METABOLISM OF BIOPTERIN AND ITS DERIVATIVES

IN MAN AND THE RAT

A THESIS

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SUMMARY

METABOLISM OF BIOPTERIN AND ITS DERIVATIVES IN MAN AND THE RAT

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In-vitro studies of tetrahydrobiopterin synthesis and dihydropteridine reductase activity have shown that these are affected by pharmacological preparations, phenylalanine and tryptophan metabolites, neurotransmitters and metal ions. Tetrahydrobiopterin given orally to man does not appear in the serum at a measurable rate. A small amount of biopterin passes unaltered across the bowel wall. Crithidia factor is synthesised in the foetus as early as the twelfth week of gestation and there is evidence for early variation in concentrations within the brain. In adults a range of tissues contain Crithidia factor with the pineal gland being especially rich. All the evidence points to the essential supply of tetrahydrobiopterin being endogenous. Males have significantly ($p = < 0.05$) higher serum Crithidia factor levels ($1.75 \pm 0.03 \mu g/L.$) than unmedicated females ($1.53 \pm 0.4 \mu g/L.$) and females taking oral contraceptives ($1.44 \pm 0.05 \mu g/L.$). There were significant changes in serum Crithidia factor levels during the menstrual cycle.

Hyperphenylalaninaemia produces high levels of serum Crithidia factor by the competitive inhibition of dihydropteridine reductase by phenylpyruvic acid. In malignant hyperphenylalaninaemia due to defective synthesis of dihydrobiopterin the serum Crithidia factor is low and does not respond to phenylalanine loading. In dihydropteridine reductase deficiency the serum Crithidia factor is raised before phenylalanine loading. In coeliac disease and malignant disease serum Crithidia factor is low. In carcinoid disease tissue levels of Crithidia factor are high although serum levels are low.

Women in the third trimester of pregnancy have low serum Crithidia factor levels, probably this is the result of increased cortisol levels. In manic-depressive psychosis serum Crithidia factor levels are increased and it is suggested that this is due to an increased rate of tetrahydrobiopterin synthesis. Senile demented patients have low serum Crithidia factor levels and impaired phenylalanine clearance.

A model has been proposed for the regulation of tetrahydrobiopterin in health and disease which depends on:-

a) Dihydropteridine reductase activity
and
b) de novo synthesis of purine precursors.

Endogenous influences on cellular tetrahydrobiopterin may come from diseased states or normal physiological changes. Exogenous influences may occur in medication and poisoning.

KEY WORDS

Biopterin
Dihydropteridine reductase
Phenylketonuria

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

Robert J. Leeming

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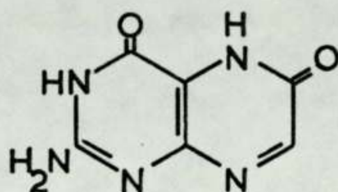
CHAPTER I

HISTORICAL INTRODUCTION

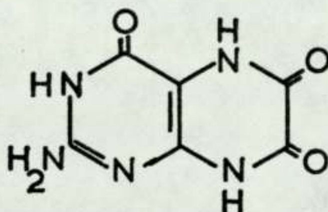
At the end of the last century Hopkins (1889) reported the extraction of a yellow pigment from butterflies' wings. Later work (Wieland and Schopf, 1925; Schopf and Wieland, 1926) confirmed his observation by the isolation of yellow and white pigments which were identified as xanthopterin

(1)



and leucopterin

(2)

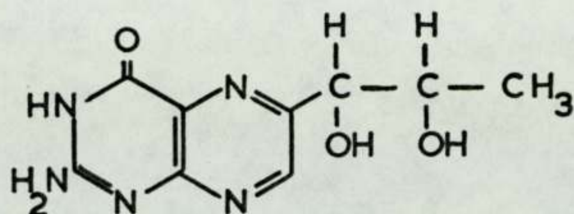


when their structure was subsequently determined (Purrman,1940). Much work has been carried out on the isolation and identification of compounds from butterflies, moths and other insects (Ziegler and Harmsen, 1969). Pteridines in amphibia and fish have also been studied extensively and reviewed (Hama, 1963).

Isolation of pterins from human sources has been a relatively recent interest. Some of the earlier attempts at the measurement of pterins in mammalian blood and serum (Frank, Baker and Sobotka, 1963) gave results some ten times greater than those now accepted, this may have had more to do with finding a suitable standard than anything else.

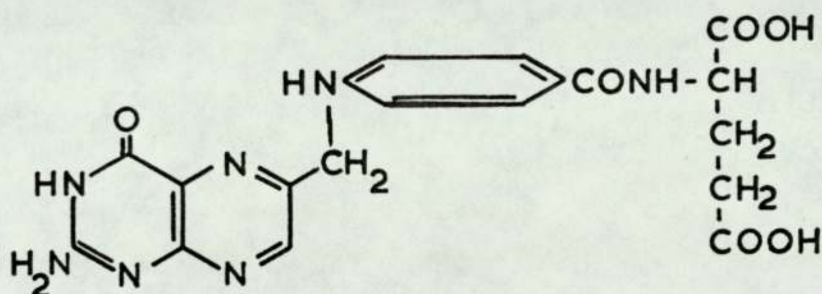
Biopterin

(3)



was also isolated from urine shortly afterwards (Pabst and Rembold,1966). Perhaps the first meaningful assays on human material with an attempt to relate the findings to a known biochemical abnormality, were those of Fleming and Broquist (1967) who used Crithidia fasciculata (Leger, 1902) in a successful microbiological assay to find out if there was a link between biopterin (3) and folic acid

(4)



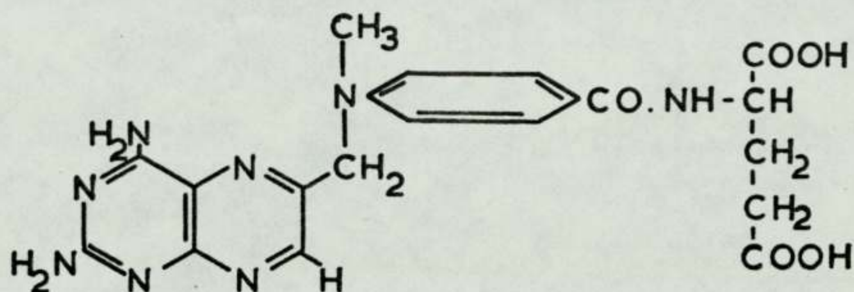
deficiency by measuring these two with Crithidia fasciculata and lactobacillus casei respectively. They did not establish a relationship between the two parameters.

The knowledge from which a microbiological assay for biopterin could be established had been available for many years. The first chemically defined medium (Cowperthwaite, Weber, Packer and Hutner, 1953) for Crithidia fasciculata described a high and 'non-physiological' folate requirement which was subsequently shown to be spared by biopterin (3) (Patterson, Broquist, Albrecht, von Saltza and Stokstad, 1955).

The first broadly based studies of biopterin derivatives in human and rat body fluids and tissues utilizing Crithidia fasciculata

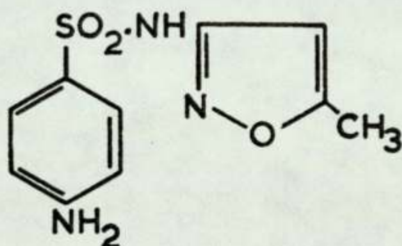
(Baker, Frank, Bacchi and Hutner, 1974; Leeming, Blair, Melikian and O'Gorman, 1976) disclosed low serum levels in a number of proliferative disorders and showed that urinary excretion produced a high gradient across the kidney with kidney disfunction increasing serum levels and decreasing urine levels very significantly. Methotrexate

(5)



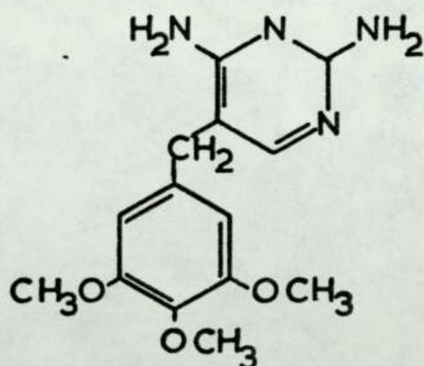
and septrin (sulphamethoxazole

(6)



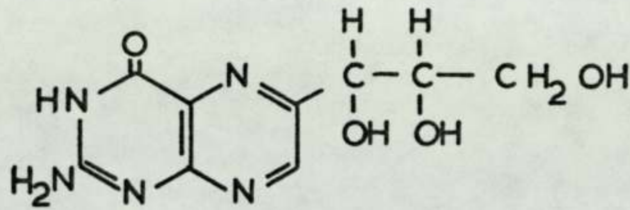
with trimethoprim)

(7)



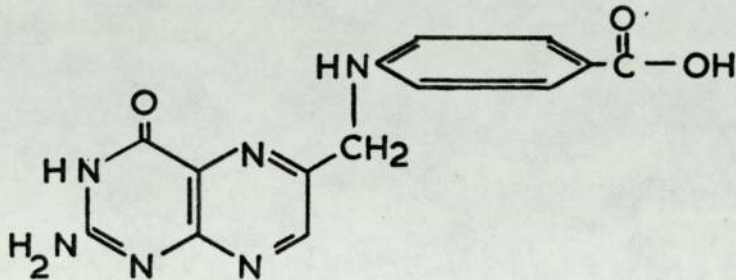
caused an increase in serum levels of Crithidia active material (Leeming, Blair, Melikian and O'Gorman, 1976). Crithidia fasciculata is very selective in its ability to utilize pteridines, with only L-neopterin

(8)



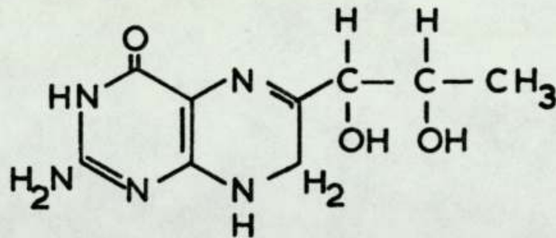
(Leeming and Blair, 1974) and pteroic acid

(9)



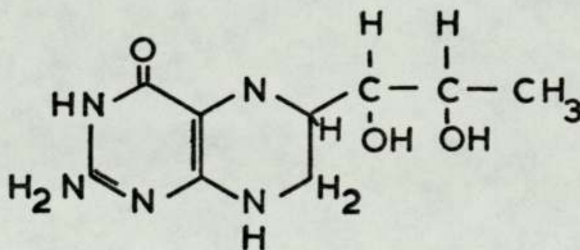
approaching the growth stimulating capacity of biopterin (3) and its reduced derivatives 7,8-dihydrobiopterin

(10)



and 5,6,7,8-tetrahydrobiopterin .

(11)

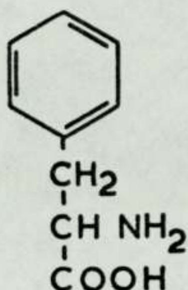


From this standpoint the Crithidia assay has proved a very useful tool for

the study of defects in biopterin synthesis.

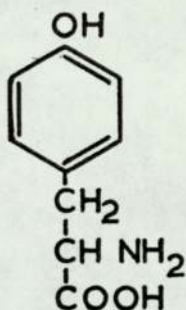
5,6,7,8-tetrahydrobiopterin (11) is the natural co-factor in the hydroxylation of phenylalanine

(12)



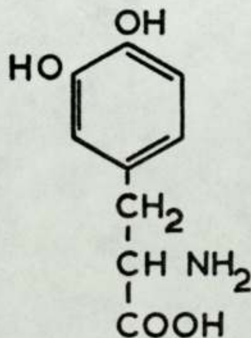
to tyrosine

(13)

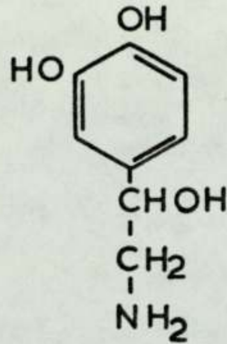


(Kaufman, 1958; Kaufman, 1963) and is similarly required in the hydroxylation of tyrosine (13) to dopa

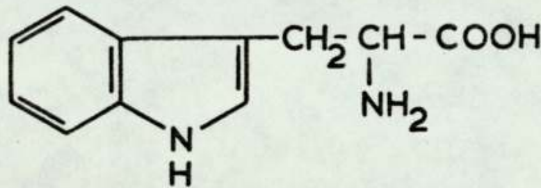
(14)



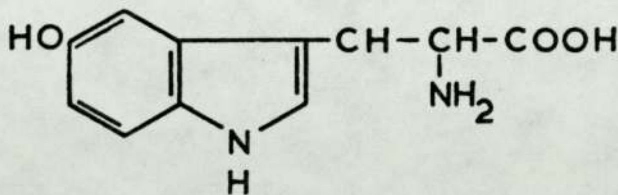
which is the rate limiting step in noradrenaline
(15)



production (Levitt, Spector, Sjoerdsma and Udenfriend, 1965).
5,6,7,8-tetrahydrobiopterin (11) is also needed for the hydroxylation of tryptophan
(16)



to 5-hydroxytryptophan
(17)

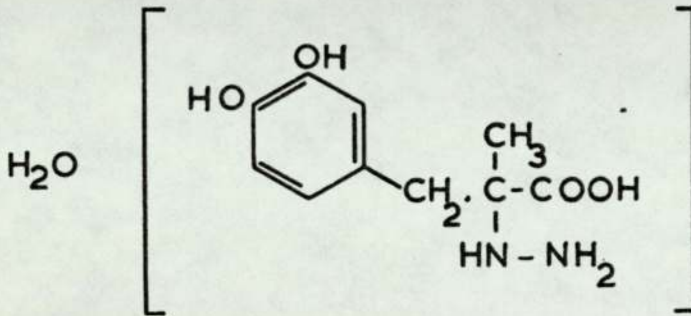


(Hosoda and Glick, 1966)

Renewed interest in the field came from a retrospective assessment of three cases of intractible phenylketonuria in which the plasma phenylalanine was easily controlled but the neurological lesion was progressive and terminated life in early childhood (Smith, Clayton and

Wolff, 1975). This report was followed quickly by the publication of case reports of living children with the same syndrome and with biochemical lesions shown to occur at the two sites, synthesis of 7,8-dihydrobiopterin (10) (Leeming, Blair and Rey, 1976; Rey, Blandin-Savoja and Rey, 1976; Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel, 1978) and dihydropteridine reductase (Kaufman,Holtzman, Milstien, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977; Grobe, Bartholome, Milstien and Kaufman, 1978). The neurological consequences of both are similar but they may be differentiated by Crithidia factor response to phenylalanine (12) load (Rey, Harpey, Leeming et al 1977; Kaufman, Berlow, Summer et al, 1978). Treatment is similar, L-dopa (14) carbidopa

(18)



and 5-hydroxytrytophan (17) (Bartholome and Byrd, 1975) although the hyperphenylalaninaemia may be corrected with 5,6,7,8-tetrahydrobiopterin (11) (Danks, Cotton and Schlesinger, 1975; Schlaub, Daumling, Curtius, Niederweiser, Bartholome, Viscontini, Schircks and Bieri, 1978). It is improbable that this syndrome (malignant hyperphenylalaninaemia) is a new disease, most likely the afflicted were previously classified with other neurological defects of obscure aetiology as an adjunct to diagnosis as classical phenylketonuria until the relevant biochemical facts were determined.

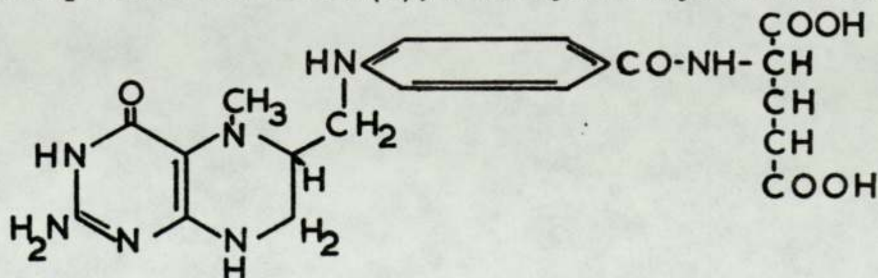
5,6,7,8-tetrahydrobiopterin (11) is a labile compound which is rapidly oxidized to 7,8-dihydrobiopterin (10) and then less quickly to biopterin (3); the kinetics of this process have been determined (Blair and Pearson, 1974). Tetrahydropterins have been reported as non-specific hydroxylation agents for aromatic amino acids (Coulson, Powers and Japson,

1970; Woolf, Jakobovic and Chan-Henry, 1971). 5,6,7,8-tetrahydrobiopterin (11) effects the non-enzymatic hydroxylation of phenylalanine (12) to tyrosine (13) (Blair and Pearson, 1975) which may have to be taken into account when dealing with its specific relationship to enzymes and the interpretation of data.

Several generations of rats fed on a biopterin (3) free diet produced 30 μg biopterin (3) per day in their urine (Pabst and Rembold,1966), thereby convincingly demonstrating that its source was endogenous. Rembold and Metzger (1967) showed that radio-active biopterin (3) administered parentally was excreted rapidly and totally in the urine whereas 5,6,7,8-tetrahydrobiopterin (11) was taken up by the tissues and retained. Orally given tetrahydrobiopterin (11) was not absorbed but biopterin (3) was absorbed and retained by the tissues. The inference was that biopterin (3) wasreduced during transport. However, tetrahydrobiopterin (11) administered intravenously (Kettler, Bartholini and Pletscher, 1974) does not pass into the brain where it is required for the enzymatic hydroxylation of tyrosine (13) to dopa (14) which is the rate limiting step in noradrenaline (15) synthesis (Levitt, Spector, Sjoerdsma and Udenfriend, 1965). Therefore dietary biopterin (3) may contribute to phenylalanine (12) hydroxylation in the liver and thereby give part of the answer to the observation that there is no linear relationship between phenylalanine (12) intake and brain damage in phenylketonuria (BMJ, 1971). Additionally the very low levels of Crithidia factor in the serum and liver of patients with a defect in 7,8-dihydrobiopterin (10) synthesis may be due to dietary biopterin (3) rather than residual biopterin (3) synthesis; with just sufficient passing the blood brain barrier to permit minimum survival levels to be achieved.

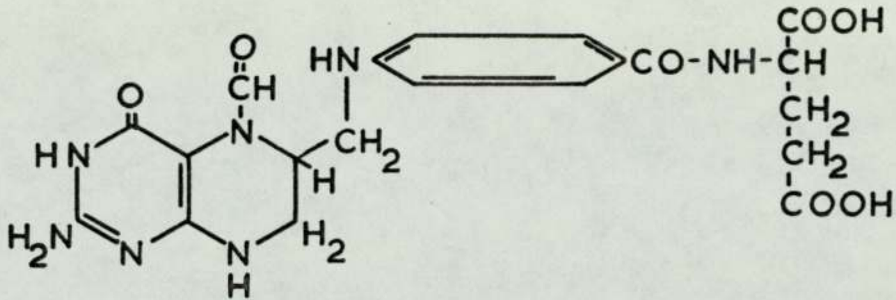
Absorption of folic acid (4), 5-methyltetrahydrofolic acid

(19)



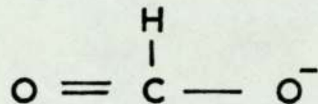
and 5-formyltetrahydrofolic acid

(20)



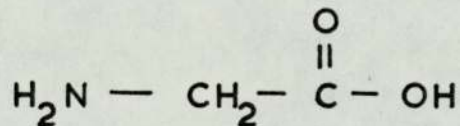
is distinctive from absorption of biopterin (3), tetrahydrobiopterin (11) and pteroic acid (9) with very high serum levels occuring rapidly (Ratanasthien, Blair, Cooke, Leeming and Melikian. 1974). The difference in the absorption of these structurally related compounds has been used to prepare a model for intestinal absorption (Blair, Ratanasthien and Leeming, 1974) based on an intestinal acid microclimate at the glycocalyx (Blair and Matty, 1974). The injection or feeding of precursors of purines, such as formate

(21)



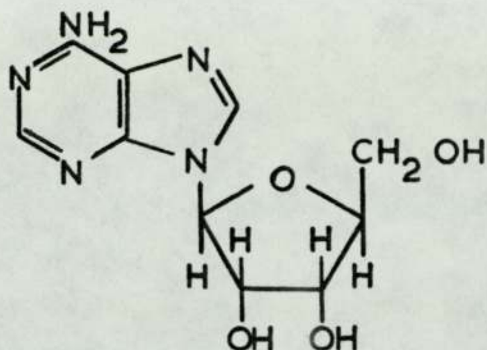
glycine

(22)



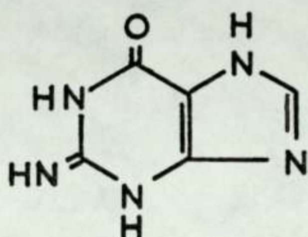
or possible pteridine precursors such as adenosine

(23)



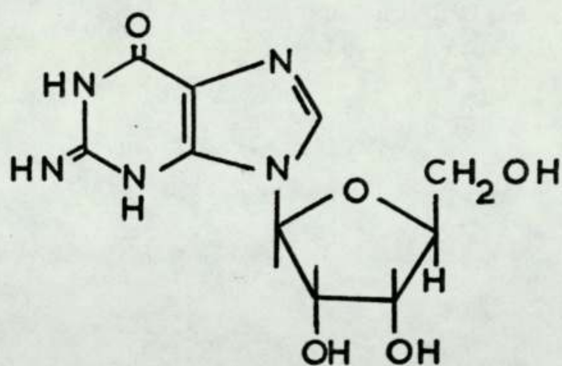
guanine

(24)



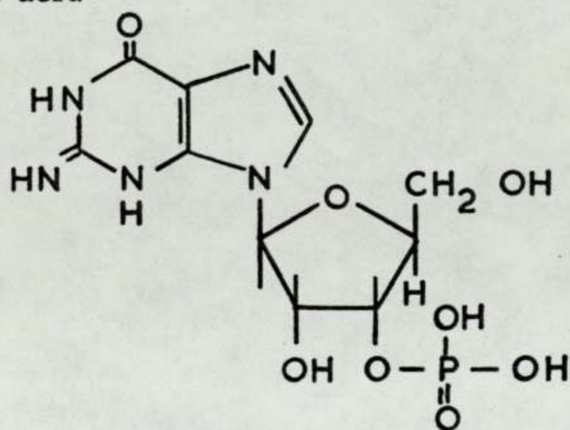
guanosine

(25)



or guanylic acid

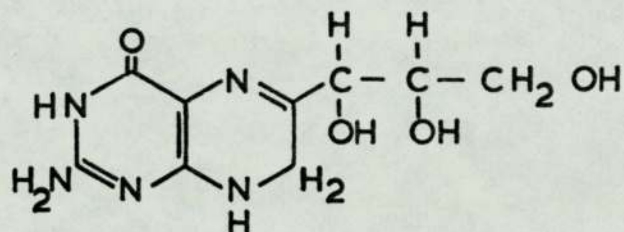
(26)



produced only slight rises in biopterin (3) in the rat (Rembold and Gyure, 1972).

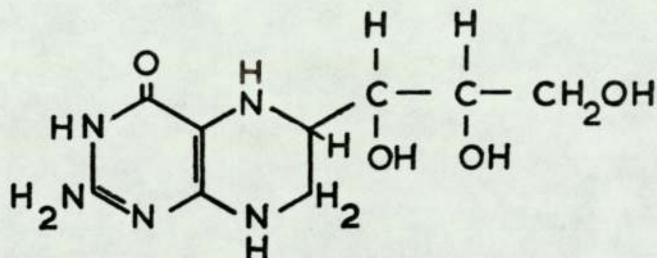
Radio-active dihydroneopterin

(27)



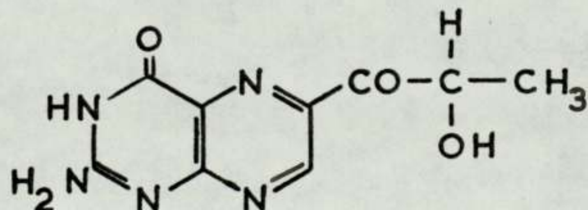
injected into tadpoles caused a low level of incorporation of the label into biopterin (3) (Fukushima, 1970), but radio-active neopterin (8) and and tetrahydroneopterin

(28)



injected into rats did not confirm the observation (Rembold, Chandrashakar and Sudershan, 1971). However sepiapterin reductase in both the erythrocytes and liver of rats is greater than in several other species (Katoh, Arai, Taketani and Yamada, 1974) and sepiapterin

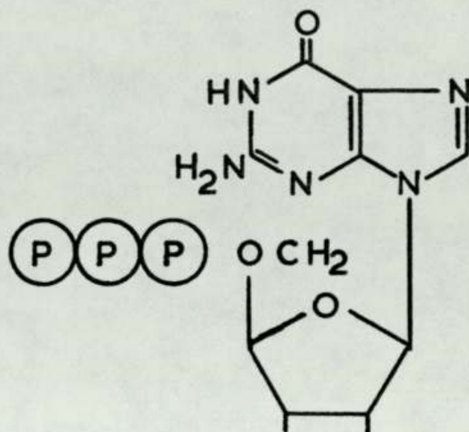
(29)



has been proposed as an intermediary in dihydrobiopterin (10) synthesis (Eto, Fukushima and Shiota, 1976) between dihydroneopterin (27) and dihydrobiopterin (10).

The synthesis of the pteridine ring from guanosine triphosphate

(30)



in the production of biopterin (3) derivatives, has been convincingly demonstrated in mammalian systems utilizing cell cultures derived from liver, brain, kidney, lung and ovary of mouse, rat and hamster (Fukushima, Eto, Richter, Goodson and Shiota, 1975). The role of dihydropteridine reductase in the reduction of 7,8-dihydrobiopterin (10) to 5,6,7,8-tetrahydrobiopterin (11) and a technique for its measurement has also been described (Craine, Hall and Kaufman, 1972). With these two systems isolated in-vitro the effects of drugs on tetrahydrobiopterin (11) synthesis and its maintenance in a reduced form can be studied. Methotrexate (5) is already known to decrease dihydropteridine reductase in-vitro (Craine et al, 1972) and with the rising doubts about long term neurological effects of the use of this valuable drug in the treatment of childhood leukaemia (Meadows and Evans, 1976, Eiser, 1978) association with the neurological effects of other drugs, particularly neuroleptics, and action against dihydropteridine reductase could help to elucidate iatrogenic disturbance of neurological function.

By extrapolating the foregoing premise a little further, it follows that effects of drugs on tetrahydrobiopterin (11) synthesis and the relationship of those drugs to the control of mental disorders could give insight into the underlying biochemical processes and their

alteration in disease. Much work on mental diseases has been carried out by observing changes in response to therapy and relating the site and method of drug action to the biochemical lesion thought to exist. This approach is not without its own peculiar problems; amitriptyline is a proven drug in the treatment of depressive illness yet clinical response as measured on the Hamilton Scale (Hamilton, 1960), cannot be correlated to blood levels as shown in the recent World Health Organization collaborative study (Coppen, Ghose, Montgomery, Rama-Rao et al, 1978). An almost identical finding has been reported with nortriptyline (Burrows, Maguire, Scoggins, Stevenson and Davies, 1977).

To date, most of the work carried out on biopterin (3) derivatives in man has concerned itself with serum levels and there are obvious ethical reasons for this being so. However, tetrahydrobiopterin (11) is needed in the brain for the hydroxylation of tyrosine (13) and tryptophan (16) but if it is not transported across the blood/brain barrier (Kettler, Bartholini and Pletscher, 1974) and there are strong indications of regional brain concentration differentials (Leeming, Blair, Melikian and O'Gorman, 1976) then serum levels will tell little of subtle changes in cerebral synthesis in individuals. Serum changes have been associated with disease by statistical evaluation of large groups and one of these groups comprised schizophrenics who had a significantly lower mean serum level than controls (Leeming, Blair, Melikian and O'Gorman, 1976). It must be pointed out that this group was medicated, indeed it is difficult to find unmedicated patients in any category as most have been treated empirically by a general practitioner prior to hospitalization. In the absence of acceptable alternatives, work on human subjects must continue with the examination of large numbers of sera from carefully selected cases and serial samples from individuals where the advantage lies in them acting as their own controls. This may be supplemented by the study of post-mortem tissues, or better from biopsy at operation. Laboratory animals will also be used although their usefulness may be limited by metabolic differences from the human and difficulties in providing disease models, particularly in mental disorders.

A crude method has been devised for the measurement of Crithidia factor synthesized by rat brain extract and a method exists (Craine, Hall and Kaufman, 1972) for the measurement of dihydropteridine reductase activity. These two systems will be used to test the effects of a number of compounds on the de novo synthesis of Crithidia factor and on dihydropteridine reductase activity. In the light of previous work (Leeming, 1975; Leeming, Blair, Green and Raine, 1976) the measurement of phenylalanine (12) and Crithidia factor in the serum of fasting patients and following phenylalanine (12) loads is also proposed.

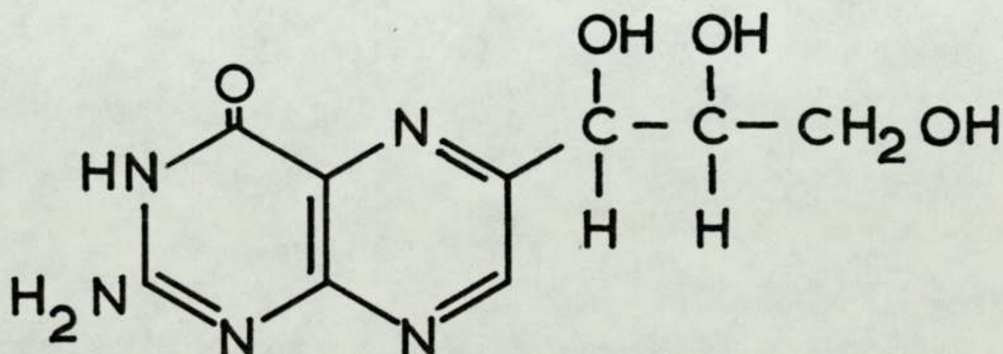
CHAPTER II

THE EFFECTS OF PHARMACEUTICAL PREPARATIONS AND OTHER COMPOUNDS ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO.

INTRODUCTION

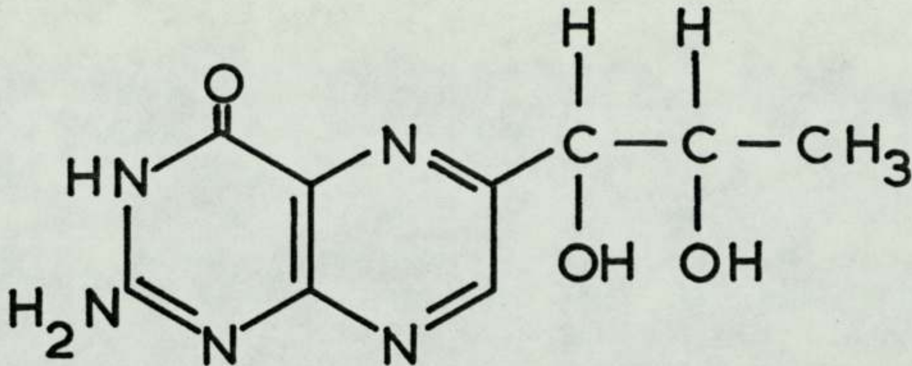
The synthesis of Crithidia active substances from guanosine triphosphate in cultures of cells obtained from liver, kidney, brain lung and ovary of the mouse, rat and hamster has been convincingly demonstrated (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota, 1975). These authors showed that the products formed from U^{14C}-guanosine triphosphate by preparations of Syrian golden hamster organs had, after phosphatase treatment and oxidation with iodine, the chromatographic properties of authentic D-neopterin

(1)



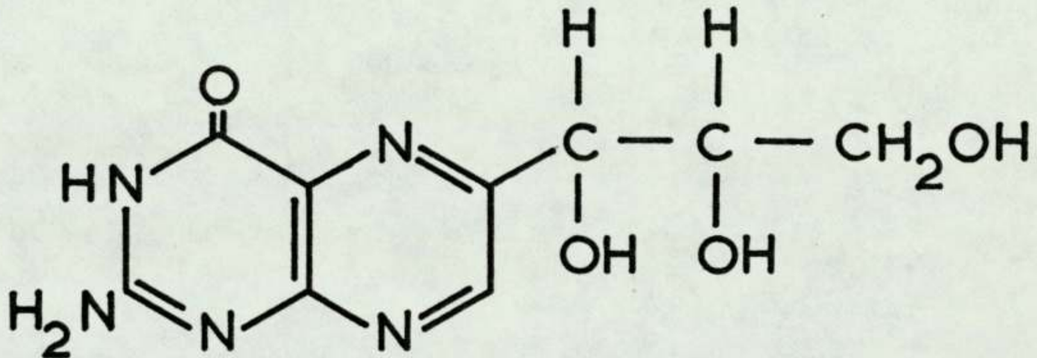
and L-biopterin

(2)



The low activity of the neopterin which they found in the Crithidia assay compared with its radio-activity, support its stereoisomeric identity, L-neopterin

(3)

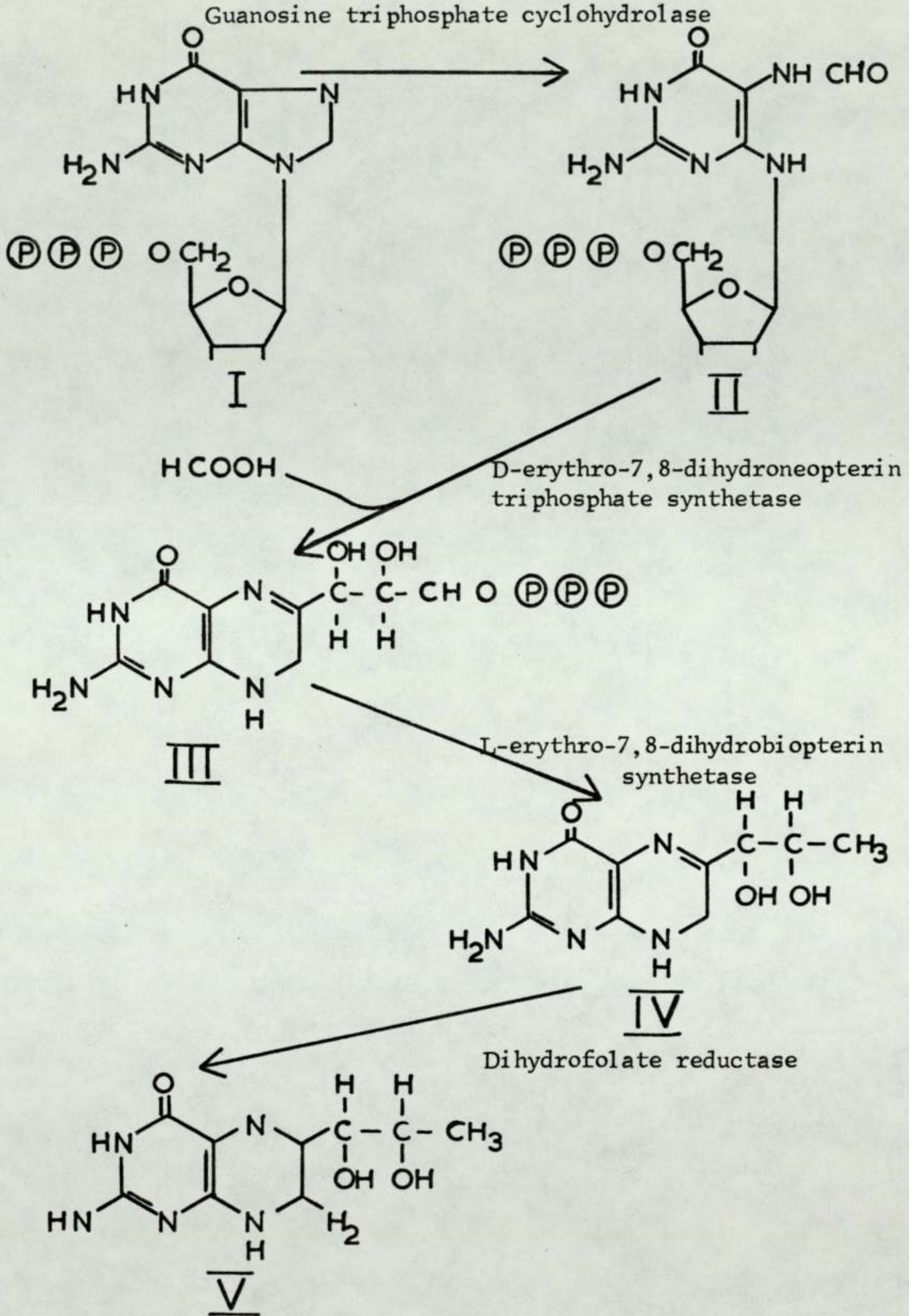


is much more active than D-neopterin for Crithidia fasciculata (Leeming and Blair, 1974). The proposed pathway from 7,8-dihydroneopterin triphosphate to 7,8-dihydrobiopterin has been studied using extracts of kidneys from Syrian golden hamsters and it was shown that non-phosphorylated neopterin was not converted to biopterin but sepiapterin was converted to biopterin if NADPH was present (Eto, Fukushima and Shiota, 1976).

Three specific enzymes (Figure 2-1) have been characterized in the synthesis of 7,8-dihydrobiopterin from guanosine triphosphate (I) in the brain. Guanosine triphosphate

FIG. 2 - 1

THE DE NOVO SYNTHESIS OF 5,6,7,8-TETRAHYDROBIOPTERIN



cyclohydrolases 1 and 2 (Mg^{2+} dependent) hydrolyse guanosine triphosphate to 2-amino-6 (5-triphosphoribosyl)-amino-5-formamido-6- hydroxypyrimidine (II) which in turn is cyclized to D-erythro-7,8-dihydroneopterin(III) by its synthetase and from there to L-erythro-7,8-dihydrobiopterin (IV) by L-erythro-7,8-dihydrobiopterin synthetase (Gal, Nelson and Sherman, 1978) - see Figure 1. It is only after 7,8-dihydrobiopterin has been produced that it is subject to reduction, by dihydrofolate reductase, (Kaufman, 1967) to 5,6,7,8-tetrahydrobiopterin (V).

The essential role of tetrahydrobiopterin in the production of neurotransmitters is clear from the dire consequences of its absence in the rare conditions where synthesis of the pterin ring is defective (Leeming, Blair and Rey, 1976) or dihydropteridine reductase is absent (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975). The sensitivity of the rate of tyrosine hydroxylation to changes in tetrahydrobiopterin has been shown in the work of Kettler, Bartholini and Pletscher (1974). Further, tyrosine hydroxylation is the rate limiting step in noradrenaline biosynthesis (Levitt, Spector, Sjoerdsma and Udenfriend, 1965). Tetrahydrobiopterin is also required for the production of 5-hydroxytryptophan from tryptophan (Hosoda and Glick, 1966). Therefore any defect in biopterin synthesis, either inherited, acquired or iatrogenic could have far reaching consequences on central nervous system function.

Administration of phenylalanine causes serum levels of Crithidia factor to rise (Leeming, Blair, Green and Raine, 1976) though serum biopterin appears to be independent of the plasma tyrosine (Leeming, 1975) and tryptophan in most cases (Leeming and Green, 1976). Therefore phenylalanine and the metabolites of phenylalanine which are raised in hyperphenylalaninaemia might act on the mechanism of biopterin synthesis.

The cause of neurological malfunction in phenylketonuria is still not known although it has been shown that phenylalanine in high concentration competes with tyrosine and 5-hydroxytryptophan for transport into brain cells (McKean, Schanberg and Giarman, 1962). Methotrexate (amethopterin)

and cotrimaxazole (sulphamethoxazole with trimethoprim) are both drugs which cause rises in the serum Crithidia factor of treated patients (Leeming, Blair, Melikian and O'Gorman, 1976). The action of co-trimoxazole on tetrahydrobiopterin synthesis has been confirmed by its reduction of phenylalanine clearance following an oral load of phenylalanine (Andrews, Purkiss, Chalmers and Watts, 1976) an identical effect in patients receiving methotrexate has been reported (Goodfriend and Kaufman, 1961). Parachlorphenylalanine has been used to block formation of serotonin and cats treated with this compound show gross behavioural disturbance and some electroencephalic similarities to schizophrenia (Antun, Eccleston and Smythies, 1971). 2,4-diamino-6-hydroxypyrimidine has been shown to reduce rat urinary biopterin by 80% without affecting the growth of the animal (Pabst and Rembold, 1966) and derivatives of the 2,4-diaminopyrimidine ring system inhibit dihydrofolate reductase (Hitchings, Elion, Falco, Russell, Sherwood and Vanderwerff, 1950). The effects of all these compounds on in-vitro synthesis of 7,8-dihydrobiopterin might disclose or eliminate a site of their action. For a similar reason other drugs used in the treatment of psychiatric disorders and commercially available neurotransmitters are included in the work described in this chapter.

The effect of copper on the biosynthesis of dihydrobiopterin is important because of the sensitivity of reduced pteridines to catalytic oxidation by low concentrations of copper (Blair and Pearson, 1974). The toxic effects of copper are haemolysis, hepatic necrosis, hypotension and convulsions (Scheinberg and Steinlieb, 1976) with serum levels of 4.5×10^{-5}M and brain levels of 3.65µmol/g reported (Hamlyn, Gollan, Douglas and Sherlock, 1977) (allowing for 80% water a rough calculation for comparison with other data 0.73×10^{-3}M) but fortunately severe systemic effects are rare as vomiting and diarrhoea usually protect the patient from ingested copper. The same protection is not afforded to patients with defects in copper metabolism although Wilson's disease (Wilson, 1912) is rare.

Other metals, especially lead and mercury, have known neurological effects (Flink, 1975) and aluminium has been shown to accumulate in the brain to six times the concentration of controls during haemodialysis for renal failure (Alfrey, Le Gendre and Kaehny, 1976). In Scotland serum levels have been shown to correlate significantly with aluminium sulphate levels in the water supply (Elliot, Dryburgh, Fell, Sabet and MacDougall, 1978). Any effect therefore on tetrahydrobiopterin synthesis could well be important in the neuropathology of metal poisoning. Mercury poisoning may arise from environmental pollution, as an occupational hazard (Joselow, Louria and Browder, 1972) or even from house paint (Hirschman, Feingold and Boylen, 1963) and causes fatigue, loss of memory, visual disturbances, acrodynia and incoordination with microscopic changes in cerebellar and ganglion cells (Hirschman, Feingold and Boylen, 1963; Flink, 1975). Cadmium poisoning (Flink, 1975) is rarely fatal but produces pulmonary oedaema and headache among its symptoms and is primarily a disease of certain manufacturing industries. Lead poisoning causes anaemia, headaches, irritability, tremor, ataxia, visual field changes and bowel spasms (Flink, 1975) and with the ubiquitous use of lead may arise in a variety of situations (Guinea, 1972) although children are more susceptible (Byers, 1959). Lead is currently an environmental issue because of possible toxicity arising from atmospheric pollution from petrol fumes (Nat.Inventory Air Pol.Emission and Control (U.S.A.),1970).

Aluminium has recently attracted attention because of high aluminium levels in brains of patients suffering from the usually fatal, dialysis dementia (or dialysis associated with encephalopathy). Levels of aluminium in the brains of patients who had died from dialysis dementia were 8.91 mg/Kg ($\pm$ 4.29 s.d.) dry weight aluminium ($\equiv 0.6 \times 10^{-4}$M in wet tissue). Undialysed controls had 1.30 mg/Kg $\pm$ 0.68 (0.96×10^{-5}M). The amount of aluminium in the grey matter was highest at 24.98 mg/Kg $\pm$ 9.10 (controls 2.18 $\pm$ 0.69), allowing for water 1.85×10^{-4} M and was significantly correlated with duration of dialysis ($r = 0.71$, $p = < 0.01$) although this did not apply to the white matter (Alfrey, Le Gendre and Kaehny, 1976). Other workers found

similar brain levels, 2.0×10^{-5}M in undialysed patients, 3.26×10^{-5}M in non demented dialysed patients and 1.18×10^{-4}M in dialysis dementia (McDermott, Smith, Ward, Parkinson and Kerr, 1978).

MATERIALS AND METHODS

Guanosine triphosphate, nicotinamide adenine dinucleotide (NAD) and aminopterin were purchased from the Sigma Chemical Company. Methotrexate (amethopterin) was purchased from Lederle Laboratories. Amitriptyline and protriptyline were gifts from Merck, Sharp and Dohme Development Laboratories. Clomipramine, desipramine, imipramine and opipramol were gifts from Geigy Pharmaceuticals. Maprotiline was a gift from Ciba Laboratories. Librium, Nobrium and Valium were gifts from Roche Research Laboratories.

Fresh rat brain was homogenized at $0^{o}C$ - $4^{o}C$ in three volumes of 0.01 M Tris - 0.04 M KCl buffer adjusted to pH 8.0 with 2M HCl. The homogenate was centrifuged at 17,500 g for 1 hour at $0^{o}C$, the supernatant was decanted (Fukushima, Eto, Mayumi et al, 1975) and the total protein estimated (automated Biuret, 'Technicon' N14a). The extract was stored at $-80^{o}C$ for up to one month from the date of preparation and tested at weekly intervals for residual activity. The following constituted the incubation mixture and were added in the order given, any reagent omitted was replaced with deionized water to maintain the final volume at 1.0 cm^3.

1. Additives (detailed in Table 2-1) in distilled water at concentrations given under Results	0.1 cm^3
2. 0.083 M Tris HCl buffer pH 8.0	0.6 cm^3
3. Methotrexate 0.1 mg/cm^3	0.1 cm^3
4. NAD 20 m.mol/cm^3	0.05 cm^3
5. Guanosine triphosphate 60 m.mol/cm^3	0.05 cm^3
6. Brain extract thawed immediately prior to use	0.1 cm^3

Incubation was carried out for three hours at $37^{o}C$ in a shaking water bath with the lid in place to exclude light. At the end of three hours 2.0 cm^3 0.1 M HCl was added to stop the reaction and the mixture was diluted a further 1 in 10 with 0.2M phosphate buffer pH 5.0. 0.5 cm^3 volumes were assayed for Crithidia factor (Leeming, Blair, Melikian and O'Gorman, 1976) and the result calculated as ng biopterin/mg protein.

The optimum concentration of methotrexate was arrived at by making serial dilutions of methotrexate and adding these to NAD, GTP and brain extract in the standard procedure, but dilutions at both 1/10 and 1/20 were made for the Crithidia fasciculata assay. The inhibitory effect of methotrexate under assay conditions on Crithidia fasciculata was shown by a lower calculated Crithidia factor value for the incubation mixture when the 1/10 dilution was used. 0.1 cm^3 of methotrexate at 0.1mg/cm^3 diluted out the inhibitory effect and gave maximum observable stimulation to the in-vitro synthesis. Similar experiments with aminopterin gave virtually identical results.

TABLE 2-1

COMPOUNDS TESTED FOR THEIR EFFECTS ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO

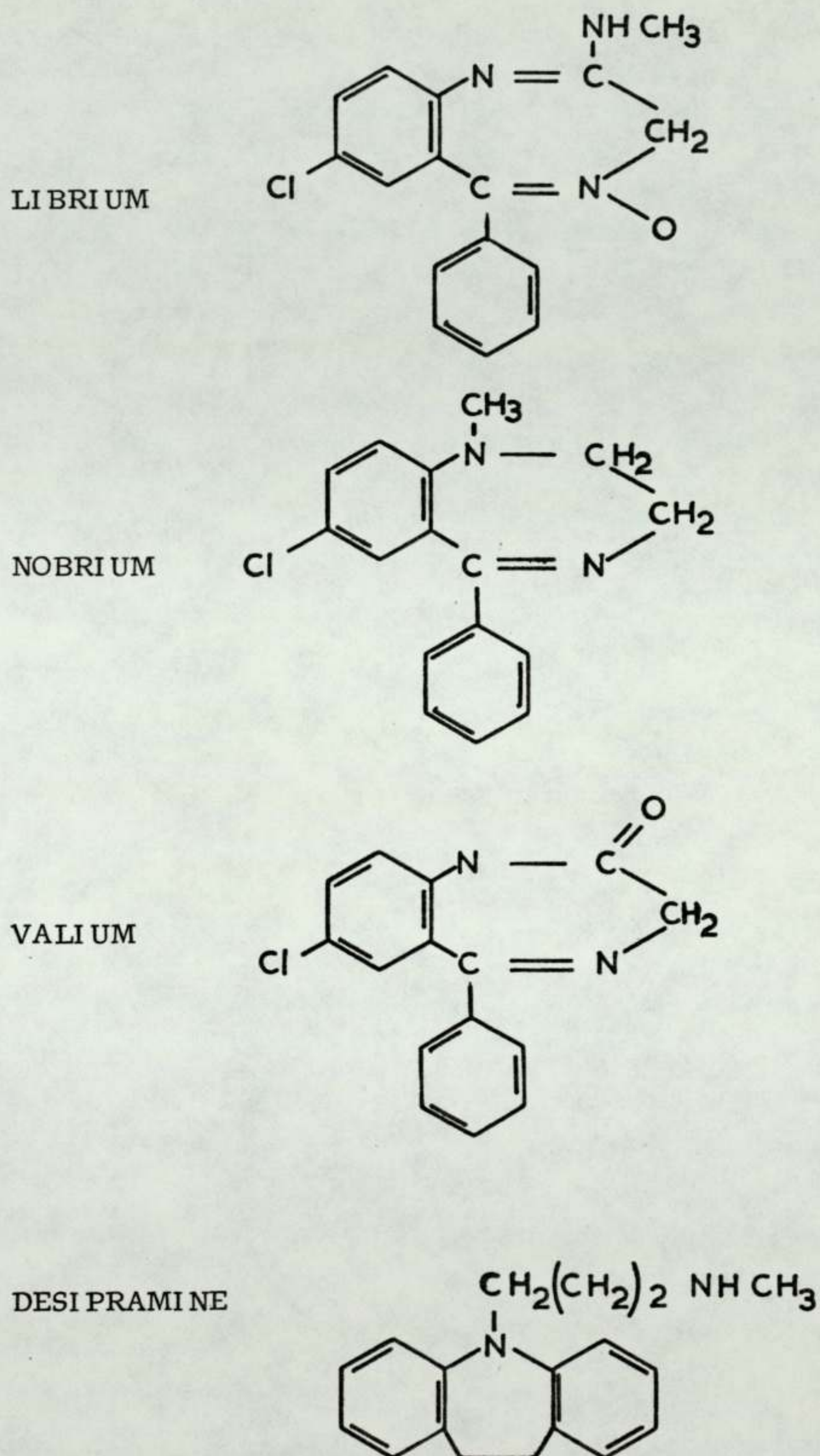


TABLE 2-1 (continued)

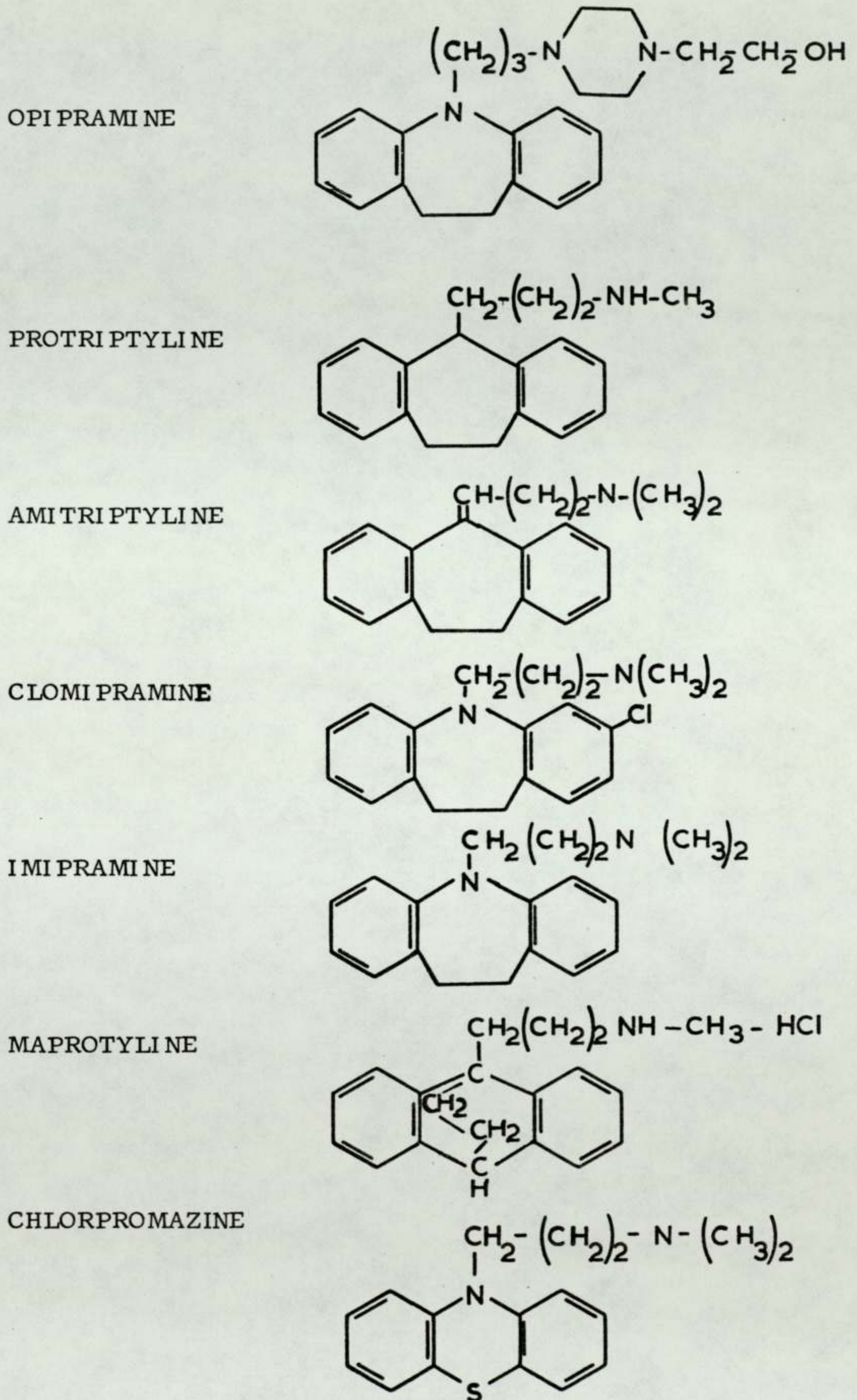
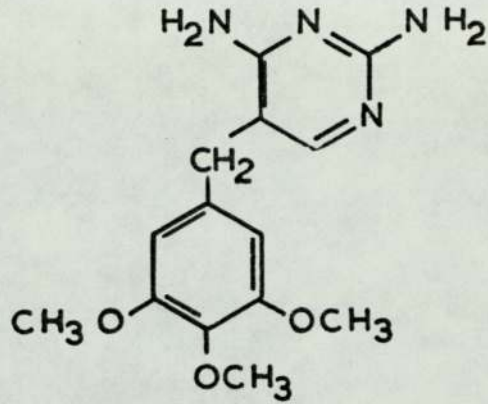
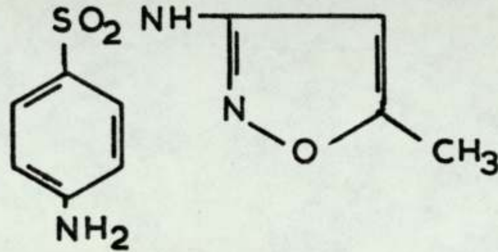


TABLE 2-1 (continued)

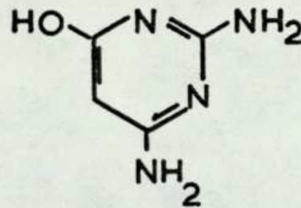
TRIMETHOPRIM



SULPHAMETHOXAZOLE



2,4-DIAMINO-6-HYDROXYPYRIMIDINE



PARACHLORPHENYLALANINE

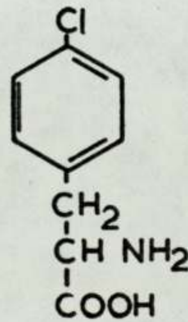
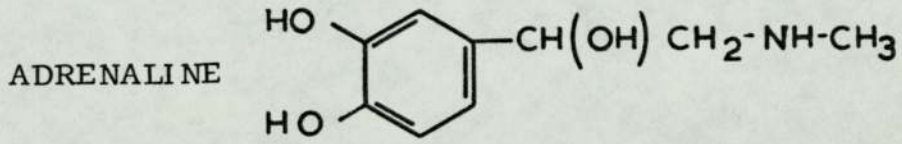
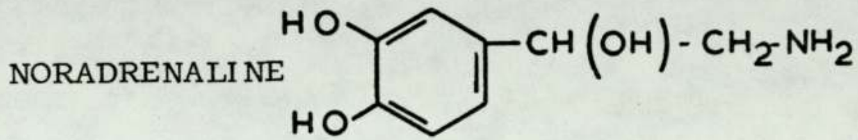


TABLE 2-1 (continued)

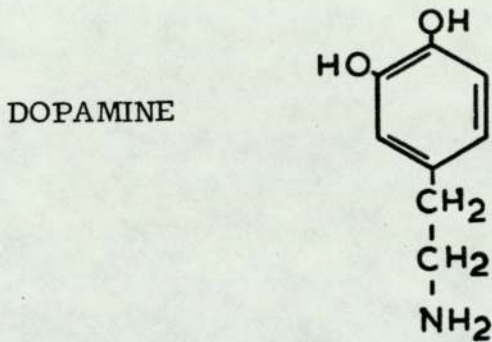
ADRENALINE



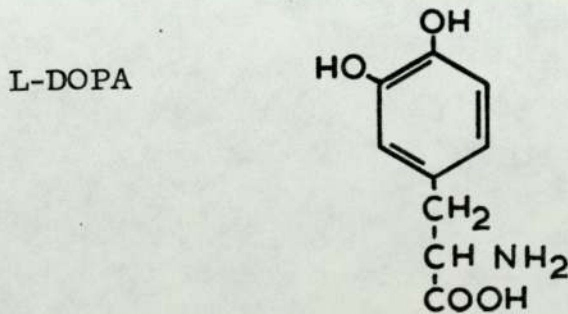
NORADRENALINE



DOPAMINE



L-DOPA



SEROTONIN

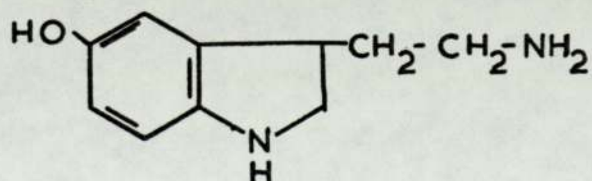
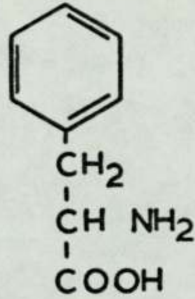
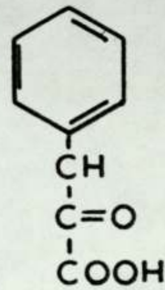


TABLE 2-1 (continued)

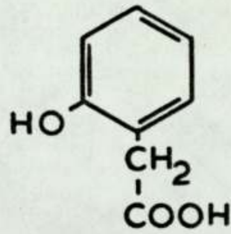
L-PHENYLALANINE



PHENYLPYRUVIC ACID



ORTHO-HYDROXYPHENYLACETIC ACID



AMINOPTERIN

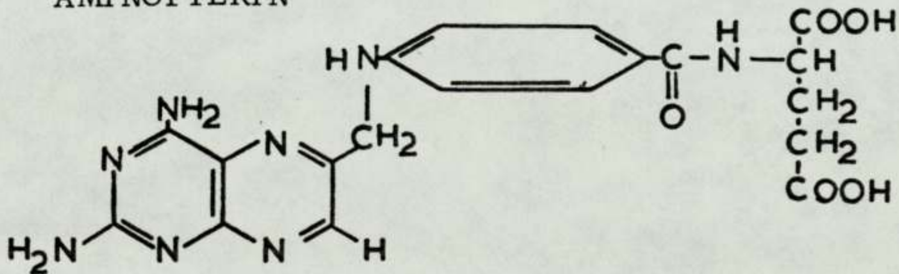
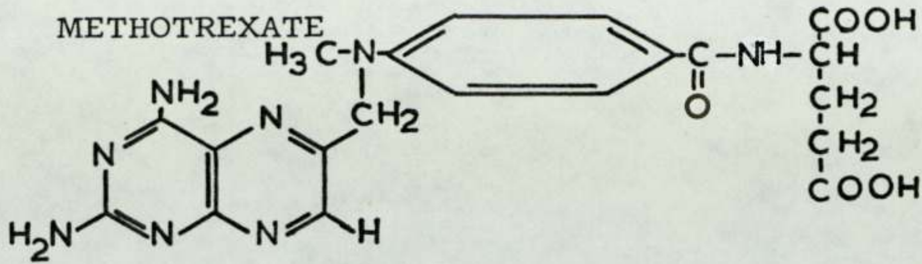


TABLE 2-1 (continued)

METHOTREXATE



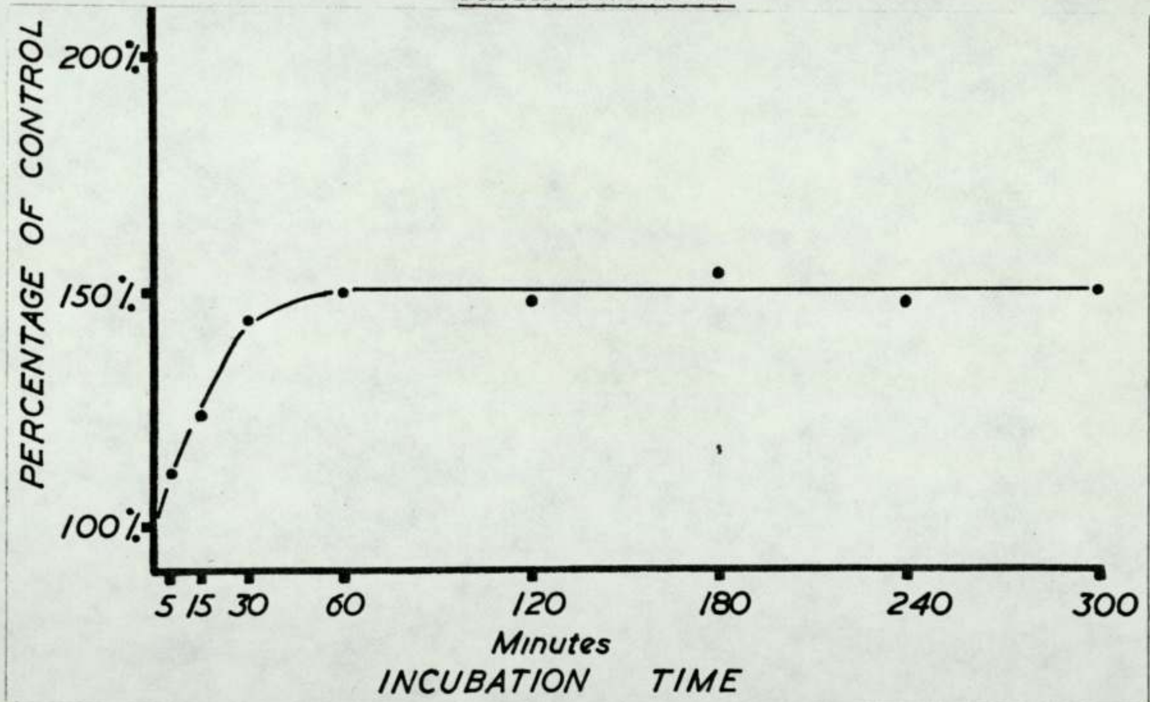
ALUMINIUM SULPHATE	-------	$Al_2(SO_4)_3 \cdot 16H_2O$
CADMIUM SULPHATE	----------	$3CdSO_4 \cdot 8H_2O$
CUPRIC SULPHATE	-------------	$CuSO_4 \cdot 5H_2O$
FERROUS SULPHATE	-----------	$FeSO_4 \cdot 7H_2O$
LEAD ACETATE	----------------	$(CH_3.COO)_2Pb \cdot 3H_2O$
LITHIUM CARBONATE	----------	Li_2CO_3
MERCURIC CHLORIDE	-----------	$HgCl_2$
ZINC SULPHATE	----------------	$ZnSO_4 \cdot 7H_2O$

RESULTS

The effects of the several components of the in-vitro biosynthesis system used in these experiments are shown in Table 2-2. It can be seen that additional NAD was not an essential requirement for the synthesis of Crithidia factor but the increase following its addition was substantial and highly significant ($p = < 0.001$) as was the further increase which occurred when methotrexate was added; aminopterin gave virtually identical results to methotrexate. The effects of incubation at different time intervals with methotrexate (Figure 2-2)

FIGURE 2-2

THE EFFECT OF THE ADDITION OF 10μg METHOTREXATE TO THE STANDARD INCUBATION MIXTURE EXPRESSED AS A PERCENTAGE OF CONTROL WITHOUT METHOTREXATE



showed the maximum stimulus to have been achieved at around half an hour. However, it was decided to use the three hour period as this would give a reasonably long exposure of the system to additives.

