TETRAHYDROBIOPTERIN METABOLISM

IN

MENTAL DISORDERS

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TETRAHYDROBIOPTERIN METABOLISM IN MENTAL DISORDERS

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SUMMARY

Changes in DHPR activity in those aged 12 and under with a variety of mental disorders were investigated using dried blood spots on Guthrie cards. DHPR activity was found to be lowered in autism and Rett's syndrome. DHPR activity was unaffected in non specific mental retardation suggesting that the deficit seen in autism and Rett's syndrome does not arise secondary to the mental dysfunction. In Down's syndrome blood biopterin levels correlated with blood spot DHPR activity.

Human brain BH_4 synthetic activity was investigated in aging and senile dementia of the Alzheimer type (SDAT). BH_4 synthetic activity and DHPR activity decline with age in non-demented controls. In SDAT, decreases in BH_4 synthetic activity were seen in temporal and visual cortices and locus coeruleus. The site of the defect is probably at 6-pyruvoyl-tetrahydropterin synthase.

Aluminium inhibits human brain BH_4 synthesis *in vitro* and produces an 'Alzheimeresque' pattern of abnormalities in rats chronically exposed to the acetate salt in drinking water. Aluminium appears to chiefly affect enzymes requiring a metal ion cofactor. Aluminium induced inhibition of BH_4 synthesis can be reversed by treatment with transferrin, an aluminium chelator. Transferrin treatment improves BH_4 synthetic activity in SDAT brains whilst having no effect on controls, further implicating aluminium as the key neurotoxin in SDAT.

Lithium inhibits human brain BH_4 synthesis *in vitro* and lowers rat brain total biopterins and inhibits rat brain BH_4 synthesis on chronic exposure to the carbonate salt in drinking water. A possible mechanism for the anti-manic actions of lithium is suggested.

Monoamine oxidase inhibitors decrease human brain BH_4 synthetic activity *in vitro*. 5-methyl-tetrahydrofolate had no effect on human brain BH_4 synthesis *in vitro* but methionine increased BH_4 synthesis *in vitro*. Oxotremorine is a potent inhibitor of BH_4 synthesis in man and the rat. This may prove useful as a tool for modelling BH_4 deficiency.

Keywords

TETRAHYDROBIOPTERIN, SENILE DEMENTIA OF THE ALZHEIMER TYPE, ALUMINIUM, LITHIUM, OXOTREMORINE

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ABBREVIATIONS

A	Absorbance (optical density)
AAD	Aromatic amino acid decarboxylase
AC	Adenylate cyclase
AMP	5'-adenosine monophosphate
BBB	Blood Brain Barrier
BH_2	7,8-dihydrobiopterin
qBH_2	quinonoid dihydrobiopterin
BH_4	5,6,7,8-L *erythro* tetrahydrobiopterin
^oC	Degrees centigrade
cAMP	cyclic adenosine 3',5'-monophosphate
CAT	Choline acetyl transferase
5-MeTHF	5-methyl-tetrahydrofolate
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DHFR	Dihydrofolate reductase
DHNTP	D *erythro* dihydroneopterin triphosphate
DHPR	Dihydropteridine reductase
$DMPH_4$	6,7-dimethyl,5,6,7,8-tetrahydropterin
L-DOPA	L-3,4-dihydroxyphenylalanine
DS	Down's syndrome
EC	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
Emax	Extinction coefficient
GSH	Glutathione - reduced form
GSSG	Glutathione - oxidised form
HPA	Hyperphenylalaninaemia
HPLC	High performance liquid chromatography
Ki	Inhibitor constant
Km	Michaelis constant
MAO	Monoamine oxidase
MHPA	Malignant hyperphenylalaninaemia
n	sample size
NAD(H)	Nicotinamide adenine dinucleotide (reduced form)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced form)
N:B	ratio of neopterin:biopterin
NH_2	D *erythro* dihydroneopterin
NSMR	Non-specific mental retardation
ODS	Octadecyl silane
PDE	cyclic nucleotide phosphodiesterase
PKU	Phenylketonuria
RS	Rett's syndrome
SD	Standard deviation
SDAT	Senile Dementia of the Alzheimer Type
SEM	Standard error of the mean
TCA	Trichloracetic acid
Tris	Tris-(hydroxymethyl)-aminoethane
UV	Ultraviolet
V	Velocity of an enzyme catalysed reaction
Vmax	Maximum initial velocity of an enzyme catalysed reaction

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CHAPTER ONE

INTRODUCTION

1.1 STRUCTURE & CHEMISTRY OF TETRAHYDROBIOPTERIN

Tetrahydrobiopterin (BH_4)(fig. 1.1 no.1 p.34) is a member of a group of compounds known as pterins. Pterin (fig. 1.1 no.2 p.34) or 2-amino-4-oxo-pteridine is the simplest of these compounds (Commission on Biochemical Nomenclature, 1965). 7,8-dihydro- (fig. 1.1 no.3 p.34) and 5,6,7,8-tetrahydro (fig. 1.1 no.4 p.34) derivatives are formed by reduction of the pyrazine moiety of the pterin ring (Pfleiderer, 1978).

BH_4 and other pterins of biological interest are derived from substitution at C(6). Many such compounds have been identified and characterised (Angier *et al.*, 1946;Patterson *et al.*, 1955;Pfleiderer, 1978).

This investigation will concentrate mainly on the pterins involved in the metabolism of BH_4, 6-(1',2'-dihydroxy-propyl)-5,6,7,8-tetrahydropterin. BH_4 is present in a wide variety of mammalian tissues and body fluids (Leeming *et al.*, 1976; Fukushima & Nixon, 1980; Abou-Donia & Viverous, 1981; Katoh & Sueoka, 1986). BH_4 is measured in this report mainly as the fully oxidised form biopterin (fig. 1.2 no.2 p.35)

1.2 FUNCTIONS OF TETRAHYDROBIOPTERIN

BH_4 is the cofactor in the *in vivo* hydroxylation of phenylalanine to tyrosine, by phenylalanine hydroxylase (EC. 1.14.16.1)(fig. 1.3 p.36). This requires molecular oxygen and a reduced pyridine nucleotide (Undenfriend & Cooper, 1952; Kaufman, 1958).

Similarly, the hydroxylation of tyrosine. to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (EC.1.14.16.2) (Brenneman & Kaufman, 1964; Shiman *et al.*, 1971), the rate limiting step of catecholamine neurotransmitter biosynthesis (fig. 1.3 p.36) (Levitt *et al.*, 1965) and the formation of 5-hydroxytryptophan (5-HTP) from tryptophan by tryptophan hydroxylase (EC.1.14.16.4) (Jequier *et al.*, 1969; Friedman *et al.*, 1972) in the synthesis of serotonin (5-HT)(Costa & Meek, 1974)(fig. 1.4 p.37) are BH_4 dependent reactions.

Thus the presence of BH_4 is necessary for maintenance of catecholamine and indoleamine neurotransmitter levels in the CNS (Leeming *et al.*, 1981; Lovenberg & Levine, 1987).

Tyrosine hydroxylase has different affinities for BH_4. Low affinity tyrosine hydroxylase has a Km of 100-600um, whilst high affinity tyrosine hydroxylase has a Km of

10-30um with respect to BH_4 (Levine *et al.*, 1981; Vulliet *et al.*, 1980). Levels of BH_4 within catecholaminergic neurones may be subsaturating for the low affinity form of tyrosine hydroxylase. This may also be the case for the high affinity form. Intracellular BH_4 levels in adrenal medulla (10-20μM),cultured chromaffin cells (4μM) and rat striatum (100μM) are below the Km of the low affinity enzyme (Abou-Donia & Viverous, 1981; Levine *et al.*, 1981; Abou-Donia *et al*, 1986).

In vivo administration of BH_4 to rat striatum and to cultured catecholaminergic neurones and rat striatum synaptosomes, enhances tyrosine hydroxylase activity and subsequent synthesis of catecholamine neurotransmitters. This occurs due to activation of the low affinity form of tyrosine hydroxylase, implying that tyrosine hydroxylase activity is mediated by cofactor availability. Numerous stimuli, both pharmacological and physiological, convert tyrosine hydroxylase from the low affinity form to the high affinity form. Ca^{2+} activates tyrosine hydroxylase *in vitro* at concentrations similar to those that occur in the cell during membrane depolarisation (Iuvone, 1984). cAMP mediated protein kinase phosphorylation activates tyrosine hydroxylase (Lovenberg *et al.*, 1975).

1.3 MODE OF ACTION OF TETRAHYDROBIOPTERIN

During hydroxylation BH_4 is converted to quinonoid dihydrobiopterin (qBH_2)(fig. 1.5 no.2 p.38). qBH_2 is converted to BH_4 by the salvage enzyme, dihydropteridine reductase (DHPR) (EC. 1.6.99.7) utilising NADH as a reductant (Kaufman, 1961; Kaufman, 1964; Craine *et al.*, 1972)(fig. 1.5 p.38).

qBH_2 is unstable and if not salvaged by DHPR will rearrange to 7,8-dihydrobiopterin (BH_2)(fig. 1.6 no.5 p.39) or can be oxidised forming 7,8-dihydropterin (PH_2)(fig. 1.1 no.3 p.34). These are lost to the plasma and are subsequently excreted in the urine (Archer & Scrimgeour,1970; Leeming *et al.*, 1976; Matsuura *et al.*, 1986).

DHPR is substrate specific for the quinonoid form of dihydrobiopterin however dihydrofolate reductase (DHFR) (EC. 1.5.1.3) will reduce BH_2 to BH_4 . In tissues with high DHFR activity this may act as a salvage pathway for BH_4 (Kaufman, 1967; Nichol *et al.*, 1983).

Though the exact mechanism by which BH_4 is involved in aromatic amino acid hydroxylation is unclear, Kaufman (1967) proposed that a hydroperoxide of BH_4, (4a-hydroperoxy-tetrahydrobiopterin: fig. 1.6 no.2 p.39) is formed by covalent addition at position 4a of

dioxygen. This decomposes, forming a 4a-carbinolamine (fig. 1.6 no.3 p.39) and hydroxide cation (OH+), the latter acting as the hydroxylation agent (Pfleiderer, 1978). Indeed studies on the hydroxylation of phenylalanine have shown the presence of a 4a-carbinolamine intermediate. This dehydrates to form qBH_2 (fig. 1.6 no.4 p.39), catalysed by phenylalanine hydroxylase stimulating protein (Lazarus *et al.*, 1981; Lazarus *et al.*, 1983).

Tyrosine hydroxylase is a metalloenzyme, containing iron. Iron is essential for activity (Fisher *et al.*, 1972). It has been suggested that an iron-oxygen intermediate is generated, and BH_4 acts as a 2-electron reductant, forming an iron-peroxide, which then acts as the hydroxylating agent (Pearson, 1974a & b).

1.4 TETRAHYDROBIOPTERIN BIOSYNTHESIS

BH_4 is poorly absorbed from the intestine and though it is salvaged after use as a cofactor by DHPR, BH_4 levels in tissue are maintained chiefly by *de novo* synthesis, as indicated by Pabst & Rembold (1966) who showed that rats fed a BH_4 deficient diet exhibit normal development and maintain urinary biopterin excretion. Early work on bacterial pteridine biosynthesis indicated that pterins were derived *in vivo* from a phosphorylated purine precursor (Vieira *et al.*, 1961; Krumdieck *et al.*, 1966;).

Burg and Brown (1968) demonstrated that the first step in the synthesis of BH_4 is the conversion of guanosine triphosphate (GTP)(fig. 1.7 no.1 p.40) to D *erythro* - dihydroneopterin triphosphate (DHNTP) by GTP-cyclohydrolase I (GTPCH-I) (EC. 3.5.4.11) proceeding via cleavage at C(8) of the imidazole ring forming 2-amino-6(triphosphoribosyl)-amino-5 or 6-formamido-4-hydroxypyramidine ($FPydP_3$) and formic acid followed by an Amadori rearrangement and ring closure (fig. 1.7 p.40). No intermediates have been isolated and there are no cofactors required. GTP cyclohydrolase consists of three subunits A_1, A_2 and A_3. The A_2 subunit displays 'neopterin synthetase' activity. GTPCH-I is heat stable. DHNTP is generally detected in samples as the fully oxidised form, neopterin (fig.1.2 no.2 p.35). BH_4 synthesis from DHNTP proceeds via tetrahydropterin intermediates (Milstein & Kaufman, 1983; Heintel *et al.*, 1984; Switchenko & Brown, 1985; Milstein & Kaufman, 1986). An intramolecular electron rearrangement in the N(5) area of the pyrazine ring, coupled with a dephosphorylation gives 6-pyruvoyl-tetrahydropterin (6-PTP)(fig. 1.8 no.2 p.41). This is catalysed by a magnesium dependent enzyme, 6-pyruvoyl-tetrahydropterin synthase (6-PTPS) or phosphate eliminating enzyme (PEE). 6-PTP undergoes a reduction of the side chain keto groups by NADPH dependent sepiapterin reductase and a 2' keto reductase forming L *erythro* tetrahydrobiopterin (BH_4).

1.5 REGULATION AND CONTROL OF TETRAHYDROBIOPTERIN BIOSYNTHESIS

In contrast to the detailed study of the regulation of neurotransmitter biosynthesis, very little work has been done on the control and regulation of BH_4 synthesis. It is thought that the control of BH_4 synthesis displays interspecies variation.

Neopterin (fig. 1.2 no.2 p.35), the fully oxidised form of DHNTP is found in the tissues of primates in similar quantities to biopterin (Fukushima & Nixon, 1980; Duch *et al.*, 1984). Using HPLC, neopterin cannot be detected in the body fluids and tissues of the lower mammals, though using a very sensitive radioimmunoassay (RIA) technique, very low levels have been found in rat tissues (Nagatsu *et al.*, 1984). GTP cyclohydrolase I is the rate limiting step of BH_4 synthesis in the rat. In humans and other primates GTPCH-I activity is much lower than that in the rat (Sawada *et al.*, 1986). It is thought that in man and other primates, 6-PTPS may be the rate limiting step of BH_4 synthesis (Blau & Niederwieser, 1986; Duch *et al.*, 1984a). BH_4 exerts negative feedback control on GTPCH-I *in vitro* (Bellahsene *et al.*, 1984) and in murine neuroblastoma cell culture (Kapatos & Kaufman, 1983).

In adrenal medulla and cortex *in vivo* and in adrenomedullary chromaffin cell cultures, reserpine-induced cellular catecholamine depletion, increases GTPCH-I activity by enzyme induction, leading to raised BH_4 levels. *In vivo*, insulin-induced hypoglycaemia has a similar effect (Abou-Donia *et al*.,1981 & 1986; Viveros *et al*., 1981). There is regional variation of the control of GTPCH-I across the adrenal gland. In the medulla, splanchnic nerve discharge stimulates GTPCH-I synthesis.

Release of adrenocorticotrophic hormone (ACTH) by the pituitary gland stimulates cortical GTPCH-I synthesis. In addition to this, there may be a cyclic adenosine monophosphate (cAMP) dependent mechanism by which GTPCH-I activity and BH_4 levels are increased in response to demand (Abou-Donia *et al*., 1981).

In the CNS, BH_4 synthesis does not appear to be under hormonal control, in contrast to that in the adrenal gland (Duch *et al*., 1986). Presynaptic receptor stimulation on dopaminergic neurones increases tyrosine hydroxylase and dopamine synthesis in those neurones, however there is no effect on BH_4 levels (Galloway & Levine 1986).

Brain sepiapterin reductase is competitively inhibited *in vitro* by noradrenaline and serotonin (Katoh *et*

al., 1986). This would indicate that both BH_4 and neurotransmitter biosynthetic pathways are co-regulated.

1.6 TETRAHYDROBIOPTERIN CATABOLISM

In addition to regulation of *de novo* synthesis, BH_4 levels are maintained by catabolism and excretion by the kidney (Rembold, 1983). In rat liver preparations, BH_4 is catabolised (fig. 1.9) forming pterin (5), 7,8-dihydroxanthopterin (4), isoxanthopterin (6), lumazine (7) and 6-hydroxylumazine (10) and 7-hydroxylumazine (8) (Rembold *et al.*, 1969). BH_4 is oxidised to BH_2 and PH_2 (1). PH_2 is converted to 7,8-dihydroxanthopterin (4) by hydration followed by further oxidation. Isoxanthopterin (6) is formed from PH_2 via xanthine oxidase (EC. 1.1.3.22) following oxidation to pterin (5). Further action of a pterin deaminase (EC.3.5.4.11) leads to the formation of lumazine (7) and hydroxylumazine (8&10) derivatives. (Rembold and Gutensohn, 1968; Rembold *et al.*, 1969a; Rembold & Simmersbach, 1969; Rembold *et al.*, 1971). In man pterin deaminase has not been found but deamination of BH_4 given orally is catalysed by gut bacterial flora. The major deamination catabolite in man is 2'-deoxy-sepialumazine, in this the BH_4 side chain is retained but modified (Niederwieser *et al.*, 1986).

1.7 TETRAHYDROBIOPTERIN METABOLISM IN METABOLIC DISEASE

Gross deficiency of BH_4 causes hyperphenylalaninaemia and massive neurological impairment and if left untreated, the individual fails to meet developmental milestones and death occurs in infancy (Leeming *et al.*, 1981; Kaufman, 1987). Such a deficiency can arise as a result of a defect in either the *de novo* synthetic pathway or in the salvage pathway i.e. DHPR deficiency. These conditions are very rare, representing one in a million live births, usually being diagnosed within the first three months of life. They are known as the malignant hyperphenylalaninaemias (MHPA) (Danks, 1978; Danks *et al.*, 1978; Dhondt *et al.*, 1981; Hamon & Blair,1987).

DHPR deficiency is characterised by elevated urine biopterins and normal neopterin levels and arises from failure to synthesise the enzyme or from the synthesis of a mutant form of the enzyme which has no catalytic activity (Firgaira *et al.*, 1983). BH_4 synthesis deficiency can arise due to a defect at either GTPCH-I, usually indicated by lowered levels of both biopterin and neopterin in urine (Niederwieser *et al.*, 1984) or at 6-PTPS where urinary levels of biopterin are lowered and levels of neopterin are markedly raised (Niederwieser *et al.*, 1985). Patients with MHPA show greatly reduced levels of biogenic amines in CSF and urinary excretion of their metabolites is also decreased markedly (Brewster *et al.*, 1979, Niederwieser *et al.*, 1984).

MHPA is routinely screened for by the analysis of erythrocyte DHPR activity, and blood biopterin concentration in dried blood spots on Guthrie cards (Leeming *et al.*, 1987). It is treated by giving a low phenylalanine diet, neurotransmitter precursors in conjunction with a peripheral dopa decarboxylase inhibitor e.g. carbidopa. In some cases oral administration of 6-methyl-tetrahydropterin has been tried with some success in a few individuals (Lovenberg and Levine, 1987)

1.8 TETRAHYDROBIOPTERIN METABOLISM IN SENILE DEMENTIA OF THE ALZHEIMER TYPE (SDAT)

The involvement of BH_4 metabolism in various neurological disorders has been investigated. Tissue and body fluid levels of BH_4 derivatives have been measured in these disorders (Table 1.1 & 1.2). Senile dementia of the Alzheimer type (SDAT) is a neurological disorder occuring in the later years, typically around 60 and beyond. It is characterised by a progressive loss of cognitive and intellectual function, usually commencing with the failure of short term memory, leading to general failure of memory and lack of spatial awareness (Reisberg 1983). Neuropathological changes include characteristic amyloid deposits known as senile plaques and neurofibrillary tangles (Reisberg, 1983; Review:British Medical Bulletin, 1986). The initial event is protein precipitation

followed by plaque generation with tangle formation occurring later. The plaque core consists of a colocalized mass of aluminium and silicon probably as the aluminosilicate (Candy *et al.*, 1986).

In SDAT, serum and CSF levels of total biopterins are decreased (Leeming *et al.*, 1979; Young *et al.*, 1982; Aziz *et al.*, 1983; Morar *et al.*, 1983).

Biopterin levels in brain tissue exhibit regional differences (Bullard *et al.*, 1978, Leeming *et al.*, 1976, Fukushima and Nixon 1980, Nagatsu *et al.*, 1986), similarly, the neuropathological alterations seen in SDAT are not generalised but are confined to certain areas, for example the temporal cortex (Brodmann 20/21) is severely affected by plaques and tangles as is the hippocampus but the frontal cortex is relatively spared (Brun, 1983). In SDAT subjects CSF, the neopterin:biopterin (N:B) ratio is elevated compared to controls indicating failure to synthesise BH_4 from DHNTP. A similar elevation was shown by Barford *et al* (1984), in temporal cortex. They also reported a diminished BH_4 synthetic capacity in temporal cortex.

Biopterin levels in both the locus coeruleus, a known noradrenergic nucleus, and the substantia nigra, a dopaminergic centre, are decreased in SDAT (Nagatsu *et al.*, 1986).

GTPCH-I sepiapterin reductase and DHPR activities are not impaired in SDAT. This in conjunction with the elevation of N:B ratio in CSF and temporal cortex would suggest that the defect lies at the second stage of BH_4 synthesis, the conversion of DHNTP to 6-PTP by 6-pyruvoyl-tetrahydropterin synthase.

Aluminium is a neurotoxin. Subjects on chronic renal dialysis develop a encephalopathy known as dialysis dementia. These individuals have a very high tissue burden of aluminium (Sideman and Manor 1982), arising as a result of aluminium containing dialysates, and the use of aluminium hydroxide gel orally as a phosphate binder. Initially the syndrome is reversible, but on repeated continuous aluminium exposure becomes permanent, with a high mortality (Sideman & Manor, 1982).

In SDAT there are raised levels of aluminium in the brain (Candy *et al.*, 1986; Ward *et al.*, 1986). Using sensitive electron probes it has been shown the senile plaque core consists mainly of aluminosilicate. Aluminium inhibits DHPR both *in vivo* and *in vitro* (Leeming & Blair, 1979; Brown, 1981; Dhondt & Bellahsene, 1983; Cutler, 1986). Patients on maintenance dialysis display elevated serum neopterin and biopterin levels as well as an increased N:B ratio (Dhondt *et al.*, 1982)

1.9 AIM OF THESIS

BH_4 metabolism is disturbed in SDAT and other mental disorders (Hamon & Blair, 1986; Anderson, 1987; Jones, 1988). DHPR activity is decreased in a variety of neurological disorders (Sahota *et al.*, 1985 & 1986; Leeming *et al.*, 1987). This thesis aims to elucidate further the mechanism of the BH_4 synthesis defect in the temporal lobe of SDAT subjects, and to investigate any role of aluminium in causing such a defect, by utilisation of *in vivo* animal models and *in vitro* investigations with human tissue preparations. Further, it aims to determine the effect of aluminium on metalloenzymes involved in neurotransmitter metabolism in addition to the effects of other metal ions on such systems. It was the aim of this thesis to clarify the role of DHPR in mental disorders and investigate the effects of psychoactive drugs on BH_4 metabolism.

Table 1.1 BH_4 metabolism in neurological dysfunction

Disorder	Tissue	Observation	Reference
Down's syndrome	serum	$+BH_2$	Aziz *et al* 1982
		=DHPR	Barford *et al* 1983
Huntington's disease	CSF	$-BH_4$	Williams *et al* 1980
Parkinson's Disease	CSF	$-BH_4$	Lovenberg *et al* 1979
	CSF	$-BH_4$	Williams *et al* 1980
	CN	-GTPCH-I	Sawada *et al* 1986
Unipolar depression	urine	+N,+B	Duch *et al* 1984
	urine	=B	Blair *et al* 1984
Bipolar Depression	urine	-B	Blair *et al* 1984
	urine	=B	Duch *et al* 1984
Depression	urine	+B	Garbutt *et al* 1985
	TL	$-BH_4$ synthesis	Blair *et al* 1984

Abbreviations:- '+'increase '-'decrease '=' normal

CN:- caudate nucleus; TL:- temporal lobe;

B:-total biopterins; N:-total neopterins;

CSF:- cerebrospinal fluid

Table 1.2 BH_4 metabolism in SDAT

Tissue	Observation	Reference
serum	-biopterin	Leeming & Blair 1980
serum	-biopterin	Aziz *et al* 1983
plasma	-biopterin	Young *et al* 1982
	=neopterin	" "
lymphocyte	-DHPR	" "
erythrocyte	=DHPR	Jeeps *et al* 1986
CSF	=neopterin	Morar *et al* 1983
	-biopterin	" "
	-biopterin	LeWitt *et al* 1985
	=neopterin	" "
temporal lobe	-BH_4 synthesis	Barford *et al* 1984
	+N:B ratio	" "
	=DHPR	" "
LC	-biopterin	Nagatsu *et al* 1986
	-BH_4 synthesis	Anderson *et al* 1986
SN	-biopterin	" "

Abbreviations:

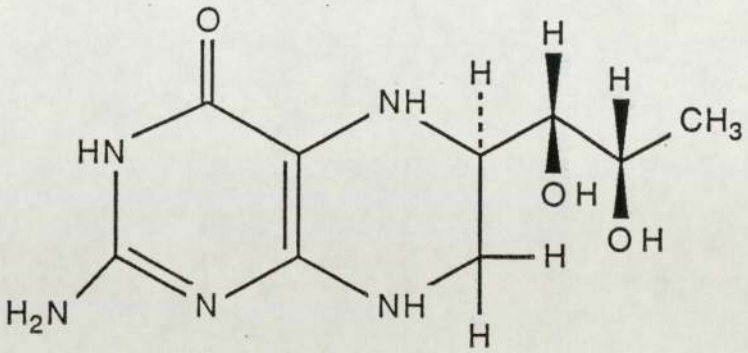
'+' increase '-' decrease '=' normal

LC:- locus coeruleus

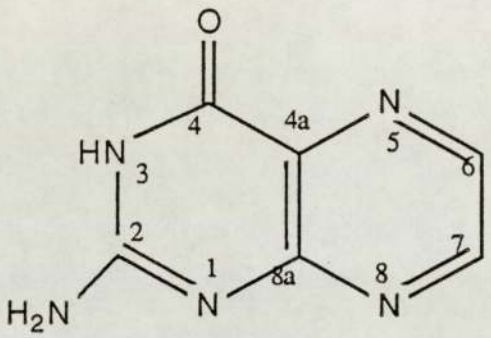
SN:- substantia nigra

biopterin and neopterin refer to total oxidised pterin

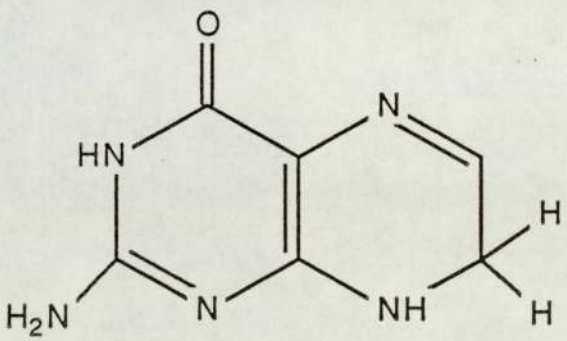
Fig. 1.1 Tetrahydrobiopterin and Other Pterins



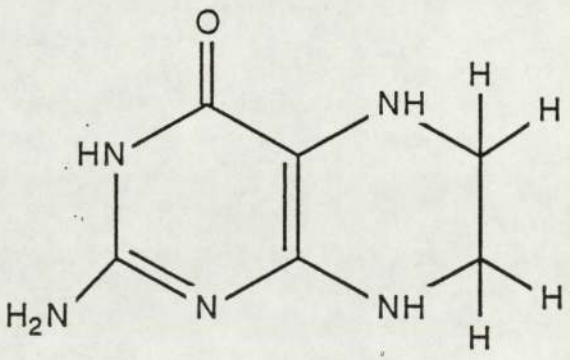
1 L-erythro-tetrahydrobiopterin



2 Pterin

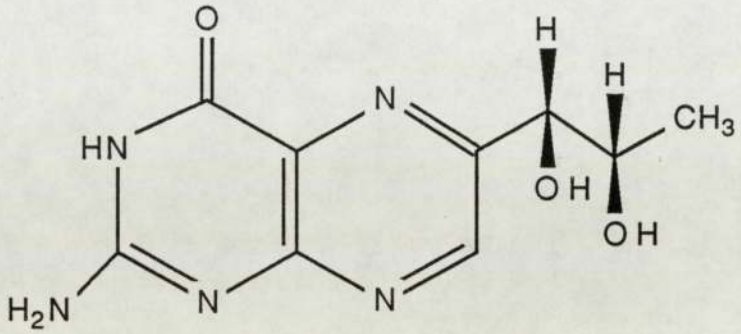


3 7,8-dihydropterin

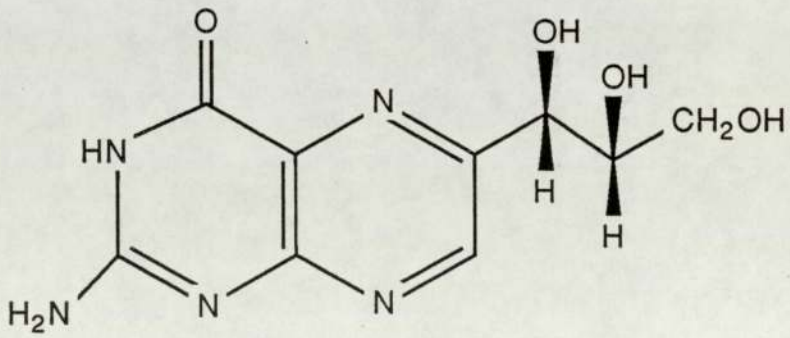


4 5,6,7,8-tetrahydropterin

Fig. 1.2 Biopterin and neopterin



1 Biopterin



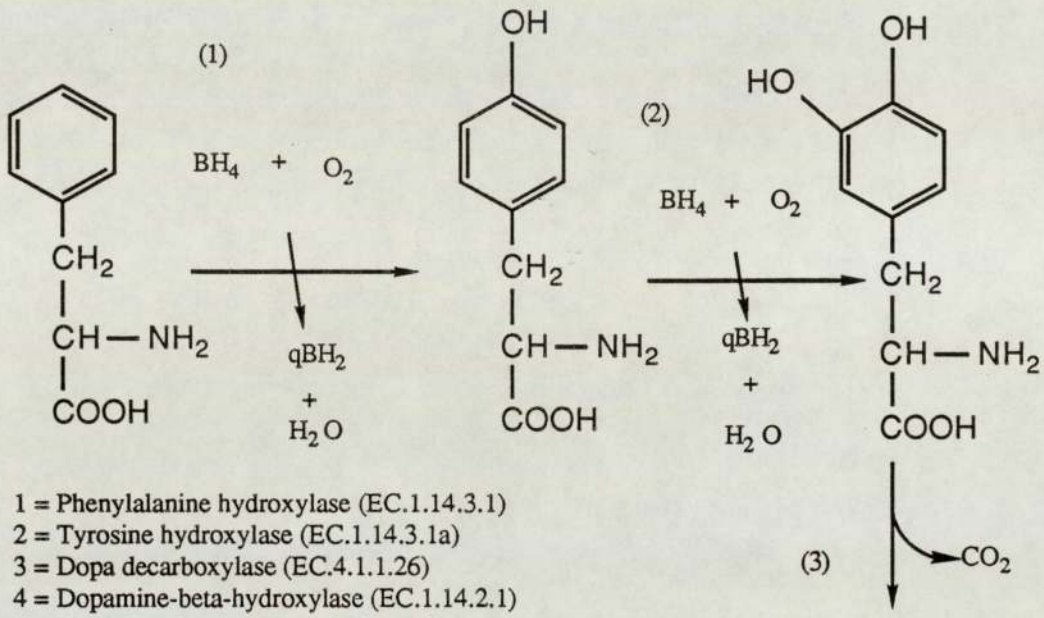
2 Neopterin

Fig. 1.3 Catecholamine Biosynthesis and Tyramine

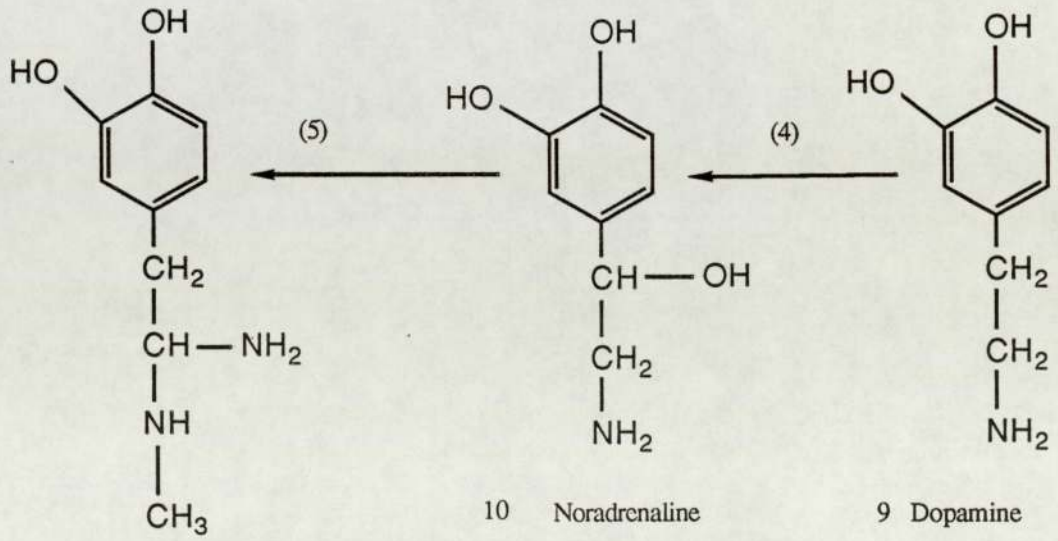
6 Phenylalanine

7 Tyrosine

8 L-Dopa



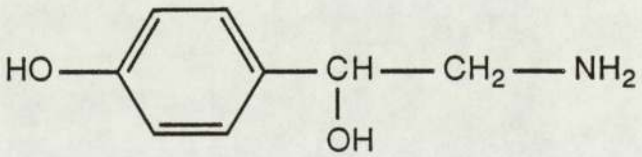
1 = Phenylalanine hydroxylase (EC.1.14.3.1)
2 = Tyrosine hydroxylase (EC.1.14.3.1a)
3 = Dopa decarboxylase (EC.4.1.1.26)
4 = Dopamine-beta-hydroxylase (EC.1.14.2.1)
5 = Phenethanolamine-N-methyl-transferase



