THE BIOSYNTHESIS OF TETRAHYDROBIOPTERIN IN THE RAT

by

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A thesis submitted for the degree of

Doctor of Philosophy

at

The University of Aston in Birmingham

April 1981

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SUMMARY

THE BIOSYNTHESIS OF TETRAHYDROBIOPTERIN IN THE RAT

De novo tetrahydrobiopterin biosynthesis has been studied in vitro using rat brain supernatants. G.T.P. was shown to be the best precursor for the biosynthetic pathway. NADPH, magnesium ions, pyrimidine antagonists and 5-methyltetrahydrofolic acid, particularly when coenzyme B_{12} was present, were shown to stimulate tetrahydrobiopterin production. The de novo biosynthesis of tetrahydrobiopterin was inhibited by metal ions, particularly lead, purine antagonists and a range of dihydrofolate reductase inhibitors which led to the postulation of a feedback control mechanism of its biosynthesis by tetrahydrobiopterin.

The activity of dihydropteridine reductase has been measured by an in vitro assay after purification from rat liver by affinity chromatography using sodium 1,2-naphthoquinone-4-sulphonate as the ligand. The enzyme was purified 900 fold over the original supernatant and quinonoid dihydrobiopterin and NADH were found to be the best substrates with apparent K_m values of $9.2. 10^{-6}$M and $3.2. 10^{-5}$M respectively. Dihydropteridine reductase was inhibited by a range of dihydrofolate reductase inhibitors, aromatic amino acids, phenylalanine metabolites, neurotransmitters and metal ions. The enzyme was shown to exhibit co-operativity and to have more than one binding site per enzyme molecule. Dopamine and noradrenaline are probably feedback inhibitors of dihydropteridine reductase to control their own biosynthesis and inhibit by binding to an allosteric site. It is suggested that this enzyme may be a regulatory point for dopamine biosynthesis. Phenylalanine and phenylpyruvate inhibit dihydropteridine reductase at concentrations which occur in untreated phenylketonurics and could be the cause of the neurological defects associated with this disease due to decreased catecholamine neurotransmitter production as a result of decreased cellular tetrahydrobiopterin levels.

A model for the regulation of cellular tetrahydrobiopterin levels was proposed and discussed in the light of the results of in vivo studies as well as the in vitro results presented in this thesis.

KEY WORDS

Tetrahydrobiopterin Biosynthesis

Dihydropteridine reductase Neurotransmitter

TO MY PARENTS

The work described in this thesis has been carried out between 1977-1980 at the University of Aston in Birmingham. It was done independently and has not been submitted for any other degree.

Sheila E. Brown

Sheila E. Brown

ACKNOWLEDGEMENTS

I wish to thank the following for their encouragement and invaluable support during this study.

Professor J.A. Blair, Chemistry Department, University of Aston in Birmingham, for valued guidance and many helpful discussions.

Dr R.J. Leeming, Department of Haematology, General Hospital, Birmingham, for exellent co-operation with the Crithidia fasciculata Assay.

Dr M. Connor, La Jolla, California, for preparation of the 10 Formyl Folate.

Dr R.F. Long and his colleagues in the Departments of Drug Metabolism and Biochemistry at Roche Products Ltd., Welwyn Garden City, for their co-operation during the part of this study which was carried out at Roche Research Laboratories and for gifts of many samples of various drugs and pterins.

Professor D.G.Wibberly, Pharmacy Department, University of Aston in Birmingham, for measurements of lead by Atomic Absorption.

Roche Products Ltd., Welwyn Garden City, for financial support in the form of a S.R.C. CASE award.

Mrs Lynda Perkins for typing the manuscript and finally but not least my husband and family.

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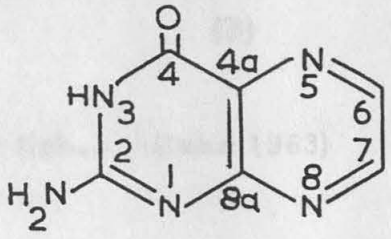
LIST OF ABBREVIATIONS

ATP	-Adenosine Triphosphate
BH_4	-Tetrahydrobiopterin
5 CH_3 THFA	-5 Methyl Tetrahydrofolic Acid
CNS	-Central Nervous System
DHFR	-Dihydrofolate Reductase
DHPR	-Dihydropteridine Reductase
DICPIP	-5,6 Dichlorophenolindophenol
$DMPH_4$	-6,7 Dimethyl-5,6,7,8 tetrahydropterin
DNA	-Deoxyribose Nucleic Acid
EDTA	-Ethylene Diamine Tetra-acetic Acid
GDP	-Guanosine Diphosphate
GMP	-Guanosine Monophosphate
GTP	-Guanosine Triphosphate
NAD^+	-Nicotinamide Adenine Dinucleotide
NADH	-Reduced Nicotinamide Adenine Dinucleotide
$NADP^+$	-Nicotinamide Adenine Dinucleotide Phosphate
NADPH	-Reduced Nicotinamide Adenine Dinucleotide Phosphate
N.D.	-Not Determined
Ⓟ	-Phosphate Group
PtH_4	-5,6,7,8 Tetrahydropterin
qBH_2	-Quinonoid Dihydrobiopterin
qDHPt	-Quinonoid Dihydropterin
$qPtH_2$	-Quinonoid 7,8 Dihydropterin
RNA	-Ribose Nucleic Acid
S.D.	-Standard Deviation
TCA cycle	-Tricarboxylic Acid cycle
TH	-Tyrosine Hydroxylase

CHAPTER ONE

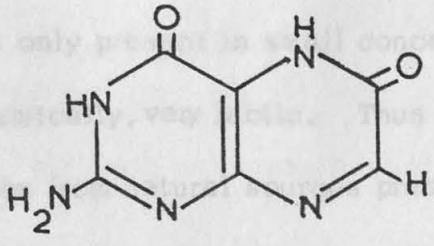
INTRODUCTION

Naturally-occurring derivatives of the parent compound 2-amino-4-oxodihydropteridine (1) are called pterins and are a widely distributed class of compounds. The numbering of the rings is as shown in figure 1. (Rembold and Gyure 1972). In 1889 Sir Frederick Gowland Hopkins



(1)

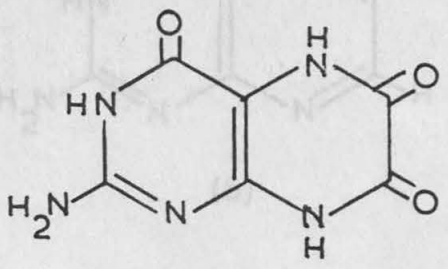
extracted a yellow pigment from butterfly wings (Hopkins 1889). This was followed in 1925 by the isolation of yellow and white pigments by Wieland and Schopf. (1925) Purrman finally determined the structures of these natural pigments after much experimental difficulty in 1940-1. They were named xanthopterin (2), leucopterin (3) and isoxanthopterin



(2)

(4). (Purrman 1940a, Purrman 1940b, Purrman 1941).

Much work on pterins has concerned their isolation and identification from butterflies, moths and other insects. (Zeigler and Harmsen 1969) Other extensively studied groups of pterins are those



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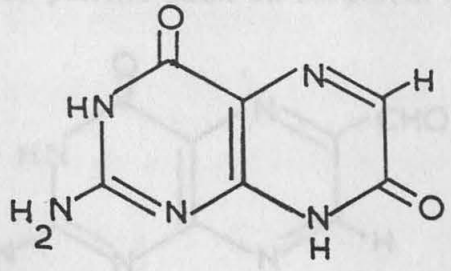
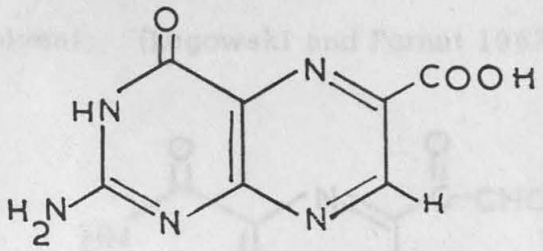
of amphibia and fish. (Hama 1963)



(4)

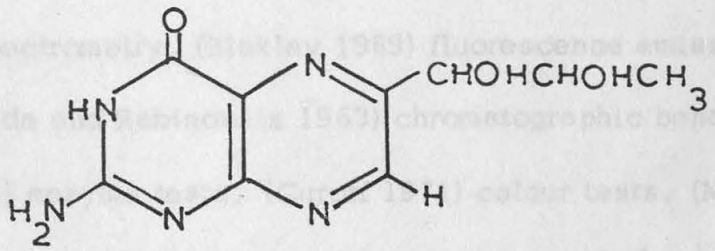
Pterins are only present in small concentrations in organisms and some are chemically, very labile. Thus working with them and their identification from natural sources presents great difficulties. The compounds isolated could be analytical artifacts as suggested with regard to xanthopterin (2) from Drosophila eyes (Koschara 1936, Zeigler 1961) and 2 amino-4-hydroxypteridine-6-carboxylic acid (5)



(5)

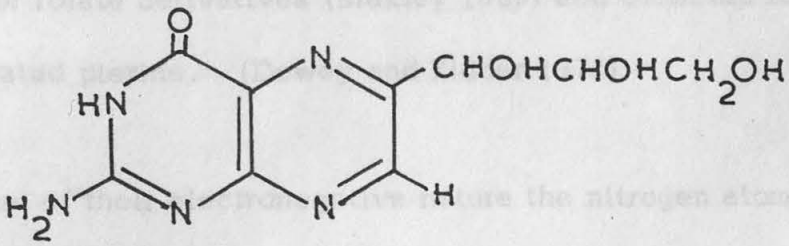
from human urine. (Blair 1958)

The method by which pterins are extracted from their natural sources depends on the solubility and lability of the pterin in question. Relatively soluble pterins such as biopterin (6), neopterin (7) and



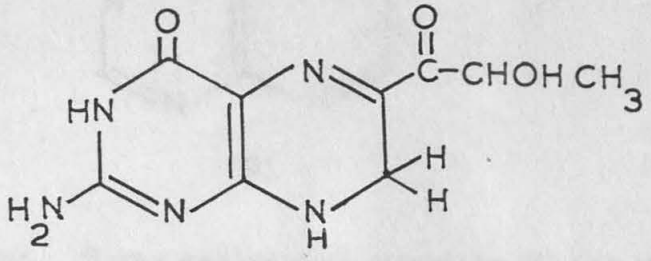
(6)

sepiapterin (8) can be isolated from trichloroacetic acid extracts. Labile hydrogenated pterins are stabilized under these acidic conditions. Acid insoluble pterins such as isoxanthopterin (4) and leucopterin (3).



(7)

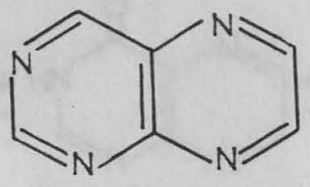
can be extracted in basic solvents. In order to prevent degradation of the reduced acid-insoluble pterins, mercaptoethanol must be added to the basic solvent. (Lagowski and Fornut 1967)



(8)

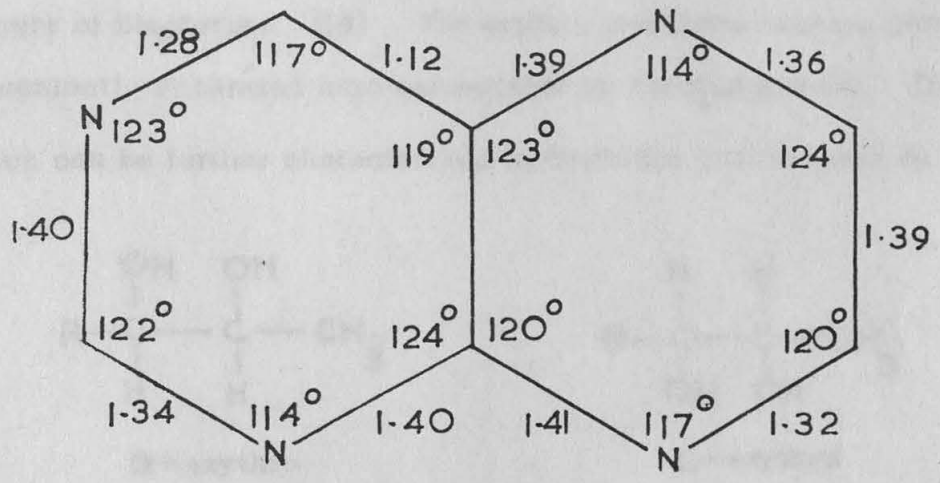
Once the pterins have been isolated they can be purified by chromatographic or electrophoretic methods (Rembold and Gyure 1972) and then identified using a variety of methods including ultra-violet absorption spectrometry, (Blakley 1969) fluorescence emission spectrometry, (Uyeda and Rabinowitz 1963) chromatographic behaviour, (Blakley 1969) enzyme tests, (Guroff 1971) colour tests, (McNutt 1964) mass spectrometry, (Van der Have-Kirchberg, de Morée, Van Laar, Gerwig, Versluis and Ebels 1977) gas chromatography, (Haug 1970, Lloyd, Markey and Weisner 1971) high performance liquid chromatography (Fukushima and Nixon 1978) and microbiological assays such as Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae for folate derivatives (Blakley 1969) and Crithidia fasciculata for unconjugated pterins. (Dewey and Kidder 1971)

Because of their electronegative nature the nitrogen atoms of the pterin rings compete with the π electrons of the system and partially



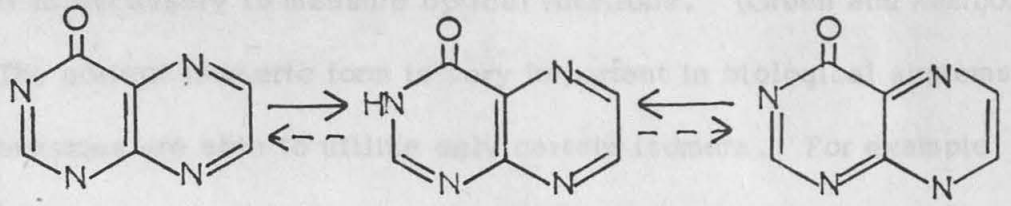
(9)

localise the latter. X-ray analysis of pteridine (9) has indicated a planar arrangement of the atoms. Bond angle and bond length measurements have shown that pteridine (9) is not centrally symmetric. (10) Pterins exist in tautomeric forms but the one favoured at equilibrium from the energy point of view is the lactam. The monohydroxypterins



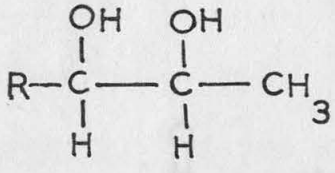
(10)

exist in the form of the true cyclic amides (11) and do not have the vinylogous amide structure (12,13). (Pfleiderer 1964)

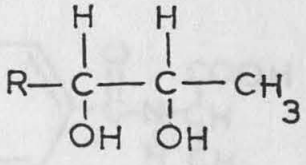


(12) (11) (13)

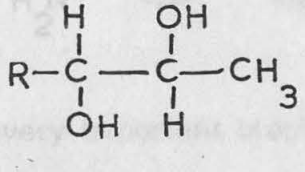
Biopterin (6) was isolated from human urine in 1956 by Patterson and his colleagues. (Patterson, Von Saltza and Stokstad 1956) This pterin bears a dihydroxypropyl side-chain on carbon atom 6. The possession of such a side-chain means that there are two chiral carbon atoms at positions 1' and 2' and therefore four optical isomers of biopterin. (14) The erythro and threo isomers can be conveniently separated from one another by chromatography. The erythro series can be further characterised by Crithidia growth tests as the L form is



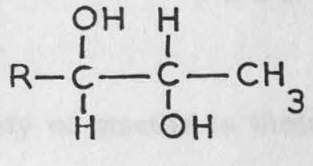
D - erythro



L - erythro



D - threo

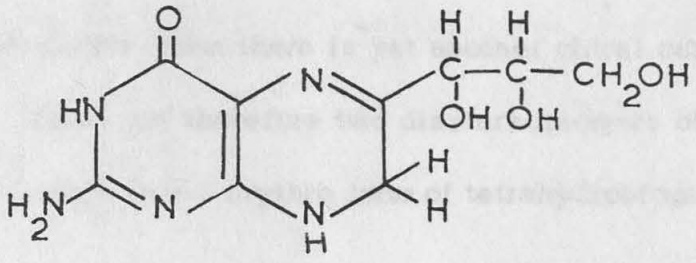


L - threo

R = 2 amino-4-hydroxypteridine group

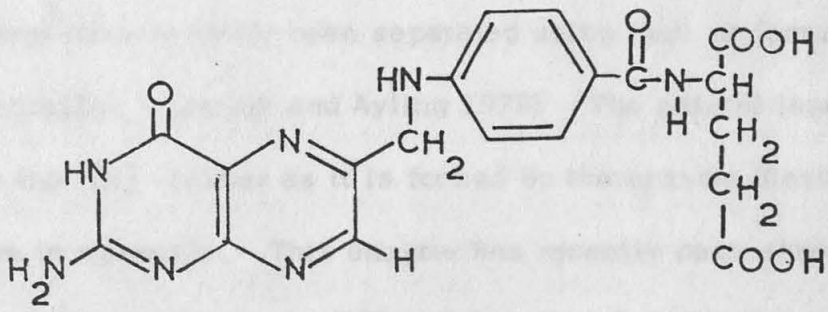
(14)

more active than the D form. For further identification of the threo series it is necessary to measure optical rotations. (Green and Rembold 1966) The correct isomeric form is very important in biological systems as enzymes are able to utilize only certain isomers. For example, the L erythro form of dihydroneopterin (15) is not only not utilized for the



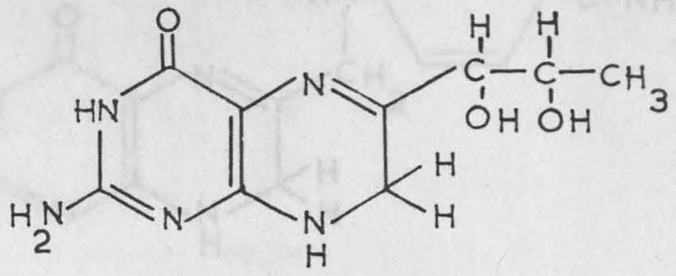
(15)

synthesis of folic acid (16) in L. plantarum but also is an effective inhibitor of the D erythro form utilization. (Rembold and Gyure 1972)



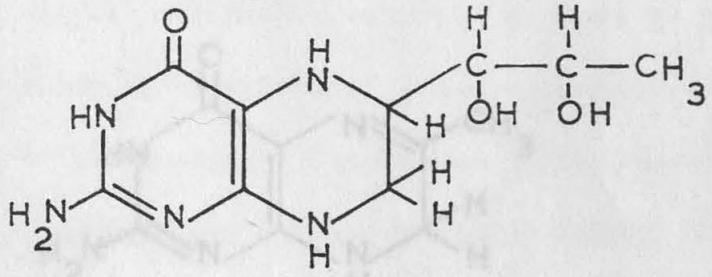
(16)

A very important biochemical property of pterins is their ability to form dihydro- and tetrhydro- derivatives for example, 7,8 dihydrobiopterin (17) and 5,6,7,8 tetrahydrobiopterin (18).



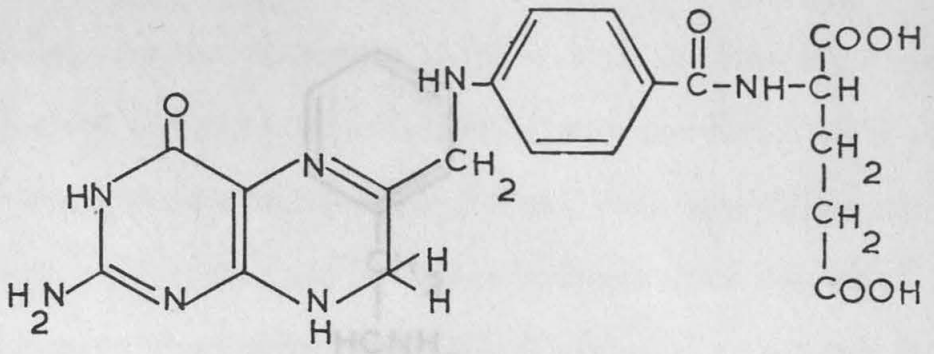
(17)

In the tetrahydro- form there is yet another chiral carbon atom, at position 6. There are therefore two diastereoisomers of the naturally occurring isomer, the L erythro form of tetrahydrobiopterin (18)



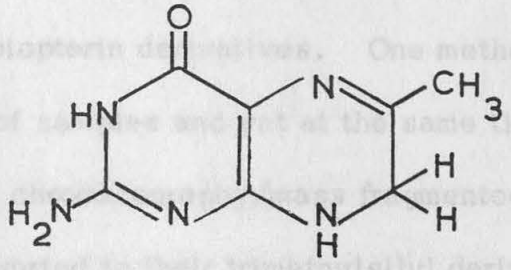
(18)

These have recently been separated using high performance liquid chromatography. (Bailey and Ayling 1978) The natural isomer is probably the [S] isomer as it is formed by the enzyme dihydrofolate reductase in mammals. This enzyme has recently been shown to reduce 7,8 dihydrofolic acid (19) and 6 methyl 7,8 dihydropterin (20) stereospecifically to the [S] configuration. (Armarego, Waring and Williams 1980) Although both the isomers of tetrahydrobiopterin (18)



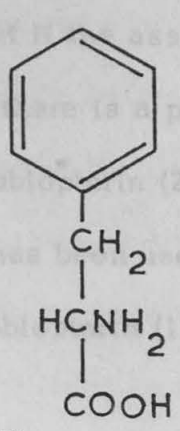
(19)

act as cofactor for phenylalanine hydroxylase with identical Km values the natural isomer gave a faster V_{max} value and also stimulated substrate inhibition by phenylalanine (21) unlike the [R] isomer.



(20)

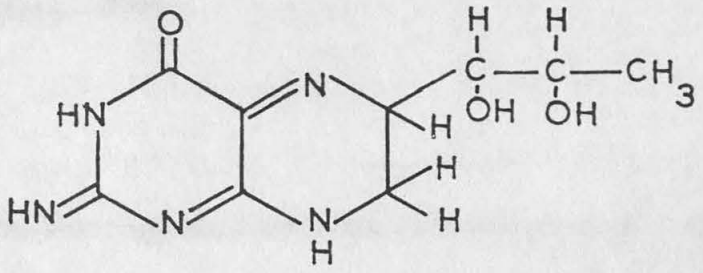
Biopterin derivatives have been measured in human and rat tissues and fluids, (Baker, Frank, Bacchi and Hutner 1974) including blood, serum (Frank, Baker and Saboka 1963) and urine. (Fukushima and Shiota 1972, Pabst and Rembold 1966) It is difficult to exactly identify the derivatives present in tissues because the reduced forms are so reactive. (Blair and Pearson 1974) Biopterin (6) isolated from mammalian tissues and fluids most probably had its origins in



(21)

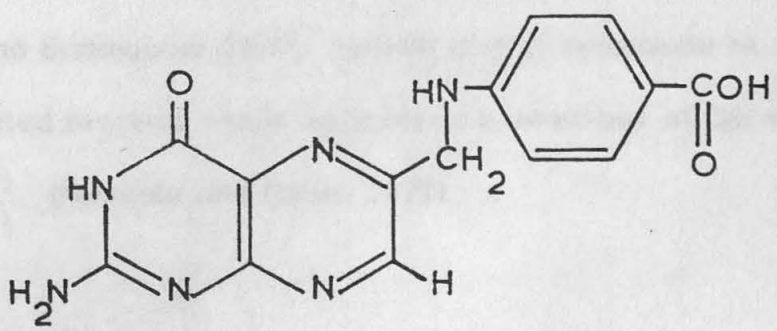
the dihydro- or tetrahydro- forms because the oxidation of 5,6,7,8-tetrahydrobiopterin (18) proceeds rapidly to 7,8 dihydrobiopterin (17) and then to biopterin (6). (Blair and Pearson 1974, Metzyer, Rembold and Gutensohn 1971) However a variety of methods are now available for measuring biopterin derivatives. One method which is useful for large numbers of samples and yet at the same time is sensitive and specific is gas chromatography/mass fragmentography. The pterins have to be converted to their trimethylsilyl derivatives initially to make them volatile enough for gas chromatography separation. (Rother and Karobath 1976) An extremely sensitive method is reverse-phase high performance liquid chromatography following initial iodine oxidation and separation by ion-exchange chromatography. This method was used recently to show that biopterin is present in mammalian tissues predominantly in the tetrahydro- form. (Fukushima and Nixon 1980) Tetrahydrobiopterin (18) can be assayed enzymatically by measuring phenylalanine hydroxylase activity as this enzyme exhibits an absolute cofactor requirement for tetrahydrobiopterin (18). (Guroff,

and Abramowitz 1967) This method is therefore very specific for tetrahydrobiopterin alone but if the assay medium contains dihydropteridine reductase and NADH there is a possibility that reduction of endogenous quinonoid dihydrobiopterin (22) may contribute to the activity measured. This assay has been used to demonstrate the regional distribution of tetrahydrobiopterin (18) in rat brain. (Bullard, Guthrie,



(22)

Russo and Mandell 1978) The Crithidia fasciculata assay has proved itself to be an invaluable tool for measuring biopterin derivatives in animal tissues and fluids. This protozoon is extremely selective in the pterins it can use for growth. Biopterin (6) and its reduced derivatives dihydrobiopterin (17) and tetrahydrobiopterin (18) are the best pterin sources and only L-Neopterin (7) and pteroic acid (23)



(23)

approach their growth - stimulating capacity. (Baker, Frank, Bacchi and Hutner 1974, Leeming and Blair 1974) This assay is also very sensitive as well as specific because Crithidia fasciculata responds to very low concentrations of the Crithidia-active pterins. This method can be supplemented with thin-layer or paper chromatography in a range of solvent systems in order to establish the identity of the particular pterins being utilized by Crithidia fasciculata. (Blakely 1969, Leeming 1975)

The reduced pterin forms are very reactive and can serve as specific reductants and can also participate in cellular electron transport reactions.

Evidence for the participation of unconjugated reduced pterins in the photosynthetic process has been put forward (Fuller and Nugent 1969) and it is suggested that they are the primary photochemical electron-acceptors for photosystem I in higher plants and for the single primary photochemical act in bacterial photosynthesis. However, pterins have not been clearly demonstrated in chloroplasts and the standard reduction potential for tetrahydropterins is + 0.15 V. (Archer and Scrimgeour 1970) Since that of ferredoxin is -0.42 V the expected reaction would be a reverse sequence of the electron flow proposed. (Rembold and Gyure 1972)

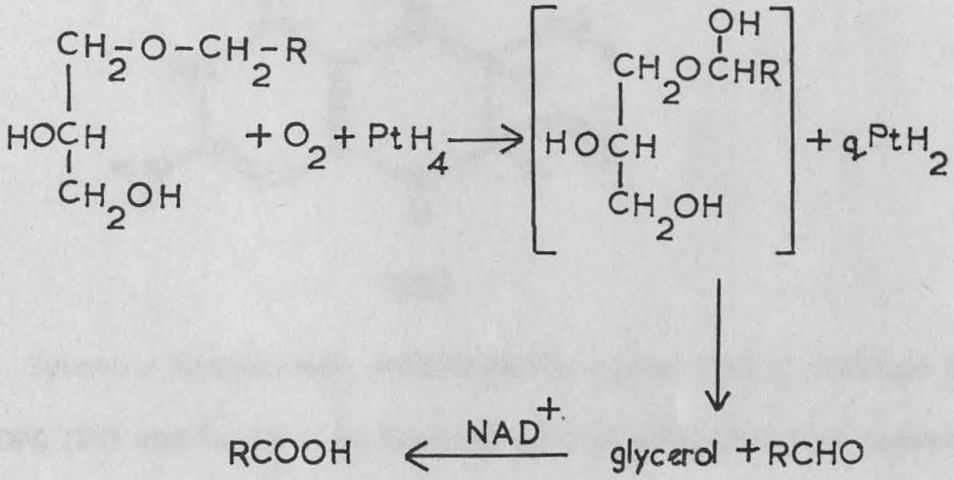
However, the function of pterins in mitochondria is much clearer. Tetrahydrobiopterin (18) has been found in the mitochondrial matrix in concentrations similar to those of the cytochromes. (Rembold and Metzger 1967) Mitochondrial respiration can be stimulated by reduced pterins at physiological concentrations and the utilization of oxygen is linearly proportional to the amount of tetrahydrobiopterin (18) present. Cytochrome C is readily reduced by tetrahydrobiopterin (18) according to its more positive redox potential of + 0.22 V. (White, Handler and Smith 1973) A soluble electron transport system can be formed but the tetrahydrobiopterin-catalysed respiration is not coupled to ATP production. The pathway would be of importance if cells must regenerate pyridine nucleotides under high hydrogen pressure without the production of ATP but the physiological significance of this pathway is not really known. (Rembold and Buff 1972a , Rembold and Buff 1972b, Rembold 1975)

The best-studied reductant function of tetrahydrobiopterin (18) is its role in the oxidation of aromatic amino acids. Tetrahydrobiopterin (18) was shown to be the natural cofactor for the enzymes phenylalanine hydroxylase, (Kaufman 1957, Kaufman 1958) tyrosine hydroxylase (Nagatsu, Mizutani, Nagatsu, Matsura and Sugimoto 1972) and tryptophan hydroxylase. (Hoseda and Gluk 1966) Other reductants such as ascorbate, glutathione, ferrous ions etc. cannot replace tetrahydrobiopterin (18), therefore, it has been established as the specific cofactor for these aromatic amino acid hydroxylations. Of the four

isomers of reduced biopterin, the L erythro form, the naturally-occurring one, has been shown to be the best cofactor. (Osanai and Rembold 1971) Other tetrahydropteridine-dependent oxygenase reactions are cinnamic acid hydroxylation, (Nair and Vining 1965)

$$\text{Cinnamic acid} + PtH_4 + O_2 \longrightarrow \text{p-coumaric acid} + qPtH_2 + H_2O$$

and the oxidation of long chain alkyl ethers of glycerol to fatty acids and glycerol. (Tietz, Lindberg and Kennedy 1964) Others have been reported but not conclusively proved and need validation especially as

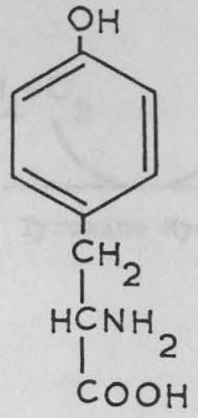


reduced pterins can indirectly catalyse an enzyme reaction by protecting the enzyme from inactivation (Zamoni, Brown and La Du 1963)

Phenylalanine hydroxylase catalyses the conversion of phenylalanine (21) to tyrosine (24). Certain regulatory features displayed by the natural cofactor but not by the unnatural cofactors often used such

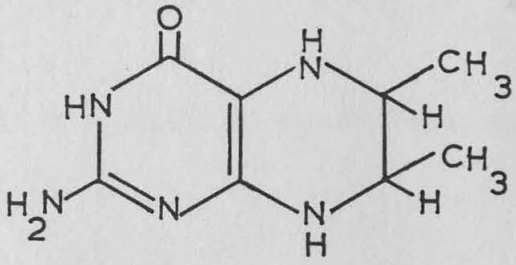
$$\text{Phenylalanine} + BH_4 + O_2 \longrightarrow \text{Tyrosine} + qBH_2 + H_2O$$

as dimethyltetrahydropterin (25) indicate that there may be a regulatory



(24)

site for tetrahydrobiopterin (18) on phenylalanine hydroxylase as well as the catalytic site.

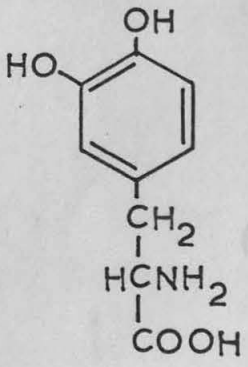


(25)

Tyrosine hydroxylase catalyses the conversion of tyrosine (24) to L-DOPA (26) and is the rate-limiting step in catecholamine biosynthesis.

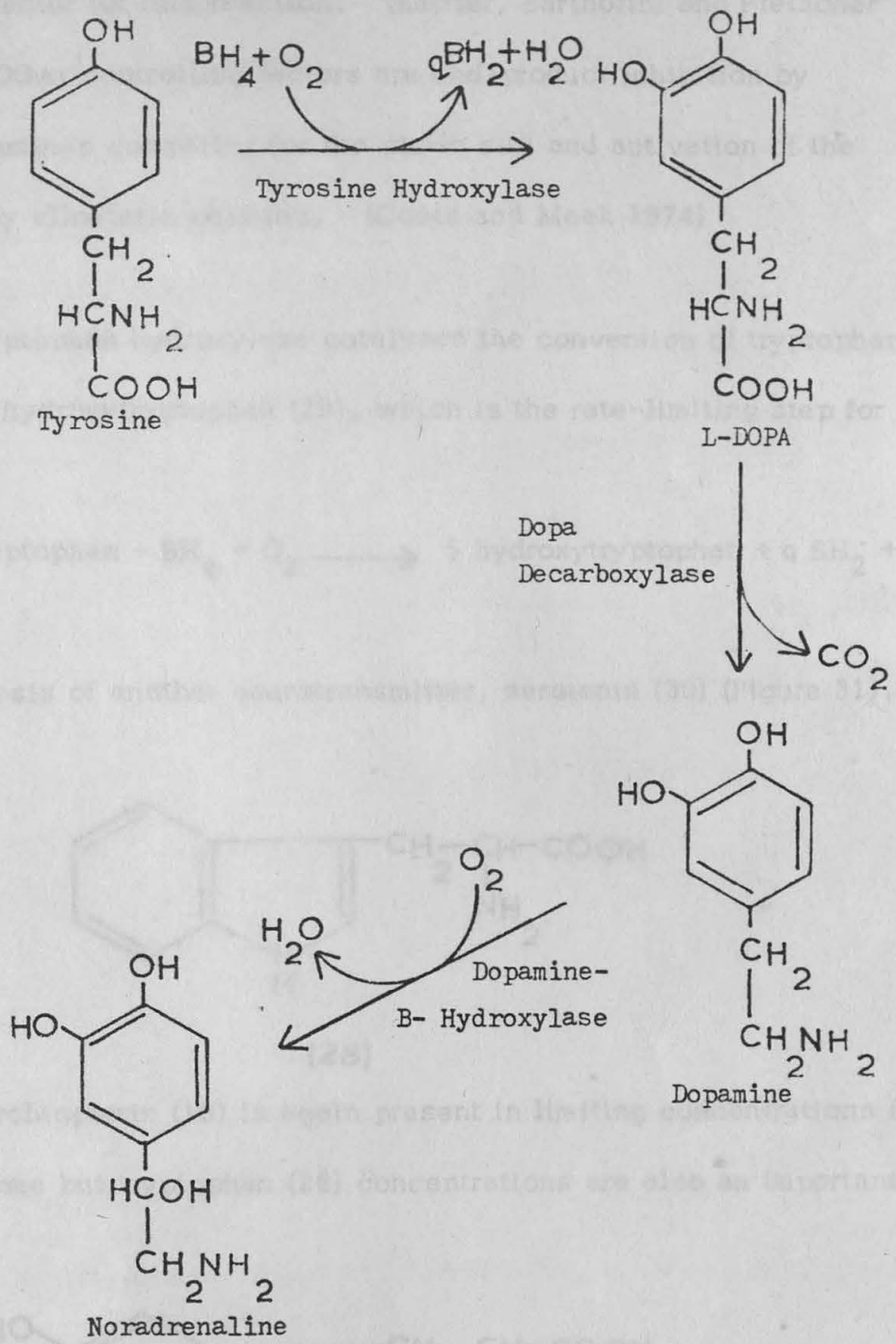
$$\text{Tyrosine} + BH_4 + O_2 \longrightarrow \text{L-DOPA} + qBH_2 + H_2O$$

(Figure 27) (Levitt, Spector, Sjoerdsman and Udenfriend 1965) It has



(26)

BIOSYNTHESIS OF CATECHOLAMINES



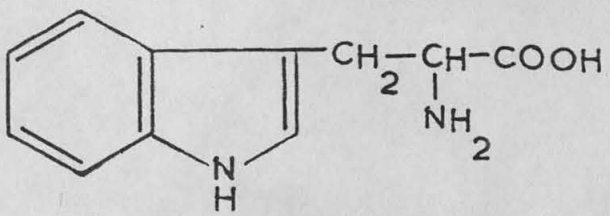
(27)

been shown that the amount of tetrahydrobiopterin (18) is the rate-limiting factor for this reaction. (Kettler, Bartholini and Pletscher 1974) Other controlling factors are end-product inhibition by catecholamines competing for the pterin site and activation of the enzyme by allosteric changes. (Costa and Meek 1974)

Tryptophan hydroxylase catalyses the conversion of tryptophan (28) to 5 hydroxytryptophan (29), which is the rate-limiting step for the

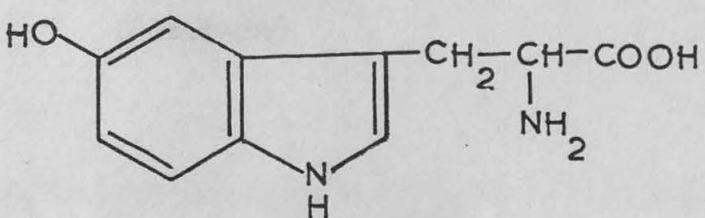
$$\text{Tryptophan} + BH_4 + O_2 \longrightarrow 5\ \text{hydroxytryptophan} + q\ BH_2 + H_2O$$

biosynthesis of another neurotransmitter, serotonin (30) (Figure 31).



(28)

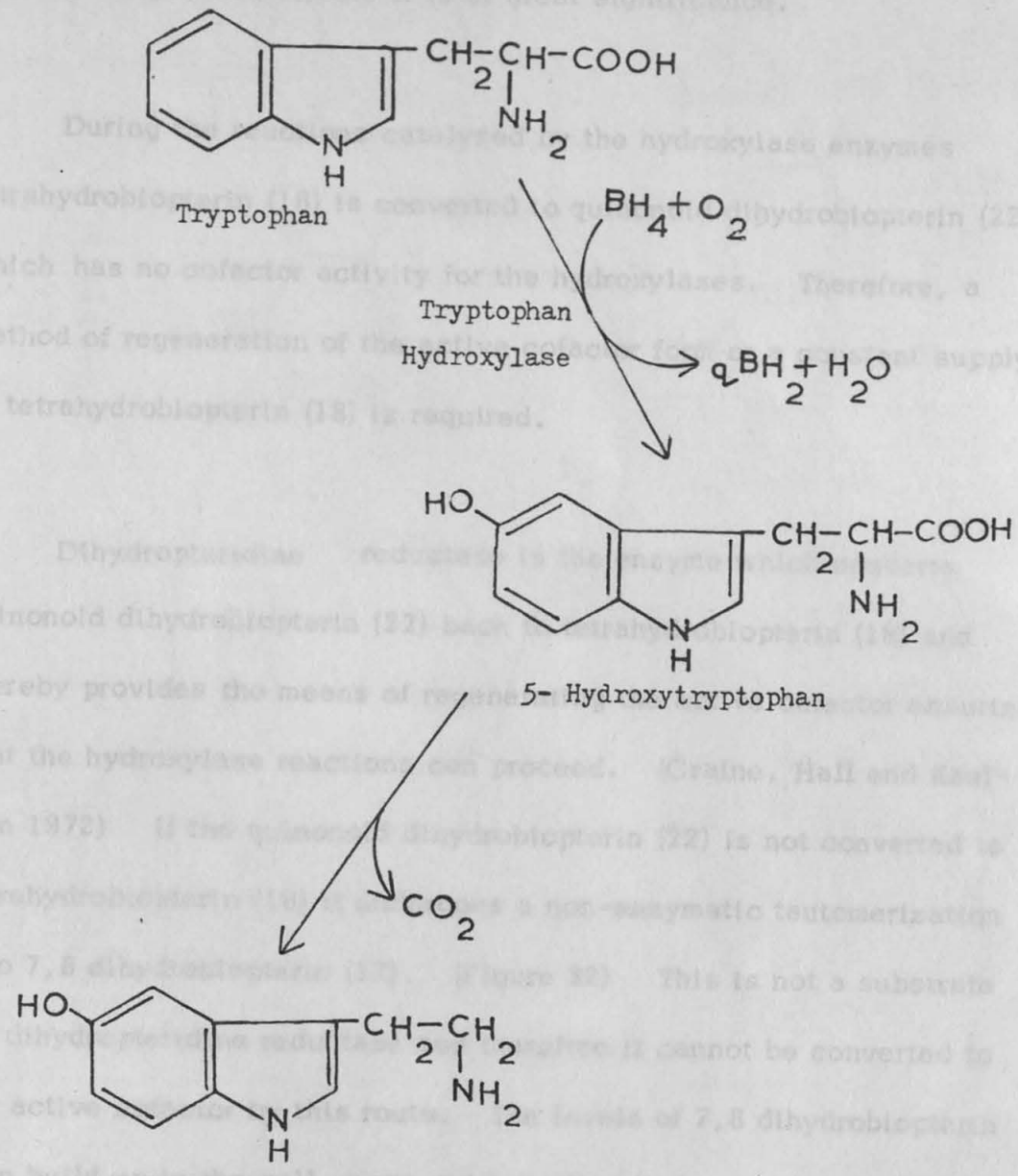
Tetrahydrobiopterin (18) is again present in limiting concentrations for this enzyme but tryptophan (28) concentrations are also an important



(29)

rate-limiting factor. (Costa and Meek 1974)

BIOSYNTHESIS OF SEROTONIN



(31)

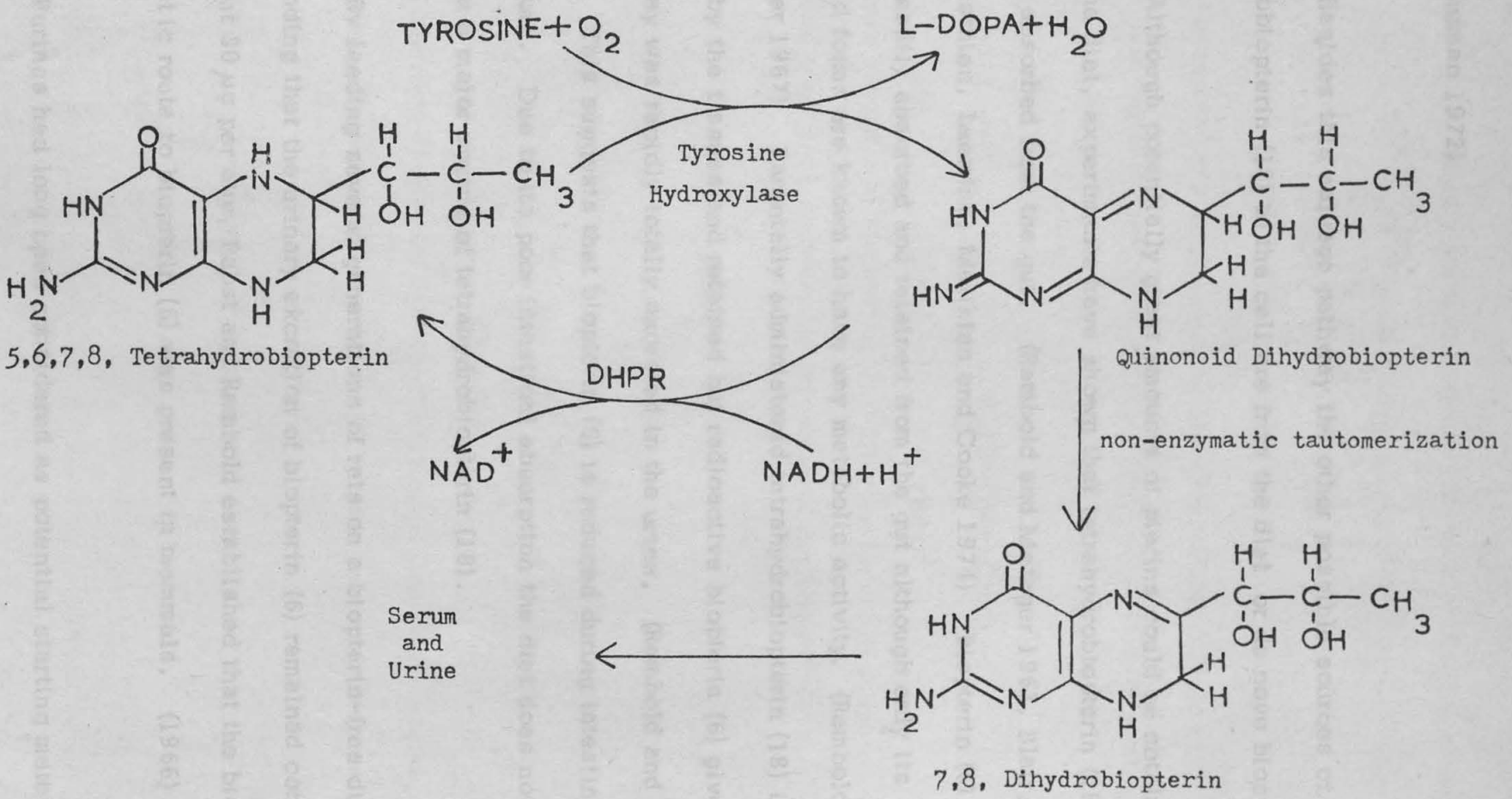
As tetrahydrobiopterin (18) plays such an important role in neurotransmitter biosynthesis the metabolism of tetrahydrobiopterin (18) itself and anything which effects it is of great significance.

During the reactions catalysed by the hydroxylase enzymes tetrahydrobiopterin (18) is converted to quinonoid dihydrobiopterin (22) which has no cofactor activity for the hydroxylases. Therefore, a method of regeneration of the active cofactor form or a constant supply of tetrahydrobiopterin (18) is required.

Dihydropteridine reductase is the enzyme which converts quinonoid dihydrobiopterin (22) back to tetrahydrobiopterin (18) and thereby provides the means of regenerating the active cofactor ensuring that the hydroxylase reactions can proceed. (Craine, Hall and Kaufman 1972) If the quinonoid dihydrobiopterin (22) is not converted to tetrahydrobiopterin (18) it undergoes a non-enzymatic tautomerization into 7,8 dihydrobiopterin (17). (Figure 32) This is not a substrate for dihydropteridine reductase and therefore it cannot be converted to the active cofactor by this route. The levels of 7,8 dihydrobiopterin then build up in the cell, move out into the serum and are excreted in the urine. (Leeming 1979)

The dihydropteridine reductase reaction is rapid and provides a so-called salvage pathway for quinonoid dihydrobiopterin thereby maintaining the tetrahydrobiopterin (18) levels in the cells. (Craine, Hall

TETRAHYDROBIOPTERIN / QUINONOID DIHYDROBIOPTERIN CYCLE



(32)

and Kaufman 1972)

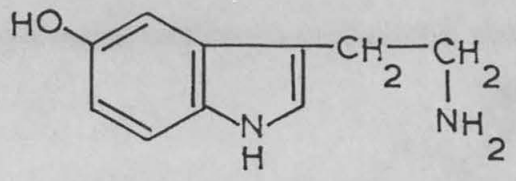
Besides this salvage pathway the other possible sources of tetrahydrobiopterin (18) to the cell are from the diet or de novo biosynthesis.

Although potentially great amounts of pterins could be obtained from the diet, experiments have shown that tetrahydrobiopterin (18) is poorly absorbed from the gut. (Rembold and Metzger 1967, Blair, Ratanasthien, Leeming, Melikian and Cooke 1974) Biopterin (6) is more readily absorbed and retained from the gut although only its reduced forms are known to have any metabolic activity. (Rembold and Metzger 1967) Parentally administered tetrahydrobiopterin (18) is taken up by the tissues and retained but radioactive biopterin (6) given in this way was rapidly totally excreted in the urine. (Rembold and Metzger 1967) This suggests that biopterin (6) is reduced during intestinal transport. Due to its poor intestinal absorption the diet does not provide a major source of tetrahydrobiopterin (18).

By feeding several generations of rats on a biopterin-free diet and finding that the urinary excretion of biopterin (6) remained constant at about 30 µg per day, Pabst and Rembold established that the biosynthetic route to biopterin (6) was present in mammals. (1966)

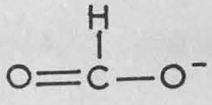
Purines had long been considered as potential starting materials for the biosynthesis of the pterin ring because of structural similarities.

When purine precursors such as formate (33) or glycine (34) or purines

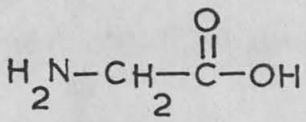


(30)

and their derivatives such as adenosine (35), guanine (36), guanosine (37) or guanylic acid (38) were injected or fed to rats they produced

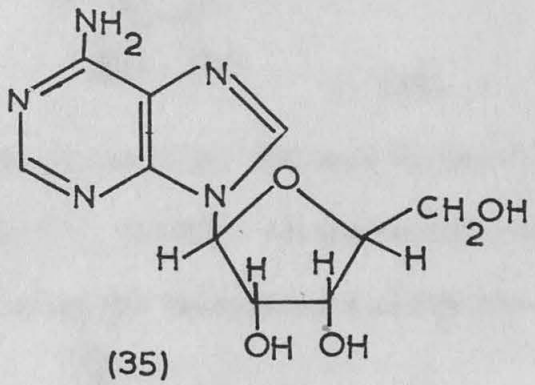


(33)



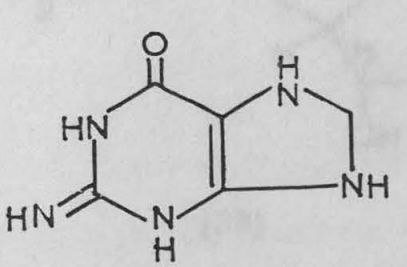
(34)

only trace amounts of biopterin (6). This synthesis of biopterin was increased with the simultaneous injection of actinomycin D to



(35)

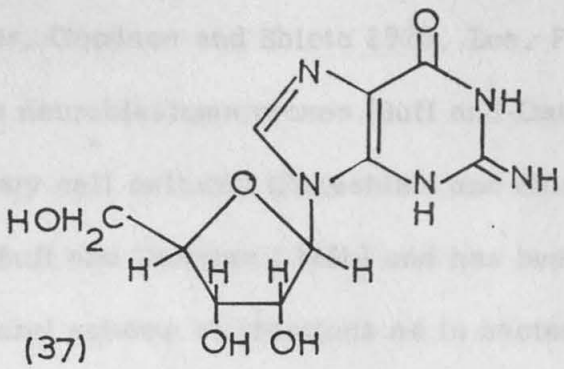
reduce the flow of purines and their precursors into nucleic acid synthesis. (Rembold and Gyure 1972) Other evidence supporting



(36)

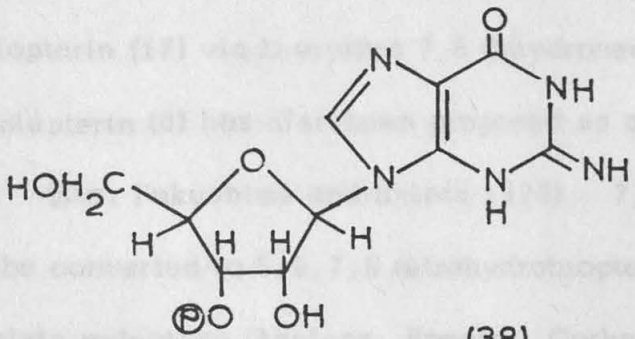
purines or their derivatives as the precursors of pterin biosynthesis is

that ^{14}C from universally labelled guanine (36) was shown to be incorporated into 2 amino-4-hydroxypteridine-6-carboxylic acid (5). (Ziegler-



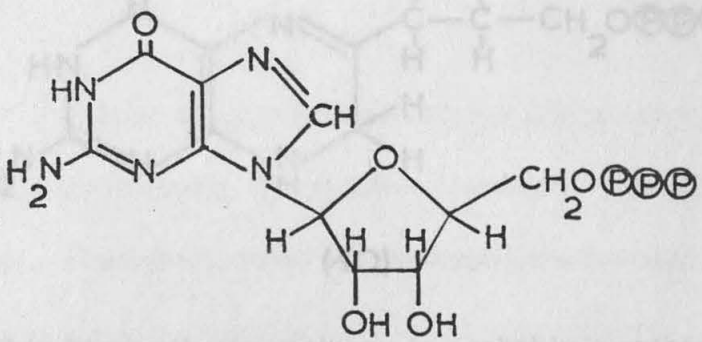
(37)

Günder 1956) Dahal and Gots showed that of all the guanine compounds,



(38)

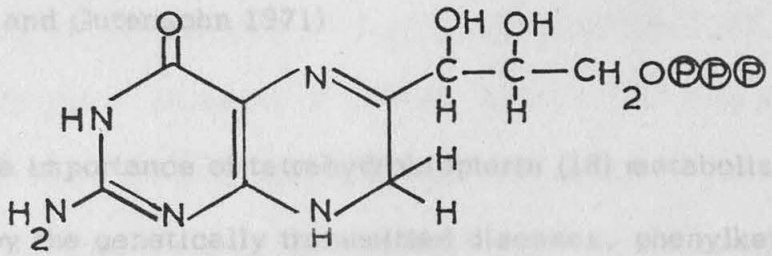
guanosine triphosphate (GTP) (39) was the most efficient substrate for pterin biosynthesis. (1965) All the initial biosynthetic work was carried out studying the biosynthesis of the pterin ring of folic acid (16)



(39)

in bacterial species. (Shiota 1971, Eto, Fukushima and Shiota 1976) Unconjugated pterin biosynthesis has now been studied in a variety of

animal cells including the skin of bullfrog tadpoles (Fukushima 1970), Drosophila melanogaster (Brown and Fan 1975), rat tissues (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota 1975, Lee, Fukushima and Nixon 1978), mouse neuroblastoma clones (Buff and Dairman 1975a), Chinese Hamster ovary cell cultures (Fukushima and Shiota 1974) and even the intact rat (Buff and Dairman 1975b) and has been shown to follow the same general scheme of reactions as in bacteria. The biosynthesis proceeds from guanosine triphosphate (39) to L erythro- 7,8 dihydrobiopterin (17) via D erythro 7,8 dihydroneopterin triphosphate (40). Sepiapterin (8) has also been proposed as an intermediate in this pathway. (Eto, Fukushima and Shiota 1976) 7,8 dihydrobiopterin (17) can be converted to 5,6,7,8 tetrahydrobiopterin (18) by the enzyme dihydrofolate reductase (Abelson, Spector, Gorka and Fosburg 1978 Pollock and Kaufman 1978, Spector, Levy and Abelson 1977, Spector, Fosburg, Levy and Abelson 1978) and hence the active cofactor is



(40)

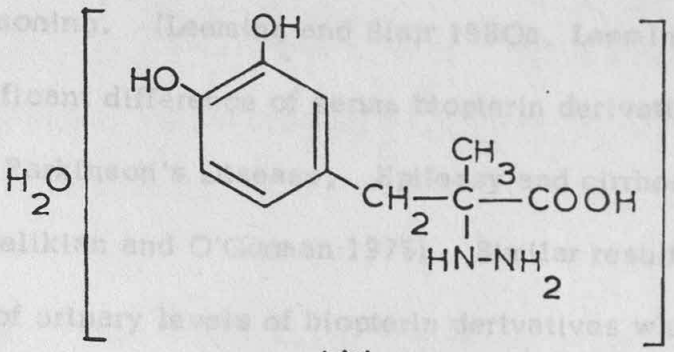
formed. This biosynthetic pathway is probably a rather slow process and performs a topping-up function of the cellular levels of tetrahydrobiopterin (18).

