TETRAHYDROBIOPTERIN METABOLISM

By

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SUMMARY

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A technique for the measurement of biopterin using HPLC has been developed and compared against the established Crithidia fasciculata assay. The two assays give a similar reading when samples are unoxidised but after acid/iodine oxidation Crithidia reads higher, probably due to the presence of other pterins.

Using HPLC as a measuring technique an oral dose of phenylalanine has been found to elevate the level of biopterin derivatives in the blood whilst tyrosine had no effect.

With control brain obtained from humans the biosynthesis of biopterin from its precursors has been investigated. Optimal assay conditions were found to be 3 mM for GTP and NADPH the optimal pH being 7.5. These conditions hold for rat brains except the pH optimum is 8.0. The level of synthesis has been found to be dependent on age and brain area examined.

The biosynthesis of biopterin has been found to be much reduced in patients dying from senile dementia, Down's Syndrome and depression. In all these subjects dihydropteridine reductase was unaffected.

Using rat brain it was found that 5-methyltetrahydrofolate greatly enhanced the biosynthesis of biopterin and is possibly an essential requirement for optimal biopterin biosynthesis. By the use of B_{12} inhibitors, nitrous oxide and nitrite, it has been found that vitamin B_{12} is possibly an essential component.

In rats, the oestrogen diethylstilboestrol has been found to inhibit biopterin biosynthesis and enhance dihydropteridine reductase activity. Long term lead exposure at low levels has been found to enhance biopterin biosynthesis but at high levels found to reduce it.

Biopterin metabolism is disrupted in tumours of breast and bowel with elevated synthesis in the bowel but increased reductase in the breast.

Key Words :

Tetrahydrobiopterin, HPLC, Brain, Neurotransmitter, Senile Dementia

To

Mum, Dad, Peter,

Doreen and Roger

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INTRODUCTION

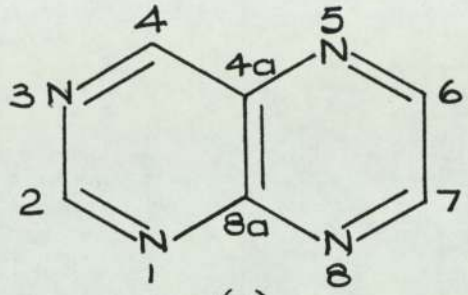
The name 'Pteridine' is given to the compound pyrazo [2-3-d] pyrimidine (1). Pterins are pteridines derived from 2-amino-4-oxodihydroxypteridine (2) (Rembold and Gyure 1972). The pteridine ring is numbered as shown (1).

It has been shown by Xray analysis that the pteridine molecule is almost coplanar and that the molecule is asymetrical (3) (Hamor and Robertson 1956).

Studies of the tautomeric relationships existing in hydroxypteridines have shown that the favoured equilibrium in aqueous solution at pH 7.0 is the ketone (5). At alkaline pH the ionised hydroxy form is the more stable. Because monohydroxypteridines exist in the form of true cyclic amides and do not have the vinylogous amide structure (4 + 6) it can be concluded that the mesomeric stablization is greatest in compound (5) (Pfleiderer 1964).

Pterins have low solubility at neutral pH, well defined UV spectra and the ability to form chelates with metal ions (Elderfield and Metita 1967). When fully oxidised pterins exhibit strong blue to red fluorescence when irradiated with ultra violet light at 365 nm. In their reduced state pterins are photolabile and subject to oxidative degradation making working with them difficult and introducing the possibility that isolated compounds could be artefacts of the system (Ziegler 1961, Blair 1958).

A yellow pigment from a butterfly wing was the first reported pterin (Hopkins 1889). Later further pterins were discovered and their structures elucidated as xanthopterin (7), isoxanthopterin (8) and leucopterin (9). An historical review is given by Albert (1975).


4
5
4a
3
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6
2
7
8a
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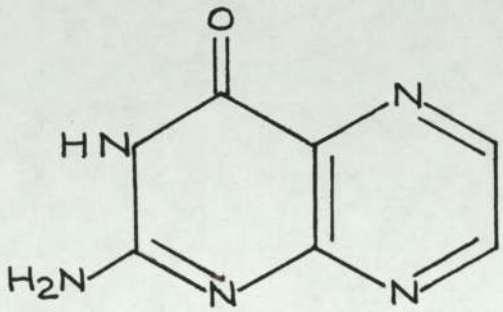
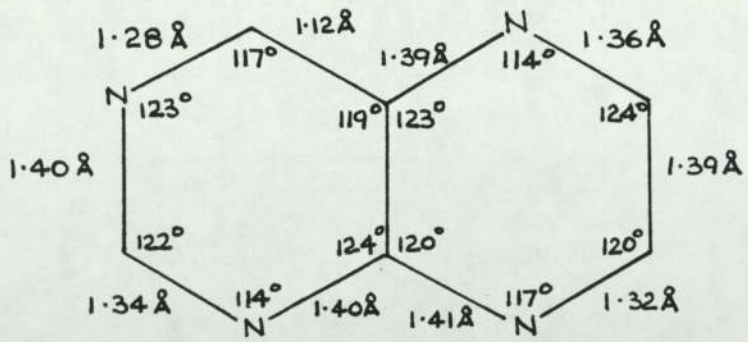
N
1
8

(1)


O
N
HN
H2N
N
N

(2)


1·28 Å
1·12 Å
N
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117°
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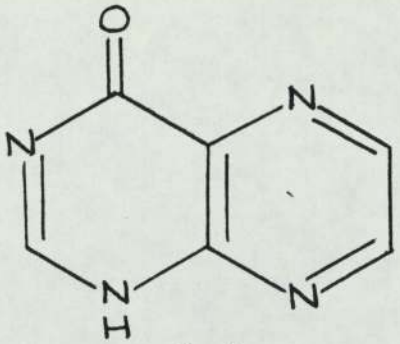
N

(3)



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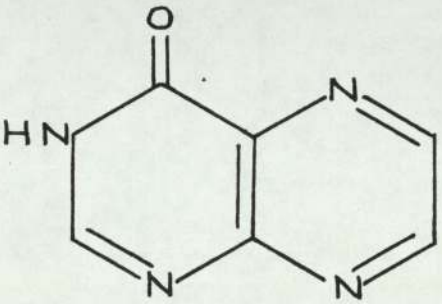

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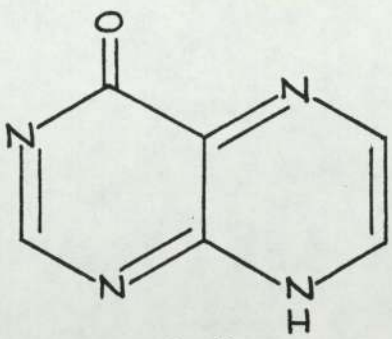

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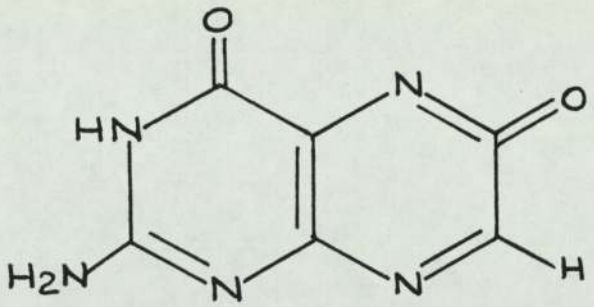

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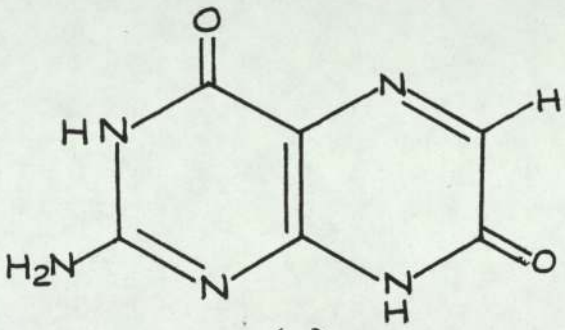

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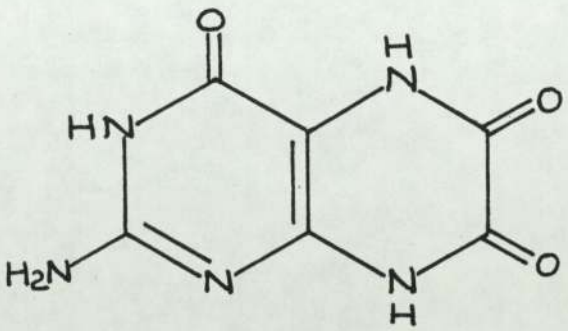
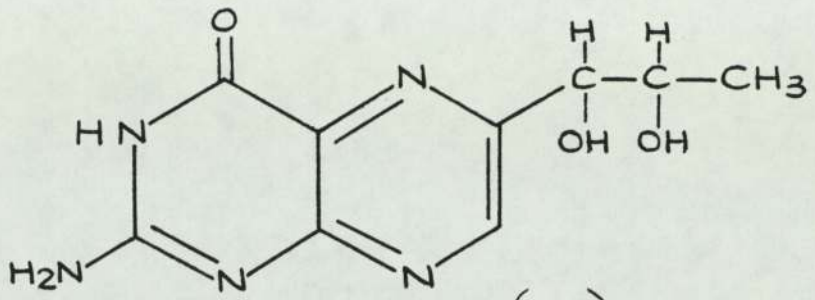


(9)

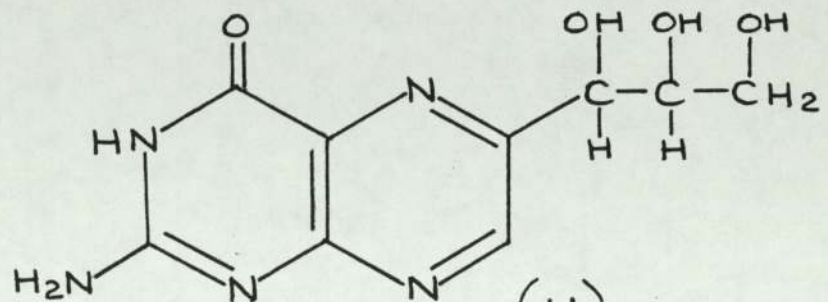
Pterins are only present in small concentrations in biological systems and are difficult to isolate. Isolation of pterins depends upon their solubility and lability. Relatively soluble pterins such as biopterin (10) and neopterin (11) can be extracted with trichloroacetic acid. Under these conditions labile hydrogenated pterins are stabilized. Acid insoluble pterins can be extracted by solubilizing using basic solvents and when used in conjunction with alkaline precipitation gives quantitive results (Rembold and Gyure 1972). The addition of mercaptoethanol prevents degradation of labile reduced pterins.

Once isolated pterins can be identified using several techniques such as their chromatographic behaviour on thin layer, paper and column chromatography (Blakley 1969, Rembold 1971), ultraviolet spectra (Blakley 1969), fluorescence emission spectra (Uyeda and Rabinowitz 1963), colorimetric methods (McNutt 1964), mass spectrometry (Blair and Foxall 1969, Hague 1970, Lloyd et al 1971), gas chromatography-mass spectrometry (Lloyd et al 1971, Kuster and Niederwiesser 1983), nuclear magnetic resonance (Blakley 1969), high performance liquid chromatography with fluorescent detection (Fukushima and Nixon 1980), high performance liquid chromatography with electrochemical detection (Brautigam and Dreesen 1982). They can also be detected using a microbiological assay such as Crithidia fasciculata (Dewey and Kidder, 1971).

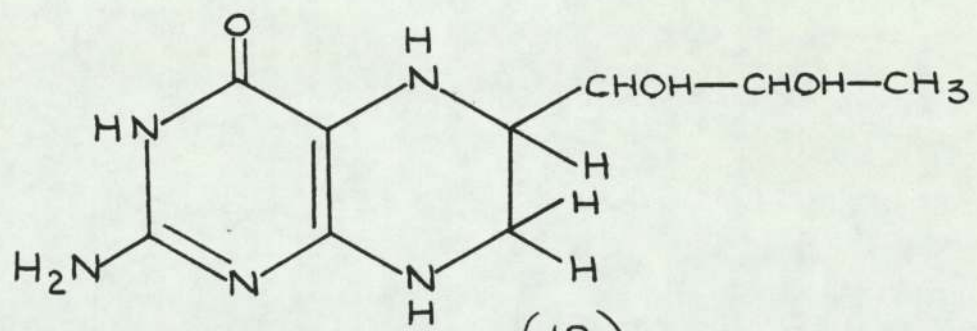
Biopterin, 4-pteridinone-2-amino -6-(1,2-dihydroxypropyl)-[S-(R*S*)], (10) was first isolated and identified from human urine (Patterson et al 1956). Due to the labile nature of reduced pteridines this biopterin probably arose from tetrahydrobioterin (12) and/or dihydrobiopterin (13), since tetrahydrobiopterin (12) is rapidly oxidised to 7,8-dihydrobiopterin (13) and then biopterin (10) (Blair and Pearson 1973, Blair and Pearson 1974). Biopterin (10) and/or its reduced derivatives have been reported in body fluids, organs and in different



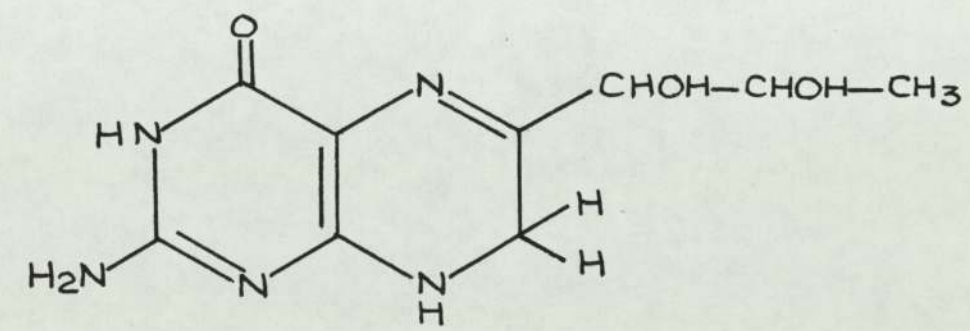
(10)



(11)



(12)

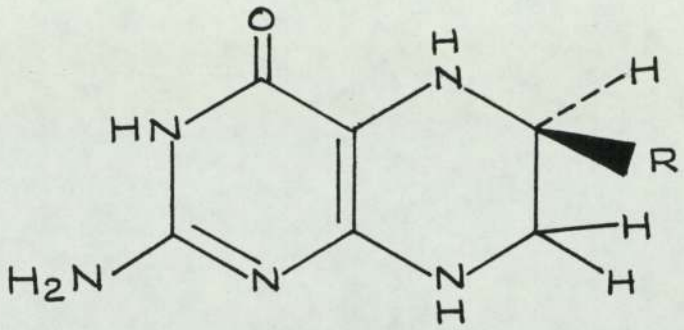


(13)

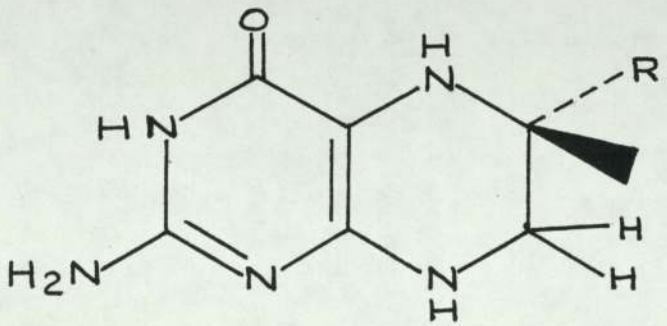
regions of some organs. (Frank et al 1963, Baker et al 1974 Leeming et al 1976, Leeming and Blair 1980, Nagatsu et al 1981).

When biopterin (10) is reduced to its tetrahydro derivative a chiral centre is introduced at C6 on the pteridine ring. This gives two diastereoisomers of the naturally occuring cofactor (fig.1). These have been reported to have been separated using high performance liquid chromatography (Bailey and Ayling 1978) but other workers have been unable to repeat this (Kato et al 1980). It has been suggested and confirmed by Xray crystallography that the natural isomer is the [6R] (Prewo et al 1982, Viscontini 1983). Both the [6R] and [6S] cofactors give identical Kmvalues but the natural isomer gave a higher Vmax with phenylalanine hydroxylase and also stimulated substrate inhibition, (Bailey and Ayling 1978).

The dihydroxypropyl sidechain on tetrahydrobiopterin (12) has two chiral centres, C1' and C2', and consequently four optical isomers as designated (fig.2). Their naming is derived partly from the spatial arrangement of the hydroxyls on C1' and C2' vis threo where the hydroxyls are diagonally opposed and erythro where they are adjacent and also on their absolute configuration at C2' related to glyceraldehyde (D&L). An examination of the cofactor activity of the four tetrahydro isomers with tyrosine hydroxylase showed that the L erythro isomer (fig.2) exhibited the greatest cofactor activity (Kato et al 1980). Using 6-L-hydroxyethyl and 6-L-dihydroxyethyl tetrahydropterins, both having the same L-configuration at C1' of the sidechain as the putative L-erythrotetrahydrobiopterin (fig.2), similar cofactor characteristics were found. They also found the L-erythro (b) and D-threo (c) exhibited similar and more active characteristics as compared with D-erythro (a) and L-threo (d) isomers, L-erythro (b) and D-threo (c) having the same configuration at C1', the other two having reversed configuration (fig.2).



[6 R]



[6 S]

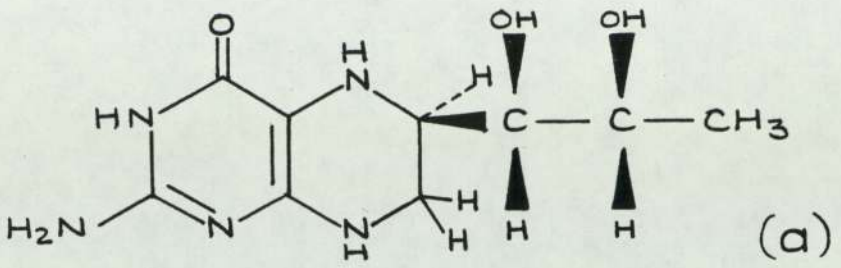
◣ Rising out of plane of paper.

---- Going into the plane of paper.

R = $CH_2OH—CH_2OH—CH_3$

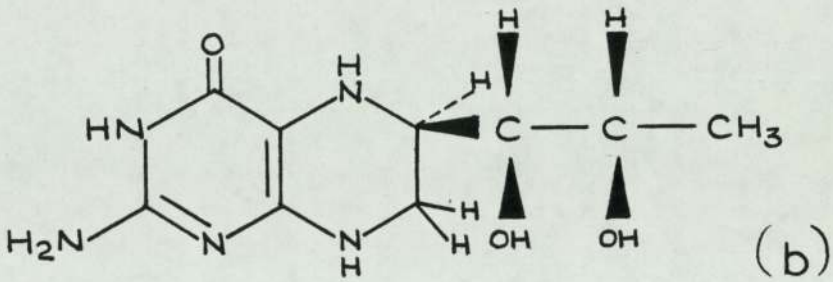
fig. 1

THE STEREOCHEMISTRY OF BIOPTERIN AT CARBON 6.



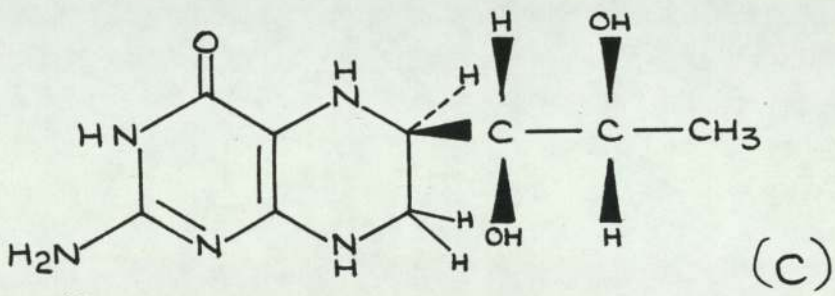
(a)

D – erythrotetrahydrobiopterin



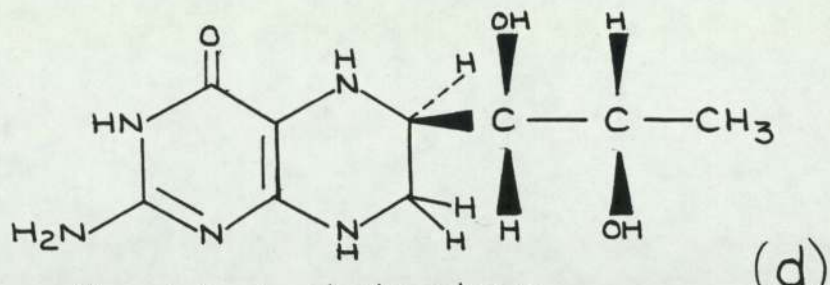
(b)

L erythrotetrahydrobiopterin



(c)

D – threotetrahydrobiopterin



(d)

L – threotetrahydrobiopterin

fig 2

▬◀ bonds rising from the plane of the paper

---- bonds going into the plane of the paper

—— bonds in the plane of the paper

THE STEREOCHEMISTRY OF THE TETRAHYDROBIOPTERIN SIDECHAIN.

From their results they concluded that the Km values for tyrosine are controlled mostly by the configuration at C1' of the sidechain.

There are several methods available for quantitive determination of biopterin derivatives. One which is sensitive and specific is gas chromatography /mass fragmentography. For this method the pterins must be converted to their trimethyl silyl derivatives to increase their volatility for separation (Rother et al 1976). Another method is a radioimmunoassay which is claimed to be specific for natural L-erythrobiopterin (fig.2) and can distinguish between the stereochemical isomers of biopterin. Its specificity is dependent on the preparation of antibodies for a specific pterin and this can be changed by changing the antibodies (Nagatsu et al 1981). The assay can be used to determine both total and reduced forms of biopterin by oxidation of the sample using iodine under acidic conditions to convert

$$BH_4 \quad + \quad BH_2 \xrightarrow{I_2 \,/\, H^+} B$$

both dihydro (13) and tetrahydrobiopterin (12) to biopterin (10), giving a measure of total biopterin, or by oxidising with iodine under alkaline

$$BH_2 \xrightarrow{I_2 \,/\, OH^-} B$$

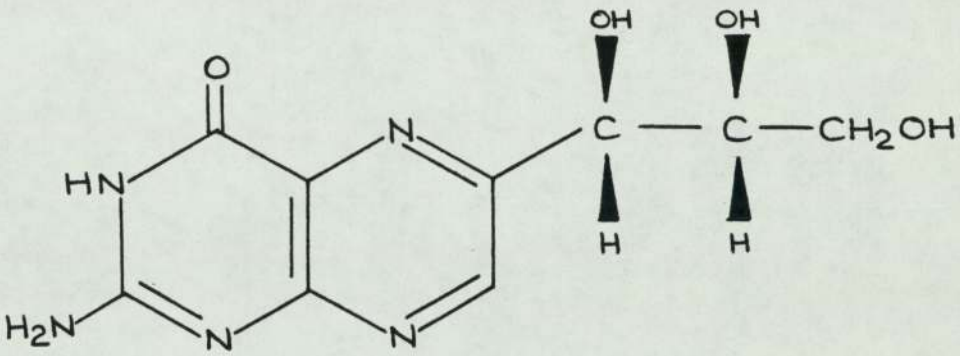
conditions to convert dihydrobiopterin (13) only to biopterin (10). The assay is suitable for large screening as it is simple and rapid (Nagatsu et al 1981). A third quantitative method is one in which phenylalanine hydroxylase activity is measured. This is specific for

$$\text{phenylalanine} \xrightarrow[\text{phenylalanine hydroxylase}]{BH_4} \text{tyrosine}$$

tetrahydrobiopterin (12) since it has a cofactor requirement for it. It can give inaccurate results if dihydropteridine reductase and a reduced cofactor is present (Guroff et al 1967). An extensively used assay is that of Crithidia fasciculata a protozoon with a specific growth requirement for pterins.

The best growth sources are biopterin 100% (10) tetrahydrobiopterin 80% (fig.2) dihydrobiopterin 100%, D-neopterin 56% (14) and sepiapterin 100% (15). The assay has several failings in that it is unable to determine between several pterins if they are present together (this can be overcome by bioautography (Leeming & Blair 1974), that tetrahydrobiopterin is destroyed by autoclaving at pH 4.5 (Milstein 1983) and that it is not able to distinguish between reduced and oxidised derivatives. It is also a rather lengthy assay to perform. It does have several advantages in that it is suitable for large numbers of samples and also that it is extremely sensitive (Dewey and Kidder 1971), Leeming et al 1974). The method most commonly used is high performance liquid chromatography (HPLC) in which the pterins are separated using a chromatography column followed by fluorescent detection. When used with iodine under either acidic or alkaline conditions it is very selective and can distinguish between the various reduced forms of the pterin present. It is a relatively quick assay and only requires small volumes for analysis (Fukushima and Nixon 1980). A comparison of Crithidia and HPLC suggests that both assays have advantages over each other. Firstly the high sensitivity of Crithidia means it can be used to estimate the levels of biopterin derivatives from Guthrie cards thus providing a means for mass screening in young children. The Crithidia assay is, however, less specific and consistently indicates lower levels due to a breakdown of tetrahydrobiopterin to inactive forms during autoclaving at pH 4.5 and during the four day incubation at 25°C (Blair et al 1983). The two assays seem to be complementary.

Whilst pterins could be obtained from the diet experiments on rats and man have shown that tetrahydrobiopterin (12) is poorly absorbed from the gut (Rembold and Metzger1967, Blair et al 1974 (a) Blair et al 1974 (b) Leeming et al 1983). It has been found that biopterin (10) is more readily



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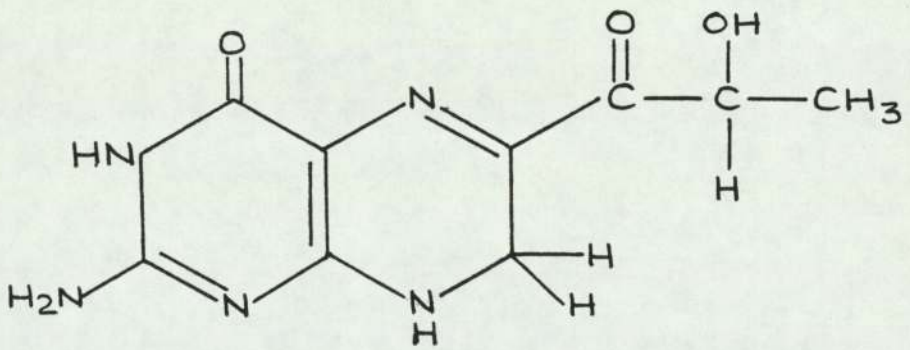
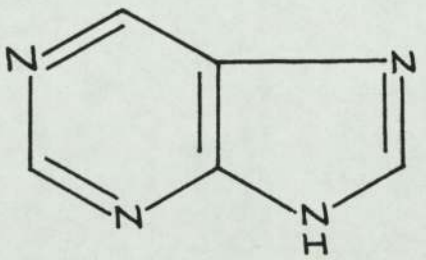


(15)

absorbed and retained. Labelled biopterin (10) given intraperitoneally to rats is excreted in a short time nearly quantitatively in the urine whilst labelled tetrahydrobiopterin (12) is retained (Rembold and Metzger 1963). This suggests that biopterin is reduced during intestinal transport. Because of its poor intestinal absorption little tetrahydrobiopterin (12) is obtained from the diet. It has also been found that on feeding rats a biopterin free diet, urinary excretion of biopterin remains constant (Pabst and Rembold 1966).

Due to structural similarities purines (16) had long been considered as a potential starting material for the biosynthesis of the pteridine ring. Using bacteria it was found that labelled guanine (17), its nucleoside (18), guanosine, and nucleotides (19) guanosine monophosphate, diphosphate and triphosphate were the best precursors for the pteridine ring (Shiota and Palumbo 1965). It was also found that the presence of a ribose moiety on guanine (18) increased the efficiency of pterin formation (Reynolds and Brown 1964) and that guanosine - 5' - triphosphate (20) was the best precursor (Dalal and Gottes 1965). These results were confirmed when GTP cyclohydrolase, which catalyses the conversion of guanosine-5'-triphosphate to D-erythro-7,8-dihydroneopterin-3'-triphosphate (21), was isolated from Escherichia coli (Burg and Brown 1968). Using $8^{14}C$ GTP it was found that C8 was not included in the final pteridine but was eliminated as a one carbon fragment believed to be formic acid (22) (Burg and Brown 1965).

Using knowledge obtained from bacterial systems the biosynthesis of pteridines was investigated in mammals where it was found that rats could be maintained on a biopterin free diet and still excrete biopterin (10) indicating biopterin biosynthesis. It was found that when purines (16) or their precursors such as formate (22) or glycine (23) were injected or fed to rats trace amounts were converted to biopterin (Rembold and Gyune 1972). It was



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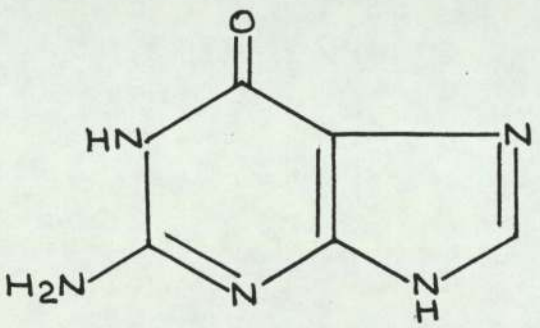

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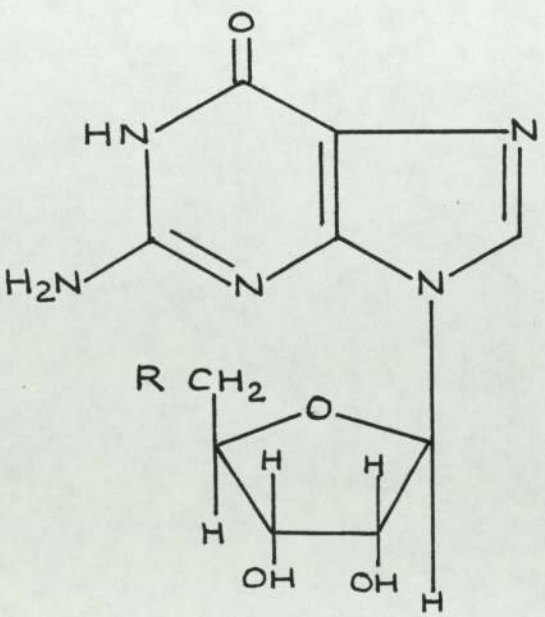


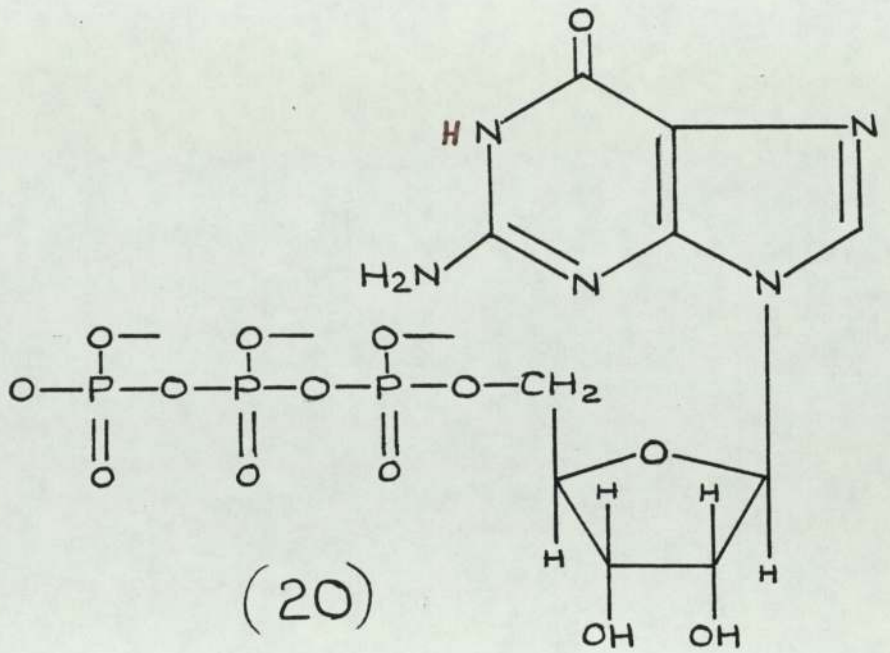
R = OH (18)

R = $(PO_4)_n$ (19)

also found that the formation of biopterin (10) was increased when nucleic acid synthesis was inhibited by an injection of actinomycin D and the accompanying flow of purine precursors into nucleic acid biosynthesis is decreased. Fukushima and Shiota found that similar to bacteria labelled 2-^{14}C GTP was incorporated into biopterin (10) whilst C8 from 8-^{14}C GTP was lost from the system suggesting the non-incorporation of C8. Using extracts from Syrian golden hampsters it was found that GTP (20) or GDP were the most active in producing Crithidia active substances (Fukushima et al 1975).