10 Noradrenaline

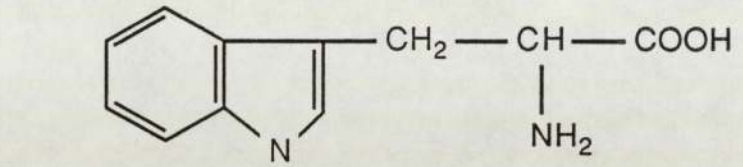
9 Dopamine

11 Adrenaline



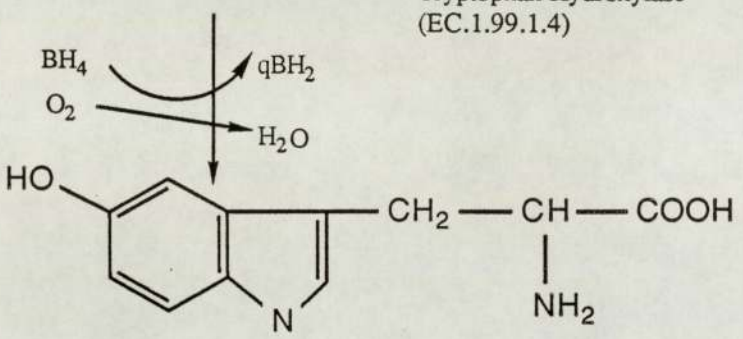
12 Tyramine

Fig. 1.4 Serotonin Metabolism



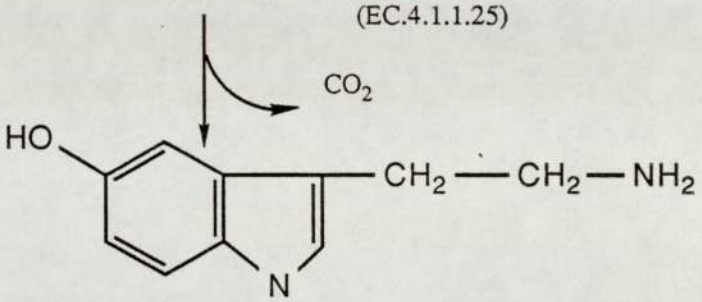
1 · L-Tryptophan

Tryptophan Hydroxylase
(EC.1.99.1.4)



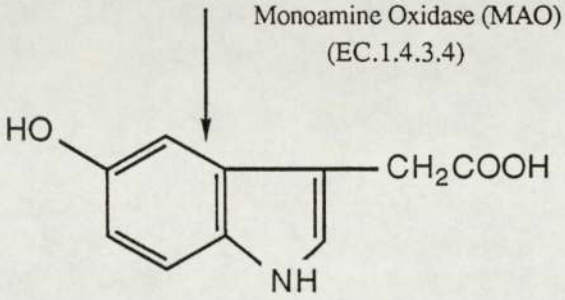
2 5-Hydroxytryptophan

Aromatic L-amino acid decarboxylase
(EC.4.1.1.25)



3 5-Hydroxytryptamine
(Serotonin)

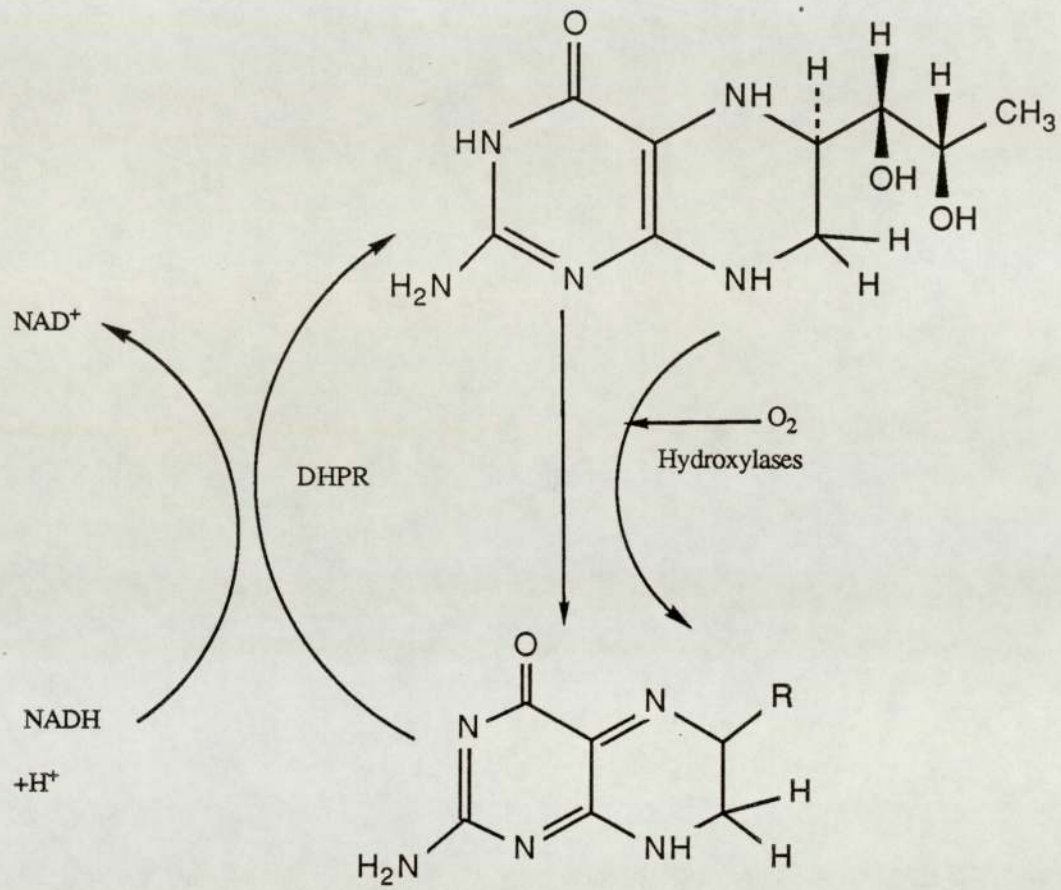
Monoamine Oxidase (MAO) + Aldehyde Dehydrogenase
(EC.1.4.3.4) (EC.1.2.1.3)



4 5-Hydroxyindoleacetic acid (5-HIAA)

Fig. 1.5 DHPR & BH_4 Catabolism

1 L-erythro-tetrahydrobiopterin



2 Quinonoid dihydrobiopterin

Fig. 1.6 Mechanism of cofactor function

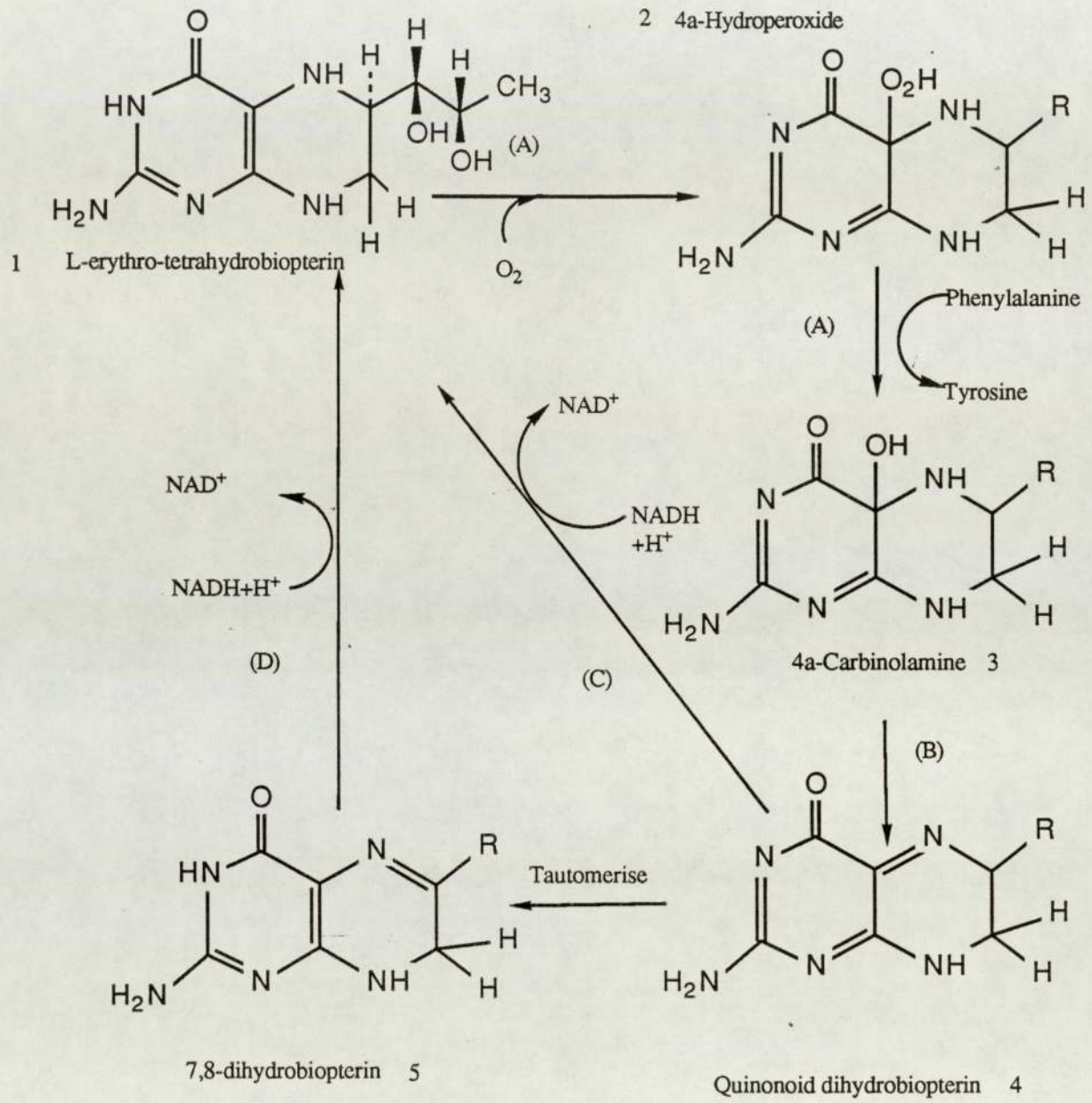


Fig. 1.7 Biosynthesis of DHNTP from GTP

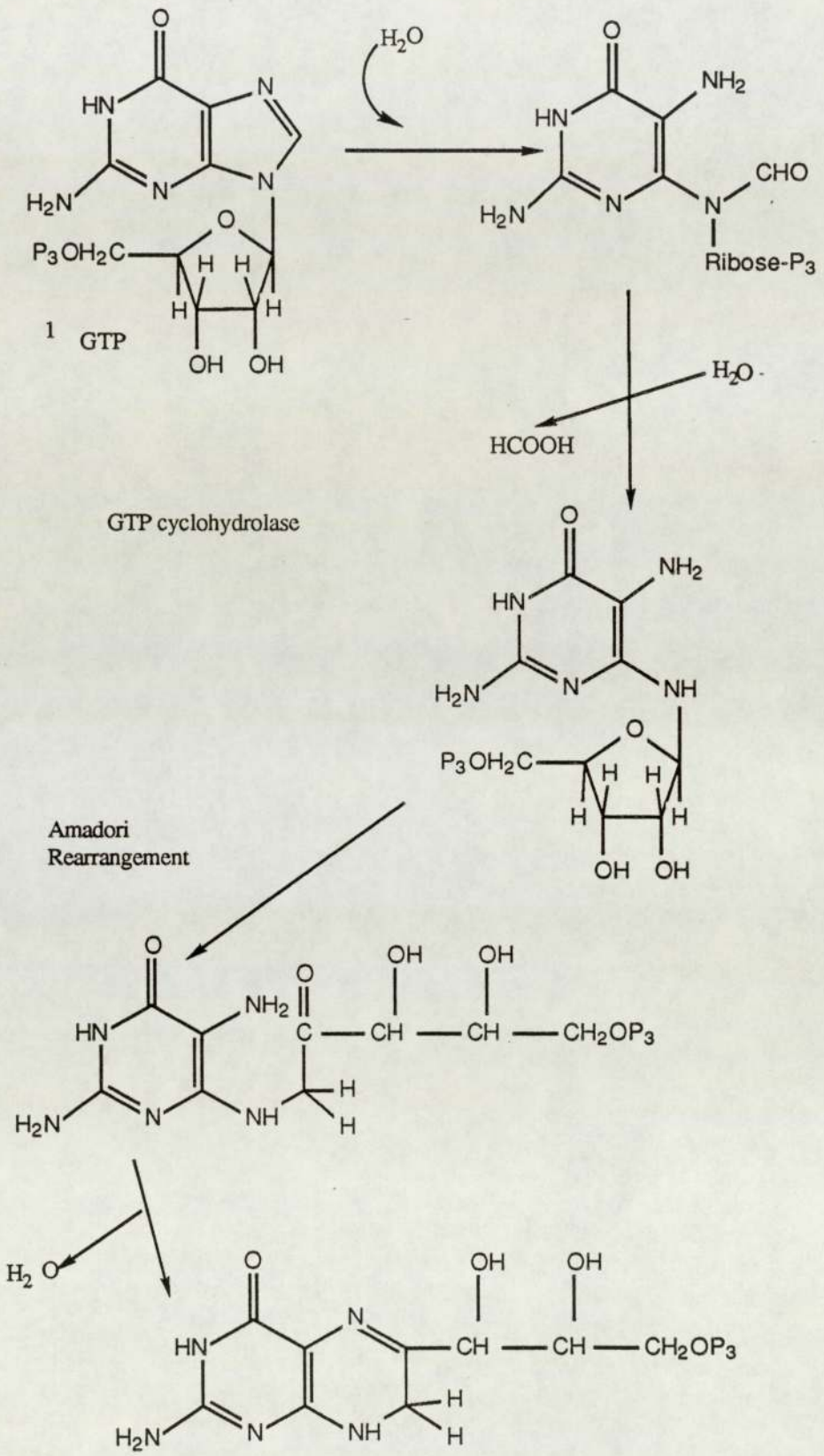
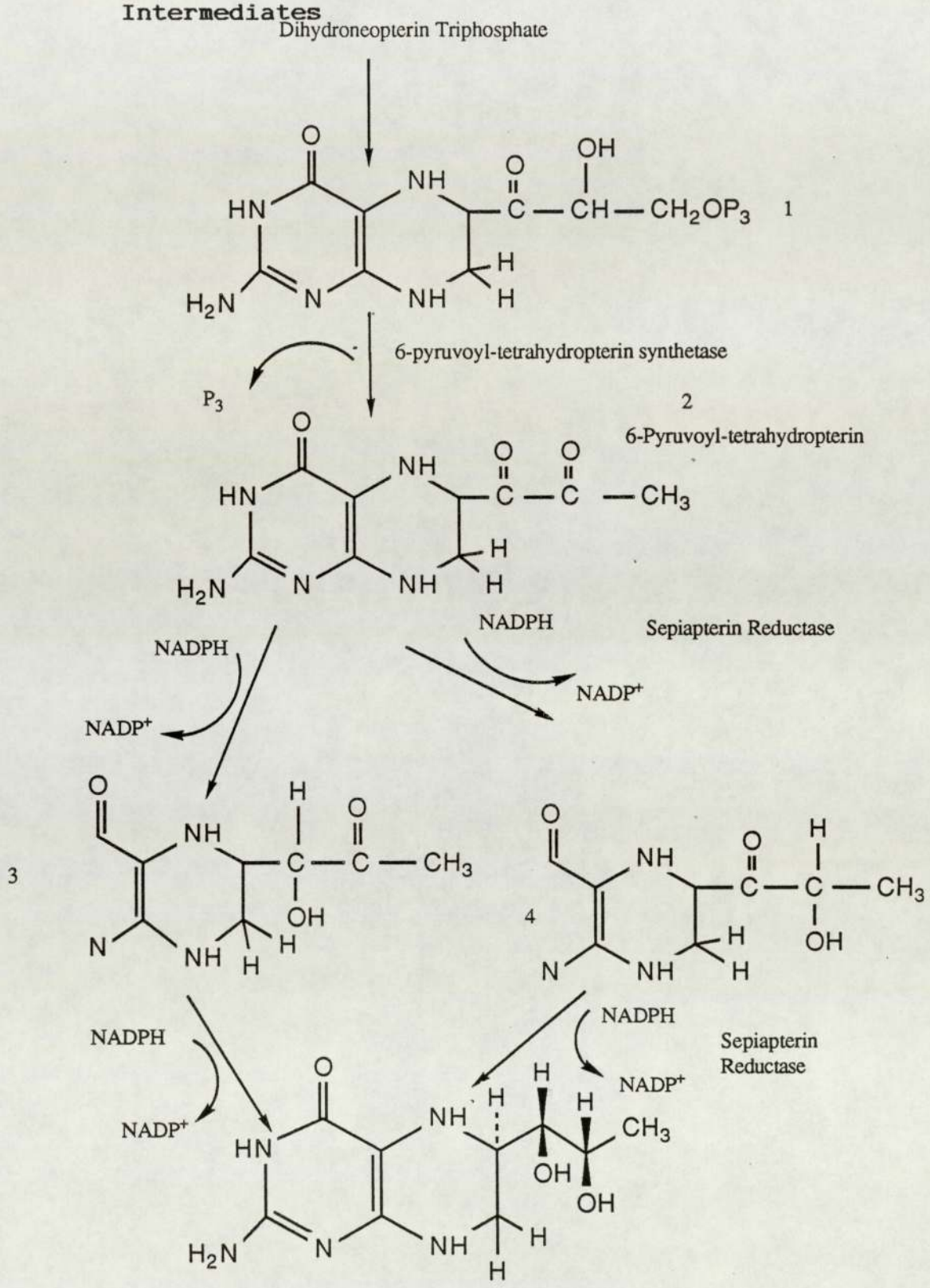
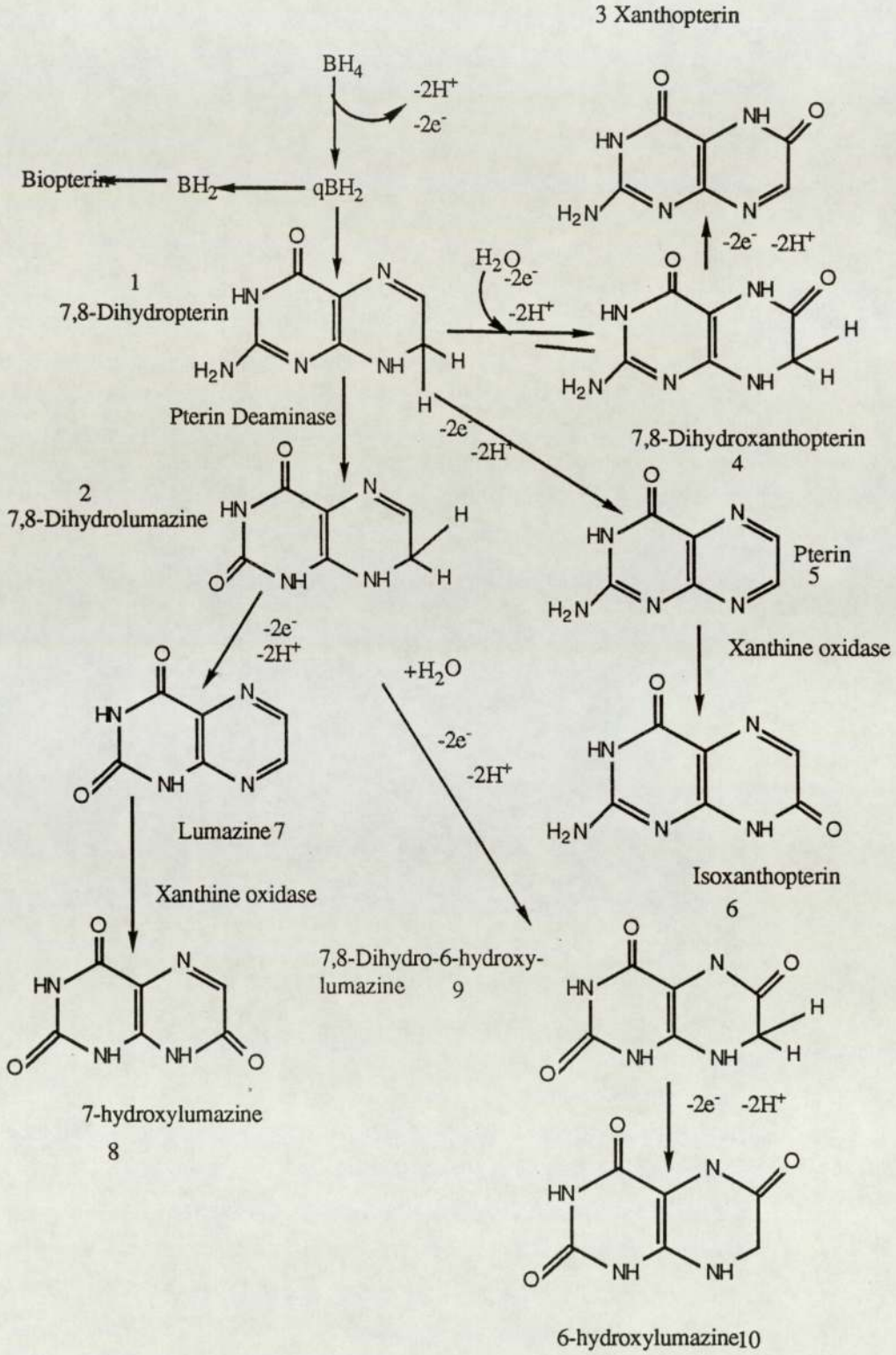


Fig. 1.8 BH_4 Biosynthesis from DHNTP via Tetrahydropterin Intermediates



5 L-erythro-tetrahydrobiopterin

Fig 1.9 Catabolism of BH_4 in the Rat



CHAPTER TWO

MATERIALS

AND

METHODS

2.1 ANIMALS

All animals used were male Wistar rats (grade 4) supplied by Bantin & Kingman Ltd, Grimston, Hull, England (weaners & 200 g). Animals were maintained on Pilsbury's rat and mouse breeding diet (table 2.1), and tap water *ad libitum*. Rats were housed in light controlled conditions with a 12:12 hour dark:light cycle at 26°C. Experimental groups were in supplier's batches and compared to age and sex matched controls.

Intragastric (I.G.) dosing was performed using a sterile hypodermic syringe fitted with an olive bulbed dosing needle. Oral dosing was by dissolving or suspending the compound of interest in the drinking water, the solution being available *ad libitum* .

Animals were killed by stunning and cervical dislocation, brain and liver were excised onto ice and stored at -70°C until preparation and analysis. Blood was removed by opening the thoracic cavity, and collected into a heparinised syringe, and stored in polythene vials until preparation and analysis.

2.2 HUMAN TISSUE SAMPLES

Human brain samples were removed at *post-mortem* , stored at -70°C, until analysis. Tissue samples of frontal cortex (Brodmann area 9) and temporal cortex (Brodmann areas 20/21) from non-demented control subjects, were provided by Dr. P. Altmann, The London Hospital, Whitechapel, London. Tissue samples of frontal cortex (Br.9 & 10), temporal cortex (Br. 20/21, Br. 38), Br.4 and visual cortex (Br.17) from control and SDAT subjects, were provided by Dr. C.M. Wyschik of the MRC Brain Bank, Cambridge. Tissue samples of temporal cortex (Br.20/21) were supplied from Dr. Whittington, Coroners Department, Birmingham and Dr.R.J. Leeming, Department of Haematology, General Hospital, Birmingham.

Dried blood spot samples on Guthrie cards were supplied by Prof. M. Rutter, Maudsley Hospital, London, Dr. A.V. Mehta, Centre for Research in Mental Retardation (CREMERE), Bombay, India and Prof. B. Hagberg, Barnklinikerna, Ostra Sjuket, Gothenberg, Sweden. These were sent by airmail and stored on arrival at -20°C until assay. Samples were eluted in 750ul of distilled water from a 8mm diameter disc punched from the card. Whole blood samples were obtained from Prof. B. Hagberg, packed in dry ice and sent to the UK by airfreight. On arrival they were frozen at -70°C until analysis.

2.3 CHEMICALS

L-biopterin, D-neopterin, L-sepiapterin and pterin were obtained from Dr. B. Schircks', Buechstrasse 17a, 8645 Jona, Switzerland.

5-methyl-tetrahydrofolate (DL-5-methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid)(calcium salt), was obtained from Eprova Research Laboratories, Switzerland.

Albumin (bovine, fraction V),alkaline phosphatase, type III from *E.coli* , amitriptyline hydrochloride, L-ascorbic acid, atropine methyl nitrate, cyclic 3',5'-adenosine monophosphate (cAMP), catalase (isolated from bovine liver), cytochrome c (from horse heart, type III), desipramine hydrochloride, 6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$), dopamine, ethylenediamine, ethylenediamine tetraacetic acid (EDTA) (disodium-calcium salt), *N* ethylmaleimide, glutathione-reduced form, guanosine 5'-triphosphate (GTP)(type II-S, sodium salt), heparin (grade II, isolated from porcine intestinal mucosa), horseradish peroxidase, hippuric acid, hippuryl-L-arginine, hippuryl-L-lysine, imipramine hydrochloride, 6-methyl-5,6,7,8tetrahydropterin ($MMPH_4$), nicotinamide adenine nucleotide-reduced form (NADH)(grade III, disodium salt), nicotinamide adenine dinucleotide phosphate - reduced form (NADPH)(type III, tetrasodium salt, noradrenaline, nortriptyline hydrochloride,

oxotremorine (sesquifumarate), hydrochloride, phenelzine (sulphate salt), L-phenylalanine, transferrin (bovine), Trizma (tris(hydroxymethyl)aminoethane) base, *p* tyramine hydrochloride were supplied by the Sigma Chemical Company, Poole, Dorset, England.

HPLC grade 'Hypersolv' methanol, $MgCl_2$, KCL, glycerol and L-tyrosine were purchased from Fisons, Loughborough, England.

Sephadex G-25M columns (PD-10) were obtained from Pharmacia, Uppsala, Sweden.

Aluminium acetate (basic), L-cysteine, lithium carbonate and lithium sulphate were obtained from BDH Ltd, Poole Dorset, England

Helium was supplied by BOC Ltd., Wolverhampton, England.

All other reagents were of AnalaR grade or equivalent.

2.4 UV/VISIBLE SPECTROPHOTOMETERS

Protein measurements, dopamine-B-hydroxylase activity, sepiapterin reductase activity, carboxypeptidase activity and pterin stock solution concentration determinations were carried out using a Shimadzu UV 240, UV/visible recording double beam spectrophotometer and Shimadzu PR-1 graphic printer (Shimadzu Corporation, Japan).

DHPR activity in Guthrie card dried blood spot samples was determined using a Pye PU 8800, UV/visible recording double beam spectrophotometer equipped with a 4-cell automatic cell programmer and changer (Pye Unicam, England).

All other DHPR measurements were carried out using a Pye Unicam SP 1700 UV/visible double beam spectrophotometer and a Pye Unicam AR 55 linear recorder (Pye Unicam, England).

2.5 CENTRIFUGES

Refrigerated centrifugation was carried out in a MSE Superspeed 50 ultracentrifuge (MSE; Measuring and Scientific Equipment Ltd., England) using the MSE 10x10ml angle rotor head. Bench centrifugation was carried out in a MSE swing bucket centrifuge.

2.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC separation of pterins was carried out using the equipment detailed below. The equipment was calibrated by the use of standard solutions of known concentration, determined by UV spectoscopy (table 2.2). Calibration curves of peak area against concentration were plotted. Retention times of standards were determined as time from injection to appearance of peak (table 2.3).

HPLC EQUIPMENT

Pump	LDC Constametric Model III
Injection	Waters Intelligent Sample Processor Model 710B
Pre-Column	LichroCart ODS 5u
Column	Phase Separations Spherisorb octadecylsilane 5u (internal dimensions)250mm x 4mm
Detectors	Kontron SFM 23 Spectrofluorometer LDC Fluoromonitor III
Recorders	W & W model 302 pen recorder J.J. CR652A chart recorder LDC CR 1 chart recorder

Solvent Flow Rate :- 1ml min-1

Solvent :- 5% methanol : glass distilled water

Excitation wavelength = 360nm

Emission wavelength = 450nm

SUPPLIERS

Waters Millipore Ltd., USA

LDC, Laboratory Data Control/Milton Roy Ltd., Stone, Staffordshire, UK.

Kontron Instruments (UK), Watford, UK.

J.J. Lloyd Instruments Ltd., Southampton, UK.

Phase Separations Ltd, Cardiff, Wales, UK.

2.7 DETERMINATION OF BRAIN AND LIVER TOTAL BIOPTERINS AND BRAIN DERIVED PTERIN.

Prior to freezing, rat brains were divided into right and left hemispheres. Right hemispheres were used for the determination of total biopterins and pterin and left hemispheres were used for enzyme assays (see later).

Brain and liver total biopterins and brain derived pterin were determined essentially by the method of Fukushima & Nixon (1980). A 25% (w/v) homogenate of the hemisphere was prepared in 0.1M HCl and 200µl 20% (w/v) trichloracetic acid (TCA), using a Potter-Elvehjem homogeniser. Brain homogenates were ultracentrifuged at 100,000g for 5 minutes, liver homogenates were spun at maximum speed in a bench centrifuge for 10 minutes.

The supernatants were decanted into test tubes, noting the volume and the pellet discarded. To 1ml of supernatant, 50µl of 3% (w/v) iodine in 6% (w/v) KI was added, and the tubes left in the dark for 1 hour, after which time the iodine was reduced by addition of excess solid ascorbate. The samples were then run on the HPLC in duplicate (liver samples were diluted 1:10). Total biopterins and pterin levels were calculated from the calibration curve and the results were expressed as ng g^{-1} wet weight for brain and µg g^{-1} wet weight for liver

2.8 DETERMINATION OF PLASMA TOTAL BIOPTERINS.

Blood was collected from the opened thoracic region into heparinised (1000U ml^{-1} 0.9% (w/v) saline) syringes. After bench centrifugation at maximum speed for 10 minutes the plasma was decanted off and used for assay or stored at -70°C until required. To 300µl of plasma, 30µl of 60% (w/v) perchloric acid and 10µl 3% (w/v) iodine in 6% (w/v) KI were added. Samples were vortex mixed and left in the dark for 1 hour, after which the iodine was reduced with excess solid ascorbate and the samples centrifuged at maximum speed for 10 minutes in a bench centrifuge. Supernatants were analysed by HPLC in duplicate and results calculated from the calibration curves and expressed as µg $litre^{-1}$ plasma.

2.9 ENZYME SOURCE PREPARATION.

20% (w/v) homogenates of human or rat tissue were prepared in 0.1M Tris/HCL pH 7.6 using a Potter-Elvehjem homogeniser, the homogenates were centrifuged at 100,000g for 45 minutes at 4°C. Supernatants were decanted and stored at -70°c until analysis for various enzyme activities. Supernatant protein concentration was determined by the Biuret method (Gornall *et al.*, 1949) (see 2.14).

2.10 DETERMINATION OF *IN VITRO* BH_4 SYNTHETIC ACTIVITY IN BRAIN AND LIVER

BH_4 synthesis was determined by a modified method of Barford *et al.*, (1984) as described below in which the freeze drying stage of sample concentration is eliminated and an antioxidant, glutathione is added (see appendix 2), increasing BH_4 yield making the assay more precise and reproducible. For rat tissue the optimum pH was 8.0 and for human tissue it was pH 7.6. Optimum substrate concentrations for rat brain were $6x10^{-3}$M GTP and $6x10^{-3}$M $MgCl_2$ and $4x10^{-3}$M GTP and $4x10^{-3}$M $MgCl_2$ for human brain. 100ul of tissue preparation was incubated for three hours at 37°C in the dark in a medium containing GTP, $MgCl_2$ $1.07x10^{-2}$M Tris/HCL buffer, $1x10^{-3}$M glutathione and $3x10^{-3}$M NADPH in a total volume of 500µl.

To determine the contribution from endogenous BH_4, blanks were run containing no $MgCl_2$ or GTP. The incubation was terminated by the addition of 980ul of 0.1M HCl and 20µl 3% (w/v) iodine in 6% (w/v) KI. After one hour in the dark, the iodine was reduced with excess solid ascorbate and the samples run in duplicate on the HPLC. Biopterin produced was calculated from the calibration curve and results expressed as pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$.

2.11 DETERMINATION OF DHPR ACTIVITY

DHPR activity was measured by the method of Craine *et al.*, (1972). The following components were placed in a cuvette and incubated at 37°C for 90 seconds, 0.05M Tris/HCl buffer, pH 6.8, $2.5x10^{-4}$M sodium azide, 8ug horseradish peroxidase, $1x10^{-4}$M NADH, $1x10^{-3}$M hydrogen peroxide and 20ul enzyme source, in a total volume of 900µl.

After incubation, the reaction was started by addition of 100µl of $1x10^{-3}$M 6,7-dimethyl-5,6,7,8tetrahydropterin ($DMPH_4$). After a 30 second equilibration, the rate of reaction was monitored by measuring the rate of decrease in absorbance at 340nm. Blanks contained no enzyme source.

Enzyme activity was calculated using a molar extinction coefficient of 6.22×10^{3} M^{-1} cm^{-1} for NADH. This was related to the protein concentration of the supernatant as measured by the biuret method, and the specific activity expressed as nmoles NADH oxidised min^{-1} mg $protein^{-1}$.

2.12 DETERMINATION OF GTP CYCLOHYDROLASE-I ACTIVITY

GTPCH-I activity was assayed by a method based on that of Duch *et al.*, (1984). The enzyme source was desalted by running it through a Sephadex G-25 minicolumn (PD-10), equilibrated with 0.1M Tris/HCl buffer,pH 7.8, containing 2.5×10^{-3}M EDTA, 0.3M KCl and 10% glycerol.

50ul of desalted enzyme source was incubated with 60mM GTP in a total volume of 62.5µl for 90 minutes at 37°C. Blanks were run in which GTP and enzyme source were separately omitted. After 90 minutes, 6.5µl of 1M HCl containing 1% (w/v) iodine and 2% (w/v) KI was added, and incubation continued for an additional 30 minutes. Iodine was reduced by the addition of 6.5µl of 2% (w/v) ascorbate. The sample was made alkaline by the addition of 6.5µl of 1M NaOH, and the neopterin triphosphate was dephosphorylated by incubation with 0.5 units of alkaline phosphatase for 60 minutes. This was terminated by addition of 12.5µl of 1M acetic acid.

The sample was centrifuged at maximum speed in a bench centrifuge and the supernatant analysed for neopterin by HPLC as described earlier. Neopterin was calculated from the standard curve, protein concentration of enzyme source was determined by the biuret method and enzyme activity expressed as pmoles neopterin produced $hour^{-1}$ $mg\ protein^{-1}$.