The brain extract, stored at -80°C (Table 2-3) showed the preparation remained viable for at least 5 weeks although there was a decrease at 4 weeks and 5 weeks which was significant by Student's 't' test ($p = < 0.02$). The maximum length of time for which brain extract was stored and used

TABLE 2-2

THE EFFECTS OF THE COMPONENTS IN THE STANDARD PROCEDURE ON THE FINAL CRITHIDIA FACTOR CONTENT

Component(s)	(No.of observations)	Mean $\pm$ SEM/mg.protein
Homogenate* alone	(6)	< 0.23 ng
Homogenate +GTP**	(6)	0.57 $\pm$ 0.01 ng
Homogenate + GTP + NAD***	(6)	1.44 $\pm$ 0.03 ng
Homogenate + GTP + NAD+ Methotrexate****	(6)	2.17 $\pm$ 0.01 ng

* 25% rat brain homogenate

** GTP (guanosine triphosphate) 3.0M

*** NAD 1.0M

**** Methotrexate 2.2×10^{-4}M

TABLE 2-3

THE VIABILITY OF BRAIN EXTRACT AFTER STORAGE AT -80°C

Crithidia factor as biopterin ng/mg protein in standard procedure

No.of weeks of storage	(No.of observations)	Without methotrexate	With methotrexate
0	(3)	1.48 $\pm$ 0.07	1.90 $\pm$ 0.02
1	(3)	1.29 $\pm$ 0.02	1.62 $\pm$ 0
2	(3)	1.65 $\pm$ 0.10	2.38 $\pm$ 0.09
3	(3)	1.44 $\pm$ 0.03	2.17 $\pm$ 0.01
4	(3)	1.12 $\pm$ 0.05	1.70 $\pm$ 0.05
5	(3)	1.09 $\pm$ 0.03	1.52 $\pm$ 0.02

under these conditions was therefore kept to 4 weeks.

Results from the tests of pharmaceutical preparations are shown in Tables 2-4, 2-5 and 2-6. Table 2-4 lists the benzodiazepine tranquillisers, Table 2-5 the tricyclic antidepressants and Table 2-6 the remaining miscellaneous drugs which it would be inconsistent to place with the neurotransmitters (Table 2-7) or elsewhere.

Most striking was the consistency of the rise with the benzodiazepines (Table 2-4) which increased the Crithidia factor content of the incubation mixture by 27% to 54% at higher concentration even though nobrium and valium were not in complete solution at the beginning of the experiment.

All the tricyclic antidepressants (Table 2-5) increased the Crithidia factor by around 20% at their most concentrated, with desipramine still being active at 3.3×10^{-6} M.

Chlorpromazine (Table 2-6) did not have any significant effect, this drug is usually given to a different category of patient from those who are given benzodiazepines and tricyclic antidepressants which are usually reserved for affective psychiatric disorders.

Trimethoprim (Table 2-6) had no effect although one might have expected an effect from a known dihydrofolate reductase inhibitor. Surprisingly, sulphamethoxazole did cause a rise and this was of the order of 30% when compared with controls. When 2,4-diamino-6-hydroxypyrimidine and parachlorphenylalanine were tested then less Crithidia factor was produced, but this only became significant with parachlorphenylalanine without added methotrexate ($p = < 0.01$) at 5.01×10^{-4}M.

L-Dopa (Table 2-7) produced a significant increase ($p = < 0.02$) with and without methotrexate. Neurotransmitters, dopamine, serotonin and noradrenaline also produced increases which were significant ($p = < 0.05$, $p = < 0.001$ and $p = < 0.01$, respectively), whereas adrenaline caused a

substantial and very significant decrease ($p = < 0.001$).

Phenylalanine (Table 2-8) had no discernible effect over a wide range of concentrations but ortho-hydroxyphenylacetic acid showed a significant increase ($p = < 0.05$) only with methotrexate present. Phenylpyruvic acid, on the other hand, produced a more significant decrease both with methotrexate ($p = < 0.05$) and without methotrexate ($p = < 0.01$).

All the metal salts (Table 2-9) except aluminium sulphate, showed significant decreases in the amount of Crithidia factor produced, cupric sulphate being by far the most potent. Indeed, cupric sulphate was so active at low concentration that it could account for the decreased Crithidia factor when other metals were tested if one assumes the maximum permitted copper contamination of most of the 'Analar' compounds at between 0.0005% and 0.001%. When a concentration of 1.0×10^{-3} M cupric sulphate was tested for its effect on the biosynthesis in the absence of NAD the resulting Crithidia factor was not significantly different ($p = 0.4 - 0.5$) from the level obtained when cupric sulphate was added in the presence of NAD. The effect of NAD on the system without added copper (Table 2-1) was highly significant ($p = < 0.001$).

None of the compounds tested stimulated the growth of Crithidia fasciculata at the concentrations used in these experiments, nor did they have any synergistic or antagonistic effect when added to the protozoological assay with biopterin.

TABLE 2-4

THE EFFECTS OF BENZODIAZEPINES ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO WITH AND WITHOUT METHOTREXATE (MTX)

Additives and concentrations	(No. of observations)	% of control	Student's 't' test
Librium 3.35×10^{-4} M	(6)	126.78 ± 2.26	$p = < 0.002$
" + MTX	(6)	127.55 ± 1.79	$p = < 0.001$
Librium 3.36×10^{-5} M	(6)	115.96 ± 1.31	$p = < 0.01$
" + MTX	(6)	98.79 ± 3.19	$p = 0.4 - 0.5$
Nobrium 3.69×10^{-4} M	(6)	144.23 ± 10.71	$p = < 0.01$
" + MTX	(6)	154.02 ± 3.44	$p = < 0.001$
Nobrium 3.69×10^{-5} M	(6)	102.70 ± 4.93	$p = 0.6 - 0.7$
" + MTX	(6)	116.96 ± 0.67	$p = 0.05 - 0.4$
Valium 3.51×10^{-4} M	(6)	128.87 ± 7.01	$p = < 0.02$
" + MTX	(6)	136.00 ± 5.55	$p = < 0.001$
Valium 3.51×10^{-5} M	(6)	129.46 ± 2.71	$p = < 0.01$
" + MTX	(6)	96.02 ± 3.67	$p = 0.4 - 0.5$

TABLE 2-5

THE EFFECTS OF TRICYCLIC ANTIDEPRESSANTS ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO WITH AND WITHOUT METHOTREXATE (MTX).

Additives and concentrations	(No.of observations)	% of control	Student's 't' test
Desipramine 3.30×10^{-4} M	(6)	116.15 ± 4.56	$p = < 0.02$
" + MTX	(6)	114.52 ± 5.78	$p = < 0.05$
Desipramine 3.30×10^{-5} M	(3)	120.70 ± 0.50	$p = < 0.01$
" + MTX	(3)	102.07 ± 0.43	$p = < 0.05$
Desipramine 3.30×10^{-6} M	(3)	113.13 ± 3.07	$p = < 0.05$
" + MTX	(3)	102.50 ± 0	$p = < 0.05$
Opipramine 2.29×10^{-4} M	(6)	132.56 ± 3.11	$p = < 0.001$
" + MTX	(6)	107.28 ± 2.15	$p = < 0.05$
Opipramine 2.29×10^{-5} M	(3)	117.50 ± 1.30	$p = < 0.01$
" + MTX	(3)	101.67 ± 0.83	$p = 0.1 - 0.2$
Opipramine 2.29×10^{-6} M	(3)	99.47 ± 6.32	$p = 0.9 - 1.0$
" + MTX	(3)	100.83 ± 0.83	$p = 0.4 - 0.5$
Protriptyline 3.34×10^{-4} M	(6)	116.23 ± 3.95	$p = < 0.02$
" + MTX	(6)	118.10 ± 7.53	$p = 0.05 - 0.1$
Protriptyline 3.34×10^{-5} M	(3)	107.77 ± 2.48	$p = 0.1 - 0.2$
" + MTX	(3)	100.40 ± 0.40	$p = 0.6 - 0.7$
Protriptyline 3.34×10^{-6} M	(3)	99.47 ± 5.36	$p = 0.9 - 1.0$
" + MTX	(3)	99.03 ± 2.33	$p = 0.7 - 0.8$

TABLE 2-5

(Continued)

Amitripyline 3.19×10^{-4}M	(3)	94.20 ± 1.10	$p = 0.3 - 0.4$
" + MTX	(3)	115.60 ± 9.22	$p = 0.1 - 0.2$
Clomipramine 2.85×10^{-4}M	(3)	116.87 ± 4.48	$p = 0.05 - 0.1$
" + MTX	(3)	123.53 ± 4.07	$p = < 0.02$
Imipramine 3.16×10^{-4}M	(3)	112.60 ± 2.19	$p = 0.05 - 0.1$
" + MTX	(3)	119.47 ± 4.07	$p = < 0.02$
Maprotyline 3.19×10^{-4}M	(3)	124.50 ± 4.48	$p = < 0.05$
" + MTX	(3)	115.10 ± 1.82	$p = < 0.01$

TABLE 2-6

THE EFFECTS OF MISCELLANEOUS DRUGS ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO WITH AND WITHOUT METHOTREXATE (MTX).

Additives and concentrations	(No.of observations)	% of control	Student's 't' test
Chlorpromazine 2.82×10^{-4}M	(3)	101.10 ± 0	p = 0.8 - 0.9
" + MTX	(3)	104.17 ± 2.92	p = 0.2 - 0.3
Trimethoprim 3.45×10^{-4}M	(9)	106.01 ± 2.57	p = 0.05 - 0.1
" + MTX	(9)	99.30 ± 1.24	p = 0.6 - 0.7
Sulphamethoxazole 3.95×10^{-4}M	(12)	129.47 ± 5.10	p = < 0.001
" + MTX	(6)	136.17 ± 4.67	p = < 0.001
2,4-diamino-6-hydroxy-pyrimidine 7.92×10^{-4}M	(3)	93.1 ± 0	p = 0.2 - 0.3
" + MTX	(3)	88.7 ± 5.98	p = 0.1 - 0.2
Parachlorphenylalanine 5.01×10^{-4}M	(12)	89.71 ± 2.38	p = < 0.01
" + MTX	(6)	99.45 ± 2.48	p = 0.9 - 1.0

TABLE 2-7

THE EFFECTS OF NEUROTRANSMITTERS AND L-DOPA ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO WITH AND WITHOUT METHOTREXATE (MTX).

Additives and concentrations	(No.of observations)	% of control	Student's 't' test
Adrenaline 5.46×10^{-4}M	(6)	76.49 ± 3.36	p = < 0.001
" + MTX	(6)	54.61 ± 0.49	p = < 0.001
Noradrenaline 5.94×10^{-4}M	(9)	109.63 ± 2.09	p = < 0.01
" + MTX	(3)	103.32 ± 0	p = 0.05 - 0.1
Dopamine 5.27×10^{-4}M	(6)	123.36 ± 2.65	p = 0.05 - 0.1
" + MTX	(6)	125.19 ± 9.05	p = < 0.05
L-Dopa 5.07×10^{-4}M	(6)	121.87 ± 4.12	p = < 0.02
" + MTX	(6)	116.08 ± 3.88	p = < 0.02
Serotonin 5.68×10^{-4}M	(6)	134.23 ± 1.40	p = < 0.01
" + MTX	(6)	135.00 ± 3.15	p = < 0.001

TABLE 2-8

THE EFFECTS OF L-PHENYLALANINE, PHENYLPYRUVIC ACID AND ORTHO-HYDROXYPHENYLACETIC ACID ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO WITH AND WITHOUT METHOTREXATE (MTX).

Additives and concentrations	(No. of observations)	% of control	Student's 't' test
Phenylalanine 6.05×10^{-4}M	(9)	98.39 ± 5.18	$p = 0.7 - 0.8$
" + MTX	(9)	107.89 ± 4.33	$p = 0.1 - 0.2$
Phenylalanine 6.05×10^{-5}M	(6)	82.45 ± 14.01	$p = 0.3 - 0.4$
" + MTX	(6)	93.43 ± 7.44	$p = 0.4 - 0.5$
Phenylalanine 6.05×10^{-6}M	(6)	94.04 ± 10.09	$p = 0.5 - 0.6$
" + MTX	(6)	90.58 ± 6.58	$p = 0.1 - 0.2$
Phenylpyruvic acid 4.90×10^{-4}M	(9)	90.67 ± 1.28	$p = < 0.01$
" + MTX	(3)	93.66 ± 1.23	$p = < 0.05$
Orthohydroxyphenylacetic acid 6.57×10^{-4}M	(9)	104.21 ± 3.13	$p = 0.2 - 0.3$
" + MTX	(3)	114.22 ± 1.23	$p = < 0.05$

TABLE 2-9

THE EFFECTS OF METALLIC SALTS
ON THE SYNTHESIS OF CRITHIDIA FACTOR IN-VITRO
WITH AND WITHOUT METHOTREXATE (MTX).

Additives and concentrations	(No.of observations)	% of control	Student's 't' test
Aluminium sulphate 10^{-3}M	(6)	80.76 ± 11.55	p = 0.1 - 0.2
" + MTX	(6)	104.98 ± 2.03	p = 0.1 - 0.2
Aluminium sulphate 10^{-5}M	(3)	90.74 ± 2.55	p = 0.3 - 0.4
" + MTX	(3)	101.69 ± 2.56	p = 0.5 - 0.6
Cadmium chloride 10^{-3}M	(6)	70.47 ± 6.70	p = < 0.05
" + MTX	(6)	70.76 ± 2.35	p = < 0.001
Cadmium chloride 10^{-5}M	(3)	90.74 ± 2.55	p = 0.05 - 0.1
" + MTX	(3)	81.72 ± 3.53	p = < 0.01
Cupric sulphate 10^{-3}M	(6)	52.91 ± 4.33	p = < 0.001
" + MTX	(6)	52.37 ± 5.25	p = < 0.001
Cupric sulphate 10^{-4}M	(3)	21.21 ± 0	p = < 0.001
" + MTX	(3)	21.15 ± 3.22	p = < 0.001
Cupric sulphate 10^{-5}M	(6)	66.85 ± 8.34	p = < 0.01
" + MTX	(6)	87.75 ± 3.77	p = < 0.05
Cupric sulphate 10^{-6}M	(3)	69.70 ± 0	p = < 0.02
" + MTX	(3)	61.34 ± 3.36	p = < 0.01

TABLE 2-9

(Continued)

Cupric sulphate 10^{-7}M	(6)	76.55 $\pm$ 4.65	p = < 0.01
" + MTX	(6)	91.02 $\pm$ 3.78	p = 0.05 - 0.1

Cupric sulphate 10^{-3}M in incubation mixture with and without NAD; - In the presence of cupric sulphate the omission of NAD gave a value of 105.33% $\pm$ 2.67 of the figure obtained with NAD. (p = 0.4 - 0.5) although the difference of both from controls was highly significant (p = < 0.001).

Ferrous sulphate 10^{-3}M	(3)	90.51 $\pm$ 5.32	p = 0.2 - 0.3
" + MTX	(3)	90.94 $\pm$ 1.63	p = < 0.02
Ferrous sulphate 10^{-5}M	(3)	92.36 $\pm$ 2.23	p = 0.1 - 0.2
" + MTX	(3)	83.41 $\pm$ 1.84	p = < 0.01
Lead acetate 10^{-2}M	(9)	100.11 $\pm$ 5.37	p = 0.9 - 1.0
" + MTX	(3)	62.67 $\pm$ 2.18	p = < 0.001
Lead acetate 10^{-3}M	(12)	98.82 $\pm$ 3.12	p = 0.6 - 0.7
" + MTX	(6)	90.98 $\pm$ 3.51	p = 0.05 - 0.1
Lead acetate 10^{-5}M	(9)	101.63 $\pm$ 2.12	p = 0.6 - 0.7
" + MTX	(3)	94.78 $\pm$ 7.97	p = 0.5 - 0.6
Lithium carbonate sat'd (circa 2 x 10^{-2}M)	(6)	71.37 $\pm$ 0.8	p = < 0.001
" + MTX	(6)	93.96 $\pm$ 2.27	p = < 0.05
Lithium carbonate 10^{-2}M	(3)	89.41 $\pm$ 5.15	p = 0.1 - 0.2
" + MTX	(3)	94.65 $\pm$ 1.03	p = < 0.05
Lithium carbonate 10^{-4}M	(3)	96.64 $\pm$ 3.91	p = 0.4 - 0.5

TABLE 2-9

(Continued)

Lithium carbonate 10^{-4}M + MTX	(3)	100.82 ± 0.82	$p = 0.4 - 0.5$
Mercuric chloride 10^{-3}M	(6)	66.77 ± 6.36	$p = 0.01$
" + MTX	(6)	55.72 ± 4.79	$p = < 0.001$
Mercuric chloride 10^{-5}M	(3)	93.28 ± 2.55	$p = 0.1 - 0.2$
" + MTX	(3)	94.31 ± 2.01	$p = 0.05 - 0.1$
Zinc sulphate 10^{-3}M	(6)	87.20 ± 5.83	$p = 0.05 - 0.1$
" + MTX	(6)	93.46 ± 3.94	$p = 0.2 - 0.3$
Zinc sulphate 10^{-5}M	(6)	83.18 ± 5.24	$p = < 0.05$
" + MTX	(6)	93.60 ± 5.69	$p = 0.3 - 0.4$

DISCUSSION

Both methotrexate and aminopterin stimulated the production of Crithidia factor from guanosine triphosphate in a crude extract of rat brain. What remained uncertain in work on human patients taking methotrexate (Leeming, Blair, Melikian and O'Gorman, 1976) was the cause of high levels of Crithidia factor in plasma. It was suggested that the trigger for de novo synthesis of the pteridine ring was an increase in unreduced biopterin derivatives accumulated by inhibition of dihydropteridine reductase or a minor pathway to biopterin being fed by unmetabolized folate. There was the added difficulty of not knowing the relationship of serum and tissue concentrations. Biopterin is excreted rapidly in the urine when given parentally (Rembold and Metzger, 1967) and is presumably synthesized locally according to need. How the serum biopterin level reflects tissue concentrations is therefore crucial in deducing, with any degree of certainty, the meaning of change in serum levels. When dealing with the intact animal, complications include kinetics of excretion and tissue/protein binding of biopterin, 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin. What is certain, however, is that raised levels of Crithidia factor in the presence of dihydropteridine reductase inhibitors do not mean an increase in active cofactor and this is reinforced by reports of children with dihydropteridine reductase deficiency (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977; Grobe, Bartholome, Milstien and Kaufman, 1978) who invariably have raised serum Crithidia factor levels even when the plasma phenylalanine is controlled by diet.

The maximum concentration of librium following therapeutic administration is around 1.7×10^{-5}M (Martindale, 1977) which is lower than the effective concentration here, but only by half. Of the three benzodiazepines, librium, nobrium and valium, only librium was sufficiently soluble in the short term of the experiment to reach a concentration which inhibited dihydropteridine reductase (Chapter III) but this does not exclude their action as being similar to methotrexate as a

cause of increased Crithidia factor synthesis. The additive effect with methotrexate could be used as an argument against this hypothesis but saturation of the active part of the system with methotrexate may not have been achieved as higher concentrations inhibited the growth of Crithidia fasciculata and increased synthesis (if any) was not sufficient to enable the inhibition effect to be diluted out during the assay.

The consistent effects of tricyclic antidepressants in raising the Crithidia factor synthesized, cannot be explained in the same way as with the benzodiazepines as the former had no effect on the dihydropteridine reductase assay (Chapter III). It is more likely that the tricyclic anti-depressants act at a separate site in the pathway from guanosine triphosphate via dihydroneopterin triphosphate to dihydrobiopterin proposed by Eto, Fukushima and Shiota (1976). The lowest concentration found to be active was that of desipramine at 3.3×10^{-6}M which is not dissimilar to the maximum concentration found in plasma, 4.56×10^{-6}M following the normal dose of up to 150 mg/day (Martindale, 1977). If use of these drugs in patients increases the production of tetrahydrobiopterin in tissue, and dihydrofolate and dihydropteridine reductases are not saturated, the level of tetrahydrobiopterin would be increased. In either event, over-flow into serum or maintenance of constant tissue concentration by some homeostatic mechanism might result in raised serum values and there is some evidence to support this in the raised mean serum Crithidia factor value of patients treated with tricyclic antidepressants (2.44 ± 0.26 μg/L compared with controls 1.88 ± 0.09μg/L) (Chapter X) although it just failed to reach the 5% level of significance in Student's 't' test.

The action of sulphamethoxazole in raising the Crithidia factor level (Table 2-6) was surprising but highly significant ($p = < 0.001$) and reproducible. The site of its action is unclear as it did not affect the dihydropteridine reductase assay (Chapter III). If the same action occurs in-vivo and cotrimoxazole (trimethoprim with sulphamethoxazole) is known to reduce phenylalanine clearance in humans following loading doses (Andrews, Purkiss, Chalmers and Watts, 1976)

then the increase in Crithidia factor following cotrimoxazole (Leeming, Blair, Melikian and O'Gorman, 1976) does not mean an increase in active cofactor unless there is separate action on phenylalanine hydroxylase. Trimethoprim, sulphamethoxazole's partner in the pharmaceutical preparation cotrimoxazole, did not affect the dihydropteridine reductase assay when 3.13×10^{-4}M was used, this was quite a substantial concentration which would approximate to the peak serum level following therapeutic administration.

The neurotransmitters tested caused an increased level of Crithidia factor, with the exception of adrenaline which caused a decrease. It is interesting to speculate on the role of adrenaline as a feedback inhibitor. The concentrations described here are less than concentrations found in argentaffine cells (1.2×10^{-2}M) but more than may be found in serum (5.5×10^{-10}M) (Documenta Geigy, 1969). The rate limiting step in noradrenaline synthesis is the hydroxylation of tyrosine (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) and the rate limiting factor in tyrosine hydroxylation in rat striatum is tetrahydrobiopterin (Kettle, Bartholini and Pletscher, 1974). If tetrahydrobiopterin is controlled by the balance of neurotransmitters then the effects of stress suggested previously in the decreased brain Crithidia factor levels in rats, kept in individual cages, (Leeming, 1975) and shown indirectly (Chapter XII) increases in credibility and it is a short step to linking this concept with mental disorders. Iverson (1976) suggested increased dopamine levels in schizophrenia although it is further suggested (Crow, Deakin, Johnson and Longden, 1976) that dopaminergic overactivity is not a necessary concomitant of schizophrenic illness and that the primary defect does not lie in the dopaminergic neurone. More recently (Bird, Barnes, Iverson, Spokes, Mackay and Shepherd, 1977) the brains of 41 patients diagnosed as schizophrenics were compared with a control group of 61 and demonstrated increased dopamine with reduced glutamic acid decarboxylase and choline acetyl transferase in the nucleus accumbens. These findings may have been related to the illness or may have been a consequence of prolonged treatment with neuroleptic drugs. The decrease in dopamine-β-hydroxylase (DBH) in schizophrenic patients (Fujita, Ito, Maruto, Teradaira, Beppu, Nakagami and Kati, 1978) and the mental

changes induced in normal men by dopamine-B-hydroxylase inhibitors plus L-dopa (Hartmann and Kellet-Teschke, 1977) go some way to supporting a link between schizophrenia and abnormal dopamine metabolism. The lack of action of the proven drug chlorpromazine on either dopamine-B-hydroxylase in-vivo (Fujita et al, 1978) or dihydrobiopterin synthesis in-vitro (Table 2-6) does not challenge the linkage of schizophrenia and tetrahydrobiopterin as phenothiazines act by blocking the synaptic uptake of dopamine (Iverson, 1976).

Although in earlier studies (Leeming, 1975) it was suggested that phenylalanine itself triggered the synthesis of Crithidia factor, the evidence presented here refutes this. Phenylalanine in-vitro had no effect over a wide range of concentrations and of the two metabolites of phenylalanine tested here, phenylpyruvic acid at 4.9×10^{-4}M caused a decrease ($p = < 0.01$) whilst ortho-hydroxyphenylacetic acid at 6.6×10^{-4}M caused a rise although this was only in the presence of methotrexate ($p = < 0.05$). In view of the closeness with which serum Crithidia factor mirrors the plasma phenylalanine (Leeming, Blair, Green and Raine, 1976) then whatever causes the serum Crithidia factor to increase or decrease must itself vary closely on the heels of phenylalanine variation and tyrosine has no such effect in-vivo (Leeming, Blair, Green and Raine, 1976). Phenylpyruvic acid in the plasma of fasting untreated adult phenylketonurics is around 2.45×10^{-5}M compared with 4.9×10^{-4}M used in these experiments. 100 mg/Kg body weight loading doses of L-phenylalanine or D-phenylalanine to adult phenylketonurics will produce plasma levels of up to 6.27×10^{-5}M phenylpyruvic acid, whilst DL-phenylalanine at 200 mg/Kg body weight generated 1.1×10^{-4}M plasma phenylpyruvic acid (Jervis and Drejza, 1966). These same authors showed that the maximum plasma level of ortho-hydroxyphenylacetic acid achieved by 100 mg/Kg body weight phenylalanine loading was 4.40×10^{-5}M which was much less than that required to produce the most modest effect on Crithidia factor synthesis. Only with D-phenylalanine and DL-phenylalanine was the plasma phenylpyruvic acid of non-phenylketonuric controls raised to a level at which it could be measured, but phenylketonurics on normal diet (as the above were) already had substantially raised plasma phenylalanine levels before loading. If the

phenylpyruvic acid level is readily raised in phenylketonuria it is a candidate for the cause of raised serum Crithidia factor levels in hyperphenylalaninaemia, but phenylpyruvic acid decreases Crithidia factor synthesis in the in-vitro synthesis (Table 2-8). A possible mode of action could be for phenylpyruvic acid to compete with q--dihydrobiopterin for dihydropteridine reductase (Chapter III), and thus act in a similar way to methotrexate whilst not affecting the rate of NADH clearance on the assay.

Copper sulphate had a dramatic effect on the synthesis of Crithidia factor in-vitro and remained active at 10^{-7}M which was well below reported serum levels of patients with Wilson's disease 4.5×10^{-5}M (Hamlyn, Gollan, Douglas and Sherlock, 1977) as well as on the dihydropteridine reductase assay. In the dihydropteridine reductase assay the apparent acceleration of the reaction was caused by the action of copper sulphate on NADH (Chapter III). There is confirmation of this action in the biosynthesis where the presence of copper sulphate reduced the amount of Crithidia factor produced in the presence of NAD to a figure not significantly different from that obtained without NAD. One cannot exclude additional action of copper on any tetrahydrobiopterin or dihydrobiopterin produced as this metal catalyses the auto-oxidation of these to biopterin even at low concentration (Blair and Pearson, 1974). Copper is present in other metal salts and is permitted in 'Analar' compounds at concentrations of up to 0.001% and the effects shown by solutions of these salts in the synthesis can be explained by this level of contamination.

Of the other metallic salts tested, lithium carbonate has a use in the treatment of manic-depressive psychosis although it is poisonous at a higher than therapeutic dosage. Lead, cadmium and mercury are all recognised poisons (Flink, 1975). There is a suggestion that lithium carbonate is associated with raised serum Crithidia factor levels in treated patients (Chapter X) but this is tempered by the fact that the patients had other therapy, with few exceptions. In-vitro lithium appeared to cause a decrease as did the other metals investigated. This may have been a copper contamination effect as already mentioned. The slight effect of aluminium ($p = >0.05$) on the biosynthesis suggests that the site of action, if indeed it

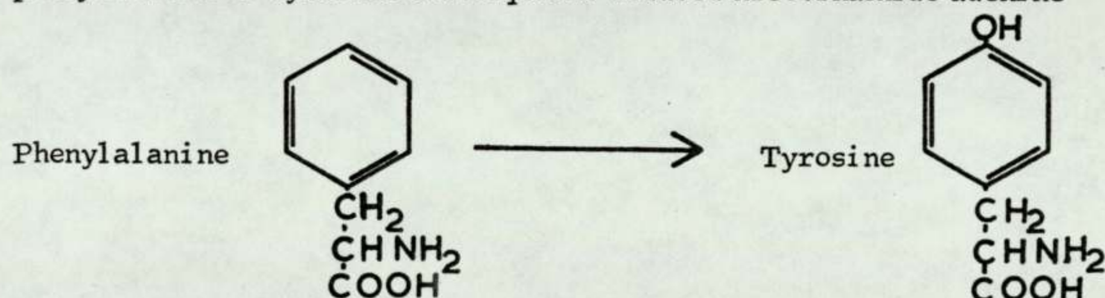
has one, in dialysis dementia is not the synthesis of tetrahydrobiopterin.

CHAPTER III

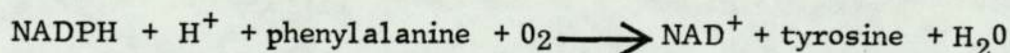
THE EFFECTS OF PHARMACEUTICAL PREPARATIONS AND OTHER COMPOUNDS ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO.

INTRODUCTION

Dihydropteridine reductase (DHPR) was first reported as 'sheep liver enzyme' (Kaufman, 1964) which with 'rat liver enzyme' (phenylalanine hydroxylase) effected the enzymatic conversion of phenylalanine to tyrosine and required reduced nicotinamide adenine

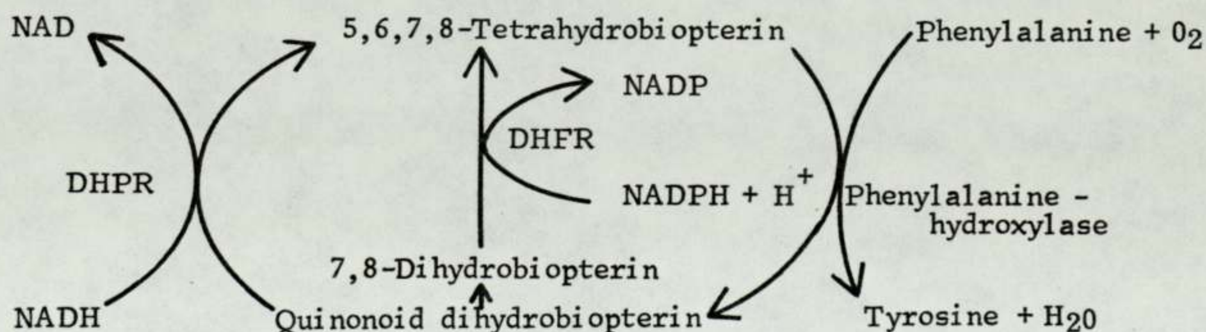


dinucleotide phosphate (NADPH) according to the formula:-

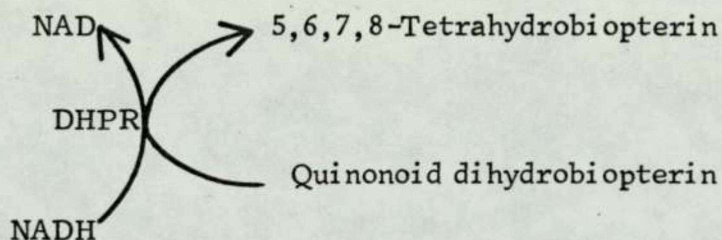


$$NADPH + H^+ + \text{phenylalanine} + O_2 \longrightarrow NAD^+ + \text{tyrosine} + H_2O$$

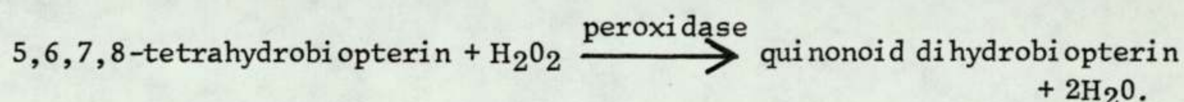
It was later shown (Kaufman, 1958; Kaufman, 1963) that tetrahydrobiopterin was an essential cofactor in the hydroxylation of phenylalanine and that the equation could be set down in detail (Craine, Hall and Kaufman, 1972) with dihydrofolate reductase (DHFR) salvaging any 7,8-dihydrobiopterin back to tetrahydrobiopterin. It followed that provided one could generate quinonoid



dihydrobiopterin then one could use the reaction:-



to determine the rate of dihydrobiopterin reduction by measuring the disappearance of NADH spectrophotometrically. This is the technique described by Craine, Hall and Kaufman (1972) who further, replaced 5,6,7,8-tetrahydrobiopterin by 6,7-dimethyl-5,6,7,8-tetrahydrobiopterin which was commercially available. They produced quinonoid dihydrobiopterin with peroxidase according to the equation:-



$$\text{5,6,7,8-tetrahydrobiopterin} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{quinonoid dihydrobiopterin} + 2H_2O.$$

These same workers purified dihydropteridine reductase from sheep liver by ammonium sulphate and zinc-ethanol fractionation followed by adsorption and elution from calcium phosphate gel, DEAE cellulose chromatography and 'Sephadex' gel filtration, achieving a 9% yield and 156% increase in specific activity (per mg protein). By polyacrylamide gel electrophoresis they showed that the enzyme existed as a dimer of molecular weight 41,000 to 42,800. Dihydropteridine reductase first assumed a more immediate importance in clinical medicine when it was retrospectively suggested that dihydropteridine reductase deficiency might have been associated with an atypical form of phenylketonuria in which progressive neurological illness was unresponsive to dietary control of hyperphenylalaninaemia and concluded in death (3 cases) in early childhood (Smith, Clayton and Wolff, 1975). Confirmation of the hypothesis rapidly came from detailed biochemical studies of other cases (e.g. Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977).

Folic acid antagonists such as aminopterin can inhibit phenyl-

alanine hydroxylation in the range 10^{-5}M to 10^{-6}M in-vitro and can be demonstrated in-vivo in the rat (Kaufman and Levenberg, 1959). Methotrexate has been shown to inhibit dihydropteridine reductase in-vitro (Craine, Hall and Kaufman, 1972) with a Ki of 4×10^{-5}M and that the inhibition is competitive with pterin cofactor. Another pharmaceutical preparation which reduces phenylalanine clearance following an oral load, presumably by inhibition of phenylalanine hydroxylation, is cotrimaxazole - sulphamethoxazole with trimethoprim - (Andrews, Purkiss, Chalmers and Watts, 1976). The importance of long term effects of dihydropteridine reductase inhibitors has been raised in connection with high dose regimes of methotrexate (Cotton, 1978; Leeming and Blair, 1978). Effects of methotrexate on the central nervous system might explain the profound dementia and dependence following chemotherapy in some cases of childhood leukaemia (Meadows and Evans, 1976) which are not explained by concurrent irradiation of the central nervous system (Eiser, 1978).

If the effects of methotrexate on neurological function are similar to those resulting from the late treatment with L-dopa, carbidopa and 5-hydroxytryptophan of dihydropteridine reductase deficient children who nevertheless had phenylalanine controlled diets from early infancy then dihydropteridine reductase deficiency may form a model from which clinical predictions may be made. For example the duration of exposure to methotrexate may be equated to the length of time before neurotransmitter therapy was instigated. One major criticism is that children with leukaemia and therefore candidates for methotrexate therapy, form an older age group and brain development will be at a different stage as will their learning programme so that comparisons of intellectual ability, even if made at a fixed age, must take into account the age when neurological insult occurred.

From the point of view of the clinician using potent, long-acting dihydropteridine reductase inhibitors such as methotrexate a clear decision may have to be taken on the balance of benefit and harm to different parts of the human organism or the use of alternative forms of therapy. Further consideration may have to be given to drugs used to mitigate side-effects, 5-formyltetrahydrofolic acid is given in high dose regimes of methotrexate.

This procedure is adopted in selected cases , for example osteogenic sarcoma where tumour tissue is less readily penetrable (Frei, Jaffe, Tattersall, Pitman and Parker, 1975). The subsequent 5-formyltetrahydrofolic acid 'rescues' more accessible tissue. If one wishes to take into account neurological effects then 5-methyltetrahydrofolic acid may be a better choice as 'rescue' agent as it is proven in the mouse (Searle and Blair, 1970) and does not appear to affect biopterin metabolism when given after methotrexate (Leeming, Blair, Melikian and O'Gorman, 1976) . When either 5-formyltetrahydrofolic acid or 5-methyltetrahydrofolic acid are given alone they do not affect the serum Crithidia factor concentration but following methotrexate, 5-formyltetrahydrofolic acid is consistently associated with a serum Crithidia factor increase whether orally or intravenously administered whereas 5-methyltetrahydrofolic acid has no such effect. Dopa, carbidopa and 5-hydroxytryptophan may be indicated, as they are in inherited dihydropteridine reductase deficiency (Bartholome and Byrd, 1975) . The use of tetrahydrobiopterin is unlikely to be effective if given orally or intravenously as it does not readily pass the blood/ brain barrier (Kettler, Bartholini and Pletscher, 1974) and is poorly transported across the intestine (Blair, Ratanasthien and Leeming, 1974) .

However deleterious the neurological effects of methotrexate may be, they are side-effects of the drug's use at other sites and do not contribute to its effectiveness. On the other hand there are many psycho-active drugs whose precise mechanism of action is uncertain. Should these drugs affect dihydropteridine reductase or dihydrobiopterin synthesis (Chapter II) then it follows that the supply of active cofactor will be diminished and as the rate-limiting determinant in the hydroxylation of tyrosine to dopa (Kettler, Bartholini and Pletscher, 1974) which itself is the rate-limiting step on which noradrenaline levels are dependent (Levitt, Spector, Sjoerdsma and Udenfriend, 1965), neurological consequences might be anticipated. There are a number of possibilities, of which competition between phenylalanine, tyrosine and tryptophan for available cofactor is one which could alter the essential balance between neurotransmitters. On such a critical area the additional role of neurotransmitters and their metabolites is important if they act in feedback control mechanisms which control tetrahydrobiopterin levels, whether by acting

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on DHPR or on de novo synthesis of the pteridine ring.

In this chapter the effects of pharmaceutical preparations, phenylalanine and some of its metabolites; neurotransmitters, metal salts and a number of pteridines on dihydropteridine reductase will be assessed.

MATERIALS AND METHODS

Dihydropteridine assays were carried out using the method of Craine et al, (1972) with the addition of sodium azide as catalase inhibitor. Each assay was made up as follows: -

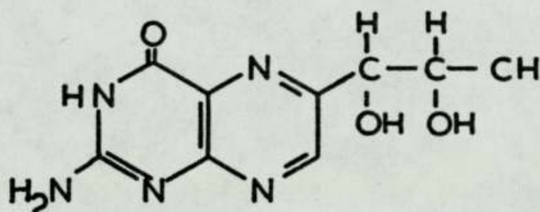
1. Distilled water	0.1 cm^3
2. Tris(hydroxymethyl) aminomethane 0.1 M with sodium azide 5×10^{-4}M adjusted to pH 7.2	0.5 cm^3
3. Horse radish peroxidase (Calbiochem Ltd.) 100 mg/L.	0.1 cm^3
4. Hydrogen peroxide 0.1 M	0.1 cm^3
5. 6,7-dimethyl-5,6,7,8-tetrahydropterin (Calbiochem Ltd.) 1×10^{-4}M	0.1 cm^3
6. Rat brain homogenate prepared as in the previous chapter and with known protein content. Then diluted a further 2:3 with tris buffer.	0.1 cm^3
7. NADH (Sigma London Ltd.) from pre-weighed vials 1×10^{-3}M	0.1 cm^3

NADH was always the last addition and accompanied by mixing. Reagents tested for their effect on the system replaced the distilled water in equal volume (0.1 cm^3). The rate of reaction was measured by decrease in absorbance at 340 nm in a Pye Unicam S.P. spectrophotometer from one minute of the addition of NADH to ensure that mixing was complete and the reaction established. Readings were taken directly onto a chart recorder and were observed for a minimum of five minutes. Results calculated as n.mol. NADH/min./mg.protein and subsequently expressed as a percentage of controls without additives but an otherwise complete reaction mixture. This was done to interpret and compare data from experiments carried out at different times with different brain extracts. In addition to the compounds described in the previous chapter (Table 2-1), the pteridines in Table 3-1 were tested.

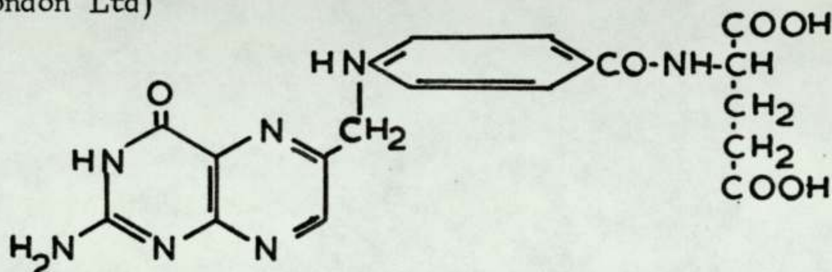
TABLE 3 - 1

PTERIDINES TESTED FOR THEIR EFFECT ON THE ACTIVITY OF DIHYDROPTERIDINE REDUCTASE IN-VITRO

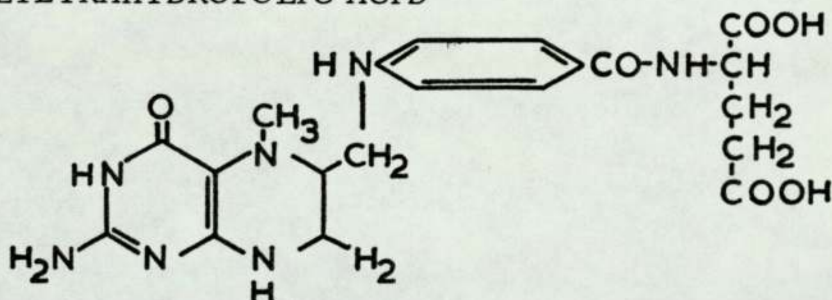
BIOPTERIN
(Roche Research Laboratories)



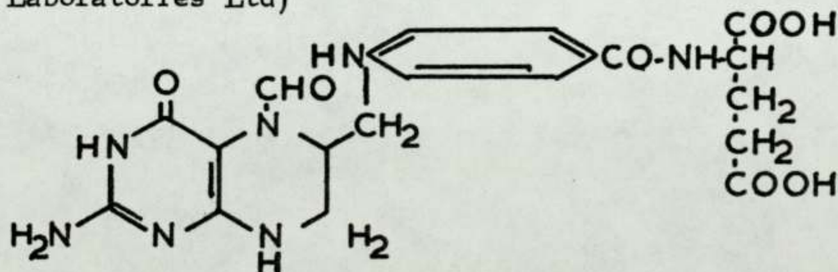
PTEROYL-L-MONOGLUTAMIC ACID (FOLIC ACID)
(Sigma London Ltd)



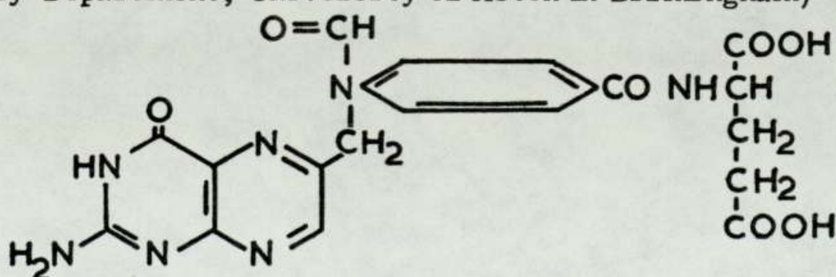
5-METHYLTETRAHYDROFOLIC ACID
(Eprova)



5-FORMYLTETRAHYDROFOLIC ACID
(Lederle Laboratories Ltd)



10-FORMYLFOLIC ACID
(Chemistry Department, University of Aston in Birmingham)

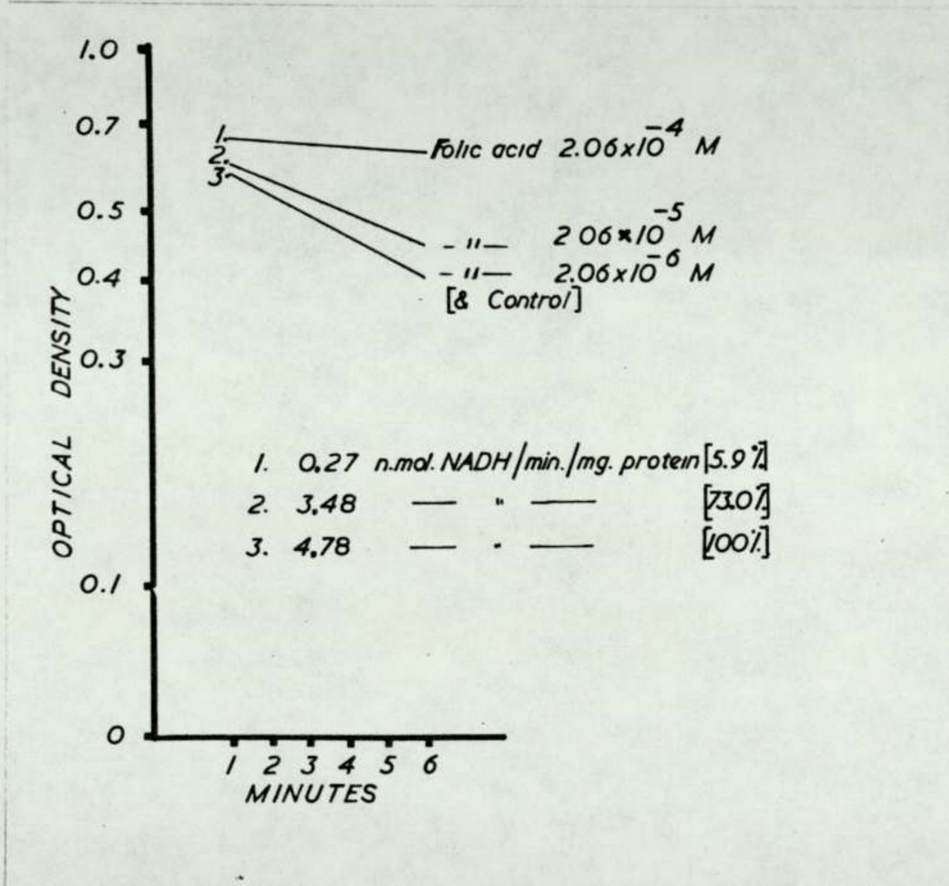


RESULTS

A typical assay is shown in figure 3-1 with folic acid at three concentrations and control without folic acid. Results were invariably

FIGURE 3-1

A TYPICAL DIHYDROPTERIDINE REDUCTASE ASSAY
SHOWING THE EFFECT OF FOLIC ACID AT THREE CONCENTRATIONS.



very close when repeated and each figure is a mean of two.

The pteridines (Table 3-2) produced some interesting results. In addition to the more predictable effects of aminopterin and methotrexate; biopterin, folic acid and 10-formylfolic acid all inhibited dihydropteridine reductase whilst the reduced folates 5-methyltetrahydrofolic acid and 5-formyltetrahydrofolic acid had no effect. The inhibition by folic acid was almost equimolar with methotrexate and disappeared at 2×10^{-6}M whilst 10-formylfolic acid at 1.737×10^{-4}M produced an identical effect to that of methotrexate at

TABLE 3-2

THE EFFECTS OF SOME PTERIDINES ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO.

Additive	Concentration of additive in reaction mixture	Rate of NADH oxidized as a % of control (mean of 2)
Methotrexate	2.00×10^{-4}M	5.9%
	2.00×10^{-5}M	74.0%
	2.00×10^{-6}M	100.0%
Aminopterin	2.06×10^{-4}M	7.3%
	2.06×10^{-5}M	66.7%
	2.06×10^{-6}M	100.0%
Biopterin	3.84×10^{-4}M	68.0%
	3.84×10^{-5}M	100.2%
Pteroyl-l-monoglutamic acid	2.06×10^{-4}M	5.6%
	2.06×10^{-5}M	73.0%
	2.06×10^{-6}M	100.0%
10-formylfolic acid	1.74×10^{-4}M	5.6%
	1.74×10^{-5}M	100.0%
5-methyltetrahydrofolic acid	1.64×10^{-4}M	99.6%
	1.64×10^{-5}M	100.0%
5-formyltetrahydrofolic acid	1.72×10^{-4}M	100.0%
	1.72×10^{-5}M	99.8%

2.001×10^{-4}M, the inhibition was diluted out by a further 10-fold dilution with 10-formylfolic acid but persisted with methotrexate.

Of the benzodiazepines (Table 3-3) only librium had a marked effect on the rate of the dihydropteridine reductase reaction. However only librium was completely soluble in the short term of these experiments unlike the biosynthesis experiments (Chapter II) where there was a three hour incubation at 37°C which allowed greater solution.

None of the tricyclic antidepressants (Table 3-4) affected the reaction although they consistently had the effect of increasing the biosynthesis (Chapter II) pointing to a different site of action to the benzodiazepines.

Chlorpromazine, trimethoprim, sulphamethoxazole, 2,4-diamino-6-hydroxypyrimidine and parachlorphenylalanine (Table 3-5) also had no action on dihydropteridine reductase and of these drugs, chlorpromazine, 2,4-diamino-6-hydroxypyrimidine and trimethoprim had no effect on the biosynthesis whilst sulphamethoxazole caused an increase and parachlorphenylalanine caused a decrease in synthesis.

L-dopa, dopamine and serotonin all increased the rate of the dihydropteridine reductase reaction (Table 3-6) at around 5.0×10^{-4}M whilst similarly increasing the biosynthesis whereas noradrenaline which had increased the in-vitro synthesis had no effect on dihydropteridine reductase. At the highest concentration dopamine and serotonin appeared to oxidize NADH without the presence of dihydropteridine reductase but not sufficiently to account for more than a very small fraction of the total effect in the complete reaction mixture.

Phenylalanine (Table 3-7) had no effect, nor did ortho-hydroxyphenylacetic acid but with phenylpyruvic acid at 4.5×10^{-4}M the rate of NADH disappearance was nearly three times that of the control (without additive) but when brain extract was omitted the reaction rate was

TABLE 3-3

THE EFFECTS OF BENZODIAZEPINES ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO.

Additive	Concentration of additive in reaction mixture	Rate of NADH oxidized as a % of control (mean of 2)
Librium	3.03×10^{-4}M	8.0%
	1.57×10^{-4}M	40.7%
	6.06×10^{-5}M	87.2%
Nobrium	0.1 cm^3 saturated soln./1.1cm^3	86.0%
	0.1cm^3 ½ sat'd soln./1.1 cm^3	100.6%
	0.1cm^3 1/5 sat'd soln./1.1cm^3	100.0%
Valium	0.1cm^3 sat'd soln/1.1cm^3	100.0%
	0.1cm^3 ½ sat'd soln./1.1cm^3	101.0%
	0.1cm^3 1/5 sat'd soln./1.1cm^3	100.3%

TABLE 3-4

THE EFFECTS OF TRICYCLIC ANTIDEPRESSANTS ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO.

Additives	Concentration of additive in reaction mixture	Rate of NADH oxidized as a % of control (mean of 2)
Amitriptyline	2.90×10^{-4}M	100.0%
Clomipramine	2.59×10^{-4}M	99.6%
Desipramine	3.00×10^{-4}M	100.2%
Imipramine	2.87×10^{-4}M	100.0%
Maprotyline	2.90×10^{-4}M	100.4%
Opipramine	2.08×10^{-4}M	100.2%
Protriptyline	3.03×10^{-4}M	99.7%

TABLE 3-5

THE EFFECTS OF MISCELLANEOUS DRUGS
ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO

Additive	Concentration of additive in reaction mixture	Rate of NADH oxidized as a % of control (mean of 2)
Chlorpromazine	2.56×10^{-4}M	initial reaction circa 100% overtaken by increase in O.D.
	2.56×10^{-5}M	99.8%
Trimethoprim	3.13×10^{-4}M	98.6%
Sulphamethoxazole	3.59×10^{-4}M	99.3%
2,4-diamino-6-hydroxy-pyrimidine	7.20×10^{-4}M	98.0%
Parachlorphenylalanine	4.55×10^{-4}M	100.4%

TABLE 3-6

THE EFFECTS OF NEUROTRANSMITTERS AND L-DOPA
ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO.

Additive	Concentration of additive in reaction mixture	Rate of NADH oxidized as a% of control (mean of 2)	
Adrenaline	4.96×10^{-4}M	79.8%	
	4.96×10^{-5}M	100.0%	
Noradrenaline	5.37×10^{-4}M	99.0%	
Dopamine	4.79×10^{-4}M	143.4%	*
	4.79×10^{-5}M	100.0%	
L-Dopa	4.61×10^{-4}M	Increase in O.D.	
	4.61×10^{-5}M	152.9%	**
	4.61×10^{-6}M	102.0%	
Serotonin	5.16×10^{-4}M	191.2%	***
	5.16×10^{-5}M	104.2%	

Reaction rate without brain extract

* 8.7%

** 1.8%

*** 4.1% Control 1.3%

TABLE 3-7

THE EFFECTS OF
L-PHENYLALANINE, PHENYLPYRUVIC ACID
AND ORTHO-HYDROXYPHENYLACETIC ACID
ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO

Additive	Concentration of additive in reaction mixture	Rate of NADH oxidized as a % of control (mean of 2)
L-Phenylalanine	5.50×10^{-4}M	100%
Phenylpyruvic acid	4.45×10^{-4}M	286.5% *
	4.45×10^{-5}M	99.6%
Ortho-hydroxyphenylacetic acid	5.97×10^{-4}M	100%

Reaction rate without brain extract

* 0.5%

control 0.8%

similar to a control without brain extract, showing that this was not caused by direct action on NADH. It must be pointed out that the maximum plasma concentration of phenylpyruvic acid in phenylketonurics loaded with L-phenylalanine, 6.27×10^{-5}M (Jervis and Drejza, 1966) was near to the lower inactive concentration of phenylpyruvic acid used in these experiments.

The metallic salts (Table 3-8) varied in their action. Cupric sulphate increased the apparent rate of the reaction even at very low concentration (1.82×10^{-6}M) although this activity could largely be ascribed to direct oxidation of NADH as the rate of NADH clearance was 57.1% of a control with dihydropteridine reductase whereas without either dihydropteridine reductase or cupric sulphate this was only 0.8%. Aluminium sulphate, cadmium chloride and lead acetate were similar to each other and inhibited dihydropteridine reductase at 1.82×10^{-4}M whilst having little or no effect at 1.82×10^{-5}M. Ferrous sulphate and mercuric chloride were ten times more active than aluminium, cadmium and lead whilst zinc sulphate was only active at 1.82×10^{-3}M.

TABLE 3-8

THE EFFECTS OF METALLIC SALTS ON DIHYDROPTERIDINE REDUCTASE ACTIVITY IN-VITRO

Additive	Concentration of additive in reaction mixture	Rate of NADH oxidized as a % of control (mean of 2)
Aluminium sulphate	1.82×10^{-4}M	59.3%
	1.82×10^{-5}M	98.0%
Cadmium chloride	1.82×10^{-4}M	45.3%
	1.82×10^{-5}M	99.0%
Cupric sulphate	1.82×10^{-4}M	355.4% *
	1.82×10^{-5}M	171.3%
	1.82×10^{-6}M	107.5%
Reaction rate without brain extract		
* 57.1% Control 0.8%		
Ferrous sulphate	1.82×10^{-4}M	33.9%
	1.82×10^{-5}M	57.2%
	1.82×10^{-6}M	98.8%
Lead acetate	1.82×10^{-4}M	59.3%
	1.82×10^{-5}M	100.2%
Lithium carbonate	0.1cm^3 sat'd soln. added circa 18.18 $\times 10^{-3}$M	92.6
	9.09×10^{-3}M	99.8%
Mercuric chloride	1.82×10^{-4}M	1.3%
	1.82×10^{-5}M	28.3%
	1.82×10^{-6}M	102.0%
Zinc sulphate	1.82×10^{-3}M	27.6%
	1.82×10^{-4}M	91.7%

DISCUSSION

The fact that methotrexate inhibited dihydropteridine reductase confirmed earlier work (Craine, Hall and Kaufman, 1972) and is in accord with the in-vivo observation of reduced phenylalanine clearance in patients being treated with methotrexate and who were subjected to phenylalanine loading tests (Goodfriend and Kaufman, 1961). Raised serum Crithidia factor levels when the plasma phenylalanine was not elevated in patients on methotrexate and in patients with malignant hyperphenylalaninaemia caused by dihydropteridine reductase deficiency (Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977) is a further pointer to dihydropteridine reductase as a target for methotrexate.

The action of the remaining pteridines tested divides them into tetrahydro forms which had no action and unreduced forms which inhibited dihydropteridine reductase, two questions arise from this observation. The first is concerned with the method of action, for example competitive inhibition, the second relates to possible neurotoxicity of unreduced folates. Although no attempt has been made unequivocally to establish competition between quinonoid dihydrobiopterin and unreduced folates for DHPR it seems a very reasonable supposition in view of their similar chemical structures. That dihydropteridine reductase could have a role in the maintenance of tetrahydrofolate levels in brain tissue is confirmed by the DHPR mediated NADH stimulation of the serine-hydroxymethyl-transferase reaction and the reduced level of folate in brain tissue in a case of congenital DHPR deficiency (Pollock and Kaufman, 1978). This competition is not equimolar with folate in-vitro when the concentrations used in the experiments described here are taken into account. Only 9.09×10^{-6}M pterin was added, yet when 2.06×10^{-5}M folic acid (or a similar amount of methotrexate) was added, dihydropteridine reductase activity was still 73% of the uninhibited reaction. There has been a report of neurological and gastrointestinal symptoms in patients on the standard dose of 5mg. three times daily (Hunter, Barnes, Oakley and Matthews, 1970) but this has not been

confirmed (Sheehy, 1973). Larger doses have not been tried and a proportion of folic acid is converted to 5-methyltetrahydrofolic acid on absorption (Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974). The total serum folate level reaches around 4.5×10^{-7}M following a standard 5mg. dose which is considerably less than the amount of folic acid required to affect dihydropteridine reductase activity in-vitro.

