DIHYDROPTERIDINE REDUCTASE FROM MAN AND THE RAT

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The work described in this thesis has been carried out independently and has not been submitted for any other degree.

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SUMMARY

"The Properties of Dihydropteridine Reductase From Man and the Rat"

by

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For the degree of Doctor of Philosophy

May 1985

Dihydropteridine reductase has been purified from rat liver and human brain tissue samples by affinity chromatography using sodium 1,2-naphthoquinone-4-sulphonate as the ligand. The rat liver dihydropteridine reductase was purified over 960 fold over the original supernatant and Km's for the two substrates NADH and quinonoid dimethyldihydropterin ($qDMPH_2$) were 1.7×10^{-5}M and 2.1×10^{-5}M respectively. Human brain dihydropteridine reductase was purified 20 fold over the original supernatant and Km's for NADH and $qDMPH_2$ were 1.9×10^{-5}M and 2.9×10^{-5}M respectively.

The effect of lead on dihydropteridine reductase activity in vivo was investigated using rat brains from animals subjected to a leaded water regime from conception. Lead at subclinical lead poisoning levels significantly inhibited enzyme activity in these brain samples. In vitro experiments using human brain dihydropteridine reductase showed lead to significantly inhibit enzyme activity in an irreversible manner.

Dihydropteridine reductase activity in crude tissue preparations was increased in rat liver as a consequence of oestrogen dosing, whilst purified rat liver enzyme was strongly inhibited by oestrone, oestradiol and their catechol derivatives. The inhibition of dihydropteridine reductase by these oestrogens possibly being related, by its subsequent effects on neurotransmitter synthesis, to the mood changes observed in pre-menstrual tension.

A variety of human tumour samples along with normal tissue, were assayed as crude preparations for dihydropteridine reductase activity; breast tumours showed a highly significantly increased activity as compared to normal breast tissue but tumours of the gut did not appear to have any change in enzyme activity.

Human brain dihydropteridine reductase activity was elevated in temporal lobe samples from patients suffering from senile dementia of the Alzheimer type as compared to age matched controls, but this elevation was not significant.

Dihydropteridine reductase activity was measured in several human brain regions.

Key Words

Dihydropteridine Reductase Tetrahydrobiopterin Human Brain SDAT

ACKNOWLEDGEMENTS

I would like to thank Dr. Pat. Barford and Professor J.A. Blair for their guidance, supervision and useful discussions throughout this work.

I dedicate this my thesis to my wife Marion

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CHAPTER ONE

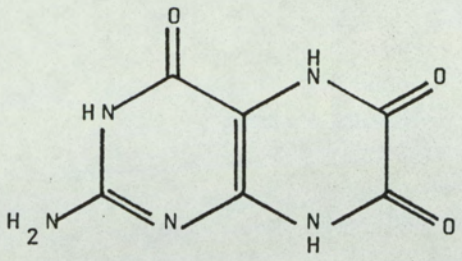
INTRODUCTION

INTRODUCTION

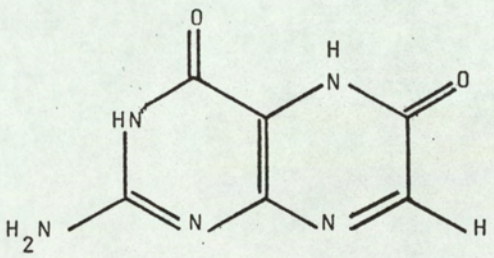
"OVERVIEW" :

The extraction of a yellow pigment from butterfly wings by Sir Fredrick Gowland Hopkins (Hopkins, 1889) was the beginning of research into a group of compounds widely distributed in nature, two of which are now known to be essential to man. Work on butterfly wing pigments continued and Wieland and Schopf (1925) purified two compounds, one white the other yellow, the structures of which were eventually elucidated by Purrman (1940). These were leucopterin (**1**) and xanthopterin (**2**). Pteridines have now been shown to be present not only in insects such as butterflies and moths, but extensive work has also been carried out on amphibians and fish (Hama, 1963).

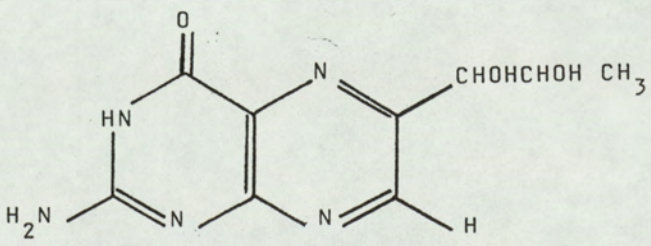
Patterson et al (1955), were the first group to isolate and identify a pterin from a human source. This group isolated biopterin (**3**), from human urine. Since this discovery biopterin and its derivatives have been measured in human urine and serum by many research groups (Fleming and Broquist, 1967; Baker et al, 1974; Leeming et al, 1976a; Rey et al, 1977; Leeming and Blair, 1980), the ease of obtaining such samples being an advantage. However, other body fluids and tissues have been examined (Baker et al, 1974; Leeming et al, 1976b; Kaufman et al, 1978; Nagatsu et

(1) 

LEUCOPTERIN

(2) 

XANTHOPTERIN

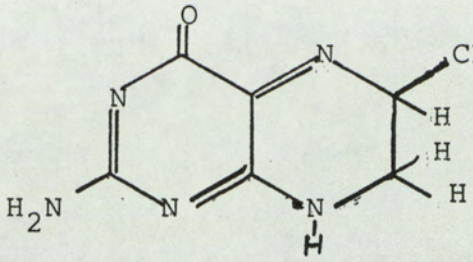
(3) 

BIOPTERIN

al, 1979; Lovenberg et al, 1979; Williams et al, 1979; Morar et al, 1983). Variations in biopterin concentration between anatomical regions of an organ (Baker et al, 1974) and the brain (Leeming et al, 1976a; Bullard et al, 1978) have been reported and this variability in the brain has been confirmed in neuroblastoma lines (Albrecht et al, 1978).

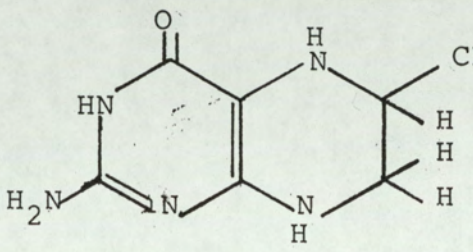
The identification of biopterin derivatives in tissue or fluid samples is made difficult due to the high reactivity of the reduced forms (Blair and Pearson, 1974). Biopterin isolated from mammalian tissue sources has as its origin the tetrahydro (5) or q dihydro (4) forms which oxidise rapidly to 7,8 dihydrobiopterin (6) and then to biopterin. However, a variety of sensitive methods are available for the measurement of derivatives such as radio-immuno assay (Nagatsu et al, 1979; Rokos & Rokos, 1983) enzymatic methods (Guroff and Abramowitz, 1967; Kaufman et al, 1978), gas chromatography/mass fragmentography (Rothler and Karobath, 1976), high performance liquid chromatography (Fukushima and Nixon, 1980), or by protozoological bioassay using Crithidia fasciculata (Leeming and Blair, 1974). C. Fasciculata is a haemoflagellic parasite of the mosquito, which has an absolute requirement for biopterin or its reduced species for growth, only L neopterin (7) and pteroic acid (8) approach this growth stimulating capacity (Baker et al, 1974; Leeming and Blair, 1974; Leeming,

(4)



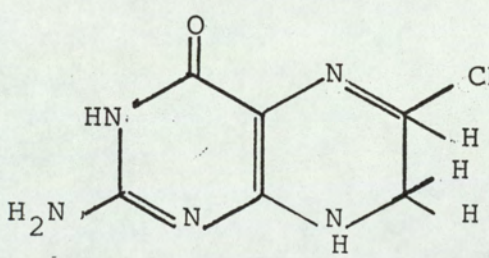
QUINONOID DIHYDROBIOPTERIN

(5)



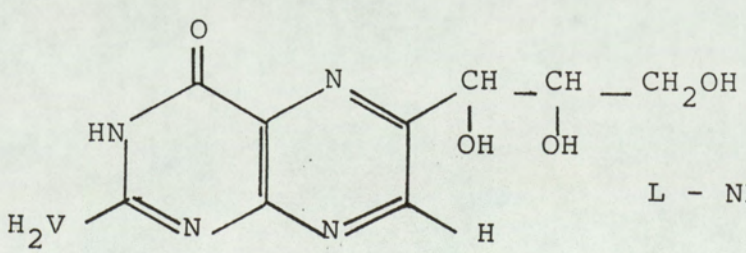
5,6,7,8 TETRAHYDROBIOPTERIN

(6)



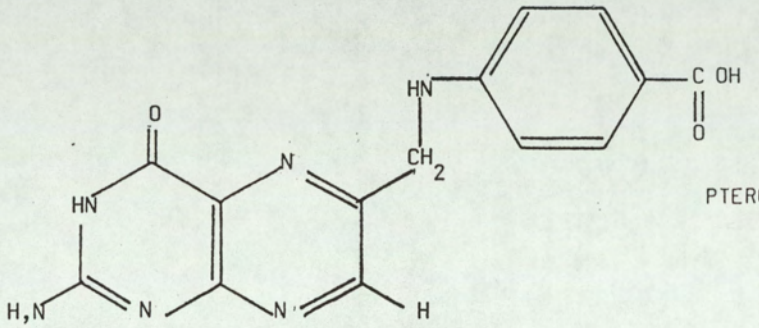
7,8 DIHYDROBIOPTERIN

(7)



L - NEOPTERIN

(8)



PTEROIC ACID

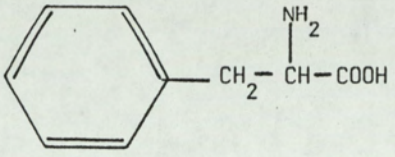
1979). Fukushima and Nixon (1980), using reverse phase high performance liquid chromatography (HPLC) of samples after iodine oxidation and ion-exchange chromatography, have shown that the biopterin present in mammalian tissues is predominantly in the tetrahydro form.

In a recent paper several of the quantitative methods were compared (Blair et al, 1983). Crithidia assay was found to be much more sensitive than the present HPLC assay for biopterin. However, it was found to be less specific than HPLC even when associated with bioautography. It was also reported that the enzymic assay for biopterin in urine could be influenced by a contaminant, thought to be Fenton's reagent, which gave rise to elevated estimates of biopterin when compared to Crithidia or HPLC.

In normal subjects serum biopterin derivative levels are usually maintained within a very narrow range (Leeming et al, 1981), mean serum level for males being 1.75 $\pm$ 0.03 ug/l and for females 1.53 $\pm$ 0.04 ug/l. Women do, however, exhibit changes during the menstrual cycle and levels increase with age in both sexes (Leeming and Blair, 1980).

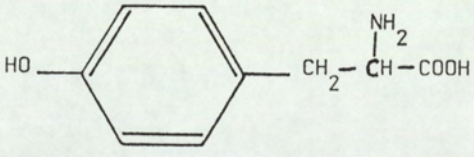
The first specific role that 5,6,7,8 tetrahydrobiopterin (BH_4) was shown to have was as a cofactor in the hydroxylation of phenylalanine (9) to tyrosine (10) (Kaufman, 1958; Kaufman 1963). It has been subsequently shown that BH_4 is a cofactor in the hydroxylation of

(9)



PHENYLALANINE

(10)



TYROSINE

tryptophan (11) to 5-hydroxytryptophan (12) (Hosoda and Glick, 1966) and tyrosine to dopa (13) (Nagatsu et al, 1972), these reactions being of importance in neurotransmitter synthesis. Two other oxygenase reactions dependent on BH_4 participation have been reported; namely the oxidation of long chain alkylethers of glycerol to fatty acids and glycerol (Tietz et al, 1964) and the hydroxylation of cinnamic acid to p-coumaric acid (Nair and Vining, 1965).

In the early 1970's several papers were published which suggested a role for BH_4 in the mitochondria (Rembold and Buff, 1972a; Rembold and Buff, 1972b; Rembold 1975). It had previously been shown that the concentration of BH_4 in the mitochondrial matrix was similar to that of the cytochromes (Rembold and Metzger, 1967), and the administration of reduced pterins at physiological concentrations stimulated mitochondrial respiration. The utilization of oxygen being linearly proportional to the amount of BH_4 present. Thus, as BH_4 could readily reduce cytochrome c, a soluble electron transport system could be formed but this BH_4 catalysed respiration is not coupled to ATP production. However, these ideas about the role of BH_4 in the mitochondrion have been disputed as other workers have failed to repeat these results. Thus the role of BH_4 in the mitochondrion remains unclear.

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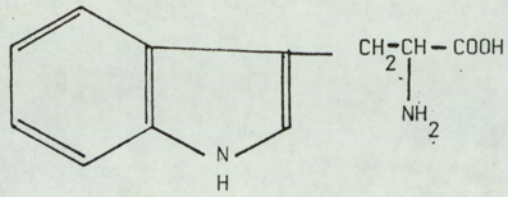
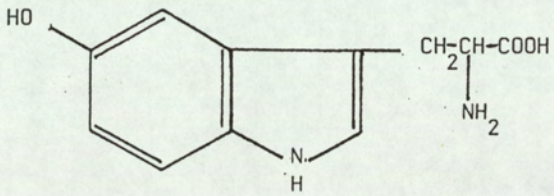


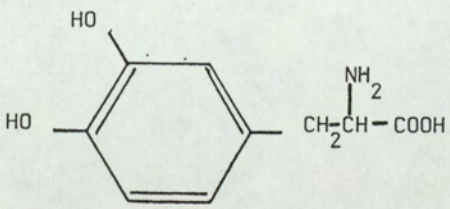
TRYPTOPHAN

(12)



5-HYDROXYTRYPTOPHAN

(13)



DOPA

More recently a "putative" indirect role for BH_4 in steroid biosynthesis has been proposed by Abou-Donia et al (1983). They found a significantly elevated level of BH_4 and an increased activity of guanosine triphosphate cyclohydrolase (GTP-CH) (the first and putative rate - limiting enzyme in BH_4 biosynthesis), in rat adrenal cortex after reserpine treatment, insulin-induced hypoglycemia or stress. Further work on hypophysectomized rats, which do not show the reserpine-dependent increase in GTP-CH activity and BH_4 content, implied the involvement of the anterior pituitary. This appears to be the case with adrenocorticotropic hormone (ACTH), acting as the messenger between the anterior pituitary and the adrenal cortex (Abou-Donia and Viveros, 1981; Viveros et al, 1981). Thus, as the enzymes known at present to require BH_4 as a cofactor are not present in the adrenal cortex, and as ACTH stimulates the production of the adrenal cortical steroids, the role that BH_4 plays is unclear. However, Abou-Donia et al (1983) believe it may have an indirect role in the adaptive changes of the gland after a period of intense stimulation.

Although the diet could potentially provide substantial amounts of pterins, experiments have shown that orally administered BH_4 is poorly absorbed from the gut (Rembold and Metzger, 1967; Blair et al, 1974). Biopterin however, is absorbed by the gut and retained and it is inferred that this is reduced during transport (Rembold and Metzger, 1967). Thus, the body's requirement for BH_4 could be

filled from the diet. BH_4 administered intravenously, however, cannot cross the blood brain barrier (Kettler et al, 1974). Since the brain is constantly active BH_4 will be required at constant levels for the production of the catecholamines and serotonin, implying an endogenous supply for BH_4. This was found to be so when Pabst and Rembold (1966) convincingly demonstrated that rats were capable of the de novo biosynthesis of BH_4, by feeding the rats on a biopterin free diet for several generations, and showing that the rats still produced biopterin in their urine. It is now known that BH_4 is synthesised from guanosine triphosphate (G.T.P.) (14) in mammalian cells (Sugiura and Goto, 1973; Fukushima and Shiota, 1974) and some details of the biosynthetic pathway have been elucidated (Gal et al, 1978; Brown et al, 1979; Lee et al, 1979; Tanaka et al, 1981). Figure **1.1** shows the most commonly accepted biosynthetic pathway in mammals, in which GTP is transformed by GTP cyclohydrolase to dihydroneopterin triphosphate (15) which is in turn converted to sepiapterin (16) via a unknown intermediate (X) (Tanaka et al, 1981). Sepiapterin is then thought to be converted by sepiapterin reductase to 7,8 BH_2, which is then reduced to BH_4 by the enzyme dihydrofolate reductase. Recently, however, an alternative pathway for BH_4 biosynthesis has been proposed by the workers at the Wellcome Research Laboratories, North Carolina, U.S.A. The research group, using the potent dihydrofolate reductase (DHFR) inhibitor methotrexate (17), have shown that de novo BH_4 biosynthesis

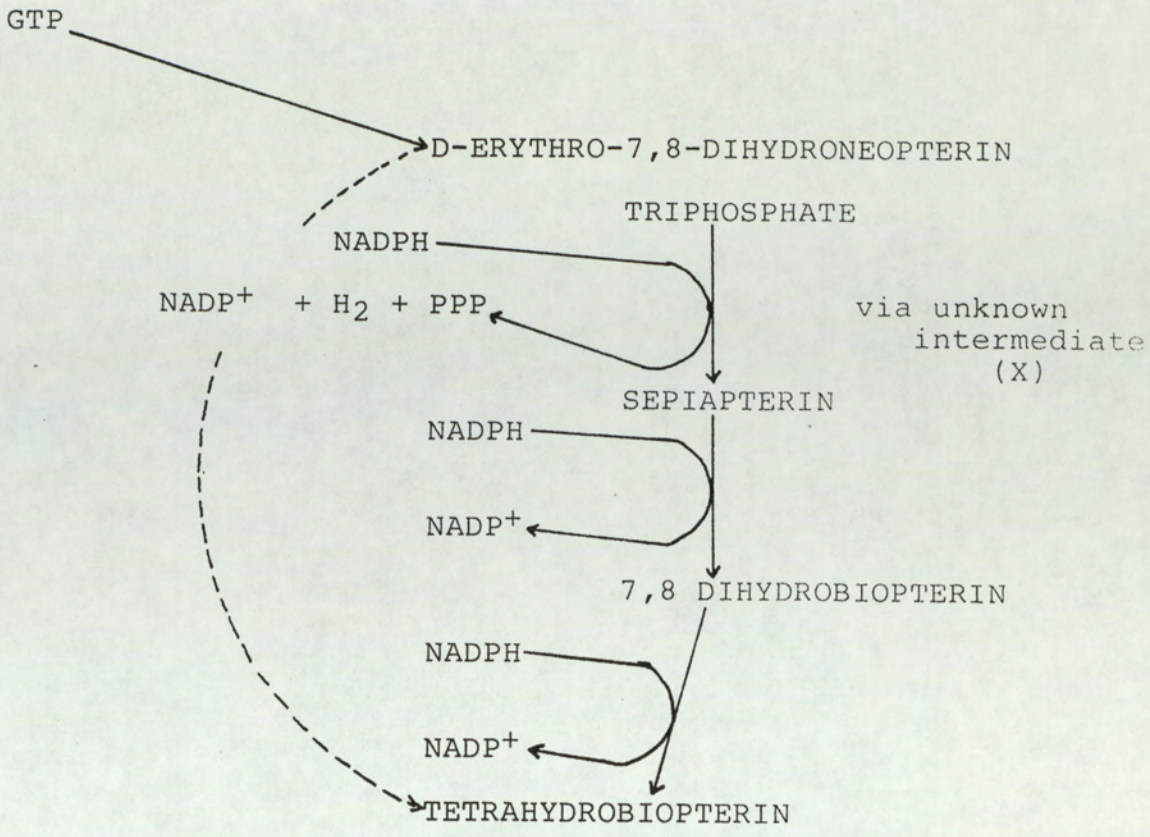


FIGURE 1.1

POSSIBLE BIOSYNTHETIC PATHWAY FOR TETRAHYDROBIOPTERIN (MAIN PATHWAY PROPOSED BY TANAKA ET AL (1981) : ALTERNATIVE DOTTED PATHWAY SUGGESTED BY THE WELLCOME RESEARCH LABORATORIES (NICHOL ET AL., 1983).

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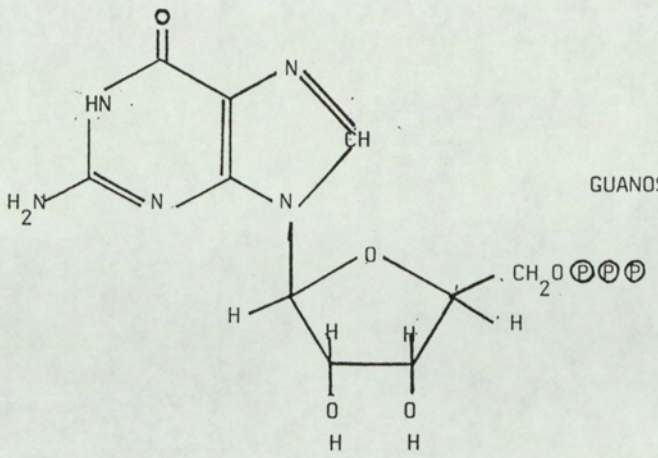
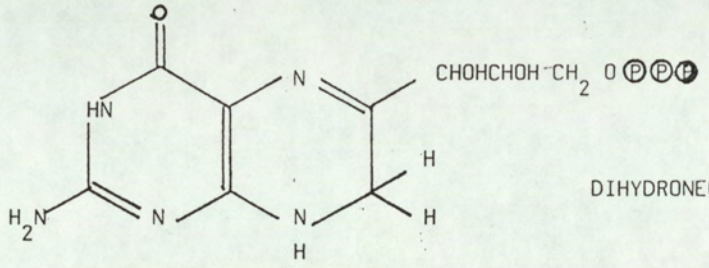


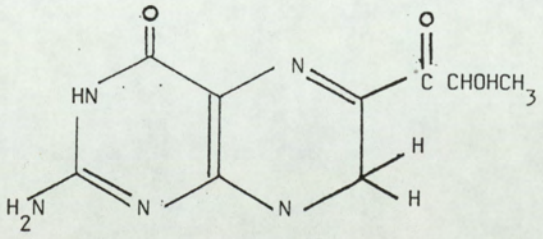
GUANOSINE TRIPHOSPHATE

(15)



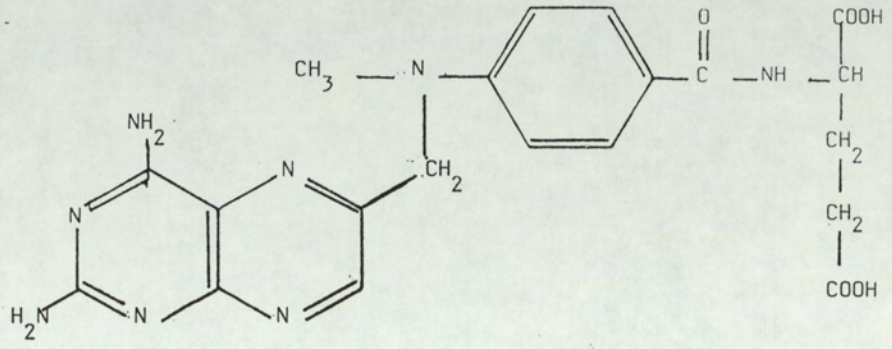
DIHYDRONEOPTERIN TRIPHOSPHATE

(16)



SEPIAPTERIN

(17)



METHOTREXATE

proceeds as normal even when the DHFR would be totally inhibited. Futhermore, their results suggest that the de novo synthesis of BH_4 is not only methotrexate - insensitive, but also that sepiapterin and 7,8 BH_2 do not act as intermediates on the de novo pathway from GTP (Nichol et al, 1982; Duch et al, 1982; Duch and Nichol, 1983; Nichol et al, 1983).

The major role for BH_4 in mammals is in the enzymic hydrolysis of the aromatic amino acids, the BH_4 being oxidised to quinonoid dihydrobiopterin (q BH_2). The salvage of the q BH_2 thus produced is achieved by the enzyme dihydropteridine reductase, and the remainder of this introduction is concerned with this enzyme.

Dihydropteridine reductase (DHPR NADH : quinonoid 6,7 dihydropteridine oxidoreductase, EC 1.6.99.7) was first reported as "sheep liver enzyme" (Kaufman 1958), which, with "rat liver enzyme" (phenylalanine-4-monoxygenase; EC 1.14.16.1), effected the enzymatic conversion of phenylalanine to tyrosine. This reaction required reduced nicotinamide adenine dinucleotide (NADH), according to the equation :-

phenylalanine + NADH + H^+ + O_2 -----> NAD^+ + tyrosine +H_2O.

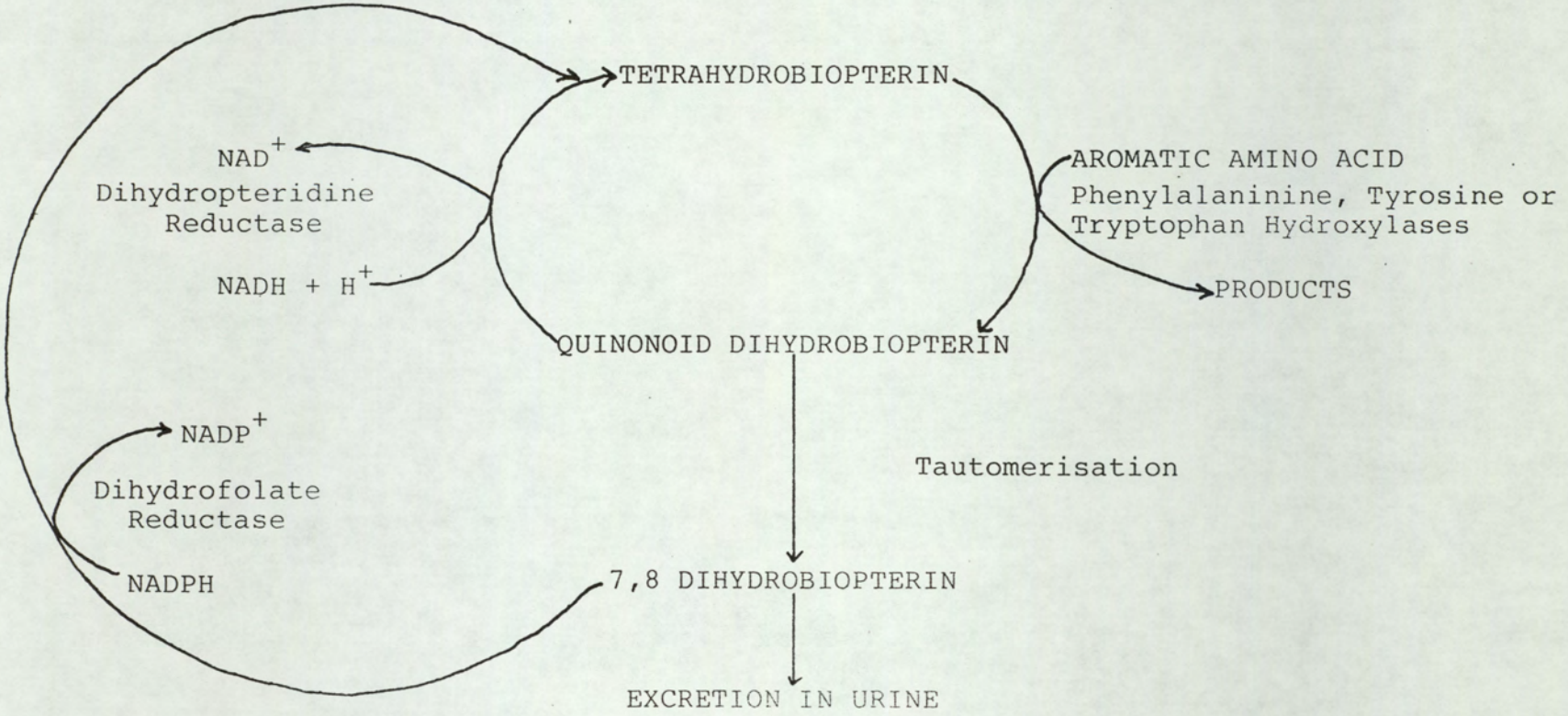
It was subsequently shown (Kaufman, 1963), that tetrahydrobiopterin (BH_4) was an essential cofactor for the hydroxylase, and that the "sheep liver enzyme" (DHPR), was responsible for maintaining the levels of BH_4 as the

phenylalanine hydroxylase (phenylalanine-4-monoxygenase) oxidises the BH_4 to quinonoid dihydrobiopterin (q BH_2). DHPR reduces the q BH_2 to BH_4 (See Scheme **1.1**). Thus, DHPR provides a means for the regeneration/salvage of BH_4, ensuring a constant supply of this essential cofactor for the hydroxylase (Craine et al, 1972). Should the q-BH_2 not be salvaged by DHPR it undergoes tautomerization to 7,8 dihydrobiopterin (7,8-BH_2) which is not a substrate for DHPR. As this cannot be converted to BH_4 by this route it may be lost to the cell by diffusion out into the serum, and eventually excreted in the urine (Leeming, 1979). 7,8-BH_2 is not automatically destined to be excreted as the enzyme dihydrofolate reductase (EC 1.5.1.3.) is capable of reducing the 7,8 BH_2 to BH_4 using reduced nicotinamide adenine dinucleotide phosphate (NADPH) (See Scheme **1.1**).

Subsequent research has shown that BH_4 is not only the natural cofactor for phenylalanine hydroxylase, but is also required by two other aromatic amino acid hydroxy-lases; tyrosine hydroxylase (tyrosine-3-monooxygenase EC 1.14.16.2) for the conversion tyrosine to dopa (Levitt et al, 1965; Nagatsu et al, 1972), and tryptophan hydroxylase (tryptophan 5-monooxygenase EC 1.14.16.4) for the conversion of tryptophan to 5-hydroxytryptophan (Hosoda and Glick, 1966). This connection between BH_4 and tyrosine and tryptophan hydroxylases is interesting as these two enzymes catalyse the initial rate limiting steps to the biosynthesis of the catecholamines and serotonin

SCHEME 1.1

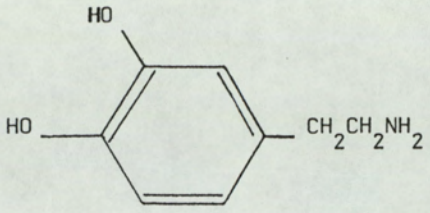
PATHWAYS FOR THE SALVAGE OF TETRAHYDROBIOPTERIN

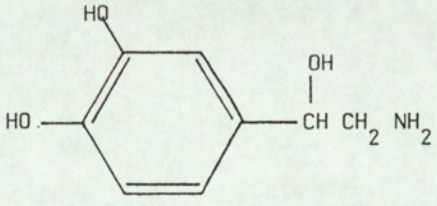


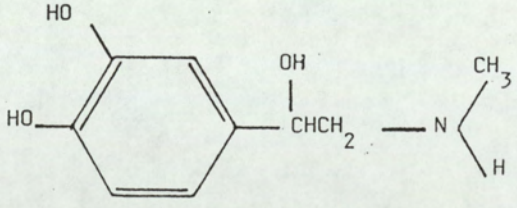
respectively.

Tyrosine hydroxylase is the rate limiting enzyme in the production of the catecholamine neurotransmitters dopamine **(18)**, noradrenalin **(19)** and adrenalin **(20)** (Nagatsu et al, 1964). The actual rate limiting factor being the concentration of BH_4, rather than the levels of the enzyme, as BH_4 is present in tissues at concentrations lower than its "normal" Km value for tyrosine hydroxylase (Kettler et al, 1974). Tyrosine hydroxylase, however, can be activated by phosphorylation through a cyclic adenosine monophosphate (c AMP) protein kinase system, the activated enzyme having a lowered Km for BH_4 (Lovenberg et al, 1975; Pollock et al, 1981). The activation of tyrosine hydroxylase by a c AMP-dependent phosphorylation process has led to the proposal that this process can be responsible for the in vivo activation of tyrosine hydroxylase resulting from electrical stimulation of neurons (Murrin et al, 1976; Roth and Salzman 1977; Weiner et al, 1981). However, El Mestikawy et al (1983), suggest that there is a calcium (Ca^{2+}) dependent phosphorylation process that causes tyrosine hydroxylase activation in dopaminergic terminals.

The rate limiting step for serotonin biosynthesis is catalysed by tryptophan hydroxylase (Costa and Meek, 1974). In this case not only is BH_4 concentration rate limiting, but also the concentration of the substrate tryptophan plays a rate limiting role (Jequier et al, 1967). As with

(18)  DOPAMINE

(19)  NORADRENALIN

(20)  ADRENALIN

tyrosine hydroxylase, tryptophan hydroxylase can be activated; when serotoninergic neurones are depolarized the enzyme's activity is increased and recent evidence suggests that this is a calcium (Ca^{2+}) dependent phosphorylation process which causes enzyme activation (Hamon et al, 1979). Thus, the production of the catecholaminergic neurotransmitters and serotonin are under complex fine control. Yet, at the simplest level, it is the level of BH_4 which controls the production of these neurotransmitters. This is one reason why DHPR initially attracted interest for study.

DHPR has been isolated from several mammalian tissues; sheep liver (Craine et al, 1972; Cheema et al, 1973; Webber et al, 1978) and brain (Cheema et al, 1973) bovine liver (Aksnes et al, 1979; Aksnes and Ljones, 1980), adrenal medulla (Cheema et al, 1973; Aksnes et al, 1979) kidney (Chauvin et al, 1979) and brain (Snady and Musacchio, 1978), and rat liver (Webber et al, 1978; Brown 1981). DHPR activity has also been isolated from a species of Pseudomonas (Williams et al, 1976) and from the algal flagellate Crithidia fasciculata (Hirayama et al, 1980). There has been increasing interest in human DHPR and it has also been isolated from the liver (Craine et al, 1972; Cotton and Jennings, 1978; Firgaira et al, 1979; Firgaira et al, 1981; Nakanisi et al, 1982; Shen, 1983). DHPR activity has also been measured in cultured human cells (Firgaira et al, 1979). The DHPR from these diverse

sources show a quite remarkably similar structure the enzyme being a dimer of molecular weight in the range 42,000-52,000 daltons. These DHPRs in general demonstrate a strong preference for NADH as a cofactor, although an NADPH as well as an NADH requiring reductase has been isolated from beef liver (Nakanishi et al, 1977).