The first step in the formation of L-erythro-5,6,7,8-tetrahydrobiopterin (fig.2) from guanosine-5'-triphosphate (20) is the opening of the imidazole ring between carbon 8 and nitrogen 9 followed by the ejection of carbon 8 as a one carbon fragment proposed to be formic acid (22) (Shiota and Palumbo 1965, Burg and Brown 1966). After the loss of the one carbon fragment the ribose moiety opens and undergoes an Amadori type rearrangement to yield a deoxypentulose derivative which contributes carbon 1' and 2' to the pterin ring as carbons 7 and 8 with the remaining ribose carbons giving rise to the pteridine sidechain at carbon 6 forming D-erythro-7,8-dihydroneopterin triphosphate (21) (Reynolds and Brown 1964).

An Amadori rearrangement (Hodge 1955) is the isomerisation of an aldosylamine to a 1-amino-1-deoxy-2-ketose. In the proposed mechanism the nitrogen accepts a proton to form an ammonium ion (b) which is in equilibrium with the action of the Schiff base (c) (fig.3). A flow of electrons makes C1 transiently positive and a secondary flow of electrons from C_2 to C1 weakens the C-H bond on C2 causing the expulsion of the proton to give the enol form of the ketose derivative (d) and thence to the keto form (e). (Hodge 1955)



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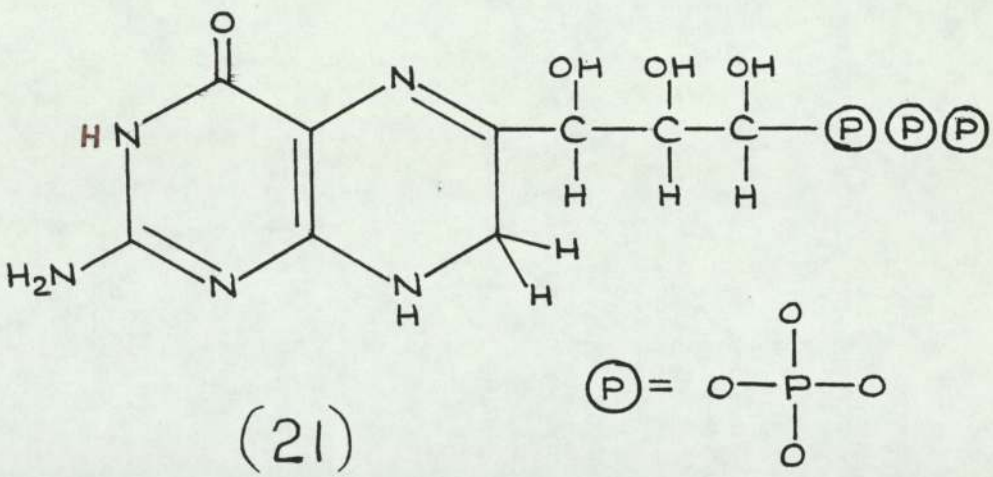

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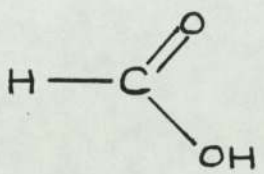

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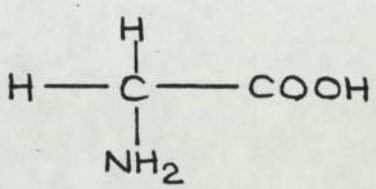


(23)

AMADORI REARRANGEMENT

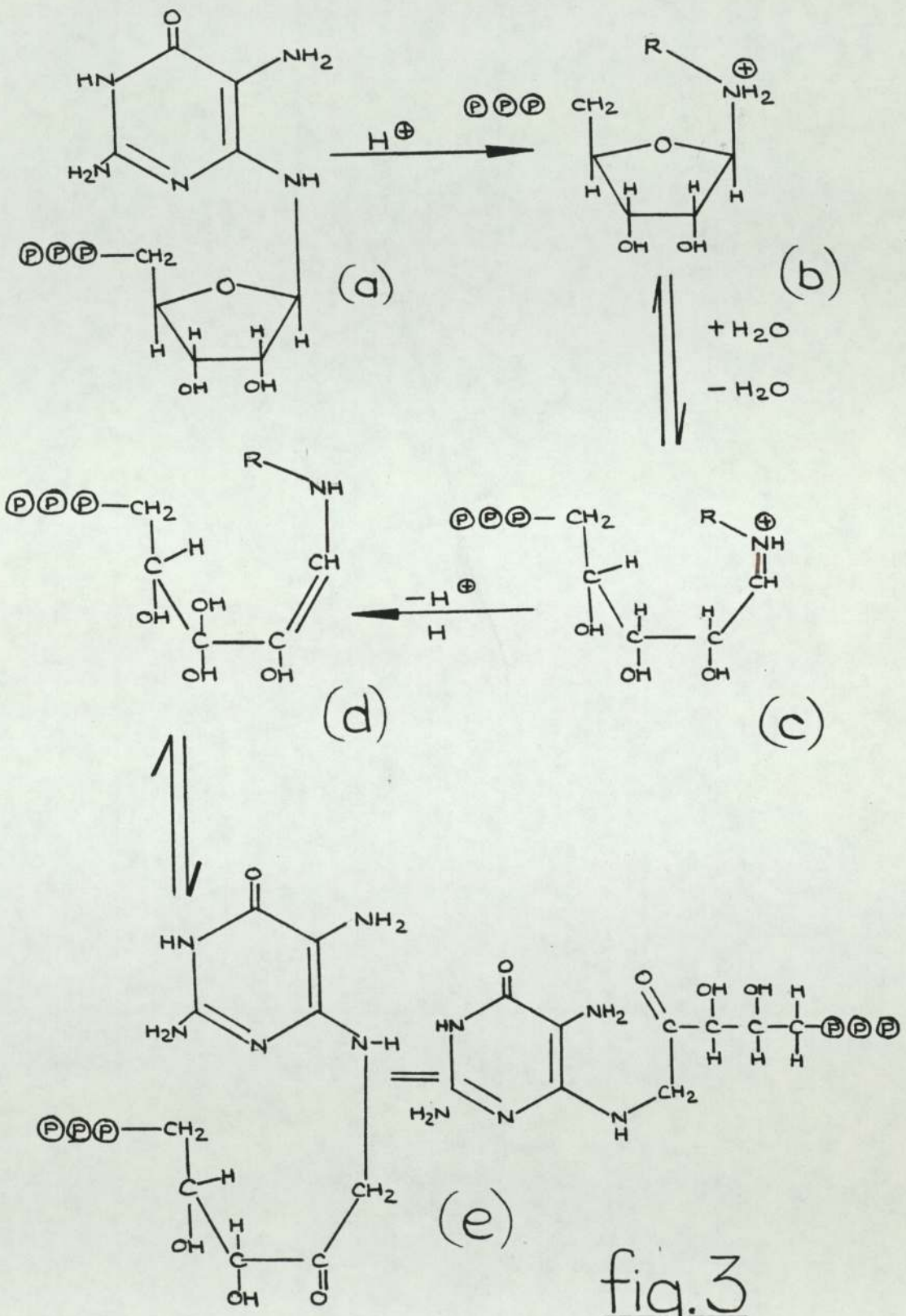


fig.3

Evidence of an Amadori rearrangement was presented by Woolf and Brown (1969) using 7-methylguanosine triosphate (24) as a substrate. The presence of a methyl group on N7 prevents final ring closure and yields a methylated 1-deoxy-2-ketopentulose derivative. This derivative reacts with phenylhydrazine (25) to give a phenylosazone of ribose (26) which is typical of Amadori rearrangement products. (Woolf and Brown 1969).

In bacteria it has been postulated that the formation of D-erythro-7,8-dihydroneopterin triphosphate (21) from guanosine-5'-triphosphate (20) is a single enzyme process catalyzing all the above reactions although none of the intermediates have been found since they are probably enzyme bound (Woolf and Brown 1969).

Gal and co-workers suggest that the formation of D-erythro-7,8-dihydroneopterin triphosphate (21) is a two enzyme step comprising the formation of an open chain formamidopyrimidine ribotide and then the formation of the end product (Gal et al 1978). There is at present, no other corroborating evidence in mammals for this proposal (Curtius et al 1983, Fukishima et al 1977).

The overall formation of L-erythro-5,6,7,8-tetrahydrobiopterin (fig.1) from D-erythro-7,8-dihydroneopterin triphosphate (21) has not been completely elucidated and several possible pathways have been proposed.

One proposal is the direct conversion of D-erythro-7,8-dihydroneopterin triphosphate (21) to L-erythro-7,8-dihydrobiopterin (13) by an enzyme L-erythro -7,8-dihydrobiopterin synthetase (fig.4) (Gal et al 1978). Contrary to the evidence of other workers on other systems it was found that this enzyme had no requirement for Mg^{2+} or pyridine nucleotides (Eto et al 1976,

A SUGGESTED PATH FOR TETRAHYDROBIOPTERIN BIOSYNTHESIS.

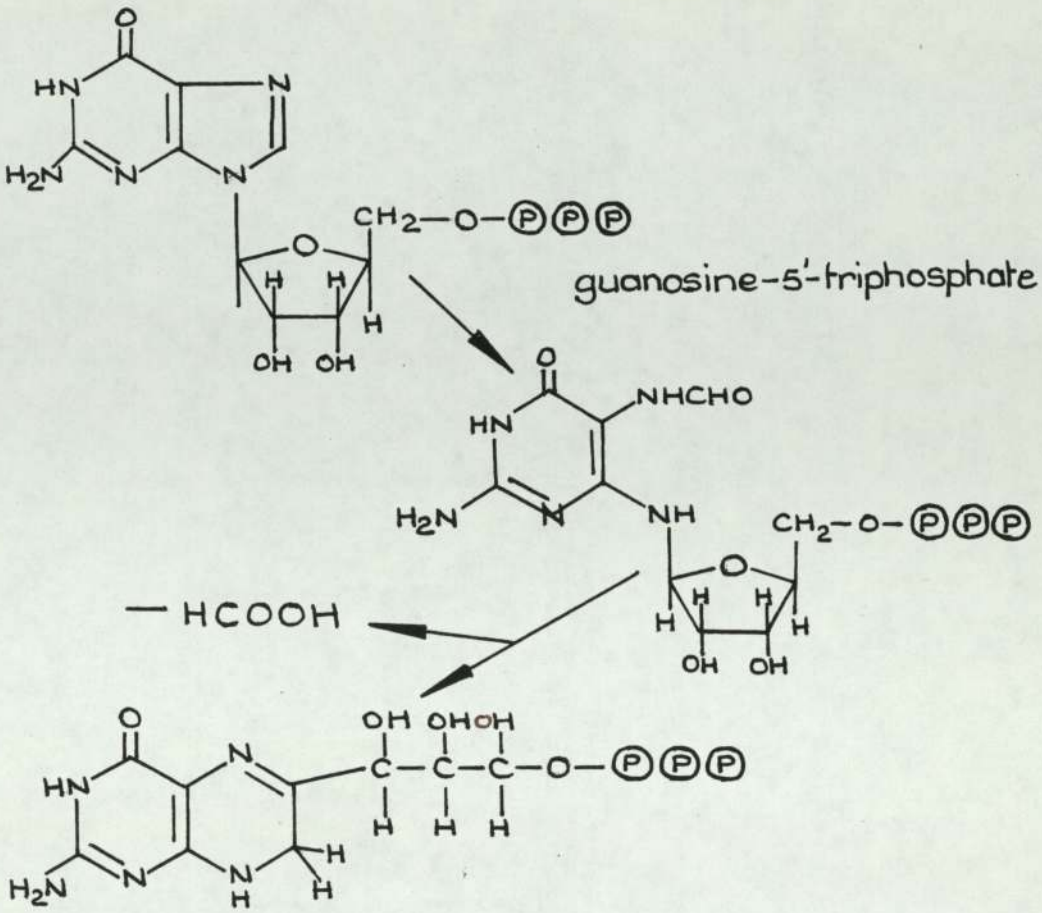
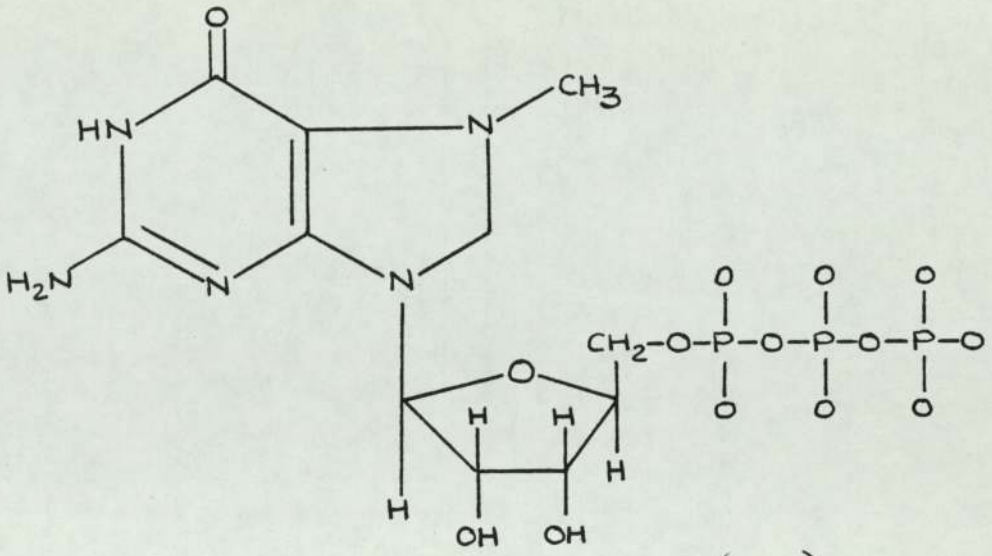


fig. 4



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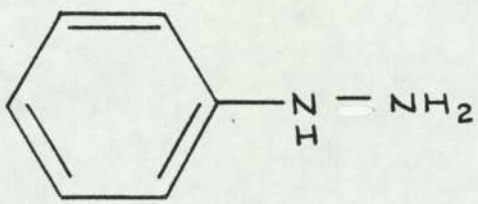

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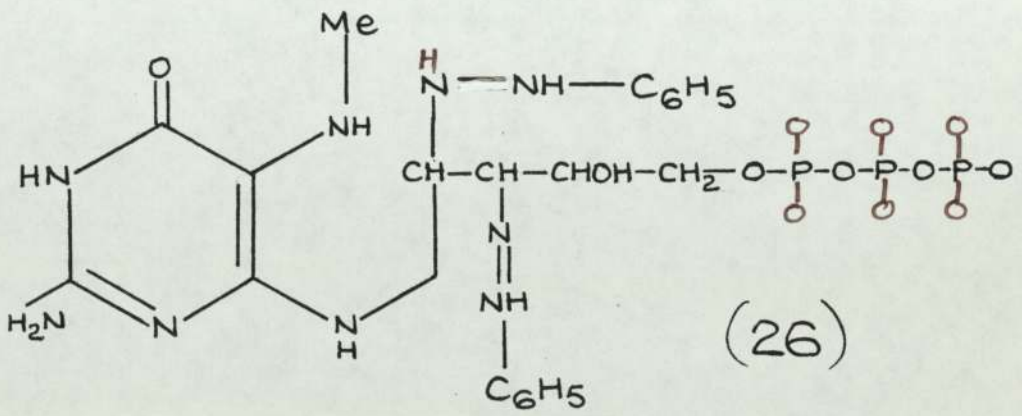


(26)

Gal et al 1978, Kapatos et al 1982). Evidence has been put forward to suggest that a quinonoid form of dihydrobiopterin (27) is the biopterin derivative formed which is reduced to L-erythro-5,6,7,8-tetrahydrobiopterin (fig.2) by the enzyme dihydropteridine reductase (Gal et al 1979).

Another suggested pathway is one in which L-erythro-7,8-dihydrobiopterin (13) is formed via sepiapterin (15) and an intermediate 'X', postulated to be 6-(1,2-dioxopropyl) dihydropterin (28). This path has been found to be dependent on both Mg^{2+} and nicotinamide adenine dinucleotide phosphatide reduced NADPH (29). It has been reported that in chicken kidney the conversion to sepiapterin (15) is a two enzyme process one catalyzing the formation of 'X' (28) and the other the conversion of 'X' to sepiapterin (15). The sepiapterin (15) formed is converted to dihydrobiopterin (13) by the NADPH dependent sepiapterin reductase and then to tetrahydrobiopterin (fig.2) by dihydrofolate reductase (Tanaka et al 1981). Similar results have been found in rat (Kapatos et al 1981) and man (Hauserman et al 1981) (fig.5).

The suggestion by Gal et al (1979) that the formation of L-erythro-5,6,7,8-tetrahydrobiopterin (fig.2) was not dihydrofolate reductase dependent has been confirmed by recent studies. Through the use of methotrexate (30), a dihydrofolate reductase inhibitor, it has been found that L-erythro-5,6,7,8-tetrahydrobiopterin (fig.2) is still formed. (Nichol et al 1983). This has led to two further suggested paths. One suggests that sepiapterin (15) is not on the synthetic route but that L-erythro-5,6,7,8-tetrahydrobiopterin (fig.2) is formed from D-erythro-7,8-dihydroneopterin triphosphate (21) via labile intermediates which can decompose to form sepiapterin (15) which can then be salvaged by sepiapterin reductase and dihydrofolate reductase (Nichol et al 1983b). It has been reported that sepiapterin (15), dihydrobiopterin (13), pterin (31) and pyruvic acid (32) are formed from D-erythro

A SUGGESTED PATH FOR TETRAHYDROBIOPTERIN BIOSYNTHESIS

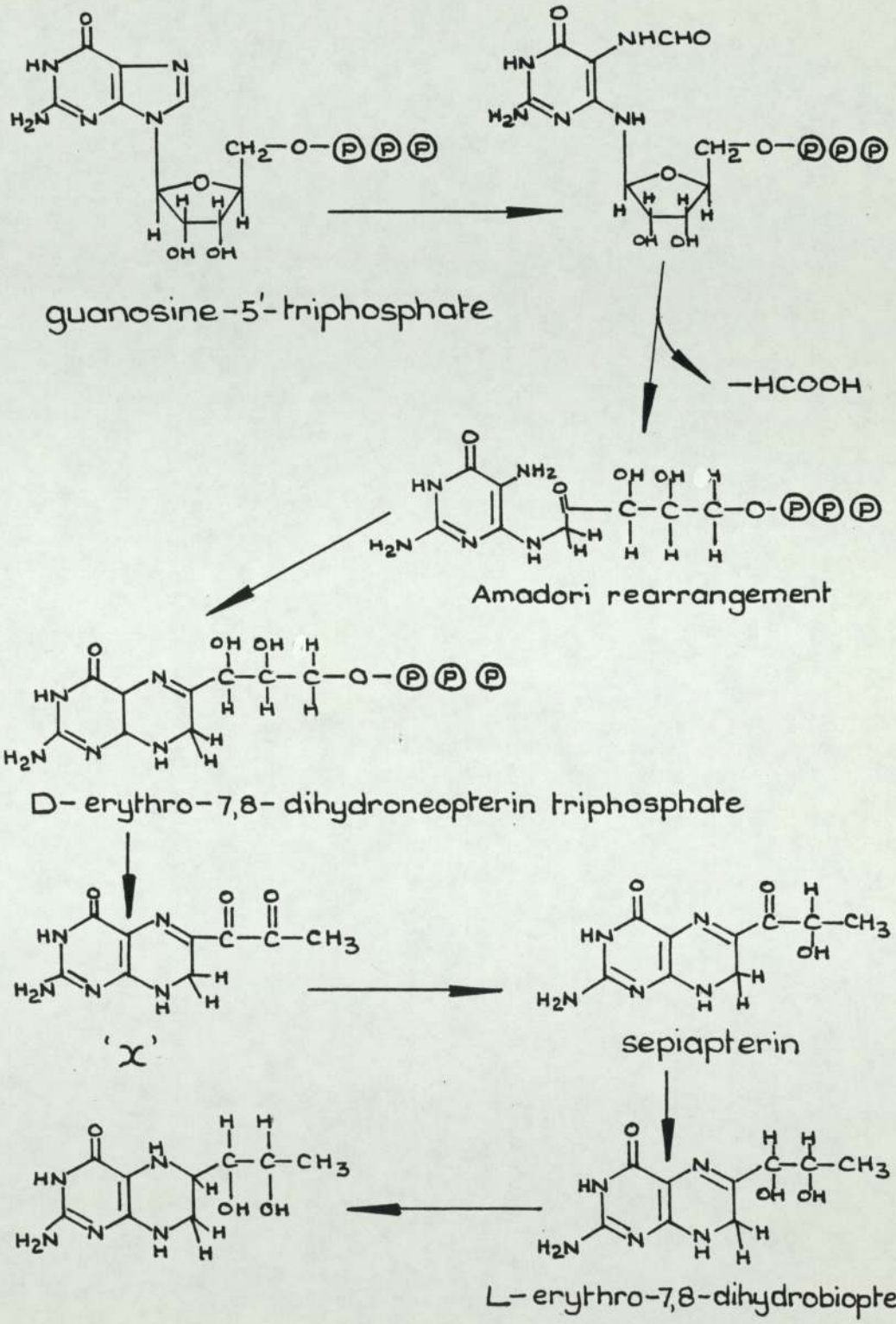
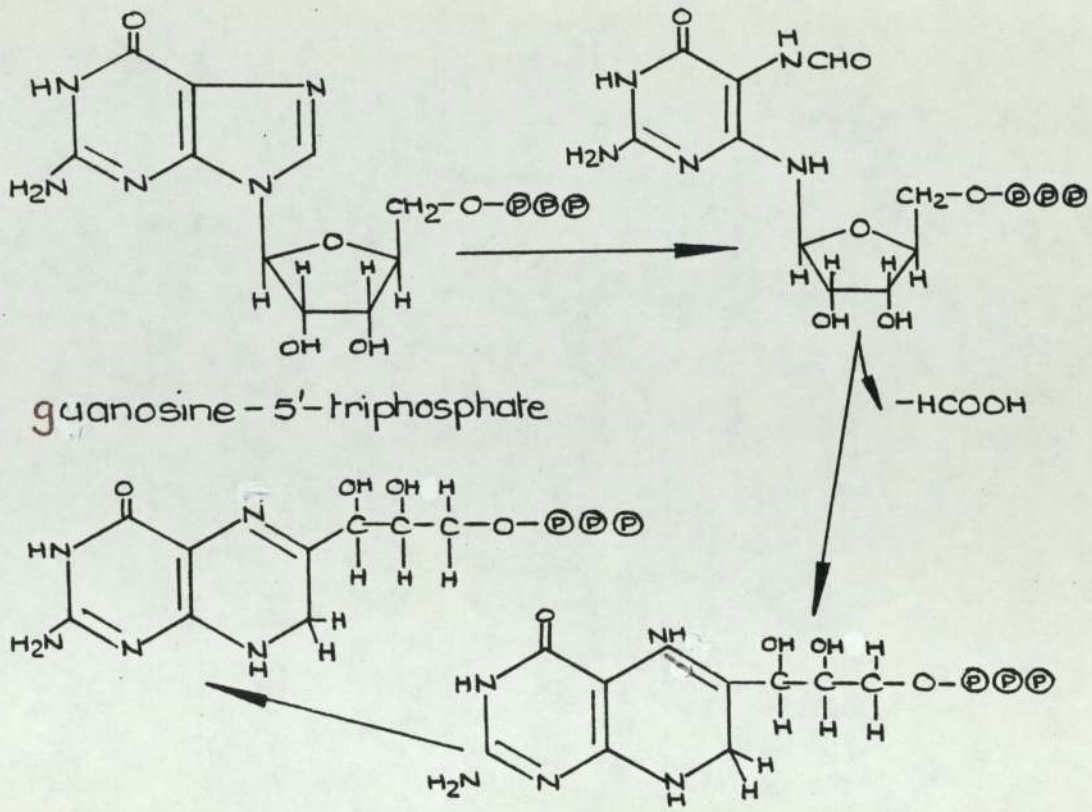


fig. 5

A SUGGESTED PATH FOR TETRAHYDROBIOPTERIN BIOSYNTHESIS



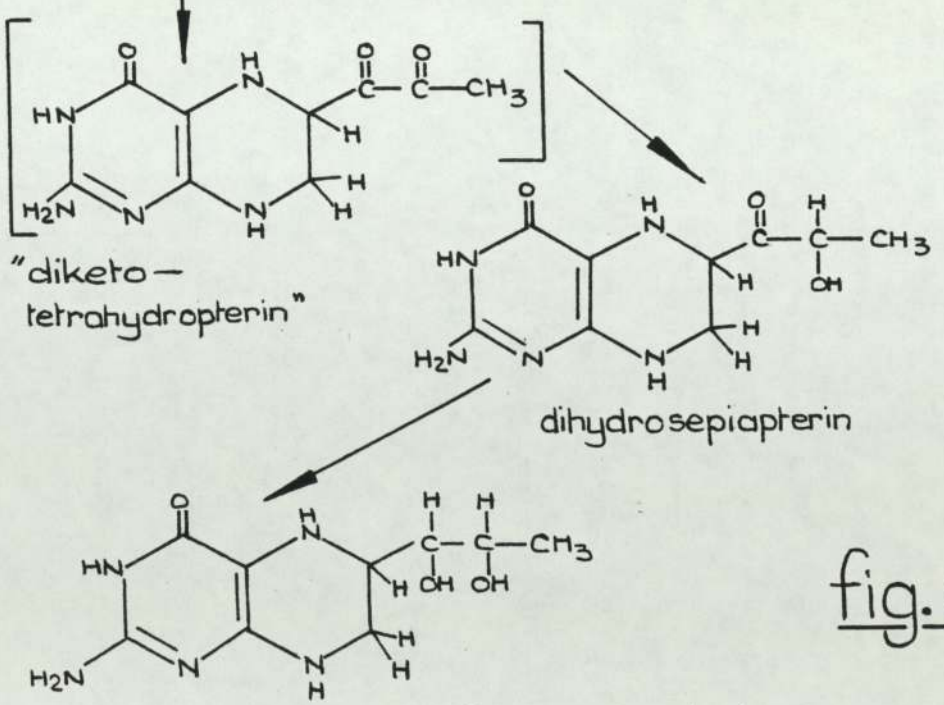
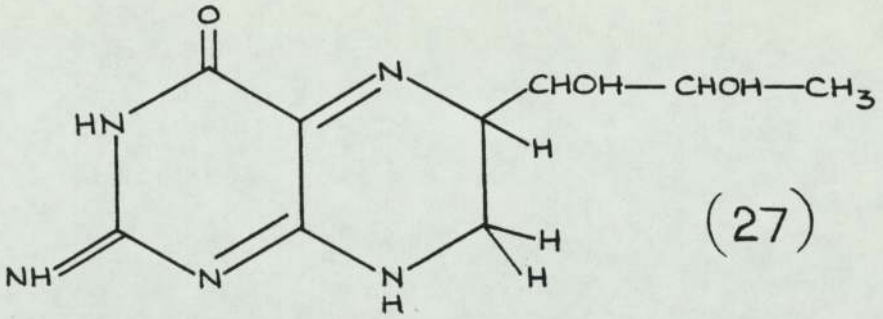


fig. 6



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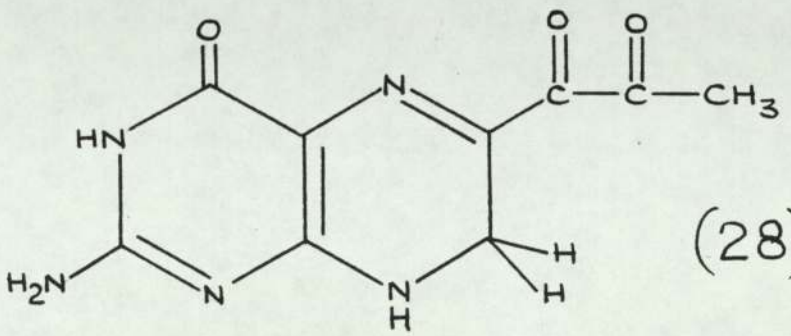

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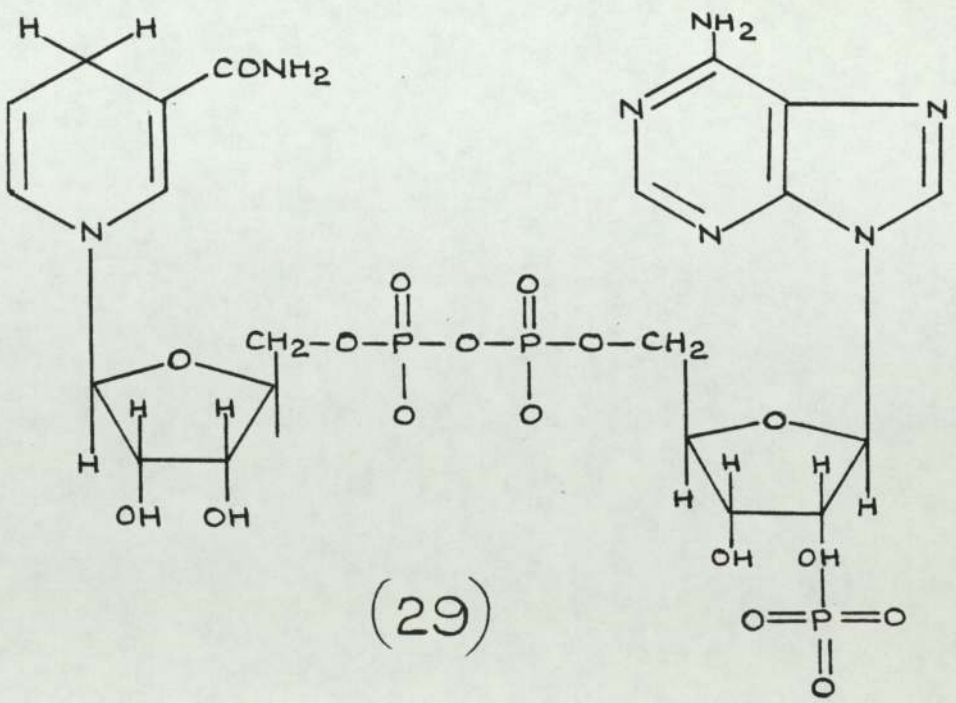

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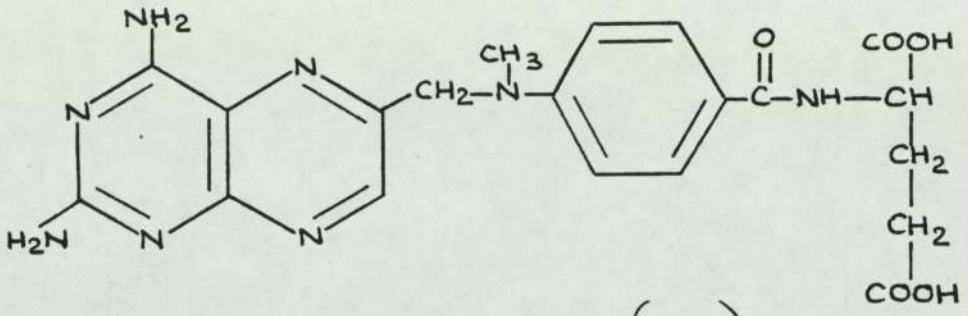

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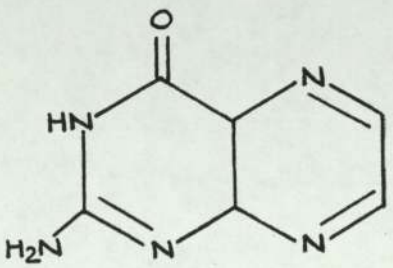

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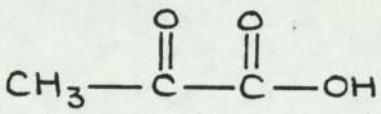

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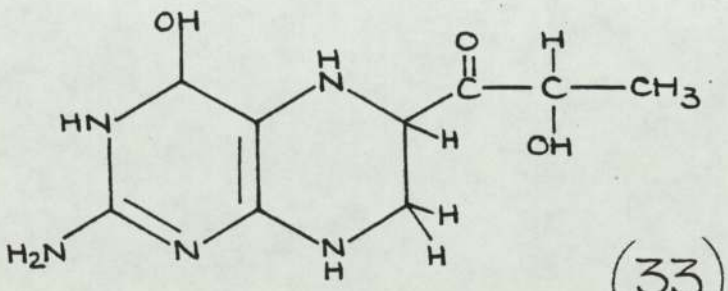


(33)

7,8-dihydroneopterin triphosphate in rat and human liver. The detection of pterin and pyruvic acid indicates the presence of some intermediate which could have the dioxo configuration (28) but their studies indicate that the synthesis occurs via an unstable intermediate. (Curtius et al 1983).

