BIOPTERIN METABOLISM
IN HEALTH AND DISEASE

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To

My Husband, Majid

and

my son, Ali

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

A.Y. Albier

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CONTENTS

University of Aston in Birmingham

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SUMMARY

BIOPTERIN METABOLISM IN HEALTH AND DISEASE

Tetrahydrobiopterin metabolism in man has been studied in three ways. Firstly the serum concentration of biopterin derivatives was measured using a Crithidia fas iculata assay. Secondly the urine concentration of neopterin and biopterin was estimated by high performance liquid chromatography. Thirdly the urine concentration of tetrahydrobiopterin was determined by an enzymatic assay using phenylalanine hydroxylase. The results obtained have been correlated with various disease states and previous studies of tetrahydrobiopterin metabolism so as to extend our understanding of tetrahydrobiopterin metabolism.

The mean serum biopterin concentration in normal males was significantly higher than in age matched normal females. No significant changes were observed with smoking, alcohol consumption or oral contraceptive taking. Pregnant women in the 36th week of gestation had significantly lower concentrations than normal women. Pregnant women later diagnosed as having post parturition depression had significantly higher serum biopterin concentrations than pregnant women not so affected. Significantly reduced serum biopterin concentrations were found in coeliac disease, and patients with elevated serum copper. Dystonia patients had significantly increased serum biopterin concentrations. Elderly patients with dementia show a bimodal distribution of serum biopterin concentrations. Each group was statistically significantly different from the other and from normal subjects but no correlation could be found with the clinical diagnosis or psychometric rating. Serum biopterin concentrations increased significantly after oral doses of Septrin to normal subjects.

Biopterin and neopterin concentrations relative to creatinine were measured in urine by high performance liquid chromatography without any prior treatment of the urine. In normal subjects neopterin and biopterin concentrations were rather lower than some previously recorded, probably because of reduced oxidation of dihydro and tetrahydro precursors. Neopterin concentrations were significantly increased in both types of malignant hyperphenylalaninaemia and in malignant disease. Insignificant increases were found in dementia, Down syndrome and in non specific mental retardation. Biopterin concentrations were changed to a much smaller amount so that the neopterin: biopterin ratios in these conditions were greater than in normal subjects.

Neopterin concentrations increased after phenylalanine loading but decreased after oral tetrahydrobiopterin administration.

Measurement of tetrahydrobiopterin concentrations in urine using a phenylalanine hydroxylase assay gave results much higher than those obtained by Crithidia fas iculata assay, probably due to non-specific hydroxylation reactions.

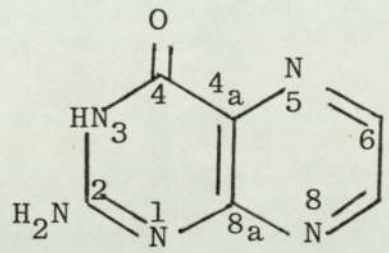
Neopterin and biopterin derivative concentrations in serum and urine are significantly altered in a number of disease states. From these observations it is suggested that tetrahydrobiopterin concentration is also significantly changed in these disease states. The similar or increased urine neopterin concentrations in dementia and non-specific mental retardation compared to normals suggests that the low serum concentrations of biopterin derivatives found in these disorders is not caused by substantial loss of functioning tissue. The changes in urine neopterin levels found in loading experiments and disease are consistent with a feed back inhibition of tetrahydrobiopterin biosynthesis in which tetrahydrobiopterin inhibits guanosine cyclohydrolase, the enzyme required for dihydroneopterin triphosphate synthesis.

KEYWORDS: BIOPTERIN NEOPTERIN
DISEASE URINE
SERUM

CHAPTER ONE

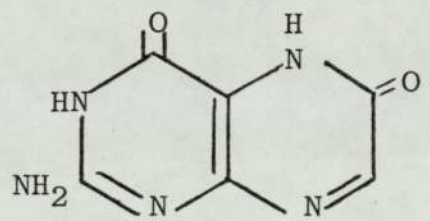
GENERAL INTRODUCTION

The term pterin was suggested for derivatives of 2-amino-4-hydroxypteridine by Pfleiderer (1964).



2-amino-4-hydroxypteridine

The first pterin to be isolated in a pure state was xanthopterin in 1925 by Wieland and Schöf, and its structure was determined in 1940 by Purrman.



xanthopterin

Much work on pterins has been concerned with their isolation and identification from butterflies, moths and other insects (Zeigler and Harmsen 1969). Others have studied pteridines from amphibia and fish (Hama 1963).

Pterins have also been isolated from human urine by Patterson et al. (1956), and are measured and identified by many methods including u.v. absorption spectrometry (Blakley 1969), and gas chromatography (Haug 1970).

Biopterin derivatives in tissues and fluids have been measured by a number of workers in different organs and different regions in the same organ. (Dewey and Kidder 1971; Baker *et al.* 1974; Leeming 1975; Kaufman *et al.* 1978; Bullard *et al* 1978). Leeming et al (1976b)measured biopterin derivatives in urine, serum, milk, cerebrospinal fluid, brain and liver by *Crithidia fasiculata* assay, This method is very useful in the study of defects of biopterin metabolism.

Another method which is sensitive and specific is gas chromatography/mass fragmentography. The pterins have to be converted to their trimethylsilyl derivatives initially to make them volatile enough for gas chromatographic separation (Rothler and Karobth 1976).

In 1972 Fukushima and Shiota reported a column chromatographic method for the separation and measurement of urinary pterins which involved the use of a weak ion exchanger, aceteola-Sephadex and phospho Sephadex.

Radioimmuno assay has also been used to determine biopterin in human urine (Nagatsu *et al* 1979) and in body fluids and tissues (Nagatsu et al 1981). In this system the recovery of biopterin and tetrahydrobiopterin (BH_4) was nearly 100%. Specific antibodies were prepared against biopterin, neopterin and 6, 7-dimethylpterin.

More recently reverse phase high performance liquid chromatography following initial iodine oxidation and separation by ion-exchange chromatography has been introduced as a method of estimating biopterin derivatives in rat tissue (Fukushima and Nixon 1980) and in human liver and urine (Stea *et al* 1980;

Stea *et al* 1981; Nixon *et al* 1980; Milstien *et al* 1980 Dhondt *et al* 1981b).

Biopterin 2-amino-4-hydroxy-6-(1,2-dihydroxy -propyl) pteridine was first isolated and identified in human urine (Patterson *et al* 1955) and most probably had its origins in the tetra- and dihydro-forms of the compound as the oxidation of tetrahydrobiopterin (BH_4) proceeds rapidly to 7,8 dihydrobiopterin and then to biopterin (Rembold *et al* 1971; Blair and Pearson 1974) (Fig. 1.1).

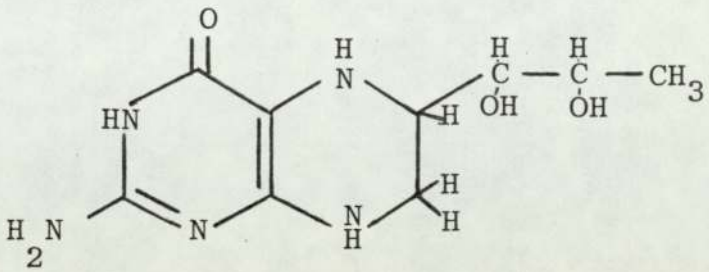
Biopterin and its biologically active reduced derivatives 7,8 dihydrobiopterin and -BH_4 differ from folic acid in the substituent at C_6 of the pteridine ring.

In (1967) Fleming and Broquist used *Crithidia fasiculata* to find out if there was a link between biopterin and folate deficiency by measuring these two with *Crithidia fasiculata* and *Lactobacillus casei*. They found there is no link between them because some of the megaloblastic patients showed high levels of Crithidia factor even when suffering from folate deficiency. Also Fukushima and Shiota (1972) showed that a large oral dose of folic acid did not affect urinary levels of biopterin derivatives.

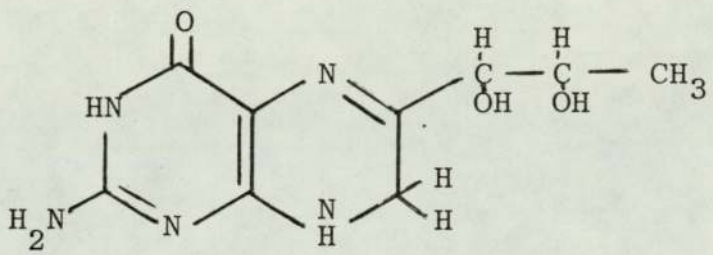
BH_4 in the cell could be derived from the diet or *denovo* synthesis (Brown 1971; Gal et al. 1978).

In 1967 Rembold and Metzger showed that radioactive biopterin administered parenterally was excreted rapidly and totally in the urine whereas BH_4 was taken up by the tissues and retained, Orally administered BH_4 was not absorbed but oral

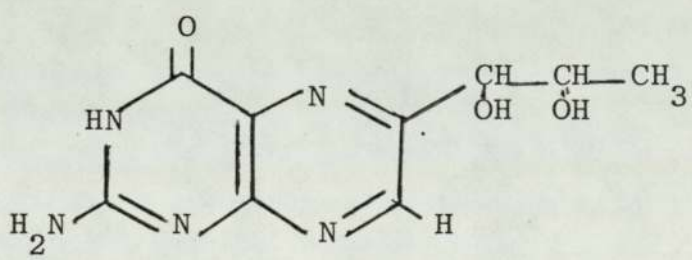
FIG. 1.1 AUTOOXIDATION OF TETRAHYDROBIOPTERIN



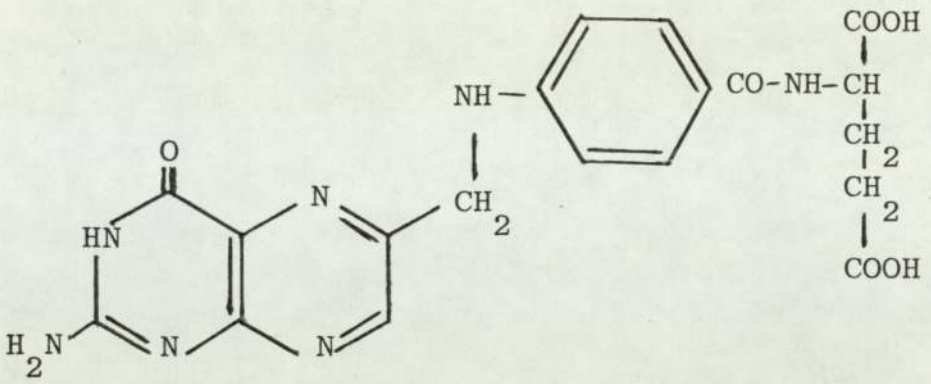
Tetrahydrobiopterin



7,8-dihydrobiopterin



Biopterin



Folic acid

biopterin was absorbed and retained by the tissues, suggesting poor absorption of BH_4 from the gut (Leeming 1975; Blair *et al* 1974).

So the diet probably cannot provide an adequate supply of BH_4. Pabstand and Rembold (1966) reported feeding several generations of rats a biopterin free diet and finding a constant biopterin level in urine of about 30 µg/day and so proved its source was endogenous.

Purines are considered to be possible starting materials for the synthesis of the pterin ring because of their structural similarities. Dalal and Gots (1965) and Brown (1981) showed that guanosine triphosphate (GTP) was the most effective substrate for pterin biosynthesis.

The synthesis of the pteridine ring from GTP by a series of reactions catalyzed by three enzymes [dihydroneopterin triphosphate synthetase, sepiapterin reductase, dihydrofolate reductase] has been demonstrated in hamster liver (Fukushima et al. 1975b) mouse lung and kidney, rat lung, hamster lung, kidney brain and liver (Fukushima *et al* 1975a) in rat brain (Gal and Sherman 1976, Lee et al. 1979).

The first step consists of the opening of the imidazole ring between C_8 and N_9 followed by removal of C_8 as a one carbon compound identified as formic acid by Shiota and Palumbo (1965). The enzyme catalysing this reaction was named GTP cyclohydrolase by Burg and Brown (1968). ^{14}C labelled GTP yields labelled D-erythro-7,8 dihydroneopterin triphosphate whereas GTP specifically labelled at C_8 yields

radioactive formic acid and unlabelled D-erythro-7,8-dihydroneopterin triphosphate (Reynolds and Brown 1964). Hence it was shown that C_8 is lost from GTP by the action of GTP cyclohydrolase.

D-erythro-7,8-dihydroneopterin triphosphate synthetase (Gal *et al* 1978) converts D-erythro-7,8-dihydroneopterin triphosphate to sepiapterin and it has been suggested that sepiapterin reductase catalyses the conversion of sepiapterin to L-erythro-7,8-dihydrobiopterin (Nagai 1968; Matsubara *et al* 1966). This reaction requires NADPH. In 1979 Lee *et al* showed that there is one hundred fold increase in biopterin synthesis when both NADPH and Mg^{++} are present. So sepiapterin may be an intermediate between dihydroneopterin and dihydrobiopterin (Eto *et al* 1976; Fukushima and Shiota (1974) (Fig. 1.2). The activity of sepiapterin reductase in erythrocytes and liver of rats is greater than in several other species (Katoh *et al* 1974).

7,8-dihydrobiopterin is reduced to BH_4 by dihydrofolate reductase (DHFR) (Abelson *et al* 1978) in the presence of NADPH, (Kaufman 1967) and this is presumed to be the final step in the biosynthetic pathway Fig. 1.3. However, the details of the biosynthesis of BH_4 remain to be fully elucidated.

Many metals show significant inhibition of the pathway from GTP especially lead and it has been suggested that lead may cause its neurotoxic effects (Rutter 1980, Needleman *et al* 1979) at low concentration by its action on this pathway. Maximum inhibition was obtained with $10^{-4}MPb^{2+}$ (Purdy *et al* 1981). Mercury, copper and cadmium are also toxic metals i.e.

Key for Figure 1.2

(I)	Guanosine triphosphate
(II)	Formylated intermediate
(III)	D-erythro-7,8-dihydroneopterin triphosphate
(IV)	Sepiapterin
(V)	7,8-dihydrobiopterin
P	Phosphate group
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide dinucleotide phosphate

Fig. 1.2

THE BIOSYNTHETIC PATHWAY OF BH_4

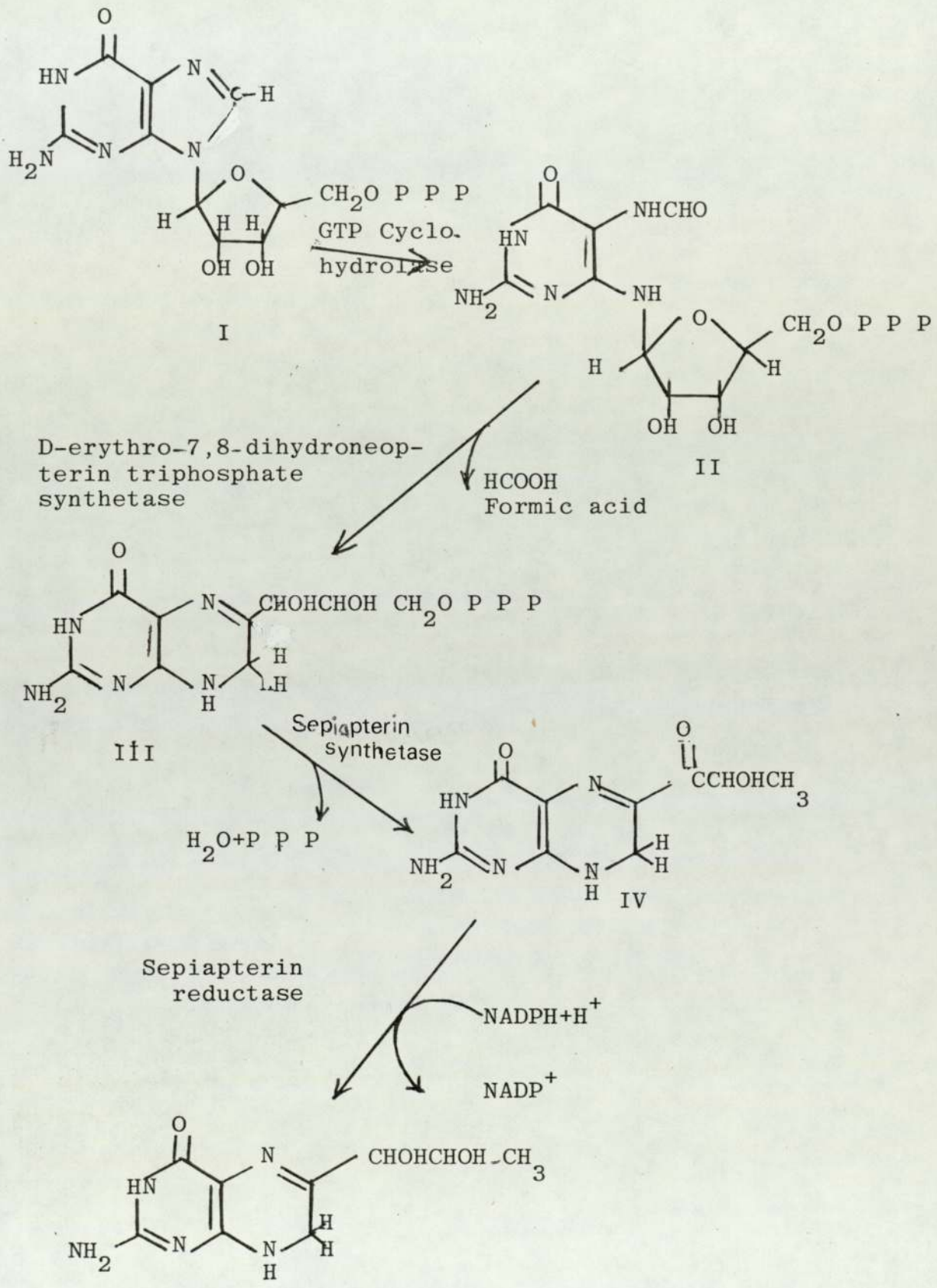
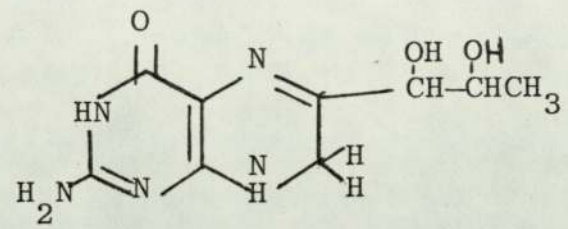
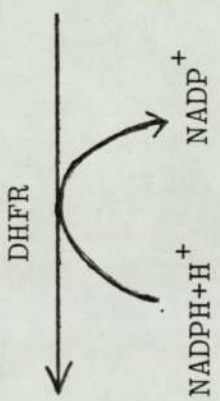


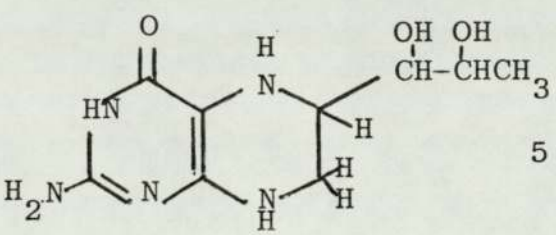
Fig. 1.3

THE FRACTION CATALYSED BY DIHYDROFOLATE REDUCTASE



7,8–dihydrobiopterin

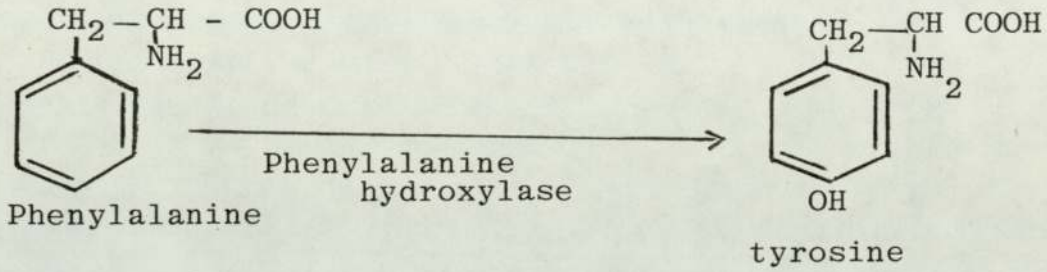




5,6,7,8–tetrahydrobiopterin

neurotoxic agents (Flink 1975) that alter the rate of BH_4 synthesis (Brown 1981).

BH_4 is a natural cofactor which acts as an electron donor for several aromatic amino acids hydroxylases e.g. hydroxylation of phenylalanine to tyrosine by phenylalanine hydroxylase (Kaufman (1963).



Tyrosine hydroxylase catalyses the conversion of tyrosine to L-DOPA and is the rate limiting step in catecholamine biosynthesis (Levitt *et al* 1965) (Fig. 1.4)

$$\text{tyrosine} + BH_4 + O_2 \rightarrow \text{L-DOPA} + \text{q-dihydrobiopterin} + H_2O$$

It has been shown that the amount of BH_4 is the rate limiting factor in this reaction (Kettler *et al* 1974). Waggoner *et al* (1980) have shown that dopamine inhibits tyrosine hydroxylase activity when the enzyme is in synaptosomes and when it had been solubilized. However, the inhibition in the synaptosomal preparation is greater than would be expected.

Tryptophan hydroxylase catalyses the conversion of tryptophan to 5-hydroxytryptophan which is the rate limiting step for the biosynthesis of another neurotransmitter, serotonin (Hosoda and Glick 1966) (Fig. 1.5). Tryptophan

Fig. 1.4

BIOSYNTEHSIS OF CATECHOLAMINES

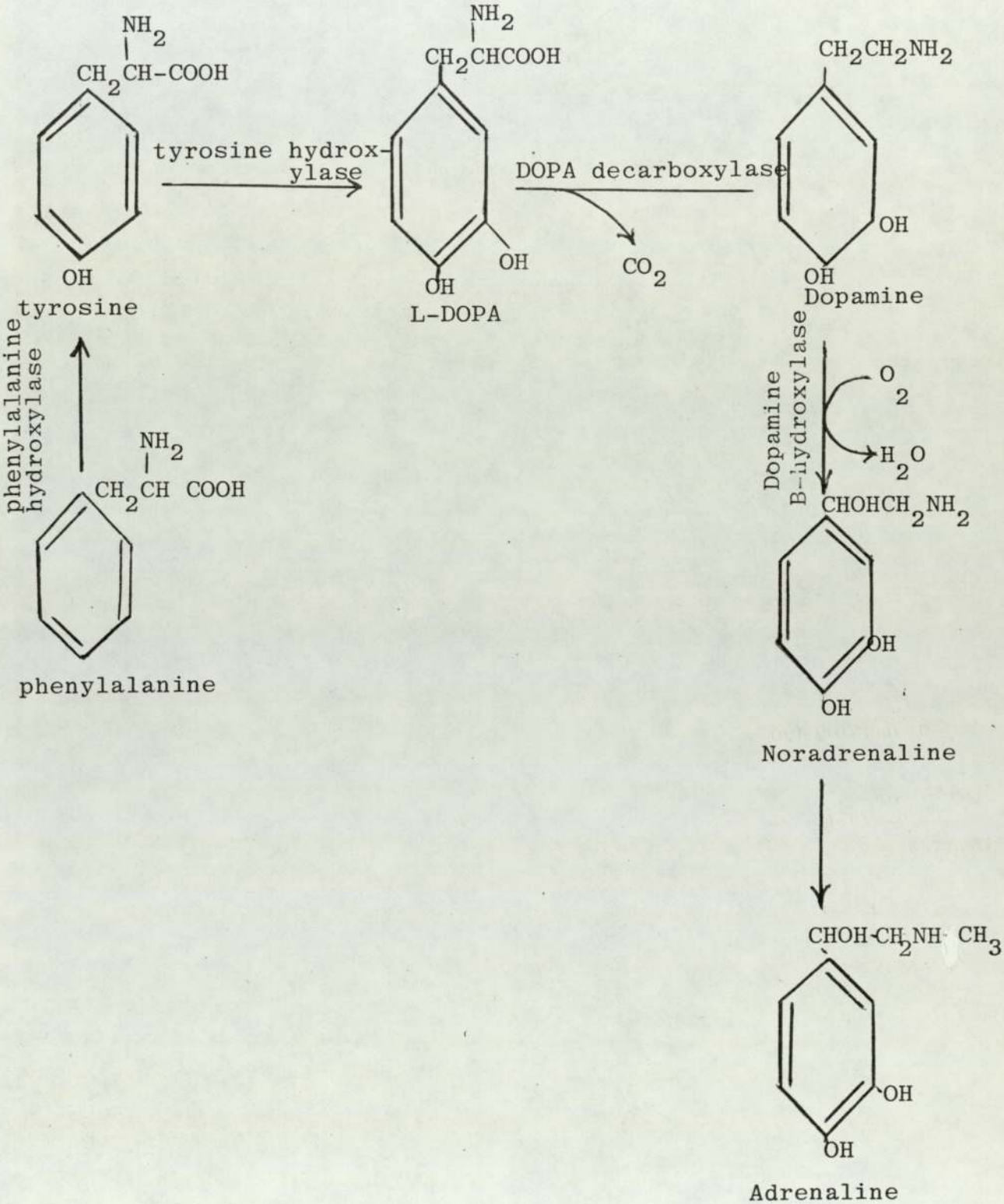
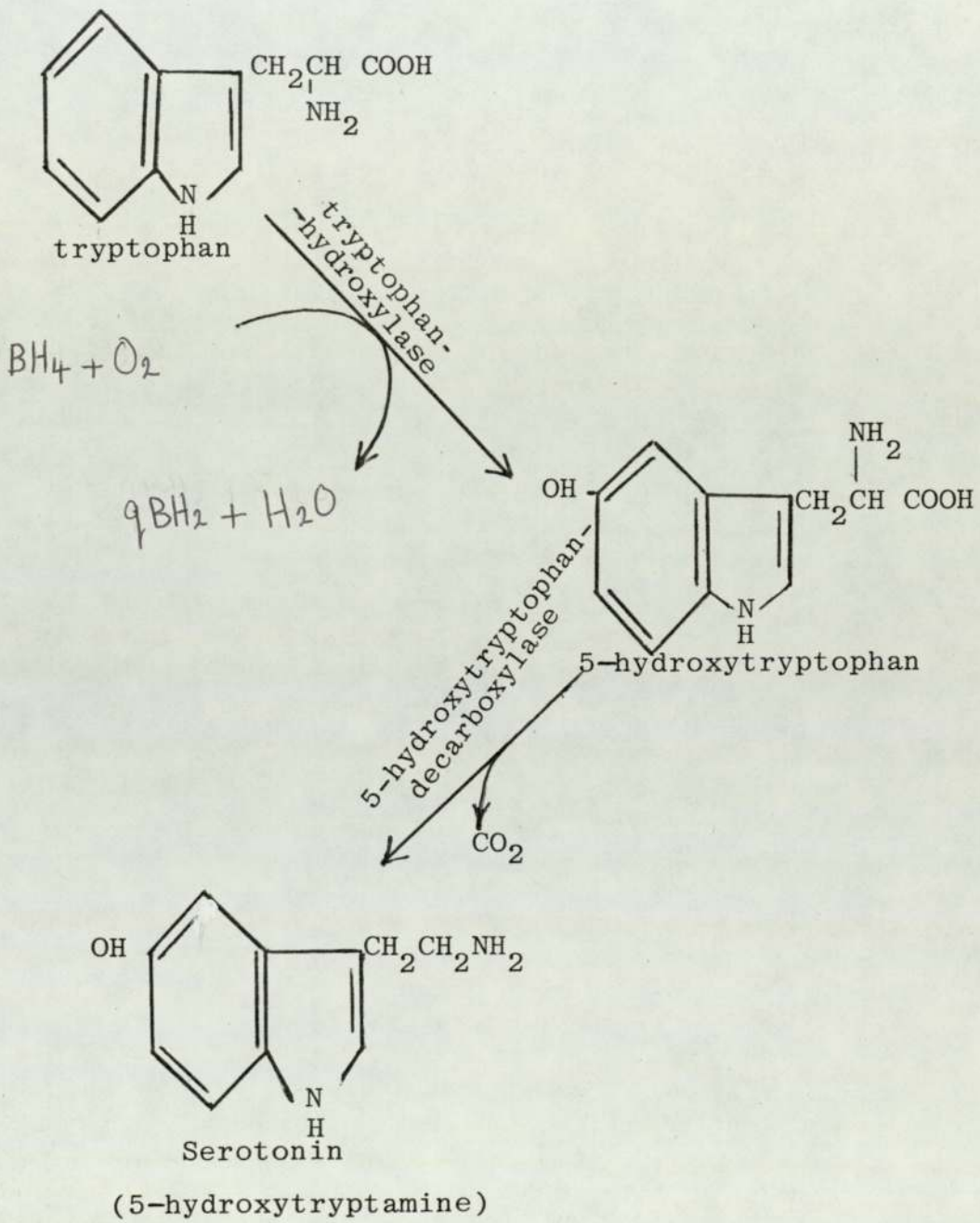


Fig. 1.5

BIOSYNTHESIS OF SEROTONIN



concentration is usually the rate limiting factor for this reaction (Costa and Meek 1974). Thus BH_4 plays an important role in neurotransmitter biosynthesis and during all these reactions is converted to quinonoid dihydrobiopterin. Therefore regeneration of BH_4 is required and this regeneration is carried out by the enzyme dihydropteridine reductase which utilizes either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine diphosphonucleotide (NADPH) but is much more active with NADH (Nielson *et al* 1969; Webber and Whiteley 1978).