Some batches of alkaline phosphatase have GTP cyclohydrolase activity, and thus are unsuitable for use in the assay, where this has been the case, the enzymatic dephosphorylation step is omitted and the acid oxidation step, is used to dephosphorylate the neopterin triphosphate by acid hydrolysis, giving lower but still measureable yields of neopterin. Assays using acid hydrolysis are indicated in the text.

2.13 DETERMINATION OF SEPIAPTERIN REDUCTASE ACTIVITY

The method of Katoh (1971) was used to assay for sepiapterin reductase except that Tris/HCl buffer, pH 7.4, was used. The reaction cuvette contained 50µM sepiapterin, 100uM NADPH, 0.085M Tris/HCl buffer, pH 7.4, and 50µl of enzyme source in a total volume of 1ml. Blank cuvettes had no enzyme source. The reaction was followed by measuring the decrease in absorbance at 420nm. Using the molar extinction coefficient of

sepiapterin, 10.4×10^{-3} M^{-1} cm^{-1}, and enzyme source protein concentration as obtained from biuret assay, the specific activity was calculated and expressed as nmol sepiapterin reduced min^{-1} mg $protein^{-1}$.

2.14 BIURET METHOD FOR PROTEIN DETERMINATION (Gornall *et al.*, 1949)

To 2ml of biuret reagent (copper sulphate pentahydrate, 0.15% (w/v); sodium potassium tartrate, 0.6% (w/v); NaOH, 3% (w/v); KI, 0.1% (w/v) in distilled water), 0.4 ml distilled water and 0.1 ml protein source were added, vortex mixed and left at room temperature for 30 minutes. Absorbance at 540nm was measured against a protein blank. The protein concentration was calculated from a calibration curve of bovine serum albumin standards (2 -10 mg ml^{-1}).

2.15 DETERMINATION OF TYROSINE HYDROXYLASE ACTIVITY.

The method of Yamamuchi & Fujisawa (1978) was used to determine tyrosine hydroxylase activity in tissue samples. The assay medium contained 0.05M Tris/HCl buffer, pH 6.1, 10µmol tyrosine, 0.4µmol of 6-methyl-5,6,7,8-tetrahydropterin, 20µl 2-mercaptoethanol, 100µg catalase and enzyme source in a total volume of 500µl. This was incubated in a shaking water bath at 30°C for 10 minutes after which the reaction was terminated by addition of 500ul of 6% (w/v) TCA. After bench centrifugation for 5 minutes at maximum speed, the supernatant was transferred to a test tube and 100µl of 10% (w/v) EDTA, 400µl 1M Tris and 100µl of 0.2M N-ethylmaleimide successively, at which point the pH should be about 8.4.

To the mixture, 60mg of dried alumina was added, and vortex mixed for 30 seconds to keep the alumina in suspension, this must be done quickly as dopa is unstable in an alkaline environment. Once the alumina has settled the supernatant can be discarded and the alumina washed twice with 2ml of water. Dopa is extracted from the alumina with 1.6ml of 0.1M HCl and 15 seconds vortex mixing. The extract is then neutralised to pH6.5 with 500µl of 0.5M K_2HPO_4. To this was added 100µl of 0.25% (w/v) $K_3Fe(CN)_6$ and after 3 minutes 1.0ml of freshly prepared alkaline ascorbate solution (9 vol

of 9M NaOH, 1 vol 2% (w/v) ascorbic acid and 0.2 vol ethylenediamine). After 5 minutes the sample was run on the HPLC (see above). Levels of dopa were calculated from a calibration curve of known standards. and expressed in terms of protein concentration as determined by the biuret method (see above) and results expressed as nmol dopa produced 10 min^{-1} mg $protein^{-1}$.

2.16 DETERMINATION OF DOPAMINE-B-HYDROXYLASE ACTIVITY

Dopamine-B-hydroxylase activity in brain tissue was determined by the method of Liones *et al.*, (1976). Tissue samples were prepared as described earlier. The assay is based on the tyramine dependent oxidation of $[Fe(CN)_6]^{4-}$ to $[Fe(CN)_6]^{3-}$. The increase in absorbance at 420nm was measured with a Shimadzu UV/vis 240 recording spectrophotometer. Results were expressed as umoles octopamine formed min^{-1} mg $protein^{-1}$.

2.17 STATISTICS

Data are presented as mean and standard deviations. Comparison of data groups was by a two tailed Student's unpaired t-test or Wilcoxon's signed ranks, assuming probabilities of 0.05 or less to be significant. Linear correlations and regression analysis were by least squares method, and lines of best fit. Outliers were tested with the Q-test.

2.18 DETERMINATION OF CARBOXYPEPTIDASE N1 ACTIVITY

Carboxypeptidase N1 activity was determined by the method of Schwiessfurth *et al.*, (1983). There are two types of carboxypeptidase N. CN1 cleaves the synthetic substrate hippuryl-L-arginine, whilst CN2 cleaves hippuryl-L-lysine. 0.05M hippuryl-L-arginine solution was prepared in 0.5M potassium phosphate buffer (pH 8.4). The buffer was prepared from 100 ml of solution A (0.5M K_2HPO_4 & 0.75M NaCl) and sufficient solution B (0.5M KH_2PO_4 & 0.75M NaCl) to achieve pH 8.4 at 25°C. 0.1M hippuryl-L-lysine was prepared in the same buffer.

50µl of supernatant prepared as decribed earlier (2.8), was added to 200µl of substrate buffer mixture, tightly covered, vortex mixed for 15 seconds and incubated for 60 minutes at 37°C in a shaking water bath. The reaction was stopped by addition of 250µl of 1M HCl and vortex mixing for 15 seconds. Blanks were treated identically except that HCl was added before incubation. After removal from the water bath, samples were placed on crushed ice for 5 minutes. 1500 µl of ethyl acetate were added and the mixture vortexed for 30 seconds, followed by bench centrifugation for 10 minutes. 1ml of the upper organic layer was removed and evaporated the ethyl acetate completely. 3ml of 1m NaCl was added to the residue and this was vortexed and then placed in a water bath at 70°C to dissolve the hippuric acid.

After standing for 15 minutes the absorbance at 228nm of the hippuric acid was read in a Shimadzu spectrophotometer (2.6). Activity was expressed as umol hippuric acid released min^{-1} $mg\ protein^{-1}$.

2.19 DETERMINATION OF cAMP LEVELS

cAMP levels were determined by radioimmunoassay using the **RIANEN cAMP [^{125}I] RADIOIMMUNOASSAY KIT cat no. NEK-033** from New England Nuclear, DuPont Diagnostics. The method followed was that supplied with the kit.

2.20 FOLATE ASSAYS.

These were carried out by Dr. R.J. Leeming, Haematology, General Hospital, Birmingham. Determination of total folate and tetrahydrofolate was performed by use of *Lactobacillus casei* after neutral iodine or acid iodine oxidation.

Table 2.1 Composition of Rat Diet

Crude oil	%	3.26
Crude protein	%	21.23
Crude fibre	%	3.48
Digestible crude oil	%	2.48
Digestible crude protein	%	17.60
Digestible crude fibre	%	2.10
Digestible carbohydrate	%	46.80
Gross energy	cals/kg	4073.00
Metabolisable energy	cals/kg	3666.00
Saturated fatty acids	%	0.73
Linoleic acid	%	0.99
Other unsaturated acids	%	1.54
Calcium	%	1.30
Phosphorous	%	1.00
Sodium chloride	%	0.64
Magnesium	%	0.24
Potassium	%	0.80
Sulphur	%	0.23
Iron	mg/kg	171.60
Copper	mg/kg	14.50
Manganese	mg/kg	86.60
Cobalt	ug/kg	104.70
Zinc	mg/kg	39.60
Iodine	ug/kg	600.00
Arginine	%	1.31
Lysine	%	1.14
Methionine	%	0.36
Cysteine	%	0.33
Tryptophan	%	0.23
Glycine	%	1.57
Histidine	%	0.51
Threonine	%	0.71
Isoleucine	%	0.84
Leucine	%	1.49
Phenylalanine	%	0.89
Valine	%	1.07
Tyrosine	%	0.69
Aspartic acid	%	1.64
Glutamic acid	%	3.96
Proline	%	1.53
Serine	%	1.00
Vitamin A	i.u.	11,587.00
Carotene	mg/kg	0.79
Vitamin B1	mg/kg	9.10
Vitamin B2	mg/kg	9.90
Vitamin B6	mg/kg	10.10
Vitamin B12	mg/kg	17.00
Vitamin E	mg/kg	77.00
Vitamin X	mg/kg	3.00
Folic acid	mg/kg	0.70
Nicotinic acid	mg/kg	78.00
Pantothenic acid	mg/kg	27.80
Choline chloride	mg/kg	2.22
Biotin	mg/kg	0.12
Vitamin D3	i.u./kg	859.00

Table 2.2 Relative retention times for neopterin, biopterin and pterin on a reverse phase ODS 5u HPLC column (250mm x 4mm) using 5% (v/v) methanol eluent at 25oC.

Pteridine	Retention Time (mins)	Relative Retention Time (biopterin =1)
Neopterin	4.50	0.60
Biopterin	7.50	1.00
Pterin	12.50	1.70

Table 2.3 Molar extinction coefficients (Emax) for neopterin, biopterin and pterin, at pH 13.

Pteridine	Wavelength (nm)	Emax $x10^{3}$ M^{-1} cm^{-1}
Neopterin	362	8.3
Biopterin	362	8.3
Pterin	358	6.6

CHAPTER THREE

DIHYDROPTERIDINE REDUCTASE IN MENTAL DISORDERS

3.1 INTRODUCTION

Dihydropteridine reductase (DHPR, EC.1.6.99.7) is responsible for the conversion of quinonoid dihydrobiopterin (qDHB) to tetrahydrobiopterin (BH_4)(Craine *et al* 1972). BH_4 serves as a cofactor in the synthesis of the catecholamine and indolamine neurotransmitters (Lovenberg & Levine 1987), during which qDHB is generated, thus DHPR acts to salvage the cofactor after use. The salvage pathway is important in the maintenance of BH_4 levels in the body. DHPR is found in erythrocytes and it is this enzyme that this study centres on.

3.1.1 DHPR DEFICIENCY

Gross deficiency of DHPR causes malignant hyperphenylalaninaemia (MHPA) and severe mental retardation (Kaufman *et al.*, 1975). Erythrocyte DHPR activity is measured routinely in dried blood spot samples on Guthrie cards (Arai *et al.*, 1982, Sahota *et al.*, 1985). Using the *Crithidia fasiculata* assay it is possible to determine the serum biopterin concentration in the dried blood spots (Leeming *et al.*, 1984). Using a group of 500 subjects, all under 12 years of age, the distribution of DHPR activity in the population was investigated (Sahota *et al.*, 1986). These controls were compared to a group of subjects with hyperphenyl-

alaninaemia (HPA) and a group of DHPR deficient subjects (Table 3.1).

Table 3.1 DHPR Activity in Newborn Children

Group	Number	Mean ± s.d.	Range
Controls	500	232 ± 60	64-440
HPA	48	206 ± 72	78-370
DHPR Deficiency	4	0	------

units nmol NADH min^{-1} ml whole $blood^{-1}$

Taken from Sahota *et al.*, (1985)

Initially it seemed that there was a biphasic distribution of activity (Sahota *et al.*, 1985), however in a subsequent investigation in which a further 500 subjects were included the biphasic pattern was not reproduced though there was a significant tailing towards the bottom end of the distribution (Armstrong *et al.*, 1986). Other studies indicated that in certain types of mental retardation, there was a concomitant reduction in DHPR activity (Table 3.2)(Sahota *et al.*, 1985). In subjects with Rett's syndrome, a disorder occuring only in

females, and autistic individuals there were similar decreases in DHPR activity compared to controls (Table 3.3).

Table 3.2 DHPR Activity in Blood Samples from Control Children and Children With a Variety of Mental Disorders

Group	No.	Mean ± s.d.	Range	p
Control	17	184 ± 52	61-278	
IMR	12	128 ± 43	70-197	<0.01
FMR	11	185 ± 71	89-301	ns
UNS	12	127 ± 69	31-234	<0.05
MBD	5	123 ± 34	74-154	<0.05
Autism	7	119 ± 48	38-181	<0.01
HPA	33	132 ± 59	43-323	<0.01

units nmol NADH min^{-1} ml whole $blood^{-1}$

IMR Idiopathic mental retardation
FMR Familial mental retardation
UNS Unusual neurological syndromes
MBD Minimal brain dysfunction
HPA Hyperphenylalaninaemia

Taken from Sahota *et al.*, 1985

Table 3.3 DHPR Activity in Controls, Subjects with Autism & Rett's Syndrome and Heterozygotes

Group	No.	Mean ± s.d.	p
Controls	32	182 ± 55	
Autism	13	126 ± 32	<0.01
Rett's syndrome	23	139 ± 37	<0.01
Heterozygotes	8	71±25	<0.01

units:- nmol NADH min^{-1} ml whole $blood^{-1}$

Taken from Leeming *et al.*, 1987

Investigations into DHPR activity have revealed that there are a number of individuals who are heterozygotes for the DHPR gene. The gene is located on chromosome 4 and these subjects were identified by the fact that they gave birth to a totally DHPR deficient child, i.e. homozygous recessive. Such children are indeed very rare, but a few have been identified. DHPR activity in the heterozygotes is approximately half that of the homozygous dominant normal subjects investigated, similar to that seen at the bottom end of the normal distribution of DHPR activity seen in normal individuals (Leeming *et al.*, 1987).

It was noted that despite having lowered DHPR activity, their IQ was well within the normal range. In contrast, subjects developing MHPA as a result of total DHPR deficiency have a lowered IQ, even if the condition is recognised early on in infancy and treated appropriately, with a low phenylalanine diet and neurotransmitter precursors. The reduction in IQ is not as severe as in the untreated condition, but is still significantly lower than non-MHPA subjects.

In this study DHPR activity was investigated in groups of subjects with various mental disorders in order to add to the data already obtained. Possible sources of variation in the study were removed by using the same type of card and having the samples assayed by the same operator.

Earlier studies used a volume baseline. Some of the data presented here is expressed on a haemoglobin (Hb) baseline, in addition to the volume baseline, in order to detect any baseline dependent effect.

DHPR activity and serum biopterin concentration were investigated in blood spots on Guthrie cards from subjects with a wide range of non-specific mental disorders and Down's syndrome. These were received from the Centre for Research in Mental Disorders (CREMERE), Bombay, India. Samples from Rett's syndrome subjects were received from

the Barnklinikerna, Gothenberg, Sweden. The autistic subject samples were provided by the Maudsley Hospital, London.

These groups provided a large number of subjects with non-specific mental retardation (NSMR), Down's syndrome (DS) and with dementias such as autism (A) and Rett's syndrome (RS).

3.1.2. NON-SPECIFIC MENTAL RETARDATION (NSMR)

For the purpose of this study, non-specific mental retardation (NSMR) was defined as any type of mental dysfunction caused by any means other than Down's syndrome, Rett's syndrome, Autism and malignant hyper-phenylalaninaemia. Common causes included anoxia or trauma at birth, head injury and viral induced encephalitis.

3.1.3 AUTISM

Autism has only recently been recognised as a type of mental dysfunction. Autistic subjects appear withdrawn and have marked difficulties with communication, whilst possessing other skills commensurate with their age. As yet there is very little known about the condition.

3.1.4 RETT'S SYNDROME

Rett's syndrome is a juvenile dementia that was first reported in 1966 (Rett 1966 and 1977). To date only females are affected. These individuals display normal intellectual development until about twelve months of age when there occurs a rapid regression to a static state of anxious isolation similar to that seen in autism. The disease is characterized by a loss of all purposeful hand function and adoption of stereotyped behaviour such as finger squeezing and tense hand clasping frequently accompanied by bursts of hyperventilation. Such individuals have a wide based jerky gait. Microcephaly and epilepsy are common findings later on in life (Hagberg *et al.*, 1983). The role of tetrahydrobiopterin metabolism in Rett's syndrome is not clear. Sahota *et al.*, (1985) report blood DHPR in Rett's syndrome to be within the normal range. Serum and urine biopterins were normal. Later studies report that Guthrie card DHPR activity in Rett's syndrome subjects was lower than that of controls (Leeming *et al.*, 1987). Zoghbi *et al.*, (1989) report that levels of CSF biogenic amine metabolites were significantly reduced in Rett's syndrome subjects. CSF biopterin was elevated compared to controls. Brain DHPR activity did not differ significantly from controls.

3.1.5. DOWN'S SYNDROME

Down's syndrome or trisomy 21 occurs in individuals who have an extra copy of chromosome 21. These individuals are mentally retarded and have certain phenotypical features such as a 'moonlike face' giving rise to the common description of the condition as 'mongolism'. Serum dihydrobiopterin levels are reported to be elevated in Down's syndrome (Aziz *et al.*, 1982). Serum neopterin levels were lower than controls (Blair *et al.*, 1984a) Barford *et al.*, (1983) report that blood DHPR activity was normal in Down's syndrome. Blair *et al.*, (1984) have shown brain DHPR activity and BH_4 synthesis to be reduced in Down's syndrome.

3.2 MATERIALS AND METHODS

Materials were as described in chapter 2. The enzyme was assayed by the method of Sahota *et al.*, (1985), at 37°C. The assay mixture in a total volume of 1ml contained 50mmol l^{-1} Tris/HCl pH 7.5, 50umol l^{-1} ferricytochrome c (type III), 100umol l^{-1} NADH, 10umol l^{-1} 6-methyl-5,6,7,8 tetrahydropterin and 100ul haemolysate. The formation of ferrocytochrome c is proportional to the formation of BH_4 from q-BH_2 and is followed at 550nm on a double beam spectrophotometer using a temperature cell at 37°c and sample changer.

Enzyme activity was expressed as nmol NADH oxidised min^{-1} ml^{-1} whole blood. Each 8mm disc typically containing 16.7 µl whole blood. The blood was eluted from the disc by vortex mixing for 10 minutes in 750µl distilled water. The resulting solution was used as the enzyme source.

Where determined, haemoglobin concentration in the blood spot samples was measured by Ian Surplice in the Haematology department General Hospital Birmingham.

Serum biopterin concentration in the blood spot samples was measured by Dr. R.J. Leeming at the General Hospital, Birmingham, using the protozoon based *Crithidia fasiculata* microbiological assay (Leeming *et al.*, 1976). The aim of this study was to follow on from those carried out previously and to clarify, from the investigation of a large number of samples, the involvement of DHPR in various types of mental disorder.

3.3 RESULTS

DHPR activity and serum biopterin concentration in several groups of subjects are shown in table 3.4. Both Rett's syndrome and autistic groups have significantly lower mean DHPR activity compared to both healthy controls and NSMR groups (Details of individuals are given in appendix 1). In all groups where data was available, except for the

Down's subjects, there was no correlation between DHPR activity and serum biopterin concentration. In the Down's subjects (table 3.5) there was a significant negative correlation between DHPR activity and serum biopterin concentration ($r=-0.60$, $p< 0.05$).

A similar negative correlation is seen on a plot of log DHPR activity versus log serum biopterin in patients on renal dialysis with high body burdens of aluminium (Altmann personal communication). The data in table 3.5 also correlates significantly on both linear and log plots ($p< 0.05$). DHPR activity in Retts syndrome subjects was measured in terms of a volume and haemoglobin baseline. In both instances the Rett's syndrome group had significantly lower mean DHPR activities compared to controls (Table 3.6)

3.4 DISCUSSION

Previous studies have shown the distribution of DHPR activities in the population to be normal, though having a large spread (Sahota *et al* 1985). Erythrocyte DHPR activity correlates with brain DHPR activity in rats and humans (Cutler 1986).

The gene for DHPR activity resides on chromosome 4 and is inherited as an autosomal dominant. Heterozygotes for the gene show activity approximately half that of normal as do subjects at the bottom end of the normal distribution of DHPR activities. Heterozygotes have no apparent lowering of IQ. Recent studies on DHPR with regard to dialysis encephalopathy (DE) induced by aluminium containg dialysates have shown there to be a significant loss of DHPR activity in subjects with DE and there is a good correlation between DHPR activity and tests of mental ability, such as the symbol digit coding test (SDCT) (Altmann *et al.*, 1987).

Some observers have noted that although the heterozygotes have lowered DHPR activity compared to normal subjects, they appear phenotypically normal, thus refuting the hypothesis of DHPR being implicated in the pathogenesis of the neurological dysfunction seen in aluminium induced encephalopathy (Kaufman *et al.*, 1987). However to date no-one has carried out subtle tests of psychomotor function on the heterozygotes. Such an investigation may reveal that in such subjects there is some loss of aptitude in SDCT tests.

The genetic aspects of DHPR expression are further highlighted in Rett's syndrome, in which there is a deletion on the X chromosome (Hagberg *et al.*, 1977). This appears to occur exclusively in females, at an

incidence of around 1 in 150,000 births. DHPR activity may be polygenically controlled with a further gene on the X chromosome modulating DHPR activity. (Armstrong *et al.*, 1986).

Previous investigations into DHPR activity in mental dysfunction (Leeming *et al.*, 1987; Sahota *et al.*, 1986) used healthy controls. In these studies it was not clear whether the DHPR activity deficit seen in the RS and autistic individuals was an epiphenomenon occuring as a result of the retardation rather then being a causal agent.

The NSMR group and Down's syndrome subjects (Table 3.4) have a mean DHPR activity which is not significantly different from that of the healthy controls. Thus the deficit of DHPR in Rett's syndrome and autism seen in the groups used in this study is not merely a consequence of the decreased mental capacity of the subject.

Using both volume and haemoglobin baselines, the RS group had significantly lowered DHPR activity compared to controls, indicating that the effect is not baseline dependent (Table 3.6). The results presented here indicate that the loss of DHPR activity in Rett's syndrome and autism is not merely a result of the impaired brain function and the deficit of DHPR activity is independent of the baseline used for the assay.

Table 3.4 DHPR activity and whole blood (Guthrie card) total biopterin concentration in controls and subjects with mental dysfunction

Group (age 1-12yrs)	[biopterin] $\mu g\ litre^{-1}$	DHPR activity **
Healthy controls	3.78 ± 1.28(4)	180 ± 54 (6)
NSMR	3.87 ± 1.15(65)	182 ± 56 (95)
DS	4.00 ± 1.40(12)	171 ± 56 (24)
A	n.d.a	136 ± 27 (11)*
RS	n.d.a	120 ± 40 (9) *

data expressed as mean ± s.d. (n)

** units nmol NADH oxidised min^{-1} ml whole $blood^{-1}$

n.d.a. no data available

* $p < 0.001$ compared to NSMR group

Table 3.5 Whole blood (Guthrie card) total biopterin concentration and DHPR activity in 12 Down's syndrome subjects

no.	[biopterin]	log	DHPR activity	log
1	3.8	0.58	106	2.02
2	3.8	0.58	205	2.31
3	6.9	0.84	75	1.88
4	6.9	0.84	70	1.85
5	3.1	0.49	138	2.14
6	3.1	0.49	202	2.31
7	3.4	0.53	287	2.46
8	4.3	0.63	165	2.22
9	3.8	0.58	122	2.09
10	4.1	0.61	128	2.11
11	2.9	0.46	106	2.02
12	2.4	0.38	234	2.37
mean	4.0	0.58	155	2.15
±s.d.	1.4	0.13	64	0.19

units:-

DHPR- nmole NADH oxidised min^{-1} ml whole $blood^{-1}$
[biopterin] - µg litre whole blood $^{-1}$

[biopterin] v. DHPR r=-0.60 $p<0.05$
log [biopterin] v log DHPR r=-0.69 $p<0.05$

Table 3.6 DHPR activity in Rett's syndrome & controls measured on two different baselines.

Baseline	Controls	Rett's Syndrome
haemoglobin(Hb)	0.97±0.28 (228)	0.73±0.20 (6)*
volume	182 ± 56 (95)	120± 40 (9)**

* $p<0.01$ ** $p<0.001$

Units

Hb µmol NADH min^{-1} g Hb^{-1}
volume nmol NADH min^{-1} ml whole $blood^{-1}$

CHAPTER FOUR

TETRAHYDROBIOPTERIN METABOLISM IN AGING AND ALZHEIMER'S DISEASE

4.1 INTRODUCTION

There is a progressive decline in cognitive and intellectual function with age and this has been described as 'benign senescent forgetfulness (Reisberg, 1983). Several studies have shown that there are marked alterations in the catecholaminergic neurotransmitter system in the aging brain. Certainly, levels of dopamine and noradrenaline are reduced in several brain areas (Winblad *et al.*, 1985; Robinson *et al.*, 1977; Yates *et al.*, 1983).

Tyrosine hydroxylase and dopa decarboxylase activity decline with age (Winblad *et al.*, 1985; Mayeux *et al.*, 1983), in contrast to monoamine oxidase whose activity correlates positively with age (Mayeux *et al.*, 1983).

These disturbances occur in conjunction with atrophic changes and cell loss of neurones in the locus coeruleus and substantia nigra (Mann *et al.*, 1980; Tomlinson *et al.*, 1981; Mann *et al.*, 1983; Carlsson *et al.*, 1985). The levels of homovanillic acid (HVA), a monoamine metabolite, in the CSF correlate positively with age, though there is a decrease in HVA turnover with age. Other catecholamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylglycol (MHPG) show no age related correlations (Carlsson *et al.*, 1985).

The effect of aging on the serotonergic neurotransmitter system has not been as widely examined as the catecholamines but levels of serotonin (5-HT) are reported to decline with age (Mackay *et al.*, 1978; Carlsson *et al.*, 1985). Levels of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the CSF, show no correlation with age.

4.1.1 TETRAHYDROBIOPTERIN METABOLISM IN AGING

The involvement of BH_4 levels in the neurological aspects of aging is not clear. Serum biopterin levels increase with age (Leeming and Blair, 1980) suggesting a loss of DHPR activity with age. The neopterin : biopterin ratio increases in the CSF with age (Lewitt *et al.*, 1982) suggesting impaired synthesis of BH_4 from DHNTP. These observations suggest that the neurochemical deficits characteristic of aging may arise due to a deficiency of BH_4. BH_4 synthesis and DHPR activity decline with age (Anderson *et al.*, 1986).

4.1.2 SENILE DEMENTIA OF THE ALZHEIMER TYPE (ALZHEIMER'S DISEASE)

Dementia afflicts 5% of those over the age of 65 and of these 60% are thought to be suffering from Alzheimer's disease (AD) or Senile Dementia of the Alzheimer Type (SDAT) (Roth, 1985; Gottfries, 1985; Review article in British Medical Bulletin 1986).

Alzheimer's disease is a progressive disorder of cognitive and intellectual function with insidious onset. The diagnosis of AD is difficult. The early stages are characterised by loss of short term memory and recall abilities, similar to those seen as part of the normal aging process. Later, all memory is lost and there are marked changes in the behaviour and personality resulting in the individual being unable to carry out normal day to day tasks, resulting in total incapacitation (Reisberg *et al.*, 1982).

AD is usually confirmed *post-mortem* by the presence of numerous extracellular senile plaques, and intracellular neurofibrillary tangles in the neocortex, hippocampus and some subcortical nuclei (Alzheimer, 1907). Similar neurochemical and neuropathological changes are seen to a lesser extent in the normal aging brain, suggesting that AD may be an exacerbation of the normal aging process.

In AD, the major atrophic and neuropathological changes, primarily involve the hippocampus and neocortex, in particular the inferior temporal and post-central parietal cortices (Brun, 1983). In patients with early onset AD there are degenerative changes in the subcortical nuclei including the locus coeruleus and raphe nuclei as well as those described above (Ishii, 1966; Tomlinson, 1981; Marcyniuk *et al.*, 1986).

Early studies demonstrated the presence of a cholinergic deficit in AD (Perry *et al.*, 1981; Bowen, 1983). More recently, noradrenergic and serotonergic deficits have been found in AD.

Serotonin (5-HT) levels are extensively reduced in AD brains (Arai *et al.*, 1984). The 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) is reduced in the neocortex and hippocampus of AD patients (Cross *et al.*, 1983; Gottfries *et al.*, 1983; Arai *et al.*, 1984).

The pre- and post-synaptic receptor density is diminished, particularly in the temporal cortex and hippocampus in early onset AD, suggesting that 5-HT synaptic transmission is impaired in AD (Bowen *et al.*, 1983; Crow *et al.*, 1984).

There are marked reductions in noradrenaline levels in several brain regions (Rossor *et al.*, 1984; Arai *et al.*, 1984). The degenerative changes seen in the locus

coeruleus occur concomitantly with reductions in cerebral cortex dopamine-β-hydroxylase activity (Perry *et al.*, 1981; Cross *et al.*, 1981).

These systems were implicated in the pathogenesis of AD due to the presence of neurofibrillary tangles in the raphe nuclei and locus coeruleus (Ishii, 1966; Tomlinson, 1981; Mann *et al.*, 1983).

The dopaminergic system, however, seems to be intact in AD. In the substantia nigra, a dopaminergic nucleus, there is no cell loss or atrophy (Mann *et al.*, 1980). In the caudate nucleus dopamine levels are reduced in AD (Winblad, 1985) in contrast to areas of maximal neuropathological change, such as neocortex and hippocampus, where dopamine levels are normal (Mann *et al.*, 1980; Arai *et al.*, 1984)

There is a significant neuronal loss in AD locus coeruleus compared to age matched controls, particularly in those with early onset AD (Forno, 1978; Tomlinson *et al.*, 1981; Bondareff *et al.*, 1982). The remaining cells are markedly atrophied (Forno, 1978) and have diminished protein synthetic capacity (Mann *et al.*, 1981; Mann *et al.*, 1984).

4.1.3 TETRAHYDROBIOPTERIN METABOLISM IN SDAT

It is now widely understood that a defect arises in the BH_4 synthetic pathway in SDAT. CSF biopterins are lowered in AD (Leeming and Blair, 1979; LeWitt *et al.*, 1985; Williams *et al.*, 1980; Morar *et al.*, 1983) as are serum biopterins (Leeming and Blair, 1980).

More recently, lowered total biopterins in locus coeruleus and substantia nigra and elevated neopterin : biopterin ratios in temporal cortex have been demonstrated (Barford *et al.*, 1984; Nagatsu *et al.*, 1986).

Impaired BH_4 synthesis in SDAT temporal cortex (Brodmann area 20/21) compared to age matched controls has been reported (Barford *et al.*, 1984), indicating that lack of BH_4 may be involved in AD neurotransmitter deficits.

The use of controls which have been matched for age and where data is available, prior medical treatment, is of paramount importance in studies such as these. These parameters can exert a marked effect on the biochemistry of the brain and must be taken into account.

This study examined BH_4 metabolism especially *in vitro* synthesis in several areas of brain from control subjects and subjects known to have had SDAT. In all comparisons, subjects were matched for age.

4.2 MATERIALS AND METHODS

Enzyme activity determination and the collection of tissues and sample preparation was as described in chapter 2.

Samples of frontal and temporal cortex from 15 non-demented subjects were obtained from Dr. P. Altmann at the London Hospital, Whitechapel, London. Patient details are given in appendix 1.

The first batch of tissue samples from the MRC Brain Bank, Cambridge, comprised portions of frontal (BA 9), visual (BA 17) and temporal (BA 20 & BA 21) cortices and locus coeruleus. These are designated MRC Batch 1. They were taken at *post-mortem* from control and SDAT subjects and stored as described in chapter 2. Age, sex, *post-mortem* delay and cause of death are shown in appendix 1.

Tissue samples were assayed for DHPR activity, sepiapterin reductase activity and *in vitro* BH_4 synthetic activity, as described in chapter 2.

The second batch of tissue samples obtained from the MRC Brain Bank, were designated MRC Batch 2. These comprised portions of frontal cortex (BA 10), temporal cortex (BA 38), BA 4 and locus coeruleus. Details of age,

sex, *post-mortem* delay and cause of death are shown in appendix 1 These samples were also accompanied by details of drug treatment recieved by the subject prior to death.

Batch 2 samples were also used to assess the effects of transferrin on *in vitro* BH_4 synthetic activity in control and SDAT brain preparations (Chapter 5).

Samples of temporal and frontal cortex from non-demented subjects were also obtained from Dr. Leeming, Haematology Department, General Hospital, Birmingham. These samples were also used to assess the effects of several psychoactive drugs on BH_4 metabolism *in vitro*. (Chapter 7). The control data from this study is presented in this chapter to shed light on the effects of aging on BH_4 metabolism in the temporal and frontal cortices.

4.3 RESULTS

DHPR, *in vitro* BH_4 synthesis and sepiapterin reductase activity were determined in samples of frontal and temporal cortex from 15 subjects (samples obtained from Dr. P. Altmann, London Hospital, Whitechapel) (Tables 4.1 and 4.2) and are summarised in Table 4.3. Age, sex, cause of death and *post mortem* delay of these subjects are given in appendix 1.

There were no significant intercortical differences for any of the parameters measured. *Post mortem* delay did not correlate with any of parameters measured.

Tables 4.4 to 4.6 show DHPR, sepiapterin reductase and *in vitro* BH_4 synthetic activities in samples of 4 brain areas (BA 9, frontal cortex; BA 17, visual cortex; BA20, temporal cortex; BA21, temporal cortex) from control subjects (MRC Batch 1). For each parameter there were no significant inter-area differences.

DHPR activity correlated negatively with age in BA 21 ($r=-0.949$ $n=5$ $p<0.05$)

Tables 4.7 to 4.9 show the DHPR, sepiapterin reductase and *in vitro* BH_4 synthetic activity in samples from the same 4 brain areas but from SDAT subjects (MRC Brain Bank, Cambridge). For each parameter there were no significant inter-area differences though BA21 had considerably lower mean *in vitro* BH_4 synthetic activity, though this was not statistically significant.

DHPR activity correlated negatively with age in BA 17 ($r=-0.957$ $n=5$ $p<0.05$).

Age, sex, cause of death and *post-mortem* delay for the controls and SDAT subjects are given in appendix 1.

There was no significant difference in DHPR and sepiapterin reductase activities in any of the brain areas analysed between SDAT and control subjects (Tables 4.10 and 4.11). *In vitro* BH_4 synthetic activity however was significantly lowered in BA17 ($p<0.01$), BA20 ($p<0.001$) and BA21 ($p<0.001$)(Table 4.12).

