TETRAHYDROBIOPTERIN METABOLISM IN MENTAL RETARDATION

by

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A thesis submitted for the degree of

Doctor of Philosophy

at

The University of Aston in Birmingham

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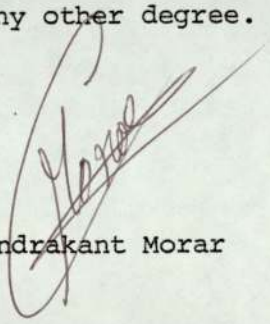
TO MY PARENTS

The work described in this thesis has been carried out between 1981-1984 at the University of Aston in Birmingham. It was done independently and has not been submitted for any other degree.



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Thesis : "Tetrahydrobiopterin Metabolism in Mental Retardation"

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Submitted for the degree of Doctor of Philosophy (1984)

SUMMARY :

Optimal conditions for the separation and detection of pteridines by high performance liquid chromatography (HPLC) were obtained. The retention time, reproducibility relative fluorescence and minimal detection limits of various pterins under fixed conditions was noted.

The stability of pterins under different oxidation conditions was investigated. Acid- and alkaline-oxidation of reduced biopterins produced almost stoichiometric conversion to biopterin or pterin and was used to quantitate dihydrobiopterin and tetrahydrobiopterin levels. Atmospheric oxidation of tetrahydrobiopterin at various pH showed it to be most unstable, especially at alkaline pH. Tetrahydrobiopterin oxidation was rapid in the presence of superoxide ions and was suppressed by superoxide dismutase. Ascorbate in the presence of iron acted as a potent oxidising agent; probably by a free-radical mechanism.

The analysis of tetrahydrobiopterin (THB) by HPLC and by Crithidia-assay was compared. Crithidia-assay values were usually lower. Tetrahydrobiopterin losses probably occur in Crithidia-assay during incubation / autoclaving procedures.

Biological samples from phenylketonurics, malignant hyperphenylalaninaemics, and healthy subjects were analysed. Phenylketonuric urine showed elevated biopterin level and normal neopterin-biopterin ratio. Dihydropteridine reductase (DHPR) deficient subjects showed low urine THB level with low blood DHP R activity. Acute oral phenylalanine loading in man produces a transient rise in total biopterin and in the rat a decrease in brain per cent THB.

Reduced THB synthesis with low biopterin level and high neopterin-biopterin ratio was noted in senile dementia brains. Down's syndrome patients showed low plasma neopterin indicating a possible synthesis defect. Acute lymphoblastic leukaemia children on methotrexate showed elevated urinary biopterin excretion whilst fragile-X patients showed no change from controls. Affective bipolar patients had low urine pterin levels. The implications of these changes are discussed.

Animal experiments show lead, aluminium and methotrexate to reduce brain per cent THB level and diethylstilboestrol to lower total biopterin pool. The mechanisms and consequences of such changes are discussed.

Key words : Tetrahydrobiopterin; phenylketonuria; senile dementia

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CHAPTER ONE : INTRODUCTION

1.1 Naturally Occuring Pteridines

Pteridines are a group of compounds containing the pyrimido-[4,5b] pyrazine ring :

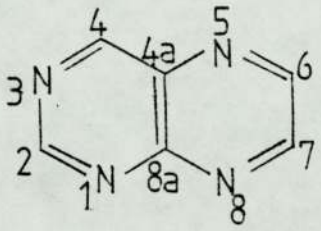
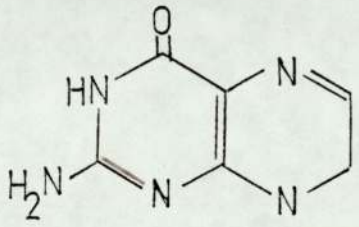


Fig.1.1
Pteridine

Naturally occurring pteridines are chemically related to pterin (2-amino-4-oxodihydropteridine)

Fig.1.2 Pterin



Pteridines including pterin are widely distributed in the animal kingdom. In 1889 Sir Frederick Gowland Hopkins isolated some yellow pigment from butterfly wings (Hopkins 1889) which was later identified as containing leucopterin and xanthopterin by Wieland and Schopf (1926)

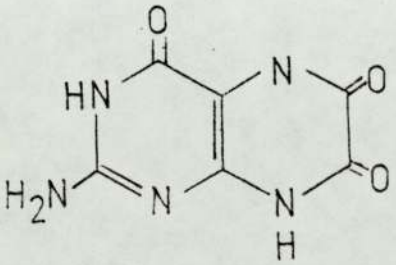
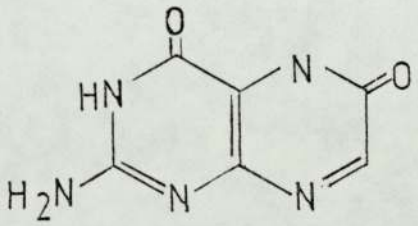


Fig.1.3 Leucopterin



Xanthopterin

The yellow eye pigments of Drosophila have been attributed to sepiapterin and related pterins (Dorsett, 1979)

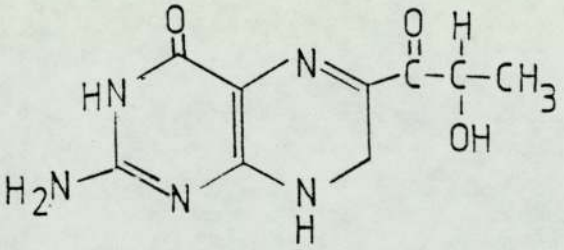
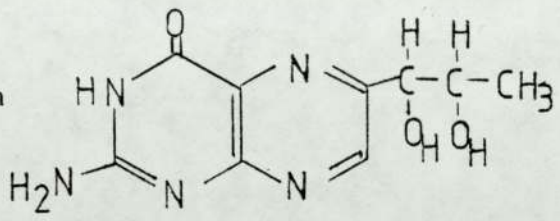


fig.1.4
Sepiapterin

Other pteridines have been isolated from sources such as fish scales (e.g. of the goldfish Carassius auratus), larvae of newts (Triturus pyrrhogaster) and the skin of the frog Buffo vulgaris (Hama et al 1962).

Pterins and in particular, biopterin have been isolated from all mammalian tissues investigated to date. The kidney actively synthesises biopterin and human and animal urines are readily accessible sources of pterins (Haberle et al 1978).

Fig.1.5 Biopterin

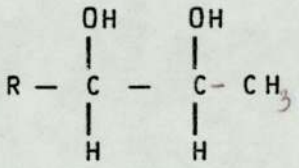


The historical findings have been more exhaustively reviewed by Brown 1981 and will not be attempted here.

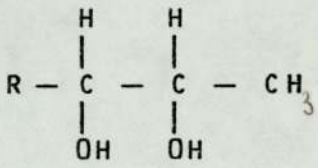
The presence of a dihydroxypropyl side-chain at carbon atom 6 means that four optical isomers of biopterin exist due to chiral carbon atoms at positions 1' and 2'.

Fig.1.6 Isomeric forms of biopterin

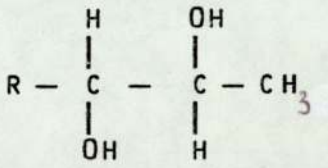
(R=2-amino-4-hydroxy pteridine moiety)



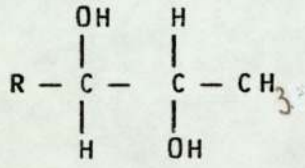
D-erythro



L-erythro



D-threo



L-threo

The correct isomeric form is important in biological systems as enzymes are able to utilise only certain isomers. For example, the L-erythro form of dihydroneopterin is not utilised for the synthesis of folic acid in L-plantarum and is also an inhibitor of the D-erythro form utilization (Rembold and Gyure 1972).

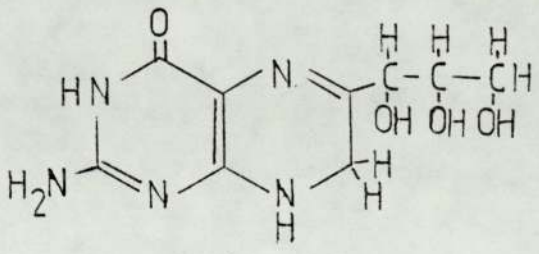
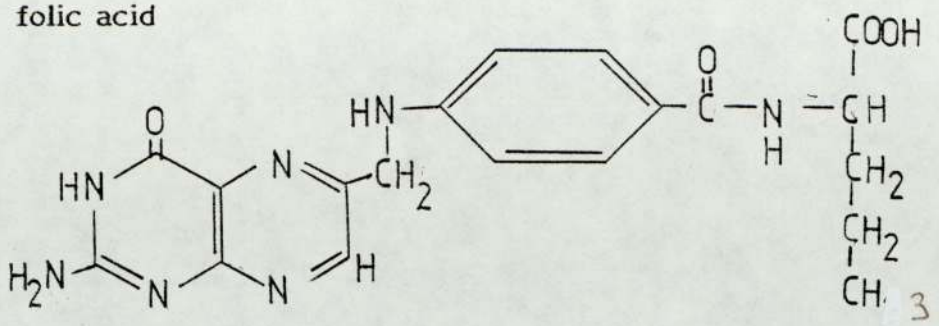


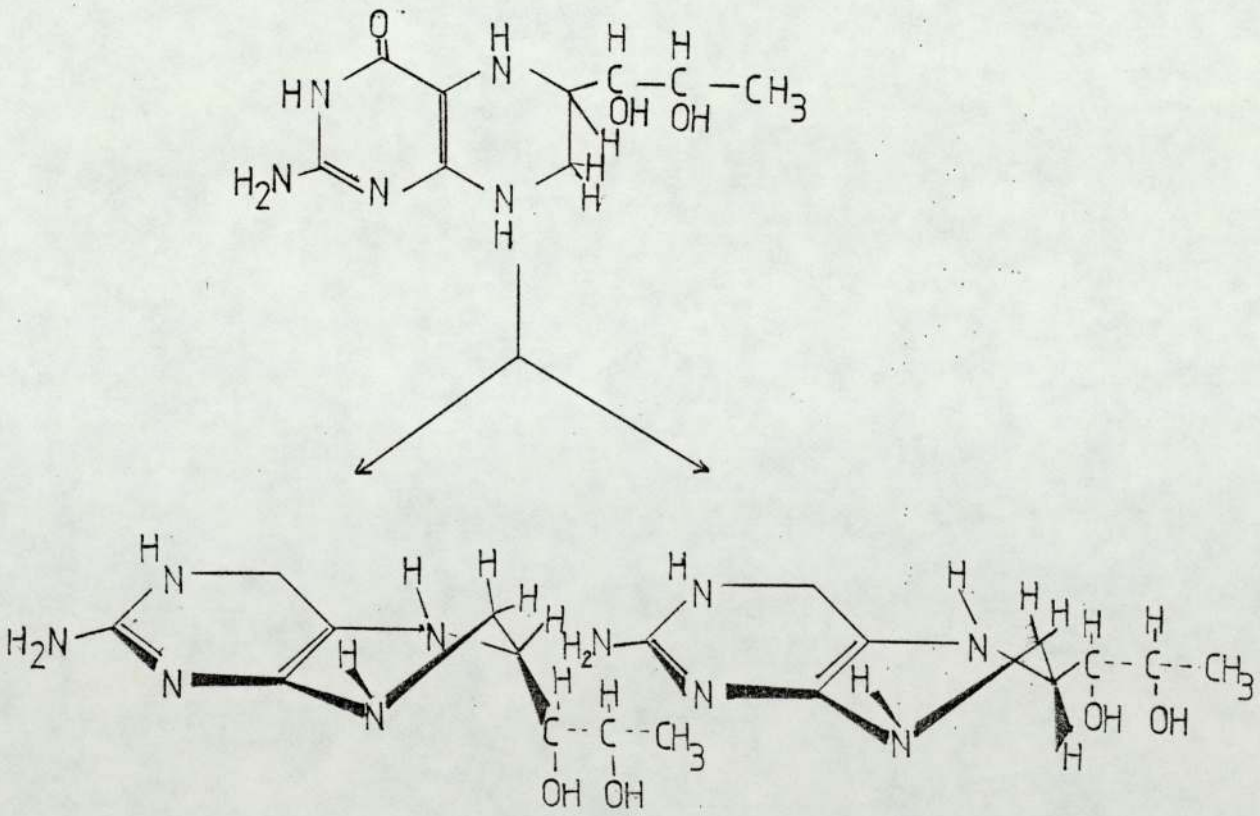
fig.1.7
dihydroneopterin

Fig. 1.8
folic acid



The fully reduced form of biopterin, 5,6,7,8-tetrahydrobiopterin has an additional chiral atom at carbon 6 giving rise to two diastereoisomers. The naturally occurring L-erythro-tetrahydrobiopterin (fig. 1.9) therefore exists as an [S]-isomer and an [R]-isomer, (Bailey and Ayling 1978).

Fig. 1.9 5,6,7,8-tetrahydrobiopterin and its diastereoisomers



(6S) -5,6,7,8-tetrahydro-L-biopterin

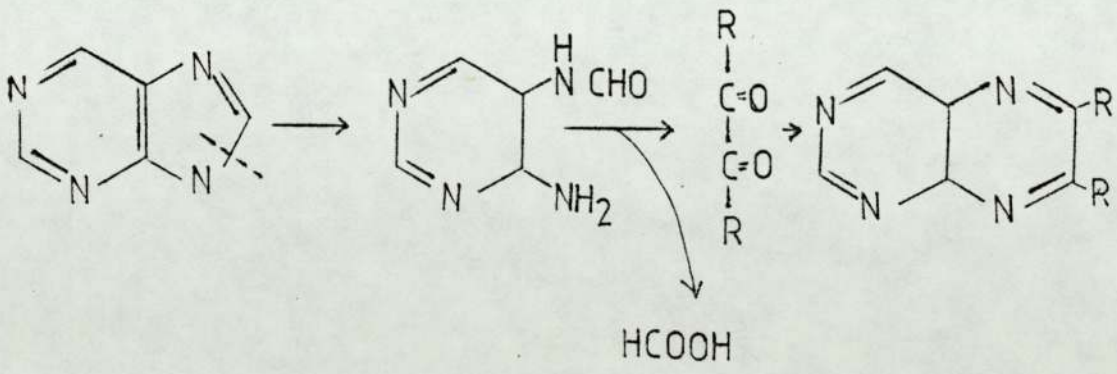
(6R) -5,6,7,8-tetrahydro-L-biopterin

Both diasterioisomers serve as cofactor for the enzyme phenylalanine hydroxylase, exhibiting identical Km values. However, the [S] isomer has a higher Vmax and shows substrate inhibition with the hydroxylase and phenylalanine and is suggested to be the natural cofactor (Bailey and Ayling 1978).

1.2 Tetrahydrobiopterin biosynthesis :

The structural similarities between purines and pteridines (fig. 1.10) initially produced speculation that purines may be precursors of pteridines. Albert (1954) showed that purines may be transformed to pteridines by incubation with 1,2-dicarbonyl compounds. In suitably substituted purines chemical treatment leads readily to the imidazole ring opening giving rise to a pyrimidine analogue (fig. 1.10). The latter may then yield a pterin by loss of the formyl group and subsequent reaction with a dicarbonyl compound.

Fig. 1.10 conversion of a purine to pterin



Evidence to support such a reaction in biological systems was presented by Weygand et al (1955) who supplied Pieris brassica caterpillars with ^{14}C-formate and ^{14}C-glycine and subsequently isolated labelled pteridine from the adult butterflies. Positions 4 and 5 of the pteridine ring were labelled in a comparative manner to incorporation of glycine into purines. Labelled-guanine was shown to be incorporated into pterin-6-carboxylic acid in the skins of Xenopus Laevis giving further proof of purines being pteridine precursors (Ziegler-Gunder et al 1956).

Additional carbon atoms needed to transform purine to pteridine were suggested to come from the ribose moiety of guanylic acid (Krumdieck et al 1966). When a culture of Corynbacterium was cultivated in a media containing uniformly labelled guanylic acid, the label was incorporated into the pteridine ring. Examination of the specific radioactivity of the individual carbon atoms showed good correlation between the specific activity of carbon atoms 6,7 and 9 of the pteridine ring and carbon atoms 2', 1' and 3' of the ribose moiety of guanylic acid. These workers suggest that all the carbon and nitrogen atoms of the pteridine ring arise from guanylic acid. Furthermore the "parent" pteridine to be synthesised by this reaction was suggested to be 2-amino-4-hydroxy-6-trihydroxypropyl pteridine (neopterin) in a reduced and phosphorylated form, (Fig. 1.12).

Evidence for the phosphorylated form of neopterin was given by Goto et al (1961) who isolated neopterin as a phosphate ester from cultures of E.Coli. Vierra et al (1961) showed that carbon-8 of the purine ring is not incorporated into the pteridine ring by growing Corynbacterium sp in ^{14}C-adenine labelled either at position 2 or 8. Very little of the label from adenine-8-^{14}C was incorporated into the pteridine.

For the conversion of guanylic acid to pteridine to be authenticated it needed to be shown that the pyrimidine so obtained following imidazole ring opening and loss of carbon-8, underwent the Amadori re-arrangement and ring-closure. Stuart et al (1962) successfully isolated the intermediate generated by the Amadori re-arrangement as the 1-(substituted amino)-1-deoxypentulose (fig. 1.11). This compound is labile and readily undergoes cyclization to the pteridine.

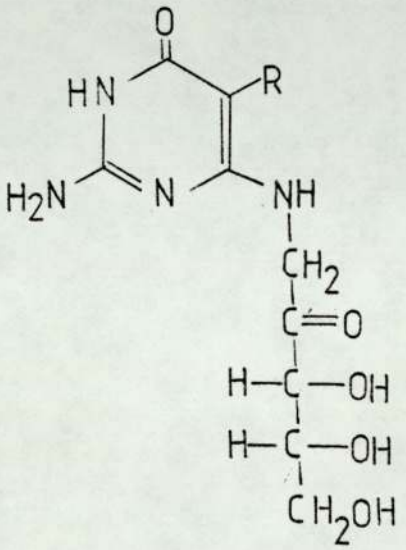
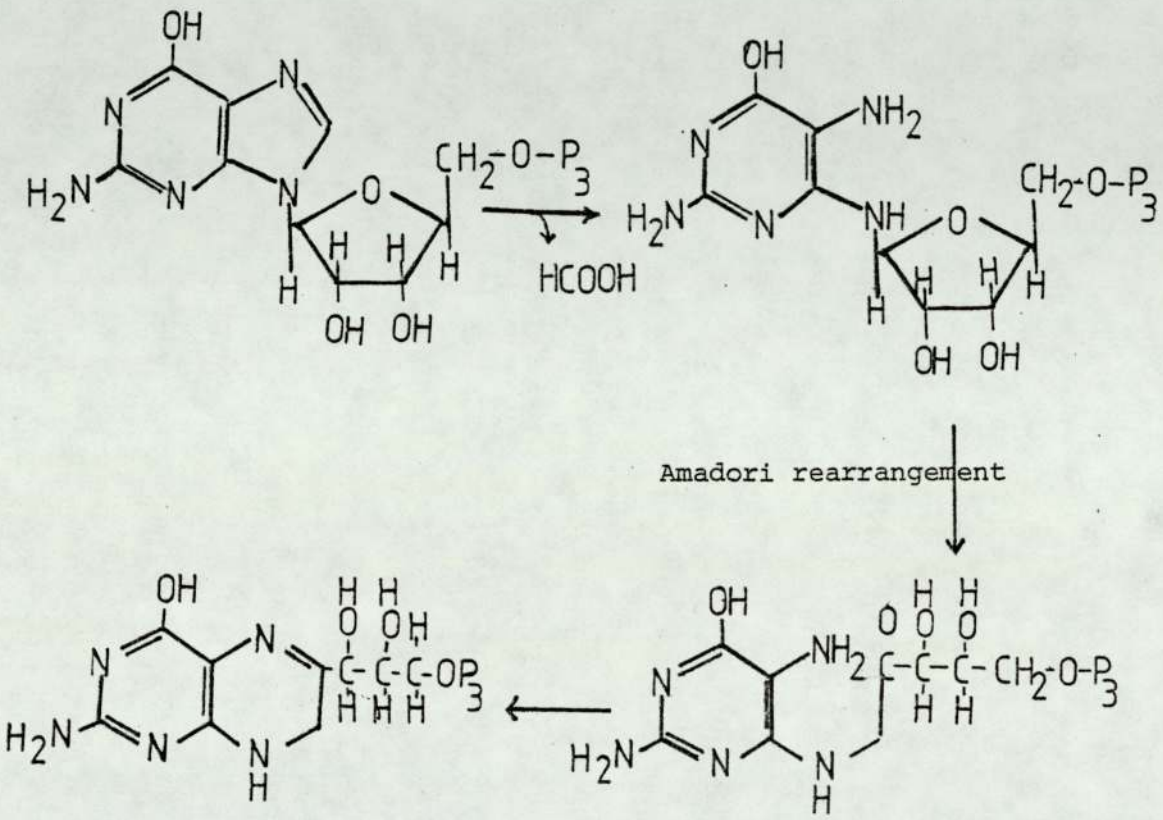


Fig.1.11 1-(Substituted amino)-1-deoxypentulose



7,8-dihydroneopterin
triphosphate

fig.1.12: 7,8-DIHYDRONEOPTERIN TRIPHOSPHATE BIOSYNTHESIS

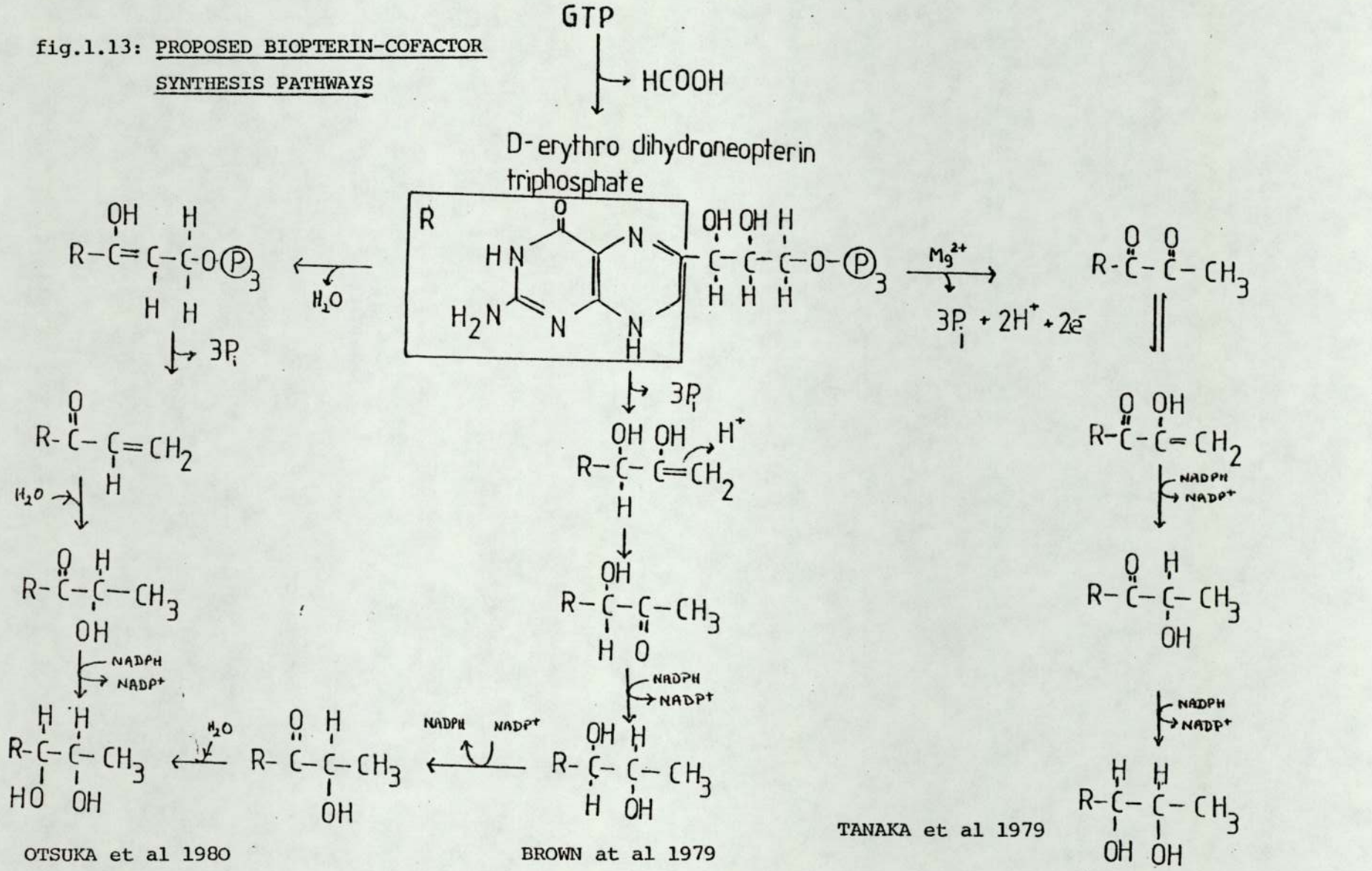
Subsequent to dihydroneopterin triphosphate (NH_2P_3) synthesis, conflicting reports exist. Nichol et al (1983) suggest that sepiapterin does not participate in the de novo biosynthesis of tetrahydrobiopterin (THB). They observe that methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR) does not affect THB biosynthesis when administered intracisternally into rat brains. Nor does it affect in vitro biosynthesis of THB from GTP. However, it does inhibit the in vitro conversion of sepiapterin and 7,8-dihydrobiopterin to THB in bovine adrenal medulla. Thus they conclude that both sepiapterin and 7,8-dihydrobiopterin are not intermediates in the de novo THB biosynthesis which is methotrexate-insensitive. They suggest that a "salvage" pathway exists which is MTX-sensitive and DHFR-dependent and which utilises sepiapterin and 7,8-dihydrobiopterin.

This would appear to confirm the earlier work of Gal et al (1978) who suggest synthesis of quinonoid-dihydrobiopterin from GTP, via quinonoid-dihydroneopterin triphosphate. This proposal omits sepiapterin from the de novo biosynthesis. However, these results have not been confirmed by other workers.

The above mechanism differs markedly from the one proposed by (Tanaka et al 1979; Brown et al 1979; and Otsuka et al 1980). These workers suggest that sepiapterin is an essential intermediate in THB biosynthesis. They differ from each other in their approach to the conversion of the D-erythro side chain of the NH_2P_3 to the L-erythro form of biopterin. The mechanisms are summarised in fig. 1.13.

Support for the involvement of sepiapterin in the biosynthesis of L-erythro-dihydrobiopterin (DHB) comes from a report by Niederwieser et al (1980) of a patient with defective dihydrobiopterin biosynthesis. The

fig.1.13: PROPOSED BIOPTERIN-COFACTOR SYNTHESIS PATHWAYS



patient was unable to convert dihydroneopterin triphosphate to dihydrobiopterin and this lead to elevated urinary excretion of 3'-hydroxysepiatpterin. The blockage was suggested as being prior to L-sepiapterin synthesis.

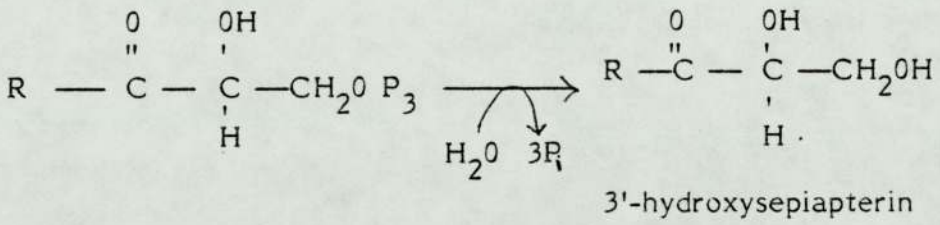


Fig. 1.14 3'-hydroxysepiapterin triphosphate

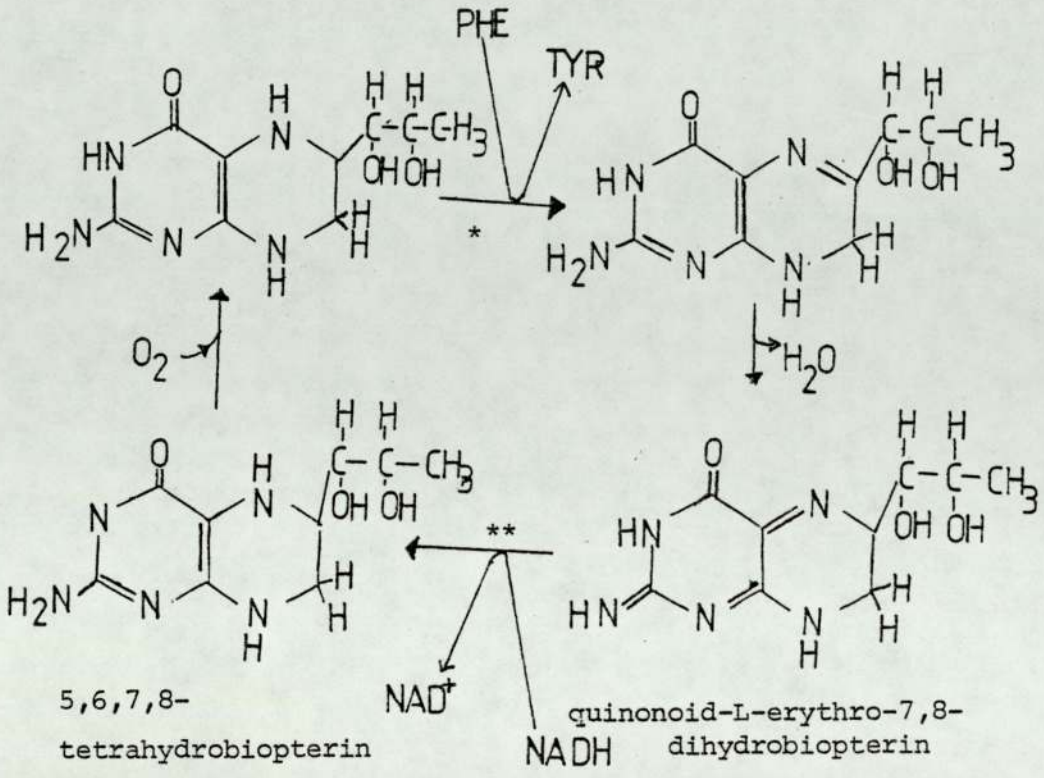
Such a pathway is, however, difficult to realise since the enzyme dihydropteridine reductase has been shown not to use 7,8-dihydrobiopterin as substrate but uses the quinonoid form of this molecule. Also, suggestions of DHFR being responsible for the conversion of DHB to THB are also unrealistic since maintenance of active cofactor levels in the brain would not be feasible as brain DHFR levels are too low (Makuln et al 1973).

Hientel et al (1984) propose tetrahydropterin intermediates being formed immediately after dihydroneopterin triphosphate synthesis. These workers suggest that sepiapterin, dihydrobiopterin and DHFR are not involved in THB biosynthesis in vivo. These findings are supported by Switchenko et al (1984) and Smith and Nichol (1984) who also report observation of tetrahydropterin intermediates that give rise to THB. However, Smith and Nichol (1984) have found that removal of sepiapterin reductase causes accumulation of a tetrahydropterin intermediate without THB formation. Further work should precise the mechanism of THB biosynthesis.

3.1 Tetrahydrobiopterin Involvement in Neurotransmitter Synthesis and other Reactions :

The involvement of tetrahydrobiopterin has been implicated in a number of biological systems. For example, Tietz (1964) showed it to be involved in the oxidation of long-chain alkylethers of gylcerol to fatty acids and free glycerol. Hagerman (1964) implicated tetrahydrobiopterin in the ω-hydroxylation of progesterone.

It has also been reported that the reduction of cytochrome C (from rat liver mitochondria) by tetrahydrobiopterin results in ATP synthesis (Taylor and Hochstein, 1975). This reaction is not diminished by the presence of superoxide dismutase and therefore unlikely to be due to superoxide radical generation as a result of tetrahydrobiopterin oxidation. Rembold and Metzger (1967) have shown that mitochondrial respiration is stimulated by physiological concentrations of tetrahydrobiopterin and the utilization of oxygen is proportional to the tetrahydrobiopterin concentration. The ubiquitous presence of tetrahydrobiopterin in the body favours such a role, as an electron-carrier in the electron-transport chain.



* phenylalanine hydroxylase

** dihydropteridine reductase

fig.1.15: SALVAGE OF TETRAHYDROBIOPTERIN COFACTOR BY DIHYDROPTERIDINE REDUCTASE

It is now well accepted that tetrahydrobiopterin is an essential cofactor for the enzymic reactions involving phenylalanine- , tyrosine- and tryptophan hydroxylases. The latter two enzymes being involved in the biosynthesis of catecholamines and 5-hydroxytryptamine, respectively (fig.1.16, 1.17).

It was first established by Kaufman (1959) that tetrahydrobioptein is involved in the hydroxylation of phenylalanine to tyrosine, in the presence of oxygen. It was later shown by the same author (Kaufman, 1964) that the reaction results in the production of a quinonoid dihydrobiopterin, which in turn needs to be reduced to tetrahydrobiopterin by the enzyme dihydropteridine reductase (fig.1.15).

Phenylalanine hydroxylase is highly specific for L-phenylalanine although under some circumstances it will also act on tyrosine as substrate (e.g. in the presence of lysolecithin (Fisher and Kaufman, 1973). With regards to cofactor specificity, the enzyme has been shown to utilise 5, 6, 7, 8-tetrahydrobiopterin or its 6, 7-dimethyl or 6-methyl synthetic analoges (Kaufman, 1963). The apparent Km of the enzyme with respect to tetrahydrobiopterin is 2.6×10^{-6}M at pH 6.9, 25°C, (Fisher and Kaufman, 1973), and the cofactor levels are suggested to be of the same order under physiological conditions in the brain, (Fisher and Kaufman, 1973).

The enzyme has been shown to be a tetramer (Fisher and Kaufman, 1973), with the subunits associating in a co-operative manner to increase enzyme activity. The quaternary structure and hence the enzyme activity are thus readily influenced by various agents. For example, lysolecithin and α-chymotrypsin enhance the activity. The former by inducing conformational change leading to sulphydryl group exposure and the latter by partial hydrolysis of the enzyme. (Fisher and Kaufman 1973).

Abita et al (1980) have found that glucagon and noradrenaline enhance phenylalanine hydroxylase activity. These authors suggest that hormones such as glucagon regulate the hydroxylase activity by elevating cellular cAMP levels which in turn produce changes in cAMP-dependant protein kinase. The kinase in turn is suggested to phosphorylate phenylalanine hydroxylase and thereby increase the affinity of the enzyme for its substrate.

The existence of the enzyme in subunit form gives rise to the possibility of phenylketonuric variants with partially active enzyme (Friedman et al 1973). A condition of hyperphenylalaninaemia may also be caused by defective tetrahydrobiopterin biosynthesis or regeneration, (Dhondt 1984).

The primary and rate-limiting step in the synthesis of the catecholamines involves the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa), by the enzyme tyrosine hydroxylase (E.C. 1.10.3.1) fig.1.16. Nagatsu et al (1964) have shown that the reaction requires tetrahydrobiopterin as cofactor.

The involvement of tetrahydrobiopterin as a cofactor in tyrosine hydroxylation has been established by fulfillment of the following criteria : (a) there is a specific requirement for tetrahydrobiopterin which is substrate (tyrosine) dependant and (b) the pterin functions catalytically and is regenerated by the enzyme dihydropteridine reductase (Kaufman 1973).

The Km of the brain enzyme with respect to tetrahydrobiopterin is 2.5×10^{-4}M (Kaufman, 1973). The actual brain concentration of the cofactor has been estimated at 1×10^{-6}M by the same author. The evidence that THB levels are rate-limiting to biogenic amine synthesis is also shown by

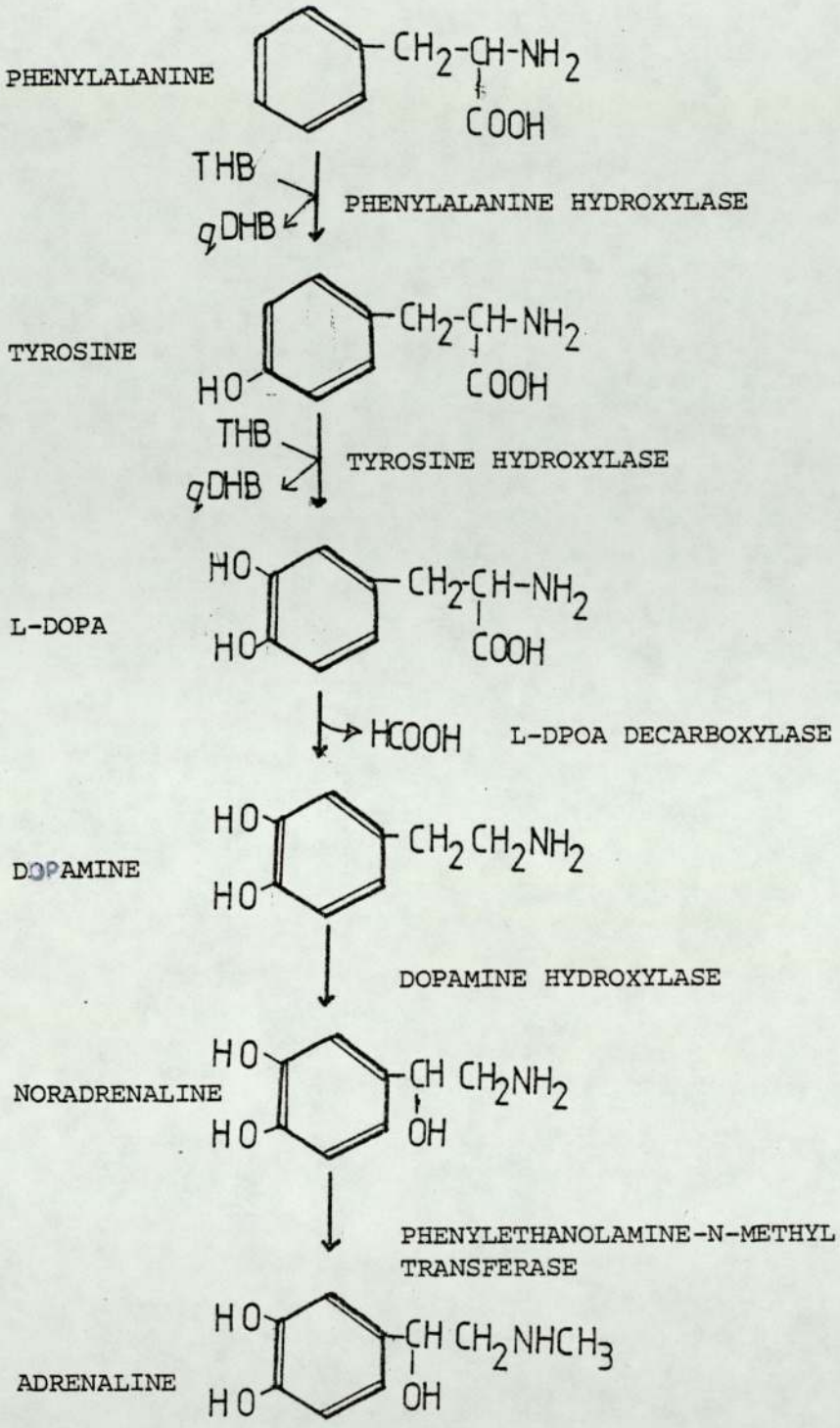


Fig.1.16: CATECHOLAMINE BIOSYNTHESIS

Cote et al (1975). Addition of biopterin to sympathetic nerve tissue cultures results in increased conversion of tyrosine to catecholamines, and dihydropteridine-reductase inhibitors abolish noradrenaline fluorescence in neuronal processes, Cote et al 1975.

A peculiar property of the enzyme is its ability to readily use phenylalanine as a substrate in the presence of tetrahydrobiopterin but not in the presence of the 6,7-dimethyl analogue of THB (Shiman et al, 1971). The importance of this to phenylalanine hydroxylase deficiency condition has yet to be established, (Karobath et al, 1972). Another THB-dependant property is the inhibition of the enzyme by excess substrate (both tyrosine and oxygen) and by product L-dopa (Kaufman, 1973).

Hormonal control of tyrosine hydroxylase activity is shown by glucagon administration which increases the conversion of phenylalanine to tyrosine in the rat liver (Fuller et al, 1974). In addition, cAMP has been shown to mediate activation of tyrosine hydroxylase in vitro by reducing the Km of the enzyme for the pterin cofactor (Lovenberg et al, 1975). The mechanism may involve a direct phosphorylation of tyrosine hydroxylase by a cyclic-AMP-dependant protein kinase, which would alter affinity of enzyme for pterin-cofactor.

Kuczenski et al (1972) maintain that dopamine and noradrenaline inhibit tyrosine hydroxylase competitively with respect to the pterin cofactor. This mechanism of end-product inhibition is suggested to regulate catecholamines synthesis.

Hence a number of mechanisms have been reported to alter the affinity of the pterin-cofactor for tyrosine-hydroxylase. In addition, the rate-limiting levels of the cofactor provide a means of regulating catecholamines synthesis at this stage, as well as making it vulnerable to various inhibiting agents.

Tryptophan hydroxylase (EC 11.14.16.4) is the other neurotransmitter generating enzyme which has a requirement for tetrahydrobiopterin (Nakamura et al, 1965). The enzyme catalysis the conversion of tryptophan to 5-hydroxytryptophan in the biosynthesis of 5-hydroxytryptamine (fig. 1.17).

Despite the concentration of brain tetrahydrobiopterin (1×10^{-6}M) being less than the Km of the enzyme with respect to tetrahydrobiopterin (3.5 to 5×10^{-5}M), it is suggested that the cofactor level isn't rate-limiting for the enzyme (Gal, 1981). This is supported by the evidence that substantial induced elevation of brain tetrahydrobiopterin does not produce comparative increases in brain catecholamine levels. However Kettler et al 1974 suggest brain cofactor levels to be rate-limiting.