Dihydropteridine reductase activity can be measured in platelets (Abelson *et al* 1979; Firgaira *et al* 1979) in lymphocytes and granulocytes which are easily obtainable from blood samples (Firgaira *et al* 1979), in cultured fibroblast and in liver biopsies (Kaufman *et al* (1974). Dihydropteridine reductase activity has been measured by Crain *et al* (1972) by measuring the disappearance of NADH spectrophotometrically when using 6,7-dimethyltetrahydrobiopterin which is commercially available as an artificial substrate.

Dihydrofolate reductase has also been isolated from rabbit and rat brain (Abelson *et al* 1978; Pollock and Kaufman 1978); The rat brain and liver enzymes have been compared and found to be similar in structure (Spector *et al* 1977).

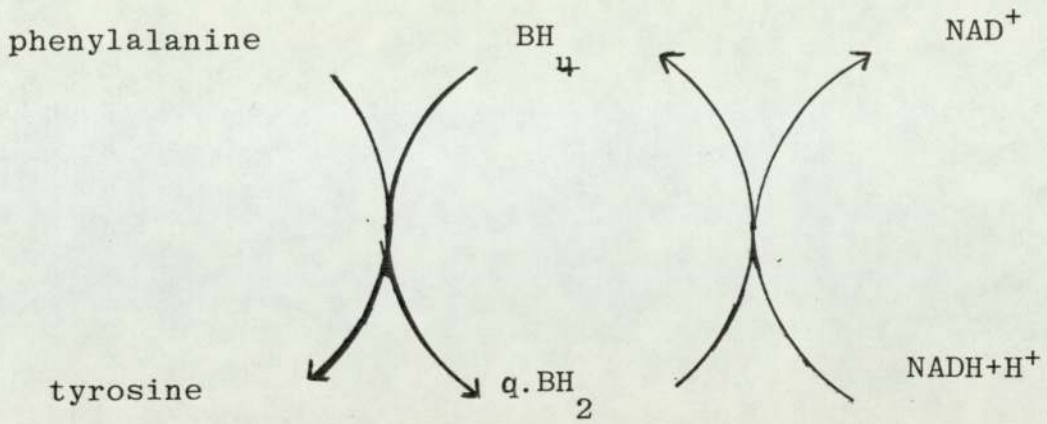
Dihydropteridine reductase has been purified from sheep liver by ammonium sulphate and zinc ethanol fractionation followed by absorption on to and elution from calcium phosphate gel and chromatography on DEAE-cellulose and Sephadex gel

filtration achieving 9% yield and 156% increase in specific activity (Craine *et al* 1972). Using acrylamide gel electrophoresis they showed that the enzyme existed as a dimer of molecular wt 41,000-42000 and it appears that the enzyme is composed of two identical subunits each mwt 21.000.

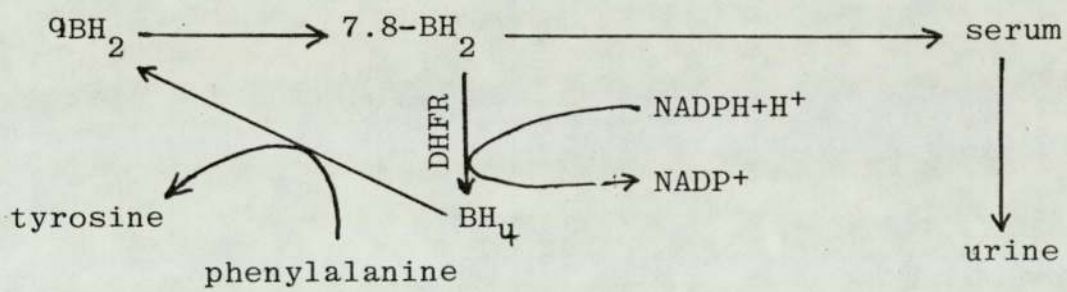
Hirayama *et al* (1980) found dihydropteridine reductase in an extract of *Crithidia fasiculata* to have a molecular weight of 55.000 by Sephadex G100 gel filteration. More recently an affinity absorbent of sodium 1,2 naphthoquinone 4-sulphonate has been used as a ligand to isolate dihydropteridine reductase from rat liver with 18% yield and 404 fold purification. This enzyme had a molecular weight 100,000 (Cotton and Jenning 1977) but was thought to consist of four subunits. It is unusual in this repsect and aggregation may have occured.

Dihydropteridine reductase also has been purified from human liver by naphthoquinone affinity absorbent, DEAE Sephadex and CM Sephadex chromatography with a yield of 25% and 1000 fold purification. This enyzme had mw 50,000 (Firgaira *et al*. 1981).

The enzymatic conversion of phenylalanine to tyrosine involves two essential enzymes, phenylalanine hydroxylase first purified from rat liver which converts phenylalanine to tyrosine (Kaufman 1964) and dihydropteridine reductase (purified from sheep liver) to convert quinonoid dihydrobiopterin (qBH_2) to BH_4 (Curtius *et al* 1979).



q-dihydrobiopterin is unstable and is not salvaged, it rapidly rearranges to 7,8-dihydrobiopterin which is not a substrate for dihydropteridine reductase.



This hydroxylation of phenylalanine requires phenylalanine hydroxylase, BH_4 and O_2. Failure of this reaction as seen in the genetic disorder phenylketonuria leads to raised serum phenylalanine levels,(serum phenylalanine ≥ 150 µmol /l (2.5mg/100ml) at day 5-7 of life by Guthrie test (Guthrie and Susie 1963).

Phenylketonuria is characterized by high levels of phenylalanine and its metabolites in blood and urine (Jervis and Drejza 1966) and reduced levels of catecholamines (Weil, Malherb and Bone 1955; Nadler and Hsia 1961; Hoeldtke 1974) and Serotonin (Pare *et al* 1957) which are accompanied by

severe mental retardation (Folling 1934).

The common form of phenylketonuria is called classical phenylketonuria and is caused by phenylalanine hydroxylase deficiency (Jervis 1947), and the incidence of this disease was calculated to be relatively high i.e. in the order of 1 in 20,000 live births.

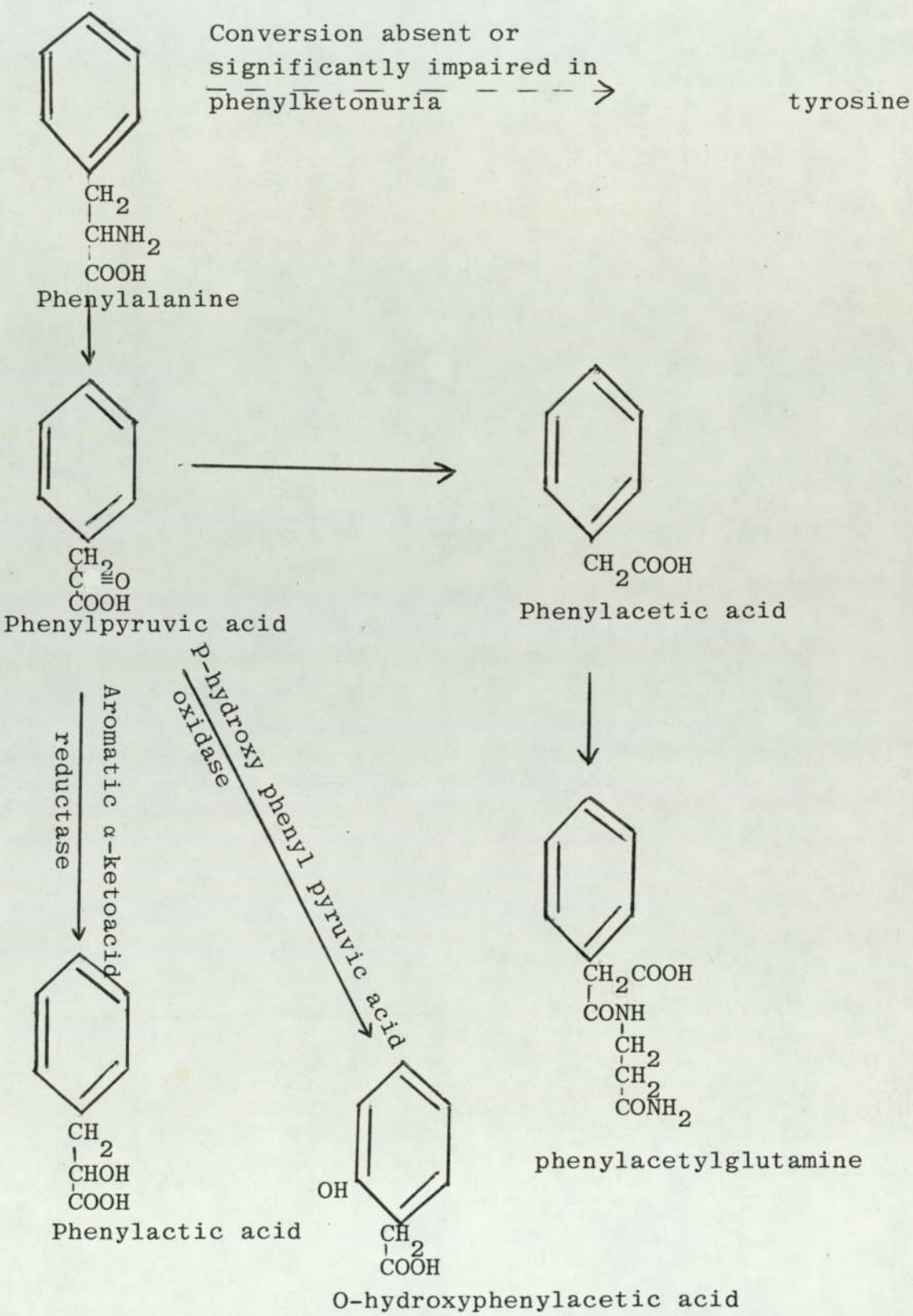
The abnormal metabolites formed in this disorder are phenylpyruvate, O-hydroxyphenylacetic acid, phenylactic acid and phenylacetylglutamine (Fig. 1.6) (Cowie 1951; Woolf and Vulliamy 1951; Partington 1978). Phenylacetic acid is responsible for the mousy odour characteristic of the urine of these patients (Centerwall *et al* 1963).

A low phenylalanine diet appears to reduce the neurological damage in phenylketonuric patients (Bickel *et al* 1953; Smith *et al* 1978). So a phenylketonuric child should be put on low phenylalanine diet as soon as possible to minimize the mental defects. However it is difficult to know how long to keep them on diet because there is evidence now that children put on a normal diet at the age of six showed a decrease in mental development compared to phenylketonurics who remained on diet (Brown and Warner 1976; Partington and Leverty 1978).

It has also been shown that phenylketonuric children placed on a normal diet between the age 5-15 years showed a lower IQ by about six points than before (Smith *et al* 1978).

Fig. 1.6

PHENYLALANINE METABOLISM IN PHENYLKETONURIA



There are many suggestions as to the cause of the mental retardation in phenylketonuric children.

One of the most likely theories is that phenylalanine and its metabolites specially phenylpyruvate inhibit dihydropteridine reductase (Purdy and Blair 1980) which will reduce BH_4 concentration in the cell. This would result in less dopamine, noradrenaline and serotonin and this has been observed in phenylketonuric patients (Wiel Malherb and Bone 1955; Nadler and Hsia 1961; Pare *et al* 1957).

It has also been suggested that high concentrations of phenylalanine and its metabolites inhibit the hydroxylation of amino acids by inhibiting the enzymes tyrosine hydroxylase and 5-hydroxytryptophan. Ikeda *et al* (1967) showed in *vitro* high concentrations of phenylalanine inhibit tyrosine hydroxylase the enzyme which convert tyrosine to dopa and also high concentrations of phenylalanine inhibit tryptophan 5-hydroxylase the enzyme which convert tryptophan to 5-hydroxytryptophan (Graham Smith and Mahoney 1965).

It has also been reported that phenylalanine metabolites (phenylpyruvate, phenylacetate and phenylactate) inhibit decarboxyation of amino acids. They inhibit Dopa decarboxylase the enzyme which catalyses the second step in catecholamine biosynthesis (Fellman 1956) and inhibit the decarboxylation of 5-hydroxytryptophan to serotonin (Davison and Sandler 1958). All this will lead to the depletion of catecholamines in the brain which is reported in phenylketonuria (Wooley and Hoeven 1964).

Another suggestion is that phenylpyruvate inhibits the transport of pyruvate into mitochondria in the brain and hence inhibits pyruvate oxidation and upsets brain function by interfering with the metabolism. The concentration of phenylpyruvate required to produce this inhibition were 2.5-5mM (Halestrap *et al* 1974).

During the last few years there have been several reports describing forms of phenylketonuria in which neurological impairment occured despite control of blood phenylalanine. These children have been shown to have abnormal biopterin metabolism: they have either dihydropteridine reductase deficiency (Kaufman *et al* 1975; Rey *et al* 1977, Grobe *et al* 1978; Milstien *et al* 1980) or they have a deficiency in the biosynthetic pathway from GTP (Leeming *et al* 1976a, Kaufman 1978, Niederwieser *et al* 1979a). Both these types of disorder are called malignant hyperphenylalaninaemia (MHPA).

Diagnosis of MHPA due to dihydropteridine reductase deficiency can be made by measurement of the activity of the enzyme in cultured fibroblasts or liver biopsy (Kaufman *et al* 1975; Firgaira *et al* 1979)

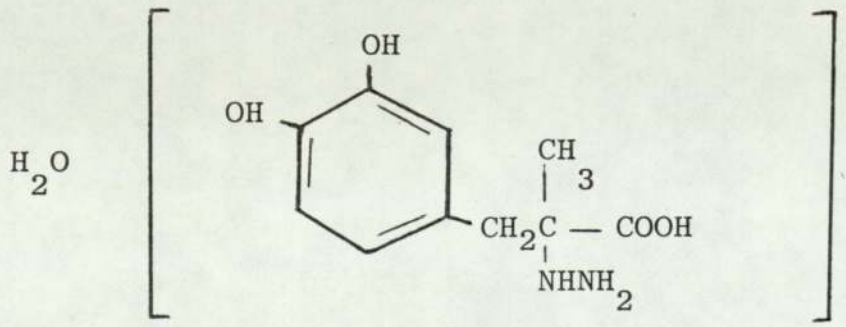
We can distinguish between MHPA due to dihydropteridine reductase deficiency and MHPA due to biopterin synthesis deficiency since children with a metabolism block in BH_4 biosynthesis have low serum levels of dihydrobiopterin whereas those with dihydropteridine reductase deficiency have higher levels than normal (Leeming and Blair 1980a; Leeming and Blair 1980b).

Deficient production of neurotransmitters from tyrosine and tryptophan is regarded as the cause of the neurological effects (Butler *et al* 1978). The treatment is low pheylalanine diet with administration of L-DOPA, carbidopa and 5-hydroxytryptophan (Bartholome and Byrd 1975; Curtius *et al* 1979; Grob *et al* 1978). Carbidopa is an essential part of the treatment because it increases the quantity of neurotransmitter precursors entering brain by inhibiting the peripheral decarboxylation. (Van Woert and Sethy 1975).

A defect in sepiapterin synthetase the enzyme which converts dihydroneopterin triphosphate to L-sepiapterin Fig. (1.7) has also been suggested as a cause of a typical phenylketonuria (Niederwieser *et al* 1979a).

In this study we are concerned to measure biopterin derivatives in serum samples from normal people and patients suffering from different diseases.

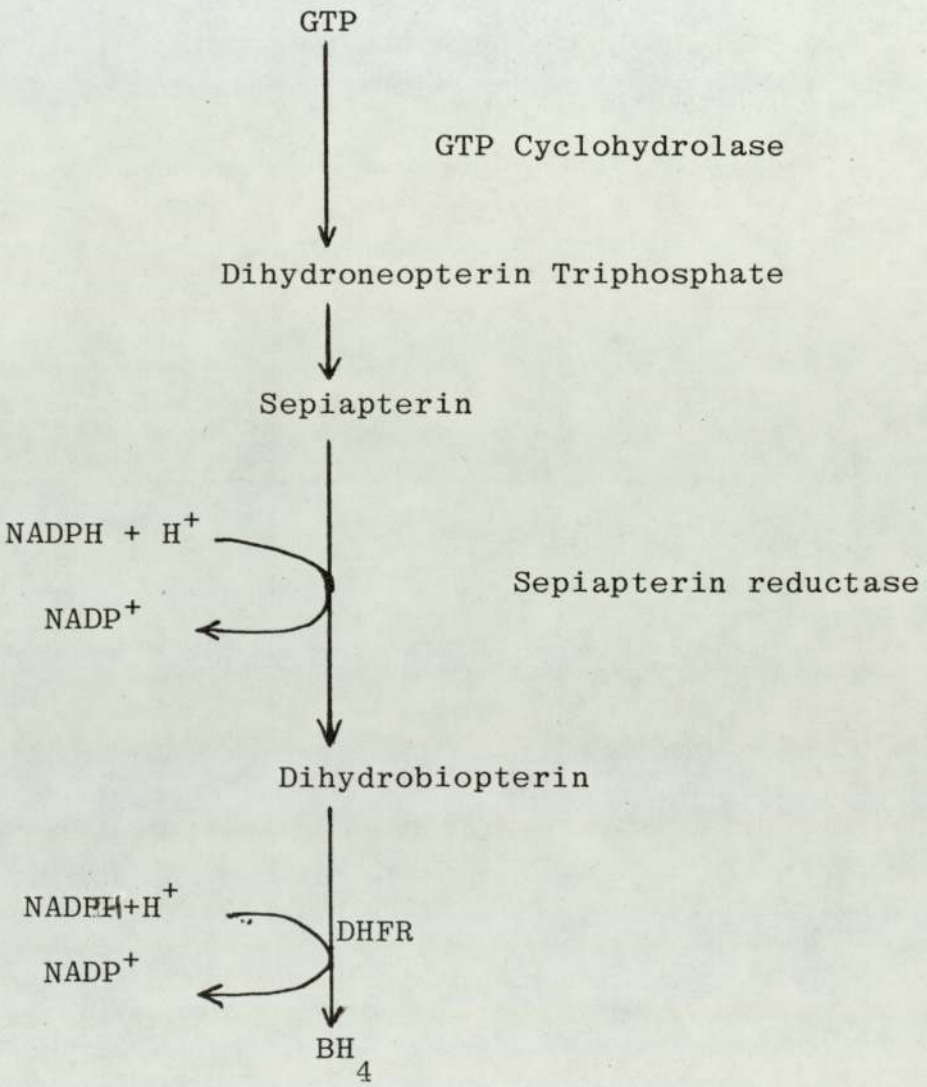
Also we tried to identify more precisely Crithidia factor in urine by radioenzymatic method in normal subjects. We also looked for precursors of BH_4 in the urine such as neopterin by using high performance liquid chromatography and compared our results with those reported before.



carbidopa

Fig. 1.7

METABOLIC PATHWAYS OF TETRAHYDROBIOPTERIN FROM GUANOSINE TRIPHOSPHATE



CHAPTER TWO

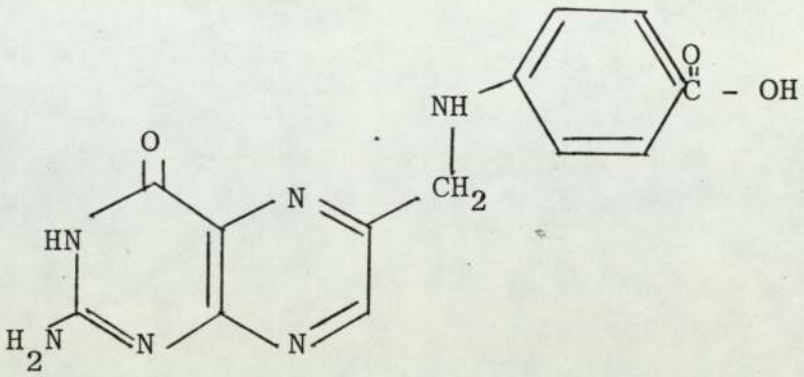
THE MEASUREMENT OF SERUM CRITHIDIA FACTOR

The first description of an organism called *Crithidia fasiculata* was presented by Leger (1902) to signify a flagellate from the gut of the mosquito. Novy *et al* (1907) described Leger's *Crithidia fasiculata* and isolated it in a pure culture, and Patterson *et al* (1955) showed that Crithidia fasiculata required biopterin for its growth.

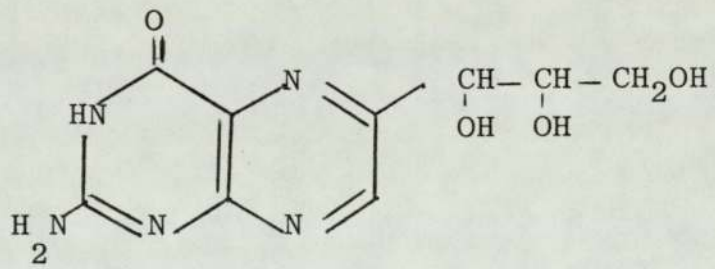
Crithidia fasiculata are monoxenous parasites ranging from 4–10 μm in length, which are very selective in their ability to utilize pteridines. Most pteridines prove unable to spare Crithidia fasiculata's need for biopterin, tetrahydrobiopterin (BH_4). 7,8 dihydrobiopterin except for L-neopterin and pteroic acid. L-neopterin is much more active than D-neopterin for Crithidia fasiculata (Leeming and Blair 1974) having 50% of the activity of biopterin.

Tyrosine and phenylalanine and metabolites of phenylalanine were unable to spare biopterin except at high concentration. So Crithidia fasiculata assay is a very selective tool for studying defects in biopterin metabolism.

Values of Crithidia factor in human body fluids and tissues have been reported (Baker *et al* 1974; Leeming and Blair 1974, Leeming *et al* 1976b; Kaufman *et al* 1978; Fukushima and Shiota 1972).



pteroic acid



L-neopterin

Tetrahydrobiopterin (BH_4) can be quantitated by its effect on the growth of the Crithidia fasiculata and an assay based on this has been used to measure biopterin derivatives in urine. It has been shown by Leeming and Blair (1974) that the principle pteridine derivatives assayed are 7,8-dihydrobiopterin and BH_4 The assay has since been modified to be applied to serum (Leeming 1975).

In this study we are concerned to study Crithidia factor (c.f.) in serum of PKU patients, pregnant women, coeliac patients, dystonia, subjects with high blood copper and geriatric patients. The effect of DHFR inhibitors on c.f of a group of normal subjects was investigated in order to determine whether BH_4 metabolism was altered. Variation of BH_4 in the cell may play an important role in disease.

Materials and Methods

Serum samples were frozen until the day of the assay at $-20^{o}C$, diluted with phosphate buffer 0.2M pH5, autoclaved at 115^{o} for 3 min to effect deproteinization and centrifuged.

Supernatant ($0.5cm^3$) was added to each of three 12x75mm assay tubes, containing distilled water ($1.5\ cm^3$). Double strength medium ($2cm^3$) was then added to each assay tube to make a total volume of $4\ cm^3$. Two standard curves were also set up in triplicate and were included with each batch of samples. The standard was prepared by addition of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1 and 1.15ng of biopterin in $0.5cm^3$ of distilled water and $2.0cm^3$ double strength medium as for the serum. All the assay tubes were then auto-

claved at 115° for 5 min using a steam pressure autoclave and inoculated aseptically.

The samples and standard were incubated at 29°C in the dark for four days and the growth of Crithidia fasiculata in each tube measured turbidometrically using a Gilford micro-sample spectrophotometer 300. This instrument was equipped with sample changer and chart recorder for automatic read-out of growth as described by Leeming and Portman-Graham (1973). All the assays were read as the absorption at 590nm against uninoculated medium. The amount of biopterin in each sample was calculated (ng biopterin/cm^3) using the standard curve. The culture medium for *Crithidia fasiculata* was that described by Guttman and Wallace (1964) with the addition of 1% casamino acidsfrom (DIFCO Laboratories Ltd.) (Iwai 1970). Experiments showed that casamino acidsproduces a considerable increase in the maximum growth and that it was not necessary to pretreat with charcoal. The medium was prepared without vitamins, haemin , and triethanolamine, steamed for 20min. and stored in screw capped bottles at 4°C. The vitamins were prepared as a dry mixture with the exception of folic acid which was added from stock kept for folic acid assay.

The addition of buffered biological material or standards produced a final of pH 6.5. This optimum pH value was also arrived at independently by Baker *et al* (1974). Urine samples were frozen alone or with the addition of ascorbate to a final concentration 2% w/v until the day of the assay. Five ten fold dilutions were made of urine in distilled water from $1/_{10^2}$ - $1/_{10^6}$. Cerebrospinal fluid (CSF) samples were diluted as serum but not autoclaved. Biopterin was a gift from Roche

Products Ltd. Stock culture of *Crithidia fasiculata* American type culture was obtained from Dr. Hunter of Hoskins Laboratory, New York, U.S.A. This was subcultured every week into maintenance medium, incubated for two days at 29°C in the dark and placed in the refrigerator.

The inoculum was prepared by taking one drop of two day culture in maintenance medium (Leeming and Blair 1974) and adding to it 15 ml single strength medium, incubating for four days to exhaust endogenous growth factor and diluting 1:100 in single strength medium. One drop was added to each tube of the assay. Ampicillin (25 mg/ml) added to this inoculum assisted in preventing bacterial contamination.

Paper chromatography was performed to identify the pterin derivatives by using 3% ammonium chloride as solvent.

The determination of phenylalanine and tyrosine was carried out in Biology Department, University of Aston in Birmingham using a Locarte amino acid analyzer (ion exchange chromatography). The amino acids are eluted from the column resin by series of buffers of increasing pH and then detected by ninhydrin. The separation depends on the adsorption of amino acids to the resin material as well ion exchange properties. Amino acids eluted are collected in an automatic fraction collector, individual amino acids are quantitated by reaction with ninhydrin and measuring the colour intensity.

PREPARATION OF MAINTENANCE MEDIUM

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

The pH was adjusted to between 6.8-7.6, the medium was autoclaved at $120^{o}C$ for 15 min. and stored in a refrigerator at $4^{o}C$.

PREPARATION OF STOCK ASSAY MEDIUM

PART A

L-arginine hydrochloride	5.0g
L-glutamic acid	10.0g
L-histidine hydrochloride	3.0g
DL-isoleucine	1.0g
D-leucine	1.0g
L-lysine hydrochloride	4.0g
DL-methionine	1.0g
DL-phenylalanine	0.6g
DL-tryptophan	0.8g
L-tyrosine	0.6g
DL-valine	0.5g
Ethylene-diamine-tetra-acetic acid	6.0g
Boric acid (H_3BO_3)	0.005g
Calcium chloride	0.005g
Cobalt sulphate	0.025g
Copper sulphate	0.025g
Ferric ammonium sulphate	0.01g
Manganese sulphate	1.4g
Manganese sulphate	6.5g
Tri-potassium phosphate	1.5g
Zinc sulphate	0.5g
Sucrose	150.0g
Distilled water	1000cm^3

The mixture was steamed at 100^{o}C for 20 minutes to dissolve the constituents and then distributed into sterile containers and stored in the refrigerator at 4^{o}C in the dark for upto 3 months.

