OXIDATION EFFECTS ON TETRAHYDROPTERIN METABOLISM

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SUMMARY:
The susceptibility of tetrahydropterins to oxidation was investigated *in vitro* and related to *in vivo* metabolism. At physiological pH, tetrahydrobiopterin (BH_4) was oxidized, with considerable loss of the biopterin skeleton, by molecular oxygen. The hydroxyl radical ($\cdot OH$) was found to increase this oxidation and degradation, whilst physiological concentrations of glutathione (GSH) retarded both the dioxygen and $\cdot OH$ mediated oxidation. Nitrite, at acid pH, oxidized BH_4 to biopterin and tetrahydrofolates to products devoid of folate structure. Loss of dietary folates, from the stomach, due to nitrite mediated catabolism is suggested.

The *in vivo* response of BH_4 metabolism to oxidizing conditions was examined in the rat brain and liver. Acute starvation depressed brain biopterins and transiently BH_4 biosynthetic and salvage (dihydropteridine reductase, DHPR) pathways. Loss of biopterins, in starvation, is suggested to arise primarily from catabolism, due to oxygen radical formation and GSH depletion. *L*-cysteine administration to starving rats was found to elevate tissue biopterins, whilst depletion of GSH in feeding rats, by *L*-buthionine sulfoximine, decreased biopterins. An *in vivo* role for GSH to protect tetrahydropterins from oxidation is suggested.

The *in vivo* effect of phenelzine dosing was investigated. Administration lowered brain biopterins, in the presence of dietary tyrosine. This loss is considered to arise from *p*-tyramine generation and subsequent DHPR inhibition. Observed elevations in plasma biopterins were in line with this mechanism. In conditions other than gross inhibition of DHPR or BH_4 biosynthesis, plasma total biopterins were seen to be poor indicators of tissue BH_4 metabolism.

Evidence is presented indicating that the pterin formed in tissue samples by acid iodine oxidation originates from the tetrahydrofolate pool and 7,8-dihydropterin derived from BH_4 oxidation. The observed reduction in this pterin by prior *in vivo* nitrous oxide exposure and elevation by starvation and phenelzine administration is discussed in this light.

The biochemical importance of the changes in tetrahydropterin metabolism observed in this thesis are discussed with extrapolation to the situation in man, where appropriate. An additional role for BH_4 as a tissue antioxidant and reductant is also considered.

Keywords: TETRAHYDROBIOPTERIN GLUTATHIONE STARVATION
OXIDATION PHENELZINE

ACKNOWLEDGEMENTS

I would like to thank Professor J.A. Blair (Department of Pharmaceutical Sciences, Biology Division) for his valued supervision throughout, and the Scientific and Engineering Research Council for financial support.

ABBREVIATIONS

A	Absorbance (optical density)
AAD	Aromatic amino acid decarboxylase
BBB	Blood brain barrier
BH_2	7,8-dihydrobiopterin
qBH_2	quinonoid dihydrobiopterin
BH_4	5,6,7,8-L-*erythro*-tetrahydrobiopterin
L-BSO	L-Buthionine sulfoximine
°C	Degrees centigrade
cAMP	Cyclic adenosine 3',5'- monophosphate
$5CH_3FH_4$	5-methyl-tetrahydrofolate
$5,10CH_2FH_4$	5,10-methylene-tetrahydrofolate
CNS	Central nervous system
CSF	Cerebrospinal fluid
DHPR	Dihydropteridine reductase
$DMPH_4$	6,7-Dimethyl,5,6,7,8-tetrahydropterin
DNA	Deoxyribonucleic acid
L-DOPA	L-3,4-Dihydroxyphenylalanine
EC	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
Emax	Extinction coefficient
GSH	Glutathione - reduced form
GSSG	Glutathione - oxidized form
HPLC	High performance liquid chromatography
IEM	Inborn error of metabolism
K_i	Pseudo rate constant
ki	Inhibitor constant
Km	Michaelis constant
LNAA	Large neutral amino acid
MAO	Monoamine oxidase
MHPA	Malignant hyperphenylalaninaemia
n	Sample size
NAD(H)	Nicotinamide adenine dinucleotide (reduced form)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced form)
NH_2	D-*erythro*-dihydroneopterin
NH_2P_3	D-*erythro*-dihydroneopterin triphosphate
ODS	Octadecyl silane
PKU	Phenylketonuria
RNA	Ribonucleic acid
SAM	S-Adenosylmethionine
SD	Standard deviation
SDAT	Senile Dementia of the Alzheimer type
SEM	Standard error of the mean
$t_{1/2}$	Half life
TAT	Tyrosine amino transferase
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultra violet
V	Velocity of an enzyme-catalysed reaction
Vmax	Maximum initial velocity of an enzyme catalysed reaction
XH_2	7,8-dihydroxanthopterin

CONTENTS

TABLES

FIGURES

CHAPTER ONE

INTRODUCTION

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INTRODUCTION.

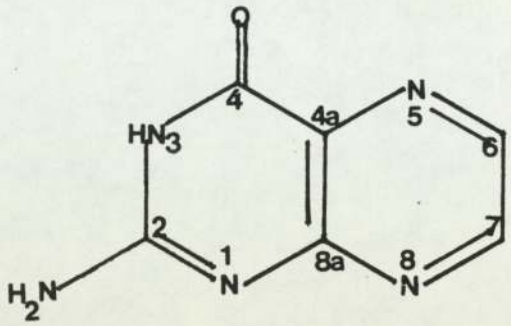
1.1 PTERINS.

Derivatives of 2-amino-4-oxo-pteridine are known as pterins (Pfleiderer, 1964). The simplest being pterin itself, the structure and numbering of which is shown (i) (Commission on biochemical nomenclature, 1965). Reduction of the pyrazine moiety of the pterin ring system produces 7,8-dihydro- (ii) and 5,6,7,8-tetrahydropterin (iii) (Pfleiderer, 1978).

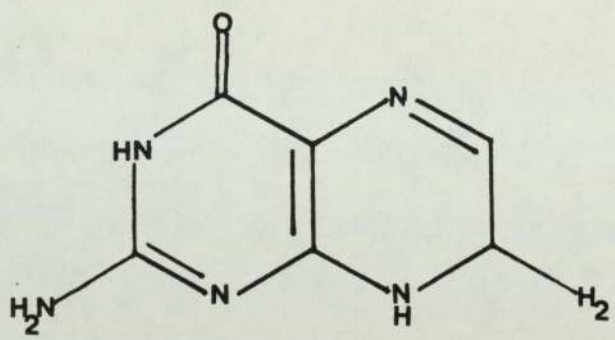
Substitution of the pterin ring at C(6) leads to the formation of a number of biologically important pterins. Many substituted pterins have been identified and characterised (Angier *et al.*, 1946; Patterson *et al.*, 1955; Pfleiderer, 1978). This study will consider those pterins associated primarily with tetrahydrobiopterin, 6-(1',2'-dihydroxypropyl) -5,6,7,8-tetrahydropterin, (iv) metabolism. However some aspects of tetrahydrofolate, 5,6,7,8-tetrahydropteroyl-glutamic acid, (v) metabolism will be considered.

1.2 ROLE OF TETRAHYDROBIOPTERIN AND TETRAHYDROFOLATE.

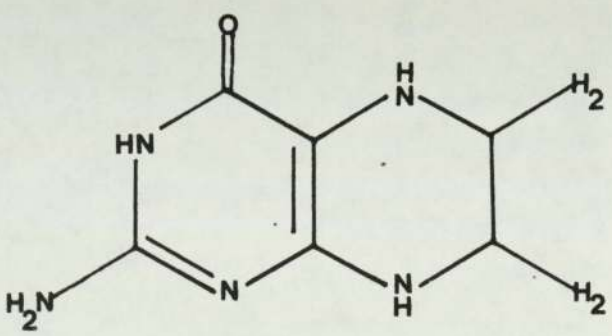
Tetrahydrobiopterin (BH_4) and tetrahydrofolate (FH_4) have different cofactor roles. BH_4 is involved in reactions



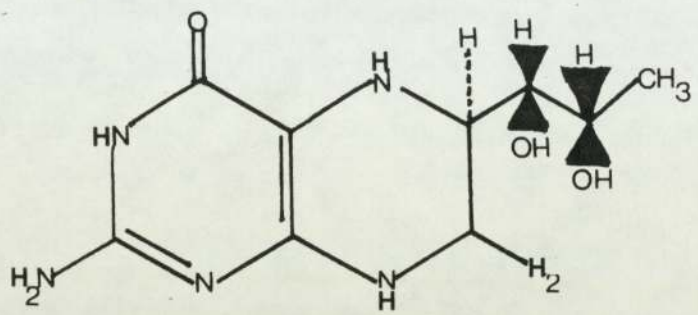
(i) Pterin



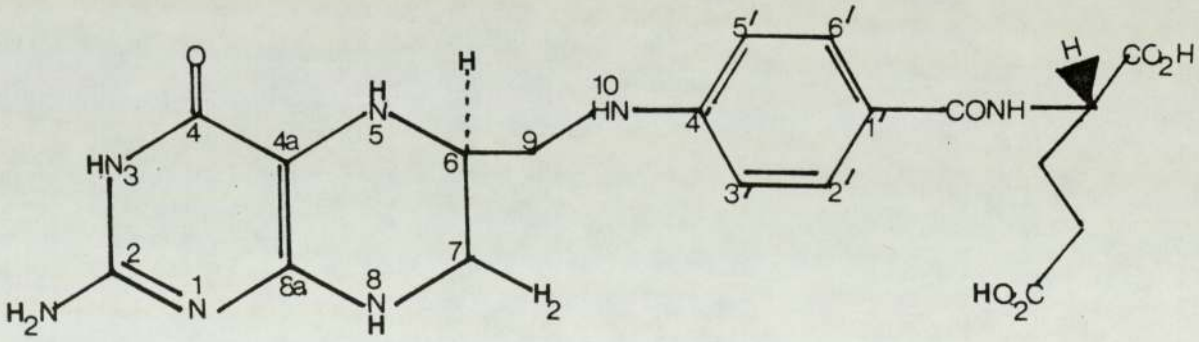
(ii) 7,8-Dihydropterin



(iii) 5,6,7,8-Tetrahydropterin



(iv) L-Erythro-Tetrahydrobiopterin



(v) Tetrahydrofolic acid

utilizing molecular oxygen (Kaufman, 1963), whereas FH_4 is involved in the transfer of single carbon groups (Rowe, 1983).

The carbon group carried by FH_4 is bonded to its N5 or N10 atom or both, giving rise to a number of FH_4 derivatives, e.g. 5-methyltetrahydrofolate and 10-formyltetrahydrofolate. These FH_4 derivatives are interconvertible *via* important enzymatic reactions, e.g. purine and pyrimidine biosynthesis (Hoffbrand, 1976). Because of the key role tetrahydrofolates play in cell division and growth, deficiency leads to a number of pathological disorders, e.g. megaloblastic anaemia (Rowe, 1983). Within the central nervous system (CNS) deficiency is associated with psychiatric disorders including forgetfulness, irritability, sleeplesness and paranoid behaviour (Botez and Reynolds, 1979).

In dividing cells folate metabolism studies have focused on DNA production, but in the CNS folates may be related to RNA metabolism, associated with memory function, and with the availability of the inhibitory neurotransmitter glycine (Nichol *et al.*, 1983).

The folate molecular skeleton, in contrast to BH_4, cannot be synthesized in mammalian tissues and is obtained in the diet. The minimum adult daily requirement for folates is 50ug (Sullivan and Herbert, 1964), increasing to 300ug during pregnancy (Willoughby, 1967).

The *in vivo* hydroxylation of phenylalanine to tyrosine, in the liver, is an enzymatic process requiring molecular oxygen, a reduced pyridine nucleotide and at least two enzymes (Undenfriend and Cooper, 1952). Kaufman (1958) identified an additional cofactor requirement for this reaction, which was later identified as a tetrahydropterin (Kaufman, 1959). BH_4 was shown to be the natural cofactor for the phenylalanine hydroxylase (EC. 1.14.16.1) component of this system (Kaufman, 1963).

BH_4 is also the cofactor for the enzymatic hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (EC. 1.14.16.2) (Brenneman and Kaufman, 1964; Shiman *et al.*, 1971). The hydroxylation of tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase (EC. 1.14.16.4) similarly requires BH_4 as a cofactor (Jequier *et al.*, 1969; Friedman *et al.*, 1972).

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

Within the CNS, the presence of BH_4 is therefore a necessary requirement for synthesis of catecholamine and serotonin neurotransmitters (Figs. 1.1 and 1.2) (Leeming *et al.*, 1981).

1.3 MODE OF ACTION OF TETRAHYDROBIOPTERIN.

Hydroxylation of aromatic amino acids results in oxidation of BH_4 to quinonoid dihydrobiopterin (qBH_2). BH_4 is regenerated from qBH_2 enzymatically (salvage pathway) by dihydropteridine reductase (DHPR) (EC. 1.6.99.7), a reaction requiring NADH (Kaufman, 1961; Kaufman, 1964; Craine *et al.*, 1972). Failure to salvage qBH_2 results in non enzymatic rearrangement to 7,8-dihydrobiopterin (BH_2) and 7,8-dihydropterin which are lost from the cell and move from the plasma to the urine in which they are excreted (Archer and Scrimgeour, 1970; Leeming *et al.*, 1976; Matsuura *et al.*, 1986). Whilst BH_2 is not a substrate for DHPR, dihydrofolate reductase (EC. 1.5.1.3) is able to reduce BH_2 back to BH_4 and may therefore have a salvage role in tissues with high activity of this enzyme (Kaufman, 1967; Nichol *et al.*, 1983) (Fig. 1.3).

The detailed mechanism by which BH_4 participates in hydroxylation of aromatic amino acids has not been fully elucidated. Molecular oxygen activation may arise from the covalent addition of dioxygen at position 4a forming a 4a-hydroperoxide, which decomposes giving a 4a-carbinolamine and the hydroxide cation (OH+) which acts as the hydroxylating agent (Pfleiderer, 1978). A 4a-carbinolamine has been identified as an intermediate in phenylalanine hydroxylation. This intermediate dehydrates to give qBH_2, a reaction catalysed by a protein called phenylalanine hydroxylase stimulating protein (PHS) which acts as a 4a-carbinolamine dehydratase (Lazarus *et al.*, 1981; Lazarus

et al., 1983) (Fig. 1.3).

The role of iron is not included in the above mechanism. Iron is essential for aromatic amino acid hydroxylase activity (Fisher *et al.*, 1972). An iron-oxygen intermediate has been suggested with BH_4 acting as a 2-electron reducing agent to form an iron-peroxide complex. The latter acting as the hydroxylating agent (Pearson, 1974b).

1.4 BIOSYNTHESIS OF TETRAHYDROBIOPTERIN.

Tissue levels of BH_4 are maintained by endogenous *de novo* biosynthesis (Pabst and Rembold, 1966). The initial step in the synthesis of BH_4 is the conversion of guanosine triphosphate (GTP) to D-*erythro*-dihydroneopterin triphosphate (NH_2P_3) by GTP cyclohydrolase (EC 3.5.4.11), a reaction involving an Amadori rearrangement (Burg and Brown, 1968) (Fig. 1.4).

It is likely that BH_4 is synthesized *via* tetrahydropterin intermediates and that NH_2P_3 is converted to a tetrahydropterin by an intramolecular rearrangement of electrons to the N(5) region of the pyrazine ring. This intermediate is dephosphorylated to give 6-pyruvoyl-tetrahydropterin by a magnesium dependent enzyme, 6-pyruvoyl tetrahydropterin synthetase (phosphate eliminating enzyme). Reduction of the keto groups of the latter by NADPH dependent sepiapterin and 2' keto reductases results in the production of L-*erythro*-tetrahydrobiopterin (BH_4) (Milstien and Kaufman,

1983; Heintel *et al.*, 1984; Switchenko and Brown, 1985; Milstien and Kaufman 1986) (Fig 1.5).

The synthesis of BH_4 is regulated by BH_4 acting as a potent inhibitor of GTP cyclohydrolase, i.e a negative feedback loop exists (Kapatos and Kaufman, 1983; Blau and Niederwieser, 1986). High levels of the neurotransmitters, serotonin and noradrenaline may also regulate BH_4 biosynthesis by acting as competitive inhibitors of sepiapterin reductase (Katoh *et al.*, 1983).

1.5 CATABOLISM OF TETRAHYDROPTERINS.

Tissue levels of BH_4, in addition to regulation by biosynthesis, are maintained by catabolism and excretion by the kidney (Rembold, 1983). BH_4 catabolism occurs in rat liver resulting in the formation of pterin, 7,8-dihydroxanthopterin, isoxanthopterin, lumazine and 6- and 7-hydroxylumazine (Rembold *et al.*, 1969). Catabolism is initiated by oxidation to BH_2 and 7,8-dihydropterin. The latter is converted to 7,8-dihydroxanthopterin, by hydration and subsequent oxidation, and to isoxanthopterin, following oxidation to pterin, by xanthine oxidase (EC. 1.1.3.22). Lumazine and 6- and 7-hydroxylumazine are formed by the additional action of a pterin deaminase (EC. 3.5.4.11) (Rembold and Gutensohn, 1968; Rembold *et al.*, 1969a; Rembold and Simmersbach, 1969b; Rembold *et al.*, 1971) (Fig. 1.6). Niederwieser *et al.* (1986) failed to detect pterin deaminase in man but reports deamination of orally administered BH_4

occurring as a result of bacterial action in the gut. In contrast to rat, the side chain of BH_4 is retained but modified to give 2'-deoxy sepialumazine as the major deamination catabolite in faeces (Niederwieser *et al.*, 1986)

The catabolism of reduced folates arises by the non-enzymatic oxidation of the C9-N10 bond of the molecule, giving rise to p-amino-benzoyl glutamate derivatives, xanthopterin and a number of unidentified pterins (Pheasant *et al.*, 1981; Pheasant *et al.*, 1983; Al-Haddad *et al.*, 1986).

1.6 TETRAHYDROBIOPTERIN METABOLISM AND DISEASE.

Lack of BH_4 leads to hyperphenylalaninaemia and neurological impairment (Leeming *et al*, 1981). Deficiency arises from diminished ability to synthesize BH_4 *de novo* or failure to salvage qBH_2 as a result of DHPR deficiency. Such disorders, having a frequency of occurrence of one in a million births, are termed malignant hyperphenylalaninaemias (MHPA) and are usually diagnosed during the first three months of life (Danks, 1978; Danks *et al.*, 1978; Dhondt *et al.*, 1981; Hamon and Blair, 1987).

Decreased BH_4 synthesis occurs as a result of deficiency of either 6-pyruvoyl tetrahydropterin synthetase (phosphate eliminating enzyme) or GTP cyclohydrolase (Niederwieser *et al.*, 1984; Niederwieser *et al.*, 1985). DHPR deficiency arises from failure to synthesize the enzyme or from production of a

mutant form of DHPR which lacks catalytic activity (Firgaira *et al.*, 1983).

Treatment of MHPA is by dietary restriction of phenylalanine and administration of neurotransmitter precursors (Danks *et al.*, 1978).

Changes in BH_4 metabolism have been noted in many other diseases (for reviews see Leeming *et al.*, 1981; Blair, 1985) including senile dementia of the Alzheimer type (SDAT). Significantly decreased levels of total biopterins (oxidized and reduced) have been reported in plasma and cerebrospinal fluid (CSF) of patients dying with SDAT (Leeming *et al.*, 1979; Young *et al.*, 1982; Aziz *et al.*, 1983; Morar *et al.*, 1983). A diminished ability to synthesize BH_4 occurs in the temporal lobe of patients with SDAT, with the biochemical lesion reported to occur at the level of phosphate eliminating enzyme (Barford *et al.*, 1984; Anderson *et al.*, 1986).

The neurotoxic metals, lead and aluminium, inhibit both salvage and synthesis of BH_4, suggesting that prolonged exposure to either metal would impair neurotransmitter synthesis (Purdy *et al.*, 1981; Cutler, 1986; Cowburn and Blair, 1987).

1.7 AIM OF THESIS.

Tetrahydrobiopterin and other reduced pterins are readily oxidized (Pearson, 1974). Since a number of oxidizing agents

and conditions may arise biologically, resulting in loss of tetrahydropterin cofactors, it was the aim of this thesis to investigate the effects of a number of such factors on BH_4 and related pterins *in vitro* and *in vivo*.

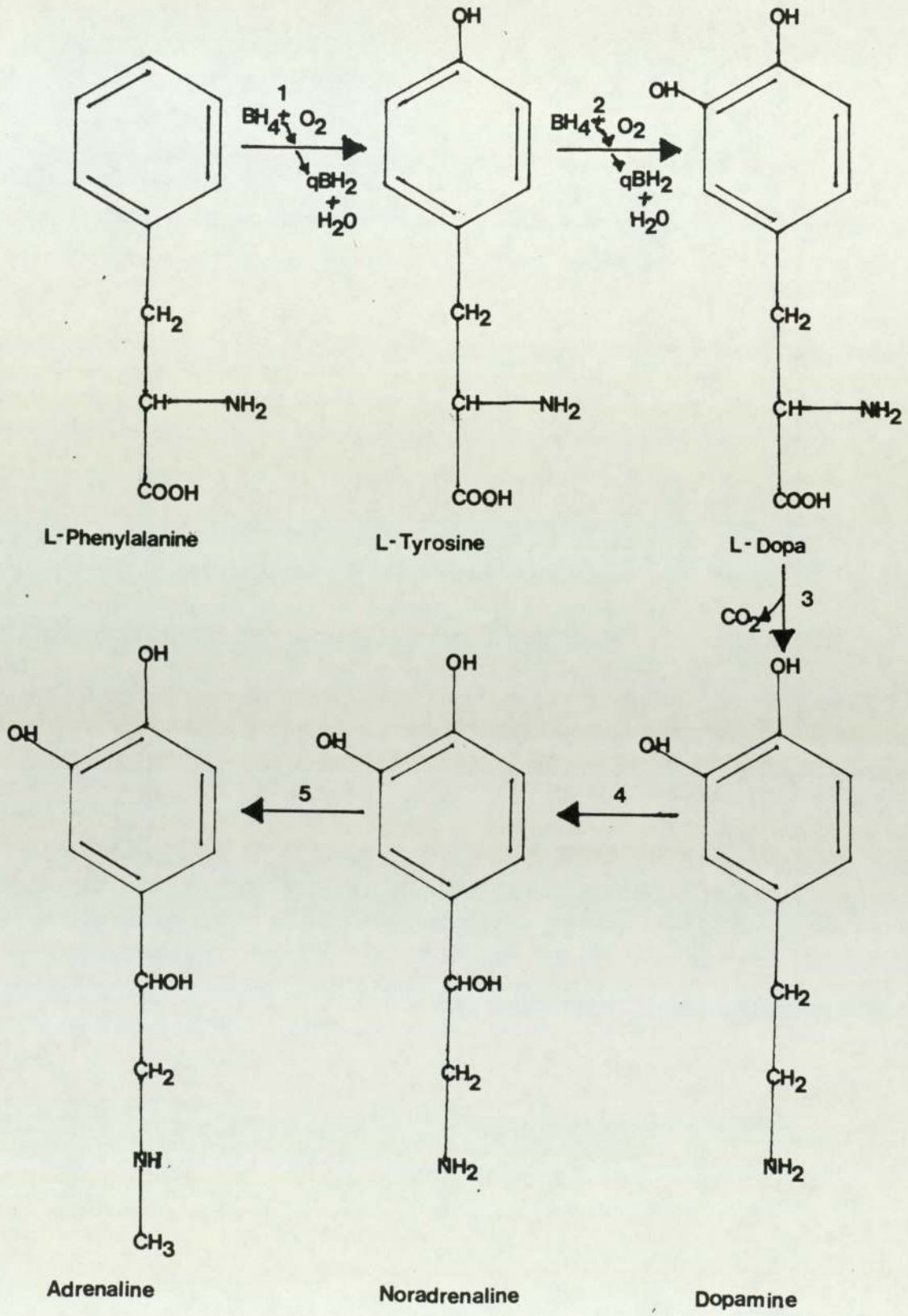


Fig 1.1 Biosynthesis of catecholamines.

1. Phenylalanine hydroxylase. 2. Tyrosine hydroxylase.
3. Aromatic amino acid decarboxylase. 4.Dopamine hydroxylase.
5. Phenethanolamine-N-methyl-transferase.

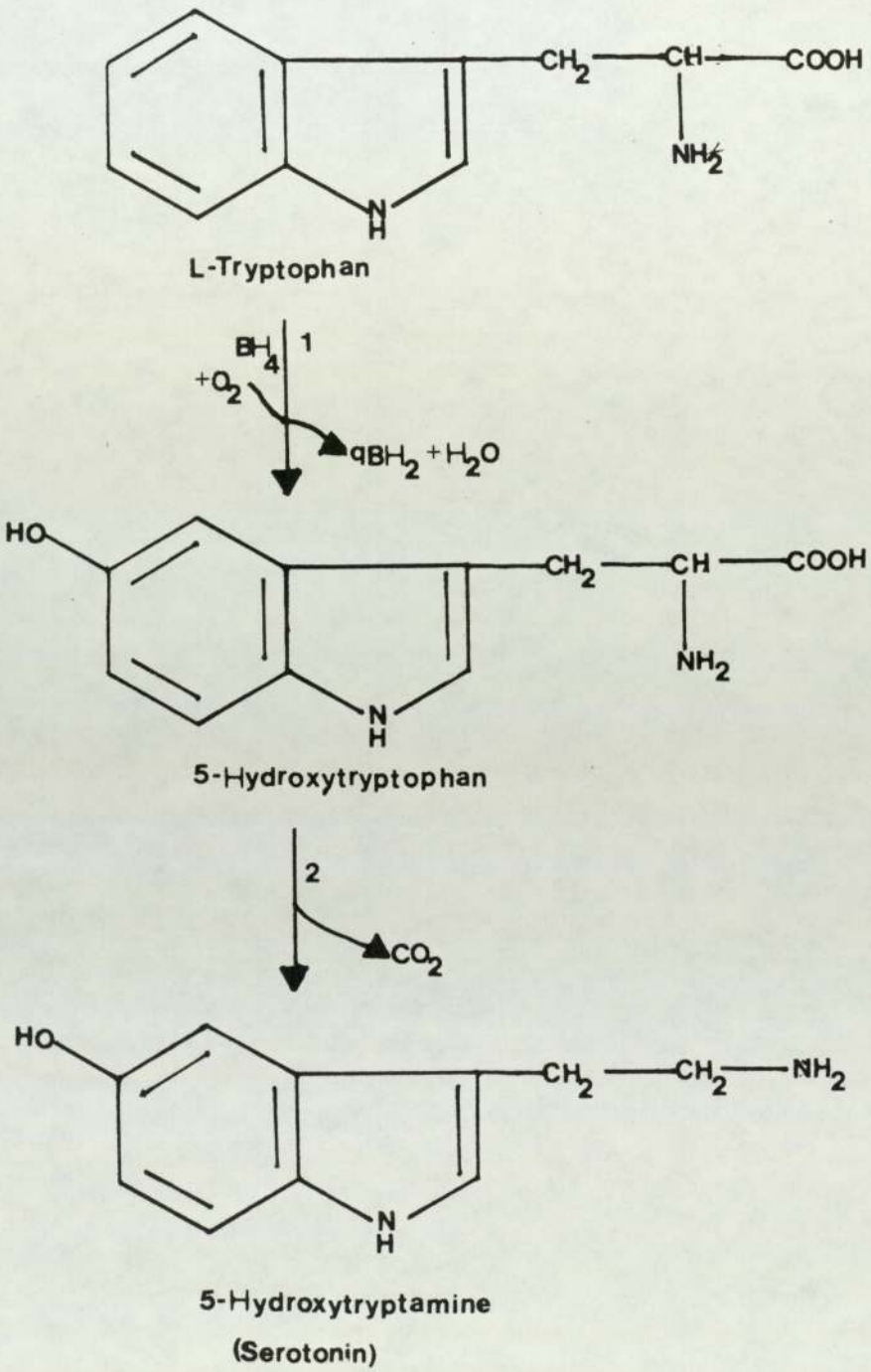


Fig. 1.2 Biosynthesis of serotonin.

1. Tryptophan hydroxylase.
2. Aromatic amino acid decarboxylase.

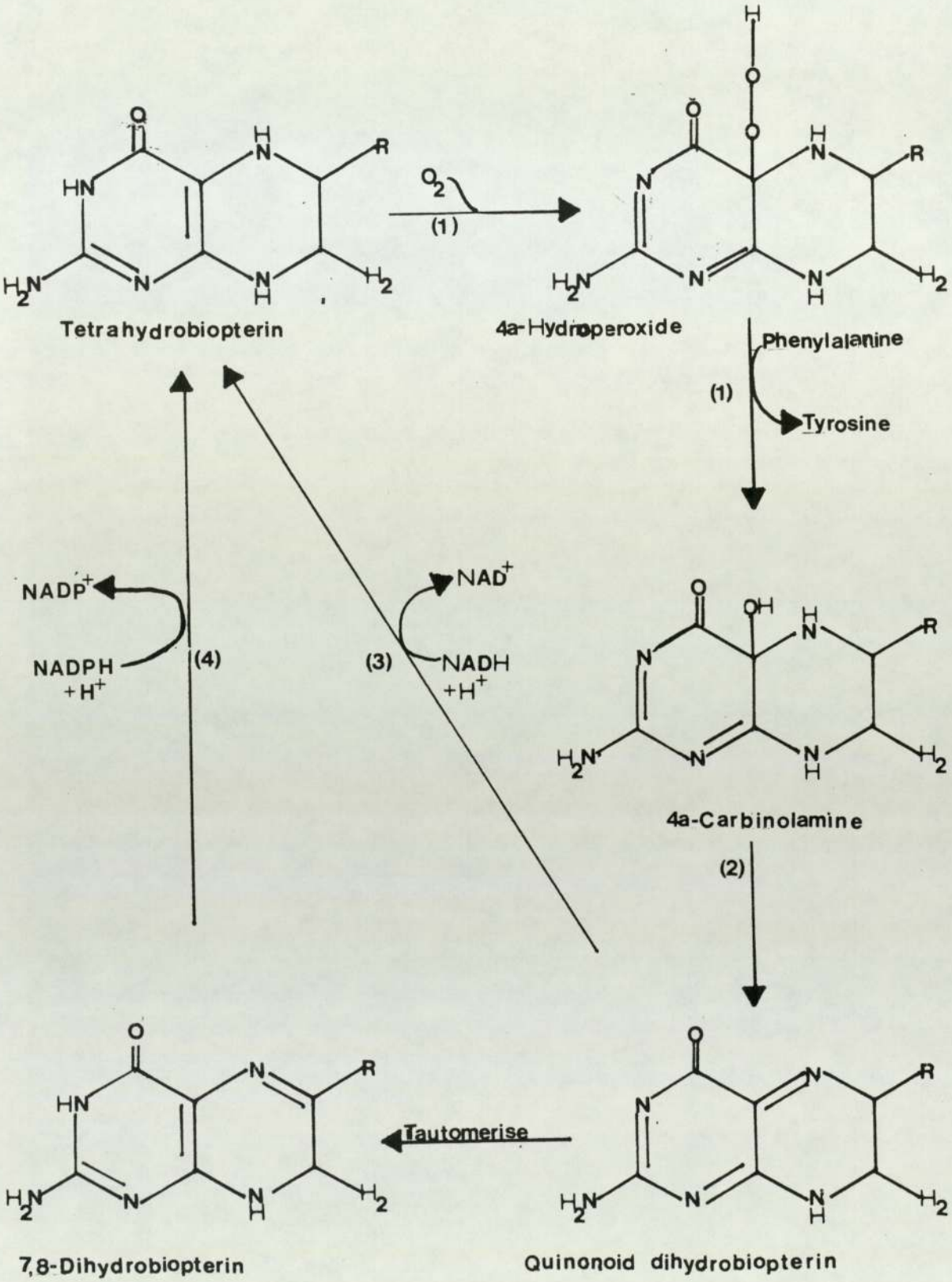


Fig. 1.3 Components of the phenylalanine hydroxylating system.

R= CHOH CHOH CH_3

1. Phenylalanine hydroxylase.
2. Phenylalanine hydroxylase stimulating protein.
3. Dihydropteridine reductase.
4. Dihydrofolate reductase.

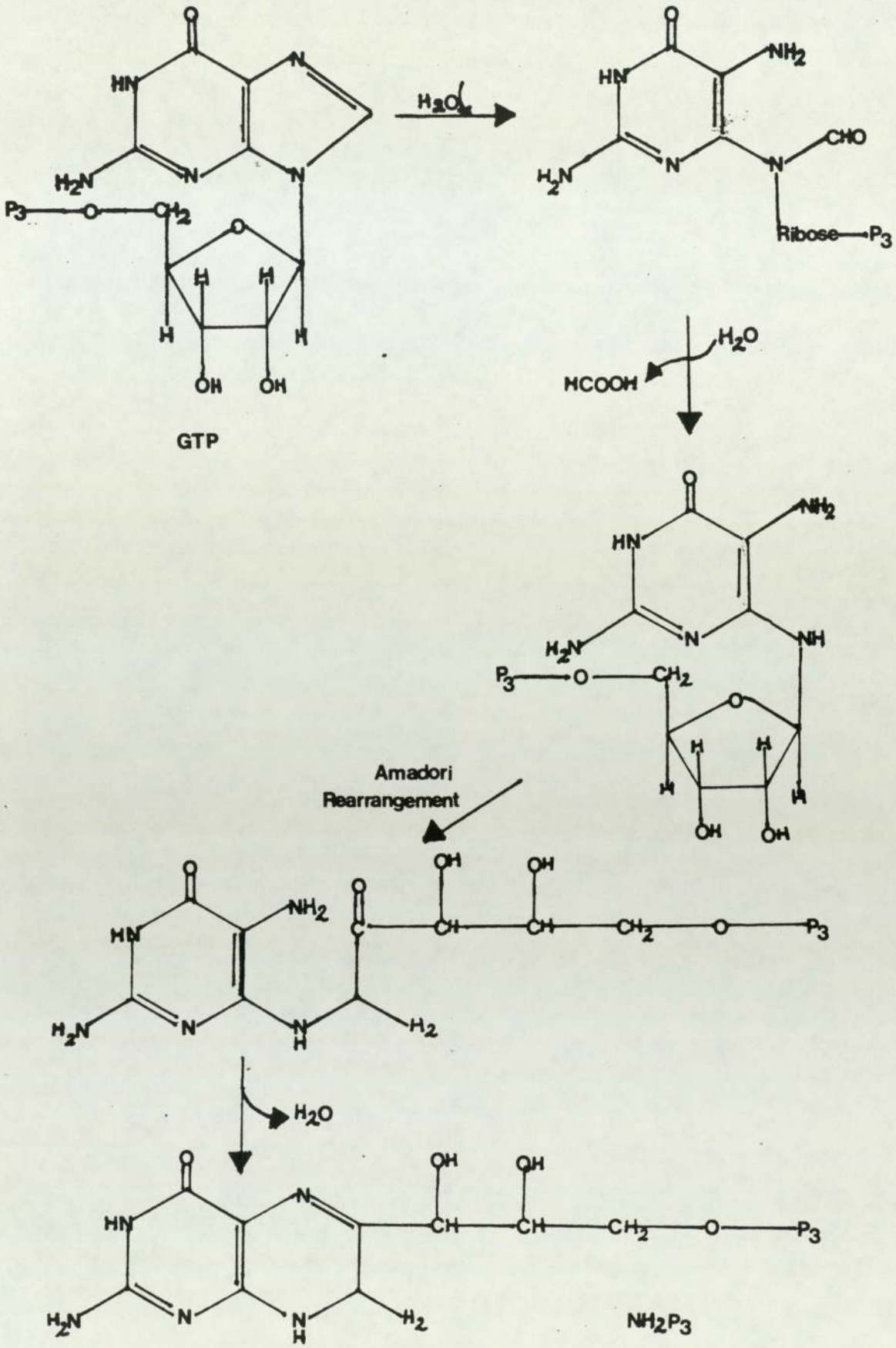


Fig. 1.4 The enzymatic conversion of guanosine triphosphate (GTP) to D-*erythro*-diydroneopterin triphosphate (NH_2P_3) by GTP cyclohydrolase.