The other suggestion is that the conversion to L-erythro-5,6,7,8-tetrahydrobiopterin goes via a dihydrosepiapterin (33) and that all the intermediates are in the tetrahydro form formed by a shift of electrons to the N5 nitrogen and protons to C6 of the pyrazine ring. It has been found that dihydrosepiapterin (33) is a substrate for sepiapterin reductase and is able to mediate the formation of the end product (fig.6) (Milstien and Kaufman 1983). This has also been reported by Heintel et al (1984) but they further reported the formation of sepiapterin (15) in the absence of NADPH and that the presence of it stimulated the formation of L-erythro-5,6,7,8-tetrahydrobiopterin (fig.2) and reduced the formation of sepiapterin (15) to barely detectable levels. They concluded that the formation of sepiapterin (15) came as a result of a breakdown of an intermediate.

Because of the confusion extensive work is still required to elucidate the biosynthetic pathway and also to determine how the loss of phosphate ester in D-erythro-7,8-dihydroneopterin triphosphate occurs to yield a terminal methyl group on the side chain.

There have been several reported functions of biopterin (10) or pteridines of similar structure.

It has been reported by Tietz (1964) that an enzyme system has been found in liver tissue which catalyzes the oxidation of long chain alkyl ethers of glycerol to fatty acids and free glycerol. Glycerol ethers have been isolated

in the marrow of the long leg bone of cattle where they are believed to play a role in haemopoiesis and a protective role in leukopaenia caused by irradiation. It is suggested that these ethers are oxidised by an enzyme system requiring a reduced pyridine nucleotide, a tetrahydropteridine and molecular oxygen. It is proposed that the ether is first oxidised by molecular oxygen and reduction by the reduced pteridine results in the formation of glycerol and an aldehyde with the aldehyde undergoing oxidation to the fatty acid. The reduced pyridine is required for the reduction of the pteridine and is oxidised during the process (fig.7). It was found that of the pteridines used D-erythro-5,6,7,8-tetrahydroneopterin was the most active. L-erythro-5,6,7,8-tetrahydrobiopterin was not available.

It has been found that the αoxidation of fatty acids is greatly enhanced when a pteridine cofactor, dimethyl-6,7-tetrahydropteridine, is present. (Macdonald and Mead 1968). It is thought that the origin of odd chain fatty acids found in sphingolipids may be related to the α oxidation sequence.

$$R\ CH_2\ COOH \longrightarrow R\ CH\ OH\ COOH \longrightarrow R\ COOH + CO_2$$

A possible involvement of αoxidation is Refsum's disease, a rare inherited neurological disease with large amounts of phytanic acid in the serum and liver, due to a marked reduction in the ability to oxidise phytanic acid in the tissues, (Stumpf 1969).

Rembold and Buff (1972a, 1972b) suggested that tetrahydrobiopterin is a possible cofactor in mitochondrial electron transport mediating the entry of electrons at cytochrome c and a/a_3. It was found that in the presence of tetrahydropterins mitochondria exhibit a strong increase in oxygen consumption. It was found that cytochromes c and a/a_3 are reduced by tetrahydrobiopterins whilst cytochrome b is not. This can be extended further in the knowledge that oxidised dihydropterins can be reduced to tetrahydropterins by high concentrations of pyridine nucleotide or

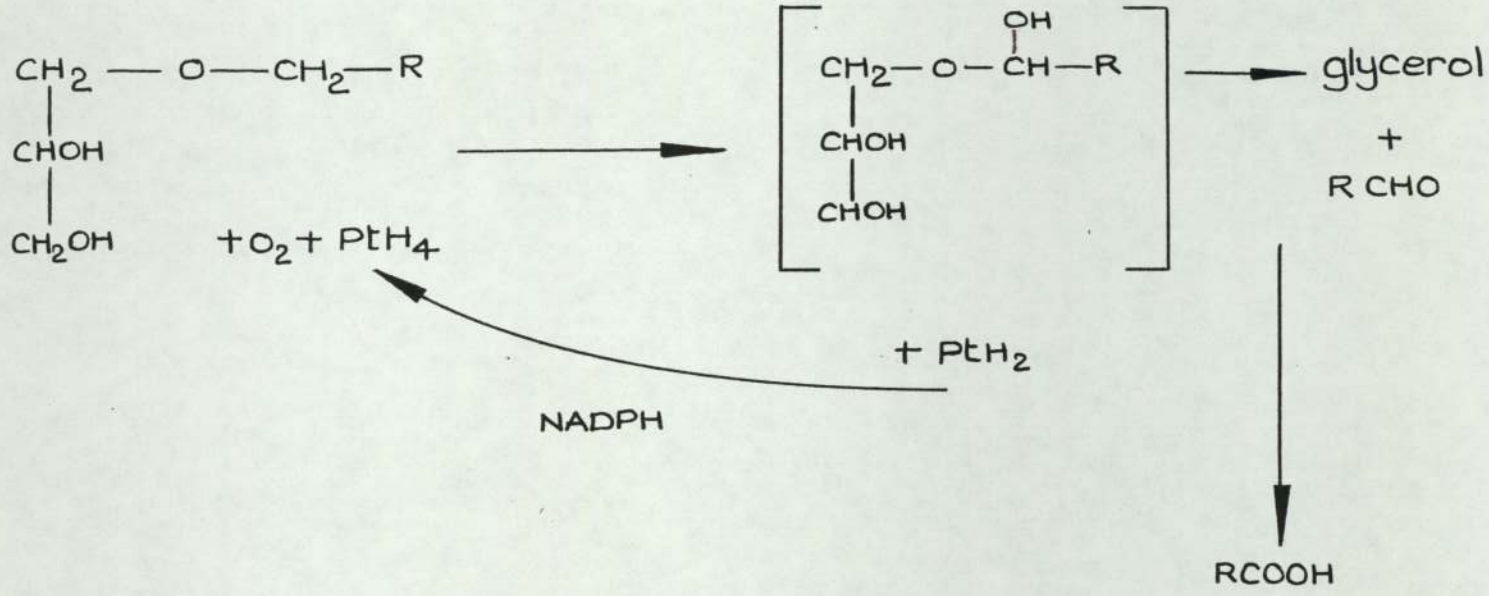


fig.7 THE OXIDATION OF GLYCEROL ETHERS

enzymatically at lower concentration. It suggested that the activating effect of 5,6,7,8-tetrahydrobiopterin could be a shuttle mediating electron transport from extramitochondrial tetrahydrobiopterin pool via cytochrome c to the intramitochondrial cytochrome c system. Another possibility is the direct diffusion of tetrahydrobiopterin through the mitochondrial membrane (fig.8). A scheme of the electron transport is shown (fig.9).

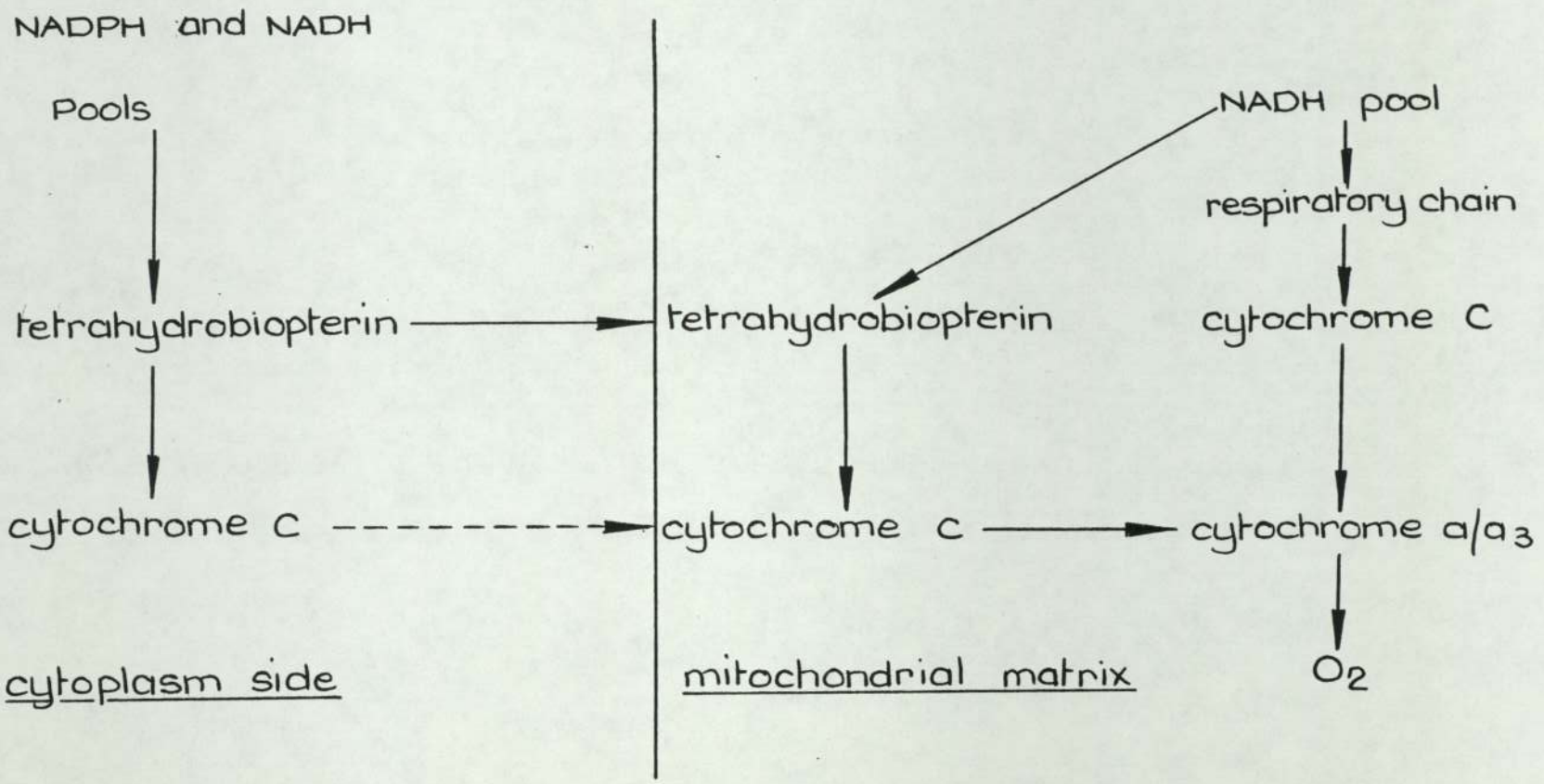
The overall function of this transport chain is obscure but it may regenerate pools of oxidising pyridine nucleotide in cells with high reductive power via the production of heat. Blair and Coleman, using 5-methyltetrahydrofolic acid (34) found that it could reduce cytochrome c and increased the rate of 0_2consumption. They considered that a possible explanation for both their results and those of Rembold and Buff for the increase in oxygen consumption may not be due to an effect on mitchondria but to the phenomenon of autoxidation by both 5-methyltetrahydrofolic acid and reduced pterins which would explain the failure to observe oxidative phosphorylation (Blair and Coleman 1981).

Pterins have been proposed to act as stabilized reductants of the primary photochemical act of photosynthesis (Fuller and Nugent 1969). It has been found that biopterin related compounds are able to stimulate transport in photosynthesis by being the primary site at which the reducing power of photosynthesis is stabilized in system 1 of higher plants. It has been found that 2-amino-4-hydroxy-6 substituted pteridines fulfill the biological, chemical and physiochemical requirements of a primary photochemical electron acceptor in photosynthesis. The mechanism is unclear but is proposed as shown (fig.10).

Melatonin biosynthesis is proposed to be regulated by a pteridine. The pteridine is proposed to be broken photolytically to 6-formylpterin which is

TETRAHYDROBIOPTERIN IN MITOCHONDRIAL ELECTRON TRANSPORT.

fig. 8



A SCHEME OF ELECTRON TRANSPORT.

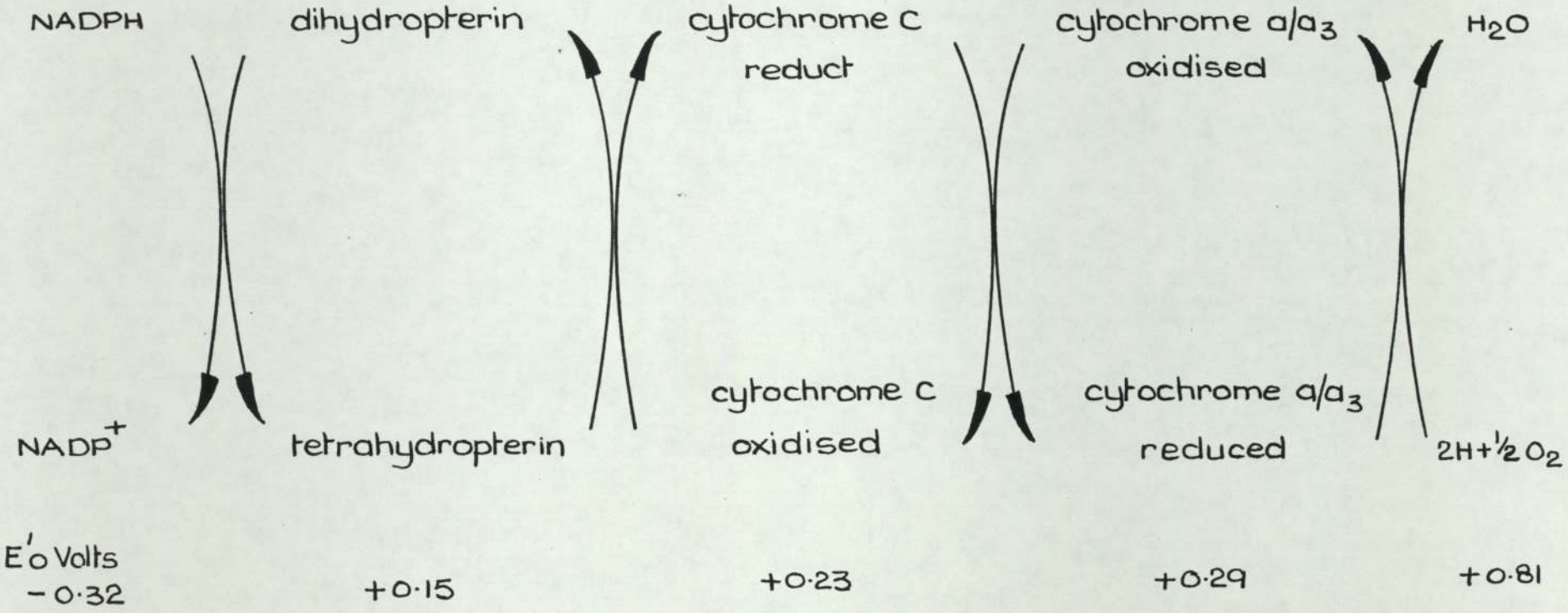
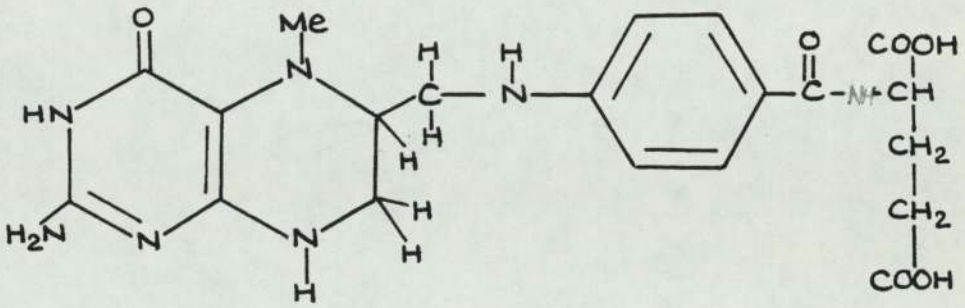


fig 9



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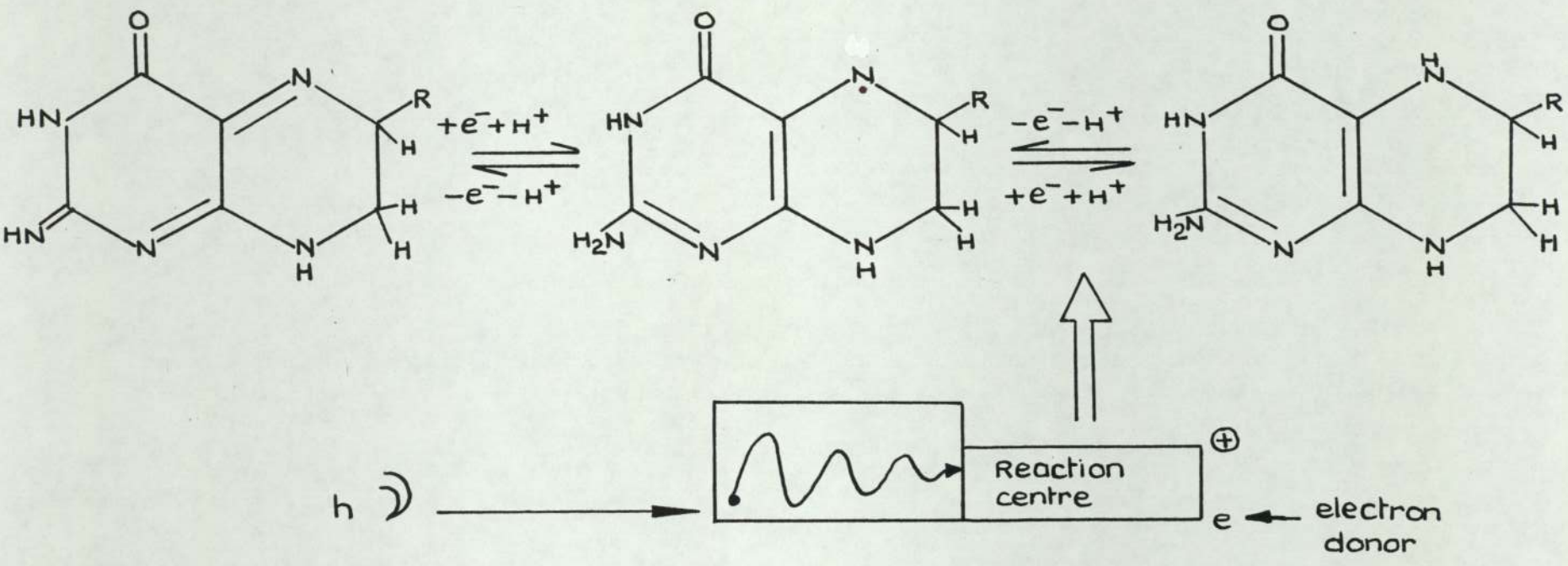


A PTERIN AS A PHOTOCHEMICAL ELECTRON ACCEPTOR

fig. 10

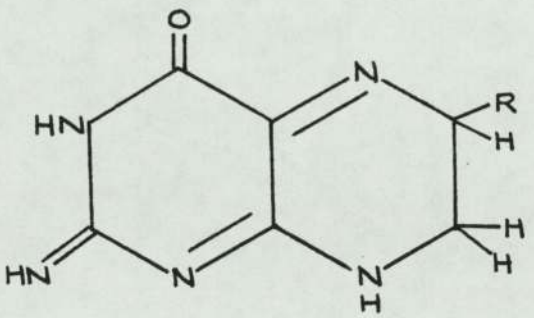
then transported to the pineal gland where it inhibits hydroxyindole-o-methyltransferase, the rate-limiting enzyme in melatonin biosynthesis (Cremer-Bartels and Ebels 1980). Recently Milstien and Kaufman (1983) have suggested that melatonin and N-acetylserotonin could regulate biopterin biosynthesis in the pineal gland making it light dependent.

The function of tetrahydrobiopterin (fig.2) which has been clearly established is its role in the oxidation of aromatic acids, where it functions as a reducing agent and is resultingly oxidised to quinonoid dihydrobiopterin (27) which is inactive as a cofactor. Quinonoid dihydrobiopterin (27) is unstable and rapidly rearranges to dihydrobiopterin (13) (Kaufman 1967). This is lost from the system in the urine.

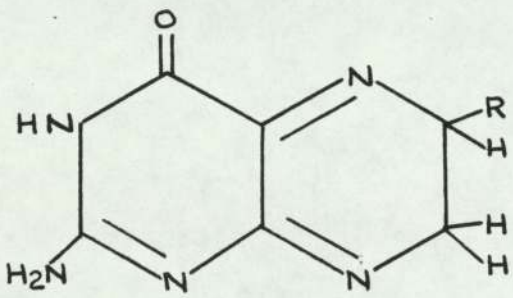
The tautomeric form in which quinonoid dihydrobiopterin exists has received some attention of late (fig.11). Armarego and Waring (1983) have presented evidence which suggests that structure c (fig.11) is the state in which it exists and they put forward a mechanism, based on this structure, for its enzymatic reduction with NADH. Lazarus et al (1983) prefer form a and suggest that this is formed during the oxidation of tetrahydrobiopterin (fig.2) via a 4a hydroxy intermediate (35). They reported evidence for this intermediate during the formation of L-tyrosine (36) from L-phenylalanine (37). Further evidence for this intermediate has been put forward by Wallick et al (1984) who propose a mechanism for the function of phenylalanine hydroxylase requiring the pre-reduction of the hydroxylase by a pterin followed by the formation of the 4a intermediate during the convertion of phenylalanine (37) to tyrosine (36). The phenylalanine hydroxylase is pre-reduced by the reduction of a non haem iron ligand from Fe^{3+} to Fe^{2+}.

Quinonoid dihydrobiopterin (fig.11) is reduced to tetrahydrobiopterin (fig.2) by

THE SUGGESTED TAUTOMERS OF QUINONOID DIHYDROBIOPTERIN



(a)



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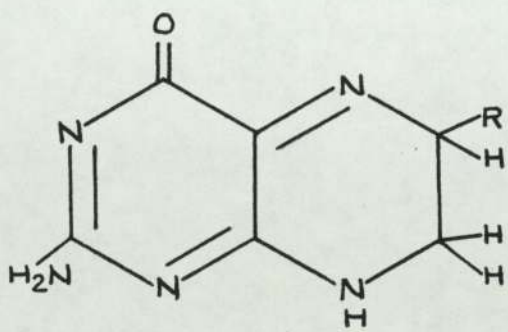
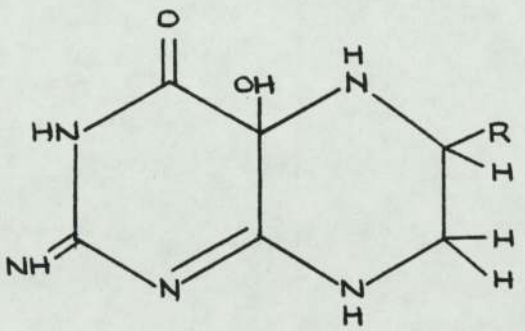


(c)

fig. 11



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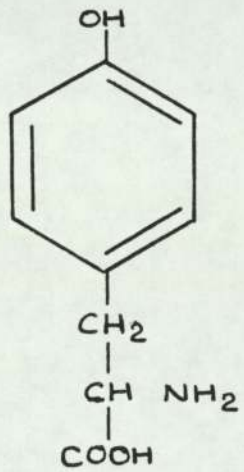

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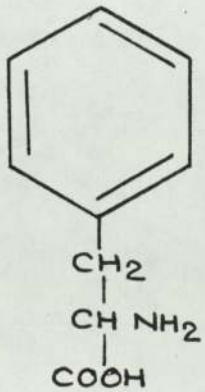


(37)

'sheep liver enzyme' (dihydropteridine reductase) and a reduced pyridine nucleotide (Kaufman 1964b). The enzyme system is able to use both reduced nicotinamide adenine dinucleotide phosphatide (NADPH) (29) or reduced nicotinamide adenine dinucleotide (NADH) (38) but is much more active with NADH (Nielson et al 1969). The activity of dihydropteridine reductase can be measured by following the disappearance of NADH (38) spectrophotometrically provided quinonoid dhydrobiopterin is present (Craine et al).

A second enzyme, dihydrofolate reductase, is able to convert dihydrobiopterin (13) to tetrahydrobiopterin (fig.2) in the presence of NADPH (29) (Kaufman 1967, Spector et al 1977). This enzyme is present only in very low concentration or absent in the brain. Tetrahydrobiopterin is the cofactor for phenylalanine hydroxylase which catalyses the conversion of phenylalanine (37) to tyrosine (36) (Kaufman 1958, Kaufman and Lovenburg 1959, Kaufman 1963),

$$\text{phenylalanine} + O_2 + BH_4 \longrightarrow \text{tyrosine} + q\,BH_2 + H_2O$$

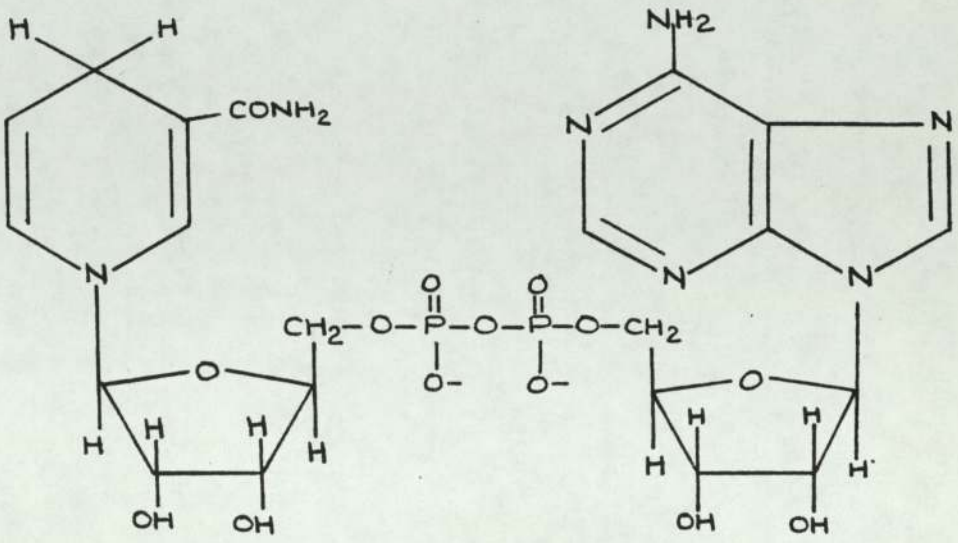
tyrosine hydroxylase, catalysing the conversion of tyrosine (36) to L-dopa (40) (Kaufman 1964, Nagatsu et al 1964)

$$\text{tyrosine} + O_2 + BH_4 \longrightarrow \text{L-dopa} + q\,BH_2 + H_2O$$

and tryptophan hydroxylase catalysing the conversion of tryptophan (41) to 5-hydroxytryptophan (42)

$$\text{tryptophan} + O_2 + BH_4 \longrightarrow \text{5-hydroxytryptophan} + q\,BH_2 + H_2O$$

(Jequier et al 1969).