PART B

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

These vitamins were ground together and stored dry in the refrigerator at $4^{o}C$.

PART C

Haemin $5mg/cm^3$ in 50% triethanolamine this was prepared freshly as required

PART D

Folic acid ng/cm^3. This was prepared freshly as required.

PREPARATION OF DOUBLE STRENGTH ASSAY MEDIUM

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

This was adjusted to pH 7.5 with concentrated sulphuric acid.

Preparation of Samples

(1) Normal serum samples were taken from a total of 168 members of hospital staff and university students aged between 15-67 years; there were 44 males and 124 females. Sex, age and alcohol consumption in previous 24 hours were recorded. Women were asked for the starting date of their last monthly period and whether they were on the contraceptive pill; 33 women were taking contraceptives and 90 were not.

(2) High blood copper 9 Specimens were obtained from people with high blood level of copper; three of them were diagnosed to be suffering from Hodgkins disease. Whole blood copper and caeruloplasmin were given for each of them as shown in Table 2.1).

TABLE 2.1 PATIENTS WITH HIGH Cu IN BLOOD

Name	Caeruloplasmin	Cu	Diagnosis
A.H	0.48	28.8	Hodgkins
M.J	0.55	39.1	Jaundice
E.A.	-	28.4	Hodgkins
E.A.	1.1	42.8	-
A473524	-	28.4	-
A796469	1.66	54.0	Hodgkins
M.B	1.12	35.6	-
D.H	0.6	31.1	-
D.B.	0.93	27.8	-

Normal range of caeruloplasmin 0.2-0.45 g/l
and for Cu level 13-27 µmol /l

(3) Dystonia patients 5 Serum samples and 4 cerebrospinal fluid samples from dystonia patients were obtained from Dr. Isabel Smith, Institute of Child Health in London. One of these patients PK and his parents received an oral dose of phenylalanine (100mg/Kgm body wt). Crithidia factor, phenylalanine, and tyrosine in serum were measured within six hours of the dose and compared with five normal people who had taken 7gm of phenylalanine and given blood samples at 0, 1, 2, 3, 4, hours following administration.

(4) Coeliac patients Serum samples from 10 out-patients with coeliac disease attending the gastrointestinal unit, General Hospital, Birmingham, were supplied by Dr. Allan; three of these patients were not on a gluten free diet.

Crithida factor was measured for all these specimens.

(5) Pregnant women serum samples were obtained from 24 pregnant women in their third trimester and supplied by Dr. S. Handly the Pharmacy department, University of Aston. 7 of them were considered by the attending psychiatrist to have had mild postnatal depression (the blues). Serum Crithidia factor for 14 of them were measured by Dr.R.J.Leeming, General Hospital, Birmingham.

(6) Geriatric patients, Serum samples were obtained from 76 patients (18 males and 56 females) in Selly Oak Hospital aged between 66-91 years old. Their scores in psychometric test (shown below) were known. This was used to show their mental state; scores below 6 are indicative of dementia. Crithida factor was measured for all of them.

Psychometric test

1. How old are you?
2. When is your birthday?
3. What year were you born?
4. What is today's date?
5. What day of the week ?
6. What is the name of this place?
7. What sort of place is this?
8. What is it for?
9. What is the name of this town?
10. What is the name of the Queen?
11. What is her number?
12. What is the name of the last King?
13. What was his number?
14. What is the name of the Prime Minister?
15. Who was the last Prime Minister?
16. What were the dates of the first world war?
17. What were the dates of the second world war?

(7) Phenylketonuric patients 21 phenylketonuric patients in the care of Dr. R.N. Raine, Children's Hospital, Birmingham. Dr. I. Smith, Institute of Child Health, London, Dr. I. Rey, Hospital des Enfants, Malades, Paris.

The information about these patients was not uniform and phenylalanine levels were available for some samples but not for all.

10 serum samples were obtained from patients diagnosed to be new cases of phenylketonuria on a normal diet; nine were aged between 2-weeks-3 weeks and one was 6 weeks old. Urine samples were obtained from five of them.

Two brothers C.D. and E.D. had an oral dose of phenylalanine (100mg/Kg body wt); serum phenylalanine and Crithidia factor were measured before and after 3 hrs, 10 hrs. for C.D. and after 3, 16 hrs. for E.D. Bioautography of serum for C.D. was carried out by streaking across the width of 20cm thin layer cellulose plates, running to $15cm^3$ in the dark in 3% ammonium chloride scraping at 1.5cm intervals and eluting with 0.5M phosphate buffer pH 5.0. The eluates were assayed with Crithidia fasiculata.

3 serum samples and 2 urine samples were obtained from 3 phenylketonurics who were on a low phenylalanine diet, their ages were 3 weeks, 5 months and 10 months.

[G.L] and [C] are a typical phenylketonuria patients [G.L] was 4 months old; his phenylalanine level was 0.24 mmol /L and tyrosine 0.07 mmol /L. [C] was 39 months old.

Crithidia factor was measured for both of them.

L.B and P.B. were two adults treated with anti-convulsant drugs and tranquillisers, and put on a low phenyl-alanine diet to control their behaviour; serum phenylalanine, tyrosine and Crithidia factor were measured after 0, 21, 47 days.

[G.T] was an untreated PKU: his serum phenylalanine was >30 mmol /L, Serum Crithidia factor was measured, and [M.A.] was two years old diagnosed to be classical phenylketonuria had an oral dose of phenylalanine (100 mg/Kg body wt). Serum phenylalanine level and Crithidia factor were measured.

(8) Trimethoprim and sulphathiazole studies

A number of normal volunteers, hospital staff and university students agreed to take part in this investigation.

5 had an oral dose of 0.3gm trimethoprim

5 had an oral dose of 1.5gm of sulphathiazole

5 had an oral dose of both drugs together

Blood samples were taken at 0, 2, 6, 24, 48 hr and 1 week after administration of the drugs.

Results

All values given as mean ± SEM.

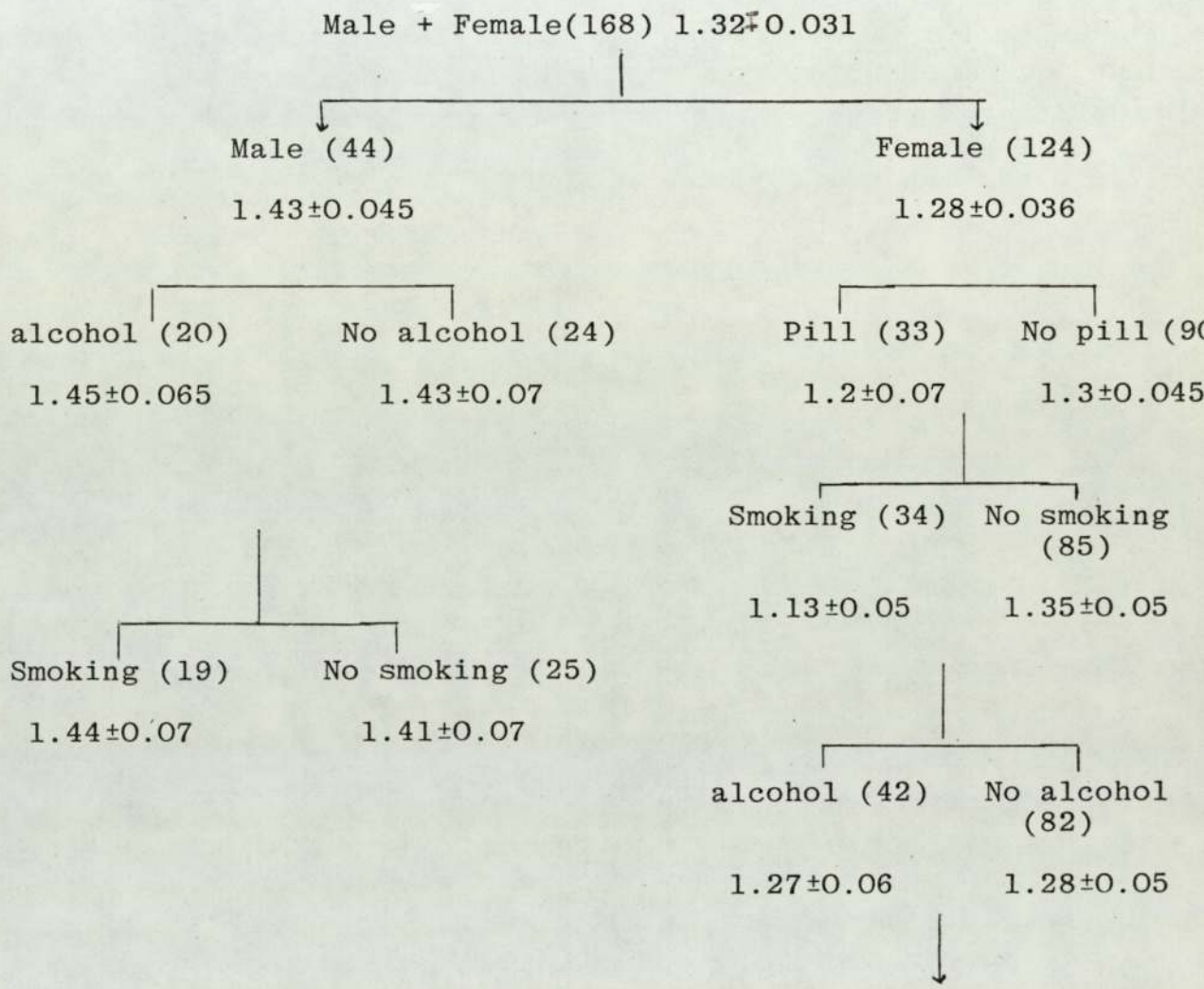
(1) Normal subjects: The mean level of serum biopterin derivatives (Crithidia factor C.F) for males was 1.43±0.045 ng/cm^3 and for the females, 1.28±0.036 ng/cm^3; a Student "t" test showed the difference between these two values to be significant $0.01<p<0.02$. There were 33 females who were known to be taking oral contraceptives; they did not show any significant difference from total female samples ($p>0.3$). The distribution of values is given in Table 2.2. The results from specimens paired within batches showed the coefficient of variation to be 12.5% around a mean of 1.03 ng/cm^3 and a coefficient of variation between batches of 15.9% around a mean of 1.0 ng/cm^3.

(2) Patients with raised blood copper levels: There was no correlation between whole blood copper and serum Crithidia factor or between caeruplasmin (the protein which carries the Cu) and Crithida factor in 9 patients with high blood copper levels (Fig. 2.1). Serum Crithidia factor for this group of patients was 0.64±0.118 ng/cm^3 which was significantly lower than normal (1.32±0.031 ng/cm^3) ($p<0.001$).

(3) Dystonia patients: The dystonia patients had a mean serum Crithidia factor of 1.76±0.081 ng/cm^3 which is significantly higher than the normal control value (1.32±0.031 ng/cm^3) ($p<0.01$). Also their cerebrospinal fluid showed a high value of 3.73±0.176 ng/cm^3 which is significantly higher

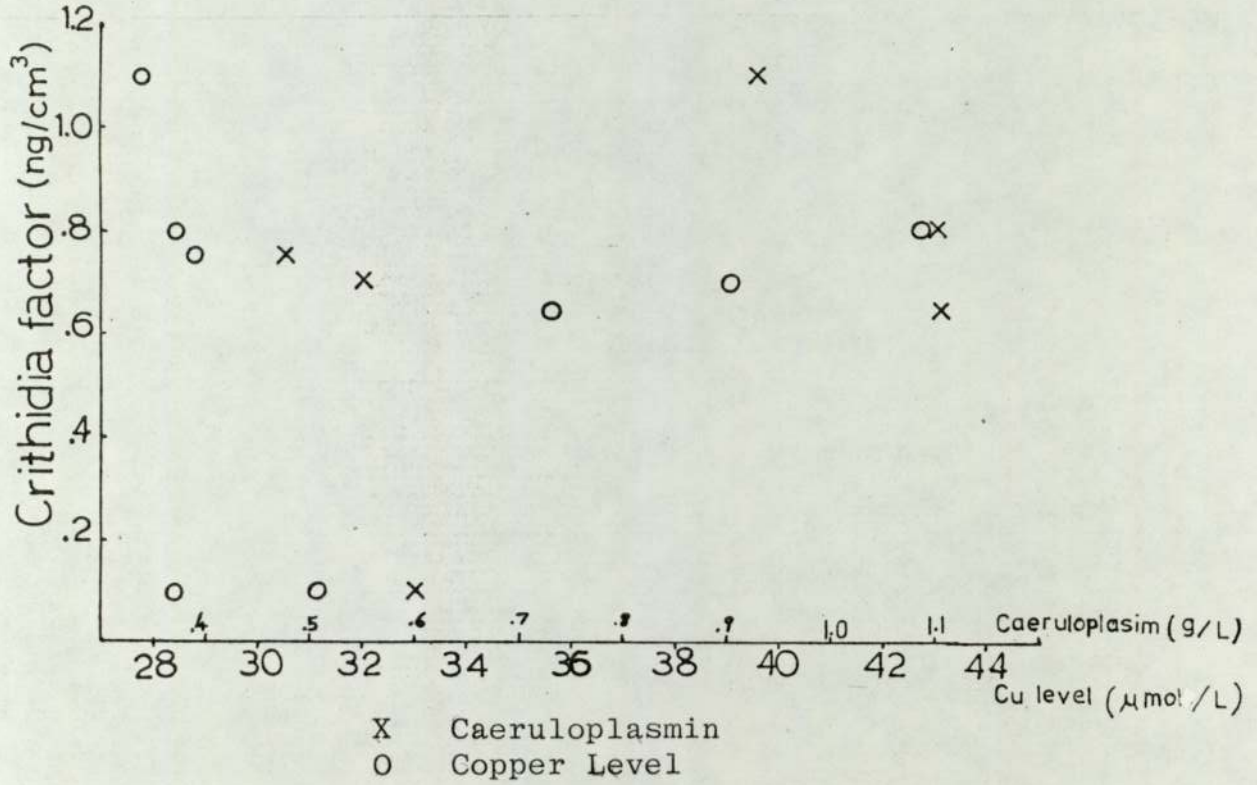
TABLE 2.2

CRITHIDIA FACTOR VALUES FOR NORMAL SUBJECTS



Results given as mean ∓ S.E.M. in ng/cm^3. Figures in brackets are numbers of subjects.

Fig. 2.1 Mean serum Crithidia factor plotted against caeruloplasmin or blood copp er in 9 patients with high (Cu) level.



than the normal (1.9±0.13 ng/cm^3) (Leeming *et al* 1976b) ($p<0.001$).

Serum Crithidia factor, phenylalanine and tyrosine were measured for the patient P.K. and his parents after they had taken an oral dose of phenylalanine (100 mg/Kg body weight) and compared to values for five normal subjects who had 7gm phenylalanine (Leeming *et al* 1976c).

However the phenylalanine values showed no significant differences from the normal (Fig. 2.2). The mean tyrosine level for all three was 0.192±0.013mmol /L after 1 hr. which was significantly higher than normal ($p<0.05$), but after 2hrs the tyrosine level was still rising whilst in the normal subjects it was falling. So it becomes significantly higher at this time ($0.01<p<0.02$ (Fig. 2.3), the mean serum Crithidia factor level was 4.2±0.016 ng/cm^3 after two hours which was significantly lower from normal ($0.01<p<0.05$) as shown in Fig. 2.4.

(4) Coeliac patients: Patients with coeliac disease showed mean serum Crithidia factor of 1.02±0.13 ng/cm^3 which is lower than normal control (1.32±0.031 ng/cm^3) ($0.02<p<0.05$). Amongst this group there were three pregnant women who showed a mean serum Crithidia factor value of 0.73±0.235 ng/cm^3 which is lower than normal females (1.28±0.038 ng/cm^3) but not significantly so ($0.1<p<0.2$). Also there were three untreated coeliac patients who showed a mean serum Crithidia factor of 1.05±0.076 ng/cm^3 which also is lower than normal control value (1.32±0.03 ng/cm^3) ($p<0.05$).

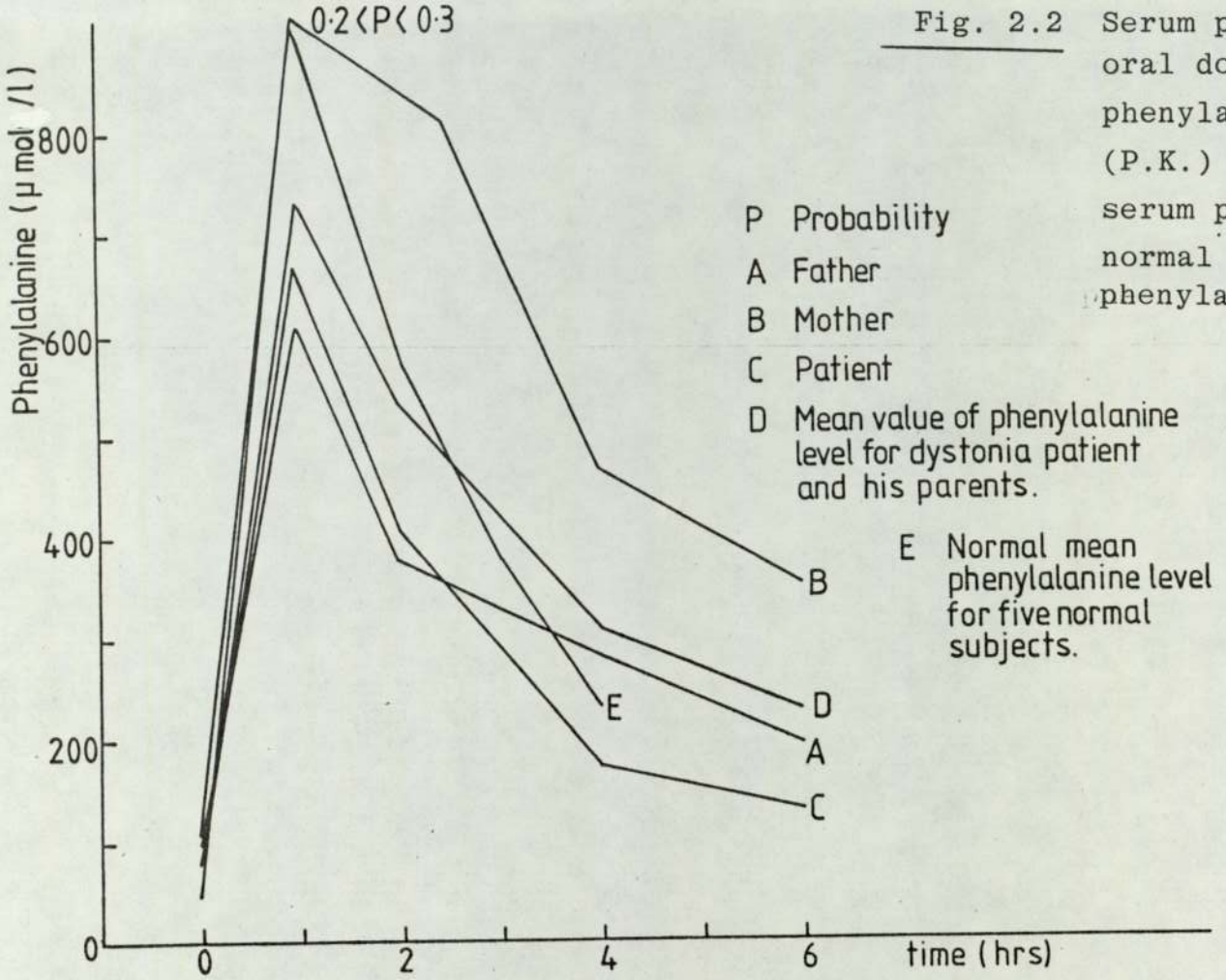


Fig. 2.2 Serum phenylalanine level following oral dose of 100 mg/Kg body wt. phenylalanine to dystonia patients (P.K.) and his parents, compared to serum phenylalanine levels for five normal people receiving 7gm phenylalanine.

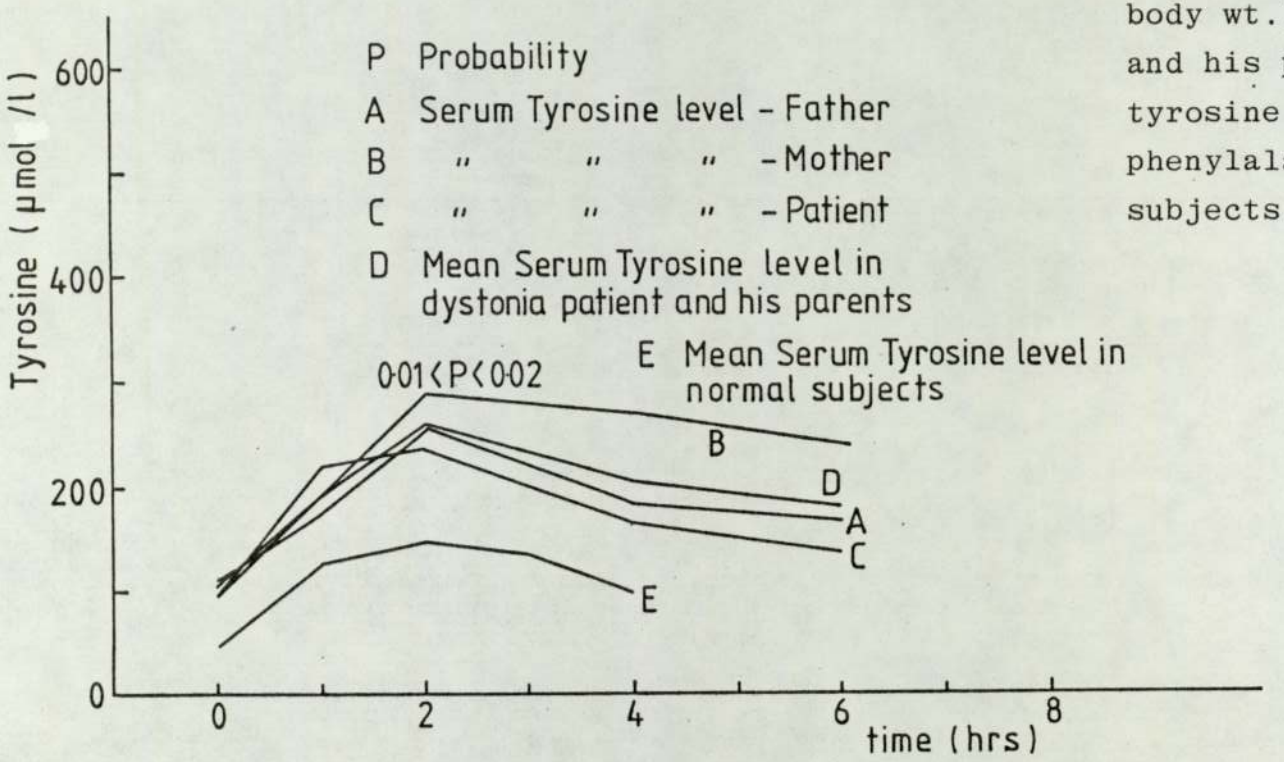


Fig. 2.3 Serum tyrosine level following an oral dose of phenylalanine (100 mg/Kg body wt.) to a dystonia patient and his parents compared with serum tyrosine levels following 7 gm phenylalanine give to five normal subjects.

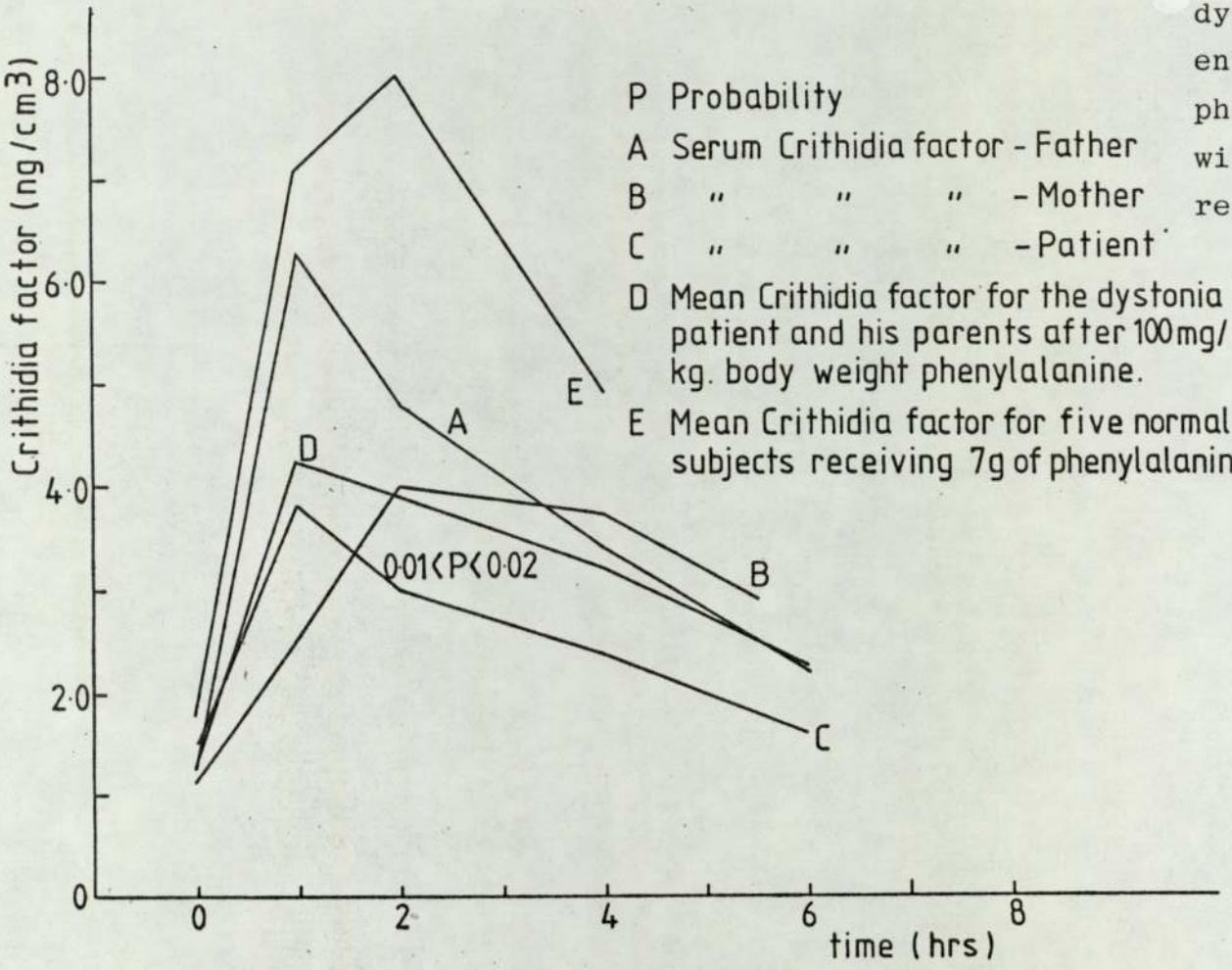


Fig. 2.4 Serum Crithidia factor for a dystonia patient and his parents after 100 gm/Kg phenylalanine compared with five normal people receiving 7gm phenylalanine.

(5) Pregnant women

Mean serum Crithidia factor for seven pregnant women who were diagnosed later as having postparturition depression was 1.129±0.04 ng/cm^3. This value is higher than the seventeen other normal pregnant women (0.99±0.03 ng/cm^3)($p<0.05$).

(6) Phenylketonuric patients: phenylalanine loading test: Oral doses of phenylalanine (7gm) given to five normal subjects produced a large increase in serum phenylalanine followed by smaller rises in Crithidia factor and tyrosine (Leeming and Blair 1976c)

This test was performed with the two brothers C.D. and E.D., In their case the serum Crithidia factor did not parallel the rise in serum phenylalanine as in the case of classical phenylketonuria and in normal adults. In the case of C.D. serum phenylalanine levels peaked after 3hrs. whereas the Crithidia factor was maximised after 10 hrs. Fig. 2.5.