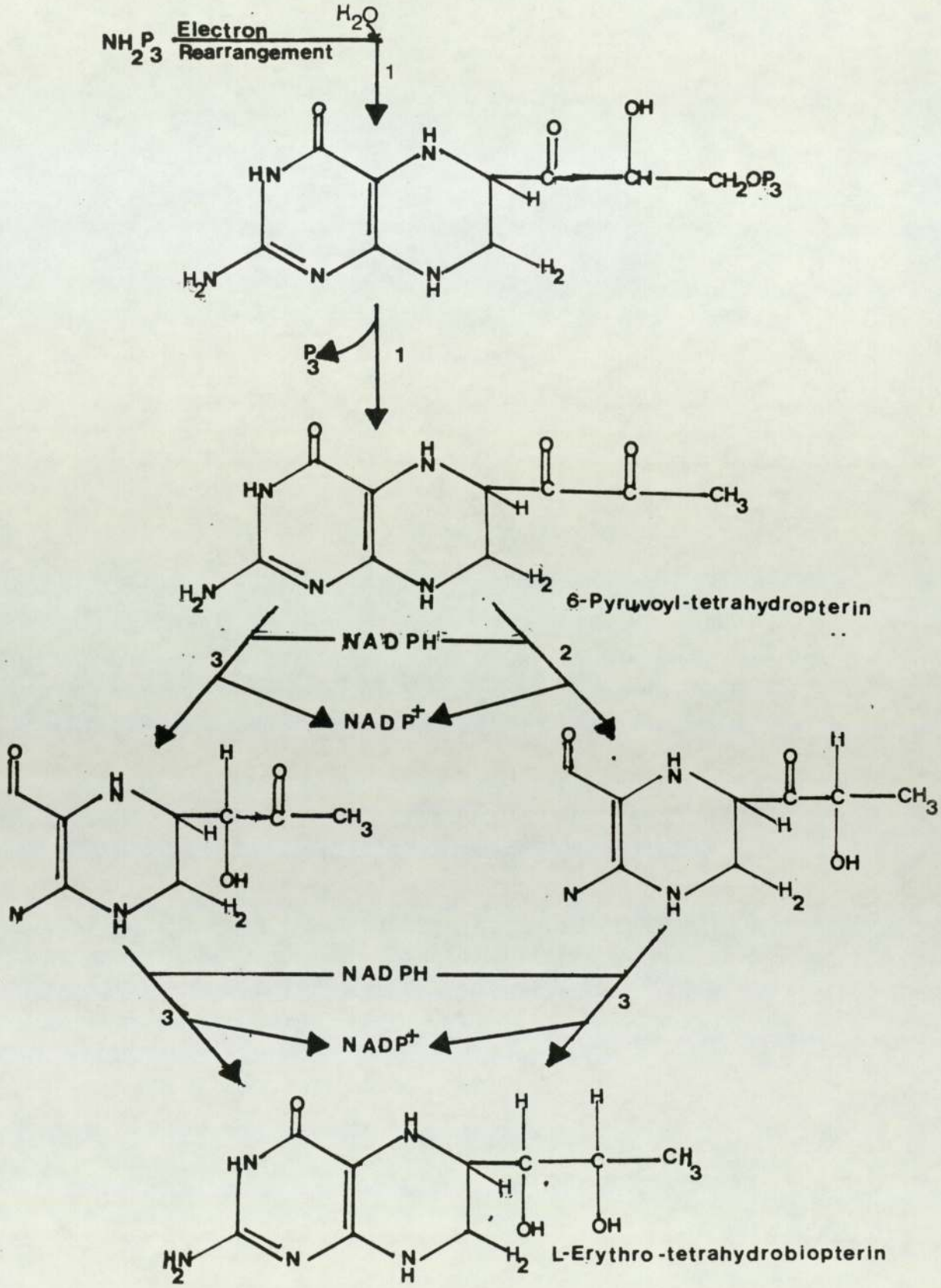


Fig. 1.5 Biosynthesis of L-*erythro*-tetrahydrobiopterin.

1. Pyruvoyl tetrahydropterin synthetase.
2. 2' Keto reductase.
3. Sepiapterin reductase.

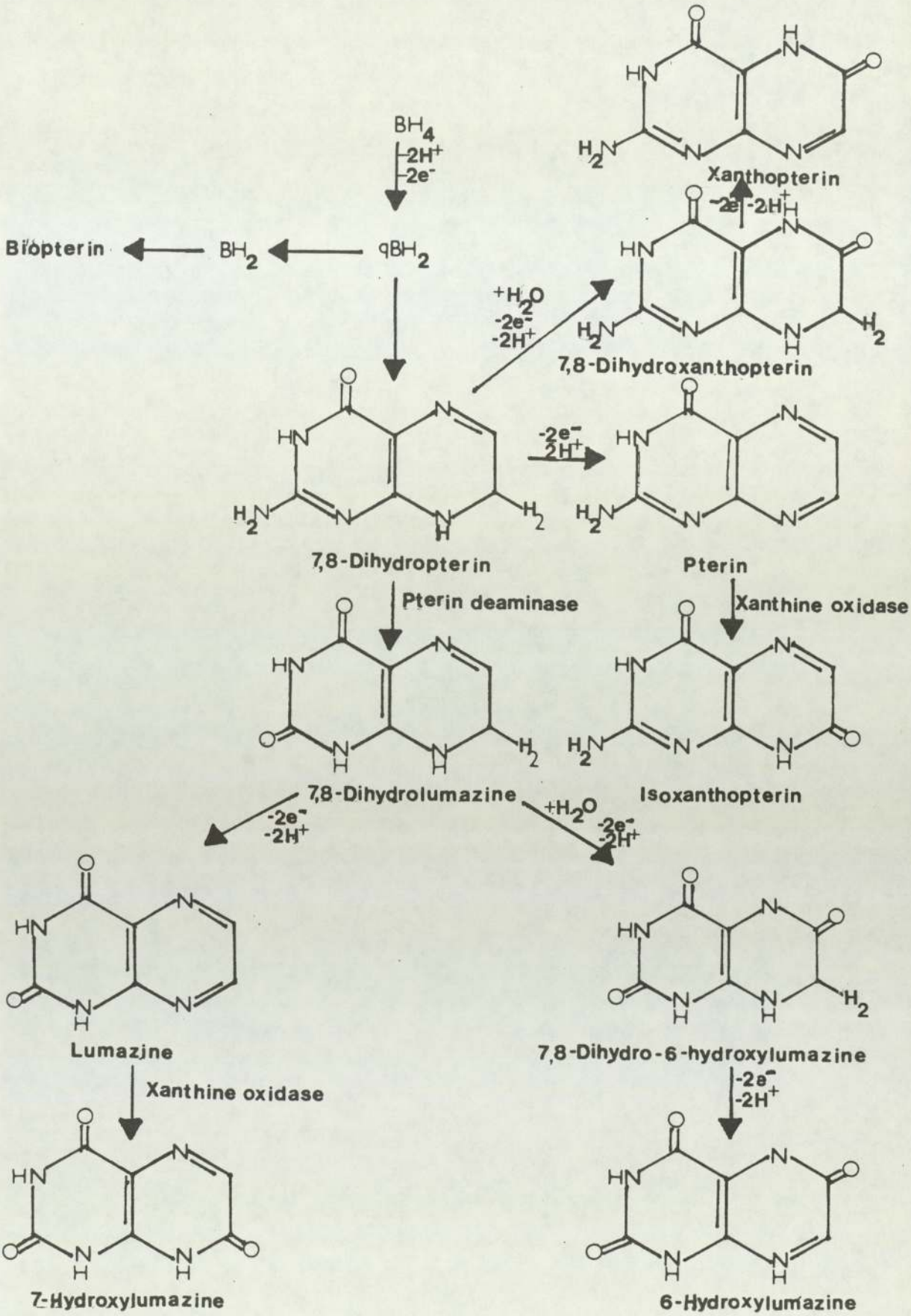


Fig. 1.6 Catabolism of BH_4 in the rat.

CHAPTER TWO

MATERIALS AND METHODS

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2.1 CHEMICALS.

5,6,7,8-Tetrahydro-L-biopterin diyhdrochloride, 7,8-dihydro-L-biopterin, 7,8-dihydro-D-neopterin, L-biopterin, D-neopterin, L-sepiapterin and pterin were obtained from Dr. B. Schircks, Switzerland.

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

Tetrahydrofolate (5,6,7,8-tetrahydropteroyl-L-glutamic acid), xanthopterin, albumin (bovine, fraction V), L-ascorbic acid, L-buthionine-(S,R)-sulfoximine, catalase (isolated from bovine liver), 6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$), ethylenediamine tetraacetic acid (EDTA) (disodium- calcium salt), glutathione - reduced form, guanosine 5'- triphosphate (GTP) (type II-S, sodium salt), heparin (grade II, isolated from porcine intestinal mucosa), horseradish peroxidase, mannitol, monoamine oxidase (isolated from bovine plasma), nicotinamide adenine dinucleotide - reduced form (NADH) (grade III, disodium salt), nicotinamide adenine dinucleotide phosphate - reduced form (NADPH) (type III, tetrasodium

salt), phenelzine (sulphate salt), streptozotocin, Trizma (tris-(hydroxy-methyl)-aminomethane) base, *p*-tyramine hydrochloride and uric acid were supplied by the Sigma Chemical Company, England.

Benzylamine, ferric chloride (Analar), ferrous sulphate (Analar), HPLC grade methanol and L-tyrosine were purchased from Fisons, England.

Sodium nitrite (Analar) was supplied by Griffin and George Ltd., England.

N-nitrosodimethylamine was supplied by the Aldrich chemical company, England.

L-cysteine was obtained from BDH Ltd., England.

Nitrous oxide (medical grade), oxygen and helium were supplied by BOC Ltd., England.

All other reagents and buffers were of standard laboratory grade.

2.2 UV/VISIBLE SPECTROPHOTOMETERS

Spectral data on reduced pterins, protein measurements, monoamine oxidase and sepiapterin reductase activity were determined using a Schimadzu uv/visible recording double beam

spectrophotometer uv-240 and graphic printer Pr-1 (Schimadzu Corporation, Japan).

Dihydropteridine reductase activity was determined using a Pye Unicam SP 1700 double beam spectrophotometer and a Pye Unicam AR 55 linear recorder (Pye Unicam, England).

2.3 CENTRIFUGES.

Centrifugation at 100,000g. was performed using a MSE Superspeed 50 ultracentrifuge (MSE Ltd., England) and a MSE 10x10ml angle head rotor. Bench centrifugation was done using a MSE swing bucket bench centrifuge.

2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

Separation of pterins was achieved using a 5 micron Spherisorb octadecyl silane (ODS) reverse phase column (25cm x 6 mm) and a short 5 micron precolumn. Injection of 20 ul samples was by manual injection or by autosampler - WISP 710B (Waters, Millipore Ltd., USA). The flow rate was maintained at 1ml min^{-1} by an LDC Constametric dual reciprocating pump (Model III) (Laboratory Data Control/Milton Roy Ltd., U.K). Detection was by an LDC Fluromonitor III, or a Kontron Spectrofluoromonitor SFM 23 (Tegimentia, Switzerland), excitation 360nm, emission 450nm. Peaks were recorded by a Spectra Physics SP 4290 recording integrator (Spectra

Physics, USA), a J.J. CR652A chart recorder (J.J. Lloyd Instruments Ltd., England), or a W.W. 302 chart recorder (W.W Scientific Instruments, Switzerland). All separations were achieved using a 5% (v/v) methanol eluent, degassed by helium prior to use. Retention times of pterin standards were determined as the time (minutes) from injection to formation of peak by recorder (Table 2.1).

Using known amounts of standard, calculated by uv spectroscopy (Table 2.2), calibration curves were produced by plotting peak area against concentration. Peak area was determined either by a computing integrator or by hand (1/2 base x peak height).

2.5 THIN LAYER CHROMATOGRAPHY (TLC).

Chromatography of pterin standards was achieved using 0.1mm Polygram cellulose MN300/UV 254 precoated plates (Macherey-Nagel and Co., West Germany). Samples were spotted, 1.0 cm from origin, on plates using capillary tubes. Spots were allowed to dry and the process was repeated 5 to 6 times. Chromatograms were developed using either 5% (w/v) ammonium chloride, 5% (v/v) acetic acid or 0.05M phosphate buffer pH 7.1, in the ascending direction. Chromatography was terminated when the solvent front reached 18.0cm. Developed chromatograms were read under uv light (366nm). Rf values (distance moved by pterin / distance moved by solvent) were calculated for pterin standards in each solvent system (Table

2.3).

2.6 ANIMALS.

All animals used were male Wistar rats (supplied by Bantam and Kingman Ltd., England.) and maintained on a standard rat breeding diet (Pilsburys Ltd., England) (Table 2.4). Tap water was available *ad libitum*. Rats were housed under light controlled conditions with a 12:12 hour dark:light controlled cycle.

Experimental groups were in supplier's batches and compared to age and sex matched controls.

Oral dosing was performed using a sterile hypodermic syringe fitted with an olive bulbed oropharyngeal needle. Care was taken to deliver the dose to the stomach.

Intraperitoneal dosing (I.P.) was performed using a sterile hypodermic syringe fitted with a 0.5mm diameter needle. Animals were lightly anaesthetised, with ether, prior to dosing.

All starved rats were allowed water but no food and placed on grids (to prevent coprophagy).

Exposure to nitrous oxide/oxygen and helium/oxygen mixtures was achieved by use of sealed metabowls, with inlet and exit holes. Regulation of the flow of gases was by means of a

Boyle's apparatus.

Rats were killed by concussion and cervical dislocation.

2.7 MEASUREMENT OF RATE OF OXIDATION OF TETRAHYDRO- AND DIHYDROPTERINS AND IDENTIFICATION OF OXIDATION PRODUCTS.

Oxidation of tetrahydro- and dihydropterins was determined over a wide pH range using the following systems: 0.1M HCl (pH 1.6), 0.1M acetic acid (pH 3.0), 0.1M sodium acetate buffers (pH 3.8, 4.6, and 5.3.), 0.1M sodium phosphate buffer (pH 7.0) and 0.075M sodium phosphate buffer (pH 7.6).

Oxidation rates were determined, at 37°C using a constant temperature cell, by uv spectroscopy using the following reaction mixture: 5.00ml reduced pterin, 4.00ml buffer and 1.00ml oxidant/inhibitor/buffer(control). After rapidly mixing, 4.00ml were transferred immediately to a quartz cuvette. Blank mixture contained: 9.00ml buffer and 1.00ml oxidant/inhibitor/buffer. Final concentration of reduced pterin was 50uM. Matched quartz cuvettes were used in all cases (path length 1cm), and changes in absorbance monitored at the appropriate wavelength (Table 2.5), from which first order plots were derived.

Product analysis was by HPLC and TLC, after 3 hours incubation in a shaking water bath (37°C) for autoxidation studies. For HPLC, dilution of samples with distilled water

was necessary, the amount varying with the degree of oxidation of the pterin under investigation. Samples incubated with oxidants were diluted (1/1000) and assayed immediately.

Autoxidation products of 5,6,7,8-tetrahydrobiopterin were measured directly by HPLC. In addition 2.00ml of sample was oxidised by 0.20ml 3%(w/v) iodine under acidic conditions (by the addition of a known volume of conc. HCl) and under alkaline conditions (by the addition of a known volume of 0.2M NaOH). After 1 hour the iodine was reduced, in both cases, by an excess of ascorbic acid. After dilution the biopterin content was measured by HPLC (alkaline samples were neutralised prior to injection). Autoxidation products of 7,8-dihydrobiopterin and 7,8-dihydroneopterin were subjected to acid-iodine oxidation only.

Under acidic conditions, iodine oxidises both 5,6,7,8-tetrahydrobiopterin and 7,8-dihydrobiopterin to biopterin, whereas in an alkaline environment only 7,8-dihydrobiopterin is oxidised to biopterin (Fukushima and Nixon, 1980). This differential oxidation, coupled with a measurement for biopterin present prior to oxidation, permits calculation of the amount of biopterin in each oxidation state.

2.8 MONOAMINE OXIDASE ACTIVITY AND ITS EFFECTS ON 7,8-DIHYDROBIOPTERIN.

Monoamine oxidase activity (MAO) was determined using the method of Dietrich and Erwin (1969). 0.01ml enzyme source was incubated, in a quartz cuvette (path length 1cm) with (final concentrations): 0.05M sodium phosphate buffer (pH 7.6), 3mM benzylamine and distilled water to give a final volume of 3.00ml. The mixture was inverted to mix.

The increase in absorbance at 250nm was monitored at 25°C against an enzyme blank, and activity calculated. A molar extinction coefficient of 12 x 10^3 M^{-1} cm^{-1} was used for benzaldehyde, and results expressed as nmoles benzylamine oxidised min^{-1} ml^{-1} enzyme source.

The effect of MAO activity on 7,8-dihydrobiopterin (BH_2) was investigated by replacing the buffer in the above mixture with a BH_2 buffer solution, giving a final concentration of 50uM. The change in absorbance was monitored at a wavelength corresponding to the oxidation of the pteridine ring system and away from contamination by benzylamine or benzaldehyde (375nm).

The effects of a ferrous sulphate / EDTA solution (final concentration 60uM) on this system was studied by replacing a proportion of the distilled water in the above incubation. Catalase or phenelzine solutions were subsequently added by replacement of the remaining distilled water, to give final

concentrations of 5U ml^{-1} and 1mM respectively.

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

Brains and portions of liver were removed immediately after death and stored at -70°C until required. Prior to freezing the brains were divided into right and left hemispheres. Right hemispheres were used for total biopterins and pterin analysis. Left hemispheres were used for the biosynthesis and dihydropteridine reductase assays.

Measurement of total biopterins and pterin was by HPLC and based on the method of Fukushima and Nixon (1980).

Right brain hemispheres were weighed and 25% (w/v) homogenates prepared in 0.1M HCl and 1.00ml 20% (w/v) trichloroacetic acid (TCA), using a Potter-Elvehjem homogeniser. Liver samples were prepared in a similar way except 10% (w/v) homogenates were produced.

Brain homogenates were spun at 100,000g. for 5 minutes, whilst liver homogenates were spun at full speed in a bench centrifuge for 10 minutes. Following centrifugation supernatants were removed and volumes noted. To 2.00ml of supernatant, 0.50 ml 3% (w/v) iodine was added and samples left in the dark. After 1 hour the iodine was reduced by the addition of an excess of ascorbic acid and samples run, in

duplicate, on the HPLC (liver samples were diluted by 1/10).

Total biopterins and pterin levels were calculated from HPLC calibration curves and related back to the weight of tissue used. Results were expressed as ng g^{-1} wet weight, for brain tissue, and ug g^{-1} wet weight, for liver.

2.10 MEASUREMENT OF PLASMA TOTAL BIOPTERINS.

Blood was removed from the thoracic region with heparinised (1000U ml^{-1} 0.9% (w/v) saline) needles, and bench centrifuged for 10 minutes. Following centrifugation supernatants (plasma) were decanted off and stored at -70° C.

To 0.30ml plasma, 0.03ml 60% (w/v) perchloric acid and 0.01ml 3% (w/v) iodine were added. After mixing, samples were left in the dark. Excess ascorbic acid was added after 1 hour, and samples centrifuged for 10 minutes in a bench centrifuge. Supernatants were assayed, in duplicate, by HPLC and plasma biopterins calculated. Results were expressed as ug $litre^{-1}$ plasma.

2.11 MEASUREMENT OF TETRAHYDROBIOPTERIN BIOSYNTHESIS IN BRAIN AND LIVER.

Measurement of the biosynthetic capacity of brain and liver was based on the method described by Hamon (1984), except

that the freeze drying step was omitted giving clearer, more reproducible results.

Left brain hemispheres were weighed and 35% (w/v) homogenates were prepared in 0.1M Tris-HCl (pH 7.6) buffer using a Potter-Elvehjem homogeniser. 25% (w/v) homogenates were prepared for liver.

Homogenates were spun at 100,000g. for 45minutes. Supernatants were decanted off and split into two (half were used for the dihydropteridine reductase assay), and stored at -70°C until required.

0.10ml of supernatant was incubated with (final concentrations): 70mM Tris-HCl (pH 8.0), 28mM KCl, 6mM GTP, 3mM NADPH, 6mM $MgCl_2$ and distilled water (or inhibitor) in a final volume of 1.00ml. This system was run in parallel with a blank assay which contained: 0.10ml supernatant, 70mM Tris-HCl (pH 8.0), 28mM KCl, 6mM $MgCl_2$ and distilled water (or inhibitor) in a final volume of 1.00ml.

Both were incubated in a shaking water bath at 37°C in the dark. The reaction was terminated after 3 hours by the addtion of 2.00ml 0.1M HCl. 0.50ml 3% (w/v) iodine was added and samples placed in the dark. After 1 hour, excess ascorbic acid was added, and the amount of biopterin in each sample measured by HPLC.

The amount of biopterin produced by the tissue supernatant

was determined by subtracting blank values from those obtained with full incubations, and related to the protein content of the supernatant. Protein was determined by the biuret method. Results were expressed as ng 5,6,7,8-tetrahydrobiopterin (BH_4) synthesised h^{-1} mg^{-1} protein.

There was a significant positive correlation between the amount of BH_4 produced and protein content, over the range 10-40mg ml^{-1} , (r= 0.917, P <0.05, n=5).

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

Brain and liver homogenates were prepared as for the biosynthesis assay.

Assay of DHPR activity was essentially the method described by Craine *et al.*, (1972).

The assay mixture contained (final concentrations), added directly into a plastic cuvette (1cm path length): 50mM Tris-HCl (pH 6.8), 0.25mM sodium azide, 8ug peroxidase, 0.1mM NADH, 0.1mM hydrogen peroxide, 0.02ml tissue supernatant and distilled water (or inhibitors) to give a volume of 0.90ml. The mixture was inverted to mix and incubated at 37°C for 90 seconds. The reaction was initiated by the addition of 0.10ml 1mM 6,7-dimethyl,5,6,7,8-tetra-hydropterin.

After a 30 second delay the rate of reaction was followed by measuring the rate of decrease in absorbance at 340nm. This was measured against an enzyme free mixture.

A molar extinction coefficient of 6.22×10^{3} M^{-1} cm^{-1} for NADH was used to calculate the enzyme activity. This activity was related to the protein content of the original supernatant, as determined by the biuret method. Results were expressed as specific activities- nmoles NADH oxidised min^{-1} mg^{-1} protein.

There was a significant positive correlation between DHPR activity and protein content, over the range 1-10mg ml^{-1}, ($r = 0.956$, $P < 0.01$, $n=5$).

2.13 DETERMINATION OF THE KINETIC PARAMETERS OF SEPIAPTERIN REDUCTASE AND THE EFFECT OF *p*-TYRAMINE.

Liver supernatant was prepared as described for the biosynthesis assay.

Sepiapterin reductase activity was based on the method described by Katoh *et al.*(1983).

0.02ml supernatant was incubated, in a plastic cuvette (path length 1cm), with (final concentrations): 150mM Tris-HCl (pH 6.8), 100uM NADPH, 8, 12, 16, 20, and 30uM sepiapterin, and

distilled water (or inhibitor) to give a final volume of 2.00ml. The mixture was inverted to mix.

The decrease in absorbance at 420nm was determined with respect to an enzyme blank, at 25°C, for each concentration of sepiapterin. A molar extinction coefficient of 10.4 x 10^3 M^{-1} cm^{-1} was used for sepiapterin and data expressed as nmoles sepiapterin reduced min^{-1} ml^{-1} enzyme source. Lineweaver-Burk plots from this data enabled calculation of Vmax and Km.

The above was repeated in the presence of *p*-tyramine (7.5mM), and the kinetics compared. Such a comparison revealed the type of inhibition and allowed the calculation of K1.

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

To 2.00ml 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), 0.40ml distilled water and 0.10ml protein source were added, mixed well and left at room temperature for 30min. The absorbance at 540nm was measured against a protein blank. The protein concentration was calculated from a calibration curve using bovine serum albumin (2-10mg ml^{-1}).

2.15 STATISTICS.

All rate constants and half-lives are expressed as arithmetic means only, as variation was negligible (coefficient of variation < 2%).

Data derived from animals are shown as the arithmetic means with standard deviations. Comparisons between test and control data was by a two tailed Student's unpaired t test and probabilities of 0.05 and less regarded as significant.

Linear regressions were by least squares analysis and lines of best fit and statistical probabilities are shown.

Table 2.1. Relative retention times for neopterin, biopterin, xanthopterin and pterin on a reverse phase ODS HPLC column using a 5% (v/v) methanol eluent, 25°C.

Pterin.	Retention time (mins).	Relative retention time (biopterin = 1.00).
Neopterin	6.10	0.58
Xanthopterin	8.80	0.84
Biopterin	10.40	1.00
Pterin	12.80	1.23

Table 2.2. Molar extinction coefficients (Emax) for neopterin, biopterin, xanthopterin and pterin, at pH 13.0.

Pterin.	Wavelength (nm).	Emax $x10^{-3}$ M^{-1} cm^{-1}
Neopterin	362	8.3
Biopterin	362	8.3
Xanthopterin	394	7.2
Pterin	358	6.6

Table 2.3. Thin layer chromatography. Rf values for pterin standards on 0.1mm cellulose plates.

Pterin.	Solvent system.	Rf
Neopterin	(i)	0.70
	(ii)	0.46
Xanthopterin	(i)	0.48
	(ii)	0.48
	(iii)	0.48
Biopterin	(i)	0.66
	(ii)	0.66
	(iii)	0.66
Pterin	(i)	0.53
	(ii)	0.65
	(iii)	0.53

(i) 5% (w/v) ammonium chloride.

(ii) 5% (v/v) acetic acid.

(iii) 0.05M sodium phosphate buffer (pH 7.1).

Table 2.4. 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 carbohydrates	%	46.80
Gross energy	Cals/kg	4073.00
Metabolisible energy	Cals/kg	3666.00
Saturated fatty acids	%	0.73
Linoleic acid	%	0.99
Other unsaturated acids	%	1.54
Calcium	%	1.30
Phosphorus	%	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
Cystine	%	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./kg	1,1587.00
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 2.5. Wavelengths for determining the rates of oxidation of 5,6,7,8-tetrahydrobiopterin (BH_4), 7,8-dihydrobiopterin (BH_2), 7,8-dihydroneopterin (NH_2), tetrahydrofolate (FH_4) and 5-methyl-tetrahydrofolate ($5CH_3FH_4$).

Pterin.	pH	Wavelength (nm).
BH_4	1.6	267
BH_4	3.0	265
BH_4	3.8	265
BH_4	4.6	265
BH_4	5.3	300
BH_4	7.0	300
BH_4	7.6	300
BH_2/NH_2	1.6	275
BH_2/NH_2	3.0	275
BH_2/NH_2	5.3	230
BH_2/NH_2	7.6	230
FH_4	1.6	250
FH_4	3.0	250
FH_4	7.0	300
$5CH_3FH_4$	1.6	217
$5CH_3FH_4$	3.0	240

CHAPTER THREE

AUTOXIDATION OF TETRAHYDRO- AND DIHYDROPTERINS

CHAPTER THREE.

AUTOXIDATION OF TETRAHYDRO AND DIHYDROPTERINS.

3.1 INTRODUCTION.

The susceptibility of tetrahydropterins to autoxidation, *in vitro*, has been known for some time (Zakrzewski and Nichol, 1956). The mechanism of this oxidation has been studied by a number of groups (Viscontini and Weilenman, 1959; Stocks-Wilson, 1971; Blair and Pearson, 1974)

Tetrahydropterin (I) autoxidation is suggested to be initiated by generation of a free radical at C(4a) of the pterin ring (II), occurring by electron abstraction followed by proton loss (Bobst, 1967; Blair and Pearson, 1974). This radical reacts with dioxygen resulting in further electron abstraction, yielding a N(5)-protonated quinonoid intermediate (III) and the peroxide ion (O_2^-). Loss of the proton at N(5) to O_2^- gives a hydroperoxyl radical ($HO_2^\bullet$) and the quinonoid intermediate (IV) described by Kaufman (1964). Propagation is achieved by the interaction of $HO_2^\bullet$, at acid pH, with other tetrahydropterin molecules. At neutral pH and above the chain carrier would be superoxide ($O_2^{\bullet-}$) (Fig. 3.1) (Blair and Pearson, 1974).

Evidence for this scheme comes from the detection of hydrogen

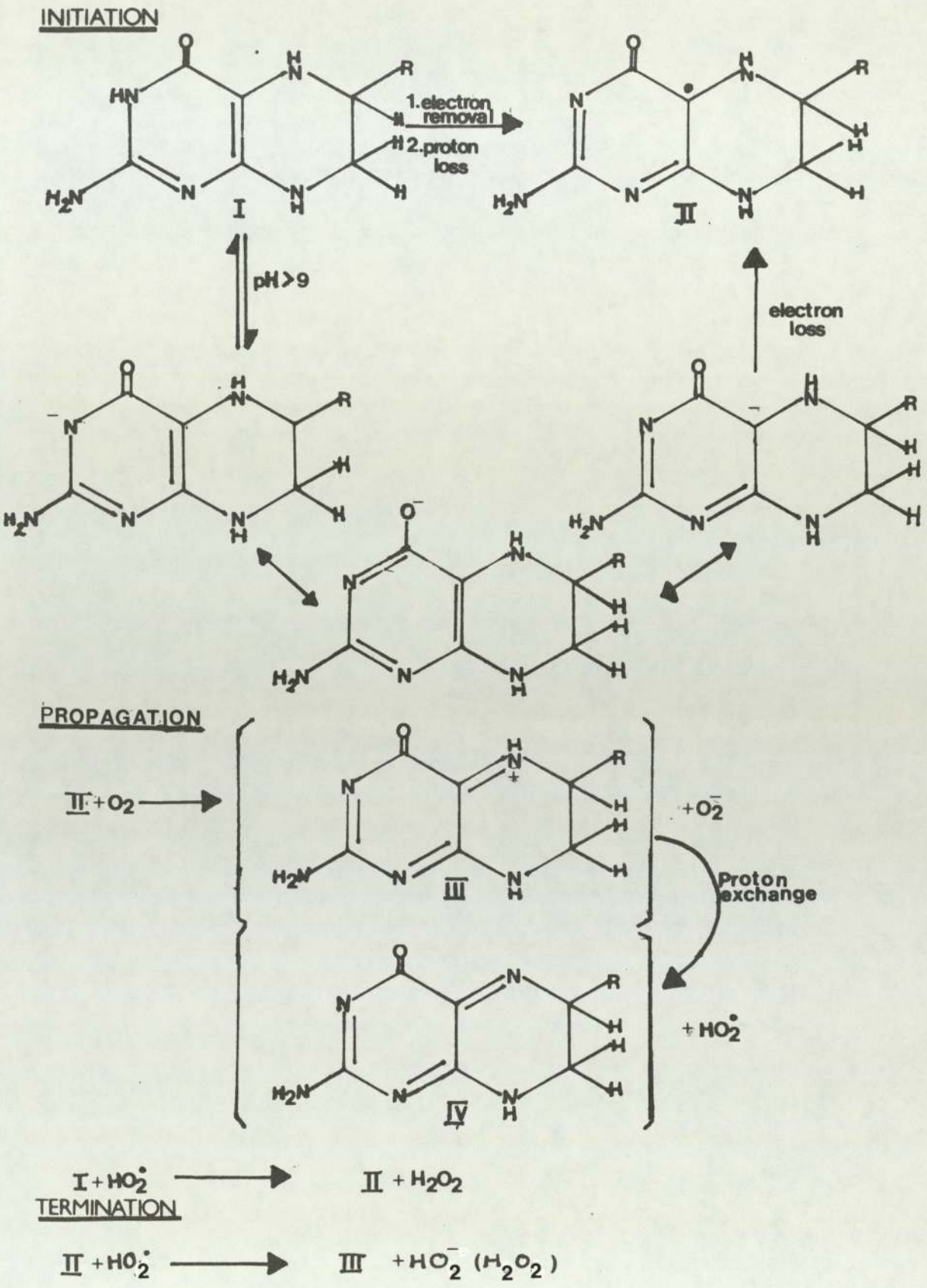


Fig. 3.1 Autoxidation of tetrahydropterins (Blair and Pearson, 1974).

peroxide, but not organic peroxides, during autoxidation and the inhibition of oxidation by free radical scavengers such as phenol (Blair and Pearson, 1974; Pearson, 1974b; Armarego and Waring, 1982).

Generation of a C(4a) hydroperoxide intermediate (V) via the addition of dioxygen to the free radical (II) has been suggested to occur during tetrahydropterin autoxidation (Fig. 3.2) (Mager and Brends, 1965; Blair and Pearson, 1973; Pfleiderer, 1978). Studies on the reaction of the free radical (II) with dioxygen and activation energy calculations do not support such a mechanism (Blair and Pearson, 1974).