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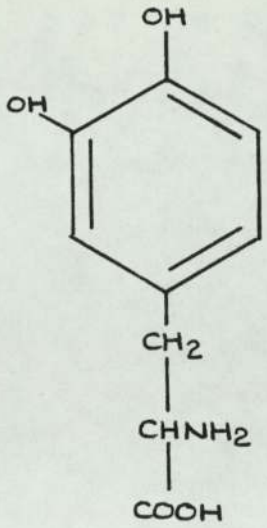

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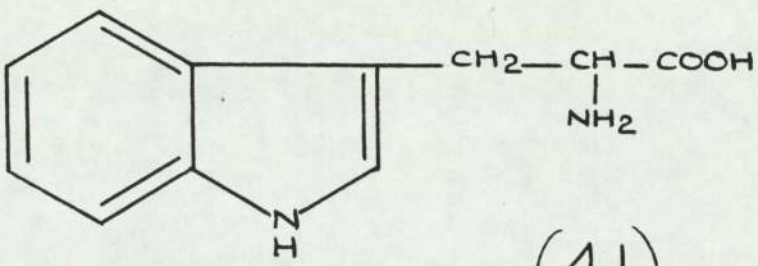

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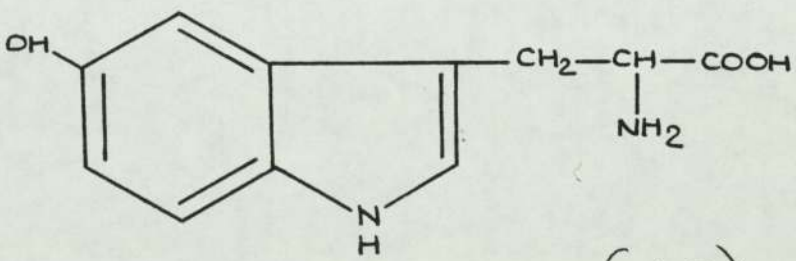


(42)

The conversion of tyrosine to L-dopa is the first step in the biosynthesis of the catecholamines, dopamine (43), adrenalin (44) and noradrenalin (45), a step which has been found to be rate limiting (fig.12) (Levitt et al 1964). It has been found that the concentration of tetrahydrobiopterin (fig.2) is the rate determining factor for tyrosine hydroxylase (Kettler et al 1974). It is also suggested that there is end product inhibition by competing with pterin cofactor (Costa and Meek 1974).

The formation of 5-hydroxytryptophan (42) from tryptophan (41) is the first step in the biosynthesis of the neurotransmitter serotonin (46). This step is rate-limiting and tetrahydrobiopterin concentration is also rate-limiting as is that of tryptophan (Costa and Meek 1974) (fig.13).

The neurotransmitters noradrenalin (45) adrenalin (44) serotonin (46) and dopamine (44) play a vital role in the function of the brain innervating large areas of the organ between them.

The brain is a very complex organ of about 1.4kg. in an average adult and is a semisolid pinkish grey. It is protected by the skull and three membranes, meninges. Whilst its external appearance is that of a simple organ, comprising of a cerebral hemisphere, cerebellum and medulla (fig.14), internally it is much more complex (fig.15) having many interconnecting parts and fluid filled ventricles, containing cerebral spinal fluid which bathes the whole brain, which are continuous with the central canal of the spinal cord. 0_2 and nutrients are supplied to the brain via the bloodstream. The nutrients then pass from the blood into the cerebral spinal fluid through which they are distributed to the brain proper. There is no direct interchange between the blood and the brain. The interchange of nutrients from the blood is somewhat restricted and many nutrients, including tetrahydrobiopterin are unable to reach the brain in any quantity.

BIOSYNTHESIS OF CATECHOLAMINES.

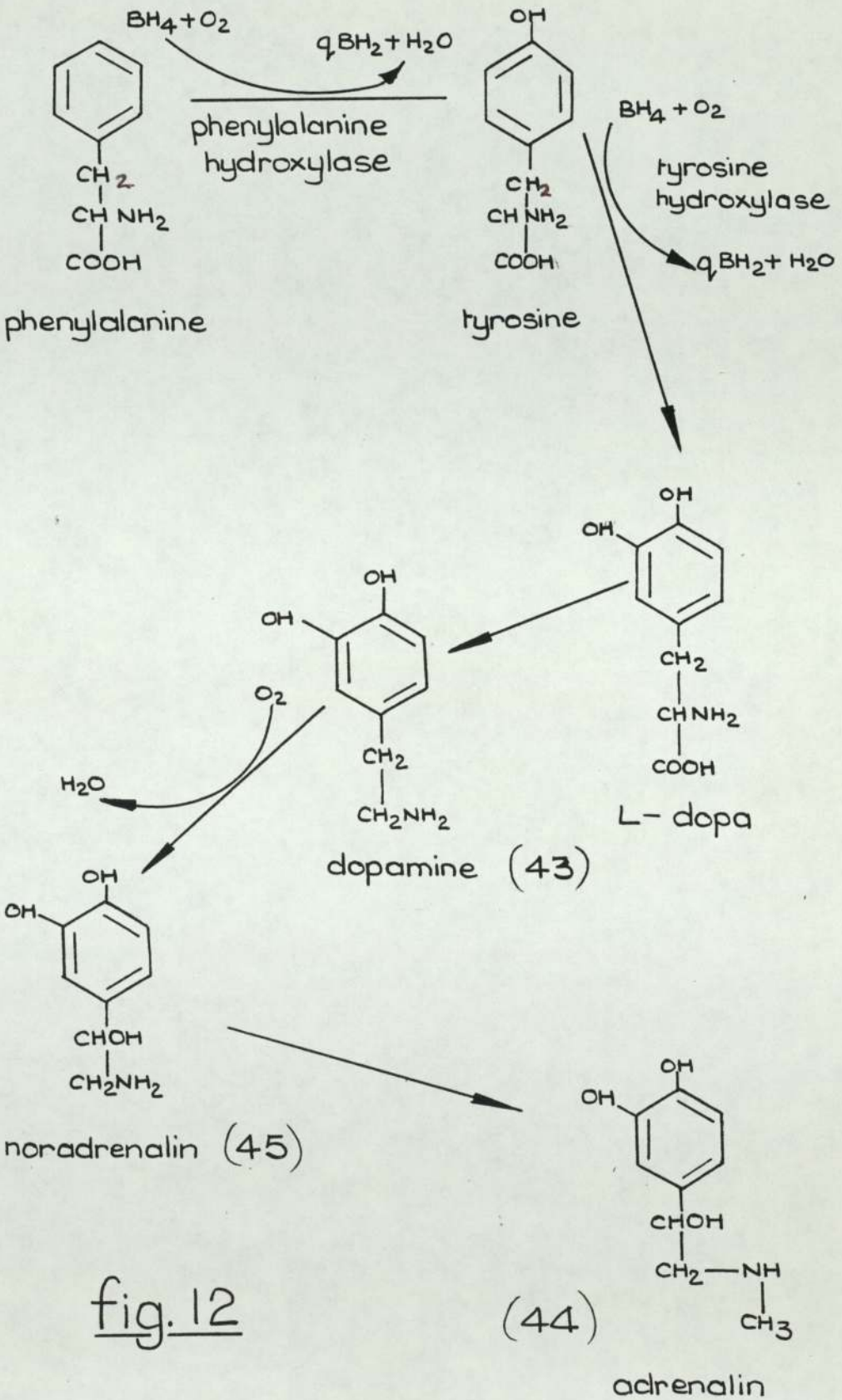
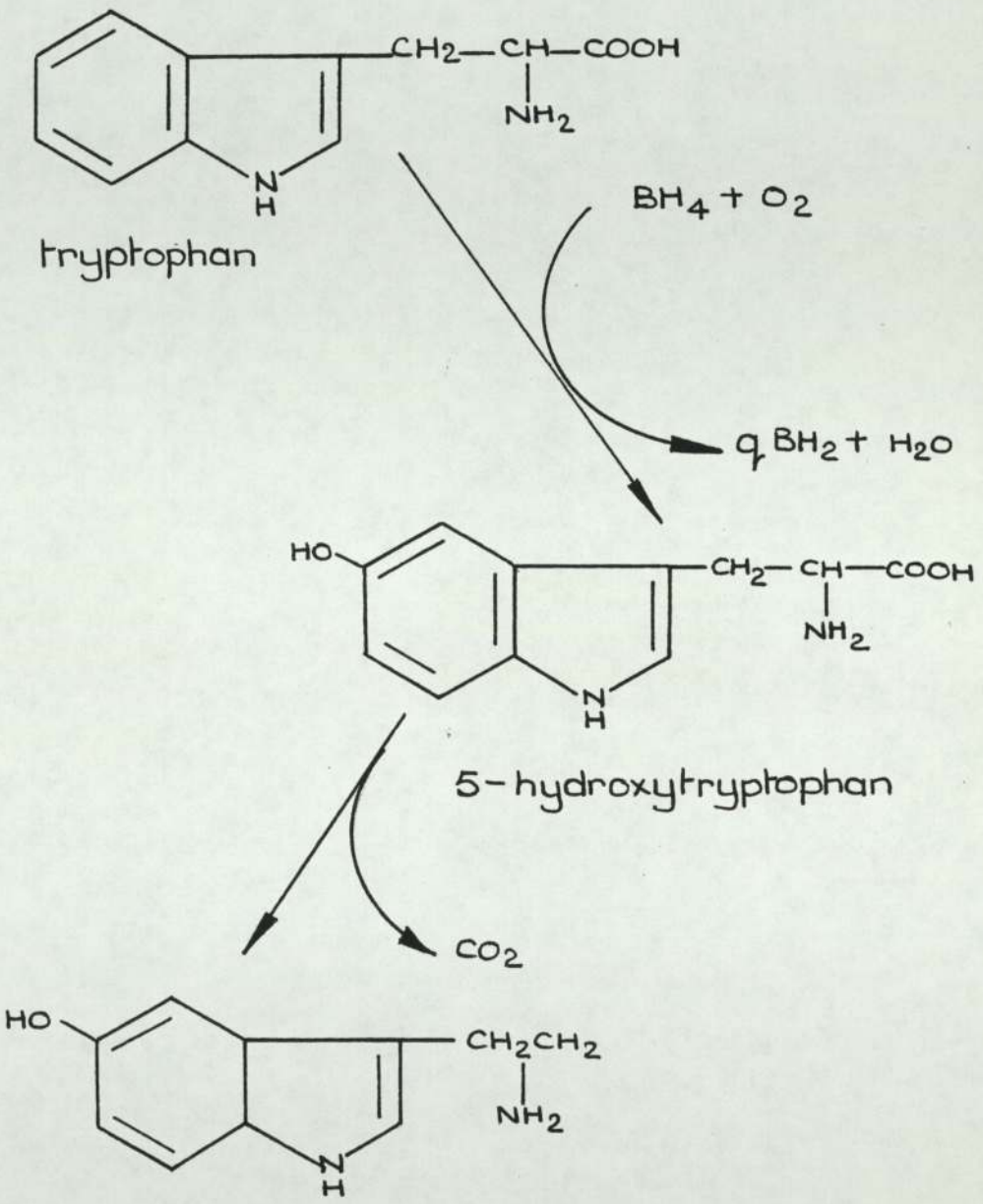


fig. 12

BIOSYNTHESIS OF SEROTONIN

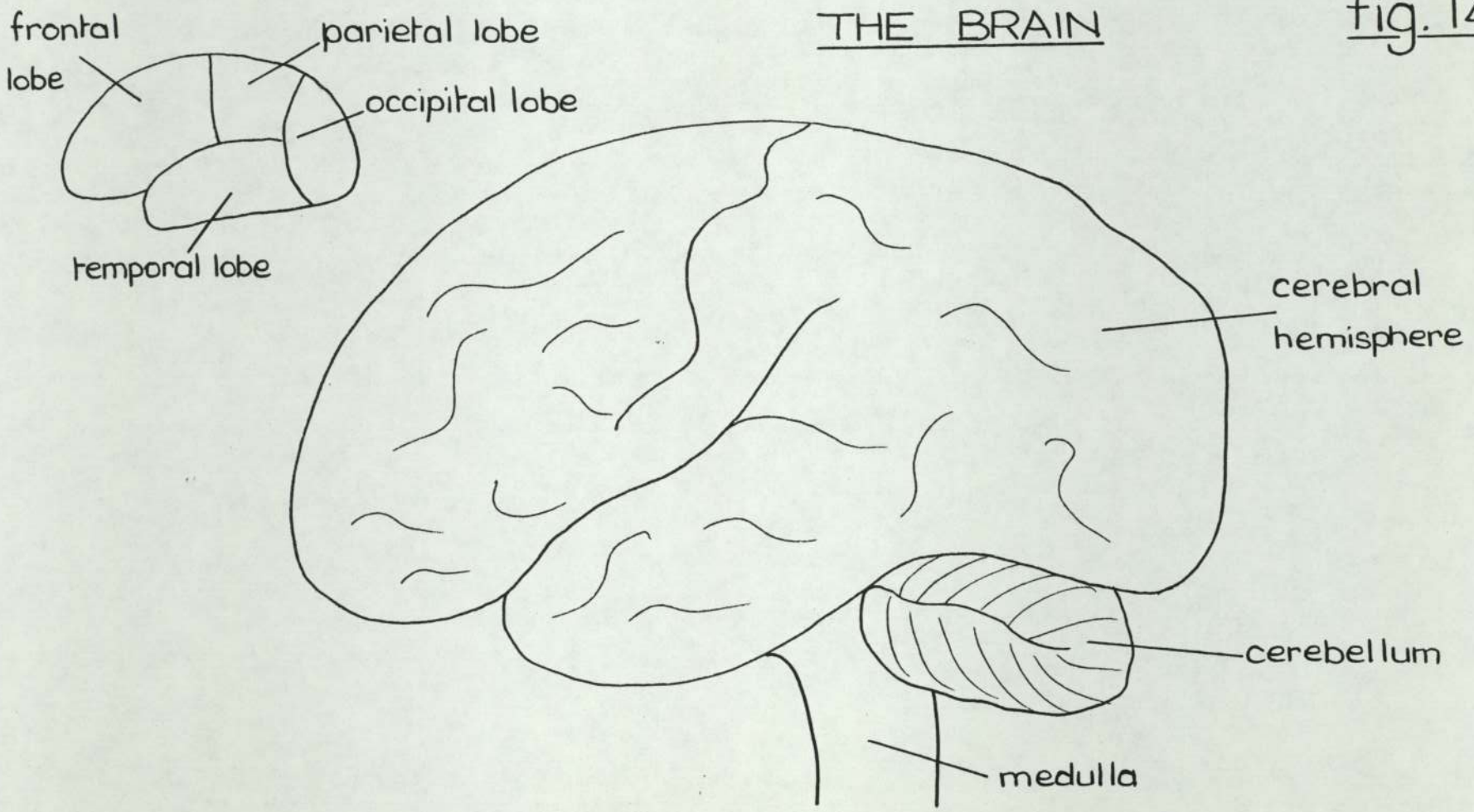


(46)

fig. 13

THE BRAIN

fig. 14



THE BRAIN

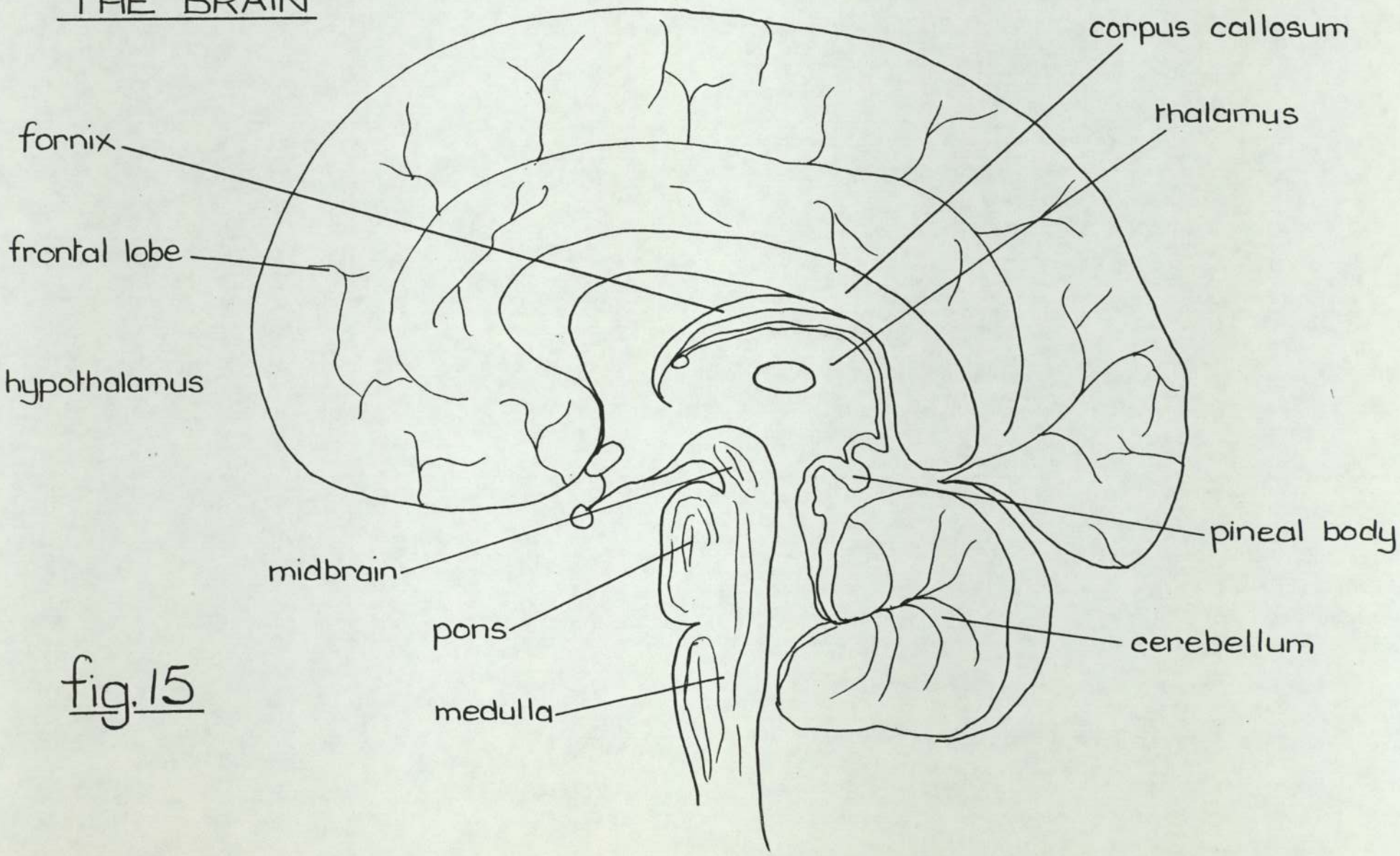


fig. 15

Noradrenalin (45) is found in almost all areas of the brain and spinal cord and originates from small groups of cells in the medulla, the largest of which is the locus coeruleus. From this group of 20,000 cells the whole of the dorsal forebrain is innervated especally the cerebral cortex and hippocampus (fig. 16) (Iverson 1982). Adrenalin (44) is localised in the pons/medulla and innervates other brain stem structures. A particularly dense innervation is the nucleus tractus solitarius (Iverson 1982).

The Corpus striatum is richly innervated by dopamine (43) which originates from dopaminergic cells in the pars compacta of the substantia nigra. Other dopaminergic neurones arising in the ventral tegmentum innervate structures in the limbic forebrain including the amygdaloid nucleus, nucleus accumbens septi and limbic areas of the frontal entorhinal cortex. Dopamine (43) in the nigrostriatal pathway plays some role in modulating the control of voluntary movement and its actions in the basal ganglia are antagonistic to acetylcholine. When dopamine (43) is absent or blocked a Parkinson syndrome ensues (fig. 17) (Iverson 1982).

Serotonin (46) projects from a series of cell groups in the raphe nuclei to many forebrain sites with especially high densities of fibres in the hypothalamus, basal ganglia, and medial forebrain bundle. It is thought to play a role in the sleep/wake cycle and has been implicated in temperature regulation and the control of agressive behaviour (Iverson 1982) (fig.17).

Changes in the levels of the catecholamines or serotonin are obviously likely to affect the overall brain function and as these depend on tetrahydrobiopterin (fig. 2) it is of considerable interest.

THE NORADRENERGIC PATHWAYS

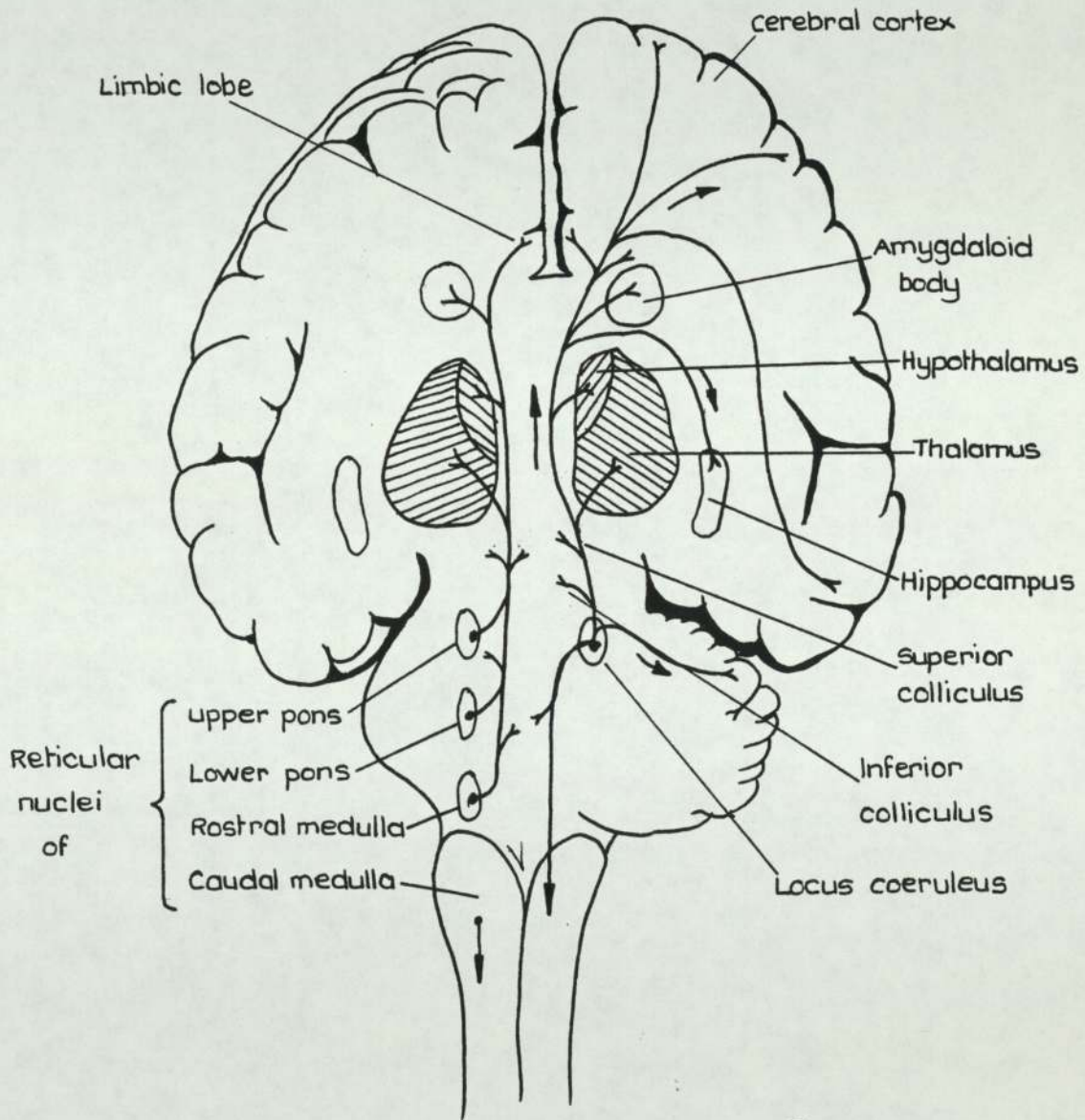


fig. 16

THE DOPAMINERGIC PATHWAYS (left) AND THE SEROTONINERGIC (5-HT) PATHWAYS (right)

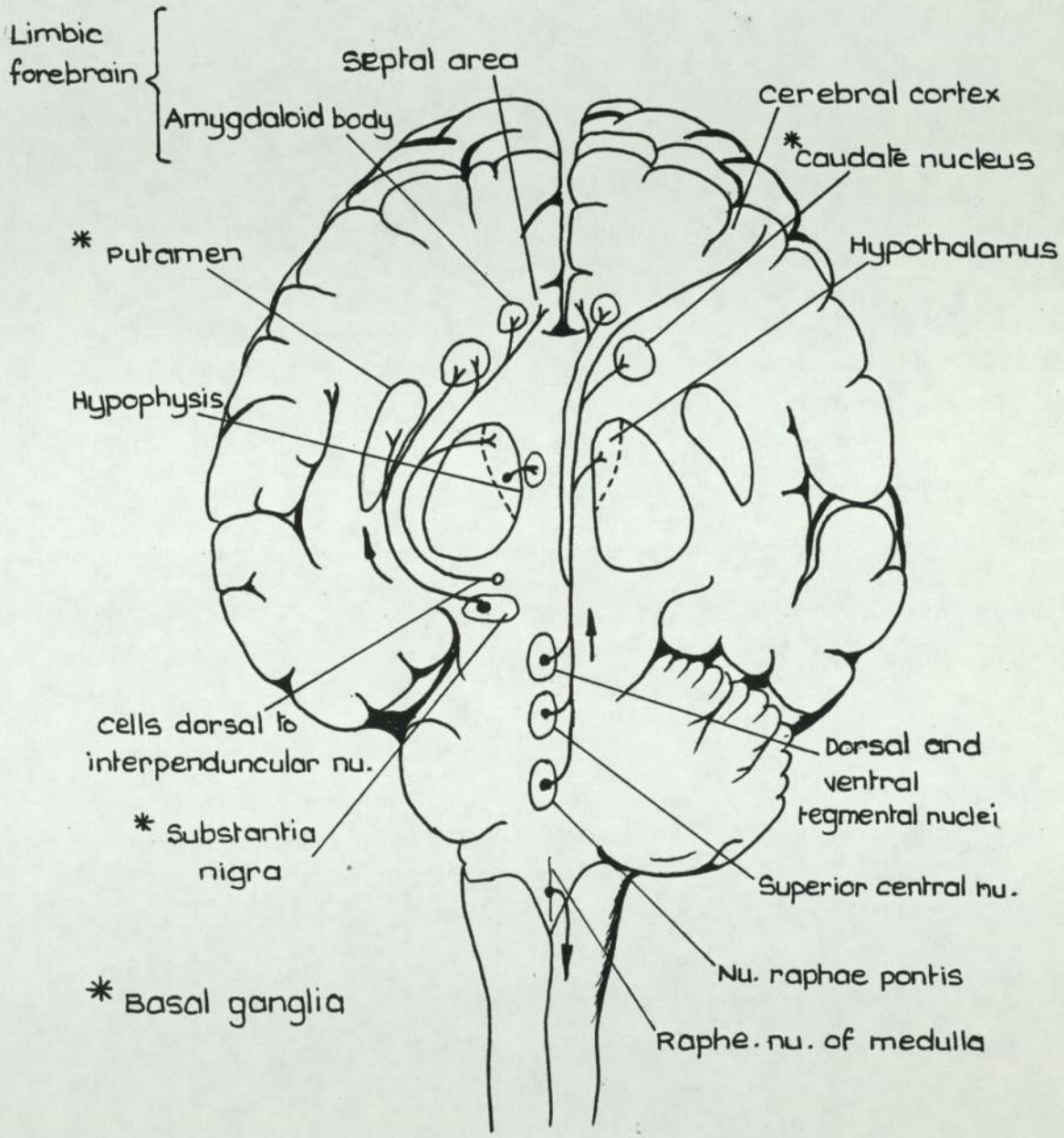
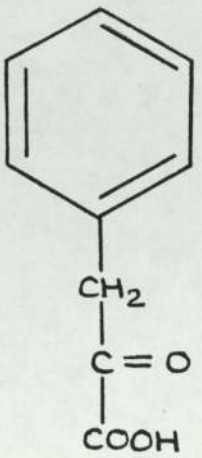


fig. 17

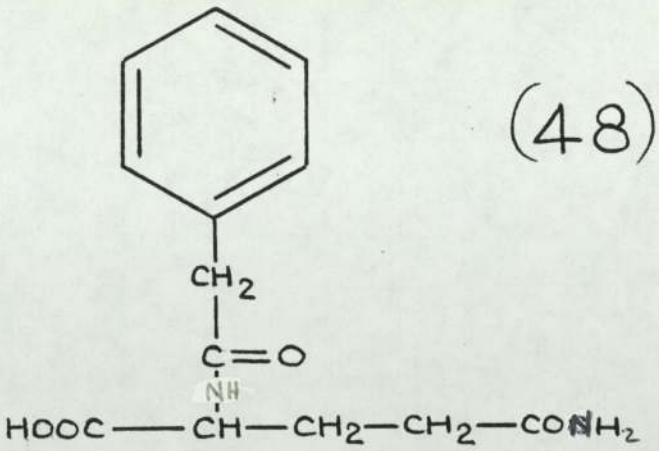
The importance of tetrahydrobiopterin (fig.2) was observed through the genetically transmitted disease phenylketonuria. This disease is characterised clinically by mental retardation and convulsive seizures. Phenylpyruvic acid (47) is observed in the urine. The disease is due to an inability to metabolise phenylalanine (37) to tyrosine (36) due to the absence of phenylalanine hydroxylase. This gives rise to high levels of phenylalanine (37) in the blood and this becomes apparent in the urine as phenylpyruvic acid (47) and phenylacetylglutamine (48) (Woolf 1951). Raised blood or serum levels of biopterin (10) have been observed in these patients (Leeming 1975, Leeming et al 1974). The disease is controllable with a low phenylalanine diet.

An atypical form of phenylketonuria in which a defect of tetrahydrobiopterin (fig.2) metabolism occurs was reported (Danks et al 1978). It was noted that there are lowered neurotransmitter levels and progressive neurological disease with death in early childhood (Smith et al 1975). This was called malignant hyperphenylalaninaemia.

Deficiency of tetrahydrobiopterin (fig.2) can arise from failure to synthesise de novo or through failure to salvage the quinonoid dihydrobiopterin (fig.11) by dihydrobiopteridine reductase. Examples of both disorders have been reported (Kaufman et al 1978, Leeming et al 1976, Rey et al 1980). The two types can be distinguished with a lowering of biopterin derivatives (13) appearing in the serum (0.2 - 0.8 µg/l in syntheis deficient patients whilst in dihydropteridine reductase deficient patients there is a marked elevation (5.5 - 12.0 µg/l compared to normals (1.1 - 3.7 µg/l). Both forms can be treated with neurotransmitter precursors, L-dopa (40) and 5-hydroxytryptophan (44) (Leeming et al 1980). It has been found that tetrahydrobiopterin (fig.2) administration can also correct hyperphenylalaninaemia (Kaufman et al 1982), Kaufman et al 1983). This



(47)



(48)

has to be administered in relatively large doses as there is poor transport across the blood-brain barrier (Kettler et al 1974, Kapatos and Kaufman 1981).