In the case of E.D. serum Crithidia factor was low even when serum phenylalanine was high but the trend in Crithidia factor and phenylalanine was similar (Fig. 2.6). All values are shown in Table 2.3.

Chromatography revealed the material in serum of patient C.D. to be the same as in normal serum i.e. 7,8 dihydrobiopterin.

Fig. 2.5 Serum Crithidia factor and phenylalanine in phenylketonuric patient (C.D.) following an oral load of phenylalanine (100 mg/kgm body wt.

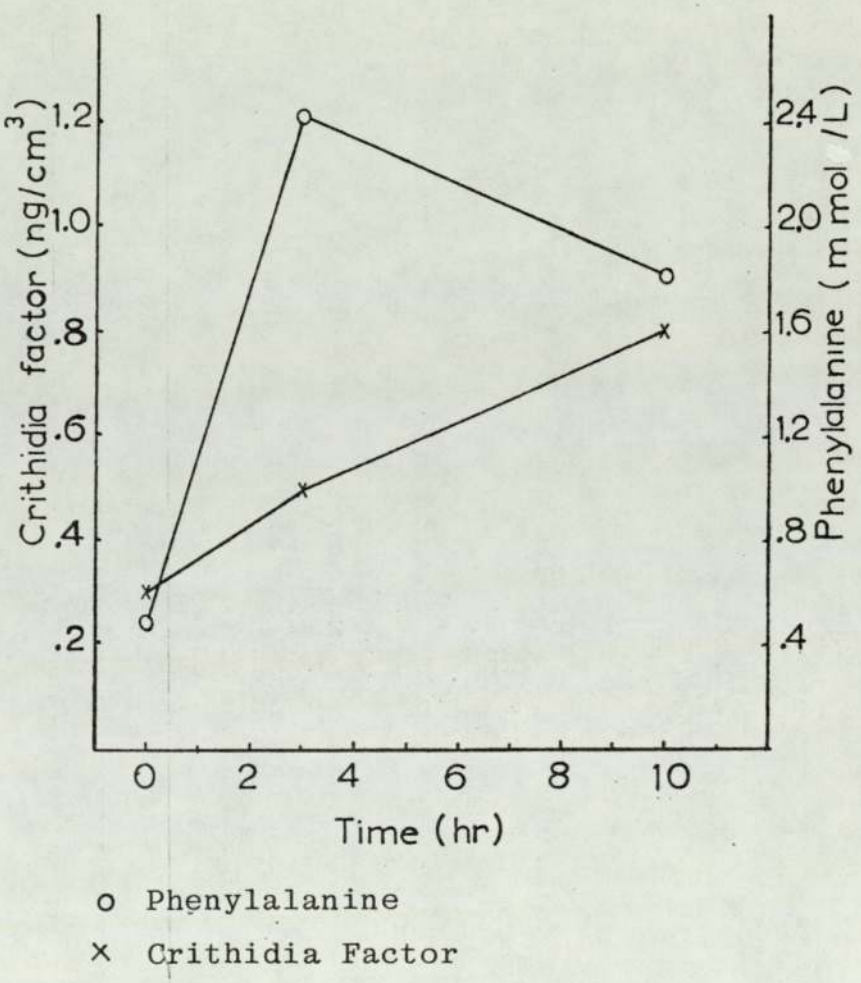


Fig. 2.6 Serum Crithidia factor and phenylalanine in phenylketonuric patient (E.D.) following an oral dose of phenylalanine (100 mg/Kgm body wt.)

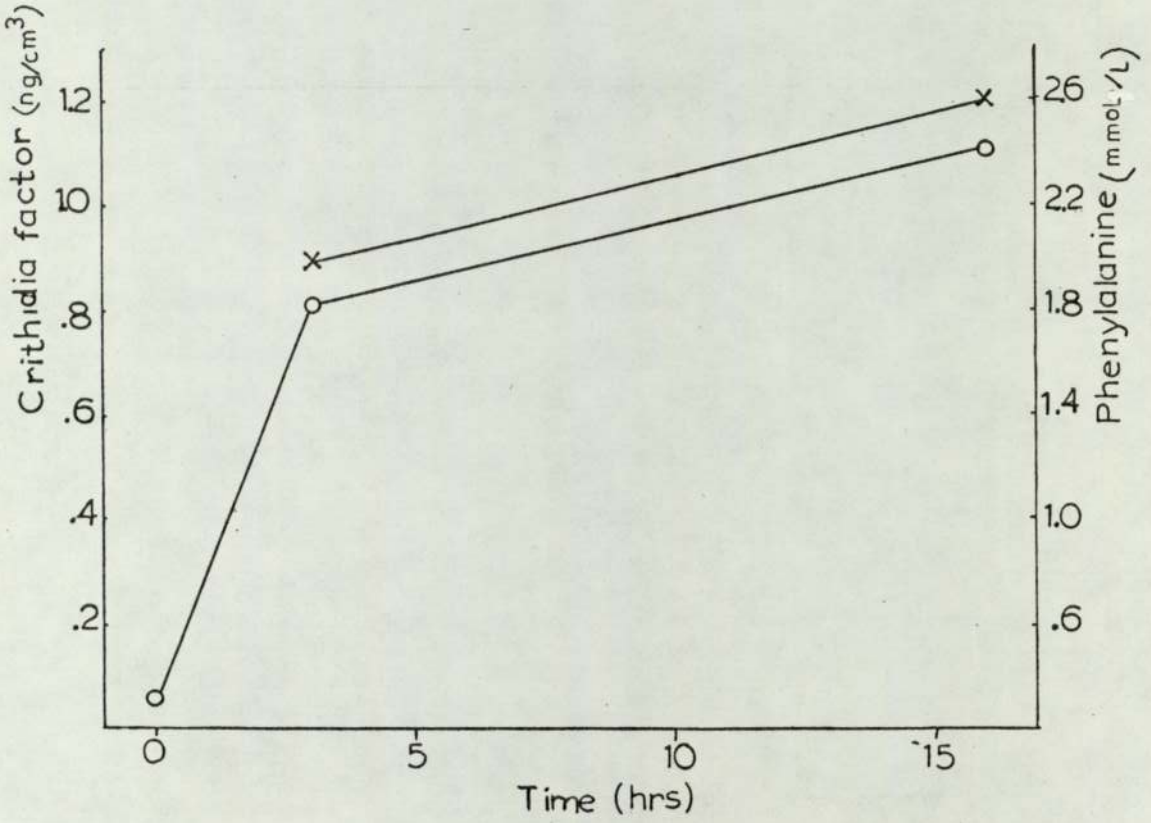


TABLE 2.3

SERUM CRITHIDIA FACTOR AND PHENYLALANINE LEVEL IN TWO PHENYLKETONURIC PATIENTS C.D. AND E.D. BEFORE AND AFTER 100 mg L-PHENYLALANINE . ORALLY/kg BODY WEIGHT.

	Time	Crithidia Factor	Phenylalanine
C.D.	0	0.3 ng/cm^3	0.485 mmol /l
	3 hrs.	0.5 ng/cm^3	2.424 mmol /l
	10 hrs.	0.8 ng/cm^3	1.818 mmol /l
E.D.	0	——	0.303 mmol /l
	3 hrs.	0.9 ng/cm^3	1.818 mmol /l
	16 hrs.	1.2 ng/cm^3	2.442 mmol /l

M.A. a patient with classical phenylketonuria (2 years old) had oral load of phenylalanine. Serum Crithidia factor and phenylalanine were raised and both fell with time but remained above the fasting level, values shown in Table 2.4.

TABLE 2.4

SERUM CRITHIDIA FACTOR AND PLASMA PHENYLALANINE IN CLASSICAL PHENYLKETONURIC PATIENT (M.A.) BEFORE AND AFTER ORAL PHENYLALANINE LOAD.

	Time	Crithidia Factor	Phenylalanine
M.A.	24.1.1980	5.2 ng/cm^3	0.727 mmol /l
	same day	9.6 ng/cm^3	3.030 mmol /l
	24 hrs. later	7.5 ng/cm^3	1.515 mmol /l

The mean serum Crithidia factor for children with newly diagnosed phenylketonuria on a normal diet was 7.87±0.62 ng/cm^3 which is significantly higher than normal (non-phenylketonuria) children aged between 2 years-12 years (Leeming and Blair 1980b) 1.78±0.25 ng/cm^3 ($p<0.001$). Their urine Crithidia factor was 0.669±0.065 µg/ml which is significantly lower than the value obtained for normal children (2.26±0.18 µg/ml) (Leeming 1975). ($p<0.001$).

The mean serum Crithidia factor 2.82±1.174 ng/cm^3 for the three phenylketonuric patients on a low phenylalanine diet was higher than normal (1.78±0.25 ng/cm^3) but not significantly so ($0.4<p<0.5$). The mean serum Crithidia factor for their urine was 0.21±0.15 ng/cm^3 which is significantly lower than normal controls ($0.01<p<0.02$)

The two patients |GL| and |C| showed serum Crithidia factor values of 4.5 ng/cm^3 and 5.4 ng/cm^3 and urine Crithidia factor values 0.69 ng/cm^3 and 0.25 ng/cm^3.

The two adults L.B. and P.B. who were on a low phenylalanine diet did show a significant decrease in serum Crithidia factor, phenylalanine and tyrosine after onset of the diet.

In the case of L.B. phenylalanine, tyrosine and Crithidia factor fell to a minimum after about 21 days (fig. 2.7) but P.B. tyrosine level did not show any major decrease until 47 days (Fig. 2.8). Values shown in Table 2.5.

Crithidia factor for G.T. who was an untreated phenylketonuria patient with a serum phenylalanine level >30 mmol /l was 12.2 ng/cm^3.

Fig. 2.7 Mean serum Crithidia factor, phenylalanine and tyrosine level in LB which on low phenylalanine diet.

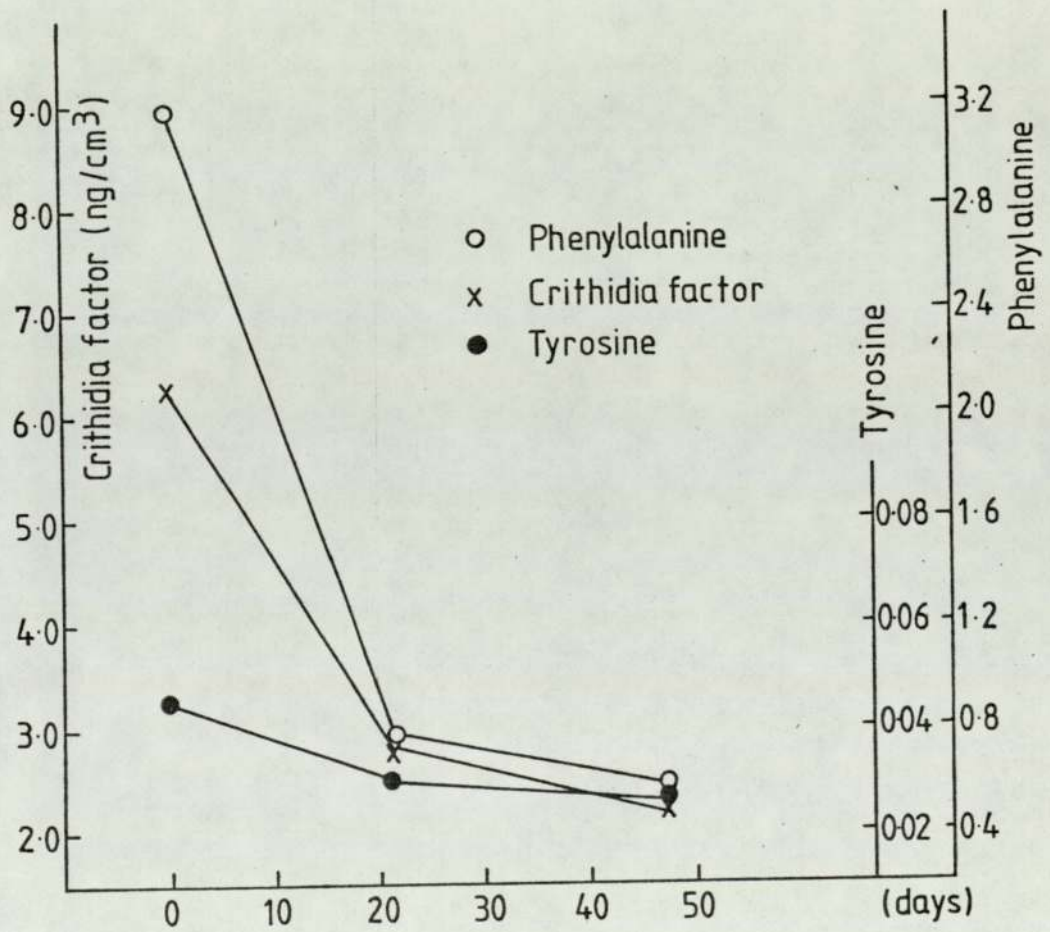


Fig. 2.8 Mean serum Crithidia factor, phenylalanine and tyrosine in P.B. which on low phenylalanine diet.

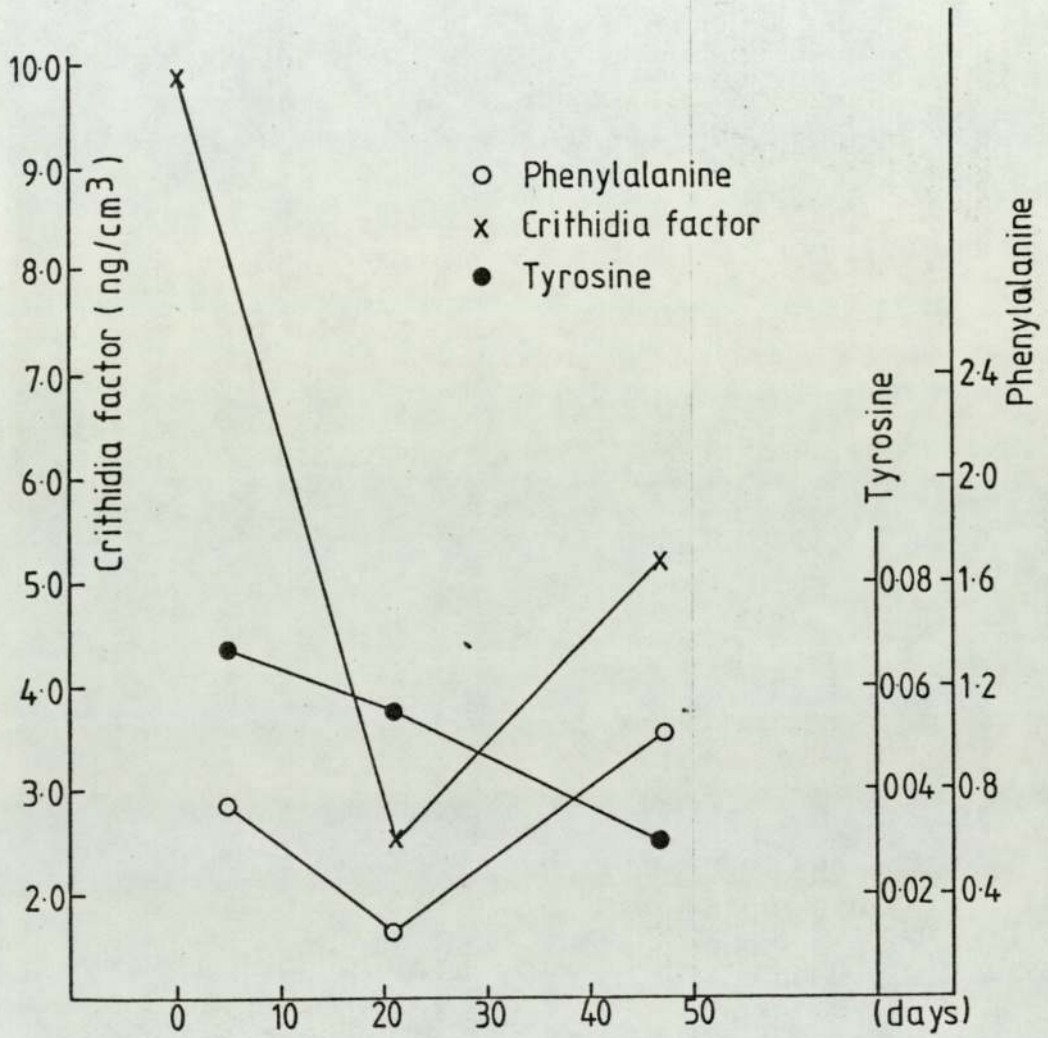


Table 2.5

SERUM CRITHIDIA FACTOR, PHENYLALANINE AND TYROSINE IN TWO CASES OF MALIGNANT HYPERPHENYLALANINAEMIA |L.B| AND [P.B.] ON LOW PHENYLALANINE DIET.

	Time	Crithidia factor	Phenylalanine	Tyrosine
L.B.				
	0	6.3 ng/cm^3	3.168 mmol /l	0.045 mmol /l
	21 days	2.8 ng/cm^3	0.743 mmol /l	0.030 mmol /l
	47 days	2.2 ng/cm^3	0.595 mmol /l	0.026 mmol /l
P.B.				
	0	9.9 ng/cm^3	0.729 mmol /l	0.067 mmol /l
	21 days	2.5 ng/cm^3	0.265 mmol /l	0.055 mmol /l
	47 days	5.2 ng/cm^3	1.033 mmol /l	0.030 mmol /l

(7) Geriatric people

Geriatric people had a mean serum Crithidia factor of 1.26±0.044 ng/cm^3 which is lower than normal control (1.32±0.031 ng/cm^3) but not significantly different ($0.3>p>0.2$) and not significantly lower ($p>0.1$) than a group of normals aged more than 50 years (1.37±0.05 ng/cm^3).

These old people can be divided into two groups; those with low Crithidia factor level and those with high Crithidia factor level (Fig. 2.9). These two groups show significantly ($p<0.001$) different Crithidia factor values from each other.

The first group showed an average value significantly lower ($p<0.001$) than normal controls and the second group a value significantly ($p<0.001$) higher than normal. Distribution of the normal values is shown in Fig. 2.10. We can also divide them into two groups according to the ratio of phenylalanine/tyrosine, the first group had a ratio more than one, and Crithidia factor 1.23 ± 0.09 ng/cm^3 which is not significantly lower than the second group in which they had a ratio of phenylalanine/tyrosine less than one with a mean serum Crithidia factor 1.49 ± 0.17 ng/cm^3 ($0.1<p<0.3$). All values are shown in table 2.6. 23 patients originally diagnosed to be suffering from senile dementia had a mean serum level of Crithidia factor of 1.29 ± 0.052 ng/cm^3. The patients were reassessed at a later date and these showed a mean level of Crithidia factor of 1.3 ± 0.076 ng/cm^3,

These elderly patients were divided into many groups:-

(7.1) Senile dementia:- They showed a mean serum Crithidia factor of 1.29 ± 0.052 ng/cm^3 which is lower than normal but not significantly so. ($0.3<p<0.4$). This group can be divided into two groups according to the psychometric test score

(a) Those who scored (0-3) show a mean Crithidia factor of 1.17 ± 0.055 ng/cm^3 which is lower than normal control (1.32 ± 0.31 ng/cm^3) ($0.02<p<0.05$).

(b) And those who scored (4-5) had a mean serum Crithidia factor of 1.506 ± 0.06 ng/cm^3 which is significantly higher than the first group (p 0.02-0.01) and higher than normal control (1.32 ± 0.031 ng/cm^3). ($0.02<p<0.05$).

Table 2.6

SERUM CRITHIDIA FACTOR CONCENTRATION IN GERIATRIC PEOPLE

Diagnosis	Number of subjects	Mean ± S.E.M.
Normal male+female	(168)	1.32±0.031 ng/cm^3
Normal Aged+50 years	(22)	1.37±0.052 ng/cm^3
Geriatric People	(75)	1.26±0.044 ng/cm^3
(1) Senile Dementia	(23)	1.29±0.052 ng/cm^3
(a) S.D. score (0-3)	(15)	1.17±0.055 ng/cm^3
(b) S.D. score (4-5)	(8)	1.506±0.052 ng/cm^3
(2) Vascular Dementia	(12)	1.97 ±0.134 ng/cm^3
(3) Diabetics	(7)	0.98 ±0.073 ng/cm^3
(4) Hemiplegia	(9)	1.26±0.151 ng/cm^3
(5) Rheumatoid arthritis	(5)	1.0±0.089 ng/cm^3
(6) Non Demented	(17)	1.18±0.086 ng/cm^3
Geriatric People	(75)	
Crithidia factor $<$ 1.4 ng/cm^3	(51)	1.06±0.026 ng/cm^3
Crithidia factor $\geq$ 1.4 ng/cm^3	(24)	1.7±0.066 ng/cm^3
phenylalanine/tyrosine $>$ 1	(13)	1.23±0.09 ng/cm^3
phenylalanine/tyrosine $<$ 1	(7)	1.49±0.17 ng/cm^3

(7.2) Vascular Dementia:- these patients had a mean serum Crithidia factor of 1.97±0.134 ng/cm^3 which is not significantly higher than normal. ($p<0.3$) and not significantly higher ($p<0.6$) than normal 1.37±0.052 ng/cm^3 in their age group, and it is also not significantly different from the senile dementia patients. ($0.2<p<0.3$)

(7.3) Psoriasis:- one of these geriatric people was diagnosed to have psoriasis and gave a value of crithidia factor 1.05 ng/cm^3.

(7.4) Diabetic patients:- seven of these geriatric people were diagnosed to have diabetis mellitus. They had a mean serum Crithidia factor of 0.98±0.073 ng/cm^3 which is significantly ($0.001<p<0.01$) lower than the normal control and lower than in senile dementia ($p<0.001$)

(7.5) Hemiplegia:- 9 of these patients had a mean serum Crithidi factor of 1.26±0.15 ng/cm^3 which is not significantly different from normal.

(7.6) Rheumatoid arthritis:- 5 of them showed a mean serum Crithidia factor of 1.0±0.039 ng/cm^3 which significantly lower ($0.01<p<0.02$) than normal control and lower value than found in senile dementia ($0.02<p<0.05$), and also lower ($0.02<p<0.05$) than the vascular dementia.

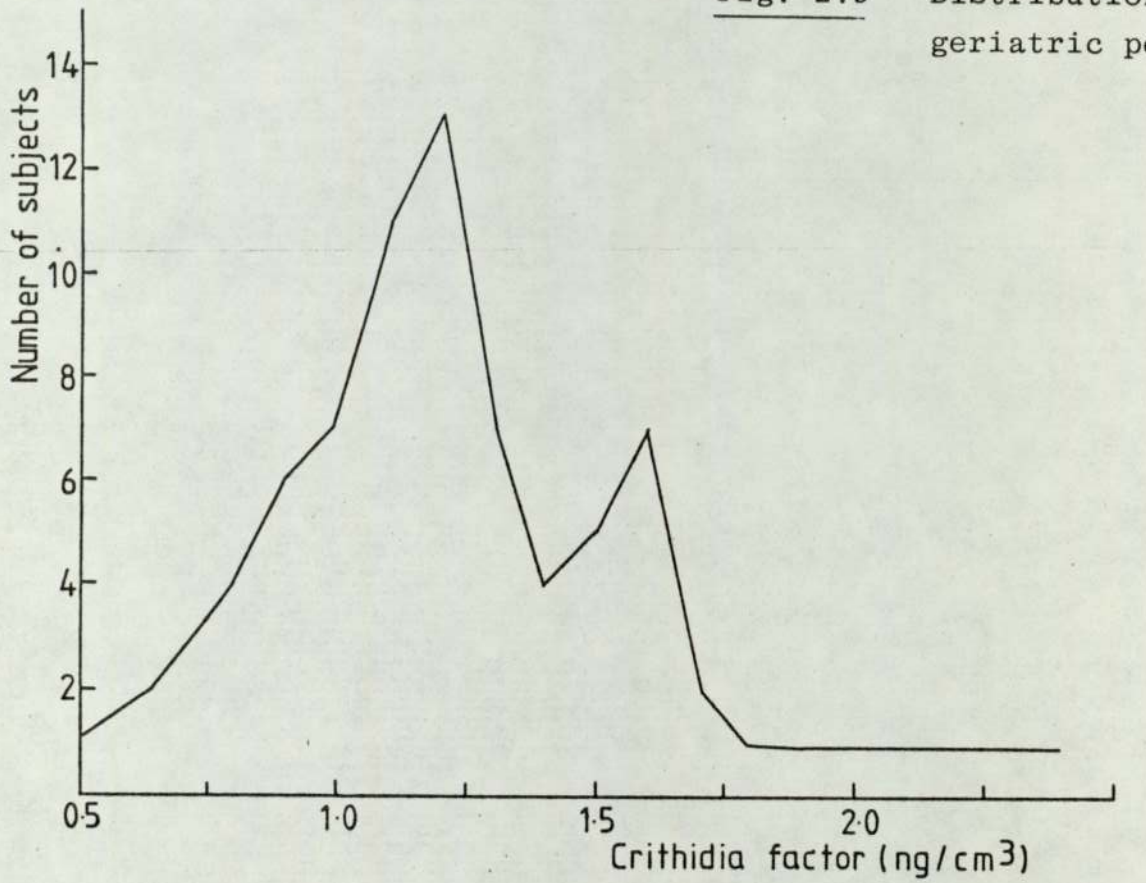
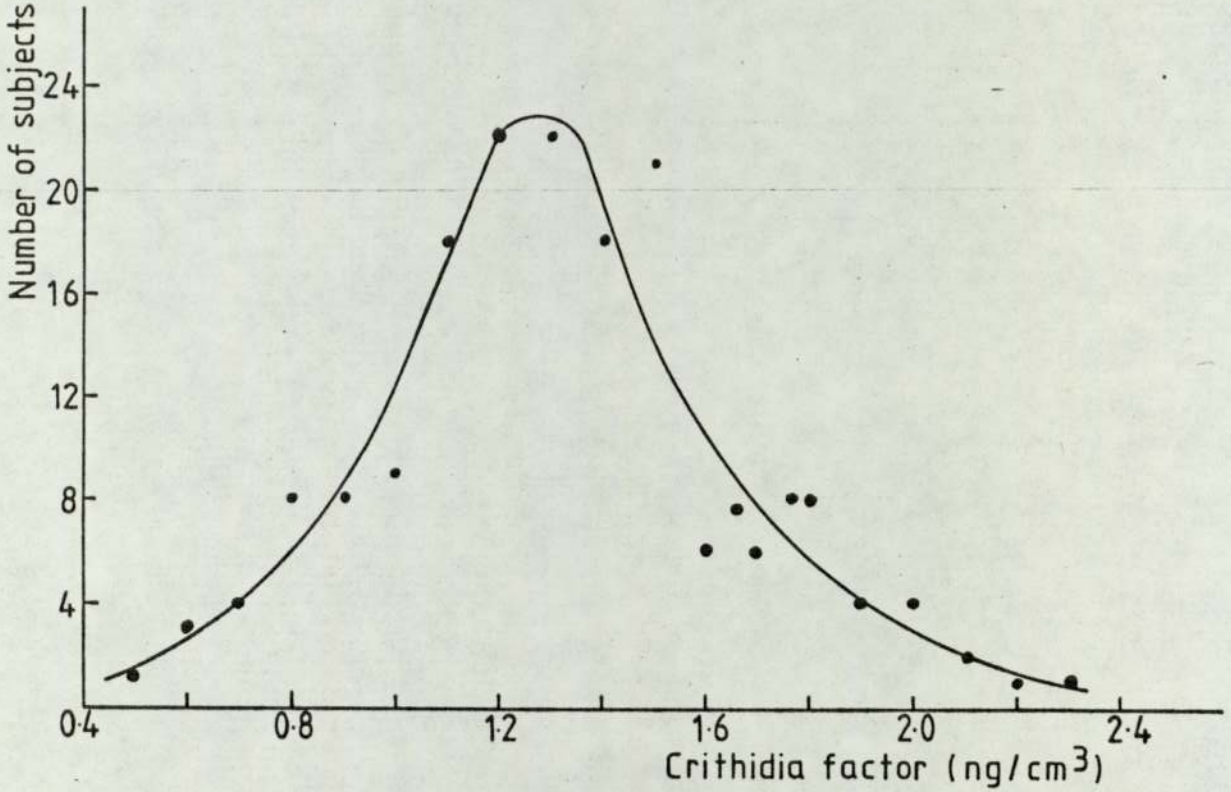


Fig. 2.9 Distribution of Crithidia levels of 76 geriatric people.

