DIHYDROPTERIDINE REDUCTASE FROM MAN AND FROM THE RAT

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Thesis: "Dihydropteridine Reductase from Man and from the Rat"

by

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Summary:

A reproducible purification procedure has been used to purify dihydropteridine reductase (DHPR) from human brain. The kinetic constants and M.Wt. were determined for the purified enzyme. Purified DHPR was found to be inhibited in the presence of phenylpyruvate, 6-hydroxydopamine, l-methyl-4-phenyl-l, 2, 5, 6-tetrahydropyridine (MPTP) and aluminium.

The in vivo effect of the neurotoxins lead and aluminium on DHPR has been studied. Human blood DHPR activity was reduced in lead workers and haemodialysis patients with chronic renal failure.

Animal experiments showed that a brief hyperphenylalaninaemia in healthy rats following an administration of phenylalanine or para-chlorophenylalanine prior to a load of phenylalanine, produces a decrease in rat brain BH_4% and actual BH_4 levels compared to controls.

In rats, the oestrogen diethylstilboestrol has been found to significantly decrease brain total biopterin, actual BH_4 and BH_4% level compared to matched controls dosed with corn oil only.

Tetrahydrobiopterin metabolism is disrupted in a number of disease states. DHPR activity in the brain of patients dying from senile dementia and Down's syndrome, has been found to be higher than controls. The kinetic studies showed that Km values are significantly higher in the demented patients as correlated with controls. Breast neoplastic tissue showed a significant increase in DHPR activity in the basis of protein and DNA level, as compared to apparently normal tissue from the same breast.

A single case of partial heterozygote DHPR deficiency was investigated. Whole blood DHPR activity was less than half that of normal subjects. The kinetic studies showed significantly increased Km values for this case in comparison to normals.

The mechanism and consequences of such change in tetrahydrobiopterin metabolism are discussed in the light of in vivo studies as well as the in vitro results presented in this thesis.

KEY WORDS:

DIHYDROPTERIDINE REDUCTASE TETRAHYDROBIOPTERIN

LEAD ALUMINIUM HETEROZYGOTE DHPR DEFICIENCY

To

My Dear Wife, Dhamya

and my Sons Samir and Saif

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LIST OF ABBREVIATIONS

Al	Aluminum
BH_4	Tetrahydrobiopterin
DHFR	Dihydrofolate Reductase
DHPR	Dihydropteridine Reductase
$DMPH_4$	6, 7-Dimethyl-5, 6, 7, 8-tetrahydropterine
DNA	Deoxyribose Nucleic Acid
Dopa	3, 4-Dihydroxyphenylalanine
GTP	Guanosine Triphosphate
HPA	Hyperphenylalaninaemia
K_i	Inhibition constant
K_i'	Dissociation constant
Km	Michaelis Constant
MPTP	1-Methyl-4-phenyl-1, 2, 5, 6-Tetrahydropyridine
M.Wt.	Molecular Weight
NADH	Reduced Nicotinamide Adenine Dinucleotide
NH_2TP	D-Erythro-Dihydroneopterin Triphosphate
P	Phosphate Group
PCPA	p-Chlorophenylalanine
$q\text{-}BH_2$	Quinonoid dihydrobiopterin
S.D.	Standard Deviation
SDAT	Senile Dementia of Alzheimer Type

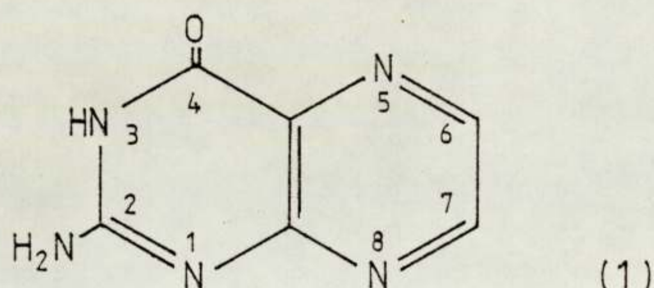
CHAPTER 1

INTRODUCTION

CHAPTER (1) :

INTRODUCTION

Pterins, derivatives of 2-amino-4-oxodihydro-pteridine (1), are a widely distributed class of naturally occuring heterocyclic compounds (Rembold and Gyure, 1972).

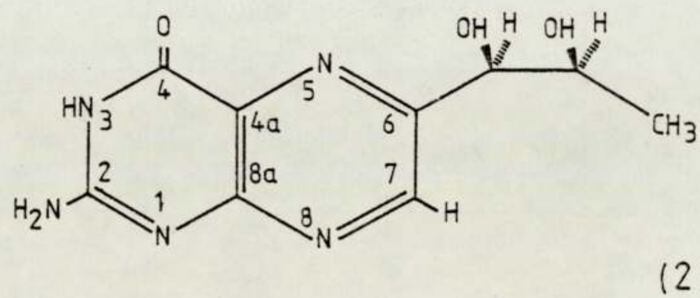


A pterin derivative, biopterin [6-(2, 3-dihydroxypropyl) pterin] (2) was isolated from urine in 1955 (Patterson et al. 1955). It was probably derived from the dihydro-(3) and tetrahydroforms (4) of the compound, since tetrahydrobiopterin is rapidly oxidized to 7, 8-dihydrobiopterin and then to biopterin (Blair and Pearson, 1973; Blair and Pearson, 1974).

Biopterin derivatives have been measured in human and rat tissues and fluids (Baker et al, 1974; Leeming et al, 1976b; Leeming and Blair, 1980a), including blood, serum (Frank et al, 1963; Leeming and Blair, 1980a) and urine (Fukushima and Shiota, 1972; Pabst and Rembold, 1966; Leeming et al, 1976b).

1.1 Analysis of Biopterin Derivatives :

There are several methods available for quantitive determination of biopterin derivatives. Gas chromatography/mass spectroscopy in which the pterins



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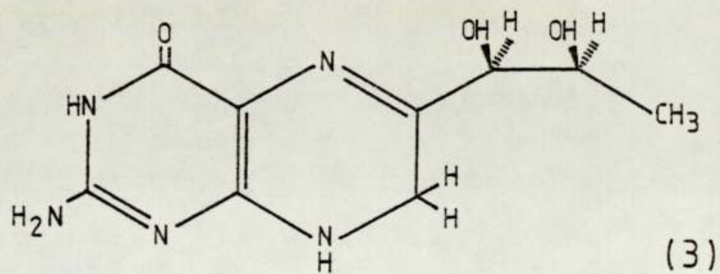

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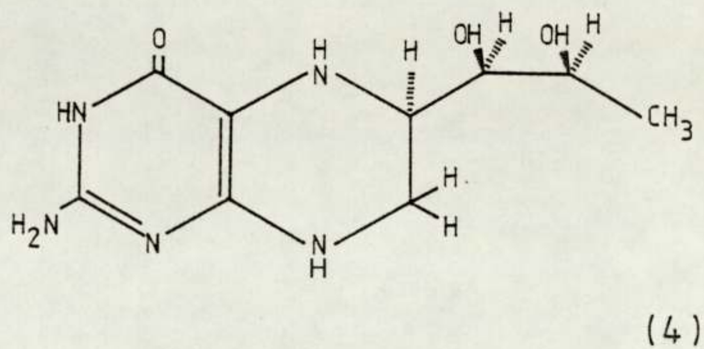
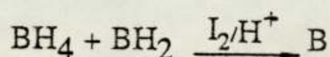


(4)

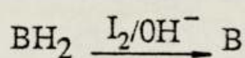
- Bonds rising out of the plane of the paper.
- Bonds going into the plane of the paper.
- Bonds in the plane of the paper.

must be converted to their trimethyl silyl derivatives to increase their volatility for separation, is sensitive and specific (Rother and Karobath, 1976). Radioimmuno-assay with an antibody raised against biopterin, can be used to determine both total and reduced forms of biopterins, by oxidation of the sample using iodine under acidic conditions to convert both dihydrobiopterin (3) and tetrahydrobiopterin (4) to biopterin (2):



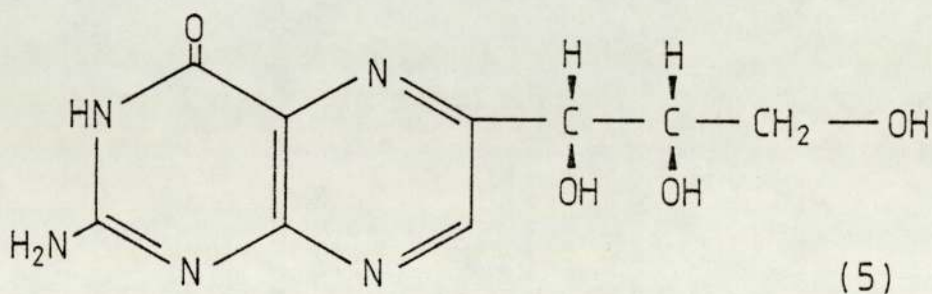
$$BH_4 + BH_2 \xrightarrow{I_2/H^+} B$$

and by oxidizing with iodine under alkaline conditions to convert dihydrobiopterin (3) only to biopterin (2) (Nagatsu et al, 1981):



$$BH_2 \xrightarrow{I_2/OH^-} B$$

A third method is using phenylalanine hydroxylase (Kaufman et al, 1978). An extensively used assay is a protozoological bioassay using Crithidia fasciculata (Leeming and Blair, 1974). This haemoflagellate parasite of the mosquito has a specific requirement for biopterin (2) or its reduced species which can only be spared at similar concentrations by L-neopterin (5) and pteroic acid (6).



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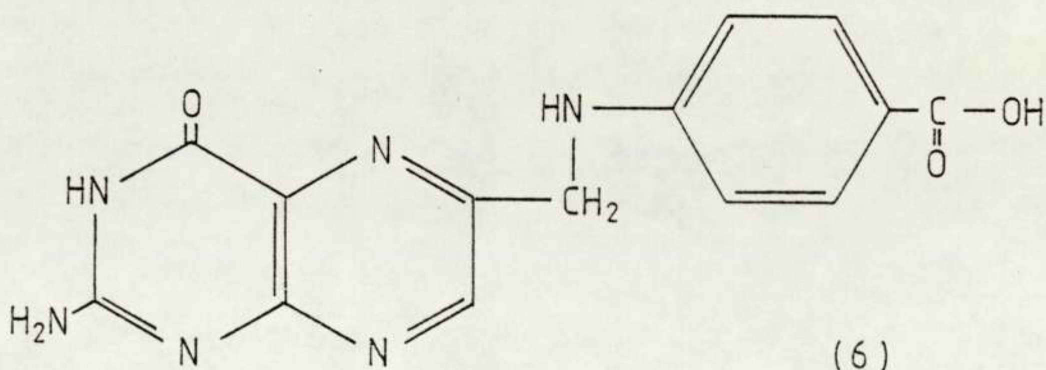


(6)

The method now most commonly used is high performance liquid chromatography (HPLC), in which the biopterins (2) are estimated after acid and alkaline oxidation, using a reversed phase column followed by fluorescent detection (Fukushima and Nixon, 1980).

1.2 Functions of Biopterin :

5, 6, 7, 8-Tetrahydrobiopterin (4) (BH_4) is a powerful reducing agent similar to ascorbate, and may function in the cell in many reducing reactions. The best studied function of BH_4 is its role in the oxidation of aromatic amino acids.

BH_4 (4) was shown to be the natural cofactor of phenylalanine hydroxylase which catalyses the conversion of phenylalanine (7) to tyrosine (8) (Kaufman, 1958; Kaufman, 1963), tyrosine hydroxylase catalysing the conversion of tyrosine (8) to L-Dopa (9) (Kaufman, 1964; Nagastu et al, 1964) as shown in Fig. (1.1), and tryptophan hydroxylase catalysing the conversion of tryptophan (12) to 5-hydroxytryptophan (13) (Jequier et al, 1969), as shown in Fig. (1.2).

The conversion of tyrosine (8) to L-Dopa (9) is a first step in the biosynthesis of the catecholamines, dopamine (10) and noradrenaline (11), a step which has been found to be rate-limiting (Levitt et al, 1964) (Fig. 1.1). The formation of 5-hydroxytryptophan (13) from tryptophan (12) is the first step in the biosynthesis of the neurotransmitter serotonin (14), and this step is rate-limiting (Costa and Meek, 1974) (Fig. 1.2).

Tetrahydrobiopterin (4), therefore has a key role in the formation of the neurotransmitters; dopamine (10), noradrenaline (11) and serotonin (14) (Leeming et al, 1981).

It has been reported by Tietz et al (1964) that the long chain alkyl ethers of glycerol are oxidized by an enzyme system requiring a reduced pyridine nucleotide, a

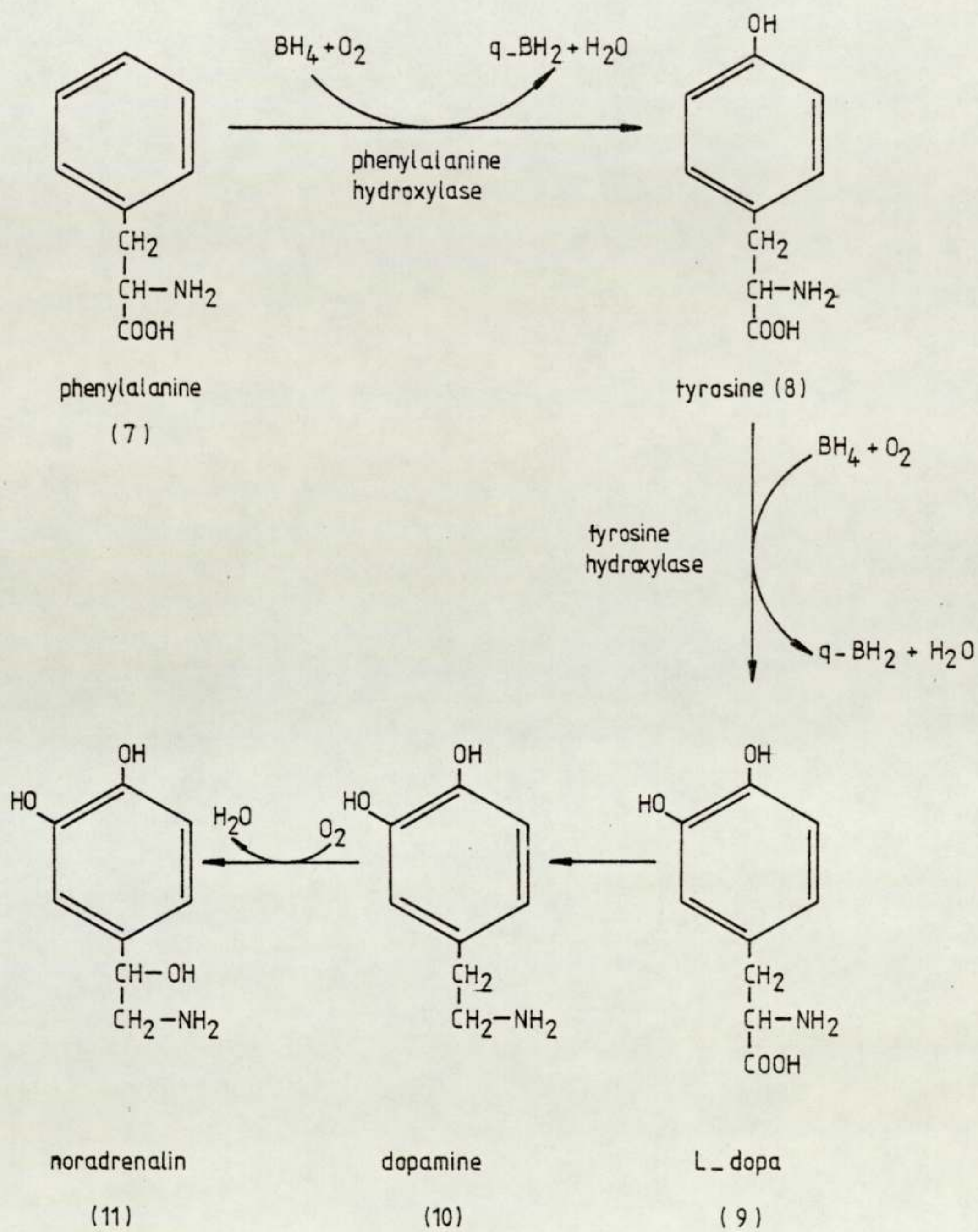


Figure 1·1 Biosynthesis of catecholamines

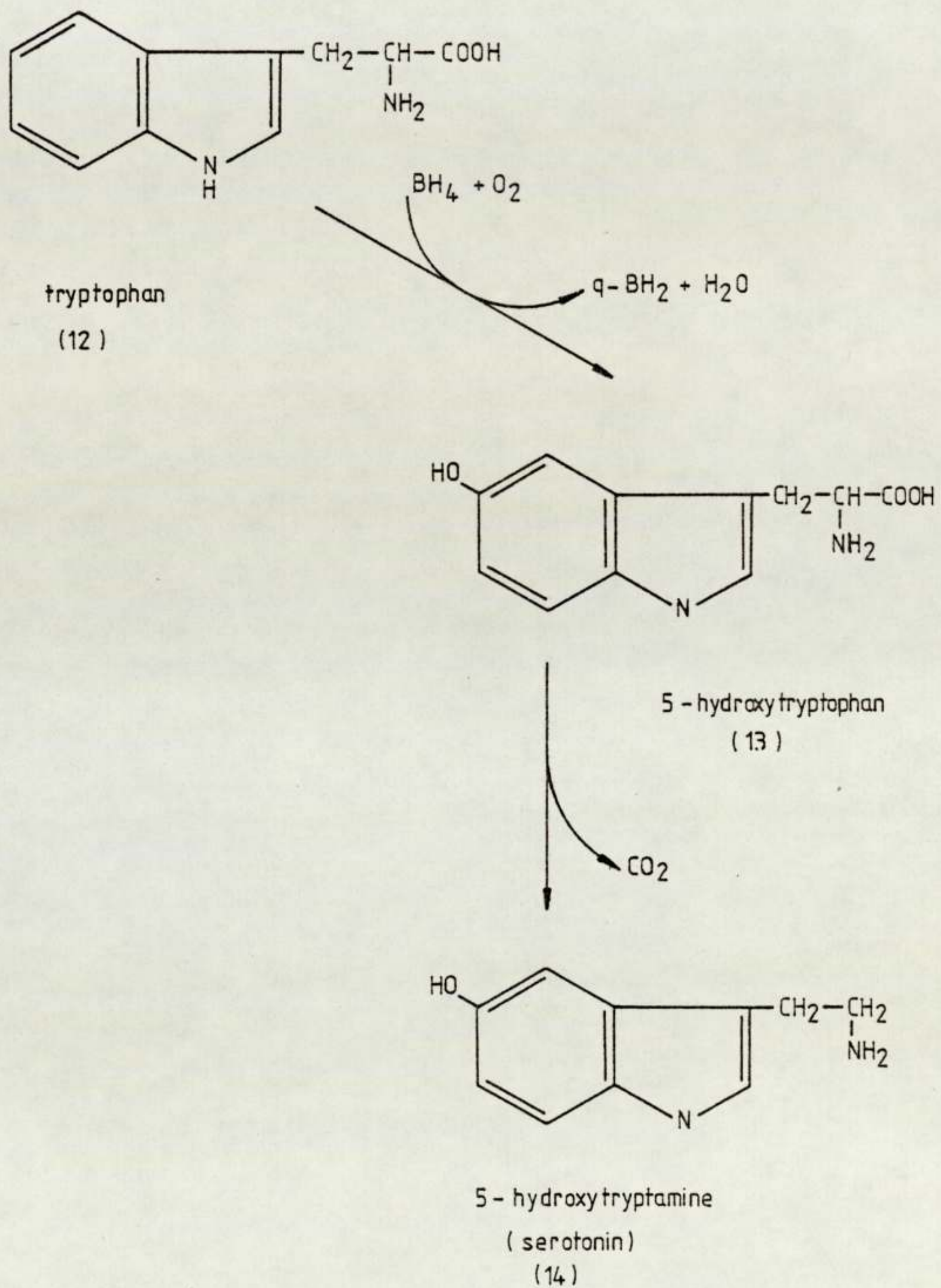
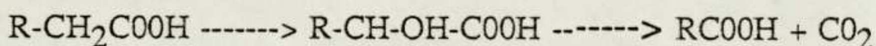


Figure 1·2 Biosynthesis of serotonin.

tetrahydropterin and molecular oxygen (Fig. 1.3). Also it has been found that the α-oxidation of fatty acids is greatly enhanced when a pteridine cofactor, 6, 7-dimethyl-5, 6, 7, 8-tetrahydropteridine is present (Macdonald and Mead, 1968). It is thought that the origin of odd chain fatty acids found in sphingolipids may be related to α-oxidation sequence.



$R\text{-}CH_2COOH$ -------> R-CH-OH-COOH -------> $RCOOH + CO_2$

Rembold and Buff (1972) suggested that BH_4 (4) is a possible cofactor in mitochondrial electron transport mediating the entry of electron at cytochrome c and a/a_2. It was found that in the presence of BH_4, mitochondria exhibit a strong increase in oxygen consumption. It suggested that the activating effect of BH_4 could be a shuttle mediating electron transport from extramitochondrial BH_4 pool via cytochrome c to intramitochondrial cytochrome c system. Another possibility is the direct diffusion of BH_4 through the mitochondrial membrane (Fig. 1.4). A scheme of the electron transport is shown in Fig. (1.5).

However Blair and Coleman (1981), using 5-methyltetrahydrofolic acid (15) found that it could reduce cytochrome c and increase the rate of O_2 consumption. They considered that a possible explanation for both their results and those of Rembold and Buff for the increase in O_2 consumption may not be due to an effect on mitochondria, but to the phenomenon of autoxidation by both 5-methyltetrahydrofolic acid (15) (5-MeTHF) and reduced pterins, which would explain the failure to observe oxidative phosphorylation. Blair and Coleman (1981), reported that 5-MeTHF appears to be incapable of acting as a carrier of reducing equivalents, and this may be result of the inability of 5-MeTHF to traverse the mitochondrial inner membrane.

Pterins have been proposed to act as stabilized reductants of the primary photochemical act of photosynthesis (Fuller and Nugent, 1969). It has been found that

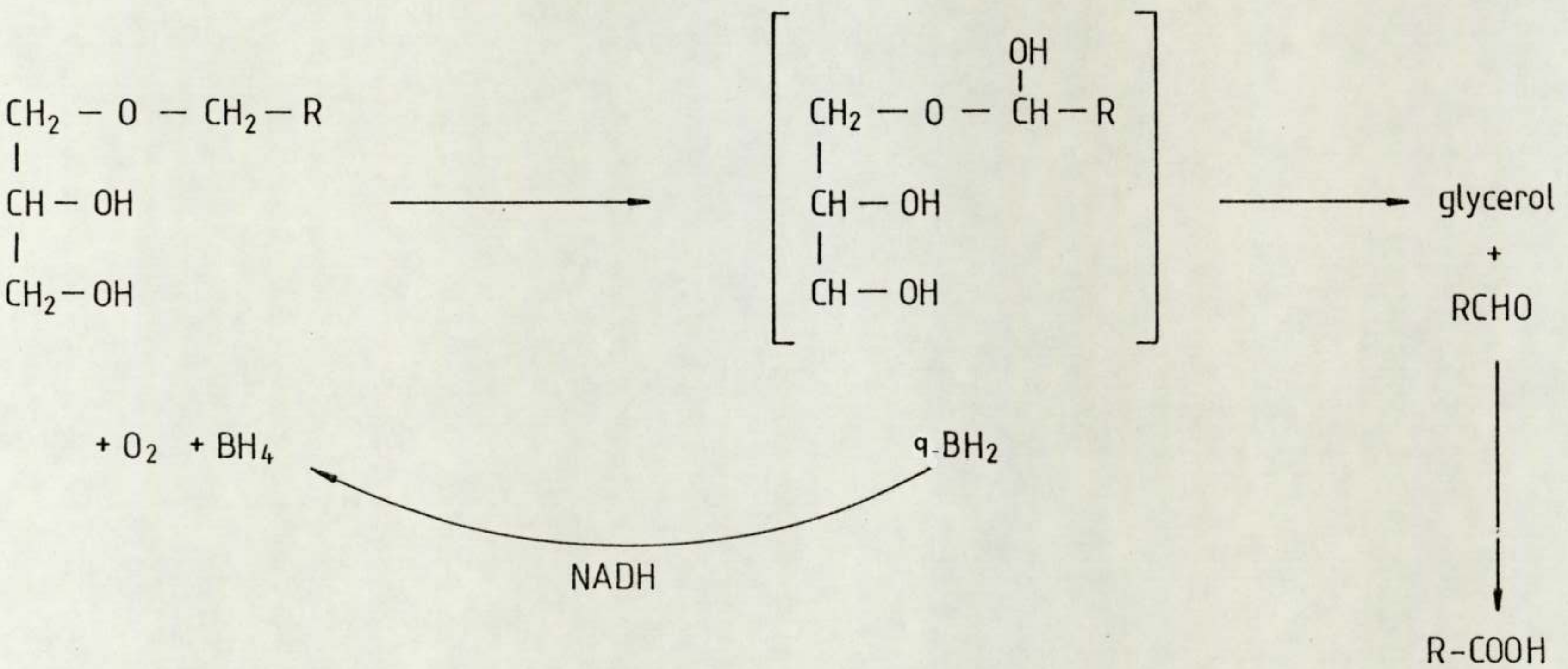


Figure 1·3 The oxidation of glycerol ethers. (Tietz et al, 1964)

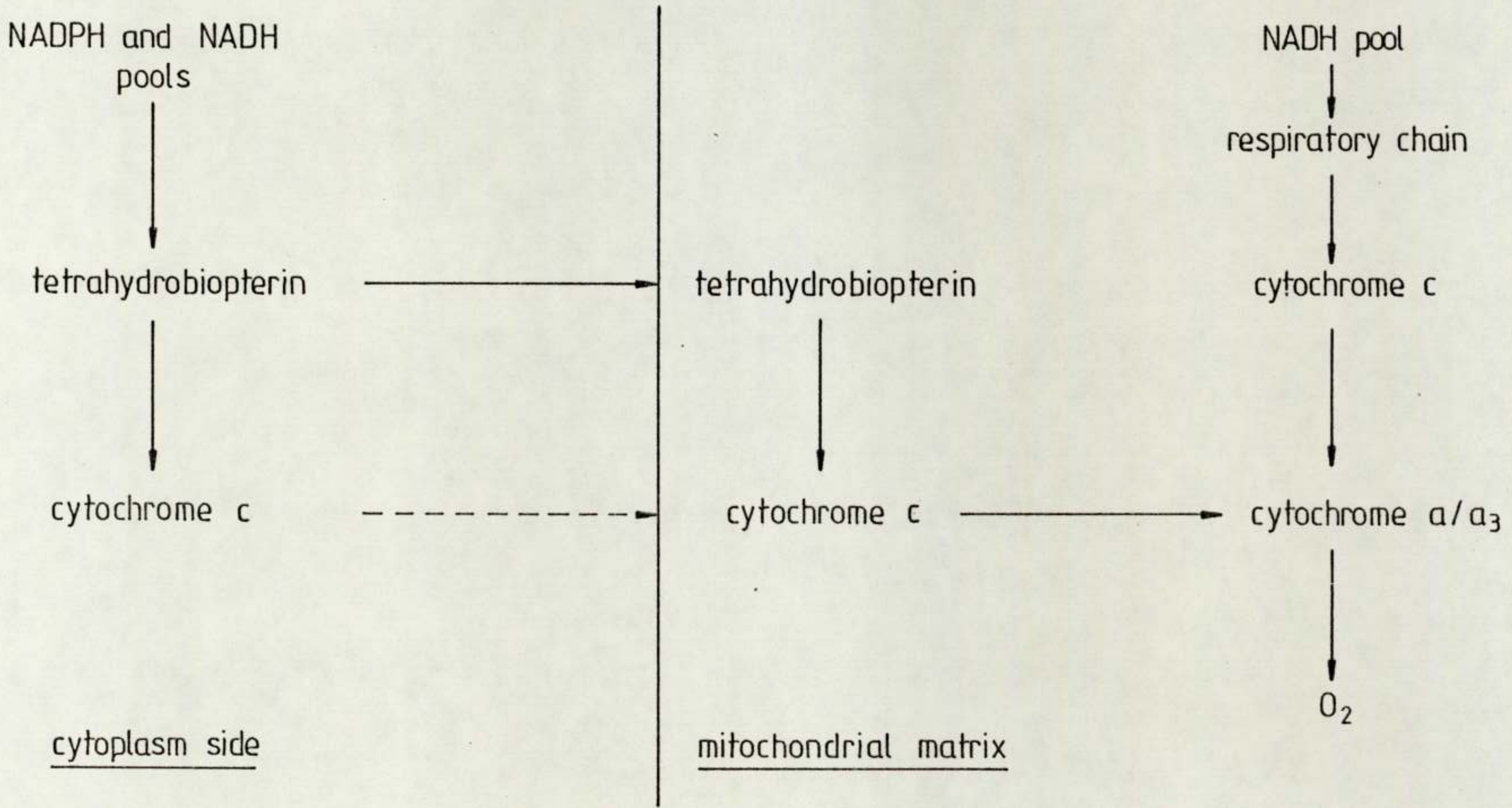


Figure 1·4 Tetrahydrobiopterin in mitochondrial electron transport.

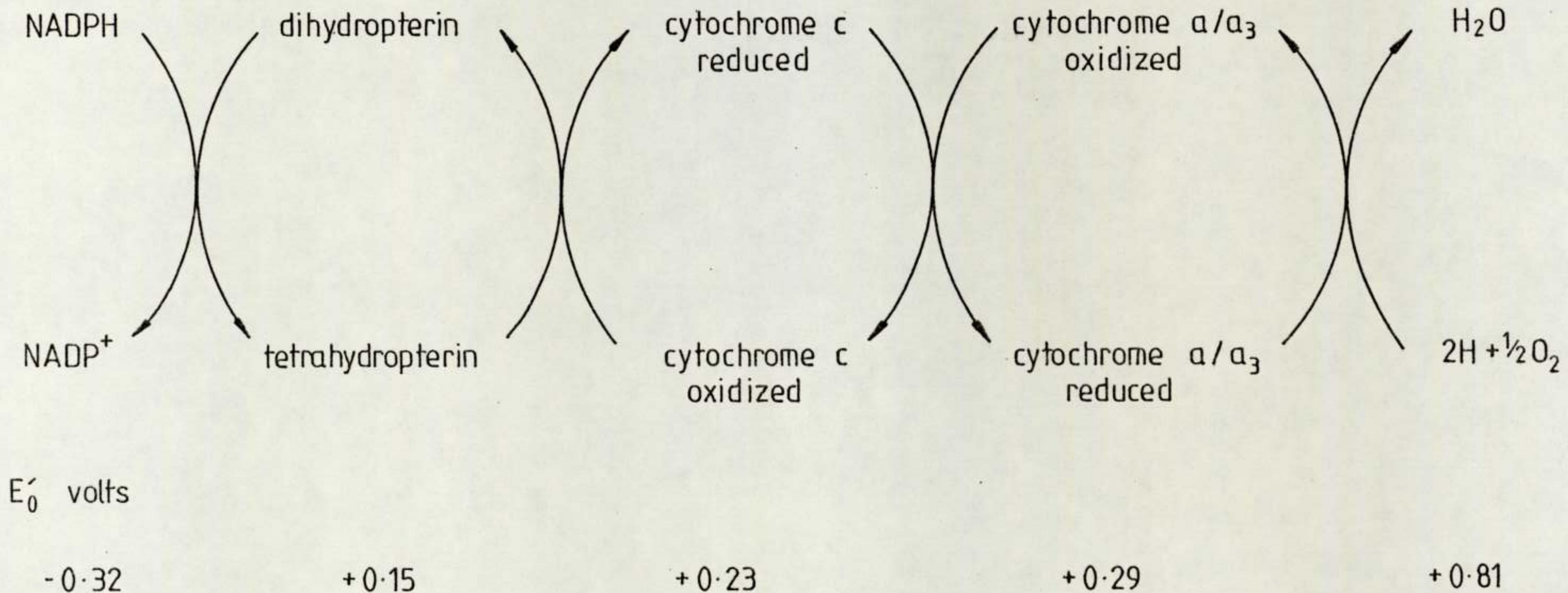
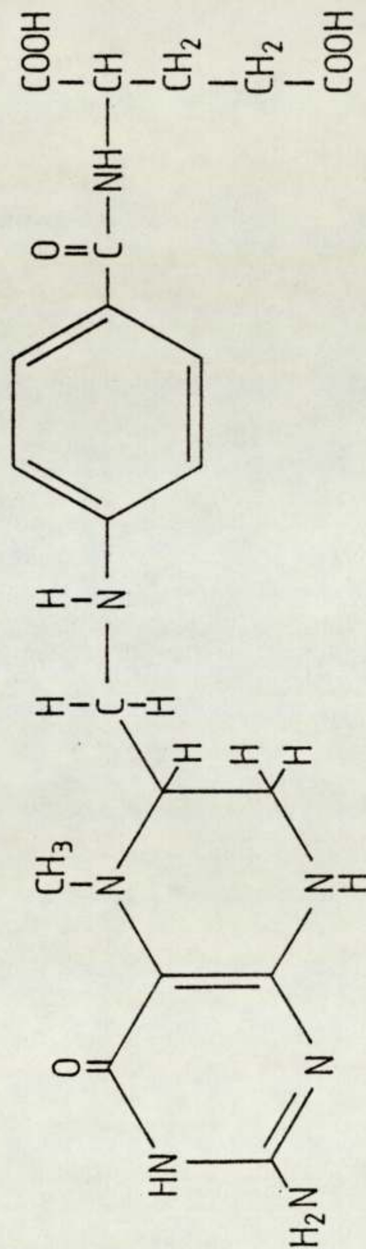


Figure 1·5 A scheme of electron transport



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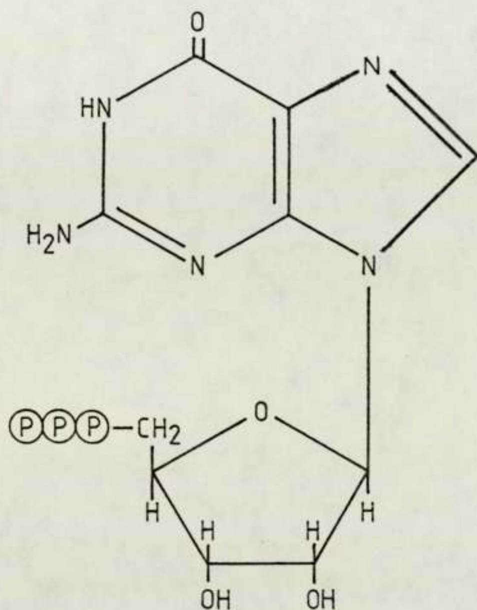
2-amino-4-hydroxy-6-substituted pteridines fulfill the biological, chemical and physiochemical requirements of a primary photochemical electron acceptor in photosynthesis.

Melatonin biosynthesis is proposed to be regulated by a pteridine. The pteridine is proposed to be broken photolytically to 6-formylpterin, which is then transported to the pineal gland, where it inhibits hydroxyindole-O-methyl-transferase, the rate-limiting enzyme in melatonin biosynthesis (Cremer-Bartels and Ebels, 1980). Recently Milstien and Kaufman (1983) have suggested that melatonin and N-acetyl serotonin could regulate biopterin biosynthesis in the pineal gland making it light dependent.

1.3 Biosynthesis of Tetrahydrobiopterin :

Because of structural similarities, purines and pyrimidines were early considered as potential starting materials for pterin biosynthesis. The conversion of guanosine triphosphate (GTP)(16) to D-erythro-dihydroneopterin triphosphate (NH_2TP) (17), is generally accepted as the initial step in biopterin synthesis. This step is catalysed by the enzyme GTP cyclohydrolase (D-erythro-7, 8-dihydroneopterin triphospate synthetase). The mechanism by which the transformation of GTP (16) to NH_2TP (17) occurs was originally proposed by Burg and Brown (1968), which involves an Amadori type rearrangement (Fig. 1.6).

The current hypothesis for the biosynthesis of BH_4 is that NH_2TP, produced enzymically from GTP, can be converted directly to an intermediate with a tetrahydropterin ring system (Milstien and Kaufman, 1983; Smith and Nichol, 1984; Heintel et al, 1984; Switchenko et al, 1984; Milstien and Kaufman, 1985: Switchenko and Brown, 1985), as shown in Fig.(1.7).



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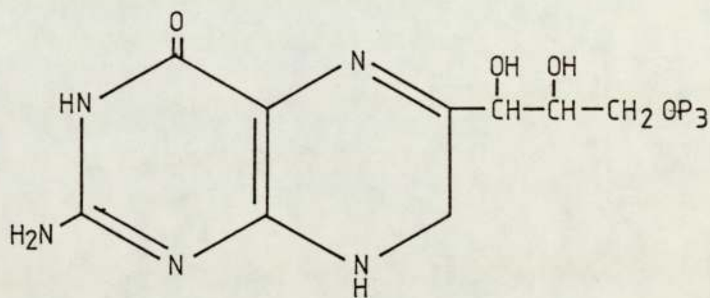

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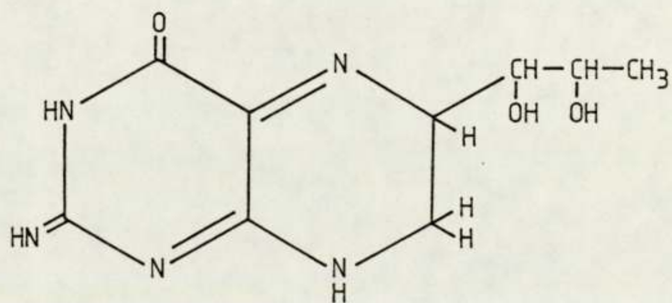

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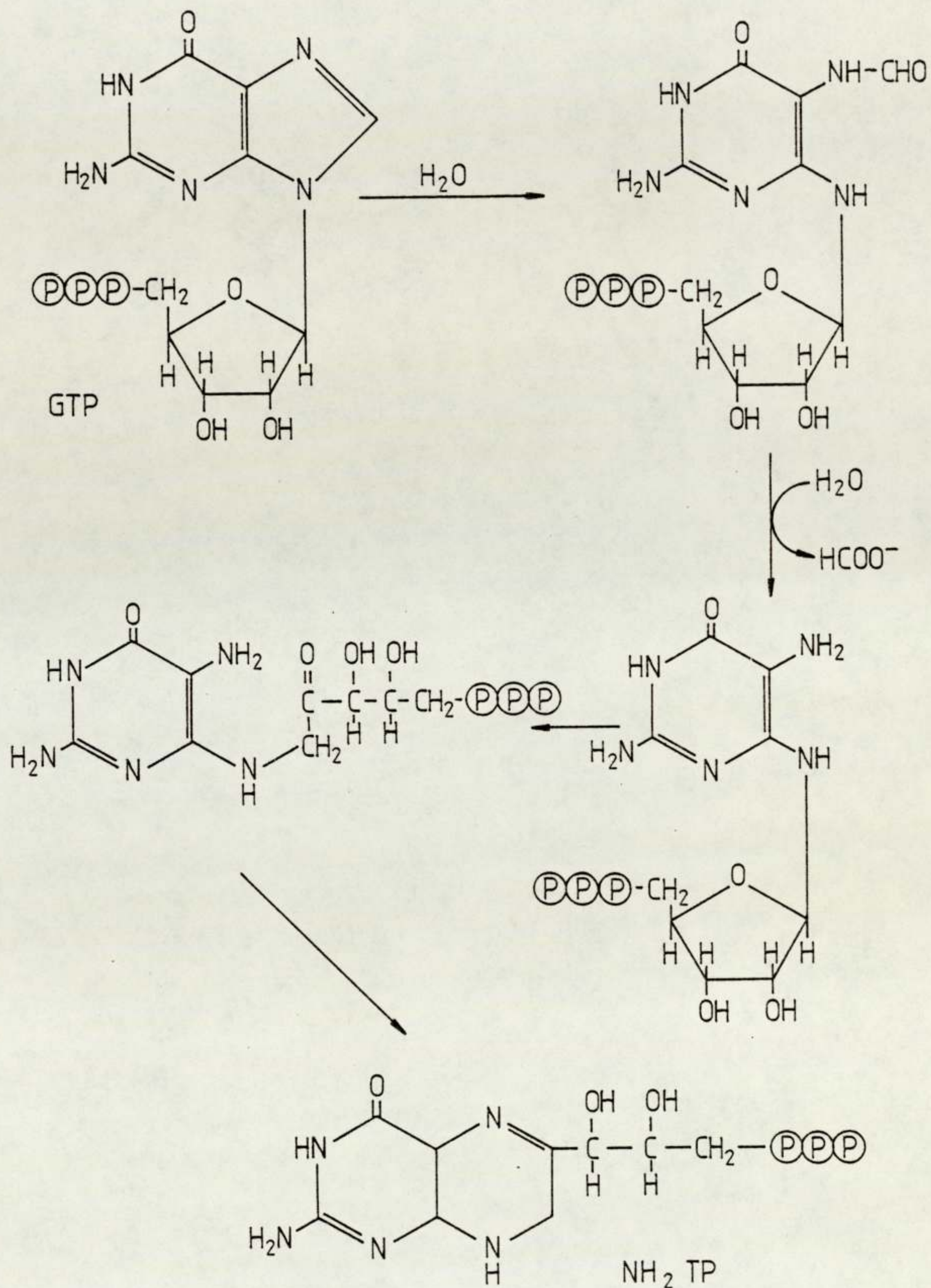


Figure 1·6 The conversion of GTP to NH_2TP as catalyzed by GTP cyclohydrolase.

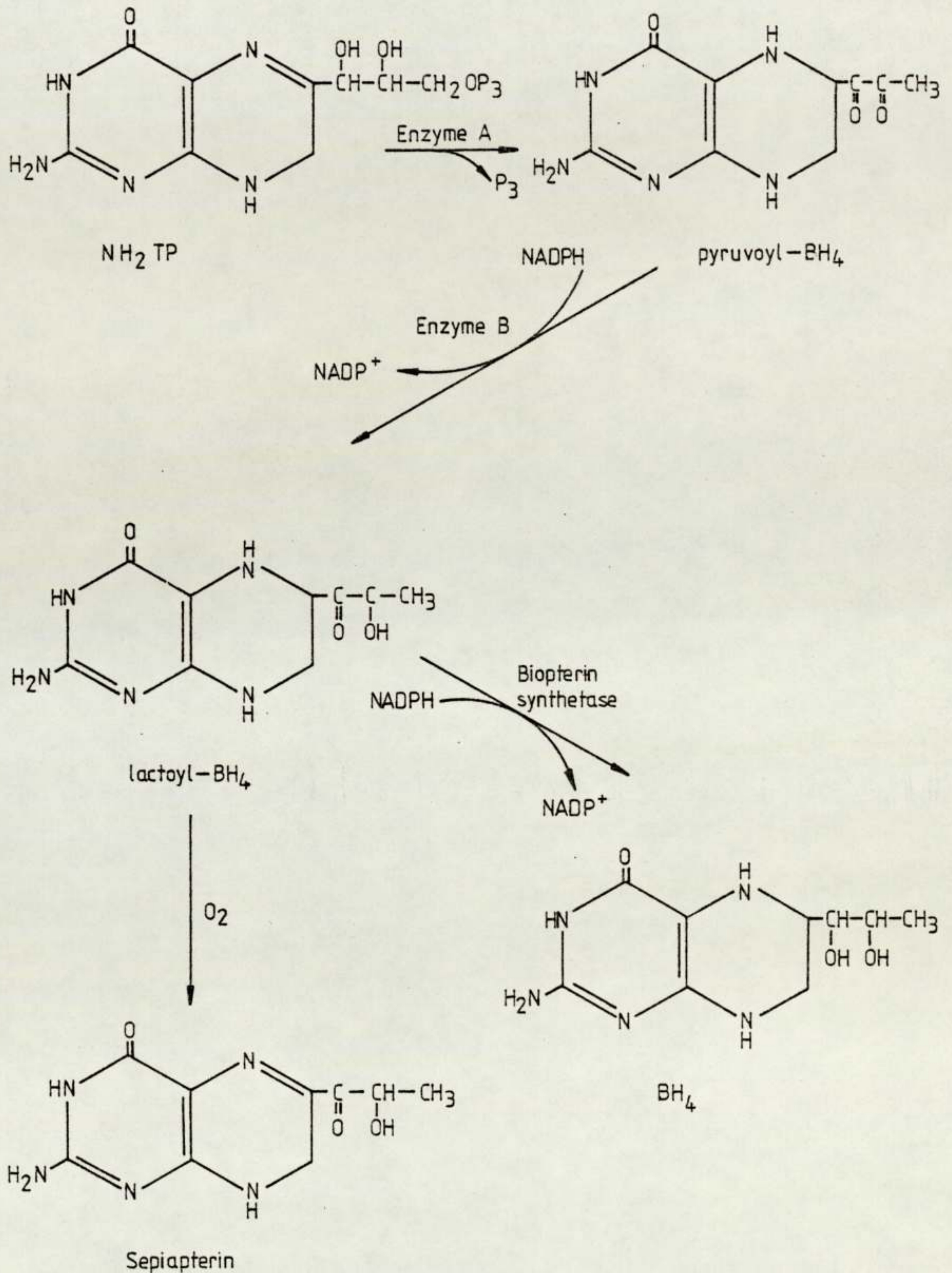


Figure 1·7 Enzymic reactions for the conversion of NH_2TP to BH_4 (Switchenko <u>et al</u> ,1984)

1.4 Oxidation of Tetrahydrobiopterin :

Tetrahydrobiopterin (BH_4) is oxidized quantitatively to quinonoid dihydrobiopterins (q-BH_4) at physiological pH by several reagents including : molecular oxygen (Viscontini and Bobst, 1965; Archer et al , 1972; Pearson and Blair, 1975; Mager, 1975), peroxidase in the presence of hydrogen peroxide (Nielsen et al, 1969) or oxygen (Armarego et al, 1983), bromine (Lazarus et al, 1982), 2, 6-dichlorophenolindophenol (Nielsen et al, 1969; Archer et al, 1972), ferric iron (Archer et al, 1972), potassium ferricyanide (Nielsen et al, 1969; Archer et al, 1972) and ferricytochrome c (Hasegawa et al, 1978). The rate of oxidation by oxygen alone is relatively slow and can be catalysed by trace metals such as iron (III) (Viscontini and Okada, 1967) and copper (II) (Blair and Pearson, 1974).

The detailed mechanisms of the conversion of BH_4 (4) to quinonoid dihydrobiopterin (q-BH_2) (18) by the various oxidants are not known. However, some work on the eludication of the mechanism of aerobic oxidation has been attempted. There is evidence that on oxidation, BH_4 (4) forms a free radical (Bobst, 1967), that the radical is localized mainly at the bridgehead carbon C4a (Blair and Pearson, 1974), that the oxidation is inhibited by radical scavengers (e.g. phenol) (Armarego and Waring, 1982; Blair and Pearson, 1974), and is catalysed by certain metal ions (Fe^{+++} , Cu^{++}) (Viscontini and Okada, 1967; Blair and Pearson 1974), that as the reaction proceeds it becomes autocatalytic (Mager and Berends, 1965), that hydrogen peroxide is formed as oxidation proceeds (Blair and Pearsons, 1974), that superoxide may be formed (autoxidation rate is decrease in the presence of superoxide dismutase) (Kaufman and Fisher, 1974; Blair and Pearson, 1975), and that the initial rate of autoxidation is decreased by addition of catalase and bovine serum albumin (Ayling et al, 1973).

A mechanism whereby the C-4a radical is further oxidized by a one electron transfer (with formation of hydrogen peroxide) to yield q-BH_2 (18) has been proposed (Blair and Pearsons 1974) (Fig. 1.8).

1.5 Role of Dihydropteridine reductase in Biopterin metabolism :

Dihydropteridine reductase DHPR [EC 1.6.99.7] is the enzyme which converts quinonoid dihydrobiopterin (18) back to BH_4 (4), in the presence of reduced pyridine nucleotide (NADH) (Craine et al, 1972).

If the quinonoid dihydrobiopterin (18) is not converted to BH_4 (4), it undergoes a non-enzymatic tautomerization into 7,8-dihydrobiopterin (3) which is not a substrate for DHPR and therefore it cannot be converted to the active cofactor by this route. The levels of 7, 8-dihydrobiopterin then build up in the cell, move out into the serum and are excreted in the urine (Fig. 1.9). DHPR recycles BH_4 (4) not only for phenylalanine hydroxylase (Kaufman, 1958), but also for the related enzymes tyrosine hydroxylase and tryptophan hydroxylase (Kaufman and Fisher, 1974). So the physiological role of DHPR is the regeneration of BH_4, which is essential for the biosythesis of neurotransmitters, as shown in Fig.(1.9); thus a lack of DHPR leads to impaired biosynthesis of neurotransmitters and is the cause the neurological deterioration observed in patients with this disorder.

