TETRAHYDROBIOPTERIN DEFICIENCY AND BRAIN NITRIC OXIDE METABOLISM

By

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A thesis submitted as partial fulfilment for
the degree of Doctor of Philosophy
in the Faculty of Science at the
University of London.

March 1997

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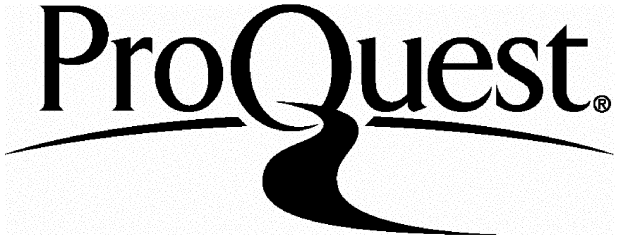

Abstract.

Tetrahydrobiopterin is an essential cofactor for the aromatic amino acid mono-oxygenase group of enzymes. Inborn errors of tetrahydrobiopterin metabolism result in hyperphenylalaninaemia and impaired catecholamine and serotonin turnover. Tetrahydrobiopterin is also a cofactor for all known isoforms of nitric oxide synthase (NOS). The effect of tetrahydrobiopterin deficiency on brain nitric oxide metabolism has to date been given little consideration. In this thesis the effect of tetrahydrobiopterin deficiency on brain nitric oxide metabolism has been studied using a mouse model of tetrahydrobiopterin deficiency, the *hph-1* mouse.

Tetrahydrobiopterin was measured in 10 and 30 day old mice in whole brain and cerebellum. At both age points there was a significant ~50% reduction in tetrahydrobiopterin content for whole brain and cerebellum in the *hph-1* mouse compared to corresponding control mice.

NOS activity was measured in whole brain from 10 and 30 day old *hph-1* and control mice. No difference was observed in enzyme activity when tetrahydrobiopterin was included in the incubation medium. However, omission of tetrahydrobiopterin from the reaction buffer resulted in significantly lower NOS activity in the *hph-1* mouse group compared to controls. Tetrahy~ drobiopterin was also shown to have a potent effect on the affinity of brain NOS for arginine. The K_m for arginine was virtually identical for the control and *hph-1* mouse when tetrahydrobiopterin was included in the reaction buffer. In the absence of cofactor, the K_m for arginine was three fold greater for control and five fold higher for *hph-1* preparations.

The accumulation of cGMP from slices prepared from the cerebellum was measured in both groups of mice at both 10 and 30 days using the glutamate analogue, kainate. In the 10 day old *hph-1* mouse there was a significant 50% reduction in cGMP levels under basal and stimulated conditions. In the 30 day old *hph-1* mouse there was a significant 30% reduction in cGMP accumulation in both basal and activated states.

Whole brain amino acid levels were measured. In the 10 day old *hph-1* mouse

confounding hyperphenylalaninaemia may affect the availability of the NOS substrate, arginine. In the 30 day old *hph-1* mouse, which has normal phenylalanine levels, reduced citrulline levels may indicate reduced NOS activity.

Reduced levels of tetrahydrobiopterin and also arginine, have been shown to lead to superoxide formation by nitric oxide synthase. Superoxide can react with nitric oxide to form the oxidising species, peroxynitrite, which has been shown to damage of the mitochondrial electron transport chain. Mitochondrial function together with the anti-oxidant, glutathione, were analysed in both *hph-1* and control mice at 10 and 30 days to measure oxidative stress. However, no differences were observed between the two groups.

In summary, partial deficiency of tetrahydrobiopterin appears to lead to impaired brain NOS function leading to an impairment of the nitric oxide/cGMP pathway.

Acknowledgements.

I would like to thank my two supervisors, Dr Simon Heales and Professor John Clark for allowing me this opportunity to study for a PhD and for their advice, encouragement and support during the duration of this project. As one of the grantholders for this project I would like to thank Dr John Land for his help, guidance and being available whenever I needed his advice.

I would like to thank all the members of the Department of Neurochemistry for their support during the last three years but I would particularly like to thank Dr Jane Barker and Dr Roger Hurst. This thesis could not have been prepared with out the expert assistance of June Smalley who helped with the more complicated figures.

I would like to extend my gratitude to the staff of the Denny Brown Laboratories, especially John Frogley, Janet Cunningham and Wendy Rooke.

I must thank Mr Tony Briddon and Mr Robert Courtney for their expertise and help in the analysis of whole brain amino acids and brain slice morphology respectively.

Finally, I must thank Dr Isabel Smith, Dr Mark Goss-Sampson and Dr David Müller for setting me on the right path.

I am indebted to the Brain Research Trust for their generous funding of this project.

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Abbreviations

BH_4	6,R-(5,6,7,8)-L-erythro-tetrahydrobiopterin
CSF	Cerebrospinal fluid
cGMP	Guanosine 3',5'-cyclic monophosphate
DRD	L-Dopa responsive dystonia
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GTP	Guanosine 3',5'-triphosphate
5HIAA	5-hydroxyindoleacetic acid
HPLC	High performance liquid chromatography
5HTP	5-hydroxy-tryptophan
HVA	Homovanillic acid
IFN-γ	Inter feron-γ
L-Dopa	L-dihydroxyphenylalanine
LPS	Lipopolysaccharide
$ONOO^-$	Peroxynitrite
NO	Nitric oxide
NOS	Nitric oxide synthase
P-450	Cytochrome P-450
SNAP	S-nitroso-N-acetyl-D-L-penicillamine
SNOG	S-nitroso-L-glutathione
DOPAC	Dihydroxyphenylacetic acid
IUPAC	(International Union for Pure and Applied Chemistry)

Chapter 1.
Introduction.

1. Introduction

1.1. Tetrahydrobiopterin

1.1.1. Pteridines

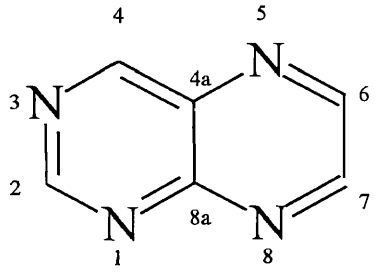
Pteridines are a widely distributed class of naturally occurring heterocyclic compounds first reported as the wing pigment of the common English brimstone butterfly and the cabbage white butterfly (Hopkins, 1889). In 1940 work by Purrmann elucidated the structure of the three butterfly wing pigments and the name "pterine" from the Greek word for wing "pteron", was coined in 1941 by Wieland. (For review, see Pfleiderer, 1987) Pteridine consists of a condensed pyrazine-(2,3-d) pyrimidine ring system and is numbered according to IUPAC rules (Figure 1.1.).

Most naturally occurring pteridines have an amino group in the 2 position and a oxy group in the 4 position, these 2-amino-4-oxypteridine derivatives are given the term "pterin" as a final syllable (Figure 1.1.) (Pfleiderer, 1984). Structural variations of pterin are limited to substituents in position 6 and 7 on the ring. The class of compounds is further subdivided into either unconjugated where the substituent at carbon 6 is a simple alkyl group or conjugated where the substituent is more complex (Pfleiderer, 1984).

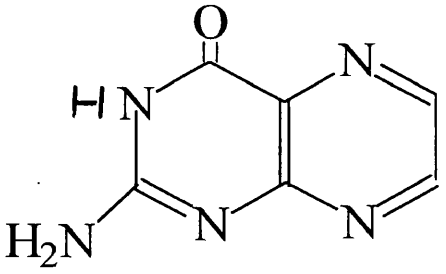
1.1.2. Biological importance of pterins

Three groups of pterins are utilized as enzyme cofactors by man; the conjugated pterins, eg folates and the unconjugated pterins eg tetrahydrobiopterin (BH_4) and the molybdenum cofactor (Figure 1.2.). Folate and BH_4 can exist in a number of different oxidation states ie tetrahydro-, dihydro- and fully oxidised forms (Figure 1.3.). Both cofactors are only active in the fully reduced tetrahydro- form (Kaufman, 1987; Steegers-Theunissen, 1995).

Folates are an essential requirement of the human diet and are only synthesised in plants and micro-organisms from guanosine triphosphate (GTP). Folate comprises a pterin, *p*-aminobenzoic acid and glutamic acid. In man, tetrahydrofolate acts as a substrate in the transfer of one carbon moieties and thereby plays an essential role in the synthesis of amino acids such as methionine, nucleic acids and provision of methyl



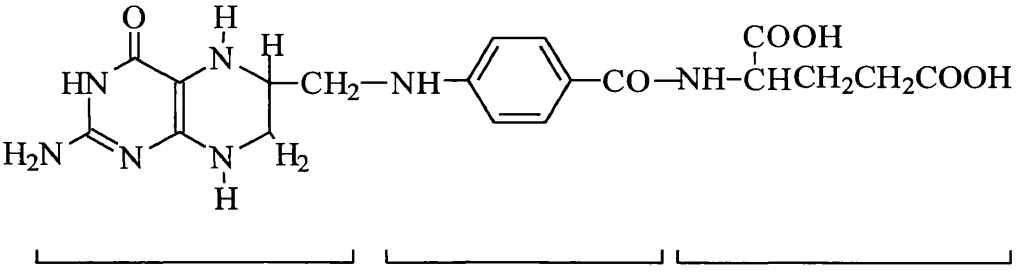
Pteridine



Pterin

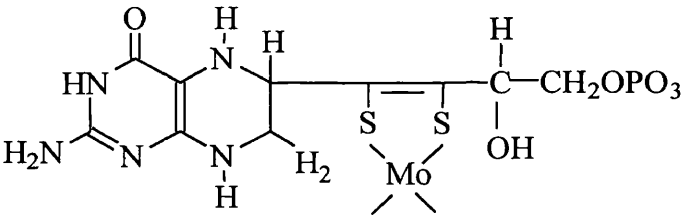
The atoms of pteridine are depicted with the numbering according to IUPAC rules. The numbering sequence is common for all pteridine derivatives.

Figure 1.1. Structure of pteridine and pterin.

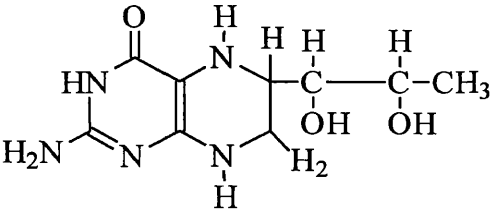


2-amino-4-oxy-6-methyl pterin | p-amino benzoic acid | glutamic acid

Tetrahydrofolate

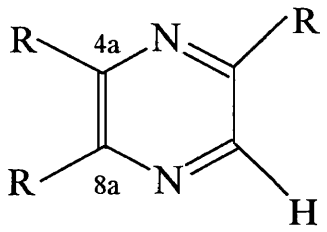


Molybdenum cofactor

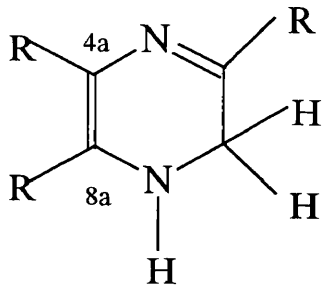


L-erythro-5,6,7,8,-tetrahydrobiopterin

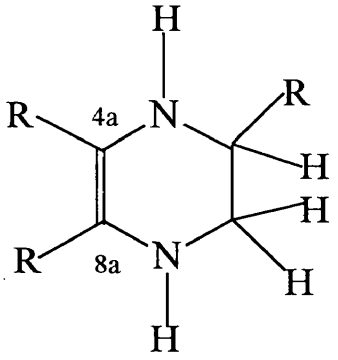
Figure 1.2. Structural formulae of the pterin biological cofactors.



Oxidised pteridine group



7,8-dihydropteridine group



5,6,7,8-tetrahydropteridine group

Figure 1.3. Oxidation states of tetrahydropterins.

groups for methylation reactions (Steegers-Thenunissen, 1995).

The molybdenum cofactor is a position 6 substituted pterin found in a variety of molybdenum containing enzymes such as xanthine oxidase (EC 1.1.3.22), nitrate reductase (EC 1.6.6.2), formate dehydrogenase (EC 1.2.1.2) and aldehyde oxidase (EC 1.1.3.13) (Nichol et al., 1985). These enzymes are widely distributed in bacteria, fungi, plants and animals (Nichol et al., 1985). The cofactor has not yet been isolated due to it's extreme lability when released from it's protein environment but results of X-ray absorbance on enzymes containing the molybdenum cofactor showed ligation of molybdenum to two or three thiolate ligands in all cases (Rajagopalan et al., 1993).

The third type of pterin cofactor is BH_4. As in folate it is synthesised from GTP but synthesis is complete in mammalian cells (Duch and Smith, 1991). This cofactor can exist in the fully reduced tetrahydro-, dihydro and the fully oxidised biopterin forms. Biopterin does not occur except as a degradation product *in vivo*. Total biopterin, usually abbreviated to biopterin, is the collective name for tetrahydro-, dihyro and biopterin (Smith, 1985).

Biopterin was first isolated and synthesised by Patterson et al. (1956). However, it was culmination of several years research that BH_4 was shown to be the obligatory cofactor for phenylalanine hydroxylase (EC 1.14.16.1) (Kaufman, 1958; Kaufman and Levenberg, 1959; Kaufman, 1964). BH_4 is also an essential cofactor for tyrosine hydroxylase (EC 1.14.16.2) (Brenneman and Kaufman, 1964), tryptophan hydroxylase (EC 1.14.16.4) (Friedman et al., 1972), glycerol ether mono-oxygenase (EC 1.14.16.5) (Tietz et al., 1964) and nitric oxide synthase (EC 1.14.13.39) (Tayeh and Merletta, 1989).

1.1.3. Biosynthesis of tetrahydrobiopterin

The biosynthesis of BH_4 has been the subject of vigorous investigation. It was originally thought that BH_4 might be derived from dietary folates, a hypothesis supported by the fact that folate antagonists, such as methotrexate could inhibit phenylalanine hydroxylation (Kaufman and Levenburg, 1959). However, failure of

dietary folate deficiency to decrease hepatic levels of phenylalanine hydroxylase cofactor and the failure of large doses of folate to increase urinary output of the stable degradation product biopterin indicated that BH_4 was not derived from dietary folate (Fukushima and Shiota, 1972). BH_4 was shown to be synthesised from GTP with the initial reaction catalysed by GTP cyclohydrolase I (EC 3.5.4.16) leading to the formation of 7,8-dihydroneopterin triphosphate (Fukushima and Shiota, 1974; 1975; Yim and Brown, 1976). The pathway for the synthesis of BH_4 is shown in Figure 1.4.

GTP cyclohydrolase I catalyses the first reaction of the biosynthetic pathway for BH_4. The enzyme has no apparent need for any cofactors (Nichol et al., 1985). It has been purified from a number of sources; *Drosophila,* 575 kDa (Weisberg and O'Donnell, 1986), rat, 300 kDa (Hatakeyama et al., 1989) and human, 440-453 kDa (Shen et al., 1989). The purified enzyme is unstable (Duch and Smith, 1991). Three different cDNA forms have been reported for human GTP cyclohydrolase I (Togari et al., 1992), however, only the recombinant protein corresponding to the longest reading frame can catalyse the conversion of GTP into dihydroneopterin triphosphate (Gütlich et al., 1994a). In organisms that can synthesise folate, dihydroneopterin triphosphate is also the first intermediate in the formation of dihydrofolate and GTP cyclohydrolase I is also the first enzyme in the biosynthetic pathway of folate (Nichol et al., 1985). The mechanism of the reaction is proposed to be imidazole ring opening followed by loss of carbon 8 of GTP as formate (Burg and Brown, 1968). This compound undergoes an Amadori type rearrangement whereby carbon 1 of the sugar is reduced and carbon 2 is oxidised to form a carbonyl group (Burg and Brown, 1968). The intermediate has an open chain structure (Burg and Brown, 1968). Closure of the pyrazine ring by formation of the N^5-C^6 double bond yields 7,8-dihydroneopterin triphosphate (Nicholl et al, 1985).

The conversion of 7,8-dihydroneopterin triphosphate to 6-pyruvoyl tetrahydropterin involves the removal of the phosphate groups and is catalysed by the enzyme 6-pyruvoyl tetrahydropterin synthase (EC 4.6.1.10) which was originally known as phosphate eliminating enzyme. This reaction requires magnesium ions as a cofactor. This enzyme has been purified from a number of sources including

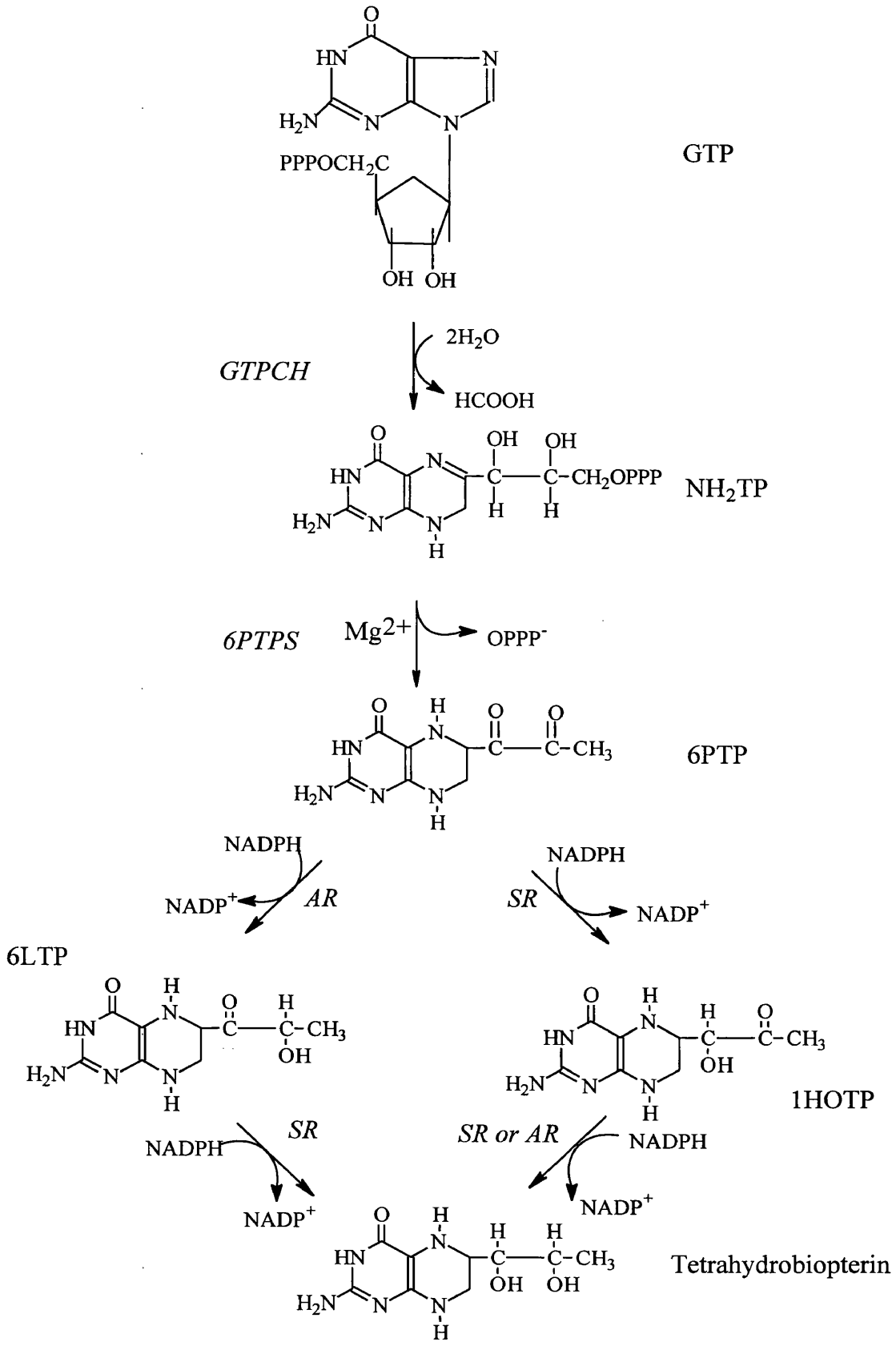


Figure 1.4. Biosynthesis pathway of tetrahydrobiopterin.

Figure 1.4. Biosynthesis pathway of tetrahydrobiopterin.

Abbreviations used are as folows:

GTP:	guanosine triphosphate
NH_2TP:	7,8-dihydroneopterin triphosphate
6PTP:	6-pyruvoyl tetrahydropterin
6LTP:	6-lactoyltetrahydropterin
1HOTP:	1'-hydroxy-2'-oxypropyl tetrahydropterin
GTPCH:	GTP cyclohydrolase I
6PTPS:	6-pyruvoyl tetrahydropterin synthase
AR:	Aldose reductase
SR:	Sepiapterin reductase

Defects in the metabolic pathway have been identified at the GTP cyclohydrolase I step and 6-pyruvoyl tetrahydropterin synthase step.

Drosophila (Park et al., 1990) and human liver (Takikawa et al., 1986). Both forms of the enzyme have a molecular weight of 83 kDa.

The next steps in the biosynthesis of BH_4 involve the NADPH dependent reduction of the keto groups on the side chain. Using the sepiapterin reductase (EC 1.1.1153)inhibitor, N-acetylserotonin, Smith (1987) has shown that this enzyme is capable of catalysing both of these reduction steps. The intermediate compound in this reaction is 1'-hydroxy-2'-oxypropyltetrahydropterin. Therefore, biosynthesis of BH_4 seems to proceed via GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase.

However, a further compound, 6-lactoyl tetrahydropterin, has also been proposed as an intermediate. Sepiapterin reductase can catalyse the formation of BH_4 from this intermediate but it cannot catalyse it's formation (Smith 1987). The formation of this intermediate was shown to be catalysed by an enzyme initially known as pyruvoyltetrahydropterin reductase but now known as aldose reductase (no EC number listed) (Steinerstauch et al, 1989; Milstein and Kaufman, 1989). Sepiapterin reductase is essential for BH_4 biosynthesis but it has been suggested that the conversion of 6-pyruvoyl tetrahydropterin by aldose reductase and sepiapterin reductase may be tissue specific (Milstien and Kaufman, 1983; Duch and Smith, 1991). In rat brain aldose reductase accounts for approximately 60% of BH_4 synthesis whereas in liver aldose reductase was shown to be unnecessary for BH_4 production (Milstien and Kaufman, 1989).

1.1.4. Regulation of the biosynthesis pathway.

Administration of phenylalanine has been reported to stimulate whole blood total biopterin levels in humans and rats but tyrosine and tryptophan loading does not produce the same effect (Leeming et al, 1976a; Milstein and Kaufman, 1983). Administration of labelled guanosine, in order to label GTP, together with phenylalanine led to formation of labelled BH_4 suggesting that phenylalanine stimulated BH_4 synthesis (Milstein and Kaufman, 1983). The regulation of the biosynthetic pathway is largely regulated by changes in GTP cyclohydrolase I activity,

the rate limiting enzyme in the biosynthesis of BH_4, since changes in it's activity usually mediate or closely correlate with changes in BH_4 (Duch and Smith, 1991). The mechanism of the stimulation of GTP cyclohydrolase I activity was complicated as phenylalanine was found to have no effect on GTP cyclohydrolase activity *in vitro* or on any other of the biosynthetic enzymes of the BH_4 biosynthetic pathway (Milstien et al., 1996). Activity of GTP cyclohydrolase I is thought to be regulated by feedback inhibition. In crude rat homogenates enzyme activity is inhibited by BH_4 (Bellahsene et al., 1984). This inhibition was found to depend specifically on BH_4 and the presence of another protein, p35 (Harada et al., 1993). Inhibition of hepatic GTP cyclohydrolase I occurs via formation of a complex between p35, BH_4 and GTP cyclohydrolase I (Harada et al., 1993). Inhibition occurs by reducing the V_{max} of the enzyme without affecting the affinity for GTP. Furthermore, this inhibition is reversed by phenylalanine which changes the GTP cyclohydrolase I enzyme to an active form (Harada et al., 1993). Thus, GTP cyclohydrolase I activity could be increased following a phenylalanine load leading to BH_4 synthesis thereby augmenting the cofactor available for phenylalanine hydroxylase activity. When hepatic phenylalanine levels fall, the inhibition of the BH_4-p35-GTP cyclohydrolase I complex is reduced and enzyme activity is again suppressed (Figure 1.5.). Recently, the presence of the regulatory protein has been demonstrated in a variety of other tissues including kidney, testis, heart and brain suggesting that this protein may play a role in the regulation of not only phenylalanine hydroxylase metabolism but also in the production of biogenic amine neurotransmitters (Milstien et al., 1996).

In the adrenal medulla, BH_4 is required for tyrosine hydroxylation in catecholamine synthesis and BH_4 levels are regulated by the splanchnic nerve through GTP cyclohydrolase I (Nicholl et al., 1987). These increases are inhibited by cycloheximide and, therefore, the increase in GTP cyclohydrolase I activity is due to new protein synthesis. Recently, reserpine, which had previously been found to increase BH_4 levels and GTP cyclohydrolase I activity in the adrenal cortex, was shown to increase enzyme levels in peripheral and central neurones (Abou-Donia and Viveros, 1981; Hirayama et al., 1993). Reserpine acts on the adrenal medulla by blocking the uptake of catecholamines into the storage vesicles (Viveros et al., 1981).

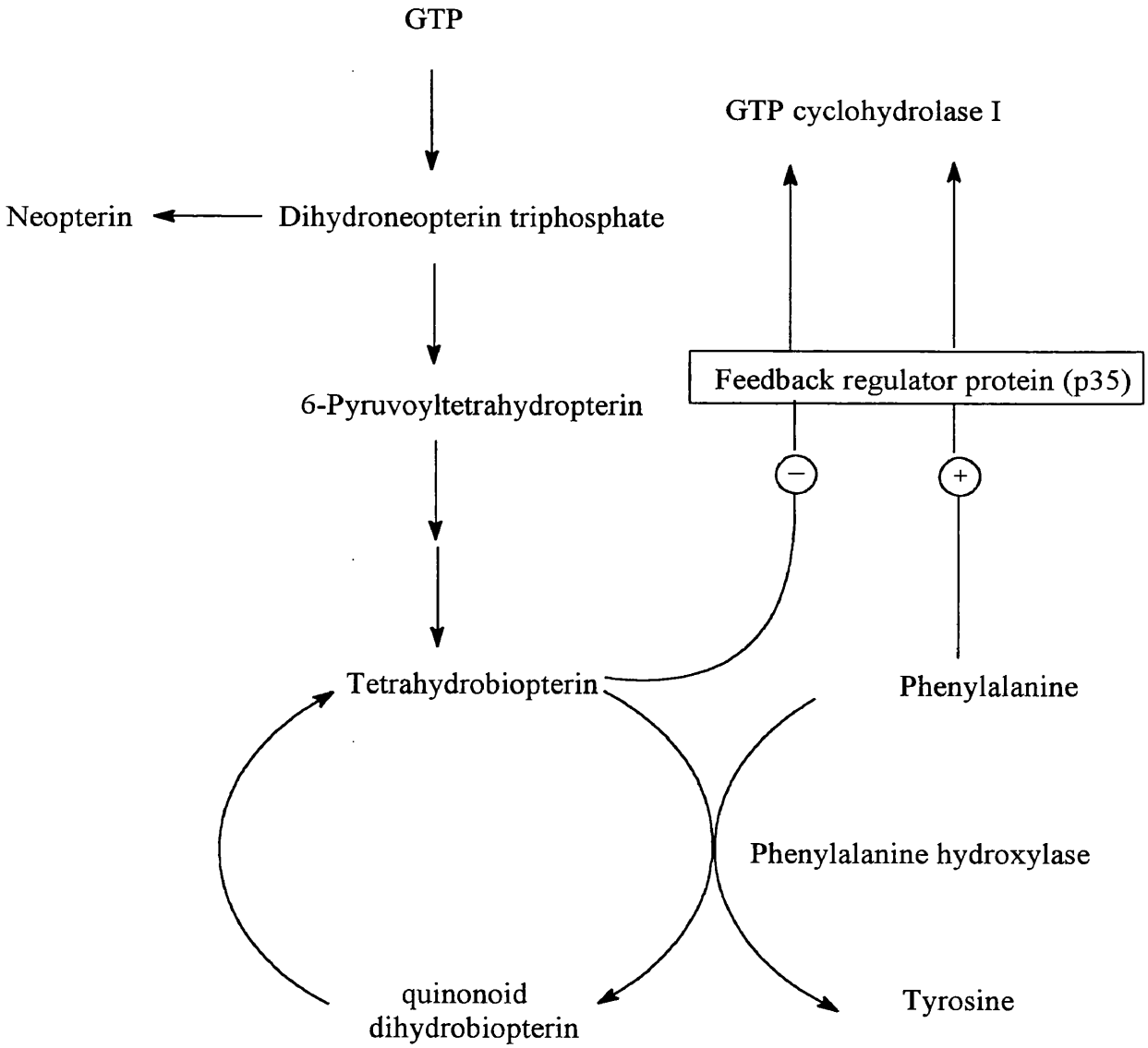


Figure 1.5. The feedback mechanism of control of tetrahydrobiopterin synthesis.

Decrease in catecholamines leads to an increase in GTP cyclohydrolase I levels and subsequent increase in BH_4 concentration (Abou-Donia et al., 1986; Nicholl et al., 1987). Catecholamines and serotonin have also been suggested to be competitive inhibitors of sepiapterin reductase but do not affect any other enzyme in the BH_4 biosynthetic pathway (Katoh et al., 1983). High concentration of catecholamines or serotonin may play a role in the regulation of BH_4 synthesis through direct inhibition of this enzyme (Katoh et al., 1983).

Another mechanism for control of BH_4 synthesis is by cytokine stimulation. Research on stimulation of BH_4 synthesis by cytokines began with the finding that patients with tumours and viral infections excreted neopterin in the urine (for review see Fuchs et al., 1993). The source of the neopterin was interferon-γ (IFN-γ) stimulated macrophages (Huber et al., 1984). Tumour necrosis factor-α (TNF-α) has no stimulatory effect but synergistically amplifies neopterin formation induced by IFN-γ (Fuchs et al., 1993). IFN-γ acts by inducing GTP cyclohydrolase I, most likely to be by *de novo* enzyme synthesis (Werner et al., 1993). 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase are constitutively present in the cells investigated (Werner et al., 1993). Due to a relative deficiency of 6-pyruvoyl-tetrahydropterin synthase in the macrophages of humans and primates an excess of neopterin triphosphate is formed in macrophages (Figure 1.6.) (Fuchs et al., 1993). Phosphatases cleave the phosphate groups leading to an accumulation of 7,8-dihydroneopterin within the cells (Werner et al., 1993). Between 40-90% of the total neopterins in body fluids are present in this form (Smith, 1988) and may explain the high levels of total neopterin seen in infectious and pathological conditions (Fuchs et al., 1993). However, whereas neopterin is exported from the cell, BH_4 is efficiently retained within the cell (Werner et al., 1993). In contrast to humans, 6-pyruvoyl-tetrahydropterin synthase is not limiting in rodents and stimulation of GTP cyclohydrolase I with lipopolysaccharide (LPS) leads to production of BH_4 in brain, liver, spleen and adrenals of rats (Werner-Felmayer et al., 1993). Pterin synthesis, linked to cytokine stimulation, is suggested to be linked to inducible nitric oxide synthase (iNOS) synthesis as this is also induced by a wide range of cytokines and bacterial products (Knowles and Moncada, 1994). In macrophages, BH_4 produced by

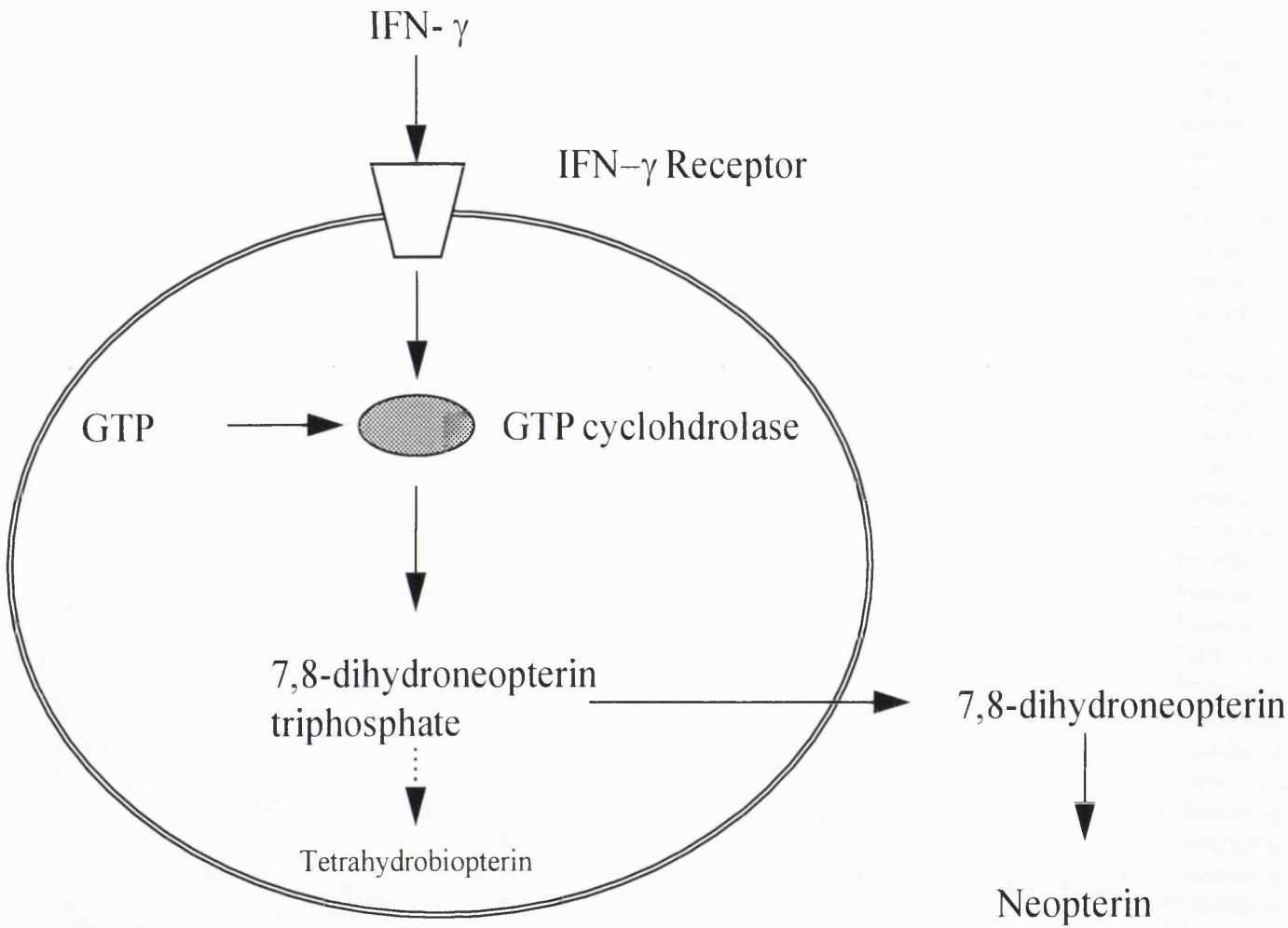


Figure 1.6. GTP cyclohydrolase I stimulation and neopterin formation in macrophages. Adapted from Fuchs et al. (1993).

cytokine stimulation may, therefore, play a role in iNOS activity (Mayer and Werner, 1995). In humans and primates, BH_4 is produced by cytokine stimulation of GTP cyclohydrolase I but also an excess of total neopterin which may be involved in enhancing the effects of reactive oxygen species in antimicrobial toxicity (Fuchs et al., 1993). Various human tumour cell lines including kidney, liver, fibroblast and glio-blastoma have also been shown to synthesise BH_4 as well as neopterin following cytokine stimulation (Werner et al., 1989).

1.1.5. Catabolism of Tetrahydrobiopterin.

Turnover of the dihydro- and tetrahydro-forms is linked to a catabolic pathway. During oxidation of BH_4 quinonoid dihydrobiopterin is formed (Figure 1.7.), this may be salvaged by dihydropteridine reductase to form BH_4 (Figure 1.10.). However, tautomerisation of quinonoid dihydrobiopterin forms 7,8-dihydrobiopterin which will give rise to biopterin after further oxidation (Figure 1.7.) (Rembold et al., 1971). An alternative mechanism for the catabolism of BH_4 is via cleavage of the dihydroxypropyl side chain by an unspecific oxidation reaction (Figure 1.7.) (Rembold et al., 1969). The resultant 7,8-dihydropterin can be oxidised by xanthine oxidase (EC 1.2.3.2) or it can serve as a substrate for pterin deaminase (EC 3.5.4.11) (Figure 1.7.) (Rembold, 1982). Oxidation by xanthine oxidase results in formation of xanthopterin, leucopterin or isoxanthopterin (Figure 1.7.) (Rembold et al., 1982). Pterin deaminase deaminates 7,8 dihydropterin, further oxidation by xanthine oxidase results in formation of lumazine, 7-hydroxy-lumazine or 7,8-dihydroxy-lumazine (Figure 1.7.) (Rembold et al., 1982). End products of BH_4 catabolism are excreted in the urine.

1.1.6. Cofactor role in tetrahydrobiopterin dependent mono-oxygenases.

BH_4 is the established cofactor for the aromatic amino acid mono-oxygenase group of enzymes. The first metabolic role for BH_4 was established as the essential cofactor for phenylalanine hydroxylase in the hepatic conversion of phenylalanine to tyrosine (Figure 1.8.) (Kaufman, 1958). It was later established as the cofactor for tyrosine hydroxylase in the conversion of tyrosine to L-dihydroxyphenyalanine (L-Dopa) (Figure 1.8.) (Brenneman and Kaufman, 1964). Tyrosine hydroxylase is primarily a soluble enzyme located in the cytosol of catecholamine containing neuronal processes

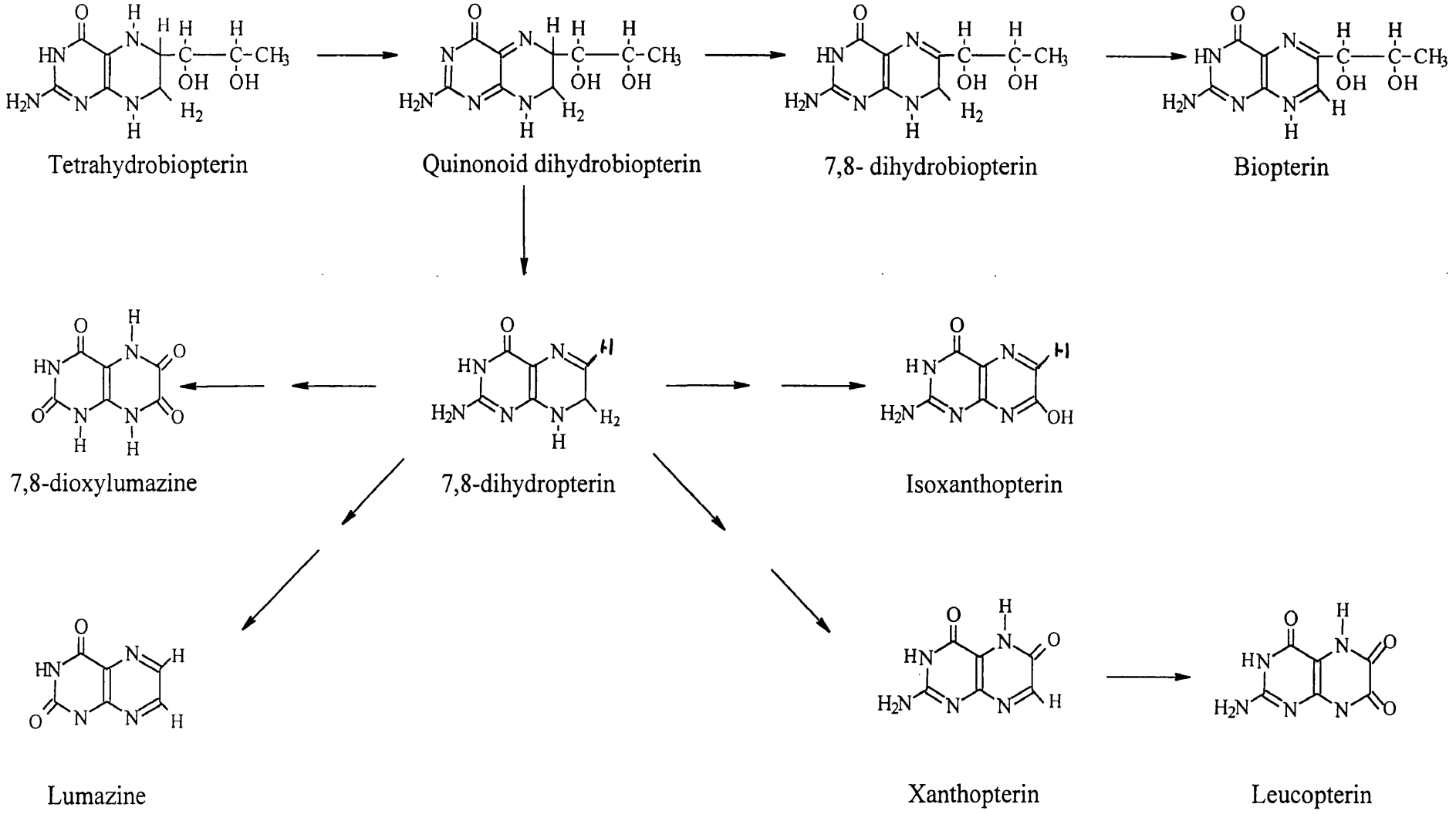


Figure 1.7. Main catabolic pathways of tetrahydrobiopterin.

Figure 1.7. Main catabolic pathways of tetrahydrobiopterin.

Tautomerisation of quinonoid dihydrobiopterin gives rise to 7,8-dihydrobiopterin, further oxidation results in biopterin.

Cleavage of the dihydroxpropyl side chain is by a non enzymatic oxidation and results in 7,8-dihydropterin (Rembold et al., 1969). Deamination by pterin deaminase is followed by conversion to 6 hydroxy derivatives by xanthine oxidase (Rembold et al., 1971). Further oxidation by xanthine oxidase results in formation of 7,8-dioxylumazine (Rembold et al., 1971). The action of xanthine oxidase on non-hydrogenated pterins results in lumazine (Rembold et al., 1971).

In the absence of a deaminating enzyme the action of xanthine oxidase results in formation of isoxanthopterin, xanthopterin and leucopterin.

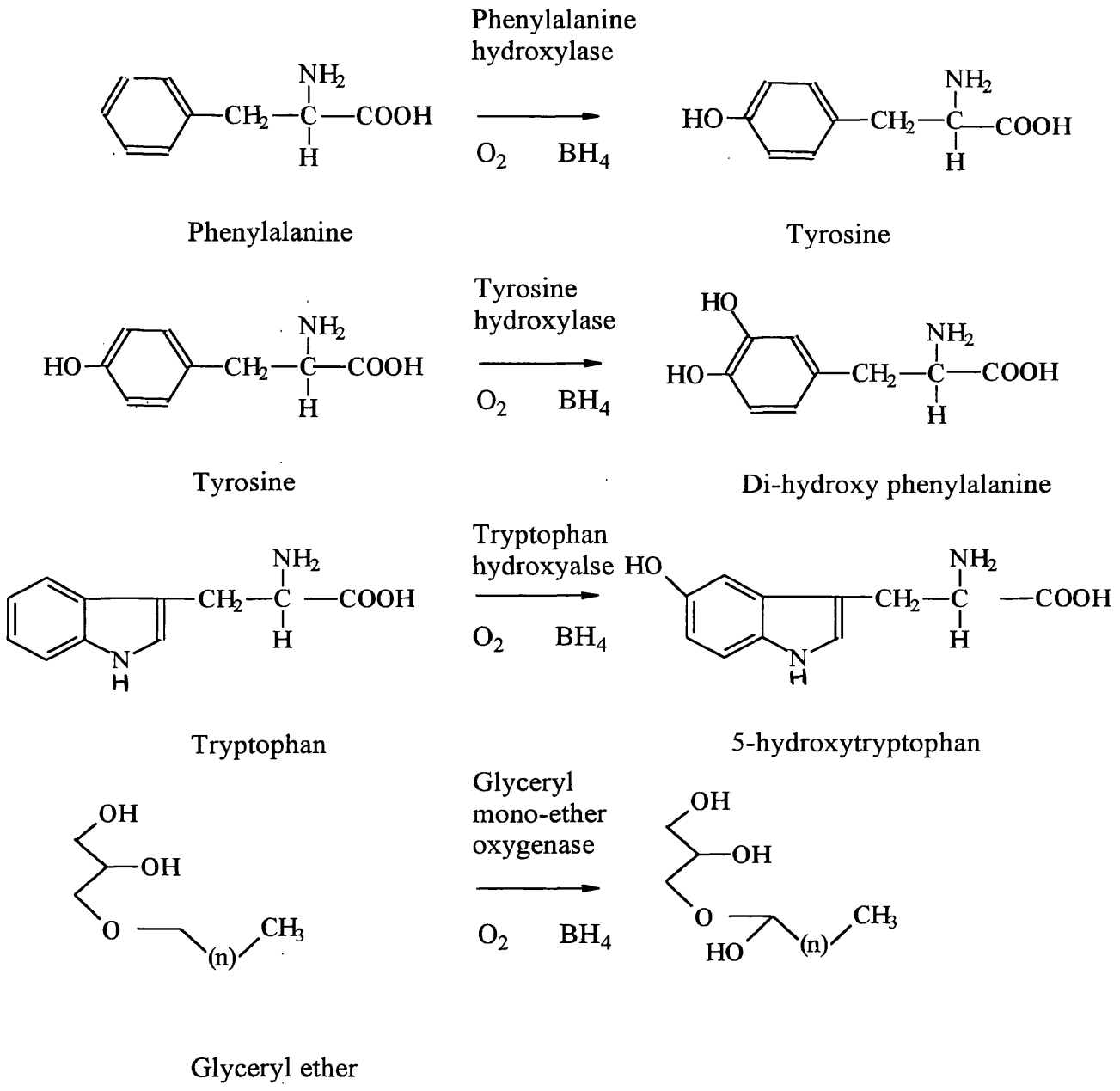


Figure 1.8. Tetrahydrobiopterin dependent hydroxylases.

(Weiner and Molinoff, 1994). L-dopa is the precursor of dopamine, noradrenaline (norepinephrine) and adrenaline (epinephrine) (Figure 1.9.). BH_4 is also the established cofactor for tryptophan hydroxylase located in the serotonergic neurones, in the conversion of tryptophan to 5-hydroxy-tryptophan (5HTP) (Figure 1.8.) (Friedman et al., 1972). 5HTP is the precursor for serotonin (Figure 1.9.).

The cofactor role of BH_4 has only been studied in detail for phenylalanine hydroxylase and is related to its ability to activate molecular oxygen. The detail of the reaction was studied using the synthetic tetrahydropterin analogue, 6-methyl-tetrahydropterin. It is suggested that BH_4 provides the electrons to activate molecular oxygen by formation of a 4a-hydroperoxy-tetrahydropterin which links to an iron atom at the active site of phenylalanine hydroxylase (Hufton et al., 1995). Formation of this hydroxylation intermediate is followed by oxygen atom transfer to phenylalanine. During this reaction phenylalanine is oxidised to tyrosine and BH_4 is oxidised to the corresponding 4a-hydroxytetrahydropterin (Figure 1.10.). At neutral pH this product breaks down to form quinonoid dihydropterin. An enzyme, 4a-carbinolamine dehydratase has recently been discovered that catalyses this reaction (Citron et al., 1992; Thön y et al., 1993). Quinonoid dihydrobiopterin is then reduced back to BH_4 by dihydropteridine reductase (Figure 1.10.) (Craine et al., 1972). It has been suggested that dihydropteridine reductase may play a role in the conversion of quinonoid species of folate to tetrahydrofolates *in vivo* and thus may play a role in folate metabolism (Pollock and Kaufman, 1978).

1.1.7. Other functions of tetrahydrobiopterin.

A further cofactor role for BH_4 was suggested as the cofactor for the glyceryl ether mono-oxygenase (Figure 1.8.) (Tietz et al., 1964). This is a microsomal enzyme which hydroxylates the ∝-methylene group of the O-alkyl substituent of glyceryl ethers leading to cleavage of the ether bond (Kosar-Hashemi et al., 1994). This is the first step in the catabolism of alkyl glyceryl ethers in which the hydroxylated ethers are then hydrolysed to glycerol and the respective fatty aldehydes (Taguchi et al., 1994). However, there have been no reports of the effect of BH_4 deficiency on the activity of this enzyme.

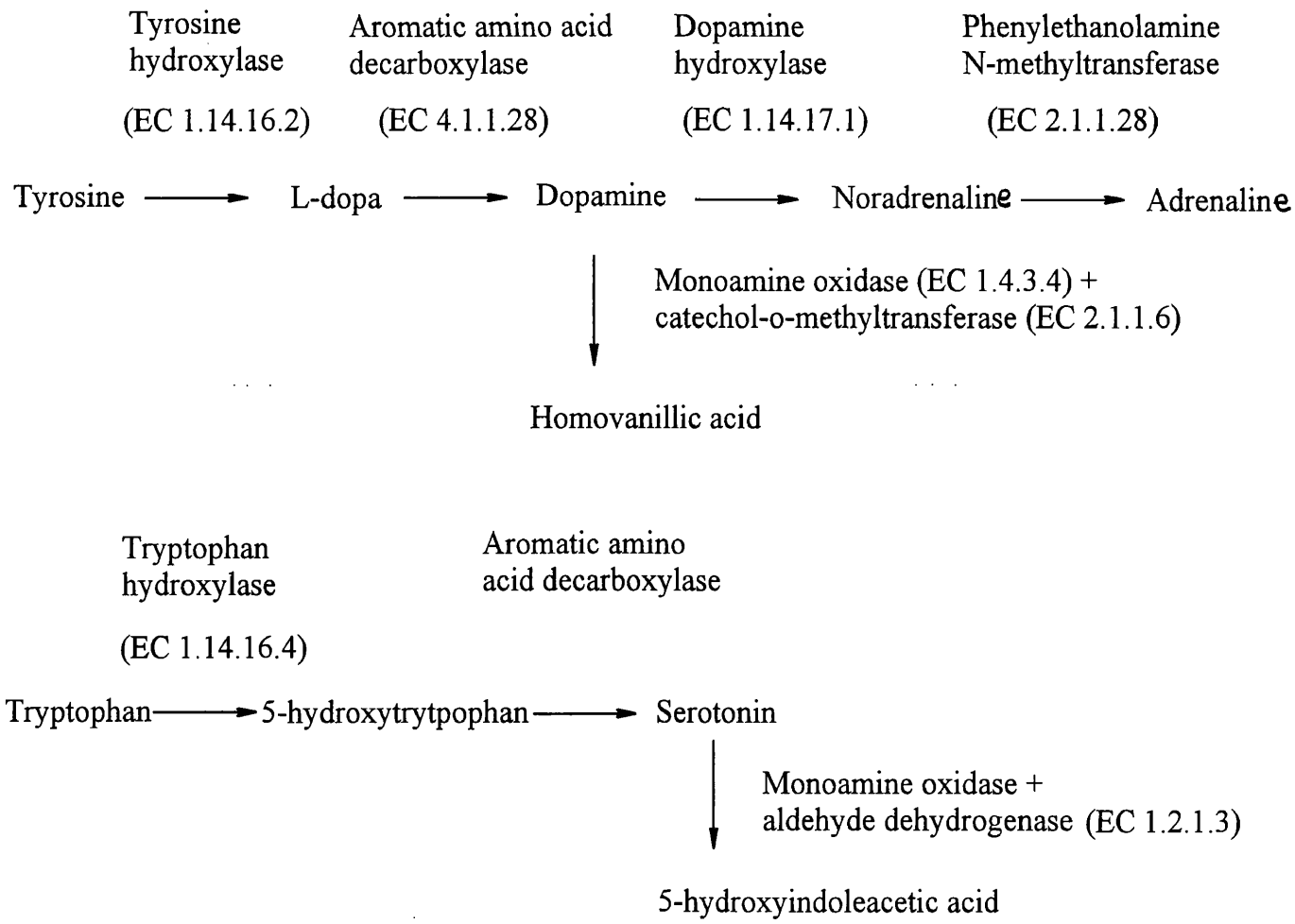


Figure 1.9. Biosynthetic and degradation pathways for catecholamines and serotonin.

Figure 1.9. Biosynthetic and degradation pathways for catecholamines and serotonin.

Tyrosine is first hydroxylated to form L-Dopa, which in turn is decarboxylated by aromatic amino acid decarboxylase to form dopamine. This may be further hydroxylated to form noradrenaline by dopamine hydroxylase. A methyl group may then be donated by S-adenosylmethionine to form adrenalaine. Dopamine is deaminated to form dihydroxyphenylacetic acid (DOPAC). DOPAC is a substrate for catechol-o-methyltransferase resulting in formation of homovanillic acid.

Serotonin is hydroxylated to form 5-hydroxytryptophan, this is decarboxylated to form serotonin. This is metabolised by monoamine oxidase and aldehyde dehydrogenase to form 5-hydroxyindole-acetic acid.