The autoxidation rates of tetrahydropterins exhibit marked pH dependence. With decreasing pH there is decreased rate of oxidation. Over the pH range 3.0 - 6.0 protonation of N(5) (pKa, 4.82) decreases the ease of electron abstraction, by removing the N(5) lone pair of electrons. At alkaline pH the rate of oxidation depends on the degree of ionisation of the 3,4-amide group (pKa, 10.82) (Fig. 3.1) (Archer *et al.*, 1972; Blair and Pearson, 1974).

pH also affects the nature of the autoxidation products when considering the substituted tetrahydropterins, tetrahydrobiopterin (BH_4) and tetrahydrofolic acid (FH_4). Decreasing the pH of the oxidizing medium for FH_4 results in decreased 7,8-dihydrofolic acid (FH_2) (VIII) and increased 7,8-dihydropterin formation (VII), as a result of the acid-base catalysed rearrangement of the quinonoid dihydrofolic acid

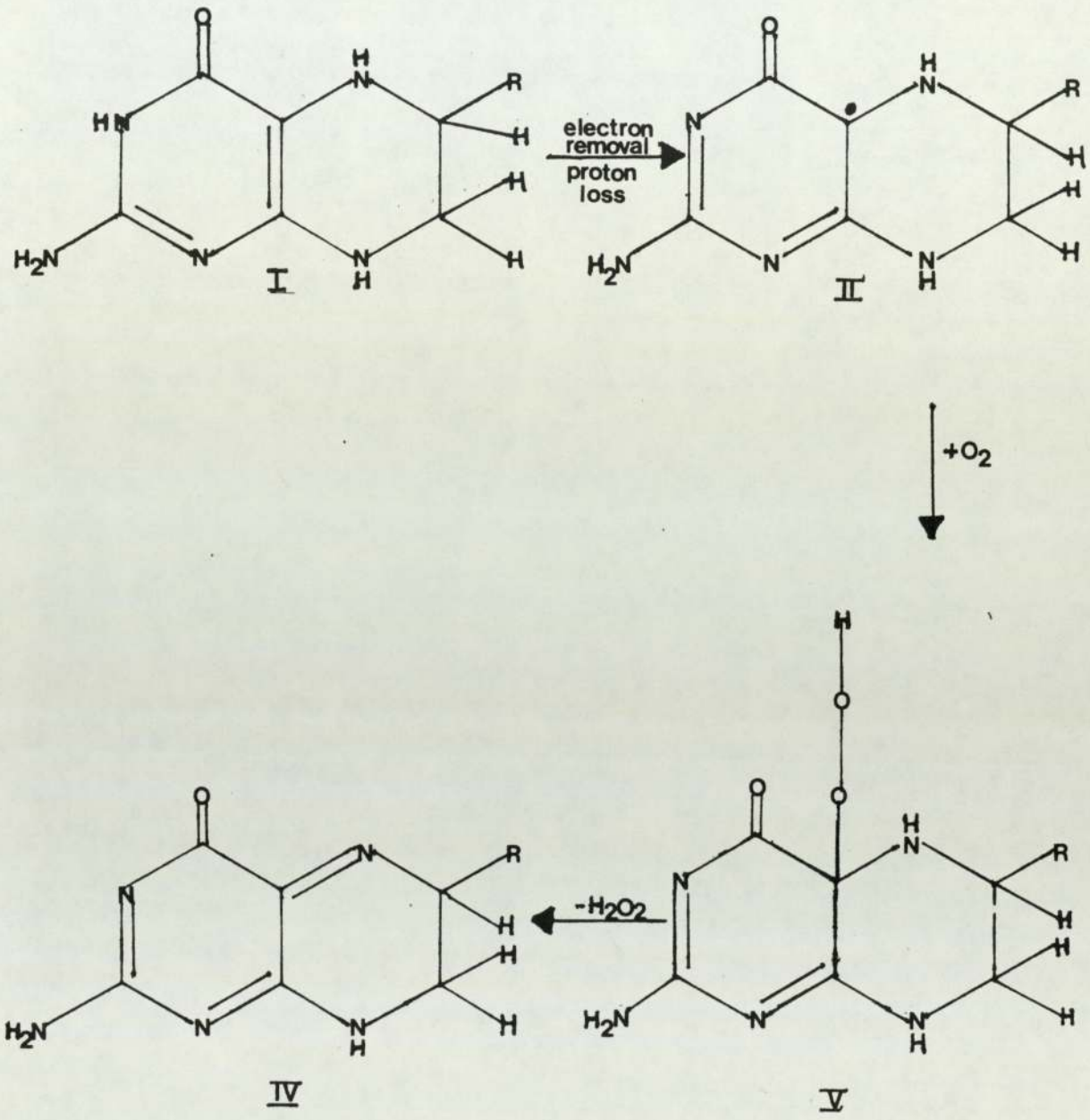


Fig. 3.2 Suggested involvement of hydroperoxide intermediate in tetrahydropterin autoxidation (Blair and Pearson, 1973; Pfleiderer, 1978)

intermediate (VI) (Fig. 3.3) (Chippel and Scrimgeour, 1970; Stocks-Wilson, 1971; Blair and Pearson, 1974). The autoxidation products for BH_4 have been reported at different pH levels with biopterin being suggested as the major oxidation product at pH 13.0 (Pearson, 1974). Pfleiderer (1978) reports that the oxidation of BH_4 at pH 7.5 does not give a quantitative conversion to biopterin and describes the formation of 7,8-dihydroxanthopterin (XH_2) (XII) and pterin in addition to biopterin.

7,8-dihydroxanthopterin can arise from the addition of water to 7,8-dihydropterin (VII) giving 6-hydroxy-5,6,7,8-tetrahydropterin (IX). The subsequent oxidation of this tetrahydropterin followed by rearrangement of the quinonoid intermediate (X) gives a tautomeric form of XH_2 (XI) (Fig 3.4) (Armarego and Randles, 1983).

The autoxidation of 7,8-dihydropterins is considerably slower compared to tetrahydropterins (Archer *et al.*, 1972; Pearson, 1974). The reaction proceeds with abstraction of an electron from N(8) (pKa, 10.4), followed by homolysis of the C(7) - H bond. Since N(8) is conjugated to the electron withdrawing oxo group at position C(4) of the pterin ring, electron abstraction is retarded (Archer *et al.*, 1972).

pH affects the autoxidation rate of 7,8-dihydropterins. When the pH approaches the pKa of N(8) there is an increase in the rate of oxidation. In direct contrast to tetrahydropterins, as the pH falls below 7.0 the rate of reaction increases.

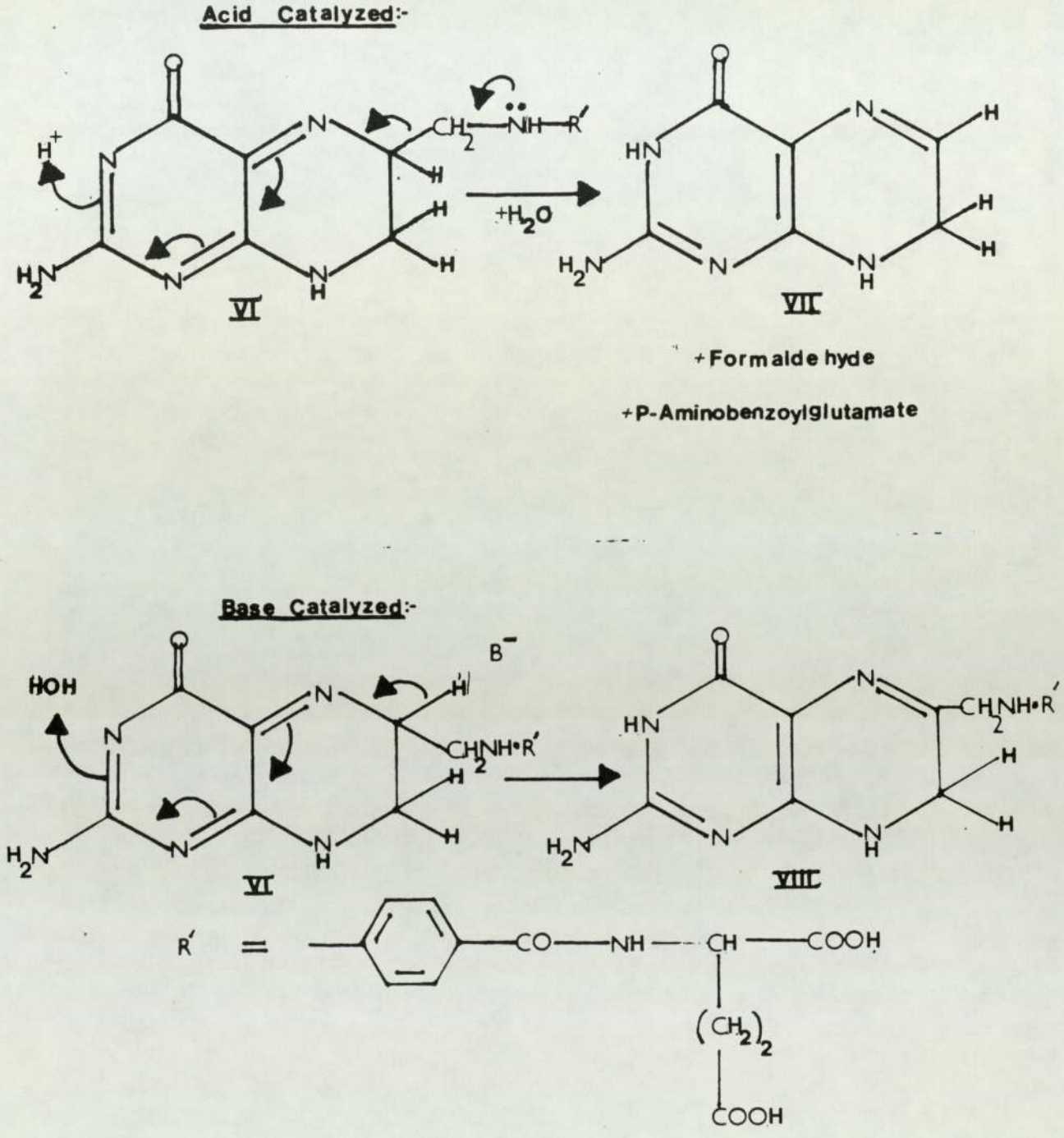


Fig. 3.3 Rearrangement of quinonoid dihydrofolate (Blair and Pearson, 1974).

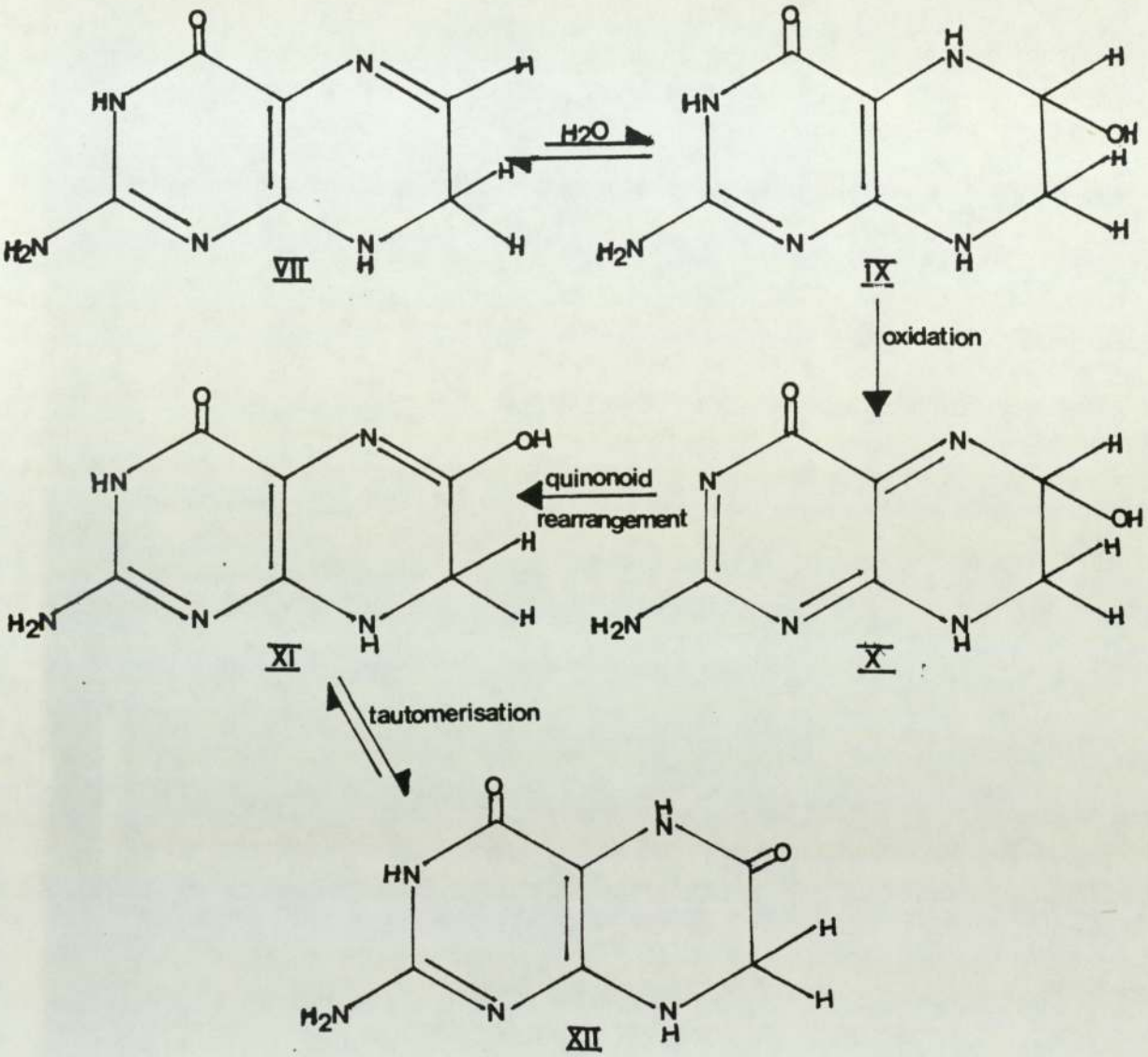


Fig. 3.4 Formation of 7,8-dihydroxanthopterin at pH 7.6 (Armarego and Randles, 1983).

This latter observation is not readily explained (Archer *et al.*, 1972).

Analysis of the autoxidation products for 7,8-dihydrobiopterin (BH_2) over a wide pH range reveals no side chain loss (Milstien, 1983). The oxidation products for FH_2 are affected by pH with folic acid being the major product at high pH, and considerable side chain loss occurring at lower pH levels (Chippel and Scrimgeour, 1970).

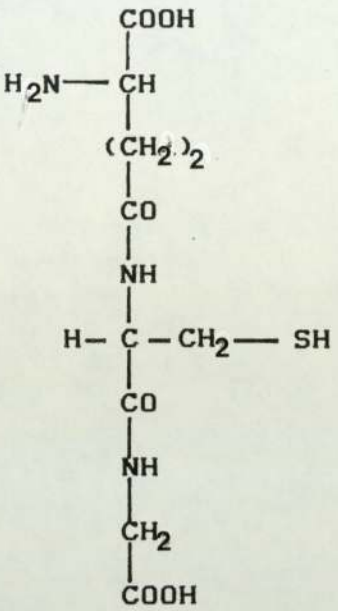
Since neither comparative studies of rates of autoxidation, nor quantitative measurements of oxidation products have been performed on tetrahydrobiopterin (BH_4), 7,8-dihydrobiopterin (BH_2) and 7,8-dihydroneopterin (NH_2), such an investigation was carried out. This study was necessary to provide information both on relative stabilities and nature of oxidation products, prior to subsequent investigations into factors affecting their stability.

Information gained from such a study is also important in providing optimum conditions for isolation, storage and measurement of the above compounds. Additionally explanations may be provided for observations of reduced pterin metabolism *in vivo*.

3.2 MATERIALS AND METHODS.

The rate of autoxidation of BH_4, BH_2 and NH_2 were determined

by uv spectroscopy as described in Chapter Two. The effect of glutathione (reduced form) (XIII) addition on the autoxidation of BH_4 was also investigated.



(XIII)

Product analysis was performed, after 3 hours incubation in a shaking water bath, 37° C, by TLC and HPLC, using the methods described in Chapter Two.

3.3 RESULTS AND DISCUSSION.

The oxidation of dihydro- and tetrahydropterins can be monitored by uv spectroscopy (example, Fig. 3.6), from which kinetic data can be derived. The autoxidation of BH_4 in the presence of an excess of dioxygen gave first order kinetics (example, Fig. 3.7) at all pH levels.

The initial autoxidation rate of BH_4 was affected by the pH of the oxidising medium (Table 3.1; Fig. 3.8). Over the pH range 1.6 - 7.6 this is explained by the varying degrees of protonation of the N(5) region of the pterin ring, as described by Blair and Pearson (1974). Ionic strength calculations suggested little contribution to this observation (Appendix 3).

Physiological concentrations of the reduced form of glutathione (GSH) inhibited the autoxidation of BH_4 (Table 3.2; Fig. 3.9). GSH may retard oxidation by acting as a chain breaker due to its ability to scavenge free radicals (Meister, 1981). Alternatively inhibition may arise since GSH, and other reduced thiols, are able to non-enzymatically reduce the quinonoid intermediate (Ayling and Bailey, 1983).

Identification of autoxidation products, following acid iodine oxidation and HPLC, reveals their identity (Table 3.3). For BH_4, with increasing pH there was increased loss of the biopterin side chain, i.e. increased formation of pterin and xanthopterin. Possibly, at acid pH, protonation of the biopterin side chain prevents electron donation to the pterin ring during quinonoid rearrangement (XIV) resulting in side chain conservation and BH_2 (XV) formation. At higher pH levels, an electron may be donated from the side chain which would subsequently be lost leaving 7,8-dihydropterin (VII). This would be detected, following acid iodine oxidation, by HPLC as pterin (Fig. 3.5).

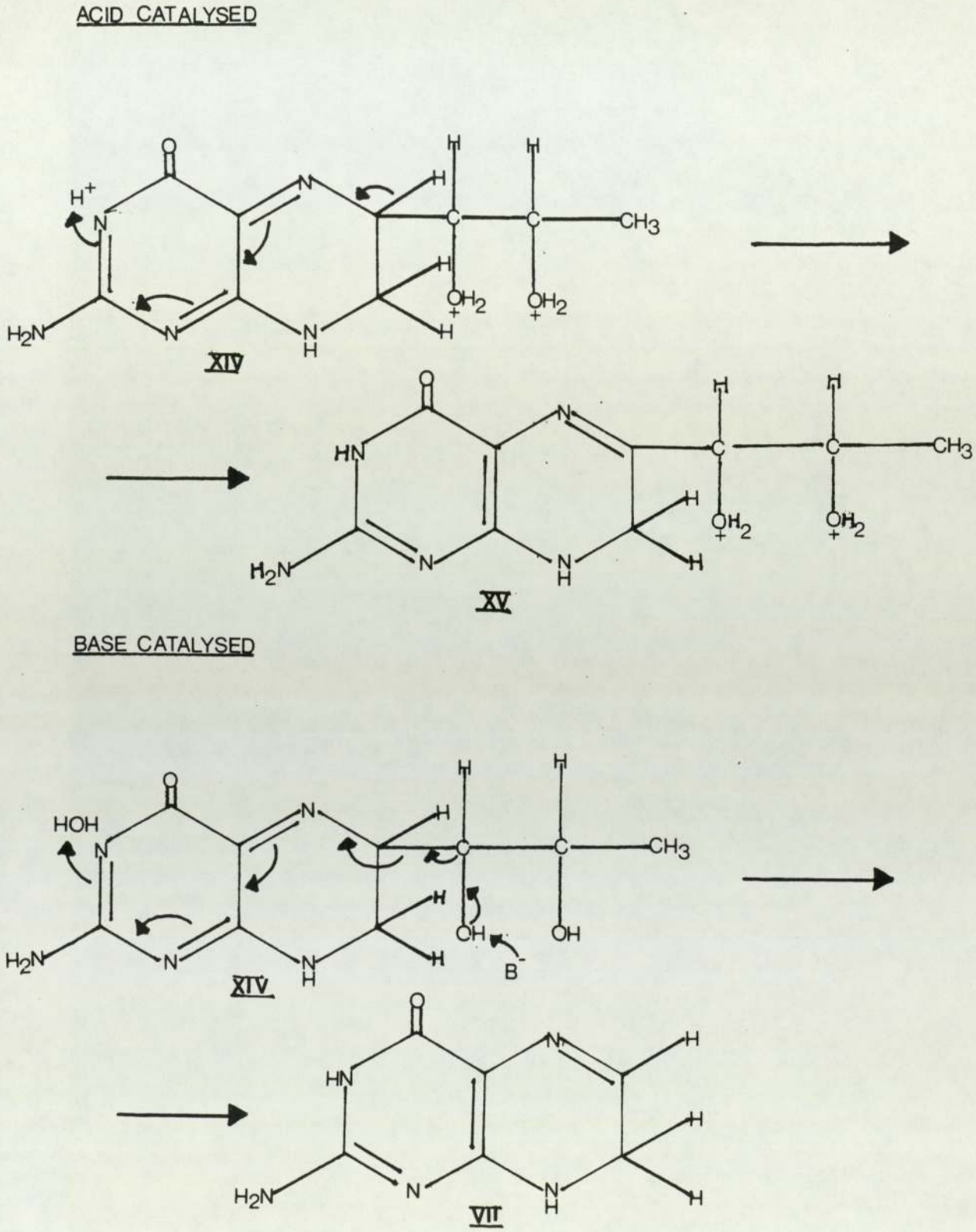


Fig. 3.5 Rearrangement of quinonoid dihydrobiopterin.

Xanthopterin may arise by the hydration of 7,8-dihydropterin as described above (Fig. 3.4).

Differential acid/alkaline iodine oxidation enables the determination of the oxidation state of identified products after 3 hours incubation (Table 3.4). As expected from the kinetic data, most of the biopterins arising from BH_4 above pH 1.6 were distributed between the dihydro- and fully oxidized forms. The largest proportion of pterin derived from BH_4 was in the fully oxidized form suggesting that 7,8-dihydropterin is readily oxidized under these conditions.

Whilst uv studies on BH_2 and NH_2, for 30 minutes, failed to detect any reaction, prolonged incubation indicated some oxidation occurring over a 3 hour period. Furthermore, decreasing the pH resulted in an increased proportion of products in the oxidized form, confirming the findings of Archer et al. (1972) (Table 3.5).

Product analysis revealed destruction of the pterin ring system with decreasing pH for NH_2 (Table 3.3). This observation is not readily explained but is attributed to a property of the side chain, since little destruction occurred with BH_2.

This study confirms the unstable nature of reduced pterins and in particular of BH_4. The results obtained suggest, for optimum storage of BH_4 and measurement of biopterins derived from BH_4, the acidification of samples to below pH 1.6.

Under conditions where BH_4 or other tetrahydropterins are required and acidification is not appropriate, e.g. in enzyme analysis, the addition of an antioxidant, such as GSH, should be considered.

BH_2 does not autoxidise readily and is little affected by the pH of the medium. However, the results obtained suggest optimum storage at pH 7.6.

When considering NH_2, the pH of the medium is more critical and neutral pH is recommended for optimum storage.

The inhibition of BH_4 autoxidation by GSH, at concentrations found intracellularly, may indicate an important role in protecting tetrahydropterins from oxidation, *in vivo*.

This study may also explain the increased excretion of 7,8-dihydroxanthopterin and xanthopterin in classical phenylketonuria (PKU) (Watson *et al.*, 1977). Since metabolites of phenylalanine inhibit dihydropteridine reductase (Hamon and Blair, 1987), this will result in accumulation of quinonoid dihydrobiopterin. Rearrangement of the latter, at physiological pH, will produce BH_2, and 7,8-dihydropterin. 7,8-dihydroxanthopterin may then be formed from the latter as described above.

Table 3.1. Effect of pH on the initial rate of autoxidation of BH_4 at 37°C.

pH	Pseudo rate constant, (K_1). min^{-1}	Half - life ($t_{1/2}$). mins.
1.6	0.0028	247.50
3.0	0.0084	82.50
3.8	0.0230	30.13
4.6	0.0360	19.25
5.3	0.0759	9.13
7.0	0.0820	8.45
7.6	0.0870	7.96

Each rate is the mean of 3 observations.
BH_4 = 50uM.

Table 3.2. Effect of glutathione (GSH) addition on the initial autoxidation rate of BH_4 at pH 7.6, 37° C.

	Pseudo rate constant (k_1). min^{-1}	Percentage inhibition.
BH_4	0.0500	-
BH_4 + GSH (mM)		
0.1	0.0418	16
0.5	0.0315	37
1.0	0.0170	66
5.0	0.0065	87

Each rate is the mean of 3 observations.
BH_4 = 50uM.

Table 3.3. Percentage conversion to identifiable oxidation products for BH_4, BH_2, and NH_2 at pH 1.6, 5.3 and 7.6, at 37° C.

	Neo-pterin	Bio-pterin	Xantho-pterin	Pterin
BH_4 pH 1.6	-	90	1	2
BH_4 pH 5.3	-	74	15	7
BH_4 pH 7.6	-	31	15	60
BH_2 pH 1.6	-	90	3	-
BH_2 pH 5.3	-	96	4	1
BH_2 pH 7.6	-	97	2	-
NH_2 pH 1.6	63	-	-	-
NH_2 pH 5.3	81	-	-	-
NH_2 pH 7.6	94	-	-	-

Products identified by TLC and HPLC after 3 hours .
Quantified by HPLC following acid-iodine oxidation.
Each conversion is the mean of 4 observations.
BH_4, BH_2, NH_2 = 50uM.

Table 3.4. Distribution of reduced to oxidized products after 3 hours oxidation at 37°C, for BH_4.

	Biopterin			Pterin	
pH	%BH_4	%BH_2	%B	%dihydro-	%pterin
1.6	40	33	26	-	-
5.3	7	68	25	15	85
7.6	18	44	38	30	70

Each product is the mean of 3 observations.
Quantified by HPLC following differential iodine oxidation.
BH_4 = 50uM.

Table 3.5. Distribution of reduced to oxidized products after 3 hours oxidation at 37°C for BH_2 and NH_2.

	Biopterin		Neopterin	
	%BH_2	%B	%NH_2	%N
BH_2 pH 1.6	82	18	-	-
BH_2 pH 5.3	97	3	-	-
BH_2 pH 7.6	97	3	-	-
NH_2 pH 1.6	-	-	76	24
NH_2 pH 5.3	-	-	84	16
NH_2 pH 7.6	-	-	96	4

Each product is the mean of 3 observations.
Quantified by uv spectroscopy.
BH_2, NH_2 = 50uM.

Fig. 3.6 UV time spectra for BH_4^- (50uM), pH 7·0, 37°C.
Time shown in minutes.

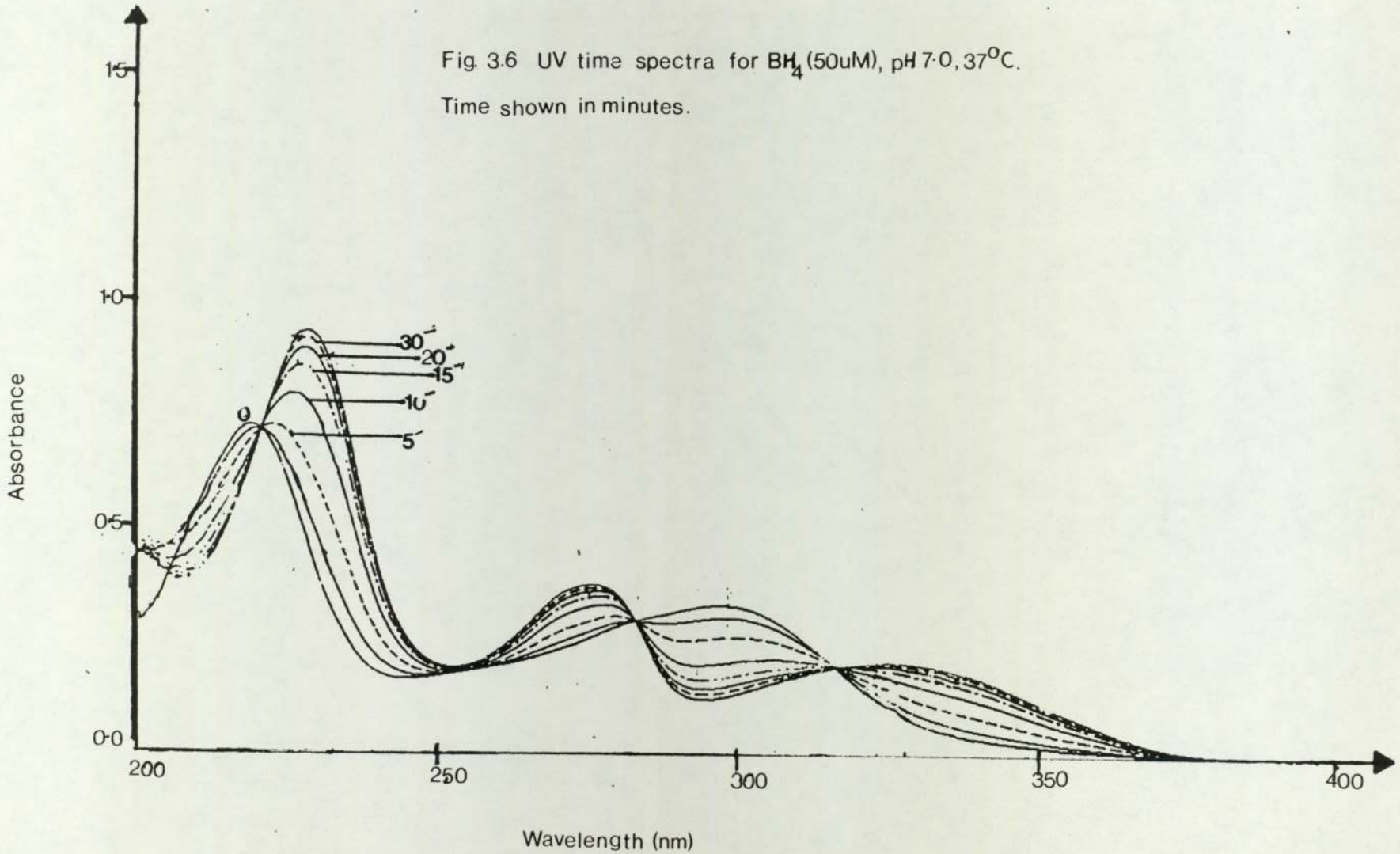
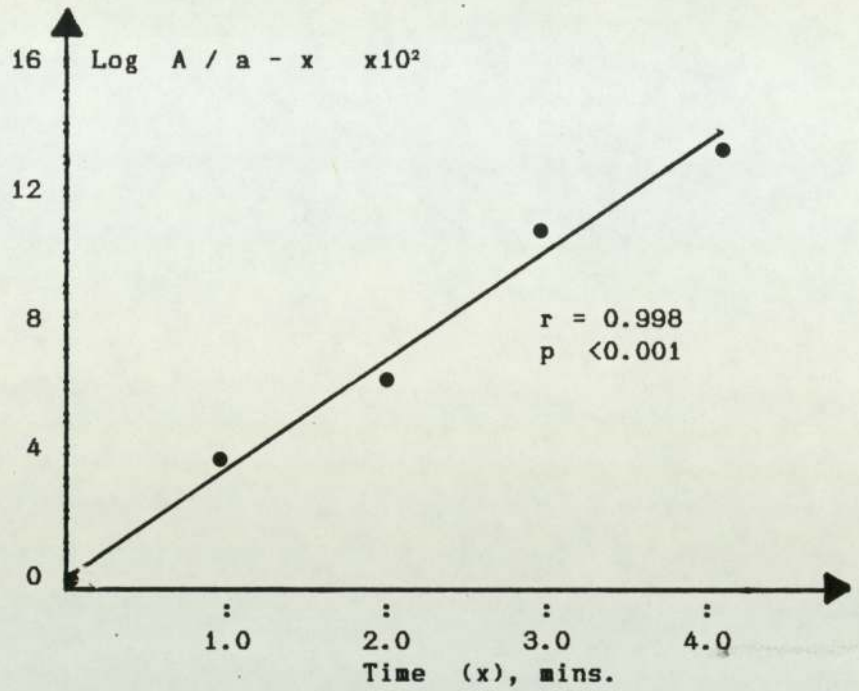
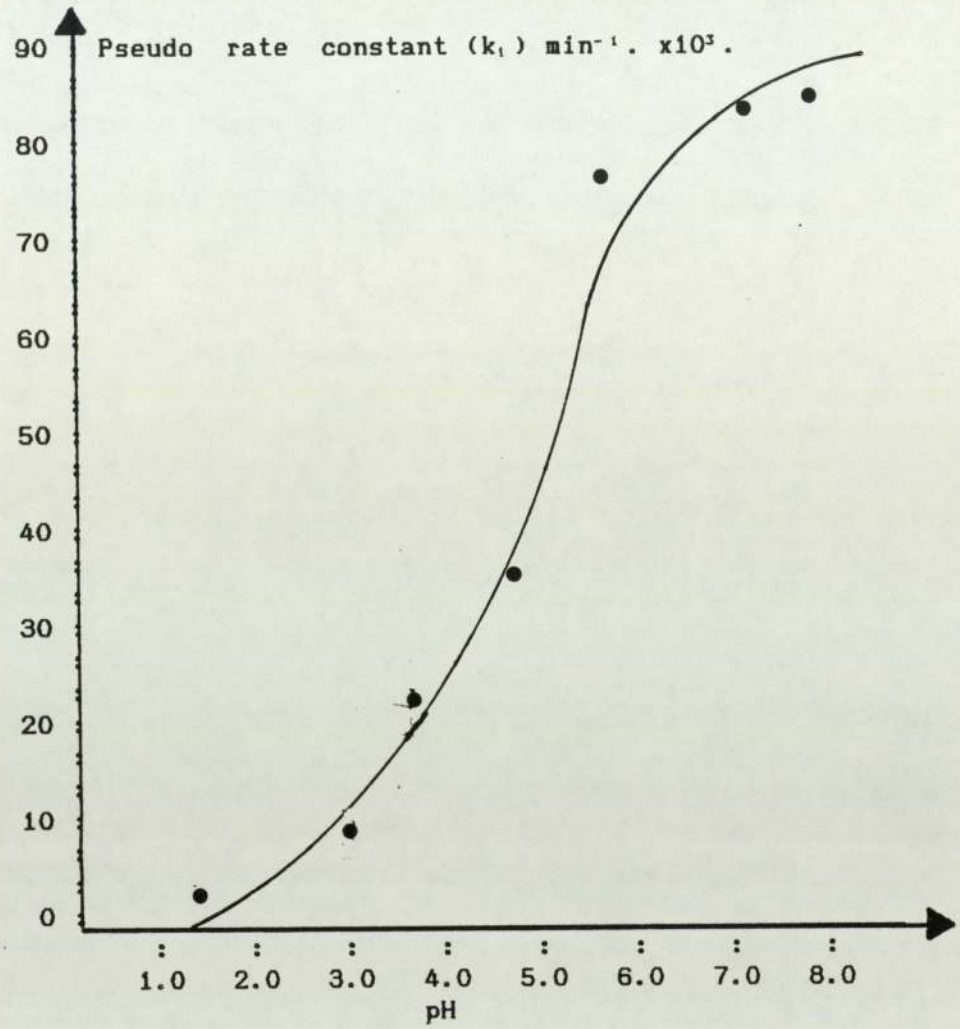


Fig. 3.7. First order plot for autoxidation of BH_4 at pH 5.3, 37° C.