Another role for DHPR is the salvaging of oxidized tetrahydrofolate, this role may be especially important in the brain, which only has low dihydrofolate reducase activity. Polleck and Kaufman (1978) reported that the folate content of the brain of a patient lacking DHPR was about 40 fold lower than that of control. Clinical evidence suggests that folate deficiency in brain may be related to severe neurological and mental deterioration.

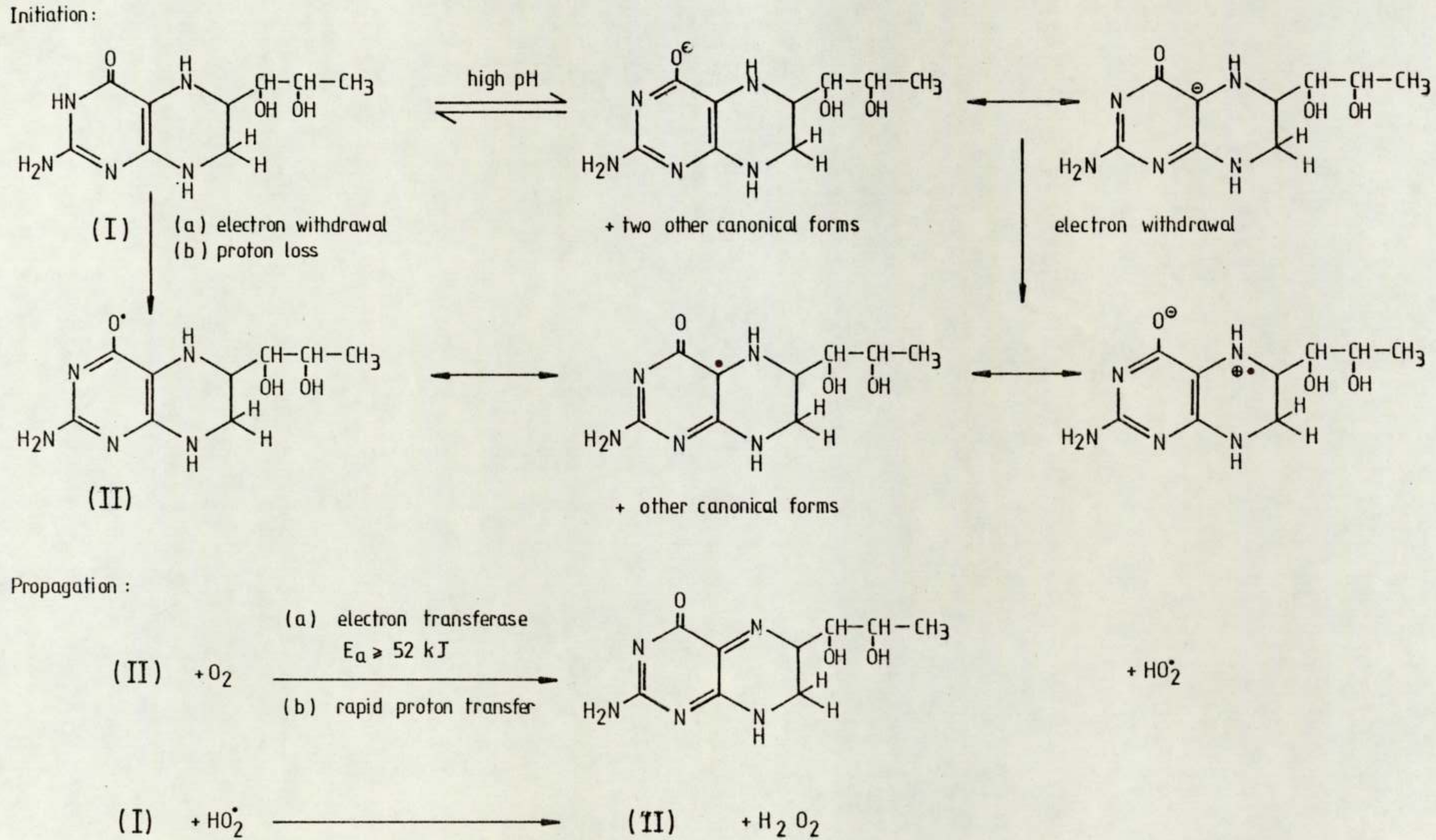


Figure 1·8 Mechanism of autoxidation of tetrahydropterin (Blair and Pearson, 1974)

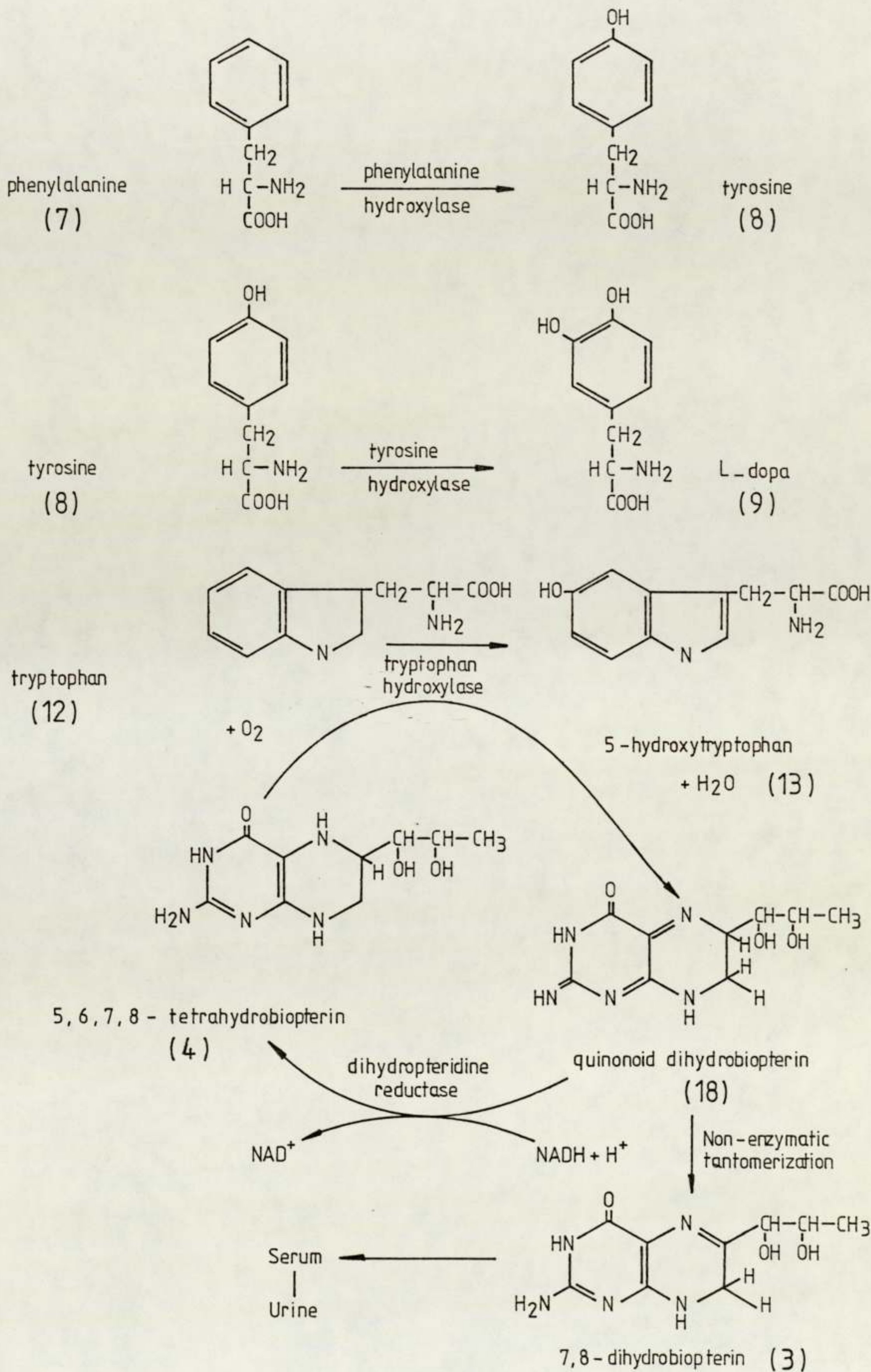


Figure 1·9 The role of tetrahydrobiopterin in the hydroxylation of phenylalanine, tyrosine and tryptophan, showing the salvage of quinonoid dihydrobiopterin by DHPR. (Leeming, Pheasant and Blair, 1981)

1.6 Biopterin Metabolism and Human Diseases :

The hydroxylation of phenylalanine (7) requires phenylalanine hydroxylase, BH_4 (4) and molecular oxygen. Failure of this reaction leads to raised serum phenylalanine levels which, uncontrolled, are accompanied by severe mental retardation (Fölling, 1934). The common form of phenylalaninaemia (classical phenylketonuria) is caused by a gross deficiency of phenylalanine hydroxylase (Jervis, 1947); treatment is by a restriction of phenylalanine intake (Bickel et al, 1953). Recently attention has been drawn to BH_4 by reports of inherited defects in BH_4 metabolism which lead to "malignant hyperphenylalaninaemia" (Danks et al, 1978). These cases, unlike classical phenylketonuria, do not develop normally if phenylalanine intake is restricted. It was noted that there are lowered neurotransmitter levels and progressive neurological disease with death in early childhood (Smith et al, 1975).

A deficiency of BH_4 can arise from a failure to salvage q-BH_2(18) by DHPR or a failure to synthesis BH_4 de novo (Fig.1.7). Examples of both these types of inherited disorder have been reported (Kaufman et al, 1975; Leeming et al, 1976a; Rey et al, 1977; Bartholomé et al, 1977; Danks et al, 1979; Danks and Cotton, 1980). In these cases of BH_4 deficiency in which the de novo biosynthesis is impaired, the block was between NH_2TP (17) and BH_4, but recently yet a new variant of phenylketonuria was found, in which the block was between GTP (16) and NH_2TP (17), i.e. with GTP cyclohydrolase deficiency (Niederwieser et al, 1983).

Biopterin metabolism appears to be distributed in a number of disease states other than hyperphenylalaninaemia. Elevation in blood and serum biopterin levels has been reported in patients with kidney dysfunction (Leeming et al, 1976b).

Lowered serum levels have been demonstrated in pernicious anaemics, schizophrenics, leukaemia, malignant carcinoid disease, senile dementia, coeliac disease and lead poisoning (Leeming et al, 1976b; Leeming and Blair, 1980 a; Leeming and Blair, 1980 b).

Barford et al (1984) have found that the temporal lobes from subjects with senile dementia of Alzheimer type (SDAT) had impaired ability to synthesis BH_4 compared to age-matched controls. The defects in BH_4 synthesis was not due to loss of brain cells, but to loss of ability to convert NH_2TP to BH_4.

Many metals have been found to inhibit both de novo biopterin biosynthesis and the salvage pathway catalysed by DHPR, in particular lead has received attention (Purdy et al, 1981). Aluminium also has been found to inhibit DHPR activity in vitro at concentrations close to those found in the brains of patients with dialysis dementia (Leeming and Blair, 1979; Brown, 1981).

1.7 Aim of the thesis:

Recent studies have indicated the involvement of DHPR in human biopterin metabolism, particularly in relation to a variety of neurological disorders, as mentioned earlier.

Although DHPR has been studied extensively in animal tissues, very few studies have been done in human tissues. An understanding of the human enzyme is needed to elucidate the importance of this enzyme in human pathological conditions.

This thesis describes the isolation of DHPR enzyme from human brain followed by detailed activity and inhibition studies on this purified enzyme, as well as the assay and kinetics of this enzyme in various human tissues; blood, breast, large intestine and brain with a variety of pathological and environmental conditions. The effect of various agents on biopterin derivatives in vivo, has also been studied by their

administration to rats follwed by direct analysis of the brain biopterin.

The results obtained from human and rat material will be used in relationship with the results obtained by others to provide further information into the metabolism of this vital pteridine, and to follow the administration of some potentially neurotoxic agents.

CHAPTER 2

PURIFICATION OF HUMAN BRAIN DHPR

CHAPTER (2) :

PURIFICATION OF HUMAN BRAIN DHPR:

2.1 INTRODUCTION

DHPR has been isolated from several different sources by a variety of methods. Recently affinity chromatography methods have been used to isolate DHPR, as they are much simpler to perform and high yield of enzyme is obtained.

Affinity columns used include ; methotrexate-amino-hexyl-sepharose (Webber et al, 1978), immobilized Cibacron blue (blue dextran agarose) (Chauvin et al, 1979), 5-AMP Sepharose (Aksnes et al, 1979, Armarego and Waring, 1983), Matrix gel blue (Shen and Abell, 1981) and a naphthoquinone AH-Sepharose column (Firgaira et al, 1981; Armarego and Waring, 1983, Cotton and Jennings, 1978, Purdy et al, 1981, Blair et al, 1984).

The molecular weight for DHPR from the different sources ranges from 41,000 to 55,000 except for the NADPH dependent enzyme which is 70,000 (Nakanishi et al, 1977). The enzyme exists as a dimer composed of 2 subunits of M.Wt. between 21,000 and 27,000 (Firgaira et al, 1981, Aksnes et al, 1979, Craine et al, 1972, Brown, 1981), the subunits appear to be identical as shown by their identical electrophoretic behaviour under a number of different conditions (Firgaira et al, 1981).

Recently, the effect of various potential inhibitors on DHPR activity has been investigated. A number of reports have appeared concerning the inhibition of DHPR by catecholamines and related compounds. In 1980, Purdy and Blair reported the inhibition of rat liver DHPR by the neurotransmitters; noradrenaline (1) , dopamine (2) and serotonin (3) (Purdy et al, 1981). However, Armarego and Waring (1983) have shown that dopamine (2), adrenaline (4) and noradrenaline (1) do not inhibit DHPR at concentration below 200 μM, but their oxidation products, the respective

aminochrome (5, 6, 7) are inhibitors.

Phenylalanine (8) has been shown to have no effect on the activity of DHPR in skin fibroblasts from either normal children or patients with classical phenylketouria (Schlesinger et al, 1976, Guttler et al, 1977, Firgaira et al, 1978). However, phenylalanine (8), phenyllactate (9) and phenylpyruvate (10) are inhibitory to DHPR purified from rat liver (Purdy and Blair, 1980).

Blair et al (1984b) have found that 1-methyl-4-phenyl- 1,2,5,6 - tetrahydropyridine (MPTP) (11), the specific potent neurotoxin causing Parkinsonism in man (Langston et al, 1983; Langston and Ballard, 1983), is a competitive inhibitor of DHPR with respect to NADH and a mixed inhibitor with respect to $DMPH_4$.

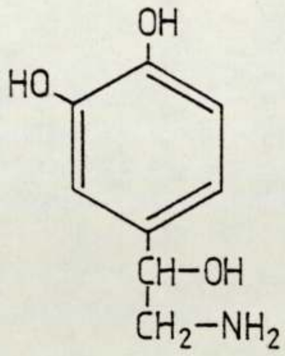
Aluminum inhibits the salvage of quinonoid dihydrobiopterin in vitro at concentrations close to those found in the brains of patients with dialysis dementia (Leeming and Blair, 1979; Brown, 1981), and the inhibition by aluminum of DHPR has been proposed as a mechanism for its neurotoxicity (Leeming and Blair, 1979).

To date, the inhibitory effects of the above mentioned potential inhibitors; phenylpyruvate (10), 6-hydroxydopamine (12), MPTP (11) and aluminium on DHPR purified from human brain, have not been mentioned in the literature. Accordingly, we have studied the inhibitory effects of those potential inhibitors on DHPR from human brain, in an attempt to describe in detail the changes in biopterin metabolism that accompany various neurological disease states.

2.2 Materials and Methods:

2.2.1 Chemicals:

Nicotinamide adenine dinucleotide reduced (NADH) (disodium salt type III), 6, 7-dimethyl-5, 6, 7, 8-tetrahydropterin ($DMPH_4$), carbodiimide, dithiothreitol, peroxidase and Tris [tris(hydroxymethyl) amino ethane] were purchased from the



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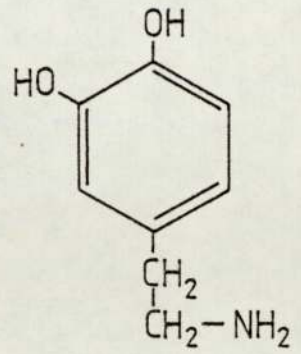

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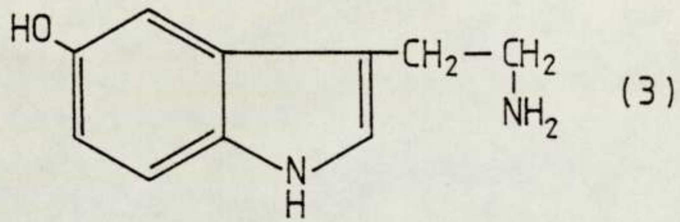

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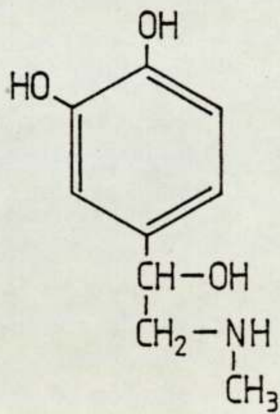

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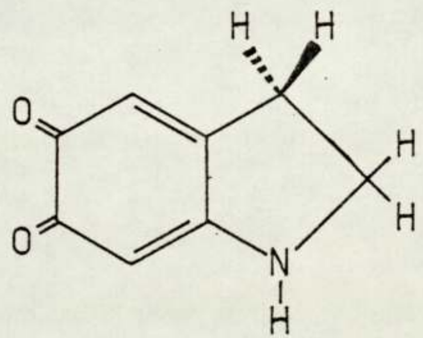

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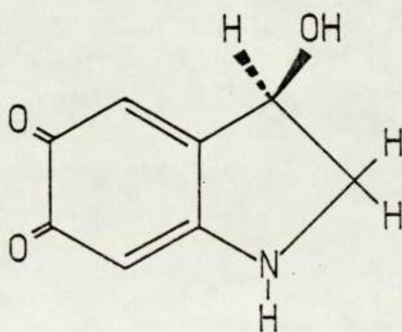

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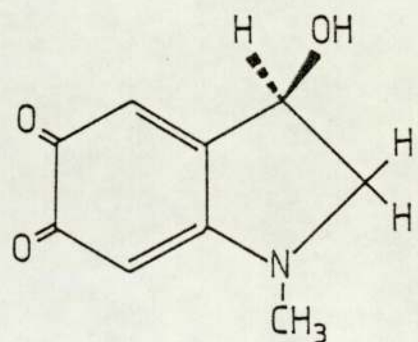

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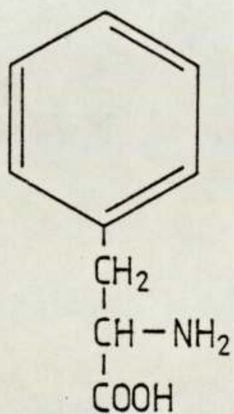

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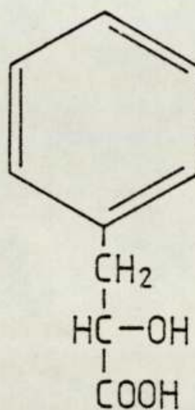

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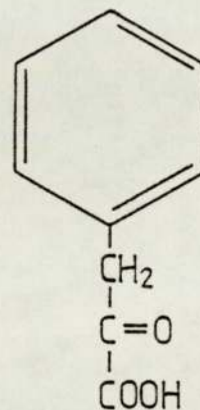

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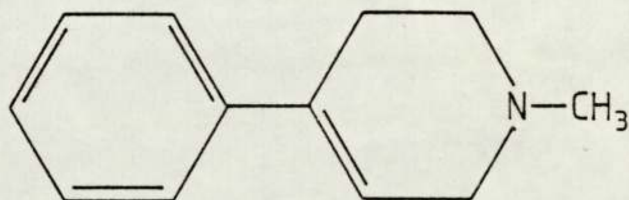

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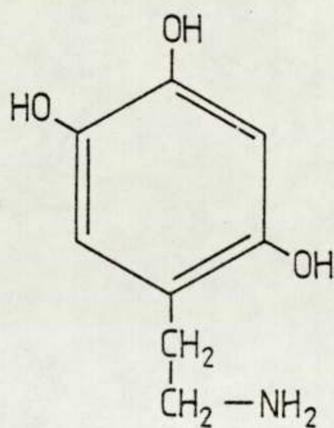

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Sigma Chemical Company. AH- Sepharose 4B and Blue-Sepharose were obtained from Pharmacia fine chemicals, sodium-1, 2-naphthoquinone-4-sulphonate was obtained from Calbiochem. All other reagents were from BDH (Poole) and were of analytical grade.

2.2.2 Methods:

Dihydropteridine reductase was isolated from human brain using a modified version of the affinity chromatography method of Armarego and Waring (1983).

1 - Preparation of the affinity adsorbents:

a - AH - Sepharose 4B :

7.5 gm. of AH-Sepharose 4B was swollen in excess 0.5 M NaCl, lactose and dextran were removed by washing the Sepharose in 1500 ml. of 0.5 M NaCl. The Sepharose was then washed in 1500 ml. of distilled water to remove the NaCl.

0.375 gm. of sodium-1, 2-naphthoquinone-4-sulphonate was dissolved in 10 ml. water and the solution was added to the gel. The pH was adjusted to between 4.5 and 6.0 and the slurry was stirred gently at room temperature for 1 hour.

Carbodiimide (1.86 gm.) was dissolved in a small amount of distilled water, and then added dropwise to the ligand/gel slurry maintaining the pH at 4.0-6.5 for 1 hour. The mixture was then shaken in the dark for 24 hours. The gel was then washed with 75 mls. of 0.1 M sodium acetate buffer (pH 4.0) containing 1M NaCl, followed by 750 mls. of distilled water and finally 750 ml. of 0.1 M Tris/HCl buffer (pH 7.6) containing 0.8 M NaCl. The gel was stored in the latter buffer in the refrigerator.

b - Blue-Sepharose :

2 gm. of Blue-Sepharose was swollen in excess 50 mM potassium phosphate buffer (pH 6.8), and then the gel washed in 500 ml. of phosphate buffer. The gel was stored in the same buffer in the refrigerator.

2 - Preparation of the tissue extract:

Human brain tissues from the temporal lobe were obtained from patients institutionalised at St. Lawrences Hospital (Caterham, Surrey), courtesy of Dr. P. Sylvester. These subjects were mentally subnormal but of undefined aetiology.

A 20% (w/v) homogenate of human brain was prepared in 0.1 M Tris/maleate buffer (pH 6.8) using a Potter Elvejhem homogeniser. The homogenate was centrifuged at 40,000 x g for 40 minutes at $0^{o}C$. in a M.S.E. Superspeed 50 centrifuge. The protein content of the supernatant was measured using the Lowry method (Lowry et al, 1951), DHPR activity was measured using a modified assay (Craine et al, 1972).

3 - Assay of dihydropteridine reductase:

Each incubation contained 10^{-4} M NADH, 10^{-3}M H_2O_2, 8mgm. horseradish peroxidase, 2.5 x 10^{-4}M sodium azide, 10^{-4}M 6, 7-dimethyltetrahydropterin, in 0.05M Tris-maleate buffer pH 6.8 and 0.02 ml. of brain extract in a total volume of 1 ml. All the components were put into plastic cuvettes (1 ml., 1 cm. pathlength), with the exception of $DMPH_4$, inverted and incubated for 90 sec. in the spectrophotometer to allow the assay to equilibrate to 37 ^{o}C. The assay was started by the addition of $DMPH_4$. The blanks contained no enzyme preparation as control assays were run for the reductase assay but in the

absence of $DMPH_4$.

The rate of reaction was followed by measuring spectrophotometrically the decrease in optical density at 340 nm, due to the disappearance of reduced NADH at 37 ^{o}C. in a Pye Unicam PU 8800 spectrophotometer with constant temperature cell holder. After a delay of 30 seconds the instrument gave the rate as change in concentration/minute (nmoles/min.) taking five readings at 10 second intervals and giving the mean. The results were then corrected to 1 mg. of protein and finally calculated as nmoles NADH oxidised per minute per mg. protein. A molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH at 340 nm, was used to calculate the enzyme activity.

4 - Purification of DHPR:

The human brain extract was fractionated with ammonium sulfate, when all the activity precipitated at 30-55% saturation. The precipitated protein was dissolved in 5 ml. of 0.1 M Tris-HCl (pH 7.6), made 0.8 M in NaCl and 0.1mM in NADH, and 4 ml. of that applied to the naphthoquinone affinity column (18 x 1.0 cm.i.d.) pre-equilibrated with this buffer. The column was washed with 20 ml. of 0.1 mM in NADH (buffer A); 20 ml. of 0.1 M $NaHCO_3$-NaOH buffer (pH 10), made 0.8 M in NaCl and 0.1 mM in NADH; 15 ml. of buffer A without NADH and finally with 30 ml. of buffer A containing no NADH but made 10 mM in DTT.

Active fractions were pooled and applied to a Blue Sepharose CL-6B column (6 x 1.0 cm.i.d.) equilibrated with 50 mM potassium phosphate buffer (pH 6.8). After washing with 15 ml. of the same buffer, DHPR was eluted with 30 ml. of 50 mM potassium phosphate buffer (pH 6.8) containing 100 mM NADH. Approximately 3 ml. fractions were collected, the protein content and DHPR activity of each fraction was measured as before. The fractions were made 2mM DTT and

0.02 mM NADH before storing in the freezer.

The naphthoquinone affinity absorbent was regenerated by washing the column in 15 ml. of 0.1 M sodium acetate buffer (pH 4.0) containing 1 M NaCl, and then 1.5 ml. of 0.1 M Tris/HCl buffer (pH 7.6) containing 0.8 M NaCl, while the Blue-Sepharose gel was regenerated by washing the column in 30 ml. of 50 mM potassium phosphate buffer (pH 6.8).

2.2.3 Measurement of Km Values :

The effect of 6, 7-dimethyl-5, 6, 7, 8-tetrahydropterin and NADH concentration on the DHPR activity were examined. Km Values were determined by variation of the concentration of one substrate at satuarating levels of the other substrate.

2.2.4 Estimation of the Molecular Weight:

The M.Wt. of the purified enzyme was estimated using a Sephadex G-100 column (60 x 1.5 cm.i.d.), calibrated with cytochrome c (M.Wt. 12,400), horseradish peroxidase (M.Wt. 40,000) and ovalbumin (M.Wt. 43,000). Samples were applied to the column equilibrated with 0.05 M Tris-HCl buffer (pH 7.6) containing 2mM DTT and were eluted in the same buffer, 5 ml. fractions were collected.

2.2.5 Inhibition Studies :

Inhibition studies were performed with different concentrations of the potential inhibitors; phenylpyruvate, 6-hydroxydopamine and MPTP. The type of inhibition, inhibition constant K_i and and dissociation constant K_i' were estimated using Dixon (1953) and Cornish-Bowden (1974) methods.

With aluminium as inhibitor, DHPR activity was also measured by pre-incubating the enzyme for various lengths of time in the presence of various concentrations of aluminium at 37 ^{o}C, before the substrates were added. Appropriate controls were also examined by pre-incubating the enzyme at 37 ^{o}C for various lengths of time before adding the substrates and measuring the activity in the usual way.

In the case of aluminium, the removal of aluminium from DHPR was investigated, after the enzyme had been incubated with 10^{-4} M aluminium sulphate, by dialysing at 4 ^{o}C for up to 24 hours against 0.05 M Tris-HCl buffer (pH 7.6). Again appropriate controls were examined by dialysing enzyme against the same buffer at 4 ^{o}C for the same lengths of time with 5 changes of buffer.

2.2.6 Spectra:

Spectra were run on a Shimadzu U.V. 240 U.V. visible recording spectrophotometer with constant temperature cell holder. Quartz cuvettes were used.

2.3 Results and Discussion :

Different procedures to purify DHPR from human brain, were attempted with limited success, as shown in tables (2.1, 2.3, 2.4) in which a purification of 58.7 fold is obtained with specific activity of 16847.8 nmole NADH/min/mg. protein.

In addition, Fig.(2.1) shows DHPR activity in two fractions when apply to naphthoquinone affinity column, first fraction with specific activity of 3068.5 nmole NADH/min/mg.protein and the other with specific activity of 6866.7 nmole NADH/min./mg. protein. Kinetic parameters of these two different DHPR forms were examined by varying the $DMPH_4$ concentration. Km values were graphically determined from Lineweaver-Burk and Hanes plots, as shown in Figs.

(2.2, 2.3) in which the Km value for first DHPR fraction is 62.5 μM and for second fraction is 42 μM.

The molecular weights of these two DHPR forms were examined by gel filtration through a Sephadex G-100 column. The M.Wt. of first fraction was 30,000 and the second 54,500 (See Fig. 2.4). These results confirmed the dimeric form of the enzyme, as the native enzyme of M.Wt. 54,500 exists as a dimer and composed of 2 subunits of M.Wt. 30,000.

Recently, we have obtained partially purified DHPR enzyme from human brain in 20% yield, using the procedure described previously by Armarego and Waring, (1983). However, a new step employing a column of Blue Sepharose has been included, which has given 338 purification fold with a high specific activity (227,750 nmole NADH/min./mg.protein), as shown in Fig. (2.5, 2.6) and table (2.7).

The Michaelis constant (Km) obtained with the substrates $DMPH_4$ and NADH, of the purified enzyme according to this procedure were 73 and 46 μM respectively (see Fig. 2.7 and Fig. 2.8).

The results obtained here indicate that it is possible to isolate DHPR from human brain by this three step method, involving ammonium sulfate precipitation, affinity chromatography with sodium naphthoquinone as ligand and Blue Sepharose gel. Hence, inhibition studies with various metal ions and metabolites, can be performed.

DHPR is inhibited by phenylpyruvate, 6-hydroxydopamine and 1-methyl-4-phenyl-1, 2, 5, 6-tetrahydropyridine (MPTP). Table (2.8) displays the results of inhibition studies by those inhibitors, 37.1%, 67.4% and 51.4% inhibition were observed with 10^{-4} M phenylpyruvate, 6-hydroxydopamine and MPTP respectively.

Dixon plot was used to determine the inhibition constant K_i, and

Cornish-Bowden plot was used to determine the dissociation constant K_i', as shown in Figs. (2.9 - 2.14). The constants K_i and K_i' are shown in tables (2.9, 2.10). Phenylpyruvate is mixed inhibitor of DHPR with respect to the pterin and NADH substrates. 6-Hydroxydopamine is mixed inhibitor of DHPR with respect to the pterin substrate and non-competitive inhibitor with respect to NADH substrate. And MPTP is a mixed inhibitor with respect to $DMPH_4$ and competitive inhibitor with respect to NADH substrate.

As can be seen from Fig. (2.15), when DHPR is incubated at 37 ^{o}C for different intervals, with two concentrations of 6-hydroxydopamine before measuring the enzyme activity, there is an immediate inhibition and a maximum inhibition of the enzyme was obtained by pre-incubation for 5 minutes. There is no relation between the degree of inhibition and the length of time the enzyme is pre-incubated with the 6-hydroxydopamine, which means that the inhibition is reversible.

In the inhibition studies of 6-hydroxydopamine, it was dissolved in Tris-maleate buffer (pH 6.8), in which 6-hydroxydopamine was rapidly oxidized to respective red product. The oxidation rate is decreased when 6-hydroxydopamine was dissolved in diluted HCl or distilled water.

As shown in table (2.11), DHPR inhibition % with 6-hydroxydopamine was higher in Tris-maleate buffer, which is probably due to the formation of the respective oxidation product (6-hydroxydopachrome) (see Fig. 2.16), and its rate of formation was decreased as the pH decreased, as shown in Figs. (2.17, 2.18).

Fig. (2.19) shows the immediate inhibition of DHPR when incubated at 37 ^{o}C with various concentrations of aluminium sulphate before measuring the enzyme activity. The degree of inhibition depends on the length of time, the enzyme is pre-incubated with the aluminium, and the concentrations of aluminum sulphate used, which raises the possibility of the inhibitor being irreversible.

Table (2.12) displays the results of attempting to remove the aluminum by dialysis against 0.05M Tris/HCl buffer (pH 7.6). As can be seen no recovery of enzyme activity was obtained which confirmed the irreversiblity of aluminum inhibition.

TABLE 2.1 SUMMARY OF THE PURIFICATION OF HUMAN BRAIN DHPR (1)

Step	Total Volume (mls)	Protein Conc. mg./ml.	DHPR/ml.	DHPR/mg. Protein	Purification fold	Yield %
Brain extract	10	10.5	3800	361.9		100%
Naphthoquin-one column Fraction I	6	0.56	1718.4	3068.5	8.5	27%
Fraction II	6	0.225	1545	6866.7	19.0	24.4%

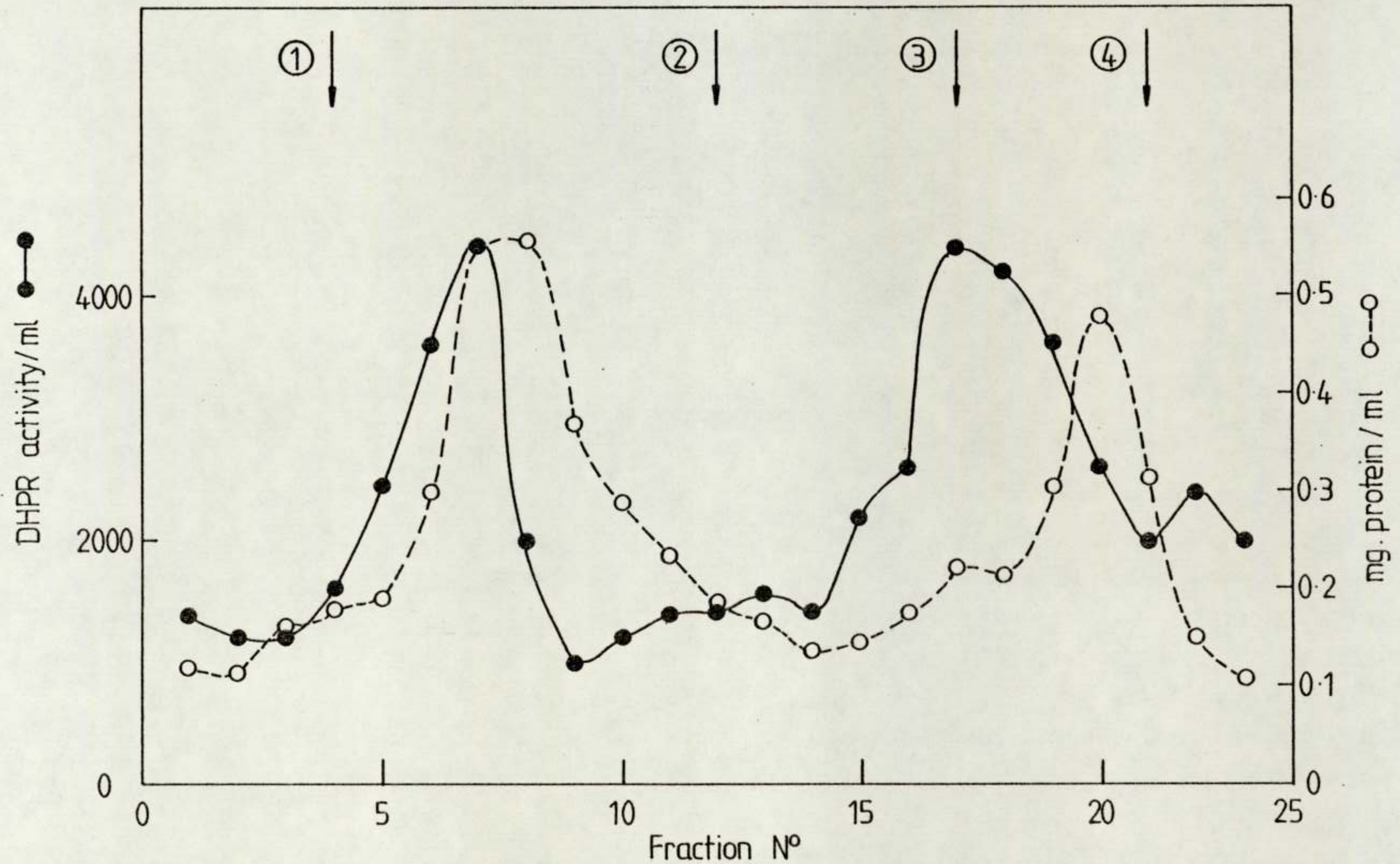


Figure 2·1 Chromatogram from Naphthoquinone affinity column. (See table 2·2 for key)

Table (2.2)

Key for Fig. (2.1)

The column was eluted with:

Arrow 1. 0.05 M Tris/HCl buffer (pH 7.6) made 0.8 M NaCl, 0.1 mM EDTA, 20% glycerol and 0.1 mM NADH.

Arrow 2. 0.1 M $NaHC0_3$/Na0H buffer (pH 10.9) made M NaCl.

Arrow 3. 0.05 M Tris/HCl buffer (pH 7.6) made 0.8 M NaCl, 0.1 mM EDTA and 20% glycerol.

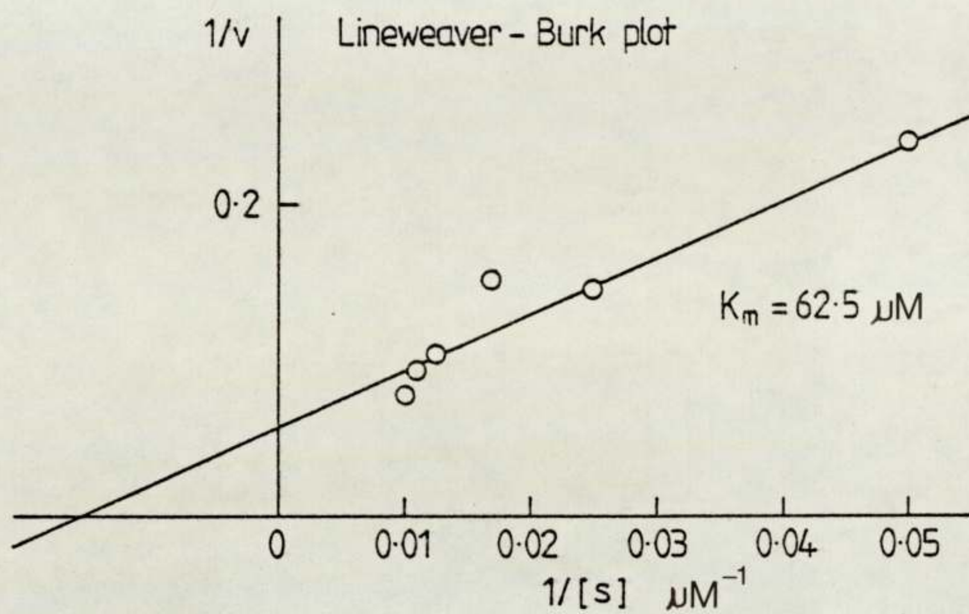
Arrow 4. 0.1 M $NaHC0_3$/Na0H buffer (pH 10.9) made M NaCl.

TABLE 2.3 SUMMARY OF THE PURIFICATION OF HUMAN BRAIN DHPR (2)

Step	Total Volume (mls)	Protein Conc. mg./ml.	DHPR/ml.	DHPR/mg. Protein	Purification fold	Yield %
Brain extract	3.5	12.5	1850	148	-	100%
Ammonium Sulfate	2.0	0.8	1950	2437.5	16.5	60.2
Naphthoquin-one Column	6.0	0.028	200	4142.9	48.3	18.5

TABLE 2.4 SUMMARY OF THE PURIFICATION OF HUMAN BRAIN DHPR (3)

Step	Total Volume (mls)	Protein Conc. mg./ml.	DHPR/ml.	DHPR/mg. Protein	Purification fold	Yield %
Brain extract	5	12.2	3500	286.9	—	100%
Ammonium Sulfate	2	1.86	5000	2688	9.4	57.2%
Blue Sepharose	9	0.046	775	16847.8	58.7	39.9%



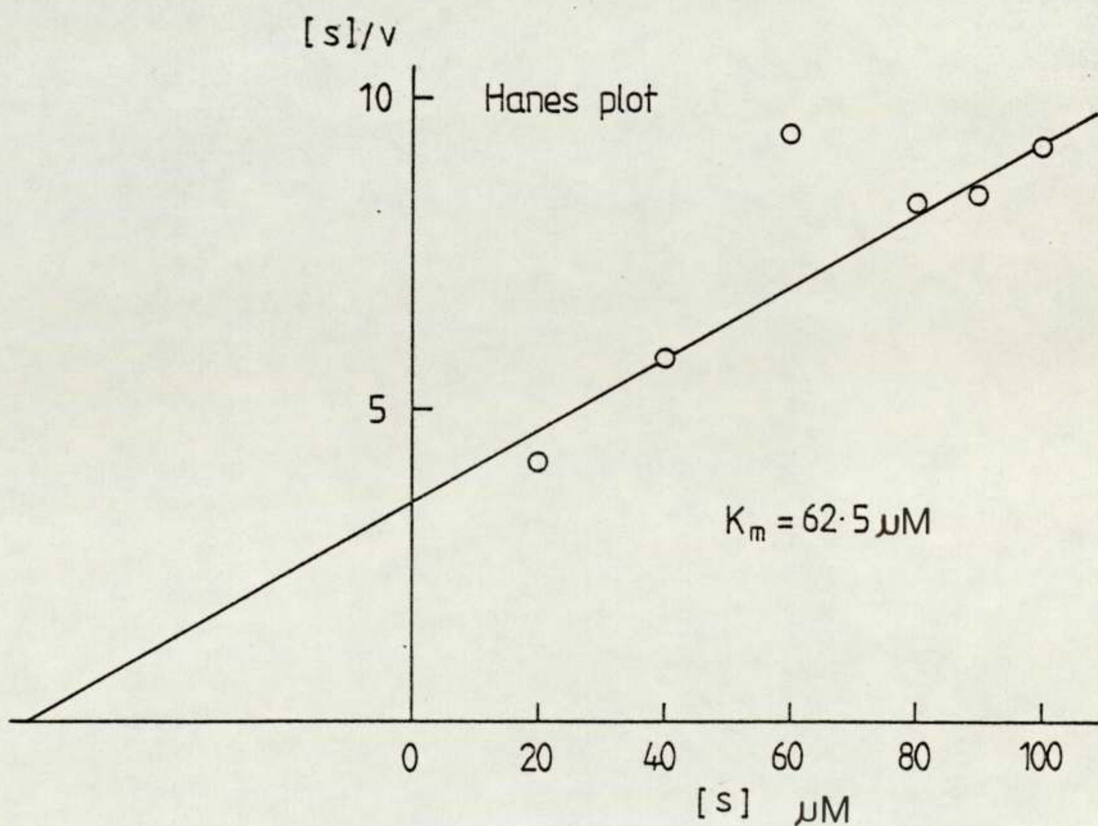
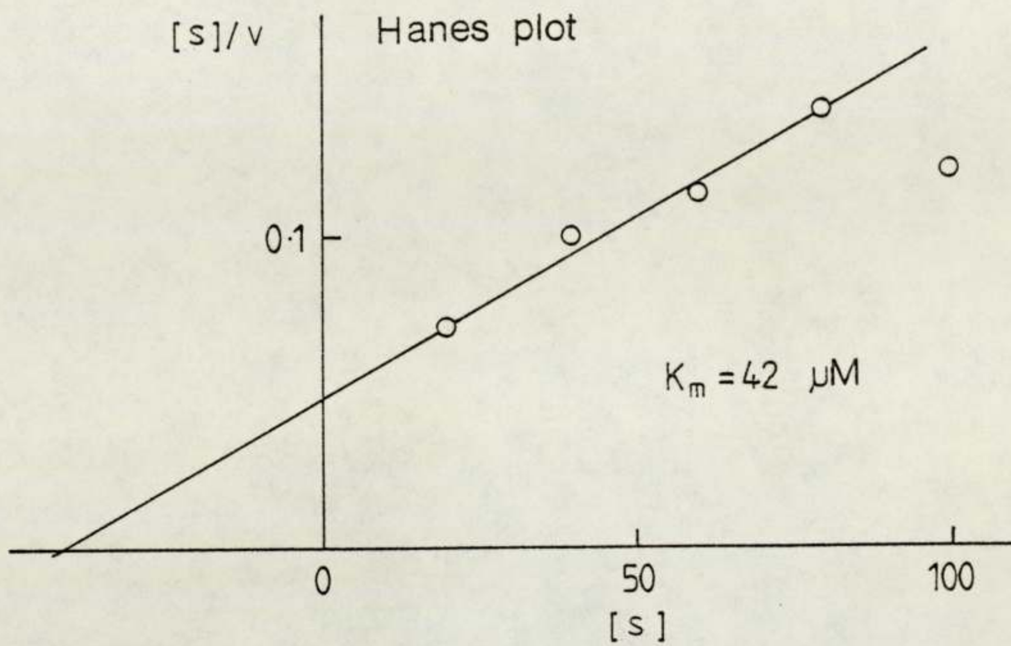


Figure 2·2 Effect of $DMPH_4$ concentration on the activity of DHPR (Fraction I)



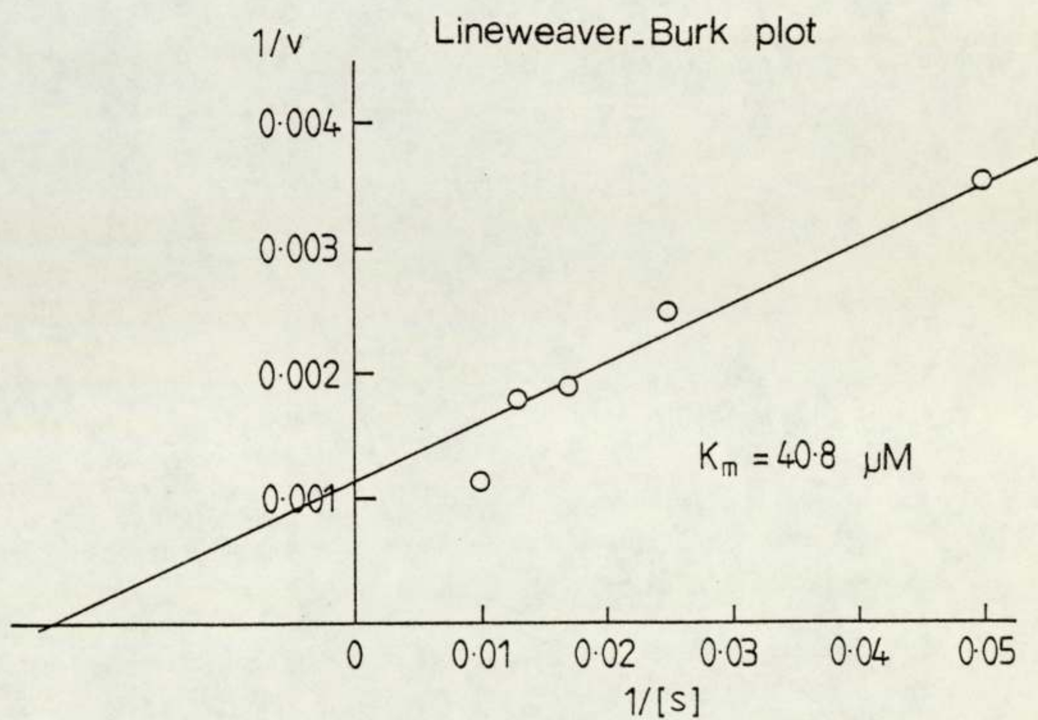


Figure 2·3 Effect of $DMPH_4$ concentration on the activity of DHPR (Fraction II)