Appendix 1 gives the case histories of the 2nd batch of brain samples obtained from the MRC Brain Bank (MRC Batch 2). *In vitro* BH_4 synthesis was lowered in SDAT BA38 compared to controls ($p<0.01$;Table 4.13). In BA4 and BA10 there was no significant difference in *in vitro* BH_4 synthetic activity between SDAT and controls (Tables 4.14 and 4.15). Locus coeruleus displayed lowered *in vitro* BH_4 synthetic activity in SDAT compared to controls ($p<0.01$)(Table 4.16).

Control locus coeruleus had a significantly higher *in vitro* BH_4 synthetic activity than the other control brain areas (Table 4.17). In SDAT subjects locus coeruleus had significantly greater *in vitro* BH_4 synthetic activity than BA4 ($p<0.001$) and BA38 ($p<0.001$) (Table 4.17).

In vitro BH_4 synthesis correlated negatively with age in control BA38 ($r=-0.947$ $n=5$ $p<0.05$). In samples c1 to c8 (temporal cortex obtained from Dr. Leeming, Haematology, General Hospital, Birmingham) *in vitro* BH_4 synthesis showed a negative correlation with age ($r=-0.7748$ $n=8$

$p<0.05$). Combining these data with MRC Batch 1, BA 21, the correlation retains statistical significance ($r=-0.556$ $n=19$ $p<0.05$; Table 4.18).

4.4 DISCUSSION

Tetrahydrobiopterin (BH_4) plays a crucial role in the synthesis of the catecholamine and indoleamine neurotransmitters (Lovenberg & Levine, 1987). There are alterations in turnover and levels of these neurotranmitters with aging (Mann *et al.*, 1980; Tomlinson *et al.*, 1981). The extent to which BH_4 is involved in these alterations is far from clear. What is clear is that serum biopterin levels increase with age indicating diminishing DHPR activity (Leeming & Blair, 1980).

4.4.1 TETRAHYDROBIOPTERIN METABOLISM IN AGING

Anderson *et al.*, 1986 have shown that DHPR in the temporal cortex declines linearly with age ($r=-0.57$, $p<0.01$ $n=24$) and in the frontal cortex the decline in DHPR activity is best described by a quadratic (curvilinear) equation ($r=-0.795$ $p<0.001$ $n=20$). In the frontal cortex DHPR activity remains fairly constant until the seventh decade when it starts to decline.

In this study DHPR shows a significant negative linear correlation in temporal cortex (BA21; r=-0.949 n=5 $p<0.05$)(Table 4.4), but no correlation was found in the frontal cortex. BH_4 synthesis has been shown to decline with age in the temporal and frontal cortices (Anderson *et al.*, 1987). This study has shown a similar correlation in temporal cortex (Table 4.18 r=-0.556 n=19), but no correlation in frontal cortex.

Anderson *et al.*, (1987) report a deficit in sepiapterin reductase activity in the aging temporal cortex. The results of this study did not show any significant age related decline in either frontal or temporal cortex.

4.4.2 TETRAHYDROBIOPTERIN METABOLISM IN ALZHEIMER'S DISEASE

A defect in temporal cortical BH_4 synthesis has been reported previously (Anderson *et al.*, 1987, Barford *et al.*, 1984). Reported here are defects in BH_4 synthesis in temporal cortex (BA21 & BA20) and in visual cortex (BA17) (MRC Batch 1;Table 4.12).

A similar defect is seen in BA38 (temporal cortex; $p<0.01$; Table 4.13) and locus coeruleus ($p<0.01$; Table 4.16), MRC Batch 2 samples. Anderson *et al.*, (1986) have shown diminished BH_4 synthesis in locus coeruleus.

BH_4 synthetic activity in locus coeruleus was significantly higher than BA4 ($p<0.001$) and BA 38 ($p<0.001$) in the controls (Table 4.17) and SDAT subjects (Table 4.17). This is in agreement with Anderson (1987).

For a limited number of the SDAT samples we have data for plaque and tangle density (appendix 1). No correlations could be detected between plaque density and any of the parameters determined, however this was only a very small sample.

The chief observations of this study are deficits in BH_4 synthesis in temporal cortex (BA20, BA21 and BA38) and locus coeruleus and this is in agreement with earlier work (Anderson *et al.*, 1987). Additionally there is a BH_4 synthesis deficit in the visual cortex (BA17). BH_4 synthesis was not impaired in frontal cortex (BA9 & BA10) in agreement with earlier reports nor was there any reduction in activity in BA4.

The declines in BH_4 synthesis and DHPR activity with age and the inter-area variations of BH_4 synthetic activity, illustrate the necessity for selection of appropriate age matched controls and use of matching brain areas, when carrying out any study of BH_4 metabolism in a disease state especially one that occurs primarily in the sixth decade onwards such as Alzheimer's disease.

Table 4.1 BH_4 biochemistry in non-demented controls (frontal cortex)

Patient	DHPR	BH_4 synthesis	S.R.
1	253	3.74	0.25
2	553	4.76	0.30
3	226	4.04	0.17
4	372	4.97	0.17
5	253	2.82	0.18
6	275	5.70	0.25
7	322	5.20	0.35
8	313	3.10	0.56
9	242	5.80	0.28
10	464	2.70	0.20
11	327	1.85	0.29
12	230	2.17	0.19
13	296	1.32	0.21
14	246	1.00	0.22
15	410	1.12	0.30
mean	319	3.35	0.26
±s.d.	95	1.67	0.09

units:-
DHPR; nmoles NADH oxidised min^{-1} mg $protein^{-1}$

BH_4 synthesis; pmoles BH_4 synthesised $hour^{-1}$ mg protein $^{-1}$

S.R.; nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$

(S.R. :- sepiapterin reductase)

Samples obtained from Dr. P. Altmann, The London Hospital Whitechapel, London.

See appendix 1 for patient details

Table 4.2 BH_4 biochemistry in non-demented controls (temporal cortex)

Patient	DHPR	BH_4 synthesis	S.R.
1	314	5.08	0.28
2	413	3.45	0.24
3	314	3.70	0.18
4	107	1.91	0.16
5	385	3.90	0.23
6	313	5.20	0.20
7	388	2.80	0.29
8	352	5.70	0.32
9	276	4.80	0.20
10	396	6.10	0.20
11	315	1.89	0.19
12	284	1.17	0.14
13	416	1.01	0.13
14	299	2.34	0.34
15	262	0.63	0.21
mean	322	3.31	0.22
±s.d.	78	1.79	0.06

units:-

DHPR; nmoles NADH oxidised min^{-1} mg $protein^{-1}$

BH_4 synthesis; pmoles BH_4 synthesised $hour^{-1}$ mg protein $^{-1}$

S.R.; nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$

(S.R. :- sepiapterin reductase)

Samples obtained from Dr. P. Altmann, The London Hospital, Whitechapel, London.

See appendix 1 for patient details

Table 4.3 Summary of tables 4.1 & 4.2

	cortex	
parameter	frontal (n=15)	temporal (n=15)
DHPR	319 ± 95	322 ± 78
BH_4 synthesis	3.35 ± 1.67	3.31 ± 1.79
sepiapterin reductase	0.26 ± 0.09	0.22 ± 0.06
age (yrs)	61.8 ± 16.7	

units:- expressed as mean ± s.d.

DHPR:- nmoles NADH oxidised min^{-1} mg protein $^{-1}$

BH_4 synthesis:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

sepiapterin reductase:- nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$ (S.R.)

Table 4.4 DHPR activity in control subjects (MRC Batch 1)

	area			
subject	BA 9	BA 17	BA 20	BA 21
D-74	398	409	415	367
C-293	341	239	361	259
D-90	256	276	289	232
C-295	309	347	245	298
D-77	426	421	509	374
mean	346	338	364	306
±s.d.	68	81	104	63

units:- nmoles NADH min^{-1} mg $protein^{-1}$

Table 4.5 Sepiapterin reductase activity in control subjects (MRC Batch 1)

	area			
subject	BA 9	BA 17	BA 20	BA 21
D-74	0.31	0.19	0.30	0.27
C-293	0.24	0.20	0.31	0.28
D-90	0.36	0.20	0.28	0.34
C-295	0.20	0.25	0.30	0.30
D-77	0.20	0.38	0.25	0.26
mean	0.26	0.24	0.29	0.29
±s.d.	0.07	0.08	0.02	0.03

units:- nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$

Table 4.6 *In vitro* BH_4 synthesis activity in control subjects (MRC Batch 1)

	area				
subject	BA9	BA 17	BA 20	BA 21	LC
D 74	16.69	12.05	7.19	13.00	7.61
C 293	8.75	9.96	7.14	3.64	7.01
D 90	10.43	3.34	10.59	4.31	---
C 295	0.00	12.13	6.47	3.24	3.88
D 70	9.29	10.56	12.45	3.98	15.88
mean	9.03	9.61	8.77	5.44	8.80
±s.d.	5.96	3.62	2.61	4.13	5.12

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

See appendix 1 for patient details
LC = locus coeruleus

Table 4.7 DHPR activity in SDAT subjects (MRC Batch 1)

	area			
subject	BA 9	BA 17	BA 20	BA 21
D-64	394	327	427	431
D-67	295	264	331	297
D-69	241	259	201	326
D-70	264	342	260	298
D-73	356	451	385	379
mean	310	329	321	346
±s.d.	64	78	92	58

units:- nmoles NADH oxidised min^{-1} mg $protein^{-1}$

Table 4.8 Sepiapterin reductase activity in SDAT subjects (MRC Batch 1)

	area			
subject	BA 9	BA 17	BA 20	BA 21
D-64	0.25	0.34	0.31	0.34
D-67	0.28	0.26	0.30	0.35
D-69	0.31	0.22	0.20	0.21
D-70	0.21	0.29	0.21	0.26
D-73	0.22	0.29	0.23	0.22
mean	0.25	0.28	0.25	0.28
±s.d.	0.04	0.04	0.05	0.06

units:- nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$

Table 4.9 *In vitro* BH_4 synthesis in SDAT subjects (MRC Batch 1)

	area				
subject	BA 9	BA 17	BA 20	BA 21	LC
D 64	5.78	0.00	4.42!	0.14	25.71
D 67	11.11	5.27	0.64	0.00	19.06
D 69	0.05	1.67	1.53	0.00	19.06
D 70	0.00	2.17	0.00	0.00	18.42
D 73	0.00	0.00	0.25	1.88	14.54
mean	3.36	1.82	1.37	0.40	19.36
±s.d.	4.95	2.16	1.80	0.83	4.02

units:- pmoles $hour^{-1}$ mg $protein^{-1}$
! value fails Q-test
:- recalculated mean for BA 20 = 0.61 ± 0.67 n=4
See appendix 1 for patient details
LC = locus coeruleus

Table 4.10 Comparison of DHPR data in control and SDAT subjects (MRC Batch 1)

Area	Control	SDAT
BA 9	346±68	310±64
BA 17	338±81	329±78
BA 20	364±104	321±92
BA 21	306±63	346±58

n=5
units:- nmoles NADH oxidised min^{-1} mg $protein^{-1}$
mean±s.d

Table 4.11 Comparison of sepiapterin reductase in control and SDAT subjects (MRC Batch 1)

Area	Control	SDAT
BA 9	0.26±0.07	0.25±0.04
BA 17	0.24±0.08	0.28±0.04
BA 20	0.29±0.02	0.25±0.05
BA 21	0.29±0.03	0.28±0.06

n=5
units:- nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$
mean±s.d

Table 4.12 Comparison of *in vitro* BH_4 synthesis in control and SDAT subjects (MRC Batch 1)

Area	Control	SDAT	
BA 9	9.03±5.96(5)	3.36±4.95(5)	
BA 17	9.61±3.62(5)	1.82±2.16(5)	*
BA 20	8.77±2.61(4)	0.61±0.67(4)	***
BA 21	3.81±0.48(4)	0.04±0.07(4)	***
Locus coeruleus	8.80±5.12(4)	19.36±4.02(5)	!

n = 5
units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
mean±s.d

lower than control *p < 0.01 **p < 0.02 ***p < 0.001
! higher than control p<0.001
See appendix 1 for patient details

Table 4.13 *In vitro* BH_4 synthesis in SDAT and control brain samples from BA 38 (MRC Batch 2)

SDAT subjects

Patient no.	Plaque Density	BH_4 synthesis
D-57	4.29	0.00
D-59	0.90	2.61
D-61	2.69	2.69
D-62	1.26	0.00
D-78	3.35	1.21
mean	2.50	1.30
±s.d.	1.41	1.33
excl D-61	mean	0.96
	±s.d.	1.24

Controls

Patient no.	BH_4 synthesis
C-345	5.00
C-393	6.83
C-430	6.50
C-476	4.16
C-486	5.13
C-504	5.64
mean	5.54
±s.d.	1.00

SDAT significantly lower than controls
excl. D-61 ($p<0.001$) incl. D-61 ($p<0.001$)

See appendix 1 for patient details

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 4.14 *In vitro* BH_4 synthesis in SDAT and control brain samples from BA 4 (MRC Batch 2)

SDAT subjects

Patient no.	Plaque Density	BH_4 synthesis
D-57	4.29	4.66
D-59	0.90	5.08
D-61	2.69	3.68
D-62	1.26	3.39
D-78	3.35	4.45
mean	2.49	4.25
±s.d.	1.42	0.70
excl D-61	mean	4.40
	±s.d	0.72

Controls

Patient no.	BH_4 synthesis
C-345	4.81
C-393	4.73
C-430	3.55
C-476	5.62
C-486	6.06
C-504	5.64
mean	5.07
±s.d	0.91

n.s

See appendix 1 for patient details
units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 4.15 *In vitro* BH_4 synthesis in SDAT and control brain samples from BA 10 (MRC Batch 2)

SDAT subjects

Patient no.	Plaque Density	BH_4 synthesis
D-57	4.29	9.38
D-59	0.90	10.84
D-61	2.69	8.52
D-62	1.26	7.47
D-78	3.35	6.12
mean	2.49	8.47
±s.d.	1.42	1.80
excl D-61	mean	8.45
	±s.d.	2.08

Controls

Patient no.	BH_4 synthesis
C-345	8.03
C-393	9.66
C-430	10.76
C-476	7.21
C-486	7.53
C-504	7.64
mean	8.47
±s.d.	1.42

n.s.

See appendix 1 for patient details
units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 4.16 *In vitro* BH_4 synthesis in SDAT and control brain samples from Locus coeruleus (MRC Batch 2)

SDAT subjects

Patient no.	Plaque Density	BH_4 synthesis
D-57	4.29	10.48
D-59	0.90	7.76
D-61	2.69	12.51
D-62	1.26	6.64
D-78	3.35	9.25
mean	2.49	9.33
±s.d.	1.42	2.30
excl D-61	mean	8.53
	±s.d.	1.68

Controls

Patient no.	BH_4 synthesis
C-345	16.91
C-393	17.85
C-430	11.54
C-476	18.60
C-486	11.80
C-504	13.91
mean	15.10
±s.d.	3.10

SDAT significantly lower than controls
excl. D-61 $p<0.001$ incl. D-61 $p<0.01$

See appendix 1 for patient details
units:- pmoles BH_4 synthesised $hour^{-1}$ $mg\ protein^{-1}$

Table 4.17 *In vitro* BH_4 synthetic activity in control and SDAT subjects (BA 4, BA10, BA 38 & locus coeruleus)

Area	control	SDAT
BA 4	5.07±0.91 (6)	4.25±0.70(5)
BA 10	8.47±1.42 (6)*	8.47±1.80(5)**
BA 38	5.43±1.00 (6)	1.30±1.33(5)
locus coeruleus	15.10±3.10 (6)**	9.33±2.30(5)**

* significantly higher than BA 4 & BA 38 ($p<0.01$)

** significantly higher than BA 4, BA 10 & BA 38 ($p<0.001$)

mean±s.d. (n)

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

See appendix 1 for patient details

Table 4.18 Age v. BH_4 synthesis activity in human temporal cortex from non-demented subjects

No.	Sex	Age	BH_4 synthetic activity
c1	f	67	11.28
c2	m	83	2.27
c3	m	80	2.06
c4	m	85	2.12
c5	m	66	9.13
c6	f	79	4.75
c7	m	69	7.74
c8	m	72	16.92
D74	f	72	13.00
C293	m	82	3.64
D90	f	87	4.31
C295	f	75	3.25
D70	f	72	3.98
C345	f	81	5.00
C393	f	63	6.83
C430	f	64	6.50
C476	m	81	4.16
C486	m	73	5.13
C504	f	72	5.64
mean		75	6.19
±s.d.		7	3.94

r= -0.556 n=19 $p<0.05$

samples c1 to c8 control temporal cortex (Dr. Leeming, Haematology, General Hospital)

samples D-74 to D-70 (BA21) controls MRC Batch 1

samples C345 to C504 (BA38) controls MRC Batch 2

See appendix 1 for case histories and patient data

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

CHAPTER FIVE

ALUMINIUM AND TETRAHYDROBIOPTERIN METABOLISM

5.1 INTRODUCTION

Aluminium is now widely understood to be a neurotoxic metal ion. Intracisternal and intracranial injection of aluminium salts into rabbits produces an encephalopathy that is similar in neuropathology and clinical course to AD (Crapper *et al.*, 1980; Bruce-Martin, 1986).

The encephalopathy is characterized by neurofibrillary degeneration, decreased RNA content and nucleolar size (Crapper *et al.*, 1980). These changes are similar to those seen in AD, however the neurofilaments of Al induced encephalopathy are of a different structure to those of AD (Wisniewski *et al.*, 1980).

5.1.1 ALUMINIUM AND DIALYSIS DEMENTIA

The involvement of Al in progressive dialysis encephalopathy or 'dialysis dementia' has been investigated extensively (Arieff *et al.*, 1979; Sideman & Manor, 1982). Aluminium is absorbed from the intestine and in normal subjects the bulk of it is eliminated from the body (Alfrey, 1983). However in uraemic patients, aluminium accumulates in the body (Arieff *et al.*, 1979; Alfrey, 1986).

In the mid 1970s several renal care units reported that some patients on long term renal dialysis developed a

progressive, fatal, neurological disorder (Burks *et al.*, 1976; Alfrey, 1972; Chokroverty *et al.*, 1976). The condition was associated with the accumulation of massive amounts of aluminium in all body tissues including the brain (Arieff *et al.*, 1979). Alfrey *et al.* (1972) found that grey matter Al concentrations were significantly higher in patients dying with dialysis dementia (mean 24.98µg/g dry weight) than those dialysis pateints dying of other causes (mean 6.5 µg/g dry weight) or non-uraemic subjects (mean 2.18µg/g dry weight)

The aluminium was found to arise from two sources. The main source of aluminium was the dialysate fluid used in haemodialysis was found to contain high concentrations of aluminium (Wills & Savory, 1985). Secondly, to counteract the hyperphosphataemia, prevalent amongst uraemic individuals, they are given large daily oral doses of aluminium hydroxide gel, as a phosphate binder (Alfrey *et al.*, 1986; Sideman and Manor, 1982; Masselot *et al.*, 1978). High body aluminium burdens are also associated with continuous ambulatory peritoneal dialysis (CAPD) (Alfrey *et al.*, 1986) and in children with renal insufficiency treated with large amounts of aluminium hydroxide to counteract hyperphosphataemia. Such individuals often have symptoms of neurological dysfunction (Altmann *et al.*, 1987).

Confirmation of aluminium's causative role in dialysis dementia was achieved when the incidence of the condition fell dramatically with the adoption of rigorous water treatment techniques such as reverse osmosis and ion exchange.

Individuals maintained on high dose oral aluminium hydroxide treatment despite appearing to have no outward signs of neurological dysfunction, show subtle alterations in psychomotor function as revealed by symbol digit coding tests (SDCT) (Altmann *et al.*, 1987; Altmann *et al.*, 1989).

Treatment of these subjects with an aluminium chelator, desferrioxamine, produced significant improvements in psychomotor test scores.

In contrast to AD, there is no neurofibrillary degeneration in dialysis encephalopathy. This would indicate that the toxic effect of aluminium is not a result of such neuronal damage (Arieff *et al.*, 1979). Due to limited availability of samples, there are few reports of neurochemical analysis in dialysis encephalopathy. Levels of catecholaminergic and serotonergic metabolites, MHPG, HVA and 5-HIAA were normal in an area of high Al burden, frontal cortex (Perry *et al.*, 1985) suggesting that the activity of these neurochemical pathways is not affected in dialysis encephalopathy.

5.1.2 ALUMINIUM AND SENILE DEMENTIA OF THE ALZHEIMER TYPE

There is strong evidence that aluminium is a major causal factor in Alzheimer's disease (AD) or senile dementia of the Alzheimer type (SDAT) (Martyn *et al.*, 1989; Editorial, Lancet (i)p82, 1989; Lione, 1985)

Levels of aluminium in the brains of SDAT subjects are reported as increased (Crapper *et al.*, 1976) or normal (McDermott *et al.*, 1979). The discrepancy may arise due to differences in sample size (Crapper *et al.*, 1980). Aluminium levels are highest in the areas of greatest neuropathological damage (Crapper *et al.*, 1976).

In AD, levels of aluminium in certain areas, approach those which induce encephalopathy in animals (Crapper *et al.*, 1976). Aluminium has been shown to be localised in the neurones containing neurofibrillary tangles (Perl and Brody, 1980 a & b).

Ward *et al* (1986) using neutron activation analysis have shown high levels of aluminium around 10^{-5}M in temporal cortex and hippocampus of AD brain. Sensitive analysis of AD brain by use of electron microprobe x-ray analysis has demonstrated the presence of an aluminosilicate body in the core of the amyloid plaques characteristic of AD. Aluminium binds to chromatin granules in the nuclei of affected cells and as such may interfere with protein

synthesis, cell replication and other aspects of cellular metabolism (Wisniewski *et al.*, 1980).

In tangle bearing cells of normal aging individuals there is aluminium accumulation (McDermott *et al.*, 1979). It has been proposed that as the brain ages, there is decreased homeostatic control and this leads to the entry and accumulation of toxins, such as aluminium, in the brain (Mann, 1983). Aluminium levels in control and diseased tissue samples are summarised in Table 5.1.1.

Studies on the effects of aluminium on BH_4 metabolism have shown that aluminium inhibits DHPR both *in vivo* and *in vitro* (Leeming and Blair, 1979; Brown, 1981; Dhondt and Bellahsene, 1983). Patients on maintenance haemodialysis demonstrate elevated levels of neopterin and biopterin in the serum (Dhondt *et al.*, 1982), indicating that there is an impairment of BH_4 metabolism in chronic uraemia. Increased plasma DHPR activity correlates with improved psychometric performance in SDCT tests. Furthermore, cumulative oral aluminium load correlates with decreased response to a visually evoked stimulus, indicating reduced brain function (Altmann *et al.*, 1987). This report investigates the effects of aluminium on BH_4 metabolism and other biochemical parameters *in vivo* and *in vitro* in the rat, in conjunction with parallel *in vitro* studies of the effects of aluminium on several biochemical parameters in human brain tissue samples.

Table 5.1.1 Aluminium levels in control and diseased tissue samples

1 Uraemia

Brain (grey matter) aluminium (mg/kg dry weight)	
control	2.4±1.3(10)[1];0.9±0.9(19)[2]
non-dialysed uraemic	4.1±1.7(3)[1] ;6.6±4.2(8) [2]
dialysed uraemic	8.5±3.5(21)[1];3.8±1.8(5) [2]
dialysed uraemic (demented)	24.5±9.9(34)[1];12.4±9.7(4)[2]
Bone aluminium (mg/kg dry weight)	
control	3.3±2.9(16)[3]
non-dialysed uraemic	27.4±20.9(30)[3]
dialysed uraemic	116±107(30)[3]
dialysed uraemic (demented)	281±143(38)[3]
Serum aluminium (µg/litre)	
control	6.2±3.1(31)[4];10.8±8.1(21)[5]
non-dialysed uraemic	19.0±8.0(13)[6];13.4±6.6(45)[4]
dialysed uraemic	40±12(24)[6];109±10.6(47)[7]
dialysed uraemic (demented)	614.5(8)[8];165±40(17)[9]
uraemic on oral aluminium	94±36(24)[6];165±40(17)[6]

2 Alzheimer's Disease

Brain aluminium (mg/kg dry weight)	
Hippocampus (control)	2.684(30)[10]
Hippocampus (Alzheimers)	7.462(22)[10]
Cerebral cortex (control)	2.826(30)[10]
Cerebral cortex (Alzheimers)	9.140(22)[10]
Serum aluminium (µg/litre)	
control	5.9±2.3(8)[11]
Alzheimers	7.1±5.9(15)[11]
Cerebrospinal fluid (µg/litre)	
control	35.3±10.8(9)[11]
Alzheimers	30.7±7.3(5)[11]

data expressed as:- mean (n) or mean±s.d.(n)

References

[1] Alfrey *et al.*, 1979; [2] Arieff *et al.*, 1979 [3] Alfrey, 1980; [4] Marsden *et al.*, 1979; [5] Fleming *et al.*, 1982; [6] Boukari *et al.*, 1978; [7] McKinney *et al.*, 1982; [8] Elliot *et al.*, 1978; [9] Pogglitsch *et al.*, 1981; [10] Freudlich *et al.*, 1985; [11] Griswold *et al.*, 1983.

5.2 MATERIALS AND METHODS

The effect of aluminium salts on *in vitro* BH_4 synthesis was determined using human brain tissue samples of temporal cortex and whole brains from male wistar rats (150g).

In vivo studies used male weaner rats, dosed orally for three months by addition of aluminium acetate to drinking water forming a suspension. For the first month at a concentration of 0.75mM litre^{-1}, for the second month at 1% (w/v) and for the final month at 2%. (w/v). Controls received normal tapwater. Animals had access to food and water/aluminium acetate suspension *ad libitum* .

Chemicals, methods of enzyme analysis and assay of *in vitro* BH_4 synthetic capacity are as described in chapter 2.

5.3 RESULTS

Aluminium salts (incubation concentration 10^{-3}M) inhibited *in vitro* BH_4 synthesis in human brain preparations ($p<0.001$, Table 5.1). The effect of aluminium salts (sulphate, acetate and hydroxide) on *in vitro* synthesis in human brain preparations was investigated over a concentration range of 10^{-3}M to $5x10^{-6}$M. All inhibited BH_4 synthesis at concentrations between 10^{-3}M and 10^{-5}M (Table 5.2, $p<0.01$).

Aluminium acetate (1mM) had no effect on rat brain BH_4 synthesis *in vitro* (Table 5.3).

Chronic administration of aluminium as the acetate to rats was carried out over a three month period as described in 'Materials & Methods'. The results are shown in tables 5.4 *et seq*. Chronic dosing with aluminium acetate caused significant reductions in brain total biopterins ($p<0.001$, Table 5.4), liver total biopterins ($p<0.02$, Table 5.5) and plasma total biopterins ($p<0.01$, Table 5.7). *In vitro* BH_4 synthetic activity in the brain was also diminished ($p<0.002$, Table 5.8). The activities of sepiapterin reductase (Table 5.9) and GTP cyclohydrolase (Table 5.10) showed no significant alteration in the Al dosed group.

Dopamine-B-hydroxylase ($p<0.001$, Table 5.13) and tyrosine hydroxylase ($p<0.05$, Table 5.14) activities were significantly lowered in the Al dosed group compared to controls, whereas carboxypeptidase N1 (Table 5.15) activity was unaltered. The levels of cyclic adenosine monophosphate (cAMP) in the brains of the Al dosed group were significantly higher than those in the control group ($p<0.05$, Table 5.16).

The addition of transferrin (TF) (50μM) to rat brain preparations from Al dosed and control groups increased the *in vitro* BH_4 synthetic activity in the Al dosed brain

preparations. TF had no effect on brain preparations from the control group. Al dosed samples treated with TF had significantly higher BH_4 synthetic activity than untreated Al dosed samples ($p<0.01$, Table 5.17) and did not differ significantly from control brain preparations.

Analysis of samples of brain tissue from Alzheimer subjects showed a deficit of BH_4 synthesis in temporal cortex BA 38 ($p<0.001$) and locus coeruleus ($p<0.001$) (Tables 5.18 & 5.21). BH_4 synthesis did not differ significantly from controls in BA 4 and BA10 (Tables 5.19 & 5.20).

Treatment of these samples with TF (50μM) produced an improvement in BH_4 synthetic activity in the SDAT samples ($p<0.001$ n=20 Wilcoxon signed ranks test) whilst having no effect on the controls. TF elevated BH_4 synthesis in SDAT locus coeruleus ($p<0.05$, n=4, Students t-test).

5.4 DISCUSSION

Many studies have now established that in man, BH_4 levels are significantly reduced by inhibiting its formation from the precursor dihydroneopterin triphosphate at the level of 6-pyruvoyl-tetrahydropterin synthase (Hamon & Blair, 1987).

5.4.1 EFFECTS OF ALUMINIUM *IN VITRO*

Addition of aluminium salts to human temporal cortex preparations from non-demented controls produced a substantial reduction in BH_4 synthesis (Tables 5.1 & 5.2). This concentration is similar to levels found in brains of subjects who have died from Alzheimer's disease. (Ward et al, 1986). The lack of effect in rat brain (Table 5.3) probably reflects the difference in rate limiting steps between the two species. In man BH_4 synthesis is controlled at 6-PTPS, catalysing the magnesium dependent dephosphorylation of dihydroneopterin triphosphate, whereas in the rat, GTP cyclohydrolase is the rate limiting step of BH_4 synthesis, this reaction needing no metal ion cofactor.

5.4.2 EFFECTS OF ALUMINIUM *IN VIVO*

The pattern of observations seen in rats chronically dosed with Al is similar to that seen in AD. BH_4 levels and synthesis are diminished, but this is not a result of neuronal loss as sepiapterin reductase and GTP cyclohydrolase activities are unchanged (Tables 5.4 to 5.10). Thus the defect must lie at 6-PTPS.

The difference between the effects of Al on rat brain BH_4 synthesis *in vitro* and *in vivo* probably reflects the slow kinetics of magnesium ion displacement from the enzyme

molecule. Over the short three hour incubation of the *in vitro* studies little ion exchange between aluminium and magnesium will occur. However over the three month chronic aluminium exposure a greater degree of magnesium displacement and subsequent inhibition of BH_4 synthesis may occur.

Tyrosine hydroxylase (TH) employs iron as a cofactor and has reduced activity in AD. Dopamine-β-hydroxylase has copper as a cofactor and it too is reduced in activity in AD. Chronic administration of Al further mimics AD pathology in that, the activities of dopamine-β-hydroxylase, tyrosine hydroxylase (Tables 5.13 & 5.14) and choline acetyl-transferase (CAT) (Edwards *et al.*, 1987) are also reduced. Reduced CAT activity in temporal cortex is a classical lesion of AD (Perry *et al.*, 1980).

Aluminium elevates levels of cAMP (fig 5.1) in the cerebral cortex, hippocampus, striatum and cerebellum in rats (Ebstein *et al.*, 1986). Al stimulates cAMP production from ATP, by adenylate cyclase (fig 5.1) *in vitro* (Ebstein *et al.*, 1986), it also inhibits cAMP breakdown to 5'-AMP by cyclic nucleotide phosphodiesterase (fig 5.1) *in vitro* . Similar observations are reported here (Table 5.16). Adenylate cyclase and phosphodiesterase are magnesium dependent enzymes.

5.4.3 ALUMINIUM AND METALLOZYMES

Dosing with aluminium causes a pattern of reduction in the activity of metal ion dependent enzymes or metallozymes similar to that seen in AD. Why then are metalloenzymes primarily affected in AD ?

One possible explanation is that aluminium displaces the metal ion from the enzyme and thus renders it inactive. During metallozyme activity the metal ion commonly changes its valency, as in tyrosine hydroxylase, Al however, cannot change valency, thus if enzyme activity is dependent on such a change, catalysis will be inhibited if the usual ion is replaced by Al.

The Al cation is small enough to replace any of the common biologically relevant cations from their enzymes and Al substitution may inhibit the enzymes (Table 5.4.1.). The similarity between the biochemical lesions induced in the rat by giving high oral doses of Al and some biochemical stigmata of SDAT is shown in Table 5.4.2.

Fig 5.1 cyclic AMP metabolism

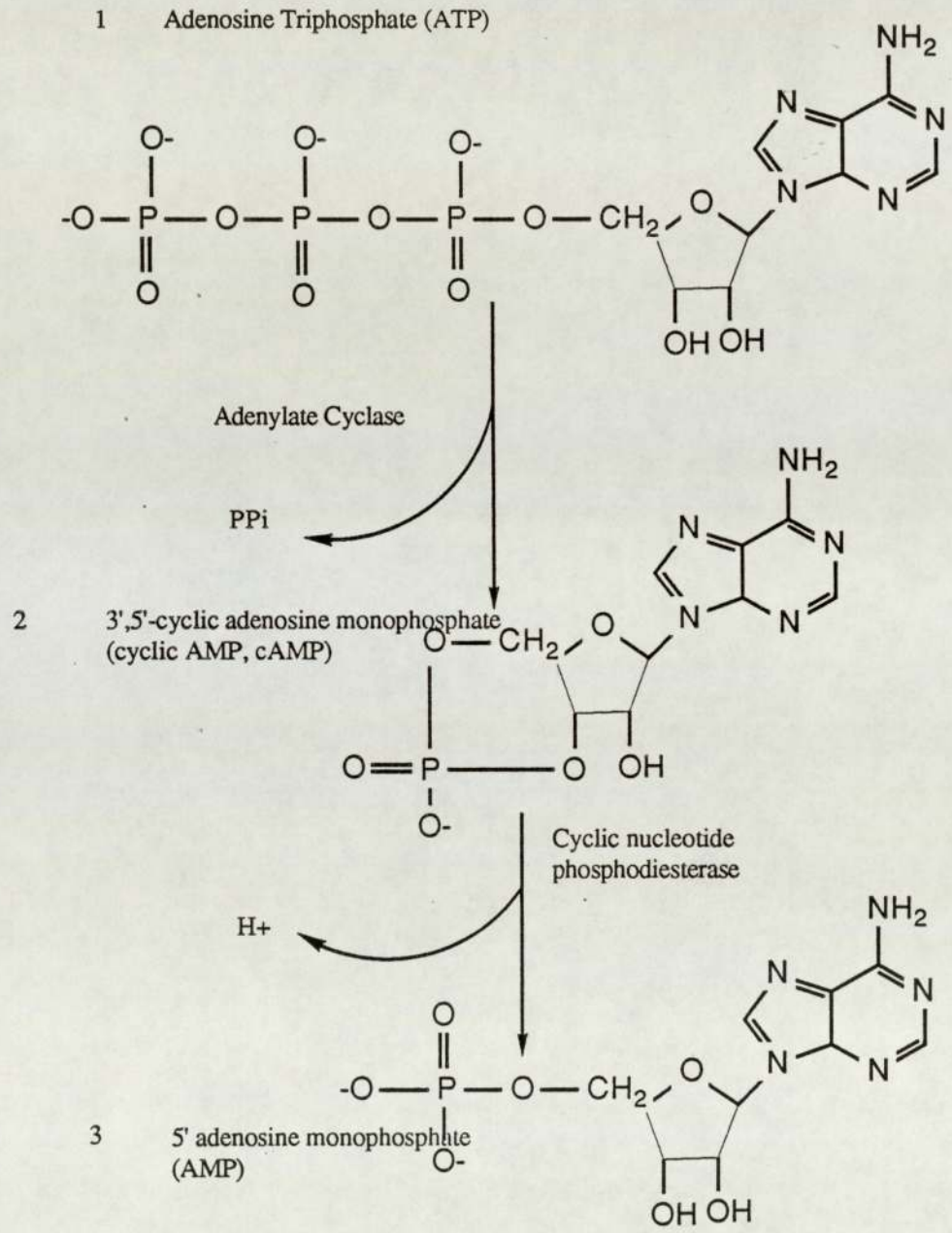


Table 5.4.1 Ionic Radii & Valency of Biologically Important Cations and Aluminium.