The level of tetrahydrobiopterin (fig.2) has been reported to be altered in some other disease states. Elevation in blood and serum biopterin levels has been reported in classical phenylketonurics and patients with kidney dysfunction (Leeming et al 1976). Lowered serum levels have been demonstrated in pernicious anaemics, schizophrenics, leukaemia, malignant carcinoid disease, senile dementia coeliac, disease and lead poisoning (Leeming et al 1976, Leeming et al 1980a, Leeming et al 1980b). Many metals have been found to inhibit de novo biopterin biosynthesis, in particular lead has received attention (Purdy et al 1981), and several drugs have been found to elevate it. (Brown 1981).

Because of the difficulty of obtaining human tissues rat models must be used. Together both rats and human tissue will be used in an attempt to investigate the biosynthesis of tetrahydrobiopterin (fig.2) under diseased conditions, the synthesis conditions for human brain and the effect when drugs are administered. Whilst the measurement of biopterin derivatives in cerebral spinal fluid gives some indications of neurotransmitter levels within the brain it is probably less reliable than direct measurements within the brain since the biopterin is compartmentalised within brain cells and we are only measuring a cellular loss in cerebral spinal fluid.

Results obtained from human and rat brains will be used in relationship with the results obtained by others to provide further information into the metabolism of this vital pteridine.

MATERIALS AND METHODS

Chemicals

Guanosine - 5-triphosphate (sodium salt type II), nicotinamide adenine dinucleotide phosphate reduced (tetrasodium salt type II), nicotinamide adenine dinucleotide reduced (disodium salt type III) 6,7-dimethyl-5,6,7,8-tetrahydropterin, peroxidase, adenosyl B_{12}, diethylstilboestrol, Tris (tris (hydroxy methyl) amino ethane) and diphenylamine were obtained from the Sigma Chemical Company (Poole). 5-Methyltetrahydrofolic acid (calcium salt) was obtained from Eprova Research Laboratories (Switzerland). Biopterin, neopterin, sepiapterin and D-erythro-5,6,7,8-tetrahydrobiopterin were obtained from Dr. B. Shircks (Switzerland). HPLC grade methanol was obtained from Fisons (Loughborough). Liver fraction L was from the Nutritional Biochemical Company (Ohio) and vitamin free casamino acids from the Difco Laboratories (Michigan). All other reagents were from BDH (Poole) and were of analytical grade.

Animals

The rats used were albino Wistar rats (grade 4) supplied by Bantin and Kingman (Hull). The animals were of both sexes and of several ages 21 days, weight about 100g, approximately 12 weeks, weight about 200g and 270 days, weight about 500g. Brains from Dr. Winder (Royal Postgraduate Medical School) were from 30 days Wistars. The animals were maintained on rat breeder diet 41B supplied by Pilsbury's (Birmingham). The animals received food and water ad libitum and were housed on sawdust.

Details of treatment, age and sex are described with the appropriate experiment.

Human Tissue

Breast and gut tissue was supplied courtesy of Mr. Oates (Birmingham General Hospital) from patients with tumors. The tissue was removed from the patients during operation after which the tumours were dissected out and portions supplied, with control tissue from the same subject, for assay. This was frozen at -20°C until it was required. Preparation was done as soon as was practical.

Human brain tissue was obtained from several sources. Tissue samples from subjects with senile dementia and 18 controls (those dying with no apparent neurological involvement in the diseased state) were removed from the temporal lobe (Brodmann area 20) at necropsy. The samples were matched for age, drug therapy and time to necropsy. They were also analysed for plaques and tangles. These were obtained from Dr. G. Reynolds at the MRC Brain Bank (Addenbrooks Hospital, Cambridge). Tissue was also obtained from four chronic depressives and matched controls (Brodmann area 21) from Dr. Reynolds. Specimens of cerebral cortex of mixed region were obtained from three foetuses diagnosed as Down's (Trisomy 21) and four age matched controls following prostaglandin E_2 abortion supplied by Dr. B. Brooksbank at the MRC Developmental Neurobiology Unit (Institute of Neurology, London). Also supplied by Dr. Brooksbank were specimens of frontal cortex and one temporal cortex obtained from five children of seven months or less. Specimens of temporal cortex were obtained from patients institutionalised at St. Lawrences Hospital (Caterham, Surrey), courtesy of Dr. P. Sylvester. Three of these were diagnosed as Down's Subjects and two as having Alzheimer disease. The other five were mentally subnormal but of undefined aetiology. Two samples of control temporal cortex were supplied by Prof. Brewer at Birmingham General Hospital. A specimen of

temporal cortex taken from a subject dying from methylenetetrahydrofolate reductase deficiency was supplied by Dr. Isobel Smith and her colleagues at the Institute of Child Health (London).

Brain tissue was stored at minus 70°C until use.

Human Blood

Venous blood was drawn from the arms of healthy volunteers, staff and students from Aston University and staff from the General Hospital, Birmingham. Blood was obtained from patients at the hospital as part of routine clinical diagnosis.

The blood was drawn using a syringe and needle and immediately diluted by 50% using a solution of 4% trichloracetic acid, to rupture the cells, and 2% ascorbate to reduce the oxidation of any unoxidised pterins. The samples were centrifuged to remove precipitated proteins. The samples were frozen at minus 20°C until required. Portions of the sample were then assayed for biopterin using the Crithidia assay (see later) or were first subject to iodine oxidation outlined later and then underwent the Crithidia assay. For the HPLC assay the samples were run both prior to and after iodine oxidation but in both cases they were lyophilised to dryness and made up to a quarter of their original volume with distilled water and then assayed by HPLC.

For iodine oxidation 1 ml of sample was taken and oxidised after first being made acid or alkaline. The sample was oxidised by the addition of 1 drop of 3% (W/V) iodine solution from a pasteur pipette and left for one hour after which the oxidation was terminated by the addition of dry ascorbic acid until the yellow colour disappeared.

Tissue Preparation

Brain tissue was removed from rats freshly killed by stunning and then breaking the neck. Human brain, breast and bowel tissue was first thawed and used as supplied. Breast and bowel was often broken into small bits first by chopping with a scalpel. 20% (W/V) Homogenates were prepared in 0.1 molar Tris/HCl buffer pH 7.6 using a Potter Elvjhen homogeniser. These homogenates were centrifuged at 0.°C at 100 000 x g in a M.S.E. superspeed centrifuge for 45 minutes. The supernatants were collected and used in the incubation and for dihydropteridine reductase assays. Protein was measured by the Biuret method (Layne 1957).

Incubation Methods

The capacity for tissue preparations to synthesis biopterin was measured by the method of Fukushima et al 1975.

The incubation contained the following :

a) 700 µl of 75 mM Tris/HCl pH 8.0
b) 50 µl of 6 mM guanosine-5'-triphosphate (GTP)
c) 50 µl of 3 mM magnesium chloride
d) 50 µl of 3 mM nicotinamide adenine dinucleotide phosphatide reduced (NADPH)
e) 50 µl of additives (if included) or distilled water to make the volume up to 1ml.
f) 100 µl supernatant (added last to initiate the reaction).

The assay was done in pyrex 4 inch x 1/2 inch test tubes. The tubes were incubated at 37°C in the dark for 3 hours after which the reaction was terminated by the addition of 2.0 ml of 0.1 molar HCl. When only small volumes of supernatant were available the volumes of the above were halved.

The samples were then used directly for biopterin assay or prepared as follows for high performance liquid chromatography. 1 ml of the assay medium was removed and placed into a vial. This was then treated by the addition of 1 drop 3% (W/V) iodine solution (3g I_2 and 6 g KI in 100 mls of distilled water) and stood in the dark for 1 hour to oxidise any reduced pterins present. The oxidation was terminated by the addition of dry ascorbic acid until the yellow colouration disappeared. The sample was then dried in a Virtis 10-030 freezdryer until dry after which 0.25ml of distilled water was added. The sample was then assayed by HPLC.

Crithidia Fasiculata Assay

Crithidia fasciculata culture (American type culture collection n° 12857) was supplied by Dr. S.H. Hutner (Hoskins Laboratory, Pace College, New York, U.S.A.) and maintained by Dr. R.J. Leeming (General Hospital, Birmingham).

The culture was subcultured weekly into maintenance medium prepared as summarised in table 2-1, incubated in the dark for two days at 20°C and then refrigerated. To prevent contamination 1 mg/ml of ampicillin was added without affecting the growth of the flagellate.

The stock assay medium, and double strength assay medium were prepared as per tables 2-2 and 2-3. The inoculum was prepared by aseptically adding 1 drop of a 2 day culture to 15 mls of single strength assay medium and incubated at 29°C for four days to exhaust the flagellate's endogenous pteridines (Dewey and Kidder 1971). 0.2 ml of the resultant growth was added aseptically to 20 ml of single strength medium. It was advantageous to add 500 mg of ampicillin to this inoculum. One drop of inoculum was added to each assay tube using a sterile pasteur pipette.

TABLE 2.1 Crithidia Maintenance Medium (Hutner 1971)

Yeast extract (Oxoid)	0.3 g
Trypticase	0.3 g
Sucrose	0.25 g
Liver fraction L (Nutr. Biochem.Co)	0.01 g
Haemin (5 mg/ml in 50% triethanolamine)	0.5 ml.
Distilled water	100 ml.

pH to 6.8 - 7.6, autoclaved 120°C for 15 minutes and store in the refrigerator at 4°C.

TABLE 2.2 Crithidia Stock Assay Medium

Part A.

L-arginine hydrochloride	5.0	grams
L-glutamic acid	10.0	
L-histidine hydrochloride	3.0	
DL-isoleucine	1.0	
DL-leucine	1.0	
L-lysine hydrochloride	4.0	
DL-methionine	1.0	
DL-phenylalanine	0.6	
DL-tryptophan	0.8	
L-tyrosine	0.6	
DL-valine	0.5	
Ethylene diamino tetra-acetic acid	6.0	
Boric acid (H_3BO_3)	0.005	
Calcium chloride ($Ca\ Cl_2$)	0.005	
Cobalt sulphate ($CoSO_4 7H_2O$)	0.025	
Copper sulphate ($CuSO_4 5H_2O$)	0.025	
Ferric ammonium sulphate ($Fe\ (NH_4)_2SO_4)_2 6H_2O$)	0.010	
Manganese sulphate ($Mn\ SO_4H_2O$)	1.4	
Magnesium sulphate ($Mg\ SO_4 7H_2O$)	6.5	
Tri-potassium phosphate ($K_3\ PO_4$)	1.5	
Zinc sulphate ($ZnSO_4 7H_1O$)	0.5	
Sucrose	150.0	
Distilled water	1000	ml.

Steamed at 100°C for 20 minutes to dissolve and distributed into sterile bottles - stored at 4°C in the dark for up to 3 months.

TABLE 2.2 ...continued

Part B

Adenine	1.0	grams
Biotin	0.001	
Calcium pantothenate	0.3	
Nicotinic acid	0.3	
Pyridoxamine dihydrochloride	0.1	
Riboflavin	0.06	
Thiamine hydrochloride	0.6	

Ground together and stored dry at 4°C in the dark.

Part C.

Haemin 5 mg/ml in 50% triethanolamine - freshly prepared.

Part D

Folic acid 100 ng/ml - freshly prepared.

TABLE 2.3 Crithidia Double Strength Assay Medium (100 ml)

Distilled water	78	ml
Assay medium Part A	20	ml
Assay medium Part B	4.8	mg
Vitamin free casamino acids (Difco)	2.0	g
Triethanolamine (Must be added before Part C)	0.5	ml
Assay medium Part C	1.0	ml
Assay medium Part D	0.5	ml

Adjusted to pH 7.5 with sulphuric acid.

Samples for biopterin assay were diluted, by trial and error, with 0.2 molar phosphate buffer pH 5.0. Biopterin was determined by adding 0.5 ml of diluted sample to each of three sterilized rimless tubes and incubated with 1.5 ml of distilled water; 2 ml of double strength assay medium was then added to each tube giving a total volume of 4 ml.

With each batch of samples two standard curves were set up in triplicate. The curve consisted of eleven tubes containing a range of 0 - 0.1 ng of standard biopterin in 0.5 ml of 0.2 molar phosphate buffer pH 5.0. The standards were treated in the same way as the samples. All the assay tubes were autoclaved at 115°C for 5 minutes using a steam pressure autoclave and then innoculated aseptically. The samples were incubated at 20°C for 72-90 hours. The growth of Crithidia in each tube was measured turbidometrically using a Gilford Micro-sample spectrophotometer 300 equiped with sample changer and chart recorder for automatic readout of growth. Assays were read as absorption at a wavelength of 590 nm against uninoculated medium. The amount of biopterin in each sample was calculated using the standard curve in ngbiopterin/ml.

High Performance Liquid Chromatography

The system comprised of a Constametric III pump from Laboratory Data Control, a Waters intelligent sample processor, a Kontron spectrofluoromonitor SFM 23/3 with SFM/23 powersupply (the spectrofluoromonitor had a 20 µl flowcell), a programable 308 integrator from Laboratory Data Control and a JJ dual pen CR652 chart recorder. The column used was a Spherisorb ODS reverse phase column with a particle size of 5µ and column size of 25 cm x 4.6 mm. A pre-column with similar packing was also used. The solvent used was a 5% methanol 95% distilled water made volumetrically and degassed under reduced pressure. The solvent

composition was found to have an effect on the retention time of neopterin (graph 2-1) so accuracy in solvent preparation was important. Variation in retention time was also found to vary with changes in laboratory temperature (graph 2-2) and it was found to be advantageous to lag the column to reduce this to a minimum.

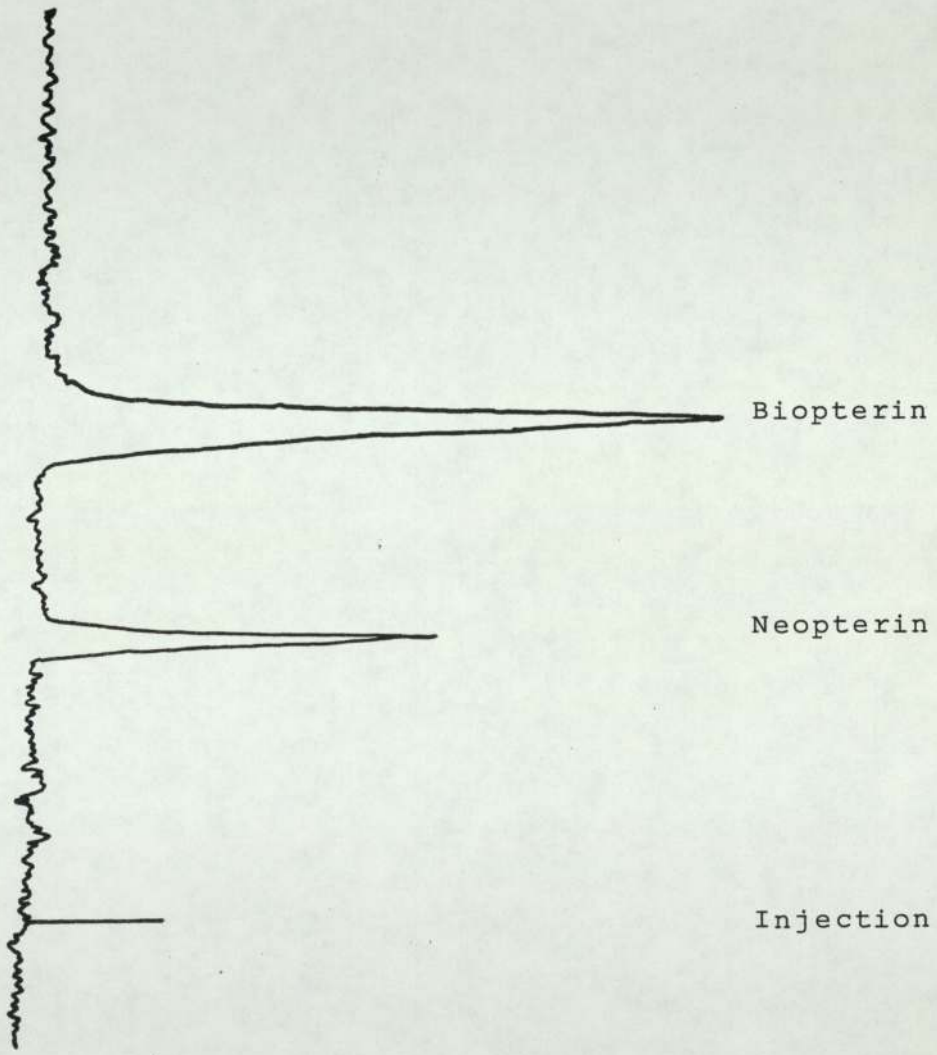
The 308 integrator was evaluated as to the optimal settings for its programme. This instrument can be programmed for noise, the amount of background to be eliminated, threshold, the distance above the baseline over which the peak must pass before integration begins and peak width, the time interval between samples of peak height from the baseline. When first switched on the instrument has its own pre-set programme and to find the optimum settings two parameters were kept fixed and the third varied. The number of peaks prior to the neopterin standard were noted. The results can be seen (graphs 2.3-2.5). The final programme was as follows :-

Peak width	20
Noise	900
Threshold	800

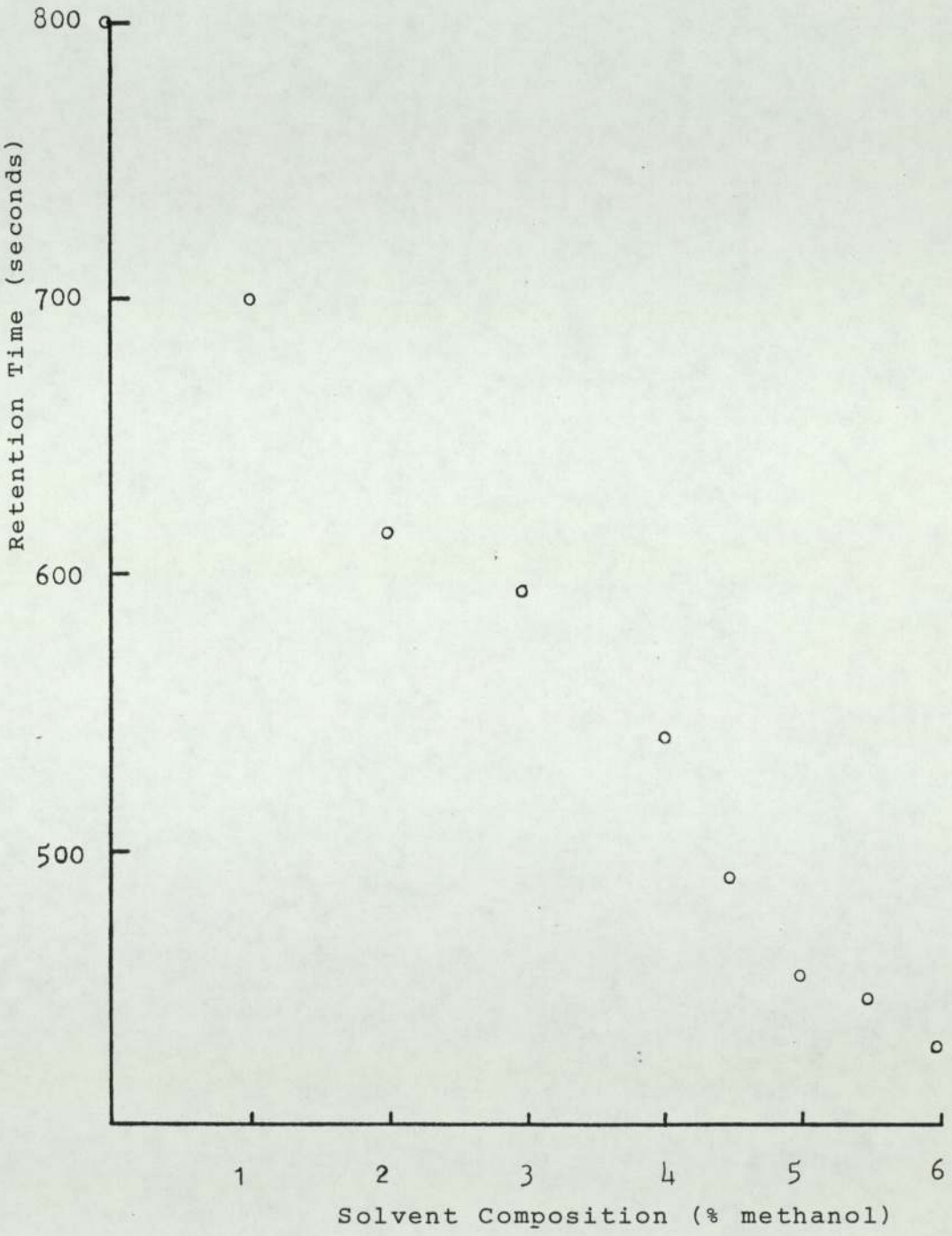
Even after setting up the instrument as above it was found that the results it presented were not so reproducible at higher sensitivity ranges of the detector as were results obtained by the chart recorder and calculated by triangulation ($\frac{1}{2}$ base x height) of the peak area. Consequently the integrator was rejected in favour of triangulation (half base x height).

The system was calibrated forthe quantitive estimation of pterins and for their retention time by using series of standards of known concentration of which 20µl were injected onto the column; injections were 6 replicates.

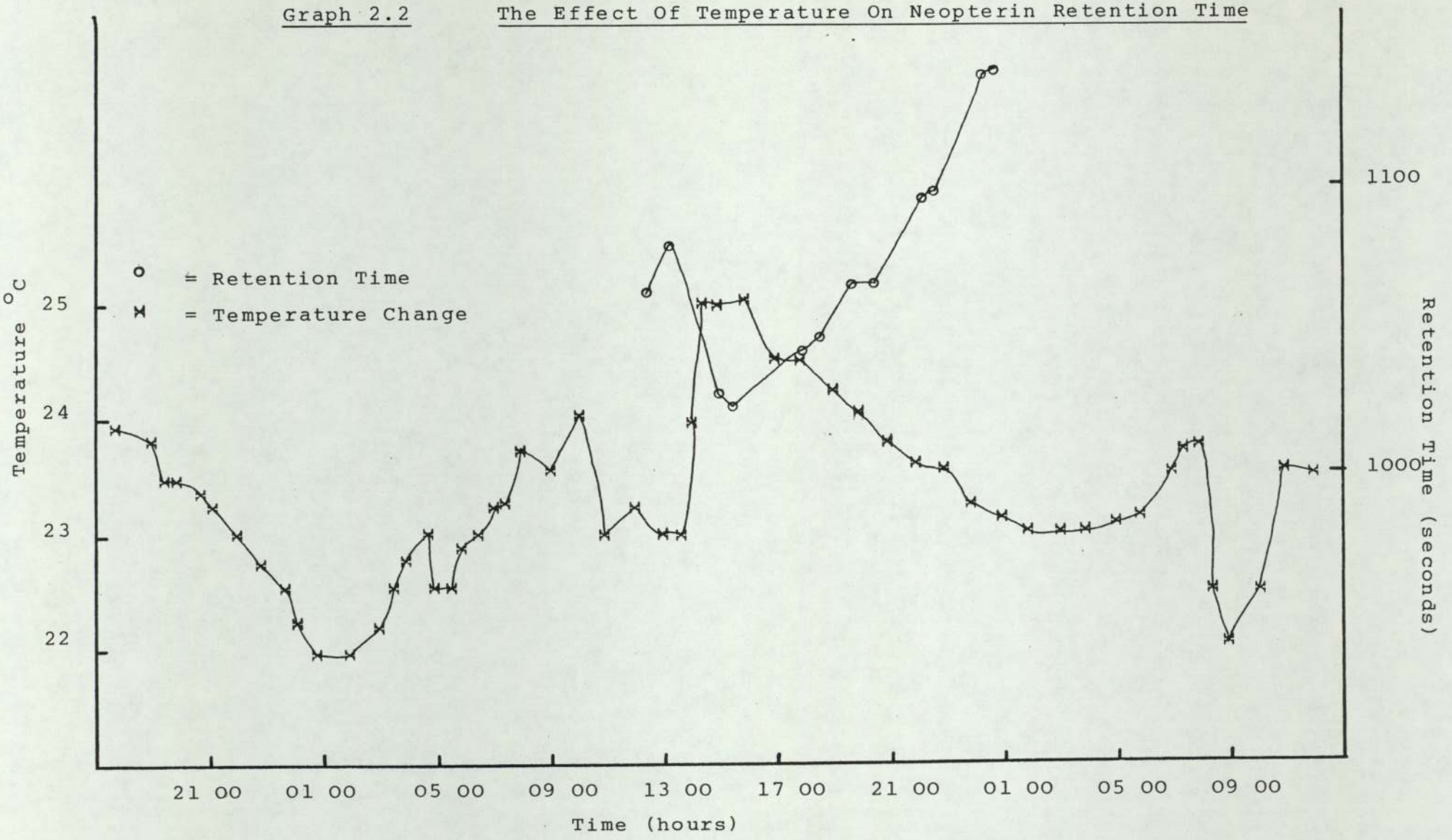
Figure 2.1 Trace From A Neopterin And Biopterin Standard



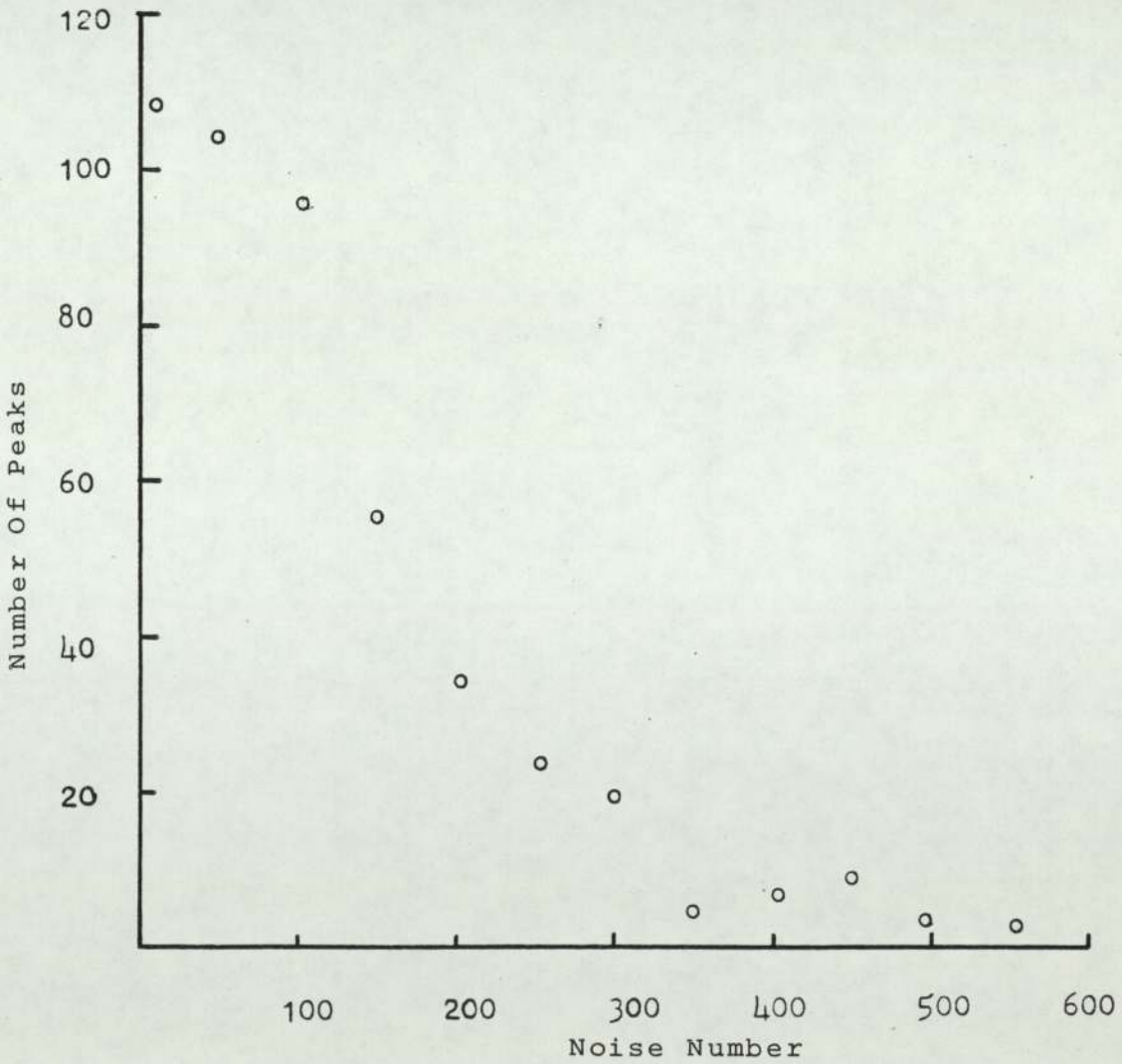
Graph 2.1 The Effect Of Solvent Composition On The Retention Time Of Neopterin



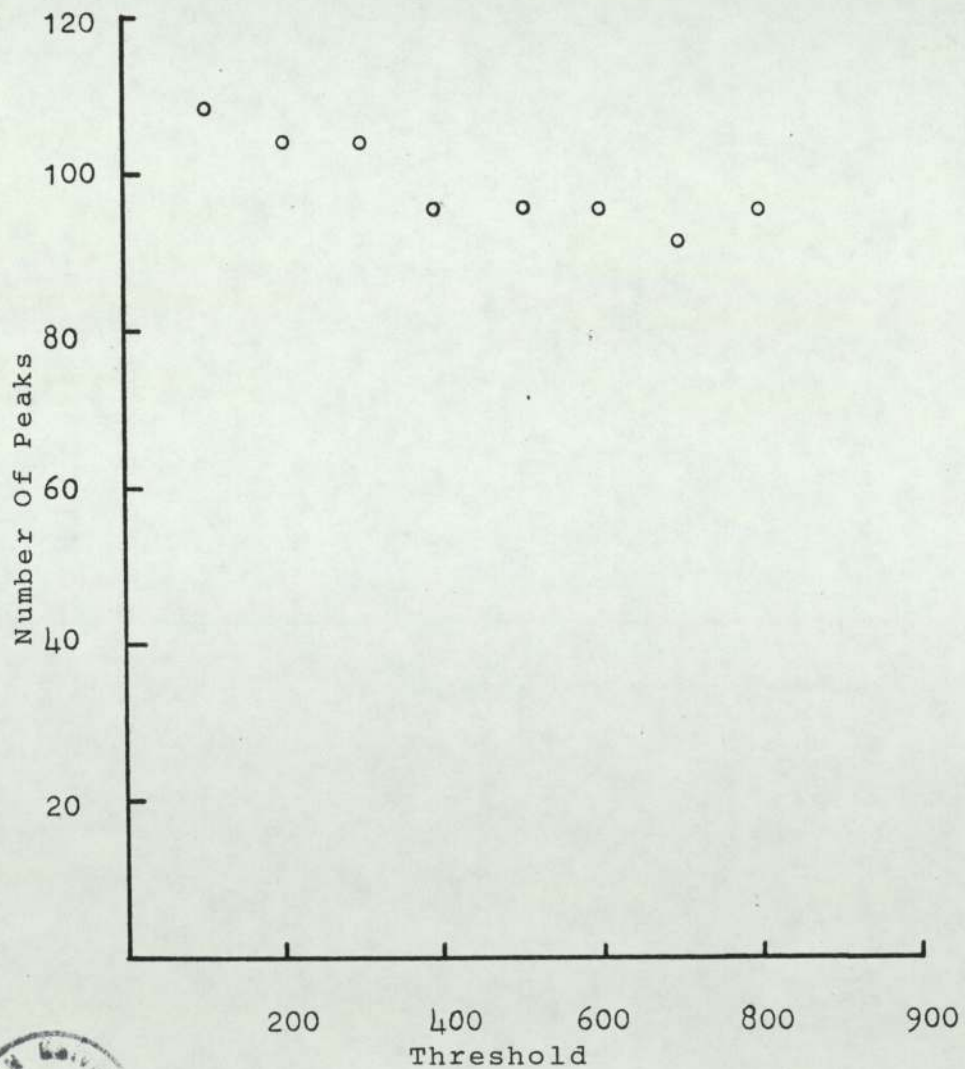
Graph 2.2 The Effect Of Temperature On Neopterin Retention Time