Results from various groups (Cotton and Jennings, 1978; Aksnes and Ljones, 1980), indicate that DHPR may follow a compulsory order mechanism in which NADH has to bind to the enzyme, before the q BH_2 can be bound. This sort of mechanism has been reported for several NADH - dependant dehydrogenases and reductases, such as horse liver alcohol dehydrogenase and lactate dehydrogenase (Dalziel, 1975; Dixon and Webb, 1979).

There have been several reports on the amino acid residues thought to be involved in DHPRs mechanism. This work has taken the form of using reagents which bind with a high degree of specificity to amino acid side chains and then observing the effects on the activity of the enzyme. In this way the evidence for thiol group involvement has been strongly implied in sheep liver, rat liver and human liver DHPRs (Cheema et al, 1973, Webber et al, 1978; Webber and Whiteley, 1981; Firgaira et al, 1981). Firgaira et al (1981), suggest that human liver DHPR activity is dependent on accessible thiol groups. Two groups, however, have reported no inhibition of rat and bovine liver DHPR by

thiol blocking agents (Craine et al, 1972; Aksnes et al, 1979), suggesting that cystine is not a part of the active site. Another amino acid residue which may play an important role in human liver DHPR activity is arginine. Firgaira et al, (1981) using the arginine specific reagent butane-2,3-dione, observed a 90% inhibition of enzyme activity at 10mM. Further experi-mentation showed that preincubation of the enzyme with NADH completely protected the enzyme from inactivation suggesting a role for arginine in the NADH binding site. This is in general agreement with reports that several dehydrogenase have arginine in the nucleotide-binding sites. (Lange et al, 1974; Bleile et al, 1975; Nagradorva and Asryants, 1975).

Most of the work on the amino acid residues of various species DHPR's has focussed on the actual numbers of each amino acid residue present in the monomeric subunit of DHPR. The results of some of these investigations are given in table 1.1. These results show that DHPR amino acid composition is very similar across these species.

In the reaction catalysed by the aromatic amino acid hydroxylases a BH_4 derived intermediate is formed which is believed to be a 4a-hydroxy adduct (21) (Kaufman, 1975). The existence of a 4a adduct is now widely accepted, but there is still much active research aimed at ascertaining the actual structure of this adduct (Bailey et al, 1982; Ayling and Bailey, 1983; Lazarus et al, 1983). This adduct

TABLE 1.1

AMINO ACID COMPOSITION OF DIHYDROPTERIDINE REDUCTASE FROM VARIOUS SOURCES

(number of residues for a subunit of molecular weight of 25,000).

Residue	Human Liver (1)	Rat Liver (2)	Bovine Liver (3)	(4)	Sheep Liver (5)	(6)
Ala	31	29	26	32	30	32
Arg	9	8	8	9	8	10
Asp	16	19	17	15	18	19
Cys	-	-	-	-	1	-
1/2 Cys	-	4	9	1	-	3
S-Cm-Cys	3	-	-	-	1	-
Glu	22	20	23	21	26	26
Gly	26	26	20	22	26	24
His	5	5	5	4	5	5
Ile	8	9	7	6	6	7
Leu	21	23	21	22	20	25
Lys	14	16	14	14	15	14
Met	4	8	5	5	4	5
Phe	7	6	6	6	6	7
Pro	9	9	10	9	10	9
Ser	18	20	18	22	21	20
Thr	17	16	15	18	15	18
Trp	5	4	3	8	2	9
Tyr	3	3	3	2	2	3
Val	17	18	16	17	17	17

(1) Firgaira et al, (1981)

(2) Webber and Whiteley, (1981)

(3) Korri et al, (1977)

(4) Hasegawa (1977)

(5) Craine et al, (1972)

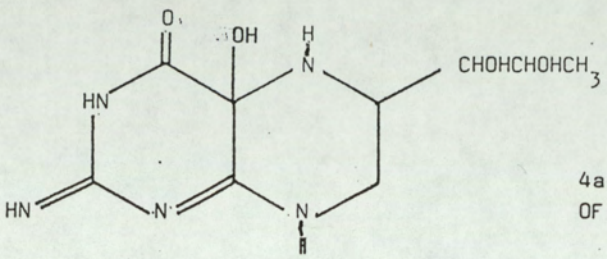
(6) Cheema et al, (1973)

is rapidly dehydrated to q BH_2 the substrate for DHPR. The structure of this is still a matter of some controversy, but evidence strongly supports the structure shown (**4**) (Armarego and Waring, 1983). DHPR reduces the q BH_2 using a hydride ion (H^-) and a proton (H^+), one of which adds to N5 (as 5,6,7,8-tetrahydrobiopterin is formed), while the other ion may add to the extracyclic N atom or to N_3. In a recent paper (Armarego and Waring, 1983), it is suggested that the hydride ion from NADH is transfered to N5 whilst the proton adds to N3 of the q BH_2.

The structure of these various intermediates are thus still a source of active research, but with the increasing ability to produce more stable mono and disubstituted tetrahydropterins (Bailey and Ayling, 1983) it would seem only a matter of time before these structures are elucidated.

One of the stimuli that prompted recent interest in DHPR and BH_4 metabolism, followed the report by Smith, Clayton and Wolff (1975), of a variant form of phenylketonuria (PKU), in which the sufferers did not respond to the usual regime of lowered phenylalanine intake. The prognosis was very poor, there was a progressive neurological deterioration which led to hypotonia, and death ensued from the consequent respiratory complications. In classical PKU there is a gross deficiency of phenylalanine hydroxylase (Jervis, 1947), which causes hyperphenylalaninaemia and, if

(21)



4a HYDROXY ADDUCT
OF TETRAHYDROBIOPTERIN

these high serum phenylalanine levels are left uncontrolled the sufferer has severe mental retardation (Folling, 1934). The variant form of PKU reported by Smith et al (1975), was due to an inherited defect in BH_4 metabolism. Such cases are now recognised as having malignant hyperphenylalaninaemia (Danks, 1978; Danks et al, 1978).

Clinical and laboratory studies of the few patients recognised as having malignant hyperphenylalaninaemia (MHPA) have described two main defects in BH_4 metabolism. The most common of these being a failure to salvage q BH_2 by DHPR (Kaufman et al, 1975; Milstein et al, 1976; Grobe et al, 1978; Danks et al, 1978). Several patients have been shown to be deficient in the de novo biosynthesis of BH_4 (Leeming et al, 1976; Rey et al, 1976; Kaufman et al, 1978; Niederwieser et al, 1983). Differentiation between these two forms of MHPA is possible as in DHPR deficiency there is an elevation of 7,8-BH_2 in serum due to the rearrangement of BH_2 to 7,8 BH_2 and its subsequent loss into the serum. Whilst in the impaired synthesis form of MHPA the low levels of BH_4 formed (if any), give rise to low levels of 7,8 BH_2 in the serum.

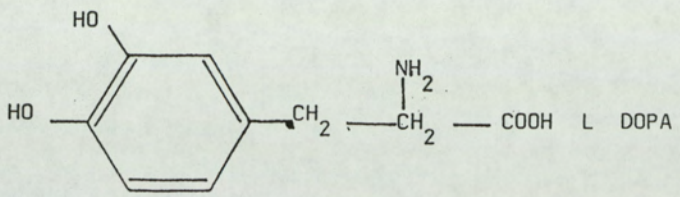
The assay of tissue samples for DHPR activity can distinguish between DHPR deficiency or de novo biosynthesis deficiency. To this end DHPR activity has been measured in cultured skin fibroblasts (Kaufman et al, 1975; Milstein et al, 1976; Grobe et al, 1978; Firgaira et al, 1979),

cultured lymphoid cells (Firgaira et al, 1979), liver biopsies (Kaufman et al, 1975; Brewster et al, 1979) and perphferal leucocytes (Narisawa et al, 1980). These procedures, however, are time consuming and some involve risks to the patient. On the other hand the successful assay of DHPR activity from the eluate from Guthrie card blood spots (Leeming et al, 1984) provides an easy, cheap and quick method for the rapid assessment of possible DHPR deficiency.

There have also been reported "partial" defects in de novo biosynthesis and in BH_4 salvage. A severe reduction in de novo biosynthesis may appear as a transient hyperphenylalaninaemia until it is stressed by a high phenylalanine load (Rey et al, 1980). A reduced level of DHPR giving only mild hyperphenylalaninaemia, however, gives rise to clear neurological symptoms (Brewster et al, 1979). These two partial forms seem to indicate that, provided an efficient salvage system is present, minimal synthesis of BH_4 is sufficient.

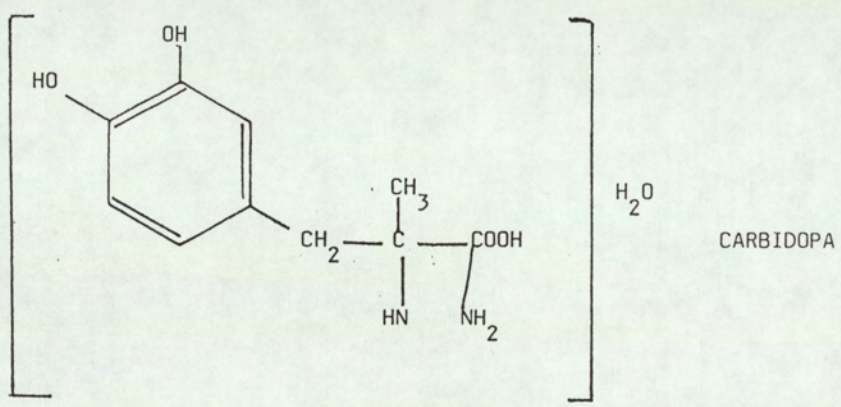
Treatment of both forms of MHPA is usually the administration of L-dopa **(22)** carbidopa, **(23)** and 5-hydroxytryptopan **(12)** in conjunction with a low phenylalanine diet (Bartholome and Byrd, 1975). This regime halts the progressive neurological deterioration and near normal development is observed. BH_4 therapy can elevate liver aromatic amino acid hydroxylase activity and, in massive

(22)



L DOPA

(23)



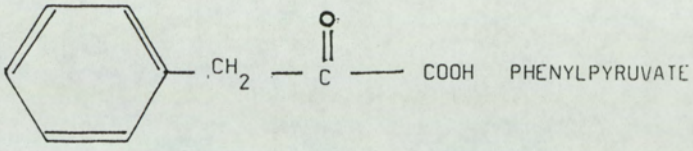
CARBIDOPA

doses, BH_4 therapy alone can alleviate all the central nervous system - related symptoms (Niederwieser et al, 1982; Kaufman et al, 1982). However, the prohibitive cost of BH_4 at the moment means the majority of treatment is through neurotransmitter precursor dosing.

The genetic disorder malignant hyperphenylalaninaemia clearly demonstrates the essential role of BH_4 in neurotransmitter biosynthesis, and strongly supports the hypothesis that any agent which effects biopterin metabolism may cause mental impairment (Leeming et al, 1981).

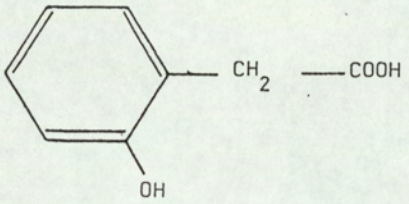
The connection between the mental retardation observed in untreated classical (PKU), and biopterin metabolism is not so clear as that observed in MHPA. The evidence for a connection, however, seems quite strong; initially it was noticed that in untreated phenylketonurics the serum biopterin levels were highly significantly elevated compared to normal controls (Leeming et al, 1976a; Kaufman et al, 1978). It was also noticed that serum dihydrobiopterin levels paralleled the serum phenylalanine levels after an oral load of phenylalanine (Leeming et al, 1976a), suggesting that either phenylalanine itself or a metabolite was effecting the DHPR salvage system. Untreated phenylketonurics excrete large amounts of unusual phenylalanine metabolites, such as phenypyruvate (24) and o-hydroxyphenylacetic acid (25) (Blastovics and Nelson,

(24)



PHENYLPYRUVATE

(25)



O-HYDROXYPHENYLACETIC ACID

1971). Phenylalanine itself has little effect on biopterin metabolism *in vitro*, but phenylpyruvate inhibits DHPR with a k_i of 5×10^{-5}M (Purdy and Blair, 1980), which effectively gives 20% inhibition of normal DHPR activity at 5.4×10^{-5}M (heterozygotes with only low levels of DHPR would have zero DHPR activity). This concentration is similar to those recorded in the serum of phenylketonuric patients, even those on controlled diets (Jervis and Drejza, 1966; Langenbeck *et al*, 1980). (In normal controls the plasma phenylpyruvate concentration is about $5\text{-}8 \times 10^{-6}$M (Langenbeck *et al*, 1980). Thus, if phenylpyruvate were to reach similar concentrations in the tissues which require fairly constant levels of BH_4 such as catecholaminergic/serotoninergic neurons, the resultant inhibition of DHPR would decrease tissue levels of BH_4 and give rise to an elevation of serum BH_2. Neurotransmitter biosynthesis would then be disrupted in a similar manner to that surmised in the DHPR deficient form of MHPA, and could be responsible for the mental deficit observed in untreated PKU.

There have been several other explanations proposed for the mental retardation observed in PKU (for reviews see Kaufman, 1976; Youdim, (1979), but none of these theories explain the effects on serum BH_2.

Noradrenaline, dopamine, adrenaline and serotonin (monoamines), have been described as neuromodulators due to

their generalised effects on brain metabolism. They have diffuse inervations and imping on very large terminal fields. Estimates suggest that less than 10% of the terminal release sites of serotonin or noradrenalin terminals in the brain make morphologically specialised synaptic contacts with target cells (Beaudet and Descarries, 1978). Thus, their release can be envisioned as a diffuse cloud of neurotransmitter, and it is generally accepted that these compounds have modulatory functions of various types (Iversen, 1982). Any depressive influences on BH_4 levels may therefore have detrimental effects on the whole brain metabolism.

Investigations into several neurological disorders have implied the participation of disturbances in biopterin metabolism. Many more disorders are associated with disturbances in monoamine levels, which may themselves be due to a defective biopterin metabolism.

One of the neurological disorders for which a disturbance of biopterin metabolism has been shown is senile dementia of Alzheimer Type (SDAT) (Aziz et al, 1983; Morar et al, 1983). Most cases of dementia are associated with degenerative disease of the CNS. Post mortem studies of geriatric dements have attributed about 70% of cases to SDAT, either alone or in combination with vascular disease (Tomlinson et al, 1970). Diagnosis of SDAT is usually comfirmed at post mortem by the widespread distribution of

senile plaques and neurofibrillary tangles thoughout the cerebral cortex. In SDAT there is a depression of serum BH_2 levels (Leeming et al, 1979; Leeming and Blair, 1980; Young et al, 1982), brain BH_4 levels are lower (Nagatsu et al, 1979) and CSF biopterin levels are lowered (Morar et al, 1983). The partial failure of the phenylalanine hydroxylating system is indicated by a slight rise in the serum phenylalanine to tyrosine ratio (Leeming et al, 1979). The lowered concentration of BH_4 in the brain might be expected to lead to a depression of monoamine synthesis, and there is evidence for such a depression of brain noradrenaline levels (Adolfsson et al, 1979; Berger et al, 1980). It has also been reported that there is a large loss of cells of the locus coeruleus, a nucleus in the dorsal brainstem from which noradrenergic fibres emerge to enervate the cortex (Bondareff et al, 1981). Serotonin levels are also reduced in post mortem SDAT hippocampus (Winblad et al, 1982) and its uptake is reduced in temporal cortical biopsy samples (Benton et al, 1982). Dopamine is also reduced in some subcortical areas (Winblad et al, 1982).

The majority of research on SDAT has been aimed at showing a specific cholinergic deficiency as the outstanding pathological finding, similar to the dopamine deficiency associated with Parkinsonism. Such evidence is quite convincing : a decrease in choline acetyltransferase (ChAT), associated with damage to the ascending cholinergic

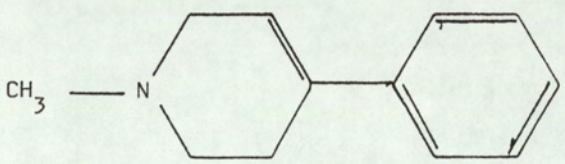
projection to the cortex, is the most commonly found abnomality in SDAT brains (Rossor, 1982). Also there is a correlation between cortical senile plaques and the reduction of ChAT and the degree of dementia at death and the reduction of ChAT, such correlations have yet to be shown with other markers. However, the increasing evidence for a possible BH_4 deficiency in SDAT, "... brings to the field of work on sanile dementia of Alzheimer Type a new dimension to consider and contend with" (Aziz, Leeming and Blair (1983).

SDAT is not the only neurological disease for which a reduction of CSF levels of BH_4 has been reported. Others include Parkinson's disease (Lovenberg et al, 1979; Levine et al, 1979), Huntingdon's Chorea, Steel-Richardson Syndrome (Williams et al, 1980) and dystonia (Williams et al, 1979; Le Witt et al, 1983). These are all due to some impairment of neurotransmitter functioning, Parkinsonism and dystonia being associated with decreased monoamine function. Several groups have reported the effect of high doses of BH_4 on patients with Parkinson's disease (Narabayashi et al, 1982; Curtius et al, 1982; Le Witt et al, 1983). In general there is a slight improvement of patients with moderate symptons. The effects however, are not so pronounced in longstanding Parkinsonian patients. Thus, for the newly diagnosed patient, BH_4 therapy may provide an alternative to the compensation therapy (levodopa dosing) used now. BH_4 therapy would also be

extremely helpful to patients who suffer side effects whilst on levodopa therapy.

Calne and Langston (1983), suggested that Parkinson's disease may be caused by the influence of a possibly toxic environmental factor on the normal slow sustained neuronal loss associated with aging. There is strong evidence that 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) (26) is one such causative agent in both monkeys and Man (Burns et al, 1983; Langston et al, 1983; Langston et al, 1984). This work has at least provided an animal model of Parkinson's disease, and could be the start of a new approach to ascertaining the cause of Parkinson's disease. It is interesting to note that this possible causative agent for Parkinsonionism provides a direct link with biopterin metabolism, as MPTP is an inhibitor of DHPR (Blair et al, 1984). This inhibition would, by feedback activation of the biosynthetic pathway, tend to increase cellular BH_4 levels, which can rapidly react with oxygen to give hydrogen peroxide and the extremely cytotoxic superoxide anion (Blair and Pearson, 1974). Thus, this inhibition could lead to increased neuronal loss and hence exacerbate the situation.

Le Witt et al (1983) have reported the improvement of patients with dystonia following large doses of (6R,S) 5,6,7,8 tetrahydro-2-biopterin. Since some patients with dystonia respond to dopaminomimetic agents, a biopterin

(26)  1-METHYL-4-PHENYL-1,2,5,6-TETRAHYDROPYRIDINE

deficiency may play a role in the pathophysiology of dystonia.

There is strong evidence that disturbances of central monoamine metabolism in depression are causal (Van Praag, 1982). Patients with depression have responded well to BH_4 therapy (Curtius et al, 1982; Curtius et al, 1983). However, Kellner et al (1983) found no disturbance in biopterin levels in the CSF of depressed patients. A possible disturbance in BH_4 metabolism is therefore unproven at present.

In other diseased states there appears to be a disturbed biopterin metabolism. For example in coeliac disease the low serum BH_2 levels observed (Leeming et al, 1976a; Leeming and Blair, 1980), return to normal during treatment, implying that the decrease is as a consequence of the disease process (Cooke, 1976). Low serum BH_2 is also observed in malignant disease, where the ratio of neopterin to biopterin in the urine is increased. This suggests either a block in BH_4 biosynthesis after the production of neopterin or increased neopterin production or possibly both of these (increased neopterin production as a feedback effect of decreased BH_4 biosynthesis).

Lead poisoning causes a disturbance in BH_4 metabolism, not only by significantly inhibiting BH_4 biosynthesis, but also by significantly inhibiting DHPR activity (Purdy et al,

1981), the inhibition of synthesis occurring at lower lead levels than the inhibition of DHPR. The lowered cellular levels of BH_4 may thus play a contributory role in the neurotoxicity of lead and, in lead dosed rats 300 and 1,000 ppm lead causes inhibition of DHPR in brain tissue (Barford *et al*, 1983). Since DHPR activity in whole blood from workers in the lead industry is raised, when compared to normal males, this increase in activity may be in response to the inhibition of *de novo* BH_4 synthesis (Barford *et al*, 1983). The difference between rat brain and human serum DHPR levels may be due to the fact that, although the rats developed in a high lead environment, they were not exposed to high lead levels in adult life. Leeming and Blair (1980), found low serum BH_2 levels in subjects with lead poisoning, suggesting that the major effect *in vivo* is the inhibition of BH_4 synthesis.

Elevated levels of aluminium have been found in the brains of patients dying with SDAT (Crapper *et al*, 1973). It was associated with the neurofibrillary tangles characteristic of Alzheimers disease (Perl and Brody, 1981). The neurofibrillary degeneration caused by the aluminium in rats, however, is not identical to that observed in SDAT (Bulgiani and Ghetti, 1982). Aluminium is now thought to be a causal agent in a form of dementia, namely that suffered by patients undergoing dialysis, dialysis dementia (Alfrey *et al*, 1976; McDermott *et al*, 1978; Arieff *et al*, 1979). Aluminium can inhibit DHPR *in vitro* at

concentrations close to those found in the brains of patients with dialysis dementia (Leeming, 1979; Brown, 1981), and this inhibition of DHPR has been proposed as a mechanism of aluminium neurotoxicity (Leeming and Blair, 1979). In chronic uraemic patients, both on maintenance dialysis and on aluminium hydroxide gel medication, serum neopterin and biopterin levels were significantly higher than in control patients (Dhondt et al, 1982), suggesting an attempt by the biosynthetic pathway to maintain BH_4 levels. Thus an impairment of BH_4 metabolism may play some role in the disturbed neurological function observed in dialysis dementia. Additional factors cannot, however be ruled out.

Leeming and Blair (1980), noted that serum biopterin concentrations fall in the menstrual cycle when oestrogen levels rise. Changes in BH_4 metabolism may thus be related to the performance changes observed during the menstrual cycle. The factor changing appears to be DHPR activity, which is increased in women compared to men, and is further increased in women on the contraceptive pill. In pregnant women the activity is increased still further (Barford et al, 1983). Thus, the physiological stimuli of increasing oestrogen levels increases DHPR activity, and in human breast tumour tissue Dhondt et al (1981) have shown a positive correlation between DHPR activity and oestrogen receptor levels. It is reasonable therefore, that high oestrogen levels will increase DHPR activity in sensitive

tissues. As DHPR is believed to play a role in maintaining cellular tetrahydrofolate levels (Pollock and Kaufman, 1978), and as oestrogens cause proliferation in certain target tissues, it may be that DHPR is helping to facilitate the cellular proliferation, by altering the folate metabolism of the cell.

A further possible association between oestrogens and BH_4 is observed in a form of chorea occurring as a rare complication of oral contraceptive medication (Bickerstaff and Holmes, 1967; Malcolm, 1971; Pulsinelli and Hammil, 1978; Nausieda et al., 1979). The evidence suggests the chorea arises from altered central dopaminergic activity.

The studies reported in this thesis are on both rat and human DHPR purified by affinty chromatography (Cotton and Jennings). These isolated enzymes have been used in kinetic studies to examine the effect of molecules and ions thought to effect biopterin metabolism in vivo. These studies will provide information about tetrahydrobiopterin metabolism and its control, which will be used to try to explain changes observed in diseased states. Further work on human tumour and control tissue will provide new information on DHPR activity in the neoplastic state, and hence BH_4 metabolism. It is hoped to provide an insight into the possible alterations in BH_4 metabolism, which have been previously reported, by measurement of DHPR activity in human brain samples.

CHAPTER TWO

METHODS AND MATERIALS

MATERIALS AND METHODS

MATERIALS

(i) Chemicals :

6,7-dimethyl-5,6,7,8-tetrahydropterin, 2-hydroxyoestrone, 2-hydroxyoestradiol, oestradiol, oestrone, 17 ethynyl-oestradiol, diethylstilboestrol, B-nicotinamide adenine dinucleotide, reduced form, nicotinamide adenine dinucleotide phosphate, reduced form, cyanamide (carbodiimide; hydrogen cyanamide), 2,6-dichlorophenolindophenol, Tris (tris [hydroxymethyl] aminomethane), horseradish peroxidase and phenylpyruvate were purchased from Sigma, London Chemical Co., Poole, Dorset U.K. A-H Sepharose 4 B and Sephadex G150 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium 1,2-naphthoquinone 4-sulphonate was purchased from Cambrian Chemicals, Croydon, CRO 4XB, U.K. Hydrogen peroxide was obtained from Fisons Scientific, Loughborough LE11 ORG, U.K. All other chemicals and reagents were of Analar grade or its equivalent. Initial samples of the catecholoestrogens were a gift from Professor R. Knuppen, Lubeck Institut fur Biochemische Endokrinology, Lubeck, West Germany.

(ii) Human Tissue Samples :

Human tumour samples were provided by Mr. G. Oates, The General Hospital, Steelhouse Lane, Birmingham, B4 6NH. Human brain temporal lobes and other brain tissue were kindly supplied by Dr. P.E. Sylvester, St. Lawrence's Hospital, Caterham, Surrey, CR3 SYA. Further temporal lobe samples were supplied by the Brain Tissue Bank, MRC Neurochemical Pharmocology Unit, Addenbrooke's Hospital, Cambridge.

(iii) Rat Tissues :

Rats used for the isolation of DHPR were Wistars supplied by Bantam and Kingman Limited, The Field Station, Grimstone, Aldbrough, Hull, HU11 4QE. Male rats were used between 90-120 gm.

Female rats used in oestrogen/diethylstilbesterol dosing were WOB/**Not** supplied by Dr. M. Pymm, Cancer Research Laboratories, The University of Nottingham, Nottingham. These were between 80-100 gm in weight at the initial dosings.

Rat brains after perinatal dosing with lead in drinking water (Carmichael et al, 1981), were a gift from Mr. C. Winder, Royal Postgraduate Medical School, London, U.K.

METHODS

Preparation of tissue extracts :

Rat liver extracts were prepared by two methods, in both methods the liver of a rat was removed and washed in ice cold 0.1M Tris/HCl buffer pH7.6, a 20% homogenate (w/v) was prepared in the same buffer.

Method 1 : The homogenate was centrifuged at 25,000xg for 1 hour in an MSE Superspeed 50 centrifuge. The supernatant obtained was filtered through muslin to remove lipids, the protein content measured by the Biuret method, and DHPR activity was measured using a modified method of Craine et al, (1972). The supernatant was then applied to a sephadex G 150 column (volume 40ml), at 0-2°C, which had been equilibrated with 0.05M Tris/HCl, buffer, pH7.6. The column was eluted with the same buffer, 5ml fractions were collected and assayed for protein content (Lowry et al, 1951), and DHPR activity. The most active fractions were pooled and made 0.8M NaCl, 0.1mM EDTA and 0.1mM NADH, in preparation for affinity chromatography.

Method 2 : The homogenate was centrifuged at 104,000xg for 45mn in a MSE Superspeed 50 centrifuge. The supernatant was filtered through muslin, protein content measured by the Biuret method, and DHPR activity was measured by the modified method of Craine et al, (1972). 10mls of the

supernatant were made 0.1mM NADH, 0.1mM EDTA and 0.8M Na Cl for direct application to the affinity column. The remaining supernatant was stored frozen (-20°C), in aliquots.

Measurement of Protein Concentration

The protein content of the supernatant and elutants from columns were measured by the Biuret and Lowry methods, against standard curves constructed using Bovine Serum Albumin (BSA).

Affinity Chromatography

The affinity chromatographic method of Cotton and Jennings (1978) was used to purified DHPR. The affinity gel used was AH Sepharose 4B, to which the ligand, sodium-1,2-naphthoquinone 4-sulphonic acid, was attached using the manufacturers recommendations. The gel, when prepared, was a dark red-brown colour in 0.05M Tris/HCl, pH7.6, in which it was stored between 0-4°C until required.

When isolating DHPR, 10mls of the affinity adsobent were degassed, packed into a jacketed column, and cooled with ice water. The gel was equilibrated with 0.05M Tris/HCL buffer, pH7.6, 20% glycerol, containing 0.8M Na Cl and

0.1mM EDTA (Buffer A). The tissue preparation was then run onto the column, which was eluted with the following buffers :

(i) 30-40mls buffer A + 0.1mM NADH.

(ii) 20mls 0.1M Na_2CO_3/NaOH buffer, pH 10.9, M NaCl, (buffer B).

(iii) 15ml buffer A.

(iv) 20mls buffer B.

3-4ml fractions were collected, the pH of each fraction being checked and adjusted to pH7.6 using M HCl if necessary. The protein content (Lowry et al, 1951), and DHPR activity (Craine et al, 1972), were measured, the most active fractions were pooled and made 2mM dithiothreitol and 0.02mM NADH before being stored frozen, in aliquots.

The affinity adsorbent was regenerated by washing the column with 20ml of 0.1M sodium acetate buffer, pH 4, containing M Na Cl, followed by 15ml of buffer A, the gel was then stored in 0.05M Tris/HCl, pH7.6, containing 0.2M Na Cl, below 4°C in the dark. After 3 or 4 uses the gel could be regenerated using a dithiothreitol treatment; the column was washed with buffer A + 2mM dithiothreitol for

16h followed by buffer A until the red-brown colour reappeared.

A typical elution profile for the affinity chromatography of rat liver DHPR is given in figure **2.1** Rat liver enzyme was purified up to 960 fold using the tissue preparation outlined in method **2** (see above), with recoveries between 10-20%.

Preparation of Human Brain Tissue :

Human brain tissue was prepared by method **2** described above, the processing taking place in a Bassaire biological safety cabinet. A portion of temporal lobe cortex was used for the isolation of human DHPR by the affinity chromatographic method.

Assays for Dihydropteridine Reductase :

Two assay methods were used, both utilizing $DMPH_4$ as the pterin substrate source, the actual method of generating the quinonoid dihydropterin being different. The method of Craine *et al*, uses horseradish peroxidase and hydrogen peroxide as the generating system, whilst the method of Cheema *et al* uses 2,4-dichlorophenolindophenol (DCPIP).

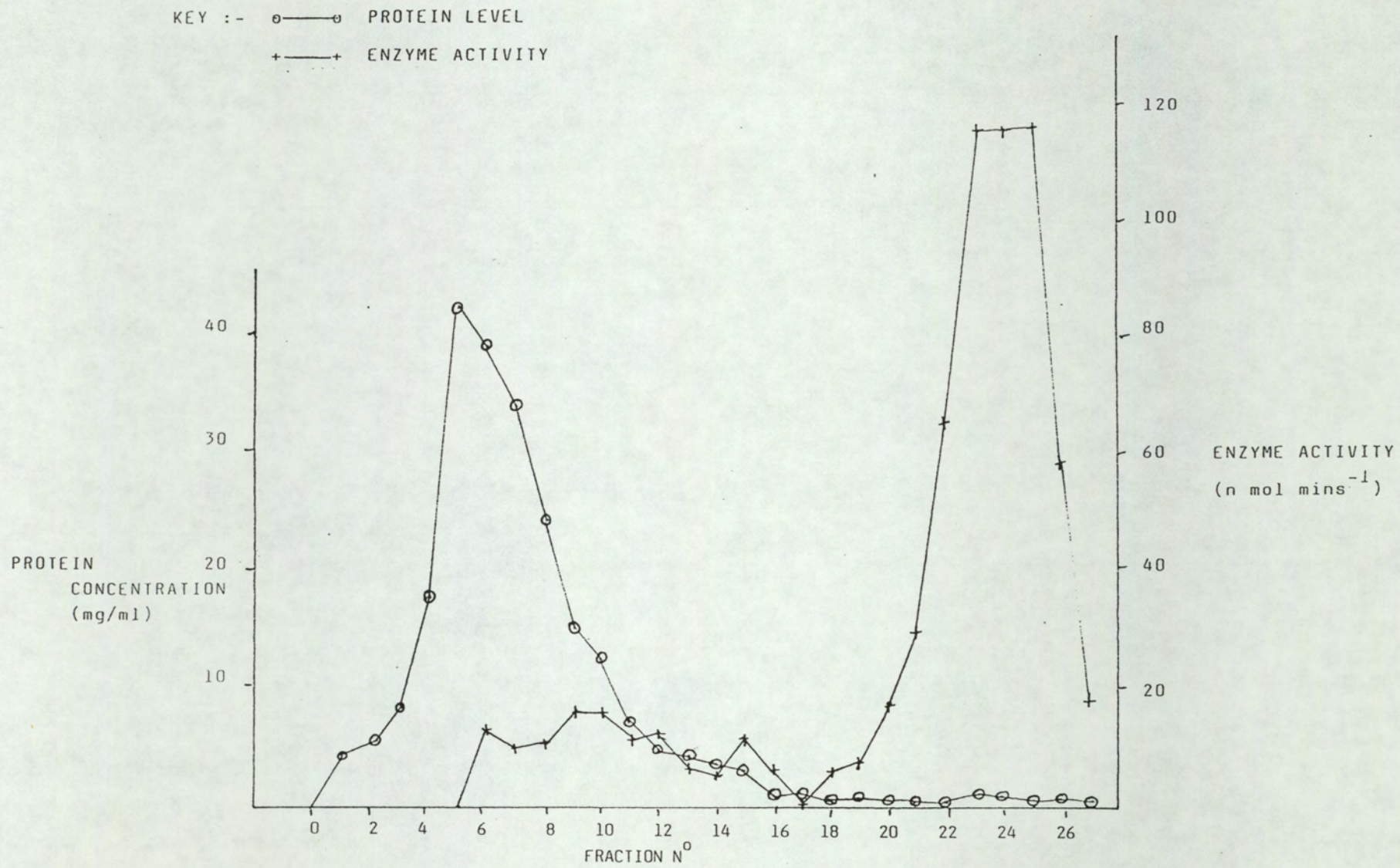


FIGURE 2.1 THE ELUTION PROFILE FOR THE AFFINITY CHROMATOGRAPHY OF RAT LIVER DHPR

The modified method of Craine et al used, had the following components in a standard 1ml assay : 0.05M Tris/HCl buffer pH7.6, 2.5×10^{-4}M sodium azide, 10^{-3}M H_2O_2, 8ug horseradish peroxidase, 10^{-4}M NADH, 10^{-4}M $DMPH_4$ and 0.02ml of the tissue extract with distilled water to make up the volume. Control assays were run without $DMPH_4$.