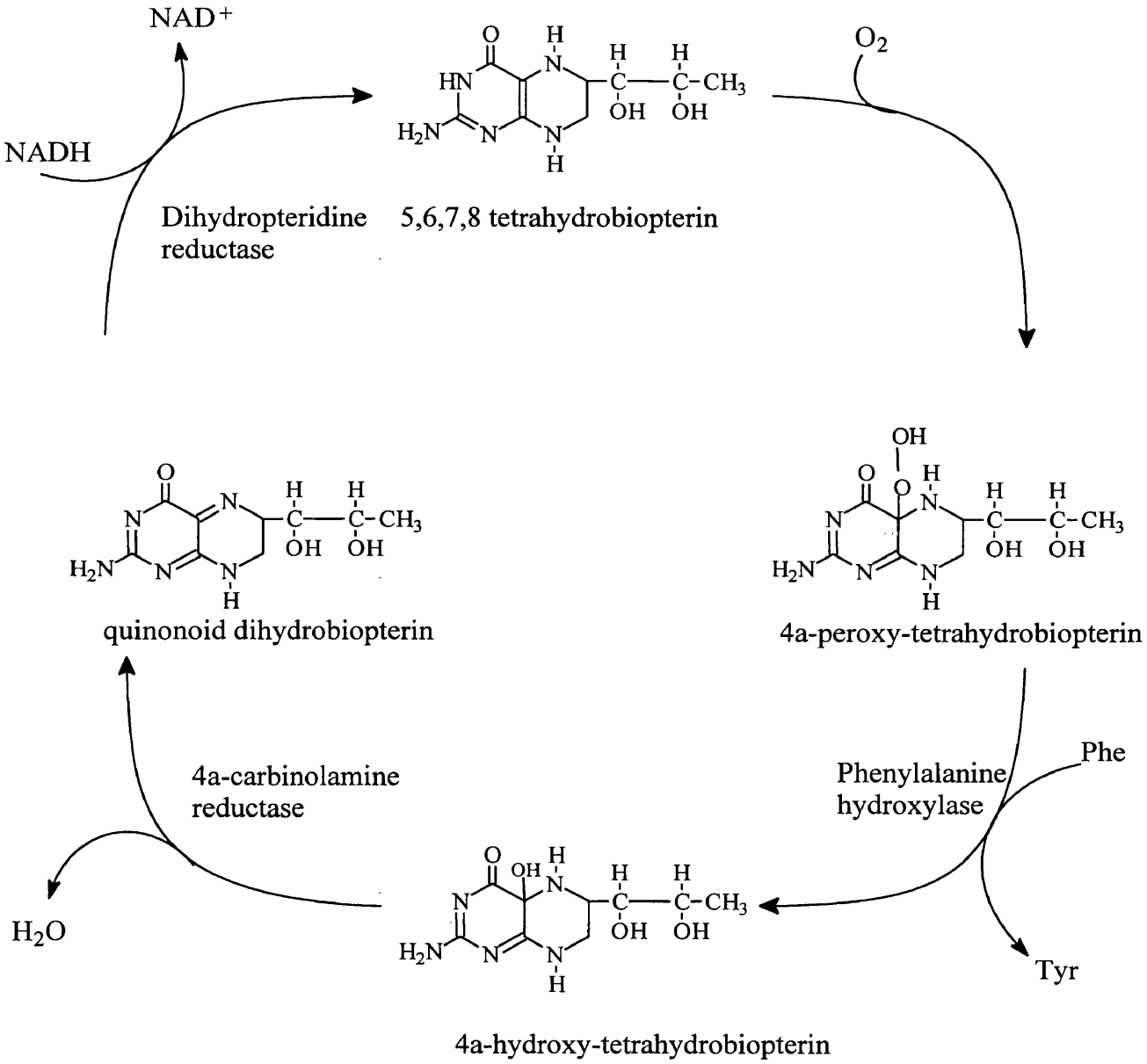


Figure 1.10. Phenylalanine hydroxylating system demonstrating the tetrahydrobiopterin salvage pathway.

A recent function of BH_4 has been suggested as a neurotransmitter releasing factor (Koshimura et al., 1990). Using micro-dialysis with a probe situated in the striatum, perfusion of BH_4 (0.25 - 1.0 mM) led to an increase in dopamine and serotonin levels in the striatal dialysates in a concentration dependant manner (Koshimura et al., 1990; Mataga et al., 1991). The possibility that BH_4 was stimulating tyrosine hydroxylase to increase dopamine synthesis was discounted by including the tyrosine hydroxylase inhibitor, ∝-methyl-*p*-tyrosine, in the perfusion buffer and this did not block the release of dopamine (Koshimura et al., 1990). Including sepiapterin, a stable dihydropterin that can be converted by sepiapterin reductase to dihydrobioperin and then to BH_4, in the perfusion buffer to increase intracellular BH_4 levels had no effect on dopamine release demonstrating that dopamine dependent BH_4 release acted independently of the cofactor role (Koshimura et al., 1994). Addition of NOS inhibitors to the reaction buffer had no effect on the neurotransmitter release thus eliminating the possibility that BH_4 was acting as a cofactor for NOS and that the neurotransmitter release might be nitric oxide dependant (Koshimura et al., 1994).

An additional role for BH_4, and other reduced pterin species, has been suggested as an anti-oxidant. BH_4 is readily oxidised by hydroxyl radicals, the susceptibility of BH_4 to free radical attack may, therefore, suggest an additional role as a free radical scavenger (Heales et al., 1988). The scavenging effect of reduced pterin species has been suggested to be related to their reducing capabilities (Reibnegger et al., 1995).

A role for BH_4 has also been suggested in mitochondrial electron transport (Rembold and Buff, 1972a; 1972b). Under state 4 (resting) respiration, BH_4 strongly activates mitochondrial respiration whereas it has little effect on state 3 (active) respiration (Rembold and Buff, 1972a). High levels of cytosolic NADH or NADPH were suggested to reduce quinonoid dihydrobiopterin back to BH_4 which can then cross to the mitochondrial matrix side where it can reduce cytochrome c (Rembold and Buff, 1972b). The function of BH_4 was suggested to be to regenerate NAD or NADP in cells with high reducing capability via production of heat as addition of BH_4 does not activate mitochondrial ATPase (Rembold and Buff, 1972b). However, in this work there was no indication of the purity of the mitochondrial preparation and the results

may be caused by tetrahydrobiopterin contamination from the cytosol. Furthermore, no work since this date has investigated the role of BH_4 on the mitochondrial electron transport chain.

1.2. Hyperphenylalaninaemia due to phenylalanine hydroxylase deficiency

In normal individuals phenylalanine is converted to tyrosine in the liver by the enzyme phenylalanine hydroxylase, this is the controlling step of phenylalanine homeostasis (Smith and Brenton, 1995). However, in approximately one in 10,000 live births in the United Kingdom there is a manifestation of hyperphenylalaninaemia (where plasma phenylalanine is greater than 240 μmol/L) and a relative tyrosine deficiency. The vast majority of these individuals have a recessively inherited deficiency of the hepatic phenylalanine hydroxylase enzyme (MRC working Party on Phenylketonuria, 1993). This disease was first reported by in 1934 by Asbjörn Følling who reported two severely mentally retarded children who excreted phenylpyruvic acid in their urine (For review, see Følling, 1994). The disease was renamed phenylketonuria by Penrose due to the excretion of phenylketones due to a combination of transamination, decarboxylation and deamination (For review, see Scriver, 1994). Efficient neonatal screening and the availability of phenylalanine free diets have virtually eliminated the severe mental handicap from the disease (MRC Working Party on Phenylketonuria, 1993).

1.3. Inborn errors of tetrahydrobiopterin metabolism.

As already discussed, hyperphenylalaninaemia in the majority of cases arises from mutations in the phenylalanine hydroxylase gene causing a deficiency of the phenylalanine hydroxylase enzyme. However, in 1-2% of cases the cause of the hyperphenylalaninaemia is due to a deficiency of BH_4 (MRC Working Party on Phenylketonuria, 1993). Patients suffering from this variant of hyperphenylalaninaemia were originally reported to display progressive neurological impairment despite instigation of a restricted phenylalanine diet (Bartholomé 1974; Smith et al., 1975). It was proposed that this neurological impairment was due to a deficiency of neurotransmitter amines (Bartholomé et al., 1977). This was confirmed by the finding that patients displayed reduced levels of cerebrospinal fluid (CSF)

neurotransmitter amine metabolites (Butler et al., 1978). Patients with inborn errors of BH_4 metabolism, who remain untreated, commonly display mental retardation, convulsions, microcephaly, disturbances of tone and posture, drowsiness, abnormal movements, recurrent hyperthermia without infection and swallowing difficulties (Smith et al., 1975; Blau et al.,1993). If untreated, death in early childhood is common with this disorder (Smith, 1985).

The molecular basis of hyperphenylalaninaemia due to BH_4 deficiency is either due to a defect in the biosynthesis (Figure 1.4.) or salvage of BH_4 (Figure 1.10.). All of the disorders are inherited in an autosomal recessive fashion (Blau et al., 1993). Defects in the biosynthesis and salvage of BH_4 have been identified at the level of GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, dihydropteridine reductase and pterin-4a-carbinolamine dehydratase. To date there has been no report of a defect of sepiapterin reductase activity. This may be due to an alternative pathway leading to BH_4, namely via aldose reductase (Figure 1.4.) (Park et al., 1991).

Currently treatments of disorders of BH_4 cofactor synthesis consist of administration of L-dopa with 5HTP to compensate for the catecholamine and serotonin deficiency together with a peripheral decarboxylase inhibitor (Smith and Brenton, 1995). A low phenylalanine diet or administration of BH_4 (4-8 μmol/kg/day) to control the hyperphenylalaninaemia is also necessary (Smith and Brenton, 1995).

Oral administration of BH_4 has been shown to be useful for controlling the plasma phenylalanine concentration in children with BH_4 biosynthesis defects (Blau et al., 1993). Treatment of the neurological features of the disease with BH_4 has been less successful and responses have been variable, some patients show no response of amine metabolism despite entry of BH_4 into the CSF whereas others show an increase in these metabolites (McInnes et al., 1984; Smith et al., 1985). The reason for the variability of effect of cofactor replacement therapy has been suggested to be due to the poor ability of the natural cofactor to cross cell membranes (Kapatos and Kaufman., 1981; Levine et al., 1985). Recently, it has been demonstrated that in a mouse model of BH_4 deficiency, *hph-1*, there is a decrease in the steady state

concentration of phenylalanine and tyrosine hydroxylase (Hyland et al., 1996a). Furthermore, decreased phenylalanine hydroxylase activity has been reported in a patient with a BH_4 biosynthesis defect (Dhondt, 1991). These observations suggest that BH_4 may control the *de novo* synthesis of aromatic amino acid monooxygenase enzymes or may be required for their stability (Hyland et al., 1996a). Using the *hph-1* mouse, acute administration of BH_4 in doses that have been used in human studies leads to "normalisation" of brain cofactor concentration but had no effect on monoamine metabolism over 24 hours (Brand et al., 1996). In view of this, any response to cofactor replacement may depend on the initial concentration/stability of the aromatic amino acid mono-oxygenases or chronic BH_4 administration may be required before any effect is seen.

1.3.1. GTP cyclohydrolase I deficiency.

Deficiency of GTP cyclohydrolase I, the first step in the synthesis of BH_4, is characterised by a deficiency of all pterins including 7,8-dihydroneopterin and BH_4 (Blau et al., 1993). Serotonin and catecholamine metabolism is severely impaired and the respective neurotransmitter metabolites 5-hydroxyindole-acetic acid (5HIAA) and homovanillic acid (HVA) are generally low (Blau et al., 1993). This inborn error of metabolism is very rare and in the 18 year period up to 1993, only seven cases were recorded worldwide (Blau et al., 1993).

1.3.2. 6-pyruvoyl-tetrahydropterin synthase deficiency.

A defect in the next step in the biosynthesis of BH_4, 6-pyruvoyl-tetrahydropterin synthase, is the most common cause of BH_4 deficiency, 58% of all reported patients worldwide in the 18 year period up to 1993 (Blau et al., 1993). Because 7,8-dihydroneopterin triphosphate cannot be converted to 6-pyruvoyl tetrahydropterin, this leads to an accumulation of the former compound in tissues. The 7,8-dihydroneopterin triphosphate is readily dephosphorylated by pyrophosphatase to form 7,8 dihydroneopterin. This is excreted in the urine together with its oxidation product, neopterin (Blau et al., 1993). Two phenotypes of this disease have been reported, a severe form where the neurotransmitter status of the individual is impaired as in GTP cyclohydrolase I deficiency and a peripheral or partial form where normal levels of

neurotransmitters are reported (Blau et al., 1993).

1.3.3. Dihydropteridine reductase deficiency.

Deficiency of dihydropteridine reductase was the first reported defect of BH_4 metabolism (Smith et al., 1975; Kaufman et al., 1975). During the hydroxylation of the aromatic amino acids, phenylalanine, tyrosine or tryptophan, BH_4 is oxidised to quinonoid dihydrobiopterin and this accumulates in this condition (Kaufman, 1987). Quinonoid dihydrobiopterin tautomerises to 7,8-dihydrobiopterin and this is excreted in the urine together with the oxidation product, biopterin (Rembold et al., 1971). The amount of BH_4 synthesised by *de novo* synthesis is insufficient for hepatic phenylalanine hydroxylase and this leads to the hyperphenylalaninaemia. Similarly, these patients are characterised by deficiency of neurotransmitters (Blau et al., 1993) The mechanism of neurotransmitter deficiency may also be due to accumulation of 7,8-dihydrobiopterin which may inhibit the aromatic amino acid mono-oxygenase enzymes (Heales and Hyland 1991). As there is a general deficiency of BH_4 there is no feedback control mechanism for GTP cyclohydrolase I, as the first committed step in BH_4 biosynthesis is active, this leads to accumulation of neopterin in this condition as 6-pyruvoyl tetrahydropterin synthase is the rate limiting step (Blau et al., 1993).

Patients with dihydropteridine reductase deficiency accumulate dihydro-species of biopterin and this may lead to a secondary inhibition of 5-methyl-tetrahydro-folate synthesis due to competition between quinonoid dihydrobiopterin and the natural substrate for 5-10-methylene-tetrahydro-folate reductase (Smith et al., 1985). This may explain the low levels of folate seen in patients with dihydropteridine reductase deficiency (Smith, 1985). Two patients reported with dihydropteridine reductase deficiency displayed clinical symptoms of amine deficiency despite adequate replacement therapy (Smith et al., 1985). This progressed to fits, deterioration in speech, signs of pyramidal tract involvement and ataxia (Smith et al., 1985). Low CSF folate was diagnosed, treatment with 5-formyl-tetrahydro-folate (Folinic acid) prevents the folate deficiency and both patients made an immediate improvement in the symptoms of amine deficiency, abolished the fits and halted the progression in neurological deterioration (Smith et al., 1985).

1.3.4. Pterin-4a-carbinolamine dehydratase deficiency.

The most recently discovered variant of hyperphenylalaninaemia and is characterised by excretion of the 7-substituted pterins in the patients urine (Blau et al., 1993). A deficiency of pterin-4a-carbinolamine dehydratase has been suggested as the cause for this variant as in the absence of the dehydratase 4a-hydroxy-tetrahydrobiopterin rearranges to derivatives with the side chain in the 7- rather than the 6- position (Davis et al., 1991). This variant of the BH_4 inborn errors of metabolism does not require any treatment as 4a-hydroxy-tetrahydrobiopterin will break down non-enzymatically to quinonoid dihydrobiopterin (Blau et al., 1993; Kaufman et al., 1993).

1.3.5. L-Dopa responsive dystonia.

Dystonia is the name given to involuntary spasmodic movements of the muscles causing unusual posture of different parts of the body. L-Dopa responsive dystonia (DRD) or hereditary progressive dystonia, is a rare treatable condition inherited in a autosomal dominant manner (Williams, 1995). DRD is a childhood onset disease with marked diurnal fluctuation, the symptoms aggravated in the evening and alleviated in the morning after sleep (Goldstein et al., 1996).

The condition was first reported in 1971 by Segawa et al. and the gene responsible for the condition was mapped to chromosome 14q (Ichinose et al., 1994). This corresponds to a mutation of the GTP cyclohydrolase I gene, the first committed step in the biosynthesis of BH_4 (Ichinose et al., 1994). Even though there is one normal allele in DRD, GTP cyclohydrolase I activity is only approximately 20% of normal control activity (Ichinose et al., 1994; Williams, 1995). Nagatsu and Ichinose (1996) have suggested that the defect in GTP cyclohydrolase I, although essential, is not sufficient for DRD by itself and that some regulatory genes may be necessary for the lower expression of GTP cyclohydrolase I activity. Patients with DRD display reduced levels of total biopterin (approximately <20% of normal controls) and neopterin (approximately 20% of controls) in the CSF due to the reduction in GTP cyclohydrolase I activity (Fink et al., 1988). Reduced CSF levels of HVA and 5HIAA, the stable end products of dopamine and serotonin respectively (Figure 1.9.), have also been demonstrated in DRD patients (Fink et al., 1988). Reduced levels of

HVA and 5HIAA indicate reduced activity of tyrosine and tryptophan hydroxylase which may be due to reduced levels of BH_4 (Hyland et al., 1996b). Dopamine deficiency is considered the basis for the dystonia (Hyland et al., 1996b). Oral L-Dopa administration dramatically improves the neurological situation (Goldstein et al., 1996). Patients with DRD do not display hyperphenylalaninaemia under normal conditions, however, following phenylalanine loading serum phenylalanine levels are raised for up to six hours post-load compared to normal controls (Hyland et al., 1996b). Tyrosine levels are also decreased following a phenylalanine load compared to normal patients (Hyland et al., 1996b). These results are consistent with decreased liver phenylalanine hydroxylase activity due to defective BH_4 metabolism (Hyland et al., 1996b). DRD should therefore be included as an inborn error of BH_4 metabolism and may, therefore, reflect the importance of BH_4 metabolism in this variant of dystonia.

1.4. Tetrahydrobiopterin deficiency in other diseases

Alterations in BH_4 have been observed in other neurological diseases including Alzheimer's (Barford et al., 1984) and Parkinson's (Williams et al., 1980). It is not known if these changes represent primary defects in BH_4 biosynthesis or salvage pathways or damage to BH_4 synthesising cells by some other process.

1.4.1. Alzheimer's disease.

Alzheimer's disease is one of a group of neuro-degenerative cerebral disorders of unknown aetiology in which there is a progressive deterioration of personality and intellect. The dementia is thought to reflect loss of cholinergic neurones in the cerebral cortex. This is associated with loss of cerebrospinal fluid (CSF) total biopterin in patients suffering with Alzheimer's (Williams et al., 1980). Reduced levels of BH_4 have also been shown together with raised levels of neopterin in the cerebral cortex from post mortem samples (Barford et al., 1984). Normal dihydropteridine reductase activity was shown and Barford et al. (1984) concluded that there may be some degree of impairment of the BH_4 biosynthetic pathway after GTP cyclohydrolase as neopterin levels were raised. Tyrosine and tryptophan hydroxylase were both reported to be reduced in the substantia nigra of post mortem patients who

suffered from Alzheimer's disease (Sawada et al., 1987). The observed decrease in BH_4, tyrosine and tryptophan hydroxylase may, therefore, reflect destruction of monoaminergic neurones (Sawada et al., 1987).

1.4.2. Parkinson's disease.

In Parkinson's disease, lesions in the nigro-striatal dopaminergic pathways cause severe depletion of dopamine. This is responsible for the movement disorders exhibited by these patients (Kopin, 1993). In Parkinson's there is a 50% decrease in CSF total biopterin (Williams et al., 1980) in contrast to serum total biopterin levels where there is no significant variation from normal control subjects (Leeming et al., 1976b). Normal serum biopterin levels together with the fact that BH_4 is also reduced in other neurological diseases might, therefore, indicate that the low levels of cofactor seen in Parkinson's patients may be due to loss of dopaminergic neurones rather than a primary metabolic defect of BH_4 metabolism (Williams et al., 1980). This is supported by recent work where post mortem studies revealed GTP cyclohydrolase I activity to be 19% of normal controls (Nagatsu and Ichinose, 1996). This decrease in enzyme activity was parallel with tyrosine hydroxylase activity and dopamine levels (Nagatsu and Ichinose, 1996). Therefore, depletion in BH_4 levels may be secondary to the observed cell death in Parkinson's disease.

1.5. Nitric oxide and nitric oxide synthase.

1.5.1. Historical Perspective

Nitric oxide (NO) is a free radical gas and is one component of polluted air, it is, however, also now known to be synthesized enzymatically by a wide variety of tissues and species and has a variety of biological functions. NO is a gas with limited solubility in water (1 mM) but can cross cell membranes by diffusion. NO is highly reactive free radical species with a half life in biological tissue of approximately four seconds (Garthwaite, 1991).

The first suggestion that there might be a pathway for nitrogen oxide biosynthesis in mammals was by Mitchell et al. (1916) with their findings that urine of rats, pigs and humans contained more nitrate than was present in the diet. It was not until 1981 that

it was shown that nitrogen oxides were normal mammalian metabolites (Green et al., 1981; Witter et al, 1981). In 1985 Stuehr and Marletta showed that activated macrophages stimulated *in vitro* with LPS expressed a nitrogen oxide synthase activity and produced nitrite and nitrate. Further studies showed that macrophages form nitrite and nitrate by enzymatic oxidation of one of the two guanidino nitrogens of arginine and that citrulline is a by product (Hibbs et al., 1987a; Iyengar et al, 1987). It was also demonstrated that N^G-methyl-L-arginine (NMA) was a potent, reversible and stereo selective inhibitor of nitrite and nitrate production by macrophages (Hibbs et al, 1987a; Hibbs et al, 1987b).

Independently of these immunological findings Furchgott and Zawadzki (1980) had shown that endothelial cells had a obligatory role in acetylcholine-mediated relaxation of vascular smooth muscle. The molecule produced by the endothelium responsible for this relaxation was termed endothelium derived relaxing factor (EDRF). Endothelial cells were shown to be capable of generating NO from arginine (Palmer et al, 1987). In 1988 two groups concluded that EDRF and NO were one and the same (Furchgott, 1988; Ignarro et al, 1988). Soon after, macrophages were shown to generate NO as a primary product (Marletta at al, 1988; Hibbs et al, 1988). It was also demonstrated that the cerebellum was capable of producing NO (Garthwaite et al, 1988).

1.5.2. The role of nitric oxide

NO is a ubiquitous biological messenger molecule that has been suggested to have a variety of functions. The first reports of NO were within the cardiovascular system and concerned the relaxation of vascular smooth muscle. The endothelium is ideally situated to transduce chemical signals from within the blood stream and also to respond to changes in physical signals. Action on endothelial receptors by a number of vasoactive substances eg acetylcholine and bradykinin leads to the production of NO (Furchgott and Zadwadski, 1980). The ability of NO to relax vascular smooth muscle appears to be linked to raised levels of cGMP following activation of soluble guanylate cyclase (for review, see Moncada et al., 1991).

Within the central nervous system (CNS) NO is produced in post synaptic structures in response to activation of excitatory amino acid receptors (Garthwaite, 1991). The NO produced diffuses out and may act on neighbouring cellular processes (Garthwaite, 1991). NO may also be formed presynaptically and thus may act as a neurotransmitter (Garthwaite, 1991). NO stimulates soluble guanylate cyclase leading to production of cyclic GMP (cGMP) (Vincent, 1994). Neurotransmitter release may be regulated through NO activation of cGMP dependent protein phosphorylation cascades (Garthwaite and Boulton, 1995). As well as being necessary for the synthesis of secondary messengers NO has been suggested to be involved in synaptic plasticity and learning (Garthwaite and Boulton, 1995) . NO has also been hypothesised to play a role in nervous system morphogenesis and synaptic plasticity as NOS is transiently expressed in the developing rat brain (Dawson and Dawson, 1996). Long term potentiation and long term depression are two forms of synaptic modification that have been suggested to be regulated by NO (For review see Garthwaite and Boulton, 1995).

Within the peripheral autonomic nervous system NO has also been suggested to carry out a number of functions including peristalsis that occurs through relaxation of smooth muscle (Dawson et al., 1991a). NO has also been suggested to be involved in penile erection (Ignarro et al., 1990).

In addition to its the above roles NO may play an important role in the immune response to various pathogens (O'Donnell and Liew, 1994). As already mentioned, following cytokine stimulation there is an induction of the inducible form of NOS together with GTP cyclohydrolase I in macrophages (Werner et al., 1993). This leads to an excessive production of NO which has been proposed to play a role as a cytotoxic agent. This may occur by NO penetrating the target cell and disrupting critical cell function by binding to complex essential metals (For review see Fukuto and Chaudhuri, 1995). Along with NO, activated macrophages also produce superoxide these two free radicals can react to form the peroxynitrite ion ($ONOO^-$) which is an extremely potent oxidising agent (Beckman et al., 1990). $ONOO^-$ has been shown to react with a variety of cellular components and may contribute to the overall toxic effects of macrophage released NO (Beckman and Crow, 1993).

When present in high concentrations, NO can initiate a neurotoxic cascade and has been implicated in the neurotoxicity associated with glutamate receptor stimulation (Dawson et al., 1991b). This observation has led to speculation that glutamate receptor stimulation may be involved in the pathogenesis of certain neurodegenerative disorders (Meldrum and Garthwaite, 1990). An alternative mechanism for NO mediated neurotoxicity is from the synthesis of NO by glial cells (Murphy et al., 1990). Induction of inducible NOS within rat glial cells can lead to mitochondrial respiratory chain damage and this may represent a plausible mechanism for NO mediated neurotoxicity (Bolaños et al., 1994).

1.5.3. Nitric oxide synthase

The enzymes responsible for the synthesis of NO from arginine in mammalian systems are known as nitric oxide synthases (NOS). The enzyme was first reported in 1989 by various groups (For review see Knowles and Moncada, 1994), the neuronal isoform was the first to be purified by Bredt and Snyder (1990) and cloned by Bredt et al. (1991). NOS was originally described as requiring arginine and NADPH for the synthesis of NO and citrulline (Knowles et al. 1989). Kwon et al. (1990) later demonstrated, using $^{18}O_2$, that the oxygen atom in citrulline and NO is derived from molecular oxygen.

The first NOS was purified from cerebellum, during purification a loss of activity was noticed and it was noticed that calmodulin was required for NOS purified from cerebellum (Bredt and Snyder, 1990). It was subsequently shown all forms of the enzyme had a requirement for calmodulin. A number of other cofactors were also shown to be necessary for NOS. Mayer et al (1991) demonstrated that the flavins flavin mono nucleotide (FMN) and flavin adenine dinucleotide (FAD) were needed for the synthesis of NO from arginine. The requirement of a protoporphyrin IX haem prosthetic group that participates in the turnover of arginine was also described (Klatt et al, 1992; White and Marletta, 1992). It was also shown that BH_4 was required as a cofactor for NOS (Tayeh and Marletta, 1989).

As already discussed, the first studies on NO synthesis were conducted on endothelial

tissue, macrophages and the brain. It was immediately apparent that the macrophage form of the enzyme was different from the other forms as it appeared to be calcium independent. Further cloning and characterisation of the enzyme led to the conclusion that there were three isoforms of the enzyme. These have been labelled by Knowles and Moncada (1994) as neuronal constitutive NOS (nNOS or type I NOS), endothelial constitutive NOS (eNOS or type III NOS) and inducible NOS (iNOS or type II NOS).

1.5.4. Neuronal nitric oxide synthase (Type I NOS).

This isoform of NOS is a calcium and calmodulin dependent enzyme (Knowles et al, 1989; Bredt and Snyder, 1990) and is constitutively expressed in the brain. Enzyme activity is present in varying degrees in all areas of brain examined (cerebellum, hippocampus, striatum, cortex, hypothalamus, midbrain and medulla) with the cerebellum having the highest activity and the medulla the lowest (Förstermann et al., 1990). The nNOS gene contains an N-terminal leader sequence coding for about 290 amino acids which is absent from the eNOS and iNOS isoforms (Liu and Gross, 1996). This isoform has also been demonstrated to be present in the peripheral nervous system and also in human skeletal muscle but not rat (Nakane et al., 1993). This isoform of NOS is devoid of membrane associating elements and, as expected, the enzyme is found mainly in the cytosolic fraction (Griffith and Stuehr, 1995).

Following purification of nNOS by Bredt et al (1991), a nNOS cDNA was cloned. This coded for a protein of 1429 amino acids with a mass of 160 kDa. The enzyme has homologies with cytochrome P-450 reductase (P-450), together with a mono-oxygenase domain within one polypeptide suggests that the NOS enzyme is a catalytically self sufficient P-450 (Klatt et al., 1994). Both enzymes have consensus sequences for FMN, FAD and NADPH. However, NOS appears to be unique in that it has a BH_4 binding site as well as a calmodulin binding sequence allowing for the regulation by calcium. The regulation of the neuronal form of the enzyme appears to be very dependent on calcium levels (Knowles et al., 1989). At normal resting cytosolic free calcium levels (~ 50 nM) nNOS appears to be virtually inactive and maximally active at 0.4-1.0 μM calcium concentration (Knowles et al, 1989).

1.5.5. Endothelial nitric oxide synthase (Type III NOS).

This constitutive form of the enzyme appears to be similar to the neuronal form of the enzyme in that it is calcium and calmodulin dependent. The eNOS isoform has a molecular mass of 135 kDA (Knowles and Moncada, 1994). Cloning of the bovine form of the enzyme reveals that it shows only 57% homology with the neuronal form of the enzyme (Knowles and Moncada, 1994). As opposed to nNOS and iNOS, this isoform is largely associated with endothelial cell membranes (Pollock et al., 1991). Because eNOS can be activated by the shear stress of blood flow, it has been suggested that association with the luminal plasma membrane may facilitate activation (Griffith and Stuehr, 1995). This isoform of the enzyme has a consensus N-myristoylation site and is myristoylated which may explain why it is partially membrane associated (Knowles and Moncada, 1994). Although eNOS expression has been found in almost all tissues studied, such activity appears to be related to vascular endothelium associated with these tissues (Griffith and Stuehr, 1995).

1.5.6. Inducible nitric oxide synthase (Type II NOS).

This form of the enzyme has been identified in liver, macrophages, astrocytes, vascular endothelium, smooth muscle cells and myocardium following exposure to cytokines or bacterial products (Griffith and Stuehr, 1995). In contrast to eNOS and nNOS this isoform appears to be insensitive to calcium/calmodulin activation. However, iNOS has a consensus sequence for the binding of calmodulin where it is very tightly bound in an apparently calcium free manner (Cho et al., 1992). The iNOS isoform, as already discussed, is induced by cytokine stimulation (Werner et al., 1993). Stimuli for iNOS induction include products of gram-negative and gram positive bacteria, IFN-γ, interleukin-1 and tumour necrosis factors (Gross and Wolin, 1995). Synergism in induction of iNOS activity has been observed with the combination of IFN-γ and any other of these agents (Gross and Wolin, 1995). It is now thought that any cell type is able to express iNOS if the appropriate cytokine or LPS stimulation is delivered (Griffith and Stuehr, 1995).

1.5.7. Biosynthesis of nitric oxide

The three different NOS isoforms catalyse the same reaction and show similar

biochemical properties. The reaction catalysed by NOS is a five electron oxidation of the guanidium nitrogen of arginine to generate the free radical, NO (Fukuto and Mayer, 1996). The mechanism of this reaction is not completely understood but the first step of the reaction is thought to proceed via the hydroxylation of arginine to the intermediate N^G-hydroxy-L-arginine, this is not released from the enzyme complex but immediately undergoes oxidative cleavage to yield NO and citrulline (Figure 1.11.) (Stuehr et al., 1991; Klatt et al., 1993). The oxygen atoms incorporated in the reaction come from molecular oxygen (Kwon et al., 1990).

The role of NADPH appears to be necessary for the reduction of molecular oxygen with one atom of oxygen being incorporated into the substrate to form the N^G-hydroxy-L-arginine intermediate and the other atom being reduced to water (Fukuto and Mayer, 1996). Thus, the initial reaction of NOS appears to be the formation of N^G-hydroxy-L-arginine (Figure 1.11.) (Fukuto and Mayer, 1996). A similar reaction occurs with P-450 (White and Marletta, 1992; Stuehr and Ikeda-Saito, 1992). This initial reaction is a two electron oxidation, the next part of the reaction involves an odd three electron chemistry reaction.

Two possible roles have been suggested for the flavin cofactors, FAD and FMN; they may act as ultimate oxygen activators and participate in the oxidation of substrate or they may act as a conduit in the transport of electrons to the haem group (Mayer and Fukuto, 1996). If, as suggested, NOS is a P-450 type protein the flavin groups may act as they do in this type of reaction with the electrons from NADPH shuttled from NADPH via FAD and FMN to the haem group (White and Marletta, 1992). Further evidence for this mechanism comes from the demonstration that calcium/calmodulin regulates NOS activity by enabling electron transfer from reduced flavins to the haem group (Abu-Soud and Stuehr, 1993).

The role of the haem iron in NOS appears to be to bind, reduce and activate molecular oxygen to generate the oxidant (Fukuto and Mayer, 1996). The haem-iron complex appears to be involved in the entire multi step process as carbon monoxide (CO) causes inhibition of arginine oxidation by NOS (For review, see Fukuto and Mayer,

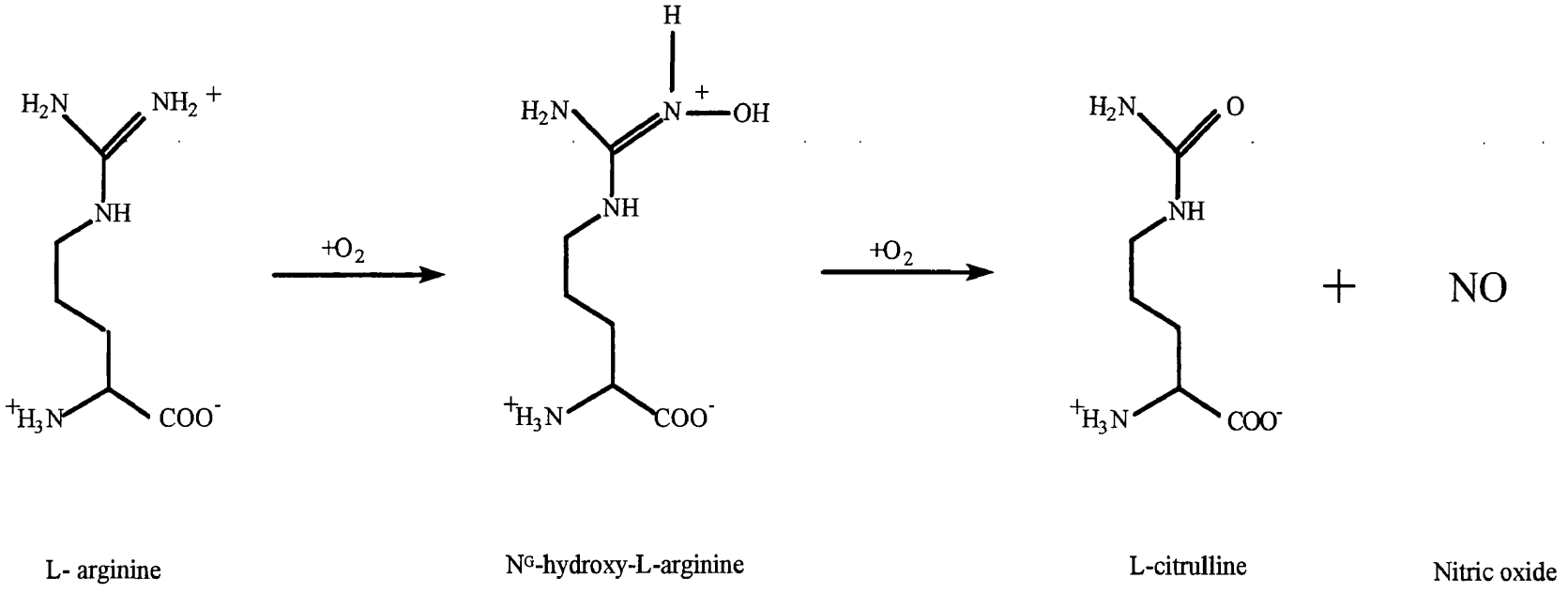


Figure 1.11. The conversion of arginine to citrulline and NO showing the N^G-hydroxy-arginine formation.

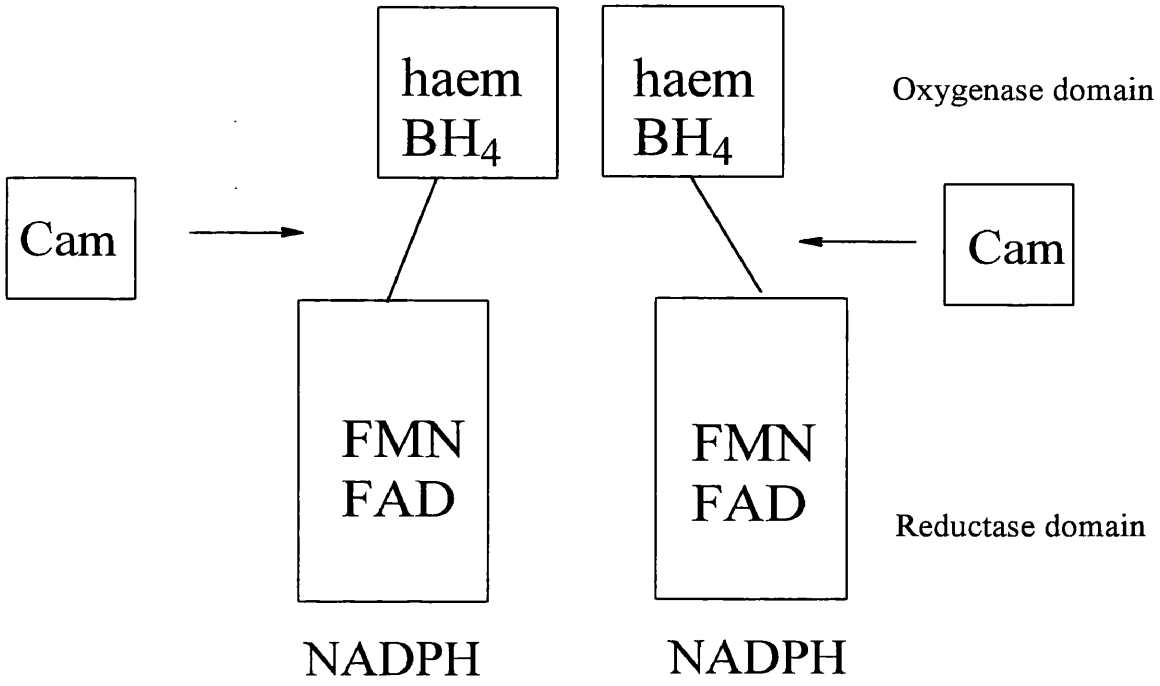
Adapted from Kaufman, 1993.

1996). The NOS bound haem reacts with CO to form a species that absorbs at 450 nm reinforcing the analogy of NOS with P-450 (Zhang and Snyder, 1995). Under conditions where the substrate, arginine, and/or BH_4 is limiting NOS will produce superoxide or hydrogen peroxide (Heinzel et al., 1992; Pou et al., 1992). This is considered to occur through an iron bound dioxygen intermediate (Fukuto and Mayer, 1996). Under certain conditions P-450 may "uncouple" from substrate oxidation to produce superoxide, again similar to NOS (Fukuto and Mayer, 1996).

1.5.8. The role of tetrahydrobiopterin in the NOS complex.

NOS shows an absolute requirement for BH_4 but the role of BH_4 is unclear and open to some speculation. BH_4 was originally thought to act in an analogous fashion in the NOS mechanism to that which has been proposed in the aromatic amino acid mono-oxygenase enzymes ie BH_4 was involved in the initial hydroxylation of arginine and would be recycled by dihydropteridine reductase. However, the nNOS reaction was found not to be stoichiometric with respect to BH_4, more than 15 moles of citrulline were formed per mole of BH_4 consumed and no salvage pathway for BH_4 was detected (Giovanelli et al., 1991). An allosteric role for BH_4 was suggested (Giovanelli et al., 1991). Klatt et al. (1995) have suggested an allosteric role for BH_4 and arginine in the formation of an SDS-resistant homodimer. BH_4 was also found to be necessary for the initial hydroxylation of arginine to N^G-hydroxy-arginine and the conversion of this intermediate to citrulline and NO (Klatt et al., 1993). Recent work has also demonstrated that there may be a recycling mechanism for the regeneration of BH_4 from dihydrobiopterin (Witteveen et al., 1996). Whether BH_4 is recycled in the NOS complex or not, there is still no consensus agreement on the functional role of this cofactor in the NOS catalysed reaction.

Active NOS consists of a oxygenase domain and a reductase domain (Figure 1.12.). Only the dimeric form of iNOS has been shown to exhibit enzyme activity and it has been suggested that BH_4, arginine and haem are essential for the dimerisation of the iNOS subunits (Baek et al., 1993). However, purified NOS from porcine cerebellum has been shown to exist as a stable dimer in the absence of BH_4 and substrate but lacking in NO synthesising ability (Klatt et al., 1995).



NOS consists of an oxygenase (haem and BH_4) domain and a reductase domain (FMN and FAD). Calmodulin (Cam) binding activates constitutive forms of NOS by increasing the electron flow between NADPH and the flavins and by allowing electron transfer between the flavins and the haem. The inducible form of NOS has calmodulin bound to the enzyme reductase domain.

Figure 1.12. Structure of the active NOS dimer.

Adapted from Witteveen et al. (1996) and Albakri and Stuehr (1996).

At physiological pH, arginine will exist with a protonated guanidine because the pK_a is above 12 (Mayer and Fukuto, 1996). Electron transfer will still take place but protonation may be necessary for the reaction to proceed (Fukuto and Mayer, 1996). As already mentioned, the first step in this reaction is the formation of the intermediate, N^G-hydroxy-arginine, followed by further oxidation of this intermediate generating citrulline and the free radical NO (Stuehr et al., 1991). This reaction consumes NADPH and oxygen and proceeds with C-N bond scission and incorporation of an atom from molecular oxygen into arginine to form N^G-hydroxy-arginine (Fukuto et al., 1993a). It is the mechanism of the further oxidation of N^G-hydroxy-arginine that is still the subject of some debate. Chemical oxidation of N^G-hydroxy-arginine leads to liberation of nitroxyl (HNO) by a two electron oxidation process (Fukuto et al., 1993a). The HNO formed can act as a vasorelaxant and is also readily converted to NO by a variety of ubiquitous biological oxidants including oxygen, methaemoglobin and flavins (Fukuto et al., 1993b). Thus, the chemistry of the NOS reaction may be a more simple four electron oxidation followed by a one electron oxidation which may occur under physiological conditions.

Other mechanisms for the enzymic generation of NO all involve five electron oxidation schemes. N^G-hydroxy-arginine may donate one electron to the enzyme and as a radical cation this intermediate may then undergo further NOS catalysed reactions (Feldman et al., 1993). A further mechanism in which enzyme reduction by the intermediate occurs via an iron-oxo species has been suggested by Klatt et al. (1993).

The generation of NO from arginine by NOS is clearly still the matter of some speculation and the role that BH_4 plays in this reaction is also unclear. However, recent suggestions for the role of BH_4 in the NOS complex have suggested a superoxide generating role for the cofactor (Mayer and Werner, 1995). This will then react with the NO produced to form $ONOO^-$, the NOS enzyme will then be a $ONOO^-$ generating enzyme (Mayer and Werner, 1995). Formation of S-nitrosylated compounds, for example S-nitroso-glutathione, may then occur and these may then release NO (Mayer and Werner, 1995). A further suggestion for the role of BH_4 in the NOS complex is to prevent inhibition of the enzyme by it's own product, NO

(Griscavage et al., 1994). This inhibition may occur by reaction of NO with the haem group of NOS (Griscavage et al., 1994).

1.6. Aims.

From the work described above it is clear that despite the explosion of interest in NO and NOS (over 3,000 publications relating to NO metabolism and over 1,500 publications relating to NOS in 1996 alone) it is clear that there is no satisfactory explanation for the role that BH_4 plays in the NOS complex. The effect of persistent BH_4 deficiency on brain NO metabolism is unknown. Furthermore, it is not clear if a partial deficiency of BH_4, as may occur in the inborn errors of BH_4 metabolism, is sufficient to result in a significant disturbance of brain NOS metabolism

The aims of this thesis are, using the BH_4 deficient *hph-1* mouse (Chapter 3) to:

- Determine whether BH_4 regulates NOS specific activity to the same extent as reported for phenylalanine and tyrosine hydroxylase.

- Ascertain if BH_4 availability influences the affinity of NOS for arginine.

- Evaluate the NO/cGMP pathway in BH_4 deficient states.

- Determine if increased oxidative stress and mitochondrial damage occurs as a result of "uncoupling" of the NOS reaction.

Chapter 2.
Materials and methods.

Chapter 2. Materials and Methods.

2.1 Chemicals.

All chemicals were of the highest possible grade and unless otherwise stated were obtained from BDH Ltd (Leicester, UK).

(6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride salt (BH_4) was obtained from Dr B. Schirks (Jona, Switzerland).

Dithioeryritol (DTE) and potassium cyanide were obtained from Aldrich (Poole, UK).

Cytochrome c was obtained from Boehringer (Lewes, UK)

Acetyl CoA, agar Noble, antimycin A, bovine serum albumin (BSA), diethylenetriaminepentacetic acid (DETAPAC), 5, 5'dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), phosphodiesterase 3'5' cyclic nucleotide activator (calmodulin), ferricyanide, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), glutamate, kainate, haemoglobin-A_0 (Ferrous), N-methyl-D-aspartic acid (NMDA), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), N-methyl-arginine (NMA), N-nitro-arginine (NNA), N-arginine-methyl ester (NAME), oxaloacetate, rotenone and succinate were obtained from Sigma (Poole, UK).

L-[2,3,4,5 ^{3}H] arginine and cGMP radio-immunoassay kits were obtained from Amersham (Amersham, UK).

Lowry protein analysis kits and cation exchange resin, Dowex AG50W-X8, 100-200 mesh, were obtained from Bio-Rad (Hemel Hempstead, UK).

Ultima Gold liquid scintillation counting fluid, a blend of alkylnaphthalene with scintillators 2,5-diphenyloxazole and *p*-bis(o-methylstyryl) benzene, was obtained from Packard (Pangbourne, UK).

S-nitroso-N-acetyl-D,L-penicilamine (SNAP), S-nitroso-L-glutathione and 6-cyano-7-

nitroquinoxaline-2,3-dione (CNQX) were obtained from Alexis (Nottingham, UK).

Ubiquinone-1 was a kind gift of the Eisai Chemical Company (Tokyo, Japan).

2.2. Animals.

Control mice (C57Bl x CBA) and *hph-1* (Chapter 3) were used from established breeding colonies. The mice were maintained at 21°C on a light/dark cycle of 12 hours/12 hours with access to food and water *ad libitum.* Diet consisted of rat and mouse No. 1 breeding diet (Special Diet Services, Witham, UK). For all experiments mice were killed by cervical dislocation.

2.3. Tetrahydrobiopterin (BH_4) analysis.

Measurement of BH_4 is complicated because the molecule is very unstable and can exist in the tetrahydro-, dihydro- and fully oxidised forms. Howells and Hyland (1987) have shown that 100% of BH_4 from cerebrospinal fluid (CSF) is lost within 3.5 hours when left at room temperature. Addition of dithioeryritol (DTE) and diethylenetriaminepentacetic acid (DETAPAC) and storage at 4^0C can reduce this lose of BH_4 to less than 5% over 2 hours (Howells and Hyland, 1987). Analysis of BH_4 was previously carried out by conversion of the cofactor into the stable, fully oxidised biopterin form of the compound which has strong fluorescent properties. Using acid and alkali iodine oxidation of biological pterins an estimation could be made of the concentration of the dihydro-and tetrahydro- species using high performance liquid chromatography (HPLC) linked to fluorescence detection (Fukushima and Nixon, 1980). This method has the disadvantages that it involves considerable sample preparation and does not allow direct measurement of the oxidation states of the pterin species. A simpler method using manganese dioxide together with 6 M HCl as the oxidising agent has been used extensively for the screening of children with hyperphenylalaninaemia (Niederwieser et al., 1982). Again, this method has the disadvantage in that it only measures total biopterin and gives no direct indication of the fully reduced active cofactor concentration. Lunte and Kissinger (1983) described direct measurement of oxidised and reduced pterins using HPLC linked to dual electrode, amperometric, electrochemical detection. However, this method involved

extensive sample preparation under anaerobic conditions. Direct measurement of BH_4 was reported by Bräutigam et al. (1982) using HPLC linked to dual electrode electrochemical detection but not in any biological samples. In 1985 Hyland described the direct measurement of BH_4 in urine by HPLC linked to a coulometric electrochemical detector. Using a modification of this method, Howells et al. (1986) measured the same species in CSF. This method has the advantage that it allows the sensitive measurement of the natural cofactor, BH_4, directly without the need for extensive sample preparation. The method also allows the measurement of dihydrobiopterin and biopterin in the same chromatographic run. This is accomplished by post column oxidation of dihydrobiopterin by an electrochemical conditioning cell. Detection of the fully oxidised pterins species after post column oxidation are then measured by fluorescence (Howells et al., 1986).

Analysis of BH_4, in this study, was carried out using a modification of the method of Howells et al. (1986). The chromatographic system (Figure 2.1.) consisted of a P100 isocratic pump (Thermo Separation Products, Fremont, Ca, USA), 210A sample injection valve (Beckman, San Ramon, Ca, USA), HPLC Technology (Macclesfield, UK) Techsphere (1cm x 3.0 mm) reverse phase guard column and a Techsphere 5µm ODS (25 x 0.46 cm) reverse phase column (HPLC Technology). Detection was by an ESA coulochem II electrochemical detector (Bedford, Ma, USA) using a model 5011 analytical cell. Mobile phase consisted of 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA and 160 µM DTE, pH 5.2. Mobile phase was degassed using a DEG-103 degasser (Kontron Instruements, Watford, UK). Column temperature was maintained at 35°C by a Model 7970 block heater (Jones Chromatography, Hengoed, UK) and flow rate was set at 1.3 ml/minute. Peak heights were determined by using a Thermo Separation Products SP4400 computing integrator.

Analysis of BH_4 was carried out out in the redox mode. This is where an electrolysis reaction is chemically reversible or generates products which can be further oxidised or reduced by a second electrode (E2). BH_4 is redox reactive, in constrast to

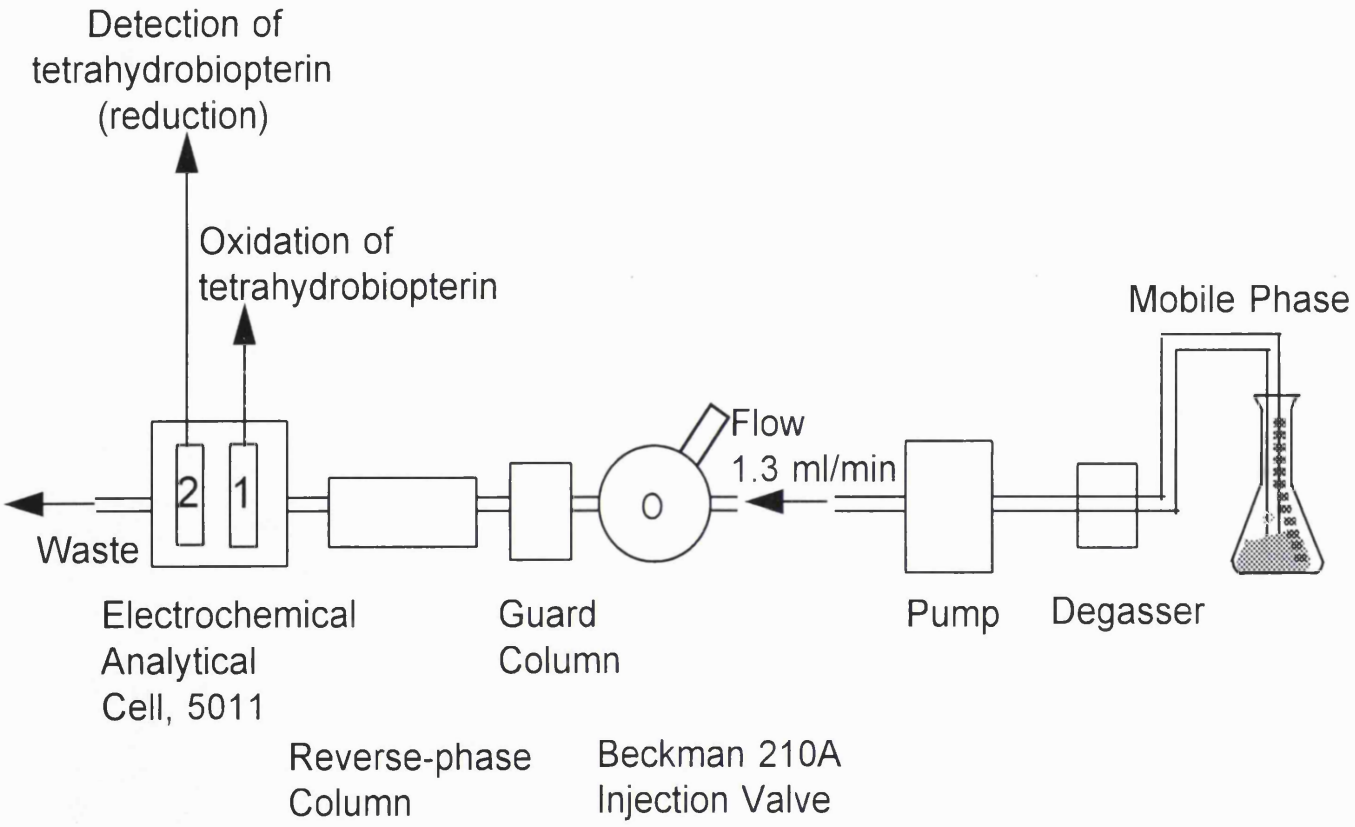


Figure 2.1, HPLC equipment configuration for the analysis of tetrahydrobiopterin

numerous other molecules, and it will be reduced back by the E2 following oxidation at the first electrode (E1). The current consumed at E2 was recorded as a peak on the integrator. This method allows for clearer chromatography, ie numerous other compounds may be oxidised at E1, but are not reduced back at E2, thus allowing resolution of BH_4. In order to optimise detection of BH_4 a current-voltage curve (C-V curve) or voltamogram was generated. This involves altering the applied potential at both electrodes until optimum peak height is obtained. In order to do this, E1 is varied in order to obtain maximum response. E1 is then set at this level and E2 varied in order to obtain maximal response. E2 was set at -0.01v and E1 was varied between 0.4 - 0.95 v. Using a concentrated BH_4 sample (318 μM) maximum peak height was obtained at 0.85 v (Figure 2.2.). E1 was then set at 0.85 v and E2 varied between -0.01 and -0.20. Maximum current generated was recorded at -0.02 (Figure 2.2.).

BH_4 standards, 318 μM, were made up in 0.1 M HCl and stored at -70^{0}C. Working standards, 63.6 nM (15.5 ng/ml) , were then made up in HPLC grade water containing 1 mg/ml DTE and DETAPAC. A standard curve was produced (Figure 2.3.) and showed that detection was linear up to 127 nM (31 ng/ml). Detection of a 30 day old control mouse whole brain sample which was spiked with 127 nM BH_4 showed 96.9% recovery. Brain tissue was homogenised (25% wt/vol) in 0.1 M perchloric acid containing 6.5 mM DTE and 2,5 mM DETAPAC. Samples were centrifuged at 15,000g for 2 minutes, 50 µl of supernatant was injected onto the HPLC system. Chromatograms are shown in Figure 2.4. for BH_4 standard and for the BH_4 peak from the cerebellum of a 30 day old control mouse.