Graph 2.3 The Effect Of Noise Number On Noise Level From Integrator



Graph 2.4 Effect Of Threshold on Noise Level From Integrator



Graph 2.5 Effect Of Peak Width on Noise Level From Integrator

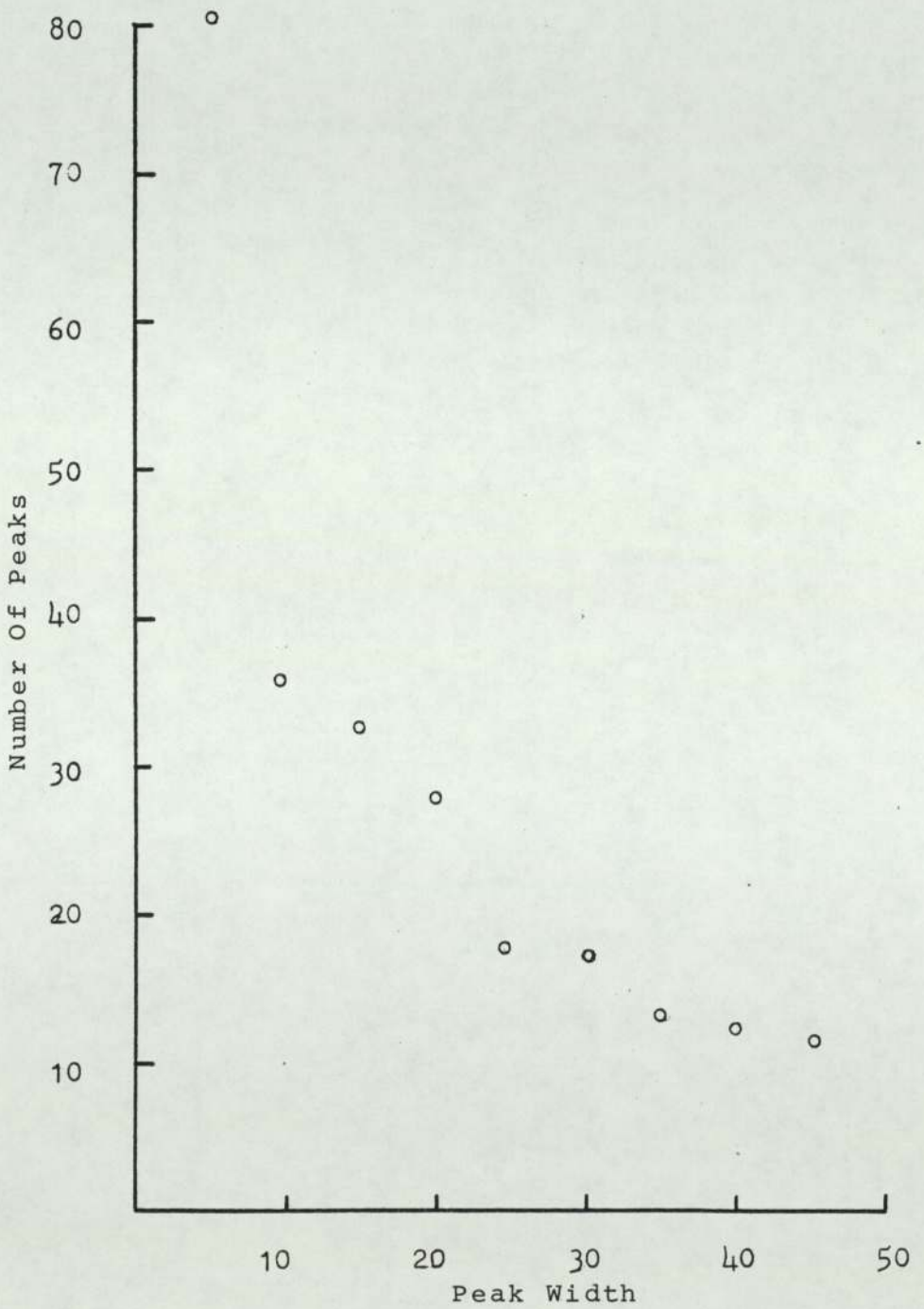
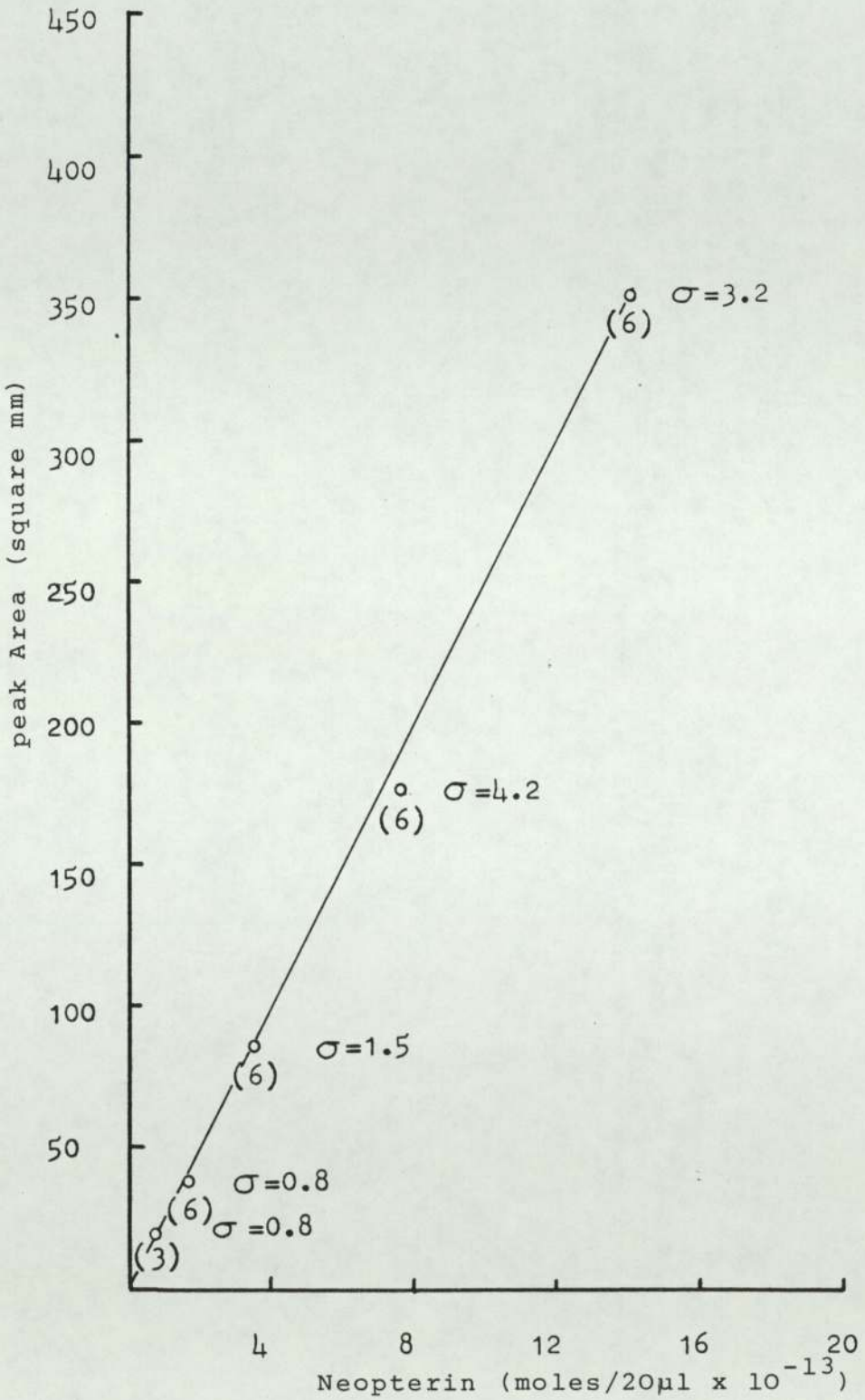


TABLE 2.4 Emax Values For Various Pterins at pH 13.

Pterin	E max		Source
biopterin	8.3×10^3	363 nm	Fukushima etal 1980
neopterin	8.3×10^3	362 nm	Fukushima etal 1980
pterin	6.6×10^3	358 nm	R.L. Blakley 1969
Xanthopterin	7.2×10^3	394 nm	R.J. Blakley 1969
isoxanthopterin	10.6×10^3	340 nm	R.J. Blakley 1969
pterin-6-COOH	9.2×10^3	365 nm	R.J. Blakley 1969

Graph 2.6 Calibration Of HPLC For Neopterin



Graph 2.7 Calibration OF HPLC For Biopterin

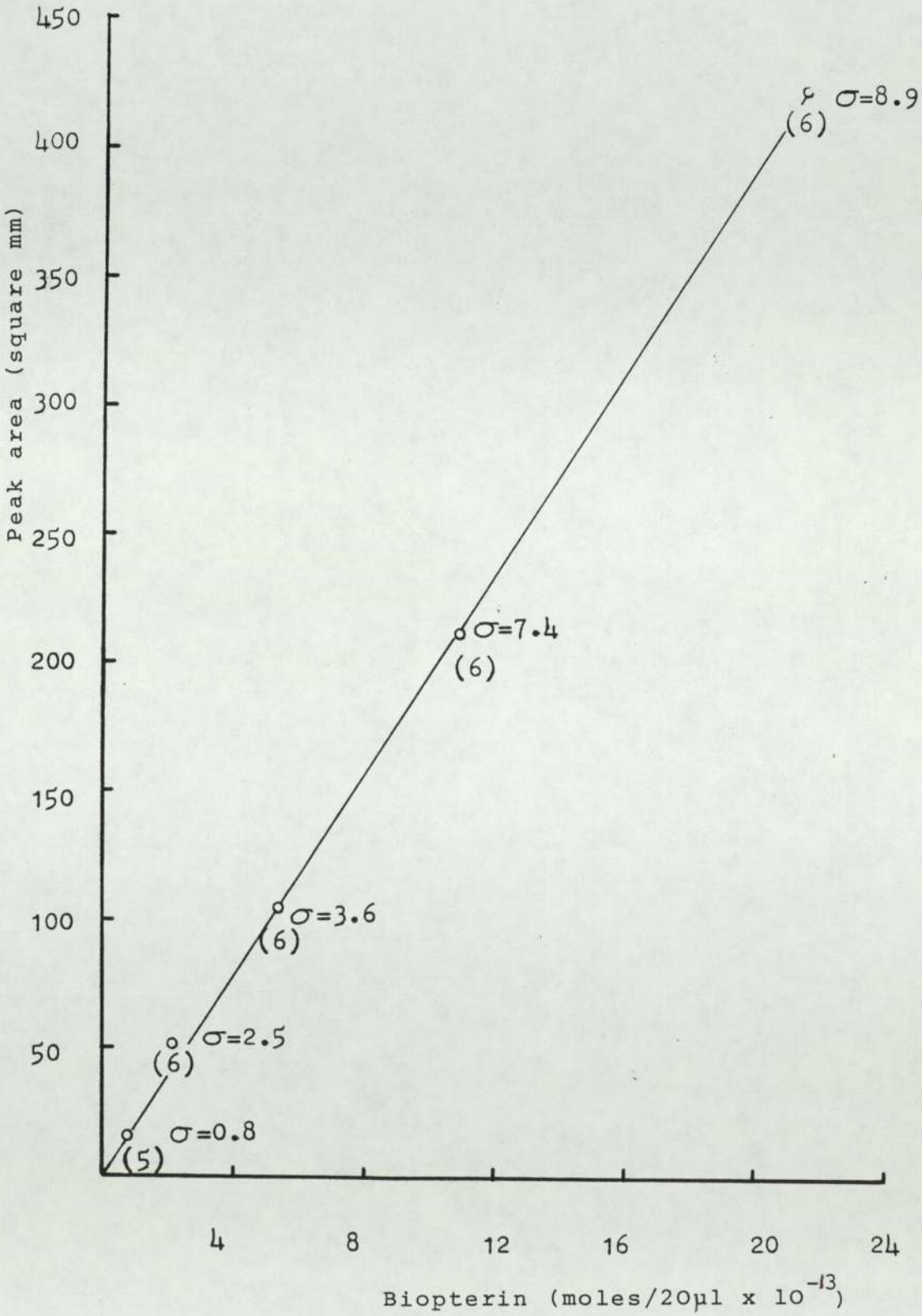


TABLE 2.5 Relative Fluorescence Of Pterins To Biopterin

Excitation 360 nm Emission 450 nm.

Pterin	fluorescence
L biopterin	100%
isoxanthopterin	14%
D„neopterin	110%
pterin„6„carboxylicacid	182%
pterin	85%
xanthopterin	6%

Calculated from calibration graphs and compared with biopterin.

TABLE 2.6 Table Of Pterin Retention Times In 5% Methanol / Water

Pterin	Retention (sec.)	S.D	Coefficient of variation
pterin-6-COOH	266.9	6.6 (n=25)	2.4%
L-neopterin	481	23.4 (n=5)	4.8%
D-neopterin	496	6.09 (n=27)	1.22%
L-biopterin	861	13.2 (n=27)	1.5%
xanthopterin	944.5	9.66 (n=16)	1.02%
isoxanthopterin	1049	20.4 (n=9)	1.94
pterin	1424	22.4 (n=13)	1.6%
sepiapterin	5325		

Because of their low concentration and consequently the small amount of pteridine required the concentration was determined by measuring the absorbance at pH 13.0 at wavelength shown in table 2.4 and calculating the concentration with the extinction coefficient (table 2.4). Results are presented as moles/20 µl relative to peak area. Examples are shown for biopterin and neopterin (graphs 2.6 and 2.7.) The system was also quantitativly calibrated for isoxanthopterin, pterin -6-carboxylic acid, pterin and xanthopterin. From these calibrations their fluorescences have been calculated relative to biopterin (table 2.5).

Using standards the retention times for various pterins were determined (table 2.6). at a flow rate of 0·75 ml / minute

The fluorescence wavelengths were those of Woolf et al (1983).

Dihydropteridine Reductase Assay

This was based on the method of Craine et al (1972). Each incubation contained the following

a) 100 µl 1 x 10^{-3} molar nicotinamide adenine dinucleotide reduced (NADH)
b) 100 µl 2.5x 10^{-3} molar sodium azide
c) 100 µl 80mg/l horseradish peroxidase
d) 100µl 1.0 x 10^{-2} molar hydrogen peroxide
e) 20 µl enzyme preparation
f) 100 µl 1x10^{-3} molar 6,7-dimethyl-5,6,7,8-tetrahydropterin
g) 100 µl 0.5 molar Tris/HCL buffer pH 6.8
h) distilled water to make volume up to 1 ml

All the components were put into plastic cuvettes (1ml, 1cm pathlength), with the exception of 6,7-dimethyl-5,6,7,8-tetrahydropterin, inverted and incubated for 90 seconds in the spectrophotometer to allow the assay to equilibrate to 37°C. The assay was started by the addition of 6,7-dimethyl-5,6,7,8-tetrahydropterin. The blanks contained no enzyme preparation. Control assays were run for the reductase assay but in the absence of 6,7-dimethyl-5,6,7,8-tetrahydropterin.

The rate of reaction was followed by measuring spectrophotometrically the decrease in optical density at 340nm due to the disappearance of reduced nicotinamide adenine dinucleotide (NADH) at 37°C in a Pye Unicam PU8800 spectrophotometer with constant temperature cell holder. After a delay of 30 seconds the instrument gave the rate as change in concentration/minute (nmols / minute) reading this five times at 10 second intervals and giving the mean. The results were then corrected to 1 mg of protein and finally calculated as n moles NADH oxidised per minute per mg protein.

Spectra

Spectra were run on a Shimadzu U.V. 240 UV.Visible recording spectrophotometer with constant temperature cell holder. Quartz cuvettes were used.

DNA Estimation

This method was based on that of Burton et al (1956). The tissue was made into a homogenate using citrate buffered saline 0.15 M NaCl with 0.015 M citrate buffer pH 4.5. A known weight of tissue was made into a 25%

homogenate and the final volume of homogenate noted. The DNA was extracted as follows. 0.95 ml of homogenate was mixed with 0.05 ml of 6 molar perchloric acid and chilled. This was centrifuged in a M.S.E benchtop centrifuge for 20 minutes at 1500 x g and the supernatant discarded. The pellet was broken up using a glass rod in 0.5ml of 0.5 molar perchloric acid and then a further 3.5ml of 0.5 molar perchloric acid added. This was heated at 70°C for 15 minutes and then centrifuged for 20 minutes in a benchtop centrifuge at 1500 x g. The supernatant was decanted off into a graduated test tube. The pellet was resuspended in 3.0ml of 0.5 molar perchloric acid and centrifuged for 20 minutes at 1500 x g. The supernatant was decanted off, pooled in the graduated test tube and the volume noted.

The DNA was estimated by taking 1 ml of the extracted DNA solution and reacting it with 2 mls of diphenylamine solution prepared by taking 1 gram of diphenylamine and dissolving it in 100 mls of glacial acetic acid and adding 2.75 mls of concentrated sulphuric acid. (These reagents must be analar). The tube was then placed into a boiling waterbath for 15 minutes after which it was removed and cooled in cold water. The colours were then estimated by determining their optical density at 600 nm against a water blank.

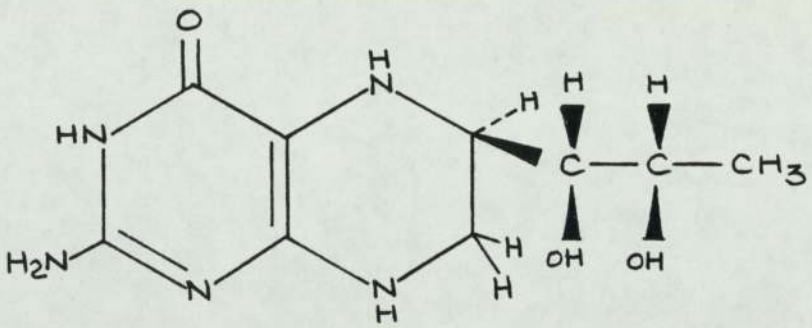
Standards were prepared by dissolving 40 mg DNA in 100 mls 0.005 molar sodium hydroxide. This was then diluted with 0.005 molar sodium hydroxide to give a range of standards. These standards were diluted 50/50 with 1.0 molar perchloric acid and then heated at 70°C for 15 minutes. They were then treated with diphenylamine solution as before. DNA results were quoted as mg DNA / wet weight of tissue or mg DNA / mg protein.

CHAPTER 3 BIOPTERIN METABOLISM IN HUMAN BRAIN

INTRODUCTION

Tetrahydrobiopterin (1) is the cofactor essential for the hydroxylation of phenylalanine (2) tyrosine (3) and tryptophan (4) and therefore necessary for the biosynthesis of adrenalin (5), noradrenalin (6), dopamine (7) and serotonin (8) (Kaufman 1963, Nagatsu et al 1964 Jequier et al 1968). Within the cell its level is maintained by its synthesis de novo from guanosine -5'-triphosphate (9) and salvage by dihydropteridine reductase which reduces quinonoid dihydrobiopterin (10) to tetrahydrobiopterin (1), this having been formed from tetrahydrobiopterin (1) during its coenzyme reaction. Both pathways are necessary for normal neurological function and this is demonstrated in patients suffering from the various forms of the genetic disease malignant hyperphenylalaninaemia where there is a failure in either the salvage or biosynthesis of tetrahydrobiopterin (1) (Leeming et al 1981).

Reports have been presented over the last few years on disturbances of tetrahydrobiopterin metabolism in various states including neurological diseases such as Parkinson's disease, (Lovenburg et al 1979), dystonia (Williams et al 1979), Huntington's chorea and Steel-Richardson syndrome (Williams et al 1980). All these investigations have been made using either serum or cerebral spinal fluid. Recently significantly reduced serum biopterin levels were reported for subjects diagnosed as senile dementia of the Alzheimer type (Leeming and Blair 1979, Young et al 1982, Aziz et al 1983). It has also been reported (Morar et al 1983) that total biopterins are lowered in the cerebral spinal fluid Alzheimer patients. A reduction in neopterin was also found but this was not significant (Morar et al 1983). This suggests a role for tetrahydrobiopterin in the pathology of senile dementia of the Alzheimer type.



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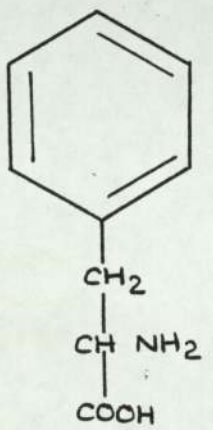

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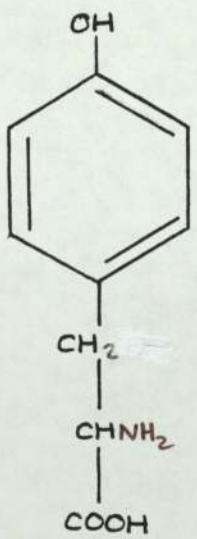

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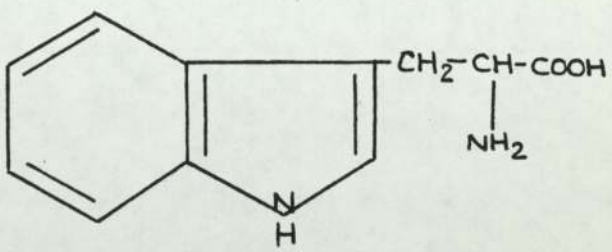

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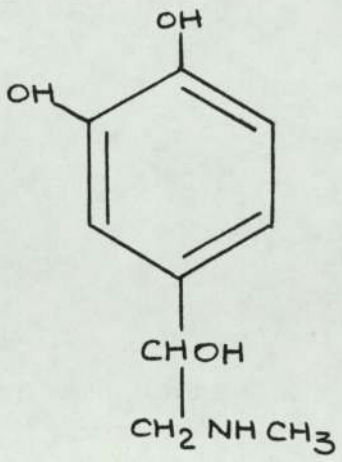

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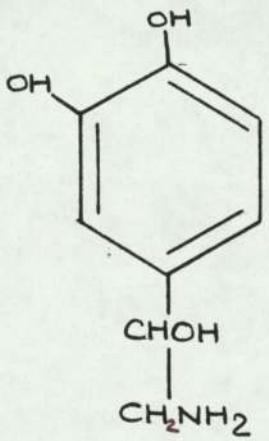

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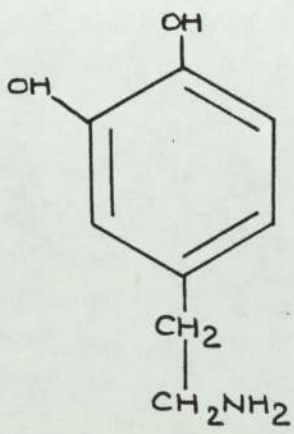

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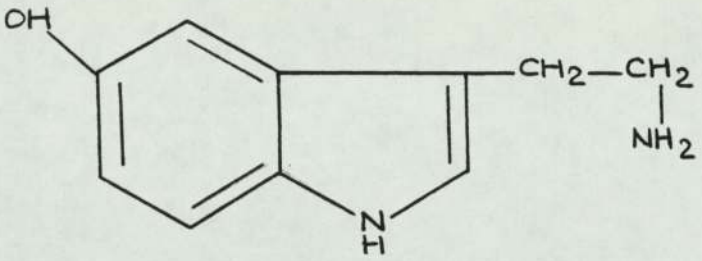

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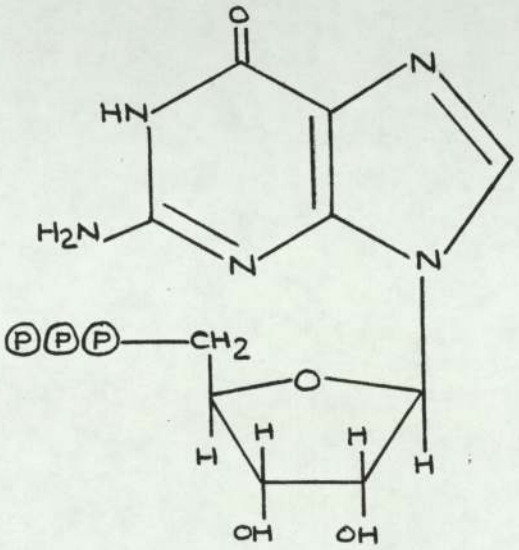

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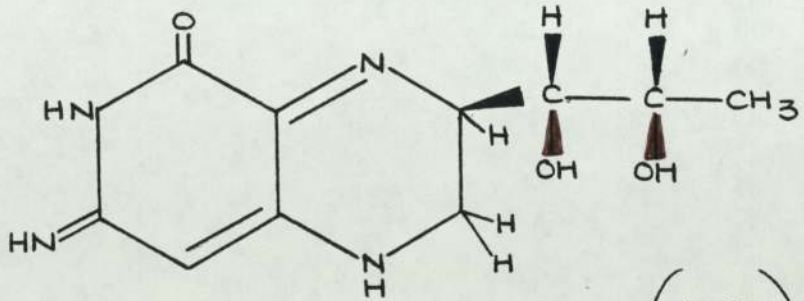

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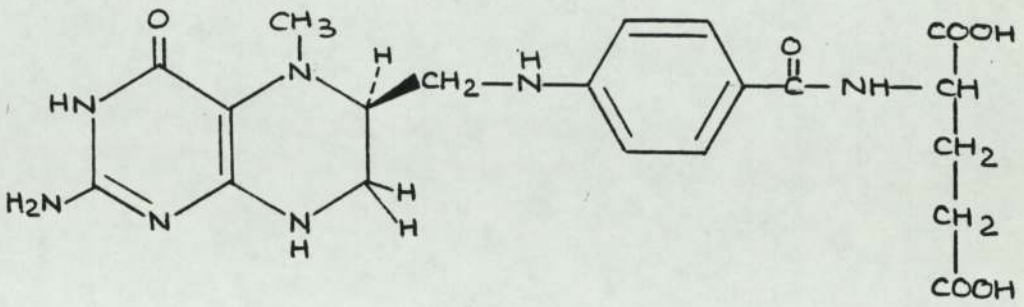
Williams et al (1980) have found that there is a decline in cerebral spinal fluid tetrahydrobiopterin (1) with age. Algeri et al (1977) found that with age the dihydropteridine reductase activity in senescent rats rises whilst the tyrosine hydroxylase activity declines. Algeri et al (1977) also reported that dihydropteridine reductase activity is detectable in rat brain at very early stages of life, the specific activity being 29% of the activity found in the adult. The activity rises rapidly at the end of the second third of foetal life and continues to increase gradually until two months of age. Kapatos et al (1983) found that brain tetrahydrobiopterin (1) levels rose prior to birth in rat brains and also that guanosine triphosphate cyclohydrolase activity correlated with developmental changes in tetrahydrobiopterin (1).

Bullard et al (1978) have investigated the regional distribution of reduced pterins, dihydropteridine reductase, tyrosine hydroxylase and tryptophan hydroxylase and found that the regional distribution varied for all pterins and enzymes. They found the distribution of reduced pterins correlated with tryosine hydroxylase but not with tryptophan hydroxylase nor with dihydropteridine reductase. The major part of the reduced pteridine appears to be catecholaminergic in origin.

Recently it has been reported that there is an increase in serum dihydrobiopterin in Down's syndrome which could indicate a lowering of tetrahydrobiopterin levels within the cell and a corresponding lowering of neurotransmitter formation. This could be responsible for the lack of mental development in these patients (Aziz et al 1982). Levine et al (1983) have reported an improvement in some patients with endogenous depression treated with tetrahydrobiopterin (1) and that neurotransmitter metabolites and tetrahydrobiopterin (1) were elevated correspondingly in cerebral spinal fluid.

5-Methyl tetrahydrofolic acid (11) and vitamin B_{12} have been found to stimulate tetrahydrobiopterin biosynthesis is rat brain by upto 200% (Leeming et al 1982). It has been found that prolonged exposure to nitrous oxide produces bone marrow depression and megaloblastic change. With prolonged nitrous oxide exposure a neurological disorder developed (Layzer et al 1978, Amess et al 1978). Nitrous oxide is known to inhibit vitamin B_{12} by oxidising it from Co^{I} and Co^{II} to inactive Co^{III} stopping methionine synthetase for which B_{12} is a cofactor (Perry et al 1983). The enhancement of biopterin biosynthesis by 5-methyl tetrahydrofolate (11) and vitamin B_{12} and the neurological abnormalities which occur when B_{12} is inhibited suggests a possible involvement of both in tetrahydrobiopterin (1) biosynthesis. It has also been observed that in senile dementia of the Alzheimer type the mean red cell folate of the patients was significantly lower and may possibly be leading to the impairment of the patient (Sneath et al 1973).

All of the previous work has been done on serum, cerebral spinal fluid or rat brains and the results extrapolated to human brains. This is indirect and not necessarily accurate. In this chapter results are presented using measurements of human biopterin biosynthesis and dihydropteridine reductase with the effects of age, vitamin B_{12}, 5-methyl tetrahydrofolic acid and nitrous oxide in an attempt to see if results already obtained can be related to human brains and to get a further insight into human brain biopterin metabolism.



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MATERIALS AND METHODS

The known information on brain samples, age, postmortem delay, cause of death of the patients and medication the patients were receiving are presented in table 3.1.