The action of librium, nobrium (and perhaps valium given greater solubility) in inhibiting dihydropteridine reductase is a factor they have in common with known dihydropteridine reductase inhibitors aminopterin and methotrexate. Their precise mode of action is not known, nor for certain, is the way in which known dihydropteridine reductase inhibitors increase biosynthesis. However there is support for the hypothesis that benzodiazepines by affecting dihydropteridine reductase reduce the amount of available pterin cofactor - intraperitoneal diazepam (valium) at 1 - 10mg/Kg to rats has been shown to cause a decrease in dopa and 5-hydroxytryptophan (Biswas and Carlsson, 1978) and these compounds are the immediate products of the hydroxylations of tyrosine and tryptophan respectively, both of which are dependent on tetrahydrobiopterin.

The tricyclic antidepressants which increased biosynthesis had no effect on the dihydropteridine reductase assay and there are no parallels to be drawn on the way these compounds reacted with the two systems. Nor is there further information from the way in which tricyclic antidepressants appear to act by inhibiting neuronal uptake of noradrenaline and 5-hydroxytryptamine (serotonin) (Horn, 1976; Waldmeier, Baumann, Greengrass and Maitre, 1976). Chlorpromazine which is a blocker of central dopamine receptors (Crow, Deakin, Johnstone and Longden, 1976) similarly had no effect on dihydropteridine reductase but neither did it have any effect on the synthesis.

Increased dihydropteridine reductase activity in the presence of dopa, dopamine and serotonin contrast with the inhibition of dihydropteridine reductase by adrenaline and total lack of action by noradrenaline. The balance

of neurologically active compounds might be concerned in the maintenance of adequate tetrahydrobiopterin in brain tissue. It is difficult to reconcile raised dihydropteridine reductase activity and increased synthesis of the pterin ring with raised synthesis in the presence of dihydropteridine reductase inhibitors.

The method by which serum Crithidia factor is raised following increased plasma phenylalanine levels in loaded normal subjects or in phenylketonurics and decreased in phenylalanine depletion (Leeming, Blair, Green and Raine, 1976) is still uncertain. It has been suggested that serum Crithidia factor levels should be examined with knowledge of concurrent plasma phenylalanine levels (Kaufman, Berlow, Summer, Milstien, Schulmann, Orloff, Spielberg and Pueschel, 1978) because of the intimate relationship of the two parameters. The results in this and the previous chapter show that phenylalanine itself has no action on the synthesis of dihydrobiopterin or its reduction to tetrahydrobiopterin. Phenylpyruvic acid is interesting as the only compound (other than copper) which increases the rate of NADH clearance in the dihydropteridine reductase reaction whilst decreasing the synthesis. The phenylpyruvic acid levels effective were higher than those found in adult phenylketonurics and in normal controls loaded with phenylalanine (Jervis and Drejza, 1966), but the exposure was short. Although there is no evidence to show that phenylpyruvic acid does not increase the rate of pterin reduction one must question this and look for credible alternatives. One such alternative is that phenylpyruvic acid utilises dihydropteridine reductase or some other enzyme in the crude brain extract in a reaction which consumes NADH. If its site of action was dihydropteridine reductase then it acts as a competitive inhibitor of dihydrobiopterin reduction and as such would fit the description required of the agent responsible for the rise in serum Crithidia factor in hyperphenylalaninaemia.

The effect of copper was largely directed at NADH itself which was clearly demonstrated by the NADH clearance when dihydropteridine reductase was removed from the reaction. The apparent increase in the

reaction rate may, however, have masked a direct effect on dihydropteridine reductase which was shown by the other metals. One of the most toxic of these, mercury, decreased dihydropteridine reductase activity to a greater extent and at lower concentration than the other metals tested.

Aluminium was active against dihydropteridine reductase at 1.82×10^{-4}M which is less than the concentration found in brain dialysis dementia (1.85×10^{-4}M - Alfrey, LeGendre and Kaehny, 1976; 1.18×10^{-4}M - McDermott, Smith, Ward, Parkinson and Kerr, 1978). Lead also inhibited dihydropteridine reductase at the same molarity.

CHAPTER IV

THE EFFECTS OF PHARMACEUTICAL PREPARATIONS AND OTHER COMPOUNDS ON THE SYNTHESIS AND MAINTENANCE OF 5,6,7,8-TETRAHYDROBIOPTERIN

In the previous two chapters the effects of a number of agents on dihydrobiopterin synthesis and dihydropteridine reductase activity were described and discussed. The biosynthesis of 7,8-dihydrobiopterin, its reduction to 5,6,7,8-tetrahydrobiopterin and its participation in phenylalanine hydroxylation are shown in Figure 4-1. The hydroxylation of tryptophan (Hosoda and Glick, 1966) to 5-hydroxytryptophan and the hydroxylation of tyrosine to dopa (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) both require 5,6,7,8-tetrahydrobiopterin and can replace the phenylalanine hydroxylation in the figure.

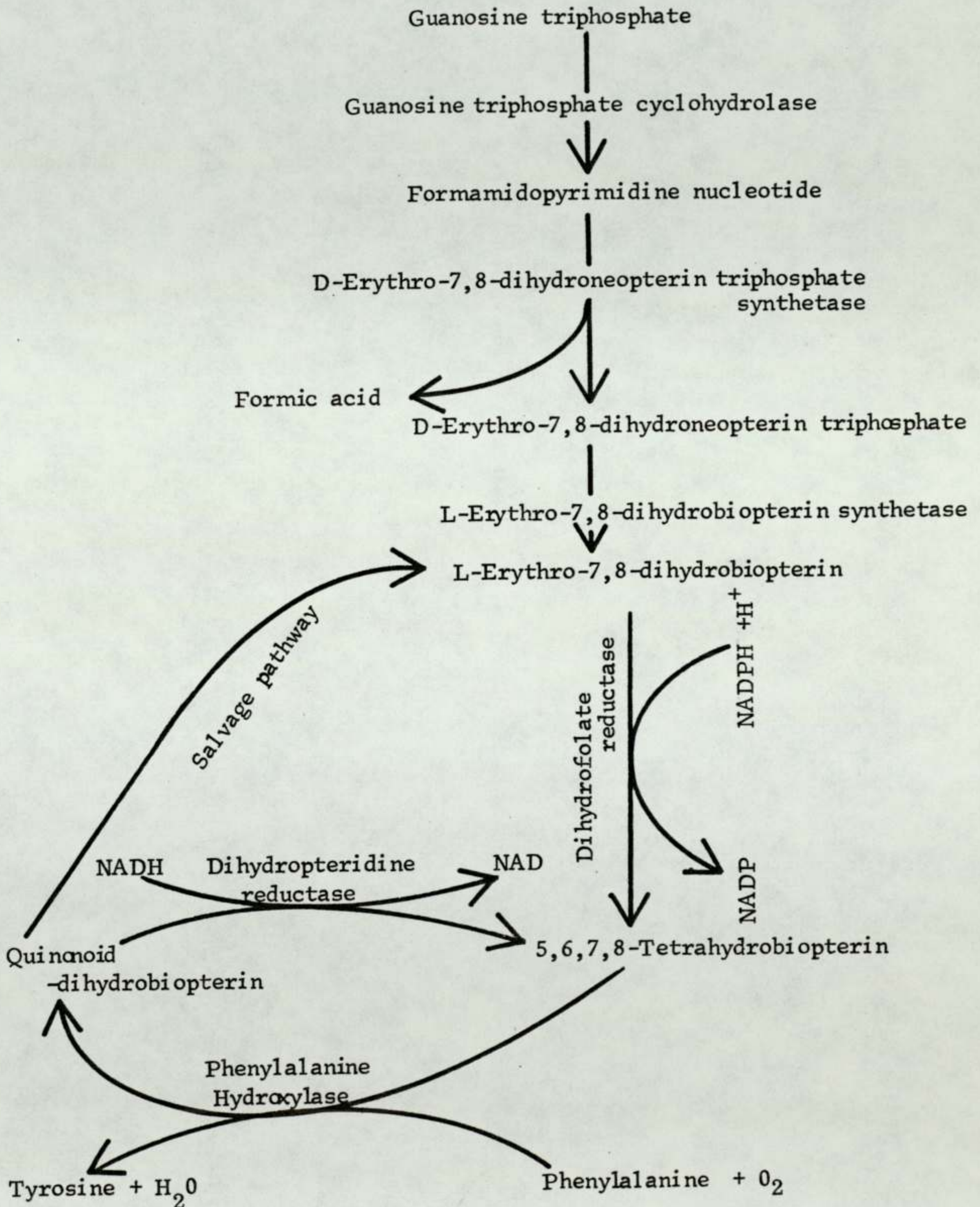
Serum levels of Crithidia factor are remarkably constant and even allowing for a wide variety of diseases the range is narrow (Leeming, 1975; Leeming, Blair, Melikian and O'Gorman, 1976; Baker, Frank, Bacchi and Hutner, 1974). In the biosynthesis experiments (Chapter II) very little change was seen in response to stimuli and the yield from guanosine triphosphate was small, suggesting mechanisms for the maintenance of tetrahydrobiopterin levels within close limits.

In malignant hyperphenylalaninaemia due to defective dihydropteridine reductase (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975) the symptoms are virtually identical to those in cases of deficient dihydrobiopterin synthesis (Leeming, Blair and Rey, 1976). Therefore dihydropteridine reductase must have a major role in the maintenance of active, reduced cofactor, in the brain as well as in other tissues.

It follows that a rate limiting determinant in maintaining tetrahydrobiopterin levels in the cell is most probably dihydropteridine

FIGURE 4-1

SCHEME FOR THE SYNTHESIS AND MAINTENANCE OF 5,6,7,8-TETRAHYDROBIOPTERIN IN THE MAMMAL



reductase.

Any material which affects the production of 5,6,7,8-tetrahydrobiopterin may do so at any of several points in the schema. The advantages of looking at tetrahydrobiopterin synthesis and dihydropteridine reductase separately in-vitro is that some of the sites of action in-vivo may be excluded. There are some sites of action which are unlikely targets for drugs given for therapeutic reasons unless that target was known. For example, dihydrofolate reductase is certainly affected by methotrexate and aminopterin (Futterman and Silverman, 1957) which reduces the available folate pool and prevents DNA synthesis; this action is used in the chemotherapy of malignancies. Unless there are different inhibition constants for drugs against folate and dihydrobiopterin, action on dihydrofolate reductase would soon be evident from the ensuing systemic effects.

The neurological consequences of methotrexate therapy in childhood leukaemia are becoming apparent (Meadows and Evans, 1976; Eiser, 1978) and more important now that the prognosis in leukaemia with more sophisticated therapy has increased life expectancy considerably. Methotrexate and aminopterin act on dihydropteridine reductase as well as on dihydrofolate reductase and give rise to an increase in Crithidia factor synthesis (Chapter II), inhibit dihydropteridine reductase (Chapter III; Craine, Hall and Kaufman, 1972) and reduce the phenylalanine clearance rate in loaded subjects (Goodfriend and Kaufman, 1961).

A summary of the several agents tested on Crithidia factor synthesis and dihydropteridine reductase is given in Table 4-1 with the various compounds grouped according to their medical use of chemical classification as appropriate. The same compounds are grouped differently in Table 4-2, this time according to their effects on the two systems.

Only librium and nobrium mimicked methotrexate and aminopterin in reducing dihydropteridine reductase activity and increasing the synthesis. On the other hand adrenaline and the metallic salts (excluding copper) reduced

TABLE 4-1

THE EFFECTS ON DIHYDROBIOPTERIN SYNTHESIS AND DIHYDROPTERIDINE REDUCTASE ACTIVITY OF A NUMBER OF COMPOUNDS GROUPED ACCORDING TO THEIR CLINICAL USE/CHEMICAL STRUCTURE.

	Biosynthesis			Dihydropteridine reductase reaction		
	Increased	Decreased	No effect	Increased	Decreased	No effect
Librium	*				*	
Nobrium	*				*	
Valium	*					*
Tricyclic antidepressants	*					*
Chlorpromazine			*			*
Trimethoprim			*			*
Sulphamethoxazole	*					*
2,4-diamino-6-hydroxypyrimidine			*			*
Parachlorphenylalanine		*				*
Adrenaline		*			*	
Noradrenaline	*					*
Dopa	*			*		
Dopamine	*			*		
Serotonin	*			*		
Phenylalanine			*			*
Phenylpyruvic acid		*		*		
Orthohydroxyphenylacetic acid	*					*

TABLE 4-1
(Continued)

	Biosynthesis			Dihydropteridine reductase reaction		
	Increased	Decreased	No effect	Increased	Decreased	No effect
Aluminium sulphate			*		*	
Cadmium chloride		*			*	
Cupric sulphate		*		*		
Ferrous sulphate		*			*	
Lead acetate		*			*	
Lithium carbonate		*			*	
Mercuric chloride		*			*	
Zinc sulphate		*			*	
Biopterin	not tested				*	
Folic acid	not tested				*	
10-formylfolic acid	not tested				*	
Methyltetrahydrofolic acid	not tested					*
10-formyltetrahydrofolic acid	not tested					*
Methotrexate	*				*	
Aminopterin	*				*	

TABLE 4-2

THE EFFECTS ON DIHYDROBIOPTERIN SYNTHESIS AND DIHYDROPTERIDINE REDUCTASE ACTIVITY OF A NUMBER OF COMPOUNDS GROUPED ACCORDING TO THEIR ACTION ON DIHYDROBIOPTERIN SYNTHESIS AND DIHYDROPTERIDINE REDUCTASE ACTIVITY

Dihydropteridine reductase reaction	Biosynthesis: Increased	Decreased	No effect	Not known
Increased	Dopa Dopamine Serotonin	Phenylpyruvic acid Cupric Sulphate	-	-
Decreased	Librium Nobrium Methotrexate Aminopterin	Adrenaline Mercuric Chloride Ferrous sulphate Cadmium chloride Lead acetate Lithium carbonate Zinc sulphate	Aluminium Sulphate -	Folic acid 10-formyl-folic acid Biopterin
No effect	Valium All tricyclic antidepressants Sulphamethoxazole Noradrenaline Orthohydroxy-phenylacetic acid	Parachlor-phenylalanine	L-phenylal-anine Chlorprom-azine Trimeth-oprim 2,4-diamino-6-hydroxy-pyrimidine	5-methyl-tetrahydro-folic acid 5-formyl-tetrahydro-folic acid

dihydropteridine reductase activity and reduced the synthesis. Therefore it is not possible to predict the effect of compounds on dihydropteridine reductase from their effect on the synthesis or vice versa. There was one correlation however, all the compounds which increased or decreased the rate of dihydropterin reduction had some effect on the synthesis although aluminium sulphate did not have a significant effect ($p = > 0.05$) at the concentration used. Also, when the synthesis was decreased then with the exceptions of phenylpyruvic acid and copper sulphate, dihydropteridine reductase activity was depressed. There are several possible points of action in this complex system where nórmal levels in man are apparently closely controlled (Leeming, Blair, Melikian and O'Gorman, 1976) and action in one part of the biosynthesis of tetrahydrobiopterin does not preclude simultaneous action at another site.

Increased folate levels in the serum of subjects to whom folic acid or 10-formylfolic acid has been given do not increase serum Crithidia factor levels (Leeming, Blair, Melikian and O'Gorman, 1976), therefore the 'back-up' of folate by dihydrofolate reductase blockade by methotrexate would not appear to be a major cause of increased synthesis. But folates, with the exception of 5-methyltetrahydrofolic acid, increase the serum Crithidia factor level if they are given following methotrexate. This effect may be due to the accumulation of unreduced folate producing a concentration which would act on dihydropteridine reductase if the enzyme was unsaturated by methotrexate. Biopterin, folic acid and 10-formylfolic acid are active against dihydropteridine reductase in-vivo (Chapter III) with folic acid mol for mol as effective as methotrexate. The folates were not tested against the synthesis as they would have been active for Crithidia fasciculata at the concentrations being used (Leeming and Blair, 1974)

Increase in synthesis and dihydropteridine reductase rate in the presence of dopa, dopamine and serotonin poses a number of important questions about the role of these potent, naturally occurring, neuro-active compounds. If they increase dihydropteridine reductase activity thereby depleting the available quinonoid dihydrobiopterin, there would be less 7,8-dihydrobiopterin passing down the salvage pathway to be reduced by

dihydrofolate reductase or excreted. Any fall in serum levels might be difficult to detect. From the results presented here one must assume that dopa, dopamine and serotonin have more than one site of action in the generation and maintenance of 5,6,7,8-tetrahydrobiopterin levels. Adrenaline decreased both synthesis and dihydropteridine reductase whilst noradrenaline had no effect on dihydropteridine reductase but increased the synthesis. Therefore, in the intact animal, dopa, dopamine and serotonin should increase the available 5,6,7,8-tetrahydrobiopterin as also should noradrenaline (provided that there is unsaturated dihydrofolate reductase), whereas adrenaline should decrease tissue tetrahydrobiopterin concentration by acting as feed-back inhibitor in both synthesis of 7,8-dihydrobiopterin and the maintenance of 5,6,7,8-tetrahydrobiopterin. Taking this to its natural conclusion, in the pathway from dopa through to adrenaline, the first action to be lost is that on dihydropteridine reductase following the β-hydroxylation of dopamine to noradrenaline after which N-methylation to adrenaline provides inhibition of both de novo synthesis and dihydropteridine reductase. It could be interesting to look at N-acetyl serotonin using the same kind of logic.

Concentrations of dopamine, noradrenaline and serotonin in human brain are given in Table 4-3, adrenaline accounts for only 4 to 12%

TABLE 4-3

CONCENTRATIONS OF DOPAMINE, NORADRENALINE AND SEROTONIN IN THE BRAIN

(McIlwain and Bachelard, 1971)

	Dopamine	Noradrenaline	Serotonin
Cerebral cortex	0.5×10^{-3}M	0.3×10^{-3}M	0.2×10^{-3}M
White matter	0.3×10^{-3}M	0.05×10^{-3}M	-
Caudate nucleus	24.0×10^{-3}M	0.45×10^{-3}M	1.8×10^{-3}M
Thalamus	1.6×10^{-3}M	0.65×10^{-3}M	1.3×10^{-3}M
Hypothalamus	4.8×10^{-3}M	6.0×10^{-3}M	2.6×10^{-3}M
Medulla oblongata	0.1×10^{-3}M	0.8×10^{-3}M	2.1×10^{-3}M

of cerebral catecholamine. These levels are of the same order as those required to act on synthesis and dihydropteridine reductase in-vitro.

A possible role for phenylpyruvic acid as a competitive inhibitor of dihydropteridine reduction by utilisation of dihydropteridine reductase has been discussed in the previous chapter. Orthohydroxyphenylacetic acid, a metabolite of phenylpyruvic acid, had the effect of increasing the synthesis in-vitro, albeit only slightly and at a non-physiological concentration. Nevertheless, the increased metabolism of phenylalanine to phenylpyruvic acid in phenylketonuria (Fölling, 1934) with urinary excretion of about one gram a day, dependent on diet, and increased phenylpyruvic acid (Penrose and Quastel, 1937) and Crithidia factor (Leeming, Blair, Green and Raine, 1976) following large oral doses of phenylalanine with consequent increased throughput of orthohydroxyphenylacetic acid (Jervis and Drejza, 1966) make this one of the most likely causes of increased Crithidia factor levels in the absence of a direct effect by phenylalanine in-vitro (Chapters II and III) or tyrosine in-vivo (Leeming, Blair, Green and Raine, 1976).

Although plasma phenylpyruvic acid in fasting adult phenylketonurics is around 1/20 of the concentration used here it rises following phenylalanine load to 6.27×10^{-4}M which is greater than the concentration at which an effect was observed in these experiments (4.9×10^{-4}M). Orthohydroxyphenylacetic acid, even after phenylalanine load only reaches 4.40×10^{-5}M (Jervis and Drejza, 1976). However these concentrations are not those measured at sites of synthesis and reduction which would be much more informative.

The lack of effect by 2,4-diamino-6-hydroxypyrimidine on the two systems in spite of its reported action of reducing urinary biopterin in rats by 80% (Pabst and Rembold, 1966) suggests that it has no direct effect on the synthesis of 5,6,7,8-tetrahydrobiopterin although it still may play a peripheral or indirect role by acting elsewhere, for example on excretion.

The neuroleptic drugs, with the exception of chlorpromazine which had no effect on dihydropteridine reductase or synthesis, all increased the synthesis. The benzodiazepines decreased dihydropteridine reductase activity depending on their solubility and were the only drugs which had this

particular action apart from methotrexate and aminopterin. The tricyclic antidepressants increased biosynthesis, but unlike the benzodiazepines did not have any effect on dihydropteridine reductase. One must assume different effects on tetrahydrobiopterin synthesis by the two classes of drugs although they are both used in depressive illness. If this increased synthesis noted in-vitro potentiates hydroxylation of tyrosine in-vivo then increased dopa and its metabolites would further stimulate biosynthesis until adrenaline inhibited the reaction.

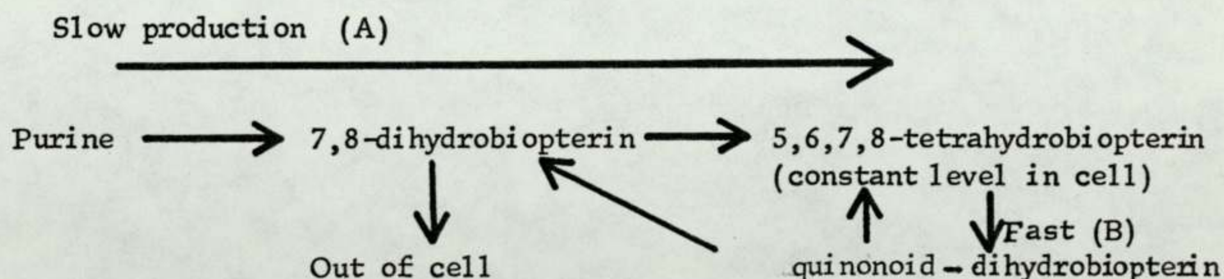
The action of cotrimoxazole in decreasing phenylalanine clearance following oral loads (Andrews,Purkiss, Chalmers and Watts, 1976) suggests that one of its ingredients (sulphamethoxazole or trimethoprim) is either a dihydropteridine reductase inhibitor or inhibits synthesis of dihydrobiopterin and serum Crithidia factor is raised following therapeutic use of cotrimoxazole (Leeming, Blair, Melikian and O'Gorman, 1976). Trimethoprim had no effect on either system although it has been reported that reduction of 7,8-dihydrobiopterin to 5,6,7,8-tetrahydrobiopterin by rat liver tetrahydrofolate dehydrogenase was competitively inhibited by trimethoprim (Stone, 1975). Moreover the reduction of dihydrobiopterin was more susceptible to inhibition than the reduction of dihydrofolate. The consistant effect of increased synthesis by sulphamethoxazole might therefore only add to accumulated 7,8 -dihydrobiopterin caused by selective and competitive inhibition of dihydrofolate reductase by trimethoprim.

The metallic salts decreased the synthesis and, with the exception of cupric sulphate, inhibited dihydropteridine reductase. If inhibition of synthesis was solely due to copper, which is possible because of its effect at low concentration and the "Analar" limits of copper content of other metallic salts, then the action of the other metals at the relatively high concentrations effective would be directed at dihydropteridine reductase alone. The effect on dihydropteridine reductase could not be attributed to copper contamination. The evidence presented here suggests that cupric sulphate was principally active against NAD as the NADH disappearance in the dihydropteridine reductase assay was substantially increased independently of dihydropteridine reductase, whilst in the synthesis Crithidia

factor produced in the presence of cupric sulphate and NAD was almost identical to that produced without cupric sulphate and NAD. Copper is medically important in Wilson's disease (Wilson,1912) although there may be other situations in which copper may have clinical significance. Copper containing intrauterine contraceptive devices, (Chantler, Critoph and Elstein, 1977), the addition of copper to animal feeds and to fungicides (Scheinberg and Steinlieb, 1976) are possible sources of increased copper intake.

The principal component of Crithidia factor in serum and urine is, 7,8-dihydrobiopterin and in view of the higher tissue levels (Leeming, Blair, Melikian and O'Gorman, 1976) and the very active urinary excretion (Leeming and Blair, 1974) it is suggested that the Crithidia factor (7,8-dihydrobiopterin) is not retained by tissues as 5,6,7,8-tetrahydrobiopterin is. Therefore low serum levels may be caused by low production and low tissue concentration or by more efficient conversion of quinonoid dihydrobiopterin back to tetrahydrobiopterin. Conversely, high serum levels may reflect either a high production rate or a poor return to tetrahydrobiopterin via dihydropteridine reductase thus compounding the difficulties inherent in the interpretation of in-vitro experimental data.

A scheme is proposed as follows:-



There is evidence in human subjects that 7,8-dihydrobiopterin in serum is low if there is slow production (A) of dihydrobiopterin (Leeming, Blair and Rey, 1976) and raised when dihydropteridine reductase is inactive (Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977) or inhibited by methotrexate (Leeming, Blair, Melikian and O'Gorman, 1976). Tetrahydrobiopterin concentration in the cell will fall if synthesis (A) is slow and serum levels will be low, and will be low while serum levels will be high

if (A) is at a normal level when (B) (dihydropteridine reductase) is slow. These predictions are confirmed by similarity of symptoms and pattern of serum levels in patients with these abnormalities (Rey, Harpey et al, 1977; Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975). With this model before us we may re-divide the compounds in a different way from that shown in Tables 4-1 and 4-2 (Table 4-4)

TABLE 4-4

PROBABLE EFFECTS ON IN-VIVO CELL TETRAHYDROBIOPTERIN OF THE COMPOUNDS TESTED AGAINST IN-VITRO SYNTHESIS OF CRITHIDIA FACTOR AND DIHYDROPTERIDINE REDUCTASE.

Increased	Decreased
Dopa	Mercuric chloride
Dopamine	Ferrous sulphate
Serotonin	Cadmium sulphate
Tricyclic antidepressants	Copper sulphate
Sulphamethoxazole	Lead acetate
Noradrenaline	Lithium carbonate
Orthohydroxyphenylacetic acid	Aluminium sulphate
	Zinc sulphate
	Adrenaline
	Librium
	Nobrium
	Folic acid
	10-Formylfolic acid
	Biopterin
	Methotrexate
	p-Chlorphenylalanine
	Phenylpyruvic acid

These three chapters precede work carried out in the rat and in human health and disease where ethical considerations prevent investigations which are too invasive. Therefore the results and conclusions, particularly

in drug administration, will have to be related to the foregoing in-vitro experiments; the roles of the compounds tested may be different in the intact animal and in chronic exposure. There are obvious dangers in a too literal translation of these results in the interpretation of effects of administration of the same compounds on intact mammalian systems, selective exclusion, metabolism and oxidative processes would all have to be taken into account. However, some of the materials could well reach sites of tetrahydrobiopterin synthesis and if they do so in the concentrations described, some prediction may be made as to their action.

Levels of heavy metals in brain tissue of Japanese who had died from causes not associated with metal poisoning, were as follows when converted into molarity: Cadmium 1.1×10^{-6}M, copper 9.8×10^{-5}M, lead 1.7×10^{-6}M, mercury 5.0×10^{-7}M, zinc 1.8×10^{-4}M (Sumino, Hayakawa, Shibata and Kitamura, 1975). Even in these apparently normal tissues, levels of copper and zinc were reached which had an effect on synthesis of tetrahydrobiopterin in-vitro. The maximum brain aluminium level in dialysis dementia at approximately 0.6×10^{-4}M (Alfrey, Le Gendre and Kaehny, 1976) would not have any effect on in-vitro synthesis but it is approaching the level active in inhibiting dihydropteridine reductase (1.82×10^{-4}M). Any material which inhibits dihydropteridine reduction must produce a similar condition to malignant hyperphenylalaninaemia (Chapter VIII) but this would depend on it being generally dispersed. The fact that dialysis using aluminium-free water and interruption of alumium containing gel allowed a patient with dialysis dementia to recover (Poisson, Mashaly and Lebkiri, 1978) suggests an inhibitory process rather than toxic permanent damage.

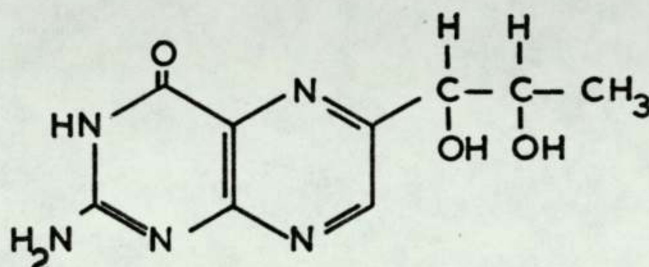
CHAPTER V

INTESTINAL ABSORPTION OF BIOPTERIN AND 5,6,7,8-TETRAHYDROBIOPTERIN

INTRODUCTION

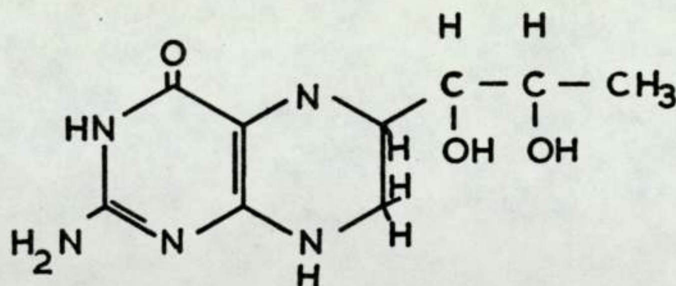
Intestinal absorption in man of biopterin (1) and 5,6,7,8-tetra-

(1)



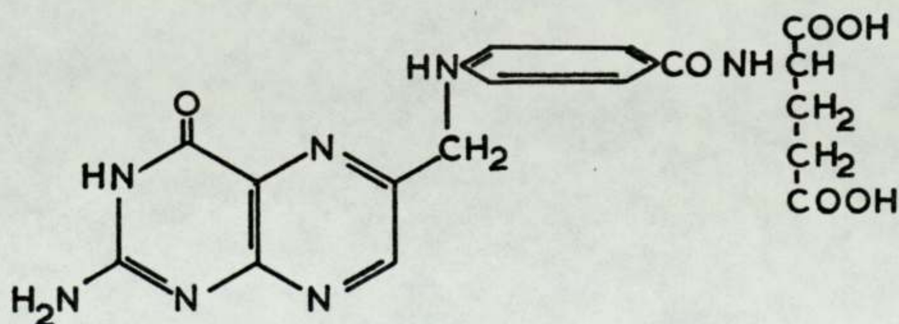
hydrobiopterin (2) has been studied (Leeming, 1975; Rembold and Metzger,

(2)



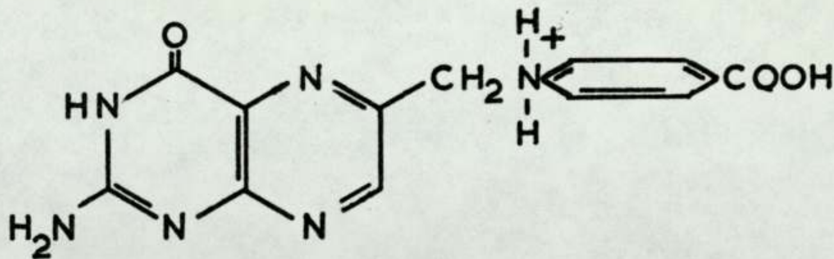
1967) but not to the exhaustive extent which has been applied to folic acid

(3)

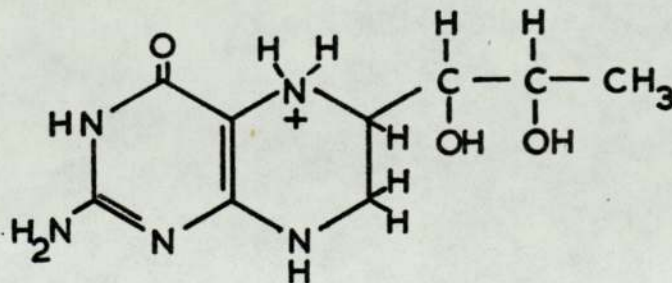


(Butterworth, Nadel, Perez Santiago, Santini and Gardner, 1957; Chanarin, Anderson and Mollin, 1958; Dormandy, Waters and Mollin, 1963; Melikian, Paton, Leeming and Portman-Graham, 1971; Leeming, Portman-Graham and Blair, 1972; Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974) and folic acid derivatives (Baker, Frank, Feingold, Ziffer, Gellene, Leevey and Sobotka, 1965; Perry and Chanarin, 1970; Nixon and Bertino, 1972; Brown, Scott, Foster and Weir, 1973; Weir, Brown, Freedman and Scott, 1973; Ratanasthien et al, 1974; Ratanasthien, Blair, Leeming, Cooke and Melikian, 1977). Weir, Brown, Freedman and Scott (1973) proposed a specific permease for transporting the pteridine moiety, dependent on the stereochemistry at C6 of the pterin ring, which would suppose uniformity of absorption for tetrahydrofolates. Hogben, Tocco, Brodie and Schanker (1959), in their work on the absorption of drugs, pointed out that to account for observed intestinal absorption of some compounds a pH of 5.0 would be required. Blair and Matty (1974) postulated an acid micro-climate of about pH 3.5 at the absorptive surface of the small intestine; at pH3 to pH4, folic acid occurs largely as the neutral species and would be absorbed, whereas pteroic acid (4) and 5,6,7,8-tetrahydrobiopterin (5)

(4)



(5)



would be charged species, poor absorption of the latter two pteridines has been described (Blair, Ratanasthien and Leeming, 1974; Leeming,1975; Ratanasthien, 1975).

Table 5-1 shows the proportion of charged to neutral species over a wide range of pH values and it is clear that at the normal pH of the small intestine lumen, pH 6.5 (Benn, Swan, Cooke, Blair, Matty and Smith, 1971), tetrahydrobiopterin would exist largely as the neutral species and should be transported easily, whereas if it had to pass through a microclimate of pH 3.5 only 5% would be in the neutral form and little would be absorbed.

TABLE 5-1

PERCENTAGE OF TETRAHYDROBIOPTERIN PRESENT AS THE CHARGED AND NEUTRAL SPECIES FROM pH 1.8 TO pH 7.8 CALCULATED ON A pKa OF 4.8

pH	% Charged Species	% Neutral Species
1.8	99.9	0.1
2.4	99.6	0.4
3.0	98.4	1.6
3.6	94.1	5.9
4.2	79.9	20.1
4.8	50.0	50.0
5.4	20.1	79.9
6.0	5.9	94.1
6.6	1.6	98.4
7.2	0.4	99.6
7.8	0.1	99.9

In coeliac disease the microclimate is less acidic (Lucas, Cooper, Lei, Johnson, Holmes, Blair and Cooke, 1978) and the gut lumen becomes alkaline (Benn, Swan, Cooke, Blair, Matty and Smith, 1971) probably due to a decrease in adenosine triphosphatase (Riekan, Stewart, Booth and Pearse, 1966) which is the major enzyme in hydrogen ion production in the small bowel (Blair, Lucas and Matty, 1972 and 1974);

inhibition of adenosine triphosphatase by sodium azide also inhibits folate transport (Kesavan and Noronha, 1978). Folate absorption in active coeliac disease is decreased (Been, Swan, Cooke et al, 1971) and there are significant inverse correlations between the height of villi and jejunal surface pH (Lucas, Cooper, Lei et al, 1978) and between jejunal surface pH and folate levels (Kitis, Lucas, Cooper, Dunne, Cooke, Allan and Blair, 1978). When the coeliac patient is on a gluten free diet, the adenosine triphosphatase returns to normal (Riekan et al, 1971) and folate absorption returns to normal (Benn and Cooke, 1971).

The ileum is usually unaffected in coeliac disease (Stewart, Pollock, Hoffbrand, Mollin and Booth, 1967) and folate transport rate is the same in the ileum of normal and coeliac subjects (Lucas, Kitis and Blair, 1979). There is evidence of an adaptive increase in absorption from the distal bowel in coeliac disease (Halsted, Reisenauer, Shane and Tamura, 1978). Serum Crithidia factor increases slowly following orally administered tetrahydrobiopterin in normal subjects (Leeming, 1975) as might be expected if microclimate pH becomes more alkaline as one moves down the gut (Lucas and Blair, 1978) and it would therefore be of value to know if this was altered in coeliac disease.

Sodium hydrogen carbonate in large doses neutralises the acid microclimate and depresses folate absorption (Benn et al, 1971). Acute ethyl alcohol intake reduces the acid microclimate (Lucas, Swanston, Lei, Mangkornthong and Blair, 1978). Intestinal alkalinity and ethyl alcohol, if they decrease folate absorption by neutralising the acid microclimate might be expected to increase intestinal absorption of 5,6,7,8-tetrahydrobiopterin because at higher pH more of the compound would exist as the neutral species. (Table 5-1).

Previous absorption studies with biopterin and tetrahydrobiopterin (Leeming, 1975) were carried out with oral doses protected from oxidation by ascorbic acid. Normal alimentary transport of pterin to the site of absorption, following an oral load, would effect dilution of pterin and anti-

oxidant which could result in the oxidation of labile tetrahydrobiopterin. Uniformity between patients is also reduced to a degree by variation in time taken to reach the small bowel as well as by dilution effects. Similarly, larger doses of alkaline reagent and alcohol would be needed to demonstrate an acute effect on the microclimate and it is undesirable to give fasting patients larger measures of alcohol than absolutely essential, particularly if they are being investigated as out-patients. Therefore there would be advantage in delivering measured doses of biopterin and 5,6,7,8-tetrahydrobiopterin in a precise way to the proximal jejunum with and without anti-oxidant, ethyl alcohol and sodium hydrogen carbonate. This became possible with access to a gastroscopy clinic and the co-operation of a consultant physician.

MATERIALS AND METHODS

Fasting patients who were undergoing gastroscopy for diagnostic purposes were informed of the nature of the proposed investigation by the consultant physician in charge of their case. Where the patient was judged to understand and had agreed to participate, selection was made if there was no evidence of disease in the stomach or small bowel. Those who had a previous history of malabsorption were also excluded. One patient was subsequently found to have scleroderma with intestinal involvement and here the results are included because of their possible relevance.

Following gastroscopic examination the Ryle's tube was re-positioned in the proximal jejunum and all materials were delivered down the tube by syringe. 10mg. doses of 5,6,7,8-tetrahydrobiopterin and 5mg. doses of biopterin were prepared immediately before administration by dissolving them in 10 cm^3 distilled water, which in some cases contained 200 mg. of ascorbic acid as anti-oxidant. One group received 10 cm^3 of 8.4% sodium hydrogen carbonate immediately prior to tetrahydrobiopterin. Two groups had 18 cm^3 of 30% ethyl alcohol followed by either tetrahydrobiopterin or biopterin. Blood samples were taken immediately before dosing and then at ½, 1,2,3 and 4 hours afterwards; the serum was separated and frozen at -20°C until assayed for biopterin derivatives with Crithidia fasciculata. A group of untreated coeliac patients with flat jejunal biopsies agreed to take oral 5,6,7,8-tetrahydrobiopterin with ascorbic acid and to give blood samples. Volunteers were normally in groups of five, but in some instances this was not possible because supplies of 5,6,7,8-tetrahydrobiopterin became exhausted.

Included for comparison are oral absorption experiments from a previous series of normal volunteers (Leeming, 1975) with the data expressed in the same way.

Chromatography was carried out on some of the serum samples by streaking them across cellulose TLC plates, running to 15 cm in three solvent systems; 3% ammonium chloride, 5% acetic acid and 0.5% sodium

carbonate. The plates were scraped in horizontal bands, eluted in 0.2M phosphate buffer pH 5.0 and then centrifuged, eluates were assayed for Crithidia factor.

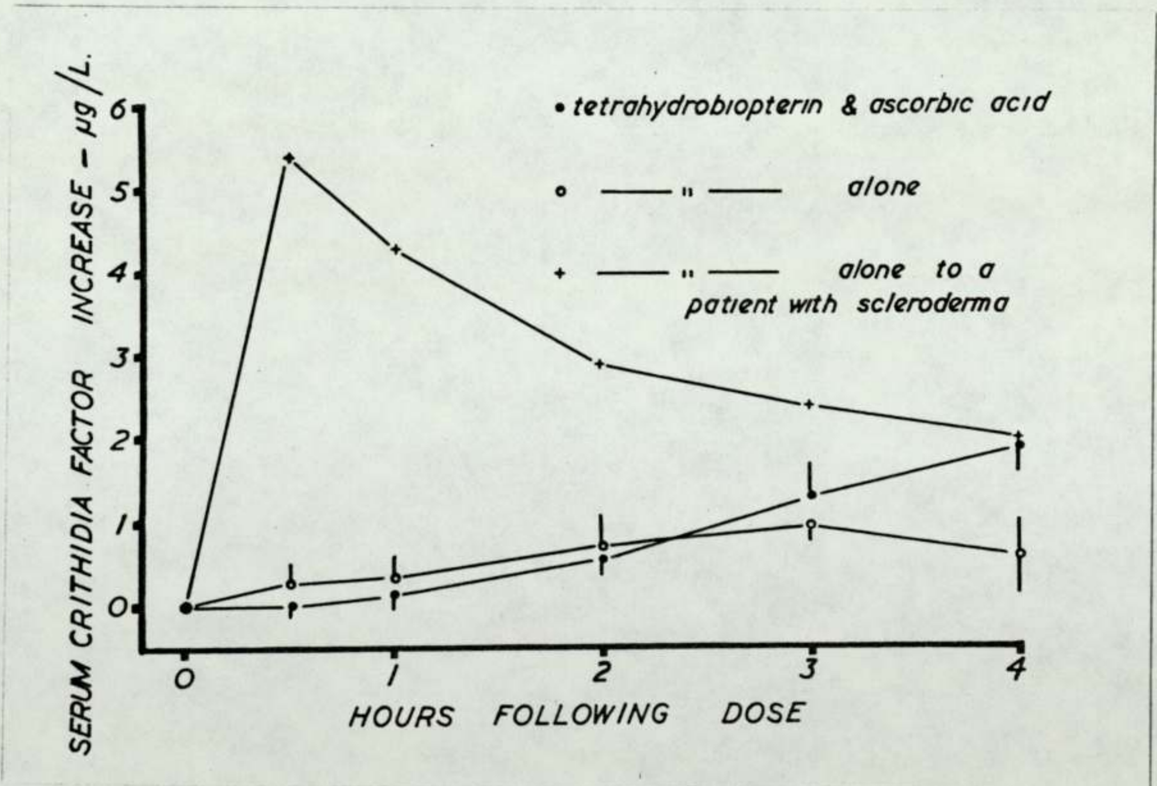
RESULTS

All the mean values of serum Crithidia factor are set out in Tables 5-2 and 5-3 with standard errors of means. To obtain greater uniformity increments above the initial serum levels were calculated and used in Figures 5-1 to 5-5.

The poor serum Crithidia factor response to tetrahydrobiopterin was not substantially altered by concurrent ascorbic acid (Figure 5-1) but

FIGURE 5-1

10MG 5,6,7,8-TETRAHYDROBIOPTERIN ADMINISTERED WITH 200MG ASCORBIC ACID TO 5 PATIENTS WITHOUT INTESTINAL DISEASE AND TO ONE PATIENT WITH SCLERODERMA INVOLVING THE SMALL BOWEL



the slight downward trend at 4 hours without ascorbic acid was significantly different from the group who took ascorbic acid (Student's 't' test - p = < 0.05). The patient who subsequently proved to have scleroderma (Figure 5-1) produced a serum response strikingly similar to two of the patients who were given ethyl alcohol immediately before the tetrahydrobiopterin (Figure

TABLE 5-2

SERUM CRITHIDIA FACTOR FOLLOWING 5,6,7,8-TETRAHYDROBIOPTERIN ADMINISTERED ORALLY AND BY RYLE'S TUBE

		HOURS					
		0	½	1	2	3	4
Administered by Ryle's tube	5,6,7,8-tetrahydrobiopterin (5)	1.14 ± 0.129	1.36 ± 0.250	1.48 ± 0.263	1.84 ± 0.286	2.08 ± 0.165	1.72 ± 0.220
		(increment)	(0.220 ± 0.195)	(0.34 ± 0.204)	(0.7 ± 0.205)	0.94 ± 0.16	0.58 ± 0.177
	5,6,7,8-tetrahydrobiopterin (1) patient with scleroderma	1.1	6.5	5.4	4.0	3.5	3.1
		(increment)	(5.4)	(4.3)	(2.9)	(2.4)	(2.0)
	5,6,7,8-tetrahydrobiopterin (5) with ascorbic acid	1.72 ± 0.292	1.70 ± 0.270	1.86 ± 0.246	2.26 ± 0.294	3.02 ± 0.431	3.62 ± 0.575
		(increment)	(0.02 ± 0.086)	(0.14 ± 0.154)	(0.54 ± 0.181)	(1.3 ± 0.335)	(1.9 ± 0.503)
	5,6,7,8-tetrahydrobiopterin (3) with ascorbic acid following sodium hydrogen carbonate	1.233 ± 0.177	1.30 ± 0.174	1.37 ± 0.219	1.60 ± 0.264	1.90 ± 0.208	2.2 ± 0.404
		(increment)	(0.07 ± 0.088)	(0.137 ± 0.167)	(0.367 ± 0.296)	(0.67 ± 0.185)	(0.97 ± 0.273)
	5,6,7,8-tetrahydrobiopterin (3) with ascorbic acid following ethyl alcohol	1.0 ± 0.153	6.63 ± 2.881	5.8 ± 2.22	4.93 ± 1.84	4.57 ± 1.68	3.90 ± 1.18
		(increment)	(5.63 ± 2.80)	(4.80 ± 2.14)	(3.93 ± 1.79)	(3.57 ± 1.65)	(2.90 ± 1.17)
Oral	5,6,7,8-tetrahydrobiopterin (5) with ascorbic acid	1.68 ± 0.273	1.90 ± 0.300	1.92 ± 0.292	2.28 ± 0.407	2.14 ± 0.347	2.40 ± 0.237
		(increment)	(0.22 ± 0.143)	(0.24 ± 0.112)	(0.60 ± 0.270)	(0.46 ± 0.266)	(0.87 ± 0.137)
	5,6,7,8-tetrahydrobiopterin (5) with ascorbic acid to coeliac disease.	1.02 ± 0.086	1.2 ± 0.084	1.46 ± 0.166	1.4 ± 0.110	1.58 ± 0.193	1.50 ± 0.063
		(increment)	(0.18 ± 0.080)	(0.44 ± 0.087)	(0.38 ± 0.111)	(0.48 ± 0.191)	(0.55 ± 0.032)

TABLE 5-3

SERUM CRITHIDIA FACTOR FOLLOWING BIOPTERIN

ADMINISTERED ORALLY AND BY RYLE'S TUBE

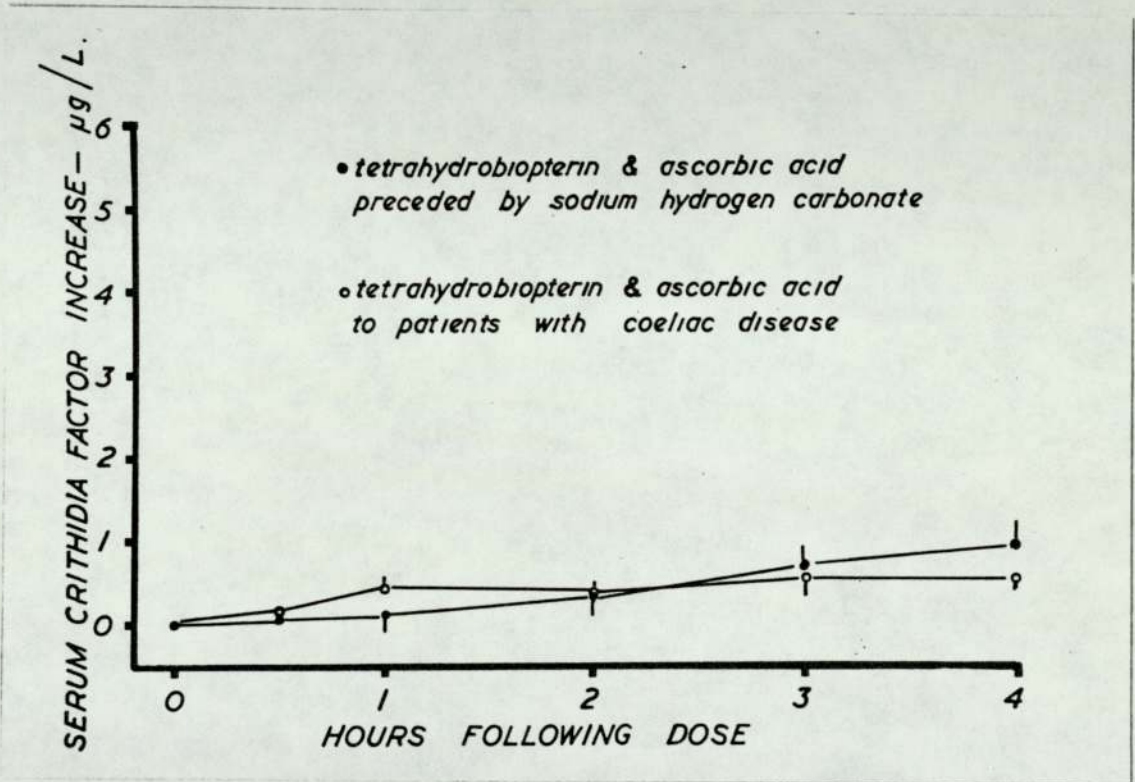
		HOURS					
		0	½	1	2	3	4
Biopterin (by Ryle's tube)	(5)	1.02 ± 0.086	2.66 ± 0.577	3.92 ± 0.787	4.84 ±1.136	4.50 ± 1.020	4.28 ± 0.693
		(increment)	(1.64 ± 0.546)	(2.90 ±0.771)	(3.82 ±1.100)	(3.48 ± 1.011)	(3.26 ± 0.676)
Biopterin (by Ryle's tube) following ethyl alcohol	(5)	1.72 ± 0.086	2.68 ± 0.183	3,38 ± 0.332	3.6 ± 0.318	4.02 ± 0.371	4.24 ± 0.683
		(increment)	(0.96 ± 0.186)	(1.66 ± 0.364)	(1.88 ± 0.310)	(2.30 ± 0.321)	(2.52 ± 0.628)
Biopterin (oral)	(5)	1.40 ± 0.228	1.66 ± 0.254	2.40 ± 0.131	3.50 ± 0.205	3.52 ± 0.116	2.43 ± 0.169
		(increment)	(0.26 ± 0.144)	(1.02 ± 0.272)	(2.1 ± 0.407)	(2.12 ± 0.282)	(1.03 ± 0.099)

5-3) with a peak maximum in the first sample, half an hour after dosing.

Sodium hydrogen carbonate did not affect the serum Crithidia factor response to tetrahydrobiopterin (Table 5-2 and Figure 5-2) and the

FIGURE 5-2

10MG 5,6,7,8-TETRAHYDROBIOPTERIN ADMINISTERED WITH 200MG ASCORBIC ACID TO 3 PATIENTS WITHOUT INTESTINAL DISEASE FOLLOWING 10 CM^3 8.4% SODIUM HYDROGEN CARBONATE AND TO 5 PATIENTS WITH COELIAC DISEASE.



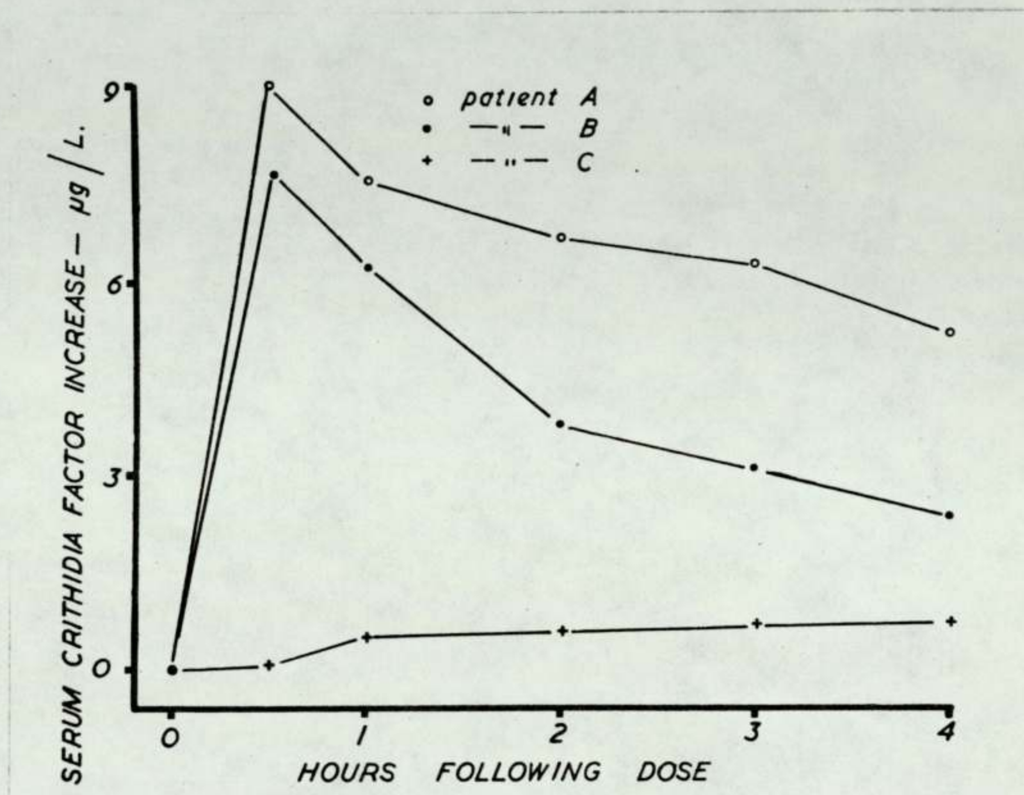
graphs of serum increments in presumed absorptively normal patients without pre-treatment with sodium hydrogen carbonate (Figure 5-1) closely resemble those of both pre-treated patients and those with coeliac disease (Figure 5-2) although there was a slight increase at one hour in coeliac patients which was not significant ($p = > 0.05$) and the increments at three and four hours was almost identical whilst in normal subjects, both with and without sodium hydrogen carbonate, the serum level was still rising. None of these differences reached a statistically significant level.

Ethyl alcohol followed by tetrahydrobiopterin stimulated a serum

Crithidia factor response in two patients (Figure 5-3, Patients A & B) higher than those achieved by other similar doses of either tetrahydro-

FIGURE 5-3

10MG 5,6,7,8-TETRAHYDROBIOPTERIN ADMINISTERED WITH 200MG ASCORBIC ACID TO 3 PATIENTS FOLLOWING 18CM^3 30% ETHYL ALCOHOL

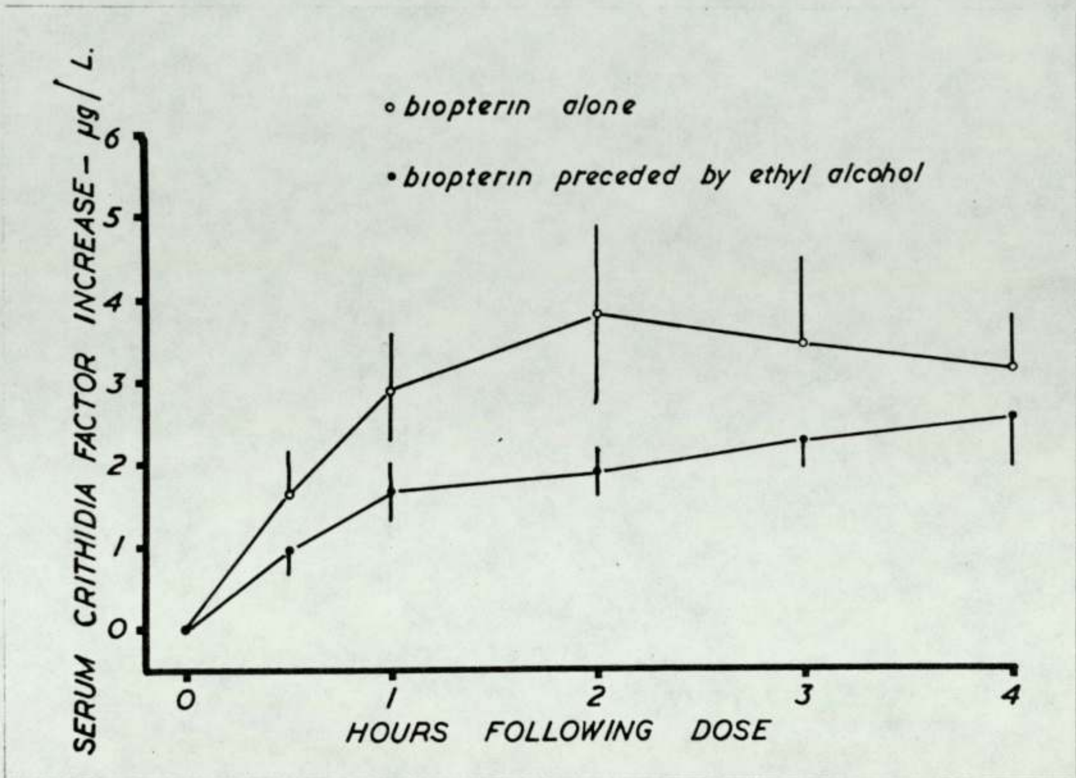


biopterin or biopterin however administered. Moreover the peak level occurred at 30 minutes, that is the first sample following dosage, whereas the peak serum value following biopterin came at 2 hours (Figures 5-4 & 5-5, Table 5-3). Patient C (Figure 5-3) did not have any clinical condition to which could be ascribed the cause of the difference from patients A and B. The mean of all three values at half an hour was significantly different from normal by Student's 't' test ($p = < 0.05$).

Biopterin given by Ryle's tube (Figure 5-4) produced a rise in serum Crithidia factor similar to that following oral biopterin (Figure (5-5). Ethyl alcohol depressed the serum level but this did not reach the 5% level of significance at any time, the maximum difference from

FIGURE 5-4

5MG BIOPTERIN ALONE AND 5MG BIOPTERIN FOLLOWING 18CM^3 30% ETHYL ALCOHOL ADMINISTERED TO 2 GROUPS OF 5 PATIENTS WITHOUT INTESTINAL DISEASE.



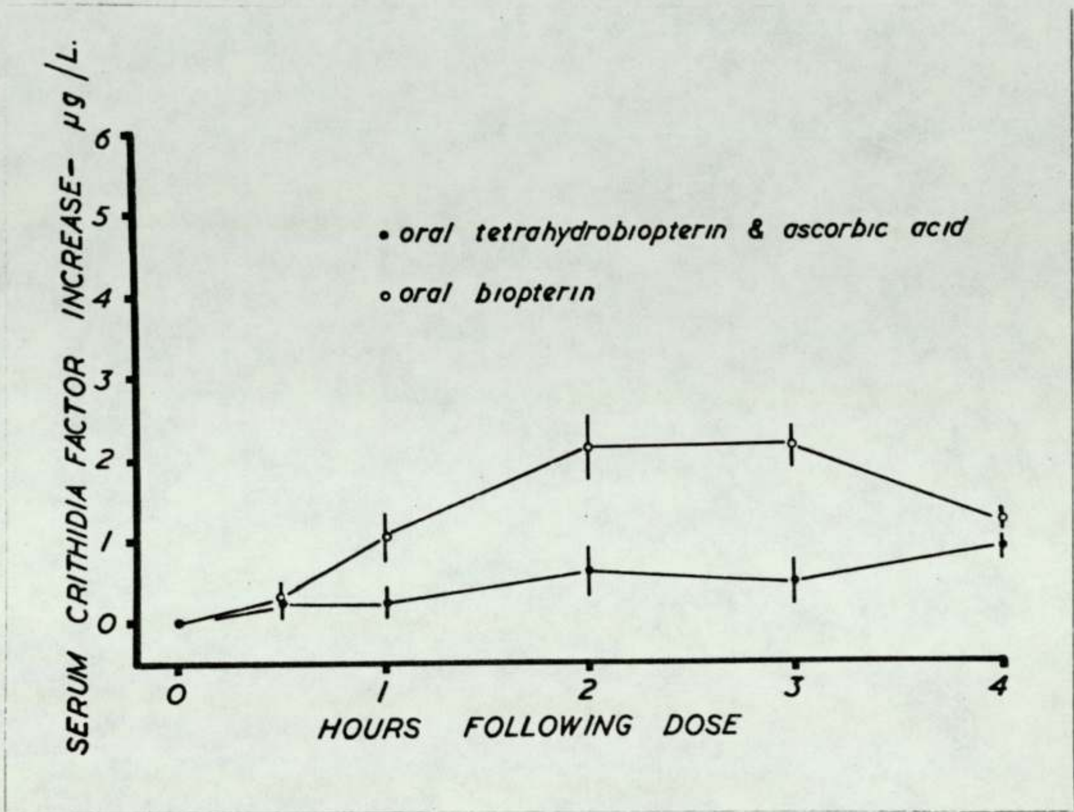
biopterin administered alone was at two hours when by Student's 't' test $p = 0.1 - 0.2$.