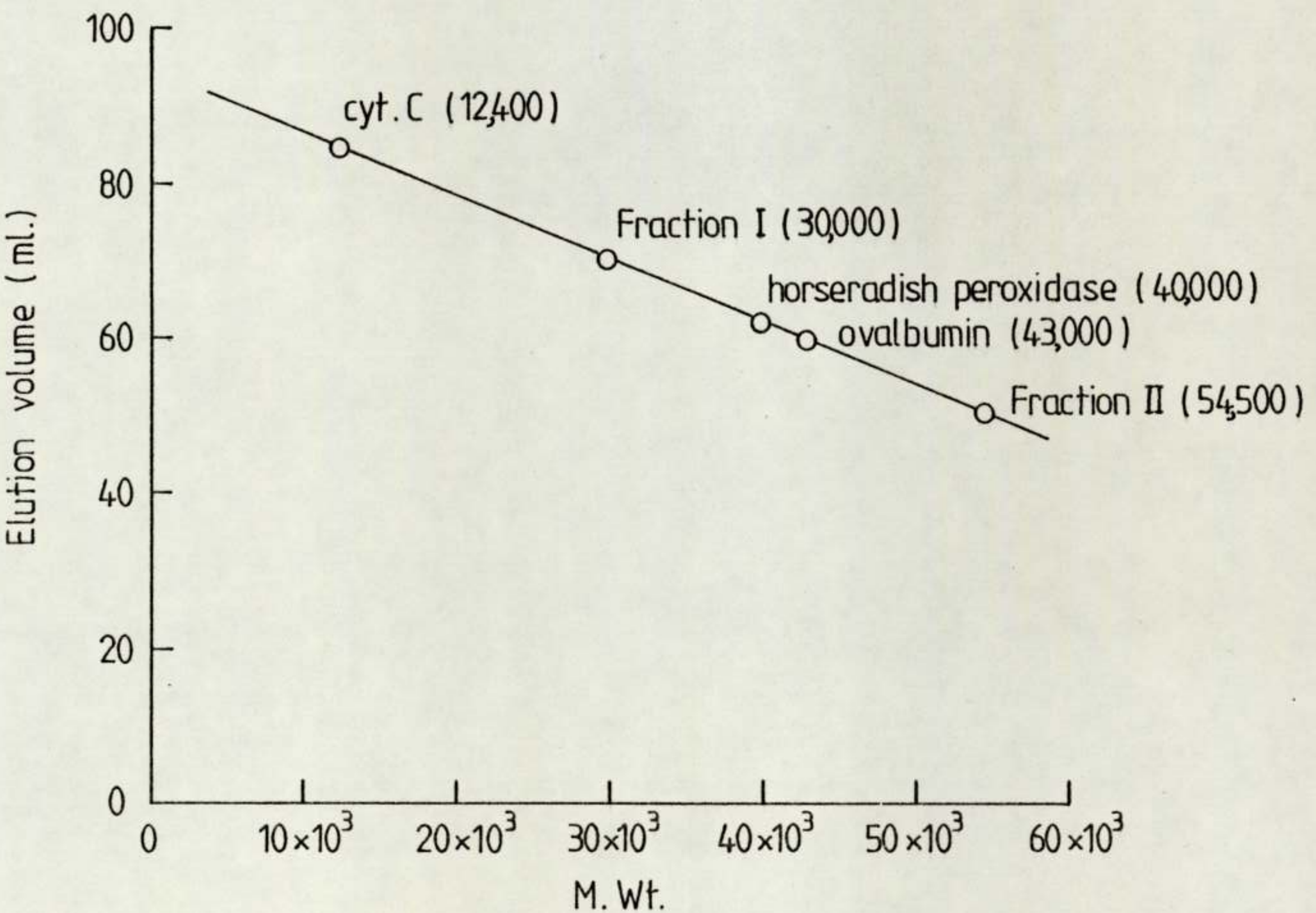


Figure 2·4 Determination of M.Wt. of DHPR by Sephadex G - 100 chromatography

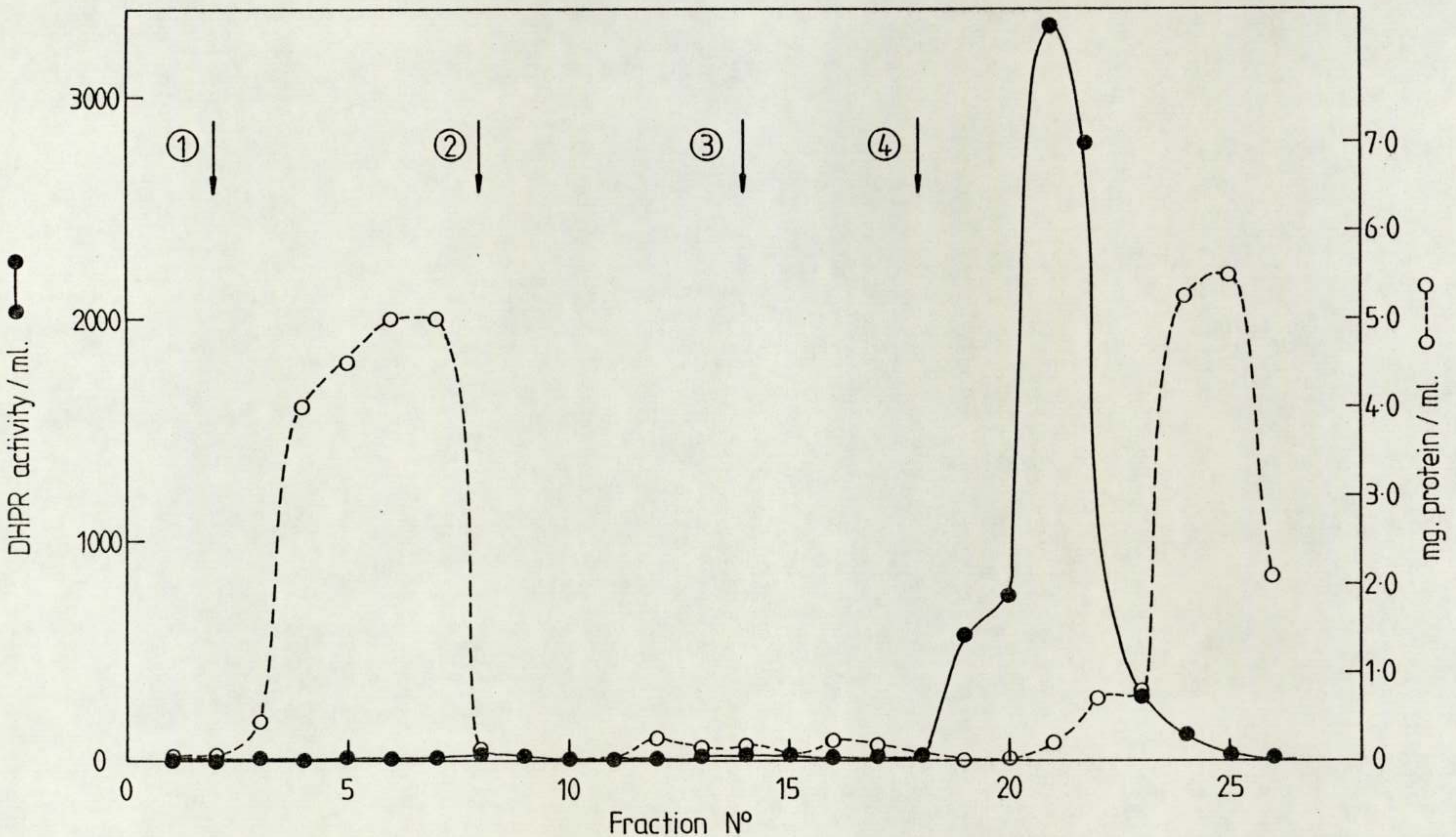


Figure 2·5 Chromatogram from Naphthoquinone affinity column. (See table 2·5 for key)

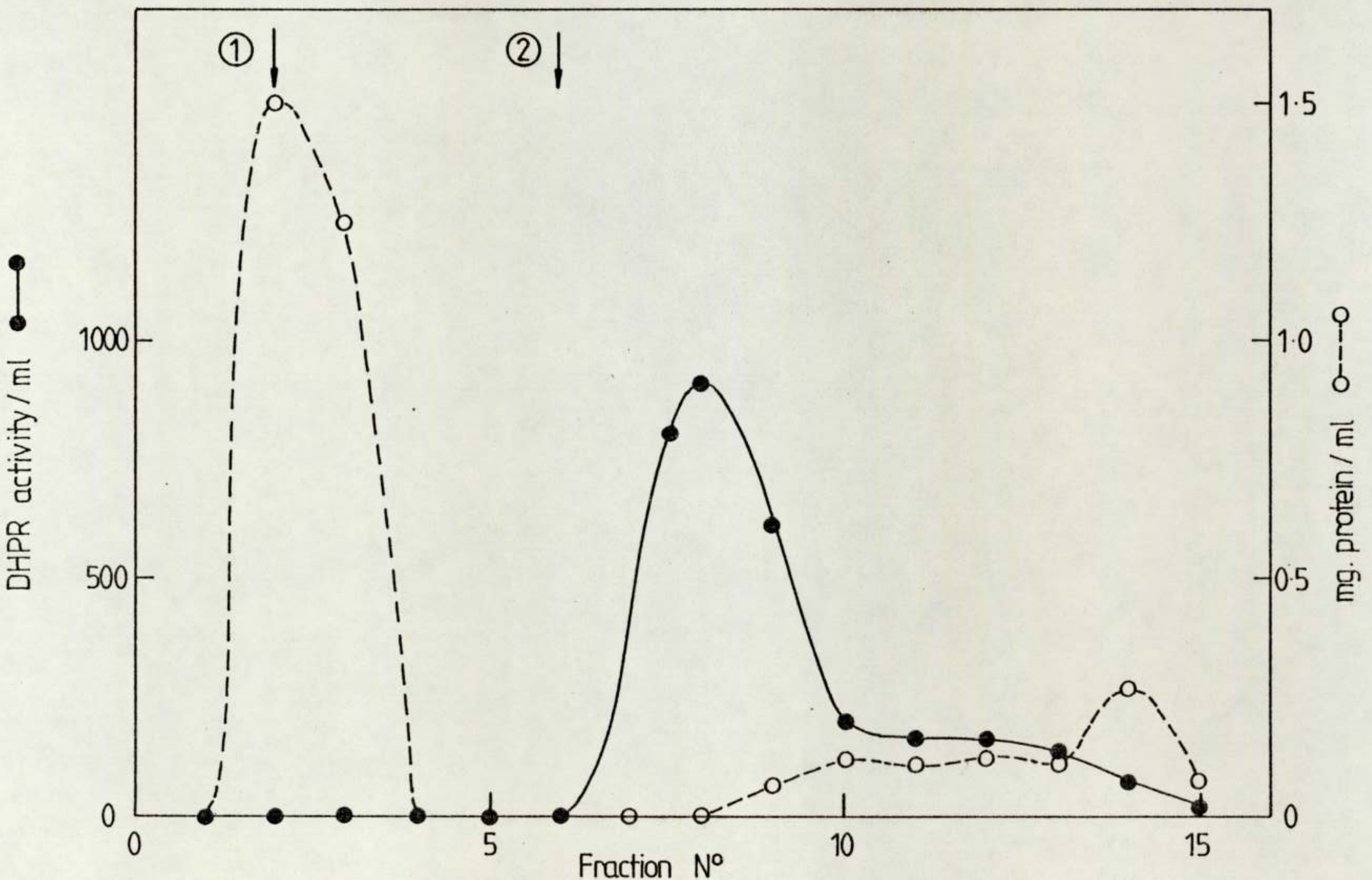


Figure 2·6 Chromatogram from Blue Sepharose column. (See table 2·6 for key)

Table (2.5)

Key for Fig. (2.5):

The column was eluted with:

Arrow 1. 0.1 M Tris/HCl buffer (pH 7.6) made 0.8 M NaCl and 0.1 mM NADH

Arrow 2. 0.1 M $NaHCO_3$/NaOH buffer (pH 10) made 0.8 M NaCl and 0.1 mM NADH.

Arrow 3. 0.1 M Tris/HCl buffer (pH 7.6) made 0.8 M NaCl.

Arrow 4. 0.1 M Tris/HCl buffer (pH 7.6) made 0.8 M NaCl and 10 mM DTT.

Table (2.6):

Key for Fig. (2.6):

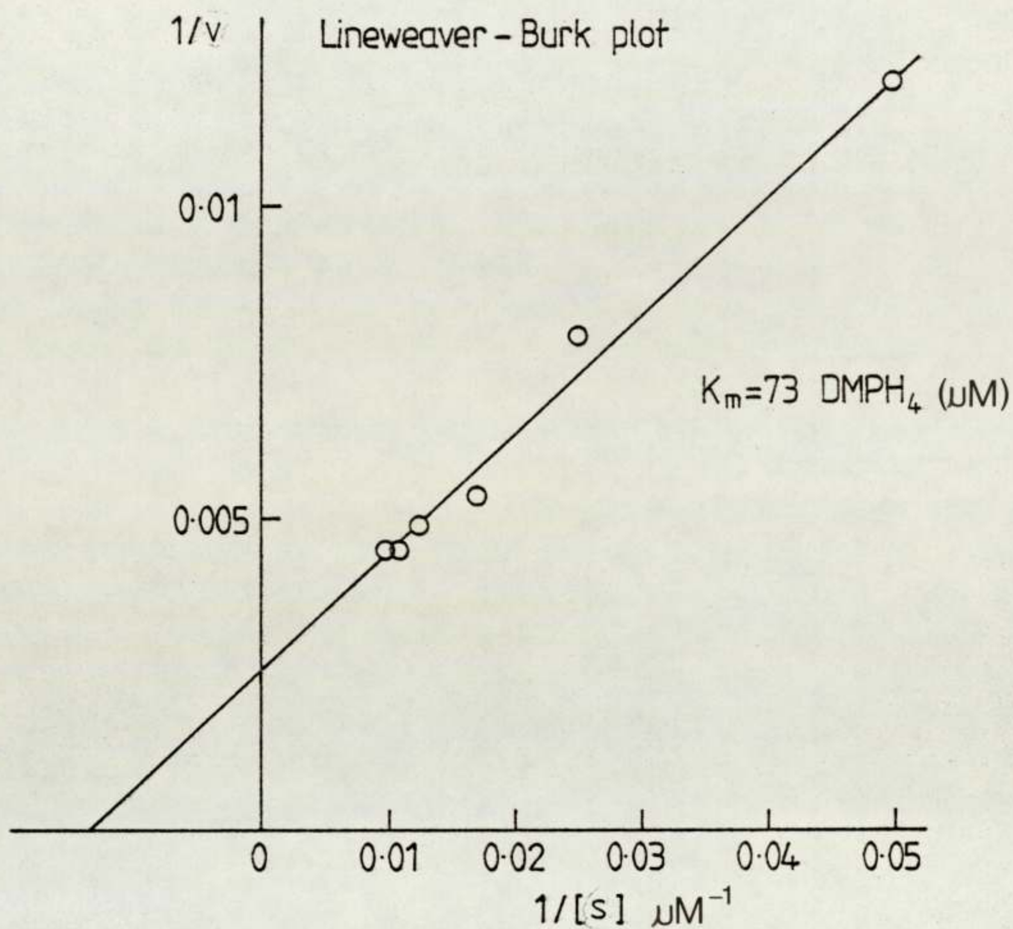
The column was eluted with:

Arrow 1. 50 mM potassium phosphate buffer (pH 6.8).

Arrow 2. 50 mM potassium phosphate buffer (pH 6.8) made 100 μM NADH.

TABLE 2.7 SUMMARY OF THE PURIFICATION OF HUMAN BRAIN DHPR (4)

Step	Total Volume (mls)	Protein Conc. mg./ml.	DHPR/ml.	DHPR/mg. Protein	Purification fold	Yield %
Brain extract	4	15.25	10268.6	673.4	-	100%
Ammonium Sulfate	3	5.0	8175.0	1635	2.43	59.7%
Naphthoquin-one Column	6	0.21	3322.6	15821.9	23.5	48.5
Blue Sepharose	9	0.004	911.0	227750	338.2	20%



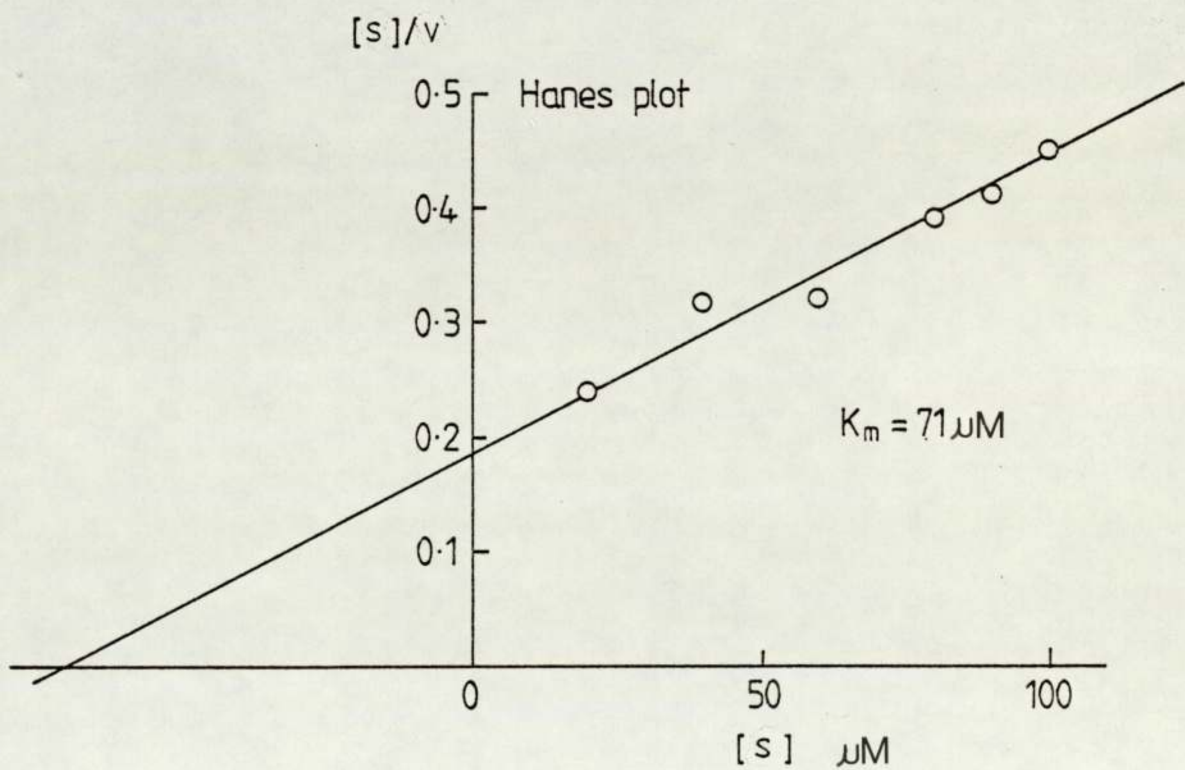
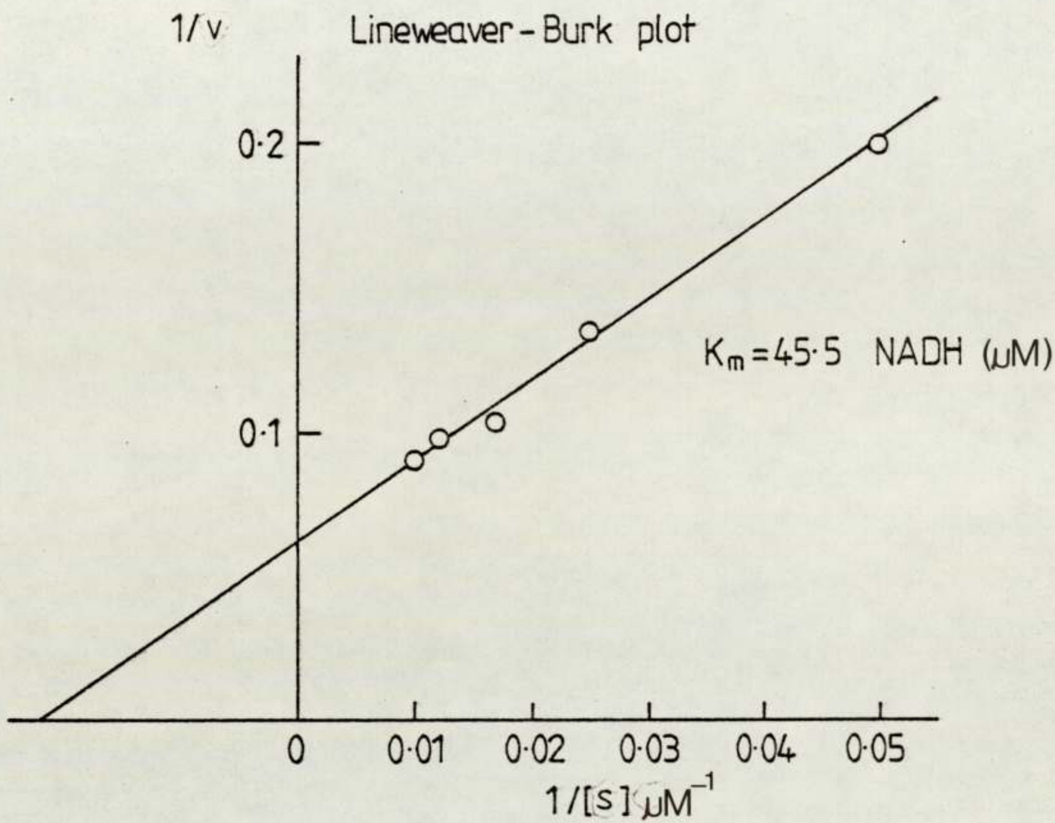


Figure 2·7 Effect of $DMPH_4$ concentration on the activity of purified DHPR



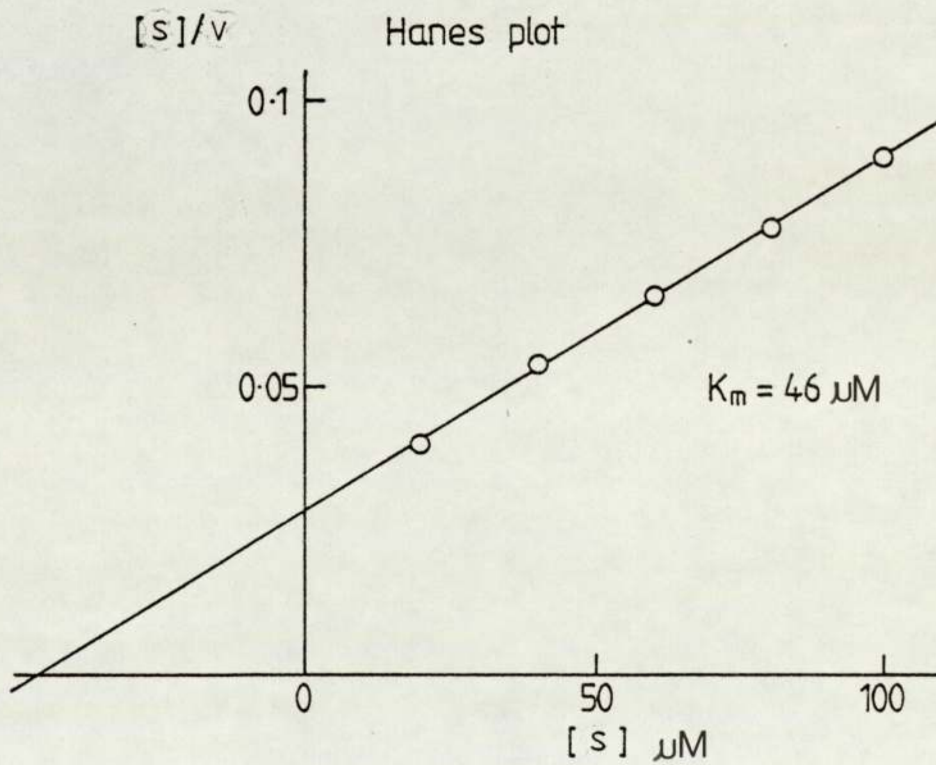
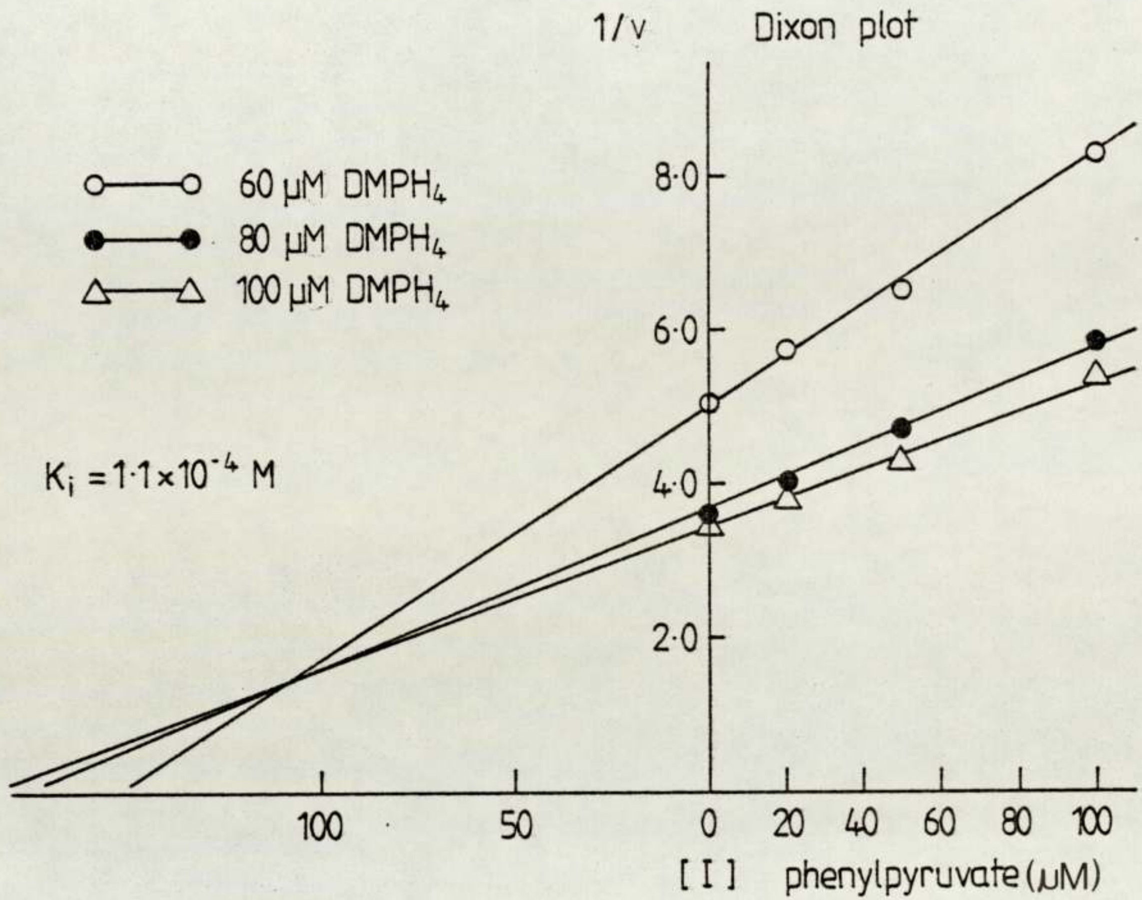


Figure 2·8 Effect of NADH concentration on the activity of purified DHPR

TABLE 2.8 : Effect of different agents on human brain DHPR

Inhibitor	Concentration	Inhibition %
phenylpyruvate	$5 \times 10^{-5}M$ $1 \times 10^{-4}M$	20.7% 37.1%
6-OH-dopamine	$5 \times 10^{-5}M$ $1 \times 10^{-4}M$	51.1% 67.4%
MPTP	$5 \times 10^{-5}M$ $1 \times 10^{-4}M$	29.1% 51.4%



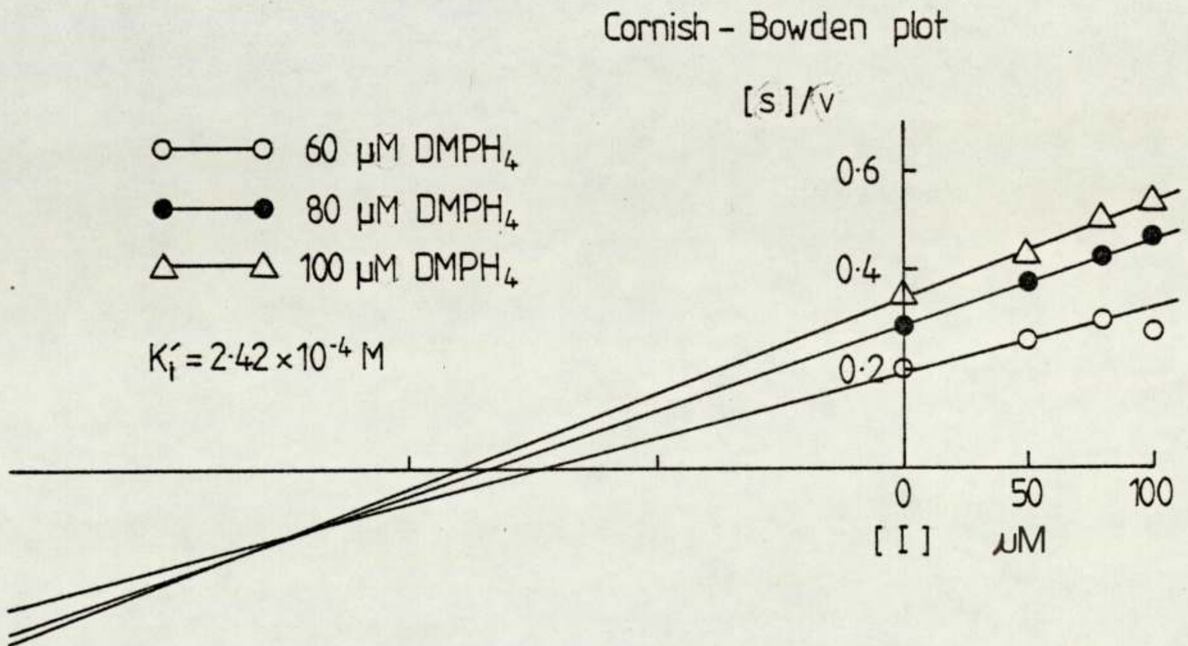
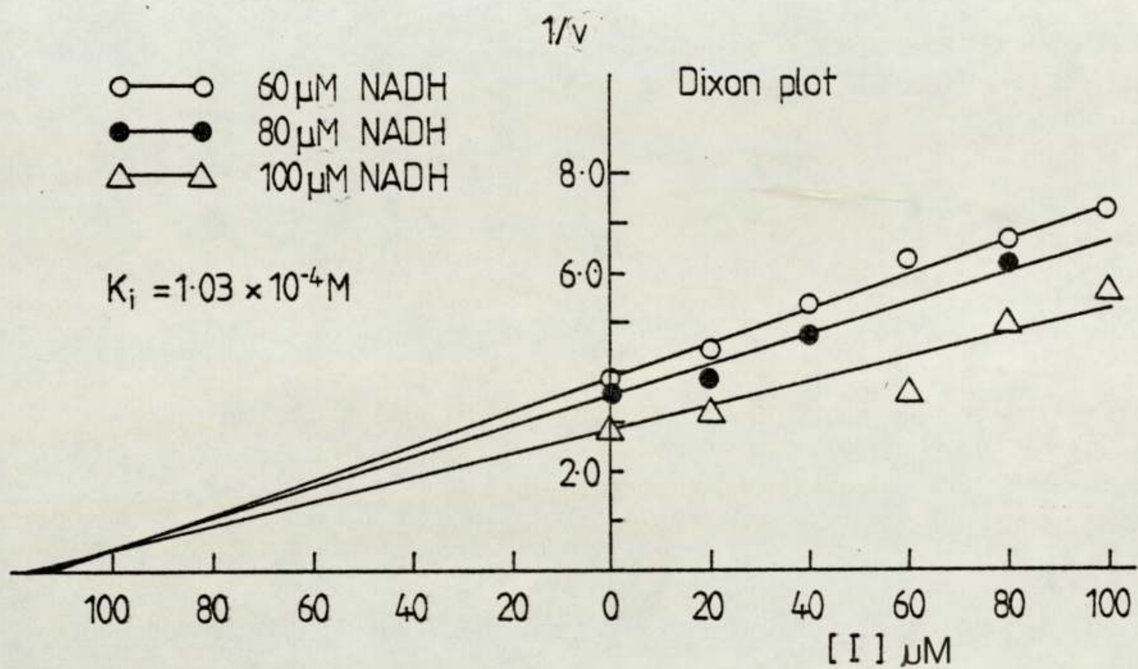


Figure 2·9 Effect of phenylpyruvate on human brain DHPR at constant NADH concentration



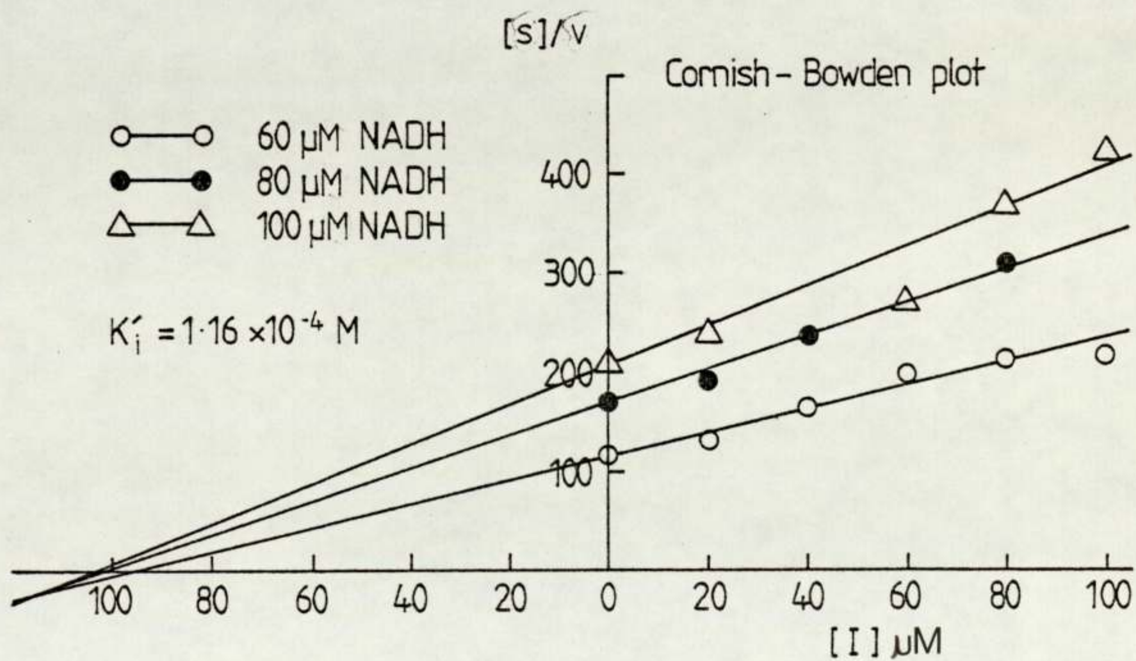
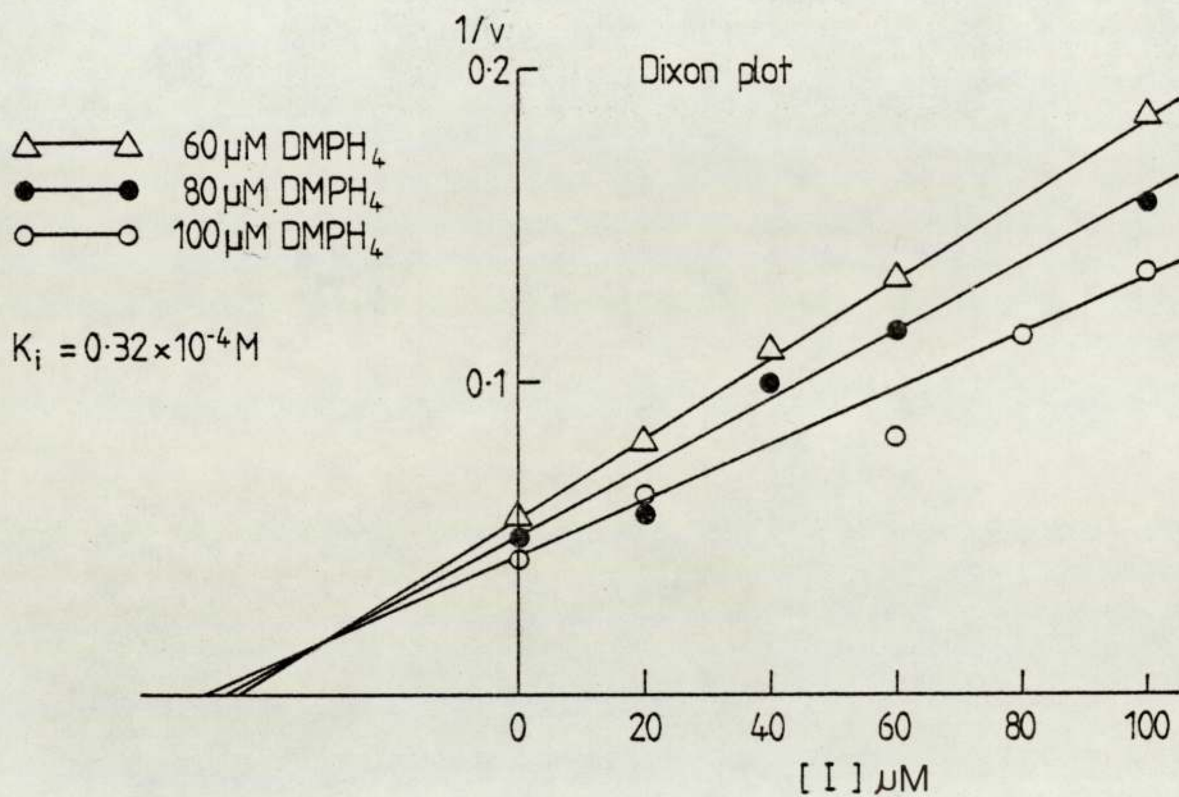


Figure 2·10 Effect of phenylpyruvate on human brain DHPR at constant $DMPH_4$ concentration.



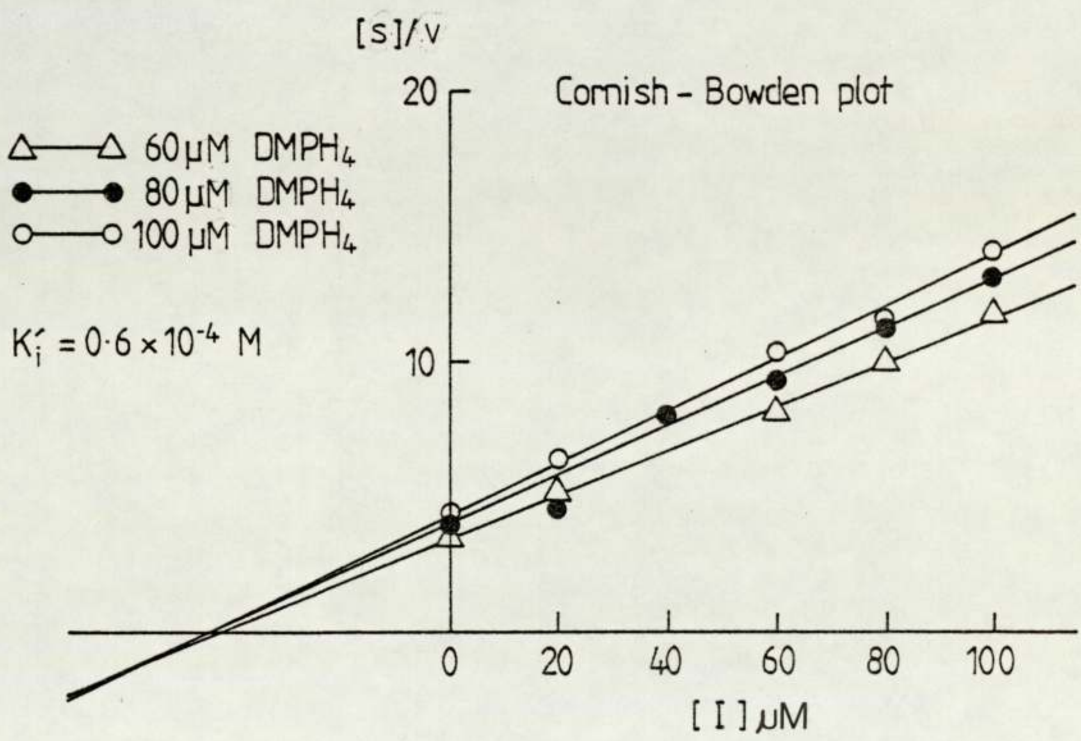
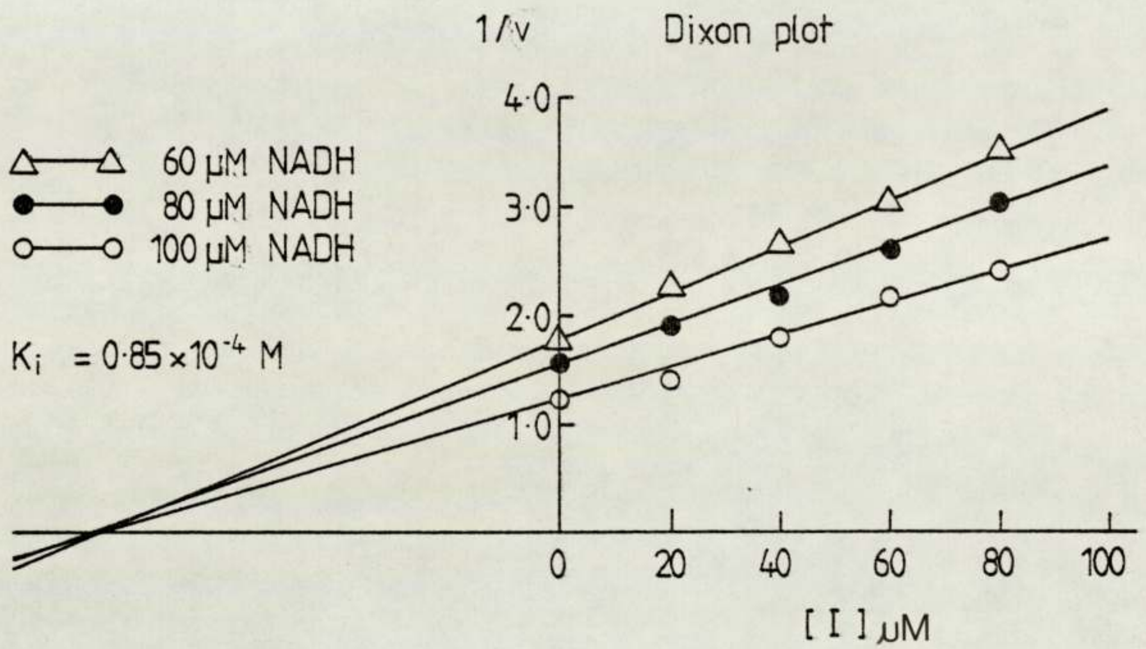
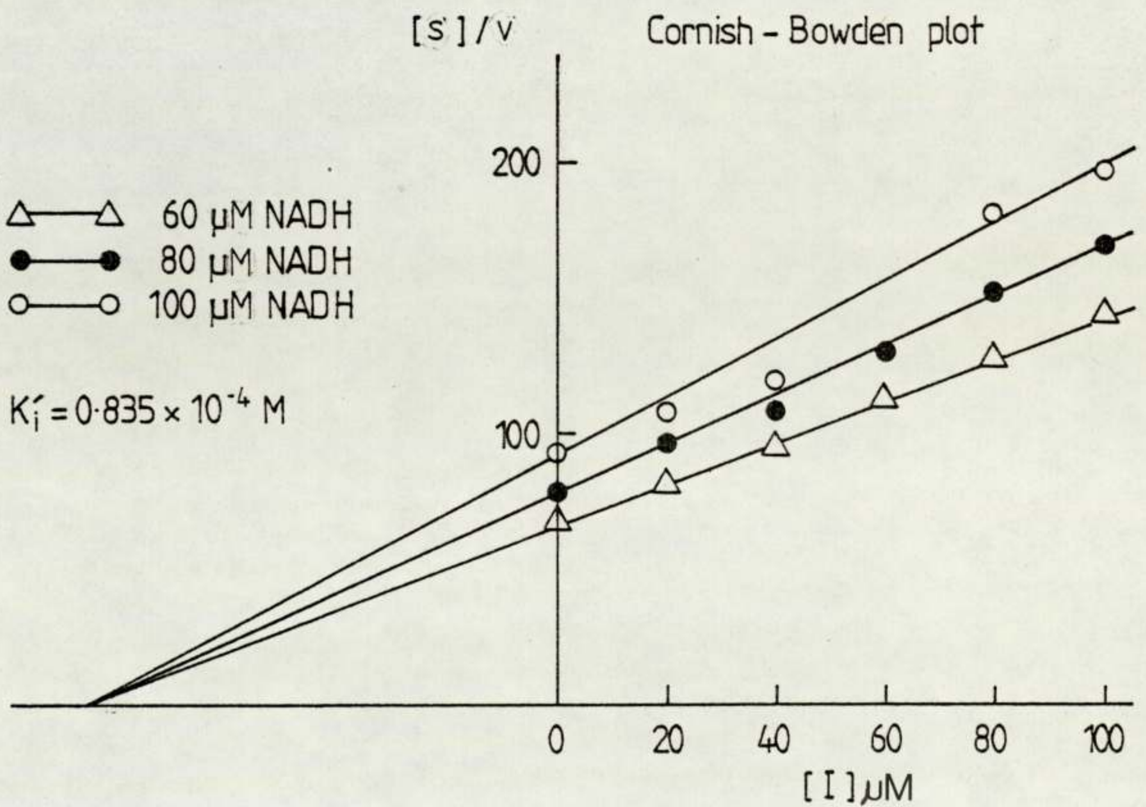
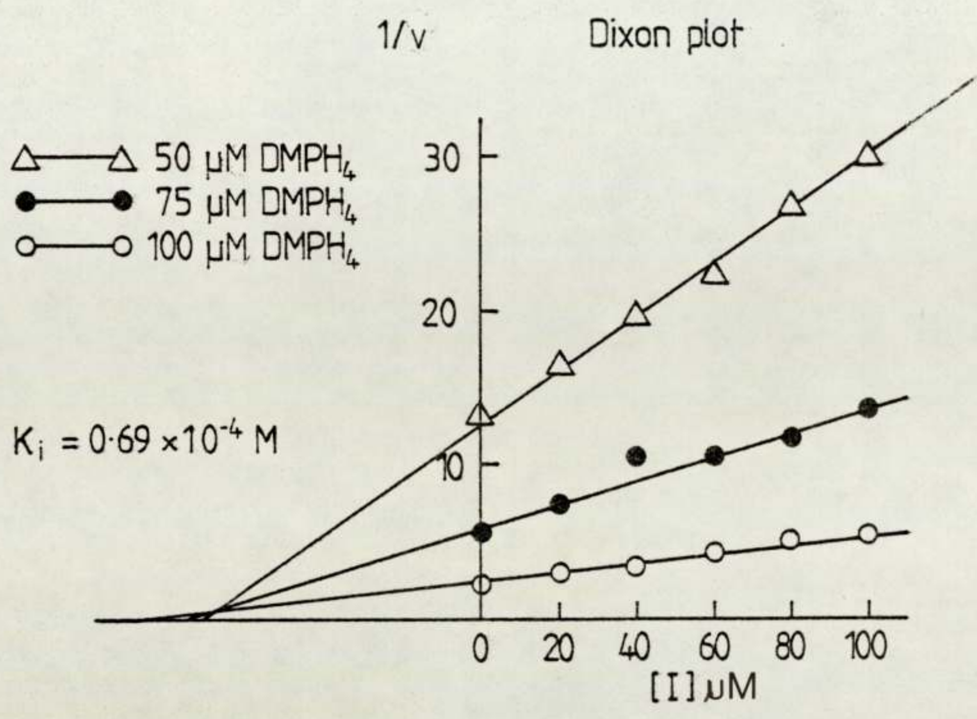


Figure 2·11 Effect of 6-hydroxydopamine on human brain DHPR at constant NADH concentration.





Figure·2·12 Effect of 6-hydroxydopamine on human brain DHPR at constant $DMPH_4$ concentration.