Fig. 2.10 Distribution of Crithidia factor in normal subjects.



(6.7) Non Demented:- Sixteen of these elderly people were not demented, they showed a mean serum crithidia factor 1.18±0.086 ng/cm^3 which is not significantly different than normal.

These results are summarised in Table 2.6.

(8) Trimethoprim and sulphathiazole studies

Five normal people who took 0.3 gm trimethoprim showed a significantly rise in serum Crithidia factor value within 6 hrs. which fell after 24 hrs. All values of serum Crithidia factor are shown in Table 2.7, below.

Table 2.7.

SERUM CRITHIDIA FACTOR FOR 5 SUBJECTS BEFORE AND 2HRS., 6HRS., 24HRS, 48HRS AND 1 WEEK AFTER 0.3gm OF TRIMETHOPRIM.

Time	Crithidia Factor Level all values are in ng/cm^3	Mean±S.E.M.
0	1.3, 1.7, 0.7, 1.8, 1.6	1.42±0.0198
2 hrs.	1.6, 2.7, 1.7, 4.8, 2.7	2.6 ±0.578
6 hrs.	2.7, 4.8, 1.5, 4.8, 2.4	3.3 ±0.65
24 hrs	2.4, 2.4, 1.0, 2.1, 2.4	2.06±0.27
48 hrs.	1.6, 1.9, 0.9, 1.5, 1.5	1.48±0.162
1 week	2.7, 2.0, 1.2, 1.8, 1.2	1.78±0.28

Five people received 1.5 gm sulphathiazole; their mean serum Crithidia factor showed a decreased value in 6 hrs. and returned to its fasting level after 24 hrs. The value of individual subjects are shown below.

Table 2.8

SERUM CRITHIDIA FACTOR FOR 5 SUBJECTS BEFORE AND 2 HRS. 6 HRS. 24 HRS. 48HRS. and 1 WEEK AFTER 1.5 gn SULPHATHIAZOLE.

Time	Serum Crithidia factor values are in ng/cm^3	MEAN±S.E.M.
0	3.6, 2.0, 1.0, 1.2, 0.9	1.74±0.504
2 hrs.	3.3, 1.8, 1.2, 0.9, 0.9	1.62±0.45
6 hrs.	2.1, 1.6, 1.0, 1.5, 1.6	1.56±0.175
24 hrs.	1.8, 3.4, 0.9, 1.8, 1.1	1.8 ±0.439
48 hrs.	2.1, 1.8, 1.0, 3.0, 1.2	1.46±0.304
1 week	2.1, 1.8, 1.0, 0.9, 1.2	1.82±0.356

Five normal people received both these drugs (0.3 gm trimethoprim + 1.5 gm sulphathiozole); mean serum Crithidia factor for four of them showed significant rise in 24 hrs. and fell to normal after 48 hrs. Table 2.9.

The fifth subject showed a greater response than the other four subjects, and serum Crithidia factor peaked after 6 hrs. (Fig. 2.10.).

Fig. 2.11 Mean serum c.f for five people given 0.3 gm trimethoprim, 5 people had 1.5 gm sulphathiazole and 4 people given both drugs.

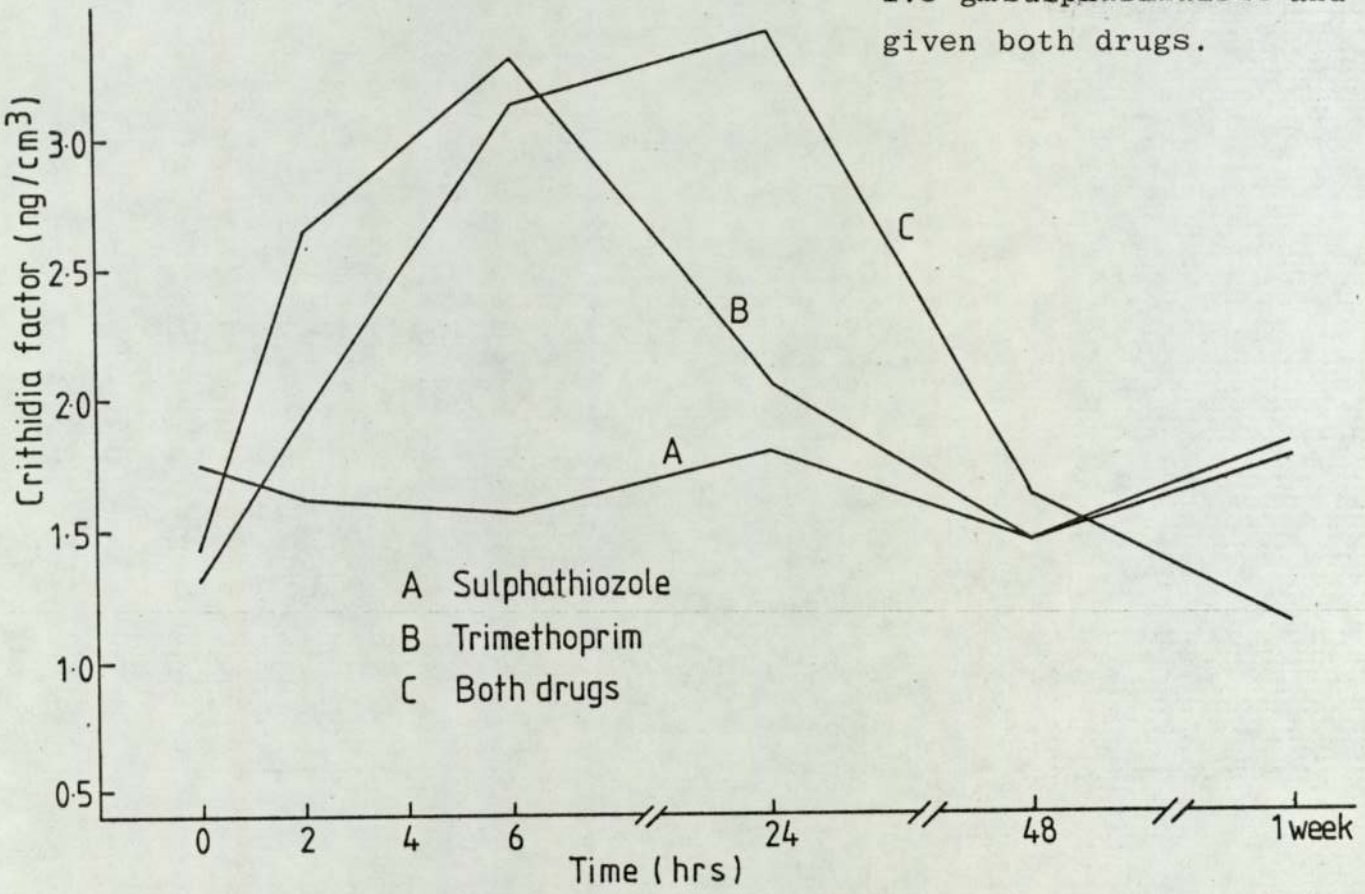


Table 2.9

SERUM CRITHIDIA FACTOR FOR 5 SUBJECTS BEFORE AND 2 HRS. 6 HRS. 24 HRS. 48 HRS. AND 1 WEEK AFTER (0.3 gn TRIMETHOPRIM + 1.5 SULPHATHIAZOLE).

Time	Serum Crithidia factor values are in ng/cm^3	MEAN±S.E.M.
0	2.3, 1.0, 0.6, 1.3, 2.1*	1.3 ±0.65
2 hrs.	2.3, 2.4, 1.5, 1.5, 3.0*	1.93±0.97
6 hrs.	3.3, 2.6, 3.0, 3.6 >12.0*	3.13±1.56
24 hrs.	2.5, 2.7 1.5, 6.9, 8.1*	3.4 ±1.7
48 hrs.	1.8, 2.3, 1.2, 1.2, 2.1*	1.63±0.81
1 week	1.2, 1.5, 0.9, 1.0, 3.0*	1.15±0.58

* Values of the last subject excluded from the mean.

Discussion

The normal values of serum Crithidia factor obtained in this study were 1.32±0.031 ng/cm^3 for the total male and female volunteers. The mean serum Crithidia factor for males being 1.43±0.045 ng/cm^3 which is significantly higher than female value (1.28±0.035 ng/cm^3) $0.01<p<0.02$. However these values are lower than those reported by Leeming and Blair (1980b) which were mean serum Crithidia factor levels for total male and female of 1.6±0.03 ng/cm^3 for the males 1.75±0.03 ng/cm^3 and for females 1.53±0.04 ng/cm^3. In this case the difference between the sexes was not statistically significant.

In this study we also found that women on the contraceptive pill showed lower values of mean serum Crithidia factor than women not taking the pill, but not significantly so ($p>0.3$).

The levels of unconjugated pteridines in serum have been reported by others to be 11-13 ng/cm^3 (Baker *et al* 1974), 27 ng/cm^3 (Frank and Baker 1963) and 11.2-38.4 ng/cm^3 (Guttman 1963) and 1.8 µg/L by Kaufman *et al* (1978). These values are higher than the value reported by Baker *et al* (1974) which was 0.9 ng/cm^3 in plasma, and values reported by Leeming and Blair (1976b) and those determined in this study.

The biopterin used in these three later studies was from Roche Product Ltd., and one expects that preparation of samples and standards for the assay may effect the apparent levels.

Leeming and Blair (1974) reported mean Crithidia factor for urine to be 2.1±0.038µg/cm^3 and for cerebrospinal fluid 1.9±0.13 ng/cm^3, which is close to serum value (Leeming *et al* 1976b).

The results obtained here from different groups of subjects will be discussed in two sections, those with serum Crithidia factor lower than normal, and those with a high value.

Tetrahydrobiopterin metabolism can be summarised as follows:

In normal cells the levels of tetrahydrobiopterin (BH_4) are maintained at a constant level by the synthesis and salvage pathways. Any disruption of BH_4 metabolism will lead to impaired neurotransmitter synthesis and thus effect mental functions.

In this study effects on BH_4 metabolism are detected by serum Crithidia factor level. There are two major groups of conditions apparent in these results. Those in which serum Crithidida factor is lower than normal and those in which it is raised.

Low serum Crithidia factor

These can be caused by

a. impaired synthesis of BH_4
b. increased salvage of q-dihydrobiopterin.

Any factor which increases the availability of NADH should increase the salvage of q-dihydrobiopterin provided NADH is limiting factor.

(1) High blood copper

Patients with high blood copper levels showed significantly lower Crithidia factor value ($p<0.001$) than normal. Leeming (1979) reported that Cu^{2+} interfered with both synthesis of BH_4 and dihydropteridine reductase activity.

Mercury, cadmium, copper, aluminium and lead have all been suggested as neurotoxic agents (Flink 1975) and copper toxicity causes convulsions (Scheinber and Steinlieb 1976).

So the low value observed may be due to inhibition of the synthesis of BH_4 which may in turn lead to the neurological symptoms.

(2) Coeliac patients

Coeliac patients showed a mean serum Crithidia factor lower than normal controls ($0.02<p<0.05$). Untreated patients showed a value which was significantly lower than normal ($p<0.05$).

Leeming and Blair(1980b)found a mean serum Crithidia factor for untreated coeliac patients of 1.02 ± 0.09 ng/cm^3 which was significantly lower than normal controls. In untreated coeliac patients glucagon level are raised (Walsh *et al* 1979). In the rat Fuller and Baker (1974) reported that glucagon increase dihydropteridine reductase activity but this was not confirmed by Donlon and Kaufman (1978). An increase in dihydropteridine reductase activity could lead to low level of 7,8-dihydrobiopterin because the dihydropteridine reductase would convert most of the quinonoid dihydrobiopterin to tetra hydro-form leaving less to rearrange to 7,8 dihydrobiopterin.

(3) Pregnant women

The mean level of serum biopterin derivatives for the pregnant women was significantly lower than the mean level of Crithidia factor for normal women (not pregnant) ($0.001<p<0.01$). Depressed pregnant women showed mean serum Crithidia factor higher than those who did not ($p<0.05$). This is consistent with the observation of Leeming (1979).

In pregnant women there is no evidence of neurological changes, therefore increased salvage of quinoniod dihydrobiopterin seems the more likely explanation for low serum crithidia factor.

(4) Geriatric people

Geriatric patients showed a low value of serum Crithidia factor. It has been reported that dihydropteridine reductase increases with age in rat (Algeri *et al* 1977) and this could explain the low levels of serum Crithidia factor in old people. It is also possible that synthesis of tetrahydrobiopterin is impaired particularly in senile dementia, patients and this could decrease levels of tetrahydrobiopterin in the cell and this explains the mental deterioration.

In diabetic old people, we found mean serum Crithidia factor 0.98±0.073 ng/cm^3 which is significantly lower than normal (p 0.01-0.001). Diabetic subjects are known to have a higher NADH/NAD ratio (Gumaa *et al* 1971). This shifts the redox state of the cell cytoplasm and could lead to more efficient salvage of quinoniod dihydrobiopterin and hence lower serum 7,8-dihydrobiopterin.

Leeming and Blair (1980a and 1980b) showed that demented patients had a mean serum crithidia factor of 1.01 ±0.12 ng/cm^3 which is significantly lower than their control ($p<0.001$)

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High serum crithidia factor values

These may be caused by decreasing the salvage of quinonoid dihydrobiopterin, so that more 7,8-dihydrobiopterin appears in serum, this could arise by:

a. inhibition of dihydropteridine reductase
b. decreased availability of NADH

Inhibition or decreasing the rate of the dihydropteridine reductase salavage path way would result in increase quinonoid dihydrobiopterin levels, which would lead to increase levels of 7,8-dihydrobiopterin which could be measured in the serum and urine.

1. Dystonia

The mean serum Crithidia factor for dystonia patients was 1.76 ± 0.08 ng/cm^3 which is significantly higher than normal ($p<0.01$) and this may represent dihydrobiopterin lost from the cell. Williams *et al* (1979) reported reduced levels of BH_4 in cerebrospinal fluid of dystonia patients; this finding of lowered tetrahydrobiopterin is consistent with our observation of raised serum Crithidia factor assuming that the derivatives present in serum is 7,8-dihydrobiopterin. These results suggest that the salvage pathway is impaired in dystonia. Similar observations have been made in Parkinson's disease. Lovenberg *et al* (1979) reported low BH_4 in cerebrospinal fluid of these patients, and Leeming (1975) has found raised serum Crithidia factor (2.05 ± 0.17 ng/cm^3).

2. Phenylketonuria (PKU)

(a) Classical PKU: This is due to phenylalanine hydroxylase deficiency (Jervis 1947), Abnormal metabolites of phenylalanine are excreted in the urine including phenylpyruvic acid (Folling 1934; Blastovics and Nelson 1971), These patients become mentally retarded unless the levels of phenylalanine are controlled by a restricted phenylalanine diet (Bickel, Gerrard *et al* 1953). Phenylalanine loading shows that the observed increase in serum phenylalanine (and presumably phenylpyruvate) is accompanied by an increase in serum Crithidia factor. This is probably 7,8-dihydrobiopterin lost into the serum because of the inhibition of salvage of quinonoid dihydrobiopterin by phenylpyruvate (Purdy and Blair 1980).

In all cases a high level of phenylalanine is associated with raised Crithidia factor. In new untreated PKU a mean serum Crithidia factor of 7.87 ± 0.62 ng/cm^3 was found which is significantly higher than normal children (non-phenylketonuria) and the three phenylketonuric patients on diet show a mean serum Crithidia factor of 2.8 ± 1.17 ng/cm^3 which is higher than normal control.

Also this is shown in cases of G.T., untreated phenylketonuria with serum phenylalanine >30 mmol /1 and Crithidia factor is high. In the case of M.A. diagnosed to be classical phenylketonuria who had an oral dose of pheylalanine Crithidia factor rose in parallel with phenylalanine level. Both phenylalanine and Crithidia factor achieved levels higher than normal.

Thus there is ample evidence to show that in people possessing the enzymes of biopterin metabolism, serum dihydrobiopterin levels parallel serum phenylalanine (Leeming *et al* 1976c). The high level of 7,8-dihydrobiopterin in serum are due to the inhibition of dihydropteridine reductase by phenylalanine and phenylpyruvate. Lowering phenylalanine levels by the recommended diet lowers the phenylalanine metabolite levels as well and so will reduce the neurological damage. In phenylalanine loading test, serum phenylalanine rises the inhibition of dihydropteridine reductase increases and so the level of serum dihydrobiopterin will increase.

(b) Atypical phenylketonuria

Malignant hyperphenylalanaemia MHPA due to

(1) Dihydropteridine reductase deficiency

(2) Defective synthesis of biopterin

Two patients with a typical phenylketonuria showed a mean serum Crithidia factor of 4.95±0.45 ng/cm^3 which is significantly higher than normal; these are presumed to have dihydropteridine reductase deficiency leading to impaired salvage of quinonoid dihydrobiopterin.

The two brothers E.D. and C.D. which are similar neurologically and biochemically showed a response to oral phenylalanine, but the rise in Crithidia factor was lower and slower than normal. This suggests that they have partial block in BH_4 synthesis. The material found on chromatography of C.D. serum was 7,8 dihydrobiopterin which supports the

hypothesis that synthesis was not totally inactive.

(3) Septrin (= trimethoprim + sulphathiazole)

Trimethoprim is an inhibitor of bacterial DHFR and also has much a smaller effect on mammalian DHFR. Brown (1981) found it also inhibits DHPR.

Sulphathiazole also inhibits DHPR but less effectively. *In vitro*, the drug Septrin was found to inhibit DHPR and to have a synergestic effect i.e. the inhibition achieved with Septrin was greater than the inhibition achieved by the sum of the two components. So we carried out an investigation of the effects of these drugs on BH_4 metabolism *in vivo*.

Volunteers who were given oral trimethoprim showed an increase in mean serum c.f. in 6 hrs whereas those who had taken sulphathiazole showed no great response. The people who had both drugs showed a significant increase in 24 hrs. except one subject showed a high response in 6 hrs.

Only trimethoprim is a significant inhibitor *in vivo*.

It has been claimed that trimethoprim inhibits the conversion of 7,8 dihydrobiopterim to BH_4 by dihydrofolate reductase (Stone 1976) and this would also add to increased serum 7,8 dihydrobiopterin levels and lower BH_4 levels.

CHAPTER THREE

ANANALYSIS OF URINARY PTERINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Introduction

Various laboratories have shown there is a correlation between disease states and pteridine content in body tissues and fluids. A variation in biopterin content in serum or urine of known types of phenylketonuria (Leeming *et al* 1976a; Kaufman *et al* 1978; Danks *et al* 1978), decreased biopterin in urine of patients with rheumatoid arthritis, leukaemia and coeliac patients, and an increase in serum biopterin level during kidney dysfunction and dystonia (Leeming *et al* 1976b) have all been reported.

Separation of pteridines has been accomplished by variety of chromatographic techniques including paper chromatography, unidimensional thin layer chromatography (Blakley 1969), two dimensional thin layer chromatography (Wilson *et al* 1975) ion exchange chromatography (Rembold 1971) gas chromatography/mass spectrometry (Liloyd *et al* 1971) high performance liquid chromatography (Halpern *et al* 1977; Stea *et al* 1980; Fukushima and Nixon 1979, Dhondt *et al* 1981b) and column chromatography (Fukushima and Shiota 1972).

The pteridines found in human urine are mainly biopterin and neopterin either in a reduced or oxidized state (Fukushima and Shiota 1972) smaller amounts of pterin 6-carboxylic acid, pterin-6-carboxaldehyde, xanthopterin, isoxanthopterin and sepiapterin are also present (Stea *et al* 1980).

Neopterin is likely to be derived from the normal precursor of biopterin, neopterin triphosphate, because neopterin and it's derivatives carry 3 carbon chain on C_6, and cannot be derived from folic acid.

This newly developed technique of HPLC is employed to separate rapidly and to determine readily the level of unconjugated pterins in biological fluids. This fast and sensitive method might be used to examine defects in pterin metabolism. Since 1977 several reports refer to elevated pterin levels in urine of cancer patients (Halpern *et al* 1977; Rokos *et al* 1980). Elevated pterins have been also shown in urine of mice bearing Ehrlich ascites tumour cells (Watcher *et al* 1979a) and in the blood of patients affected by a variety of cancers (Kokolis and Ziegler 1977). These workers also showed that there is no correlation between high or low pteridine values and antibiotic or radiation therapy, and they did not observe any significant effect of inhibitors of dihydrofolate reductase (trimethoprim or methotrexate) on urinary pteridine excretion.

Malignant hyperphenylalanimia (MHPA) due to dihydropteridine reductase deficiency or due to biopterin synthesis deficiency could be diagnosed by the urinary level of biopterin, neopterin or the ratio of neopterin to biopterin (Dhondt *et al* 1980b). MHPA due to dihydropteridine reductase deficiency can also be diagnosed by measurement of enzyme activity in culutred fibroblasts or in liver biopsies (Kaufman *et al* 1975) but neither assay is ideal as the growth of enough cells in culture requires several weeks and biopsy of the liver involves a certain degree of risk to the patient. So high performance liquid chromatography (HPLC) analysis of urine would be a

a better and less invasive way to diagnose these two forms of MHPA.

In this study we looked at the level of neopterin (D-neopterin + L-neopterin) and biopterin (as biopterin + xanthopterin) and the ratio of Neopterin/biopterin+xanthopterin in norma subjects, variant phenylketonuria, Down's syndrome and cases of non specific mental retardation, demented patients, cancer and dystonia patients.

MATERIALS AND METHOD

Biopterin and neopterin were supplied by Dr. B. Schircks (postfach CH8623 Wetzikon, Zurich). High pressure liquid chromatography was used to separate the components of the mixtures. These compounds are first dissolved in a liquid solvent and forced to flow through a chromatographic column under high pressure by pump LDC III. Pteridines were separated on a reversed phase columns packed with Spherisorb ODS 5 µM (25 cm x 4.6mm) (Jones Chromatography Ltd.).

The detector used in this study was an LDC fluoromonitor III (Model 1311) and the recorder was JJ CR 652 single channel. The pteridines were identified by their retention times in 5% aqueous methanol as a solvent in the HPLC.

Pteridines were quantitated by comparison of the peak areas at a flow rate of 1ml/min on a sensitivity setting of 0-100 according to a calibration curve. Calibration curves were constructed using a selection of sensitivity settings as follows 0-50, 0-100, 0-200. From these we found 0-100 to be the

best sensitivity and it was used through out the experiment; standard curves for neopterin and biopterin are shown in Fig. 3.1 and 3.2. The calibration curves were performed by injection of 20 µl of a standard solution of biopterin (0.15, 0.2, 0.4, 0.5, 0.6 and 0.7 µg/ml) and neopterin (0.2, 0.5, 1.0, 1.2 and 1.4 µg/ml). Filtration of urine through 0.22 Millipore filter was performed before injecting the sample in a 20 µl loop.

Pteridine concentrations are given relatively to urinary creatinine concentration (in ng/mg creatinine). Creatinine levels were measured in Clinical chemistry, General Hospital, Birmingham. Elution positions of various standard pterins are shown in table 3.1.

Biopterin results include biopterin + xanthopterin. Neopterin results include (L-neopterin + D-neopterin).

Varying concentrations of acetonitrile were also tested as a solvents and the best separation for biopterin from neopterin was found at 5% acetonitrile (elution position of biopterin 2.4 cm, xanthopterin 2.3 cm and neopterin 1.6 cm). The separation achieved with 5% acetonitrile was inferior to that achieved with 5% methanol.

PREPARATION OF SAMPLES

(1) Normal subjects: Normal urine samples were taken from a total of 14 university staff and students, there were 5 females and 9 males aged between 23-57 years.

Fig. 3.1 High performance liquid chromatography biopterin standard curve.

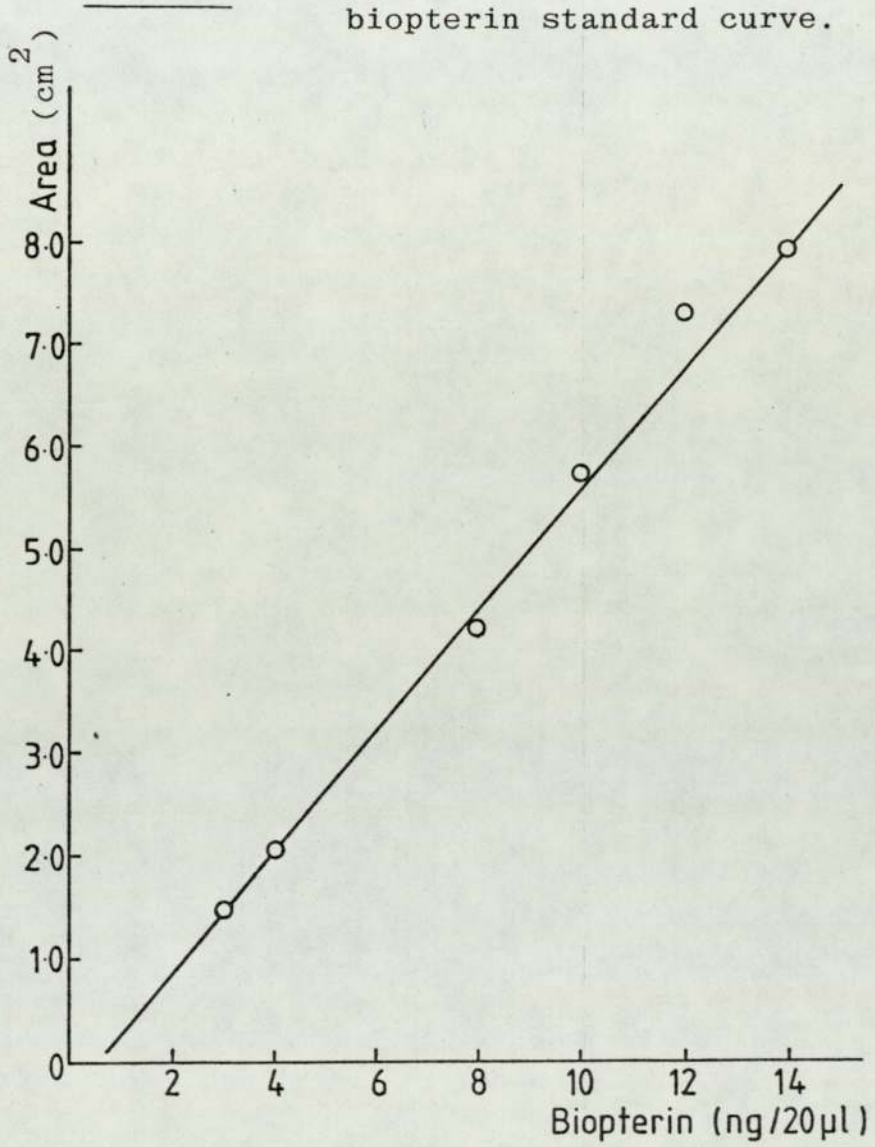


Fig. 3.2 High performance liquid chromatography neopterin standard curve.