2.4. Glutathione Analysis.

Analysis of glutathione (GSH) was based on the method of Harvey et al. (1989) and measured both reduced (GSH) and the oxidised form (GSSG) of glutathione using ion-paired reverse phase HPLC coupled to a coulometric electrochemical detector. The HPLC system used for the analysis of GSH/GSSG was the same as was used for the analysis of BH_4. The mobile phase consisted of 10 mM sodium phosphate monobasic monohydrate, 0.05 mM octansulphonic acid and 2% acetonitrile, pH 2.7. Separation

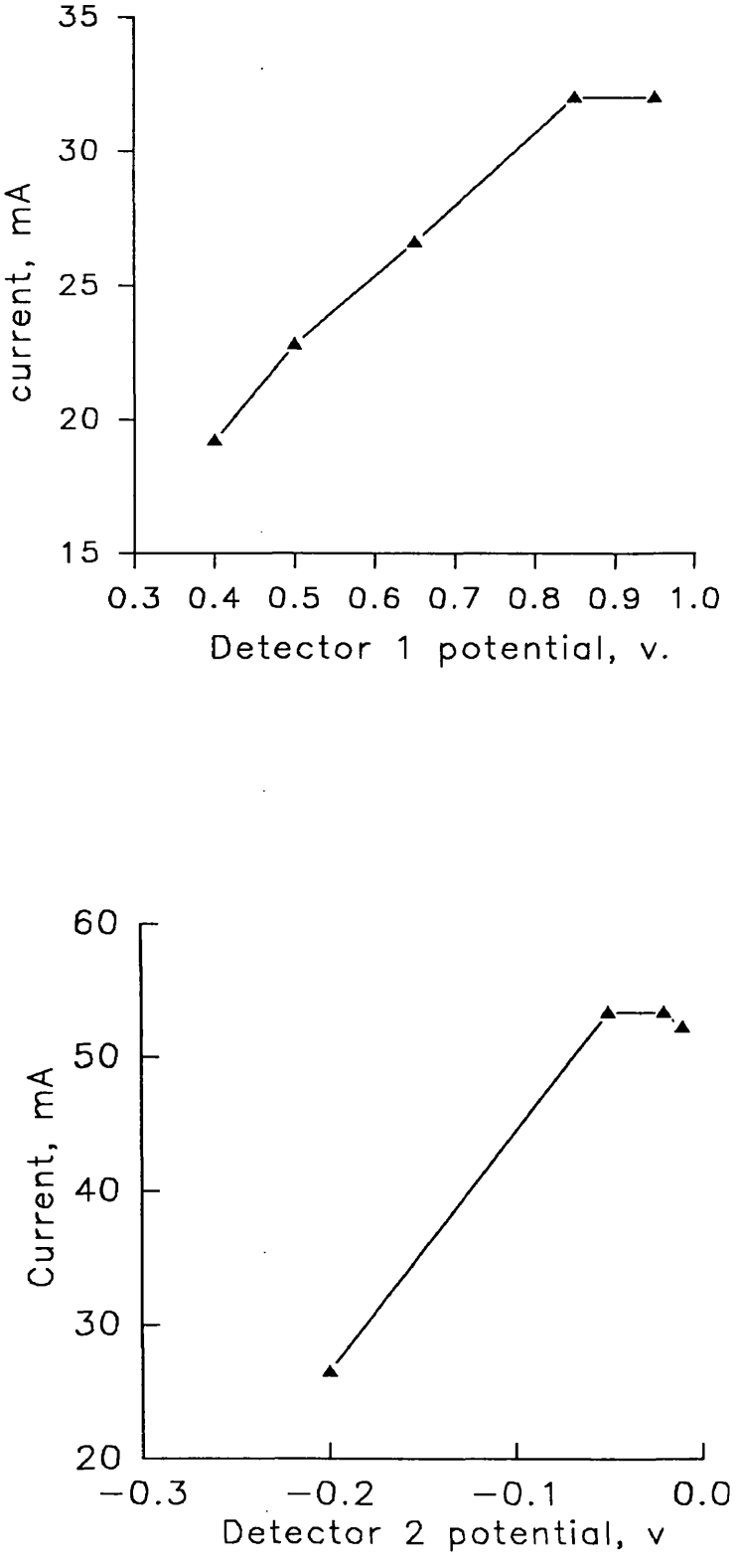


Figure 2.2. C-V curves for tetrahydrobiopterin.

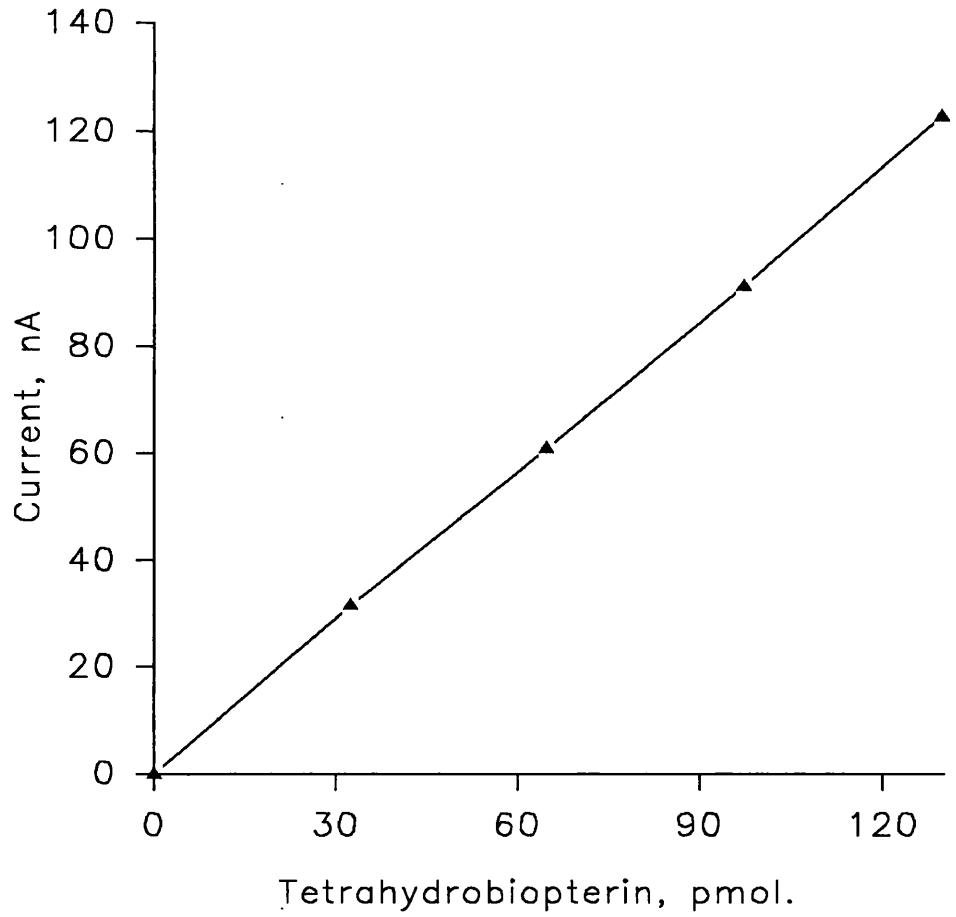
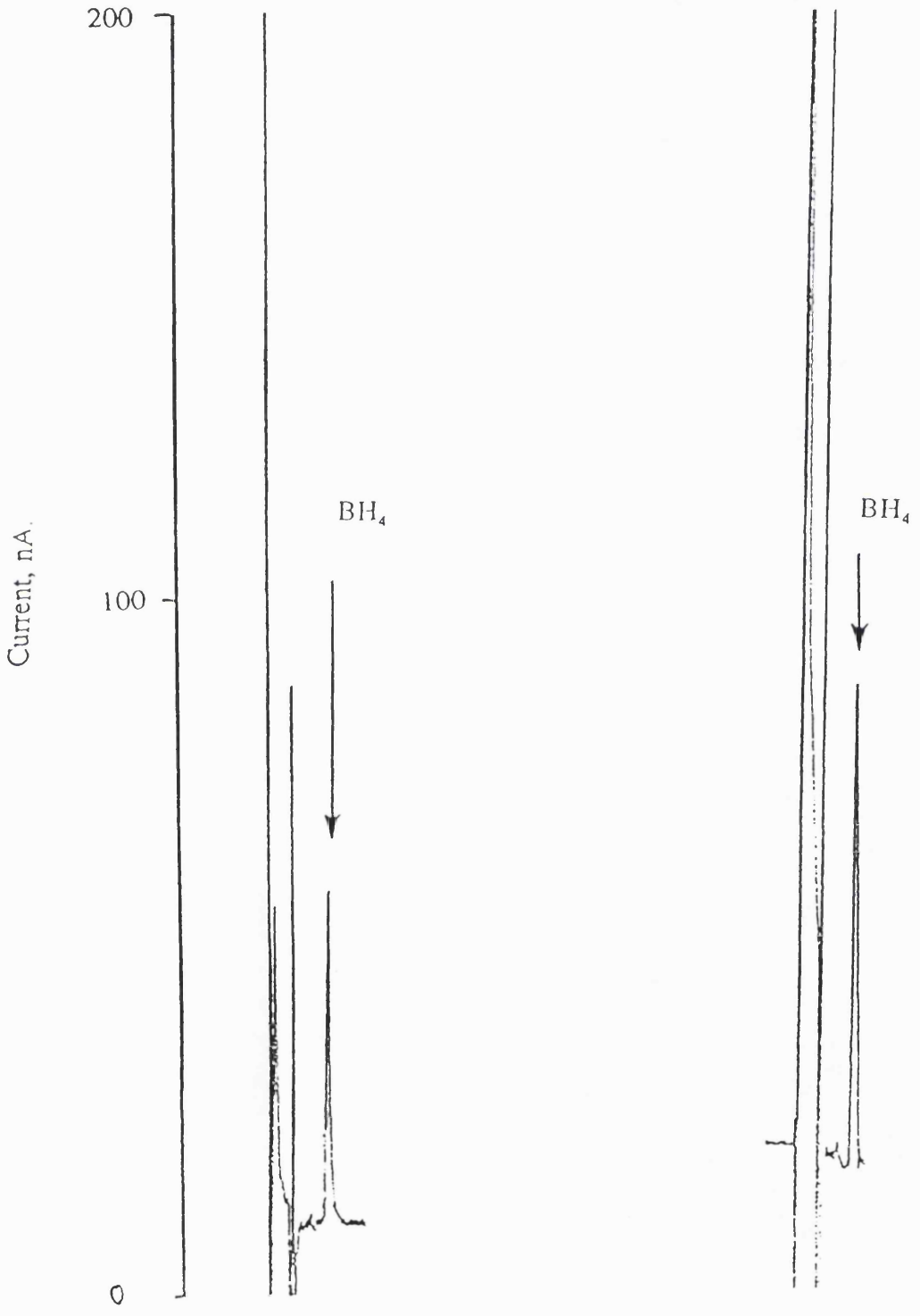


Figure 2.3. Tetrahydrobiopterin standard curve.



64 pmol BH_4 standard **30 day old control cerebellum**

Figure 2.4. Tetrahydrobiopterin chromatograms.

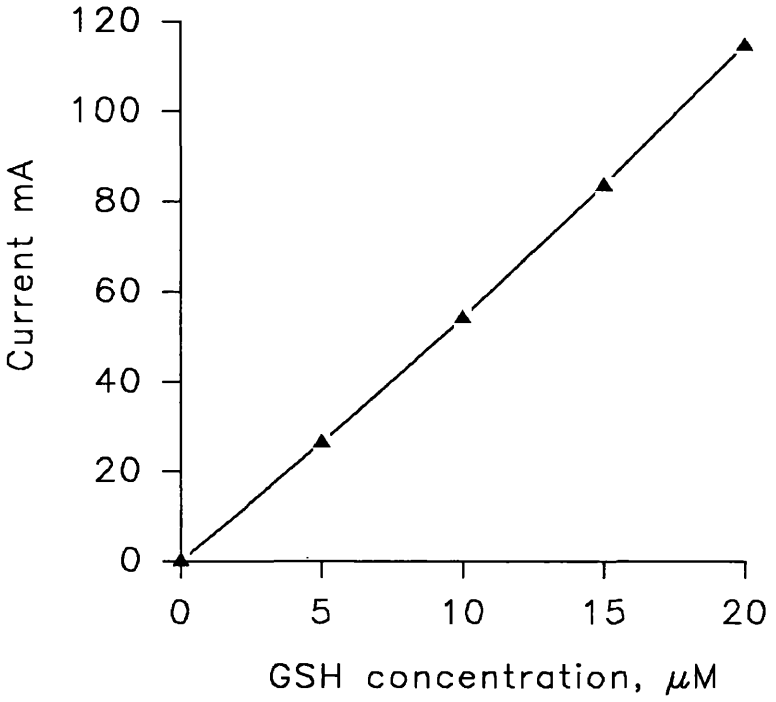
was performed at ambient temperature with a flow rate of 1 ml/minute. Detection of GSH and GSSG was performed in the oxidation mode with electrode 2 screening out the oxidised products of the electrode 1. A C-V curve was generated to find the optimal potentials and these were established as +0.2 for electrode 1 and +0.8 for electrode 2. Standards were prepared of 10 μM for GSH and 1 μM for GSSG. Samples were homogenised 1:10 in 16 mM phosphoric acid and spun at 14,000 RPM for 2 minutes to remove protein, supernatant was then further diluted 1:10 prior to injection onto the HPLC system. 10 μl of supernatant was injected onto the system. Detection of a 30 day old *hph-1* sample when spiked with 20 μM GSH showed 97.7 % recovery and a standard curve showed that detection was linear up to 20 μM for both GSH and GSSG (Figure 2.5.).

2.5. Nitric oxide synthase activity.

NOS activity can be measured by a variety of methods and techniques including

1. Spectrophotometrically by the reaction of NO with oxyhaemoglobin.
2. Measurement of nitrite and nitrate, the breakdown products of NO.
3. Quantification of [^{3}H] citrulline from [^{3}H] arginine.

The oxyhaemoglobin assay has the disadvantage that BH_4 at concentrations above 12 μM causes interference and so this assay is not suitable for studying the effects of BH_4 availability on NOS activity. Measurement of nitrite and nitrate is simple but this spectrophotometric assay is relatively insensitive and only allows micromolar concentrations of nitrite and nitrate to be measured. The [^{3}H] citrulline assay is a sensitive, discontinuous assay which uses cation exchange columns to separate radio-labelled citrulline from arginine. As citrulline and NO are formed in an equimolar ratio, citrulline formation allows a calculation of NOS activity (Bush et al., 1992). This assay can only be used as a quantitative step if there is no arginine present in the sample, but this can easily be removed by using an ion exchange resin. NOS activity was measured in the whole brain cytosolic fraction. Whole brain was homogenised (25% wt/vol) in buffer (320 mM sucrose, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol) and the homogenate was centrifuged at 100,000g for 60 minutes.



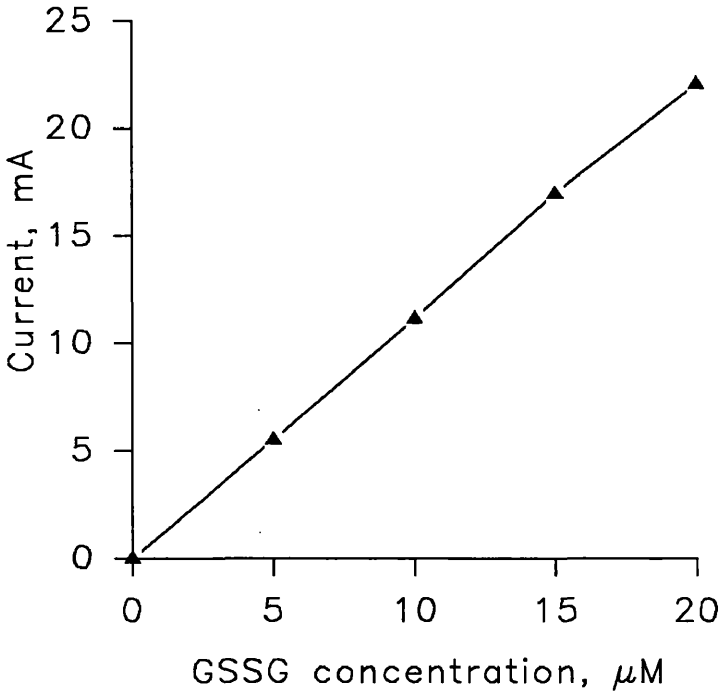


Figure 2.5. Glutathione (GSH) and oxidised glutathione (GSSG) standard curves.

Endogenous arginine was removed from the cytosol by cation exchange. Cation exchange resin, AG50W-X8 100-200 mesh, was converted from the hydrogen form to the sodium form by mixing with 1M sodium hydroxide and then washing with distilled water until the pH of the resin was ≤ 9.0. Initial experiments were carried out using approximately three mls of cytosol passed through a 2.5 ml column containing 2 mls of ion exchange resin. However, where the cytosol volume was reduced to 1 ml, the column size was reduced to 1 ml column containing 0.5 ml of cation exchange resin.

NOS measurement was carried out by quantification of ^3H citrulline formed from ^3H arginine (Hevel and Marletta, 1993). 100 µl of cytosol was added to 100 µl of reaction buffer containing 100 mM HEPES, pH 7.5, 100 µM NADPH, 1 mM Calcium chloride, 1mM magnesium acetate, 10 µg/ml calmodulin, 50 µM BH_4, 5 µM FAD, 5 µM FMN, 100 µM arginine and 1 µCi/ml ^3H arginine. Incubation temperature was 37°C. The assay was terminated using 50 µl TCA (1.5 M) and then neutralised using 1.5 M HEPES, pH 6.0. Blank values were determined where TCA was added before the protein to the reaction buffer. Arginine was removed from assay buffer by passing 1 ml through an ion exchange column, containing 2 mls of cation exchange resin and eluting the citrulline with 4 mls of water. The amount of labelled citrulline was measured by liquid scintillation counting using a Packard (Pangbourne, UK) 2500TR liquid scintillation counter. Specific activity of the reaction buffer was also determined in order to calculate enzyme activity. Disintegrations per minute (DPM) was used for all calculations.

Enzyme activity was calculated as μmol of arginine incorporated into citrulline per minute per mg of protein.

≡ μmol of NO produced per minute per mg of protein.

$$\equiv \mu\text{mol/min/mg} = \frac{\text{Activity (DPM)}}{\text{specific activity (DPM) x time x protein}}$$

$$\text{specific activity} = \frac{\text{DPM}}{\mu\text{mol}} = \frac{\text{DPM/ml}}{\mu\text{mol/ml}} = \frac{\text{DPM/ml}}{\text{mM}}$$

50 μl of reaction buffer is counted to obtain specific activity, this is equivalent to 100 μl of buffer as the reaction buffer was diluted 1:1 with cytosol.

Therefore 100 μl reaction buffer gives, for example, 200,000 DPM/100 μl.
$\equiv$ 2,000,000 per ml.

$$\text{Specific activity} = \frac{2{,}000{,}000 \text{ DPM/ml}}{0.1 \text{ mM}} \equiv \frac{2{,}000{,}000 \text{ DPM/ml}}{0.1 \ \mu\text{mol/ml}}$$

$\equiv$ 20,000,000 DPM/μmol

$$\text{Enzyme activity} = \frac{\text{Activity (DPM)}}{20{,}000{,}000 \text{ x incubation time x protein (mg)}}$$

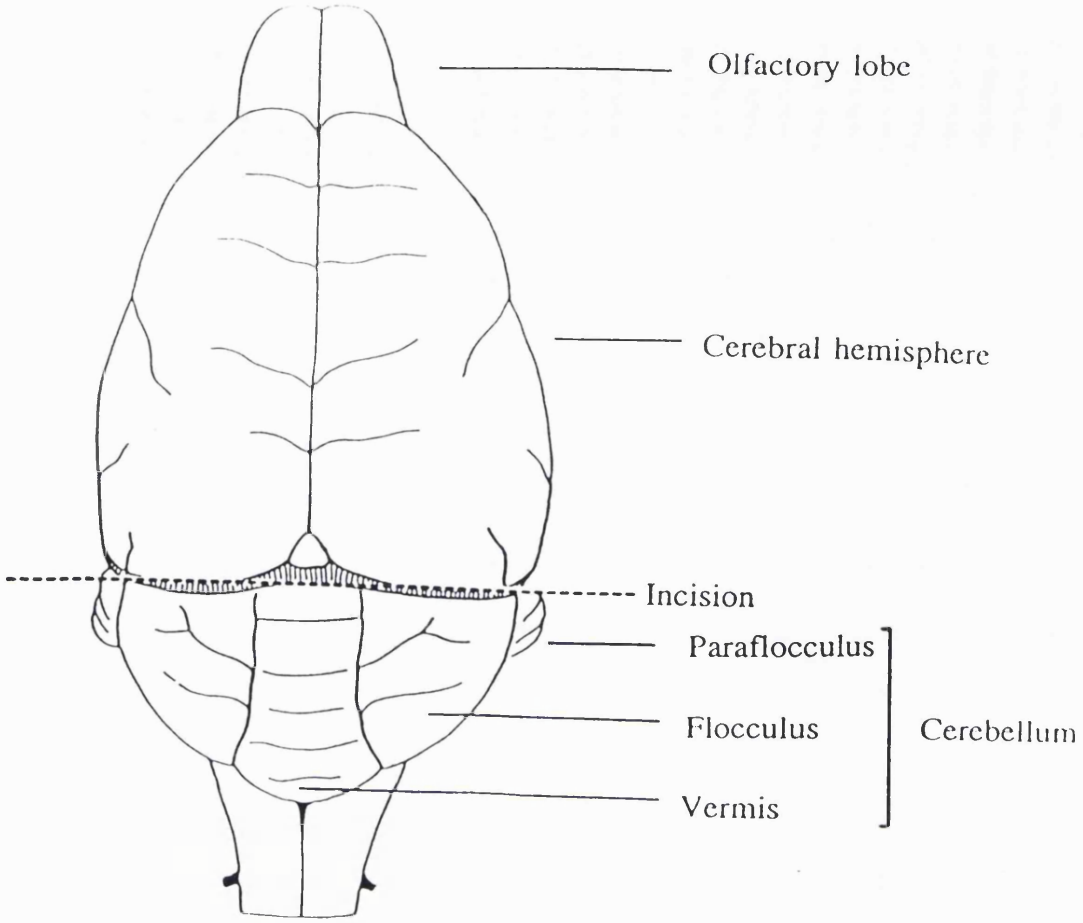
Enzyme activity expressed as pmol of citrulline formed per minute per mg of protein.

2.6. Preparation of Cerebellar Slices.

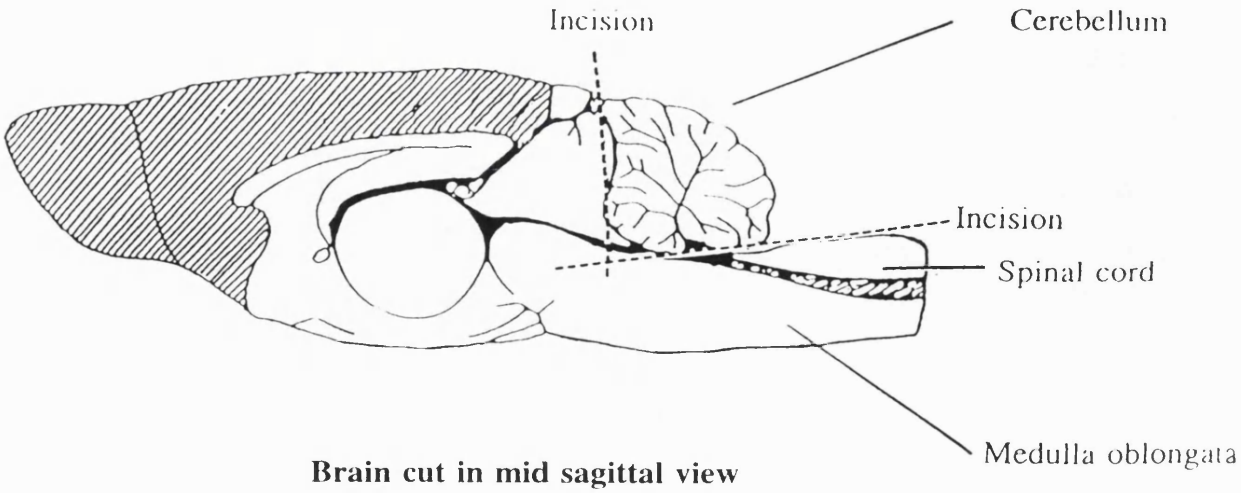
Slices of brain tissue incubated provide relatively intact preparations in which local synaptic circuitry is at least partially preserved *in vitro*. The cerebellum contains the highest level of NOS in the brain and also the lowest levels of phosphodiesterases (Garthwaite, 1991). It, therefore, provides a good model for the examination of the NO/cGMP pathway. Slices of mouse cerebellum were prepared from 10 and 30 day old mice.

2.6.1. 10 day old mice

Mice, of either group, were killed by cervical dislocation and the head severed. The cerebellum was removed by making lateral scissor-cuts through the occipital and parietal bones and the upper skull levered off carefully and bent back exposing the cerebellum which was excised using forceps and a scalpel (Figure 2.6.). The cerebellum was then placed on the chopping table of a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK) and sliced at 400 μm intervals. The



Dorsal view



Brain cut in mid sagittal view

Figure 2.6. Diagram representing dissection of the cerebellum

cerebellar slices were then dispersed in a petri dish containing 10 mls of room temperature Krebs-Henseleit buffer containing 120 mM sodium chloride, 2 mM potassium chloride, 2 mM calcium chloride, 1.19 mM magnesium sulphate, 1.18 mM potassium dihydrogen phosphate, 26 mM sodium hydrogen carbonate and 11 mM glucose. Buffer was gassed with 5% carbon dioxide in oxygen for 30 minutes prior to use to produce a pH 7.4 buffer. Each cerebellum produced two to three slices from the vermis. Slices from the vermis of the cerebellum were then pooled in a conical flask containing 20 mls of Krebs-Henseleit buffers. Slices were then incubated for 90 minutes at 37^0C in a shaking water bath whose speed was adjusted to keep the slices in gentle motion. The surface of the buffer was continuously gassed with 5% carbon dioxide in oxygen and the buffer changed after 30 minutes. After this pre-incubation slices were redistributed into 10 ml conical flasks containing 5 mls of fresh Krebs-Henseleit buffer with four slices per flask.

2.6.2. 30 day old mice

It has been previously shown in rats that preservation of cells after mechanical chopping (McIlwain tissue chopper) of the cerebellar varies considerably with the age of the rat (Garthwaite et al., 1979; Garthwaite et al., 1980). Up to 14 days of age mechanical chopping of the cerebellum provides tissue showing good morphological preservation (Garthwaite et al., 1979). However, in adult rats greater than 90% of the cell number appeared pyknotic and much vacuolation was seen in all areas of the cerebellum (Garthwaite et al., 1979; Garthwaite et al., 1980). An alternative to mechanical chopping is to slice the cerebellum using a Vibroslice (Campden Instruments, Loughborough, UK). The Vibroslice uses a vibrating blade to slice fixed tissues without the traumatic damage produced by mechanical chopping. For 30 day old mice, cerebellum was removed as before and hemispheres removed by parasagital scalpel cuts, the vermis was then fixed to the stage of the Vibroslice using cyanacrylate. The vermis was embedded to the stage using 3% Agar Noble (containing 0.9% sodium chloride and kept stirred at 37^0C), this provides a firm exoskeleton which stabilises brain tissue during sectioning. Slices are cut at 400 μm intervals in cool Krebs-Henseleit buffer (12 - 14^0C) previously gassed with 5% carbon dioxide in oxygen. The slices from different animals were then pooled in a conical

flask containing 20 mls of Krebs-Henseleit buffer and pre-incubated as for the 10 day old mice. They were then redistributed into 10 ml flasks containing 5 mls of fresh Krebs-Henseleit buffer with three to four slices per flask.

2.7. Morphology of brain slices.

Slices of brain tissue incubated are used extensively for a variety of neurochemcial investigations *in vitro*. When using slices it is important to know to which the tissue in isolation resembles that *in vivo*. One of the least ambiguous ways of assesing the preservation of cells is to examaine the morphology of the slices in order to determine that preservation of the cells in incubated slices was adequate. Staining with Haematoxylin, which denotes staining of the nuclei, and Eosin, which stains the cytoplasm, gives an indication of the preservation of the cells. Staining was carried out as follows: Slices were incubated for 90 minutes at 37^{0}C in oxygenated Krebs-Henseleit buffer with a change in buffer after 30 minutes. Slices were treated as follows:

1. Dehydrated and fixed in formal saline for 30 minutes.
2. Fixed in formal ethanol for 75 minutes.
3. Fixed in 90% ethanol for 60 minutes.
4. Fixed in 100% ethanol for five hours.
5. Fixed in 100% chloroform for three hours 30 minutes.
6. Immersion in wax for three hours 30 minutes.
7. Slices were then cut at 7 μm intervals in a cryostat.
8. Slices were then dewaxed in xylene and rehydrated by washing twice in 100% ethanol.
9. Washed twice with 70% ethanol
10. Washed twice with water.
11. Slices were stained with Harris' Haematoxylin for 3-5 minutes
12. Washed in water.
13. Slices were then washed in acid alcohol (1% hydrochloric acid in 70% ethanol) until the nuclei are strongly stained and the background was clear.
14. Slices were stained in Eosin for one minute.
15. Washed with tap water.

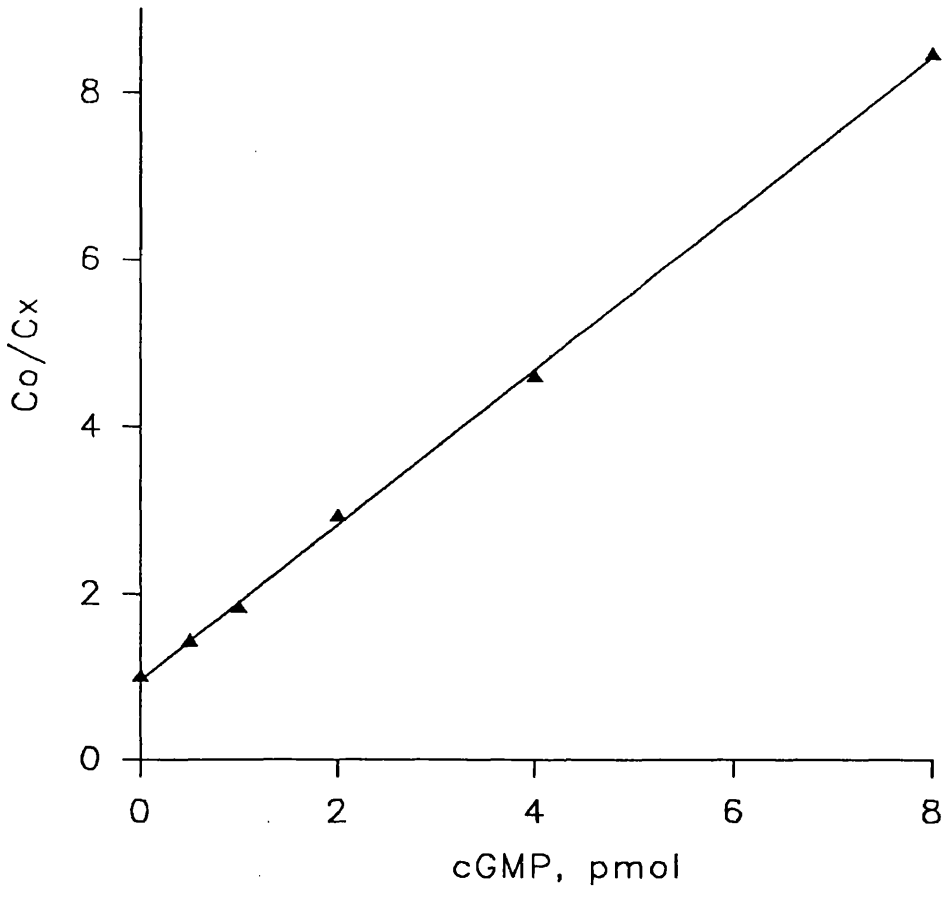
16. Slices were finally dehydrated and excess Eosin removed with washes with 70% ethanol and then twice with 100% ethanol.

17. Slices were cleared in xylene and mounted.

This analysis was kindly carried out by Mr R Courtney, Department of Neuropathology, Institute of Neurology, London, UK.

2.8. Analysis of cGMP.

The assay of guanosine 3'5'-cyclic monophosphate (cGMP) was measured using a radioimmunoassay kit (Amersham). This assay is based on the competition between unlabelled cGMP and a fixed quantity of [^{3}H] labelled compound for binding to an antiserum with high specificity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely proportional to the level of cGMP present in the assay sample. All reagents were prepared according to manufacturers recommendations. Measurement of cGMP was carried out in Eppendorf centrifuge tubes. To each tube, standard and test, 50 μl of [^{3}H] cGMP was added. A standard curve was constructed in duplicate across the range 0 - 8 pmol unlabelled cGMP (Figure 2.7.). Standard, 100 μl, was added to the tritiated cGMP, in addition two tubes were used for the determination of zero dose binding (binding in absence of unlabelled cGMP) using blank reagent. Brain slice samples were prepared by heating the slices in a boiling water bath for three minutes in 250 mM Tris / 4 mM EDTA, pH 7.6 followed by sonication using a Soniprep 150 Sonicator (Fisons Scientific Equipment, Loughborough, UK). 100 μl of each sample was added to the tritiated cGMP. To each standard or unknown, 50 μl of antiserum was added, samples were then vortexed and then incubated on ice for 90 minutes. After this incubation period 1 ml of ice cold 2.9 M ammonium sulphate was added to as many tubes as could be centrifuged in one batch. Tubes were vortexed and incubated on ice for a further five minutes, they were then centrifuged at 14,000 RPM for two minutes in an Eppendorf bench 5415c centrifuge. Tubes were then removed from the centrifuge and the supernatant decanted, excess liquid was carefully removed using tissue paper taking care not to disturb the precipitate. After removal of the supernatant, 1.1 mls of distilled water was added, tubes were then vortexed until all precipitate had dissolved. 1 ml of



Co is equal to counts per minute (CPM) bound in the absence of cGMP.

Cx is equal to the CPM bound in the presence of standard cGMP.

Co/Cx is determined for each standard, a linear graph with intercept at 1.0 is the result. To determine unknown samples, Co/Cx is calculated and cGMP concentration calculated from the standard curve.

Figure 2.7. cGMP standard curve.

sample was then removed and added to 10 mls of Ultima Gold (Packard) scintillation fluid. After mixing, counts per minute (CPM) were determined using a Packard (Pangbourne, UK) 2500TR liquid scintillation counter.

2.9. Mitochondrial electron transport chain enzymes.

The measurement of the mitochondrial electron transport chain enzymes (Figure 2.8.) was carried out using spectrophotometric techniques which use electron acceptors in order to study the activity of the individual mitochondrial electron transport chain enzymes.

2.9.1. Complex I (NADH-CoQ_1 reductase EC 1.6.99.3.).

Mitochondrial complex I catalyses the oxidation of NADH, ie electrons are transferred from NADH through complex I to ubiquinone which is reduced to ubiquinol. This assay is based on the method of Ragan et al. (1987) which measures the rotenone sensitive decrease in NADH at 340 nm. Tissue homogenates were freeze thawed three times using liquid nitrogen and samples diluted 1:5 prior to analysis. Reaction and reference cuvettes were set up and consisted of (final concentration) 25 mM phosphate buffer pH 7.2, 10 mM magnesium chloride, 2.5 mg/ml bovine serum albumin, 0.2 mM NADH, 1 mM potassium cyanide and 20 μl of sample. To the test cuvette, 80 μl of distilled water was added, 90 μl added to the reference. The cuvettes were mixed by gentle inversion using Parafilm to seal the cuvette and were placed in a dual wavelength Kontron Uvikon 940 spectrophotometer (Watford, UK) and absorbance measured at 340 nm. After two minutes, 10 μl of 5 mM ubiquinone (50 μM final concentration) was added to the test cuvette and mixed. Change in absorbance was measured for five minutes. After this period 20 μl of 1mM rotenone (50 μM final concentration) was added to the test cuvette, mixed and absorbance measured for a further five minutes.

The results are expressed as rotenone sensitive NADH - ubiquinone oxido-reductase specific activity. This is calculated by subtracting the rotenone insensitive rate from the overall initial rate. Enzyme activity was calculated using the Beer Lambert law and expressed as nmol/min/mg:

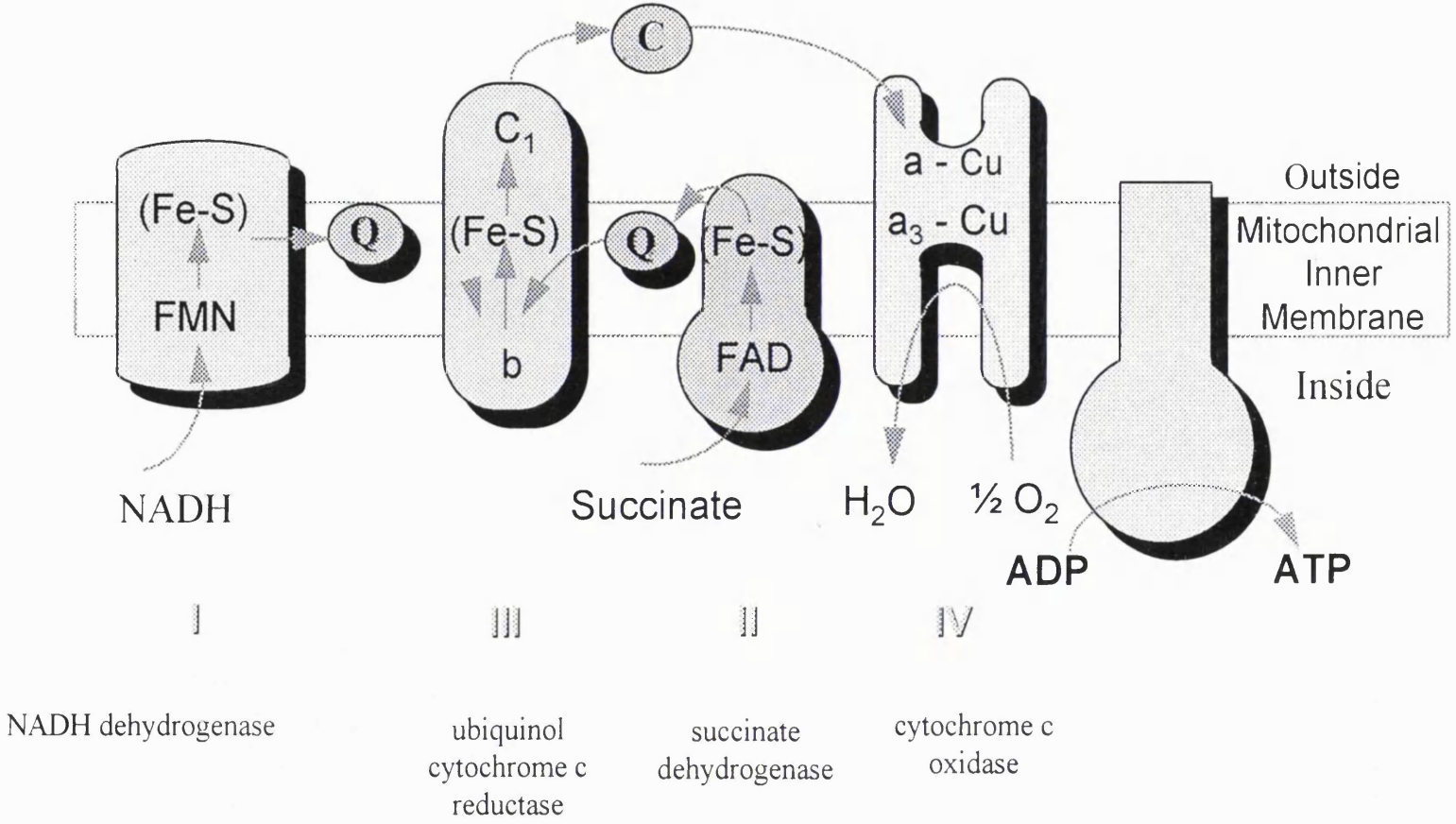


Figure 2.8. Mitochondrial electron transport chain.

$$A = \varepsilon \times c \times l$$

where: A = change in absorbance

ε = molar absorbance coefficient, 6.81×10^3 for NADH

c = concentration

l = pathlength, 1cm

2.9.2. Complex II/III (succinate cytochrome c reductase EC 1.8.3.1).

Succinate is oxidised by complex II. Electrons are transferred from complex II by ubiquinone which is reduced to ubiquinol which is then reoxidised by complex III with the transfer of electrons to cytochome c. Complex II/III activity was measured by the succinate dependent antimycin a sensitive reduction of cytochrome c (King, 1967). Samples were freeze thawed three times as for complex I and 20 μl added to a reaction buffer made up in a 1 ml cuvette which consisted of (final concentration) 100 mM phosphate buffer, pH 7.4, 0.3 mM EDTA, 1 mM potassium cyanide and 0.1 mM cytochrome c. Cuvettes were set up in duplicate with one used as a reference cuvette, 185 μl of distilled water was added to the test cuvette, 225 μl of distilled water was added to the reference cuvette. Cuvettes were mixed as for Complex I and placed in a Kontron Uvikon 940 spectrophotometer set at wavelength 550 nm. After two minutes, 40 μl of 0.5 M succinate (20 mM final concentration) was added to the test cuvette, mixed and replaced in the spectrophotometer. After 5 minutes 10 μl of 1 mM antimycin (10μM final concentration) was added to the test cuvette, mixed and absorbance measured for 5 minutes.

Complex II/III activity was expressed by subtracting the rate prior to addition of antimycin a from the rate after antimycin a addition. Activity was calculated as for complex I, molar absorbance coefficient of cytochrome c is 19.2×10^3.

2.9.3. Complex IV (cytochrome oxidase EC 1.9.3.1).

Complex IV catalyses the oxidation of cytochrome c from reduced cytochrome c to molecular oxygen. The assay of complex IV activity relies on measuring absorbance change as reduced cytochrome c is oxidised and is dependent on cytochrome c

concentration. The assay for determination of complex IV activity is expressed as a first order reaction because the reaction is first-order with respect to cytochrome c. The first order constant, *k*, relates reaction rate, V, to concentration of substrate [S] thus:

$$V = k\,[S]$$

The assay is based on the method of Wharton and Tzagoloff (1967) and measures the oxidation of reduced cytochrome c at 550 nm. Cytochrome c (10 mg/ml) was prepared in distilled water and reduced using a few crystals of ascorbate. The ascorbate was removed from the cytochrome c by passing it through a PD-10 column containing 8.5mls of Sephadex G25 gel filtration media (Pharmacia). Cytochrome c elutes faster than ascorbate due to its larger molecular mass and can easily be separated from the ascorbate. The column was washed with 10 mM phosphate buffer, pH 7.0, before and after the application of cytochrome c. Concentration of reduced cytochrome c was determined by placing 50μl of cytochrome c in test and reference cuvettes together with 950 μl of water. To the reference cuvette 10 μl of 100mM ferricyanide was added which oxidised the cytochrome c. The absorbance reading was used in the Beer Lambert equation to determine the concentration of cytochrome c:

$$c = \frac{\text{absorbance reading} \times 20 \text{ (Dilution factor)}}{19.2 \times 10^3 \text{ (molar absorption coefficient)}}$$

Reaction buffer consisted of (final concentration) 10 mM phosphate buffer, pH 7.0, 50 μM reduced cytochrome c and 1 mM ferricyanide in the reference cuvette. Ferricyanide was omitted from the test cuvette. Both cuvettes were placed in a Kontron 940 Uvikon spectrophotometer and the reaction was initiated with 20 μl of homogenate added to the test cuvette and absorbance measured at 550 nm for five minutes.

To calculate enzyme activity, maximal absorbance after addition of homogenate was found. This was designated as time = 0. Absorbance at time = 1, 2 and 3 minutes after time = 0 were then determined. The first order reaction rate constant, *k*, was

calculated from these by the following equation:

$$k = \frac{\ln (A)t=0}{(A)t=x}$$

Where k = rate constant

ln natural log

At=0 Absorbance at time = 0.

At=x Absorbance at 1 minute.

Absorbance at two and three minutes was divided by two or three respectively to give *k*/minute. The mean of the calculated *k* values were taken and the calculated activity was expressed in *k*/minute. This was then divided by protein to give enzyme activity as *k*/minute/mg protein.

2.9.4 Citrate synthase (EC 4.1.3.7).

Citrate synthase catalyses the condensation of oxaloacetate and acetyl CoA to form citrate and free CoA. Citrate synthase is a mitochondrial matrix marker enzyme. The activity of citrate synthase was measured by the reaction of free CoA with 5'dithio-bis(2-nitrobenzoic acid) (DTNB) which has a maximal absorbance at 412 nm (Shepherd and Garland, 1969). The assay was measured using sample and reference 1 cm cuvettes as before. The reaction buffer was (final concentration) 95 mM Tris buffer containing 0.1% Tris, pH 8.0 for the sample cuvette. To each of the cuvettes, 10 μl of 10 mM acetyl CoA (100 μM final concentration), 10 μl 20 mM DTNB (200 μM final concentration) and 20 μl of sample (freeze thawed three times) were added. The cuvettes were mixed and placed in a Kontron 940 Uvikon spectrophotometer, the reaction is started by the addition of 10 μl of 20 mM oxaloacetate (200 μM final concentration), absorbance was then read for 5 minutes.

Enzyme activity was calculated as for complex I, the molar absorbance coefficient for DTNB is 13.6×10^3.

2.10. Amino acid analysis

Whole brain was homogenised in 0.1 M perchloric acid and spun at 14,000 g for two minutes. Supernatant was mixed with an equal volume of 10% sulphosalicylate containing 200 μM norleucine as internal standard and spun at 4,000 g for ten minutes. Supernatant was passed through a 0.22 μm membrane filter. Analysis of amino acids was carried out using automated ion exchange chromatography on a Biochrom 20 amino acid analyser (Pharmacia Biotech, Cambridge, UK). Separation took place in a column of sulphonated cross linked polystyrene resin, a strong cation exchanger. At the low pH used for separation, the column is strongly anionic and positively charged amino acids will be attracted to the column. Injection volume was 70 μl and elution was carried out using a standard physiological fluid elution programme. When the pH of the buffer passed through the column was gradually raised and the amino acids will come off the column according to their p*I* values. This analysis was kindly carried out by Mr A Briddon, Department of Clinical Biochemistry, National Hospital for Neurology and Neurosurgery, London, UK.

2.11. Protein analysis

Protein determination was based on the method of Lowry et al. (1951) and was carried out using the Bio Rad DC Protein Assay kit. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. In brief, bovine serum albumin was used to prepare a standard curve across the range 0 - 100 μg protein. To 200 μl of standard or unknown 100 μl of solution A, alkaline copper tartrate, was added. After vortex mixing, 800 μl of solution B, dilute Folin reagent, was added and vortex mixed. After standing for 15 minutes, absorbance was measured at 750 nm using a Beckman DU-6 spectrophotometer (San Ramon, Ca, USA).

2.12. Statistical analysis.

Statistical significance for the comparison of two groups was by a Student's unpaired *t* test. Multiple comparisons were made by one-way analysis of variance followed by the least significant multiple range test. All results are expressed as the mean $\pm$ standard error of the mean (SEM). All statistical analysis was carried out using

Statgraphics statistical programme (Statistical Graphics Corporation, Maryland, USA).

Chapter3.

The *hph-1* mouse.

Chapter 3. The *hph-1* mouse.

3.1. Development of the *hph-1* mouse.

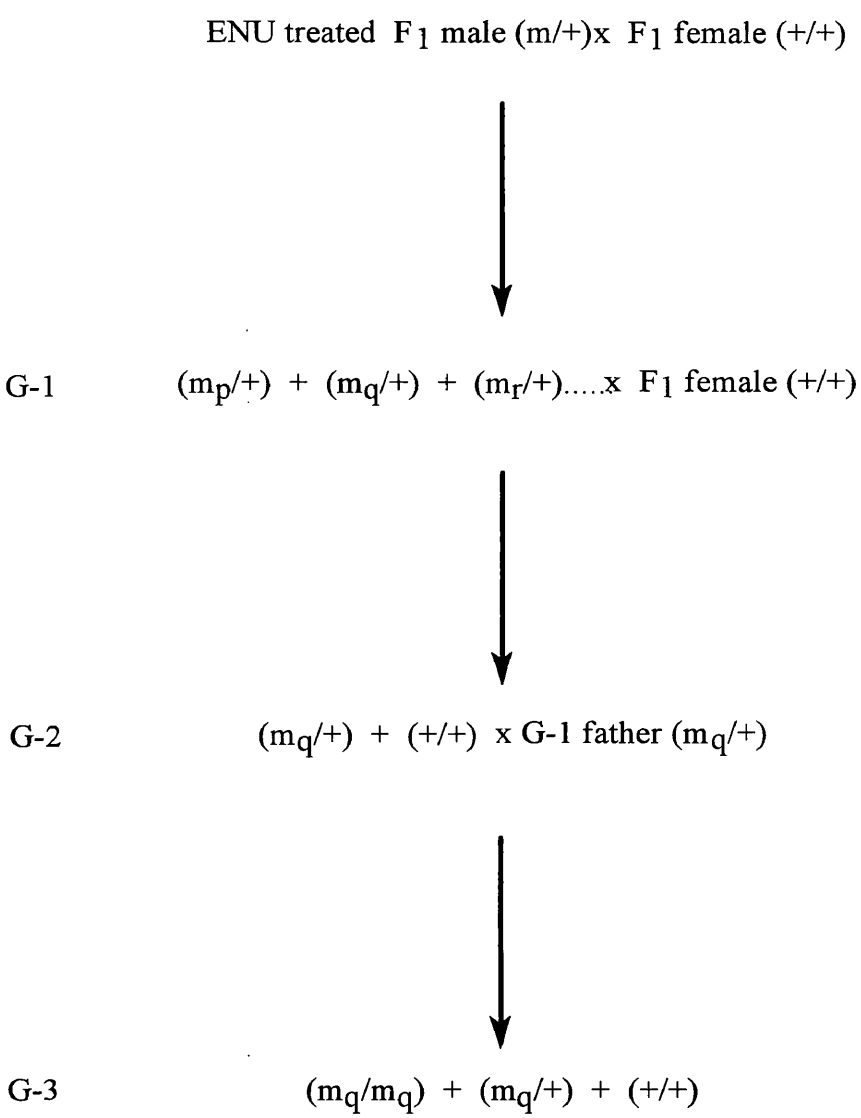
The *hph-1* mouse was originally developed in response to the demand for a model of phenylketonuria which is defined as persistent hyperphenylalaninaemia (>240 μmol/l), relative tyrosine deficiency and excretion of phenylketones (MRC working party on phenylketonuria, 1993). In the United Kingdom, this disease affects approximately 1:10 000 live births, the cause is usually due to a recessively inherited defect in the hepatic phenylalanine hydroxylase enzyme. However, 1-2% of subjects with phenylketonuria arise from deficiency of tetrahydrobiopterin (BH_4), the cofactor for phenylalanine hydroxylase (Smith and Brenton, 1995).

Previous animal models of hyperphenylalaninaemia have used a variety of techniques with partial or limited success. Auerbach et al. (1958) fed large amounts of phenylalanine together with tyrosine to rats in order to inhibit the hydroxylation reaction and so bring about hyperphenylalaninaemia. Phenylalanine hydroxylase levels were claimed to be a third of normal rats and behavioural disturbances were claimed in the treated group. However, such findings could not be substantiated. Dolan and Godin (1967) fed weanling rats a 5% phenylalanine diet for a week followed by a 7% phenylalanine diet. At the end of their experimental period they concluded that a high phenylalanine diet did not fulfil the requirements of a PKU model because the high plasma levels of phenylalanine induced were transient and were accompanied by high plasma levels of tyrosine.

Chemically induced models of hyperphenylalaninaemia have been produced where phenylalanine hydroxylase is inhibited. Using ρ-chlorophenylalanine (PCPA), an irreversible inhibitor of phenylalanine hydroxylase, together with phenylalanine, Lipton et al. (1967) showed a large increase in the phenylalanine to tyrosine ratio. However, PCPA was found to lead to an immediate reduction of food intake and subsequent weight loss (Copenhaver et al., 1970). Because PCPA must be given continuously in order to maintain the hyperphenylalaninaemia, the use of this chemical does not produce a good model of the disease because of the side effects caused.

An alternate mechanism to produce hyperphenylalaninaemia is to produce peripheral BH_4 deficiency, the cofactor of phenylalanine hydroxylase. This can be carried out using diamino-hydroxypyrimidine (DAHP), which inhibits the first committed step of BH_4 synthesis, GTP cyclohydrolase I (Gál and Whitacre, 1981). Using DAHP mixed with the diet (0 - 7.0% by weight) Cotton (1986) produced almost undetectable levels of liver BH_4. In this model, BH_4 deficiency was suspected to be confined to the periphery so that only liver phenylalanine hydroxylase is affected thus leading to hyperphenylalaninaemia (Cotton, 1986). In the adult mice of the study, no affect was observed on brain BH_4 levels leading to the conclusion that DAHP did not cross the blood brain barrier (Cotton, 1986). However, Cox et al. (1992) reported that DAHP mixed with the diet at a 5.0% by weight level, does effect rat brain levels of total biopterin. However, use of DAHP lead to lower dietary intake and weight loss (Cotton, 1986; Cox et al., 1992). Reduced dietary intake itself can lead to lower brain biopterin levels and can confound the use of DAHP in experimental animals (Cox et al., 1992). Therefore, this model does not appear suitable for the study of hyperphenylalaninaemia.

Another mouse model for hyperphenylalaninaemia was presented by Bode et al. in 1988. This model was produced from C57Bl/6 x CBA/Ca mice using the sperm mutagen, N-ethyl-N-nitrosourea (ENU) (Bode et al., 1988). Male mice were given intraperitoneal injections of ENU and were then allowed to recover for 7/8 weeks in order to regain fertility. After the recovery period the ENU treated mice were mated with wild type females on a weekly basis. The F_1 progeny of the ENU treated males were tested for hyperphenylalaninaemia using the bacterial inhibition method of Guthrie and Sussi (1963) which gives an indication of raised phenylalanine levels in the blood. However, in the initial testing of 7015 first generation offspring no positive tests for high phenylalanine were found (Bode et al., 1988). It was concluded that it was necessary to produce homozygous mutant mice because this system only produced heterozygotes (Bode et al., 1988). Therefore, a three generation breeding scheme was established to produce homozygous mutant mice (Figure 3.1.). ENU mutagenised males were mated with wild type females to produce approximately 25 male progeny, generation 1 (G-1). These G-1 progeny would contain heterozygotes



m - mutant

(+/+) - wild type control mice

(m/+)- ENU treated male mice

(m_p/+), (m_q/+), (m_r/+) -mutant heterozygotes for any given locus

(m_q/m_q) - mutant homozygotes for a given locus

G-1, G-2, G-3 - Generation 1, 2 and 3

Figure 3.1. Outline of three generation breeding scheme to produce homozygous mutant mice.