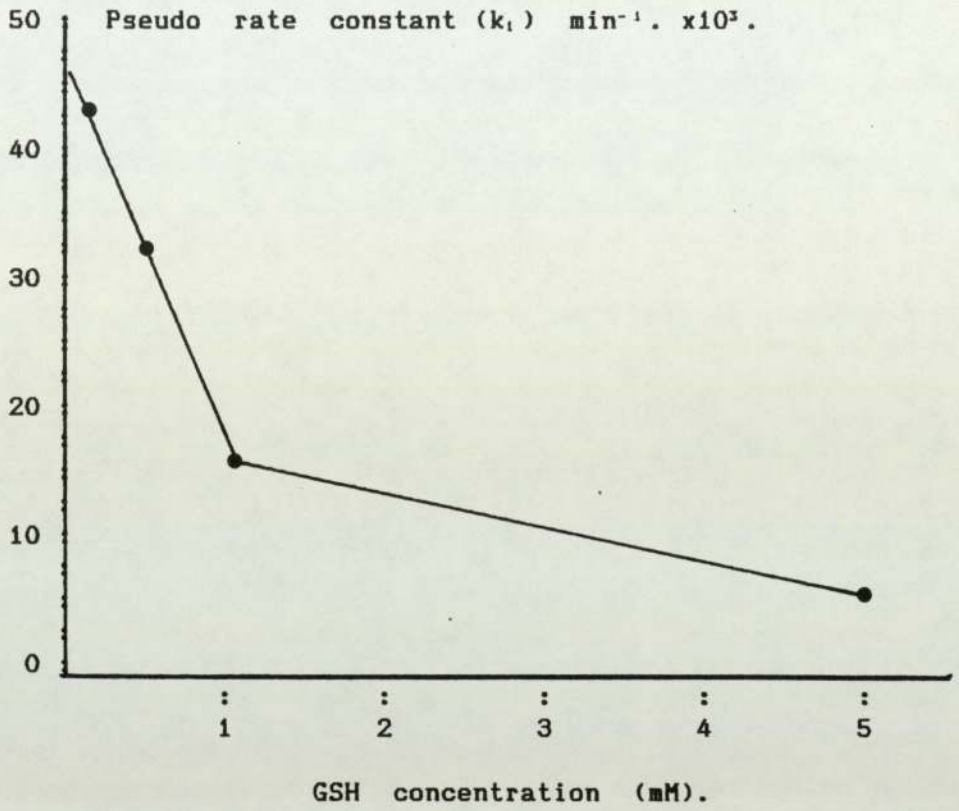
A = initial absorbance of BH_4.
a - x = absorbance of BH_4 at time x.
BH_4 = 50uM.
Measured at 300nm.
slope = 0.033 min^{-1}.
Pseudo rate constant = 0.0759 min^{-1}.
Half - life = 9.13 mins.

Fig. 3.8 Plot of pseudo rate constant (k_1) vs pH for autoxidation of BH_4 at 37° C.



Each point is the mean of 3 observations.
BH_4 = 50uM.

Fig. 3.9. Plot of initial autoxidation rate for BH_4 vs. glutathione concentration at pH 7.6, 37° C.



Each point is the mean of 3 observations.
BH_4 = 50uM.

CHAPTER FOUR

FREE RADICAL OXIDATION OF TETRAHYDRO- AND DIHYDROPTERINS

CHAPTER FOUR.

FREE RADICAL OXIDATION OF TETRAHYDRO- AND DIHYDROPTERINS.

4.1 INTRODUCTION.

Reduced pterins, such as tetrahydrobiopterin, in the presence of dioxygen readily oxidize (Blair and Pearson, 1974). Whilst dioxygen is a potent oxidizing agent, it is unable to react directly with many organic compounds due to the parallel spin of its unpaired electrons restricting it to accepting electrons one at a time (Halliwell and Gutteridge, 1984; Nunn, 1985). The reactivity of dioxygen is increased by the formation of oxygen derived radicals such as superoxide ($O_2^{\cdot-}$), and the hydroxyl radical ($^{\cdot}OH$) (Aust *et al.*, 1985).

Acceptance of a single electron by dioxygen produces $O_2^{\cdot-}$. Superoxide has been shown to cause oxidative damage to a number of biological molecules, *in vitro*, (Halliwell and Gutteridge, 1984, 1985). The exact mechanism of this damage is unclear but is suggested to involve $^{\cdot}OH$ production *via* hydrogen peroxide (H_2O_2) (Aust, 1985). Superoxide readily disproportionates to form H_2O_2, a reaction catalysed, *in vivo*, by the superoxide dismutases (EC. 1.15.1.1) (Fridovich, 1975; Ingraham and Meyer, 1985):-

$$2O_2 + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide has no unpaired electrons and reacts slowly (Meneghini and Hoffmann, 1980; Halliwell and Gutteridge, 1985). In the presence of transition metals such as copper or iron (Fenton's reagent), it readily decomposes to give the highly reactive ·OH (Haber and Weiss, 1934):-

$$H_2O_2 + Fe^{++} \longrightarrow {\cdot}OH + OH^- + Fe^{+++}$$

Hydrogen peroxide is generated directly, *in vivo*, *via* a number of metabolic processes; e.g. monoamine oxidase activity (EC. 1.4.3.4) and indirectly from $O_2^{\cdot-}$ generating reactions e.g. xanthine oxidase activity (EC. 1.1.3.22) (Sinet, 1980; Halliwell and Gutteridge, 1984).

Hydrogen peroxide formed, *in vivo*, is removed by the enzyme catalase (EC 1.11.1.6) and/or by the action of glutathione (GSH) and glutathione peroxidase (EC. 1.11.1.9):-

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

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

The reduced form of glutathione is regenerated by the NADPH dependent glutathione reductase (EC. 1.6.4.2) (Christophersen, 1969; Fridovich, 1976; Hothersall *et al.*, 1982).

Brain levels of glutathione peroxidase and catalase are low compared with other tissues (Hartz *et al.*, 1973; De Marchena *et al.*, 1974). Unscavenged H_2O_2 may, in the

presence of free iron, generate ·OH. Free iron has been detected in cerebrospinal fluid (CSF) (Gutteridge *et al.*, 1981). The potential therefore exists for ·OH production within the central nervous system (CNS).

The hydroxyl radical reacts readily with most organic molecules. Its reactions are of three main types; addition, hydrogen abstraction and electron transfer (Wilson, 1978). Because of the high reactivity of ·OH, involvement has been inferred in a number of disease processes. Within the CNS production is implicated in the pathogenesis of a number of neurological diseases including Parkinson's disease (Cohen, 1985). Generation of ·OH is also implicated as the cause of the neurological damage following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) (Cohen, 1985).

Oxygen radical formation is essential for functioning of the immune response. Within the phagocytic cell, radicals are produced for the destruction of endocytosed bacteria (Babior, 1978). Following accumulation of phagocytes, these highly reactive molecules may be released into the extracellular environment where the concentrations of protective enzymes are several times lower than those found intracellularly. This release has been implicated in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (Mc Cord, 1974).

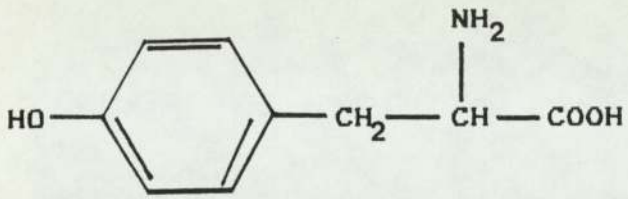
Activation of macrophages by γ-interferon results in

release of neopterin (Huber *et al.*, 1984). The mechanism of this neopterin production is not clear, but has been suggested to arise from the increased oxidative environment within the macrophage (Fuchs *et al.*, 1985). γ-interferon stimulates H_2O_2 production in macrophages (Nathan *et al.*, 1985).

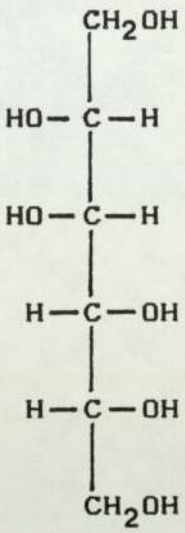
Because of the key role reduced pterins play in neurotransmitter production, and the suggested involvement of oxygen radicals in neurological disease, the *in vitro* effect of hydroxyl radical attack on tetrahydrobiopterin (BH_4), 7,8-dihydrobiopterin (BH_2) and 7,8-dihydroneopterin (NH_2) was investigated. Such a study may also explain neopterin formation following immune response activation.

4.2 MATERIALS AND METHODS.

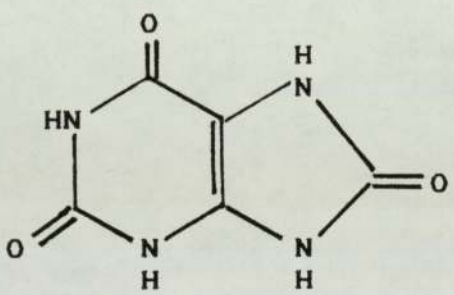
The initial rates of oxidation were determined, by uv spectroscopy, for BH_4, BH_2 and NH_2 in the absence and presence of hydroxyl radicals generated by a Fenton's reagent; hydrogen peroxide, EDTA and ferrous sulphate, as described in Chapter two. The effect of the substitution of a ferric salt was investigated. The effectiveness of the free radical inhibitors; tyrosine (i), D-mannitol (ii), uric acid (iii) and glutathione (reduced form) (iv), was also studied. Product analysis was by HPLC and TLC as described in Chapter Two. Monoamine oxidase activity and it effects on the oxidation of BH_2 was determined using the method in Chapter Two.



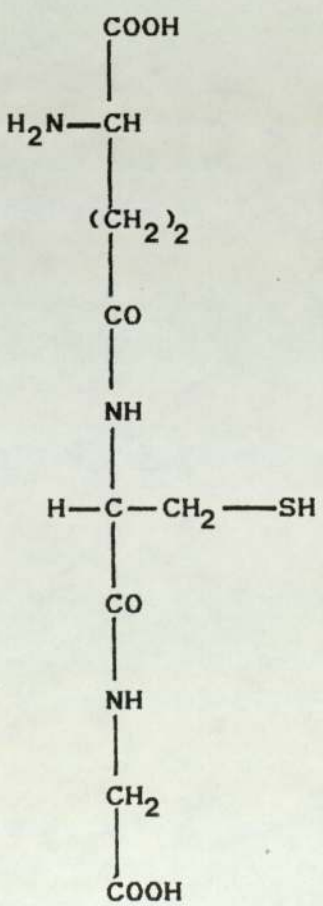
(i) L-Tyrosine



(ii) D-Mannitol



(iii) Uric acid



(iv) L-Glutathione

4.3 RESULTS AND DISCUSSION.

The oxidation of BH_4, BH_2 and NH_2 in the presence of an excess of oxidant gave pseudo first order kinetics, at all pH levels (example, Fig. 4.1).

The generation of ·OH enhanced the oxidation of BH_4, BH_2 and NH_2. The rate of oxidation increased with decreasing pH for BH_2 and NH_2 and was too rapid for accurate determination at pH 1.6. No clear pH dependence was observed for BH_4 (Table 4.1).

BH_4 readily autoxidizes, which is a process that produces and is propagated by free radicals (Blair and Pearson, 1974). The oxidation rate will therefore be determined by the free radical concentration derived from autoxidation as well as from the Fenton's reagent.

Since BH_2 and NH_2 do not readily autoxidize, results obtained with these pterins should reflect characteristics of ·OH oxidation only.

The increased rate of oxidation for BH_2 and NH_2, with decreasing pH (Table 4.1) may be explained by the fact that autoxidation of ferrous-EDTA is inhibited at acid pH (Aust et al, 1985). Since the rate of ·OH production from H_2O_2 and ferric-EDTA is considerably slower (Aust et al., 1985), the rate of oxidation of BH_2 and NH_2 by the Fenton's reagent would decrease as the pH increases due to ferrous-EDTA

autoxidation. Evidence for this comes from comparing the rates of oxidation of BH_2 over a wide pH range using H_2O_2 and either ferrous or ferric-EDTA (Table 4.2). At pH 1.6 the rates of oxidation were considerably different, but at 7.6 the rates were comparable suggesting the existence of similar systems, i.e as a result of the autoxidation of ferrous-EDTA.

The rate of oxidation of NH_2 by ·OH was faster than BH_2 (Table 4.1), suggesting that the neopterin side chain conveys increased susceptibility to oxidation by ·OH. The side chains of NH_2 and BH_2 differ in the fact that the terminal methyl group of BH_2 is replaced by a primary alcohol group in NH_2. Alcohols react with ·OH to produce organic radical intermediates (Cederbaum and Cohen, 1984). Possibly increased organic radical formation with NH_2 enhances its oxidation rate by inter- or intramolecular effects.

Many hydroxylated compounds act as scavengers of ·OH (Hochstein *et al.*, 1984). Neither tyrosine nor mannitol, in high concentrations, greatly inhibited the oxidation of BH_2 (Table 4.3), illustrating the ease of oxidation of BH_2 by ·OH. Uric acid gave better inhibition of the oxidation of BH_2 (Table 4.3). In contrast to the other inhibitors investigated, uric acid can form complexes with free iron and so directly inhibit the generation of ·OH (Hochstein *et al.*, 1984). The reduced form of glutathione (GSH) inhibited the oxidation of BH_4 by Fenton's reagent (Table 4.4). GSH is readily oxidized by free radicals (Meister and Anderson,

1983), due presumably to the ease of oxidation of its sulphydryl group.

No further oxidation was necessary, following reaction with Fenton's reagent, for detection of products by HPLC. Product analysis for BH_4, BH_2 and NH_2 revealed a pH dependence (Table 4.5). With increasing pH there was increased destruction of the pterin ring system, suggesting that the protonated form of the pterin ring is not readily attacked by ·OH. The NH_2 side chain appeared to convey increased susceptibility, compared with BH_2, to destruction of the pterin ring by ·OH above pH 1.6.

Xanthopterin is suggested to arise from hydration of 7,8-dihydropterin, a slow process (Armarego and Randles, 1983). Alternatively, as ·OH can readily add across double bonds (Czapski, 1984), xanthopterin may also be formed from the direct addition of ·OH to 7,8-dihydropterin. Such a mechanism would explain the formation of xanthopterin in the absence of any detected pterin (Table 4.5).

Variation of the H_2O_2 concentration in the Fenton's reagent had no effect on the rate of oxidation of NH_2 at pH 5.3 (Table 4.6). As H_2O_2 itself is reported to be unreactive (Halliwell and Gutteridge, 1985) excess amounts, which cannot participate in the Fenton's reaction, will have no effect.

Neither monoamine oxidase (MAO) itself nor the generation of

H_2O_2 by MAO activity oxidized BH_2. MAO activity in the presence of ferrous sulphate caused oxidation. Inhibition of this BH_2 oxidation was achieved by catalase and phenelzine, an inhibitor of MAO (Table 4.7). This result indicates that, in the presence of free iron, generation of small amounts of H_2O_2 are sufficient to cause oxidation of reduced pterins.

This study has shown that the reduced pterins BH_4, BH_2 and NH_2 are readily oxidized by ·OH, with substantial destruction of the pterin ring at near physiological pH. Possibly production of oxygen radicals, *in vivo*, in regions dependent on BH_4 for neurotransmitter synthesis, may, if not scavenged, contribute to neurological impairment by depletion of the cofactor pool.

The inhibition of the free radical oxidation of BH_2 by uric acid suggests that, *in vivo*, it may act as an antioxidant in urine and plasma, where it is present in high concentrations (2,100 and 450uM respectively) (Hochstein *et al.*, 1984).

Inhibition of the ·OH oxidation of BH_4 by physiological concentrations of GSH confirms the importance of this tripeptide as a free radical scavenger, and may suggest an important role, *in vivo*, for protecting reduced pterins from free radical oxidation.

The extensive destruction of the pterin ring system of NH_2 by ·OH, above pH 1.6, suggests consideration of different

conditions, e.g. an excess of NH_2 over ·OH, to explain the neopterin release from activated macrophages, where the intracellular pH is typically 5.0 - 5.5 (Babior and Crowley, 1983)

Table 4.1. Pseudo rate constants (k_1) and half lives ($t_{1/2}$) for BH_4, BH_2, and NH_2 in the presence and absence of a Fenton's reagent, at 37° C.

			Air oxidation		Air oxidation + Fenton's reagent	
			k_1 (min^{-1})	$t_{1/2}$ (mins)	k_1 (min^{-1})	$t_{1/2}$ (mins)
BH_4	pH	1.6	0.0028	247.50	0.2126	3.26
BH_4	pH	5.3	0.0750	9.24	0.1529	4.53
BH_4	pH	7.6	0.0805	8.61	0.2772	2.50
BH_2	pH	1.6	*	*	>>0.6930	<<1.00
BH_2	pH	5.3	*	*	0.0154	45.00
BH_2	pH	7.6	*	*	0.0126	55.00
NH_2	pH	1.6	*	*	>>0.6930	<<1.00
NH_2	pH	5.3	*	*	0.1255	5.52
NH_2	pH	7.6	*	*	0.0277	25.00

*No measurable reaction within 30 minutes.
BH_4, BH_2 and NH_2 = 50uM.
Each rate is the mean of 4 observations.
Fenton's reagent = ferrous sulphate / EDTA (0.1mM), H_2O_2 (0.3mM).

Table 4.2. Comparison of the rates of reaction for BH_2 in the presence of a ferrous or ferric salt and H_2O_2, at 37°C.

	Half - life ($t_{1/2}$) mins.	
	Fe^{++}	Fe^{+++}
BH_2 pH 1.6	<1.00	24.80
BH_2 pH 5.3	45.00	54.06
BH_2 pH 7.6	55.09	63.05

Each rate is a mean of 3 observations.
BH_2 = 50uM.
Reaction mixture contained ferrous sulphate or ferric chloride (0.1mM), EDTA (0.1mM) and H_2O_2 (0.3mM).

Table 4.3. Effect of a number of potential free radical scavengers on the initial oxidation rate of BH_2, at pH 7.6, in the presence of a Fenton's reagent at 37°C.

	Pseudo rate constant. (min^{-1})	Percentage inhibition.
BH_2	0.0125	-
BH_2 + tyrosine (1mM)	0.0117	6
BH_2 + mannitol (1mM)	0.0105	16
BH_2 + mannitol (3mM)	0.0105	16
BH_2 + uric acid (1mM)	0.0105	16
BH_2 + uric acid (3mM)	0.0076	39

Each rate is the mean of 3 observations.
BH_2 = 50uM.
Fenton's reagent = ferrous sulphate / EDTA (0.1mM), H_2O_2 (0.3mM)

Table 4.4. Effect of glutathione (GSH) on the initial oxidation rate of BH_4, at pH 7.6, in the presence of a Fenton's reagent at 37° C.

	Pseudo rate constant. (min^{-1})	Percentage inhibition.
BH_4	0.2900	-
BH_4 + GSH (mM)		
2.0	0.2210	24
4.0	0.1560	47
6.0	0.0960	67

Each rate is the mean of 3 observations.
BH_4 = 50uM.
Fenton's reagent = ferrous sulphate / EDTA (0.1mM), H_2O_2 (0.3mM).

Table 4.5. Percentage conversions to identifiable products for the reactions of BH_4, BH_2 and NH_2 in the presence of a Fenton's reagent at 37°C.

	Neopterin	Biopterin	Xantho-pterin	Pterin
BH_4 pH 1.6	-	100	1	2
BH_4 pH 5.3	-	31	-	3
BH_4 pH 7.6	-	5	20	3
BH_2 pH 1.6	-	100	-	-
BH_2 pH 5.3	-	45	15	-
BH_2 pH 7.6	-	23	20	-
NH_2 pH 1.6	100	-	-	-
NH_2 pH 5.3	14	-	-	-
NH_2 pH 7.6	10	-	-	-

Products identified after 1 hour incubation, by HPLC and TLC, quantified by HPLC,.
BH_4, BH_2 and NH_2 = 50uM.
Each percentage conversion is the mean of 4 observations.
Fenton's reagent = ferrous sulphate / EDTA (0.1mM), H_2O_2 (0.3mM).

Table 4.6. Effect of varying the H_2O_2 concentration on the initial rate of oxidation of NH_2, at pH 5.3, in the presence of ferrous sulphate and EDTA, at 37°C.

H_2O_2 concentration mM.	Half - life ($t_{1/2}$) mins.
0.10	5.20
1.00	4.41
10.00	5.56
16.00	5.52

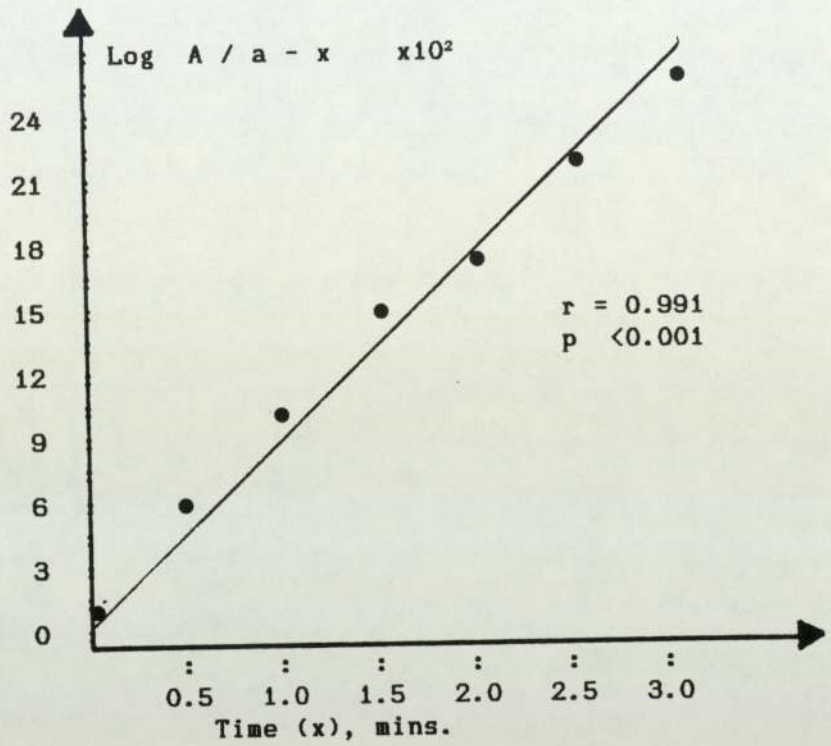
Each rate is the mean of 3 observations.
NH_2 = 50uM.
Ferrous sulphate/EDTA = 0.1mM.

Table 4.7. Effect of monoamine oxidase activity (MAO) on the oxidation of BH_2 in the presence and absence of ferrous sulphate, plus the effects of phenelzine and catalase addition.

	Rate constant (min^{-1})	Percentage inhibition.
BH_2 + MAO + EDTA	*	-
BH_2 + MAO + benzylamine + EDTA	*	-
BH_2 + MAO + benzylamine + EDTA + ferrous sulphate (1)	0.0041	-
(1) + catalase (5U ml^{-1})	0.0015	63
(1) + phenelzine (1mM)	0.0000	100

BH_2 = 50um.
MAO activity = 4nMoles benzylamine oxidised min^{-1} ml^{-1} enzyme source at pH 7.6, 25°C.
Benzylamine final concentration = 3mM.
Ferrous sulphate and EDTA final concentration = 60uM.
Each rate is the mean of 3 observations.
* No reaction over a 30 minute period.

Fig 4.1. First order plot for BH_4 in the presence of a Fenton's reagent at pH 1.6, 37°C.



A = initial concentration of BH_4.
a - x = concentration of BH_4 at time x.
Slope = 0.092 min^{-1}.
Pseudo rate constant = 0.2126 min^{-1}.
Fenton's reagent = ferrous sulphate / EDTA (0.1mM), H_2O_2 (0.3mM).
Half-life = 3.26 mins.
BH_4 = 50uM.

CHAPTER FIVE

NITRITE OXIDATION OF TETRAHYDRO- AND DIHYDROPTERINS

CHAPTER FIVE.

NITRITE OXIDATION OF TETRAHYDRO- AND DIHYDROPTERINS.

5.1 INTRODUCTION.

Reduced pterins in the presence of suitable oxidizing agents readily oxidize (Pearson, 1974). Nitrite (NO_2^-) is a widely distributed environmental oxidizing agent, and it is estimated that the average US citizen ingests 11mg of nitrite a day from various sources (Green et al., 1982).

The largest daily source of nitrite arises from saliva, resulting from the bacterial reduction of nitrate (NO_3^-) in the oral cavity (Goaz and Biswell, 1961). Whilst nitrate, *per se*, is not toxic, reduction of a nitrate rich source can lead to toxicity (Wolff and Wasserman, 1972; Green *et al.*, 1982). The major dietary contribution of nitrite, in the Western diet, is cured meats. Nitrite is added for three reasons; (1) colour formation, (2) production of flavour, and (3) antibacterial activity (Wolff and Wassermann, 1972).

Ingestion of nitrite is associated with a number of clinical disorders, the most conspicuous and longest recognized action being the oxidation of the ferrous haem centre of haemoglobin resulting in methaemoglobin formation (Haldane *et al.*, 1897). Methaemoglobin is unable to transport oxygen and the increased susceptibility of foetal haemoglobin to oxidation

has implicated nitrite in the blue baby syndrome (Martin and Huisman, 1963). Methaemoglobin formation is suggested to arise from destabilisation of the oxygen-haem complex, by nitrite, resulting in oxidation by molecular oxygen (Cohen *et al.*, 1964).

Under acidic conditions, nitrite forms nitrous acid (HNO_2) (Zimmermann, 1975):-

$$NO_2^- + H_2O \rightleftharpoons HNO_2 + {}^-OH$$

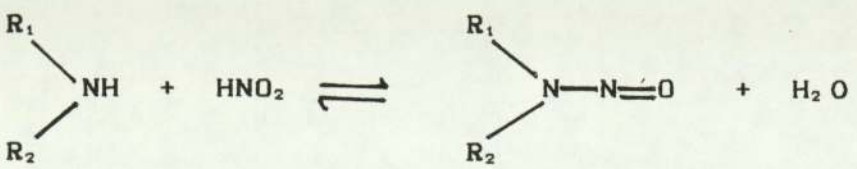
Nitrous acid can act as an oxidizing agent, oxidizing a wide range of molecules, due to formation of the electrophiles, dinitrogen trioxide (N_2O_3) and the nitrosonium ion (NO^+) (Turney and Wright, 1959; Mirvish, 1975):-

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$HNO_2 + H^+ \rightleftharpoons NO^+ + H_2O$$

Formation of the nitrous acidium ion ($H_2NO_2^+$) is also suggested under conditions of high acidity (Turney and Wright, 1959). The ratio of N_2O_3 to NO^+ is altered in favour of NO^+ with low concentrations of nitrous acid and high acidity. (Turney and Wright, 1959; Mirvish, 1975).

Nitrous acid has been implicated in carcinogenesis due to its ability to form carcinogenic N-nitrosocompounds from a number of nitrogen containing compounds encountered *in vivo* e.g. secondary amines:-



If R_1 and R_2 are alkyl or aryl moieties a N-nitrosamine will

be formed which requires metabolic activation, e.g. by P450 cytochrome system, before exerting carcinogenic activity. Replacement of R_2 with an acyl group will result in N-nitrosamide formation which requires no activation. Carcinogenesis is suggested to arise from production of reactive electrophilic species which interact with cellular nucleic acids (Challis and Challis, 1982; Preussmann and Stewart, 1984).

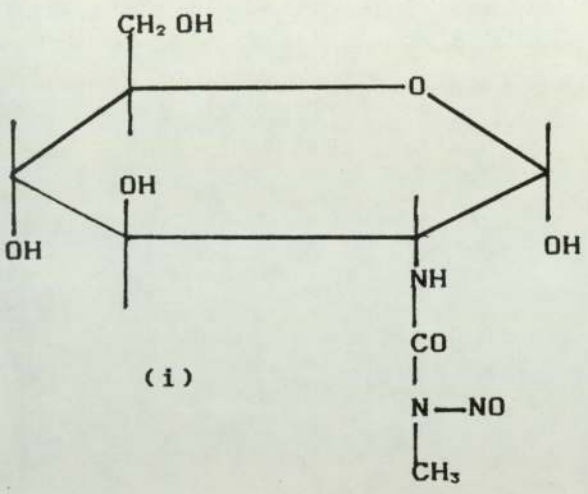
Because nitrite can form oxidizing agents and biological systems are constantly at risk to exposure, its effect on the oxidation rates of tetrahydrobiopterin (BH_4), and 7,8-dihydrobiopterin (BH_2) was investigated, over a wide pH range. Since the majority of nitrite enters the body via the oral route, the effect on the important dietary reduced folates, tetrahydrofolate (FH_4) and 5-methyl-tetrahydrofolate ($5CH_3FH_4$), was also studied.

N-nitrosamines and N-nitrosamides can, under acidic conditions, give rise to nitrous acid (Challis and Challis, 1983). Such compounds may therefore act as indirect oxidants of reduced pterins. Consequently their effect on the oxidation of BH_4 was examined.

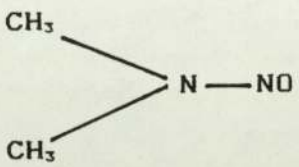
5.2 MATERIALS AND METHODS.

The initial rates of oxidation were determined, by uv spectroscopy, for BH_4, BH_2, FH_4 and $5CH_3FH_4$ in the absence and presence of sodium nitrite, as described in Chapter two.

The effect of 2-deoxy-2-(N-methyl-N-nitrosoureido)-D-glucopyranose (streptozotocin) (i), a N-nitrosamide, and N-nitrosodimethylamine (ii), a N-nitrosamine, were similarly investigated on the oxidation rate of BH_4. Product analysis was by HPLC and TLC as described in Chapter Two.



(i)



(ii)

5.3 RESULTS AND DISCUSSION.

The oxidation rates of BH_4, BH_2, FH_4 and $5CH_3FH_4$ were enhanced in the presence of nitrite at pH 1.6 and 3.0 (Table 5.1). The rate of reaction at these pH levels was first order with respect to nitrite concentration (example Fig. 5.1). In the presence of excess nitrite the oxidation of all the reduced pterins gave overall first order kinetics (example Fig. 5.2). Nitrite had no effect at pH 7.0 (Table 5.1).

Compared to FH_4 both the autoxidation and nitrite oxidation rates of $5CH_3FH_4$ were considerably slower (Table 5.1). The slow autoxidation rate of $5CH_3FH_4$ is attributed to steric

hindrance. A methyl group at N(5) decreases the number of effective collisions between the pterin ring system and dioxygen (Pearson, 1974). A similar situation may occur with nitrite oxidation.

The rate of nitrite oxidation exhibited a marked pH dependence, increasing with decreasing pH (Table 5.1). This pH dependence, first order nitrite kinetics and comparison with other systems (Challis and Challis, 1982) suggest the oxidizing agent in this study is NO^+ or $H_2NO_2^+$ and a possible mechanism to be as illustrated for BH_4 :-

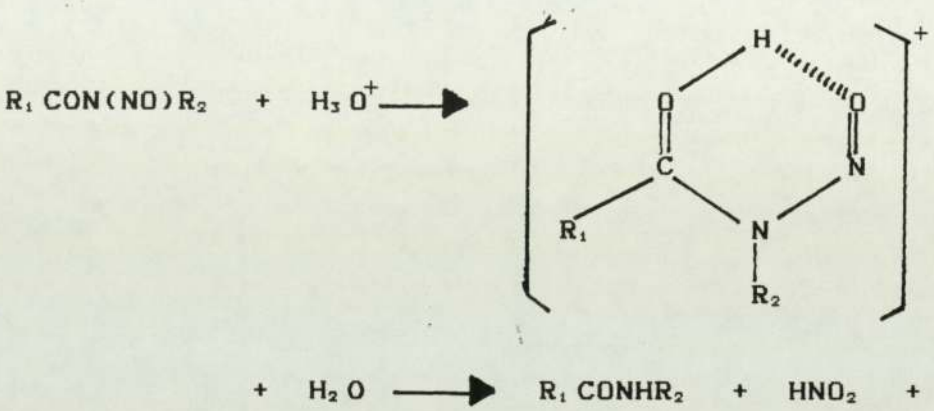
$$BH_4 + \underset{(NO^+)}{H_2NO_2^+} \longrightarrow BH_3^{\bullet} + NO + H_2O + H^+$$

$$BH_3^{\bullet} + \underset{(NO^+)}{H_2NO_2^+} \longrightarrow qBH_2 + NO + H_2O + H^+$$

$$qBH_2 \xrightarrow{\text{rearrange}} BH_2$$

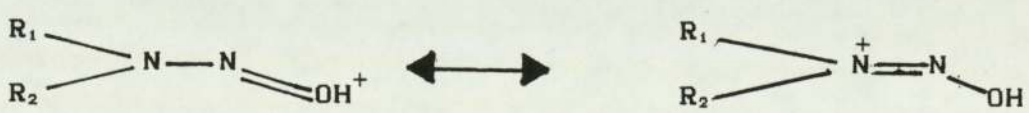
Product analysis for BH_4 and BH_2 showed, at pH 1.6 and 3.0, that nitrite oxidized both compounds to biopterin (Table 5.2). Some loss of the pterin ring system occurred with BH_2 (Table 5.2). FH_4 was oxidized by nitrite to give pterin as the only identifiable product, in common with air oxidation (Table 5.2). The oxidation products for $5CH_3FH_4$ were complex and not identified. Autoxidation of $5CH_3FH_4$ results in formation of 5,methyl-5,6-dihydrofolate which is acid labile forming a number of oxidation products (Gupta and Huennekens, 1967; Blair *et al.*, 1975). Formation of 5,methyl-5,6-dihydrofolate by nitrite oxidation of $5CH_3FH_4$ may explain the complexity of the oxidation products.

Streptozotocin oxidized BH_4 giving biopterin as the major oxidation product at pH 1.6 only (Table 5.4). The rate of oxidation increased with time, suggesting oxidation is not mediated by the parent compound (Table 5.3). N-nitrosamides, in acid, undergo denitrosation resulting in nitrous acid formation (Challis and Challis, 1982):-



It therefore appears that oxidation of BH_4 by streptozotocin is dependent on the rate of denitrosation and hence nitrous acid formation.

N-nitrosodimethylamine had no effect on BH_4 at any of the pH levels. N-nitrosamines require concentrated acids for denitrosation to occur. In acid media the most stable conjugate acid is that arising from O-protonation, allowing resonance stabilisation:-



For denitrosation to occur formation of an N-protonated conjugated acid is necessary, which is not resonance stabilized and is unstable (Challis and Challis, 1982):-

$$R_1R_2NNO \overset{H_3O^+}{\rightleftharpoons} R_1R_2\underset{H}{N}NO \overset{Y}{\rightleftharpoons} R_1R_2NH + YNO$$

$$YNO \overset{H_2O}{\rightleftharpoons} HNO_2 + HY$$

$Y = Cl^-, Br^-, SCN^-, H_2O.$

The apparent unreactivity of N-nitrosodimethylamine towards BH_4, under the conditions employed, may be due to lack of or inappropriate protonation of the nitrosamine.

The results from this study suggest that nitrite ingestion would not affect BH_4 metabolism directly, since BH_4 is synthesized endogenously and hence the pH would not be appropriate.