Oral biopterin and tetrahydrobiopterin (Figure 5-5) behaved in a similar way to when they were administered by jejunal intubation.

Chromatography of normal serum or serum before administration of biopterin or tetrahydrobiopterin showed a Crithidia active compound which co-chromatographed with 7,8-dihydrobiopterin. Chromatography in two patients following biopterin revealed a material which co-chromatographed with biopterin. In two patients where an increase in serum Crithidia factor was noted following tetrahydrobiopterin with ascorbic acid there was an increase in the material which co-chromatographed with 7,8-dihydrobiopterin.

FIGURE 5-5

5MG ORAL BIOPTERIN AND 10MG ORAL 5,6,7,8-TETRAHYDROBIOPTERIN ADMINISTERED TO 2 GROUPS OF NORMAL VOLUNTEERS.



DISCUSSION

Neither biopterin nor 5,6,7,8-tetrahydrobiopterin gave rise to increases in serum Crithidia factor levels which approached serum folate levels following oral folates (Ratanasthien et al, 1974). Rembold and Metzger (1967) demonstrated that 90% of orally administered $8a^{14}C$ biopterin was absorbed by rats and distributed throughout the body, with higher concentrations in the liver and adrenals where it could still be measured after three days. Intraperitoneally injected biopterin was rapidly and almost totally excreted in the urine. These same workers showed that orally administered 5,6,7,8-tetrahydrobiopterin was excluded by the bowel although parental doses were distributed throughout the body. This latter observation on parental tetrahydrobiopterin has been confirmed radiochemically (Rembold, 1970) and enzymatically (Stone, 1974). Poor absorption of tetrahydrobiopterin into serum has already been described (Leeming, 1975) and, with pteroic acid, presented as a model (Blair, Ratanasthien and Leeming, 1974) for the intestinal, acid microclimate hypothesis (Hepner et al, 1959; Blair and Matty, 1974).

The small rise in serum Crithidia factor following oral biopterin could be explained if unreduced biopterin was absorbed into the epithelial tissue of the bowel where it was reduced and from which it was released slowly into the circulation. Changes in absorption of biopterin would not be very obvious in the presence of this mechanism where absorption is not reflected by an immediate substantial rise in serum concentration. The shape of the Crithidia factor curve following administration of biopterin after ethyl alcohol resembled the serum curves following tetrahydrobiopterin with a prolonged, gentle rise not significantly different from its origin. This could be explained by dilution effect by 18 cm^3 of diluted alcohol. It is possible that ethyl alcohol inhibits intestinal reduction of biopterin during transport or that it effects its reduction in the bowel lumen. If the acid microclimate is decreased by acute alcohol administration (Lucas, Swanston, Lei, Mangkornthong and Blair, 1978) then one might expect a reversal of the process which excludes

tetrahydrobiopterin, but this would not necessarily have any effect on biopterin absorption.

There was a marked similarity in serum Crithidia factor levels following tetrahydrobiopterin with and without anti-oxidant, both in patients without gastro-intestinal disease and those with coeliac disease; a slow rise continued at a time when the unabsorbed dose would have passed through the small bowel. The only observable differences were firstly that without ascorbic acid the level fell at four hours when it was still rising in those subjects who had had concurrent ascorbic acid, secondly there was a slight rise at one hour in coeliac patients with the third and fourth hour specimens having almost identical levels of Crithidia factor. The difference noted between subjects with and without ascorbic acid may simply be the result of oxidation as the dose passed down the bowel. The slight (but not significant $p = > 0.05$) rise at one hour in coeliac patients is in keeping with evidence for adaptive folate absorbance in the ileum (Halsted, Reisenauer, Shane and Tamura, 1978), in normal subjects folate absorption decreases down the bowel which is not so in coeliac disease (Hepner, Booth, Cowan, Hoffbrand and Mollin, 1968). Arguing from this evidence and the premise that folate absorption and tetrahydrobiopterin absorption are inversely related one might expect rather better absorption of tetrahydrobiopterin in the jejunum of coeliac patients with poor absorption in the ileum.

The one patient with scleroderma who showed increased tetrahydrobiopterin absorption comparable to two patients given prior ethyl alcohol lends support to the association of increased tetrahydrobiopterin transport with decreased folate absorption, although folate status was not established in this case. Serum folate is frequently low in scleroderma with intestinal involvement (Peachey, Creamer and Pierce, 1969).

The jejunal pH in coeliacs is greater than pH 7.0 as opposed to the normal pH 6.5 and folate absorption is decreased (Benn et al, 1971; Dormandy et al, 1963) and one would therefore expect similar absorptive

spectra in coeliac disease and when the small bowel was rendered alkaline with sodium hydrogen carbonate, provided that absorption was solely dependent on the physico-chemical properties at the lumen surface. The surface area of the small bowel is reduced in coeliac disease which could account for some decrease in absorption but this would not apply to a transitory alkaline state produced by sodium hydrogen carbonate. Although it was thought at the outset that the amount of alkali needed might be less when applied with precision down a Ryle's tube it is highly probable that a much larger quantity would be required to neutralise the acid microclimate. Oral absorption experiments with folates have required 3 to 5g. before any effect was noted (Ratanasthien, Blair, Leeming, Cooke and Melikian, 1977).

Chromatography of serum following tetrahydrobiopterin showed 7,8-dihydrobiopterin increased which gives some support for absorption of unaltered 5,6,7,8-tetrahydrobiopterin. However, following biopterin the major increase was in the moiety which co-chromatographed with biopterin which suggested absorption of unaltered biopterin.

The most outstanding feature of the large increase in serum Crithidia factor when tetrahydrobiopterin followed ethyl alcohol, was its timing. The highest value came in the first sample following the dose, suggesting rapid unaltered absorption. There is a reduction in the acid microclimate and folic acid transfer in rat jejunum following ethanol (Lucas,Swanston, Lei et al, 1978) which is in keeping with the above findings.

In normality one would not expect ingested biopterin derivatives to play a significant role as liver levels are independent of dietary biopterin (Pabst and Rembold, 1966) although folate deficiency might decrease liver biopterin levels slightly (Goodfriend and Kaufman, 1961). The one disease which might be affected by what would otherwise be trivial amounts of tetrahydrobiopterin is the atypical phenylketonuria with defective tetrahydrobiopterin synthesis in whom very low levels of

Crithidia activity have been measured in serum, urine and liver (Leeming, Blair and Rey, 1976) (Chapter VIII). Unless the biosynthetic process was incompletely blocked in these patients, the small amount of biopterin derivative detectable in these patients can only have come from a minor synthetic pathway or from the diet. Although these afflicted children have little sentient existance without therapy, it is hardly likely that they could survive totally devoid of neurotransmitter substances derived from tyrosine and tryptophan. Recent work on a child with defective dihydrobiopterin synthesis showed that tetrahydrobiopterin dissolved in deaerated water, supplemented with ascorbic acid and administered through a gastric tube resulted in a striking decrease in serum phenylalanine (Schlaub, Daumling, Curtius, Niederwieser, Bartholome, Viscontini, Schirks and Bieri, 1978). If phenylalanine hydroxylation took place in the liver in the normal manner then tetrahydrobiopterin must have been released into the circulatory system from the bowel. There is evidence that similar results might have been obtained by the administration of biopterin which decreases plasma phenylalanine levels in classical phenylketonuria when administered orally (Jacobson, 1967) and has the advantage of being commercially available and more stable.

The foregoing data may be resolved if biopterin is substantially transported into the epithelial cells of the jejunum, reduced and then slowly released into the portal circulation with a small amount of unaltered biopterin bypassing this effect and appearing in the serum, transport being unaffected by changes in the microclimate. Likewise, tetrahydrobiopterin would have to be taken up by bowel tissue, normally in the ileum, but in the jejunum when the microclimate is reduced by disease or ethanol and slowly released. Ethanol also appears to facilitate transport across the membrane and social alcohol consumption apparently has a positive association with increased serum Crithidia factor levels (Chapter VI) suggesting a cumulative effect of enhanced, reduced biopterin derivative absorption.

CHAPTER VI

CRITHIDIA FACTOR IN HUMAN BODY FLUIDS AND TISSUES

INTRODUCTION

Values for Crithidia factor in human body fluids and tissues have been reported (Baker, Frank, Bacchi and Hutner, 1974; Leeming, 1975; Leeming and Blair, 1974; Leeming, Blair, Melikian and O'Gorman, 1976; Kaufman, Berlow, Summer, Milstien et al, 1978; Fukushima and Shiota, 1972) and are summarised in Table 6-1. The term Crithidia factor has been coined to denote growth stimulating factors which will spare Crithidia fasciculata's need for biopterin. As these in man are principally biopterin, 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin (Leeming and Blair, 1974), the term biopterin derivatives is used synonymously with Crithidia factor.

It can be seen from Table 6-1 that there is a wide distribution of biopterin derivatives in the body and concentrations vary considerably from site to site. The brain has different concentrations in separate easily identifiable areas. Serum biopterin derivative levels are within a narrow range and may not give precise information on tissue levels. It would require considerable argument to overcome ethical scruples against free use of biopsy techniques therefore serum samples and urine samples are frequently all that are available in normal subjects. A previous study of serum biopterin derivatives in 114 volunteers (Leeming, 1975) divided subjects by age and sex without finding any significant differences. Comparatively small numbers were produced in some groups and subsequently it was considered that more detail might have revealed differences. Personal information was difficult to assess, for example it was not always possible to be certain that some unmarried women were being honest about their oral contraceptive status. At this later date oral contraception is a more generally accepted practice among

TABLE 6-1

PUBLISHED LEVELS OF BIOPTERIN DERIVATIVES IN HUMAN BODY FLUIDS AND TISSUES

	Baker et al, 1974			Leeming, 1975; Leeming et al, 1976.		
	(No.)	Range	Mean ± S.E.M.	(No.)	Range	Mean ± S.E.M.
Plasma - Adult	(31)	0.7 - 1.7μg/L	0.9 ± 0.2	(10)	1.2 - 1.8μg/L	1.44 ± 0.08
* Serum - Adult		-	-	(114)	0.4 - 3.6μg/L	1.81 ±0.06
** Serum - children < 10yrs.		-	-	(10)	1.1 - 3.7μg/L	1.78 ± 0.25
Red Blood Cells	(31)	0.8 - 2.2μg/L	1.2 ± 0.4	(10)	5.7 -11.1μg/L	7.8 ±0.55
Whole Blood	(31)	1.2 - 2.8μg/L	1.9 ± 0.9	(10)	3.2 - 5.2μg/L	4.0 ± 0.18
Cerebrospinal Fluid	(27)	0.25 - 0.7μg/L	0.4 ± 0.21	(19)	1.2 - 3.4μg/L	1.9 ± 0.13
Human Milk		-	-	(6)	85 - 163μg/L	131 ±13.6
Urine		-	-	(60)	0.3 - 6.9mg/L	2.1 ± 0.38
*** Urine/24hrs.	(19)	1.5 - 2.4 mg	2.1 ± 0.3	(5)	0.8 - 2.2mg	1.3 ± 0.3
Brain (dry wt.)		130-520ng/g	340 ± 230		-	-
Brain - grey matter (wet wt.)		-	-	(4)	20 - 35 ng/g	25.5 ± 3.3
Brain - white matter (wet wt.)		-	-	(4)	20 -112ng/g	46.3 ± 22.0
Brain Substantia Nigra (wet wt.)		-	-	(4)	53 -500ng/g	225.8 ±95.8
Liver (dry wt.)	(26)	360-900ng/g	520 ±160		-	-
Liver (wet wt.)		-	-	(4)	41 -163ng/g	112.0 ±36.3

	Range	Mean
*Kaufman et al 1978	(4) 1.3 - 2.4μg/L	1.7μg/L
**Kaufman et al 1978	(3) 1.0 - 2.6μg/L	2.1μg/L
***Fukushima and Shiota 1972	-	1.5mg

single women and it was considered that greater accuracy could be obtained by asking volunteers to fill in forms which maintained their anonymity, rather than giving details verbally to be recorded against their name.

Biopterin derivatives are widely distributed in nature (Rembold and Gyure, 1972), consequently they are present in normal diets. However, tetrahydrobiopterin is transported only slowly across the bowel (Blair, Ratanasthien and Leeming, 1974; Chapter V) and although a small amount of biopterin may be transported unaltered across the bowel and excreted, the remainder is reduced during transport and slowly released from the intestine (Rembold and Metzger, 1967; Chapter V).

Rats fed on a biopterin free diet for several generations continued to develop normally and to excrete about 30μg of biopterin each day in their urine (Pabst and Rembold, 1966). Tetrahydrobiopterin does not pass the blood/brain barrier readily (Kettler, Bartholini and Pletscher, 1974). Difficulty in passing cell membranes, independence from dietary sources and regional concentration within the body demonstrate that, normally, tetrahydrobiopterin is synthesised locally in tissues and that exogenous sources do not play a significant role in meeting demand for this essential cofactor.

Although in the previous study (Leeming, 1975) the possibility was examined of diurnal variation in serum biopterin derivatives, the impact of the menstrual cycle was not taken into consideration. If serum biopterin derivative levels alter during the normal menstrual cycle then differences between unmedicated females and those taking oral contraceptives could be exposed.

The relationship of serum and tissue biopterin derivative levels has not been established although normal ranges in brain and liver have been established post-mortem. It is one of the intentions of this chapter to measure biopterin derivatives in liver biopsies taken for diagnostic purposes and to examine serum samples collected at the same time. Regional variation in brain biopterin derivatives (Leeming, Blair, Melikian and O'Gorman, 1976) is particularly important, tetrahydrobiopterin is the rate limiting factor in

the hydroxylation of tyrosine to dopa (Kettler, Bartholini and Pletscher, 1974) and cofactor in the enzymatic hydroxylation of tryptophan to 5-hydroxytryptophan (Hosoda and Glick, 1966; Friedman, Kappelman and Kaufman, 1972). High levels of biopterin have been reported in sheep pineal glands (van der Have-Kirchberg, de Moree, Vanhaar, Gerwig, Versluis, Ebels, Hus-Citharel, Heritier, Roseau, Zurburg and Moszkowska, 1977).

In summary, serum samples from (apparently) normal volunteers together with other body fluids and tissues made available by clinical colleagues are to be examined with the broad objective of shedding further light on normal biopterin metabolism.

MATERIALS AND METHODS

All samples were obtained with informed consent. Biopsies and bile samples were obtained by a clinician for diagnostic purposes. Post-mortem material was obtained by a pathologist at autopsy.

Blood samples were taken from a total of 331 members of hospital staff, university staff and students. The serum was separated by centrifugation and stored at -20°C until assayed using Crithidia fasciculata as previously described (Leeming, Blair, Melikian and O'Gorman, 1976). Sex, age and alcohol consumed in the previous 24 hours were recorded for each volunteer, women were also asked for the starting date of their last monthly period and whether they were taking oral contraceptives. Anonymity was given by allocating numbers to samples and forms and by not recording names. It was hoped in this way to obtain truthful personal details.

Ages ranged from 16 to 66 years. Two of the women were pregnant and 98 were taking oral contraceptives, the remaining 156 were unmedicated. The series included 75 men.

The results from the three main groups (i.e. males, unmedicated females and females taking oral contraceptives) were divided by age. The results from the two female groups were recorded against the day in their menstrual cycle, excluding of course, post menopausal females, those on whom hysterectomies had been performed, those whose periods were irregular or who suffered from amenorrhoea and a few who did not know the date on which they last started to menstruate. One 19 year old female had her serum Crithidia factor measured daily throughout the whole of one menstrual cycle, samples were separated for phenylalanine and tyrosine and sublingual temperature was taken at the same time.

45 cerebrospinal fluids with normal cell counts were assayed

for Crithidia factor. Bile samples were obtained from nine subjects, seven of these were duodenal bile and the other two were obtained at cholecystectomy. Twelve patients having liver biopsies for diagnostic purposes had blood samples taken at the same time, liver and serum Crithidia factor levels were measured.

Pineals were taken at post-mortem examination from ten cases where there was no involvement of the central nervous system in disease processes, ages at death ranged from 44 to 82.

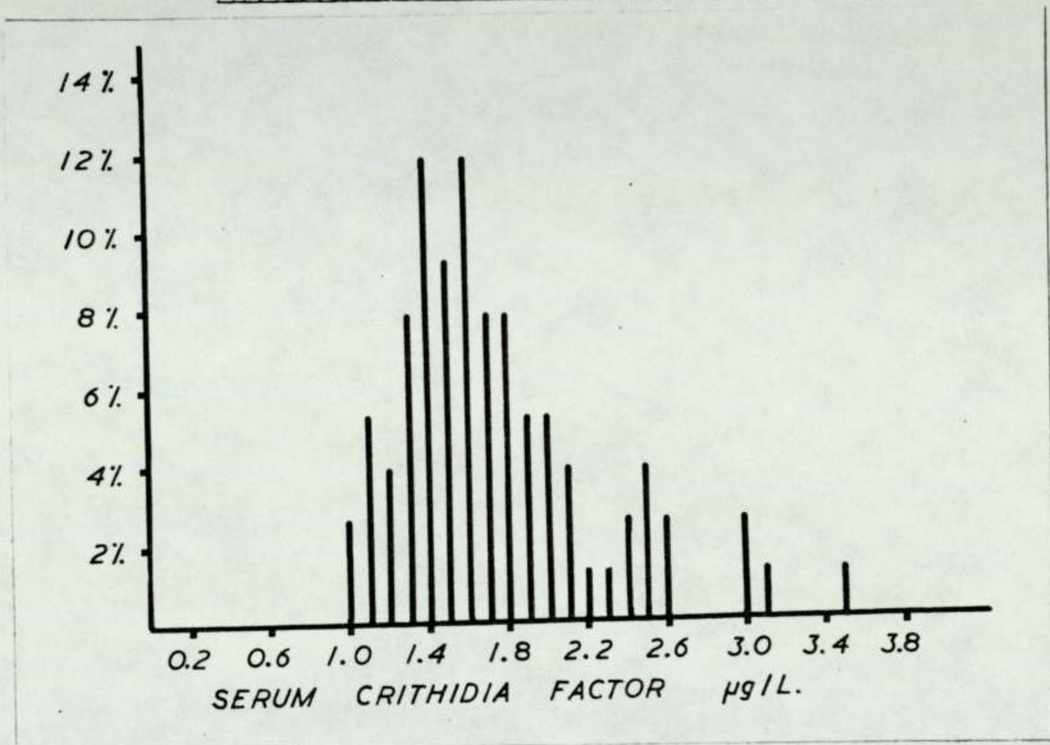
Samples of carcinoid tissue taken at operation were obtained from three cases and Crithidia factor was assayed.

RESULTS

The distribution of serum Crithidia factor levels in males, unmedicated females and females taking oral contraceptives are shown in Figures 6-1 to 6-3. The mean level for males was 1.75μg/L ± 0.03 (s.e.m.),

FIGURE 6-1

DISTRIBUTION OF SERUM CRITHIDIA FACTOR LEVELS IN 75 MALES EXPRESSED AS A PERCENTAGE OF THE TOTAL



for unmedicated females 1.53μg/L ± 0.04 and for females on oral contraceptives 1.44μg/L ± 0.05. The difference between unmedicated females and males was significant by Student's 't' test ($p = <0.05$). The effect of oral contraceptives on serum Crithidia factor in females was not significant ($p = 0.1 - 0.2$).

Serum Crithidia factor increased with age in both males and females (Table 6-2), with the greatest difference between the sexes occurring between 21 and 30 years of age ($p = < 0.01$). Differences between sexes at all other ages failed to satisfy Student's 't' test at the 5% level of significance. Mean values on each day of the menstrual cycle, counting

TABLE 6-2

SERUM CRITHIDIA FACTOR IN 75 MALES, 156 UNMEDICATED FEMALES AND 98 FEMALES TAKING ORAL CONTRACEPTIVES DISTRIBUTED ACCORDING TO AGE

Age	Males (N) mean ± s.e.m.	Females (N) mean ± s.e.m.	Females on 'pill' (N) mean ± s.e.m.
16 - 20	(12) 1.542 ± 0.085μg/L (range 1.1 - 2.1)	(31) 1.465 ± 0.073μg/L (range 0.6 - 2.3)	(24) 1.467 ± 0.090μg/L (range 0.7 - 2.4)
21 - 30	(45) 1.727 ± 0.072 (range 1.0 - 2.5)	(68) 1.434 ± 0.052 (range 0.7 - 2.4)	(68) 1.412 ± 0.060 (range 0.4 - 2.5)
31 - 40	(8) 1.650 ± 0.124 (range 1.3 - 2.3)	(24) 1.700 ± 0.078 (range 1.0 - 2.4)	(5) 1.560 ± 0.087 (range 1.3 - 1.7)
41 - 50	(5) 1.860 ± 0.314 (range 1.1 - 3.0)	(25) 1.940 ± 0.111 (range 1.1 - 3.1)	(1) 1.8 -
51 - 66	(5) 2.540 ± 0.300 (range 1.6 - 3.5)	(8) 1.838 ± 0.145 (range 1.6 - 2.2)	-
Total	(75) 1.747 ± 0.030 (range 1.0 - 3.5)	(156) 1.534 ± 0.041 (range 0.6 - 3.1)	(98) 1.442 ± 0.086 (range 0.4 - 2.5)

FIGURE 6-2

DISTRIBUTION OF SERUM CRITHIDIA FACTOR LEVELS IN 98 FEMALES TAKING ORAL CONTRACEPTIVES EXPRESSED AS A PERCENTAGE OF THE TOTAL

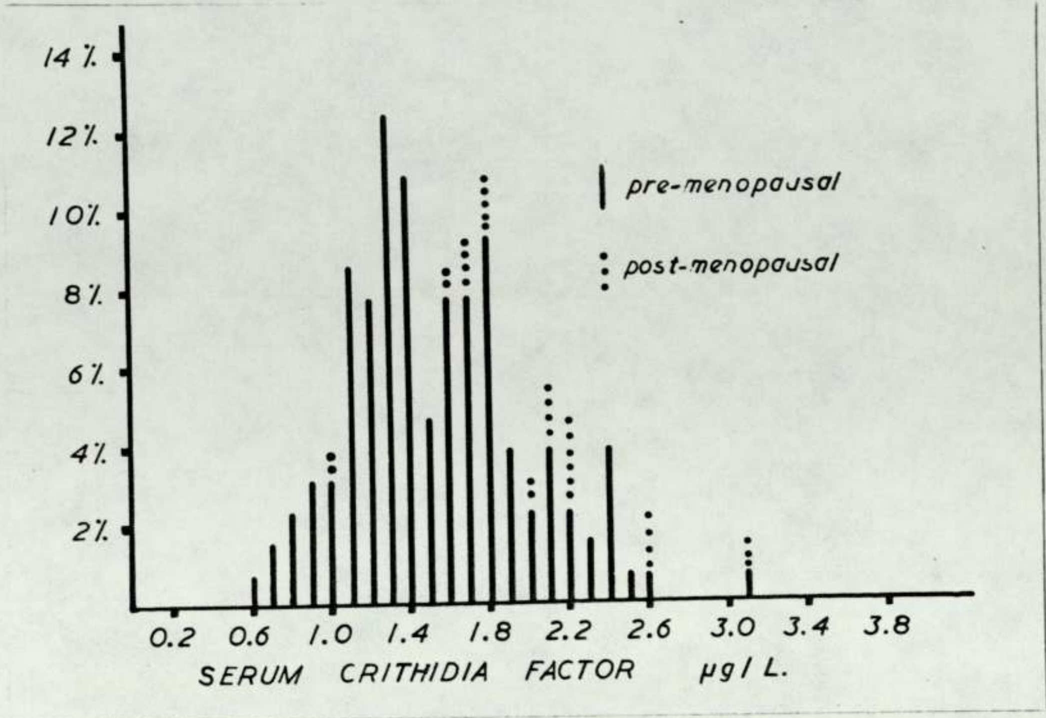
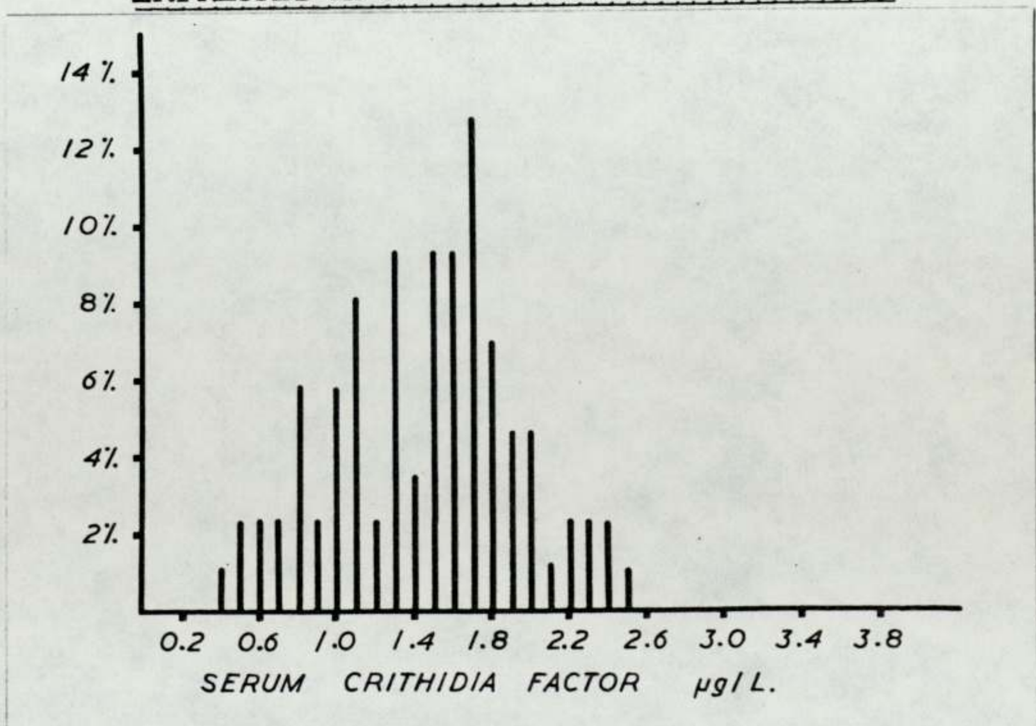


FIGURE 6-3

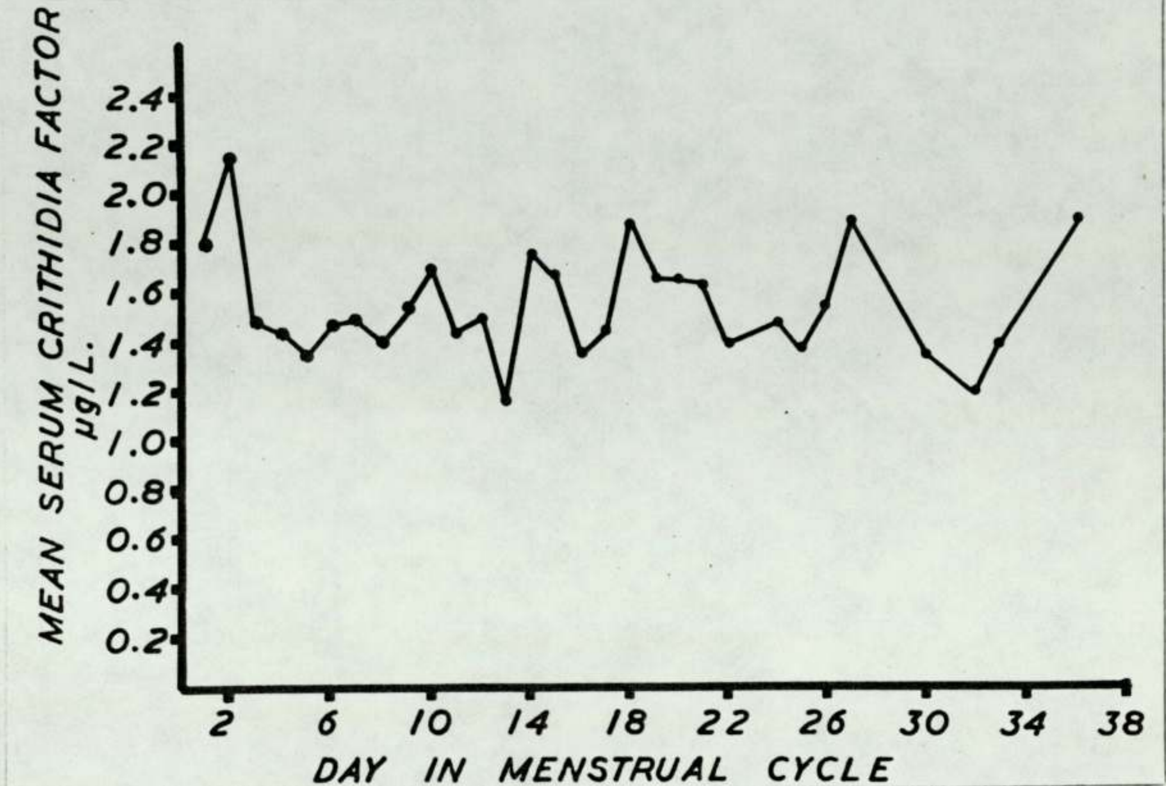
DISTRIBUTION OF SERUM CRITHIDIA FACTOR LEVELS IN 156 FEMALES NOT TAKING ORAL CONTRACEPTIVES EXPRESSED AS A PERCENTAGE OF THE TOTAL



day one as the first day of menstruation, are shown in Figures 6-4 and 6-5.

FIGURE 6-4

MEAN SERUM CRITHIDIA FACTOR LEVELS PLOTTED AGAINST THE DAY IN THE MENSTRUAL CYCLE OF 129 FEMALES NOT TAKING ORAL CONTRACEPTIVES.



There was no obvious pattern although at an intermediate analysis of unmedicated females, as data was being accumulated, there appeared to be a marked dip at the middle of the cycle and a rise at the end. Both these observations could have been caused by analysis of a more homogenous age-group within the whole; the longer cycles were predominantly in the higher age groups, therefore females below the age of 30 and not taking oral contraceptives were looked at separately. In view of the distribution in this relatively small sample a three point moving average was plotted (Figure 6-6), trends appeared with the values decreasing from the beginning of the cycle and increasing sharply at the end with the lowest value appearing on day 12. The young woman whose serum Crithidia factor level was followed throughout her menstrual cycle showed a similar pattern (Figure 6-7) to the unmedicated females below the age of 30. The highest value occurred immediately before menstruation (Table 6-3), the seven days up to and including day 14 were significantly higher than the seven days following ($p = \leq 0.05$). There was

FIGURE 6-5

MEAN SERUM CRITHIDIA FACTOR LEVEL PLOTTED AGAINST THE DAY IN THE MENSTRUAL CYCLE OF 92 FEMALES TAKING ORAL CONTRACEPTIVES

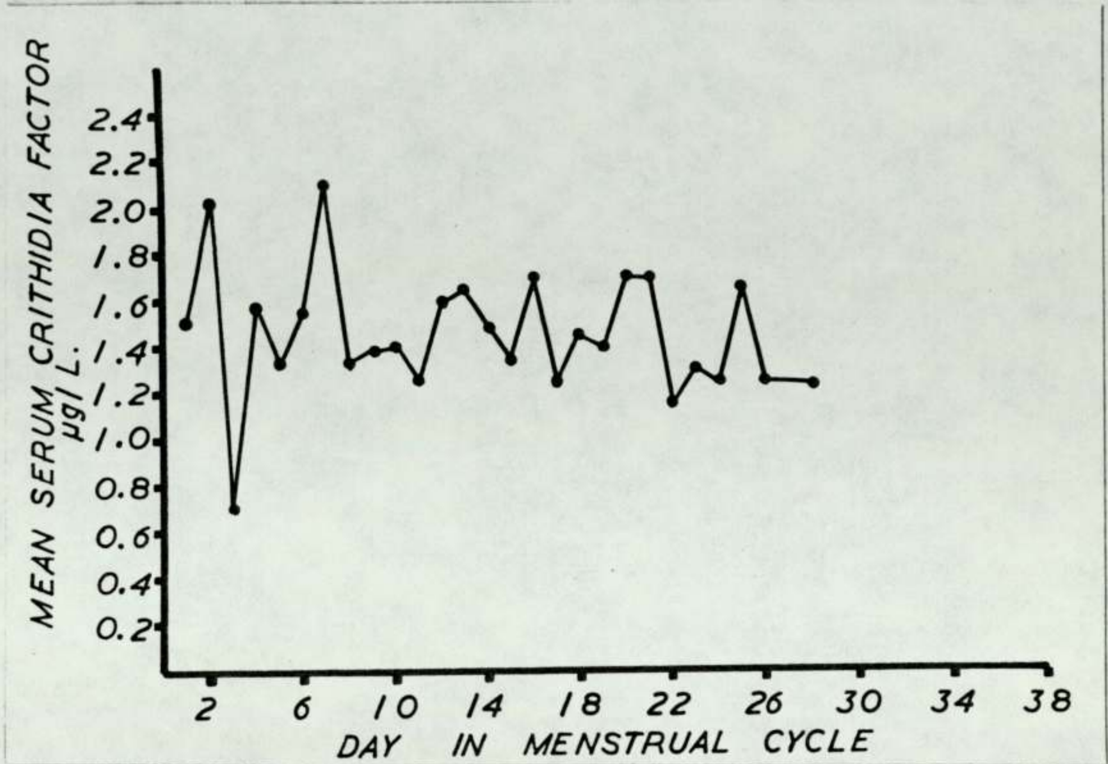


FIGURE 6-6

THREE-POINT MOVING AVERAGE OF SERUM CRITHIDIA FACTOR PLOTTED AGAINST THE DAY IN THE MENSTRUAL CYCLE OF 99 FEMALES UP TO THE AGE OF 30 YEARS AND NOT TAKING ORAL CONTRACEPTIVES.

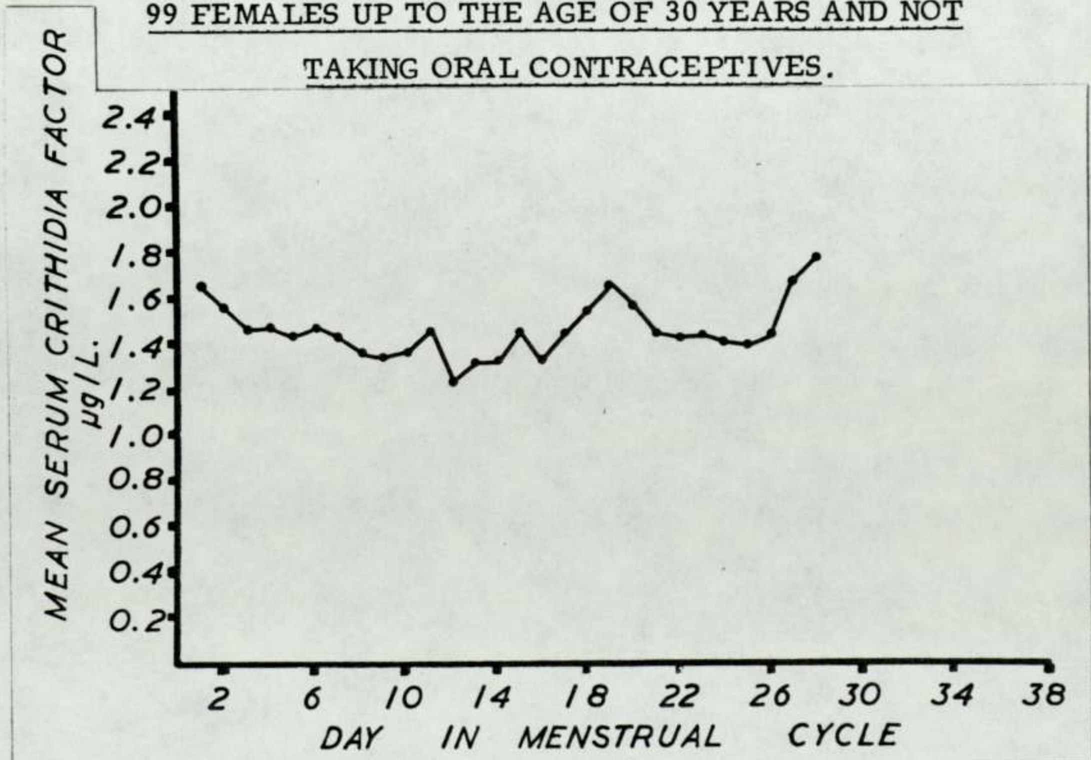


TABLE 6-3

SERUM CRITHIDIA FACTOR, PHENYLALANINE AND TYROSINE, IN AN UNMEDICATED FEMALE AGED 19 YEARS THROUGHOUT ONE MENSTRUAL CYCLE

Day	Menstruating	Crithidia factor μg/L	Phenylalanine μmol/L	Tyrosine μmol/L
1	+	1.7	44.6	46.9
2	+	1.2	49.4	37.5
3	+	1.2	43.0	51.6
4	+	1.2	62.1	71.9
5	-	1.4	27.1	37.5
6	-	1.2	35.0	39.1
7	-	0.8	36.6	32.8
8	-	1.4	57.3	57.8
9	-	1.4	47.8	50.0
10	-	1.3	47.8	42.2
11	-	1.5	58.9	57.8
12	-	1.6	44.6	40.6
13	-	1.3	41.4	35.9
14	-	1.4	71.6	75.0
15	-	1.0	63.7	71.9
16	-	1.1	54.1	51.6
17	-	1.1	41.4	37.5
18	-	1.2	49.4	59.4
19	-	1.2	46.2	31.3
20	-	1.3	38.2	42.2
21	-	1.3	43.0	43.7
22	-	1.2	41.4	45.3
23	-	1.4	40.6	45.3
24	-	1.3	50.9	43.7
25	-	1.2	51.7	42.2
26	-	1.5	58.9	62.5
27	-	1.2	31.8	32.8
28	-	1.2	46.2	43.7
29	-	1.7	50.9	53.1

TABLE 6-3

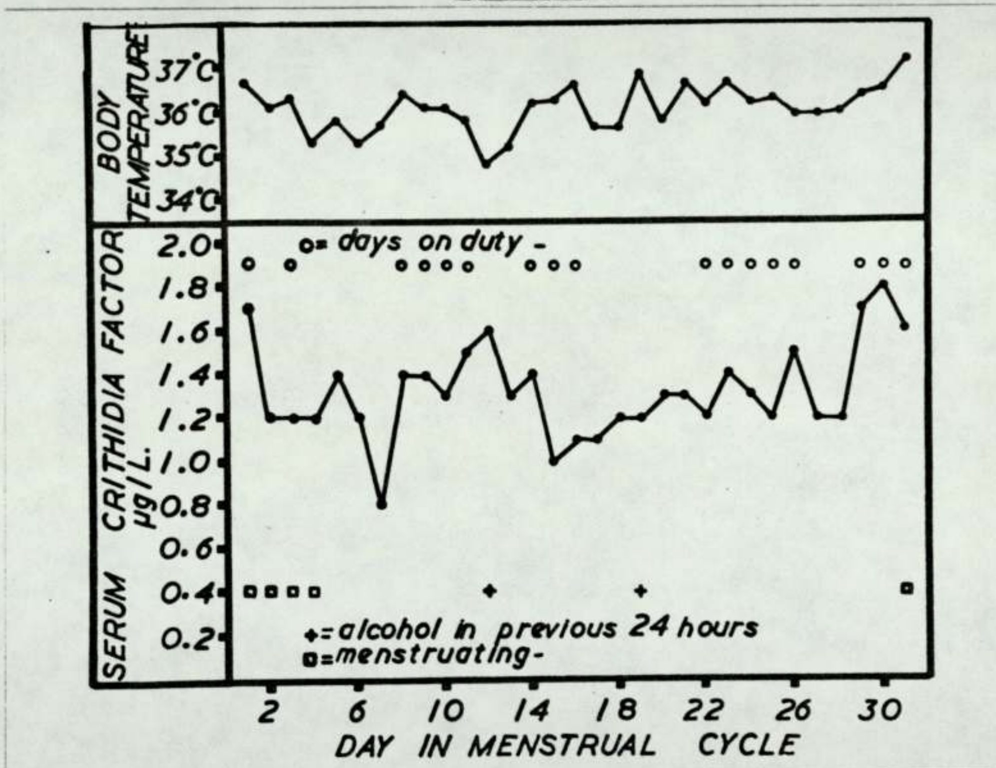
(Continued)

30	-	1.8	47.8	50.0
31	+	1.6	63.7	54.7

FIGURE 6-7

SERUM CRITHIDIA FACTOR IN A 19 YEAR OLD FEMALE NURSE THROUGHOUT ONE MENSTRUAL CYCLE

TEMPERATURE, ALCOHOL CONSUMPTION AND DAYS ON DUTY WERE RECORDED



no obvious correlation with sublingual temperature.

The effects of alcohol consumption in the 24 hours prior to sampling are shown in Table 6-4. In all three groups those who had taken alcohol in the previous 24 hours had higher serum Crithidia factor levels but this never attained a significant level.

The twelve liver samples had values ranging from 105ng/g to 862 ng/g (wet weight) Crithidia factor with mean and standard error of mean -

TABLE 6-4

THE EFFECTS OF ALCOHOL CONSUMPTION (IN PREVIOUS 24 HOURS) ON SERUM CRITHIDIA FACTOR LEVELS IN MALES AND FEMALES IN THE AGE RANGE 18 - 30 YEARS.

	No	Mean $\pm$ s.e.m.	Student's 't' test
Males - no alcohol	19	1.63 $\pm$ 0.12μg/L	p = 0.3 - 0.4
Males + alcohol	22	1.78 $\pm$ 0.10μg/L	
Females - no alcohol	68	1.51 $\pm$ 0.07μg/L	p = 0.05 - 0.1
Females + alcohol	34	1.73 $\pm$ 0.11μg/L	

375ng/g $\pm$ 67, with serum levels in the same patients ranging from 0.8μg/L to 2.5μg/L, mean and s.e.m. 1.58μg/L $\pm$ 0.17. There was no significant correlation between serum and liver concentration r = 0.036

The cerebrospinal fluids had Crithidia factor levels ranging from 1.2μg/L to 3.6μg/L, mean 2.03μg/L $\pm$ 0.10.

Bile samples had from 2.7 to 200μg/L Crithidia factor, mean 48.4μg/L $\pm$ 22.6μg/L.

The pineals had from 31.8 to 388.8 ng Crithidia factor total content, mean 157.6ng $\pm$ 38.2. Measured in concentration per gram wet tissue the values were - range 87.4 to 1318ng/g, mean 525.9ng/g $\pm$ 120.1. These can be compared with total folate levels measured using L. casei. Total folate/pineal range - 7.6ng to 148.0ng, mean 24.7ng $\pm$ 13.7 and in concentration - range 24.7 to 365.4ng/g, mean 71.2ng/g $\pm$ 32.9. Correlation between pineal folate and Crithidia factor was not significant (r = 0.449, p = 0.1).

Concentrations of Crithidia factor in carcinoid tissues in the three samples were 365ng/g., 596ng/g. and 898ng/g. wet tissue.

DISCUSSION

The general difference between serum biopterin derivative levels in males and unmedicated females is made significant by the large difference in the age group from 21 to 30 years. The distribution of biopterin derivatives in women not taking oral contraceptives was similar to that found in males (Figures 6-1 and 6-3) with the older, post menopausal women identified as being nearly always above the mean One distinction which contributed to the difference between the sexes was the number of females with values below 1.0μg/L. These all occurred below the age of 30 years which was a phenomenon shared with women who were taking oral contraceptives (Figure 6-2, Table 6-2) although it must be emphasised that only six out of ninety-eight women in the latter group were above 30 years of age.

Mean values during the menstrual cycle of women not taking oral contraceptives (Figure 6-4) followed no coherent pattern and times of up to 36 days from commencement of the last period were recorded. The women below 30 years of age (Figure 6-6) gave a similar pattern to the one young woman aged 19 who was followed throughout the cycle (Figure 6-7), the most striking similarity was the rise in the few days immediately before menstruation commenced. The mean serum biopterin derivative level for the week commencing on the 15th day of the cycle of the woman who gave daily samples was significantly lower ($p = <0.01$) than for the preceding week, with the lowest value occurring on day 15.

It has been suggested (Kaufman, Berlow and Summer et al, 1978) that serum biopterin derivative levels should be 'normalised', that is considered in relationship to plasma phenylalanine concentration and it has been shown that there is a significant relationship between serum biopterin derivatives and phenylalanine levels in phenylketonurics, normal adults who have had phenylalanine loads and a normal male adult on a low phenylalanine diet (Leeming, Blair, Green and Raine, 1976). Serum biopterin derivatives in the blood samples of the subject who gave samples throughout her menstrual cycle (Table 6-4) did not show any

meaningful relationship with phenylalanine or tyrosine.

The women taking oral contraceptives were mostly below the age of 30 years and the effect of this social medication on serum biopterin derivatives during the menstrual cycle (Figure 6-5) was therefore assessed by comparison with unmedicated females below 30 years of age (Figure 6-6). Excluded were those who had altered the recommended pattern of oral contraception, for example two subjects who were taking the 'pill' continuously. There was no trend except perhaps a slight fall in the mean level from beginning to end with the most widely dispersed values during the seven days abstention followed by a closer range when oral contraceptive pills were resumed.

Biopterin derivatives in cerebrospinal fluids were at very similar levels to those reported earlier (Leeming, Blair, Melikian and O'Gorman, 1976) and although significantly higher than serum levels ($p = < 0.02$) the difference was not great and in no way matches brain levels even in areas of low concentration. Transfer of biopterin derivatives between brain and cerebrospinal fluid is then similar to that between other tissues and blood thereby demonstrating again that synthesis is carried out locally.

Pineal organs had higher biopterin derivative levels than those found elsewhere in the brain (to date) although more discrete dissection may be more revealing. Others have found high biopterin levels in pineals (van der Have-Kirchberg et al, 1977) which would fit in with the large amounts of serotonin found in mammalian pineal tissue, Giarman and Day (1959) found 0.4μg/g of serotonin in bovine pineal but the proportions of noradrenaline and serotonin vary with individual mammalian species (Owman, 1965; Wurtman, Axelrod and Kelly, 1968).

The lack of correlation between liver and serum biopterin derivatives is not conclusive; as stated earlier it would have been unethical to take liver samples from normal subjects and as these samples were from diseased patients, processes could have been at work affecting

synthesis or dihydropteridine reductase. It is important to note however that these levels are very similar to those obtained from post-mortem tissue (Leeming, Blair, Melikian and O'Gorman, 1976), thereby increasing the validity of the results from other post-mortem tissue such as brain and the pineals which one would have difficulty in obtaining fresh from human sources.

Bile samples had a wide distribution of levels and it is difficult to find an explanation for this.

Biopterin derivatives in carcinoid tissue were high and again in this tissue serotonin is synthesised with tissue serotonin reaching 1 - 3mg/g. and serum levels of $0.5\mu g/cm^3$ to $2.7\mu g/cm^3$ compared with the normal 0.1 to $0.3\mu g/cm^3$ (Sjoerdsman, Weissbach and Udenfriend, 1956).

In conclusion, as previously found (Baker, Frank, Bacchi and Hutner, 1974; Leeming, Blair, Melikian and O'Gorman, 1976; Leeming, 1975) Crithidia active derivatives of biopterin are ubiquitous in the human body with a wide variation in concentration. Serum levels are within a close range except in a very few diseases but there is a suggestion that age, menstrual cycle and perhaps oral contraceptives have a minor effect on serum biopterin derivative concentrations.

CHAPTER VII

CRITHIDIA FACTOR IN THE FOETUS AND NEONATE

INTRODUCTION

The issue of foetal material for research by the nursing home concerned was in accordance with Department of Health and Social Security regulations and the recommendations of the Peel Committee. The pregnant woman's consent was given in all cases. Permission to carry out work on foetuses at the General Hospital, Birmingham was granted by the Research Ethical Committee of the Central Birmingham Health District (Teaching) following an application by Dr. A. Pollock, Consultant Haematologist, head of the department where this work was carried out. The application precedes the section on Materials and Methods.

Phenylalanine hydroxylase is produced in the human foetal liver as early as the eighth week of gestation (Räihä, 1973) and has been reported as half the adult value between the eleventh and twenty-second weeks without any correlation between foetal age, weight and hydroxylase level (Delvalle and Greengard, 1977). Bessman, Wapnir and Towell (1977) showed significantly negatively correlated liver phenylalanine hydroxylase levels and gestational age in foetuses of up to twenty weeks gestation. Hydroxylation of phenylalanine in the liver of a twelve week foetus without added pteridine cofactor (Jacubovic, 1971) points to endogenous cofactor being present at an early stage, the same author deduced that dihydropteridine reductase was present in a seventeen week old foetal liver by showing enhanced tyrosine formation when NADH was added to the incubation mixture. Jacubovic (1971) failed to show tyrosine formation in human foetal brain between the eleventh and twentieth weeks whereas Bessman et al,(1977) showed, in foetal brain homogenates, phenylalanine hydroxylase from the sixteenth to the thirty-third week which, unlike liver levels, did not correlate with foetal age. The latter found phenylalanine

hydroxylase, in the brain of a thirty-three week old foetus, without being able to detect it in the liver.

Tyrosine hydroxylase is low or undetectable in the human foetal brain from the eleventh to the thirty-third week of gestation (Bessman et al, 1977). In the striatum of the newborn rat, the activity of tyrosine hydroxylase is around 10% of that found in the adult rat, reaching 75% of the adult level in four weeks (Coyle and Compachiaro, 1976).

Biopterin derivative levels in human foetal tissue have not been reported although there is documented information on adult human and rat brain, liver and serum levels (Baker, Frank, Bacchi and Hutner, 1974; Leeming, Blair, Melikian and O'Gorman, 1976; Chapters VI and XII). The hydroxylations of phenylalanine, tyrosine and tryptophan need active cofactor, therefore the synthesis of 5,6,7,8-tetrahydrobiopterin is essential.

Amniotic fluids are taken from pregnant women at risk for the diagnosis of conditions in which therapeutic abortion is indicated to prevent the live births of anencephalics, spina bifidas and similar conditions. Amniotic fluids are more readily available, therefore, than foetal tissue and it is appropriate that they are included in this chapter. With clinical co-operation samples of cord blood are available in a number of deliveries at term.

A more adequate knowledge of the development of systems necessary for the hydroxylation of phenylalanine and the synthesis of neurotransmitters is important if neurological damage to the foetus is to be avoided in maternal phenylketonuria, where early dietary control is required (Smith,Macartney, Erdohazi, Pincott et al, 1979). When tetrahydrobiopterin is not synthesised (Leeming, Blair and Rey, 1976; Rey, Blandin-Savoja and Rey; 1976; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977; Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschal, 1978) or dihydropteridine reductase is deficient (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977), gross hypotonia and death in infancy will follow (Smith, Clayton and Wolff, 1975) if neurotransmitter replacement therapy (Bartholome and Byrd, 1975) is not given.

APPLICATION TO ETHICAL COMMITTEE
OF
CENTRAL BIRMINGHAM HEALTH DISTRICT

PROPOSED STUDY OF FOETAL MATERIAL

We wish to measure levels of folates in foetal blood serum after different gestational periods. Experiments in this hospital indicate raised levels of 10-formyltetrahydrofolic acid in pathological conditions of high cellular replication, such as malignancy and psoriasis. If the elevated level of this folate is a function of cell turnover then its measurement should help monitor the efficacy of cytotoxic therapy. The measurement of different members of the folate pool in the foetus at different stages of development should confirm these observations in normal cell division and give more information on the relationship.

Additionally we would like to identify biopterin derivations in foetal tissue to determine the pattern of synthesis before parturition. These pteridines are cofactors in the hydroxylation of essential amino acids en route to central neurotransmitters. Recent published work has shown (and been confirmed in our laboratory) that a condition exists where cofactor metabolism is defective and which is associated with severe neurological degeneration and death in infancy. Further studies into the origin of the biosynthetic process could give insight into this condition and possibilities of treatment.

The work proposed is an extension of what has already been carried out in this laboratory. The necessary expertise exists and has been well proved by our publications in the scientific literature.

MATERIALS AND METHODS

Fifteen foetuses were obtained following prostaglandin induced abortion. Each foetus was weighed and sexed. The brains and livers were removed, weighed and homogenised in 0.2M phosphate buffer pH 5.0 and serially diluted in buffer for assay with Crithidia fasciculata. Liver from one foetus was homogenised, frozen, thawed and centrifuged. The resultant extract was divided into two parts. One aliquot was autoclaved for three minutes at 115°C and then stirred under a stream of oxygen for two hours. Both were chromatographed on thin layer cellulose with 3% ammonium chloride.

Specimens from amniocentesis on 32 pregnant women at risk for various reasons were assayed for biopterin derivatives, urea and in some cases alpha feto-protein.

Seventeen samples of cord blood were taken at term in normal pregnancies when infants did not have known abnormalities. Nine of these were assayed for serum vitamin B_{12} as well as for serum Crithidia factor.

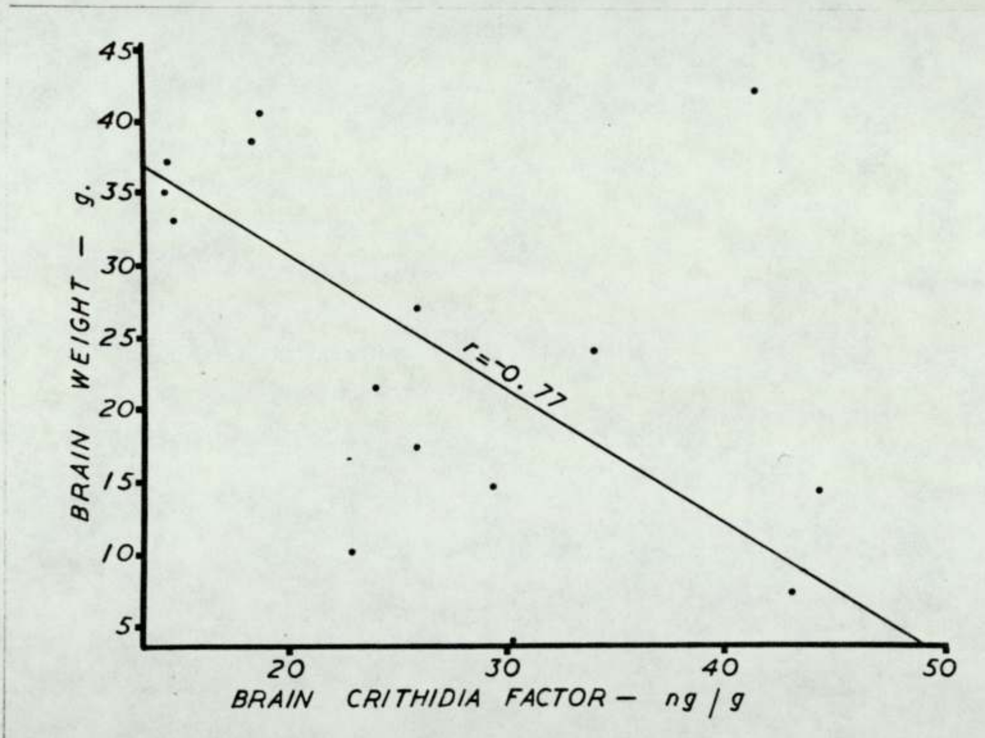
Thin-layer chromatography was carried out on tissue, blood and amniotic fluids in 0.5% sodium carbonate, 3% ammonium chloride and 5% acetic acid. After development the plates were cut horizontally and eluted. The eluates were assayed by Crithidia fasciculata.

