscopy, Phosphatase Histochemistry and Electron Probe Analysis.
J. Neuropath. Exp. Neurol. 1004-1010.

Tourian A., Sidbury J.B. (1983).
Phenylketonuria and Hyperphenylalaninaemia.
Ch. 12. 5th Edition The Metabolic Basis of Inherited Disease. Stanbury J.B., Wyngaarden J.B. Fredrickson D.S., Goldstein J.L., Brown M.S., (McGraw-Hill).

Trapp G.A., Miner G.D., Zimmerman R.L., Mastri A.R., Heston L.L. (1978).
Aluminium Levels in Brain in Alzheimer's Disease.
Biol. Psychiatr. 13, 709-718.

Udenfriend S., Cooper J.R. (1952).
The Eymatic Conversion of Phenylalanine to Tyrosine.
J. Biol. Chem. 194, 503-514.

Udenfriend S., Zaltzman-Nirenberg P., Nagatsu J. (1965).

Inhibitiors of Purified Beef Adrenal Tyrosine Hydroxylase.
Biochem. Pharmac. 14, 837-845.

Ward N. (1987).
Alzheimer's - the Saucepan Link.
Chemistry in Britain 307.

Wen G.Y., Wisniewski H.M., Shek J.W., Loo Y.H., Fulton J.R. (1980).
Neuropathology of Phenylacetate Poisoning in Rats: An Experimental Model of Phenylketonuria.
Ann. Neurol. 7, 557-566.

Wiener C.D., Collins M.A. (1978).
Tetrahydroisoquinolines Derived from Catecholamines on DOPA: Effects on Brain Tyrosine Hydroxylase.
Biochem. Pharmacol. 27, 2699-2703.

Williams A., Ballenger J., Levine R., Lovenberg W., Calne D. (1980).
Hydroxylase Cofactor Aging and CSF.
Neurol. 30, 1244-1246.

Williams A., Eldridge R., Levine R., Lovenberg W., Paulson G. (1979).
Low C.S.F. Hydroxylase Cofactor Levels in Inherited Dystonia.
Lancet ii, 410-411.

Williams A.C., Levine R.A., Calne J.N., Lovenberg W., Calne D.B. (1980).
C.S.F. Hydroxylase Cofactor Levels in some Neurological Diseases.
J. Neurol. Neurosurg. Psychiatr. 43, 735-738.

Wilson S.D., Horne D.W. (1984).
High Performance Liquid Chromatographic Determination of the Distribution of Naturally Occurring Folic Acid Derivatives in Rat Liver.
Anal. Biochem. 142, 529-535.

Wilson S.D., Horne D.W. (1986).
Effect of Nitrous Oxide Inactivation of Vitamin B_{12} on the Levels of Folate Coenzymes in Rat Bone Marrow, Kidney, Brain and Liver.
Arch. Biochem. Biophys. 244, 1, 248-252.

Wisniewski H.M., Sturman J.A., Shek J.W., Iqbal K. (1985).
Aluminium and the Central Nervous System.
J. Environ. Pathol. Toxicol and Oncology 6, 1, 1-8.

Woolf L.J. (1951).
Excretion of Conjugated Phenylacetic Acid in Phenylketonuria.
Biochem. J. 49, IX-X.

Wright C.E., Harding G.F.A., Owin A. (1984).
Presenile Dementia - the Use of the Flash and Pattern VEP in Diagnosis.
Clin. Neurophysiol. 57, 405-415.

Yamaguchi J., Nagatsu J., Sugimoto J., Matsuura S., Kondo J., Izuka R., Narabayashi M. (1981).
Effect of Tyrosine Administration on Serum Biopterin in Normal Controls and Patients with Parkinson's Disease.

Science 219, 75-77.

Yates C.M., Simpson J., Russell D., Gordon A. (1980).
Cholinergic Enzymes in Neurofibrillary Degeneration Produced by Aluminium.
Brain. Res. 197, 269-274.

Young J.H., Kelly B., Clayton B.E. (1982).
Reduced Levels of Biopterin and Dihydropteridine Reductase in Alzheimer Type Dementia.
J. Clin. Exp. Gerentol. 4, 389-402.