However, dietary tetrahydrofolates in the acid medium of the stomach, typically pH 2.5 rising to 4.5 (Knowles *et al.*, 1974), may be destroyed by the large daily intake of nitrite. Nitrite ingestion is calculated to exceed that of folate by about 300 fold on a molar basis (assuming the molecular weight of nitrite is 46 and folate 434 and daily intake is 11mg and 300ug respectively).

In addition to nitrite, ingestion of N-nitrosamides could contribute to the oxidative breakdown of tetrahydrofolate.

Table 5.1. Pseudo rate constants (k_1) and half lives ($t_{1/2}$) for BH_4, BH_2, FH_4 and $5CH_3FH_4$ in the presence and absence of an excess of sodium nitrite, at 37°C.

	Air oxidation		Air oxidation + nitrite.	
	k_1 (min^{-1})	$t_{1/2}$ (mins)	k_1 (min^{-1})	$t_{1/2}$ (mins)
BH_4 pH 1.6	0.0028	247.50	0.1320	5.23
BH_4 pH 3.0	0.0084	82.44	0.0450	15.36
BH_4 pH 7.0	0.0820	8.41	0.0820	8.41
BH_2 pH 1.6	*	*	0.9090	0.76
BH_2 pH 3.0	*	*	0.0679	10.20
BH_2 pH 7.0	*	*	*	*
FH_4 pH 1.6	0.0123	56.34	0.9240	0.75
FH_4 pH 3.0	0.0409	16.90	0.3680	1.88
FH_4 pH 7.0	0.0529	13.10	0.0529	13.10
$5CH_3FH_4$ pH 1.6	*	*	0.1796	3.85
$5CH_3FH_4$ pH 3.0	0.0118	58.72	0.0748	9.25

*No measurable reaction within 30 minutes.
Each rate is the mean of 3 observations.
BH_4 and BH_2 = 50uM.
FH_4 and $5CH_3FH_4$ = 20uM.
Sodium nitrite = 0.1mM.

Table 5.2. Percentage conversions to biopterin and pterin for the reactions of BH_4, BH_2, and FH_4 with sodium nitrite, at 37° C.

			Biopterin.	Pterin.
BH_4	pH	1.6	96	-
BH_4	pH	3.0	96	-
BH_2	pH	1.6	83	-
BH_2	pH	3.0	83	-
FH_4	pH	1.6	-	77
FH_4	pH	3.0	-	70

Products identified by TLC and HPLC, quantified by HPLC after 30 minutes incubation.
Each conversion is the mean of 3 observations.
BH_4 and BH_2 = 50uM.
FH_4 = 20uM.
Sodium nitrite = 0.1mM.

Table 5.3. Pseudo rate constant (k_1) and half life ($t_{1/2}$) for BH_4 in the presence of streptozotocin at pH 1.6, 37°C.

Time period of measuring rate (mins).	k_1 (min^{-1})	$t_{1/2}$ (mins)
0 - 10	0.0056	123.6
20 - 30	0.0172	40.0

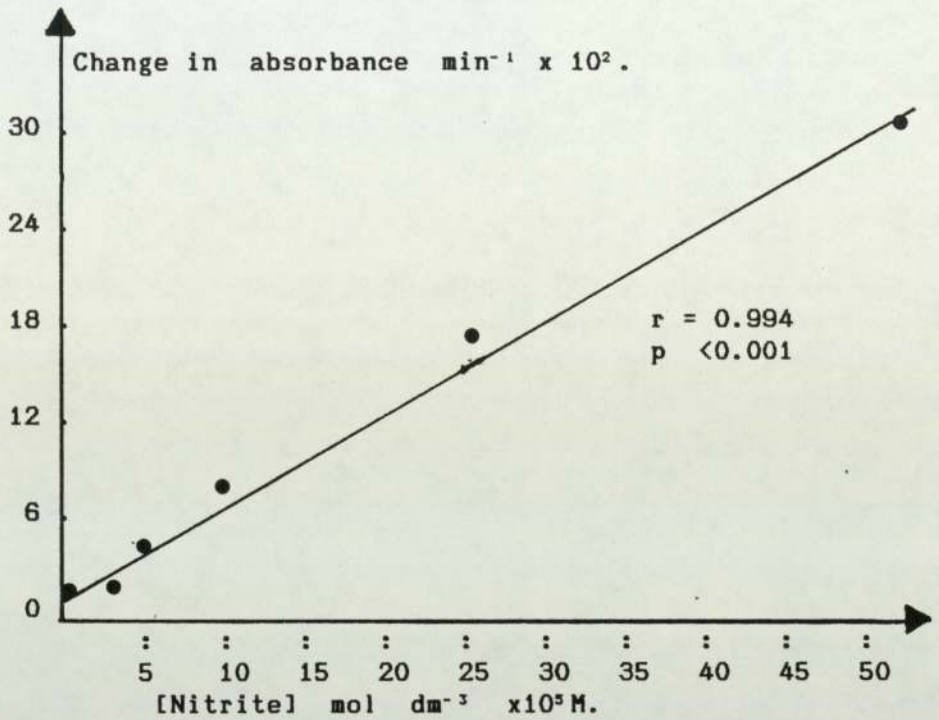
Each rate is the mean of 3 observations.
BH_4 = 50uM.
Streptozotocin = 0.1mM.

Table 5.4. Percentage conversion to biopterin for the reaction of BH_4 with streptozotocin, at pH 1.6, 37°C.

	% Biopterin
BH_4 + Streptozotocin.	73

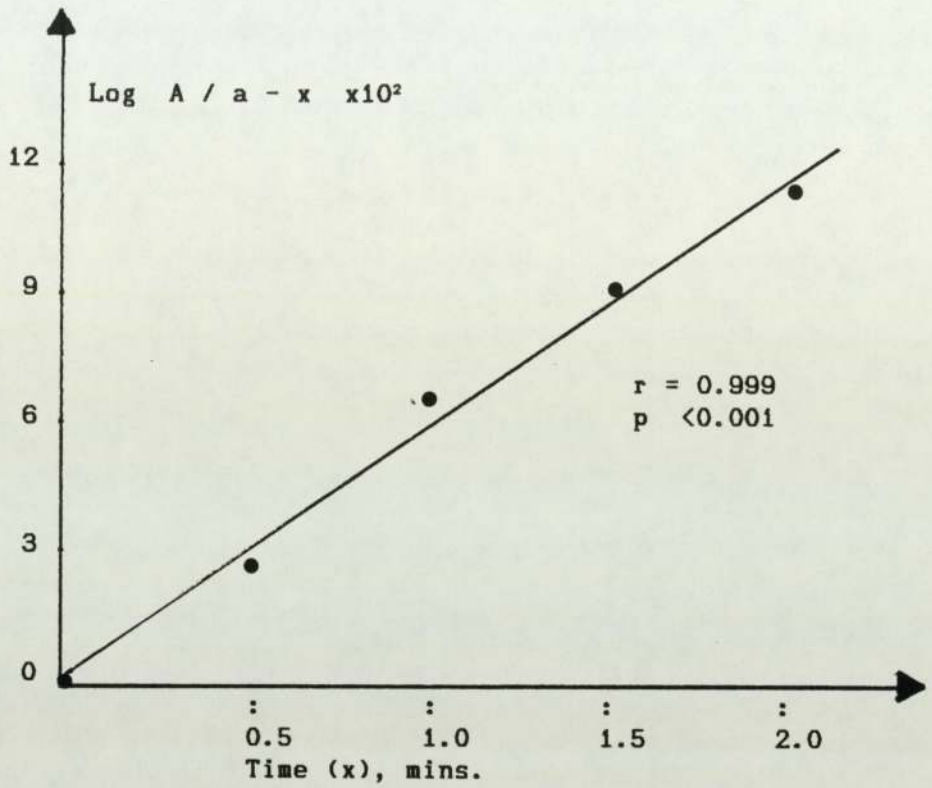
Conversion is mean of 3 observations.
Product identified by TLC and HPLC, quantified by HPLC.
BH_4 = 50uM.
Streptozotocin = 0.1mM.

Fig. 5.1. Plot of initial change in absorbance, at 267nm, for BH_4 against sodium nitrite concentration at pH 1.6, 37° C.



Each point is the mean of 3 observations.
BH_4 = 50uM.

Fig. 5.2. First order plot for BH_4 in the presence of sodium nitrite at pH 1.6, 37°C.



A = initial concentration of BH_4.
a - x = concentration of BH_4 at time x.
Slope = 0.0575 min^{-1}.
Pseudo rate constant = 0.1320 min^{-1}.
Half-life = 5.23 mins.
BH_4 = 50uM.
Sodium nitrite = 0.1mM.

CHAPTER SIX

EFFECT OF NITROUS OXIDE ON TETRAHYDROPTERIN METABOLISM IN THE RAT

CHAPTER SIX.

EFFECT OF NITROUS OXIDE AND OXYGEN ON TETRAHYDROPTERIN METABOLISM IN THE RAT.

6.1 INTRODUCTION.

The anaesthetic gas, nitrous oxide (N_2O), oxidizes the cobalt I centre of vitamin B_{12} (cob(I)alamin) resulting in selective inactivation, *in vivo*, of methionine synthetase (EC. 2.1.1.13) for which cob(I)alamin is a cofactor (Banks *et al.*, 1968; Deacon *et al.*, 1978):-

$$Co^{+} - B_{12} + N_2O \longrightarrow Co^{++} - B_{12} + H_2O + N_2$$

Inactivation, *in vivo*, is a rapid process with 95 per cent inhibition occurring in the mouse liver after 4 hours (Koblin *et al.*, 1981).

Methionine synthetase catalyses the transfer of a methyl group from 5,methyl-tetrahydrofolate ($5CH_3FH_4$) (mono or polyglutamate, the monoglutamate being the major transport folate) to homocysteine yielding methionine and tetrahydrofolate (FH_4) (Fig. 6.1) (Taylor and Weissbach, 1973; Smith *et al.*, 1986). In rats, N_2O inhibition of methionine synthetase for 18 hours, results in decreased tissue levels of FH_4 and an accumulation of $5CH_3FH_4$ (mono and polyglutamates) (Wilson and Horne, 1986).

Exposure to N_2O in man and rats results in decreased nucleic

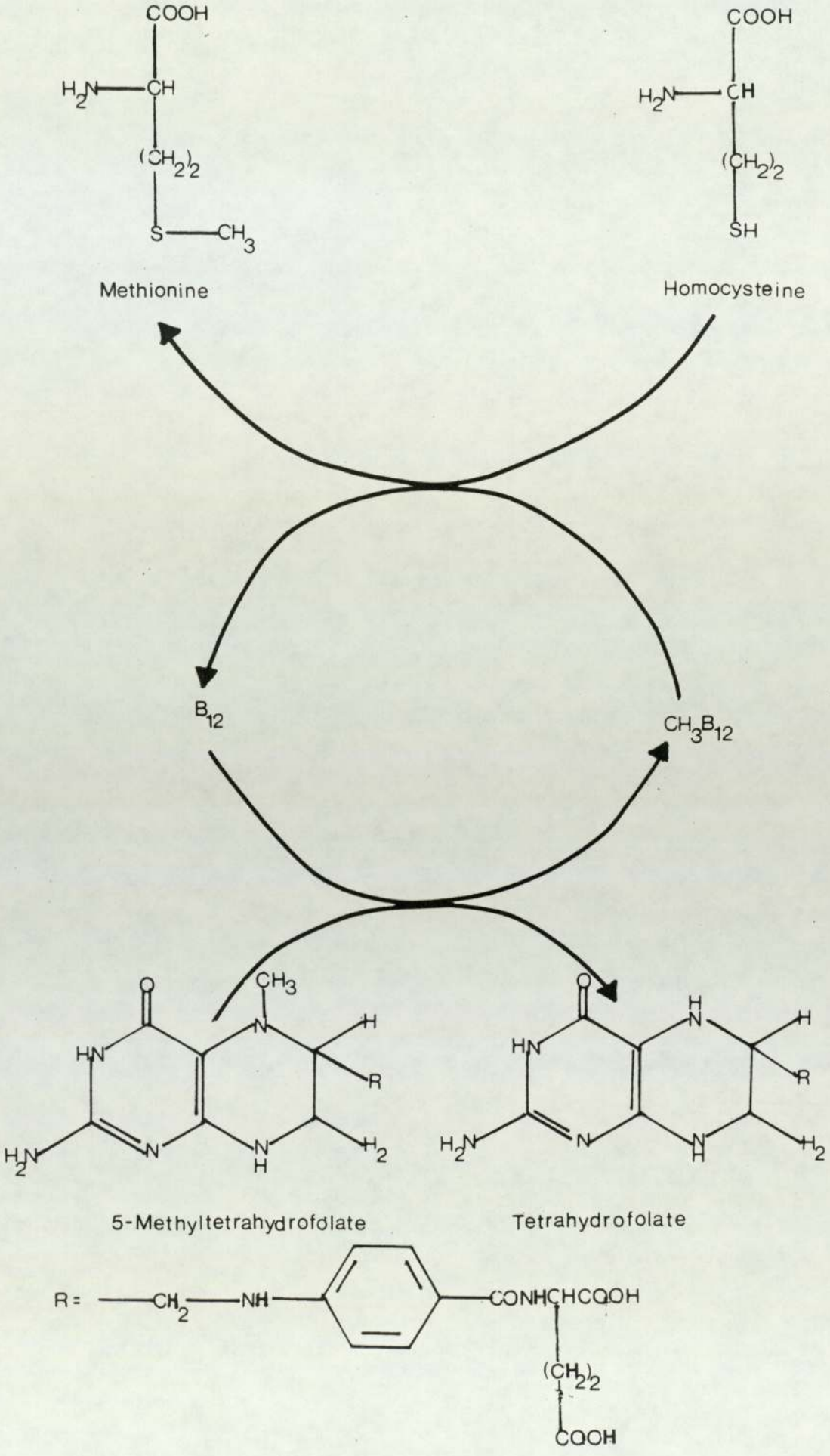


Fig. 6.1 Action of methionine synthetase.

acid synthesis. This is due to lack of formate, derived from methionine, and FH_4 required for production of the tetrahydrofolate derivatives necessary for purine and pyrimidine biosynthesis (Chanarin *et al.*, 1981; Koblin *et al.*, 1981). Synthesis of folate polyglutamates, the active tissue cofactors, is also inhibited, following N_2O induced vitamin B_{12} deficiency, due to reduced synthesis of 10-formyl tetrahydrofolate and FH_4 the substrates for polyglutamate synthesis (Chanarin *et al.*, 1980; Rowe, 1983).

Defective $5CH_3FH_4$ turnover, as in vitamin B_{12} deficiency, results in demyelination of the spinal cord. This is suggested to arise from deficiency of methionine, S-adenosyl-methionine (SAM) or their derivatives (Clayton *et al.*, 1986). SAM is required in numerous transmethylation reactions including the post translational methylation of the basic protein of myelin, which is associated with the myelination process (Crang and Jacobson, 1980).

Decreased levels of total biopterins are reported in the cerebrospinal fluid (CSF) of a patient with 5,10-methylene-tetrahydrofolate ($5,10CH_2FH_4$) reductase deficiency (EC. 1.1.1.58), the enzyme which converts $5,10CH_2FH_4$ to $5CH_3FH_4$. Deficiency therefore results in diminished turnover of $5CH_3FH_4$ (Clayton *et al.*, 1986). *In vitro*, tetrahydrobiopterin (BH_4) biosynthesis is enhanced by $5CH_3FH_4$ and Vitamin B_{12} and inhibited by N_2O. These observations suggest a link between FH_4 and BH_4 metabolism (Leeming *et al.*, 1982; Hamon *et al.*, 1986). Consequently the effect of

N_2O was examined on BH_4 metabolism *in vivo* using the rat.

Exposure to high levels of dioxygen (O_2) may inactivate methionine synthetase by a similar mechanism to N_2O (Deacon *et al.*, 1980; Koblin *et al.*, 1981). Tetrahydropterins are also susceptible to oxidation by O_2 (Pearson, 1974) and an increased *in vivo* concentration may result in enhanced oxidative catabolism. Inhalation of 100 per cent O_2, by rats, results in an approximate trebling of the brain O_2 concentration (Jamieson and Van Den Brenk, 1963). The effect of increased O_2 exposure was therefore investigated on BH_4 metabolism in the rat.

The rat is a convenient model for studying BH_4 metabolism *in vivo* (Hamon, 1984). Though differences exist between BH_4 metabolism in rat and man, notably the failure to detect neopterin in rat tissue (Rokos *et al.*, 1985), many findings are observable in both species (Barford *et al.*, 1983; Cutler, 1986).

Any comprehensive *in vivo* study of BH_4 metabolism requires reliable methods for determining tissue cofactor levels, biosynthetic and salvage capacities. Since the male Wistar rat was used in this study, these parameters were measured in brain and liver to provide details of BH_4 metabolism in the rat, prior to studying the effects of N_2O and O_2 exposure.

In addition to total biopterins, pterin levels were measured following acid-iodine oxidation. This pterin is derived from

the FH_4 pool (Fukushima and Nixon, 1980) or may originate from tissue 7,8-dihydropterin arising form BH_4 oxidation (Chapter 3).

6.2 MATERIALS AND METHODS.

Derived pterin, total biopterins (tetrahydro, dihydro, and fully oxidized biopterins), biosynthesis and DHPR activity were determined in the liver and brain of normal 150g male Wistar rats and rats exposed to $N_2O:O_2$ (1:1 and 4:1) and helium:O_2 (1:1, 4:1 and 1:4) mixtures and 100 per cent O_2 using the methods described in Chapter 2.

Because of the large inter assay variability that occurs with the techniques employed, all test and control samples were assayed together and no between batch comparisons were made.

The oxidation rates of BH_2 and $5CH_3FH_4$ were monitored *in vitro* in N_2O saturated buffers at pH 1.6 and 7.0 using UV spectroscopy. The percentage conversions of $5CH_3FH_4$ and FH_4 to pterin following acid-iodine oxidation were also determined using the methods described in Chapter 2.

6.3 RESULTS AND DISCUSSION.

Measurement of total biopterins in brain and liver revealed highest levels occurring in the liver (Table 6.1). This

difference is explained by the large biosynthetic capacity of this organ which exceeds that of the brain by a factor of approximately 20 (Table 6.1).

Pterin was not detectable in brain and liver samples prior to acid-iodine oxidation indicating that no endogenous source directly contributes to the measurable pterin following oxidation. This derived pterin was greater in the liver compared with the brain (Table 6.1), due to the larger FH_4 pool (Horne and Wilson, 1986). 7,8-dihydropterin arising from oxidation of the large BH_4 pool may also contribute to this pterin.

N_2O in *vitro* did not oxidize the pterin ring system of BH_2 or $5CH_3FH_4$ at pH 1.6 and 7.0 and so is unlikely to have a direct oxidative effect on reduced pterins either *in vivo* or during acid-iodine oxidation of tissue samples.

Exposure to a 1:1 $N_2O:O_2$ mixture for 24 hours had no effect, compared to appropriate controls, on either total biopterin levels or DHPR activity in the brain and liver (Tables 6.2 - 6.4). Derived pterin levels in both organs were significantly reduced (Tables 6.2 and 6.4). Repeating with a higher concentration of N_2O (4:1 $N_2O:O_2$) resulted in a similar pattern of results i.e only the derived pterin was significantly depressed in both liver and brain (Tables 6.5 - 6.7). No effect was observed on the BH_4 biosynthetic capacity of the brain (Table 6.8).

The decrease in liver derived pterin by N_2O exhibited a dose response effect with 21 and 42 percent reduction, compared to controls, at the lower and higher levels respectively (Tables 6.4 and 6.7). No such effect was observed in the brain, but at the lower level of exposure this organ appeared to be more susceptible to the action of N_2O with 54 percent reduction occurring compared to 21 percent in the liver (Tables 6.2 and 6.4). This difference may be explained by the higher absorption coefficient (carrying capacity of organ blood supply per round / absorptive capacity of whole organ) of the brain for N_2O, which exceeds that of the liver over 5 times (Harris, 1951).

Short term, i.e 24 hours, N_2O exposure results in decreased FH_4 and increased $5CH_3FH_4$ tissue levels (mono and polyglutamates) (Lumb et al., 1981; Wilson and Horne, 1986). $5CH_3FH_4$ is poorly converted to pterin by acid-iodine oxidation, whereas FH_4 gives a 98 per cent yield (Table 6.9). The changes in derived pterin observed in this study therefore reflect changes in the FH_4 pool arising from N_2O exposure and are comparable with the percentage decrease in brain and liver total FH_4 reported by Wilson and Horne (1986). (Brain decreased by 43 and liver by 42 percent following 18 hours N_2O (4:1 with oxygen) exposure .

In vitro N_2O is reported to inhibit BH_4 biosynthesis and it is suggested that demethylation of $5CH_3FH_4$ by methionine synthetase is a necessary requirement for the biosynthesis of BH_4 (Hamon *et al.*, 1986). This observation implies that

methionine or derivatives of methionine are involved in BH_4 biosynthesis, rather than FH_4, which inhibits GTP cyclohydrolase (Kapatos and Kaufman, 1983; Bellahsene *et al.*, 1984). *In vivo* inactivation of methionine synthetase, for 24 hours, had no effect on the measured parameters of BH_4 metabolism (Tables 6.2 - 6.8). This may be due to the fact that the methionine requirement indicated by Hamon *et al.* (1986) can be met from betaine methyl transferase (EC. 2.1.1.5) activity which is induced following methionine synthetase inhibition (Chanarin, 1981; Chanarin *et al.*, 1985).

Exposure to a 1:4 $He:O_2$ mixture or 100 per cent O_2 for 24 hours had no effect on brain DHPR activity, total biopterins or derived pterin levels (Tables 6.10 - 6.13). Brain levels of O_2 rise under such conditions (Jamieson and Van Den Brenk, 1963) suggesting, because of the susceptibility of BH_4 to oxidation by O_2, either tissue cofactor levels are maintained by *de novo* biosynthesis or oxidative catabolism is prevented by the action of antioxidants such as glutathione (GSH). GSH inhibits BH_4 autoxidation *in vitro* (Chapter 3) and oxidation by O_2 derived radicals such as the hydroxyl radical ($^{\cdot}OH$) (Chapter 4).

Table 6.1. Tetrahydrobiopterin metabolism in brain and liver of the male 150g Wistar rat.

	Mean	SD	n
Brain:			
Total biopterins. (ng g^{-1} wet weight)	84	11	16
Derived pterin. (ng g^{-1} wet weight)	56	10	16
DHPR. (nmoles NADH oxidized min^{-1} mg^{-1} protein)	210	19	16
Biosynthesis. (ng BH_4 synthesized h^{-1} mg^{-1} protein)	0.95	0.19	5
Liver:			
Total biopterins. (ng g^{-1} wet weight)	1480	400	16
Derived pterin. (ng g^{-1} wet weight)	520	130	16
DHPR. (nmoles NADH oxidized min^{-1} mg^{-1} protein)	302	38	5
Biosynthesis. (nmoles BH_4 synthesized h^{-1} mg^{-1} protein)	20.10	5.70	6

Food and water were available *ad. lib.*

Table 6.2. Effect of nitrous oxide (50%) on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	75	10	6	
Nitrous oxide	78	17	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	42	2	6	
Nitrous oxide	19	2	6	<0.001

Table 6.3. Effect of nitrous oxide (50%) on brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	263	66	6	
Nitrous oxide	215	35	6	NS

150g male Wistar rats were used in each case.
Tests were exposed to 1:1 N_2O:O_2 mixture for 24 hours.
Controls were exposed to 1:1 He:O_2 mixture for 24 hours.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 6.4. Effect of nitrous oxide (50%) on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.15	0.09	6	
Nitrous oxide	1.21	0.21	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.44	0.07	6	
Nitrous oxide	0.34	0.08	6	<0.05

150g male Wistar rats were used in each case.
Tests were exposed to 1:1 N_2O:O_2 mixture for 24 hours.
Controls were exposed to 1:1 He:O_2 mixture for 24 hours.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 6.5. Effect of nitrous oxide (80%) on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	83	10	6	
Nitrous oxide	77	7	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	60	5	6	
Nitrous oxide	34	4	6	<0.001

Table 6.6. Effect of nitrous oxide (80%) on brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	207	87	6	
Nitrous oxide	173	35	6	NS

150g male Wistar rats were used in each case.
Tests were exposed to 4:1 $N_2O:O_2$ mixture for 24 hours.
Controls were exposed to 4:1 $He:O_2$ mixture for 24 hours.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 6.7. Effect of nitrous oxide (80%) on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.22	0.43	6	
Nitrous oxide	1.17	0.11	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.66	0.13	6	
Nitrous oxide	0.38	0.05	6	<0.001

150g male Wistar rats were used in each case.
Tests were exposed to 4:1 $N_2O:O_2$ mixture for 24 hours.
Controls were exposed to 4:1 $He:O_2$ mixture for 24 hours.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 6.8. Effect of nitrous oxide (80%) on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein)*.

	Mean	SD	n	Probability
Control	0.70	0.27	6	
Nitrous oxide	0.60	0.18	5	NS

150g male Wistar rats were used in each case.
Tests were exposed to 4:1 $N_2O:O_2$ mixture for 24 hours.
Controls were exposed to 4:1 $He:O_2$ mixture for 24 hours.
NS = Not significantly different.
Food and water were available *ad. lib.*
* Assayed by CGB Hamon.

Table 6.9. Percentage conversion of tetrahydrofolate and 5-methyl-tetrahydrofolate to pterin following 1 hour acid - iodine oxidation.

Folate	% Pterin
Tetrahydrofolate*	98.0
5-methyl-tetrahydrofolate*	0.3

* 20uM in 0.1M HCl.

Table 6.10. Effect of oxygen (80%) on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	83	7	6	
Oxygen	81	15	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	52	5	6	
Oxygen	53	6	6	NS

Table 6.11. Effect of oxygen (80%) on brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	124	32	6	
Oxygen	121	28	6	NS

150g male Wistar rats were used in each case.
Tests were exposed to 1:4 $He:O_2$ mixture for 24 hours.
Controls were air breathers.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 6.12. Effect of oxygen (100%) on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	78	8	6	
Oxygen	81	12	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	53	7	6	
Oxygen	48	6	6	NS

Table 6.13. Effect of oxygen (100%) on brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	179	21	6	
Oxygen	176	14	6	NS

150g male Wistar rats were used in each case.
Tests were exposed to 100 % O_2 for 24 hours.
Controls were air breathers.
NS = Not significantly different.
Food and water were available *ad. lib.*

CHAPTER SEVEN

EFFECT OF STARVATION AND CHANGES IN TISSUE GLUTATHIONE LEVELS ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

CHAPTER SEVEN.

EFFECT OF STARVATION AND CHANGES IN TISSUE GLUTATHIONE LEVELS ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT.

7.1 INTRODUCTION.

The availability of the neurotransmitter precursors, tyrosine and tryptophan within the central nervous system (CNS) influence the rate of synthesis and release of dopamine, noradrenaline and serotonin (Wurtman, 1982).

Tyrosine, tryptophan and other large neutral amino acids (LNAAs) are transported across the blood brain barrier (BBB) by the same transport mechanism. Consequently brain levels of these amino acids depend on the concentration ratio of the individual amino acid to the other LNAAs in the plasma (Fernstorm and Wurtman, 1972).

In rats, 24 hour starvation significantly reduces plasma tyrosine but not phenylalanine levels (Arola *et al.*, 1984), suggesting diminished hepatic hydroxylation of phenylalanine. Plasma levels of the essential amino acid, tryptophan are also significantly lower (Arola *et al.*, 1984). The decreased availability of tyrosine is implicated as a factor contributing to the reduced turnover and synthesis of noradrenaline (NA) which accompanies acute starvation (Scweiger *et al.*, 1985).

Reduction of brain and liver dihydropteridine reductase (DHPR) activity is also reported in acute starvation (Cutler, 1986). Possibly, loss of tetrahydrobiopterin (BH_4), due to diminished salvage, is a contributing factor to the impaired brain NA synthesis and turnover in starvation.

24 hour starvation in rats also results in decreased liver catalase and glutathione (GSH) levels, and an increased formation of oxygen radicals in the brain and liver (Tateishi et al., 1974; Isaacs and Binkley, 1977; Stankiewicz, 1987). Liver GSH is suggested to act as a cysteine reservoir during periods of inadequate cysteine and cystine intake (Cho et al., 1981). On refeeding GSH levels increase directly with the amount of dietary cysteine (Tateishi et al., 1977).

The activities of the NADPH producing enzymes, glucose-6-phosphate dehydrogenase (EC. 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC. 1.1.1.44) are also significantly reduced in the liver by starvation (Martins *et al.*, 1985). NADPH is required in numerous biosynthetic pathways including BH_4 biosynthesis and for the regeneration of GSH from its oxidized form (Meister, 1981; Milstien and Kaufman, 1986).

In vitro, physiological concentrations of GSH inhibit BH_4 autoxidation and oxidation by the hydroxyl radical ($^{\bullet}OH$) (Chapters 3 and 4). The large decrease, *in vivo*, of tissue GSH and increased oxygen radical production occurring in

acute starvation may therefore disrupt BH_4 metabolism by enhancing catabolism. Consequently the effect of starvation on rat BH_4 metabolism was studied.

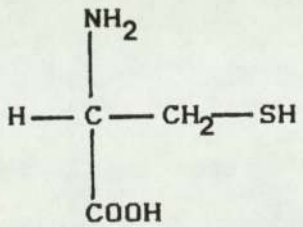
In order to assess the *in vivo* role of GSH, with regards BH_4 metabolism, the effects of *L*-cysteine (i) administration to starving rats and *L*-buthionine sulfoximine (*L*-BSO) (ii) to feeding rats were subsequently investigated. *L*-BSO is a specific inhibitor of GSH biosynthesis resulting in over 80 per cent depletion of liver GSH in 3 hours (Griffith and Meister, 1979).

Alterations in tissue BH_4 synthesis and salvage are reflected by plasma total biopterins (Leeming *et al.*, 1981). This parameter was therefore monitored.

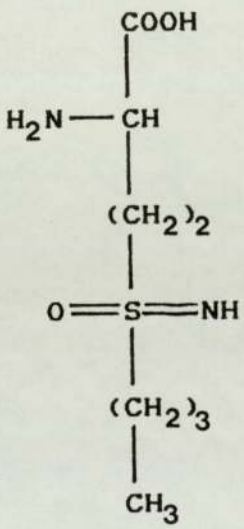
7.2 MATERIALS AND METHODS.

Wistar rats were denied food, but not water, for 24 and 48 hours on grids. Animals receiving *L*-BSO were dosed intraperitoneally and *L*-cysteine orally as described in Chapter 2.

Plasma total biopterins, liver and brain total biopterins (tetrahydro, dihydro and fully oxidized biopterin), derived pterin, biosynthetic capacity and DHPR activity were determined as described in Chapter 2. All test and control samples were assayed together and no between batch comparisons made.



(i) L-Cysteine



(ii) L-Buthionine Sulfoximine

7.3 RESULTS AND DISCUSSION.

7.3.1 STARVATION.

Fasting for 24 and 48 hours significantly decreased brain total biopterins (Table 7.1 and 7.6). Liver total biopterins were significantly reduced by 24 but not 48 hours starvation (Tables 7.4 and 7.9).

Reduction in total biopterins may arise from diminished *de novo* synthesis, salvage or enhanced oxidative degradation of BH_4. The measured biosynthetic capacity of the brain was significantly reduced by 24 but not 48 hours starvation (Tables 7.2 and 7.7).

Lowered brain biosynthetic capacity occurring after 24 hours may reflect reduced synthesis and/or irreversible inhibition of one or more of the enzymes involved in BH_4 biosynthesis. However, care must be taken when interpreting results from this assay since it is sensitive to oxidation due to the unstable nature of BH_4 that is formed (Chapter 3). Furthermore the limited availability of NADPH occurring in starvation (Martins *et al.*, 1985) may restrict BH_4 biosynthesis despite unaffected brain and liver biosynthetic capacities occurring after 48 hours starvation (Tables 7.7 and 7.10).

Brain DHPR was unaffected by 24 hours starvation but reduced by 48, whilst liver activity was unaffected (Tables 7.3, 7.8 and 7.11). Depression in DHPR activity may represent reduced

enzyme synthesis, irreversible inhibition or oxidation of the thiol groups described by Cheema *et al.* (1973) essential for activity. Cutler (1986) describes a change in the molecular form of brain DHPR, accompanying this reduction in activity, from a monomer in the fed state to a dimer in the fasted.

The protein content of the brain and liver supernatants used to measure DHPR and biosynthetic capacity were unaffected by 48 hours starvation (Table 7.13), i.e. changes in enzymatic activity following starvation are not due to alterations in the protein base line.

The transient reduction of liver biopterins by 24 but not 48 hours starvation may indicate increased *de novo* biosynthesis of BH_4, salvage or diminished oxidative degradation of BH_4 due to changes in liver GSH levels. Whilst liver GSH is significantly reduced by 24 hours fasting, levels return to those of feeding controls after 48, despite the continued loss in other tissues. This increase is facilitated by the catabolism of liver protein and conversion of liberated cysteine into GSH (CHO *et al.*, 1981).

Despite changes in brain and liver BH_4 metabolism occurring in starvation no significant changes in plasma total biopterins were observed (Tables 7.5 and 7.12).

Pterin may be derived from the tissue tetrahydrofolate (FH_4) pool (Fukushima and Nixon, 1980) or 7,8-dihydropterin arising from oxidation of BH_4 at physiological pH (Chapter 3).

Derived pterin was significantly reduced in the brain by 24 hours starvation but not 48 (Tables 7.1 and 7.6). Due to the non specific origin of this pterin it is not possible to explain this observation. Liver derived pterin was elevated by 24 and 48 hours starvation (Tables 7.4 and 7.9). This increase may reflect oxidation of BH_4 to 7,8-dihydropterin or an increase in the tissue FH_4 pool due to a change in the partitioning of liver folates.

7.3.2 *L*-CYSTEINE DOSING.

Oral dosing of *L*-cysteine (4 mmoles kg^{-1}, twice a day for two days) to starving rats significantly increased brain and liver total biopterins (Table 7.14 and 7.17). Brain DHPR and biosynthetic capacity were unaffected by this treatment (Tables 7.15 and 7.16), but in the liver both these parameters were significantly increased (Tables 7.18 and 7.19).

In vitro, *L*-cysteine and GSH inhibit liver DHPR activity, presumably by removing hydrogen peroxide required for assay (Tables 7.27 and 7.28). GSH also increases the measured protein concentration of the liver supernatant (Table 7.29). The increase in liver DHPR activity following *L*-cysteine administration is therefore not an assay effect and may represent increased enzyme synthesis or protection of the thiol groups of this enzyme from oxidation.

The measured increase in liver biosynthetic capacity following *L*-cysteine administration may represent increased

enzymatic activity or an *in vitro* antioxidant effect. Measurable biosynthetic activity increases directly with supernatant GSH concentration (Cowburn, 1986), due to the antioxidant properties of GSH towards BH_4 (Chapter 3).