The catabolism of pterins is more fully understood than any other section of their biochemistry. Rembold and his coworkers showed that tetrahydrobiopterin (18) is degraded in vitro using rat liver homogenates and in vivo after intraperitoneal injections into the rat, in a similar manner, by a series of deaminations and oxidations to yield lumazine derivatives and hydroxylated pterin species. Principally two enzymes are involved : xanthine oxidase and a specific pterin deaminase. (Rembold and Gutensohn 1968, Rembold, Metzger, Sudersham and Gutensohn 1969) More recent studies in mice receiving intraperitoneal injections of a variety of pterins with and without the xanthine oxidase inhibitor, allopurinol suggest that aldehyde oxidase can also play a part in pterin oxidation. (Knipe and McCormack 1977) These enzymes plus non-enzymatic degradations provide not only a catalytic pathway for unconjugated pterins but also a pathway for the production of simple lumazines and ring hydroxylated pterins. (Rembold, Metzger and Gutensohn 1971)

The importance of tetrahydrobiopterin (18) metabolism is demonstrated by the genetically transmitted diseases, phenylketonuria and its variants. Phenylketonuria is characterised by high levels of phenylalanine (21) and its metabolites in the blood and urine. It is due to a lack or deficiency of the enzyme phenylalanine hydroxylase and is transmitted by a recessive gene. Although the disease is not fatal it causes mild to severe mental retardation if untreated. Treatment is by a controlled low-phenylalanine diet. (Woolfetel 1951) Cases of

phenylketonuria where the plasma phenylalanine (21) levels were easily controlled by the low-phenylalanine diet but where the neurological defects were progressive and terminated life in early childhood have been described. (Smith, Clayton and Wolff 1975 a and b) More recently the same syndrome was reported where the children were shown to have biochemical lesions at one of two sites, either in dihydropteridine reductase (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975, Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977, Grobe, Bartholome, Milstien and Kaufman 1978) or in the biosynthetic pathway for 7,8 dihydrobiopterin (17). (Leeming, Blair and Rey 1976, Rey, Blandin-Saroja and Rey 1976, Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel 1978) In both these types of syndrome, named malignant hyperphenylalaninaemia, the neurological defects are similar but the two types may be distinguished by measuring their Crithidia factor response to an oral phenylalanine (21) load (Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977, Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel 1978) and their serum dihydrobiopterin (17) levels. These children with a metabolic block in the tetrahydrobiopterin biosynthetic pathway from guanosine triphosphate (39) had low serum levels of dihydrobiopterin of 0.4 µg/L compared to the normal level of 1.78 µg/L. (Leeming and Blair 1980 b) Those children lacking the dihydropteridine reductase salvage pathway had higher than normal serum dihydrobiopterin (17) levels of 4.9 µg/L. (Leeming and Blair 1980 b) Treatment of both

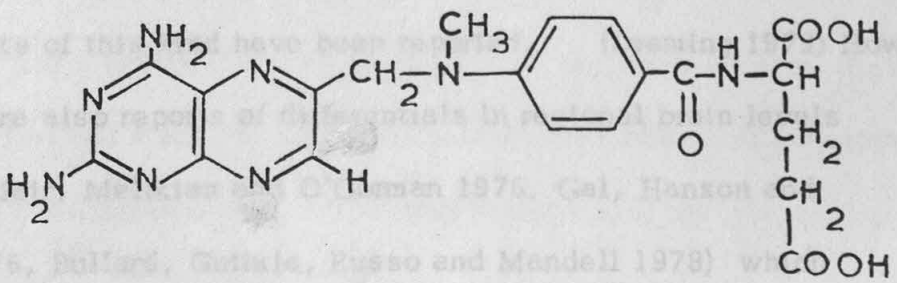
types of the syndrome is by a low phenylalanine diet with the administration of L-DOPA (26), carbidopa (41) and 5 hydroxytryptophan (29). (Bartholome and Byrd 1975) It has been shown that the hyperphenylalaninaemia can be corrected by the administration of 5,6,7,8 tetra-



(41)

hydrobiopterin (18). (Danks, Cotton and Schleisinger 1975, Schaub, Daumling, Curtius, Niederweiser, Bartholome, Viscontini, Schiniks and Biers 1978)

The study of these variants of phenylketonuria provides an <u>in vivo</u> demonstration of tetrahydrobiopterin (18) metabolism. Other studies of serum and urine levels of biopterin (6) and its derivatives have been undertaken for a number of different disease states. Raised blood or serum levels of biopterin derivatives have been reported in tumour patients (Kokolis and Ziegler 1977) classical phenylketonurics, patients with kidney disfunction and patients taking the drug methotrexate (42). (Leeming, Blair, Melikian and O'Gorman 1976, Leeming and Blair 1980a)



(42)

whereas lowered serum levels have been demonstrated in pernicious anaemics, schizophrenics, rheumatoid arthritics patients with regional enteritus, leukaemia, (Leeming, Blair, Melikian and O'Gorman 1976) malignant carcinoid disease, senile dementia, coeliac disease and lead poisoning. (Leeming and Blair 1980a, Leeming and Blair 1980 b) No significant difference of serum biopterin derivative levels were found in Parkinson's Disease, Epilepsy and cirrhosis. (Leeming, Blair, Melikian and O'Gorman 1976) Similar results are reflected in studies of urinary levels of biopterin derivatives where epileptics, rheumatoid arthritics (Leeming and Blair 1974) and patients with kidney dysfunction all had lowered levels whereas in schizophrenics there was no significant change. (Leeming, Blair, Melikian and O'Gorman 1976)

The major role that tetrahydrobiopterin (18) plays in the production of catecholamine neurotransmitters indicates that changes in brain levels of tetrahydrobiopterin (18) in disease could have a significant effect. Tetrahydrobiopterin(18) is not effectively transported across the blood-brain barrier, (Kettler, Bartholini and Pletscher 1974) so the relevance of these serum and urine measurements with respect to brain levels must be regarded with caution. Levels in cerebral spinal fluid may provide a better indication of brain concentrations and recently measurements of this kind have been reported. (Leeming 1979) However there are also reports of differentials in regional brain levels (Leeming, Blair, Melikian and O'Gorman 1976, Gal, Hanson and Sherman 1976, Bullard, Guthrie, Russo and Mandell 1978) which

indicates that even cerebral spinal fluid measurements are not an ideal indication of the biopterin status of the various brain regions.

For ethical reasons, only so much information can be obtained from the in vivo measurement studies. Yet this information can be supplemented by that obtained from in vitro studies such as those reported in this thesis.

The de novo biosynthesis of tetrahydrobiopterin (18) from guanosine triphosphate (39) in rat brain homogenates will be measured using the Crithidia fasciculata assay. (Leeming and Blair 1974) Isolated enzyme studies may also add information to the in vivo results. Methods exist for the isolation of dihydropteridine reductase from mammalian liver (Cheema, Soldin, Knapp, Hofman and Scrimgeour 1973, Hasegawa 1977, Webber, Deits, Synder and Whiteley 1978, Aksnes, Skotland, Flatmark and Ljores 1979) and its activity can be measured using a spectrophotometric assay. (Craine, Hall and Kaufman 1972) An affinity chromatographic method using a sodium naphthoquinone sulphonate adsorbent will be used to prepare dihydropteridine reductase from rat liver. (Cotton and Jennings 1978)

These two systems isolated in vitro will therefore be used to look at the effect of a number of drugs, metabolites and other compounds on the de novo biosynthesis of tetrahydrobiopterin (18) and its main-

tenance in the reduced form by the dihydropteridine reductase salvage pathway. These studies should provide more information about tetrahydrobiopterin (18) metabolism and how it is controlled. The results obtained can then be used together with the in vivo measurements to try to explain the changes observed in diseases, though extrapolation of results from animals to humans must be limited because of the metabolic differences involved. Comparison of the changes in biopterin derivative levels observed when drugs are administered to patients with the effects of the same drugs in the in vitro experiments will indicate which metabolic pathway of tetrahydrobiopterin, if any, is being affected by the drug. Hence these studies may give an indication of how the drugs are acting to control the disease or produce side-effects associated with the drug. These results may also provide further insight into the underlying biochemical processes and how these are altered in disease.

CHAPTER TWO

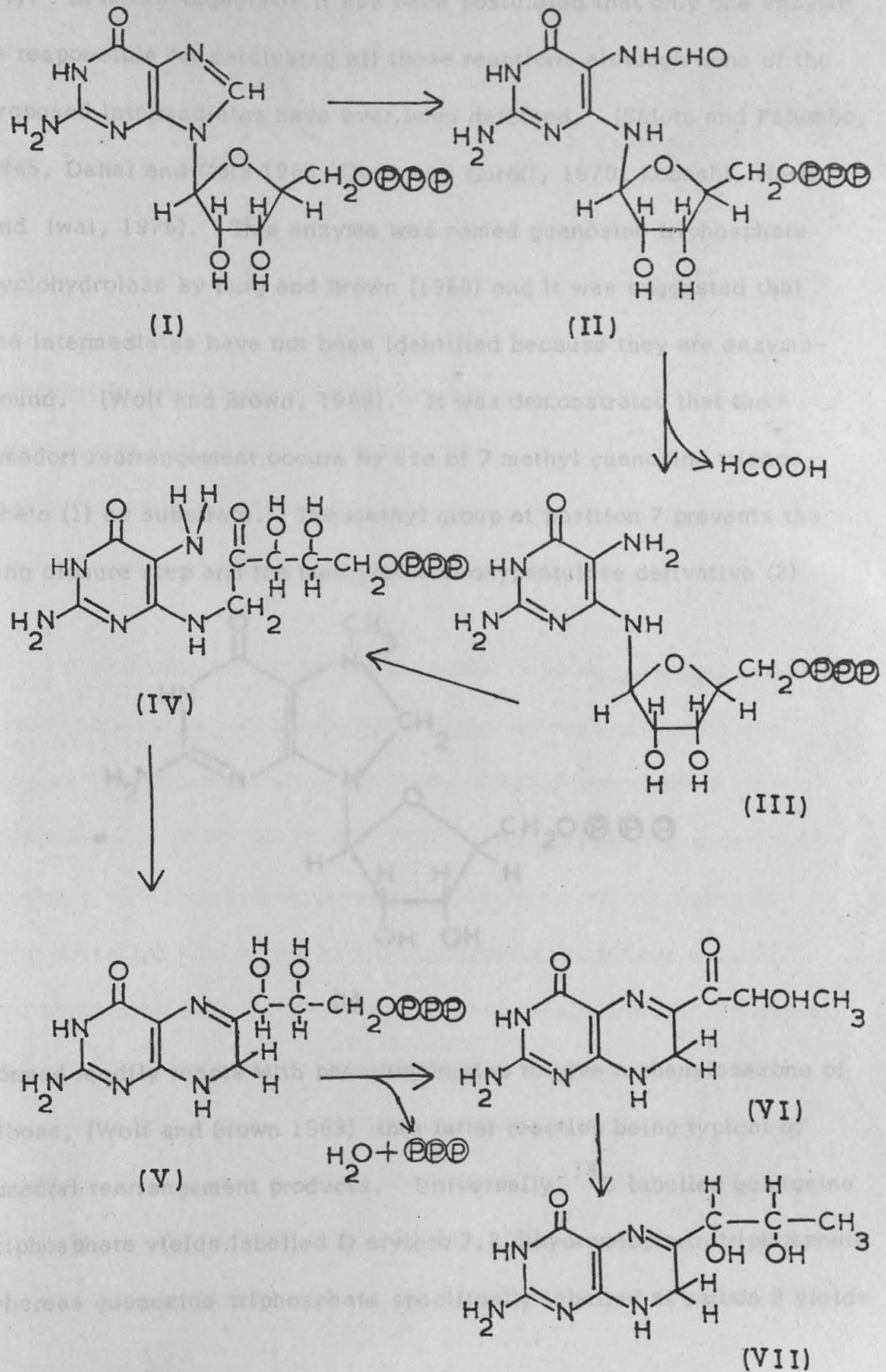
THE IN VITRO MEASUREMENT OF THE BIOSYNTHESIS OF BIOPTERIN DERIVATIVES

INTRODUCTION

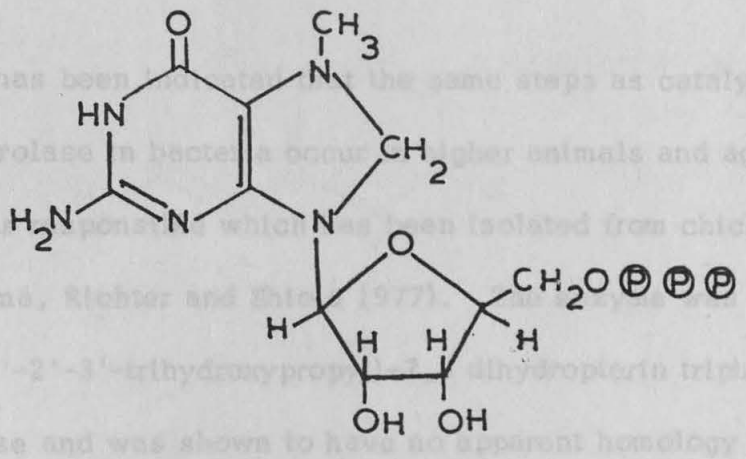
The de novo biosynthesis of biopterin derivatives from guanosine triphosphate has been demonstrated in many different mammalian tissues such as rat brain, hamster kidney, liver and brain (Lee, Fukushima and Nixon 1978, Fukushima, Eto, Saliba and Shiota 1975, Fukushima, Eto, Mayumi, Richter, Goodson and Shiota, 1975) and also in cell cultures of rat lung and ovary, hamster kidney, lung and ovary and mouse kidney, lung, ovary and neuroblastoma clones. (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota, 1975, Fukushima and Shiota, 1974, Buff and Dairman, 1975B.).

Figure 2-1 shows the steps which have been postulated for the biosynthetic pathway from guanosine triphosphate (I) to L erythro 7,8 dihydrobiopterin (VII). The first step consists of opening of the imadazole ring between carbon 8 and nitrogen 9 followed by removal of carbon 8 as a one carbon compound identified as formic acid by Shiota and Palumbo, (1965). The ribose moiety of the compound formed (III) then undergoes an Amadori rearrangement resulting in the formation of a deoxypentulose derivative (IV). This compound contributes its carbons 1' and 2' to form the pterin ring as carbons 7 and 8 respectively. The remaining ribose carbons form the pterin side chain at

Figure 2-1 Postulated Biosynthetic Pathway of Dihydrobiopterin

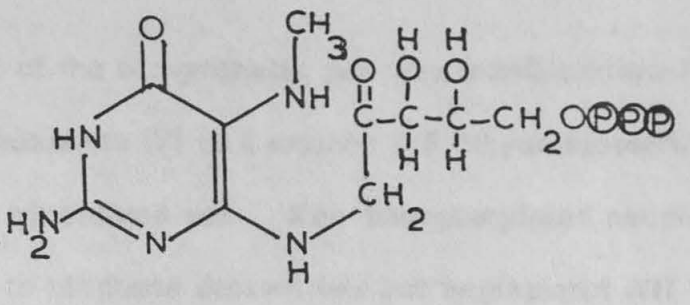


position 6, thus forming D erythro 7,8 dihydroneopterin triphosphate (IV). In micro-organisms it has been postulated that only one enzyme is responsible for catalysing all these reactions although none of the proposed intermediates have ever been detected. (Shiota and Palumbo, 1965, Dahal and Gots 1965, Cone and Guroff, 1970, Kobashi, Hariu and Iwai, 1976). This enzyme was named guanosine triphosphate cyclohydrolase by Burg and Brown (1968) and it was suggested that the intermediates have not been identified because they are enzyme-bound. (Wolf and Brown, 1969). It was demonstrated that the Amadori rearrangement occurs by use of 7 methyl guanosine triphosphate (1) as substrate. The methyl group at position 7 prevents the ring closure step and the methylated deoxypentulose derivative (2)



(I)

formed readily reacts with phenylhydrazine to give a phenylosazone of ribose, (Wolf and Brown 1969) this latter reaction being typical of Amadori rearrangement products. Universally ^{14}C labelled guanosine triphosphate yields labelled D erythro 7,8 dihydroneopterin triphosphate whereas guanosine triphosphate specifically labelled at carbon 8 yields



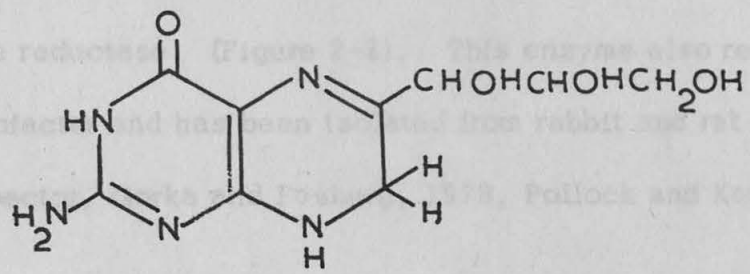
(2)

radioactive formic acid and unlabelled D erythro 7,8 dihydroneopterin triphosphate. (Reynolds and Brown 1964). Hence, it was shown that it is carbon 8 which is lost from guanosine triphosphate (I) by the action of guanosine triphosphate cyclohydrolase.

It has been indicated that the same steps as catalysed by GTP cyclohydrolase in bacteria occur in higher animals and again one enzyme is responsible which has been isolated from chicken liver (Fukushima, Richter and Shiota 1977). The enzyme was named 6-(D erythro 1'-2'-3'-trihydroxypropyl)-7,8 dihydropterin triphosphate synthetase and was shown to have no apparent homology with GTP cyclohydrolase from E. coli using immunological studies. However, Gal and co-workers have characterized two enzymes responsible for these steps in rat brain. The first enzyme they named GTP cyclohydrolase which catalyses the hydrolysis of guanosine triphosphate (I) to formamidopyrimidine ribotide, (II). The second enzyme converts the formamidopyrimidine ribotide (II) to D erythro 7,8 dihydroneopterin

triphosphate (V) and was called D erythro 7,8 dihydroneopterin triphosphate synthetase. (Gal, Nelson and Sherman 1978).

The rest of the biosynthetic pathway from D erythro 7,8 dihydroneopterin triphosphate (V) to L erythro 7,8 dihydrobiopterin (VII) has not been fully elucidated yet. Non-phosphorylated neopterins are not converted to biopterin derivatives but sepiapterin (VI) is. (Eto, Fukushima and Shiota 1976). Therefore, the immediate fate of D erythro dihydroneopterin triphosphate (V) is not the removal of the triphosphate ester to form dihydroneopterin. (3) If universally ^{14}C-labelled guanosine triphosphate is used as the precursor with cold



(3)

sepiapterin (VI) the amount of biopterin produced increases but the incorporated radioactivity decreases compared to if the labelled GTP is used alone. (Fukushima and Shiota 1974) This indicates that sepiapterin (VI) is probably a biosynthetic intermediate of biopterin.

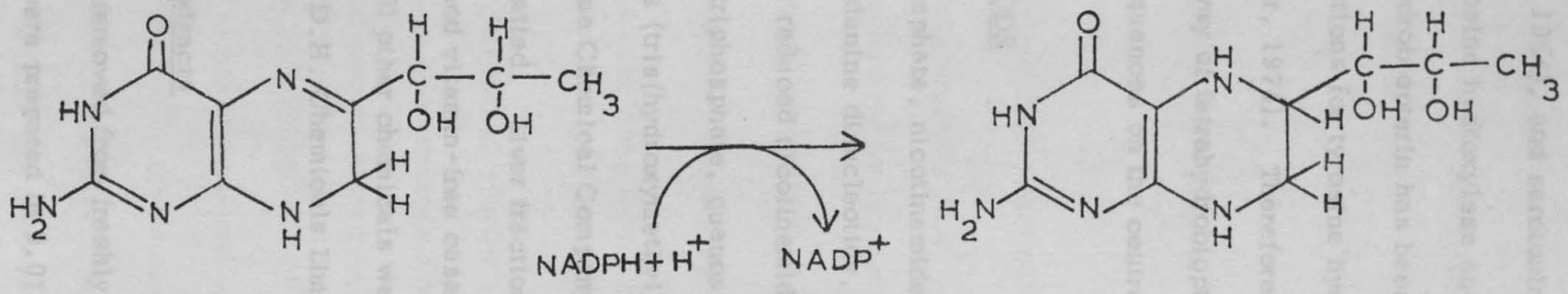
It was shown that NADPH was required for the conversion of D erythro 7,8 dihydroneopterin triphosphate (V) to L erythro 7,8 dihydro-

biopterin (VII) (Eto, Fukushima and Shiota 1976) and it was suggested that sepiapterin reductase which is known to require NADPH may be involved. This enzyme catalyses the conversion of sepiapterin (VI) to L erythro 7,8 dihydrobiopterin (VII). (Nagai and Matsubara 1968, Matsubara, Katoh, Akino and Kaufman, 1966). But also an enzyme which was named L erythro 7,8 dihydrobiopterin synthetase has been isolated from rat brain and shown to catalyse the whole step from D erythro 7,8 dihydroneopterin triphosphate (V) to L erythro 7,8 dihydrobiopterin (VII). (Gal, Nelson and Sherman, 1978).

It has been shown that L erythro 7,8 dihydrobiopterin (VII) can be converted to 5,6,7,8 tetrahydrobiopterin (VIII) by the enzyme dihydrofolate reductase. (Figure 2-2). This enzyme also requires NADPH as cofactor and has been isolated from rabbit and rat brain. (Abelson, Spector, Gorka and Fosburg, 1978, Pollock and Kaufman, 1978).

The importance of this biosynthetic pathway is clear when the consequences due to its absence in the rare condition, malignant hyperphenylalaninaemia are considered. (Leeming, Blair and Rey, 1976, Rey, Blandin-Saroja and Rey 1976, Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel, 1978). Tetrahydrobiopterin is the cofactor required for the rate-limiting steps for the production of both the catecholamine neurotransmitters, dopamine and noradrenaline (Nagatsu, Mizutani, Nagatsu, Matraw and Sigimote,

Figure 2-2 Conversion of Dihydrobiopterin to Tetrahydrobiopterin



(VII) (VIII)

1972, Levitt, Spector, Sjoerdsma and Udenfriend, 1965, Nagatsu, Levitt and Udenfriend, 1964), and serotonin, (Hosoda and Glick, 1966) catalysed by tyrosine hydroxylase and tryptophan hydroxylase respectively. Tetrahydrobiopterin has been shown to be present in rate-limiting concentrations for tyrosine hydroxylase. (Kettler, Bartholini and Pletscher, 1974). Therefore, anything which effects this biosynthetic pathway of tetrahydrobiopterin in any way could have significant consequences on the central nervous system.

MATERIALS AND METHODS

Guanosine triphosphate, nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate, adenosine triphosphate, guanosine diphosphate, guanosine monophosphate and Tris (tris(hydroxymethyl)aminomethane) were purchased from the Sigma Chemical Company. Biopterin was a gift from Roche Products Limited. Liver fraction L was from the Nutrition Biochemical Company and vitamin-free casamino acids was from the Difco Laboratories. All other chemicals were of analytical grade and were purchased from B.D.H. Chemicals Limited.

Preparation of Tissue Extracts

Brain tissue was removed from freshly killed male Wistar rats and 25% homogenates were prepared in 0.01M Tris/HCl, 0.04M KCl

buffer pH 8. These homogenates were centrifuged at 0°C at 19,000 xg in a M.S.E. Superspeed 50 centrifuge for 1 hour. The supernatants were collected and used in the incubations. Protein was measured by the Biuret method. (Layne 1957).

Incubation Methods

The incubation mixture contained the following in a total volume of 1 cm^3 - :

(i) 75 µmoles of Tris/HCl buffer pH 8.

(ii) Guanosine triphosphate (GTP) at various concentrations to provide substrate for biopterin biosynthesis.

(iii) 0.1 cm^3 supernatant (added last to initiate the reaction).

(iv) 0.1 cm^3 additives (if included)

(v) Distilled water to make up the right volume.

The tubes were incubated in a water bath at 37°C in the dark for 3 hours. The reaction was terminated by addition of 2 cm^3 of 0.1M HCl. Any reduced pterins formed were oxidised to their more stable forms by addition of 0.01 cm^3 of iodine solution (2g of KI and 1g of iodine in 100 cm^3 of distilled water) in the presence of one drop of 1% starch solution. After 15 minutes the excess iodine was reduced using 0.1M ascorbic acid. (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota 1975). The mixture was then assayed for biopterin

derivatives using the Crithidia fasciculata assay. (Leeming 1975, Leeming and Blair 1974). The results were calculated as ng biopterin per mg of protein.

The incubation period was varied to discover an appropriate length of time.

Other substrates were tested such as adenosine triphosphate (ATP), guanosine monophosphate (GMP) and guanosine diphosphate (GDP) to discover which was the best precursor for biopterin biosynthesis.

The effect of nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) on the biosynthesis of biopterin derivatives was tested by including a range of concentrations of these pyridine nucleotides in the incubations.

Similarly the effect of magnesium ions and ATP was investigated.

Crithidia Fasciculata Assay

Crithidia fasciculata culture (American Type culture collection No. 12857) was supplied by Dr. S. H.Hutner of Hoskins Laboratory, Pace College, New York, U.S.A. and maintained by Dr. R. J. Leeming,

General Hospital, Birmingham.

This was subcultured weekly into maintenance medium prepared as summarized in Table 2-1. It was incubated for two days at 29°C in the dark and then placed in the refrigerator at 4°C. Prevention of contamination was assisted by the addition of 1 mg/cm^3 ampicillin when inoculating and this did not affect the growth of the flagellate.

The stock assay medium and the double strength assay medium were prepared as summarized in Tables 2-2 and 2-3. The inoculum was prepared by aseptically adding 1 drop of a two day culture to 15 cm^3 of single strength assay medium. This was incubated at 29°C for four days to exhaust the endogenous pteridines. (Dewer and Kidder 1971) 0.2 cm^3 of the resultant growth was added aseptically to 20cm^3 of single strength medium. It was found to be advantageous to add 500 mg of ampicillin to this inoculum. One drop of the inoculum was added to each assay tube using a sterile pasteur pipette.

The samples to be assayed for biopterin derivatives were diluted appropriately with 0.2 M phosphate buffer pH 5. Dilutions were determined by a method of trial and error. 0.5 cm^3 of the diluted sample was added to each of three sterilized rimless 12 x 75 mm assay tubes containing 1.5 cm^3 of distilled water. 2 cm^3 of double strength assay medium was then added to each assay tube to make a total volume of 4 cm^3. Two standard curves were also set up in triplicate and were included with each batch of samples. The

standard curve consisted of eleven tubes containing a range of standard biopterin solutions from 0 - 0. lng in 0.5 cm^3 of 0.2 M phosphate buffer pH 5. These standards were treated exactly the same as the other samples. Where additives were included in the incubations, these were also tested to see if they had a direct effect on the growth of the Crithidia fasciculata itself. For these control tests 0.05 ng of biopterin standard in 0.25 cm^3 of 0.2 M phosphate buffer pH 5 was added to each assay tube in triplicate together with 0.25 cm^3 containing the amount of the additive corresponding to that included in the incubations but appropriately diluted as before. All the assay tubes were then autoclaved at 115°C for 5 minutes using a steam pressure autoclave and inoculated aseptically as described above. The samples were incubated at 29°C for a period of 72-90 hours. The growth of Crithidia fasciculata in each tube was measured turbidometrically using a Gilford micro-sample spectrophotometer 300 with direct digital concentration read-out. This instrument was equipped with sample changer and chart recorder for automatic read-out of growth as described by Leeming and Portman-Graham, (1973). All assays were read as absorption at a wavelength of 590 nm against uninoculated medium. The amount of biopterin in each sample was calculated using the standard curve in ng biopterin/cm^3.

Chromatography

Paper chromatography was performed to identify the pterin

derivatives being measured in the following solvent systems :-

(i) n-butanol/acetic acid/water 4:1:1

(ii) 3 % ammonium chloride

(iii) n-propanol/1% ammonia solution 2:1

(Goto, Forrest, Dickerman and Urushibara 1965,

Viscontini, Schoeller, Loeser, Karrer and Hadorn, 1955)

TABLE 2-1

PREPARATION OF MAINTENANCE MEDIUM

Yeast extract (Oxoid)	0.3 g
Trypticase	0.3 g
Sucrose	0.25g
Liver Fraction L	0.01g
Haemin (5 mg/cm^3 in 50% triethanolamine)	0.5 cm^3
Distilled water	100 cm^3

The pH was adjusted to between 6.8 - 7.6. The medium was autoclaved at 120°C for 15 minutes and stored in the refrigerator at 4°C.

TABLE 2 - 2

PREPARATION OF STOCK ASSAY MEDIUM

PART A

L-arginine hydrochloride	5.0g
L-glutamic acid	10.0g
L-histidine hydrochloride	3.0g
DL-isoleucine	1.0g
DL-leucine	1.0g
L-lysine hydrochloride	4.0g
DL-methionine	1.0g
DL-phenylalanine	0.6g
DL-tryptophan	0.8g
L-tyrosine	0.6g
DL-valine	0.5g
Ethylene-diamine-tetra-acetic acid	6.0g
Boric acid (H_3BO_3)	0.005g
Calcium chloride ($CaCl_2$)	0.005g
Cobalt sulphate ($CoSO_4 7H_2 0$)	0.025g
Copper sulphate ($CuSO_4 5 H_2 0$)	0.025g
Ferric ammonium sulphate ($Fe(NH_4)_2(SO_4)_2 6H_2 0$)	0.010g
Manganese sulphate ($MnSO_4 H_2 O$)	1.4g
Magnesium sulphate ($MgSO_4 7H_2 O$)	6.5g
Tri-potassium phosphate (K_3PO_4)	1.5g

Continued...:.

TABLE 2-2 (Continued..)

PREPARATION OF STOCK ASSAY MEDIUM

PART A

Zinc sulphate ($Zn\ SO_4\ 7\ H_2 0$)	0.5g
Sucrose	150.0g
Distilled water	1000 cm^3

This mixture was steamed at 100°C for 20 minutes to dissolve the constituents and then distributed into sterile bottles and stored in the refrigerator at 4°C in the dark for up to 3 months.

PART B

Adenine	1.0g
Biotin	0.001g
Calcium pantothenate	0.3g
Nicotinic acid	0.3g
Pyridoxamine dihydrochloride	0.1g
Riboflavin	0.06g
Thiamine hydrochloride	0.06g

These vitamins were ground together and stored dry in the refrigerator at 4°C.

PART C

Haemin 5 mg/cm^3 in 50% triethanolamine

This was prepared freshly as required.

TABLE 2-2 (continued..)

PART D

Folic acid 100 ng/cm^3

This was prepared freshly as required.

TABLE 2 - 3

PREPARATION OF DOUBLE STRENGTH ASSAY MEDIUM

For 100 cm^3 ____ :

Distilled water	78 cm^3
Stock assay medium part A	20 cm^3
Stock assay medium part B	4.8 mg
Vitamin-free casamino acids	2.0 g
Triethanolamine (must be added before part C)	0.5 cm^3
Stock assay medium part C	1.0 cm^3
Stock assay medium part D	0.5 cm^3

This was adjusted to pH 7.5 with concentrated sulphuric acid.

RESULTS

A typical standard curve for the growth of Crithidia fasciculata with a range of different biopterin concentrations is shown in Figure 2-3.

The effects of varying the GTP concentration is shown in Table 2-4. It can be seen that guanosine triphosphate alone did not produce any biopterin but when the brain supernatant was present as well there were biopterin derivatives produced. As the concentration of guanosine triphosphate was increased the amount of biopterin derivatives produced also increased until it levelled off at approximately 6mM GTP. Similar results were obtained by varying the guanosine triphosphate concentrations in the presence of 1 mM NADPH. (Figure 2-4) 3mM was chosen as a convenient concentration of GTP to use in the standard incubation.

The results from varying the incubation time are shown in Figure 2-5 and demonstrate that the maximum amount of biopterin was produced at around 2 hours. However, three hours was chosen as the standard incubation time to ensure that the reaction was complete. These results also show that without GTP in the incubation the endogenous biopterin breaks down after about 1 hour.

Table 2-5 shows the effect of different substrates on the production of biopterin derivatives. Guanosine triphosphate was found

to be the best substrate in brain supernatant for biopterin synthesis.

The results using the various pyridine nucleotide additives are shown in Tables 2-6, 2-7, 2-8 and in figure 2-6 in the presence of a constant concentration of GTP (3mM). They all stimulated the production of biopterin derivatives significantly ($p < 0.001$) and it was decided to include a pyridine nucleotide, NADPH in the standard incubation mixture.

As can be seen from Table 2-9 adenosine triphosphate had no significant effect on the production of biopterin derivatives from GTP.

The effect of magnesium chloride on the synthesis of biopterin derivatives is shown in Table 2-10. Magnesium increased the production of biopterin from GTP and this was a significant effect if concentrations of 10^{-5}M or above were used in the presence of 1mM NADH. There was still a significant stimulation by magnesium chloride if the NADH was left out of the incubation.

The paper chromatography showed that a compound was produced when GTP was included in the incubations which co-chromatographed with standard biopterin in the three solvent systems used. However, if boiled supernatant plus GTP or supernatant alone were used in the incubations, this compound could not be detected.

Figure 2-3 STANDARD CURVE FOR CRITHIDIA FASCICULATA ASSAY FOR BIOPTERIN

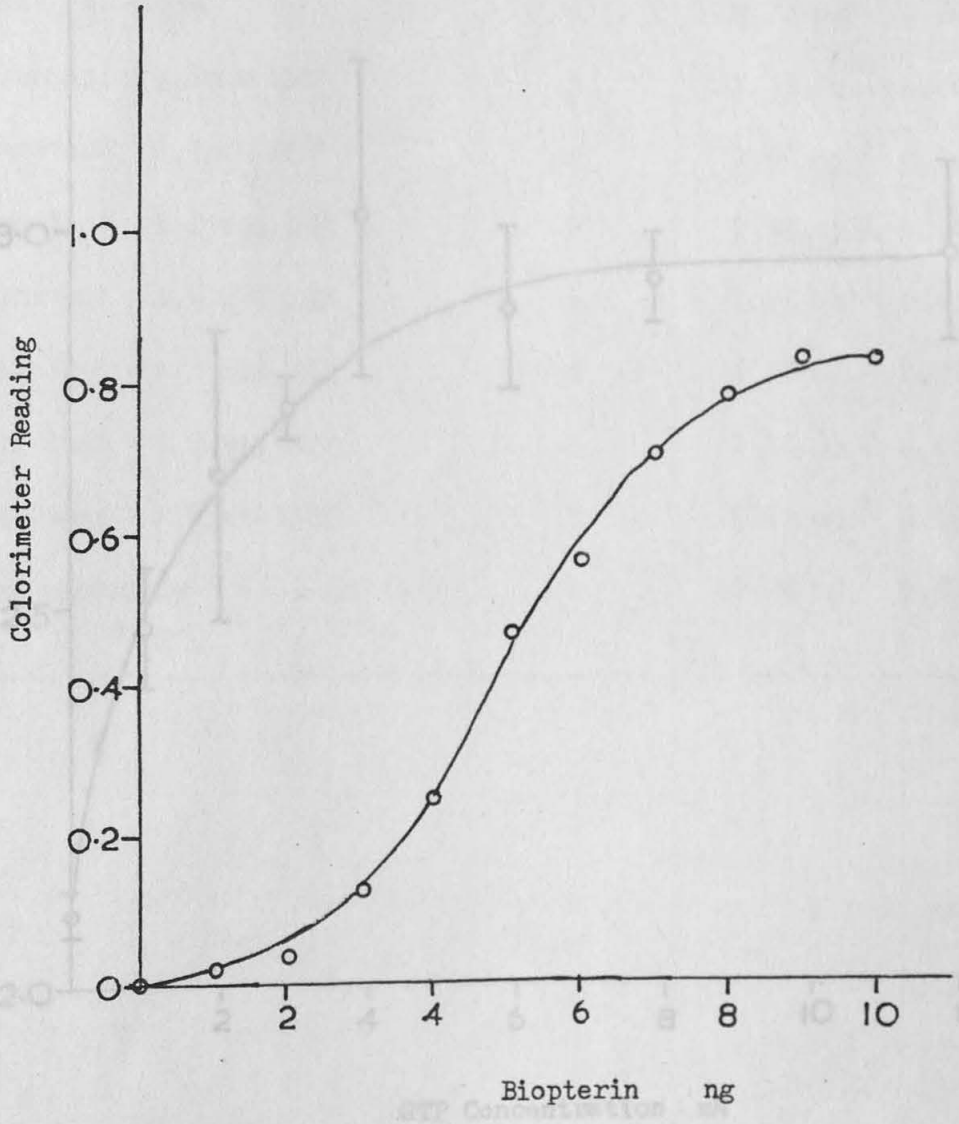


Figure 2-4 EFFECT OF GUANOSINE TRIPHOSPHATE CONCENTRATION ON THE SYNTHESIS OF BIOPTERIN IN THE PRESENCE OF 1 mM NADPH

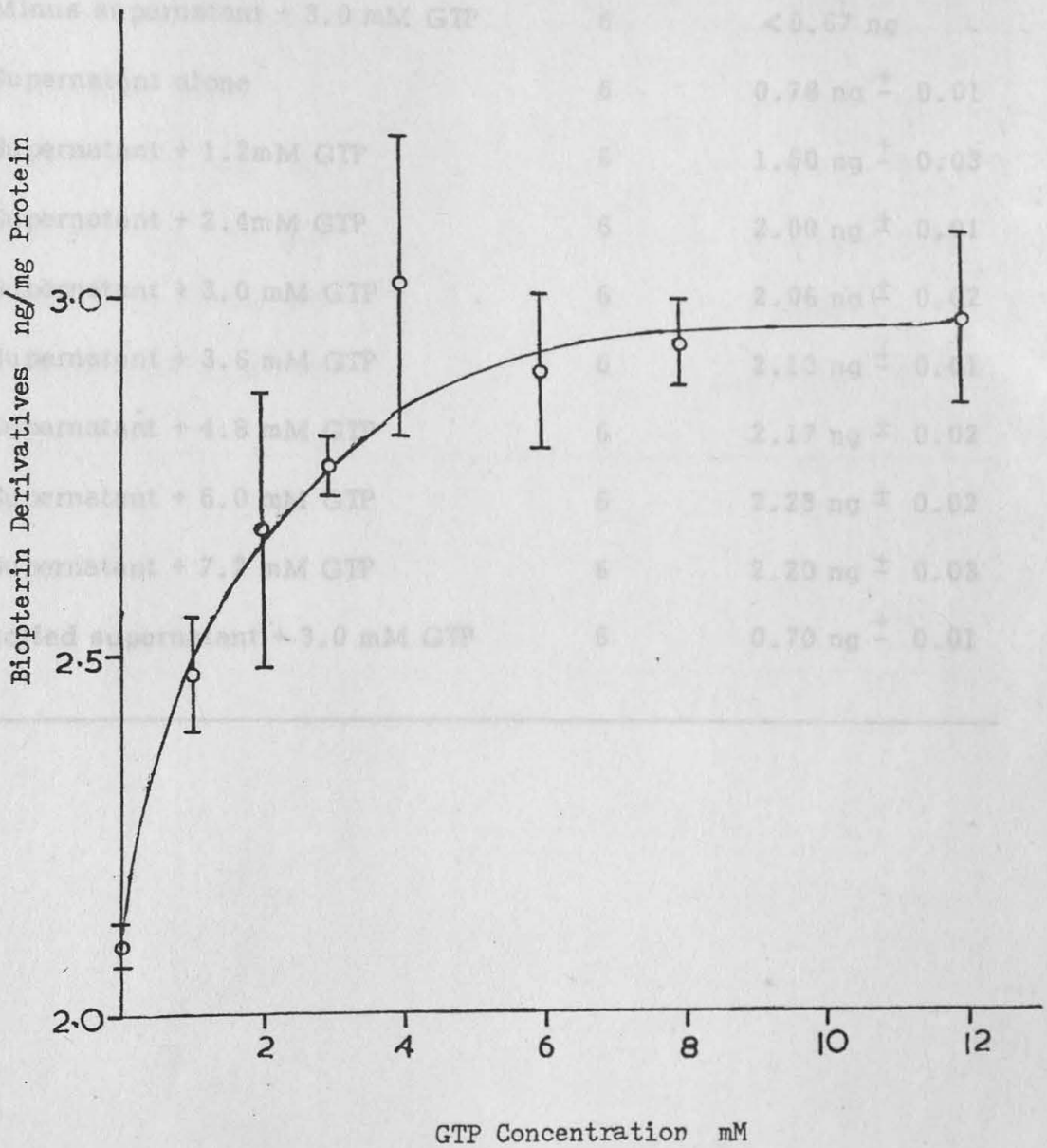


TABLE 2-4

THE EFFECT OF CONCENTRATION OF GTP ON THE SYNTHESIS OF BIOPTERIN DERIVATIVES in vitro

Components	No. of observations	Mean ± SD/mg protein
Minus supernatant + 3.0 mM GTP	6	< 0.67 ng
Supernatant alone	6	0.78 ng ± 0.01
Supernatant + 1.2mM GTP	6	1.50 ng ± 0.03
Supernatant + 2.4mM GTP	6	2.00 ng ± 0.01
Supernatant + 3.0 mM GTP	6	2.06 ng ± 0.02
Supernatant + 3.6 mM GTP	6	2.13 ng ± 0.01
Supernatant + 4.8 mM GTP	6	2.17 ng ± 0.02
Supernatant + 6.0 mM GTP	6	2.23 ng ± 0.02
Supernatant + 7.2 mM GTP	6	2.20 ng ± 0.03
Boiled supernatant + 3.0 mM GTP	6	0.70 ng ± 0.01

TABLE 2-5

EFFECT OF SUBSTRATE TYPE ON SYNTHESIS OF BIOPTERIN DERIVATIVES in vitro

Substrate	No. of Observations	Mean ± SD/mg protein
Supernatant alone	6	0.78ng ± 0.01
Supernatant + 3mM GTP	6	2.06ng ± 0.02
Supernatant + 3mM GDP	5	1.32ng ± 0.01
Supernatant + 3mM GMP	5	1.00ng ± 0.03
Supernatant + 3mM ATP	5	1.03ng ± 0.03

TABLE 2-6

EFFECT OF NICOTINAMIDE ADENINE DINUCLEOTIDE ON BIOPTERIN SYNTHESIS FROM GTP

NAD^+ Concentration	No. of Observations	Mean $\pm$ SD/mg protein
None	6	2.10ng $\pm$ 0.03
0.5 mM	6	2.87ng $\pm$ 0.02
1.0 mM	6	3.71ng $\pm$ 0.08
1.5 mM	6	3.68ng $\pm$ 0.05
2.0 mM	6	3.58ng $\pm$ 0.07
3.0 mM	6	3.64ng $\pm$ 0.02
4.0 mM	6	3.50ng $\pm$ 0.06
1 mM minus GTP	6	0.88ng $\pm$ 0.02

TABLE 2-7

EFFECT OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE ON BIOPTERIN SYNTHESIS FROM GTP

NADH Concentration	No. of observations	Mean $\pm$ SD/mg protein
None	6	2.10ng $\pm$ 0.03
0.5 mM	6	3.47ng $\pm$ 0.05
1.0 mM	6	4.00ng $\pm$ 0.07
1.5 mM	6	4.70ng $\pm$ 0.01
2.0 mM	6	3.93ng $\pm$ 0.06
3.0 mM	6	3.85ng $\pm$ 0.07
4.0 mM	6	3.98ng $\pm$ 0.02
1 mM minus GTP	6	0.85ng $\pm$ 0.03

FIGURE 2-5 THE EFFECT OF INCUBATION TIME ON THE SYNTHESIS OF BIOPTERIN DERIVATIVES FROM GTP

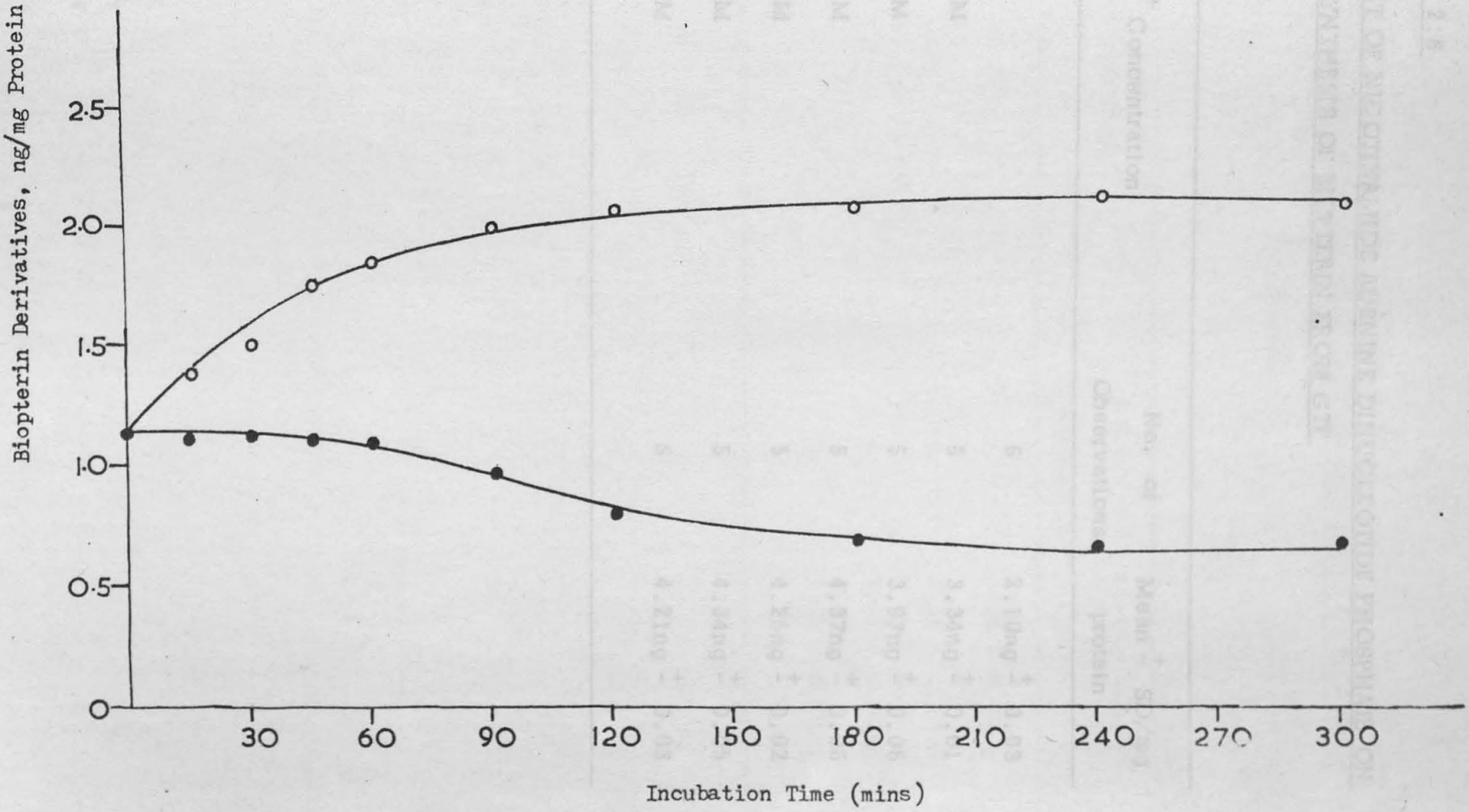


TABLE 2.8

EFFECT OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE ON THE SYNTHESIS OF BIOPTERIN FROM GTP

$NADP^+$ Concentration	No. of Observations	Mean $\pm$ SD/mg protein
None	6	2.10ng $\pm$ 0.03
0.5 mM	5	3.34ng $\pm$ 0.01
1.0 mM	5	3.97ng $\pm$ 0.06
1.5 mM	5	4.37ng $\pm$ 0.06
2.0 mM	5	4.25ng $\pm$ 0.02
3.0 mM	5	4.34ng $\pm$ 0.05
4.0 mM	5	4.21ng $\pm$ 0.03

TABLE 2-9

EFFECT OF ADENOSINE TRIPHOSPHATE ON SYNTHESIS OF BIOPTERIN FROM GTP

ATP Concentration	No. of Observations	Mean ± SD/mg protein
None	6	2.07 ± 0.02ng
1 mM	6	2.00 ± 0.03ng
3 mM	6	2.05 ± 0.02ng
6 mM	6	2.03 ± 0.01ng

FIGURE 2-6 THE EFFECT OF NADPH CONCENTRATION ON THE PRODUCTION OF BIOPTERIN DERIVATIVES FROM GTP

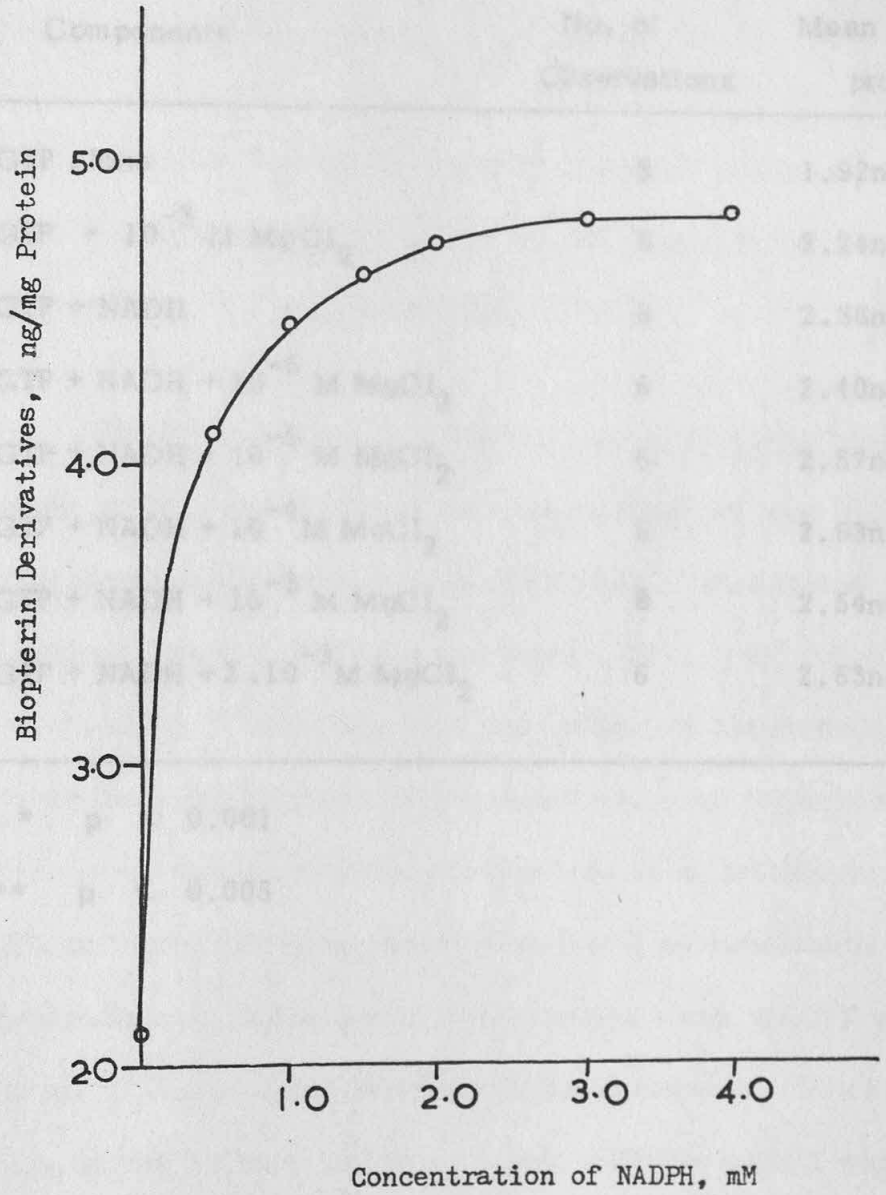


TABLE 2-10

EFFECT OF MAGNESIUM CHLORIDE ON THE PRODUCTION OF BIOPTERIN DERIVATIVES FROM GTP

Components	No. of Observations	Mean $\pm$ SD/mg protein
GTP alone	8	1.92ng $\pm$ 0.09
GTP + 10^{-3} M $MgCl_2$	6	2.24ng $\pm$ 0.02 *
GTP + NADH	8	2.36ng $\pm$ 0.01
GTP + NADH + 10^{-6} M $MgCl_2$	6	2.40ng $\pm$ 0.01
GTP + NADH + 10^{-5} M $MgCl_2$	6	2.57ng $\pm$ 0.03**
GTP + NADH + 10^{-4} M $MgCl_2$	6	2.63ng $\pm$ 0.07**
GTP + NADH + 10^{-3} M $MgCl_2$	8	2.54ng $\pm$ 0.08 *
GTP + NADH + 2.10^{-3} M $MgCl_2$	6	2.63ng $\pm$ 0.03 *

* $p < 0.001$

** $p < 0.005$

DISCUSSION

The results obtained here for the biosynthesis of biopterin in rat brain supernatants do not differ substantially from results obtained with different mammalian tissues (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota, 1975, Eto, Fukushima and Shiota 1976, Fukushima, Eto, Saliba and Shiota, 1975) and cell cultures. (Buff and Dairman 1975a, Fukushima and Shiota 1974).