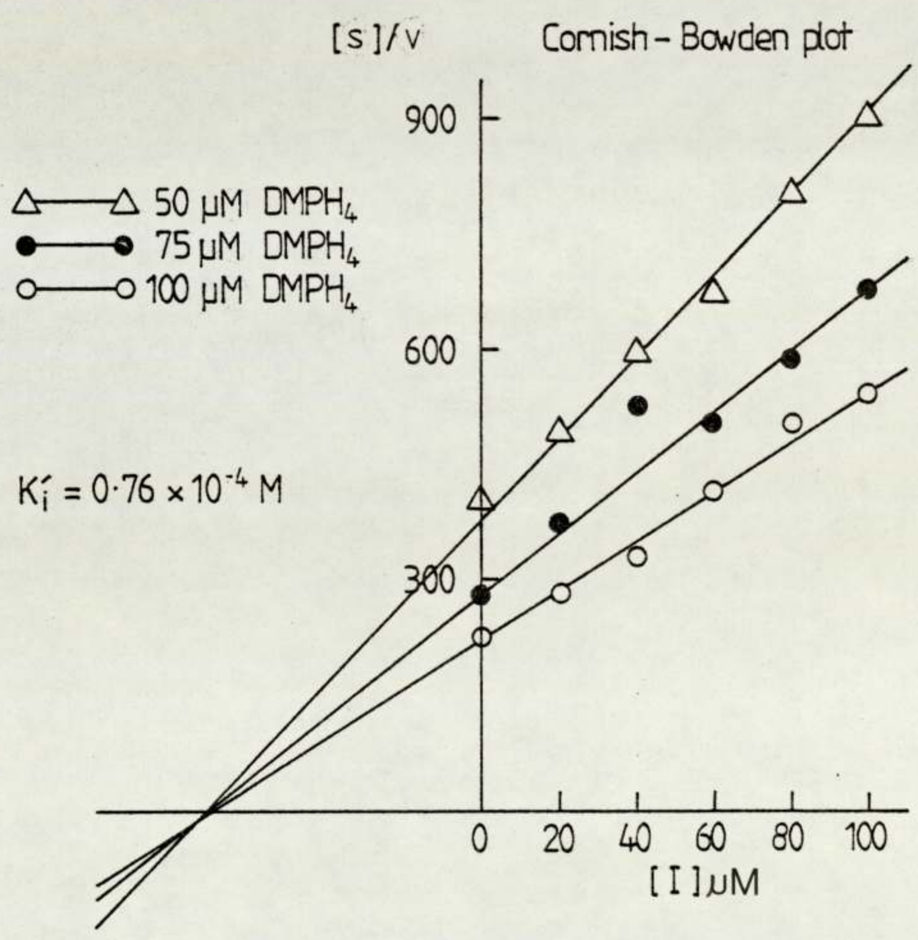
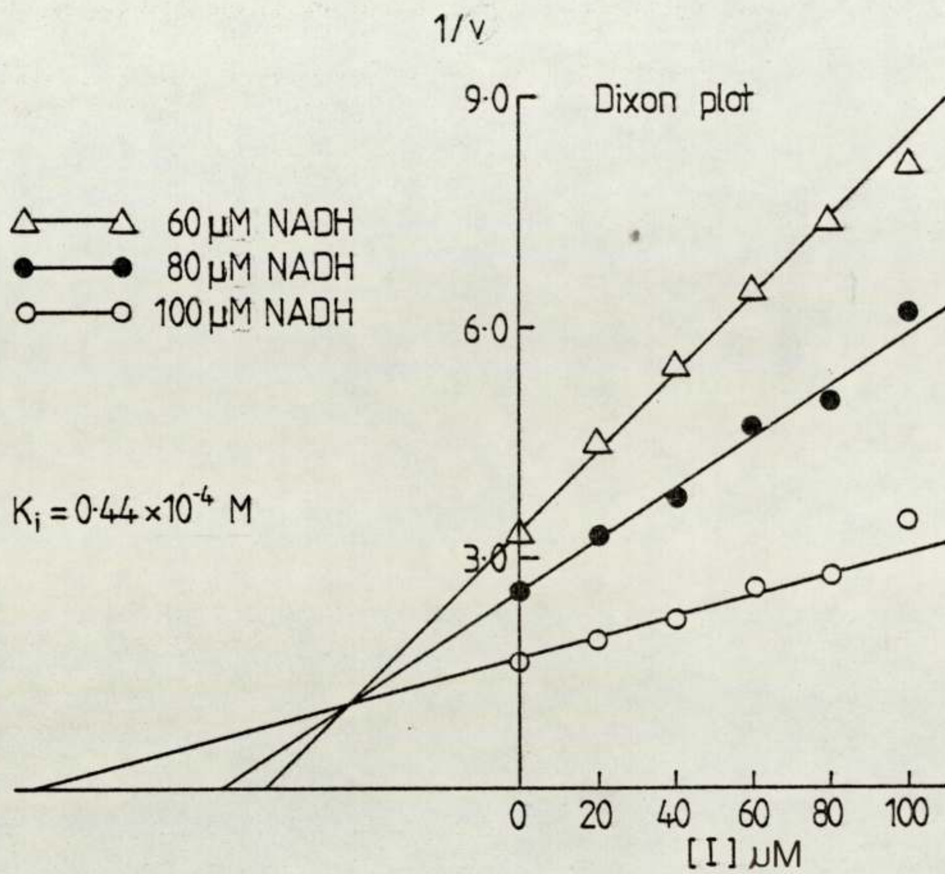


Figure 2·13 Effect of MPTP on human brain DHPR at constant NADH concentration.



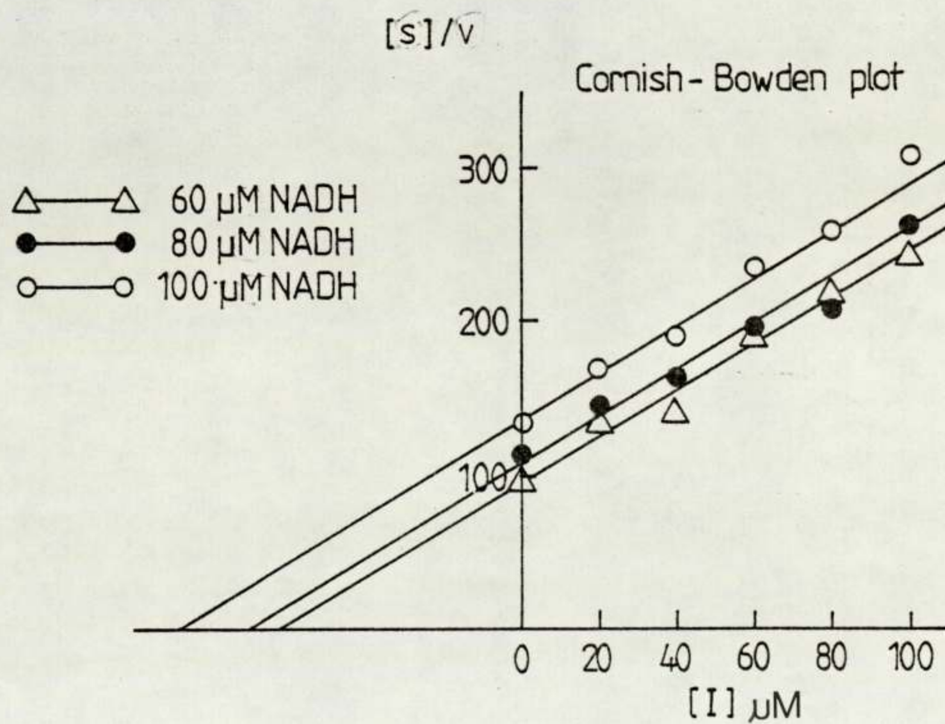


Figure 2·14 Effect of MPTP on human brain DHPR at constant $DMPH_4$ concentration.

TABLE 2.9: Inhibition constants for human brain DHPR with respect to $DMPH_4$

Inhibitor	Apparent type of Inhibition	K_i Value	K_i' Value
phenylpyruvate	Mixed	1.1×10^{-4} M	2.4×10^{-4} M
6-OH-dopamine	Mixed	0.32×10^{-4} M	0.6×10^{-4} M
MPTP	Mixed	0.69×10^{-4} M	0.76×10^{-4} M

TABLE 2.10: Inhibition constants for human brain DHPR with respect to NADH

Inhibitor	Apparent type of Inhibition	K_i Value	K_i' Value
phenylpyruvate	Mixed	1.03×10^{-4} M	1.16×10^{-4}M
6-OH-dopamine	Non-Competitive	0.85×10^{-4} M	0.84×10^{-4} M
MPTP	Competitive	0.44×10^{-4} M	---

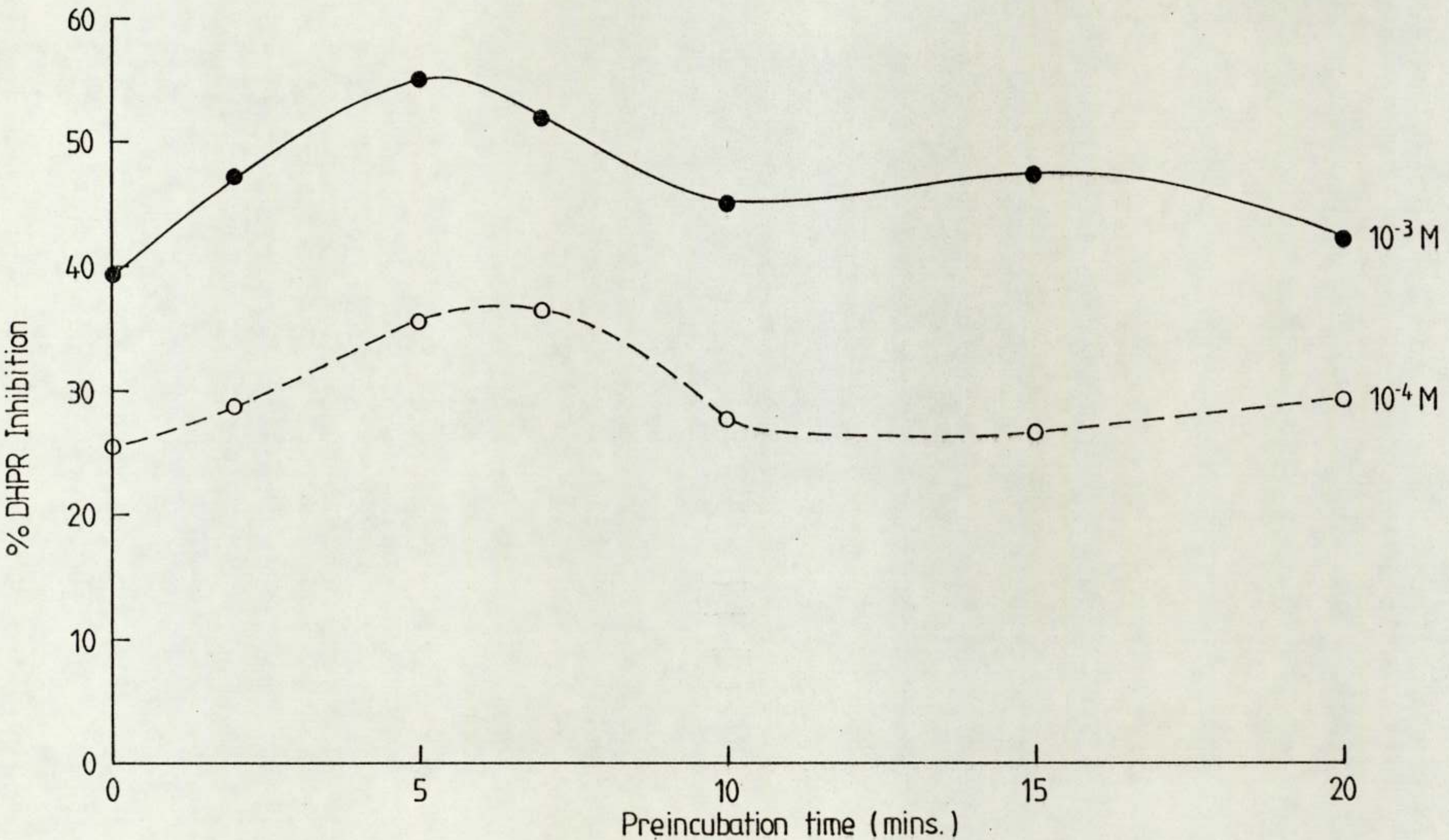
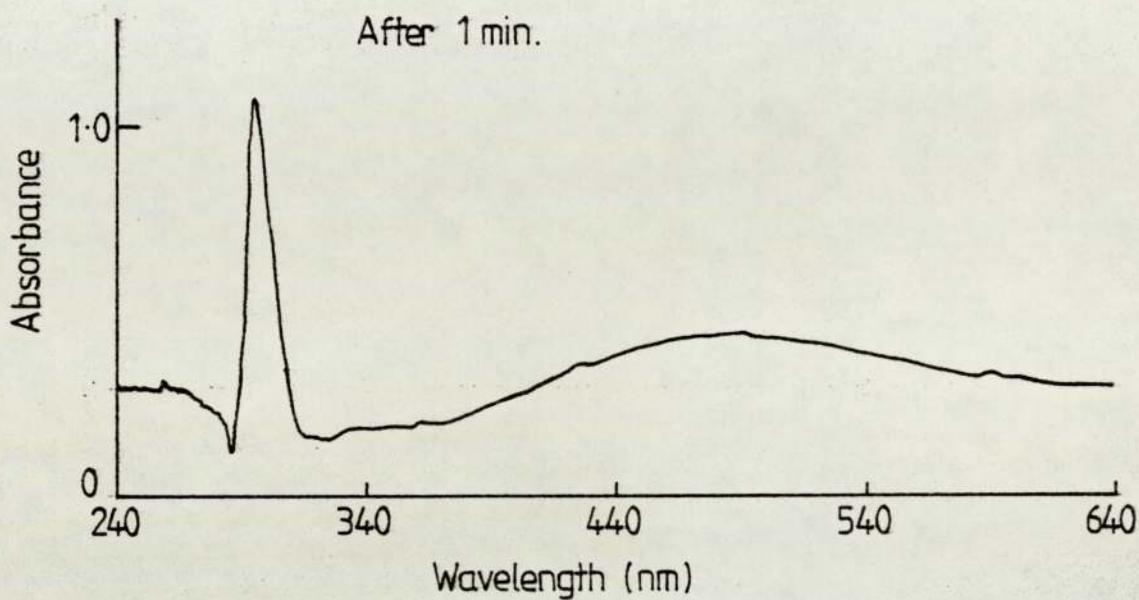


Figure 2·15 Effect of preincubation of the enzyme with 6-hydroxydopamine on human brain DHPR activity

TABLE 2.11: Effect of 6-hydroxydopamine on DHPR purified from human brain

	DHPR activity*	Inhibition%
Control	235.3	
10^{-3}M 6-hydroxydopamine in Tris-maleate (pH 6.8)	73.5	68.8%
in dil. HCl (pH 2.0)	220.6	6.2%
in H_20 (pH 6.5)	147.1	37.5%

*nmole NADH/min./mg. protein



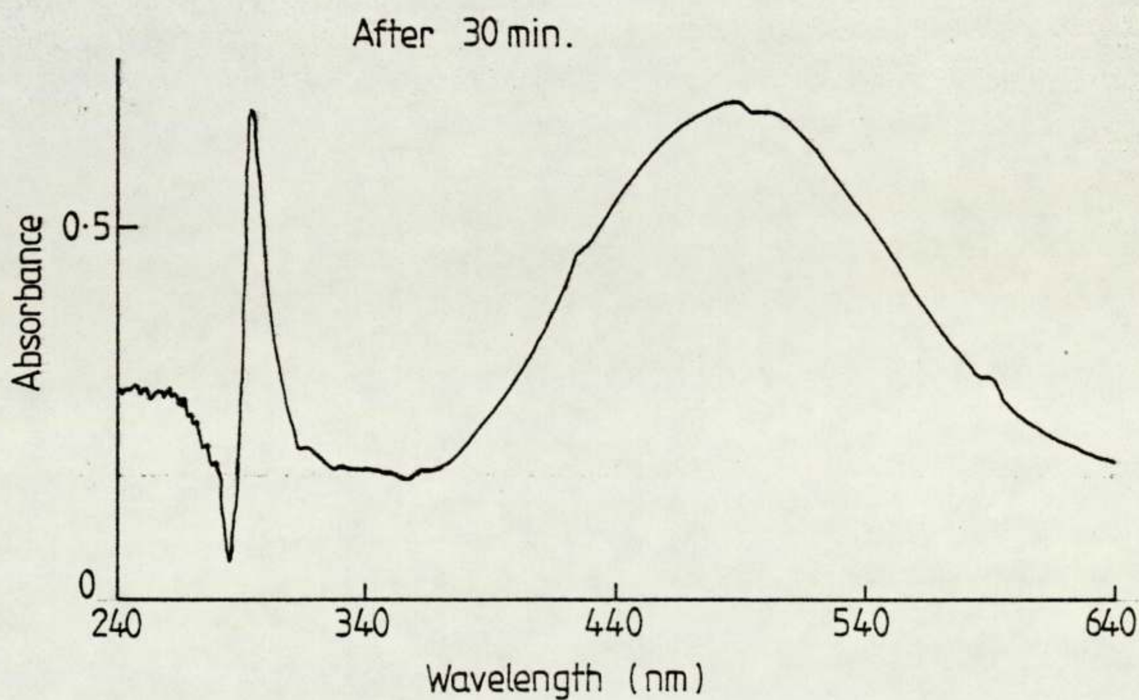


Figure 2·16 Absorption spectrum of 6-hydroxydopamine in Tris-maleate buffer (pH 6·8)

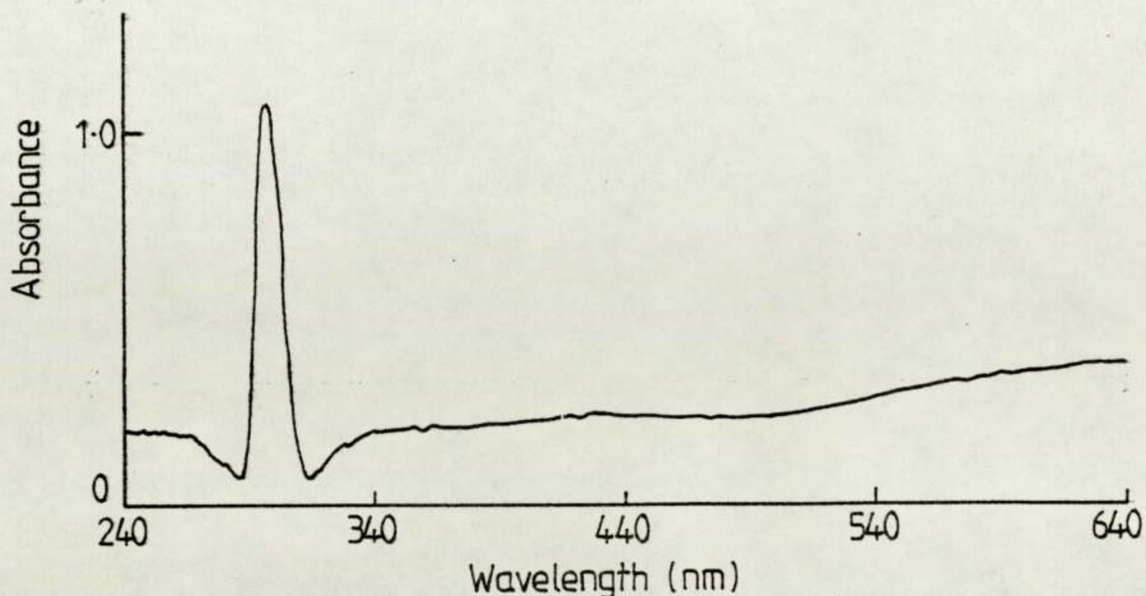


Figure 2·17 Absorption spectrum of 6-hydroxydopamine in diluted HCl (pH 2·0) after 13 min.

Absorbance
1·0
0·5
0
240
340
440
540
640
Wavelength (nm)

Figure 2·18 The effect of HCl on absorption spectrum of 6-hydroxydopamine in Tris-maleate buffer (pH 6·8) final pH 2·0.

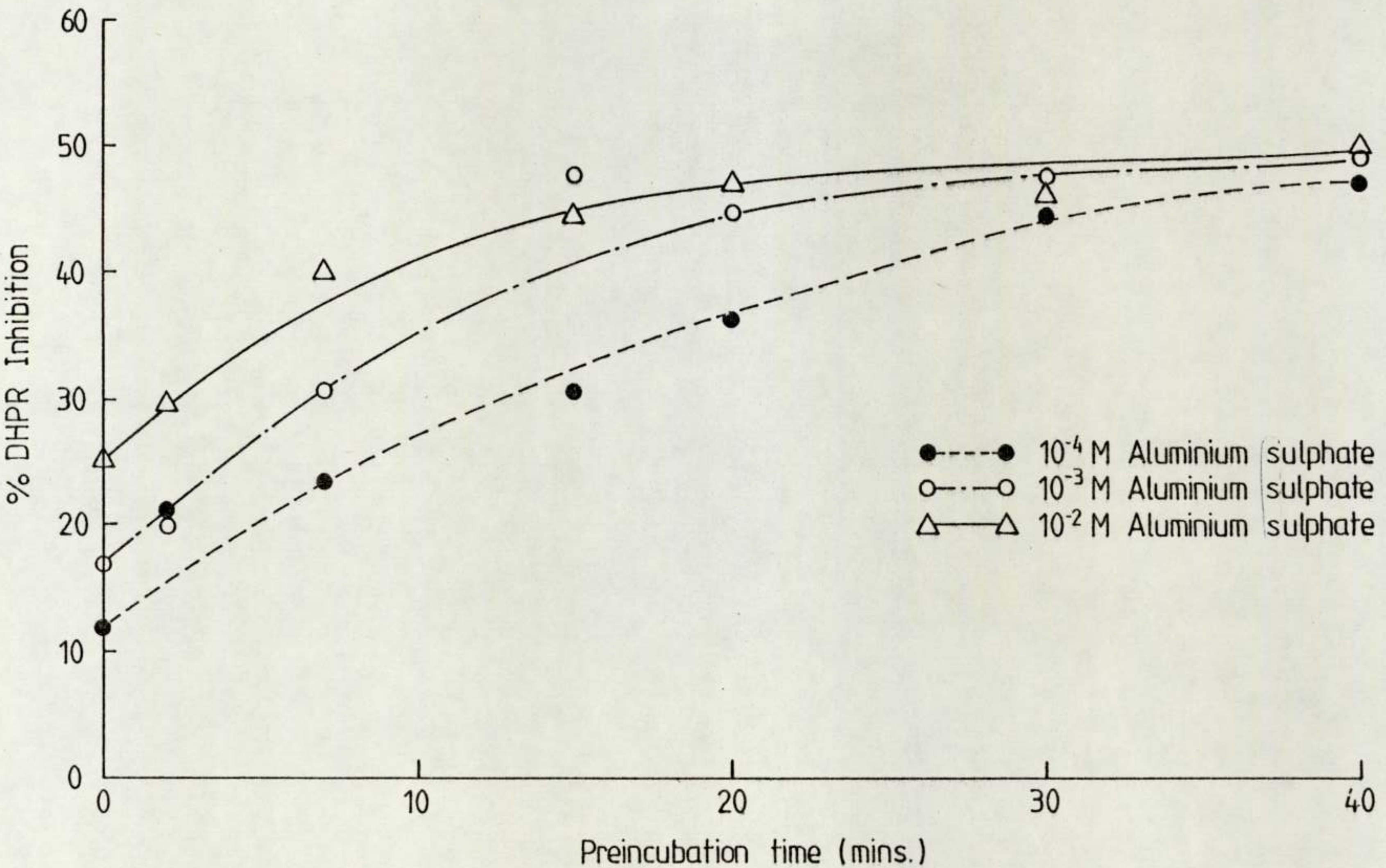


Figure 2·19 Effect of preincubation of the enzyme with aluminium sulphate on human brain DHPR activity

TABLE 2.12 EFFECT OF DIALYSIS ON PURIFIED HUMAN BRAIN DHPR AND ALUMINUM TREATED DHPR

	* CONTROL DHPR		** ALUMINUM TREATED DHPR		
Dialysis time (hrs)	Enzyme Actvity +	% of undialysed control	Enzyme Activity	% of undialysed control	% of control in the same time
before dialysis	1102.4	100%	557.8	50.6%	50.6%
2	1061.0	96.2%	538.4	48.9%	50.7%
4	1014.1	92%	497.0	45.1%	48.9%
8	1020.2	92.5%	520.0	47.2%	51.0%
24	975.6	88.5%	483.9	43.9%	49.6%

* Control DHPR : a mixture of 0.05M Tris-HCl buffer (pH 7.6) and enzyme with 7 changes of buffer.

** Aluminum treated DHPR : a mixture of buffer and enzyme that had been incubated with 10^{-4}M aluminum sulphate at 37 C for 40 min.

+ nmole NADH oxidized/min./mg. protein.

CHAPTER 3

DHPR ACTIVITY IN HUMAN BLOOD

CHAPTER (3);

DHPR ACTIVITY IN HUMAN BLOOD

3.1 Introduction

BH_4 deficiency can arise from DHPR deficiency (Kaufman et al, 1975; Rey et al, 1977), or from defective synthesis of tetrahydrobiopterin (Leeming et al, 1976c; Rey et al, 1977). Deficiency of DHPR has resulted in new interest in its detection in a diagnostic manner, when applied to human fibroblasts (Kaufman et al, 1975, Milstien et al, 1976), liver biopsies (Kaufman et al, 1975; Rey et al, 1977) or in continuous lymphoid cell cultures transformed by Epstein-Barr virus (Firgaira et al, 1979).

It has been reported that DHPR deficiency could be diagnosed by DHPR assay in peripheral leukocytes (Firgaira et al, 1979; Narisawa et al, 1980) and platelets (Abelson et al, 1979), as well as by high perfomance liquid chromatographic measurement of the various forms of biopterin in urine (Milstien et al, 1980). Arai et al (1982) have shown that the assay of DHPR activity can be carried out on blood eluted from dried spots on Guthrie cards. Recently, the Guthrie card technique has been used as a routine procedure for the measurement of DHPR activity and total biopterin in a series of infants with hyperphenylaninaemia detected on routine neonatal screening (Leeming et al, 1984, Sahota et al,1985).

A number of materials affect BH_4 in vitro, some of these occur in the environment and have known neurological sequelae. Lead ions inhibit both the synthesis of biopterin and the salvage pathway catalysed by DHPR (Purdy et al, 1981). In addition, Barford et al (1983) found reduced DHPR activity in the brains of rats born to mothers drinking leaded water.

Aluminum is now well documented as a casual agent in dialysis dementia (Alfrey et

al, 1976; McDermott et al, 1978). Aluminium also inhibits the salvage of q-BH_2 in vitro at concentration close to those found in the brains of patients with dialysis dementia (Leeming and Blair, 1979; Brown, 1981).

It is possible that prolonged exposure to one or more of the agents that disturb BH_4 metabolism, is involved in the pathology of senile dementia (Leeming et al, 1981).

Kinetic studies of DHPR from human whole blood and DHPR activity in human blood samples from workers in the lead industry and haemodialysis patients with high aluminum levels, have not been mentioned in the literature to date. Accordingly, we described here the application of an optimised assay to study the kinetics of human whole blood DHPR and the effect of the neurotoxins lead and aluminum on the enzyme.

3.2 Materials and Methods;

3.2.1 Chemicals;

Nicotinamide adenine dinucleotide reduced (disodium salt type III) (NADH), 6, 7-dimethyl-5,6,7, 8-tetrahydropterin ($DMPH_4$), Tris [Tris (hydroxymethyl) aminoethane], cytochrome c (from horse heart type III), total hemoglobin determination kit and human hemoglobin were obtained from Sigma Chemical Company (Poole). All other reagents were from BDH (Poole) and were of analytical grade.

3.2.2 Human Blood;

Venous blood was drawn from the arms of healthy volunteers; staff and students from Aston University, blood was obtained also from patients at The General Hospital - Birmingham, as a part of routine clinical diagnosis.

Blood samples from a group of workers in the lead industry were obtained from IMI Refiners Ltd workers (James Bridge Copper Works), Darlston Rd, Walsall, courtesy of

Dr. G. Krishnan (Health and Safety Executive, West Midlands area). Blood samples from haemodialysis patients with high blood aluminium levels were obtained from Queen Elizabeth Hospital - Birmingham, courtesy of Dr R A Braithwaite (Regional Laboratory for Toxicology-Dudley Rd. Hospital, Birmingham), and by Dr P Altmann (The London Hospital - Whitechapel, London). A blood sample from one female heterozygote for DHPR deficiency was supplied by Dr R Leeming (The General Hospital - Birmingham).

Blood lead levels for normals and lead workers were performed using atomic absorption spectrometry, courtesy of Dr. G. Krishnan and Dr. R. A. Braithwaite. Blood aluminium levels for haemodialysis patients with chronic renal failure were performed, courtesy of Dr. Altmann and Dr. Braithwaite.

3.2.3 Assay of DHPR Activity in Whole Blood;

DHPR activity was assayed by the modified method of Narisawa et al (1980). Each incubation contained 35×10^{-6} M NADH, 80×10^{-6}M ferricytochrome c, 5×10^{-2} M Tris-HCl buffer (pH 7.6), 18×10^{-6}M $DMPH_4$ and 0.1 ml. of the enzyme solution in a total volume of 1 ml. Control assays were run without $DMPH_4$. Whole blood samples were diluted 10 times with distilled water for assay.

The rate of reaction was followed by measuring spectrophotometrically the increase in optical density at 550 nm, due to formation of ferrocytochrome c at 37°C in a Pye Unicam PU 8800 spectrophotometer with a constant temperature cell holder. A molar extinction coefficient of 21 mM^{-1} cm^{-1} for cytochrome c at 550 nm was used to calculate the enzyme activity. The results were then corrected to 1 mg. of hemoglobin, which was determined according to the method of Drabkin and Austin (1935).

3.2.4 Total Hemoglobin Determination in Whole Blood;

Total hemoglobin in whole human blood was determined by the cyanomethemoglobin method, using Drabkin's reagent (Drabkin and Austin, 1935), in which 20 µl. whole blood added to 5 ml. Drabkin solution, mix well and allow to stand 15 minutes at room temperature. Then the absorbance from the sample was recorded vs. blank (Drabkin's reagent only), and the total hemoglobin concentration (mg./ml.) was determined directly from the calibration curve of standard hemoglobin solution.

3.2.5 Measurement of Km Values:

The effect of $DMPH_4$ and NADH concentrations on the DHPR activity from whole blood were examined. Km values were determined by variation of the concentration of one substrate at saturating level of the other substrate.

3.2.6 Effect of pH on DHPR Activity:

The effect of pH on DHPR activity were examined, and the whole blood DHPR activity was assayed using 0.05 M Tris-maleate buffer (pH 5.8-7.6) and Tris-HCl buffer (pH 7.2-9.0).

3.3 Results and Discussion:

DHPR activity was assayed in whole blood of normal adults (Table 3.1, 3.2). Normal adult males (n = 45) have DHPR activity of 1.72 ± 0.41 nmole cytochrome c/min./mg. Hb. (mean $\pm$ S.D.). The activity in the blood of normal adult females (n = 37) is significantly higher (1.95 ± 0.45 nmole cytochrome c/min./mg. Hb.) (student's "t" test $p < 2\%$). As shown in table (3.2), DHPR activity in the whole blood of normal adults decreases as the age increases above 40, but not significantly. DHPR activity in normal adult

female is still higher than male even when the samples were matched for age.

The effect of varying the concentration of $DMPH_4$ and NADH on the measured DHPR activity from human whole blood, was determined. This showed a hyperbolic relation for enzymatic activity versus substrate concentrations. Substrate inhibition was observed at concentration of $DMPH_4$ and NADH greater than 18 and 35 μM respectively (as shown in Figs. 3.1, 3.2). Michaelis constants were determine from Hanes and Eadie-Hofstee plots (see Fig. 3.3), and the Km values for normal subjects (n = 5) were 2.60 ± 0.93 (mean $\pm$ S.D.) and 15.63 ± 3.5 μM for $DMPH_4$ and NADH respectively.

Fig. (3.4) demonstrates the optimal cytochrome c concentration that can be used in DHPR activity assay, and this clearly shows cytochrome c inhibition observed at concentration greater than 80 μM.

Table (3.3) shows the effect of dilution on whole blood DHPR activity, in which the activity increased as the blood becomes more diluted. From Dixon plot with using dilution factor as inhibitor concentration, the dilution effect seems to be as uncompetitive inhibitor to DHPR (as shown in Figs. 3.5, 3.6).

We have studied the effect of the blood components on DHPR activity, in an attempt to describe in detail the relationship between DHPR activity and the blood dilution. Fig. (3.7) shows the effect of dilution of heat inactivated blood at 50°C with untreated blood, in which DHPR activity increased as % of heat inactivated blood decreases.

Table (3.4) shows the effect of hemoglobin on the activity of whole blood DHPR, and this clearly shows DHPR inhibiiton as 0.1 ml. of human hemoglobin solution added to the assay mixture according to modified Narisawa or Craine methods. Table (3.5) shows the effect of hemoglobin on the activity of DHPR purified from human brain, in which the DHPR inhibition % increases as the concentration of hemoglobin added to the assay mixture increased.

The effect of other blood components on DHPR activity has been studied, as shown

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in table (3.6), in which 0.1 ml. of blood plasma, serum, bovine serum albumin and heat inactivated blood at 50°C have been added to the assay mixture consecutively, and it clearly shows the maximum inhibition with using blood plasma or serum, while there is no inhibition with bovine serum albumin.

Table (3.7) shows that one female heterozygote for DHPR deficiency has a whole blood DHPR activity of 0.74 nmole cyt.c/min./mg. Hb., which is less than half that of normal female (1.95 ± 0.45).

The effects of varying the concentration of $DMPH_4$ and NADH on the measured DHPR activity from the female heterozygote for DHPR deficiency, was determined (Fig. 3.8). As shown in table (3.8), Km values for female heterozygote are significantly higher than that of normal subjects.

DHPR activity was measured in whole blood samples from workers in the lead industry, together with blood lead levels and mean cell hemoglobin (picogm.) (M.C.H. pg) (Table 3.9). As the blood lead level of workers in the lead industry is significantly increased ($p < 0.1\%$ student's "t" test), there is a significant decrease in DHPR activity/mg. Hb. and DHPR activity/cell ($p < 0.1\%$). Fig. (3.9) demonstrates the correlation between DHPR activity/mg. Hb. and the blood lead level (µg./dl.), (Correlation coefficient = 0.42) and the regression line in which there is a significant decrease in DHPR activity as the blood lead level increases ($p < 0.1\%$).

Michaelis constants for lead workers were determined from Hanes and Eadie-Hofstee plots (see Fig. 3.10), and are summarized in table (3.10), in which there is no difference in Km values for lead workers and normal subjects. The effect of pH on the enzymatic activity of both normal and lead workers is shown in Fig. (3.11), in which 0.05 M Tris-maleate and 0.05 M Tris-HCl buffers were used. The enzyme showed a plateau of peak activity from pH 6.8 to 8.4.

DHPR activity was measured in the whole blood samples from haemodialysis patients with chronic renal failure. Hemoglobin concentration for these samples was

determined , and in the majority found to be too low to correlate with the normal samples (as shown in table (3.11). This problem led us to dilute the normal blood samples and to prepare a standard curve of DHPR activity with different dilution of blood (different hemoglobin concentrations), as in Fig. (3.12), in order to correlate DHPR activity/mg.Hb. for blood samples of haemodialysis patients with normal samples in the same level of hemoglobin. There is a significant difference between DHPR activity/mg. Hb. for blood samples of haemodialysis patients and that for correlated normal blood samples ($p < 5\%$ Student's "t" test), as shown in table (3.11).

In addition, table (3.12) shows the highly significant difference between DHPR activity/mg. Hb. for blood samples of haemodialysis patients supplied by Dr. Braithwaite and that for correlated normal blood samples ($p < 1\%$ Student's "t" test).

The plot of DHPR activity/mg.Hb. versus aluminum level gives a significant correlation (correlation coefficient = 0.52, $p < 2\%$ Student's "t" test), see Fig. (3.13).

In contrary, if the blood samples supplied by Dr. Altmann, split in separate group, there is a good correlation between DHPR activity/mg.Hb. and aluminum level (Correlation coefficient = 0.64) and the difference is highly significant ($p < 0.1\%$ Student 's "t" test). Meanwhile, as we plot DHPR activity/ml. versus hemoglobin concentration, the correlation coefficient is 0.6 ($p < 0.1\%$ Student's "t" test), as shown in Fig. (3.14, 3.15).

The results are also very significant if we split the two groups, one with patients having aluminum levels less than 50 and the other with levels more than 50 ($p < 0.2\%$ Student's "t" test) as in table (3.13).

So far, there is a significant difference in DHPR activity/mg.Hb. between blood sample from haemodialysis patients with high aluminum level and normal hemoglobin levels, supplied by Dr. Braithwaite and normal blood samples ($p < 2\%$ Student 's "t" test), as shown in table (3.14).

TABLE 3.1 DHPR activity* in whole blood from normal subjects (I)

Subject	Age	mg.Hb./ml	DHPR activity
Male (45)	17 - 89	14.92 ± 1.86	$1.72 \pm 0.41^{+}$
Female (37)	11 - 54	13.75 ± 1.60	$1.95 \pm 0.45^{+}$

*nmole cytochrome c/min./mg, Hb.
$^{+}p < 2\%$ (Student's "t" test)
All values as mean $\pm$ S.D.
Figures in parenthesis refer to number of subjects studied.

TABLE 3.2 DHPR ACTIVITY IN WHOLE BLOOD FROM NORMAL SUBJECTS (2)

Subject	Age	mg.Hb./ml.	DHPR activity / mg.Hb.	M.C.H.pg.$^{+}$	DHPR activity/cell
Male	17-36	15.85$\pm$ 1.30(15)	1.77$\pm$ 0.36 (15)	29.99 $\pm$ 2.23 (15)	52.41 $\pm$ 11.97 (15)
	40-89	14.81$\pm$ 1.50(19)	1.59$\pm$ 0.35 (19)	30.66 $\pm$1.27 (19)	48.61 $\pm$ 10.48 (19)
Female	11-39	13.42$\pm$ 1.71(22)	1.94$\pm$ 0.45 (22)	55.02 $\pm$ 11.61 (13)	55.02 $\pm$ 11.61 (13)
	40-71	14.20$\pm$ 1.29(5)	1.78$\pm$ 0.32 (5)	32.05 $\pm$ 1.48 (2)	57.00 $\pm$ 6.51 (2)

* nmole cytochrome c/min./mg.Hb.

$^{+}$ Mean cell hemoglobin (pgm.)

All values as mean $\pm$ S.D.

Figures in parenthesis refer to number of subjects studied.

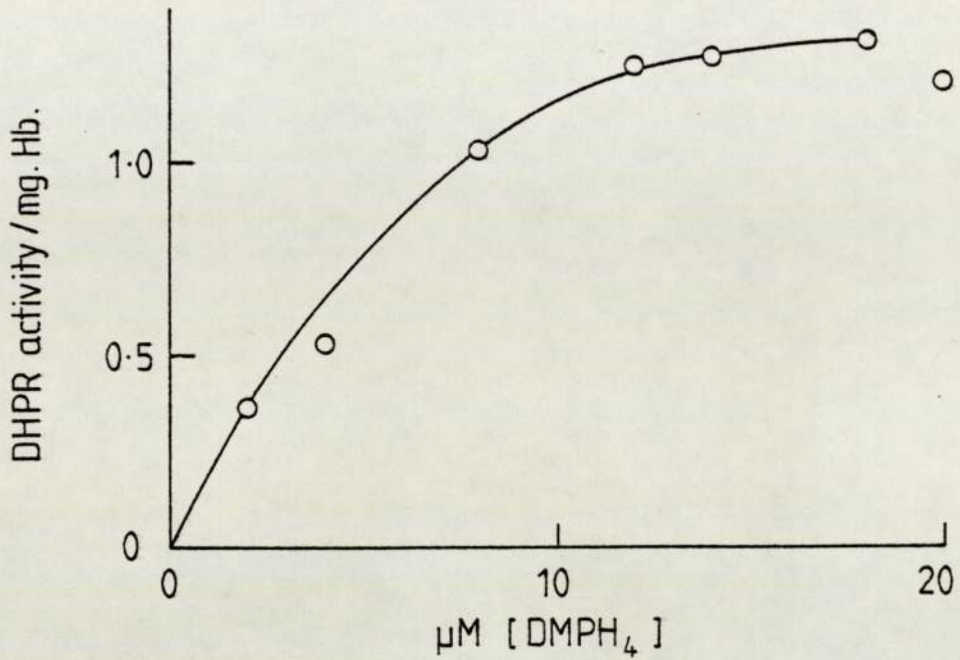


Figure 3·1 Effect of $DMPH_4$ concentration on whole blood DHPR activity.

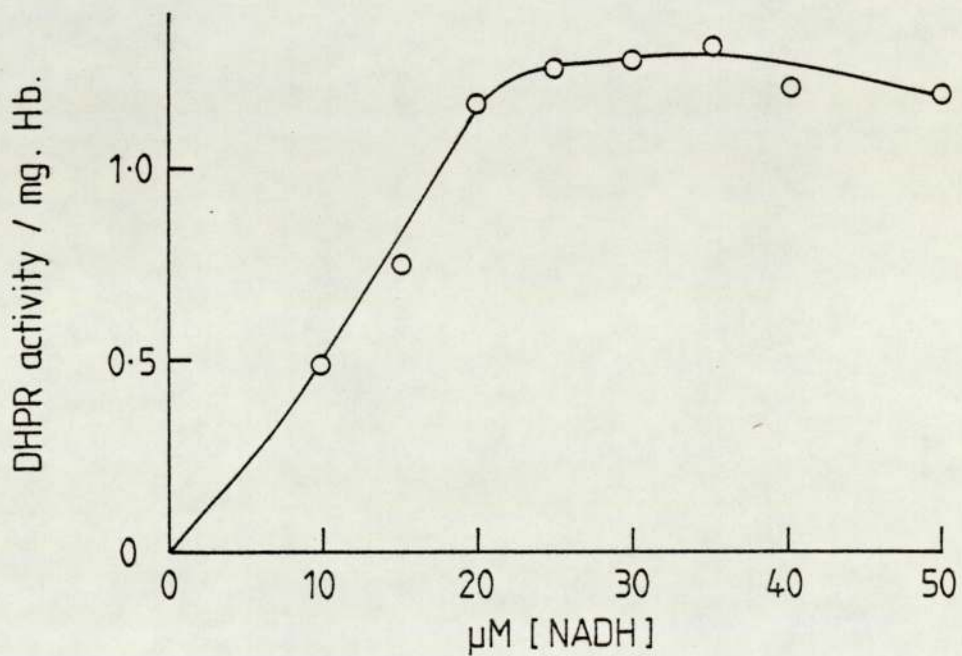
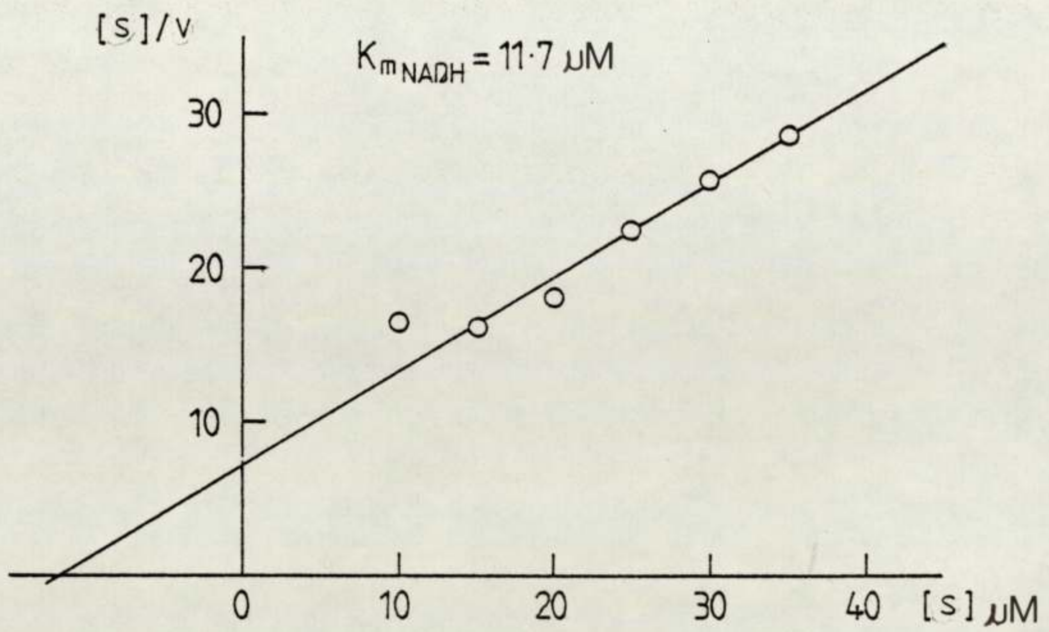


Figure 3·2 Effect of NADH concentration on whole blood DHPR activity.



v

$K_{m_{DMPH_4}} = 3{\cdot}6 \mu M$

2·0

1·0

0

0 0·1 0·2 0·3 0·4 0·5 0·6

v/[S]

Figure 3·3 Effect of $DMPH_4$ and NADH concentrations on whole blood DHPR activity from normal subjects.

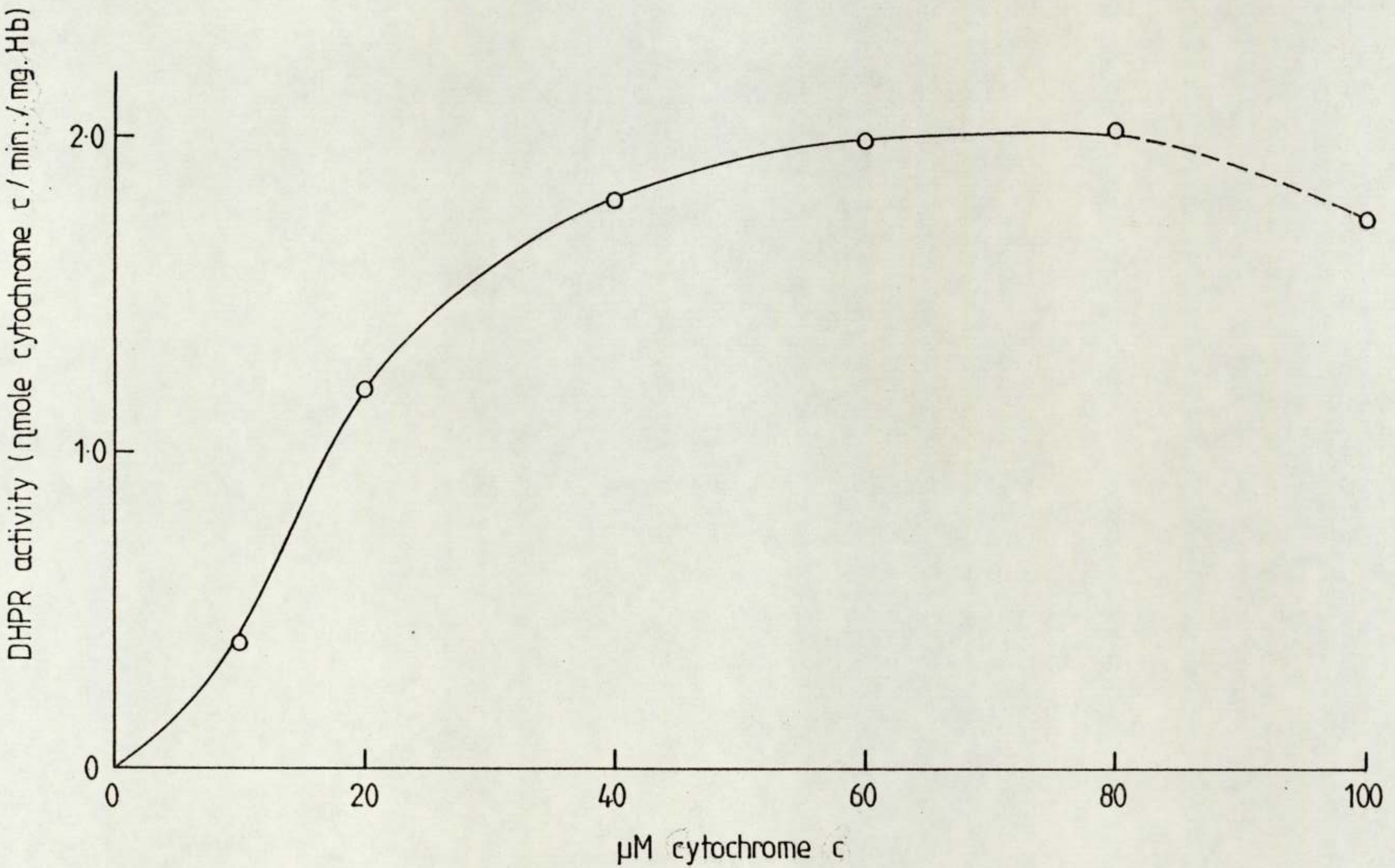


Figure 3·4 Effect of cytochrome c concentration on whole blood DHPR activity.

TABLE 3.3: The effect of dilution on whole blood DHPR activity*.

Dilution	mg.Hb./ml.	DHPR activity/mg.Hb.
1:5	21.8	1.03
1:10	12.1	3.70
1:20	6.3	5.22
1:50	2.3	7.04
1:100	1.37	6.61
1:1000	0.20	7.15

* μmole cytochrome c/min. mg.Hb.

TABLE 3.4 EFFECT OF HEMOGLOBIN ON THE ACTIVITY OF WHOLE BLOOD DHPR

Assay Method	Blood Dilution	DHPR activity/mg.Hb.		mg.Hb./ml. after addition of Hb.	DHPR Inhibition %
		before addition of Hb.	after addition of Hb.		
modified Narisawa (Narisawa _et al_, 1980)	1 : 20	5.2 +	2.5	10.2	51.9%
	1 : 100	6.5	0.43	8.8	93.4%
Modified Craine (Craine _et al_, 1972)	1 : 50	60.7 ++	3.06	12.0	95%
	1 : 100	128.6	3.5	12.5	97%

+ nmole cytochrome c/min./mg.Hb.

++ nmole NADH/min./mg.Hb.