The affinity of tryptophan hydroxylase for pterin cofactor has been reported to vary under various conditions. Drugs such as reserpine, haloperidol and methiothepin increased the affinity of the enzyme for the cofactor and the actions of these neuroleptics may partially be mediated through such an effect (Zivkovic et al, 1975). In the pres ence of Ca^{++} ions a protein kinase is suggested to phosphorylate tryptophan hydroxylase and increase its affinity for the pterin-cofactor, (Hamon et al, 1981).

Phospholipids may also regulate tyrosine hydroxylase activity by enhancing cofactor affinity for enzyme (Hamon et al, 1981).

In general the pterin-dependant hydroxylases appear to be under the influence of many agents which may act in a modulatory capacity to regulate neurotransmitter biosynthesis, or which may inhibit to the detriment of neuronal homeostasis. A number of modulatory effects are mediated through alteration in the affinity of THB cofactor for the enzyme.

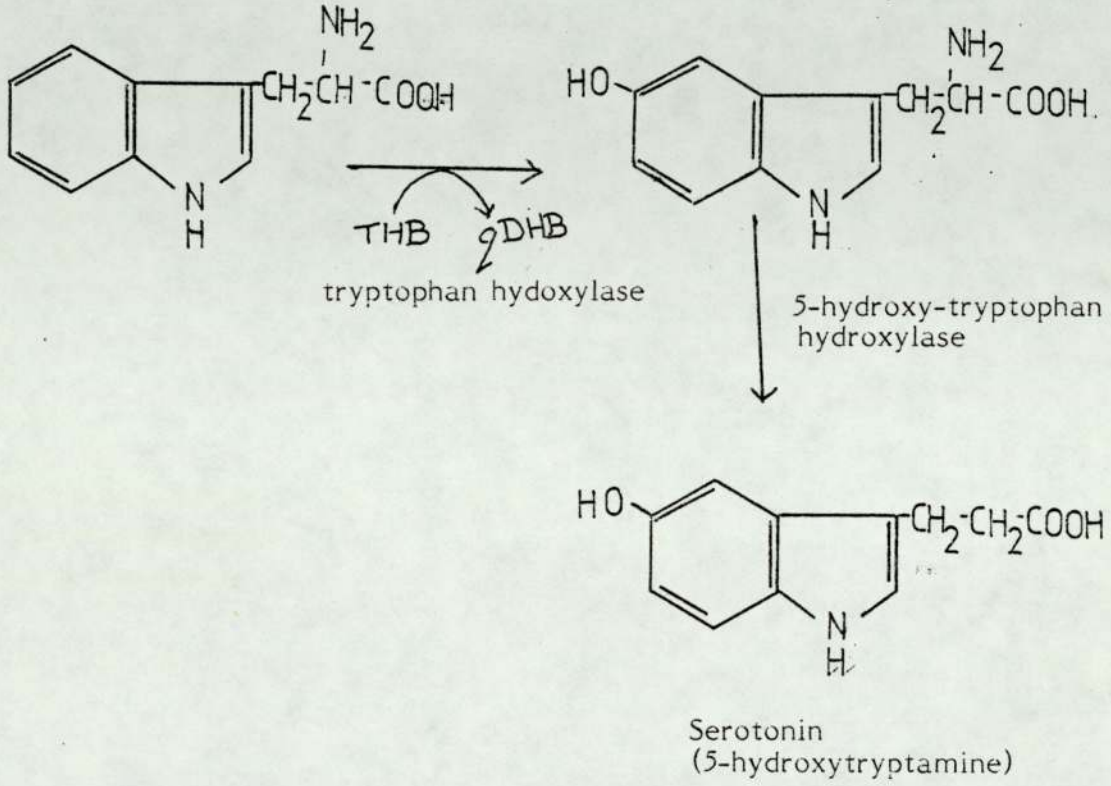


Fig. 1.17. Seroto nin Biosynthesis

1.4 Tetrahydrobiopterin Metabolism In Health And Disease.

The discovery of pteridines has been accompanied by a prolific amount of data on pteridine levels in various body compartments under various conditions of health and disease. A review of this literature is necessary to discuss the implications of change in pteridine levels in the body.

In normal subjects serum biopterin levels are suggested to remain fairly constant (Leeming et al, 1981). This is probably also true of most other tissues under normal conditions. Males appear to have a higher serum biopterin compared to females (Leeming and Blair, 1980). However, Bichler et al (1982) report the converse to be true when urinary neopterin values are assessed. Leeming et al (1980) report elevation of serum biopterin with age whilst Bichler et al (1982) report elevation in urinary neopterin with age, in both sexes.

In normal subjects, the urinary biopterin levels are the highest when compared to the other body fluids (whole blood and csf), Leeming et al, 1976. This is probably due to the contribution of biopterin by the kidney which actively synthesis the pterin (Haberle et al, 1978). The csf biopterin levels are higher than serum probably due to the restricted transfer of pterins across the blood-brain barrier or a high brain synthesis. Within the brain biopterin levels vary within different anatomical regions (Leeming et al, 1976; Bullard et al 1978). The substantia nigra has a particularly high biopterin concentration which reflects its role as a dopaminergic nucleus.

Leeming and Blair (1980) report a variation in serum biopterin during the menstrual cycle with a high value immediately prior to menstruation. A similar pattern is observed by Bichler et al (1982) in urinary neopterin excretion.

TABLE 1.1 : BIOPTERIN LEVELS IN NORMAL SUBJECTS AND DISEASED PATIENTS.

Clinical condition	Sample	Biopterin level	Reference
Parkinson's Disease (11)*	csf	9.1 pmol./ml	Williams et al 1980.
Shy-Drager Syndrome (4)	"	4.5 "	"
Steele-Richardson Syndrome (7)	"	8.5 "	"
Pre-Senile dementia (8)	"	7.8 "	"
Controls (13)	"	17.5 "	"
Depressed (unipolar + bipolar) (13)	csf	16.4 ± 2.4**	Kellner et al 1983.
Controls (24)	"	19.7 ± 1.6	"
age-corrected depressives	"	18.4 ± 2.0	"
Torsion dystonia (-)	csf	3 pmol./ml	Williams et al 1979.
Controls	"	20.2 "	"
Parkinson's Disease (10)	csf	8.9 ± 0.95	Lovenberg et al 1979.
Controls (10)	"	17.7 ± 1.69	"
Acute lymphocytic leukaemia (children)(28)	Serum	18.5 ± 2.3 pmol./ml.	Leeming et al 1980
Senile dementia (15)	Serum	4.2 ± 0.5 pmol./ml	"
Controls (age matched for dementia) (21)	"	8.2 ± 0.8 pmol./ml	"
Coeliac disease untreated (5)	"	4.3 ± 0.4 pmol./ml	"
Healthy adults (231)	"	6.7 ± 0.1	"

** ± SEM.
* Number of subjects studied.

TABLE 1.1 : BIOPTERIN LEVELS IN NORMAL SUBJECTS AND DISEASED PATIENTS. (continued.)

Clinical condition	Sample	Biopterin level	Reference
Control (26)	serum	6.8 ± 2.4 pmol./ml	Wachter et al (1982)
Breast Cancer (57)	"	5.5 ± 1.6 pmol./ml	"
Controls (39)	Urine	699.8 ± 288.2+	Rokos et al (1983)
Renal disease (6)	"	306.2 ± 88.8	"
Leukaemia + lymphoma (15)	"	623.8 ± 399.3	"
Breast Cancer (7)	"	701.3 ± 533.7	"
Gastrointestimal cancer (10)	"	912.5 ± 396.4	"
Controls (53)	"	679 ± 186	Wachter et al (1982)
Breast Cancer (54)	"	554 ± 191	"
Parkinson's disease (21)	"	660 ± 66.7	Nagatsu et al (1981)
Controls (13)	"	738 ± 76.8	"

+ µmol./mol. creatinine.

During gestation the urinary neopterin value increases with advancing pregnancy reaching a peak prior to puerperium (29-42 weeks). The foetal amniotic fluid neopterin levels are low until 34th week of gestation after which they increase to more than two-fold (over 50 nmol./l), Bichler et al 1982. This may either be due to increased foetal synthesis of pteridines or due to cross-placental transfer from the mother who also shows a high urinary neopterin level at this stage. Leeming and Blair (1982) observe a rise in amniotic biopterin level at an earlier stage (24th week).

Newborn infants tend to show high neopterin to biopterin ratio (e.g. urine 4.4, serum 11.4) compared to adults (urine 0.5, serum 2.4), Dhondt et al 1982a. This reflects the high biopterin synthesis accompanying the rapid tissue growth in the neonate. As the "biopterin synthetase" appears to be rate-limiting, dihydroneopterintriphosphate accummulates and leaves the cell after dephosphorylation. Hence the high neopterin levels in newborn infants (Dhondt et al 1982b).

The major role that tetrahydrobiopterin (THB) plays in neurotransmitter biosynthesis (Leeming et al 1981) means that neuropathological disease states could arise due to impaired THB synthesis. A number of disease states have been reported where biopterin levels have been found to be altered (Table 1.1). Whether these pterin-level changes are part of the aetiology of the disease is uncertain.

Hyperphenylalaninaemia is one condition where a change in THB metabolism has been shown, implicitly. Since the early report of Folling (1934) that raised serum phenylalanine level is accompanied by severe mental retardation further observations have subclassified this condition.

The most commonest form of hyperphenylalaninaemia (classical phenylketonuria) is due to a deficiency of phenylalanine hydroxylase (Jervis, 1947). These subjects exhibit a high urinary THB level and a low neopterin/biopterin ratio (Dhondt et al 1981). Treatment of these infants is by phenylalanine-restricted diet, without which mental retardation ensues. The possible mechanisms of mental retardation associated with phenylketonuria have been reviewed by Kaufman (1976).

The malignant forms of hyperphenylalaninaemia are so classified because of the lethal potential of such states if untreated. In 1974, Bartholome reported a child with hyperphenylalaninaemia whose condition did not ameliorate on a phenylalanine-restricted diet. In 1975, Smith et al reported a child with dihydropteridine-reductase deficiency. The subject showed normal liver phenylalanine hydroxylase activity, raised blood phenylalanine level and progressive neurological impairment. Other similar cases have since been reported (Leeming et al, 1976 ; Schlesinger et al, 1979 ; Milstein et al, 1980).

An error in biopterin synthesis at a stage beyond dihydroneopterin triphosphate synthesis has also been reported in some patients (Kaufman et al, 1975 ; Ray et al 1976). These subjects exhibit normal DHPR and phenylalanine hydroxylase activities but show elevated neopterin and low biopterin levels in their urine.

More recently a new variant, involving GTP-cyclohydrolase 1 deficiency has been reported by Niederwieser et al (1984). The patient responded to tetrahydrobiopterin treatment but not to tetrahydroneopterin.

Such discoveries have given new impetus for research on tetrahydrobiopterin metabolism and its relation to neurological function.

In general voluntary movements are under the control of the basal ganglia (Calne 1983). The nigro-striatal pathway therein has a dopaminergic component. Malfunction of this pathway has been suggested in tardive-dyskinesia, Huntington's disease and Parkinson's disease (De Vaugh-Geiss, 1982).

Since dopamine biosynthesis utilises pterin-cofactor during the hydroxylase reaction it is conceivable that some dopaminergic disorder could arise due to THB-synthesis impairment.

Parkinsonian patients show the characteristic symptoms of tremor, rigidity and bradykinesia, disorders of movement which are under the influence of the basal ganglia (Marsden 1982). Parkinsonian patients have been treated with dopaminergic precursor (L-dopa) and dopa-decarboxylase inhibitors (e.g. carbidopa) as well as dopaminergic agonists (e.g. bromocryptine). Short-term therapy with these agents ameliorates the symptoms of abnormal movement. However prolonged therapy produces adverse reactions and declining efficacy (Calne, 1983). Furthermore, some patients with Parkinson's disease exhibit a dementia component. Hence the disease may be more complex than at first envisaged. Current reports have implicated a disturbance in tetrahydrobiopterin metabolism in this disorder. Lovenberg et al (1979) have shown that csf hydroxylase activity in Parkinson's disease patients (8.9 $\pm$ 0.95 pmol./ml.) is 50% of the activity observed in the csf of control group. Csf cofactor activity correlated well with homovanillic acid (dopamine metabolite) level. Nagatsa et al (1981) found tyrosine-hydroxylase activity in the caudate nucleus and putamen to be 50% of control, with significant reductions also in locus coeruleus and other regions. Furthermore, the biopterin levels in the caudate nucleus were

significantly lowered compared to healthy control subjects. The low csf hydroxylase-cofactor observed by Williams et al (1980) in Parkinson's disease is suggested to be a consequence of aminergic neuronal loss due to unspecified causes. Recently Curtius et al (1982) claimed amelioration of clinical symptoms when a single dose of tetrahydrobiopterin was administered to each of two Parkinsonian patients.

Dystonia is a disorder of posture induced by slow, sustained alternating contraction and relaxation of agonist and antagonist muscles (Eldridge 1970). A disturbance of the dopaminergic system has been implicated. Recently, Williams et al (1979) found much reduced csf pterin cofactor levels in these patients and suggested that a primary disorder of THB-metabolism is involved. LeWitt et al (1983) confirm the low biopterin findings in dystonic patients. They have also treated dystonic individuals with THB and found a substantial improvement in their condition.

Other neuropathological disorders where tetrahydrobiopterin metabolism has been shown to be impaired are senile dementia of Alzheimer type (Morar et al 1983), Down's syndrome (Aziz et al 1982), dialysis encephalopathy (Dhondt et al 1983a), and affective disorder (Blair et al, 1983). These will be discussed more fullly later (Chapter 4).

Not all neuropathic disorders affect the body pterine levels. Leeming et al (1976) investigated schizophrenic patients and found that whilst the serum levels of biopterin was slightly lower than control, the urinary biopterin level was normal. Garbutt et al (1982) have found no change in the csf pterin-cofactor levels of schizophrenic subjects compared with controls.

Apart from the brain, the kidney and the liver are the two other major tetrahydrobiopterin-synthesising organs, and pathology of these organs would be expected to affect pteridine discharge. Leeming et al (1976) observed elevated serum biopterin levels and reduced urinary discharge of biopterin following kidney dysfunction. This could be the result of reduced capacity of the kidney to excrete the biopterin, probably accompained by impaired synthesising ability. Haberle et al, 1978, have shown that a significant percentage of the urinary load of biopterin is contributed as a direct result of renal synthesis of this pterin. In uraemia, a condition of renal insufficiency accompanied by increased retention of nitrogenous waste, Dhondt et al (1983b) have found elevated neopterin and biopterin levels in the serum. Whilst high biopterin levels would be explained by reduced glomerular filtration rate, the increase in neopterin/biopterin ratio suggested an impaired THB synthesis, probably caused by the uraemigenic agent.

Apart from the enzyme-deficiency induced changes in tetrahydrobiopterin levels in the liver, few reports exist about pteridine metabolism in liver dysfunction. Leeming et al (1976) report no significant change in serum biopterin levels in liver cirrhosis. This is surprising since systemic tetrahydrobiopterin levels would be expected to be regulated by the liver in the same way that it metabolises other endogenous substance (e.g. insulin, glucagon and gastrin, Rappaport, 1979). Dhondt et al (1983c) report no changes in total biopterin levels during liver regeneration. The enzymes involved in THB-metabolism were also of normal activity apart from the elevated phenylalanine hydroxylase activity. This could affect THB levels but was not investigated.

Pteridine levels have been shown to be altered in several immunostimulant disease states (Leeming et al 1980). Fuchs et al (1983) report elevated urinary neopterin in coeliac disease, whilst Rokos et al (1983) reported raised urinary neopterin in various neoplasms, viral diseases and in renal insufficiency Dhondt et al (1982) report elevated serum and urine neopterin and low biopterin in breast cancer. Grunewald et al (1983) describe elevated urinary neopterin during haematologic neoplasia with reversion towards normal on remission. A number of hypothesis have been put forward to explain this phenomenon. Fuchs et al (1983) suggest it is the direct result of immune system activation since t-lymphocyte stimulation is always associated with increased neopterin production. Dhondt et al (1982) suggest that since the neopterin/biopterin ratio in breast cancer increases to a level seen in newborns, the cancerous tissue is reverting to a dedifferentiated cell-form typical of neoplastic tissue. Grunewald et al (1982) suggest cell proliferation activity such as t-lymphocyte hyperplasia is accompanied by increased pterin-synthesis.

It is clear that a disturbance of tetrahydrobiopterin metabolism occurs in many disease states. Whether its a primary, biochemical lesion or a secondary effect is debatable in many cases. This research was directed at elucidating more clearly the relationship between tetrahydrobiopterin metabolism and neurotransmitter synthesis under various pathological conditions. A number of human case studies have been undertaken and this work has been supplemented by research on animals.

The aims of this research project were

- to evaluate methods for the rapid, sensitive and accurate determination of pterins ;
- to apply a suitable method to the detection of pterins in various body fliuds and tissues in health and disease;
- to investigate changes in pterin levels in animal models following exposure to various agents in everyday use.

The overall aim has been to deduce pathogenic, diagnostic, and therapeutic significance of changes in pteridine levels in the body, both in health and disease.

CHAPTER TWO

THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF REDUCED PTERIDINES AND THEIR OXIDATION PRODUCTS :

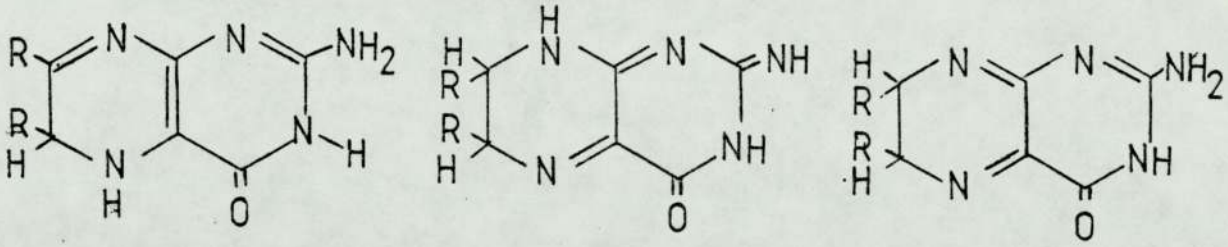
The oxidation of tetrahydrobiopterin (THB) and related pterins has been investigated for many reasons. Tetrahydrofolate is investigated with a view to analysing the vitamin content of processed foods, for example. Tetrahydrobiopterin oxidation is of interest in evaluating its stability in biological samples during storage and analysis, for investigating its interaction with molecular oxygen and for analysing the structure of the product, quinonoid-dihydrobiopterin.

The labile nature of the pteridine molecule was demonstrated by Albert et al (1952). The instability of the pteridine was attributed to the loss of aromatic character caused by the high N:C ratio and the electron attracting nature of the four ring-nitrogen atoms. Substitution on the pteridine nucleus by electron-releasing groups (e.g. -OH, -SH or $-NH_2$), especially at positions 2- or 4- increases their stability, whilst decreasing their solubility in aqueous solution. The reduced forms of these pteridines exhibit greater lability, with the tetrahydro-derivative being more unstable than the dihydro-compounds (Albert et al, 1962).

Non-enzymatic oxidation of tetrahydrobiopterin in the presence of 2,6-dichlorophenolindophenol causes bleaching of the dye (Kaufman 1961). The immediate product, unlike 7,8-dihydropteridine can be reduced back to tetrahydropteridine by reduced triphosphopyridine nucleotide (TPNH) and is active in the phenylalanine hydroxylase system in the presence of TPNH (Kaufman, 1961). The intermediate readily oxidises to 7,8-dihydropteridine. The possible structures of the primary oxidation products

were suggested to be either 5,6-dihydropteridine, or para - or ortho-quinonoid dihydropteridine (Kaufman 1964).

Fig. 2.1 : Possible Intermediates of THB Oxidation

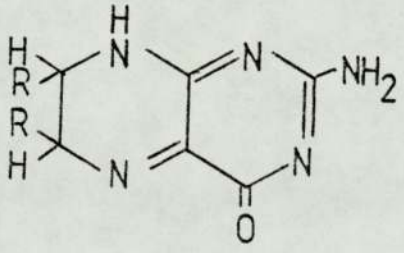


5,6-dihydropteridine **para-quinonoid dihydropteridine** **ortho-quinonoid dihydropteridine**

R=CH_3

Tritium-labelled tetrahydropteridine experiments showed that little if any of the tritium at carbon 7 is lost during oxidation and this suggests 5,6-dihydropteridine not to be the intermediate candidate in the oxidation. Spectral analysis of the oxidation products of 2-alkylamino-tetrahydropteridine shows a pronounced bathochromic shift, higher than with the unalkylated compound. Kaufman suggests that this would be expected if the 2-amino group were a part of the extended conjugation system involving the other double bonds in the pteridine ring. This would therefore support the para-quinonoid-dihydropteridine as an intermediate. Recent ^{1}H-NMR studies confirm this (Lazarus, 1982b). However, Armarego and Waring, 1983, support an alternate structure shown in fig. **2.2** based on spectral and kinetic analysis.

Fig. 2.2

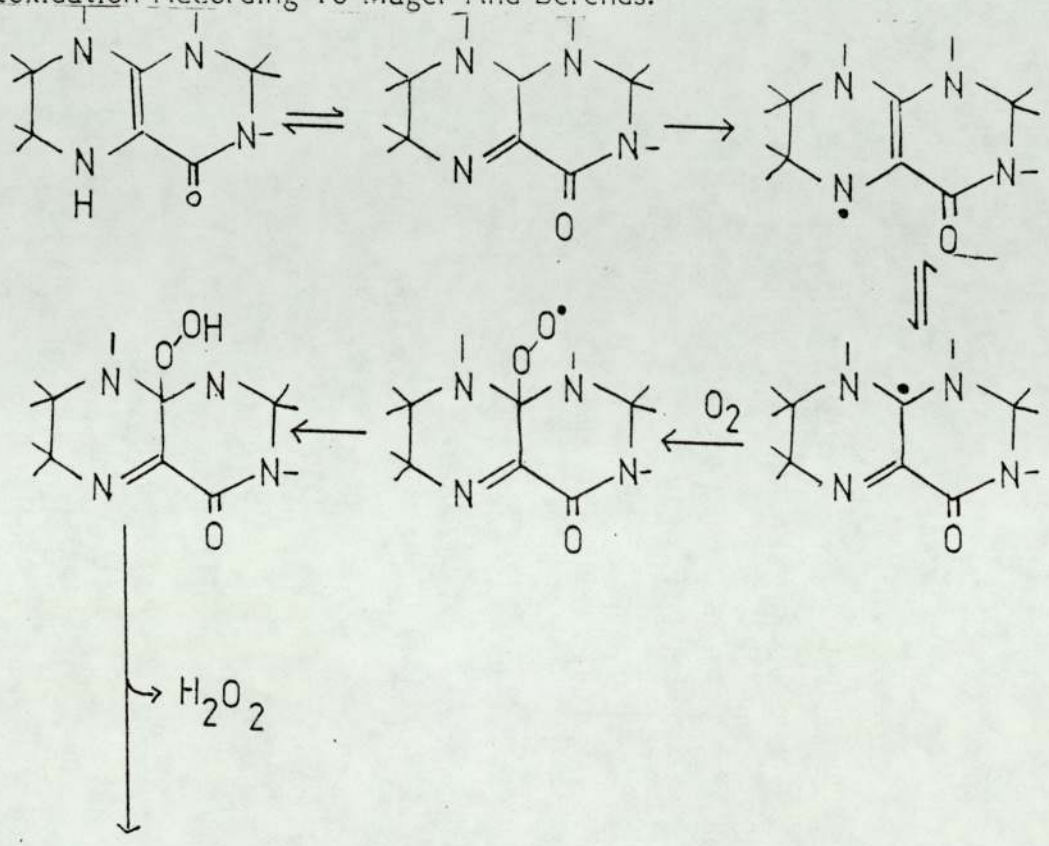


quinonoid dihydropterin. R= CH_3.

A number of attempts have been made to detail the mechanism of tetrahydropterin oxidation. Mager and Berends (1965) propose the mechanism in fig. 2.3, involving hydroperoxide intermediates.

FIG. 2.3

Autoxidation According To Mager And Berends.



QUINONOID DIHYDROPTERIDINE

The above mechanism was experimentally supported by the finding that N(5) substituted compounds were more resistant to oxidation by air whereas N(8) substituted compounds were sensitive to oxygen. Furthermore using isoalloxazine as a pterin analogue, Mager and Berends observed hydrogen peroxide as a decomposition product of the hydroperoxide intermediate. The findings of Jakubovic et al (1971) would tend to support the hydroperoxide theory. Hydrogen peroxide generated by the oxidation of 6,7,dimethyltetrahydropterin ($DMPH_4$) was found to inhibit phenylalanine hydroxylase in vitro. The inhibition was found to be relieved by the presence of catalase or dithiothreitol.

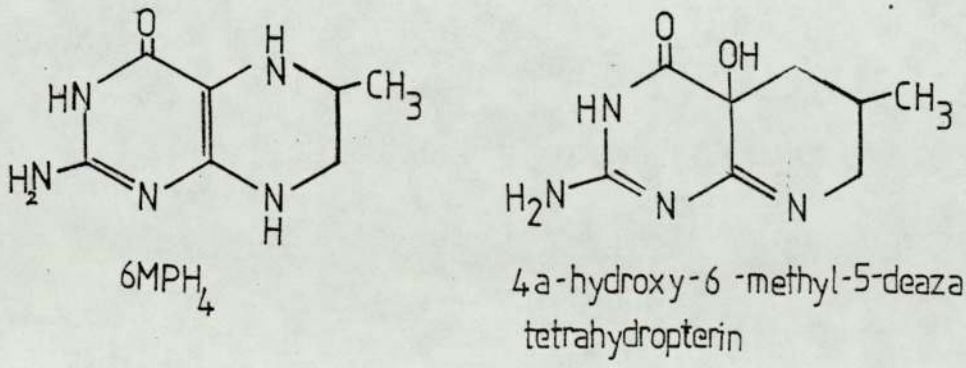
Mager and Berends suggest that a similar mechanism exists for biological oxidations. It is generally held that the oxidation of THB under enzymic conditions is similar to its autoxidation under non-enzymic conditions. Kaufman (1967) tends to question the involvement of the hydroperoxide species.

Other mechanisms have been proposed. For example, Pearson (1974) envisages a radical mechanism in which peroxy-intermediates do not play kinetically significant parts. Armarego and Waring (1982) suggest a free-radical mechanism with peroxy-intermediates.

More recently, it has been shown by UV spectra analysis and NMR studies (Lazarus et al 1981, Lazarus et al 1982a) that at least in the enzymic oxidation the pterin-intermediate is highly likely to be a 4a-hydroxy adduct. UV spectra of the intermediate generated by 6-methyltetrahydropteridine ($6MPH_4$) in the presence of phenylalanine and phenylalanine-hydroxylase was similar to the spectrum of its analogue, 4a-hydroxy-6-methyl-5-deazatetrahydropterin.

FIG. 2.4

6-methyltetrahydropterin ($6MPH_4$) and its analogue.

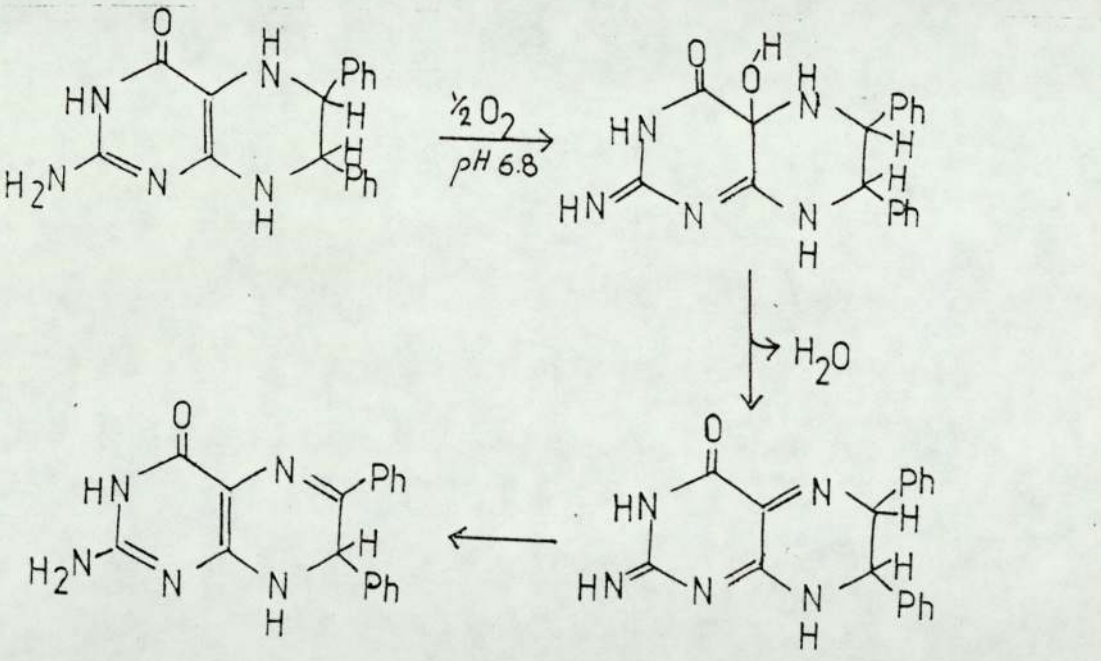


Attempts to trap potential hydroperoxide intermediate by thioxane, or glutathione with glutathione peroxidase were unsuccessful. Hence these workers are of the opinion that the 4a-hydroxy adduct is more likely to exist during tetrahydropterin oxidation. The 4a-hydroperoxide adduct is suggested to be more reactive and would lead to an uncoupled reaction with the release of hydrogen peroxide or a hydroperoxide species.

The involvement of 4a-hydroxy adduct would support the earlier proposal of Viscontini and Okada (1967) (fig. 2.5).

FIG. 2.5

THB oxidation proposed by Viscontini and Okada.



7,8-dihydrobiopterin

quinonoid dihydrobiopterin

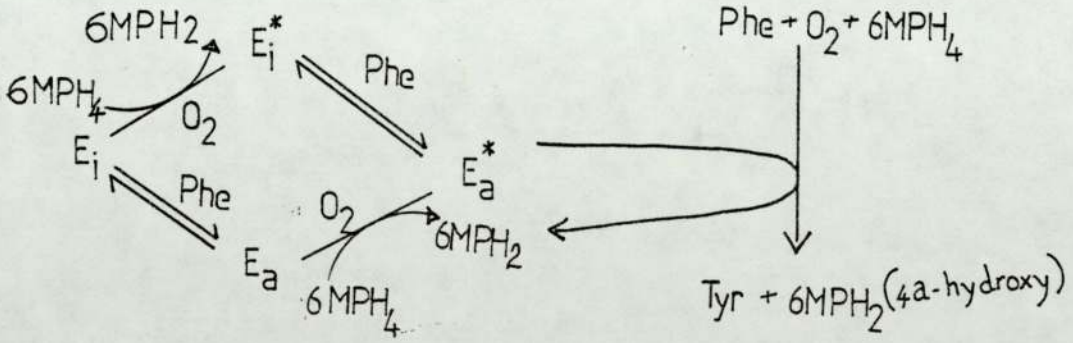
Ph = phenyl group

Marota and Shiman (1984) have investigated rat liver phenylalanine hydroxylase and found that the enzyme oxidised stoichiometric amounts of

the cofactor independent of the presence of phenylalanine. There was no evidence for the presence of the 4a-hydroxydihydropterin intermediate. Neither hydrogen peroxide nor superoxide ions were detected as products of the oxidation. The hydroxylase appeared to be the sole electron acceptor from the biopterin cofactor and in the process is suggested to become "activated", showing altered chemical and physical properties. A quinonoid dihydropterin is formed in the process. The activation of the enzyme is unusual in that the electrons transferred from the biopterin cofactor to the enzyme are not subsequently used for the hydroxylation of the amino acid. A second molecule of cofactor is needed for the hydroxylation reaction. In addition the hydroxylase is not full activated until a molecule of phenylalanine (or lysolecithin) interacts with the enzyme, (fig. 2.6). Marota and Shiman (1984) suggest that the quinonoid dihydropterin may be a direct product of the reaction or arise by dehydration of a 4a-hydroxydihydropterin which rapidly dehydrates with a half-life of 3.5s.

FIG. 2.6

Proposed Phenylalanine Hydroxylase-cofactor interaction (Marota and Shiman, 1984).



$6MPH_4$ = 6-methyltetrahydropterin.

$6MPH_2$ = 6-methyldihydropterin.

E_i = enzyme in unactivated state.

E_a = enzyme in activated state.

E* = enzyme in "reduced" state.

The above observations could fit in with the proposal of Wallick et al (1984) that phenylalanine hydroxylase undergoes an obligatory pre-reduction step from (Fe^{3+})-enzyme to (Fe^{2+})-enzyme prior to hydroxylation of phenylalanine to tyrosine.

Further oxidation of the quinonoid-dihydropterin species may give rise to a number of other products depending on the conditions. In general, reduced biopterins have been found to be as labile as reduced folates (Chippel and Scrimgeour, 1970). Under mild oxidising conditions reduced folates readily undergo side-chain cleavage It appears that the bonding of a moiety at C6 via a lone pair carrying species (e.g. N.,O) destabilises the molecule (Pearson, 1974), promoting loss of the side chain. However, the decomposition products of THB can be variable depending on the conditions. Pfleiderer, 1978, obtained biopterin, 7,8-dihydroxanthopterin and pterin at pH 7.5, and sepiapterin, isosepiapterin and pterin-6-carboxylic acid at pH 4. A pH effect has also been observed during oxidation of tetrahydrofolate (Blair and Pearson 1974). These workers also observed enhanced oxidation due to the presence of trace amounts of transition metal ions in solution. Armarego and Wang (1982) have observed enhanced oxidation due to tris-buffer and diminished oxidation in the presence of protein. Under strong oxidising conditions such as acid-iodine oxidation (Fukushima and Nixon 1980) or boiling in air, THB may undergo breakdown to pterin and dihydroxanthopterin (Rembold 1969). These observations reflect the variability of the oxidation of reduced pterins under differing conditions. This chapter discusses the oxidation products observed by HPLC under various conditions.

Materials :

D-Biopterin, L-neopterin, pterin, 7,8-dihydrobiopterin, 5,6,7,8-tetrahydrobiopterin, sepiapterin, xanthopterin, isoxanthopterin and 6-methylpterin were obtained from Dr. B. Schircks, Schachenstrasse 4, Wettswill, Switzerland. HPLC solvents were from Fisons PLC (U.K.), except for distilled water which was laboratory-produced. All other chemicals were of analytical grade and obtained from the Sigma Chemical Company.

Sample filtration was carried out using cellulose-acetate filters (Millipore Corporation, USA). All UV and visible spectra were recorded with a Pye-Unicam SP1700 spectrophotometer.

HPLC specifications (fig. 2.3) :

Dual-Piston Pump	:	Constametric III, Laboratory Data Control (L.D.C.) U.K.
Auto-sampler	:	Wisp 710B, Waters Associate Inc. USA.
Column	:	Spherisorb s5.ODS 5µ. 25 cm. x 4.6 mm. (LCD).
Fluorescence Detectors	:	Spectrofluormeter SFM 23/3 (Kontron USA). LDC Fluoromonitor III model. 1311.
Recorder	:	CR652 Recorder, JJ instruments UK.
Integrator	:	Model 308 LDC - peak integrator.

Biological Samples : urines from affective disorder patient and controls were obtained from Dr. A. Coppen at West Park Hospital, Epsom in Surrey. Adult phenylketonuria and tuberose sclerosis patient urine was obtained from Dr. P.E. Sylvester at St. Lawrence's Hospital, Caterham.

Sample Preparation :

The standard solutions of pteridines were prepared fresh when required and quantitated using the following extinction coefficients :

Biopterin	E_{362nm}	= 8.3×10^{-3} M^{-1} cm^{-1} in 0.1 N NaOH (Fukushima and Nixon 1980)
Neopterin	E_{362nm}	= 8.3×10^{-3} M^{-1} cm^{-1} in 0.1N NaOH. Fukushima and Nixon 1980.
5,6,7,8-tetrahydrobiopterin	E_{267nm}	= 16×10^{7} M^{-1} cm^{-1} in 0.1NHCl. Fukushima and Nixon 1980.
7,8-Dihydrobiopterin	E_{330nm}	= 6.2×10^{3} M^{-1} cm^{-1} pH 6.8. Fukushima and Nixon 1980.
Pterin	E_{251nm}	= 20.4×10^{3} M^{-1} cm^{-1}, 0.1N NaOH. Blakeley 1969.
Sepiapterin	$E_{281}nm$	= 10.4×10^{3} M^{-1} cm^{-1} 0.1N HCl. Blakeley 1969.
7,8-dihydroneopterin	E_{330nm}	= 6.2×10^{3} M^{-1} cm^{-1} pH 6.8. Fukushima and Nixon.
Tetrahydrofolate	E_{298}	= 28×10^{3} M^{-1} cm^{-1} pH 7. Blakely 1969.
Cytochrome C	E_{550}	= 27.7 mol^{-1} cm^{2} pH 7. Lehninger 1978.

Reduced pteridines were protected from exposure to light. Oxidation of standards was according to the method of Fukushima and Nixon (1980), unless stated otherwise.

1ml. of the pteridine solution was mixed with 1ml of 0.2N HCl and 0.1 ml of 0.1% iodine in 0.2% potassium iodide (w/v in 0.2N HCl). The iodine was present in excess as noted by visual observation and starch test. The solution was left to stand in the dark for 1 hour. At the end of this period excess iodine was reduced by addition of excess of 1% ascorbate (w/v) solution which decolourised the sample. Following dilution, the resulting solution was analysed by HPLC. The experiment was repeated under alkaline conditions using 0.2N NaOH.

Xanthine Oxidase assay : This assay was according to the method of Fridovich (1962). All solutions were made up in potassium phosphate buffer (0.1M) pH 7.8 containing 0.1mM ethylene diamine tetracetic acid, disodium dihydrate (EDTA). The rate of urate production from xanthine was followed at 295 nm and at 25°C. An equivalant amount of superoxide ions were assumed to be produced at the same time. 1 unit of xanthine oxidase activity was taken to represent the conversion per minute of 1 µmole of xanthine into urate at 25°C an pH 7.8. The Sigma xanthine oxidase was diluted 250-fold for the purpose of the assay.

Cytochrome C assay : This assay was carried out according to the method of McCord and Fridovich (1969). All assays were carried out in potassium phosphate buffer (0.1M) pH 7.8, containing 0.1mM disodium EDTA, at 25°C. The reduction of cytochrome C at 550 nm was observed over initial 30s of reaction.