RESULTS

Foetal Crithidia factor levels are set out in Table 7-1. Concentration in the liver ranged from 63.6ng/g to 396 ng/g with a mean and standard error of 240 $\pm$ 25.8ng/g. The concentration in the brain was lower, ranging from 14.8ng/g to 44.4ng/g with a mean of 26.8 $\pm$ 2.6ng/g. The concentration of Crithidia factor in the brain was significantly and negatively correlated with brain weight ($p = < 0.001$; Figure 7-1) and

FIGURE 7-1

BRAIN WEIGHT PLOTTED AGAINST BRAIN CRITHIDIA FACTOR CONCENTRATION IN FIFTEEN FOETUSES



gestational age ($p= < 0.05$; Figure 7-2). There was no correlation with foetal weight ($p = > 0.05$). A positive correlation between gestational age and total Crithidia factor in the brain was shown to be significant ($p = < 0.05$; Figure 7-3). Liver Crithidia factor did not show any correlation with liver weight (Figure 7-4), gestational age (Figure 7-5), brain Crithidia factor (Figure 7-6) or total foetal weight ($p = > 0.01$ in all cases). The total liver Crithidia factor in liver did not correlate with

TABLE 7-1

CRITHIDIA FACTOR LEVELS IN THE LIVERS AND BRAINS OF FIFTEEN FOETUSES

Foetus No.	Sex	Wt. (g)	Gestational Period (weeks)	Liver Wt. (g)	Liver Biopterin Derivatives ng/g wet wt.	Liver (Total ng/liver)	Brain Wt. (g)	Brain Biopterin Derivatives ng/g wet wt.	Brain (Total ng/brain)
1	M	300	18	12.7	319	(4051)	37.2	14.8	(550)
2	F	635	22	12.2	103	(1259)	38.7	18.1	(690)
3	M	176	18	7.2	326	(2347)	24.1	34.0	(819)
4	M	296	19	13.7	183	(2407)	40.6	18.5	(751)
5	F	225	19	9.4	170	(1598)	33.4	15.0	(501)
6	M	245	20	11.0	636	(6996)	35.0	14.3	(501)
7	F	31	16	1.5	233	(350)	4.3	30.2	(130)
8	M	215	18	8.7	172	(1496)	27.1	25.8	(699)
9	M	63	16	2.6	279	(725)	10.3	23.3	(240)
10	M	297	20	11.9	240	(2856)	42.3	41.4	(1751)
11	F	106	17	3.4	368	(1251)	14.8	29.4	(435)
12	M	100	17	4.9	199	(975)	15.9	25.8	(410)
13	M	46	12	2.4	396	(950)	7.2	43.1	(310)
14	F	104	17	3.8	184	(699)	14.5	44.4	(644)
15	M	175	18	6.8	370	(2516)	21.6	24.0	(518)

FIGURE 7-2

GESTATIONAL AGE PLOTTED AGAINST BRAIN CRITHIDIA FACTOR CONCENTRATION IN FIFTEEN FOETUSES

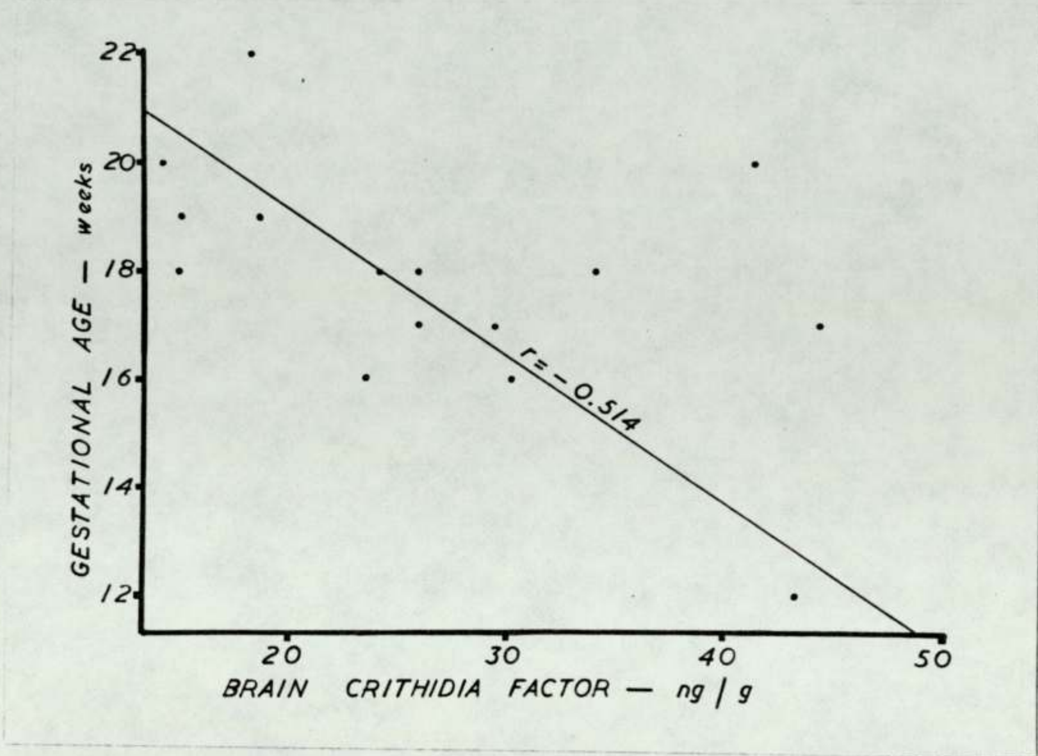


FIGURE 7-3

GESTATIONAL AGE PLOTTED AGAINST TOTAL BRAIN CRITHIDIA FACTOR IN FIFTEEN FOETUSES

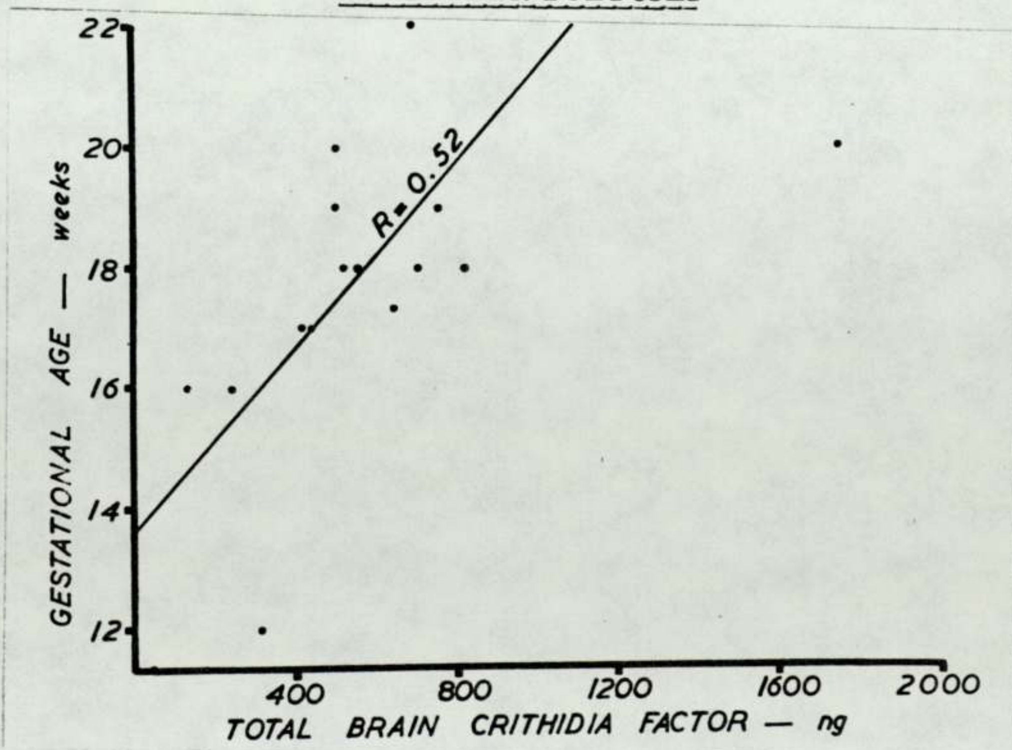


FIGURE 7-4

LIVER WEIGHT PLOTTED AGAINST LIVER CRITHIDIA FACTOR CONCENTRATION IN FIFTEEN FOETUSES

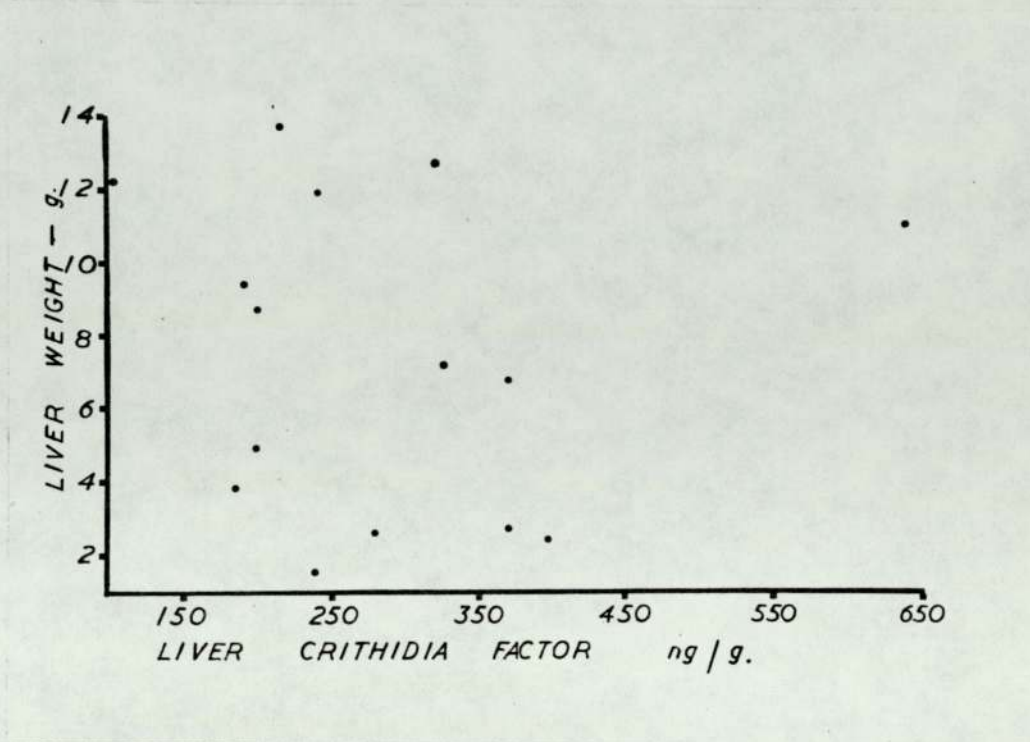


FIGURE 7-5

GESTATIONAL AGE PLOTTED AGAINST LIVER CRITHIDIA FACTOR CONCENTRATION IN FIFTEEN FOETUSES

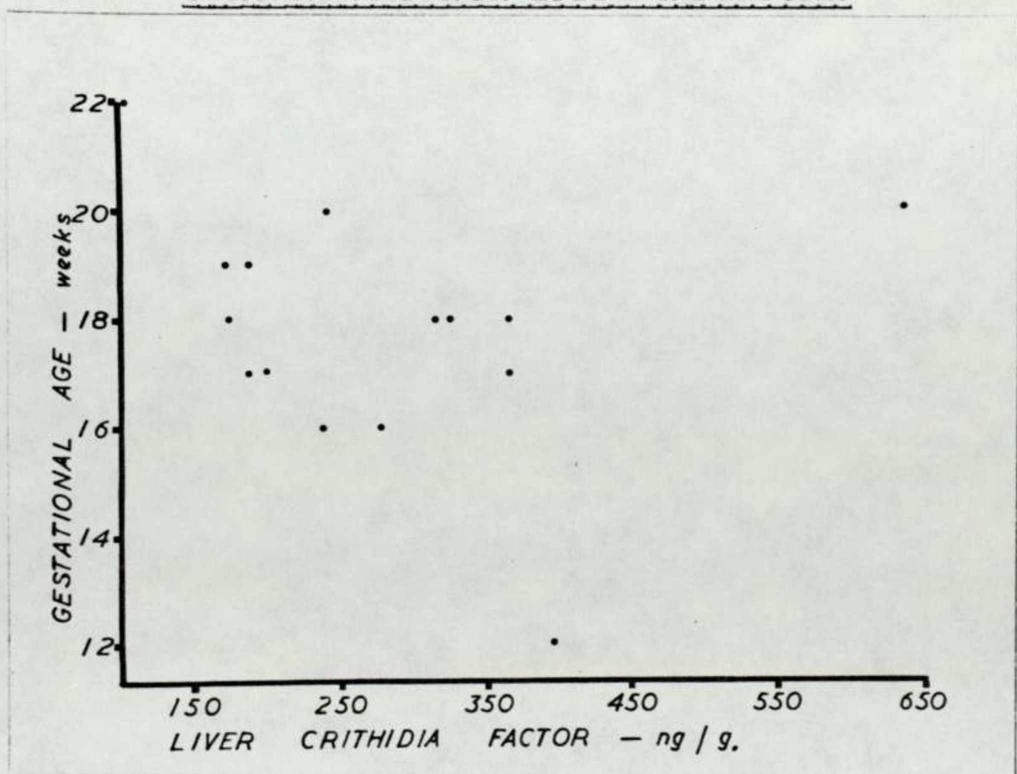
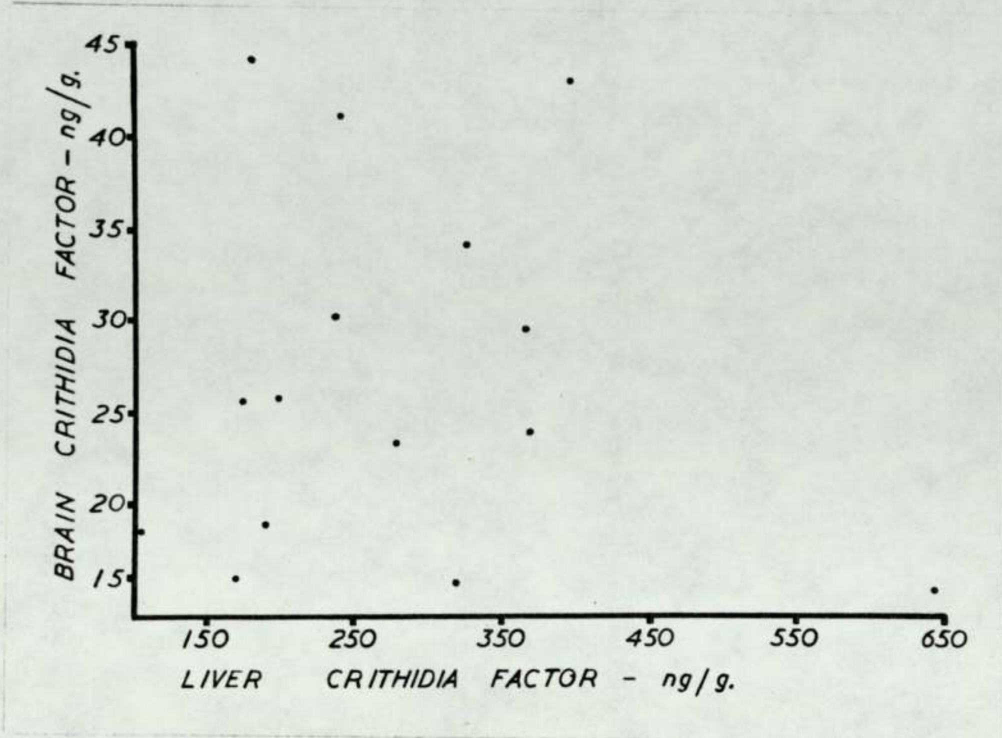


FIGURE 7-6

BRAIN CRITHIDIA FACTOR CONCENTRATION PLOTTED AGAINST LIVER CRITHIDIA FACTOR CONCENTRATION



the gestational age of foetuses ($p = > 0.05$).

Chromatography showed a Crithidia active material in the brain which produced an elongated spot straddling biopterin and 7,8-dihydrobiopterin. A similar spot occurred with liver, but liver autoclaved for three minutes at 115°C and stirred in a stream of oxygen for two hours produced a spot which co-chromatographed with biopterin only.

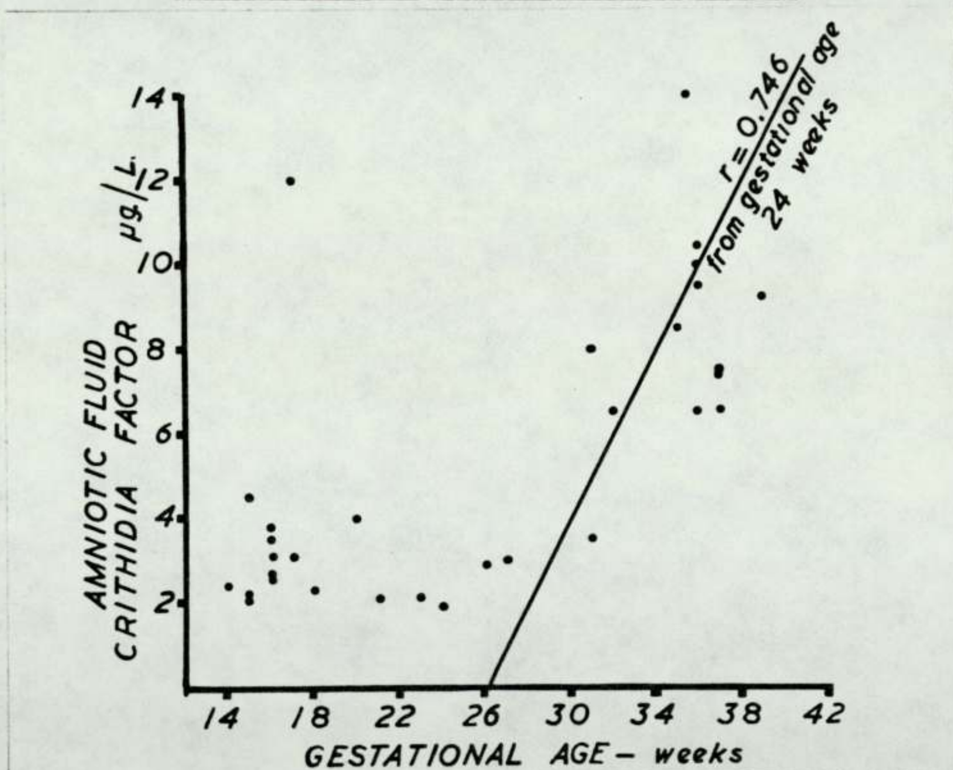
Serum Crithidia factor in cord blood ranged from 3.8µg/L to 8.3 µg/L (mean 5.8 ± 0.65µg/L) and was significantly different from normal (1.75µg/L for males and 1.53µg/L for females - Chapter VI) by Student's 't' test ($p = < 0.001$). There were two chromatographically distinct materials in foetal cord blood serum, the major material ran with 7,8-dihydrobiopterin and the minor one ran with biopterin. Phenylalanine levels in cord blood ranged from 0.06m.mol/L to 0.14 m.mol/L, mean 0.083 ± 0.016 m.mol/L

which was higher than fasting levels in five adults - 0.049 ± 0.0053 m.mol/L but was not significantly different ($p = > 0.05$). Tyrosine levels ranged from 0.014 to 0.14 m.mol/L, mean 0.063 ± 0.022 m.mol/L. Vitamin B_{12} levels were 550, 780, and 1000 ng/L in the three assayed sera.

Crithidia factor concentrations in amniotic fluids ranged from 1.9μg/L to 10.4μg/L and urea levels from 2.2 m.mol/L to 8.7 m.mol/L. Those specimens taken after 30 weeks gestation were significantly higher than those before ($p = < 0.001$) and the correlation between gestational age and Crithidia factor level is shown in Figure 7.7. It was observed that the

FIGURE 7-7

CRITHIDIA FACTOR IN 32 AMNIOTIC FLUIDS PLOTTED AGAINST GESTATIONAL AGE



urea levels also rose with gestational age (Figure 7-8) and the relationship ($p = < 0.01$) between amniotic urea and Crithidia factor is shown in Figure 7-9. A table of normal alpha feto-protein levels in amniotic fluids is given (Table 7-2). Two patients had very high levels of alpha feto-protein (104 mg/L and 108 mg/L) and were aborted. One produced an anencephalic and the other a spina bifida. A marginally raised alpha feto-protein (40 mg/L at 23 weeks) was allowed to go to term and a spina bifida resulted. In these

FIGURE 7-8

UREA IN 32 AMNIOTIC FLUIDS

PLOTTED AGAINST GESTATIONAL AGE

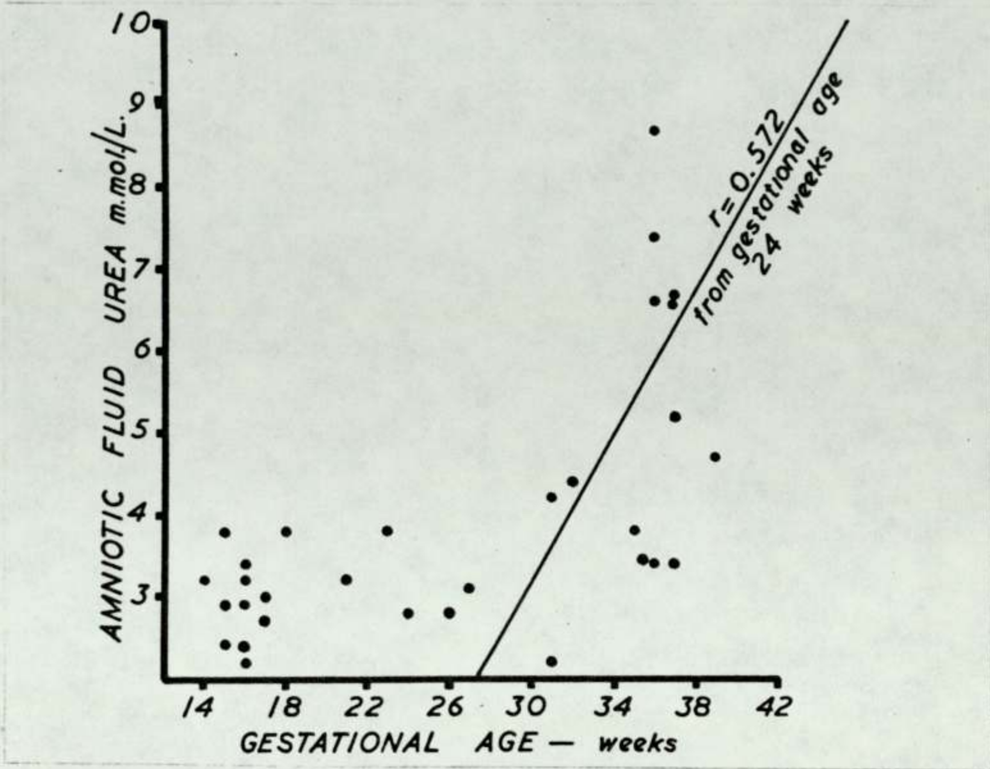


FIGURE 7-9

UREA IN 32 AMNIOTIC FLUIDS

PLOTTED AGAINST CRITHIDIA FACTOR

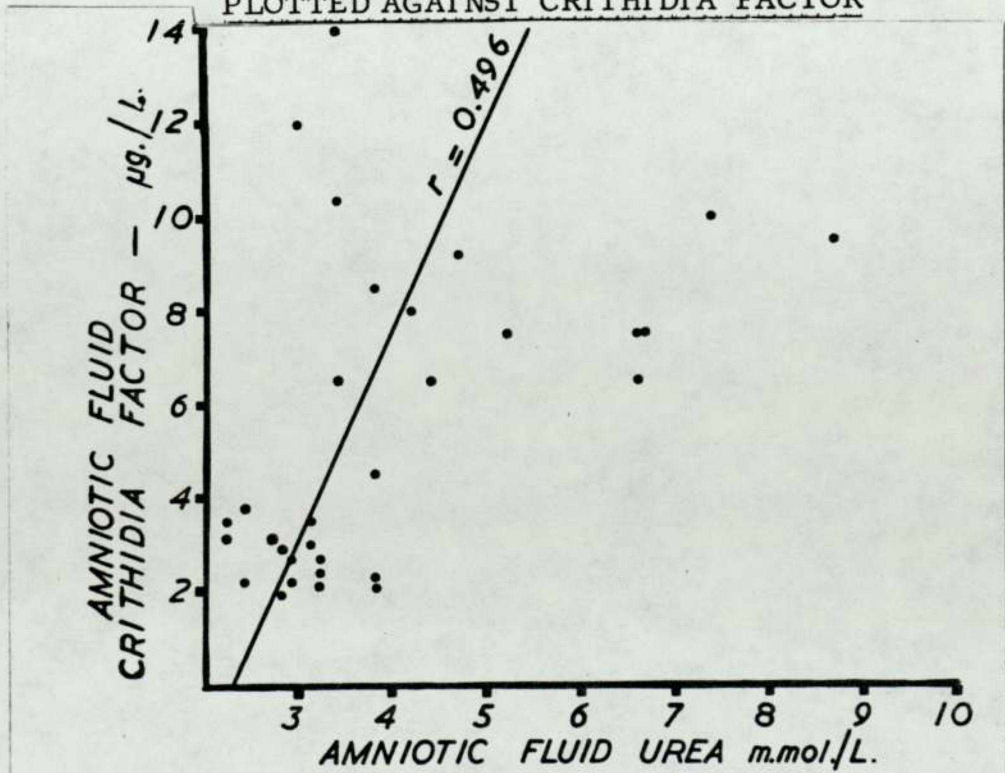


TABLE 7-2

ALPHA FETO PROTEIN - NORMAL RANGES

11 - 16 weeks	0	-	50 mg/litre
17 weeks	0	-	42 mg/litre
18 weeks	0	-	36 mg/litre
19 weeks	0	-	30 mg/litre
20 weeks	0	-	24 mg/litre
21 weeks	0	-	20 mg/litre
22 weeks	0	-	16 mg/litre
23 weeks	0	-	10 mg/litre
24 weeks	0	-	8 mg/litre
28 weeks	0	-	6 mg/litre
34 weeks	0	-	2 mg/litre

three cases the biopterin derivative levels were not raised. Only one material was detected in amniotic fluid by chromatography this material co-chromatographed with 7,8-dihydrobiopterin.

Cord blood samples from seventeen babies at term showed Crithidia factor values ranging from 1.1μg/L to 7.2μg/L mean 2.46 ± 0.37μg/L. Two of the specimens had raised levels (5.4μg/L and 7.2μg/L) but no reason could be found for this. Chromatography of cord blood at term showed a major material which chromatographed with 7,8-dihydrobiopterin and a minor spot which chromatographed with biopterin. Vitamin B_{12} levels ranged from 100 to 800 ng/L three of these were between 100 and 150 ng/L.

DISCUSSION

Mean foetal brain and liver concentration of Crithidia factor were similar to adult post-mortem values (Baker et al, 1974; Leeming, Blair, Melikian and O'Gorman, 1976) and adult liver biopsy specimens (Chapter VI) Thus biosynthesis of cofactor, shown here from twelve weeks gestation, precedes tyrosine hydroxylation. Tyrosine hydroxylase is not produced even at week 33 (Bessman et al 1977). In the rat, tyrosine hydroxylase at birth is only 10% of the adult level and rises to 75% of adult levels by the fourth week (Coyle and Compachiaro, 1976). 5,6,7,8-tetrahydrobiopterin is the rate-determining factor in tyrosine hydroxylation in adult rat brain (Kettler, Bartholini and Pletscher, 1974) but tyrosine hydroxylase must be the determinant in the foetus and neonate.

The fact that Crithidia factor concentration appears to decrease with gestational age (Figure 7-2) may be due to select site(s) of production maturing at an early stage. The pineal gland is particularly rich in biopterin (Have-Kirchberg, Moree, van Laar et al, 1977; Chapter VI) but it is unlikely as sole candidate because of its small size. The total brain Crithidia factor increases ($r = 0.524$) with gestational age which just reaches the 5% level of significance; this would fit in still with the maturation of areas of brain particularly active in Crithidia factor synthesis.

The higher serum phenylalanine level in cord blood of the aborted foetuses was higher than in normal controls but not significantly so, $p = > 0.05$. However the ratio 1: 1.7 was identical to that found by others (Butterfield and O'Brien, 1963) and a persistently high transplacental gradient may explain the high Crithidia factor levels in the cord blood of aborted foetuses, which were much higher than in cord blood at term ($p = < 0.01$), the normal adult (Chapter VI) or children (Leeming, 1975). The few vitamin B_{12} levels on foetal blood (760 $\pm$ 291 ng/L) were higher than in cord blood at term (298 $\pm$ 73 ng/L) but not at a significant level ($p = > 0.05$).

Amniotic fluids until 30 weeks gestation showed levels of Crithidia factor not far removed from normal adult serum levels (Chapter VI). One of the highest values (14.0µg/L) was in a patient with hypertensive renal disease, it would have been convenient to afford an explanation on grounds of the patient's condition (Leeming, Blair, Melikian and O'Gorman, 1976) but the amniotic fluid urea was only 3.4 m.mol/L. However there was a positive correlation between urea and Crithidia factor which on examination of the data (Figures 7-6, 7-7 and 7-8) was caused by a rise in both parameters from around week 24. As the kidneys would have begun to function (Holton, 1977) and the amniotic fluid is a repository for waste material, the association has its probable explanation in excretion; Leeming and Blair (1974) reported high levels of Crithidia factor in urine.

One patient had a high amniotic Crithidia factor level and had previously produced a spina bifida child, it was considered that the high biopterin derivative level may have been associated with a lesion leaking cofactor into the amniotic fluid. However, the alpha feto-protein was not raised and the pregnancy was allowed to go to term producing a normal child. Other amniotic fluids with high alpha feto-proteins and subsequently shown to be bathing spina bifida and anencephalic foetuses did not have raised Crithidia factor levels.

CHAPTER VIII

MALIGNANT HYPERPHENLALANINAEMIA

INTRODUCTION

Phenylketonuria was first described in severely retarded children over 40 years ago by Fölling (1934). He screened the urines of institutionalised, retarded children for phenylpyruvic acid with ferric chloride and found other subjects with similar clinical courses, phenylpyruvic acid in their urines and defective phenylalanine metabolism. Jervis (1947) showed greatly reduced activity inthe enzyme phenylalanine hydroxylase which converts phenylalanine to tyrosine. The Guthrie test (Guthrie and Susi, 1963) for the detection of elevated serum phenylalanine levels made screening programmes easier and was more reliable than the ferric chloride test for urinary phenylpyruvic acid which was not very sensitive to small changes in phenylalanine and missed patients who were screened too early (Armstrong and Low, 1957).

When it was reported that phenylalanine restriction, started in infancy, prevented mental retardation and improved the behavioural control of children with phenylketonuria (Bickel, Gerard and Hickman, 1953) screening and dietary control rapidly became standard procedures in the western world. The precise cause of brain damage in phenylketonuria is not known and there is no clear evidence of a linear relationship between phenylalanine intake and brain damage. (B.M.J., 1971). In broad terms, phenylketonuria is characterised clinically by mental retardation and convulsive seizures; biochemical diagnosis is by measurement of increased plasma phenylalanine and decreased phenylalanine clearance after loads in appropriate cases. Incidence is around 1; 20,000 to 1; 25,000 in a mixed European population (Knox,1966). The natural history of phenylketonuria is now largely obscured by the effect of early dietary treatment (Smith,1971) but has been summarised in Table 8-1 (Blastovics and Nelson,

TABLE 8-1

COURSE OF UNTREATED PHENYLKETONURIA

Clinical	Laboratory
Birth Physical: Normal Neurological: Normal Development: Normal	Serum: ± elevated serum Phe. normal serum Tyr. Urine: ± elevated Phe. metabolites E.E.C.: normal
15 days to 3 months Physical: Eczema may be present, musty or vinegary odour of PAC. Neurological: normal to variable increased irritability or lethargy, seizures ± Development: normal to questionable delay in motor milestones	Serum: Phe. > 1.5 m.mol/L. Tyr. - normal or decreased. Urine: elevated Phe. PPYA, O-HPAA + $FeCl_3$ + DNPH. E.E.C: may be abnormal, i.e: hypsarrhythmia, petit mal variant, multifocal spiking.
3 months to 6 months Physical: Eczema may be present, 30% microcephaly. Neurological: 50% with seizures, increased irritability or lethargy. Development: delays usually present and becoming more obvious.	Serum: Phe. 1.8 - 6.1 m.mol/L Tyr. normal or decreased. Urine: elevated Phe. PPYA, O-HPAA + $FeCl_3$, + DNPH. E.E.C.: 90-95% with abnormal pattern, hypsarrhythmia, petit mal variant, multifocal spiking.
6 months to 1 year Physical: Eczema frequently present, odour present, 30% mild microcephaly Neurological: 50% with seizures, increased irritability, rarely apathy and lethargy Development: obviously delayed motor milestones.	Serum: Phe. 1.8 - 6.1 m.mol/L Tyr. normal or decreased. Urine: elevated Phe. PPYA, O-HPAA + $FeCl_3$, + DNPH E.E.C: 90-95% with abnormal pattern, hypsarrythmia, petit mal variant, slow waves, multifocal spiking.
1 year to 3 years Physical: Eczema may be present, odour present, skin, hair and iris may be lighter than family, 30% mild to moderate microcephaly. Neurological: 30-50% with seizures, increased tremours and irritability. Development: moderate to severe M.R., may have destructive and autistic behaviour.	Serum: Phe. 1.8 - 3.0 m.mol/L Tyr. normal or decreased. Urine: elevated Phe. PPYA, O-HPAA + $FeCl_3$, + DNPH. E.E.C: 90% moderately to severely abnormal hypsarrhythmia, petit mal variant, slow waves, multifocal spiking.

TABLE 8-1

(Continued)

3 years to 6 years	
Physical: Eczema uncommon, odour present, skin, hair and iris lighter than family, 30% mild to moderate microcephaly. Neurological: 30-50% with seizures increased tremours, irritability. Development: moderate to severe M.R., may have destructive and autistic behaviour.	Serum: Phe. usually 1.2 m.mol/L Tyr. normal Urine: elevated Phe.PPYA,O-HPAA + $FeCl_3$ + DNPH. E.E.C: 30-50% abnormal, petit mal variant, slow waves, multifocal spiking.
6 years to adult	
Physical: Eczema uncommon, odour present, skin, hair and iris lighter than family, 30% mild to moderate microcephaly. Neurological: 10-20% with seizures increased tremours, irritability. Development: moderate to severe M.R., may have destructive and autistic behaviour.	Serum: Phe. usually 1.2m.mol/L Tyr. normal Urine: elevated Phe.PPYA,O-HPAA + $FeCl_3$ + DNPH. E.E.C: 10-20% abnormal, petit mal variant, multifocal spiking.

Phe. = phenylalanine. Tyr. = tyrosine. PPYA = phenylpyruvic acid. O-HPAA = orthohydroxyphenylacetic acid. PAC = phenylacetic acid. FeCls = ferric chloride. M.R. = mental retardation.

1971), patients normally survive into adult life.

Among patients diagnosed as phenylketonurics there is a small proportion (about 1%) who have malignant hyperphenylalaninaemia (Danks, Bartholome, Clayton et al, 1978). These infants are externally similar to 'classical' phenylketonurics but, if treated similarly, neurological symptoms appear within the first few weeks and develop. In malignant hyperphenylalaninaemia the plasma phenylalanine level is easily controlled but abnormal limb movements and axial hypotonia precede a generalised hypotonia becoming profound by the age of around one year. Convulsions are often seen but are not a consistent feature. If untreated, death results in infancy from recurrent respiratory infection exacerbated by

by hypersalivation, difficulty in swallowing and inhalation of food (Smith, Clayton and Wolff, 1975).

This metabolic disorder has been reported in England (Smith, Clayton and Wolff, 1975), Western Germany (Bartholome, 1974), America (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975), Australia (Danks, Cotton and Schlesinger, 1975), France (Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977) and Austria (Leeming and Schreibenreiter, 1978). Patients of British, German, French, Italian, Maltese, Austrian, Palestinian Arab, Portugese and Chinese origin are known.

There are two known variants of malignant hyperphenylalaninaemia, in one there is defective synthesis of tetrahydrobiopterin (Leeming, Blair and Rey, 1976: Rey, Blandin-Savoja and Rey, 1976; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977; Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel, 1978) and in the other, dihydropteridine reductase deficiency (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977).

Consanguinity has been present in approximately one third of the families affected and afflicted siblings have been observed in half the families with dihydropteridine reductase deficiency. One of the females with defective tetrahydrobiopterin synthesis had an unaffected sister so X-linked inheritence can be ruled out. Autosomal recessive inheritance of both forms of the disease seems most probable.

Progressive neurological deterioration may be halted by administration of L-dopa, carbidopa (to prevent extra-cerebral decarboxylation) and 5-hydroxytryptophan (Bartholome and Byrd, 1975). The dramatic effects of therapy, even when administered after several months' delay are well illustrated in Figures 8-1 and 8-2.

Gestational progress, parturition and birth weight in these

children is unremarkable. The untreated clinical progress is inexorable and similar; a brief typical history of one child untreated for three years follows this introduction. The degree of permanent impairment of brain function is related to the length of time before control of neurological symptoms is achieved by neurotransmitter replacement therapy (Danks, Bartholome, Clayton et al, 1978).

FIGURE 8-1

MALIGNANT HYPERPHENYLALANINAEMIC CHILD
(M.K.) AT 12 MONTHS, BEFORE TREATMENT

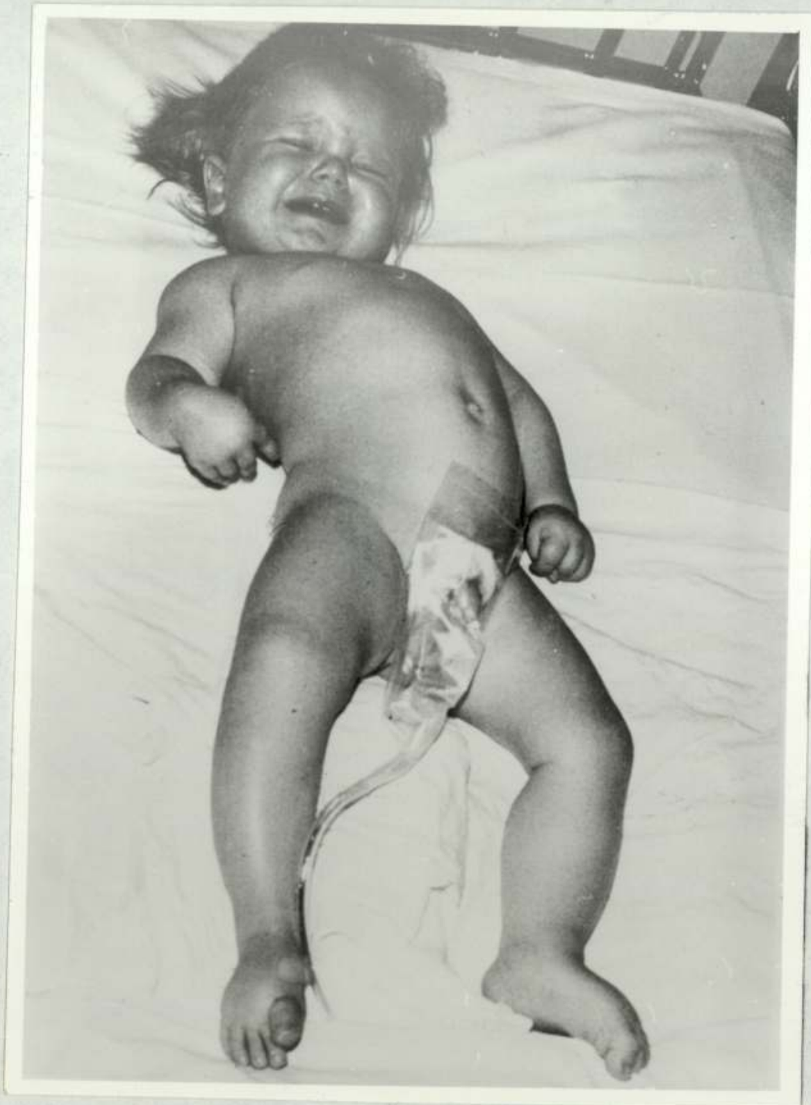


FIGURE 8-2

MALIGNANT HYPERPHENYLALANINAEMIC CHILD (M.K.)
AT 27 MONTHS ON TREATMENT WITH L-DOPA,
CARBIDOPA, AND 5-HYDROXYTRYPTOPHAN FOR 14 MONTHS

CASE HISTORY (C.A.)

Female, first child of unrelated parents, birth weight 3100 g. At seven days the plasma phenylalanine was 1.82 m.mol/L. and at thirteen days 2.97 m.mol/L., tyrosine 0.088 m.mol/L. The hyperphenylalaninaemia was controlled from fourteen days with a low phenylalanine diet, after forty-eight hours on diet the plasma phenylalanine fell to 2.0 m.mol/L., after five days 1.1 m.mol/L. and after eight days 0.5 m.mol/L. Thereafter plasma phenylalanine was perfectly under control at between 0.1 and 0.3 m.mol/L.

During the first two months she responded poorly to stimuli. At three months axial hypotonia with muscular hypotonia of the limbs with brisk tendon reflexes was present. At six months spontaneous motility was much reduced, hypotonia of the trunk had increased and abnormal movements of the limbs appeared; "pedalage" of the legs, athetotic arm movements, myoclonia, arythmic and swift movements of the extremities. Frequent yawning, hyper-sialorrhoea and difficulty in swallowing were associated with recurrent respiratory infection. At ten months, febrile (42°C) bronchopneumonia produced grand mal. The abnormal movements disappeared around one year of age. At two and a half years of age there was complete and gross hypotonia and passivity; the child was unresponsive with limbs completely relaxed and no tendon reflexes to be found. Hypotonia affected the abdomen and respiratory muscles. The electroencephalogram was diffusely abnormal but there was no evidence of peripheral nerve damage. Growth was normal.

At the age of three years four months treatment was commenced - L-dopa (10mg/Kg body weight), carbidopa (1mg/Kg.) and 5-hydroxytryptophan (20mg/Kg.). Simple although unco-ordinated voluntary movements appeared, the L-dopa was increased to 16 mg/Kg. and the 5-hydroxytryptophan to 20mg/Kg. She is still alive, severely retarded with a few spontaneous movements and slight interest in her environment.

MATERIALS AND METHODS

Serum and urine specimens from five children suspected on clinical grounds as suffering from malignant hyperphenylalaninaemia were sent to the Haematology Department of the General Hospital, Birmingham, England for biopterin studies. These children were in the care of paediatricians in specialised units in different parts of Europe to which some had been referred from other hospitals. The patients and referring paediatrician are identified as follows: - M.K. (Male) Dr. K. Bartholome, Universität Kinderklinik, Heidelberg. A.M. (Male) Dr. S. Scheibenreiter, Universität Kinderklinik, Wien, Austria. C.A. (Female), D.P. (Male) and Y.T. (Female) Dr. F. Rey, Hopital des Enfants Malades, Paris.

One (A.M.) had an oral load of phenylalanine at 100mg/Kg. body weight and three of the others (C.A., D.P. and Y.T.) had phenylalanine by intravenous infusion. A number of random samples were also obtained, phenylalanine levels were available on some samples but not on all of them. The ways in which sampling was carried out, phenylalanine loading was performed and treatment was given differed considerably between centres. The availability of information was not uniform. Nevertheless, their discrete identity was made plain from characteristic neurological signs and subsequently confirmed by their distinctive biopterin metabolism. One (D.P.) was diagnosed as dihydropteridine reductase deficient by enzyme studies (Craine, Hall and Kaufman, 1972). One child (M.K.) had a Crithidia assay carried out on liver taken by needle biopsy and this was compared with a control taken, stored and transported under identical conditions. The parents of C.A. had an oral phenylalanine load.

Serum and urine were chromatographed in three solvent systems - 3% ammonium chloride, 5% acetic acid and 0.5% sodium carbonate. Some of the urines were chromatographed in 3% ammonium chloride, dried, turned through 90° and run again in propanol/1% ammonia (2:1). Propanol /ammonia will distinguish between biopterin and neopterin but not between 7,8-dihydrobiopterin and biopterin, therefore prior separation of

these latter two is necessary. Liver homogenate from M.K. was chromatographed in 3% ammonium chloride.

RESULTS

The five patients can be divided into two groups, those with low serum Crithidia factor levels (C.A., M.K., and Y.T.) and those with high levels (A.M. and D.P.) (Table 8-2). The low levels in two (C.A. and Y.T.) did not increase to any great extent when phenylalanine was infused intravenously although the phenylalanine levels rose in both cases (Figures 8-3 and 8-4) to heights which would have produced significant Crithidia factor increases in normal adults and in classical phenylketonuria. A phenylalanine load was not carried out on M.K.

FIGURE 8-3

SERUM CRITHIDIA FACTOR AND PLASMA PHENYLALANINE IN MALIGNANT HYPERPHENYLALANINAEMIA FOLLOWING INTRAVENOUS PHENYLALANINE PATIENT C.A.

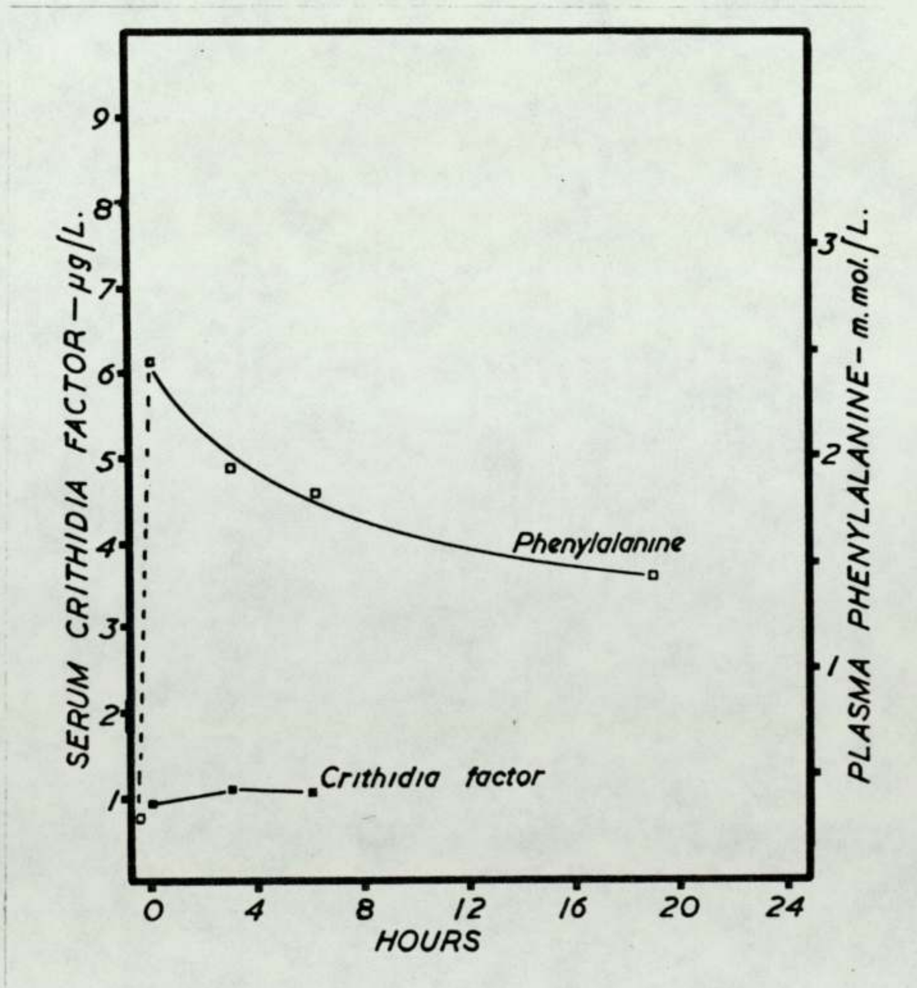
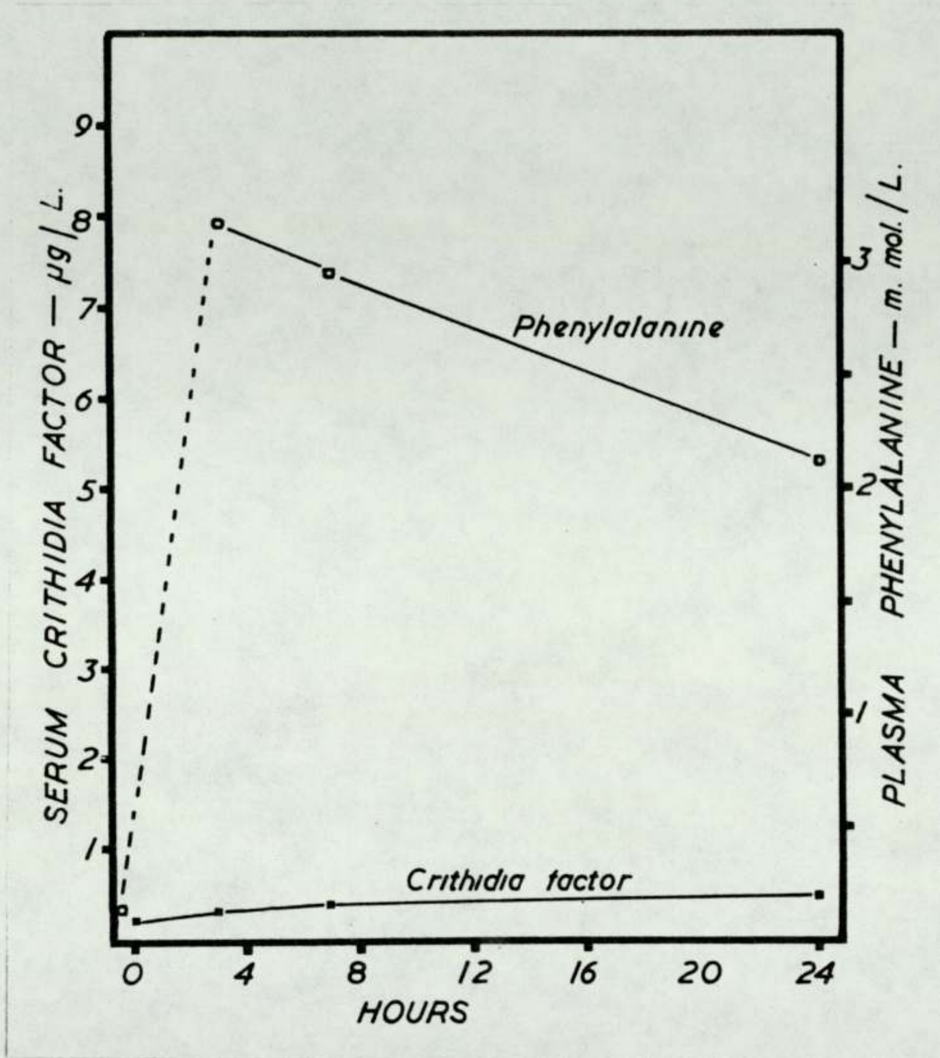


FIGURE 8-4

SERUM CRITHIDIA FACTOR AND PLASMA PHENYLALANINE IN MALIGNANT HYPERPHENYLALANINAEMA FOLLOWING INTRAVENOUS PHENYLALANINE PATIENT Y.T.

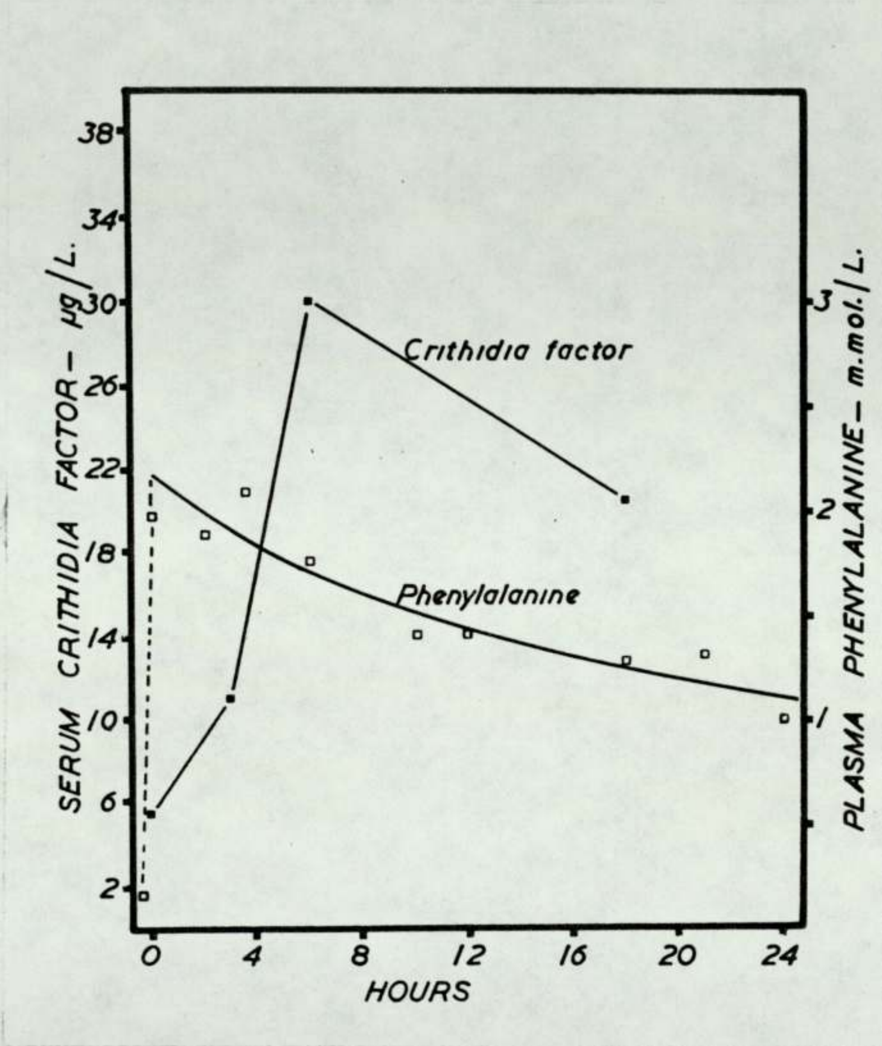


D.P. (Figure 8-5) and A.M. (Figure 8-6) both showed serum Crithidia factor increases higher than had been stimulated by phenylalanine in any other patient or volunteer. D.P. did not have any measurable dihydropteridine reductase activity, A.M. had a very high serum Crithidia factor level when the plasma phenylalanine level was not unduly elevated (Figure 8-6, Table 8-2).

Random samples of serum are shown in Table 8-2. The two patients (C.A. and M.K.) from whom two samples were obtained separated

FIGURE 8-5

SERUM CRITHIDIA FACTOR AND PLASMA PHENYLALANINE IN MALIGNANT HYPERPHENYLALANINAEMIA FOLLOWING INTRAVENOUS PHENYLALANINE PATIENT D.P.

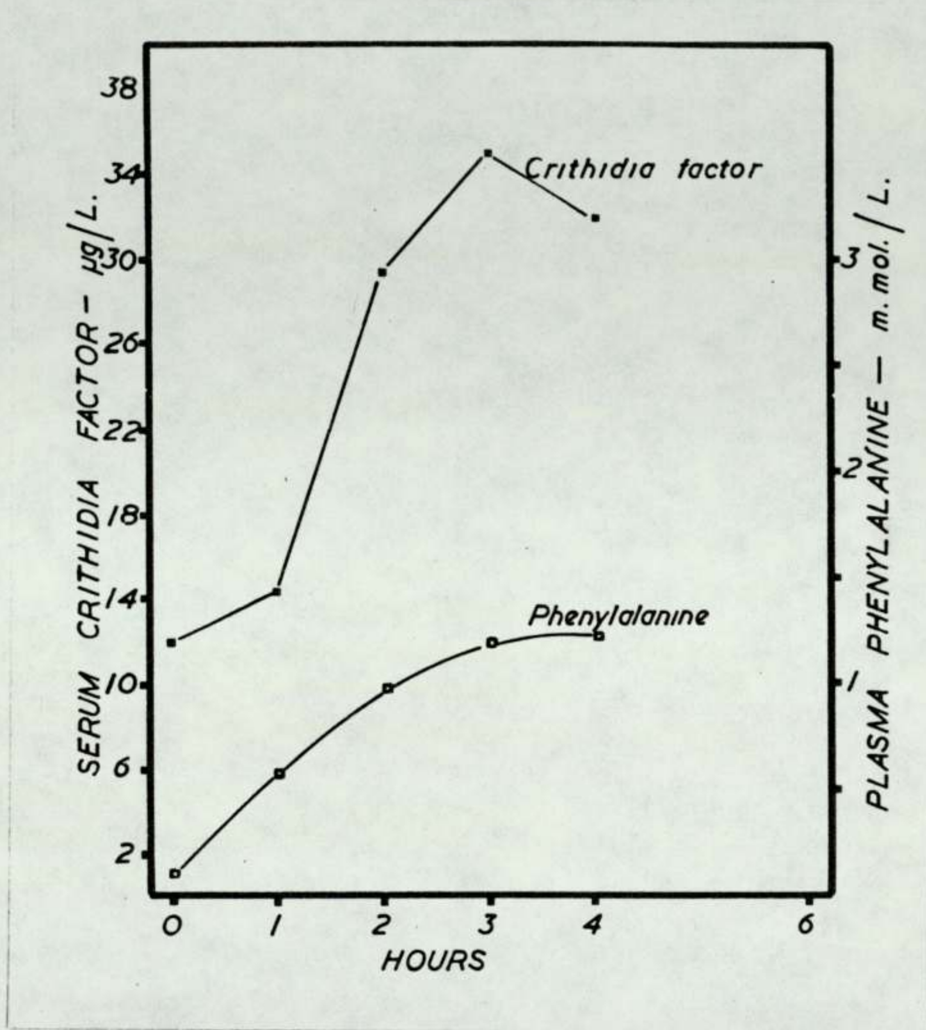


by approximately a year produced a slight rise with time. The liver value (M.K.) was lower at 16.7 ng/g than any obtained in previous samples. In a series of thirteen adult liver biopsies the range was from 105 ng/g to 862 ng/g (mean and s.e.m. 347 ± 63). The urinary excretion of Crithidia factor (Table 8-3) again demonstrated the division into two groups but there was a greater degree of variation and some overlap which was not apparent with sera. Urinary output, to some extent, depends on urinary flow (Leeming, 1975).

Chromatography of urine from C.A. showed two moieties which

FIGURE 8-6

SERUM CRITHIDIA FACTOR AND PLASMA PHENYLALANINE IN MALIGNANT HYPERPHENYLALANINAEMIA FOLLOWING ORAL PHENYLALANINE PATIENT A.M.



chromatographed with biopterin and 7,8-dihydrobiopterin respectively. When compared with normal urine the lower total concentration could be ascribed to loss of 7,8-dihydrobiopterin. The serum of C.A. showed only biopterin in contrast to normal serum where the major material is 7,8-dihydrobiopterin and there is only a small amount of biopterin at about the same level as found in C.A. Serum samples from M.K. and Y.T. were similar and the liver from M.K. showed only the compound which was chromatographically identical with biopterin.

Chromatography of sera from D.P. and A.M. showed large

TABLE 8-2

PHENYLALANINE HYDROXYLASE, DIHYDROPTERIDINE REDUCTASE, CRITHIDIA FACTOR AND PHENYLALANINE LEVELS IN MALIGNANT HYPERPHENYLALANINAEMIA COMPARED WITH CONTROLS AND CLASSICAL PHENYLKETONURIA IN FASTING AND PHENYLALANINE LOADED SUBJECTS AFTER 3 HOURS

			Liver			Serum Before Phenylalanine		Serum After Phenylalanine	
Patients	Age	Sex	Phenylalanine Hydroxylase*	Dihydropteridine Reductase**	Crithidia Factor	Phenylalanine ****	Crithidia Factor***	Phenylalanine ****	Crithidia Factor***
A.M.	11mths	M	79.0	Not detected	-	0.12	12.0	1.21	35.0
C.A.	2yrs	F	-	-	-	0.10	0.3	-	-
	3yrs		49.1	61.2	-	0.29	0.8	1.98	1.1
D.P.	3mths	M	61.2	Not detected	- -	0.07	5.5	1.99	11.0
Y.T.	2mths	F	51.0	76.0	-	0.18	0.2	2.91	0.3
M.K.	1yr	M	74.9	95.1	16.7ng/g (control 550)	-	0.2	-	-
	2yrs		-	-		0.07	0.7	-	-
*****11 Normal children 6-12 months:			37.9 -105	60.3-141	-	-	-	-	-
******10 Normal children 1 mth - 3yrs:			-	-	-	0.03-0.09	1.1-3.7	-	-
*******30PKUchildren 6mths - 16yrs: (on diet)			-	-	-	0.13-2.36	1.3-13.3	-	-
*******5 Normal adults			-	-	-	0.04 -0.07	1.5-2.1	0.26-0.49	5.5-7.5

*μ.mol. tyrosine/hr/g protein ** μ.mol NADH oxidised/min/g protein ***μg/L **** m.mol/L

*****(Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977) ******(Leeming, Blair, Green and Raine, 1976)*******(Leeming, 1975)

TABLE 8-3

URINARY CRITHIDIA FACTOR LEVELS IN FIVE CASES OF MALIGNANT HYPERPHENYLALANINAEMIA BEFORE AND AFTER PHENYLALANINE ADMINISTRATION

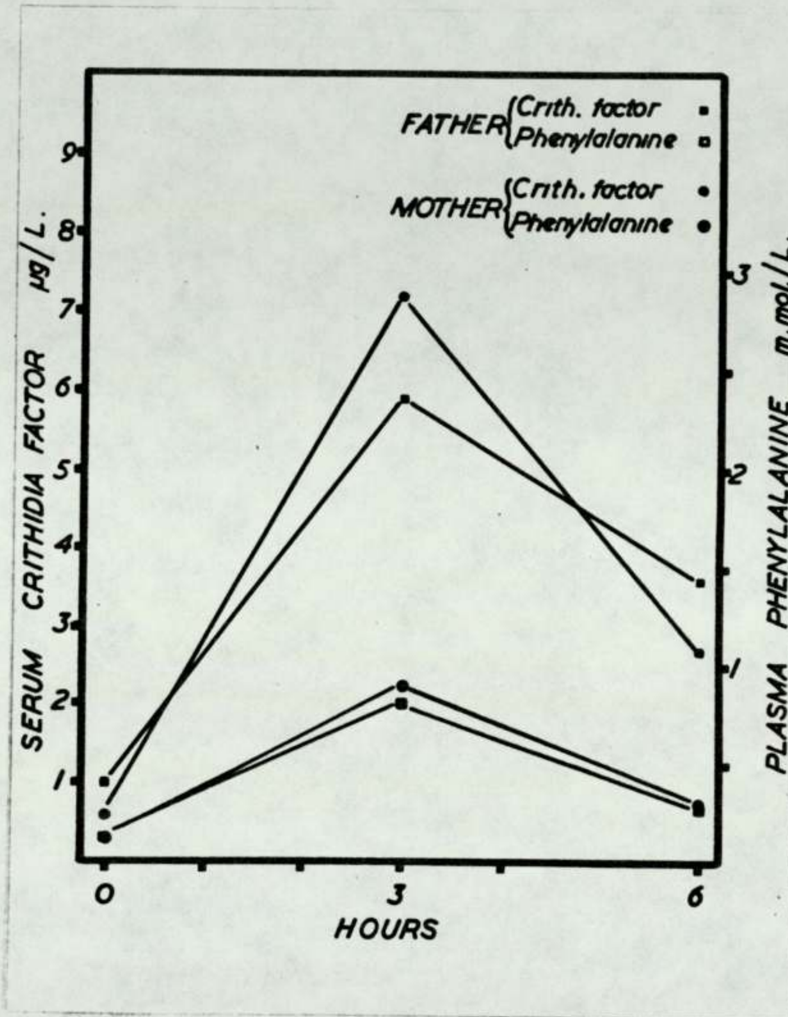
Patient	(Age)	Period of Collection	Crithidia factor Concentration mg/L	Crithidia factor total for period µg
C.A.	(2yrs)	unknown	0.175	-
	(3yrs)	24 hrs	0.76	43.3
		24 hrs	1.0	18.0
		24 hrs	0.85	38.3
		2 hrs pre-load	0.83	24.1
		3-8½ hrs post infusion	0.88	30.8
		8½-32½ " "	0.37	62.9
		32½-56½ " "	0.39	54.6
Y.T.	(2mths)	0-3hrs post infusion	0.16	3.52
		3-7 " "	0.155	2.48
		7-24 " "	0.065	3.58
M.K.	(2yrs)	6 hrs	0.09	5.4
D.P.	(3mths)	24 hrs	1.43	443
		24 hrs	0.27	124
		0-3hrs post infusion	2.75	-
		3-9 " "	0.95	46
		9-21 " "	0.83	208
		21-27 "	1.43	171
		27-48 "	1.50	345
		48-57 "	1.38	83
A.M.	(11mths)	24hrs pre oral load	7.5	1200
		24hrs post oral load	18.75	3375
Controls (5)	(0-2yrs)	Random	1.9-2.8	-

amounts of both 7,8-dihydrobiopterin and biopterin, both materials increased in quantity following phenylalanine loads. In both these cases the principal component was 7,8-dihydrobiopterin. Urinary Crithidia factor in these two again chromatographed with 7,8-dihydrobiopterin (the major component) and biopterin. Chromatograms which had been run in 3% ammonium chloride were exposed to pure oxygen in a moist chamber for two hours at room temperature and re-run at 90° in propanol/1% ammonia (2:1) or in 3% ammonium chloride. The 7,8-dihydrobiopterin and biopterin ran together after oxidation and could be distinguished from authentic L-neopterin, thus giving further evidence of the identity of 7,8-dihydrobiopterin by its oxidation to biopterin rather than to neopterin.

The parents of C.A. had oral phenylalanine loads of 100mg/Kg body weight, the results (Figure 8-7) show low initial Crithidia factor values of 0.6μg/L and 1.0μg/L. Handling of phenylalanine was normal.

FIGURE 8-7

SERUM CRITHIDIA FACTOR AND PLASMA PHENYLALANINE
IN THE PARENTS OF C.A.
FOLLOWING ORAL PHENYLALANINE



DISCUSSION

Initially, malignant hyperphenylalaninaemia is clinically indistinguishable from classical phenylketonuria and further distinction is not forthcoming from the standard Guthrie screening test (Guthrie and Susi, 1963). However, liver biopsy, when it is carried out, reveals that phenylalanine hydroxylase is active and that in some cases dihydropteridine reductase (Craine, Hall and Kaufman, 1972) is decreased (Table 8-2). Rey, Harpey, Leeming et al, (1977) listed seven published cases where phenylalanine hydroxylase has been measured, six had normal levels and one had an activity 25% of normal.