Ion	Valency	Radius (nm)
Lithium	1+	0.068
Sodium	1+	0.098
Potassium	1+	0.133
Magnesium	2+	0.065
Calcium	2+	0.094
Iron	2+	0.076
Iron	3+	0.064
Copper	1+	0.096
Copper	2+	0.069
Zinc	2+	0.074
Aluminium	***3+***	***0.045***

Source:- Nuffield book of data. p.54 Penguin Books

Table 5.4.2 Enzyme defects and metabolite alterations in SDAT and in rats after treatment with Al.

Parameter	SDAT	Aluminium
GTP cyclohydrolase	no change	no change
sepiapterin reductase	no change	no change
BH_4 synthesis	inhibited	inhibited
Brain biopterin levels	lowered	lowered
tyrosine hydroxylase	inhibited	inhibited
choline acetyl transferase	inhibited	inhibited
dopamine-β-hydroxylase	inhibited	inhibited
cAMP levels	elevated	elevated

5.4.4 EFFECTS OF TRANSFERRIN

Inhibition of BH_4 synthesis in rats after chronic Al dosing was reversed by treatment with a powerful Al chelator, transferrin (Huebers and Finch, 1987; Larson *et al.*, 1981), further suggesting that Al is the causative agent of the inhibition (Table 5.17). Treatment with transferrin improved BH_4 synthesis in SDAT brain samples (Tables 5.18 to 5.21).

To conclude, the biochemical evidence now available lends further support to the hypothesis that aluminium is a major cause of the Alzheimer pathology. Al induced neurotoxicity may be mediated via the inhibition or activation of metal ion requiring enzymes or metallozymes, as the Al ion displaces the ion normally required for correct enzyme function. Aluminium is far from innocuous and is a known neurotoxin. The evidence presented here further strengthens the case for its involvement in Alzheimer's disease by shedding some light on a possible mechanism for enzyme inhibition and demonstrating a reversal of a biochemical deficit in Alzheimer brain samples by treatment with an aluminium chelator.

Table 5.1. Effect of aluminium on *in vitro* BH_4 biosynthesis in human brain preparations

Al salt incubation concentration = $10^{-3}M$

	Control	Al sulphate	Al acetate
mean	3.95	0.92*	1.43*
±s.d.	0.98	0.21	0.69

* $p<0.001$ n=6

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 5.2. The dose response effect of aluminium on *in vitro* BH_4 synthesis in human brain preparations

	Al salt		
[Added Al]	sulphate	acetate	hydroxide
control	3.98±1.68	5.24±1.32	5.71±2.10
$10^{-3}M$	0.00±0.00*	0.05±0.01*	0.00±0.00*
$5x10^{-4}M$	1.31±1.77*	2.13±0.56*	2.26±1.08*
$10^{-4}M$	1.14±0.77*	2.56±1.12*	2.19±0.34*
$5x10^{-5}M$	0.52±0.74*	1.58±0.52*	1.95±0.53*
$10^{-5}M$	1.25±1.31*	1.72±0.89*	2.29±0.72*
$5x10^{-6}M$	3.28±0.87	4.62±1.39	5.89±1.46

* $p<0.01$ mean±s.d. (n=6)

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 5.3 Effect of aluminium on *in vitro* BH_4 biosynthesis in rat brain preparations

Al salt incubation concentration = 10^{-3}M

	Control	Al acetate
mean	1.95	1.65
±s.d.	0.98	1.90
n=6		n.s.

units:- pmoles BH_4 synthesised $hour^{-1}$ $mg\ protein^{-1}$

Tables 5.4 to 5.17.

The *in vivo* effects of chronic dosing with aluminium acetate (as suspension) in drinking water for three months.

1st month @ 0.75mM
2nd month @ 1% (w/v)
3rd month @ 2% (w/v)

Table 5.4 Brain total biopterins (ng/g wet weight tissue)

	Control	Aluminium
mean	88.1	59.4*
±s.d.	6.2	7.1

* $p<0.001$

(n=5)

Table 5.5 Liver total biopterin
(µg g wet weight^{-1})

	Control	Aluminium
mean	0.87	0.75*
±s.d.	0.08	0.04

* $p<0.02$

(n=5)

Table 5.6 Liver derived pterin
(µg g wet weight^{-1})

	Control	Aluminium
mean	0.12	0.10
±s.d.	0.04	0.03

(n=5)

Table 5.7 Plasma total biopterin
(ng ml plasma^{-1})

	Control	Aluminium
mean	33.5	27.7*
±s.d.	3.2	2.0

*$p<0.01$

(n=5)

Table 5.8 Brain *in-vitro* BH_4 synthesis
(pmoles BH_4 synthesised hour^{-1} mg protein^{-1})

	Control	Aluminium
mean	14.71	5.35*
±s.d.	4.15	0.97

* $p<0.002$

(n=5)

Table 5.9 Brain sepiapterin reductase (nmol sep. reduced min^{-1} mg $protein^{-1}$)

	Control	Aluminium
mean	0.34	0.33
±s.d.	0.02	0.03

(n=5)

Table 5.10 Brain GTP cyclohydrolase (ng neopterin $hour^{-1}$ mg $protein^{-1}$)

	Control	Aluminium
mean	0.28	0.31
±s.d.	0.07	0.06

(n=5)

acid iodine method (See chapter 2)

Table 5.11 Brain protein concentration (mg protein ml $homogenate^{-1}$)

	Control	Aluminium
mean	13.86	12.18*
±s.d.	0.94	0.18

* $p<0.01$

(n=5)

Table 5.12 Brain weight (g)

	Control	Aluminium
mean	1.84	1.80
±s.d.	0.09	0.24

(n=5)

Table 5.13 Brain dopamine β-hydroxylase (μmoles octopamine produced min^{-1} mg $protein^{-1}$)

	Control	Aluminium
mean	7.58	0.87*
±s.d.	1.25	0.24

* $p<0.001$

(n=5)

Table 5.14 Brain tyrosine hydroxylase activity (nmol/min/g wet wt.)

	Control	Aluminium
mean	4.99	2.47*
±s.d.	0.95	1.77

*$p<0.05$

(n=5)

Table 5.15 Liver carboxypeptidase N1 activity (μmol/min/mg protein.)

	Control	Aluminium
mean	23.3	19.3
±s.d.	4.3	2.7

(n=5)

Table 5.16 cAMP levels in rat brain (pmoles cAMP ml $supernatant^{-1}$)

	Control	Aluminium
mean	0.58	1.41*
±s.d.	0.16	0.74

*$p<0.05$

(n=5)

Table 5.17 The effect of transferrin (TF) on *in vitro* BH_4 synthesis in tissue from control rats and those dosed with Al

control group

	Control	Control + TF (50μM)
mean	11.67	10.97
±s.d	1.81	2.07

(n=5)

Al dosed group

	Al	Al+ TF(50μM)
mean	6.56	9.60*
±s.d	1.24	1.42

* Al+TF › Al ($p<0.01$)

(n=5)

Al group significantly lower than both control groups ($p<0.001$)

Al + transferrin (50uM) not significantly different from either control group.

Table 5.18 The effect of transferrin (TF) on *in vitro* BH_4 biosynthesis in human brain preparations from controls and SDAT subjects (BA 38)

SDAT subjects

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF (50μM)
D-57	64	f	0.00	0.34
D-59	82	f	2.61	4.11
D-61	71	f	2.69	2.42
D-62	63	f	0.00	0.37
D-78	72	m	1.21	1.00
		mean	1.30	1.65
		±s.d.	1.33	1.61

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Controls

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
C-345	81	f	5.00	3.57
C-393	63	f	6.83	3.99
C-430	64	f	6.50	6.06
C-476	81	m	4.16	6.29
C-486	73	m	5.13	4.30
C-504	72	f	5.64	4.67
		mean	5.54	4.81
		±s.d.	1.00	1.11

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

SDAT < Control $p<0.001$

Table 5.19 The effect of transferrin (TF) on *in vitro* BH_4 biosynthesis in human brain preparations from controls and SDAT subjects (BA 4)

SDAT subjects

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
D-57	64	f	4.66	4.97
D-59	82	f	5.08	5.79
D-61	71	f	3.68	3.15
D-62	63	f	3.39	3.64
D-78	72	m	4.45	5.78
		mean	4.25	4.67
		±s.d.	0.70	1.22

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Controls

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
C-345	81	f	4.81	3.30
C-393	63	f	4.73	5.35
C-430	64	f	3.55	3.69
C-476	81	m	5.62	5.69
C-486	73	m	6.06	5.96
C-504	72	f	5.64	4.67
		mean	5.07	4.78
		±s.d	0.91	1.09

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

n.s.

Table 5.20 The effect of transferrin (TF) on *in vitro* BH_4 biosynthesis in human brain preparations from controls and SDAT subjects (BA 10)

SDAT subjects

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
D-57	64	f	9.38	11.14
D-59	82	f	10.84	10.68
D-61	71	f	8.52	7.60
D-62	63	f	7.47	7.54
D-78	72	m	6.12	6.66
		mean	8.47	8.72
		±s.d.	1.80	2.04

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Controls

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
C-345	81	f	8.03	11.39
C-393	63	f	9.66	8.82
C-430	64	f	10.76	9.35
C-476	81	m	7.21	10.10
C-486	73	m	7.53	11.30
C-504	72	f	7.64	4.81
		mean	8.47	9.30
		±s.d.	1.42	2.42

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

n.s.

Table 5.21 The effect of transferrin (TF) on *in vitro* BH_4 biosynthesis in human brain preparations from controls and SDAT subjects (Locus coerueleus)

SDAT Subjects

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
D-57	64	f	10.48	13.53
D-59	82	f	7.76	12.27
D-61	71	f	12.51	10.89
D-62	63	f	6.64	10.81
D-78	72	m	9.25	10.34
		mean	9.33	11.57
		±s.d.	2.30	1.31

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Controls

Patient no.	Age years	Sex m/f	BH_4 synthesis control	+TF(50μM)
C-345	81	f	16.91	15.94
C-393	63	f	17.85	14.92
C-430	64	f	11.54	16.15
C-476	81	m	18.60	11.77
C-486	73	m	11.80	9.67
C-504	72	f	13.91	8.35
		mean	15.10	12.80
		±s.d.	3.10	3.35

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

SDAT ‹ Control $p<0.001$

Combining all SDAT subject data (BA38 + BA4 + BA10 + locus coeruleus: n=20)
then: SDAT+TF › SDAT $p<0.05$ (Wilcoxon sum of ranks)

CHAPTER SIX

LITHIUM, DEPRESSION AND TETRAHYDROBIOPTERIN METABOLISM

6.1 INTRODUCTION

Lithium is the first member of the group 1 or alkali metals in the periodic table of elements. Other members of this group include sodium and potassium, ions of great physiological importance, especially in the generation and conduction of neuronal and muscular electrical activity. To date there is no known biochemical role for lithium in the body.

Lithium has the smallest ionic radius of any metal ion, but in aqueous solution is effectively larger than both sodium or potassium as it becomes fully hydrated (Birch 1973). After ingestion, lithium is rapidly absorbed from the intestinal lumen. To date there is no evidence for lithium uptake being an active process, absorption occuring passively (Birch *et al.*, 1973). After absorption lithium is thought to accumulate in organs in the following decreasing order of preference after entry into the blood:- kidney, liver, bone, muscle and brain (Birch 1982).

The hydrated lithium cation is not lipid soluble and this in conjunction with the highly selective nature of the blood-brain barrier explains the poor penetration of the brain by lithium. Brain lithium levels peak 24 hours after oral administration to rats (Birch *et al.*, 1978).

Parenterally administered lithium has no effect on sodium and potassion ion levels in human CSF. Lithium has been shown to decrease levels of sodium and magnesium ions in rat brains (Birch and Jenner, 1973).

6.1.1 LITHIUM AND BIPOLAR AFFECTIVE DISORDER

Lithium was first used in the treatment of mania by Cade in 1949. Early studies showed no effect, however these were poorly controlled and used 'melancholic subjects', who would be classified today as unipolar depressives, on whom lithium has only minor beneficial effects (Johnson and Johnson 1978). Clinical trials have now shown it to produce responses better than placebo (Abou-Saleh and Coppen, 1986). Despite its widespread use in the treatment of BAD now for many years, the exact mechanism of its action is unclear.

Currently in the UK there are some 25,000 patients receiving lithium therapy, representing 1 in 2000 of the population. Despite extensive study, the biochemical basis of BAD and the effect of lithium on the disease process are still not known (Birch, 1978).

Depression can be classified as a unipolar or bipolar disorder. Unipolar depressives exhibit only a depressed mood; this is also known as true depression. Bipolar depression or bipolar affective disorder is a condition in

which the depressed mood of the subject is alternated with a manic mood characterised by exaggerated and inappropriate joyful emotion. BAD is also known as manic-depression.

Depression that occurs for no apparent reason is known as endogenous depression in contrast to reactive depression, which is a result of a stressful or traumatic life event, such as bereavement or divorce. Primary depression occurs in the absence of any underlying physiological disorder, whereas the depression occurring in those suffering from chronic pain, terminal disease, alcoholism etc, is termed secondary depression (Johnson and Johnson 1978).

To the clinician, the most commonly used definition of depression is that of the unipolar/ bipolar disorder. It is this classification that will be used in this study. It is thought that BAD is a familial condition, that is, it is transmitted genetically (Schlesser, 1979). In monozygotic twins, if one member develops BAD, then the other has an 80% chance of developing the condition. Unipolar depression appears to have no genetic component. The symptoms of depression include intense sadness, abdominal pain, headache, dizziness and lassitude. The condition has a high mortality rate, if left untreated, as the sufferer develops suicidal intentions and will attempt, usually successfully, suicide.

The biochemical nature of depressive illness has been speculated upon for many years. The current hypothesis centres on the levels of serotonin and catecholamines in the brain (Van Praag, 1982). It is thought that neurotransmission by these compounds is in some way impaired in depression. Certainly, tricyclic antidepressant drugs act to down regulate the post synaptic receptor density, by inhibiting neurotransmitter reuptake or by increasing post synaptic receptor sensitivity. These actions serve to potentiate catecholaminergic and serotonergic neurotransmission. A new drug 'Rolipram', undergoing clinical trials acts to inhibit cyclic nucleotide phosphodiesterase, the enzyme responsible for the conversion of 3',5'-cyclic adenosine monophosphate (3'5'-cAMP) to 5'-adenosine monophosphate (5'-AMP). Rolipram may therefore exert its antidepressant effect via the secondary messenger system, which is 'switched on and off' by the neurotransmitters.

6.1.2 BH_4 METABOLISM IN BIPOLAR AFFECTIVE DISORDER

As tetrahydrobiopterin (BH_4) is the cofactor for the synthesis of the catecholamine neurotransmitters and serotonin, its involvement in depression has been investigated. The bulk of studies have been on the urinary excretion of biopterin and neopterin. In unipolar depression elevated urinary levels of biopterin and neopterin have been reported (Duch *et al.*, 1984).

Blair *et al.*, (1984) found normal levels of biopterin in the urine of unipolar depressives.

Similar contradictory reports are seen for bipolar depression. Blair *et al.*, (1984) report decreased urinary biopterin, whereas normal urinary biopterin with elevated urinary neopterin was reported by Duch *et al.*, (1984).

The probable cause of the contradictory nature of these reports lies in subject selection. Some subjects were 'drug free', others were on maintenance lithium, it is important in such studies to have subjects which are standardised for pharmacological status at the time of investigation. CSF BH_4 levels in depression are not different from controls (Hamon and Blair, 1987). The BH_4 synthetic capacity of samples of temporal cortex was found to be diminished in BAD (Blair *et al.*, 1984).

This investigation set out to look at the effects of lithium on tetrahydrobiopterin metabolism in man and the rat. *In vitro* investigations were carried out using samples of human temporal cortex from individuals with no history of depressive disorder. The effects of chronic (3 months) oral administration of lithium salts in equivalent therapeutic doses to rats were investigated.

6.2 MATERIALS AND METHODS

The effect of lithium on *in vitro* BH_4 synthesis in human and rat brain preparations was investigated. *In vitro* enzyme assays were carried out as detailed in chapter 2 except that lithium was added to the incubation medium in the concentrations shown in the results tables.

In *in vivo* investigations, weaner male wistar rats were given lithium carbonate in drinking water over a three month period. For the first month the animals were given a solution of lithium carbonate assuming an average fluid intake of 15mls day^{-1}, and an equivalent dose for a 70kg human of 0.25g day^{-1}. For the second and third month, average fluid intake was assumed to be 25mls day^{-1} and an equivalent dose for a 70kg human of 1g day^{-1}. The daily water intakes may at first sight seem higher than those normally quoted for rats, but are elevated to allow for increased thirst, a known side effect of lithium.

6.3 RESULTS

Lithium (10^{-4}M) had no effect on *in vitro* BH_4 synthesis in rat brain and rat liver preparations (Tables 6.1 & 6.2). In human brain lithium inhibited *in vitro* BH_4 synthesis at concentrations as low as 50uM. Lithium concentrations below this had no significant effect (Tables 6.3 & 6.4).

Administration of lithium to rats in their drinking water for a period of three months (section 6.2) caused significant reductions in brain total biopterins (Table 6.5; $p<0.001$), liver total biopterins (Table 6.6; $p<0.001$) and plasma total biopterins (Table 6.8; $p<0.01$).

In vitro BH_4 synthetic activity was reduced in the lithium group (Table 6.9; $p<0.001$). Chronic administration of lithium had no effect on sepiapterin reductase or dihydropteridine reductase (DHPR) (Tables 6.10 and 6.11).

Brain homogenate protein concentration (Table 6.12; $p<0.001$) and brain weight (Table 6.13; $p<0.001$) were both reduced in the lithium dosed group compared to controls. Dopamine-β-hydroxylase activity was significantly reduced in the lithium dosed group compared to controls (Table 6.15; $p<0.05$).

6.4 DISCUSSION

This study has shown that lithium causes significant inhibition of *in vitro* BH_4 synthesis in human brain preparations, and is effective in this respect at concentrations as low as $5x10^{-5}M$ (tables 6.3 & 6.4). *In vitro* BH_4 synthesis correlates significantly with lithium concentration on a log v. log plot ($r=0.9388$ $p<0.05$).

In rat brain preparations, however, no such effect is seen (tables 6.1 & 6.2). The difference between the two systems may be due to there being different rate controlling steps in the synthesis of BH_4 in man and the rat.

In the rat the rate controlling step is the first enzyme in the synthetic sequence, GTP cyclohydrolase (Duch *et al.*, 1984a); in man it is 6-pyruvoyl-tetrahydropterin synthase (6-PTPS) (Lovenberg & Levine 1987). 6-PTPS requires magnesium as a co-enzyme and the lithium may interact with the magnesium causing a decrease in the activity of the enzyme and so affect the synthesis of BH_4.

Studies in the rat have shown that lithium ions do interfere with other metal ions including magnesium (Birch & Jenner 1973). Lithium inhibits dopamine-B-hydroxylase (table 6.15). This metalloenzyme has a copper ion cofactor, enzyme inhibition may be due to displacement of the copper ion by lithium.

Lithium is used prophylactically in BAD to prevent further manic episodes once the initial mania has been controlled with tranquillizers. The mechanism of action is unclear. In BAD patients, not treated with lithium, there is an increase in urinary total biopterin output compared to controls possibly reflecting increased BH_4 synthetic activity (Garbutt *et al.*, 1986).

Lithium may act in mania to inhibit metal ion dependent enzymes and other enzymes crucial to optimal noradrenergic activity such as dopamine-B-hydroxylase and 6-PTPS (Agar *et al.*, 1975). In mania there is evidence of abnormal noradrenergic metabolism characterised by elevated urinary 3-methoxy4-hydroxyphenylglycol (MOPEG) though the data do not support the idea that mania is the 'biochemical antithesis' of depression (Schildkraut *et al* 1977). If anything the conditions have very similar biochemical profiles.

Lithium has been shown to have marked effects on the inositol phosphate second messenger system. The dephosphorylation of inositol phosphates is a metal ion dependent process, requiring magnesium. Noradrenaline stimulated adenylate cyclase responsible for generation of cyclic AMP is also a magnesium dependent enzyme that is inhibited at therapeutic plasma lithium concentrations (Ebstein *et al.*, 1980) lending further support to the theory that lithium acts by interference with crucial metal ion dependent systems and subsequent alteration of the cellular response to neurotransmitters or causing a reduction in the biosynthesis of the neurotransmitters either directly or by decreasing cofactor availability.

Table 6.1 Effect of lithium on tetrahydrobiopterin (BH_4) biosynthesis in rat brain

	Control	Lithium 10^{-4}M
mean	1.95	0.90
±s.d.	0.98	0.69 n.s.

n=6

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 6.2 Effect of lithium on tetrahydrobiopterin (BH_4) biosynthesis in rat liver

	Control	Lithium 10^{-4}M
mean	31.01	24.68
±s.d.	5.36	8.86 n.s.

n=6

units pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 6.3 Dose response effect of lithium on *in vitro* BH_4 biosynthesis in human brain tissue

	Control	Li^+ 10^{-3}	Li^+ $5x10^{-4}$	Li^+ 10^{-4}	Li^+ $5x10^{-5}$
mean	5.63	1.88	1.96	2.32	1.38
±s.d.	0.95	1.61	2.12	1.22	0.64
p		‹0.002	‹0.01	‹0.001	‹0.001
n	6	4	5	6	5

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 6.4 Dose response effect of lithium on *in vitro* BH_4 biosynthesis in human brain tissue

Control	Li^+ (M)			
	10^{-6}	10^{-7}	10^{-8}	10^{-9}
mean 3.20	2.75	1.78	2.53	1.92
±s.d 1.31	0.70	1.77	1.18	1.41
p	n.s	n.s.	n.s.	n.s

n = 5

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

Table 6.5 *et seq.*
The effects of chronic (three months) lithium administration on rats *in vivo*.
Dose regimen
1st month:- human equivalent dose 0.25g day^{-1}
2nd & 3rd months:- human equivalent dose 1g day^{-1}
see section 6.2 for details.

Table 6.5 Brain total biopterin (ng biopterin g wet $weight^{-1}$.)

	Control	Lithium
mean	88.1	65.1*
±s.d.	6.5	8.7

* $p<0.001$

Table 6.6 Liver total biopterin (µg g wet $weight^{-1}$)

	Control	Lithium
mean	0.87	0.48*
±s.d.	0.08	0.14

* $p<0.001$

Table 6.7 Liver derived pterin
(μg g wet weight^{-1})

	Control	Lithium
mean	0.12	0.08
±s.d.	0.04	0.01

Table 6.8 Plasma total biopterin
(ng ml plasma^{-1})

	Control	Lithium
mean	33.5	23.9*
±s.d.	3.2	5.2

* $p<0.01$

Table 6.9 Brain *in-vitro* BH_4 synthesis
(pmol hour^{-1} mg protein^{-1})

	Control	Lithium
mean	14.71	4.28*
±s.d.	4.15	1.33

* $p<0.001$

Table 6.10 Brain sepiapterin reductase activity
(nmol sepiapterin reduced min^{-1} mg protein^{-1})

	Control	Lithium
mean	0.34	0.31
±s.d.	0.02	0.02

Table 6.11 Brain DHPR activity
(nmol NADH reduced min^{-1} mg $protein^{-1}$)

	Control	Lithium
mean	304	332
±s.d.	56	75

Table 6.12 Brain protein concentration
(mg protein ml $homogenate^{-1}$)

	Control	Lithium
mean	13.86	10.77*
±s.d.	0.94	0.86

* $p<0.001$

Table 6.13 Brain weight (g)

	Control	Lithium
mean	1.84	1.40*
±s.d.	0.09	0.20

* $p<0.001$

Table 6.14 Body weight (g)

	Control	Lithium
mean	582	549
±s.d.	21	45

Table 6.15 Dopamine β-hydroxylase
(μmoles octopamine produced min^{-1} mg $protein^{-1}$)

	Control	Lithium
mean	7.58	5.86*
±s.d.	1.25	0.37

* $p<0.05$

CHAPTER SEVEN

PSYCHOACTIVE DRUGS BIOMOLECULES AND TETRAHYDROBIOPTERIN METABOLISM

7.1 INTRODUCTION

As seen in the previous chapter, the metabolism of BH_4 is affected in depression, a common psychiatric disorder. In view of the effects of the lithium ion on BH_4 metabolism, and the possibility that this may possibly contribute to the therapeutic effect of lithium in the treatment of bipolar affective disorder, this chapter sets out to investigate the effects of other psychoactive drugs on BH_4 metabolism. Neurotransmitters and their precursors are reported to affect BH_4 metabolism in the rat (Sueoka and Katoh, 1986). This report looks at the effects of these compounds on human brain BH_4 metabolism. Hamon *et al.*, (1986) suggest that 5-methyl-tetrahydrofolate is necessary for BH_4 synthesis. This observation has proved difficult to repeat. In view of this, this report looks at the effects of 5-MeTHF and the compounds associated with methionine synthetase, on human brain BH_4 synthesis in order to clarify the earlier work.

7.1.1 TRICYCLIC ANTIDEPRESSANTS

The tricyclic antidepressants (fig 7.1) are common drugs of choice used in the treatment of depression. A major method of neurotransmitter inactivation at the nerve ending is by a high affinity, energy requiring, uptake system (Iversen, 1975), and the tricyclics inhibit this.

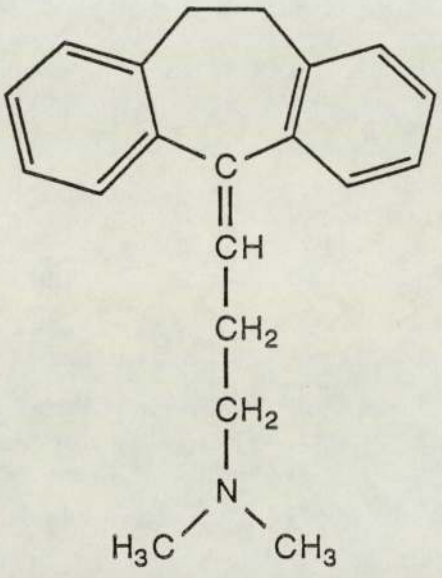
The mechanism of action of the tricyclics is not clear but it has been suggested that they act by causing an elevation of neurotransmitter levels in the synaptic cleft following inhibition of reuptake, leading to increased monoamine function (Iverson and Mackay, 1978).

However tricyclics also inhibit neurotransmitter biosynthesis. When synaptic neurotransmitter levels exceed a certain threshold, there is stimulation of presynaptic alpha adrenoceptors and this causes inhibition of neurotransmitter biosynthesis (Langer, 1977).

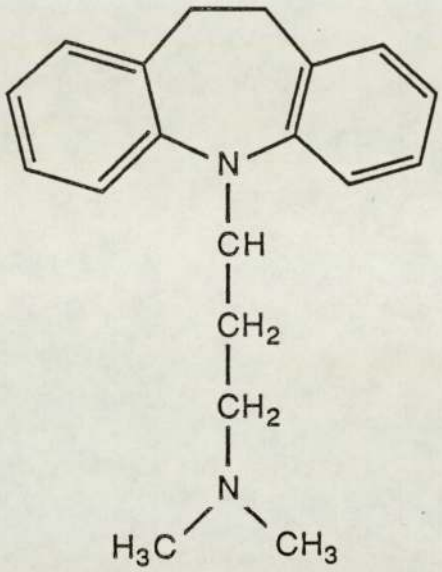
Furthermore, changes in monoamine synthesis and blockade of neuronal reuptake occur very soon after drug administration. In marked contrast, the drugs take 2 to 3 weeks to produce their antidepressant effects (Green and Costain, 1981).

Thus their efficacy cannot be a result of simple uptake blockade. It is thought that they may act by antagonizing presynaptic alpha 2 noradrenergic receptors (U'Pritchard *et al.*, 1978) or by alteration of the sensitivity of the forebrain noradrenergic sensitive adenylate cyclase. These mechanisms would explain the delay in onset of therapeutic effect (Vetulani *et al.*, 1976).

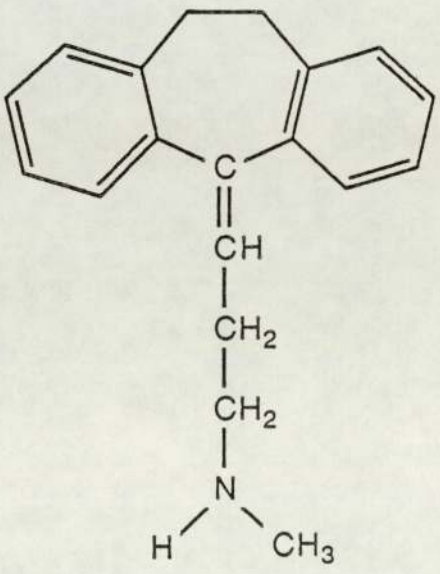
1 Amitriptyline



2 Imipramine



3 Nortriptyline



4 Desipramine

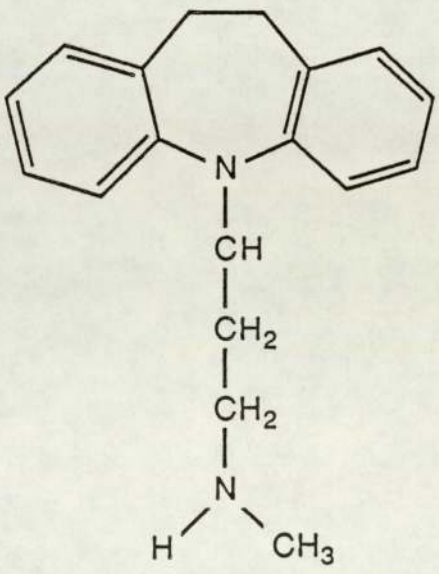


Fig 7.1 tricyclic antidepressants

Mianserin (fig 7.2) was developed as a possible 5-HT antagonist in the treatment of migraine. It was used as an antidepressant after EEG studies showed it induced changes in the EEG similar to those seen after amitriptyline administration (Itil *et al.*, 1972).

Midalcipran (fig 7.2) is a new potential antidepressant with equipotent inhibition of noradrenaline and serotonin reuptake but with no antagonistic effects at post-synaptic receptors (Moret *et al.*, 1985).

Rolipram (fig 7.2) is currently in clinical trials to assess its antidepressant activity (Cattell, 1988). This drug has no uptake blocking activity but instead is a phosphodiesterase inhibitor. Four of the tricyclics in addition to the newer antidepressants mianserin, midalcipran and rolipram have been used in *in-vitro* and *in-vivo* experiments to determine their effects, if any on BH_4 metabolism.

7.1.2 MONOAMINE OXIDASE INHIBITORS

In the early 1960s it was noted that patients being treated with the antituberculosis drug, iproniazid developed a euphoric state of mind (Marley, 1977). This drug was modified to form isoniazid and this was the first monoamine oxidase inhibitor antidepressant. MAOIs (fig 7.3) are thought to act by increasing monoamine function

by inhibition of the major degradative enzyme of the monoamine neurotransmiiters. However, the inhibition occurs quite soon after administration, in contrast to the beneficial effects which take two to three weeks to appear. This is similar to the delay in action that is characteristic of the tricyclics (Marley 1977).

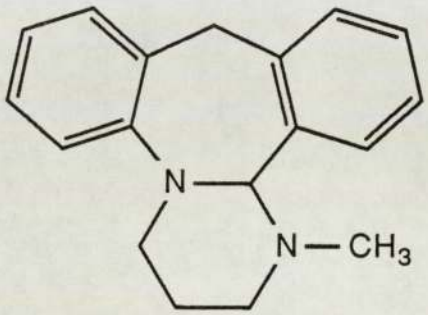
7.1.3 PARKINSONS DISEASE AND L-DOPA

Parkinson's disease was first described as a 'shaking palsy' or paralysis agitans' by James Parkinson in 1817.

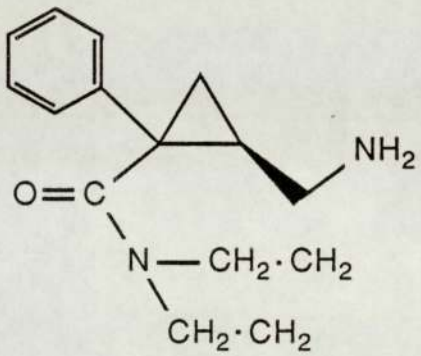
Today, the term 'Parkinsonism' is used to describe those individuals who show the major features of Parkinson's disease but do not have idiopathic Parkinson's disease (i.e. of unknown origin), for example subjects who are suffering from the extrapyramidal side effects of neuroleptic treatment for psychoses such as schizophrenia, and those who suffered neurological damage to the substantia nigra by MPTP a neurotoxic by-product of illicitly synthesised opiate analogues (Heikkila, 1988).

The clinical features of Parkinson's disease are well documented and include tremor, rigidity, akinesia, postural abnormality, psychiatric disturbances and alimentary disorders (Green & Costain, 1981).