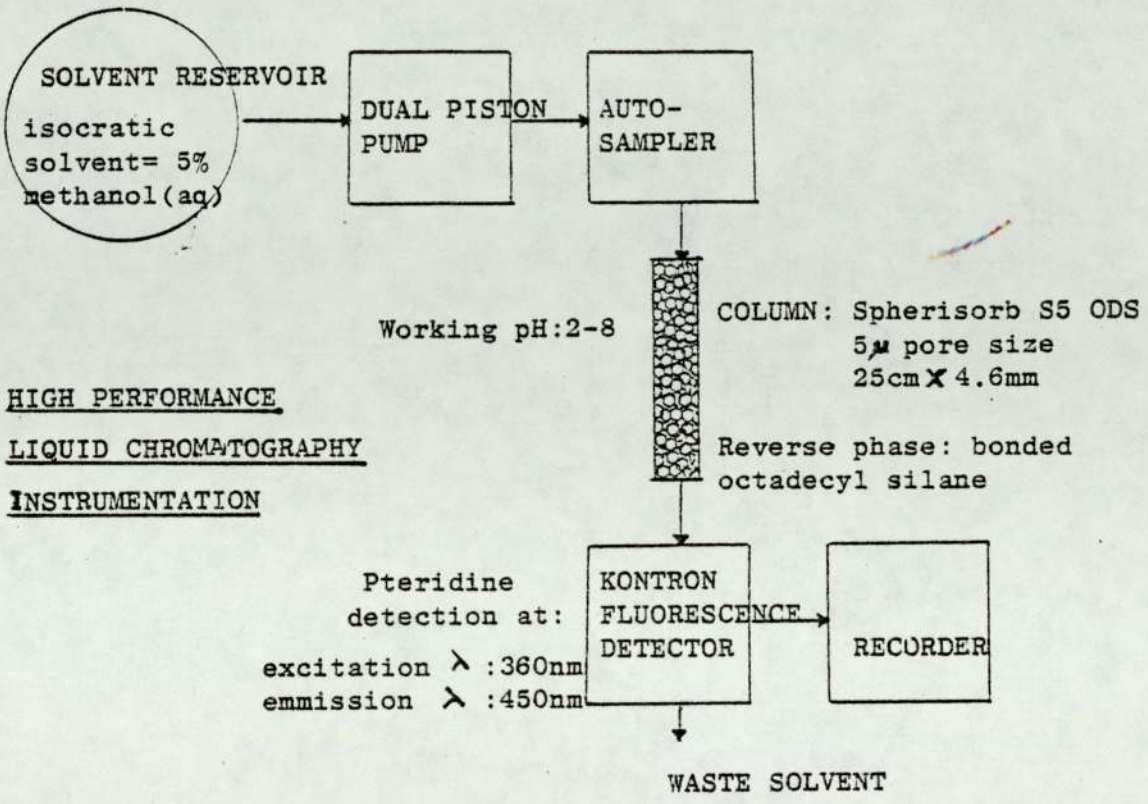


fig 2.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY INSTRUMENTATION

fig.2.8:
EFFECT OF FLOW RATE ON COLUMN EFFECIENCY (N)

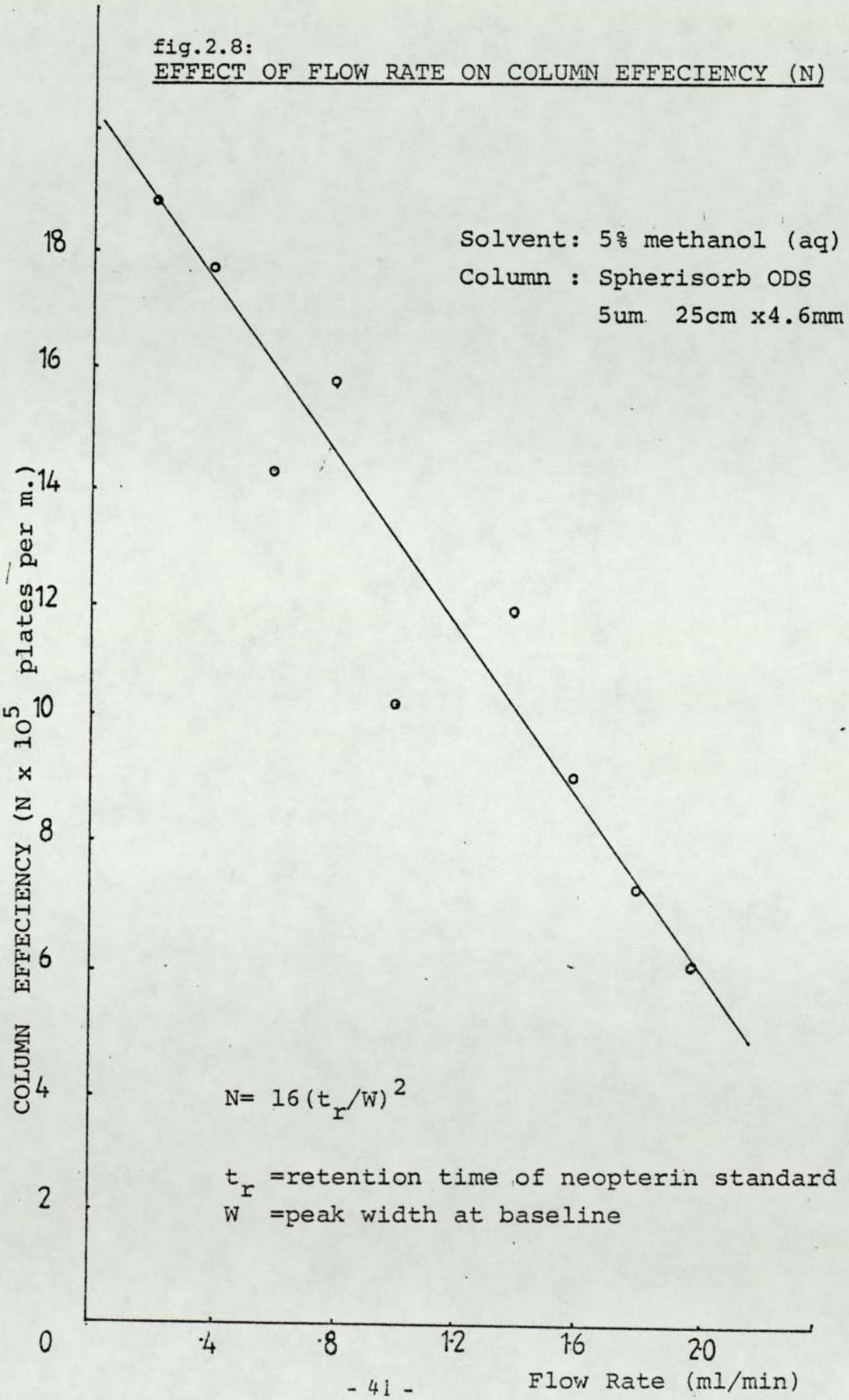


fig.2.9:

EFFECT OF FLOW RATE ON THE RETENTION TIME OF NEOPTERIN

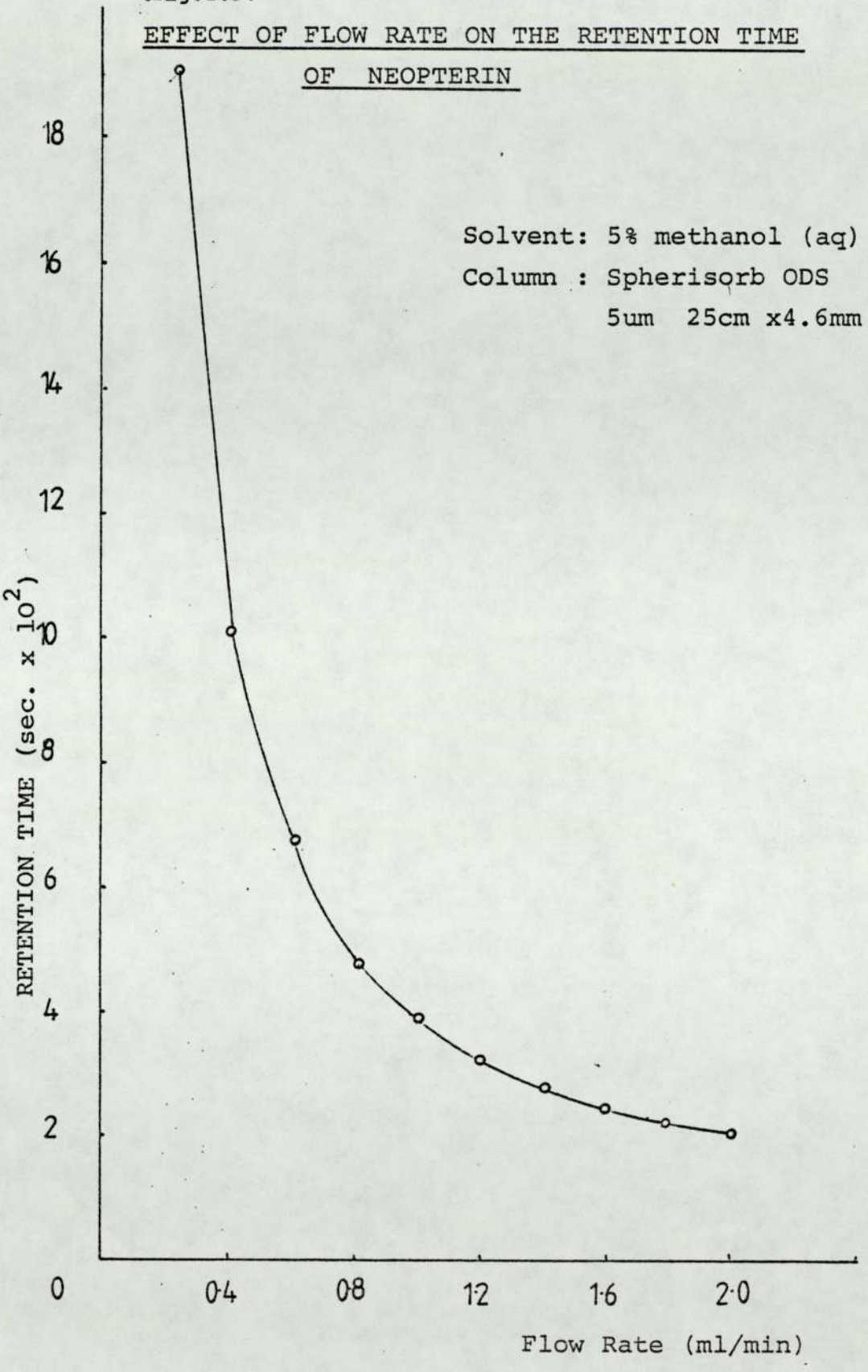


fig. 2.10
EFFECT OF FLOW RATE ON NEOPTERIN PEAK SIZE

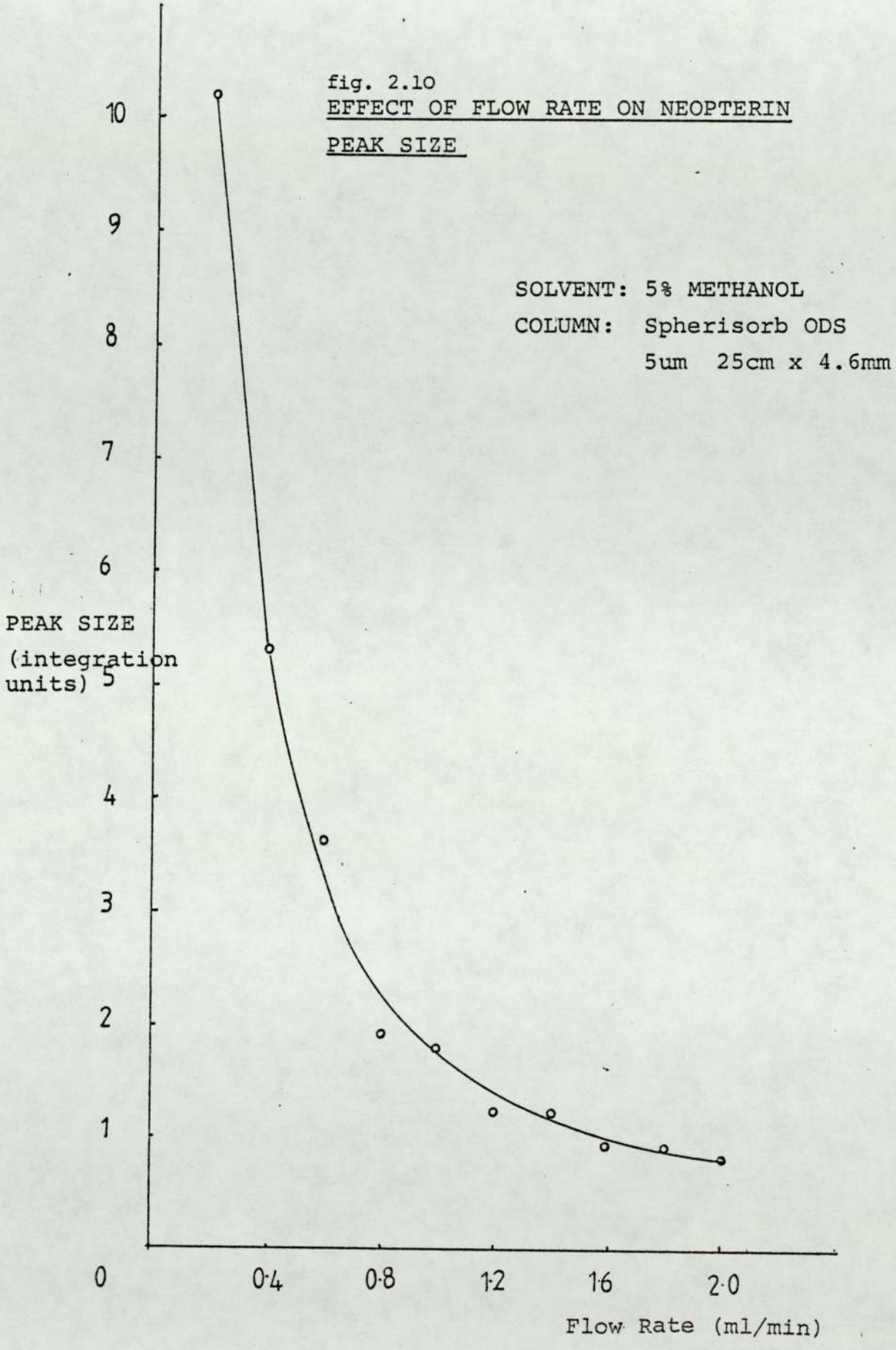
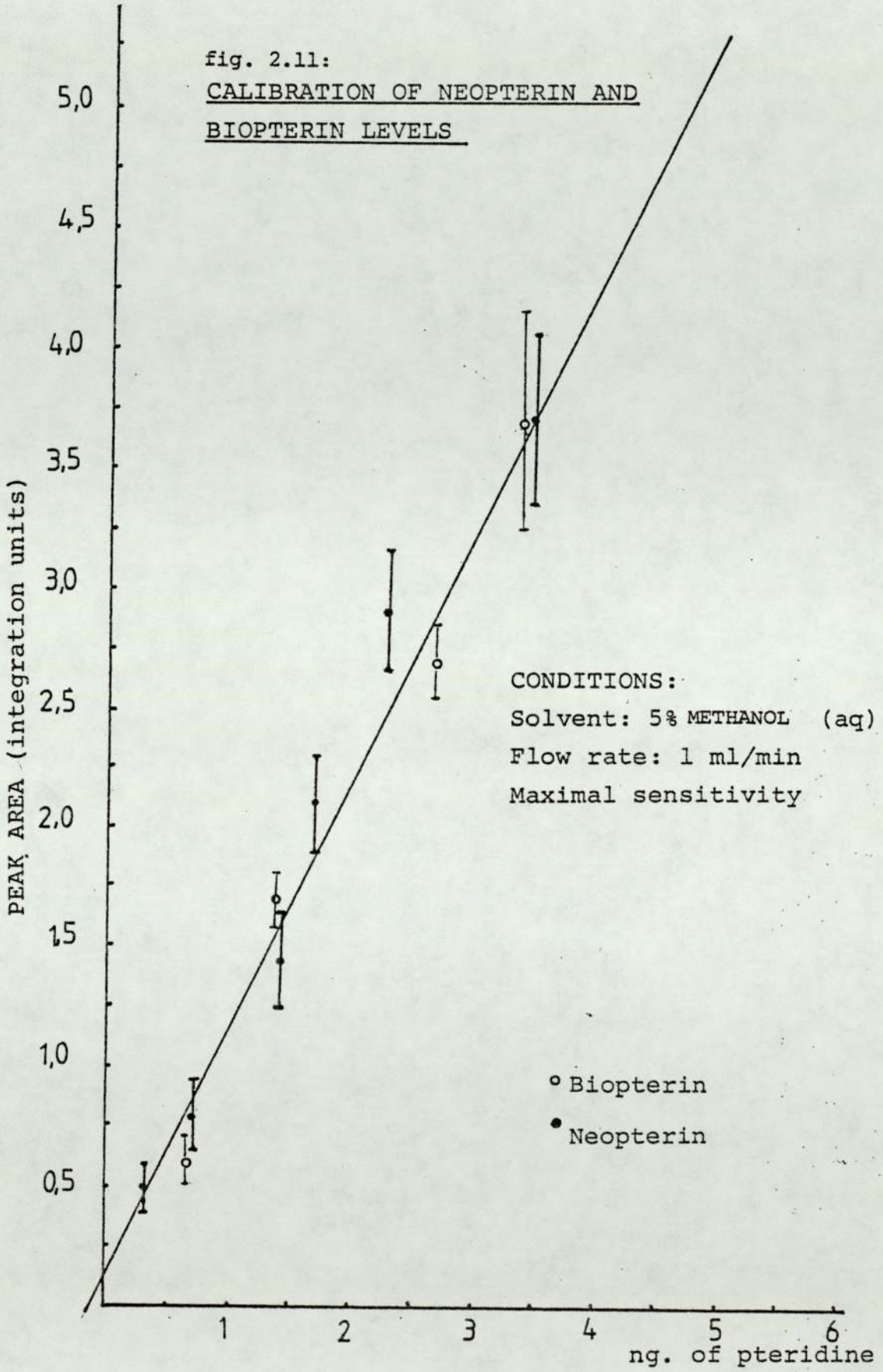


fig. 2.11:
CALIBRATION OF NEOPTERIN AND BIOPTERIN LEVELS



Results :

With the spectrofluorometer maintained at fixed emission wavelength of 450nm. the excitation wavelength of individual pteridines was investigated. The excitation wavelength for all pteridines was found to be optimal at 360nm. The optimal emission wavelength is 450nm.

All HPLC separations were carried out at ambient (20-25°C) temperature using 5% HPLC methanol in water. Fig. 2.8, 2.9, 2.10 show the effect of solvent flow rate on column efficiency, solute retention time and solute peak size, respectively. Column efficiency was measured according to Stevens (1980) using :

$$N = 16 \left(tr_{/W}\right)^2$$

where N = Column efficiency

tr = Retention time (s).

W = peak width at base line.

Using flow rate of 1ml./min. the pteridine standards were calibrated. Fig. 2.11. shows calibration plots for neopterin and biopterin which were equifluorescent. The minimal detectable amount of standard was 10 ng/ml. of neopterin or biopterin.

TABLE 2.1 : RELATIVE FLUORESCENCE OF PTERIDINES.

Compound	Relative Fluorescence.
L-Biopterin	100 %
D-Neopterin	100
L-Neopterin	100
Pterin	65
L-erythro-7,8-dihydrobiopterin	12
Pterin-6-aldehyde	5.5
Sepiapterin	2
Tetrahydrobiopterin	0

TABLE 2.2. : RELATIVE RETENTION TIMES OF PTERIDINES.

Sample	Retention Time (s)
L-Biopterin	714 $\pm$ 192 (S.D)
D-Neopterin	360 $\pm$ 68
L-Neopterin	360
7,8-dihydrobiopterin	720
Xanthopterin	660
Pterin	1140 $\pm$ 222
Pterin-6-aldehyde	1140
Sepiapterin	2750
Pterin-6-carboxylic acid	195
I_2/H^+ oxidised sepiapterin	705

TABLE 2.3 : REPRODUCIBILITY OF RETENTION TIME OF STANDARDS.

Chromatography session of 18 : 1 : 83 : 18 hour duration. Standard sample containing neopterin, biopterin and pterin injected after every two injections of biological sample.

Standard Retention Time (Rt) (min).

Neopterin	Biopterin	Pterin	
6.25	11.5	18.25	
7.25	13.25	20.75	
7.25	13.25	20.75	
5.75	12.50	20.00	
6.75	12.50	19.75	
7.00	12.50	20.00	
5.75	10.50	17.00	
7.00	12.50	20.00	
5.75	12.50	20.25	
6.50	12.00	19.25	
7.25	12.75	20.50	
6.75	12.00	19.25	
6.60	12.31	19.65	: Mean
± 0.57	± 0.72	± 1.05	: Standard deviation
8.6 %	5.8 %	5.3 %	: coefficient of variation

Pearson's Correlation coefficients : (r)

Neopterin R_t vs. biopterin R_t : $r = 0.6390$ $(p < 0.05)$.

biopterin R_t vs. pterin R_t : $r = 0.9851$ $(p < 0.001)$.

Table 2.4. Reproducibility of Peak size.

Conditions as in Table 2.3.

Peak area (mm^2) :

Neopterin	Biopterin	Pterin	
224	198	611	
222	180	660	
224	195	600	
224	198	600	
228	202	594	
214	195	594	
230	173	660	
234	205	611	
224	186	600	
200	186	611	
200	183	660	
200	186	650	
218.7	190.6	620.9	: Mean
12.2	9.7	27.8	: Standard deviation
5.6 %	5.1 %	4.5 %	: Coefficient of Variation

Tables 2.3. and 2.4. show reproducibility of chromatograms over an 18 hour session. The biological samples showed similar reproducibility of retention time. Pterin/biopterin retention time ratio of standards was 1.596 $\pm$ 0.0179; a comparable ratio of 1.609 $\pm$ 0.0142 was found for the sample pteridines. Since the pterin peak was always prominent in the sample, the authenticity of the biopterin peak could be checked by investigating the retention time ratio. Peaks were also identified as biopterin by "spiking" the sample with standard biopterin and observing for co-chromatography between standard and suspected biopterin.

Table 2.4 shows the stability of pteridines at room temperature for almost a day.

Table 2.5. Iodine - oxidatoin Of Reduced Pteridines :

Pteridine	Oxidation System.	Initial Concentration nmol./ml.	Yield of Biopterin nmol./ml.	Yield of Pterin nmol./ml	% Conversion
7,8-dihydro-biopterin	HCI+I_2+KI	3.09	2.79	0	90
	NaOH+I_2+KI	3.09	2.77	0	90
5,6,7,8-tetrahydro biopterin	HCI,I_2,KI.	2.0	1.94	0	97
	NaOH,I_2.KI.	2.4	0.5	1.90	79.2*
Tetrahydrofolate	HCI,I_2KI.	2.3	0	2.44	108 +
			Neopterin yield.		
7,8-dihydro-Neopterin.	HCI,I_2KI	4.7	4.7	"	100
	NaOH,I_2KI	2.8	1.68	"	60**

+3 other minor peaks at retention time 6 min., 9.5 min., 15 min.

* % conversion to pterin.

** 3 other minor peaks at retenti on time 4.9 min., 5.6 min., 10.8 min.

Table 2.6. **Effect of Iodine Concentration On Oxidation Of Tetrahydrobiopterin :**

Conditions : 1ml. THB was mixed with 0.1ml. of potassium iodide containing varying concentration of iodine in 0.1N HCl. Oxidation was allowed to take place for 30 min. after which solutions were analysed.

Stock I_2-solution.	[Iodine] in reaction	[THB] nmol./ml.	% Conversion to biopterin	Pterin peak
0.1% (w/v.)	0.72	2.9	46	0
0.2%	1.43	2.9	47	+
0.3%	2.15	2.9	54	+
0.4%	2.86	2.9	59	++
0.5%	3.58	2.9	73	+++

+ small pterin peak detected but not quantifiable.

TABLE 2.7 : THIN LAYER CHROMATOGRAPHY OF OXIDATION PRODUCTS OF TETRAHYDROBIOPTERIN.

Sample	Observation	Rf value
Pterin	Single white spot	0.68
Biopterin	Single, bluish white spot	0.789
Tetrahydrobiopterin	Single, bluish white spot	0.833
I_2/H^+ oxidised THB	Single, bluish white spot	0.782
I_2/OH^- oxidised THB	Major spot	0.635
	Minor spot	0.783

Conditions : 5% acetic acid (aq).
cellulose acetate plates.
10 cm. solvent front.
Observations at 366 nm.

TABLE 2.8 : BIOTERIN STABILITY UNDER ACID AND ALKALINE IODINE TREATMENT.

Sample	Biopterin Recovery. (nmol./ml.)
Biopterin.	6.9 $\pm$ 0.7
Biopterin + 0.1%I_2 + 0.2N HCI	6.3 $\pm$ 2.2
Biopterin + 0.5%I_2 + 0.2N HCI.	7.4 $\pm$ 0.3
Biopterin + 0.1%I_2 + 0.2N NaOH	6.9 $\pm$ 0.6
Biopterin + 0.5%I_2 + 0.2N NaOH	7.1 $\pm$ 0.7

TABLE 2.9 : QUALITATIVE ANALYSIS OF OXIDATION PRODUCTS OF SEPIAPTERIN.

	Product Retention Time (min)	
Oxidation conditions :.	0.1%I_2 + KI in 0.2N HCI	0.1%I_2 in KI + 0.2N NaOH
Product :		
Pterin.6.carboxylic acid	3.1	3.1
Unidentified minor peak.	5.7	.
6.lactyl Pterin (?) Major peak	9.0 min.	9

Biopterin retention time on the day = 10.3 min.

TABLE 2.10 ATMOSPHERIC OXIDATION OF TETRAHYDROBIOPTERIN UNDER SPECIFIED CONDITIONS

OXIDATION MEDIUM AND pH	OXIDATION TIME	RECOVERY OF BIOPTERIN AS % OF INITIAL THB LEVEL	OTHER PRODUCTS OBSERVED (% YIELD IN PARENTHESIS)
pH 4: POTASSIUM HYDROGEN PHTHALATE (0.05M)/ NaOH BUFFER + 5mg/100ml EDTA	4 h	58.6	PTERIN-6-CARBOXYLIC ACID
pH 5: POTASSIUM HYDROGEN PHTHALATE (0.05M) / NaOH BUFFER + 5mg/100ml EDTA	4 h	21.8	XANTHOPTERIN + PTERIN (0.6)
pH 6: POTASSIUM DIHYDROGEN PHOSPHATE (0.05M) / NaOH BUFFER + 5mg/100ml EDTA	4 h	26.0	PTERIN (9.3)
pH 7: POTASSIUM PHOSPHATE BUFFER (0.05M) + 9mg/100ml EDTA	1 h 45min.	trace	PTERIN-6-CARBOXYLIC ACID XANTHOPTERIN, PTERIN (15)
pH 7.8: POTASSIUM PHOSPHATE BUFFER (0.1M) + 5mg/100ml EDTA	3 h	24.3	PTERIN, +(UNIDENTIFIED PRODUCT)

OXIDATION AT AMBIENT TEMPERATURE IN THE DARK

HPLC analysis of oxidation products of reduced biopterins shows that under acid-iodine oxidation, after 1 hour in the dark, almost all of dihydrobiopterin and tetrahydrobiopterin is oxidised to biopterin (Table 2.5.). The 10% loss in biopterin following oxidation of dihydrobiopterin may have been due to handling. Other peaks were not detected under these conditions. Alkaline-iodine oxidation at ambient temperature converts almost all of dihydrobiopterin to biopterin and 80% of tetrahydrobiopterin to pterin. HPLC analysis of alkaline-iodine oxidised THB yielded only a pterin peak but thin layer chromatography indicated some conversion to biopterin (Table 2.7.). When the oxidation time was reduced to 30 min. the percentage conversion of reduced biopterins was also diminished (Table 2.6.). Increasing the iodine concentration, enhanced the oxidation of tetrahydrobiopterin to biopterin but it also yielded trace amounts of pterin (Table 2.6.). Biopterin was found to be stable under the oxidising conditions used above (Table 2.8.). Even at high temperatures biopterin was remarkably stable producing 8-12% loss at 100°C, in the presence of alkali or acid and iodine. Dihydroneopterin was found to be more labile yielding 100% neopterin under acid conditions but only 60% neopterin under alkaline conditions. The latter condition also producing other products which were not identified. Tetrahydrofolate yielded 108% pterin when oxidised with acid-iodine solution, but it was also produced other peaks. Qualitative analysis of oxidation of sepiapterin under acid-iodine conditions shows a major peak with a retention time of 9 min., which is slightly shorter then that of biopterin. It also yields pterin-6-carboxylic acid and an unidentified peak.

Atmospheric oxidation at ambient temperature shows a gradual decline in percentage oxidation to biopterin and an increase in percentage conversion to pterin with increase in pH (Table 2.10).

Since the iodine-oxidation primarily yields biopterin from dihydrobiopterin and THB under acid conditions and pterin from THB but biopterin from dihydrobiopterin under alkaline conditions one can use simple arithmetic to estimate the percentage of reduced biopterins in a sample :

$$\% \text{ tetrahydrobiopterin} = \frac{[\text{acid-}I_2 \text{ oxidised sample biopterin}] - [\text{alkaline } I_2 \text{ oxidised sample biopterin}]}{[\text{Acid } I_2 \text{ oxidised sample Biopterin}]} \times 100$$

$$\% \text{ dihydrobiopterin} = \frac{[\text{alkaline-}I_2 \text{ oxidised Biopterin}] - [\text{neat, unoxidised sample biopterin}]}{[\text{Acid } I_2 \text{ oxidised sample biopterin}]} \times 100$$

No correction was made for the small amount of THB undergoing oxidation to biopterin under alkaline oxidation conditions.

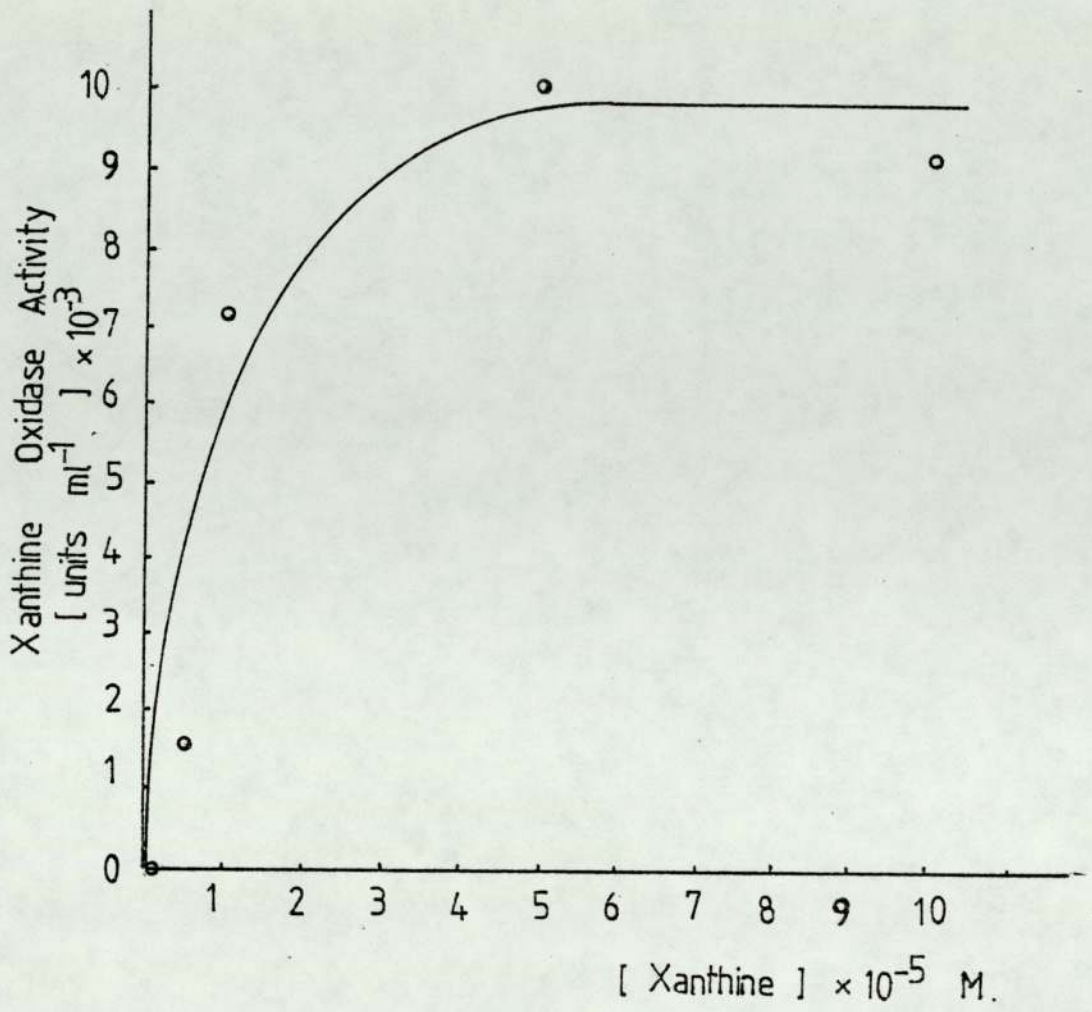


fig.2.12: XANTHINE OXIDASE ACTIVITY AS A FUNCTION OF SUBSTRATE CONCENTRATION

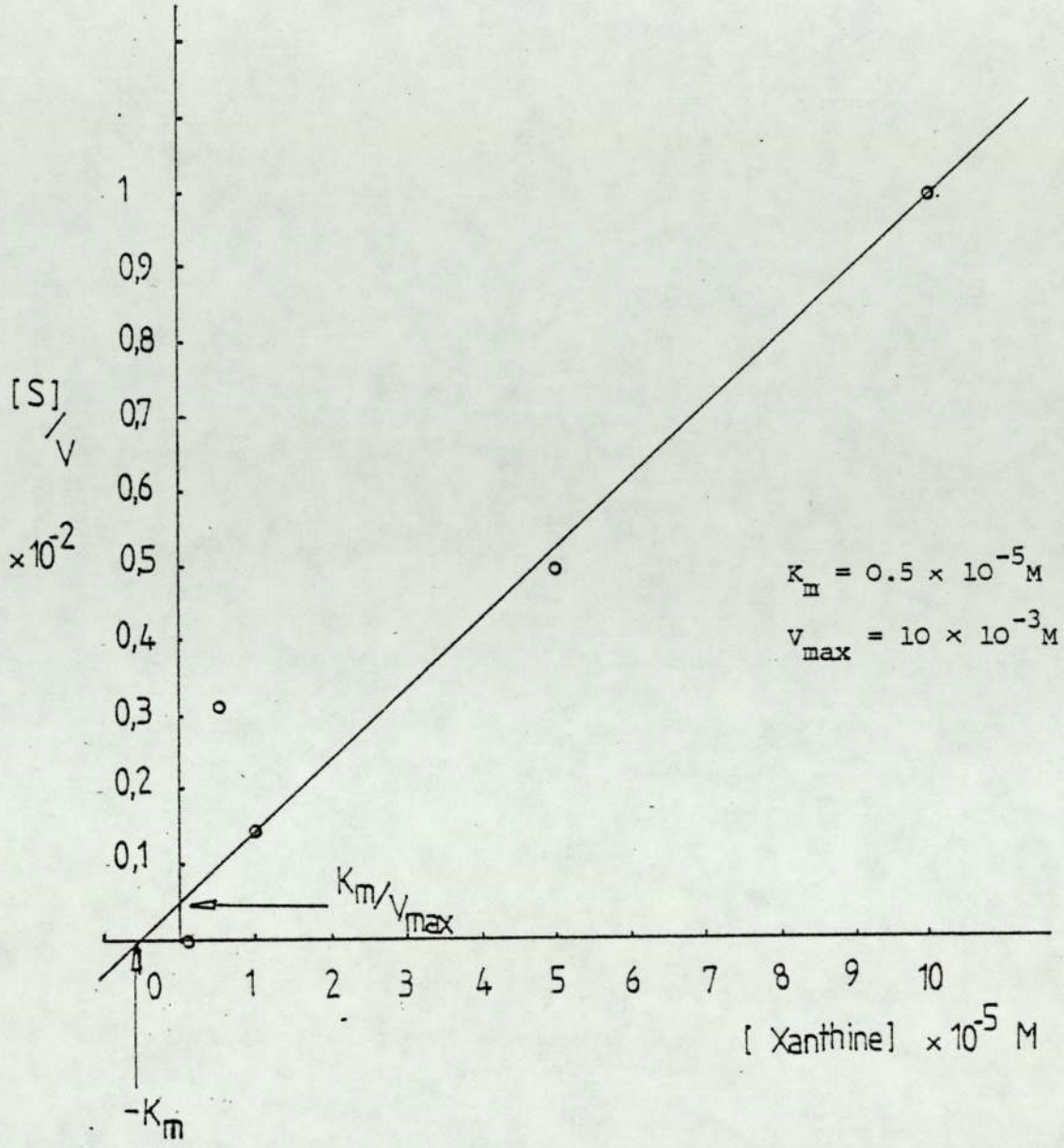
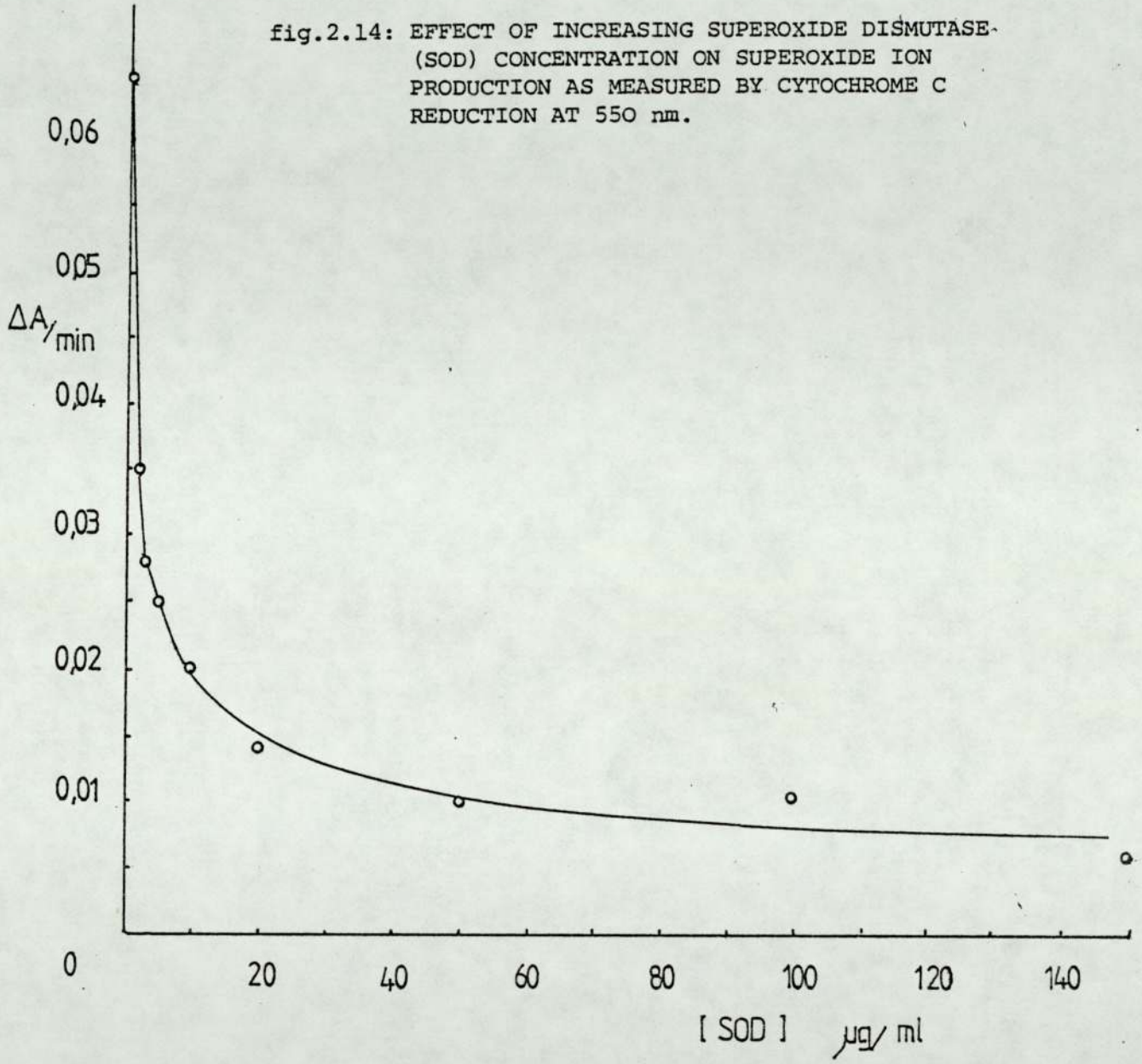


fig.2.3 WOOLF PLOT: XANTHINE OXIDASE ACTIVITY

fig.2.14: EFFECT OF INCREASING SUPEROXIDE DISMUTASE (SOD) CONCENTRATION ON SUPEROXIDE ION PRODUCTION AS MEASURED BY CYTOCHROME C REDUCTION AT 550 nm.



OXIDATION OF TETRAHYDROBIOPTERIN BY SUPEROXIDE RADICALS :

Table 2.11 : Xanthine - Xanthine Oxidase System : Effect of substrate Concentration On Enzyme Activity.

[Xanthine] M	Enzyme Activity. (units)
0.1×10^{-5}	0
0.5×10^{-5}	1.6×10^{-3}
1×10^{-5}	$(7.14 \pm 0.51) \times 10^{-3}$
5×10^{-5}	$(10.07 \pm 0.41) \times 10^{-3}$
10×10^{-5}	$(9.14 \pm 0.35) \times 10^{-3}$

Table 2.12 : Xanthine - Xanthine Oxidase System : Effect of change In Enzyme concentration. Substrate Concentration Fixed at 1 $\times 10^{-4}$M.

Relative Enzyme Concentration.	Xanthine Oxidase Activity (units).
2x	$(13.80 \pm 0.56) \times 10^{-3}$
1x	$(9.14 \pm 0.33) \times 10^{-3}$
0.5x	$(4.76 \pm 0.65) \times 10^{-3}$
0.33x	$(3.30 \pm 0.24) \times 10^{-3}$
0.25x	$(1.60 \pm 1.39) \times 10^{-3}$

Using the xanthine oxidase concentration which gave 9.14×10^{-3} units of activity at 1×10^{-4}M xanthine concentration, the effect of superoxide dismutase (SOD) on the superoxide ions generated by this system was investigated. The reduction of cytochrome C by superoxide was used to monitor SOD activity.

By monitoring the absorbance at 500 nm the cytochrome C that could be totally reduced in the prescence of xanthine/xanthine oxidase was deduced as 8.7×10^{-6}M. The same solution of cytochrome C in the absence of xanthine/xanthine-oxidase system was calculated to contain 0.9×10^{-6}M reduced cytochrome C. (i.e 10% of the total was ferricytochrome C). Over a 30s. assay time a further 10% of cytochrome C was calculated to undergo reduction in the prescence of xanthine/xanthine oxidase. Hence an excess of cytochrome C was present during the cytochrome C/SOD assay.

Table 2.13 : The Effect of Superoxide Dismutase Concentration On the reduction of cytochrome C by Superoxide at 550 nm.

[SOD] µg/ml	A/min. at 550nm
0	0.065 ± 0.003
2	0.035 ± 0.005
2.5	0.028 ± 0.005
5	0.025 ± 0.003
10	0.020 ± 0.004
20	0.014 ± 0.001
50	0.010 ± 0.002
100	0.011 ± 0.002
150	0.006 ± 0.001

Table 2.14 : Effect of Tetrahydrobiopterin On Xanthine Oxidase Activity

Assay condition as previously described.

[THB] pmol./ml.	Xanthine Oxidase Activity ($\times 10^{-3}$) units.
0	7.83
21	7.30
42	8.22
63	6.89
84	7.14

Tetrahydrobiopterin left at room temperature (25°C) exposed to light and atmospheric oxidation for a period of 18 min. was found not to alter the xanthine oxidase activity. Hence any breakdown products of THB oxidation had little effect during this time on the xanthine oxidase activity.

Using xanthine-xanthine oxidase as a superoxide-generating system the effect of superoxide ions on THB oxidation was investigated by HPLC (Table 2.15). The conditions used were as for the enzymic assays; 100 µg/ml of SOD was used where required. Aliquots of the reaction mixture were taken at time 5 min., 10 min., and 20 min. and added to acid-iodine and alkaline-iodine reagents. The latter solutions acted as "reaction - quenchers".