GTP was found to be the best precursor of the purine derivatives tested for rat brain biosynthesis of biopterin as in hamster liver. (Fukushima, Eto, Saliba and Shiota 1975).

Although not absolutely necessary for biopterin biosynthesis in rat brain, the addition of pyridine nucleotides was found to be stimulating as in hamster liver and kidney. (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota, 1975, Eto, Fukushima and Shiota 1976). Since this work was done, the requirement for NADPH by rat brain for biopterin biosynthesis has been published (Lee, Fukushima and Nixon 1978) but this was using a different assay system where GTP cyclohydrolase from E.coli was used with the rat brain supernatant. Of the pyridine nucleotides used, NADPH was found to be the best stimulator for biopterin biosynthesis. This has also been shown for hamster kidney where NADH or NADPH was shown to be essential for the conversion of D erythro dihydroneopterin triphosphate to biopterin and of the two, NADPH was the more efficient.

(Eto, Fukushima and Shiota 1976). It seems likely from these results that a reduced pyridine nucleotide is required at one or more points along the biosynthetic pathway for biopterin in rat brain. There are several possible points where these reduced pyridine nucleotides may be involved assuming the biosynthetic pathway is similar to that in bacteria. These cofactors may keep the D erythro dihydroneopterin triphosphate in the dihydro form, protecting it from being converted to an inactive form. They may be required by the enzyme sepiapterin reductase which catalyses the conversion of sepiapterin to dihydrobiopterin and requires the cofactor NADPH (Nagai 1968, Matsubara, Katsh, Akino and Kaufman 1966) and has been shown to be present in rat brain. (Rembold and Metzger 1963). This assumes that this enzyme is necessary for the biosynthesis of biopterin in rat brain. Or the cofactors might be required for unknown enzyme reactions which are involved in this pathway.

Magnesium chloride was also found to be stimulating for the production of biopterin in rat brain as in hamster kidney biopterin biosynthesis (Eto, Fukushima and Shiota, 1976) and using the mixed assay for rat brain biosynthesis. (Lee, Fukushima and Nixon 1978). Magnesium ions could be required for the efficient activity of one or more of the enzymes involved in this pathway.

These results indicate that the biosynthetic pathway for biopterin is present in rat brain and that GTP is necessary for this

synthesis. Pyridine nucleotides and magnesium ions were shown to stimulate this pathway.

These results enabled a standard incubation mixture for biopterin biosynthesis to be assembled which included GTP as substrate, NADPH and magnesium ions.

CHAPTER THREE

THE EFFECT OF METAL IONS, DRUGS AND OTHER COMPOUNDS ON THE IN VITRO BIOSYNTHESIS OF BIOPTERIN DERIVATIVES

INTRODUCTION

Tetrahydrobiopterin is an essential cofactor for tyrosine hydroxylase (Nagatsu, Mizutani, Nagatsu, Matsura and Sugimoto, 1972) and for tryptophan hydroxylase, (Hosada and Glick 1966) the rate-limiting steps in catecholamine biosynthesis (Levitt, Spector, Sjoerdsma and Udenfriend 1965, Nagatsu, Levitt and Udenfriend 1964) and serotonin biosynthesis (Costa and Meek 1974) respectively. Tetrahydrobiopterin is present in tissues at concentrations lower than the Km value for tyrosine hydroxylase and therefore its concentration is rate-limiting in controlling the activity of this enzyme (Kettler, Bartholini and Pletscher 1974) and hence catecholamine biosynthesis. (Cote et al., 1975) Tetrahydrobiopterin is also present in rate-limiting amounts for tryptophan hydroxylase activity (Costa and Meek 1974) but tryptophan concentrations are an important controlling factor for this enzyme. (Jequier, Lovenberg and Sjoerdsma 1967) Obviously tetrahydrobiopterin concentrations are going to have important consequences on the production of these neurotransmitters. The importance of the biosynthetic pathway of tetrahydrobiopterin is demonstrated by the neurological defects which occur in the form of malignant hyperphenylalaninaemia where there is a lesion in this pathway. (Leeming, Blair and Rey 1976, Rey, Blandin-Saroja and Rey 1976, Kaufman, Berlow, Summer, Milstien

Schulman, Orloff, Spielberg and Pueschel 1978)

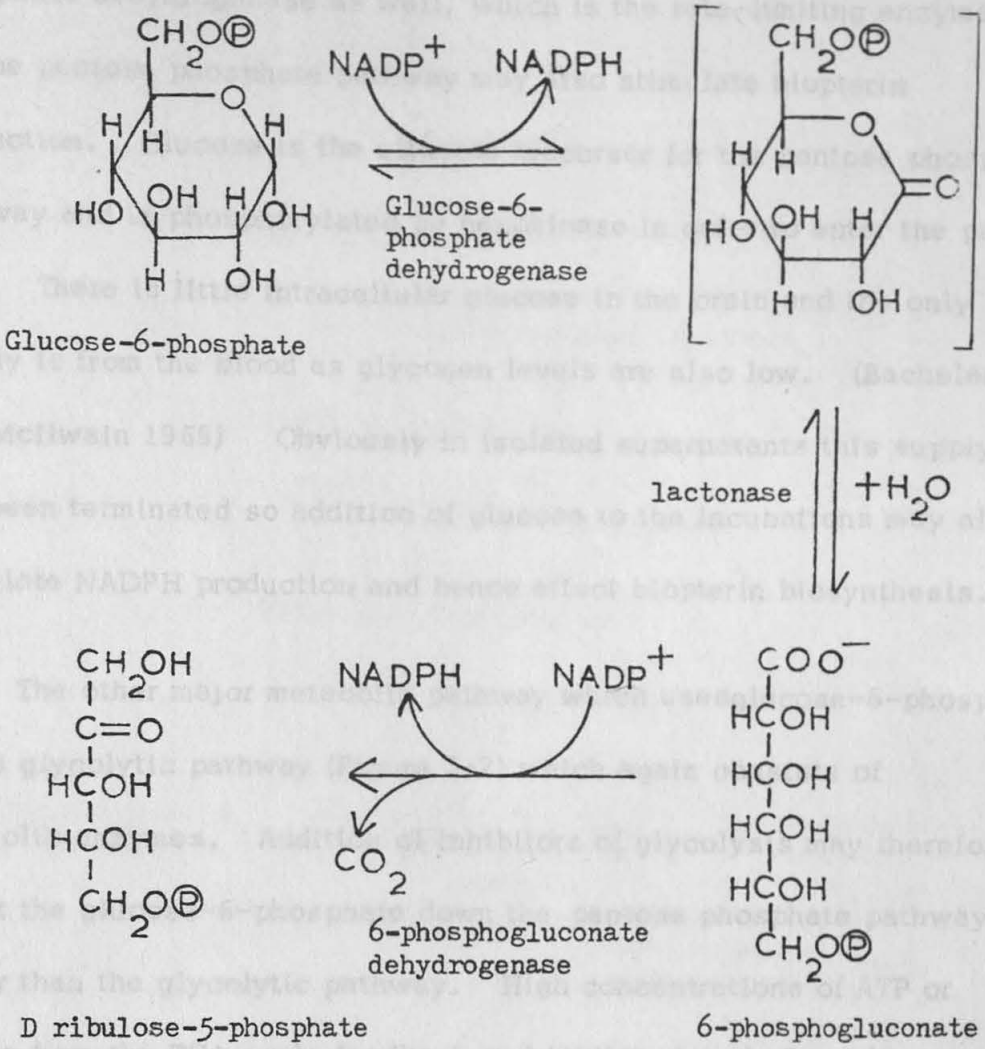
Anything which affects the biosynthesis of tetrahydrobiopterin in any way would therefore cause considerable changes in neurotransmitter biosynthesis and hence, have serious implications on the central nervous system.

Reduced pyridine nucleotides stimulate the biosynthesis of biopterin. (Chapter 2) Therefore, the supply of these nucleotides could consequently cause changes in the amount of biopterin produced. NADPH can be provided by various methods such as glutamate dehydrogenase and the pentose phosphate pathway. (Lehninger 1970) Glutamate dehydrogenase is a pyridine-linked enzyme which catalyses the deamination of glutamate. It can use either NAD^+ or $NADP^+$.

$$\text{L-glutamate} + NADP^+ \rightleftharpoons \alpha\text{-ketogluturate} + NH_4^+ + NADPH$$

As well as unloading the amino groups in the form of NH_4^+ it serves as a supply of NADPH. The pentose phosphate pathway (Figure 3-1) is also a good source of reduced nicotinamide adenine dinucleotide phosphate. The enzymes of the pentose phosphate pathway are cytosolic but are not present in large amounts in brain tissue. (Glock and McLean 1954) Assuming that glutamate dehydrogenase and the enzymes of the pentose phosphate pathway are present in the brain supernatant preparations, addition of the substrates, glutamate and glucose-6-phosphate might have an effect on biopterin biosynthesis by stimulating NADPH production. Addition of these substrates with the cofactor

FIGURE 3-1 THE PENTOSE PHOSPHATE PATHWAY



Glucose-6-phosphate

D ribulose-5-phosphate

6-phosphogluconate

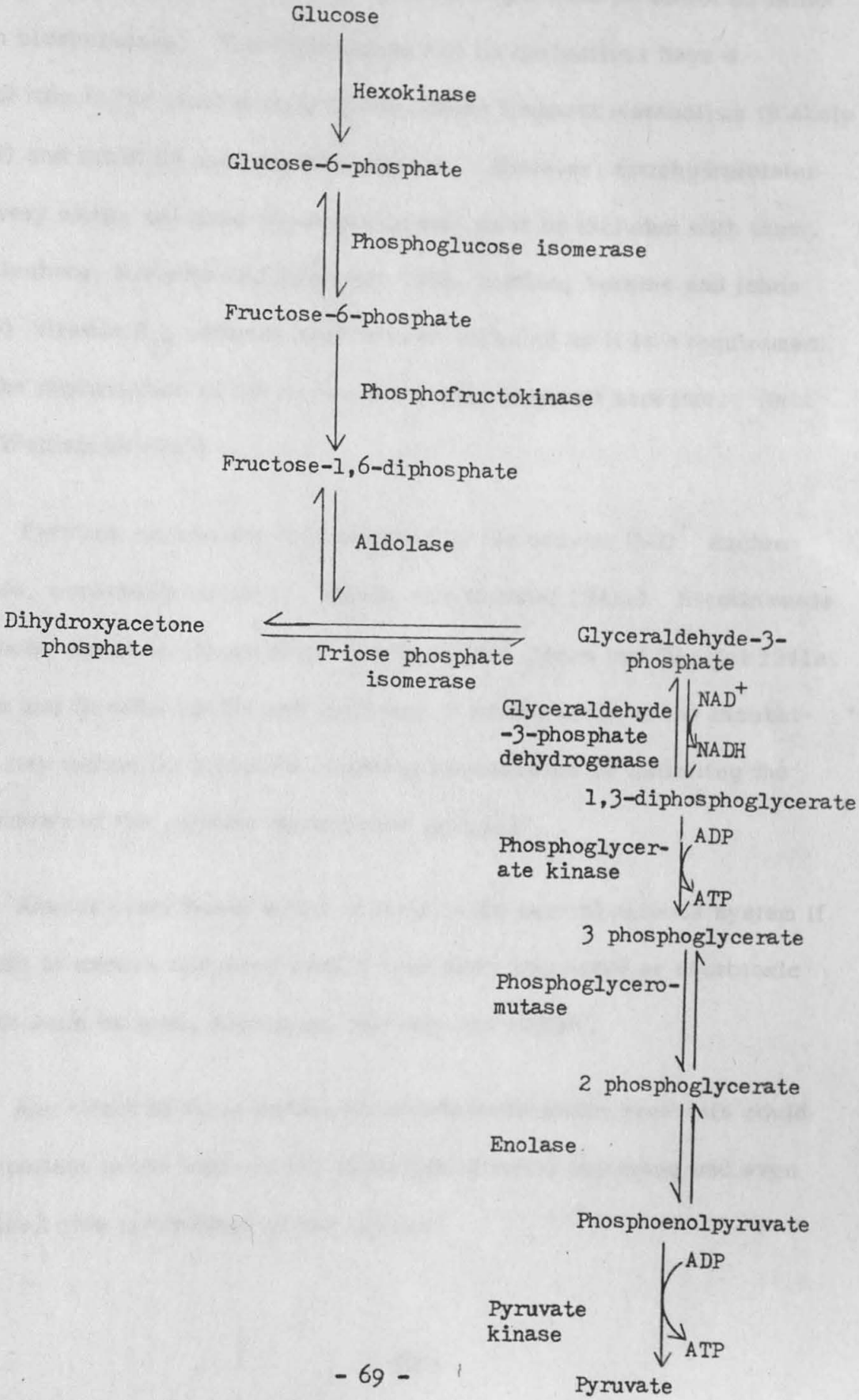
D ribose-5-phosphate

$NADP^+$ and in the case of glucose-6-phosphate some glucose-6-phosphate dehydrogenase as well, which is the rate-limiting enzyme for the pentose phosphate pathway may also stimulate biopterin production. Glucose is the ultimate precursor for the pentose phosphate pathway and is phosphorylated by hexokinase in order to enter the pathway. There is little intracellular glucose in the brain and the only supply is from the blood as glycogen levels are also low. (Bachelard and McIlwain 1969) Obviously in isolated supernatants this supply has been terminated so addition of glucose to the incubations may also stimulate NADPH production and hence effect biopterin biosynthesis.

The other major metabolic pathway which usesglucose-6-phosphate is the glycolytic pathway (Figure 3-2) which again consists of cytosolic enzymes. Addition of inhibitors of glycolysis may therefore direct the glucose-6-phosphate down the pentose phosphate pathway rather than the glycolytic pathway. High concentrations of ATP or citrate from the TCA cycle feedback and inhibit phosphofructokinase. (Webb 1966a, Webb 1966b) Iodoacetate inhibits glyceraldehyde-3-phosphate dehydrogenase which is an enzyme with free thiol groups. (Webb 1966c, Velick and Furfine 1963) Fluoride ions inhibit the enzyme, enolase especially if phosphate and magnesium ions are also present due to the formation of a magnesium-fluoride-phosphate complex (Wold 1971) and phenylalanine inhibits pyruvate kinase. (Boyer 1962)

As the biosynthetic pathway includes the elimination of a one

FIGURE 3-2 THE GLYCOLYTIC PATHWAY



carbon fragment after the initial ring-opening step (Buff and Dairman 1975) the addition of a one carbon unit acceptor might have an effect on biopterin biosynthesis. Tetrahydrofolate and its derivatives have a major role in the biochemistry of one carbon fragment metabolism (Blakely 1969) and could be used for this purpose. However, tetrahydrofolates are very easily oxidised so an antioxidant must be included with them. (Wittenberg, Noronha and Silverman 1962, Bertino, Perkins and Johns 1965) Vitamin B_{12} cofactor must also be included as it is a requirement for the regeneration of the active one carbon fragment acceptor. (Brot and Weissbach 1966)

Pyridine nucleotides are degraded by the enzyme NAD^+ nucleosidase, especially in brain. (Mann and Quastel 1941a) Nicotinamide has been shown to inhibit NAD^+ nucleosidase (Mann and Quastel 1941a, Mann and Quastel 1941b) and inclusion of nicotinamide in the incubations may indirectly stimulate biopterin biosynthesis by inhibiting the breakdown of the pyridine nucleotides included.

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

Any effect of these metals on tetrahydrobiopterin synthesis could be important in the study of the symptoms of metal poisoning and even perhaps, give information on the causes.

Exposure to high levels of lead has been shown to cause neurotoxicity. (Byers and Lord 1943, Chisolm 1971, Rutter 1980) Behavioural changes and hyperactivity occur in animals (Sauerhoff and Michaelson 1973, Silbergeld and Goldberg 1974) and humans (David, Clark and Voeller 1972, Baloh, Sturm, Green and Gleser 1975, Beevers, Erskini, Robertson, Beattie, Campbell, Goldberg, Moore and Hawthorne 1976) when blood lead levels are greater than at least 80 µg/dl. At present no biochemical explanation for the neurological effects of lead has been presented and the few studies on the interaction of neurochemical components and lead have produced conflicting results. (Sauerhoff and Michaelson 1973, Silbergeld and Goldberg 1975, Golter and Michaelson 1975, Michaelson, Greenland and Roth 1976, Satija and Seth 1978, Shih and Hanin 1978) Children are more susceptible to lead poisoning than adults. (Byers 1959) Lead poisoning can arise in a variety of situations (Guinea 1972) due to its ubiquitous use. Lead in paint and lead water pipes present a major hazard, (Egan 1972, MAFF 1972, MAFF 1975, Campbell 1977, Barltrop 1969, Airborne Lead in Perspective 1972) and lead from petrol fumes is thought to be an environmental problem. (National Inventory Air Pollution Emission and Control, 1970)

Aluminium has recently become a neurotoxic issue due to the high levels which have been found in the brains of patients suffering from Dialysis Dementia. (dialysis associated with encephalopathy) (Flendrig, Kruis and Das 1976, Alfrey, LeGendre and Kaehny 1976, Parkinson, Ward,

Feest, Fawcett and Kerr 1979, Wing, Brunner, Brynger, Chantler Donckerwolcke, Gurland, Jacobs, Kramer and Selwood 1980) In this, usually fatal disease, aluminium in the brain was shown to be accumulated to levels about six times the concentration found in undialysed controls during haemodialysis for renal failure. (Alfey, LeGendre and Kaehny 1976) The levels of aluminium in patients who had dialysis dementia were about $1.18 \cdot 10^{-4}$M compared to those of undialysed controls which were about $2.0 \cdot 10^{-5}$M whereas non-demented dialysis patients had levels of about $3.26.10^{-5}$M. (McDermott, Smith, Ward, Parkinson and Kerr 1978) Aluminium has also been implemented in Alzheimer's disease where raised aluminium concentrations in the brains of patients dying from this form of dementia have been reported. (Crapper, Krishman and Quittkat 1976, Crapper, Krishman and Dalton 1973) In normal subjects the mean concentration of aluminium in grey matter was 1.9 ± 0.7 µg/g and in the patients with Alzheimer's disease it ranged from 2.8 ± 2.7 to 4.3 ± 1.8 µg/g. (Crapper, Krishman and Quittkat 1976) Intracranial injection of aluminium salts to cats produced cerebral function disorders such as impaired short-term acquisition and retention of conditional behavioural responses. (Crapper, Dalton 1973a, Crapper and Dalton 1973b) The degree of functional disorder was related to the number of neurofibrillary tangles and the amount of aluminium present in the cerebral cortex. (Crapper and Dalton 1973b) Neurofibrillary tangles are a striking histological feature of the brain in Alzheimer's disease.

Mercury is associated with severe CNS toxicity. (Flink 1975, Cumings 1959) The symptoms of mercury poisoning are fatigue, incoordination, acrodynia, loss of memory, visual disturbances and there are microscopic changes in cerebellar and ganglionic cells. (Flink 1975, Hirschman, Feingold and Boyler 1963) Mercury poisoning may arise as an occupational hazard, from house paint or environmental pollution. (Joselow, Louvia and Browder 1972, Hirschman, Feingold and Boyler 1963) Mercury damages the blood-brain barrier and this leads to incorporation of mercury into the brain quite readily. (Magos 1968) The mean mercury concentration in seventeen post-mortem brain specimens was 0.28 µg/g in the cerebellum (Glomski, Brody and Pulay 1971) but levels of 1 or 2 µg/g have been reported. (Olszewski, Pillay, Glomski and Brody 1974)

Cadmium poisoning is mainly caused by exposure from manufacturing industries and is rarely fatal. Its symtoms include headaches and pulmonary oedema. (Flink 1975)

Copper is normally present in the central nervous system at concentrations of approximately 100 µg/g dry weight. (Scheinberg 1978) Copper is an essential element for life but is also potentially toxic. Therefore, mechanisms for the conservation when uptake is low and homeostasis of copper have had to be evolved along with mechanisms to exclude unwanted excess copper. These homeostatic processes are not well understood and in fact their existence is only known from

patients in whom one of the mechanisms is defective or genetically absent. For example, a fatal intracellular deficiency of copper known as Menkes' Disease (Nat. Acad. Sci. Washington D.C. 1977) is caused by a defect in transporting copper across membranes. Another rare genetic defect causes chronic copper toxicity known as Wilson's Disease (Wilson 1912, Nat. Acad. Sci. Washington D.C. 1977) due to a fault in normal excretory pathway for daily copper balance. The toxic symptoms of copper are haemolysis, hepatic necrosis, hypotension and convulsions. (Scheinberg and Steinlieb 1976) Brain levels of copper of 3.65 μmol/g have been reported. (Hamlyn, Gollan, Douglas and Sherlock 1977) Reduced pteridines are sensitive to catalytic oxidation by low concentrations of copper. (Blair and Pearson 1974) So the effect of copper on tetrahydrobiopterin biosynthesis could prove to be an important effect.

Phenylalanine given in phenylalanine-loading tests causes serum levels of dihydrobiopterin to rise. (Leeming, Blair, Green and Raine 1976) The levels of serum dihydrobiopterin appear to correlate with those of serum phenylalanine but seem to be independent of serum tyrosine (Leeming 1975) and tryptophan levels. (Leeming 1979) In hyperphenylalaninaemia the levels of phenylalanine are raised dramatically. (Letendre, Nagaiah and Guroff 1980) Hence phenylalanine may have an affect on biopterin biosynthesis.

The drugs, methotrexate (amethopterin) and co-trimoxazole (sulphamethoxazole with trimethoprim) have both been shown to cause

rises in serum dihydrobiopterin levels of patients treated with them. (Leeming, Blair, Melikian and O'Gorman 1976) Co-trimoxazole and methotrexate were further shown to interfere with phenylalanine metabolism by reducing its clearance after an oral load. (Andrews, Purkiss, Chalmers and Watts 1976, Goodfriend and Kaufman 1961) Methotexate and one component of co-trimoxazole, trimethoprim have been shown to inhibit dihydrofolate reductase (Futterman and Silverman 1957, Osborn & Huennekens 1958, Buchall and Hitchings 1965) which could be involved in the last step of tetrahydrobiopterin biosynthesis. (Chapter 2) Other drugs which inhibit dihydrofolate reductase are aminopterin, (Osborn and Huennekens 1958) tetroxoprim, (Burchell 1979) triampterene (Bertino, Perkins and Johns 1965) a 'potassium sparing' diuretic and pyrimethamine (Waxman and Herbert 1969, Hitchings 1972) a drug used to treat malaria (Covell 1953) toxoplasmosis (Grisham 1962) and polycythaemia. (Issacs 1954) The effect of any of these drugs on biopterin biosynthesis may help to disclose their site of action.

6-mercaptopurine, azathioprine and thioguanine are all drugs which interfere with purine synthesis and hence may interfere in the supply of guanine nucleotides for biopterin biosynthesis. (Stebbins, Scott and Herbert 1973)

MATERIALS AND METHODS

L-phenylalanine, L-tyrosine, L-tryptophan and nicotinamide (niacinamide) were purchased from the Sigma Chemical Company.

Sulphamethoxazole, trimethoprim and mercaptopurine were obtained from Burroughs Wellcome. Upjohn Limited supplied cytosine arabinoside and thioguanine was obtained from the Wellcome Foundation. Fluoro-uracil was a gift from Roche Products Limited. Aminopterin, triampterene, tetroxoprim, pyrimethamine and azathioprine were gifts from Dr. R. J. Leeming of the General Hospital, Birmingham. Methotrexate (amethopterin) was purchased from Lederle Laboratories. All other chemicals were of analytical grade and purchased from B.D.H. Chemicals Limited, or as described in Chapter 2.

Brain supernatant from freshly killed male Wistar rats was prepared as described in Chapter 2.

The standard incubation mixture now contained the following in a total volume of 1 cm^3 -:

(i) 75 μmoles of Tris/HCl buffer pH 8.0.

(ii) 3 μmoles of G.T.P. as substrate.

(iii) 1 μmole of NADPH.

(iv) 2 μmoles of $MgCl_2$.

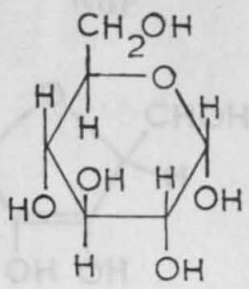
(v) 0.1 cm^3 of additive (if required).

(vi) 0.1 cm^3 of brain supernatant (added last to initiate the reaction).

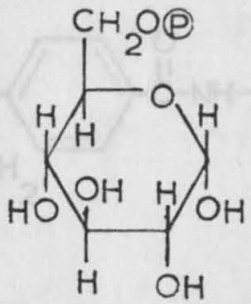
(vii) Distilled water to make up the volume and replace any component which was omitted.

TABLE 3-1 COMPOUNDS TESTED FOR EFFECTS ON THE in vitro BIOSYNTHESIS OF BIOPTERIN

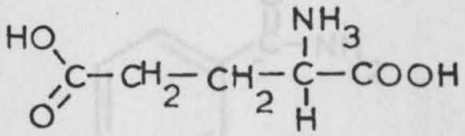
D Glucose



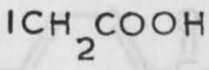
Glucose-6-phosphate



L Glutamate



Iodoacetate



Citrate

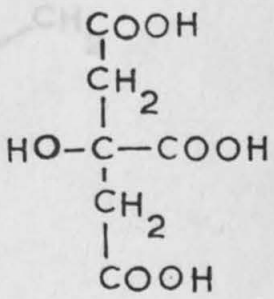
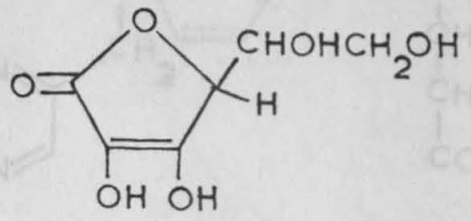


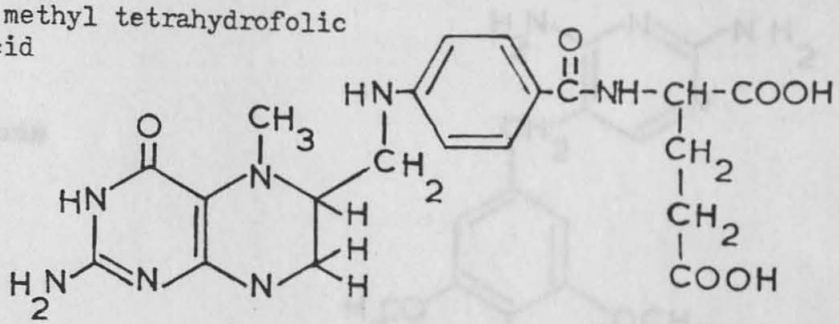
TABLE 3-1 continued

Sodium fluoride

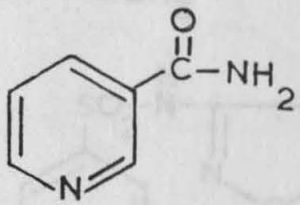
Ascorbate



5 methyl tetrahydrofolic acid



Nicotinamide



Methotrexate

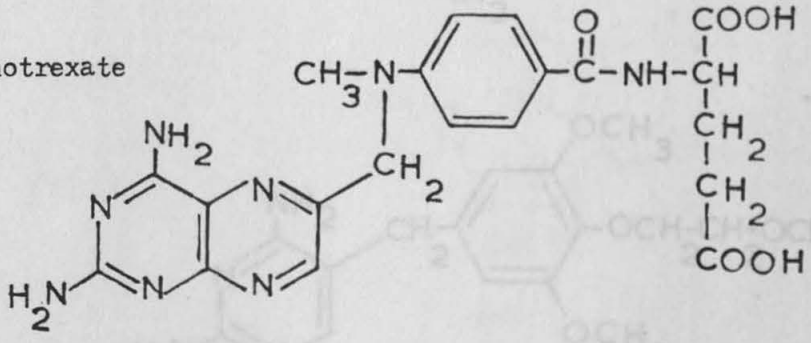
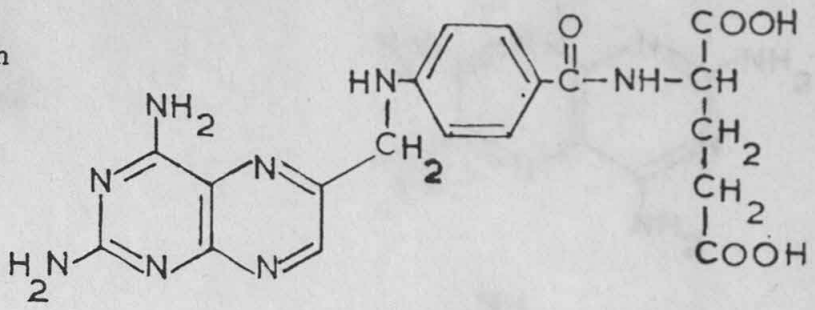
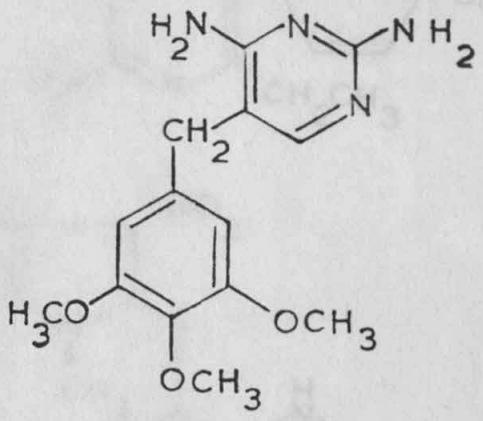


TABLE 3-1 continued

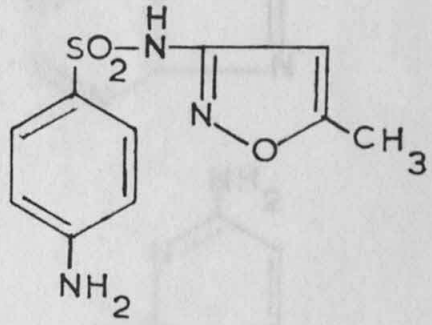
Aminopterin



Trimethoprim



Sulphamethoxazole



Tetroxoprim

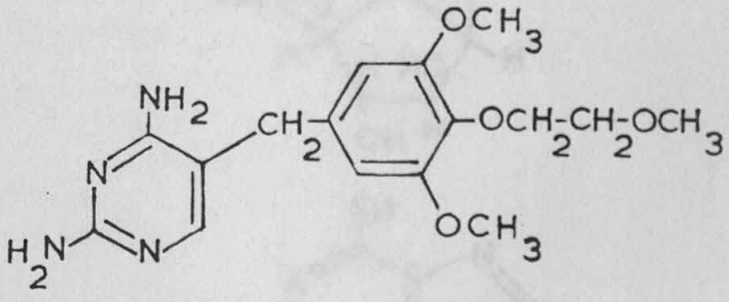
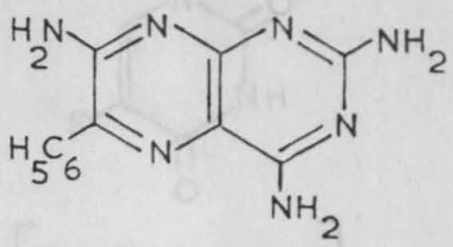
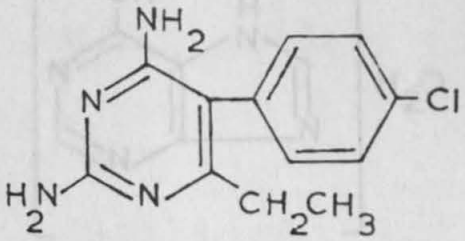


TABLE 3-1 continued

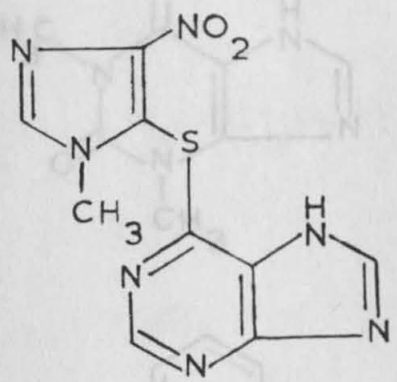
Triampterene



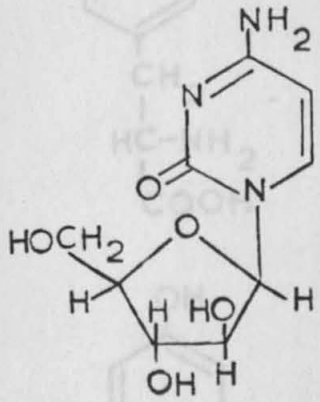
Pyrimethamine



Azathioprine



Cytosine arabinoside



Thioguanine

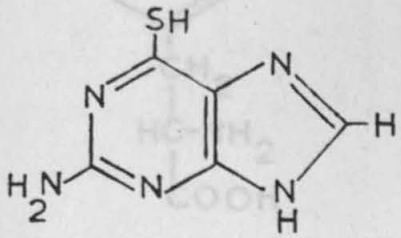
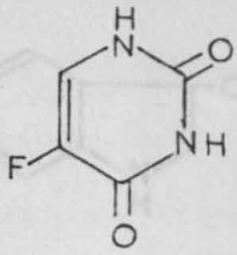
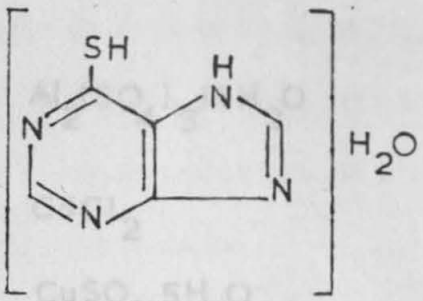


TABLE 3-1 continued

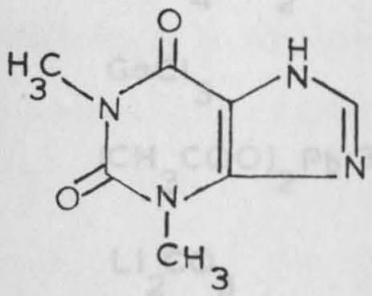
Fluoro-uracil



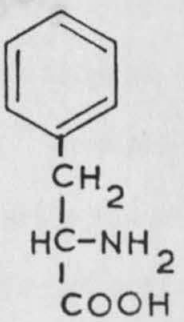
Mercaptopurine



Theophylline



L phenylalanine



L tyrosine

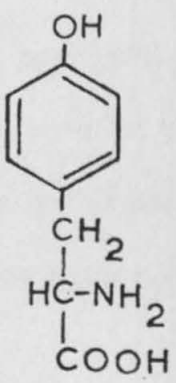
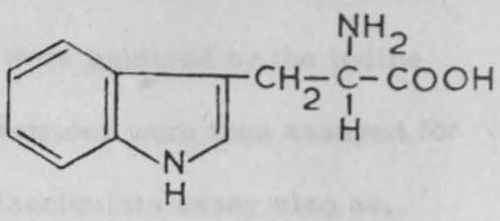


TABLE 3-1 continued

L tryptophan	
Aluminium sulphate	$Al_2(SO_4)_3.16H_2O$
Cadmium chloride	$CdCl_2$
Cupric sulphate	$CuSO_4.5H_2O$
Gallium chloride	$GaCl_3$
Lead acetate	$(CH_3COO)_2Pb.3H_2O$
Lithium carbonate	Li_2CO_3
Mercuric chloride	$HgCl_2$

The tubes were incubated in a water bath at 37°C in the dark for 3 hours when the reaction was terminated by the addition of 2 cm^3 of 0.1 M HCl. Any reduced pterins formed were oxidised by the iodine method as described in Chapter 2. The samples were then assayed for biopterin derivatives using the Crithidia fasciculata assay also as described in Chapter 2 and the results were calculated in ng of biopterin per mg of protein. Appropriate controls for the effect of the various additives used in order to test the effect of these compounds on the growth of Crithidia fasciculata itself were also included.

The compounds tested for their effects on biopterin biosynthesis are listed in Table 3-1. (The Pharmaceutical Codex 1979)

RESULTS

The results of the tests of the agents used to stimulate NADPH production are shown in Table 3-2. Glutamate only gave a significant increase in biopterin biosynthesis when $NADP^+$ was present also. However, this increase was not as great as that produced with NADPH alone. Glutamate and $NADP^+$ with NADPH did not give an additive effect so the NADPH could be sparing the glutamate and $NADP^+$. Glucose-6-phosphate alone did not change the amount of biopterin biosynthesis and also glucose-6-phosphate dehydrogenase alone only increased the biopterin biosynthesis significantly if 7 units of the enzyme were provided. However, together they gave a significant

increase in biopterin biosynthesis with 2.5 units of the enzyme. Providing $NADP^+$ with glucose-6-phosphate and glucose-6-phosphate dehydrogenase gave a further stimulation to biopterin biosynthesis which increasedwith increasing $NADP^+$ concentrations. Again this mixture did not produce an effect of the same magnitude as NADPH although they did not give an additive result if NADPH was provided as well. Glucose significantly inhibited the in vitro production of biopterin and the inhibition increased as the glucose concentration increased.

The results of the glycolytic inhibitors are shown in Table 3-3. All three compounds gave significant increases in biopterin biosynthesis and as the concentration of inhibitor was raised the effect also increased. Iodoacetate and sodium fluoride raised the synthesis of biopterin by about 20% whereas citrate only increased the synthesis by about 8% which is much less than the approximately 130% increase with NADPH. Again these reagents did not give additive results with NADPH.

Table 3-4 shows the results from the addition of ascorbate, 5-methyl tetrahydrofolic acid and coenzyme B_{12} to the standard assay mixture. Ascorbate increased the biosynthesis of biopterin significantly by as much as 50% and the degree of rise corresponded to the amount of ascorbate added. However, there was no additive effect with ascorbate and NADPH together. 5-methyl tetrahydrofolic acid had no effect alone but with ascorbate there was an additional rise and the amount of biop-

terin formed increased as the amount of 5-methyl tetrahydrofolate added increased. 5-methyltetrahydrofolate also gave an additional increase when NADPH was present as well as compared to NADPH alone. Coenzyme B_{12} had no effect alone or additional effect with ascorbate. However, if ascorbate and 5-methyltetrahydrofolate were added with coenzyme B_{12} there was a further increase to give an approximate doubling of biopterin biosynthesis. If coenzyme B_{12} was added with 5-methyltetrahydrofolate and NADPH there was a further increase in biopterin biosynthesis of approximately 50%.

The results of adding nicotinamide to the standard assay mixture areshown in Table 3-5. The significant increases in biopterin biosynthesis shown are related to the nicotinamide concentration used and the maximum increase achieved was about 20% with 10^{-1} M nicotinamide.

Table 3-6 shows the results of the metallic salts on biopterin biosynthsis. The most dramatic effect was the decrease caused by the lead acetate which was significant at concentrations as low as 10^{-8} M. The maximum effect with lead acetate was a 45% reduction of biopterin biosynthesis. All the other metals also showed significant decreases in the biopterin produced but to lesser extents except for lithium carbonate in which 10^{-2} M was required to produce a decrease significant at the 1% level.

The results for the drugs are shown in Table 3-7. All the dihydrofolate reductase inhibitors increased the amount of biopterin produced.

Of these, methotrexate appeared to be the most potent giving an increase of 135% at 1.1. 10^{-4}M.

All the purine inhibitors gave significant decreases in the amount of biopterin formed. The pyrimidine inhibitors gave increases in the amount of biopterin produced, that of cytosine arabinoside being significant and that of fluoro-uracil not being significant by the student 't' test.

Table 3-8 shows the effect of the aromatic amino acids on biopterin biosynthesis. Tryptophan had no effect but both tyrosine and phenylalanine significantly increased the amount of biopterin formed.

No inhibitory or stimulatory effect on the growth of the Crithidia fasciculata itself was measured with any of these compounds used as additives at the concentrations used in these experiments.

TABLE 3-2

EFFECTS OF AGENTS USED TO STIMULATE NADPH PRODUCTION OF BIOPTERIN BIOSYNTHESIS IN VITRO

The incubation tubes contained the standard incubation mixture minus NADPH unless stated otherwise. The control was the standard incubation mixture minus NADPH and the amount of biopterin produced in the control was 2.10 ± 0.03 ng/mg protein which was taken as 100%

Additive and Concentration	No. of Observations	Mean $\pm$ S.D ng/mg protein	% of Control	Student 't' test results
10^{-3}M NADPH	12	4.77 ± 0.05	227.1	$P = < 0.001$
2.10^{-5}M glutamate	6	1.89 ± 0.09	90.0	$P = < 0.005$
2.10^{-4}M glutamate	6	2.10 ± 0.04	100	———
2.10^{-3}M glutamate	6	2.16 ± 0.04	102.8	$P = < 0.01$
2.10^{-5}M glutamate + 2.10^{-3}M $NADP^+$	6	2.07 ± 0.02	98.6	$P = < 0.1$
2.10^{-4}M glutamate + 2.10^{-3}M $NADP^+$	6	2.23 ± 0.08	106.2	$P = < 0.01$
2.10^{-3}M glutamate + 2.10^{-3}M $NADP^+$	6	2.43 ± 0.05	115.7	$P = < 0.0025$
2.10^{-3}M glutamate + 2.10^{-3}M $NADP^+$ + 10^{-3}M NADPH	6	4.65 ± 0.03	221.4	$P = < 0.001$

TABLE 3-2 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student 't' test results
2.10^{-5}M glucose-6-phosphate	12	2.08 ± 0.04	99.1	P = <0.1
1 unit of glucose-6-phosphate dehydrogenase	6	2.10 ± 0.02	100	———
3 units of glucose-6-phosphate dehydrogenase	12	2.08 ± 0.05	99.1	P = >0.1
5 units of glucose-6-phosphate dehydrogenase	6	2.15 ± 0.08	102.4	P = <0.1
7 units of glucose-6-phosphate dehydrogenase	6	2.16 ± 0.05	102.9	P = <0.05
2 10^{-5}M glucose-6-phosphate + 1 unit of glucose-6-phosphate dehydrogenase	6	2.15 ± 0.08	102.4	P = <0.1
2.10^{-5}M glucose-6-phosphate + 2.5 units of glucose-6-phosphate dehydrogenase	6	2.21 ± 0.08	105.2	P = <0.01

TABLE 3-2 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student 't' test results
2.10^{-5}M glucose-6-phosphate + 3 units of glucose-6-phosphate dehydrogenase	12	2.23 ± 0.10	106.2	P = < 0.005
2.10^{-5}M glucose-6-phosphate + 5 units of glucose-6-phosphate dehydrogenase	12	2.30 ± 0.06	109.5	P = < 0.0025
2.10^{-5}M glucose-6-phosphate + 7 units of glucose-6-phosphate dehydrogenase	6	2.26 ± 0.04	107.6	P = <0.0025
2.10^{-5}M glucose-6-phosphate + 2.10 M $NADP^+$	12	2.13 ± 0.05	101.4	P = <0.05
5 units of glucose-6-phosphate dehydrogenase + 2. 10^{-3}M NADP+	6	2.17 ± 0.08	103.3	P = <0.05

TABLE 3-2 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student 't' test results
5 units of glucose-6-phosphate dehydrogenase + 2.10^{-5}M glucose-6-phosphate + 2.10^{-5}M NADP+	6	2.21 ± 0.08	105.2	P = <0.01
5 units of glucose-6-phosphate dehydrogenase + 2.10^{-5}M glucose-6-phosphate + 2.10^{-4}M NADP+	6	2.24 ± 0.03	115.7	P = <0.0025
5 units of glucose-6-phosphate dehydrogenase + 2.10^{-5}M glucose-6-phosphate + 2.10^{-3}M NADP+	12	2.45 ± 0.04	116.7	P = <0.001
5 units of glucose-6-phosphate dehydrogenase + 2.10^{-5}M glucose-6-phosphate + 2.10^{-3}M NADP+ + 10^{-3}M NADPH	6	4.74 ± 0.05	225.7	P = <0.001

TABLE 3-2 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student 't' test results
6.10^{-4}M glucose	6	1.89 ± 0.08	90.0	P = < 0.005
6.10^{-3}M glucose	6	1.81 ± 0.02	86.2	P = < 0.0025
6.10^{-2}M glucose	6	1.77 ± 0.10	84.3	P = < 0.0025
6.10^{-2}M glucose + 10^{-3}M NADPH	6	4.77 ± 0.07	227.1	P = < 0.001

1 unit of glucose-6-phosphate dehydrogenase is the enzyme activity which transforms 1 µmole of glucose-6-phosphate per minute under optimal conditions.

TABLE 3-3

EFFECT OF GLYCOLYSIS INHIBITORS ON IN VITRO BIOSYNTHESIS OF BIOPTERIN

The incubation tubes contained the standard incubation mixture minus NADPH unless otherwise stated. The control was the standard incubation mixture minus NADPH and the amount of biopterin produced in the control was 2.10 ± 0.03 ng/mg protein and was taken as 100%

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student 't' test results
10^{-3}M NADPH	12	4.77 ± 0.05	227.1	P = < 0.001
3.10^{-6}M citrate	6	2.12 ± 0.03	101.0	P = < 0.1
3.10^{-5}M citrate	6	2.14 ± 0.05	101.9	P = < 0.1
3.10^{-4}M citrate	12	2.25 ± 0.08	107.1	P = < 0.0025
3.10^{-3}M citrate	6	2.23 ± 0.05	106.2	P = < 0.005
3.10^{-2}M citrate	6	2.27 ± 0.04	108.1	P = < 0.0025
3.10^{-2}M citrate + 10^{-3}M NADPH	6	4.72 ± 0.05	224.8	P = < 0.001
3.10^{-6}M Iodoacetate	6	1.93 ± 0.12	91.9	P = < 0.025
3.10^{-5}M Iodoacetate	12	2.13 ± 0.06	101.4	P = < 0.1

TABLE 3-3 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student 't' test results
3.10^{-4}M Iodoacetate	18	2.42 ± 0.07	115.2	P = < 0.0025
3.10^{-3}M Iodoacetate	12	2.56 ± 0.09	121.9	P = < 0.0025
3.10^{-2}M Iodoacetate	6	2.58 ± 0.09	122.9	P = < 0.0025
3.10^{-2}M Iodoacetate + 10^{-3}M NADPH	6	4.77 ± 0.03	227.1	P = < 0.001
10^{-4}M Sodium fluoride	12	2.26 ± 0.06	107.6	P = < 0.0025
10^{-3}M Sodium fluoride	12	2.29 ± 0.05	109.1	P = < 0.0025
10^{-2}M Sodium fluoride	12	2.52 ± 0.02	120.0	P = < 0.002
10^{-2}M Sodium fluoride + 10^{-3}M NADPH	6	4.77 ± 0.01	227.1	P = < 0.01

TABLE 3-4

EFFECT OF ASCORBATE, 5 METHYLTETRAHYDROFOLIC ACID AND COENZYME B_{12} ON THE IN VITRO BIOSYNTHESIS OF BIOPTERIN

The incubation tubes contained the standard incubation mixture minus NADPH unless otherwise stated and this was the control. The amount of biopterin produced in the control was 2.10 ± 0.03 ng/mg protein and this was taken as 100%.

Additive and Concentration	No. of Observations	Mean $\pm$ S.D. ng/mg protein	% of Control	Student 't' test results
10^{-3} M NADPH	12	4.77 ± 0.03	227.1	$P = < 0.001$
$2.8.10^{-4}$ M ascorbate	12	2.19 ± 0.12	104.3	$P = < 0.05$
$5.7\ 10^{-4}$ M ascorbate	18	2.23 ± 0.09	106.2	$P = < 0.0025$
$1.1\ 10^{-3}$ M ascorbate	12	2.25 ± 0.11	107.1	$P = < 0.005$
$2.3\ 10^{-3}$ M ascorbate	12	2.28 ± 0.07	108.6	$P = < 0.0025$
$4.6\ 10^{-3}$ M ascorbate	6	2.30 ± 0.06	109.5	$P = < 0.0025$
$2.8\ 10^{-2}$ M ascorbate	6	2.78 ± 0.04	132.4	$P = < 0.0025$
$2.8\ 10^{-1}$ M ascorbate	6	3.15 ± 0.05	150.0	$P = < 0.002$

TABLE 3-4 continued..