Timed studies on biopterin biosynthesis were done by stopping the synthesis assay, outlined in chapter two, at timed intervals with 2mls of 0.1 molar HCl. Assay for pH were done using 0.075 molar Tris/HCL titrated to varying pH. The effect of GTP and NADPH concentration were found by keeping all concentrations fixed and changing the one of interest.

Assays with 5-methyl tetrahydrofolic acid were done by adding 5-methyl tetrahydrofolic acid in 25µl to make the 1ml assay 1.1×10^{-4} molar. The 5-methly tetrahydrofolic acid was prepared in 0.1 molar ascorbic acid.

Vitamin B_{12} (adenosyl cobalamin) was made 4.4×10^{-6} molar of which 25µl was added to the 1ml assay giving a final molarity of 1.1×10^{-7} M.

The effect of nitrous oxide was studied by by incubating for 3 hours under an atmosphere of 100% nitrous oxide. The effect of nitrite was studied with the addition of 50µl of 1.1×10^{-4} molar sodium nitrite to give a resultant 5.5×10^{-6} molar in the assay.

The assay used was that outlined in the materials and methods (chapter 2) altered, where necessary, as above.

Adenosyl cobalamin, tetrahydrobiopterin and NADPH spectra were run in a Shimadza UV 240 UV/visible spectrophotometer. The solutions were assayed

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS

Patient	Age	P.M. Delay	Sex	
D38	80	11hr	F	All subjects were presented as demented. They were matched for age, sex, medication with controls C234-C248 below. All were Brodmann area 20 and obtained from Dr. Reynolds.
D39	71	32hr	M	
D40	72	20hr	F	
D41	76	54hr	M	
D42	71	74hr	M	
D43	79	4hr	M	
D45		50hr	F	
D46	82	46hr	M	
C240	89	72hr	F	Control subjects matched with samples D38 - D46 for age, sex, postmortem delay and medication. All were Brodmann area 20 and obtained from Dr. Reynolds.
C241	71	57hr	M	
C242	79	63hr	F	
C244	74	49hr	M	
C234	44	92hr	F	
C248	74	65hr	M	
C236	72	69hr	M	
C238	74	24hr	F	
D48	74	11hr	F	All subjects were presented as demented and were matched with controls C247 - C278 for age, sex, medication and postmortem delay. All were Brodmann area 20 and obtained from Dr. Dreynold
D49	85	12hr	F	
D50	80	36hr	M	
D51	97	43hr	F	
D52	87	28hr	M	
D53	89	58hr	F	
D58	97	60hr	F	
D59	82	20hr	F	
D60	76	19hr	F	
D61	71	72hr	F	

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS ....continued

Patient	Age	PM Delay	Sex	
C247	34	48hr	F	Control matched with samples D48-D61 for age, sex and medication. Brodmann area 20 obtained from Dr. Reynolds.
C249	35	44hr	F	
C253	74	144hr	F	
C254	76	47hr	F	
C256	79	89hr	M	
C257	83	4_hr	F	
C258	85	87hr	F	
C267	87	79hr	F	
C273	84	46hr	M	
C278	87	73hr	F	
X5	80	45hr	F	Depression. No evidence of SDAT Died in coma of bronchopneumonia. Brodmann area21 obtained from Dr. Reynolds.
X7	78	28hr	F	Depression no evidence of SDAT. Good cognative function died from pneumonia. Obtained from Dr. Reynolds Brodmann area 21.
X8	81	48hr	F	Depression No indication of SDAT recent acute schizophrenic episode. Suicide (asphyxia + drugs) Brodmann area 21. obtained from Dr. Reynolds.

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS ....continued

Patient	Age	P.M Delay	Sex	
9404 9398 9169 9397			? ? ? ?	Control foetus of 21,21,23, 19.5 weeks gestation respectivly. Abortion for social reasons with prostaglandin E_2 injection. Obtained from Dr. Brooksbank. Cerebral cortex mixed.
CFC 22	6 wk	18hr	F	Control infant. Heart defect died on operating table. Corvexity of frontal cortex. Obtained from Dr. Brooksbank.
CFC 53	5 day	40hr	M	Control infant. Congenital heart defect hypoxic from birth. Convexity of frontal cortex. Obtained from Dr. Brooksbank.
CFC 59	4mth	3hr	M	Control infant. Fallot's tetralogy. Hypoxic 36 hrs before death. Convexity of frontal Cortex. Obtained from Dr. Brooksbank.
CFC 96	7mth	55	F	Control infant. Sudden infant death syndrome. Convexity of frontal cortex obtained from Dr. Brooksbank.

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS ....continued

Patient	Age	P.M Delay	Sex	
TC/CFC 99	6mth	74	F	Control infant. Sudden infant Death Syndrome. Convexity of frontal cortex and Temporal cortex. Obtained from Dr. Brooksbank.
GF	72	72hr	F	Control. Large disseminated bowel carcinoma No brain secondary. Had cystic softening. Medication unknown Temporal cortex, pineal, Amygdala, Hippocampus, putamen Globus Pallidus, Locus Coerules, Frontal cortex. Obtained from Professor Brewer.
GH			?	Control aetiology unknown; Temporal cortex. Obtained from Professor Brewer.
A.L.	31	7hrs	M	Control. No evidence of aging. Diplegic originally thought to have Hunter-Hurler syndrome. Death from Bronchopneumonia + chronic pyelonephritis Received, Ampicillin, Amoxil septrin Carbenicellin. Temporal cortex obtained from Dr. Sylvester.

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS ....continued

Patient	Age	P.M Delay	Sex	
W. H	51		M	Downs Syndrome. Brain showed typical features of Down's. Death due to bronchopneumonia. Temporal cortex Obtained from Dr. Sylvester.
D.T	49	60hr	F	Down's Syndrome. Brain had plaques and tangles. Death due to bronchopneumonia, acute trachebronchitis. Received Ceporin 500mg + Sodium Valporate syrup 200mg. Temporal Cortex. Obtained from Dr. Sylvester.
M.L	50		M	Down's Syndrome. Evidence of plaques and tangles. Received Epantuim, Sodium Valporate, Abidec and Diamorphine elix. Temporal cortex obtained from Dr. Sylvester
W.N	31		F	Sub Normal. Death due to coronary artery occlusion. Hypertensive ischaemic heart disease. Temporal cortex. Obtained from Dr. Sylvester.
S.H	72		?	Subnormal. Temporal cortex from Dr. Sylvester.

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS ....continued

Patient	Age	P.M Delay	Sex	
Y12.	86	32hr	F	Depression. Few if any plaques and tangles. Brodmann area 21. Obtained from Dr. Reynolds.
C254 C256 C257 C273	76 79 83 84	47hr 89hr 48hr 46hr	F M F M	Control. Matched with depressives. Death from cerebral infaction, [redacted] broncho-pneumonia and acute left ventricular failure respectively. Brodmann area 21 obtained from Dr. Reynolds.
L.C.	3		F	Methylene tetrahydrofolate reductase deficiency with cerebral atrophy Received 5-hydroxytryptamine and L-dopa. Treatment with N^5 methyl-tetrahydro-folic acid with limited success. Temporal Cortex. Obtained from Dr. Smith.
T249 T248 104			? ? ?	Downs Foetus of 24, 21 and 23 weeks gestation Absorbed with prostaglandin E_2 injection. Cerebral cortex mixed. Obtained from Dr. Brooksbank.

TABLE 3.1 THE CLINICAL STATUS OF HUMAN BRAINS ....continued

Patient	Age	P.M Delay	Sex	
ST	65		?	Subnormal. Temporal cortex from Dr. Sylvester.
SDAT			?	Temporal cortex from Dr. Sylvester.
MS	64		F	Subnormal Cortex atrophy of right frontal lobe. Ventricular dilation. No evidence of plaques and tangles. Received Septrin , Sodium Valporate, Ethosuxamide. Temporal Cortex obtained from Dr. Sylvester.

at pH 8.0 in 0.1 molar Tris/HCl thermostated at 37°C over a 3 hour period in 3ml cuvettes at intervals of 15 minutes. When assayed with sodium nitrite 1ml of the assay was replaced by 1ml of $1x10^{-4}$ molar sodium nitrite. Reduction of adenosyl cobalamin was by the addition of a trace of zinc dust and a few drops of concentrated hydrochloric acid from a Pasteur pipette to a solution of $1.1x10^{-7}$ molar adenosyl cobalamin (vitamin B_{12}).

RESULTS AND DISCUSSION

The recovery of neopterin and biopterin after freezedrying was investigated for loss. This was done with standards of known concentration and are presented as shown in table 3.2. 1ml of sample was taken to dryness and then 0.25ml of water was added. They were then injected into the HPLC.

The assay conditions used were those found for the rat brain and it was thought advisable to ensure the conditions were the same for human brains. This was done for GTP, NADPH, pH and linearity of synthesis with respect to time. This was done holding all but one of the conditions constant. The results are presented in Graphs 3.1 - 3.4. The recovery of the synthesising enzyme from the homogenate in the supernatant was examined by observing the biopterin synthesis under normal assay conditions and comparing the synthesis in the supernatant with that of the homogenote. A recovery of 91% was observed in the supernatent (table 3.3).

The biosynthesis of biopterin was investigated in brains from patients suffering from senile dementia and these were compared against matched controls. The result are presented in table 3.4 and 3.5. It was found that the biosynthesis in dements was reduced, in many being zero or below the detectable limits (0.5 ng/1ml) of the instrument, whilst the control brains synthesised.

The dihydropteridine reductase activity of the brains was also examined. The DHPR's for samples C234-C244 and D38-D46 were done by Mr. C. Eggar. The results are presented in table 3.6 from which it can be seen that the controls have higher activity although this is not significant.

Because of the large amounts of potential information available on these samples the results we obtained were sent to Dr. Mountjoy in Cambridge (Addenbrooks Hospital) to see if they could correlate with any of the information he had. He investigated the correlation of biopterin biosynthesis with age, plaques, tangles, dementia score, neurones per square mm in the temporal gyrus and neurones in 4 columns of the temporal gyrus. The only correlation found was when both the dements and control results were pooled where it was found they correlated with neuronal fall out in the temporal gyrus, using a Pearsons Correlation, the correlation being -0.369 P=0.011. As the neurones fall out the synthesis rises.

The additions of 5-methyl-5,6,7,8-tetrahydrofolate ($1.1x10^{-4}$ molar in $5x10^{-3}$ molar ascorbic acid) had a definate enhancing effect in four out of the five samples. (Table 3.7). If all five samples are taken together there is no significant effect. If the sample with no synthesis is omitted and the four compared against all seventeen dements then they are significant at 0.2% using Wilcoxon's sum of ranks. When adenosyl cobalamin was added ($1.1x10^{-7}$ molar) with 5-methyl tetrahydrofolate no effective change was found. (Table 3.7).

When 5-methyl tetrahydrofolate was added to control brains the effects were limited and not significant using a sum of ranks test (Table 3.8).

In a subject who died with 5,10-methylene tetrahydrofolate reductase deficiency the effect of 5-methyl-5,6,7,8-tetrahydrofolic acid boosted zero synthesis into a range falling between the biosynthetic capcity of young infants and control adults. The subject's dihydropteridine reductase activity was found to be within the range considered to be normal (table 3.9).

The biosynthesis of biopterin in patients dying with Down's syndrome was very low. There was evidence of plaques and tangles in all three subjects (Dr. Sylvester personal communication). There were no samples from young Down's subjects to examine and samples from Down's foetus's showed no synthesis. Foetal controls did not synthesis biopterin either (table 3.11).

In depressed patients it was found that the synthesis was below the detectable limit of the system and that in one sample the synthesis was raised by the addition of 5-methyl-tetrahydrofolate. A second was raised by the addition of adenosyl cobalamine. The dihydropteridine reductase levels fell within normal range (table 3.13).

An examination of biopterin biosynthesis and dihydropteridine reductase for several brain regions is presented in table 3.14. It can be seen that the temporal cortex, frontal cortex, Locus Coeruleus, pineal and Amygdala had biosynthetic capacity whilst the hippocampus, putamen and Globus Pallidus did not. All those assayed had dihydropteridine reductase activity.

When biopterin biosynthesis is examined with respect to age it is observed that no biopterin biosynthesis is found in foetus's of up to 24 weeks in age. This then rises after birth to a maximum at some point in life before 31 but due to a lack of suitable tissue it is not possible to say at

what age this occurs. After this maximum the synthesis tails off to a lower level with increased age (Graph 3.5). A similar effect is seen with dihydropteridine reductase although there does not seem to be a tail-off with age. Once again there is a shortage of samples between infancy to middle age. (Graph 3.6).

Because the addition of adenosyl cobalamin was found to have no effect (table 3.17) the possibilty of sufficient amounts of the vitamin in the supernatent was investigated. When synthesis was carried out under an atmosphere of nitrous oxide it was found that synthesis fell to zero (Table 3.18). Nitrous oxide is known to inhibit adenosyl cobalamin (Amess et al 1979).

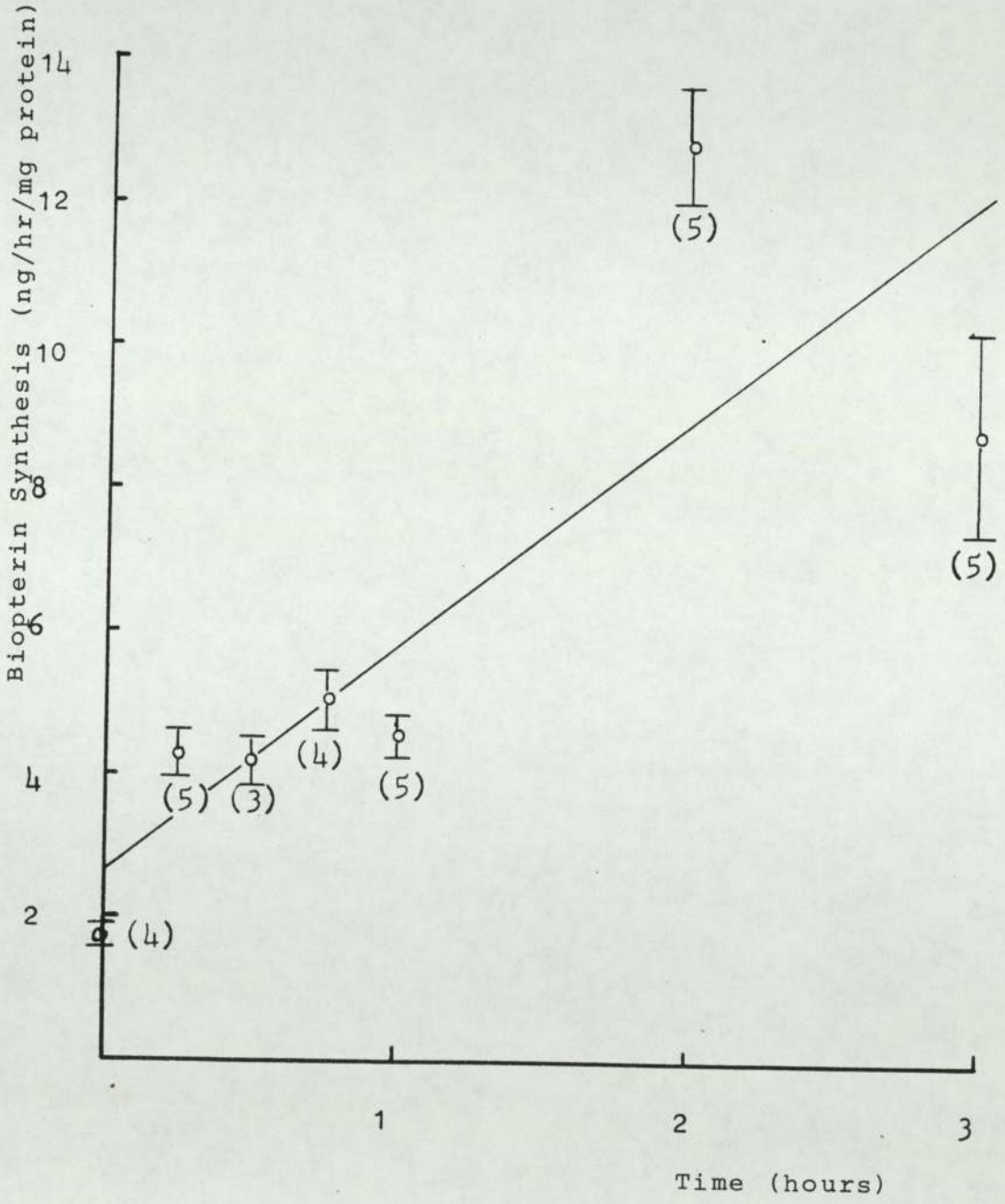
Whilst it was known that nitrous oxide will inhibit adenosyl cobalamin no reports could be found on the effect of nitrite. When it was present in the biosythetic system no biopterin synthesis was found (Table 3.19).

To see the effect of nitrite on adenosyl cobalamin spectral assays were undertaken. A spectrum was run of adenosyl Co^{III} cobalamin and this was found to have maxima at 525 nm and 350 nm at pH 8.0. When adenosyl Co^{III} cobalamin was reduced using either Zn/ HCl it was found that the 525 nm peak decreased and peak at 475 nm observed. If this solution was left it was found that it oxidised very slowly. It was not found possible to reduced Co^{III} to Co^{I}.

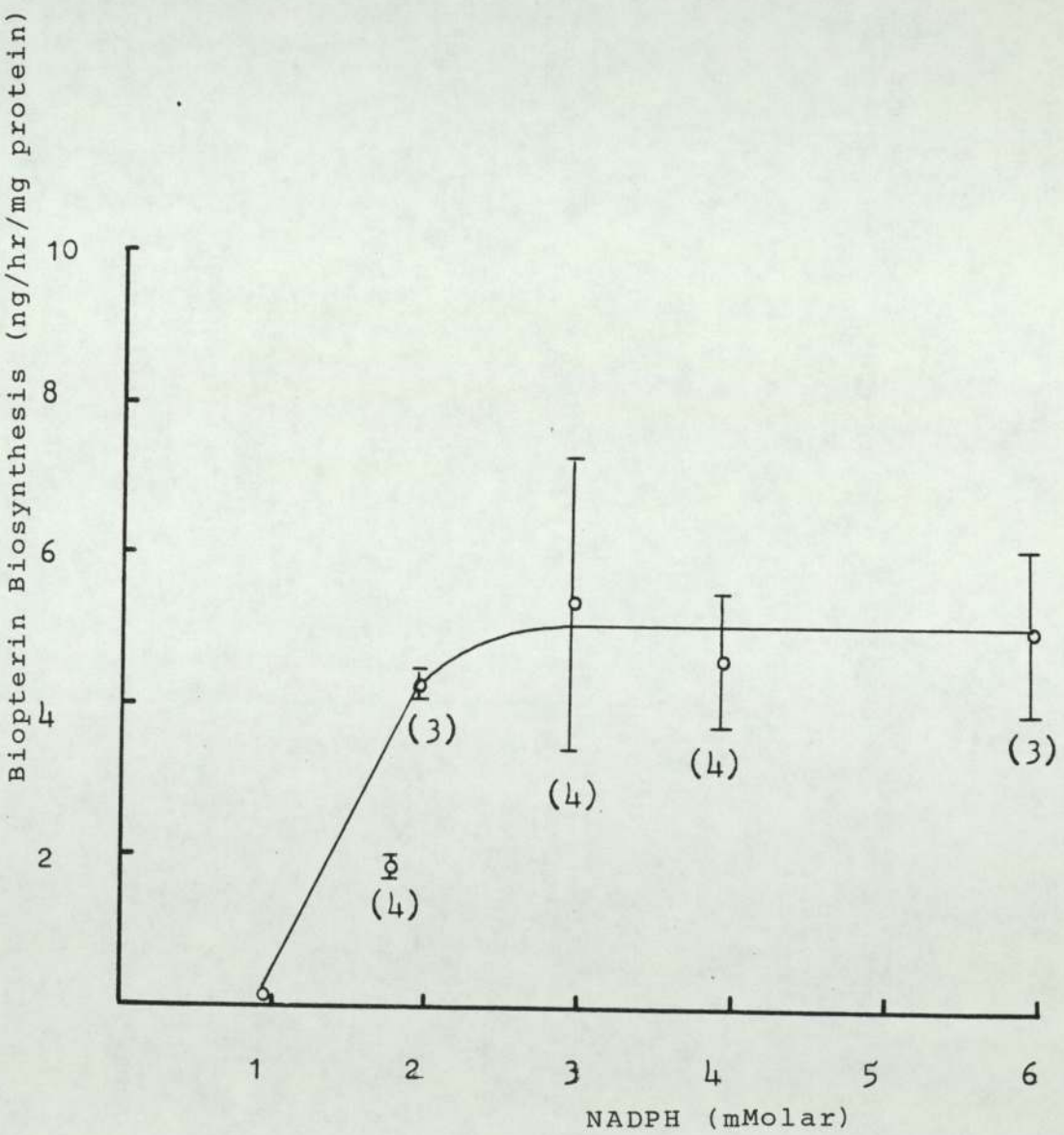
When 10^{-4} m nitrite was added to the reduced adenosyl cobalamin it was found that the spectrum rapidly reverted back to that of Co^{III}.

To ensure that nitrite was having no effect on any other substrates in the biosynthetic assay NADPH and tetrahydrobiopterin were assayed with and without nitrite over a period of 2 hours at 37°C pH 3.0. It was found that nitrite had no effect on NADPH and had no effect on the slow oxidation of tetrahydrobiopterin to biopterin. It was assumed, therefore, that nitrite inhibited in the same way as nitrous oxide by keeping Co in adenosyl cobalamin in oxidation state III. A cobalt - nitrite complex has been reported for vitamin B_{12} (Kaczka et al 1951).

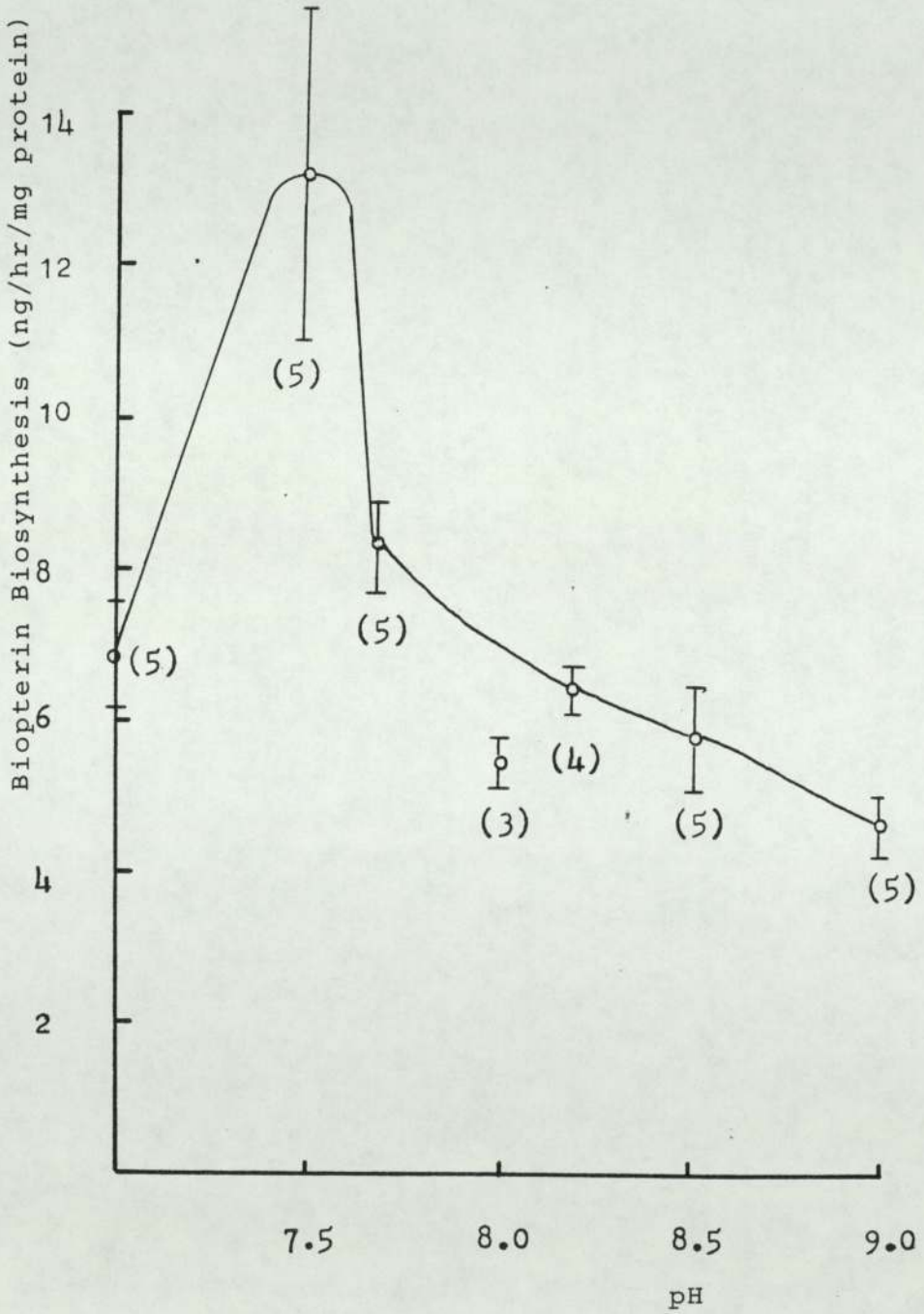
Graph 3.1 Linearity Of Biopterin Biosynthesis With Time (GF Temporal Lobe)



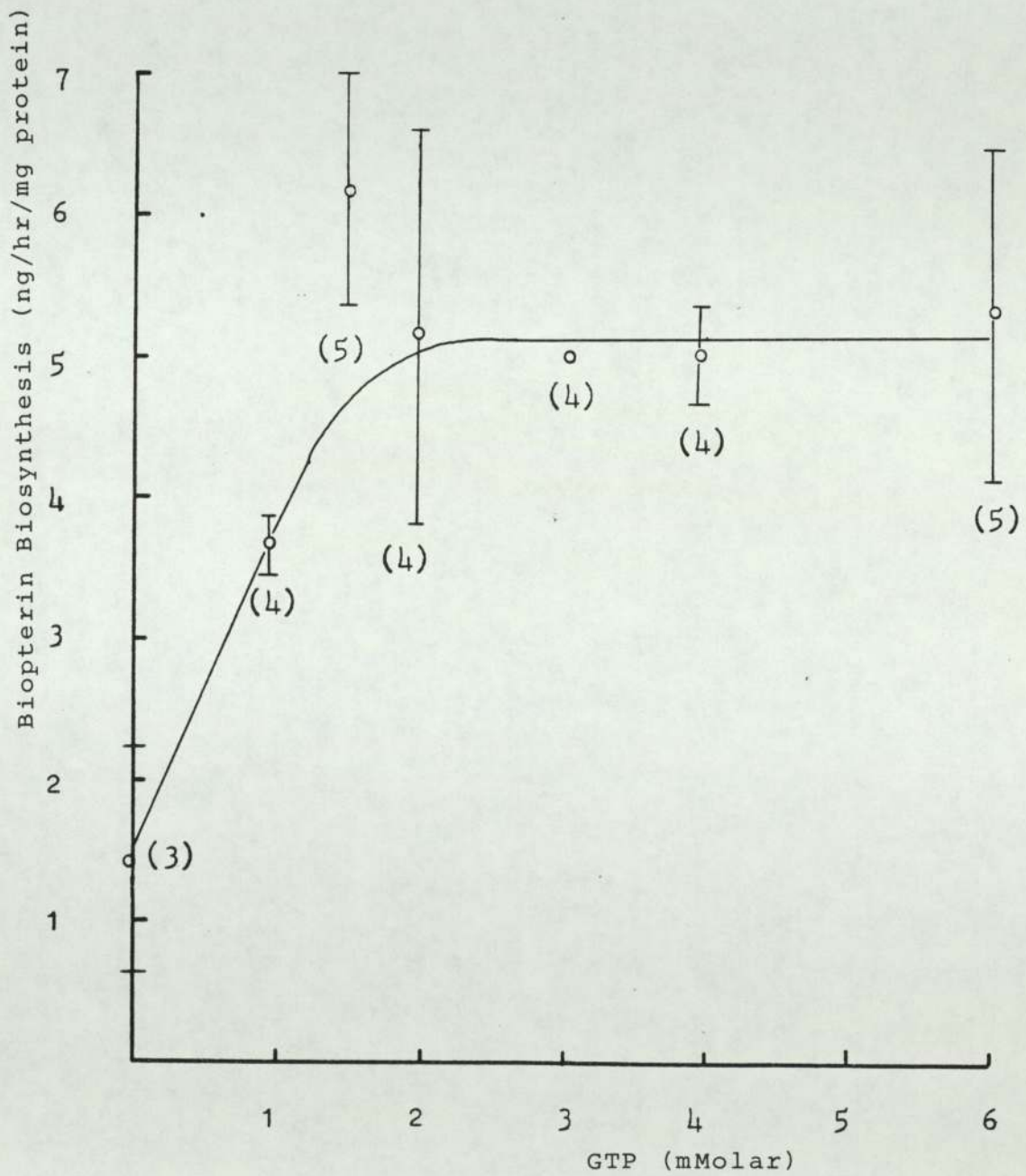
Graph 3.2 Effect Of NADPH On Biopterin Biosynthesis (GH Temporal Lobe)