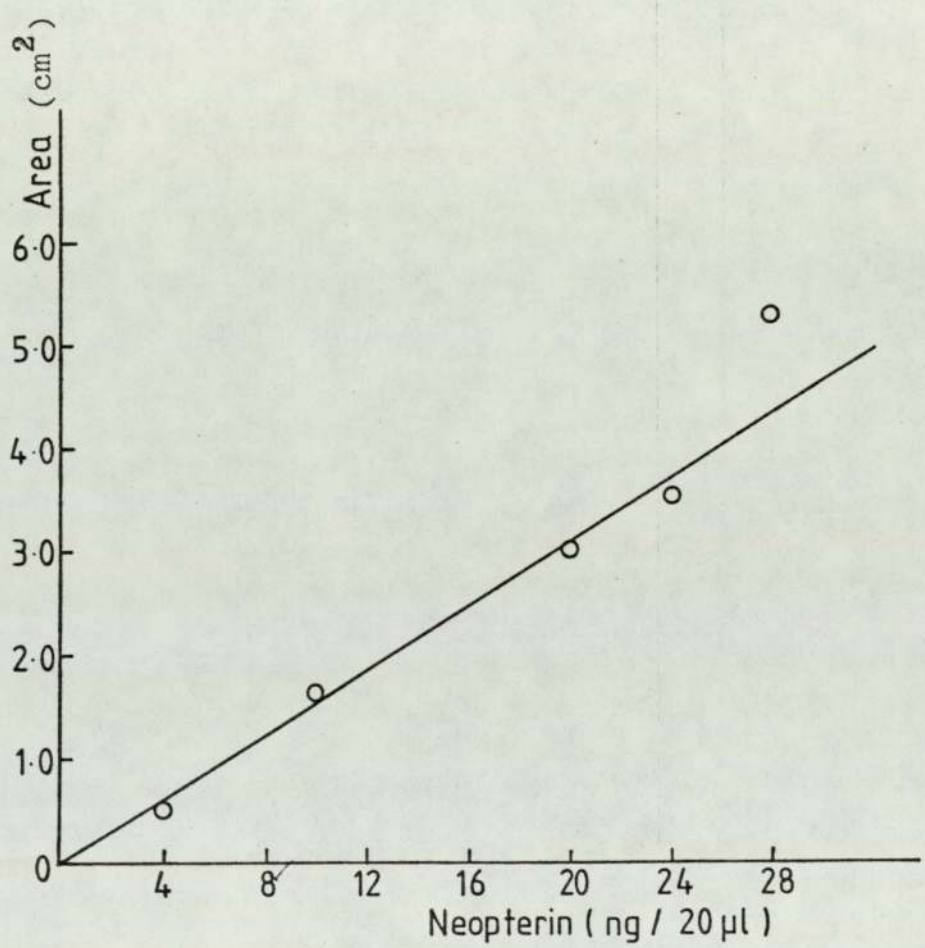


TABLE 3.1

ELUTION POSITION OF DIFFERENT PTERINS

IN 5% METHANOL

biopterin	3.5 cm
xanthopterin	3.3 cm
D-neopterin	2.0 cm
L-neopterin	2.1 cm
pterin-6 CHO	0.7 cm
pterin 6 COOH	0.7 cm
pteroic acid	0.7 cm
tetrahydrobiopterin-dihydrochloride	6.8 cm
pterin	10.4 cm
2-amino-4-hydroxy 6-hy droxy methyl-pterin	4.0 cm

(2) Malignant hyperphenylalaninaemic patients: samples were obtained from Dr. R.J. Leeming, General Hospital, Birmingham.

(a) dihydropteridine reductase deficients.

P.D. this patient has been reported by Rey *et al* (1977). The child received a phenylalanine load, 500 mg/m^2 surface area of the child pure phenylalanine was dissolved in physiological saline and sterilized by passing through a millipore filter and was infused intravenously during 3 hrs. Urine samples were obtained before and 12hrs and 18hrs after the dose.

K.H. this patient was 18 months old; phenylalanine level at birth 0.22 mmol /l. When he was on a low phenylalanine diet phenylalanine dropped to 0.18 mmol /l and serum biopterin was 5.9 µg/l. Following an oral load of phenylalanine, phenylalanine were maximised at 4 hrs.(1.07mmol /l) serum biopterin became 15.3 µg/l. He had a liver biopsy, Phenylalanine hydroxylase was normal, but dihydropteridine reductase activity was zero. They put him on neurotransmitter replacement therapy but he continued to regress. He received 100 mg/Kg body wt phenylalanine orally. Urine samples were obtained before dosing and 3hrs. after the dose. On a different occasion a fasting urine sample was obtained at 6.30 am. Then at 8.0 o'clock the patient received 100 mg/Kg body wt phenylalanine orally, followed at 10.00 a.m. by 40mg tetrahydrobiopterin. Urine samples were obtained at 11.30 a.m.

(b) Synthesis deficient.

Y.L. He was two months old. This patient received an intravenous load phenylalanine in the same way as P.D, serum

biopterin and phenylalanine level before and after the dose shown in Table 3.2

TABLE 3.2

SERUM DIHYDROBIOPTERIN AND PLASMA PHENYLALANINE IN MALIGNANT HYPERPHENYLALANINAEMIC PATIENT [Y.L.] BEFORE AND AFTER INTRAVENOUS LOAD OF PHENYLALANINE.

	Biopterin	Phenylalanine
Y.L. before dose	0.2 µg/l	0.18 mmol /l
after 3 hrs	0.28µg/l	3.33 mmol /l
24 hrs after dose	0.51 µg/l	2.242 mmol /l

urine samples were obtained 3hrs and 24 hrs after the dose.

C.A. Described by Rey *et al* (1977), She was on phenylalanine restricted diet.

(c) Partial deficiency of synthesis.

L.A. Described by Rey *et al* (1979 and 1980), Urine samples were obtained before and 12hrs. and 18hrs. after an intravenous dose of phenylalanine.

(d) ?Heterozygotes, Partial defect in dihydropteridine reductase.

S.H. She is the sister of K.H. She was five months old. In the fasting state phenylalanine level was normal, 0.06 mmol /l and 0.05 mmol/l , while biopterin measured in two occasions was 3.1 µg/l, and 2.7 µg/l which are at the upper end of the normal range. It is probable she is heterozygote.

Mr. and Mrs. H., parents of S.H. and K.H. Urine samples were obtained from S.H. before and after a 200 mg phenylalanine load orally. Urine samples were also obtained from their parents before and after 100 mg/Kg body wt. phenylalanine.

(3) Down's syndrome and non specific mentally retarded

15 urine samples were supplied from Dr. Sylvester in St. Lawrence Hospital, Caterham, Surrey. 6 of them were diagnosed to be Down's syndrome and 9 were suffering from non-specific mental retardation

(4) Senile dementia

9 urine samples from demented elderly subjects were obtained from All Saints Hospital, Birmingham and 4 others from St. George's Hospital, Stafford.

(5) Dystonia patients

2 urine samples from dystonia patients were supplied by Dr. I. Smith, Institute of Child Health, London.

(6) Malignant disease

17 urine samples from cancer patients.

13 from Dudley Road Hospital, Birmingham.

3 from the General Hospital, Birmingham.

1 from All Saints Hospital, Birmingham.

Urine samples from normals and patients were used immediately or stored at -20°C, urine was collected at any time of the day. Stea *et al* (1980) established that excretion of pterins remains relatively constant throughout the day. Analysis of urinary pterins was performed without knowlege of diagnosis. Urine samples were filtered and analysed by HPLC.

RESULTS

(1) NORMAL SUBJECTS

TABLE 3.3

DISTRIBUTION OF BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND N/B+X RATIO IN URINE OF 14 NORMAL SUBJECTS

(a) Female	(B+x)	N	N/(B+X)
A.G.	239.4	359.3	1.43
O.S.	239.4	239.4	1.00
A.P.	98.9	152.0	1.54
J.H.	184.9	462.0	2.5
P.R.	81.9	397.6	4.86
Mean±S.E.M.	169±33.7(5)	321.9±55.7(5)	2.3±0.69 (5)

(b) Male	(B+x)	N	N(B+x)
D.E.	75	225	3.0
N.K.	69.7	132.6	1.9
M.C.	245.6	361.9	1.49
D.H.	127.7	397.1	3.1
A.A.	103.1	360.8	3.5
J.B.	269.3	631.4	2.3
C.E.	63.6	448.5	7.0
R.L.	56.1	126.3	2.25
R.C.	68.6	142.7	2.08
Mean±S.E.M.	119.6±26.9(9)	314±57.2 (9)	2.96±0.57(9)
TOTAL	137±21.3(14)	316.9±40.4(14)	2.7±0.45(14)

All values are in ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

Number of subjects in brackets.

(Biopterin + xanthopterin) level in females (169±33.7 ng/mg creatinine) was higher than in males (119.6±26.9 ng/mg creatinine) $\Delta(B+x) = 49.4$, but not significantly so ($p>0.3$).

Neopterin [D-neopterin + L-neopterin] for the females 321.9±55.7 ng/mg creatinine also higher than males (314±57.2 ng/mg creatinine) $\Delta N=7.9$ but again not significantly so ($p<0.9$).

The range of the ratio $N/_{B+x}$ for the normal subjects was 1.0-7.07 with only one subject above 3.5. The mean of the ratio for all normal subjects was 2.7±0.45. All values are shown in Table 3.3.

(2) MALIGNANT HYPERPHENYLALANINAEMIA (MHPA)

TABLE 3.4

(BIOPTERIN+XANTHOPTERIN), NEOPTERIN AND N/(B+X) RATIO FOR MALIGNANT HYPERPHENYLALANINAEMIC PATIENTS.

(a) REDUCTASE DEFICIENT

Subject	B+x	N	N(B+x)
*K.H	1435.0	1932.4	1.4
K.H	1921.6	1647.1	0.86
P.D	1857.0	865.7	0.46

(b) SYNTHESIS DEFICIENT

Subject	B+x	N	$N/_{B+x}$
C.A.	526.1	32123.1	61.0

(c) PARTIAL SYNTHESIS DEFICIENT

Subject	B+x	N	N/B+x
L.A.	147.1	3591.1	24.4

(d) (HETEROZYGOTES) PARTIAL REDUCTASE DEFICIENT

Subject	B+x	N	N/B+x
S.H.	1460.1	295.7	0.2
Father of S.H. and K.H.	23.7	128.0	5.4
Mother of S.H. and K.H.	78.9	434.1	5.5

All values are given in ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

* Two urine samples were obtained in different occasions.

K.H. and P.D. were diagnosed to be dihydropteridine reductase deficient, they had high levels of (B+x) and N, the ratio of $N/_{B+x}$ was lower than one for P.D. and for K.H. on one occasion.

C.A. Patient diagnosed to be synthesis deficient had a very high value of neopterin 32123.1 ng/mg creatinine which is very much higher than normal. The biopterin + xanthopterin level was also higher than normal, but the rise was not so marked. The ratio of $N/_{(B+x)}$ was 61.0 which is extremely high.

L.A. A patient diagnosed to be partially synthesis deficient had a high level of neopterin, and the ratio of $N/_{(B+x)}$ was also high compared to normal and fell between the normal value and that shown by C.A.

S.H. She is a sister of K.H. and a definitive diagnosis of her condition is not yet available. However the measurement of serum Crithidia factor suggests that she may have a partial defect in dihydropteridine reductase. She had a high level of (B+x) in the urine at 1460 ng/mg creatinine and a low level of neopterin giving a ratio of N/(B+X) of 0.2 which is lower than normal and similar to values of dihydropteridine reductase deficiency. Both parents of S.H. and K.H. had a higher neopterin level than biopterin level, and (B+x) values for both of them were lower than normal (23.7 and 78.9 ng/mg creatinine).

Values are shown in Table 3.4.

Loading experiments with malignant hyperphenylalaninaemic subjects.

(1) Dihydropteridine reductase deficiency+phenylalanine load

TABLE 3.5

BIOPTERIN+XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO IN TWO PATIENTS K.H. AND P.D. BEFORE AND AFTER PHENYLALANINE LOAD

	B+x	N	$N/_{B+x}$
K.H. Before dose	1435.0	1932.4	1.4
2 hrs. after dose	1327.6	4209.3	3.2
P.D. before dose	1857.0	865.7	0.47
12 hrs.-18hrs.	2975.4	1669.1	0.56
18hrs.-24hrs.	6475.1	4532.9	0.7

All values are in ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

From Table 3.5 it can be seen that an oral dose of phenylalanine given to K.H. caused lower (B+x) and higher neopterin exertion, but an intravenous dose of phenylalanine caused higher (B+x) and neopterin levels in P.D. The overall effect in both cases was to raise the $N/_{B+x}$ ratio.

(2) Dihydropteridine reductase deficiency + tetrahydrobiopterin + phenylalanine load

TABLE 3.6

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO IN URINE OF PATIENT K.H. BEFORE AND AFTER 100 mg PHENYLALANINE ORALLY/Kg BODY WT. + 40 mg TETRAHYDROBIOPTERIN ORALLY

	B+x	N	$N/_{B+x}$
K.H. before dose	1921.6	1647.1	0.86
4½ hrs. (after dose)	1993.0	1329.3	0.67

values are in ng/mg creatinine

B+x = Biopterin + xanthopterin

N = Neopterin

(Biopterin + xanthopterin) level and neopterin was higher than normal levels but after (40 mg orally tetrahydrobiopterin + 100 mg/Kg body wt. orally phenylalanine). (B+x) rose and neopterin excretion fell, thus the ratio of $N/_{B+x}$ became lower also (Table 3.6). This is in contrast to the result obtained following phenylalanine alone (Table 3.5).

(3) Synthesis deficiency + phenylalanine load

TABLE 3.7

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO IN PATIENT Y.L. BEFORE AND AFTER INTRAVENOUS LOAD OF PHENYLALANINE

	B+x	N	$N/_{B+x}$
Y.L. 3 hrs after the dose	95.3	8339.0	87.5
Next day	1485.6	17084.0	11.5

All values in ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

In this patient (B+x) and neopterin levels were lower 3hrs after an intravenous dose of phenylalanine than on the following day. Phenylalanine load seem to increase excretion of (B+x) and neopterin, in Table 3.2 phenylalanine load increased serum biopterin derivatives 3 hrs after the dose and was still rising 24 hrs. after the dose. One might expect the same happened here.

(4) Partial synthesis deficiency + phenylalanine

TABLE 3.8

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+X}$ RATIO IN PATIENT L.A. BEFORE AND AFTER INTRAVENOUS LOAD OF PHENYLALANINE

	B+x	N	$N/_{B+x}$
L.A. before dose	147.1	3591.1	24.4
12hr-18hr	73.1	4676.9	64.6
18hr-24hr	167.0	16407.9	98.0

All values ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

(B+x) level decreased 12 hrs. after an intravenous dose of phenylalanine and became higher after 24 hrs. but neopterin level and the ratio of $N/_{B+x}$ became higher after 12 hrs. and was still rising 24 hrs. after the dose (Table 3.8).

(5) ?Heterozygotes for dihydropteridine reductase deficiency +phenylalanine dose.

TABLE 3.9

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO BEFORE AND AFTER PHENYLALANINE LOADS GIVEN TO S.H. AND PARENTS OF K.H. AND S.H.

	B+x	N	$N/_{B+x}$
S.H. before dose	1460.0	296	0.2
after dose	721.6	451	0.63
Father of K.H. and S.H. before dose	23.7	128.1	5.4
after dose	123.3	331.4	2.69
Mother of K.H. and S.H. before dose	78.9	434.1	5.5
after dose	157.8	410.4	2.6

All values are in ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

S.H. showed high levels of (B+x) in urine which fell after a dose of phenylalanine and neopterin output became higher after the dose, and the ratio of $N/_{(B+x)}$ became higher also. Parents showed changes in (B+x) and neopterin which resulted in fall in the $N/_{(B+x)}$ ratio in both cases unlike their children (Table 3.9).

(3) Down's syndrome and non-specific mental retardation

TABLE 3.10

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO IN URINE OF PATIENTS WITH DOWN'S SYNDROME AND NON-SPECIFIC MENTAL RETARDATION.

Down's Syndrome	B+x	N	N/B+x
M.	99	185.7	1.9
C.B.	101.9	183.3	1.8
B.V.	136.5	716.7	5.25
M.G.	202.0	732.4	3.63
A.W.	122.5	471.1	3.85
M.B.	307.6	2007.4	6.53
Mean±S.E.M.	161.6±32.9(6)	716±276.4(6)	3.8±0.76(6)

Non-specific mental Retardation	B+x	N	N/B+x
A.B.	350.3	731.4	2.0
E.T.	232.6	277.6	3.0
P.J.	52.6	59.1	1.1
W.P.	78.5	314.1	4.0
P.M.	193.6	791.3	4.09
E.S.	147.7	698.6	4.7
W.G.	210.4	252.6	1.2
B.S.	85.3	256.0	3.0
S.E.	171.3	1947.1	11.38
Mean±S.E.M.	169.1±30.9(9)	591.9±197.3(9)	3.85±1.28(9)

All values are in ng/mg creatinine
(B+x) = Biopterin + xanthopterin
N = Neopterin

Number of subjects in brackets

Neopterin levels for Down's syndrome patients was 716±276.4 ng/mg creatinine and for non-specific mental retardation 591±197.3 ng/mg creatinine. These values are higher than normal neopterin levels ($0.1<p<0.2$) for both. (B+x) values for Down's syndrome was 161.6±33 ng/mg and for none-specific mental retardation 169±31 ng/mg creatinine; these values are slightly higher than normal but not significantly so ($p>0.5$ and $p>0.4$) respectively (Table 3.10). Two of these patients had a particularly high level of neopterin. One diagnosed as Down's syndrome patient M.B. with a neopterin level of 2007.4 ng/mg creatinine and the other with non specific mental retardation had a neopterin level of 1947 ng/mg. If we exclude these two values, neopterin level for the Down's syndrome patients will be 457±120.8 ng/mg creatinine, and 423±97 ng/mg creatinine for non-specific mentally retarded which are also higher than neopterin level in normal subjects.

(4) Senile dementia

TABLE 3.11

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO IN 9 SENILE DEMENTED PATIENTS

St. George's Hospital	B+x	N	$N/_{B+x}$
D.P.	341.3	801.4	2.4
L.B.	131.1	2039.9	14.6
G.P.	114.9	918.4	8.0
C.D.	130.1	313.7	2.4
M.A.	132.9	292.4	2.2
Mean±S.E.M.	169.7±43(5)	873.1±317.7(5)	6.1±2.6(5)

All Saint's Hospital	B+x	N	$N/_{B+x}$
H.H.	76.6	937.6	12.3
L.W.	103.7	546.6	5.3
E.M.	68.1	247.5	33.6
W.	119.8	414.7	3.6
Mean±S.E.M.	92±12(4)	536.6±147(4)	6.2±2.07(4)

All values are in ng/mg creatinine
(B+x) = Biopterin
N = Neopterin
Number of subjects in brackets

Neopterin level for the demented patients was higher than neopterin level in normal subjects, and the ratio of $N/_{B+x}$ significantly higher than normal ($p<0.05$).

(5) Dystonia Patients

TABLE 3.10

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+x}$ RATIO IN TWO DYSTONIA PATIENTS F.O. AND S.W.

Dystonia	B+x	Neopterin	N/(B+x)
F.O.	99.5	183.4	1.8
S.W.	112.3	322.7	2.75

All values in ng/mg creatinine

(B+x) = Biopterin + xanthopterin

N = Neopterin

Dystonia patients had lower (Biopterin + xanthopterin) and neopterin levels than normal. As excretion of both pterins was low the $N/_{B+x}$ ratios fell within the normal range.

(6) Malignant disease

TABLE 3.13

BIOPTERIN + XANTHOPTERIN, NEOPTERIN AND $N/_{B+X}$ RATIO IN 17 PATIENTS WITH MALIGNANT DISEASES

	Diagnosis	(B+X)	Neopterin	N/(B+X)
H.	Acute myeloid leukaemia	178.1	404.7	2.27
T.	T. cell lymphoma	131.4	1446.9	11.0
W.	Leukaemia	76.5	238.0	3.1
N.	Carcinoma bronchus	126.3	4690.3	38.6
P.J.	Carcinoma bronchus	58.9	276.7	4.7
W.P.	Carcinoma bronchus	45.3	1255.7	27.7
D.K.	Carcinoma bronchus	189.1	1939	10.25
E.R.	Carcinoma breast	90.4	1377.6	15.0
D.A.	Carcinoma breast	90.1	500.0	5.6
E.U.	Carcinoma rectum	135.3	676.4	5.0
A.D.	Carcinoma endometrium	37.3	1320.1	35.4
A.B.	Carcinoma posterior 1/3 pharynx	64.1	887.8	13.8
L.	Carcinoma squamous cell	861	1396.7	16.2
(1)	Carcinoma primary hepatoma	1008.4	19292.0	19.1
(2)	Carcinoma anaplastic retroperitoneal	594.7	1830.1	30.77
V.T.	Liver metastases unknown primary	89.6	644.7	7.2
H.	Carcinoma oesophagus	36.3	817.6	22.5

All values are in ng/mg creatinine

(B+X) = Biopterin + xanthoperin

N = Neopterin

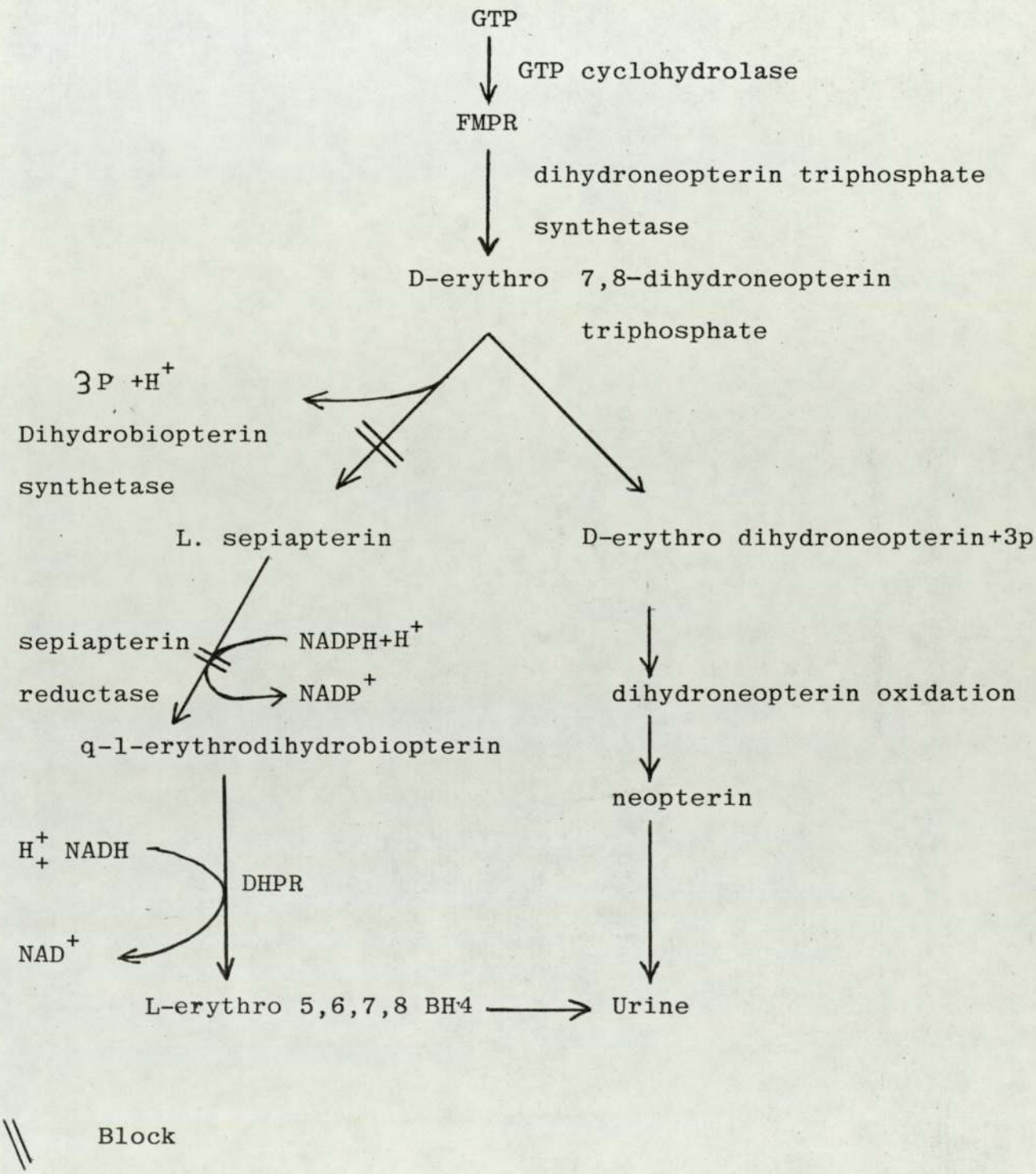
These patients showed low urinary levels of biopterin + xanthopterin with the exception of two cases one of them diagnosed as primary hepatoma had a value of 1008.4 ng/mg creatinine, and the other had a value of 594.7 ng/mg creatinine diagnosed to be anaplastic retroperitoneal. A high level of neopterin was found in these patients except in two cases. One a leukaemic patient had a value of 238.0 ng/mg creatinine and the other with carcinoma of the bronchus had a value of 276.7 ng/mg creatinine. The ratio of N/B+x were high except in some cases which showed ratios within the normal range.

Discussion

Recently there have been several reports of the measurement and identification of pteridines in tissues and biological fluids, but the methods employed were not as efficient as the HPLC method used here. Paper chromatography has been used (Blakely 1969; Halpern *et al* 1977). This is fast but difficult to quantitate and the paper did not have enough resolving power.

Column chromatography (Fukushima and Shiota 1972) requires a large amount of sample, gas chromatography, mass fragmentography (Rother and Karbth 1976) was applied to the analysis of pteridines in urine. This method appears simple and rapid but requires the silylation of pteridines. It is questionable whether this method could be used for the analysis of pteridines in the tissue extracts.

FIG 3.3 THE BIOSYNTHETIC PATHWAY OF TETRAHYDROBIOPTERIN



Tetrahydrobiopterin (BH_4) synthesised from guanosine triphosphate (GTP) (Dalal and Gots 1965; Brown 1981) by a series of reactions (Fig. 3.3) as described earlier in Chapter (1). Any defect in this system will alter the level of pterins in the tissues and biological fluids, because this pathway involves many essential enzymes (GTP cyclohydrolase, dihydrobiopterin synthetase, sepiapterin reductase, dihydrofolate reductase, dihydropteridine reductase). Deficiency of any one of these enzymes would case a disturbance in tetrahydrobiopterin metabolism.

(1) Normal subjects

Neopterin levels which are reported in this study for males and females are very similar to values reported by Watcher *et al* (1979b) which were for the male 280±117 ng/mg creatinine and for the female 340±116 ng/mg creatinine. They are lower than those reported by Rokos *et al* (1980) which were for the male 469±52.3 ng/mg creatinine and for the female 522±55.2 ng/mg creatinine. (Biopterin + xanthopterin) levels reported in this study for the male and for the female were lower than values reported by Rokos *et al* (1980), (B+x) for the male 512±44.5 ng/mg creatinine and for the female 677±82.6 ng/mg creatinine.

The difference between our values and those reported by Rokos *et al* (1980) is probably because of the reduced oxidation of dihydro and tetrahydro precursors.

(2) Malignant Hyperphenylalaninaemia (MHPA)

The ratio of neopterin/$_{(B+x)}$ can be of value in discriminating between MHPA, one due to dihydropteridine reductase deficiency the other caused by a defect in synthesis from GTP.

K.H. and P.D. are diagnosed to be dihydropteridine reductase deficient, (enzyme activity studied by method of Craine *et al* (1972)), this leads to an accumulation of dihydrobiopterin and biopterin because of failure to regenerate tetrahydrobiopterin from quinonoid dihydrobioptern. These subjects showed high levels of biopterin in urine probably due to their dihydropteridine reductase deficiency. Interestingly the urinary neopterin levels were also higher than normal.

The ratio of N/$_{B+x}$ is low and is similar to that reported by Kaufman *et al*(1975);Neiderwiesser *et al* (1979b); Nixon *et al* (1980) and Dhondt *et al* (1981b).

Phenylalanine load decreased urine (B+x) level in the case of K.H. and increased(B+x) level in the case of P.D. This might be due to the different way of administration of phenylalanine; K.H. had it orally and P.D. intravenously. Neopterin levels increase in both cases after phenylalanine load this might be because phenylalanine and its metabolites such as phenylpyruvate inhibit dihydropteridine reductase (Purdy and Blair 1980). Tetrahydrobiopterin levels are reduced, this might increase the conversion of GTP to dihydroneopterin triphosphate which accumulated intracellularly and hydrolysed to neopterin.

Oral tetrahydrobiopterin load increased (B+x) and decreased neopterin in the urine, because high concentration of BH_4 will inhibit GTP cyclohydrolase the first enzyme in synthetic pathway. So less neopterin will be released.

C.A. and L.A.

C.A. was diagnosed to be synthesis deficient. She did not show 7,8 dihydrobiopterin in the serum.