Table 2.15 : THB oxidation by superoxide Ions and the effect of SOD.

System	Time : (min). 0	5	10	20
	A. THB yield as % of initial THB			
THB	100	84.5	58.8	13.8
THB + Xanthine		65.2	22.5	12.8
THB + Xanthine + XO		64.1	22.3	13.1
THB + Xanthine + XO + SOD		105.0	81.9	66.5
	B. Biopterin yield (acid/I_2 oxidation) (nmol./ml).			
THB	7.33	6.33	5.01	3.91
THB + Xanthine		5.18	2.97	2.50
THB + Xanthine + XO		5.42	3.20	3.27
THB + Xanthine + XO + SOD		7.73	6.46	6.10
	C. Pterin yield (acid/I_2 oxidation) nmol./ml.			
THB	0	1.12	1.54	2.34
THB Xanthine		1.15	1.57	3.20
THB Xanthine XO		0.87	3.14	3.39
THB Xanthine XO SOD		0.44	0.94	1.90

XO = Xanthine Oxidase.
SOD = Superoxide dismutase.

Conditions : Potassium phosphate buffer (0.1 M), pH 7.8, 0.1 mM EDTA, at 25 C, contained 10^{-4} M Xanthine, 0.1 ml of commercial Xanthine oxidase diluted 250 x and 100 μg/ml SOD where required. Aliquots taken at stated times and oxidised to determine per cent THB, biopterin and pterin levels.

Table 2.15 shows that the presence of xanthine-xanthine oxidase appears to make very little difference to the oxidation of THB over a 20 min. period. However, if one looks at the oxidation for the initial 10 min only then it can be seen that the presence of superoxide ions produces a greater yield of pterin following acid-iodine oxidation, and a low residue of tetrahydrobiopterin.

The presence of SOD appears to retard the oxidative degradation of tetrahydrobiopterin. This could either be due to its superoxide-dismutating effect, a protein effect or an entirely novel biological effect. To observe whether there was a protective anti-oxidant effect due to the presence of protein in solution the experiment was repeated in the absence of a superoxide generating system (Table 2.16). Additional protection was not afforded by the presence of protein.

Table 2.16 : Effect of Xanthine Oxidase, SOD and albumin on THB oxidation in the absence of Xanthine.

System	Time (min)			
	0	5	10	20
	A. THB yield as % of initial THB			
THB	100	83	54	25
THB + XO		74	41	23
THB + SOD		98	54	43
THB + albumin		82	41	32
	B. Biopterin yield (acid/I_2 oxidation) (nmol./ml)			
THB	6.61	6.04	5.04	2.91
THB + XO		5.13	4.13	4.27
THB + SOD		6.58	4.99	3.77
THB + albumin		5.66	3.75	2.94
	C. Pterin yield (acid/I_2 oxidation) (nmol./ml)			
THB	0	0.58	1.95	0.95
THB + XO		0.46	2.24	1.28
THB + SOD		0.25	1.14	0.92
THB + albumin		0.49	1.02	0.69

XO = Xanthine oxidase
SOD = Superoxide dismutase

Conditions : Potassium phosphate buffer (0.1M), pH7.8, 0.1mM EDTA, at 25 C, containing 0.1ml of commercial Xanthine oxidase diluted 250 fold or 100 µg/ml SOD or 100 µg/ml albumin Aliquots taken at stated times and oxidised to determine % THB, biopterin and pterin levels.

OF EDTA.

	10 min.			20 min.		
NO EDTA.	Biopterin* (nmol./ml)	% THB	% Pteridine*	Biopterin* (nmol./ml)	% THB	% Pteridine*
1. THB	1.91	13.2	62.6	1.107	1.6	76.1
2. THB + Ascorbate	0.025	0	0.25	0.429	0.4	4.2
3. THB + Fe(II)	0.091	0	0.90	2.287	19.6	35.9
4. THB + Fe(II) + Ascorbate	0.021	0	0.20	0.286	0	7.8
EDTA.						
THB	2.680	20.4	58.5	1.936	14	55.9
THB + Ascorbate	2.605	20.9	31.6	2.027	15.9	21.3
THB + Fe(II)	1.488	3	85.8	1.352	4.5	79.1
THB + Fe(II) + Ascorbate	2.489	22	27.7	2.243	18	23.0

* After acid, iodine oxidation.

Conditions : Potassium phosphate buffer (0.05M), pH 7.4 at 37 C
Ascorbate = 100 mH (sodium salt.
EDTA = 0.1mM $FeSO_4 . 7H_2O$.
THB = 0.1mM.

Table 2.18 : Comparison of Results Obtained by HPLC and by Crithidia fasciculata Assay : Urine Analysis.

Clinical status	Crithidia active factor levels (ng/ml)	Biopterin level* by HPLC (ng/ml)
Healthy adults (13)	820 ± 31	1470 ± 86
Affective Disorder subjects (19)	510 ± 138	800 ± 28
Adult PKU (6)	1430 ± 33	1580 ± 36
Tuberose Sclerosis patients (6)	1360 ± 94	890 ± 82

* After acid-iodine oxidation.

Results as mean ± SEM.

figures in parenthesis = number of subjects studied

Table 2.19 : Camparison of Results Obtained by Crithidia fasciculata Assay : Urine Biopterin Levels.

Subject	Clinical status	Biopterin * level by HPLC (ng/ml)	Crithidia active factor level (ng/ml)
IH	Tuberose Sclerosis	438	580
MT	Tuberose Sclerosis	538	780
WJ	"	1500	1820
MT	"	413	1340
SV	"	1150	1940
JT	"	1275	1720
JS	phenylketonuria.	1638	1620
AU	"	1600	1320
JS	"	1638	1550
JG	"	1550	1440
CH	"	1188	1100
WP	"	1863	1540

* After acid I_2 oxidation of sample.

During routine analysis an anomalous behaviour of THB was observed in the presence of ascorbate. An investigation of the effect of iron and ascorbate on THB oxidation was therefore carried out. (Table 2.17).

The presence of EDTA affords protection as can be seen by the higher yield of biopterin, total pteridine and THB, in its presence. THB by itself also tends to yield the most product (in terms of bioptein, THB and total pteridine). Ascorbate tends to have a protective effect when EDTA is present but not otherwise. Presence of ascorbate and/or iron leads to conversion to non-fluorescent, unidentified products. Presence of iron is most destructive, yielding low biopterin and THB but substantial pterin which is more stable than biopterin to further oxidation. Iron and ascorbate together act in a similar manner to ascorbate alone.

Discussion:

In acid-iodine solution iodine undergoes the following reaction in the presence of potassium iodide,

$$I_{2\,(aq)} + I^{(-)}_{(aq)} = I_3^{(-)}{}_{(aq)}$$

producing the tri-iodide ion. The oxidation of tetrahydropterin has been investigated over a wide pH range. The observation that protonation at N(5) leads to a large decrease in the rate of oxidation with ferricyanide (Archer et al 1972) has led to postulation that oxidation occurs primarily by electron withdrawal from the lone pair at N(5). On this basis the reaction mechanism for the oxidation of THB can be suggested to occur as in fig. 2.15.

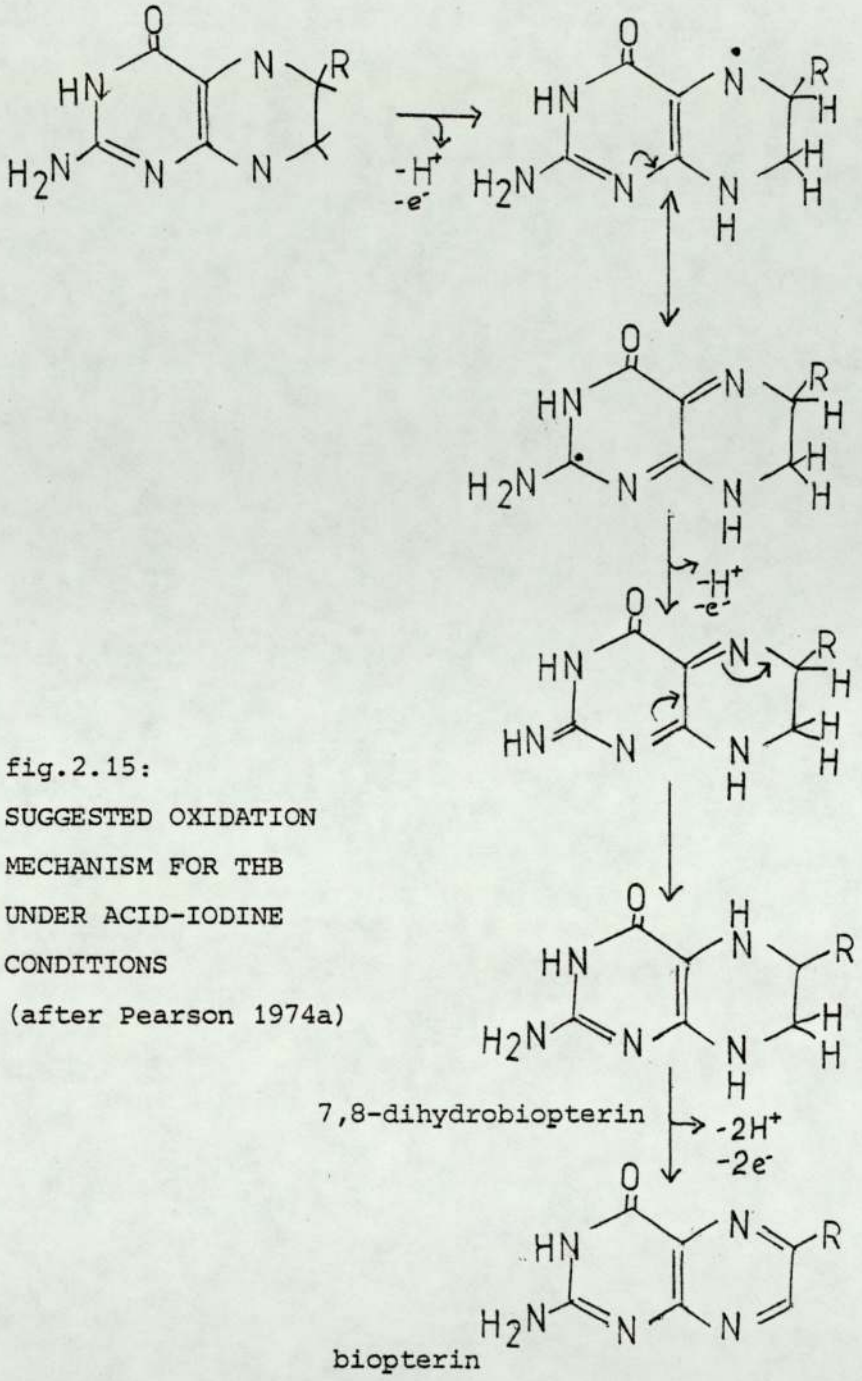


fig.2.15:
SUGGESTED OXIDATION MECHANISM FOR THB UNDER ACID-IODINE CONDITIONS
(after Pearson 1974a)

However the detailed mechanism may be more complex than suggested here and may involve other forms of electron loss. For example Pearson (1974a) states that heterolytic fission of bonds is favoured over homolysis in solvents with high dielectric constants such as aqueous media. Whatever the mechanism, iodine oxidation under acid conditions leads to almost 100% yield of biopterin.

Under alkaline conditions, iodine undergoes the following reactions :

$$I_{2\,(aq)} + 2OH^-_{(aq)} \longrightarrow I^{(-)}_{(aq)} + IO^{(-)}_{(aq)} + H_2O \quad ....(3)$$

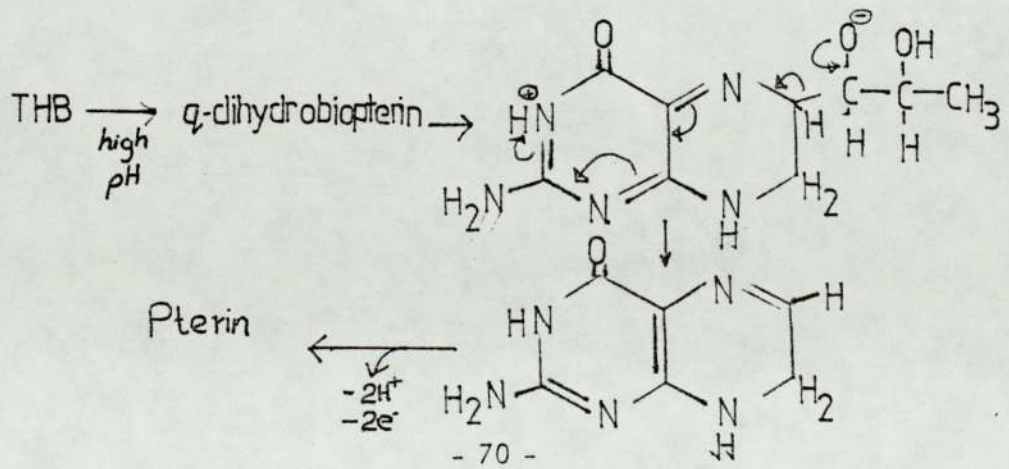
hypoiodite ion.

$$3IO^{(-)} \longrightarrow 2I^{(-)}_{(aq)} + IO_3^{(-)} \quad ....(4)$$

iodate ion.

According to Li and White (1943) the half-life of the hypoiodite ion is approximately 30 min. The iodine solutions used in the experiment were at least a day old and it is therefore assumed that the major species is the iodate ion. Under alkaline-iodine oxidation conditions THB is more labile yielding 80% pterin and some biopterin. The proposed mechanism of oxidation under these conditions is as laid out in fig. 2.16.

Fig. 2.16 THB Oxidation under alkaline conditions



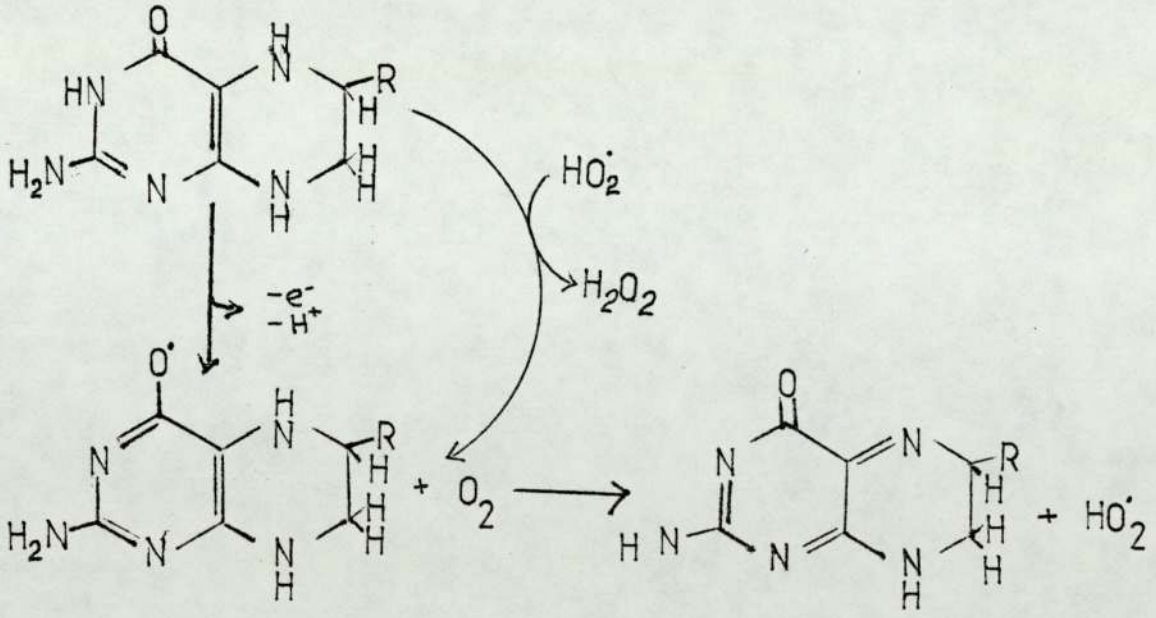
The oxidative degradation of tetrahydrofolate under acid-iodine conditions yields pterin almost entirely. Chippel and Scrimgeour (1970) have identified dihydroxanthopterin and pterin as the oxidation products of ferricyanide oxidation of tetrahydrofolate at pH 5.6. At pH 6.2 there was some conversion to 6-formyldihydropterin. The contribution to pterin by oxidation of tetrahydrofolate in a biological sample would exclude the quantification of pterin as a means of estimating tetrahydrobiopterin level following alkaline-iodine oxidation.

Acid-iodine oxidation of sepiapterin produced three peaks, of which the major peak, which could be clearly resolved from biopterin-standard, is suggested to be 6-lactylpterin. Katoh et al (1977) have observed the oxidation of sepiapterin to 6-lactylpterin using mass and NMR spectra. 6-lactylpterin is suggested to be unstable degrading to give pterin-6-carboxylic acid, which was also observed.

The determination of the levels of sepiapterin and its oxidised product in the urine does not appear to be of direct diagnostic value because of its low fluorescence relative to biopterin and neopterin. However sepiapterin is almost equipotent compared to biopterin in its stimulatory effect on Crithidia fasciculata growth (Blair et al, 1983). Hence significant differences may be observed in values of total urinary biopterin observed by HPLC and by Crithidia-active factor levels. Recently, Neiderwieser et al (1980) have observed a patient with biopterin-synthesis deficiency suggested to be due to a metabolic block at GTP-cyclohydrolase level. The patient excreted high levels of sepiapterin in the urine. In such rare instances knowledge of sepiapterin and its oxidation products would be useful.

Observations of atmospheric oxidation of tetrahydrobiopterin over a wide-range of pH shows that at high pH the oxidation yields less biopterin and more pterin (Table 2.10). There was no clearcut evidence of an increase in xanthopterin to pterin ratio towards alkaline pH as observed by Stocks-Wilson (1971). The xanthopterin remained a minor product of the oxidation. Blair and Pearson (1974) have examined the kinetics of the autoxidation of tetrahydrobiopterin. Observation of the initial rates show the reaction to be first order with regards to oxygen and tetrahydropterin. It is suggested that atmospheric oxidation of tetrahydropterins is due to a free-radical chain reaction, initiated by electron removal at N(3) followed by rapid deprotonation at this site to give the free radical. Propagation would occur by chain carriers such as hydroperoxy radicals (Fig 2.17).

Fig. 2.17 **Autoxidation Of Tetrahydrobiopterin (Blair and Pearson, 1974).**



The free-radical mechanism proposed by Blair and Pearson (1974) would explain the observation of hydrogen peroxide during oxidation of 6,7-dimethyltetrahydropterin ($DMPH_4$), (Shiman et al 1971).

The generation of superoxide ions observed by Nishikimi 1975, and Heikkila and Cohen 1975, during oxidation of tetrahydrobiopterin could be due to further reactions of the hydroxyl radicals.

The protective effect of superoxide dismutase on the oxidation of tetrahydrobiopterin by superoxide ions is shown in Table 2.16. After a 10min. oxidation period the yield of pterin is highest in the superoxide-generating system. Oxidation of THB is also observed to be rapid in the presence of xanthine alone. This may be due to trace metal impurities present in the xanthine solution. The oxidising capability of the medium is reflected in the biopterin-pterin ratio. Where the conditions favour oxidation by strongly oxidising species the ratio is low (e.g. in the presence of xanthine-xanthine oxidase), whereas in the presence of SOD the ratio is high. Heikkila and Cohen (1975) are of the opinion that THB-mediated superoxide generation may be an essential step for the hydroxylation of tyrosine by tyrosine-hydroxylase. It remains to be shown if this is the case in vivo.

Tetrahydrobiopterin has been shown to be bound to plasma albumin in the blood. This binding property has been shown to preserve the tetrahydrobiopterin from aerobic oxidation in vitro (Ayling et al 1973). Such protective effect was not seen when serum albumin or superoxide dismutase was used (Table 2.16). This could be due to the fact that the concentration of protein used was 50% of the level used by Ayling et al.

Table 2.17 shows the oxidation of THB by ascorbate and iron in the presence and absence of EDTA. This investigation is of importance because of the generally held belief that addition of ascorbate to biological samples during storage will preserve the THB by way of the reducing action of ascorbate. However when trace metal impurities are present the tendancy for ascorbate is to act as a powerful oxidising agent. This can be seen from Table 2.17. when the chelating agent is absent.

Oxidations mediated by metal ions have been observed as early as 1893 when Fenton exhibited the oxidation of D-tartaric acid by H_2O_2 and Fe^{2+}. Haber and Weiss (1934) have proposed the following Fenton-type reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{-} + HO^{\cdot}$$

The hydroxyl free radical acts as a strong oxidising species. Oxidation of quinoline mediated by ascorbate in the presence of ferrous sulphate and oxygen have been reported by Udenfriend et al (1954). They proposed a reaction of the type :

$$2Fe^{2+} + 2H^{+} + O_2 \longrightarrow 2Fe^{3+} + H_2O_2$$

The H_2O_2 was suggested to undergo further (possibly Fenton-type reactions) to yield stronger oxidising species.

Tetrahydrobiopterin may be oxidised by similar mechanisms. The presence of EDTA with ascorbate solution conserves a substantial proportion of THB, it is assumed by chelating metal ion impurities. That iron + EDTA does not afford protection to THB-oxidation may be due to the fact that EDTA levels were only ten times higher then the iron concentration. Also under some circumstances the EDTA may be unable to completely encompass the metal ion to form a true octahedral complex, (Hoard et al, 1961). This may be the case when ferrihdroxyl

species occur as suggested by Breslow and Lukens (1960) :

$$Fe^{2+} + H_2O_2 \longrightarrow FeOH^{++} + OH^{\cdot}$$

It is interesting to note that iron has been shown to be an integral part of phenylalanine hydroxylase (Fisher et al 1972) and that oxidation of tetrahydrobiopterin by iron under non-enzymatic conditions has been observed by Vonderschmitt and Scrimgeour (1967) and others. Furthermore superoxide ions have been proposed to be substrate in various reactions involving tetrahydrobiopterin(Nishikimi, 1974). It remains to be confirmed that species which are involved in in vitro degradation of THB may function as co-substrates in-vivo.

Comparison of HPLC and Crithidia fasciculata Assay Results :

As rapid, routine analysis of biological samples is especially relevant to the early diagnosis of the condition of hyperphenylalaninaemia, two popular methods of biopterin analysis will be compared here. Biopterin is an essential dietary requirement of the trypanosomatid insect parasite Crithidia fasciculata (Baker et al 1980). The other unconjugated pteridines, dihydrobiopterin, tetrahydrobiopterin, sepiapterin and neopterin are also active to differing extent. (Blair et al 1983).

Comparison of HPLC results with Crithidia fasciculata assay of urines shows that in the case of tuberose sclerosis patients, (Table 2.19), the Crithidia results are significantly elevated at 1313 ± 232 compared with HPLC mean of 886 ± 195 (all results as mean $\pm$ SEM in ng/ml.); $p < 0.01$ by paired t-test. However the phenylketonuric urines consistently show elevated results by HPLC measurement compared to Crithidia assay. The difference is significant ($p<0.025$ by paired t-test); the HPLC mean being

1580 ± 89 and the Crithidia mean being 1428 ± 78. The correlation coefficient between HPLC and Crithidia is highly significant for both tuberose sclerosis samples and phenylketonuria ($r = 0.83$, $p < 0.005$) ; the Crithidia values being conistently higher in tuberose patients and consistently lower in phenylketonurics when compared with HPLC measurements.

Crithidia total biopterin concentration in urine of 13 normal subjects was significantly low compared to HPLC values (820 ± 110 vs. 1470 ± 310, $p < 0.002$ by Wilcoxon signed ranks test) and in 19 patients with affective disorder (510 ± 60 vs 800 ± 120, $p < 0.002$ by Wilcoxon test), (Blair et al 1983). It is found in these patients that the higher the percentage of reduced biopterins in the urine (as dihydro- and tetrahydrobiopterin) the greater is the elevation of HPLC results over Crithidia results. For example, the percentage reduced biopterin in the 13 normal subjects was 70%, in the affective disorder patients 65%, in the phenylketonuric patients 55% and in tuberose sclerosis patients 12% (Blair et al 1983).

The discrepancy between the HPLC and Crithidia results is suggested to be directly related to the percentage reduced biopterin present in the sample (Blair et al 1983). When the reduced biopterin content is low the Crithidia results tend to agree with HPLC results and when high the HPLC results tend to be elevated. This discrepancy arises due to the autoclaving procedure and the incubation of the sample at 29-32°C for three or four days that is required for the Crithidia assay (Baker et al 1980). It is suggested that the reduced biopterins oxidise and in the process breakdown to Crithidia-inactive products. Milstein (1983) has investigated the oxidation of tetra - and dihydrobiopterin at pH 4.5 under

conditions used in the Crithidia assay and found that at this pH a substantial proportion of reduced pterin is lost. If the biological sample is oxidised with iodine prior to the autoclaving procedure then the Crithidia results tend to agree well with HPLC results (Milstein 1983).

Where Crithidia assay results are higher than HPLC (e.g. as for the tuberose sclerosis patients) it is suggested that other Crithidia-active material such as sepiapterin is responsible for the discrepancy (Blair et al 1983).

Hence although Crithidia-assay is suitable for analysing large numbers of samples its sensitivity has to be questioned on the grounds of non-specificity and inability to detect reduced biopterins unless the latter are first oxidised.

CHAPTER THREE

Tetrahydrobiopterin Metabolism In Phenylketonuria And Related Hyperphenylalaninaemia

Phenylketonuria is an inborn error of phenylalanine metabolism first described by Folling in 1934. It is characterised by clinical symptoms of mental retardation, accompanied by an excessive urinary excretion of phenylalanine and its metabolites (phenylpyruvate, phenylacetate, o-hydroxyphenylacetate and phenyl lactate), fig. 3.1, and a distinct pattern of urinary pterin excretion.

The condition is inherited as an autosomal recessive gene-contribution by both parents (Jervis, 1939). Phenylalanine accumulates in the body fluids due to an inability to hydroxylate phenylalanine to tyrosine. An inactive phenylalanine hydroxylase has been implicated as a causal defect (Jervis, 1953). Extensive clinical, biochemical and neuropathological studies have been performed but the pathogenesis of the associated mental retardation remains obscure (Scriver and Clow, 1980).

The condition as described by Bartholome et al, 1976, and others (Reynolds et al, 1979 Lassala et al 1979) has been termed "classical phenylketonuria" and is caused by an inactive or partially active phenylalanine hydroxylase. The defective metabolism of phenylalanine in the brain results in inadequate levels of tyrosine being present in the neuronal tissue. The latter amino acid in such cases becomes an essential amino acid. Tyrosine is a precursor of the neurotransmitters dopamine, dihydroxyphenylalanine aid (dopa) and noradrenaline. Elevated systemic levels of phenylalanine

competitively inhibit the uptake of tyrosine and tryptophan across the blood-brain barrier (Pratt, 1982). Tryptophan is the amino-acid precursor of the neurotransmitter 5-hydroxytryptamine. The defective neuronal levels of the various neurotransmitters may lead to progressive mental retardation if the condition is left untreated.

The incidence of phenylketonuria in the United Kingdom has been estimated to be 10.4 per 100,000 live births from the 1974-8 period, by the Medical Research Council committee on PKU (1981). The upper limit of normal plasma phenylalanine concentration has been taken as 240 nmol/l (4 mg/100 ml), and anyone on a normal diet with a persistent hyperphenylalanaemia (HPA) of 480 nmol/l or above is suspected of phenylketonuria or a disorder of biopterin metabolism (MRC report 1981). Patients with a severe degree of hydroxylase deficiency show plasma phenylalanine concentrations of over 1800 nmol./l (30 mg/100 ml.), when untreated.

Of the infants born with a condition of HPA, 1-5% have a biopterin-defect rather than a phenylalanine hydroxylase deficiency. In the UK the incidence of this "malignant hyperphenylalaninaemia" is one in 500,000 (MRC report, 1981).

Such patients are unable to maintain adequate levels of cellular tetrahydrobiopterin; the condition arising either due to a genetic defect in the enzymic de novo synthesis of tetrahydrobiopterin (Kaufman et al 1975) or of its salvage by the enzyme dihydropteridine reductase (DHPR) (Leeming et al 1976). In 1974, Bartholome reported a child with HPA whose condition did not ameliorate with a phenylalanine-restricted diet. In 1975, Smith et al reported a similar case and attributed it to

dihydropteridine reductase deficiency. This patient showed normal liver phenylalanine hydroxylase activity, raised blood phenylalanine levels and progressive neurological impairment. Other DHPR-defective cases have been reported subsequently (Leeming et al 1976, Milstein et al 1980, Schlesinger et al 1979). It has been suggested that the defect in these patients is one of reduced conversion of quinonoid dihydrobiopterin to tetrahydrobiopterin. These patients are more likely to show milder HPA (4-10 mg/100 ml.) than the synthesis-deficient patients (Dhondt 1984), but have the same age of onset of neurological symptoms as the synthesis-deficient patients (about 4 months), Dhondt 1984.

Patients with hyperphenylalanaemia exhibiting normal DHPR and phenylalanine hydroxylase activity have been reported (Kaufman et al 1975; Rey et al 1976; Niederwieser et al 1979). Such patients show high neopterin and relatively low biopterin and dihydrobiopterin levels in their urine (Niederwieser et al 1979). Hence a defect in the synthesis pathway subsequent to neopterin-triphosphate synthesis has been implicated. These patients show a median age of onset of neurologic symptoms of four months and blood phenylalanine levels usually greater than 20 mg/100 ml, (Dhondt 1984). The situation is further complicated by the appearance of a partial-defect in synthesis, in some patients, which is transient in nature. These patients only exhibit clinical symptoms following administration of a phenylalanine-load (Dhondt 1984). Also a peripheral form of synthesis-defect has been reported with mild HPA which was corrected by THB administration (Dhondt 1984). The cerebrospinal fluid biopterin and neurotransmitter levels were normal and neurological symptoms lacking in this patient.

A defect in GTP-cyclohydrolase I activity has been reported by Niederwieser et al (1984) in a child with hyperphenylalaninaemia with progressive neurological deterioration and lack of cyclohydroxylase activity in the liver. Dihydroneopterin and its pterin metabolites were markedly diminished in the liver suggesting defect at an early biosynthesis-stage.

Patients with malignant hyperphenylalaninaemia have normal liver phenylalanine hydroxylase activity and elevated plasma phenylalanine levels. However, in some patients with DHPR deficiency the plasma phenylalanine levels may not be excessively high due to some compensatory conversion of dihydrobiopterin to THB by dihydrofolate-reductase (DHFR), Guthrie, 1963. This enzyme is present in large amounts in the liver and in much smaller amounts in the brain (Makulu et al, 1973). Such patients may give a false-negative result with the Guthrie test. In such circumstances the patient continues to undergo progressive neurological damage since the central nervous system does not have adequate levels of DHFR to generate the biopterin-cofactor.

Furthermore, patients with malignant HPA do not benefit from dietary restriction of phenylalanine intake since the defect is one of biopterin-cofactor deficiency. Since THB is also a cofactor for tyrosine-3-hydroxylase and tryptophan hydroxylase its absence leads to deficiency of the monoamine neurotransmitters and the treatment plan for these patients involves administration of relevant neurotransmitter precursors (Curtius et al 1979). Hence an early, differential diagnosis of the various forms of HPA is important. Various biochemical tests have been formulated to diagnose these patients.

The simplest preliminary test is the Guthrie test (1963). This can be carried out in the first few days of life when the baby's blood sample collected on a Guthrie card is added to a culture of Bacillus subtilis containing an inhibitor thi-enylamine. Phenylalanine in elevated amounts counteracts this inhibition and evokes a growth response from the bacteria indicating the hyperphenylalaninaemic condition.

Measurement of the patients blood phenylalanine level after administration of tetrahydrobiopterin differentiates between atypical and classical PKU. The phenylalanine level decreases in the former whilst remaining unchanged in the latter (Curtius et al 1979, Danks et al 1979). However, THB is expensive as it is not manufactured on a large scale.

Niederwieser et al (1979) have used high voltage electrophoresis to separate urinary pteridines. This method does not allow quantitative analysis of reduced pteridines which would be susceptible to atmospheric oxidation during separation .

Leeming et al (1976) have successfully used Crithidia fasciculata assay for the determination of Crithida-active factors but this method does not allow investigation of the individual pteridines or the quantitative determination of the reduced forms of biopterins.

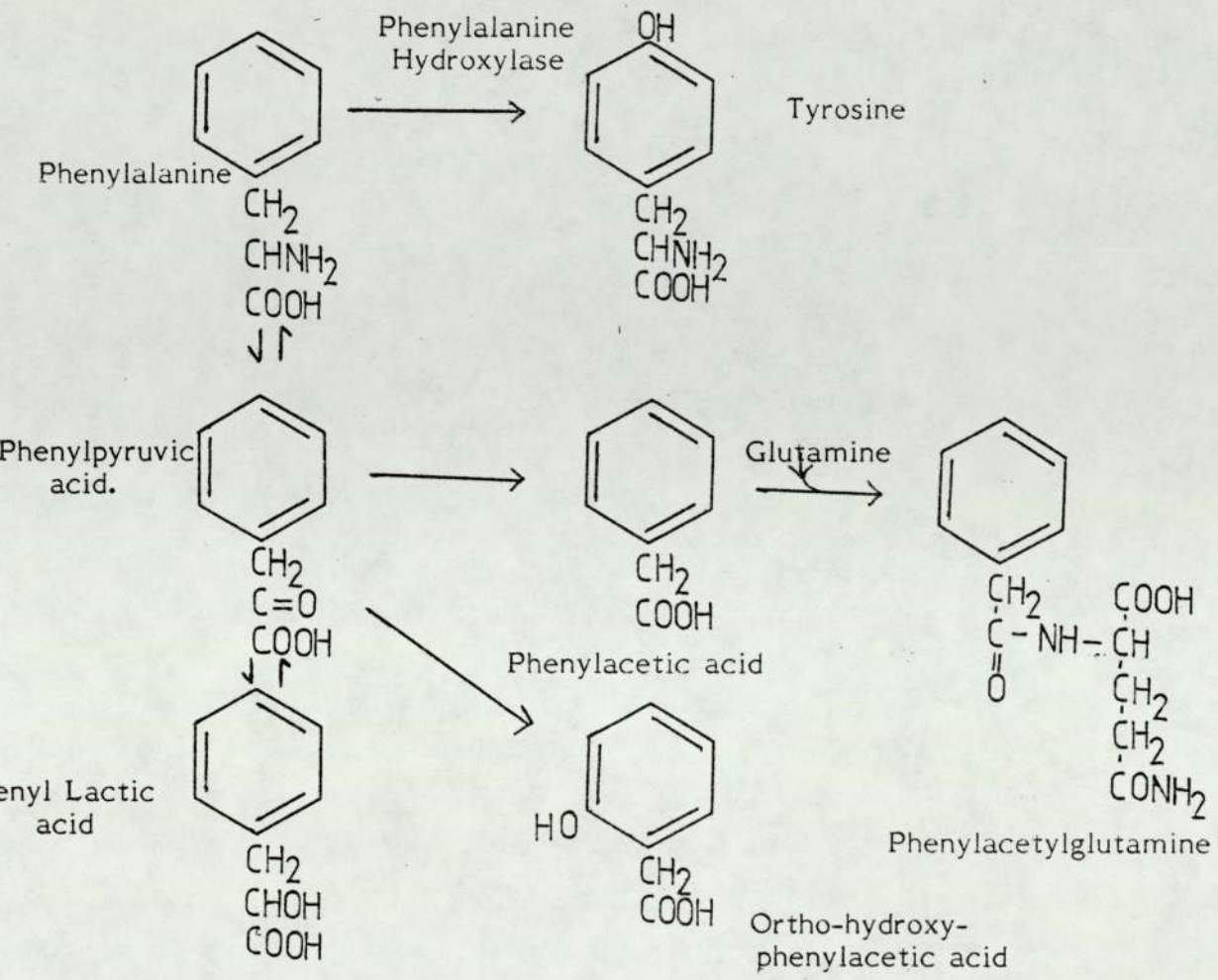
Other methods such as gas-chromatography (Rothler et al, 1976) and radio-immunoassay (Nagatsu 1981) have also been suggested but their usefulness in screening large populations is questionable or the method open to criticism.

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

Quantitation of the levels of tetrahydrobiopterin, dihydrobiopterin and fully oxidised biopterin as well as the level of neopterin, is crucial if differential diagnosis is to be made between normal subjects, classical PKU and atypical hyperphenylalaninaemic patients. Fukushima and Nixon (1980) have utilised iodine-oxidation under acid and alkaline conditions as a means of distinguishing between the oxidised and reduced forms of biopterins. Patients with classical PKU exhibit high levels of THB with a low neopterin / biopterin ratio in their urine. Patients with DHPR-deficiency accummulate quinonoid-dihydrobiopterin which is readily converted to dihydrobiopterin and excreted in the urine. These patients have low tetrahydrobiopterin levels and a high total urinary biopterin concentration (Dhondt et al 1981). In contrast, patients who are biopterin-synthesis deficient exhibit markedly low levels of total biopterin as well as elevated neopterin levels in their urine (Nixon et al 1980). Hence measurement of the neopterin/biopterin ratio as well as levels of various reduced biopterin levels offers a means of diagnosing the HPA conditions.

This study was directed at evaluating, the versatility of the HPLC system in measuring pterins and collating data about the various conditions. An animal model of HPA at the adult stage was also used to investigate the

relationship between biopterin metabolism and plasma phenylalanine level.



Deficiency of phenylalanine hydroxylase or biopterin cofactor reduces conversion of phenylalanine to tyrosine and phenylalanine metabolism by other pathways becomes predominant. Phenylalanine accumulates in the blood and tissues and the levels of phenylpyruvate, O-hydroxy-phenylacetate, phenylacetate and phenyllactate become elevated both in the serum and urine.

Fig. 3.1. PHENYLALANINE METABOLISM

Materials :

Ion-exchange resin, Dowex 50 [H^+], 12% cross-linked, mesh size 200-400 was obtained from Sigma, as were 6-methylpterin, phenylalanine and p-chlorophenylalanine. Fiso-solv tissue solubiliser and scintillation coctail were obtained from Fisons (UK).

L-(U - ^{14}C) phenylalanine was obtained from Amersham (UK) and had 88% isotopic abundance in all carbon atoms. Beckman LS 7500 liquid scintillation counter was used for beta-counting.

Urine and blood samples were obtained from Dr. P.E. Sylvester at St. Lawrence's Hospital, Caterham and from Dr. R.M. Veall at Tatchbury Mount Hospital, Southampton. Healthy control samples were from laboratory personnel at the University. Dr. I.N. Smith supplied csf samples from leukaemia patients (and urine samples of subjects A.K., D.T., A.C.) from Gt. Ormond St. Children's Hospital, London.

Methods :

Urine sample analysis :

Urine samples were either investigated neat or after acid-iodine or alkaline-iodine oxidation (Fukushima and Nixon, 1980). 1 ml of the solution was mixed with 1 ml. of 0.1% iodine (w/v, IN, HCl) which also contained 2% potassium iodide. The iodine was present in excess as noted by visual observation and by starch test. The solution was left to stand in the dark for 1 hour. At the end of this period the excess iodine was reduced by addition of excess of 1% ascorbate. The resulting solution was further diluted prior to analysis by HPLC. The experiment was repeated under alkaline conditions (IN. NaOH).

Heparinised whole blood analysis :

0.5 ml. of the whole blood sample was mixed with 0.2 ml of 1% iodine solution in 2% potassium iodide made up in IN HCl and left to oxidise for 1 hour at 21°C (room temperature). Excess iodine was then reduced by 0.3mls of 2% ascorbic acid. The resulting solution was centrifuged for 30 min. at 4000 rpm and 0.5mls. of resultant supernatant mixed with 0.5ml. of 6-methylpterin of known concentration. The 1ml. sample was then passed through a Dowex 50[H^+] column (5 x 5mm) and the column washed with 2.5mls of distilled water. The pteridines were eluted with 1.4 mls of IN ammonium hydroxide, and the eluate neutralised with 0.1 ml. of glacial acetic acid. A 50 ul. sample was analysed by HPLC. Neopterin and biopterin levels were corrected for the recovery of the standard 6-methylpterin.

Acute hyperphenylalaninaemia models :

Adult male, Wistar rats (200-250g.) were starved overnight and then administered phenylalanine (300 mg/kg.) by gavage, as a suspension in corn-oil (0.4 ml.). Rats were sacrificed half hour, 1 hour or 4 hours later and the brain and liver pteridines analysed. The animals were killed by cervical transection with prior anaesthesia. The brain and liver samples were quickly removed and placed in ice at 0°C. A 20% homogenate in 20% trichloroacetic acid (w/v) was prepared and centrifuged at 3000 rpm for 2 min. The supernatant was analysed neat and by iodine oxidation (as per urines). A further six rats were dosed with phenylalanine containing 25% (v/v) of U-^{14}C-phenylalanine and sacrificed half-hour later. Brain and liver radiolabel distribution was investigated by liquid-scintillation experiments.

Six starved rats were also dosed with p-chlorophenylalanine in corn-oil (300

mg/kg.) and hour later with phenylalanine (300 mg./kg.) and sacrificed half hour later. Liver and brain biopterins were investigated.

Control rats were given 0.4 ml. dose of corn-oil only and killed in a similar manner to test-animals after appropriate time intervals (1/2 hr, 1 hr and 4 hr).

Liquid Scintillation Counting :

0.1g. of tissue was dissolved in 1 ml. of Fiso-solv solubiliser and incubated at 60°C in water bath for 3 hrs, complete solubilization was ensured by shaking the sample constantly. Three drops of 30% H_2O_2 (u/v) were added to each sample to decolourise it. The scintillation coctail was made up as 7ml. per litre of glacial acetic acid. Ten ml. of coctail was added to each sample and to blank and standard solutions. Blank solutions contained 0.1 ml distilled water and standard vials contained 0.1 ml of dose which had been diluted ten-fold. Each sample was counted for ten minutes in the counter.