Measurement of Crithidia factor in the five cases cited in this chapter differentiated malignant hyperphenylalaninaemics into two groups. The first group (A.M. and D.P.) had high serum Crithidia factor even when the plasma phenylalanine was controlled at a low level, phenylalanine loading increased the serum Crithidia factor further (Tables 8-2 and 8-3). D.P. had low dihydropteridine reductase. In the second group (C.A., M.K., and Y.T.), dihydropteridine reductase was normal, the serum Crithidia factor low and phenylalanine loading only produced a minimal rise in serum Crithidia factor (Tables 8-2 and 8-3).

Increase in serum Crithidia factor may arise from administration of a known dihydropteridine reductase inihibitor such as methotrexate (Chapter XI ; Leeming, Blair, Melikian and O'Gorman, 1976). Inherited deficiency of dihydropteridine reductase would therefore be demonstrated by a much increased serum Crithidia factor relative to phenylalanine level as in A.M. and D.P. Patients with dihydropteridine reductase deficiency would have to have residual dihydropteridine reductase for this to be the mechanism which produces a further increase in serum Crithidia factor following phenylalanine as with A.M. and D.P. There is evidence that phenylpyruvic acid is a competitive inhibitor of dihydropteridine reductase (Chapter IV). There has been a suggestion that phenylalanine itself inhibited dihydropteridine reductase in fibroblast cultures of phenylketonuric cell lines (Schlesinger, Cotton and Danks, 1976) but this was not substan tiated

(Guttler, Kaufman and Milstien, 1977; Firgaira, Schlesinger, Cotton and Danks, 1978).

The results of phenylalanine loading in other types of hyperphenylalaninaemia will follow (Chapter IX), it is only with malignant hyperphenylalaninaemia in which there is deficient synthesis of tetrahydrobiopterin that there is clear identification before dietary stabilisation of plasma phenylalanine levels. This identification is achieved by demonstration of low serum Crithidia factor even when the phenylalanine is raised. The poor response of serum Crithidia factor to phenylalanine in such cases has been confirmed (Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel, 1978). Serum and liver only had demonstrable biopterin in these cases and a synthesis proceeding to biopterin and not to dihydrobiopterin and tetrahydrobiopterin would be unaffected by interference with dihydropteridine reductase if this is the mechanism in phenylalanine loading. One case has been reported (Niederwieser, Curtius, Bettoni, Bieri, Schircks, Viscontini and Schaub, 1979) in which biopterin was not demonstrated but 10-20 mg neopterin/litre of urine was found. The techniques used were high voltage electrophoresis, chromatography and identification by fluorescence in ultra-violet light. The amount of biopterin demonstrated here would be too small for these methods to detect and Crithidia fasciculata is relatively insensitive to D-neopterin although L-neopterin would be detectable (Leeming and Blair, 1974).

The early laboratory diagnosis of these atypical cases of phenylketonuria, as opposed to clinical differentiation from classical phenylketonuria when irreversible neurological damage has taken place, is of obvious importance. If liver biopsies were to be taken from all cases of phenylketonuria then phenylalanine hydroxylase would be normal in both forms of malignant hyperphenylalaninaemia, unless the unfortunate infant had more than one defect in phenylalanine metabolism, and dihydropteridine reductase would be depressed in one form of malignant hyperphenylalaninaemia but not in the other. However, the procedure is not without its own dangers

and the incidence of malignant hyperphenylalaninaemia is perhaps 1% of all neonatal hyperphenylalaninaemias. The technique would probably be criticised as too invasive as the risk would be greater than the diagnostic gain.

Measurement of Crithidia factor levels alone in serum and urine (Leeming, Blair and Rey, 1976; Kaufman, Berlow, Summer, Milstien et al, 1978) can no longer be regarded as a guide to prognosis, in cases with low levels (e.g. C.A., M.K., and Y.T.) with absolute certainty in view of the child (A.L.) to be described in the following chapter, but it is a valuable aid to diagnosis if only by elimination. A.L.'s development was relatively normal but his serum and urine Crithidia factor levels were low even following oral phenylalanine although there was a rise in serum Crithidia factor greater in relationship to the fasting level than those of the two children with defective synthesis described here (C.A. and Y.T.). A.L. (following chapter) showed a small amount of 7,8-dihydrobiopterin in his serum whereas C.A., M.K., and Y.T. showed only biopterin. Therefore A.L. may have had reduced synthesis of dihydrobiopterin but sufficient to function normally under normal conditions whereas C.A., M.K., and Y.T. synthesised the pterin ring but failed to produce 7,8-dihydrobiopterin.

When serum Crithidia factor is high before diet is started, Crithidia factor will be high in patients with dihydropteridine reductase deficiency but low in patients with defective synthesis. If serum Crithidia factor is measured when dietary control has been established, then the serum Crithidia factor will still be high in patients with dihydropteridine reductase deficiency. Intravenous administration of 5,6,7,8-tetrahydrobiopterin would normalise the serum phenylalanine in malignant hyperphenylalaninaemia (Danks, Cotton and Schlesinger, 1975; Schaub, Däumling, Curtius, Niederwieser et al, 1978) which might aid diagnosis and the peripheral biochemistry but not the synthesis of neuro transmitters (Kettler, Bartholini and Pletscher, 1974). These cases are rare and represent perhaps 1/2000,000 live births. They offer an insight

into the effects of materials which interfere with biopterin metabolism (Chapters II to IV) either at the point of synthesis of the biopterin ring or dihydropteridine reductase. They may ultimately be shown to have features in common with patients treated for long periods with methotrexate or other dihydropteridine reductase inhibitors (Meadows and Evans, 1976; Eiser, 1978). A number of pharmaceutical preparations and other compounds have been shown to have effects on synthesis and dihydropteridine reductase (Chapters II to IV).

CHAPTER IX

PHENYLKETONURIA

INTRODUCTION

From the first description of phenylketonuria (Fölling, 1934) the disease has been treated as one in which the apoenzyme, phenylalanine hydroxylase, is defective (Jervis, 1947). The majority of cases have classical phenylketonuria in which there is virtually no active phenylalanine hydroxylase. Apart from malignant hyperphenylalaninaemia in which there is a deficiency of tetrahydrobiopterin (Chapter VIII) and first investigated quite recently (Bartholome, 1974), a number of intermediate states exist which provide problems of diagnosis, treatment and counselling (Smith and Francis, 1975).

There are atypical phenylketonurics of normal intelligence and without history of epilepsy who are able to convert phenylalanine to tyrosine at a rate higher than in classical phenylketonuria though much lower than normal individuals (Cowie, 1951; Woolf, 1963). A form of phenylalanine hydroxylase has been suggested which loses its activity at high concentrations of phenylalanine (Woolf, Cranston and Goodwin, 1967). To add to this grey area, transient hyperphenylalaninaemia (Scriver, 1967) in the post-natal period may resolve itself during the first months of life. It is valuable to have a firm diagnosis as soon as possible after birth so that dietary restriction of phenylalanine (Bickel, Gerrard and Hickmans, 1953) can be considered. Error in diagnosis and therefore treatment may cause brain damage.

Throughout pregnancy plasma amino acid levels in the foetus are higher than those of the mother with cord blood phenylalanine reaching 1.5 to 3 times the concentration in the mother's blood (Butterfield and

O'Brien, 1963). Immediately after birth, in normal children, they fall only to rise again when milk is given, peaking on the sixth day and returning to levels similar to adult levels by day fourteen (Hambraeus and Wranne, 1968). If infants are fed on undiluted cows' milk then plasma amino acids are often higher than in children fed on human milk which contains four or five times less of these amino acids (Smith and Francis, 1976). Tyrosine and phenylalanine are the amino acids most commonly found to be raised (Clow, Scriver and Davies, 1969).

During the first two years of life, the human brain is in a very active growth phase (Dobbing and Sands, 1973). Anderson, Rowe and Guroff (1971) treated rats below the age of twenty-one days with subcutaneous doses of phenylalanine at 0.33 mg/g. body weight and demonstrated that their brains did not show the same weight gain as controls, 76% at day ten and 86% at day twenty. Moreover rats which reached adulthood gave poor problem solving performances even long after cessation of hyperphenylalaninaemia. Pensky, Fishman and Daftar (1974), gave 4mg/g. per day of phenylalanine subcutaneously to rats between the eighth and twelfth days of life and showed permanent impairment of cellular replication in the cerebellum. Dietary phenylalanine is more effective in producing experimental phenylketonuria in weanling rats than in adult rats which was due in part to higher food consumption per unit body weight. But not all differences are dependent on phenylalanine intake; 100mg/Kg body weight injections of phenylalanine caused a brain serotonin decrease of 0.068μg/hr/g. brain in weanlings and 0.044μg/hr/g. brain in adult rats (Geller and Yuwiler, 1969).

Although man's development cannot be equated to the development of the rat, because of different time scales, the implications are clear; early control of amino acid levels is very important if the developing infant is to reach its full potential. Woolf and Vulliamy (1951) suggested that the mental abnormalities in phenylketonuria were the result of intoxication by phenylalanine or its metabolites which would exact their toll most severely during the first year of life when fundamental learning was taking place and myelin was being laid down at

the maximum rate. Further emphasis on the importance of dietary control at birth comes from the report of lower I.Q. ratings in children at the age of seven years, who, as premature infants, had been fed on milk containing 4% protein rather then 2% protein (Goldman, Goldman, Kaufman and Liebman, 1974) high blood concentrations of amino acids were noted in low-birth-weight infants on high protein diets. Neonatal tyrosinaemia, which is caused by an inability to metabolise tyrosine normally, may also cause intellectual impairment which becomes apparent only when I.Q. is measured later in life (Menkes, Welcher, Levi, Dallas and Gretsky, 1972) and is of interest as tyrosine is the major metabolite of phenylalanine except in phenylketonuria.

The clinical significance of transient phenylketonuria is difficult to assess but it is reasonable to assume some intellectual loss, however small, in view of the foregoing observations. If a permanent endogenous deficit in amino acid control presents itself then the only way to prevent gross brain damage is to impose external control by dietary sanction. Overcorrection, by giving too little phenylalanine, may lead to wasting and death (Moncrief and Wilkinson, 1961).

The work of Bickel, Gerrard and Hickman (1953) showed quite clearly that there was a behavioural and reversible component of phenylketonuria in man. This could be the result of competition of excess phenylalanine with tyrosine and 5-hydroxytryptophan for transport sites into brain cells (McKean, Schanberg and Giarman, 1962) reducing the production of neurotransmitters. Alternatively a reduction in the level of tetrahydrobiopterin in brain tissue could follow inhibition of dihydropteridine reductase by phenylpyruvic acid.

It is difficult to know how long to proceed with a low phenylalanine diet. In a group who discontinued restriction between the ages of five years and fifteen years, I.Q. dropped an average of six points (Smith, Lobascher, Stevenson, Wolff, Schmidt, Grubel-Kaiser and Bickel, 1978). Eleven children taken off diet at the age of six years showed significant decreases in the rate of mental development when compared

with twenty-six control children of comparable I.Q. and seventeen phenylketonuric children of comparable I.Q. who remained on diet (Brown and Warner, 1976).

With the early diagnosis and treatment of phenylketonuria there will be an increase in the number of phenylketonuric women conceiving. This presents the problem of controlling phenylalanine levels in the mother, which can be achieved successfully, but more importantly, in the foetus (Allan and Brown, 1966). There may be a further difficulty in maintaining adequate tyrosine levels, for protein synthesis, which may be low in some cases of phenylketonuria (Koepp and Held, 1977). The stigmata of foetal exposure to maternal phenylketonuria, cardiac defects and microcephaly, have been reported when a low phenylalanine diet commenced five weeks after conception (Smith, Macartney, Erdohazi, Pincott, Wolff, Brenton, Biddle, Fairweather and Dobbing, 1979) showing the foetus to be vulnerable at a very early stage in development. Phenylalanine challenge has been used as a method for the detection of heterozygotes in families with histories of phenylketonuria and have been oral (Hsai and Driscoll, 1956; Jervis, 1960) and intravenous (Woolf, Cranston and Goodwin, 1967; Lambert, Vrailhet, Monot, Lepaire, Baradel, Nabet, Martin and Pierson, 1973). These tests measure the ability of patients to clear phenylalanine normally by metabolism to tyrosine. Normal random samples of plasma have phenylalanine levels of between 0.03 and 0.09m.mol/L. (Leeming, Blair, Green and Raine, 1976) whilst urine levels are around 0.15m.mol/L. (Documenta Geigy, 1962) with a total excretion of approximately 25mg. per day in the adult. Minimum dietary requirement in young adult males is 1.1 gram per day although 70-75% can be replaced by tyrosine.

Serum biopterin derivative levels are increased in normal adults following oral phenylalanine and the variation in biopterin response, measured protozoologically with Crithidia fasciculata, has been suggested as a method of improving the identification of certain atypical forms of phenylketonuria (Leeming, Blair, Green and Raine, 1976). Following oral loads of phenylalanine, plasma phenylalanine and serum Crithidia factor increase (Leeming, Blair, Green and Raine, 1976; Kaufman, Berlow, Summer,

Milstien et al, 1978). In phenylketonuria the raised plasma phenylalanine persists longer than in normal subjects. In malignant hyperphenylalaninaemia (Chapter VIII), inadequate synthesis of tetrahydrobiopterin or dihydropteridine reductase results in decreased and increased Crithidia factor response respectively. In the event of abmormal biopterin metabolism in atypical forms of phenylketonuria, Crithidia factor studies could be informative.

With the availability of patients undergoing routine phenylalanine loads for diagnostic purposes, opportunity was taken to measure Crithidia factor in serum and, in some cases, in urine.

MATERIALS AND METHODS

Five normal volunteers, in whose families there was no history of phenylketonuria, had 7g. oral doses of L-B-phenylalanine. Blood samples were taken for up to four hours, serum Crithidia factor, phenylalanine and tyrosine were measured.

The following children with hyperphenylalaninaemias were variously in the care of Dr.D.N. Raine, Children's Hospital, Birmingham; Dr.I.Smith, Institute of Child Health, London; Dr.F. Rey, Hopital Des Enfants Malades, Paris.

Ten children with classical phenylketonuria on diet had oral phenylalanine loads of 100mg/Kg body weight as did the parents of one child with classical phenylketonuria. Two mild phenylketonurics (R.G. and K.J.) and two brothers (A.S. and P.M.) with atypical phenylketonuria whose tolerance to dietary phenylalanine decreased during the first year of life then commenced to increase, were given oral phenylalanine loads. These last two were older than one year at the time of the challenge.

Four mild phenylketonurics whose plasma phenylalanine on normal diet was less than 0.6 m.mol/L. (Et, Ta, Ro and To) were challenged by intravenous infusion with phenylalanine, urines were available from three.

A six month old child (R.M.) who appeared, clinically, to have classical phenylketonuria had a phenylalanine hydroxylase of 16.8 units (μmol tyrosine/hour/g protein) - normal range 35-50 and a dihydropteridine reductase of 80 units (normal range 60.3 - 141 μmol NADH/min/g protein - Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977), was taken off diet for four days. Phenylalanine and Crithidia factor were measured before dietary relaxation and at two and four days afterwards.

A five year old child (Z) had convulsive seizures whenever the phenylalanine level exceeded 0.9 m.mol/L. but whose level was normally

0.08 to 0.09 m.mol/L. with normal mental and neurological development apart from the seizures, had an intravenous load of phenylalanine, Crithidia factor and phenylalanine were estimated.

Another five year old child (Y.L.) who had had a transient hyperphenylaninaemia which had resolved itself in the first few months of life, was given an oral phenylalanine load.

Finally a further five year old child (A.L.) had a mild form of phenylketonuria and was judged on clinical and biochemical grounds to be sufficiently stable at four months for dietary restriction to be discontinued, was noted to have surprisingly low Crithidia factor levels in both serum and urine (serum 0.4 μg/L., urine on two consecutive days 0.10 mg/L. and 0.19 mg/L - volumes 290 cm^3 and 166 cm^3 per twenty-four hours respectively). An oral phenylalanine load of 100mg/Kg. body weight was administered. The serum and urine from this child was chromatographed in 3% ammonium chloride, 0.5% sodium carbonate and 5% acetic acid. Additionally, two-dimensional chromatography was carried out, firstly with 3% ammonium chloride and then with propanol/1% ammonia (2:1) to distinguish between biopterin and L-neopterin.

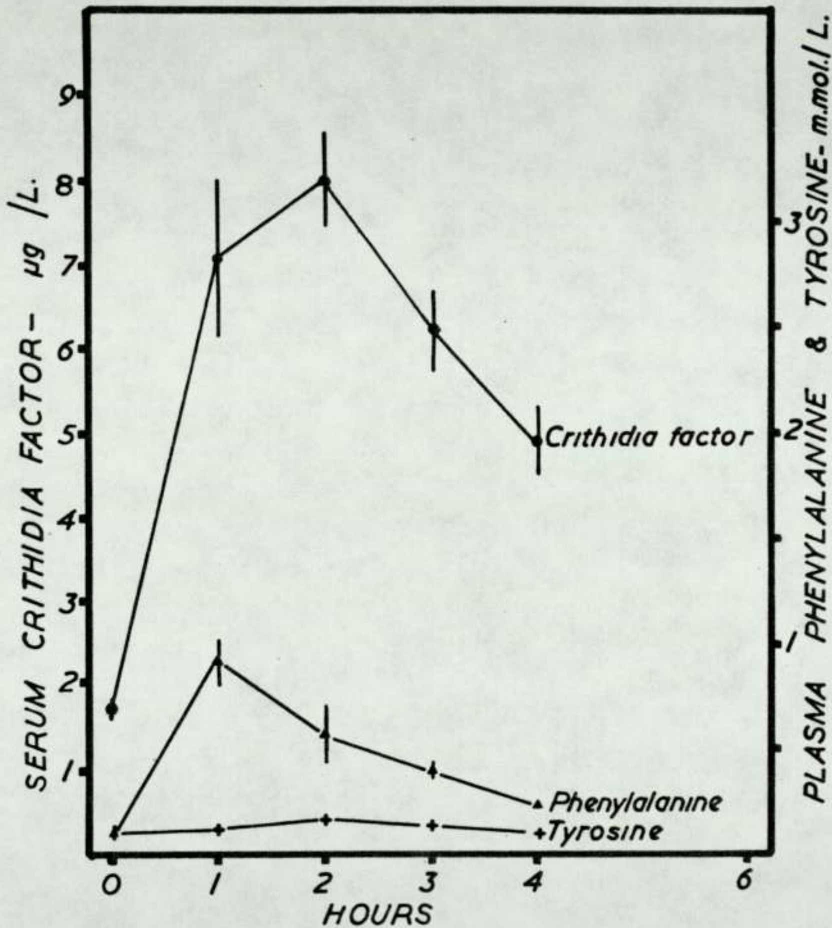
Sera from four classical phenylketonurics were chromatographed in 3% ammonium chloride, 0.5% sodium carbonate and 5% acetic acid.

RESULTS

Oral phenylalanine loads given to five normal adults (Figure 9-1)

FIGURE 9-1

MEAN SERUM CRITHIDIA FACTOR, PHENYLALANINE AND TYROSINE IN FIVE NORMAL ADULTS GIVEN 7g. L-PHENYLALANINE ORALLY



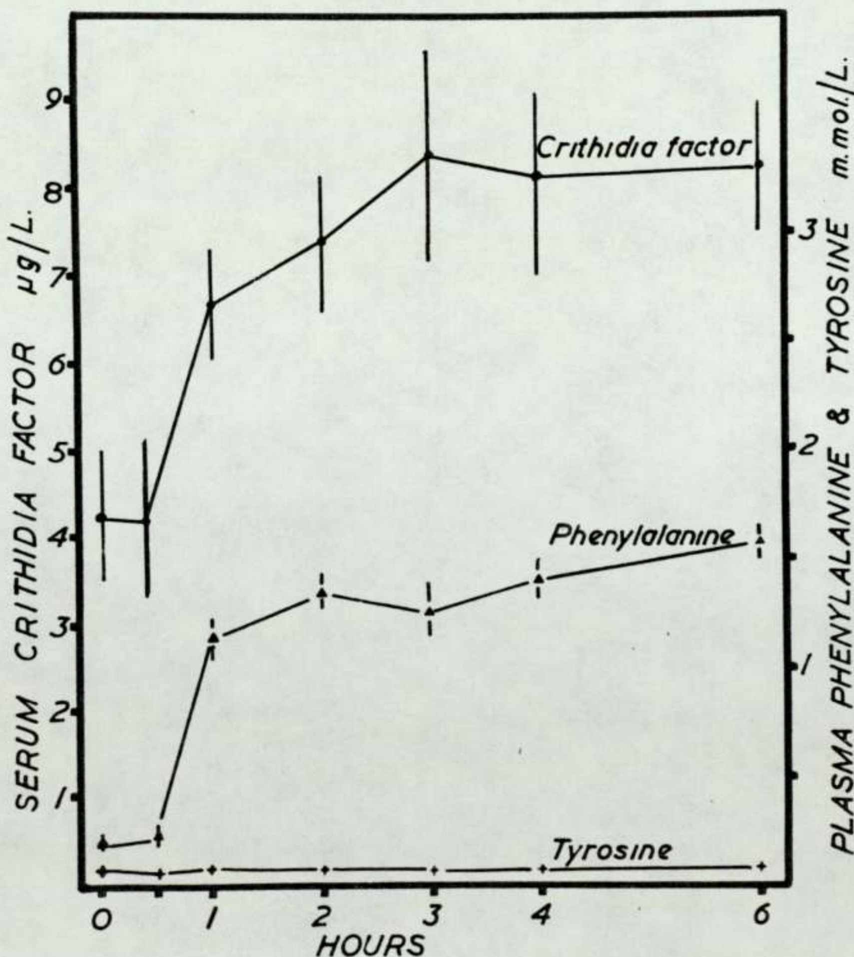
produced a response in serum Crithidia factor which followed the phenylalanine level. The peak serum Crithidia factor was obtained at two hours whereas the highest phenylalanine level occurred at one hour; thereafter both values fell but were still above the fasting level when sampling was discontinued at four hours. The tyrosine level rose slightly peaking at two hours, thus showing hydroxylation of phenylalanine to tyrosine. Similar loads to ten classical phenylketonurics produced a rise in serum Crithidia factor which levelled out

at around three hours although the phenylalanine level was still rising and remained high at six hours; there was no increase in tyrosine (Figure 9-2). Classical phenylketonurics have phenylalanine hydroxylase levels of less than 1 unit (μmol tyrosine/hour/g.protein) whilst normal controls have 35-50 units (Rey, 1979).

The two cases of mild phenylketonuria (R.G. and K.J.) cleared phenylalanine slightly better than classical phenylketonurics but the Crithidia factor response showed no obvious variation from that of children with lower phenylalanine tolerance (Figure 9-3), except that in the case of R.G. the peak

FIGURE 9-2

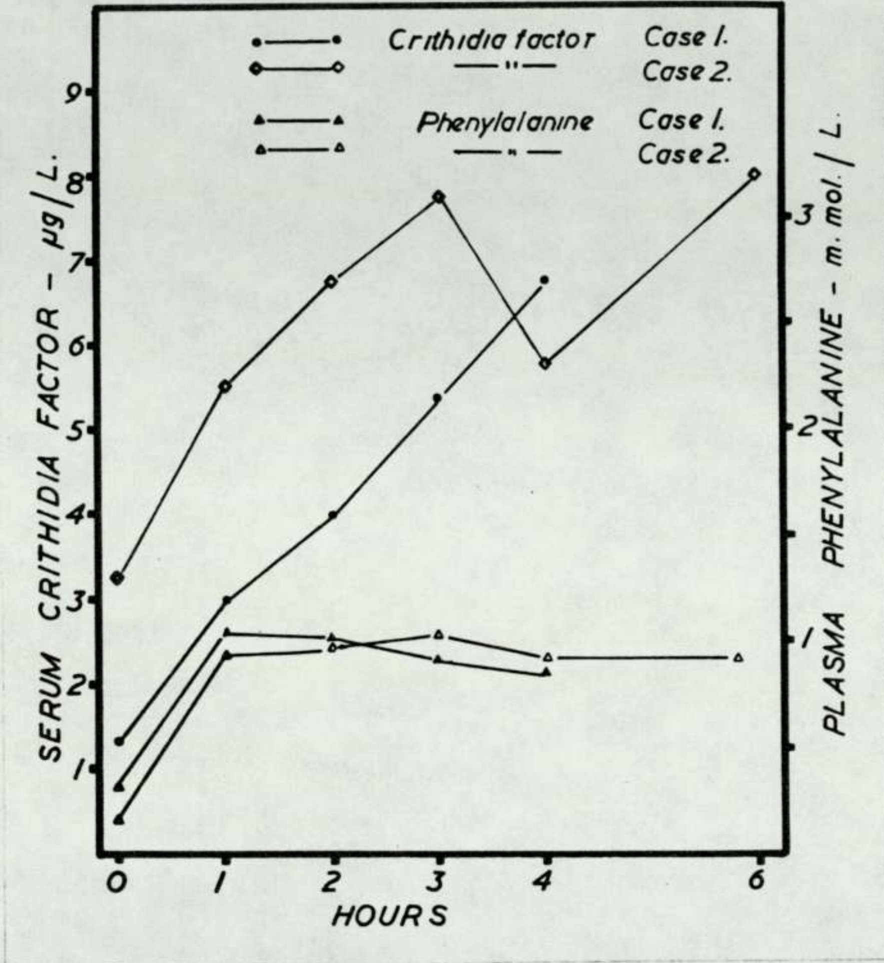
MEAN SERUM CRITHIDIA FACTOR, PHENYLALANINE AND TYROSINE IN TEN CASES OF CLASSICAL PHENYLKETONURIA GIVEN 100mg L-PHENYLALANINE ORALLY/Kg BODY WEIGHT



Crithidia factor level had clearly not been reached at four hours.

FIGURE 9-3

SERUM CRITHIDIA FACTOR AND PHENYLALANINE IN TWO MILD CASES OF PHENYLKETONURIA (CASE 1 R.G., CASE 2 K.J.) GIVEN 100mg L-PHENYLALANINE ORALLY/Kg BODY WEIGHT

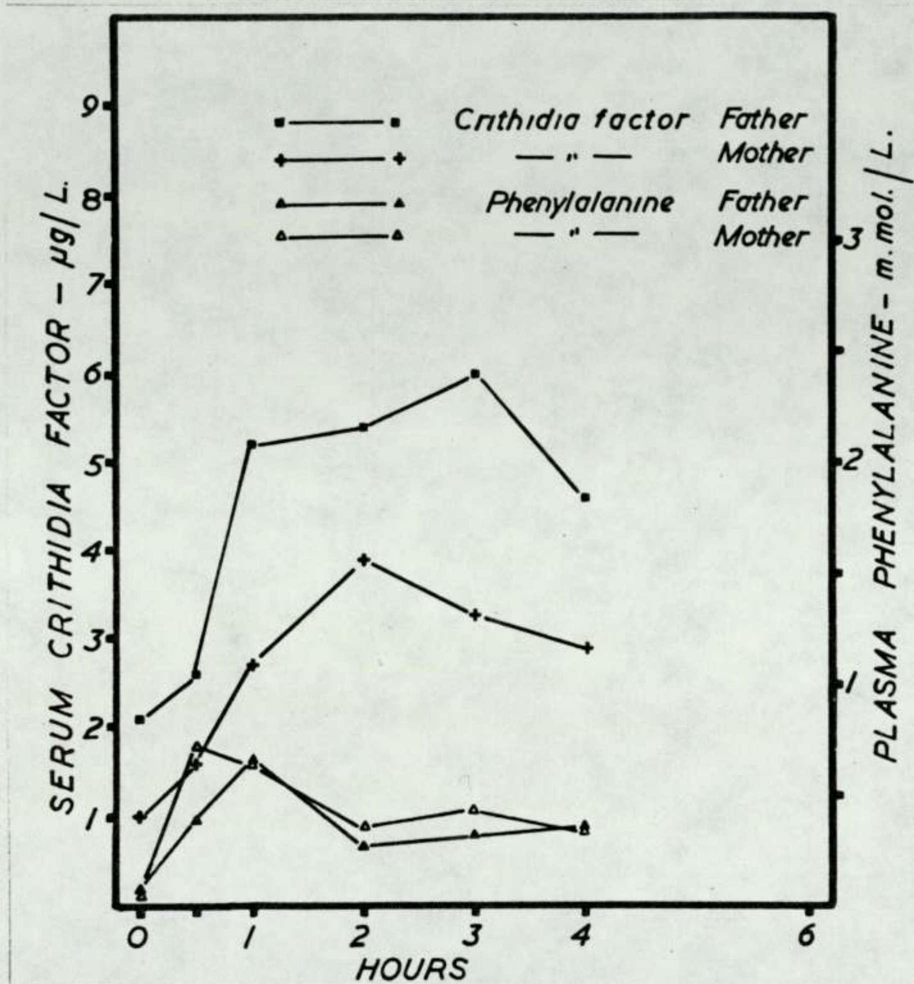


The heterozygotes, parents of a child with classical phenylketonuria, were very similar (Figure 9-4) to the series of normal adults (Figure 9-1). The two brothers (P.M. and A.S.) with mild atypical phenylketonuria where the phenylalanine tolerance had decreased during the first year of life and subsequently improved, showed very different (from each other) Crithidia factor responses, this may well have been caused by the much higher pre-load plasma phenylalanine level in one case (P.M.) (Figure 9-5) P.M.'s Crithidia factor level reached the remarkable value of 30.5μg/L at two hours.

The four mild phenylketonurics intravenously infused with

FIGURE 9-4

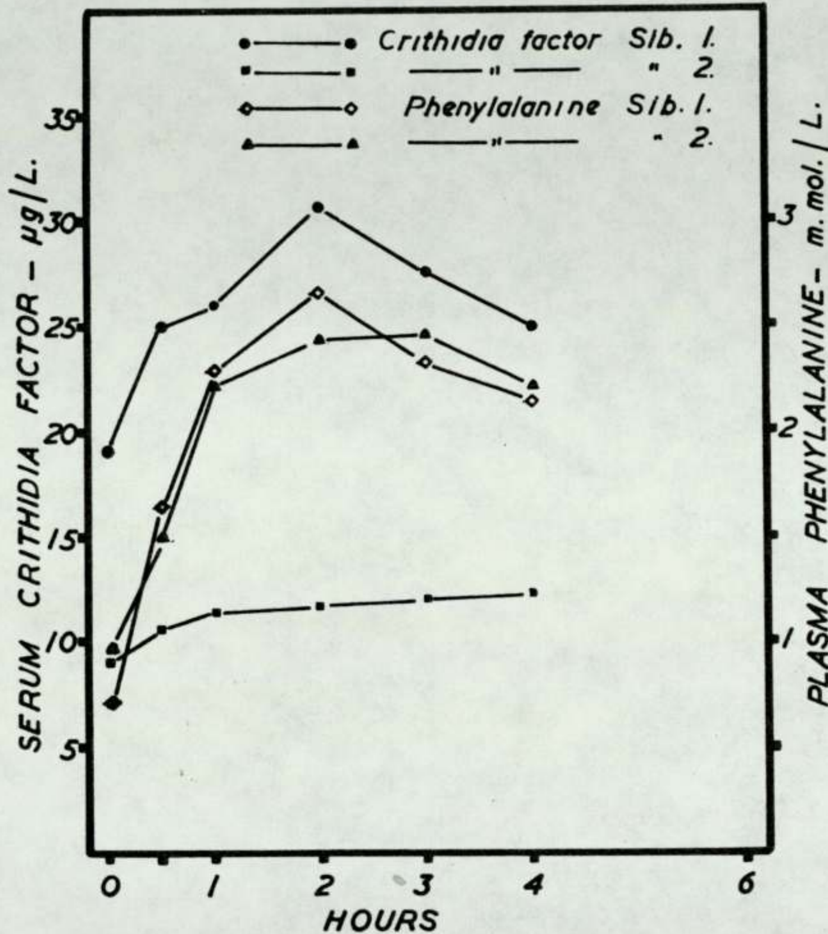
SERUM CRITHIDIA FACTOR AND PHENYLALANINE FOLLOWING 100mg ORAL L-PHENYLALANINE/Kg BODY WEIGHT IN (HETEROZYGOTE) PARENTS OF A CHILD WITH CLASSICAL PHENYLKETONURIA



phenylalanine, Et. (Figure 9-6), Ta. (Figure 9-7), Ro. (Figure 9-8) and To. (Figure 9-9) showed Crithidia factor increases in keeping with previous findings although the clearance of phenylalanine was not uniform. Phenylalanine hydroxylase levels on Et. and To. were 2.4 and 3.5 units respectively. Urine levels were available from three of these cases, Et. excreted 400μg Crithidia factor in 500 cm^3 of urine during the 24 hours prior to loading, 91μg in 29 cm^3 in the three hours following infusion and 525μg in 300 cm^3 in the subsequent fifteen hours. Ta. produced 76μg Crithidia factor in 27 cm^3 urine in the first two hours, 141μg in 27 cm^3 urine in the next twelve hours followed by 31μg in 9 cm^3 in the following ten hours. Ro. produced 696μg Crithidia factor in 200 cm^3 in the first six hours followed by 379μg in 112 cm^3 in the following fifteen hours.

FIGURE 9-5

SERUM CRITHIDIA FACTOR AND PHENYLALANINE FOLLOWING 100mg ORAL L-PHENYLALANINE/Kg BODY WEIGHT IN 2 BROTHERS (SIB.1 A.S. SIB. 2 P.M.) WITH MILD PHENYLKETONURIA WHOSE TOLERANCE TO PHENYLALANINE DECREASED DURING THE FIRST YEAR OF LIFE AND THEN IMPROVED.

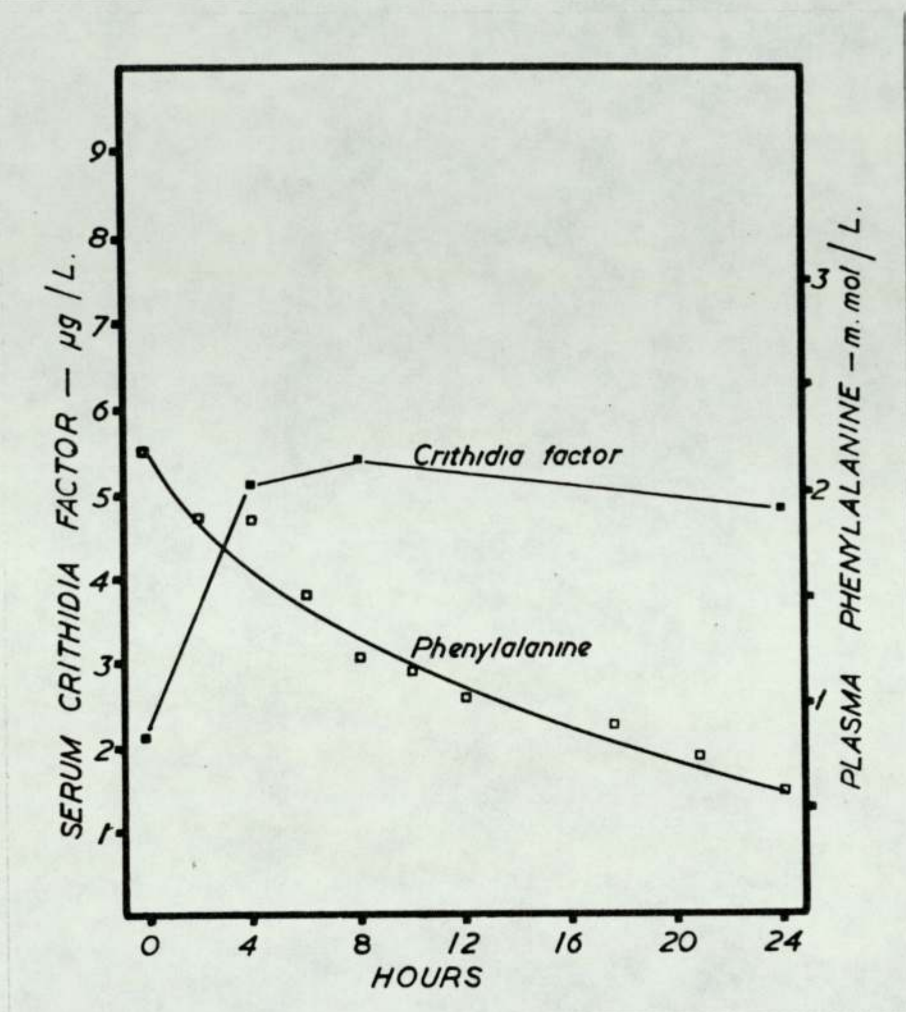


The child (R.M.) with phenylalanine hydroxylase of 16.8 units and dihydropteridine reductase of 80 units showed a response (Figure 9-10) which was not substantially different from classical phenylketonuria (Figure 9-2). The resolved transient hyperphenylalaninaemic child, Y.L., differed in response (Figure 9-11) to normals in that the Crithidia factor level continued to rise when the phenylalanine level reached a plateau and the phenylalanine tolerance was reduced.

The child (Z.) who had convulsive seizures whenever the plasma phenylalanine rose to 0.9m.mol/L. produced a strange result with the plasma

FIGURE 9-6

SERUM CRITHIDIA FACTOR AND PHENYLALANINE
IN A MILD CASE OF PHENYLKETONURIA (Et.)
FOLLOWING INTRAVENOUS INFUSION WITH L-PHENYLALANINE

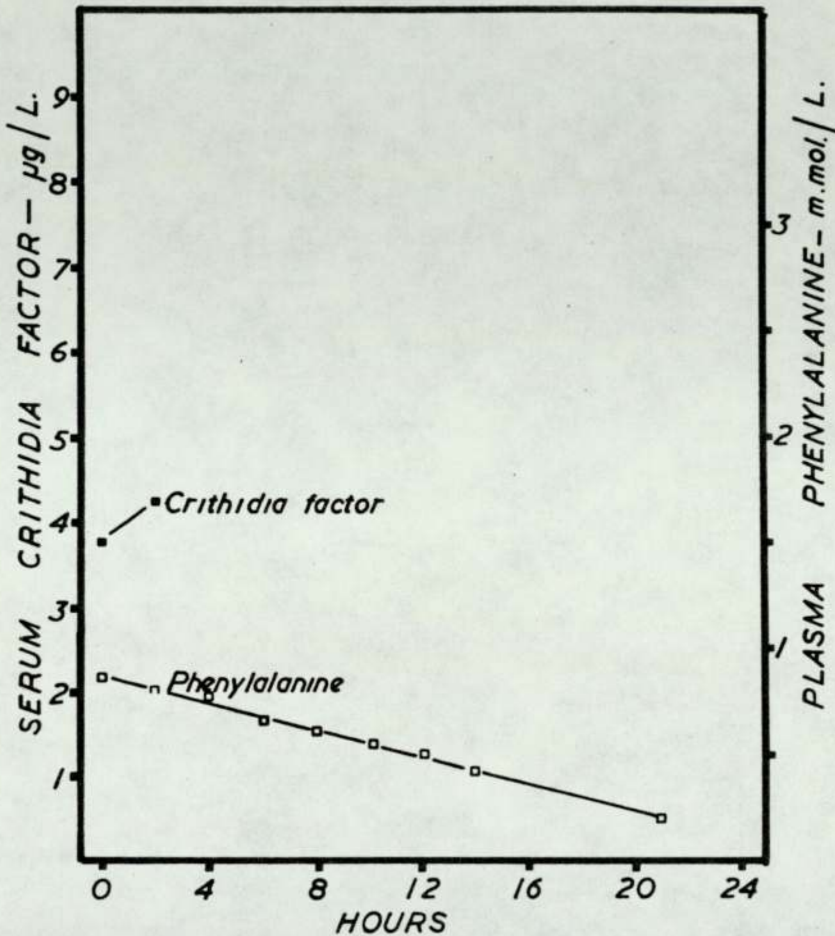


phenylalanine level rising steadily but the Crithidia factor level was high before load and did not alter much during the load (Figure 9-12).

The mildly hyperphenylalaninaemic child aged 5 years (A.L.), off diet and with low Crithidia activity in serum and urine, showed hardly any increase in serum Crithidia factor following oral phenylalanine (Figure 9-13) and the urinary excretion in the six hours immediately following the load was 30μg in 80 cm^3 and for the subsequent 24 hours 208μg in 640 cm^3. Chromatography of his urine revealed Crithidia active compounds which co-chromatographed with biopterin and 7,8-dihydrobiopterin respectively as in normal subjects although there was an apparent decrease in 7,8-dihydrobiopterin.

FIGURE 9-7

SERUM CRITHIDIA FACTOR AND PHENYLALANINE
IN A MILD CASE OF PHENYLKETONURIA (Ta.)
FOLLOWING INTRAVENOUS INFUSION WITH L-PHENYLALANINE



Two-dimensional chromatography showed there to be no L-neopterin. His serum showed only 7,8-dihydrobiopterin whilst the serum of classical phenylketonurics showed 7,8-dihydrobiopterin and a trace of biopterin.

In contrast C.A. and Y.T. (Chapter VIII) had similar low levels of Crithidia factor which showed little response to phenylalanine load and only biopterin was demonstrated in the serum. They had poor phenylalanine clearance and clinically C.A. even on diet was severely brain damaged.

FIGURE 9-8

SERUM CRITHIDIA FACTOR AND PHENYLALANINE
IN A MILD CASE OF PHENYLKETONURIA (Ro.)
FOLLOWING INTRAVENOUS INFUSION WITH L-PHENYLALANINE

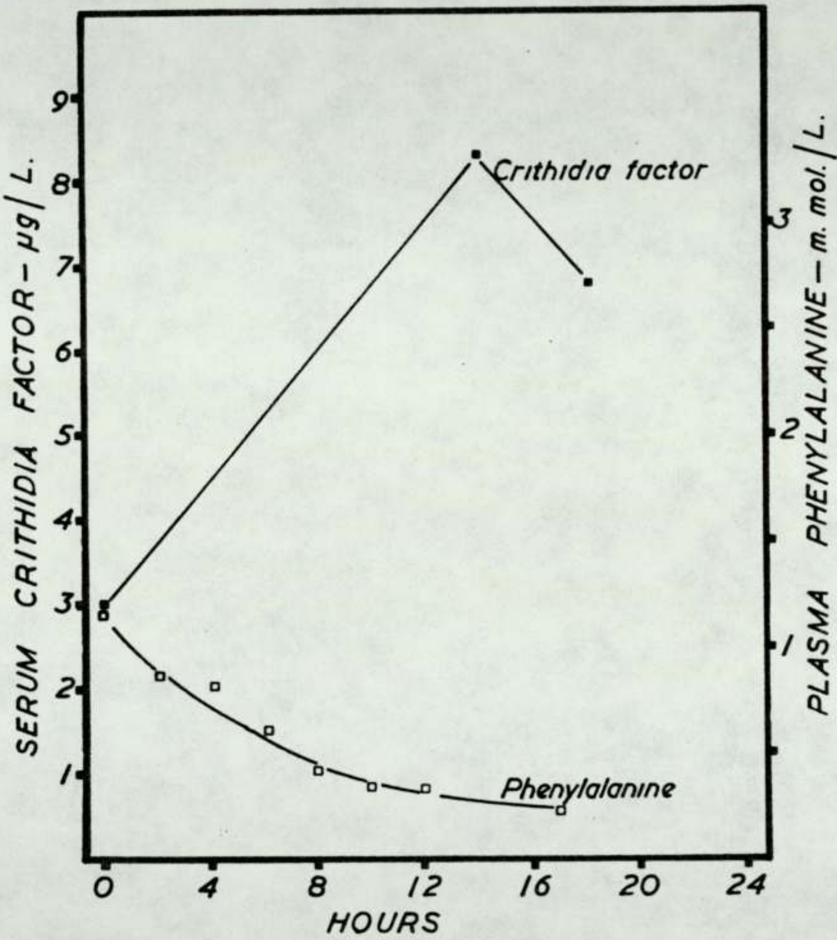


FIGURE 9-9

SERUM CRITHIDIA FACTOR AND PHENYLALANINE
IN A MILD CASE OF PHENYLKETONURIA (To.)
FOLLOWING INTRAVENOUS INFUSION WITH L-PHENYLALANINE

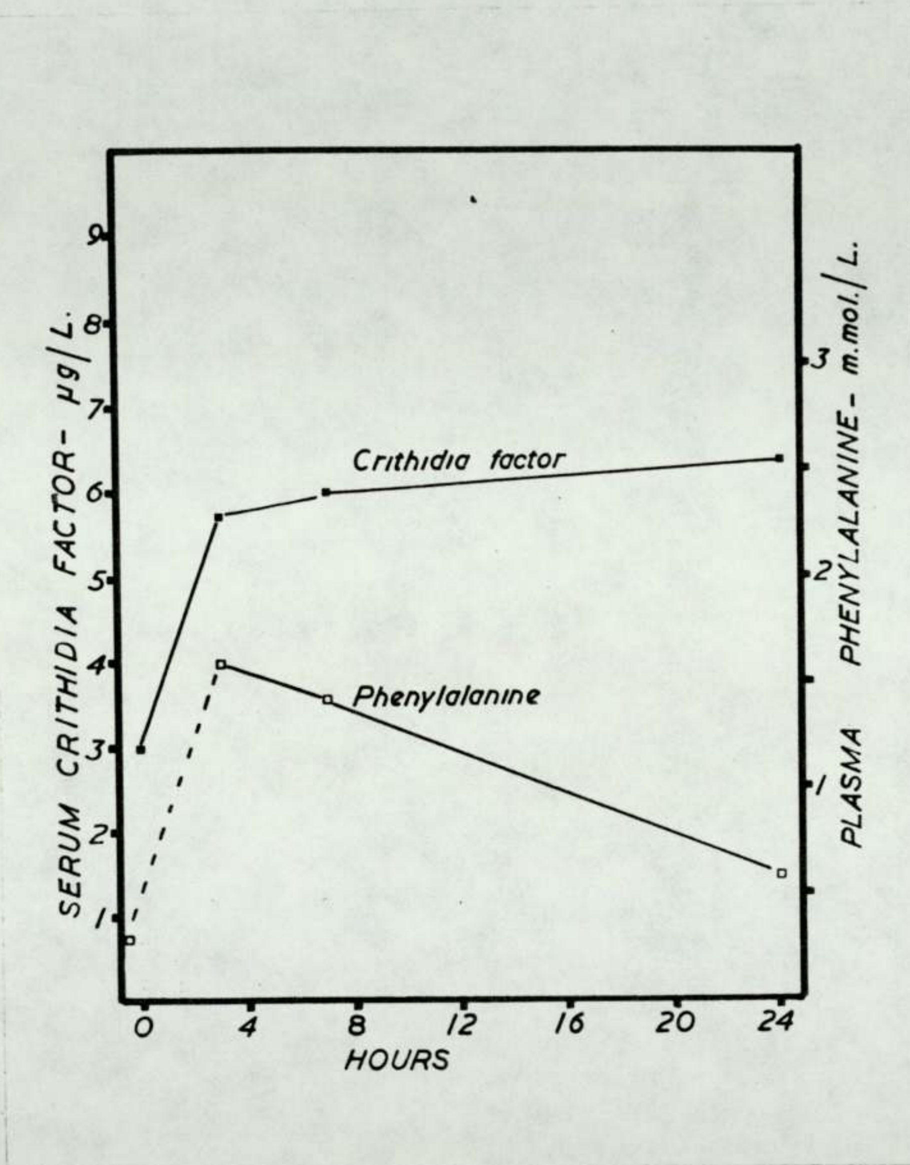


FIGURE 9-10

SERUM CRITHIDIA FACTOR AND PHENYLALANINE FOLLOWING REMOVAL OF DIETARY RESTRICTION IN A CASE OF PHENYLKETONURIA (R.M.) CLINICALLY CLASSICAL BUT WITH PHENYLALANINE HYDROXYLASE OF 16.8 UNITS AND DIHYDROPTERIDINE REDUCTASE OF 80 UNITS.

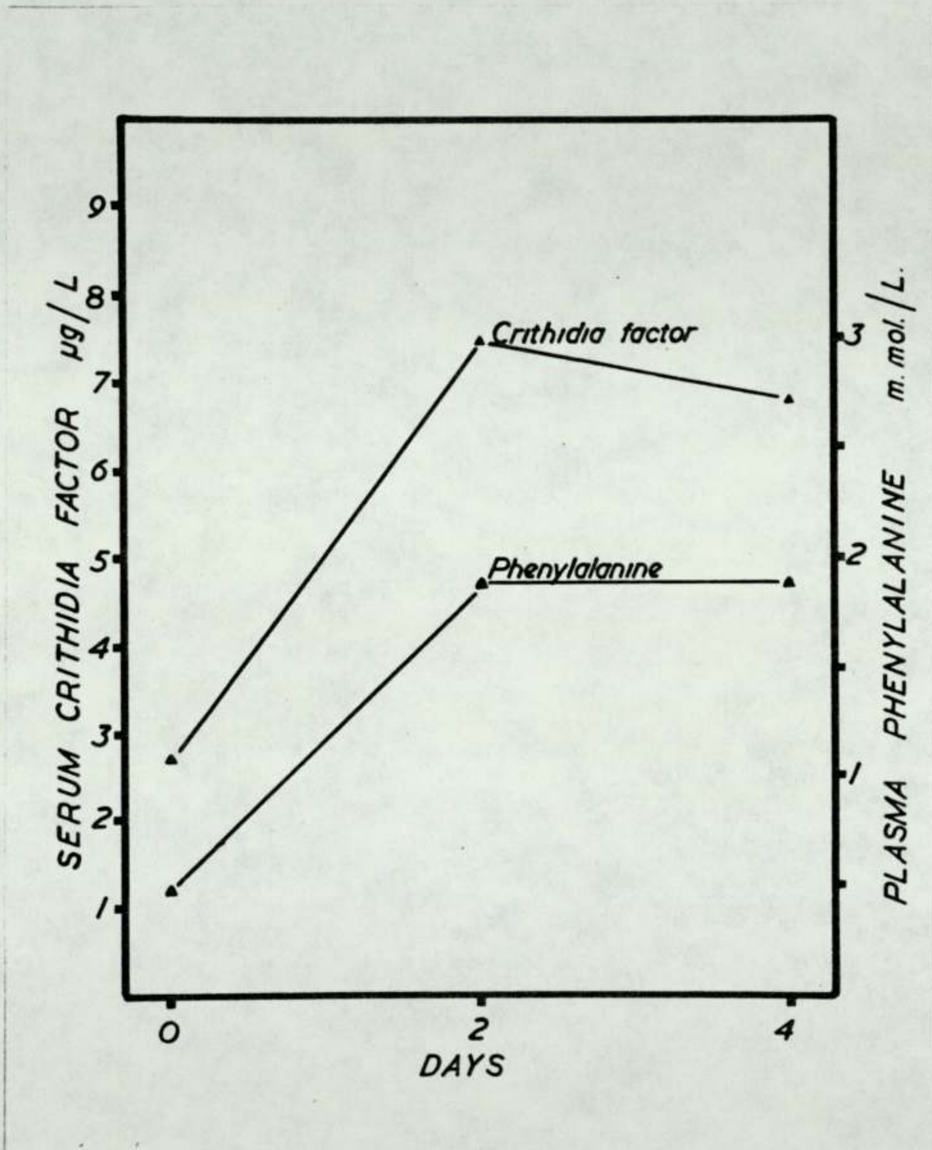


FIGURE 9-11

SERUM CRITHIDIA FACTOR AND PHENYLALANINE
IN A RESOLVED CASE OF TRANSIENT HYPERPHENYLALANINAEMIA (Y.L.)
FOLLOWING 100mg ORAL L-PHENYLALANINE/Kg BODY WEIGHT.

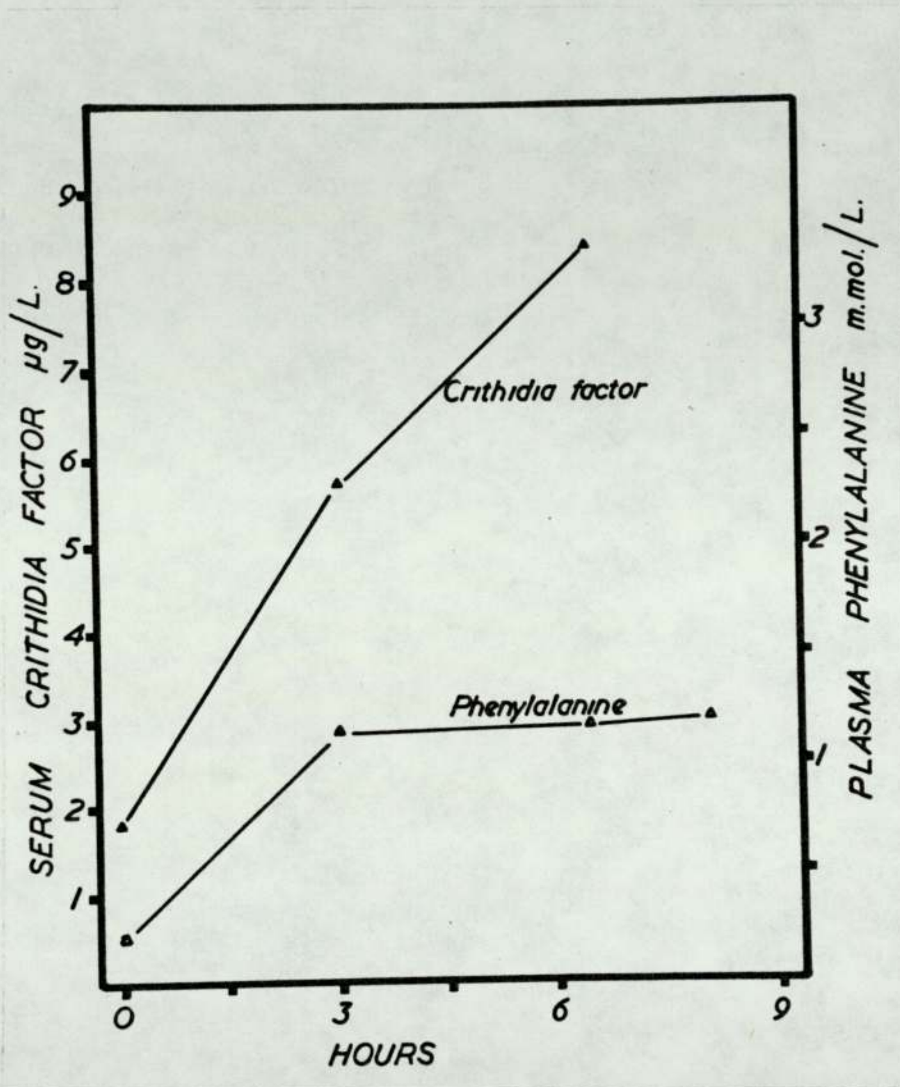


FIGURE 9-12

SERUM CRITHIDIA FACTOR AND PHENYLALANINE FOLLOWING INTRAVENOUS L-PHENYLALANINE IN A CHILD (Z) WHOSE PHENYLALANINE WAS NORMALLY 0.08 TO 0.09 m.mol/L. BUT WHO HAD CONVULSIVE SEIZURES WHEN THE PLASMA PHENYLALANINE EXCEEDED 0.9 m.mol/L.

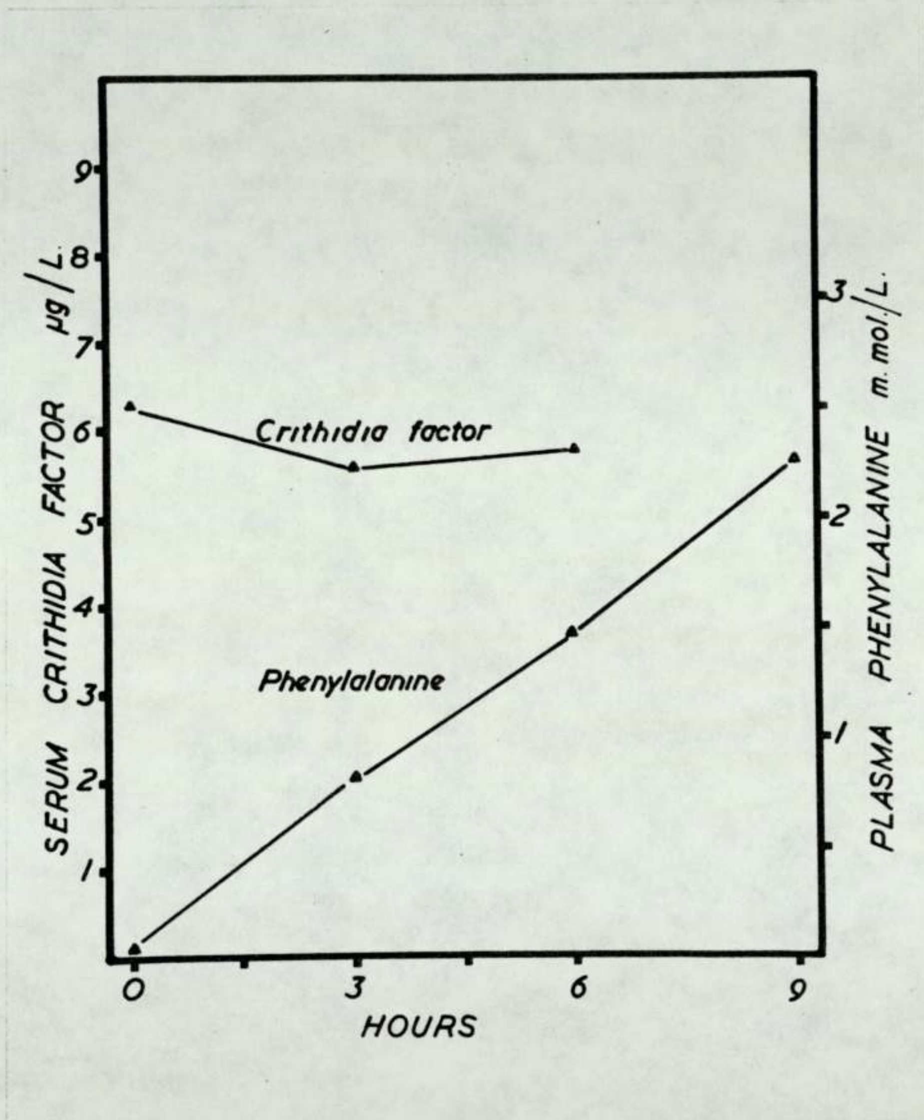
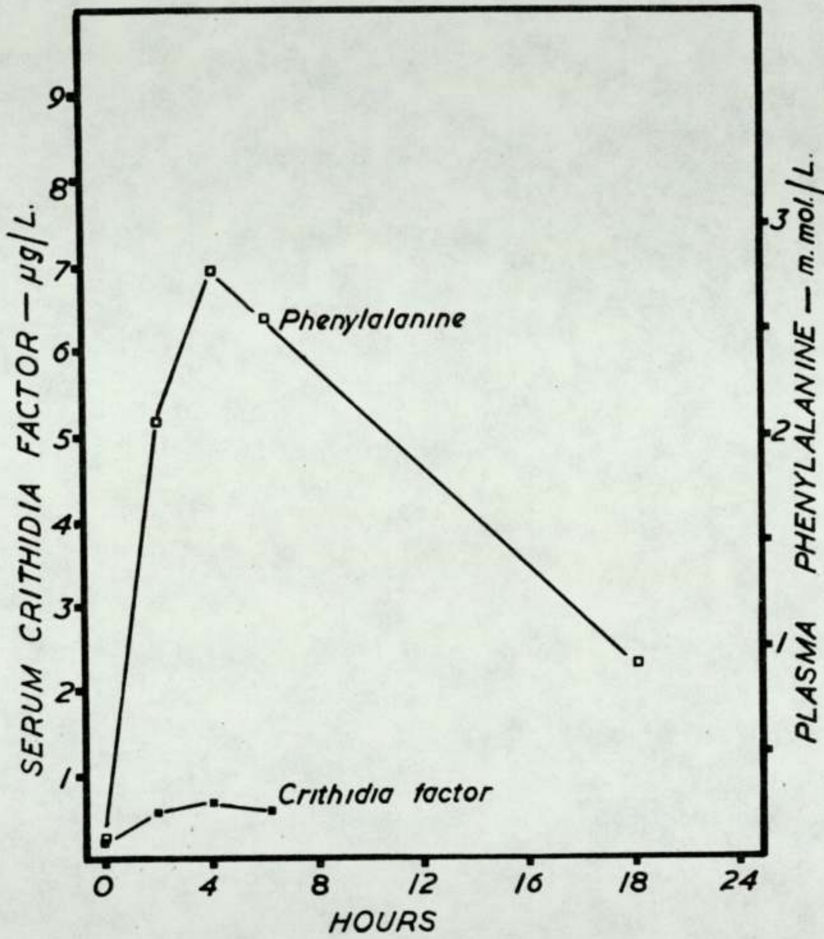


FIGURE 9-13

SERUM CRITHIDIA FACTOR AND PHENYLALANINE FOLLOWING 100mg L-PHENYLALANINE ORALLY IN A CASE (A.L.) OF MILD PHENYLKETONURIA WHO WAS NOTED TO HAVE LOW SERUM AND URINE CRITHIDIA FACTOR LEVELS IN RANDOM SAMPLES.