Adapted from Bode et al. (1988).

for many mutations (m_p, m_q, m_r) as ENU is a very potent sperm mutagen and will produce many different mutations. The G-1 males were then mated with wild type females. For any given mutant locus, m_q, 50% of the G-2 daughters will be mutant heterozygotes, 50% will be wild type. The daughters from this G-2 progeny were then back crossed with their G-1 fathers. Therefore, the G-3 progeny will consist of 25% wild type, 50% mutant heterozygotes and 25% homozygous mutants, m_q. The G-3 progeny were tested at one week for hyperphenylalaninaemia by culling the litter and testing for high phenylalanine levels using the test of Guthrie and Susi (1963). When a positive test was encountered other litters are produced from the relevant parents. For G-1 and G-2 litters average litter size was 7.18 and 7.02 respectively. However, average G-3 litter size was 4.56. The reduction in G-3 litter size by approximately a third may be due to induction of new induced lethal mutations (Bode et al., 1988). Using 19 ENU treated male mice, 105 G-1 male progeny were produced. Among this group was a carrier for hyperphenylalaninaemia which when back crossed with one of the G-2 daughters produced a litter of ten, of which there were three positive tests for elevated phenylalanine. The inheritance of hyperphenylalaninaemia is as expected for a single Mendelian gene and acts in a recessive manner (Figure 3.1.).

The mice produced were termed *hph-1* with *hph* being an abbreviation of hyperphenylalaninaemia. The *hph-1* mouse has a mutation that leads to a deficiency in GTP cyclohydrolase I (McDonald et al., 1988), the first committed step in BH_4 synthesis. In the liver GTP cyclohydrolase I activity is less that 90 % of control wild type mice (McDonald et al., 1988). The mutation in the *hph-1* mouse may involve alteration of the catalytic site but it does not detectably alter the whole enzyme structure (Cha et al., 1990). The mutation does not alter the sequence within the reading frame of GTP cyclohydrolase I but affects the steady state levels of the enzyme mRNA produced (Gutlich et al., 1994b). It is, therefore, assumed that the defect resides in the regulatory region of the GTP cyclohydrolase gene (Gutlich et al., 1994b). The effect of the mutation is that it leads to reduced tissue levels of BH_4 (Hyland and Bola, 1989).

Because the *hph-1* mouse only displays hyperphenylalaninaemia until age 12 days,

it was only of limited use for the study of classical phenylketonuria and a further mouse model, *hph-5*, was produced in 1990 (for review see McDonald, 1994). This mouse displays a mutation of the phenylalanine hydroxylase gene leading to a specific inability to clear phenylalanine (McDonald, 1994). In this respect this mouse is very similar to the human disease, phenylketonuria and therefore provides a good model for the investigation of hyperphenylalaninaemia caused by phenylalanine hydroxylase deficiency. However, the *hph-1* mouse provides a good model for the study of BH_4 deficiency.

3.2. Biochemical characteristics of the *hph-1* mouse.

The primary defect in the *hph-1* mouse is deficiency in GTP cyclohydrolase I (McDonald et al., 1988). The animals display hyperphenylalaninaemia until about 12 days when phenylalanine levels gradually drop to normal levels at 20 days (Hyland and Bola, 1989). Plasma tyrosine levels are also reduced when compared to control mice up to 20 days of age in the *hph-1* mouse but by 30 days have returned to normal (Hyland and Bola, 1989). Normal serum phenylalanine and tyrosine levels after 20 days are probably due to a slow accumulation of BH_4 by the liver during the first month of life (Hyland et al., 1996a). At 4 days liver BH_4 levels in the *hph-1* mouse are 10% of those seen in control mice (Hyland and Bola, 1989; Hyland et al., 1996a). At 30 days liver BH_4 levels in the *hph-1* mouse are 45% of those seen in controls (Hyland and Bola, 1989). Together with the increase in liver BH_4 at 30 days is the change in diet from milk to a solid diet which is not so high in phenylalanine so this will also contribute to the reduction in plasma phenylalanine levels. However, although plasma phenylalanine concentration is normal in adult *hph-1* mice, a phenylalanine challenge results in two fold higher levels in plasma phenylalanine than control mice (McDonald and Bode, 1988). The half life of phenylalanine in the plasma after a phenylalanine challenge is 5.5 hours in the *hph-1* mouse whereas in wild type control mice the hyperphenylalaninaemia is transient and rapidly abates, plasma phenylalanine returns to normal after 1.5 hours (McDonald and Bode, 1988). This decreased ability to hydroxylate phenylalanine may be due to a combination of two factors, decreased level of liver BH_4 and a reduced level of phenylalanine hydroxylase (Hyland et al., 1996a). Liver phenylalanine hydroxylase and BH_4 levels

are usually approximately equal and most BH_4 is bound to the enzyme (Mitnaul and Shiman, 1995). Therefore, this observation suggests that BH_4 may control the *de novo* synthesis of phenylalanine hydroxylase or may be required for the stability (Hyland et al., 1996a). Decreased phenylalanine hydroxylase specific activity has also been reported in a child with a BH_4 synthesis defect (Dhondt, 1991).

BH_4 levels are also decreased in whole brain in the *hph-1* mouse at all ages up to at least 30 days (Hyland et al., 1996a). In post natal control mice, whole brain BH_4 levels decreased with age (Hyland et al., 1996a). In contrast , whole brain BH_4 levels in the *hph-1* mouse are essentially unaffected by age being 44% of control values at 30 days of age (Brand et al., 1995; Hyland et al., 1996a). BH_4 is also the essential cofactor for tyrosine and tryptophan hydroxylase which are the rate limiting steps in the production of dopamine and serotonin within monoamine producing neurones (Leeming et al., 1981). At 30 days of age there is a significant reduction in dopamine and serotonin metabolism in whole brain of the *hph-1* mouse (Hyland et al., 1996a). The greatest effect was seen on serotonin metabolism, serotonin itself was reduced by 22% and it's metabolite 5-hydroxyindoleacetic acid was decreased by 44% (Hyland et al., 1996a). Dopamine levels were reduced by 14% and the metabolites homovanillic acid and 3,4-dihydroxyphenylacetic acid reduced by 26% and 34% respectively(Hyland et al., 1996a). Noradrenaline levels were only reduced by 5% (Hyland et al., 1996a). Endogenous activities of tyrosine and tryptophan hydroxylase are also decreased in the *hph-1* mouse leading to decreased turnover of dopamine and serotonin (Hyland et al, 1996a). A reduction in the steady state concentration of brain tyrosine hydroxylase protein has also been shown in the *hph-1* mouse (Hyland et al., 1996a). Similarly, reduced levels of tyrosine hydroxylase enzyme protein has been shown in patients suffering from DRD (Hyland et al., 1996a). It is not known if the levels of tryptophan hydroxylase are similarly affected. These observations may explain why the acute peripheral administration of tetrahydropterins had little effect on dopamine or serotonin turnover in the *hph-1* mouse despite normalisation of brain BH_4 levels (Brand et al, 1996).

The *hph-1* mouse provides a good model for the study of BH_4 synthesis defects and

has been used for the study of the effect of BH_4 deficiency on biogenic amine metabolism (Hyland et al., 1996a; Brand et al., 1996). This study uses the *hph-1* mouse to study the effect of BH_4 deficiency on brain nitric oxide metabolism.

3.3. General characteristics and development of the *hph-1* mouse.

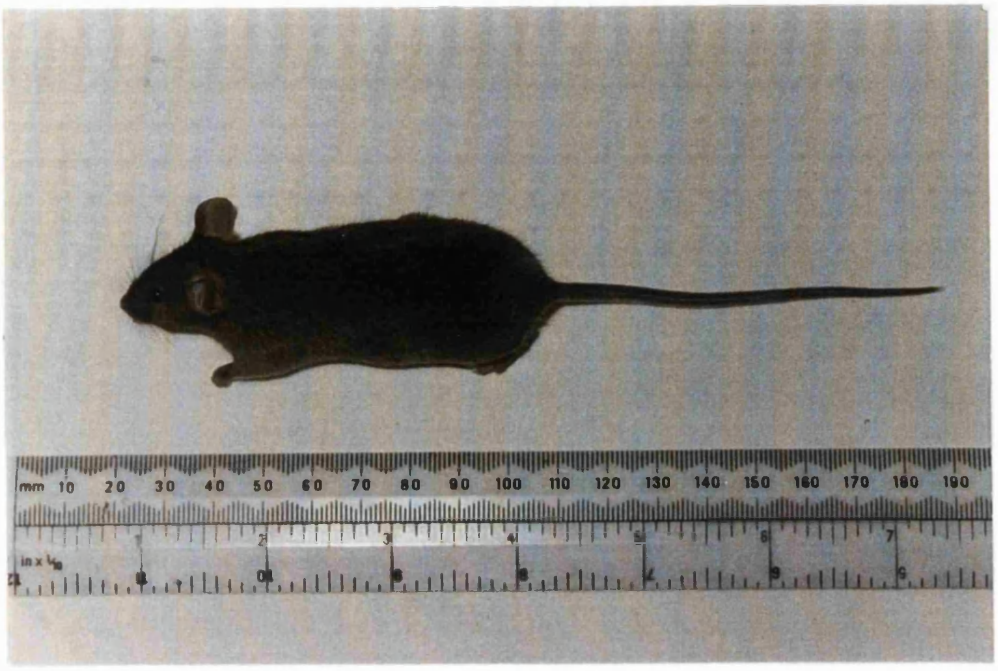
In 1987 a *hph-1* mouse colony was brought into the United Kingdom by Dr Keith Hyland at the Institute of Child Health, London. The colony was subsequently transferred to the Institute of Neurology, London, by Dr Simon Heales in 1993. Thirty breeding pairs are currently maintained and to conserve the GTP cyclohydrolase I mutation, in-breeding is carried out by brother-sister mating. A colony of control mice (C57BL/6 x CBA/Ca) are also maintained by seven breeding pairs. In breeding of the control colony is also by brother-sister mating.

Despite biochemical characterisation of the mice little documentation of the general characteristics has been reported. The *hph-1* mouse colony are mottled brown in colour. Control mice (C57/Bl/6 x CBA/Ca), ie the strain used to produce the *hph-1* mouse, are either mottled brown or black because a black mouse is crossed with a brown mouse to produce the control strain. The *hph-1* mouse appears morphologically normal when compared to appropriate control mice apart from being smaller in size at 10 days (Figure 3.2.) and also at 30 days (Figure 3.3.). Control mice were compared to *hph-1* mice and body weight measured over a period of 69 days (Figure 3.4.). From 18 days post birth, male *hph-1* mice weighed significantly less than male control mice (Figure 3.4.). Similarly, female mice, *hph-1* mice weighed significantly less than female control mice from 10 days post birth. At 10 and 30 days there is no significant difference between the weight of male and female *hph-1* mice. The food intake to weight gain ratio was monitored for both groups of mice and no significant difference was observed between the two groups: control, 1.57 ± 0.01, n=8; *hph-1* 1.50 ± 0.07, n=7 (Data shown as mean $\pm$ SEM) indicating that the reduced weights of the *hph-1* mouse maybe attributed to growth retardation rather than due to malnutrition. Brain weight was measured for both groups of mice at 10 and 30 days of age (Table 3.1.). At both age points brain weight was significantly reduced in the *hph-1* group when compared to controls. When brain weight was compared to body

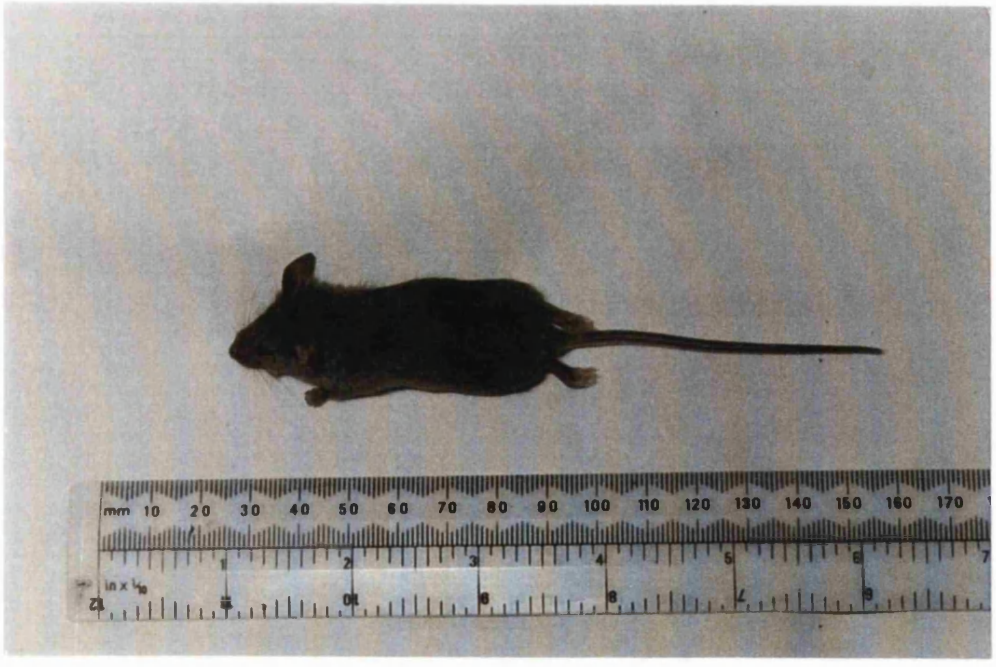
Control

hph-1

Figure 3.2. Control and *hph-1* mice at 10 days of age.

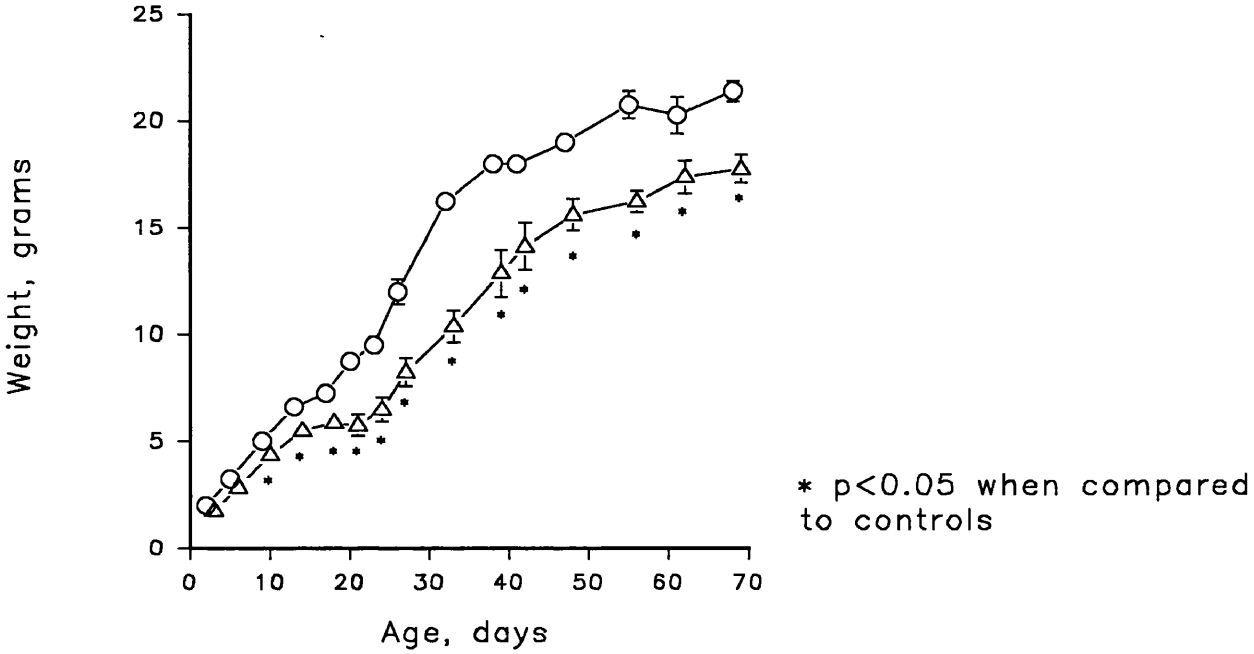


control

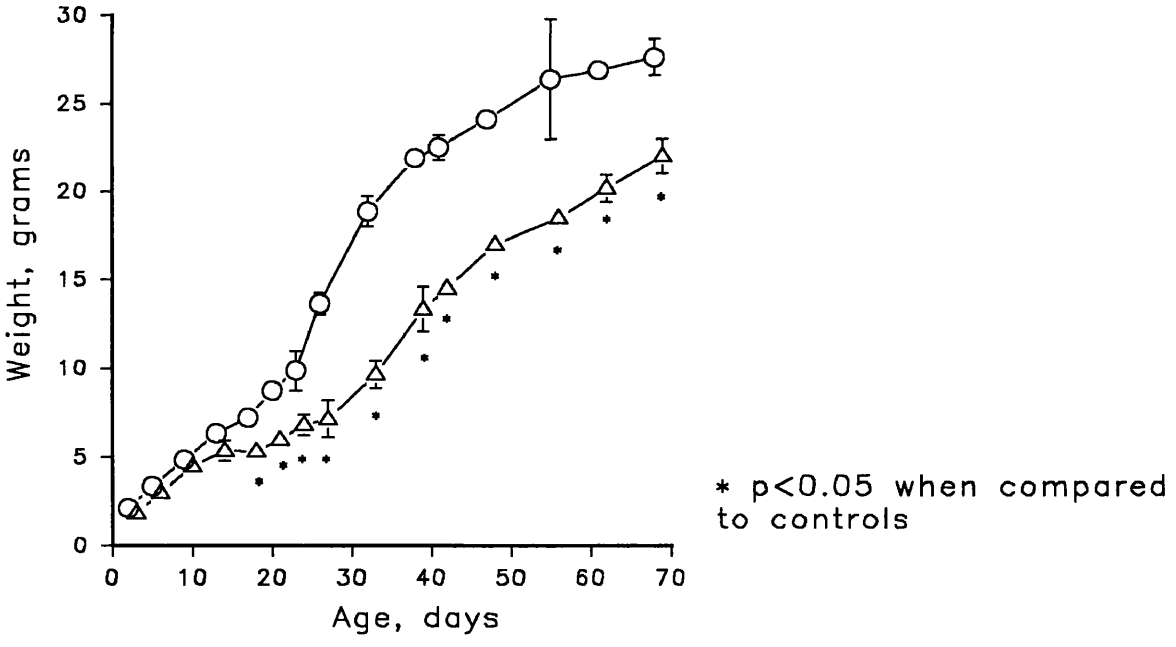


hph-1

Figure 3.3. Control and *hph-1* mice at 30 days of age.



Female mice



Male mice

Figure 3.4. Body weight of control and *hph-1* mice.

	Control		*hph-1*	
	Whole brain weight, g.	Brain: Body weight %.	Whole brain weight, g.	Brain: Body weight %.
10 day old mice	0.32 ± 0.04 (21)	6.5 %	0.27 ± .02 (28)	6.1 %
30 day old mice	0.41 ± 0.02 (53)	3.2 %	0.34 ± .02 (32)	4.4 %

Data displayed as mean ± SEM. Numbers in parentheses are equal to sample size.

Table 3.1. Brain weight in control and *hph-1* mice.

weight, no difference was observed in brain to body weight ratio (Table 3.1.)

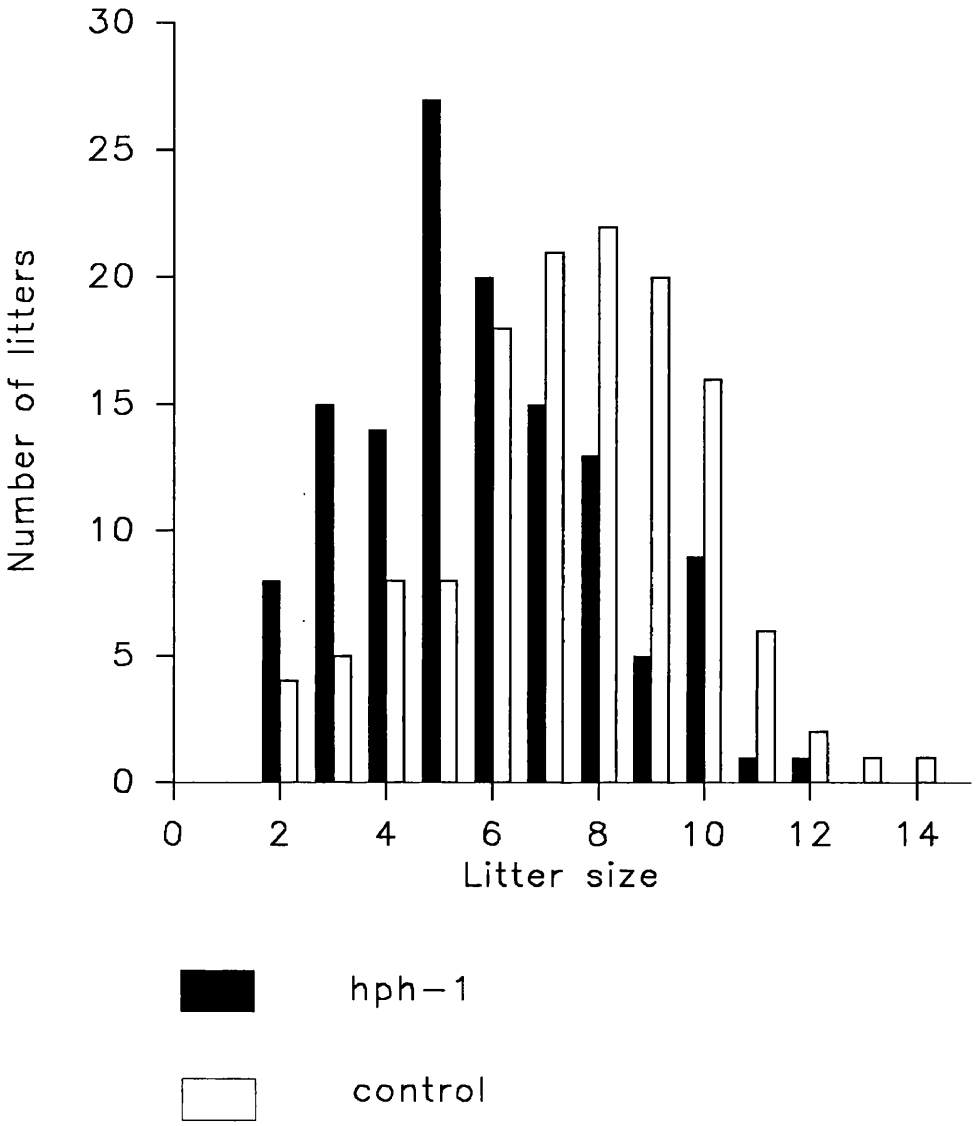
Litter sizes were measured from 128 litters for *hph-1* mice and 132 litters for control mice (Figure 3.5.). Mean litter size was 5.8 ± 0.2 for the *hph-1* mice colony and was significantly lower ($p<0.001$) than for the control mice, 7.5 ± 0.2 (Data shown as mean ± SEM). The number of still births and neonate mortality was also significantly higher ($p<0.001$, standard error of the difference between the two populations) in the *hph-1* mouse with 39% of all births not surviving past 1 day. This compares to 5.5% in the control mice colony.

3.4. Discussion

From this data it can be seen that the *hph-1* mouse weighs less and appears smaller than the control mice from which they were derived. Brain size appears smaller in the *hph-1* mouse but as a percentage of body weight, no significant difference was observed between the two groups. No difference was observed in food intake ratio between the different groups of mice. The *hph-1* mouse colony, therefore, appears to display growth retardation not attributable to malnutrition.

The *hph-1* mouse produces smaller litters than control mice. Recent evidence has shown that foetal *hph-1* mice, although not suffering from hyperphenylalaninaemia, display a 50% deficiency of dopamine and serotonin (Hyland et al., 1996a). Therefore, deficiency of BH_4 leads to reduction in monoamine neurotransmitter turnover which may in turn lead to malformation of the neuronal circuitry and account for the increase in neonatal mortality observed in the *hph-1* mice colony (Hyland et al., 1996a).

The *hph-1* mouse appears to be a good model for the study of the inborn errors of BH_4 metabolism which now also include hereditary Dopa (Dihydroxy-phenylalanine) responsive dystonia (DRD). In conclusion, the *hph-1* mouse is a good biochemical model of the inborn errors of BH_4 metabolism. As such, it has been utilised in this study to investigate the effect of BH_4 deficiency on brain NO metabolism.



Histogram showing an approximate normal distribution for litter size for both control and *hph-1* mice.

Total number of litters: control - 132 litters, 983 births

hph-1 - 128 litters, 739 births.

Litter size data collected between November 1993 and November 1994.

Figure 3.5. Litter size in control and *hph-1* mice.

Chapter 4.

Brain nitric oxide synthase activity in the *hph-1* mouse.

Chapter 4. Brain Nitric oxide synthase activity in the *hph-1* mouse.

4.1. Introduction.

Tetrahydrobiopterin (BH_4) is an established cofactor for the aromatic amino acid mono-oxygenase group of enzymes. Consequently, it is required for the hepatic hydroxylation of phenylalanine to tyrosine and within monoamine producing neurones it is required for the conversion of tyrosine to dihydroxyphenylalanine and tryptophan to 5-hydroxytryptophan (Leeming et al., 1981).

Recently, BH_4 has been shown to be a cofactor for all known isoforms of nitric oxide synthase (NOS) which catalyse the conversion of arginine to citrulline with the subsequent liberation of nitric oxide (NO) (Tayeh and Marletta, 1989). In addition to BH_4, all of the isoforms of NOS have a requirement for a number of cofactors which include a requirement for protoporphyrin haem iron and the reduced flavins FMN and FAD (Mayer et al., 1991). Reducing equivalents are provided by NADPH and the amino acid arginine is required as the substrate (Knowles et al., 1989).

The exact role of BH_4 in the NOS enzyme complex is not known. In the hydroxylation reaction of the aromatic mono-oxygenase enzymes BH_4 provides reducing equivalents to the enzyme leading to activation of molecular oxygen and ultimately hydroxylation of the substrate. In the process, BH_4 is oxidised to quinonoid dihydrobiopterin which is devoid of cofactor activity and must be reduced back to the fully active tetrahydro- form by the enzyme dihydropteridine reductase (Kaufman, 1993). An analogous role for BH_4 in the NOS complex was suggested by Tayeh and Marletta (1989). The first step in the NOS reaction is the conversion of arginine to N^G-hydroxy-L-arginine (Stuehr et al., 1991) and, therefore, it was suggested that BH_4 may be involved in this initial hydroxylation step as in the aromatic amino acid mono-oxygenase (Scott-Burden, 1995). The oxidised pterin species then formed could be recycled at the expense of NADPH. However, this hypothesis was contradicted by Giovanelli et al. (1991) who demonstrated that initial activity of purified NOS is independent of BH_4. Addition of BH_4 to the purified nNOS led to a catalytic effect with each mole of BH_4 leading to the formation of more than 15 moles of product. Recycling of BH_4 by dihydropteridine reductase was also discounted as the enzyme

inhibitor, methotrexate, had no effect on NOS activity (Giovanelli et al., 1991). The role of BH_4 within the NOS complex was suggested either as an allosteric modifier or to maintain some groups in a reduced state (Giovanelli et al., 1991). Structural analysis of porcine cerebella NOS showed that BH_4 had no effect on the structure of NOS but BH_4 acted synergistically with arginine to convert nNOS into a stable, non-covalently bound dimer (Klatt et al., 1995).

It was shown that BH_4 co-purified with both constitutive and inducible NOS suggesting that initial NOS activity was not independent of BH_4 (Mayer et al., 1991; Hevel and Marletta, 1992). This lead to the suggestion that NOS depends on BH_4 as a cofactor which can be recycled by NADPH within the NOS complex during formation of the intermediate N^G-hydroxy-L-arginine (Mayer et al., 1991). BH_4 was reported to bind with high affinity to the NOS complex and enzyme activity is maximal when the pterin subunit ratio is 1:1 (Hevel and Marletta, 1992). Because BH_4 is tightly bound to the NOS complex, the pterin may be recycled with the NOS complex (Hevel and Marletta, 1992). This suggestion was supported by recent findings that quinonoid dihydrobiopterin was recycled at the expense of NADPH by NOS (Witteveen et al., 1996).

A suggestion for the role of BH_4 is for the generation of superoxide auto-oxidation of BH_4 leads to formation of superoxide (Mayer et al., 1995). Purified cerebellar NOS, in the presence of 30 μM BH_4, does not produce NO but instead peroxynitrite ($ONOO^-$) is formed (Mayer et al., 1995). Thus, it has been proposed that the NOS enzyme may be a $ONOO^-$ producing enzyme instead (Mayer and Werner, 1995). Further support for this suggestion comes from the fact that $ONOO^-$ may act as a vasodilator and inhibit platelet aggregation (Liu et al., 1994; Moro et al., 1994). This may be due to the formation of S-nitrosylated species, in particular S-nitroso-glutathione which will then release NO (Moro et al., 1994; Mayer and Werner, 1995). An alternative suggestion for the role of BH_4 is to protect the NOS complex from inhibition by it's own product, NO (Griscavage et al., 1994). NO can inhibit NOS by interacting with the haem group, this inhibition is prevented by 50 μM BH_4 (Griscavage et al., 1994). The mechanism of this protection may be that BH_4 favours

the maintenance of the oxidation state of enzyme bound haem iron in the Fe^{2+} state and this diminished the effect of NO (Griscavage et al., 1994).

The function of BH_4 within the aromatic amino acid mono oxygenase enzymes is relatively well established but clearly the role of the cofactor in the NOS complex is not fully understood. However, one striking difference between the former group of enzymes and NOS is the K_m for BH_4. For phenylalanine hydroxylase the K_m is 2 μM (Kaufman, 1993) whereas for purified cerebellar NOS, the K_m is a hundred fold lower (0.02-0.03 μM) (Giovanelli et al., 1991; Stuehr and Griffith, 1992). This low K_m for BH_4 led to the suggestion that inborn errors of BH_4 metabolism leading to deficiency of BH_4, while severe enough to affect phenylalanine hydroxylase and the other aromatic amino acid mono-oxygenase enzymes, would not affect NOS (Kaufman, 1993).

A new finding for the role of BH_4 appears to be in the maintenance of the steady state concentration of the aromatic amino acid mono-oxygenases (Hyland et al., 1996a). Using the *hph-1* mouse decreased levels of phenylalanine and tyrosine hydroxylase enzyme protein were found (Hyland et al., 1996a). It is not known if BH_4 is required for the stability of these enzymes or is required for *de novo* enzyme synthesis.

The experiments described in this chapter measure NOS specific activity in whole brain and the effect of chronic BH_4 deficiency on enzyme activity using the *hph-1* mouse. In addition, NOS activity was measured against age to determine the effect of BH_4 deficiency on the development of the enzyme.

4.2. Methods

Control and *hph-1* mice of various ages were killed by cervical dislocation and the whole brain removed.

For the measurement of BH_4, whole brain was immediately frozen at -70^{0}C prior to analysis. Analysis of BH_4 was carried out as described in Chapter 2. on whole brain from 10 and 30 day old mice

NOS activity was determined using whole brain which was homogenised immediately after removal as described in chapter 2. NOS activity was then carried out on the cytosolic fraction as described in chapter 2. The NOS assay was validated by demonstrating dependence on calcium. This was carried out by replacing the 1 mM calcium chloride in the reaction buffer with 1 mM EGTA. Linearity with protein was measured by diluting the cytosolic fraction with buffer (320 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) to produce a variety of protein concentrations. The effect of time on the NOS assay was measured by assessing the linearity with time, 15 - 90 minutes. Sensitivity to NOS inhibitors was also measured by including the NOS inhibitors N-methyl-arginine (NMA), N-nitro-arginine (NNA) and N-arginine-methyl ester (NAME) (1 - 1000 μM) in the incubation medium together with arginine (100 μM).

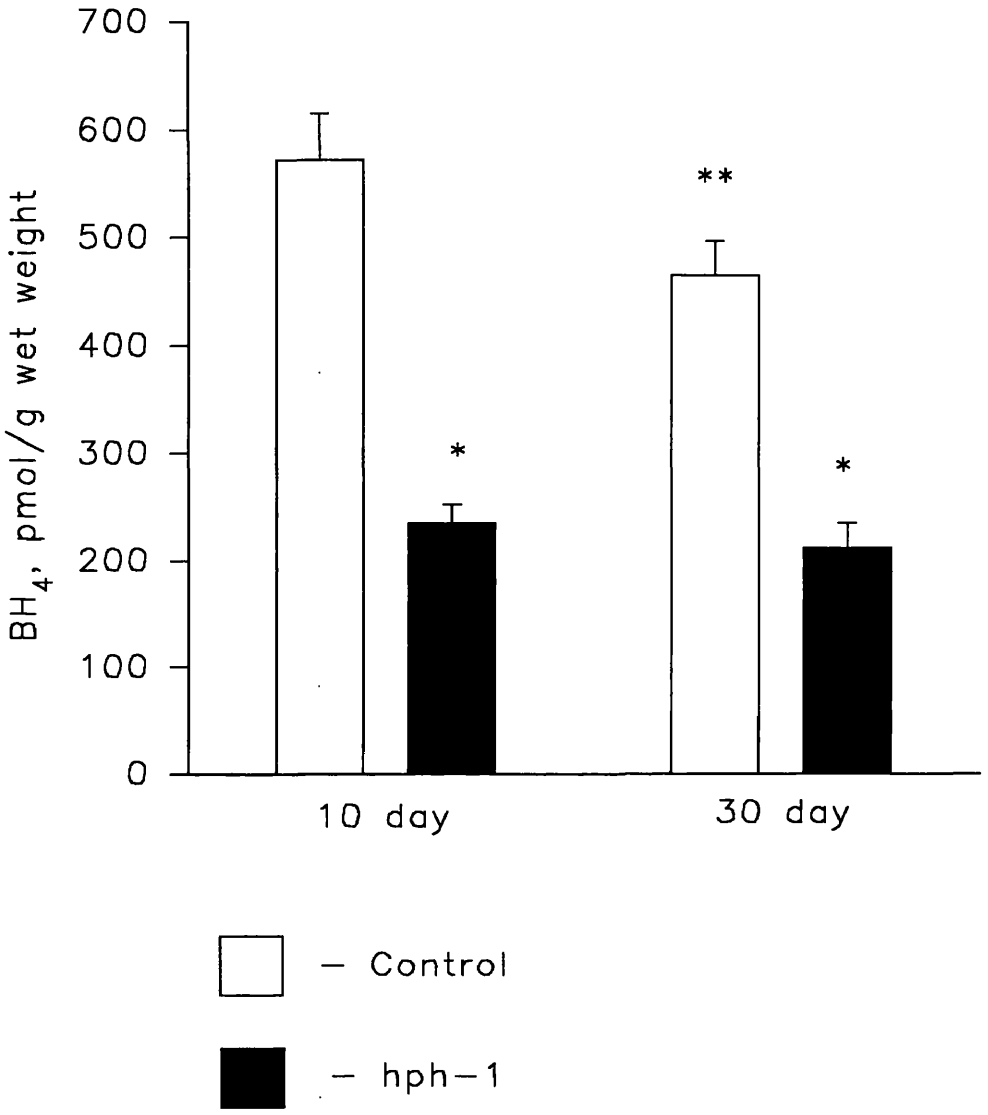
The development of NOS activity was also measured in both groups of mice with enzyme activity measured between 1 and 30 days in the presence and absence of exogenous BH_4 (50 μM).

The K_m of NOS for arginine was determined in the cytosolic fraction for control and *hph-1* mice in the presence and absence of exogenous BH_4. The K_m was determined using Hanes plots which plots substrate (s) against substrate/velocity (s/v). The Lineweaver-Burk plot (1/v against 1/s) where reciprocals are plotted gives large experimental error bars and the plot can conceal a poor fit between the data and a straight line. The Eadie-Hofstee plot (v against v/s) can result in angular distortion of error bars, therefore, the Hanes plot may be considered to give the best estimate of the true K_m (Henderson, 1992).

4.3. Results

4.3.1. Tetrahydrobiopterin Analysis.

In control mice the concentration of whole brain BH_4 decreased with age from 10 to 30 days (Figure 4.1.). In contrast, whole brain BH_4 levels in the *hph-1* mouse were relatively unaffected by age with no significant decrease observed in BH_4 concentration between 10 and 30 days (Figure 4.1.). BH_4 levels were significantly



Data displayed as mean ± SEM. For 30 day old mice, n = 7, for 10 day old mice, n = 6. *Significantly lower than appropriate age matched control, $p<0.01$. **Significantly lower from 10 day old control mice, $p<0.01$.

Figure 4.1. Whole brain tetrahydrobiopterin concentration in control and *hph-1* mice.

lower in the *hph-1* mouse being 41% of control values at 10 days and 45% at 30 days.

4.3.2. NOS assay validation.

Time course reactions were carried out for the NOS reaction with BH_4 included in the reaction buffer for 30 day old mice. The reaction appeared to be linear over the first 30 minutes (Figure 4.2.), after this time there appeared to be a gradual decline in enzyme activity up to 90 minutes when both control and the *hph-1* group showed an approximate 30% loss of enzyme activity. No difference was observed between the control and *hph-1* group when 50 μM BH_4 was included in the reaction buffer (Figure 4.2.). All further NOS assays were measured over 15 minutes to ensure that the reaction was on the linear part of the curve.

NOS activity was measured in the presence and absence of exogenous calcium in whole brain cytosolic fraction from 10 and 30 day old mice (Table 4.1.). At both age points NOS activity showed a strong dependence on calcium, however, the results from the 10 day old group of mice showed that there was a higher residue activity in the absence of calcium in the control mice when compared to the *hph-1* mouse (Table 4.1.)

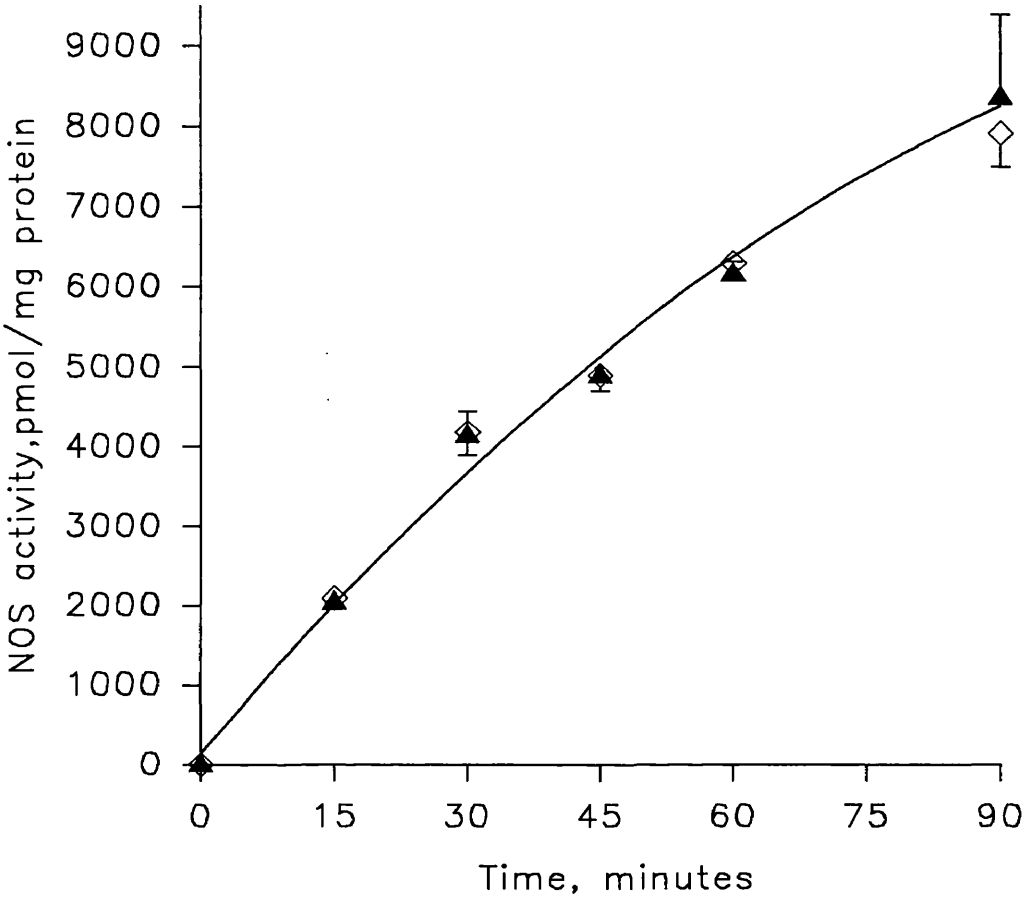
The NOS assay was shown to be linear with protein (Figure 4.3.).

4.3.3. NOS inhibitors

NOS activity was measured using the enzyme inhibitors, NMA, NNA and NAME at concentrations between 1 - 1000μM in both groups of mice (Figures 4.4, 4.5. and 4.6.). For control mice enzyme activity was inhibited with a half maximal effect at 40 μM for NMA, 7.4 μM for NNA and 8.0 μM for NAME. For the *hph-1* mouse enzyme activity was inhibited with half maximal effect at 46 μM for NMA, 7.1 μM for NNA, 7.7 μM for NAME.

4.3.4. NOS activity ± BH_4.

When BH_4 (50 μM) was omitted from the reaction buffer, in the control group there was no difference in NOS activity at 15 minutes in the presence and absence of BH_4



▲ control mice.

◊ *hph-1* mouse.

Data shown as mean ± SEM, n = 3. Where no error bars are indicated they lie within the area of the symbol.

Figure 4.2. Plot of NOS activity against time in 30 day old control and *hph-1* mice. 50μM tetrahydrobiopterin included in the reaction buffer.

	NOS activity, pmol/min/mg protein	
	+ calcium	- calcium
10 day old control	89.3 ± 2.8 (12)	9.7 ± 0.7 (6)[1]
10 day old *hph-1*	89.8 ± 3.5 (13)	5.8 ± 0.6 (6)[1,2]
30 day old control	136.1 ± 9.2 (17)	1.8 ± 1.7 (3)[1]
30 day old *hph-1*	139.3 ± 4.4 (16)	1.2 ±0.3 (3)[1]

Data displayed as mean ± SEM. Figure in brackets = *n*. [1] Significantly lower than corresponding value in the presence of calcium, $p<0.001$. [2] Significantly lower than 10 day old control mice in the absence of calcium.

Table 4.1. Brain NOS activity ± calcium.

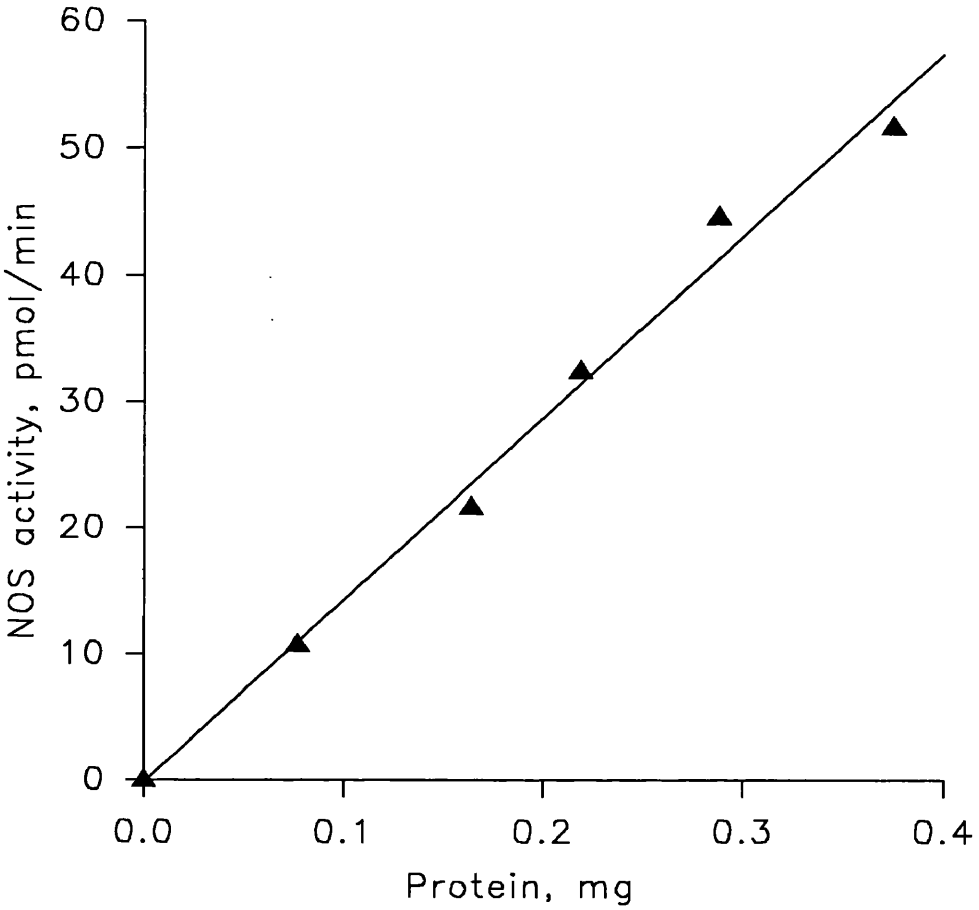
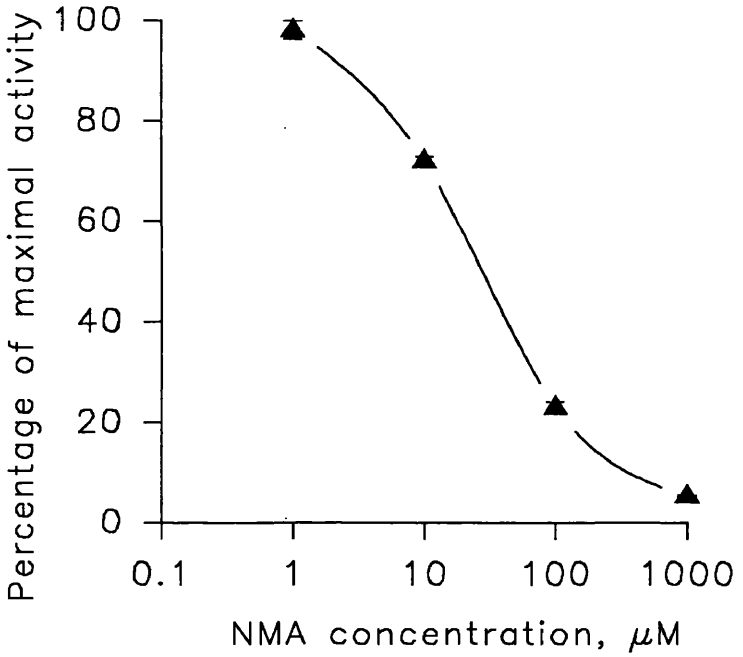
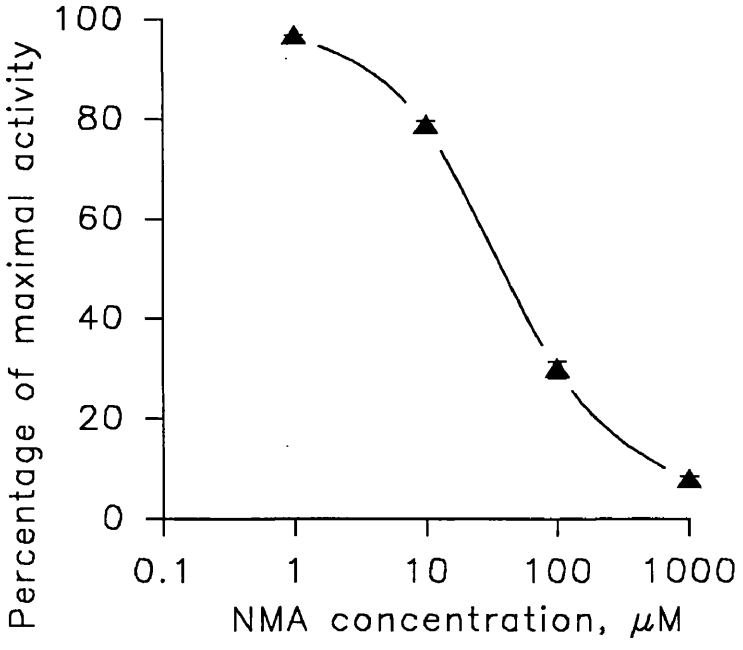


Figure 4.3. Plot of NOS activity vs assay protein concentration in 30 day old control mice.



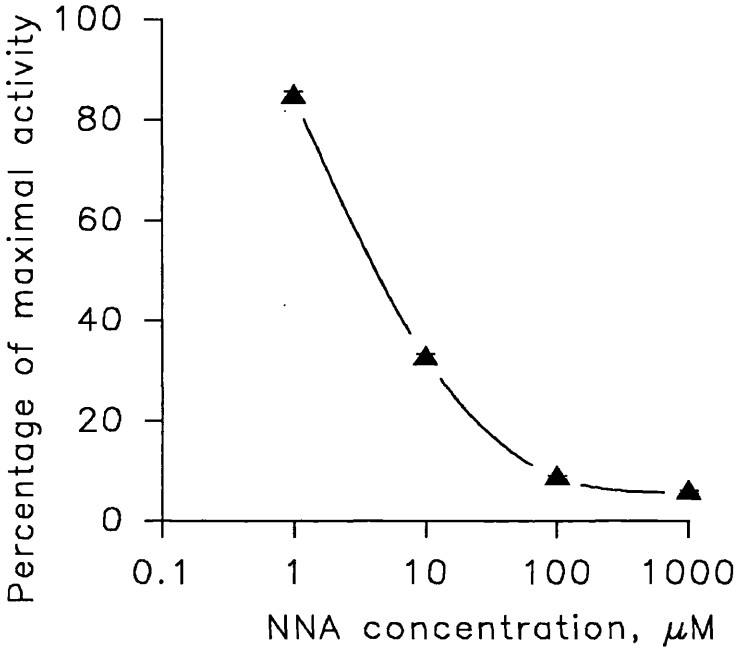
Control



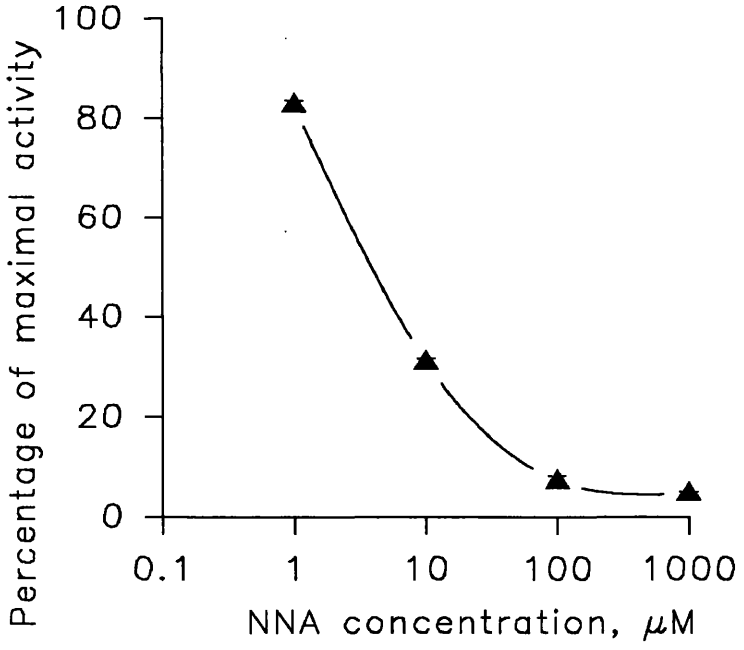
hph-1

Data displayed as mean ± SEM, n = 3. Where no error bars are indicated, they lie within the symbol.

Figure 4.4. NOS activity with N-methyl-arginine (NMA) in 30 day old control and *hph-1* mice



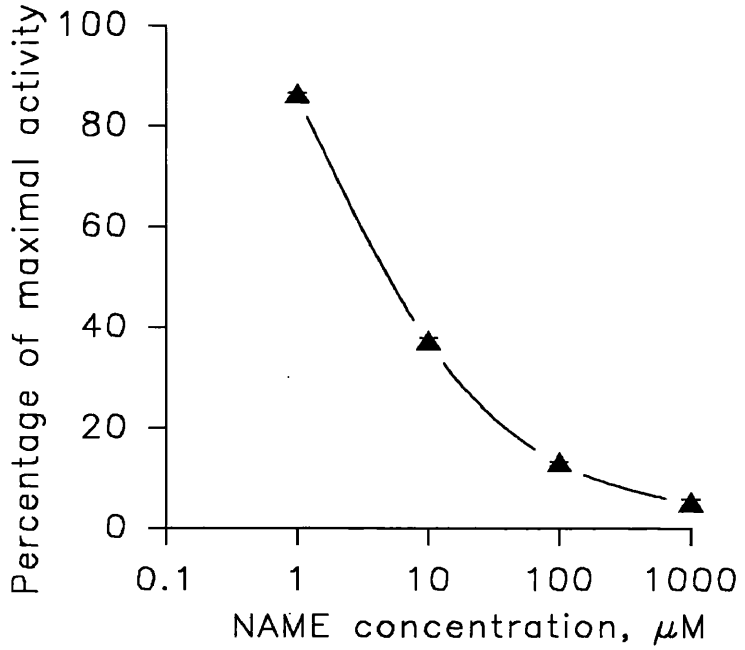
Control



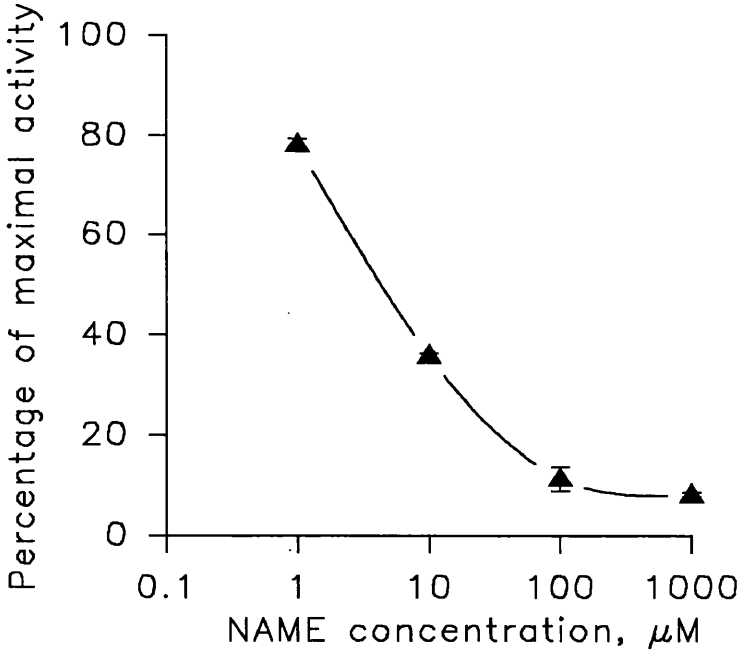
hph-1

Data displayed as mean ± SEM, n = 3. Where no error bars are indicated, they lie within the synbol

Figure 4.5. NOS activity with N-nitro-arginine (NNA) in 30 day old control and *hph-1* mice



Control



hph-1

Data displayed as mean ± SEM, n = 3. Where no error bars are indicated they lie within the synbol.

Figure 4.6. NOS activity with N-arginine-methyl ester (NAME) in 30 day old control and *hph-1* mice.