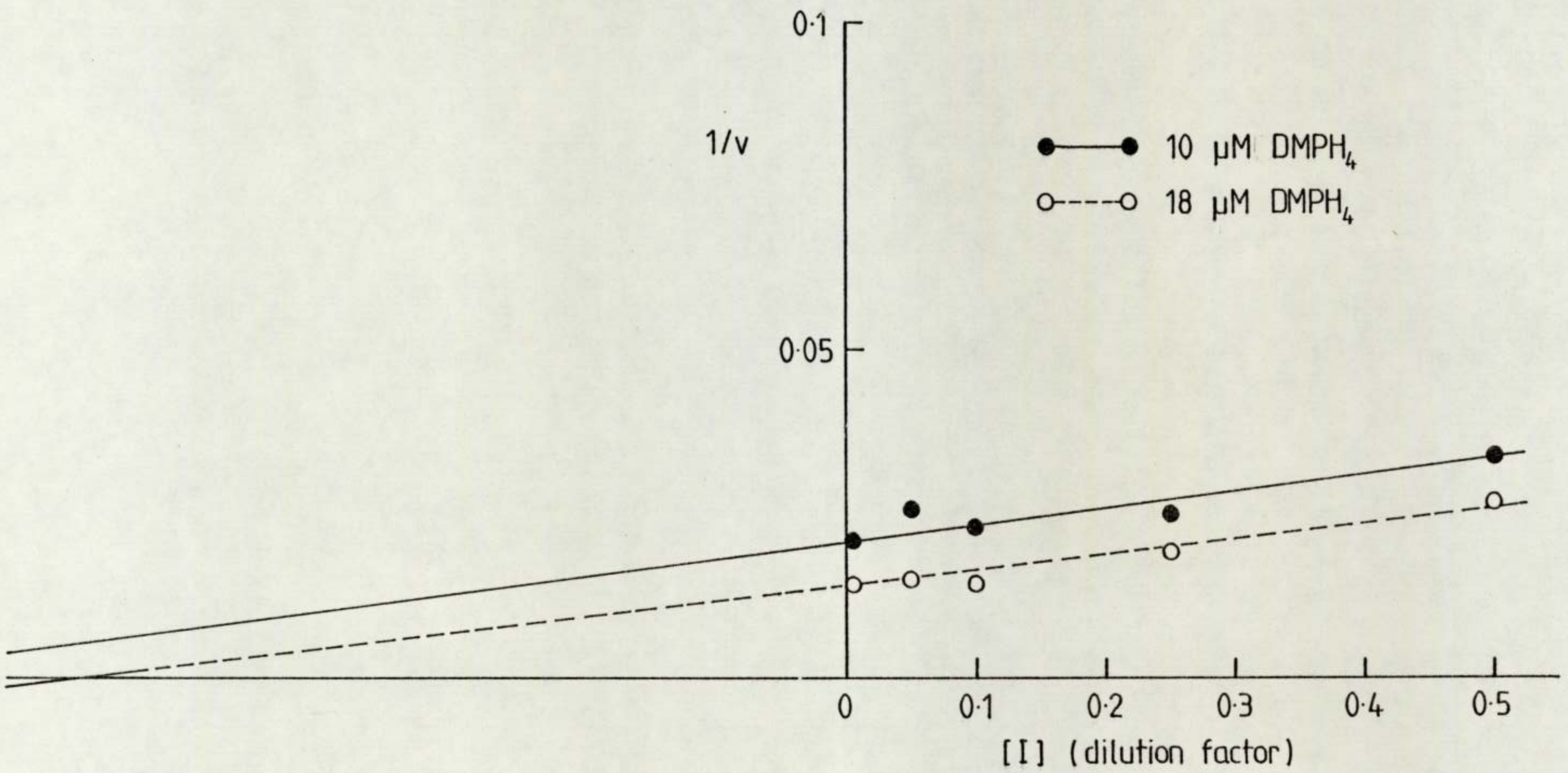


Figure 3·5 Effect of dilution on whole blood DHPR activity (Narisawa method) (1)

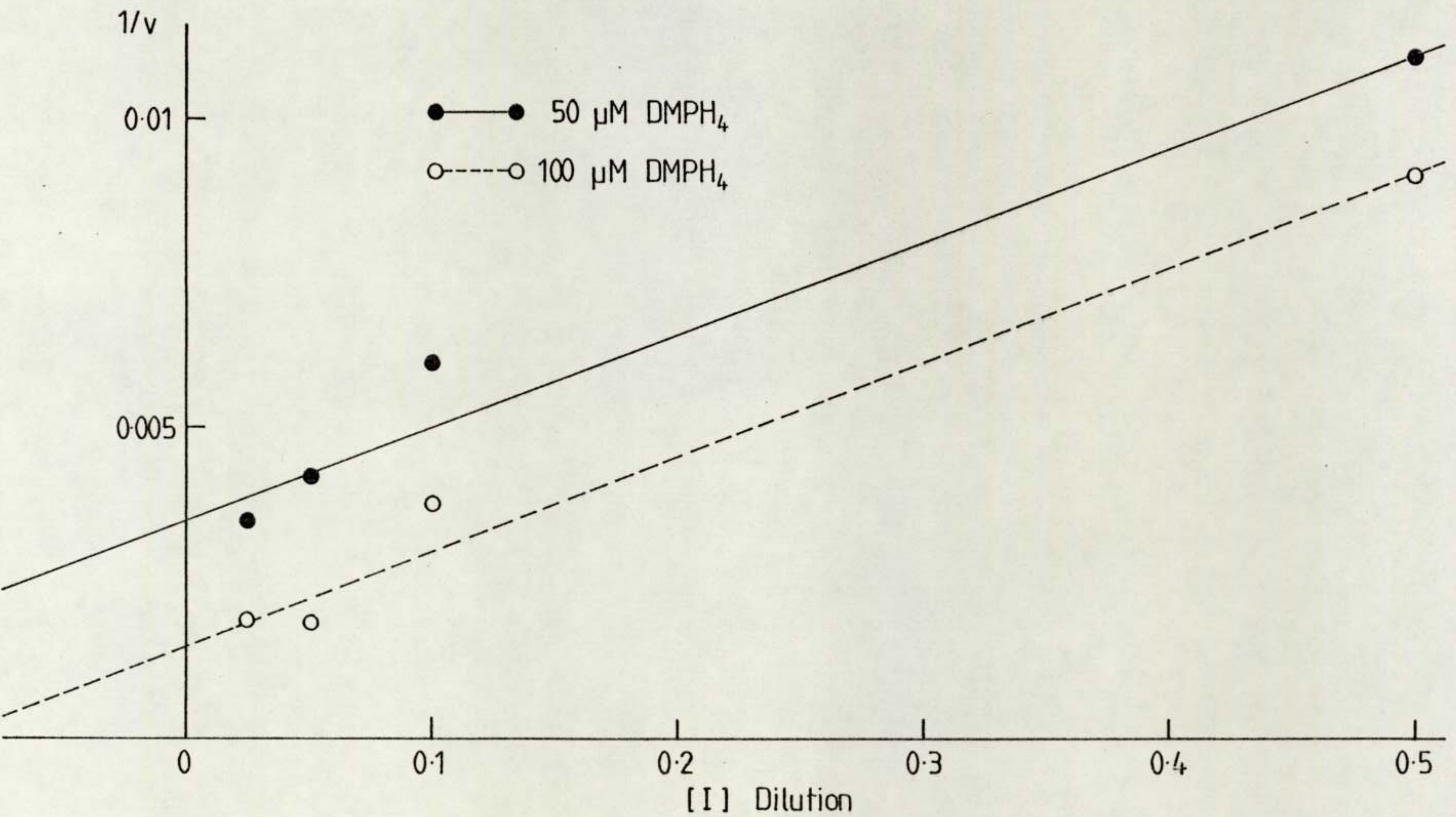


Figure 3·6 Effect of dilution on whole blood DHPR activity (Craine method) (2)

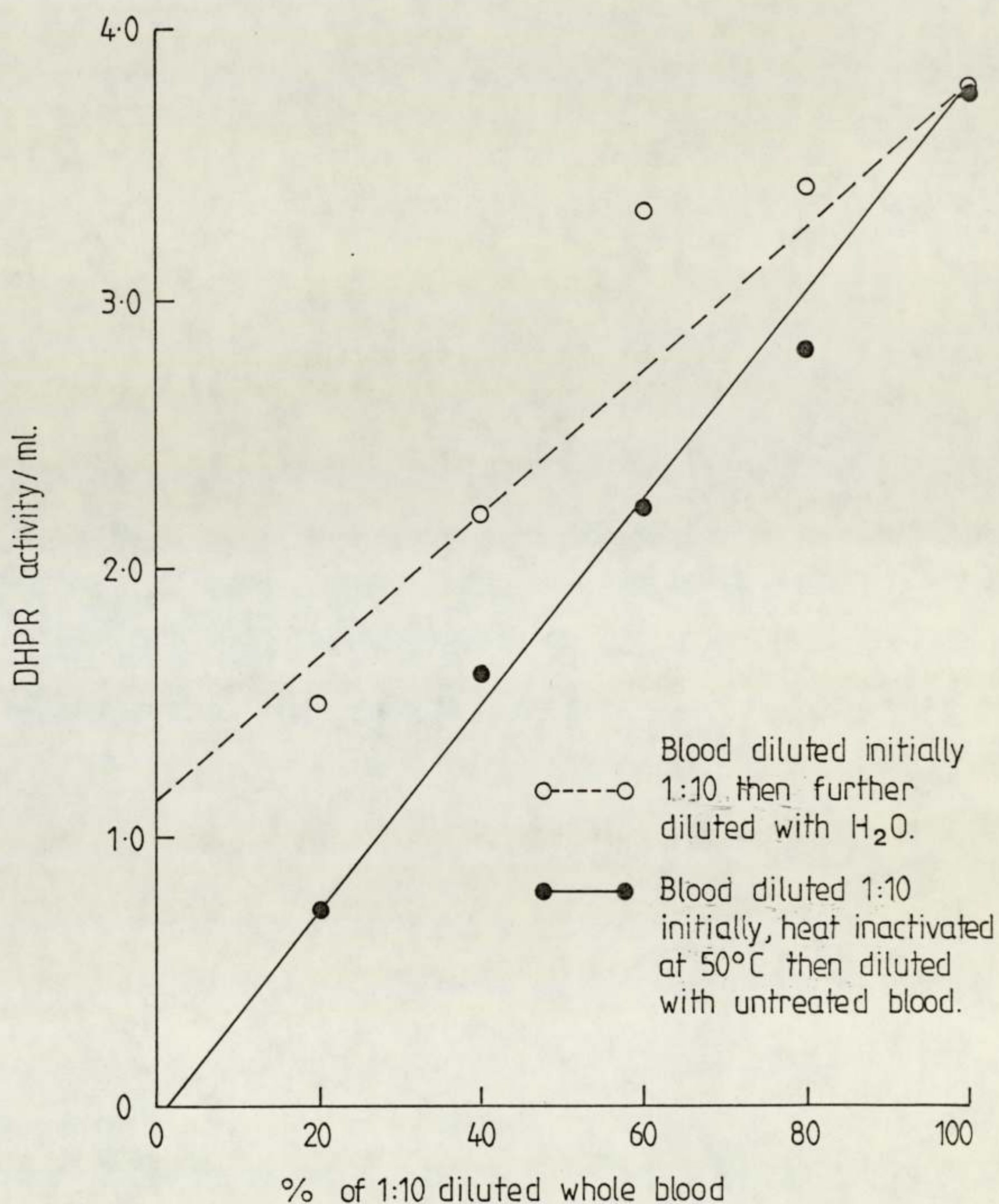


Figure 3·7 Effect of dilution on whole blood DHPR activity. (3)

TABLE 3.5 Effect of hemoglobin on the activity of DHPR* purified from human brain.

Conc. of Hb. added (mg./ml.)	Control DHPR activity	DHPR activity after Hb. addition	Inhibition %
3	54.6	46.8	14.3%
10	54.6	21.5	45.7%

*µmole cytochrome c/min./mg. protein

TABLE 3.6: The effect of blood components on DHPR activity*.

	DHPR activity	DHPR inhibition %
whole blood (1:10 dilution)	2.08 ± 0.14 (4)	
with blood plasma	1.20 ± 0.11 (4)	42.3%
with blood serum	1.04 ± 0.08 (4)	50%
with BSA (10 mg./ml.)	2.08 ± 0.06 (4)	------
with heat inactivated blood (1:10 dilution)	1.56 ± 0.10 (4)	25%

* µmole cytochrome c/min. /mg. Hb.
All values as mean ± S.D.

TABLE 3.7 : DHPR activity* in the whole blood from normal female subjects and female heterozygote for DHPR deficiency.

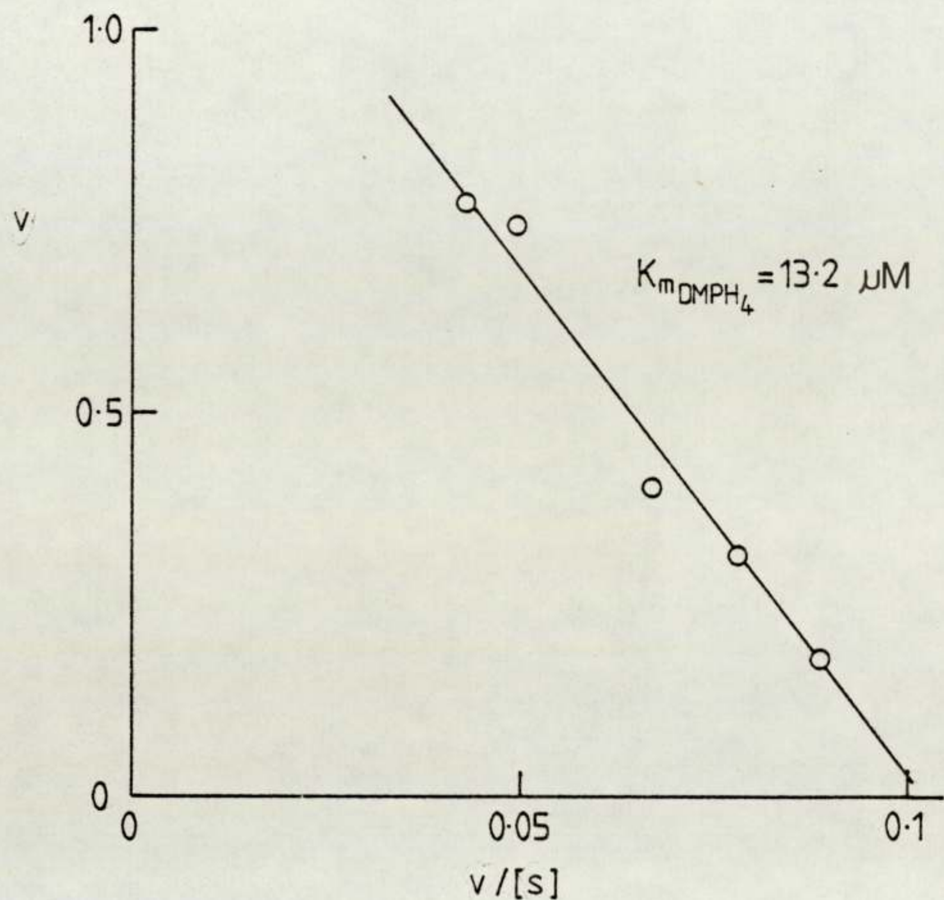
Subject	Age	mg.Hb./ml	DHPR activity
Normal (5)	11 - 54	13.75 ± 1.60	1.95 ± 0.45
Female Heterozygote (1)	2	20	0.74

*nmole cytochrome c/min./mg.Hb.
All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.

TABLE 3.8: Km values for DHPR from female heterozygote for DHPR deficiency.

Subject	Km $DMPH_4$	Km NADH
Normal (5)	2.60 ± 0.93	15.63 ± 3.5
Female heterozygote (1)	13.2 , 13.9	38.0 , 40.1

All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.



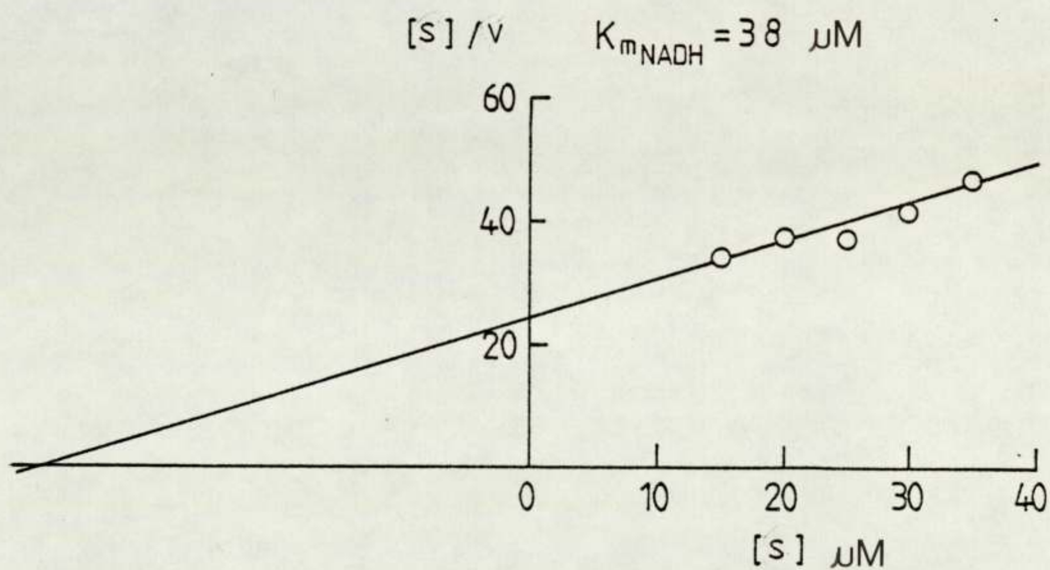


Figure 3·8 Effect of $DMPH_4$ and NADH concentrations on whole blood DHPR activity from female heterozygote for DHPR deficiency.

TABLE 3.9 DHPR ACTIVITY* IN THE WHOLE BLOOD FROM WORKERS IN LEAD INDUSTRY

Subject	Age	No.	mg.Hb./ml.	M.C.H.pg.**	DHPR Activity/mg.Hb.	DHPR Activity/cell	blood lead conc. μg./dl.
Normal Male	24 - 83	20	14.69 ± 2.22	30.89 ± 1.46	$1.66 \pm 0.36^{+}$	$51.26 \pm 11.40^{+}$	$13.55 \pm 5.09^{+}$
Lead workers	22 - 61	46	15.83 ± 1.55	28.67 ± 3.03	$1.23 \pm 0.25^{+}$	$35.94 \pm 7.20^{+}$	$48.0 \pm 15.4^{+}$

* nmole cytochrome c/min./mg.Hb.

** Mean cell hemoglobin (pgm.)

All values as mean $\pm$ S.D.

$^{+}$ $p < 0.1\%$ (Student's "t" test).

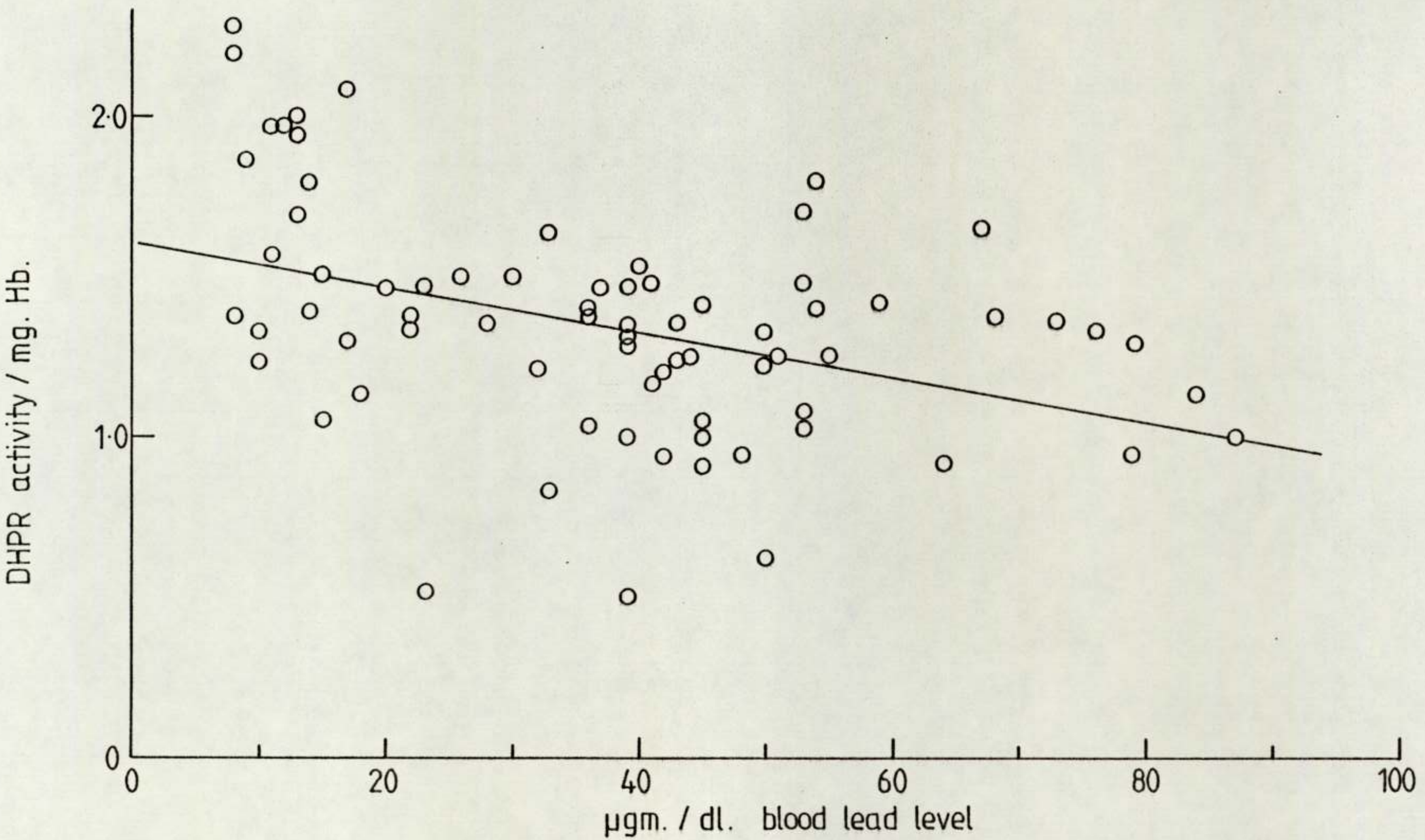
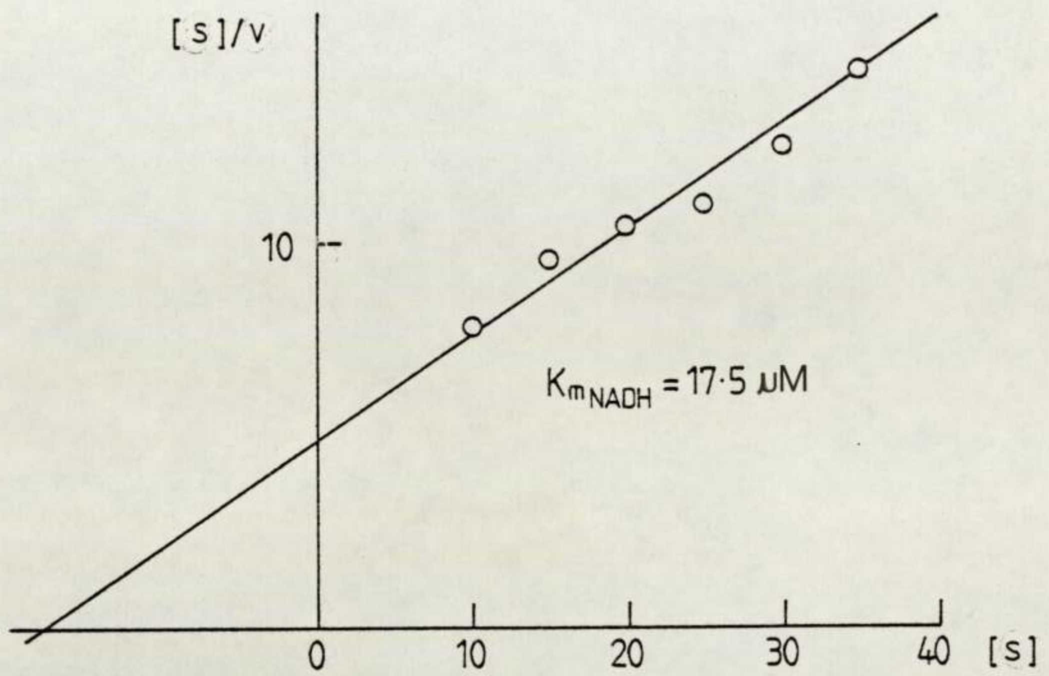


Figure 3·9 The correlation of blood lead level with DHPR activity / mg.Hb. in human whole blood. (Correlation coefficient = 0·42 $p < 0{\cdot}1\%$)



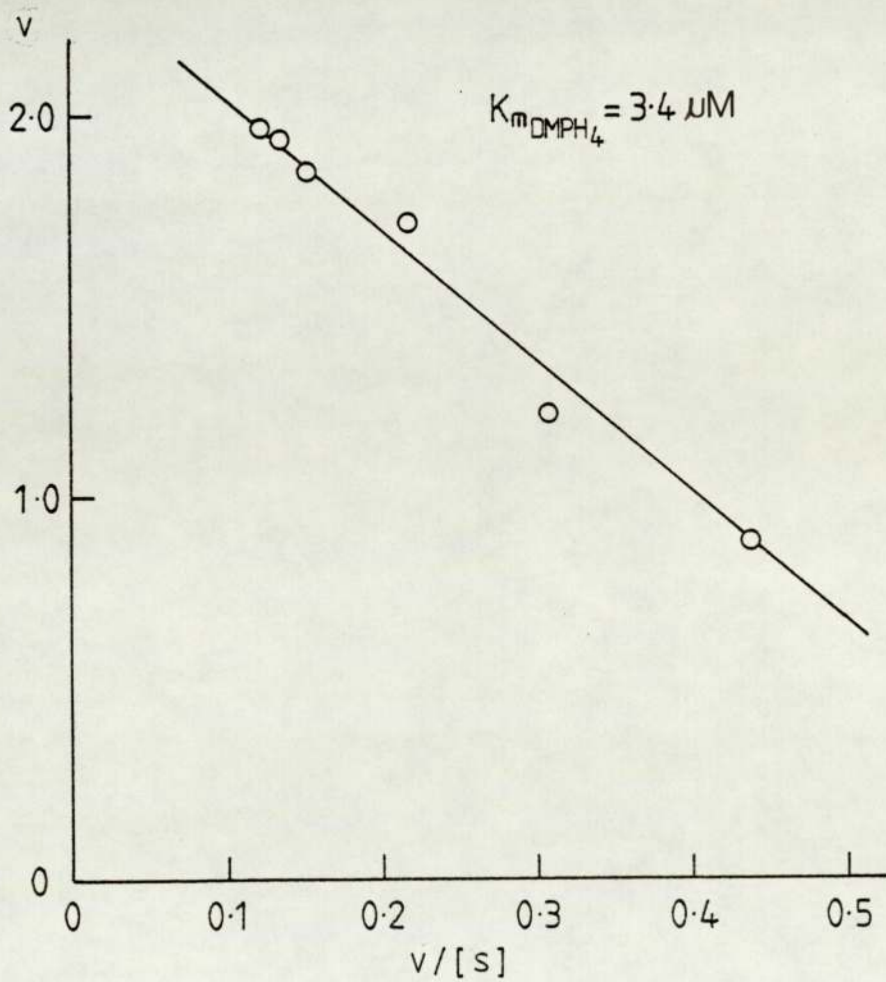


Figure 3·10 Effect of $DMPH_4$ and NADH concentrations on whole blood DHPR activity from lead workers.

TABLE 3.10: Km Values for DHPR from whole human blood

Subject	Km $DMPH_4$	Km NADH
Normal (5)	2.60 ± 0.93	15.63 ± 3.5
lead workers (5)	3.0 ± 0.68	16.2 ± 2.31

All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied

TABLE 3.11: DHPR activity* in the whole blood from haemodialysis patients with chronic renal failure. (1).

Subject	mg.Hb./ml.	DHPR activity	blood aluminum level (μg./l.)
correlated normal	8.52 ± 1.71 (47)	2.68 ± 0.17 (47)+	5.9 ± 2.3 (8) **
Haemodialysis patients	8.52 ± 1.71 (47)	2.37 ± 0.80 (47)+	91.91 ± 69.98 (47)

* μmole cytochrome c/min./mg.Hb.
** Shore et al. 1983
+ < 5% Student's "t" test

All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.

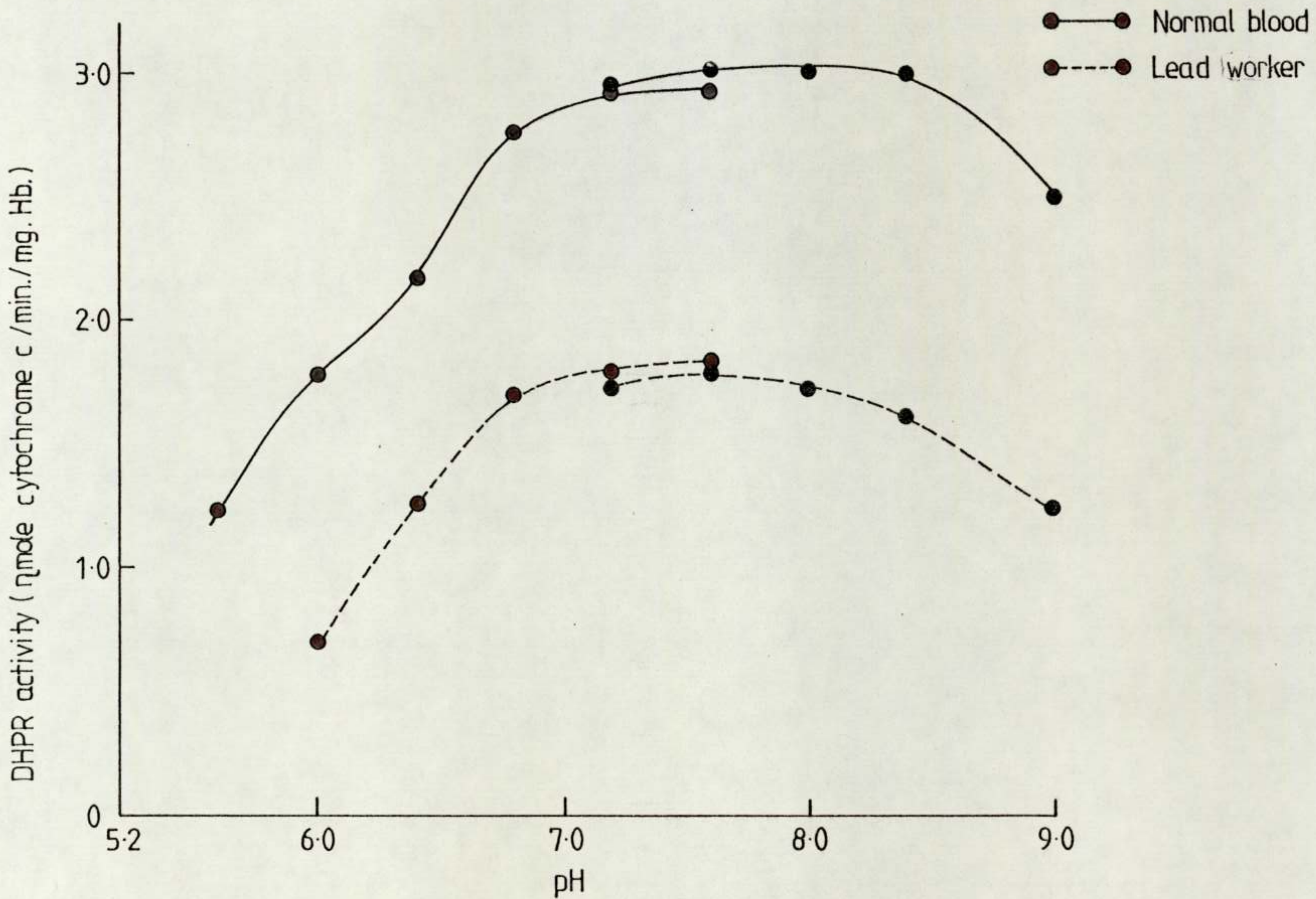


Figure 3·11 Effect of pH on whole blood DHPR from normal subjects and lead workers.

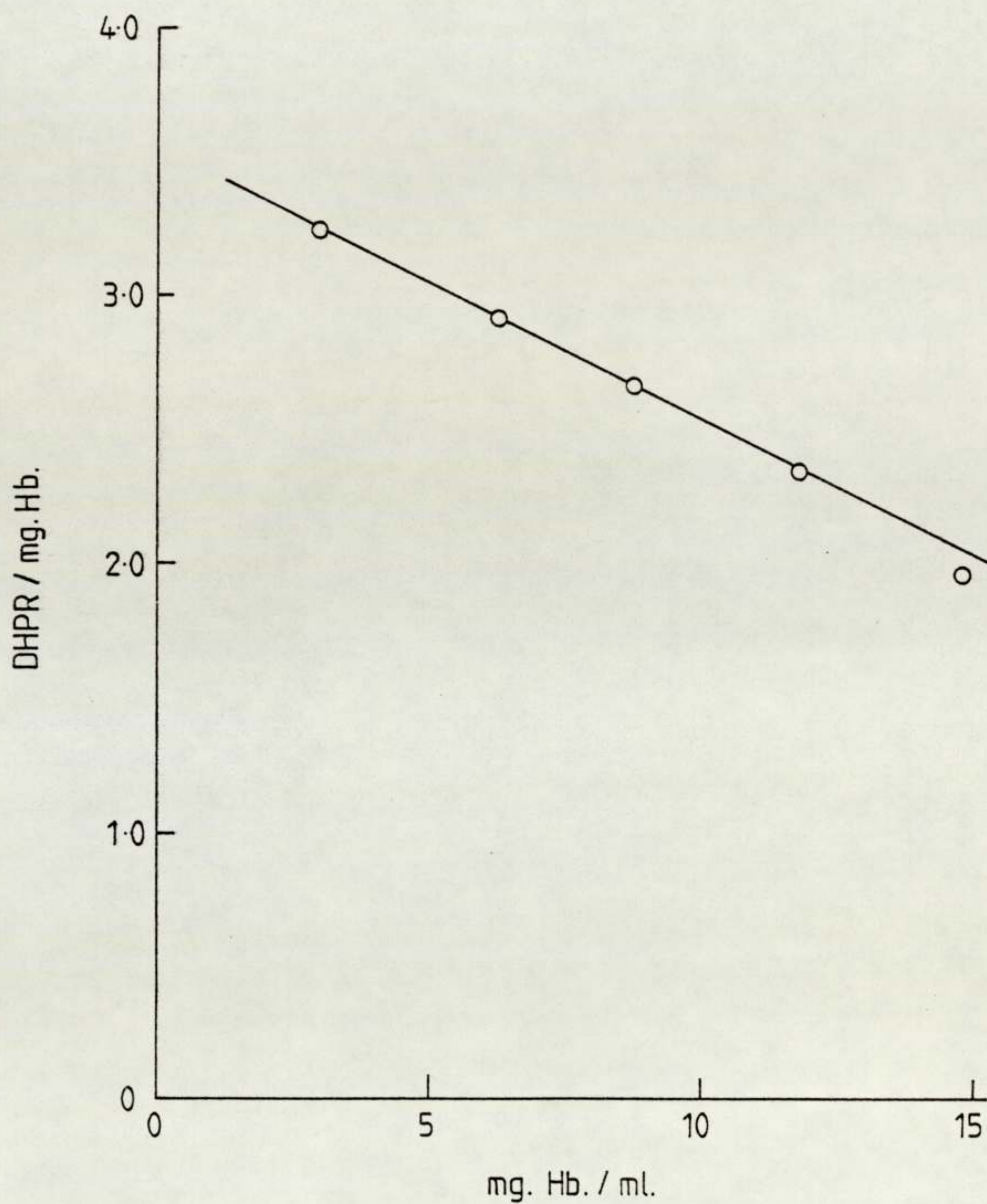


Figure 3·12 Standard curve of whole blood DHPR activity with Haemoglobin concentration.

TABLE 3.12: DHPR activity in the whole blood from haemodialysis pateints with chronic renal failure (2)*.

Subject	mg.Hb./ml.	DHPR activity**	Blood aluminum level (µg./l.)
Correlated Normal	9.17± 1.72(17)	2.61 ± 0.18 + (17)	5.9 ± 2.3 (8) ++
Haemodialysis pateints	9.17 ± 1.72(17)	2.16 ± 0.62 + (17)	143.44 ± 70.30 (18)

* Blood samples supplied by Dr. Braithwaite

** µmole cytochrome c/min./mg.Hb.

+p < 1% (Student's "t" test)

++Shore et al., 1983

All values as mean ± S.D.

Figures in parenthesis refer to number of subjects studied.

TABLE 3.13: Effect of blood aluminum level on DHPR activity from the whole blood of haemodialysis patients.

blood aluminum level (µg/l.)	DHPR activity (µmole cytochrome c/min./mg.Hb.)
15 - 50	2.59 ± 0.87 (23)*
55 - 288	1.82 ± 0.59 (26)*

All values as mean ± S.D.

Figures in parenthesis refer to number of subjects studied.

*P < 0.2% (Student's "t" test).

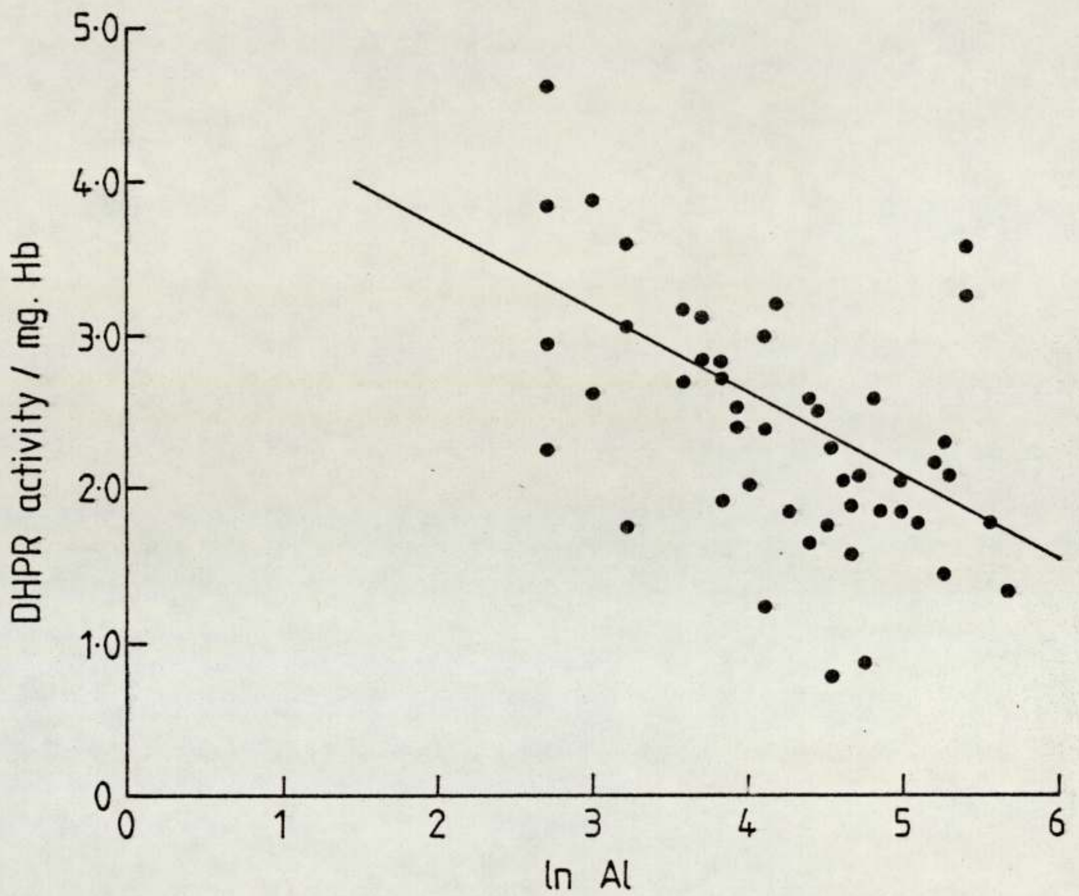


Figure 3·13 The correlation of blood Al level with DHPR activity / mg. Hb. in human whole blood from haemodialysis patients.
(Correlation coefficient = 0·52 $p < 0{\cdot}1\%$)

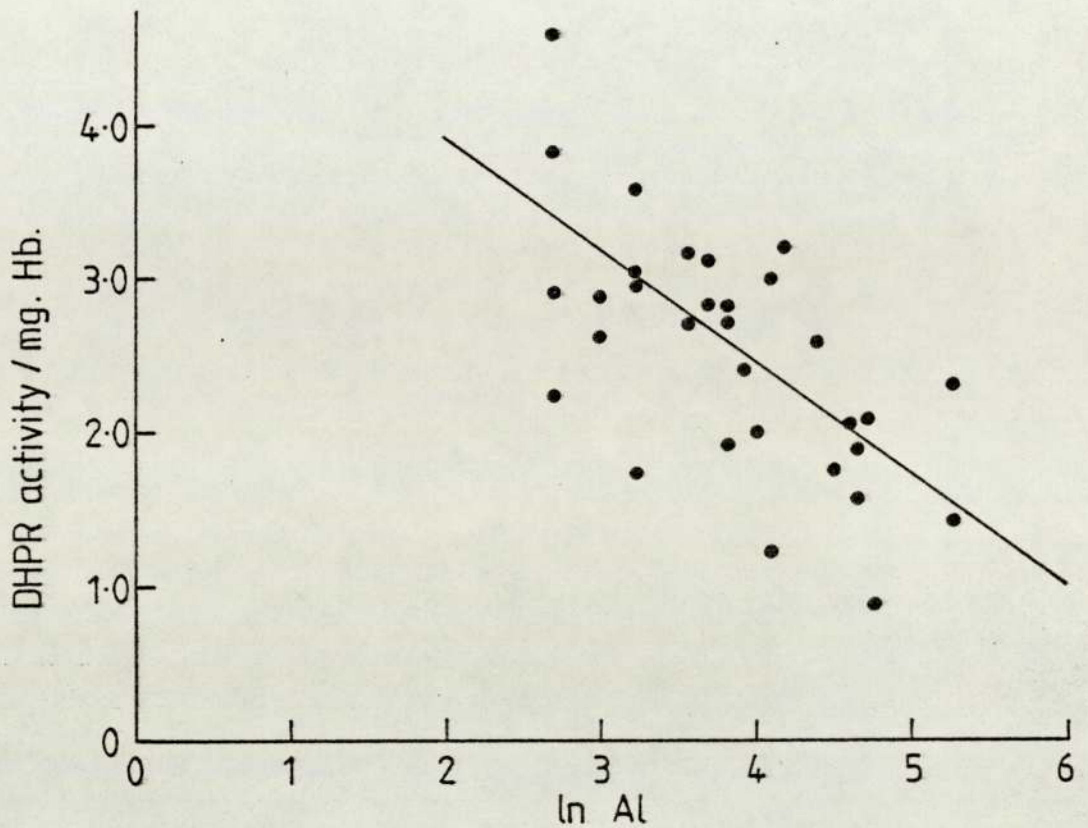


Figure 3·14 The correlation of blood Al level with DHPR activity/mg. Hb in human whole blood from haemodialysis patients. (Supplied by Dr. Altmann). (Correlation coefficient = 0·64 $p < 0{\cdot}1\%$)

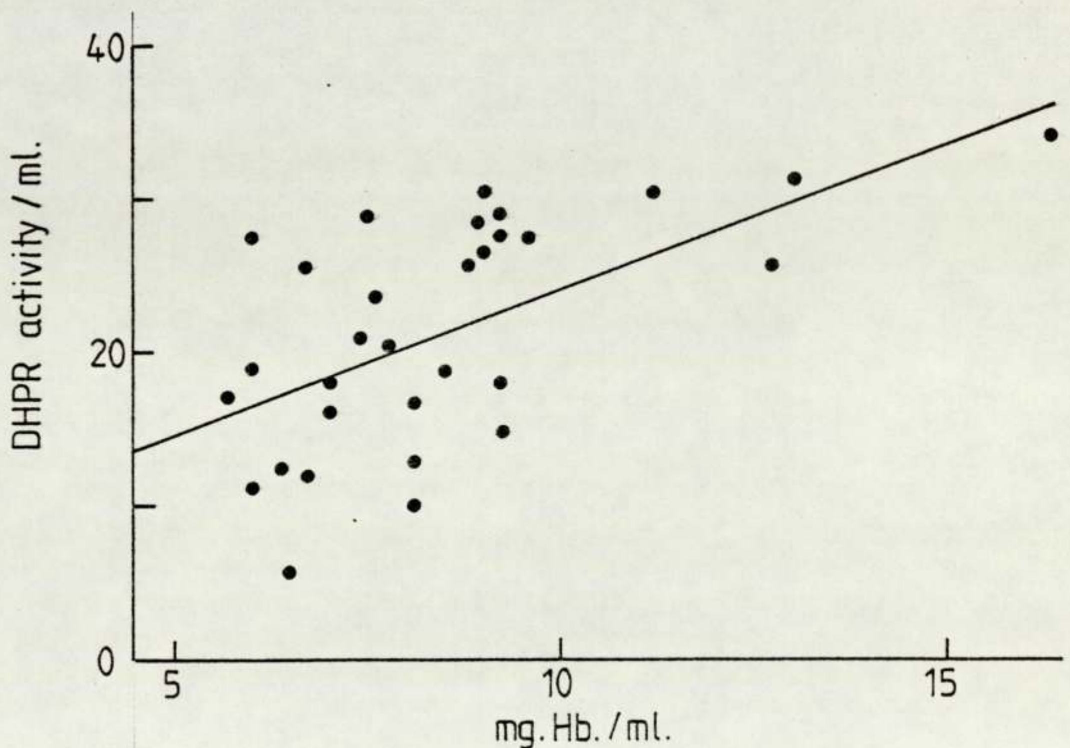


Figure 3·15 The correlation of blood Hb. level with DHPR activity / ml. in human whole blood from haemodialysis patients.
(Supplied by Dr. Altmann).
(Correlation coefficient = 0·61 $p < 0{\cdot}1\%$)

TABLE 3.14 : DHPR activity in the whole blood from haemodialysis patients with normal hemoglobin levels*.

Subject	mg.Hb./ml.	DHPR activity**	Blood aluminum level (μgm./l.)
Normal	14.39 ± 1.83 (81)	1.82 ± 0.44 (81)+	5.9 ± 2.3 (8)++
Haemodialysis patients	14.92 ± 2.52 (10)	1.46 ± 0.44 (10)+	75.40 ± 54.27 (10)

* Blood samples supplied by Dr. Braithwaite
** μmole cyctochrome c/min./mg. Hb
+ $P < 2\%$ (Student's "t" test).
++ Shore et al. 1983
All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.

CHAPTER 4

DHPR ACTIVITY IN TUMOURS

CHAPTER (4):

DHPR ACTIVITY IN TUMOURS:

4.1 Introduction:

The interest in the relationship of pteridines and diseases has been growing since defects in pteridine biosythesis have been described in atypical PKU. Pteridine levels have been reported to be altered in either blood or urine of cancer, viral disease and renal insufficiency patients (Leeming et al, 1976b; Leeming and Blair, 1980a; Rokos and Rokos, 1983; Wachter et al, 1979, Dhondt et al, 1983), BH_4 is accumulated in the blood of all tumour patients tested, and the major part was found in the erythrocyte fraction (Kokolis and Ziegler, 1977).

Leeming et al (1976b) have shown low levels of serum and urine biopterin derivatives in malignant carcinoid diseases, and the high levels of biopterin derivatives in carcinoid tissues match the increased biosynthesis of serotonin in this tumour (Grahame-Smith, 1972).

The urinary excretion of neopterin in patients with breast cancer has not been found to be significantly different from controls, whilst in gastrointestinal cancer urinary neopterin was elevated (Rokos and Rokos, 1983). Dhondt et al (1981), pointed out a large variation of DHPR activity in breast tumours in which the main group has activities of same magnitude as that in cultured fibroblasts. Meanwhile a significant correlation appeared between DHPR activites and oestrogen receptors. Eggar et al (1983) have shown that DHPR activity is significantly raised in neoplastic breast tissue, but not in neoplastic tissue from the large intestine.

To extend these observations in an attempt to identify steps in pteridine metabolism which may be disturbed in neoplastic disease, we have determined the kinetics of DHPR in the neoplastic breast tissue.

This thesis records for the first time the relation betweeen DHPR activity and DNA levels in the neoplastic breast tissues.

4.2 Materials and Methods:

4.2.1 Chemicals:

Nicotinamide adenine dinucleotide reduced (disodium salt type III) NADH, 6, 7-dimethyl-5, 6, 7, 8-tetrahydropterin ($DMPH_4$), Tris [tris (hydroxymethyl) aminoethane], cytochrome c, peroxidase and diphenylamine were obtained from the Sigma Chemical Company. Deoxyribonucleic acid-sodium salt (DNA) (from calf thymus gland) was obtianed from BDH chemicals Ltd. (Poole), and all other reagents were from BDH chemicals Ltd. (Poole) and were of analytical grade.