Additive and Concentration	No. of Observations	Mean ± S. D. ng/mg protein	% of Control	Student 't' test results
$2.8\ 10^{-1}$M ascorbate + 10^{-3}M NADPH	6	4.76 ± 0.07	226. 7	P = < 0.001
$1.1\ 10^{-4}$M 5 methyl tetrahydrofolic acid (5 CH_3 THFA)	6	2.14 ± 0.06	101.9	P = < 0.1
10^{-3}M NADPH + $1.1\ 10^{-4}$M 5 CH_3 THFA	6	5.42 ± 0.02	258.2	P = < 0.001
$1.1\ 10^{-7}$M B_{12}	6	2.04 ± 0.08	97.1	P = < 0.1
$2.8\ 10^{-1}$M ascorbate + 1.1 10^{-7}M B_{12}	6	3.15 ± 0.08	150.0	P = < 0.001
$2.8\ 10^{-1}$M ascorbate + $1.1\ 10^{-7}$M 5 CH_3THFA	6	3.15 ± 0.02	150.0	P = < 0.001

TABLE 3-4 continued..

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student 't' test results
$2.8\ 10^{-1}$M ascorbate + 1.1 10^{-5}M 5 CH_3 THFA	6	3.28 ± 0.01	156.2	P = < 0.001
$2.8\ 10^{-1}$M ascorbate + 1.1 10^{-5}M 5 CH_3 THFA	6	3.39 ± 0.03	161.4	P = < 0.001
$2.8\ 10^{-1}$M ascorbate + 1.1 10^{-4}M 5 CH_3 THFA	12	3.46 ± 0.05	164.8	P = < 0.001
$2.8\ 10^{-1}$M ascorbate + 1.1 10^{-4}M 5 CH_3 THFA + 1.1 10^{-7}M B_{12}	6	4.36 ± 0.05	207.6	P = < 0.001
10^{-3}M NADPH 2.8 10^{-1}M ascorbate + 1.1 10^{-4}M 5 CH_3 THFA + 1.1 10^{-7}M B_{12}	6	6.45 ± 0.03	307.1	P = < 0.001

TABLE 3-5

EFFECT OF NICOTINAMIDE ON THE IN VITRO BIOSYNTHESIS OF BIOPTERIN

The incubation tubes contained the standard incubation mixture which was the control. The amount of biopterin produced in the control was 4.77 $\pm$ 0.06 ng/mg protein and this was taken as 100%

Concentration of nicotinamide	No. of Observations	Mean $\pm$ S.D. ng/mg protein	% of Control	Student 't' test results
10^{-7}M	6	4.39 $\pm$ 0.16	92.0	P = < 0.005
10^{-6}M	6	4.58 $\pm$ 0.12	96.0	P = < 0.01
10^{-5}M	12	4.70 $\pm$ 0.08	98.5	P = < 0.01
10^{-4}M	12	5.07 $\pm$ 0.11	106.3	P = < 0.0025
10^{-3}M	24	5.24 $\pm$ 0.04	109.8	P = < 0.001
10^{-2}M	12	5.34 $\pm$ 0.04	112.0	P = < 0.0025
10^{-1}M	12	5.72 $\pm$ 0.04	119.9	P = < 0.001
M	6	5.63 $\pm$ 0.06	118.0	P = < 0.0025

TABLE 3-6

EFFECT OF METAL IONS ON THE IN VITRO BIOSYNTHESIS OF BIOPTERIN

The incubation tubes contained the standard incubation mixture which was taken as the control and which produced 4.77 ± 0.08 ng/mg protein of biopterin which was taken as 100%

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student 't' test results
10^{-9}M lead acetate	6	4.48 ± 0.32	93.9	P = < 0.05
10^{-8}M lead acetate	12	4.15 ± 0.38	87.0	P = < 0.005
10^{-7}M lead acetate	30	3.58 ± 0.35	75.0	P = < 0.001
10^{-6}M lead acetate	24	3.10 ± 0.25	65.0	P = < 0.001
10^{-5}M lead acetate	24	2.86 ± 0.22	60.0	P = < 0.001
10^{-4}M lead acetate	6	2.62 ± 0.05	54.9	P = < 0.001
10^{-3}M lead acetate	6	2.67 ± 0.12	56.0	P = < 0.001
10^{-7}M aluminium sulphate	6	4.83 ± 0.18	101.3	P = > 0.1
10^{-5}M aluminium sulphate	6	4.71 ± 0.29	98.7	P = > 0.1
10^{-3}M aluminium sulphate	6	4.40 ± 0.14	92.2	P = < 0.005

TABLE 3-6 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student 't' test results
10^{-7}M mercuric chloride	6	4.88 ± 0.18	102.3	P = < 0.1
10^{-5}M mercuric chloride	6	4.51 ± 0.15	94.5	P = < 0.01
10^{-3}M mercuric chloride	6	4.23 ± 0.09	88.7	P = < 0.001
10^{-7}M cadmium chloride	6	4.75 ± 0.12	99.6	P = > 0.1
10^{-5}M cadmium chloride	6	4.47 ± 0.16	93.7	P = < 0.005
10^{-3}M cadmium chloride	6	4.27 ± 0.12	89.5	P = < 0.0025
10^{-7}M copper sulphate	6	4.64 ± 0.21	97.3	P = < 0.1
10^{-6}M copper sulphate	12	4.46 ± 0.32	93.5	P = < 0.01

TABLE 3-6 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student 't' test results
10^{-5}M copper sulphate	12	4.22 ± 0.12	88.5	P = < 0.0025
10^{-4}M copper sulphate	12	4.00 ± 0.16	83.8	P = < 0.0025
10^{-3}M copper sulphate	6	3.77 ± 0.13	79.0	P = < 0.001
10^{-4}M lithium carbonate	6	4.94 ± 0.22	103.6	P = < 0.1
10^{-3}M lithium carbonate	6	4.58 ± 0.32	96.0	P = > 0.1
10^{-2}M lithium carbonate	6	4.31 ± 0.31	90.4	P = < 0.01

TABLE 3-6 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of control	Student 't' test results
10^{-4}M gallium chloride	6	4.84 ± 0.18	101.4	P = > 0.1
10^{-3}M gallium chloride	6	4.88 ± 0.18	102.3	P = < 0.1
10^{-2}M gallium chloride	6	4.46 ± 0.15	93.5	P = <0.005
2.10^{-2}M gallium chloride	6	4.39 ± 0.14	92.0	P = <0.005

TABLE 3-7

EFFECT OF A VARIETY OF DRUGS ON IN VITRO BIOPTERIN BIOSYNTHESIS

The incubation tubes contained the standard assay mixture which was taken as the control. This control produced 4.77 $\pm$ 0.06 ng/mg protein of biopterin and this was taken as 100%

Additive and Concentration	No. of Observations	Mean $\pm$ S.D ng/mg protein	% of Control	Student "t" test results
$5.5\ 10^{-6}$M methotrexate	12	4.53 $\pm$ 0.45	94.9	P = < 0.1
$1.1\ 10^{-5}$M methotrexate	18	5.37 $\pm$ 0.15	112.5	P = < 0.0025
$5.5\ 10^{-5}$M methotrexate	18	6.69 $\pm$ 0.11	140.3	P = < 0.001
$1.1\ 10^{-4}$M methotrexate	12	11.21 $\pm$ 0.07	235.0	P = < 0.001
$2.3\ 10^{-5}$M aminopterin	6	4.47 $\pm$ 0.36	93.7	P = < 0.05
$1.1\ 10^{-4}$M aminopterin	12	6.35 $\pm$ 0.84	133.1	P = < 0.005
$2.3\ 10^{-4}$M aminopterin	12	8.31 $\pm$ 0.44	174.2	P = < 0.002

TABLE 3-7 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student "t" test results
$1.1\ 10^{-3}$M aminopterin	6	16.21 ± 0.33	339.8	P = < 0.01
$1.1\ 10^{-5}$M pyrimethamine	6	4.66 ± 0.36	97.7	P = > 0.1
$1.1.\ 10^{-4}$M pyrimethamine	6	5.18 ± 0.32	108.6	P = < 0.025
$1.1\ 10^{-3}$M pyrimethamine	6	6.67 ± 0.33	139.8	P = < 0.0025
$1.1\ 10^{-4}$M triampterene	6	4.72 ± 0.23	99.0	P = > 0.1
$1.1\ 10^{-3}$M triampterene	6	5.41 ± 0.18	113.4	P = < 0.0025
$1.1\ 10^{-2}$M triampterene	6	7.24 ± 0.23	151.8	P = < 0.001

TABLE 3-7 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student "t" test results
$2.3\ 10^{-4}$M tetroxoprim	6	5.02 ± 0.24	105.2	P = < 0.05
$2.3\ 10^{-3}$M tetroxoprim	6	5.55 ± 0.31	116.3	P = < 0.005
$2.3\ 10^{-2}$M tetroxoprim	6	5.89 ± 0.22	123.5	P = < 0.0025
$3.4\ 10^{-4}$M trimethoprim	12	5.25 ± 0.27	110.1	P = < 0.005
$3.4\ 10^{-3}$M trimethoprim	12	5.43 ± 0.33	113.8	P = < 0.005
$3.4\ 10^{-2}$M trimethoprim	12	5.43 ± 0.35	113.8	P = < 0.005
$1.3\ 10^{-5}$M sulphamethoxazole	12	4.95 ± 0.36	103.8	P = > 0.1
$1.3\ 10^{-4}$M sulphamethoxazole	12	5.13 ± 0.62	107.6	P = < 0.1
$1.3\ 10^{-3}$M sulphamethoxazole	12	5.13 ± 0.33	107.6	P = < 0.01

TABLE 3-7 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student "t" test results
1.3 10^{-3}M sulphamethoxazole + 3.4 10^{-2}M trimethoprim	12	5.25 ± 1.09	110.1	P = > 0.1
1.2 10^{-4}M mercaptopurine	6	4.88 ± 0.28	102.3	P = > 0.1
1.2 10^{-3}M mercaptopurine	6	4.59 ± 0.21	96.2	P = < 0.05
1.2 10^{-2}M mercaptopurine	6	4.06 ± 0.34	85.1	P = <0.005
1.5 10^{-5} thioguanine	6	4.75 ± 0.22	99.6	P = > 0.1
1.5 10^{-4}M thioguanine	6	4.42 ± 0.36	92.7	P = < 0.05
1.5 10^{-3}M thioguanine	6	3.91 ± 0.33	81.9	P = < 0.005
7.2 10^{-5}M azathioprine	6	4.91 ± 0.27	104.0	P = < 0.1

TABLE 3-7 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student "t" test results
$7.2\ 10^{-4}$M azathioprine	6	4.28 ± 0.33	89.7	P = < 0.01
$7.2\ 10^{-3}$M azathioprine	6	3.75 ± 0.29	78.6	P = < 0.0025
$8.2\ 10^{-6}$M cytosine arabinoside	6	4.89 ± 0.25	102.5	P = > 0.1
$8.2\ 10^{-5}$M cytosine arabinoside	6	5.18 ± 0.32	108.6	P = < 0.025
$8.2\ 10^{-4}$M cytosine arabinoside	6	5.52 ± 0.29	115.7	P = < 0.005
$1.5\ 10^{-4}$M fluorouracil	6	4.92 ± 0.24	103.1	P = < 0.1
$1.5\ 10^{-3}$M fluorouracil	6	5.16 ± 0.54	108.2	P = < 0.1
$1.5\ 10^{-2}$M fluorouracil	6	5.32 ± 0.62	111.5	P = < 0.05

TABLE 3-7 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D. ng/mg protein	% of Control	Student "t" test results
10^{-5}M theophylline	12	5.08 ± 0.55	106.5	P = < 0.1
10^{-4}M theophylline	12	5.16 ± 0.51	108.2	P = < 0.05
10^{-3}M theophylline	12	5.39 ± 0.57	113.0	P = < 0.01

TABLE 3-8

EFFECT OF AROMATIC AMINO ACIDS ON THE IN VITRO BIOSYNTHESIS OF BIOPTERIN

Additive and Concentration	No. of Observations	Mean $\pm$ S.D ng/mg protein	% of Control	Student "t" test results
10^{-5}M L-tryptophan	6	4.75 $\pm$ 0.22	99.6	P = > 0.1
10^{-4}M L-tryptophan	6	4.49 $\pm$ 0.44	94.1	P = < 0.1
10^{-3}M L-tryptophan	6	4.88 $\pm$ 0.31	102.3	P = > 0.1
10^{-5}M L-tyrosine	12	4.64 $\pm$ 0.22	97.3	P = < 0.1
10^{-4}M L- tyrosine	12	5.11 $\pm$ 0.47	107.1	P = < 0.05
10^{-3}M L-tyrosine	12	5.55 $\pm$ 0.42	116.4	P = < 0.002

The incubation tubes contained the standard assay mixture which was taken as the control. The control tubes produced 4.77 $\pm$ 0.06 ng/mg protein of biopterin and this was taken as 100%

TABLE 3-8 (continued)

Additive and Concentration	No. of Observations	Mean ± S.D ng/mg protein	% of Control	Student "t" test results
10^{-5} M L-phenylalanine	12	5.18 ± 0.42	108.6	$P = < 0.025$
10^{-4}M L-phenylalanine	18	5.22 ± 0.52	109.4	$P = < 0.01$
10^{-3}M L-phenylalanine	18	5.38 ± 0.46	112.8	$P = < 0.001$

DISCUSSION

The stimulation of biopterin biosynthesis by glutamate plus $NADP^+$ and glucose-6-phosphate plus glucose-6-phosphate dehydrogenase plus $NADP^+$ and the fact that both these systems did not give an additional increase when NADPH was also included adds to the evidence that NADPH is a required cofactor of de novo biopterin biosynthesis. The pentose phosphate pathway and glutamate dehydrogenase are recognised routes of NADPH production and stimulation of these reactions will result in increased NADPH levels which in turn, increases biopterin production. (Chapter 2) Hence the availability of NADPH to the de novo biosynthetic pathway for tetrahydrobiopterin could be a limiting factor on this pathway's activity in vivo.

The inhibition of biopterin biosynthesis by glucose was surprising. It could not be accounted for by contamination of the Analar glucose by lead ions which were shown to be present in concentrations less than 0.033 ppm as measured by atomic absorption. (Wibberley 1978) It could be that addition of glucose stimulated the glycolytic pathway rather than the pentose phosphate pathway and hence, caused decreased biopterin biosynthesis due to decreased NADPH availability. This is supported by the disappearance of the inhibition when NADPH was added with the glucose.

The increased biopterin production caused by the glycolytic inhibitors also points to the importance of the pentose phosphate path-

way for providing NADPH for biopterin biosynthesis. The fate of glucose-6-phosphate will depend on the activities of the two metabolic pathways: glycolysis and the pentose phosphate shunt. When the glycolytic pathway is blocked by inhibitors the glucose-6-phosphate will be channelled into the pentose phosphate pathway and hence, NADPH production will increase. This stimulates the biosynthesis of biopterin. (Chapter 2) The relative activities of these two major metabolic pathways, which can be assessed by measuring Bloom and Stetton ratios, (1953, 1955) could have a significant effect on the rate of de novo biopterin biosynthesis. In the adult brain the participation of the pentose phosphate pathway has been shown to be small compared to that of glycolysis with Bloom and Stetton ratios close to 1. (Bloom 1955, Sacks, 1957, Hoskin 1960). However, the pentose phosphate pathway makes a greater contribution in the immature brain when more NADPH is needed. (Guerra, Melgar and Villavincencio 1967)

Ascorbate caused a significant increase in biopterin biosynthesis which was not additive with NADPH and so the ascorbate is sparing the need for additional NADPH. The ascorbate could be stabilizing the NADPH or NADH present endogenously acting in an antioxidant capacity. Alternatively the ascorbate could be actively stimulating the biopterin biosynthesis either directly or indirectly. If the ascorbate was acting directly on the biosynthetic pathway an additive result with the NADPH might be expected. Ascorbate and methylene blue have been shown to increase purine biosynthesis and it was suggested that this increase was achieved by these compounds increasing the activity of the pentose-phosphate

pathway by acting as hydrogen acceptors. (Baskin and Sperling 1978) Ascorbate could be acting in the same way here to increase the NADPH production by stimulating the pentose phosphate pathway and hence, increasing biopterin biosynthesis.

The effect of 5-methyl tetrahydrofolic acid and coenzyme B_{12} to significantly increase the amount of biopterin synthesized indicates that the folate status of the cell may be important in determining the rate of de novo biopterin biosynthesis. Folate levels in the brain have been reported to be low. (Blakely 1969) The fact that the increase caused by 5 methyl tetrahydrofolic acid was further increased when coenzyme B_{12} was also included indicates that these reagents may be acting via stimulation of the step involving the loss of a one carbon fragment. They could however, be having an affect on some other step such as the dihydrofolate reductase step.

Nicotinamide increases de novo biopterin biosynthesis most likely by inhibiting the enzyme NAD nucleosidase which degrades NADH (Mann and Quastel 1941a, Mann and Quastel 1941b) and NADPH. (Handler and Klein 1942) This enzyme is particularly plentiful in brain extracts. (Mann and Quastel 1941b) Nicotinamide therefore protects the NADPH present and this stimulates biopterin production. (Chapter 2)

Lead acetate showed the most dramatic decrease in biopterin biosynthesis of the metals tested. 10^{-8}M lead acetate, a concentration that is similar to that found in normal adult brains (Schroeder and Tipton 1968) gave a significant 13% decrease in the amount of biopterin produced.

10^{-9} M gave a 6% decrease which although was only significant at the 5% level by the 'student t' test, fits the trend of increased inhibition of biopterin biosynthesis with increased lead acetate concentrations. These results show that lead has a potent effect on this pathway. When a severe inhibition of tetrahydrobiopterin biosynthesis occurs such as in malignant hyperphenylalaninaemia due to a lesion in this pathway (Leeming, Blair and Rey 1976, Rey, Blandin-Saroja and Rey 1976, Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel 1978) mental retardation results. Therefore, perhaps the effects of lead on biopterin biosynthesis seen here could partly explain neurotoxic effects of lead. A severe decrease in tetrahydrobiopterin synthesis could over a period of time, cause a reduction in cellular levels of tetrahydrobiopterin. As this is rate-limiting for tyrosine hydroxylase (Kettler, Bartholini and Pletcher 1974) a reduction would significantly reduce the activity of this enzyme which in turn would reduce dopamine and noradrenaline synthesis as it is the rate-limiting step for this pathway. (Levitt, Spector, Sjoerdsma and Udenfriend 1965) Reduction in the synthesis of these two central neurotransmitters could be related to neurological impairment. What is not clear from these results is on which step of the biosynthetic route for tetrahydrobiopterin lead is acting.

None of the other metals tested were as potent as lead but all showed reductions in biopterin biosynthesis. Lead, mercury and cadmium are all recognised neurotoxic agents. (Flink 1975)

Aluminium has been linked with dialysis dementia (Flendrig, Kruis and Das 1976) and Alzheimer's dementia (Crapper, Krishman and Quittkat 1976) and excess copper with convulsions. (Schienberg and Steinlieb 1976) Lithium has been used to treat such mental disorders as manic-depressive psychosis and is poisonous at really high levels. (Schou 1958, Schou 1968) So perhaps these metals are acting in a similar way to lead but exceedingly high concentrations would be required to produce comparable effects. The effect of these metals on biopterin could not be accounted for by lead contamination in the analar reagents.

All the drugs which are dihydrofolate reductase inhibitors increased the amount of biopterin produced, methotrexate being the most potent doubling the amount of biopterin produced at $1.1\ 10^{-4}$M concentration. The order of increasing degree of stimulation for the drugs tested is triampterene, tetroxoprim, trimethoprim, pyrimethamine, aminopterin and methotrexate. This order correlated fairly well with the ability of drugs to bind rat dihydrofolate reductase as trimethoprim and tetroxoprim do not have a great affinity for the mammalian enzyme whereas methotrexate, aminopterin and pyrimethamine have a much greater affinity for this enzyme. (Burchall 1979). The only exception is triampterene which has an intermediate affinity for the enzyme and is the least potent biopterin biosynthesis stimulator. It appears that this increase in biopterin biosynthesis could be related to the effect of these drugs on dihydrofolate reductase which probably catalyses the final step in tetrahydrobiopterin synthesis. If these drugs inhibit this final step

7,8 dihydrobiopterin levels in the cell would increase. However as the Crithidia fasciculata assay measures total biopterin including 7,8 dihydrobiopterin, 5,6,7,8 tetrahydrobiopterin and biopterin itself this should not alter the level of biopterin biosynthesis measured. Therefore, dihydrofolate reductase inhibition must be causing a stimulation of the biosynthetic pathway. The trigger for this stimulation could either be the reduction in 5,6,7,8 tetrahydrobiopterin levels or an increase in 7,8 dihydrobiopterin levels. If the trigger for the stimulation is a reduction in cellular 5,6,7.8 tetrahydrobiopterin levels, this could work by the synthesis pathway being under feedback inhibition by 5,6,7,8 tetrahydrobiopterin. If there is plenty of 5,6,7.8 tetrahydrobiopterin in the cell it will feedback to inhibit the synthesis pathway but if the cellular levels of 5,6,7,8-tetrahydrobiopterin fall, this inhibition will be reduced and more 5,6,7,8 tetrahydrobiopterin will be synthesised from G.T.P. Hence the stimulation observed would be due to reduced feedback inhibition of the synthesis pathway by tetrahydrobiopterin. If the trigger is an increase in cellular levels of 7,8 dihydrobiopterin, this would have to work by the 7,8 dihyrobiopterin stimulating the activity of the synthetic pathway. Thismethod seems unlikely and feedback inhibition is a common mechanism of biological regulation of metabolic pathways. Methotrexate, pyrimethamine and trimethoprim also inhibit dihydropteridine reductase. (Chapter 5) Therefore with both reductases inhibited the cellular levels of 5,6,7,8 tetrahydrobiopterin would certainly decrease. The increase in 7,8 dihydrobiopterin levels caused by these two effects would be expected to cause an increase in serum dihydro-

biopterin levels which in fact has been reported with patients on methotrexate (Leeming, Blair, Melikian and O'Gorman 1976) and in children with dihydropteridine reductase deficiency. (Kaufman, Holtzman, Milstien, Butler and Krumholz 1975, Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977, Grobe, Bartholome, Milstien and Kaufman 1978)

Sulphamethoxazole also increased biopterin biosynthesis. This drug had a slight inhibitory effect on dihydropteridine reductase (Chapter 5) so this could perhapsexplain this effect. This would lead to a small reduction in 5,6,7,8 tetrahydrobiopterin which could be the trigger for biopterin biosynthesis stimulation. Sulphamethoxazole and trimethoprim are the two active components of the pharmaceutical preparations, co-trimoxazole, bactrim and septrin. Therefore, an additive effect might be expected with both trimethoprim and sulphamethoxazole together but this was not observed.

Of the metabolic inhibitors, the pyrimidine antagonists, fluorouracil and cytosine arabinoside increased the synthesis of biopterin. These both inhibit DNA synthesis which will probably be a major route for GTP utilization. If this pathway is blocked it follows that more GTP will be available for biopterin biosynthesis. The purine antagonists all inhibit biopterin biosynthesis. As these also inhibit DNA and RNA synthesis, a similar result as the pyrimidine antagonists might be expected but presumably these analogs of purines some how block GTP utilization by the biopterin biosynthetic enzymes. They could be competing with GTP for the GTP site on GTP cyclohydrolase as they are

of a similar structure.

Of the aromatic amino acids, tryptophan had no effect and phenylalanine and tyrosine both stimulated biopterin synthesis. As all three of these amino acids inhibited dihydropteridine reductase (Chapter 5) all of them might be expected to stimulate biopterin biosynthesis via release from the feedback inhibition of 5,6,7,8 tetrahydrobiopterin in the same way as methotrexate. Serum dihydrobiopterin levels mirror closely serum phenylalanine levels (Leeming, Blair, Green and Raine 1976) so an effect on biopterin metabolism by phenylalanine should be expected. The inhibition of dihydropteridine reductase could explain this correlation. (Chapter 5) Phenylalanine also inhibits pyruvate kinase and so could be acting in the same way as the other glycolytic inhibitors to stimulate the production of NADPH by diverting the flow of glucose down the pentose phosphate pathway. Alternatively tyrosine and phenylalanine could be acting to stimulate the biopterin biosynthesis by a direct method. Normal human brain concentrations of tyrosine and phenylalanine are about 10^{-4}M for both (Letendre, Nagaiah and Guroff 1980) which is the same as the concentrations used here which are producing a 7% and 9% stimulation respectively. In the case of tyrosine this stimulation is only a significant one if the levels of tyrosine are raised to 10^{-3}M.

The results shown here add to the evidence that NADPH is a required cofactor for tetrahydrobiopterin biosynthesis and indicate that the activity of the biosynthetic pathway for tetrahydrobiopterin from GTP

can be altered depending on the levels of folate, metal ions especially lead ions, the amino acids; phenylalanine and tyrosine and dihydrofolate reductase inhibitors, which probably occur via changes in tetrahydrobiopterin cellular levels, suggest that the activity of this pathway can be controlled by its product, tetrahydrobiopterin.

CHAPTER FOUR

THE ISOLATION OF DIHYDROPTERIDINE REDUCTASE FROM RAT LIVER

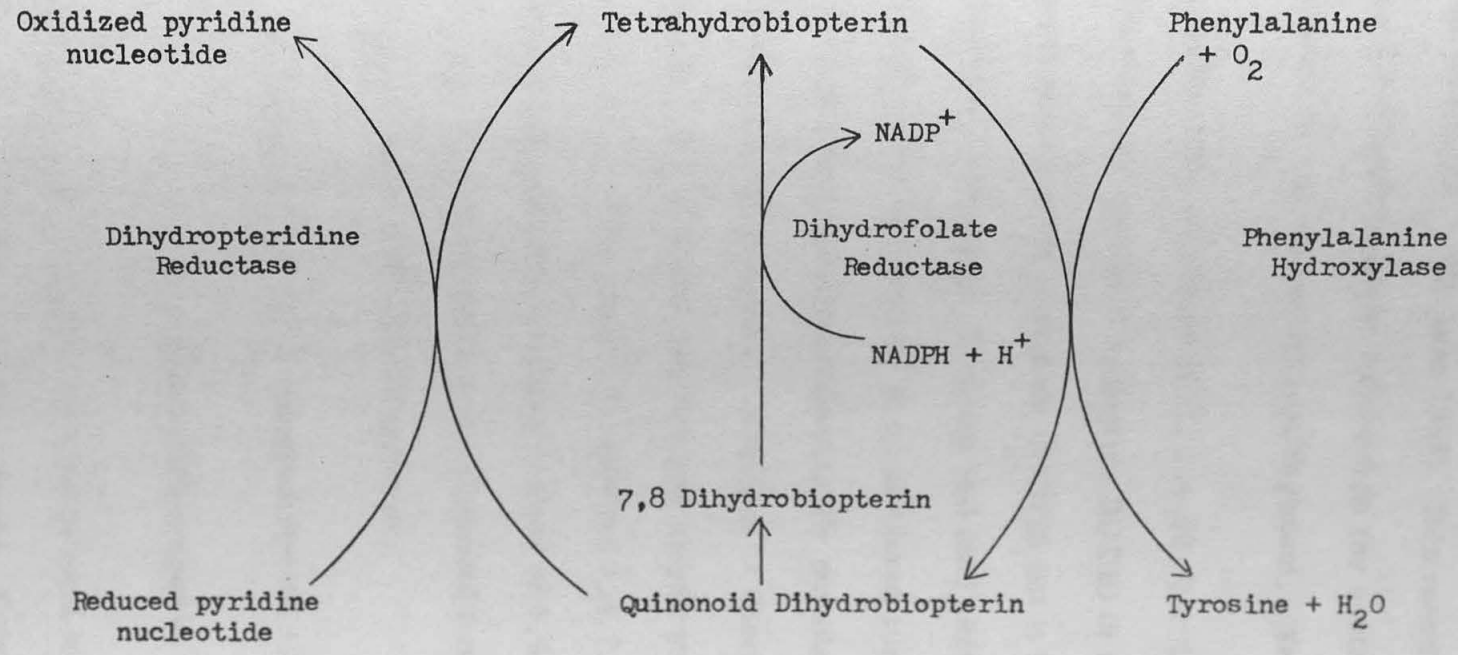
INTRODUCTION

The enzymatic conversion of phenylalanine to tyrosine was shown to involve two essential enzymes (Kaufman 1957, Mitoma 1956): one purified from rat liver extracts (phenylalanine hydroxylase) (Kaufman 1969) and one purified from sheep liver extracts (dihydropteridine reductase) (Kaufman 1969) In this coupled enzyme system reduced nicotinamide adenine dinucleotide phosphate (NADPH) was also shown to be necessary. (Kaufman 1957)

$$NADPH + H^+ + \text{phenylalanine} + O_2 \longrightarrow \text{tyrosine} + NADP^+ + H_2O$$

It was later shown that tetrahydrobiopterin was an essential cofactor for this hydroxylation and that the above equation is in fact, the sum of at least two reactions. (Figure 4-1) (Kaufman 1958, Kaufman 1963) The first of these reactions is catalyzed by the 'rat liver enzyme' (phenylalanine hydroxylase) and converts phenylalanine to tyrosine and tetrahydrobiopterin to quinonoid dihydrobiopterin. (Kaufman 1964) The second reaction is catalyzed by 'sheep liver enzyme' (dihydropteridine reductase) and converts quinonoid dihydrobiopterin back to tetrahydrobiopterin and requires a reduced pyridine nucleotide. (Kaufman 1964) More recently it was shown that a third enzyme was an essential component for this hydroxylation if the cofactor

FIGURE 4-1 REACTION SCHEME FOR THE CONVERSION OF PHENYLALANINE TO TYROSINE



form isolated from rat liver, 7,8 dihydrobiopterin was used. This enzyme was dihydrofolate reductase which reduces 7,8 dihydrobiopterin to tetrahydrobiopterin in the presence of reduced nicotinamide adenine dinucleotide phosphate. (Kaufman 1967) This reaction serves to salvage the 7,8 dihydrobiopterin formed from the unstable quinonoid dihydrobiopterin by non-enzymatic rearrangement. (Kaufman 1961)

Dihydropteridine reductase (E.C. 1.6.99.7) utilizes either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) but it is much more active with NADH. (Nielsen, Simonson and Lind 1969) This enables dihydropteridine reductase activity to be measured simply following the disappearance of NADH spectrophotometrically provided quinonoid dihydrobiopterin could be generated. A method is described by Craine, Hall and Kaufman (1972) where they measure dihydropteridine reductase activity in this way. They used 6,7, dimethyl 5,6,7,8 tetrahydropterin which was commercially available instead of 5,6,7,8 tetrahydrobiopterin as substrate and generated the quinonoid form by the use of horseradish peroxidase and hydrogen peroxide.

$$\text{6,7 dimethyl, 5,6,7,8 tetrahydropterin} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{quinonoid}$$

$$\text{6,7 dimethyldihydropterin} + 2\,H_2O$$

Dichlorophenolindophenol (DICPIP) can also be used to generate the quinonoid substrate. (Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973)

Dihydropteridine reductase has been isolated from several different sources by a variety of methods. It was isolated from sheep liver extracts by a method involving ammonium sulphate, zinc-ethanol and alkaline ammonium sulphate fractionations, followed by adsorption and elution from calcium phosphate gel and chromatography on DEAE cellulose and Sephadex G.100 to give a 9% yield and a 156 fold purification. (Craine, Hall and Kaufman 1972) This method was modified by additional chromatographic steps to produce a yield of 2.4% and a purification of 433 fold for the isolation of the enzyme from bovine liver. (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977) A simpler method of chloroform-ethanol and ammonium sulphate fractionations followed by chromatography on DEAE-Sephadex A-50. Hydroxylapatite and CM-Sephadex C-50 was used for dihydropteridine reductase purification from bovine liver to give a 15% yield and a 1724 fold purification. The enzyme prepared in this way was crystallized both as free dihydropteridine reductase and as a reductase-NADH complex. (Hasegawa 1977)

More recently affinity chromatographic methods have been used to isolate dihydropteridine reductase as they are much simpler to perform. The enzyme was isolated from bovine kidney extracts by using columns of immobilized Cibacron Blue (Blue Dextan - Agarose) to which the dihydropteridine reductase bound by its dinucleotide (NADH) domain. This gave a 16% yield and a 359 fold purification. (Chauvin, Korri, Tirpak, Simpson and Scrimgeour 1979, Scrimgeour, Tirpak and Chauvin 1978) Another method utilizing the reductase's NADH binding domain involved

chromatography on 5'-AMP-Sepharose 4B and was used to isolate the enzyme from bovine liver and bovine adrenal medulla giving 16% and 17% yields respectively. (Aksnes, Skotland, Flatmark and Ljones 1979) These methods are not very specific for dihydropteridine reductase as many enzymes have NADH binding domains and so still involve many other steps to aid the purification. An alternative method was to use an affinity chromatographic step involving binding to an inhibitor of the enzyme. Methotrexate-Aminohexyl-Sepharose was used to give a 27% yield and 148 fold purification of dihydropteridine reductase from rat liver and similar results for sheep liver enzyme. (Webber, Deits, Snyder and Whiteley 1978) As methotrexate is also a potent inhibitor of dihydrofolate reductase this method is likely to co-purify this enzyme. Use of an affinity adsorbent of sodium 1,2 naphthoquinone-4-sulphonate, a phenylalanine hydroxylase inhibitor, gave an 18% yield and 404 fold purification of dihydropteridine reductase from human liver obtained at autopsy. This was a single step method and the sodium 1,2-naphthoquinone-4-sulphonate was believed to be effective because of structural similarities to the quinonoid substrate. (Cotton and Jennings 1978)

All the above methods showed that the enzyme existed as a dimer of molecular weight of about 40,000-50,000 except the naphthoquinone method which indicated a tetratmer of a molecular weight of about 100,000.

To date, it is believed that the enzymic reaction proceeds by a

compulsory ordered mechanism. (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977, Aksnes and Ljones 1980)

It was suggested retrospectively that dihydropteridine reductase deficiency may be associated with a form of phenylketonuria which was unresponsive to dietary control of phenylalanine and in which there was progressive neurological illness which was terminated by death in early childhood. (Smith, Clayton and Wolff 1975) This theory was confirmed by detailed biochemical studies of similar cases. (Kaufman, Holtzman, Milstien, Butler and Krumholz 1975, Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977) This form of malignant hyperphenylalaninaemia and the serious consequences of this inherited disease emphasize the importance of dihydropteridine reductase activity.

MATERIALS AND METHODS

NADH, NADPH, 6,7, dimethyl 5,6,7,8 tetrahydropterin, carbodiimide, dithiothreitol, dichlorophenolindophenol, tetrahydrofolic acid, cytochrome C, α chymotrypsinogen, equine apoferritin, bovine serum albumin, hexokinase and dextran blue 2000 were all purchased from the Sigma Chemical Company. AH-Sepharose 4B and Sephadex G-150 were obtained from Pharmacia Fine Chemicals, Sodium 1,2 naphthoquinone-4-sulphonate was purchased from Cambrian Chemicals. Horseradish peroxidase was obtained from Calbiochem. Tetrahydrobiopterin was a gift from Roche Products Limited. All other chemicals were obtained as described before or from BDH and were of Analar grade.

METHODS

Dihydropteridine reductase was isolated from rat liver using a modified version of the affinity chromatographic method of Cotton and Jennings, (1978).

Preparation of the Affinity Adsorbent

7.5 g of AH-Sepharose 4B was allowed to swell in excess 0.5M NaCl at 4°C Lactose and dextran were removed by washing the Sepharose in 1500 cm^3 of 0.5 M NaCl. The Sepharose gel was then washed in 1500 cm^3 of distilled water to remove NaCl.

0.375g of sodium 1,2 naphthoquinone-4-sulphonate was dissolved in a small amount of distilled water. This ligand solution was added to the gel and the pH of the mixture was adjusted to between 4.5 - 6.0. The slurry was stirred gently at room temperature for 1 hour in the dark.

1.86g of carbodiimide was dissolved in a small amount of distilled water and this was added dropwise to the slurry with gentle stirring. The pH was maintained at between 4.5 and 6.0 for 1 hour. This mixture was then shaken in the dark for 24 hours. The adsorbent was then washed with 750 cm^3 of 0.1M sodium acetate buffer pH4 containing 1M NaCl followed by 750 cm^3 of distilled water and finally with 750 cm^3 of 0.05M Tris/HCl buffer pH 7.6 containing 0.2M NaCl (Buffer A) keeping the adsorbent as cold as possible and in the dark as much as possible. The adsorbent was stored in the latter buffer in the dark at 4°C and proved to be stable for at least 12 months. It was a rich mahogany

colour in Buffer A.

Preparation of Tissue Extract

A 20% homogenate of rat liver from freshly killed male Wistar rats was prepared in 0.1M Tris/HCl buffer pH 7.6. The homogenate was centrifuged at 25,000 xg for 1 hour at $0^{o}C$ in a M.S.E. Superspeed 50 centrifuge. The resulting supernatant was filtered through muslin to remove lipids. The protein content of the supernatant was measured using the Buiret method. (Layne 1957) The dihydropteridine reductase (DHPR) activity was measured using a modified method of Craine, Hall and Kaufman (1972). 5 cm^3 of the remaining supernatant were applied to a Sephadex G-150 column (1.5 x 25 cm) at $2^{o}C$ and equilibrated in 0.05 M Tris/HCl buffer pH 7.6. The column was eluted using the same buffer collecting approximately 7 cm^3 fractions. The protein content and DHPR activity of each fraction were measured as before. The most active fractions were pooled and made 0.8 M NaCl 0.1 mM NADH and 0.1 mM EDTA ready for application to the affinity adsorbent.

Purification of DHPR by Affinity Chromatography

All operations were performed at below $4^{o}C$. 10 cm^3 of the affinity adsorbent were packed into a column and equilibrated with 0.05 M Tris/HCl buffer pH 7.6 containing 0.8 M NaCl, 0.1 mM EDTA and 20% glycerol. (Buffer 1) The prepared active Sephadex G-150 fractions were applied to the column which was eluted with the following :-

(i) 30 cm^3 of Buffer 1 + 0.1 mM NADH

(ii) 15 cm^3 of 0.1 M Na_2CO_3 /Na OH buffer pH 10.9 containing M NaCl + 0.1 mM NADH

(iii) 10 cm^3 of Buffer 1

(iv) 15 cm^3 of 0.1 M Na_2CO_3/NaOH buffer pH 10.9 containing M NaCl.

Approximately 7 cm^3 fractions were collected. The pH of each fraction was checked and adjusted, if necessary, to pH 7.6 using M HCl. The protein content of each fraction was measured using the Lowry method (Lowry, Rosebrough, Farr and Randall 1951) and the DHPR activity of each fraction was measured as before. The fractions were made 2 mM dithiothreitol and 0.02 mM NADH before storing in the freezer. The affinity adsorbent was regenerated by washing the column with 15 cm^3 of 0.1 M sodium acetate buffer pH_4 containing M NaCl and then 15 cm^3 of buffer 1. To prevent deterioration in the performance of the column it was regenerated after every 4 runs using a dithiothreitol treatment. This consisted of washing the column adsorbent with Buffer 1 + 2 mM dithiothreitol for 16 hours followed by the same buffer minus the dithiothreitol until the colour reappeared. The adsorbent was always stored in Buffer A at 4°C in the dark.

Assay for Dihydropteridine Reductase Activity

This method was essentially that of Craine, Hall and Kaufman (1972) but with the addition of sodium azide to the buffer as a catalase inhibitor. Each assay cuvette (1 cm^3, 1 cm path length) contained the following :-

(i) 0.5 cm^3 of 0.1 M Tris/HCl buffer pH 7.0 containing 5.10^{-4} M sodium azide.

(ii) 0.1 cm^3 of 10^{-2} M hydrogen peroxide

(iii) 0.1 cm^3 of 80 mg/L horseradish peroxidase

(iv) 0.1 cm^3 of 10^{-4} M 6,7 dimethyl 5,6,7,8 tetrahydropterin

(v) 0.02 cm^3 of enzyme preparation

(vi) 0.1 cm^3 of 10^{-3} M NADH

(vii) Distilled water to make the volume up to a total of 1 cm^3.

The NADH was always added last with mixing to initiate the reaction after the other reagents had been allowed to equilibrate at 37°C. The rate of reaction was measured spectrophotometrically by following the decrease in optical density at 340 nm due to the disapperance of reduced nicotinamide adenine dinucleotide (NADH) at 37°C in a Pye Unicam SP 1700 spectrophotometer linked to a variable recorder and constant temperature water bath. Readings of the decrease in optical density against time were measured directly on the chart recorder and then the enzyme activity was calculated in μ moles NADH oxidised per minute per cm^3 of enzyme. 1 unit of enzyme activity is defined as that amount of enzyme which will catalyze the oxidation of 1 μ mole of reduced pyridine nucleotide at 37°C in 1 minute.

Measurement of Km Values

The effect of 6,7 dimethyl 5,6,7,8 tetrahydropterin and NADH

concentrations on the reductase activity were examined. The Km values were determined for those substrates by variation of the concentration of one substrate at saturating levels of the other substrate.

Effect of Ionic Strength

The effect of differing ionic strengths on the enzyme activity was investigated by including sodium chloride in the assay mixture at a range of concentrations from 10^{-8}M to 2M.

Effect of pH

The effect of pH on the enzyme activity was studied by using a range of buffers at different pHs in the assay mixture. 0.1 M Tris/HCl buffer with 5.10^{-4} M sodium azide was used in the range of pH5 to pH 8.5 and 0.1 M phosphate buffer with 5.10^{-4}M sodium azide was used in the same pH range.

Estimation of Molecular Weight

The molecular weight of the enzyme was estimated using a Sephadex G-150 column (1.5 x 60 cm) calibrated with the following protein standards, molecular weights shown in brackets :- cytochrome C (12,400) $\propto$ chymotrypsinogen (25,000), bovine serum albumin (fraction V) (65,000), hexokinase (100,000), horseradish peroxidase (40,000) and dextran blue 2000 (average of 2,000,000) as described by Andrews (1966). Samples were applied to the column equilibrated with 0.05 M phosphate buffer pH 7.0 containing 2 mM dithiothreitol and were eluted in the same buffer, 40 x 3 ml fractions were collected.

Effect of Substrate Type

Km values were determined for alternative substrates with the enzyme. NADPH was used instead of NADH at a constant saturating concentration of 6,7 dimethyl 5,6,7,8 tetrahydropterin and tetrahydrobiopterin and tetrahydrofolic acid were used in place of 6,7 dimethyl 5,6,7,8 tetrahydropterin at a constant saturating concentration of NADH.

Use of Dichlorophenolindophenol (DICPIP)

DICPIP was used as an alternative method of generating the quinonoid substrate form from the tetrahydro- form in place of the horseradish peroxidase and hydrogen peroxide to check the results of the Km values. (Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973)

RESULTS

The results of the purification of rat liver dihydropteridine reductase are shown in Tables 4-1, 4-2 and 4-3 and in figure 4-2. The only changes in experimental procedure between tables 4-1 and 4-3 are that for the Table 4-3 results :-

(i) a smaller volume of Sephadex G-150 peak fractions was applied to the affinity column;

(ii) the affinity column was run at a slower speed and

(iii) the affinity column fractions collected were of a smaller volume, 5 cm^3 instead of 7 cm^3.

These changes in experimental conditions improved the purification of dihydropteridine reductase over the original supernatant from about 400 fold to about 900 fold and gave a corresponding increase in specific activity from 33.5 units/mg to 55.1 units/mg. However, the percentage recovery of the activity applied to the affinity column dropped from 61% to 41%.

The effect of quinonoid 6,7 dimethyl dihydropterin and NADH concentrations on dihydropteridine reductase activity are shown in figures 4-3 and 4-4 and gave hyperbolic velocity versus substrate concentration curves. Both Lineweaver-Burk and Eadie Hofstee plots gave similar results for the kinetic constants. The apparent Km values for quinonoid 6,7- dimethyldihydropterin and NADH were found to be $1.2.10^{-5}$ M and 3.210^{-5} M respectively and the apparent V_{max} value was 24.2 μ moles/min/mg of protein.

The effect of changes in the ionic strength on dihydropteridine reductase are shown in Table 4-4 and it can be seen that very little change in activity occurs over the wide range of sodium chloride concentrations used.

The results of varying the pH of the assay buffer are shown in figure 4-5. There was measurable dihydropteridine reductase activity over the whole range of pH tested and with both Tris/HCl and phosphate buffers. As can be seen in figure 4-5 there was a plateau of peak activity over the pH range of 6.3 to 7.0.

The results of the molecular weight determination are shown in figure 4-6. The dihydropteridine reductase eluted as a single band from the Sephadex G-150 column corresponding to a molecular weight of 51,000.

The kinetic data obtained using alternative substrates for NADH and 6,7 dimethyl 5,6,7,8 tetrahydropterin are summarized in Tables 4-5 and 4-6 respectively. Table 4-5 shows that NADPH gave an apparent Km value which was greater than that for NADH by approximately five times and an apparent V_{max} value which was about one-third that obtained with NADH. Tetrahydrofolate gave a much lower apparent V_{max} value than that of 6,7 dimethyl 5,6,7,8 tetrahydropterin and an apparent Km value which was about 30 times greater, as can be seen in Table 4-6. However, 5,6,7,8 tetrahydrobiopterin gave a slightly lower apparent Km value than that of 6,7 dimethyl 5,6,7,8 tetrahydropterin and an apparent V_{max} value which was about a third more again.

Table 4-7 summarizes the kinetic data obtained when DICPIP is used to generate the quinonoid pterin substrate instead of Horseradish peroxidase and hydrogen peroxide. If these results are compared with the corresponding ones in Tables 4-5 and 4-6 it can be seen that the apparent Km values are all slightly elevated but the apparent V_{max} values are about the same.