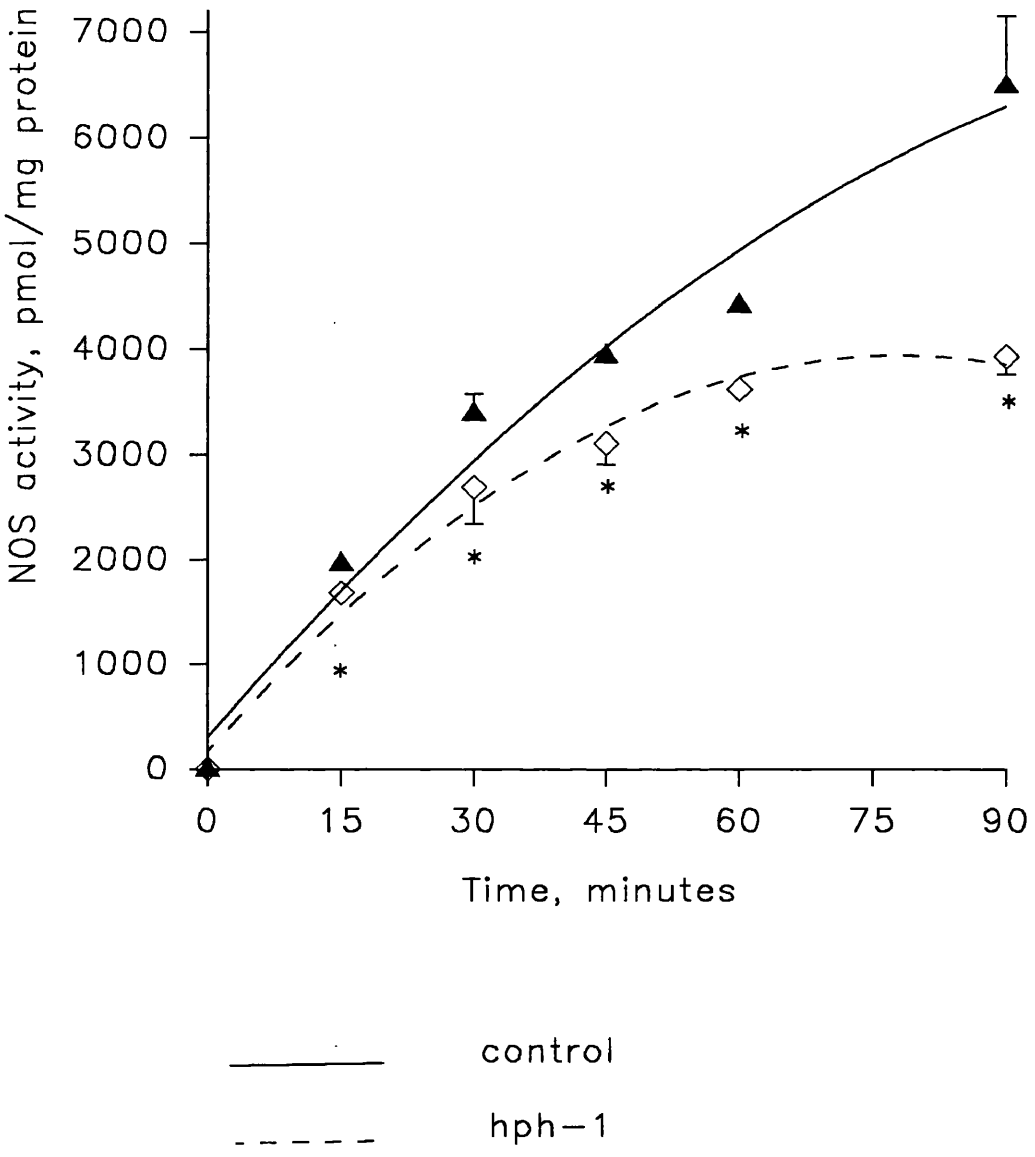
but from 30 minutes there was a significant decrease in NOS activity (Figure 4.7.). However, in the *hph-1* mouse group there was a significant drop in NOS activity from 15 minutes (Figure 4.7.). This decrease in activity was significantly lower than for the control group at all points (Figure 4.7.). NOS activity was also measured in 10 day old mice, enzyme activity measured over 15 minutes. In the presence of 50 μM BH_4 enzyme activity was virtually identical in the control and *hph-1* mouse at 10 and 30 days (Table 4.2.). When BH_4 was omitted from the reaction buffer in control preparations there was a minimal 6.2% decrease in NOS activity at 10 days compare to a significant 19.7% drop in enzyme activity in the *hph-1* mouse. A similar result was obtained at 30 days with only a 3.7% drop in whole brain NOS activity in the controls compared with a 19.5% significant decrease in enzyme activity in the *hph-1* mouse (Table 4.2.).

4.3.5. Whole brain NOS activity developmental profile.

NOS activity was measured at ages 1, 5, 10, 15, 20 and 30 days in the presence and absence of BH_4 in both groups of mice. In the presence of BH_4, control mice enzyme activity increased by approximately 50% between 10 and 15 days (Figure 4.8.). When BH_4 was omitted from the reaction buffer, again there was an increase in activity between 10 and 15 days in the control mice with a minimal loss in NOS activity (Figure 4.8.). In the *hph-1* mouse, with BH_4 included in the incubation buffer, there was a similar increase in enzyme activity between 10 and 15 days (Figure 4.9.). However, when BH_4 was omitted from the reaction buffer there was a significant decrease in enzyme activity at all age points for the *hph-1* mouse (Figure 4.9.).

4.3.6. Determination of K_m for arginine $\pm$ BH_4.

The K_m for arginine was determined for both control and *hph-1* mice in the presence and absence of exogenous BH_4 (50μM) at both 10 and 30 day old age points. For the control and *hph-1* mice groups at both age points there was no difference in K_m when BH_4 was present in the reaction buffer (Table 4.3. and Figures 4.10. - 4.13.). The omission of BH_4 from the reaction buffer led to a significant three fold increase in K_m for control mice at both 10 and 30 days (Table 4.3. and Figures 4.10. and 4.12.). Omission of BH_4 from the reaction buffer led to a four fold increase in K_m for the



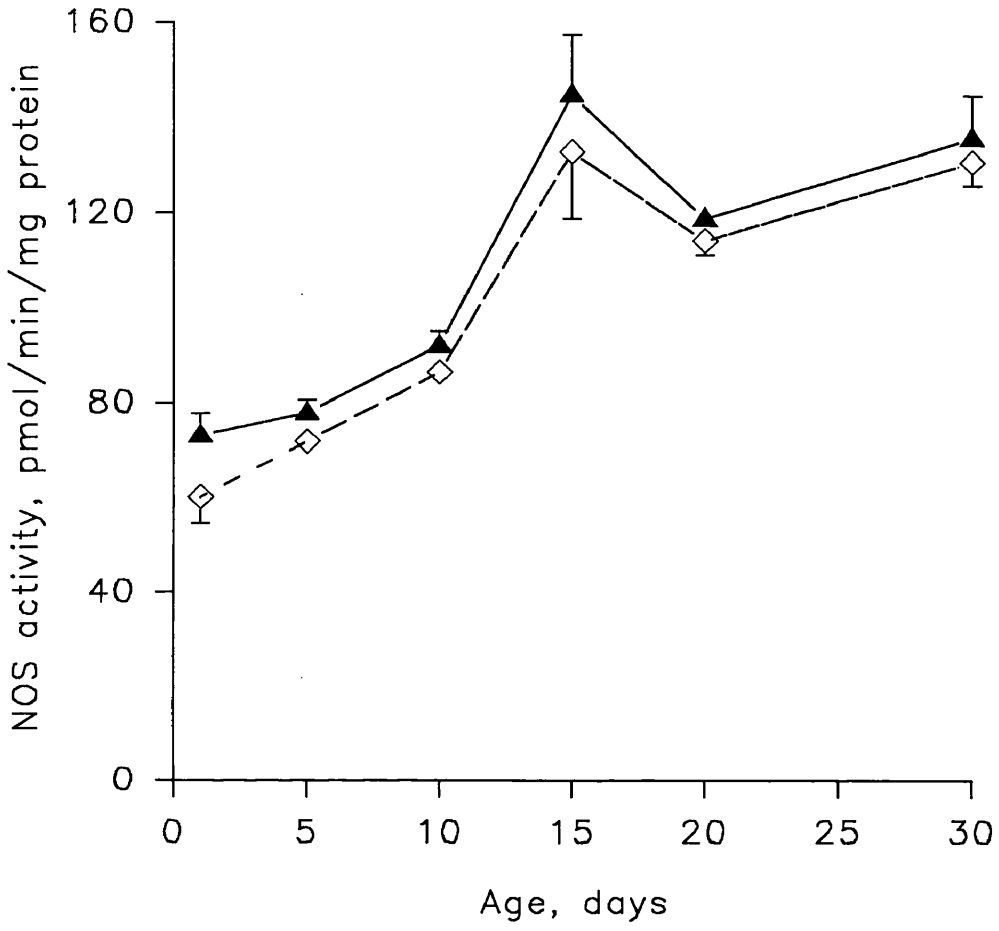
Data displayed as mean ± SEM, n = 3. Where no error bars are indicated thay lie witin the symbol. * NOS activity in the *hph-1* mouse is significantly lower than control mice at each time point in the absence of BH_4 ($p<0.05$).

Figure 4.7. Plot of NOS activity against time in 30 day old control and *hph-1* mice. BH_4 omitted from reaction buffer.

	NOS activity, pmol/min/mg protein	
	+ BH_4	- BH_4
10 day old control	89.3 ± 2.8 (12)	86.6 ± 2.0 (9)
10 day old *hph-1*	89.8 ± 3.5 (12)	75.2 ± 3.1 (10)*
30 day old control	136.1 ± 9.2 (17)	131.1 ± 5.3 (14)
30 day old *hph-1*	139.3 ± 4.4 (16)	112.1 ±12.3 (13)*

Data expressed as mean ± SEM. Figures in parentheses is sample size. * Significantly lower than NOS activity in control mice ± BH_4 and *hph-1* mice in the presence of BH_4, $p<0.05$.

Table 4.2. Whole brain NOS activity ± BH_4

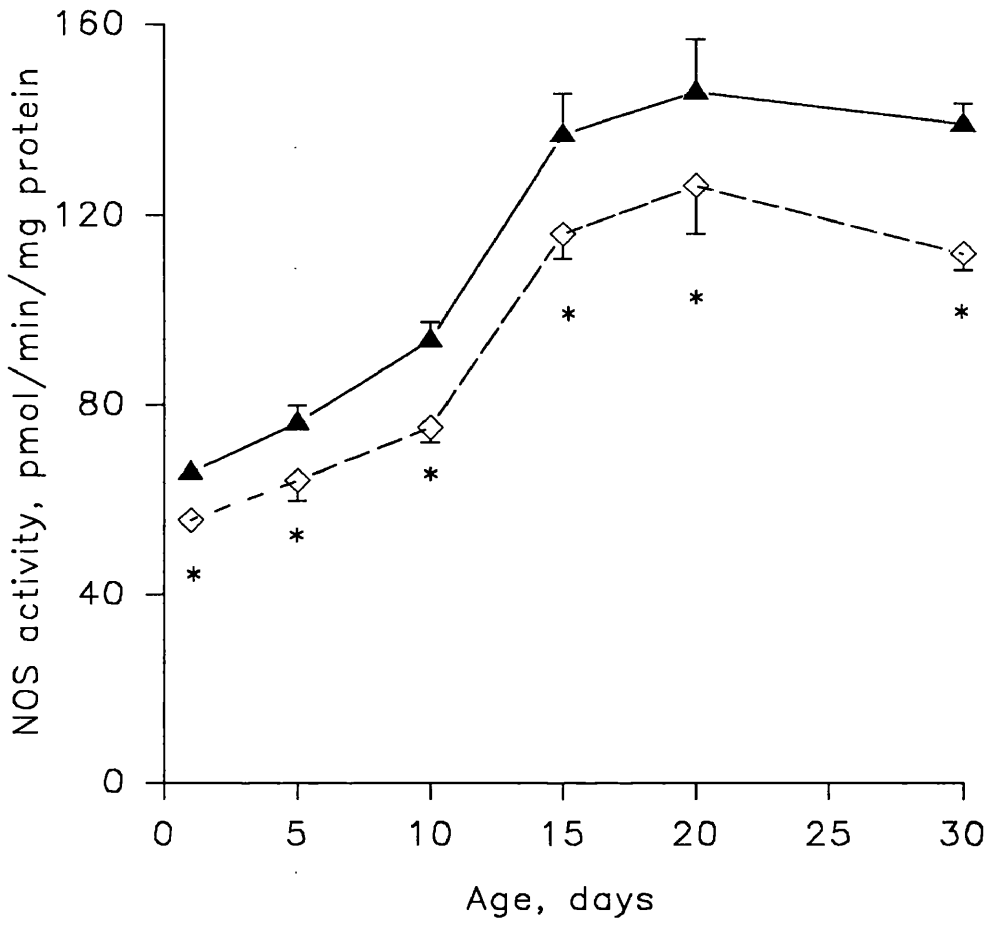


▲ + BH_4

◊ - BH_4

Data shown as mean ± SEM. n is equal to at least 3. Where no error bars are indicated they lie within the symbol.

Figure 4.8. Change in NOS activity in control mice with age ± 50 μM tetrahydrobiopterin.



▲ + BH_4

◊ - BH_4

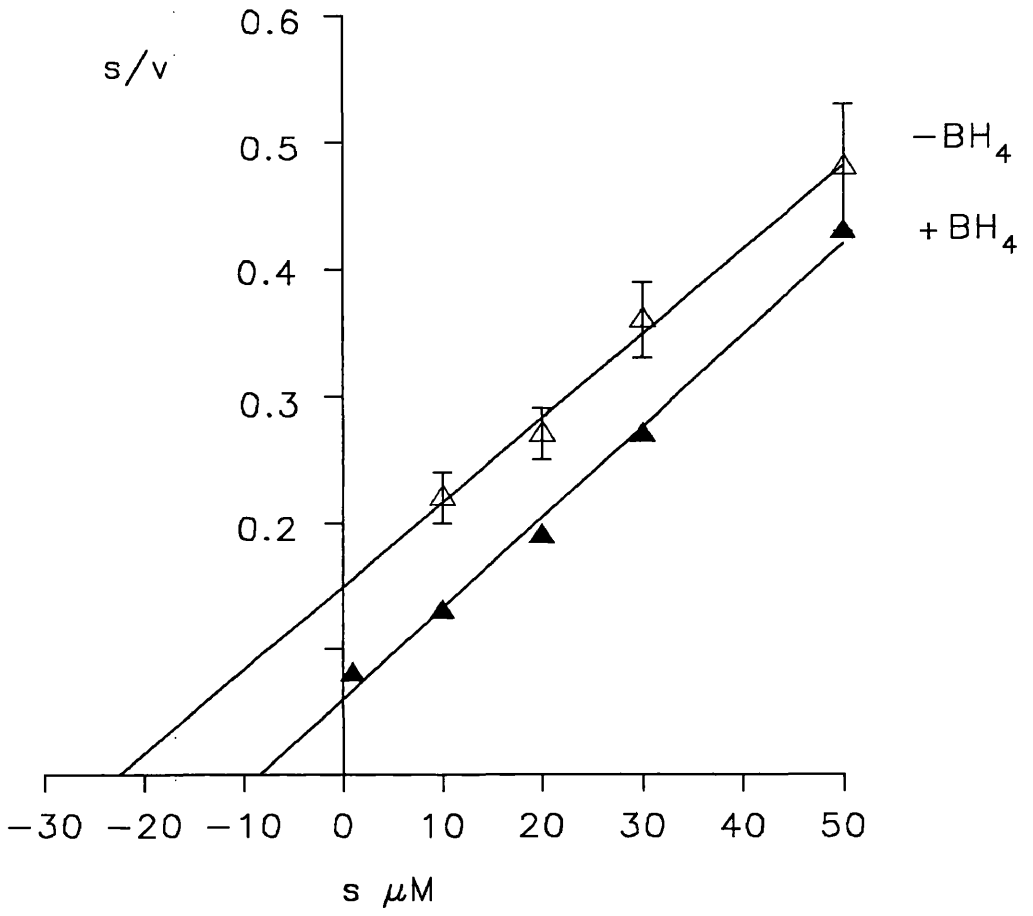
Data shown as mean ± SEM. n is equal to at least 4. Where no error bars are indicated they lie within the symbol. *Significantly lower than enzyme activity in the presence of BH_4 ($p<0.05$).

Figure 4.9. Change in NOS activity in *hph-1* mice with age ± 50 μM tetrahydrobiopterin.

	K_m values for arginine (μmol/L)	
	+ BH_4	- BH_4
10 day old control	8.0 ± 3.4 (3)	22.6 ± 3.0 (3)[1]
10 day old *hph-1*	9.6 ± 3.0 (3)	38.4 ± 1.8 (3)[1,2]
30 day old control	8.9 ± 1.2 (3)	25.6 ± 2.1 (5)[1]
30 day old *hph-1*	8.2 ± 1.9 (3)	45.5 ± 7.2 (5)[1,2]

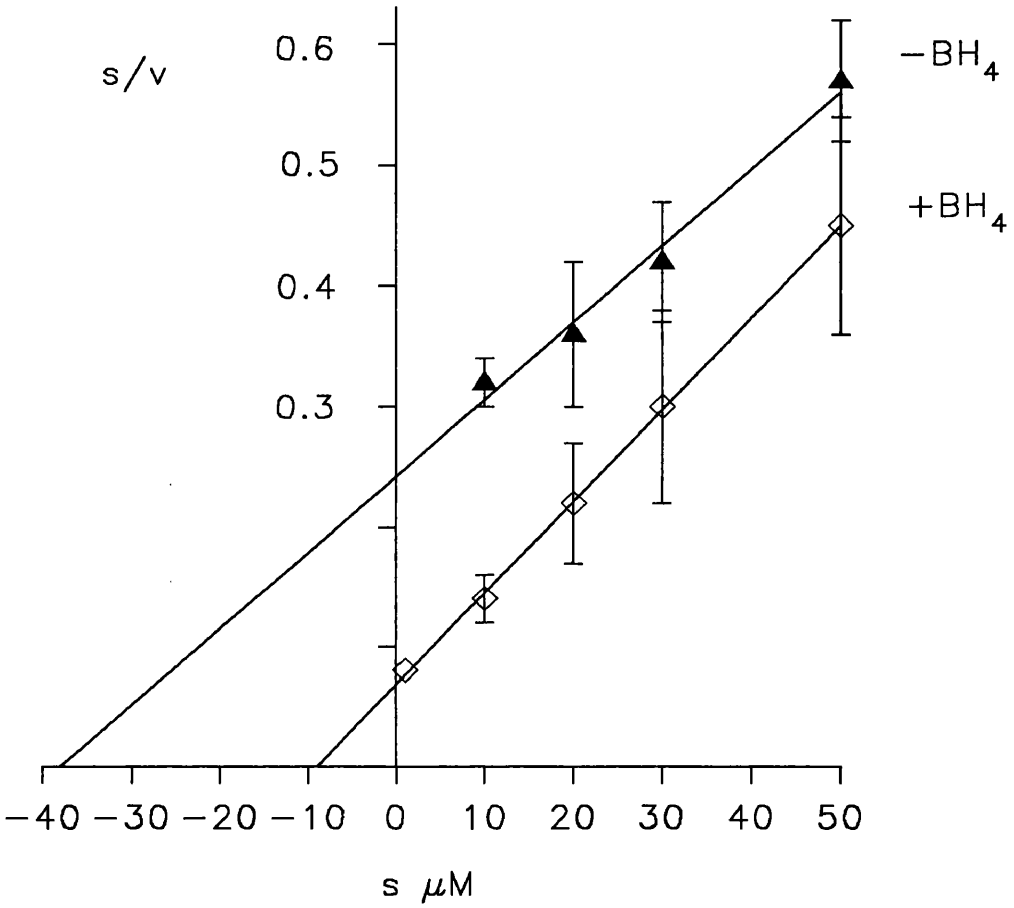
Data displayed as mean ± SEM. Number in parentheses is equal to sample size. K_m for arginine was determined by Hanes plots in the presence and absence of 50 μM BH_4. [1] Significantly different from corresponding value in presence of BH_4.
[2] Significantly different from control mice ± BH_4. $p<0.01$ in both cases.

Table 4.3. K_m values for arginine for whole brain NOS ± BH_4.



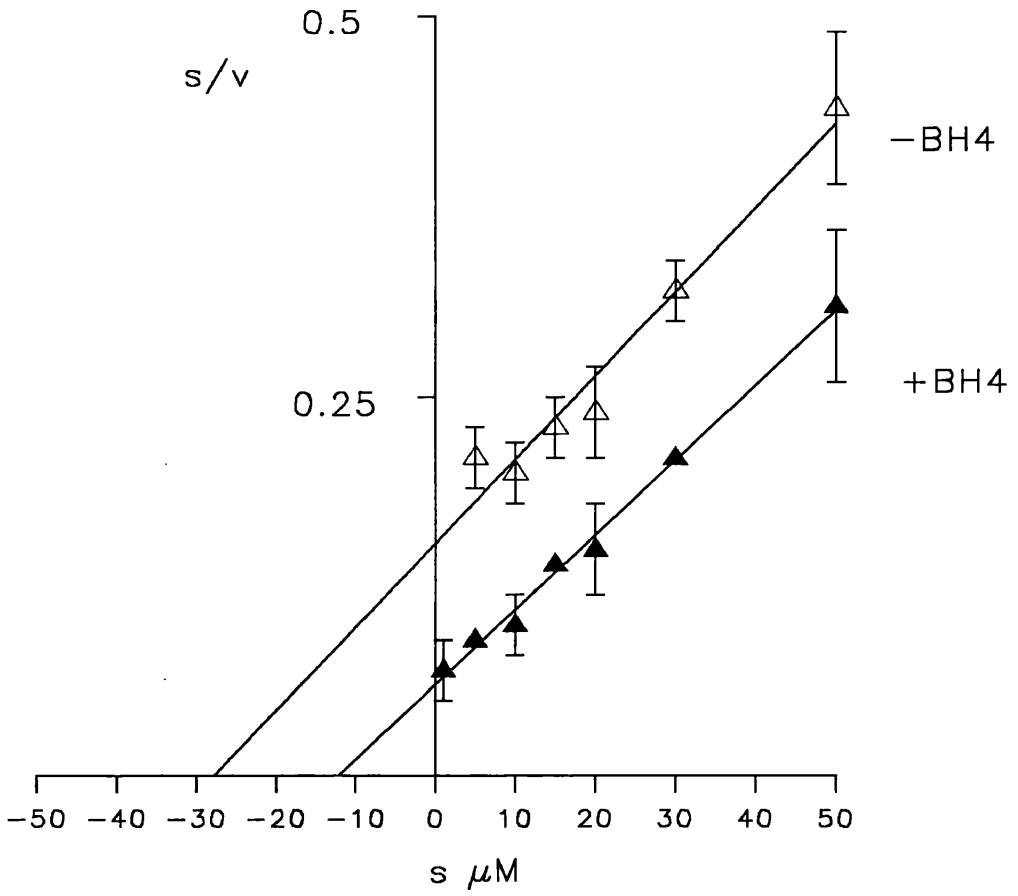
Data shown as mean ± SEM, n = 3. s is the substrate concentration, v is the initial velocity of the reaction (pmol/min/mg protein). Where no error bars are indicated they lie within the symbol.

Figure 4.10. Hanes plot illustrating the effect of BH_4 on the kinetic properties of NOS from whole brain of 10 day old control mice.



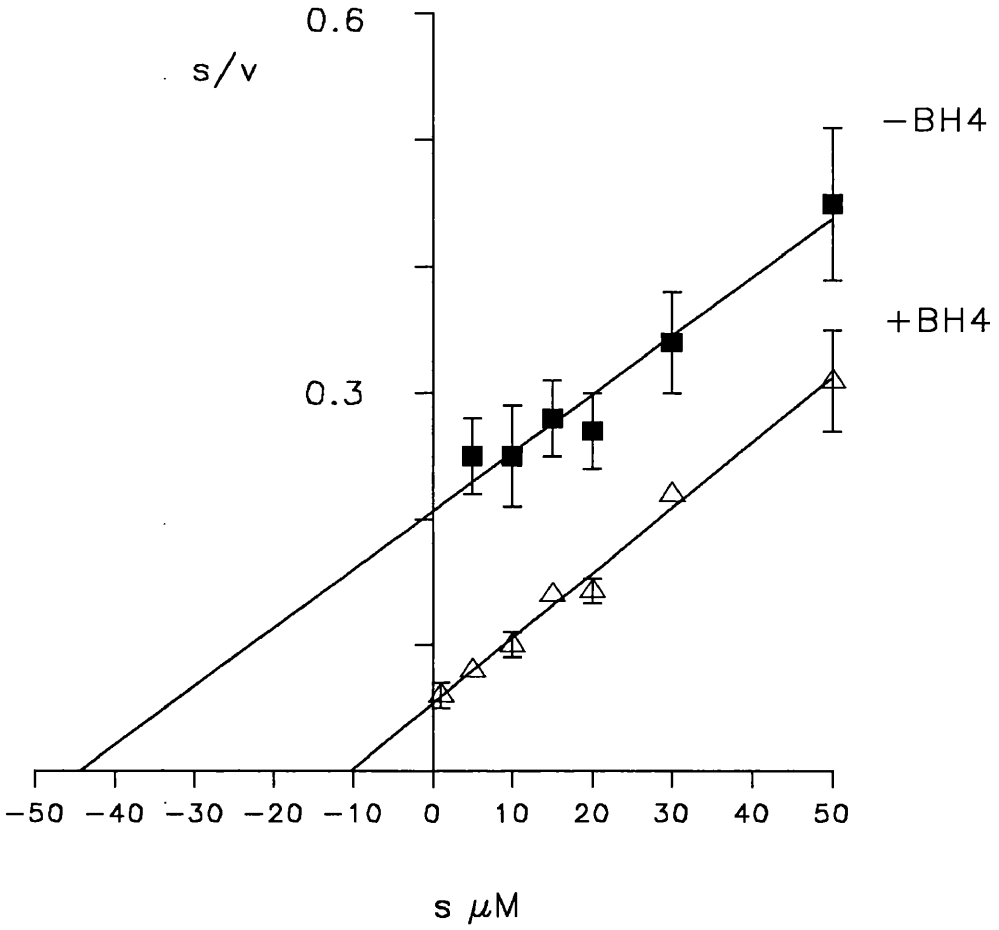
Data shown as mean ± SEM, n = 3. s is the substrate concentration, v is the initial velocity of the reaction (pmol/min/mg protein). Where no error bars are indicated they lie within the symbol.

Figure 4.11. Hanes plot illustrating the effect of BH_4 on the kinetic properties of NOS from whole brain of 10 day old *hph-1* mice.



Data shown as mean ± SEM, n = 3 for determinations + BH_4 and n = 5 for determinations - BH_4. s is the substrate concentration, v is the initial velocity of the reaction (pmol/min/mg protein). Where no error bars are indicated they lie within the symbol.

Figure 4.12. Hanes plot illustrating the effect of BH_4 on the kinetic properties of NOS from whole brain of 30 day old control mice.



Data shown as mean ± SEM, n = 3 for determinations + BH_4 and n = 5 for determinations - BH_4. s is the substrate concentration, v is the initial velocity of the reaction (pmol/min/mg protein). Where no error bars are indicated they lie within the symbol.

Figure 4.13. Hanes plot illustrating the effect of BH_4 on the kinetic properties of NOS from whole brain of 30 day old *hph-1* mice.

hph-1 mouse (Table 4.3. and Figures 4.11. and 4.13.).

4.4. Discussion.

The observation that the *hph-1* mouse displays significantly lower whole brain BH_4 concentrations, 45% of control values at 30 days, is in agreement with Hyland et al. (1996a) who report whole brain BH_4 levels being 44% of control values at 30 days. Whole brain BH_4 decreases with age from birth in control mice whereas the levels in the *hph-1* mouse are relatively unaffected by age (Hyland et al., 1996a). Similarly in this study, at 10 days of age whole brain BH_4 levels are 41% of the control mice group.

Initial validation experiments demonstrated that the arginine-citrulline radio-isotope assay was linear with protein and showed an absolute dependence on calcium in both groups of mice. This dependence on calcium indicates that the NOS enzyme activity being measured is a constitutive isoform as the inducible form of the enzyme is considered to be calcium independent (Knowles and Moncada, 1994). In the 10 day old mice there is a higher enzyme activity in the absence of calcium, this may be due to the presence of two NOS isoforms during development (Southam et al., 1991). If there is a second isoform of NOS present during development it is likely to be an calcium independent form as it demonstrates activity in the absence of calcium.

The NOS inhibitors NMA, NNA and NAME all inhibited the enzyme reaction with NNA and NAME being the most effective. No obvious difference was apparent between NNA and NAME, this may be because it has been suggested that NAME is converted to NNA in biological systems and this may then act as the enzyme inhibitor (Pfeiffer et al., 1996). NMA was the least effective NOS inhibitor and this may be because it is considered to be an endothelial NOS inhibitor rather than NNA which is considered to be a neuronal NOS inhibitor (Knowles and Moncada, 1994). There was no difference in the effectiveness of the NOS inhibitors between the two groups of mice. Therefore, the predominant isoform present in the brain of both groups of mice appears to be the calcium dependent, NNA sensitive constitutive form.

NOS activity was measured in whole brain against time in both groups of mice. When 50 μM BH_4 was included in the reaction buffer no difference was detected between control and *hph-1* mice. The reaction appeared to be linear over the first 30 minutes of the reaction with a gradual decrease in enzyme activity. This may be due to substrate limitation or product inhibition in the assay medium. When exogenous BH_4 was omitted from the reaction buffer there was a decline in enzyme activity after the first 15 minutes of the reaction in control mice. A more dramatic loss of enzyme activity was observed in the *hph-1* group with a loss in enzyme activity at 15 minutes and at every time point measured thereafter. Similar results were obtained by Giovanelli et al. (1991) using purified neuronal NOS who suggest that BH_4 plays a role in prevention of progressive loss of enzyme activity. Giovanelli et al. (1991) suggested that BH_4 is needed to maintain NOS in an active form and that initial enzyme activity is independent of BH_4 by their finding that each added mole of cofactor can support the synthesis of 15 moles of product at low concentrations. However, this group did not consider that BH_4 may remain tightly bound to the enzyme during purification (Mayer and Werner, 1995).

NOS activity increases from birth to reach a plateau at about 15 days of age in both groups of mice. A marked increase in NOS activity has also been reported in rat cerebrum and cerebellum after two weeks (Matsumoto et al., 1993). A similar increase is observed in rats (Keelan et al., 1996). Lower NOS activity in pups is suggested as one reason why immature pups display a greater resistance to ischaemia than adults ie less NO derived free radicals being produced in pups following ischaemia and reperfusion (Keelan et al., 1996)

Recently, Hyland et al. (1996a) has reported a 30% decrease in specific activity of tyrosine hydroxylase in the brain of the *hph-1* mouse together with a 50% decrease in liver phenylalanine hydroxylase activity. Furthermore, it has been reported that a patient with an inborn error of BH_4 biosynthesis displayed reduced activity of phenylalanine hydroxylase (Dhondt, 1991). In the *hph-1* mouse it has been shown that this loss of mono-oxygenase activity was due to reduced levels of enzyme protein (Hyland et al., 1996a). This suggests that BH_4 is involved in the maintenance of the

steady state concentration of these enzymes. However, in contrast to these mono-oxygenase enzymes BH_4 does not appear to regulate intracellular concentrations of NOS as enzyme specific activity is not reduced in the *hph-1* mouse when BH_4 is included in the reaction buffer.

The results show that omission of BH_4 from the reaction buffer has a minimal effect on NOS activity in control mice. This result is in agreement with that of Klatt et al. (1994) who report that BH_4 is very tightly bound to NOS and remains bound to the enzyme even after purification. The observed loss of NOS enzyme activity in the *hph-1* preparations when BH_4 is excluded from the reaction buffer may suggest that there is less binding of cofactor to the enzyme when compared to control animals *in vivo*.

Chronic BH_4 deficiency of the order of 50% appears to have no long term effect on the affinity of NOS for arginine. This is demonstrated by the similar K_m in control and the *hph-1* mouse when analysed in the presence of saturating exogenous BH_4 at both 10 and 30 days. These K_m values agree well with those reported by Knowles et al. (1990) who report a K_m of 8.4 ± 2.7 μmol/L for NOS from forebrain of the rat. However, in the absence of BH_4 there is a three fold increase in K_m in preparations prepared from whole brain of control mice at both 10 and 30 days. There is a greater increase in preparations from whole brain of the *hph-1* mouse where there is a four fold increase in K_m. The mechanism for this effect is not known but BH_4 is reported to be an allosteric modifier of phenylalanine hydroxylase (Døskeland et al., 1987) and has recently been shown to increase the affinity of purified NOS for the inhibitor, NNA (Klatt et al., 1994). This work suggests that both arginine and BH_4 influence a conformation change in NOS resulting in enhanced affinities of the two binding domains for their respective ligands (Mayer and Werner, 1995). Similar results were obtained by Baek et al. (1993) who demonstrated that arginine was needed for the BH_4 induced dimerisation of macrophage iNOS.

Whole brain concentration of arginine are reported to be 100 μmol/L in rats and 70 μmol/L in humans (Perry et al., 1981). In view of the observed relationship between

BH_4 and the affinity of NOS for arginine it is possible that even under normal conditions arginine may become a limiting factor for NO formation especially as arginine has been reported to be predominantly located in glial cells in the central nervous system (Aoki et al., 1992). Therefore, it is conceivable that this situation may become exaggerated when BH_4 is limited as in the *hph-1* mouse and inborn errors of BH_4 biosynthesis. The suggestion that the low K_m of NOS for BH_4 would result in no impairment of the enzyme in BH_4 deficient states appears to be contradicted by these results, but it is not known if the observed loss of NOS activity in the *hph-1* mouse will result in impaired brain NO metabolism *in vivo*.

Chapter 5.

The nitric oxide/cyclic GMP pathway in the *hph-1* mouse.

Chapter 5. The nitric oxide/cyclic GMP pathway in the *hph-1* mouse.

5.1. Introduction.

As already discussed BH_4 is tightly bound to NOS but the exact mechanism whereby it participates in the production of NO is still unclear. The previous chapter provided evidence to suggest that less binding of the pterin cofactor occurs in BH_4 deficient states *in vitro* and that BH_4 modifies the ability of neuronal NOS to bind it's substrate, arginine again *in vitro*.

A well documented action of NO within the CNS is to stimulate guanylate cyclase and increase cGMP formation following glutamate receptor activation (Garthwaite et al., 1988). NO synthesis appears calcium dependent following glutamate receptor activation (Figure 5.1.) (Garthwaite et al., 1988). Activation of these receptors leads to an influx of calcium into the cell (Figure 5.1.). There are two broad categories of glutamate receptor, those with an integral ion channel (ionotropic) and those coupled to G proteins (metatrobic) (Garthwaite, 1991). The latter group of receptors activate phospholipase C and generate inositol 1,4,5-triphosphate leading to calcium mobilization from intracellular stores (Miller, 1991). Ionotropic receptors can be divided into three broad groups after their selective agonists; N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. AMPA and kainate receptors are often grouped together and labelled as non-NMDA receptors. Stimulation of non-NMDA receptors leads to activation of an ion channel that is permeable to sodium and potassium ions although some reports indicate that these receptors are permeable to calcium (Miller, 1991). Indeed, kainate toxicity in cultured hippocampal neurons and cerebellar slices is dependent on extracellular calcium ions (Cheng and Sun, 1994). In addition, Weiss et al. (1990) have shown that a voltage gated calcium channel blocker, nifedipene, appeared to protect cultured cortical neurones from kainate excitotoxic damage. Therefore, activation of kainate receptors appears to lead to influx of calcium into the cell (Miller, 1991).

At normal resting cytosolic calcium concentration, ~ 50 nM, NOS is virtually inactive and is maximally active at 0.4 - 1.0 μM calcium (Knowles et al., 1989). Neuronal

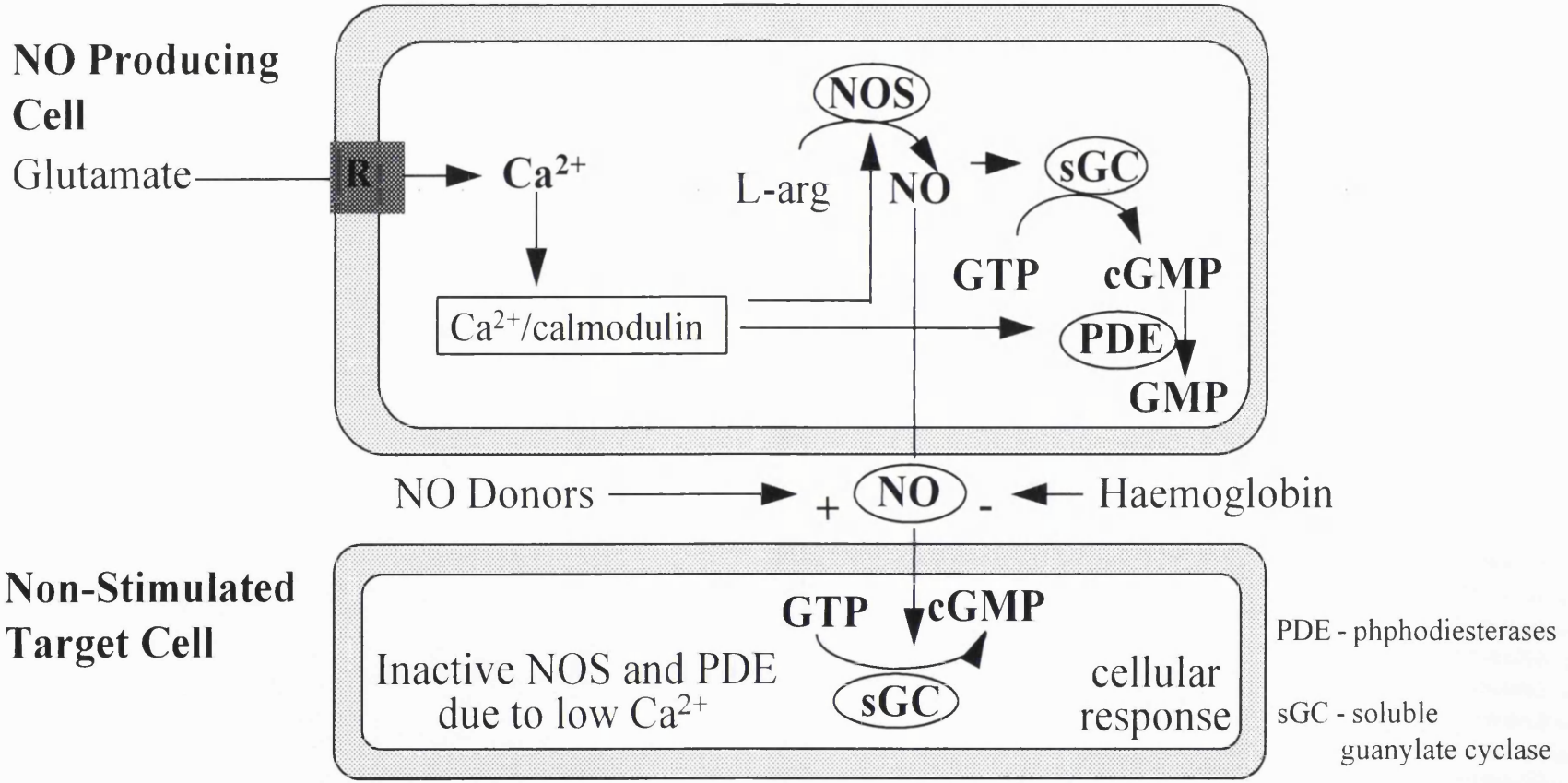


Figure 5.1. The nitric oxide/cGMP pathway.

NOS requires the binding of calmodulin and calcium for activity and produces NO from arginine (Figure 5.1.). The NO produced binds to the prosthetic haem group of guanylate cyclase thereby causing a conformation change and activating the enzyme (Mayer, 1993). Activated guanylate cyclase can, thus, produce cGMP from GTP in the stimulated target cell. However, increase in calcium concentration in the NO producing cell can also stimulate a calcium/calmodulin phosphodiesterase iso-enzyme, thus leading to breakdown of cGMP to GMP (Figure 5.1.) (Mayer et al., 1992). Therefore, stimulation of glutamate receptors in a NO producing cell may lead to a minimal increase in cGMP in the stimulated target cell. However, NO is readily diffusible and it can activate guanylate cyclase in neighbouring cells leading to cGMP accumulation and a cellular response in the absence of phosphodiesterase activation (Figure 5.1.)

NOS activity is present in all areas of the brain studied thus far, the area with the highest enzyme activity is the cerebellum (Garthwaite, 1991). Southam and Garthwaite (1993) suggest that the main function of NO is to act as an intercellular messenger as cells that can generate NO may be separate from those that can synthesize cGMP. In the cerebellum the main location of NMDA receptors are the granule cells (Garthwaite, 1991; Southam and Garthwaite, 1993). Granule cells of the cerebellum can both generate NO and cGMP but may be prevented from doing both simultaneously by activation of phosphodiesterases but as NO may diffuse from these cells and activate guanylate cyclase in neighbouring Purkinje cells (Mayer, 1993; Southam and Garthwaite, 1993). This scenario may, therefore, provide a model of the NO/cGMP pathway.

In cerebellar slices from young rats Garthwaite et al. (1989) have shown that the glutamate analogue, kainate, induced a large accumulation of cGMP. This response is calcium dependent as incubation of slices in a calcium free buffer did not lead to any increase in cGMP. Clearly activation of non-NMDA receptors also leads to calcium influx into the cell (Garthwaite et al, 1989). This accumulation of cGMP is also inhibited by the NOS inhibitor, N-monomethyl-arginine (Garthwaite et al., 1989). In addition, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), an antagonist with

preferential activity against non-NMDA receptors (Honoré et al., 1988) potently inhibited this response (Garthwaite et al., 1989). Stimulation of kainate receptors, therefore, leads to activation of NOS and subsequent stimulation of guanylate cyclase in cerebellar slices in a calcium dependent manner. Kainate has a significant advantage over exogenous glutamate in cerebellar slice preparations in that the action of glutamate is impaired because of a restricted access of the amino acid to the neurones imposed by cellular uptake mechanisms (Garthwaite et al., 1989).

The aim of the experiments described in this chapter was to compare cGMP accumulation following NMDA receptor activation in cerebellar slices from control and *hph-1* mice in order to determine whether BH_4 deficiency leads to impairment of the NO/cGMP pathway. Experiments were carried out on 10 and 30 day old mice.

5.2. Methods.

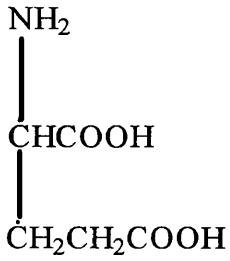
Control mice (CBA/C57BL) and *hph-1* mice at 10 and 30 days of age were used in these experiments and were killed by cervical dislocation.

For the measurement of BH_4, cerebella were immediately frozen at -70^{0}C prior to analysis. Analysis of BH_4 was carried out as described in Chapter 2 on cerebellum from 10 and 30 day old mice. Two cerebella were used for each BH_4 assay.

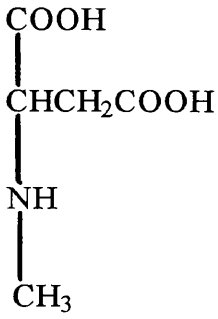
Measurement of cGMP was carried out using cerebellar slices which were prepared as described in Chapter 2. Slices were redistributed into 10 ml conical flasks so that there were four slices per flask, cGMP was measured by radio-immunoassay as described in Chapter 2.

In order to determine that the slices prepared were viable and that the integrity of the individual cells is not compromised, slice morphology was determined by light microscopy as described in Chapter 2.

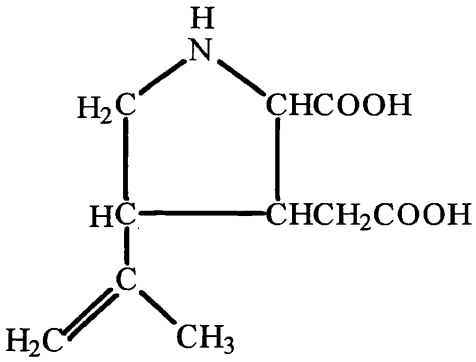
NMDA receptor agonists glutamate, NMDA and kainate (Figure 5.2.) were used to stimulate cGMP synthesis in the slice preparations from 10 and 30 day old mice. In



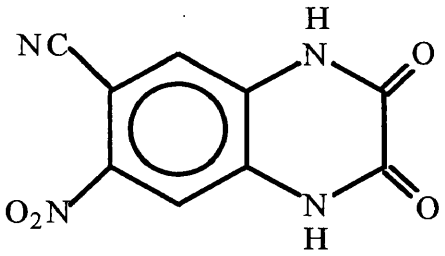
Glutamate



N-methyl-D-aspartate (NMDA)



Kainate



6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)

Figure 5.2. Structure of NMDA receptor agonists and antagonist.

addition to validate the kainate stimulation of cerebellar slices, experiments were set up as described in Table 5.1. for slices prepared from 30 day old mice. The effect of kainate (500 μM) over time was then investigated (1-5 minutes). Various kainate concentrations (1 - 1000 μM), were then used to stimulate cGMP production over a five minute period.

Amino acid analysis was carried out on whole brain as described in chapter 2.

Protein concentration was determined as described in Chapter 2.

Statistical significance for the comparison of two groups was by Student's unpaired t-test. All results are expressed as the mean ± standard error of mean (SEM) with the number of slice preparations represented by n.

5.3. Results.

5.3.1. Tetrahydrobiopterin analysis.

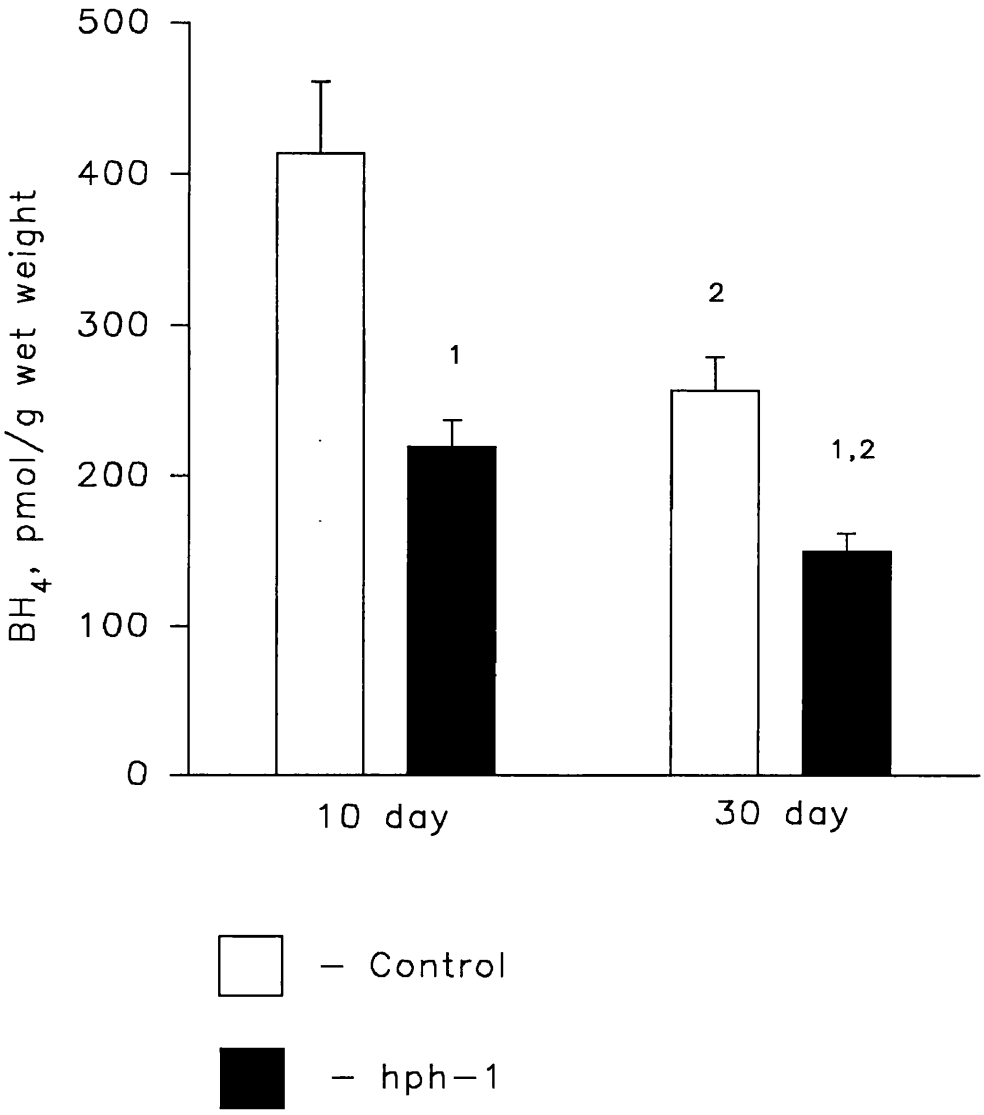
In control mice there was a significant 38% decrease in the BH_4 concentration from 10 to 30 days in the cerebellum (Figure 5.3.). In the *hph-1* mouse there was also a significant 32% decrease in cerebellar BH_4 levels from 10 to 30 days (Figure 5.3.). BH_4 levels were significantly lower in the *hph-1* mouse being 53% of control values at 10 days and 58% at 30 days.

5.3.2. Morphology of cerebellar slices.

Figure 5.4. shows the light microscope appearance of chopped slices of cerebellum from 10 day old mice following 90 minute incubation in Krebs-Henseleit buffer. The normal landmarks of the developing cerebellum are evident with the external germinal layer followed by the relatively cell free molecular layer with the aligning Purkinje cells and then the internal granule layer. No difference was observed between the two groups of mice with regard to the differentiation or the preservation of the cerebellum. Figure 5.5. shows the light microscope appearance of cerebellar slices from 30 day old mice. The cerebellum shows normal development characteristics in both groups with no external germinal layer, also the molecular layer appears deeper in the 30 day

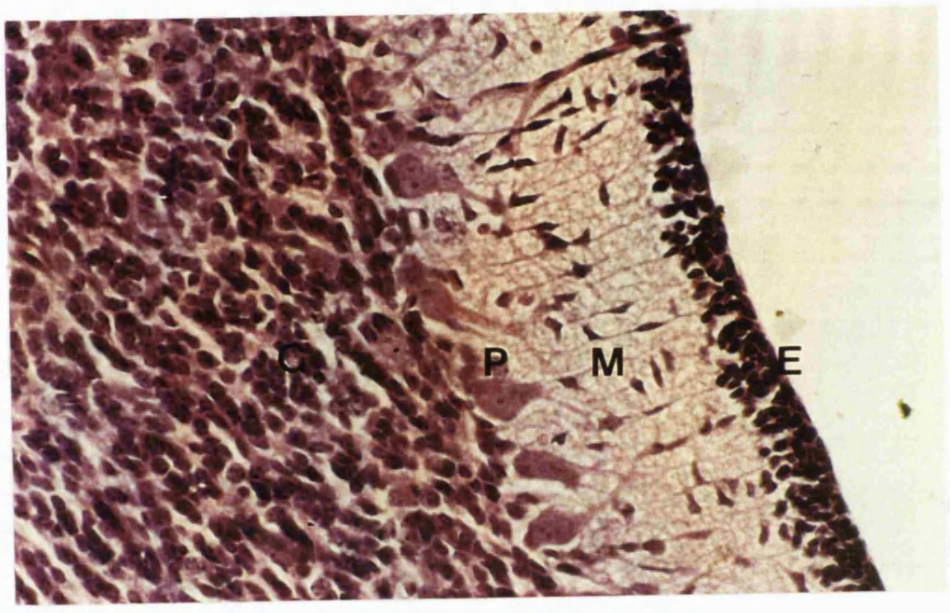
INCUBATION	AIM
No additions	Determine basal cGMP levels.
500 μM kainate	Determine cGMP levels following non-NMDA receptor stimulation.
500 μM kainate + 500μM NNA	Measure the effect of a NOS inhibitor on kainate stimulated cGMP response.
500 μM CNQX	Measure the effect of a kainate receptor antagonist on basal cGMP levels.
500 μM kainate + 500 μM CNQX	Measure the effect of a kainate receptor antagonist on kainate stimulated cGMP response.
500 μM SNAP	Determine cGMP response following exogenous NO exposure.
500 μM SNOG	Determine cGMP response following exogenous NO exposure.
500 μM SNOG + 25 μM haemoglobin	Determine the influence of the residual compound following NO release.

Table 5.1. Experimental design for the investigation of cGMP response in cerebellar slices following kainate exposure.

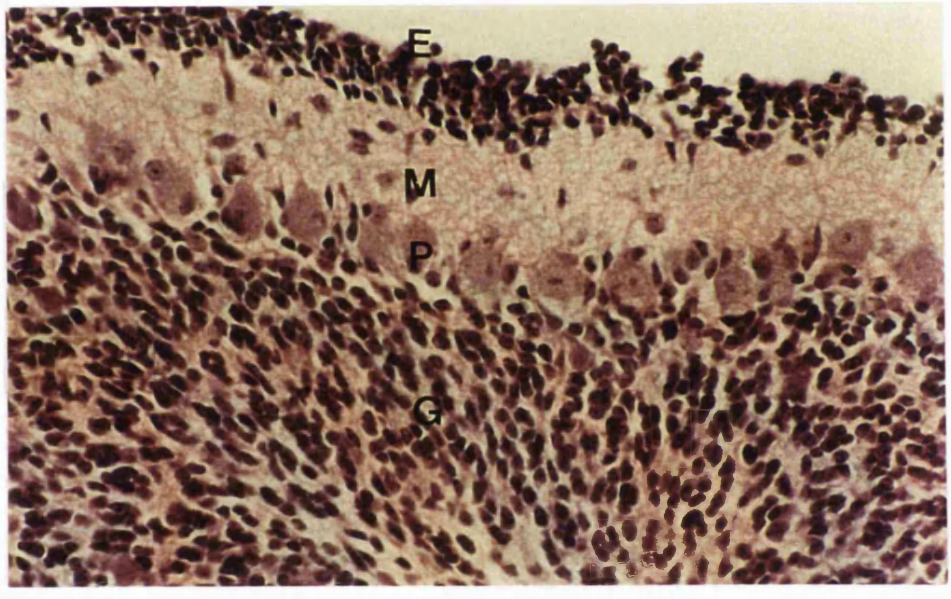


Data displayed as mean ± SEM, n = 4 for all points. [1] Significantly lower than age matched control, $p<0.01$. [2] Significantly lower than equivalent 10 day old result, $p<0.01$.

Figure 5.3. Cerebellar tetrahydrobiopterin concentration.