L-cysteine administration, to starving rats, will increase tissue levels of this amino acid and GSH. The elevation of brain biopterins reported here may therefore arise from decreased BH_4 catabolism, and in the liver by the additional increase in salvage and possibly synthesis pathways.

Repeating with a higher dose of *L*-cysteine (7 mmoles Kg^{-1}, twice a day for two days) raised but not significantly brain and liver total biopterins (Tables 7.20 and 7.23). The measurable effectiveness of *L*-cysteine is likely to depend on the magnitude of tissue GSH loss and hence oxidative stress induced in the starved controls. Reduction in the rate of GSH depletion in an animal or a number of animals within this group may result in a loss of statistical significance. Coprophagy in rats has been shown to act as a cystine source (Kwong and Barnes, 1975). Although rats were housed on wire grids this practise could not always be prevented.

Liver DHPR and biosynthetic capacity were again increased by *L*-cysteine administration (Tables 7.24 and 7.25). Brain activities were unaffected by this treatment (Tables 7.21 and 7.22).

L-cysteine administration significantly increased brain

derived pterin at the lower but not higher dose (Table 7.14 and 7.20). Liver derived pterin was elevated but only significantly by the higher *L*-cysteine dose (Tables 7.17 and 7.23). The origin of this increase in pterin is not clear but may reflect decreased FH_4 catabolism. Folate catabolism occurs by chemical oxidation (Al-Haddad *et al.*, 1986) and administration of antioxidants *in vivo* decreases this catabolism (Stankiewicz, 1987). *L*-cysteine administration to starving rats may restrict FH_4 catabolism and so increase the amount of pterin formed by acid-iodine oxidation.

Administration of *L*-cysteine did not significantly affect measured plasma total biopterins (Table 7.26).

7.3.3. *L*-BSO ADMINISTRATION.

Acute dosing of *L*-BSO to feeding rats (4 mmoles kg^{-1} 4 times over a 12.5 hour period) had no effect on brain total biopterins but levels in the liver were significantly reduced (Tables 7.30 and 7.33). This organ difference is explained by considering the mode of action of *L*-BSO. *L*-BSO depletes tissue GSH by inhibiting biosynthesis at the level of γ-glutamylcysteine synthetase (EC. 6.3.2.2) (Meister, 1978). The rate of GSH depletion in a particular tissue will therefore be related to the rate of turnover. The half life of GSH is 4 hours in the liver and 70 in the brain (Douglas and Mortensen, 1956). Over the time period of this study it is unlikely that significant depletion of brain GSH occurred. Furthermore *L*-BSO is poorly transported across the BBB (Griffith and Meister, 1979).

Reduction in liver total biopterins following *L*-BSO administration may represent increased oxidative catabolism of BH_4 resulting from the oxidizing environment created by GSH depletion. Derived pterin, DHPR and biosynthetic capacity were not affected in the brain or liver of animals receiving an acute dose of *L*-BSO (Tables 7.30 - 7.35).

Chronic administration of *L*-BSO to developing rats (4m moles Kg^{-1} day^{-1} for 17 days) had no effect on brain total biopterins, derived pterin or biosynthetic capacity (Tables 7.36 and 7.37). DHPR activity was significantly increased (Table 7.38). In the liver total biopterins were unaffected but derived pterin, DHPR and biosynthetic capacity were significantly elevated (Tables 7.39 - 7.41).

Elevation in DHPR and biosynthetic capacity suggests enzyme activation or increased enzyme synthesis, and is not attributable to alterations in the protein content of the liver and brain supernatants used for assay (Table 7.43). A measurable increase in DHPR activity may also arise as a result of an assay effect, due to reduction of tissue GSH levels. GSH inhibits DHPR activity *in vitro* (Table 7.28). However, acute GSH depletion had no effect on liver DHPR activity (Table 7.35). Maintenance of total biopterins in this case appears to be facilitated by increased salvage and *de novo* synthesis of BH_4 in the liver and increased salvage in the brain.

The origin of the increased liver derived pterin is unclear but may reflect oxidative catabolism of BH_4 to 7,8-dihydropterin, or a redistribution of liver folates resulting in increased FH_4.

Plasma biopterins were unaffected by chronic *L*-BSO exposure, despite changes in brain and liver BH_4 metabolism (Table 7.42).

The final body weights of animals receiving *L*-BSO chronically were significantly reduced (Table 7.44). This may be due to the reduction in daily food, but not water, consumption (Tables 7.45 and 7.46) or indicates a requirement for GSH in development. Reduction in the growth rate of mice treated with *L*-BSO has been reported (Calvin *et al.*, 1986).

Reduction in dietary intake may be a contributing factor to the observed changes in BH_4 metabolism occurring in animals receiving *L*-BSO for 17 days. Further work with this compound should therefore ensure the matching of food consumption in test and control groups.

7.3.4. CONCLUSIONS.

Acute starvation decreases brain and transiently liver total biopterins. Reduction of brain total biopterins by starvation is likely to be a contributing factor to the decreased synthesis and turnover of noradrenaline occurring in this condition. Depression of liver total biopterin levels after

24 hours starvation may be part responsible for the decreased plasma tyrosine levels accompanying this period of starvation.

GSH may have a role in maintaining tissue BH_4 levels. Depression of brain and liver biopterins by acute starvation may therefore arise from oxidative catabolism of BH_4 due to GSH depletion and increased oxygen radical formation. Limited availability of NADPH and possible reduction in DHPR and biosynthetic capacity will also contribute to this loss.

Alterations in tissue BH_4 catabolism are not reflected by changes in rat plasma biopterins. This observation questions the ability of this parameter to predict disruptions in tissue BH_4 metabolism.

Table 7.1. Effect of 24 hours starvation on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	92	6	4	
Starved	77	2	5	<0.002

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	49	4	5	
Starved	42	5	5	<0.05

Table 7.2. Effect of 24 hours starvation on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Fed	0.21	0.09	5	
Starved	0.06	0.06	4	<0.05

150g male Wistar rats were used in each case.
Animals were starved on grids.
Food was removed at 4.00pm.
Water was available *ad. lib.*

Table 7.3. Effect of 24 hours starvation on rat brain DHPR activity (nmoles NADH oxidized $min^{-1} mg^{-1}$ protein).

	Mean	SD	n	Probability
Fed	210	19	6	
Starved	201	19	6	NS

Table 7.4. Effect of 24 hours starvation on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	2.00	0.13	4	
Starved	1.69	0.21	5	<0.05

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	0.65	0.11	4	
Starved	0.90	0.15	5	<0.05

150g male Wistar rats were used in each case.
Animals were starved on grids.
Food was removed 4.00pm
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.5. Effect of 24 hours starvation on rat plasma total biopterins (ug litre^{-1}).

	Mean	SD	n	Probability
Fed	27.9	8.6	5	
Starved	32.5	15.7	5	NS

Table 7.6. Effect of 48 hours starvation on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	73	6	6	
Starved	65	6	6	<0.05

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	55	6	6	
Starved	59	10	6	NS

150g male Wistar rats used in each case.
Animals were starved on grids.
Food was removed on day 1 at 4.00pm.
Water was available *ad. lib.*

Table 7.7. Effect of 48 hours starvation on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Fed	0.84	0.31	5	
Starved	0.69	0.26	4	NS

Table 7.8. Effect of 48 hours starvation on rat brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein)*.

	Mean	SD	n	Probability
Fed	172	12	6	
Starved	142	24	6	<0.05

Table 7.9. Effect of 48 hours starvation on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	1.51	0.19	6	
Starved	1.46	0.24	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Fed	0.54	0.08	6	
Starved	1.02	0.07	6	<0.001

150g male Wistar rats used in each case.
Animals were starved on grids.
Food was removed on day 1 at 4.00pm.
NS = Not significantly different.
Water was available *ad. lib.*
* Assayed by J. Cox.

Table 7.10. Effect of 48 hours starvation on rat liver BH_4 biosynthesis (ng BH_4 synthesized $h^{-1} mg^{-1}$ protein)

	Mean	SD	n	Probability
Fed	15.75	4.48	6	
Starved	14.38	5.52	6	NS

Table 7.11. Effect of 48 hours starvation on rat liver DHPR activity (nmoles NADH oxidized $min^{-1} mg^{-1}$ protein)*.

	Mean	SD	n	Probability
Fed	586	88	6	
Starved	542	47	6	NS

Table 7.12. Effect of 48 hours starvation on rat plasma total biopterins (ug $litre^{-1}$).

	Mean	SD	n	Probability
Fed	33.5	12.6	5	
Starved	24.6	6.0	6	NS

Table 7.13. Effect of 48 hours starvation on protein content of brain and liver supernatants (mg^{-1} ml)

	Mean	SD	n	Probability
Brain:-				
Fed	6.58	2.75	6	
Starved	5.97	2.43	6	NS
Liver:-				
Fed	19.26	5.14	6	
Starved	18.10	6.58	6	NS

150g male Wistar rats were used in each case.
Animals were starved on grids.
Food was removed at 4.00pm
NS = Not significantly different.
Water was available *ad. lib.*
* Assayed by J. Cox.

Table 7.14. Effect of *L*-cysteine (4mmoles Kg^{-1}) administration, during 48 hours starvation, on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	73	13	6	
L-cysteine	89	11	6	<0.05

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	27	3	6	
L-cysteine	32	2	6	<0.01

Table 7.15. Effect of *L*-cysteine (4mmoles Kg^{-1}) admimistration, during 48 hours starvation, on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	0.33	0.15	6	
L-cysteine	0.42	0.16	6	NS

150g male Wistar rats were used in each case.
Animals were starved on grids.
L-cysteine administered 4mmoles Kg^{-1} orally.
Vehicle; distilled water.
Dosed 9.30am and 9.30pm for 2 days
Controls received 0.3mls distilled water orally.
Food was removed at 4.00pm on day 1.
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.16. Effect of *L*-cysteine (4mmoles Kg^{-1}) administration, during 48 hours starvation, on rat brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	150	17	6	
L-cysteine	162	17	6	NS

Table 7.17. Effect of *L*-cysteine (4mmoles Kg^{-1}) administration, during 48 hours starvation, on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.43	0.14	6	
L-cysteine	1.63	0.11	6	<0.025

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.65	0.06	6	
L-cysteine	0.80	0.21	6	NS

150g male Wistar rats were used in each case.
Animals were starved on grids.
L-cysteine administered 4mmoles Kg^{-1} orally.
Vehicle; distilled water.
Dosed 9.30am and 9.30pm for 2 days
Controls received 0.3mls distilled water orally.
Food was removed at 4.00pm on day 1.
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.18. Effect of *L*-cysteine (4mmoles Kg-1) admimistration, during 48 hours starvation, on rat liver BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	4.68	2.03	6	
L-cysteine	8.42	1.32	6	<0.01

Table 7.19. Effect of *L*-cysteine (4mmoles Kg^{-1}) administration, during 48 hours starvation, on rat liver DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	443	64	6	
L-cysteine	535	74	6	<0.05

150g male Wistar rats used in each case
Animals were starved on grids.
L-cysteine administered 4mmoles Kg^{-1} orally.
Vehicle; distilled water.
Dosed 9.30am and 9.30pm for 2 days
Controls received 0.3mls distilled water orally.
Food was removed at 4.00pm on day 1.
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.20. Effect of *L*-cysteine (7mmoles Kg^{-1}) administration, during 48 hours starvation, on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	83	10	5	
L-cysteine	86	13	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	50	8	5	
L-cysteine	55	13	6	NS

Table 7.21. Effect of *L*-cysteine (7mmoles Kg-1) admimistration, during 48 hours starvation, on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	1.18	0.17	4	
L-cysteine	1.31	0.23	5	NS

150g male Wistar rats were used in each case.
Animals were starved on grids.
L-cysteine administered 7mmoles Kg^{-1} orally.
Vehicle; distilled water.
Dosed 9.30am and 9.30pm for 2 days
Controls received 0.3mls distilled water orally.
Food was removed at 4.00pm on day 1.
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.22. Effect of *L*-cysteine (7mmoles Kg^{-1}) administration, during 48 hours starvation, on rat brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	146	28	5	
L-cysteine	153	30	5	NS

Table 7.23. Effect of *L*-cysteine (7mmoles Kg^{-1}) administration, during 48 hours starvation, on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.49	0.23	6	
L-cysteine	1.64	0.33	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.64	0.15	6	
L-cysteine	0.87	0.09	6	<0.01

150g male Wistar rats were used in each case.
Animals were starved on grids.
L-cysteine administered 7mmoles Kg^{-1} orally.
Vehicle; distilled water.
Dosed 9.30am and 9.30pm for 2 days
Controls received 0.3mls distilled water orally.
Food was removed at 4.00pm on day 1.
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.24. Effect of *L*-cysteine (7mmoles Kg^{-1}) admimistration, during 48 hours starvation, on rat liver BH_4 biosynthesis (ng BH_4 synthesized $h^{-1} mg^{-1}$ protein).

	Mean	SD	n	Probability
Control	7.14	1.70	6	
L-cysteine	11.53	3.23	6	<0.05

Table 7.25. Effect of *L*-cysteine (7mmoles Kg^{-1}) administration, during 48 hours starvation, on rat liver DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	308	78	5	
L-cysteine	451	86	5	<0.05

Table 7.26. Effect of *L*-cysteine (7mmoles Kg^{-1}) administration, during 48 hours starvation, on rat plasma total biopterins (ug $litre^{-1}$).

	Mean	SD	n	Probability
Control	28.8	9.3	6	
L-cysteine	36.2	11.2	6	NS

150g male Wistar rats used in each case
Animals were starved on grids.
L-cysteine administered 7mmoles kg^{-1} orally.
Vehicle; distilled water.
Dosed 9.30am and 9.30pm for 2 days
Controls received 0.3mls distilled water orally.
Food was removed at 4.00pm on day 1.
NS = Not significantly different.
Water was available *ad. lib.*

Table 7.27. Effect of *L*-cysteine on rat liver DHPR activity *in vitro* (nmoles NADH oxidized min^{-1} ml^{-1} enzyme).

	Mean	SD	n	Probability
Control	611	28	3	
L-cysteine	515	28	3	<0.02

L-cysteine final concentration = 10^{-3}M

Table 7.28. Effect of glutathione (reduced form) on rat liver DHPR activity *in vitro* (nmoles NADH oxidized min^{-1} ml^{-1} enzyme).

	Mean	SD	n	Probability
Control	482	97	3	
Glutathione	209	28	3	<0.01

Glutathione final concentration = 10^{-3}M

Table 7.29. Effect of *L*-cysteine and glutathione on the mesurable protein of liver supernatant, judged by biuret (mg protein ml^{-1}).

	Mean	SD	n
Control	1.15	0.18	2
L-Cysteine	1.13	0.04	2
Glutathione	3.10	0.10	2

L-cysteine final concentration = 2×10^{-4}M
Glutathione final concentration = 2×10^{-4}M

Table 7.30. Effect of *L*-buthionine sulfoximine (*L*-BSO) on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	96	8	6	
L-BSO	96	10	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	38	2	6	
L-BSO	41	4	6	NS

Table 7.31. Effect of *L*-buthionine sulfoximine (*L*-BSO) on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	1.94	1.16	6	
L-BSO	1.72	0.19	6	NS

90g male Wistar rats were used in each case.
L-BSO administered 4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3mls distilled water I.P.
Dosed 4 times over a 12.5 hour period.
Animals sacrificed 4.5 hours after last dose.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 7.32. Effect of *L*-buthionine sulfoximine (*L*-BSO) on rat brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	96	14	5	
L-BSO	91	19	5	NS

Table 7.33. Effect of L-buthionine sulfoximine (*L*-BSO) on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.70	0.12	5	
L-BSO	1.46	0.08	5	<0.01

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.37	0.07	5	
L-BSO	0.35	0.11	5	NS

90g male Wistar rats were used in each case.
L-BSO administered 4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3mls distilled water I.P.
Dosed 4 times over a 12.5 hour period.
Animals sacrificed 4.5 hours after last dose.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 7.34. Effect of *L*-buthionine sulfoximine (*L*-BSO) on rat liver BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	8.08	3.55	5	
L-BSO	7.00	0.75	5	NS

Table 7.35. Effect of *L*-buthionine sulfoximine (*L*-BSO) on rat liver DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	350	58	5	
L-BSO	340	55	5	NS

90g male Wistar rats were used in each case.
L-BSO administered 4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3mls distilled water I.P.
Dosed 4 times over a 12.5 hour period.
Animals sacrificed 4.5 hours after last dose.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 7.36. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat brain total biopterins and derived pterin.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	95	8	5	
L-BSO	90	5	5	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	28	3	5	
L-BSO	28	3	5	NS

Table 7.37. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat brain BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	2.52	0.94	5	
L-BSO	2.45	0.96	5	NS

Male Wistar rats were used in each case.
Initial weight 40g.
L-BSO administered in drinking water (16mM).
Each rat received approx. 4mmoles kg^{-1} day^{-1} for 17 days.
Controls received drinking water.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 7.38. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat brain DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	144	14	5	
L-BSO	191	28	5	<0.01

Table 7.39. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat liver total biopterins and derived pterin.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.99	0.20	5	
L-BSO	1.87	0.15	5	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.70	0.12	5	
L-BSO	0.93	0.14	5	<0.05

Male Wistar rats were used in each case.
Initial weight 40g.
L-BSO administered in drinking water (16mM).
Each rat received approx. 4mmoles kg^{-1} day^{-1} for 17 days.
Controls received drinking water.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 7.40. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat liver BH_4 biosynthesis (ng BH_4 synthesized h^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	8.47	1.25	5	
L-BSO	13.93	2.54	5	<0.01

Table 7.41. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat liver DHPR activity (nmoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	302	38	5	
L-BSO	393	62	5	<0.05

Table 7.42. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on rat plasma total biopterin levels (ug $litre^{-1}$).

	Mean	SD	n	Probability
Control	58.5	25.4	5	
L-BSO	35.7	5.9	4	NS

Male Wistar rats were used in each case.
Initial weight 40g.
L-BSO administered in drinking water (16mM).
Each rat received approx. 4mmoles kg^{-1} day^{-1} for 17 days.
Controls received drinking water.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 7.43. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on supernatant protein levels (mg ml^{-1}).

	Mean	SD	n	Probability
Brain:-				
Control	9.68	2.63	5	
L-BSO	8.89	2.59	5	NS
Liver:-				
Control	15.75	3.16	5	
L-BSO	15.08	3.56	5	NS

Table 7.44. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on final body weight (g).

	Mean	SD	n	Probability
Control	157	8	5	
L-BSO	105	18	5	<0.001

Table 7.45. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on daily food consumption, estimated over final three days of experiment (g kg^{-1}).

	Mean	SD	n	Probability
Control	140	7	5	
L-BSO	117	20	5	<0.05

Table 7.46. Effect of chronic dosing of *L*-buthionine sulfoximine (*L*-BSO) on daily water consumption, estimated over final three days of experiment (ml kg^{-1}).

	Mean	SD	n	Probability
Control	229	11	5	
L-BSO	233	40	5	NS

Male Wistar rats were used in each case.
Initial weight 40g.
L-BSO administered in drinking water (16mM).
Each rat received approx. 4mmoles kg^{-1} day^{-1} for 17 days.
Controls received drinking water.
NS = Not significantly different.
Food and water were available *ad. lib.*

CHAPTER EIGHT

EFFECT OF PHENELZINE ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

CHAPTER EIGHT.

EFFECT OF PHENELZINE ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

8.1 INTRODUCTION.

Monoamine oxidase (MAO) (EC. 1.4.3.4) catalyses the oxidative deamination of biologically active amines according to the general equation (Meyers *et al.*, 1980):-

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + NH_3 + H_2O_2$$

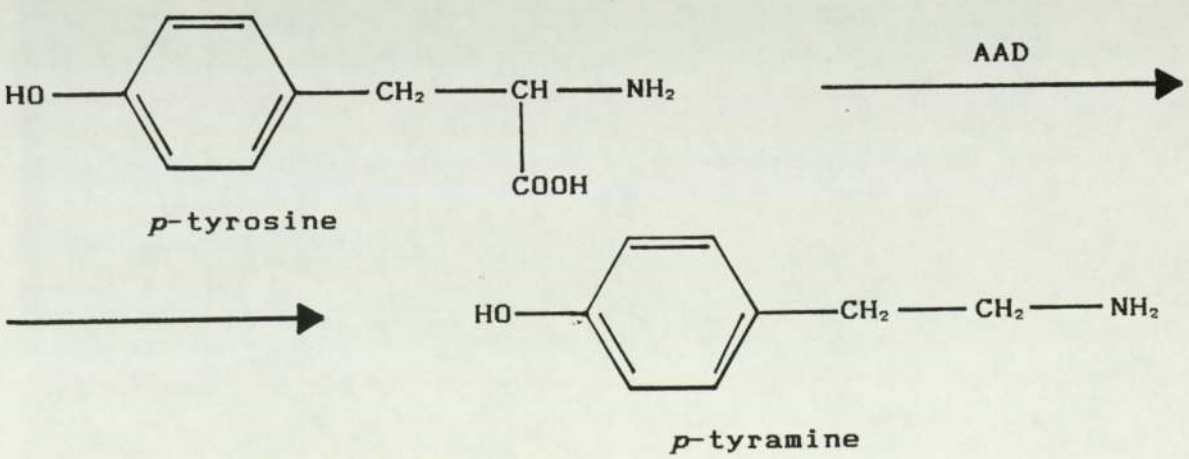
MAO has been detected in a number of tissues including the brain. Within the cell MAO is located predominantly on the outer mitochondrial membrane (Mayanil and Baquer, 1984).

MAO exists in two forms, MAO A and MAO B, classified according to substrate and inhibitor selectivities (Knoll, 1978). MAO A preferentially deaminates serotonin, noradrenaline and in rodents dopamine, whilst β-phenylethylamine, benzylamine and dopamine (in primates) are among the amines deaminated by MAO B. A number of amines e.g. *p*-tyramine are deaminated equally by both forms of the enzyme (Murphy *et al.*, 1981). The proportion of MAO present in each form is dependent on the tissue and species (Garrick *et al.*, 1979). MAO A is selectively inhibited by clorgyline, and MAO B by deprenyl, whilst phenelzine is a non-selective MAO

inhibitor (MAOI) (Murphy *et al.*, 1981). Inhibition of MAO has been used in the treatment of clinical depression and a number of double blind studies suggest MAO A inhibition is responsible for the antidepressant action (Raft *et al.*, 1979; Mendis *et al.*, 1981; Murphy *et al.*, 1981).

Clinical depression is suggested to arise from decreased availability of monoamine neurotransmitters, within the central nervous system (CNS), and the antidepressant effect of MAOIs results from increased catecholamine and serotonin levels (Knoll, 1978; Van Praag, 1982).

P-tyramine and other trace amines are inactivated by MAO. Inhibition of this enzyme therefore results in an increase in brain *p*-tyramine levels (Philips and Boulton, 1979). The principal pathway for *p*-tyramine synthesis is decarboxylation of *p*-tyrosine by aromatic amino acid decarboxylase (AAD) (EC. 4.1.1.28) (Dyck *et al.*, 1983):-



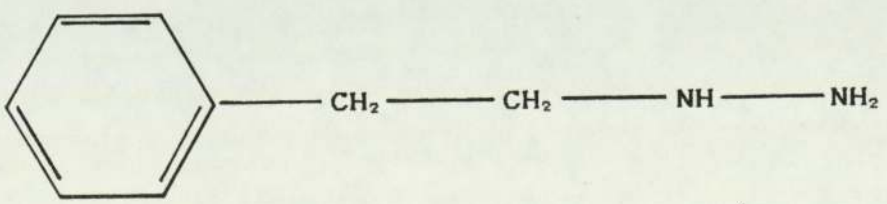
p-tyrosine

p-tyramine

As well as irreversibly inhibiting MAO, phenelzine reversibly inhibits liver tyrosine amino transferase (TAT) (EC. 2.6.1.5), the major hepatic tyrosine catabolizing enzyme, and

brain AAD. However, compared to other MAOIs, phenelzine administration causes a greater accumulation of brain *p*-tyramine (Dyck and Dewar, 1986). Inhibition of TAT, following administration of phenelzine, increases the amount of *p*-tyrosine transported into the central nervous system. Initially *p*-tyrosine decarboxylation is prevented due to AAD inhibition, but as phenelzine levels decrease in the brain only MAO remains inhibited resulting in *p*-tyramine accumulation (Dyck and Dewar, 1986).

In vitro, *p*-tyramine has been shown to be a non-competitive inhibitor of dihydropteridine reductase (DHPR) having a Ki of 50uM (Shen, 1984). Possibly, *in vivo*, an increase in *p*-tyramine levels may deplete tissue tetrahydrobiopterin (BH_4) due to failure of the salvage pathway. Increased plasma and urine *p*-tyramine levels occur in tyrosinaemias, which are accompanied by mental retardation (Goldsmith, 1983). Consequently the effect of phenelzine administration on rat BH_4 metabolism was investigated. Phenelzine was administered at a dose shown by Dyck and Dewar (1986) to significantly elevate brain *p*-tyramine levels.



Phenelzine

Because of the structural similarity of *p*-tyramine to catecholamines, which are inhibitors of sepiapterin reductase (Katoh et al., 1983), the effect of this amine on sepiapterin reductase was investigated, *in vitro*.

8.2 MATERIALS AND METHODS.

Male Wistar rats were dosed with phenelzine and / or *p*-tyrosine by intra peritoneal injection as described in Chapter 2. Starved rat were denied food and placed on grids for the duration of the experiment. Brain and liver total biopterins, derived pterin, biosynthetic capacity, sepiapterin and DHPR activity, and plasma total biopterins were determined as described in Chapter 2.

8.3 RESULTS.

Administration of phenelzine (0.4mmoles kg^{-1}) to fed rats at 3.00pm. decreased brain total biopterins on assay 20 hours later, but had no effect on DHPR activity (Tables 8.1 and 8.2). Phenelzine dosing at 10.00am. reduced brain total biopterins 12 but not 6, 24, or 48 hours after dosing. Brain derived pterin was significantly elevated at all time intervals except 24 hours after phenelzine administration (Table 8.3; Fig. 8.1).

Dosing of phenelzine at 10.30pm. had no effect on brain or liver total biopterins, when assayed 12 hours later, but plasma total biopterins and liver derived pterin were

significantly elevated (Tables 8.4 - 8.6). Phenelzine administration to starved rats resulted in a similar pattern of results (Tables 8.7 - 8.9).

Administration of phenelzine at 9.00am., followed by *p*-tyrosine (1.3mmoles Kg^{-1}) two hours later, to starved rats significantly reduced brain total biopterins, elevated the derived pterin level, but had no effect on DHPR activity 12 hours after the *p*-tyrosine dose (Tables 8.10 and 8.11). Liver total biopterins were unaffected by this treatment but derived pterin was elevated (Table 8.12). Plasma total biopterins were elevated but not significantly (Table 8.13). *p*-tyrosine administration (1.3mmoles kg^{-1}) to starving rats at 9.00am. had no effect on any of these parameters 12 hours later (Tables 8.14 - 8.16).

In vitro *p*-tyramine appeared to have limited effect on the capacity of the liver to synthesize BH_4 (Table 8.17) and is a weak uncompetitive inhibitor of liver sepiapterin reductase (Fig 8.2).

8.4 DISCUSSION.

Increased plasma total biopterins following phenelzine administration to fed and starved animals is consistent with DHPR inhibition (Leeming *et al.*, 1981). Elevated brain and liver derived pterin may reflect accumulation of quinonoid dihydrobiopterin (qBH_2) due to inhibition of the salvage pathway, since this intermediate rearranges to give

predominantly 7,8 dihydropterin at physiological pH (Chapter 3). Acid iodine oxidation of the latter forms pterin (Fukushima and Nixon, 1981). Due to the non specific origin of this pterin, alterations in the tetrahydrofolate pool may also contribute to this increase.

Inhibition of DHPR is likely to arise from the production of *p*-tyramine that follows phenelzine administration (Dyck and Dewar, 1986). No measurable decrease in brain DHPR activity was observed in this study, indicating dilution of this reversible inhibitor during sample preparation.

In feeding animals, reduction of brain total biopterins following phenelzine administration depends on the time of dosing. Loss of tissue biopterins is likely to depend on the degree of DHPR inhibition by *p*-tyramine, as the enzymes of the biosynthetic pathway are poorly inhibited by this amine. Since the rate of synthesis of *p*-tyramine is dependent on the *p*-tyrosine concentration (Dyck and Dewar, 1986), and rats exhibit nocturnal behaviour, the availability of dietary derived *p*-tyrosine is likely to be greatest in the morning. The maximum reduction of brain total biopterins occurring 12 hours after phenelzine dosing at 10.00am corresponds to the peak in tyramine concentration, whereas the level of phenelzine peaks 15 minutes after dosing (Dyck and Dewar, 1986).

Starvation decreases brain total biopterins (Chapter 7.), but phenelzine administration to starving animals had no further

effect on levels despite evidence of DHPR inhibition. Lack of dietary *p*-tyrosine and phenylalanine is likely to limit *p*-tyramine synthesis and hence the magnitude of DHPR inhibition. Increasing *p*-tyramine synthesis by the co-administration of *p*-tyrosine and phenelzine to fasting rats resulted in loss of brain total biopterins and further elevation of brain and liver derived pterin, due to increased DHPR inhibition. In the absence of phenelzine, *p*-tyrosine had no effect on BH_4 metabolism as accumulation of *p*-tyramine and other *p*-tyrosine derived inhibitors of DHPR can not occur.

Liver total biopterins were unaffected by any of the treatments in this study, suggesting replacement of lost cofactor by *de novo* synthesis or minimal DHPR inhibition. Peripherally tissue phenelzine levels drop at a slower rate compared with the CNS, resulting in prolonged inhibition of hepatic TAT and accumulation of *p*-tyrosine (Dyck and Dewar, 1986). Compared to *p*-tyramine, *p*-tyrosine is a poor inhibitor of DHPR (Shen, 1984), however the increased liver derived pterin levels occurring throughout this study may indicate DHPR inhibition.

In conclusion, this study demonstrates that inhibition of DHPR, *in vivo*, by *p*-tyramine leads to eventual loss of brain biopterins. Continued BH_4 loss may therefore occur in tyrosinaemias and contribute to the mental retardation associated with this disorder, due to reduction in catecholamine and serotonin synthesis. Similarly, elevation

of brain *p*-tyramine levels by MAOIs (Philips and Boulton, 1979) may limit the effectiveness of this therapy in treating chronic depression.

Table 8.1. Effect of phenelzine administration on rat brain total biopterins, 20 hours after dosing at 3.00pm.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	93	11	6	
Phenelzine	77	12	6	<0.05

Table 8.2. Effect of phenelzine administration on rat brain DHPR activity (nMoles NADH oxidized min^{-1} mg^{-1} protein), 20 hours after dosing at 3.00pm.

	Mean	SD	n	Probability
Control	167	28	6	
Phenelzine	176	12	6	NS

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 8.3. The effect of time, after dosing with phenelzine, on rat brain total biopterins and derived pterin (dosed 10.00am. on day 1).

Brain total biopterins (ng g^{-1} wet weight).

Time(hours)	Mean	SD	n	Probability
0	71	6	3	
6	64	12	3	NS
12	53	1	3	<0.01
24	65	14	3	NS
48	68	4	3	NS

Brain derived pterin (ng g^{-1} wet weight).

Time(hours)	Mean	SD	n	Probability
0	33	2	3	
6	48	7	3	<0.05
12	45	3	3	<0.01
24	42	9	3	NS
48	41	1	3	<0.01

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
Killed at times indicated.
NS = Not significantly different, compared to time 0.
Food and water were available *ad. lib.*

Table 8.4. Effect of phenelzine administration on rat brain total biopterins (ng g^{-1} wet weight). Animals killed 12 hours after dosing at 10.30pm.

	Mean	SD	n	Probability
Control	74	7	6	
Phenelzine	77	13	6	NS

Table 8.5. Effect of phenelzine administration on rat plasma total biopterins (ug $litre^{-1}$). Animals killed 12 hours after dosing at 10.30pm.

	Mean	SD	n	Probability
Control	25.9	2.4	6	
Phenelzine	33.9	7.4	6	<0.05

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 8.6. Effect of phenelzine administration on rat liver total biopterins and derived pterin. Animals killed 12 hours after dosing at 10.30pm.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.71	0.13	6	
Phenelzine	1.62	0.05	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.58	0.09	6	
Phenelzine	0.77	0.10	6	<0.01

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
NS = Not significantly different.
Food and water were available *ad. lib.*

Table 8.7. Effect of phenelzine administration on rat brain total biopterins and derived pterin. Animals starved overnight, killed 12 hours after dosing at 10.30am.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	56	3	6	
Phenelzine	53	5	6	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	49	6	6	
Phenelzine	56	7	6	NS

Table 8.8. Effect of phenelzine administration on rat plasma total biopterins (ug $litre^{-1}$). Animals starved overnight and killed 12 hours after dosing at 10.30am.

	Mean	SD	n	Probability
Control	23.2	5.9	6	
Phenelzine	31.5	6.7	6	<0.05

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
All animals starved on grids for a total of 30 hours.
NS = Not significantly different.
Water was available *ad. lib.*

Table 8.9. Effect of phenelzine administration on rat liver total biopterins and derived pterin. Animals starved overnight, killed 12 hours after dosing at 10.30am.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.29	0.07	6	
Phenelzine	1.38	0.14	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.60	0.12	6	
Phenelzine	0.80	0.04	6	<0.01

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
All animals starved on grids for a total of 30 hours.
NS = Not significantly different.
Water was available *ad. lib.*

Table 8.10. Effect of phenelzine and p-tyrosine administration on rat brain total biopterins and derived pterin. Animals starved overnight.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	69	4	6	
Phenelzine + p-tyrosine	60	7	6	<0.05

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	36	5	6	
Phenelzine + p-tyrosine	51	2	6	<0.001

Table 8.11. Effect of phenelzine and p-tyrosine administration on rat brain DHPR activity (nMoles NADH oxidized min^{-1} mg^{-1} protein).

	Mean	SD	n	Probability
Control	228	25	6	
Phenelzine + p-tyrosine	207	50	6	NS

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P, at 9.00am.
p-tyrosine administered 1.3mmoles kg^{-1} I.P, at 11.00am.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
Animals killed 11.00pm.
All animals starved on grids for a total of 30 hours.
NS = Not significantly different.
Water was available *ad. lib.*

Table 8.12. Effect of phenelzine and p-tyrosine administration on rat liver total biopterins and derived pterin. Animals starved overnight.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.40	0.31	6	
Phenelzine + p-tyrosine	1.53	0.17	6	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.57	0.11	6	
Phenelzine + p-tyrosine	1.07	0.22	6	<0.001

Table 8.13. Effect of phenelzine and p-tyrosine administration on rat plasma total biopterins (ug $litre^{-1}$). Animals starved overnight.

	Mean	SD	n	Probability
Control	35.5	6.8	6	
Phenelzine + p-tyrosine	40.8	25.4	6	NS

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles kg^{-1} I.P., at 9.00am.
p-tyrosine administered 1.3mmoles kg^{-1} I.P., at 11.00am.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
Animals killed 11.00pm.
All animals starved on grids for a total of 30 hours.
NS = Not significantly different.
Water was available *ad. lib.*

Table 8.14. Effect of p-tyrosine administration on rat brain total biopterins and derived pterin. Animals starved over night.