Subject A G : Fig. **3.2.** describes the urinary pterin excretion pattern of a healthy laboratory personnel who had become pregnant. The subject was not on any medication at the time of analysis. The sample of urine was collected at 10.00 a.m. each morning at weekly intervals.

Phenylalanine - loading experiment : four healthy laboratory personnel (2 males and 2 females) participated in a phenylalanine-loading test. A mid-stream, mid-morning sample of urine was collected in ascorbate (1-5% w/v urine). The subjects were then given on oral load of 7g phenylalanine and

urine samples taken two hours later. Urinary neopterin and biopterin levels were analysed by HPLC; urinary Crithidia-factor levels were determined by Dr. R. Leeming at Birmingham General Hospital.

RESULTS :

Further Information On Individual Cases from Table : 3.1

Patient N.P : (from Tatchbury Mount Hospital, Southampton) is a Caucasian male, born of non-consangueous parents, after a 39 week pregnancy. The patient weighed 2.5 kg. at birth and showed normal initial development and was breast-fed. Initial signs of developmental delay appeared at eight months when hypotonia and poor head control was observed. Plasma phenylalanine at ten months was 900 umol./l. Urine exhibited phenylalanine and small amounts of o-hydroxyphenylacetic acid, but no phenylpyruvic acid. On a phenylalanine-restriction diet the plasma phenylalanine ranged between 60-120 umol/l. Phenylalanine-restricted diet was discontinued at 5 years and thereafter levels ranged between 180-480 umol./l. The patient had febrile convulsions at 14 months and a single "fit" at four years and grand-mal convulsions at eight years and thereafter. At age 19 (1982) the patient had a social age of about 7 years on Vineland Social Maturity Scale. At present the patient shows some physical and mental development, is fully ambulent and has some useful speech, is continent and able to feed and dress himself.

In the present study the patient showed no DHPR activity in his blood. Of two urine samples assessed for biopterins one showed low total biopterin level containing 60% tetrahydrobiopterin. The other urine sample showed high total biopterin but very low percentage THB. Plasma phenylalanine level was elevated at 300 umol/l, but tyrosine level was normal at 40 umol./l. (tyrosine range in normals is between 32-76 umol/l; Young et al 1983). The patient showed zero DHPR activity and was classified as deficient in this

enzyme.

Patient D.T. : is a twelve year old Caucasian male. Present analysis shows urine neopterin and biopterin to be within normal range with a neopterin/biopterin ratio of 0.3. This might be expected as the patient was on a restricted but relaxed PKU-diet. However, the Guthrie card whole blood analysis showed elevated biopterin level at 7.2 ug/l. Phenylalanine (917 umol/l) and tyrosine (97 umol./l) were both elevated in the plasma. The cerebrospinal fluid showed 86.3 ng/ml of biopterin and 28.8 ng/ml neopterin. The neopterin/biopterin reatio is 0.3 and 60% of the csf biopterin was estimated to be THB. The csf values are similar to those of leukaemic children; (values for healthy controls of similar age are not available.). Four leukaemic children had mean values of 20 $\pm$ 5 ng/ml neopterin 50 $\pm$ 8 ng/ml biopterin in their csf.

The neurotransmitter metabolite levels in the patient are as follows, with adult range in parenthesis (courtesy of Dr, I. Smith).

csf	5-hydroxyindole acetic acid	:	5.74 ng/ml	(20-100 ng/ml).
csf	Homovanillic acid	:	43.14 ng/ml	(50-200 ng/ml)
csf	dihydroxyphenylacetic acid	:	not detectable	
	blood 5-hydroxytryptamine	:	49.7 ng/ml	(100-150 ng/ml).

Hence all the csf values reported are low compared to healthy controls, and probably reflect an impaired monomine neurotransmitter synthesis in the brain.

Patient A.K : (Male). No further information is available on this patient but on the basis of the high neopterin value and therefore high

neopterin/biopterin ratio, yet normal blood DHPR activity, it is suggested that this patient is possibly synthesis deficient.

Patient A.C :

Male. This patient showed a significantly high plasma phenylalanine level (340 μmol./l) even though he was on a phenylalanine-restriction diet. Urine neopterin to biopterin ratio is slightly elevated and percent THB very low. The urine crithidia activity too was low. It would therefore seem that the patient is synthesis-deficient. However a low DHPR activity was also recorded in the blood . Since blood tends to be a better indcator than urine it is more likely that the subject is DHPR-deficient.

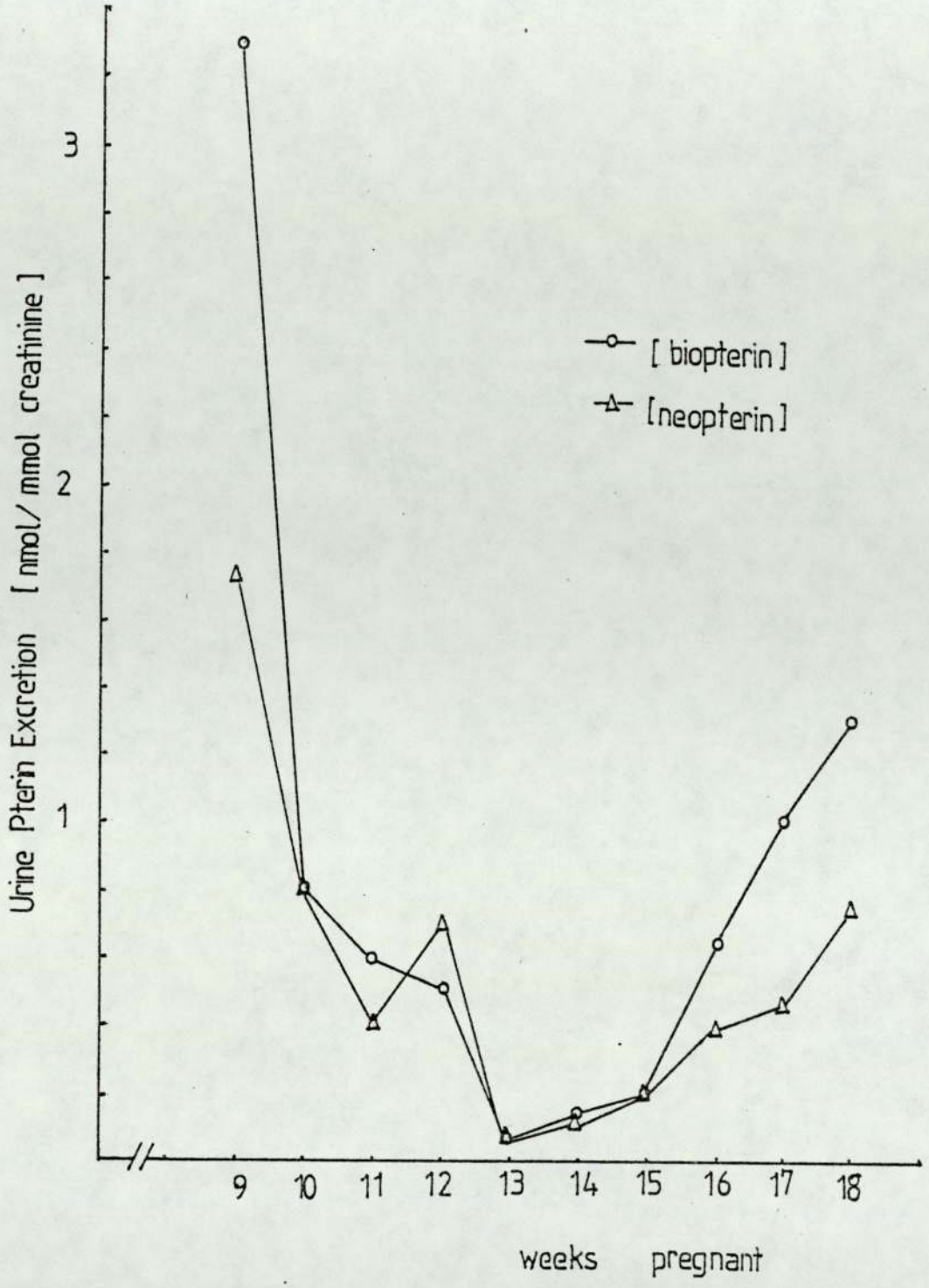


fig. 3.2: URINE PTERIN EXCETION DURING PREGNANCY: AN EXAMPLE OF VARIABLE EXCRETION DURING NORMAL PHYSIOLOGICAL CHANGE

TABLE 3.1 TETRAHYDROBIOPTERIN METABOLISM IN HYPERPHENYLALANINAEMIA AND IN NORMAL HEALTH

SUBJECT	URINE NEOPTERIN* nmol / mmol Creatinine	URINE BIOPTERIN* nmol/mmol Creatinine	% BIOPTERIN	% DIHYDRO-BIOPERIN	% THB	NEOPTERIN/ BIOPTERIN	WHOLE BLOOD DHPR ACTIVITY nmol NADH/min mg. protein	CLINICAL STATUS
NORMAL ADULTS	1516± 373**	3550±962	29±3.5	9.6±3.1	62.5 ±4.2	0.5±0.1	-	NORMAL
N.P.	108++	513	40	0	60	0.2	0	DHPR
	950	2475	15	44	41	0.4	-	DEFICIENT
D.T.	1100	3450	4	14	72	0.3	-	PKU
A.K.	5325	475	91	8	1	11.2	0.99	SYNTHESIS DEFICIENT
A.C.	1440++	420	65.5	28.1	6.4	3.4	0.26	DHPR
	5125	725	87	5.5	7.5	7.1	-	DEFICIENT
B.A.	238	650	5.9	28	66	0.4	0.42	DHPR DEFICIENT

* following acid-iodine oxidation
** mean±SEM
++ two samples

Table 3.2 : Blood Pterin levels and DHPR Activity in Healthy Controls.

Subject	sex	Blood neopterin nmol./l	Blood biopterin nmol./l	Blood DHPR activity nmol. NADH/min mg. Hb.	neopterin / biopterin
FAS	M	166	Trace	1.6	-
CH	M	113	19	2.2	6
MS	M	69	25	1.5	3
CM	M	89	24	1.6	4
SM	M	9.6	3	1.1	3
SP	M	195	38	1.9	5

Subject	sex	Urine * Neopterin. nmol./mmol. Creatinine	Urine Biopterin * nmol./mmol Creatinie.	% Urine tetrahydrobiopterin.	Neopterin / Biopterin
CM	M	257	1281	57	0.2
JH	F	289	970	68	0.3
SP	M	609	835	44	0.7
MS	M	534	1010	80	0.5
CE	M	479	1199	59	0.4
CH	M	315	598	61	0.5
AG	F	429	739	74	0.6
CE	M	380	315	-	1.2
SM	M	287	347	-	0.8
JH	F	184	285	-	0.7
PB	F	464	206	-	2.3

* pterin values represent oxidised + reduced pool.

TABLE 3.4 URINE PTERIN EXCRETION AND PLASMA PHENYLANINE AND TYROSINE LEVELS IN PHENYLKETONURICS

SUBJECT	SEX	URINE NEOPTERIN+ nmol/mmol. creatinine	URINE BIOPTERIN+ nmol./mmol. creatinine	% URINE THB	NEOPTERIN/ BIOPTERIN	PLASMA PHENYLALANINE mmol/l	PLASMA TYROSINE mmol/l
DL	M	182	896	80	0.2	1.86	0.12
PW	M	819	1279	72	0.6	1.33	0.10
SJ	M	598	1102	53	0.5	1.43	trace
SJ	F	397	1330	90	0.3	2.00	trace
SJ	M	714	1720	92	0.4	-	-
UA	F	1256	3360	-*	0.4	1.68	0.22
SJ	F	710	1323	-	0.6	-	-
GJ	M	671	845	-	0.9	-	-
HC	M	445	1188	-	0.4	1.55	trace
PW	M	683	1263	-	0.6	-	-

*no data available
+pterin values represent oxidised + reduced pterins
M= male
F= female

Table 3.5 : Daily total excretion of biopterin and neopterin in urine.

Daily total excretion = 24 hour urine volume x acid-I_2 oxidised pterin level.

Subject	Clinical Status.	Neopterin. (µg)	Biopterin. (µg.)
IH	Tuberose sclerosis	414	964
MT	"	72	59
WJ	"	518	690
MT	"	37	39
SV	"	173	345
JT	"	351	701
JS	phenylketonuria	1189	2686
AU	"	1735	4352
JS	"	169	295
JG	"	2088	2465
CH	"	133	333
WP	"	1097	1900

Table 3.6 : Urinary Pterin Excretion In Phenylketonuric Individuals And Normal Adults Pre- and Post-oral- phenylanine-load.

		Neopterin nmol./mmol. creatinine.	Biopterin nmol./mmol. creatinine.
Phenylketonurics	(6)	747 ± 110	1617 ± 660
Normal adults	(4)	329 ± 61	545 ± 59
Normal adults after phenylalanine intake.	(4)	589 ± 211	1085 ± 233
Values by Dhondt et al (1981).			
Normal adults	(12)	280 ± 14	709 ± 47
Phenylketonurics	(32)	2027 ± 182	3808 ± 243
Values by Crithidia fasciculata assay :			
Classic PKU	(6)	1524 ± 321	
Normal adults	(4)	288 ± 30	
Normal adults after phenylalanine intake.	(4)	676 ± 126	

All values as mean ± SEM

Numbers in parenthesis indicate number of subjects studied.

Results represent total pterin level after acid-iodine oxidation.

TABLE 3.7 PHENYLALANINE LOADING EXPERIMENTS IN ADULT MALE WISTAR RATS

	BRAIN					LIVER			
	n	TOTAL BIOPTERIN (oxidised+reduced) pmol/g.wet wt.	% BIOPTERIN	% DIHYDRO-BIOPTERIN	% THB	TOTAL BIOPTERIN (oxidised+reduced) pmol/g.wet wt.	% BIOPTERIN	% DIHYDRO-BIOPTERIN	% THB
½ h. PHE-DOSED RATS	12	234.5±16	6.1±0.6	12.5±2.8	81.4 ±3.3	2945±159	3.5±0.8	3.8±0.7	92.6 ±0.9
½ h.CONTROLS	6	198.2±8	4.1±0.3	1.5±0.5	94.4 ±0.5	1998±66	8.9±1.5	5.5±2.2	85.6 ±2.5
1 h. PHE-DOSED RATS	6	209.3±20	5.2±0.5	9.2±3.4	85.6 ±3.7	2361±259	7.1±0.8	7.7±2.4	85.1 ±3.0
1 h.CONTROLS	6	203.9±5	1.4±0.9	7.4±0.6	91.3 ±1.4	1837±61	8.9±1.7	4.2±1.1	86.9 ±2.0
4 h. PHE-DOSED RATS	6	179.5±20	0	9.2±2.5	90.8 ±2.5	2081±130	4.4±0.7	5.1±2.1	90.6 ±0.9
4 h. CONTROLS	6	168.8±8	1.3±0.7	6.0±2.1	92.7 ±1.8	1878±122	6.4±0.7	2.1±1.0	91.6 ±0.9

results as mean ± SEM
control rats were administered drug-vehicle (corn-oil)
drug-dose: 300 mg/kg phenylalanine in corn-oil by gavage.
brain and liver pterins investigated by HPLC
n = number of experimental rats

Table 3.8 Effect of P-Chlorophenylalanine and Phenylalanine Treatment On Liver And Brain Tetrahydrobiopterin Metabolism.

		PCPA + Phe dosed Rats (n=6)	Control Rats (n=6)	P
Brain :				
1.	Total Biopterin pmol./g.wet.wt.	247.4 ± 27.8	198.2 ± 7.9	N.S
2.	% THB	90.5 ± 2.4	94.4 ± 0.6	N.S
Liver :				
1.	Total Biopterin. pmol./g.wet.wt.	4159.2 ± 217	1997.8 ± 66	$p < 0.001$
2.	% THB	91.5 ± 1.7	85.6 ± 2.5	N.S

Adult male Wistar rats dosed orally with p-chlorophenylalanine in corn-oil (300 mg/kg.) and one hour later given 300 mg/kg. phenylalanine in corn-oil, and sacrificed half-hour later. Brain and liver biopterins analysed by HPLC.

All values as mean ± SEM.
Statistics : Student's t-test.
N.S. = not significant.

Adult Male Wistar Rat Model of Hyperphenylalaninaemia

Half an hour after an oral dose of phenylalanine (100 mg/kg) the brain and liver total biopterin levels reach a peak. The brain total biopterin is normal. However, the brain per cent THB level is significantly depressed ($p<0.02$) and this is reflected in an equivalent rise in per cent dihydrobiopterin in these rats.

At one hour and four hours after phenylalanine dosing the brain total biopterin is equivalent to levels in the control rats (at the respective time intervals). The brain per cent THB at one hour is low compared with control rats but not statistically significantly so.

The liver appears to be more resistant to changes in per cent THB levels following phenylalanine intake which may be due to the high DHPR activity of this organ. At half hour after phenylalanine dosing the liver biopterin is significantly elevated ($p<0.001$, Student's t-test), being 50% higher then in control rats. This elevated liver total biopterin is still present after 1 hour, (30% more than control) and at 4 hours (10% more than controls).

Hence a brief hyperphenylalaninaemia in healthy rats produces a rise in biopterin levels which is still present after four hours in the liver, being gradually reduced back to normal by homeostatic mechanisms. The rise in liver biopterin is much greater than in the brain. This is partly due to the close relationship between the gut and the liver, via the hepatic portal vein, which exposes the liver to the bulk of the absorbed phenylalanine and partly due to the protection of the brain by the blood-brain barrier.

As the phenylalanine was administered as a suspension in corn-oil, control

animals also were treated with corn-oil at the stated time intervals. The corn-oil dosed control rats show some variation in biopterin levels (Table 3.7). The total biopterin in the brain and liver is higher half-hour after dosing then at subsequent time intervals. In the liver this is not statistically significant. However, the half hour brain total biopterin is significantly elevated compared to the four hour level ($p<0.05$). In contrast the brain per cent THB does not show any significant change but the liver % THB does. The liver half-hour % THB is significantly depressed compared to the four hour level ($p<0.05$). Hence corn-oil appears to stimulate synthesis and depress DHPR activity.

Six of the twelve half-hour dosed rats were administered ^{14}C-labeled phenylalanine. The brain and liver radioactivity was measured half-hour later and 0.2% of the administered label was found in the brain whilst 2.6% was present in the liver. If this is taken to reflect the amount of administered phenylalanine that entered these organs then the brain concentration is estimated at 268 uM and the liver at 1418 uM.

Para-chlorophenylalanine (PCPA), an inhibitor of phenylalanine hydroxylase and tryptophan-hydroxylase (Gal et al , 1982) when given prior to a load of phenylalanine produces an even higher elevation in the brain and liver total biopterin then when phenylalanine is administered alone. The brain total biopterin at 247.4 $\pm$ 28 pmol/g. wet wt. is elevated compared to controls but not significantly so. The liver total biopterin is two-fold higher than controls ($p<0.001$ by Student's t-test). The administration of PCPA plus phenylalanine has no effect on the per cent THB level in the brain and the liver. This is in contrast to the effect of phenylalanine alone which produced a significant fall in brain per cent THB, with no equivalent effect in the liver.

Dicussion :

HPLC methods are becoming increasingly popular for the identification and quantitation of pteridines in biologic fluids. A careful sampling is essential because of the extreme lability of the reduced pterins. Furthermore, care is needed in the interpretation of results as wide variation in values can occur during normal health as well. This is illustrated by the urine pterin values during pregnancy (fig. 3.2) and by values in Table 3.3. Females in particular appear to show a greater variation compared with males. A large neopterin/biopterin ratio may falsely indicate biopterin-synthesis deficiency.

Comparison of pooled phenylketonuric group against healthy controls (Tables 3.3, 3.4) shows that both urinary neopterin and biopterin are significantly elevated ($p<0.02$, $p<0.01$ respectively; Student's t-test). The mean neopterin level in phenylketonurics (674.5 $\pm$ 90 nmol/mmol creatinine) is some 70% higher than in controls (384.3 $\pm$ 39 nmol./mmol creatinine). The mean urine biopterin level in phenylketonurics (1440 $\pm$ 226 nmol./mmol. creatinine) is two-fold higher than control values. These findings of elevated pterin elemination in PKU patients is confirmed by previous reports (Leeming et al 1976, Schlesinger et al 1979). The elevated pterin excretion is probably in response to elevated body biopterin synthesis. This is supported by the observed rise in neopterin level. The elevated body phenylalanine level may possibly stimulate a step in THB biosynthesis.

A similar situation is observed in healthy adults given a phenylalanine load who subsequently excreted elevated levels of neopterin and biopterin. Following a 100 mg/kg phenylalanine dose, the mean urine neopterin level increased from 329 $\pm$ nmol/mmol creatinine to 589 $\pm$ 211 nmol./mmol

creatinine. However the rise is not statistically significant. The mean urinary biopterin level almost doubles following phenylalanine intake, but again without statistical significance. Dhondt and Farriaux (1982) have some evidence for reduced pteridines having a negative feedback influence onthe biosynthesis of THB. If elevated phenylalanine leads to enhanced hydroxylation of the amino-acid than reduced pterin levels will fall and THB biosynthesis will be stimulated. Also, breakdown products of phenylalanine such as phenylpyruvic acid act as inhibitors of DHPR (Purdy and Blair, 1980) and would therefore lead to decreased THB level and thereby stimulate biosynthesis.

Comparison of the per cent reduced biopterin (THB) shows no statistically significant difference between control and phenylketonuric groups (Tables 3.3., 3.4). However, the phenylketonuric group does show a slightly higher percentage THB (77%) compared to controls (63%). The wide variability in the reduced pterin levels in the urine suggests that this is not a good indicator of pathological change. This is partly due to the variability in the bladder-storage time for urine from person to person. The bladder-urine being saturated with oxygen. the control urines were all collected as early morning samples, but not first-pass urines, and in all cases a "mid-stream" sample was requested.

Several experimental models of hyperphenylalaninaemia (HPA) have been used for in-vivo studies of clinical conditions such as phenylketonuria. Most of these studies have been based on long term administration of inhibitors such as p-chlorophenylalanine and alpha-methylphenylalanine, together with phenylalanine to suckling rats (Delvalle et al 1978). Whilst these chronic models are useful as long-term conditions of HPA, they may not truely represent the chinical condition as the drug produces a 'selective poisoning'

of the developing animal (Lane et al 1980). Furthermore long term treatment with these inhibitors is toxic and non-specific. For example, chronic treatment with p-chlorophenylalanine gives rise to cataracts, loss of body hair and skin lesions, and an incidence of high mortality, not seen in the clinical condition (Lane et al 1980). Both p-chlorophenylalanine and methyltyrosine are potent inhibitors of phenylalanine hydroxylase (Delvalle et al 1978). However L-methylphenylalanine is less toxic during chronic treatment, whilst PCPA is more potent (Lane et al , 1980). Both inhibitors produce some biochemical and symptomatological effects which mimic the condition of phenylketonuria. For example, L-methylphenylalanine inhibits phenylalanine hydroxylase resulting in HPA and also increases the excretion of phenylketones. Long term treatment also produces learning-impairment and some reduction in brain size (Delvalle et al , 1976) which is also observed in clinical PKU.

Since chronic studies in neonatal rats are complex and due to diverse effects of enzyme inhibition, blood-brain barrier disruption and impaired neural tissue development (Lane et al 1980), the present study was restricted to short-term effects of PCPA and phenylalanine in adult animals. Hence the effects are limited largely to competitive amino-acid uptake inhibition across the blood-brain barrier (Lane et al 1980) and to inhibition of the enzyme phenylalanine hydroxylase and tyrosine hydroxylase (Edwards and Blau 1972).

High dose phenylalanine administration alone produces an elevated biopterin level both in healthy individuals as well as some cases of HPA. In fact a phenylalanine-loading test can be used to differentiate between individuals with a biopterin-synthesis defect from other forms of HPA since in the former there is a failure of the blood biopterin level to rise (Kaufman 1980).

Acute dosing with phenylalanine produces an elevation in total brain and liver biopterins which reaches a peak approximately half hour after administration of the amino-acid. Whilst total biopterin is elevated the per cent THB level drops significantly by 13% after half hour. The elevation in total biopterin is most likely due to stimulation of biopterin synthesis. Brown (1981) observed approximately 10% increase in biopterin biosynthesis at 10^{-5} M phenylalanine in vitro . Long term elevated phenylalanine intake decreases hepatic phenylalanine hydroxylase activity (Woods and McCormick 1964). If this also occurs after acute dose, than an increase in per cent THB should have occured . However, long-term effects are more likely due to a fall in enzyme-protein level. In the acute dose experiment a fall in % THB was observed. Purdy and Blair (1980) have found that phenylalanine and phenyllactate in mM concentrations and phenylpyruvate at 50 uM concentration produce a 50% inhibition of dihydropteridine reductase activity. According to the radiolabel studies the liver phenylalanine levels could have reached 1.4 mM, but the brain phenylalaanine levels are too low to account for the fall in % THB. However if phenylpruvate is produced in significant amounts in the brain then an inhibition of DHPR activity could occur resulting in the observed fall in active cofactor levels. It should be noted that the Km of tryptophan hydroxylase, phenylalanine hydroxylase and tyrosine hydroxylase for phenylalanine is 287 uM, 200 μM and 0.3 mM respectively (Taylor et al 1983, Milstein et al 1975, Ikeda et al 1965). Hence certainly in the liver phenylalanine could have competitively inhibited the hydroxylation of tyrosine and tryptophan and in the brain approached concentrations which were similarly inhibitory.

PCPA is a competitive inhibitor of phenylalanine-hydroxylase in vitro. (Ayling and Helfand 1974), but after long term administration to rats is suggested to become incorporated into the enzyme molecule producing an

altered phenylalanine hydroxylase of low activity (Goodwin 1979). Hence inhibition of the hydroxylase would exacerbate the HPA effect observed with phenylalanine alone. Following PCPA plus phenylalanine administration the total biopterin becomes much more highly elevated. However PCPA treated rats do not show any effect on the brain and liver per cent THB whilst rats treated with phenylalanine-alone do. This could come about if PCPA were to block the other metabolic pathways of phenylalanine as well. In this case the DHPR-inhibitors such as phenylpyruvate would not be produced in amounts that effectively depress the % THB level.

Difficulties in solubilising PCPA and phenylalanine in aqueous or saline solutions restricted their administration by the oral route only. Information about the disposition of PCPA via this route is lacking but it is assummed to be transferred across the gut by the same mechanisms as phenylalanine. However, once inside the body, liver phenylalanine hydroxylase may convert 4-chlorophenylalanine to 3-chlorotyrosine (Counsell et al 1970). The resulting product is not known to be an inhibitor of phenylalanine hydroxylase. In addition, Edwards and Blau (1972) have found substantial amounts of PCPA being converted to p-chlorophenylethylamine which is a specific depletor of 5-hydroxytryptamine and may be responsible for some of the behavioural effects observed in long term studies. However, p-chlorophenylethylamine is rapidly oxidised further by monoamine oxidase and its effects in acute experiments would be minimal.

CHAPTER FOUR :

Tetrahydrobiopterin Metabolism In Senile Dementia and Other Diseases

Senile dementia is a progressive degenerative, neuropathological disease state characterised by impaired memory function (Gottfries et al 1969). The morphological changes found in senile dementia are also observed in normal aged brain but to a much lesser extent (Tomlinson, 1979). Since normal ageing is not associated with the severe mental impairment that is observed in senile dementia the latter is suggested to be a distinct pathological condition (Gottfries et al 1969). The condition of presenile dementia as first described by Alzheimer (1907) produces similar morphological changes as senile dementia and therefore may be an identical disease being distinguished only by the age of onset of the condition (Burger and Vogel 1973). Presenile dementia is arbitrarily suggested to occur in subjects before the age of 65 (Gottfries et al 1969). The presenile and senile dementias are distinct from dementia due to cerebrovascular degeneration where the incidence of infarction and haemorrhage produce the degenerative changes in the brain, (Tomlinson 1979). Also unlike other dementias due to physical trauma (dementia pugilistica) or viral infection (e.g. Jacob-Cruetfelt's disease) the aetiology of the senile and presenile dementia remains obscure.

Structural observations of brain tissue at autopsy reveals essentially identical changes in senile dementia of Alzheimer type (SDAT) and Alzheimer's disease (Terry, 1980). There is general loss of brain weight with widening of sulci and narrowing of gyri and enlargement of the ventricles (Terry 1980). There is a small but significant loss of neurones and certain cells (e.g. pyramidal cells) show loss of dendrites (Terry 1980). Senile plaques and neurofibrillary tangles are observed in significant numbers in Alzheimer-

type dementia. Neurofibrillary tangles are lesions within the cytoplasm of affected neurones in the cortex which under light miscroscope show up as masses of coarse fibres (Terry 1980). Under electron miscroscope the neurofibillary tangles appear as clusters of paired helical filaments with each filament having a diameter of 100 Å and a pitch of 800 Å (Terry 1980). Neurofibrillary tangles are also observed during normal aging but to a lesser extent and in other pathological conditions such as Guam-Parkinson-dementia complex and older Down's syndrome patients (Terry 1980). Neuritic plaques are lesions that occupy the neuropil with approximately the same distribution as that of the neurofibrillary tangles (Terry, 1980). Their concentration in the neocortex has been directly correlated with the degree of dementia and inversely with the tissue concentration of choline acetyltransferase (Terry 1980). Plaques contain a central dense core of amyloid surrounded by a translucent region containing granules and fibres (Terry, 1980). Abnormal, unmyelinated neurites with a high lysosomal content, as well as astrocyte (supportive cells) are found surrounding these structures. Granulovacuolar bodies are also found in Alzheimer's disease and SDAT, occuring almost exclusively in the hippocampal pyramidal cells (Terry 1980). These are vacuoles within the neuronal cytoplasm containing small granules and their concentration has been directly correlated with the degree of dementia (Terry 1980). Such histopathological changes suggest Alzheimer-type dementias to be distinct from the process of aging.

In the human brain there is a progressive increase in DNA with age which is suggested to be due to glial cell proliferation. However lipids such as neutral fats, cerebrosides and phosphotides show decrease with age (Cote et al 1983). The glycolysis enzyme hexokinase shows a slightly increased activity with age whilst fructose-6-phosphate kinase decreases by two-fold from age 20 to 80 years (Cote et al 1983). There is a net decrease in

glucose utilization by the brain with age. This decrease is more pronounced in subjects with the loss of cognitive function, (Cote et al 1983). The rate of energy metabolism may have an impact on cognitive function and vice-versa. Another important consideration is the ability of the organism to alter activities of enzymes to various stimuli. For example, Kremzner et al (1978) have observed that older mice have a greater delay time in initiating an increase in ornithine decarboxylase and other enzyme activities when re-introduced to normal diet following food-deprivation. Senescence is accompanied by a decrease in the activities of most enzymes involved in neurotransmitter synthesis and degradation (Cote et al 1983). For example tyrosine hydroxylase activity decreases significantly with age in the substantia nigra, neostriatum and globus pallidus. Notable exceptions are the increases in the activity of monoamine oxidase A and B (Cote et al 1983). These changes will affect neurotransmitter levels leading to the observed alterations in sleep pattern, mood, appetite, neuroendocrine function, memory function and so on (Cote et al 1983). Hence with increasing age there is perturbation of neurotransmitter metabolism which appears to be more severe in some people who exhibit the features of dementia. It is unknown whether senile dementia is simply an accelerated form of aging. In dementia, various neurotransmitter systems have been reported to be altered. Substantial evidence has been presented for a cholinergic defect in Alzheimer disease (which is discussed as being similar to SDAT here). For example, post mortem brain tissue from Alzheimer disease (AD) cases revealed that whilst there was no change in cholinergic muscarinic receptor level in the caudate nucleus, putamen and frontal cortex, the hippocampus showed significant reduction (Reisine et al, 1978). All four regions showed 60-80% reduction in choline-acetyltransferase activity in dementia. This enzyme is suggested to be unique to cholinergic neurones within mammalian brains and therefore an indicator of cholinergic neurone (Reisine et al

(1978). Rosser et al (1980), have investigated post-mortem brain tissue of SDAT patients and controls of equivalent age and found a significant fall in choline acetyltransferase (CAT) activity in the cerebral cortex of SDAT cases, with a maximal reduction of CAT activity in the temporal lobe. The cortical CAT activity is reported to be present exclusively in cholinergic afferents arising from the magnocellular forebrain nuclei and the reduced CAT activity is suggested to be due to loss or damage of afferent terminals rather than of intrinsic cortical neurones (Rosser et al 1980). Reports of reduction in acetylcholinesterase activity in AD and SDAT have also been made, but as this enzyme is not specific to cholinergic neurones the observation holds less significance (Perry and Perry, 1980).

The evidence for a cholinergic defect in AD is compelling since experimental observations show a role for cholinergic system in memory function. For example, scopolamine a muscarinic receptor antagonist impairs learning ability in man (Perry and Perry 1980). In animals lesioning of the hippocampal region impairs some forms of learning and since acetylcholinesterase and CAT are reduced in this region in AD a relation between the hippocampal-cholinergic system and memory function has been implicated, (Perry and Perry 1980). The medial-septal nucleus of the central cholinergic system is known to innervate the hippocampal pathway and lesioning of septal region produces a fall in acetylcholinesterase activity in the hippocampus (Mellgren and Srebro 1973).

In view of the widespread degeneration, however, it has been considered unlikely that a single neurotransmitter would be affected selectively (Dayan, 1974). Evidence has also been presented for a catecholaminergic defect both in AD and SDAT (Adolfsson et al 1979). In 7 out of 10 regions of demented brain there was a fall in dopamine level which was significant in the

thalamus and pons (Adolfsson et al 1979). The homovanillic acid levels in putamen and caudate nucleus were also found to be reduced, significantly. However, the changes in brain dopamine levels showed no correlation with intellectual or motor impairment (Adolfsson et al, 1983). The levels of serotonin and its breakdown product 5-hydroxy-indoleacetic acid were also found to be reduced in 6 of 8 brain regions investigated but without reaching significance when compared with controls. Adolfsson et al (1983) argue that since the patients had been on neuroleptic treatment in the last year or so the difference between control and demented brains was less marked than expected. Neuroleptics accelerate dopamine and noradrenaline turn-over (Adolfsson et al 1983). It is suggested that the nigro-striatal pathway is impaired in dementia of Alzheimer-type even though only a minority of patients have marked extrapyramidal symptoms. The appearance of these symptoms is suggested to be related to patient-age and duration of illness. Dopamine and noradrenaline projections from the mesencephalon are essential for normal behaviour and learning (Adolfsson et al 1979) as indicated by the reduced drive, lack of initiative, lowered psychomotor speed and reduced learning capability in Parkinsonian patients (Adolfsson et al 1979).

The locus coeruleus (LC) is considered to be the major source of noradrenergic neurones in the cerebral cortex (Perry et al 1981), fig **4.1.** The neurone density in this region is reduced to less then 50% in Alzheimer-type dementia compared with controls (Perry et al, 1981). Although no correlation was found in this study between cortical dopamine-B-hydroxylase (DBH) activity and locus coeruleus neurone loss, there was a tendency for a reduced DBH activity with increasing plaque count. Perry et al (1981), suggest this to indicate a secondary abnormality of the noradrenergic system in Alzheimer's disease. However, the surviving neurones of the LC may compensatorily increase their activity in which case no correlation would be

observed between DBH activity and mental test score. Also the locus coeruleus is a, complex three dimensional structure in itself (Mann 1983) with different parts of it innervating specific higher structures, and hence the usual sampling of mid-nuclear cells of such a nucleus would not give a detailed insight into damage to a specific region of the LC. In any case the central noradrenergic system comprises many nuclei (fig. **4.1**) and damage to any one of these could impair central nervous function. The involvement of the noradrenergic system has been strongly implicated in Korsakoff's psychoses (alcohol-poisoning syndrome) where a significant reduction in CSF levels of noradrenaline correlated well with memory loss (McEntee et al 1984). Hence the amnesia component of diseases such as Alzheimer's dementia may also be due to an impaired monoaminergic system (McEntee et al 1984).

Some of the histopathological features of Alzheimer's disease are also present in such diseases as Down's syndrome (in cases reaching middle age), dementia pugilistica and the Guam-Parkinson dementia complex (Perry and Perry, 1980).

Parkinson's disease is characterised primarily by motor symptoms of akinesia, tremor, and rigidity, but Parkinsonians show symptoms of mental disorder as well (Agid et al, 1984). A general slowness of thought together with other forms of intellectual disturbance are noted. Correlations have been found between akinesia and impaired performance in visual-spatial reasoning and psychomotor tests and therefore a dopaminergic component to intellectual function has been suggested (Agid et al 1984). This is further supported by reports of improvement in psychological performance following L-DOPA therapy especially in the early stages of the disease (Agid et al, 1984). A loss of dopaminergic innervation of the prefrontal cortex as well as impaired

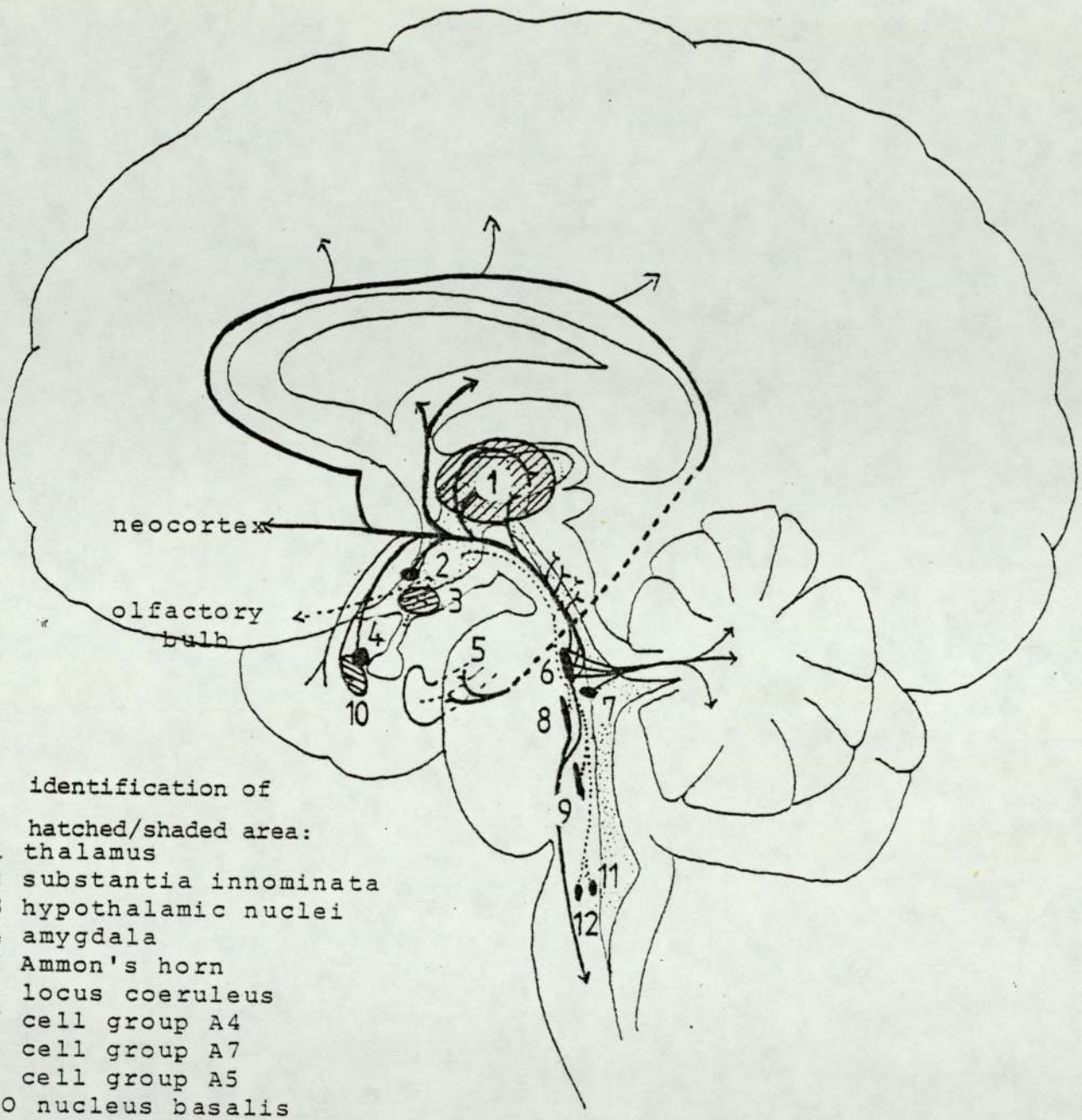
cholinergic function in the substantia innominata and the cortex has been implicated (Agid et al 1984). Tetrahydrobiopterin treatment has been carried out in several trials with at least one reporting some amelioration of symptoms in Parkinson's disease (Curtius et al 1984). However the effect of THB treatment on the dementia component in this disease has not been investigated.

Pathological evidence of Alzheimer-type changes in Down's Syndrome (DS) have been reported (Lott, 1982), with evidence of neuronal loss, senile plaques and neurofibrillary tangles present in the brain. The hippocampus shows neurofibrillary tangles and loss of pyramidal cells with remaining neurones showing loss of dendritic spines, (Lott 1984). These observations are similar to the ones reported in AD. These changes are particularly prevalent in the older DS subjects and obvious clinical symptoms of recent-memory loss, aphasia, agnosia and personality changes are noted (Lott 1984).