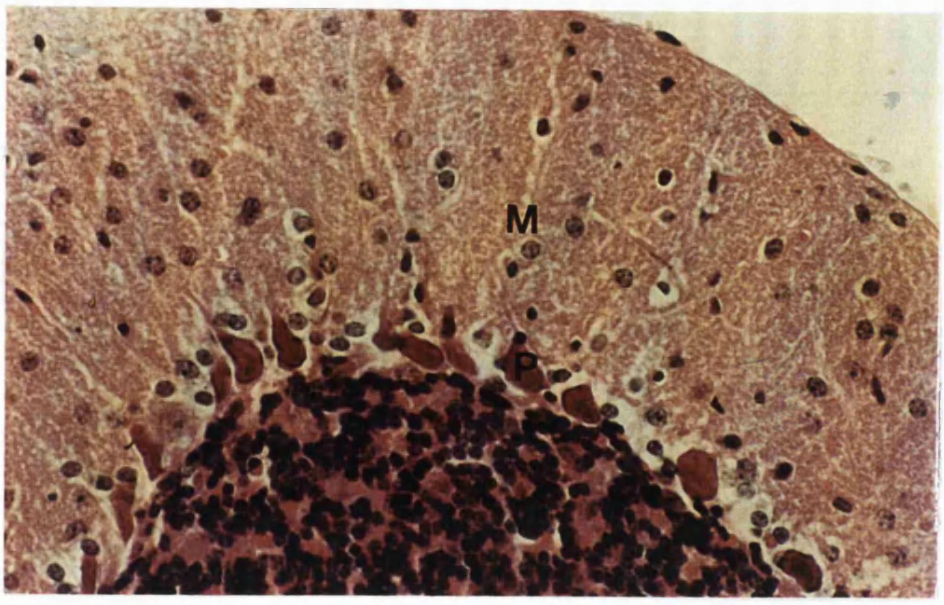
Control



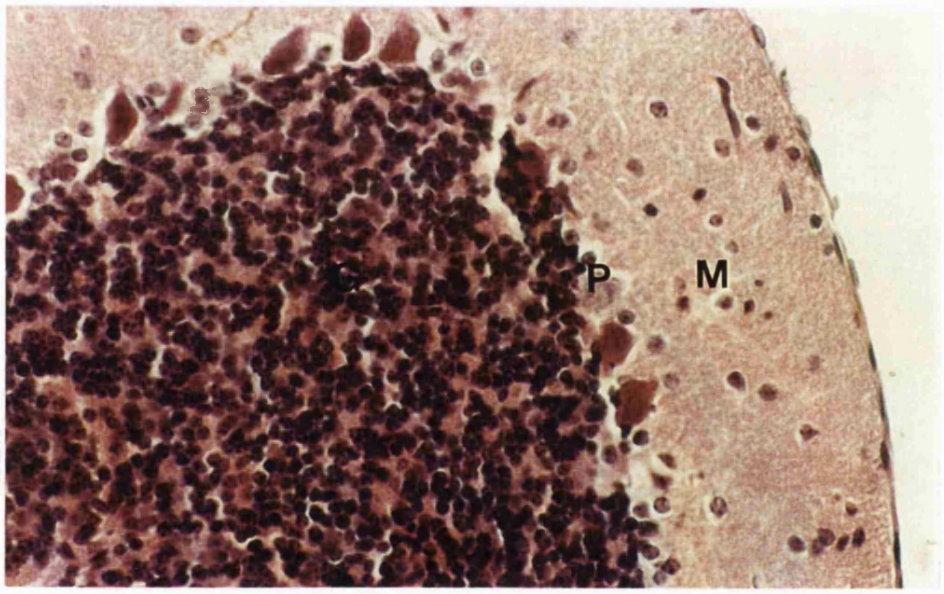
hph-1

E: external germinal layer, M: molecular layer, P: Purkinje layer, G: granular layer.

Figure 5.4. Cerebellar slices at low magnification (x100) from 10 day old mice.



Control



hph-1

M: molecular layer, P: Purkinje layer, G: granular layer.

Figure 5.5. Cerebellar slices at low magnification (x100) from 30 day old mice.

old slices. No difference was observed between the two groups of mice with regard to the differentiation or the preservation of the cerebellum.

Cerebellar slices from both groups of mice at both age points, therefore, appear to contain viable cell populations and appear suitable for the studies carried out in this chapter.

5.3.3. Effect of NMDA receptor agonists on cGMP response.

At 10 days of age, the mean cGMP level in unstimulated *hph-1* cerebellar slices was approximately 40% of that seen in control mice (Table 5.2.). The effects of the NMDA receptor agonists, glutamate, NMDA and kainate were investigated over five minutes (Table 5.2.). The cGMP response to each of these was significantly lower in the *hph-1* mouse than for the control group (Table 5.2.). NMDA gave the best cGMP response for both the *hph-1* and control groups at this age point with glutamate giving the poorest response (Table 5.2.).

At 30 days of age the basal level of cGMP in the *hph-1* group was approximately 70% of the levels seen in the control mice group (Table 5.3.). At this age point following five minutes stimulation with glutamate, NMDA and kainate, the cGMP response to each of these was lower in the *hph-1* group (Table 5.3.). Glutamate produced very little stimulation of cGMP levels with no difference between the glutamate treated group and the basal levels. At this age point kainate produced the best cGMP response in both groups of mice (Table 5.3.).

Kainate gave a good cGMP response in both 10 and 30 day old mice and was, therefore, used in all further investigations.

5.3.4. Time course response to kainate.

In 10 day old mice there was a prompt response to kainate by one minute in both groups of mice (Figure 5.6.). This remained at a plateau for at least five minutes (Figure 5.6.). Similar results were obtained for the 30 day old mice groups with both sets of mice recording a response at 1 minute after addition of kainate which remained

Treatment	pmol cGMP/mg protein	
	control	*hph-1*
Basal	4.64 ± 0.61 (30)	1.87 ± 0.22 (32)*
500 μM Glutamate	8.83 ± 1.86 (5)	4.25 ± 0.06 (3)*
500 μM NMDA	22.54 ± 2.68 (5)	16.32 ± 1.19 (4)*
500 μM Kainate	10.46 ± 0.91 (23)	5.59 ± 0.36 (27)*

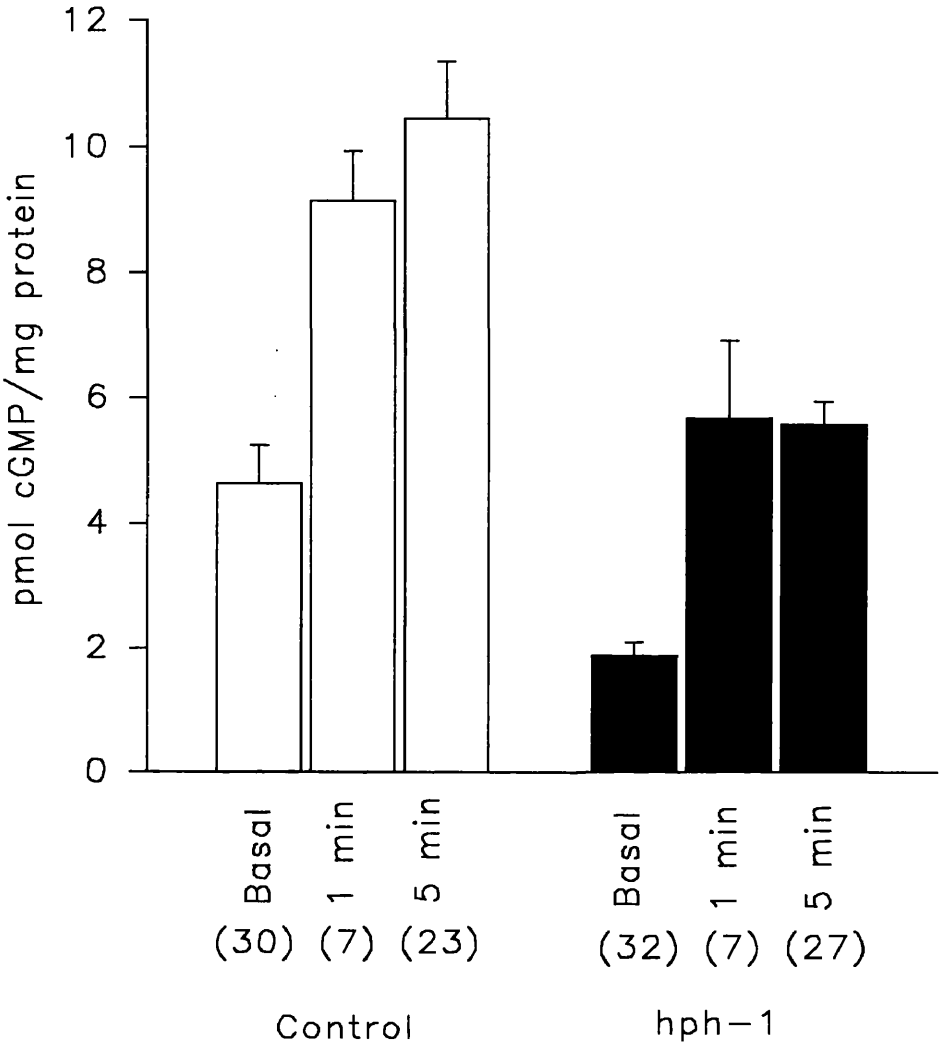
Incubation time = 5 minutes. Data is expressed as mean ± SEM. Number in parentheses is equal to sample size. * Significantly lower than control group, $p<0.01$.

Table 5.2. The effect of NMDA receptor agonists on cerebellar slices from 10 day old mice.

Treatment	pmol cGMP/mg protein	
	control	*hph-1*
Basal	12.54 ± 0.62 (19)	9.09 ± 0.62 (21)*
500 μM Glutamate	13.40 ± 2.24 (4)	7.72 ± 0.33 (3)*
500 μM NMDA	20.46 ± 5.09 (3)	10.50 ± 1.32 (3)*
500 μM Kainate	24.55 ± 2.58 (11)	14.51 ± 1.00 (14)*

Incubation time = 5 minutes. Data is expressed as mean ± SEM. Number in parentheses is equal to sample size. *Significantly lower than control group, $p<0.001$.

Table 5.3. The effect of NMDA receptor agonists on cerebellar slices from 30 day old mice.



Data shown as mean ± SEM, number in parentheses is equal to sample size. At each time point cGMP levels are significantly lower in the *hph-1* mouse compared with the control, $p<0.05$.

Figure 5.6. Time course response to kainate in cerebellar slices prepared from 10 day old mice.

at this level for at least five minutes (Figure 5.7.). At both time points the maximal response to kainate was decreased in the *hph-1* mouse.

5.3.5. Dose response to kainate.

In 10 day old mice half maximal response to kainate was approximately 8 μM in control mice but approximately 50 μM in the *hph-1* mouse (Figure 5.8.). However in the 30 day old group of mice the half maximal response to kainate was comparable for both groups, 8 μM for the control group and 7 μM for the *hph-1* mouse (Figure 5.9.).

5.3.6. Kainate stimulation and the involvement of NO in cerebellar slices.

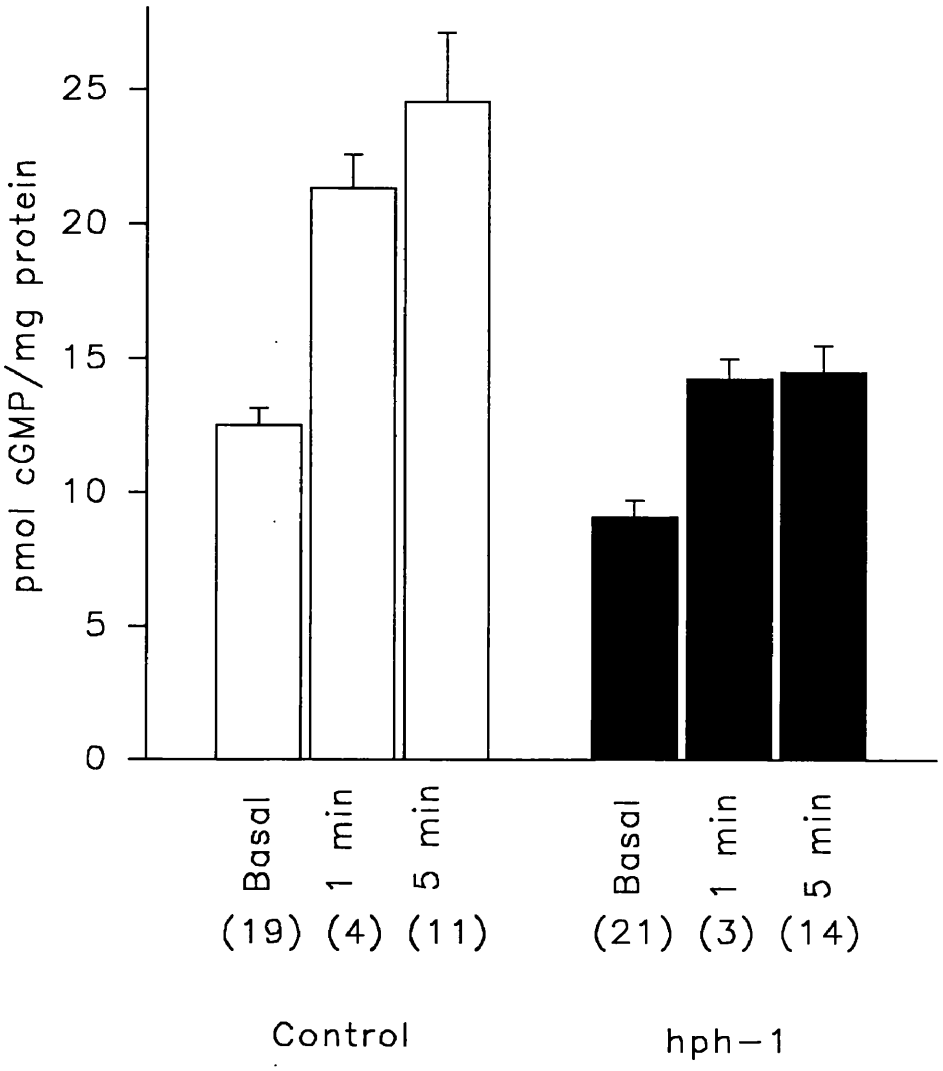
In cerebellar slices from 30 day old mice, addition of kainate led to an increase in cGMP levels in both groups of mice (Table 5.4.). Five minutes preincubation with CNQX, an antagonist with preferential activity at non-NMDA receptors, potently inhibited this response (Table 5.4.).

Incubation of cerebellar slices with the NOS inhibitor, NNA, together with kainate led to a significant inhibition of the cGMP response to kainate to below basal levels in 30 day old control and *hph-1* mice (Table 5.4.).

Using the NO donors SNAP and SNOG there was a marked increase in the cGMP concentrations observed in the cerebellar slices from both 30 day old control and *hph-1* mouse groups (Table 5.5.). No difference was observed between the stimulation with SNAP or SNOG in either group.

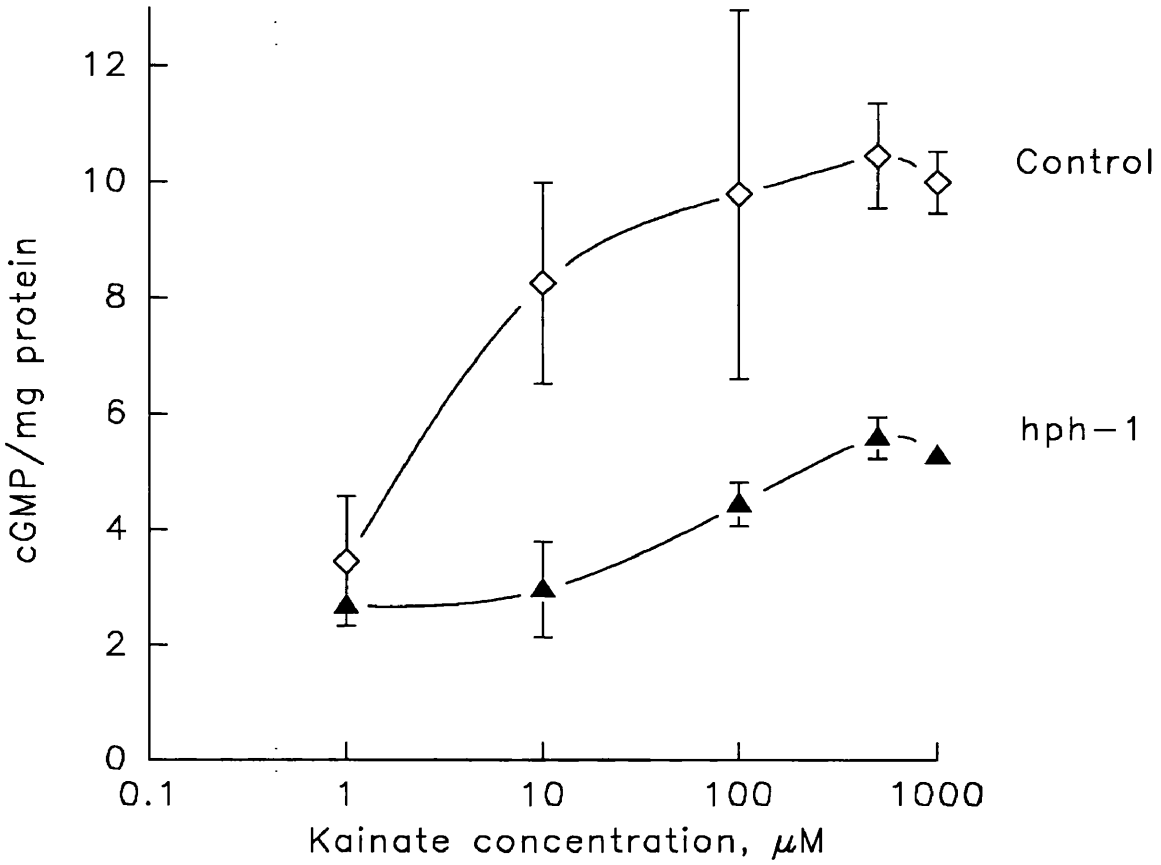
In order to determine that the cGMP response was due to NO release and not the residual compound, cerebellar slices from 30 day old mice were incubated with 500 μM SNOG in the presence of 25 μM haemoglobin (Table 5.5.). Haemoglobin attenuated the cGMP response and cGMP levels returned to basal levels (Table 5.5.).

The effect of the NOS inhibitor, NNA, together with 500 μM kainate was also investigated in 10 day old mice (Table 5.6.). A similar effect was observed to the 30



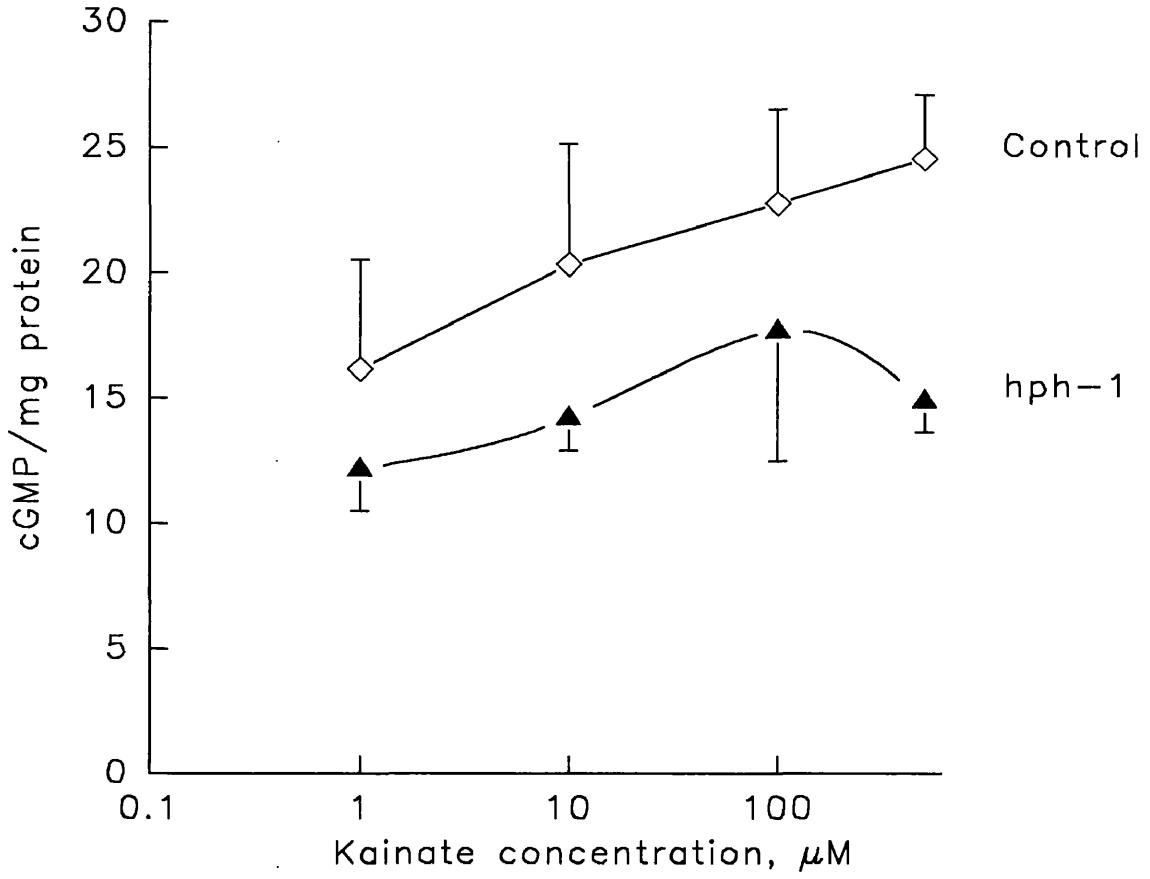
Data shown as mean ± SEM, number in parentheses is equal to sample size. At each time point, cGMP levels are significantly lower in the *hph-1* mouse compared with the control group, $p<0.05$.

Figure 5.7. Time course response to kainate in cerebellar slices prepared from 30 day old mice.



Data shown as mean ± SEM, each point represents the mean of at least 3 determinations.

Figure 5.8. Dose response curves to kainate in 10 day old mice.



Data shown as mean ± SEM, each point represents the mean of at least 3 determinations.

Figure 5.9. Dose response curves to kainate in 30 day old mice.

Treatment	pmol cGMP/mg protein	
	control	*hph-1*
Basal	12.54 ± 0.62 (19)	9.09 ± 0.62 (21)[1]
500 μM Kainate	24.55 ± 2.58 (11)	14.51 ± 1.00 (14)[1]
500 μM Kainate + 500 μM NNA	1.39 ± 0.24 (3)[2]	0.89 ± 0.29 (3)[2]
Basal + 500 μM CNQX	7.55 ± 1.96 (3)[3]	5.38 ± 0.54 (3)[3]
500 μM kainate + 500 μM CNQX	11.68 ± 2.27 (3)[4]	7.83 ± 1.51 (3)[4]

Incubation time = 5 minutes.

Data displayed as mean ± SEM. Number in parentheses is equal to sample size.

[1] Significantly different than corresponding control values., $p<0.05$.

[2] Significantly different than 500 μM kainate alone, $p<0.001$.

[3] Significantly different than basal levels in the absence of CNQX, $p<0.05$.

[4] Significantly different than 500 μM kainate alone, $p<0.05$.

Table 5.4. The effect of the NOS inhibitor, NNA and the kainate antagonist, CNQX on the cGMP response to kainate in 30 day old mice.

Treatment	pmol cGMP/mg protein	
	control	*hph-1*
Basal	12.54 ± 0.62 (19)	9.09 ± 0.62 (21)
500 μM SNAP.	46.87 ± 1.49 (4)	51.54 ± 8.87 (4)
500 μM SNOG.	44.60 ± 8.03 (7)	41.46 ± 3.53 (3)
Basal + 25 μM haemoglobin	7.25 ± 0.85 (3)[1]	2.78 ± 0.93 (3)[1]
500 μM SNOG + 25 μM haemoglobin	17.73 ± 2.99 (3)[2]	13.25 ± 7.60 (3)[2]

Data shown as mean ± SEM. Figures in parentheses are equal to sample size.

[1] Significantly lower than basal values in the absence of haemoglobin, $p<0.01$.

[2] Significantly lower than 500 μM SNOG alone, $p<0.05$.

Table 5.5. The effect of NOS donors and haemoglobin on cGMP response in cerebellar slices from 30 day old mice.

Treatment	pmol cGMP/mg protein	
	control	*hph-1*
Basal	4.64 ± 0.61 (30)	1.87 ± 0.22 (32)[1]
500 μM kainate	10.46 ± 0.91 (23)	5.59 ± 0.36 (27)[1]
500 μM kainate + 500 μM NNA	0.78 ± 0.13 (3)[2]	0.72 ± 0.04 (3)[2]
500 μM SNAP	58.45 ± 4.07 (3)	51.48 ±2.39 (3)

Data expressed as mean ± SEM, numbers in parentheses is equal to sample size. [1]Significantly different from control group ($p<0.01$). [2]Significantly different from kainate stimulation alone ($p<0.01$).

Table 5.6. The effect of kainate, NNA and SNAP on cerebellar slices prepared from 10 day old mice.

day old mice groups with cGMP levels being reduced to below basal levels (Table 5.6.). No difference was observed between the two groups of mice with NNA (Table 5.6.). The NO donor, SNAP, was used to stimulate a cGMP response in 10 day old mice, again no difference was detected between the control and *hph-1* mouse at this age point (Table 5.6.).

5.3.7. Amino acid analysis.

In order to determine further if BH_4 deficiency impairs brain NOS activity, whole brain concentrations of arginine and citrulline was determined in control and the *hph-1* mouse. Citrulline production in brain can only occur via NOS since the urea cycle is incomplete in the brain (Ratner et al., 1960). For comparison, phenylalanine and tyrosine levels were also determined. In 10 day old mice, as expected, brain phenylalanine levels were significant elevated but tyrosine and arginine levels were significantly lower in the *hph-1* mouse (Table 5.7.). No difference was observed in citrulline concentration between control and *hph-1* mice in 10 day old mice (Table 5.8.). However, in 30 day old mice citrulline concentration was significantly decreased in the *hph-1* mouse whereas arginine, phenylalanine and tyrosine concentrations were comparable between the two groups (Table 5.8.).

5.4. Discussion.

The concentration of BH_4 in the cerebellum is significantly reduced in the *hph-1* mouse at both age points when compared to control mice.

The morphology of the incubated slices provides important information that the incubated slices used in these experiments showed good preservation as it has been previously reported that mechanical chopping of the cerebellum in adult mice results in pyknotic cells and vacuolation of the slices (Garthwaite et al., 1979; Garthwaite et al., 1980). Slices produced for this work from 30 day old mice, therefore, provide viable tissue suitable for the experiments carried out.

The NMDA receptor agonist, glutamate, NMDA and kainate all elicited a cGMP response in 10 day old mice with NMDA being the most effective agonist. Similar

	amino acid concentration, nmol/g wet weight	
	control	*hph-1*
phenylalanine	74.0 ± 14.8 (4)	1338.0 ± 46.6 (6)*
tyrosine	117.0 ± 13.4 (4)	35.3 ± 3.3 (6)*
arginine	154.0 ± 7.6 (4)	83.2 ± 4.2 (5)*
citrulline	35.0 ± 1.0 (4)	40.0 ± 3.9 (6)

Data expressed as mean ± SEM. Number in parentheses is equal to sample size.
*Significantly different from control mice ($p<0.001$).

Table 5.7. Whole brain amino acid concentration in 10 day old mice.

	amino acid concentration, nmol/g wet weight	
	control	*hph-1*
phenylalanine	58.0 ± 15.1	63.5 ± 8.1
tyrosine	65.3 ± 13.1	56.0 ± 10.7
arginine	110.5 ± 33.3	95.5 ± 20.4
citrulline	37.0 ± 10.8	21.0 ± 5.1 *

Data shown as mean ± SEM, n = 8. *Significantly different from control mice ($p<0.05$).

Table 5.8. Whole brain amino acid concentration in 30 day old mice.

finding are reported by Southam et al. in a study of rat cerebellum slices (1991). However, in 30 day old mice the most effective agonist is kainate. This, again, agrees with the observation of Southam et al. (1991) who report that the response to kainate remains effective in both immature and adult tissues whereas the response to NMDA declines with age. The poor cGMP response to glutamate may be due to restricted access of the amino acid to neurones due to cellular uptake mechanisms in the slices (Garthwaite et al., 1989). Kainate provides a good agonist for the investigation of the NO/cGMP pathway in both 10 and 30 day old mice.

Under basal conditions, levels of cGMP were significantly lower in the *hph-1* mouse when compared to controls at both age points. Furthermore, following kainate exposure, the concentration of cGMP attained was again lower in slices prepared from the *hph-1* mouse. When the cerebellar slices were preincubated with the non-NMDA receptor antagonist, CNQX, the effect of kainate was completely inhibited. Previous work using kainate to stimulate a cGMP response in rat cerebellar slices in a calcium free buffer demonstrated that the kainate induced rise in cGMP is absolutely dependant on calcium (Garthwaite et al., 1989). Formation of cGMP in the cerebellar slices is clearly NOS dependent as the NOS inhibitor, NNA, prevented formation of cGMP following kainate exposure and decreased cGMP levels to below the basal concentration. The decreased capacity of the *hph-1* mouse cerebellar slices to form cGMP is unlikely to be related to an impairment of guanylate cyclase since in the presence of the NO donors, SNAP or SNOG, comparable levels of cGMP were obtained in cerebellar slices from both groups of mice.

One difference between the 30 day old and 10 day old *hph-1* mouse is in the dose response to kainate. In the adult 30 day old mice there is almost an identical half maximal response to kainate to that seen in control mice. However, in 10 day old mice the half maximal response to kainate is almost six times higher in the control mice group. Southam et al (1991) have suggested that there may be two NO synthases in the brain, one of which is inhibited more potently by NNA. The activity of the high inhibition isoform is proposed to lessen during maturation leaving a dominant lower inhibition isoform. The presence of two isoforms of NOS in the

immature brain may explain the difference in the response to kainate. Matsumoto et al. (1993), however, found no evidence of two brain isoforms of NOS during their studies of developmental changes. These findings appear to indicate an impaired ability to synthesize NO in *hph-1* cerebellar slices which is particularly reduced in the 10 day old *hph-1* mouse. This may also be due to the result of partial BH_4 deficiency which has been shown to increase the K_m of NOS for arginine (Chapter 4). An increased K_m for arginine combined with decreased arginine levels in the 10 day old *hph-1* mouse may, therefore, lead to impaired NOS activity in the cerebellum.

Further support for this suggestion comes from the whole brain amino acid analysis. It has been known for some time that high concentrations of phenylalanine will inhibit the transport of other aromatic amino acids but it also appears to inhibit transport of arginine (Neame, 1961; Hyland et al., 1985). This is clearly demonstrated by this study with a very high brain phenylalanine level leading to reduced brain tyrosine and arginine concentration. Interestingly, citrulline, an intermediate of arginine metabolism and a product of the NOS reaction, is not reduced in the 10 day old *hph-1* mouse. This may be because of free transport of citrulline across the developing blood brain barrier in the 10 day old mouse. The reduced levels of arginine in the brain of the *hph-1* mouse may lead to lower NOS activity and, consequently, lower cGMP synthesis. This effect may be exaggerated because arginine is predominantly stored in the astrocytes and must be transported to the neurones as a substrate for NOS (Aoki et al., 1991). It is conceivable that whole brain phenylalanine levels may interfere with this transport leading to less arginine in the neurone which in turn may lead to reduced production of NO. In the brain of the 30 day old *hph-1* mouse, phenylalanine levels are comparable with control mice as the *hph-1* mouse do not display hyperphenylalaninaemia at this age point. As expected brain tyrosine and arginine concentrations are normal. However, whole brain citrulline levels are significantly reduced in the *hph-1* mouse at 30 days. NOS is likely to be the source of citrulline in the brain as the urea cycle is incomplete in the brain (Ratner et al., 1960). NO and citrulline are produced in equimolar quantities from arginine by NOS (Bush et al., 1992). This reduced level of citrulline may indicate a reduced level of NOS activity in the 30 day old *hph-1* mouse brain.

The physiological effects of cGMP are not well understood, although Southam and Garthwaite (1993) have suggested that cGMP may act directly on ion channels or on phosphodiesterases to increase/decrease cAMP levels. Therefore, partial reduction in the cerebellar BH_4 concentration may as a result of impairment of the NO/cGMP pathway have important neurochemical effects

In addition to the effects of BH_4 deficiency on the NO/cGMP pathway, is the effect of arginine deficiency on NOS activity. It has been reported that in situations where arginine and BH_4 are limiting NOS will generate superoxide and/or hydrogen peroxide (Pou et al., 192; Heinzel et al. 1992). Superoxide can react with nitric oxide to form $ONOO^-$ (Beckman et al., 1990). In cultured astrocytes and neurones, one of the targets for $ONOO^-$ is the mitochondrial electron transport chain (Bolaños et al., 1995). The activity of the mitochondrial electron chain in control and *hph-1* mice will be measured in order to determine if there is any mitochondrial damage which may be due to excess free radical production.

Chapter 6. Brain mitochondrial function and glutathione status in the *hph-1* mouse.

Chapter 6. Brain mitochondrial function and glutathione status in the *hph-1* mouse.

6.1. Introduction.

In the brain, NO has been implicated in many different physiological processes including synaptic plasticity, regulation of the cerebral circulation and neuronal and neuroendocrine function (Lizasoain et al., 1996). Within the central nervous system, a major function of NO is activation of guanylate cyclase, following activation of glutamate receptors, resulting in an increase in cGMP (Garthwaite, 1991; Garthwaite and Boulton, 1995). However, studies on purified NOS where arginine and/or BH_4 are limiting, have revealed that molecular oxygen will act as the electron acceptor rather than arginine leading to formation of oxygen species, either superoxide or hydrogen peroxide (Heinzel et al., 1992; Pou et al., 1992). Deficiency of BH_4, as shown in Chapter 4, decreases the affinity of NOS for arginine, a small decrease in arginine may result in the amino acid becoming limiting which in turn may result in formation of oxygen free radicals (Heinzel et al., 1992). Furthermore, intact cerebellar granule cells, which have been depleted of arginine through the use of arginase (EC 3.5.3.1), release superoxide following NMDA receptor stimulation (Culcasi et al., 1994). This superoxide release was also responsible for a limited but significant cell death that was suppressed by either arginine or the NOS inhibitor, NNA (Culcasi et al., 1994).

As stated, at low concentrations of arginine and/or BH_4, NOS may produce superoxide ($O_2^{\cdot -}$) as well as NO (Heinzel et al., 1992: Pou et al., 1992). These two compounds react rapidly together to form peroxynitrite ($ONOO^-$) (Beckman et al., 1990):

$$O_2^{\cdot -} + NO \rightarrow ONOO^-$$

This reaction occurs at a near diffusion limited rate of 6.7×10^9 $M^{-1}s^{-1}$ and will effectively compete with the enzymatic dismutation of superoxide which has been calculated to have a rate constant of 5×10^5 $M^{-1}s^{-1}$ (Hogg et al., 1992; Beckman and Tsai, 1994). $ONOO^-$ is an unstable species at physiological pH and has a half life of up to 1.9 seconds, even so this will allow diffusion over several cell diameters

(Beckman et al., 1990). Lipton et al. (1993) have suggested that the agent responsible for the cell mediated toxicity may be $ONOO^-$. It may decompose into toxic products including nitronium ion, nitrogen dioxide and the hydroxy radical (Halliwell, 1995). It directly oxidises many important biological molecules including lipids, sulphydryls and nitrosation of several functional groups of amino acids including tyrosine (Beckman and Tsai, 1994). It has also been shown that acute exposure of cultured neurones to $ONOO^-$ led to mitochondrial damage suggesting that neurotoxicity may also be mediated through mitochondrial dysfunction (Bolaños et al., 1995).

Efficient energy supply is essential for the cell and mitochondria produce much of the useful energy in the form of ATP. Disruption of mitochondrial function is a common cause of cell dysfunction and cell death (Jones and Lash, 1993). Mitochondrial dysfunction can be defined as defective ATP production due to enzymatic, transport, structural or regulatory failure (Jones and Lash, 1993). The mitochondrial electron transport chain conserves the energy of electron transfer in the form of a proton gradient which is then used to drive the synthesis of ATP via the ATP synthetase (complex V) (Figure 2.8.). Electron transfer is carried out by the mitochondrial electron transport chain complexes (Figure 2.8.). The lipid soluble co-enzyme Q (ubiquinone) transfers reducing equivalents from complex I and II to complex III. Electrons are then transferred from complex III to complex IV via the water soluble cytochrome c. Exposure of $ONOO^-$ to cultured neurones led to a dose dependent decrease in complex II/III and complex IV activity (Bolaños et al., 1995). Because $ONOO^-$ can initiate lipid peroxidation and complex IV requires cardiolipin for maximal activity, it is possible that this process is responsible for the observed inactivation of complex IV (Beckman et al., 1990; Bolaños et al., 1995). Mitochondrial inhibition occurs before cell death, therefore, suggesting that mitochondrial damage may be a cause of the $ONOO^-$ mediated cell death (Bolaños et al., 1995). Mitochondrial damage has also been suggested to be an important part in the pathology of Parkinson's and Alzheimer's diseases (Beal et al., 1993).

Glutathione (GSH) is an intracellular antioxidant capable of reacting with a number of oxidising agents including $ONOO^-$. GSH has a number of well established roles

including protection of the cell against hydrogen peroxide formation (For review, see Halliwell and Gutteridge, 1989). Glutathione peroxidase (EC 1.11.1.9) catalyses the oxidation of glutathione (GSH) to oxidised glutathione (GSSG) at the expense of hydrogen peroxide:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

The GSSG formed is converted back to GSH by glutathione reductase (EC 1.6.4.2):

$$GSSG + NADPH \rightarrow 2GSH + NADP^+$$

GSH is a simple tri-peptide (Glutamic acid - cysteine - glycine) in its reduced form. In the oxidised form, two GSH molecules join together as the -SH groups of the cysteine are oxidised to form a disulphide bridge. GSH is a well documented intracellular anti-oxidant and can react directly with $ONOO^-$ (Bolaños et al., 1995). In support for this, exposure of cultured neurones to $ONOO^-$ led to a 40% decrease in neuronal intracellular GSH (Bolaños et al., 1995). Cultured astrocytes are relatively resistant to $ONOO^-$ and this appears to be related to higher intracellular levels of GSH than neurones (Bolaños et al., 1995). Depletion of intracellular astrocytic GSH followed by exposure to $ONOO^-$ led to a decrease in mitochondrial complex I and II/III leading to the conclusion that GSH is an important defence against $ONOO^-$ (Barker et al., 1996).

In view of the "uncoupling" of the NOS reaction when BH_4 and/or arginine concentration is limiting the aim of the experiments was to determine if deficiency of brain BH_4 results in damage to the mitochondrial electron transport chain and a loss of GSH. In addition these parameters were measured in cerebellar slices following exposure to kainate to evaluate the effects following NMDA receptor stimulation.

6.2. Methods

Control mice and *hph-1* at 10 and 30 days of age were used in these experiments and were killed by cervical dislocation.

Whole brain mitochondrial electron transport chain complexes and citrate synthase activity was measured in 10 and 30 day old mice as described in Chapter 2. Brain was removed and homogenised in 320 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4 (10% wt:vol) (Heales et al., 1995). Homogenates were then freeze-thawed three times before enzymic analysis using liquid nitrogen. Prior to analysis, samples were diluted 1 in 5 in the appropriate assay buffer.

Cerebellar slices from 10 and 30 day old mice were prepared and preincubated for 90 minutes as described in Chapter 2. Slices were then incubated for five minutes in the presence or absence of 500 μM kainate as described in Chapter 5. After this five minute incubation, slices were transferred to an Eppendorf centrifuge tube containing 250 μl of 320 mM sucrose. 10 mM Tris, 1 mM EDTA, pH 7.4 and then frozen in liquid nitrogen. Samples were freeze-thawed three times and then homogenised using a Soniprep 150 sonicator (Fisons Scientific Equipment, Loughborough, UK). Individual mitochondrial complexes and citrate synthase were then measured as described in Chapter 2.

Analysis of GSH and GSSG was carried out on whole brain as described in Chapter 2. Whole brain was removed from the mouse and homogenised in 15 mM phosphoric acid (25% wt:vol) (Heales et al., 1995). Homogenate were centrifuged at 15,000 RPM for 10 minutes and the supernatant used for GSH/GSSG analysis.

6.3. Results.

6.3.1. Whole brain mitochondrial electron transport chain and citrate synthase activity.

Mitochondrial complex activities in whole brain homogenates are shown in Table 6.1. Both citrate synthase and mitochondrial complexes activity were significantly lower ($p<0.05$) in the 10 day old mice of both groups when compared to 30 day old mice of both groups (Table 6.1.). In the 10 day old age group there was a significant 8.5% decrease ($p<0.05$) in the activity of citrate synthase in the *hph-1* mouse when compared to control mice (Table 6.1.). However, in this age group there was no significant difference in any mitochondrial complex activity between the two groups

	Complex I nmol/min/mg protein	Complex II/III nmol/min/mg protein	Complex IV k/min/mg protein	Citrate synthase nmol/min/mg protein
10 day old control	30.2 ± 2.5 (6)	34.6 ± 2.4 (6)	5.9 ± 0.1 (3)	327.0 ± 7.2 (6)
10 day old *hph-1*	30.4 ± 2.5 (6)	30.8 ± 2.8 (6)	6.3 ± 0.7 (4)	299.0 ± 5.8 (6)*
30 day old control	52.4 ± 3.8 (6)	51.0 ± 3.5 (6)	8.1 ± 0.3 (6)	421.4 ± 18.9 (6)
30 day old *hph-1*	52.2 ± 3.4 (6)	50.2 ± 3.1 (6)	9.1 ± 0.4 (6)	439.0 ± 9.7 (6)

Data displayed as mean ± SEM. Numbers in parentheses is equal to the number of samples. *Significantly lower than 10 day old control mice when compared to 30 day old mice ($p<0.05$). For all mitochondrial complexes and citrate synthase, enzyme activity was significantly lower at 10 days in both groups of mice when compared to 30 day old mice ($p<0.05$).

Table 6.1. Mitochondrial electron transport chain complexes and citrate synthase from whole brain homogenates in 10 and 30 day old mice.

of mice (Table 6.1.). No significant difference was observed in any enzyme activity between the control and *hph-1* mouse in the 30 day old age groups (Table 6.1.).

6.3.2. Mitochondrial electron transport chain and citrate synthase activity in cerebellar slices from 10 day old mice.

There was no significant difference in enzyme activity between the control and *hph-1* group for any mitochondrial complex or citrate synthase in homogenates prepared from cerebellar slices at this age point (Table 6.2.). The effect of incubating slices with 500 μM kainate for five minutes had no effect on enzyme activity in either the control mice or the *hph-1* mouse (Table 6.2.).

6.3.3. Mitochondrial electron transport chain and citrate synthase activity in cerebellar slices from 30 day old mice.

No significant difference was observed in enzyme activity between the control and *hph-1* group for any mitochondrial complex or citrate synthase in homogenates prepared from cerebellar slices in 30 day old mice (Table 6.3.). The effect of incubating slices with 500 μM kainate for five minutes had no significant effect on enzyme activity in either group of mice (Table 6.3.).

6.3.4. Whole brain glutathione analysis

There was no significant difference in GSH levels between the control and the *hph-1* mouse groups in 10 day old mice (Table 6.4.). GSSG could not be detected in samples prepared from whole brain of either group of the 10 day old mice (Table 6.4.). The GSH levels in 30 day old mice, from both groups, were significantly higher than the values seen in 10 day old control and *hph-1* mice (Table 6.4.). No significant difference was observed in whole brain GSH concentration between the control and the *hph-1* mouse at 30 days of age (Table 6.4.). GSSG levels were less than 5% of the whole brain GSH concentration in both sets of mice, no significant difference was observed in GSSG between the control and *hph-1* mice groups (Table 6.4.).

6.4. Discussion.

In 10 day old mice there was no significant difference in any whole brain

	Complex I nmol/min/mg protein	Complex II/III nmol/min/mg protein	Complex IV k/min/mg protein	Citrate synthase nmol/min/mg protein
10 day old control basal.	41.3 ± 2.0 (3)	30.5 ± 4.8 (5)	4.7 ± 0.6 (3)	257.0 ± 60.4 (3)
10 day old control 500 μM kainate.	41.9 ± 1.1 (3)	31.4 ± 5.1 (5)	5.4 ± 0.4 (3)	244.7 ± 5.0 (3)
10 day old *hph-1* basal.	36.6 ± 4.7 (3)	20.0 ± 2.4 (6)	3.6 ± 0.4 (3)	237.3 ± 36.4 (3)
10 day old *hph-1* 500 μM kainate	45.6 ± 6.2 (3)	26.0 ± 4.6 (6)	5.0 ± 1.0 (3)	289.7 ± 41.7 (3)

Data displayed as mean ± SEM. Figures in parentheses is equal to sample size.

Table 6.2. The effect of kainate on the mitochondrial electron transport chain complexes and citrate synthase activity in cerebellar slices from 10 day old mice.

	Complex I nmol/min/mg protein	Complex II/III nmol/min/mg protein	Complex IV k/min/mg protein	Citrate synthase nmol/min/mg protein
30 day old control basal.	72.5 ± 2.4 (4)	57.9 ± 4.4 (6)	8.0 ± 0.5 (4)	403.6 ± 24.0 (4)
30 day old control 500 μM kainate.	67.1 ± 2.2 (3)	64.4 ± 2.9 (5)	8.6 ± 0.3 (3)	357.3 ± 3.8 (3)
30 day old *hph-1* basal.	76.7 ± 3.2 (3)	55.5 ± 4.1 (5)	8.6 ± 0.2 (3)	435.0 ± 38.3 (3)
30 day old *hph-1* 500 μM kainate	74.1 ± 1.4 (3)	62.5 ± 6.0 (4)	8.1 ± 0.2 (3)	440.7 ± 19.9 (3)

Data displayed as mean ± SEM. Figures in parentheses is equal to sample size.

Table 6.3. The effect of kainate on the mitochondrial electron transport chain complexes and citrate synthase activity in cerebellar slices from 30 day old mice.

	Glutathione μmol/g wet weight.		Oxidised Glutathione μmol/g wet weight.	
	Control	*hph-1*	Control	*hph-1*
10 day	1.22 ± 0.12 (6)	1.22 ± 0.15 (6)	ND	ND
30 day	1.64 ± 0.09 (6)*	1.66 ± 0.20 (5)*	0.077 ± .009 (5)	0.069 ±0.012 (3)

Data displayed as mean ± SEM. Number in parentheses is equal to sample size. ND = not detectable. *Significantly higher than appropriate 10 day old group ($p<0.05$).

Table 6.4. Whole brain concentration of glutathione and oxidised glutathione in whole brain.

mitochondrial complex enzyme activity between control and the *hph-1* mouse. There was, however, a significant 8.5% decrease in citrate synthase activity in the *hph-1* mouse. At 10 days of age the *hph-1* mouse displays hyperphenylalaninaemia (McDonald and Bode, 1988). This situation causes excess phenylalanine to be metabolised by alternative minor pathways, namely by transamination to form phenylpyruvate or decarboxylated to form phenylethylamine (Scriver et al., 1989). It has been demonstrated that phenylpyruvate can inhibit 14 day old rat brain citrate synthase activity in a competitive fashion with respect to acetyl CoA with a K_i of 700 μM (Land and Clark, 1973). This could explain why citrate synthase activity is reduced in the 10 day old *hph-1* but not in the 30 day old *hph-1* mouse group. However, in the citrate synthase assay, the brain homogenate sample is diluted 250 times in the reaction buffer and there is also an excess of acetyl CoA in the assay system. This explanation is unlikely to be the cause of the decrease in citrate synthase specific activity. Elevated levels of phenylalanine have also been shown to decrease the transport of the large neutral amino acids into the central nervous system (Neame, 1961; Hyland et al., 1985). This altered brain concentration of amino acids has been suggested to play a role in inhibition of neural protein synthesis (Hughes and Johnson, 1977). A further suggestion for the inhibition of neural protein synthesis is that phenylalanine may inhibit may inhibit the aminoacylation reaction of specific tRNA molecules by either direct inhibition or because the specific amino acids are now limiting (Hughes and Johnson, 1977). Inhibition of neural protein synthesis may explain reduced citrate synthase activity in the 10 day old *hph-1* mouse whole brain. Reduced activity of citrate synthase may impair lipid biosynthesis since not only is cytosolic citrate the source of acetyl-CoA for fatty acid synthesis but acetyl -CoA carboxylase itself has a definite requirement for citrate for maximal activity (Land and Clark, 1973). However, it remains to be demonstrated if a 8.5% decrease in citrate synthase activity will result in a significant decrease in citrate availability.

In whole brain homogenates mitochondrial complexes and citrate synthase activity increased with age from 10 to 30 days. Similar results have been obtained with brain homogenates and isolated rat non-synaptic mitochondria where there is an increase in complex activity between 10 and 30 days (Land et al., 1977; Bates et al., 1994). In

synaptic mitochondria there is a comparable increase in mitochondrial complex activity with the exception of complex I which does not appear to show any developmental increase in activity after 10 days post partum (Almeida et al., 1995).

In 10 and 30 day old mice there was no difference in activity for any of the mitochondrial complexes between the control and *hph-1* mice. $ONOO^-$ has previously been shown to irreversibly damage mitochondrial complexes II/III and IV in neurones and astrocytes in culture (Bolaños et al., 1994; Bolaños et al., 1995). No observed decrease in activity of the mitochondrial complexes in the *hph-1* mouse may indicate that basal level of NOS activity in the mouse brain are insufficient to generate $ONOO^-$ at high enough concentrations to inhibit mitochondrial complexes. $ONOO^-$ can react with sulphydryls including the intracellular antioxidant, GSH ((Beckman and Tsai, 1994; Bolaños et al., 1995). Barker et al. (1996) have demonstrated that GSH is an important intracellular defence against $ONOO^-$ and depletion of intracellular GSH makes the mitochondria a vulnerable target and this may lead to cell death. The comparable levels of GSH observed in the *hph-1* mouse compared to the control mouse group may indicate that under normal conditions generation of oxidising species by NOS is minimal. Alternatively, any free radical generation may be adequately handled by the anti-oxidant system.

A possible role of BH_4 within the NOS complex is that it provides a specific means of preventing NO mediated inhibition of NOS (Mayer and Werner, 1995). As a potent activator of guanylate cyclase, NO binds to the prosthetic haem group causing a conformation change leading to guanylate cyclase activation (Mayer, 1993). A similar binding mechanism to the haem group in the NOS complex may lead to enzyme inactivation. It has, therefore, been suggested that enzyme bound BH_4 may play a role in the protection of the haem group from NO (Mayer and Werner, 1995). In the *hph-1* mouse, lower levels of BH_4 may lead to inactivation of NOS by NO. Decreased activity of NOS by this mechanism may, thus, explain the lack of mitochondrial complex inhibition seen in the *hph-1* mouse since free radical generation may, in fact, be less than control mice.

In homogenates prepared from cerebellar slices from 10 and 30 day old mice, there was no difference in any mitochondrial complex or citrate synthase activity at either age point between the two groups of mice. Citrate synthase activity was the same as control mice values in cerebellar slices from the 10 day old *hph-1* mouse as opposed to whole brain homogenates (Tables 6.1. and 6.2.). This may be because that the cerebellar citrate synthase is either unaffected by high phenylalanine levels or cerebellar phenylalanine levels are not as high as whole brain thus allowing normal citrate synthase expression.

Using the NMDA antagonist, 500 μM kainate, in the cerebellar slices, there was no effect on the mitochondrial electron transport chain (Tables 6.2 and 6.3). This maybe because concentration of kainate or exposure time may be insufficient to cause sufficient generation of peroxynitrite to inhibit mitochondrial complexes in the slices. In culture systems NMDA receptor stimulation has been shown to lead to cell death (Culcasi et al., 1994). Direct application of excitatory amino acids allows direct access to NMDA receptors, whereas in intact slice preparations transport of the excitatory amino acid is necessary across the slice and this may also account for the lack of response over the period of the experiment.

In summary, these results suggest that even in conditions where there is a greater than 50% reduction in brain BH_4 concentration, there appears to be no evidence of loss mitochondrial electron transport chain activity in the *hph-1* mouse. Reduction of citrate synthase activity in the 10 day old *hph-1* mouse appears to be related to high phenylalanine level.

Chapter 7.
Discussion and further work.

7. Discussion and further work.

7.1. Discussion.

The inborn errors of BH_4 metabolism were first reported in the mid-1970's when deficiency of dihydropteridine reductase was described (Bartholomé 1974; Smith et al., 1975). Reports of the deficiency of the biosynthetic enzymes soon followed this work (for review see Blau et al., 1993). These patients were unresponsive to a low phenylalanine diet, the traditional treatment for "classical phenylketonuria" (Smith et al., 1975). Currently, patients with disorders of BH_4 metabolism are treated with a phenylalanine restricted diet to control the hyperphenylalaninaemia together with administration of L-Dopa and 5-HTP because of the catecholamine and serotonin deficiency in this disorder (Smith and Brenton, 1995). Although this therapy is beneficial it appears incomplete as a number of patients continue to display neurological impairment despite early onset of treatment (Blau et al., 1993). This may be related to other roles of BH_4 which are not corrected with this treatment regime.

The role of BH_4 in the NOS complex has been the subject of much investigation but is still unclear. The effect of BH_4 deficiency on brain NOS activity has been given scant consideration. Kaufman (1993) stated that because of the 100 fold lower K_m for BH_4 in NOS compared to phenylalanine hydroxylase, BH_4 deficiency, as seen in the inborn errors of metabolism, would not be severe enough to impair NOS activity. When all substrates and cofactors including BH_4 are present in excess, brain cytosolic NOS specific activity is not decreased in the *hph-1* mouse, *in vitro* (Chapter 4). This is in contrast to the aromatic amino acid mono-oxygenase enzymes, phenylalanine and tyrosine hydroxylase, which show reduced specific activity and enzyme levels in the *hph-1* mouse (Hyland et al., 1996a). Furthermore, decreased specific activity of phenylalanine hydroxylase has been reported in a patient with an inborn error of BH_4 biosynthesis and decreased tyrosine hydroxylase specific activity and enzyme protein in a patient suffering from DRD (Dhondt, 1991; Rajput et al., 1994). The mechanism for the loss of the aromatic amino acid mono-oxygenase activity is not known but it is suggested that BH_4 may be required for enzyme stability or for *de novo* enzyme synthesis (Hyland et al., 1996a). BH_4 does not, therefore, appear to regulate NOS to

the same extent as for the aromatic mono-oxygenase enzymes. In the absence of BH_4, purified cerebellar NOS has been shown to exist as a stable dimer but lacking the ability to synthesise NO (Klatt et al., 1995). This stability is independent of substrate (arginine) or cofactor binding (Klatt et al., 1995). These findings may explain why, when BH_4 is added to the reaction buffer and all other substrates and cofactors are present in excess, there is no difference in enzyme activity between control and the *hph-1* mouse. Indeed there is a normal developmental profile of the NOS enzyme in the *hph-1* mouse compared to the control mouse when all substrates/cofactors are present in excess (Chapter 4). Omission of BH_4 from the reaction buffer, however, results in a significant ~20% decrease in NOS activity in the *hph-1* mouse only. This decrease in enzyme activity is apparent in both the 10 day old and the 30 day old *hph-1* mice. This data suggests that there may be less binding of BH_4 in the *hph-1* mouse *in vivo* as there was no effect on the control mice when BH_4 was omitted from the reaction buffer (Chapter 4). In the control mice maximum activity of NOS is achieved without the need for exogenous BH_4 which may be due to the high affinity of NOS for BH_4.