TABIE 4-1

SUMMARY OF PURIFICATION (1)

	Volume cm^3	Enzyme Activity units/cm^3	Total Enzyme Activity units	Recovery %	Protein mg/cm^3	Specific Activity units/mg protein	Purification
Crude Extract (Supernatant)	100	3.05	305.0	100	40.00	0.08	1
Sephadex G-150 peak fractions	12	9.63	115.6	38	1.12	8.60	107.5
Affinity chromatography peak fraction	7	10.05	70.4	23	0.30	33.50	418.8

TABLE 4-2

KEY FOR FIGURE 4-2

The column was eluted with :-

Arrow①	0.05M Tris/HCl pH 7.6, 0.8M NaCl 0.1 mM EDTA, 20% glycerol buffer, (Buffer 1) + 0.1 mM NADH
Arrow②	0.1 M Na_2CO_3/NaOH pH 10.9, M NaCl + 0.1 mM NADH
Arrow③	Buffer 1 with no NADH
Arrow④	0.1 M Na_2CO_3/NaOH pH 10.9, M NaCl with no NADH

FIGURE 4-2 CHROMATOGRAM FROM AFFINITY CHROMATOGRAPHY ON SODIUM 1,2 NAPHTHOQUINONE-4-SULPHONATE ATTACHED TO AH-SEPHAROSE 4B

(see table 4-2 for key)

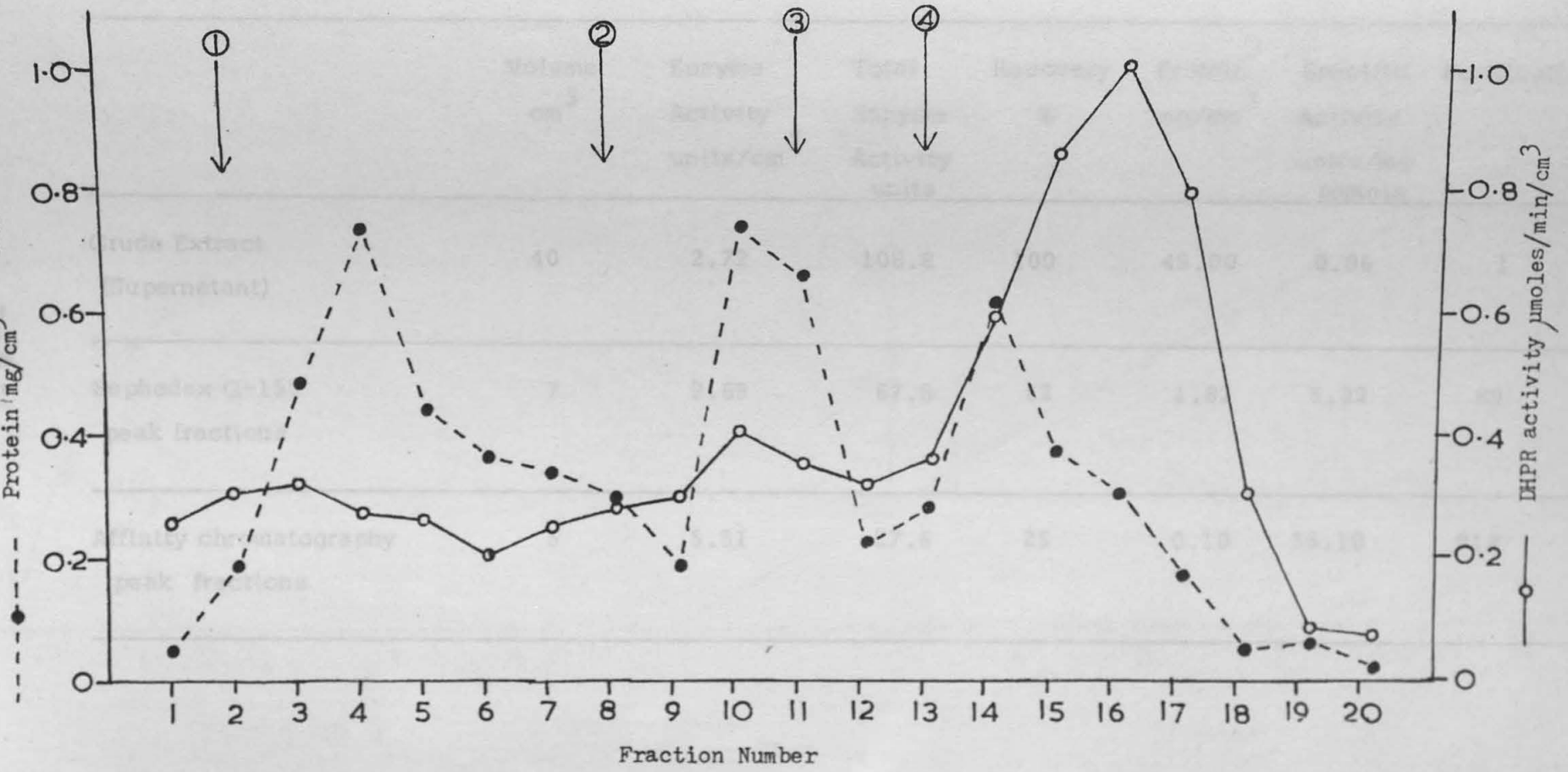


TABLE 4-3

SUMMARY OF PURIFICATION (2)

	Volume cm^3	Enzyme Activity units/cm^3	Total Enzyme Activity units	Recovery %	Protein mg/cm^3	Specific Activity units/mg protein	Purification
Crude Extract (Supernatant)	40	2.72	108.8	100	49.00	0.06	1
Sephadex G-150 peak fractions	7	9.69	67.8	62	1.82	5.32	89
Affinity chromatography peak fractions	5	5.51	27.6	25	0.10	55.10	918

FIGURE 4-3 EFFECT OF CONCENTRATION OF QUINONOID DIHYDRO 6,7 DIMETHYLPTERIN ON THE ACTIVITY OF DIHYDROPTERIDINE REDUCTASE

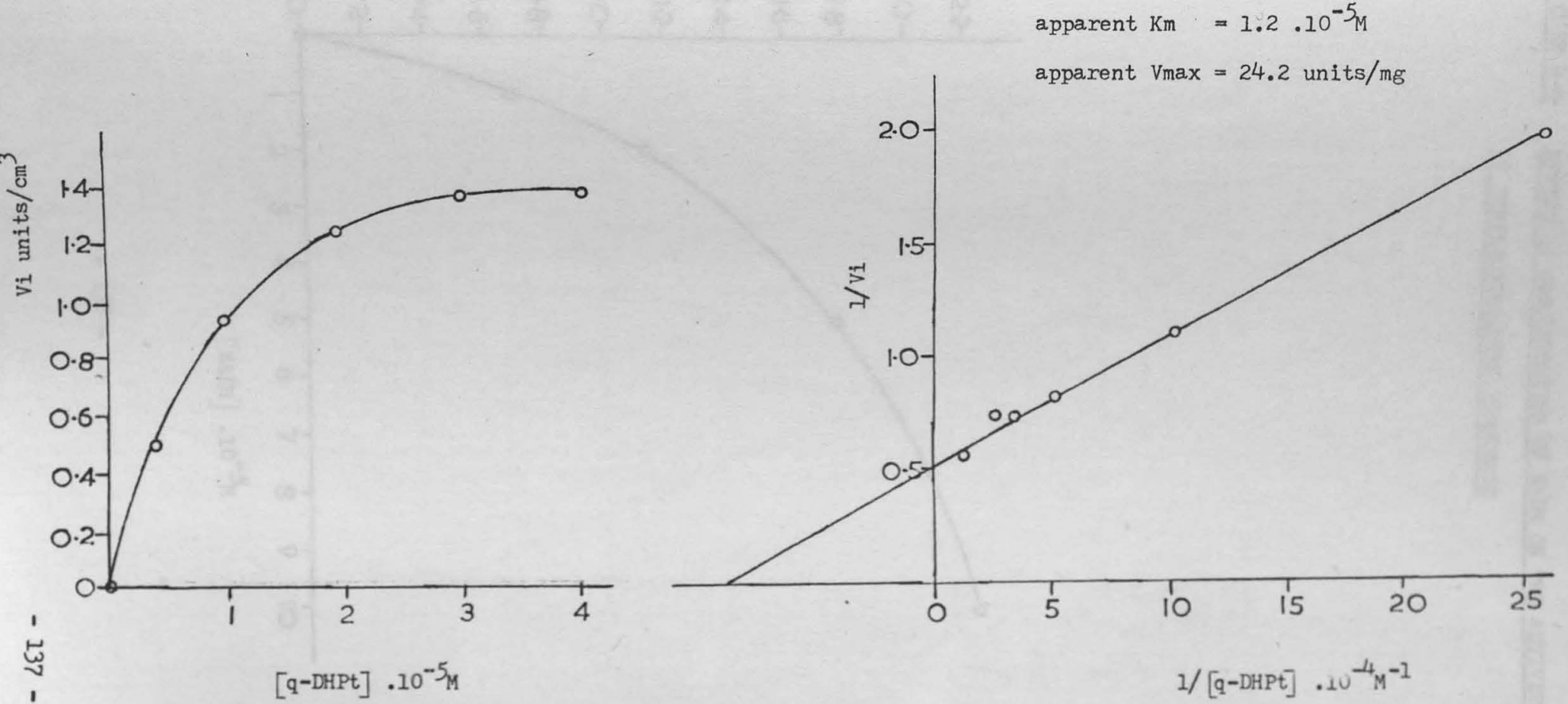


FIGURE 4-4 EFFECT OF CONCENTRATION OF NADH ON THE ACTIVITY OF DIHYDROPTERIDINE REDUCTASE

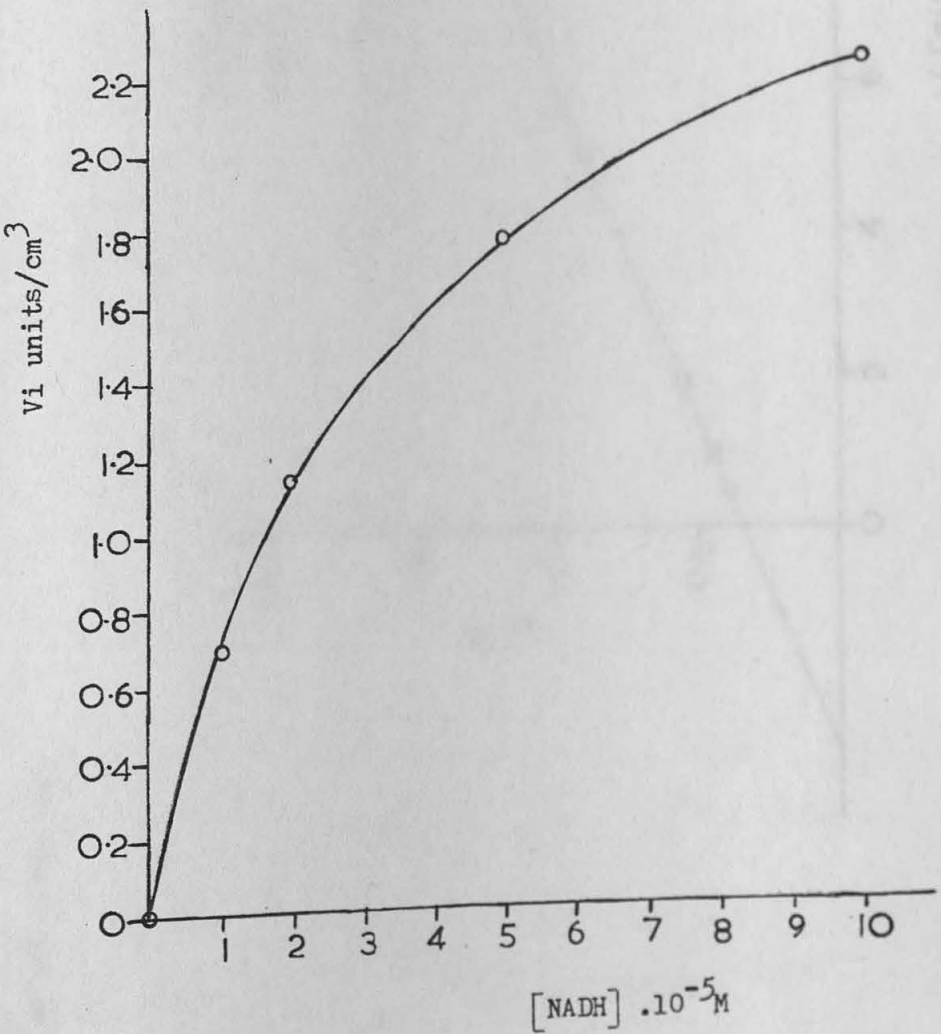


FIGURE 4-4 continued

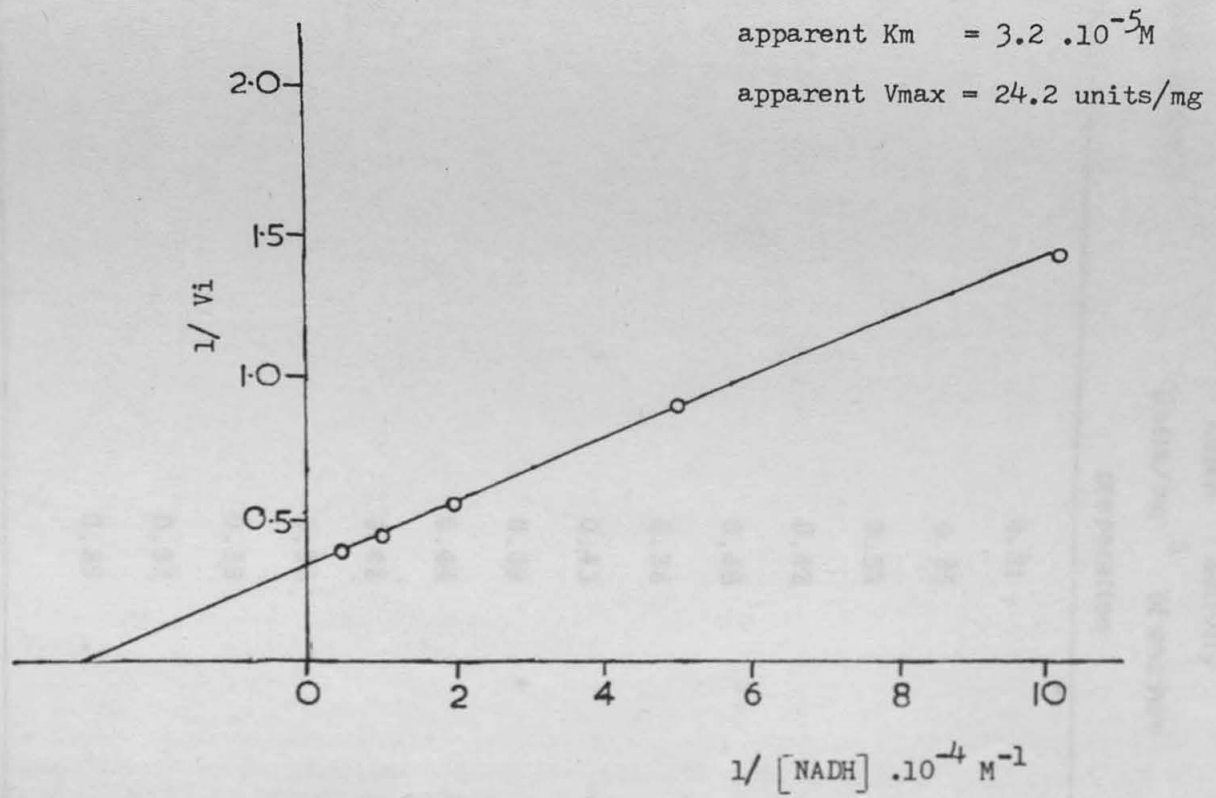


TABLE 4-4

EFFECT OF IONIC STRENGTH ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

Concentration of NaCl	DHPR Activity Units/cm^3 of enzyme preparation
10^{-8}M	0.31
10^{-7}M	0.35
10^{-6}M	0.35
10^{-5}M	0.42
10^{-4}M	0.46
10^{-3}M	0.36
10^{-2}M	0.43
5.10^{-2}M	0.39 *
10^{-1}M	0.44
2.10^{-1}M	0.42
4.10^{-1}M	0.36
5.10^{-1}M	0.38
M	0.37
2M	0.35

* The ionic strength in the normal assay mixture

FIGURE 4-5 EFFECT OF pH ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

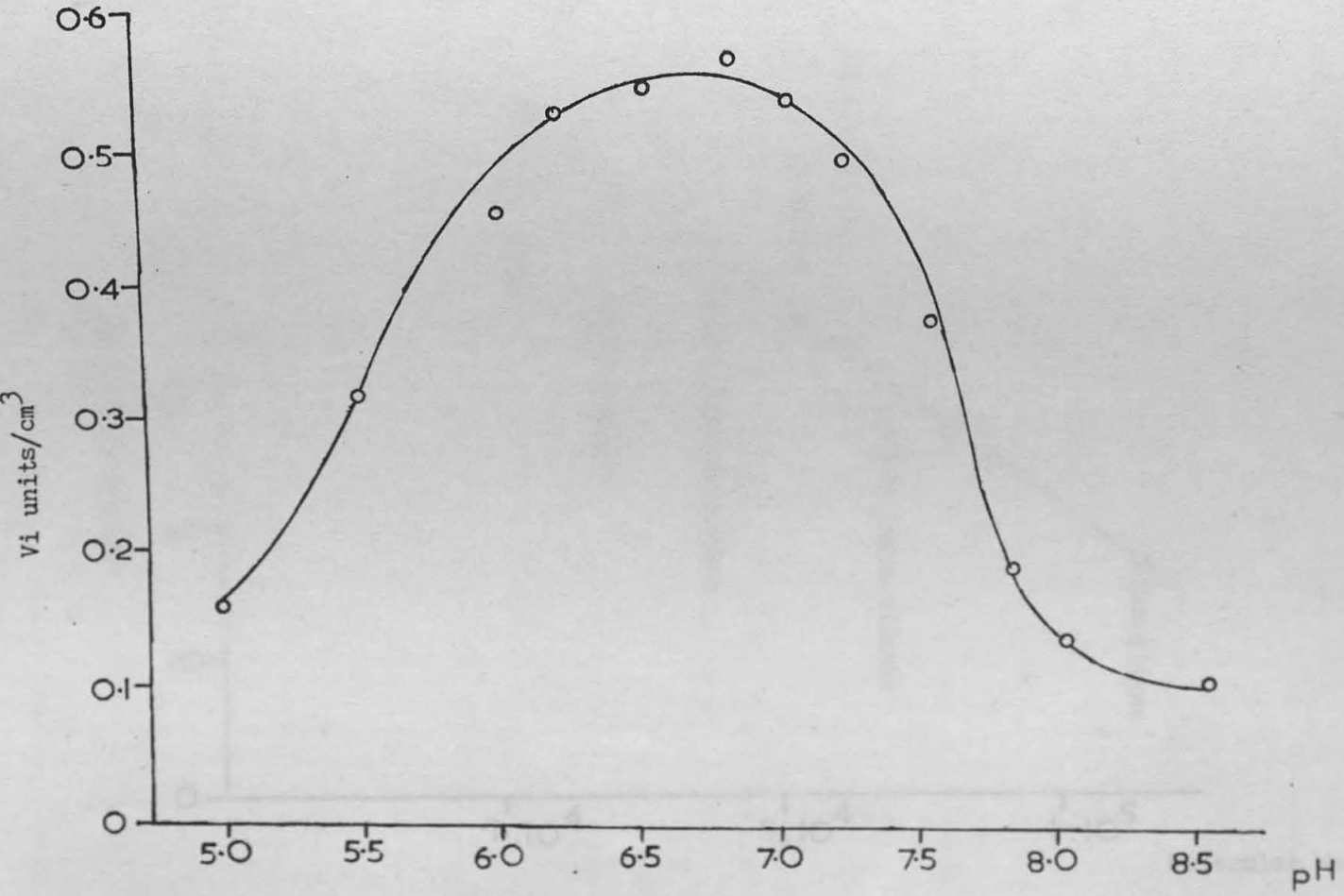


FIGURE 4-6 DETERMINATION OF MOLECULAR WEIGHT OF DIHYDROPTERIDINE REDUCTASE BY SEPHADEX G-150 CHROMATOGRAPHY

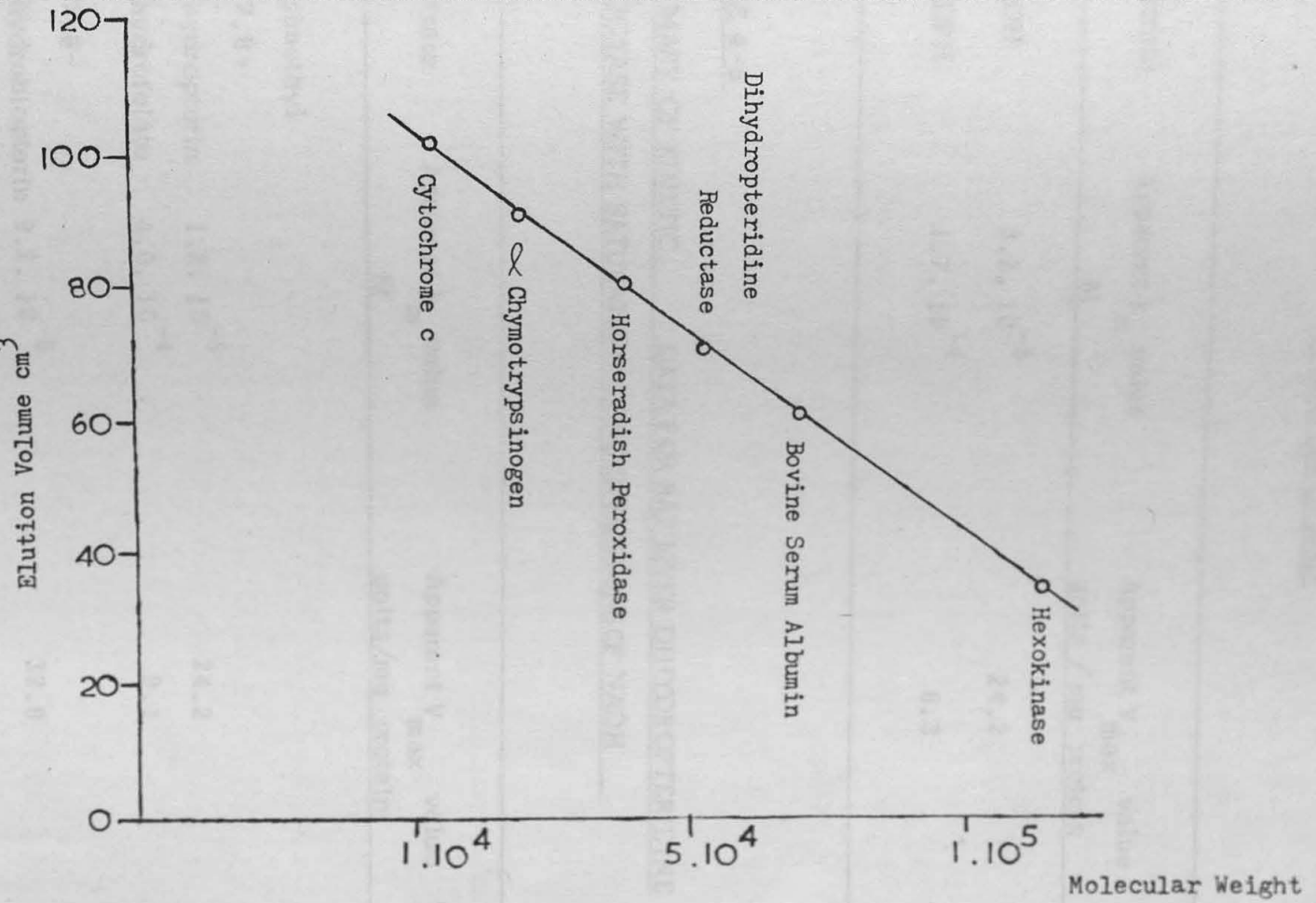


TABLE 4-5

SUMMARY OF KINETIC DATA FOR RAT LIVER DIHYDROPTERIDINE REDUCTASE WITH SATURATING CONCENTRATIONS OF 6,7 DIMETHYL 5,6,7,8-TETRAHYDROPTERIN

Substrate	Apparent k_m value M	Apparent V_{max} value units / mg protein
NADH	$3.2 \cdot 10^{-5}$	24.2
NADPH	$1.7 \cdot 10^{-4}$	8.3

TABLE 4-6

SUMMARY OF KINETIC DATA FOR RAT LIVER DIHYDROPTERIDINE REDUCTASE WITH SATURATING CONCENTRATIONS OF NADH

Substrate	Apparent k_m value M	Apparent V_{max} value units/mg protein
6,7 dimethyl 5,6,7,8-tetrahydropterin	$1.2 \cdot 10^{-5}$	24.2
tetrahydrofolate	$4.0 \cdot 10^{-4}$	9.1
5,6,7,8-tetrahydrobiopterin	$9.2 \cdot 10^{-6}$	32.8

TABLE 4-7

SUMMARY OF KINETIC DATA FOR RAT LIVER DIHYDROPTERIDINE REDUCTASE USING DICPIP AS THE METHOD OF GENERATION OF THE QUINONOID SUBSTRATE

Saturating Substrate	Substrate	Apparent k_m M	Apparent V_{max} units/mg protein
NADH	6,7 dimethyl 5,6,7,8-tetrahydropterin	$4.2.10^{-5}$	24.1
NADH	5,6,7,8-tetrahydrobiopterin	$1.0.10^{-5}$	31.2
6,7, dimethyl 5,6,7,8-tetrahydropterin	NADH	$5.2.10^{-5}$	24.1

DISCUSSION

The method of purification used here is a modified version of that of Cotton and Jennings (1978). Although a slightly longer method it gave a higher purification of about 900 fold over the original supernatant compared to about 400 fold and a slightly better recovery of the enzyme activity in the most active fraction of 25% compared to 18%. (Cotton and Jennings 1978) Like the adsorbent of Cotton and Jennings, the adsorbent used here could be regenerated and used repeatedly if stored correctly. If the results obtained here are compared to those of other affinity chromatographic methods of dihydropteridine reductase purification, it can be seen that with a Blue Dextran-agarose adsorbent (Chauvin, Korri, Tirpak, Simpson and Scrimgeour 1979) and a 5' AMP-Sepharose 4B adsorbent (Aksnes, Skotland, Flatmarck and Ljones 1979) better specific activities of 230 units/mg and 155 units/mg respectively were obtained. These can be compared to the 55 units/mg obtained by the method used here but smaller yields of activity were recorded, 16% and 17% respectively. The purification was only 359 for the Blue Dextran adsorbent and 705 for the 5'AMP-Sepharose 4B adsorbent. With a Cibacron blue F3GA - agarose adsorbent, Webber and Whiteley obtained a better specific activity of 94 units/mg and a similar 26% recovery of enzyme activity but lower purification of 223 (1978). The results obtained here are very similar to the 62 units/mg specific activity and, 27% recovery of enzyme activity but there was a 148 fold purification recorded with a methotrexate-sepharose adsorbent. (Webber, Deits, Snyder and Whiteley 1978) Even though the results obtained here are not

quite as good as some of those recorded with other affinity chromatographic methods the method used has the advantage that it is a much shorter process consisting of only two steps.

Cotton and Jennings suggested that the sodium 1,2-naphthoquinone-4-sulphonate ligand may be effective for dihydropteridine reductase purification by affinity chromatography due to structural similarities to the quinonoid pterin substrate. This would explain its specificity for dihydropteridine reductase and why a single affinity chromatography step gave them such good purification results. (Cotton and Jennings 1978) As can be seen from Figure 4-2 and Table 4-2, NADH has to be present for the dihydropteridine reductase to bind to the adsorbent and use is made of this property in the purification by firstly binding the enzyme onto the adsorbent in a buffer containing NADH. Then after the impurities have been washed off the column the enzyme is eluted from the adsorbent using a buffer minus NADH when the enzyme is released from the adsorbent being unable to bind without the NADH. This method also suggests that dihydropteridine reductase may follow a compulsory order mechanism where the NADH has to bind to the enzyme before the quinonoid pterin substrate can be bound at all, hence the binding of the NADH substrate probably produces a conformational change in the enzyme which then enables it to bind the quinonoid pterin substrate or in the case of the purification method used here, the 1,2 sodium naphthoquinone-4-sulphonate. Therefore, this purification technique adds more evidence to the theory of dihydropteridine reductase having a

compulsory order mechanism, (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977, Aksnes and Ljones 1980) as do several NADH-dependent dehydrogenase and reductase enzymes such as liver alcohol dehydrogenase and lactate dehydrogenase. (Dalziel 1975, Dixon and Webb 1979)

The rat liver dihydropteridine reductase isolated here had a pH optimum between pH 6.3 and 7.0 and this result agrees well with those appearing in the literature. (Webber, Deits, Snyder and Whiteley1978, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973) The pH optimum of this rat liver dihydropteridine reductase is therefore near the pH value at which the quinonoid dihydropterin substrate is most stable. (Archer and Scrimgeour 1970)

The molecular weight of the dihydropteridine reductase isolated by the method used here was 51,000 which again agrees well with the values quoted in the literature which range from 48,000 (Webber, Deits, Snyder and Whiteley 1978) through 49 000 (Hasegawa 1977) and 50,000 (Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973) to 52,000 . (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977, Aksnes, Skotland, Flatmarck and Ljones 1979) In all these cases the enzyme was found to be a dimer consisting of two identical subunits but the dihydropteridine reductase isolated by the similar method as used here from monkey and human liver by Cotton and Jennings (1978) had a molecular weight of 100,000 and was a tetramer. In all the studies the monomer molecular weight was found to be about 25,000 which suggests that the dihydro-

pteridine reductase isolated from rat here is probably a dimer. Based on the molecular weight of 51,000 and the specific activity of 55.1 units/mg of protein the turnover number of dihydropteridine reductase is $1.71.10^3$, (molecules of product synthesized per minute by each molecule of enzyme)

The Michaelis constants obtained with the substrates NADH and 6,7, dimethyl 5,6,7,8 tetrahydropterin of $3.2\ 10^{-5}$M and $1.2.10^{-5}$M respectively are again in good agreement with values quoted in the literature. (Webber, Deits, Snyder and Whiteley 1978, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Webber and Whiteley 1978, Craine, Hall and Kaufman 1972) The higher apparent Km and lower apparent V_{max} values obtained with NADPH suggest that the enzyme has a distinct preference for NADH as the cofactor. Most work on dihydropteridine reductase has indicated that NADH is the preferred cofactor. (Webber, Deits, Snyder and Whiteley 1978, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Craine, Hall and Kaufman 1972, Webber and Whiteley 1978, Nielsen, Simonsen and Lind 1969) except for a dihydropteridine reductase isolated from bovine liver by Nakanishi and his coworkers which was shown to have a preference for NADPH rather than NADH but it is thought to be a different type or form of the enzyme. (1978) Of the three pterin substrates studied here, the 5,6,7,8 tetrahydrobiopterin appears to be the most favoured substrate for the dihydropteridine reductase as it has the lowest apparent Km value and highest apparent V_{max} value but 6,7, dimethyl 5,6,7,8 tetrahydropterin has similar kinetic

constants. Tetrahydrofolate gave a higher apparent Km value and a much lower apparent V_{max} value but still can act as a substrate for this enzyme. However, these results may also be due not to tetrahydrofolate acting as a substrate for dihydropteridine reductase but due to a small amount of an impurity in the tetrahydrofolate which is a substrate for dihydropteridine reductase such as 6 methyl tetrahydropterin which has been used in assays of this enzyme. (Kaufman and Levenberg 1959, Kaufman 1975) Pollock and Kaufman suggested that dihydropteridine reductase may play a role in folate metabolism in the brain by preserving tetrahydrofolate from oxidation. (1978) These results show that tetrahydrofolate could indeed be a substrate for dihydropteridine reductase but the comparative apparent Km values of the enzyme for tetrahydrofolate and tetrahydrobiopterin indicate that the enzyme has a much greater affinity for tetrahydrobiopterin.

The results obtained here indicate that it is possible to isolate dihydropteridine reductase from rat liver by this two step method involving affinity chromatography with sodium 1,2 napthoquinone-4-sulphonate as ligand and hence, inhibition studies with various metal ions, metabolites and drugs can be performed.

CHAPTER FIVE

THE EFFECT OF METAL IONS, DRUGS AND OTHER COMPOUNDS ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

The cellular concentration of tetrahydrobiopterin will have a significant effect on the production of catecholamine neurotransmitters because it is present in rate-limiting amounts (Kettler, Bartholini and Pletscher 1974, Patrick and Barchas 1976) for the action of the rate-limiting step, tyrosine hydroxylase. (Levitt, Spector, Sjoerdsma and Udenfriend 1965, Nagatsu, Levitt and Udenfriend 1964) Tetrahydrobiopterin is also an essential cofactor for the enzyme tryptophan hydroxylase (Hosoda and Glick 1966, Friedman, Kappelman and Kaufman 1972) which is the rate-limiting step for the biosynthesis of another neurotransmitter, serotonin. (Costa and Meek 1974) Dihydropteridine reductase (E.C. 1.6.99.7) is the enzyme whose main function is to maintain the cellular levels of tetrahydrobiopterin (Craine, Hall and Kaufman 1972) by recycling the quinonoid dihydrobiopterin formed by phenylalanine, tyrosine and tryptophan hydroxylases ready to be used again. Any factor reducing the activity of dihydropteridine reductase therefore, could have serious consequences on neurotransmitter production via tetrahydrobiopterin levels.

Deficiency of dihydropteridine reductase results in raised serum biopterin levels, (Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977, Danks, Bartholome, Clayton, Curtius, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978) because the quinonoid dihydrobiopterin

is not being reduced and therefore rearranges to the 7,8 dihydrobiopterin form which appears in the blood and urine. (Leeming 1975, Leeming 1979, Kaufman 1964, Curtius, Niederwieser, Viscontini, Otten, Schaub, Scheibenreiter and Schmidt 1979) Dihydroxanthopterin is also detectable in the urine of these patients. (Watson, Schlesinger and Cotton 1977) Methotrexate therapy produces a similar raised serum biopterin level and raised levels of biopterin in urine as well as the appearance of dihydroxanthopterin. (Leeming, Blair, Melikian and O'Gorman 1976, Watson, Schlesinger and Cotton 1977, Cotton 1978) Methotrexate is a potent dihydrofolate reductase inhibitor (Ki below 10^{-9}M) (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977) but the literature is controversial on its effect on dihydropteridine reductase. It has been shown to inhibit dihydropteridine reductase of sheep liver with a Ki value of 3.8. 10^{-5}M and this inhibition was competitive with respect to the pterin. (Craine, Hall and Kaufman 1972) Inhibition of beef liver dihydropteridine reductase by methotrexate which was uncompetitive with respect to NADH and non-competitive with respect to the quinonoid pterin substrate has also been described. (Chauvin, Korri, Tirpak, Simpson and Scrimgeour 1979) It was suggested that the methotrexate could bind to either substrate site but appears to bind most strongly to the quinonoid dihydropterin site. (Chauvin, Korri, Tirpak, Simpson and Scrimgeour 1979) However, it has also been claimed that methotrexate has no effect on dihydropteridine reductase activity. (Gal, Bybee and Sherman 1979) The effect of methotrexate on dihydropteridine reductase is important because of the long term side effects of methotrexate on the central nervous system which

have been reported in some cases of childhood leukaemia. (Meadows and Evans 1976) Dihydropteridine reductase inhibition may be the cause (Cotton 1978) and these neurological symptoms would then be due to a reduction in neurotransmitter production, as is proposed for the cause of the mental defects which occur in malignant hyperphenylalaninaemia due to dihydropteridine reductase deficiency. (Cotton 1978)

If this theory is correct then any drug which has an effect on dihydropteridine reductase activity will be of importance.

There are many dihydrofolate reductase inhibitors which, like methotrexate, could be dihydropteridine reductase inhibitors. Aminopterin and co-trimoxazole (trimethoprim and sulphamethoxazole) reduce phenylalanine clearence after an oral load as methotrexate does, (Andrews, Purkiss, Chalmers and Watts 1976, Leeming, Blair, Melikian and O'Gorman 1976, England and Coles 1972) most probably by reduction of phenylalanine hydroxylation. Aminopterin has been shown to inhibit phenylalanine hydroxylation in vitro and in vivo. (Kaufman and Levenberg 1959)

Serum dihydrobiopterin has been shown to parallel serum phenylalanine after oral loads. (Leeming, Blair, Green and Raine 1976) Therefore either phenylalanine itself or a metabolite of phenylalanine may have an effect on the dihydropteridine reductase system. Phenylalanine occurs at concentrations of about 10^{-4}M in normal human brain. (Letendre,

Nagaiah and Guroff 1980) Langenbeck and his co-workers have determined by studying phenylketonurics on and off the phenylalanine-restricted diet that there is a high correlation between plasma phenylalanine levels and phenylpyruvate levels where the ratio of amino acid/keto acid is about 12:1 at a plasma phenylalanine value of 1.7 mM. The range of serum phenylpyruvate was 10^{-5}M to 1.6. 10^{-4}M in controlled phenylketonurics. (Langenbeck, Behbehani and Luthe 1980b) The normal (non-phenylketonuric) plasma phenylpyruvate concentration is about 5 to 8 μM. (Langenbeck, Behbehani and Luthe 1980a, Langenbeck, Behbehani and Luthe 1980b) Jervis and Drejza found both phenylpyruvate and o-hydroxyphenylacetic acid in the plasma of every case of phenylketonuria they examined in the ranges of 10^{-5}M to 6.4. 10^{-5}M and 7.9. 10^{-6}M to 4.0. 10^{-5}M respectively. (1966) Langenbeck's group showed that phenylpyruvate appears in the urine when plasma phenylalanine levels rise to 1.1mM or higher. (Langenbeck, Behbehani, Mench-Hoinowski and Petersen 1980) A non-linear relationship between the urinary levels of both phenylpyruvate and o-hydroxyphenyl acetic acid and the plasma levels of phenylalanine was also demonstrated where levels of phenylpyruvate were typically 5 times higher than those of o-hydroxyphenylacetic acid. (Langenbeck, Behbehani, Mench-Hoinowski and Petersen 1980) It has been demonstrated that the amount of phenylpyruvate and o-hydroxyphenylacetic acid excreted in the urine of patients with phenylketonuria or its variants increases with age up to about 2 years of age with only small amounts being excreted in the first few months. This is thought to be due to delayed maturation of phenylalanine transaminase. (Rey, Pellie, Sivy, Blandin-Saroja, Rey and

Frezal 1974) The lumbar cerebrospinal fluid of a patient with untreated phenylketonuria had only traces of phenylpyruvate, of about 1 μM. (Langenbeck, Mench-Hoinowski and Rod-Urban 1978) An *in vivo* metabolic pathway in rabbit brain has been demonstrated which linked L-phenylalanine to phenylethylamine and tyramine. (Mosnaim, Silkaitis and Wolf 1980) So these two metabolites of phenylalanine might be expected to rise in phenylketonuria as well as the usual, quoted metabolites, phenylpyruvate and o-hydroxyphenylacetic acid. The concentration of phenylethylamine in brain has been quoted as 5nM, (Fischer, Spatz and Heller 1972) and as 9.1. 10^{-9}M in rat brain. (Martin and Baker 1977) The level of para-tyramine in rat brain was measured and found to be 1.3. 10^{-8}M.

5-formyltetrahydrofolic acid, which is given as a rescue therapy after methotrexate, (Frei, Jaffe, Tattersall, Pitman and Parker 1975) is also associated with rises in serum dihydrobiopterin whether given orally or intravenously. If the clinician wished to avoid the possible neurological consequences, then 5-methyl tetrahydrofolic acid which has no effect on serum dihydrobiopterin levels when given orally might be a better choice as has been shown in the mouse. (Blair and Searle 1970) Also 5-methyl tetrahydrofolic acid has been demonstrated to have no measurable effect on serum dihydrobiopterin levels when administered after methotrexate. (Leeming, Blair, Melikian and O'Gorman 1976) The effect of various folates on dihydropteridine reductase could therefore be of interest.

As tetrahydrobiopterin plays such an important role in the production of the catecholamine neurotransmitters, dopamine and noradrenaline, (Kettler, Bartholini and Pletscher 1974, Nagatsu, Levitt and Udenfriend 1964) it might be expected that these neurotransmitters or an intermediate on their biosynthetic pathway or one of their metabolites may have a regulatory or controlling influence on tetrahydrobiopterin metabolism. Tetrahydrobiopterin is also involved in the production of serotonin (Friedman, Kappelman and Kaufman 1972, Hosoda and Glick 1966) so this neurotransmitter also may have an influence on tetrahydrobiopterin metabolism. Dopamine has been shown to be present in the central nervous system in relatively high amounts but it is not evenly distributed (Glowinski and Sversen 1966, Popov, Pahle, Rosler and Matthies 1967) The neostriatum (the caudate nucleus and putamen) contains about 70-80% of the brain content of dopamine. (Berler and Rosengren 1959, Sano, Gamo, Kakimoto, Taniguch, Takesada and Nichiuuma 1959) The concentration of noradrenaline in the central nervous system is generally higher than that of dopamine. (Pal Kovits 1979) It has been shown by fluorimetry and electrochemical methods that whole rat brain contains about $3.6.\ 10^{-6}$M dopamine and about $2.1.10^{-6}$M noradrenaline plus about $4.0.\ 10^{-7}$M homovanillic acid and about $3.7.10^{-7}$M dihydroxyphenylacetic acid, the metabolites of dopamine. (Adams 1979, Taylor 1979) However the concentrations in specific brain areas can be greater such as in the striatum where there is about $5.0.\ 10^{-5}$M dopamine, $2.1.\ 10^{-6}$M noradrenaline, $5.5.\ 10^{-6}$M homovanillic acid and $6.6.\ 10^{-6}$M dihydroxyphenylacetic acid. McKellwin et al., quoted dopamine concentrations

of $5.0.\ 10^{-4}$M in the cerebral brain tissue, $1.6.\ 10^{-3}$M in the thalamus and $8.0.\ 10^{-4}$M in the hypothalamus. (1971) Serotonin levels in whole rat brains have been shown to be about $1.83.\ 10^{-6}$M. (Kamata, Okada, Watanabe, Kawashima and Wada 1980)

The effect of these neurotransmitters and their metabolites, phenylalanine and its metabolites, certain folates and dihydrofolate reductase inhibitors plus other drugs and metal ions associated with neurotoxicity form the basis of the results presented in this chapter.

MATERIALS AND METHODS

Folic acid, dopamine, tyramine, phenylpyruvic acid, phenyllactic acid, o-hydroxyphenylacetic acid, phenylacetic acid and phenylethylamine were all purchased from the Sigma Chemical Company. 10-formyl folate was prepared in the department of chemistry, at Aston University in Birmingham by Dr. M. J. Connor. Noradrenaline, adrenaline, serotonin and L-DOPA were all gifts from Roche Products Limited. All other chemicals were obtained as listed before or from B.D.H. and were of Analar grade.

Methods

The basic assay method used in these studies was essentially the same as that used by Craine, Hall and Kaufman (1972) as described in Chapter 4. The enzyme source was the purest fraction from the affinity column. Again 1 unit of enzyme activity was defined as that amount of enzyme which will catalyse the oxidation of 1 μmole of reduced

pyridine nucleotide at 37°C in 1 minute. The apparent K_m and apparent V_{max} values were measured and calculated as described in Chapter 4, 5, 6, dichlorophenolindophenol (DICPIP) was used as before to check the results with this alternative method of regenerating the quinonoid substrate. (Cheema, Soldin, Knapp, Hofmannand Scrimgeour 1973)

Inhibition Studies

In addition to some of the reagents used in Chapter 3, (Table 3-1) those shown in Table 5-1 were also used for inhibition studies on dihydropteridine reductase.

Inhibition studies were performed with at least three different concentrations of the potential inhibitor. The concentration of dimethyltetrahydropterin was varied and that of NADH was kept constant at 10^{-4}M in order to determine the type of inhibition with respect to NADH. Types of inhibition were assigned using the descriptions in 'Enzymes' by Dixon and Webb (3rd Edition), after plotting the results on velocity versus substrate plots, Lineweaver-Burk plots and Eadie-Hofstee plots. (Dixon and Webb 1979)

With the potential metal ion inhibitors, dihydropteridine reductase activity was also measured by preincubating the enzyme for various lengths of time in the presence of various concentrations of the metal ions at 37°C before the substrates were added. Appropriate controls were also examined by preincubating the enzyme at 37°C for various lengths of time

before adding the substrates and measuring the activity in the usual way.

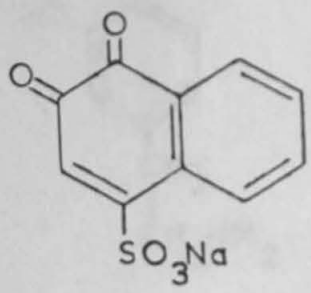
In the case of lead ions, the removal of the lead from the dihydropteridine reductase was investigated, after the enzyme had been incubated with 10^{-4}M lead acetate, by dialysing at 4°C for up to 72 hours against 0.05 M Tris/HCl buffer at pH 7 or against the same buffer containing 10^{-4}M EDTA with twelve changes of buffer for each dialysis. Again appropriate controls were examined by dialysing enzyme against the same buffers at 4°C for the same lengths of time with twelve changes of buffer.

The K_i values were measured for methotrexate and phenylpyruvic acid on the normal dihydropteridine reductase enzyme and on dihydropteridine reductase which had been incubated with 10^{-4}M lead acetate in order to see if the values were altered in the modified enzyme system. The effect of dopamine on the leaded enzyme was also investigated.

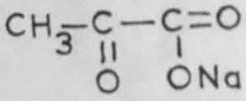
Inhibition studies were also performed using the following mixtures of inhibitors to see if any synergistic action could be detected :- dopamine and phenylpyruvic acid; methotrexate and phenylpyruvic acid; phenylethylamine and phenylpyruvic acid; dopamine and methotrexate; and trimethoprim and sulphamethoxazole. In each case at least one of the inhibitors was present in concentrations which produced maximal inhibition when used alone.

TABLE 5-1 FORMULAE OF REAGENTS USED FOR DIHYDROPTERIDINE REDUCTASE INHIBITION STUDIES

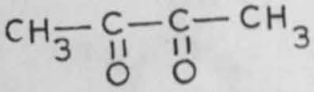
Sodium 1,2 naphthoquinone-4-sulphonate



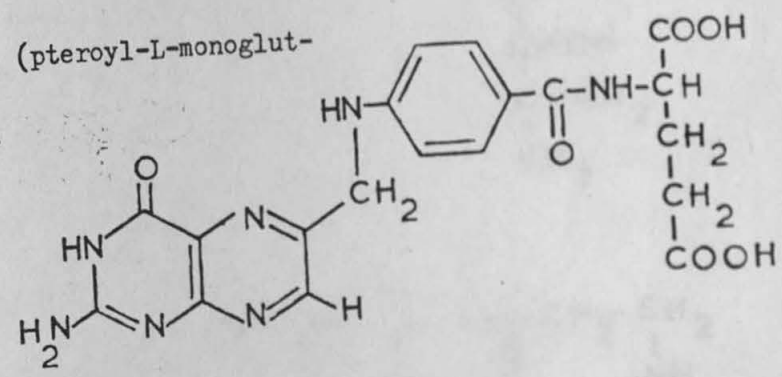
Sodium pyruvate



Diacetyl (butan-2,3-dione)



Folic acid (pteroyl-L-monoglutamic acid)



10 formyl folic acid

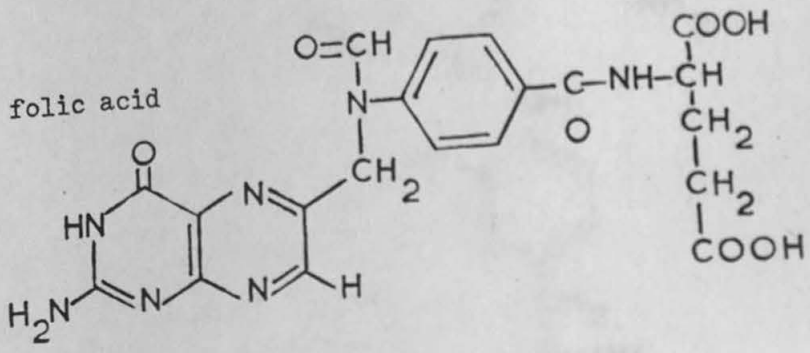
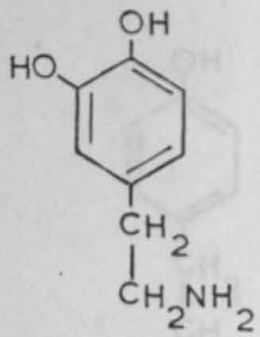
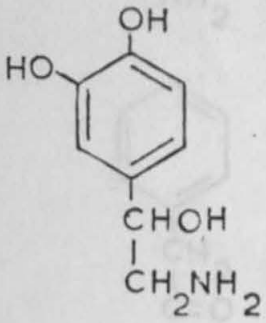


TABLE 5-1 continued

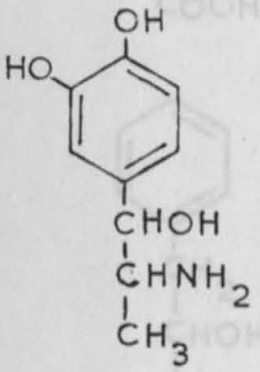
Dopamine



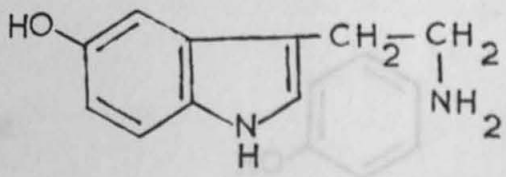
Noradrenaline



Adrenaline



Serotonin



L- DOPA

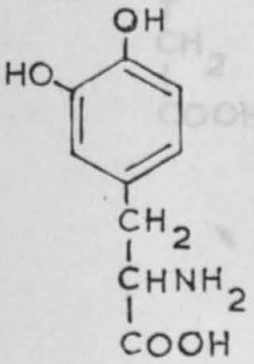
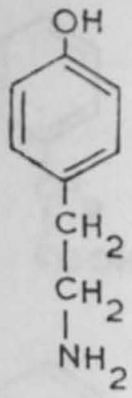
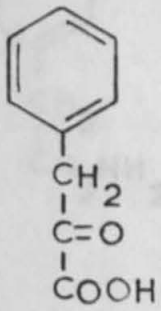


TABLE 5-1 continued

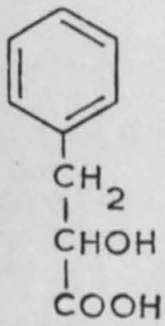
Tyramine



Phenylpyruvate



Phenyllactate



o-hydroxyphenylacetic acid

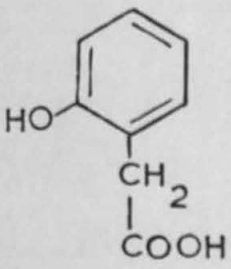
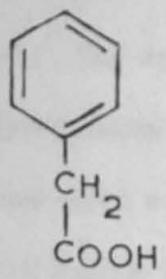
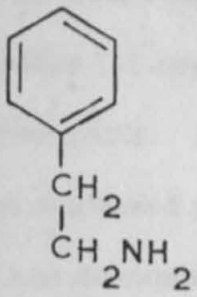


TABLE 5-1 continued

Phenylacetic acid



Phenylethylamine



RESULTS

The results of sodium 1,2 naphthoquinone-4-sulphonate inhibition are shown in Figures 5.1 and 5.2, and in Table 5-2. The apparent K_m values increased with increasing sodium 1,2-naphthoquinone-4-sulphonate concentrations but the V_{max} value remained the same when a constant NADH concentration of 10^{-4}M was used. Therefore sodium 1,2 naphthoquinone-4-sulphonate is a competitive inhibitor of rat liver dihydropteridine reductase with respect to the pterin substrate. Sodium 1,2 naphthoquinone-4-sulphonate showed mixed inhibition with respect to NADH. This means that both the apparent V_{max} and apparent K_m values decreased and increased respectively with increasing sodium 1,2 naphthoquinone-4-sulphonate concentrations. The Dixon plot (Figure 5.2) showed the K_i value for sodium 1,2 naphthoquinone-4-sulphonate to be $3.7. 10^{-4}$M.

Table 5-3 displays the results of inhibition studies by the dihydrofolate reductase inhibitors. All those tested showed competitive inhibition with respect to the pterin substrate but mixed inhibition with respect to NADH. The same results were obtained if 5,6 dichlorophenolindolphenol (DICPIP) were used in the assay system instead of horseradish peroxidase and hydrogen peroxide to generate the quinonoid pterin substrate. The K_i values ranged from $6.4. 10^{-5}$M to $5.8. 10^{-4}$M. Sulphamethoxazole, the other drug which appears with trimethoprim in the pharmaceutical preparations co-trimoxazole, bactrin and septrin showed mixed inhibition with respect to the pterin and competitive inhibition with respect to NADH. The K_i value for sulphamethoxazole was $8.7. 10^{-4}$M. When inhibition by both

trimethoprim and sulphamethoxazole was studied the inhibition obtained by the two drugs together appeared greater than would be expected by the separate drugs (Table 5.4). For example, $3.5. 10^{-4}$M trimethoprim had no effect and $3.9. 10^{-4}$M sulphamethoxazole gave a 24% inhibition but together the same concentrations of these drugs gave a 40% inhibition. This is a more than additive effect. However, if the concentrations of the drugs were increased this effect disappears. For example, $6.9. 10^{-4}$M trimethoprim and $7.8. 10^{-4}$M sulphamethoxazole both gave 38% inhibitions when used separately but together gave 46% inhibition.

The results of various folates as inhibitors of dihydropteridine reductase are shown in Table 5-5. 5-methyltetrahydrofolic acid had no effect on the enzyme but both folic acid and 10-formyl folate showed competitive inhibition with respect to the pterin substrate and mixed inhibition with respect to NADH. The K_i values for folic acid and 10-formylfolate were $7.3. 10^{-5}$M and $8.5. 10^{-5}$M respectively.

Table 5.6 shows the inhibition results of the aromatic amino acids and phenylalanine metabolites. All those determined showed mixed inhibition with respect to NADH. Tryptophan, phenylalanine, phenyllactate, phenylpyruvate, o-hydroxyphenylacetic acid, phenylacetic acid and phenylethylamine displayed mixed inhibition with respect to the pterin substrate as well whereas tyrosine and tyramine showed non-competitive inhibition with respect to the pterin. Non-competitive inhibition is when the apparent V_{max} value decreases and the apparent K_m value remains the same with increasing inhibitor concentrations. Again the same results

were obtained for phenylalanine, phenylpyruvate, phenyllactate and phenylethylamine when DICPIP was used in the enzyme assay. There was a wide range of K_i values obtained with this set of inhibitors from the tyramine value of $1.8. 10^{-5}$M to the value for both phenylalanine and phenyllactate, $1.0. 10^{-3}$M. Table 5.7 shows the percentage inhibition which occurred at 10^{-5}M dimethyltetrahydropterin with various concentrations of phenylpyruvate. It can be seen that a significant 16% inhibition was obtained with a phenylpyruvate concentration of $2.7. 10^{-5}$M. Some problems were encountered with the phenylpyruvate inhibition experiments at first when fractions, which were not so pure as the 900 fold purification fractions, or crude supernatant was used in the studies. The phenylpyruvate appeared to accelerate the dihydropteridine reaction. However, if the pterin substrate and/or the quinonoid generation system were omitted from the reaction mixture, a rate was still obtained. This method is measuring a decrease in NADH so it appeared that some contaminent in the enzyme preparation was causing the phenylpyruvate to be reduced at the expense of the NADH. The phenylpyruvate without the enzyme preparation would not oxidise the NADH so the contaminent was not in the phenylpyruvate solutions. By measuring the specific activity of the dihydropteridine reductase activity with the pterin substrate and the activity of the enzyme preparation to reduce phenylpyruvate at various stages of the purification it was determined that the reduction of the phenylpyruvate was not being brought about by the dihydropteridine reductase enzyme itself. This was because the specific activity of the dihydropteridine reductase activity increased as the purification was

carried out whereas, the specific activity of the 'phenylpyruvate - reducing -enzyme' decreased as the purification proceeded (Table 5-8). This problem was overcome at first by subtracting the rate of the 'phenyl-pyruvate-reducing-enzyme' from the rate of dihydropteridine reductase in the presence of phenylpyruvate but later the problem did not arise as the 900 fold purification fraction from the affinity chromatography had no 'phenylpyruvate-reducing activity.

The results of inhibition studies of the catecholamines and serotonin are shown in Table 5-9. Adrenaline and L-DOPA had no effect but noradrenaline showed non-competitive inhibition and serotonin showed competitive inhibition with respect to the pterin substrate and both these inhibitors showed mixed inhibition with respect to NADH. The same results were obtained if DICPIP was used in the assay. The K_i values for noradrenaline and serotonin were $4.9. 10^{-4}$M and $1.2. 10^{-3}$M respectively.

The results of dopamine inhibition of dihydropteridine reductase are shown in Figures 5.3, 5.4, 5.5, 5.6 and 5.7. As can be seen from Figure 5.3, in the presence of dopamine the usual hyperbolic velocity versus pterin substrate concentration curve becomes sigmoidal in shape. The symoidicity does not disappear if DICPIP is used to generate the quinonoid dimethyl dihydropterin substrate instead of horseradish peroxid-ase and hydrogen peroxide, (Figure 5.4) showing that the effect is on dihydropteridine reductase rather than the quinonoid regeneration method. The velocity versus NADH concentration curve also becomes sigmoidal in the presence of dopamine. (Figure 5.5) The Hill plot (Figure 5.4)

shows positive co-operativity for this enzyme because the Hill coefficient determined from the slope of the middle part of the curve is 2.26. This will approximate to 2 binding sites per enzyme molecule as there has to be a whole number of binding sites. The slope at the top and botton ends of the curve approach values of unity. These deviations from the theoretical linear prediction of the Hill equation plot occur because it is assumed that the substrate binding does not occur in steps but in practice it is possible to obtain partly liganded intermediates. At very low ligand concentration when proceeding from the state where no sites are occupied on the enzyme there would be no co-operative effect and similarly when only one ligand site remained to be filled at high levels of saturation there would be no co-operativity and hence, the values of the Hill-co-efficient approach unity at high and low levels of saturation. The Scatchard plot (Figure 5.7) also shows that the number of substrate molecules bound per molecule of enzyme is greater than 1, in this case, 1.52.

Tables 5.10 and 5.11 show inhibition results with sodium pyruvate and diacetyl respectively. In both cases very high concentrations of the compound were required in order for any inhibition to be measured, $1.8. 10^{-3}$M for sodium pyruvate and $3.5. 10^{-2}$M for diacetyl. The percentage inhibition for each of these concentrations was 23% for sodium pyruvate and 70% for diacetyl.