Tetrahydrobiopterin metabolism is suggested to be disturbed in some affective disorders (Levine et al 1983). Affective disorders are those pertaining to a change in mood and feeling, of a degree that is detrimental to the normal functioning of a person. These disorders include schizophrenia, depression and mania. Affective disorders have been classified in a number of ways (Andreasen, 1982). Those depressive states that cannot be attributed to external forces or events have been classified as endogenous and these are further sub-classified as unipolar or bipolar. Unipolar patients are true depressives exhibiting signs of sleep disturbance, psychomotor retardation, severely depressed mood, weight loss and an inability to concentrate and so on (Andreason 1982). Bipolar subjects exhibit episodes of both mania and depression. An episode of mania may include mood changes of euphoria, irritabilty and anger, and disinhibition accompanied by increased sleep, sexual overactivity, and poor

judgement (Tyrer and Shopsin 1982). A wide spectrum of conditions exists between these two extremes of depression and mania (Andreasen 1982). Genetic studies show a high incidence of bipolar psychoses amongst relatives of a bipolar patient. A similar relationship is also observed for unipolar patients but to a lesser extent (Perris 1982). Such observations suggest the two disorders to be distinct.

Observation of a precipitation of mania or a stimulant effect with drugs that increase the output of catecholamines and conversely a sedative or antimanic effect ...with drugs that suppress the catecholaminergic output has led to a biogenic amine hypothesis of affective disorders (Zis and Goodwin, 1982). This hypothesis states that depression is associated with a funcional deficit of one or more neurotransmitter amines at critical synapses in the central nervous system and mania is associated with a functional excess of these amines (Zis and Goodwin, 1982). Support for this hypothesis comes from clinical observations of drugs such as monoamine oxidase inhibitors and tricyclic antidepressants which elevate brain catecholamine levels and thereby have their pharmacological effects. However, a discrepancy exists in the effect of Lithium which has an inhibiting effect on noradrenaline output and yet is effective as an antidepressant in some patients and as an antimanic in other patients (Zis and Goodman, 1982). However, drugs without effect on the neurotransmitter system itself could act by more novel methods to elevate synaptic amine levels. For example, mainserin is a potent α_2-receptor blocker and may act as an antidepressant by inhibiting noradrenaline re-uptake (Zis and Goodwin, 1982).

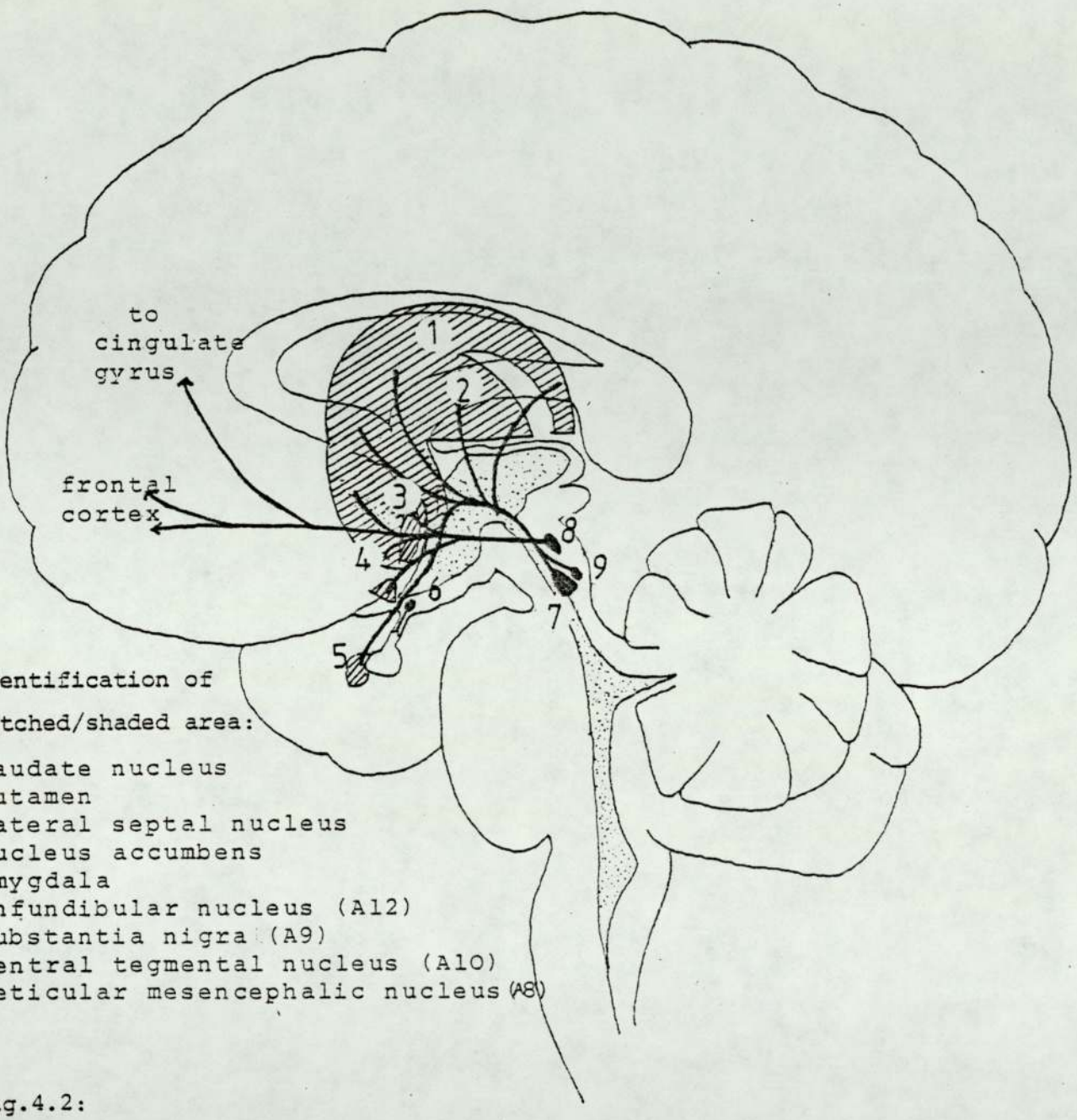


identification of hatched/shaded area:

1 thalamus
2 substantia innominata
3 hypothalamic nuclei
4 amygdala
5 Ammon's horn
6 locus coeruleus
7 cell group A4
8 cell group A7
9 cell group A5
10 nucleus basalis
11 cell group A2
12 cell group A1

(modified from Nieuwenhuys et al 1978)

fig.4.1:
SOME CONNECTIONS OF THE NORADRENERGIC SYSTEM



identification of
hatched/shaded area:

1 caudate nucleus
2 putamen
3 lateral septal nucleus
4 nucleus accumbens
5 amygdala
6 infundibular nucleus (A12)
7 substantia nigra (A9)
8 ventral tegmental nucleus (A10)
9 reticular mesencephalic nucleus (A8)

fig.4.2:

DOPAMINERGIC CONNECTIONS

(modified from Nieumenhuys et al 1978)

The biogenic amine hypothesis is further supported by finding of decreased baseline level of serotonin metabolite 5-hydroxyindoleacetic acid (5HIAA) in cerebrospinal fluid of depressives compared with controls (Zis and Goodman 1982). However, Asberg et al 1976 have found a bimodal distribution of 5HIAA in depressed subjects. One group exhibited low 5HIAA levels whilst a second group exhibited normal 5HIAA level. Within the former group there was a negative correlation between the severity of depression and the concentration of the metabolite. These patients were also more prone to suicidal attempts (Asberg et al 1976). Hence distinct subgroups of depressives may exist with disturbance of a particular neurotransmitter pathway. Recently an involvement of tetrahydrobiopterin system in endogenous depression has been reported (Levine et al 1983). These workers observed an improvement in endogenous depression following THB therapy.

The above is an account of some of the disease states where a possible disturbance of tetrahydrobiopterin metabolism has been reported. The present study further investigates these diseases and discusses the implications of any change in biopterin cofactor levels.

Materials And Methods.

All brain samples were obtained from Dr. G.P. Reynolds at the MRC brain bank, Cambridge. The samples were stored at -70°C until required.

and Fragile X Syndrome
The senile dementia urine and serum samples and plasma of control and Down's syndrome patients were obtained from Dr. P.E. Sylvester at St. Lawrence's Hospital, Caterham and kept at -20°C until required.

The SDAT csf samples were obtained from Dr. G.K. Wilcock at Radcliffe Infirmary, Oxford.

Urine samples from affectively ill patients and hospitalised control subjects were obtained from Dr. A. Coppen, MRC Neuropsychiatry Research Laboratory, West Park Hospital, Epsom, Surrey. The affectively ill subjects were on Lithium therapy and were euthymic. No other drug treatment was being undertaken. Creatinine measurements were carried out by Dr. Leeming at the General Hospital, Birmingham.

Cerebrospinal fluid samples from leukaemic children were obtained from Great Ormond Street Children's Hospital, London, courtesy of Dr. I. Smith. The samples were taken by lumbar puncture and unless stated otherwise the children were not administered intrathecal methotrexate around the time of csf sampling.

Iodine-oxidation of samples was as previously described for urines in chapter 3.

Results :

Senile Dementia of Alzheimer type :

No significant difference is obtained if the brain neopterin and biopterin is estimated per mg. of protein. The brain biopterin of the dementia group when expressed per ml. of homogenate is significantly depressed compared to controls (2.58 ± 1.15 and 9.37 ± 2.69, respectively). Values given as ng/ml ± SEM. If subjects D41 and D42 (Table **4.2**) with vascular dementia are excluded then the dementia brain biopterin level (1.81 ± 0.47) is still significantly depressed ($p < 0.05$ by Student's t-test). If however the subjects with dementia but without

excluded then the dementia brain biopterin level (1.81 ± 0.47) is still significantly depressed ($p < 0.05$ by Student's t-test). If however the subjects with dementia but without plaques and tangles in the brain are also excluded then brain biopterin level loses statistical significance although the dementia group still exhibits extremely low biopterin level compared with controls. In contrast the brain neopterin level shows an elevation in the dements compared to controls but without statistical significance. The control group has a mean biopterin level of 6.26 ± 2.31. The total demented group has a mean neopterin level of 18.23 ± 6.99, and 9.98 ± 4.96 if subjects D41 and D42 are excluded, and 11.03 ± 5.60 if in addition subjects D43 and D46 are excluded. The neopterin : biopterin ratio of the control group is 0.67 compared to 7.60 for the SDAT subjects. Hence synthesis up to neopterin-triphosphate stage appears to be stimulated in dementia, or there is a metabolic block thereafter.

The synthesis (Tables 4.1, 4.2) as measured by biopterin produced following incubation of brain tissue with guanosine triphosphate shows a drastically low activity in dementia (0.046 ± 0.040 nmol./hr. mg. protein) compared with controls (0.55 ± 0.19), $p < 0.02$ by Student's t-test. If the vascular dementia is excluded the synthesis activity (0.048 ± 0.05) is still significantly reduced ($p < 0.01$) but if the subjects with dementia without plaques and tangles are excluded then the statistical significance no longer exists, although the synthesis is still low.

The brain DHPR activity (Tables 4.1, 4.2) of the dementia group (excluding, D41, D42) is significantly elevated at 348.1 ± 45.6 compared with control group (196.5 ± 65.2), $p < 0.02$. Values as nmol. NADH used/min. mg. protein ± SEM. The DHPR activity becomes more significantly elevated at 369.2 ± 33.8 if subjects D43 and D46 are also excluded ($p < 0.01$ by Student's t-test).

Table 4.1. Brain Pterin Levels in Healthy Subject At Autopsy.

Subject code	Sex	Age	Post mortem delay time. hr.	Neopterin * ng/ml homogenate.	Biopterin * ng/ml homogenate.	DHPR activity nmol.NADH/ min.mg.Protein.	Synthesis activity $ng.h^{-1}$ $mg.Protein^{-1}$
C240	F	89	72	91.44	30.70	223.1	0.11
C241	M	71	47	18.29	9.99	117.0	0.28
C242	F	79	63	0.35	2.65	167.5	0.47
C244	M	74	49	9.90	18.00	194.4	0.52
C234	F	44	92	5.05	2.38	265.5	1.05
C248	M	74	65	5.20	17.60	124.0	1.42
C236	M	72	69	3.00	2.00	283.7	0
C238	F	74	24	2.00	13.00	460.0	1.01

Brain area investigated : Brodmann area 21.

Tissue prepared as 20% homogenate in Tris buffer (0,05M) pH 7.6

* Total pterin after acid-oxidation.

TABLE 4.2 BRAIN PTERIN LEVELS IN DEMENTED SUBJECTS

SUBJECT CODE	CLINICAL STATUS	SEX	AGE	POSTMORTEM DELAY TIME	NEOPTERIN* ng/ml homogenate	BIOPTERIN* ng/ml homogenate	DHPR ACTIVITY nmol NADH/min mg.protein	SYNTHESIS ACTIVITY $ng.h^{-1}$ mg. protein
D38	SDAT	F	90	11	1.51	2.43	335.5	0.29
D39	SDAT	M	71	32	28.7	0	315.0	0
D40	SDAT	F	72	20	2.81	2.60	359.6	0
D41	VASCULAR DEMENTIA	M	76	54	25.2	0	222.8	0.08
D42	"	M	71	74	52.5	9.0	52.7	0
D43	MODERATE DEMENTIA no P+T	M	79	4	70.71	82.82	158.5	0
D45	SDAT	F	-	50	11.1	1.8	466.6	0
D46	HIGH DEMENTIA no P+T	M	80	46	5.8	2.2	453.3	0

brain area investigated: Brodmann area 21
tissue prepared as a 20% homogenate in Tris buffer (0.05M), pH 7.6
* total pterin after acid-iodine oxidation

TABLE 4.3 URINE AND SERUM BIOPTERIN LEVELS IN SDAT SUBJECTS

Subject	Status	Urine*		Serum
		Neopterin µmol./mmol creatinine	Biopterin µmol./mmol creatinine	Crithidia µg/ml
Wx	C	1.28	0.11	2.5
WS	C	0.64	0.23	2.5
Ds	C	2.50	0.08	1.6
Hl	C	1.06	0.08	1.0
Wr	C	1.17	0.05	1.7
Wn	D	0.53	0.08	1.4
Od	D	0.79	0.08	2.2
Ps	D	0.42	0.06	0.9
Wn	D	0.33	0.05	2.2
Or	D	1.93	0.03	2.7

C = control subject

D = Demented subject

* = Urine pterins without iodine oxidations

TABLE 4.4 CSF PTERIN LEVEL IN SDAT AND CONTROL SUBJECTS

Subject	Status	Neopterin * pmol./ml	Biopterin * pmol./ml
Md	SDAT	19.7	52.5
Cx	"	9.1	58.8
Kg	"	51.2	31.5
Hn	"	167.3	63.0
Ds	"	147.6	136.6
Ml	"	92.5	144.9
Dr	"	122.0	128.2
Wt	"	72.8	42.0
Nn	Control	78.7	111.3
Kt	"	118.1	241.6
Gd	"	54.1	241.6
Pe	"	68.9	231.1
Ml	"	138.8	121.8
Js	"	295.3	142.9
Wr	"	413.4	378.2

* Pterin levels after acid.iodine oxidation.

By Spearman's Rank correlation test there was no correlative trend with neopterin and bioptern . and age (dementia and controls pooled together). There was no correlation of neopterin or biopterin with post-mortem delay time. The DHPR activity too, shows no correlation with age or post-mortem delay. Any trends, however, may be obscured by sparsity of data.

There was no difference between males and females in the brain neopterin and biopterin levels. However, the females tended to show a higher DHPR activity both in the control group and demented group.

The neuronal density per four columns of the inferior temporal gyrus and neurofibrillary tangles in this region were measured at Addenbrooks Hospital (courtesy of Dr. Reynolds) and correlation tests undertaken. There was no correlation found with DHPR activity, neopterin level or biopterin level. However, the biopterin-synthesis activity gave a negative linear correlation when compared with neurone density (Pearson correlation coefficient $r = -0.87$, $p < 0.01$). This suggests a compensatory increase in cofactor synthesis activity in remaining neurones in the brain.

The csf biopterin level (Table 4.4) in dements, at autopsy, is reduced compared to controls (82.2 ± 16.4, 145.2 ± 31.5, respectively). Values as pmol./ml. $\pm$ SEM. The difference is not statistically significant. The neopterin level too is reduced in dementia compared to controls (82.3 ± 20.5, 165.4 ± 58.5 respectively), again without statistically significant difference. The neopterin to biopterin ratio of control subjects was 0.7 whilst that of SDAT subjects was 6.5. This suggests that a blockage at the "neopterin-synthetase" stage occurs in SDAT patients. The spead of results observed is most likely due to variability in the post-mortem

delay time and the subsequent handling of the csf samples prior to pterin analysis.

The unoxidised urine neopterin and biopterin level (Table 4.3) of SDAT subjects are approximately 50% of control values but these differences are not statistically significant. The serum Crithidia-factor levels in the same subjects show no change, with the controls and SDAT subjects both showing a mean value of 1.9 ± 0.3 g/ml.

Affective Disorder :

Nine females and eight male patients with affective psychosis were studied. Thirteen of these were unipolar and four bipolar with a mean of 58 ± 8. Ten females and three male controls from the same hospital were also studied (mean age 53 ± 8), tables 4.5, 4.6.

Mean urinary neopterin level of control subjects (0.343 ± 0.04) was no different from the levels found in unipolar patients (0.309 ± 0.04). All values as μmol./mmol creatinine ± SEM. In contrast, the bipolar patients showed a considerable reduction in urinary neopterin (0.183 ± 0.04). However, this decrease was not statistically by student's t-test. the above results suggest that the bipolar patients may be deficient in THB synthesis following dihydro-neopterin triphosphate synthesis. This is further supported by the low biopterin levels found in bipolar patients (0.281 ± 0.08) compared to controls (0.558 ± 0.07). The reduction is significant ($p < 0.05$ by Student's t-test). The unipolar subjects showed no change in their biopterin excretion (0.499 ± 0.05) compared with controls.

TABLE 4.5 : URINE NEOPTERIN AND BIOPTERIN IN AFFECTIVE DISORDER PATIENTS

Subject	Sex	Neopterin μmol./mmol creatinine	Biopterin μmol./mmol creatinine	Status
N.B	F	0.265	0.565	Unipolar
W.B	F	0.111	0.703	"
N.D	M	0.319	0.390	"
J.G	F	0.154	0.434	"
B.H	M	0.174	0.417	"
A.E	M	0.377	0.265	"
M.L	F	0.312	0.792	"
R.L	M	0.251	0.514	"
A.D	M	0.294	0.446	"
M.M	F	0.386	0.727	"
F.L	F	0.597	0.503	"
R.J	M	0.463	0.255	"
W.H	M	0.324	0.484	"
D.C	F	0.276	0.309	Bipolar
E.M	F	0.165	0.403	"
A.N	F	0.203	0.358	"
L.C	M	0.091	0.054	"

* Acid . iodine oxidated urine samples

TABLE 4.6 : URINE NEOPTERIN AND BIOPTERIN LEVELS IN HOSPITALISED CONTROL PATIENTS WITHOUT NEUROLOGICAL SYMPTOMS

Subject	Sex	Neopterin * μmol/m mol creatinine	Biopterin * μmol/m mol creatinine
FC	M	0.103	0.232
MM	F	0.517	0.857
AC	M	0.298	0.855
MH	F	0.342	0.497
JR	F	0.448	0.994
EB	M	0.211	0.363
VH	F	0.229	0.612
JF	F	0.471	0.104
BG	F	0.398	0.773
MW	F	0.417	0.536
TL	F	0.296	0.489
MR	F	0.587	0.613
AM	F	0.145	0.339

* Acid„iodine oxidised urine sample

There was no significant change in the biopterin excretion between males and females, either in the patients or in the controls.

The above urinary differences were not reflected in the serum Crithidia-factor levels (Table 4.8). However, Crithidia assay may not truely reflect biopterin levels in the sample. Investigation of post-mortem brain tissue of patients with a history of severe depression shows that the biopterin synthesis is severely reduced in Brodmann area 21 (0.01 ± 0.04 ng. h^{-1}. mg. protein $^{-1}$) compared with controls (0.41 ± 0.30 ng. h^{-1} mg. $protein^{-1}$), table 4.7.

Acute Lymphoblastic leukaemia :

Analysis of data by Williams et al 1980, shows that subjects up to the age of 20 years have a csf biopterin level between 24 - 28 pmol./ml. Subjects with acute lymphoblastic leukaemia (ALL) exhibited a mean csf biopterin level of 42.71 ± 8.96 pmol./ml. (Table 4.9). Hence biopterin levels in these patient are elevated. These subjects were all below the age of 14 and control csf samples from children of equivalent age were not available. Kostron-Krainz et al (1982) have found an elevated urinary neopterin level in ALL children compared with age-matched controls. Leeming et al (1980) have found an increase in serum biopterin levels in children with ALL on methotrexate therapy.

By Spearman's Rank Correlation there was no correlative trend between biopterin or neopterin level and the neurotransmitter metabolites 5HIAA, DOPAC and HVA (Table 4.10).

TABLE 4.7 : THB SYNTHESIS IN TEMPORAL CORTEX (BRODMAN AREA 20, 21) OF PATIENTS WITH A HISTORY OF SEVERE DEPRESSION

Group	THB synthesis ($ng.hr^{-1}.mg.protein^{-1}$)
Controls : Brodmann Area 20 (44-89 yr.) n=14	0.68 ± 0.055
Controls : Brodmann Area 21 (76-84 yr.) n=3	0.41 ± 0.30
Depressed patients : Brodmann Area 21 (78-86 yr.) N=4	0.01 ± 0.04

TABLE 4.8 : SERUM CRITHIDIA LEVELS IN CONTROLS AND AFFECTIVE DISORDER PATIENTS :

Control			Affective Disorder Patients		
Subject	Sex	Crithidia level µg/ml	Subject	Sex	Crithidia level µg/ml
			Unipolar Subjects		
FC	M	1.8	NB	F	1.3
MM	F	2.9	WB	F	2.2
AC	M	2.3	ND	M	2.2
MH	F	3.0	JG	F	1.8
JR	F	2.8	BH	M	2.5
EB	M	1.9	AE	M	2.5
VM	F	1.7	ML	F	2.9
JF	F	2.1	RL	M	4.0
BG	F	1.8	AD	M	4.0
MW	F	1.6	MM	F	2.4
TL	F	2.0	FL	F	2.0
MR	F	2.0	RJ	M	3.2
AM	F	1.7	WH	M	1.4
			Bipolar Subjects		
			DC	F	1.7
			EM	F	2.2
			AN	F	1.9
			LC	M	2.3

TABLE 4.9 : NEOPTERIN AND BIOPTERIN LEVELS IN CSF OF CHILDREN WITH ACUTE LYMPHOCYTIC LEUKAEMIA

Subject	Neopterin pmol./ml	Biopterin pmol./ml
MH	17.3	25.21
PW	17.3	78.9
ET	31.5	40.3
HA	31.5	16.8
MH	14.2	40.3
JA	22.0	16.8
WF (I)	72.4	30.3
WF (II)	31.5	60.5
SC	14.2	35.3
SR	14.2	30.3
WF (III)	9.4	25.2
CH	47.2	35.3
HR	47.2	35.3
SF	9.4	10.1
SM	17.3	35.3
LM	9.4	53.8
RP	44.0	30.3
DK	31.5	35.3
DH	47.2	25.2
IC	31.5	35.3
DW	67.7	218.5
MB	14.2	25.2

Tuberose Sclerosis :

Patients with tuberose sclerosis (TS), Table 4.11, showed a mean urinary neopterin excretion of 0.33 ± 0.13 μmol./mmol creatinine (± SEM) which was not different from control value of 0.34 ± 0.04 μmol./nmol creatinine and this too was similar to healthy urine biopterin level of 0.56 ± 0.07.

Fragile X Chromosome Disorder :

Patients with fragile X syndrome (Table 4.11) showed an elevated urine neopterin level (0.50 ± 0.22 μmol./mmol creatinine) compared with controls ($p < 0.05$). However the biopterin excretion was normal at 0.50 ± 0.09 compared to a mean control value of 0.56 ± 0.07.

Down's Syndrome :

Down's syndrome patients (Table 4.13) showed a marked reduction in plasma neopterin level at 0.49 ± 0.37 pmol./ml, compared with control level of 9.05 ± 1.71 pmol./ml (Table 4.12). The biopterin level in these patients was highly variable ranging from 0 to 106.7 pmol./ml. but the mean of 21.86 ± 4.66 pmol./ml. was not statistically different from control level of 13.26 ± 2.39 pmol./ml. Aziz et al (1982) report an elevation of serum biopterin level in Down's subjects and suggest this to be due to greater cellular efflux following enhanced oxidation of reduced biopterins.

TABLE 4.10 : MONOAMINE NEUROTRANSMITTER METABOLITES IN CSF OF ACUTE LYMPHOCYTIC LEUKAEMIA PATIENTS

Subject	5 HIAA pmol./ml	DOPAC pmol./ml	HVA pmol./ml
MH	65.4	7.38	198.35
PW	98.5	9.76	286.26
ET	170.5	14.16	394.95
HA	159.3	9.52	478.57
MH	196.1	14.64	522.74
JA	77.7	4.88	328.13
WF (I)	97.2	7.38	389.18
WF (II)	121.0	7.14	480.77
SC	60.1	1.19	183.13
SR	183.1	26.79	474.73
WF	51.3	1.19	213.74
CH	136.5	22.20	431.32
HR	86.1	13.75	292.31
SF	111.3	7.38	427.47
SM	292.0	9.46	769.23
LM	128.2	11.90	516.48
RP	166.7	11.01	350.71

5HIAA = 5-hydroxyindole acetic acid

DOPAC = dihydroxyphenylacetic acid

HVA = Homovanillic acid

TABLE 4.11 : URINARY PTERIN IN TUBEROSE SCLEROSIS AND FRAGILE X PATIENTS :

Subject	Sex	Status	Neopterin (μmol./nmol. creatinine)	Biopterin (μmol./nmol. creatinine)
JW	F	Tuberose Sclerosis	0.662	0.995
HI	F	"	0.037	0.164
TJ	M	"	0.602	0.764
CE	F	"	0.191	0.671
ML	F	"	0.164	0.385
HC	M	Fragile X	0.718	0.818
MJ	M	"	0.548	0.495
BJ	M	"	0.391	0.217
WM	M	"	0.285	0.162
WK	M	"	1.009	0.536
WP	M	"	0.291	0.305
LA	M	"	0.176	0.247
LH	M	"	0.570	1.166
JL	M	"	0.397	0.086
RE	M	"	0.501	0.773
JS	M	"	0.498	0.644
JSA	M	"	0.586	0.519

TABLE 4.12 : PLASMA PTERIN LEVEL FROM HOSPITALISED CONTROL SUBJECTS

Subject	Sex	Neopterin pmol./ml	Biopterin pmol./ml.
ME	F	0	28.6
JC	M	9.4	20.2
AT	F	17.3	36.6
AM	F	7.9	25.2
MK	M	14.2	15.1
PQ	M	24.8	6.3
RE	M	14.2	21.4
PH	F	20.1	6.3
JB	M	24.8	6.3
AW	F	12.2	36.1
EN	F	7.1	19.3
DF	F	7.9	8.4
HH	M	12.2	2.8
AD	F	3.9	1.7
EDS	F	1.6	8.4
PM	F	2.0	7.6
ES	F	1.6	8.8
DT	F	2.8	6.7
SC	M	2.0	7.6
AW	M	2.0	2.1
EW	F	2.0	2.9

* Total pterin levels after acid.iodine oxidation

TABLE 4.13 : PLASMA PTERIN LEVELS IN DOWN'S SUBJECTS

Subject	Sex	Neopterin * pmol./ml	Biopterin * pmol./ml.
MH	F	0	15.1
DP	F	0	0
SC	M	0	10.1
"P	"	0	10.1
DS	M	0	0
MH	F	0	0
DB	F	0	0
L"	F	0	0
GD	M	0	0
W	F	0	0
LS	F	0	0
JW	F	0	5.0
MS	M	4.7	5.5
DM	F	0	0
GH	F	0	0
FG	F	10.9	24.4
SS	F	0	31.9
JM	F	0	0
JP	F	0	36.1
SE	M	0	60.0
MP	M	0	53.4
PC	M	0	5.0
JP	M	0	19.7
LD	F	0	5.0
GC	M	0	106.7
CR	M	0	63.0
SS	F	0	36.1
WD	M	0	55.5
JB	M	0	19.7
LJ	M	0	52.9
MH	M	0	38.7
MG	M	0	45.8

* Total pterin levels after acid"iodine oxidation.

DISCUSSION :

Dementia of the Alzheimer type has been suggested to be a pathological condition which is distinct from normal aging and resulting in severe mental impairment. A number of structural as well as biochemical changes in the brain have been reported which support this view (Terry 1980).

A number of investigations have shown defective monoamine neurotransmitter metabolism in Alzheimer-type dementia (Mann 1983, Gottfries 1969). More recently, reports have been made of an impaired THB metabolism in these conditions (Aziz et al 1983, Morar et al 1983, Barford et al 1984). Hence a primary disturbance may be of a cofactor-deficiency type leading to an impaired monoamine synthesis in the brain.

Results show that the THB synthesis activity is drastically reduced in the temporal lobe area of the brain (Table 4.2), Barford et al 1984. This is reflected by the decreased biopterin and neopterin levels in this region of the brain. An increase in DHPR activity is noted which may be a compensatory reaction to the diminished cofactor synthesis. This reduced brain neopterin and biopterin levels are also reflected in the csf and urine but to a lesser extent. These results confirm previous reports by Aziz et al 1983 and others.

The cofactor synthesis may be impaired as a result of regional vitamin deficiency as has been cited for idiopathic dementia and we have observed THB synthesis in dementia brain following 5-methyltetrahydrofolate addition, in vitro (Blair et al 1984). This is supported by

observation of an inverse correlation between the mental assessment score of some dementia patients and red blood cell folate level (Sneath et al 1983). Hence the demented subjects may have the capacity to synthesis THB, provided adequate levels of folate are present. Hence dementia is some patients may simply be due to a lack of appropriate precursors (including THB) for neurotransmitter synthesis.

The selective loss of locus coeruleus (LC) neurones reported by Mann (1983) may play a significant role in SDAT. Such a loss could be elicited as reduced brain biopterin level or the latter could give rise to noradrenegic neurones degeneration. The LC makes a substantial noradrenergic contribution to the rest of the brain, giving rise to tracts that ramify the hypothalamus, hippocampus, the neocortex, cerebellum and the spinal cord (Mann 1983). This nucleus plays an important regulatory role in sleep, behaviour, mood, learning and memory (Mann 1983) and its degeneration could therefore manifest as dementia. Furthermore, the cholinergic defect observed in the hippocampus and which is suggested to be an important aetiological factor in the memory defect of SDAT (Perry and Perry 1980) may arise as a result of LC degeneration. Segal (1982) reports that the LC innervates both the hippocampus and the medial septum. The latter giving rise to the cholinergic septal-hippocampal pathway. The modulation of activity in these higher centres by LC (Segal 1982) could become impaired should the LC undergo a degenerative change.

The LC also gives rise to noradrenergic fibres that innervate the capillary bed (Mann 1983) and therefore the noradrenergic system is important in the regulation of blood flow and capillary permeability. Hence a noradrenergic disturbance could also manifest as impaired brain

homeostasis as a result of altered blood-brain barrier properties. Such a change could give rise to the CNS vitamin deficiency aspect discussed earlier, as well as other changes which could culminate in the observed mental deterioration in SDAT.

Hence an impaired THB synthesis reported here, could play a significant role in the aetiology of Alzheimer-type dementias. However, it still remains to be clarified whether the observed changes in THB metabolism are a cause or an effect of SDAT.

Down's syndrome (DS) is a genetic disorder which results in mental retardation. An increased predisposition towards Alzheimer disease has been reported in these subjects (Roy Breg 1977). The neuropathologic changes are essentially the same as those described for Alzheimer patients (Roy Breg 1977). The brain oxygen uptake in these patients is normal and therefore the derangement of the CNS cannot be attributed to generalised reduction in metabolic activity (Roy Breg, 1977). A possible disorder of the catecholaminergic system has been suggested as urinary noradrenaline output is reduced and in addition, serum dopamine-β-hydroxylase activity is diminished (Roy Breg 1977).

Aziz et al (1982) report an elevation in serum dihydrobiopterin level in DS and suggest that the active cofactor level may be reduced in these subjects. Blair et al (1984) report that THB synthesis activity in DS subjects is considerably reduced compared with healthy controls with no significant change in DHPR activity. The present results support these findings as plasma neopterin levels were very low in DS compared to healthy individuals. However no change in the mean biopterin level was obtained. DS foetuses show no detectable synthesis activity as do

control foetuses and therefore it is not clear whether DS patients have a congenital biopterin defect or a predisposition towards biopterin-defect in later life (Blair et al 1984). The observed synthesis defect must only manifest in the CNS and not peripherally as the DS patients do not develop hyperphenylalaninaemia (Blair et al 1984).

Another inheritable disorder investigated was the fragile X syndrome. This is an X-chromosome linked, recessive, mental retardation which is associated with a fragile site on the sex chromosome (Glower 1981). Unlike other fragile sites in human chromosomes the marker X-chromosome is associated with phenotypic abnormalities. Most consistent of these in males is mental retardation but macro-orchidism, characteristic facial features and a distinctive speech disorder have also been described in some affected males (Glower 1981). The connection between the fragile site on the X chromosome and mental retardation is unknown.

Lymphocytes from fragile X patients when cultured in a medium deficient in folic acid and thymidine express a constriction on the long arm of the X chromosome, (Sutherland 1979). The inhibiting effect of folic acid on expression of fragile X is negated by 2'-deoxy-5-fluorouridine which is an inhibitor of thymidylate synthetase (Glower 1981). This effect was not observed when thymidine replaced folic acid. Hence it is suggested that X-chromosome fragile site is expressed because of depletion of deoxythymidine monophosphate available for DNA synthesis.

The present investigation into the urinary excretion of pterins in these subjects shows that urinary neopterin level is elevated whilst biopterin is not. This could be due to an enhanced pterin synthesis activity which lead to an accumulation of dihydron eopterin triphosphate. There

is a bottle-neck effect at the 'dihydrobiopterin synthetase' stage because this enzyme(s) is rate-limiting. Hence excess neopterin would be excreted following dephosphorylation. The enhanced pterin synthesis could simply be a means of restoring the balance between the purine and pyrimidine levels since thymidine may not be synthesised in fragile X and therefore purines such as guanine might accummulate.

Not all neurological disorders are accompanied by changes in pterin levels in the body. Urine neopterin and biopterin excretion in five subjects with tuberose sclerosis show no change compared to healthy controls. However, these subjects have a congenital anatomical abnormality of brain cells and the associated mental retardation may not be due to a biochemical lesion.

The disorder of endogenous depression is another neuropathological condition where an impaired THB metabolism has been implicated. Endogenous depression has been shown to ameliorate with THB treatment (Levine and Ghisla 1983). The present study shows that there is a disturbance of biopterin metabolism in bipolar depressives which is not found in unipolar subjects. The urinary output of biopterin was significantly reduced in bipolar subjects compared with healthy controls. The observation of no significant change in the neopterin output in these patients suggestd a blockage at a post dihydroneopterin-triphosphate stage. Impaired THB synthesis is found in post-mortem analysis of brain tissue of depressed patients who showed considerably less synthesising ability compared to control subjects. Blair et al (1984) suggest that the THB defect may involve folate deficiency since one of the four brain tissue samples showed stimulation of THB synthesis on addition of 5-methyltetrahydro-folate. An impaired THB synthesis

could lead to a disturbance in biogenic amine neurotransmitters reported in depression (Zis and Goodman 1982), in subjects with a bipolar affective psychoses.

Leukaemia is a chronic or acute disease of unknown aetiological factors characterised by unrestrained growth of leukocytes and their precursors in the tissue (Ruddon, 1981). Acute lymphocytic leukaemia primarily strikes children, almost two-thirds of whom are below the age of 15. There is a large increase in proliferation of lymphoblast cells in the bone-marrow which leads to reduced production of other haemotopoetic cells (Ruddon 1981).

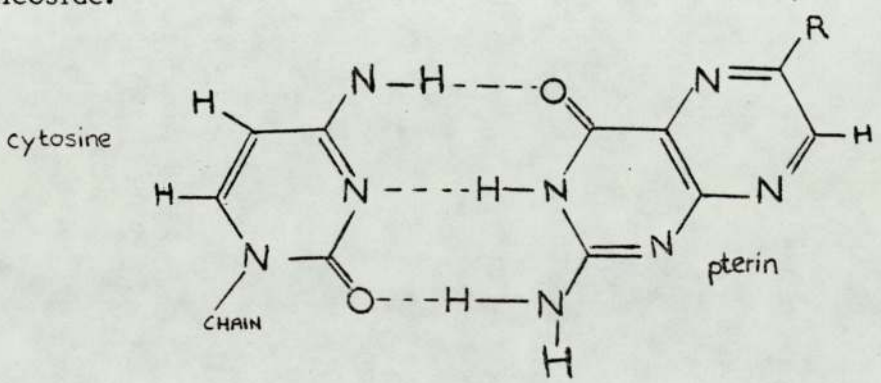
The present study shows that there is a significant elevation of biopterin levels in the cerebrospinal fluid of ALL subjects. These results support previous findings of elevated serum biopterin (Leeming et al 1980) and elevated urinary neopterin (Grunewald et al 1982) in ALL patients. Hence an increased synthesis or salvage of the pterin cofactor may occur in these patients. The significance of such a change is unclear.

Grunwald et al (1982) suggests that the enhanced neopterin levels in haematological neoplasias may be either due to neopterin production by the malignant cell itself, or may be produced by the host organism in response to the transformed cells. The observation of an increased phosphoribosylpyrophosphate (PRPP) synthetase activity in ALL white blood cells (Danks and Scholar 1979) suggests that the elevated serum and csf pterin levels are due to enhanced synthesis in the malignant cells themselves. PRPP-synthetase catalyses the formation of PRPP from ATP and ribose-5-phosphate (Danks and Scholar 1979), and the PRPP so

formed than serves as an essential substrate in purine and pyrimidine synthesis. Hence an elevation of PRPP could lead to enhanced GTP synthesis and thereby stimulate pterin synthesis.

The significance of the elevation in pterin levels to the pathogenesis of ALL is unclear but one can speculate as to the possible role pterins could play. A characteristic feature of leukaemia is the failure of cell differentiation with resultant accummulation of immature leukocytes (Grunewald et al 1982). Cellular dedifferentiation involves the restriction of genes from transcripting. Wachter et al (1982) suggest that pterins may bind to nucleic acids and thereby mask sites and inhibit translation (fig. 4.3). This could be the case in acute lymphoblastic leukaemia.

It is clear that many of the disease states become apparent as a result of the involvement of both environmental and genetic factors. Causation in many diseases (especially of a psychiatric nature) is often due to multiple factors and subgroups may exist within a given diseased population where patients show distinct aetiologies. Disturbance of tetrahydrobiopterin metabolism may play an important role in the aetiology of some diseases and the above has been a brief investigation into the involvement of biopterin metabolism in some diseases afflicting our society. Fig.4.3 Hydrogen bonds between a pteridine and a nucleoside:



CHAPTER FIVE :STUDIES ON TETRAHYDROBIOPTERIN METABOLISM IN WISTAR RATS FOLLOWING EXPOSURE TO CERTAIN XENOBIOTICS

5.1 The Effect of Acute Lead Acetate Administration On Rat Brain Tetrahydrobiopterin Metabolism

Lead poisoning is still an important cause of illness both in industrial workers and in those, often children, who are not occupationally exposed. Supposed 'safety levels' of exposure have been questioned and subclinical effects have been reported (Needleman et al 1979). An increasing volume of evidence from subjects with lead levels beneath those at which encephalopathy occurs suggests that the nervous system may be very sensitive to this metal, (Needleman et al 1979).

The World Health Organization (WHO) report on lead (1977) shows that the greatest consumption of lead is by the car industry in the form of lead-containing electric-batteries and as alkyl-lead additives in motor-fuels. Other users include the cable-industry, the chemical industry (e.g. leaded paints) and the construction industry (e.g. for roofing, cladding etc). Lead-pollution of the environment is largely due to the mining, smelting and refining of the lead-ore; during manufacture of lead containing goods; release of car exhaust fumes and disposal of leaded car-oil; and domestic contamination due to leaded water-pipes and leaded paints (WHO, 1977). The general population is exposed to lead by ingestion of contaminated food and water and by inhalation of lead-particles. Soil contamination due to vehicular emissions, particularly in urbanised area, is suggested to be a major source of

ingested lead in children at the crawling stage, who engage in hand-to-mouth exploratory behaviour (Mielke et al, 1984, Royal Commission Report 1983).

The absorption of lead from the environment is dependant on a number of parameters including the physical and chemical state of the mental and the sex, age and physiological well being of the individual (WHO, 1977). Absorption by the respiratory route is more rapid and complete then by any other route (Paterson 1965). Some 9% of the dietary lead has been found to be absorbed (Rabinowitz et al 1973). However, the diet is suggested to contribute two-thirds or more of the total lead intake (Royal Commission Report 1983). The daily uptake of lead through the gut, lungs and skin ranges from 200-500 μg but only 10% of this is retained (Niklowitz 1977), the rest being excreted.

Blood lead levels in urban children without known lead exposure may range up to 400 μg/l which is also considered to be the approximate upper limit of acceptable lead exposure (Needleman et al, 1979). However the margin between safe and toxic level is suggested to be narrow, especially in children. Children with blood lead levels as low as 350 μg/l have been reported to show signs of distractibility, dependency, day-dreaming tendency and an inability to persist with a task (Needleman et al 1979.) David et al 1972 report hyperactive behaviour in children with blood lead levels as low as 260 μg/l. Some of these children showed an elevated body-store of lead as elicited by post-penicillamine treatment levels of lead in urine. Lowering of blood-levels by penicillamine also produces marked improvement in behavioural characteristics, suggesting a direct causal relationship between elevated lead levels and hyperactivity (David et al, 1984).