In both the 10 and 30 day old *hph-1* mouse there was an approximate five fold increase in K_m for arginine in the absence of exogenous BH_4 *in vitro*. This compared to a three fold increase in the K_m for arginine in the control mouse in the absence of added BH_4 *in vitro*. Recent work has demonstrated that purified cerebellar NOS exhibits a highly specific binding site for BH_4 and that the cofactor enhanced the affinity of the enzyme for the inhibitor, N^G-nitro-arginine (Klatt et al., 1994). Therefore, deficiency of BH_4 may well also lead to decreased binding of the cofactor to NOS resulting in a decreased affinity for arginine.

The results from Chapter 4 suggest that BH_4 may play a dual role in the NOS complex. Omission of BH_4 from the reaction buffer leads to lower NOS activity in the *hph-1* mouse suggesting that the pterin appears to play a cofactor role in the reaction and it also appears to be necessary for the binding of the substrate to the enzyme. Indeed, recent evidence has suggested that quinonoid dihydrobiopterin can be recycled to BH_4 by NOS and that BH_4 may be involved in electron transfer between

flavins and NADPH to the haem centre of the enzyme (Witteveen et al., 1996).

The proposed decrease in NOS activity in the *hph-1* mouse is corroborated in a reduction in the activity of the NO/cGMP pathway (Chapter 5). In the 10 day old *hph-1* mouse, there was an approximate 50% reduction in cGMP levels under both basal and kainate stimulated states compared to control mice. In adult 30 day old *hph-1* mice there was only a 30% reduction in cGMP levels under basal and stimulated conditions compared to control mice. The greater reduction in the NO/cGMP pathway observed in the 10 day old *hph-1* mouse compared to the 30 day old *hph-1* mouse may be related to the high phenylalanine levels displayed in the 10 day old *hph-1* mouse (Chapter 5). High serum phenylalanine levels have been shown to interfere with transport of amino acids across the blood brain barrier ie proline, histidine, ornithine, tyrosine and arginine (Neame, 1961; Hyland et al., 1985). In addition, high brain phenylalanine concentrations can interfere with the efflux of these amino acids from brain cells as well as the transport into cells (Hughes and Johnson, 1977). Such a mechanism may, therefore, explain why whole brain arginine levels in the *hph-1* mouse are approximately 50% of the control mouse at 10 days of age.

Arginine is predominantly stored in the astrocytes and must be transported to the neurones (Aoki et al., 1991). Competition between phenylalanine and arginine for transport into the neurone may, therefore, result in low neuronal arginine levels. In the 10 day old *hph-1* mouse the whole brain arginine concentration is approximately 50% of the control mouse value, neuronal levels of arginine may be even lower than the whole brain value. As shown in the present study, decreased levels of BH_4 result in an increase in K_m for arginine in the *hph-1* mouse *in vitro*. This increase in K_m for arginine in the absence of exogenous BH_4 may also result in arginine becoming limiting and result in less NO synthesis in the 10 day old *hph-1* mouse.

In the inborn errors of BH_4 metabolism, where the deficiency of BH_4 is typically greater than 90%, the increase in K_m for arginine may be higher. This situation may also become exaggerated in hyperphenylalaninaemic states where competition between phenylalanine and arginine may cause lower neuronal arginine concentration. It is

also possible that children suffering from hyperphenylalaninaemia, caused by phenylalanine hydroxylase deficiency, where serum phenylalanine levels are not under adequate control, may also suffer from low brain arginine concentration leading to decreased neuronal arginine levels. This in turn may lead to decreased NO production.

In the 30 day old *hph-1* mouse, where whole brain arginine levels are normal compared to control mice, there is still a significant 30% decrease in the NO/cGMP pathway when compared to control mice. At this age point, serum phenylalanine levels are not elevated and transport of amino acids into the brain should be normal. Subsequently, whole brain phenylalanine, tyrosine and arginine levels are normal with respect to the control mouse. These findings suggest that there is an impaired ability to synthesise cGMP in the *hph-1* mouse which is most likely to be related to the reduction in brain BH_4 levels. The low citrulline levels observed in whole brain of the 30 day old *hph-1* mouse may indicate reduced NO formation as NO and citrulline are formed in an equimolar ratio as a result of NOS activity (Bush et al., 1992). In the 10 day old *hph-1* mouse, citrulline levels are comparable with control mice. This may be because of a free movement of citrulline across the blood brain barrier as the latter has been reported to be more permeable in younger animals (for review see Statz and Felgenhauer, 1983).

In the 30 day old *hph-1* mouse there is no confounding high phenylalanine levels to interfere with arginine transport, but the amino acid must still be transported from the astrocyte to the NO producing neurone. Furthermore, most NO synthesizing neurones lack the enzyme necessary for the recycling of citrulline to arginine, arginosuccinate synthetase (EC 6.3.4.5) (Collard, 1995). This is especially true of the cerebellum where the NO synthesis occurs in the granule and basket cells but arginosuccinate synthetase activity appears to be located in adjacent neurons (Arnt-Ramos et al., 1992). However, most glial cells contain high amounts of arginosuccinate lyase (EC 4.3.2.1) which cleaves arginosuccinate to form arginine and fumarate (Nakamura et al., 1990). Thus, there appears to be a significant degree of compartmentalisation of the NO pathway in the cerebellum, ie storage of arginine in astrocytes, production of

NO in NOS containing neurones, conversion of citrulline to arginosuccinate in another group of neurones and finally conversion of arginosuccinate to arginine in glial cells (Figure 7.1.) (Collard, 1995). Because of the separation of the various parts of the NO/arginine pathway, there must be a mechanism of transport of arginine to the neurones from the glia cells. Depolarisation of the glial cells seems to lead to arginine release from these cells (Hansel et al., 1992). Synaptosomes prepared from rat cerebellum and cortex appeared to show a high affinity carrier for arginine (Aldridge and Collard, 1996). Cultured neurones have previously been shown to take up extracellular arginine with a K_m of approximately 100μM (Westergaard et al., 1993). Arginine concentration in the CSF has been reported to be 20 μM and it may be that uptake of arginine could well regulate NO synthesis (Westergaard et al., 1993). Control mice display a K_m for arginine of approximately 25 μmol/L in both 10 and 30 day old mice in the absence of exogenous BH_4 *in vitro*. At this level arginine concentration may be limiting for efficient NO synthesis in the brain but in the *hph-1* mouse where the K_m is approximately 40 μmol/L in 10 and 30 day old mice the situation may be exaggerated. This may result in the neuronal NOS "uncoupling" and producing superoxide and NO at the same time, resulting in $ONOO^-$ formation.

A recent suggestion for the role of BH_4 in the NOS complex is that the enzyme bound BH_4 may be required to generate superoxide and to transfer the free radical to the NO generated by the enzyme (Mayer and Werner, 1995). In this case the enzyme would be a $ONOO^-$ generating enzyme rather than a NO synthase (Mayer and Werner, 1995). Several reports have suggested that $ONOO^-$ may be released from cells and that $ONOO^-$ may be involved in the formation of S-nitrosoylated species, eg S-nitroso-glutathione, which then release NO (for review, see Mayer and Werner, 1995). Compartmentalisation of the brain arginine-citrulline-NO system may result in low neuronal arginine levels causing superoxide/hydrogen peroxide formation (Heinzel et al., 1992; Pou et al., 1992). Thus, in effect it is possible that the neuronal form of NOS may even under normal conditions generate significant amounts of $ONOO^-$and may indeed be a $ONOO^-$ generating enzyme due to limiting arginine concentration. In the *hph-1* mouse, because of the higher K_m for arginine, there may be increased formation of superoxide and/or $ONOO^-$.

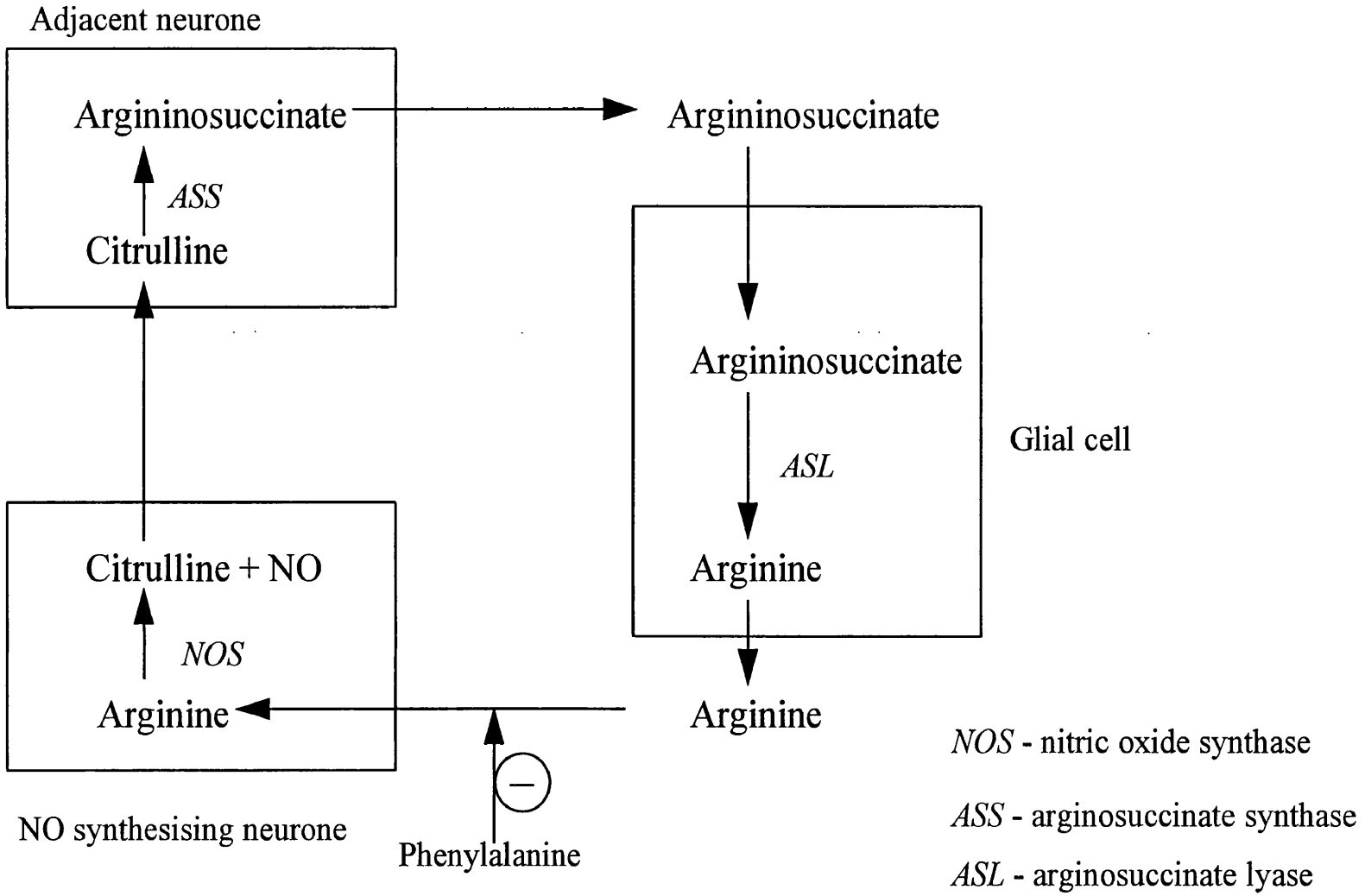


Figure 7.1. Proposed compartmentalisation of arginine-citrulline-NO system in the CNS. Adapted from Collard, 1995.

In cultured neurones, one target for excess $ONOO^-$ is the mitochondrial electron transport chain with complex II/III and IV being particularly sensitive (Bolaños et al., 1995). In whole brain homogenates and cerebellar slice homogenates, there was no difference in activity of the mitochondrial electron transport chain between control and the *hph-1* mouse (Chapter 6). This may be because any oxidising species produced may be adequately handled by the anti-oxidant systems in the *hph-1* mouse. There was no difference in GSH levels between the control and the *hph-1* mouse which may suggest that excess free radical formation in the *hph-1* mouse is not excessive or is coped with by the anti-oxidant defences. Another hypothesis for the role of BH_4 in the NOS complex has suggested that BH_4 plays a role in protecting the enzyme from auto-inhibition by NO (Griscavage et al., 1994). If this is so, decreased levels of BH_4 in the *hph-1* mouse may favour inactivation of NOS by NO and, thus, prevent excess free radical generation.

Conclusion

The field of BH_4 metabolism appeared to be reaching maturity until the discovery that NOS displayed an absolute requirement for BH_4 (Kaufman, 1993). The effect of BH_4 deficiency on the aromatic amino acid mono-oxygenase enzymes has been well documented (for review, see Blau et al., 1993) but, as stated, the effect of this cofactor deficiency on NOS does not seem to have been extensively considered. As opposed to the suggestion of Kaufman (1993) that BH_4 deficiency in the inborn errors of metabolism would have no effect on NOS, an approximate 50% reduction in whole brain concentration of BH_4 would appear to lead to alterations in the kinetic properties of NOS and an impairment of the NO/cGMP pathway. In patients with the inborn errors of BH_4 metabolism, where BH_4 levels are typically reduced by greater than 90%, this situation may be exaggerated. The findings of this study may also have implications for other neurological disorders where BH_4 metabolism may be impaired, for example in Parkinson's and Alzheimer's Diseases (Williams et al., 1980; Barford et al., 1984). Also methotrexate, a folate antagonist used in the treatment of leukaemia, has been shown to inhibit dihydropteridine reductase which in turn may lead to a loss of BH_4 (Craine et al., 1972; Hyland et al., 1989).

7.2. Further work.

The results of this thesis have raised several new questions with regard to the effect of BH_4 deficiency on brain NO metabolism.

The findings that purified brain NOS produces either superoxide or hydrogen peroxide in the absence of arginine and/or BH_4 suggests that NOS may be an important source of intracellular oxidising species (Pou et al., 1992; Heinzel et al., 1992). In order to determine if an intact cellular system would also produce these species it would be of interest to produce cerebellar slices from control and the *hph-1* mouse, as described in Chapter 2, incubate them under basal conditions and with kainate, as described in Chapter 5. After the incubation period, cerebellar slices would be homogenised and hydrogen peroxide levels determined as described by Bates et al. (1994). Hydrogen peroxide could be measured in slice preparations from 10 and 30 day old mice. If superoxide/hydrogen peroxide are produced by an "uncoupled" NOS reaction it is hypothesised that there will be an increased hydrogen peroxide concentration in slices prepared from the *hph-1* mouse. An alternative method to test the hypothesis that brain NOS may be a $ONOO^-$ producing enzyme is to measure $ONOO^-$ directly using either 2,7-dichlorodihydrofluorescein or dihydrorhodamine, both of these compounds have been shown to react with $ONOO^-$ and can be measured by fluorescence (Crow, 1996). Whole brain cytosol can be used to measure $ONOO^-$ production, after removal of endogenous arginine, in control and *hph-1* mice.

In normal animals, there is a decrease in the activity of the mitochondrial electron chain enzymes with age (Beal et al., 1993). It is possible that if superoxide or hydrogen peroxide are produced in the *hph-1* mouse by NOS, there may be no adverse effects until they add to or potentiate the effect of normal ageing, by doing so it may go over a threshold of damage which then has detrimental effects on cell metabolism. It has been suggested that acute exposure of $ONOO^-$ seriously damages neurones with astrocytes being relatively unaffected, ie mitochondrial electron transport chain damage may be a cause of neuronal death (Bolaños et al., 1995). Using one year old control and *hph-1* mice, mitochondrial electron transport chain complexes could be measured to assess if there is any evidence of mitochondrial damage.

The degradation products of NO metabolism are the relatively stable compounds, nitrite and nitrate (Hevel and Marletta, 1994). Measurement of these products from the whole brain and also urine and whole blood from both groups of mice may give further insights as to whether NO metabolism is impaired *in vivo*. It has been suggested that measurement of CSF concentration of nitrite and nitrate may be important in the clinical investigation of neurological disorders (Clelland et al., 1996). Recently, a significant positive correlation between nitrite and nitrate concentration and BH_4 levels has been demonstrated (Heales et al., 1996). In the inborn errors of BH_4 metabolism, the determination of CSF nitrite and nitrate levels may also be useful to assess if there is a significant disturbance of brain NO metabolism.

In the *hph-1* mouse there is normal NOS activity in the presence of exogenous BH_4 suggesting that there are normal levels of NOS protein (Chapter 4). Klatt et al. (1995) have suggested that NOS can exist as stable dimer and long term stability of the NOS protein is independent of cofactor binding. This is the opposite of the aromatic amino acid mono-oxygenase enzymes where BH_4 appears to be necessary for the stability of the protein (Hyland et al., 1996a). Using the *hph-1* mouse it has been previously demonstrated that BH_4 replacement therapy using 100 μmol/kg corrects the BH_4 deficiency for up to four hours (Brand et al., 1996). Measurement of NOS activity and amino acid levels in whole brain and cGMP synthesis in cerebellar slices prepared from mice given the cofactor replacement could be carried after BH_4 administration. Further studies involving chronic administration of BH_4 may be considered if there is no apparent stimulation of NO metabolism.

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Appendix.

Publications.

J. Inher. Metab. Dis. 18 (1995) 33–39

Tetrahydrobiopterin deficiency and brain nitric oxide synthase in the *hph1* mouse

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MS received 29.9.94 Accepted 28.11.94

Summary: Tetrahydrobiopterin (BH_4) is the cofactor for the aromatic amino acid monoxygenase group of enzymes and for all known isoforms of nitric oxide synthase (NOS). Inborn errors of BH_4 metabolism lead to hyperphenylalaninaemia and impaired catecholamine and serotonin turnover. The effects of BH_4 deficiency on brain nitric oxide (NO) metabolism are not known. In this study we have used the *hph-1* mouse, which displays GTP cyclohydrolase deficiency, to study the effects of BH_4 deficiency on brain NOS. In the presence of exogenous BH_4, NOS specific activity was virtually identical in the control and *hph-1* preparations. However, omission of BH_4 from the reaction buffer led to a significant 20% loss of activity in the *hph-1* preparations only. The K_m for arginine was virtually identical for the control and *hph-1* NOS when BH_4 was present in the reaction buffer. In the absence of cofactor, the K_m for arginine was 3-fold greater for control and 5-fold greater for *hph-1* preparations. It is concluded that (a) BH_4 does not regulate the intracellular concentration of brain NOS; (b) less binding of BH_4 to NOS occurs in BH_4 deficiency states; (c) BH_4 has a potent effect on the affinity of NOS for arginine; and (d) the availability of arginine for NOS activity may become severely limiting in BH_4 deficiency states. Since, in the presence of suboptimal concentrations of BH_4 or arginine, NOS may additionally form oxygen free-radicals, it is postulated that in severe BH_4 deficiency states NO formation is impaired and the central nervous system is subjected to increased oxidative stress.

Tetrahydrobiopterin (BH_4) is an established cofactor for the aromatic amino acid monoxygenase group of enzymes. Consequently, BH_4 is required for the intracellular hydroxylation of phenylalanine to tyrosine, tyrosine to dihydroxyphenylalanine (L-DOPA) and tryptophan to 5-hydroxytryptophan (5-HTP) (Leeming et al 1981).

Intracellular concentrations of BH_4 are maintained by both biosynthetic and salvage pathways. BH_4 is synthesized from guanosine triphosphate (GTP) via four enzymatic

steps (Blau et al 1993). The activity of this pathway is regulated by BH_4, which is a potent feedback inhibitor of GTP cyclohydrolase (EC 3.5.4.16), the enzyme catalysing the first committed step of BH_4 synthesis (Blau and Niederwieser 1986). During the hydroxylation of amino acid substrates, BH_4 is oxidized to quinonoid dihydrobiopterin (qBH_2), which is devoid of cofactor activity. However, qBH_2 is reduced back to the active cofactor by the enzyme dihydropteridine reductase (EC 1.6.99.10) (Craine et al 1972).

Inborn errors of BH_4 metabolism, arising from impaired BH_4 biosynthetic (McKusick 261640 and 233910) or salvage capacity (McKusick 262630), result in hyperphenylalaninaemia and impaired catecholamine and serotonin synthesis (Leeming et al 1981). Consequently, if untreated, such patients remain severely mentally retarded (Smith 1990). The current treatment regime comprises administration of L-DOPA and 5-HTP, with a peripheral decarboxylase inhibitor, and dietary restriction of phenylalanine or administration of low doses of BH_4 to control hyperphenylalaninaemia (Smith 1990; Blau et al 1993). Although such therapy is beneficial, it appears incomplete, since in a significant number of patients neurological impairment is apparent despite early intervention (Blau et al 1993). The reason for this failure of treatment is not known but may be related to other cofactor roles of BH_4, the CNS deficiency of which is not adequately corrected.

Recently, BH_4 has been shown to be a cofactor for the nitric oxide synthase (NOS) family of enzymes (EC 1.14.13.39), which catalyse the conversion of arginine to citrulline and nitric oxide (NO) (Tayeh and Marletta 1989; Giovanelli et al 1991). Three isoforms of NOS have been documented: a calcium-independent form (iNOS), a calcium-dependent vascular endothelial constitutive form (eNOS), and a calcium-dependent neuronal constitutive form (nNOS) (Knowles and Moncada 1994). BH_4 is tightly bound to NOS, but the exact function of BH_4 in this enzyme is not known (Klatt et al 1994). Giovanelli et al (1991) have suggested that BH_4 is an allosteric modifier to maintain NOS in an active form or acts to provide reducing equivalents for a functional group in the active site. More specifically, Hevel and Marletta (1992) propose that BH_4 is necessary to facilitate electron transfer from NADPH to the haem centre of the active site of NOS.

Recently, a mouse model of BH_4 deficiency has been developed using the sperm mutagen, *N*-ethyl-*N'*-nitrosourea. The *hph-1* mouse displays a 90% deficiency of GTP cyclohydrolase when compared to wild type (C57BL×CBA) mice (McDonald et al 1988). As a consequence, the *hph-1* mouse has BH_4 deficiency, impaired phenylalanine tolerance and decreased catecholamine and serotonin turnover (Hyland and Bola 1989). The *hph-1* mouse is therefore biochemically similar to children with inborn errors of BH_4 biosynthesis and provides a useful model for studying the effects of chronic BH_4 deficiency.

Under normal conditions, the predominant form of NOS in the brain is the calcium-dependent constitutive isoform, nNOS (Knowles and Moncada 1994). NO has a number of proposed functions within the central nervous system, the best documented being activation of guanylate cyclase following stimulation of the *N*-methyl-D-aspartate receptor by glutamate (Bredt and Snyder 1989). In view of this, an impaired ability to synthesize NO is likely to have important neurological consequences.

In this study we have used the *hph-1* mouse as a model for the inborn errors of BH_4 biosynthesis to study the effects of chronic BH_4 deficiency on brain nNOS.

METHODS

Materials: BH_4 (dihydrochloride salt) was obtained from Dr B. Schircks (Jona, Switzerland). [^{3}H]Arginine was supplied by Amersham (Amersham, UK). All other chemicals and reagents were of Analar grade and were obtained from Sigma (Poole, UK). Control (C57BI×CBA) and *hph-1* mice 30 days of age were used throughout and were obtained from established breeding colonies. Animals were killed by cervical dislocation.

Analysis of BH_4: After sacrifice, the brain was removed and homogenized (25% w/v) in 0.1 mol/L perchloric acid containing 6.5 mmol/L dithioerythreitol, and 2.5 mmol/L diethylenetriaminepentaacetic acid. Following centrifugation (15 000*g* for 2 min) BH_4 was measured in the supernatant, derived from control and *hph-1* mice, by HPLC and electrochemical detection (Howells et al 1986).

nNOS activity: Activity was determined in the brain cytosolic fraction prepared from control and *hph-1* mice. The brain was homogenized (25% w/v) in isolation buffer (320 mmol/L sucrose, 10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, pH 7.4) and centrifuged at 100 000*g* for 60 min at 4°C. The resulting supernatant was used for the determination of nNOS activity.

Measurement of nNOS activity was as described by Bolaños et al (1994) and is based on quantification of the rate of [^{3}H]citrulline formation from [^{3}H]arginine. In brief, following removal of endogenous arginine by cation exchange, 100 μl of cytosol was added to 100 μl of reaction buffer containing (final concentration) 100 mmol/L Hepes, pH 7.5, 100 μmol/L NADPH, 1 mmol/L calcium chloride, 1 mmol/L magnesium acetate, 10 μg/ml calmodulin, 50 μmol/L BH_4, 1 mmol/L dithiothreitol, 5 μmol/L FAD, 5 μmol/L FMN, 100 μmol/L arginine and 1 μCi/ml [^{3}H]arginine). After 15 min the reaction was terminated by the addition of 50 μl trichloroacetic acid (1.5 mol/L) followed by 1 ml of Hepes buffer, pH 6.0. Arginine was then removed from the reaction mixture by ion-exchange chromatography and the amount of labelled citrulline present was determined by liquid scintillation counting. The assay was validated by demonstrating linearity with protein and time up to 15 min, absolute dependence on calcium, and demonstration of sensitivity towards the NOS inhibitors *N*-methylarginine and *N*-nitroarginine. Activity of nNOS was also determined in the absence of exogenous 50 μmol/L BH_4. The affinity constant (K_m) for arginine was determined, in the presence and absence of exogenous BH_4, by varying the final concentration of arginine (1 – 50 μmol/L) in the reaction buffer. The K_m was determined by the Hanes plot (s/v vs s) (Hendersen 1992). Protein concentration was determined by the method of Lowry et al (1951).

Statistics: Statistical significance for the comparison of two groups was by a Student's unpaired two-tailed *t*-test. Multiple comparisons were made by one-way

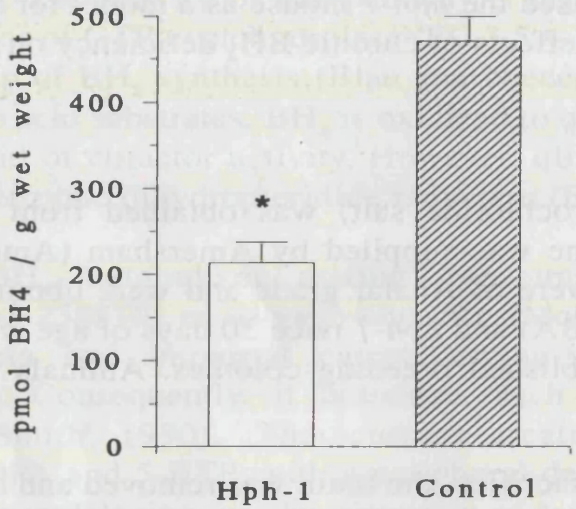

Figure 1 Brain BH_4 concentration in control and *hph-1* mice. $*p<0.005$; $n=6$ in both cases

Table 1 Whole-brain nNOS activity (pmol/min per mg protein) in control and *hph-1* mice in the presence and absence of BH_4 (50 μmol/L)

	Control	*hph-1*
$+BH_4$ (9)[a]	155 ± 28	151 ± 11
$-BH_4$ (6)[a]	144 ± 22	121 ± 9*

$*p<0.05$ when compared to control mice ± BH_4 and *hph-1* mice in the presence of BH_4
[a]Number in parentheses is sample size

analysis of variance followed by the least significant difference multiple range test. All results are expressed as the mean ± standard deviation.

RESULTS

The brain concentration of BH_4 was decreased by approximately 50% in the *hph-1* mice when compared to the control group (Figure 1).

In the presence of exogenous BH_4, nNOS activity was virtually identical in the control and *hph-1* mice (Table 1). For control preparations, omission of BH_4 from the reaction buffer resulted in minimal loss (7%) of activity (Table 1). However, a significant loss (20%) occurred in the *hph-1* preparations (Table 1).

The K_m for arginine, for both control and *hph-1* mice, was determined in the presence and absence of exogenous BH_4. For both groups, the K_m was virtually identical when BH_4 was present in the reaction buffer (Table 2). Omission of exogenous BH_4 resulted in a highly significant ($p<0.01$) increase in K_m of approximately 3-fold for control and

Table 2 K_m values (μmol/L) for arginine for whole-brain nNOS in control and *hph-1* mice and the effects of exogenous BH_4 (50 μmol/L). K_m was determined from Hauer plots (*s/v* vs *s*)

	Control	*hph-1*
$+BH_4$ (3)[c]	10.4 ± 1.7	9.5 ± 2.6
$-BH_4$ (5)[c]	31.3 ± 5.3[a]	53.3 ± 13.5[a,b]

[a]Significantly different from corresponding value in presence of BH_4
[b]Significantly different from control mice ± BH_4
[c]Number in parentheses is sample size
$p < 0.01$ in all cases

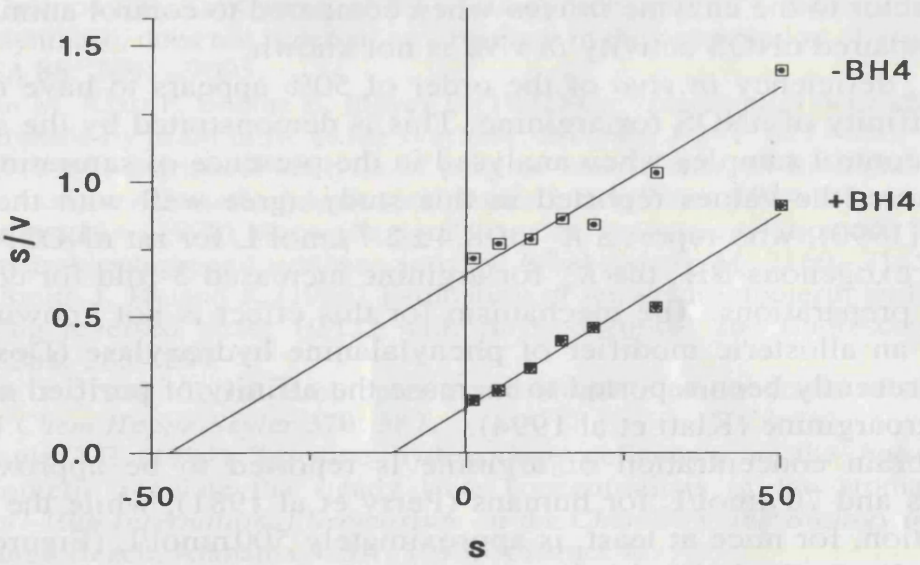

Figure 2 Representative Hanes plot illustrating the effect of BH_4 on the kinetic properties of *hph-1* nNOS: *s* is the substrate concentration (μmol/L) and *v* is the initial velocity of the reaction (pmol/min)

5-fold for *hph-1* preparations (Table 2). Representative Hanes plots illustrating this effect of BH_4 in the *hph-1* mouse are shown in Figure 2.

Determination of the K_m of nNOS for BH_4 was not possible because of the tight binding of the cofactor to the enzyme (Klatt et al 1994), and hence the difficulty in obtaining activities over a range of known cofactor concentrations.

DISCUSSION

Our observation that the *hph-1* mouse displays a 50% reduction in brain BH_4 concentration when compared to wild-type mice is in agreement with the results of Hyland and Bola (1989). These animals are therefore a useful model for the inborn errors of BH_4 biosynthesis and for studying the biochemical consequences of chronic BH_4 deficiency.

Hyland and Engle (1993), in a preliminary communication, report decreased specific activity of tyrosine hydroxylase (EC 1.14.16.2) in the brain of the *hph-1* mouse.

Furthermore, reduced activity of phenylalanine hydroxylase (EC 1.14.16.1) has been reported in the liver of these animals and in a patient with an inborn error of BH_4 biosynthesis (McDonald and Bode 1988; Dhondt 1991). Whilst the mechanism for these observations is not known, it is possible that BH_4 either may control the *de novo* synthesis of these enzymes or may be required for their stability. In contrast to the aromatic amino acid monoxygenases, nNOS specific activity is not decreased in the *hph-1* mouse, i.e. when all substrates and cofactors are present in excess. This finding suggests that BH_4 does not regulate the intracellular concentration of nNOS to the same extent as for tyrosine and phenylalanine hydroxylase.

Omission of BH_4 from the reaction buffer had minimal effect on nNOS activity in the control mice. This observation is in agreement with those of Klatt et al (1994), who report that BH_4 is so tightly bound to nNOS that it remains associated with the enzyme even after purification. The observed loss of nNOS activity in the *hph-1* preparations when BH_4 is excluded from the reaction buffer may therefore suggest that there is less binding of cofactor to the enzyme *in vivo* when compared to control animals. Whether this leads to impaired nNOS activity *in vivo* is not known.

Chronic BH_4 deficiency *in vivo* of the order of 50% appears to have no long-term effect on the affinity of nNOS for arginine. This is demonstrated by the similar K_m in the *hph-1* and control samples when analysed in the presence of saturating exogenous BH_4. Furthermore, the values reported in this study agree well with the findings of Knowles et al (1990), who report a K_m of $8.4 \pm 2.7\ \mu$mol/L for rat nNOS. However, in the absence of exogenous BH_4 the K_m for arginine increased 3-fold for control and 5-fold for *hph-1* preparations. The mechanism for this effect is not known, but BH_4 is reported to be an allosteric modifier of phenylalanine hydroxylase (Doskeland et al 1987) and has recently been reported to increase the affinity of purified nNOS for the inhibitor, *N*-nitroarginine (Klatt et al 1994).

The whole-brain concentration of arginine is reported to be approximately 100 μmol/L for rats and 70 μmol/L for humans (Perry et al 1981), while the whole-brain BH_4 concentration, for mice at least, is approximately 500 nmol/L (Figure 1). In view of this and the observed relationship between BH_4 availability and the affinity of nNOS for arginine (Table 2), it is possible that even under normal conditions arginine may be a limiting factor for NO formation and that this situation is made worse in the inherited disorders of BH_4 biosynthesis where deficiency of cofactor may be greater than 90% (Blau et al 1993).

In the presence of suboptimal concentrations of arginine or BH_4, nNOS may form reactive oxygen species in addition to NO (Heinzel et al 1992). Whether this phenomenon occurs in the central nervous system in patients with inborn errors of BH_4 metabolism remains to be demonstrated. However, it is possible that part of the reason for the failure of current treatment regimes for these conditions is a failure not only to correct impaired NO formation but also to prevent increased oxidative stress. In view of this, the use of chronic cofactor replacement therapy should be re-evaluated together with antioxidant and arginine administration.

ACKNOWLEDGEMENTS

The authors are grateful to the Worshipful Company of Pewterers and the Brain Research Trust for funding to carry out this research.

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J. Inher. Metab. Dis. 18 (1995) 40–47

An HPLC assay for detection of elevated urinary *S*-sulphocysteine, a metabolic marker of sulphite oxidase deficiency

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MS received 1.8.94 Accepted 22.9.94

Summary: Sulphite oxidase deficiency occurs in man in two forms, as the isolated deficiency and as a syndrome of combined molybdoenzyme deficiency. This latter pleiotropic condition has as its underlying cause a defect in the synthesis of the molybdenum cofactor required for the activity of all molybdoenzymes in humans. Difficulties in diagnosis of sulphite oxidase deficiency are often encountered. A new method for detection of a key diagnostic metabolite, *S*-sulphocysteine, is outlined. The procedure is based on precolumn derivatization of urinary amino acids with dimethylaminoazobenzene sulphonyl chloride (Dabsyl-Cl) and resolution of the modified *S*-sulphocysteine by reversed-phase HPLC. A number of affected patients and control individuals with similar clinical symptoms have been studied, and a clear demarcation between the two groups has been noted.

Isolated sulphite oxidase deficiency (McKusick 272300) is an inborn error of metabolism now characterized in at least 13 patients (Johnson and Wadman 1989). A more common variant of the deficiency disease (Johnson and Wadman 1989) is an inherited deficiency of the molybdenum cofactor required for the function of this and several other molybdoenzymes (Rajagopalan and Johnson 1992). At least 54 patients have been identified with molybdenum cofactor deficiency (McKusick 252150) since the first case was described (Duran et al 1978; Johnson et al 1980). Molybdenum cofactor-deficient patients exhibit a lack of xanthine dehydrogenase (EC 1.2.1.37) and aldehyde oxidase (EC 1.2.3.1), as well as sulphite oxidase (EC 1.8.2.1). Both the isolated and the cofactor-based forms of sulphite oxidase deficiency are characterized by an array of characteristic but non-specific clinical symptoms that include seizures and other neurological abnormalities, mental retardation with failure to achieve developmental milestones, and dislocated ocular lenses. Both classes of patients can show considerable variation in the age of onset and severity of symptoms, making detection and diagnosis difficult in some instances.

A deficiency of sulphite oxidase results in altered levels of certain sulphur

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BRAIN RESEARCH

Brain Research 735 (1996) 169–172

Short communication

Impairment of the nitric oxide/cyclic GMP pathway in cerebellar slices prepared from the *hph-1* mouse

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Accepted 16 July 1996

Abstract

In this study, the effect of tetrahydrobiopterin deficiency on the nitric oxide/cGMP pathway has been investigated in cerebellar slices derived from the *hph-1* mouse. This animal displays a partial deficiency of tetrahydrobiopterin. Basal levels of cGMP were significantly reduced (−29.5%) in the *hph-1* mouse cerebellum compared to controls. Following kainate stimulation (500 μM) cGMP levels increased in both control and *hph-1* preparations but were again significantly lower (−29.1%) in the *hph-1* mouse. Exposure of slices to the nitric oxide donors, *S*-nitroso-*N*-acetylpenicillamine and *S*-nitroso-glutathione, revealed no difference in cGMP accumulation between the two groups. These findings suggest that the cerebellar nitric oxide/cGMP pathway may be impaired in partial tetrahydrobiopterin deficiency states due to diminished nitric oxide formation.

Keywords: cGMP; *hph-1* mouse; Kainate; Nitric oxide; Tetrahydrobiopterin

Tetrahydrobiopterin (BH_4) is an established cofactor for the aromatic amino acid mono-oxygenase group of enzymes. Consequently, it is required for the hepatic hydroxylation of phenylalanine to tyrosine and within the CNS, conversion of tyrosine to dihydroxyphenylalanine (L-DOPA) and tryptophan to 5-hydroxytryptophan (5-HTP) [14]. BH_4 is also an essential cofactor for all known isoforms of nitric oxide synthase (NOS) (EC 1.14.13.39) which catalyses the conversion of arginine to citrulline resulting in the liberation of nitric oxide (NO) [20,25,26]. BH_4 is tightly bound to NOS but the exact mechanism whereby it participates in the production of NO is unclear. However, we have recently provided evidence to suggest that BH_4 modifies the ability of neuronal NOS to bind it's substrate, arginine [2].

BH_4 is synthesised from GTP by at least four enzymatic steps and intracellular levels are maintained by both synthetic and salvage pathways [1]. Inborn errors of BH_4 metabolism lead to cofactor deficiency, hyperphenylalaninaemia and neurological impairment due to reduced catecholamine and indoleamine turnover [16]. Currently, treatment of disorders of BH_4 metabolism consist of administration of L-DOPA and 5-HTP with a peripheral decarboxylase inhibitor and either dietary restriction of phenylalanine or administration of BH_4 (4–8 μmol/kg per day) to control hyperphenylalaninaemia [1,22]. Such treatment is beneficial but appears incomplete since in a significant number of patients neurological impairment is still apparent [1]. The reason for this failure of treatment is unknown but may be related to the cofactor role of BH_4 in NOS, the deficiency of which is not adequately corrected for under current treatment regimes [1].

Recently a mouse model of BH_4 deficiency, *hph-1*, has been developed [18]. This mouse displays 90% deficiency in GTP cyclohydrolase activity (EC 3.5.4.16), the enzyme catalysing the first committed step in BH_4 synthesis [19]. This results in a deficiency in tissue BH_4 concentrations when compared to wild type control mice, hyperphenylalaninaemia until weaning (at 21 days) and persistent impairment of monoamine turnover [11]. The *hph-1* mouse therefore provides a good biochemical model for the inborn errors of BH_4 metabolism and also for hereditary DOPA-responsive dystonia, a disorder caused in some instances by deficiency of GTP cyclohydrolase [13].

A well documented action of NO within the CNS is to stimulate guanylate cyclase and increase cGMP synthesis

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following glutamate receptor activation [6]. In cerebellar slices from young rats Garthwaite et al. [7] have shown that the glutamate analogue, kainate, induced a large accumulation of cGMP. This response is calcium dependent and is also inhibited by NOS inhibitors [7]. Stimulation of kainate receptors, therefore, leads to activation of NOS and subsequent stimulation of guanylate cyclase in cerebellar slices.

In this study we have compared cGMP accumulation following kainate activation in cerebellar slices from control and *hph-1* mice to determine whether a partial BH_4 deficiency leads to impairment of the NO/cGMP pathway.

Control mice (CBA/C57BL) and *hph-1* mice at 30 days of age were used in these experiments and were killed by cervical dislocation. All of the experiments were carried out with permission from the Secretary of State under appropriate project and personal licences issued by the Home Office under the Animals (Scientific Procedures) Act 1986.

BH_4 was obtained from Schircks (Jona, Switzerland), dithioerythritol DTE) was from Aldrich (Gillingham, UK), Kainate, *N*-nitro-arginine (NNA), oxyhaemoglobin (HbO_2) and 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX) were obtained from Sigma (Poole, UK), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitroso-glutathione (SNOG) were from Alexis (Nottingham, UK). All other chemicals were of highest possible grade from BDH (Leicester, UK). cGMP radioimmunoassay kits were obtained from Amersham (Amersham, UK).

Measurement of cGMP was carried out using cerebellar slices. The cerebellum was excised and the hemispheres removed. Cerebellar slices were then prepared from the vermis as described by Southam et al. [23] and were sliced at 400-μm intervals. Slices were pre-incubated for 90 min in oxygenated Krebs-Henseleit buffer at 37°C and the buffer was changed after 30 min of this pre-incubation. Slices were redistributed into 10 ml conical flasks so that there were four slices per flask. The glutamate analogue, kainate (10–1000 μM), was used to stimulate cGMP production over a five min period, in addition the effect of pre-treatment with the kainate antagonist, CNQX (500 μM) was investigated [9]. These experiments were repeated in the presence of the NOS inhibitor, NNA at a concentration of 500 μM. The effects of the NO donors, SNAP and SNOG, 500 μM, were also investigated. In order to confirm that any cGMP formation was due to NO liberation some incubations were also performed in the presence of HbO_2 (25 μM). After the incubation period (5 min), slices were inactivated by boiling in 250 mM Tris/4 mM EDTA, pH 7.6, for 3 min. Inactivated slices were homogenised using a Soniprep 150 Sonicator (Fisons Scientific Equipment, Loughborough, UK). Levels of cGMP were measured by radioimmunoassay (Amersham).

BH_4 levels were measured in cerebella from both groups of mice. Cerebella were homogenised (25% w/v) in 0.1 M perchloric acid containing 6.5 mM DTE and 2.5 mM diethyltriaminepentaacetic acid. Samples were centrifuged at $15000 \times g$ for 2 min and 50 μl of supernatant was injected onto the HPLC system. Analysis of BH_4 was by HPLC with electrochemical detection [10].

For amino acid analysis, whole brain was homogenised in 0.1 M PCA and spun at $14000 \times g$ for 2 min. Supernatant was mixed with an equal volume of 10% sulphosalicylate, containing 200 μM norleucine as internal standard, and spun at $4000 \times g$ for 10 min. Supernatant was then passed through a 0.22 μm membrane filter. Analysis of arginine, citrulline, tyrosine and phenylalanine was carried out using automated ion exchange chromatography on a Biochrom 20 amino acid analyser (Pharmacia Biotech, Cambridge, UK). Injection volume of supernatant was 70 μl onto the column and elution was carried out using lithium elution buffers with a standard physiological fluid elution programme.

Protein concentration was determined by the method of Lowry et al. [17].

Statistical significance for the comparison of two groups was by Student's unpaired *t*-test. All results are expressed as the mean ± S.E.M. with the number of determinations represented by *n*.

The cerebellar concentration of BH_4 was, as expected, significantly lower in the *hph-1* mouse compared to control mice (control; 219 ± 8.9. *hph-1*; 150.0 ± 6.1 pmol/g wet weight, $n = 4$ in both cases, $P < 0.01$).

Analysis of cGMP revealed that under basal conditions, cerebellar slices prepared from the *hph-1* mouse had 70% of cGMP levels seen in control cerebellar slices (Table 1). Following kainate stimulation, cGMP accumulation was dose-dependent and reached a plateau at 500 μM kainate for both control and *hph-1* mice cerebellar slices (data not shown). Furthermore, this cGMP accumulation could be totally prevented by the kainate antagonist, CNQX. With 500 μM kainate both groups showed a two fold increase in cGMP concentration, however, levels in the *hph-1* mouse cerebellar slices were approximately 30% lower than in the control group (Table 1). Activation of slices with 500 μM kainate in the presence of 500 μM NNA, decreased cGMP concentration to below basal levels in both *hph-1* and control slice preparations (Table 1). When 500 μM SNAP or SNOG were used to stimulate guanylate

Table 1
cGMP levels in cerebellar slices

Treatment	pmol cGMP/mg protein	
	control	*hph-1*
Basal	11.2 ± 1.1 (9)	7.9 ± 0.8 (9) *
500 μM kainate	20.6 ± 2.2 (10)	14.6 ± 2.2 (9) *
500 μM kainate + 500 μM NNA	1.4 ± 0.2 (3)	0.9 ± 0.3 (3)
500 μM SNAP	46.9 ± 1.5 (4)	51.5 ± 8.9 (4)
500 μM SNOG	44.8 ± 1.6 (3)	43.3 ± 14.3 (5)

Data shown as mean ± S.E.M. Figures in parentheses equal sample size.
* $P < 0.05$ when compared to control mouse group.

Table 2
Whole brain amino acid concentration

	Amino acid concentration, nmol/g wet weight	
	control	*hph-1*
Phenylalanine	58.0 ± 15.1	63.5 ± 8.1
Tyrosine	65.3 ± 13.1	56.0 ± 10.7
Arginine	110.5 ± 33.3	95.5 ± 20.4
Citrulline	37.0 ± 10.8	21.0 ± 5.1 *

Data shown as mean ± S.E.M., $n = 8$.
* $P < 0.05$ when compared to control group.

cyclase there was no significant difference in cGMP accumulation between control and *hph-1* slices (Table 1). This response was attributed to NO release by these compounds since HbO_2, a potent scavenger of NO [21], inhibited cGMP formation by up to 95%. HbO_2 alone had no effect on basal levels of cGMP formation.

In order to determine further if BH_4 deficiency impairs brain NOS activity, whole brain concentrations of arginine and citrulline was determined in control and the *hph-1* mouse. Citrulline production in brain can only occur via NOS since the urea cycle is incomplete in the brain [8]. For comparison, phenylalanine and tyrosine levels were also determined. Citrulline concentration was significantly decreased in the *hph-1* mouse whereas arginine, phenylalanine and tyrosine concentrations were comparable between the two groups (Table 2).

The *hph-1* mouse is a good model for examining the effects of a partial deficiency of BH_4 on brain metabolism. Previous work by our group and others have shown that brain monoamine metabolism is impaired in the *hph-1* mouse [3,11]. Kaufman [15] has previously suggested that in view of the fact that NOS has a high affinity for BH_4, a partial defect of BH_4 may have little effect on NOS activity. However, we have previously provided evidence using the *hph-1* mouse to suggest decreased binding of BH_4 to brain NOS in vivo [2]. Furthermore, we also demonstrated that BH_4 has a potent effect on the affinity of brain NOS for arginine [2].

Using slices prepared from the cerebellum, the area of the brain with the greatest concentration of NOS activity [4], we have shown that under basal conditions, levels of cGMP were significantly lower in the *hph-1* mouse when compared to controls. Furthermore, following kainate exposure, the concentration of cGMP attained was again lower in slices prepared from the *hph-1* mouse. Formation of cGMP is clearly NO dependent as the NOS inhibitor, NNA, decreased basal cGMP levels and prevented formation of cGMP following kainate exposure. The decreased capacity of the *hph-1* mouse cerebellar slices to form cGMP is unlikely to be related to an impairment of guanylate cyclase since in the presence of the NO donors, SNAP or SNOG, comparable levels of cGMP were obtained in cerebellar slices from both groups of mice.

These findings therefore suggest an impaired ability to synthesize NO in *hph-1* cerebellar slices. In view of the metabolic defect in the *hph-1* mouse and our previous observations [2], it is likely that this is as a result of partial BH_4 deficiency leading to impaired NOS activity in the cerebellum. Further support for this suggestion comes from the additional finding of this study that whole brain citrulline, but not arginine, concentration is significantly reduced in the *hph-1* mouse. NO and citrulline are produced in equimolar quantities from arginine by NOS [5].

The physiological effects of cGMP are not well understood, although Southam and Garthwaite [24] have suggested that cGMP may act directly on ion channels or on phosphodiesterases to increase/decrease cAMP levels. Therefore, partial reduction in the cerebellar BH_4 concentration may as a result of impairment of the NO/cGMP pathway have important neurochemical effects.

In addition, the results presented here may have implications for our understanding of the pathogenesis of neurological disorders associated with BH_4 deficiency, ie the inborn errors of BH_4 metabolism, hereditary DOPA-responsive dystonia [13] and possibly Parkinson's Disease [27]. In contrast to the aromatic amino acid mono oxygenase group of enzymes, BH_4 deficiency states do not appear to be accompanied by a loss of NOS protein [2,12]. Consequently, cofactor replacement therapy to correct the impaired NO/cGMP pathway should be considered. Recently, we have provided evidence demonstrating that acute peripheral administration of BH_4 to the *hph-1* mouse can return brain cofactor concentration to normal [3].

Acknowledgements

M.P.B. was funded by the Brain Research Trust. The authors would like to thank Miss Wendy Rooke for her technical assistance.

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Neurochemical Research, Vol. 21, No. 8, 1996, pp. 923–928

Nitric Oxide and Antioxidant Status in Glucose and Oxygen Deprived Neonatal and Adult Rat Brain Synaptosomes

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(Accepted May 7, 1996)

Nitric oxide ($NO^{\cdot}$) has been implicated in the process of cerebral ischemia/reperfusion injury. We have examined the production of $NO^{\cdot}$, as reflected by nitrite (NO_2^-) + nitrate (NO_3^-) accumulation, from synaptosomes isolated from neonatal or adult rat brain and subjected to a period of glucose and oxygen deprivation. There was a significant increase in the amount of $NO_2^- + NO_3^-$ production from adult synaptosomes under these conditions, whereas there was no difference compared to control in the production of $NO_2^- + NO_3^-$ from the neonatal synaptosomes. The total antioxidant status of the synaptosomes at these different stages of brain development was found to be the same. These data suggest that the vulnerability of the adult brain to ischemia/reperfusion injury may be associated with the production of $NO^{\cdot}$ from nerve terminals. The ratios of antioxidant capacity to $NO^{\cdot}$ production under such conditions have been shown here to be different between the neonatal and adult nerve terminals. Thus the well documented resistance of neonatal brain to ischemia/reperfusion injury may involve the neonatal nerve terminal being under less oxidative stress than the adult.

KEY WORDS: Nitric oxide ($NO^{\cdot}$), brain, synaptosomes, ischemia/reperfusion, neonatal, antioxidant.

INTRODUCTION

The adult mammalian brain is known to be vulnerable to damage during episodes of ischemia and reperfusion such as those experienced during a stroke or following cardiac arrest. It is well documented that the neonatal brain is more tolerant to such periods of ischemia/reperfusion (1), however the basis of this resistance is not fully understood. The biochemical mechanisms of neuronal injury that occur during an ischemic period and subsequent reperfusion are incompletely defined both in the neonate and adult.

Ischemia and subsequent reperfusion favour the increased formation of free radicals such as superoxide ($O_2^{\cdot -}$) from sites including the mitochondrial respiratory chain (2), and the enzymatic action of xanthine oxidase (3). The conversion of superoxide by the enzyme superoxide dismutase produces hydrogen peroxide which is able to diffuse across cell membranes and in the presence of iron forms the highly reactive hydroxyl radical ($OH^{\cdot}$). Another free radical, nitric oxide ($NO^{\cdot}$), has recently received much attention in studies of normal and postischemic events (2,4). $NO^{\cdot}$ has been postulated to be a prime mediator (2,4) of neuronal injury, possibly as a result of peroxynitrite ($ONOO^-$) formation, particularly following stroke (4). However the precise mechanism of that injury is not known.

In view of the known relative resistance of the immature brain to ischemia/reperfusion, we decided to investigate the response of nerve terminals (synaptosomes) from immature and adult rat brain to a period of glucose

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0364-3190/96/0800-0923$09.50/0

and oxygen deprivation (akin to ischemia) followed by reintroduction of oxygen (reperfusion). Previous work using these preparations has shown that as a result of lack of oxygen and glucose adult synaptosomes demonstrate a significant increase in cytosolic calcium concentration which did not occur in the synaptosomes prepared from immature brain (5). Because the activity of the neuronal form of nitric oxide synthase (nNOS) is known to be calcium-calmodulin dependent (6), we have focused on the $NO^{\cdot}$ production from synaptosomes prepared from both neonatal and adult rats, and their inherent ability to cope with free radical production, i.e. their total antioxidant capacity.

EXPERIMENTAL PROCEDURE

Synaptosome Preparation. 10 day old Wistar rat pups, and adult male Wistar rats age 60 days (B and K Universal, Aldborough, England) were used for this study. Brains were rapidly removed and placed in isolation medium (320 mM sucrose, 1 mM K^+EDTA, 10 mM Tris-HCl, pH 7.4 at 4°C) within 30–60 seconds. Purified synaptosomes were prepared from 4 adult rats per experiment by the method of Booth and Clark (7), or from 8–12 rat pups using essentially the same method but with a slight modification of the density gradient. (5) In brief, 1 volume of scissor-chopped forebrains were homogenised in nine volumes of isolation medium at 4°C. The homogenate was centrifuged at 1,500 g_{av}, for 3 min using a Beckman JA-20 rotor at 4°C, and the resultant supernatant was centrifuged at 17,400 g_{av} for 10 min. Purified synaptosomes were produced by density gradient centrifugation using Ficoll, as in the original method for adults (7), and by a slight modification of the gradient for younger age pups (5). It is of utmost importance that any functional differences observed between synaptosomes from adults and neonates are not due to inherent differences in the preparations. Therefore the density gradient was altered to 10%, 6%, 4% Ficoll for all pups younger than 21 days. This gave a comparable integrity and purity to the adult preparation (<8% free mitochondria and <20% total contamination for all preparations) as determined by marker enzymes detailed in the original method (7). All synaptosomes were pelleted (14,000 g_{av}, 10 min) in a modified Kreb's phosphate buffer (KPB) (141 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 10 mM Na_2HPO_4, 10 mM glucose, 1.2 mM $CaCl_2$, pH 7.4) which had been equilibrated with air (maximum oxygen concentration in KPB = 195 μM O_2 at 37°C).