Brain total biopterins (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	70	10	5	
p-tyrosine	78	14	4	NS

Brain derived pterin (ng g^{-1} wet weight).

	Mean	SD	n	Probability
Control	32	2	5	
p-tyrosine	32	3	5	NS

Table 8.15. Effect of p-tyrosine administration on rat plasma total biopterins (ug $litre^{-1}$). Animals starved overnight.

	Mean	SD	n	Probability
Control	33.7	8.3	5	
p-tyrosine	38.4	16.5	5	NS

150g male Wistar rats were used in each case.
p-tyrosine administered 1.3mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
Dosed 8.00am and killed 8.00pm.
All animals were starved on grids for a total of 30 hours.
NS = Not significantly different.
Water was available *ad. lib.*

Table 8.16. Effect of p-tyrosine administration on rat liver total biopterins and derived pterin. Animals starved overnight.

Liver total biopterins (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	1.28	0.11	5	
p-tyrosine	1.25	0.25	5	NS

Liver derived pterin (ug g^{-1} wet weight).

	Mean	SD	n	Probability
Control	0.56	0.06	5	
p-tyrosine	0.67	0.09	5	NS

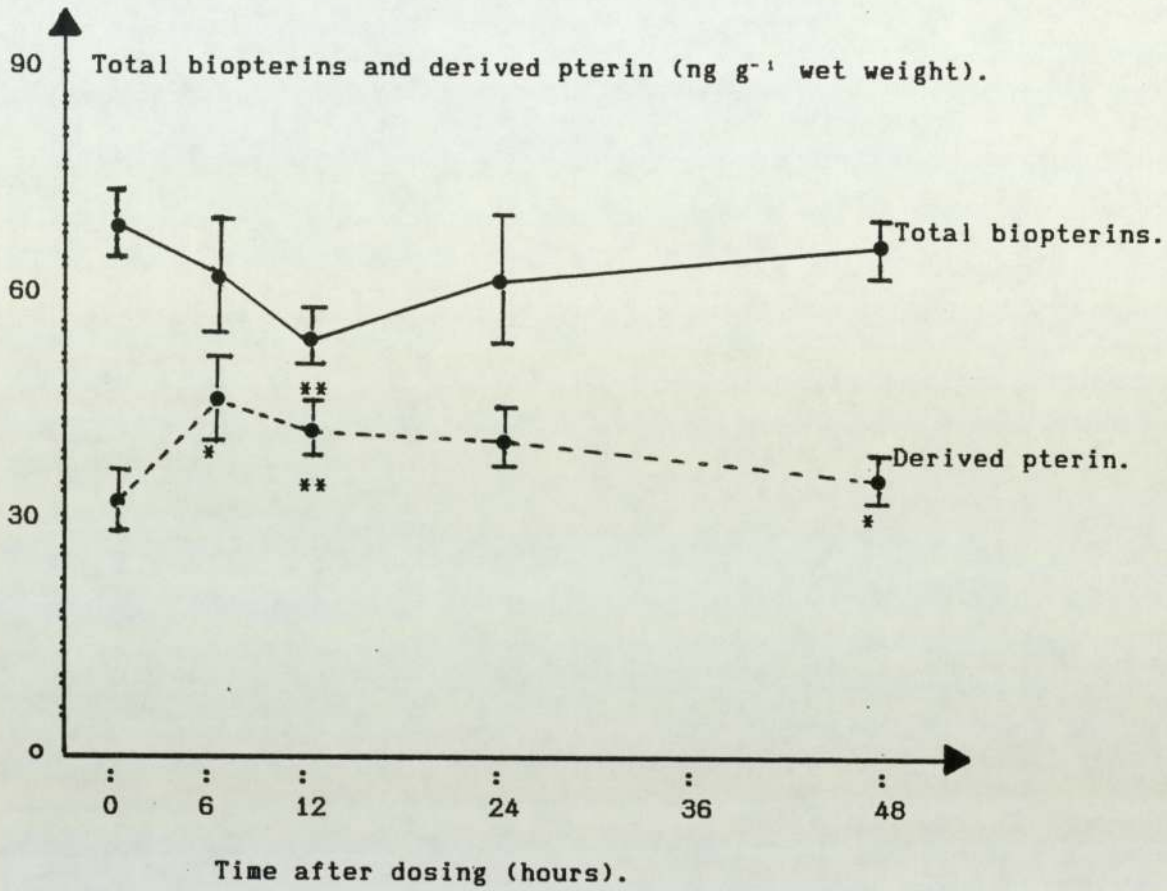
150g male Wistar rats were used in each case.
p-tyrosine administered 1.3mmoles kg^{-1} I.P.
Vehicle; distilled water.
Controls received 0.3ml distilled water I.P.
Dosed 8.00am. and killed 8.00pm.
All animals starved on grids for a total of 30 hours.
NS = Not significantly different.
Water was available *ad. lib.*

Table 8.17. Effect of p-tyramine on rat liver BH_4 biosynthesis *in vitro* (ng BH_4 synthesized h^{-1} ml^{-1} enzyme).

	Mean	SD	n
Control	169	31	2
p-tyramine	157	7	2

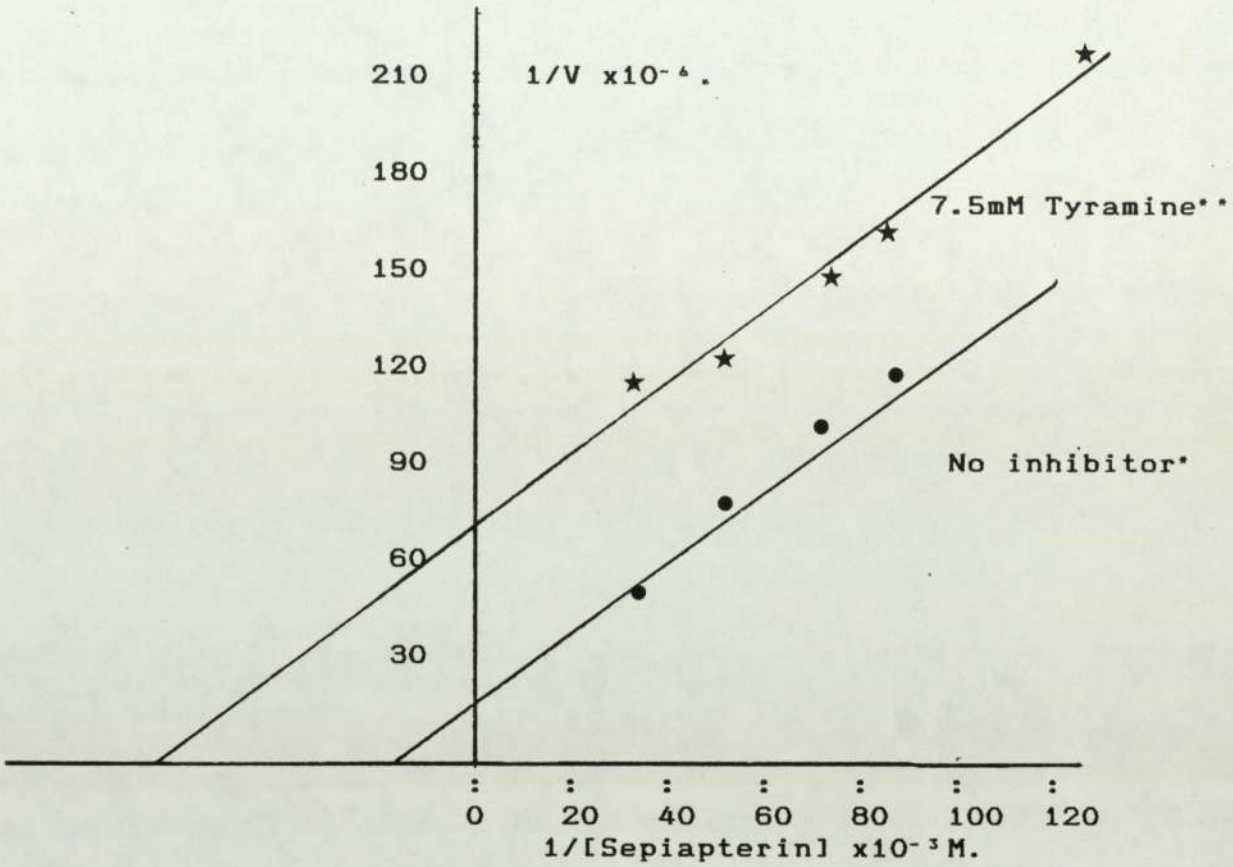
p-tyramine final concentration = 10^{-4}M.

Fig. 8.1. Plot of rat brain total biopterins and derived pterin levels against time after dosing with phenelzine.



150g male Wistar rats were used in each case.
Phenelzine administered 4mmoles kg^{-1} I.P.
Vehicle; distilled water.
Dosed 10.00am. on day 1 and killed at times indicated.
Food and water were available *ad. lib.*
Each point is the mean of 3 $\pm$ SEM.
* p <0.05 compared with time 0.
** p <0.01 compared with time 0.

Fig. 8.2. Effect of p-tyramine on sepiapterin reductase activity (partially purified from liver). NADPH concentration constant at 100uM.



Km for sepiapterin = 71.0uM.
Vmax = 66.0nMoles sepiapterin reduced min^{-1} ml^{-1} enzyme.
Ki for p-tyramine = 2.5mM, uncompetitive inhibition.
* r= 0.9803, P<0.02
** r= 0.9902, p<0.01

CHAPTER NINE

GENERAL DISCUSSION

CHAPTER NINE.

GENERAL DISCUSSION.

9.1 TETRAHYDROBIOPTERIN AND TETRAHYDROFOLATES.

Tetrahydrobiopterin (BH_4) and tetrahydrofolate (FH_4) are tetrahydropterin cofactors. FH_4 and its derivatives are essential for purine and pyrimidine biosynthesis and synthesis of glycine from serine. Glycine is required for protein and glutathione synthesis and within the central nervous system (CNS) it functions as a neurotransmitter (Nichol *et al.*, 1983; Meister, 1981). Deficiency of tetrahydrofolates lead to a number of pathological disorders e.g. megloblastic anaemia and psychiatric disorders including forgetfulness, irritability, sleeplessness and paranoid behaviour. As the folate molecular skeleton cannot be synthesized in mammalian tissues it is obtained from dietary sources (Botez and Renolds, 1979; Rowe, 1983).

BH_4 is the natural cofactor for the hydroxylation of phenylalanine to tyrosine, tyrosine to DOPA and tryptophan to 5-hydroxytryptophan, reactions of importance in the synthesis of the neurotransmitters, noradrenaline, dopamine, and serotonin (Kaufman, 1986). Depletion of BH_4 leads to neurological disorders as illustrated by the inborn errors of BH_4 metabolism, the malignant hyperphenylalaninaemias (Leeming *et al.*, 1981). Tissue levels of BH_4 are maintained

by *de novo* synthesis from guanosine triphosphate (GTP) and a salvage pathway, dihydropteridine reductase (DHPR), which regenerates BH_4 from quinonoid dihydrobiopterin (qBH_2) formed as a result of cofactor activity (Craine *et al.*, 1972; Milstien and Kaufman, 1986).

Tetrahydropterins are unstable and readily oxidized by a number of oxidizing agents (Pearson, 1974). Since oxidizing environments arise *in vivo*, the aim of this thesis was to identify agents and conditions which may be encountered biologically resulting in oxidative loss of BH_4. The impact of some of these factors on FH_4 metabolism was considered where appropriate.

9.2. AUTOXIDATION OF TETRAHYDROBIOPTERIN.

The susceptibility of tetrahydropterins to autoxidation has been known for some time (Zakrzewski and Nichol, 1956) and is illustrated here by the *in vitro* studies on BH_4. The term autoxidation refers to air oxidation, i.e. oxidation by molecular oxygen, as air saturated buffers were used in all cases.

In vitro, the rate of BH_4 autoxidation, in common with FH_4, is affected by the pH of the oxidizing medium and decreases with increasing acidity (Table 9.1). Protonation of the N(5) region of the pterin ring system, under acidic conditions, retards electron abstraction and consequently the oxidation rate.

Table 9.1. Stability of BH_4 with pH at 37°C.

pH	Pseudo Rate Constant (Min^{-1}).	Half-life (mins).
1.6	0.0028	247.50
3.0	0.0084	82.50
4.6	0.0360	19.25
5.3	0.0759	9.13
7.6	0.0870	7.96

Each rate is the mean of 3 observations.
BH_4 = 50uM.

The nature of the autoxidation products of BH_4 are also affected by pH, i.e. with decreasing acidity there is increased side chain loss, as illustrated by the formation of xanthopterin and pterin (Table 9.2). The autoxidation of BH_4 proceeds *via* generation of qBH_2 which is unstable and tautomerises (Matsuura *et al.*, 1986). Under acidic conditions, protonation of the hydroxyl groups of the side chain prevents electron donation to the pterin ring system during quinonoid rearrangement and hence conservation of the side chain (Fig. 3.5, Chapter 3).

In vivo, the initial step in BH_4 catabolism is non-enzymatic oxidation to 7,8-dihydrobiopterin (BH_2) and 7,8-dihydropterin. Hydration and subsequent oxidation of the latter results in xanthopterin formation which is excreted in the urine (Rembold, 1983). Xanthopterin also arises from the oxidative catabolism of tetrahydrofolates (Pheasant *et al.*, 1981). The daily excretion of xanthopterin in man is between 0.3 and 3.0 mg day^{-1} (Rembold, 1983), suggesting substantial oxidative catabolism of tetrahydropterins under normal conditions.

Due to the unstable nature of BH_4, careful manipulation is required and acidification of BH_4 samples to below pH 1.6 is required for optimum storage and measurement.

Table 9.2. Effect of pH on the percentage conversion to identifiable oxidation products and recovery of pterin ring system for BH_4 at 37° C.

pH	Biopterin.	Xantho-pterin.	Pterin.	Total recovery
1.6	90	1	2	92
5.3	74	15	7	96
7.6	31	15	60	106

Products identified by TLC and HPLC after 3 hours autoxidation, at indicated pH, followed by 1 hour acid iodine oxidation. Each observation is the mean of 3 observations. BH_4 = 50uM.

The unstable nature of BH_4, particularly at physiological pH, implies that variation of the oxidizing environment *in vivo* may influence the catabolism of BH_4. The rate of oxidation of BH_4 is enhanced by a number of inorganic agents which are encountered *in vivo*, e.g. transition metals (Armarego and Waring, 1982), nitrite, and the hydroxyl radical.

9.3 NITRITE OXIDATION.

Nitrite is a widely distributed environmental oxidizing agent, and *in vitro* increases the measurable oxidation rates of BH_4, FH_4, (Table 9.3), BH_2 and 5-methyltetrahydrofolate ($5CH_3FH_4$) at acid, but not neutral, pH. This oxidation is mediated by generation of the electrophilic nitrosonium ion (NO^+) or the nitrous acidium ion ($H_2ONO_2^+$) *via* nitrous acid.

Table 9.3. Nitrite oxidation of BH_4 and FH_4 at 37°C.

	Autoxidation rate	Autoxidation rate + nitrite.
	Pseudo rate constant (min^{-1})	
BH_4		
pH 1.6	0.0028	0.1320
3.0	0.0084	0.0450
7.0	0.0820	0.0820
FH_4		
pH 1.6	0.0123	0.9240
3.0	0.0409	0.3680
7.0	0.0529	0.0529

Each rate is the mean of 3 observations.
BH_4 = 50uM, FH_4 = 20uM, sodium nitrite = 0.1mM.

As nitrite has no affect on the oxidation rate of reduced pterins at neutral pH, it is unlikely that ingestion will directly affect BH_4 metabolism. In contrast, the folate molecular skeleton is obtained from dietary sources as $5CH_3FH_4$, FH_4, 10-formyltetrahydrofolate and their derivatives (Ratanasthien *et al.*, 1977). Therefore, some dietary folates (*ca* 300ug day^{-1}) in the acid medium of the stomach (pH 2.5 - 4.5) (Knowles *et al.*, 1974) may be oxidized by the relatively large daily intake of nitrite (*ca* 300 fold molar excess over total folate ingestion). Since the nitrite mediated oxidation of $5CH_3FH_4$ results in formation of a number of acid labile products and of FH_4 formation of pterin (Table 9.4), loss in this way can be replenished only by further dietary intake. Oxidative destruction, enhanced by nitrite, may be a contributing factor to the substantial loss of oral $5CH_3FH_4$ occurring in man (Ratanasthien *et al.*, 1977).

Table 9.4. Formation of pterin from FH_4 in the presence of nitrite at 37° C.

pH	% Pterin
1.6	77
3.0	70

Pterin identified by TLC and HPLC, after 30 minutes incubation.
Conversion is the mean of 3 observations.
FH_4 = 20uM, sodium nitrite = 0.1mM.

Ascorbate and α-tocopherol react with nitrous acid resulting in nitric oxide formation (Mirvish *et al.*, 1972; Kamm *et al.*, 1977), indicating possible antioxidant roles in the stomach for protecting tetrahydrofolates from nitrite oxidation. Oral administration of ascorbate along with $5CH_3FH_4$ significantly elevates plasma total levels of this folate, compared to controls receiving only $5CH_3FH_4$ (Ratansthien *et al.*, 1977). Thus, adequate dietary intake of antioxidants coupled with the rapid absorption of nitrite from the stomach (85% of an oral dose of sodium nitrite is absorbed from the mouse stomach in 10 minutes) (Friedman *et al.*, 1972) will limit folate lost in this way. However, high nitrite ingestion and/or low dietary intake of antioxidants may lead to folate deficiency.

Because of the high incidence of folate deficiency, particularly in pregnant women, estimated in the UK at 25% (US, 24%) (Chanarin, 1979), a large dietary intake of nitrite should be considered as a possible contributing factor to this deficiency.

9.4 HYDROXYL RADICAL OXIDATION.

The hydroxyl radical (·OH) is a powerful oxidizing agent and reacts at high rate with most organic molecules (Ingraham and Meyer, 1985). *In vitro*, the observed rate of oxidation of BH_4 (Table 9.5), 7,8-dihydrobiopterin (BH_2) and 7,8-dihydroneopterin (NH_2) are enhanced by ·OH. Furthermore substantial destruction of the pterin ring occurs above pH 1.6, as illustrated for BH_4 (Table 9.6).

Table 9.5. Effect of ·OH generation on the initial oxidation rate of BH_4 at 37° C.

pH	Autoxidation Rate	Autoxidation Rate + ·OH
	Pseudo Rate Constants (min^{-1}).	
1.6	0.0028	0.2126
5.3	0.0750	0.1529
7.6	0.0805	0.2272

Each rate is the mean of 4 observations.
·OH generated by reaction of H_2O_2 (0.3mM) with ferrous sulphate and EDTA (0.1mM), BH_4 = 50uM.

Table 9.6. Effect of ·OH on the percentage conversion to identifiable products and recovery of pterin ring system for BH_4 at 37° C.

pH	Biopterin.	Xantho-pterin.	Pterin.	Total Recovery.
1.6	100	1	2	103
5.3	31	-	3	34
7.6	5	20	3	28

Each percentage conversion is the mean of 4 observations.
Products identified after 1 hour incubation, by HPLC and TLC.
·OH generated by reaction of H_2O_2 (0.3mM) with ferrous sulphate and EDTA (0.1mM), BH_4 = 50uM.

The finding that the ·OH oxidation of reduced pterins is poorly inhibited by an excess of free radical scavengers such as mannitol (Table 4.3, Chapter 4), illustrates their

susceptibility to oxidation by •OH. However, BH_4 autoxidation and •OH oxidation are inhibited by physiological concentrations of the reduced form of glutathione (GSH), indicating a possible protective role against tetrahydropterin oxidation *in vivo* (Table 9.7). GSH may inhibit this oxidation of BH_4 by the non-enzymatic scavenging of oxygen radicals and reduction of qBH_2 to BH_4. The brain GSH concentration is estimated at 0.9 - 3.4mM (Mc Ilwain and Bachelard, 1985).

Table 9.7. Inhibition of BH_4 autoxidation and •OH oxidation by GSH at pH 7.6, 37° C.

	Pseudo Rate Constant (min^{-1}).	Percentage Inhibition.
BH_4	0.0500	-
+ GSH (mM)		
0.1	0.0418	16
0.5	0.0315	37
1.0	0.0170	66
5.0	0.0065	87
BH_4 + •OH	0.2900	-
+ GSH (mM)		
2.0	0.2210	24
4.0	0.1560	47
6.0	0.0960	67

Each rate is the mean of 3 observations.
•OH generated by reaction of H_2O_2 (0.3mM) with ferrous sulphate and EDTA (0.1mM). BH_4 = 50uM.

The hydroxyl radical may be formed, *in vivo*, from the reaction of hydrogen peroxide (H_2O_2) with free (unbound) transition metals such as iron (Halliwell and Gutteridge, 1985). Free iron has been detected in the cerebrospinal fluid (CSF) (2.2 $\pm$ 1.3 uM) (Gutteridge *et al.*, 1981), and increased production of •OH, *via* monoamine oxidase (MAO) activity, is suggested in the pathogenesis of Parkinson's disease (Cohen, 1985).

The *in vitro* observation that MAO activity, in the presence of ferrous sulphate, results in BH_2 oxidation indicates that generation of small amounts of H_2O_2 (4 nmoles min^{-1} ml^{-1} enzyme) are sufficient to generate ·OH (Table 9.8). Inhibition by catalase and phenelzine, a MAO inhibitor, confirm that this oxidation is dependent on the generation of H_2O_2 from MAO.

Table 9.8. Effect of monoamine oxidase activity on BH_2 oxidation in the presence and absence of ferrous sulphate, plus the effects of phenelzine and catalase addition, 25°C.

	Pseudo Rate Constant (min^{-1}).	Percentage Inhibition.
BH_2 + MAO + benzylamine + EDTA (1)	*	-
(1) + ferrous sulphate (2)	0.0041	-
(2) + catalase	0.0015	63
(2) + phenelzine	0.0000	100

MAO activity = 4nmoles H_2O_2 formed min^{-1} ml^{-1} enzyme.
Benzylamine = 3mM; BH_2 = 50uM; ferrous sulphate/EDTA = 60uM.
Catalase = 5Uml^{-1}; phenelzine = 1mM.
* = No oxidation observed over a 30 minute period.

The extensive destruction of reduced pterins by ·OH at physiological pH indicates that production *in vivo*, in regions dependent on BH_4 for neurotransmitter synthesis, may, if not scavenged, lead to cofactor depletion. The suggested production of ·OH in Parkinson's disease (Cohen, 1985) may therefore contribute to the reduced CSF levels of BH_4 reported in untreated patients with this disease (Controls 17.7 $\pm$ 1.69 (n=10), Parkinsonian patients 8.9 $\pm$ 0.95 pmole ml^{-1} (n=9); $P < 0.001$) (Lovenburg *et al.*, 1979).

In vivo, formation of oxygen radicals is essential for functioning of the immune response, and production and utilization of ·OH occurs within stimulated macrophages (Babior, 1978). Activation of macrophages, by γ-interferon, results in synthesis of NH_2 triphosphate, but not BH_4, and the release of NH_2 and neopterin. Between 25-43% of the neopterins released are in the fully oxidized form (Werner *et al.*, 1987).

The *in vitro* observation that NH_2 is relatively stable to oxidation by molecular oxygen, i.e no detectable rate of oxidation occurring over a 30 minute period (Table 4.1, Chapter 4), suggests that any neopterin formed from NH_2 arises as a result of the oxidizing radicals formed within the activated macrophage. The amount of neopterin released by macrophages correlates positively with the amount of H_2O_2 subsequently released (Nathan, 1986).

Whilst generation of •OH, *in vitro*, increases the measurable oxidation rate of NH_2 (Table 4.1, Chapter 4), the extensive destruction of the pterin system above pH 1.6 (Table 4.5, Chapter 4) suggests consideration of different conditions e.g. an excess of NH_2 over ·OH to explain the release of neopterins following macrophage activation.

Because of the susceptibility of the pterin ring to ·OH attack, it is likely that tetrahydrofolates will be sensitive to formation of this radical. Production of ·OH *in vivo* may therefore deplete this cofactor pool in addition to

the BH_4 pool.

The persistent activation of macrophages by γ-interferon is suggested to be responsible for the decreased CSF total folates occurring in two patients with aquired immune deficiency syndrome (AIDS) (Smith *et al.*, 1987). Since γ-interferon stimulates macrophage H_2O_2 production which readily crosses the cell membrane (Halliwell and Gutteridge, 1984; Nathan *et al.*, 1985), it is possible that depletion of CSF folates arises from enhanced catabolism due to generation of ·OH, *via* an *in vivo* Fenton's reagent.

9.5. REDUCED PTERINS: TISSUE ANTIOXIDANTS ?

The susceptibility of reduced pterins to oxidation by oxidizing species such as nitrite and the hydroxyl radical, suggests that these compounds may have the capacity to act as antioxidants.

In vitro, BH_4 inhibits the Luminol-dependent chemiluminescence of activated monocytes in a concentration dependent manner and to an extent which exceeds that of the established antioxidants, ascorbate and dithioerythritol (Table 9.9). Chemiluminescence arises from production of high energy oxygen compounds, including the hydroxyl radical, and oxidation of 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol) by oxygen radicals is used to increase the amount of measurable light (Easmon *et al.*, 1980). As BH_4 is readily

oxidized by the hydroxyl radical, it is likely that inhibition of this chemiluminescence arises from scavenging of oxygen radicals by BH_4.

Table 9.9. Inhibition of Luminol-dependent chemiluminescence by BH_4, ascorbate and dithioerythritol (Heales *et al.*, 1987).

Inhibitor Concentration. uM.	% Luminescence of Control Control = 100
BH_4	
10	80
20	55
30	41
40	35
50	28
Ascorbate	
100	80
Dithioerythritol	
100	60

Luminescence recorded with respect to control for 60 minutes, 37°C.

The effectiveness of BH_4 as a free radical scavenger illustrated here, coupled with the ubiquitous presence of BH_4 and related pterins in tissues (Fukushima and Nixon, 1980), indicates a possible role for reduced pterins as antioxidants. However, scavenging of oxidizing species in regions requiring tetrahydropterin cofactors may lead to disruption of biochemical pathways utilizing BH_4 and FH_4 due to cofactor depletion.

9.6 OXIDATIVE STRESS AND TETRAHYDROBIOPTERIN METABOLISM.

9.6.1 TETRAHYDROBIOPTERIN METABOLISM AND THE RAT.

Because of the susceptibility of BH_4 to oxidation, *in vitro*,

the effect of alterations in the *in vivo* oxidizing environment was examined on BH_4 metabolism.

Due to the limited availability of human samples and the inability to monitor the *in vivo* effects of certain agents and conditions on BH_4 metabolism in human tissues, the male Wistar rat was used as an experimental model. The rat is a convenient model for studying BH_4 metabolism *in vivo* and, despite differences occurring between BH_4 metabolism in rat and man, notably the failure to detect neopterins in rat tissue, many findings are observable in both species (Barford *et al.*, 1982; Hamon, 1984; Rokos *et al.*, 1985; Cutler, 1986).

Any *in vivo* study of BH_4 metabolism requires reliable methods for determining tissue cofactor levels, biosynthetic and salvage capacities. Measurement of these parameters, using the methods described in chapter 2, in apparently identical control animals, but separate batches, results in significant differences in the mean values obtained, i.e there is a high inter, but not intra, assay variability associated with the techniques employed (Table 9.10).

Because of the lability of BH_4 and 6,7-dimetyl-tetrahydropteridine ($DMPH_4$, used in the dihydropteridine reductase assay), subtle changes in the handling of tissue samples and $DMPH_4$ solutions may contribute to this variation. Consequently, in order to minimize error, no between batch comparisons were made and control and test samples were assayed alternately.

Table 9.10. Between batch comparison of measured brain total biopterins, DHPR and biosynthetic capacity.

	Batch A		Batch B
Total biopterins (ng g^{-1} wet weight).	69 ± 4 (6)	P <0.05	78 ± 8 (6)
DHPR. (nmoles NADH ox. min^{-1} mg^{-1} protein).	210 ± 19 (6)	P <0.01	172 ± 12 (6)
Biosynthesis (ng BH_4 formed h^{-1} mg^{-1} protein).	1.18 ± 0.17 (5)	P <0.05	2.52 ± 0.94 (5)

150g Wistar rats used in all cases. Food and water available *ad. lib.*
Each observation is expressed as the mean ± SD.
Number in brackets indicates sample size.

Measurement of total biopterins in brain and liver reveals higher levels occurring in the liver. This difference is explained by the large biosynthetic capacity of the liver which exceeds that of the brain by a factor of approximately 20 (Table 9.11).

The biopterin measured in brain and liver samples, following acid iodine oxidation arises from BH_4, BH_2 and any oxidized biopterin present. Under normal conditions over 90% of tissue biopterins are BH_4 (Al-Salihi, 1985). Therefore, measurement of total biopterins can be used as an indicator of tissue availability of the BH_4 cofactor, but slight changes in the relative partitioning of the biopterins in each oxidation state may not be detected. However, an

increase in the oxidative catabolism of BH_4 will result in a lowering of total biopterins, if occurring in excess to the rate of BH_4 *de novo* synthesis, due to loss of the biopterin molecular skeleton at physiological pH (Tables 9.2 and 9.6).

Table 9.11. Comparison of brain and liver total biopterins, DHPR and biosynthetic capacities

	Brain.	Liver.
Total biopterins (ng g^{-1} wet weight.	84 ± 11 (16)	1480 ± 400 (16)
DHPR (nmoles NADH ox. min^{-1} mg^{-1} protein).	210 ± 19 (16)	302 ± 38 (16)
Biosynthesis (ng BH_4 formed h^{-1} mg^{-1} protein).	0.95 ± 0.19 (5)	20.10 ± 5.70 (5)

150g male Wistar rats used in each case.
Each observation is expressed as the mean ± SD.
Number in brackets = sample size.
Food and water available *ad. lib.*

9.5.2 OXYGEN EXPOSURE.

Despite the susceptibility of BH_4 to oxidation by dioxygen, *in vitro*, inhalation of 100% oxygen, by rats, for 24 hours has no effect on brain total biopterin levels (Table 9.12).

Table 9.12. Effect of 100% oxygen exposure for 24 hours on rat brain total biopterins.

ng g^{-1} wet weight

Air breathers		100% oxygen
78 ± 8 (6)	NS	81 ± 12 (6)

150g male Wistar rats used in all cases.
Food and water available *ad. lib.*
Each observation is expressed as the mean ± SD.
Number in brackets = sample size
NS = Not significantly different.

Under these conditions the mean brain oxygen concentration trebles (Jamieson and Van Den Brenk, 1963) suggesting cofactor levels are maintained by salvage and *de novo* synthesis or oxidative catabolism is prevented by the action of antioxidants such as GSH. There is a large molar excess, of the order of 8×10^3, of GSH over BH_4 in rat brain (Mc Ilwain and Bachelard, 1985; Fukushima and Nixon, 1980).

In the rat, inhalation of 100% oxygen results in elevated catecholamine and serotonin synthesis (Diaz *et al.*, 1968; Neff and Costa, 1967). The maintenance of brain biopterins under such conditions suggests that this elevation in neurotransmitter synthesis arises from the increased availability of oxygen.

9.5.3 NITROUS OXIDE EXPOSURE.

Nitrous oxide (N_2O) is a mild oxidizing agent and CNS depressant. Exposure *in vivo* selectiviely inactivates the enzyme methionine synthetase by oxidation of the cobalt centre of the cob(1)alamin (vitamin B_{12}) cofactor. Methionine synthetase catalyses the transfer of a methyl group from $5CH_3FH_4$ to homocysteine, forming methionine and FH_4 (Banks *et al.*, 1968; Deacon *et al.*, 1978).

Demethylation of $5CH_3FH_4$ is reported to be necessary for the biosynthesis of BH_4. *In vitro* N_2O abolishes BH_4 biosynthesis (Hamon *et al.*, 1986), but exposure *in vivo* for 24 hours has no effect on total biopterin levels or biosynthetic capacity

in the rat brain (Table 9.13).

Table 9.13. Effect of 24 hours nitrous oxide exposure on rat brain total biopterins and biosynthetic capacity.

	Control		Nitrous oxide
Total biopterins (ng g^{-1} wet weight).	83 ± 10 (6)	NS	77 ± 7 (6)
Biosynthesis (ng BH_4 formed h^{-1} mg^{-1} protein)*.	0.70 ± 0.27 (6)	NS	0.60 ± 0.18 (5)

150g male Wistar rats used in all cases. Food and water available *ad. lib.*
Controls received 4:1 He:O_2 mixture. Test 4:1 N_2O:O_2 mixture.
Each observation is expressed as the mean ± SD.
Number in brackets = sample size. * = Assayed by CGB Hamon.
NS = Not significantly different.

This result suggests that the products derived from the methionine synthetase reaction, required for BH_4 biosynthesis, are available *in vivo* following inactivation of this enzyme for at least 24 hours.

9.5.4. STARVATION, GLUTATHIONE AND OXIDATIVE STRESS.

Acute starvation results in an increased tissue oxidizing environment due to a reduction in catalase and GSH levels (Tateishi *et al.*, 1974; Isaacs and Binkley, 1971). Furthermore, overnight starvation results in an increased production of oxygen radicals in the brain and liver, as determined by the reaction of nitroblue tetrazolium with oxygen derived radicals (Stankiewicz, 1987).

Starvation is also accompanied by other metabolic changes including a reduction in catecholamine turnover in the

peripheral organs and brain. In addition, reduction of noradrenaline and dopamine levels occurs in the brain of rats undernourished from mid gestation and killed at weaning. Abnormal catecholamine metabolism has also been noted in primary anorexia nervosa (Young and Landsburg, 1977; Shoemaker and Wurtman, 1971; Gross *et al.*, 1979).

The mechanism of this reduction in catecholamine turnover is not well understood, but a limitation in the availability of tyrosine is implicated as a factor (Schweiger *et al.*, 1985). However, the reduction of brain total biopterins by acute starvation (Table 9.14) indicates that neurotransmitter synthesis may be regulated by BH_4 levels in addition to precursor amino acid availability.

Table 9.14. Effect of starvation on rat brain total biopterins.

Period of starvation (hours).	Fed		Starved.
	ng g^{-1} wet weight.		
24	93 ± 6 (4)	P <0.02	77 ± 2 (5)
48	73 ± 6 (6)	P <0.05	65 ± 6 (6)
72*	96 ± 16 (6)	P <0.002	65 ± 7 (6)

150g male Wistar rats were used in each case.
Animals starved on grids for period indicated.
Water available *ad. lib.*
Controls received food and water *ad. lib.*
Results expressed as mean ± SD.
Number in brackets indicates sample size.
* Assayed by J Cox.