Children with mental retardation of unknown aetiology consistently showed elevated (but subclinical) lead levels compared to those with mental retardation of probable aetiology (David et al 1976).

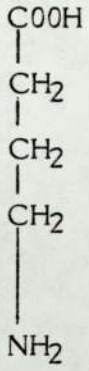
The brain may be more susceptible to lead toxicity then has been hitherto accepted. Cerebral uptake of lead occurs at any level of blood lead (Goldstein et al 1974) and there appears to be no threshold to protect the brain against absorption and storage. Furthermore, brain levels of lead persist whilst blood levels fall to acceptable values and therefore the blood levels cannot be used as a guide to the brain content (Goldstein et al, 1974).

Biochemical studies of lead toxicity have revealed a number of effects on the central nervous system. Chronic low level lead exposure in rat pups produces detrimental effects on the dopaminergic system (Wince et al 1980). There is suppression of dopamine receptor-mediated activation of adenylate cyclase in the neostriatum and diminished potassium-chloride induced release of dopamine. Silbergeld and Adler (1978), however, report enhanced release of dopamine in the presence of lead. No changes in the activity of tyrosine hydroxylase (the rate-limiting enzyme for dopamine synthesis) were observed in vivo (Wince et al, 1980). However, Govoni et al (1978) have observed a decrease in the synthesis of dopamine in the nigrostriatal system in lead-exposed rats.

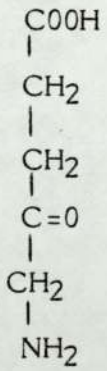
Electrophysiological experiments on the noradrenaline-induced stimulation of cerebellar neurones shows diminished sensitivity following exposure to lead (Taylor et al, 1978). In contrast, inhibition of receptor-mediated firing of Purkinje cells by

noradrenaline is antagonised by lead. This may be due to inhibition of noradrenaline-sensitive adenylate cyclase by Pb^{++}. Nathanson and Bloom (1975) report inhibition of rat cerebeller adenylate cyclase by micromolar levels of lead chloride.

Gamma aminobutyric acid (GABA) the inhibitory neurotransmitter in the brain, has been found to show increased high-affinity receptor binding in the cerebellum and reduced binding in the striatum following lead-intoxication (Govoni et al, 1980). The implications of these findings are uncertain. Silbergeld and Lamon (1980) suggest inhibition of GABAergic function is consistent with clinical observations of excitability and hyperactivity. Furthermore these workers suggest that inibition of the GABA system may be indirect through disturbance in the heme synthesis. Lead inhibits the enzyme δ-aminolevulinic acid dehydrase which converts δ-aminolevulinic acid (ALA) to porphobilinogen, a precursor of heme. The similarity in structure between GABA and ALA results in the latter acting as a competitive inhibitor of GABA-binding to receptors.



y-aminobutyric



d-amino levulinic acid

Shih and Hanin (1978) have reviewed the effects of chronic exposure of neonatal rats to lead and report an increase of up to 48% in cortical

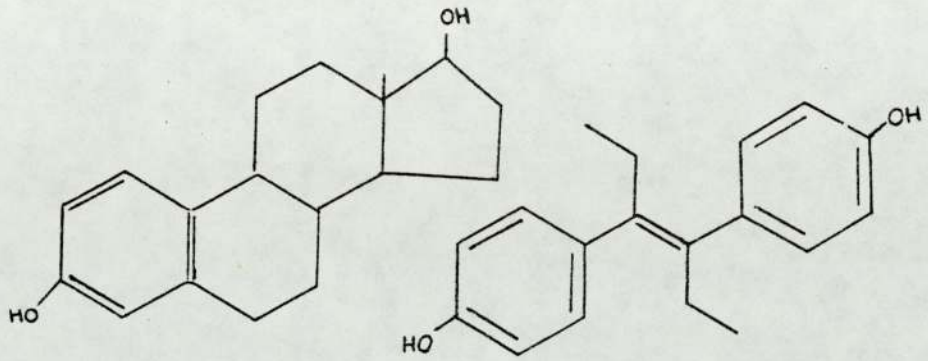
acetylcholine levels with the turnover rate reduced by approximately 50% in the hippocampus and midbrain. There was no change reported in choline transport and choline acetyltransferase activity was unchanged in most parts of the brain. Acetylcholinesterase activity was decreased except in the forebrain and cerebellum where a slight increase or no change was observed. These authors suggest that possible neurotoxic effects of lead may be by interference with calcium stores, the latter being involved in the regulation of high affinity uptake of acetylcholine, choline and also dopamine.

Other neurotoxic effects may be mediated by inhibition of heme synthesis leading to reductions in hemoproteins such as cytochrome P450, an important component of the hepatic drug-metabolising apparatus, and cytochrome C of the oxidative phosphorylation chain in the mitochondria. This raises the possibilty that lead may exert its neurotoxic effect by impairing the general metabolism (Silbergeld and Lamon 1980).

Until recently reports of the effects of lead on tetrahydrobiopterin metabolism have been restricted to in-vivo studies and few observations on the systemic effects in man, (Purdy et al 1981, Barford et al 1983). An investigation of the acute effects of orally administered lead on the brain tetrahydrobiopterin metabolism was therefore carried out.

5.2 Neurological Effects of Diethylstilboestrol By Interference With Tetrahydrobiopterin Metabolism.

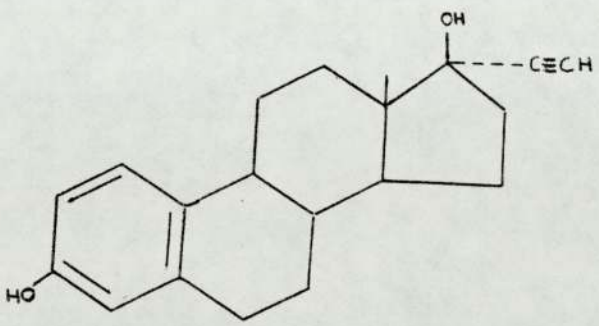
Oestrogens are female sex hormones derived by hydroxylation of the parent molecule Estradiol - 17β.



Estradiol - 17β Diethylstilboestrol.

Oestrogens are produced in the Graafian follicles of the ovary, in the corpus luteum and during pregnancy by the placenta (Begley et al, 1980). Small quantities of oestrogens are also produced in the male gonads. In the female they control the course of the menstrual cycle and in conjunction with progesterone and the gonadotrophins are responsible for the proliferation of the uterine mucosa, growth of the mammary glands and the appearance of the secondary sexual characteristics (Begley et al, 1980).

A number of substituted phenols (including diethylstilbesterol) have oestrogenic activity. Diethylstilboestrol has been used in the past as a post-coital contraceptive but has been superseded by less toxic synthetic oestrogens such as ethinyloestradiol (Begley et al, 1980).



ethinyloestradiol

Naturally occuring oestrogens and synthetic analoges are suggested to medicate their effect by entering target cells by active and passive processes. Within the cell they interact with cytopplasmic receptors to form activated complexes which can then enter the nucleus and influence the synthesis of specific ribonucleic acids (Katznellenbogen, 1978). This leads to synthesis of specific proteins which produce the physiological responses of the hormone. The contraceptive effect occurs by inhibition of ovulation by negative feedback action at the hypothalamus and anterior petuitary (Begley et al, 1980).

Apart from their effects at the major target sites, oestrogen and its analogoes also act on other tissues producing a number of side effects. The majority of the side effects of the "pill" have been attributed to the oestrogenic - component and not progesterone (Connell, 1978), hence the advent of the progestin-only containing pill. Oestrogen influences liver metabolism and thereby affects the production of blood-clotting factors and influences blood pressure (Katznellenbogen, 1978). The contraceptive pill has been reported to induce chorea in subjects with a predisposition to Sydenham's chorea (Nausieda et al, 1979). Furthermore oestrogens are known to be antidopaminergic, lowering striatal brain dopamine concentrations and compensatorily increasing post-synaptic dopamine-receptors (Di Paulo et al, 1982). A number of females on the pill have also been reported to suffer from depression. Improvement in their condition is found either after removal from the pill or after low-dose progesterogen therapy (Dennis et al, 1968).

Hence the oestrogenic component of the pill has been implicated in psychotic or motor defects which are regulated by the monoamine neurotransmitter in the brain. A number of observations have shown

body biopterin levels to alter during the menstrual cycle, in women on the pill and during pregnancy (Leeming and Blair 11980, Barford et al, 1983). As the oestrogen levels rise the biopterin level falls and there is a compensatory increase in dihydropteridine reductase activity (Barford et al 1983). Eggar et al (1983) have investigated the effect of diethylstilboestrol (DES) on rat liver DHPR activity and found this to increase significantly following oral dosage with DES. A similar experiment has been repeated here to observe the effect of DES on brain biopterin levels to give further insight into the possible involvement of tetrahydrobiopterin in the psychosomatic effects of oestrogens.

5.3 Metabolic Aspects of Aluminium Toxicity : Effects On Tetrahydrobiopterin Metabolism.

Aluminium toxicity has been implicated in the pathogenesis of a number of clinical disorders. Patients with chronic renal failure, on long-term haemodialysis treatment have been shown to suffer from osteodystrophy (McClure and Smith 1983) and dialysis-encephalopathy, (Schreeder et al 1983). Aluminium is found in these patients to be localised in the bone tissue and brain in amounts that are significantly higher than in healthy controls (McLure and Smith 1983, Crapper et al 1978). Aluminium has been implicated in the pathogenesis of Alzheimer's disease (Crapper et al 1978) and a progressive encephalopathy with neurofibrillary degeneration has been induced in animals by intracranial injection of a soluble aluminium salt (Kaltzo et al 1965). The potential toxicity of this ubiquitous metal has been underestimated until recently.

Aluminium comprises approximately 8% of the earths crust and is the most abundant metal (King et al, 1981). It's highly reactive nature allows it to be present in 300 different mineral forms including silicates, feldspars and micas. It occurs naturally in water in varying amounts but may be present in elevated amounts in treated drinking water, when aluminium sulphate is used as a flocculent (King et al, 1981). In plant foodstuffs its concentration varies depending on soil-content but some sub-tropical plants (e.g. Euphorbiaceae) have a natural ability to accumulate aluminium (Ondreicka et al 1971).

The ability of aluminium to form complexes within the digestive tract results in low absorption of aluminium across the gut. Hence aluminium

concentration in foodstuffs of animal origin is much lower ranging between 1.6 and 16 mg/kg according to Ondreicka et al 1971. The liver, heart, brain and gut show a higher aluminium content compared with other tissues (Ondreicka et al 1971). Moderate increases in aluminium content of food increases the faecal elimination and does not affect the tissue levels significantly. However, excessively large doses (200 mg/kg) cause a considerable retention of aluminium in the rat, particularly in the liver, testes and bone (Ondreicka et al 1971). Part of the absorbed load is incorporated into long-term storage pool whilst the rest is eliminated by urinary excretion.

The possible routes of entry of aluminium into the brain are suggested to be via axoplasmic flow along the olfactory nerves, or by passage through the blood-brain barrier or by a vascular route (Crapper et al, 1976). The olfactory bulb contains considerably higher aluminium than other regions of the brain and neurofibrillary degeneration is often found in close proximity to the olfactory system in demented brains (Crapper et al 1976). Systemic injection of sodium aluminium tartarate produces an elevation in brain levels without increases in the cerebrospinal fluid or choroid plexus. Within the brain the metal binds to the nuclear chromatin material and its toxic effect may be via alteration in the transcription of genetic information (Crapper et al 1976).

Aluminium is involved in the pathogenesis of dialysis encephalopathy and also, possibly, Alzheimer's disease. Alzheimer's disease is a condition of progressive dementia, with specific necropsy findings of brain atrophy, neurofibrillary degeneration, senile plaques and granulovacuolar degeneration in the absence of vascular lesions (Crapper

et al, 1976). The cytotoxic effects of aluminium have been shown by Crapper and workers who have demonstrated neurofibrillary degeneration in cats by intracranial application of aluminium chloride. Areas free of neurofibrillary degeneration had concentrations of less than 4 μg/g dry weight and fibrillary lesion density paralleled concentrations in excess of 4 μg/g. dry weight. In live animals, aluminium-induced behavioural changes were restricted to short-term memory loss and to delayed acquisition of avoidance response (Crapper and Dalton 1973). The histological findings are similar to post-mortem findings in Alzheimer brains. Normal human brains exhibited an aluminium concentration of 1.9 $\pm$ 0.7 S.D. μg/g. dry weight whilst Alzheimer brain samples showed a range of 0.4 - 107 μg/g, (Crapper et al 1976). Two cases of Alzheimer's disease showed a positive correlation between neurofibrillary degeneration and aluminium concentration in various anatomical regions of the brain. Furthermore elevated aluminium content was not found in various cortical regions in two cases of vascular dementia (Crapper et al, 1976).

Dialysis dementia is a more recently recognised disorder in patients with renal dysfunction maintained on haemodialysis for some time. The disorder is characterised by the onset of altered behaviour, dementia, speech disturbance, myoclonus and convulsions (Dunea et al, 1978). Aluminium intoxication has been attributed as the causal agent (Alfrey et al 1976), based on findings of high brain grey-matter aluminium content in these patients compared with control subjects. These findings are supported by observations of dialysis-dementia outbreaks in patients on home-dialysis equipment, exposed to high water aluminium levels (Dunea et al, 1978 ; Davison et al 1982). Futhermore, the condition is not observed if the patient is maintained on fluid-free of

aluminium (Davison et al 1982).

The condition of dialysis, if uncontrolled, is lethal. In a four year period, 19 out of 20 subjects in one study died within 16 months of being identified as suffering from dialysis dementia (Dunea et al 1978). Davison et al (1982) have observed an exponential relationship between time to death from dementia and mean water aluminium content. An inverse relationship exists between degree of exposure to aluminium and the time of death from dementia.

Aluminium from the dialysate is now accepted as the basis for dialysis encephalopathy but other sources of aluminium may also give rise to aluminium toxicity. Randall (1983) reports a child with renal dysplasia who was maintained on a regular oral dose of aluminium carbonate (as a phosphate binder gel). This patient deteriorated with signs of renal osteodystrophy, grand mal seizures, hypotonia and poor motor skills and eventual death at eleven months. The encephalopathy was attributed to aluminium as high serum and cerebrospinal fluid levels were observed. Hence aluminium-containing phosphate binder gels can be a significant source of the metal and give rise to non-dialysis related toxicity. Furthermore, the gastrointestinal absorption of aluminium is suggested to be stimulated by parathyroid hormone (PTH) and patients with persistently elevated levels of PTH may be more susceptible to aluminium toxicity (Randall 1983) by the oral route.

Very little is known about the effect of aluminium on tetrahydrobiopterin metabolism. In vitro studies on rat liver tissue show that aluminium, in concentrations comparable to those found in clinical conditions, causes inhibition of the enzyme dihydropteridine

reductase (Brown 1981). This study investigated the in vivo effects of orally administered aluminium on brain and liver tetrahydrobiopterin levels.

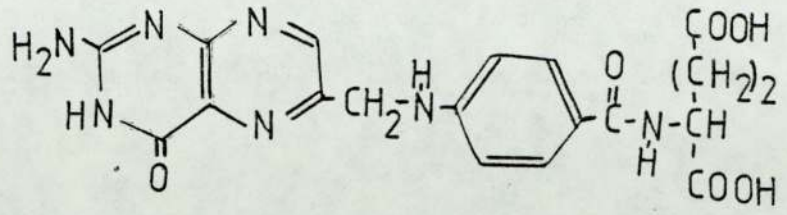
5.4 Biochemical Effects of Methotrexate On Tetrahydrobiopterin Metabolism

Methotrexate is an antimetabolite drug which has been successfully used in the treatment of acute lymphocytic leukaemia, choriocarcinoma and psoriasis.

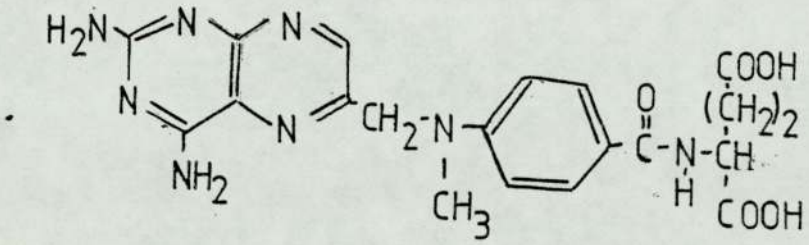
The principle mechanism of action of this drug is the inhibition of dihydrofolate reductase (DHFR) the enzyme that reduces dihydrofolate to tetrahydrofolate in the presence of NADPH (Bertino et al 1979). Tetrahydrofolate is converted to a variety of coenzymes that are necessary for one-carbon transfer reactions involved in the synthesis of thymidylate, purines, methionine and glycine (Bertino 1971) (fig. 5.3) Hence inhibition of DHFR leads to inhibition of DNA, RNA and protein synthesis. In this way cell-division is inhibited.

Thymidine monophosphate is synthesised by a one-carbon transfer, from N^5, N^{10} - methylene-tetrahydrofolate, to deoxyuridine monophosphate under the direction of thymidylate synthetase . The dihydrofolate generated in the process has to be reduced to tetrahydrofolate by DHFR to maintain the level of the active cofactor. Inhibition of DHFR by methotrexate leads to depletion of active cofactor and cessation of thymidine monophosphate synthesis. There is some evidence that methotrexate may also act by inhibiting thymidylate synthetase directly, by occupying the folate coenzyme site (Borsa et al 1969).

fig.5.1: STRUCTURAL SIMILARITY BETWEEN FOLIC ACID AND METHOTREXATE

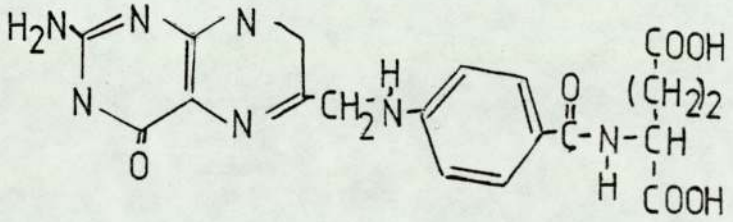


FOLIC ACID



METHOTREXATE

fig.5.2: THE DIHYDROFOLATE REDUCTASE REACTION



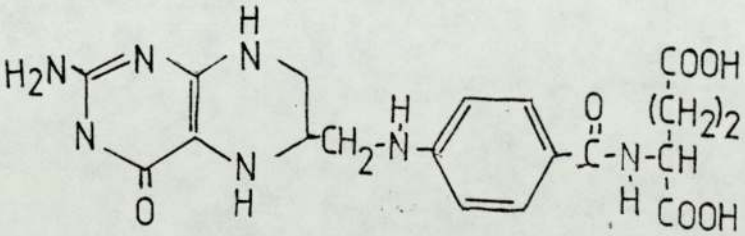
DIHYDROFOLATE
dihydrofolic acid

dihydrofolate reductase

NADPH

$NADP^+$

methotrexate inhibition



tetrahydrofolic acid

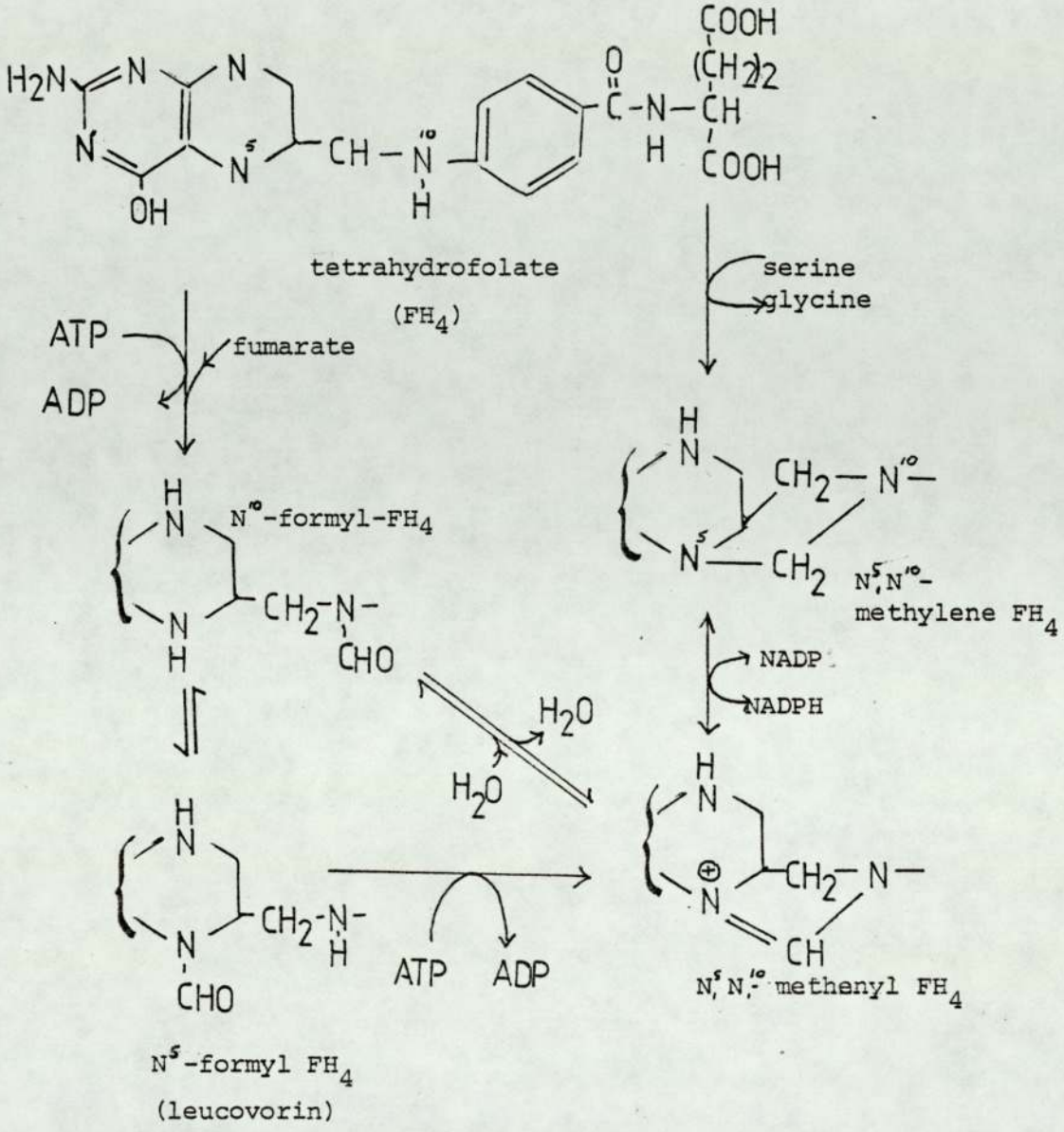


fig. 5.3: INTERCONVERSION OF THE TETRAHYDROFOLATE COENZYMES NECESSARY FOR THE SYNTHESIS OF THE PURINES AND THYMIDINE MONOPHOSPHATE

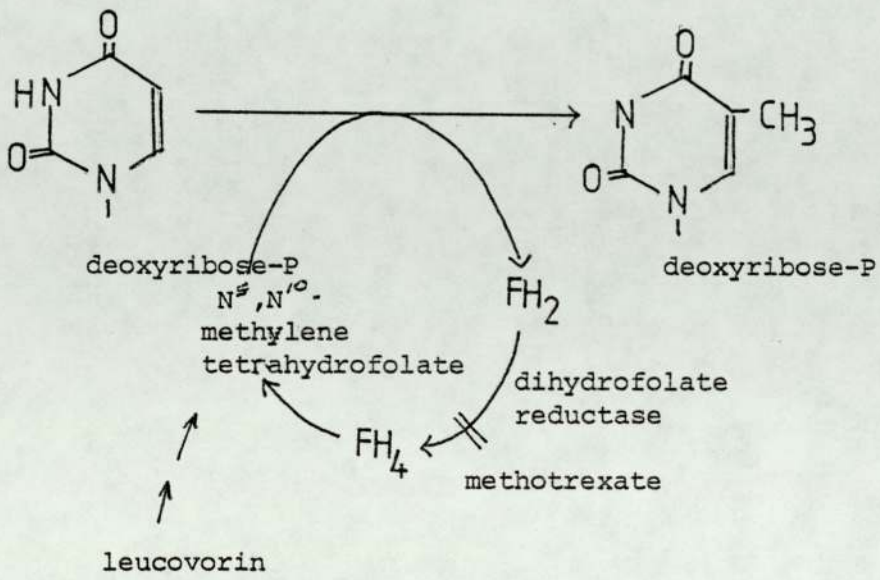
Actively proliferating tissues such as malignant cells, bone marrow, fetal cells and epithelial cells are in general more sensitive to methotrexate. Cellular proliferation in malignant tissue is greater than in most normal tissue and thus methotrexate may impair growth without irreversible damage to normal tissue (Bertino et al, 1971).

Methotrexate (MTX) can be administered by the oral, intra-venous, intra-arterial or intra-thecal route. Similar plasma levels are seen after either oral or intravenous administration of low doses of methotrexate (Henderson et al 1965). As much as 50% of an oral dose can be accounted for as the parent compound in the plasma in rats (Zaharko and Oliverio 1970). There is some experimental evidence that methotrexate and some folate coenzymes share the same transport system in certain types of cells (Goldman 1971). It is possible that a similar situation exists in the small intestine where uptake at low concentrations involves a transport system and when drug concentrations are high absorption by diffusion is enhanced. In the systemic circulation as much as 50% of the drug may be bound to serum protein (Henderson et al 1965). The csf concentrations of the drug correlate well with the unbound-methotrexate levels in the serum for a given individual (Evans et al 1983). However there can be a 300% range in the csf MTX concentrations in children given identical intermediate dose methotrexate therapy (Evans et al 1983). The brain uptake of methotrexate is strongly influenced by the integrity of the blood-brain barrier. Neuwelt et al (1982) have demonstrated increased uptake of methotrexate following mannitol-induced reversible disruption of the blood-brain barrier, and reduced uptake in tumour-bearing rats after adrenal steroid treatment. Steroids had no effect on methotrexate delivery to the brain in healthy rats. The transport of methotrexate

across the blood-brain barrier is important in assessing the therapeutic efficacy and neurotoxicity at a given systemic dose.

Toxic effects of methotrexate occur at both low and high dose therapy. At low doses methotrexate is more detrimental to normal proliferating tissues such as bone-marrow and the gastrointestinal tract since tumour cells often have a poor ability to transport the drug. Hence in such cases as osteogenic sarcoma high doses of methotrexate are employed intermittently in alternation with rescue therapy with N^5-formyltetrahydrofolate (leucovorin), (Tattersall et al, 1975). In such a therapy, enough of the drug penetrates the tumour cells to kill them. Leucovorin or its metabolite is transported to the cells and converted to the tetrahydrofolate-coenzymes required for thymidylate and purine synthesis. It bypasses the methotrexate blockade and rescues the normal cells (fig. 5.4). However the use of high dose protocols may produce toxic reactions in a patient even with leucovorin rescue (Nirenberg et al, 1977). This is largely due to the substantial interpatient variability in systemic clearance of methotrexate (Evans et al, 1983). Furthermore, methotrexate can cause renal damage which is a frequent complication of high dose therapy (Condit et al 1969). In such situations delayed excretion of methotrexate occurs and increases the risk of toxic reactions. Most of the methotrexate administered is excreted largely unchanged by the kidneys, under normal conditions (Zaharko and Oliverio 1970).

Neurotoxicity due to methotrexate therapy has been reported not infrequently. Weiss et al (1974) review cases with symptoms of headache, nausea, vomiting, tremor, ataxia and dementia following intrathecal methotrexate therapy. Post-mortem brain analysis often



FH_2 = dihydrofolate

FH_4 = tetrahydrofolate

fig.5.4: SYNTHESIS OF THYMIDINE MONOPHOSPHATE FROM DEOXYURIDINE MONOPHOSPHATE

shows periventricular necrosis and reactive astrocytosis (Weiss et al 1974). Bleyer et al (1973) suggest that patients with a pathological inability to eliminate methotrexate from the csf, as when suffering from meningeal leukaemia are more susceptible to the neurotoxic effect of methotrexate. The more detailed biochemical aspects of methotrexate neurotoxicity remain unclear.

5.5 Materials :

Unlabeled methotrexate was obtained from Lederle (UK). (3',5',7- H^3)-methotrexate was obtained from Amersham (UK). ^{203}Pb was obtained from the Cyclotron Research Unit, London. All other chemicals were of analytical grade and obtained from Sigma Chemical Company (UK).

All animals were supplied by Bantin and Kingman Limited, Grimston (UK).

5.6 Methods :

The Effect of Acute lead Acetate Administration On Rat Brain Tetrahydrobiopterin metabolism

Adult Male Wistar rats were used in all experiments. Rats were housed in groups of two or three in plastic cages under a 12 hour light/12 hour dark cycle. The temperature in the animal room was 20-25°C. Rats had access to pelleted rodent chow and to drinking water until the penultimate day of experiment when they were transferred to a cage with grating to act as a faecal trap, and had access to water only,

overnight.

Rats (7-8 weeks of age, 170-250 g. in weight) were given lead-acetate in distilled water by gavage on the day of experiment. The dose of lead was varied between 0.3 ml. of 10^{-2} M to 0.3 mls. of 10^{-6} M per animal. Each 0.3 ml. dose contained 1 μCi of ^{203}Pb. Control group of rats were not dosed with the drug-vehicle. The rats were maintained on water, ad libitum for a further 4 hours and then sacrificed by decapitation.

Half the brain organ was homogenised to produce a 20% homogenate in 20% trichloroacetic acid. The homogenate was centrifuged on a bench centrifuge at 3000 rpm for 2 minutes or less and the supernatant used for pterin analysis. The supernatant was analysed neat and by acid- and alkaline-iodine oxidation according to the method of Fukushima and Nixon (1981). The procedure was carried out with samples maintained in an ice-bath as far as possible, until they were oxidised with iodine-solutions at room temperature.

The other half of the brain sample was analysed for ^{203}Pb radioactivity using a Nuclear Enterprise gamma-counter.

Aluminium Toxicity Experiment :

The experimental procedure was essentially the same as for lead-dosed rats. No radiolabel was present in the administered dose. Six rats were given 0.3 mls of 0.05 M. aluminium acetate and a further six rats were administered 0.5M aluminium sulphate ($Al_2(SO_4)_3 . 16 H_2O$). After a further 4 hours each rat was killed by decapitation and the brain and liver analysed for biopterins by HPLC as above.

Oestrogen-treatment of Rats :

Twenty-one day old female weaning Wistar rats were dosed orally with a suspension of diethylstilboestrol in corn-oil (500 mg/kg body weight daily for 4 days). Twenty-four hours after the last dose of DES the animals were killed and the brains removed for analysis. The rats had free access to rodent chow and drinking water at all times and the litters of dose rats were maintained together in one cage. Control rats were given corn-oil only.

Methotrexate Treatment of Rats :

Six adult male Wistar rats were administered methotrexate (100 mg/kg.) by gavage, having been starved overnight. Each dose contained 4 μCi/0.4 ml dose of 3',5',7-^{3}H methotrexate. Nine more were dosed similarly but without radiolabel.

Levels of biopterins (oxidised and reduced) were determined four hours later, by HPLC. Brain radioactivity was measured by liquid scintillation counting.

5.7 Results

TABLE 5.1 : RESIDUAL BIOPTERIN LEFT IN PELLET FROM PREPARATION OF BRAIN HOMOGENATES

Pellet	Biopterin in Pellet pmol./g. wet wt.	% of total Biopterin
P_1	9.27	4.10
P_2	10.65	3.49
P_3	31.94	11.58
P_4	19.56	6.85
P_5	18.93	6.30
P_6	24.20	8.50
Mean :	**19.10**	**6.80%**

Brain tissue was prepared as a 20% homogenete (w/v) in 20% trichloraecetic acid and centrifuged at 3000 rpm for 2 min. The supernatant was used for biopterin analysis. The pellet surface was gently washed and pellet re suspended in 1ml of acid/I_2 solution for 1hr. The resultant homogenate was centrifuged at 3000 rpm and the supernatant, so yieldedwas tested for biopterin.

TABLE 5.2 : DISRIBUTION OF PTERIDINES IN THE LEFT AND RIGHT HALF OF CONTROL ADULT RAT BRAINS :

	Left half	Right half	P
Total Biopterin (pmol./g. wet wt.)	254.9±15.9	242.2±11.7	N.S
% Biopterin	0	0	N.S
% Dihydrobiopterin	4.3± 1.4	1.5± 0.7	N.S
% THB	95.3± 1.1	96.5± 0.7	N.S

Results as mean ± SEM.

Statistics : paired t-test.

N.S. = no significant difference.

TABLE 5.3 : THE EFFECT OF ORALLY ADMINISTERED LEAD ACETATE ON BRAIN BIOPTERIN LEVELS IN ADULT MALE WISTAR RATS.

$[Pb^{2+}]$ M	Total Biopterin pmol/g. wet wt.	Tetrahydrobiopterin pmol/g. wet wt.	% Biopterin	% Dihydrobiopterin	% Tetrahydrobiopterin
0 (12)+	227.5 ± 12.6	216.2 ± 12.8	1.97 ± 0.5	3.3 ± 0.8	97.7 ± 0.86
10^{-6}M (6)	219.1 ± 28.4	180.8 ± 29.9	5.2 ± 2.9	12.6 ± 4.0**	82.2 ± 3.5*
10^{-5}M (6)	282.6 ± 16.6*	221.6 ± 22.6	7.1 ± 1.8**	15.5 ± 4.8**	77.5 ± 4.7***
10^{-4}M (6)	260.3 ± 11.2	210.0 ± 12.7	6.9 ± 1.8**	12.5 ± 3.0**	80.6 ± 2.8***
10^{-3}M (6)	229.9 ± 22.3	188.1 ± 16.3	10.4 ± 3.5**	6.7 ± 3.5**	82.9 ± 3.7***
10^{-2}M (6)	223.3 ± 15.3	199.1 ± 14.6	6.1 ± 1.3**	4.8 ± 1.7	89.1 ± 0.9***

Total Biopterin = Biopterin + dihydrobiopterin + tetrahydrobiopterin
+ figures in parenthesis = number of test animals.
Students t-test: comparison with controls :
* $p < 0.02$.
** $p < 0.01$.
*** $p < 0.001$.
Results as mean ± SEM.

TABLE 5.4 : LEAD UPTAKE INTO BRAIN AT VARYING LEAD ACETATE CONCENTRATIONS USING ^{203}Pb AS RADIOTRACER.

[lead acetate] M	Dose PbAc ng/rat	Brain Uptake ng/g.wet wt	Brain Uptake / Dose
10^{-6}	62	1.05 ± 0.12	0.017
10^{-5}	620	8.07 ± 2.02	0.013
10^{-4}	6200	85.74 ± 39.57	0.014
10^{-3}	62000	697.5 ± 116.25	0.011

TABLE 5.5 : THE EFFECT OF AN ACUTE ORAL DOSE OF ALUMINIUM ON BRAIN BIOPTERIN LEVEL IN ADULT MALE WISTAR RATS.

	Control Rats (n=12)	Rats acutely dosed with 4 mg/kg Al.+ (n=6)	Rats acutely dosed with 40 mg/kg Al.± (n=6)
Total Biopterin. (pmol./g. wet wt)	227.5 ± 12.6	323.7 ± 14.7 *	307.7 ± 14.3 *
% Biopterin.	1.9 ± 0.5	4.9 ± 1.5 **	1.4 ± 0.4
% Dihydrobiopterin.	3.3 ± 0.8	3.1 ± 1.6	11.8 ± 5.3 **
% Tetrahydrobiopterin.	94.7 ± 0.9	92.0 ± 1.9	87.1 ± 5.0
Actual Tetrahydrobiopterin level. pmol./g. wet wt.	216.2 ± 12.8	298.6 ± 17.5 *	266.8 ± 17.0 *

One tailed t-test comparison of control rats with Al-dosed rats :

* $p < 0.01$
** $p < 0.05$

values as mean ± SEM

\+ aluminium as aluminium acetate
± aliminium as aluminium sulphate.

TABLE 5.6 : THE EFFECT OF AN ACUTE ORAL DOSE OF ALUMINIUM ON LIVER BIOPTERIN LEVELS IN ADULT MALE WISTAR RATS.

	Control Rats (n=6)	Rats acutely dosed with 4 mg/kg. Al.+ (n=6)	Rats acutely dosed with 40 mg/kg. Al.± (n=6)
Total Biopterin (pmol./g. wet wt)	2651.1 ± 254.5	3174.5 ± 244	3900.3 ± 401 *
% Biopterin	4.8 ± 2.1	2.3 ± 0.8	1.3 ± 0.2
% Dihydrobiopterin	1.9 ± 1.4	6.2 ± 2.7	3.9 ± 0.7
% Tetrahydrobiopterin	93.3 ± 1.9	91.6 ± 2.6	94.8 ± 0.7
Actual Tetrahydrobiopterin. (pmol./g. wet wt)	2464.9 ± 225.9	2916.7 ± 266.8	3706.7 ± 395 *

One tailed t-test comparison of control and Al-dosed rats :

* $p < 0.05$

Results as mean ± SEM

\+ aluminium as aluminium acetate.
± aluminium as aluminium sulphate.

TABLE 5.7: THE EFFECT OF DIETHYLSTILBOESTROL ON RAT BRAIN BIOPTERIN METABOLISM.

An oral 500 mg/kg dose of diethylstilboestrol in corn-oil was given to female weanling rats for 4 consecutive days. The animals were killed on the fifth day. Control animals were given corn-oil only.

	Controls (n=6)	Diethylstilboestrol dosed (n=6) animals	P
THB synthesis (ng biopterin/ hr. x mg.protein.)	2.02 ± 1.5	0.17 ± 0.3	$p < 0.01$
Total biopterin. (pmol./g. wet wt.)	289 ± 24.0	160 ± 19.5	$p < 0.001$
% THB	95%	95%	N.S.
Actual THB level (pmol./g. wet wt.)	278.4 ± 25.0	153.2 ± 21.8	$p < 0.001$

N.S. = not statistically significant.

THB synthesis is significantly reduced in the rats pretreated with oestrogen compared to matched controls killed at the same time ($p<0.001$). This is reflected in a fall in the total brain biopterin concentration of 45%. In both groups 95% of the brain biopterins is as THB, demonstrating that salvage of quinonoid dihydrobiopterin is not affected by oestrogens in the brain. This is consistent with the increase in DHPR activity reported in the liver (Eggar et al 1983). The reduction in the synthesis is the probable cause of the lower serum biopterins reported in women (Leeming et al 1980).

TABLE 5.8 : CONCENTRATION OF BRAIN BIOPTERINS IN RATS TREATED WITH METHOTREXATE (100 mg/kg) AND CONTROL RATS

Adult male Wistar rats were given an oral dose of methotrexate in corn oil and sacrificed 4 hr. later. Control rats were given corn oil only

Results as pmol./g. wet wt. ± SEM	MTX-treated Rats (N=15)	Control Rats (n=12)
Total Biopterins oxidised + reduced	250.4 ± 30.6	227.5 ± 12.6
Biopterin	12.4 ± 2.9	4.0 ± 1.2 *
Dihydrobiopterin	26.2 ± 8.9	7.1 ± 1.6 *
Tetrahydrobiopterin	211.8 ± 29.7	216.1 ± 12.8

* p <0.05 (Student's t-test)

TABLE 5.9 : BRAIN BIOPTERINS AS A PERCENTAGE OF THE TOTAL BIOPTERIN (Oxidised & reduced) POOL

	MTX-treated Rats (N=15)	Control Rats (n=12)
% Biopterins	4.5 ± 0.6	2.0 ± 0.5 **
% Dihydrobiopterin	11.9 ± 3.7	3.3 ± 0.8
% THB	83.6 ± 3.5	94.7 ± 0.9 *

Results as mean ± SEM

** p <0.01 (Student's t-test)

* p <0.02.