The standard assay for the method of Cheema et al contained : 0.05M Tris/HCl buffer pH7.6, 2.5×10^{-4}M sodium azide, 1.2×10^{-4}M DCPIP, 10^{-4}M $DMPH_4$, 10^{-4}M NADH and 0.02ml tissue preparation with distilled water to give a final volume of 1ml.

In both methods the reaction components minus the $DMPH_4$ and reductase source were mixed in 1ml semi-micro cuvettes, and incubated at 37°C for 1.5min. The $DMPH_4$ was then added to both cuvettes (blank and assay cuvettes), and mixed by repeated inversions of the cuvette, then the tissue preparation was added to the assay cuvette, with mixing, to initiate the reaction.

Enzymatic activity was measured, against blanks minus the DHPR source, at 37°C, using either a Pye-Unicam 1750 dual beam recording spectrophotometer or a Pye-Unicam PU 8800 UV/VIS dual beam recording spectrophotometer with the program as in figure **2.2.** Enzyme activity is expressed as nmol NADH used min^{-1}, and specific activity is expressed as NMOL NADM used $min^{-1}mg^{-1}$ protein.

WAVELENGTH nm 340.0	F: 160.8 U/l 0.000 ABSORBANCE
BANDWIDTH : 2 nm	GRAPHS SPAN : 0.5 A OFFSET : AUTO
RATE DELAY : 03 : 00 TIME : 04 : 00 READS : 05 : 00	
	SAMPLE : 000

FIGURE 2.2

PROGRAM USED WITH PYE-UNICAM PU. 8800 UV/VIS SPECTROPHOTOMETER.

As a result of work on human tissue extracts a sequential assay system using the Craine generating system was developed. The first assay measured the general reductase activity not due to DHPR in the tissue extract in the normal assay procedure but in the absence of pterin. The second assay was the one outlined above and DHPR activity was calculated by subtracting the rate measured in the first assay from that measured in the second assay.

Measurement of Kinetic Constants :

The effect of $DMPH_4$ and NADH concentrations on the purified DHPR activity were determined by varying the concentration of one substrate at saturating levels of the other substrate. Hanes and Lineweaver-Burk plots were used in the determination of the Michaelis constant (Km) for both substrates. Lines of "best fit" were calculated using the "least squares" method.

Measurement of the effects of oestogens on rat DHPR in vitro and in vivo :

Purified rat liver DHPR was used to assess the effects of oestrogens *in vitro*. The oestrogens were dissolved in dimethyl sulfoxide (DMSO) to the required concentration, such that the DMSO was 0.1% of the assay volume (1.4×10^{-2}M DMSO), this level of DMSO did not have any effect on the

DHPR activity. Several concentrations of the oestrogens were used, and comparison was made between the oestrogens and their catechol derivatives. The modified Craine assay was used, and the oestrogens were added prior to the 1.5 min preincubation.

The in vivo effect of oestrogens was investigated by dosing female rats with diethylstilbestrol in corn oil (50mg/Kg body weight), for 3 days. On day 5 the animals were killed and livers and uterus were removed and prepared by method **2.** The control animals were dosed with corn oil alone. A further group of rats were dosed with 17∝ ethynyloestradiol (2mg/0.2ml corn oil per day) for 5 days. Animals were killed at 24, 48 and 72 h after the final dose, and portions of the liver were prepared by method **2.**

Measurement of the effect of metal ions on DHPR activity :

DHPR was preincubated at 37°C with solutions of the metal ion, and aliquots were removed and immediately assayed by the Craine assay. The preincubation was for up to 30mins and the results were compared to a control incubation of enzyme and distilled water.

CHAPTER THREE

INITIAL ISOLATION OF RAT LIVER DHPR

INTRODUCTION

Dihydropteridine reductase (DHPR:EC.1.6.99.7) is responsible for the salvage of quinoniod dihydrobiopterin (qBH_2) in order to maintain the cellular levels of tetrahydrobiopterin (BH_4). The reducing power is obtained by the concomitant oxidation of NADH. It is the oxidation of NADH which enables the accurate measurement of the enzyme activity, by monitoring the change in absorption of samples at 340nm, provided that the enzymes second substrate, qBH_2, is present. The natural substrate for DHPR is now thought, almost certainly, to be qBH_2, and it can be easily generated from BH_4 using Horseradish peroxidase and hydrogen peroxide;

$$BH_4 + H_2O_2 \xrightarrow{\text{PEROXIDASE}} qBH_2 + 2H_2O$$

(Craine et al, 1972), or by using dichlorophenolindophenol (DCPIP), (Cheema et al, 1973). It is also possible to assess DHPR activity by measuring the pterin dependent reduction of ferri-cytochrome C (Hasegawa, 1977). However, BH_4 although now fairly readily available commercially is expensive and very labile, so the artifical substrate precurser 6,7,dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) has been widely used, (Craine et al, 1972; Cheema et al, 1973; Chauvin et al, 1979; Aksnes et al, 1979; Purdy and Blair, 1980; Firgaira et al, 1981; Purdy et al, 1981; Shen, 1983). Not only is $DMPH_4$ more stable than BH_4,

but also the $qDMPH_2$ is substantialy more stable than qBH_2, thus reducing the possibility of substrate degradation giving rise to low enzyme activities during enzyme assays. The effectiveness of $qDMPH_2$ as an appropriate substitute for qBH_2 has been indicated in inhibition studies with amethopterin which inhibits DHPR equally well, regardless of whether qBH_2 or $qDMPH_2$ is used as the substrate (Craine et al, 1972; Chauvin et al, 1979). Furthermore, Shen (1983) measured K_i values for $qDMPH_2$ and NADH and found them to be similar, which indicated the effectiveness of $qDMPH_2$ as an appropriate substitute for qBH_2. However, that qBH_2 is the favoured substrate is indicated by the enzyme lower Km for qBH_2 than the Km for the artificial substrates for DHPR isolated from a variety of sources (Craine et al, 1972; Brown, 1981; Firgaira et al, 1981; Firgaira et al, 1979).

DHPR has been isolated and characterised from a wide range of higher organisms; sheep liver (Craine et al, 1972; Cheema et al; 1973; Webber et al, 1978) and brain (Cheema et al, 1973), bovine liver (Hasegawa, 1977; Aksnes et al, 1979; Aksnes and Ljones, 1980; Nakanisi, et al, 1982), adrenal medulla (Cheema et al, 1973; Aksnes et al, 1979), kidney (Chauvin et al, 1979) and brain (Snady and Musacchio, 1978), rat liver (Webber et al, 1978; Purdy et al, 1981; Brown, 1981), human liver (Craine et al, 1972; Cotton and Jennings, 1978; Firgaira et al, 1981), as well as from the algal flagellate Crithidia fasciculata (Hirayama et al, 1980) and from the bacterium species Pseudomonas (Williams

et al, 1976). On the whole these studies have shown a similarity of properties and structure for DHPR from all these diverse sources. The enzyme having a molecular weight in the range 42,000-52,000, made up of 2 subunits of half this molecular weight. One significant variation was the enzyme isolated from human liver (Cotton and Jennings, 1978), which was reported to have a molecular weight of 100,000, and to be tetrameric. The enzyme was obtained by using an affinity adsorbent and was a 400 fold purification. However, when later workers repeated these experiments on enzyme purified 1,000 fold (Firgaira et al, 1981), they recorded a molecular weight of between 47,500-50,000 made up of 2 identical subunits. The reason for the anomalous molecular weight recorded by Cotton and Jennings, was due to the behaviour of the enzyme in the gradient polyacrylamide gel system they used to elucidate their enzymes molecular weight (Firgaira et al, 1981).

Many forms of chromatography have been used in the purification of DHPR, the affinity chromatographic method devised by Cotton and Jennings (1978) providing a quick and efficient one step method for the isolation of highly purified DHPR. The affinity ligand used is sodium 1,2-naphthoquinone-4-sulphonate, which is believed to be effective as the affinity matrix because of structural similarities with the quinonoid substrate (Cotton and Jennings, 1978). These workers as well as others (Brown, 1981), noted that the affinity method was not effective if

NADH was omitted from the crude extract prior to chromatography. This suggests that the enzyme needs to bind NADH before it can bind the pterin/affinity ligand. Since the enzymatic reaction is believed to proceed via a compulsory ordered mechanism, with NADH binding first (Korri et al 1977; Aksnes and Ljones 1980), this is only to be expected. The enzyme is eluted by a buffer at the operational pH of the enzyme minus NADH.

The purpose of the research reported here was to use and adapt the purification method of Cotton and Jennings (1978) to purify rat liver DHPR. Subsequent assays were performed to ascertain the kinetic constants for this enzyme, and the purfied enzyme was used to test the effects of manganese on the enzymic activity.

RESULTS

Purification of Rat Liver Dihydropteridine Reductase

Two methods were used to isolate rat liver DHPR. The first used a low speed centrifugation of a liver homogenate followed by gel chromatography on Sephadex G150 and the subsequent affinity chromatography of the most active G150 fractions using the affinity adsorbent sodium 1,2 naphthoquinone 4-sulphonate. A typical elution profile for DHPR from a Sephadex G150 column is shown in figure 3.1 (Enzyme activity was measured using the modified method of Craine et al (1972) as outlined in Chapter 2). The most active fractions were pooled and applied to a column of the affinity adsorbent. The purified DHPR was then eluted after washing with buffers. Figure 3.2 shows a typical elution profile for the DHPR from the affinity chromatography column. Table 3.1 gives a summary of the purification of rat liver DHPR by this method. This procedure was not found to be very reliable since large variations in both recovery and purification factor were found in subsequent preparations of the enzyme. Method 2. was tried in order to reduce these variations, the high speed centrifugation step removing any lysosomes and thus hopefully reducing any loss in enzyme activity due to release of proteolytic enzymes from these organelles. Figure 3.3 shows the elution profile for DHPR after the

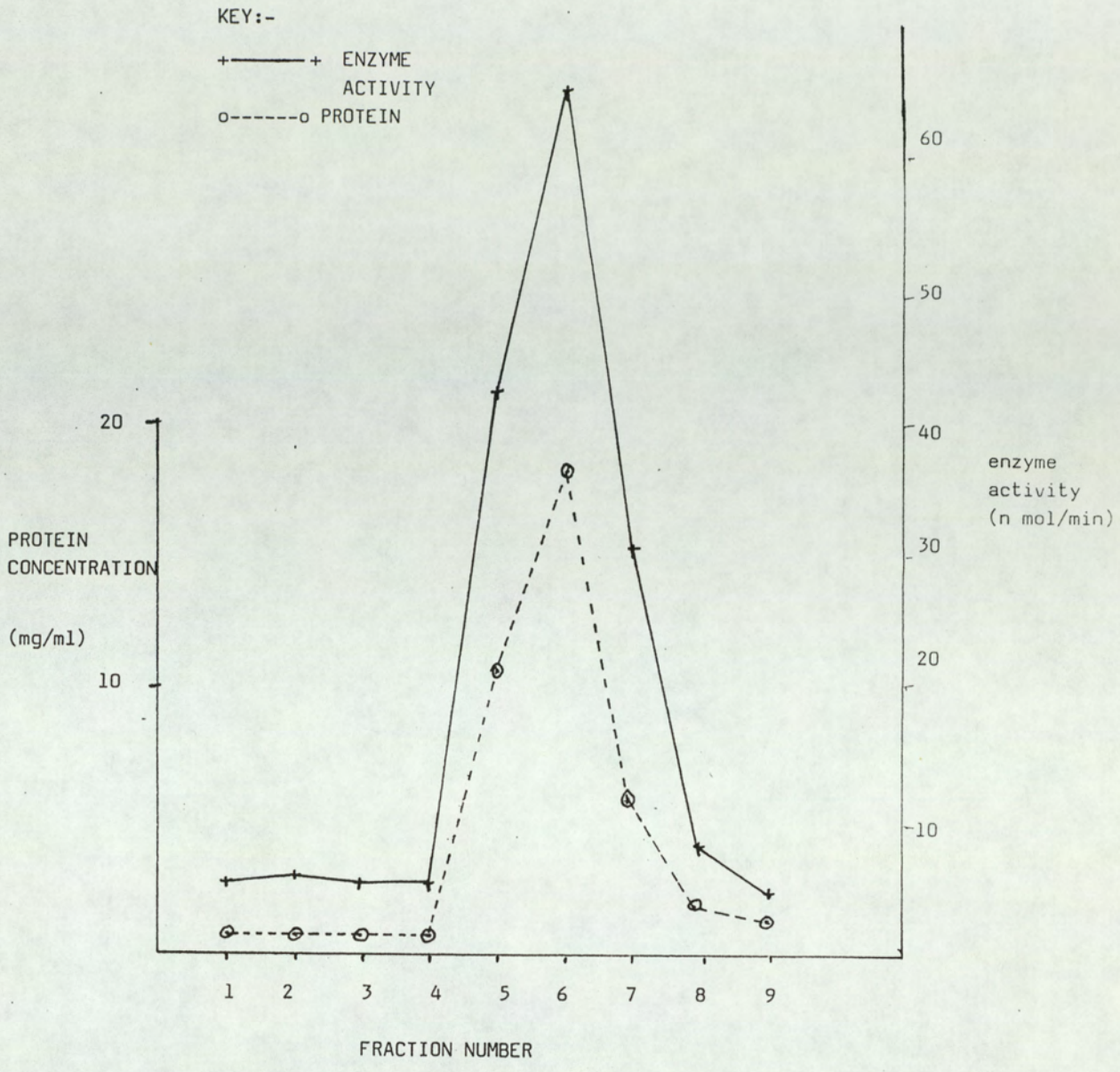


FIGURE 3.1 ELUTION PROFILE FROM SEPHADEX G150 OF A RAT LIVER PREPARATION AFTER 25,000 g CENTRIFUGATION.

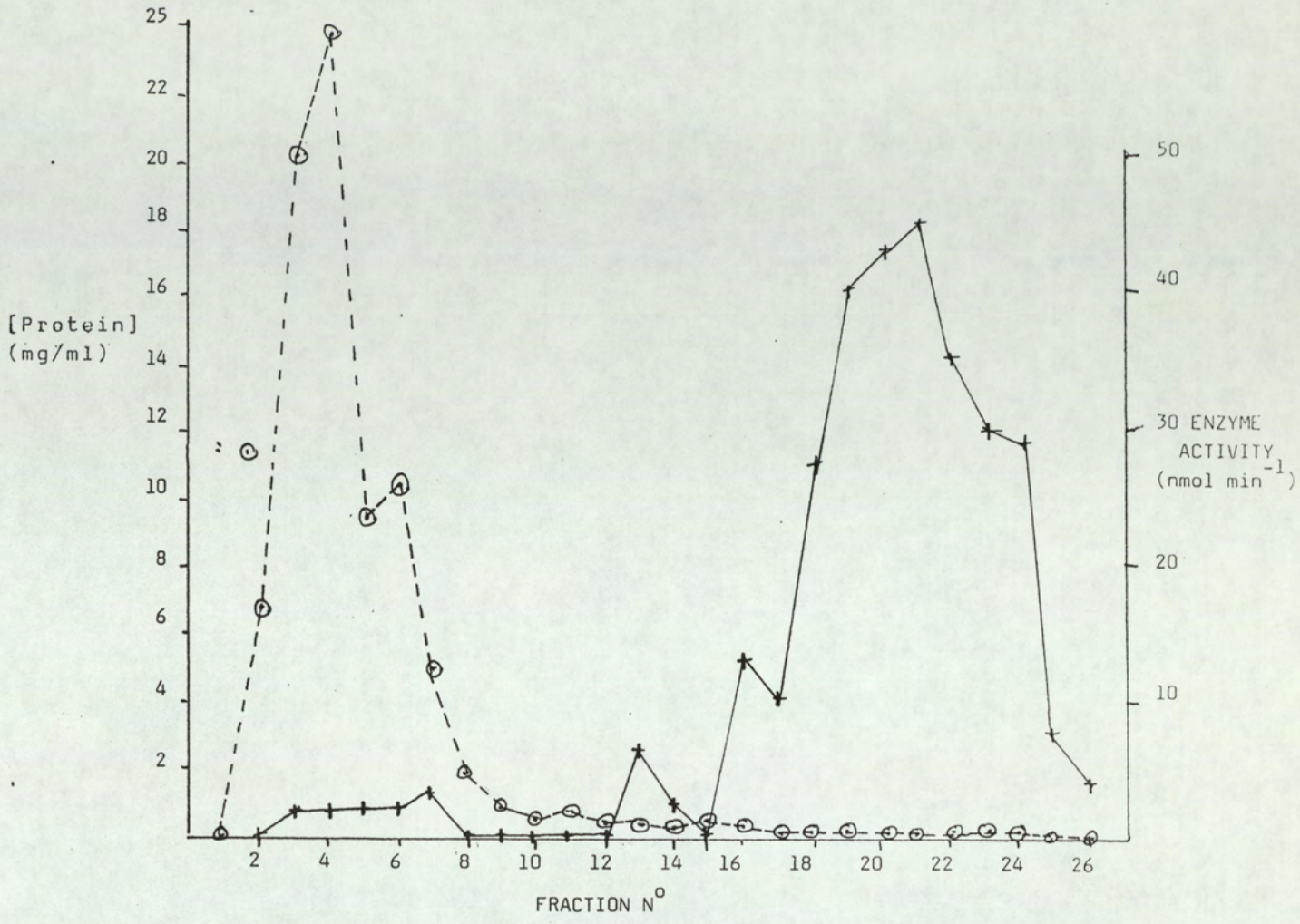


FIGURE 3.2 ELUTION PROFILE OF RAT LIVER DHPR FROM AFFINITY GEL AFTER GEL CHROMATOGRAPHY ASSAYED WITH 10^{-4}M NADH and $DMPH_4$

TABLE 3.1

SUMMARY OF PURIFICATION OF RAT LIVER DHPR USING SEPHADEX G150 FILTRATION STEP

	Volume (ml)	Enzyme Activity ($nmol.min^{-1} ml^{-1}$)	Total Enzyme Activity ($nmol.min^{-1}$)	Recovery (%)	Protein Concentration ($mg.ml^{-1}$)	Specific Activity ($nmol.min^{-1} mg^{-1}$)	Purification Factor
Crude Extract (Supernatant)	42	52.5	2205	100	43.4	1.21	1
Sephadex G-150 Peak Fractions	9	137.6	1238.4	56	11.5	11.9	9.8
Affinity Chromatography (Pooled Fractions)	8	36.8	294.4	13.4	0.4	9 2	76.0

high speed centrifugation step was introduced, and table 3.2 gives a summary of a purification of DHPR by Method 2. Subsequent purifications by Method 2 consistently gave a more highly purified enzyme (as measured by the purification factor), and the recovery of enzyme was usually between 20-35%, (the example in table 3.2 is exceptional). It was noticed however, that the best results were obtained if the affinity column's filtration rate was slower than 2.5min per ml.

Evaluation of Kinetic Constants

It is possible to obtain values for the kinetic constants of a 2 substrate enzyme by measuring enzyme activity (velocity) at different concentrations of one substrate whilst the other remains constant at a high concentration. The concentration of the non varying substrate is kept high so that its part in the general equation for two substrate kinetics becomes insignificant. These data can then be represented in a number of ways in order to evaluate the kinetic constants for the substrate which was varied. Initial experiments on rat liver DHPR showed that both substrates were saturating at 10^{-4}M, and Figure 3.4A and B show the expected rectangular hyperboles obtained when plots of velocity (v) versus substrate concentration ([s]) were drawn for NADH varying (A) and qDMPH$_2$ varying (B). Since it is easier to obtain values for the Km from a

Figure 3.3

Typical elution profile for dihydropteridine reductase from a rat preparation after high speed centrifugation (104,000xg). Assayed with 10^{-4}M NADH and $DMPH_4$.

+---------+ Dihydropteridine Reductase Activity.

⊙————⊙ Protein Concentration.

(*) Arrows mark the points when buffers were changed (for details see Methods and Materials).

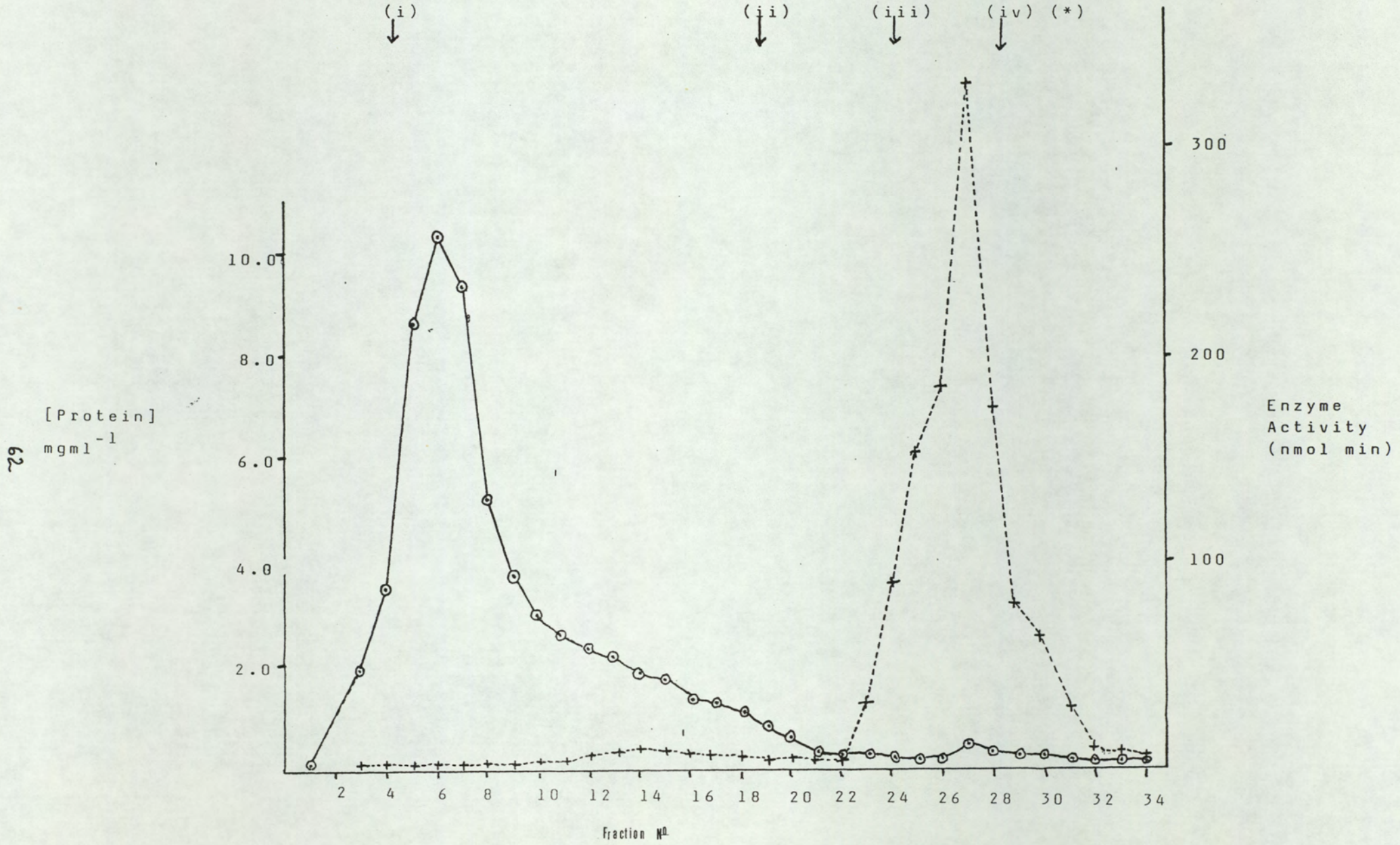

(i)
(ii)
(iii)
(iv)
(*)
[Protein]
mgml^{-1}
10.0
8.0
6.0
4.0
2.0
300
200
100
Enzyme
Activity
(nmol min)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Fraction No.

Figure 3.3

TABLE 3.2

PURIFICATION OF RAT LIVER DHPR AFTER HIGH SPEED CENTRIFUGATION

	Volume (ml)	Enzyme Activity $nmol.min^{-1} ml^{-1}$	Total Enzyme Activity (units)	Recovery (%)	Protein Concentration $mg.ml^{-1}$	Specific Activity $nmol.min^{-1} mg^{-1}$	Purification Factor
Crude Extract	60	1668.0	100,080	100	63	26.5	1
Affinity Chromatography Pooled Fractions	6	11,438.0	68,628	68.6	0.45	25417	960

FIGURE 3.4 Velocity versus substrate concentration curves

A VARYING THE CONCENTRATION OF NADH AT SATURATING $qDMPH_2$ CONCENTRATION (10^{-4}M)

B VARYING THE CONCENTRATION OF $qDMPH_2$ AT A SATURATING NADH CONCENTRATION (10^{-4}M)

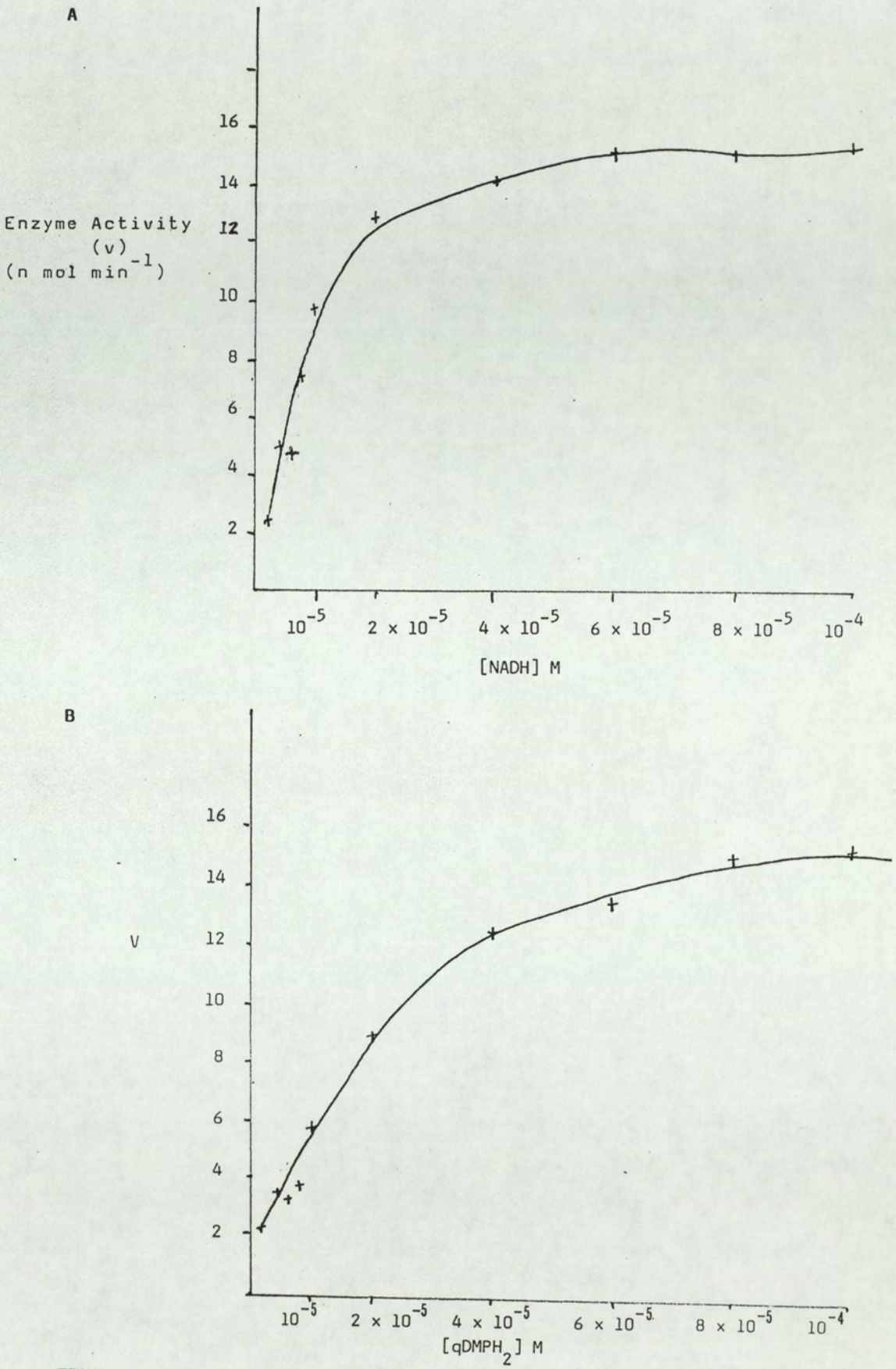
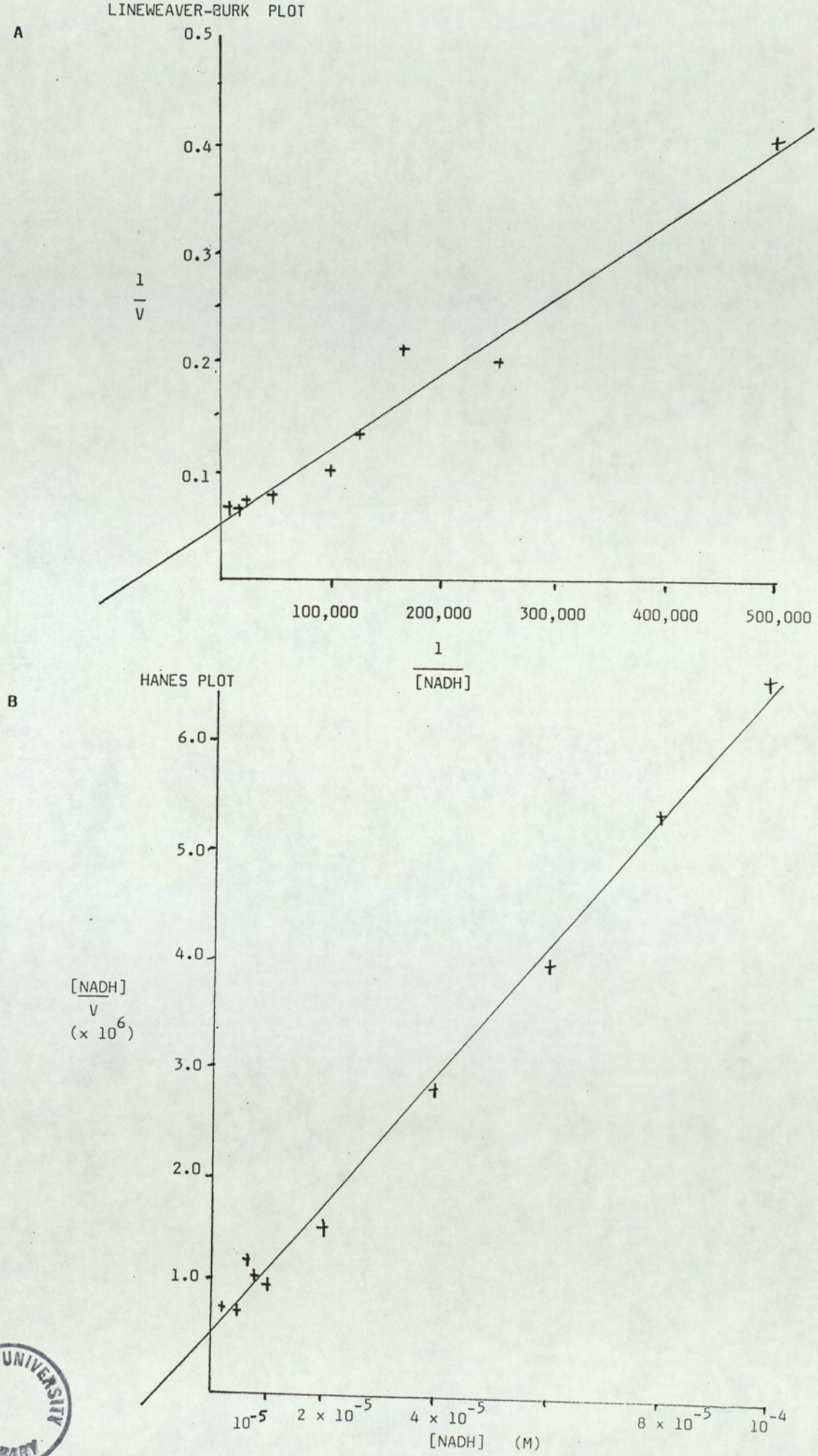

A
Enzyme Activity
(v)
(n mol min^{-1})
16
14
12
10
8
6
4
2
10^{-5}
2×10^{-5}
4×10^{-5}
6×10^{-5}
8×10^{-5}
10^{-4}
[NADH] M
B
V
16
14
12
10
8
6
4
2
10^{-5}
2×10^{-5}
4×10^{-5}
6×10^{-5}
8×10^{-5}
10^{-4}
[qDMPH$_2$] M

FIGURE 3.4

straight line, these results are also shown in Lineweaver-Burk (1/v versus 1/[s]) and Hanes ([s]/v versus [s]) plots. These plots for NADH varying are shown in figures **3.5A** and **B**, whilst those for $qDMPH_2$ varying are shown in figures **3.6A** and **B**. From a series of several experiments Km's for NADH and DMPH were obtained, these are given in table **3.3**.