1 Mianserin



2 Midalcipran



3 Rolipram

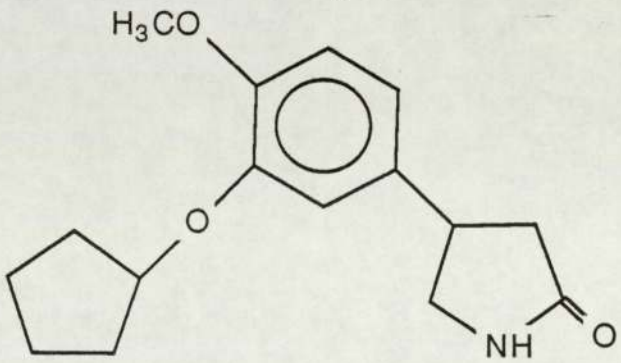
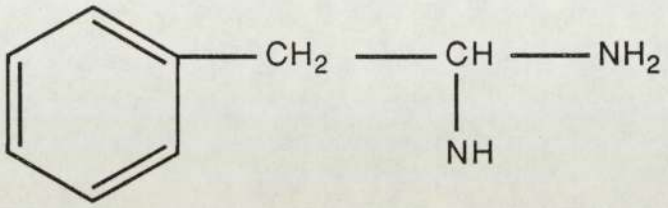
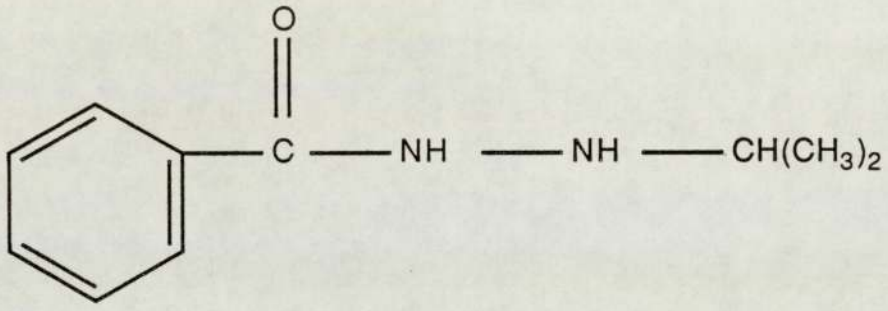


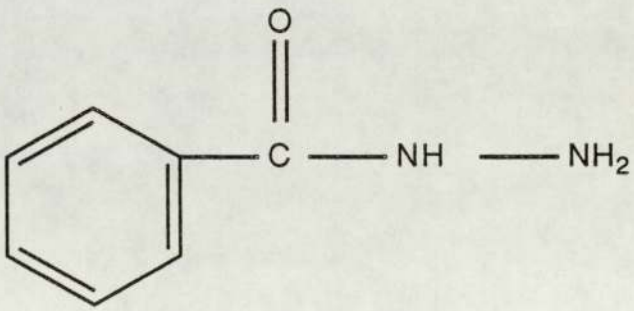
Fig 7.2 Newer Antidepressants



1 Phenelzine



2 Iproniazid

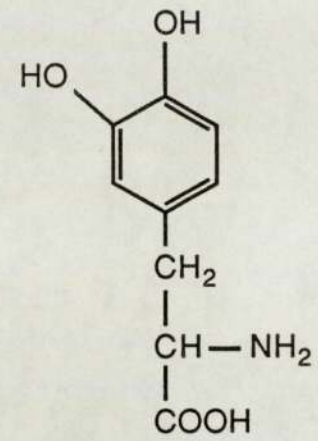


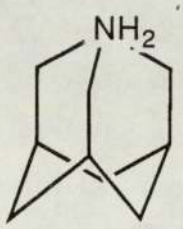
3 Isoniazid

Fig. 7.3 Monoamine Oxidase Inhibitors

The classical neurological deficit of Parkinson's disease is a marked decrease in dopamine (fig 7.5) and its metabolites in the nigrostriatal system (Ehringer & Hornykiewicz, 1960), and treatment has been designed to remedy this. Dopamine does not cross the blood-brain-barrier (BBB), and administration of high doses of tyrosine does not markedly affect dopamine formation, presumably because it is tyrosine hydroxylase that is the rate limiting enzyme of catecholamine synthesis. Administration of L-dopa (fig 7.4) does however produce significant increases in dopamine levels as the activity of dopa decarboxylase is high (Hornykiewicz, 1973). L-dopa is given in doses of around 0.5-1g/day (Martindale, 1977), however this can cause side effects probably as a result of peripheral decarboxylation. The co-administration of a decarboxylase inhibitor that is peripherally selective reduces the side effects, and enhances drug activity, such combinations as L-dopa and benserazide ('Madopar') or L-dopa and carbidopa ('Sinemet') (Glover *et al.*, 1977).

1 L-Dopa





2 Amantadine

3 Bromocriptine

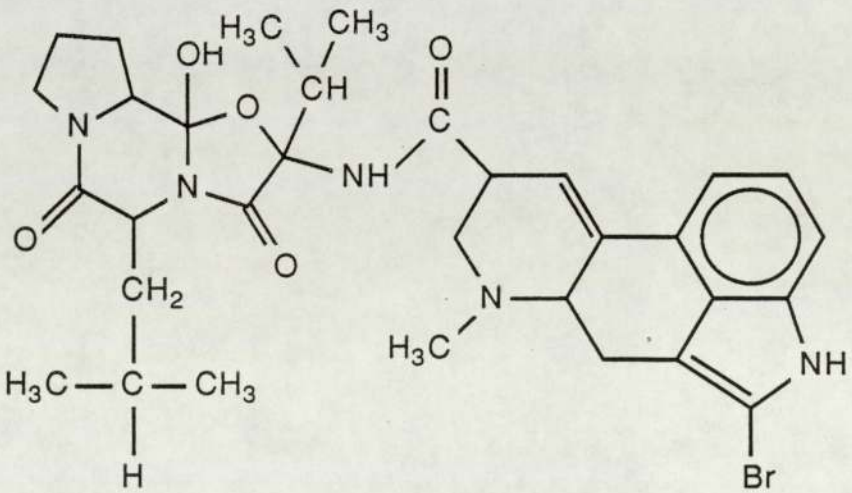


Fig 7.4 Antiparkinson Drugs

BH_4 metabolism in Parkinson's disease has been studied previously (Nagatsu *et al.*, 1984). Edwards (1988) has shown that bromocriptine (fig 7.4) causes a marked decrease in the synthesis of BH_4 in the rat in both *in vivo* and *in vitro* systems. Bromocriptine is essentially a dopamine agonist, however it appears to function optimally in the presence of intact presynaptic dopamine stores (Calne *et al.*, 1974; Lees *et al.*, 1975; Lipscey and Peres 1984; LeWitt *et al.*, 1983).

The effects of L-dopa on BH_4 metabolism in human brain preparations *in vitro* were investigated. Amantadine (fig 7.4) is used as an antiviral drug, and is also beneficial in mild variants of Parkinson's disease, especially in reducing akinesia. Amantadine appears to act by releasing dopamine from nerve terminals. The effects of L-dopa and amantadine are additive. The effects of amantadine on BH_4 metabolism in human brain were investigated *in vitro* .

7.1.4 OXOTREMORINE AND BH_4 METABOLISM

Oxotremorine (fig 7.5) causes an increase in brain acetylcholine concentration, and also possesses direct cholinergic activity. It is used to create a Parkinsonian syndrome in laboratory animals for the screening of anti-parkinsonian drugs. It causes tremor, hypokinesia, rigidity and pronounced parasympathetic

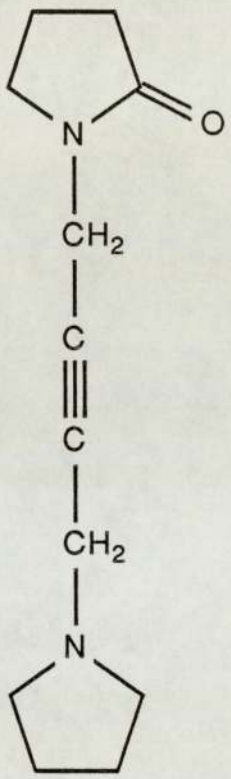
effects including salivation and defaecation, these are probably due to enhanced central cholinergic function. The effects are mediated centrally as proved by spinal cord section experiments (Bowman & Rand, 1982).

Indirect evidence suggests that dopaminergic neurones have muscarinic cholinergic receptors and stimulation of these causes a reduction in dopamine release. This may be an additional mechanism by which oxotremorine causes its effects (Green & Costain, 1981).

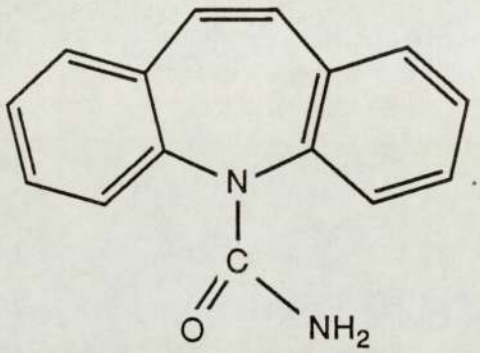
The cholinergic deficit in Alzheimer's disease is one of the characteristic lesions of the condition (Perry *et al.*, 1981). The cholinergic system is thought to be involved in memory and general cognitive function, both of which are severely impaired in AD.

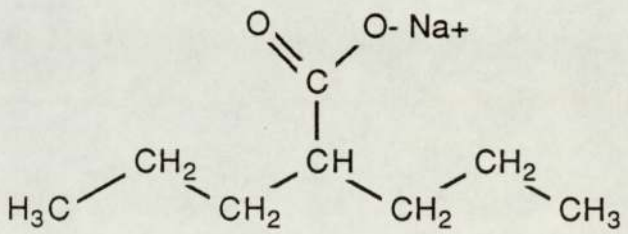
Oxotremorine was used in AD subjects with the intention of restoring compromised cholinergic function, and possibly alleviating the symptoms of memory loss and cognitive dysfunction (Davis *et al.*, 1986). Instead, the individuals developed a severe depression. AD and depression are both conditions in which there are documented disturbances of BH_4 metabolism. This study aimed to investigate the effects of oxotremorine on BH_4 metabolism *in vivo* and *in vitro* .

1
Oxotremorine



2
Carbamazepine



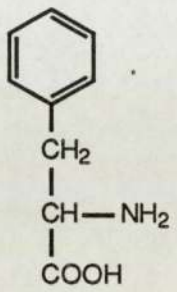


Sodium Valproate

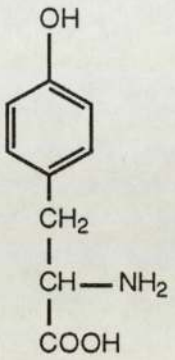
3

Fig 7.5 Oxotremorine and Anticonvulsants

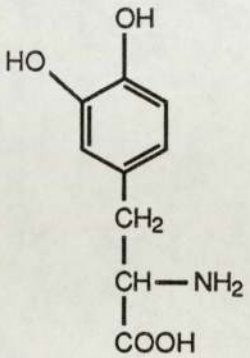
1 Phenylalanine

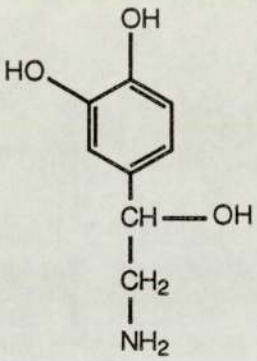


2 Tyrosine

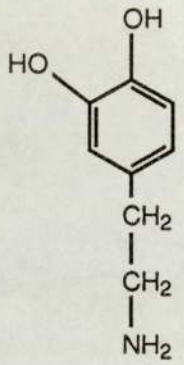


3 L-Dopa

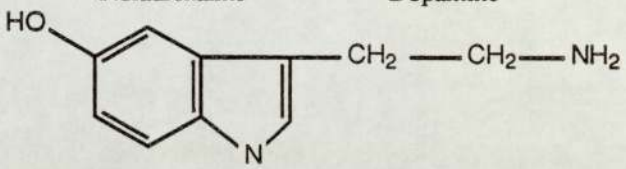




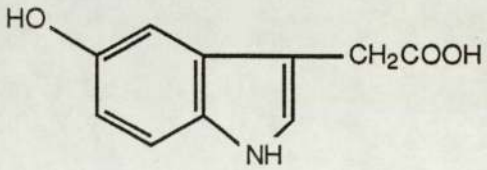
4 Noradrenaline



5 Dopamine



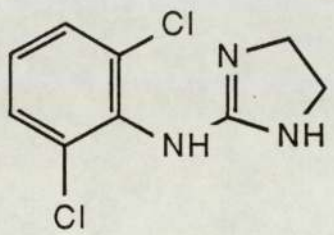
6 5-Hydroxytryptamine (Serotonin)

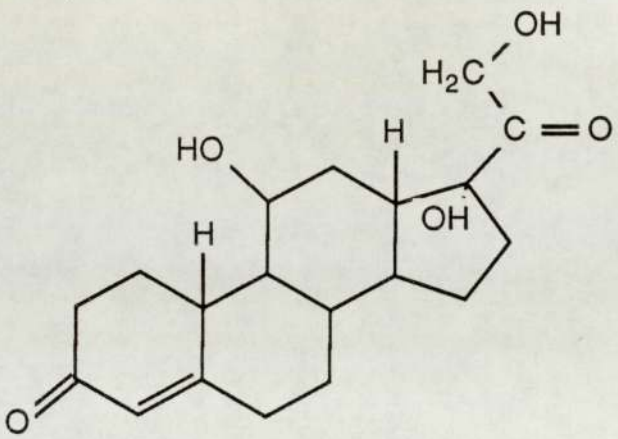


7 5-Hydroxyindoleacetic acid (5-HIAA)

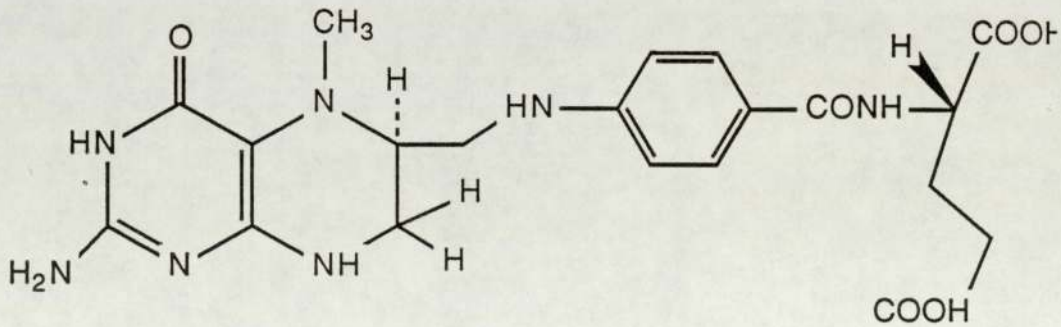
Fig 7.6 Catecholamines and Indolamines

1 Clonidine



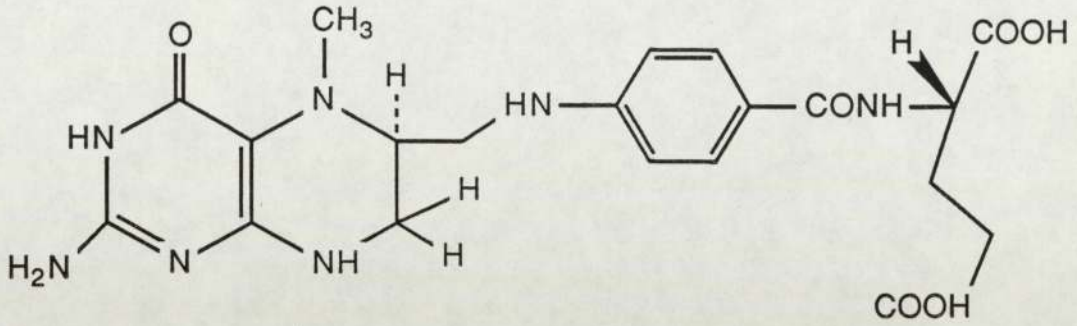


2 Cortisol

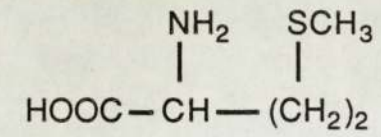


3 5-methyltetrahydrofolic acid

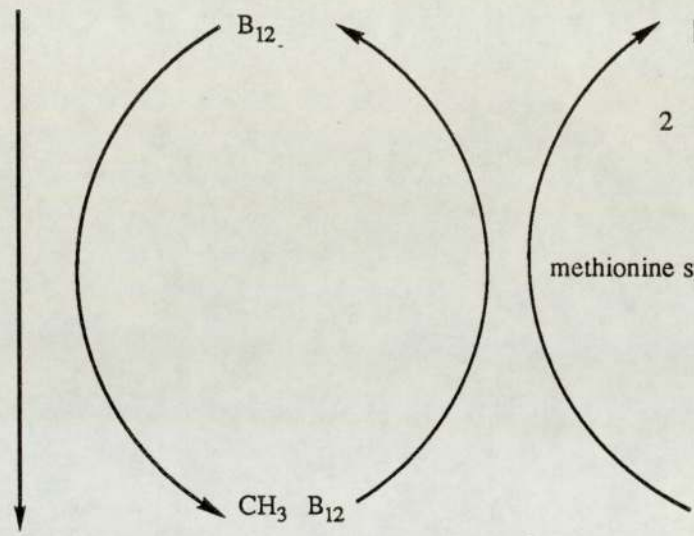
Fig 7.7 Clonidine, cortisol and 5-methyl-tetrahydrofolic acid



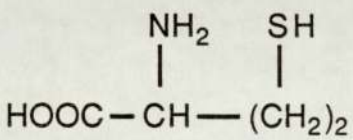
1 5-methyltetrahydrofolic acid



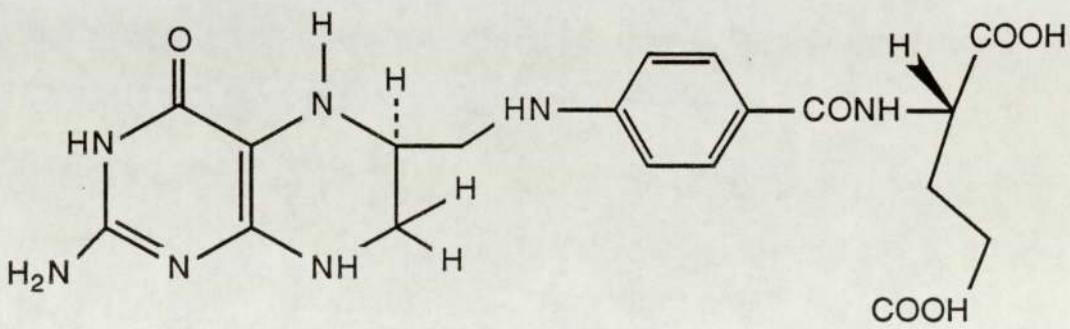
2 Methionine



methionine synthetase EC.2.1.1.13



3 Homocysteine



4 Tetrahydrofolic acid

Fig 7.8 5-Methyltetrahydrofolate & methionine synthetase

7.1.5 NEUROTRANSMIITERS, AMINO ACIDS AND BH_4 METABOLISM

BH_4 is necessary for the synthesis of the catecholamine and indoleamine neurotransmitters (fig 7.6) (Lovenberg and Levine 1986). It has been shown that these neurotransmitters can exert an inhibitory effect on the synthesis of BH_4 at the level of sepiapterin reductase in rat brain (Katoh and Sueoka, 1986).

In this study the effects of catecholamine and indolamine neurotransmitters and the metabolite 5-HIAA (fig 7.6). on human brain BH_4 metabolism were investigated *in vitro* using preparations of human temporal cortex from subjects who had no manifest psychiatric abnormalities and had died from non-pyschiatric related disorders.

7.1.6 FOLATES METHIONINE AND BH_4 METABOLISM

Methionine (fig 7.8 no.2) is involved in the metabolism of folic acid, and folates are thought to be involved in depression (Jones, 1988). Hamon *et al.*, (1986)report that 5-methyl tetrahydrofolate (5-MeTHF) increased BH_4 synthesis in brain preparations from SDAT subjects. but this finding has proved difficult to repeat (Heales, 1987; Cattell, 1988).

Methionine is formed from homocysteine by the action of methionine synthetase (EC.2.1.1.13)(fig 7.8). The methyl group is obtained from 5-MeTHF and is carried via vitamin B_{12}(fig 7.8). This study sets out to determine if the stimulation of BH_4 synthesis by 5-MeTHF as reported by Hamon *et al.*, (1986) is mediated by methionine.

7.1.7 ANTICONVULSANTS CORTISOL AND BH_4 METABOLISM

Sodium valproate and carbamazepine (fig 7.5) are used in the treatment of epilepsy (Green & Costain, 1981). It has been reported that they may be of benefit in the treatment of the manic phase of depression. Clonidine (fig 7.7) is an alpha-2 adrenoceptor agonist (Bowman & Rand, 1982), and is used to treat menopausal flushing. It is also thought to have some central action in reducing the severity of the panic attacks common in menopausal women.

Cortisol (fig 7.7) levels are reported as being disturbed in depression (Jones, 1988). This study sought to see if the disturbances in BH_4 metabolism in depression were in any way related to the elvation in cortisol levels.

7.2 MATERIALS AND METHODS

All enzyme activity determinations, chemicals and assays of tissue biopterins were carried out as detailed in chapter 2.

Rolipram was obtained from Schering Aktiengesellschaft, Berlin. Midalcipram was obtained from Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, F-81106, Castres, France. 5-methyl-tetrahydrofolate was obtained from Eprova, Switzerland. All other drugs and compounds were obtained from Sigma Chemical Company, Poole, Dorset.

Samples of human temporal cortex from non-demented subjects was obtained from Dr. R.J. Leeming, Haematology Department, General Hospital, Birmingham.

In vivo experiments with nortriptyline and oxotremorine used male wistar rats (150g) dosed orally (see footnotes to relevant tables for doses and time period of experiments).

In the *in vitro* studies the compounds were tested initially at an incubation concentration of 1mMolar. Those which had an effect, were then used in a dose-response investigation to see if the effect was still evident at physiologically relevant concentrations and was dose related.

Folate assays were performed by Dr. R.J. Leeming, Haematology Department, General Hospital, Birmingham, using the *Lactobacillus casei* assay coupled with acid iodine oxidation for determination of 5-methyl-tetrahydrofolate.

7.3 RESULTS

7.3.1 *In vitro* effects of compounds on BH_4 metabolism in human brain

In initial screening tests *in vitro* using human brain preparations at 1mM incubation concentration the following compounds had no effect on BH_4 synthesis and were not investigated further, imipramine, desipramine (Table 7.1), amitriptyline, nortriptyline (Table 7.2), sodium valproate, carbamazepine (Table 7.6), 5-hydroxyindole-acetic acid (Table 7.5), clonidine, mianserin (Table 7.7) cortisol (Table 7.8), and Rolipram (Table 7.12).

The monoamine oxidase inhibitors, phenelzine(1mM) ($p<0.02$), iproniazid(1mM) ($p<0.05$) and isoniazid(1mM) ($p<0.002$) all caused significant reductions in BH_4 synthesis in human temporal cortex *in vitro* . Phenelzine(1mM) inhibited DHPR ($p<0.05$) in human temporal cortex. None of these compounds had any effect on sepiapterin reductase (Table7.4).

Noradrenaline($p<0.002$), dopamine($p<0.02$) and L-dopa ($p<0.002$) all inhibited BH_4 synthesis in human temporal cortex *in vitro* at an incubation concentration of 1mM. No effect was seen on DHPR activity, but sepiapterin reductase was inhibited by all three ($p<0.02$) (Table 7.5).

Serotonin(1mM) inhibited BH_4 synthesis and sepiapterin reductase in human temporal cortex but had no effect on DHPR activity. Its main metabolite 5-hydroxyindole acetic acid (5-HIAA)(1mM) had no effect on any of the measured activities (Table 7.6).

Amantadine(1mM) ($p<0.05$) and midalcipran(1mM) ($p<0.05$) inhibited BH_4 synthesis *in vitro* but had no effect on DHPR or sepiapterin reductase.

Oxotremorine(1mM) was a potent inhibitor of BH_4 synthesis in human temporal cortex reducing activity by approximately 90% ($p<0.01$). Oxotremorine did not have any effects on DHPR or sepiapterin reductase (Table 7.10).

5-MeTHF (0.1mM) and homocysteine (1mM) had no effect on BH_4 synthesis in human brain preparations. Methionine(1mM) produced an increase in BH_4 synthesis in human temporal cortex ($p<0.05$) (Table 7.10).

Midalcipran inhibits BH_4 synthesis at concentrations between 10^{-3}M and 10^{-4}M (Table 7.11). Methionine

elevates BH_4 synthesis over the same range (Table 7.12). Amantadine only inhibits BH_4 synthesis at a concentration of $10^{-3}M$ (Table 7.13).

Oxotremorine (Table 7.14) and dopamine (Table 7.15) produce a marked inhibition of BH_4 synthesis in human temporal cortex between $10^{-3}M$ and $10^{-6}M$ incubation concentrations.

Noradrenaline, L-dopa and serotonin only inhibit BH_4 synthesis between $10^{-3}M$ and $10^{-4}M$ (Tables 7.16 to 7.18).

Phenelzine inhibits BH_4 synthesis between $10^{-3}M$ and $10^{-5}M$ (Table 7.19). Iproniazid produces a decrease in BH_4 synthetic activity at concentrations of $10^{-3}M$ and $10^{-4}M$ whilst isoniazid exerted an inhibitory action only at $10^{-3}M$ (Tables 7.20 and 7.21).

7.3.2 *In vivo* effects of oxotremorine on rat BH_4 metabolism

The effects of oxotremorine were investigated *in vivo* using male wistar rats (150g) dosed i.g. once daily for 7 days with oxotremorine 0.25mg/kg and atropine methyl nitrate 1mg/kg. Co-administration of atropine was neccesary to block any peripheral effects of prolonged cholinergic stimulation by oxotremorine.

The results are shown in table 7.22. Brain total biopterins were significantly decreased in the dosed animals compared to controls ($p<0.01$) as was brain derived pterin ($p<0.01$). Brain *in vitro* BH_4 synthesis was lowered in the dosed animals compared to the controls ($p<0.05$). No effects were observed in either liver or plasma total biopterin levels. DHPR and sepiapterin reductase activities were unaltered in either brain or liver. Dosing with oxotremorine had no discernible effect on tissue protein concentrations or body weight.

The experiment was repeated using four groups of animals: control, oxotremorine (0.25mg/kg)alone (oxo), oxotremorine (0.25 mg/kg) plus atropine methyl nitrate (1mg/ kg) (oxo + atr) and atropine methyl nitrate alone (1mg/kg) (atr), to ascertain the effects, if any, of atropine on BH_4 metabolism.

The results are given in Tables 7.23 to 7.35. As in the previous experiment animals dosed with the oxotremorine and atropine methyl nitrate combination displayed reductions in brain total biopterins (Table 7.23, $p<0.01$), brain derived pterin (Table 7.24, $p<0.05$) and *in vitro* BH_4 synthesis (Table 7.34, $p<0.05$).

Animals dosed with oxotremorine alone had a similar profile of deficits. Atropine methyl nitrate singly produced no significant effects on any of the parameters

determined. None of the experimental groups showed any significant weight loss and food and water consumption appeared normal.

7.3.3 *In vivo* effects of nortriptyline on rat BH_4 metabolism

Nortriptyline has been reported to inhibit dihydrofolate reductase (DHFR) (Blake & Gould, 1985). DHFR is very similar in substrate and mode of action to DHPR (Kaufman *et al.*, 1964). The *in vivo* effects of nortriptyline in the rat were investigated. Nortriptyline (1.43mg/kg), equivalent to the normal therapeutic dose used in humans for treatment of depressive illness, was given i.g. to male wistar rats (150g) once daily for 17 days.

The results are given in Table 7.36. Brain derived pterin was significantly lowered in the dosed animals compared to the controls ($p<0.01$). No other significant alterations were seen in the dosed group.

7.4 DISCUSSION

7.4.1 *IN VITRO* EFFECTS OF COMPOUNDS ON HUMAN BRAIN BH_4 METABOLISM

Of the antidepressant agents tested for effects on human BH_4 metabolism *in vitro* using human temporal cortex preparations, only the monoamine oxidase inhibitors exerted any effects (Table 7.4) and these were only apparent at high concentrations ($>10^{-5}$M)(Tables 7.19 to 7.21). Of these phenelzine was the most potent producing a 30% inhibition of BH_4 synthetic activity at 10^{-5}M ($p<0.01$, Table 7.19). Phenelzine also inhibited DHPR activity *in vitro* ($p<0.05$, Table 7.4).

Phenelzine (0.4mmol/kg) lowers brain total biopterins and causes elevated plasma total biopterins (Heales 1987) in rats. These observations are consistent with DHPR inhibition. Heales (1987) has shown that these effects result from p-tyramine accumulation due to inhibition of tyrosine amino transferase (EC.2.6.1.5) by phenelzine. The effect reported here may be due to formation of excess p-tyramine or may be a direct effect on DHPR. The effect on BH_4 synthesis may be due to inhibition of sepiapterin reductase. This enzyme is inhibited by catecholamines as part of the homeostatic control of BH_4 synthesis (Katoh and Sueoka, 1986). Phenelzine is very similar in structure to the catecholamines (Fig 7.1 no. 1)

and so may inhibit sepiapterin reductase. Phenelzine and p-tyramine (fig 7.1 no.2) are similar in structure and p-tyramine inhibits sepiapterin reductase (Ki=2.5mM, uncompetitive) (Heales 1987). Alternatively, inhibition of MAO in the brain preparation will lead to an increase in the levels of catecholamines within the preparation and these will feedback on sepiapterin reductase and possibly GTP cyclohydrolase and so reduce BH_4 synthetic activity.

The catecholamines and serotonin inhibited human BH_4 synthesis *in vitro* at the level of sepiapterin reductase. This is similar to the effects of these compounds on rodent BH_4 metabolism (Katoh and Sueoka, 1986). The serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) did not inhibit sepiapterin reductase thus these compounds may inhibit the enzyme by interaction via the terminal amino group, though more work needs to be done to clarify this.

Sodium valproate and carbamazepine have been reported to have antimanic effects(Bowman and Rand, 1980). Lithium, the most common antimanic agent, inhibits BH_4 synthesis *in vivo* in the rat and *in vitro* in human brain preparations (Chapter 6). However these agents had no effect on BH_4 synthesis, DHPR or sepiapterin reductase (Table 7.7) thus it is unlikely that their antimanic action is mediated via BH_4. Clonidine and mianserin are used as antidepressants. They had no effect on any of the paramters measured, thus

their antidepressant actions are not mediated by any impact on BH_4 biochemistry.

Oxotremorine is a potent inhibitor of human BH_4 synthesis *in vitro* causing a 30% reduction in synthetic activity at 10^{-6}M incubation concentration ($p<0.05$, Table 7.14). The site of action is not at sepiapterin reductase as this is not inhibited *in vitro* at 10^{-3}M (Table 7.10).

Methionine increased BH_4 synthesis in human brain preparations (Table 7.12). 5-MeTHF and B_{12} are reported to increase BH_4 synthesis *in vitro* in human brain preparations (Hamon *et al.*, 1986). Subsequent investigations using rat preparations *in vivo* and *in vitro* have been unable to reproduce this observation (Heales, 1987; Cattell, 1988). 5-MeTHF had no effect on human brain BH_4 synthetic activity (Table 7.10).

5-MeTHF may cause increased levels of methionine and this then may increase BH_4 synthetic activity. The lack of reproducibility of the 5-MeTHF stimulatory effect may reflect differences in B_{12} levels or methionine synthetase activity between the species used.

7.4.2 *IN VIVO* EFFECTS OF OXOTREMORINE ON RAT BH_4 METABOLISM

In vivo in the rat, oxotremorine (0.25mg/kg + atropine methyl nitrate 1mg/kg) i.g.) causes a 30% reduction in the levels of brain total biopterins and derived pterin. Brain BH_4 synthesis was reduced by 50% in the dosed animals compared to controls. Sepiapterin reductase activity was unaffected, thus the site of inhibition must lie at GTP cyclohydrolase or 6-pyruvoyl-tetrahydropterin synthase (6-PTPS). Co-administration of atropine was necessary to avoid peripheral manifestations of enhanced cholinergic stimulation and parasympathetic activation. These may have resulted in behavioural alterations or decreased food intake. The latter particularly is of note as it leads to lowered total tissue biopterins (Cox and Blair, 1987). Repetition with the inclusion of a group of animals dosed with atropine alone (1 mg/kg i.g.) showed that atropine exerted no effects on any of the measured parameters. It would appear then that oxotremorine acts as a specific inhibitor of BH_4 synthesis. Further investigations into this compound would be useful as it would provide a model for BH_4 deficiency. Diaminohydroxypyrimidine (DAHP) has been suggested as a tool for generating such a deficiency (Cotton, 1986) but is associated with problems of solubility and gastrointestinal interactions (Hasegawa *et al.*, 1988). Rats dosed with oxotremorine appeared to

eat normally and at the end of the experiment weighed the same as controls. Oxotremorine is highly soluble and is easily administered in distilled water as the sesquifumarate salt solution.

7.4.3 *IN VIVO* EFFECTS OF NORTRIPTYLINE ON RAT BH_4 METABOLISM

Nortriptyline has been reported as having an inhibitory effect on bovine DHFR activity (Blake and Gould, 1985). It is widely used as an antidepressent belonging to the dibenzocycloheptene group. Patient folate status has been linked to depressive illness (Jones 1988) and were this drug to have a deleterious effect on folate metabolism by decreasing availability of the tetrahydrofolate species then it may act to aggravate the condition.

Dosing with nortriptyline 1.43 mg/kg daily for 17 days produced a decrease in brain derived pterin ($p<0.01$, Table 7.35) but not in brain total biopterin (Table 7.34). Edwards (1988) has proposed that brain pterin arises from two sources, dihydropterin derived from quinonoid dihydrobiopterin, and from the tetrahydrofolate (THF) pool. In the brain the derived pterin mainly arises from dihydropterin whereas in the liver the main source of derived pterin is the THF pool (Edwards 1988, Heales 1987). Reduced brain derived pterin may therefore

reflect reduced throughput of BH_4 through tyrosine hydroxylase and DHPR.

Nortriptyline acts to increase synaptic concentrations of the catecholamine and indoleamine neurotransmitters by reuptake blockade (Bowman and Rand, 1980). As less of these are required less will be synthesised and thus BH_4 turnover will decrease. The findings of this investigation agree with the theory.

Furthermore elevated synaptic levels of catecholamines feedback onto tyrosine hydroxylase causing a reduction in activity and subsequent lowering of BH_4 turnover. The lack of synaptic uptake systems in the liver would explain the absence of any effect on hepatic BH_4 metabolism.

It is unlikely that the lowering of brain derived pterin occurs secondary to reversible inhibition of DHPR as there are no changes in plasma total biopterins where we would expect an increase following DHPR inhibition (Leeming *et al.*, 1981).