4.2.2 Human Tissues:

Breast and large intestine tissues were supplied courtesy of Mr Oates (Birmingham General Hospital), from patients with tumours. The tissues were removed from the patients during operation, after which the tumours were dissected out and portions supplied, with apparently normal tissue adjacent to the tumour from the same subject, for assay. This was frozen at -20°C until it was required.

Breast and large intestine tissues were broken into small bits first by chopping with a scalpel . 20% (w/v) Homogenates were prepared in 0.5M Tris/HCl buffer pH 7.6 using a Potter Elvejhem homogeniser. These homogenates were centrifuged at 0°C at 40,000 xg in a M.S.E. Superspeed centrifuge for 45 minutes. The supernatants were collected and used for the assay of DHPR activity. Protein was measured by the Biuret method (Layne 1957).

4.2.3 DHPR Activity Assay:

DHPR activity in the supernatant of neoplastic human tissue was measured according to the assay method of Craine et al (1972) and Narisawa et al (1980), as shown previously in chapters (2) and (3).

4.2.4 DNA Estimation:

DNA level in the breast neoplastic tissue was estimated according to the method of Burton (1956). The tissue was made into a homogenate using citrate buffer saline (0.15 M NaCl with 0.15 M sodium citrate buffer pH 4.5).

A known weight of tissue was made into a 25% homogenate and the final volume of homogenate noted. The DNA was extracted as follows: 0.95 ml. of homogenate was mixed with 0.05 ml. of 6 M perchloric acid and chilled. This was centrifuged in a M.S.E. benchtop centrifuge for 20 minutes at 1500 xg and the supernatant discarded. The pellet was broken up using a glass rod in 0.5 ml. of 0.5 M perchloric acid and then a further 3.5 ml. of 0.5 M perchloric acid added. This was heated at 70°C for 15 minutes and then centrifuged for 20 minutes in a benchtop centrifuge at 1500 xg. The supernatant was decanted off into a graduated test tube. The pellet was resuspended in 3 ml. of 0.5 M perchloric acid and centrifuged for 20 minutes at 1500 xg. The supernatant was decanted off, pooled in the graduated test tube and the volume noted. The DNA was estimated by taking 1 ml. of the extracted DNA solution and reacting it with 2 ml. of diphenylamine solution, prepared by taking 1 gm. of diphenylamine and dissolving it in 100 ml. of glacial acetic acid and adding 2.75 ml. of conc. H_2S0_4 (these reagents must be Analar). The tube was then placed into a boiling water bath for 15 minutes, after which it was removed and cooled in cold water. The colours were then estimated by determining their optical density at 600 nm against a water blank.

Standards were prepared by dissolving 40 mg. DNA (from calf thymus gland) in 100 ml. 0.005 M sodium hydroxide. This was then diluted with 0.005 M sodium hydroxide to give a range of standards. These standards were diluted 50/50 with 1 M perchloric acid and then heated at 70°C for 15 minutes. They were then heated with diphenylamine solution as before. DNA results were quoted as µg. DNA/gm. wet weight of tissue or µg. DNA/mg. protein.

4.3 Results and Discussion:

DHPR activity was measured in the neoplastic tissue from human breast. It was significantly increased compared to apparently normal tissue from the same breast ($p < 0.2\%$ by Wilcoxon's signed ranks test) as the activity measured according to Craine method (1972), table (4.1) . To confirm these results, the activity was measured by using two different assay methods; Craine et al (1972) and Narisawa et al (1980), and both show a significant increase ($p < 2\%$ by Wilcoxon's signed ranks test) as shown in table (4.2).

DHPR activity was measured also in the neoplastic tissue from human large intestine, and as shown in table (4.3), there is no significant increase as compared to apparently normal tissue from the same intestine.

The effect of 6, 7-dimethyl-5, 6, 7, 8-tetrahydropterin concentration on DHPR activity from neoplastic tissue was examined (see Fig. 4.1). Km values were measured from Hofstee-Eadie and Cornish-Bowden plots, as shown in Figs. (4.2-4.4). The apparently normal breast tissue has lower Km values than that of neoplastic tissue, but the difference is not significant (see table 4.4).

The effect of pH on the enzymatic activity of DHPR from human breast tissue is shown in Figs. (4.5 - 4.7), in which 0.05 M Tris-maleate and 0.05 M phosphate buffers were used. The enzyme showed a peak of activity at pH 6.8 for both normal and neoplastic tissues.

DHPR activity was measured also on the basis of DNA level, as shown in table (4.5), in which DHPR activity/μg. DNA in neoplastic tissue is significantly increased as compared to apparently normal tissue from the same breast ($p < 5\%$)

TABLE 4.1: DHPR activity* in apparently normal and neoplastic tissue form human breast

Patient	Age	Apparently normal tissue		neoplastic tissue	
		mg.protein/ml.	DHPR activity/ mg. protein	mg. protein/ml	DHPR activity/ mg. protein
G.I.	40	10	24.8	11.2	50.9
E.W.	64	13.8	2.47	11.3	6.94
A.B.		12.5	18.0	10.0	24.0
H.W.		12.5	1.2	8.75	9.74
M.F.		12.5	2.05	13.75	10.14
F.J.	70	4.1	3.7	7.5	7.5
M.K.		11.5	22.5	13.5	27.0
D.N.	70	6.75	8.7	9.0	10.9
M.P.	36	12.25	7.55	16.75	7.89
M.T.	75	13.35	2.79	11.25	25.04
G.H.	73	14.5	0.73	15.6	21.3
I.H.	60	8.85	9.6	9.6	14.3
F.H.		7.6	2.96	18.3	6.44
O.Q.	42	5.6	4.91	15.5	5.33
M.A.	80	9.0	1.4	13.5	30.2
D.C.	50	7.75	5.2	13.65	18.7
F.M.	43	8.75	11.42	11.75	40.22
B.J.	72	7.0	2.50	11.85	0.84
I.K.	38	8.0	7.5	10.35	34.8
T.N.		12.75	1.96	22.75	2.64
Z.S.	45	12.5	4.0	16.75	31.0
J.I.	53	9.5	6.05	22.5	31.6
C.D.	67	14.9	12.10	17.0	329.4

* DHPR activity (µmole NADH/min./mg. protein) measured according to Craine assay method. (1972)

TABLE 4.2: DHPR activity in apparently normal and neoplastic tissue from human breast, according to different assay methods.

Patient	Craine method (µmole NADH/min./mg. protein)		Narisawa method (µmole cytochrome c/min./mg.protein)	
	Apparently normal tissue	Neoplastic tissue	Apparently normal tissue	Neoplastic tissue
E. W.	50.5	73.0	3.9	5.3
H. W.	51.1	112.6	3.1	12.5
M. F.	19.0	63.0	3.7	5.8
F. J.	3.7	7.5	0.7	2.3
A. B.	18.0	24.0	3.8	8.1
G. I.	24.8	50.9	4.9	8.4

TABLE 4.3 : DHPR activity* in apparently normal and neoplastic tissue from human large intestine.

Patient	source of tumour	apparently normal tissue		neoplastic tissue	
		mg. protein/ ml.	DHPR activity/ mg.protein	mg. protein/ ml.	DHPR activity/ mg. protein
E.M.	Colon	19.5	33.7	19.7	13.7
C.J.	Colon	18.8	8.7	11.9	80.4
A.H.	Colon	17.8	41.8	18.3	7.6
A.T.	Rectum	14.5	35.4	19.2	30.0
B.L.	Rectum	6.65	2.05	10.75	22.6
E.T.	Rectum	10.5	18.2	14.2	26.1
J.C.	Caecum	26.7	40.2	23.5	27.3
V.T.	Omentum	11.25	25.4	13.0	33.7

* DHPR activity (nmole NADH/min./mg. protein) measured according to Craine assay method (1972).

TABLE 4.4: Km $DMPH_4$ values for human breast tissue

Patient	Craine method		Narisawa method	
	apparently normal tissue	Neoplastic tissue	apparently normal tissue	Neoplastic tissue
H.W.	57 μM	39 μM	10.8 μM	9.0 μM
E.W.	70	52	5.3	2.7
F.J.	55	40	9.7	3.5
M.F.	42	51	3.6	4.9
D.P	67	43	4.6	2.8

Each value is the mean of 3 experimental results.

TABLE 4.5 DHPR activity* in relation to DNA level, in apparently normal and neoplastic tissue from human breast.

Patient	Apparently normal tissue			Neoplastic tissue		
	μg. DNA/ gm. wt.	μg. DNA/ mg. protein	DHPR activity/ μg DNA	μg. DNA/ gm. Wt.	μg. DNA/ mg.protein	DHPR activity/ μg DNA
F. H.	16.58	0.06	3.3	47.89	0.124	6.67
O. Q.	27.0	0.25	2.47	564.9	1.74	0.395
M. A.	22.5	0.05	1..49	96.4	0.09	12.68
D. C.	38.5	0.049	2.77	95.5	0.073	8.01
F. M.	17.2	0.092	11.6	78.6	0.203	16.3
B. J.	16.0	0.176	2.69	36.8	0.113	0.85
I. K.	24.56	0.160	5.05	135.09	0.405	7.7
Z. S.	138.8	0.64	0.86	228.4	0.46	5.77
C. D.	24.7	0.014	26.11	109.3	0.36	51.24

* DHPR activity measured according to Craine assay method (1972)

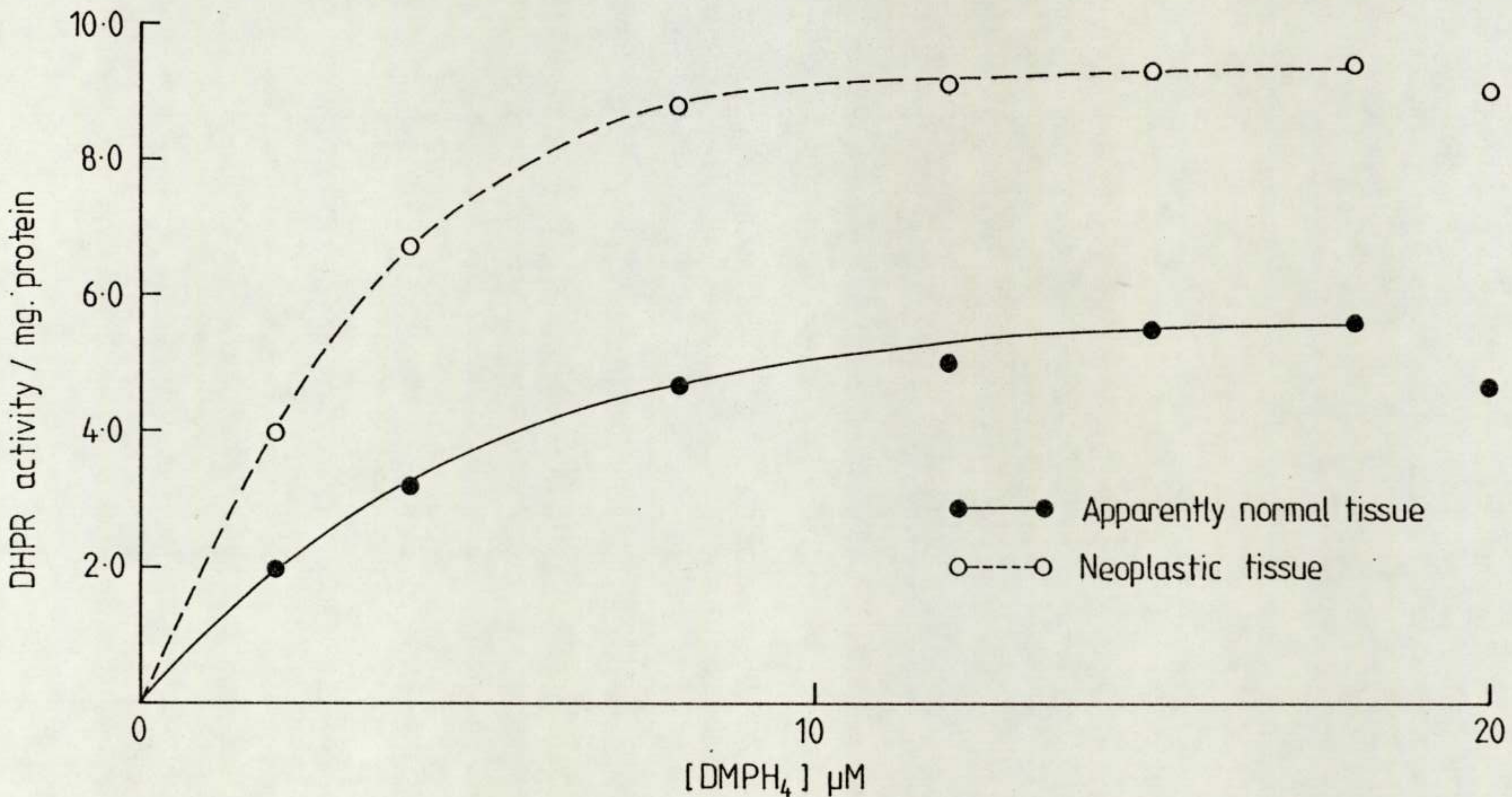
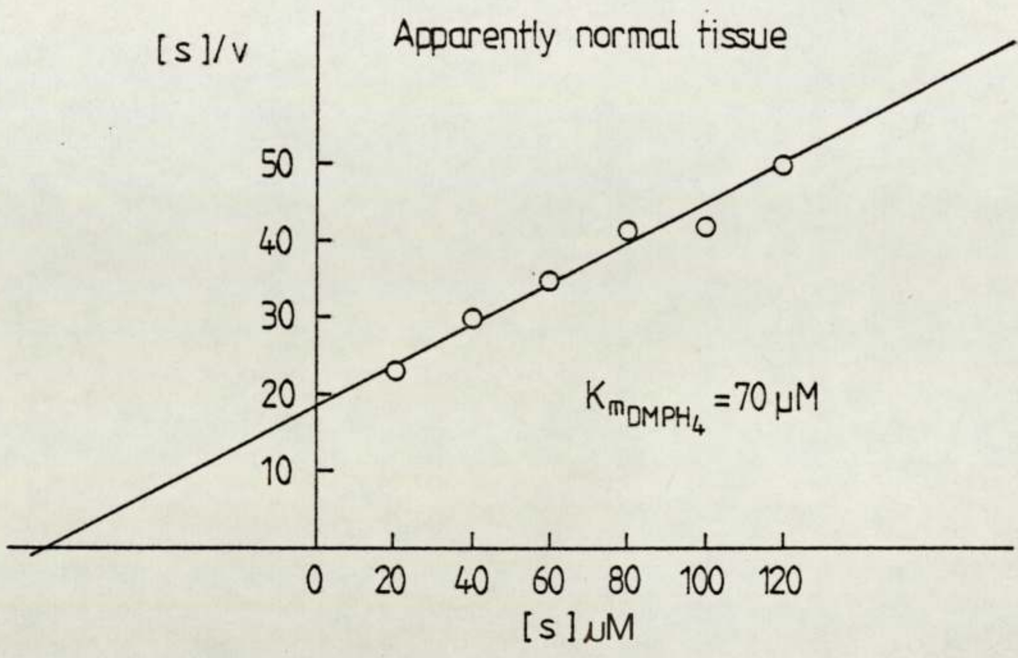


Figure 4·1 Effect of $DMPH_4$ concentration on DHPR activity from human breast tissues.



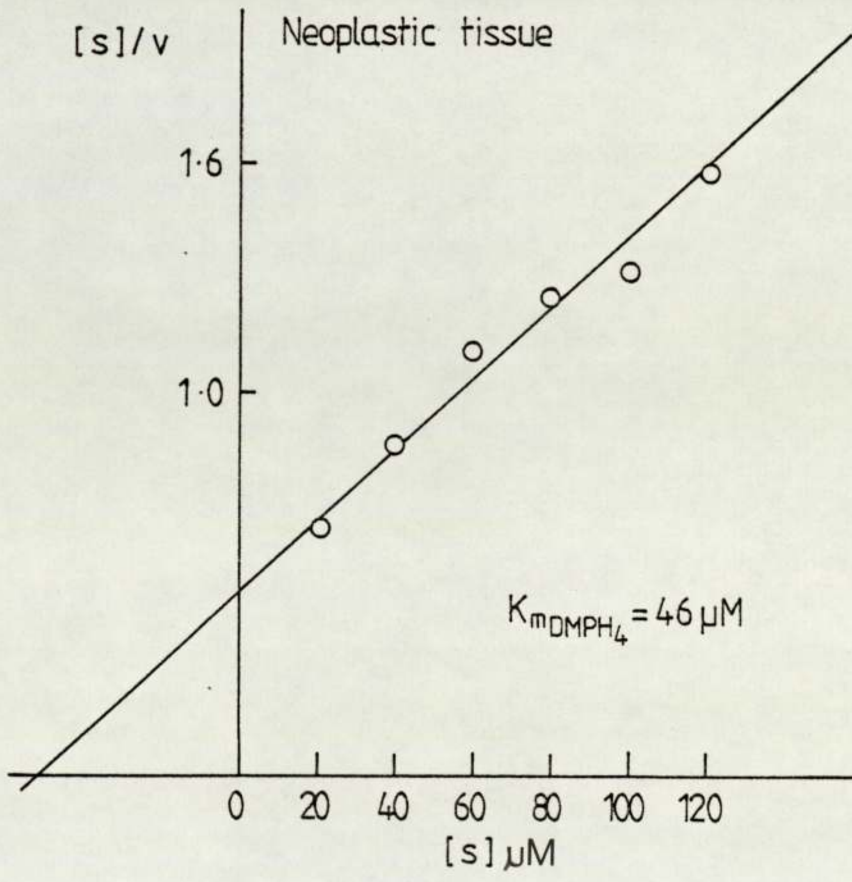
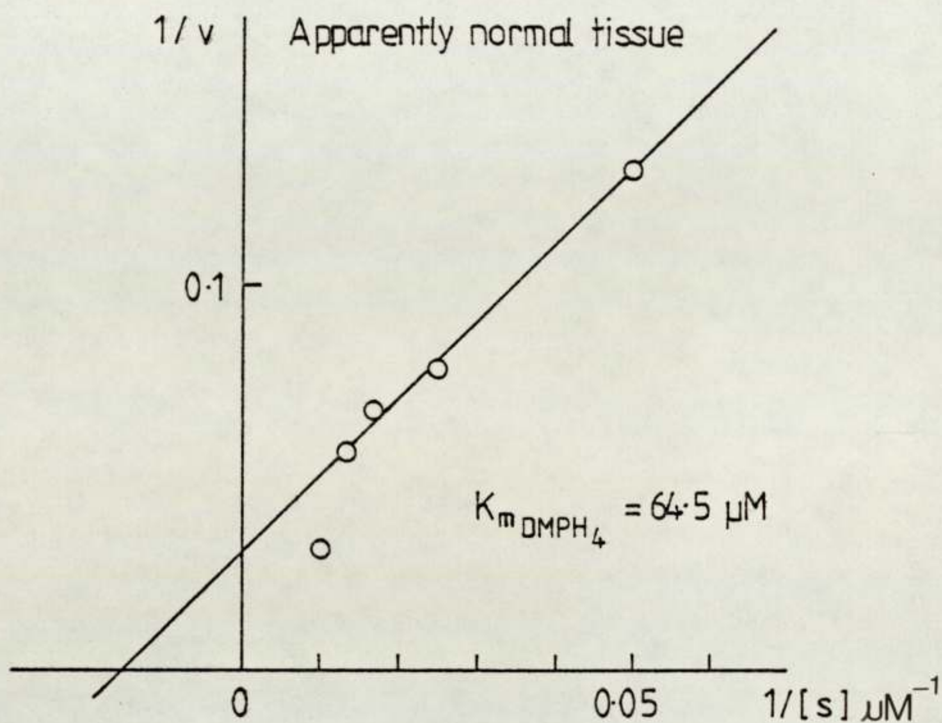


Figure 4·2 Effect of $DMPH_4$ concentration on DHPR activity from human breast tissues. (Craine method) (1)



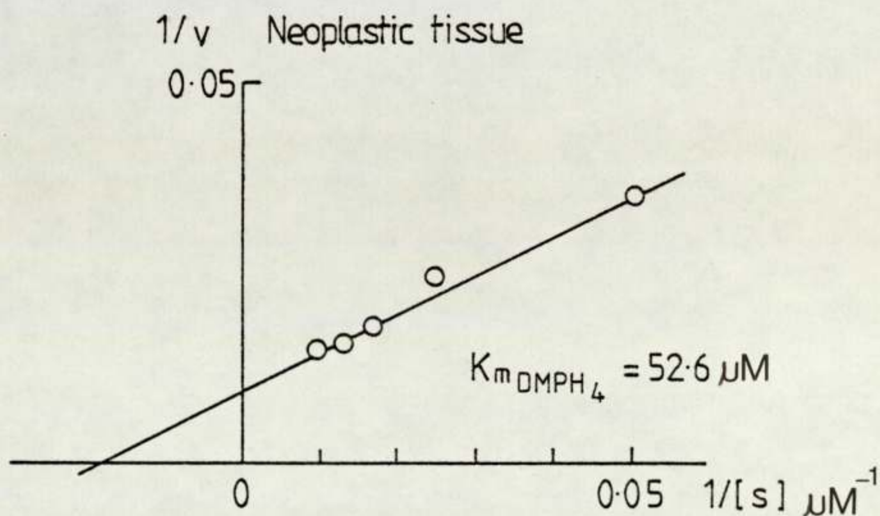


Figure 4·3 Effect of $DMPH_4$ concentration on DHPR activity from human breast tissues. (Craine method) (2)

[s]/v Apparently normal tissue

50
40
30
20
10
0
10
18
[s] μM

$K_{m_{DMPH_4}} = 9{\cdot}7\ \mu M$

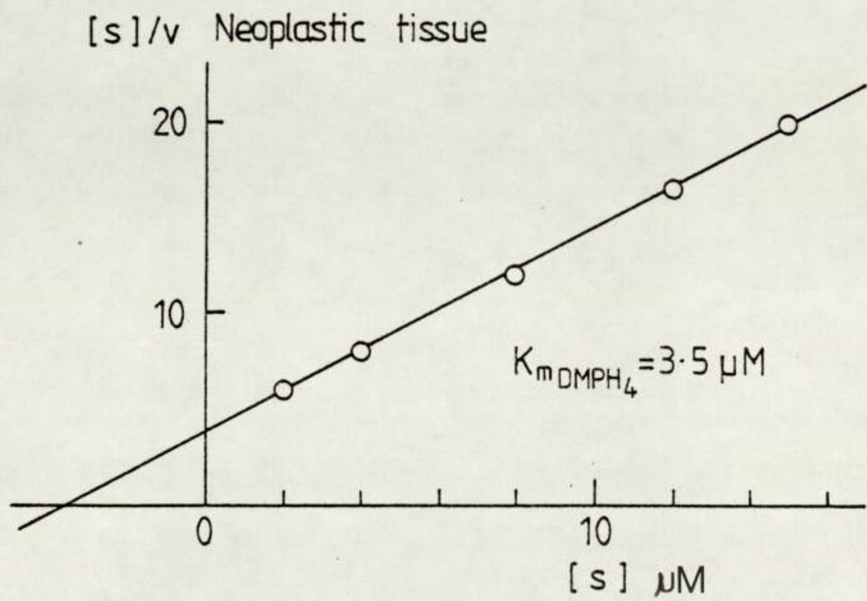


Figure 4·4 Effect of $DMPH_4$ concentration on DHPR activity from human breast tissues. (Narisawa method)

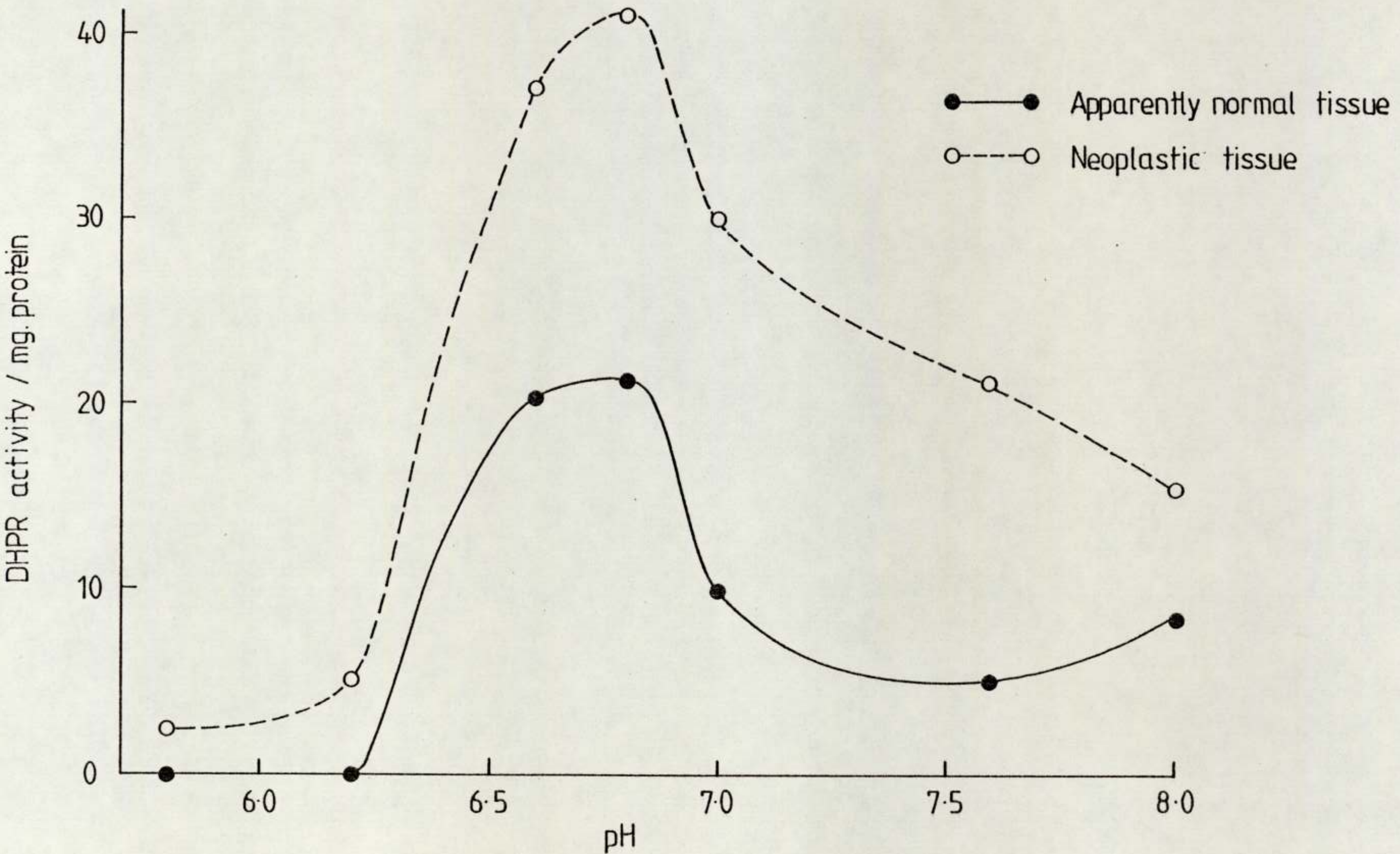


Figure 4·5 Effect of pH (phosphate buffer) on DHPR activity from human breast tissues.

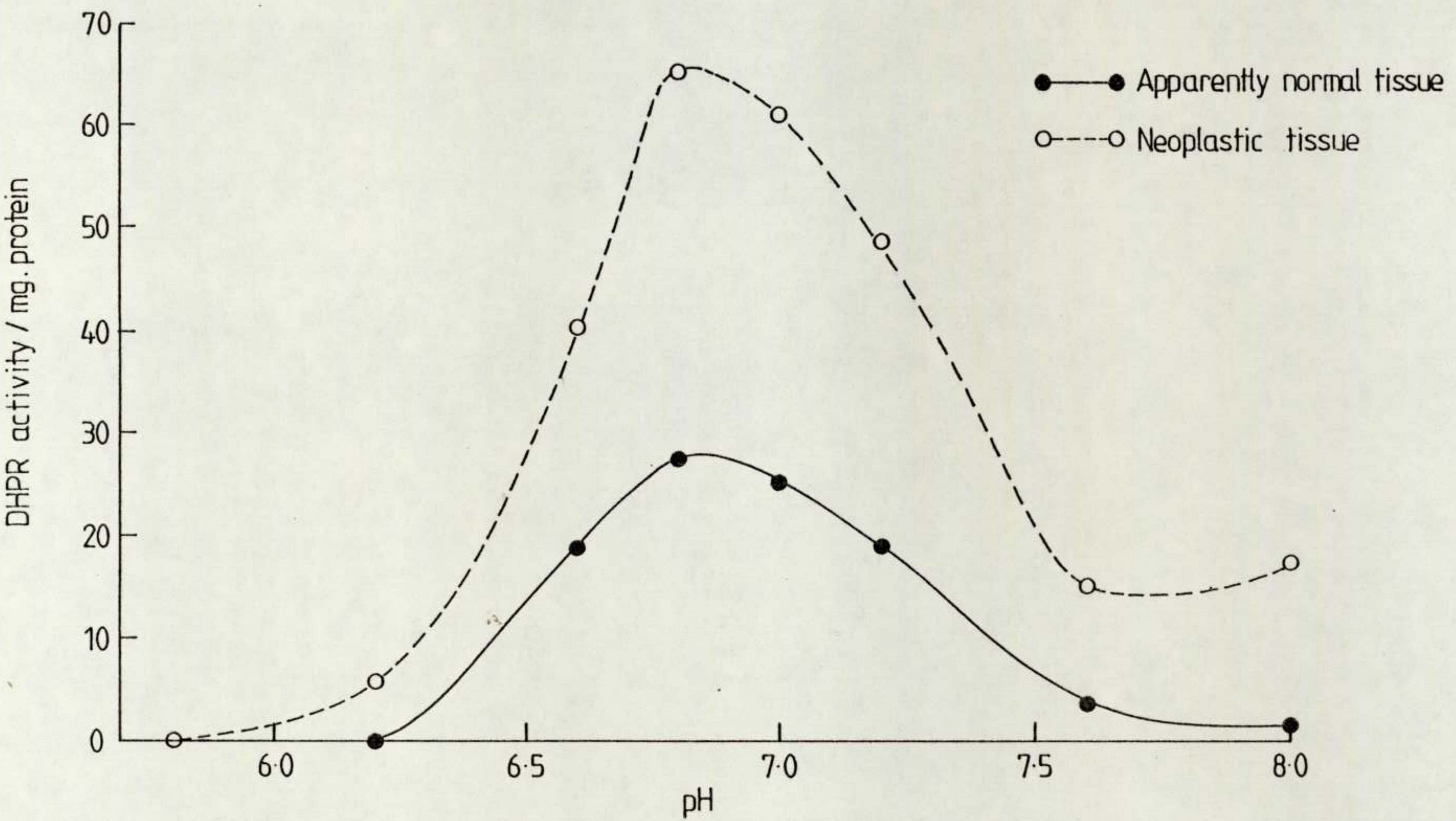


Figure 4·6 Effect of pH (tris-maleate buffer) on DHPR activity from human breast tissues.

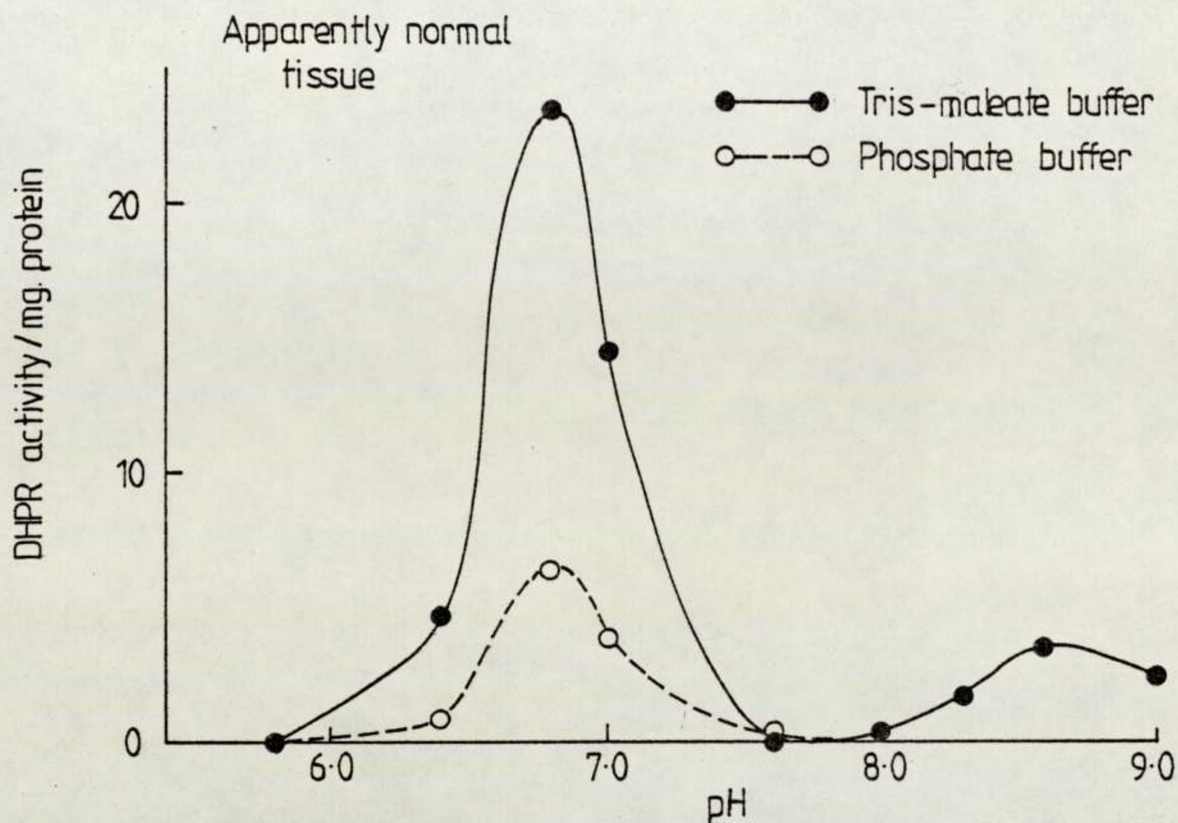


Figure 4·7 Effect of pH (tris-maleate and phosphate buffer) on DHPR activity from apparently normal and neoplastic breast tissues.

CHAPTER 5

DHPR ACTIVITY IN HUMAN BRAIN

CHAPTER (5);

DHPR ACTIVITY IN HUMAN BRAIN

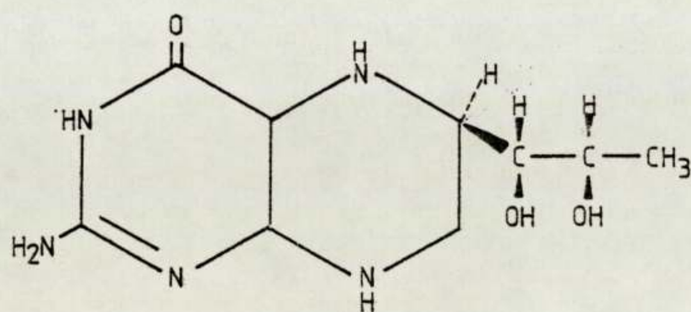
5.1 Introduction:

Tetrahydrobiopterin (BH_4) (1) is the rate-limiting factor in catecholamine synthesis (Leeming et al, 1981) and deficient BH_4 metabolism may cause reduced neurotransmitter production and hence disease of the central nervous system.

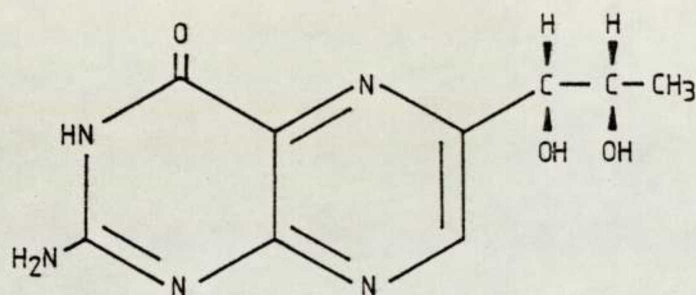
Over the last few years, reports have been presented on disturbances of BH_4 metabolism in various states including neurological diseases such as Parkinson's disease (Lovenburg et al, 1979; Williams et al, 1980; Aziz et al, 1982) and torsion dystonia (Williams et al, 1979).

A number of investigations have shown defective monoamine neurotransmitter metabolism in senile dementia of the Alzheimer type (SDAT) (Mann, 1983; Gottfries et al, 1969). Significantly reduced serum biopterin (2) levels were reported for subject diagnosed as SDAT (Leeming and Blair, 1979; Young et al, 1982; Aziz et al, 1983). It has also been reported that total biopterins are lowered in the cerebral spinal fluid of Alzheimer patients (Morar et al, 1983). A reduction in neopterins (3) was also found but this was not significant (Morar et al, 1983),which suggests a role for BH_4 in the pathology of SDAT. Morar (1984) reported that the unoxidized urine neopterin and biopterin levels of SDAT subjects are approximately 50% of control values, but these differences are not statistically significant.

Barford et al (1984) reported that BH_4 metabolism was impaired in the brains of subjects with SDAT compared to age-matched controls, and in the control



(1)



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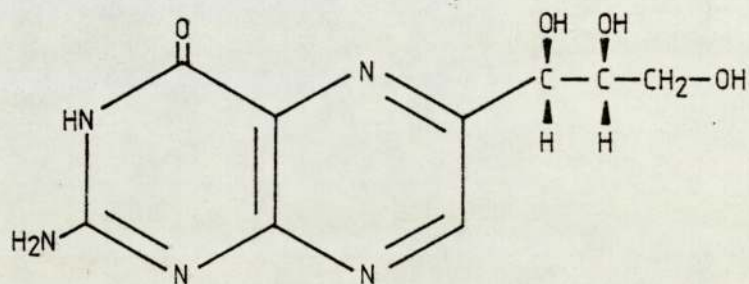

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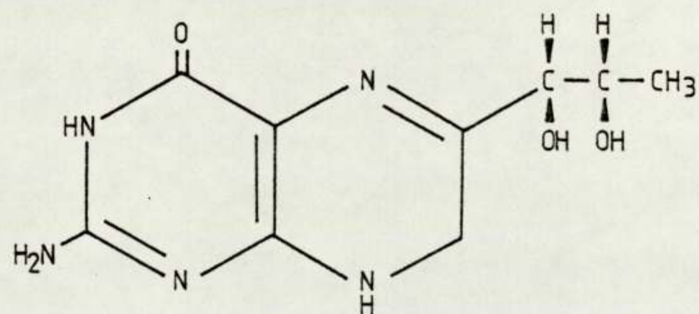

(4)

group, there was evidence for a decrease in the ability to synthesize BH_4 with age. Barford et al (1984) also reported that DHPR activity in temporal lobes from subjects with SDAT was higher than that in controls, but the elevation was not significant.

Down's syndrome is a genetic disorder which results in mental retardation. The neuropathologic changes are essentially the same as those described for Alzheimer patients (Roy Bregg, 1977). Recently it has been reported that there is an increase in serum dihydrobiopterin (4) in Down's syndrome, which could indicate a lowering of BH_4 (1) levels within the cell and a corresponding lowering of neurotransmitter formation. This could be responsible for the lack of mental development in these patients (Aziz et al, 1982).

Blair et al (1984a) reported that BH_4 synthesis activity in Down's syndrome subjects is considerably reduced compared with healthy controls, with no significant change in DHPR activity. The above is an account of some of the disease states, where a possible disturbance of BH_4 metabolism has been reported, so in this chapter results are presented using measurements of DHPR activity in the human brain with different pathological conditions, and its kinetics; in an attempt to see if results already obtained can be related to human brains and to get a further insight into human brain biopterin metabolism.

5.2 Materials and Methods:

5.2.1 Chemicals:

Nicotinamide adenine dinucleotide reduced (disodium salt type III) NADH, 6, 7-dimethyl-5, 6, 7, 8-tetrahydropterin ($DMPH_4$), Tris [tris (hydroxymethyl) aminoethane], peroxidase and cytochrome c were obtained from the Sigma Chemical Company (Poole). All other reagents were from BDH (Poole) and

were of analytical grade.

5.2.2 Human Tissues:

Human brain tissue was obtained from several sources. Tissue samples from subjects with senile dementia and controls (those dying with no apparent neurological involvement in the diseased state) were removed from the temporal lobe (Brodmann area 20) and frontal cortex (Brodmann area 9) at necropsy. The samples were matched for age, drug therapy and time to necropsy. They were also analysed for plaques and tangles. These were obtained from Dr G Reynolds at the MRC Brain Bank (Addenbrook's Hospital, Cambridge). Specimens of temporal cortex were also obtained from patients institutionalised at St. Lawrences Hospital (Caterham, Surrey), courtesy of Dr P Sylvester. Three of these were diagnosed as Down's subjects and one as having Alzheimer disease. The other five were mentally subnormal, but of undefined aetiology. Brain tissue was stored at -70°C until use. The known information of brain samples, age, postmortem delay, cause of death of the patients and medication the patients were receiving are presented in table (5.1).

5.2.3 Tissue Preparation:

Human brain tissue was broken into small bits first by chopping with a scalpel. 20% (w/v) homogenates were prepared in 0.5 M Tris/HCl buffer pH 7.6, using a Potter Elvejhem homogeniser. These homogenates were centrifuged at 0°C at 40,000 xg in a M.S.E. Superspeed centrifuge for 45 minutes. The supernatants were collected and used for the assay of DHPR, which measured according to the assay method of Craine et al (1972) and Narisawa et al (1980). Protein was measured by the Biuret method (Layne 1957).

Table (5.1) : The clinical status of human brains

Patients	Age	P.M. delay	Sex	
D38	90	11 hr	F	All subjects were presented as demented. They were matched for age, sex, medication with controls C234-C242 below. All were Brodmann area 9 and 20 and obtained from Dr Reynolds.
D39	71	32 hr	M	
D40	72	20 hr	F	
D41	76	54 hr	M	
D42	71	74 hr	M	
D43	79	4 hr	M	
D46	80	46 hr	M	
C242	79	63 hr	F	Control subjects matched with samples D38-D46 for age, sex, postmortem delay and medication. All were Brodmann area 9 and 20 and obtained from Dr Reynolds.
C234	44	92 hr	F	
C236	72	69 hr	M	
C238	74	24 hr	F	
D48	74	11	F	All subjects were presented as demented and were matched with controls C249-C278 for age, sex, medication and post-mortem delay. All were Brodmann area 9 and 20 and obtained from Dr Reynolds.
D49	85	12	F	
D59	82	20	F	
D60	76	19	F	
C249	85	44	F	Control matched with samples D48-D60 for age, sex and medication. Brodmann area 9 and 20, obtained from Dr Reynolds.
C254	76	47	F	
C256	79	89	M	
C257	83	48	F	
C258	85	87	F	
C267	87	78	F	
C278	87	73	F	

.... continued

Patients	Age	P.M. delay	Sex	
S.T.	65		?	Subnormal, Temporal cortex from Dr Sylvester.
A.L.	31	7 hr	M	Control, No evidence of aging Diplegic originally thought to have Hunter-Hurler Syndrome. Death from Bronchopneumonia and chronic pyelonephritis. Received Ampicillin, Amoxil, Septrin and Carbenicellin. Temporal cortex obtained from Dr Sylvester.
S.H.	78		?	Subnormal, Temporal cortex from Dr Sylvester.
D.T.	49	60 hr	F	Down's Syndrome, Brain had plaques and tangles. Death due to bronchopneumonia and acute trachebronchitis. Received ceporin 500 mg. and Sodium Valproate syrup 200 mg. Temporal cortex obtained from Dr Sylvester.
M.L.	50		M	Down's Syndrome, Evidence of plaques and tangles. Received Epantuim, Sodium Valproate, Abidec and Diamorphine elix. Temporal cortex obtained from Dr Sylvester.
W.N.	81		F	Subnormal, Death due to coronary artery occlusion, Hypertensive ischaemic heart disease. Temporal cortex obtained from Dr Sylvester.
SDAT			?	Temporal cortex from Dr Sylvester.
W.H.	51		M	Down's Syndrome, Brain showed typical features of Down's. Death due to bronchopneumonia. Temporal cortex obtained from Dr Sylvester.
M.S.	64		F	Subnormal, cortex atrophy of right frontal lobe. Ventricular dilation. No evidence of plaques and tangles. Received Septrin, Sodium Valproate, Ethosuxamide. Temporal cortex obtained from Dr Sylvester.

5.3 Results and Discussion:

DHPR activity was measured in human temporal cortex from normal subjects. DHPR activity is significantly higher than in other human tissues, such as breast, large intestine, placenta and whole blood, as shown in table (5.2).

An examination of DHPR activity for several human brain regions is presented in table (5.3). It can be seen that the frontal lobe has a higher activity than other brain regions used here.

DHPR activity in control tissues from Brodmann area 9, was investigated and correlated with results from Brodmann area 20, (Anderson personal communication). The activity in these is fairly similar as shown in table (5.4). Meanwhile table (5.5) demonstrates that DHPR activity in Brodmann area 9 from subjects presented as demented is significantly higher than Brodmann area 20 ($p = 2\%$ by Student's "t" test).

Clinically the demented subjects shown in table (5.5) did not all present with the same dementias. Six were senile dementia of the Alzheimer type (SDAT) with characteristics plaques and tangles, two had dementia of vascular origin and three had a medium to high dementia score but no evidence of plaques and tangles. Attempts were made to correlate the results from these different dementia groups with age-matched controls. The results are presented in table (5.6), from which it can be seen that Brodmann area 9 from the SDAT subjects have higher DHPR activity than controls, although this is not significant. As shown, there is no significant difference in the activiy of SDAT subjects and other dementias shown in the table.

DHPR activity in patients dying with Down's syndrome was higher than age-matched control (table 5.7), but there is no significant difference, probably due to the small number of Down's samples presented.

Kinetic studies on human brain DHPR in controls and patients dying with Down's syndrome and SDAT have done, as shown in Fig. (5.1-5.4). The Km values

for $DMPH_4$ and NADH substrates are significantly higher in the demented patients as correlated with controls (table 5.8).

TABLE 5.2: Dihydropteridine reductase activity in different human tissues.

Tissue	DHPR activity	
	Craine method*	Narisawa method**
Brain (temporal cortex)(6)	193.12 ± 59.41	59.27 ± 12.74
Breast (n = 6)	27.85 ± 19.09	3.35 ± 1.42
Large intestine		
Colon (n = 3)	28.07 ± 17.25	
Rectum (n = 3)	18.55 ± 16.68	
Placenta (n = 1)	48.0	2.95
Whole blood (n = 81)		1.82 ± 0.44

All values as mean ± S.D.
n = number of subjects

* nmole NADH oxidized/min./mg. protein
** nmole cytochrome c/min./mg. protein

TABLE 5.3: Dihydropteridine reductase activity (nmole NADH/min./mg. protein) in different human brain areas.

Patient	Brain area	DHPR activity
A.A.	Ammon's Horn	343.04
	Temporal Cortex	400.2
	Fontal Lobe	506.5
	Amygdala	304.9
Greeley	Ammon's Horn	393.7
	Temporal Cortex	689.1
	Frontal Lobe	721.5
	Amygdala	400.04

TABLE 5.4 : Dihydropteridine reductase activity (nmole NADH/min./mg. protein) in human brain from control subjects.

sample	DHPR activity	
	BA9	BA20
C 267	275.9	130.9
C 278	137.3	416
C 258	300	—
C 249	275.9	236
C 257	459.3	497.3
C 256	255.1	150.2
C 242	157.2	167.3
C 254	365.4	361.9
C 238	565.3	460
C 236	245.3	288.6

TABLE 5.5: Dihydropteridine reductase activity (nmole NADH/min./mg.protein) in human brain from subjects presented as demented.*

sample	DHPR activity	
	BA9	BA20
D 38	427	333.5
D 59	343.8	293
D 60	394.7	146
D 48	378.8	381
D 40	459.3	359.6
D 39	420	315
D 46	384.1	453.3
D 43	326.1	158.3
D 41	226.7	222.8
D 42	440	52.75
D 49	447.8	364.0

* D 41 and D 42 had vascular dementia
D 46 and D 49 has high dementia score but no plaques and tangles.
D 43 had moderate dementia score but no plaques and tangles.
The remainder were all presented as Alzheimer type.

Student's "t" test :

Demented BA9 vs. BA20 p = 2%

TABLE 5.6 : Dihydropteridine reductase activity (nmole NADH/min./mg. protein) of human brain in control and demented subjects.