It is also possible to gain values for the kinetic constants of a 2 substrate enzyme from plots varying the concentration of one substrate at several fixed concentrations of the second substrate. In this case Lineweaver-Burk plots are constructed initially and, from these two secondary plots can be drawn one of the intercept on 1/v axis versus 1/[B], (where [B] is the fixed concentrations of the non-varying substrate), and the other of the slope of the individual Lineweaver-Burk plots versus 1/[B]. The graphs shown in figure **3.7** are secondary plots from an experiment in which the concentration of NADH was varied at 4 set concentrations of $qDMPH_2$ (10^{-4}, 8×10^{-5}, 6×10^{-5} and 4×10^{-5}M). From figure **3.7A** it is possible to obtain values for 1/Vmax and Km $qDMPH_2$, whilst figures **3.7B** can give Km NADH and K_s^a , an apparent dissociation constant for the first substrate to be bound. These figures are given in table **3.4** with the values obtained directly from the Lineweaver-Burk and Hanes plots. The Km values for NADH are all in close agreement, whilst the Km for $qDMPH_2$ obtained from the secondary plot is closer in value to that calculated from the Hanes plot (figure 3.6B).

FIGURE 3.5 KINETIC PLOTS VARYING [NADH] AT 10^{-4}M q $DMPH_2$ (LINES OF BEST FIT BY LEAST SQUARES)



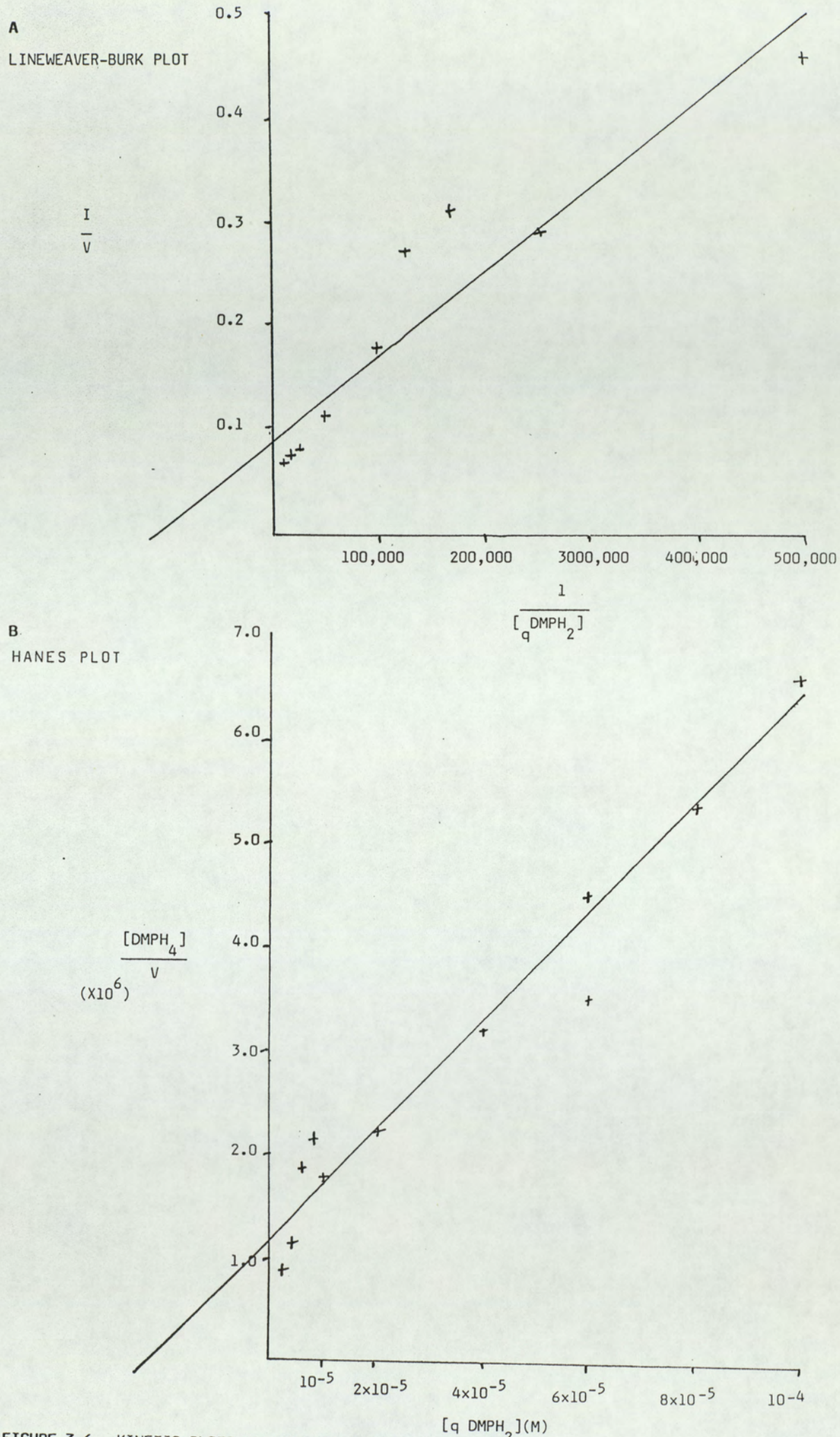


FIGURE 3.6 KINETIC PLOTS VARYING THE [$qDMPH_2$] AT 10^{-4} NADH

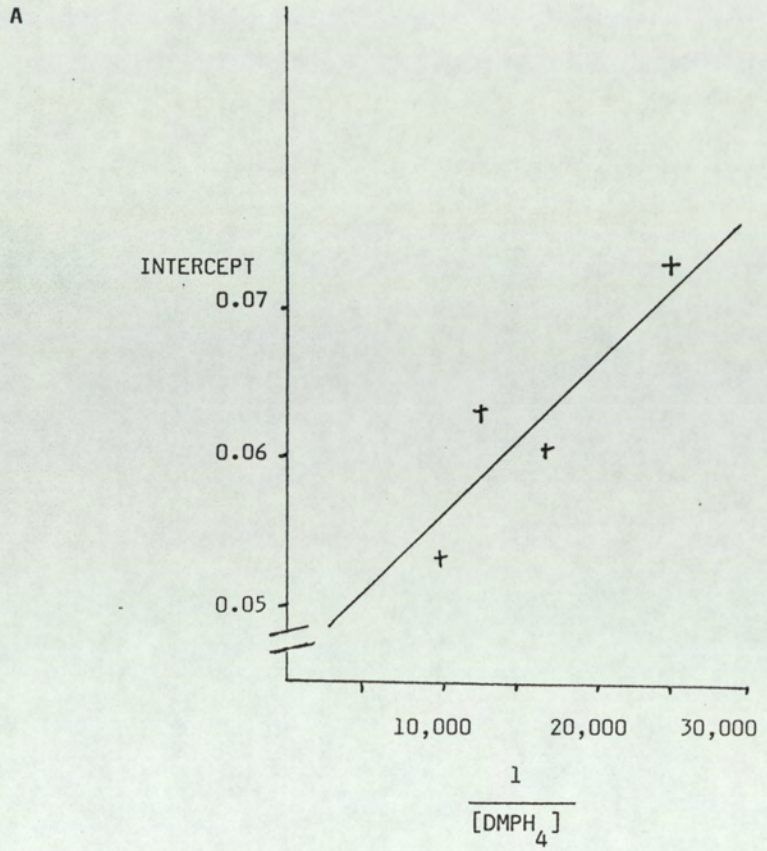
TABLE 3.3

RAT LIVER DIHYDROPTERIDINE REDUCTASE.

Michaelis constants (Km's), as calculated from the results of several experiments, from both Hanes and Lineweaver Burke plots*

NADH	$DMPH_4$
$1.7 \times 10^{-5} \pm 0.5 \times 10^{-5}$M	$2.1 \times 10^{-5} \pm 0.6 \times 10^{-5}$M
(n = 18)	(n = 16)

* See text for experimental details.



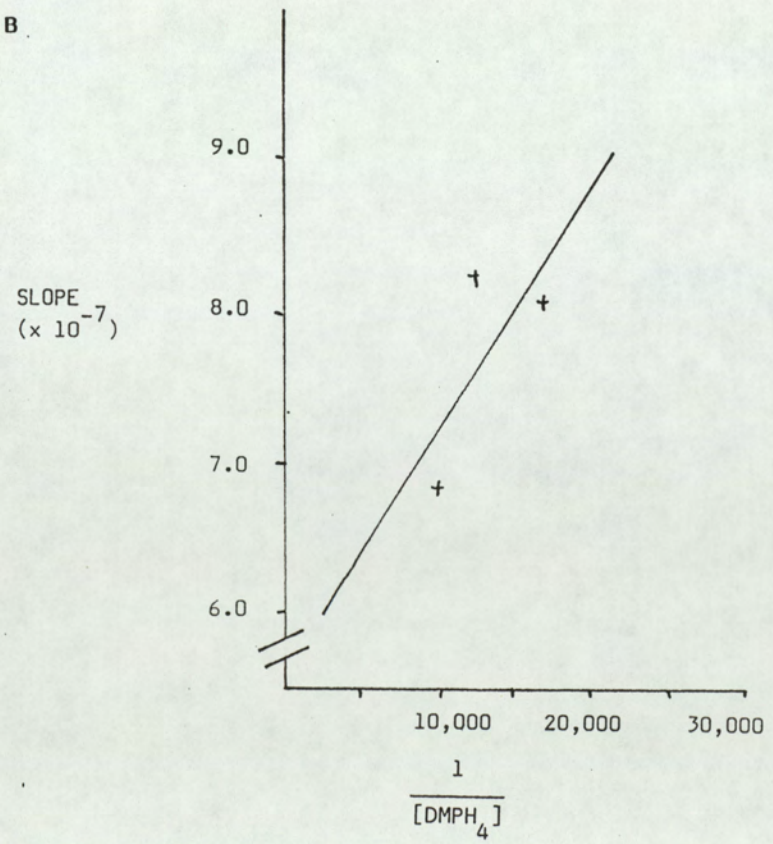


FIGURE 3.7 SECONDARY PLOTS FROM LINEWEAVER-BURK GRAPHS VARYING [NADH] AT SET CONCENTRATIONS OF $DMPH_4$ (SEE TEXT FOR DETAILS)

TABLE 3.4

KINETIC CONSTANTS FOR DHPR FROM SECONDARY PLOTS, WITH THOSE OBTAINED DIRECTLY FROM LINEWEAVER-BURK AND HANES PLOTS (Figures 3.5 & 3.6)

	Secondary Plot (Fig 3.7)	Lineweaver-Burk Plots	Hanes Plots
Km NADH	1.26×10^{-5}M	1.3×10^{-5}M	10^{-5}M
Km $DMPH_4$	2.5×10^{-5}M	9.52×10^{-6}M	2.35×10^{-5}M
K_S^A	1.45×10^{-5}M	-	-

Rat Liver DHPR Activity With NADPH and NAD^+

Figure 3.8 shows the elution profile from the same affinity chromatographic step as that shown in figure 3.3, but in this case the fractions were assayed in the presence of 10^{-4}M NADPH rather than NADH.

The main peak of NADPH activity corresponds exactly with the major fraction with NADH activity. The activity with NADPH, however, is only about 3.0% of the enzyme activity with NADH. In some of the earlier fractions the NADPH activity is almost 30.0% of the NADH activity, and is above 10% in other fractions, which may be indicative of the presence of a NADPH dependant DHPR, or may simply be due to the presence of other enzymes capable of using NADPH since in these fractions there are other proteins (see figure 3.3).

Purified rat liver DHPR showed no NAD^+ reducing capacity.

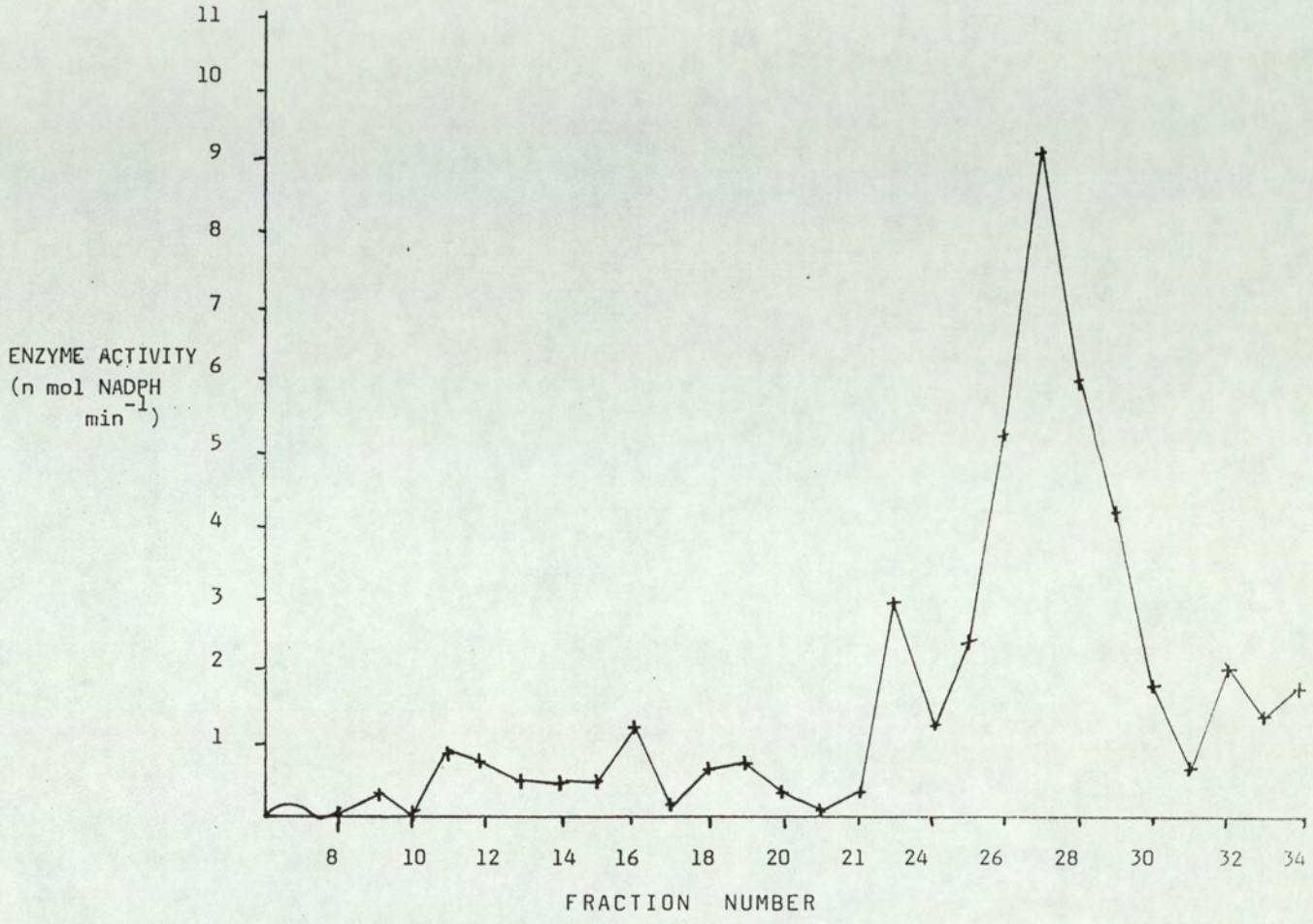


FIGURE 3.8 DIHYDROPTERIDINE REDUCTASE ACTIVITY WITH NADPH AS ELUTED FROM AFFINITY COLUMN (ELUTION PROFILE FOR NADH DEPENDENT DHPR ACTIVITY IS SHOWN IN FIGURE 3.3) ASSAYED WITH 10^{-4}M NADPH AND $DMPH_4$.

Effect of Manganese on DHPR Activity

The effect of manganese on DHPR activity was assessed by preincubating the enzyme with 10^{-2}M manganese and assaying aliquots at set time intervals for enzyme activity. Control assays were performed by preincubating enzyme and tris buffer and assaying aliquotes. The results of such experiments are shown in figure **3.9.** These results suggest that Mn^{2+} apparently increases DHPR activity, a t- test on these results show that there is a significant increase in enzyme activity in the presence of 10^{-2}M manganese ($P < 0.02$). However, experiments carried out with less highly purified enzyme did not show manganese to be having any effect on DHPR activity. This could possibly be due to the sequestation of the manganese by the contaminating proteins in this less purified sample.

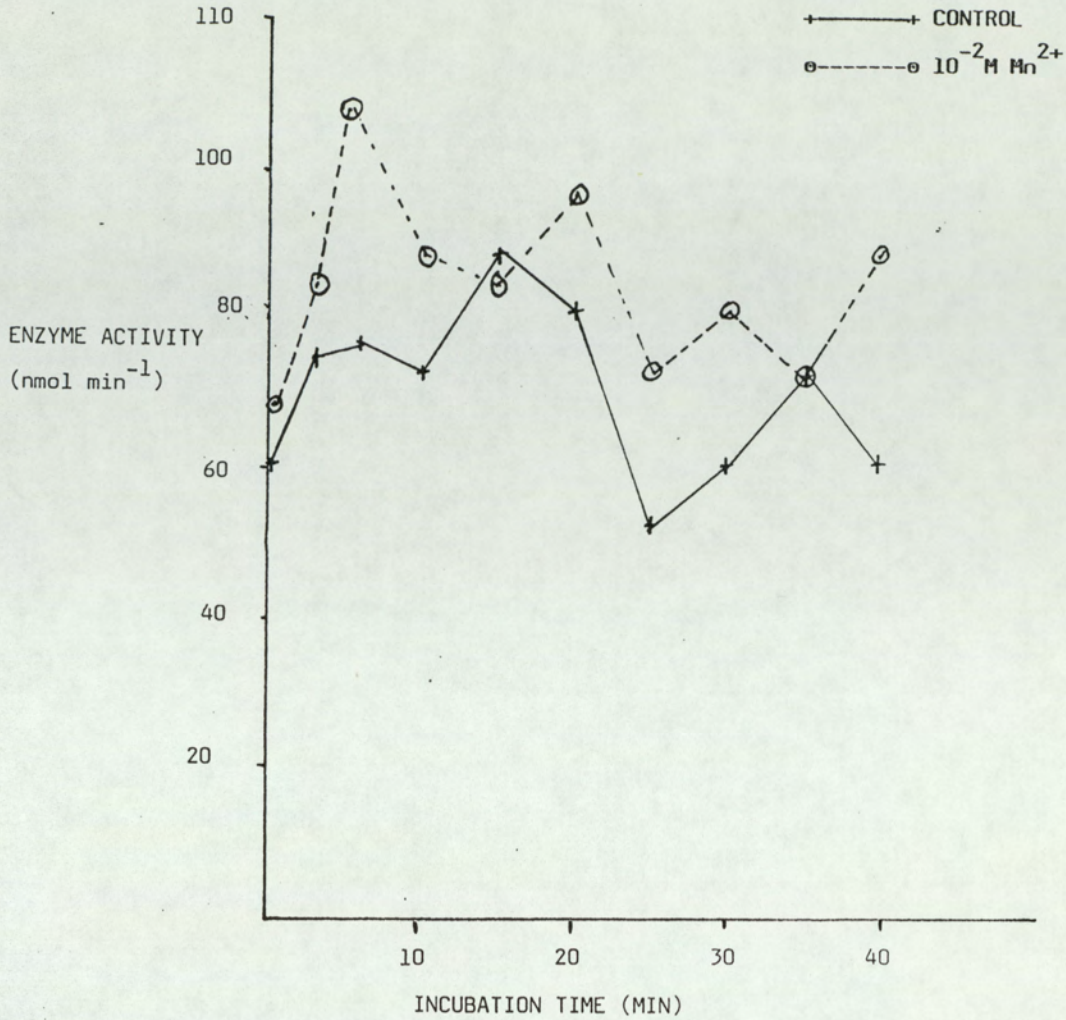


FIGURE 3.9 THE EFFECT OF INCUBATION WITH MANGANESE (10^{-2}M), ON RAT LIVER DHPR (PURIFIED 960 FOLD)

SUMMARY

1) The use of a preliminary gel filtration step on Sephadex G150 was primarily to remove excess proteins from the crude liver preparation. However, figure 3.1 illustrates that this was not being achieved, and the deep red colouration of the crude preparation and fractions implies the presence of haemoglobin, (molecular weight 64,500) which elutes at about the same time as DHPR (molecular weight 50,000).

The high speed centrifugation, after vigorous homogenation, provides a mainly cytosolic crude extract, which does not clog the affinity column and gives highly purified enzyme (500-1,000 fold). In addition it speeds up the process completing the whole purification procedure in a day. This reduces the possibility of loss of enzyme activity during storage and defrosting. The high speed centrifugation step also removes the lysosomes thus decreasing the risk of proteinases being present in the crude extract.

2) In initial experiments with enzyme purified below 300 fold it was observed that concentrations of $qDMPH_2$ above $8 \times 10^{-5}M$ produced substrate inhibition, as shown by a down turn in the plateau of velocity versus concentration $qDMPH_2$ plots. This was not apparent with the more highly purified enzyme, and a

similar substrate inhibition has been reported in crude enzyme preparations by Firgaira et al (1979).

Highly purified enzyme gave Michaelis constants (Km's) for NADH and $qDMPH_2$ of 1.7 $\pm$ 0.56 x 10^{-5}M for NADH, and 2.2 $\pm$ 0.62 x 10^{-5}M for $qDMPH_2$. Previously reported Km NADH for rat liver DHPR have been slightly higher (Webber et al, 1978; Purdy and Blair, 1980; Brown, 1981), whilst Km $qDMPH_2$ have been reported as low as 1.2 x 10^{-5}M (Purdy and Blair, 1980; Brown, 1981) and as high as 3.6 x 10^{-5}M (Webber et al, 1978). However, as experimental procedures do vary, it seems likely that these results do present reasonable values for the Michaelis constants.

The value for K_S^A on its own gives little information. As it is above zero, however, it removes any possiblity that the enzyme uses a "ping-pong" mechanism.

3) The enzyme isolated by the affinity chromato-graphy demonstrated very little activity with NADPH. This is in agreement with observations with other mammalian reductases (Nielsen et al, 1969; Craine et al, 1972; Cheema et al, 1973; Brown, 1981; Firgaira, 1981).

The presence of 2 types of DHPR one NADH-specific, the other NADPH-specific in bovine liver (Nakanishi et

al, 1977) does mean that there is the possibility of "contamination" of NADH specific with NADPH-specific, in preparations from some organs/species.

4) Previous work on the effect of manganese on DHPR activity, was carried out on the enzyme isolated from Pseudemonas (Williams et al, 1976) and 100% inhibition of enzyme activity was recorded with 10^{-4}M manganese. The results recorded here are totally at variance with this previous report, and suggest a major difference between the bacterial and vertebrate DHPR. The slight activation observed with 10^{-2}M manganese could possibly be a non specific effect brought about by the divalent cationic state of the metal ion, rather than a specific activation of DHPR. The concentration of intracellular Mn^{2+} being 0.2-1uM in hepatocytes (Schramm, 1982) would suggest that, to be on effector of DHPR activity in vivo, far lower concentrations of manganese would effect enzyme activity. This was not found to be so.

This experiment also highlighted the heat stability of rat liver DHPR.

CHAPTER FOUR

DHPR IN HUMAN NEOPLASTIC TISSUE, HORMONAL INVOLVEMENT IN DHPR ACTIVITY REGULATION

DHPR IN NEOPLASTIC TISSUE, HORMONAL INVOLVEMENT IN DHPR ACTIVITY REGULATION

INTRODUCTION

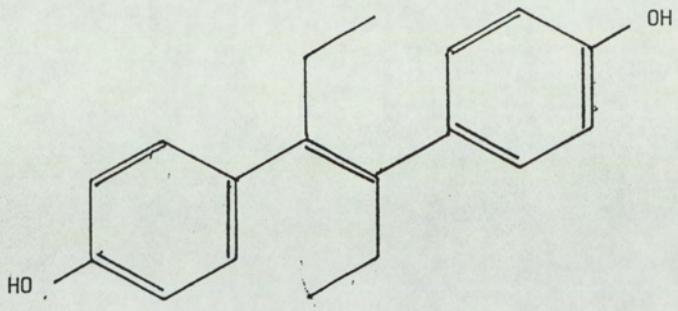
Evidence for an altered metabolism of pteridines in malignant disease has been accumulating since the mid 1970's. Such alterations have been demonstrated in animals with experimental tumours, (Kokolis and Ziegler, 1977; Wachter et al, 1979a), as well as in cancer cells in culture (Albrecht et al, 1978; Stea et al, 1981; Hausen et al, 1981). In man a significant elevation of urinary neopterin concentration in many, but not all, cancer patients has been reported (Rokos et al, 1980; Stea et al, 1981; Hausen et al, 1981). Alterations in biopterin concentrations have been recorded but biopterin levels do not vary as much as neopterin levels (Rokos et al, 1981; Leeming and Blair, 1980; Stea et al, 1981). The frequency of raised ratios of neopterin to biopterin in the urine of cancer patients suggests a block in BH_4 biosynthesis.

Sanchez-Urretia et al (1978) have investigated DHPR activities in human neoplastic tissue. In pulmonary tumours as well as in rat neoplasms the DHPR activity was lower than that in the non-neoplastic tissue. These results were taken to be a reflection of the undifferentiated enzymic composition of neoplasms. However, Dhondt et

al (1981) working on breast tumour DHPR activity, found large variations in the DHPR activity with 6% of the tumours they assayed having DHPR activities close to those noted in the liver, a tissue which has an extremely high DHPR activity. These workers also report a significant correlation between DHPR activities and hormonal dependence, as measured by cytosolic oestrogen receptor sites. In a later paper (Dhondt et al, 1983), the correlation was confirmed and a correlation between DHPR activity and progesterone receptors was reported. It would thus appear that DHPR activity may be under oestrogenic/ progesteronic control.

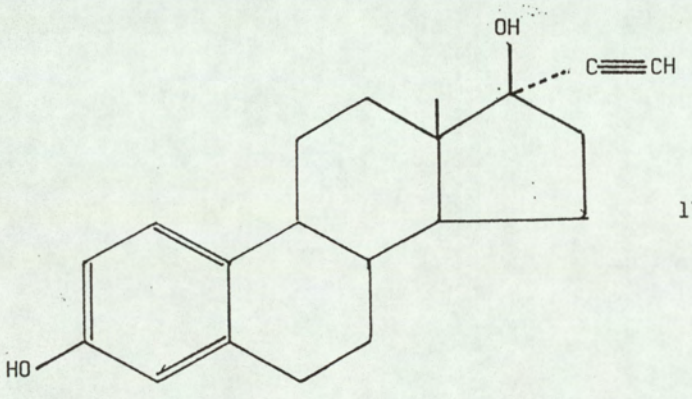
To investigate the possible effects of oestrogenic molecules it is customary to use either an oestrogenic molecule, such as diethylstilbestrol (DES), (I) or an artificial (man made) oestrogen such as 17 ∝ ethynyl-oestradiol (II). These molecules were developed as contraceptives, and it is their prolonged biological half life (and therefore slower metabolisation), which makes them ideal for experiments to assess the effect of oestrogens on metabolic pathways. It is well documented that oestrogens affect folate metabolism in humans (Shojania et al, 1971; Larson-Cohn, 1975) and 7,8 dihydrobiopterin levels have been shown to follow a cyclic pattern throughout the menstrual cycle, the pattern correlating roughly with serum oestogen levels (Leeming and Blair, 1980). Further evidence suggesting a link between

(I)



DIETHYLSTILBOESTROL

(II)



17 ∝ ETHYNYLOESTRADIOL

oestrogen levels and catecholamine production is that hypothalamic tyrosine hydroxylase fluctuates during the menstrual cycle (Voogt et al, 1979).

The concentration and turnover of catecholamines in the mammalian central nervous system is altered by gonadal hormones, whilst gonadotrophin release and reproductive behaviour are altered by manipulations of brain catecholamines (Wurtman, 1970; Wilson, 1974; Sandler and Gessa, 1975). It is also well established that the catecholaminergic system of the preoptic-hypothalamic brain is involved in the feedback control of estradiol on gonadotrophin release (McCann et al, 1979). These effects, of oestrogens on catecholamine neurotransmitter function, may occur as a direct effect of the oestrogens on members of the catecholaminergic biosynthetic pathway rather than by the generally assumed involvement of translocation of the hormone to the nucleus followed by a chain of biochemical events involving RNA production resulting in de novo protein synthesis (O'Malley et al, 1976). The evidence for such a direct effect is that oestrogens can be 2 hydroxylated by brain tissue (Fishman and Norton, 1975; Fishman, 1976), this gives the catechol structure which makes these molecules potent inhibitors of catechol-O-methyltransferase (Breuer and Koster, 1975) one of the enzymes responsible for the inactivation of dopamine and noradrenaline. The first evidence that oestrogens can influence catecholamine biosynthesis via the catechol-

oestrogens was the inhibition of tyrosine hydroxylase by 2-hydroxyoestradiol, reported by Lloyd and Weisz (1978), and confirmed by Foreman and Porter (1980).

In this chapter, DHPR activity in neoplastic and normal breast and large intestine tissue will be presented. Also the effect of oestrogen dosing on rat liver and uteri DHPR activity will be reported, together with the results of various experiments to determine the effect of oestrogens and their catechol derivatives on purified rat liver DHPR.

RESULTS

DHPR ACTIVITY IN HUMAN NEOPLASTIC AND NORMAL TISSUE

Human tissue samples were obtained immediately after surgical removal of the tumour, with apparently normal tissue used as controls. The tissue samples were prepared by method 2 outlined in Chapter 2 and DHPR activity was taken as the difference between the rate in the presence of pterin and that in the absence of pterin, using the modified Craine assay. Initially however, the tumour and control tissue preparations were assayed by the Cheema assay using DCPIP. It was, however, noticed that in some of the samples there was a considerable oxidation of NADH in the absence of pterin, giving rise to an apparent elevation in DHPR activity in the presence of pterin. Breast tissues assayed in this way showed a significant elevation of DHPR activity in the neoplastic breast tissue as compared to apparently normal tissue from the same breast ($P < 1\%$ by paired t test). When some of these samples were assayed by the Craine assay, the general trend was continued but the DHPR activites recorded were reduced, so the Craine assay was used as this method overcame the oxidation of NADH observed in the DCPIP assays. The high levels of NADH used probably arose as a consequence of the presence of diaphorase in these crude tissue preparations. This enzyme is able to oxidise NADH in the presence of an

electron acceptor such as DCPIP or methylene blue. The DHPR activities of a number of breast tissue samples are given in table **4.1.** These results show a highly significant elevation of the DHPR activity in neoplastic breast tissue when compared to normal breast tissue (P <0.2% Wilcoxon's signed ranks test; P <5% paired t test). To verify that the enzyme activity being measured was due to DHPR the assays were repeated with NADH being replaced by equimolar NADPH. In none of these tissue samples did the activity with NADPH exceed 4% of the activity recorded with NADH.

The enzyme activity in the normal tissue showed no age related change, and the menopausal status of the patient did not seem to correlated in anyway with the DHPR activity measured.

Table **4.2** gives the DHPR activities for the breast samples as $nmol.min^{-1}gm^{-1}$ wet weight of tissue prepared. This change from the usual protein baseline against which the enzyme activity is normaly reported, is to overcome the possibility of there being a variation in the protein level in the neoplastic samples as a consequence of the neoplastic state, which might have a gross effect on the DHPR activity being reported. The significant elevation in tumour DHPR activity is still observed (P <0.2% Wilcoxon's sum of ranks; P <5% paired t test), suggesting that there is a real elevation in DHPR activity in breast tumours and

TABLE 4.1

DIHYDROPTERIDINE REDUCTASE ACTIVITY IN APPARENTLY NORMAL AND NEOPLASTIC TISSUE FROM HUMAN BREAST*

Patient	DHPR specific activity (nmol. min^{-1}. mg^{-1}.).	
	Apparently normal tissue	neoplastic tissue
1. B.S.	24.4	25.2
2. B.L.	16.3	99.0
3. B.T.	13.3	19.8
4. J.H.	8.8	28.9
5. R.M.	15.7	27.2
6. H.E.	11.5	20.9
7. M.B.	26.7	151.2
8. D.W.	8.5	5.3
9. G.L.	16.3	127.0
10. A.G.	17.6	39.3
11. D.P.	13.1	21.0
12. E.Y.	50.3	341.0
13. G.B.	0.8	16.6
14. L.T	40.6	48.6
15. M.K	51.5	52.3

* DHPR activity measured at 10^{-4}M NADH and $DMPH_4$.

$P < 0.2$ % Wilcoxon's signed ranks test.