L.A.was diagnosed as partial synthesis deficient. There was a small amount of 7,8 dihydrobiopterin in his serum and urine. Both these patients had extremely high levels of neopterin in urine indicating that there is a block in the biosynthetic pathway between neopterin and biopterin involving the step between dihydroneopterin triphosphate and dihydrobiopterin. The ratio of $N/_{B+x}$ was high and this is similar to that reported by Niederwieser *et al* (1979b); Curtius *et al* (1979), Kaufman (1979) and Nixon *et al* (1980).

L.A. had low Crithidia factor even after phenylalanine load this confirms the finding here, (B+x) level did not change much, but neopterin became higher after the dose. The rise in neopterin level after the dose of phenylalanine might be due the inhibition of dihydropteridine reductase which cause less tetrahydrobiopterin which release the inhibition of GTP cyclohydrolase So tetrahydrobiopterin concentration controls the cyclohydrolase activity in all cases.

Also we notice in both cases L.A. and Y.L. neopterin levels around the same value after 24 hrs.

S.H.

She is the sister to K.H. She showed a high level of biopterin in urine suggesting that she also has dihydropteridine reductase deficiency. After phenyl alanine load she showed similar pattern to her brother K.H., (B+x) lower and neopterin level higher. Parents of K.H. and S.H., are presumably hetrozygotes for dihydropteridine reductase deficiency. They had slightly high ratio of $N/_{B+x}$. Unlike their children, after a dose of phenylalanine, (B+x) becomes higher and neopterin higher in the father and slightly lower in the mother.

(3) Down's syndrome and non-specific mental retardation

These patients showed elevated levels of neopterin and that might caused by a disturbance of BH_4 metabolism. One of the Down's patients had a very high level of neopterin. She was not known to have either a virus infection or a malignant disorder both of which have been reported to raise neopterin excretion (Watcher *et al* 1979). The other patient with non-specific mental retardation had a high level of neopterin and we found that she was receiving 125 mg Dopa/day which has been shown to inhibit dihydropteridine reductase (Purdy *et al* 1981).

Disturbance in tetrahydrobiopterin metabolism might cause the high level of neopterin in both groups. This disturbances were confirmed by high levels of serum Crithidia factor for patients having Down syndrome from the same institute which is significantly higher than normal ($p<0.01$) and the low value of serum Crithida factor for mentally retarded non-Down syndrome which is significantly lower than normal ($p<0.01$) (Aziz *et al*

1981a).

(4) Senile dementia

Patients from St. George's hospital showed a higher level of neopterin in urine than normal subjects and we have details that their dementia was not vascular in origin. Two patients L.B, G.P. had high level of neopterin and we found that they had neither malignant disease nor arthritis.

The high level of serum Crithidia factor which was found in St. George's Hospital patients might be due to haemolysis which elevated the level.

These alterations in biopterin and neopterin levels might be due to the disturbance of BH_4 metabolism. This suggestion is supported by the observation that serum Crithidia factor for demented patients is significantly lower than normal (Leeming and Blair 1980a). Also Nagatsu *et al* (1979) reported low BH_4 levels in the brain of demented people by radioimmunoassay and Williams et al. (1980) showed low hydroxylase cofactor level in presenile dementia.

(5) Dystonia

These two patients showed lower levels of (Biopterin+ xanthopterin) than normal subjects. This is consistent with reports by Williams *et al* (1979) of low cerebrospinal hydroxylase cofactor (BH_4). In inherited dystonia this low level of BH_4 in CSF might cause reduced neurotransmitter synthesis.

(6) Malignant disease

Cancer patients showed high levels of neopterin and low levels of biopterin in urine. These results and others reported by other investigators (Watcher *et al* (1974); Kokolis *et al* 1977, Rokos *et al* 1980; Stea *et al* 1980) indicate that malignant disease is accompanied by disturbance in the biosynthesis and/or catabolism of some pterins. The high concentration of GTP in malignant cells correlates with tumour growth (Kraml *et al* 1976), and increased GTP synthetase activity, quoted as 2.5 fold in renal cell carcinoma (Williams *et al* 1979). This might cause an intracellular accumulation of dihydroneopterin triphosphate which would be hydrolysed and oxidized to neopterin.

Sanachez *et al* (1978) reported low dihydropteridine reductase activity in neoplastic disease which was not confirmed by Dhondt *et al* (1981a) who reported that breast cancer patients had a high dihydropteridine reductase activity. A high activity of this enzyme might explain the low level of biopterin+xanthopterin which we found in cancer patients and also the low level of serum and urine Crithdia factor in malignant patients (Leeming and Blair 1980b).

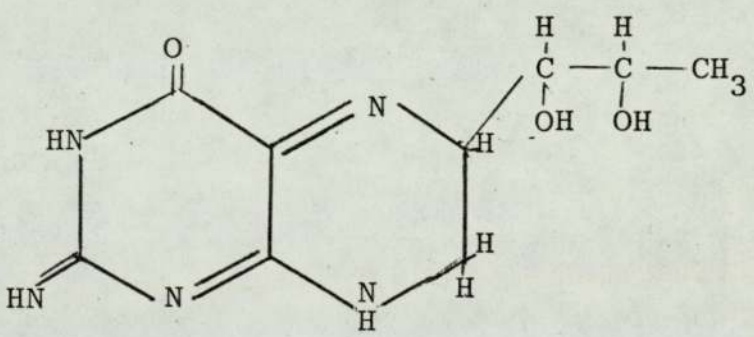
Additional evidence for defective hydroxylation of aromatic amino acids in malignant disease comes from the observation of Zeigler *et al* (1979) who found phenylalanine: tyrosine ratio to be raised in the blood of tumour bearing rats.

CHAPTER FOUR

MEASUREMENT OF TETRAHYDROBIOPTERIN IN URINE

Introduction

Tetrahydrobiopterin (BH_4) act as a cofactor in hydroxylation of phenylalanine to tyrosine (Blakley 1969) in the presence of phenylalanine hydroxylase (Kaufman 1963) During the hydroxylation BH_4 is converted to quinonoid dihydrobiopterin (qBH_2) (Kaufman 1964).

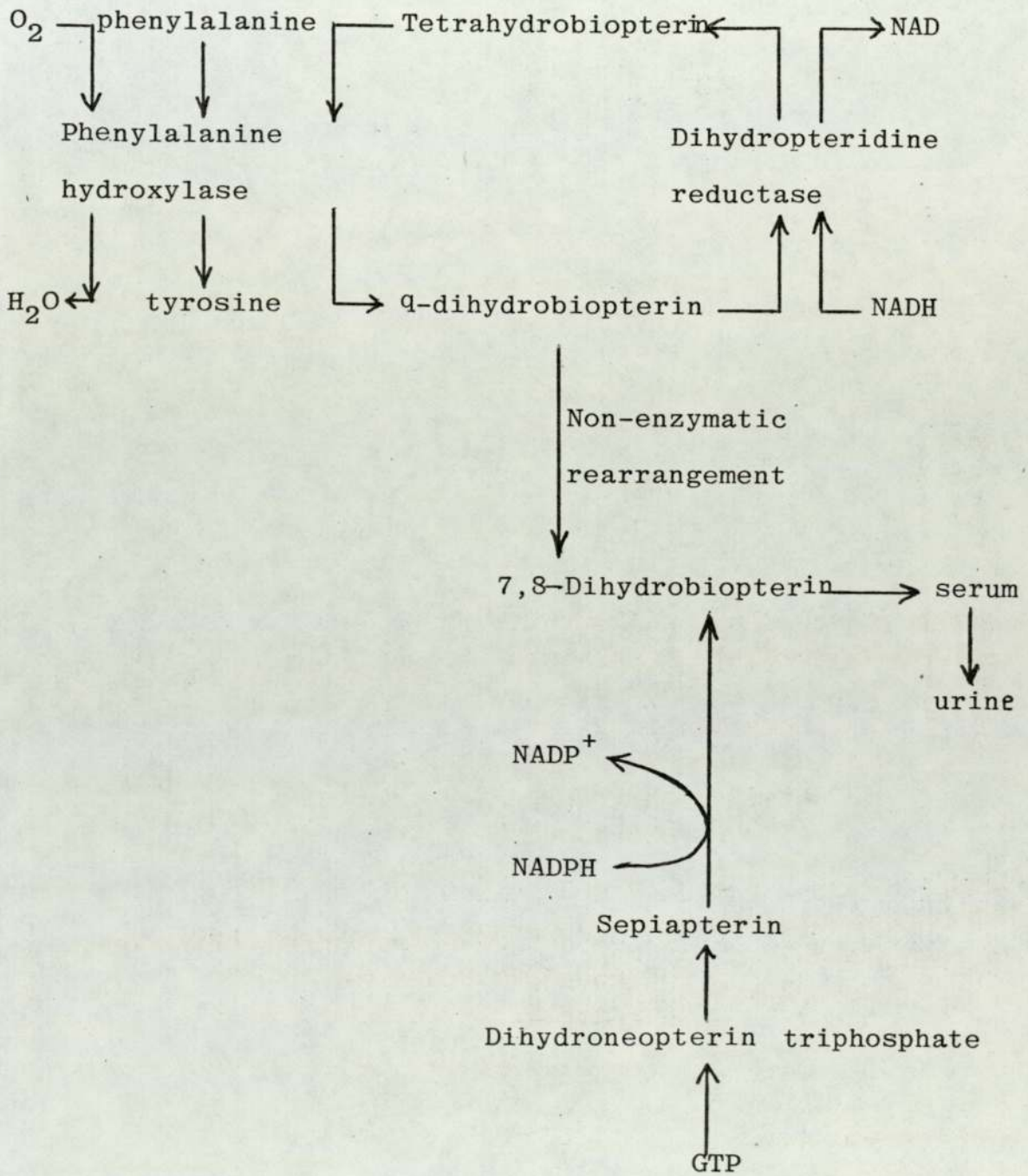


quinonoid dihydrobiopterin

which is reduced back to BH_4 by dihydropteridine reductase (Griffen *et al* 1973; Kaufman 1967). This system is shown in Fig. 4.1.

Hydroxylation of phenylalanine to tyrosine has been shown by perfusing dog liver with a solution of phenylalanine and finding tyrosine in the perfusate (Embden and Baldes 1913). In 1940 Moss and Schoenheimer fed labelled phenylalanine to rats and they found tyrosine to contain some of the label that was originally in the phenylalanine.

FIG. 4.1 SUMMARY OF THE METABOLIC PATHWAYS OF TETRAHYDROBIOPTERIN

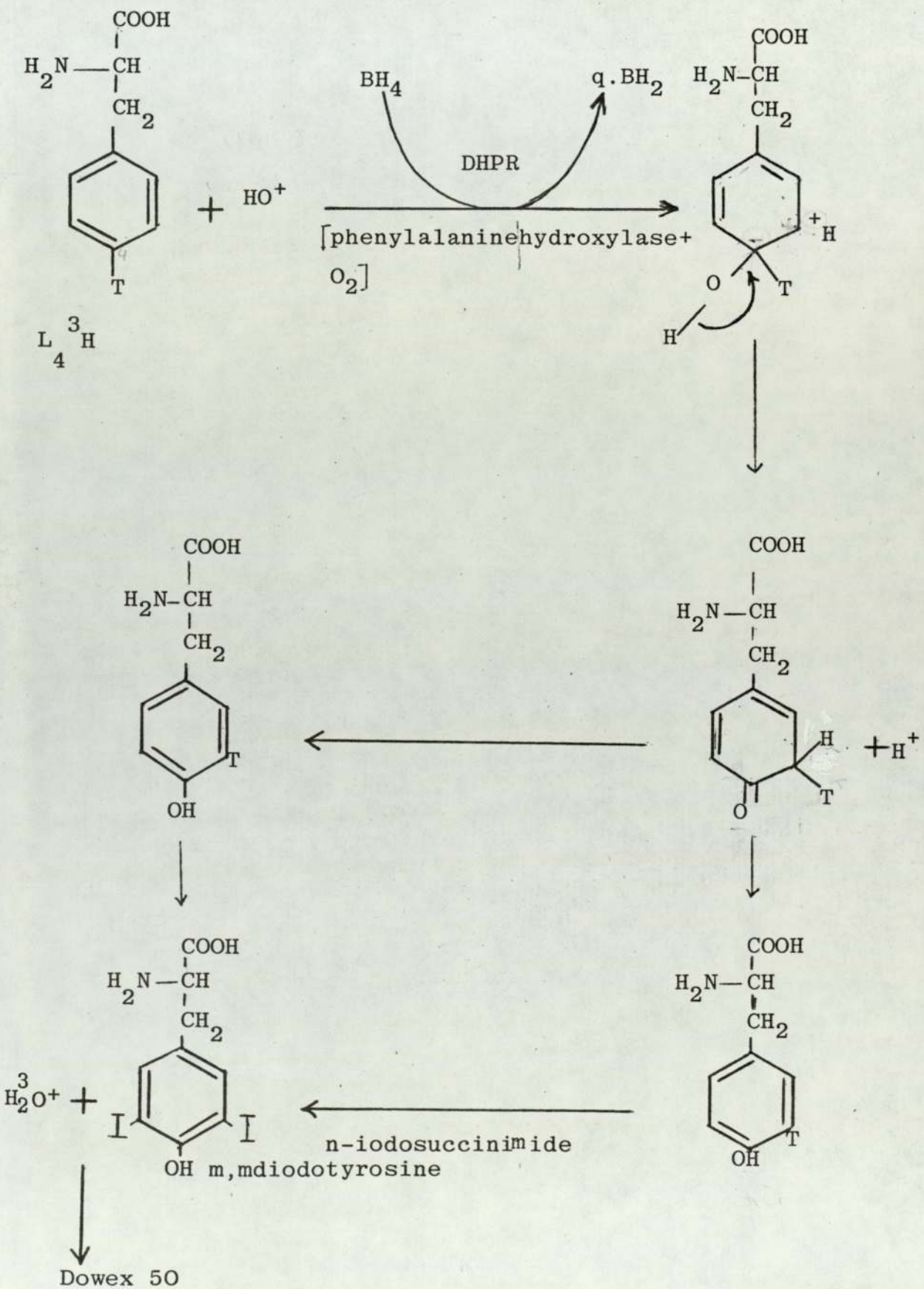


The dependence of hydroxylation on BH_4 has been used to measure BH_4 concentration in cerebrospinal fluid (Lovenberg *et al* 1979; Williams *et al* 1980) in rat liver and brain (Mandell *et al* 1980; Guroff *et al* 1967) in guinea pig liver, brain and kidney (Guroff *et al* 1967) and human liver (Kaufman *et al* 1975; Kaufman *et al* 1978).

In this study we used the same system with some modification to measure BH_4 in urine, whereas Crithidia factor measures biopterin, dihydrobiopterin and BH_4. The method involves hydroxylation of p-tritiophenylalanine and iodination of m-tritiotyrosine by N-iodosuccinimide and determination of the tritiated water formed by these two processes, The tritiated water which is formed is separated from ^3H phenylalanine by passing the mixture through an ion exchange column. Tritiated water released is proportional to BH_4 concentration. The mechanism of this reaction is outlined in Fig. 4.2.

In this the substrate is attacked by OH^+ at the *para*-position and a pair of electrons becomes localized at the *para* position which results in a net unit position charge at the meta position. Coulson *et al* (1968) reported that 0.2% of the substrate oxidized to m-tyrosine, which formed by the attack of OH^+ at *meta* position. Iodination of tyrosine is known to occur exclusively in the *meta* position to form 3-5 diiodotyrosine (Oswald and Zeit 1909), with ^3H release. In this study we tried to measure BH_4 concentration in urine of normal subjects and compare this value with the total biopterin measured by Crithidia assay.

Fig. 4.2 Mechanism of phenylalanine hydroxylation



Preparation of samples and method

Serum samples were obtained from three normal subjects, sodium ascorbate was added immediately to the blood to prevent the oxidation of BH_4. Urine samples were obtained from 10 normal subjects half an hour after they had emptied their bladders and assayed immediately to minimize the oxidation of BH_4. Tetrahydrobiopterin was obtained from Dr. B. Schirks (Postfach CH 8623 Wetzikon). Dimethyl tetrahydrobiopterin ($DMPH_4$) was obtained from Aldrich chemical corporation, phenylalanine hydroxylase from Sigma 4-[^{3}H] phenylalanine from the Radiochemical centre Amersham, England. Calibration curves in duplicate were prepared using fresh BH_4 or $DMPH_4$ ranging from 0-0.5 nM.

The method which we used was essentially that used by Mandell *et al* 1980). The incubation mixture contained:

Standard or urine sample		200 µl
Phenylalanine hydroxylase	2.4 mg/ml	20 µl
Dihydropteridine reductase	2.2 mg/ml	40 µl
Phenylalanine	1.75 mM	20 µl
[4-H^3] phenylalanine	40 mCi/mmol	10 µl
NADH	35 mM	20 µl
Catalase	10 mg/ml	20 µl
Buffer (Tris HCL pH 8.0)	2.1 M	20 µl

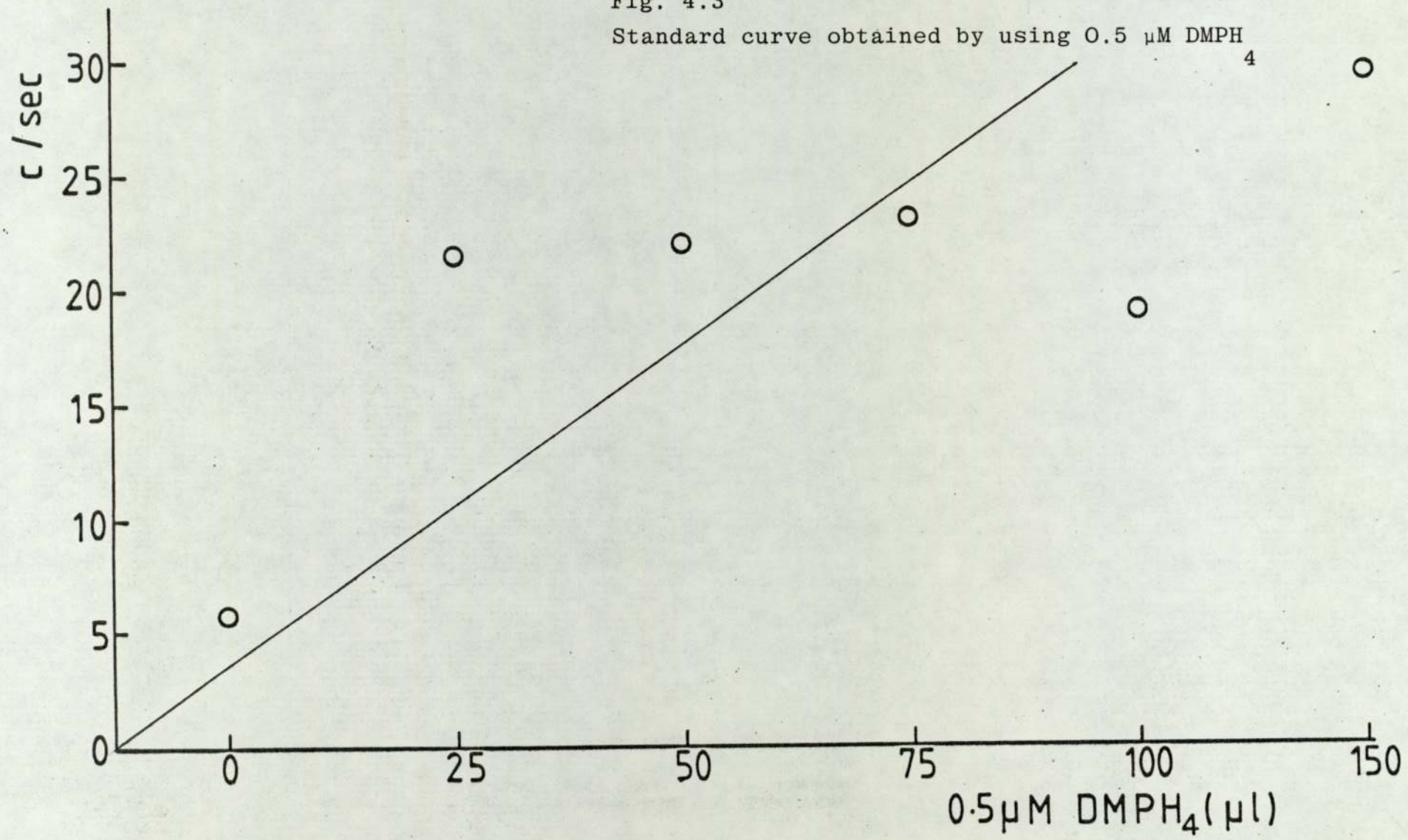
and distilled water to give a final volume 0.35ml. The mixtures were incubated for 30 min at 37°C. The incubation time start by adding standard or samples. After the incubation the mixture was stopped by adding 100 µl of 1.2 M sodium acetate pH 5.5

and then cooled to $0^{o}C$ in ice bath. To release any tritium in position 3 or 5 of the tyrosine formed 20 µl of 1 N iodosuc cinamide (50 mg/ml dimethyl sulfoxide) was added to cooled samples. After five mins. the tritium released as ^{3}HOH, was separated from the remaining phenylalanine by passing a 300 µl aliquot through a column (0.4 x 3.5 cm) containing Dowex $5OH^{+}$ exchange resin. The column was washed twice with 0.65 ml water, and 50 µl of the eluate was counted in a glass scintillation vial with 10 ml scintillation fluid and placed in Beckman LS 7500 liquid scintillation counter to estimate the tritium. Crithidia factor for 9 urine samples were measured by Dr. R. Leeming, General Hospital, Birmingham.

Results.

In the beginning we used 0.5 µM of $DMPH_4$ (the same concentration which used by Lovenberg *et al* (1979) to obtain standard curve (Fig. 4.3) but it did not work. So we used higher concentration 0.5 mM $DMPH_4$ fig 4.4. We obtained two standard curves by using 0.5mM $DMPH_4$ and BH_4 Fig. 4.5. We tried to quantitate BH_4 concentration in the serum but we found the value is low, below the standard curve, so we used urine. Urine BH_4 concentrations with $DMPH_4$ is higher (2.5-4) times than values obtained by using BH_4 standard curve. The values of BH_4 concentration for the individual normal subjects by this method and total biopterin measured by Crithidia fasiculata assay were listed in table 4.1. We notice from the table that the BH_4 values were much higher than the total biopterin measured by Crithidia assay except in two cases. In the case of C.E. total biopterin in urine measured by crithida assay was 3.38 µg/ml

Fig. 4.3
Standard curve obtained by using 0.5 μM $DMPH_4$



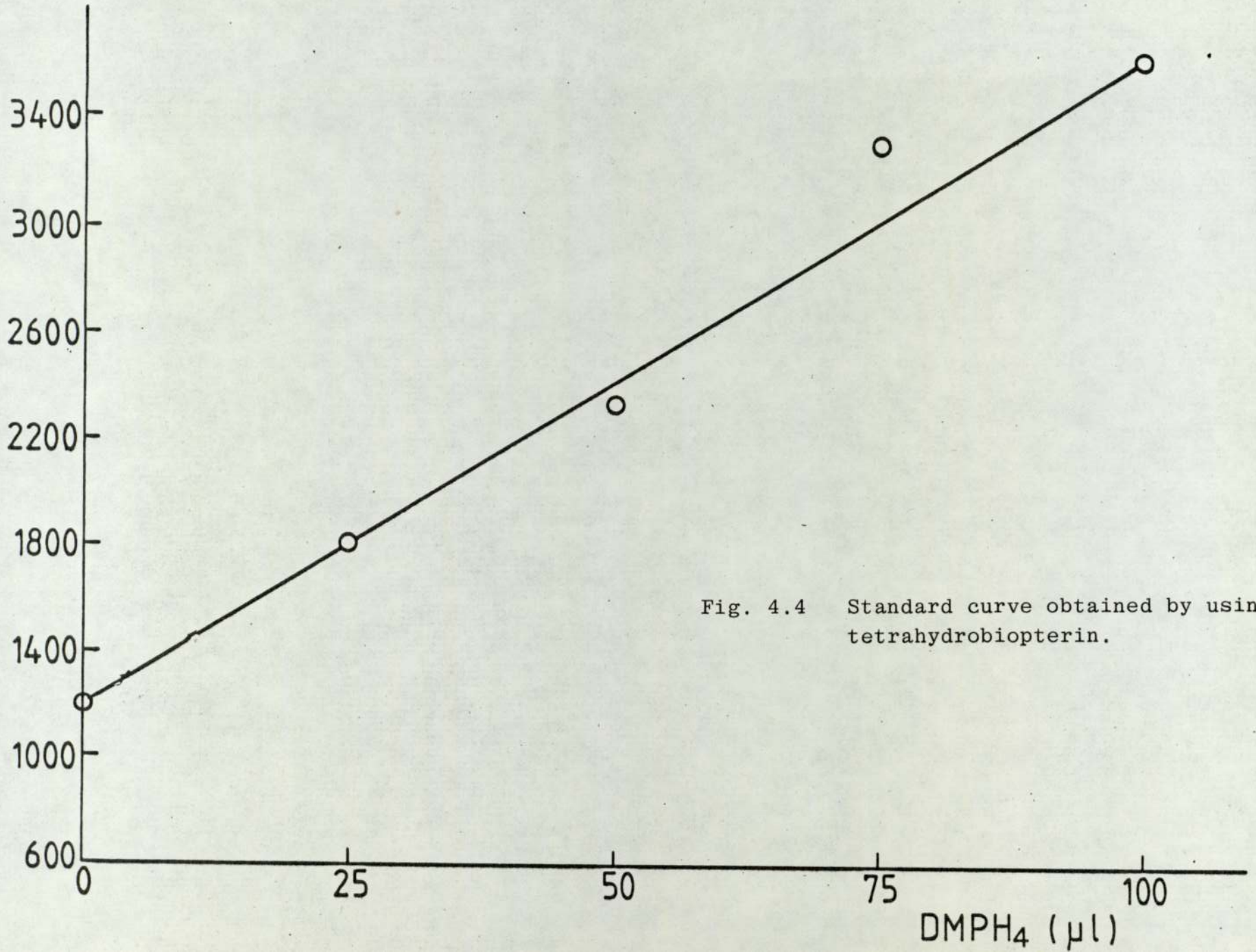


Fig. 4.4 Standard curve obtained by using 0.5 mM tetrahydrobiopterin.