Subject	Age	DHPR activity*	
		BA9	BA20
Control	72 - 87	303.67 ± 130.36 (10)	300.91 ± 139.05 (9)
Senile dementia of Alzheimer type (SDAT)	71 - 90	403.93 ± 40.47 (6)	304.68 ± 83.80 (6)
Vascular dementia	71 - 76	333.35 ± 150.83 (2)	137.78 ± 120.24 (2)
Medium-high dementia score without plaques and tangles	79 - 85	386.00 ± 60.87 (3)	325.20 ± 151.28 (3)

* Mean ± S.D.
Figures in parenthesis refer to number of subjects studied.

Student's "t" test :

BA9 Control Vs. SDAT No significance
BA20 Control Vs. SDAT No significance

BA9 Control Vs. BA20 Control No significance
BA9 SDAT Vs. BA20 SDAT $p < 5\%$

TABLE 5.7 : Dihydropteridine reductase activiy (nmole NADH/min./mg. protein) of human brain in Down's and Control patients.

Subject	Age	DHPR activity*
Control (n = 10)	44 - 87	297.37 ± 131.57
Down's (n = 3)	49 - 81	369.97 ± 92.04

* Mean ± S.D.
n = number of subjects.

Student's "t" test :

Control Vs. Down's No significance

TABLE 5.8: Km values for DHPR from human brain.*

Patient	Km value	
	μM $DMPH_4$	μM NADH
S.H.	4.4	13.2
S.T.	3.5	16.5
A.L.	5.2	11.8
Control (n = 3)	4.37 ± 0.85	13.83 ± 2.41
SDAT	7.90	26.2
Down's	7.95	30.1

* DHPR activity determined using Narisawa method (1980).
Each single value is the mean of 3 experimental results.

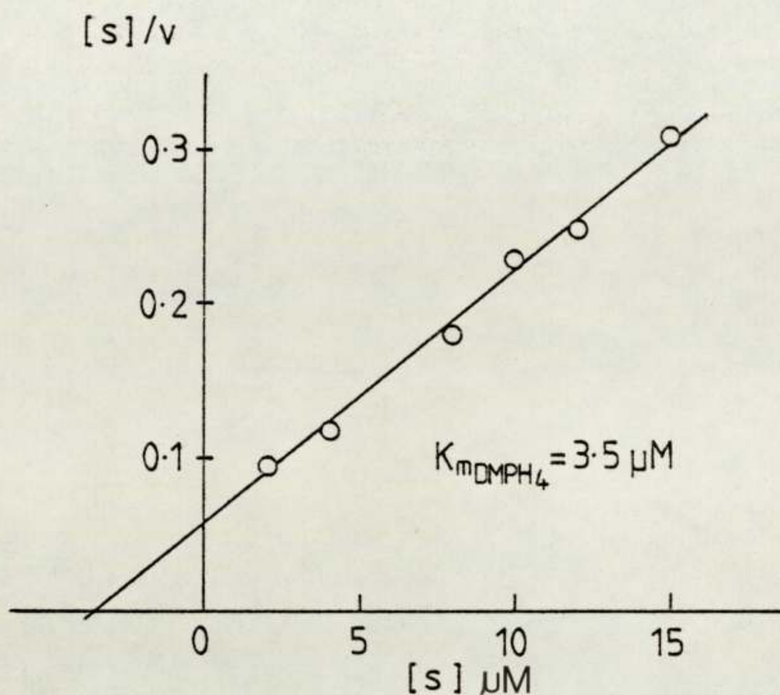

Figure 5·1 Effect of $DMPH_4$ concentration on DHPR activity from control human brain.

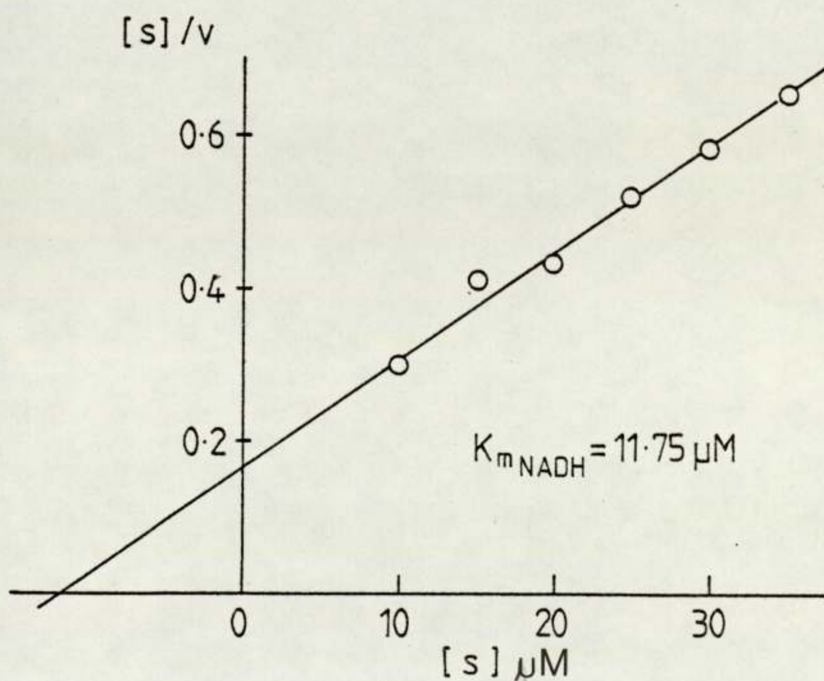

Figure 5·2 Effect of NADH concentration on DHPR activity from control human brain.

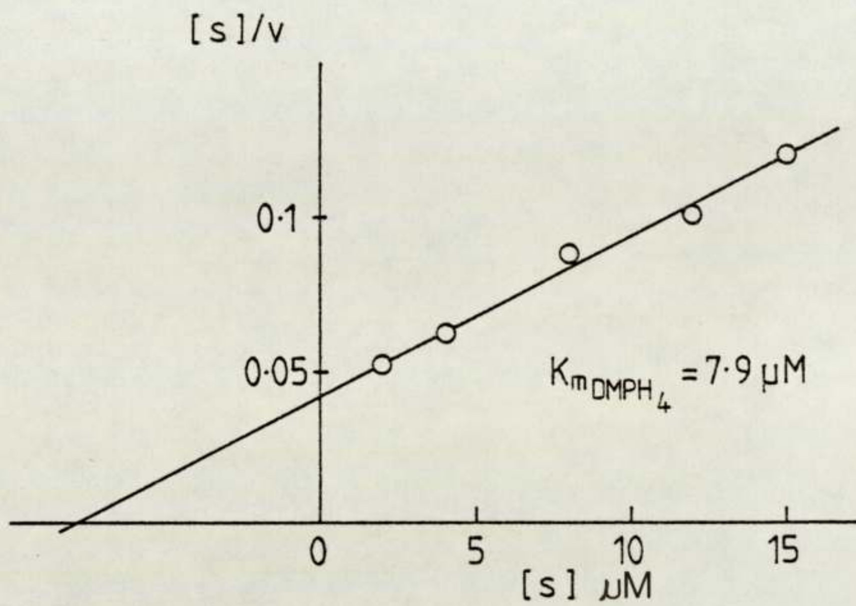

Figure 5·3 Effect of $DMPH_4$ concentration on DHPR activity from SDAT human brain.

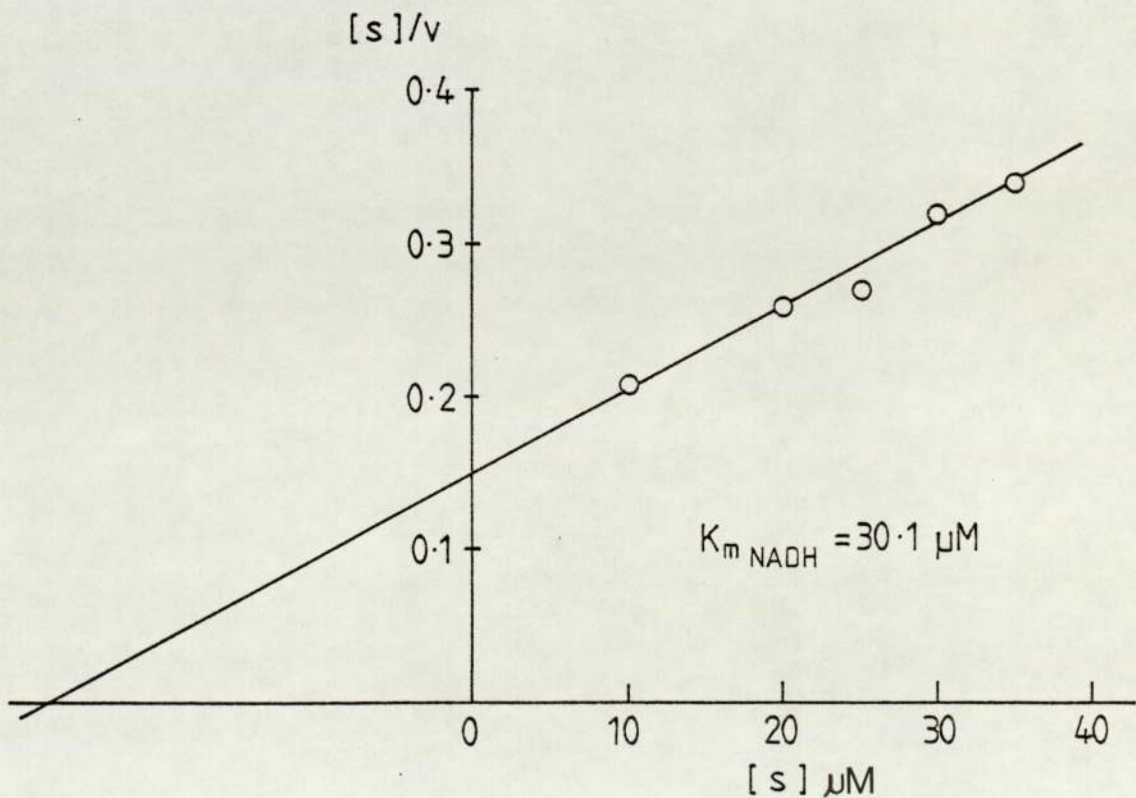

Figure 5·4 Effect of NADH concentration on DHPR activity from Down's syndrome brain.

CHAPTER 6

TETRAHYDROBIOPTERIN METABOLISM IN THE RAT BRAIN

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TETRAHYDROBIOPTERIN METABOLISM IN THE RAT BRAIN

6.1 Introduction

Various laboratories have studied the relationship between disease states and pteridine content in tissues or body fluids (Kaufman et al, 1978, Danks et al, 1975; Stea et al, 1978; Leeming et al, 1976b). The majority of the quantitative data on biopterin compounds has been collected by either the bioassay for the Crithidia growth factor (Leeming and Blair, 1974; Danks et al; 1975 Leeming et al, 1976b) or the cofactor assay in the phenylalanine hydroxylation system (Kaufman et al, 1978). Other methods such as gas-chromatography (Rothler and Karobath, 1976) and radioimmunoassay (Nagatsu et al, 1981) have also been used.

More recently, the use of high performance liquid chromatography (HPLC) has gained popularity (Fukushima and Nixon, 1980; Stea et al, 1980; Blair et al, 1983; Blair et al, 1984a). Such a system incorporating a fluorescence detector is capable of detecting fluorescent substances, such as most of the pteridines in the picomole range (Stea et al, 1980). The high sensitivity, good resolution and reduced analysis time with this technique, offers a means of obtaining metabolic profiles without complex sample preparation procedures.

Leeming et al (1976a) reported that plasma biopterin derivatives, studied in normal and phenylketonuric children, showed a significant high concentration in the latter group. Biopterin derivatives correlated with plasma phenylalanine concentration, but in normal adults given an oral phenylalanine load the rate of increase with phenylalanine differed from that in phenylketonuric patients.

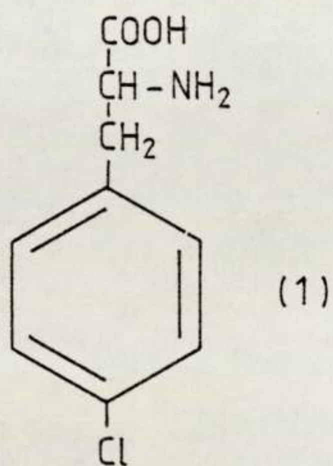
Several experimental models of hyperphenylalaninaemia (HPA) have been used for in vivo studies of clinical conditions such as phenylketonuria. Most of these studies have been based on long term administration of inhibitors such as p-chlorophenylalanine (PCPA) (1) and alpha-methylphenylalanine (2), together with phenylalanine (3) to suckling rats (Delvalle et al, 1978). Intraperitoneal injection of a single dose of PCPA into rats produces selective irreversible inactivation of phenylalanine and tryptophan hydroxylase in vivo (Koe and Weissman, 1966; Jaquier et al, 1967). The effect in vitro of PCPA is that of a competitive inhibitor (Jacquier et al, 1967).

Morar (1984) reported that PCPA when given in corn oil prior to a load of phenylalanine produces an elevation in the rat brain and liver total biopterin, in contrast to a reduction produced when phenylalanine is adminstered alone.However, the mechanism of these effects was not completely elucidated. We have thereforere examined the relationships between hyperphenylalaninaemia (HPA) and pteridine metabolism. An animal model of HPA was used to investigate.

Oestrogens are female sex hormones derived by hydroxylation of the parent molecule estradiol-17 ß (4). A number of substituted phenols (including diethylstilboestrol (5)) have oestrogenic activity. Diethylstilboestrol (5) has been used in the past as a post-coital contraceptive, but has been superseded by a less toxic synthetic oestrogen such as ethinyloestradiol (6) (Begley et al, 1980)

Plasma biopterin levels alter during the menstrual cycle, in women on the contraceptive pill and during pregnancy (Leeming and Blair, 1980a; Barford et al, 1983). As the oestrogen levels rise the biopterin level falls and there is a compensatory increase in DHPR activity (Barford et al, 1983). Eggar et al (1983) reported that oral administration of diethylstilboestrol to rats significanly raised lever levels of DHPR.

Recently, Blair et al (1984a) reported that orally administered diethylstilboestrol drastically reduced the total rat brain biopterin pool without altering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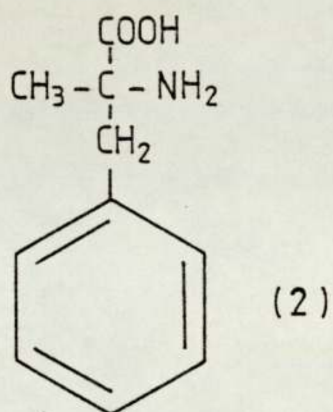
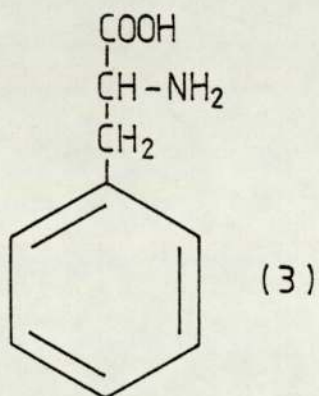
CH - NH2
CH2
Cl
(1)


COOH
CH3 - C - NH2
CH2
(2)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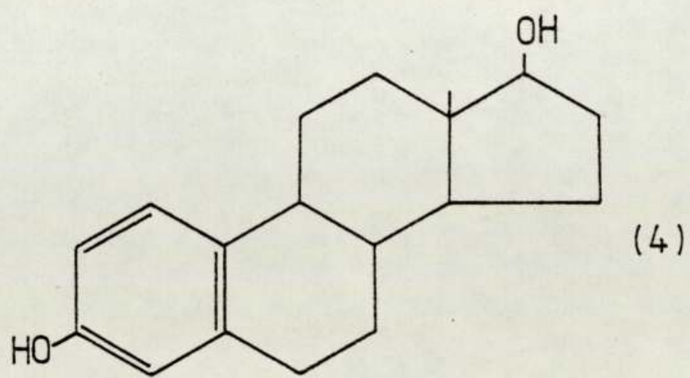
CH2
(3)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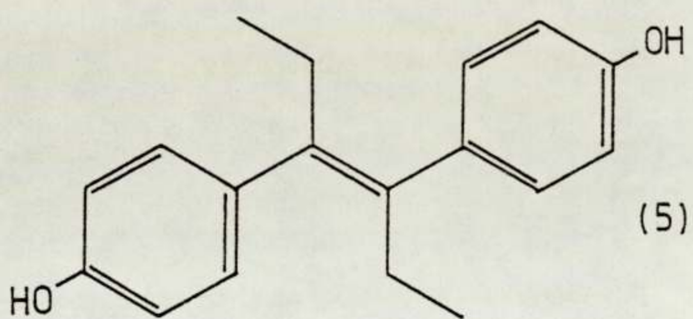
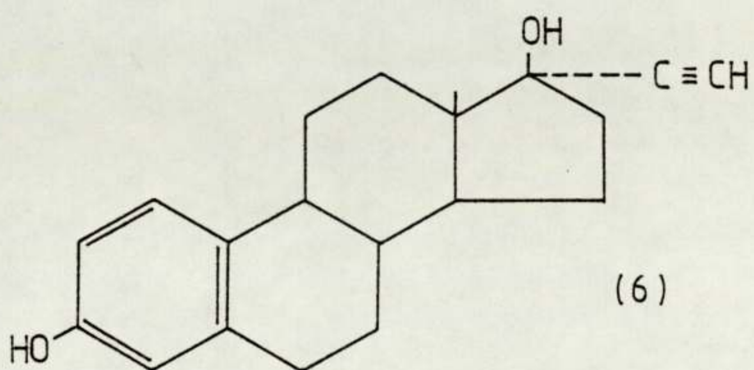
HO
(4)


OH
HO
(5)


OH
C ≡ CH
HO
(6)

the percent BH_4 level. A similar experiment has been repeated here with various diethylstilboestrol concentrations, to observe the effect of diethylstilboestrol on brain biopterin levels, to give further insight into the possible involvement of BH_4 in psychosomatic effects of oestrogens.

6.2 Materials and Methods

6.2.1 Chemicals

L-biopterin, D-neopterin and pterin were obtained from Dr B Shircks (Switzerland), HPLC solvents were from Fisons PLC (UK), except for distilled water which was laboratory produced. All other chemicals were of analytical grade and obtained from the Sigma Chemical Company.

6.2.2 Tissue Preparation

Brain tissue was removed from rats freshly killed by cervical transection with prior anaesthesia. A 20% homogenate in 20% trichloroacetic acid (w/v) was prepared and centrifuged at 3000 rpm. for 2 min. The supernatant was analysed neat by using HPLC and by acid-iodine and alkaline-iodine oxidation (Fukushima and Nixon, 1980).

0.5 ml. of the supernatant was mixed with drops of 0.2 N HCl or 0.2 N NaOH. Then, each mixture was oxidized by 0.1 ml. of 0.1% iodine (w/v) in 0.1 N HCl which also contained 2% KI, or 0.1 N NaOH, respectively. After standing for 1 hour in the dark, the mixture was neutralized with NaOH or HCl and excess iodine was reduced by the addition of excess of 1% ascorbic acid solution, which decolourised the sample. After dilution, the resulting solution was analysed by HPLC.

6.2.3 High Performance Liquid Chromatography (HPLC)

The system comprised of a dual-piston pump (Constametric III pump) from Laboratory Data Control (L.D.C.) UK, auto sampler (Wisp 71 OB) from Waters Associate Inc., U.S.A., a spectrofluoromonitor SFM 23/3 (Kontron LDC fluoromonitor III model 1311, U.S.A.) with SFM/23 power supply and a W +W chart recorder (Model 302).

The column used was a Spherisorb ODS reverse phase column with a particle size of 5μ and column size of 25 cm x 4.6 mm. A pre-column with similar packing was also used. The solvent used was 5% aqueous methanol which was degassed under reduced pressure.

The system was calibrated for the quantitive estimation of pterins and for their retention time, by using series of standards of known concentration, of which 50μl. were injected into the column, injections were 6 replicates (as shown in Fig. 6.1). Because of their low concentration and consequently the small amount of pteridine required, the concentration was determined by measuring the absorbance at pH 13.0 at wavelength shown in table (6.1) and calculating the concentration using the extinction coefficient (table 6.1). Results are presented as pmole/50μl. relative to peak area, examples are shown for biopterin, neopterin and pterin (Fig. 6.2, 6.3).

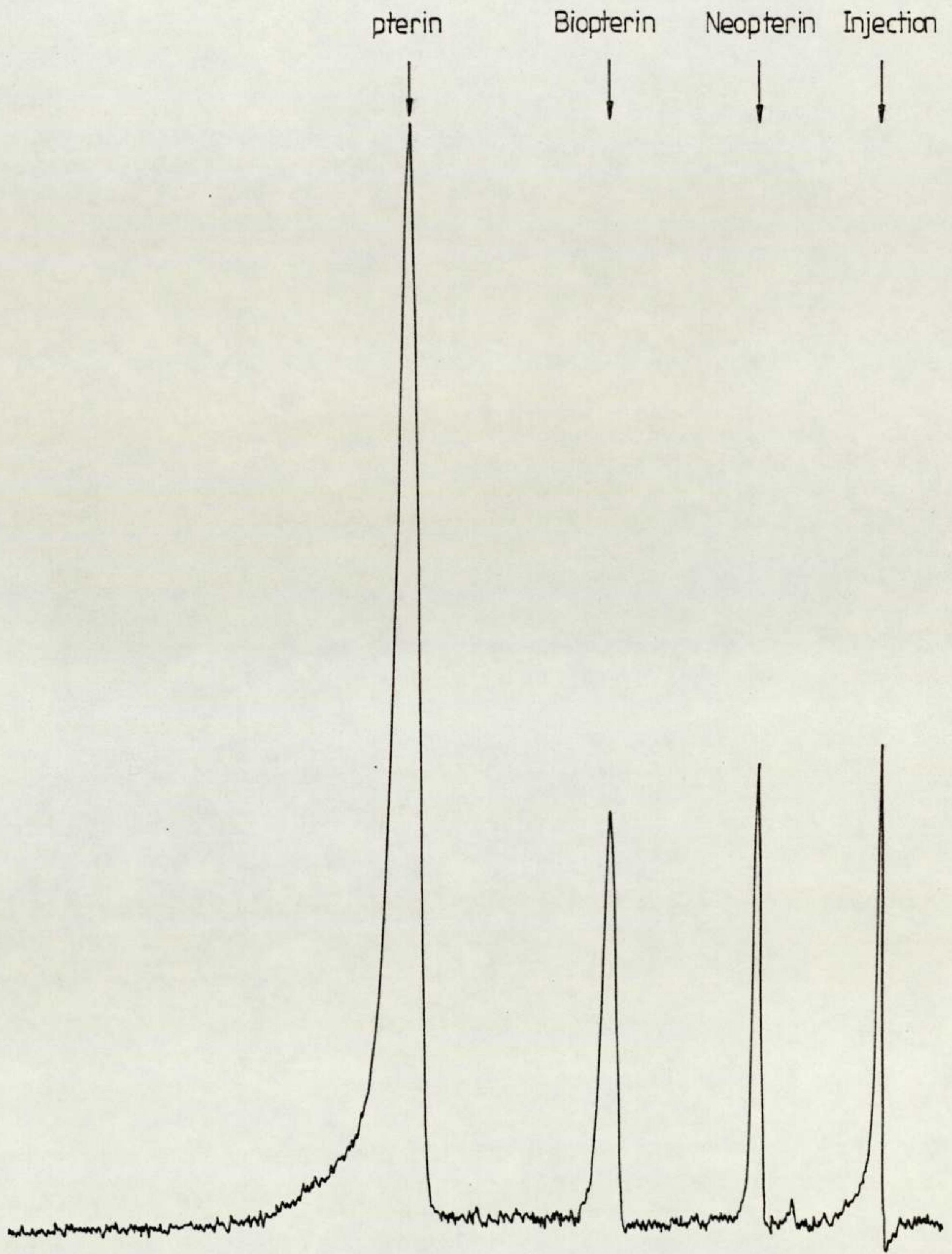


Figure 6·1 Trace from a neopterin, biopterin and pterin standard.

TABLE 6.1 : The Molar extinction coefficient (Σmax) values for various pteridines at pH 13.0.

pteridine	Σmax (M^{-1} cm^{-1})	Wavelength (nm)	Reference
Biopterin	8.3×10^3	363	Fukushima and Nixon (1980)
Neopterin	8.3×10^3	362	Fukushima and Nixon (1980)
Pterin	6.6×10^3	358	Blakley (1969)

6.2.4 Spectra

Spectra were run on a Shimadzu UV 240/UV visible recording spectrophotometer with constant temperature cell holder, quartz cuvettes were used.

6.2.5 The Effect of Phenylalanine Administration on Rat Brain BH_4 Metabolism

A group of 6 adult male Wistar rats (100 gm.wt.) were given an oral dose of phenylalanine (300, 600 and 900 mg./kg. body wt.) in distilled water and killed 30 min., 1 hour, 2 hours, 3 hours and 4 hours later, one at each time. The brains were removed and the brain biopterins analysed by HPLC. Control animals received distilled water only.

Another group of 6 adult rats were injected intraperitoneally with phenylalanine (300 mg./kg. body wt.) in 0.4 ml. isotonic saline solution. A control

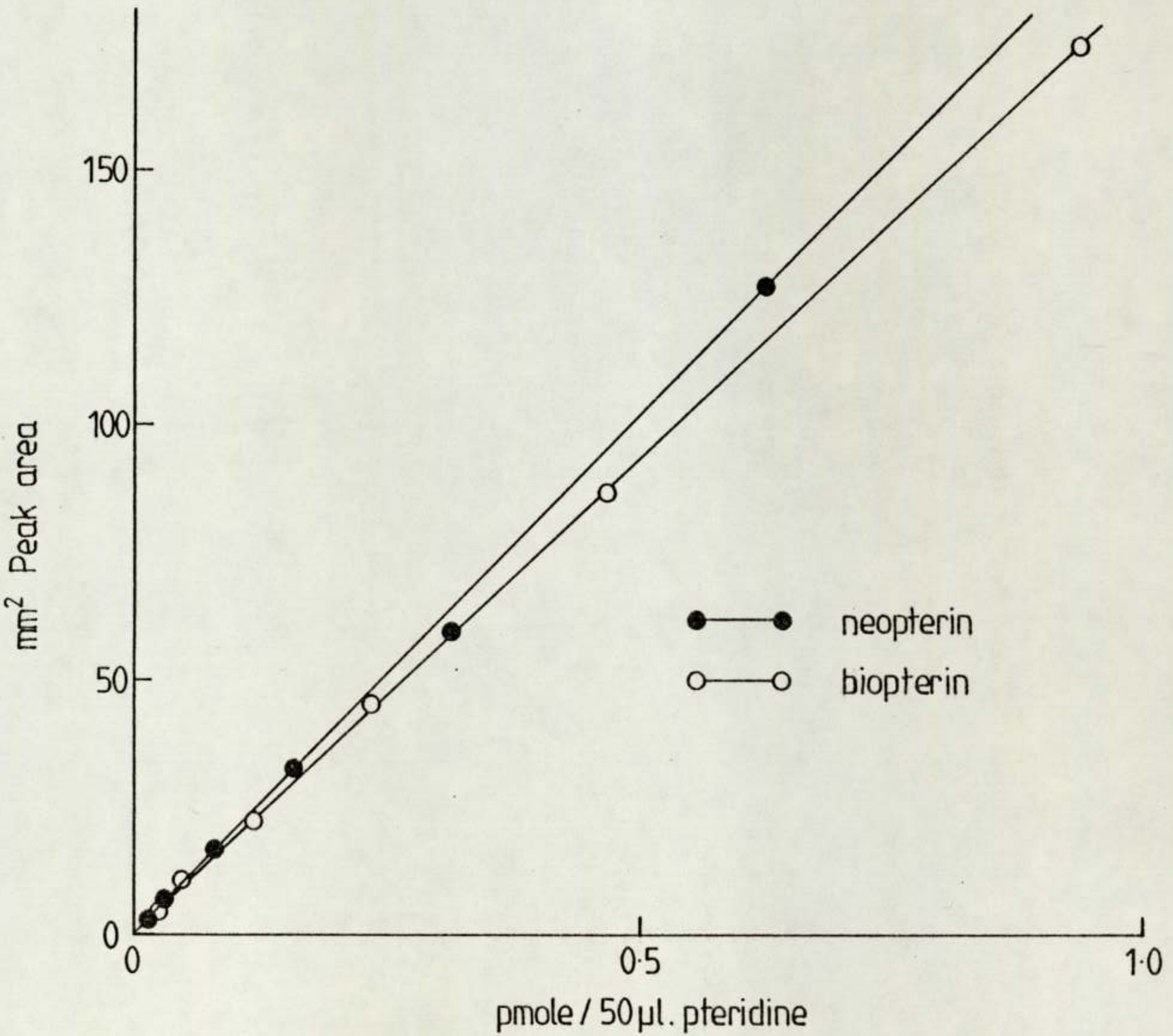


Figure 6·2 Standard curve of neopterin and biopterin.

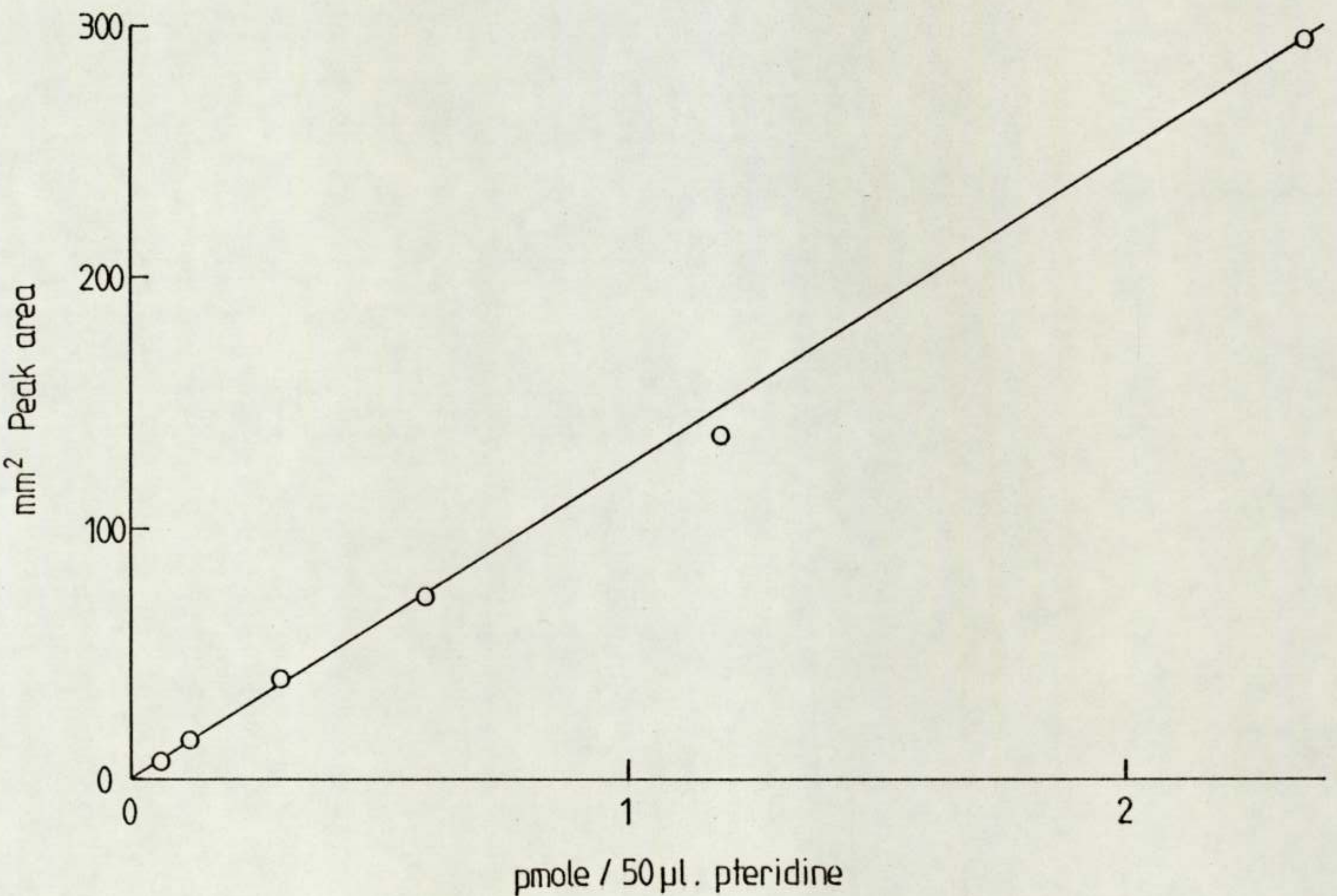


Figure 6·3 Standard curve of pterin .

group was injected with an isotonic saline solution.

6 rats were also dosed with p-chlorophenylalanine (300 mg./kg. body wt.) in 0.4 ml. corn oil and 1 hour, 2 hours, 3 hours, 4 hours, 6 hours and 24 hours later with phenylalanine (300 and 600 mg./ kg. body wt.) and killed 2 hours later. Brain biopterins were investigated. Control rats were given 0.4 ml. dose of corn oil only, another control group were given 0.4 ml. dose of corn oil and then treated with phenylalanine and killed in a similar manner to test animals.

6.2.6 The Effect of Diethylstilboestrol Administration on Rat Brain BH_4 Metabolism

Female Wistar rats (100 gm. wt.) were dosed orally with a suspension of diethylstilboestrol dipropionate (DES) (0.43 µg., 1, 10 and 50 mg./kg. wt.) in 0.4 ml. corn oil daily for 4 days. 24 hours after the last dose of DES, the animals were killed and brains removed for analysis. Control rats were given the same volume of corn oil for the same period.

6.3 Results and Discussion

In order to investigate whether the dosing time of phenylalanine affects BH_4 metabolism in the rat brain *in vivo*, groups of rats were given an oral dose of phenylalanine (300, 600 and 900 mg./kg. body wt.) as shown in Fig. (6.4). 2 hours after an oral dose of phenylalanine, the brain BH_4% and actual BH_4 level is significantly depressed and this reflected in an equivalent rise in BH_2% level (tables 6.2, 6.3 and 6.4).

In addition, at the concentration of 600 and 900 mg./kg. phenylalanine,

the BH_4% level is significantly decreased (p = 0.1%, Student's "t" test) and the actual BH_4 and total biopterin level is significantly decreased (p = 0.1% for actual BH_4 level and p = 1% for total biopterin level, Student's "t" test) (table 6.5).

A brief hyperphenylalaninaemia in healthy rats produces a decrease in BH_4% and actual BH_4 level, which is still present after 3 and 4 hours in the brain and is gradually reduced back to normal.

Difficulties in dissolving phenylalanine in aqueous solution restricted their administration by the oral route, so phenylalanine was dosed intraperitoneally to the rats (300 mg. phenylalanine/kg. body wt.). The BH_4% and actual BH_4 level was significantly decreased (p = 0.1%, student T test), as shown in table(6.6).

Para-chlorophenylalanine (PCPA), an inhibitor of phenylalanine hydroxylase and tryptophan hydroxylase (Gal et al, 1982) when given 4 hours prior to a load of phenylalanine produces the greatest depression in the brain BH_4% level than at earlier time intervals (see Fig. 6.5), and is subsequently stable. The administration of PCPA plus phenylalanine has the same effect on BH_4% level in the brain as the administration of phenylalanine alone, which produced a significant decrease in brain BH_4% level (p = 0.1% Student's "t" test), compared to controls dosed with corn oil only (table 6.7).

With doses of diethylstilboestrol in corn oil, brain total biopterin concentration is significantly decreased, actual BH_4 and BH_4 % level is significantly decreased as well compared to matched controls dosed with corn oil only, as shown in table (6.8).

The brain total biopterin and actual BH_4 levels are significantly increased in the rat dosed with diethylstilboestrol (0.43 µg./kg. wt.) (p = 5% Student's "t" test), and as the dose of diethylstilboestrol increased up to 50 mg./kg. wt., the decrease in

total biopterin and actual BH_4 level are highly significant ($p < 0.1\%$).

Later on, we report the effect of diethylstilboestrol on DHPR activity from rat brain, DHPR is significantly elevated in the brain of rats dosed with diethylstilboestrol (50 mg./kg wt.) compared to control rats ($p < 5\%$) as shown in table (6.9).

Animals given a single dose of corn oil had a significant elevation in the rat brain $BH_4\%$ level ($p < 1\%$, student 's "t' test) and a reduction in actual BH_4 level, although it was not significant, compared to controls dosed with water (table 6.10).

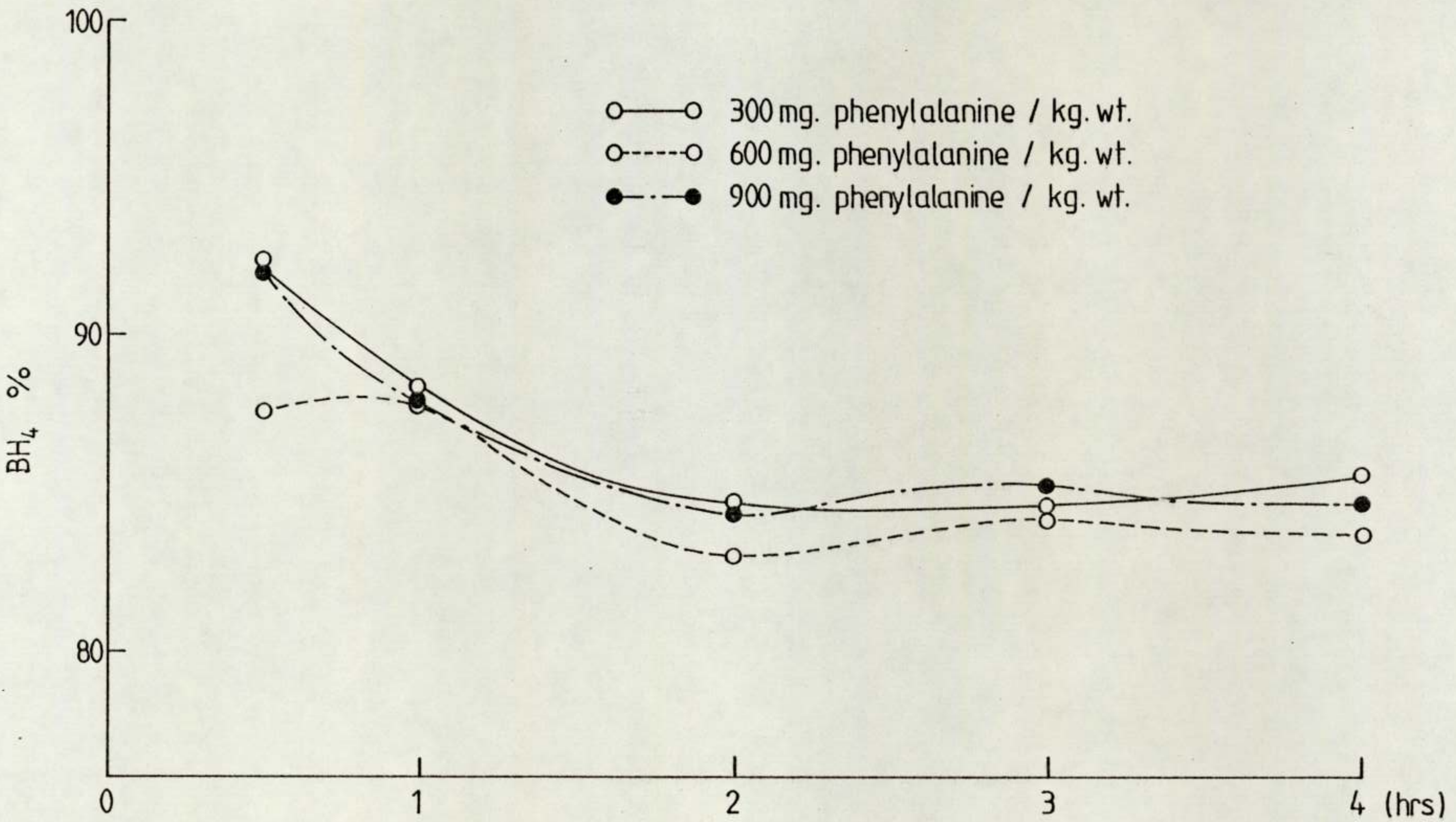


Figure 6·4 Effect of dosing time of phenylalanine on BH_4 metabolism.

TABLE 6.2 EFFECT OF PHENYLALANINE DOSING TIME ON RAT BRAIN BH_4 METABOLISM

(PHENYLALANINE DOSE 300mg./kg. WT.)

Dosing Time	Actual BH_4 pmol/gm. wet wt.	BH_4 %	BH_2 %	Total Biopterin (oxidized + reduced) pmole/gm. wet wt.	Fully Oxidized Biopterin %	Neopterin pmole/gm.wet.wt.	Pterin pmole/gm.wet.w
Control	240.38 ± 5.75 (6)	91.4±2.39 (6)	6.58±2.39(6)	263.0 ± 24.59 (6)	2.01 ± 1.55(6)	3.77 ± 0.99(6)	182.0 ± 130.3(6
30 min	223.1 (1)	92.0 (1)	7.6 (1)	242.5 (1)	0.5 (1)	2.25(1)	132.8 (1)
1 hour	211.20 (1)	88.39 (1)	7.5 (1)	240.0 (1)	2.0 (1)	2.25(1)	217.3(1)
2 hours	180.0 (1)	84.71(1)	14.1 (1)	212.5 (1)	1.4(1)	4.8(1)	117.7(1)
3 hours	175.55 (1)	84.6 (1)	15.4 (1)	207.5 (1)	1.97(1)	3.37(1)	260.6(1)
4 hours	179.55 (1)	85.5 (1)	14.26 (1)	210.0 (1)	0.2(1)	2.79(1)	254.7(1)

All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.
Control rats were administered water only.

TABLE 6.3 EFFECT OF PHENYLALANINE DOSING TIME ON RAT BRAIN BH_4 METABOLISM

(PHENYLALANINE DOSE 600mg./kg. WT.)

Dosing Time	Actual BH_4 pmol/gm. wet wt.	BH_4 %	BH_2 %	Total Biopterin (Oxidized + reduced) pmol/gm. wet wt.	Fully Oxidized Biopterin %	Neopterin pmol/gm.wet wt.	Pterin pmol/gm.wet wt.
Control	240.38 ± 5.75 (6)	91.4±2.34(6)	6.58±2.39(6)	263.0 ± 24.59 (6)	2.01±1.55(6)	3.77±6.99 (6)	182.0±130.3(6)
30 min.	227.8 (1)	87.6 (1)	10.3 (1)	260 (1)	2.07 (1)	4.75 (1)	275.2(1)
1 hour	216.9 (1)	87.8 (1)	9.5 (1)	247 (1)	2.67 (1)	7.72 (1)	313.6 (1)
2 hours	181.0 (1)	83.02 (1)	17.0 (1)	225.8 (1)	4.13 (1)	5.37 (1)	209.3 (1)
3 hours	181.45 (1)	84.2 (1)	12.8 (1)	215.5 (1)	3.0 (1)	8.7 (1)	188.1 (1)
4 hours	183.92 (1)	83.6 (1)	10.9 (1)	220 (1)	5.5 (1)	9.9 (1)	225.5 (1)

All values as mean ± S.D

Figures in parenthesis refer to number of subjects studied.

Control rats were administered water only.

TABLE 6.4 EFFECT OF PHENYLALANINE DOSING TIME ON RAT BRAIN BH_4 METABOLISM

(PHENYLALANINE DOSE 900mg./kg. WT.)

Dosing Time	Actual BH_4 pmole/gm. wet wt.	BH_4 %	BH_2 %	Total Biopterin (oxidized + reduced) pmole/gm. wet wt.	Fully Oxidized Biopterin %	Neopterin pmole/gm. wet wt.	Pterin pmol/gm. wet wt.
Control	240.4 ± 5.75 (6)	91.4±2.34(6)	6.58±2.39(6)	263.0 ± 24.59 (6)	2.01 ± 1.55 (6)	3.77 ± 0.99 (6	182.0±130.3(6)
30 min.	225.89 (1)	92.2 (1)	7.8 (1)	245 (1)	4.8 (1)	3.1 (1)	382.1 (1)
1 hour	183.71 (1)	87.9 (1)	12.1 (1)	209 (1)	3.0 (1)	2.2 (1)	312.4 (1)
2 hours	180.83 (1)	84.5 (1)	15.5 (1)	214 (1)	2.36 (1)	2.7 (1)	256.2 (1)
3 hours	183.9 (1)	85.2 (1)	14.8 (1)	215.8 (1)	3.4 (1)	1.6 (1)	242.1 (1)
4 hours	184.0 (1)	84.6 (1)	15.4 (1)	217.5 (1)	0.94 (1)	2.76 (1)	170.3 (1)

All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.
Control rats were administered water only.

TABLE 6.5 : EFFECT OF PHENYLALANINE CONCENTRATION ON RAT BRAIN BH_4 METABOLISM

Dose	n	Actual BH_4 *	BH_4 %	BH_2 %	Total Biopterin * (oxidized + reduced)	Fully Oxidized Biopterin %	Neopterin *	Pterin *
Control	6	240.29±22.31	91.38± 2.34	6.58±2.39	263.00 ± 24.54	2.01 ± 1.56	3.77 ± 0.99	245.37±34.81
300mg/kg wt. phenylalanine	6	205.42±39.26	83.12± 2.10	10.0±2.99	246.67 ± 43.98	4.1 ± 2.24	6.78 ± 3.34	214.92±38.23
p^+		N.S.	<0.1%	N.S.	N.S.	N.S.	N.S.	N.S.
600mg/kg.wt. phenylalanine	6	184.59±12.55	83.02±12.55	13.75±3.09	222.19 ± 8.34	4.54 ± 1.33	5.37 ± 0.34	209.65±18.20
p^+		<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	5%
900mg/kg wt. phenylalanine	6	183.92±12.44	83.38± 3.10	12.47±1.55	220.51 ± 11.35	1.65 ± 0.28	4.80 ± 1.29	353.80±61.26
p^+		<0.1%	<0.1%	<0.1%	<0.1%	N.S.	N.S.	<0.1%

All values as mean ± S.D.
Control rats were administered water only
n = number of experimental rats
N.S. = No significant difference
* pmole/gm wet wt.   + Student's "t" test.

TABLE 6.6: EFFECT OF INTRA-PERITONEAL ADMINISTRATION OF PHENYLALANINE ON RAT BRAIN BH_4 METABOLISM

Dose	n	Actual BH_4 *	BH_4 %	BH_2 %	Total Biopterins * (oxidized + reduced)	Fully Oxidized Biopterin %	Neopterin *	Pterin *
Control	6	246.89±42.64	90.87±3.37	7.33±2.92	255.35 ± 23.31	1.88 ± 0.35	4.45 ± 1.08	246.7 ± 10.08
Phenylalanine (300mg./kg.)	6	210.50±23.47	81.07±3.68	17.30±3.42	260.40 ± 34.88	2.44 ± 1.02	5.63 ± 2.25	282.07 ± 16.25
P (Student's "t" test)		N.S.	0.1%	0.1%	N.S.	N.S.	N.S.	0.1%

Adult male Wistar rats dosed inta-peritonally with phenylalanine (300 mg./kg.) in water and sacrificed 30 min. later.

All values as mean ± S.D.

n = number of experimental rats.

* pmole/kg. wet wt.