TABLE 4.2

DIHYDROPTERIDINE REDUCTASE LEVELS IN APPARENTLY NORMAL AND NEOPLASTIC TISSUE FROM HUMAN BREAST CALCULATED AS PER GRAM WET WEIGHT OF TISSUE PREPARED

Patient	Enzyme activity nmol min^{-1}. g wet wt^{-1}.	
	Normal tissue	Neoplastic tissue*
1. B.S.	2.0	5.9
2. B.L.	1.7	18.0
3. B.T.	3.3	4.9
4. J.H.	2.4	7.0
5. R.M.	2.6	4.9
6. H.E.	1.9	6.3
7. M.B.	8.8	121.5
8. D.W.	1.2	4.7
9. G.L.	1.9	28.0
10. A.G.	2.5	7.0
11. D.P.	1.8	3.3
12. E.Y.	10.4	77.5
13. G.B.	0.2	7.6
14. L.T.	9.5	19.4
15. M.K.	7.8	15.0

* $P < 0.2$ % by Wilcoxon's ranks test.

that the elevation is not due to a faulty baseline brought about by the neoplastic state.

In all the breast tissue samples assayed there was no increase in the non-specific oxidation of NADH in neoplastic tissue as compared to appparently normal tissue (Table **4.3**).

DHPR activity in neoplastic tissue from human large intestine is not elevated when compared with normal adjacent tissue (Table **4.4**). These results indicate that there may be a hormonal effect involved in the elevation of the DHPR activity observed in the neoplastic breast tissue, and Dhondt et al (1981)have reported a correlation between breast tumour DHPR activity and oestrogen receptor levels. Table **4.5** gives the oestrogen and progesterone receptor levels measured in the cytosol of the tumour samples by Dr. Hughes, Queen Elizabeth Hospital, Birmingham, with their corresponding DHPR activity. These results do not show any correlation between DHPR activity and oestrogen receptor levels. However, there is a significant correlation between enzyme activity and progesterone receptor levels ($P < 1\%$ Spearman's correlation test).

TABLE 4.3

NON SPECIFIC OXIDATION OF NADH BY NEOPLASTIC AND NORMAL HUMAN BREAST TISSUE

PATIENT	RATE OF NON SPECIFIC OXIDATION OF NADH ($nmol^{-1}_{min^{-1}}mg^{-1}$)	
	NORMAL TISSUE	NEOPLASTIC TISSUE
1. B.S.	11.0	7.0
2. B.L.	9.5	8.2
3. B.T.	6.5	15.6
4. J.H.	7.3	5.4
5. R.M.	11.0	8.1
6. H.E.	4.3	6.0
7. M.B.	11.0	9.1
8. D.W.	4.2	16.6
9. G.L.	3.3	7.4
10. A.G.	7.0	8.0
11. D.P.	3.2	4.0
12. E.Y.	11.3	2.7
13. G.B.	9.2	3.2
14. L.T.	4.6	6.5
15. M.K.	5.3	4.8

TABLE 4.4

DHPR ACTIVITY IN APPARENTLY NORMAL AND NEOPLASTIC TISSUE FROM HUMAN LARGE INTESTINE

Patient	Source of Tumour	DHPR specfic activity (nmol min^{-1}. mg^{-1}.)	
		Apparently normal	tumour tissue*
1. B.L.	Rectum	8.8	22.8
2. E.H.	Colon	24.4	7.1
3. C.J.	Colon	10.2	39.5
4. H.S.	Colon	16.7	19.6
5. J.C.	Caecum	45.6	30.0
6. A.H.	Colon	28.2	18.7
7. A.Y.	Rectum	33.1	34.4

* No significant difference between normal and tumour DHPR activities.

TABLE 4.5

OESTROGEN AND PROGESTERONE RECEPTOR LEVELS IN HUMAN BREAST NEOPLASTIC TISSUE WITH THE CORRESPONDING DHPR ACTIVITY

Patient	DHPR Activity ($nmol.min^{-1}mg^{-1}$.)	Oestrogen receptors		Progesterone receptors	
		f mol ml^{-1} Cytosol	f mol mg^{-1} Protein	f mol ml^{-1} * Cytosol	f mol mg^{-1} * Protein
1. B.S.	25.2	202.2	33.7	ND	ND
2. B.L.	99.0	291.5	45.4	998.3	155.5
3. B.T.	19.8	328.9	54.2	68.6	11.3
4. J.H.	28.9	202.2	60 9	ND	ND
5. R.M.	27.2	422.9	63.7	317.4	47.8
6. H.E.	20.9	ND	ND	ND	ND
7. M.B.	151.2	627.6	81.4	474.9	61.6
8. D.W.	5.3	ND	ND	ND	ND
9. G.L.	127.0	225.1	29.9	1781.0	208.8
10. A.G.	39.3	528.2	113.1	588.4	126.8
11. D.P.	21.0	ND	ND	58.1	19.7
12. E.Y.	341.0	419.4	_	365.9	-
13. G.B.	16.6	ND	ND	9.4	1.5
14. L.T.	48.6	ND	ND	84.7	20.9
15. M.K.	52.3	45.9	9.89	361.3	77.9

ND = NON DETECTED

* Direct correlation as shown by Spearman's correlation test ($P < 1\ \%$).

The Effect of Diethylstilboestrol on Rat Liver and Ulteri DHPR Activity.

To further investigate the effect of oestrogens on DHPR activity, young female rats were dosed with diethylstilboestrol (DES) (50mg/kg) for 3 days and then the livers and uteri were prepared and assayed for DHPR activity. The liver is the principale site for the metabolic destruction of oestrogens, and the blood can be completely cleared of oestrogens by a single passage through the liver (Goodman, 1974), whilst the cells of the uterus of the rat are known to contain oestrogen receptors. The results for two experiments are shown in table **4.6** the difference between the two experiments being the age and strain of the rats. In the first experiment the rats were 70-90g and were Wob/NOT's, whilst in the second experiment the rats were 90-120g Wistars supplied by Bantam and Kingman Ltd. Both experiments showed that DES caused a significant elevation in the DHPR activity in the liver ((i) ($P < 1\%$ Student's t test; (ii) $P < 0.1\%$ Student's t test), but the uteri although showing increased volume due to water uptake as a consequence of the DES dosing, did not show any elevation in the DHPR activity (control rats 60.1 ± 2.7 vs DES dosed 51.7 ± 2.8), compared to control rats dosed with corn oil, for 3 days.

TABLE 4.6

DIHYDROPTERIDINE REDUCTASE ACTIVITY IN NORMAL RAT LIVERS FROM RATS RECEIVING DIETHYLSTILBOESTROL (DES). NUMBER OF ANIMALS IN BRACKETS

		DHPR specific activity (n mol. min^{-1}. mg^{-1}.)
(i) Control/rats (3)	WOB/NOT's	108.2 ± 37.4
DES treated rats (8)		306.8 ± 80.1[a]
(ii) Control rats (5)	Wistars	368.0 ± 32.2
DES treated rats (5)		638.6 ± 143.2[b]

a $P = 1\%$ Wilcoxon's sum of Ranks and
$P < 1\%$ Student t test

b $P < 1\%$ Wilcoxon's sum of ranks test
$P < 0.1\%$ Student t test.

The Effect of 17 ∝ Ethynyloestradiol Dosing on Rat Liver DHPR Activity.

The results in table **4.7** are the DHPR activites measured in liver samples from young female rats (70-90g), after a 4 day course of dosing with 17 ∝ ethynyloestradiol. The animals were killed at 24 hr. intervals and the liver samples were prepared and assayed for DHPR activity. These results show that there is a large elevation in DHPR activity occuring between 24 and 48 hrs ($P < 1\%$ Student's t test), and this elevation was maintained between 48 and 72 hrs after the last dose of oestrogen. When compared to control levels the DHPR activity in the 24 hr. sample is highly significantly elevated ($P < 0.1\%$ Student's t test). These results not only show an oestrogen linked increased in liver DHPR activity, but also that this increase occurs slowly up to 72 hrs. after the final dose of oestrogen.

The Effect of DMSO on Purified Rat Liver DHPR Activity

In initial experiments investigating the effect of oestrogens on purified rat liver DHPR it was noticed that the solvent that they were dissolved in (dimethyl-sulfoxide : DMSO), was inhibiting DHPR activity.

TABLE 4.7

DIHYDROPTERIDINE REDUCTASE ACTIVITY IN RAT LIVERS FROM RATS RECEIVING 17∝ETHYNYLOESTRADIOL (2mg/0.2 ml CORN OIL FOR 4 DAYS)

Time from dose	DHPR activity (nmol. min^{-1}. mg^{-1}.) ($\pm$ S.D)
24 (5)	337.8 $\pm$ 34.6
48 (5)	558.8 $\pm$ 108.7
72 (4)	545.4 $\pm$ 44.9
24 vs 48	P < 1% } Student's t test
24 vs 72	P < 0.1% }
48 vs 72	No significant difference

Figure 4.1 shows the effect of DMSO on the Michaelis-Menton kinetics of rat liver DHPR, varying the concentration of $DMPH_4$ at a fixed concentration of NADH ($10^{-4}M$). Treatment of these data give the Lineweaver-Burk plots and Dixon plots shown in figures **4.2 A & B.** The results give a normal Km $qDMPH_2$ for the rat enzyme as $2.4 \times 10^{-5}M$, and the general disposition of the plots in figure **4.2** suggest that the DMSO may be inhibiting in either a competitive or a mixed manner. To further clarify the possible mode of inhibition a plot of [S]/V against [DMSO] (figure **4.3**) was constructed and this is strongly indicative of competitive inhibition.

The Effect of Oestrogens and Their Catechol Derivatives on Rat Liver DHPR Activity

To investigate the effects of oestrone, oestradiol, 2-hydroxyoestrone and 2-hydroxyoestradiol on DHPR activity, these compounds were dissolved in DMSO to give a final assay DMSO concentration of 0.014M. As in the substrate saturating conditions initially used this concentration of DMSO had no effect on the enzyme activity. The dose response curves obtained for these oestrogens on rat liver DHPR activity, are shown in figure **4.4**. It is possible to get I_{50} values for these oestrogens from this figure (these are the concentration of oestrogen which gave 50% inhibition of activity). These are tabulated in table **4.8**.

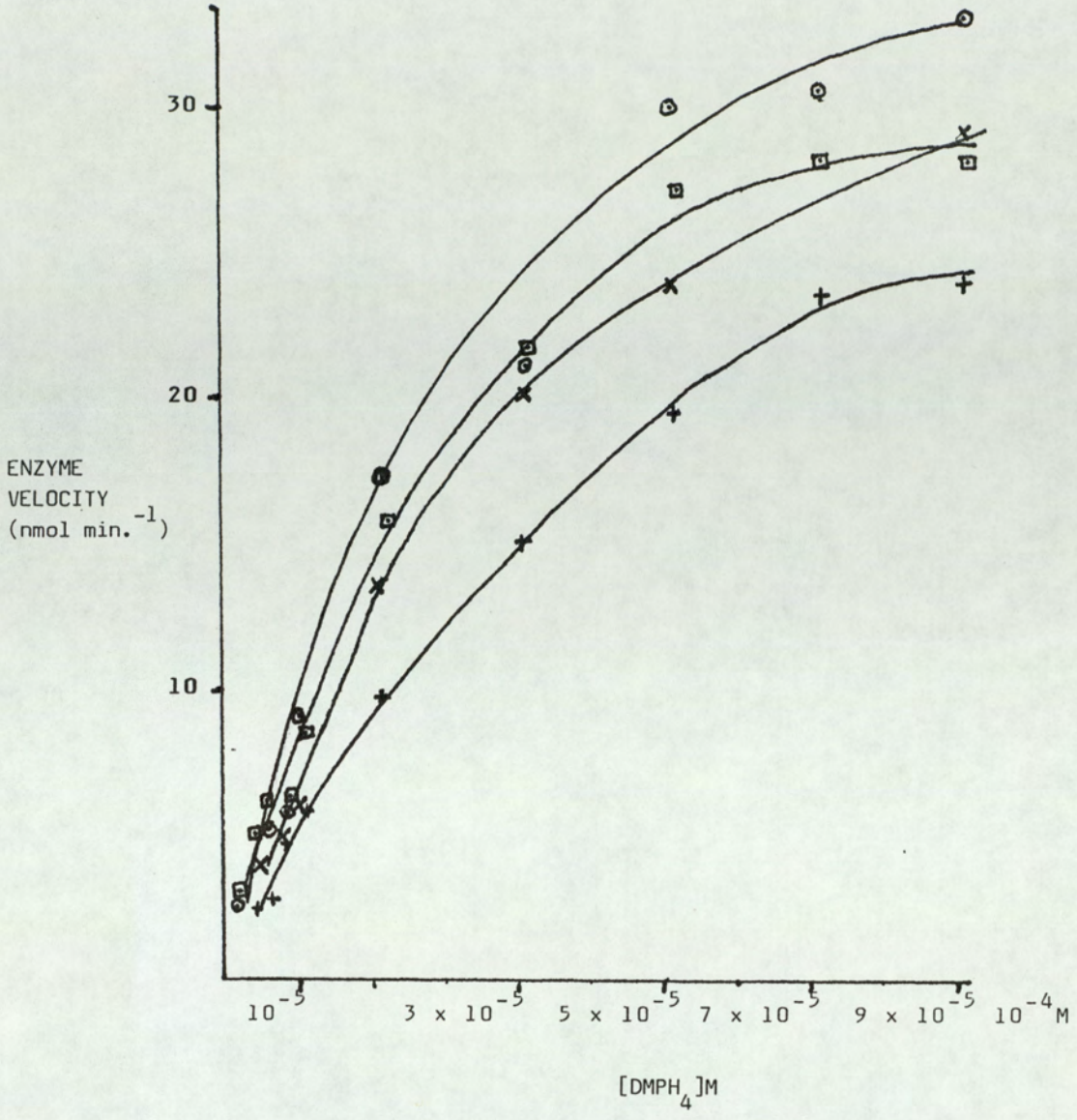


FIGURE **4.1** MICHAELIS-MENTON TYPE PLOT, OF ENZYME ACTIVITY IN THE PRESURE OF VARYING CONCENTRATION OF DMSO AT 10^{-4}M NADH (+——+, 0.7M DMSO; o——o 0.14M DMSO; X——X, 0.14M DMSO; ▣——■ CONTROL).

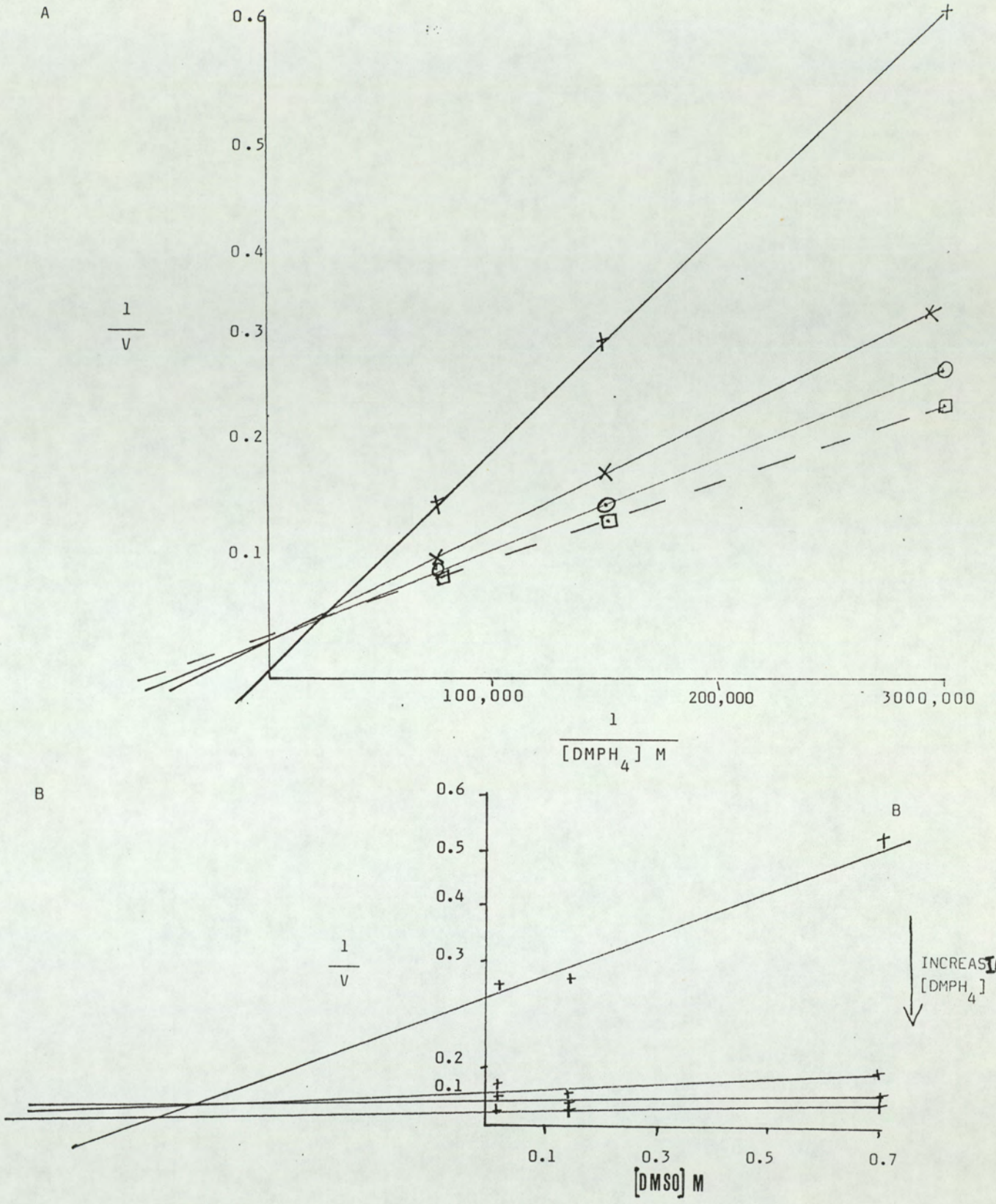


FIGURE 4.2 TWO KINETIC PLOTS OF THE EFFECT OF DMSO ON RAT LIVER DHPR AT 10^{-4}M NADH VARYING THE CONCENTRATION OF $DMPH_4$.

(A) LINEWEAVER - BURK PLOTS "LINES OF BEST FIT"

(B) DIXON PLOTS AT INCREASING CONCENTRATIONS OF $DMPH_4$. OF 1/V AGAINST [DMSO] (DIXON, 1953)

(KEY AS FIGURE 4.1)

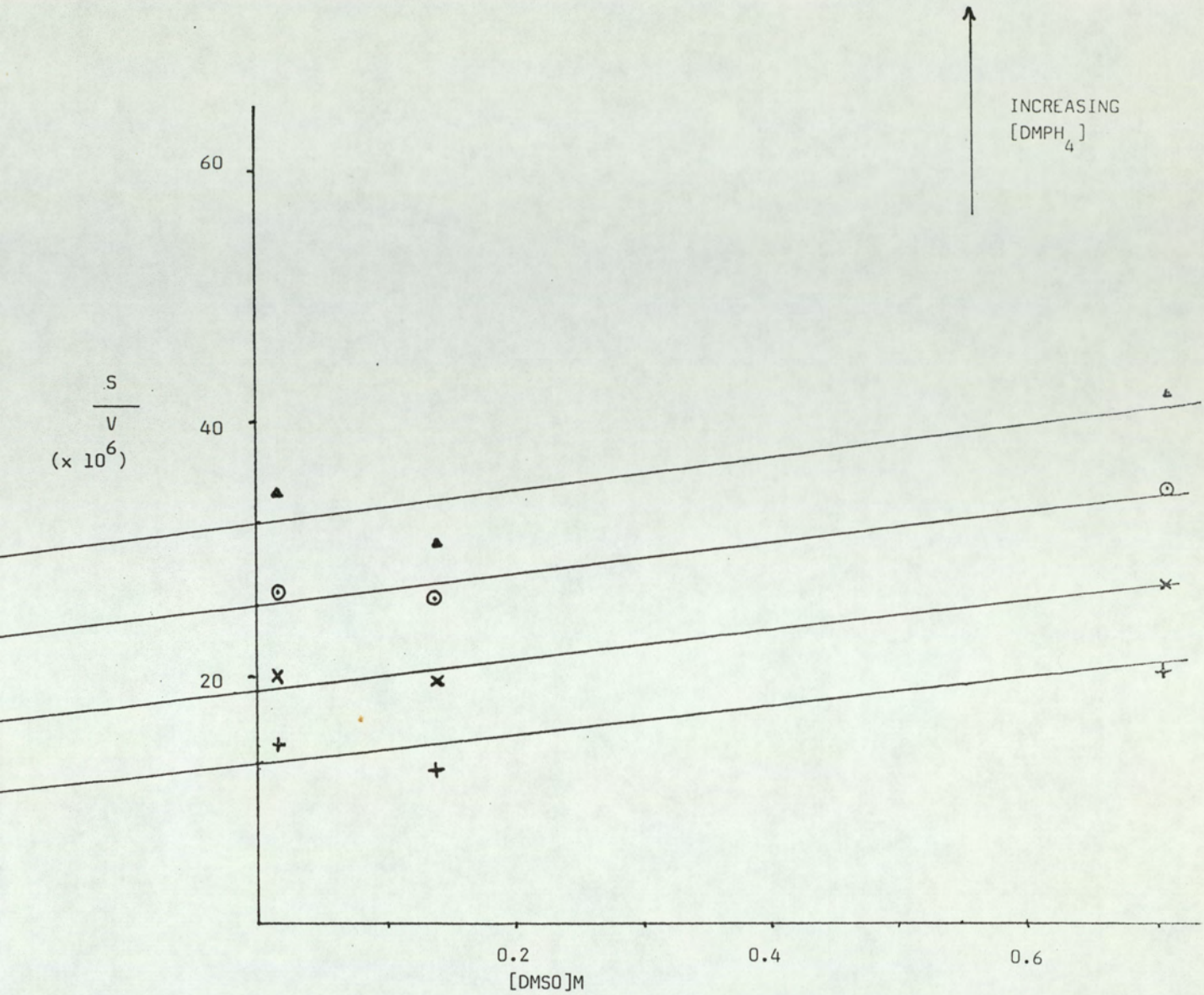


FIGURE 4.3 PLOTS OF S/V AGAINST [DMSO] AT VARIOUS $DMPH_4$ CONCENTRATIONS ON RAT LIVER DHPR (CORNISH - BOWDEN, 1974).

(KEY AS FIGURE 4.1)

EXCEPT ▲——▲ = 0.7M DMSO.).

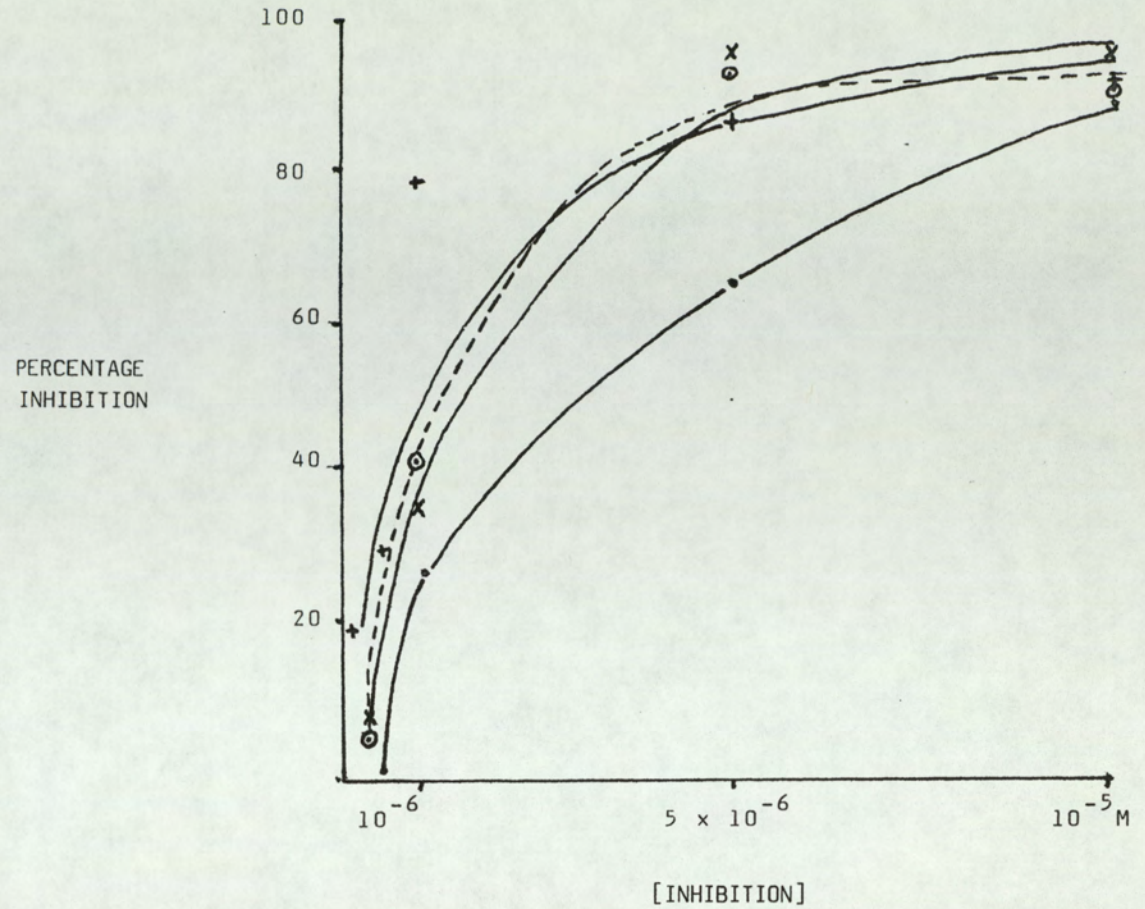


FIGURE 4.4 PERCENTAGE INHIBITION OF RAT LIVER DHPR ACTIVITY BY OESTRONE (+——+) OESTRADIOL (.——.), 2-HYDROXYOESTRONE (x——x), AND 2-HYDROXYOESTRADIOL (⊙-----⊙), ASSAYED AT 10^{-4}M NADH AND $DMPH_4$.

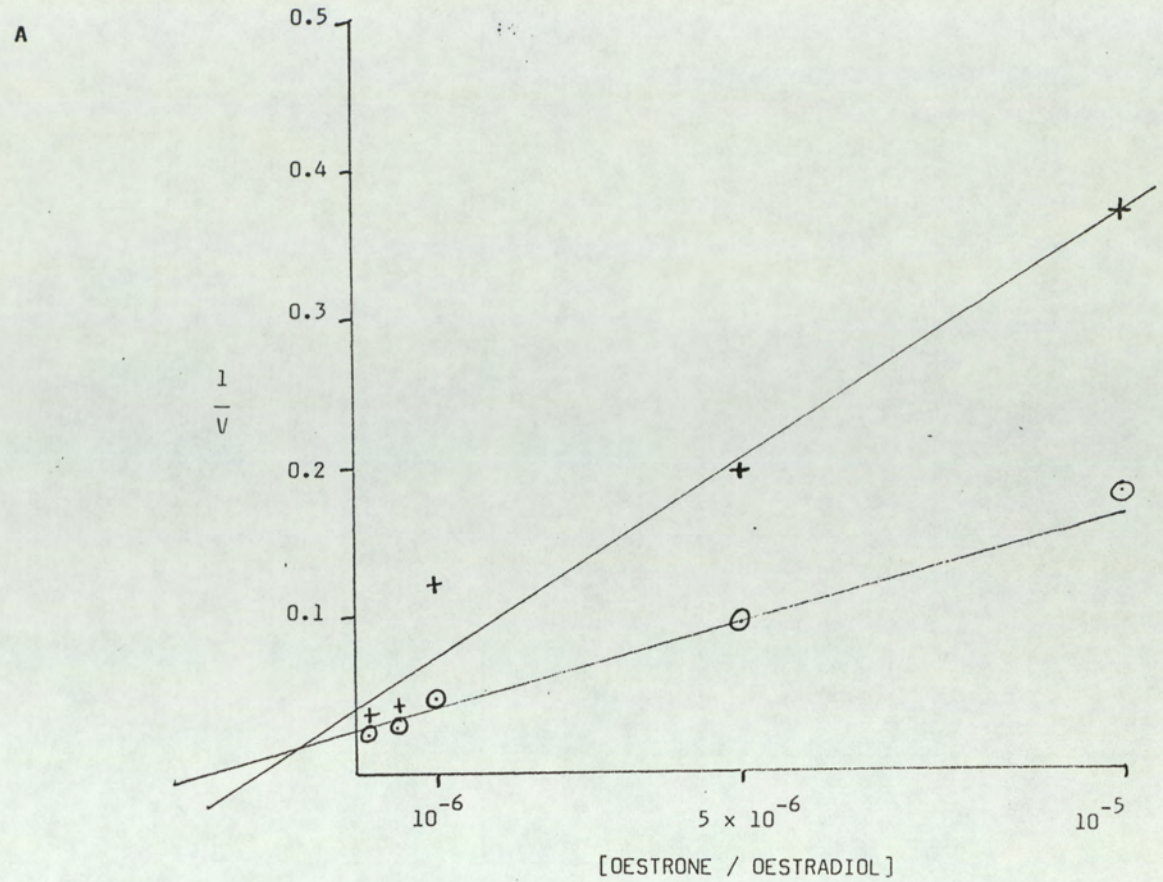
TABLE 4.8

I50 VALUES FOR OESTRONE, OESTRADIOL, 2-HYDROXYOESTRONE AND 2-HYDROXYOESTRADIOL ON PURIFIED RAT LIVER DHPR AT 10^{-4} M NADH AND $DMPH_4$ *

	I_{50} (M)
Oestrone	6×10^{-7}
Oestradiol	3×10^{-6}
2-hydroxyoestrone	1.3×10^{-6}
2-hydroxyoestradiol	1.5×10^{-6}

* Oestrogens dissolved in DMSO at a final concentration of 1.4×10^{-2}M

Oestrone has the lowest at 6 x 10^{-7}M, then its catechol derivative with 1.3 x 10^{-6}, 2 hydroxyoestradiol has an I_{50} of 1.5 x 10^{-6}M and oestradiol has the highest I_{50} at 3 x 10^{-6}M. These results may be replotted as Dixon plots (1/v against [inhibitor], and the plots for oestrone and oestradiol are shown in figure **4.5A**, whilst those for 2-hydroxyoestrone and 2-hydroxyoestradiol are shown in figure **4.5B**. (These are lines of best fit). The similarity in I_{50} values for the catecholoestrogens is reflected in their very similar Dixon plots. In further experiments to obtain K_i's for these oestrogens the inhibition by DMSO seemed to have an adverse effect. For example, figures **4.6** and **4.7** are the results for experiments on the oestradiol inhibition, whilst varying the concentration of $DMPH_4$ at 10^{-4}M NADH. The Linweaver - Burk plot (**4.6**), does seem to indicate that the inhibition by oestradiol is mixed, but the further plots in figure **4.7 A & B** are not very clear. However, as the points of intersection are above the axis in **4.7A** and below in **4.7B** this is indicative mixed inhibition (Cornish-Bowden, 1979). These graphs are not however, of such a good quality that a K_i can be obtained as they do not give a single point of intersection for all the lines. This may be due to the influence of DMSO on the enzymes activity in the assay with the lowest concentration of oestradiol, and figures **4.7 A & B** show that there are some problems at the lowest oestradiol concentrations. The intersection of the oestradiol lines would then give an apparent K_i. If this is so one could expect the maximal



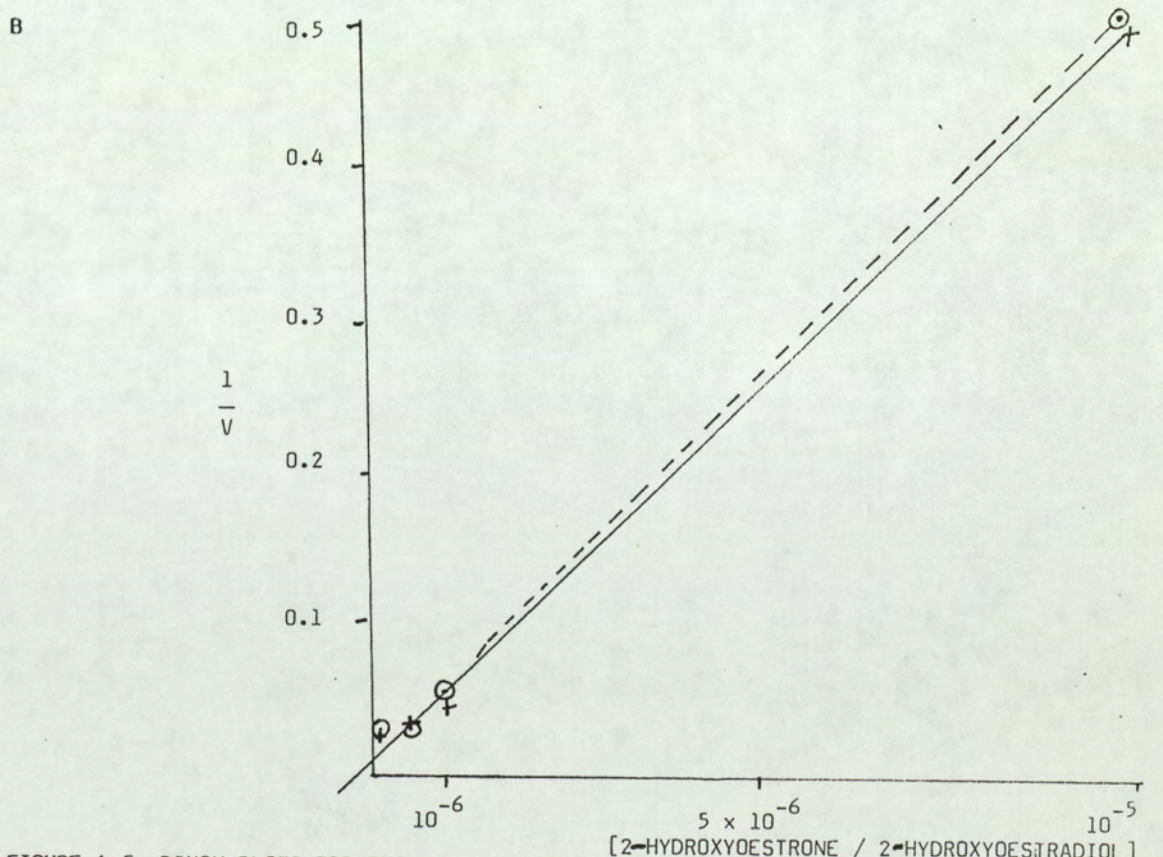


FIGURE 4.5 DIXON PLOTS FOR THE EFFECT OF OESTRADIOL ON RAT LIVER DHPR (LINES OF BEST FIT)

A. EFFECT OF OESTRADIOL (+——+) AND OESTRADIOL (⊙——⊙)

B. EFFECT OF 2-HYDROXY OESTRONE (+——+) AND 2-HYDROXYOESTRADIOL (⊙------⊙)

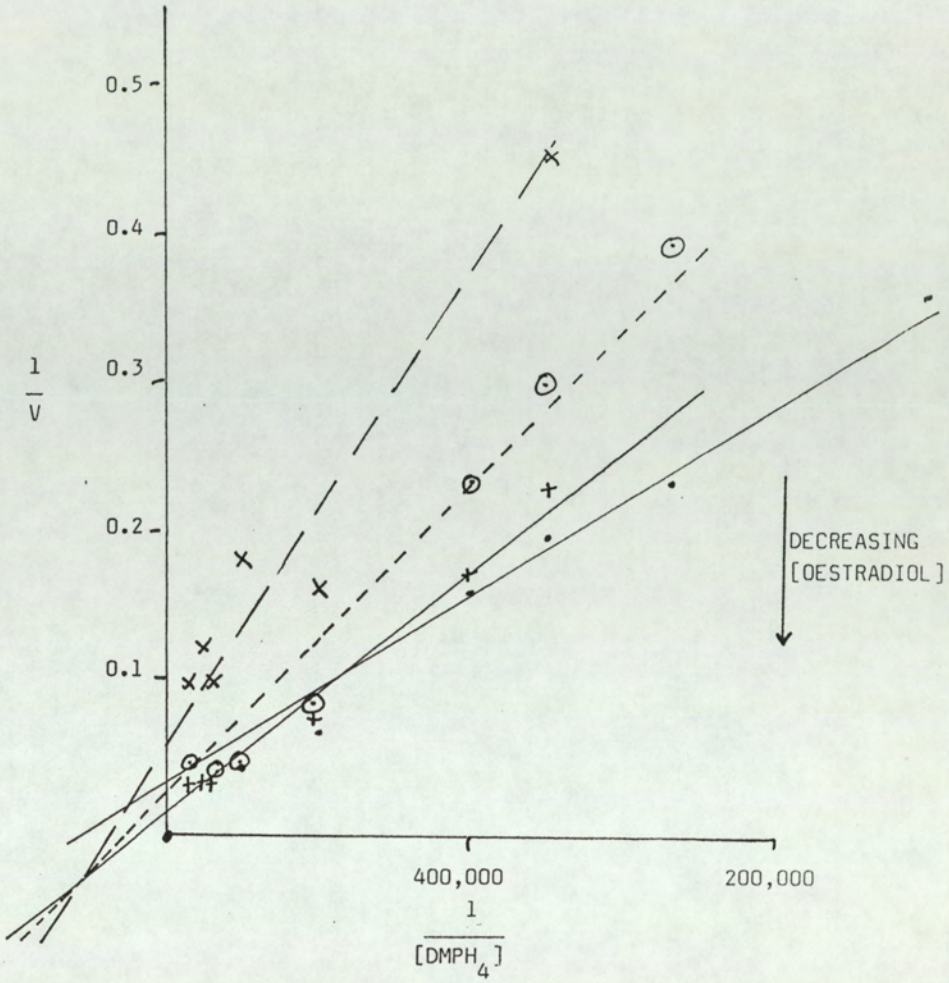


FIGURE 4.6 LINEWEAVER-BURK PLOTS VARYING THE CONCENTRATION OF $DMPH_4$, AT 10^{-4}M NADH, AT 3 CONCENTRATIONS OF OESTRADIOL:- 5×10^{-6}M (x---X), 10^{-6}M (⊙----⊙) AND 5×10^{-7}M (+———+).