DISCUSSION

Serum Crithidia factor levels, in normal individuals, increase when the plasma phenylalanine level increases and decrease when the plasma phenylalanine level falls in dietary restriction (Leeming, 1975; Leeming, Blair, Green and Raine, 1976). The results of loading experiments on classical phenylketonurics (Figure 9-2) show phenylalanine remaining at a high level in the plasma and serum Crithidia factor also elevated. The difference between serum Crithidia factor in normal subjects and in classical phenylketonuria mirrors the difference in handling of phenylalanine. The same materials, 7,8-dihydrobiopterin and biopterin are present in normals and phenylketonurics.

Apart from cases of malignant hyperphenylalaninaemia which have been dealt with in the previous chapter and in which phenylalanine hydroxylase is in the normal range, there is a wide spectrum of hydroxylase levels. Phenylalanine hydroxylase is normally present in excess and this is demonstrated by Et. and To. who had 5% - 10% residual hydroxylase activity and were able to clear phenylalanine satisfactorily on a normal dietary regime. Therefore, hyperphenylalaninaemia starts at a low level of phenylalanine hydroxylase and increases in severity until one reaches classical phenylketonuria in which there is less than 1% of the normal hydroxylase activity. Further, delayed post-natal enzyme development may cause transient difficulties in handling phenylalanine.

The difference between classical phenylketonuria and normals is clear from the clearance of phenylalanine loads but the difficulty in differentiation of phenylketonuric heterozygotes (Figure 9-4) is not resolved by the additional parameter afforded by the Crithidia assay. The results of phenylalanine loads in the mild cases of phenylketonuria demonstrates the variations to be seen in this disease. Previously (Chapter VIII) clear defects in synthesis of the pterin ring and dihydropteridine reductase were readily identified by Crithidia factor measurement and this investigation of mild cases of hyperphenylalaninaemia was carried out to

determine if these could be classified further by variation in biopterin metabolism.

Phenylalanine alone is not the trigger for maximum biosynthesis of Crithidia factor but the latter does follow the phenylalanine level most closely even when the plasma phenylalanine is depressed to abnormally low levels (Leeming, Blair, Green and Raine, 1976) or here (Figure 9-10) when a phenylketonuric is taken off diet. Phenylalanine has no effect on the synthesis of Crithidia factor to dihydropteridine reductase activity in-vitro (Chapters II and III). There is evidence that phenylpyruvic acid acts as a competitive inhibitor of dihydropteridine reductase (Chapters III and IV) and is raised in phenylketonuria (Jervis and Drejza, 1966) to levels shown to be active in-vitro.

R.M. had a dihydropteridine reductase of 80 units (normal range 60 - 90 units) and the phenylalanine hydroxylase was 16.8 (normal range 35 - 50). With substantially lower levels of phenylalanine hydroxylase, Et. and To. (2.4 and 3.5 units respectively) were maintained satisfactorily on a normal diet whereas R.M. resembled classical phenylketonuria clinically. No explanation is afforded by data to hand.

The patient A.L. whose serum Crithidia factor was raised to only 0.64μg/L. by an oral phenylalanine load is illustrated in Figure 9-13. However, because the Crithidia factor was increased to treble its initial value, which was proportionate to the rise in normals and classical phenylketonurics, the identity of the Crithidia active compounds was sought in case there was a variant pterin metabolically active in the mammal but which supports the growth of Crithidia fasciculata weakly; no evidence was found for this. A more plausible explanation is that de novo synthesis of tetrahydrobiopterin was poor, compensated for by a high level of dihydropteridine reductase. Quinonoid dihydrobiopterin would be reduced rapidly to 5,6,7,8-tetrahydrobiopterin which would be retained within the cell. There would therefore be little 7,8-dihydrobiopterin produced to pass into the bloodstream to be detected as serum Crithidia factor. The chromatographic evidence suggests that the Crithidia active compounds

produced were biopterin and 7,8-dihydrobiopterin as in normal subjects. This does not rule out finally the possibility of another metabolically active pterin which is undetectable in the Crithidia assay. The effect of methotrexate on this patient might prove informative, methotrexate, septrin and phenylalanine are the only compounds which, when administered, stimulate substantial increases in serum Crithidia factor in man (Leeming, Blair, Melikian and O'Gorman, 1976; Leeming, Blair, Green and Raine, 1976).

It is interesting to compare this last patient with two of the cases of malignant hyperphenylalaninaemia described in the previous chapter. Serum Crithidia factor levels were of the same low order and yet the clinical conditions were quite distinct. The patient described above was clinically normal without dietary control or therapy of any kind, the only evidence of a neurological defect was provided during the phenylalanine load when the patient fell into a deep and unnatural sleep. On the other hand the two with malignant hyperphenylalaninaemia were grossly hypotonic and socially unaware until neurotransmitter production was induced by the provision of L-dopa and 5-hydroxytryptophan as precursors. One certain conclusion is that low serum Crithidia factor levels are not sure indicators of malignant hyperphenylalaninaemia. Identification of the pterin constituents of serum and urine might aid differentiation of patients like A.L. who had a low serum Crithidia factor but an identifiable amount of 7,8-dihydrobiopterin, from C.A. (previous chapter) who only had a trace of biopterin in her serum.

In classical phenylketonuria, phenylalanine is not cleared because of an almost total lack of active phenylalanine hydroxylase but normal development follows dietary restriction of phenylalanine from infancy. Phenylalanine itself does not interfere with tetrahydrobiopterin synthesis in-vitro (Chapters II to IV) although it competes with tyrosine for uptake by brain cells (McKean, Schanberg and Giarman, 1962) and may limit neurotransmitter production in that way. Phenylpyruvic acid competes for dihydropteridine reductase (Chapter III) and could reduce

tetrahydrobiopterin levels and account for the increased 7,8-dihydrobiopterin in serum during hyperphenylalaninaemia.

With mild phenylketonurics in whom there is partially active phenylalanine hydroxylase, judgement on phenylalanine restriction has to be made from measurement of plasma phenylalanine. Et., Ta., Ro., and To. had plasma phenylalanine levels of less than 0.6 m.mol/L. when on a normal diet. Et. and To. had phenylalanine hydroxylase levels of 2.4 and 3.5 units with dihydropteridine reductase levels of 64 and 76 units respectively. These are in marked contrast with R.M. (see above) who had hydroxylase and reductase levels of 16.8 and 80 units respectively and who appeared more like a classical case of phenylketonuria.

The transient hyperphenylalaninaemia, Y.L., cleared phenylalanine slowly (Figure 9-11) and perhaps similarly to the mild phenylketonurics. The heterozygotes (Figure 9-4) had little to distinguish them from normal.

Apart from A.L., the only clearly abnormal serum biopterin derivative response was from one of the brothers (A.S.) with mild hyperphenylalaninaemia which appeared to worsen during the first year of life and improve (Figure 9-5). The serum Crithidia factor was high before the load but did not change much during the four hours in which samples were taken. In this respect he resembled the case of transient hyperphenylalaninaemia previously published (Leeming, Blair, Green and Raine, 1976). A decrease in phenylalanine tolerance in the first months of life in cases where phenylalanine hydroxylase is partially active would add further weight to the hypothesis that phenylpyruvic acid inactivates dihydropteridine reductase (Chapter III) as phenylpyruvic acid is not produced as readily in the first three months of life (Rey, Pellie, Sivy, Blandin-Savoja, Rey and Frezal, 1974). As food consumption/body weight also decreases with age then the dietary burden of phenylalanine would also decrease and lessen the risk of hyperphenylalaninaemia and account for the apparent increased tolerance to phenylalanine after the age of one year.

As a general observation on the value of phenylalanine loading tests, differences in the ways in which phenylalanine is handled may well prove to be best illustrated by intravenous infusion, the mode of clearance can be seen as a distinct entity in contrast to the oral load when absorption and clearance occur together initially. More useful information might be forthcoming if sampling was continued for a longer period following oral loading but this would have to be nustified if the patient had to be starved for a prolonged period. No definitive statement could be found concerning the renal threshold of phenylalanine but from the phenylalanine levels in classical phenylketonuria following load one must assume it to lie between 1.0 and 2.0 m.mol/L.

CHAPTER X

MANIC-DEPRESSIVE PSYCHOSIS

INTRODUCTION

The role of tetrahydrobiopterin as a rate determinant in tyrosine hydroxylation (Kettler, Bartholini and Pletscher, 1974) make it a potentially interesting study in its relationship to mental disorders which are notorious for their putative biochemical lesions and absence of hard experimental evidence. The problems besetting any kind of investigation in this field are legion, perhaps the most troublesome being an absence of virgin cases in or attending institutions. General practitioners invariably treat patients before referring them for specialist attention. Another difficulty lies in the diagnosis which, although in some cases defined by strict criteria, may vary depending on the medical practitioner.

The possibility of catecholamine synthesis being implicated in psychoses is heightened by the mode of action of various drugs effective in improving some symptoms of schizophrenia. These drugs are phenothiazines, of which the best known and most commonly used is chlorpromazine, which block dopamine receptors and prevent the behavioural excitation and vomitting induced in experimental animals by apomorphine which acts as dopamine mimetic at central nervous system dopamine receptors (Iverson, 1976).

In addition to beneficial effects, some drugs have side-effects which alter mood. Iatrogenic depression has been noted for around a quarter of a century with reserpine as an early candidate (Freis, 1954; Doyle and Smirk, 1954) followed by a host of others including oestrogens, corticosteroids and tryptophan (Knox and Auerbach, 1955) which stimulate tryptophan

pyrrolase and may cause depression by diverting tryptophan from monoamine production (Curzon, 1969) in the brain. Suicidal patients have been shown to consume greater quantities of potentially depressing drugs than matched controls and with the example of the extreme depression caused by fluphenazine and flupenthixol in schizophrenia, patients with a pre-disposition to mental disorders must be prescribed depressing drugs with caution (Baker, Bartholomeuz, Siskind and Whitlock, 1977).

With the increase in use and potency of psycho-active drugs there has not been an equal increase in knowledge of their in-vivo action in either the long or the short term. Therefore it would be of value to look at any part of catecholamine metabolism in relationship to drugs which affect mood and personality. It was with this in mind that this study was undertaken. The Crithidia fasciculata assay is sensitive and specific (Leeming, 1975) although there is normally the limitation of only having serum for examination which may be further complicated by the intervention of the blood/brain barrier (Kettler, Bartholini and Pletscher, 1974). Gross variation in biosynthesis may be reflected in serum levels (Leeming and Blair, 1974).

The synthesis of biopterin in man is sensitive to the administration of phenylalanine (Leeming, Blair, Green and Raine, 1976), methotrexate and septrin (Leeming, Blair, Melikian and O'Gorman, 1976). A number of drugs used in psychiatric medicine as well as neurotransmitters have been tested for in-vitro effects on synthesis and reduction of dihydrobiopterin (Chapters II to IV). Synthesis was increased by benzodiazepines, tricyclic anti-depressants, noradrenaline, dopa, dopamine and serotonin but decreased by adrenaline. Dihydropteridine reductase activity was decreased by lithium, benzodiazepines and adrenaline but increased by dopa, dopamine and serotonin. Chlorpromazine had no effect.

Identifiable groups of patients have been shown to have altered serum Crithidia factor levels (Leeming, Blair, Melikian and O'Gorman, 1976; Baker, Frank, Bacchi and Hutner, 1974) and a group of treated

schizophrenics had a statistically lowered mean value (Leeming, Blair, Melikian and O'Gorman, 1976). The main group of psychiatric patients presented here were in attendance at a lithium clinic and receiving out-patient therapy for manic-depressive and depressive illnesses.

The intracellular concentration of lithium during therapy is similar to those of sodium and magnesium and is maintained between 0.6 and 1.2 m.mol/L. At 3.0 m.mol/L. it is toxic and if sustained rapidly produces irreversible changes and death. Its beneficial effects on manic patients was first shown in 1949 (Cade). Side-effects are many and a subjective list is as follows (Ghose, 1977) in Table 10-1. The therapeutic dose depends on body size and renal efficiency.

The reported benefits of lithium in alcoholism (Merry, Reynolds, Bailey and Coppen, 1976) migraine (Kudrow, 1976) and its possible uses in thyroid disorders (Lazarus, Richards, Addison and Owen, 1974), if they do not make it a creditable panacea, suggest that its range of biological effects is wide; this has been shown in review (Schou, 1976). How these biological effects are related to mental state and lithium is problematical, for example the gain in weight by some patients may be an effect of inhibition of hormone secretion by the thyroid (Berens and Wolff, 1976) on water and electrolyte metabolism, but could equally be consequent on improved appetite because of improvement in mental health. The thirst of patients on lithium is probably associated with the lithium induced polyuria which is itself positively associated with nephrogenic diabetes insipidus (Baylis and Heath, 1977). These multiple side-effects, particularly those of electrolyte and glucose metabolism could reasonably be expected to alter the ability of tetrahydrobiopterin to pass cell wall barriers. Tetrahydrobiopterin is poorly absorbed by the bowel (Chapter V) does not pass the blood/brain barrier (Kettler, Bartholini and Pletscher, 1974). At higher levels than those attained in therapy, lithium carbonate depresses synthesis of Crithidia factor in-vitro (Chapter II) by 29% at 2×10^{-2}M and reduces dihydropteridine reductase activity by 7% at 18×10^{-3}M (Chapter III).

TABLE 10-1

STANDARDISED CHECKLIST ADMINISTERED REGULARLY TO PATIENTS ATTENDING LITHIUM CLINIC AT MRC NEUROPSYCHIATRY LABORATORY.

Sickness	Abnormal noise in ears
Vomiting	Difficulty in speech
Diarrhoea	Feeling of unreality
Constipation	Poor memory
Poor appetite	Flushing attacks
Dry mouth	Increased sweating
Indigestion	Stuffy nose
Metallic taste	Trouble starting urination
Excessive thirst	Passing urine too often
Headache	Passing more than usual quantity of urine.
Drowsiness	Palpitation
Dizziness or giddiness	Ankle swelling
Fainting attacks or lightheadedness	Weight gain
Stiffness of arms and legs	Goitre
Spontaneous twitching (Contraction) of muscles	Skin rash
Trembling hands	Aches and pains over the muscles and joints
Blurred vision	Weakness of legs and arms.
Double vision	Unsteady walk

Score according to nil (=0), mild (=1) moderate (=2) and severe (=3) symptoms.

MATERIALS AND METHODS

Specimens from patients were obtained with informed consent and the co-operation of D.H. White, Consultant Psychiatrist at the Midland Nerve Hospital.

Over a three month period eighty-two samples of blood were taken from 22 males and 36 females with either bipolar manic-depressive psychosis or unipolar depression being treated with lithium carbonate in slow release formulation ("Priadel" regd.) orally. The patients were attending an out-patient clinic. At each attendance serum lithium was measured by a standard flame-photometric method, microbiological assays were carried out for Crithidia factor (Crithidia fasciculata), folate (Lactobacillus casei) and vitamin B_{12} (Lactobacillus leichmannii). 41 of the patients had a single blood sample taken for these three estimations, 12 had two samples, 3 had three samples and 2 had four samples. As far as could be ascertained only 19 patients were taking lithium alone, the remainder were having one or more additional drugs. 11 patients were identified as 'poor responders' to lithium and 4 as 'unequivocally good responders'.

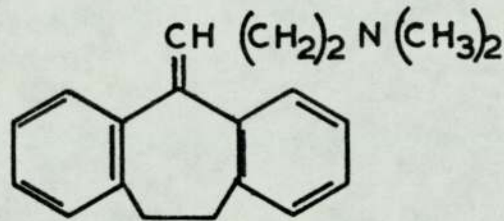
6 serum samples were taken from a woman maintained on low doses of Clomipramine (Anafranil) over a period of 8 weeks. The dose was contained in 25mg tablets of which she took one every day for a week, one on alternate days then one every third day and finally discontinued. A complete list of drugs being used on these patients is given in Table 10-2.

TABLE 10 - 2

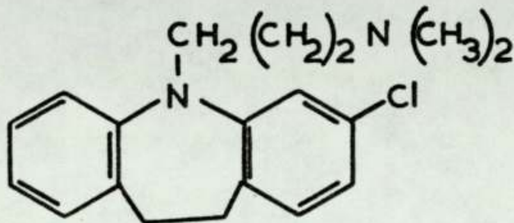
DRUGS BEING USED ON PATIENTS WITH MANIC-DEPRESSIVE AND DEPRESSIVE ILLNESS IN THIS STUDY

ANTI- DEPRESSANTS

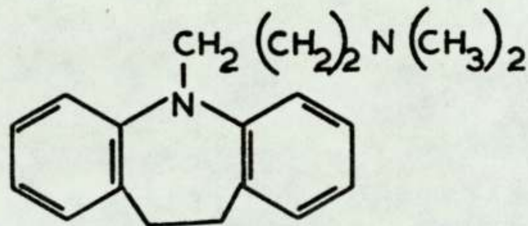
Amitriptyline



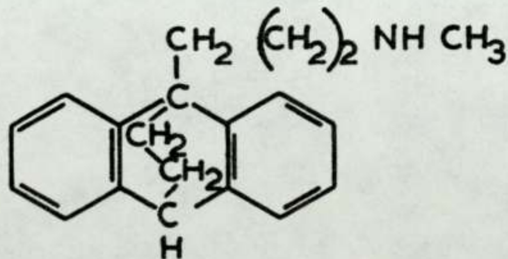
Clomipramine



Imipramine



Maprotiline



Doxepin

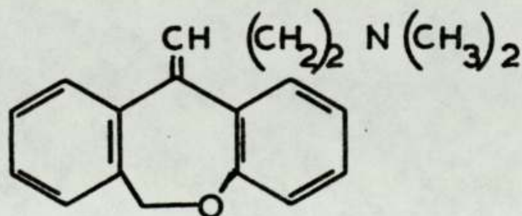
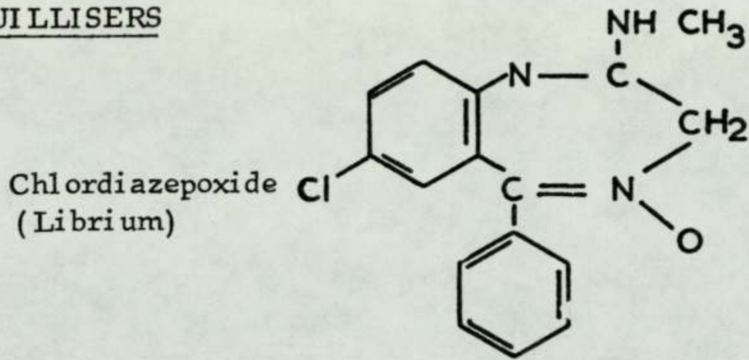


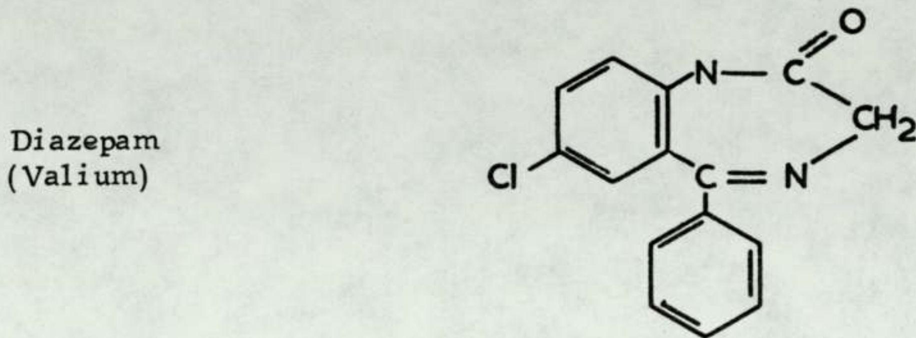
TABLE 10 - 2 (continued)

TRANQUILLISERS

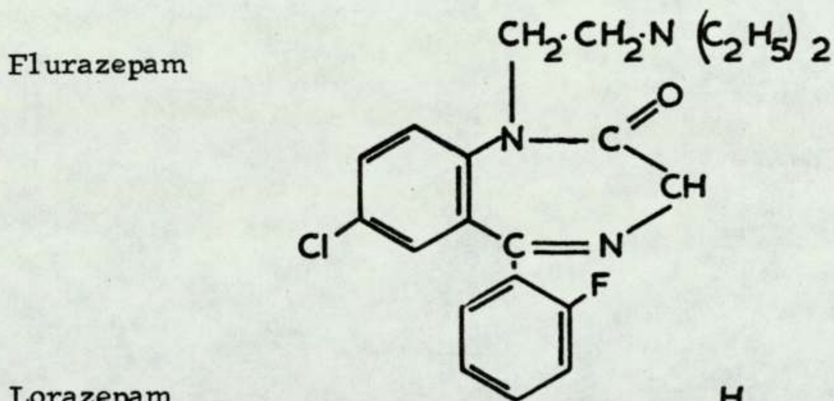
Chlordiazepoxide (Librium)



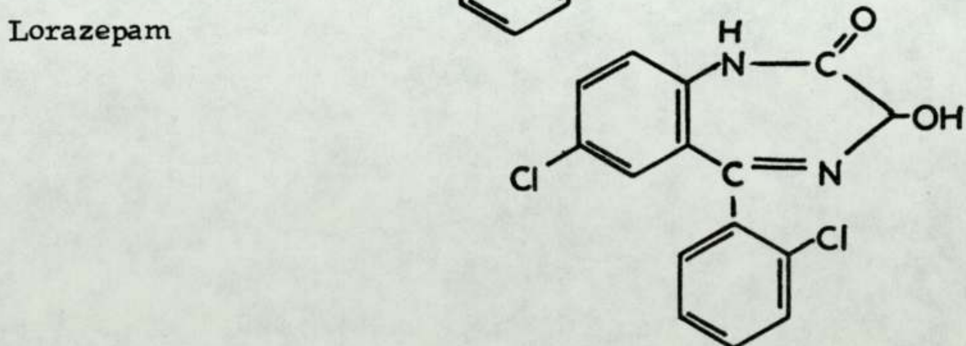
Diazepam (Valium)



Flurazepam



Lorazepam



Nitrazepam

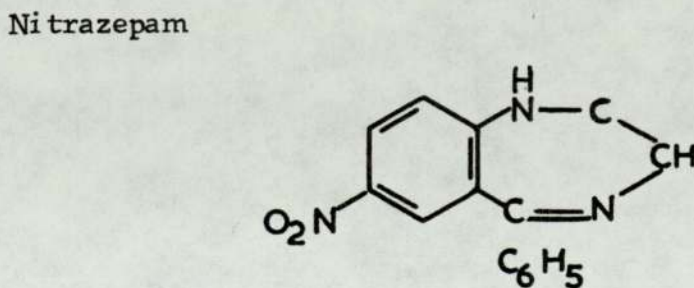
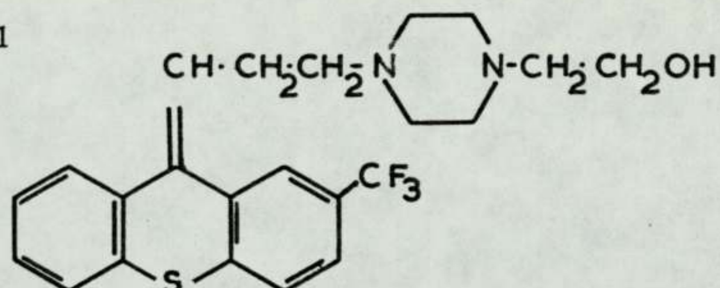


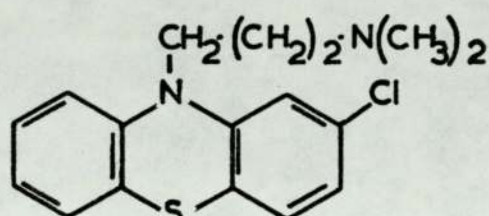
TABLE 10 - 2 (continued)

PHENOTHIAZINES

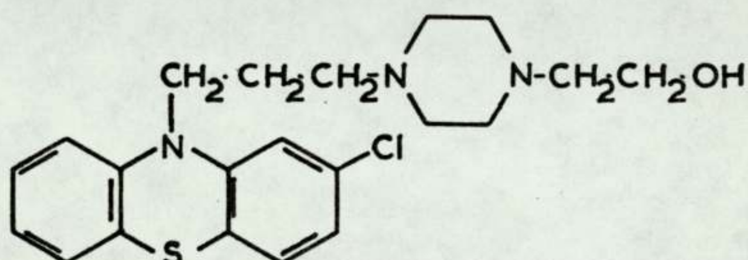
Flupenthixol



Chlorpromazine

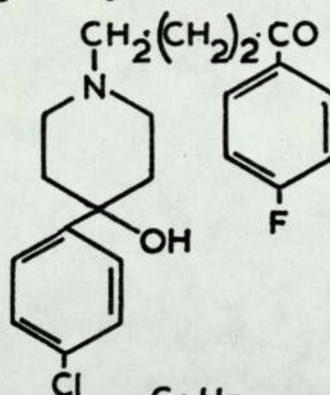


Perphenazine



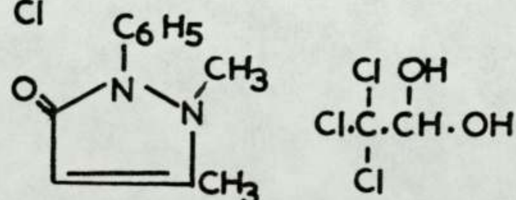
BUTYROPHENONES

Haloperidol



HYPNOTICS

Dichloralphenazone



STIMULANTS

Amphetamine

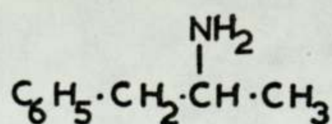
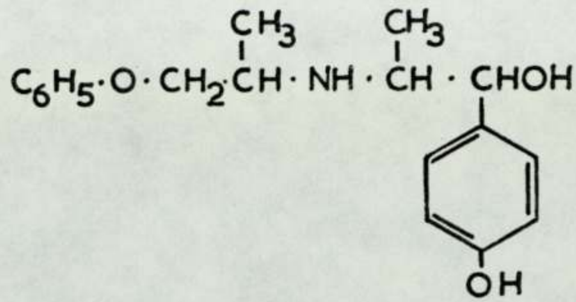


TABLE 10 - 2 (continued)

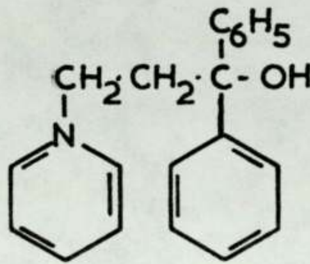
VASODILATOR

Isoxsuprine

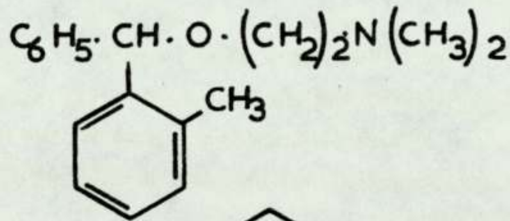


ANTI-PARKINSON DRUGS (Used to counteract side effects of other drugs)

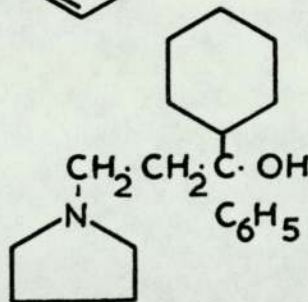
Benzhexol



Orphenadrine



Procyclidine



VITAMIN COMPLEX (B and C)

'Orovite'

RESULTS

The difference in serum Crithidia factor values for all the patients on lithium between males (2.26 ± 0.14μg/L.) and females (1.87 ± 0.08 μg/L.) was significant by Student's 't' test ($p = < 0.05$). The mean value for males was significantly above that of normal males (Chapter VI) $p = < 0.02$. The females in the survey also had a raised mean when compared with normal females - $p = < 0.001$.

Table 10-3 shows the serum Crithidia factor levels in all the patients together with the drugs being used; where several samples were obtained from one patient only the first was included to avoid idiosyncratic weighting of the data. The combinations of drugs were diverse and the inclusion of a number of patients under one heading in Table 10-3 does not mean that they were being treated exclusively with that drug and lithium, the exception being the group labelled 'Lithium alone'. Although a few high values are given, the only group large enough to give statistically useful information was the one where lithium was used with concurrent tricyclic antidepressants, but the level - 2.44 ± 0.26μg/L. just failed to reach the 5% level of significance by Student's 't' test ($p = 0.05 - 0.10$). A plot of serum Crithidia factor values against lithium (Figure 10-1) showed no correlation until a lithium level of 1.0m.mol/L. was reached when the corresponding serum Crithidia factor values were increased. Unfortunately no values of lithium above 1.2 m.mol/L. were obtained. A similar sort of relationship was noted between lithium and folate in serum (Figure 10-2). These observations have little statisically valid significance because of the short range in lithium levels available in therapeutic maintenance. The few patients with lithium values of 1.0 m.mol/L. and above were separated into those taking tricyclic antidepressants (Crithidia factor 2.67 ± 0.31μg/L) and those not taking them (Crithidia factor 2.08 ± 0.31μg/L) but the difference between the mean Crithidia factor levels was not significant ($p = 0.05 - 0.1$). Although those taking tricyclic antidepressants had a

TABLE 10-3

MEAN SERUM CRITHIDIA FACTOR AND LITHIUM LEVELS LISTED ACCORDING TO DRUGS USED

	No.	Crithidia factor	Lithium
Tricyclic antidepressants	17	2.44 ± 0.26	0.74 ± 0.07
Benzodiazepines	5	1.52 ± 0.14	0.70 ± 0.10
Phenothiazines	16	1.84 ± 0.20	0.75 ± 0.06
Barbiturates	1	2.3	1.1
Butyrophenones	5	2.2 ± 0.57	0.72 ± 0.11
Amphetamines	1	2.3	1.1
Isoxsuprine	1	3.7	0.9
Orovite	1	1.1	0.8
Trifluperazine	1	2.6	0.9
Anti Parkinson drugs (not L-dopa) Benxhexol & Procyclidine	11	1.85 ± 0.21	0.75 ± 0.07
Larazipan	1	1.8	1.0
Dichloralphenazone	1	1.8	1.0
Propanodol	2	2.05	0.7
Lithium alone	19	1.88 ± 0.09	0.72 ± 0.04
All males	(36)	2.26 ± 0.14	0.75 ± 0.04
All females	(46)	1.87 ± 0.08	0.71 ± 0.03

FIGURE 10-1

MEAN SERUM CRITHIDIA FACTOR LEVELS PLOTTED AGAINST SERUM LITHIUM

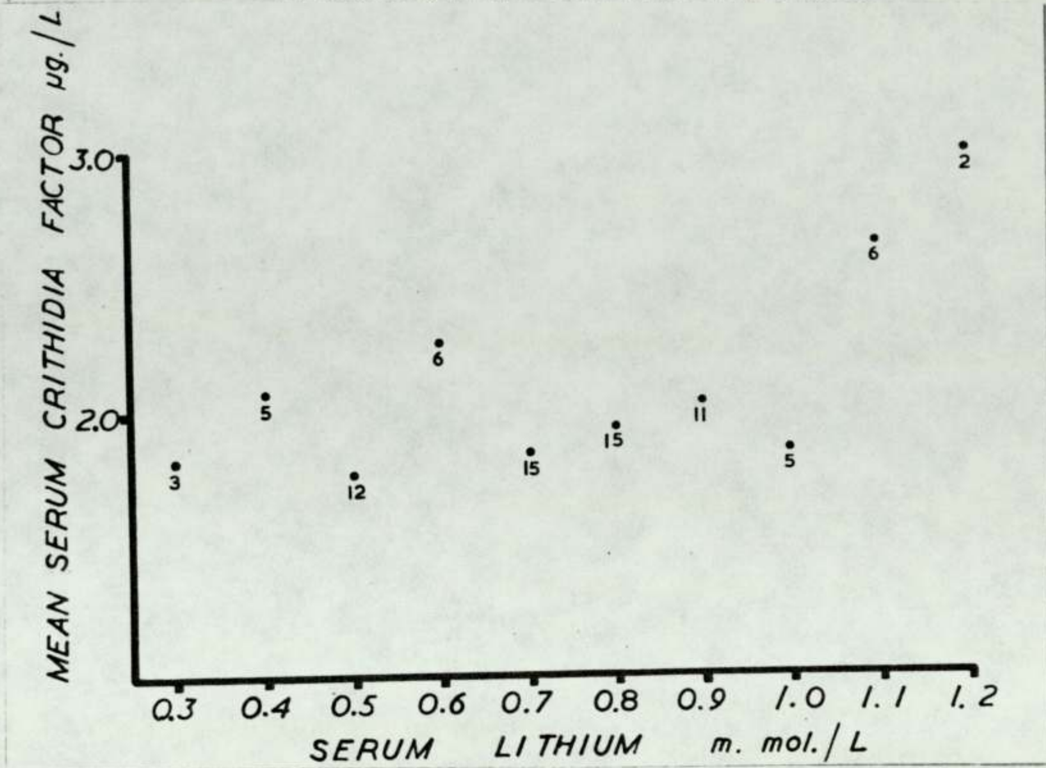
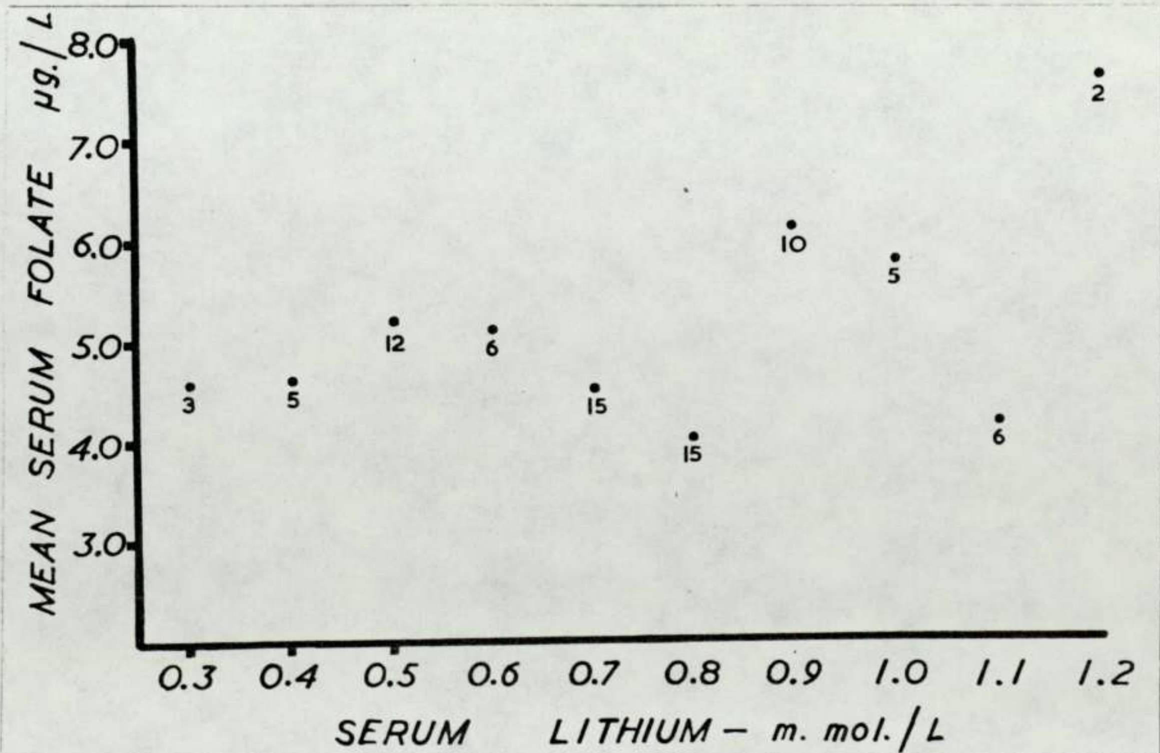


FIGURE 10-2

MEAN SERUM FOLATE LEVEL PLOTTED AGAINST SERUM LITHIUM



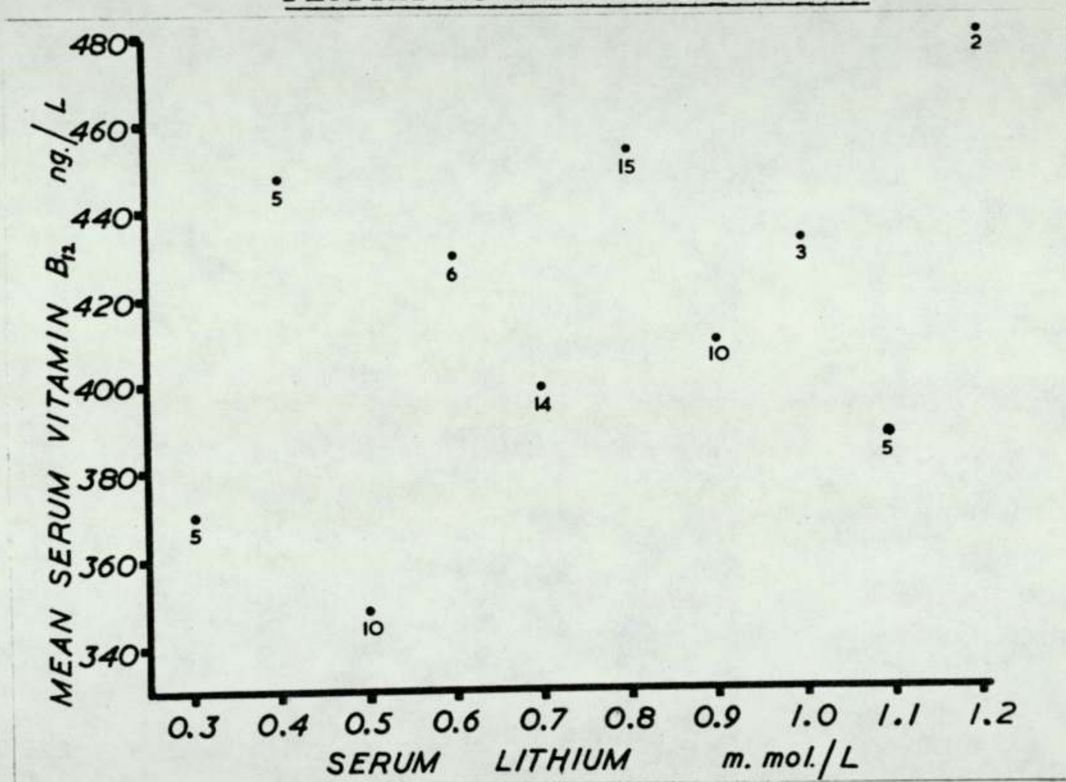
significantly higher level than normal ($p = 0.01$).

A group of eleven patients identified as unequivocally good responders were compared with four poor responders to lithium therapy; mean serum Crithidia factor values were very similar at 2.28 ± 0.15µg/L. and 2.33 ± p.45µg/L. Vitamin B_{12} levels with the exception of one patient who proved to have megaloblastic anaemia, were within the normal range and did not correlate with serum lithium (Figure 10-3).

FIGURE 10-3

MEAN SERUM VITAMIN B12 LEVEL

PLOTTED AGAINST SERUM LITHIUM



The woman who took decreasing doses of 'Anafranil' did not show dose correlated serum Crithidia factor levels and the mean for the six samples was 1.77 ± 0.16µg/L, which was close to the mean for her age and sex.(Chapter VI - 1.70 ± 0.08µg/L.).

DISCUSSION

Crithidia factor is significantly ($p = < 0.02$) increased in the serum of manic-depressive and depressive patients being treated with lithium.

It seems reasonable to suggest that the high levels of Crithidia factor found in the serum is due to an increased rate of tetrahydrobiopterin biosynthesis in this disease. If this is so then this disease is the first to be recorded in which a high serum Crithidia factor level reflects increased brain biosynthesis of tetrahydrobiopterin. Later in this thesis, theoretical arguments will be advanced that increased tetrahydrobiopterin synthesis or levels will not result in increased serum Crithidia factor levels.

Another possibility is that treatment of these psychoses with lithium results in increased serum Crithidia factor levels. Raised serum Crithidia factor with reduced neurotransmitter formation, presumably due to lowered levels of cellular tetrahydrobiopterin, are found in dihydropteridine reductase deficient malignant hyperphenylalaninaemia (Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977). Dihydropteridine reductase inhibitors increase serum Crithidia factor levels (Leeming, Blair, Melikian and O'Gorman, 1976) and reduce the rate of phenylalanine hydroxylation (Goodfriend and Kaufman, 1961); the latter suggesting that they also lower cellular tetrahydrobiopterin levels. Lithium salts at 18×10^{-3}M concentration reduce dihydropteridine reductase by 7%, but this concentration is much higher than the therapeutic level in serum (circa M^{-3}). Thus the higher levels of serum Crithidia factor in these patients is unlikely to have arisen from inhibition of dihydropteridine reductase by lithium.

From similar serum Crithidia factor levels in good and poor responders to lithium ($2.28 \pm 0.15\mu g/L.$ and $2.33 \pm 0.45\mu g/L.$, respectively) as defined by Misra and Burns (1977), the increase in serum biopterin derivatives in manic-depressive patients on lithium is the same in patients

with and without remission of their symptoms.

There is evidence that drugs which alleviate manic symptoms do so by reducing dopamine levels (Sack and Goodwin, 1974). Serum serotonin levels have been shown to be significantly lower in manic-depressive patients in depression but not those in mania, when compared with controls (Kaneko, Hayashi, Unno, Wanatabe, Takahashi, Takano and Kumashiro, 1975). Cerebrospinal fluid 5-hydroxyindolacetic acid was shown to be significantly lower than controls in depression and mania and in depressive patients after recovery (Coppen, 1971) suggesting that a constant feature of the disease both in and out of remission is a reduced biosynthesis of serotonin.

Perhaps the most reasonable suggestion for the increased serum Crithidia factor levels in manic-depressive psychoses is a reduced activity of dihydropteridine reductase confined to areas of the brain concerned with serotonin biosynthesis. This would explain why both reduced serotonin biosynthesis and increased serum Crithidia factor levels are found in both the active disease and in remission.

The drugs, other than lithium which appeared to be associated with changes in serum biopterin derivative levels were the tricyclic anti-depressants. However, all patients in this sample were taking lithium and no significant changes could be associated with any other treatment.

A serum Crithidia factor value of 4.2μg/L in a patient whose lithium level was 1.1 m.mol/L and who was taking the butyrophenone, haloperidol excited interest as this drug causes a toxic neurological reaction with lithium, manifested by rigidity, ataxia and oral dyskinesia (Loudon and Waring, 1976). No other high level of lithium or Crithidia factor was found in the few cases of this dual therapy which is avoided but for a few exceptional cases of mania or schizo-affective disorders.

CHAPTER XI

DISEASE AND PREGNANCY

INTRODUCTION

Urines and sera from a number of patients with physical and mental diseases have been assayed for Crithidia factor (Leeming, 1975; Leeming, Blair, Melikian and O'Gorman, 1976; Broquist and Fleming, 1967; Baker, Frank, Bacchi and Hutner, 1974). An earlier chapter of this thesis has dealt with situations in which specific disorders of tetrahydrobiopterin metabolism have caused a clear cut syndrome and hyperphenylalaninaemia (Chapter VIII). Availability of specimens being taken for other investigations and the co-operation of clinicians allowed a broader approach to human disease. Reduced serum levels of Crithidia factor in proliferative disorders prompted an investigation into malignant carcinoid disease where large quantities of serotonin are produced and its metabolites are excreted in the urine (Sjoerdsma, Weisbach and Udenfriend, 1956). Senile dementia which afflicts some 20% of people at 80 years of age was another obvious choice in that cerebral function is diminished.

Earlier observations on phenylalanine and Crithidia factor in the serum of a normal subject taking a low phenylalanine diet showed that as the phenylalanine level decreased so did the Crithidia factor (Leeming, 1975; Leeming, Blair, Green and Raine, 1976). In anorexia nervosa, patients are fed on normally balanced diets of 3,000 calories, under observation, (Crisp and Stonehill, 1971) and one might therefore expect to see a rise in plasma amino acids with concurrent increase in serum Crithidia factor, this would be difficult to mimic in normals.

Hartnup's disease (Baron, Dent, Harris, Hart and Jepson, 1956) is a rare condition in which there is a grossly increased renal clearance of amino acids by virtue of a defect in renal tubular reabsorption. The aminoaciduria includes phenylalanine, tyrosine and tryptophan. There is also an amino acid transport defect across the mucosal cells of the jejunum (Milne, Crawford, Giras and Loughbridge, 1960). Hartnup's disease would be useful as a comparison to phenylketonuria, as another disease in which there is abnormal handling of phenylalanine.

Phenylketonuria is one example of potentially mentally disabling disease and has already been discussed (Chapters VIII and IX). Therefore other groups of mentally handicapped patients would form a useful comparative study.

Methotrexate increases the serum Crithidia factor in normal subjects (Leeming, Blair, Melikian and O'Gorman, 1976) and increases the synthesis of Crithidia factor (Chapter II) whilst decreasing dihydropteridine reductase activity (Chapter III) (Craine, Hall and Kaufman, 1972). In view of the neurological sequelae of methotrexate therapy in children (Eiser, 1978; Meadows and Evans, 1976) further study of the action of methotrexate in-vivo could shed light on its effects on neurotransmitter production.

MATERIALS AND METHODS

Samples were made available from patients by clinicians in charge of their cases; informed consent was obtained where appropriate. In nearly every situation specimens were being taken for other diagnostic tests and aliquots were set aside for use in this study.

Specimens were obtained from a total of six patients with malignant carcinoid disease, these comprised five sera, three urines and three samples of fresh frozen tissue obtained at operation.

Serum samples from five autistic children were made available by a child psychiatrist and serum from seven patients with disseminated sclerosis and from a child with Hartnup's disease were supplied by clinicians.

Samples of serum were obtained from 63 in-patients with ages ranging from 57 to 97 years, in a district general hospital. These elderly patients were subsequently divided into three groups, without sight of the laboratory results, by the consultant in charge of their medical care. These groups were:

1. Senile dementia - A progressive and irreversible loss of coherent thought and social awareness.

2. Confusion - A state from which recovery was anticipated.

3. Normal

Six patients with senile dementia had phenylalanine loads of 7g., serum Crithidia factor, phenylalanine and tyrosine were measured at hourly intervals for four hours.

Thirty-two samples of blood were obtained from patients

attending a gastro-intestinal unit, they all had coeliac disease. Five were untreated and the others were being treated by prohibition of dietary gluten although there may have been variation in adherence to diet.

Serum specimens were obtained from ninety males and fifty-seven females in a special hospital for the mentally handicapped.

Serum samples were provided from forty-four pregnant women in the third trimester.

Five patients with anorexia nervosa and hospitalised whilst being given normally balanced 3,000 calorie diets had serum phenylalanine and Crithidia factor measured for up to six and a half months.

Serum and whole blood in E.D.T.A. were collected from forty-two workers in industries where lead was being handled. Blood lead and serum Crithidia factor were measured.

A serum sample was obtained from a twelve year old boy with lead poisoning whose whole blood lead was 50μg/100cm^3. A serum sample was also obtained from an adult male diagnosed as suffering from lead poisoning, in this case the lead value was not available as the patient's notes had been mislaid when this work was being put together. However, the serum Crithidia value was recorded.

Serum samples were available from thirty-two children receiving methotrexate therapy for acute lymphoblastic leukaemia; six of these patients had two samples taken, the first immediately before a dose and the second one hour afterwards. One adult patient aged thirty-four years had high dose methotrexate as part of his treatment for acute lymphoblastic leukaemia. 950mg. methotrexate was given by intravenous infusion followed six hours later by 25mg 5-formyltetrahydrofolic acid and three 15mg. oral doses of 5-formyltetrahydrofolic acid at six hourly intervals.

RESULTS

The results of the Crithidia assays on samples from the various groups of patients are given in Table 11-1 together with serum controls (Chapter VI) and urine controls (Leeming and Blair, 1974). The mean serum Crithidia factor of the male controls ($1.75 \pm 0.03 \mu g/L.$) was significantly ($p = < 0.001$) higher than the mean of the female control group ($1.53 \pm 0.10 \mu g/L.$).

Both serum and urine from patients with malignant carcinoid had mean Crithidia factor levels which were significantly below the levels of controls ($p = < 0.001$ and $p = < 0.05$ respectively); the serum value was $1.16 \pm 0.07 \mu g/L.$ and the urine 0.89 ± 0.09 mg/L.

The mean serum Crithidia factor of patients with active untreated coeliac disease ($1.02 \pm 0.09 \mu g/L$) was significantly less than that of normal controls ($p = < 0.001$) and below that of coeliac patients being treated ($1.38 \pm 0.10 \mu g/L$) ($p = < 0.05$) whereas the means of all the treated patients and of males alone were lower than, but not significantly different from controls ($p = > 0.05$). Treated female coeliacs had a significantly lower mean than female controls ($p = < 0.05$).

Patients with disseminated sclerosis did not have a significantly lower serum Crithidia factor than normal ($1.23 \pm 0.17 \mu g/L$). The pregnant women had a mean serum Crithidia factor of $1.20 \pm 0.03 \mu g/L$ which was significantly lower ($p = < 0.001$) than normal non-pregnant females ($1.53 \pm 0.04 \mu g/L$).

The elderly normal patients had a mean serum Crithidia factor ($1.96 \pm 0.18 \mu g/L$) higher than normal controls but not significantly so ($p = > 0.05$). The confused elderly patients had a mean serum Crithidia factor of $1.83 \pm 0.14 \mu g/L$ which was not significantly different from elderly controls ($p = > 0.05$). However, the patients with senile dementia had a significantly ($p = < 0.001$) lower mean serum value ($1.01 \pm 0.12 \mu g/L$)

TABLE 11-1

CRITHIDIA FACTOR LEVELS IN NORMAL SUBJECTS AND DISEASED PATIENTS

(No)	Clinical Condition	Sample	Range	Mean ± S.E.M.
	ADULTS 16-66years			
75	Normal males	serum	1.0 - 3.5µg/L	1.75 ± 0.03µg/L
156	" females	serum	0.6 - 3.1µg/L	1.53 ± 0.04µg/L
231	" males-females	serum	0.6 - 3.5µg/L	1.60 ± 0.03µg/L
60	" males-females	urine	0.34-6.9µg/L	2.10 ± 0.32µg/L
5	Malignant carcinoid	serum	1.0 - 1.4µg/L	1.16 ± 0.07µg/L
3	" "	urine	0.75-1.05mg/L	0.89 ± 0.09µg/L
3	" "	tissue	365-898ng/g	650 ± 182µg/L
7	Disseminated sclerosis	serum	0.8 -2.0 µg/L	1.23 ± 0.17µg/L
1	Male with lead poisoning	serum	1.0µg/L	
5	Coeliac disease -untreated	serum	0.8 - 1.3µg/L	1.02 ± 0.09µg/L
26	" " -treated	serum	0.7 - 2.9 µg/L	1.38 ± 0.10ug/L
17	Females with treated coeliac disease	serum	0.8 - 2.1	1.30 ± 0.08µg/L
9	Males " " "	serum	0.7 - 2.9	1.53 ± 0.22µg/L
44	Pregnant women	serum	0.8 - 1.4µg/L	1.20 ± 0.03µg/L
	ELDERLY 57-92 years			
21	Normal	serum	0.6 - 3.8µg/L	1.96 ± 0.18µg/L
27	Confused	serum	1.3 - 3.5µg/L	1.83 ± 0.14µg/L
15	Senile dementia	serum	0.3 - 1.7µg/L	1.01 ± 0.12µg/L
	MENTALLY DISABLED			
90	Males 10-55 years	serum	0.9 - 3.0µg/L	1.45 ± 0.02µg/L
57	Females 9-48 years	serum	1.0 - 2.5µg/L	1.65 ± 0.05µg/L

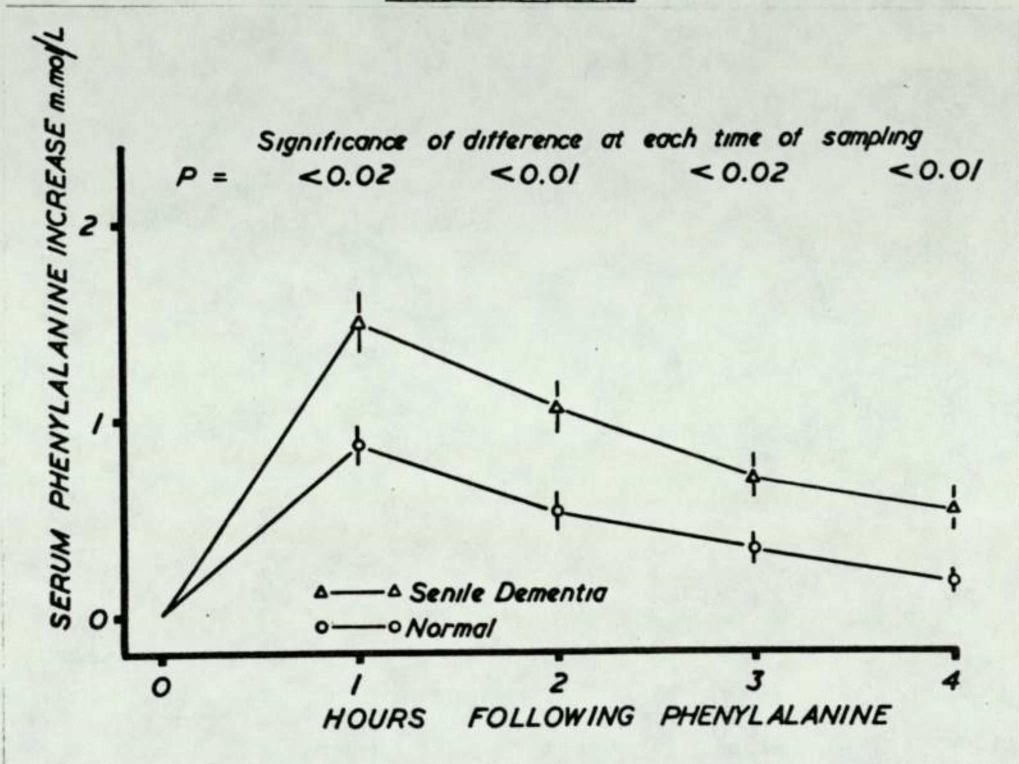
TABLE 11-1
(Continued)

	CHILDREN 2-12 years			
10	Normal	serum	1.1 - 3.7μg/L	1.78 ± 0.25μg/L
5	Autistic	serum	1.2 - 2.0μg/L	1.58 ± 0.23μg/L
1	Hartnup's disease	serum	0.9μg/L	
1	Lead poisoning	serum	0.8μg/L	
	Acute Lymphoblastic leukaemia			
28	On methotrexate	serum	1.5 -16.0μg/L	4.40 ± 0.54
6	Immediately before dose	serum	4.0 - 7.8μg/L	5.55 ± 0.53
6	1hr. after dose	serum	3.4 - 8.0μg/L	5.02 ± 0.64

than both normal controls and elderly controls. The phenylalanine clearance tests in six patients with senile dementia showed significantly higher increases ($p = < 0.02$, < 0.01, < 0.02 and < 0.01) in phenylalanine levels in serum at each hourly interval following load (Figure 11-1).

FIGURE 11-1

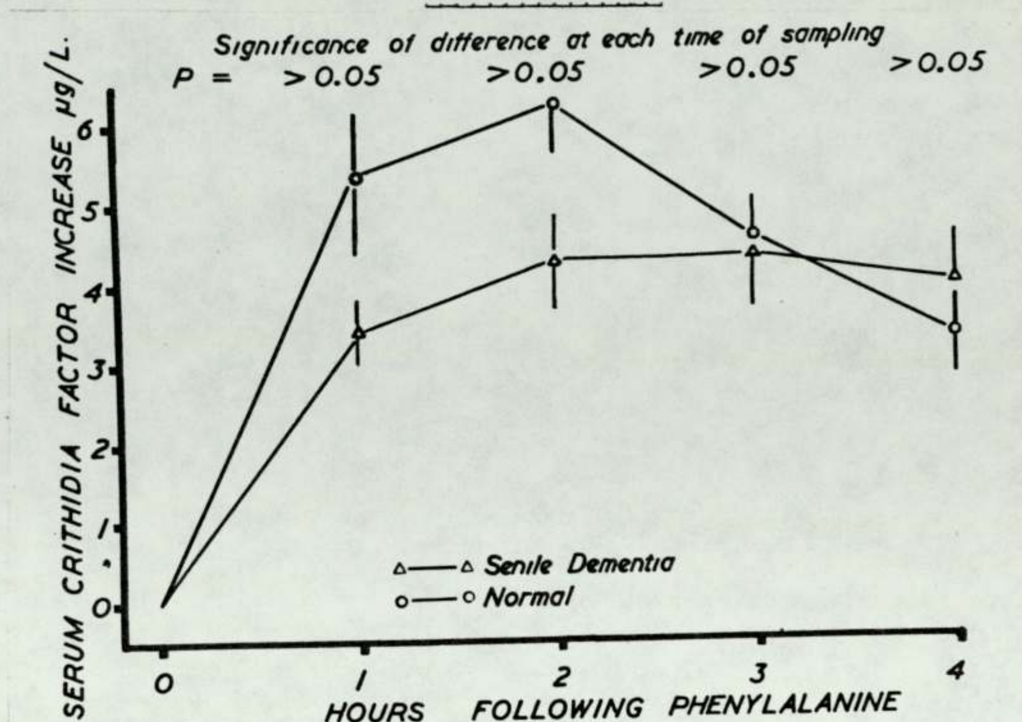
SERUM PHENYLALANINE INCREASE IN 6 PATIENTS WITH SENILE DEMENTIA FOLLOWING 7g L-PHENYLALANINE GIVEN ORALLY



The serum Crithidia factor increase (Figure 11-2) was not significantly different ($p = > 0.05$) although there was an apparently slower response in Crithidia factor in demented patients. Serum tyrosine levels were not significantly different until the fourth hour ($p = < 0.001$ - Figure 11-3) when serum tyrosine was still rising whilst the level in normal controls was falling. In the fasting serum samples of these patients with senile dementia the mean serum Crithidia factor was $1.15 \pm 0.14\mu g/L$. compared with the mean fasting level of the controls before phenylalanine load - $1.78 \pm 0.07\mu g/L$. This difference was significant ($p = < 0.02$). The fasting phenylalanine level in the demented patients was 0.099 ± 0.003 m.mol/L. compared with 0.049 ± 0.008 m.mol/L in fasting controls ($p = < 0.01$).

FIGURE 11-2

SERUM CRITHIDIA FACTOR INCREASE IN 6 PATIENTS WITH SENILE DEMENTIA FOLLOWING 7g L-PHENYLALANINE GIVEN ORALLY



The mentally disabled males (Table 11-1) in the hospital for mentally handicapped had a significantly (p = < 0.001) lower mean serum Crithidia factor (1.45 $\pm$ 0.02µg/L) than normal males and mentally disabled females. The mean serum Crithidia factor of mentally disabled females (1.65 $\pm$ 0.05µg/L) was slightly above but not significantly (p = > 0.05) different from normal females.

The serum Crithidia factor, phenylalanine and tyrosine levels in the patients with anorexia nervosa are given in Table 11-2. There was no correlation between Crithidia factor and phenylalanine, tyrosine or the phenylalanine/tyrosine ratio.

There was no correlation between whole blood lead and serum Crithidia factor in the forty-two lead workers with the sample (Figure 11-4) viewed overall. However the lead values between 15 and 27µg/100 cm^3 can be correlated with serum Crithidia factor (R = 0.9038 - p = < 0.001). The adult male with lead poisoning had a serum Crithidia factor of 1.0µg/L. The child with lead poisoning had a serum Crithidia factor level of

TABLE 11-2

SERUM CRITHIDIA FACTOR, PHENYLALANINE AND TYROSINE IN ANOREXIA NERVOSA PATIENTS, HOSPITALISED AND ON 3,000 CALORIE NORMAL DIETS

	Date	20/1	23/1	27/1	30/1	3/2	8/2	15/2	17/2	20/2	22/2	
	Crithidia factor	1.4	1.7	1.5	1.9	1.5	1.8	1.8	1.8	1.9	1.7	μg/L.
R.F.	Phenylalanine	65	67	63	69	64	85	72	84	64	69	μmol/L.
	Tyrosine	49	57	69	77	52	81	61	64	58	61	μmol/L.

	Date	18/1	20/1	23/1	27/1	30/1	3/2	6/2	8/2	10/2	17/2	20/2	22/2	6/3	12/5	19/6	21/6	27/6	7/7	
	Crithidia factor	1.6	1.9	1.5	2.2	1.6	2.8	2.0	1.4	1.6	1.8	2.2	1.8	1.7	1.8	1.8	2.1	2.1	2.2	μg/L.
P.W.	Phenylalanine	52	59	61	69	77	69	65	100	48	64	68	68	69	69	80	65	74	73	μmol/L.
	Tyrosine	64	61	71	78	83	76	71	116	66	69	67	54	71	71	70	79	74	80	μmol/L.

	Date	19/6	21/6	7/7	
	Crithidia factor	2.0	2.0	2.9	μg/L.
D.P.	Phenylalanine	144	94	98	μmol/L.
	Tyrosine	111	78	98	μmol/L.

	Date	25/3	
	Crithidia factor	2.0	μg/L.
W.B.	Phenylalanine	100	μmol/L.
	Tyrosine	120	μmol/L.

FIGURE 11-3

SERUM TYROSINE INCREASE IN 6 PATIENTS WITH SENILE DEMENTIA FOLLOWING 7g L-PHENYLALANINE GIVEN ORALLY.