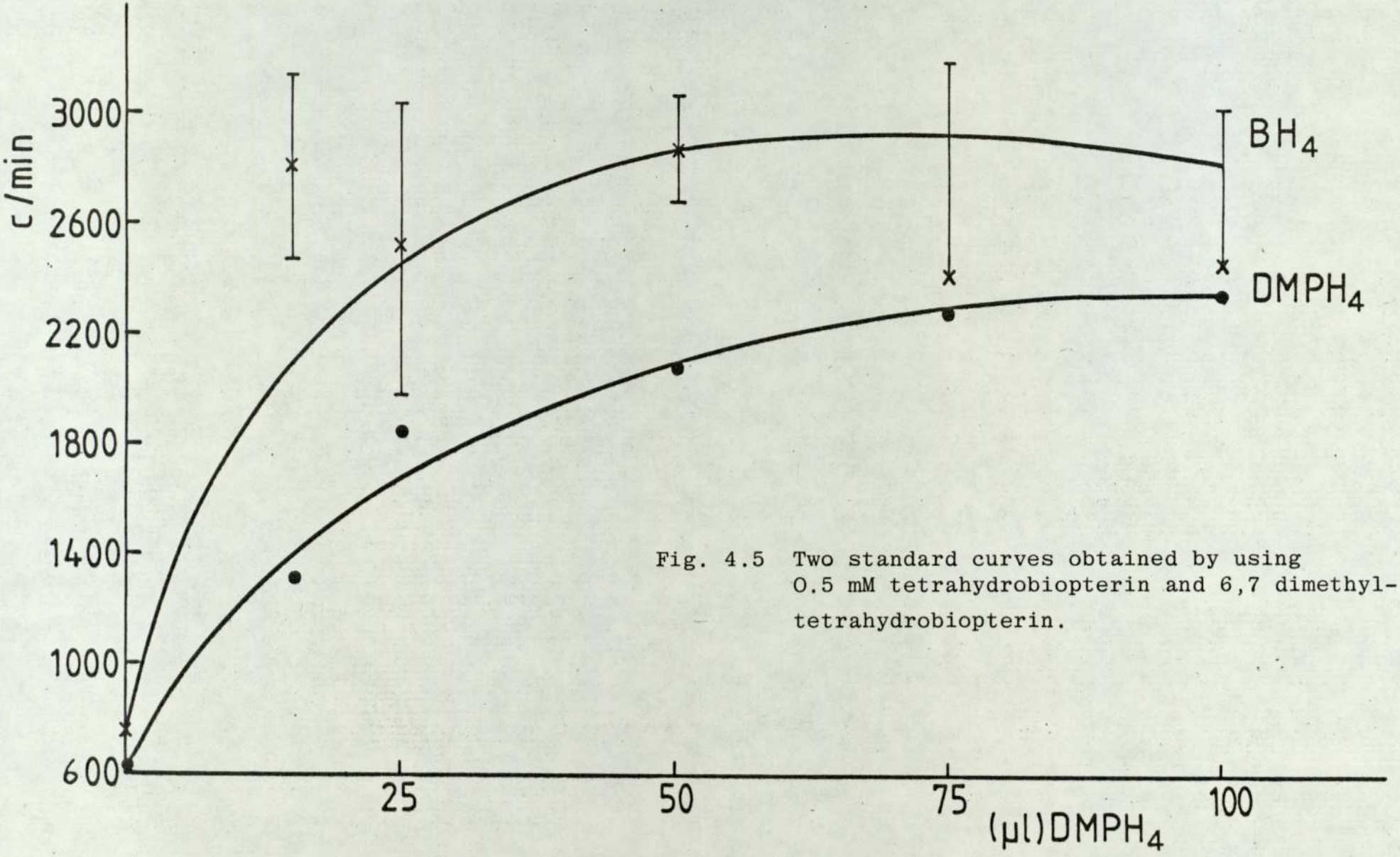


Fig. 4.5 Two standard curves obtained by using 0.5 mM tetrahydrobiopterin and 6,7 dimethyl-tetrahydrobiopterin.

and BH_4 concentration measured in the urine by enzymatic method using BH_4 standard curve was 0.85. µg/ml. The percentage of total biopterin as BH_4 was 25%. In the case of D.AL. total biopterin measured by Crithidia assay was 0.88 µg/ml and the BH_4 concentration in response to BH_4 standard curve was 0.57 µg/ml which is 64% of the total biopterin.

TABLE 4.1

URINE BIOPTERIN DERIVATIVES AND TETRAHYDROBIOPTERIN BY USING 6,7 DIMETHYTETRAHYDROBIOPTERIN AND 5,6,7,8 TETRAHYDROBIOPTERIN STANDARD CURVES.

	Crithidia factor	$DMPH_4$	BH_4
A.S	0.43	14.2	4.8
S.W.	0.05	7.1	2.27
N.J.	0.25	11.3	3.9
J.B.	0.75	9.65	3.81
J.P.	0.38	4.5	1.7
A.P.	0.1	9.0	3.7
J.H.	0.93	10.8	4.26
C.E.	3.38	3.4	0.85
D.AL.	0.88	2.27	0.57
A.G.	*	6.8	2.55
	0.79±0.34(9)	7.9±1.2(10)	2.8±0.47(10)
	Mean±S.E.M.	Mean±S.E.M.	Mean±S.E.M.

All values are in µg/ml

Discussion

Some workers think the method which is described here may be of value in studies on the biosynthesis of hydroxylase cofactor and the level of the cofactor in body fluids and tissues under various pathological conditions. The naturally occuring cofactor is BH_4, but other tetrahydropterins are also active (Storm and Kaufman 1968). The most readily available and most widely used is $DMPH_4$. So BH_4 concentration in the urine were measured with the model cofactor 6,7-$DMPH_4$ standard curve and with BH_4 standard curve. We can see from Fig. 4.2 that response of cofactor assay system to BH_4 was higher than $DMPH_4$ as more ^3HOH were released by using BH_4.

Shiman *et al* (1971) showed that hydroxylation is about twice as fast in the presence of BH_4 as it is in the presence of $DMPH_4$, and this was confirmed by Ayling *et al* (1973) who showed that the pseudocofactor 7,8-$DMPH_4$ had a four times slower rate than natural isomers. So more tritiated water formed in the presence of BH_4 because the reaction is faster. We found that our values by using radioenzymatic assay system is higher than the total biopterin measured by *Crithidia fasiculata*.

A comparison of other values in the literature has shown that other biopterin concentrations calculated for various tissues and fluids using enzymatic method are higher than values using Crithidia assay and HPLC (Fukushima and Nixon (1980) Table 4.2). Also BH_4 concentration measured enzymatically by Lovenberg *et al* (1979) in cerebrospinal fluid is 4.1 ng/ml which is higher than the value reported in humans by Leeming *et al* (1976b) for the total biopterin (1.9 ng/ml).

In general values observed using the enzyme assay are much higher than those obtained by the bioassay and also higher than values by the HPLC. Milstien *et al* (1980) reported that concentration of biopterin in urine per millimeter of urine was 3.7 times higher with enzymatic assay than with HPLC assay.

Tissue	Hydroxylation cofactor assay	Crithidia assay	HPLC
Human Liver	1.4[a]	0.52[b]	-
	4.1[c]	0.11[d]	
Rat liver	13[e]	6[f]	1.6[g]
	2.8[a]	2.3[b]	
Rat brain whole	0.75[e]	0.2[f]	0.09[g]
		0.08[b]	

Data are expressed in µg/g wet wt.

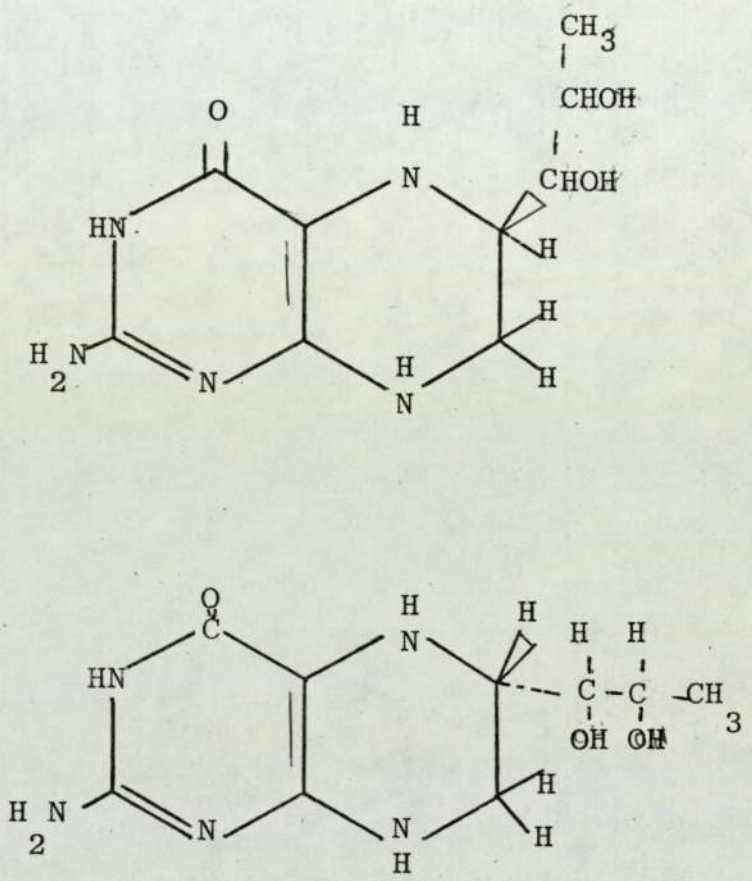
a. Kaufman *et al* (1975)
b. Baker *et al* (1974)
c. Kaufman *et al* (1978)
d. Leeming *et al* (1976b)
e. Guroff *et al* (1967)
f. Rembold and Metzger (1967)
g. Fukushima and Nixon (1980)

Bailey and Ayling (1978) reported that chemical reduction of biopterin to BH_4 produce two isomers Fig. 4.5. In the enzyme assay a mixture of two 6-diasteroisomers of BH_4 has been used as a standard. Since tissues contain only one isomer and this natural isomer is slightly more active for mediating hydroxylation reaction than the unnatural isomer. This might explain a small amount of the difference between the assays

There were some reagents which are known to hydroxylate the benzene ring. Fenton's reagent (hydrogen peroxide, ferrous ion) is known (Lindsay, Smith and Norman 1963) to hydroxylate the benzene ring through generation of the hydroxyl radical. Undenfriend/system (aqueous ascorbate, ferrous ion oxygen) (Brodie *et al* 1954) was shown to lead to non specific hydroxylation of the aromatic nucleus.

In the urine there is non-specific hydroxylation (ascorbate and ferrous ion) which might lead to non-specific hydroxylation of phenylalanine. This could explain the high values obtained in the experiment described in this Chapter and in other tissues

Fig. 4.5



Structures of the two 6-diastereoisomers of L-erythro-tetrahydrobiopterin
(6-L-erythro-2'3',dihydroxypropyltetrahydrobiopterin).

CHAPTER FIVE

GENERAL CONCLUSION

Tetrahydrobiopterin (BH_4) is the cofactor in the hydroxylation of phenylalanine, tyrosine and tryptophan. It is converted to quinonoid dihydrobiopterin (qBH_2) which is reduced to tetrahydrobiopterin by dihydropteridine reductase (DHPR) and nicotinamide adenine dincleotide (NADH). Quinonoid dihydrobiopterin is unstable and rearranges to give 7,8-dihydrobiopterin (7,8-BH_2) which may be converted to tetrahydrobiopterin by dihydrofolate-reductase (DHFR) in the presence of nicotinamide adenine diphosphonucleotide (NADPH). This is summarised in the equations:

$$\text{phenylalanine} + BH_4 + O_2 \xrightarrow{\text{phenylalanine hydroxylase}} \text{tyrosine} + qBH_2 + H2O$$

$$qBH_2 + NADH + H^+ \xrightarrow{DHPR} BH_4 + NAD^+$$

$$qBH_2 \xrightarrow{\text{rearranged}} 7.8\ BH_2$$

$$7,8\ BH_2 + NADPH + H^+ \xrightarrow{DHFR} NADP^+ + BH_4$$

In this study tetrahydrobiopterin metabolism has been examined by three approaches.

(1) estimation of the total biopterin derivatives by using *Crithidia fasiculata* in the normal and diseased subjects.

(2) Measurement of biopterin and neopterin in urine by high performance liquid chromatography (HPLC).

(3) By enzymatic assay to estimate tetrahydrobiopterin in urine.

The mean serum biopterin derivative level in 168 normal male and female subjects was 1.32±0.031 ng/cm^3. The mean serum biopterin level in 44 normal males was 1.43±0.045 ng/cm^3 is significantly higher than 124 normal women which was 1.28±0.036 ng/cm^3 ($0.01<p<0.02$) (Chapter 2)

However these values are lower than those reported by Leeming and Blair (1980a) and (1980b) which were mean serum Crithidia factor levels for total males and females 1.6±0.03 ng/cm^3; for the males 1.75±0.03 ng/cm^3 and for the females 1.53± 0.04 ng/cm^3. In this case the differences between the sexes was not statistically significant. Women on the pill had lower values of mean serum biopterin derivatives (1.2±0.07 ng/cm^3) than women not taking the pill (1.3±0.045 ng/cm^3) but not significantly so ($p>0.3$).

The mean serum biopterin derivative concentration for the pregnant women was significantly lower than that for normal women ($0.001<p<0.01$). The mean serum biopterin derivative concentration for seven pregnant women who showed clinical postnatal depression was 1.129±0.04 ng/cm^3. This value is higher than that for the seventeen other not depressed women (0.99±0.03 ng/cm^3) ($p<0.05$).

Patients with dementia had a mean serum biopterin derivatives level of 1.26±0.04 ng/cm^3 which not significantly lower than normal control (1.32±0.03 ng/cm^3) ($p>0.1$). Analysis of the individual results showed a bimodal distribution; one group with low serum dihydrobiopterin (less than 1.4 ng/cm^3) and group with high serum dihydrobiopterin (equal or higher than 1.4 ng/cm^3). Distribution of results are shown in Fig. 2.9.

The low group of 51 subjects showed an average value of 1.06±0.026 ng/cm^3 significantly lower than normal controls ($p<0.001$). The second group of 24 subjects had a mean serum dihydrobiopterin level of 1.7±0.066 ng/cm^3 significantly higher than normal ($p<0.001$). Although they divided into two groups with Crithidia factor, no correlations were achieved with their clinical state..

These elderly patients were divided into several groups.

(1) 23 subjects with senile dementia had a mean serum dihydrobiopterin of 1.29±0.05 ng/cm^3 lower than normal but not significantly so (p 0.3-0.4) similar to the finding of Leeming and Blair (1979). This group can be divided into two groups according to the psychometric test score:-

a) 15 who scored (0-3) had a mean serum dihydrobiopterin of 1.17±0.055 ng/cm^3 which is lower than normal controls (p 0.02-0.05).

b) 8 who scored (4-5) had a mean serum dihydrobiopterin of 1.5±0.06 ng/cm^3 which is significantly higher than the first group (p 0.02-0.01) and higher than normal control (p 0.05-0.02).

(2) Seven geriatric people were diagnosed to having diabetes mellitus. They had a mean serum dihydrobiopterin of 0.98±0.073 ng/cm^3 significantly lower than normal controls ($p<0.001$).

(3) Twelve patients with vascular dementia had a mean serum dihydrobiopterin 1.97±0.13 ng/cm^3 not significantly higher than normal ($p<0.3$).

(4) Nine patients diagnosed with hemiplegia had a mean serum dihydrobiopterin level of 1.26±0.15 ng/cm^3 not significantly lower than normal.

(5) Five subjects with rheumatoid arthritis had a mean serum dihydrobiopterin level of 1.0±0.089 ng/cm^3 significantly lower than normal controls (p 0.02-0.01). This is similar to the finding of Leeming *et al* (1976b).

Aziz *et al* (1982b) found in senile dementia of the Alzheimer type a significant decrease in serum dihydrobiopterin concentration compared to a control group.

Itis possible that the synthesis of tetrahydrobiopterin is impaired. in senile dementia patients and this could decrease levels of tetrahydrobiopterin in the cell as confirmed by low tetrahydrobiopterin in brain (Nagatsu *et al* 1979) and in cerebrospinal fluid (Williams *et al* 1980).

Ten patients with coeliac disease showed a mean serum dihydrobiopterin of 1.02±0.13 ng/cm^3 significantly lower than normal controls ($0.02<p<0.05$). There were three untreated coeliac patients who showed a mean serum dihydrobiopterin of 1.05±0.076 ng/cm^3 which is also lower than normal control ($p<0.05$). This agrees with Leeming and Blair (1980b).

There was no correlation between whole blood copper and serum Crithidia factor or between caeruplasmin and Crithidia factor in 9 patients with high blood copper (greater than 27×10^{-6} M) (Fig. 2.1). However the serum dihydrobiopterin level in these patients was 0.64 0.118 ng/cm^3 which is significantly lower than normal ($p<0.001$). It has been suggested that metals like copper, cadmium, lead, mercury and aluminium inhibit the synthetic pathway and act as neurotoxic agents (Flink 1975) and copper toxicity causes

convulsions (Scheinberg and Steinlieb 1976). Leeming (1979) and Brown (1981) reported that Cu^{+2} interfered with both synthesis of tetrahydrobiopterin and dihydropteridine reductase activity. If tetrahydrobiopterin levels are depleted this would decrease catecholamine neurotransmitter biosynthesis due to absolute dependence of the rate limiting step tyrosine hydroxylase on tetrahydrobiopterin (Levitt *et al* 1965) which would lead to neurological damage.

Five dystonia patients had a mean serum dihydrobiopterin of 1.76 ± 0.081 ng/cm^3 which is significantly higher than the normal control value (1.32 ± 0.03 ng/cm^3) ($p<0.01$). One patient and his parents had 100 mg/Kg body wt. phenylalanine. Mean serum dihydrobiopterin after two hours was 4.2 ± 0.016 ng/cm^3 significantly lower from normal ($0.01<p<0.05$). The high serum dihydrobiopterin in dystonia patients might be caused by dihydropteridine reductase inhibition. This confirmed by the reduced level of tetrahydrobiopterin in cerebrospinal fluid reported by Williams *et al* (1979). Similar observations had been made in Parkinson's disease, Lovenberg *et al* (1979) reported low tetrahydrobiopterin in cerebrospinal fluid in those patients and raised serum Crithidia factor has been reported (Leeming *et al* 1976b).

Volunteers given oral trimethoprim had an increase in mean serum Crithidia factor in 6hrs. Those who had sulphathiazole showed little change. The people who had both drugs showed a significant increase in 24 hrs. Only trimethoprim is a significant inhibitor *in vivo* for dihydropteridine reductase. Trimethoprim is an inhibitor of dihydrofolate reductase, Leeming (1979) and Brown (1981) found it also inhibits

dihydropteridine reductase thus causing a decrease in tetrahydriobiopterin produced and increasing dihydrobiopterin levels.

Classical phenylketonuria patients showed a high level of serum dihydrobiopterin. Ten untreated phenylketonuria patients had a mean serum dihydrobiopterin of 7.87±0.62 ng/cm^3 significantly higher than ten normal children aged between 2-12 years (1.78±0.25 ng/cm^3) (Leeming and Blair 1980a). In all cases a high level of phenylalanine is associated with raised serum dihydrobiopterin (Leeming et al 1976c), e.g. Patient G.T. who was untreated with a serum phenylalanine level >30 mmol /l had serum crithidia factor of 12.2 ng/cm^3.

Phenylalanine produces phenylpyruvic acid as a minor metabolite which does not appear in serum or urine in measurable quantities in normal circumstances. Normal human serum levels of phenylpyruvate are about $5x10^{-6}$M (Langenbeck *et al* 1980) but in untreated adult phenylketonuria, phenylpyruvate is higher due to reduced phenylalanine hydroxylase activity (Jervis (1947) around $2.45x10^{-5}$M which may increase three fold in loading experiments with 100 mg/Kg body weight L-phenylalanine (Jervis and Drejza 1966). This is shown in M.A. diagnosed to be a classical phenylketonuria who had an oral dose of phenylalanine Crithidia factor rose in parallel with phenylalanine level. Both phenylalanine and crithidia factor levels are higher than normal.

Inhibition of dihydropteridine reductase by phenylpyruvat and/or phenylalanine would explain this observation because there would be an accumulation of quinonoid dihydrobiopterin

in the cell which would rearrange to give the increased levels of serum dihydrobiopterin recorded. Lowering phenylalanine levels by use of the recommended diet, lowers the phenylalanine metabolites, and reduces the neurological damage (Bickel et al 1953). Lowering phenylalanine metabolites reduces the inhibition of dihydropteridine reductase so less quinonoid dihydrobiopterin will be available to rearrange to 7,8-dihydrobiopterin. Three phenylketonuric patients on a low phenylalanine diet had a mean serum dihydrobiopterin 2.8 ± 1.17 ng/cm^3.

Malignant hyperphenylalaninaemia is unlike classical phenylketonuria in they show severe neurological impairment despite control of blood phenylalanine. A deficiency of

tetrahydrobiopterin can arise from a failure to synthesis tetrahydrobiopterin denovo or a failure to salvage quinonoid dihydrobiopterin by dihydropteridine reductase. Impaired synthesis of tetrahydrobiopterin results in low levels of tetrahydrobiopterin in the tissues and reduced amounts of 7,8-dihydrobiopterin appearing in the serum. The two brothers E.D. and C.D. had low levels of serum dihydrobiopterin, and their response to phenylalanine load slower than normal.

In the case of dihydropteridine reductase deficiency, low tissue levels of tetrahydrobiopterin are caused by a failure of the salvage pathway, the quinonoid dihydrobiopterin rearranges to 7,8-dihydrobiopterin and there is increased loss of this biopterin derivatives into the serum. The two patients |GL| and |C| showed serum dihydrobiopterin values

of 4.5 ng/cm^3 and 5.4 ng/cm^3 are higher than normal level. This is similar to the finding of Leeming and Blair (1980a).

Reduced levels of tetrahydrobiopterin are caused by

(a) Reduced tetrahydrobiopterin synthesis

(b) Diminished regeneration of tetrahydrobiopterin from quinonoid dihydrobiopterin.

In (a) serum crithidia factor is significantly lower than normal as we found in senile dementia, diabetes, rheumatoid arthritis patients, subjects with high blood copper, pregnant women and untreated patients with coeliac disease which they had reduced serum Crithidia factor levels which returned toward normal when a gluten free diet was given. In (b) serum crithidia factor is high which is similar to patients with dystonia, or subjects receiving dihydrofolate reductase and dihydropteridine reductase inhibitors and vascular dementia.

Biopterin and neopterin concentrations relative to creatinine were measured in urine by high pressure liquid chromatography (HpLc) (Chapter 3). This very quick and easy method may be used to estimate if there is a defect in biopterin metabolism. The excreted pteridines were identified by their retention position according to a standard run every time of the assay. They were quantitatively estimated by measuring the area under the peak and comparison of peak area with standard curve obtained for biopterin and neopterin.

Biopterin and xanthopterin (B+x) level in 5 normal females 169±33.7 ng/mg creatinine and neopterin (N) as |D-neopterin + L-neopterin| were 321.9±55.7 ng/mg creatinine

with a ratio of $N/_{B+x}$ 2.3±0.45 (mean±S.E.M.) These values are higher than values for 9 male normal subjects with (B+x) 119.6±26.9 ng/mg creatinine and N of 314±57.2 ng/mg creatinine with a ratio of $N/_{B+x}$ 2.96±0.57 (Mean±S.E.M.). The range of the ratio $N/_{B+x}$ for total males+females was 1.0-7.07, with only one subject above 3.5. The values of biopterin+xanthopterin and neopterin for the male and female are very similar to values reported by Watcher *et al* (1979b) but are lower than the values reported by Rokos *et al* (1980). The differences between our value and their values is probably because of their reduced oxidation of dihydro and tetrahydro precursors.

In 5 demented patients from St. George's hospital showed a high level of neopterin 873.1±317.7 ng/mg creatinine with a ratio of $N/_{B+x}$ 6.1±2.6 (Mean±S.E.M.) Four patients with senile dementia of Alzheimer type (SDAT) from All Saints hospital had a high level of neopterin 536.6±147 ng/mg creatinine with a ratio of 6.2±2.07 (Mean ±S.E.M.) In both groups the neopterin level is higher than the neopterin level in 14 normal males + females which is 316.9±40.4 ng/mg creatinine and the ratios of $N/_{B+x}$ in both groups were significantly higher than normal ratio (2.7±0.45)(Mean±S.E.M.) ($p<0.05$).

The low serum dihydrobiopterin level in senile dementia (Leeming and Blair 1979; Aziz *et al* 1982b) could be explained as a result of loss of functioning tissue. From this, low neopterin might be expected in contrast to our finding. The high level of neopterin which we found in senile dementia patients might be caused by disturbance in tetrahydrobiopterin metabolism.

6 patients diagnosed as having Down syndrome had a mean neopterin level of 716±276.4 ng/mg creatinine and biopterin+xanthopterin level of 161.6±32.9 ng/mg creatinine giving a ratio of $N/_{B+x}$ 3.8±0.76 (Mean±S.E.M.). 9 patients non specific mentally retarded had a mean urine neopterin level of 591.9±197.3 ng/mg creatinine and biopterin+xanthopterin level of 169±30.9 ng/mg creatinine with a ratio of $N/_{B+x}$ 3.85±1.28 (Mean±S.E.M.) Neopterin levels in both groups were higher than neopterin level for normal subjects which is 316.9±40.4 ng/mg creatinine ($0.1<p<0.2$) for both. This high level of neopterin might cause by a disturbance of tetrahydrobiopterin metabolism. Two patients with dystonia showed low levels of biopterin + xanthopterin 99.5 ng/mg creatinine and 112.3 ng/mg creatinine. Neopterin level were 183.4 ng/mg creatinine and 322.7 respectively. And also the $N/_{B+x}$ ratio within the normal range.

17 patients diagnosed as having malignancy showed a low urinary levels of biopterin + xanthopterin with the exception of two cases. One of them had a value of 1008.4 ng/mg creatinine and the other 594.7 ng/mg creatinine. These patients showed a high level of neopterin except in two cases; they had a value of 238 .0 ng/mg creatinine and 276.7 ng/mg creatinine. The ratio of $N/_{B+x}$ were high except in some cases, showed a value within the normal. And the mean ratio of $N/_{B+x}$ 15.8±2.8 which is significantly higher than normal 2.7±0.45. These results are similar to others reported by Watcher *et al* (1974); Rokos *et al* 1980, Stea *et al* (1980). This high level of neopterin indicate malignant disease is accompanied by disturbance in the biosynthesis of tetrahydrobiopterin.

Both types of malignant hyperphenylalaninaemia (MHPA) had very high levels of neopterin. The ratios of $N/_{B+x}$ could be used to discriminate between the two types of MHPA; one due to dihydropteridine reductase deficiency, the other caused by a defect in synthesis from GTP. K.H. Patient diagnosed to be dihydropteridine reductase deficient had neopterin level of 1932.4 ng/mg creatinine and 1647.1 ng/mg creatinine biopterin+ xanthopterin 1435.0 ng/mg creatinine and 1921.6 ng/mg creatinine respectively on two different occasions giving $N/_{B+x}$ ratios of 1.4 and 0.86. P.D. another patient also diagnosed dihydropteridine reductase deficient had neopterin level of 865.7 ng/mg creatinine and biopterin + xanthopterin of 1857.0 ng/mg creatinine with a ratio of 0.46 which is lower than the range of ratios for the normal subjects of 1.0-7.07. These results are similar to those reported by Niederwieser *et al* (1979b); Nixon *et al* (1980). An oral phenylalanine load given to K-H increased neopterin level and decreased biopterin + xanthopterin level which is in contrast to oral tetrahydrobiopterin given to the same patient when increased biopterin + xanthopterin and reduced neopterin level where found. Neopterin levels increase after phenylalanine, load because phenylalanine and its metabolites such as phenylpyruvate inhibit dihydropteridine reductase (Purdy and Blair 1980) tetrahydrobiopterin levels in the cell are reduced, thus reducing the inhibition of GTP cyclohydrolase so the conversion of GTP to dihydroneopterin triphosphate increase and more neopterin will be released. Oral tetrahydrobiopterin load increases tetrahydrobiopterin concentrations in the cell which then inhibit GTP cyclohydrolase, decreasing the formation of neopterin.

C.A. a patient diagnosed to be synthesis deficient had extremly high level of neopterin 32123.1 ng/mg creatinine with a ratio of $N/_{B+x}$ 61.0. L.A. also diagnosed synthesis deficient had a high level of neopterin 3591.1 ng/mg creatinine with a ratio of 24.4. This high level of neopterin in both cases indicate there is a block between dihydroneopterin triphosphate and biopterin. The ratio of $N/_{B+x}$ was high and similar to that reported by Curtius *et al* (1979) Nixon *et al* (1980). Y.L. A patient also diagnosed to be synthesis deficient, after a phenylalanine load had increased biopterin + xanthopterin from 95.3 ng/mg creatinine after 3 hrs to 1485.6 ng/mg creatine in 24 hrs. and neopterin levels from 8339.0 ng/mg creatinine to 17084.0 ng/mg creatinine, and a decreased ratio of $N/_{B+x}$ from 87.5 to 11.5. L.A. after a phenylalanine load, the noepterin level and the ratio of $N/_{B+x}$ rose 12 and 24 hrs. later.

High levels of pheylalanine inhibit dihydropteridine reductase, so less tetrahydrobiopterin will be in the cell and this might release the inhibition of GTP cyclohydrolase.

In Chapter (4) tetrahydrobiopterin was measured in the urine by an enzymatic assay. The urine tetrahydrobiopterin concentration with dimethyltetrahydrobiopterin as standard was higher (2.5-4.0) times than the values obtained by using tetrahydrobiopterin as standard. Mean tetrahydrobiopterin value for ten normal subjects using the enzymatic assay 2.8±0.47 µg/l is higher than those obtained by the crithidia fasiculata assay for the total biopterin in urine 0.75±0.34µg/l.

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