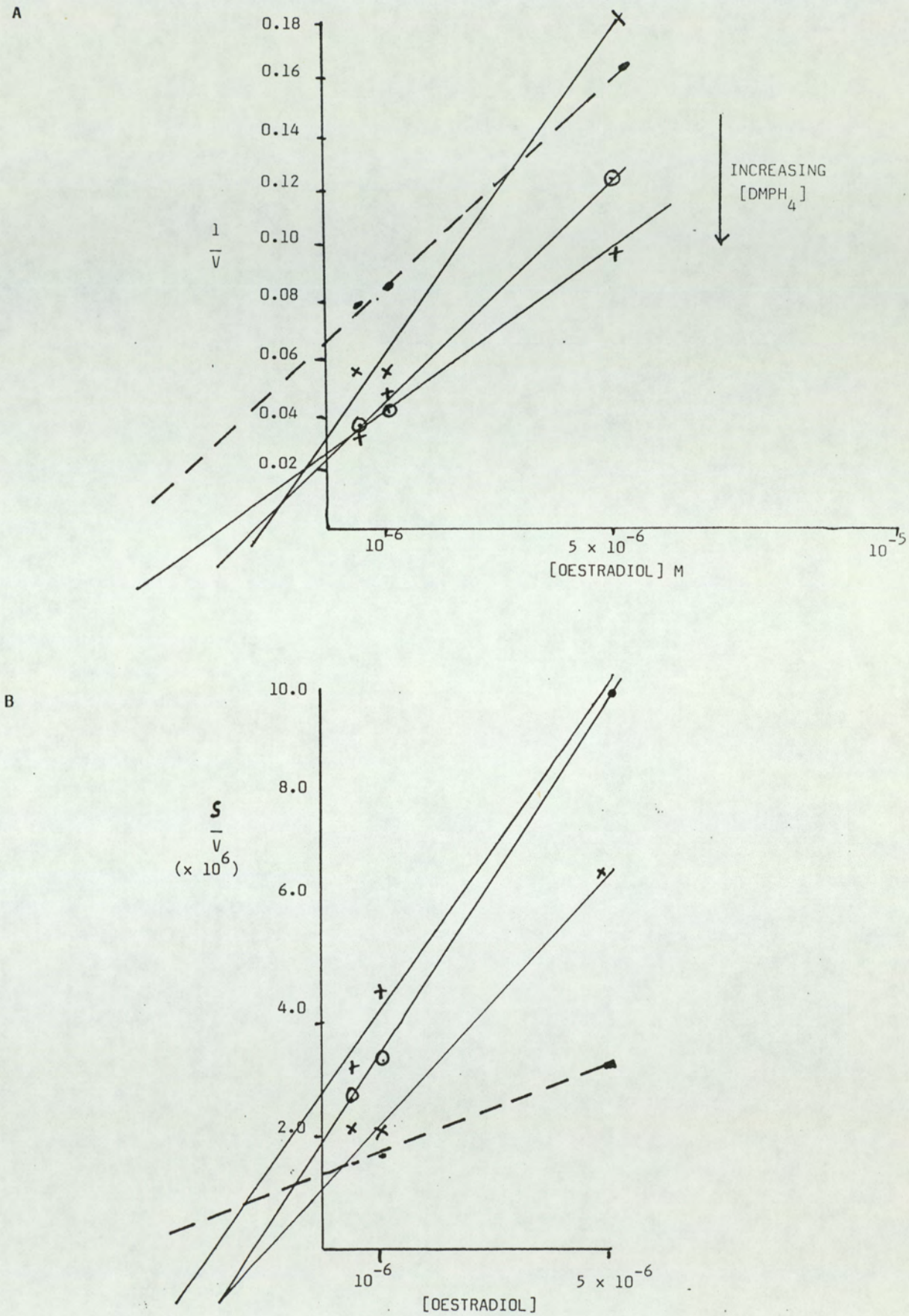


FIGURE 4.7 TWO KINETIC PLOTS OF THE EFFECT OF OESTRADIOL ON RAT LIVER DHPR ACTIVITY

A. DIXON PLOTS AT SEVERAL CONCENTRATIONS OF $DMPH_4$

B. S/V AGAINST [OESTRADIOL] PLOTS AT SEVERAL CONCENTRATIONS OF $DMPH_4$

DMSO effects to be seen for oestradiol at 1.3×10^{-5}M.

Further work with the catecholoestrogens was conducted in ethanolic solution but not only was the ethanol a slight inhibitor of DHPR, but also the catecholoestrogens did not dissolve well in ethanol. This meant that this work did not produce any utilizable results.

SUMMARY

1) DHPR activity is significantly increased in breast tumour tissue when compared to normal breast tissue. Such an elevation is not however, observed in tumours from the large intestine, implying a distinct difference between pterin metabolism within these two different tumour types. An obvious causal agent may be hormonal influence in the breast tumour samples, and Dhondt et al (1981) reported a significant correlation between DHPR activities and hormonal dependence as measured by cytosolic oestrogen receptors. In a later paper (Dhondt et al, 1983), they have also shown a significant correlation between cytosolic progesterone receptors and DHPR activity (as well as confirming their earlier results). A correlation was not found between DHPR activity and oestrogen receptors (table 4.5), in this experiment, but a significant correlation was found between enzyme activity and progesterone receptor levels.

2) DES caused a significant elevation in rat liver DHPR activity whilst having no effect on the uterii DHPR activity, although an oestrogenic effect was observed in the uteri. The liver is the site of oestrogen deactivation and breakdown so one might expect a localised increase in DES in the liver possibly causing the massive increase in DHPR activity. In

experiments not reported here DES did seem to have a direct activatory effect on purified rat liver DHPR, but it could also be increasing the actual levels of DHPR *in vivo* via an oestrogenic increase in protein production.

3) The gradual increase in DHPR activity in rat livers after 17 ethynyloestradiol dosing is also what would be expected if the actual levels of DHPR were being increased via protein production rather than by a direct activatory mechanism on the enzyme. Since the increase in activity is maintained over 72 hr. it is reasonable to assume, even with its long biological half life, that there has been an increase in enzyme protein.

4) The inhibition of DHPR by DMSO was unexpected. However, it is quite possible that DMSO is binding to a cation in the $DMPH_4$ binding site as it has quite a large dipole. This would be expected to give something like competitive inhibition as the bond formed would be weak allowing the DMSO to leave and bind quite rapidly. It is also quite possible that it is binding in the NADH site, giving rise to the problems in interpreting the results.

5) The I_{50} values for the oestrogens and their catechol derivatives show that these molecules could all be

strong inhibitors of DHPR *in vivo*. The catechol derivatives are all breakdown products of the original oestrogens and the catechol structure makes them prime possible effector molecules since several catecholamines and their derivatives have been shown to inhibit DHPR (Purdy *et al*, 1981; Shen, 1983). However, oestrone appears to be the strongest inhibitor as judged by I_{50} value and since the two catechols have almost identical I_{50} values one might believe that the catechol structure is extremely important in the control of DHPR activity. However, recent work by Armarego and Waring (1983b) suggests that it is the oxidation products of noradrenalin and adrenalin that are the actual inhibition products *in vivo*.

CHAPTER FIVE

DHPR ACTIVITY IN HUMAN BRAIN TISSUE, EXPERIMENTS ON PURIFIED HUMAN BRAIN DHPR

INTRODUCTION

The best documented role for tetrahydrobiopterin (BH_4), is as the cofactor required by the aromatic amino acid hydroxylases, phenylalanine hydroxylase (EC 1.14.16.1), tyrosine hydroxylase (EC 1.14.16.2) and tyrptophan hydroxylase (EC 1.14.16.4). (Kaufman and Fisher, 1974). Tyrosine hydroxylase is the enzyme at the rate limiting step in catecholamine biosynthesis (Levitt *et al*, 1965; Nagatsu *et al*, 1964), whilst tyrptophan hydroxylase is at the rate limiting step for serotonin biosynthesis (Costa and Meek, 1974). The tissue concentration of BH_4 is lower than the "normal" Km value for tyrosine hydroxylase and its concentration would thus appear to be rate limiting in the pathway (Kettler *et al*, 1974). There is, however, evidence that direct phosphorylation of this enzyme causes on activation of the enzyme which is expressed in kinetic terms by a lower Michaelis constant (Km) for BH_4 (Lovenberg *et al*, 1975; Pollock *et al*, 1981). The factors controlling the rate of serotonin biosynthesis are more involved, since not only is BH_4 present in rate limiting amounts, but also tryptophan concentrations are an important rate controlling factor (Jequier *et al*, 1967). Therefore, BH_4 is involved in the rate limiting steps and in a rate limiting capacity in the production of the neurotransmitters dopamine, noradrenalin and serotonin.

The importance of BH_4 for normal neurological function is highlighted in the genetic disease malignant hyperphenylalaninaemia (Danks et al, 1978). Children suffering from this disease have a progressive neurological disease with lowered neurotransmitter production, which, if left untreated, leads to hypotania and certain death in early childhood (Smith et al, 1975). The cause of the disease is a profound deficiency of BH_4, arising from a defective de novo biosynthesis of BH_4, or a failure to salvage quininoid dihydropterin due to a non functional DHPR. Examples of both these types have been reported (Kaufman et al, 1975; Leeming et al, 1976; Rey et al, 1977). Treatment of the disease is by L-dopa, carbidopa and 5-hydroxytryptophan administration (Bartholome and Byrd, 1975), which give a near normal development, though the long-term prognosis of treated patient is not known at present. If treatment is delayed, due to late diagnosis, development does take place (Leeming, 1979), but not as well as if early treatment is given (Rey et al, 1977). Malignant hyperphenylalaninaemia, therefore, acts as a model for the thesis that agents that affect the metabolism of biopterin may cause mental impairment.

The involvement of a defect in BH_4 metabolism has been suggested for a variety of mental diseases and disorders (Leeming and Blair, 1980; Leeming et al, 1981; Narabayashi et al, 1982; Le Witt et al, 1983). There is evidence in SDAT of significantly reduced levels of biopterin in

cerebro-spinal fluid (CSF) (Williams et al, 1980; Morar et al, 1983) and in serum (Aziz et al, 1983). Whilst reduced levels of BH_4 in CSF have also been reported in Parkinson's disease (Lovenberg et al, 1979), Huntingdon's chorea, Steel-Richardson syndrome (Williams et al, 1980) and dystonia (Williams et al, 1979) (The levels of BH_4 in CSF are assumed to reflect the BH_4 levels in the brain to a greater extent than serum or urine BH_4 levels). Such reports support the hypothesis that some disorders of a neurochemical nature are associated with disorders of biopterin metabolism.

The bulk of evidence relating to the neurological disorder in SDAT however, indicate a specific cholinergic neuron loss as being the outstanding pathological finding. This is analogous of the finding of dopamine in Parkinsonisam. Rossor et al (1982) reported a 71% decrease in choline acetyltransferase, the biosynthesis enzyme for acetylcholine, in the temporal cortex of SDAT sufferers. This being so, the reduced levels of biopterin in SDAT CSF suggest that research into BH_4 metabolism in SDAT temporal lobe tissue may well bring some enlightenment to this disease. The results of such a survey are reported in this chapter.

Several other brain areas have been assayed for DHPR activity and the results are reported in this chapter. DHPR activity has been reported in human tissues not known

to hydroxylate the aromatic amino acids (Craine et al, 1972; Sanchez-Urretia et al, 1978), also in rat brain tissue, DHPR has been measured in areas lacking the aromatic amino acid hydroxylases (Turner et al., 1974; Snady and Musacchio, 1978). The outcome of these experiments could not, therefore, be predicted but it would not be unreasonable to expect high DHPR activities in regions known to be catecholaminergic/serotonergic.

The majority of research on human DHPR has been on the enzyme purified from liver (Craine et al., 1972; Firgaira et al., 1979). The enzymes from liver (containing aromatic amino acid hydroxylases), cultured fibroblasts and continuous lymphoid cells (lacking aromatic amino acid hydroxylases), have been compared using immunological and molecular methods, and the results support the concept that the enzyme from all these sources in encoded in the same structural gene(s) (Firgaira et al., 1981b). It would seem likely therefore, that human brain DHPR would be the same enzyme, although there is the possibility of the brain enzyme being different. The affinity chromatographic method for DHPR of Cotton and Jennings (1978) was used to isolate human brain DHPR for kinetic characterisation studies, and for use in other assays with effector molecules.

The effect of lead and aluminium on some aspects of biopterin metabolism have been previously reported

(Leeming, 1979; Brown, 1981; Purdy *et al.*, 1981; Leeming and Blair, 1980; Barford *et al.*, 1982). Lead significantly inhabits BH_4 synthesis and DHPR in rat preparations, the resultant lowered cellular levels of BH_4 being implicated in lead neurotoxicity (Leeming, 1979; Brown, 1981; Purdy *et al.*, 1981), whilst aluminium inhibits the salvage of quinonoid dihydrobiopterin in rat preparations at levels similar to those in the brain of patients with dialysis dementia (Leeming, 1979; Brown, 1981). The neurotoxicity of these metals may therefore be due in some part to their inhibitory effects on biopterin metabolism. As this previous work has been on rat tissue preparations, there is still the need to show these effects on the human tissue preparation in order to verify that what is happening in the rat *in vitro* is applicable to the situation in man *in vitro*.

Purdy and Blair (1980), showed that phenylpyruvate, a metabolite of phenylalanine, is a good inhibitor of rat liver DHPR (K_i App = 50 uM). Plasma phenylalanine and phenylpyruvate levels show a high correlation in phenylketonurics on and off the phenylalanine restricted diet, with a ratio of 12:1 (amino acid/keto acid) at plasma phenylalanine values of 1.7mM (Langenbeck *et al.*, 1981). These results indicate that the neurological disorder observed in untreated and, to a lesser extent, in treated phenylketonurics, may be due in part to the inhibition of DHPR by phenylpyruvate. The effect of phenylpyruvate on

human DHPR was investigated.

In this chapter DHPR activity in SDAT and control temporal lobe tissue, as well as in a variety of other brain tissues will be reported. The results for the effect of metal salts on crude preparations and purified human brain DHPR activity will also be presented.

RESULTS

Dihydropteridine Reductase Activity in Human Temporal Lobe Preparations

Temporal lobe samples, some of which were specifically Brodmann area 21, were prepared by method 2 (see chapter 2). The samples were kept at -70°C until preparation, DHPR activity was measured immediately after preparation.

Three sets of data for DHPR activity are given in table 5.1, DHPR in Brodmann area 21 of temporal lobe, DHPR activity in temporal lobe cortex and DHPR in temporal lobe irrespective of the area, of normal subjects and subjects dying with senile dementia of Alzheimer type (SDAT). In all three cases the SDAT brain samples have a higher DHPR activity than the control samples, but this elevation over control activities is not significant.

The Broadmann area 21 samples were from the MRC Brain Bank and further personal data for these samples is given in table 5.2. The DHPR activity in the SDAT samples was higher than control levels, but not significantly so. There does not appear to be any age or sex related change in DHPR levels in the control group and in addition there is no correlation between the time to post mortem and DHPR activity. The brain sample with the longest post mortem

TABLE 5.1 DIHYDROPTERIDINE REDUCTASE ACTIVITY IN HUMAN TEMPORAL LOBE TISSUE. NUMBER OF SUBJECTS IN BRACKETS

AREA OF BRAIN	DHPR activity (n mol, $min^{-1}mg^{-1}$) (mean ± S.D)	
	Normal Controls	Subjects with SDAT
Brodmann area 21	229.4 ± 111.0 (8)	295.5± 143.0 (8)
Temporal lobe cortex	218.5 ± 38 (4)	382 0± 150.0 (3)
Temporal lobe irrespective of area	225.7 ± 91.0 (12)	319.1± 143.0 (11)

TABLE 5.2 CLINICAL DATA FOR MRC BRAIN BANK SAMPLES

(Brodmann area 21)

Demented Samples

	Age	Sex	PM Delay(h)	DHPR n nol $min^{-1}mg^{-1}$
1	90	F	11	335.5
2	71	M	32	315.0
3	72	F	20	359.6
4	76	M	54	222.8
5	71	M	74	52.75
6	79	M	4	158.5
7	/	F	50	466.6
8	80	M	46	453.3

Control Samples

9	89	F	72	223.1
10	71	M	47	117.0
11	79	F	63	167.5
12	74	M	49	194.4
13	44	F	92	265.5
14	74	M	65	124.0
15	72	M	69	283.6
16	74	F	24	460.0

Mean age of Demented Samples 77 $\pm$ 6.8yr.

Mean age of Control Samples 71.8 $\pm$ 12.7yr

delay (92h) had one of the highest DHPR activities recorded in the control group (265.5 ± 23.0 $nmol.min^{-1}mg^{-1}$). This suggests that the enzyme is very stable and this is in agreement with work where DHPR activity was measured in the eluate from Guthrie card spots (Barford et al., 1983). The small size of this sample does mean that any differences in DHPR levels related to age or sex might, at the moment, be masked but further control and demented brain samples are being assayed (J.A. Blair and P.A. Barford personal communication), and the results of these further samples may highlight any such differences.

All the brain samples were assayed with an equimolar concentration of NADPH in place of NADH in the 2 assay proceedure (chapter 2), to verify that the actual activity being measured was due to DHPR. The characteristic preference for NADH over NADPH has been well documented for other mammalian dihydropteridine reductase (Craine et al., 1972; Cheema et al., 1973; Webber et al., 1978; Firgaira et al., 1979; Brown, 1981), the enzyme seldom have >10% of its activity with NADPH than NADH. In these samples the control brains had 5.8% if their NADH activity with NADPH, whilst the demented samples had 5.9% of their NADH activity. So the enzyme activity measured in these brain samples was that of DHPR.

Dihydropteridine Reductase in Other Brain Areas

The areas of the brain investigated are all associated with catecholaminergic or serotonergic enervation, and the measured activities are given in table 5.3. The amygdalas and Ammons Horn were from control patients, as was the pineal gland. The DHPR activity in a Downs brain Ammons Horn is also given. The Ammons horn has a highly significantly increased activity when compared to the amygdala ($P < 0.1\%$, Students t test), and the Downs Ammons Horn has a very much lower DHPR activity than the control levels but with only one sample further analysis is not possible,

The order of activity appears to be Ammons Horn > amygdala > pineal gland. The pineal gland, however, was from an elderly patients (82yrs) and an age related decrease in enzyme activity cannot be totally ruled out.

PURIFICATION AND KINETIC CONSTANTS OF HUMAN DHPR

Figure 5.1 gives the elution profile of DHPR from human brain temporal lobe (the sample used was one from a patients dying with SDAT). The enzyme was purified 14 fold wioth a 22% recovery. The most active fractions from the column were pooled and used in various assays to assess the effects of substrate concentration and effector molecules/

TABLE 5.3 DIHYDROPTERIDINE REDUCTASE IN HUMAN BRAIN REGIONS (Mean ± SD)*

	DHPR activity n $mol.min^{-1}$ mg^{-1}
Amygdala (2)	273.8 ± 32.0
Ammons Horn (2)	359.0 ± 76.2
Pineal Gland (1)	101.5 ± 14.0
Downs Brain Ammons Horn (1)	172.2 ± 28.0

(*) Number of samples in brackets.

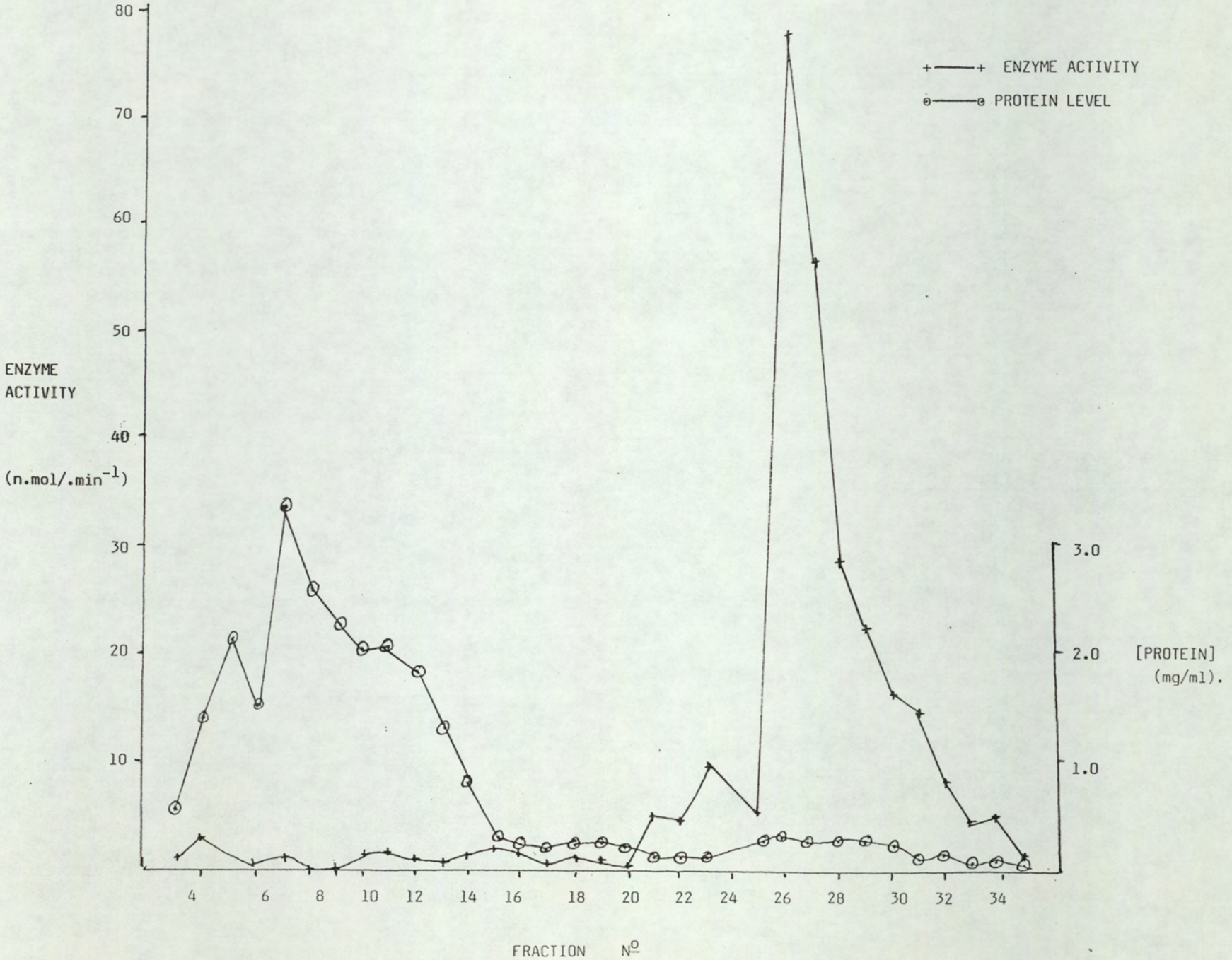


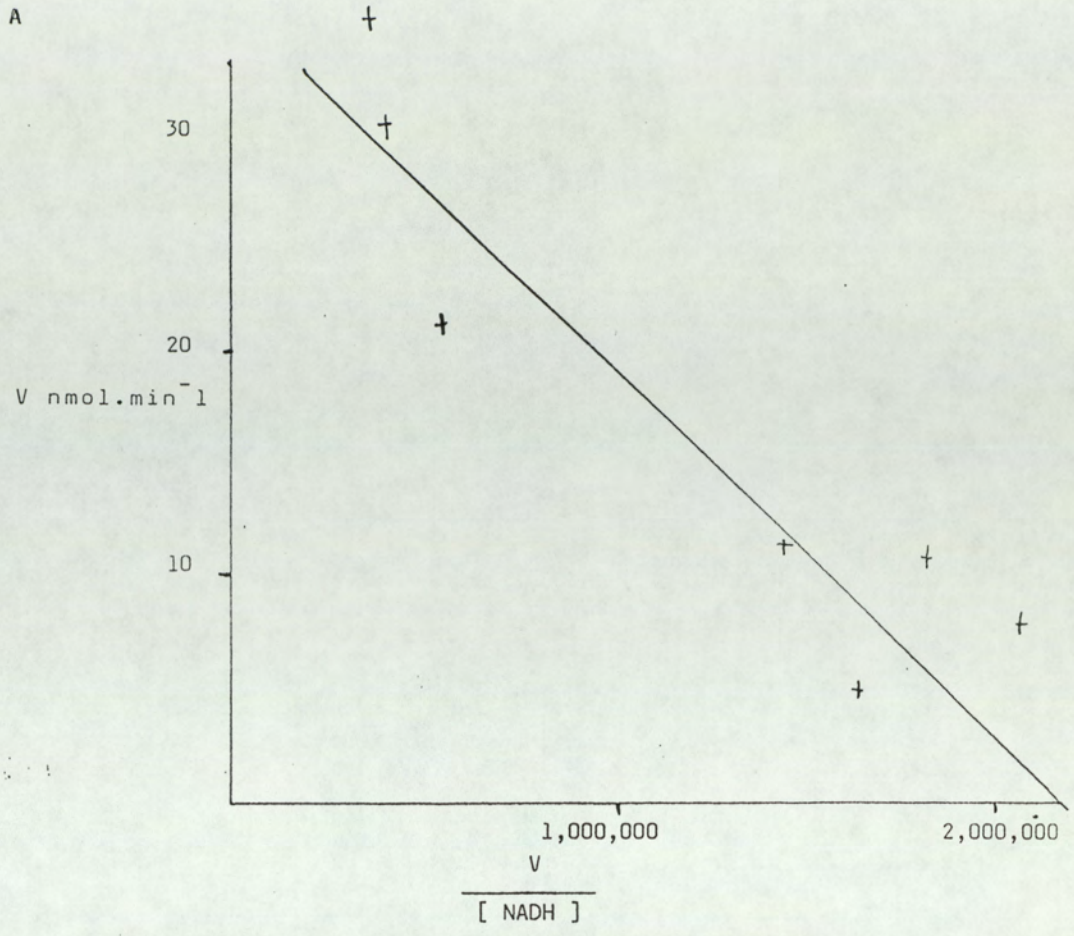
FIGURE 5.1 ELUTION PROFILE OF HUMAN ENZYME AFFINITY COLUMN (RUN AS STATED IN THE METHODS AND MATERIALS CHAPTER)

ions on human DHPR activity.

The plots in figure **5.2** are two different linearizations of experimental data in order to obtain a Km for NADH. The first plot (A) an Eadie-Hofstee plot, gives the Km for NADH as 1.6 x 10^{-5}M, whilst the Hanes plots (B) gives a slightly higher Km, at 2.1 x 10^{-5}M. The same plots as those in figure **5.2** are shown in figure **5.3** but in this case q $DMPH_2$ was the substrate being varied giving a Km for $DMPH_2$ as 2.5 x 10^{-5}M (Plot A) and 3.2 x 10^{-5}M (Plot B). (The plots drawn in Figures **5.2** and **5.3** are lines of best fit as calculated by the least squares method).

The Effect of Lead on Human DHPR Activity and in vivo on Rat Brain DHPR Activity

Initial experiments were performed on crude extracts (after 104,000xg centrifugation), from human temporal lobe. Figure **5.4** shows the results obtained on incubating the extract from Downs brain with 10^{-6} and 10^{-7}M lead acetate. This also shows the protective effect of 10^{-4}M NADH on heat denaturation over the experimental time period. Figure **5.5** shows similar results obtained using a SDAT brain preparation. In both these figures there is no apparent inhibition of DHPR activity in crude extracts over 30 mins. It is however, quite well known that the high protein concentrations in crude preparations are capable of



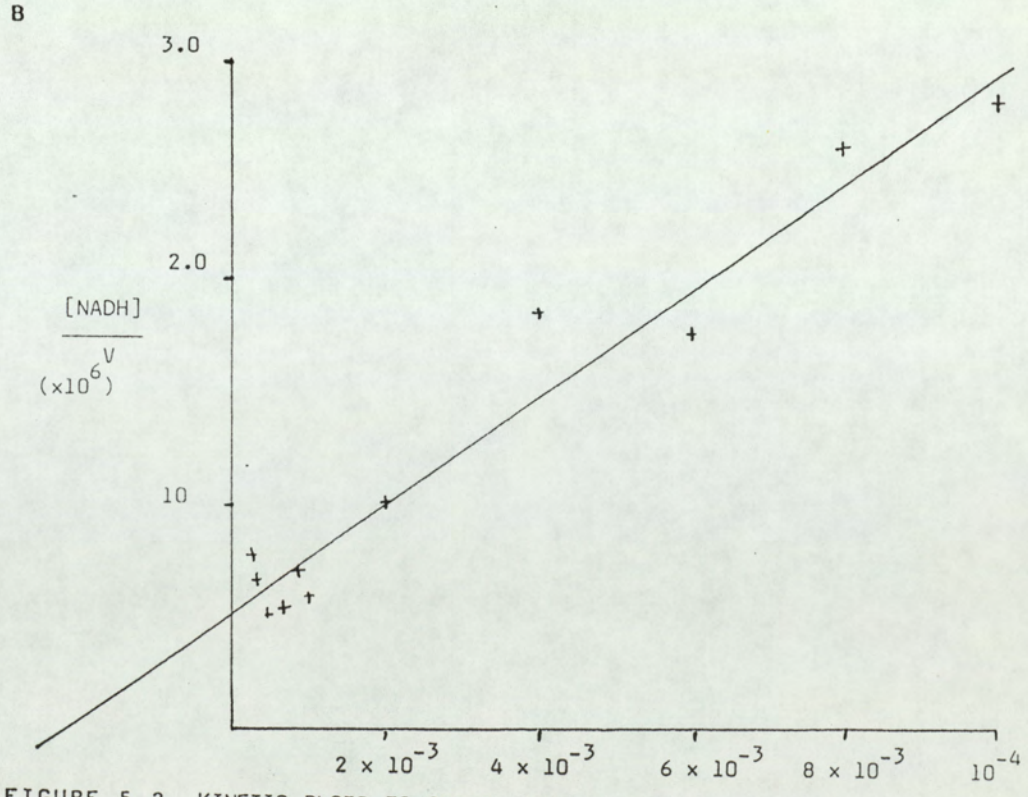


FIGURE 5.2 KINETIC PLOTS TO OBTAIN KINETIC PARAMETERS FOR NADH, ASSAYED AT 10^{-4}M $DMPH_4$ (CRAINE ASSAY).