There was no alteration in the folate status of the nortriptyline dosed group. Total folates (Table 7.38) and tetrahydrofolate levels (Table 7.39) were unchanged indicating no effect on DHFR *in vivo* . Studies on DHFR directly were not carried out here but would be of interest for further work.

To conclude, of the compounds investigated the monoamine oxidase inhibitors had significant effects on BH_4 metabolism *in vitro* at physiologically relevant concentrations. The catecholamines appear to have the same inhibitory effects on BH_4 synthesis in human brain preparations as they do in the rat. *In vivo* and *in vitro* oxotremorine would appear to be a potent BH_4 synthesis inhibitor, though the site and mode of action is not yet clear. Its potential as a tool for the generation of models of BH_4 deficiency is worthy of further investigation.

Nortriptyline, despite reports of inhibiting bovine liver DHFR, appears to have no effect on BH_4 metabolism and folate status *in vivo* in the rat but studies of its effects on DHFR would be of interest in order to obtain a complete picture.

Table 7.1 The effect of imipramine & desipramine on *in vitro* BH_4 synthesis, DHPR & sepiapterin reductase (SR) in human temporal cortex preparations

compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	4.18±1.21	167±56	0.25±0.04
imipramine	5.35±0.57	189±36	0.31±0.07
desipramine	4.60±1.04	181±49	0.22±0.06

incubation contains 1mMolar glutathione
drug incubation concentration 1mM n=6
units

[1] BH_4 synthesis:-pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] dihydropteridine reductase:-nmoles NADH min^{-1} mg $protein^{-1}$
[3] sepiapterin reductase:-nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.2 The effect of amitriptyline & nortriptyline on *in vitro* BH_4 synthesis, DHPR & sepiapterin reductase (SR) in human temporal cortex preparations

compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	7.41±0.64	132±51	0.35±0.06
amitriptyline	6.17±1.05	161±28	0.30±0.07
nortriptyline	7.03±0.64	157±39	0.27±0.05

incubation contains 1mMolar glutathione
drug incubation concentration 1mM n=6

units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.3 effect of phenelzine, iproniazid and isoniazid on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	2.12±0.52	156±51	0.24±0.04
phenelzine	0.64±0.88*	102±12$	0.23±0.02
iproniazid	0.72±1.00$	132±21	0.27±0.05
isoniazid	0.32±0.73!	127±26	0.27±0.08

*p<0.02 $p<0.05 !p<0.002
drug incubation concentration 1mMolar n=6
Incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.4 The effect of noradrenaline, dopamine and L-dopa on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

Compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	2.12±0.52	178±42	0.32±0.03
noradrenaline	0.46±0.23!	192±28	0.22±0.04*
dopamine	1.32±0.85*	167±56	0.24±0.02*
L-dopa	0.00±0.00!	137±26	0.21±0.04*

*p<0.02 !p<0.002
drug incubation concentration 1mM n=6
incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.5 The effect of serotonin and 5-HIAA on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	16.92±3.12	159±38	0.30±0.04
serotonin	5.65±2.67!	164±31	0.23±0.03!
5-HIAA	12.75±2.71	172±50	0.26±0.07

!p<0.002 5-HIAA:- 5-hydroxyindoleacetic acid
drug incubation concentration 1mM n=6
incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.6 The effect of sodium valproate and carbamazepine on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

Compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	2.27±0.52	174±49	0.23±0.04
sodium valproate	2.54±0.84	153±51	0.26±0.06
carbamazepine	2.56±0.98	162±46	0.24±0.01

drug incubation concentration 1mM n=6
incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.7 The effect of clonidine and mianserin on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

Compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	6.87±0.52	144±31	0.18±0.04
clonidine	5.94±0.89	123±28	0.17±0.06
mianserin	6.52±0.91	152±37	0.15±0.01

drug incubation concentration 1mM n=6
incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.8 The effect of cortisol, amantadine and midalcipran on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

Compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	8.39±0.73	178±32	0.22±0.05
amantadine	5.26±1.39*	189±56	0.27±0.04
cortisol	9.14±1.82	163±48	0.24±0.07
midalcipran	6.52±0.91*	175±32	0.19±0.03

* $p<0.01$ n=6
drug incubation concentration 1mM n=6
incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.9 The effect of oxotremorine and methionine on *in vitro* BH_4 synthesis, DHPR and sepiapterin reductase (SR) in human temporal cortex.

compound	BH_4 synth.[1]	DHPR[2]	SR[3]
control	7.83±1.02	147±52	0.18±0.03
oxotremorine	0.52±0.47*	152±31	0.17±0.05
methionine	9.39±0.79$	139±49	0.23±0.04

* $p<0.01$ $ $p<0.05$ n=6
drug incubation concentration 1mM n=6
incubation contains 1mMolar glutathione
units

[1] pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$
[2] nmoles NADH min^{-1} mg $protein^{-1}$
[3] nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 7.10 The effect of 5-methyl-tetahydrofolate (5-MeTHF) and homocysteine on *in vitro* BH_4 synthesis in human temporal cortex.

compound	BH_4 synthesis
control	9.11±1.10
5-MeTHF	8.18±1.93
homocysteine	8.51±1.60

n=6
drug incubation concentration 1mM
incubation contains 1mMolar glutathione

units :-pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$

Table 7.11 The effect of midalcipran on *in vitro* synthesis of tetrahydrobiopterin

	control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	7.70	4.42	5.82	7.55	7.17	6.57
±s.d.	0.57	1.10	1.15	0.67	0.78	1.52
p		<0.001	<0.01			

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
incubation contains 1mMolar glutathione
n=6

Table 7.12 The effect of Rolipram on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}
mean	9.63	9.30	8.67	9.42	8.83
±s.d.	1.96	2.19	2.50	3.16	1.79

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1mMolar glutathione
n=6

Table 7.13 The effect of methionine on *in vitro* synthesis of tetrahydrobiopterin

	control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	6.34	9.86	9.67	7.12	5.85	5.60
±s.d.	0.72	0.97	0.82	0.68	0.84	1.25
p		<0.001	<0.001	n.s.	n.s.	n.s.

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1 mMolar glutathione
n=6

Table 7.14 The effect of amantadine on *in vitro* synthesis of tetrahydrobiopterin

	control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	8.29	6.03	9.15	8.71	8.68	7.98
±s.d.	1.36	1.78	2.84	1.50	3.02	2.37
p		<0.001	n.s.	n.s.	n.s.	n.s.

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
incubation contains 1mMolar glutathione
n=6

Table 7.15 The effect of oxotremorine on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	7.74	0.14	1.22	1.07	6.18	7.85
±s.d.	0.47	0.22	0.24	0.43	1.30	0.82
p		<0.001	<0.001	<0.001	<0.05	

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1 mMolar glutathione
n=6

Table 7.16 The effect of dopamine on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	7.58	2.49	2.17	3.98	4.20	7.21
±s.d.	1.49	1.37	0.96	1.83	1.41	1.93
p		<0.001	<0.001	<0.001	<0.001	

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1 mMolar glutathione
n=6

Table 7.17 The effect of noradrenaline on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	4.11	1.69	1.94	2.85	4.60	4.39
±s.d.	0.92	0.74	0.74	1.29	2.49	1.62
p		<0.001	<0.001	n.s.	n.s.	n.s.

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1 mMolar glutathione
n=6

Table 7.18 The effect of L-DOPA on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}M	10^{-4}M	10^{-5}	10^{-6}M	10^{-7}M
mean	11.28	0.00	6.58	12.69	10.24	12.08
±s.d.	3.83	0.00	2.06	2.31	3.40	2.85
n	5	6	6	5	5	6
p		<0.001	<0.05			

units:- pmoles BH_4 synthesised hour^{-1} mg protein^{-1}
Incubation contains 1 mMolar glutathione

Table 7.19 The effect of serotonin (5-HT)on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	8.41	4.28	5.71	9.52	8.93	8.76
±s.d.	0.65	0.83	1.35	1.77	1.63	1.08
p		<0.001	<0.001	n.s	n.s.	n.s.

units:- pmoles BH_4 synthesised hour^{-1} mg protein^{-1}
Incubation contains 1 mMolar glutathione
n=6

Table 7.20 The effect of phenelzine on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	8.71	5.86	6.02	6.21	8.93	9.11
±s.d.	0.65	0.83	1.35	1.92	2.29	1.62
p		<0.001	<0.01	<0.01	n.s.	n.s.

units:- pmoles BH_4 synthesised hour^{-1} mg protein^{-1}
Incubation contains 1 mMolar glutathione
n=6

Table 7.21 The effect of iproniazid on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	4.38	1.16	1.86	4.09	4.51	3.92
±s.d.	0.47	0.83	0.72	1.28	1.13	1.62
p		<0.001	<0.001	n.s	n.s.	n.s.

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1 mMolar glutathione
n=6

Table 7.22 The effect of isoniazid on *in vitro* synthesis of tetrahydrobiopterin

	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
mean	9.97	6.51	8.63	9.40	8.02	7.68
±s.d.	1.42	1.07	1.82	1.29	2.49	2.32
p		<0.001	n.s.	n.s	n.s.	n.s.

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$
Incubation contains 1 mMolar glutathione
n=6

Table 7.23 The effects of oxotremorine on the rat (summary).

Parameter(units)	Control(n=5)	oxotremorine(n=5)
brain total biopterin (ng/g wet wt.)	113.1±16.6	74.9±21.5*
liver total biopterin (µg/g wet wt.)	0.90±0.22	1.03±0.25
plasma total biopterin (ng/ml)	36.3±4.5	37.2±5.7
brain derived pterin (ng/g wet wt.)	5.5±1.6	6.8±1.7
liver derived pterin (µg/g wet wt.)	0.24±0.06	0.24±0.08
brain *in vitro* BH_4 synthesis (pmol BH_4 $hour^{-1}$ mg $protein^{-1}$)	6.01±1.12	3.63±1.71*
liver *in vitro* BH_4 synthesis (pmol BH_4 $hour^{-1}$ mg $protein^{-1}$)	36.2±9.8	38.7±9.0
brain DHPR activity (nmol NADH min^{-1} mg $protein^{-1}$)	186±58	173±54
liver DHPR activity (nmol NADH min^{-1} mg $protein^{-1}$)	200±69	192±69
brain sepiapterin reductase activity (nmol sepiapterin min^{-1} mg $protein^{-1}$)	0.28±0.06	0.24±0.03
brain protein conc'n (mg/ml)	8.49±1.21	9.02±1.43
liver protein conc'n (mg/ml)	36.5±4.7	36.7±5.0

* $p<0.05$

Dosing Schedule

Oxotremorine group dosed i.g. with 0.25mg/kg oxotremorine & 1mg/kg atropine methyl nitrate in distilled water once daily for 7 days at 10 a.m.

Controls dosed as above but vehicle only.

Tables 7.24 *et seq*
effects of Dosing with:-
1. oxotremorine 0.25mg/kg (Oxo)
2. oxotremorine 0.25mg/kg & atropine methyl nitrate 1mg/kg (Oxo+Atr)
3. atropine methyl nitrate 1mg/kg (Atr)

doses made up in distilled water, given once daily i.g. for 7 days.

controls dosed as others but vehicle only.

Table 7.24 Brain total biopterin (ng/g wet wt.)

	control	Oxo+Atr	Oxo	Atr
mean	115	83*	75*	109
±s.d	15	12	19	25

*p<0.01

Table 7.25 Brain derived pterin (ng/g wet wt.)

	control	Oxo+Atr	Oxo	Atr
mean	10.2	6.3**	7.4*	11.4
±s.d	1.8	2.1	1.7	2.6

*p<0.01 **p<0.05

Table 7.26 Liver total biopterins (µg/g wet wt.)

	control	Oxo+Atr	Oxo	Atr
mean	8.97	9.29	10.36	11.4
±s.d	3.22	3.01	2.50	2.6

Table 7.27 liver derived pterin (µg/g wet wt.)

	control	Oxo+Atr	Oxo	Atr
mean	0.38	0.41	0.40	0.34
±s.d	0.13	0.19	0.22	0.18

Table 7.28 plasma total biopterin (ng/ml.)

	control	Oxo+Atr	Oxo	Atr
mean	36.3	34.1	37.2	33.0
±s.d	4.5	7.2	5.7	5.7

Table 7.29 Brain DHPR (nmol NADH mg protein^{-1} min^{-1}.)

	control	Oxo+Atr	Oxo	Atr
mean	186	152	173	201
±s.d	58	69	54	48

Table 7.30 Liver DHPR (nmol NADH mg protein^{-1} min^{-1}.)

	control	Oxo+Atr	Oxo	Atr
mean	200	173	192	190
±s.d	69	53	69	44

Table 7.31 Brain sepiapterin reductase (nmol sepiapterin mg protein^{-1} min^{-1})

	control	Oxo+Atr	Oxo	Atr
mean	0.29	0.23	0.26	0.31
±s.d	0.05	0.07	0.03	0.07

Table 7.32 Liver sepiapterin reductase (nmol sepiapterin mg protein^{-1} min^{-1}.)

	control	Oxo+Atr	Oxo	Atr
mean	0.32	0.26	0.32	0.37
±s.d	0.06	0.04	0.07	0.10

Table 7.33 Brain protein (mg/ml supernatant.)

	control	Oxo+Atr	Oxo	Atr
mean	8.49	9.27	9.02	7.90
±s.d	1.21	2.61	1.43	2.52

Table 7.34 Liver protein (mg/ml supernatant)

	control	Oxo+Atr	Oxo	Atr
mean	36.5	30.1	36.7	33.9
±s.d	4.7	8.4	5.0	6.1

Table 7.35 Brain *in vitro* BH_4 synthesis
(pmol BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$)

	control	Oxo+Atr	Oxo	Atr
mean	6.01	4.02*	3.63*	5.09
±s.d	1.12	0.73	1.71	2.07

* $p<0.05$

Table 7.36 Liver *in vitro* BH_4 synthesis
(pmol BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$)

	control	Oxo+Atr	Oxo	Atr
mean	36.2	39.1	38.7	32.3
±s.d	9.8	5.9	9.0	5.8

Table 7.37 The effects of nortriptyline on the rat (summary).

parameter	control(n=5)	nortriptyline(n=5)
brain total biopterin (ng/g wet wt.)	87±10	90±11
liver total biopterin (µg/g wet wt.)	10.37±3.22	11.31±1.32
plasma total biopterin (ng/ml)	36.3±6.9	35.0±5.6
brain derived pterin (ng/g wet wt.)	11.0±2.3	7.2±1.7*
liver derived pterin (µg/g wet wt.)	0.15±0.05	0.15±0.06
brain DHPR activity (nmol NADH min^{-1} mg $protein^{-1}$)	200±44	189±61
liver DHPR activity (nmol NADH min^{-1} mg $protein^{-1}$)	231±56	249±72
brain sepiapterin reductase activity (nmol sepiapterin min^{-1} mg $protein^{-1}$)	0.29±0.05	0.25±0.05
liver sepiapterin reductase activity (nmol sepiapterin min^{-1} mg $protein^{-1}$)	0.27±0.05	0.32±0.08
brain protein conc'n (mg/ml)	9.56±1.31	9.23±2.32
liver protein conc'n (mg/ml)	50.19±7.33	48.17±8.60
brain total folate (ng/ml homogenate)!	32.8±1.8	30.2±4.5
brain tetrahydrofolate (ng/ml homogenate)!	30.6±1.2	27.9±3.8

mean ±s.d. *p<0.01

! 20% (w/v) in phosphate buffer

nortriptyline group dosed with 1.43 mg/kg (i.g.) in distilled water (vol. 0.2ml) once daily at 10.00 am.

controls dosed as above vehicle only.

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 TETRAHYDROBIOPTERIN METABOLISM IN MENTAL DYSFUNCTION

Tetrahydrobiopterin (BH_4) is the natural cofactor for the hydroxylation of the aromatic amino acids, phenylalanine, tyrosine and tryptophan in the synthesis of the catecholamine and indolamine neurotransmitters, noradrenaline, dopamine and serotonin (Kaufman, 1985). Depletion of BH_4 leads to neurological dysfunction as typified by inborn errors of BH_4 metabolism, the malignant hyperphenylalaninaemias (Leeming *et al.*, 1981; Dhondt 1984; Niederwieser *et al.*, 1984).

Tissue levels of BH_4 are maintained by *de novo* synthesis from guanosine triphosphate (GTP) and by salvage after cofactor function by dihydropteridine reductase (DHPR) which converts quinonoid dihydrobiopterin (qDHB) to BH_4 (Crane *et al.*, 1972; Milstein and Kaufman, 1986).

This study investigated BH_4 metabolism in a variety of mental disorders, particularly Alzheimer's disease. It also looks at the role of aluminium in Alzheimer pathology with particular regard to its effects on BH_4 metabolism. The effects of pyschoactive drugs on BH_4 metabolism in human brain were investigated to see if their effects could be attributed to a pharmacological interaction with central BH_4 biochemistry.

8.2 DIHYDROPTERIDINE REDUCTASE IN MENTAL DISORDERS

There is a reduction in erythrocyte DHPR activity measured on Guthrie card blood spots from subjects with autism and Rett's syndrome 12 years old and under (Leeming *et al.*, 1987; Sahota *et al.*, 1986). Was this deficit merely an epiphenomenon arising secondary to the retardation?

Mean DHPR activity in the non-specific mental retardation (NSMR) and Down's syndrome groups are not significantly different from healthy controls (Table 8.1) hence the deficit seen in Rett's syndrome and autism is not a consequence of the mental dysfunction.

Rett's syndrome occurs as a result of a deletion on the X chromosome, hence explaining its occurrence solely in females (Rett, 1977). DHPR is thought to have an additional gene modulating its activity on the X chromosome in addition to the known gene on chromosome 4 (Armstrong *et al.*, 1986). The deletion on the x chromosome in Rett's syndrome may affect this 'modulation gene', and so cause the DHPR deficit seen in Rett's syndrome. Further work needs to be done to clarify the role of the X-chromosome gene in mental disorders such as Rett's syndrome.

Table 8.1 Guthrie card blood spot DHPR activity and total biopterin concentration in different subjects

Group (age 1-12yrs)	[biopterin] μg litre^{-1}	DHPR activity **
Healthy controls	3.78 ± 1.28(4)	180 ± 54 (6)
NSMR	3.87 ± 1.15(65)	182 ± 56 (95)
Down's syndrome	4.00 ± 1.40(12)	171 ± 56 (24)
autism	n.d.a	136 ± 27 (11)*
Rett's syndrome	n.d.a	120 ± 40 (9) *

data expressed as mean ± s.d. (n)

** units nmol NADH oxidised min^{-1} ml whole blood^{-1}

n.d.a. no data available

* $p < 0.001$ compared to NSMR group

NSMR = non specific mental retardation

Table 8.2 DHPR activity in Rett's syndrome & controls measured on two different baselines.

Baseline	Controls	Rett's Syndrome
haemoglobin(Hb)	0.97±0.28 (228)	0.73±0.20 (6)*
volume	182 ± 56 (95)	120± 40 (9)**

* $p<0.01$ ** $p<0.001$

Units

Hb μmol NADH min^{-1} g Hb^{-1}

volume nmol NADH min^{-1} ml whole $blood^{-1}$

all age ‹12 years

In contrast to earlier studies (Leeming *et al.*, 1987), DHPR activity in Rett's syndrome has been measured by two assay systems using different baselines, volume and haemoglobin (Hb) (Table 8.2). In both there is a significant decrease in DHPR activity in Rett's syndrome confirming that the effect is genuine and is not baseline dependent. Impairment of DHPR activity has an impact on blood biopterin levels. In the Down's syndrome subjects blood biopterin levels correlated negatively with DHPR on both linear ($r=-0.60$ $p<0.05$) and log plots ($r=-0.69$ $p<0.05$)(Table 8.3). Altmann *et al.*, (1987) report a similar correlation of serum biopterin levels with DHPR in uraemic subjects.

Table 8.3 Whole blood (Guthrie card) biopterin concentration and DHPR in Down's syndrome subjects

No.	[biopterin]	log	DHPR activity*	log
1	3.8	0.58	106	2.02
2	3.8	0.58	205	2.31
3	6.9	0.84	75	1.88
4	6.9	0.84	70	1.85
5	3.1	0.49	138	2.14
6	3.1	0.49	202	2.31
7	3.4	0.53	287	2.46
8	4.3	0.63	165	2.22
9	3.8	0.58	122	2.09
10	4.1	0.61	128	2.11
11	2.9	0.46	106	2.02
12	2.4	0.38	234	2.37
mean	4.0	0.58	155	2.15
±s.d.	1.4	0.13	64	0.19

Units:- DHPR- nmole NADH oxidised min^{-1} ml whole $blood^{-1}$

[biopterin] - µg litre whole blood $^{-1}$

[biopterin] v. DHPR $r=-0.60$ $p<0.05$

log [biopterin] v log DHPR $r=-0.69$ $p<0.05$

Further work needs to be done to determine to what extent the defects in DHPR in autism and Rett's syndrome contribute to the mental dysfunction.

8.3 TETRAHYDROBIOPTERIN METABOLISM IN AGING AND ALZHEIMER'S DISEASE

Turnover and levels of the catecholamine neurotransmitters alter with age (Mann *et al.*, 1980; Tomlinson *et al.*, 1981). Serum biopterin levels increase with age (Leeming & Blair, 1980).

8.3.1 BH_4 METABOLISM IN AGING

Anderson *et al.*, 1986 have shown that DHPR in the temporal cortex declines linearly with age ($r=-0.57$, $p<0.01$ $n=24$) and in the frontal cortex the decline in DHPR activity is best described by a quadratic (curvilinear) equation ($r=-0.795$ $p<0.001$ $n=20$). In the frontal cortex DHPR activity remains fairly constant until the seventh decade when it starts to decline. In this study DHPR shows a significant negative linear correlation in temporal cortex (BA21; $r=-0.949$ $n=5$ $p<0.05$)(Table 8.4), but no correlation was found in the frontal cortex. BH_4 synthesis has been shown to decline with age in the temporal and frontal cortices (Anderson *et al.*, 1987). This study has shown a similar correlation in temporal cortex (Table 8.5 $r=-0.935$ $n=5$ $p<0.05$), but no correlation in frontal cortex.

This investigation found no age related decline in temporal cortex sepiapterin reductase activity (Table 8.6). Anderson *et al.*, (1987) have reported such a defect.

Table 8.4 DHPR activity in control subjects(MRC Batch 1)

		cortex(BA)	
subject	age	frontal(9)	temporal(21)
D-74	72	398	367
D-77	72	426	374
C-295	75	309	298
C-293	82	341	259
D-90	87	256	232
mean	78	346	306
±s.d.	7	68	63

units:- nmoles NADH min^{-1} mg $protein^{-1}$

DHPR v age (temporal cortex) $r = -0.949$ $p < 0.05$

All subjects are controls including those with D prefix

Table 8.5 *In vitro* BH_4 synthesis activity in control subjects (MRC Batch 1)

		cortex (BA)	
subject	age	frontal(9)	temporal(21)
D 70	72	9.29	3.98
D 74	72	16.69	13.00
C 295	75	0.00	3.24
C 293	82	8.75	3.64
D 90	87	10.43	4.31
mean	78	9.03	5.44
±s.d.	7	5.96	4.13

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

BH_4 synthesis v age (temporal cortex) $r=-0.935$ $p<0.05$

All subjects are controls including those with D prefix

Table 8.6 Sepiapterin reductase activity in control subjects (MRC Batch 1)

		cortex (BA)	
subject	age	frontal(9)	temporal(21)
D-77	72	0.20	0.26
D-74	72	0.31	0.27
C-295	75	0.20	0.30
C-293	82	0.24	0.28
D-90	87	0.36	0.34
mean	78	0.26	0.29
±s.d.	7	0.07	0.03

units:- nmoles sepiapterin reduced min^{-1} mg $protein^{-1}$

All subjects are controls including those with D prefix

8.3.2 BRAIN BH_4 METABOLISM IN ALZHEIMER'S DISEASE

BH_4 synthesis in temporal cortex is known to be reduced in SDAT(Anderson *et al.*, 1987,Barford *et al.*, 1984). This study confirms the presence of a BH_4 synthesis defect in BA21 and BA20. BH_4 synthesis was also found to be impaired in SDAT visual cortex (BA17)(Table 8.7).

Table 8.7 Comparison of *in vitro* BH_4 synthesis in control and SDAT subjects (MRC Batch 1)

Area	Control	SDAT
BA 9	9.03±5.96(5)	3.36±4.95(5)
BA 17	9.61±3.62(5)	1.82±2.16(5) *
BA 20	8.77±2.61(4)	0.61±0.67(4) ***
BA 21	3.81±0.48(4)	0.04±0.07(4) ***
locus coeruleus	8.80±5.12(4)	19.36±4.02(5) **

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

mean±s.d n=5

* $p < 0.01$ ** $p < 0.02$ *** $p < 0.001$

The elevation in BH_4 synthesis in SDAT locus coeruleus shown above is in contradiction to that shown by Anderson *et al.*, (1987). Later figures in this report also show a decrease in BH_4 synthetic activity in SDAT locus coeruleus (Table 8.8). The inconsistency of data for this brain area is probably a result of difficulties arising from tissue homogenate preparation. Only 50mg of tissue are supplied by the Brain Bank for analysis and preparation of accurate homogenates with such a small amount of tissue is difficult. Case histories for these subjects are given in appendix 1.

BA38 is in the temporal cortex and like BA21 has lowered BH_4 synthetic activity in SDAT (Table 8.8, $p<0.001$). Locus coeruleus from MRC batch 2 samples showed decreased BH_4 synthetic activity in SDAT (Table 8.8, $p<0.001$), this is in agreement with the findings of Anderson *et al.*, (1987) and would concur with other alterations in the locus coeruleus such as neurotransmitter deficits and neuronal loss. Case histories are given in appendix 1.

Table 8.8 *In vitro* BH_4 synthesis in SDAT and control brain samples from BA 38 and locus coeruleus (MRC Batch 2)

Area	SDAT	control
BA38	1.30±1.33(5)*	5.54±1.00(6)
locus coeruleus	9.33±2.30(5)*	15.10±3.10(6)

units pmol BH_4 synthesised $hour^{-1}$ $mg\ protein^{-1}$

expressed as mean±s.d.(n)

* SDAT significantly lower than controls ($p<0.001$)

BH_4 synthetic activity in locus coeruleus was significantly higher than BA4 ($p<0.001$) and BA 38 ($p<0.001$) in the controls and SDAT subjects (Table 8.9). Anderson (1987) has shown interarea variations in BH_4 synthetic activity.

Table 8.9 *in vitro* BH_4 synthetic activity in control and SDAT (BA 4, BA10, BA 38 & locus coeruleus) MRC batch 2

Area	Control	SDAT
BA 4	5.07±0.91 (6)	4.25±0.70(5)
BA 10	8.47±1.42 (6)	8.47±1.80(5)
BA 38	5.43±1.00 (6)	1.30±1.33(5)
locus coeruleus	15.10±3.10 (6)**	9.33±2.30(5)*

* significantly higher than BA 4 & BA 38 ($p<0.01$)

** significantly higher than BA 4, BA 10 & BA 38 ($p<0.001$)

units pmol BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

expressed as mean±s.d. (n)

This study has confirmed the age related declines and inter-area variation in BH_4 synthetic activity SDAT involves a marked reduction in BH_4 synthetic activity in brain areas known to suffer from major neuronal damage, but this defect is not a consequence of neuronal loss as sepiapterin reductase activity is retained. Anderson *et al.*, (1987) has shown GTP cyclohydrolase to be normal in SDAT brains hence the defect must be at 6-PTPS. The age and area effects on BH_4 biochemistry illustrate the need for matched controls when carrying out any study of BH_4 metabolism in a disease state especially one that occurs in the later decades such as Alzheimer's disease.

8.4 ALUMINIUM AND TETRAHYDROBIOPTERIN METABOLISM

8.4.1. ALUMINIUM AND *IN VITRO* BH_4 SYNTHESIS IN HUMAN AND RAT BRAIN

Aluminium inhibited *in vitro* BH_4 synthesis in human temporal cortex at a concentration of 10^{-5}M (Table 8.10). This is similar to brain aluminium levels seen in those who have died from Alzheimer's disease (Ward *et al.*, 1986).

In contrast rat brain *in vitro* BH_4 synthesis was not inhibited by aluminium salts even at high concentration (10^{-3}M). The species difference probably reflects the different rate determining steps in the two species. In man the rate determining step is 6-PTPS and this requires magnesium, and thus is likely to be affected by aluminium as the former displaces the essential magnesium. The rate controlling step in the rat is GTP cyclohydrolase and this has no known metal ion cofactor requirement and so should be unaffected by aluminium.

Table 8.10 The effect of aluminium on *in vitro* BH_4 synthesis in human brain preparations

[Added Al]	sulphate	acetate	hydroxide
control	3.98±1.68	5.24±1.32	5.71±2.10
10^{-3}M	0.00±0.00*	0.05±0.01*	0.00±0.00*
$5x10^{-4}$M	1.31±1.77*	2.13±0.56*	2.26±1.08*
10^{-4}M	1.14±0.77*	2.56±1.12*	2.19±0.34*
$5x10^{-5}$M	0.52±0.74*	1.58±0.52*	1.95±0.53*
10^{-5}M	1.25±1.31*	1.72±0.89*	2.29±0.72*
$5x10^{-6}$M	3.28±0.87	4.62±1.39	5.89±1.46

* $p<0.01$ mean+s.d. (n=6)

units:-pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

8.4.2 THE EFFECTS OF CHRONIC ALUMINIUM DOSING ON THE RAT

Several enzyme systems are reported to be inhibited in Alzheimer's disease and many of these require a metal ion cofactor(in parenthesis) e.g. tyrosine hydroxylase (Fe), hexokinase (Mg), dopamine-B-hydroxylase (Cu) and angiotensin converting enzyme (Zn). The rate determining step of BH_4 synthesis in man (6-PTPS) is thought to be the site of the BH_4 synthetic defect seen in SDAT. 6-PTPS requires a magnesium cofactor for activity. Many of these enzyme defects are seen in the temporal cortex, an area of the brain that is primarily affected in AD. Alterations in adenylate cyclase and phosphodiesterase activity are reported in SDAT as are reductions in AMP levels (Ebstein *et al.*, 1980).

Chronic administration of aluminium to rats in their drinking water as an aluminium acetate suspension produces a profile of biochemical defects that is similar to that seen in humans with Alzheimer's disease. Tissue biopterin levels are reduced and BH_4 synthesis is inhibited. GTP cyclohydrolase and sepiapterin reductase activities do not differ from normal hence the site of inhibition must be the magnesium dependent 6-PTPS (Table 8.11). Dopamine-B-hydroxylase and tyrosine hydroxylase activities are also reduced in the Al dosed group. Such defects are commonly seen in SDAT brains. Levels of cAMP are elevated in the Al dosed group (Table 8.11).

Table 8.11 The effects of aluminium on the rat.

Parameter	control(n=5)	aluminium(n=5)
1 brain total biopterin	88.1±6.2	59.4±7.1*
2 liver total biopterin	0.87±0.08	0.75±0.04*
3 plasma total biopterin	33.5±3.2	27.7±2.0*
4 brain *in vitro* BH_4 synthesis	14.71±4.15	5.35±0.97*
5 brain GTP cyclo-hydrolase	0.28±0.07	0.31±0.06
6 brain sepiapterin reductase	0.34±0.02	0.33±0.03
7 brain dopamine-β-hydroxylase	7.58±1.25	0.87±0.24*
8 brain tyrosine hydroxylase	4.99±0.95	2.47±1.77*
9 cAMP levels	0.58±0.16	1.41±0.74*

* $p<0.05$

Dosing Schedule: see chapter 5 section 5.2

units:-

1 ng g^{-1} 2 μg g^{-1} 3 ng ml^{-1} 4 pmol BH_4 $hour^{-1}$ mg $protein^{-1}$

5 ng neopterin $hour^{-1}$ mg $protein^{-1}$

6 nmol sepiapterin min^{-1} mg $protein^{-1}$

7 nmol octopamine min^{-1} mg $protein^{-1}$ 8 pmol cAMP ml^{-1}

Aluminium inhibition of rat brain BH_4 synthesis *in vivo* in contrast to a lack of effect *in vitro* probably reflects the slow kinetics of magnesium displacement from the enzyme by aluminium. Over the relatively short three hour incubation of the *in vitro* experiment little, if any magnesium will be replaced by aluminium and hence there will be no appreciable enzyme inhibition.

Hexokinase is one of many enzymes known to be inhibited by aluminium. Its activity is markedly reduced at Al concnetrations similar to those found in the brains of SDAT subjects (Lai and Blass, 1984). Cerebral glycolytic activity is known to be reduced in SDAT (Gibson, 1979). This may partially explain the cholinergic deficit of SDAT as mild impairments of cerebral glycolytic activity have been shown to have profound effects on cholinergic function, reducing acetylcholine synthesis by choline acetyl transferase (Tucek *et al.*, 1981). This may be caused by a reduction in availability of the main glycolytic end product, acetyl Coenzyme A (ACoA), following Al inhibition of key glycolytic enzymes. Aluminium will increase levels of the 'second messenger' 3',5'-cyclic adenosine monophosphate (cAMP) by stimulating its production from ATP, by Mg dependent adenylate cyclase *in vitro* (Northup *et al.*, 1983). cAMP breakdown to 5'-AMP by Mg dependent cyclic nucleotide phospho-diesterase is inhibited by Al *in vitro* (Ebstein *et al.*, 1980)

The neurofibrillary tangles characteristic of AD brains are similar in structure to microtubules. Microtubule synthesis is activated by cAMP. Tangle formation in AD may be errant microtubule synthesis caused by increased levels of cAMP due to Al activation of adenylate cyclase.

Why does metalloenzyme inhibition appears as a recurrent motif in AD ? Because of its size the Al cation can replace any of the common biologically relevant cations from their metallozymes (Table 8.12). The Al substitution of the necessary cation may then inhibit the enzymes.

High doses of oral aluminium or other forms of treatment are capable of producing an 'Alzheimeresque' pattern of biochemical lesions in the rat (Table 8.13). The BH_4 synthesis defect occurring after chronic Al dosing was reversed by treatment of the brain samples *in vitro* with transferrin, a potent Al chelator, (Table 8.14). More evidence for the involvement of Al in the Alzheimer pathology. Treatment with transferrin improved BH_4 synthesis in SDAT brain samples whilst having no effect on samples from non-demented controls (see chapter 4).