The results of mixtures of the various inhibitors are shown in Tables 5.12, 5.13, 5.14 and 5.15. Using saturating concentrations of

methotrexate which produces 83% inhibition with 1.1. 10^{-3}M dopamine which produces 65% inhibition, gave 88% inhibition together and with 2.1. 10^{-3}M dopamine which produces 80% inhibition gave 92% together. So an additive effect is being produced. However, dopamine together with phenylpyruvate did not produce an additive effect because using 2.2. 10^{-3}M phenylpyruvate which produces 46% inhibition with 2.1. 10^{-3}M dopamine only gave a 78% inhibition together. Likewise, phenylethylamine with phenylpyruvate produced no additive effect but methotrexate at saturating concentrations with phenylpyruvate did produce an additive effect. The 83% inhibition usually produced by 4.4. 10^{-4}M methotrexate was increased to 87% when used with 1.1. 10^{-3}M phenylpyruvate which only produces 43% inhibition alone and 90% with 2.2. 10^{-3}M phenylpyruvate which only produces 46% inhibition when used alone.

As can be seen from Figure 5.8, when dihydropteridine reductase is incubated at 37°C with various concentrations of lead acetate before measuring the enzyme activity, there is an immediate inhibition. The degree of inhibition depends on the length of time the enzyme is preincubated with the lead, and the concentration of lead acetate used. Maximum inhibition of the enzyme was obtained by preincubation for 20 minutes. If the enzyme alone is preincubated at 37°C for similar lengths of time there is no significant loss of enzyme activity up to 25 minutes after which the enzyme activity decreases rapidly. (Table 5.16) Table 5.17 and Figure 5.9 show the results of inhibition of dihydropteridine reductase with a range of concentrations of lead acetate where the enzyme

was preincubated with the lead for 20 minutes. An inhibition, significant at the 5% level, was obtained with 10^{-6}M lead acetate. As the concentration of lead acetate was increased the level of inhibition increased up to a maximum of 54% inhibition obtained with 10^{-4}M lead acetate. The same results were obtained if DICPIP was used to generate the quinonoid pterin substrate instead of Horseradish peroxidase and hydrogen peroxide. Tables 5.18 and 5.19 show the results of attempting to remove the lead by dialysis against 0.05M Tris/HCl buffer at pH 7.0 and the same buffer containing 10^{-4}M EDTA. As can be seen very little enzyme activity was regained after dialysis. The control dialyses show that very little enzyme activity is lost because of the dialysis techniques.

The effect of various other inhibitors on dihydropteridine reductase activity after the enzyme had been incubated with 10^{-4}M lead acetate for 20 minutes are shown in Figure 5.10 and Table 5.20. The enzyme still displayed a sigmoidal velocity versus substrate concentration curve when it was inhibited by dopamine in the presence of lead acetate and the inhibition by the lead was additive to that of dopamine (Figure 5.10). The K_i values for both methotrexate and phenylpyruvate decreased if measured on the leaded enzyme so again it appears that the lead acetate inhibition is additive to those of methotrexate and phenylpyruvate. (Table 5.20).

Tables 5.21, 5.22, and 5.23 show results of inhibition of dihydropteridine reductase activity by aluminium sulphate, cadmium chloride and mercuric chloride respectively. 2.10^{-4}M aluminium sulphate gave a significant 30% inhibition and the level of inhibition

FIGURE 5-1 EFFECT OF SODIUM 1,2NAPHTHOQUINONE - 4 - SULPHONATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY AT CONSTANT NADH CONCENTRATIONS

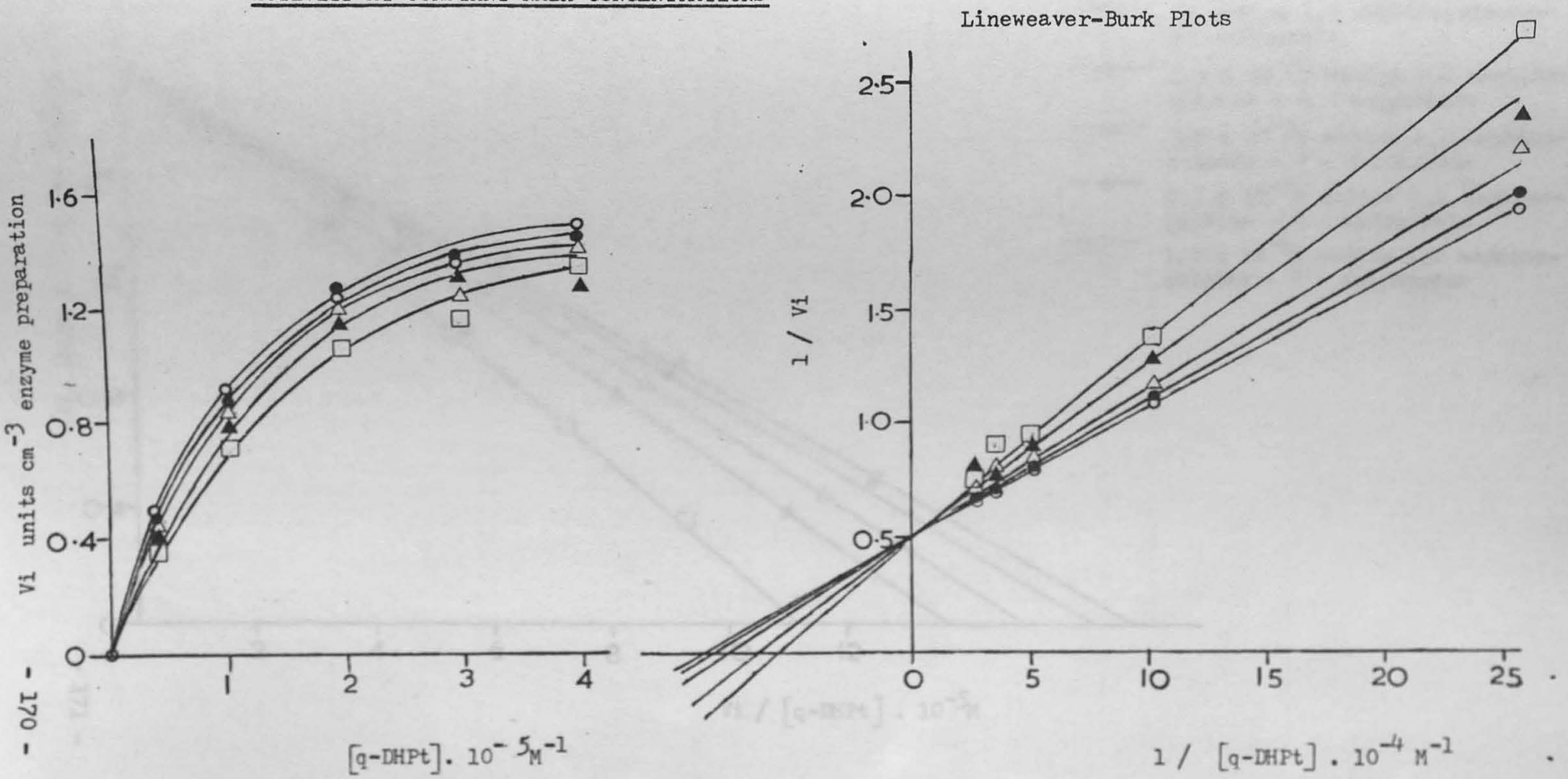


FIGURE 5-1 continued

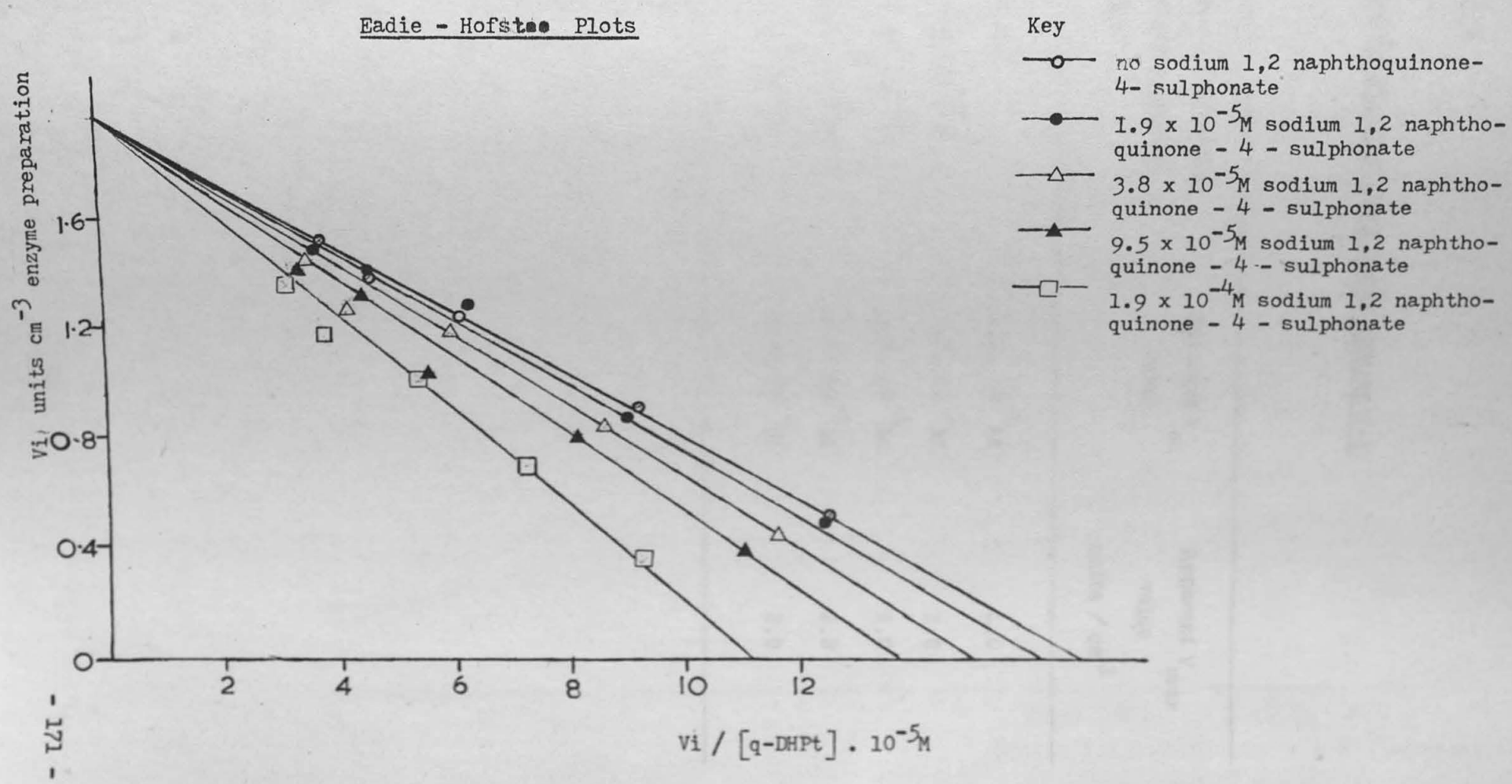


TABLE 5-2

SUMMARY OF KINETIC DATA FROM FIGURE 5-1

	Concentration of sodium 1,2-naphthoquinone-4-sulphonate	Apparent k_m value	Apparent V_{max} value units / cm^3
A.	None	$1.1. 10^{-5}$M	2.0
B.	$1.9. 10^{-5}$M	$1.2. 10^{-5}$M	2.0
C.	$3.8. 10^{-5}$M	$1.3. 10^{-5}$M	2.0
D.	$9.5. 10^{-5}$M	$1.5. 10^{-5}$M	2.0
E.	$1.9. 10^{-4}$M	$1.8. 10^{-5}$M	2.0

FIGURE 5.2 DIXON PLOT TO SHOW THE EFFECT OF SODIUM 1,2 NAPHTHOQUINONE - 4 - SULPHONATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

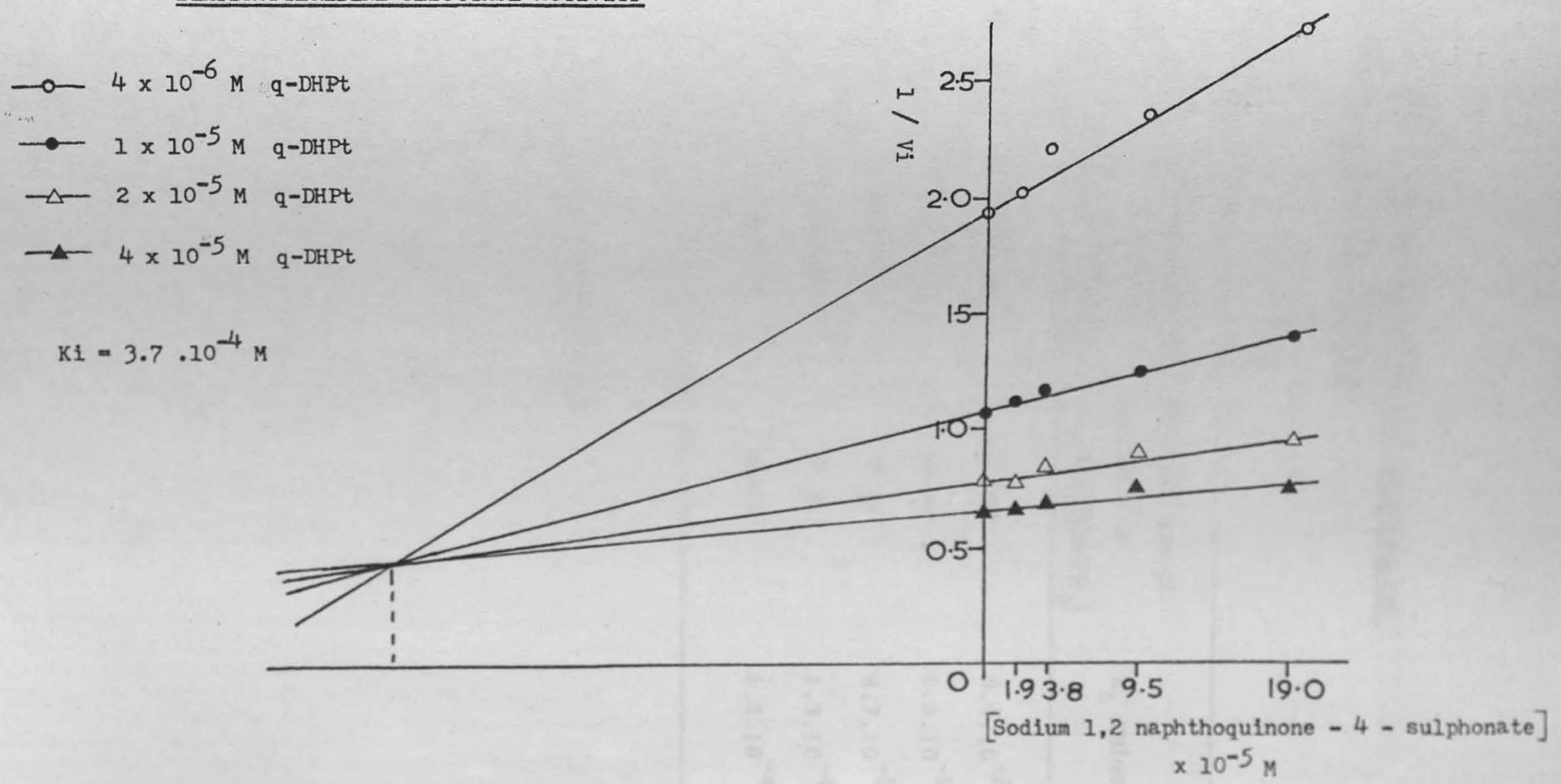


TABLE 5-3

THE EFFECT OF DIHYDROFOLATE REDUCTASE INHIBITORS ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

Inhibitor	Apparent type of inhibition at constant [NADH]	Apparent type of inhibition at constant [$DMPH_4$]	k_i values
Methotrexate	competitive	mixed	$6.4.10^{-5}$ M
Aminopterin	competitive	mixed	$6.5.10^{-5}$ M
Pyrimethamine	competitive	N.D	$8.7.10^{-5}$ M
Tetroxoprim	competitive	N.D	$1.3.10^{-4}$ M
Trimethoprim	competitive	mixed	$5.8.10^{-4}$ M

N.D = not determined .

TABLE 5.4.

THE EFFECT OF TRIMETHOPRIM AND SULPHAMETHOXAZOLE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

Inhibitor and concentration	Enzyme Activity units/mg protein	No. of observations	% Inhibition
None	2.15	12	-
$3.5.10^{-4}$M trimethoprim	2.15	6	0
$6.9.10^{-4}$M trimethoprim	1.33	6	38
$3.9.10^{-4}$M sulphamethoxazole	1.63	6	24
$7.8.10^{-4}$M sulphamethoxazole	1.33	6	38
$1.7.10^{-4}$M trimethoprim + $2.0.10^{-4}$M sulphamethoxazole	1.45	6	33
$3.5.10^{-4}$M trimethoprim + $3.9.10^{-4}$M sulphamethoxazole	1.26	6	40
$3.5.10^{-4}$M trimethoprim + $7.8.10^{-4}$M sulphamethoxazole	1.25	6	42
$6.9.10^{-4}$M trimethoprim + $3.9.10^{-4}$M sulphamethoxazole	1.26	6	42
$6.9.10^{-4}$M trimethoprim + $7.8.10^{-4}$M sulphamethoxazole	1.15	6	46

TABLE 5-5

THE EFFECT OF FOLATES ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

Inhibitor	Apparent type of inhibition at constant [NADH]	Apparent type of inhibition at constant $[DMPH_4]$	k_i value
Folic acid	Competitive	mixed	$7.3.10^{-5}$ M
10 formyl folate	Competitive	mixed	$8.5.10^{-5}$ M
5 methyl tetra--hydrofolate	no effect	no effect	-

TABLE 5-6

THE EFFECT OF AROMATIC AMINO ACIDS AND PHENYLALANINE METABOLITES ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

Inhibitor	Apparent type of inhibition at constant [NADH]	Apparent type of inhibition at constant [$DMPH_4$]	k_i value
Tryptophan	mixed	mixed	$2.0.10^{-4}$ M
Tyrosine	non-competitive	mixed	$3.2.10^{-4}$ M
Phenylalanine	mixed	mixed	$1.0.10^{-3}$ M
Phenyllactate	mixed	mixed	$1.0.10^{-3}$ M
Phenylpyruvate	mixed	mixed	$5.0.10^{-5}$ M
o-hydroxyphenyl--acetic acid	mixed	N.D	$9.6.10^{-4}$ M
Phenylacetic acid	mixed	N.D	$9.8.10^{-4}$ M
Phenylethylamine	mixed	mixed	$8.5.10^{-4}$ M
Tyramine	non-competitive	N.D	$1.8.10^{-5}$ M

N.D = not determined

TABLE 5-7

THE EFFECT OF PHENYLPYRUVATE ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

All measurements were made with 10^{-4}M NADH and 10^{-5}M $DMPH_4$

Concentration of Phenylpyruvate	Enzyme Activity units/mg protein	% inhibition	Student t test results
none	2.11	-	-
$6.1.\ 10^{-6}$M	2.00	5	P <0.10
$1.1.\ 10^{-5}$M	1.92	9	P <0.10
$2.7.\ 10^{-5}$M	1.77	16	P <0.01
$5.4.\ 10^{-5}$M	1.69	20	P <0.005
$1.1.\ 10^{-4}$M	1.60	24	P <0.005
$2.7.\ 10^{-4}$M	1.31	38	P <0.001
$5.4.\ 10^{-4}$M	1.10	48	P <0.001
$1.1.\ 10^{-3}$M	1.06	50	P <0.001

TABLE 5-8

THE SPECIFIC ACTIVITIES OF THE DIHYDROPTERIDINE REDUCTASE AND 'PHENYLPYRUVATE-REDUCING-ENZYME' AT DIFFERENT STAGES OF PURIFICATION

	Specific Activity of Dihydropteridine Reductase μmoles NADH/min/ mg protein	Specific Activity of 'phenylpyruvate-reducing-enzyme' μmoles NADH/min/ mg protein
Supernatant	0.019	0.0074
Sephadex G-150 active fractions	0.103	0.015
Affinity chromatography active fractions	2.03	0.096

TABLE 5-9

THE EFFECT OF CATECHOLAMINES AND SEROTONIN ON DIHYDROPTERIDINE REDUCTASE ACTIVITY

Inhibitor	Apparent type of inhibition at constant [NADH]	Apparent type of inhibition at constant $[DMPH_4]$	k_i value
Noradrenaline	non-competitive	mixed	$4.9.10^{-4}$ M
Adrenaline	no effect	no effect	-
L-DOPA	no effect	no effect	-
Serotonin	competitive	mixed	$1.2.10^{-3}$ M

FIGURE 5-3 INITIAL VELOCITY VERSUS $DMPH_4$ CONCENTRATION PLOTS TO SHOW THE EFFECT OF DOPAMINE CONCENTRATIONS ON DHPR USING PEROXIDASE AND HYDROGEN PEROXIDE TO GENERATE THE QUINONOID SUBSTRATE

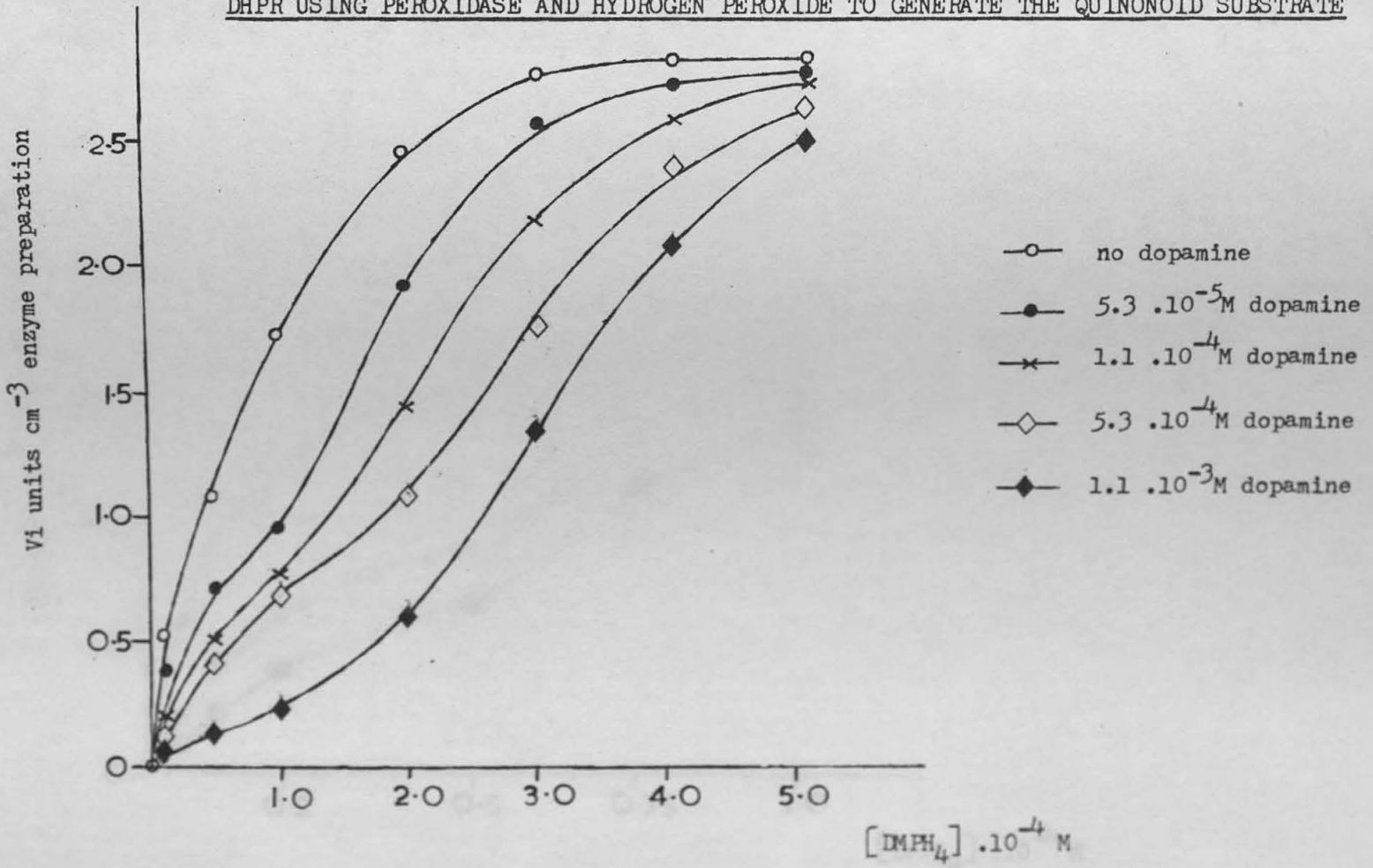
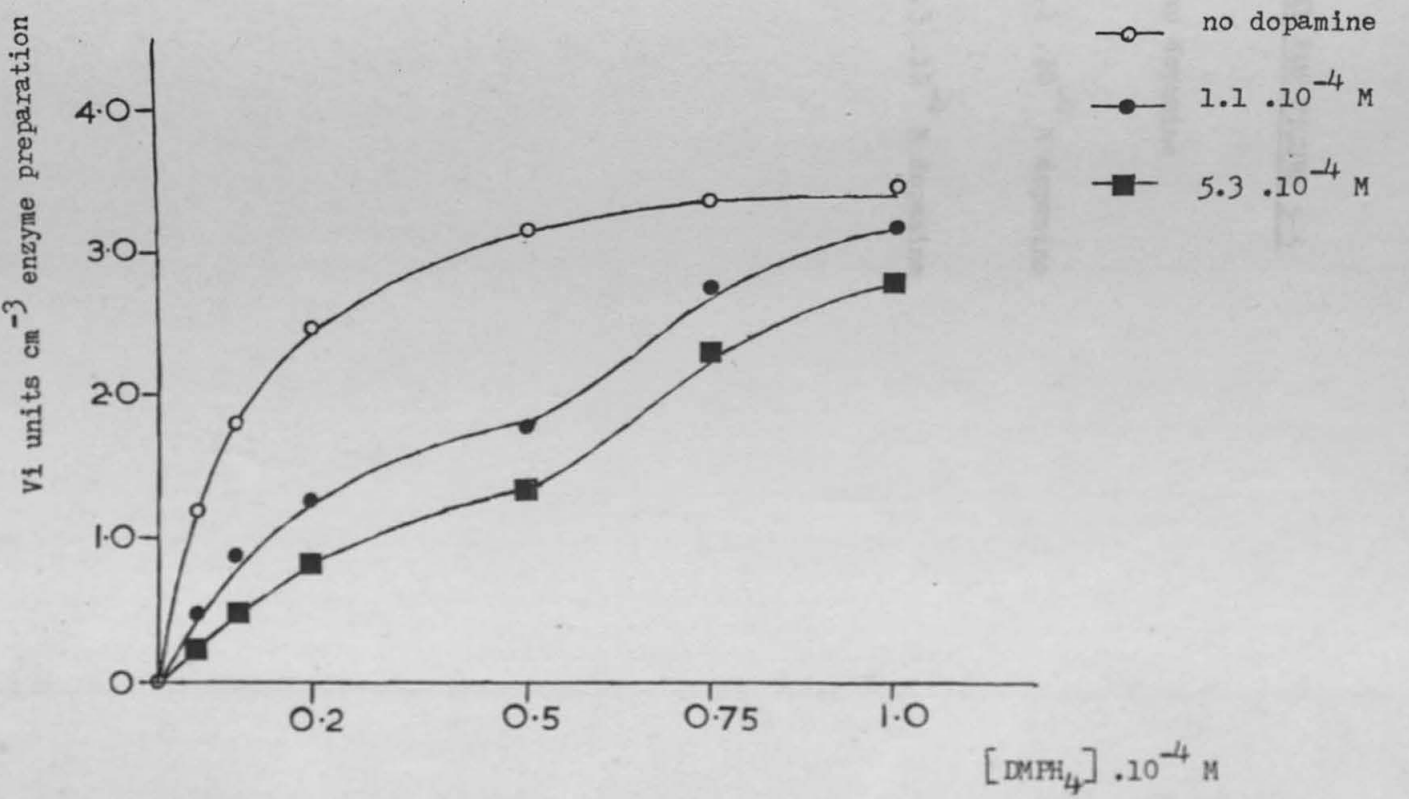
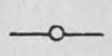


FIGURE 5-4 INITIAL VELOCITY VERSUS $DMPH_4$ CONCENTRATION PLOTS TO SHOW THE EFFECT OF DOPAMINE ON DHPR ACTIVITY USING DICPIP TO GENERATE THE QUINONOID SUBSTRATE

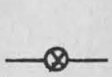


KEY FOR FIGURE 5-5



no dopamine

$1.1 \cdot 10^{-4}$ M dopamine



$5.3 \cdot 10^{-4}$ M dopamine

FIGURE 5-5 INITIAL VELOCITY VERSUS NADH CONCENTRATION PLOTS TO SHOW THE EFFECT OF DOPAMINE ON DHPR ACTIVITY

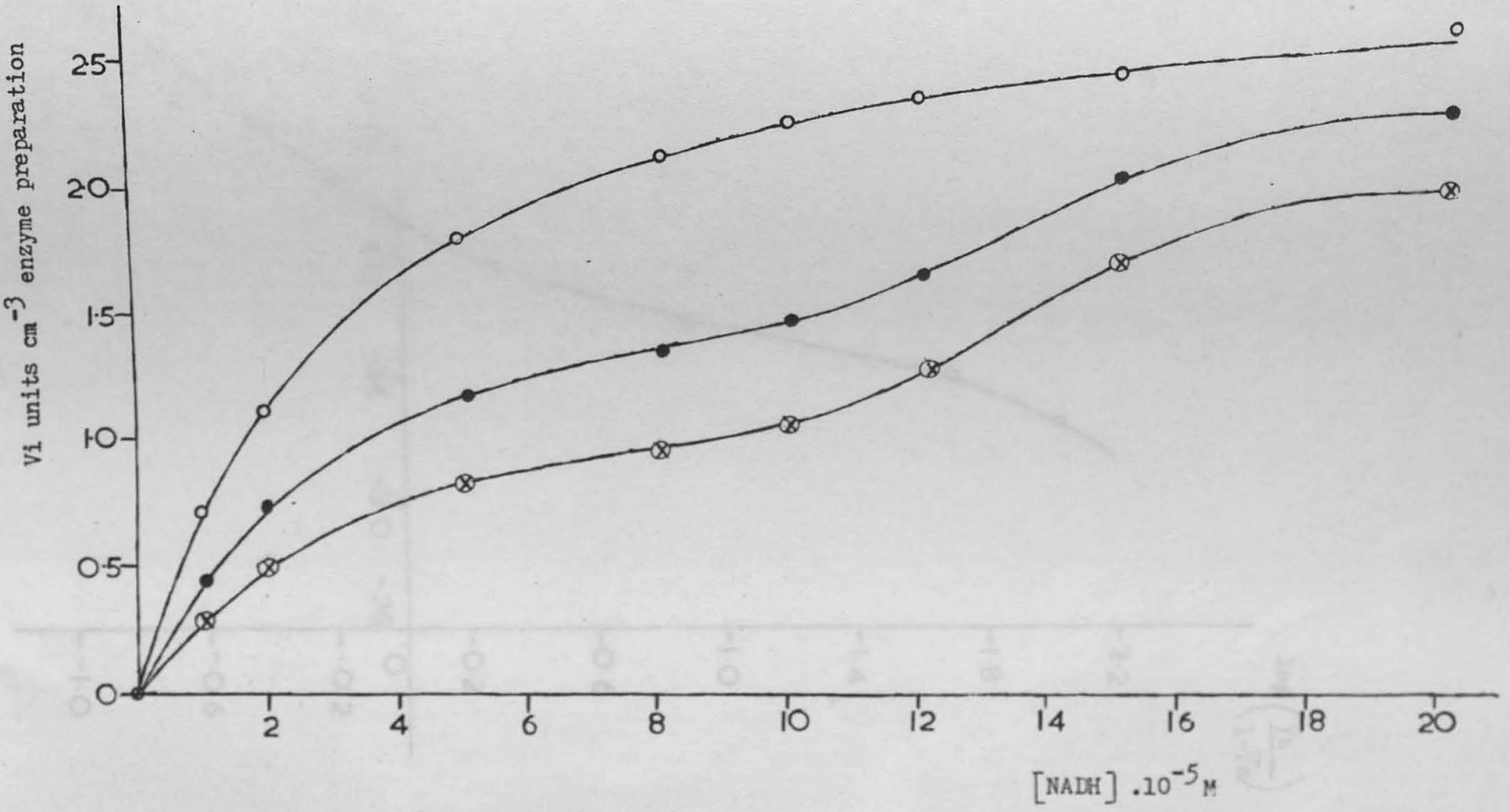


FIGURE 5-6 HILL PLOT FOR DIHYDROPTERIDINE REDUCTASE

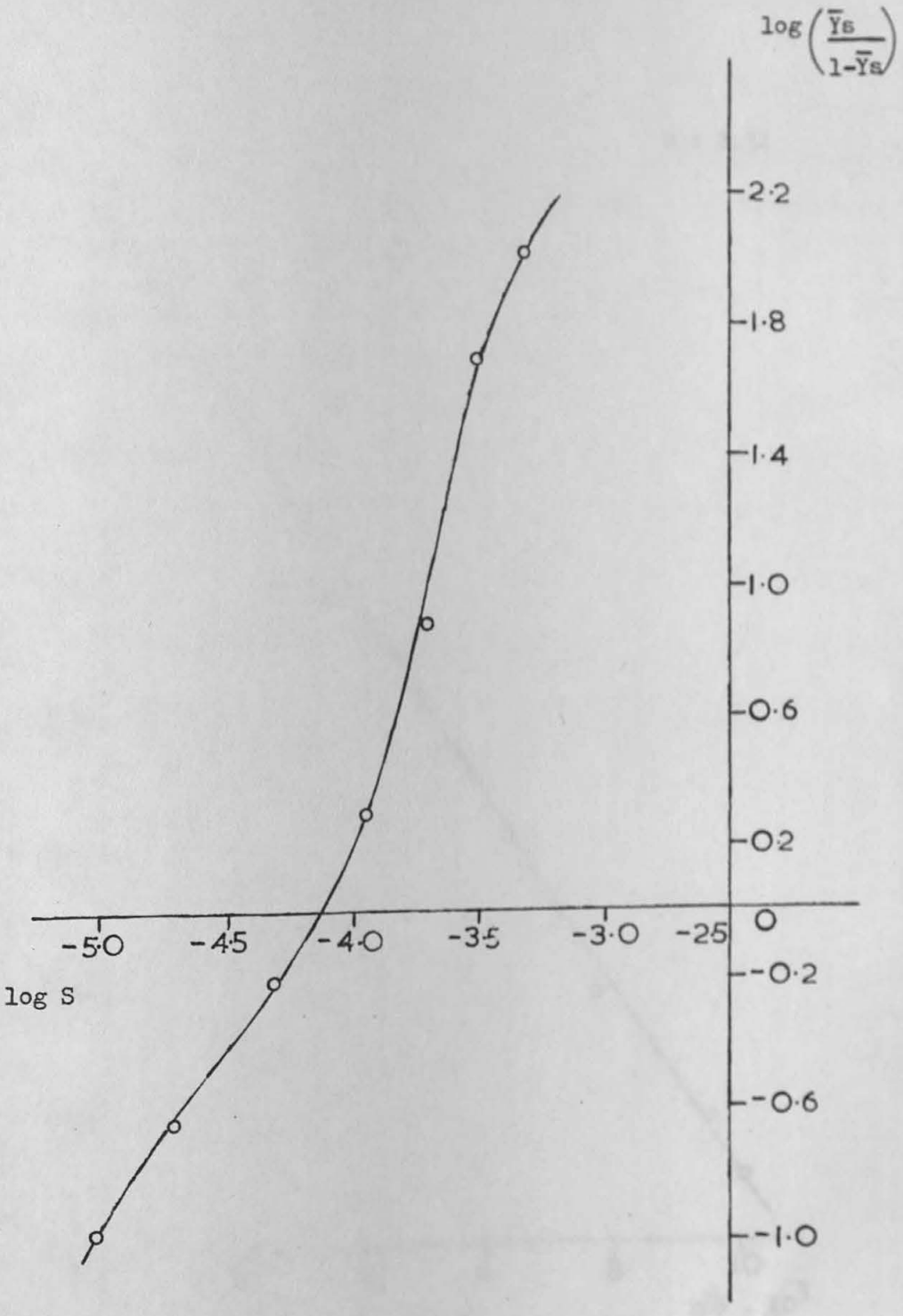


FIGURE 5-7 SCATCHARD PLOT FOR DIHYDROPTERIDINE REDUCTASE

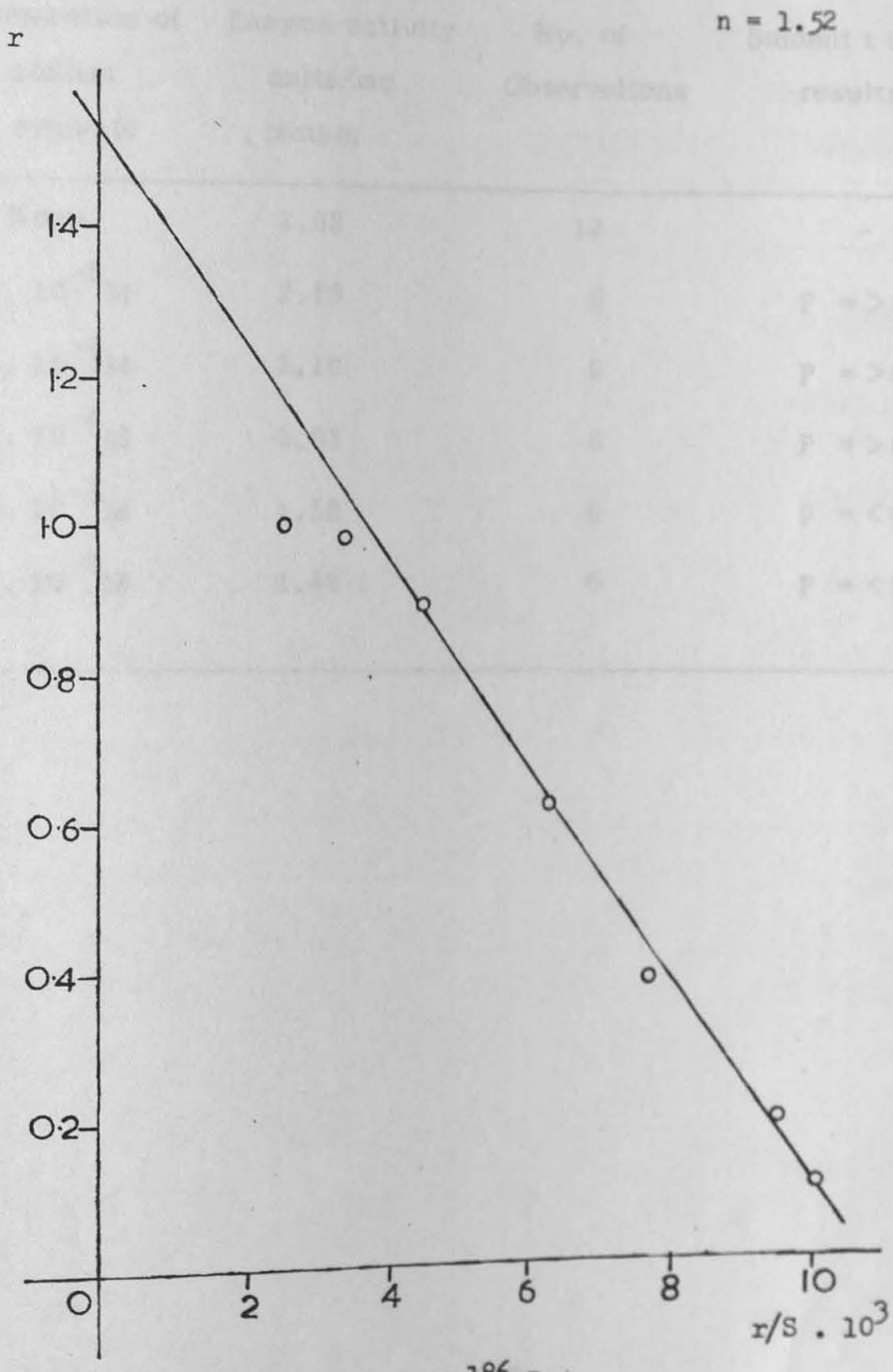


TABLE 5-10

EFFECT OF SODIUM PYRUVATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

All measurements were made with 10^{-4}M NADH and 10^{-5}M $DMPH_4$

Concentration of sodium pyruvate	Enzyme activity units/mg protein	No. of Observations	Student t test results
None	2.05	12	-
$9.1.\ 10^{-5}$M	2.13	6	P =>0.1
$4.6.\ 10^{-4}$M	2.10	6	P =>0.1
$9.1.\ 10^{-4}$M	2.03	6	P =>0.1
$1.8.\ 10^{-3}$M	1.58	6	P =<0.05
$2.7.\ 10^{-3}$M	1.48	6	P =<0.01

TABLE 5-11

EFFECT OF DIACETYL ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

All measurements were made with 10^{-4}M NADH and 10^{-5}M $DMPH_4$

Concentration of diacetyl	Enzyme activity units/mg protein	No. of Observations	Student t test results
None	2.05	12	P =>0.1
$2.3.\ 10^{-3}$M	1.95	6	P =>0.1
$5.8.\ 10^{-3}$M	1.95	6	P =>0.1
$1.2.\ 10^{-2}$M	2.01	6	P =>0.1
$2.3.\ 10^{-2}$M	1.91	6	P =<0.1
$3.5.\ 10^{-2}$M	0.68	6	P =<0.01

TABLE 5-12

EFFECT OF DOPAMINE ANDMETHOTREXATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

All estimations were with 10^{-4}M NADH and 10^{-5}M $DMPH_4$

Inhibitor and concentration	Enzyme Activity units/mg protein	No. of observations	% inhibition
None	2.93	12	-
1.1. 10^{-3}M Dopamine	1.03	6	65
1.6. 10^{-3}M Dopamine	0.60	6	79
2.1. 10^{-3}M Dopamine	0.59	6	80
3.3. 10^{-3}M Methotrexate	0.47	6	84
4.4. 10^{-3}M Methotrexate	0.50	6	83
1.1. 10^{-3}M Dopamine + 4.4. 10^{-3}M Methotrexate	0.35	6	88
2.1. 10^{-3}M Dopamine + 4.4. 10^{-3}M Methotrexate	0.24	6	92

TABLE 5-13

EFFECT OF DOPAMINE AND PHENYLPYRUVATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

All estimations were with 10^{-4}M NADH and 10^{-5}M $DMPH_4$.

Inhibitor and concentration	Enzyme Activity units/mg protein	No. of observations	% inhibition
None	2.93	12	-
$1.6. 10^{-3}$M phenylpyruvate	1.64	6	44
$2.2. 10^{-3}$M phenylpyruvate	1.58	6	46
$1.6. 10^{-3}$M dopamine	0.60	6	79
$2.1. 10^{-3}$M dopamine	0.59	6	80
$1.1. 10^{-3}$ phenylpyruvate + $2.1. 10^{-3}$M dopamine	0.62	6	79
$2.2. 10^{-3}$M phenylpyruvate + $2.1. 10^{-3}$M dopamine	0.63	6	78

TABLE 5-14

EFFECT OF METHOTREXATE AND PHENYLPYRUVATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

All estimations were with 10^{-4}M NADH and 10^{-5}M $DMPH_4$

Inhibitor and concentration	Enzyme Activity units/mg protein	No. of observations	% Inhibition
None	2.77	12	-
2.2. 10^{-4}M Methotrexate	0.67	6	76
3.3. 10^{-4}M Methotrexate	0.48	6	83
4.4. 10^{-4}M Methotrexate	0.47	6	83
1.1. 10^{-3}M phenylpyruvate	1.73	6	43
2.2.10^{-3}M phenylpyruvate	1.50	6	46
1.1. 10^{-3}M phenylpyruvate + 4.4. 10^{-4}M Methotrexate	0.37	6	87
2.2. 10^{-3}M phenylpyruvate + 4.4. 10^{-4}M Methotrexate	0.28	6	90

TABLE 5-15

EFFECT OF PHENYLETHYLAMINE AND PHENYLPYRUVATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

Inhibitor and concentration	Enzyme Activity units/mg protein	No. of observations	% Inhibition
None	2.77	12	-
$1.3.10^{-4}$M phenylethylamine	2.02	6	27
$6.5.10^{-4}$M phenylethylamine	1.77	6	36
$1.3.10^{-3}$M phenylethylamine	1.66	6	40
$2.0.10^{-3}$M phenylethylamine	1.69	6	39
$2.7.10^{-4}$M phenylpyruvate	1.72	6	38
$1.3.10^{-4}$M phenylethylamine + $2.7.10^{-4}$M phenylpyruvate	1.69	6	39
$2.0.10^{-3}$M phenylethylamine + $2.7.10^{-4}$M phenylpyruvate	1.75	6	37

FIGURE 5-8 THE EFFECT OF PREINCUBATION OF THE ENZYME WITH LEAD ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

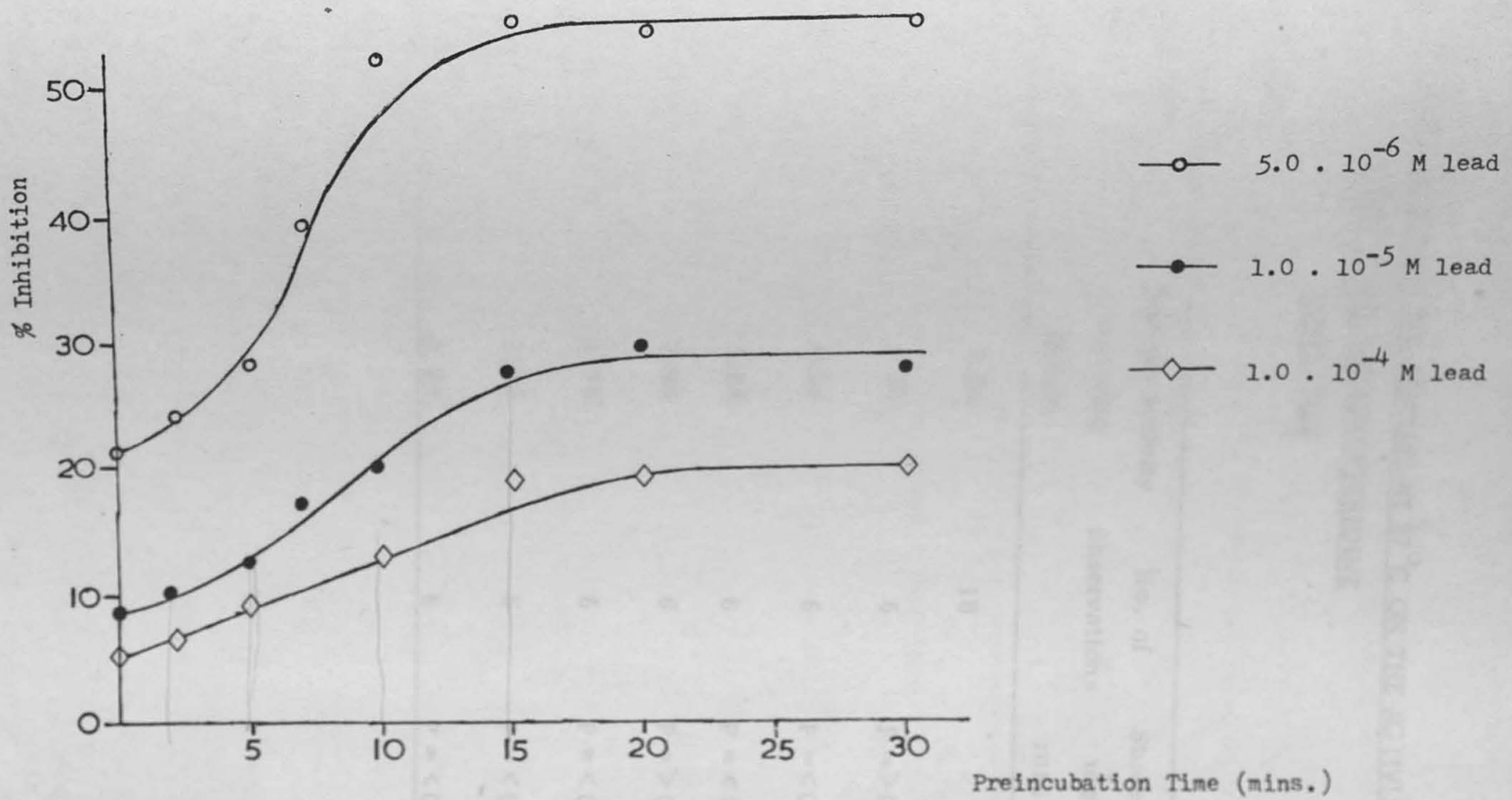


TABLE 5-16

EFFECT OF PREINCUBATION OF THE ENZYME AT 37°C ON THE ACTIVITY OF RAT LIVER DIHYDROPTERIDINE REDUCTASE

Preincubation time (mins)	Enzyme Activity units/mg protein	No. of observations	Student t test results
0	3.85	18	-
5	4.05	6	P =>0.1
10	4.05	6	P =<0.1
15	3.88	6	P =<0.1
20	3.40	6	P =>0.1
25	2.73	6	P =<0.05
30	1.20	6	P =<0.01
35	0.83	6	P =<0.005

TABLE 5-17

EFFECT OF LEAD ACETATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

The enzyme was preincubated with the lead acetate for 20 minutes before the enzyme activity was assayed. All estimations were with 10^{-4}M NADH and 10^{-4}M $DMPH_4$

Concentration of lead acetate	Enzyme activity units/mg protein	No. of Observations	% Inhibition	Student t test results
None	3.85	18	-	-
$5.0.\ 10^{-8}$M	3.73	6	3	P = >0.1
$1.0.\ 10^{-7}$M	3.58	6	7	P = >0.1
$5.0.\ 10^{-7}$M	3.46	6	10	P = <0.1
$1.0.\ 10^{-6}$M	3.31	12	14	P = <0.05
$5.0.\ 10^{-6}$M	3.00	12	22	P = <0.05
$1.0.\ 10^{-5}$M	2.77	12	28	P = <0.01
$5.0.\ 10^{-5}$M	2.08	12	46	P = <0.01
$1.0.\ 10^{-4}$M	1.77	12	54	P = <0.005
$5.0.\ 10^{-4}$M	1.81	12	53	P = <0.005
$1.0.\ 10^{-3}$M	1.73	6	55	P = <0.005
$1.0.\ 10^{-2}$M	1.77	6	54	P = <0.005

FIGURE 5-9 THE EFFECT OF LEAD ACETATE CONCENTRATION ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

The enzyme was preincubated with the lead acetate for 20 minutes before its activity was measured.