A EADIE-HOFSTEE PLOT ($\frac{V}{[NADH]}$ against V)

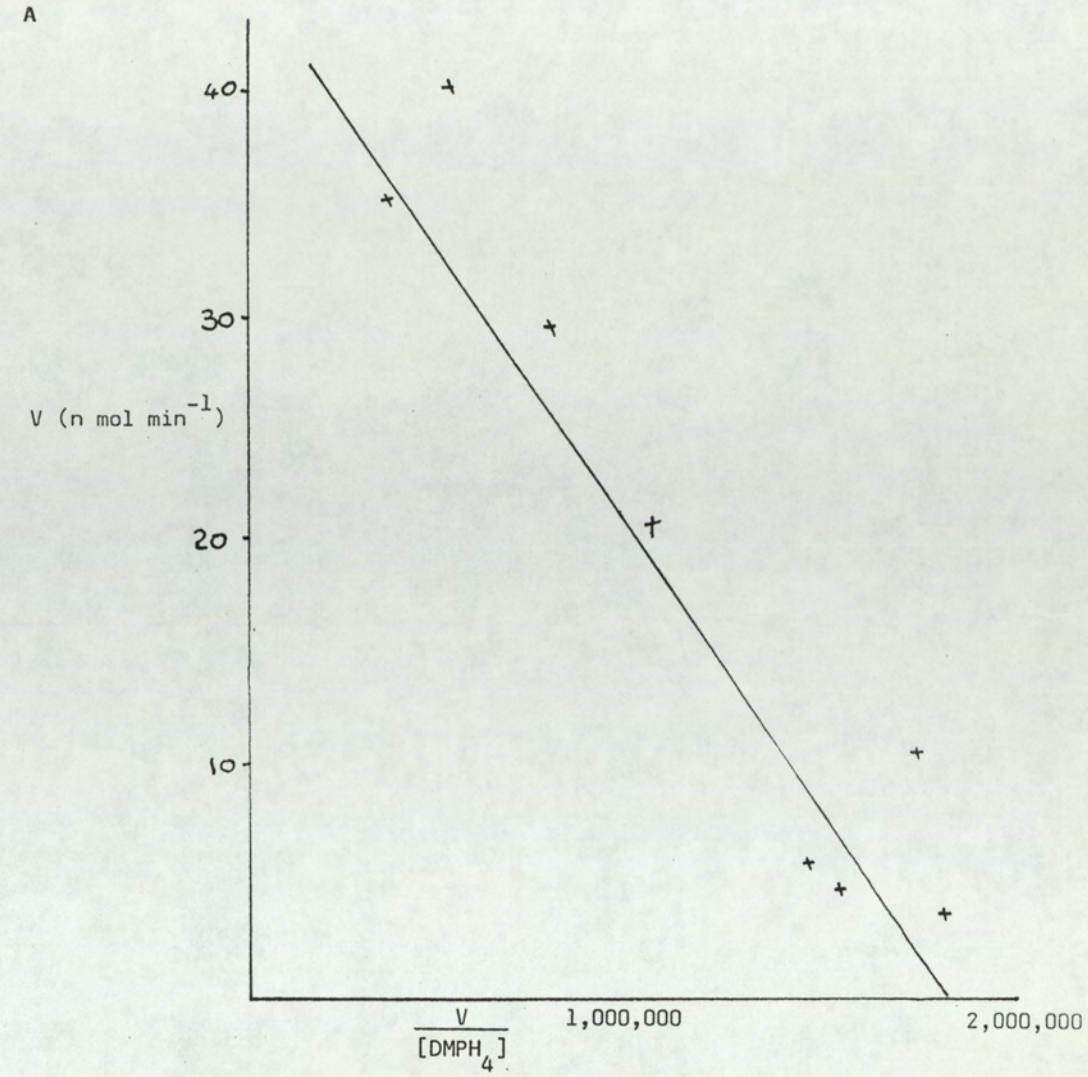
B HANES PLOT ([NADH] against $\frac{[NADH]}{V}$)

FIGURE 5.3

Graphs to obtain kinetic parameters for $DMPH_4$, the concentration of $DMPH_4$ varying at constant NADH concentration (10^{-4}M)

A Eadie-Hofstee Plot of $\frac{V}{[DMPH_4]}$ verse V

B Hanes Plot of $[DMPH_4]$ verse $\frac{[DMPH_4]}{V}$


A
40
30
20
10
V (n mol min^{-1})
$\frac{V}{[DMPH_4]}$
1,000,000
2,000,000

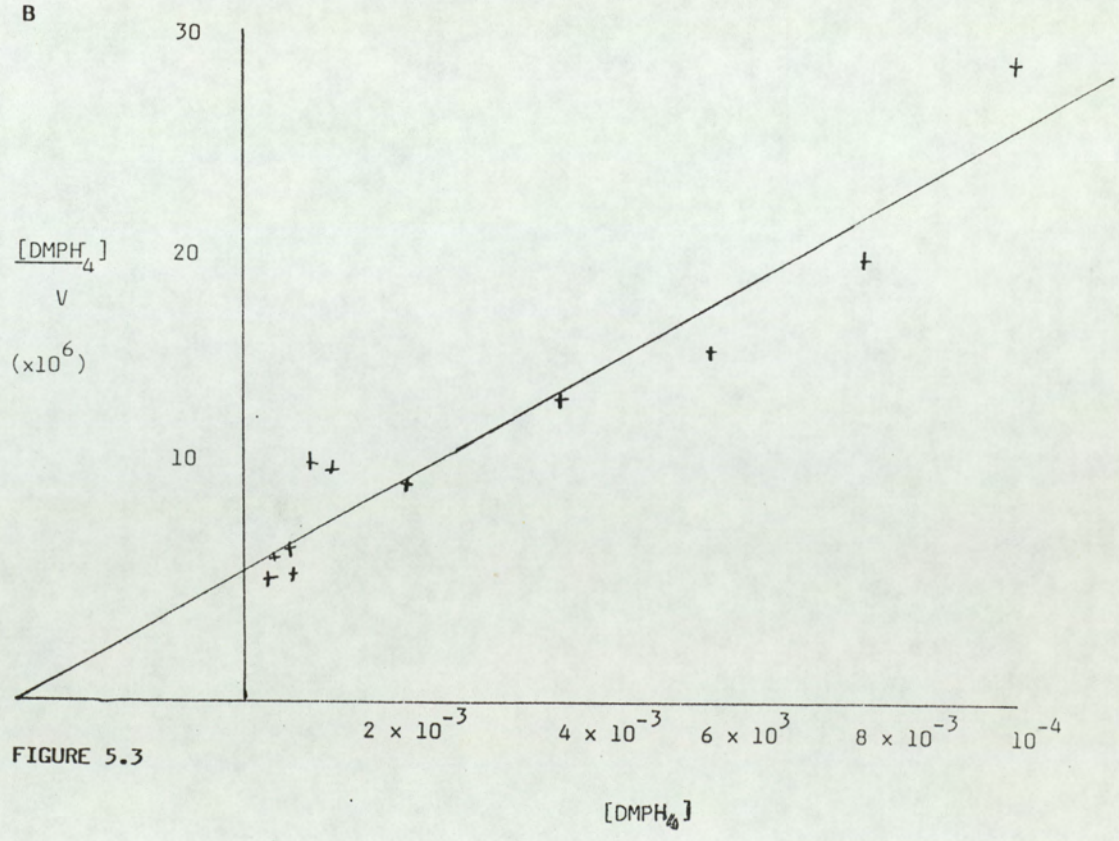

B
30
20
10
$\frac{[DMPH_4]}{V}$
(x10^6)
2 x 10^{-3}
4 x 10^{-3}
6 x 10^3
8 x 10^{-3}
10^{-4}
[DMPH$_4$]

FIGURE 5.3

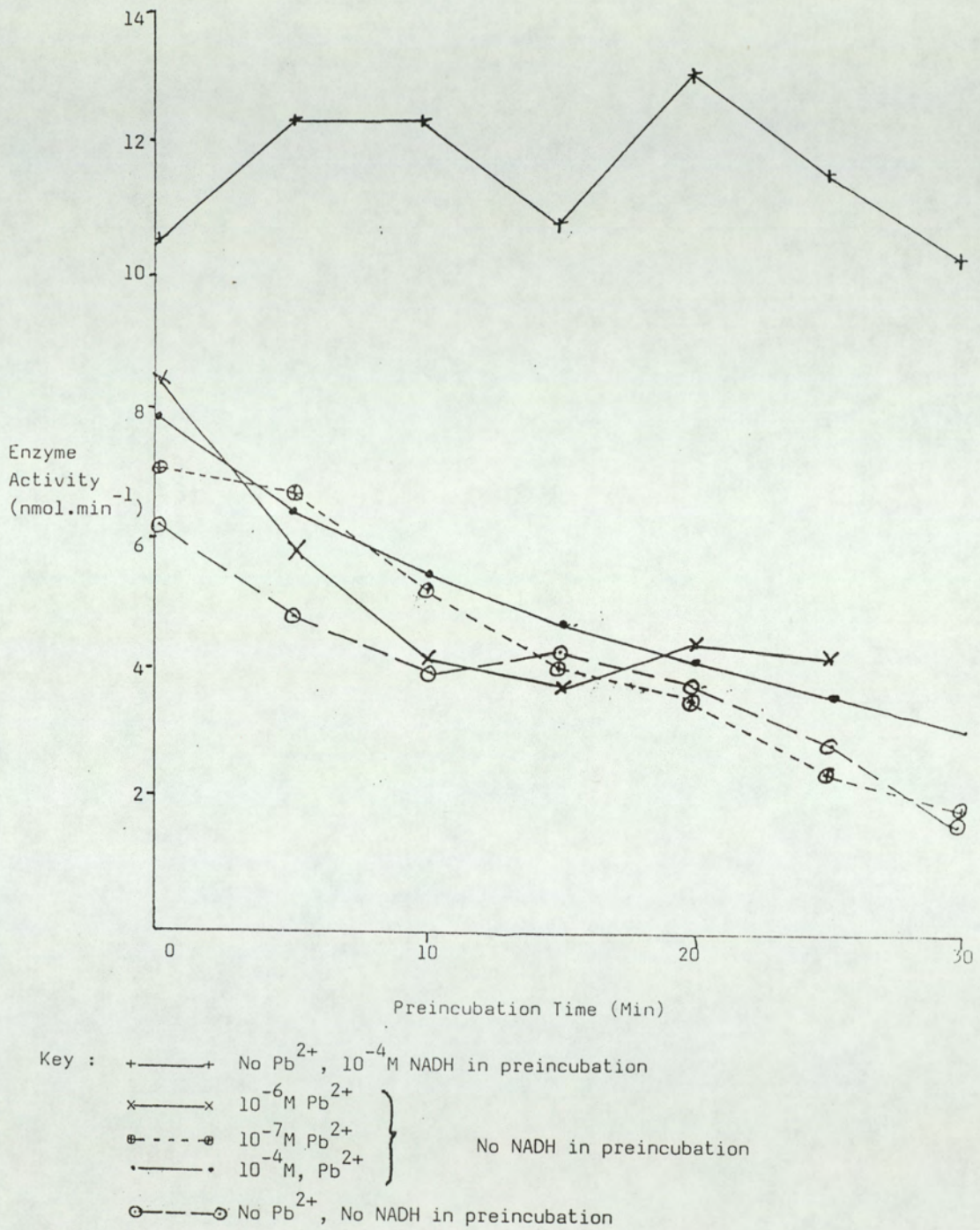


Figure 5.4

The effect of preincubation with lead acetate on Downs brain DHPR activity (Crude Preparation) Lead and enzyme solutions mixed and aliquotes assayed for enzyme activity.

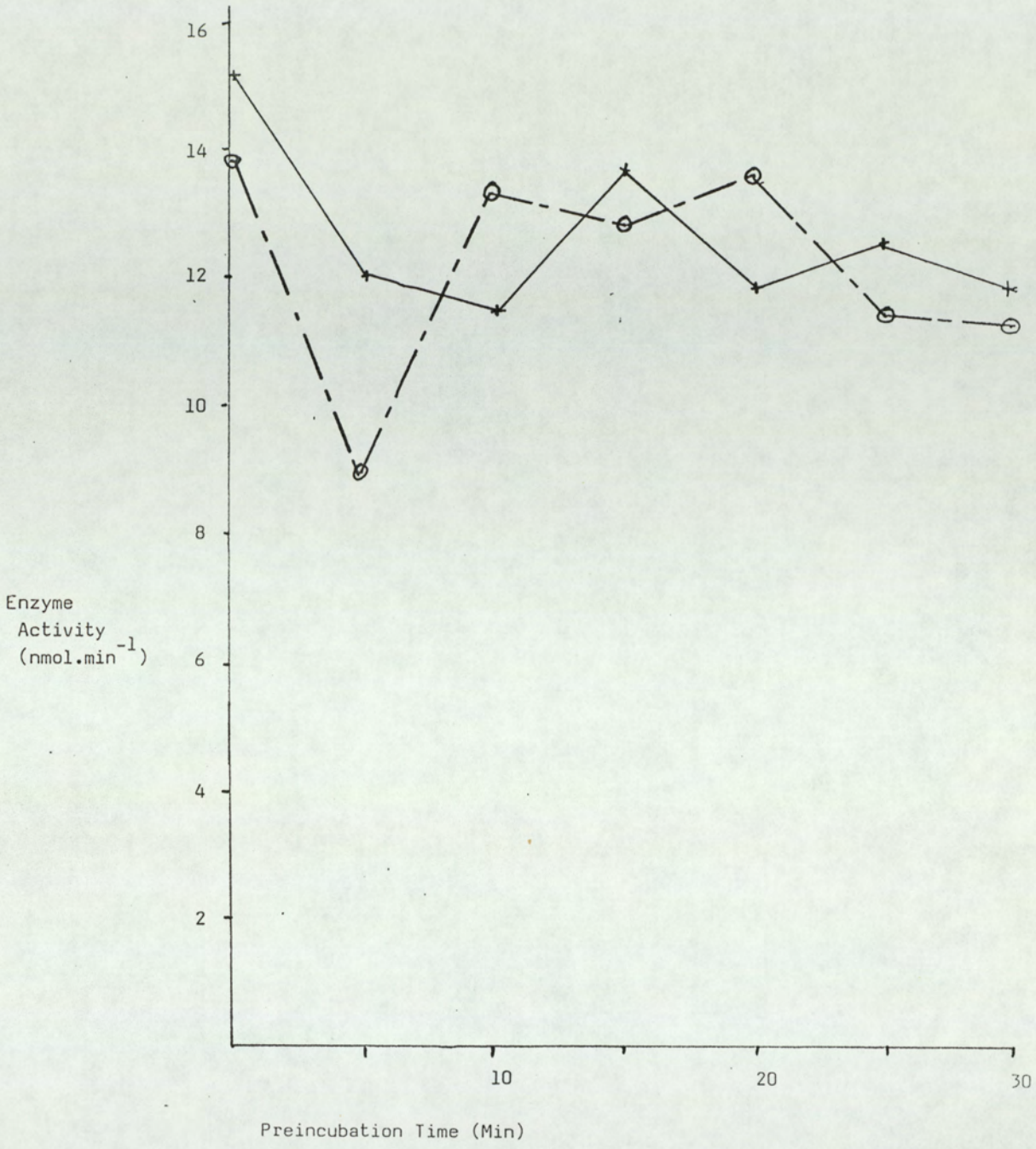


Figure 5.5

The Effect of 10^{-4}M lead acetate on SDAT DHPR activity (Crude preparation). Enzyme and lead were mixed, preincubated at 37°C, and aliquotes were removed and assayed from enzyme activity at the times shown.

sequestering a large proportion of the heavy metal ions present, thus reducing the effective concentration of the ions (Cornish-Bowden, 1979). The purer the enzyme is the less it might be protected by other proteins so these experiments were repeated with the purified enzyme.

Figure **5.6** shows the results obtained when purified human DHPR was preincubated with increasing concentrations of lead acetate. These results are also represented as percentage inhibition against time of preincubation of matched controls (Figure **5.7**). The inhibition by lead appears to be independent of the concentration of the lead ion, (61, 65 and 75% are the final percentage inhibitions recorded with 10^{-4}, 10^{-5} and 10^{-6}M lead respectively). This is strongly indicative of irreversible inhibition.

In order to ascertain whether lead can effect DHPR activity in vivo female rats were mated then kept in cages supplied with drinking water (ad libitum) containing lead at either 300, 1,000 or 2,000ppm (Carmichael, et al., 1981). The leaded water regime was continued until the pups were 21 days old, at this time they were sacrificed, the brains were removed and prepared and assayed for DHPR activity and tetrahydrobiopterin synthesis. In a series of experiments the 2 halves of the brain were prepared separately for DHPR activity assessment. A small number of striatums were also prepared for enzymic assessment the results of these experiments are shown in table **5.4** (the results for the

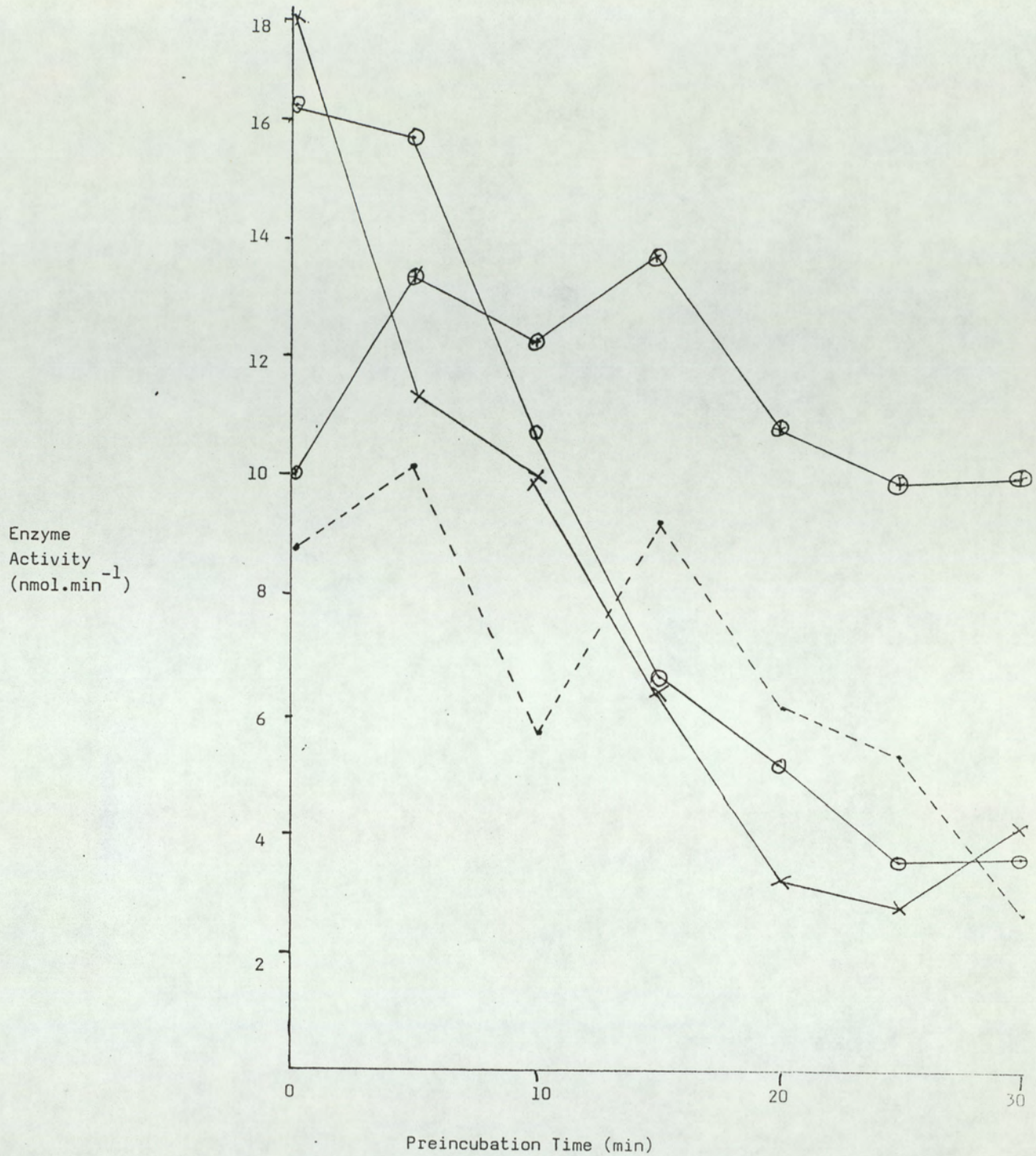
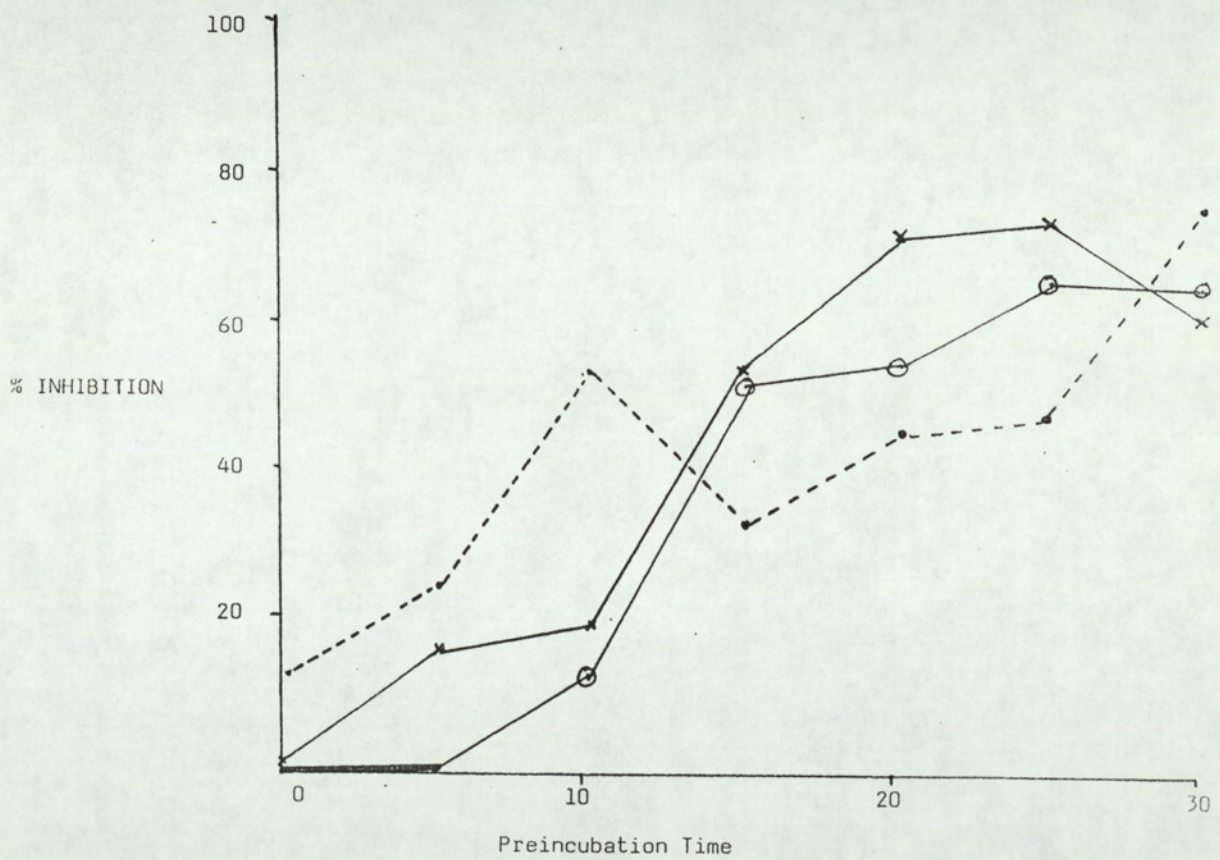


Figure 5.6

The effect of preincubation with lead acetate on purified human DHPR.



Key : See Fig. 5. 6

Figure 5.7

% Inhibition of human brain DHPR by lead acetate over 30 Min.

TABLE 5.4

THE EFFECT OF LEADACETATE DOSING ON DHPR ACTIVITY IN HALF AND WHOLE RAT BRAINS AND IN THE STRIATUM (MEAN ± SD)

REGIME	DHPR ACTIVITY ($nmol.min^{-1}$)			
	LEFT Halves	RIGHT Halves	Whole Brain	Striatum
CONTROL	224.8±35.0	237.2 ± 16.6	230.7±27.7	297.4 ± 6.15
300,ppm	177.9±21.6*	223.1 ± 14.5	197.9±29.5*	267.8 ±32.2
1000,ppm	177.4±43.0**	220.4 ± 25.2	193.0±43.6*	289.4 ±23.6
2,000,ppm	199.9±65.0	221.9 ± 10.5	209.5±49.3	

* P < 1% Students t test

** P = 5% " " "

whole brain DHPR activity were calculated by summing the results for the left and right halves). In the left half of the brain there is a significant decrease in DHPR activity observed at 300ppm ($P < 1\%$). This decrease is also seen in the brains dosed with 1,000ppm but the depression is not so significant ($P = 5\%$). The pattern is repeated in the whole brain result, with the decrease in activity at 300ppm and 1,000ppm when compared to control brain being significant ($P < 1\%$). However, the results for the right halves of brain show no decrease in activity after lead dosing. These results may be in some way related to asymmetries in the brain and this will be further examined in the general discussion (chapter 6).

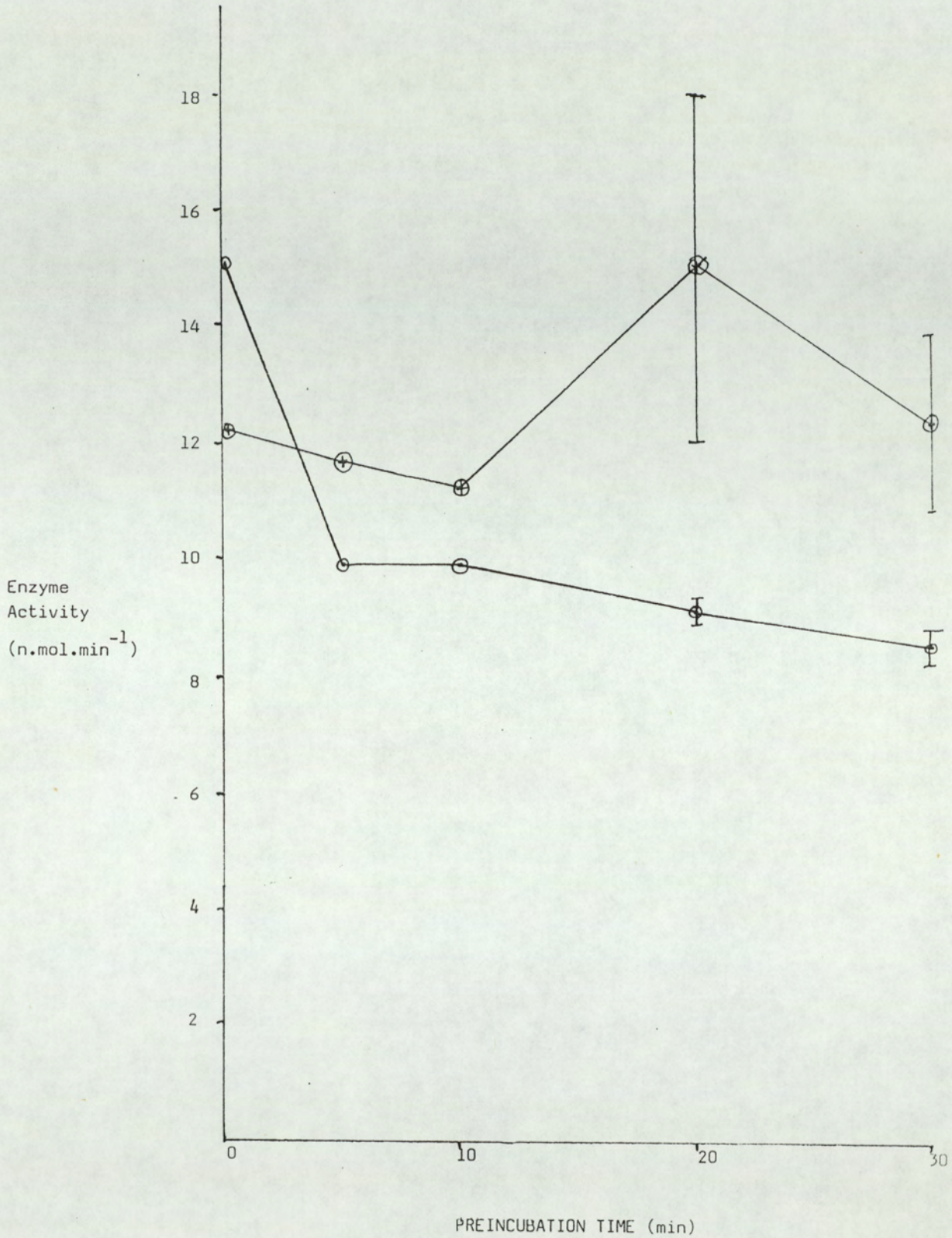
In all the brain areas assayed the activities recorded in the 2,000ppm samples were the same as the control brain activities. The apparent lack of an effect may in part be explained by some of the physiological effects of lead; at 2,000ppm brain weight, body weight and body length are lower than normally found in age matched controls (Carmichael et al., 1981). The activity measured in the 2000ppm samples may thus appear normal due to a lowered protein level in the sample masking a concomitant decrease in DHPR activity. When the brain weights of the control sample were compared to the 2,000ppm sample a significant difference was observed (1.43 ± 0.05gm versus 1.28 ± 0.09gm respectively $P = 1\%$).

The result for the striatum show the same trend as the left temporal lobe with a decrease in activity after 300ppm lead. The sample number however, was too small for statistical evaluation.

Effect of Aluminium on Human Brain DHPR

In the light of recent research, which strongly implies a link between aluminium levels and dialysis dementia, (Crapper et al, 1973; Alfrey et al, 1976; McDermott et al, 1978; Leeming, 1979; Brown 1981), an experiment was performed in which DHPR was preincubated with 10^{-4}M aluminium sulphate and aliquots taken and assayed for enzyme activity, the results are shown in figure 5.8. The results represented at 20 and 30 minutes are given with their standard deviations to illustrate the difference between the 2 sets of data, (before 20 min the standard deviations of the 2 sets of points overlap).

The results show that after 20-30 min. of preincubation in the presence of 10^{-4}M aluminium sulphate, the enzyme activity is reduced by 35% compared to control levels.



Key ⊕-----⊕ control

⊙-----⊙ 10^{-4} M Al^{3+}

Figure 5.8

The effect of preincubated with 10-4 M aluminium on purified Human DHPR Activity.

Effect of Phenylpyruvate on Human DHPR

In initial assays, the presence of 10^{-4}M phenylpyruvate gave an apparent increase in enzyme activity, compared to controls. Assays in the absence of $DMPH_4$ gave an increased activity compared to assays in the presence of $DMPH_4$ and phenylpruvate. This method measures a decrease in NADH so it would appear that a contaminent in the enzyme preparation was causing phenylpruvate to be reduced at the expense of NADH. A percentage inhibition for phenylpyruvate was obtained however, by subtracting the rate of the phenylpyruvate "reductase" from the rate of DHPR activity in the presence of phenlpyruvate, and gives a percentage inhibition at 10^{-4}M phenylpyruvate as 8%.

This apparent phenylpyruvate reductase activity has been reported before for the rat liver enzyme (Brown, 1981) and seems to be related to the low purification of the enzyme. Further work needs to be carried out to clarify the effect of phenylpyruvate on human DHPR of a higher purification.