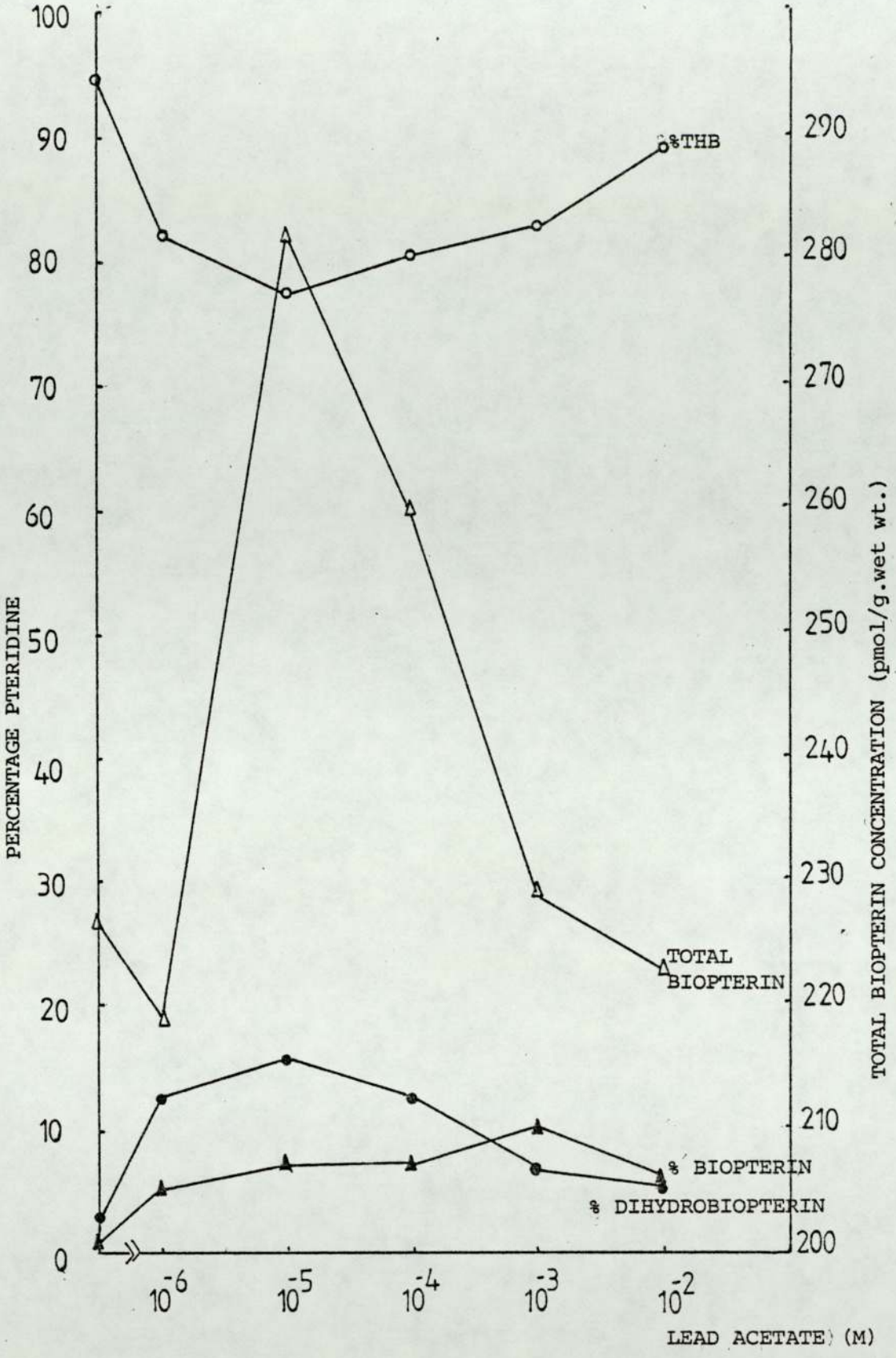


fig.5.5: THE EFFECT OF LEAD ACETATE ON RAT BRAIN BIOPTERIN POOL

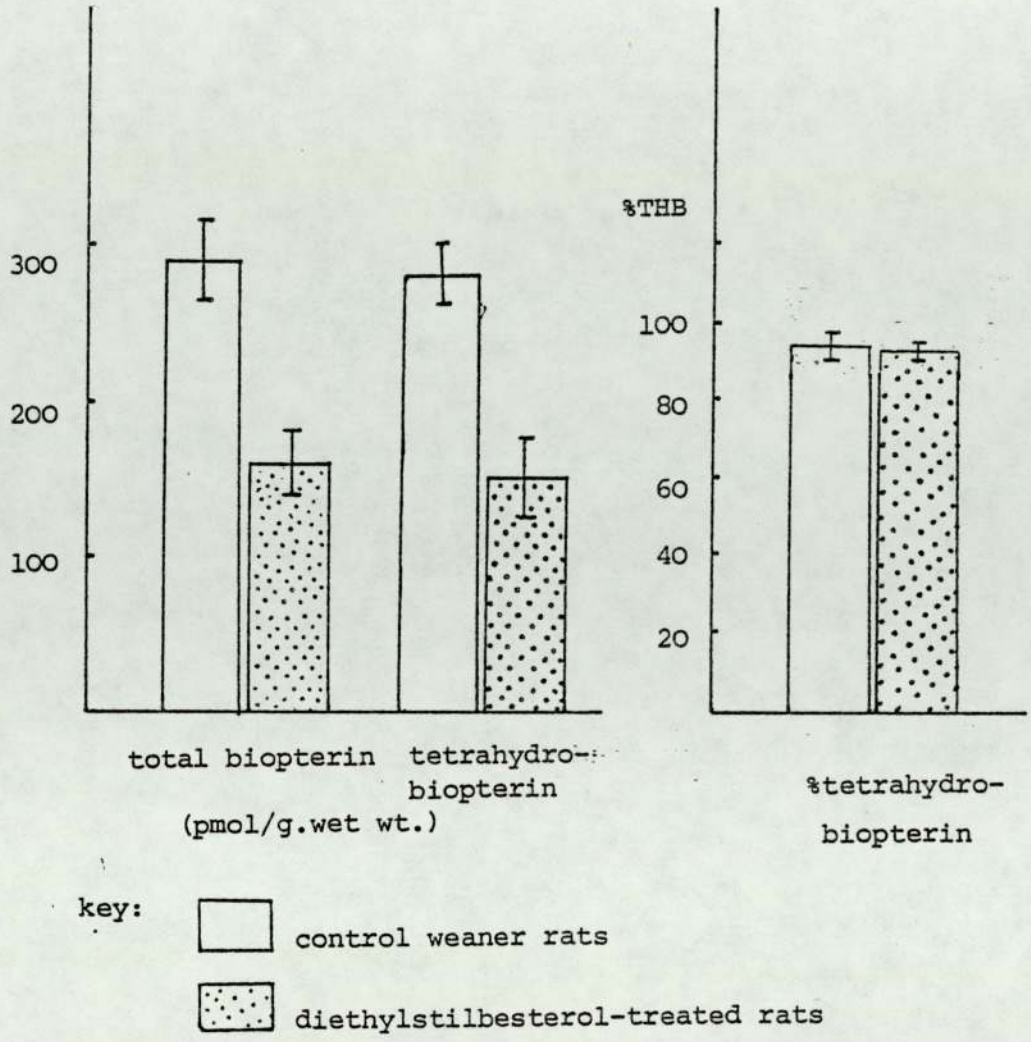


fig.5.6: HISTOGRAM: EFFECT OF DIETHYLSTILBESTEROL ON TOTAL BIOPTERIN AND TETRAHYDROBIOPTERIN LEVEL IN THE BRAINS OF WEANLING RATS.

DISCUSSION :

5.8 The Effect of Acute Lead Acetate Administration On Tetrahydrobiopterin Metabolism

Analysis of biopterin distribution in the left and right half of the brain shows that there is no significant difference in biopterin

distribution between the two halves. Hence assessment of whole brain biopterin levels using half brain samples was acceptable under the circumstances. The mean biopterin concentration of control brains was found to be 227.7 pmol./g. wet wt.

Administration of a single acute dose of lead acetate produces little change in total biopterin but the percent tetrahydrobiopterin is significantly reduced at all concentrations of lead, (Table **5.3**). This suggests that biopterin-synthesis isn't affected but salvage of the cofactor is inhibited. Purdy et al 1981 have shown lead to inhibit the enzyme dihydropteridine reductase in vitro.

Recently, McIntosh et al (1984) have observed elevated brain, tetrahydrobiopterin levels in post-weaning rats after chronic exposure to lead in drinking water. The results obtained here tend to suggest that high dose lead tends to stimulate an increase in turnover rate of THB. That is an increase in synthesis as well as elimination of cofactor. The elimination may take place by catabolic effects of such non-specific enzymes as xanthine oxidase (Bergemann et al 1977) or by increase in egress across the blood brain barrier. The latter is feasible due to the reported detrimental effects of lead on the blood brain barrier. Radioactive lead (^{210}Pb) has been shown to localise in the endothelial cells of the brain capillaries within one hour following intrapenitoneal injection (Thomas et al 1973). It is the brain capillary endothelial cells which constitute the blood-brain barrier that possess at least eight known enzyme carrier systems which regulate blood-borne essential metabolite entry into brain (Partridge et al 1975). Toxicological alterations in capillary permeability and blood-brain barrier transport have been shown for lead following high-

dose exposure (Domer and Woolf 1980). It is possible that lead may also have more subtle effects on transport at lower concentrations. Very little is known of the transport of THB across membranes. Levine et al 1981, have observed selective concentration of THB in depaminergic neurones in the striatum but this is more likely to be due to localised synthesis, rather than a membrane-effect.

Increase in lead dose increases the brain uptake of lead as shown by radiotracer study (Table 5.4). This supports observations of Goldstein et al (1974) who suggests that there is no threshold to the brain uptake of lead. At higher doses the brain-uptake to administered-dose ratio tended to fall. This may have been due to reduced per cent absorption of dose from the gut.

The above observations together with studies on plasma biopterins in lead-industry workers (Blair et al 1974) clearly show that lead exposure affects tetrahydrobiopterin metabolism. The implications of these findings have yet to be ascertained.

5.9 The Effect of Aluminium Toxication On Tetrahydrobiopterin Metabolism

The effect of aluminium on brain biopterin pool is to elevate the total biopterin (oxidised plus reduced) level at both 4 mg/kg and 40 mg/kg aluminium dose. At the higher dose there is a fall in the percentage tetrahydrobiopterin level which is not statistically significant. The fall in percentage tetrahydrobiopterin may be due to inhibition of dihydropteridine reductase reported by Brown (1981). A 30% inhibition in DHPR activity was achieved in vitro by 2×10^{-4} M aluminium salt.

If 10% of an oral dose is absorbed from the gastrointestinal tract as suggested by Fortus (1967) then it is possible for the aluminium concentration to reach inhibitory levels in the body with regards to DHPR activity.

The stimulation of biopterin synthesis as suggested by the increase in total biopterin levels is contrary to expectations since in vitro biosynthesis is inhibited at 10^{-3}M aluminium concentration (Brown 1981). Also energy metabolism has been shown to be inhibited in vivo by aluminium, as indicated by decreased ATP/ADP ratio (Ondreicka et al 1971). However chronic aluminium intoxication in rats decreased liver glycogen levels and increased lactate and pyruvate levels (Kortus 1967). Also serum aldolase activity was elevated following hyperglycaemia (Kortus 1967). If glycogenolysis and glucose metabolism are stimulated then the increased ATP and reduced pyridine nucleotide levels could stimulate biopterin biosynthesis. Reduced pyridine nucleotides are required at one or more points in the biosynthesis of biopterin (Eto et al 1976, Lee et al 1978) and enhance synthesis. The observation of decreased ATP/ADP ratio may indicate enhanced turnover of this nucelotide. Hence aluminium would indirectly stimulate biopterin biosynthesis in vivo. However it must be stated that the individual brain biopterin values show large variations between animals and difference between control and aluminium-dosed rats may be attributable to this.

5.10 The Neurological Effects of Diethylstilboestrol By Interference with Tetrahydrobiopterin Metabolism

The observation of psychosis associated with contraceptive induced chorea (Sale et al, 1981) as well as perinatal depression (Dalton 1971) strongly supports the idea of a hormonal imbalance which triggers off other events leading to the observed condition.

The depression can be explained by a disturbance in tryptophan metabolism. One important pathway for tryptophan metabolism is its hydroxylation and subsequent decarboxylation to the neurotransmitter 5-hydroxytryptamine, (Hamon et al, 1981). The hydroxylation reaction in the brain is suggested to be rate-limiting with regards to both tryptophan level and the cofactor, tetrahydrobiopterin level (Hamon et al 1981). Should oestrogenic effects reduce tetrahydrobiopterin levels then the hydroxylation of tryptophan as well as phenylalanine and tyrosine would be impaired. This could divert tryptophan to other metabolic pathways which are not normally as active. Oestrogen levels reach a peak in the third trimester immediately prior to puerperium (Begley et al 1980). Perinatal depression has been reported (Cutrona, 1983) and attributed to sudden changes in steroid levels (Dalton, 1971). It is conceivable that oestrogenic effects on tryptophan metabolism, via impaired tetrahydrobiopterin metabolism, could give rise to depression. The brain levels of 5-hydroxytryptamine have been found to be low in depressive-suicide cases (Shaw et al, 1976).

It has been found that healthy young women on the pill, as well as pregnant women excrete large amounts of xanthurenic acid (Rose 1966, Brown et al 1961), in their urine. Xanthurenic acid is a catabolite

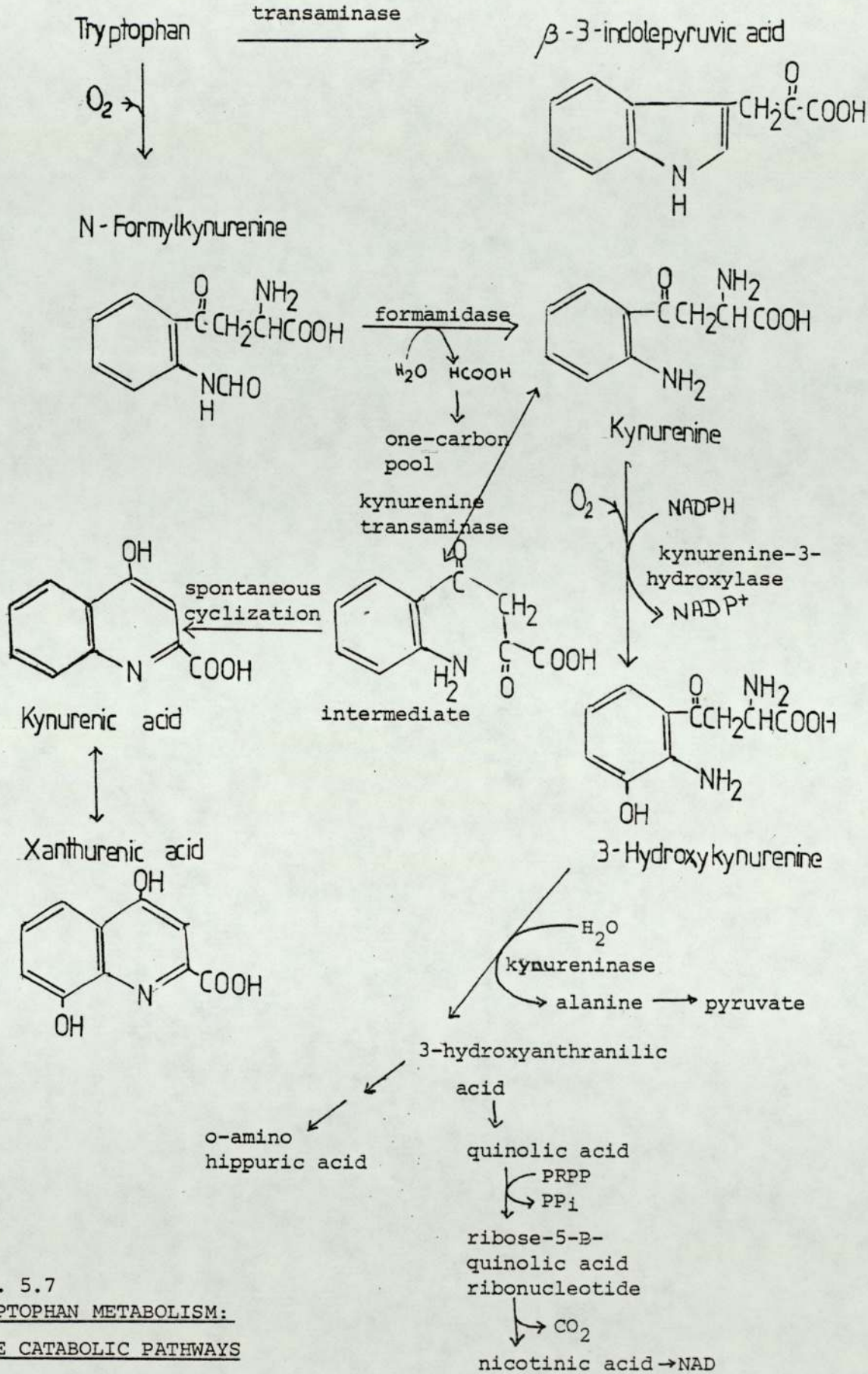


fig. 5.7
TRYPTOPHAN METABOLISM:
SOME CATABOLIC PATHWAYS

of tryptophan which is normally found in the urine (fig.5.7). Brown et al (1961) have shown that elevated xanthurenic acid is due to vitamin B_6 deficiency inasmuch as pyridoxine (B_6) supplementation in the diet restores xanthurenic acid excretion to normal levels. However, administration of B_6 (6mg. per day for 12 days) before trypotophan loading, to pregnant women, lowers xanthurenic acid excretion to that of controls without affecting the elevated excretion of other tryptophan metabolites (Kynurenine, hydroxykynurenine and pyridone). Hence it is suggested that the disorder of tryptophan metabolism may not be entirely one of vitamin B_6 deficiency.

This arguem nt is further supported by observations on excretion of o-aminohippuric acid which is the chief urinary metabolite of anthranilic acid (Brown et al, 1961) (fig. 5.7). The enzyme kynureninase which is involved in anthranilic acid synthesis is pyridoxal-phosphate dependent. A state of pyridoxine deficiency can be induced in humans by isoniazid and leads to a fall in the excretion of o-aminohippuric acid excretion which can be normalised by pyridoxine supplementation (Brown et al 1961). In contrast, the low excretion of o-aminohippuric acid by pregnant subjects is not increased by pyridoxine supplementation. Brown et al suggest that this is due to the hormonal effect on the enzyme kynureninase during pregnancy. However, the observation of elevated excretion of other tryptophan metabolites seems to suggest that the hormonal effect is not essentially one of altered kynureninase activity but one of inhibition of normal tryptophan metabolism.

These observations, together with a dopaminergic defect seems to suggest that a common substrate to both indoleamine and catecholamine system is depleted. Tetrahydrobiopterin could be that substrate. The observed effects of oestrogenic agents on tetrahydrobiopterin metabolism as well as

the changes in biopterin levels during the menstrual cycle seem to suggest that induced or normal physiological elevation in body oestrogen level contribute to depressive psychosis by impairing biopterin-cofactor metabolism in women predisposed to this condition.

5.11 Biochemical Effects of Methotrexate on Tetrahydrobiopterin Metabolism :

In the rat, an oral dose of MTX (methotrexate), 100 mg/kg, increased the brain total biopterin concentration which could be accounted for by a rise in fully oxidised biopterin and dihydrobiopterin ($p < 0.05$ Student's t-test; Table **5.7**). Actual THB level remained normal but as a percentage of the total biopterin pool was significantly reduced ($p < 0.02$, Table **5.8**).

Radiolabel studies show that 0.6% of the administered dose reached the brain. The maximal brain MTX concentration at this dose was approximately 10^{-7} M, less than the K_i value for DHPR (10^{-6} M; Saleh et al 1981).

These data suggest that inhibition of DHPR by MTX results in elevated levels of oxidised forms of biopterins with compensatory stimulation of THB synthesis and maintenance of the active cofactor levels. It is envisaged that prolonged methotrexate therapy could cause intractable damage to the brain by inhibiting THB metabolism resulting in the reported neurological symptoms. Patients recieving methotrexate therapy show elevated serum biopterin level, (Leeming and Blair 1980). This suggests that inhibition of cofactor salvage via DHPR activity results in the cofactor becoming oxidised to dihydrobiopterin and biopterin which are more able to leave the cell. In this manner, depletion of neuronal THB compromise THB-dependant neurotansmitter synthesis.

CHAPTER SIX : GENERAL DISCUSSION :

Malignant hyperphenylalaninaemia is an established condition of impaired THB metabolism with associated mental retardation (Leeming et al 1981). Brain biogenic amine synthesis could be deranged through a disturbance in the biopterin cofactor synthesis or its salvage (Leeming et al 1981). Such disturbance in THB metabolism could be prevalent in a number of neuropathological disease states. This research has tried to elucidate the causal, correlative and coincidental associations between disturbance in THB metabolism and various diseases in man.

A suitable technique for the analysis of pterins in biological samples was sought for, and the stability of the pterins under various conditions was investigated. A HPLC-fluorometric technique was used for the pterins because of the ease and sensitivity proferred by this system. Results by HPLC analysis were compared with Crithidia-assay (Table 6.1) and it was found that with the exception of one set of results (tuberose sclerosis patients), the HPLC results were always elevated compared to Crithidia values. Oxidation of tetrahydrobiopterin under various conditions showed that at room temperature (21-25 C) the loss of THB to non-fluorescent product was considerable, with greater losses at alkaline pH. THB degradation to Crithidia-insensitive substances could account for low Crithidia-assay values. The Crithidia-assay involves long incubation periods and autoclaving in an aerated environment (Baker et al 1980).

In the presence of acid-iodine, THB and dihydrobioipterin (DHB) were almost totally converted to biopterin. Under alkaline-iodine oxidation THB was 80% converted to pterin, and the rest to biopterin.

TABLE 6.1 : COMPARISON OF RESULTS OBTAINED BY HPLC AND BY CRITHIDIA FASCICULATA ASSAY :

Urine analysis

Clinical Status	Biopterin level * by HPLC (ng/ml)	Crithidia Active factor levels ng/ml.
Healthy adults (13)	1470 ± 86	820 ± 31
Affective disorder subjects. (19)	800 ± 28	510 ± 138
Adult PKU (6)	1580 ± 36	1430 ± 33
Tuberose sclerosis patients (6)	890 ± 82	1360 ± 94

* after acid iodine oxidation.

results as mean ± SEM

figures in parenthesis refer to number of subjects studied.

Dihydrobiopterin was totally converted to biopterin under these conditions. Hence from analysis of biopterin in neat sample, acid-iodine oxidised sample and alkaline-iodine oxidised sample, one would deduce the amount of THB, and DHB present.

Further oxidative studies showed that THB was unstable in the presence of ascorbate. It was suggested that the presence of trace-metal ions in solution could lead to auto-oxidative reactions in which ascorbate oxidation could lead to free-radical generation with concomittant oxidation of THB (Table **6.2**).

Free-radical mediated oxidation experiments showed that THB oxidation in the presence of Xanthine - xanthine oxidase was rapid and was suppressed by the presence of superoxide dismutase (Table **6.3.**). However, even in the absence of Xanthine - xanthine oxidase, THB oxidation was rapid suggesting an auto-oxidative mechanism of THB degradation. Blair and Pearson (1974) suggest the generation of free-radicals during atmospheric oxidation of THB and such a mechanism could operate here.

The oxidation experiments indicated the labile nature of the reduced pterins. However, the oxidation under acid- and alkaline-iodine conditions was found to be relatively straightforward and afforded a means of analysing biological samples. The pterins so oxidised were then detected by high performance liquid chromatography.

Many neuropathological conditions leading to altered behavioural states may involve an impairment of biogenic amine systems due to a disturbance of tetrahydrobiopterin metabolism. This study puts

TABLE 6.2 : THB OXIDATION IN PHOSPHATE BUFFER (0.05M) AT 37°C, pH7.4.

	% THB recovered after 10 min.	% Biopterin + pterin recovered
THB (0.1 m M)	13.2	63
THB + Ascorbate (100 m M)	0	0.3
THB + Fe^{2+} (0.1 m M)	0	0.9
THB + Fe^{2+} + Ascorbate	0	0.2

TABLE 6.3 : THE EFFECT OF SUPEROXIDE DISMUTASE ON FREE-RADICAL OXIDATION OF THB.

System.	% THB	[Biopterin]* nmol./ml	[Pterin]* nmol./ml.
THB	13.8	3.9	2.3
THB + Xanthine	12.8	2.5	3.2
THB + Xanthine + Xanthine Oxidase.	13.1	3.3	3.4
THB + Xanthine + Xanthine Oxidase + SOD	66.5	6.1	1.9

* after acid-iodine oxidation

conditions : potassium phosphate luffer (0.1M) pH 7.8 at 25 C incubation for 20 min.

TABLE 6.4 : BIOPTERIN-COFACTOR METABOLISM IN THE TEMPORAL LOBE (BRODMANN AREA 21) OF CONTROL SUBJECTS AND PATIENTS WITH SDAT, AFTER AUTOPSY.

	Normal controls	SDAT subjects
Synthesis activity ng. biopterin./mg. protein/h.	0.61 ± 0.50 (8)	0.07 ± 0.15(4)
DHPR activity nmol. NADH/mg. protein/min.	197 ± 65 (7)	369 ± 68 (4)
Total biopterin (ng/ml)	9.4 ± 7.1 (7)	1.7 ± 1.2 (4)
Total neopterin (ng/ml)	6.3 ± 6.1 (7)	11.0 ± 12.5(4)
Neopterin/biopterin	0.67	6.47

mean ± sem

TABLE 6.5 : NEOPTERIN AND BIOPTERIN LEVELS IN BODY FLUIDS IN VARIOUS DISEASE STATES :

Condition	Pterin level in Disease	Pterin level in healthy control
Down's syndrome :		
plasma neopterin (pmol./ml) :	0.49 ± 0.37 (32)	9.05 ± 1.71 (21)
plasma biopterin (pmol./ml)	21.86 ± 4.66 (32)	13.26 ± 2.39 (21)
Acute lymphoblastic leukaemia :		
csf neopterin : pmol /ml	29.18 ± 3.93 (22)	
csf biopterin : pmol /ml	42.71 ± 8.96 (22)	
Tuberose Sclerosis.		
Urine neopterin :	0.33 ± 0.13 (6)	0.34 ± 0.04 (11)
Urine biopterin :	0.60 ± 0.15 (6)	0.56 ± 0.07 (11)
Fragile X Syndrome :		
Urine neopterin	0.50 ± 0.22 (6)	as above
Urine biopterin	0.50 ± 0.09 (6)	"
Affective Disorder (bipolar)		
Urine neopterin	0.18 ± 0.04 (17)	"
Urine biopterin	0.28 ± 0.08 (17)	"
Classic PKU		
Urine neopterin	0.75 ± 0.11 (6)	"
Urine biopterin	1.62 ± 0.66 (6)	"

Urine values as μ mol./mmol. creatinine ± SEM.

TABLE 6.6 : BRAIN BIOPTERIN METABOLISM FOLLOWING ACUTE ADMINISTRATION OF VARIOUS XENOBIOTICS.

Xenobiotic administered	Experimental Animals		Control Animals	
	Total Biopterin	% THB	Total Biopterin	% THB
Pb^{2+} (0.3mg/kg)	219 ± 28	82 ± 4 (6)	227 ± 13	98 ± 6 (12)
Al^{3+} (40mg/kg)	308 ± 14	87 ± 5 (6)	227 ± 13	98 ± 6 (12)
DES (500mg/kg)	153 ± 22	95 ± 1 (6)	278 ± 25	96 ± 1.5 (6)
MTX (100mg/kg)	250 ± 31	84 ± 4 (15)	228 ± 13	95 ± 1 (12)

total biopterin as pmol./g/wet wt.

DES = diethylstiboestrol

MTX = methotrexate.

figures in parenthesis are number of animals studied.

forward some plausible arguements as to how THB metabolism may be deranged in some disease states and conditions arising from environmental exposure to various pollutants. However, one has to bear in mind the limitations of the various findings. Ideally, a neuropathological change should be investigated by directly observing changes in the brain. Such studies at post-mortem have proved invaluable in the investigation of conditions such as Alzheimer's disease and Parkinson's disease (Curzon 1972) but they have their limitations. Many neurotransmitter and some enzyme concentrations change rapidly after death (Curzon 1972). For example, serotonin levels tend to decrease with time following autopsy (Dawson, 1969). The cause of death can also influence results, for example, oxygen deficit may alter enzyme activity as a consequence of a subject dying from bronchopneumonia (Curzon 1972). The patient may further be on a regimen of drugs and this will further influence the biochemical changes, giving rise to further discrepancies. Despite such limitations the brain tissue is desirable for neuropathological investigations as it gives direct insight into the integrity of the various neuronal systems. However, the limited availability of brain tissue meant that the next most suitable alternative, the cerebrospinal fluid, was also used.

An important assumption has to be made that the cerebrospinal fluid (csf) concentrations of pterin and neurotransmitter metabolites reflect changes in the brain. They may be due to changes in the rate of egress of these compounds from the brain to csf or csf to blood, in which case the conclusion reached may not be true. Furthermore, csf sampling is usually by lumbar-puncture and it is well known that there is a gradient of metabolism down the spinal cord with the ventricles

showing the highest levels (Gerbode and Bowers 1968). Hence neuronal metabolic changes may not be reflected in the csf. However, in Parkinson's disease where a known defect in neuronal dopamine metabolism exists, low concentrations of homovanillic acid and 5-hydroxyindoleacetic acid are found in the csf (Gottfries et al 1969).

Blood and urine were the most readily available samples and therefore the most studied. Such samples do not always reflect the disturbances in the central nervous system as the brain may only contribute a minor percentage of the neurometabolite to these fluids. Despite this, the blood is a good indicator of diseases such as hyperphenylalaninaemia and routine screening of urinary neopterin has been suggested as a means of monitoring patients with acute lymphoblastic leukaemia on therapy (Kostron-Krainz et al 1982).

Tetrahydrobiopterin metabolism appears to be affected in a number of diseases, the most intensively studied of which is the condition of hyperphenylalaninaemia. In phenylketonuria it is still unknown how the resulting accumulation of phenylalanine can result in reduced intelligence. However, it is well known that the damaging effect of the toxic agent can be prevented to some extent by the earlier restriction of phenylalanine intake. A number of neurotransmitter deficits have been implicated in producing the observed mental retardation. For example, a depletion of central 5-hydroxytryptamine has been reported but such a depletion in experimental animals does not produce a state resembling phenylketonuria (Sandler 1982). An interplay of a number of toxic metabolites over a period of time is suggested to give rise to the mental disorder. For example, certain metabolites of phenylalanine, particularly phenylacetic acid inhibit

the enzyme 5-hydroxytryptophan decarboxylase and experimentally can produce a resultant decrease in urinary 5-hydroxytryptamine output in animals (Sandler 1982).

McKean (1972) has investigated the necropsied brain tissue of untreated PKU patients and found serotonin, dopamine and noradrenaline to be 30-40% of normal values. The amino acids tyrosine and tryptophan were reduced by 40-50% in the cortex and this is an important difference compared to animal models of hyperphenylalaninaemia where the tyrosine level is elevated following treatment with phenylalanine plus a phenylalanine-hydroxylase inhibitor (Taylor et al 1983). Hence the observations in the experimental model of biogenic amine depletion are not due to lack of precursor availability unlike the clinical condition (Taylor et al 1983). The investigations by McKean (1972) support the idea that elevated phenylalanine level leads to a competitive inhibition of influx of other amino acids such as tyrosine and tryptophan which share a common carrier mechanism across the blood-brain barrier (Pratt 1982). The competitive inhibition of tryptophan uptake by phenylalanine has also been demonstrated at the neuronal level and such an effect could decrease the rate of serotonin synthesis (Grahame-Smith and Parfitt 1970).

Although McKean (1972) observed a reduction in brain catecholamine levels, associated with a comparable decrease in the amino acid precursor tyrosine, the lowest concentration of latter encountered in PKU brains was only 1.2×10^{-4} M which is well above the Km of tyrosine hydroxylase (Km = 5×10^{-5} M), McKean 1972. This suggests that precursor availability may not play such a prominent role in impairing neurotransmitter synthesis.

A number of investigations show that phenylalanine and its metabolites are toxic to the brain. Phenylalanine has a high affinity for tyrosine hydroxylase (K_1 = 1.7 x 10^{-5} M), McKean 1972, and inhibits conversion of tyrosine to DOPA. Phenylethylamine an analoge of amphetamine may produce psychotic effects in PKU (Sandler 1982). Phenylacetic acid produces effects resembling alcohol intoxication and phenylpyruvate condenses with monoamines such as dopamine to produce pharmacologically active alkaloids (Sandler 1982). The resultant product with dopamine inhibits dopamine- -hydroxylase. The present study also suggests that phenylalanine metabolites could play a major role in the pathogenesis of PKU. Phenylalanine loading produced a fall in percentage THB in the brain whilst administration of p-chlorophenylalanine (PCPA) plus phenylalanine had no effect. Since inhibition of the hydroxylase by PCPA had no effect on percent THB it is more likely that with phenylalanine administered alone it is a metabolite of this amino acid that produces its toxic effect. Purdy et al (1980) have shown that phenylalanine and its metabolites are inhibitory to the enzyme dihydropteridine reductase. In hyperphenylalaninaemia, an enhanced conversion of phenylalanine to products other then tyrosine could generate toxic metabolities. The biogenic amine deficits are even greater in the atypical hyperphenylalaninaemia which are associated with a THB deficit (Sandler 1982). Hence decrease in percent THB is an important factor in the aetiology of hyperphenylalaninaemia.

Another disorder with a reported disturbance in tetrahydrobiopterin metabolism (Aziz et al 1982, Morar et al 1983, Barford et al 1984) is senile dementia of Alzheimer-type where a profound memory deficit exists. It is suggested that memory storage involves, in part, some

changes of the cholinergic system (e.g. synaptic transmission efficacy) based on observation of drugs such as physostigmine (an anticholinesterase) which facilitates learning in adult rats and scopolamine (an anticholinergic drug) which impairs memory (Squire and Davies, 1981). The involvement of the cholinergic system in senile dementia and Alzheimer's disease is well supported by observations of drastic reductions in cholineacetyltransferase and acetylcholinesterase activities in the cortex of these patients (Squire and Davies 1984). However, administration of physostigmine and/or lecithin (a source of choline) have produced inconsistent results (Peters and Levine 1979). In a study of five patients with Alzheimer's disease receiving physostigmine or lecithin or both it was observed that three patients improved intellectual function when both drugs were administered whilst two patients showed impaired performance when the drugs were given alone (Peters and Levine, 1979). In another study oral choline improved memory test performance to a small extent in three patients identified as exhibiting early-stage Alzheimer's disease, but did not affect the performance of patients with more advanced stages of the disease (Signoret et al 1978). Such reports suggest senile dementia to be more complex and involving other neurotransmitter systems as well.

Pharmacological experiments suggest that catecholamines may also play some role in memory processes. For example, the central administration of noradrenaline, dopamine or isoprenaline (a catecholamine agonist), improve retention of memory (Squire and Davies 1981). The intracerebral injection of catecholamine antagonists propanolol or alprenolol impairs acquisition and retention of memory (Squire and Davies 1981). Newman et al (1984) have subdivided memory

into various components including short-term "episodic" memory (which has an effort requiring component) and long term "semantic" memory. Cholinergic agonists are suggested to facilitate learning and memory by having a generalised effect on intellect whilst serotonergic systems facilitate learning and memory by amplifying weak memory traces (Newman et al 1984). In normal adults effortful-learning was improved by administration of levodopa (Newman et al 1984). Hence catecholaminergic systems are involved in specific types of memory processes.

The catecholamine system is impaired in senile dementia (Mann 1983). Senile dementia patients have a disorder of THB metabolism (Morar et al 1983, Barford et al 1984). Studies on temporal cortex taken from patients dying with SDAT (Barford et al 1984) showed a very much reduced capacity to synthesise THB (Table **6.4**). The DHPR activity in the SDAT brains was significantly elevated. This may be a homeostatic response to the low synthesis activity. However, the brain total biopterin level was found to be low in the dements and the neopterin to biopterin ratio was elevated. This suggests that there is a blockage at the neopterin-triphosphate stage. The low brain biopterin level is also reflected in the csf (Morar et al 1983). The addition of 5-methyltetrahydrofolate to the incubation media, containing demented brain tissue, significantly increased THB synthesis suggesting that an underlying folate deficiency may exist in these patients (Barford et al 1984). Such results also suggest that these patients have the capacity to synthesise the THB cofactor.

The mental retardation of Down's syndrome may also be due to an underlying THB disorder. Aziz et al (1984) observed diminished serum THB level and an elevated serum dihydrobiopterin level. They attributed these findings to increased oxidation of cell tetrahydrobiopterin. Blair and Pearson (1974) suggest a free-radical mechanism of oxidation of THB. If THB oxidation yields free radicals in vivo then oxidative cell damage would occur due to inactivation of enzymes, DNA damage and autolysis. Brooksbank and Balazs (1983) have observed enhanced lipoperoxidation in Down's syndrome foetal brains. However, there was also an elevated superoxide dismutase activity. The present study tends to show that a disorder of THB synthesis occurs in Down's patients as shown by the significant reduction in plasma neopterin output in adult subjects. An earlier damage or congenital abnormality of the THB system could give rise to the observed results.

A disorder of THB metabolism was also noted in affective psychoses patients. Bipolar depressives showed reduced urinary biopterin output compared with controls and post-mortem brain analysis of patients with severe depression showed diminished THB synthesis (Blair et al 1984). The primary cause of this disturbance could be a nutritional deficiency, in particular one of vitamin deficiency. Coppen et al (1982) have shown that endogenous depression ameliorates as serum folate rises. Also, synthesis is stimulated in rat brain preparations by 5-methyltetrahydrofolate and vitamin B_{12}. However, only tentative inferences as to the cause of this imbalance can be made as subjects with endogenous depression also exhibit a hormonal imbalance. For example, there is hypersecretion of cortisol in depression (Sacchar 1982) and its analogue cortisone has been shown to

reduce urinary neopterin excretion in children with acute lymphoblastic leukaemia (Kostron-Krainz et al 1982).

Children with acute lymphoblastic leukaemia on methotrexate therapy exhibited elevated csf biopterin level compared to results by Williams et al 1980. The results agree with those by Kostron-Krainz et al 1982 and appear to be due to lymphoblastic-cell proliferation (Kostron-Krainz et al 1982). The implications of these findings on the neurological status of these subjects is uncertain.

A number of other diseases which have a neurological component show altered biopterin metabolism. For example, Parkinson's disease, Steele-Richardson syndrome, Huntington's chorea and dystonia (Leeming et al 1981). Hence disorders of biopterin metabolism are associated with many neuropathological conditions.

There is a growing awareness of the potential detrimental affects to health of environmental agents such as lead and aluminium. For example, the Royal Commission on Environmental Health constantly submits reports to the British Government on occupational and environmental health hazards. This research has investigated a few of the xenobiotics which affect tetrahydrobiopterin metabolism.

Acute lead administration, orally, was found to affect brain biopterin pool. Radiotracer studies indicated that labelled lead was able to reach the brain. The total brain biopterin was not found to be affected (except at 10^{-5} M concentration). However the percentage THB was found to be reduced at all concentrations of lead studied. This would be expected as lead ions in vitro have been shown to inhibit

the enzyme dihydropteridine reductase. The reduced cofactor levels could impair intellectual function. The possible effects of lead on IQ are discussed by Blair et al (1982).

High concentrations of aluminium also produced a fall in the active cofactor level whilst increasing the total biopterin pool (Table **6.6**). Brown 1981 showed that DHPR activity was inhibited by aluminium in vitro. Aluminium toxicity has been implicated in dialysis encephalopathy, Alzheimer's disease and aluminium hydroxide gel neuropathy. The pathological mechanism underlying the neurotoxic effect of this metal is uncertain. Like lead, aluminium has a number of other effects. For example, aluminium has been shown to bind calmodulin to produce a helix-coil structure and thereby alter its biological property (Siegel and Hang 1983). This is reminiscent of the neurofibrillary tangle effect observed in feline brain by Crapper et al 1976. Hence aluminium could induce conformational change in proteins and thereby have a profound effect on their biological properties. Certainly, the high charge and small radius of this ion makes it an avid chelating agent (Albert 1973). This could explain the basis of aluminium toxicity.

Perinatal depression (Dalton 1971) and concraceptive-induced chorea (Sale et al 1981) could be attributed to a resultant imbalance of tetrahydrobiopterin metabolism, following elevated body oestrogen levels. Studies in women have shown that serum biopterin derivatives vary with hormonal states. Biopterin concentrations fall when oestrogen level rises throughout the menstrual cycle, when taking the contraceptive 'pill' and during pregnancy (Leeming and Blair 1980, Barford et al 1983). Such fluctuations in hormonal levels are often

accompanied by affective psychological changes (Dalton 1971) and may be due to the reduced THB level impairing catecholamine synthesis. The present animal study supports this arguement. Orally administered diethystilboestrol drastically reduced the total brain biopterin pool without altering the per cent THB level, (Table **6.6**). Brain biopterin-synthesis was found to be depressed. If this action of DES reflects an oestrogenic effect then women would be constantly susceptible to fluctuations in their brain biopterin level which could produce changes in personality and mood.

The antifolate drug, methotrexate was also investigated for its effects on THB metabolism in the rat brain. This drug has been found to inhibit DHPR activity in vitro (Craine et al 1972) and patients recieving methotrexate have markedly increased serum biopterin concentrations (Leeming and Blair 1980). The present study shows that the brain cofactor levels are reduced when methotrexate is administered orally. Sufficient methotrexate was able to reach the brain to make this feasible by inhibition of DHPR activity. Hence the neurological effects observed using high-dose methotrexate treatment of neoplastic conditions (Weiss et al 1974) may involve impaired THB metabolism.

In conclusion, most conditions of disturbed tetrahydrobiopterin metabolism are associated with neuropathological change and in many cases neuronal biopterin changes are reflected in altered serum and urine biopterin levels. However causal relationships are difficult to establish. In some disease states more then one neurotransmitter system may be deranged and implementation of a single neurotransmitter therapy may not be successful. This may explain the poor success in the treatment of Alzheimer's disease. Also long term structural

damage in the brain precludes any possibility of major improvements in intellectual performance in subjects with diseases such as senile dementia. In malignant hyperphenylalaninaemia, the dramatic response to neurotransmitter replacement therapy and the absence of improvement following phenylalanine restriction alone give positive evidence that the cause of this progressive neurological illness is lack of tetrahydrobiopterin. Small doses of L-dopa given to patients with Alzheimer's disease produce minimal improvement (Ferris et al 1980) but there are no reports of concurrent L-dopa, carbidopa and 5-hydroxytryptophan administration in this condition. Other neurological illnesses may also benefit from such combination therapy. Tetrahydrobiopterin therapy on an experimental basis has been implemented in patients with Parkinsonism (Narabayashi et al 1982, Curtius et al 1982), in dystonia (LeWitt et al 1983) and in depression (Curtius et al 1983) with some success. Further studies need to be undertaken for more conclusive results.

The neurological symptoms arising from tetrahydrobiopterin deficiency will vary depending upon the area of the brain or the neuronal pathway affected. The effect of environmental pollutants and other agents such as lead may have specific effects depending on the distribution of the pollutant or its metabolite in the brain.

The understanding of the involvement of tetrahydrobiopterin in psychiatic and other diseases may throw new light on the role of this ubiquitous molecule and clarify the therapeutic potential of this cofactor.

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