Incubation Conditions for Glucose and Oxygen Deprivation. Synaptosomes were deprived of oxygen and glucose by resuspension in a modified Kreb's phosphate buffer which did not contain glucose and which had been depleted of oxygen by the addition of the reducing agent sodium dithionite (0.1 mM) and continuous bubbling with 100% nitrogen gas. This is a widely accepted protocol for rendering physiological buffers anoxic (8). Synaptosomes were then incubated in a shaking water bath (37°C) for 15 or 30 minutes in vials which were capped and carrying a gassing line (delivering nitrogen gas) suspended above each incubation. This protocol was classed as in vitro ischemia. For all experiments normoxic controls were carried out similarly but using buffer containing 10 mM glucose, and gassing lines carrying pure oxygen. All incubations contained 100 μM arginine final concentration (f.c.) so that this substrate for Nitric Oxide Synthase (NOS) did not become limiting. Synaptosome integrity was not compromised using this protocol. Measurement of lactate dehydrogenase activity (LDH) (9) in the supernatant of pelleted synaptosome preparations, showed that less than 10% of total activity of this cytosolic protein had leaked from the intact synaptosomes. After the incubation period, synaptosomes were pelleted (4,000 g_{av}, 1 minute), 700 μl KPB removed and replaced with 700 μl of control KPB (which had been equilibrated with pure oxygen and kept at 37°C), to allow reintroduction of oxygen into the ischemic incubations. Synaptosomes were further incubated with access to pure oxygen for 15 minutes before pelleting (14,000 g_{av}, 3 minutes).

ATP Measurements. ATP was acid extracted from synaptosomal suspensions using 3.5% (final concentration) ice-cold perchloric acid. Extracts were neutralized with 0.7 M K_2HPO_4 (final concentration), kept on ice for 30 minutes and then pelleted (10,000 g_{av} for 2 minutes). Resultant supernatants were snap frozen in liquid nitrogen and kept frozen at −80°C until the time of assay. ATP concentration was measured with a luciferin-luciferase bioluminescence assay (10), at 30°C on a 1251 LKB Wallac Luminometer.

Nitrite+Nitrate Determination. $NO^{\cdot}$ production from the synaptosomes was measured by assaying the stable end products of $NO^{\cdot}$ degradation in aqueous solution—nitrite (NO_2^-) and nitrate (NO_3^-) (6), as the half-life of $NO^{\cdot}$ is very short (11). $NO_2^- + NO_3^-$ released from the intact synaptosomes were measured in the supernatant of the synaptosomal incubations by a colorimetric procedure using the Griess reagent as described by Green et al. (12), which has been previously used with synaptosomes (13). To validate that the assay was reflecting $NO^{\cdot}$ production the calcium ionophore, ionomycin (10 μM f.c.), was added to control synaptosomes to establish whether $NO_2^- + NO_3^-$ production would adequately reflect an increased calcium concentration and subsequent increased activity of NOS and thus NO production. Applications of the known NOS inhibitor N'. nitro-arginine (NNA) were made to the ischemic incubations (100 μM f.c.) (11) to assess whether increased $NO_2^- + NO_3^-$ production was due to increased NOS activity.

Nitric Oxide Synthase Activity. NOS activity was determined in brain cytosolic fractions prepared from 10 and 60 day old rats. Whole brain was homogenised (25% w/v) in isolation buffer (320 mM sucrose, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) and centrifuged at 100,000 g_{av} for 60 minutes at 4°C. The resulting supernatant was used for the determination of NOS activity. Measurement of NOS activity is based on the quantification of [^{3}H]citrulline converted from [^{3}H]arginine, as described by Brand et al. [14]. In brief, following removal of endogenous arginine by cation exchange, 100 μl of cytosol was added to 100 μl reaction buffer containing (f.c.) 100 mM Hepes, pH 7.5, 100 μM NADPH, 1 mM calcium chloride, 1 mM magnesium acetate, 10 μg/ml calmodulin, 50 μM tetrahydrobiopterin, 1 mM dithiothreitol, 5 μM FAD, 5 μM FMN, 100 μM L-arginine and 1 μCi/ml [^{3}H]arginine. After 15 minutes the reaction was terminated by addition of 50 μl of trichloroacetic acid (1.5 M) followed by 1 ml of Hepes (1.5 M) buffer, pH 6.0. Arginine was then removed from the reaction mixture by ion exchange chromatography and the amount of labeled citrulline present was determined by liquid scintillation counting.

Antioxidant Status. Total antioxidant status was measured in synaptosomes with glucose and oxygen supplied, from both 10 day old pups and adults. Synaptosomes (approximately 2 mg/ml) were freeze-thawed three times and homogenised in a tight glass-glass homogenizer, to release all the synaptosomal contents. After pelleting the membranes (14,000 g_{av}, 5 mins), the supernatant was assayed for an-

Table I. Concentration of ATP in Adult and Pup Synaptosomes After 15 or 30 Minutes of Hypoxia-Aglycaemia

Conditions	ATP Concentration (nmol/mg protein)	
	Pup Synaptosomes	Adult Synaptosomes
Control, 15 mins	1.31 ± 0.15 (n=4)	1.73 ± 0.15 (n=5)
Hypoxia-aglycaemia, 15 mins	0.18 ± 0.02[a] (n=4)	0.24 ± 0.03[a] (n=5)
Control, 30 mins	1.28 ± 0.27 (n=4)	1.68 ± 0.05 (n=5)
Hypoxia-aglycaemia, 30 mins	0.19 ± 0.02[a] (n=4)	0.23 ± 0.02[a] (n=5)

Control values for the appropriate incubation periods (i.e., 15 or 30 minutes) are also shown.
[a]$P < 0.01$ compared with appropriate control.

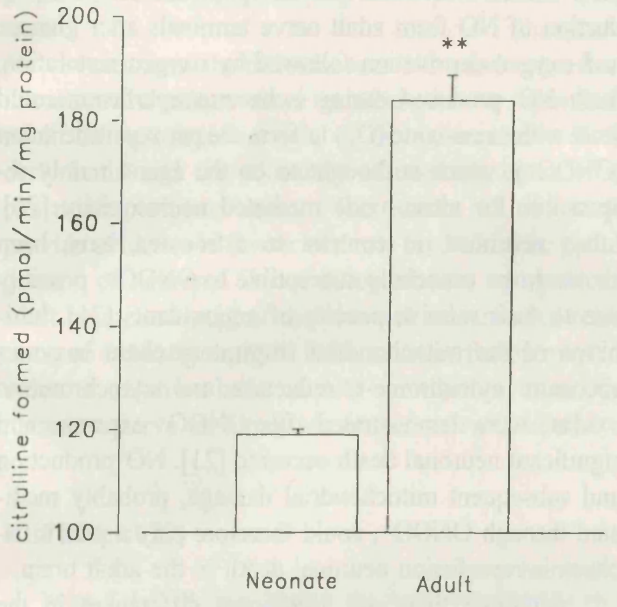

Fig. 1. Bar graph showing nitric oxide synthase (NOS) activity, as determined by [^{3}H]citrulline formed from [^{3}H]arginine, in whole brain homogenates from neonates (10 day old rat pups) and adults (60 day old rats). Results are expressed as mean ± SEM, n = 3 separate synaptosome preparations, measurements performed in triplicate. ** represents significant ($P < 0.01$) difference from neonatal value.

tioxidant status. This is a functional assay which measures the capacity of a sample to scavenge free radicals generated from the reaction between metmyoglobin and hydrogen peroxide. If unscavenged, these radicals react with 2.2'-azino-bis-3-ethylbenzo-thiaoline-6-sulphonic acid (ABTS) to form a chromophore which absorbs at 734 nm. The assay was carried out as in the original method [15].

Protein Measurement. Protein concentrations were determined according to Lowry et al, [16].

Statistical Analysis. Differences in the means of separate experiments were tested by analysis of variance (ANOVA), followed by the least significant difference multiple range test. All results are expressed as the mean ± standard error of the mean (SEM).

Table II. Concentration of NO_2^- + NO_3^- Released from Adult and Pup Synaptosomes After 15 or 30 Minutes of Hypoxia-Aglycaemia Followed by 15 Minutes Reperfusion with Oxygen Supplied

Conditions	Nitrite + Nitrate Concentration (nmol/mg protein)	
	Pup Synaptosomes	Adult Synaptosomes
Control, for 15 mins + 15 mins oxygen	0.50 ± 0.04[a] (n=4)	1.15 ± 0.11 (n=6)
Hypoxia-aglycaemia, for 15 mins + 15 mins oxygen	0.47 ± 0.02[a] (n=4)	2.24 ± 0.12[b] (n=6)
Control, for 30 mins + 15 mins oxygen	0.49 ± 0.03[a] (n=4)	1.10 ± 0.14 (n=6)
Hypoxia-aglycaemia, for 30 mins + 15 mins oxygen	0.49 ± 0.03[a] (n=4)	2.27 ± 0.15[b] (n=6)

Control values for the appropriate incubation periods (i.e., 15 or 30 minutes followed by 15 minutes reperfusion with oxygen) are also shown.
[a]$P < 0.01$ compared to adult.
[b]$P < 0.01$ compared with appropriate adult control.

RESULTS

Synaptosomal hypoxia-aglycaemia (oxygen and glucose deprivation) was confirmed by analysis of ATP. These data are detailed in Table I. Results show that both the adult and neonatal synaptosomes experienced a relatively severe degree of hypoxia-aglycaemia because ATP levels fell to 13–15% of controls by 15 minutes in both types of preparation.

In 60 day old adult rats the total brain NOS activity was significantly ($P < 0.01$) higher than in 10 day old rat pups (Fig. 1). This was also reflected in measurements of NOS activity in cytosol from synaptosomes prepared from these ages (pup value was 65% of adult value. ie. pup = 74 ± 1 pmol/min/mg, n = 3, adult = 113 ± 4 pmol/min/mg, n = 3, $P < 0.01$).

The production of $NO^\cdot$ from synaptosomes, reflected by the NO_2^- + NO_3^- concentration was also significantly lower in pups compared to adults (Table II). This data therefore suggests an increase in NOS activity and $NO^\cdot$ formation as the brain matures. A period of glucose and oxygen deprivation for 15 or 30 minutes followed by reintroduction of oxygen (akin to ischaemia/reperfusion) caused a marked increase in $NO^\cdot$ production from the adult synaptosomes (Table I), but no increase in the synaptosomes prepared from pups. The increase in NO_2^- + NO_3^- seen in adult synaptosomes was prevented by the addition of the NOS inhibitor NNA to the incubation, (hypoxia-aglycaemia + NNA, 30 min = 1.23 ± 0.11 nmol/mg protein, n = 3). Addition of ionomycin to control incubations increased the

Table III. Total Antioxidant Status of Adult and Pup Synaptosomes

Total antioxidant capacity (μmol/mg protein)	
Adult	Pup
0.11 ± 0.01 (n=9)	0.12 ± 0.01 (n=7)

NO_2^- + NO_3^- production by 40%, indicating that the assay reflected calcium-dependent NOS activity.

The total antioxidant status of immature and adult synaptosomes were not statistically different (Table III), suggesting that the adult and immature brain have similar capabilities for scavenging free radicals.

DISCUSSION

In this study, we have provided evidence to suggest that the activity of cytosolic nitric oxide synthase (NOS) in rat brain homogenates and synaptosomes is much lower in the immature brain than in the fully matured adult brain. This data is in agreement with previous findings that NOS activity in rat brain cerebellum cytosolic and particulate fractions increases with age (17). In pups it appears that the activity of NOS in the nerve terminal from immature brain would be low during the initial stages of ischaemia/reperfusion. This is due to a combination of the low intrinsic activity of NOS in immature brain, coupled with previous data demonstrating no increase in cytosolic calcium during glucose and oxygen deprivation (5), which would hence keep the NOS activity low under these conditions. This is confirmed by our NO_2^- + NO_3^- results after hypoxia-aglycaemia and oxygen restoration, where there is no significant difference in $NO^\cdot$ production compared to control levels in the nerve terminals from immature brain. This observation suggests that $NO^\cdot$ production by nerve terminals does not feature as an important part of the mechanism of damage in the neonatal brain during the immediate stages of reperfusion after ischaemia.

In a number of in vivo studies it has been reported that brain damage after ischaemia/reperfusion in can be ameliorated by administration of NOS inhibitors, suggesting a role for NO in the pathogenesis of cerebral damage in the brain (4). However there are also a number of contradictory reports concerning the possible neuroprotective effect of NOS inhibitors in ischaemia/reperfusion injury. These differences may reflect the various experimental procedures and models used but the findings of these and other studies are generally supportive of an involvement of reactive oxygen species in adult ischaemia/reperfusion injury (2). Less work has been done on the neonatal brain specifically although some in vivo models have suggested that NOS inhibitors can ameliorate damage following focal stroke (18). The model that we have used here is more relevant to global ischaemia and there are no blood vessels present, thus allowing the neuronal NOS to be studied effectively in isolation from the endothelial and inducible forms.

Recent work on developing brain showed that adult brain slices recovering from glucose and oxygen deprivation suffered a significant decrease in energy metabolites when NO was generated in the system from an NO donor, but in neonatal slices under the same conditions NO did not decrease the energy metabolites at all (19). We show here that there is significant increased production of NO from adult nerve terminals after glucose and oxygen deprivation followed by oxygen restoration. Such $NO^\cdot$ produced during ischaemia/reperfusion could react with superoxide (O_2^-) to form the peroxynitrite anion ($ONOO^-$), which is thought to be the agent mainly responsible for nitric-oxide mediated neurotoxicity [20]. Intact neurones, in contrast to astrocytes, have been shown to be especially susceptible to $ONOO^-$, possibly due to their relative paucity of antioxidants [21]. Inhibition of the mitochondrial respiratory chain enzymes succinate cytochrome-c reductase and cytochrome c oxidase were demonstrated after $ONOO^-$ exposure and significant neuronal death occurred [21]. $NO^\cdot$ production and subsequent mitochondrial damage, probably mediated through $ONOO^-$, could therefore play a part in ischaemia/reperfusion neuronal death in the adult brain.

Although there are significant differences in the amount of $NO^\cdot$ production from immature and adult nerve terminals, the inherent ability of the brain to cope with $NO^\cdot$/$ONOO^-$ and other reactive species may be an important determinant of whether damage will occur. Our data show that the antioxidant status of the nerve terminal does not change with development, at least in the membrane free fraction studied here. However, this observation correlates with the developmental profile of the major membrane bound antioxidant alpha-tocopherol which remains constant as the brain matures [22]. Thus the increased production from the adult as compared with the immature animal may not compensated for by an increased capacity of the nerve terminal to scavenge free radicals. In this respect therefore the immature brain is less likely to suffer from $NO^\cdot$ related oxidative stress than the adult brain because of the differences in the ratio of antioxidant status: $NO^\cdot$ production in the adult and neonate.

The overall development of the brain is complex. It is known that the oxygen consumption of the neonatal brain is lower than that of the adult brain, as are the resting cellular membrane potentials and EEG activity [23]. Many enzymes of the glycolytic pathway have been shown to change with development [24], and the activities of many of the complexes of the mitochondrial respiratory chain increase as the brain matures [25], [26]. There are complicated developmental profiles of a number of receptors such as the NMDA receptor in brain [27], and nerve terminal calcium homeostasis has shown to change as the brain develops [5]. All of these changes and others such as anti-oxidant status means that a variety of mechanisms may be involved in the resistance of neonatal brain to ischaemia/reperfusion. However, brain function is centred at the synapse and the data we present here may play an important part in this resistance.

In summary, the differences in $NO^{\cdot}$ production from immature and mature nerve terminals that we report here could be significant in light of the vulnerability of elderly brain and the resistance of neonatal brain to ischaemia/reperfusion injury. The results of this study provide evidence in favour of a role for $NO^{\cdot}$ in the pathogenesis of neuronal ischemia/reperfusion injury in the adult brain, and suggest that the well documented resistance of neonatal brain to ischemia/reperfusion may involve a relative lack of $NO^{\cdot}$ production from the immature nerve terminals in comparison with adults.

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **220,** 13–19 (1996)
ARTICLE NO. 0348

Inhibition of Tetrahydrobiopterin Synthesis Reduces *in Vivo* Nitric Oxide Production in Experimental Endotoxic Shock

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Received January 26, 1996

Nitric oxide synthesis requires the cofactor tetrahydrobiopterin. We have examined the effect on nitric oxide synthesis in experimental endotoxic shock of 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP cyclohydrolase I, the first and rate limiting enzyme for tetrahydrobiopterin synthesis. Rats given lipopolysaccharide (LPS, 10 mg/kg) showed a large rise in plasma nitrate at 4 and 8 hours which was significantly reduced by DAHP (1 g/kg) given at the same time as LPS. There was a 40–50% reduction in the haem-NO signal detected in kidney by electron paramagnetic resonance spectroscopy. LPS produced hypotension at 3 hours and 6 hours and this was ameliorated at 6 hours in rats given DAHP. DAHP abolished the rise in kidney tetrahydrobiopterin levels seen 4 hours after LPS but no effect was seen on induction of inducible nitric oxide synthase (iNOS) as assessed by immunohistochemistry and reverse transcriptase PCR, consistent with the effect of DAHP being by reduction of tetrahydrobiopterin levels. The results show that inhibition of tetrahydrobiopterin synthesis is an effective strategy to reduce nitric oxide synthesis by iNOS *in vivo.*

Nitric oxide (NO) is a highly reactive free radical and potent vasodilator which is synthesised from L-arginine by nitric oxide synthase (NOS). NOS exists in three different isoforms (1). Two of these are dependent for their activity on intracellular calcium levels and were first demonstrated to be constitutively expressed in nervous tissue (NOS I, ncNOS) and endothelium (NOS III, ecNOS). The other isoform (NOS II, iNOS) is independent of intracellular calcium and its activity is controlled principally at the levels of transcription and translation. iNOS is induced in a range of cells by pro-inflammatory cytokines and lipopolysaccharide (LPS) (2). NO produced by iNOS has been implicated in inflammation and in the hypotension of endotoxic shock and there is thus considerable interest in strategies for inhibiting NO *in vivo.*

The activity of NOS is dependent on the availability of co-factors including tetrahydrobiopterin (BH_4). The exact role of BH_4 is unknown but it may be important in maintaining NOS in an active configuration, or may have a regulatory redox role (3,4). The need for an adequate supply of BH_4 has been shown for purified macrophage iNOS (5,6) and for intact cells including macrophages, (7,8) fibroblasts (9) and smooth muscle cells (10,11). In human endothelial cells, inflammatory cytokines increase NO synthesis by increasing BH_4 levels (12,13). The rate-limiting enzyme for *de novo* BH_4 synthesis is GTP cyclohydrolase (GTPCH) and it is up-regulated in a variety of cells by stimuli which also induce iNOS (10,11,14). *In vivo,* increased GTPCH mRNA and enzyme activity have been demonstrated in the tissues of LPS-treated rats (15,16). 2,4-Diamino-6-hydroxypyrimidine (DAHP) is an inhibitor of GTPCH and has been used extensively *in vitro* to elucidate the role of BH_4 in NO synthesis (9,10). We have now studied the effect of administration of DAHP *in vivo* on NO synthesis, tissue BH_4 levels and blood pressure in rats given LPS.

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; BH_4, tetrahydrobiopterin; GTPCH, GTP cyclohydrolase; DAHP, 2,4-diamino-6-hydroxypyrimidine; EPR, electron paramagnetic resonance.

0006-291X/96 $18.00

MATERIALS AND METHODS

Lipopolysaccharide administration. Inbred male Lewis rats (200–300g) were given an intraperitoneal injection of lipopolysaccharide (LPS) *Escherichia coli* serotype 0127:B8 (10 mg/kg, Sigma Chemical Co., Poole Dorset).

Four or eight hours after administration, animals were sacrificed and the kidneys were frozen immediately in liquid nitrogen. Blood was taken into heparinised syringes. Samples of kidney, liver, spleen and lung were placed in formal saline for 24 hours and embedded in paraffin wax for immunohistochemistry.

DAHP administration. DAHP was dissolved in phosphate buffered saline (PBS, 10mM pH 6.5) immediately prior to use. DAHP (1 g/kg) was administered intraperitoneally with LPS or 4 hours after LPS. Animals receiving LPS only were given an equivalent amount of PBS. The animals were sacrificed 8 hours after LPS.

Immunohistochemistry. iNOS antibody was a gift from Dr V. Riveros-Moreno (Wellcome Research Laboratories, Beckenham, UK) (17). Paraffin sections were dewaxed in xylene, rehydrated and microwaved in citrate buffer (10mM, pH 6.0) for 15 minutes. Primary antibody was incubated overnight at 4°C. A biotinylated secondary antibody was enhanced with streptavidin-biotin-peroxidase (Dako, High Wycombe, Bucks, UK) and developed with diaminobenzidine. Macrophages were detected with the monoclonal antibody ED-1 (1:500, Serotec, Oxford, UK.) using the same developing system.

Plasma nitrate/nitrite levels. Blood was anticoagulated with heparin and plasma removed. Nitrate was reduced to nitrite by bacterial nitrate reductase using *Pseudomonas oleovorans* (National Collection of Type Cultures, Colindale, London, UK) (18) and the supernatant assayed via the Griess reaction as described previously (19). The lower limit of detection was 7.5 μM.

Tetrahydrobiopterin analysis. Kidneys were thawed and homogenised (20% w/v) in perchloric acid (0.1 M) containing dithioerythreitol (6.5 mM) and diethylenetriaminepentaacetic acid (2.5 mM). Following centrifugation (15 000g for 3 minutes) supernatants were diluted 1:2 with the perchloric acid (+ additives) and analysed for tetrahydrobiopterin by HPLC and electrochemical detection (20).

Blood pressure measurements. Systolic blood pressure was measured in conscious rats using a tail cuff blood pressure monitor (Harvard Apparatus, Edenbridge, Kent, UK.). Animals were accustomed for 3 days prior to LPS administration. Measurements were taken prior to LPS/DAHP administration and 3 and 6 hours subsequently.

Electron paramagnetic resonance (EPR) spectroscopy. Kidneys were thawed and homogenised in an equal weight of HEPES buffer (0.2 M, pH 7.4) containing EDTA (10 mM). Samples were placed in quartz EPR tubes (3 mm i.d.) and frozen in liquid nitrogen. X-band EPR spectra were recorded on a Bruker ESP 300 spectrometer fitted with an Oxford Instruments ESR 900 liquid helium flow cryostat at 15 K. Spectra were an average of 5 scans and normalised for direct comparison.

Protein determination. Kidney homogenates, taken at the same time as EPR sample preparation, were assayed by the modified Lowry method for total protein, using bovine serum albumin as a standard (21).

Reverse transcription polymerase chain reaction. RNA was isolated from frozen kidney by homogenisation in RNAzol B (Biogenesis, Bournemouth, UK) following manufacturers instructions. RNA (5 μg) from each sample was reverse transcribed with Moloney murine leukaemia virus enzyme (GIBCO BRL, Paisley, UK) using random hexamers. The resultant cDNA was amplified by the polymerase chain reaction (PCR) using primers for iNOS as described previously (22). PCR was carried out for 35 cycles with the annealing temperature of 61°C. PCR products were analysed on 1.5% agarose gel and visualised with ethidium bromide staining and ultraviolet transillumination.

Statistics. Data are presented as mean ± SEM. Comparisons between groups were by Mann-Whitney U test.

RESULTS

Administration of LPS (10 mg/kg) caused a large rise in plasma nitrate after 4 hours, increasing further by 8 hours (Figure 1). DAHP (1 g/kg) administered with LPS significantly reduced plasma nitrate production at both 4 and 8 hours. Normal rats or rats given DAHP alone showed no detectable nitrate. There was no reduction in plasma nitrate at 8 hours when DAHP was given 4 hours after LPS.

NO formation induced by LPS administration can be directly measured by its binding to haem proteins by EPR spectroscopy. NO binding to these proteins gives rise to a unique EPR spectrum with a characteristic triplet splitting due to the hyperfine coupling of the nitrogen nucleus of bound NO. At 40 K, the EPR spectrum of kidney homogenates from rats administered LPS alone (Figure 2a) displayed a prominent resonance centred at $g \sim 2.0$, with a peak at $g \sim 2.07$ and a triplet splitting centred at $g \sim 2.01$, typical of haem-NO formation. The broad feature at $g \sim 1.94$ is due to reduced iron-sulphur proteins. DAHP given simultaneously with LPS significantly decreased (by 40–50%) the haem-NO signal intensity (Figure 2b). However, only a small decrease in intensity of the haem-NO EPR signal was observed when DAHP was given four hours after LPS (data not shown). The haem-NO signal observed in the difference spectrum shown in Figure 2 (lower spectrum) shows a splitting of $A(^{14}N) \sim 1.7$ mT. Similar signals have been observed for the

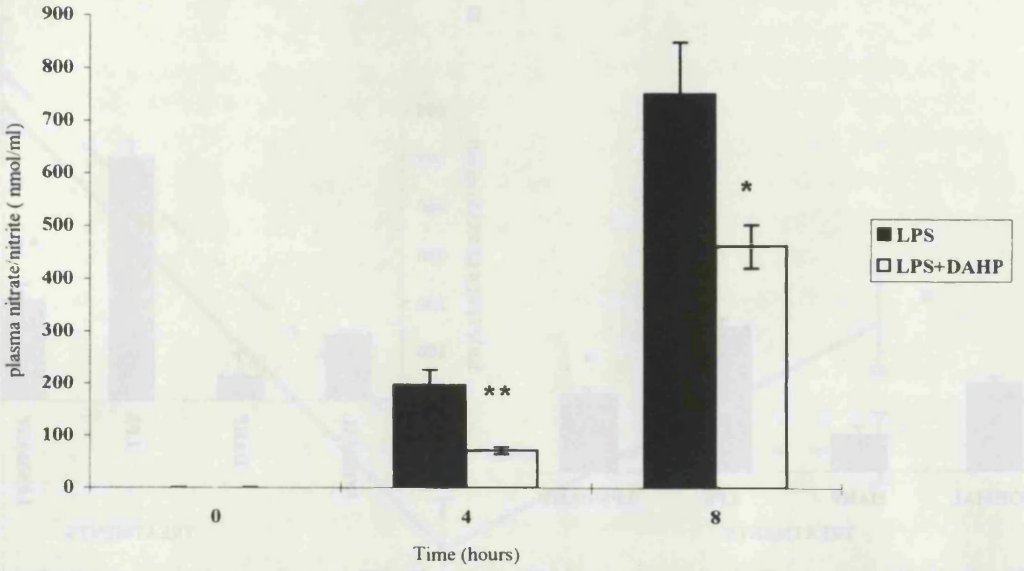

FIG. 1. Plasma nitrate/nitrite levels (nmol/ml) at varying times following LPS (10 mg/kg) and LPS with DAHP (1 g/kg) administration. Normal and DAHP treated rats had no detectable nitrate/nitrite and represent time 0 above. Plasma was incubated with *P. oleovorans,* plasma protein was precipitated and the resultant nitrite assayed via the Griess reaction. * $p < 0.05$, ** $p < 0.01$ compared with LPS nitrate/nitrite levels ($n = 6$).

NO-adducts of Type II haem proteins such as cytochrome *c* oxidase and haemoglobin (23) DAHP administration caused a significant reduction in kidney BH_4 levels compared with normal rats (Figure 3). BH_4 increased dramatically with LPS at 4 and 8 hours and this rise was completely inhibited at 4 hours by DAHP given at the same time as LPS and markedly attenuated at 8 hours.

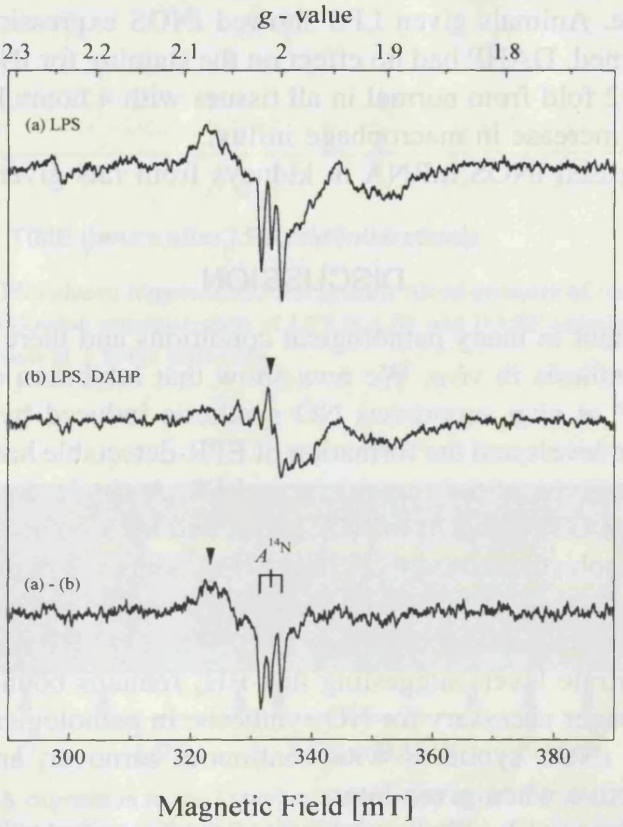

FIG. 2. X-Band EPR spectra from LPS-treated rats, demonstrating the effect of DAHP administration on endotoxin-induced NO formation. Kidney homogenate from rats treated with either LPS alone (a), or LPS and DAHP (b). Measurement conditions were: temperature, 40 K; microwave power, 20 mW; microwave frequency, 9.375 GHz; modulation amplitude, 0.49 mT; time constant, 0.082 s; sweep rate, 2.38 mT/s. The spectra are an average of two scans.

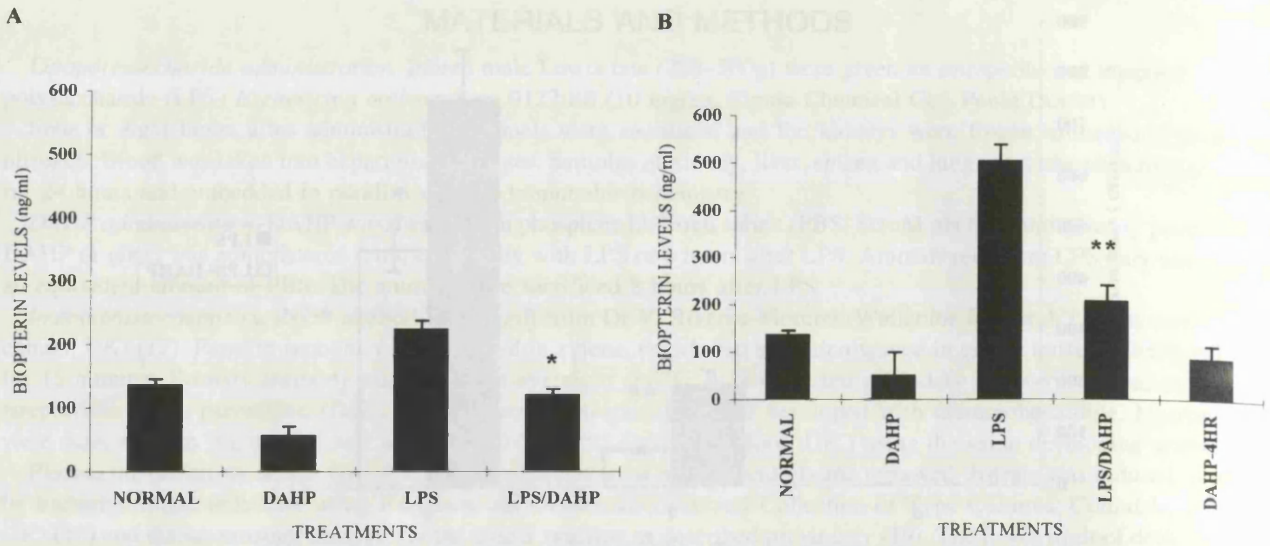

FIG. 3. Tetrahydrobiopterin levels in the kidney of LPS and/or DAHP treated rats. Kidney tetrahydrobiopterin levels (ng/ml) were measured in rats receiving LPS (10 mg/kg), DAHP (1 g/kg) or LPS and DAHP (administered simultaneously) for 4 hours (A) or 8 hours (B). DAHP-4 (B) refers to DAHP administered 4 hours after LPS. Normal rats received no treatment. * $p < 0.05$, ** $p < 0.01$ compared with LPS alone ($n = 6$).

LPS caused hypotension after 3 hours which was not significantly altered 6 hours later (Figure 4). DAHP administration with LPS had no effect at 3 hours but a significant increase in blood pressure towards normal was apparent at 6 hours following administration. DAHP given alone had no effect on blood pressure (data not shown).

Immunohistochemical staining for iNOS was negative in tissues from normal rats and rats treated with DAHP alone. Animals given LPS showed iNOS expression, increasing from 4 to 8 hours in all tissues examined. DAHP had no effect on the staining for iNOS. Macrophage numbers increased approximately 2 fold from normal in all tissues with 4 hours LPS and 3 fold at 8 hours. DAHP did not alter this increase in macrophage influx.

RT-PCR showed increased iNOS mRNA in kidneys from rats given LPS or LPS and DAHP (Figure 5).

DISCUSSION

NO synthesis is important in many pathological conditions and there is considerable interest in ways of inhibiting its synthesis *in vivo*. We now show that inhibition of BH_4 synthesis with the GTPCH inhibitor DAHP *in vivo* suppresses NO synthesis induced by a single dose of LPS as assessed by plasma nitrate levels and the formation of EPR-detectable haem-NO complexes. DAHP was most effective when given at the same time as LPS. A single dose of LPS leads to a rapid increase in iNOS (24) and GTPCH (15) mRNA levels and by 3 hours iNOS protein is detectable in many tissues by immunohistochemistry (17) but by 12 hours iNOS protein is only detectable in the spleen. Thus a single dose of LPS causes a massive reversible increase in iNOS and it is likely that, once synthesised, the enzyme rapidly binds BH_4. When DAHP was given 4 hours after LPS there was no effect on nitrate levels suggesting that BH_4 remains bound to iNOS and continuing synthesis of BH_4 is no longer necessary for NO synthesis. In pathological conditions there may be longer-lasting stimuli to iNOS synthesis with continuous turnover, and in those circumstances DAHP may remain effective when given later.

NO is a potent vasodilator and a likely mediator of the hypotension that follows LPS administration. After LPS administration there is a rapid fall in blood pressure (25) which is thought to be due to enhanced formation of NO by eNOS followed by a delayed phase in which iNOS is implicated. Hypotension in endotoxic shock can be reversed by competitive inhibitors of NOS

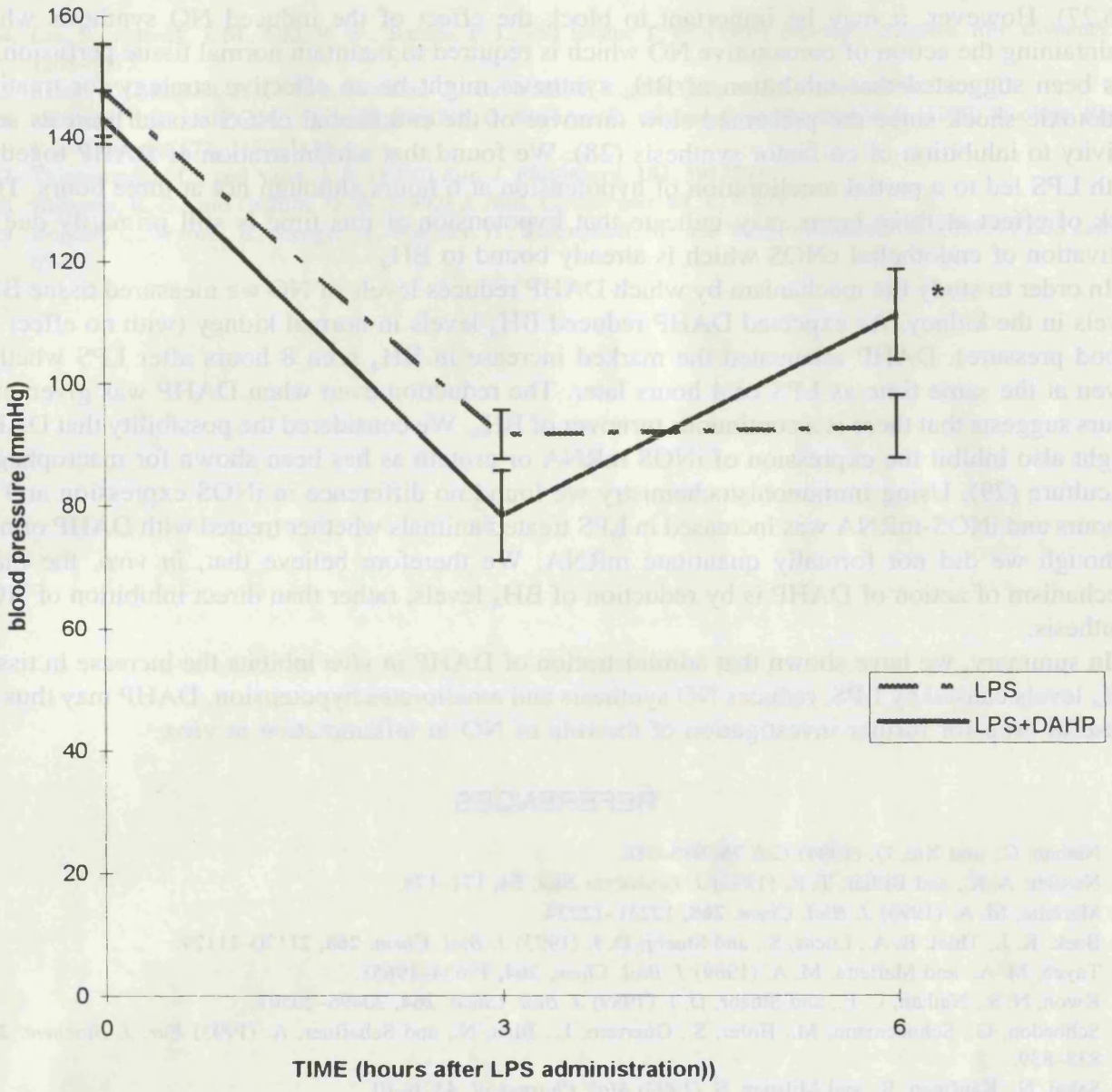

FIG. 4. Effect of DAHP on LPS induced hypotension. The systolic blood pressure of conscious rats was recorded prior to LPS (time 0), 3 and 6 hours following administration of LPS or LPS and DAHP (administered, simultaneously). * $p < 0.05$ compared with the hypotension at 3 hours post-LPS.

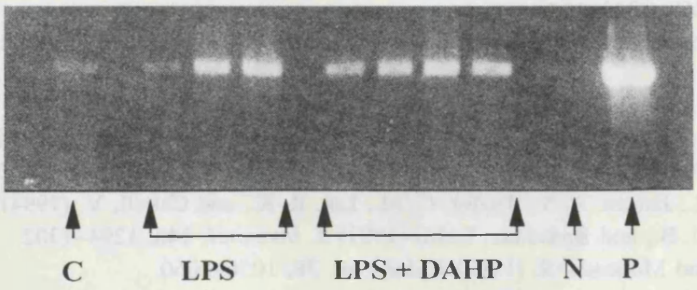

FIG. 5. Inducible NOS mRNA expression in the kidney of rats treated with LPS or LPS and DAHP. LPS (10 mg/kg) was administered for 4 hours either with or without DAHP (1 g/kg). Control (C) rats received saline only. mRNA was isolated, reverse transcribed and PCR was performed with primers for iNOS. Products were separated by gel electrophoresis and visualised with ethidium bromide and UV light. The negative control (N) was saline and the positive control (P) was iNOS cDNA.

(26,27). However, it may be important to block the effect of the induced NO synthesis while maintaining the action of constitutive NO which is required to maintain normal tissue perfusion. It has been suggested that inhibition of BH_4 synthesis might be an effective strategy for treating endotoxic shock since the presumed slow turnover of the endothelial cNOS would limit its sensitivity to inhibition of co-factor synthesis (28). We found that administration of DAHP together with LPS led to a partial amelioration of hypotension at 6 hours although not at three hours. This lack of effect at three hours may indicate that hypotension at this time is still primarily due to activation of endothelial cNOS which is already bound to BH_4.

In order to study the mechanism by which DAHP reduces levels of NO we measured tissue BH_4 levels in the kidney. As expected DAHP reduced BH_4 levels in normal kidney (with no effect on blood pressure). DAHP attenuated the marked increase in BH_4 seen 8 hours after LPS whether given at the same time as LPS or 4 hours later. The reduction even when DAHP was given at 4 hours suggests that there is a continuous turnover of BH_4. We considered the possibility that DAHP might also inhibit the expression of iNOS mRNA or protein as has been shown for macrophages in culture (29). Using immunohistochemistry we found no difference in iNOS expression at 4 or 8 hours and iNOS-mRNA was increased in LPS treated animals whether treated with DAHP or not, although we did not formally quantitate mRNA. We therefore believe that, *in vivo,* the main mechanism of action of DAHP is by reduction of BH_4 levels, rather than direct inhibition of NOS synthesis.

In summary, we have shown that administration of DAHP *in vivo* inhibits the increase in tissue BH_4 levels caused by LPS, reduces NO synthesis and ameliorates hypotension. DAHP may thus be a useful drug for further investigation of the role of NO in inflammation *in vivo.*

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Short Report *Ann Clin Biochem* 1996; 33: 71–72

Age dependent changes in the cerebrospinal fluid concentration of nitrite and nitrate

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Additional key phrases: nitrite; nitrate; nitric oxide; reference ranges; neurodegeneration; peroxynitrite

Within the central nervous system (CNS), nitric oxide (NO) is proposed to have a number of functions, the best documented being activation of guanylate cyclase following stimulation of the *N*-methyl-D-aspartate receptor by glutamate.[1] However, excessive NO formation is considered to be neurotoxic.[1] The exact mechanism whereby NO exerts its toxic effects is not known, but formation of peroxynitrite ($ONOO^-$), generated from a reaction between the superoxide anion and NO, may be responsible for the majority of neurotoxic effects.[1] *In vitro*, neurones and oligodendrocytes are particularly vulnerable to the actions of excess $NO/ONOO^-$.[1,2] The exact mechanism whereby these molecules cause cell death is not known, but the mitochondrial respiratory chain is damaged by $NO/ONOO^-$.[1,2] Excessive $NO/ONOO^-$ formation has been implicated, in adults, in the pathogenesis of neurodegenerative diseases, such as multiple sclerosis,[1,2] and is likely to be important in some neurodegenerative disorders in children.

NO is synthesized from arginine by nitric oxide synthase (NOS).[3] Within the CNS at least three isoforms of NOS exist, all of which require a number of cofactors which include NADPH and tetrahydrobiopterin.[3]

In view of the proposed functions of NO and its potential involvement in pathological conditions, an index of NO formation in the CNS may be useful. Unfortunately, NO and $ONOO^-$ are very unstable and hence not amenable to direct measurement in CSF.[4] Degradation products of $NO/ONOO^-$, nitrite (NO_2^-) and nitrate (NO_3^-) are relatively stable.[4] Measurement of these metabolites may therefore be a useful indicator of $NO/ONOO^-$ formation in the CNS. However, concentrations of NO_2^- and NO_3^- in CSF may depend on age and, therefore, we investigated the effect of age upon the CSF concentrations of NO_2^- and NO_3^-.

The CSF concentrations of NO_2^- and NO_3^- may be affected in various neurological conditions such as Parkinson's and Alzheimer's disease[5] and by infections such as meningitis.[6] In view of these observations, CSF was obtained from 35 hospitalized children (birth to 17 years) with various neurological conditions and in whom no disturbances of monoamine neurotransmitter metabolism or infection was suspected. Lumbar CSF was obtained from patients under sedation or light anaesthesia in a standardized way. The CSF was frozen at the bedside by immersion in liquid nitrogen and stored at —70°C until analysis. The first 2 mL collected was used for other purposes whilst the next 1 mL fraction was used in this study.

Determination of the CSF concentrations of NO_2^- and NO_3^- was based on the method described by Hevel and Marletta.[4] In this procedure NO_2^- reacts with Griess reagent (equal volumes of 0·1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) to form a chromophore which maximally absorbs at 550 nm.[4] Since NO_3^- does not react with the Griess reagent this is converted to NO_2^- by pre-treatment with nitrate reductase.[4]

Eighty microlitres of CSF incubated in a microtitre plate with 652 u/L (final concentration in the incubation mixture) nitrate reductase

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(Boehringer–Mannheim, Germany) and 25 μmol/L of NADPH (Sigma, Poole, UK) for 30 min at room temperature. L-glutamate dehydrogenase (2174 u/L, Sigma), α-ketoglutarate (4 mmol/L, Sigma) and NH_4Cl (100 mmol/L, BDH, Leicester, UK) were then added to remove any residual NADPH which would interfere with the assay.[4] After 10 min, 100 μL of Griess reagent were added to make a final volume of 200 μL. The absorbance at 550 nm was then measured using a plate reader (Wellcozyme, Wellcome Diagnostics, Berkhamstead, UK). The concentration of NO_2^- and NO_3^- was calculated by reference to a standard curve (0–40 μmol/L).

Peak concentrations of NO_2^- plus NO_3^- were observed in the CSF during the first year of life. After this point the concentration declined with age in a logarithmic fashion reaching an approximate plateau at 13 years (Fig. 1). Using analysis of variance for groups divided according to age the mean CSF concentration (+95% confidence intervals) were: 0–1 year, 16 (14·2–18·6) μmol/L; 1–2 years, 12 (9·3–14·1) μmol/L; 2–17 years, 8 (6·9–9·8) μmol/L.

CONCLUSIONS

The data in this study suggest that CSF concentrations of NO_2^- and NO_3^- are highest in the first year of life and decline steadily with age up to the age of approximately 13 years. Other workers[5,6] have reported that CSF concentrations in adults are similar to those reported here for adolescents. Whilst the reason for the age effect is not known, it is of interest that tetrahydrobiopterin a cofactor which regulates nitric oxide synthase activity[3] displays a similar decline in CSF concentration with age.[7]

Measurement of the CSF concentration of NO_2^- and NO_3^- may be of importance in the investigation of neurological disorders and monitoring the response to treatment regimes. The data presented in this study illustrate the importance of establishing age matched reference ranges.

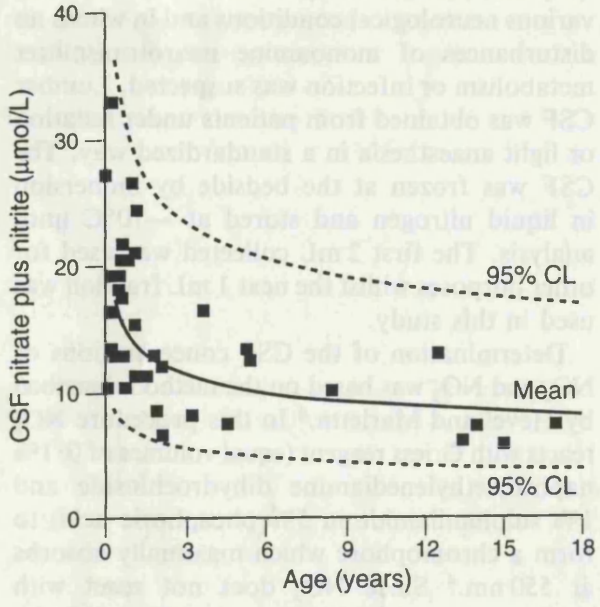

FIGURE 1. *The effect of age upon cerebrospinal fluid (CSF) nitrite and nitrate. The relationship is best fitted by a logarithmic curve* ($F_{34,1} = 24·96$, $P = 0·00002$, $R^2 = 42\%$). *The solid line is the mean concentration, dashed lines represent the 95% confidence limits.*

Acknowledgements

This work was funded by the Brain Research Trust (MPB), the Leukaemia Research Fund (JDC) and the Wellcome Trust (RAHS).

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Accepted for publication 3 August 1995

J. Inher. Metab. Dis. 19 (1996) 140–142

Short Communication

Mitochondrial damage: An important feature in a number of inborn errors of metabolism?

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The primary function of mitochondria is to generate energy in the form of ATP. This is achieved by the four complexes of the electron transport chain and the ATP synthetase. Damage to one or more of the complexes of the mitochondrial respiratory chain may therefore result in depletion of cellular energy reserves and cell death.

Increased cellular formation of oxidizing species may lead to mitochondrial damage, since the components of the mitochondrial respiratory chain are susceptible to oxidative damage *in vitro* (Zhang et al 1990). Thus it may be conjectured that mitochondrial damage may occur in a number of inborn errors of metabolism where the metabolic block increases oxidative stress. One such group are the inborn errors of glutathione (GSH) biosynthesis, which lead to GSH deficiency. Clinically they are characterized by a neurological syndrome, namely spinocerebellar degeneration, peripheral neuropathy, myopathy and ataxia (Meister and Larsson 1989). Interestingly, depletion (−60%) of brain GSH by the subcutaneous administration of L-buthionine sulphoximine (L-BSO) to pre-weanling rats results in mitochondrial swelling, damage to complex IV (cytochrome oxidase, EC 1.9.3.1) of the mitochondrial electron transport chain and neuronal damage (Jian et al 1991; Heales et al 1995).

In this study we have expanded on our previous observations (Heales et al 1995) by examining the effect of varying degrees of mitochondrial GSH depletion on complex IV activity.

METHODS

Pre-weanling Sprague–Dawley rats were used throughout. Pups were weighed every day and injected subcutaneously with L-BSO. In order to achieve varying degrees of brain GSH depletion, L-BSO was administered twice a day (2.5 mmol/kg) for 5 days or once a day (3.8 mmol/kg for 11 days). After sacrifice, free 'non-synaptic' mitochondria were isolated from the brains of these animals and analysed for complex IV activity and GSH concentration. For further details see Heales et al (1995).

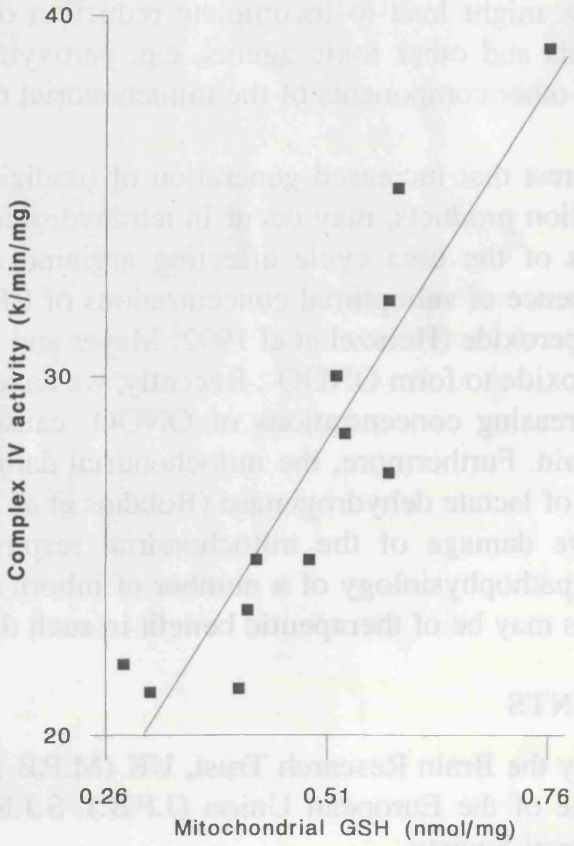

Figure 1 The activity of complex IV (cytochrome oxidase) is proportional to mitochondrial GSH concentration; *k* is the first-order rate constant

RESULTS

Administration of L-BSO as described resulted in mitochondrial GSH concentrations between 0.28 and 0.78 nmol/mg mitochondrial protein (control concentration 1.09 ± 0.08; Heales et al 1995). When complex IV activity was plotted against GSH concentration, a highly significant correlation ($r = 0.915$, $p < 0.001$) was observed (Figure 1).

DISCUSSION

Deficiency of brain GSH, as may occur in inborn errors of GSH metabolism, causes mitochondrial damage in experimental animals (Jian et al 1991; Heales et al 1995). In this study we have shown that complex IV inactivation is directly proportional to the magnitude of mitochondrial GSH depletion and hence degree of oxidative stress. The susceptibility of complex IV to damage as a result of GSH depletion may arise in part from its dependence on cardiolipin for maximal activity (Soussi et al 1990). Cardiolipin is a polyunsaturated phospholipid located in the inner mitochondrial membrane, and under conditions of high oxidative stress is prone to lipid peroxidation owing to its unusually high content of unsaturated bonds (Soussi et al 1990).

Damage to mitochondrial complex IV could in theory further increase the degree of cellular oxidative stress, since it is the terminal enzyme complex of the mitochondrial electron transport chain and is involved in the transfer of electrons from cytochrome *c* to oxygen, resulting in the formation of water. Consequently, it is possible that a reduced

capacity of this complex might lead to incomplete reduction of oxygen and increased formation of free radicals and other toxic agents, e.g. peroxynitrite ($ONOO^-$), thereby giving rise to damage to other components of the mitochondrial respiratory chain (Heales et al 1995).

It is of particular interest that increased generation of oxidizing species, in particular $ONOO^-$ and its degradation products, may occur in tetrahydrobiopterin (BH_4) deficiency states and inborn errors of the urea cycle affecting arginine availability. It has been observed that in the presence of suboptimal concentrations of BH_4 and/or arginine, nitric oxide synthase forms superoxide (Heinzel et al 1992; Mayer and Werner 1995). The latter reacts avidly with nitric oxide to form $ONOO^-$. Recently, we have shown that exposure of cultured neurons to increasing concentrations of $ONOO^-$ causes damage to the mitochondrial respiratory chain. Furthermore, the mitochondrial damage precedes cell death, as judged by the release of lactate dehydrogenase (Bolaños et al 1995).

In summary, oxidative damage of the mitochondrial respiratory chain may be an important feature in the pathophysiology of a number of inborn errors of metabolism. In view of this, antioxidants may be of therapeutic benefit in such disorders.

ACKNOWLEDGEMENTS

This work was funded by the Brain Research Trust, UK (M.P.B.) and the Human Capital and Mobility Programme of the European Union (J.P.B.). S.J.R.H. was in receipt of a travel grant from The Royal Society.

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