SUMMARY

1) Previously workers looking at variations in biopterin metabolism in senile dementia of Alzheimer type (SDAT) patients have shown that there is a significant reduction in the level of total biopterin in the serum

(Leeming et al., 1979; Aziz et al., 1983; Morar et al., 1983), and that a lowered brain BH_4 concentration (Nagatsu et al., 1979) is accompanied by a slightly raised phenylalanine to tyrosine ratio (Leeming et al., 1979). Neopterin levels however, in both urine and serum of SDAT patients are similar to those found in normal healthy controls (Al-Bier, 1982; Young et al., 1982). The sole role of neopterins in the body is the production of BH_4 (via biopterin), thus in SDAT there would appear to be a breakdown in the BH_4 biosynthetic pathway. The results presented in this chapter (table **5.1**) show that DHPR activity is elevated in SDAT brains, when compared to age matched controls (319.1 $\pm$ 143.0 versus 225.7 $\pm$ 91). This elevation in activity is probably in response to the lower levels of BH_4. The large range of DHPR activities recorded (table **5.2**), in both the SDAT and control brain temporal lobe samples would tend to mask such a feedback activation and it is hoped that a larger sample number would increase the significance of the elevation in the SDAT brains.

To verify that the enzyme activity being measured in these samples was in fact DHPR, the brain samples were all assayed in the presence of an equimolor concentration of NADH and then with NADPH, and in both control and SDAT samples there was < 6% of the enzyme activity with NADH in the presence of NADPH. In

addition the dual assay system was used (see Ch. 2) eliminating the possibility of other NADH oxidases in the samples masking/elevating the DHPR activities recorded.

2) DHPR activity was measured in several brain areas, giving the following result, Ammons Horn > amygdala > temporal lobe > pineal gland. The small sizes of all but the temporal lobe sample and the large range of activities measured in the temporal lobe samples however, does suggest that the order of DHPR activity may well be altered as sample sizes increase.

3) The purification of human DHPR was not very successful for several reasons giving only a 14 fold purification.

Typical hyperbolic curves were obtained from experiments on varying the concentration of substrates on enzyme activity, and the Km values for q $DMPH_2$ and NADH were determined from Eadie-Hofstee and Hanes plots as 2.85×10^{-5}M and 1.85×10^{-5}M respectively. These values are close to those previously reported (Firgaira et al., 1981; Shen, 1983), and illustrate the sensivity of the assay method, as this enzyme preparation was far less highly purified than those used by the other workers.

4) Lead had no inhibitory effect on human brain crude

extract DHPR activity, probably because of the sequestering of the lead ions by proteins in these crude preparations. What is noticable from these experiments (Figure 5.4), is the protective property of NADH against heat deactivation of DHPR.

However, lead was strongly inhibitory on the purified human enzyme (Figures 5.6 and 5.7) giving a 75% inhibition of activity after 30 minutes at 10^{-6}M. It is possible that this figure is not as large as it might be due to the presence of contaminating proteins in this enzyme preparation. Further work on more highly purified enzyme will elucidate this.

DHPR activity effected by lead <u>in vivo</u>, this is shown in the rat dosing experiments. These experiments however, also seem to predict that the two halves of the brain may be differentially effected by lead, as although the control brain halves have the same DHPR activity, lead dosing effects each half differently (Table 5.4). Thus it would be interesting to measure the effect of lead and the "Handedness" of the animal at a later date.

5) Human DHPR is inhibited by 10^{-4}M aluminium sulphate to 65% of its control activity after 30 minutes of preincubation. This is less than reported for the rat liver enzyme (Brown, 1981) however, that enzyme

preparation was more highly purified than the one used here and again sequestration of metal ions by contaminating proteins may give rise to this lower inhibitory effect. It has been previously reported that the salvage of q BH_2 is inhibited by levels of aluminium observed in dialysis dementia (Leeming, 1979; Brown, 1981), and the results reported here are in general agreement with the involvement of a disruption in biopterin metabolism in dialysis dementia caused by excess aluminium.

C H A P T E R S I X

GENERAL DISCUSSION

GENERAL DISCUSSION

That dihydropteridine reductase (DHPR) plays a most important role in the maintainance of normal brain functions is in no doubt, as witnessed by the gross neurological disorders observed in the form of malignant hyperphenylalaninaemia (MHPA) in which DHPR is non functional/missing. This disorder equally illustrates, of course, the importance of tetrahydrobiopterin (BH_4) for normal brain function. The role of BH_4 in the brain is as the cofactor for 3 enzymes, phenylalanine, tyrosine and tryptophan hydroxylases (Kaufman and Fisher, 1970; Lloyd and Weiner, 1971; Friedman et al., 1972), and it is as the cofactor for tyrosine hydroxylase that it acts in a rate limiting manner for the production of the neuromodulators dopamine and noradrenalin, cellular BH_4 levels being on or below its Km for the enzyme (Kettler et al., 1974). In addition the tryptophan hydroxylase catalysed step is rate limiting in the biosynthesis of the neurotransmitter serotonin. However, it appears that the concentration of tryptophan is the rate limiting factor (Costa and Meek 1974). Thus DHPR plays an extremely important role in the brain in maintaining cellular levels of BH_4 and ensuring that in normal circumstances the biosynthesis of these important neuroactive molecules can proceed.

DHPR is a salvage enzyme responsible for reducing the quinonoid dihydrobiopterin formed as a product of the

hydroxylase reaction back to BH_4. The labitily of the quinonoid species however, makes DHPR quite a strong candidate in controlling the levels of BH_4 and hence influencing the production of dopamine, noradrenalin and serotonin. Factors effecting DHPR would provide a means of fine controlling the production of these molecules in the short term however, one would expect longer term control to be effected via the BH_4 biosynthetic pathway. That BH_4 was biosynthesised in mammalian cells from GTP was reported in the early 1970's (Sugiura and Goto, 1973; Fukushima and Shiota, 1974), and although the elucidation of the individual intermediates has been investigated since then, not all the intermediates have been characterized (Kisluik and Brown, 1980; Nichol et al., 1983). Although there is still not an agreed biosynthetic pathway in mammals the first steps would seem to be conversion of GTP to dihydro-neopterin triphosphate by GTP cyclohydrolase which is then converted to sepiapterin via an unknown intermediate (Tanaka et al., 1981). However, there is some dispute as to whether sepiapterin is a biosynthetic intermediate (Duch and Nichol, 1983; Nichol et al., 1983). So although there are most probably control points for BH_4 production, until such time as the pathway is unambiguously elucidated these will have to remain open to speculation.

The aim of the work presented in this thesis was to examine dihydropteridine reductase both from the rat and from man in vitro, and to see how the enzyme activity could be

altered by various reagents some of which are thought to effect tetrahydrobiopterin levels *in vivo*. In addition DHPR activity was measured in human tissue samples from patients with a variety of disorders and compared to control tissue when available. The results from these experiments were then compared with similar results and with those from *in vivo* studies with a view to relating *in vitro* results to the *in vivo* situation.

The two assay methods employed in this study (Cheema *et al*., 1974; Craine *et al*., 1972) where found to give easily replicable results when used on purified enzyme samples. For studies to determine the effect of effector molecules on enzyme activity the method of Cheema *et al*, did appear to be the ideal choice as it does not depend on another enzyme for the generation of the quininoid substrate. However, in experiments using crude human tissue preparations a major problem was encountered, as contaminants in these samples gave elevated levels of NADH usage. This activity probably being due to the presence of diaphorase in the crude samples (the name diaphorase being applied to enzymes which catalyse the oxidation of either NADH or NADPH in the presence of an electron acceptor such as dichlorophenolindophenol (DCPIP). This problem was overcome by modifying the Craine assay into two assays as outlined in chapter 2.

There is now a third assay procedure (Hasegawa, 1977; Narisawa et al., 1980), which utilises the BH_4 dependant reduction of ferricytochrome C in the presence of NADH and uses 6 methyltetrahydropterin as the substrate source. The assay has been used to assess DHPR activity in blood samples from patients with mental disease, and in eluates from Guthrie cards from children suspected of suffering from the genetic disease MHPA (Barford et al., 1983). This work has also highlighted the stability of DHPR, as activity can be eluted from Guthrie cards which have been stored at room temperature for several weeks (Leeming et al, 1984). The method however, does have one major disadvantage since the levels of pterin used in the assay are not saturating (10^{-5}M).

Work carried out on purified enzymes is usually easier to interpret and correlate with clinical studies as only one small section of the in vivo situation is being investigated rather than a complete pathway. In the case of rat liver DHPR since the enzyme was isolated as a relatively pure fraction the results gained are unlikely to be artifacts due to contamination of the sample. However, the enzyme so obtained is in an artificial environment and, although every step is taken to ensure the enzyme has all the conditions required for optimal activity, it is unlikely that the in vitro system studied is exactly as one would find the enzyme in vivo.

The availability of an easily prepared affinity adsorbent for DHPR (Cotton and Jennings, 1978), makes it a relatively easy task to isolate the enzyme in a highly purified form. Previous workers have used a gel filtration step on Sephadex G150 for the initial purification before affinity chromatography (Brown, 1981). However, initial results were discouraging (Table **3.1**), and the isolation involved freezing and thawing a relatively impure sample, possibly containing proteinases. Hence, the simple high speed centrifugation - affinity chromatographic method was employed with great success, giving an enzyme sample purified 960 fold over the original supernatant, with a specific activity of 25,400nmol.min.$^{-1}$mg.$^{-1}$ and as high as 68% recovery. These results compare favourably with both those reported by workers using the naphthoquinone adsorbent (Cotton and Jennings, 1978; Brown, 1981), and those using other affinity chromatographic methods (Webber et al, 1978; Webber and Whitely, 1978; Aksnes et al., 1979; Chauvin et al., 1979). Affinity chromatography on the naphthoquinone adsorbent has a major advantage over the other affinity chromatographic methods. This ligand is believed to be effective due to its structural similarities to the enzyme quinonoid substrate (Cotton and Jennings, 1978), whereas some other workers have relied on ligands which attach to the enzymes NADH binding domains and thus are not DHPR specific, therefore requiring many other steps to aid purification (Scrimgeour et al., 1978; Aksnes et al., 1979; Chauvin et al., 1979; Nakanisi et al., 1982).

Since these methods use commercially available gels, however, they avoid the laborious and time - consuming preparation of the affinity gel. However, once prepared the affinity absorbent, if stored correctly, can be used repeatedly following regeneration (Cotton and Jennings, 1978).

The enzyme as isolated here conformed to all the general characteristics for rat liver DHPR, and gave Michaelis constants (Km's) of $1.7 \pm 0.5 \times 10^{-5}$M (n = 12) for NADH and $2.2 \pm 0.6 \times 10^{-5}$M (N = 9) for q $DMPH_2$, which are in agreement with previously reported values (Craine et al., 1972; Cheema et al, 1973; Webber et al., 1978; Webber and Whiteley, 1978; Brown et al, 1981).

Manganese acts as a cofactor for several mammalian liver enzymes such as; arginase, super-oxide dismutase, pyruvate carboxylase and phosphotransferases (Schramm, 1982). There have also been several other reports of enzymes being activated by Mn^{2+}, or of giving greater catalytic activity in its presence. These results have been seen as indicating that Mn^{2+} has the potential for metabolic regulation (Williams, 1982), however, the lack of any strong experimental data on these systems means that this is still only speculative. The activation of rat liver DHPR by Mn^{2+} is probably not a specific effect as a high concentration of Mn^{2+} was required to evoke the response, but these results may be indicative that the enzyme has

lost a cofactor of some type in the purifcation process which can be partially replaced by Mn^{2+}. Less purified forms of DHPR showed no effect with Mn^{2+} and this is probably due to the excess protein in these samples mopping up the managanese ions (Cornish-Bowden, 1979). The only previous report on the effect of Mn^{2+} on DHPR activity was for the enzyme isolated from Pseudemonas (Williams et al., 1976), and it was found to be strongly inhibited by Mn^{2+}. It is quite possible however, that the bacterial enzyme's relationship to the mammalian enzyme is just in the ability to reduce quinonoid dihydrobiopterin rather than any structural similarities.

Interest in manganese effects on DHPR arises from the characteristics disturbances in the extrapyramidal system associated with chronic manganese encephalopathy. These disturbances are very similar to those observed in Parkinson's disease and the theraputic effects of L DOPA administration in manganese poisoning is consistent with the idea that these two diseases have a common factor which causes the deficiency in the central dopaminergic system (Pentschew et al., 1963; Neff et al., 1969). In animal models of manganese poisoning brain levels of dopamine and homovanillic acid are decreased (Mustafa and Chandra, 1971; Bonilla and Diez-Ewald, 1974). Chandra and Shukla (1981), however, reported that manganese dosing induced an initial elevation in the levels of dopamine and noradrenalin in the striatum. These results agree with earlier results of

increased levels of catecholamines in the corpus striatum of mice exposed to managanese from birth (Chandra et al., 1979). Thus, further work on manganese poisoning will have to be carried out before it can be assumed that it can act as a laboratory model for Parkinson's disease.

There is now growing evidence for a disruption of biopterin metabolism in cancer, although the actual point of disruption has yet to be elucidated. The results presented in tables **4.1** and **4.2**, show that there is a significantly elevated level of DHPR activity in neoplastic breast tissue when compared to apparently normal breast tissue ($P < 0.2\%$, Wilcoxon's signed ranks test). An elevation of breast tumour tissue DHPR activity has previously been reported (Dhondt et al., 1981), but these workers made no measure of DHPR activity in normal breast tissue. They compared tumour DHPR levels with those recorded for human liver. There may be a precedent for comparing enzyme levels measured for tumours to the enzyme levels recorded from other organ/tissues derived from patients with no known form of cancer, as there is always the possibility that "normal" tissue from a cancer patient may contain metastasis. Histological studies performed by the Histology department, (The General Hospital, Steelhouse Lane, B'Ham), however, did not identify any tumour tissue in the "normal" tissue reported here.

There does not appear to be an age related change in DHPR level in normal breast tissue, nor does there seem to be any change in DHPR activity associated with menopausal state. But one must remember that this "normal" tissue has been excised from patients who may well be under much stress and medication, and these factors may have an effect on DHPR activity.

The increased DHPR activity in breast tumours may be in response to the increased demand for reduced folates for the biosynthesis of purines and pyrimidines and Pollock and Kaufman's suggestion (1978), that DHPR may be involved in maintaining cellular tetrahydrofolate pools by reducing any quinonoid dihydrofolate would support this idea. Further support comes from the work of Saleh et al., (1981) who looked at the effect of methotrexate on folate metabolism in normal animals and animals with tumours. They showed that folate catabolism, which is decreased in tumour bearing animals, is increased in tumour bearing animals dosed with methotrexate, and suggest that these findings could be achieved by the inhibition of DHPR as well as inhibition of dihydrofolate reductase. This being so, the results for DHPR activity in large intestinal tumours (table 4.4), are anomalous as there was no elevations of DHPR activity in tumour tissue. This could be explained if the demand for reduced folate was lower in intestinal tumour tissues or, as tetrahydrofolate is produced by micro-organisms in the intestinal tract then intestinal

tumours could have easy access to tetrahydrofolate. Intestinal tumours would therefore have no need for increased DHPR, as their normal levels of folate reductases could cope with the tumour cells increased requirement for reduced folates.

Dhondt et al., (1981), have shown a significant positive correlation between DHPR and cytosolic oestrogen receptors in human breast tumours. This correlation was not found in the tumour samples reported in table **4.5**, however, the small number of samples may have masked such a correlation, Dhondt's results being based on over 200 samples. The DHPR levels do show a significant positive correlation with progesterone receptor levels ($P < 1\%$, Spearman's correlation test; $P < 5\%$, Linear regression), and a more recent paper by Dhondt et al., (1983) also reports such a finding.

In order to ascertain whether oestrogens could effect DHPR activity experiments were performed on rats dosed with DES or 17 α ethynyloestradiol, the results of which strengthened the argument of an oestrogenic link with DHPR activity (tables **4.6** and **4.7**). Both of these oestrogenic compounds caused a significant elevation of DHPR activity in the liver of female rats. The results obtained after 17 α ethynyloestradiol dosing (table **4.7**), suggest that there has been the production of more DHPR molecules, as the increase in enzyme activity is maintained well after the

time that the oestrogen would remain unmetabolised. Oestrogens are known to cause a stimulation of RNA synthesis (Mueller et al., 1972; Balnave and Pearce, 1974), which would in turn put demands on the purine and pyrimidine biosynthetic pathways requiring folate coenzymes (Blakley, 1969). Thus, as a result of oestrogen administration one would expect to see a general increase in the folate metabolising enzymes, and as a consequence an increase in DHPR activity due to its quinonoid dihydrofolate reductase activity.

The specific increase in DHPR in breast tumours, along with its increase in oestrogen treated rats, indicates that this enzyme is responsive to oestrogens. This could explain the effectiveness of oestrogens antagonists in the treatment of oestrogen sensitive breast tumours, and suggests that the use of a DHPR inhibitor at the same time may be beneficial.

The inhibition of rat liver DHPR by dimethlysulfoxide (DMSO) was unexpected as in general DMSO behaves like water, however, as DMSO can be represented as $(CH_3)_2 - S^+ - O^-$, it is quite possible that this molecule could interact with positively charged amino acid residues in the DHPR molecule. This would account for its apparently competitive mode of inhibition, as DMSO could bind to cationic residues in the q $DMPH_2$ binding site. It is also feasible for the DMSO to bind to cationic residues in the NADH binding site, and this may in part be responsible for

complicating the kinetic plots.

The results for the inhibition of rat liver DHPR by oestrogens and their catechol derivatives suggest that these molecules are very strong inhibitors of DHPR activity. The I_{50} values for these molecules show that oestrone and its catechol derivative 2-hydroxyoestrone are better inhibitors of DHPR activity than oestradiol and 2 hydroxyoestradiol (6×10^{-7}M and 1.3×10^{-6}M against 3×10^{-6}M and 1.5×10^{-6}M). The Dixon plots for these inhibitors show that the catechol oestrogens are very similar in inhibitory power, but the lack of multiple plots for each does means a specific K_i can not be given to them. Examples of Dixon plots in Dixon and Webb (1979), and Cornish-Bowden (1979), would indicate that the K_i's for these oestrogens must be either equal to or less than the apparent value gained by the lines intercept with the x axis. Thus one would expect the following K_i's or smaller for these hormones on DHPR, oestrone 1.3×10^{-6}M, Oestradiol 2×10^{-6}M, and the 2 catecholoestrogens having a K_i of 2×10^{-7}M or less. In the case of oestradiol this estimated K_i seems to be vindicated by the K_i obtained from the plots in Fig. 4.8 as between 1.65×10^{-6}-10^{-6}M.

The value for the I_{50}'s obtained in these experiments using the catecholoestrogens, are far smaller than those obtained by workers investigating their effect on tyrosine hydroxylase. Lloyd and Weisz (1978) give an I_{50} for 2-

hydroxyoestradiol on bovine caudate tyrosine hydroxylase as 2.0×10^{-5}M, whilst Foreman and Porter (1980), working with rat brain tyrosine hydroxylase report K_i 2-hydroxyoestrone as 3.5×10^{-4}M and K_i 2-hydroxyoestradiol 2.5×10^{-4}M. It would therefore seem that in the production of catecholamines the catecholoestrogens would be having a greater effect on the level of BH_4 than on the step utilizing the BH_4. This may be reflected in some of Foreman and Porters results. They found that the membrane-bound form of tyrosine hydroxylase was inhibited to a greater extent by the catecholoestrogens, than the soluble form (as measured by their K_is), and in the membrane bound preparation there is the possibility that there will be DHPR associated/present with the tyrosine hydroxylase, hence the greater inhibition due to the inhibition of the associated DHPR.

Table 6.1 gives values for the level of 2-hydroxyoestrone in serum, such low values observed in the plasma do not preclude the possible compartmentalization and subsequent increased concentration of the 2 hydroxyoestrogens in certain areas of the brain. This in fact seems to be the case as Stumpf et al., (1975), demonstrated that hypothalamic tuberoinfundibulor dopaminergic neurons can concentrate tritiated oestrogen. The brain has the capacity to form catechol derivatives (Fishman and Norton, 1975; Fishman, 1976), so there is certainly the possibility of inhibition of catecholamine production by oestrogens and catecholoestrogens. This raises the possibility that the

TABLE 6.1

CONCENTRATION OF 2 HYDROXYOESTRONE IN PLASMA

(AFTER BALLE AND KNUPPEN, 1981)

		Pg /ml 2 hydroxyoestrone	
SUBJECT	PHASE/WEEK	UNCONJUGATED	CONJUGATED
Children	-	20-40	-
Men	-	45-60	-
Women	-	50-95	1500-2800
Menstrual Cycle	Proliferation	50-70	1600
	Periovary	65-95	2300
	Luteal	55-85	1750
	Pregnancy (10^{th}-40^{th} week)	105-220	-

depression observed in some pregnant women may be related to the inhibition of catecholamine production by oestrogens action on DHPR. It is also feasible that the large fluctuations in oestrogens levels observed in the menstrual cycle, which give rise to the mood changes in premenstrual tension syndrome, might be linked to the inhibition of DHPR by the oestrogens.

Dihydropteridine Reductase (DHPR) activity has been measured in a number of human brain temporal lobe cortex samples from patients dying with senile dementia of the Alzheimer type (SDAT), and from age matched controls. The enzyme activity in SDAT temporal lobes (319.1 $\pm$ 143.0 n mol.$min^{-1}mg^{-1}$ (n = 11) is higher than that in control temporal lobes (225.7 $\pm$ 91.0nmol.$min^{-1}mg^{-1}$ (n = 12), but this elevation is not significant. When these results are split into a group of samples where DHPR activity was measured from temporal lobe cortex (Brodmann area 21) again the elevation is observed, but the elevation is not significant, (295.5 $\pm$ 143.0 v 229.4 $\pm$ 111.0 : SDAT v control). The total neopterin concentration and total biopterin concentration of the MRC Brain Bank samples (Brodmann Area 21) were assessed by HPLC by Mr C. Morar using acid-iodine oxidation (Fukushima, and Nixon, 1980). Whilst the measurement of BH_4 biosynthesis in these samples was done by Mr C. Hamon using the method of Brown (Brown, 1981). These results are given in table 6.2. The results for the synthesis of BH_4 in Brodmann area 21 samples shows

TABLE 6.2

RATE OF TETRAHYDROBIOPTERIN BIOSYNTHESIS, TOTAL NEOPTERIN AND TOTAL BIOPTERIN LEVELS IN HUMAN TEMPORAL LOBE SAMPLES (BRODMANN AREA 21). (MENA ± SD).

	NORMAL CONTROLS	SUBJECTS WITH SDAT
BH_4 Synthesis*	0.607 ± 0.50 (n = 8)	0.046 ± 0.102 (N = 8)
Total Biopterin	0.902 ± 1.03 (n = 7)	0.232 ± 0.22 (N = 7)
Total Neopterin	0.496 ± 0.35 (n = 7)	1.237 ± 0.94 (N = 7)
NEOPTERIN/ BIOPTERIN	0.55	5.33

* Units = ngm. biopterin mg^{-1} protein hr^{-1}.

" = ngm. mg^{-1} protein.

that there is a significantly lower capacity to synthesis BH_4 in SDAT brains (0.046ngm biopterin mg^{-1} protein hr^{-1}) than control brains (0.616ngm biopterinn mg^{-1} protein hr^{-1}) (P = 1% Wilcoxon sum of ranks). The control group also showed some evidence of an age related decrease in BH_4 biosynthetic ability, the youngest brain (44yr) having a biosynthetic rate of 1.05ng biopterin mg^{-1} protein hr^{-1}, 6 brains in the age range 70-80yr having a mean rate of 0.75ng biopterin mg^{-1} protein hr^{-1}, and the oldest brain (age 89yr) with a synthetic activity of 0.11 ngm biopterin mg^{-1} protein hr^{-1}. These results are in agreement with those of Williams et al, (1980), who showed a decrease in BH_4 concentration in CSF with increasing age.

In temporal lobes from subjects with SDAT the total biopterin concentration has fallen, while the total neopterin concentration has risen when compared to age matched controls. This is reflected in the large difference between the neopterin/biopterin ratios for the SDAT samples and control samples (5.23 and 0.55 respectively).

Further statistical analysis of these results were carried out by the MRC Brain Bank, who found a positive correlation between the degree of dementia, as measured by dementia tests (Blessed et al., 1968), and DHPR activity in 6 dements (Correlation coefficient 0.95(r) P = 0.003). There was however, no correlation between DHPR activity and age

or plaques, but there appears to be a slight correlation between DHPR activity and tangle counts (as measured at death : P = 10%), tangle counts themselves correlating quite well with the degree of dementia.
The Cambridge Brain Bank also suggests that DHPR levels are higher in demented then non demented patients (7 v 14).

Recently workers have reported a lowering of biopterin concentration in serum (Aziz et al., 1983) and CSF (Morar et al., 1983) of patients with SDAT compared to age matched controls. The results reported here for the Brodmann area 21 samples gives a neopterin/biopterin ratio of 0.55 in control subject and 5.23 in SDAT subject. The large increase in SDAT being due to a decrease in the total biopterin concentration with a concomitant increase in total neopterin concentration, when compared to age matched controls. From these results it can be postulated that there is an impairment in the biopterin biosynthesis pathway in SDAT somewhere after the initial steps to produce neopterin. Such a lesion would lead to the lowering of the total biopterin concentration which, if the biosynthetic pathway is under feedback inhibition by its end product (BH_4), would in itself increase the rate of the initial steps of the biosynthetic pathway giving rise to the increased neopterin levels recorded. This tentative hypothesis is further strengthened by the results for the BH_4 biosynthetic capacity of these samples, the ability to synthesis BH_4 being significantly lower in SDAT brains

(0.046nmg biopterin mg^{-1} protein h^{-1}), than in control brains (0.616ngm biopterin mg^{-1} protein. h^{-1}) (P = 1% Wilcoxon sum of ranks).

The reduction in the level of noradrenalin, dopamine and serotonin brought about by the lowering of the rate limiting cofactor for their production in SDAT (BH_4), would alleviate the feedback inhibition on DHPR by these neurotransmitters (Nagatsu, et al., 1964; Undenfriend, et al., 1965; Purdy et al., 1981; Shen, 1983). This would account for the elevated activity of DHPR measured in SDAT brains. However, since the actual control of these pathways is far more complex this may be the reason why the DHPR activity is not consistently elevated in SDAT.

Since SDAT brains retain their ability to synthesis dihydroneopterin, but lack the ability to make BH_4, and DHPR is unimpaired, it seems unlikely that these results are due to neuronal loss in SDAT brains.

DHPR has been measured in different regions of the rat brain (Turner et al., 1974; Algeri et al., 1977), but there has not been a survey of DHPR in human brain regions. One might however, expect there to be a close association between regions of the brain associated with catecholominergic/serotonergic enervation. The regions measured here do show large differences in DHPR activity, but such a small sample can not be thought to be representative and

there is a definite need for further study of the regional distribution of DHPR in human brain. Studies have shown that BH_4 and its biosynthetic system are localized in dopaminergic nerve terminals in rat striatum (Levine et al., 1981), and the pineal gland has a very high BH_4 biosynthetic capacity (Kapatos et al., 1981) as well as being under noradrenergic enervation (Klein and Namboodi, 1982). Several area of the brain would thus be expected to have high DHPR activities.

The purification of human brain DHPR by a modified version of the method of Cotton and Jennings (1978), was not as successful as the purification of rat liver enzyme. The Michaelis constants (Km's) for the two substrate and those gained by other workers for human enzyme from a variety of sources are given in table **6.3.** The experiments to give the kinetic constants gave the typical hyperbolic curves for plots of velocity versus substate concentration and Lineweaver-Burk plots gave straight lines. As however, there is a highly non-uniform distribution of errors over the range of values of $1/[s]$ and $1/v$ in the Lineweaver-Burk plot (Cornish-Bowden, 1979), Eadie-Hofstee plots and Hanes plots were used, these having a better distribution of error over the experimental values. The values for Km's, thus obtained compare well with the values gained for other sources of human enzyme (Table **6.3**).

TABLE 6.3

MICHAELIS CONSTANTS FOR HUMAN DHPR

	ENZYME SOURCE	Km (M) ($\times 10^{-5}$) $DMPH_4$	NADH
Present Work	Temporal lobe	2.9	1.9
Firgaira et al, '79	Fibroblasts	3.8	
	Continuous Lymphoid Cells	4.1	1.2
	Liver	4.5	
Firgaira et al, '81	Liver	3.6	2.9
Craine et al, '72	Liver	-	0.80
Shen, 1983	liver	3.0	2.4

When crude enzyme preparations were preicubated with lead acetate no inhibition was observed. This was not altogether unexpected as it has been recorded that the high protein levels inherent in crude preparations can sequester heavy metal ions (Cornish-Bowden, 1979). These results also show that NADH protects against heat inactivation of DHPR (Fig. 5.6), and in experiments not reported here NADH had a protective effect against lead inhibition. This is in agreement with Brown (1981), who suggests that the NADH binding site seems the most likely target for lead attack. The inhibition of purified DHPR by lead was time dependent, raising the possibility of the inhibitor being irreversible. The time dependent inhibition curves (Fig. 5.9), all level off at around the same percentage inhibition (65-75%) which again is strongly indicative of irreversible inhibition.

The most likely cause of lead inhibition is through attachment to a thiol group on the enzyme (Ferdinand, 1976; Dixon and Webb, 1979). The human enzyme has been shown to contain sulphydryl groups (Firgaira et al., 1981a) and work on the rat enzyme using a variety of sulphydryl specific reagents has suggested that the major role for sulphydryl groups is to maintain a functional enzyme tertiary structure (Webber and Whiteley, 1981).

The effect of lead in vivo on DHPR activity in rat brains after perinatal lead administration was a significant

decrease in DHPR activity in 300 and 1000ppm treated rats. However, results from an *in vivo* experiment are open to differing interpretation, as the lead may be acting on many cellular processes not directly connected with biopterin metabolism but having a peripheral effect on biopterin levels. When protein levels were compared to DNA levels, it was found that as the lead concentration increases the protein : DNA ratio decreases, raising the possibility that the lead has decreased protein concentration if one assumes that the DNA levels have not been affected.

These results also raise the question of the effect of brain asymmetry; it is well known that in the human brain one or other of the brain hemispheres is dominant a trait that is reflected by anatomical, chemical and electrical asymmetries in the brain. More recent work indicates that these asymmetries are not unique to Man but also occur in the brains of lower animals such as birds and rats (Marx, 1982). It would seem that this is what is being observed between the left and right hemispheres of these rat brain. The lead levels measured in the blood of rats after the perinatal admimistration of 300ppm and 1,000ppm, were 39.8 g/100ml and 85.4 g/ml respectively (Carmichael *et al.*, 1981). These lead levels are within the range of blood lead values measured in children with "subclinical lead poisoning". They show no obvious neurological disturbance, but the results of these experiments indicate that quinonoid dihydrobiopterin salvage may well be

perturbed due to the decreased activity of DHPR.

Aluminium sulphate (10^{-4}M) inhibited human DHPR by 35% after 30 mins, which is in the same range as that reported by Brown (1981), who measured 30% inhibition with 2 x 10^{-4}M aluminium sulphate after 20 mins preincubation. Aluminium is well documented as a causal agent in dialysis dementia (Alfrey et al., 1976; McDermott et al., 1978; Arieff et al., 1979) and elevated aluminium concentrations are found in SDAT brains (Crapper et al., 1973). The aluminium present in the SDAT brains is associated with the neurofibrillary tangles characteristic of Alzheimer's disease (Perl and Brody, 1980), but the relationship is not so straight forward as aluminium induced neurofibrillary degeneration in rats is not indentical to the lesions of Alzheimer's disease, (Bulgiani and Ghetti, 1982).

The inhibition of human DHPR by phenylpyruvate was low (8%) at 10^{-4}M, which is lower than the inhibition reported by Brown (1981), who reported a 16% inhibition of rat liver DHPR by 2.7 x 10^{-5}M phenylpyruvate, and would not be consistent with the K_i app if 50uM for phenylpyruvate reported by Purdy and Blair (1980). But these results were gained using a 400 fold purified rat liver enzyme. Phenylpyruvate is one of the metabolites of phenylalanine which is elevated in the serum of untreated phenylketonurics, with a serum range of 10^{-5} - 1.6 x 10^{-4}M, the normal non-phenylketonuria plasma phenylpyruvate concentration is

5-8 M (Langenbeck et al., 1980a; Langenbeck et al., 1981). The inhibition of DHPR by phenylalanine and its metabolites has been proposed to be the causative factor in the elevation of serum levels of dihydrobiopterin seen in phenylketonuria (Leeming et al., 1976; Leeming, 1979). The inhibition of DHPR may also be responsible for the mental retardation and decreased neurotransmitter concentrations that occur in phenylketonuria (Purdy and Blair, 1980).

The results presented in this chapter add to the reported data on human DHPR, which at the moment seem to be limited to the human liver enzyme. The similar Km's for liver and brain DHPR indicate that the enzymes are the same even through their source is different. Although recent work does seem to suggest other functions for BH_4 (Nichol et al., 1983) there would not appear to be a need for different for different forms of DHPR. The association between a disturbance of biopterin metabolism and SDAT is strengthened by the results reported here, the actual disturbance being in the biosynthetic pathway of BH_4 rather than an effect on DHPR. The human enzyme is inhibited by two metals, both associated with neurological disorders/ disease, and by a metabolite of phenylalanine which occurs in high levels in untreated phenylketonuria.

In conclusion there is now so much evidence for the involvement of defects in tetrahydrobiopterin metabolism in several different neurological disorders that the complete

elucidation of this biosynthetic pathway is essential not only to further our understanding of these disorders but also to help alleviate the symptoms and suffering of the afflicted. The importance of dihydropteridine reductase is illustrated by one of the forms of malignant hyperphenylalaninaemia. Much more biochemical work needs to be done, however, to elucidate the mechanisms of action of the enzyme and also to determine the actual mode of inhibition by known inhibitions, as well as continuing work on tentative inhibitors.

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