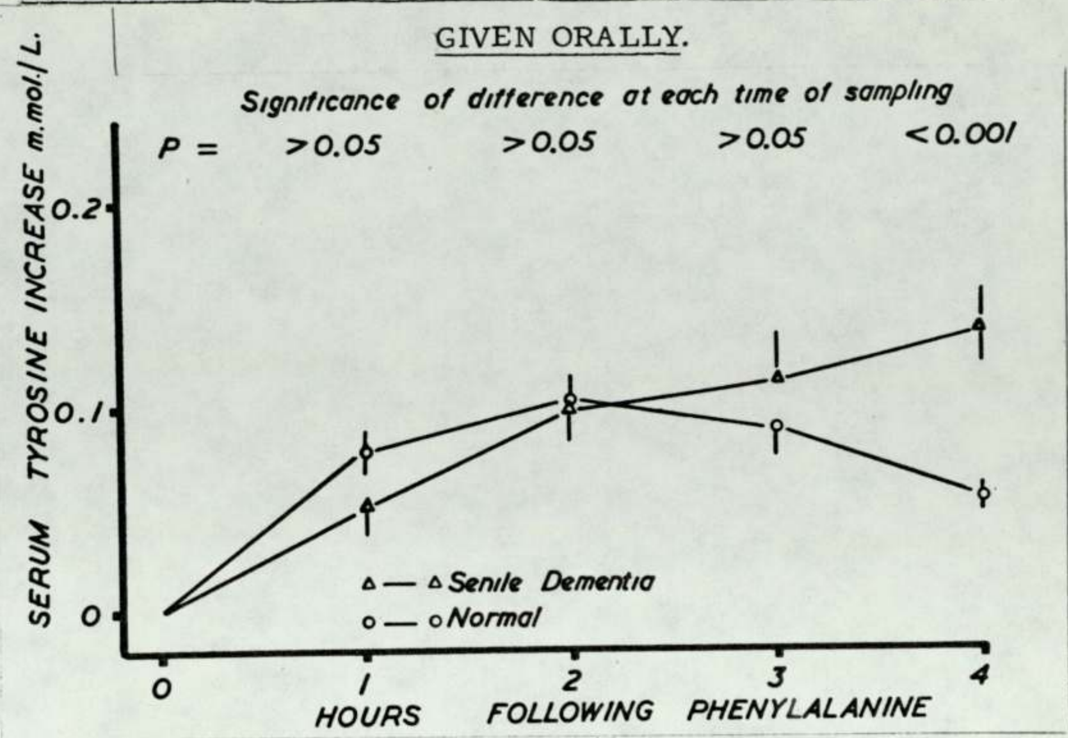
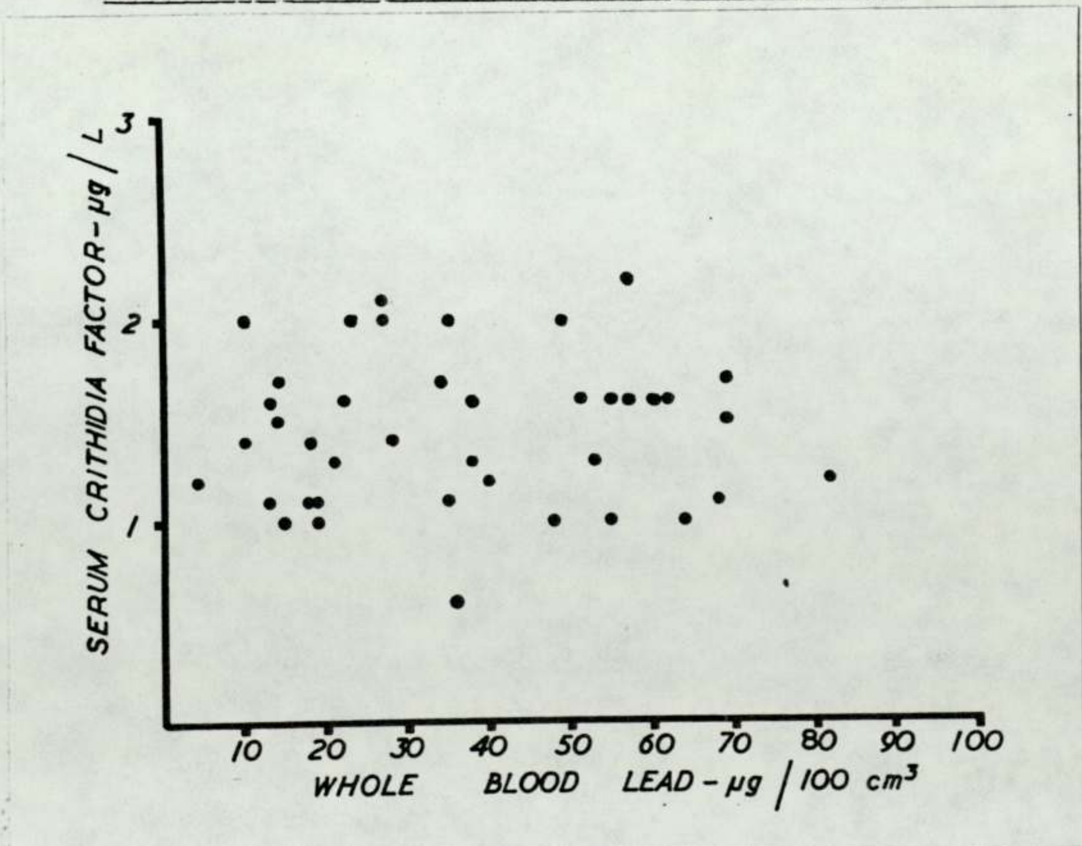


FIGURE 11-4

DISTRIBUTION OF SERUM CRITHIDIA FACTOR AND WHOLE BLOOD LEAD VALUES IN 42 WORKERS EXPOSED TO LEAD



0.8μg/L compared with the mean of 1.78$\pm$ 0.25 of his peer group controls, his phenylalanine and tyrosine levels were 0.07 and 0.05 m.mol/L.

The autistic children had a mean serum Crithidia factor level (1.58 $\pm$ 0.23μg/L.) not significantly different ($p = > 0.05$) from their peer group control (1.78 $\pm$ 0.25μg/L.).

The one patient with Hartnup's disease had a serum Crithidia factor value of 0.9μg/L which was the lowest of any child outside the group of male mentally disabled and the boy with lead poisoning.

The children with acute lymphoblastic leukaemia who were all being treated with methotrexate had a mean value of 4.40 $\pm$ 0.54μg/L. which was very significantly higher than control children ($p = < 0.001$). The difference between the serum Crithidia factor immediately before a methotrexate dose (5.55 $\pm$ 0.54μg/L.) and one hour afterwards (5.02 $\pm$ 0.64 μg/L.) was not significant ($p = > 0.05$).

The serum and urine Crithidia factor and methotrexate levels in the patient on high dose methotrexate as part of his therapy for acute lymphoblastic leukaemia are shown in Table 11-3. On two occasions the Crithidia factor level in his serum was still raised a fortnight after the last treatment. The urinary Crithidia factor reached 5.63mg/L. at twenty-four hours after the first dose of methotrexate but subsequent to later methotrexate it was within normal limits. Methotrexate was still detectable in urine two weeks after administration.

TABLE 11-3

SERUM AND URINE CRITHIDIA FACTOR AND METHOTREXATE FOLLOWING METHOTREXATE THERAPY AND 5-FORMYLTETRAHYDROFOLIC ACID RESCUE IN ADULT ACUTE LYMPHOBLASTIC LEUKAEMIA

HOURS	0	2	4	6	8	10	12	18	20	24	30	48
Methotrexate mg.	950											
5-formylTHF mg.				25			15	15		15		
5.12.77												
Serum Crithidia factor µg/L.	1.9	3.0	3.2	-	-	-	-	-	6.0	7.5	-	6.2
Serum MTX mg/L.	0	12.8	11.1	-	-	-	-	-	0.77	0.33	-	0.26
Urine Crithidia factor mg/L.	-	-	-	-	-	-	-	-	-	5.63	2.81	3.44
Urine MTX mg/L	-	-	-	-	-	-	-	-	-	276	12.4	9.2
19.12.77												
Serum Crithidia factor µg/L.	3.9	5.7	6.4	-	-	-	-	-	-	3.9	3.6	-
Serum MTX mg/L	0	8.41	16.03	-	-	-	-	-	-	0.18	0.11	-
Urine Crithidia factor mg/L.	1.60	-	-	-	-	-	-	-	-	1.13	1.83	-
Urine MTX mg/L.	0.047	-	-	-	-	-	-	-	-	23.3	14.25	-
3.1.78												
Serum Crithidia factor µg/L.	4.2	-	4.8	-	5.3	-	-	-	-	6.8	-	-
Serum MTX mg/L.	0	-	5.43	-	5.58	-	-	-	-	3.07	-	-
Urine Crithidia factor mg/L.	-	-	-	1.0	-	-	-	-	-	-	-	-
Urine MTX	-	-	-	42.65	-	-	-	-	-	-	-	-

TABLE 11-3
(Continued)

HOURS	0	2	4	6	8	10	12	18	20	24	30	48
2.2.78 (No further methotrexate)												
Serum Crithidia factor μg/L.	1.8	-	-	-	-	-	-	-	-	-	-	-
Serum MTX mg/L	0	-	-	-	-	-	-	-	-	-	-	-

DISCUSSION

Serum Crithidia factor levels are low in active cœliac disease. In treated patients the levels are not significantly different from normal. It follows that the low levels of serum Crithidia factor are a consequence of the disease process.

The mean serum Crithidia factor level in carcinoid tissue (650 ± 182 ng/g) was higher than the mean level in liver (Chapter VI, 375 ± 67 ng/g) and demonstrated that it is possible to have a bulk of tissue with high Crithidia factor content at the same time as a low serum Crithidia factor. The mean serum Crithidia factor in these patients was 1.16 μg/L. which was significantly ($p = <0.001$) below the mean level in normal controls. Carcinoid disease is distinctive in that it secretes large amounts of serotonin which are produced from tryptophan by hydroxylation to 5-hydroxytryptophan and subsequent decarboxylation to 5-hydroxytryptamine (serotonin). This process requires tetrahydrobiopterin and tryptophan hydroxylase in the initial step (Hosoda and Glick, 1966). If the hydroxylation of tryptophan is rapid and the reduction of quinonoid dihydrobiopterin by dihydropteridine reductase is slower then dihydropteridine reductase activity will determine the rate of the reaction; this may be the case in some tissues. The level of serotonin in carcinoid tissue is dependent on the supply of tryptophan in the diet (Smith, Nyhus, Dalgleish, Dutton, Lennox and MacFarlane, 1957) as well as on the number of functional cells in the tumour mass (Grahame-Smith, 1967). Therefore in carcinoid tissue, reduced pteridine cofactor is required for tryptophan 5-hydroxylation. (Grahame-Smith, 1972) but unless the tryptophan hydroxylase becomes saturated with the substrate dihydropteridine reductase will not be rate limiting and serum dihydrobiopterin levels will remain low.

The low serum Crithidia factor in pregnant women could be explained as a function of increased cellular replication as with proliferative disorders but plasma tyrosine concentrations in a group of eighteen pregnant women (Griffin and Elsas, 1975) were lower ($p = < 0.01$) than in twenty non-pregnant controls and with the use of the formula:

$\left(\frac{\text{Phenylalanine}}{\text{Tyrosine}}\right)^2$ (Griffin, Humienny, Hall and Elsas, 1973) were still significantly ($p = < 0.01$) higher than controls and did not overlap the raised values of phenylketonuric heterozygotes. This suggests lower levels of cellular tetrahydrobiopterin.

Anorexia nervosa patients showed no discernable trend in the relationship between serum Crithidia factor and amino acid level.

The elderly patients with senile dementia had a significantly low mean serum Crithidia factor level. Without a brain biopsy it is not easy to assign a definite pathology to patients with senile dementia although many may be indistinguishable from Alzheimer's disease which may prove to be identical to senile dementia in many cases (Lancet, 1976). Alzheimer's disease is a primary nerve-cell disorder (Bowen, Spillane, Curzon, Meier-Ruge, White, Goodhardt, Iwangoff and Davison, 1979) in which firm diagnosis may await autopsy. In an earlier chapter there appeared to be a positive relationship between age and serum Crithidia factor. If one of the consequences of senile dementia is a reduction in hydroxylase for phenylalanine, tyrosine and tryptophan, which themselves become rate-limiting then the tetrahydrobiopterin content of cells would increase and there would be less 7,8-dihydrobiopterin in the serum. Tyrosine hydroxylase is decreased in the senescent rat whereas dihydropteridine reductase is increased (Algeri, Bonati, Brunello and Ponzio, 1977) but whether the senescent rat has any of the hallmarks of senile dementia is unknown. The production of neurotransmitters is not solely governed by the hydroxylation of amino acids although this may prove to be the mechanism having the greatest influence. A general decrease in metabolic efficiency with advancing years and vascular degeneration may play an important part.

The significantly high levels of serum phenylalanine following phenylalanine loads in senile dementia compared with normal controls (Figure 11-1) show that some part of the phenylalanine hydroxylation system is depleted. Serum Crithidia factor was raised but was not significantly higher than normal (Figure 11-2), however the increase in serum tyrosine

(Figure 11-3) appeared later in demented patients. These results suggest that clearance of phenylalanine by metabolism to tyrosine and subsequent metabolism of tyrosine are both reduced in senile dementia. The low serum Crithidia factor could occur because of a substantially reduced de novo synthesis of tetrahydrobiopterin from purine precursors, from an increase in dihydropteridine reductase activity or from a decrease in apoenzyme. It seems reasonable to assume a decrease in either tetrahydrobiopterin or apoenzyme in view of the poor clearance of phenylalanine and tyrosine and the depressed neurological function of these patients.

The mentally disabled all had severely depressed intellectual ability, were from the same hospital, there was no known sexual bias in medication or in clinical condition. Phenylketonuria was excluded from both male and female samples.

The child with Hartnup's disease had a low serum Crithidia factor, this disease is rare and no other case was available for study. The low serum Crithidia factor could not be explained by increased dihydropteridine reductase activity unless there is decreased transport of phenylalanine into the cell with less phenylpyruvic acid being produced. In-vitro (Chapter III) the concentration of phenylpyruvic acid which would affect dihydropteridine reductase is not attained normally, the plasma phenylalanine of this child was 0.05m.mol/L. which was within normal range.

The lack of an overall correlation between whole blood lead and serum Crithidia factor suggests that unless lead poisoning is seen as a clear clinical entity then either there is no correlation between the two parameters or other factors influencing serum Crithidia factor levels mask any relationship.

The correlation between lead values of 15 to 27μg/100 cm^3 whole blood and serum Crithidia factor corresponds to the normal range in Birmingham - 22.1μg/100 cm^3 (s.d. 6.4) (Hillburn, 1979) but no explanation is offered for this observation. The two patients diagnosed

clinically as suffering from lead poisoning both had low serum Crithidia factor concentrations as did the lead treated rats (Chapter XII) thereby supplying further evidence that lead decreases the supply of tetrahydro-biopterin in total.

Patients on methotrexate have been studied before (Leeming, 1975; Leeming, Blair, Melikian and O'Gorman, 1976) when it was noted that administration of methotrexate promoted an increase in serum Crithidia factor which was maintained for up to two weeks. It was further reported that 5-formyltetrahydrofolic acid, 10-formylfolic acid and folic acid given twenty-four hours after methotrexate caused a further rise in serum Crithidia factor although 5-methyltetrahydrofolic acid had no such effect. Folates without prior methotrexate did not have any effect on serum Crithidia factor levels. Unreduced folates and biopterin as well as methotrexate decrease dihydropteridine reductase in-vitro (Chapter III).

Again (Table 11-1) it was found that serum Crithidia factor was increased significantly by methotrexate and that in patients already treated with the drug a subsequent dose did not have any effect of the serum Crithidia level when measured one hour later, suggesting a saturation effect. The serum Crithidia of the patient on high-dose methotrexate with 5-formyltetrahydrofolic acid rescue, did fall slightly between doses but the high dose of methotrexate was not matched by an equally high serum Crithidia factor level, again suggesting saturation. The fact that folates stimulate a further increase in serum Crithidia factor whereas a second dose of methotrexate does not, suggests different affinities of folates and methotrexate for dihydropteridine reductase.

The clearance of plasma phenylalanine in methotrexate treated patients is slower than normal (Goodfriend and Kaufman, 1961) clearly indicating the impact of methotrexate on phenylalanine metabolism. As 5,6,7,8-tetrahydrobiopterin is required in the hydroxylation of tyrosine (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) and tryptophan (Hosoda and Glick, 1966) as well as for phenylalanine, then the action of

methotrexate on dihydropteridine reductase and the consequent decrease in cellular tetrahydrobiopterin is likely to reduce the production of dopamine and serotonin. Children treated with methotrexate for acute lymphoblastic leukaemia have been reported as suffering from neurological damage (Meadows and Evans, 1976; Eiser, 1978). Children with dihydropteridine reductase deficiency (Kaufman, Holtzman, Milstein, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977) need neurotransmitter therapy (Bartholome and Byrd, 1975) if they are to escape gross neurological deficit. It is reasonable to predict that children treated with methotrexate might be similarly damaged and this could be avoided by neurotransmitter replacement therapy (Cotton, 1978; Leeming and Blair, 1978).

Data from this chapter is discussed in greater detail in Chapter XIII where consideration is given to metabolic processes which may be affected by the several conditions described here.

CHAPTER XII

CRITHIDIA FACTOR IN THE RAT AND THE EFFECTS OF LEAD

INTRODUCTION

Rat brain, liver and whole blood have been assayed for Crithidia active compounds (Baker, Frank, Bacchi and Hutner, 1974) and can be compared with human tissue and fluid levels (Baker et al, 1974; Leeming, Blair, Melikian and O'Gorman, 1976). The work carried out on the rat has so far been of a preliminary nature in that little attempt has been made to follow levels over a period of time or to assess the impact of environmental influences including diet. One early exception to this is the work of Pabst and Rembold (1966) who showed the excretion of biopterin by the rat to be unaffected by deficiencies of folate or riboflavin although 2,4-diamino-6-hydroxypyrimidine reduced urinary biopterin by 80% without affecting the growth of the animals.

The work described here attempts to establish baseline information on rat levels of biopterin derivatives in the laboratory environment. Experiments were being conducted by others into lead toxicity and it was thought useful to make use of these experiments to see if lead had any effect on rat Crithidia factor levels. Lead acetate decreased synthesis of Crithidia factor in-vitro at 10^{-2}M by 37% but only in the presence of methotrexate (Chapter II) and dihydropteridine reductase activity at 10^{-4}M by 41% (Chapter III). Neurological and behavioural deficits are recognised as the main severe consequences of toxic exposure to lead with the central nervous system of the developing organism being most sensitive (U.S. Environmental Protection Agency, 1977). There is a reputed relationship between lead and mental retardation in children (Moore, Meredith and Goldberg, 1977).

Diet is the major source of lead in man (Hilburn, 1977) although

airborne lead, of which 90% in the United States of America originates from petrol combustion (Nat. Inventory Air Pol. Emission and Control, 1976), is now under close scrutiny. Any information on the impact of lead on catecholamine synthesis has potential medical and social importance.

MATERIALS AND METHODS

Five Wistar rats of approximately 200g. weight were placed in individual 'Metabowls' for two days with food and water ad libitum. Urine and faeces were collected for each twenty-four hour period and measured. At the end of the second day the animals were anaesthetised with ether and dissected to remove the liver. Blood was collected direct from the heart with a syringe before coagulation had occurred, the serum was separated by centrifugation. The faeces were homogenised in 25ml. 0.2M. phosphate buffer pH 5.0 and the livers in 50ml. buffer, the serum was diluted 1/20 in buffer and all three were autoclaved for three minutes at 115°C and centrifuged. A sample of pelleted diet was treated as faeces. The serum extract was diluted a further 1/5, the liver extract was diluted 1/10 followed by four five-fold dilutions which were assayed for Crithidia factor. The faeces and food extracts were diluted through four five-fold dilutions and the urine was diluted 1/500 followed by four five-fold dilutions. 0.5ml. of each dilution was used in the Crithidia fasciculata assay.

Four rats had Crithidia factor measured in serum, plasma and whole blood on which the red cell Crithidia factor was calculated using the following formula:-

$$\text{Red cell concentration} = \frac{\text{whole blood concentration} - \text{serum concentration} \dfrac{(1 - \text{packed cell vol.})}{100}}{\dfrac{\text{packed cell vol.}}{100}}$$

Two rats were anaethetised with barbiturate and cannulated for bile after which the rats were killed before they regained consciousness, blood was taken from both; from one the brain and liver were removed for measurement of Crithidia factor.

Six rats were anaesthetised and 1.0ml. blood removed by cardiac puncture. After recovery three were placed in separate cages and three

in one cage. At days two, four and seven the solitary animals were sacrificed and blood and brain removed for Crithidia assay. On day seven the three animals sharing a cage were treated similarly. Six rats were placed in separate cages and six in one cage; after nine days three of each group were sacrificed and after a further seven days the remainder were killed, brain and serum Crithidia factor was measured.

Twelve rats were anaesthetised and bled by cardiac puncture. Radio-active lead acetate (equivalent to 68μg lead) was given to each animal intraperitoneally, a similar dose of lead was given again on the second and fourth days. Four animals were sacrificed after two, four and six days had elapsed, serum, liver, brain and kidney Crithidia factor was measured. A repeat of this experiment with control animals not treated with lead was carried out but lead values were not obtained in this second experiment.

Ten rats were given 20% ethanol in their drinking water for seventy-two hours; after the first twenty-four hours 10μg lead acetate was injected intraperitoneally into each animal (equivalent to 5.5 g. lead). Five were killed at forty-eight hours and the remaining five at seventy-two hours. Brain and serum Crithidia factor levels were measured. Four rats without ethyl alcohol but on a similar low dose of lead were examined for Crithidia factor in serum only.

RESULTS

The rat serum, liver, jejunum, colon and spleen, urine and faecal content of Crithidia factor is given in Table 12.1. Serum levels

TABLE 12.1

CRITHIDIA FACTOR IN DIET, LIVER, SERUM, URINE AND FAECES OF FIVE RATS KEPT IN 'METABOWLS' FOR TWO DAYS.

CRITHIDIA FACTOR IN THE JEJUNUM, COLON AND SPLEEN OF SIX FURTHER RATS

	Range	Mean $\pm$ S.E.M.
Serum	20-22μg/L	20.80 $\pm$ 0.34μg/L
Liver concentration	0.92-1.48μg/g	1.26 $\pm$ 0.13μg/g
Liver content	8.10 - 13.37μg	11.40 $\pm$ 1.01μg
Urine	60-131μg/day	94.66 $\pm$ 8.62μg/day
Faeces	200-938ng/day	363.90 $\pm$ 72.87ng/day
Diet	*55.8 ng/g	-
Jejunum	63.9-135-6ng/g	90.45 $\pm$ 12.46ng/g
Colon	58.0-82.1ng/g	72.60 $\pm$ 4.28ng/g
Spleen	58.1-135.2ng/g	80.32 $\pm$ 11.52ng/g

* 6g food/100g body weight (Geller & Yuwiler, 1969) = 670ng/day

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varied between 20.0 and 22.0μg/L. being approximately ten times the concentration found in human adults, whereas urinary concentration ranged from 1.9 to 12.5mg/L. with the mean of 4.35 $\pm$ 0.96mg/L. being only a little over twice the human concentration. The mean urinary output was from 60 to 131μg/day (mean 94.66 $\pm$ 8.62μg) which is about twenty times the rate/Kg body weight of the human adult. Liver concentrations were again greater than those found in man (Leeming, 1975) by a factor of 10. Faecal concentration of Crithidia factor was approximately six times that of the dry pelleted diet but calculated on an intake of 6g food/100g body weight only half was excreted in the

faeces. Bile levels of Crithidia factor (Table 12-2) were within the range

TABLE 12-2

CRITHIDIA FACTOR IN THE BILE, SERUM, LIVER AND BRAIN OF TWO CANNULATED RATS

	Rat 1	Rat 2
Bile	68.0μg/L	86.0μg/L
Serum	22.0μg/L	16.5μg/L
Liver	1.54μg/g (total 20.02μg)	
Brain	65.0ng/g (total 162.5ng)	

for human adults (Chapter VI). Red cell Crithidia factor levels were greater than in the human by a factor of 100-200 and plasma levels were similar to serum levels in the rat when measured on the same animal (Table 12-3).

TABLE 12-3

A COMPARISON OF SERUM, PLASMA, WHOLE BLOOD AND RED BLOOD CELL CRITHIDIA FACTOR IN FOUR RATS

(all in μg / L.)

Rat	Serum	Plasma	Whole blood	Red blood cells
1	36.0	40.0	350	843
2	19.0	25.0	213	534
3	20.0	22.5	150	383
4	20.0	20.0	300	986

The contrast in serum and brain Crithidia factor between animals in separate cages and those sharing a cage (Tables 12-4 and 12-5) is not statistically significant.

TABLE 12-4

BRAIN AND SERUM CRITHIDIA FACTOR IN THREE RATS KEPT IN SEPARATE CAGES AND THREE RATS KEPT IN ONE CAGE FOR UP TO SIX DAYS

	Rat No.	Serum Crithidia Factor at start (μg/L.)	Day Killed	Serum Crithidia Factor at death (μg/L.)	Brain Crithidia Factor (ng/g)	Total Brain Crithidia Factor (ng/brain)
SEPARATE CAGES	1	19.0	2	16.5	68.1	100.01
	2	12.0	4	17.5	52.0	89.96
	3	Haemolysed	7	18.0	43.9	76.4
IN ONE CAGE	4	18.8	7	21.0	61.4	112.46
	5	21.0	7	23.5 21.5 ± 1.0	74.5 63.7 ± 5.7	137.57 116.7 ± 11.0
	6	20.0	7	20.0	55.1	100.06

TABLE 12-5

BRAIN AND SERUM CRITHIDIA FACTOR IN SIX RATS KEPT IN SEPARATE CAGES AND SIX RATS KEPT IN ONE CAGE FOR UP TO SIXTEEN DAYS

	Rat No.	Day Killed	Serum Crithidia Factor at death (μg/L.)		Brain Crithidia Factor (ng/g)		Total Brain Crithidia Factor (ng/brain)	
IN ONE CAGE	1	9	2.8		146.2		247.9	
	2	9	4.5		119.3	114.0 ± 20.3	200.1	119 ± 51.1
	3	9	12.0	9.42 ± 2.15	76.4		134.0	
	4	16	9.5		76.2		132.0	
	5	16	11.0		80.6	78.7 ± 1.3	118.5	132.8 ± 8.5
	6	16	17.0		79.3		148.0	
IN SEPARATE CAGES	7	9	3.5		82.2		140.0	
	8	9	13.5		108.9	89.5 ± 9.8	189.9	156.6 ± 16.6
	9	9	9.0	8.33 ± 2.51	77.4		140.0	
	10	16	9.5		72.9		130.1	
	11	16	4.0		74.5	73.2 ± 0.68	132.0	129.4 ± 1.8
	12	16	10.5		72.2		126.0	

The effect of lead administration is demonstrated in Tables 12-6a, 12-6b and 12-7. The serum Crithidia factor is most clearly affected in Table 12-6a with serum levels dropping to less than half that of controls ($p = < 0.001$) and the values for days four and six were significantly below those on day two ($p = < 0.02$). This was confirmed by the fall in serum Crithidia factor (Table 12-6a) between days two and four ($p = < 0.05$), although in this case the fall in level was not continued into day six. Liver Crithidia factor appeared to rise with time but not significantly ($p = > 0.05$). Brain Crithidia factor fell over time both in rats given lead and those not given lead, and was highly significant by Student's 't' test ($p = < 0.01$ between days two and six), confirming the earlier observation of the effect of isolation - the rats were again in individual cages.

Rats with alcohol and concurrent 5.5μg lead (Table 12-8) showed a significant fall in serum Crithidia factor ($p = < 0.02$) not greatly different from those receiving 68μg lead alone although brain levels did not come down in this shortened time scale. Four rats given the lower dose of lead alone did not show a similar fall in serum Crithidia factor, brains were not available from these controls. One must assume therefore that the ethyl alcohol in their drinking water contributed to the fall in serum Crithidia factor.

TABLE 12-6a

SERUM, BRAIN, LIVER AND KIDNEY CRITHIDIA FACTOR

BEFORE AND AFTER INTRA PERITONEAL ADMINISTRATION OF 68μg LEAD ON THREE ALTERNATE DAYS

Rat No.	Day Sacrificed	Serum Before Lead (μg/L.)		Serum After Lead (μg/L.)		Liver μg/g		Brain ng/g		Kidney ng/g	
1	2	20		14.0		1.93		167		210	
2	2	14.5		12.5		1.24		168		245	
3	2	16.0	18.25 ± 1.83	20.0	16.0 ± 1.70	1.87	1.56 ± 0.20	157	158 ± 6.5	256	233 ± 10.5
4	2	22.5		17.5		1.20		140		222	
5	4	19.0		9.5		1.36		145		203	
6	4	19.0		11.0		2.56		143		295	
7	4	19.0	19.37 ± 0.38	10.0	9.88 ± 0.43	1.23	1.60 ± 0.32	114	133 ± 7.2	315	253 ± 30.2
8	4	20.5		9.0		1.26		128		200	
9	6	21.5		16.0		1.30		150		358	
10	6	17.5		11.0		2.32		121		223	
11	6	21.5	19.75 ± 1.03	9.0	15.0 ± 3.3	1.73	1.71 ± 0.22	127	130 ± 6.9	218	285.5 ± 37.7
12	6	18.5		24.0		1.47		121		343	

TABLE 12-6b

WHOLE BLOOD, BRAIN, LIVER AND KIDNEY LEAD VALUES

AFTER INTRA PERITONEAL ADMINISTRATION OF 68µg LEAD ON THREE ALTERNATE DAYS

Rat No.	Day Sacrificed	Whole Blood Lead (ng/g)	Liver Lead (ng/g)	Brain Lead (ng/g)	Kidney Lead (µg/g)
1	2	66.7	840.2	29.9	2.17
2	2	105.9	296.0	25.7	3.50
		77.1 ± 14.6	460.1 ± 191.2	26.1 ± 2.1	2.69 ± 0.41
3	2	-	-	-	-
4	2	58.7	244.9	22.7	2.39
5	4	85.5	435.6	35.4	5.51
6	4	50.4	523.7	24.3	1.87
		90.2 ± 14.8	472.6 ± 31.8	41.7 ± 9.3	4.21 ± 0.83
7	4	115.7	402.0	68.1	5.22
8	4	109.1	529.1	38.9	4.25
9	6	137.4	1851.0	49.3	3.38
10	6	151.2	804.0	44.4	6.72
		142.9 ± 4.2	966.3 ± 298.6	55.4 ± 7.0	4.96 ± 1.04
11	6	140.0	616.9	75.8	6.80
12	6	-	593.3	52.0	2.95

TABLE 12-7

SERUM, BRAIN, LIVER AND KIDNEY CRITHIDIA FACTOR

FOLLOWING INTRA PERITONEAL ADMINISTRATION OF 68μg LEAD ON ALTERNATE DAYS

Rat No.	Day Killed	Serum Before Lead (μg/L)	Serum After Lead (μg/L)	Liver (μg/g)	Brain (ng/g)	Kidney (ng/g)
1	2	15.5	17.5	1.437	91.9	215.7
2	2	20.0	14.0	1.311	72.7	128.7
		18.0 ± 0.98	14.88 ± 0.97	1.103 ± 0.159	90.65 ± 6.29	166.2 ± 18.12
3	2	17.5	15.0	0.794	101.4	157.5
4	2	19.0	13.0	0.871	96.6	163.0
5	2	18.0	19.0	1.481	105.9	165.7
		18.25 ± 0.25	18.5 ± 0.50	1.571 ± 0.089	94.15 ± 11.75	155.15 ± 7.46
controls 6 (no lead)	2	18.5	18.0	1.660	82.4	144.6
7	4	17.5	8.0	1.507	87.4	155.6
8	4	20.0	11.5	1.030	93.2	144.9
		18.6 ± 0.80	10.0 ± 1.17	1.338 ± 0.151	87.85 ± 3.00	147.9 ± 14.96
9	4	20.0	12.5	1.671	79.6	181.6
10	4	17.0	8.0	1.144	91.2	109.4
11	4	17.0	17.0	1.549	66.7	165.2
		17.5 ± 0.50	18.0 ± 1.0	1.749 ± 0.20	61.65 ± 5.05	149.6 ± 15.65
controls 12 (no lead)	4	18.0	19.0	1.949	56.6	133.9

TABLE 12-7
(Continued)

Rat No.	Day Killed	Serum Before Lead ($\mu g/L$)	Serum After Lead ($\mu g/L$)	Liver ($\mu g/g$)	Brain (ng/g)	Kidney (ng/g)
13	6	18.0	12.0	1.048	77.1	129.5
14	6	17.5	9.0	0.840	68.5	135.4
		18.25 $\pm$ 0.595	7.63 $\pm$ 1.77	1.303 $\pm$ 0.247	69.25 $\pm$ 3.035	133.7 $\pm$ 17.53
15	6	20.0	5.0	1.351	69.1	177.8
16	6	17.5	4.5	1.972	62.3	92.2
17	6	20.0	20.0	1.439	55.3	166.5
controls		22.5 $\pm$ 2.5	19.75 $\pm$ 0.250	1.475 $\pm$ 0.035	65.9 $\pm$ 10.6	166.55 $\pm$ 0.05
18 (no lead)	6	25.0	19.5	1.510	76.5	166.6

TABLE 12-8

THE EFFECTS OF 10% ETHANOL IN DRINKING WATER FOR 24 HOURS PRIOR TO AND UNTIL 48 HOURS AFTER INTRA PERITONEAL ADMINISTRATION OF 5.5μg LEAD

	Rat No.	Day Killed	Brain Crithidia Factor		Serum Crithidia Factor	
Alcohol given	1	2	68.22		12.5	
	2	2	68.90		11.25	
	3	2	72.82	69.89 ± 0.79 (ng/g)	12.0	12.95 ± 1.03 (μg/L)
	4	2	69.52		17.0	
	5	2	69.98		12.0	
	6	3	71.22		9.5	
	7	3	73.56		9.25	
	8	3	79.83	68.84 ± 3.95 (ng/g)	7.5	9.0 ± 0.395 (μg/L)
	9	3	59.96		9.0	
	10	3	59.64		9.75	
No Alcohol given	11	2	-		11.5	11.75 ± 0.25 (μg/L)
	12	2	-		12.0	
	13	3	-		16.5	14.25 ± 2.25 (μg/L)
	14	3	-		12.0	

DISCUSSION

When compared with previous work (Baker et al, 1974) the liver values given here are lower by a factor of two whilst whole blood levels are around five times higher and brain levels are very similar in both series. When the human values are compared, Baker et al, (1974) showed lower concentrations in plasma, red blood cells and cerebrospinal fluid, with brain, liver and urine levels roughly comparable with the work of Leeming et al (1976) and Chapter VI. The differences which exist may reflect differences in technique, sampling or strain of experimental animal.

Pabst and Rembold (1966) showed that rats on a biopterin free diet for several generations excreted around 30μg of biopterin derivatives a day in their urine thus giving conclusive evidence of endogenous synthesis supplying endogenous demand. Oral folates alone do not increase the appearance of biopterin derivatives in serum or urine (Fukushima and Shiota, 1972; Leeming et al, 1976).

The appearance of only half the dietary intake of Crithidia factor in the faeces could be accounted for in only two ways firstly that it was absorbed or secondly that it was degraded in the bowel.

High tissue and plasma levels of Crithidia factor in the rat, compared with the human, may be due to a number of factors but these were not explored in this study.

Comparison of whole brain levels between the human and the rat is not sensible as sites of synthesis have different concentrations (Leeming et al, 1976) and the sizes of different structures in the brains of the two species are not proportionate to total brain size. The consistently lower levels of Crithidia factor in whole rat brains, unrelated to serum levels, when animals were placed in separate cages deserves further study. The environmental conditions under which these experiments were carried out were not sufficiently controlled and better facilities were not available to allow

additional work. It would be interesting to observe the effects of stress and learning on brain Crithidia factor.

Lead at 68μg/animal on alternate days, although not apparently having any impact on brain levels of biopterin derivatives, produced a significant fall in serum levels which paralleled increasing whole blood lead levels. A major part of the administered lead found its way into the liver (Table 12-5) but the rise in liver Crithidia factor was not significant. It is likely that lead decreases tetrahydrobipterin synthesis and dihydropteridine reductase activity as it does in-vitro (Chapters II - IV), thereby decreasing total available tetrahydrobiopterin. This would fit in with the observation that lead decreases dopamine levels in rat brain (Satija, Seth and Tandon, 1978).

The effect of ethyl alcohol and a concurrent low dose of lead (5.5μg) also significantly lowers the serum Crithidia factor.

CHAPTER XIII

GENERAL CONCLUSIONS

It is the intention, in this final chapter, to bring together all the information which has been presented in the earlier chapters and to provide a coherent working model from these observations in the light of data from other sources. Nevertheless, it must be borne in mind that the field which has been investigated in this thesis has been explored in detail for the first time and therefore many of the conclusions are of a tentative nature.

The mechanisms proposed in this thesis link the biosynthesis of neurotransmitters with other fundamental pathways. This is a novel departure in that in most of the published work, neurotransmitter formation seems to owe little to key metabolic pathways in cell metabolism.

Tetrahydrobiopterin is not readily transported across the cell membrane. Rembold and Metzger (1967) showed, by radio-chemical studies, that orally administered tetrahydrobiopterin in the rat was not absorbed. This has been confirmed in man (Blair, Ratanasthien and Leeming, 1974; Leeming, 1975) (Chapter V). 7,8-dihydrobiopterin probably passes the cell membrane more easily and is excreted in the urine (Leeming and Blair, 1974). Biopterin present in the intestine is absorbed (Rembold and Metzger, 1967; Jacobson, 1967). Evidence presented in this thesis suggests that biopterin is rapidly transported into intestinal cells and reduced to tetrahydrobiopterin which is retained within the cell and slowly released as dihydrobiopterin.

It is clear from the foregoing absorptive evidence and the

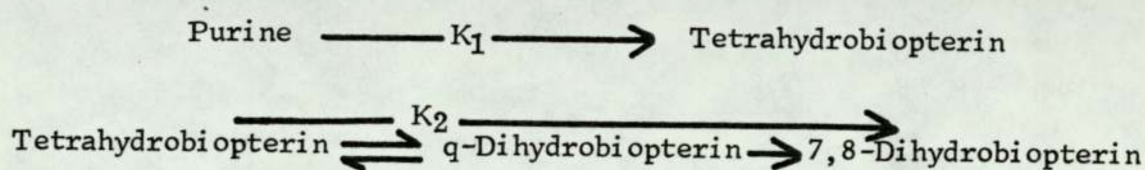
high tissue levels compared with cerebrospinal fluid and serum levels of biopterin derivatives (Baker, Frank, Bacchi and Hutner, 1974; Leeming, Blair, Melikian and O'Gorman, 1976) (Chapter VI) that tetrahydrobiopterin is formed within the cell and only lost when it is degraded to 7,8-dihydrobiopterin. This unique localisation within the cell means that in addition to a potential for overall deficiency of tetrahydrobiopterin, probably arising from inherited metabolic defects, there may be also disease states in which there is very local interference with tetrahydrobiopterin metabolism due to local concentration of a toxic material or a deficiency of metabolites for the synthesis of tetrahydrobiopterin. Because of this localised action, particularly in the brain, then what is basically the same interference with the metabolic process might result in quite distinctive neurological disorders.

The synthesis of biopterin derivatives from guanosine triphosphate has been shown in cultures of cells from brain, kidney, liver, lung and ovary of the hamster, mouse and rat (Fukushima, Eto, Mayumi, Richter, Goodson and Shiota, 1975). High tissue levels of biopterin derivatives have been found in human and rat brain and liver (Baker, Frank, Bacchi and Hutner, 1974; Leeming, Blair, Melikian and O'Gorman, 1976). Regional variation has been shown in the brain (Leeming, Blair, Melikian and O'Gorman, 1976), with pineal glands of man (Chapter VI) and sheep (van der Have-Kirchberg, de Moree, Vanhaar, Gerwig, Versluis, Ebels, Hus-Citheral, Heritier, Roseau, Zurburg and Moszkowska, 1977) being particularly rich in biopterin derivatives. The endogenous source of tetrahydrobiopterin and mammalian capacity to synthesize total bodily requirement is shown in the work of Pabst and Rembold (1966) who fed rats on a biopterin-free diet for several generations. Their rats continued to develop normally and to excrete around 30 μg of biopterin each day in their urine.

Tetrahydrobiopterin is the essential cofactor in the hydroxylation of phenylalanine to tyrosine (Kaufman, 1958), tyrosine to dopa (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) and tryptophan to

5-hydroxytryptophan (Hosoda and Glick, 1966). It donates two hydrogen atoms in the hydroxylation and is regenerated from quinonoid dihydrobiopterin by dihydropteridine reductase (Craine, Hall and Kaufman, 1972) in the presence of NADH.

There are, therefore, two mechanisms for maintaining the level of tetrahydrobiopterin in the cell. The first of these is synthesis de novo and the second is an effective salvage pathway from quinonoid dihydrobiopterin. The two processes can be put into a simple kinetic equation in which there are composite rate constants (K_1) from precursor purine to tetrahydrobiopterin and from tetrahydrobiopterin to dihydrobiopterin (K_2).



In the light of all the evidence, conversion of tetrahydrobiopterin to dihydrobiopterin (K_2) is a slow process compared with the synthesis of tetrahydrobiopterin from purines (K_1). The salvage of quinonoid dihydrobiopterin is rapid because of a normally large excess of dihydropteridine reductase. Therefore K_2, production of 7,8-dihydrobiopterin from 5,6,7,8-tetrahydrobiopterin, is the rate determining factor for the appearance of dihydrobiopterin in the serum. Only in the most exceptional circumstances will the synthesis of tetrahydrobiopterin from precursors (K_1) become the rate-limiting reaction for the synthesis of 7,8-dihydrobiopterin and its subsequent level in the serum.

Thus in the cell a situation exists whereby the level of tetrahydrobiopterin is maintained by the two processes outlined above, de novo synthesis and salvage from quinonoid dihydrobiopterin, and a further process whereby 7,8-dihydrobiopterin is formed and rapidly removed.

Assuming that only one process changes at a time, changes in the level of dihydrobiopterin in the blood and cell tetrahydrobiopterin levels

may be grouped under four headings:-

1. Inhibition of dihydropteridine reductase. This inhibition will result in an increase in the formation of dihydrobiopterin, therefore as the rate-determining stage there will be an increase in the amount appearing in the serum, the cell level of tetrahydrobiopterin will be lowered.

2. Increase in dihydropteridine reductase. Here there is a reaction which would diminish the rate of 7,8-dihydrobiopterin production and hence there will be less outflow from the cell into the serum and cell tetrahydrobiopterin levels will be raised.

3. Decrease in synthesis of tetrahydrobiopterin from purine precursors. Cell tetrahydrobiopterin levels would be lowered. For this to have any impact on the appearance of the serum 7,8-dihydrobiopterin, the rate of tetrahydrobiopterin production would have to be very slow indeed so that it would become the rate-determining process. Under extreme conditions, the slow rate of production of tetrahydrobiopterin would be reflected in a low level of cellular tetrahydrobiopterin, slow production of dihydrobiopterin and a low serum Crithidia factor.

4. Increase in production of tetrahydrobiopterin. As the synthesis of tetrahydrobiopterin from purine precursors is not the rate-limiting step in dihydrobiopterin production, this would not affect the appearance of dihydrobiopterin in the serum. Cellular tetrahydrobiopterin levels would be raised.

The problem that remains is how to judge changes in tetrahydrobiopterin levels in cells and thereby distinguish between the four possibilities proposed above. There may be particular problems in brain cells because of the the blood/brain barrier and, more specifically, discrete variation in small but neuro-functionally important sites. Differences in biopterin derivative concentration at different brain sites has been demonstrated in the human after death (Leeming, Blair,

Melikian and O'Gorman, 1976) and in cultures of human neuroblastoma cells (Albrecht, Biedler, Baker, Frank and Hutner, 1978).

Having recognised that limitations exist, a good test for assessing tetrahydrobiopterin levels would be the measurement of the plasma phenylalanine level after a pre-determined fasting period, or the resulting changes in phenylalanine level following a phenylalanine challenge. Such challenges are currently in use for the differentiation of phenylketonuric variants and heterozygotes (Lambert, Vrailhet, Monot, Lepaire, Baradel, Nabet, Martin and Pierson, 1973; Woolf, Cranston and Goodwin, 1967; Hsai and Driscoll, 1956; Jervis, 1960; Leeming, Blair, Green and Raine, 1976), therefore results of phenylalanine challenge in normal and diseased states has been well documented. If tetrahydrobiopterin levels are reduced in the liver, then there would be a much higher blood phenylalanine after a challenge than one would find in a normal subject. Other means which could be useful would be to try to correlate levels of Crithidia factor with known excess or deficit of neurotransmitters in particular cases.

In malignant hyperphenylalaninaemia in which dihydropteridine reductase is inactive (Kaufman, Holtzman, Milstien, Butler and Krumholz, 1975; Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977) (Chapter VIII) there is a very high serum Crithidia factor when the patient is on a low phenylalanine diet and a serum Crithidia factor response following a phenylalaninine loading dose as well as a severe degree of neurological disability. In the equation set out above this fits in with example 1.

Patients with the alternative variant of malignant hyperphenylalaninaemia fail to synthesize tetrahydrobiopterin (Leeming, Blair and Rey, 1976; Kaufman, Berlow, Summer, Milstien, Schulman, Orloff, Spielberg and Pueschel, 1978) (Chapter VIII), produce a rapid phenylalanine response to phenylalanine load and clear the phenylalanine only slowly and do not have a serum Crithidia factor response as in normals

and classical phenylketonuria. For these example 3 seems a good fit.

These two types of malignant hyperphenylalaninaemia demonstrate that when brain tetrahydrobiopterin levels are lowered by a large reduction in either (a) tetrahydrobiopterin biosynthesis or (b) regeneration of tetrahydrobiopterin from quinonoid dihydrobiopterin then there is hyperphenylalaninaemia, inadequate production of neurotransmitters and severe neurological disease.

However, it still remains to be determined by what amount the biosynthesis or salvage pathways for tetrahydrobiopterin have to be reduced before hyperphenylalaninaemia, diminished neurotransmitter formation and neurological disorder occur.

When patients are receiving therapeutic doses of methotrexate there is an increase in serum Crithidia factor concentration (Leeming, Blair, Melikian and O'Gorman, 1976) (Chapter XII). In L-phenylalanine loading experiments in patients with choriocarcinoma who had received methotrexate within the last four days, Goodfriend and Kaufman (1961) showed the maximum increase in serum phenylalanine correlated with total methotrexate received as shown below:-

Total methotrexate before phenylalanine	No. of patients	Increase in serum Phenylalanine m.mol/L.
0mg	3	0.417 ± 0.031
25mg	1	0.438
125mg	3	0.757 ± 0.067

The difference between those taking 125 mg methotrexate and those who had had none was significant by Student's 't' test ($p = < 0.02$). Earlier experiments (Chapter III) have shown that methotrexate at 10^{-5}M, reduces dihydropteridine reductase to 74% of its normal value. The work of Meadows and Evans (1976) and Eiser (1978) suggests that therapeutic doses of methotrexate

are neurotoxic. Thus it seems likely that only a small reduction in dihydropteridine reductase activity is sufficient to cause hyperphenylalaninaemia, give a significantly increased serum Crithidia factor and produce neurotoxic effects.

Cotrimaxazole, which contains trimethoprim and sulphamethoxazole, is also known to increase serum Crithidia factor (Leeming, Blair, Melikian and O'Gorman, 1976) and decreases phenylalanine clearance in loading experiments (Andrews, Purkiss, Chalmers and Watts, 1976). Trimethoprim did not affect either the in-vitro synthesis of Crithidia factor (Chapter II) or the activity of dihydopteridine reductase (Chapter III) whilst sulphamethoxazole increased the synthesis of Crithidia factor whilst having no effect on dihydropteridine reductase. However, the increased serum Crithidia factor and decreased phenylalanine clearance suggest that cotrimaxazole somehow reduces dihydropteridine reductase activity. If this is so then again it suggests that only a small reduction in reductase activity is sufficient to cause hyperphenylalaninaemia.

In senile dementia two principal observations have been made (Chapter XI). Firstly serum Crithidia factor was low and secondly the clearance of phenylalanine following phenylalanine loading was significantly slower than in normals. The fasting phenylalanine level (0.099 ± 0.003 m.mol /L was significantly higher ($p = < 0.01$) than that of normal controls (not age matched - 0.049 ± 0.005 m.mol./L.)

It has been reported that dihydropteridine reductase increases with age in the rat (Algeri, Bonati, Brunell and Ponzio, 1977) but although this could be used to explain the low serum Crithidia factor in senile dementia clearance of phenylalanine was poor, demonstrating that tissue tetrahydrobiopterin was low. Although normal controls for phenylalanine levels (see above) were from a much younger age group, they perhaps form a more useful comparison than age matched controls in whom there are disease processes in sub-clinical stages prior to emerging as flagrant clinical conditions.

The metabolism of the brain and its supporting blood flow vary with

age although in normal healthy men in their eighth decade, selected for good health and freedom from all disease, both blood flow and oxygen were similar to those of men 50 years younger. In elderly subjects with minimal arteriosclerosis, the blood flow was sufficiently reduced for the oxygen tension of cerebral venous blood to decline (Sokoloff, Fitzgerald and Kaufman, 1977). The brain derives almost all its energy from the aerobic oxidation of glucose, therefore depressed oxygen tension must affect glycolysis. Glucose is required as a source of NADH and NADPH, inhibitors of glycolysis, albeit at very high concentration, depress the synthesis of Crithidia factor in brain extracts (Purdy, 1979).

In Alzheimer's disease, glycolysis is much reduced in the brain (Bowen, Spillane, Curzon, Meier-Ruge, White, Goodhardt, Iwangoff and Davison, 1979). This in turn would result in a reduced level of NADH and NADPH which are required in both biosynthesis and reduction stages of tetrahydrobiopterin metabolism. The result must be a lowering of tetrahydrobiopterin in the brain. Confirmation is given by the further observation (Bowen et al, 1979) that 5-hydroxyindolacetic acid in Alzheimer's disease was reduced to 60% of controls, suggesting that neurotransmitter formation, instanced here by a defect in serotonin metabolism, mediated by tetrahydrobiopterin, is reduced.

Phenylalanine produces phenylpyruvic acid as a metabolite. Under normal circumstances phenylpyruvic acid is a minor metabolite and does not appear in the serum or urine in measurable quantities. However, in phenylketonuria, dependent upon diet, up to 1g. of phenylpyruvic acid is excreted in the urine every day (Penrose, 1955). Serum phenylpyruvic acid in untreated adult phenylketonuria is around 2.45×10^{-5}M which may increase three-fold in loading experiments with 100mg/Kg body weight L-phenylalanine (Jervis and Drejza, 1966). However at 6.1×10^{-4}M phenylpyruvate, dihydropteridine reductase activity was reduced to about 60% of normal (Purdy, 1978). This suggests that in the intact animal phenylpyruvic acid competes for dihydropteridine reductase which would then be less available for reduction of quinonoid dihydrobiopterin, which would re-arrange and appear in the serum as

7,8-dihydrobiopterin. The resulting lowered levels of tetrahydrobiopterin would explain why in untreated phenylketonuria, although tryptophan supplies are adequate and tyrosine is normal or slightly reduced (Blastovics and Nelson, 1971), there are reduced amounts of neurotransmitters and their metabolites (Hoeldtke, 1974).

In untreated classical phenylketonuria therefore, the permanent brain damage could be caused by phenylpyruvic acid acting as an inhibitor of dihydropteridine reductase. The consequences of neurotransmitter therapy without phenylalanine restriction have not been reported. It could be informative. The severe problems of dietary phenylalanine restriction (Smith and Francis, 1976) could be ameliorated if an alternative regimen existed. In malignant hyperphenylalaninaemia with dihydropteridine reductase deficiency there will be no dihydropteridine reductase to inhibit but neurotransmitter replacement therapy produces dramatic results (Rey, Harpey, Leeming, Blair, Aicardi and Rey, 1977).

Tricyclic antidepressants increase tetrahydrobiopterin levels which could explain their effectiveness in the treatment of manic-depressive psychosis in which there may be reduced levels of dihydropteridine reductase in localised areas of the brain. On the other hand, benzodiazepines would decrease tetrahydrobiopterin levels by depressing dihydropteridine reductase activity (Chapter III). These effects are, of course, in isolation from other action which these drugs may have. Tricyclic antidepressants inhibit noradrenaline uptake by neurones (Glowinski and Axelrod, 1964) and block the uptake of serotonin and dopamine into platelets (Hallstrom, Pare, Rees, Trenchard and Turner, 1976). Such a blockade in-vivo, at sites of tetrahydrobiopterin synthesis would add a further variable to the kinetics of tetrahydrobiopterin control.

Pregnant women in their third trimester had low levels of serum biopterin derivatives when compared with normals (Chapter XI) which may be associated with either a reduced level of tetrahydrobiopterin in the tissues or an enhanced level. Published data suggests that because the phenylalanine[2] : tyrosine ratio is raised in pregnancy (Griffin and Elsas, 1975) and tyrosine

is at the lower limit of normal whilst phenylalanine is at the upper limit (Butterfield and O'Brien, 1963; Documenta Geigy, 1962), the level of tetrahydrobiopterin in the tissues is low. One explanation could be that this is an effect of increased blood cortisol levels in pregnancy (Kühl, Hornnes and Klebe, 1977) which would reduce glycolysis in the tissues (Zilva and Pannall, 1975) and thereby reduce the synthesis of tetrahydrobiopterin from its precursors.

Cortisol is also raised in humans following oestrogens, in terminally ill patients (Gowenlock and Longson, 1970) and in stress. Serum Crithidia factor is low when women are taking oestrogens orally (oral contraceptives - Chapter VI). The increased cortisol levels in stress might reasonably be associated with the observations on rat brain (Chapter XII) when removal of this social animal to isolation apparently caused the brain Crithidia factor levels to fall. Stress might also explain the reduced serum Crithidia factor levels in malignant disease (Leeming, Blair, Melikian and O'Gorman, 1976) (Chapter XI).

In untreated coeliac disease glucagon levels are raised (Besterman, Sarson, Johnston et al, 1978; Walsh, Cooper, Holmes et al, 1979) and serum Crithidia factor is low (Chapter XI). Glucagon levels fall in treatment of coeliac disease as the serum Crithidia factor rises. The increased glucagon levels may explain the reduced serum Crithidia factor levels.

It has been claimed that glucagon increases dihydropteridine reductase and increases the rate of phenylalanine conversion to tyrosine (Fuller and Baker, 1974). This effect on dihydropteridine reductase was not confirmed by later work (Donlon and Kaufman, 1978) in which glucagon was described as increasing the phosphorylation of phenylalanine hydroxylase, thereby increasing its activity. Clearly, further work is necessary although the claims that these two enzymes are raised may not be very meaningful, in-vivo. However both sets of authors agree that glucagon will increase the rate of conversion of phenylalanine to tyrosine thus lowering the serum phenylalanine level. This in its turn will reduce the serum Crithidia factor level. (Leeming, Blair, Green and Raine, 1976).

There is now evidence that lead interferes not only with the biosynthesis of tetrahydrobiopterin but also in its salvage from quinonoid dihydrobiopterin and that these effects occur at levels which might be found in the human subject (Chapters II to IV). In two subjects with lead poisoning (Chapter XI) the serum Crithidia factor was found to be low. The one subject who had plasma phenylalanine measured was within the normal range though towards the upper end of the normal range. Serum Crithidia factor fell significantly in rats given high doses of lead (300 μg/Kg bodyweight) over a period of six days (Hilburn, 1977) and this fall was paralleled by an increase in whole blood levels. It has been claimed that lead causes neurotoxic effects in children (Moore, Meredith and Goldberg, 1977) and there was a highly significant trend towards higher blood-lead concentrations in phenylketonuric test cards in mentally retarded children. This, of course, would reflect lead exposure during pregnancy. It is therefore likely that lead decreases tetrahydrobiopterin synthesis and dihydropteridine reduction in-vivo as it does in-vitro, thereby decreasing total available tetrahydrobiopterin. The association of low levels of lead with mental retardation is still a controversial field but there is no doubt that high levels of lead are associated with encephalopathy.

A survey of other metals (Chapters II to IV) such as copper, cadmium and mercury, suggested that they too interfered with the level of tetrahydrobiopterin present in tissues and could therefore produce neurological effects by this interaction. All these metals are known to produce encephalopathy if not minor neurotoxic effects.

There was marked reduction in biosynthesis of Crithidia factor in the presence of cupric sulphate at low concentration. Copper oxidised NADH in the absence of dihydropteridine reductase (Chapter III) and was alone among the metals in having this effect and could therefore have a different action in-vivo by interfering with tetrahydrobiopterin precursors.

Brain aluminium levels in dialysis dementia (Alfrey, Le Gendre and Kaehny, 1976) approach the concentration effective in inhibiting dihydro-

pteridine reductase in-vitro (Chapter III). Any material which decreases dihydropteridine reductase must produce a condition similar to malignant hyperphenylalaninaemia if the effect is severe enough. Reversal of dementia when aluminium intake is stopped (Poisson, Mashely and Lekiril, 1978) suggests an inhibitory process rather than toxic permanent damage.

There has been an attempt to relate Alzheimer's disease to increased aluminium concentrations in the brain (Crapper, Krishnan and Dalton, 1973). This relationship has not been proven (Lancet Editorial, 1976) and there is an increase in brain aluminium with age (McDermott, Smith, Iqbal and Wisniewski, 1977). The body burden of lead also increases with age (Schroeder, Brattleboro and Tupton, 1968) and it is reasonable to assume that continued exposure to common elements will increase their concentration in the body with age. Their distribution will be dependent on their interaction with transport mechanisms and metabolic processes.

There appears to be little information on the effects of metals on specific neurotransmitter formation. If compounds had similar effects on a particular biochemical pathway, in this case the supply of tetrahydrobiopterin, then it would be reasonable to assume that their clinical effects would be similar. This simple approach would be appropriate in a single compartment model but the brain is a diverse compartmentalised organ with areas of highly specialised function. Compounds would accumulate in areas for which they had affinities, regional brain distribution of aluminium (Alfrey, LeGendre and Kaehny, 1976) and copper (Scheinberg and Sternlieb, 1976) illustrate this. Thus compounds with the same biochemical action would produce different symptoms by virtue of their concentration and locale.

In the brain there are marked differences in monoamine distribution at different brain sites and between brains (Mackay, Yates, Wright, Hamilton and Davies, 1978). Dopamine has become a candidate for a role in schizophrenia partly because drugs which are effective in

schizophrenia block dopamine mechanisms in the brain and the fact that such drugs prevent the stereotyped behaviour in animals, of the schizomimetic drug apomorphine which stimulates excess amine release from central dopamine neurons (Crow, Johnstone, Deakin and Longden, 1974; Snyder, Banerjee, Yamamura and Greenberg, 1974; Iverson, 1976). It is suggested that locally increased levels of tetrahydrobiopterin arise in schizophrenia from increased activity of dihydropteridine reductase from unknown cause(s), low serum Crithidia factor levels are found in schizophrenia (Leeming, Blair, Melikian and O'Gorman, 1976). In manic-depressive psychosis there are mildly elevated levels of dihydrobiopterin in the serum (Chapter X). It is proposed that there are reduced levels of dihydropteridine reductase in certain localised brain cells the result of which would be an increase in the output of dihydrobiopterin into the serum. This occurs in untreated and treated cases in and out of remission and correlates with the claims made that serotonin synthesis is similarly reduced in depressive illness in an almost identical way (Coppen, 1970; Kaneko, Hayashi, Unno, Wanatabe, Takahashi, Takano and Kumashiro, 1975). Then this failure in tetrahydrobiopterin synthesis because of a reductase defect causes a reduced serotonin output.

In this chapter it has been argued that tetrahydrobiopterin in the cell is maintained within close limits by two processes. This level of tetrahydrobiopterin is responsible for the rate of conversion of phenylalanine to tyrosine and other compounds in neurotransmitter metabolism. Variation in this level in the cell results in various malfunctions detected as hyperphenylalaninaemia and over or under production of neurotransmitters. There are two ways in which the levels can be affected. One is by interference with the synthesis itself, the other is by interference with the salvage pathway from quinonoid dihydrobiopterin. Interference with these processes, not very great interference in some cases, can influence tetrahydrobiopterin levels and give rise to appropriate disease states.

The tools for looking at these problems are as follows:-

1. Measurement of the serum Crithidia factor level.

2. The serum Crithidia factor after a phenylalanine loading dose and the phenylalanine level itself after loading, especially when this is assessed in conjunction with tyrosine changes.

3. Simple experimental systems using brain extracts in which overall biosynthesis of tetrahydrobiopterin can be measured.

4. Simple experimental systems using brain extracts in which dihydropteridine reductase can be measured.

It is generally anticipated that by using one or more of these methods of approaching problems that coherent answers can be obtained.

METABOLISM

OF

BIOPTERIN AND ITS DERIVATIVES

IN MAN AND THE RAT

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