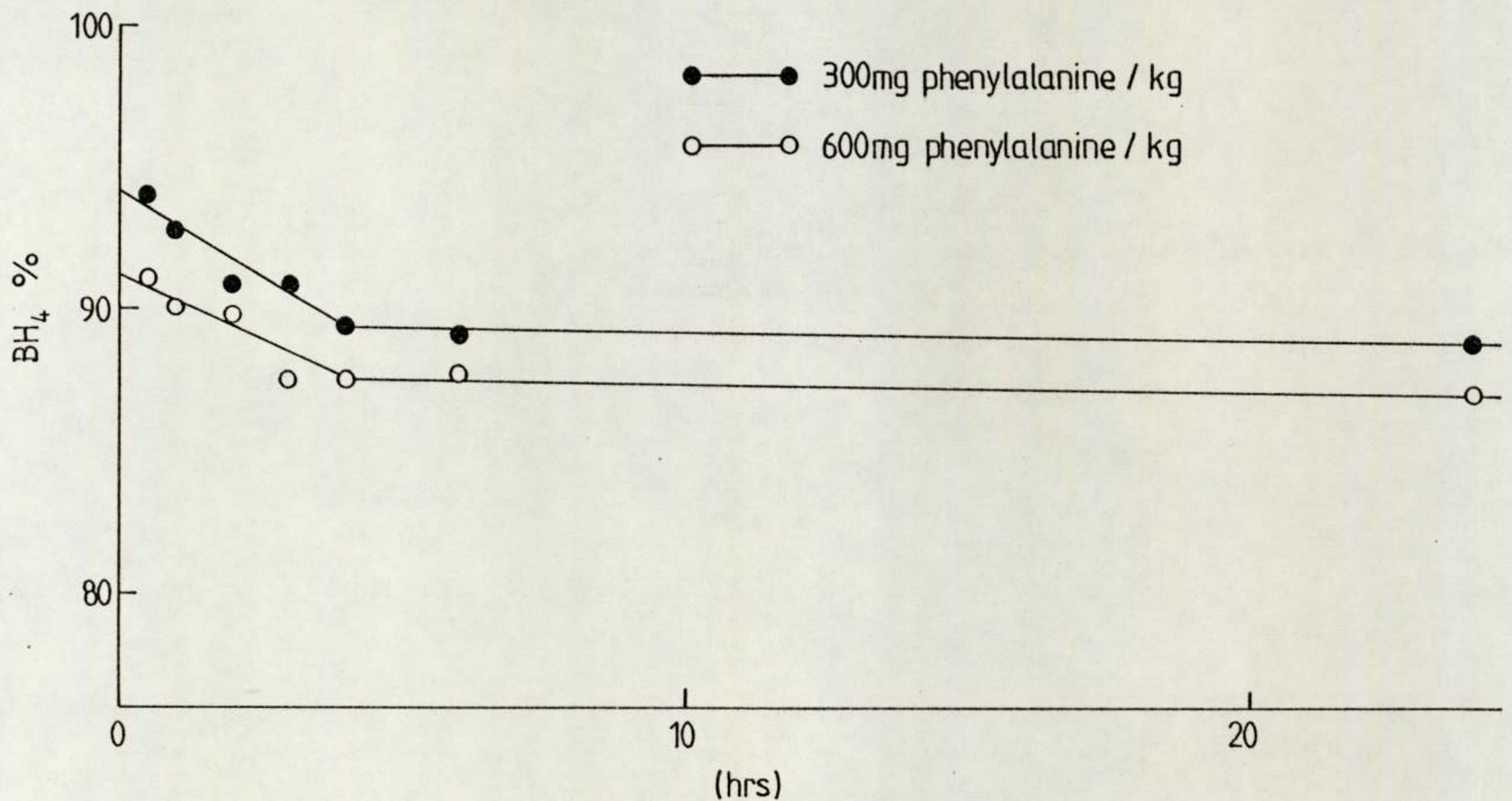


Figure 6·5 Effect of p-chlorophenylalanine (300 mg/kg) on BH_4 metabolism as given prior to a load of phenylalanine.

TABLE 6.7 EFFECT OF p-CHLOROPHENYLALANINE (PCPA) ON RAT BRAIN BH_4 METABOLISM

Dose	n	Actual BH_4 +	BH_4 %	BH_2 %	Total Biopterins +	Fully Oxidized Biopterin %	Neopterin +	Pterin +
Control (corn oil)	6	226.74±30.72	94.52±0.74	5.17±1.47	239.95±32.86	1.09 ± 0.27	4.52 ± 1.00	298.27±42.53
* Corn Oil + Phenylalanine(300mg./kg.wt.)	6	182.83±21.27	88.45±1.94	9.24±2.17	207.13± 27.67	1.43 ± 0.45	4.37 ± 0.15	286.05±28.18
p ++		2%	0.1%	1%	N.S.	N.S.	N.S.	N.S.
** PCPA (300mg./kg.wt) + Phenylalanine(300mg./kg.wt.)	6	189.44±18.35	88.28±1.00	7.64±0.93	214.58±20.15	1.83 ± 0.55	3.32 ± 1.11	339.57±31.04
p ++		5%	0.1%	1%	N.S.	2%	N.S.	N.S.
** PCPA (300mg./kg.wt.) + Phenylalanine(600mg./kg.wt.)	6	185.25±18.70	87.88±1.13	9.58±0.84	210.83±21.66	1.41 ± 0.23	5.93 ± 0.5	265.50 ± 34.76
p ++		2%	0.1%	0.1%	N.S.	5%	2%	N.S.

* Adult male Wistar rats dosed orally with 0.4ml. corn oil and 4 hours later given 300mg./kg.wt. phenylalanine in 0.4ml. water and sacrificed 2 hours later.

** Adult male Wistar rats dosed orally with PCPA (300mg./kg.wt.) in 0.4 ml. corn oil and 4 hours later given phenylalanine in water and sacrificed 2 hours later.

All values as mean ± S.D.

n = number of experimental rats N.S. = no significant difference

\+ pmole/gm.wet wt. ++ Student's "t" test

TABLE 6.8 EFFECT OF DIETHYLSTILBOESTROL ON RAT BRAIN BH_4 METABOLISM

Dose	n	Actual BH_4 *	BH_4 %	BH_2 %	Total biopterin * biopterin	Fully Oxidized biopterin %	Neopterin *	Pterin *
Control (corn oil)	6	236.26±24.38	94.28±1.16	4.78±1.55	250.55 ± 25.10	1.08 ± 0.25	4.28 ± 0.65	223.3 ± 30.34
Diethylstilboestrol 0.43μg./kg.wt.	6	204.06±21.56	92.16±2.23	6.60±1.86	221.1 ± 19.15	1.55 ± 0.46	4.97 ± 1.34	253.78 ± 39.68
p+		<5%	N.S.	N.S.	5%	N.S.	N.S.	N.S.
1mg./kg. wt.	6	142.45±17.12	86.22±5.14	13.33±5.22	164.96 ± 14.06	1.25 ± 0.37	7.83 ± 1.11	222.85 ± 17.64
p+		<0.1%	1%	1%	0.1%	N.S.	N.S.	N.S.
10mg./kg. wt.	6	128.1±7.46	87.08±5.26	11.88±5.39	147.56 ± 12.32	0.75 ± 0.34	4.85 ± 1.43	200.08 ± 39.82
p+		<0.1%	1%	1%	0.1%	N.S.	N.S.	N.S.
50mg./kg. wt.	6	131.66±8.86	88.68±3.66	9.63±3.50	149.34 ± 9.84	0.84 ± 0.22	3.65 ± 0.61	165.02 ± 12.3
p+		<0.1%	1%	1%	0.1%	N.S.	N.S.	1%

Adult female Wistar rats (100gm.wt.) dosed orally with a suspension of diethylstilboestrol dipropionate in corn oil daily for 4 days. 24 hours after the last dose the animals were killed.

All results as mean ± S.D.

n = number of experimental rats

N.S. = No significant difference

* pmole/kg. wet wt.

\+ Student's "t" test.

TABLE 6.9 : Effect of Diethylstilboestrol on DHPR activity (nmole NADH/min./mg. protein) in rat brain.

subject	n	DHPR activity
Control	4	131.34 ± 50.39
Diethylstilboestrol treated	6	291.37 ± 127.87

Adult female wistar rats (100 gm. wt.) dosed orally with a suspension of Diethylstilboestrol dipropionate in corn oil (50 mg./Kg.) for 3 days non-consecutively. 24 hours after the last dose, the animals were killed and the brain removed and DHPR activity was measured according to Craine mehtod (1972).

All results as mean ± S.D.
n = number of experimental rats.

TABLE 6.10 EFFECT OF CORN OIL ON RAT BRAIN BH_4 METABOLISM

Dose	n	Actual BH_4 +	BH_4%	BH_2%	Total Biopterin + (oxidized + reduced)	Fully Oxidized Biopterin %	Neopterin +	Pterin +
Water	6	240.71±22.99	91.52±1.79	6.75±2.56	263.0 ± 24.54	2.18 ± 1.56	3.73 ± 1.05	196.5 ± 121.91
* Corn Oil	6	217.51±59.46	94.52±0.74	5.00±1.43	246.03 ± 32.34	1.09 ± 0.27	4.43 ± 1.0	298.27± 42.53
p (Student's "t" test)		N.S.	1%	N.S.	N.S.	N.S.	N.S.	N.S.

* Adult male Wistar rats (100 gm.wt.) administered orally with 0.4ml. corn oil and 4 hours later the animals were killed.

\+ pmol/Kg. wet wt.

All results as mean ± S.D.
n = number of experimental rats.
N.S. = no. significant difference.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

BH_4 deficiency is a rare cause of hyperphenylalaninaemia, and usually leads to a progressive neurologic deterioration despite early dietary control of blood phenylalanine concentration. Among the enzymopathies responsible for such disease, DHPR deficiency appears to be the most severe cause with respect to the incidence of fatal outcome, the poor results of neurotransmitter therapy and the possible resistance to BH_4 supplementation (Dhondt, 1984).

Complete deficiency of DHPR in the neonate is generally associated with overt hyperphenylalaninaemia (600 µmol./l. or above). However, a small number of cases are known, in which there is only mild to moderate hyperphenyalaninaemia (240 - 600 µmol./l.) in the neonatal period (Dhondt, 1984). DHPR deficiency was suspected, and later confirmed, in these cases due to the failure of dietary treatment to prevent the onset of neurological symptoms.

Sahota et al (1985) reported undetectable DHPR activity in 4 cases of DHPR deficiency. Recently a new case of complete DHPR deficiency was investigated (Sahota, unpublished data).

A number of adult obligate heterozygotes for DHPR deficiency (partial deficiency) are known, and are apparently normal. They had DHPR activity in whole blood about half that of a group of normals (Barford et al, 1982). Serum biopterins in partial DHPR deficiency are within normal level.

Recently some cases of partial DHPR deficiency have been investigated

(Sahota, unpublished data), and in all cases the blood phenylalanine and biopterin levels were normal.

The significance of this partial DHPR deficiency, and its possible relationships to other disorders is under study. The only report of a pathological state with a significant reduction in DHPR activity, is senile dementia of the Alzheimer type, where DHPR activity in the lymphocytes is significantly reduced (Young et al, 1982).

However, significant partial reduction in DHPR activity was found in several children with a variety of neurological disorders associated with some degree of mental retardation (Sahota, unpublished data). Partial deficiency of DHPR may therefore predispose towards neurological disease and mental retardation.

In this thesis, we described a further case of a partial DHPR deficiency in one girl child (chapter 3).

Deficiency of DHPR means that the cofactor BH_4 is not recycled in brain. This cofactor is necessary for hydroxylating tyrosine to dihydroxytyrosine, DOPA (with tyrosine hydroxylase EC 1.14.16.2) and tryptophan to 5-hydroxytryptophan (with tryptophan hydroxylase EC 1.14.16.4), as shown in fig.(7.1). Its absence causes depletion in the brain of precursors of catecholamine and serotonin neurotransmitters with obvious consequences (Kaufman , 1976). Biogenic amine synthesis becomes defective and the levels of dopamine and serotonin and their metabolites; homovanillic acid and 5-hydroxyindolacetic acid, decrease in brain tissues and in cerebrospinal fluids (Butler et al, 1978; Koslow and Butler, 1977; Danks et al, 1976).

Accordingly brain tissue is desirable for neuropathological investigations of the defect in BH_4 metabolism, as it gives direct insight into the integrity of the various neuronal systems.

DHPR enzyme was isolated from human brain tissue and used for detailed activity and inhibition studies. Changes in DHPR in brain diseases were also investigated, to describe in detail the changes in biopterin metabolism that accompany

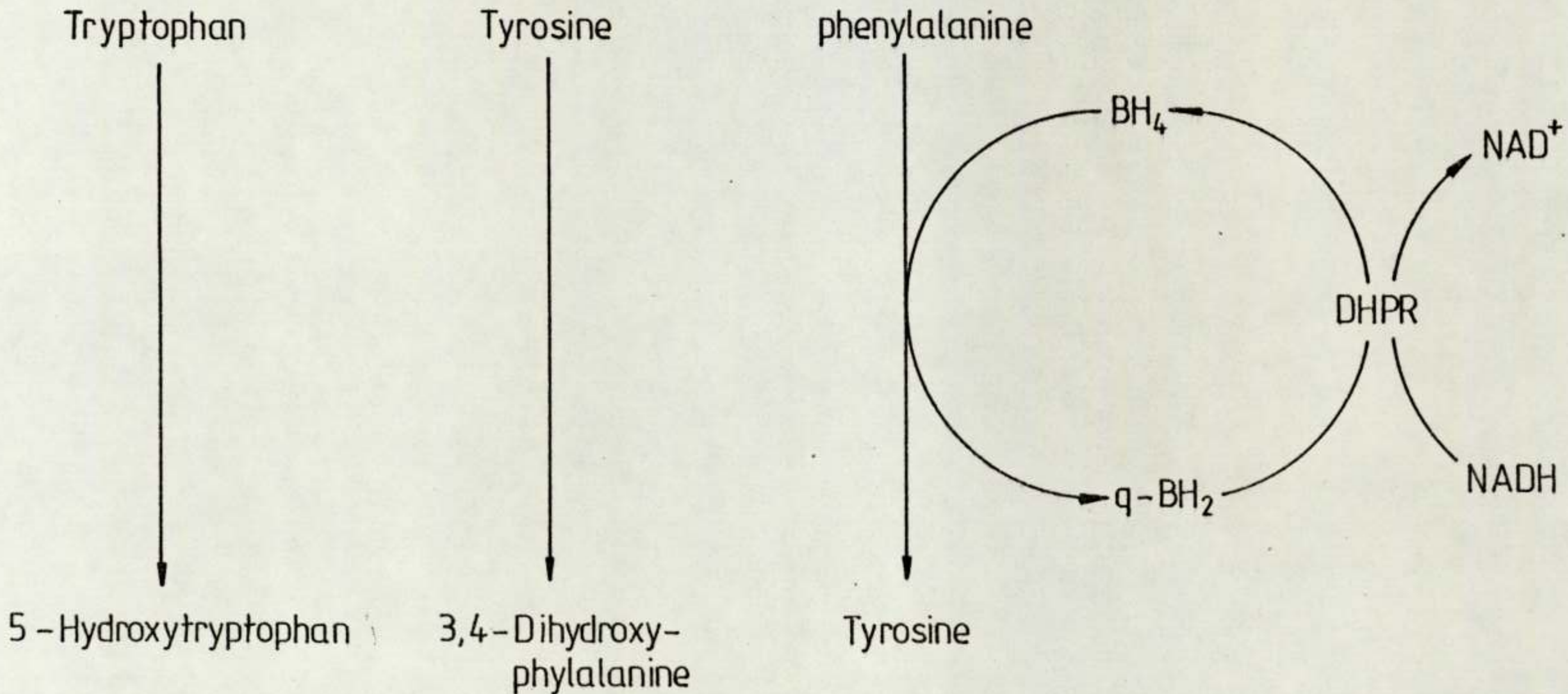


Figure 7·1 Involvement of BH_4 in the hydroxylation of the aromatic amino acids.

various neurological disease states.

Human brain tissue is limited in availability. In order to extend these investigations and to study the change in brain DHPR activity in man in neurological disorders, it is necessary to find more readily available samples from man. Cutler (personal communication) has shown that in rat normal fed, starved and dosed with a large amounts of lead, there is a significant correlation between brain DHPR activity and blood DHPR activity level (see tables 7.1, 7.2). Accordingly, we measured blood DHPR activity in a variety of human subjects, to investigate the changes in DHPR resulting from exposure to environmental agents.

The studies have been extended to rat brain, to investigate the neurotoxic effects of various agents on biopterin metabolism in vivo, by their administration to rats followed by direct analysis of the brain biopterins.

TABLE 7.1: DHPR activity in the rat brain and blood*

Tissue	n	DHPR activity		
		Fed rat	Starved rat**	p
Brain	12	$256 \pm 25.4^{+}$	$221.0 \pm 22.6^{+}$	0.1 %
Blood	12	$7.9 \pm 3.95^{++}$	$7.50 \pm 2.50^{++}$	N.S.

	Fed rat	Starved rat
correlation coefficient	0.75	0.66
Significance of correlation (p)	0.1%	0.1%

All values as mean ± S.D.

* Cutler personal communication

$^{+}$ nmole NADH oxidized/min/mg. protein

$^{++}$ nmole cytochrome c/min/mg. protein

**Adult male Wistar rats starved overnight (16 hours).

TABLE 7.2: Effect of lead on DHPR activity in the rat brain and blood.*

Tissue	DHPR activity		
	Control	Lead dosed**	p
Brain	275 ± 44.1 (12)+	212 ± 46.4 (11)+	0.1%
Blood	31 ± 7.8 (12)++	16 ± 8.1 (11)++	0.1%

correlation coefficient	0.75	0.64
significance of correlation (p)	0.1%	0.1%

All values as mean ± S.D.
Figures in parenthesis refer to number of rats.
* Cutler personal communication
**Adult male Wistar rats dosed orally with lead acetate (10^{-3}M) in 0.3 ml. water twice a day for 3 days.
+ nmole NADH oxidized /min./mg. protien
++ nmole cytochrome c/min./mg. protein

7.1 Properties of Human Brain DHPR:

7.1.1 Purification:

A reproducible purification procedure has been used here to purify DHPR from human brain, which is a modified version of Armarego and Waring method (1983), to get purified DHPR enzyme rapidly from small amounts of tissue (as shown in chapter 2).

Michaelis constant (Km) and M.Wt. for the purified DHPR were in good agreement with values quoted in the literature (Webber et al, 1978; Cheema et al, 1973, Craine et al, 1972; Hasegawa, 1977; Korri et al, 1977, Aksnes et al, 1979; Brown, 1981).

7.1.2 <u>Inhibition Studies:</u>

DHPR purified from human brain was found to be inhibited by phenylpyruvate to the extent of 37% at 10^{-4} and 20% at 5×10^{-5}M phenylpyruvate. These results agreed with results quoted in the literature (Dhondt and Bellahsene, 1983; Purdy and Blair, 1980).

The inhibition of DHPR by phenylalanine or its metabolite phenylpyruvate could be responsible for the neurological defects, which occur in phenylketonuria (PKU). Much evidence supports this theory. Inhibition of DHPR would lead to reduce cellular BH_4 level, and this would then result in less dopamine and noradrenaline being synthesized from tyrosine, and perhaps less serotonin being produced from tryptophan. This theory explains the reduced brain catecholamines and serotonin reported in PKU (Butler et al, 1978).

Children with PKU have higher than normal serum BH_2 levels, which parallel the serum levels of phenylalanine (Leeming et al, 1976a). Serum phenylpyruvate levels can be correlated with serum phenylalanine levels (Langenbeck et al, 1980). Inhibition of DHPR by phenylpyruvate or phenylalanine would explain these observations, because there would be an accumulation of quinonoid dihydrobiopterin (q-BH_2) in the cell, which would rearrange to give the increased levels of serum BH_2 recorded.

DHPR purified from human brain was inhibited to extent of 67% in the presence of 10^{-4}M of the neurotoxin 6-hydroxydopamine. Recently Armarego and Waring (1983) have shown that dopamine, adrenaline and noradrenaline do not inhibit DHPR, but their oxidation products the respective aminochromes are inhibitors. These results agree with our observations; in which 6-hydroxydopamine was rapidly

oxidized to a red product as it was dissolved in Tris-maleate buffer (pH 6.8). The oxidation rate was decreased in dilute HCl or distilled water. DHPR% inhibition was higher in Tris-maleate buffer, probably due to the formation of 6-hydroxydopachrome and its rate of formation decreased as the pH decreased (see chapter 2).

These results should be compared with an earlier report (Turner et al, 1974), in which the effect of intraventricular injections of 6-hydroxydopamine on rat brain DHPR activity was examined. Although treatment with 6-hydroxydopamine was seen to cause a significant reduction in brain noradrenaline and dopamine content and in tyrosine hydroxylase activity, no change in the activity of DHPR was observed. (Turner et al, 1974).

l-methyl-4-phenyl-l, 2, 5, 6-tetrahydropyridine (MPTP), a specific potent neurotoxin causing Parkinsonism in man, we have found to inhibit DHPR enzyme purified from human brain, to extent of 51% at 10^{-4}M. These results confirmed that reported by Blair et al (1984b), in which MPTP showed to be inhibitor of DHPR enzyme isolated from rat liver.

It is well-known that MPTP, a contaminant in an illegally manufactured drug (Langston et al, 1983), produced persistent Parkinsonian symptoms in individuals who injected the crude material (Langston et al, 1983) and in a laboratory worker who was exposed to high levels of MPTP and its analogues (Langston and Ballard, 1983). It was found that MPTP produces similar persistent pathological and neurochemical changes in rhesus monkeys (Burns et al, 1983).

The activity of DHPR enzyme purified from human brain, in the presence of aluminum cation shows a 47% reduction at 10^{-4}M, which is similar to aluminum level in the brains of dialysis dementia patients, but greater than that in normal brains (Alfrey et al, 1976; McDermott et al, 1978). This agrees with the results of Leeming and Blair (1979), in which DHPR activity in rat brain extract in the presence of aluminum cation shows a 40% reduction at 10^{-4}M.

The inhibition of DHPR by aluminum was time-dependent, which raises

the possibility of the inhibitor being irreversible. Dialysis of the aluminum treated enzyme against Tris-HCl buffer with no recovery of enzyme activity, confirmed the irreversibility of aluminum inhibition. Such irreversible inhibitions of enzyme by metals are common and usually the heavy metal attaches to a thiol group on the enzyme (Ferdinard, 1976).

The amino acid residue cysteine in which a thiol group exist on its side-chain has been determined in DHPR isolated from different sources; Craine et al (1972) showed the sheep liver enzyme to contain one cysteine per subunit and Aksnes et al (1979) showed that the bovine liver enzyme had three cysteines per subunit.

7.2 Environmental Influences on Biopterin Metabolism.

Almost every heavy metal is toxic to the central nervous system, if present in excess and many metals have been suggested as neurotoxic agents such as lead, aluminum, mercury and copper.

7.2.1 Lead

Exposure to high levels of lead has been shown to cause neurotoxicity (Byers and Lord, 1943; Rutter , 1980). The interaction between lead and BH_4 metabolism has received some attention. Purdy et al (1981) reported that lead irreversibly inhibits the activity of DHPR and reduces the synthesis of BH_4 in vitro in rat tissue preparations. Barford et al (1983) found reduced DHPR activity in the brains of rats born to mothers drinking leaded water. Measurement of plasma biopterins with blood lead in man and rat, shows that the plasma biopterin concentration correlated significantly with blood lead values (Blair et al, 1982; McIntosh et al, 1982).

Here in this thesis, these studies of biopterin have been extended to man on long term exposure to lead, to investigate the effect of lead in vivo. The DHPR activity in whole blood from a group of workers in the lead industry, has shown to be significantly reduced ($p < 0.1\%$ Student's "t" test), as shown in table (7.3). The regression curve between DHPR activity/mg.Hb. and the blood lead level, shows a correlation coefficient of 0.42 ($p < 0.1\%$).

TABLE 7.3: DHPR activity in lead workers.

Subject	Age	DHPR activity/mg.Hb.+	Blood lead level µg./dl.
Normal male (20)	24-83	1.66 ± 0.36*	13.55 ± 5.09**
Lead workers (46)	22-61	1.23 ± 0.25*	48.0 ±15.4**

All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.
+ µmole cytochrome c/min./mg. Hb.
*P = 0.1% (Student's "t" test)
** P = 0.1%

DHPR is a thiol containing enzyme (Shima, 1985) and this functional group is necessary for activity. Furthermore, it is well recognized that certain sulfhydryl containing enzymes are especially susceptible to the direct effect of lead (Kennedy et al, 1983). It is therefore likely that lead acts at this site. Serious deficiency of DHPR leads to severe neurological disease (Kaufman et al, 1975), and it has been suggested that interference with BH_4 metabolism in vivo would account, in part, for the neurotoxicity of lead (Purdy et al, 1981).

7.2.2 Aluminium:

Aluminium toxicity has, in recent years, been implicated in the pathogenesis of a number of clinical disorders in patients with chronic renal failure, on long-term intermittent haemodialysis treatment (Alfrey et al, 1976; Elliott et al, 1978; Parkinson et al, 1979). The predominant disorders have been those involving either

bone (osteomalacic dialysis osteodystrophy) or brain (dialysis encephalopathy). In nonuremic patients, an increased brain aluminium concentration has been implicated as a neurotoxic agent in the pathogenesis of Alzheimer's disease and was associated with experimental neurofibrillary degeneration in animals (Crapper et al, 1973).

The brain aluminium concentration in patients dying with the syndrome of dialysis encephalopathy (dialysis dementia) are significantly higher than in dialysed patients without the syndrome and in nondialysed patients (Alfrey et al, 1976; Mc Dermott et al, 1978).

Very little is known about the effect of Al on BH_4 metabolism. High concentrations of Al produced a fall in the rat brain and liver BH_4% level, whilst increasing the total biopterin pool and the actual BH_4 level (Morar, 1984).

We have reported earlier in this thesis (chapter 2), that Al inhibits DHPR enzyme purified from human brain, to extent of 50% at 10^{-4}M, a concentration found in the brain of those dying with dialysis dementia (Alfrey et al, 1976; Mc Dermott, et al, 1970). To investigate the in vivo effect of Al on BH_4 metabolism in man, we measured DHPR activity in the whole blood from haemodialysis patients with chronic renal failure (see chapter 3), in which the activity was significantly decreased in contrast to correlated normal subjects (table 7.4). In addition there was a good correlation between DHPR activity/mg. Hb. and Al concentration in the blood samples from haemodialysis patients ($r = 0.52$, $P = 0.1\%$).

TABLE 7.4: DHPR activity in whole blood from haemodialysis patients.

Subject	mg.Hb./ml.	DHPR activity*	Blood Al level (µgm./l.)
correlated normal	8.52 ± 1.71 (47)**	2.68 ± 0.17 (47) +	5.9 ± 2.3 (8)++
Haemodialysis patient	8.52 ± 1.71 (47)	2.37 ± 0.80 (47) +	91.91 ± 69.98 (47)

* µmole cytochrome c/min. /mg. Hb.
** mg.Hb./ml. of normal subjects as adjusted to that level of haemodialysis patients (for details see Chapter 3).
+ P = 5% (Student's "t" test)
++ Shore et al (1983).
All values as mean ± S.D.
Figures in parenthesis refer to number of subjects studied.

Like lead, aluminium has been shown to bind to sulfhydryl containing enzyme (such as DHPR) causing irreversible inhibition of the enzyme.

Thus it seems likely that at least some of the neurotoxic effect of Al is caused by loss of cell BH_4 by inhibition of DHPR and that, this loss of cell BH_4 could be factor in dialysis dementia.

The hemoglobin level was found to be too low in the majority of blood samples from haemodialysis patients, as shown in table (7.4).

The dilution of blood samples causes an elevation in the measured DHPR activity, and the dilution of heat inactivated blood with untreated blood, causes decreased DHPR activity as % of heat inactivated blood increases. This result agrees with the observation of Professor B Clayton and coworkers - The University of Southampton (Professor Clayton personal communication) , in which they suggested that the blood contained a substance which interfered with the reaction.

The study of the effect of hemoglobin on the activity of whole blood DHPR, shows DHPR inhibition as hemoglobin solution is added to the assay mixture. Furthermore, the inhibition% of DHPR purified from human brain increased as the concentration of hemoglobin added to the assay mixture increased.

7.3 Effect of Phenylalanine on BH_4 Metabolsim:

The mental defects which occur in untreated PKU, are due to the increased levels of phenylalanine or its metabolites (Bickel et al, 1953; Udenfriend, 1961). A low-phenylalanine diet appears to reduce the neurological damage of PKU (Bickel et al, 1953).

In order to study BH_4 metabolism in phenylketonuria, experimental models of hyperphenylalaninaemia have been used. These studies are based on short term administration of high phenylalanine alone or together with p-chlorophenylalanine (PCPA), to a group of rats.

Acute dosing with phenylalanine produces a significant decrease in actual BH_4 level of the rat brain, two hours after administration of the amino acid. The change was highly significant at doses of 600 and 900 mg. phenylalanine/Kg. body weight (p = 0.1% Student's "t" test), and this is reflected in an equivalent rise in BH_2% in these rats (table 7.5). This is consistent with inhibiton of DHPR by a phenylalanine metabolite, such as phenylpyruvate. Such inhibition would lead to the observed fall in active cofactor levels and increased loss of BH_2 into the blood, and hence high serum biopterin (Leeming et al, 1976).

p-Chlorophenylalanine (PCPA) is a competitive inhibitor of phenylalanine hydroxylase in vitro (Jequier et al, 1967). Recent studies have suggested that PCPA inhibits phenylalanine hydroxylase, as being incorporated into the enzyme protein during protein synthesis (Gál and Millard, 1971).

Following PCPA plus phenylalanine administration, the treated rats shows the same effect on the actual BH_4 and total biopterin levels as rats treated with phenylalanine alone (table 7.6).

TABLE 7.5 : **EFFECT OF PHENYLALANINE CONCENTRATION ON RAT BRAIN BH_4 METABOLISM**

Dose	n	Actual BH_4 *	BH_4%	BH_2%	Total Biopterins *	Fully Oxidized Biopterin %	Neopterin *	Pterin *
Control	6	240.29±22.31	91.38± 2.34	6.58±2.39	263.00 ± 24.54	2.01 ± 1.56	3.77 ± 0.99	245.37±34.81
300mg/kg wt. phenylalanine	6	205.42±39.26	83.12± 2.10	10.0±2.99	246.67 ± 43.98	4.1 ± 2.24	6.78 ± 3.34	214.92±38.23
p+		N.S.	0.1%	N.S.	N.S.	N.S.	N.S.	N.S.
600mg/kg.wt. phenylalanine	6	184.59±12.55	83.02±12.55	13.75±3.09	222.19 ± 8.34	4.54 ± 1.33	5.37 ± 0.34	209.65±18.20
p+		0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	5%
900mg/kg wt. phenylalanine	6	183.92±12.44	83.38± 3.10	12.47±1.55	220.51 ± 11.35	1.65 ± 0.28	4.80 ± 1.29	353.80±61.26
p+		0.1%	0.1%	0.1%	0.1%	N.S.	N.S.	0.1%

All values as mean ± S.D.
Control rats were administered water only
n = number of experimental rats
N.S. = No significant difference
* pmole/gm wet wt.   + Student's "t" test.

TABLE 7.6 EFFECT OF p-CHLOROPHENYLALANINE (PCPA) ON RAT BRAIN BH_4 METABOLISM

Dose	n	Actual BH_4 +	BH_4 %	BH_2 %	Total Biopterins +	Fully Oxidized Biopterin %	Neopterin +	Pterin +
Control (corn oil)	6	226.74±30.72	94.52±0.74	5.17±1.47	239.95±32.86	1.09 ± 0.27	4.52 ± 1.00	298.27±42.53
* Corn Oil + Phenylalanine(300mg./kg.wt.)	6	182.83±21.27	88.45±1.94	9.24±2.17	207.13±27.67	1.43 ± 0.45	4.37 ± 0.15	286.05±28.18
p ++		2%	0.1%	1%	N.S.	N.S.	N.S.	N.S.
** PCPA (300mg./kg.wt) + Phenylalanine(300mg./kg.wt.)	6	189.44±18.35	88.28±1.00	7.64±0.93	214.58±20.15	1.83 ± 0.55	3.32 ± 1.11	339.57±31.04
p ++		5%	0.1%	1%	N.S.	2%	N.S.	N.S.
** PCPA (300mg./kg.wt.) + Phenylalanine(600mg./kg.wt.)	6	185.25±18.70	87.88±1.13	9.58±0.84	210.83±21.66	1.41 ± 0.23	5.93 ± 0.5	265.50 ± 34.76
p ++		2%	0.1%	0.1%	N.S.	5%	2%	N.S.

* Adult male Wistar rats dosed orally with 0.4ml. corn oil and 4 hours later given 300mg./kg.wt. phenylalanine in 0.4ml. water and sacrificed 2 hours later.

** Adult male Wistar rats dosed orally with PCPA (300mg./kg.wt.) in 0.4ml corn oil and 4 hours later given phenylalanine in water and sacrificed 2 hours later.

All values as mean ± S.D.

n = number of experimental rats N.S. = no significant difference

+ pmole/gm.wet wt. ++ Student's "t" test.

As PCPA was administered as a suspension in corn oil, control animals were also treated with corn oil only at the stated time intervals. The corn oil dosed control rats shows some variation in biopterin levels, in contrast to water dosed control rats. The BH_4% level in the rat brain is significantly elevated (p = 1% Student's "t" test). Hence corn oil appears to stimulate brain DHPR activity.

Later on these results have been confirmed, as rat brain DHPR activity is significantly increased (p = 0.1% Student's "t" test) in corn oil dosed animals in comparison to water dosed animals, as shown in table (7.7) (Cuttler, personal communication).

TABLE 7.7: Effect of corn oil on rat brain DHPR activity+

Subject	n	DHPR activity (nmole NADH/min./mg. protein)
Control rats	6	193 ± 37.7**
*Corn oil dosed rats	6	581 ± 66.8**

+Cuttler personal communication

* Adult male Wistar rats administered orally with 0.3 ml. corn oil daily for 4 days. Control rats were fed with normal diet and water.

All values as mean ± S.D.
n = number of experimental rats

** p = 0.1% Student's "t" test

It is well known that the ß-oxidation of unsaturated fatty acids is due to mitochondrial enzymes, which are NAD^+ and $NADP^+$ dependent (Stumpf, 1969). So the stimulation of DHPR activity in the control oil dosed rats is probably due to the formation of NADH and NADPH cofactors.

7.4 Effect of Oestrogen on BH_4 Metabolism:

Previous studies in women have shown that serum biopterin derivatives vary with hormonal state. Their concentrations fall when oestrogens levels rise throughout the menstrual cycle, when taking the contraceptive pill and during pregnancy (Leeming and Blair, 1980a; Barford et al, 1983). DHPR activity in whole blood rises in the same situations (Barford et al, 1983).

Here in this thesis, various concentrations of diethylstilboestrol starting from 0.43 µg./Kg. body wt., which is equivalent to ethinyloestradiol concentration in the oral contraceptive pill (20-50 µgm.) (British National Formulary, 1985), up to 50 mg./Kg. body wt., were dosed to an experimental animals in order to investigate a possible effect of oestrogens on brain biopterins.

Total biopterin and actual BH_4 levels were significantly reduced in rats pretreated with diethylstilboestrol at 0.43 µg./Kg. body wt. ($p = 5\%$ Student's "t" test), compared to matched controls killed at the same time. The change became highly significant as the dose concentration increased up to 50mg./Kg. body wt. ($p = 0.1\%$), and that is reflected in an equivalent decrease in the BH_4% level (table 7.8).

TABLE 7.8 <u>EFFECT OF DIETHYLSTILBOESTROL ON RAT BRAIN BH_4 METABOLISM</u>

Dose	n	Actual BH_4 *	BH_4 %	BH_2 %	Total Biopterins *	Fully Oxidized Biopterin %	Neopterin *	Pterin *
Control (corn oil)	6	236.26±24.38	94.28±1.16	4.78±1.55	250.55 ± 25.10	1.08 ± 0.25	4.28 ± 0.65	223.3 ± 30.34
Diethylstilboestrol 0.43µg./kg.wt.	6	204.06±21.56	92.16±2.23	6.60±1.86	221.1 ± 19.15	1.55 ± 0.46	4.97 ± 1.34	253.78 ± 39.68
P^+		5%	N.S.	N.S.	5%	N.S.	N.S.	N.S.
1mg./kg. wt.	6	142.45±17.12	86.22±5.14	13.33±5.22	164.96 ± 14.06	1.25 ± 0.37	7.83 ± 1.11	222.85 ± 17.64
P^+		0.1%	1%	1%	0.1%	N.S.	N.S.	N.S.
10mg./kg. wt.	6	128.1±7.46	87.08±5.26	11.88±5.39	147.56 ± 12.32	0.75 ± 0.34	4.85 ± 1.43	200.08 ± 39.82
P^+		0.1%	1%	1%	0.1%	N.S.	N.S.	N.S.
50mg./kg. wt.	6	131.66±8.86	88.68±3.66	9.63±3.50	149.34 ± 9.84	0.84 ± 0.22	3.65 ± 0.61	165.02 ± 12.3
P^+		0.1%	1%	1%	0.1%	N.S.	N.S.	1%

Adult female Wistar rats (100gm.wt.) dosed orally with a suspension of diethylstilboestrol dipropionate in 0.4ml. corn oil daily for 4 days. 24 hours after the last dose the animals were killed.

All results as mean ± S.D.

n = number of experimental rats

N.S. = No significant difference

* pmole/kg. wet wt.

\+ Student's "t" test.

The decrease in total serum biopterins in menstruating women, women on the pill and in pregnancy reported by Leeming and Blair (1980a) and Barford et al (1983) is therefore, most probably explained by decreased BH_4 synthesis.

The administration of oestrogens to humans can have neurological consequences, e.g. "pill" induced chorea in subjects with a previous history of sydenham's chorea (Nausieda et al, 1979) and chorea gravidum. Oestrogens are known to be anti-dopaminegic, lowering striatal brain dopamine concentrations and increasing the number of post synaptic dopamine receptors as a compensation mechanism (Di Paulo et al, 1982). The effect on dopamine levels could be explained by the reduction in BH_4 level, leading to a slowing of the tyrosine hydroxylase reation, the rate-limiting step in dopamine biosynthesis.

7.5 Biopterin Metabolism in Disease:

The major role that BH_4 plays in neurotransmitter biosynthesis (Leeming et al, 1981), means that neuropathological disease states could arise due to impaired BH_4 synthesis. A number of disease states have been reported, where biopterin levels have been found to be altered.

7.5.1 Senile Dementia and Down's Syndrome:

Recent reports have suggested a defect in BH_4 metabolism in senile dementia of Alzheimer type (SDAT); biopterin concentration in serum (Aziz et al, 1982), CSF (Morar et al, 1983) and brain (Barford et al, 1984) are lowered in patients with SDAT compared to age-matched controls, suggesting a role for BH_4 in the pathology of SDAT. Blair et al (1984a) have shown that synthesis of BH_4 was significantly lower in

SDAT brain than in the control brains.

Studies on temporal and frontal cortex taken from patients dying with SDAT, showed that DHPR activity in frontal cortex (BA9) from subjects with SDAT was higher than that in frontal lobe from control subjects, but the elevation was not significant. In contrary, there is no difference in DHPR activity in BA20 for subjects with SDAT and controls. However, DHPR activity in BA9 area is significantly higher than that in BA20 area from SDAT patients (p = 5% Student's "t" test).

The biosynthesis of biopterin in brain area BA9 of dements, showed no reduction when compared to controls (Anderson personal communication), to these can be added the results from brain area BA20, in which the synthesis was significantly reduced in SDAT patients, in comparison with controls (p = 0.1%) (Hamon, 1984), as shown in table(7.9).

However, it was found that in dementia brain (BA20) samples, total biopterin is lower than that of the controls, and the neopterin to biopterin ratio is elevated (Barford et al, 1984).

DHPR activity in the brain from subjects dying with Down's syndrome, was found to be higher than controls, although the difference was not significant (chapter 5). In addition, neopterin concentration in the blood serum of Down's patients are significantly below normal (Blair et al , 1984a). These, together with the lack of BH_4 synthesis in Down's brain (Hamon, 1984), suggest that the mental retardation seen in Down's patients could be related to a failure to synthesis BH_4.

DHPR activity was examined in several regions of the brain of two subjects. The highest activity was found in the frontal lobe. This result fit in with the observations of Hamon (1984), that DHPR activity in frontal lobe was higher than in temporal lobe. He reported that human locus ceruleus and hippocampus have the highest activity, which disagreed with the results of Bullard et al (1978), in which rat hippocampus has a lower DHPR activity. The highest BH_4 synthesis was found in the

TABLE 7.9 DIHYDROPTERIDINE REDUCTASE ACTIVITY AND BIOPTERIN SYNTHESIS IN HUMAN BRAIN FROM CONTROL AND DEMENTED SUBJECTS

	BIOPTERIN SYNTHESIS (ngm./hr./mg. protein)		DHPR ACTIVITY (nmole NADH oxidized/min./mg. protein)	
Subject	BA9+	BA20++	BA9	BA20
Control	0.88 ± 0.75(10)	*0.66±0.48(8)	303.67 ± 130.36 (10)	300.91 ± 139.05 (10)
SDAT	1.04 ± 0.53(6)**	*0.09±0.14(12)**	403.93 ± 40.47 (6)***	304.68 ± 83.80 (6)***

* P = 0.1% (Student's "t" test)

** P = 0.1%

*** P = 5%

\+ Anderson personal communication

++ Hamon (1984)

All values as mean ± S.D.

Figures in parenthesis refer to number of subjects studied.

frontal lobe and no synthesis was found in the hippocampus, putamen and globus pallidus (Hamon, 1984).

The effect of the substrates $DMPH_4$ and NADH on DHPR activity was investigated and Km values were determined. Increased Km values were observed for SDAT and Down's patients (1 each) compared with 3 normal subjects. On the basis of this very small sample size, it is difficult draw any definite conclusion, but it would appear that the Km for both $DMPH_4$ and NADH is increased, which is probably due to the structural change in the enzyme from these patients.

7.5.2 Heterozygote DHPR deficiency:

100% Deficiency of DHPR leads to hyperphenylalaninaemia similar to that observed in phenylketonuria (PKU). However, these patients are unresponsive to the dietary regime with which classical PKU is treated, developing a progressive and life threatening neurological disease arising from deficiency of dopamine, noradrenaline and serotonin, neutrotransmitter products of tyrosine and tryptophan hydroxylation (Danks et al, 1978).

Heterozygotes with DHPR deficiency (parents of homozygotes with DHPR deficiency) with DHPR activity about half that of a group of normals, are known and are apparently normal (Barford et al, 1982; Sahota, unpublished data).

In this thesis, we present evidence from a single case study, that there is a central nervous system disorder, due to partial heterozygote DHPR deficiency in a female child (S.H.).

Her parents have partial DHPR deficiency, her brother (K.H.) , now dead, was a homozygote for DHPR deficiency. At the age of 2 years, DHPR activity was 0.74 nmole cytochrome c/min./mg.Hb. (normal adult female DHPR level 1.95 ± 0.45) (see table 7.10). CSF homovanillic acid level was 103 ng./ml. and 5-hydroxyindoleacetic

acid was 90 ng./ml. (normal levels of these compounds 20-100).

TABLE 7.10 DHPR activity in whole blood from heterozygote DHPR deficiency.

	Age	mg.Hb./ml.	DHPR activity/mg.Hb.*
Normal adult male (45)	17-89	14.92 ± 1.86	1.72 ± 0.41
Normal adult female (37)	11-54	13.75 ± 1.60	1.95 ± 0.45
S.H.	2	20.0	0.74
Father of S.H.		16.0	0.78
Mother of S.H.		11.0	0.94

* µmole cytochrome c/min./mg.Hb.
All values as mean ± S.D.

The computerized tomography scan was normal. Plasma and CSF biopterin levels were 1.5 µg./l. and 3.8 µg./l. respectively.

DHPR kinetic studies in whole blood from this case, showed a significantly increased Km values for both $DMPH_4$ and NADH substrates; 13.2 and 38.0 µM, in comparison to normals with 2.6 ± 0.93 and 15.65 ± 3.5 for $DMPH_4$ and NADH respectively, suggesting decreased binding of these coenzymes caused by a change in enzyme structure.

Early urine analysis in this subject showed a high level of urine biopterin, a low neopterin level and a low neopterin/biopterin ratio (table 7.11) (Al-Beir, 1982).

She is now aged 4 and is mental retarded . Her reflexes are normal and she has had no fits since the age of 3 years. She can help with dressing, but cannot dress herself. She does not handle a spoon very well, she is not toilet trained and has no speech but can walk and climb.

In the absence of any other explanation, it seems likely that the severe

mental retardation of this child, is caused by a greatly reduced capacity to salvage quinonoid dihydrobiopterin, arising from heterozygote DHPR deficiency plus changes in enzyme structure (Firgaira et al, 1981).

TABLE 7.11 : Urinary pteridines levels in S.H. family, (Al-Beir, 1982).

Subject	Biopterin	Neopterin	Neopterin/Biopterin
Normal male (n = 9)	119.86 ± 81.30	314.03 ± 171.62	2.96 ± 1.64
Normal female (n = 5)	168.9 ± 75.28	322.06 ± 124.93	2.27 ± 1.55
Homozygote DHPR deficiency K.H.*	1435.0 1921.6	1932.4 1647.1	1.4 0.86
Heterozygote DHPR deficiency S.H.	1460.1	295.7	0.2
Father of S.H. and K.H.	23.7	128.0	5.4
Mother of S.H. and K.H.	78.9	434.1	5.5

All values are given in ng./gm. creatinine
* Two urine samples were obtained in different occassions.

7.5.3 Neoplastic Diseases:

DHPR activity in breast neoplastic tissue showed a significant increase compared to apparently normal tissue from the same breast; $p = 0.2\%$ by Wilcoxon's signed ranks test, using protein content and $p = 5\%$ using DNA content as base. Meanwhile, DHPR activity/mg. protein in large intestine neoplastic tissues was evelated over controls, but the elevation was not significant.

The results for breast and large intestine tumours fit in with the observations of Eggar et al (1983). Hamon (1984) reported that the biosynthesis of BH_4 in breast neoplastic tissue showed no significant difference from control tissue. In contrast, the

level of BH_4 biosynthesis in large intestine neoplastic tissues was elevated over controls, which agreed with the observation of Baker et al (1981), that the levels of biopterin were significantly raised in these neoplastic tissues.

The description of high DHPR activity in human neoplastic tissues, is similar to Dhondt et al (1981). They showed that large variations in DHPR activity in breast cancers were found, 6% of tumors showing high values close to those noted in liver, whereas the main group has activities of same magnitude of that in cultured fibrobalsts. Significant correlation appeared between DHPR activities and hormonal dependence, as measured by cytosolic oestrogen receptor sites.

The findings of elevated DHPR activity in cancerous cells which are resistant to analogs of folic acid (Alt et al, 1976; Dolmick et al, 1980), and the results of Saleh et al (1981) which showed that folate metabolism was decreased in the tumour-bearing animals, and increased in tumour-bearing animals dosed with methotrexate, led us to suggest a model for the role of DHPR in the neoplastic tissues.

The incorporation of folic acid into the reduced folate pool increased in patients with malignant diseases (Saleh et al, 1982), and the urine excretion of its catabolites decreased. It has been suggested that these are due to the anoxia of solid tumours and to more reducing conditions prevailing in the cytosol of tumour cells (Saleh et al, 1981).

As tetrahydrofolate in vivo oxidised by bein exposed to 0_2 , then DHPR which is increased in neoplastic tissues, may play role for converting the oxidized product back to tetrahydrofolate (Pollock and Kaufman, 1978). It is known that tetrahydrofolate is oxidized via the quinonoid tautomer of dihydrofolate (Kaufman, 1961), and that this quinoniod dihydrofolate is a substrate for DHPR (Lind, 1972), an enzyme with high activity in neoplastic tissues, as we have metioned earlier.

Hence, increased DHPR activity could contribute to the neoplastic process by increasing the availability of reduced folates for the biosynthesis of purines and

pyrimidines, so increasing the rate of cellular proliferation.

Human breast DHPR has a maximum activity at pH 6.8 for both apparently normal and tumour tissues similar to the optimum pH for sheep liver DHPR (6.8-7.0) (Webber et al, 1978; Cheema et al, 1973) and rat liver (6.3-6.8) (Webber et al, 1978, Brown, 1981).

The quinonoid substrate for DHPR is unstable, and rapidly rearrange to the corresponding 7, 8-dihyropterin. The rate of rearrangment is pH dependent, hence it is fortunate that the optimum we have observed , is near the pH value at which the substrate quinonoid dihydropterin is most stable (Archer and Scrimgeour, 1970). Nielsen (1969) studied the rate of rearrangement of quinonoid-6-methyldihydroperin and the 6, 7-dimethyl derivative, and found that the rates were much slower in Tris buffer than in phosphate buffer at pH 6.8.

7.6 Conclusion and Suggestions for Further Work:

Most conditions in which deranged BH_4 metabolism occurs, whether demonstrated by direct measurement of cofactor in tissues, measurement of DHPR activity in tissue and whole blood or measurement of enzymes of the synthesio pathway can have associated neuropathies. The difficulty lies in determining causal relationships.

In addition to the known genetic defect, the pathways of BH_4 metabolism are found to be altered in a wide range of pathological states. Senile dementia of Alzheimer type and Down's syndrome are associated with a substantial reduction of BH_4 metabolism and slight elevation in DHPR activity in the brain.

The specific increase in DHPR activity in neoplastic tissues, and its increase in oestrogen treated rats, indicted that this enzyme may be responsive to oestrogen.

Furthermore DHPR is inhibited in vivo and in vitro by phenylalanine and its metabolites (such as phenylpyruvate), 6-hydroxydopamine, MPTP, lead and

aluminium. So it is clear that bioperin derivatives vary with pathological state, and in response to physiological stimuli and exogenous neurotoxins.

Severe derangement of BH_4 metabolism by genetic deficiency, leads to serious neurological damage. The changes described here, will also lead to altered neurotransmitter metabolism, and may be responsible for the mental deficient of senile dementia and Down's syndrome, and contribute to the neurotxic effects of compounds such as, phenylalanine, MPTP, lead and aluminium.

Further work with human brain has given an insight into its biopterin metabolism and shows, it to be similar to that in rats. This need to be taken further, to see if it can yield insight to brain function and dysfunction, with respect to biopterin metabolism.

More work needs to be done to confirm the inhibitory effect of 6-hydroxydopamine and its aminochrome derivative.

The results obtained from lead workers and haemodialysis patients, are of interest, and further work is required to study the BH_4 metabolism in these samples.

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