Table 8.12 Ionic radii of aluminium and other cations

Ion	Valency	Radius (nm)
Lithium	1+	0.068
Sodium	1+	0.098
Potassium	1+	0.133
Magnesium	2+	0.065
Calcium	2+	0.094
Iron	2+	0.076
Iron	3+	0.064
Copper	1+	0.096
Copper	2+	0.069
Zinc	2+	0.074
Aluminium	***3+***	***0.045***

Source:- Nuffield book of data.
Penguin books p54

Table 8.13. Biochemical defects in SDAT and in rats after treatment with Al.

Observation	SDAT	Aluminium
brain total biopterin	lowered	lowered
BH_4 synthesis	inhibited	inhibited
GTP cyclohydrolase	no change	no change
sepiapterin reductase	no change	no change
tyrosine hydroxylase	inhibited	inhibited
choline acetyl transferase	inhibited	inhibited
glutamate decarboxylase	inhibited	inhibited
dopamine-β-hydroxylase	inhibited	inhibited
monoamine oxidase A	no change	no change
hexokinase	inhibited	inhibited
adenylate cyclase	elevated	elevated
AMP levels	reduced	not known
cAMP levels	elevated	elevated

Table 8.14 The Effect of Transferrin (TF) on *in vitro* BH_4 synthesis in tissue from control rats and those dosed with Al

	Control	+ TF	Al dosed	Al +TF
mean	11.67	10.97	6.56	9.60*
±s.d	1.81	2.07	1.24	1.42

* Al + TF > Al dosed $p<0.01$

Al + transferrin (50μM) not significantly different from either control group.

units:-pmol BH_4 synthesised $hour^{-1}$ $mg\ protein^{-1}$

The epidemiological evidence now available lends further support to the hypothesis that aluminium is a major cause of the Alzheimer pathology (Martyn *et al.*, 1989). Aluminium produces a profile of biochemical changes similar to that seen in SDAT. Aluminium is known to be neurotoxic, as seen in the dialysis dementia syndrome. Removal of aluminium from Al treated rat brain preparations, with an aluminium chelator, transferrin, reverses the biochemical defect. Brain samples from SDAT subjects which demonstrated a reduced BH_4 synthetic activity showed an improvement in activity when treated with transferrin indicating that the cause of impaired synthesis is aluminium.

The use of aluminium chelators may well provide a therapeutic rationale for Alzheimer's disease. Desferrioxamine improved the psychomotor performance of subjects on renal dialysis with high plasma aluminium levels, who despite having no outward signs of mental dysfunction, scored less than healthy controls in subtle tests of psychomotor function (Altmann *et al.*, 1987).

The evidence presented here further strengthens the case for its involvement in Alzheimer's disease by shedding some light on a possible mechanism for enzyme inhibition and demonstrating a reversal of a biochemical deficit in Alzheimer brain samples by treatment with an aluminium chelator.

8.5 LITHIUM, DEPRESSION AND TETRAHYDROBIOPTERIN METABOLISM

Lithium inhibits *in vitro* BH_4 synthesis in human brain preparation at concentrations as low as $5x10^{-5}M$ (Table 8.15). The inhibitory effect was dose related, correlating with lithium concentration on a log v. log plot ($r=-0.9388$, $p<0.05$).

Table 8.15 Dose Response Effect of Lithium on *in vitro* BH_4 Biosynthesis in Human Brain Tissue

	[Li^+]			
control	10^{-3}M	$5x10^{-4}$M	10^{-4}M	$5x10^{-5}$M
mean 5.63	1.88	1.96	2.32	1.38
±s.d.0.95	1.61	2.12	1.22	0.64
p	‹0.002	‹0.01	‹0.001	‹0.001
n 6	4	5	6	5

units:- pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

In vitro in rat brain preparations, however, no such effect is seen. The difference between the two systems may be due to there being different rate controlling steps in the synthesis of BH_4 in man and the rat.

Lithium ions interfere with magnesium homeostasis in the rat (Birch & Jenner 1973). Chronic lithium administration to rats in their drinking water caused lowered brain, liver and plasma biopterins and inhibited dopamine-β-hydroxylase (table 8.16). This metalloenzyme has a copper ion cofactor, enzyme inhibition may be due to displacement of the copper ion by lithium.

In BAD patients, not treated with lithium, there is an increase in urinary total biopterin output compared to controls possibly reflecting increased BH_4 synthetic activity (Garbutt *et al.*, 1986). Lithium may act in mania to inhibit metal ion dependent enzymes crucial to optimal noradrenergic activity such as dopamine-B-hydroxylase and 6-PTPS.

Lithium has been shown to have marked effects on the inositol phosphate second messenger system. The dephosphorylation of inositol phosphates is a metal ion dependent process, requiring magnesium (Birch *et al.*, 1988).

Noradrenaline stimulated adenylate cyclase responsible for generation of cyclic AMP is also a magnesium dependent enzyme that is inhibited at therapeutic plasma lithium concentrations (Ebstein *et al.*, 1980) lending further support to the theory that lithium acts by interference with crucial metal ion dependent systems and subsequent alteration of the cellular response to neurotransmitters or causing a reduction in the biosynthesis of the neurotransmitters either directy or by decreasing cofactor availability.

Table 8.16 The effects of chronic lithium administration on the rat

Parameter	control(n=5)	lithium(n=5)
1 brain total biopterin	88.1±6.2	65.1±8.7*
2 liver total biopterin	0.87±0.08	0.48±0.14*
3 plasma total biopterin	33.5±3.2	23.9±5.2**
4 brain *in vitro* BH_4 synthesis	14.71±4.15	4.28±1.33*
5 brain sepiapterin reductase	0.34±0.02	0.31±0.02
6 brain dopamine-β-hydroxylase	7.58±1.25	6.57±1.61

* $p<0.001$ ** $p<0.01$

Dosing Schedule:- see chapter 6 section 6.2

units:-

1 ng g^{-1} wet weight

2 μg g^{-1} wet weight

3 ng ml^{-1} plasma

4 pmol BH_4 $hour^{-1}$ mg $protein^{-1}$

5 nmol sepiapterin min^{-1} mg $protein^{-1}$

6 nmol octopamine min^{-1} mg $protein^{-1}$

8.6 PSYCHOACTIVE DRUGS BIOMOLECULES AND TETRAHYDROBIOPTERIN METABOLISM

8.6.1 HUMAN BRAIN *IN VITRO* STUDIES

Monoamine oxidase inhibitors (MAOIs)inhibited human brain BH_4 synthesis *in vitro* at high incubation concentrations ($>10^{-5}$M). Additionally, phenelzine inhibited DHPR activity *in vitro* ($p<0.05$, Table 8.17). The catecholamines and serotonin inhibit human BH_4 synthesis *in vitro* at the level of sepiapterin reductase (Table 8.17)in a manner similar to the effects of these compounds on rodent BH_4 metabolism (Katoh and Sueoka, 1986).

Oxotremorine is a potent inhibitor of human brain BH_4 synthesis *in vitro* causing a 30% reduction in synthetic activity at 10^{-6}M incubation concentration ($p<0.05$, Table 8.18). The site of action is not at sepiapterin reductase as this is not inhibited *in vitro* at 10^{-3}M (Table 8.17).

Hamon *et al.*, (1986) report that 5-methyl tetrahydrofolate (5-MeTHF) produced improvements in human brain Bh_4 synthesis. Subsequent investigations (Heales, 1987; Cattell, 1988) have failed to reproduce this. This report has shown that although 5-MeTHF has no effect on human brain BH_4 synthesis *in vitro* (Table 8.17),methionine

increases *in vitro* BH_4 synthetic activity (Table 8.17). 5-MeTHF may act to increase levels of methionine via methionine synthetase and this may then elevate BH_4 synthetic activity.

Table 8.17 Effects of compounds on human brain BH_4 metabolism *in vitro*

compound	BH_4 synthesis.[1]	DHPR[2]	Sepiapterin r'ase[3]
control	2.12±0.52	156±51	0.24±0.04
phenelzine	0.64±0.88*	102±12**	0.23±0.02
iproniazid	0.72±1.00**	132±21	0.27±0.05
isoniazid	0.32±0.73**	127±26	0.27±0.08
control	2.79±0.48	178±42	0.32±0.03
noradrenaline	0.46±0.23**	192±28	0.22±0.04*
dopamine	1.32±0.85*	167±56	0.24±0.02*
L-dopa	0.00±0.00**	137±26	0.21±0.04*
control	16.92±3.12	159±38	0.30±0.04
serotonin	5.65±2.67**	164±31	0.23±0.03**
5-HIAA	12.75±2.71	172±50	0.26±0.07

n=6 *p<0.05 **p<0.01

5-HIAA:- 5-hydroxyindoleacetic acid

continued over

Table 8.17 cont'd

compound	BH_4 synthesis.[1]	DHPR[2]	Sepiapterin r'ase[3]
control	7.83±1.02	147±52	0.18±0.03
oxotremorine	0.52±0.47**	152±31	0.17+0.05
methionine	9.39±0.79*	139±49	0.23±0.04
5-MeTHF	8.18±1.93	nd	nd
homocysteine	8.51±1.60	nd	nd

n=6 * $p<0.05$ ** $p<0.01$

5-MeTHF:- 5-methyl-tetrahydrofolate

drug incubation concentration = 1mM

nd = not determined

incubation contains 1mMolar glutathione

units:-

1 pmoles BH_4 $hour^{-1}$ mg $protein^{-1}$

2 nmoles NADH min^{-1} mg protein-1

3 nmoles sepiapterin min^{-1} mg $protein^{-1}$

Table 8.18 The effect of oxotremorine on *in vitro* synthesis of tetrahydrobiopterin in human brain

	Control	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M
mean	7.74	0.14*	1.22*	1.07**	6.18	7.85
±s.d.	0.47	0.22	0.24	0.43	1.30	0.82

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

units:-

pmoles BH_4 synthesised $hour^{-1}$ mg $protein^{-1}$

n=6

Incubation contains 1 mMolar glutathione

8.6.2 OXOTREMORINE AND RAT BH_4 METABOLISM *IN VIVO*

In vivo oxotremorine (0.25mg/kg + atropine methyl nitrate 1mg/kg) i.g.) causes a 30% reduction in the levels of brain total biopterins and derived pterin. Brain BH_4 synthesis was reduced by 50% in the dosed animals compared to controls. Sepiapterin reductase activity was unaffected, thus the site of inhibition must lie at GTP cyclohydrolase or 6-pyruvoyl-tetrahydropterin synthase (6-PTPS)(Table 8.19).

As oxotremorine acts as an inhibitor of BH_4 synthesis further investigations into this compound would be useful as it would provide a model for BH_4 deficiency. Diaminohydroxypyrimidine (DAHP) has been suggested as a tool for generating such a deficiency (Cotton 1986) but is associated with problems of solubility and gastro-intestinal interactions (Hasegawa *et al.*, 1988). Rats dosed with oxotremorine appeared to eat normally and at the end of the experiment weighed the same as controls, hence oxotremorine treatment does not appear to affect food consumption. This is important as reduced food consumption causes reductions in tissue total biopterins (Heales,1987; Cox and Blair, 1988). Oxotremorine is highly soluble and is easily administered in distilled water as the sesquifumarate salt solution.

8.6.3 NORTIPTYLINE AND RAT BH_4 METABOLISM *IN VIVO*

Nortriptyline is reported to inhibit bovine DHFR (Blake and Gould, 1986). Dosing with nortriptyline 1.43 mg/kg daily for 17 days produced a decrease in brain derived pterin ($p<0.01$,Table 8.20) but not in brain total biopterin (Table 8.20).

Table 8.19 The effects of oxotremorine on the rat

parameter	control(n=5)	oxotremorine(n=5)
1 brain total biopterin	113.1±16.6	74.9±21.5*
2 liver total biopterin	0.90±0.22	1.03±0.25
3 plasma total biopterin	36.3±4.5	37.2±5.7
4 brain derived pterin	5.5±1.6	6.8±1.7
5 liver derived pterin	0.24±0.06	0.24±0.08
6 brain *in vitro* BH_4 synthesis	6.01±1.12	3.63±1.71*
7 liver *in vitro* BH_4 synthesis	36.2±9.8	38.7±9.0
8 brain DHPR activity	186±58	173±54
9 liver DHPR activity	200±69	192±69
10 brain sepiapterin reductase activity	0.28±0.06	0.24±0.03

* $p<0.05$

Dosing Schedule See chapter 7 section 7.2

units:- 1 & 4 ng/g wet weight^{-1} 2 &5 µg/g wet weight^{-1}

3 ng ml^{-1} 6 & 7 pmol BH_4 synthesised hour^{-1} mg protein^{-1}

8 & 9 nmol NADH oxidised min^{-1} mg protein^{-1}

10 nmol sepiapterin reduced min^{-1} mg protein^{-1}

Edwards (1988) has proposed that brain pterin arises from two sources, dihydropterin derived from quinonoid dihydrobiopterin, and from the tetrahydrofolate (THF) pool. In the brain the derived pterin mainly arises from dihydropterin whereas in the liver the main source of derived pterin is the THF pool (Edwards 1988, Heales 1987). Reduced brain derived pterin may therefore reflect reduced throughput of BH_4 through tyrosine hydroxylase and DHPR.

Nortriptyline acts to increase synaptic levels of catecholamines and this will feedback onto tyrosine hydroxylase causing a reduction in activity and subsequent lowering of BH_4 turnover. It is unlikely that the lowering of brain derived pterin occurs secondary to reversible inhibition of DHPR as there are no changes in plasma total biopterins where we would expect an increase following DHPR inhibition (Leeming *et al.*, 1981).

There was no alteration in the folate status of the nortriptyline dosed group. Total folates (Table 8.23) and tetrahydrofolate levels (Table 8.23) were unchanged indicating no effect on DHFR *in vivo*. Studies on DHFR directly were not carried out here but would be of interest for further work.

Table 8.20 The effects of nortriptyline on the rat

parameter	control(n=5)	nortriptyline(n=5)
1 brain biopterin	87±10	90±11
2 liver biopterin	10.37±3.22	11.31±1.32
3 plasma biopterin	36.3±6.9	35.0±5.6
4 brain pterin	11.0±2.3	7.2±1.7*
5 liver pterin	0.15±0.05	0.15±0.06
6 brain DHPR activity	200±44	189±61
7 liver DHPR activity	231±56	249±72
8 brain sepiapterin reductase activity	0.29±0.05	0.25±0.05
9 liver sepiapterin reductase activity	0.27±0.05	0.32±0.08
10 brain total folate	32.8±1.8	30.2±4.5
11 brain tetrahydrofolate	30.6±1.2	27.9±3.8

mean +s.d. *p<0.01

units:- 1 & 4 ng/g wet weight^{-1} 2 &5 µg/g wet weight^{-1}

3 ng ml^{-1}

6 & 7 nmol NADH oxidised min^{-1} mg protein^{-1}

8 & 9 nmol sepiapterin reduced min^{-1} mg protein^{-1}

10 & 11 ng ml homogenate^{-1} (20%(w/v), made up in phosphate buffer pH 7.4)

8.7 GENERAL CONCLUSIONS

This study has shed further light on the role of tetrahydrobiopterin in mental disorders. The reduction in DHPR seen in neurological dysfunctions such as autism and Rett's syndrome does not arise secondary to the mental retardation. However more work needs to be carried out to ascertain any causal relationship between DHPR deficit in autism and Rett's syndrome and their associated clinical manifestations.

In Alzheimer's disease there are deficits in BH_4 synthetic capacity in several areas of human brain. This study has demonstrated a defect in the visual cortex in addition to confirming those previously reported in the locus coeruleus and temporal cortex (Anderson *et al.*, 1987, Barford *et al.*, 1984). The importance of accurate age and area matched controls is highlighted with the observation that DHPR activity and BH_4 synthetic capacity both correlate with age in non demented subjects and also vary between brain area.

This study further implicates aluminium in the Alzheimer pathology. Chronic administration of aluminium to rats in their drinking water produced a profile of defects in tetrahydrobiopterin and neurotransmitter biochemistry similar to that seen in Alzheimer's disease sufferers, thus providing a model for the disease.

The defect in BH_4 synthesis was reversed by treatment of the tissue samples with a potent aluminium chelator transferrin. Treatment with transferrin of human brain samples taken from Alzheimer's disease victims produced improvements in BH_4 synthetic capacity.

This report suggests that aluminium exerts its toxic effect by displacing crucial cations, such as magnesium and iron, from metallozymes and so inhibits enzyme activity. By doing so it would have marked effects on the enzymes of glycolysis, neurotransmitter biosynthesis and second messenger metabolism. Such alterations would have a profound effect on the functioning of the brain.

Lithium may also interfere with metal ion dependent enzymes. It produces a similar profile of effects on chronic administration, to that produced by aluminium. Lithium is also reported to have effects on the inositol phosphate second messenger system (Birch, 1982). To what extent these effects, especially on BH_4 metabolism, contribute to its well documented anti-manic action is unclear.

Other psychoactive drugs used have effects on BH_4 metabolism, particularly the monoamine oxidase inhibitors. These probably act by decreasing monoamine turnover and this then will feedback on BH_4 turnover. Oxotremorine induces depression in Alzheimer's disease sufferers and

appears to be a potent inhibitor of brain BH_4 metabolism in the rat. Oxotremorine may be of potential use as a tool in generating a central BH_4 deficiency. Nortriptyline despite being reported as inhibiting bovine liver DHFR had no effects on folate metabolism in the rat but did cause a reduction in brain pterin levels in the absence of any other effect on biopterin metabolism.

5-MeTHF is reported to increase BH_4 synthesis (Hamon *et al.*, 1986), though this finding has proven difficult to repeat. This report shows methionine to increase human brain BH_4 synthetic activity *in vitro*. This provides us with a possible explanation of the observations of Hamon *et al.*, (1986). 5-MeTHF may cause elevation of tissue methionine levels and this then elevates BH_4 synthesis by some as yet unknown mechanism.

8.8 FURTHER WORK

(1) Analysis of other brain regions in SDAT and control subjects for defects in BH_4 synthesis. Investigation of any relationship between plaque and tangle density and BH_4 synthesis defect in affected brain areas. Confirmation that the defect in BH_4 synthesis in SDAT lies at the level of 6-PTPS using the newly developed radioimmunoassays.

(2) Investigation of brain aluminium levels after chronic dosing. This was not performed in this report because of a lack of facilities for such measurements. Experiments should be performed at different dose levels and for different durations to ascertain the pharmacokinetics of aluminium with respect to its effects on biopterin and neurotransmitter biochemistry.

(3) Experiments should be performed to see if transferrin treatment *in vitro* reverses the other effects seen after chronic aluminium dosing, such as tyrosine hydroxylase and dopamine-β-hydroxylase inhibition.

(4) Choline acetyl transferase has no metal ion cofactor requirement and is not inhibited by aluminium *in vitro* but is reduced in activity *in vivo* following aluminium dosing. The mechanism of this inhibition is in need of elucidation, especially as reduced CAT activity is a classical lesion of SDAT.

(5) Lithium inhibits BH_4 synthesis in man, probably at 6-PTPS. Investigation of its effects on other metal ion dependent enzyme systems may shed some light on its mode of action as an anti-manic agent, which is to-date unknown.

(6) Oxotremorine is potent inhibitor of BH_4 synthesis in man and the rat, both *in vivo* and *in vitro*. Determination of the site of action and the dose-response nature of the inhibition will enable this compound to be used effectively as a model for BH_4 deficiency.

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APPENDIX 1. CASE HISTORIES AND PATIENT DATA FOR SUBJECTS USED IN CHAPTERS 3 AND 4

A1.1: Case Histories of DHPR subjects; Chapter 3 (Samples from Dr. A. V. Mehta, CREMERE, Bombay.)

No.	sex (m/f)	age (yrs)	diagnosis	[biopterin] (µg/litre)	DHPR (*)
1	?	9	Down's	4.1	128
2	F	14	?	4.1	128
3	F	12	epilepsy	‹2.4	186
4	F	17	hypothyroid	3.4	240
5	F	12	meningitis	4.6	149
6	M	8	epilepsy	4.3	176
7	M	10	asphyxia	4.6	180
8	M	12	anoxia	2.4	101
9	M	8	polio	4.7	186
10	F	7	Down's	3.8	122
11	M	8	Down's	19.2	186
12	M	9	anoxia	4.3	213
13	F	11	anoxia	4.6	250
14	F	6	Down's	4.3	165
15	M	2	?	4.3	138
16	F	4	convulsions	4.6	218
17	M	8	?	3.4	138
18	M	4	meningitis	3.8	202
19	M	4	Down's	3.4	287
20	F	9	cerebral palsy	3.6	255
21	M	7	premature	3.4	212
22	M	25	Lesch-Nyhan	3.6	197
23	F	7	anoxia	3.6	207
24	M	6	?	5.5	234
25	M	8	Down's	3.8	205
26	M	11	Down's	6.9	75
27	F	11	?	3.8	95
28	M	6	microcephaly	3.4	160
29	M	8	Down's	6.9	70
30	M	15	Down's	3.8	181
31	M	5	anoxia	4.3	213
32	M	9	microcephaly	4.3	170
33	?	15	microcephaly	3.8	170
34	M	6	Down's	3.1	138
35	M	11	?	3.1	160
36	M	9	Down's	3.1	202
37	M	9	?	3.6	213
38	F	11	microcephaly	3.4	255
39	F	10	Down's	12.5	191
40	M	6	Rubella	3.1	170
41	F	4	?	3.4	266
42	F	3	Down's	3.8	106
43	M	5	low birth wt.	3.4	181
44	M	6	?	3.6	160
45	M	4	enz.deficiency	2.4	255
46	F	15	asphyxia	2.4	106
47	F	25	?	‹2.4	128
48	F	15	Down's	‹2.4	191
49	M	17	encephalitis	‹2.4	128
50	M	24	Down's	3.1	234
51	M	19	genetic	‹2.4	223

A1.1. cont'd

No.	sex (m/f)	age (yrs)	diagnosis	[biopterin] (µg/litre)	DHPR (*)
52	M	26	birth trauma	‹2.4	138
53	M	21	head injury	‹2.4	106
54	M	14	encephalitis	‹2.4	138
55	M	25	cerebral palsy	‹2.4	159
56	M	19	?	‹2.4	160
57	M	14	epilepsy	‹2.4	159
58	M	26	?	2.4	245
59	M	15	head injury	‹2.4	191
60	?	14	asphyxia	‹2.4	149
61	F	10	?	2.4	138
62	M	9	?	2.4	160
63	M	9	brain damage	‹2.4	128
64	M	5	anoxia	2.4	128
65	M	7	Down's	‹2.4	138
66	M	6	convulsions	2.4	202
67	F	12	damage/trauma	2.4	43
68	F	14	meningitis	‹2.4	234
69	F	14	anoxia	‹2.4	117
70	F	13	birth trauma	2.4	128
71	F	8	birth anoxia	2.4	159
72	M	12	?	‹2.4	266
73	M	5	Down's	‹2.4	160
74	M	4	Down's	‹2.4	181
75	M	8	encephalitis	‹2.4	245
76	F	8	autistic	‹2.4	159
77	M	9	convulsions	‹2.4	202
78	F	4	Down's	‹2.4	254
79	M	11	Rubella	‹2.4	180
80	M	10	?	‹2.4	202
81	F	11	Trauma	‹2.4	75
82	M	11	Down's	‹2.4	213
83	M	9	Down's	‹2.4	191
84	F	4	Down's	‹2.4	190
85	M	10	convulsions	‹2.4	192
86	M	13	hydrocephalus	2.4	212
87	M	16	Down's	‹2.4	170
88	M	14	Down's	‹2.4	180
89	F	14	?	2.9	117
90	M	14	?	‹2.4	202
91	M	6	Down's	‹2.4	117
92	F	13	?	2.6	191
93	F	11	?	‹2.4	154
94	F	12	Down's	‹2.4	223
95	M	27	Down's	2.6	266
96	M	15	Down's	2.4	106
97	F	11	Down's	2.9	106
98	M	1	Down's	2.4	234
99	F	5	Down's	‹2.4	212
100	F	28	microcephaly	‹2.4	212
101	F	25	microcephaly	‹2.4	170

A1.1 cont'd

No.	sex (m/f)	age (yrs)	diagnosis	[biopterin] (µg/litre)	DHPR (*)
102	F	7	meningitis	2.4	168
103	M	7	birth anoxia	‹2.4	171
104	F	18	brain damage	‹2.4	223
105	M	9	?	‹2.4	298
106	F	11	?	‹2.4	170
107	M	7	birth asphyxia	3.1	191
108	M	13	" "	4.1	234
109	M	14	Down's	‹2.4	170
110	F	9	birth anoxia	‹2.4	191
111	F	6	Down's	‹2.4	191
112	F	8	Down's	‹2.4	0
113	M	8	Down's	‹2.4	128
114	M	12	anoxia	4.3	212
115	M	10	cerebral palsy	3.6	138
116	F	?	Down's	2.9	0
117	M	14	?	3.1	106
118	M	12	?	4.1	106
119	M	12	?	3.4	234
120	M	13	Down's	‹2.4	149
121	M	15	?	‹2.4	149
122	F	11	?	4.1	64
123	F	11	?	3.8	223
124	F	13	Down's	‹2.4	191
125	F	12	birth anoxia	2.9	181
126	M	13	premature	2.6	85
127	M	12	birth anoxia	4.3	128
128	M	20	Down's	4.6	106
129	M	6	jaundice	2.6	202
130	F	5	? genetic	4.1	95
131	M	16	?	2.6	212
132	M	16	rubella	4.3	181
133	M	14	encephalitis	‹2.4	170
134	F	15	Down's	2.9	95
135	M	16	convulsions	5.7	266
136	F	?	Down's	‹2.4	0
137	F	2	drug toxicity	4.8	191
138	M	8	convulsions	5.3	330
139	M	12	premature	6.2	234
140	F	11	head injury	3.4	213
141	M	9	Down's	4.3	181
142	M	9	jaundice	3.8	128
143	F	?	asphyxia	›24.0	181
144	F	10	anoxia	8.6	181
145	M	9	anoxia	4.1	277
146	F	8	anoxia	4.6	234
147	?	9	asphyxia	4.6	213
148	M	22	? genetic	2.6	234
149	M	8	anoxia	4.6	181
150	?	?	jaundice	3.1	245

A1.1 cont'd

HEALTHY CONTROLS

No.	sex (m/f)	age (yrs)	[biopterin] (µg/litre)	DHPR (*)
1	M	14	3.4	223
2	F	14	‹2.4	202
3	F	45	5.5	170
4	M	34	3.8	223
5	M	28	7.7	223
6	M	55	‹2.4	117
7	F	4	2.4	170
8	F	5	3.1	266
9	M	6	5.3	106
10	M	8	‹2.4	170
11	M	6	4.3	213
12	F	21	3.1	191
13	F	8	‹2.4	160

* units= nmoles NADH oxidised min^{-1} ml whole $blood^{-1}$

[biopterin] measured by Dr. R.J. Leeming, Haematology,Birmingham General Hospital.

A 1.2 RETT'S SYNDROME SUBJECTS

No.	sex (m/f)	age (yrs)	Code number	DHPR activity *
1	f	?	J165093	126
2	f	?	J088428	115
3	f	?	J165096	153
4	f	?	J088442	131
5	f	?	730722/0363	101
6	f	?	470309/0284	37
7	f	?	760108/0604	181
8	f	?	720523/6248	117
9	f	?	780617/8542	128

* units= nmoles NADH oxidised min^{-1} ml whole $blood^{-1}$

Provided by Prof.B. Hagberg, Barnklinikerna, Gothenberg.

A1.3 AUTISTIC SUBJECTS (all aged 12 years or under)

No.	sex (m/f)	Code	DHPR activity*
1	?	AH	127
2	?	ML	132
3	?	EK	127
4	?	SH	106
5	?	MK	151
6	?	JB	111
7	?	PK	134
8	?	RP	64
9	?	BK	149
10	?	TN	80
11	?	KJ	170

Provided by Professor Rutter, Maudsley Hospital, London
* nmol NADH oxidised min^{-1} ml whole $blood^{-1}$

A 1.4 Cause of death, age & *post-mortem* delay in control subjects (Tables 4.1 & 4.2) Obtained from Dr. P. Altmann, London Hospital, Whitechapel

Subject (sex;m/f)	Cause of Death	Age (yrs)	*Post-Mortem* Delay (hours)
1 (m)	cardiac disease	48	48
2 (m)	cancer	66	48
3 (?)	vascular disease	45	18
4 (m)	cancer	75	72
5 (f)	embolism	56	48
6 (m)	myocardial infarct.	57	120
7 (m)	pneumonia	87	48
8 (f)	Parkinson's Dis.	70	96
9 (?)	Haemorrhage	44	144
10(f)	cancer	77	24
11(m)	heart disease	72	24
12(f)	myeloma	36	72
13(f)	cancer	73	48
14(m)	Hodgkin's Dis.	38	48
15(f)	Haemorrhage	83	24
	mean	61.8	
	+s.d.	16.7	

A 1.5 Age, sex cause of death and *post mortem* delay in SDAT subjects (MRC Batch 1)

No.	Age (yrs)	Sex (m/f)	PM delay (hours)	Cause of death
D-64	73	f	41	Pneumonia
D-67	82	f	27	Bronchopneumonia
D-69	87	m	19	NK
D-70	77	f	34	Acute heart failure
D-73	63	f	105	Bronchopneumonia

NK = not known

A 1.6 Age, sex cause of death and *post mortem* delay in control subjects (MRC Batch 1)

No.	Age (yrs)	Sex (m/f)	PM delay (hours)	Cause of death
D-74	72	f	30	NK
C-293	82	m	96	Pulmonary embolism
D-90	87	f	36	NK
C-295	75	f	26	NK
D-77	72	f	30	NK

NK = not known

Samples obtained from MRC Brain Bank Cambridge.

Samples D-74, D-90 and D-77 are controls and not dements despite the D-prefix.

A 1.7 Case histories of SDAT subjects (MRC Batch 2)

D-57 Plaque density 4.29 Tangle density 3
Age:- 64yrs PM delay:- 45 hours Sex:- F
Cause of Death:- ?
Drug History :- ?

D-59 Plaque density 0.90 Tangle density 3
Age:- 82yrs PM delay:- 22 hours Sex:- F
Cause of Death:- Congestive Heart Failure
Drug History :- ?

D-61 Plaque density 2.69 Tangle density 3
Age:- 71yrs PM delay:- 72 hours Sex:- F
Cause of Death:- ?
Drug History :- Chlorpromazine Haloperidol
since 1967 Promazine Procyclidine
Imipramine
just Electroconvulsive therapy
before death Diamorphine

D-62 Plaque density 1.26 Tangle density 3
Age:- 63yrs PM delay:- 26 hours Sex:- F
Cause of Death:- Bronchopneumonia
Drug History :- None in last week

D-78 Plaque density 3.35 Tangle density 3
Age:- 72yrs PM delay:- 26 hours Sex:- M
Cause of Death:- Bronchopneumonia, heart failure
Drug History :- Aspirin

Portions of BA4, BA10, BA38 and locus coeruleus

Samples obtained from MRC Brain Bank, Cammbridge.

Plaque and tangle densities determined in Superior Temporal Gyrus.

A 1.8 Case histories of control subjects (MRC Batch 2)

C-345
Age:- 81yrs PM delay:- 43 hours Sex:- F
Cause of Death:- ?
Drug History :- ?

C-393
Age:- 63yrs PM delay:- 39 hours Sex:- F
Cause of Death:- Myocardial Infarction,
Atherosclerosis
Drug History :- Prothiaden

C-430
Age:- 64yrs PM delay:- 10 hours Sex:- F
Cause of Death:- Haemopericardium
Drug History :- ?

C-476
Age:- 81yrs PM delay:- 3 hours Sex:- M
Cause of Death:- Acute heart failure
Drug History :- Chlormethiazole

C-486
Age:- 73yrs PM delay:- 44 hours Sex:- M
Cause of Death:- Myocardial infarction,
Kidney cancer
Drug History :- none

C-504
Age:- 72yrs PM delay:- 46 hours Sex:- F
Cause of Death:- Myocardial Infarction
Drug History :- cardiovascular therapy

Portions of BA4, BA10, BA38 and locus coeruleus

Samples obtained from MRC Brain Bank, Cambridge.

APPENDIX 2
THE IMPROVEMENT OF BH_4 YIELD IN THE *IN VITRO* BH_4 SYNTHESIS ASSAY BY ADDITION OF GLUTATHIONE

A 2.1 INTRODUCTION

The *in vitro* synthesis assay is used extensively throughout this research project and as an attempt to improve both yield and reproducibility of the assay the effect of addition of glutathione to the incubation medium was investigated. BH_4 is very labile and is rapidly broken down in an aerobic atmosphere at 37°C (Heales 1987), the conditions of the assay.

A 2.2 MATERIALS AND METHODS

The assay technique was as described in chapter 2 with the exception that prior to this investigation no antioxidants were added to the incubation medium. Glutathione (GSH) was obtained from Sigma Chemical Company, Poole, Dorset. All other reagents were as described in chapter 2.

A 2.3 RESULTS

GSH increased BH_4 yield significantly (Table A 2.1). The dose response study with GSH (10^{-2}M to 10^{-6}M) indicates that optimum protective effect is achieved at a concentration of 10-3M in the assay medium(table A2.2.), the yield being significantly higher than controls and lower concentrations of GSH. There was no increase in yield with increased GSH concentrations above this limit.

Table A2.1. *In vitro* effect of GSH on BH_4 Biosynthesis in rat brain

	Control	GSH [6mM]
mean	2.78	7.19*
±s.d.	1.10	2.77

*($p<0.01$)

abbreviations: GSH= glutathione

units= pmoles BH_4 synthesised $hour^{-1}$ $mg\ protein^{-1}$

Table A 2.2. Effect of GSH on *in vitro* BH_4 synthesis

Molarity of added GSH	biosynthetic activity (±s.d.) *	n
Control	2.45 (0.65)	6
10^{-5}	1.96 (0.70)	6
10^{-4}	2.75 (1.27)	5
10^{-3}	9.15 (3.60)	6
10^{-2}	9.13 (4.60)	6

* units pmoles BH_4 synthesised $hour^{-1}$ $mg\ protein^{-1}$

A 2.4 DISCUSSION

BH_4 is very labile and is oxidised readily at room temperature (Heales 1987). The addition of glutathione to the incubation medium improves BH_4 yield by over 300% (Tables A2.1 and A2.2) and thus enhances the sensitivity of the assay. Samples of temporal cortex from subjects with senile dementia of the Alzheimer type have very low BH_4 synthetic activity compared to controls and thus an improvement in the sensitivity of the assay allows determination of actual values for their synthetic activity.