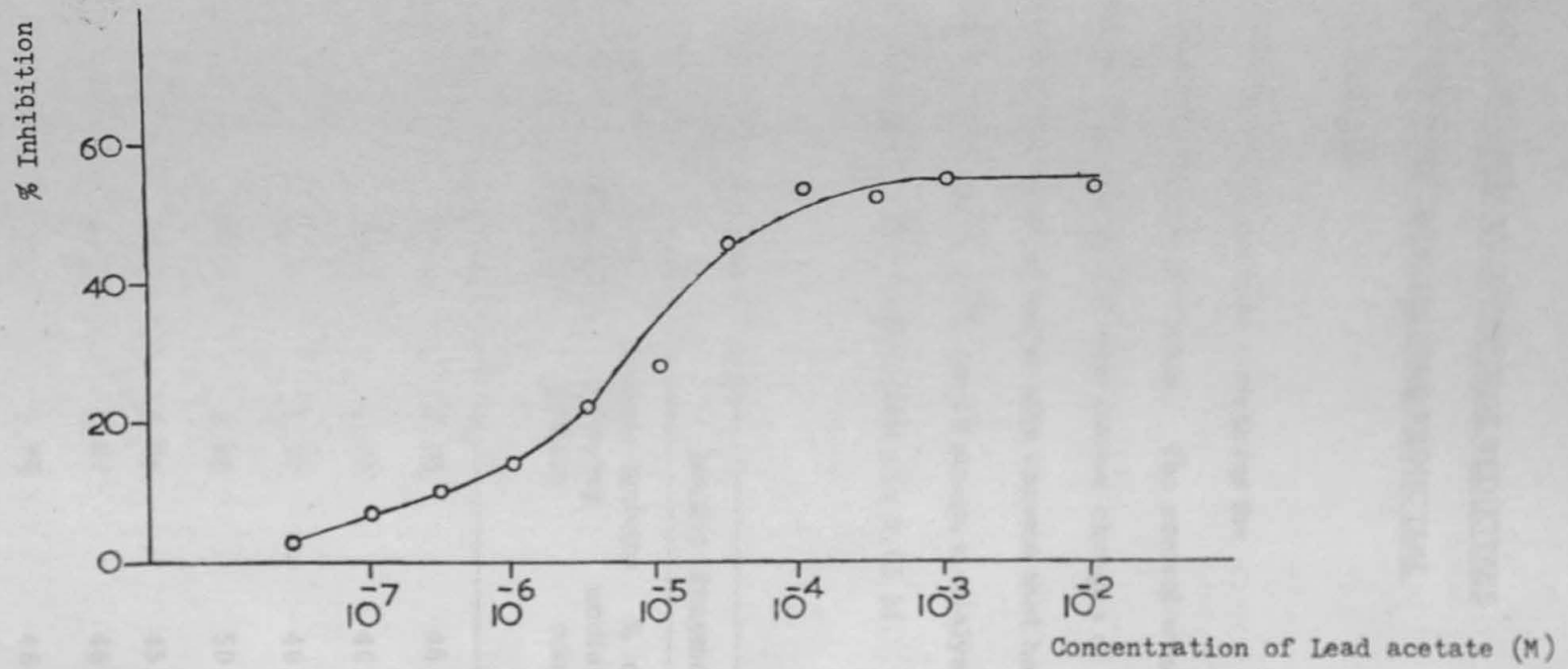


TABLE 5-18

EFFECT OF DIALYSIS ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY AND LEADED RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

6 observations were made for each dialysis time except for the undialysed control where 18 observations were made. The control was a mixture of buffer and enzyme dialysed at 4°C with twelve changes of buffer. The leaded enzyme was a mixture of buffer with enzyme that had been incubated with 10^{-4} M lead acetate at 37°C for 20 minutes, dialysed at 4°C with twelve changes of buffer. The buffer used was 0.05 M Tris/HCl pH 7.

Dialysis Time hrs	Control		Leaded Enzyme	
	Enzyme activity units/mg protein	% of undialysed control	Enzyme activity units/mg protein	% of undialysed control
0 (undialysed)	3.85	100	1.75	45.5
1	3.81	99.0	1.77	46.0
2	3.86	100	1.77	46.0
3	3.73	96.9	1.96	50.9
5	3.62	94.0	1.76	45.7
8	3.58	93.0	1.77	46.0
24	3.60	93.5	1.79	46.5
48	3.57	92.7	1.82	47.3
72	3.59	93.3	1.76	45.7

TABLE 5-19

EFFECT OF DIALYSIS IN EDTA BUFFER ON DIHYROPTERIDINE REDUCTASE ACTIVITY AND LEADED DIHYDROPTERIDINE REDUCTASE ACTIVITY

Dialysis Time hrs	Control		Leaded Enzyme	
	Enzyme Activity units/mg protein	% of undialysed control	Enzyme activity units/mg protein	% of undialysed control
0 (undialysed)	3.85	100	1.75	45.5
1	3.88	100.8	2.00	51.9
2	3.81	99.0	1.77	46.0
3	3.77	97.9	1.85	48.1
5	3.73	96.9	1.88	49.0
8	3.54	92.0	1.81	47.0
24	3.58	93.0	1.85	48.1
48	3.58	93.0	1.81	47.0
72	3.56	92.5	1.83	47.5

FIGURE 5-10 EFFECT OF DOPAMINE AND LEAD ACETATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

For the studies with lead the enzyme was preincubated with the metal for 20 mins. before the enzyme activity was measured.

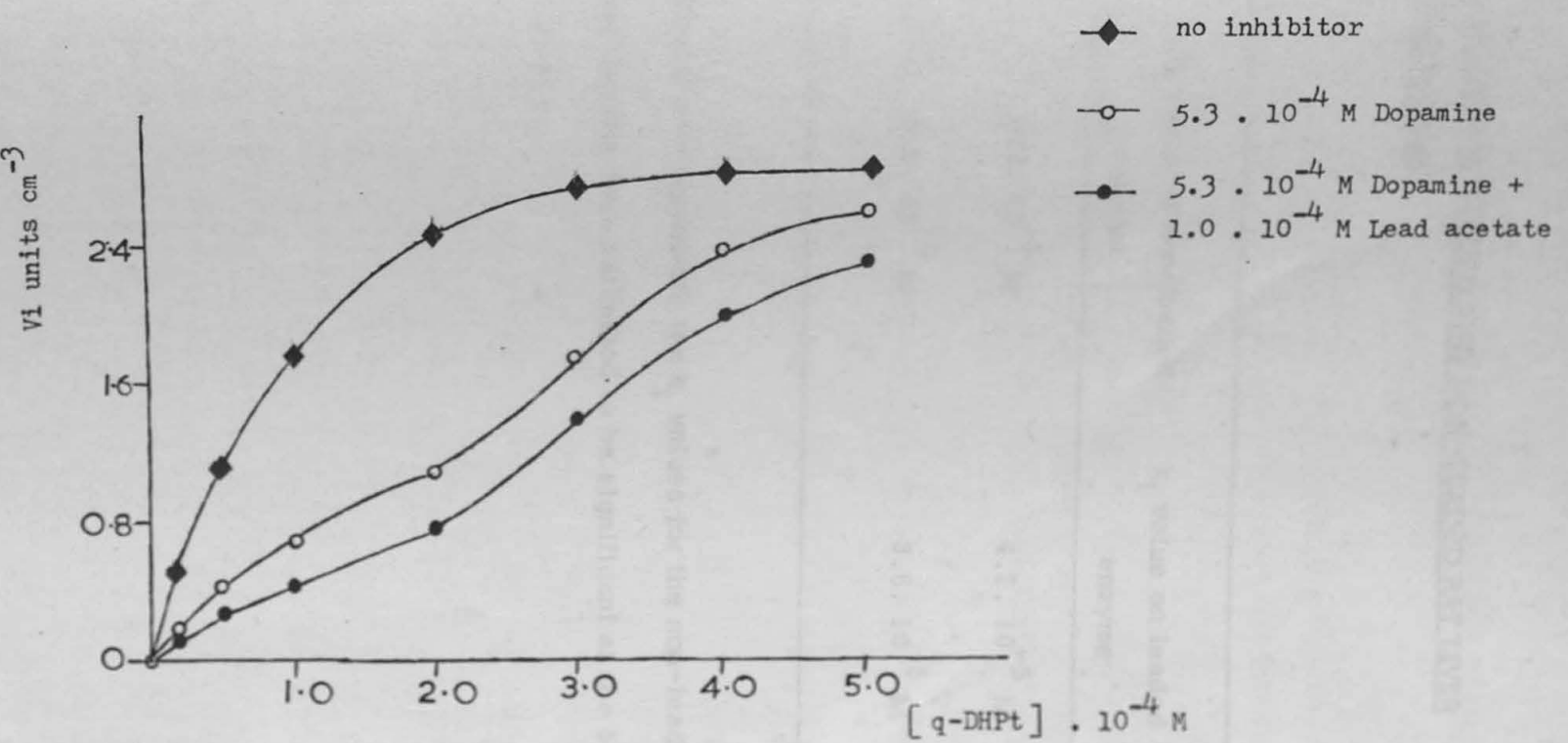


TABLE 5-20

COMPARISON OF k_i VALUES ON LEADED AND NON-LEADED RAT LIVER DIHYDROPTERIDINE REDUCTASE

Inhibitor	k_i value on non-leaded enzyme	k_i value on leaded enzyme
Methotrexate	$6.4.\ 10^{-5}$ M	$4.2.\ 10^{-5}$ M
Phenylpyruvate	$5.0.\ 10^{-5}$ M	$3.8.\ 10^{-5}$ M

For both methotrexate and phenylpyruvate the k_i values for the non-leaded enzyme and the leaded enzyme were estimated to be significant at the 5% level (i.e. $P = < 0.05$).

TABLE 5-21

EFFECT OF ALUMINIUM SULPHATE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

The enzyme was preincubated at 37°C with the aluminium sulphate for 20 minutes before the enzyme activity was assayed. All estimations were with 10^{-4}M NADH and 10^{-4}M $DMPH_4$

Concentration of Aluminium sulphate	No. of Observations	Enzyme Activity units/mg protein	% Inhibition	Student t test results
None	12	4.25	-	-
$2.0\,10^{-6}$M	6	4.00	6	P =>0.1
$2.0.10^{-5}$M	6	3.50	18	P = <0.1
$2.0.10^{-4}$M	6	3.00	30	P =<0.05
$2.0.10^{-3}$M	6	2.35	45	P =<0.01
$2.0.10^{-2}$M	6	2.00	53	P =<0.01
$2.0.10^{-1}$M	6	1.90	55	P =<0.005

TABLE 5-22

EFFECT OF CADMIUM CHLORIDE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

The enzyme was preincubated at 37°C with the cadmium chloride for 20 minutes before the enzyme activity was assayed. All estimations were with 10^{-4}M NADH and 10^{-4}M $DMPH_4$

Concentration of Cadmium Chloride	No. of Observations	Enzyme Activity units/mg protein	% Inhibition	Student t test results
None	6	4.62	-	-
$1.0.\ 10^{-6}$M	6	4.61	0	P =>0.1
$5.0.\ 10^{-6}$M	6	4.63	0	P =>0.1
$1.0.\ 10^{-5}$M	6	4.57	1	P = <0.1
$5.0.\ 10^{-5}$M	6	3.97	14	P = <0.05
$1.0.\ 10^{-4}$M	6	3.19	31	P = <0.05
$5.0.\ 10^{-4}$M	6	2.31	50	P = <0.01
$1.0.\ 10^{-3}$M	6	1.99	57	P = <0.01

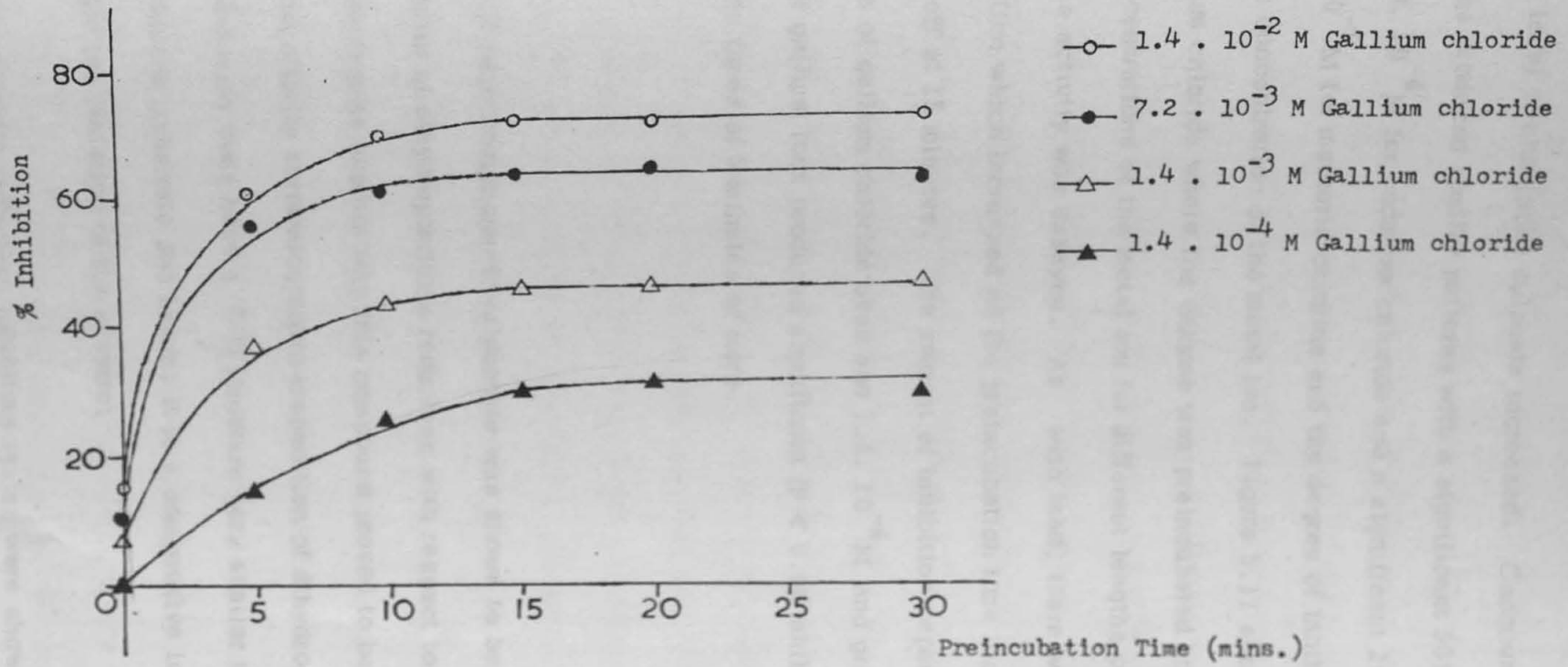
TABLE 5-23

EFFECT OF MERCURIC CHLORIDE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY

The enzyme was preincubated with the mercuric chloride at 37°C for 20 minutes before the enzyme activity was assayed. All estimations were with 10^{-4}M NADH and 10^{-4}M $DMPH_4$

Concentration of Mercuric Chloride	No. of Observations	Enzyme Activity units/mg protein	% Inhibition	Student t test results
None	12	4.25	-	-
$2.0.\ 10^{-6}$M	6	4.08	4	P =>0.1
$2.0.\ 10^{-5}$M	6	3.15	26	P =<0.05
$2.0.\ 10^{-4}$M	6	1.91	55	P =<0.01
$2.0.\ 10^{-3}$M	6	1.62	62	P =<0.01

FIGURE 5-11 THE EFFECT OF PREINCUBATION OF THE ENZYME WITH GALLIUM CHLORIDE ON RAT LIVER DIHYDROPTERIDINE REDUCTASE ACTIVITY



increased as the level of aluminium sulphate increased. Cadmium and mercuric chlorides produced similar patterns with a significant 50% inhibition at $5.0.\ 10^{-4}$M for cadmium chloride and a significant 26% inhibition at 2.10^{-5}M for mercuric chloride and the degree of inhibition depending on the concentration of the metal ion. Figure 5.11 shows the result with gallium chloride where the enzyme was preincubated at 37°C with various concentrations of the metal ion for different lengths of time before the enzyme activity was assayed. As with lead, there was an immediate inhibition which increased as the preincubation time increased up to a levelling off at 15 minutes. The amount of inhibition depended on the concentration of gallium chloride used and $1.4.\ 10^{-4}$M and greater concentrations of gallium ions produced significant ($P < 0.01$) inhibitions with preincubation times of 5 minutes or more.

DISCUSSION

Sodium 1,2 naphthoquinone-4-sulphonate was shown to be a competitive inhibitor of dihydropteridine reductase with respect to the pterin substrate. These results explain why this compound proved to be such a good ligand for the affinity chromatographic preparation of dihydropteridine reductase. It obviously does have a 3-D structure very similar to the quinonoid dihydropterin substrate and hence, it is a competitive inhibitor and a good tool for the isolation of the enzyme.

All the dihydrofolate reductase inhibitors tested were shown to be

inhibitors of dihydropteridine reductase which were competitive with the pterin substrate and showed mixed inhibition with respect to NADH. The apparent K_i value for methotrexate was $6.4. 10^{-5}$M which is of similar magnitude to the $3.8. 10^{-5}$M obtained by Craine et al., who also stated that the inhibition was competitive with respect to quinonoid dihydrobiopterin. (Craine, Hall and Kaufman 1972) Chauvin et al., found methotrexate to have a K_i value of $7.0. 10^{-5}$M but to be non-competitive with respect to the pterin. (Chauvin, Korri, Tirpak, Simpson and Scrimgeour 1979) The increasing inhibitor potency of the dihydrofolate reductase inhibitors tested for dihydropteridine reductase inhibition is as follows :- trimethoprim; tetroxoprim; pyrimethamine; aminopterin and methotrexate. This order of potency is the same as that for these inhibitors for inhibition of rat dihydrofolate reductase. (Burchall 1979) The apparent K_i value for aminopterin was $6.5. 10^{-5}$M which can be favourably compared with that obtained by Cheema et al., of $2.0. 10^{-5}$M however, they found aminopterin to be non-competitive with respect to the pterin substrate. (Cheema, Soldin, Knapp, Kaufman and Scrimgeour 1973) The apparent K_i values for pyrimethamine and trimethoprim were $8.7. 10^{-5}$M and $5.8. 10^{-4}$M respectively whereas, Cheema et al., could not detect inhibition of dihydropteridine reductase by either of these drugs even at concentrations as high as 10^{-3}M. (Cheema, Soldin, Knapp, Hofmannand Scrimgeour 1973) It seems that dihydrofolate reductase inhibitors do in fact inhibit dihydropteridine reductase but the concentrations at which they are effective are much higher than those needed for dihydrofolate reductase inhibition. (Burchall 1979, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973)

Sulphamethoxazole also inhibits dihydropteridine reductase being a competitive inhibitor with respect to NADH and a mixed inhibitor with respect to the pterin. It has an apparent K_i value of $8.7. 10^{-4}$M. Sulphamethoxazole has also been shown to be an inhibitor of E. coli dihydrofolate reductase being competitive with the substrate, dihydrofolate and having a K_i value of $2.0. 10^{-3}$M. (Poe 1976) The literature appears controversial as to the mechanism by which sulphamethoxazole and trimethoprim produce a synergistic inhibitory effect on E. coli. Poe argues in favour of the synergism of these two drugs due to simultaneous binding to dihydrofolate reductase, (Poe 1976, Poe 1977, Lacey 1979) whereas Burchall and Then are in favour of a sequential blockade of dihydrofolate reductase by trimethoprim and dihydropteroate synthetase by sulphamethoxazole as being the mechanism of synergism. (Burchall 1977, Burchall 1979, Then 1977) The results shown here do seem to suggest that trimethoprim and sulphamethoxazole have a synergistic effect on dihydropteridine reductase and because they demonstrate competitive inhibition to the different substrates, trimethoprim to the pterin and sulphamethoxazole to NADH, it is possible to envisage that this is produced by simultaneous binding to the enzyme.

5-methyltetrahydrofolic acid had no effect on dihydropteridine reductase whereas folic acid and 10-formylfolate both inhibited the enzyme competitively with respect to the pterin and with mixed inhibition with respect to NADH with apparent K_i values of $7.3. 10^{-5}$M and $8.5. 10^{-5}$M respectively. These results agree with those of Leeming on dihydropteridine

reductase whereas the unreduced forms inhibited the enzyme. (Leeming 1979) Cheema et al., found folic acid to be a non-competitive inhibitor of dihydropteridine reductase with an apparent K_i value of $2.4. 10^{-4}$M which is greater than the value obtained here by about 3 fold. (1973)

The metabolites of phenylalanine and phenylalanine itself were all shown to be inhibitors of dihydropteridine reductase as were tryptophan and tyrosine (Chapter 5). These results conflict with those of Leeming (1979) which showed phenylalanine and ortho-hydroxyphenylacetic acid to have no effect on dihydropteridine reductase and phenylpyruvate to stimulate the enzyme activity. It seems likely that this stimulation was in fact due to the same 'phenylpyruvate-reducing' activity that was obtained here with unpurified enzyme extracts as only rat brain supernatant extracts were used in Leeming's study. (1979) Of the phenylalanine metabolites the most potent inhibitors were phenylpyruvate and tyramine with apparent K_i values of $5.0. 10^{-5}$M and $1.8. 10^{-5}$M respectively. Inhibition of dihydropteridine reductase by phenylalanine and its metabolites such as phenylpyruvate, tyramine, phenylethylamine could result in raised levels of dihydrobiopterin in the cell due to increased non-enzymatic rearrangement from quinonoid dihydrobiopterin and hence raised serum dihydrobiopterin levels. It is possible that phenylalanine and its metabolites therefore are the agents responsible for the recorded rise in serum dihydrobiopterin seen in hyperphenylalaninaemia. (Leeming 1979, Leeming, Blair, Green and Raine 1974)

Adrenaline and L-DOPA had no effect on dihydropteridine reductase but the neurotransmitters noradrenaline, dopamine and serotonin all

inhibited it. Noradrenaline and serotonin had apparent K_i values of $4.9. 10^{-4}$M and $1.2. 10^{-3}$M respectively. So noradrenaline is inhibiting at concentrations which suggest that it may be involved in a feedback inhibition on dihydropteridine reductase in order to control its own production because dihydropteridine reductase is responsible for tetrahydrobiopterin production (Craine, Hall and Kaufman 1972) and tetrahydrobiopterin is rate-limiting for the first enzymatic step for noradrenaline biosynthesis. (Kettler, Bartholini and Pletscher 1974, Levitt, Spector, Sjoerdsman and Udenfriend 1965, Musacchio, D'Angelo, and McQueen 1971) Dopamine gave sigmoid velocity versus substrate concentration curves (both for pterin and NADH substrates) at all concentrations of dopamine used and whatever the system for the generation of the quinonoid substrate used. These sigmoid curves prompted the plotting of a Hill plot to see if the enzyme, which is known to be a subunit enzyme (Craine, Hall and Kaufman 1972, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Webber, Deits, Snyder and Whiteley 1978, Hasegawa 1977) demonstrated any co-operativity. The Hill plot did in fact demonstrate a positive co-operative effect. This implies that binding of the substrate increases the affinity of the enzyme for subsequent molecules of the substrate (i.e. a homotropic effect). The effect of dopamine would presumably be a heterotropic effect of co-operativity where the binding of the dopamine decreases the affinity of the enzyme for subsequent molecules of the substrate, and hence causes an inhibition. A Scatchard plot confirmed that each molecule of enzyme had more than one substrate binding site, in fact, that each molecule of enzyme had two substrate

binding sites which fits the data which shows that each molecule of dihydropteridine reductase to be a dimer. (Craine, Hall and Kaufman 1972, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Webber, Deits, Snyder and Whiteley 1978) The inhibition of dihydropteridine reductase by dopamine occurred at concentrations which suggest that it is involved in a feedback mechanism on this enzyme, like noradrenaline, to inhibit its own synthesis via control of tetrahydrobiopterin levels available for use by tyrosine hydroxylase, the rate-limiting step for catecholamine synthesis. (Nagatsu, Levitt and Udenfriend 1964, Levitt, Spector, Sjoerdsman and Udenfriend 1965)

Studies with dopamine and methotrexate together demonstrated that an additive effect was produced. Methotrexate has been shown to be a competitive inhibitor of dihydropteridine reductase and so competes with quinonoid dihydropterin for the pterin active site of the enzyme. The additive effect obtained with saturating inhibition concentrations of methotrexate used with dopamine indicated that the dopamine was probably binding to another part of the enzyme other than the pterin active site in order to bring about its inhibition. Therefore, dopamine appears to be an allosteric inhibitor of dihydropteridine reductase. Allosteric inhibitors and/or activators have been found for all enzymes known to be co-operative enzymes, (Dixon and Webb 1979) so an allosteric inhibitor of dihydropteridine reductase which demonstrates co-operativity is not surprising. No additive effect was obtained using saturating dopamine concentrations with phenylpyruvate or with saturating concentrations of phenylpyruvate

with phenylethylamine so it seems likely that these three inhibitors bind to the same part of the enzyme molecule. However, additive effects were obtained with saturating concentrations of methotrexate with phenylpyruvate adding to the evidence that phenylpyruvate binds to the same site on the enzyme as dopamine which is a different site to the pterin active site of the enzyme.

The inhibition of dihydropteridine reductase by lead was time-dependent which raises the possibility of the inhibitor being irreversible although the time-dependent inhibition curves did appear to level off as if it was coming to an equilibrium for each lead acetate concentration. (Figure 5.8) Dialysis of the leaded enzyme against tris buffer and tris buffer containing the chelating agent EDTA with no recovery of enzyme activity confirmed the irreversibility of lead inhibition. Aluminium, cadmium and mercuric salts all inhibited dihydropteridine reductase after preincubation of the enzyme with the metal presumably in an irreversible manner like lead. Gallium chloride also inhibited dihydropteridine reductase and this inhibition was time-dependent so it was most probably an irreversible inhibition. The inhibition results with lead, aluminium, cadmium and mercuric salts are at similar concentrations to those obtained by Leeming.(1979) The most likely cause of the heavy metal inhibition is attachment to a thiol group on the enzyme by those metals as lead and mercury are known to act in this way. (Ferdinand 1976, Dixon and Webb 1979) If dopamine was used with the leaded enzyme, the velocity versus substrate concentration curve remained sigmoidal in shape indicating that

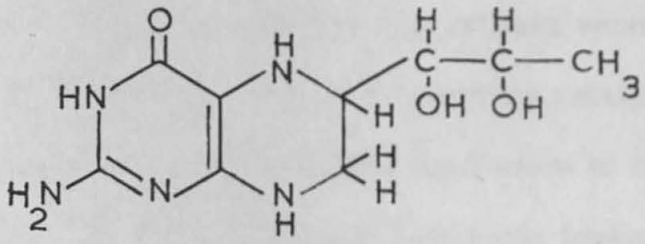
the modification of the enzyme by lead did not affect dopamine's allosteric inhibition effect. The apparent K_i values for both phenylpyruvate and methotrexate with the lead-modified enzyme were less than the corresponding values on the unmodified enzyme. This indicates that the inhibition is increased in the presence of lead which infers that the lead is not blocking the pterin active site to which methotrexate binds or the allosteric site to which phenylpyruvate appears to bind. If the lead did block either of these sites, no further inhibition on addition of these inhibitors would occur. The time dependence of the inhibition, however, implies some protection from lead inhibition by one of the substrates. As the pterin site appears to be unaffected, the NADH site seems the most likely target for lead attack.

The results presented in this Chapter add to the evidence that dihydropteridine reductase can be inhibited by dihydrofolate reductase inhibitors and indicate that dihydropteridine reductase displays co-operativity. It is shown that dopamine is likely to be an allosteric inhibitor of this enzyme. Dihydropteridine reductase can also be inhibited by a range of metals, a range of phenylalanine metabolites, aromatic amino acids and certain neurotransmitters which probably act as feedback inhibitors of this enzyme in order to control their own biosynthesis by limiting the cellular levels of tetrahydrobiopterin.

CHAPTER SIX

GENERAL DISCUSSION

Tetrahydrobiopterin (Figure 6-1) is widely distributed in tissues (Rembold and Gyure 1972) and its cofactor role in phenylalanine, tyrosine and tryptophan hydroxylases is now firmly established.



(figure 6-1)

(Kaufman and Fisher 1970, Lloyd and Weiner 1971, Friedman, Kappelman and Kaufman 1972) Tyrosine hydroxylase converts tyrosine to L-DOPA and is the rate-limiting step for dopamine and noradrenaline biosynthesis. (Levitt, Spector, Sjoerdsma and Udenfriend 1965, Nagatsu, Levitt and Udenfriend 1964, Patrick and Barchas 1976) Tryptophan hydroxylase is responsible for the conversion of tryptophan to 5 hydroxytryptophan and is the rate-limiting step for the biosynthesis of serotonin. (Hosoda and Glick 1966, Friedman, Kappelman and Kaufman 1972) Cellular tetrahydrobiopterin levels are rate-limiting for tyrosine hydroxylase activity (Kettler, Bartholini and Pletscher 1974) and may also be limiting for tryptophan hydroxylase activity although tryptophan concentrations are probably the major rate-limiting factor for the activity of this enzyme. (Costa and Meek 1974) Therefore, tetrahydrobiopterin concentrations will have significant effect on the production of the catecholamine neurotransmitters; noradrenaline and

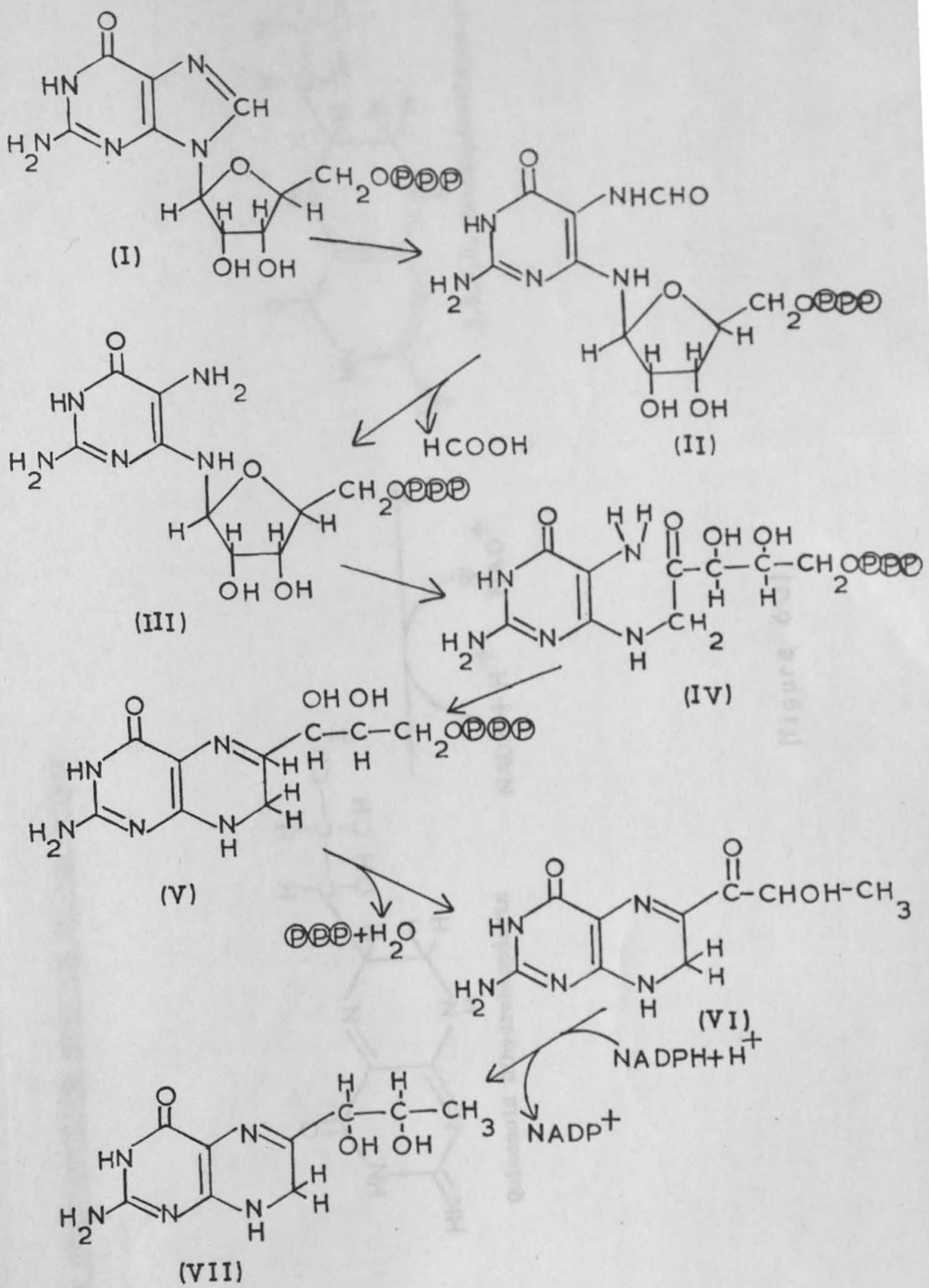
dopamine and possibly on serotonin production, to a lesser extent.

Tetrahydrobiopterin levels are maintained in the cell by two processes: the de novo biosynthetic pathway (Figure 6-2) (Brown 1971, Rembold and Gyure 1972, Gal, Nelson and Sherman 1978) and the dihydropteridine reductase salvage pathway. (Figure 6-3) (Craine, Hall and Kaufman 1972) The biosynthetic pathway serves to synthesize dihydrobiopterin from GTP by a series of reactions catalysed by three enzymes: dihydroneopterin triphosphate synthetase or GTP cyclohydrolase; an enzyme which converts dihydroneopterin triphosphate to sepiapterin and sepiapterin reductase. Dihydrobiopterin is then converted to tetrahydrobiopterin by dihydrofolate reductase. (Figure 6-4) (Abelson, Spector, Gorka and Fosburg 1978, Pollock and Kaufman 1978) Dihydropteridine reductase regenerates the active tetrahydro form of the cofactor from the quinonoid dihydrobiopterin formed in the hydroxylase reactions. The importance of tetrahydrobiopterin is demonstrated by the severe neurological consequences which occur in malignant hyperphenylalaninaemia. There are two causes of this disease. The first is a deficiency in one of the enzymes of the tetrahydrobiopterin biosynthetic pathway. (Leeming, Blair and Rey 1976, Rey, Blandin-Saroja and Rey 1976, Kaufman, Berlow, Summer, Milstien, Schulmann, Orloff, Spielberg and Pueschel 1978) The second is a deficiency of dihydropteridine reductase. (Kaufman, Holtzman, Milstien, Butler and Krumholz 1975, Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977, Grobe, Bartholome, Milstien and Kaufman 1978,

KEY FOR FIGURE 6-2

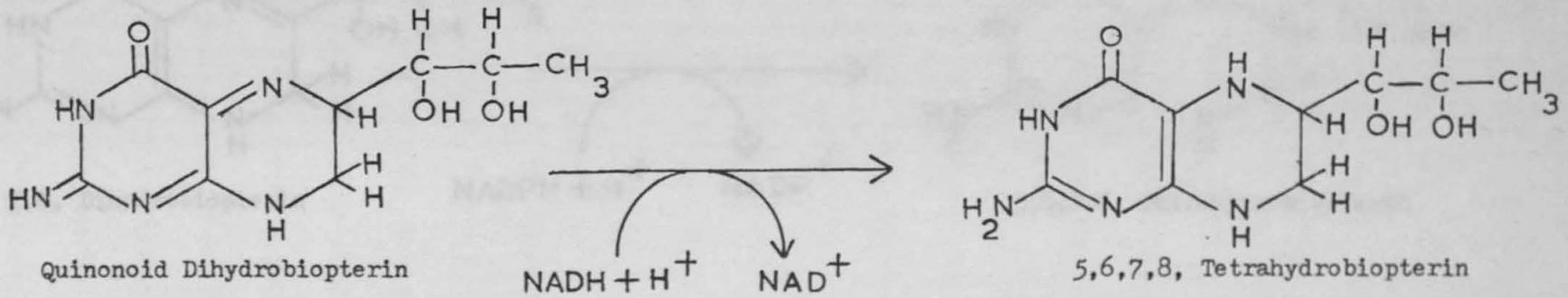
(I)	Guanosine triphosphate
(II)	Formylated intermediate
(III)	2,5, diamino-6-(5'-triphosphoribosyl)-amino-4-hydroxypyrimidine
(IV)	Deoxypentulose derivative
(V)	D erythro 7,8, dihydroneopterin triphosphate
(VI)	Sepiapterin
(VII)	7,8, dihydrobiopterin
Ⓟ	Phosphate group
HCOOH	Formic acid
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

THE BIOSYNTHETIC PATHWAY OF TETRAHYDROBIOPTERIN



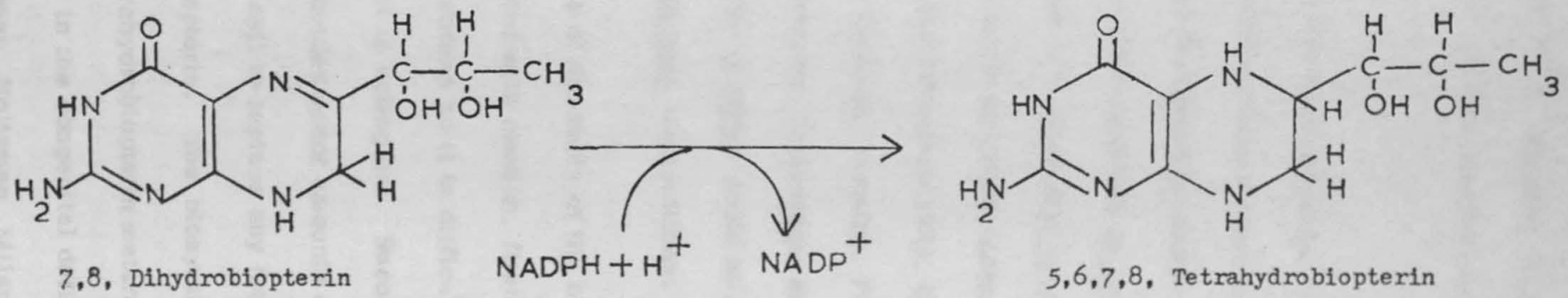
(figure 6-2)

THE DIHYDROPTERIDINE REDUCTASE SALVAGE PATHWAY



(figure 6-3)

THE REACTION CATALYSED BY DIHYDROFOLATE REDUCTASE



7,8, Dihydrobiopterin

5,6,7,8, Tetrahydrobiopterin

(figure 6-4)

Koslow and Butler 1977, Milstien, Holtzman, O'Flynn, Thomas, Butler and Kaufman 1976, Butler, Koslow, Krumholz, Holtzman and Kaufman 1978)

The aim of the work presented in this thesis was to examine he in vitro tetrahydrobiopterin production by these two pathways and to see how they can be altered by various reagents, some of which may effect tetrahydrobiopterin levels in vivo . The results were then to be compared to those of published in vivo studies, (Leeming and Blair 1980a, Leeming and Blair 1980b, Leeming 1975, Leeming 1979, Leeming, Blair, Melikian and O'Gorman 1976, Danks, Bartholome, Clayton, Curtius, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978, Kaufman 1978, Watson, Schlesinger and Cotton 1977) to see if the observations made in vitro could be related to them with a view to explaining the in vivo observations.

The results of the study of the biosynthetic pathway from GTP must be interpreted with caution, firstly because the whole pathway was studied as one process so it is difficult to tell exactly which step a particular reagent is acting on. Secondly, the biosynthetic pathway probably only provides minor amounts of tetrahydrobiopterin in the normally-active cell to replace any tetrahydrobiopterin lost by conversion to 7,8 dihydrobiopterin. The biosynthetic pathway will only play a major role in tetrahydrobiopterin metabolism when the normal metabolism is upset such as in the congenital deficiency of dihydropteridine reductase, (Kaufman, Holtzman, Milstien, Butler and Krumholz 1975,

Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977, Grobe, Bartholome, Milstien and Kaufman 1978, Koslow and Butler 1977, Milstien, Holtzman, O'Flynn, Thomas, Butler and Kaufman 1976, Butler, Koslow, Krumholtz, Holtzman and Kaufman 1978) or when dihydropteridine reductase is inhibited.

The results of the biosynthetic work show that the tetrahydrobiopterin biosynthetic pathway is present in rat brain tissue and that guanosine triphosphate (GTP) is the best precursor. (Chapter 2) This is as other workers have shown in different mammalian tissues: hamster liver; (Fukushima, Eto, Saliba and Shiota 1975) mouse lung and kidney, rat lung, hamster lung, kidney, brain and liver; (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota 1975) and rat brain. (Gal and Sherman 1976, Lee, Fukushima and Nixon 1978) The results also demonstrate that NADPH is required for the biosynthetic pathway, also as other workers have shown. (Chapters 2and 3) (Lee, Fukushima and Nixon 1978, Fukushima, Eto, Mayumi, Richter, Goodson and Shiota 1975, Fukushima, Eto, Saliba and Shiota 1975, Eto, Fukushima and Shiota 1976) The methods used to demonstrate that NADPH is required are somewhat different to those utilized by other workers, although straightforward NADPH addition was used. They consisted of protection of NADPH by the NAD nucleosidase inhibitor, nicotinamide, (Mann and Quastel 1941a, Mann and Quastel 1941b) and stimulation of production of NADPH by glutamate dehydrogenase and the pentose phosphate pathway, either directly or indirectly by glycolytic inhibition. The most likely point in the biosynthetic pathway where NADPH will be required would seem

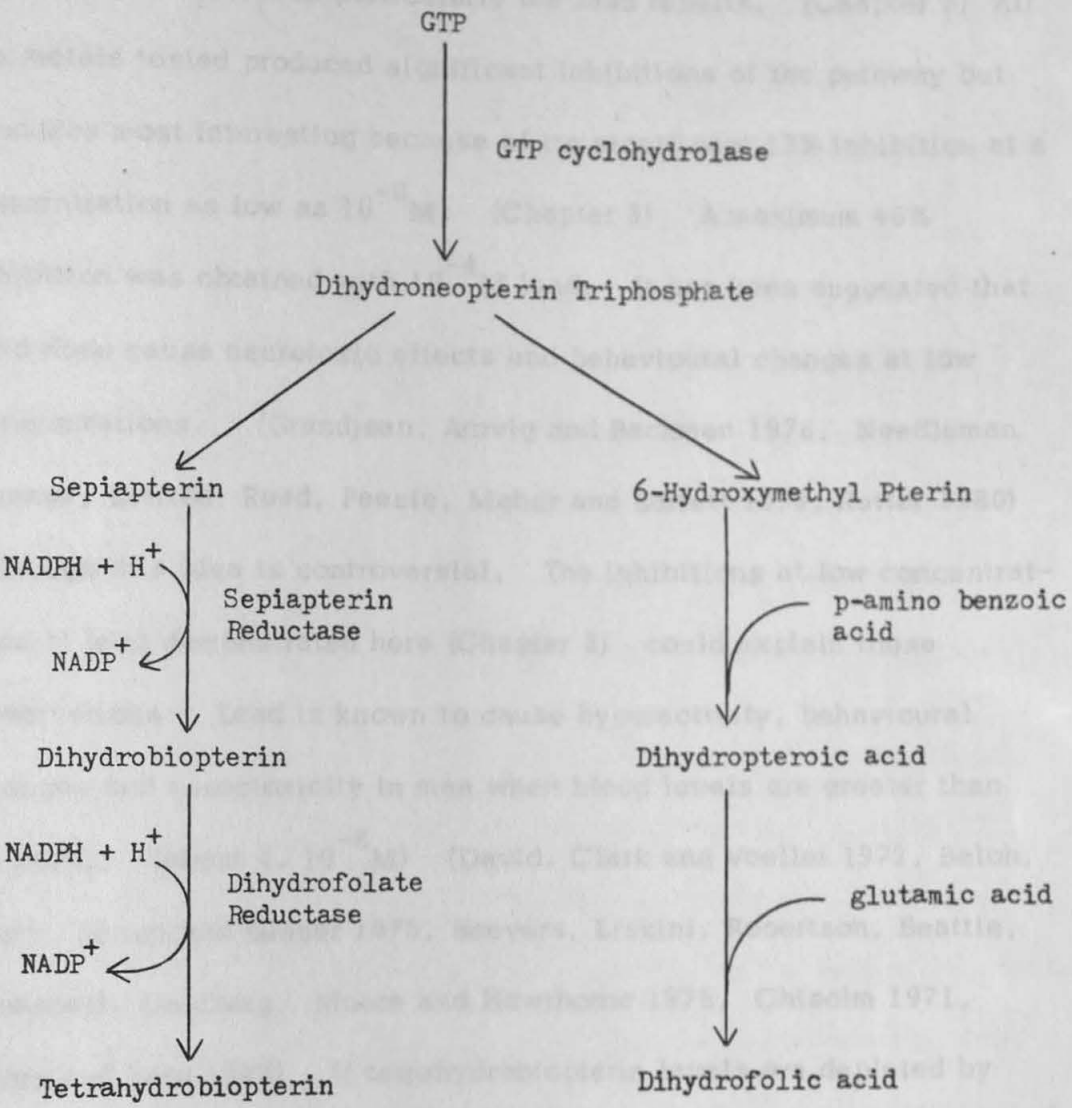
to be the conversion of sepiapterin to 7,8 dihydrobiopterin. (Figure 6-2) This reaction has been shown to be catalysed by sepiapterin reductase, (Nagai 1968, Matsubara, Katoh, Akino and Kaufman 1966) which does require NADPH. NADPH has been shown to be required for this conversion in pterin biosynthesis in a variety of animal systems. (Brown, Krivi, Fan and Unnasch 1978, Eto, Fukushima and Shiota 1976, Tanaka, Akino, Hagi and Shiota 1978) However, NADPH has also been shown to be required for the conversion of dihydroneopterin triphosphate to sepiapterin. (Brown, Krivi, Fan and Unnasch 1978, Tanaka, Akino, Hagi and Shiota 1978) but there is no evidence of NADPH being required for the conversion of guanosine triphosphate to dihydroneopterin triphosphate. (Fukushima, Richter and Shiota 1977, Fan and Brown 1976, Gal, Nelson and Sherman 1978) Magnesium ions have also been shown to be stimulating for the biosynthetic pathway activity. (Chapter 2) (Eto, Fukushima and Shiota 1976, Lee, Fukushima and Nixon 1978) It has been demonstrated that magesium ions are required for the conversion of dihydroneopterin triphosphate to sepiapterin in the presence of NADPH. (Tanaka, Akino, Hagi and Shiota 1978) Magnesium ions (Fukushima, Richter and Shiota 1977) and other divalent ions (Fan and Brown 1976) have been shown to inhibit the conversion of guanosine triphosphate to dihydroneopterin triphosphate (Brown 1971) but the enzyme GTP-cyclohydrolase A-II isolated from rat brain which converts guanosine triphosphate to 2-amino-6-(5-triphosphoribosyl)-amino-5-formamido-6-hydroxypyrimidine was shown to have an absolute dependence on magnesium ions. (Gal, Nelson and Sherman 1978)

The dihydrofolate reductase inhibitors exhibit the same order of degree of inhibition on both dihydrofolate reductase and tetrahydrobiopterin biosynthesis. (Chapter 3) (Burchall 1979) Dihydrofolate reductase is capable of reducing dihydropterins and is postulated as the final step in tetrahydrobiopterin biosynthesis. (Figure 6-4) (Kaufman 1967, Pollock and Kaufman 1978, Abelson, Spector, Gorka and Fosburg 1978, Spector, Fosburg, Levy and Abelson 1978) It appears that these drugs are acting on dihydrofolate reductase, thus causing a decrease in the tetrahydrobiopterin produced and increasing dihydrobiopterin levels. As the Crithidia fasciculata assay measures both reduced forms of biopterin and biopterin itself, this inhibition should not be detected using this method. The increases recorded must therefore be due to a stimulation of the biosynthetic pathway due to dihydrofolate reductase inhibition. The most likely explanation of this is if the biosynthetic pathway is under the control of feedback inhibition by its product. Then when the tetrahydrobiopterin levels fall due to dihydrofolate reductase inhibition, the feedback inhibition would be reduced and the activity of the biosynthetic pathway would increase as seen. The obvious target for this feedback inhibition would be the first enzyme of the pathway, guanosine triphosphate cyclohydrolase or dihydroneopterin triphosphate synthetase depending on the notation used. This is because the first enzyme in a pathway commits the precursor to that particular pathway rather than any other, for example, DNA synthesis in the case of GTP. It is therefore the best place for feedback control. GTP cyclohydrolase from E. coli was shown not to be

subject to feedback inhibition by a range of folate molecules but tetrahydrobiopterin was not tested. (Burg and Brown 1968) Dihydroneopterin triphosphate, the product of this reaction, has been shown to be the key intermediate in the biosynthesis of pterins so the GTP cyclohydrolase step is an important reaction of this pathway. (Brown and Fan 1975) However, GTP cyclohydrolase is a common enzyme for both pterin and folate biosynthesis, (Figure 6-5) (Brown 1971) so the next enzyme in the pathway which converts dihydroneopterin triphosphate to sepiapterin may be a more suitable control point for tetrahydrobiopterin feedback inhibition, so that folate biosynthesis is not inhibited at the same time. The final product, tetrahydrobiopterin is similar in structure to dihydroneopterin and so could act by competing for the pterin site on this enzyme. As stated previously, these results must be treated tentatively until further studies are done on the individual enzymes of the biosynthetic pathway.

The purine antagonists, mercaptopurine, thioguanine and azathioprine all inhibited biopterin biosynthesis probably by competing with GTP for the GTP site on GTP cyclohydrolase as they are of similar structure. (Chapter 3) The pyrimidine inhibitors, cytosine arabinoside and fluorourasil both increased biopterin biosynthesis. (Chapter 3) This could be due to their inhibition of other pathways utilizing GTP such as DNA biosynthesis which would increase the availability of GTP for biopterin biosynthesis.

FIGURE 6-5 BIOSYNTHESIS OF PTERINS AND FOLATES



Other interesting results of the biosynthetic work were those of the metal ion inhibition particularly the lead results. (Chapter 3) All the metals tested produced significant inhibitions of the pathway but lead was most interesting because of its significant 13% inhibition at a concentration as low as 10^{-8}M. (Chapter 3) A maximum 46% inhibition was obtained with 10^{-4}M lead. It has been suggested that lead does cause neurotoxic effects and behavioural changes at low concentrations. (Grandjean, Arnvig and Beckman 1978, Needleman, Gunnoe, Leviton, Reed, Peesie, Maher and Barrett 1979, Rutter 1980) although this idea is controversial. The inhibitions at low concentrations of lead demonstrated here (Chapter 3) could explain these observations. Lead is known to cause hyperactivity, behavioural changes and neurotoxicity in man when blood levels are greater than 80 µg/dl. (about $4. 10^{-6}$M) (David, Clark and Voeller 1972, Baloh, Sturm, Green and Gleser 1975, Beevers, Erskini, Robertson, Beattie, Campbell, Goldberg, Moore and Hawthorne 1976, Chisolm 1971, Byers and Lord 1943) If tetrahydrobiopterin levels are depleted by chronic inhibition of the biosynthetic pathway this would decrease catecholamine neurotransmitter biosynthesis due to the absolute dependence of the rate-limiting step, tyrosine hydroxylase, on tetrahydrobiopterin, (Levitt, Spector, Sjoerdsma and Udenfriend 1965, Nagatsu, Levitt and Udenfriend 1964) which is rate-limiting for the activity of this enzyme. (Kettler, Bartholini and Pletscher 1974) Mercury, cadmium, copper and aluminium have also been suggested as neurotoxic agents. Mercury is associated with loss of memory and

severe central nervous system toxicity whereas cadmium poisoning produces headaches and neurological disturbances. (Flink 1975) Copper toxicity causes convulsions (Scheinberg and Steinlieb 1976) and aluminium has been shown to be responsible for dialysis dementia (Alfrey, LeGendre and Kachny 1976) and to be associated with Alzheimer's dementia. (Crapper, Krishman and Quittkat 1976) The effect on tetrahydrobiopterin metabolism of these metals could be a contributing factor in the same way as lead. The effect on neurotransmitter production could only occur with chronic exposure to the metals because the dihydropteridine reductase system should be able to provide the majority of the tetrahydrobiopterin required by the hydroxylase enzymes.

5 methyl tetrahydrofolate alone increased biopterin biosynthesis and with coenzyme B_{12} increased it to 300% of normal. (Chapter 3) This suggested that the folate may be stimulating the step involving the loss of the one carbon fragment by removing the one carbon fragments as soon as they are eliminated from the molecule and so causing the equilibrium of the reaction to be in favour of dihydroneopterin triphosphate formation. It is known that B_{12} deficiency is associated with neurological defects such as occur in pernicious anaemia. (White, Handler and Smith 1973, Guyton 1971)

The work on dihydropteridine reductase should be easier to interpret and correlate with clinical studies because only one enzyme is being studied rather than a complete pathway. (Figure 6-3) Also the enzyme was isolated from rat liver in a relatively pure fraction so the

effects demonstrated are unlikely to be artifacts due to contamination by other enzymes. Such an isolation does result in studying the enzyme in an 'artificial' environment. Many steps are taken to provide the enzyme with all the conditions for optimal activity but the conditions used will probably not be exactly like those which occur in the cell, in vivo.