Depression of brain total biopterins may arise as a result of increased oxidative catabolism, due to the oxidizing environment induced by starvation. The transient reduction in brain biosynthetic capacity occurring after 24 hours and DHPR after 48 are also likely to be responsible in part for this loss of biopterins (Table 9.15). In addition, the limited availability of NADPH in starvation (Martins *et al.*, 1985) may limit BH_4 biosynthesis and possibly salvage *in vivo*.

Table 9.15. Effect of starvation on rat brain DHPR and biosynthetic capacity.

Period of starvation (hours)	Fed		Starved
DHPR*			
24	210 ± 19 (6)	NS	201 ± 19 (6)
48[J]	172 ± 12 (6)	P <0.05	142 ± 24 (6)
Biosynthesis**			
24	0.21 ± 0.09 (5)	P <0.05	0.06 ± 0.06 (4)
48	0.84 ± 0.31 (6)	NS	0.69 ± 0.26 (6)

150g male Wistar rats were used in each case.
Animals starved on grids for period indicated.
Water available *ad. lib.*
Controls received food and water *ad. lib.*
Results expressed as mean ± SD.
Number in brackets indicates sample size
* = nmoles NADH oxidized min^{-1} mg^{-1} protein.
** = ng BH_4 formed h^{-1} mg^{-1} protein.
NS = Not significantly different.
J = assayed by J. Cox.

An increased oxidizing environment, in fasting, may inhibit DHPR due to oxidation of the thiol groups required for

activity (Cheema *et al.*, 1973). Similarly one or more of the enzymes of the biosynthetic pathway may be sensitive to oxidation. Alternatively reduction in activity results from a depression in enzyme synthesis.

In contrast, liver total biopterins are significantly reduced by 24 hours starvation with no measurable effect on salvage or biosynthetic pathways (Tables 7.10 and 7.11, Chapter 7).

Depression of liver total biopterins, may limit the hepatic hydroxylation of phenylalanine. Plasma tyrosine, but not phenylalanine, levels are significantly reduced by 24 hours starvation in the rat (Arola *et al.*, 1983). However, reduction of liver biopterins in starvation appears to be a transient event, occurring only after 24 hours (Table 9.16).

Table 9.16. Effect of starvation on rat liver total biopterins.

Period of starvation (hours).	Fed		Starved
	ug g^{-1} wet weight.		
24	2.00 ± 0.13 (4)	P <0.05	1.69 ± 0.21 (5)
48	1.51 ± 0.19 (6)	NS	1.46 ± 0.24 (6)
72*	1.31 ± 0.07 (6)	NS	1.30 ± 0.12 (6)

150g male Wistar rats used in each case.
Animals starved on grids for period indicated.
Water available *ad. lib.*
Results expressed as mean ± SD.
Number in brackets = sample size.
NS= Not significantly different.
* Assayed by J Cox.

The origin of this pattern of results may be related to alterations in GSH levels and hence degree of oxidative stress and BH_4 catabolism. The liver GSH pool is reduced by up to 50% by 24 hours starvation, but levels return to those of feeding controls after 48 (Tateishi *et al.*, 1974; Cho *et al.*, 1981).

Depletion of liver GSH, in feeding rats, by L-buthionine sulfoximine (L-BSO), results in a depression of total biopterins (Table 9.17). Administration of L-BSO, as indicated, depletes the liver GSH pool by up to 80% (Griffith and Meister, 1979). This observation, coupled with the the *in vitro* findings above, indicate a role for protecting BH_4 from oxidative catabolism *in vivo*.

Table 9.17. Effect of L-BSO on rat liver total biopterins

Control	L-BSO
ug g^{-1} wet weight.	
1.70 $\pm$ 0.12 (5)	1.46 $\pm$ 0.08 (5)
P <0.01	

90g male Wistar rats used in each case.
L-BSO administered 4mmoles Kg^{-1} I.P
Dosed 4 times over a 12.5 hour period.
Controls received 0.3ml distilled water I.P
Food and water available *ad. lib.*
Results expressed as mean $\pm$ SD.
Number in brackets = sample size.

In contrast, prolonged GSH depletion, in feeding animals, results in a maintenance of brain and liver total biopterins

(Tables 7.36 and 7.39, Chapter 7), due, presumably, to the increased biosynthesis and DHPR activity occurring after such treatment (Table 9.18). This suggests that the biosynthetic and salvage components of BH_4 metabolism are sensitive to prolonged BH_4 catabolism and respond to maintain cofactor levels. Increases in the activity of these pathways have been reported following chronic exposure to lead in rats, and the existence of feedback mechanisms to maintain BH_4 levels proposed (Mc Intosh *et al.*, 1985; Eggar *et al.*, 1986). The mechanism of this activation is not clear but could involve cAMP, since GTP cyclohydrolase is induced by cAMP and DHPR activated by cAMP dependent phosphorylation (Abou-Donia, 1986; Cutler, 1986).

Table 9.18. Effect of chronic administration of L-BSO on rat brain and liver DHPR activity and liver biosynthetic capacity.

	Control		L-BSO
DHPR Activity*.			
Brain	144 ± 14 (5)	P <0.01	191 ± 28 (5)
Liver	302 ± 38 (5)	P <0.05	393 ± 62 (5)
Biosynthesis**			
Liver	8.47 ± 1.25 (5)	P <0.01	13.93 ± 2.54 (5)

Male Wistar rats used in each case.
Food and water available *ad. lib.*
L-BSO administered in drinking water
Dose = 4mmoles kg^{-1} day^{-1} for 17 days.
Results expressed as mean ± SD.
Number in brackets = sample size.
* = nmoles NADH oxidized min^{-1} mg^{-1} protein.
** = ng BH_4 formed h^{-1} mg^{-1} protein.

The elevation of brain and liver total biopterins by L-cysteine administration to starving rats (Table 9.19) is likely to arise from a lowering of the starvation induced oxidizing environment, due to the increased concentration of GSH and L-cysteine, and from the ability of these compounds to regenerate BH_4 from qBH_2 (Ayling *et al.*, 1983). Furthermore liver DHPR activity is significantly elevated by this treatment (Table 9.19), suggesting protection of the thiol groups of this enzyme from oxidation. Administration of α-tocopherol also results in elevation of brain DHPR activity (Stankiewicz, 1987).

Table 9.19. Effect of L-cysteine administration during 48 hours starvation on rat brain and liver total biopterins and liver DHPR activity.

	Starved	Starved + L-cysteine
Brain ($ng\ g^{-1}$ wet weight).	73 ± 13 (6)	89 ± 11 (6)
	P <0.05	
Liver ($ug\ g^{-1}$ wet weight).	1.43 ± 0.14 (6)	1.63 ± 0.11 (6)
	P <0.025	
Liver DHPR*	443 ± 64 (6)	535 ± 74 (6)
	P <0.05	

150g male Wistar rats were used in each case,
Animals starved on grids.
L-cysteine administered 4mmoles Kg^{-1}, orally 2 X day^{-1}.
Water was available *ad. lib.*
* = nmoles NADH oxidized min^{-1} mg^{-1} protein.
Each observation is expressed as the mean ± SD.
Number in brackets = sample size.

This elevation of tissue biopterins and liver DHPR suggests

that manipulation of the tissue oxidizing environment can influence BH_4 metabolism and indicates consideration of antioxidant therapy for conditions where there is reported oxidative stress and disruption of BH_4 metabolism, e.g. Parkinson's disease, and senile dementia of the Alzheimer type (Levine *et al.*, 1979; Cohen, 1975; Anderson *et al.*, 1986; Martins *et al.*, 1986).

9.5.5. INHIBITION OF DIHYDROPTERIDINE REDUCTASE BY *P*-TYRAMINE

Oxidation of BH_4 proceeds *via* the formation of qBH_2 (Blair and Pearson, 1974). Similarly hydroxylation of phenylalanine, tyrosine and tryptophan results in qBH_2 production. *In vivo*, DHPR functions to regenerate BH_4 from qBH_2 (Kaufman, 1985). Loss of this reducing capacity results in accumulation of qBH_2 which rearranges to give 7,8-dihydropterin and BH_2, which are lost from the cell (Matsuura *et al.*, 1986; Leeming *et al.*, 1981).

In vitro, *p*-tyramine is a non-competitive inhibitor of DHPR (Shen *et al.*, 1984) and generation of this amine, *in vivo*, *via* phenelzine administration and dietary tyrosine, results in loss of brain total biopterins (Table 9.20). A measurable reduction in brain DHPR activity is not detectable due to dilution of this reversible inhibitor during sample preparation. However, evidence for the inhibition of DHPR *in vivo* comes from the increase in plasma biopterins and tissue derived pterin occurring after phenelzine administration (Sections 9.5.6 and 9.5.7).

Table 9.20. Effect of phenelzine administration on rat brain total biopterins.

Control		Phenelzine
	ng g^{-1} wet weight	
93 $\pm$ 11 (6)		77 $\pm$ 12 (6)
	P <0.05	

150g male Wistar rats used in each case.
Phenelzine administered, 4mmoles Kg^{-1}, I.P.
Vehicle distilled water.
Controls received 0.3ml distilled water I.P.
Food and water available *ad. lib.*
Each observation is the mean $\pm$ SD.
Number in brackets = sample size.

In the absence of dietary tyrosine, as in starvation, phenelzine has no effect on brain biopterin levels, despite evidence of DHPR inhibition. This indicates that cofactor loss depends on the degree of DHPR inhibition and occurs only when the rate of loss exceeds that of *de novo* synthesis. Co-administration of tyrosine and phenelzine to starved rats results in a lowering of brain total biopterins, and illustrates the requirement for tyrosine in this study. Tyrosine in the absence of phenelzine has no effect on brain total biopterins (Table 9.21).

Generation of p-tyramine by phenelzine arises as a result of inhibition of liver tyrosine amino transferase (TAT), resulting in the transport of large amounts of dietary derived p-tyrosine into the CNS. Decarboxylation of this amino acid forms p-tyramine and inhibition of monoamine oxidase, also by phenelzine, results in accumulation (Dyck and Dewar, 1986).

Table 9.21. Effect of phenelzine, tyrosine and phenelzine plus tyrosine administration on brain total biopterin levels in the starved rat (ng g^{-1} wet weight).

Starved		Starved + Phenelzine
56 ± 3 (6)	NS	53 ± 5 (6)
Starved		**Starved + p-tyrosine**
70 ± 10 (5)	NS	78 ± 14 (4)
Starved		**Starved + Phenelzine + p-tyrosine**
69 ± 4 (6)	P <0.05	60 ± 7 (6)

150g male Wistar rats used in each case.
Animals starved on grids for a total of 30 hours.
Water available *ad. lib.*
Phenelzine dosed I.P, 0.4mmoles Kg^{-1}
Tyrosine dosed I.P, 1.3mmoles Kg^{-1}
Co-administration = sum of above.
Controls dosed 0.3ml distilled water.
NS = Not significantly different.
Each observation is the mean ± SD.
Number in brackets = sample size.

Total deficiency of DHPR, arising from an inborn error of metabolism (IEM), results in neurological impairment due to depletion of the BH_4 cofactor pool (Kaufman, 1985). The evidence presented here indicates that reduction of DHPR activity, in the rat, by inhibitors such as p-tyramine also results in loss of brain biopterins.

The IEM, Tyrosinaemia II (Richner-Hanhart Syndrome), arises from a deficiency of hepatic TAT, resulting in elevated plasma levels of tyrosine, p-tyramine and other tyrosine metabolites (Goldsmith, 1983). Since many of the metabolites of tyrosine are potent inhibitors of DHPR (Shen, 1984), the

mental retardation accompanying this disease may arise in part from depressed CNS catecholamine and serotonin synthesis.

9.5.6. PLASMA TOTAL BIOPTERINS.

Clinically, measurement of plasma biopterins and biopterin derivatives are used as indicators of tissue BH_4 metabolism and diagnosis of malignant hyperphenylalaninaemias. In general decreased plasma biopterins are indicative of a reduction in tissue BH_4 synthesis and elevated levels DHPR deficiency (Leeming *et al.*, 1976; Leeming *et al.*, 1981).

In the rat a similar situation occurs as indicated by the significant elevation of plasma biopterins following phenelzine administration and reduction after bromocriptine dosing. Bromocriptine has been shown to inhibit BH_4 biosynthesis *in vitro* (Edwards, 1987). However, plasma biopterin levels are unaffected by starvation despite reduction of tissue biopterins (Table 9.22)

These observations suggests that changes in the rate of catabolism of BH_4 and/or slight reductions in biosynthesis or salvage within the tissues are not detectable by the monitoring of plasma biopterins. Furthermore under conditions were both salvage and synthesis are depressed it is unlikely that a change will be observable in the plasma. It follows that caution must be taken when using this parameter as a predictor of tissue BH_4 metabolism and the

finding of normal plasma biopterins in some neurological disorders e.g. Rett's syndrome (Blair, 1985), is not indicative of normal tissue BH_4 metabolism.

Table 9.22. Effect of agents and conditions that affect tissue BH_4 metabolism on plasma total biopterins(ug $litre^{-1}$).

Control	25.9	±	2.4	(6)	
Phenelzine	33.9	±	7.4	(6)	P <0.05
Control	22.2	±	3.1	(6)	
Bromocriptine*	14.6	±	2.0	(6)	P <0.001
Control	27.9	±	8.6	(5)	
Starved, 24 hours	32.5	±	15.7	(5)	NS
Control	33.5	±	12.6	(5)	
Starved, 48 hours	24.6	±	6.0	(6)	NS

180g male Wistar rats were used for bromocriptine dosing.
150g male Wistar rats used in all other cases.
Phenelzine was dosed 4mmoles Kg^{-1} I.P., in distilled water.
Controls received 0.3ml distilled water I.P.
Animals were killed 12 hours after dosing.
Bromocriptine was dosed 42umoles Kg^{-1} I.P., in lactate.
Controls received 0.3ml lactate.
Animals dosed once a day for 2 days and killed on day 3.
Animals were starved on grids for the period indicated.
Water was available *ad. lib.* and food for feeding controls.
Each observation is the mean ± SD.
Number in brackets = sample size.
NS = Not significantly different.
* = Assayed by P. Edwards.

9.5.7. DERIVED PTERIN:

Pterin, 2-amino-4-hydroxypteridine, is formed in brain and liver supernatants by acid iodine oxidation and detected by HPLC, under the conditions described in Chapter 2, as a broad fluorescent peak with a retention time 1.23 times greater than that of biopterin.

This derived pterin originates, in part, from oxidation of tissue FH_4, but not other folate derivatives (Fukushima and Nixon, 1980). This is illustrated by the 98% conversion

of FH_4 to pterin and poor conversion of 5-methyltetrahydrofolate ($5CH_3FH_4$) by acid iodine oxidation (Table 9.23).

Table 9.23. Percentage conversion of FH_4 and $5CH_3FH_4$ to pterin by 1 hour acid iodine oxidation.

Folate (20uM in 0.1M HCl)	% Pterin.
FH_4	98.0
$5CH_3FH_4$	0.3

Pterin detected by HPLC.

Depression of the tissue FH_4 pool, by nitrous oxide (N_2O), results in a large decrease in the amount of brain and liver derived pterin (Table 9.24), confirming that this pterin arises from tissue FH_4. However, calculations on a molar basis show that under normal conditions derived pterin levels exceed total FH_4, i.e. mono and polyglutamates, in both the brain and liver by a factor of 2.6 and 1.6 respectively (Edwards, 1987). Additional sources therefore contribute to the formation of pterin by acid iodine oxidation.

Table 9.24. Effect of 24 hour N_2O exposure on tissue derived pterin.

	$He:O_2$	$N_2O:O_2$
Brain (ng g^{-1} wet weight).	60 $\pm$ 5 (6)	34 $\pm$ 4 (6)
	P <0.001	
Liver (ug g^{-1} wet weight).	0.66 $\pm$ 0.13 (6)	0.38 $\pm$ 0.50 (6)
	P <0.001	

150g male Wistar rats were used in each case.
Tests were exposed to 4:1 $N_2O:O_2$ mixture for 24 hours.
Controls were exposed to 4:1 $He:O_2$ for 24 hours.
Food and water available *ad. lib.*
Each observation is expressed as the mean $\pm$ SD.
Number in brackets = sample size.

In vitro, autoxidation of BH_4 results in formation of qBH_2 which is labile and rearranges with considerable side chain loss above pH 6.0 (Matsuura *et al.*, 1986). Autoxidation of BH_4 at pH 7.6 followed by acid iodine oxidation results in a 60% conversion to pterin (Table 9.2). *In vivo*, formation and rearrangement of qBH_2 from BH_4, during amino acid hydroxylation and/or oxidative catabolism, may therefore contribute to the pterin formed in tissue supernatants by acid iodine oxidation.

Following generation of the DHPR inhibitor, p-tyramine - via phenelzine and tyrosine administration, the measured brain and liver derived pterin is elevated (Table 9.25). Inhibition of this salvage pathway will result in an accumulation of qBH_2 and consequently 7,8-dihydropterin. Acid iodine oxidation of the latter forms pterin.

Table 9.25. Effect of phenelzine and p-tyrosine on brain and liver derived pterin

	Control	Phenelzine + p-tyrosine
Brain ($ng\ g^{-1}$ wet weight).	36 ± 5 (6)	51 ± 2 (6)
	P <0.001	
Liver ($ug\ g^{-1}$ wet weight).	0.57 ± 0.11 (6)	1.07 ± 0.22 (6)
	P <0.001	

150g male Wistar rats were used in each case.
Phenelzine administered 0.4mmoles Kg^{-1}.
Tyrosine administered 1mmoles Kg^{-1}.
Controls received 0.3ml water.
All animals starved on grids for total of 30 hours.
Water available *ad. lib.*
Each observation is expressed as the mean ± SD.
Number in brackets = sample size.

Due to the non specific origin of this pterin, alterations in the metabolism of FH_4, following phenelzine administration, may contribute to this elevation.

Starvation also affects derived pterin levels resulting in a consistent elevation in the liver (example, Table 9.26). Again, as tissue FH_4 status is unknown, the precise origin of this increase is unknown. However, the increased catabolism of BH_4 suggested in starvation will result in 7,8-dihydropterin formation and production of pterin after acid iodine oxidation.

Table 9.26. Effect of 48 hours starvation on liver derived pterin (ug g^{-1} wet weight).

Fed	Starved
0.54 $\pm$ 0.08 (6)	1.02 $\pm$ 0.07 (6)
P <0.001	

150g male Wistar rats used in each case.
Animals on grids for period indicated.
Water available *ad. lib.*
Each observation is expressed as the mean $\pm$ SD.
Number in brackets = sample size.

Levels of derived pterin appear to be affected by a number of diverse agents and conditions, but in the absence of information regarding tissue FH_4 levels this parameter is of limited value.

However, coupled with a knowledge of folate status, pterin measurements may be used experimentally to monitor tissue BH_4 catabolism or indicate DHPR inhibition. Many reversible DHPR inhibitors are diluted to below an effective concentration

during preparation of samples for assay of DHPR (Edwards *et al.*, 1987).

In addition, measurement of pterin may be used as an *in vivo* indicator of BH_4 turnover, since salvage appears to be incomplete under normal conditions, as is illustrated by the excess of tissue derived pterin over FH_4.

9.6 GENERAL CONCLUSIONS.

In vitro, tetrahydropterins, such as BH_4, are unstable and are readily oxidized by molecular oxygen, particularly at physiological pH. In addition, the rate of this oxidation is enhanced, over a wide pH range, by the hydroxyl radical and at acid pH, nitrite. Encounter of these oxidizing species *in vivo* may lead to deficiencies of tetrahydropterin cofactors.

However, care must be taken when extrapolating to the *in vivo* situation since a number of protective mechanisms exist for BH_4, i.e tissue levels are maintained by synthesis and salvage pathways. Furthermore, these pathways may be activated in response to prolonged BH_4 catabolism. Reduction in the efficiency of either of these pathways results in a depression of tissue BH_4 as illustrated by the lowering of brain biopterins after generation of the DHPR inhibitor, p-tyramine.

Generation of p-tyramine *in vivo*, as a result of monoamine

oxidase inhibition or tyrosinaemia II, may restrict neurotransmitter synthesis due to loss of the BH_4 cofactor. Such inhibition will also limit the effectiveness of monoamine oxidase inhibitors in the treatment of depression.

In addition to the protective mechanisms indicated, tissue levels of BH_4 and FH_4 may be maintained by GSH, as indicated by the reduction of liver total biopterins following depletion of the GSH pool. GSH would act to limit the oxidative catabolism of tetrahydropterin cofactors, as illustrated by the *in vitro* studies presented.

Acute starvation results in an increased oxidizing environment and depression of tissue biopterins, particularly in the brain. Reduction of brain BH_4 is therefore likely to be a contributing factor to the depression in catecholamine synthesis accompanying this state.

Reduction of tissue biopterins by starvation indicates that careful monitoring of dietary intake should be performed when attempting to establish the *in vivo* effect of an experimental agent on BH_4 metabolism.

Routine measurement of BH_4 metabolism in man is confined to plasma and urine. However, results obtained in this study suggest that measurement of plasma biopterins is not always a true indicator of tissue BH_4 metabolism. Assessment of tissue BH_4 metabolism in neurological disease states should be accompanied, where possible, by measurement of total

biopterins in CSF and liver biopsies. Additionally phenylalanine loading tests should be performed.

Conclusions drawn from the *in vivo* observations in this study are limited by the fact that the rat was used as an experimental model and extensions to the situation in man must bear this fact in mind.

Because of the susceptibility to oxidation by a wide range of oxidizing agents BH_4 may also function as an important tissue antioxidant.

9.7 FURTHER WORK.

(1). The susceptibility of FH_4 and derivatives to oxidation by $\cdot OH$ has been assumed. The effect of generation of this radical, *in vitro*, on tetrahydrofolates should therefore be examined.

(2). *In vitro*, N_2O inhibits the biosynthesis of BH_4 (Hamon et al., 1986). Since exposure *in vivo* for 24 hours has no effect on tissue biopterins or biosynthetic capacity, the effect of prolonged N_2O exposure on rat BH_4 metabolism should be investigated.

(3). $5CH_3FH_4$ and vitamin B_{12} are suggested to be necessary for BH_4 biosynthesis (Hamon et al., 1986). The *in vitro* effects of products derived from the methionine synthetase reaction should be studied on BH_4 biosynthesis e.g., methionine, S-adenosylmethionine and their derivatives.

(4). Further investigation into the role of GSH in BH_4 metabolism. This study should include the use of agents other than L-BSO to deplete the GSH pool, e.g 1,3-bis-(2-chloroethyl)-1-nitrosourea, and the measurement and correlation of tissue GSH levels with biopterins.

(5). Investigation further into dietary factors affecting BH_4 metabolism. Such a study should include the effect of a cysteine free diet, alteration in calorific intake, and dietary restriction but not starvation.

(6). Effect of the free radical generators, 6-hydroxy-DOPA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, on BH_4 metabolism in the normal and GSH depleted rat.

(7). Evaluation of the use of antioxidants for elevation of tissue biopterins by administration of a range of antioxidants, e.g. ascorbate, α-tocopherol, and 2,6-di-t-butyl-4-methylphenol, to starved rats.

(8). Generation of p-tyramine, in the rat, results in loss of brain biopterins and elevation of plasma biopterins due to DHPR inhibition. Since patients with tyrosinaemia II

accumulate p-tyramine and other tyrosine metabolites and are often mentally retarded (Goldsmith, 1983), measurement of plasma biopterins from patients with this condition would therefore be of interest.

(9). Since the pterin formed by acid iodine oxidation is derived from tissue FH_4 and 7,8-dihydropterin, originating from BH_4 oxidation, determination of the tissue FH_4 contribution to this pterin should be established under normal conditions, after starvation and DHPR inhibition.

(10). Derived pterin may reflect the turnover of BH_4, therefore, coupled with a knowledge of folate status, the effect of agents which may affect BH_4 turnover should be examined on derived pterin levels, e.g. L-DOPA.

APPENDIX ONE

REDUCTION OF CHROMATE BY TETRAHYDROBIOPTERIN

APPENDIX ONE.

REDUCTION OF CHROMATE BY TETRAHYDROBIOPTERIN.

A.1.1 INTRODUCTION.

The key step in the carcinogenicity and toxicity of chromium (VI) compounds is the intracellular reduction to chromium (III). The chromium (III) so produced binds to macromolecules, e.g. DNA and protein, or small molecules, e.g. glutathione and nucleotides, thereby interfering with normal cellular function (Connett and Wetterhahn, 1985).

A number of intracellular components have been proposed as sites for chromium (VI) reduction including, the cytochrome P450 and mitochondrial electron transport systems, haeme proteins, flavoproteins and a number of small molecules, e.g. cysteine, glutathione and ascorbate (Connett and Wetterhahn, 1985).

At pH 7.4 and 25°C, the standard redox potential (E_o') for the chromium (VI) - chromium (III) half reaction is + 0.52V and for the dihydropterin - tetrahydropterin couple + 0.15V (Rembold and Buff, 1972; Connett and Wetterhahn, 1985). Therefore, tetrahydrobiopterin (BH_4) should theoretically reduce chromium (VI) at physiological pH. However, the reduction of chromium (VI) at pH 7.4 is determined by kinetic as well as thermodynamic factors and may require proton, as well as electron, transfer (Connett and Wetterhahn, 1985).

Since BH_4 is a widely distributed intracellular component, its effect on chromium (VI) was investigated *in vitro*.

A.1.2. MATERIALS.

Potassium dichromate was obtained from Fisons Ltd, England, and Trizma (tris-(hydroxymethyl)-amino methane) base was supplied by the Sigma Chemical Company, England. 5,6,7,8-tetrahydro-L-biopterin was purchased from Dr. B. Schircks, Switzerland.

A.1.3. METHOD.

The method employed was based on that described by Connett and Wetterhahn (1985). The reduction of chromium (VI) (final concentration 9.0uM in 800mM tris-HCl, pH 7.4) was monitored in the presence and absence of BH_4 (final concentration; 120uM and 240uM in 800mM tris-HCl, pH 7.4) by following the decrease in absorbance of chromium (VI) at 372nm, 37°C, as a function of time with a Schimadzu uv/visible recording double beam spectrophotometer UV-240.

A.1.3. RESULTS AND DISCUSSION.

Under the experimental conditions employed over 96% of the chromium (VI) was in the form of chromate, CrO_4^{2-}, (Connett and Wetterhahn, 1985).

In the absence of BH_4 no reduction of chromate occurred, but in the presence of BH_4 (120uM) 14% of the chromate was

reduced in 4 minutes. The extent of this reduction was increased to 25% by increasing the concentration of BH_4 to 240uM (Table A.1).

Table A.1. Reduction of chromate by tetrahydrobiopterin at pH 7.4, 37° C.

BH_4 concentration. uM.	% Reduction of chromate after 4 minutes.
0	0
120	14
240	25

Initial chromate concentration = 9.0uM.
Buffer = 800mM Tris-HCl.
Each observation is the mean of 3.

The ability of BH_4 to reduce chromate suggests that BH_4, and possibly other tetrahydropterins, may also function as intracellular reductants and play a role in the metabolism of carcinogenic chromium (VI) compounds. However this reduction by BH_4 is likely to be limited, since ascorbate, cysteine and glutathione are, under comparable conditions, better chromate reductants (Connett and Wetterhahn, 1985).

APPENDIX TWO

HYPOCHLORITE OXIDATION OF REDUCED PTERINS

APPENDIX 2

HYPOCHLORITE OXIDATION OF REDUCED PTERINS.

A.2.1 INTRODUCTION.

The hypochlorite ion (OCl^-) is formed, *in vivo*, from hydrogen peroxide (H_2O_2), within activated neutrophils and monocytes by myeloperoxidase:-

$$Cl^- + H_2O_2 \longrightarrow OCl^- + H_2O_2$$

Hypochlorite may form oxidizing species, e.g. molecular oxygen and chlorine, and generation *in vitro* has been shown to oxidize a variety of biologically significant molecules. Formation of hypochlorite is suggested to play a role in the phagocyte oxygen dependent killing of endocytosed bacteria (Babior and Crowley, 1983; Greenwood and Earnshaw, 1984).

Since reduced pterins are prone to oxidation, the effect of hypochlorite on 7,8-dihydrobiopterin (BH_2) and 5-methyl-tetrahydrofolate ($5CH_3FH_4$) was investigated.

A.2.2 MATERIALS.

Sodium hypochlorite was obtained from BDH Ltd., England., 5-methyl-tetrahydrofolate was supplied by Eprova Research Laboratories, Switzerland. 7,8-dihydrobiopterin was obtained from Dr. B. Schircks, Switzerland.

A.2.3 METHOD.

The rate of oxidation of BH_2 and $5CH_3FH_4$ (50uM) was monitored, using a Schimadzu uv/visible recording double beam spectrophotometer UV-240, in the presence and absence of sodium hypochlorite (0.2mM). Rates were determined at pH 1.6 (0.1M HCl) and 7.4 (0.1M phosphate buffer) at 37°C. Pseudo rate contants (k_1) and half lives ($t_{1/2}$) were calculated from the change in absorbance at 275nm and 230nm for BH_2 and 217 and 240nm for $5CH_3FH_4$ at pH 1.6 and 7.4 respectively.

A.2.4 RESULTS AND DISCUSSION.

At pH 1.6 and 7.4, in the absence of hypochlorite, no measurable oxidation of BH_2 occurred over a 30 minute period, due to the slow autoxidation rate of 7,8-dihydropterins (Table 3.5; Chapter 3). No observable autoxidation of $5CH_3FH_4$ was recorded at pH 7.4 despite a detectable rate of oxidation at pH 3.0 (Table 5.1; Chapter 5). Since the rate of $5CH_3FH_4$ autoxidation increases with decreasing acidity, when determined by manometry (Pearson, 1974), this suggests that the first autoxidation product at pH 7.4 has an identical UV spectrum to $5CH_3FH_4$.

In the presence of hypochlorite oxidation of BH_2 and $5CH_3FH_4$ occurred (Table A.2). The rate of this oxidation was greater at pH 7.4 than 1.6.

Table A.2. Oxidation of BH_2 and $5CH_3FH_4$ by hypochlorite at 37° C.

	Air oxidation.		Air oxidation + OCl^-.	
	K_1 (min^{-1})	$t_{1/2}$ (mins)	K_1 (min_{-1})	$t^{1/2}$ (mins)
$5CH_3FH_4$ pH 1.6	*	*	0.0015	60.18
$5CH_3FH_4$ pH 7.4	*	*	0.0353	19.60
BH_2 pH 1.6	*	*	0.0479	14.40
BH_2 pH 7.4	*	*	0.2550	2.71

Each observation is the mean of 3. BH_2 and $5CH_3FH_4$ = 50uM. NaOCl = 0.2mM. * = No rate of oxidation observed in 30mins.

At pH 1.6 protonation of the pterin ring system at N(5) may retard electron abstraction by oxidising species generated from hypochlorite. However, at physiological pH N(5) will not be protonated resulting in an increased rate of oxidation.

Encounter of hypochlorite *in vivo* may result in oxidation of reduced pterins, and the antibacterial action of hypochlorite may arise partly from enhanced catabolism of bacterial tetrahydropterins. The release of neopterin from activated monocytes and macrophages has been suggested to arise from an increased intracellular oxidizing environment (Fuchs et al., 1985). Oxidation of 7,8-dihydroneopterin by hypochlorite may therefore contribute to neopterin formation in monocytes.

APPENDIX THREE

THE EFFECT OF IONIC STRENGTH ON THE AUTOXIDATION RATE OF TETRAHYDROBIOPTERIN

APPENDIX THREE

THE EFFECT OF IONIC STRENGTH ON THE AUTOXIDATION RATE OF TETRAHYDROBIOPTERIN

Ionic strength (I) is defined as:-

$$I = 1/2 \left(Z_i^2 \; C_i \right) \qquad (1)$$

Where Z_i = valence of ion i
C_i = Concentration (molar) of ion i.

The ionic strength of a solution will affect the rate of reaction between two reactants, A and B, in accordance with the Bronsted-Bjerrum equation:-

$$\text{Log } k = \log k_o + 2Q \; Z_A \; Z_B \sqrt{I} \qquad (2)$$

Where ko = rate constant in absence of ionic effect.
k = rate constant in presence of ionic effect
Q = Debye-Huckel coefficient, 0.509.
Z_A = Charge on reactant A.
Z_B = Charge on reactant B.

Equation (2) predicts a positive salt effect if A and B carry charges of the same sign; a negative effect if of opposite sign; and a zero effect if both reactants are uncharged.

Increasing the ionic strength of the medium for reactants of like sign accelerates the reaction rate. This is due to association with oppositely charged ions which reduces the electrostatic barrier for the formation of the transition state. Conversely, if reactants are of opposite charge, increasing the ionic strength reduces the number of effective collisions due to hindrance (Davies, 1961).

The ionic strength for each buffer system used to determine the rate of tetrahydrobiopterin (BH_4) autoxidation was calculated using equation (1) (Table A.3a).

Table A.3a. Ionic strengths of buffer systems used to determine the autoxidation rate of BH_4.

Buffer system.	Ionic strength. (M)
0.1M HCl, pH 1.6.	0.025
0.1M acetic acid, pH 3.0.	0.001
0.1M acetate, pH 3.8.	0.010
0.1M acetate, pH 4.6.	0.040
0.1M acetate, pH 5.3.	0.070
0.075M phosphate, pH 7.6.	0.180

The rate of BH_4 autoxidation is reported to increase with pH (Chapter 3), but ionic strength also increases with pH, i.e. there may be a positive salt effect.

In order to assess the contribution of ionic strength to BH_4 autoxidation the following equation was derived from (2):-

$$\text{Log } k_2/k_1 = 2Q\ Z_A\ Z_B \left(\sqrt{I_2} - \sqrt{I_1} \right) \qquad (3)$$

Where K_1 = rate constant at lower ionic strength.
K_2 = rate constant at higher ionic strength.
I_1 = lower ionic strength.
I_2 = higher ionic strength.

For the purpose of testing a positive effect on BH_4 autoxidation $2Q\ Z_A\ Z_B$ was assumed = 1.

The ratio obtained from (3), assumes an ionic effect in the absence of any other factors. Ratios obtained from this equation were therefore compared with actual ratios derived

from experimental data (Table A.3b).

Table A.3b. Comparison of calculated ratio of rate constants, assuming a positive salt effect, with actual ratios obtained.

pH	Observed rate constant (min^{-1})	I (M)	Calculated K_2/K_1 ratio	Actual ratio.
3.8	0.0230 (k_1)	0.010		
5.3	0.0759 (k_2)	0.070	1.46	3.30
3.0	0.0084	0.001		
5.3	0.0759	0.070	1.71	9.03
3.0	0.0084	0.001		
7.6	0.0870	0.180	2.46	10.35
1.6	0.0028	0.025		
7.6	0.0870	0.180	1.84	31.07

pH was achieved using the buffer systems in Table A.3a.

In all cases the ratio of rate constants obtained experimentally exceeds that calculated for a positive increase arising from an ionic effect. This suggests an additional factor is responsible for determining the rate of BH_4 autoxidation i.e. degree of protonation of the pterin ring system as a function of pH.

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