The affinity chromatography method using sodium 1,2 naphthoquinone-4-sulphonate as a ligand to isolate dihydropteridine reductase from rat liver resulted in an enzyme fraction purified about 900 fold over the original supernatant with a specific activity of 55 units/mg protein and 25% recovery of enzyme activity in the most active fraction. (Chapter 4) These results compare favourably with those using other methods of isolation by affinity chromatography (Chauvin, Korri, Tirpak, Simpson and Scrimgeour1979, Aksnes, Skotland, Flatmarck and Ljones 1979, Webber and Whiteley 1978, Webber, Deits, Snyder and Whiteley 1978) and the same method. (Cotton and Jennings 1978) This affinity method gave purification results near to those of the more traditional methods of enzyme isolation involving many fractionation steps (Hasegawa 1977, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977) but because the ligand was so specific for dihydropteridine reductase, had the advantage of consisting of only two steps. The affinity gel proved to be a useful tool for isolation of this enzyme and could be regenerated and used repeatedly if stored correctly as Cotton and Jennings have shown (1978). The sodium 1,2 naphthoquinone-4-

sulphonate proved to be so efficient at dihydropteridine reductase isolation because it is a competitive inhibitor of the enzyme with respect to the pterin substrate. (Chapter 5) It has a Ki value of $3.7.\ 10^{-4}$M and must resemble closely the quinonoid dihydrobiopterin substrate in structure in order that the enzyme binds to the affinity adsorbent. The enzyme will only bind to the gel when NADH is present (Chapter 4) which presumably causes a conformational change in the enzyme in order to allow the pterin to bind or in this case the sodium 1,2 naphthoquinone-4-sulphonate inhibitor. This also indicates the ligand resembles the quinonoid substrate very much or it would bind to the enzyme in the absence of NADH. This observation also implies that the enzyme follows a compulsory ordered mechanism which has been suggested in the literature. (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977, Aksnes and Ljones 1980)

The affinity chromatography method used here for the enzyme purification also gave a similar pH optimum range (6.3 to 7.0) (Chapter 4) as those quoted in the literature. (Webber, Deits, Snyder and Whiteley 1978, Cheema, Soldin, Knapp, Hofmann, and Scrimgeour 1973) This pH optimum is near the pH value at which the quinonoid dihydropterin substrate is most stable which is the most efficient pH for this enzyme. (Archer and Scrimgeour 1970)

The molecular weight of dihydropteridine reductase was demonstrated to be about 51,000 (Chapter 4) which is in good agreement with values obtained by other methods which were all shown to be dimers,

(Webber, Deits, Snyder and Whiteley 1978, Hasegawa 1977, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977, Aksnes, Skotland, Flatmark and Ljones 1979) but is about half the value obtained by Cotton and Jennings by a similar method of 100,000. (1978) They showed the enzyme molecule to consist of a tetramer where each monomer subunit was 25,000 molecular weight. This was about the same monomer subunit molecular weight as obtained for the dimers. (Webber, Deits, Snyder and Whiteley 1978, Hasegawa 1977, Aksnes, Skotland, Flatmark and Ljones 1979, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Korri, Chippel , Chauvin, Tirpak and Scrimgeour 1977) So it is probable that the enzyme isolated here is a dimer with a similar monomer subunit molecular weight. The differences in value of enzyme molecular weight and number of subunits per enzyme molecule between this study and that of Cotton and Jennings who used a similar method could be because these results are for rat liver dihydropteridine reductase and those of Cotton and Jennings were for monkey and human liver dihydropteridine reductase that is a species difference. Or Cotton and Jennings claim that they obtained a tetramer structure, which they believe to be the native form of the enzyme due to the simple one-step method of isolation used whereas in the other methods the tetramer becomes broken down into dimers due to the isolation procedures. If this is so, the dimer obtained here may have been obtained on the Sephadex G-150 fractionation step which Cotton and Jennings did not perform.

The turnover number of $1.71. 10^{-3}$ (molecules of product synthesized per minute by each molecule of enzyme) obtained and the apparent Km values for quinonoid dimethyl dihydropterin of $3.2. 10^{-5}$M and for NADH of $1.1. 10^{-5}$M are again in good agreement with literature values. (Chapter 4) (Webber, Deits, Snyder and Whiteley 1978, Cheema, Soldin, Knapp, Hofmannand Scrimgeour 1973, Webber and Whiteley 1978, Craine, Hall and Kaufman 1972) NADH was shown to be a better substrate than NADPH for the enzyme although both could function as substrate, also as demonstrated by other workers. (Chapter 4) (Webber, Deits, Snyder and Whiteley 1978, Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Craine, Hall and Kaufman 1972, Webber and Whiteley 1978, Nielsen, Simonsen and Lind 1969) Quinonoid dihydrobiopterin, the natural substrate, was the best pterin substrate studied but quinonoid dimethyl dihydropterin was nearly as active and the enzyme did display some activity with quinonoid dihydrofolate (Chapter 4) although this may have been due to an impurity present in the tetrahydrofolate such as 6 methyl tetrahydropterin, the quinonoid form of which is active as a substrate for dihydropteridine reductase. (Kaufman and Levenberg 1959, Kaufman 1975) However, Lind has shown that quinonoid dihydrofolate can act as a substrate for dihydropteridine reductase. (1972)

All these general properties of the enzyme which are similar to the characteristics of dihydropteridine reductase quoted in the literature show that this affinity chromatography method of isolation is a good

and reliable method for purifying this enzyme.

The inhibition studies on dihydropteridine reductase also produced several interesting results.

The end-products of neurotransmitter biosyntheses which involve tetrahydrobiopterin, noradrenaline, dopamine and serotonin all inhibited dihydropteridine reductase activity. (Chapter 5) Noradrenaline and serotonin had Ki values of $4.9.\ 10^{-4}$M and $1.2.\ 10^{-3}$M respectively and a Ki value for dopamine cannot be calculated due to the sigmoid curve obtained for the velocity versus substrate concentration plots. The concentration of noradrenaline in the whole brain is $2.1.\ 10^{-6}$M, (Taylor 1979) in the hypothalamus, $6.4.\ 10^{-6}$M (Westerink 1979) and in the nucleus accumbens, $4.1.\ 10^{-6}$M (Westerink 1979) whereas the concentration of serotonin in the whole brain is $1.83.\ 10^{-6}$M, (Kamaka, Okada, Watanabe, Kawashima and Wada 1980) in the hypothalamus, $7.5.\ 10^{-6}$M (Cummings, James, Soeters, Keane, Foster and Fischer 1976) and in the midbrain, $5.3.\ 10^{-6}$ (Cummings, James, Soeters, Keane, Foster and Fischer 1976) These neurotransmitters could therefore, be involved in a feedback inhibition of tetrahydrobiopterin production by dihydropteridine reductase especially as the neurotransmitters are probably concentrated in the terminal vesicles of the neurones. However, these feedback inhibitors probably work by different mechanisms as the serotonin is a competitive inhibitor and noradrenaline is a non-competitive inhibitor of dihydropteridine reductase with respect to the pterin. Therefore, serotonin inhibits

by competing with the quinonoid dihydropterin for the pterin site on dihydropteridine reductase and noradrenaline binds to some other site on the enzyme, most probably the same site as dopamine due to its structural similarity, that is, to the allosteric site. Dopamine itself, gave sigmoid velocity versus substrate concentration curves for both the pterin and NADH. (Chapter 5) This led to the discovery that dihydropteridine reductase exhibited positive co-operativity. This implies that binding of a substrate molecule to one subunit of the enzyme induces a conformational change in the enzyme which makes the other subunit more readily bind substrate molecules. The results, that there are two binding sites per enzyme molecule (The Scatchard plot, Chapter 5) is expected from the molecular weight studies which indicate that the enzyme exists as a dimer but provides evidence that each subunit of the dimer possesses an active site. $1.1.\ 10^{-4}$M dopamine gave a 46% inhibition at a pterin substrate concentration of 10 μM and 57% at 20 μM which are concentrations of dihydrobiopterin which can occur in vivo. Inhibition studies with dopamine and saturating concentrations of the pterin-competitive inhibitor, methotexate indicated that these two inhibitors bind to different sites on the enzyme molecule as both together gave an additive inhibition. (Chapter 5) Therefore, dopamine binds to an allosteric site. Dopamine inhibits dihydropteridine reductase at concentrations which occur in vivo as the concentration of dopamine in whole brain is $2.8.\ 10^{-6}$M (Taylor 1979), in the nucleus accumbens, $3.3.\ 10^{-5}$M and in the striatum, $5.0.\ 10^{-5}$M (Westerink 1979) Therefore it is a good candidate for

feedback inhibition of the dihydropteridine reductase to reduce cellular tetrahydrobiopterin and hence decrease catecholamine synthesis from tyrosine. These results suggest that dihydropteridine reductase may be a regulatory enzyme responsible for control of dopamine biosynthesis. This has already been suggested, (Musacchio, D'Angelo and McQueen 1971) but only indirect evidence was presented where an assay system for the hydroxylation of tyrosine hydroxylase was utilized, which contained dihydropteridine reductase as well as the tyrosine hydroxylase. It was shown that DOPA formation could be controlled by the concentration of dihydropteridine reductase present and that the feedback inhibition of tyrosine hydroxylase can be antagonised by increasing the concentration of dihydropteridine reductase. (Musacchio, D'Angelo and McQueen 1971) However, no direct effect of catecholamines on dihydropteridine reductase has been demonstrated before. Dopamine biosynthesis is known to be controlled by feedback inhibition of catecholamines on tyrosine hydroxylase (Nagatsu, Mizatani, Nagatsu, Matsura and Sugimoto 1972, Nagatsu, Levitt and Udenfriend 1964, Kuczenzki and Mandell 1972) where the catecholamines compete for the pterin site on tyrosine hydroxylase. As this feedback inhibition can be opposed by increasing the concentration of tetrahydrobiopterin as a result of increased dihydropteridine reductase activity (Musacchio, D'Angelo and McQueen 1971) it seems likely that in order to control dopamine biosynthesis more efficiently, the dopamine levels should control the amount of tetrahydrobiopterin available to tyrosine hydroxylase as well as tyrosine hydroxylase activity. Recently it has been

shown that the inhibition of tyrosine hydroxylase by dopamine is greater in synaptosome preparations than in soluble preparations. (Waggoner, McDermed and Leighton 1980) These workers were unable to explain these results but suggested that this phenomenon could be explained if dopamine were capable of lowering the reduced cofactor (tetrahydrobiopterin) levels in some way as well as directly inhibiting tyrosine hydroxylase as this would increase the competitive inhibition by dopamine. The results presented here explain these observations by showing that dopamine does indeed inhibit tetrahydrobiopterin formation. Dihydropteridine reductase therefore has a possible role in dopamine biosynthesis regulation.

Dihydropteridine reductase is inhibited by phenylalanine and metabolites of phenylalanine. (Chapter 5) The Ki values for these compounds range from $1.8. 10^{-5}$M for tyramine and $5.0. 10^{-5}$M for phenylpyruvate to $1.0. 10^{-3}$M for phenylalanine and phenyllactate. The concentrations of these compounds are also very variable in health and disease with normal human brain levels of phenylalanine being about $1.0. 10^{-4}$M, (Letendre, Nagaiah and Guroff 1980) normal human serum levels of phenylpyruvate being about $5.0. 10^{-6}$M (Langenbeck, Behbehani and Luthe 1980a, Langenbeck, Behbehani and Luthe 1980b) the normal rat brain levels of phenylethylamine being about 5nM (Fischer, Spatz and Heller 1972) and the normal rat brain levels of tyramine being about $1.3. 10^{-8}$M. However in phenylketonuria and its variants the levels of phenylalanine and its metabolites are much higher. (Jervis and

Drejza 1966, Bickel, Gerrard and Hickmans 1953, Udenfriend 1961) Values of 10^{-5}M to 1.6. 10^{-4}M for human serum phenylpyruvate (Langenbeck, Behbehani and Luthe 1980b) and 7.9. 10^{-6}M to 4.0. 10^{-5}M for human serum o-hydroxyphenylacetic acid (Jervis and Drejza 1966) have been measured. This is due to reduced phenylalanine hydroxylase activity due to the deficiency of the enzyme itself in normal phenylketonuria and due to a deficiency of tetrahydrobiopterin in malignant hyperphenylalaninaemias. Therefore, instead of being converted to tyrosine by phenylalanine hydroxylase, the phenylalanine accumulates and more of its metabolites such as phenylpyruvate and o-hydroxyphenylacetic acid are produced. (Partington 1978)

It was suggested that the mental defects which occur in phenylketonuria were due to the increased levels of phenylalanine or its metabolites. (Bickel, Gerrard and Hickmans 1953, Udenfriend 1961) This is supported by the fact that a low-phenylalanine diet appears to reduce the neurological damage of phenylketonuria. (Bickel , Gerrard and Hickman 1953) It is usual for phenylketonuric children to be put on the low-phenylalanine diet as soon as possible so that the possibilities of mental defects are kept to a minimum. The children are then kept on this regime until the age of about five or six when the diet is relaxed because the phase of rapid brain development is finished at the age of about two years. (Dobbing and Sands 1973, Smith, Lobascher, Stevenson, Wolff, Schmidt, Grubel-Kaiser and Bickel 1978) However, there is evidence now that children in whom the diet is relaxed or whom

are given complete freedom of diet have a lower I.Q. measurement than children who are kept on the diet with good control. (Berry, O'Grady, Perlmutter and Bofinger 1979, Brown and Warner 1976, Smith, Lobascher, Stevenson, Wolff, Schmidt, Grubel-Kaiser and Bickel 1978, Cabalska, Duezynska, Borzymowska, Korska, Koslocz-Folga and Bozkowa, 1977, Dobson, Williamson, Azen and Koch 1977, Partington and Leverty 1978, Williamson, Koch and Berlow 1977). Nowadays phenylketonuric women who wish to have children are recommended to return to the phenylalanine-restricted diet before conception in order to avoid neurological damage to the fetus which might be normal as such defects have been reported to occur. (Smith, Erdohgi, Macartney, Dincott, Wolff, Brenton, Biddle, Fairweather and Dobbing 1979, Farquhar 1979, Smith and Wolff 1979, Buist, Lis, Tuerck and Murphey 1979) This is evidence that, not only does the phenylalanine or its metabolites cause damage to the developing brain, but probably causes some damage after brain maturation is complete. Many suggestions have been made as to why phenylalanine and/or its metabolites cause neurological damage. One theory is that phenylpyruvate is responsible because it inhibits the transport of pyruvate into mitochondria in the brain and hence inhibits pyruvate oxidation and upsets brain function. The concentrations of phenyl-pyruvate used to produce this inhibition were 2.5 to 5mM. (Halestrap, Brand and Denton 1974) Phenylpyruvate is formed in the brain by transamination of phenylalanine and it has been suggested that this causes a depletion of α ketoglutarate in the brain which then has consequences on the Krebs cycle leading to defective brain metabolism

(Korey 1957) Alternatively the depletion of ∝ ketoglutarate makes it unavailable for essential transamination of other amino acids again leading to brain dysfunction. (Jervis and Drejza 1966) These hypotheses are supported by the decreased urinary output of a ∝ glutarate in phenylketonurics. (Jervis and Drejza 1966) It has also been suggested that the neurological defects in phenylketonuria are due to phenylpyruvate inhibition of aromatic amino acid decarboxylation which leads to depletion of catecholamines in the brain which has also been reported in phenylketonuric patients (Davidson and Sandler 1958, Boylen and Quastel 1961, Wolley and Van der Hoeven 1964) One of the most popular theories is that the increased phenylalanine levels inhibit the entry of other amino acids into the brain as they all have to compete for common membrane carriers for transport. (Pratt 1980, Banos, Daniel, Moorhouse, Pratt and Wilson 1974)

The inhibition of dihydropteridine reductase by phenylalanine and its metabolites could also be responsible for the neurological defects which occur in phenylketonuria. Much evidence supports this theory. Inhibition of dihydropteridine reductase would lead to reduced cellular tetrahydrobiopterin levels and this would then result in less dopamine and noradrenaline being synthesized from tyrosine and perhaps less serotonin being produced from tryptophan. This theory explains the reduced brain catecholamines and serotonin reported in phenylketonurics. (Butler, Koslow, Krumholz, Holtzman and Kaufman 1978) Therefore the mental defects are due to a lack of tetrahydrobiopterin. Treatment

of malignant hyperphenylalaninaemics usually involves giving L-DOPA and serotonin which bypass the lesion. (Curtius, Niederwieser, Viscontini, Otten, Schaub, Scheibenreiter and Schmidt 1979, Schaub, Daumling, Curtius, Niederwieser, Bartholome, Viscontini, Schircks and Bieri 1978, Danks, Bartholome, Clayton, Curtius, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978) Children with phenylketonuria have higher than normal serum dihydrobiopterin levels which in fact parallel the serum levels of phenylalanine. (Leeming, Blair, Green and Raine 1976) Serum phenylpyruvate levels can be correlated with serum phenylalanine levels. (Langenbeck, Behbehani and Luthe 1980b) Inhibition of dihydropteridine reductase by phenylpyruvate and/or phenylalanine would explain these observations because there would be an accumulation of quinonoid dihydrobiopterin in the cell which would rearrange to give the increased levels of serum dihydrobiopterin recorded. It has been reported that there is a significant inverse correlation between I.Q. measurements and serum phenylalanine levels. (Berry, O'Grady, Perlmutter and Bofinger 1979) It has also been shown that taking phenylketonurics off the low-phenylalanine diet causes I.Q. to decrease (Smith, Lobascher, Stevenson, Wolff, Schmidt, Grubel-Kaiser and Bickel 1978) and that phenylketonuric children on phenylalanine-restricted diets had I.Q. measurements more than one standard deviation below those of family members. (Berry, O'Grady, Perlmutter and Bofinger 1979) Phenylketonuria heterozygotes are also mildly hyperphenylalaninaemic and have lowered I.Q. measurements compared to normal children. (Bessman, Williamson and Koch, 1978) Not only

have lower I.Q. measurements in mild hyperphenylalaninaemic children been correlated with higher serum phenylalanine levels but also with higher serum dihydrobiopterin levels too. (Rey 1980) As, serum dihydrobiopterin and serum phenylalanine levels correlate inversely with I.Q. measurements and serum phenylalanine and phenylpyruvate levels also correlate with each other, plus that inhibition of dihydropteridine reductase by phenylalanine and/or phenylpyruvate causes increased serum dihydrobiopterin levels, then it could be said that inhibition of dihydropteridine reductase correlated inversely with I.Q. measurements. So that inhibition of dihydropteridine reductase causes decreased I.Q. measurements in young children. All the phenylalanine metabolites tested were shown to inhibit dihydropteridine reductase but at different concentrations. (Chapter 5) Obviously the most potent inhibitors, tyramine, (Ki value = $1.8. 10^{-5}$M) phenylpyruvate (Ki value = $5.0. 10^{-5}$M) and phenylethylamine (Ki value = $8.5. 10^{-4}$M) are the most likely causes of the neurological defects in phenylketonuria and concentrations of phenylpyruvate in phenylketonurics of $1.6. 10^{-4}$M are known. (Langenbeck, Behbehani and Luthe 1980b) Phenylalanine (Ki value = $1.0. 10^{-3}$M) could also contribute as high phenylalanine concentrations do occur in untreated phenylketonuria. (Jervis and Drejza 1966) Lowering phenylalanine levels by the recommended diet lowers the phenylalanine metabolite levels as well and so will reduce the neurological damage. This theory is supported by the fact that in malignant hyperphenylalaninaemia the mental defects are not relieved by a phenylalanine-restricted diet. In these children the phenylalanine hydroxylase

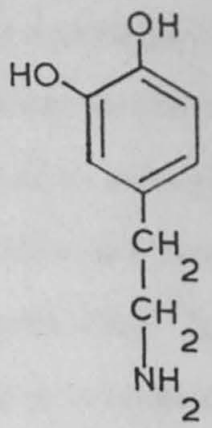
enzyme is normal but cellular tetrahydrobiopterin levels are very low. The lesion here is in tetrahydrobiopterin metabolism itself so phenylalanine restriction will not eleviate the symptoms as it does in typical phenylketonuria. The fact that the neurological damage seems to improve if the children with malignant hyperphenylalaninaemia are treated with tetrahydrobiopterin also adds to the evidence that the brain damage is due to lack of tetrahydrobiopterin. (Curtius, Niederwieser, Viscontini, Otten, Schaub, Scheiberecter and Schmidt 1979, Schaub, Daumling, Curtius, Niederwieser, Bartholome, Viscontini, Schircks and Bieri 1978) It is of course, possible that the neurological defects occurring in phenylketonuria are a result of all these postulated theories of the effects of phenylalanine and its metabolites acting together rather than one theory alone.

This proposed model for the cause of mental retardation in phenylketonuria has other wide-reaching consequences. It could be extended to suggest that any agent that inhibits dihydropteridine reductase could cause neurological damage due to reduced tetrahydrobiopterin levels in young children.

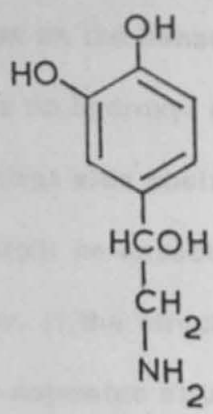
This theory may also explain why the levels of serum dihydrobiopterin parallel so closely the serum levels of phenylalanine in phenylalanine loading tests. (Leeming, Blair, Green and Raine 1976) As the levels of serum phenylalanine rise, the inhibition of dihydropteridine reductase increases and so the levels of serum dihydrobiopterin will increase as the quinonoid dihydrobiopterin is converted to the tetra-hydro form.

Dihydropteridine reductase inhibition by phenylpyruvate and phenylethylamine has been shown to occur by these reagents binding to the same site on the enzyme as dopamine, that is, the allosteric site. (Chapter 5) It is assumed that phenylalanine and its other metabolites, tyramine and noradrenaline for example, also bind to the allosteric site because of similarities of structure. A comparison of the degree of inhibition by these reagents with consideration of their structures should give an indication of the kind of structure required to produce inhibition at the allosteric site. Dopamine (Figure 6-6) is likely to be the natural inhibitor with noradrenaline (Figure 6-6) being another possibility. A Ki value for dopamine could not be derived due to the sigmoid curves it produced. Noradrenaline had a Ki value of $4.9.\ 10^{-4}$M (Chapter 5) and has a very similar structure to dopamine having only an additional hydroxyl group on the side-chain. This group must prevent noradrenaline being as potent an inhibitor as dopamine. Adrenaline (Figure 6-6) has an additional methyl group at the end of the side-chain to noradrenaline but had no effect on dihydropteridine reductase. Likewise L-DOPA (Figure 6-6) which has an additional carboxyl group on the side-chain also had no effect on the enzyme. It can be concluded from this that the addition of a large group such as a methyl or a carboxyl to the side-chain prevents these compounds binding to the allosteric site. Of the phenylalanine metabolites, tyramine (Figure 6-6) and phenylpyruvate (Figure 6-6) were the most potent inhibitors of dihydropteridine reductase followed by phenylethylamine (Figure 6-6). Tyramine is like dopamine except for the absence of one hydroxyl group from the

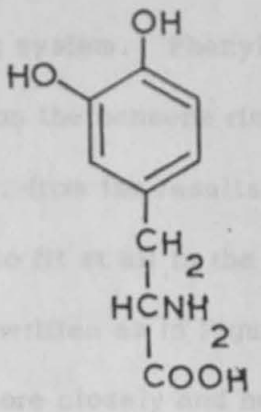
STRUCTURES OF POTENTIAL DIHYDROPTERIDINE REDUCTASE ALLOSTERIC SITE INHIBITORS



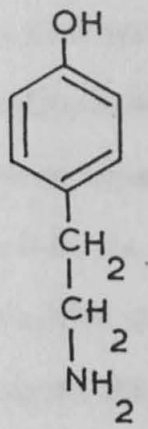
Dopamine



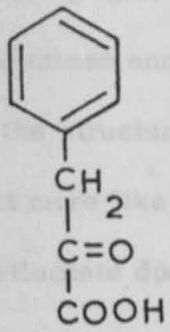
Noradrenaline



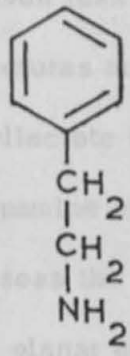
L-DOPA



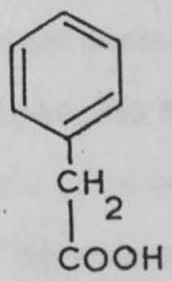
Tyramine



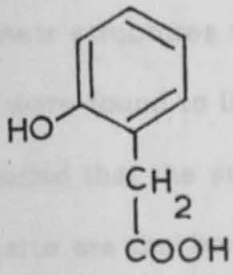
Phenylpyruvate



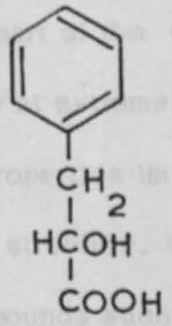
Phenylethylamine



Phenylacetic acid



o-Hydroxyphenylacetic acid

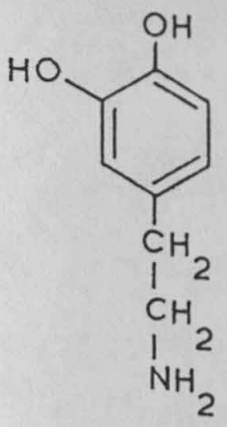


Phenyllactate

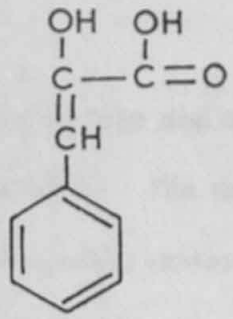
figure 6-6

benzene ring system and so is quite a potent inhibitor of dihydropteridine reductase. Phenylethylamine is structurally like dopamine except for having no hydroxy l groups on the benzene ring system. Phenylpyruvate like phenylethylamine has no hydroxyl groups on the benzene ring but also has an entirely different side chain which, from the results with adrenaline and L-DOPA might be expected not to fit at all in the allosteric site. However, if the structure is written as in Figure 6-7, it begins to resemble the dopamine structure more closely and hence its potent inhibition effect might be explained. Phenylacetic acid, o-hydroxy-phenylacetic acid and phenyllactate (Figure 6-6) are even less potent inhibitors of dihydropteridine reductase and their structures are even less like that of dopamine. If the structure of phenyllactate is written as in Figure 6-7, its structure is more like that of dopamine and phenyl-pyruvate but unlike them, phenyllactate does not possess the double bonds to keep the dihydroxyl group configuration in a planar arrangement and so still would not fit the allosteric site as well as dopamine and phenylpyruvate. Sodium pyruvate and diacetyl were tested to see if they would inhibit because their structures were like part of the dopamine structure and they were found to inhibit only at extremely high concentrations. It is concluded that the structural properties important for binding to the allosteric site are the benzene ring structure, a planar dicarbonyl group and an amine group. Dicarbonyl compounds such as 1,2 cyclohexanedione are known to attach to the amine groups at the end of the side-chains of arginyl residues on proteins. (Ferdinand 1976) So perhaps an arginyl residue is present in the allosteric site

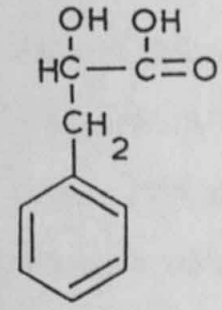
figure 6-7



Dopamine



Phenylpyruvate



Phenyllactate

of dihydropteridine reductase. The amino acid, arginine was found when the amino acid composition of dihydropteridine reductase was determined. (Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973, Craine, Hall and Kaufman 1972, Hasegawa 1977, Aksnes, Skotland, Flatmark and Ljones 1979, Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977)

Inhibition of dihydropteridine reductase by lead and other metals was probably by irreversible inhibition.(Chapter 5) The time dependent inhibition by lead and gallium suggested irreversible inhibition but the inhibition levelled off as if an equilibrium were being set up. However dialysis studies with the leaded enzyme produced no recovery of activity and so confirmed that the inhibition was irreversible. Such irreversible inhibitions of enzymes by metals are common and usually the heavy metal attaches to a thiol group on the enzyme. (Ferdinard 1976) The amino acid composition of dihydropteridine reductase has been determined by several groups from different sources. (Korri, Chippel, Chauvin, Tirpak and Scrimgeour 1977, Cheema, Soldin, Knapp, Hofmann andScrimgeour 1973, Craine, Hall and Kaufman 1972) Of these only two groups measured cysteine residues which is the amino acid residue with a thiol group on its side-chain. Craine et al., showed the sheep liver enzyme to contain one cysteine per subunit and Aksnes et al., showed that the bovine liver enzyme had three cysteines per subunit. (Craine, Hall and Kaufman 1972, Aksnes, Skotland, Flatmarck and Ljones 1979) So assuming the rat liver enzyme also

has at least one free cysteine residue, then the inhibition by heavy metals is likely by attachment to the thiol group if the cysteine occurs in an accessible position on the enzyme molecule and the results obtained suggest this is so. They also suggest that the thiol group is not in or near the pterin active site or the allosteric site to which dopamine binds as, when methotrexate, phenylpyruvate and dopamine were used with the fully-leaded enzyme further inhibition occurred. (Chapter 5) However, the time dependence of the lead and gallium inhibitions suggest that one of the substrates is causing partial or whole protection of the enzyme from lead inhibition. The pterin substrate does not appear to be fulfilling this property or further inhibition on top of the maximum lead inhibition would not be expected to occur with methotrexate which obviously binds to the pterin active site because it is a competitive inhibitor. So it is probably the NADH which is protecting the enzyme from lead inhibition suggesting the cysteine residue to which lead attaches is in or near the NADH site. Alternatively there is a possibility that the thiol group is not near the NADH active site but that the attachment of lead to the thiol group causes a conformational change in the enzyme molecule which prevents the NADH from binding so readily. Other workers have looked for the presence of thiol groups on dihydropteridine reductase to see if they are required for enzyme activity. Aksnes et al., could not detect any free thiol groups using 5'5' dithiobis-(2-nitro-benzoic acid) in the absence of denaturing agents but upon denaturation of the bovine liver enzyme with sodium dodecyl sulphate, they found three free thiol groups and concluded a free thiol group was

not part of the active site. (Aksnes,Skotland, Flatmarck and Ljones 1979) However, Webber and Whiteley found rat liver dihydropteridine reductase to be strongly inhibited by both mercuric chloride and p-chloromercuri-benzoate and that the loss of activity could be halted though not restored by addition of NADH. (1978) Cheema et al., also showed that sheep liver dihydropteridine reductase was also inhibited by these two reagents, but that iodoacetic acid gave no inhibition. They too discovered the partial protection from inhibition afforded by NADH. (Cheema, Soldin, Knapp, Hofmann and Scrimgeour 1973) It is concluded that a thiol group is involved in the maintenance of dihydropteridine reductase activity. Inhibition of dihydropteridine reductase by lead is significant at concentrations of 10^{-6}M or greater. (Chapter 5) This inhibition could lead to the same mental defects as phenylpyruvate is thought to cause by decreasing the tetrahydrobiopterin levels in the cells. Similarly, inhibition of this enzyme system by any of these metals could cause neurological damage. The dihydropteridine reductase inhibition by aluminium is significant at $2. 10^{-4}$M (Chapter 5) and the brain levels of aluminium quoted for patients with dialysis dementia were $1.18. 10^{-4}$M (McDermott, Smith, Ward, Parkinson and Kerr 1978) and for patients with Alzheimer's dementia were 2.8-4.3 µg/g. (Approximately 10^{-4}-$1.6. 10^{-4}$M) Inhibition of dihydropteridine reductase has already been suggested as a cause of dialysis dementia. (Leeming and Blair 1979)

The effects of agents on tetrahydrobiopterin metabolism can be

examined using the clinical data available from the in vivo studies to indicate what might be the in vivo effects of those agents.

If Figure 6-8 is used for reference and effects of altering the rates of the various metabolic pathways of tetrahydrobiopterin are considered, a model for tetrahydrobiopterin metabolism can be built up. Inhibition or decreasing the rate of the dihydropteridine reductase salvage pathway would result in increased quinonoid dihydrobiopterin levels, which would lead to increased levels of 7,8 dihydrobiopterin which could be measured in the serum and urine, and decreased tetrahydrobiopterin levels which would lead to decreased tyrosine hydroxylase activity and hence, decreased production of the catechol neurotransmitters. The biosynthetic pathway for tetrahydrobiopterin would probably not manage to provide enough tetrahydrobiopterin to keep the tyrosine hydroxylase activity at the normal rate even though the biosynthetic pathway would be stimulated by reduced feedback inhibition by tetrahydrobiopterin. Inhibition or decreased activity of any step including dihydrofolate reductase on the biosynthetic pathway for tetrahydrobio pterin would reduce the tetrahydrobiopterin levelsgradually because the cellular levels would no longer be being topped up. The 7,8 dihydrobiopterin levels would fall because the dihydropteridine reductase would convert most of the quinonoid dihydrobiopterin to the tetrahydro-form leaving less to rearrange to 7,8 dihydrobiopterin . Inhibition or decreased activity of tyrosine hydroxylase would result in lowered quinonoid dihydrobiopterin levels and hence, lowered 7,8-dihydrobiopterin levels in the cell, serum

SUMMARY OF THE METABOLIC PATHWAYS OF TETRAHYDROBIOPTERIN

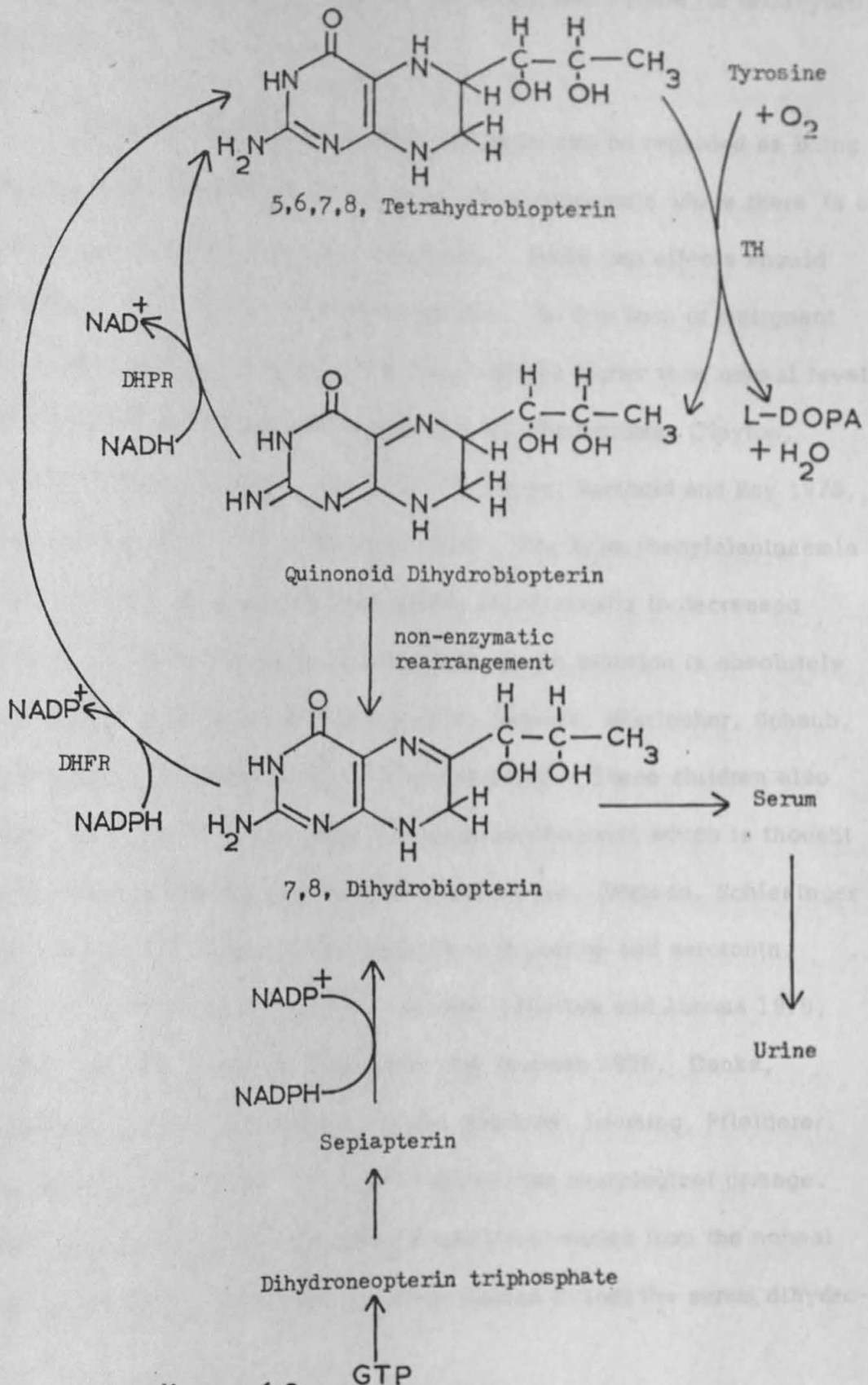


figure 6-8

and urine. The levels of tetrahydrobiopterin would be increased and would probably feedback to inhibit the biosynthetic route for tetrahydrobiopterin.

Inhibition of dihydropteridine reductase can be regarded as being similar to that form of malignant hyperphenylalanaemia where there is a deficiency of dihydropteridine reductase. These two effects should therefore, give similar clinical symptoms. In this form of malignant hyperphenylalaninaemia there are the predicted higher than normal levels of serum and urine dihydrobiopterin (Danks, Bartholome, Clayton, Curtius, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978, Leeming and Blair 1980b, Kaufman 1978) The hyperphenylalaninaemia results from lack of tetrahydrobiopterin which results in decreased phenylalanine hydroxylase activity because the reaction is absolutely dependent on tetrahydrobiopterin (Curtius, Zagalak, Baerlocher, Schaub, Leimbacker and Redweik 1977, Kaufman 1978) These children also have increased levels of urine 7,8dihydroxanthopterin which is thought to be derived from reduced biopterin derivatives, (Watson, Schlesinger and Cotton 1977) and reduced amounts of dopamine and serotonin. (Brewster, Moskowitz, Kaufman, Berlow, Milstien and Abroms 1979, Butler, Koslow, Krumholz, Holtzman and Kaufman 1978, Danks, Bartholome, Clayton, Curtius, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978) They also suffer from neurological damage. Their response to a phenylalanine loading test varies from the normal response and that of typical phenylketonurics in that the serum dihydro-

biopterin concentrations rise higher. (Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977)

Inhibition of the tetrahydrobiopterin biosynthetic pathway can be regarded as being similar to that form of malignant hyperphenylalaninaemia where there is a deficiency of the biosynthesis of tetrahydrobiopterin. Again these two effects should produce similar clinical symptoms. There is hyperphenylalaninaemia, again due to the lack of tetrahydrobiopterin as in the other form of malignant hyperphenylalaninaemia. (Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel 1978) The response to a phenylalanine loading test is different to that of normal patients and typical phenylketonurics because the serum dihydrobiopterin levels do not increase. (Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel 1978, Rey, Harpey, Leeming, Blair, Aicardi and Rey 1977) The catecholamine concentrations in these children have also been found to be low and they suffer neurological defects (Danks, Bartholome, Clayton, Curtius, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978) and it has been determined that they excrete large amounts of neopterin and small amounts of dihydroneopterin and dihydroxanthopterin but no traces of biopterins in their urine. (Curtius, Niederweiser, Viscontini, Otten, Schaub, Scheibereicter and Schmidt 1979) These children also have the predicted lowered serum dihydrobiopterin levels. (Danks, Bartholome, Clayton, Grobe, Kaufman, Leeming, Pfleiderer, Rembold and Rey 1978, Leeming and Blair 1980b, Kaufman 1978)

Therefore these two types of malignant hyperphenylalaninaemia can be distinguished by their different serum and urine 7,8 dihydrobiopterin concentrations, different urine dihydroxanthopterin levels and different responses when presented with an oral phenylalanine load. It has recently been suggested that ratios of neopterin to biopterin excreted in the urine can also be used to distinguish between these two syndromes. (Nixon, Lee, Milstein, Kaufman, Bartholome 1980) It should also be possible to distinguish between inhibitions of the various metabolic pathways of tetrahydrobiopterin by looking for these symptoms.

For example, in typical phenylketonuria, there is a deficiency of phenylalanine hydroxylase which leads to increased levels of phenylpyruvate which inhibits dihydropteridine reductase activity. (Chapter 5) This gives rise to increased quinonoid dihydrobiopterin which rearranges to give the increased levels of 7,8 dihydrobiopterin, seen in typical phenylketonuria. (Leeming, Blair, Melikian and O'Gorman 1976) 7,8 dihydroxanthopterin has also been found in the urine of untreated phenylketonurics but not in those under good dietary control (Watson, Schlesinger and Cotton 1977) A phenylalanine loading test in phenylketonuric patients also produces rises in serum dihydrobiopterin concentrations greater than in normal patients. (Leeming, Blair, Green and Raine 1976) If untreated, phenylketonurics are severely mentally retarded. (Bickel, Gerrard and Hickmans 1953) The symptoms predicted from the model of tetrahydrobiopterin metabolism for inhibition of dihydropteridine reductase are in fact found in this case.

The results presented in this thesis indicate that methotrexate should also cause increased serum 7,8 dihydrobiopterin levels because it too inhibits dihydropteridine reductase and such high serum levels have been recorded in patients on methotrexate. (Leeming, Blair, Melikian and O'Gorman 1976) Methotrexate has also been shown to increase the time taken to clear a phenylalanine load (Goodfriend and Kaufman 1961) and children with acute lymphoblastic leukaemia treated with methotrexate have been reported as suffering from neurological damage. (Eiser 1978, Meadows and Evans 1976) This might also be predicted from the model set up and it has been suggested that these children might benefit from neurotransmitter replacement therapy, (Leeming, Blair 1978, Cotton 1978) shown to help the malignant hyperphenylalaninaemics. (Curtius, Niederwieser, Viscontini, Otten, Schaub, Scheibereiter and Schmidt 1979, Schaub, Daumling, Curtius, Niederwieser, Bartholome, Viscontini, Schrirks and Bieri 1978) if they are to escape severe neurological defects.

Folic acid and 5 formyltetrahydrofolic acid following doses of methotrexate also raised serum 7,8 dihydrobiopterin levels whereas 5 methyltetrahydrofolic acid did not. (Leeming, Blair, Melikian and O'Gorman 1976) This also agrees with the prediction that can be made from the model and the results presented in this thesis as folic acid and 10 formyl folic acid inhibited dihydropteridine reductase (Chapter 5) and 5 methyltetrahydrofolic acid did not. These compounds should therefore increase the serum concentrations of dihydrobiopterin when given

alone but they do not. (Leeming, Blair, Melikian and O'Gorman 1976) It is possible that alone these compounds are rapidly metabolised and hence, *in vivo* have no effect on dihydropteridine reductase.

Trimethoprim and sulphamethoxazole inhibited dihydropteridine reductase both alone and together, when they gave a synergistic effect. (Chapter 5) These drugs would be expected to increase serum dihydrobiopterin levels and pharmaceutical preparations containing both of them such as co-trimoxazole, septrin and bactrin would be expected to give more than additive increase of serum dihydrobiopterin concentrations. Co-trimoxazole has indeed been shown to cause abnormal phenylalanine-tolerance tests in patients and slight phenylalanine intolerance occurred when the drugs were given individually which suggested that the drugs in combination had a synergistic effect on phenylalanine tolerance. Patients on co-trimoxazole also had raised fasting serum phenylalanine levels. (England and Coles 1972) Recently serum 7,8 dihydrobiopterin concentrations in volunteers who took trimethoprim or/and sulphamethoxazole have been measured and with trimethoprim alone there was a significant rise of about 1.88 μg/ml, sulphamethoxazole alone there was a small insignificant rise of about 0.06 μg/ml whereas together these drugs gave a significant rise of about 3.44 μg/ml. (Leeming 1980) So these results also fit the predictions from the model well. It has also been claimed that trimethoprim inhibits the conversion of 7,8 dihydrobiopterin to tetrahydrobiopterin by dihydrofolate reductase (Stone 1976) and this would also add to increased serum 7,8 dihydrobiopterin levels and lower

tetrahydrobiopterin levels. This would also be expected from the results presented in this thesis of the biosynthetic studies. (Chapter 3)

Patients with blood lead levels greater than 100 µg % (>$4.8.10^{-6}$M) have significantly lowered serum 7,8 dihydrobiopterin levels of 0.95 µg/L compared to the normal 1.75 µg/L (Leeming and Blair 1980a) and one child with lead poisoning had a serum level of 0.8 µg/L instead of the normal 1.78 µg/L. (Leeming and Blair 1980a) This fits well with the data presented in this thesis that lead inhibits tetrahydrobiopterin synthesis from GTP. (Chapter 3) There is experimental evidence from in vivo rat studies that also supports this hypothesis. (Hilburn 1979, Leeming 1979)

The correlation of the in vivo results with the predictions that can be made from the tetrahydrobiopterin metabolism model and the in vitro results obtained suggest that other diseases where biopterin levels are found to be abnormal may also be explained in a similar manner. However the effects on tetrahydrobiopterin metabolism could be a secondary effect of the disease and not the primary cause. Care must be taken when considering serum dihydrobiopterin levels because they are the result of tetrahydrobiopterin metabolism in all tissues and not just brain metabolism.

7,8 dihydrobiopterin levels in the serum have been shown to rise with age being 1.95 µg/L compared to the normal levels of 1.60 µg/L. (Leeming and Blair 1980a Leeming and Blair 1980b) Tyrosine hydroxy-

lase activity has been shown to decrease with age (McGeer 1978, Algeri, Bonati, Brunello and Ponzio 1977) as have dopamine and noradrenaline levels, (Austin, Connole, Kett and Collins1978) Inhibition at the level of dihydroneopterin triphosphate would give increased levels of neopterin and decreased serum dihydrobiopterin concentrations which have been measured. (Leeming and Blair 1980a, Leeming, Blair, Melikian and O'Gorman 1976, Rokos, Rokos, Frisius and Kirstaedker 1980)

Schizophrenic patients have been shown to have low serum dihydrobiopterin levels (Leeming, Blair, Melikian and O'Gorman 1976) and significantly high fasting levels of phenylalanine. (Poisner 1960) Urinary excretion of phenylethylamine has been reported to be significantly higher in paranoid chronic schizophrenics compared to normal patients. (Potkin, Karoum, Chatang, Cannon-Spoor, Phillips and Wyatt 1979) Dopamine levels in schizophrenia are raised. (O.H.E. 1979) Dopamine feeds back to inhibit tyrosine hydroxylase (Nagatsu, Mizatani, Nagatsu, Matsuura and Sugimoto 1972, Musacchio, D'Angelo and McQueen 1971) and limits its own production from tyrosine and this could explain the lowered serum dihydrobiopterin levels observed whereas the results presented in this thesis suggest that the dihydrobiopterin levels in the serum should be high due to inhibition of dihydropteridine reductase by dopamine. In a volunteer who took a monoamine oxidase inhibitor, Nardil, which should increase dopamine concentrations, the serum dihydrobiopterin concentrations decreased which agrees with the observations made in schizophrenic patients.

In Parkinson's disease the dopamine levels are low (Horynykiewicz 1966, Horynykiewicz 1973) and administration of precursor amino acids or L-DOPA have been found to be beneficial in many patients. (Cotzias, Van Woert, Schiffer 1967, Gey and Pletscher 1964) Patients are also given monoamine oxidase inhibitors which prolong the action of dopamine by inhibition of its breakdown. Levels of serum dihydrobiopterin in Parkinson's disease were found to be not significantly different from those of normal patients. (Leeming, Blair, Melikian and O'Gorman 1976) However all these patients were medicated except one in which the levels were higher than normal at 5.2 µg/L. In medicated patients the drugs increase dopamine levels which inhibit tyrosine hydroxylase and reduce dihydrobiopterin levels. Because the serum levels in Parkinson's disease are higher than normal anyway, the overall effect is that the serum levels of dihydrobiopterin are not significantly different from those of normal patients.

There is a difference in serum dihydrobiopterin levels between the sexes, normal males having higher levels (1.75 µg/L) than normal females (1.53 µg/L) with the greatest differences occurring between the ages of 21 and 30 which indicates there may be an hormonal involvement in tetrahydrobiopterin metabolism. (Leeming and Blair 1980a, Leeming and Blair 1980b, Leeming 1979) Women not taking oral contraceptives show a distinct cyclic pattern of serum dihydrobiopterin levels throughout the menstrual cycle whereas women taking oral contraceptives do not. (Leeming and Blair 1980a, Leeming 1979) If the first day of menstruation

is taken as day one of the cycle, the values of serum dihydrobiopterin in women not taking oral contraceptives decrease from day one, the lowest value occurs at around day twelve. Then there is a peak at about day twenty and the values then rise steadily at the end of the cycle to the highest value on day twenty-eight just before the onset of menstruation. These changes in levels can be loosely correlated with the changes in levels of oestrogens throughout the menstrual cycle. (Guyton 1971) Tyrosine hydroxylase activity has also been shown to vary throughout the menstrual cycle (Voogt, Carr and Levin 1979) and to increase following ovariectomy in rats (Beattie, Rodgers and Soyka 1972) whereas oestrogen replacement therapy in ovariectomized rats has been shown to decrease tyrosine hydroxylase activity in the substantia **nigra.** (Luine, McEiwen and Black 1977) The hypothalamus is capable of converting oestrogens to catechol oestrogens such as 2 hydroxyestradiol and 2 hydroxyestrone (Fishman and Norton 1975, Fishman 1976, Paul, Axelrod and Diliberto 1977) and these have been shown to inhibit the activity of tyrosine hydroxylase. (Lloyd and Weisz 1978, Foreman and Porter 1980) The catechol oestrogens are similar in structure to dopamine and hence could inhibit dihydropteridine reductase as dopamine does. (Chapter 5) This would result in high serum levels of dihydrobiopterin when the levels of catechol oestrogens are high, and hence would explain why the serum dihydrobiopterin levels correlate with oestrogen levels throughout the menstrual cycle. A form of depression known as premenstrual tension occurs at the time of the paramenstruum (Dalton 1971, Dalton 1964, Wickham 1958) and

this is the time of high serum dihydrobiopterin concentrations. It is therefore probably a time of decreased dopamine and noradrenaline biosynthesis due to decreased tyrosine hydroxylase activity because of decreased cellular tetrahydrobiopterin concentrations. This could explain the depression and in-attentiveness seen in premenstrual tension. (Dalton, 1971a, Dalton 1964, Wickman 1958, Dalton 1970, Dalton 1960)

Levels of serum dihydrobiopterin have also been shown to vary in pregnant women, being low at week 38 (1.01 µg/L) and rising rapidly post-partum. (Leeming and Blair 1980c) Post-partum depression is also common. (Dalton 1971a, Dalton 1971b) The women post-partum could be divided into two groups; i) those who became depressed and ii) those who did not. The first group had a greater serum dihydrobiopterin than the second group (Leeming and Blair 1980c) which indicates again that high levels of serum dihydrobiopterin are correlated with depression.

Finally patients diagnosed as suffering from depression also had significantly high serum dihydrobiopterin levels compared to normal patients, and this was in both male (2.26 µg/L) and female (1.87 µg/L) depressives. (Leeming 1979)

It seems therefore that the model of tetrahydrobiopterin metabolism can be used to explain the differences in serum dihydrobiopterin concentrations in disease where these have been found to alter. Increased serum dihydrobiopterin levels can be explained as decreased dihydropteridine reductase activity but decreased serum dihydrobiopterin

levels are more difficult to attribute to a defect unless further measurements and observations are made. This is because both decreased de novo biosynthesis of tetrahydrobiopterin and decreased tyrosine hydroxylase activity can lead to this condition. The tetrahydrobiopterin metabolism model explains the situation occurring in both health and certain well-defined disease states such as phenylketonuria and malignant hyperphenylalaninaemias so it can be determined that cellular tetrahydrobiopterin levels are closely maintained by two processes, the dihydropteridine reductase salvage pathway and the de novo tetrahydrobiopterin biosynthetic pathway but the activity of the hydroxylase reactions also has some effect. Interference of these pathways upsets the balance of cellular tetrahydrobiopterin and because it is required for catechol neurotransmitter production, the balance of these neurotransmitters as well. This can lead to mental defects and the appropriate disease syndrome develops.

In order to further understand the control and interaction of these pathways, further study is required and their activities should be related to various disease states. The biosynthetic pathway, particularly requires more consideration by the isolation of each enzyme involved and these being studied individually in order to discover which particular step is effected by the reagents. The measurements of serum dihydrobiopterin, tyrosine and phenylalanine concentrations in disease states needs to be related to activities of enzymes of tetrahydrobiopterin metabolism. Dihydropteridine reductase activity can now be

measured in platelets, lymphocytes and granulocytes which are easily obtainable from blood samples, so making the direct assay of this enzyme from patients with diseases easy. (Firguira, Cotton and Danks 1979, Abelson, Gorka and Beardsley 1979) Compounds suspected of being the agent responsible for interference with tetrahydrobiopterin metabolism such as the catechol estrogens in the menstrual cycle can be tested on the in vitro systems such as those which have been setup for the work presented in this thesis and also on in vivo animal models or volunteers.

By co-ordinating the results from all such studies, answers to many of the problems of tetrahydrobiopterin metabolism may be obtained.

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