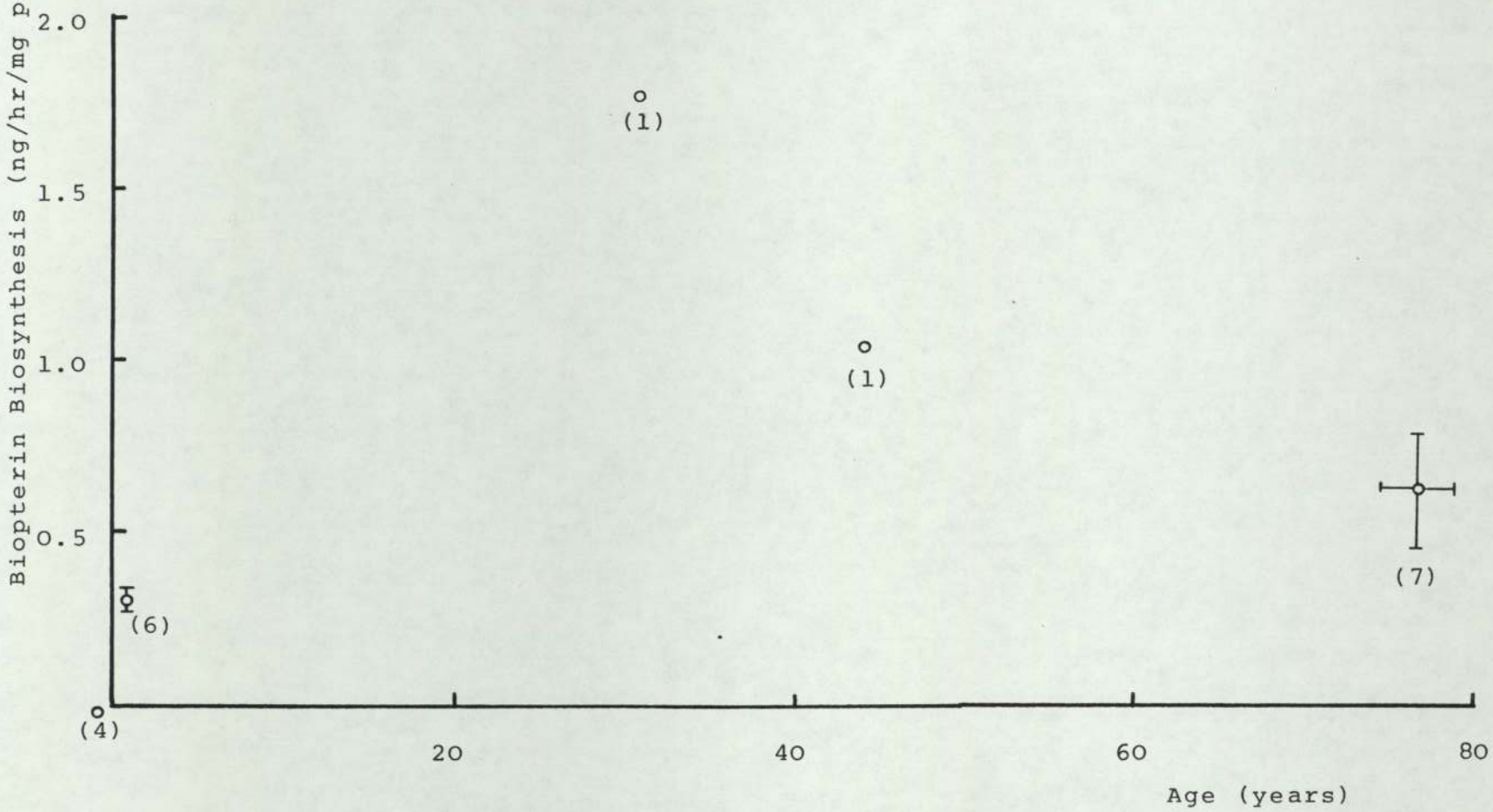
Graph 3.3 Effect OF pH On Biopterin Biosynthesis (GF Temporal Lobe)



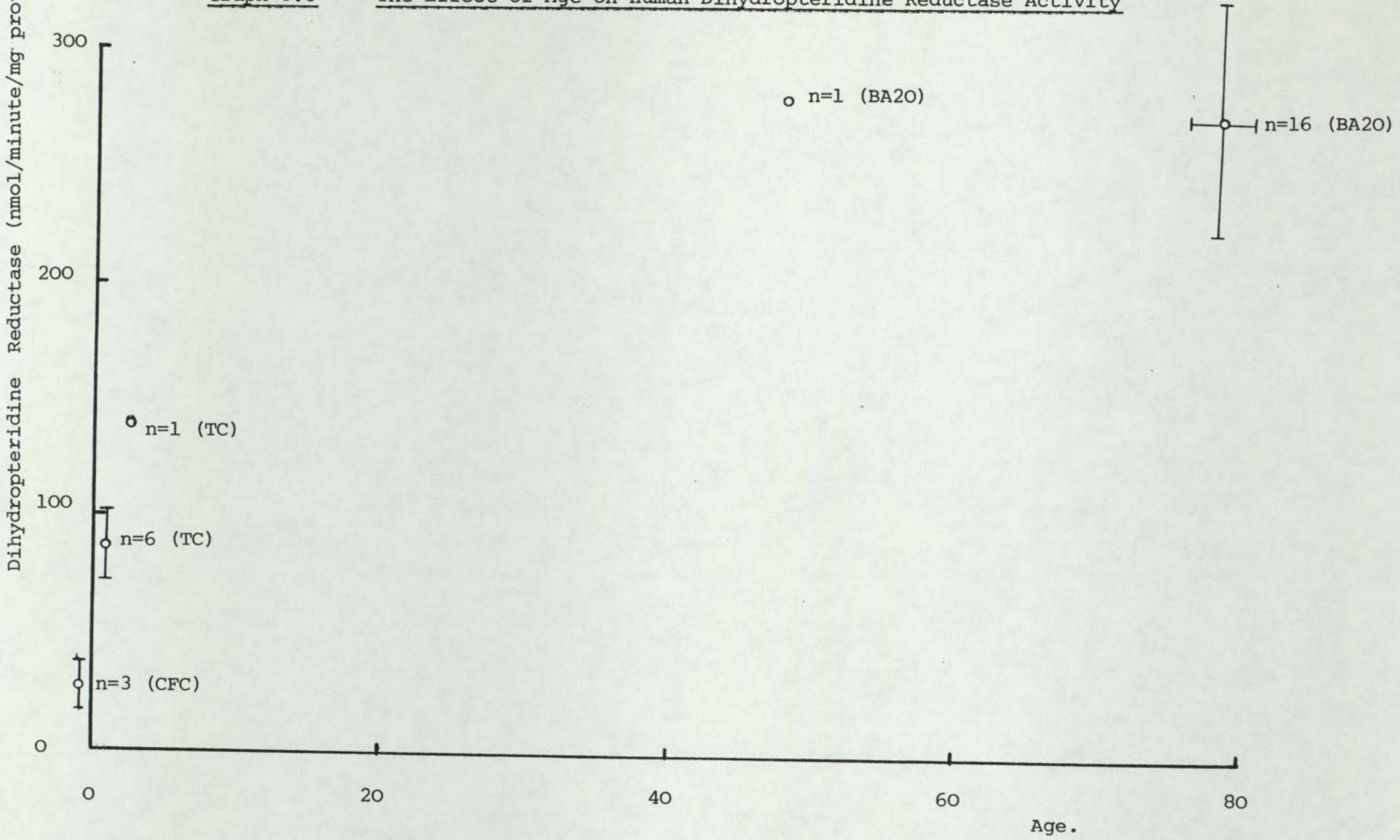
Graph 3.4 Effect Of GTP On Biopterin Biosynthesis (GF Temporal Lobe)



Graph 3.5 Effect Of Age On Biopterin Biosynthesis



Graph 3.6 The Effect Of Age On Human Dihydropteridine Reductase Activity



TABLE

TABLE 3.2 Biopterin and neopterin recovery after freezedrying related to the standards prior to drying.

	Concentration	Recovery	Recovery
neopterin	6.6×10^{-8} molar	97.5%	97.5%
	1.6×10^{-8} molar	95.8%	103%
	6.6×10^{-9} molar	80%	84%
	2.6×10^{-9} molar	83%	104%
biopterin	1.9×10^{-8} molar	72%	
	9.5×10^{-9} molar	74%	
	3.8×10^{-9} molar	98%	
	1.5×10^{-9} molar	96%	

TABLE

TABLE 3.3 Recovery of biopterin synthesis in homogenate and supernatent.

	Synthesis ng / hr / mg protein ±SD
Synthesis in homogenate	(5) 6.7 ± 0.6 ng biopterin/hr/mg protein
Synthesis in Supernatent	(4) 6.1 ± 2.9 ng biopterin/hr/mg protein

no significant difference by Wilcoxon's sum of ranks

TABLE

TABLE 3.4 Biopterin biosynthesis (ng / hr / mg protein) and dihydropteridine reductase activity (nmol / min / mg protein) in control and patients dying from senile dementia in matched subjects BA 20.

	Age	Synthesis ±SD	DHPR ±SD
Demented	71-97	0.07 ± 0.12 (n=17)	253.0 ± 122 (n=17)
Control	44-89	0.66 ± 0.48 (n=8)	280.7 ± 148 (n=17)

Significance of Synthesis is better than 0.2% using Wilcoxon's sum of ranks.

TABLE 3.5 Biopterin biosynthesis (ng / hr / mg protein) and dihydropteridine reductase activity (n mol / min / mg protein) in control and subjects presented as demented.

CONTROL				DEMENTS			
Sample	Protein	Synthesis	DHPR	Sample	Protein	Synthesis	DHPR
C240	17.3	0.11	223.1	D39	10.9	0.29	333.5
C241	19.2	0.23	117.0	D39	15.2	0.0	315
C242	10.8	0.47	167.3	D40	4.7	0.0	359.6
C244	20.0	0.52	194.4	D45	0.6	0.0	466.6
C234	8.2	1.05	265.5	D48	4.4	0.4	301
C248	5.7	1.52	124.0	D49	2.4	0.0	364
C236	9.6	0.0	280.6	D50	8.5	0.02	94.1
C238	10.9	1.008	460.0	D51	8.9	0.29	150.7
C247	1.4	N/A	607	D52	4.7	0.16	154
C249	N/A	N/A	236	D53	N/A	N/A	N/A
C253	6.6	0.33	390	D59	5.2	0.0	293
C254	2.5	N/A	361.9	D60	5.3	0.0	146
C256	6.1	N/A	150.2	D61	6.1	0.0	167.2
C257	3.1	N/A	497.3				
C267	2.8	N/A	130.9	D41	19.7	0.09	222.8
C273	3.9	N/A	153.8	D42	10.0	0.0	52.75
C270	3.1	N/A	416				
				D46	9.2	0.0	453.3
				D58	8.0	0.0	268.7
				D43	16.5	0.0	158.3

D41 and D42 had vascular dementia

D46 and D58 had high dementia score but no plaques and tangles

D43 had moderate dementia score but no plaques and tangles

the remainder were all presented as Alzheimer type. Alzheimer synthesis is significant at 0.2% using Wilcoxon's sum of ranks

TABLE

TABLE 3.6 Dihydropteridine Reductase Activity in dements and controls in Matched Subjects B.A 20

	nmol / min / mg protein ±SD			
Dements	259.7	±	122.1	n = 17
Controls	280.7	±	148.5	n = 17

TABLE

TABLE 3.7 Effect of 5-Methyltetrahydrofolate with Adenosyl Cobalamin on Demented Brains (BA 20) ng / hr / mg protein)

	Incubation	Incubation + 5-MeTHF	Incubation+ 5-MeTHF + Vitamin B_{12}
D 50	0.02	0.37	0.26
D 51	0.29	0.42	0.38
D 59	0.0	0.57	0.51
D 60	0.0	0.0	0.0
D 61	0.0	0.64	0.57
Mean $\pm$ SD	0.06 $\pm$ 0.12	0.4 $\pm$ 0.24	0.34 $\pm$ 0.02

No significance with Wilcoxon's sum of ranks if D60 is omitted there is a 5% significance between the plain incubation and the incubation with 5-MeTHF.

TABLE 3.8 Effect of 5-Methyltetrahydrofolate on Biopterin Biosynthesis on Temporal Cortex.

Brain	Age	ng/hr/mg protein	+ 5-Me THF ng/hr/mg protein
CFC 58	5 day	0.23	0.36
CFC 96	7 month	0.21	0.44
CFC 99	6 month	0.50	0.44
M	74 yr	0.33	0.29
Mean ± SD		0.317 ± 0.13	0.38 ± 0.07

no significance using Wilcoxon's sum of ranks.

TABLE

TABLE 3.9 .Synthesis of Biopterin (ng / hr / mg protein) and Dihydropteridine Reductase Activity (n mol/min/ng protein) in a Patient With Methylene Tetrahydrofolate Reductase Deficiency

	Age	Biopterin	DHPR
LC	3	0.0	131.9
LC + 5MeTHF ($1.1 \times 10^{\pm 4}$ molar)		0.62	
Controls	6wk-7mnth	0.308 ± 0.11	90.2 ± 26.3 (n=6)
Contol BA 20	44 - 89	0.65 ± 0.48	280 ± 148 (n=17)

TABLE 3.10 Bopterin Synthesis (ng / hr / mg protein) and Dihydropteridine Reductase Activity (n mol / min / mg protein) in A Patient with Methylene Tetrahydrofolate Reductase Deficiency versus Controls.

	Synthesis	DHPR
LC	0.0	121.9
LC + 5-MeTHF	0.62	
CFC 22	0.26	66.6
CFC 58	0.23	100.0
CFC 59	0.38	69.1
CFC 96	0.21	127.2
CFC 99	0.27	66.9
TC 99	0.50	75.8
C240	0.11	223.1
C241	0.28	117.0
C242	0.47	147.5
C244	0.52	194.4
C234	1.05	265.5
C243	1.52	124.0
C236	0.0	288.6
C238	1.008	460.0
C247		607
C249	0.33	236
C253		380
C254		361.9
C256		150.2
C251		497.2
C267		130.0
C273		153.3
C272		416

TABLE

TABLE 3.11 Biopterin Biosynthesis (ng / hr / mg protein) in Down's and Control patients.

		Age	Synthesis ± SD	
Down's	(n=3)	50-51	0.036	± 0.06
Control	(n=8)	44-89	0.65	± 0.48

Significant at 5% level with Wilcoxon's sum of ranks

TABLE

TABLE 3.12 Biopterin Biosynthesis (ng / hr / mg protein) in Down's and control patients

Patient	Synthesis
WH	0.11
ML	0.0
DT	0.0
C240	0.11
C241	0.28
C242	0.47
C244	0.52
C234	1.05
C248	1.52
C238	1.008
C253	0.33

TABLE

TABLE 3.13 Biopterin Biosynthesis (ng / hr / mg protein) in Depressed Patients (BA 21) and Matched Controls

	Synthesis	+ 5MeTHF	+ 5-MeTHF + B_{12}
X5	0.0	0.0	0.0
X7	0.0	0.0	1.4
X8	0.03	0.21	0.12
X12	0.0	0.0	0.0
C254	0.16	0.19	0.24
C256	0.24	0.11	0.11
C273	0.84	0.28	

These samples were run blind.

x 5, x7, x8, x 12 are depressive

TABLE 3.14 Dihydropteridine Reductase Activity in Depression and Controls.

	DHPR nmol / min / mg protein ±SD
Depressed (n=4)	259.7 ± 113.6
Controls (n=3)	207 ± 127.2
Controls (Reynolds) (n=17)	230 ± 143

TABLE

TABLE 3.15 Dihydropteridine Reductase activity in Depressives and Controls

	DHPR (n mol / min / mg / protein)
X5	166.6
X7	214.2
X8	233.3
X12	425.
C256	117.4
C257	202.0
C258	297.4

TABLE

TABLE 3.16 Biopterin Biosynthesis and Dihydropteridine Reductase Activity in Differing Brain Areas (GF)

	Biopterin ng/hr/hr/mg protein	DHPR n mol/min/mg protein
Temporal Cortex	1.3	328.1
Frontal Cortex	2.3	427.6
Hippocampus	0.0	1011.0
Putamen	0.0	538.0
Globus Pallidus	0.0	863.1
Locus Coerulus	0.49	1044.0
Pineal	0.24	N/A
Amygdala	0.35	N/A

TABLE 3.17 The Effect of Adenosyl Cobalamin on Biopterin Biosynthesis (ng / hr / mg protein)

Subject	Incubation	Incub^{n} + B_{12}
TC 99	0.5	0.5
C 254	0.16	0.24
C 256	0.24	0.11

TABLE 3.18 Effect of Nitrous Oxide and Nitrite on Biopterin Biosynthesis (ng / hr / mg protein)

Subject	Incubation	N_2O	NO_2^-
CFC 22	0.26	0	0
CFC 58	0.23	0	0
CFC 59	0.38	0	0
CFC 96	0.21	0	0
CFC 99	0.27	0	0
TC 99	0.50	0	0

SUMMARY

a) Biopterin is well recovered after freeze drying.

b) Optimum biopterin biosynthesis occurs at concentrations of 3mM NADPH and 3mM GTP at pH 7.5 and is linear over a period of 3 hours.

c) Biopterin biosynthesis is greatly reduced in senile dementia of the Alzheimer type, Down's syndrome and depression.

d) The inclusion of 5-methyl tetrahydrofolic acid enhanced biopterin biosynthesis in dementia and in one depressed but had little if any effect on control samples.

e) The addition of 5-methyl tetrahydrofolic acid to a brain sample from a subject dying from methylene tetrahydrofolate reductase deficiency boosted biopterin synthesis from zero to within the "normal" range.

f) Biopterin biosynthesis was found to reach a maximum and then decline with age whilst dihydropteridine reductase reaches a maximum and does not decline.

g) Nitrous oxide and nitrite were found to inhibit biopterin biosynthesis.

h) Nitrite probably inhibits biopterin biosynthesis by keeping adenosyl cobalamin cobalt as cobalt III.

i) Biopterin biosynthesis and dihydropteridine reductase activity vary with brain area.

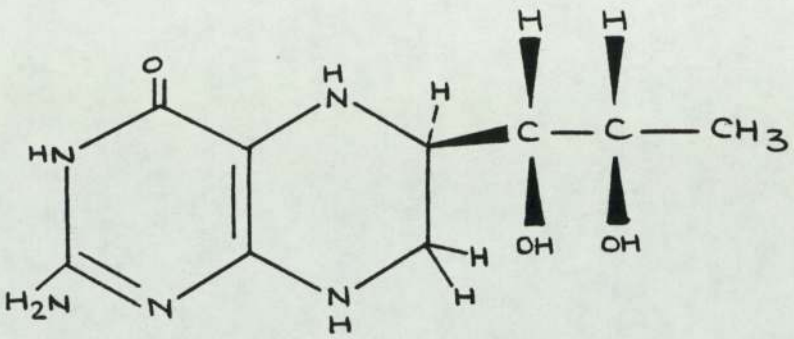
CHAPTER 4. BIOPTERIN METABOLISM IN RAT BRAIN

INTRODUCTION

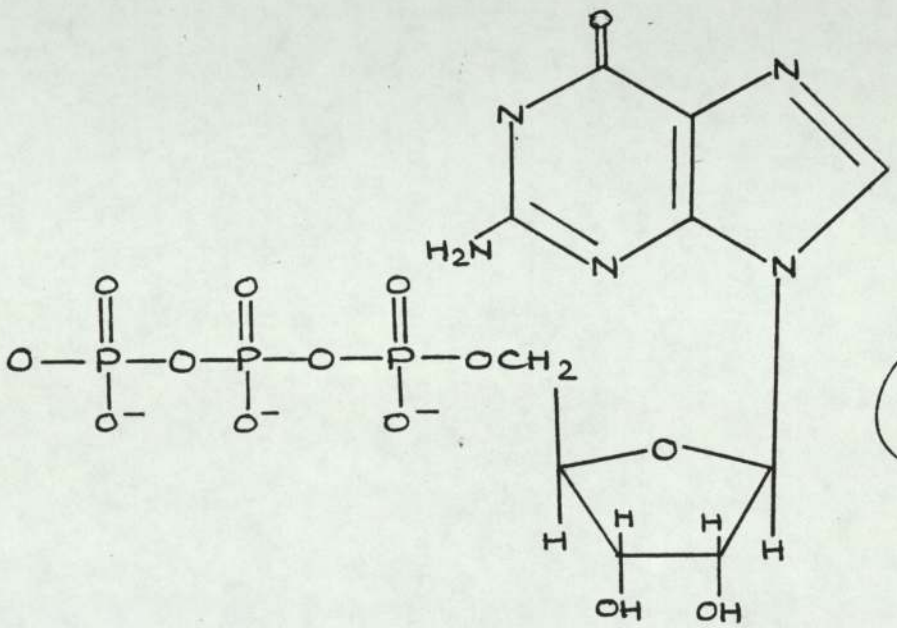
Tetrahydrobiopterin (1) is essential for the function of the aromatic aminoacid hydroxylases and consequently neurotransmitter biosynthesis. Its level is dependent on its biosynthesis from guanosine-5'-triphosphate (2) and the salvage of quinonoid dihydrobiopterin (3), formed when tetrahydrobiopterin (1) functions as a cofactor, by dihydropteridine reductase. Disruption of either synthesis or salvage can have serious consequences.

The effect of age on the synthesis of tetrahydrobiopterin has been reported for rats (Kapatos et al 1983) finding that the highest biopterin level occured two days prior to birth and then decreased threefold by the fifth day. GTP Cyclohydrolase activity, the enzyme responsible for the first step in biopterin biosynthesis, was observed and two significant activity peaks observed one of two days prior to birth and the second at 10-15 days after birth. Algeri et al (1977) investigating dihydropteridine reductase levels in rat brains found that the enzyme activity increased gradually prior to birth and reached a maximum at about 90 days.

Lead is a known neurotoxin which affects tetrahydrobiopterin metabolism in vitro and in vivo. Lead has been found to inhibit rat brain dihydropteridine reductase activity and also biopterin biosynthesis (Purdy et al 1981). Measurement of plasma biopterins with blood lead in man and rat revealed that plasma biopterin increased with blood lead levels and then fell at higher levels (McIntosh et al 1982). Measurement of whole blood dihydropteridine reductase activity in blood samples taken from workers in the lead industry has shown a reduction in in vivo activity



(1)



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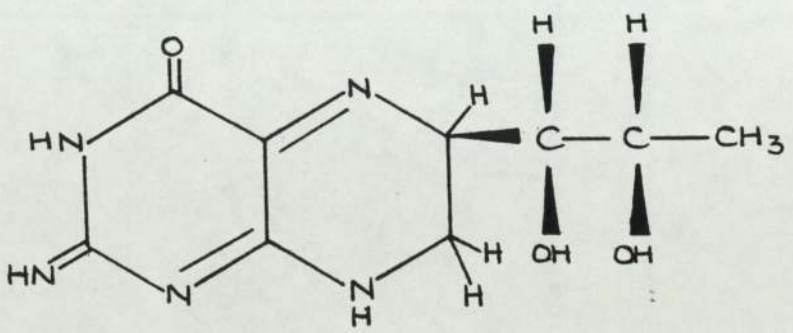


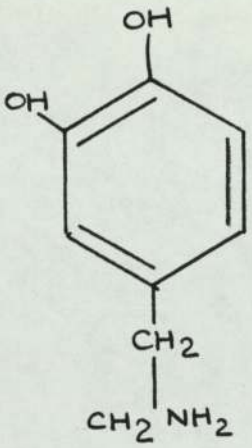
(3)

on long term exposure to lead (Blair et al 1984). When pregnant female rats are maintained on water containing lead (300 ppm and 1000 ppm) during pregnancy and until the offspring are 21 days old the dihydropteridine reductase activity in the brains of the offspring is significantly decreased (Barford et al 1983).

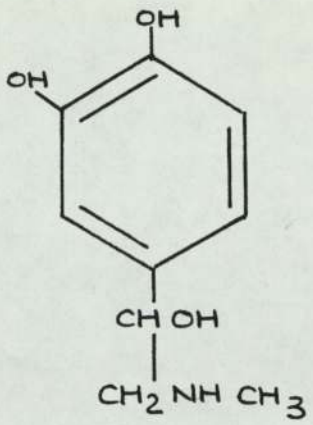
Oestrogen administration to humans is known to have neurological consequences. Nausieda et al (1979) reported an increased incidence of chorea in women taking oral contraceptives. The causative agent is thought to be an oestrogen since Barber et al (1976) have been unable to find any incidence of chorea associated with progesterone preparations. Animal studies suggest that female sex hormones may enhance central dopaminergic sensitivity giving rise to chorea (Nausieda et al 1979). Bedard et al (1979) have reported that oestrogens are antidopaminergic reducing the stimulatory effect of a dopaminergic agonist. Di Paulo et al (1982) suggest that oestrogens increase dopamine binding by increasing the number of dopamine receptors. They also found that oestrogens caused a decrease in dopamine levels. It appears that oestrogens decrease dopamine levels and this induces a compensatory increase in the number of dopamine receptors.

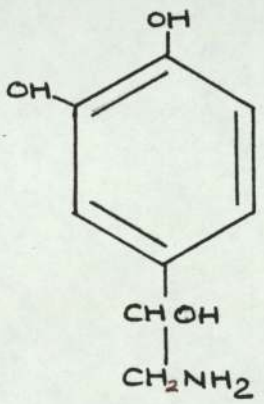
Leeming and Blair (1980) have found lower serum biopterins in pre menopausal women and women on the pill and Barford et al (1983) report lower serum biopterins in pregnancy implying an hormonal effect on tetrahydrobiopterin which would ultimately have an effect on levels of dopamine (4) noradrenalin (5) adrenalin (6) and serotonin (7). Eggar et al (1983) reported that diethylstilboestrol (8), when dosed orally to rats, raised liver levels of dihydropteridine reductase.

(4)

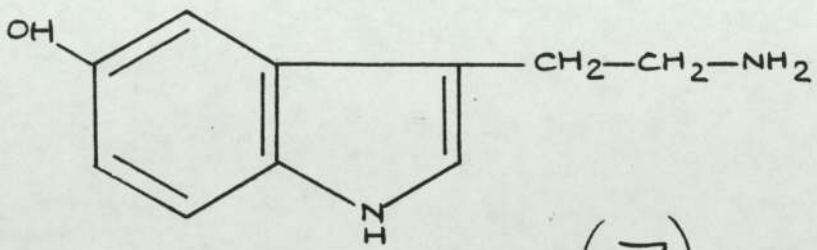

OH
OH
CH2
CH2 NH2

(5)


OH
OH
CH OH
CH2 NH CH3


OH
OH
CH OH
CH2NH2

(6)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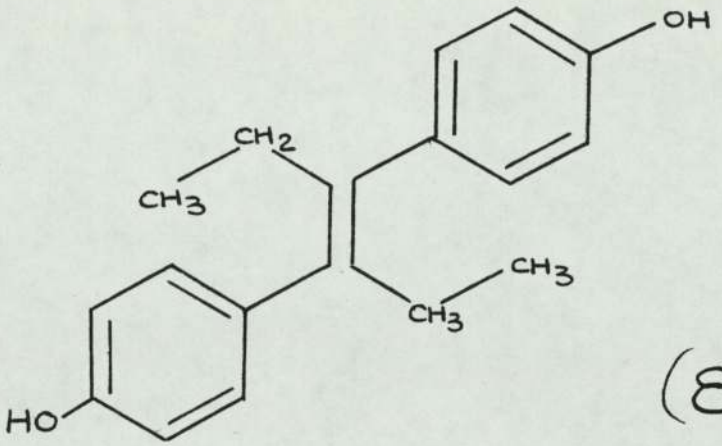
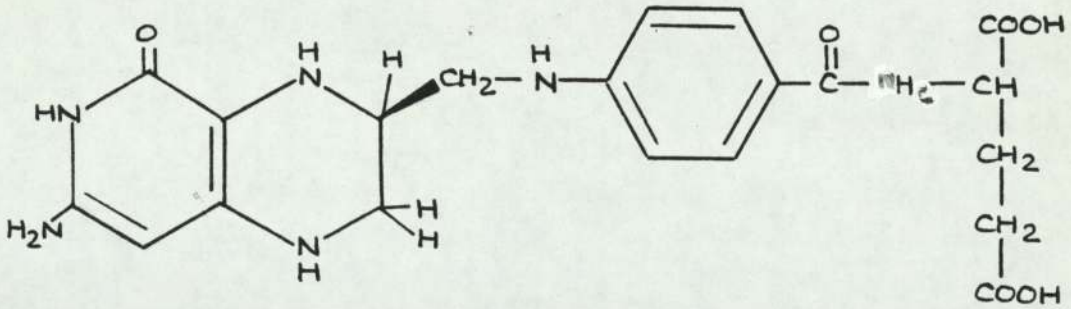
CH2—CH2—NH2
N
H

(7)


OH
CH2
CH3
CH3
CH3
HO

(8)


O
HN
H2N
N
H
H
CH2
N
H
C
O
H2
CH
COOH
CH2
CH2
COOH

(9)

5-methyl tetrahydrofolic acid (9) when present with vitamin B12 and ascorbate has been reported to enhance biopterin biosynthesis in the rat brain (Ieeming et al 1982). It has been found that folate levels in red blood cells of patients with dementia are lower than that found in normal control patients (Sneath et al 1973). Shaws et al (1971) have reported dosing folate to patients with low red blood cell folate suffering from dementia but found no effect and also found that the folate administered, folic acid, did not enter the CSF. It is possible that the folate did not reach the site at which it was required or was in the wrong form.

In this chapter results will be presented on the effect of of 5-methyl tetrahydrofolate (9) diethylstilboestrol (8) and lead on biopterin biosynthesis in rat brain preparation.

Material and Methods

Rat brains from lead dosed rats were kindly provided by Dr. C. Winder (Royal Postgraduate Medical School; Hammersmith Hospital). They were set up as a model for the study of lead effects on brain development (Charmichael et al 1981). The rats were dosed with drinking water containing lead acetate. The water was supplied to pregnant females and the supply continued until the neonates were killed at 30 days. Once killed the brains were removed and stored at minus 70°C until required. The striata were dissected out by Dr. Winder. There were twelve striata for each lead dosed group and these were pooled to make sufficient enzyme preparation for the assay. Half Brain assays were done on a group of 48 brains, twelve for each lead level (0,300,1000,2000 ppm). Of these six were taken from each group and dissected in half and the six halves pooled for the enzyme preparations. Blood leads were measured on litter mates of those from which the striata were taken. No blood

leads were done on the 'whole brain' groups.

The synthetic oestrogen diethylstilboestrol was administered orally to female Wistar rats (21 days old, 100g body weight). The dose was prepared in corn oil and the animals received 500mg stilboestrol/kg body weight daily for four days. 24 hours after the last dose the animals were killed and the brains removed for study.

The male rat receiving aluminium was dosed orally with an aqueous solution of aluminium 0.3ml of 0.5 molar/200g body weight. After four hours it was killed and its brain removed for study.

Optimal pH, GTP and NADPH concentrations were found by holding all conditions fixed and varying the condition of interest. The assay conditions used were those outlined in Materials and Methods.

The addition of lead to the assay was done by the addition of 50µl 1×10^{-4} molar lead acetate solution to the final 1ml assay system.

5-methyl tetrahydrofolate was added by preparing 2.2×10^{-3} M 5-methyl tetrahydrofolate in 0.1 molar ascorbic acid. Of this 50 µl was added to the final 1ml assay system.

All incubations were done as replicates of 5. Unless stated all results were obtained by HPLC.

Results and Discussion

Results obtained through varying the concentrations of GTP and NADPH confirmed the concentrations of both to be saturating for the enzyme

system at 3 m molar for both. An apparent Km for the system is 2.2 x 10^{-4} molar for GTP and 7.0 x 10^{-4} molar for NADPH. An optimum pH was found to be pH 8.0 (graphs 4.1 - 4.3) these results are the same as those found by Brown (1981).

Using rats of differing age the biosynthesis of biopterin and the activity of dihydropteridine reductase was examined. These results are presented in graphs 4.4 and 4.5. As can be seen the reductase activity increases with age and levelsoff, however the biosynthesis starts from a high level falls rapidly and then rises again.

The effect of increasing lead doses on rats seems to increase the biosynthesis of biopterin up to about 1000 ppm beyond which the synthesis falls towards or beyond the control value. Dihydropteridine reductase activity however falls with increasing lead concentration and then rises again about or beyond 1000 ppm (Tables 4.1 - 4.4). Dosing rats with lead causes a significant decrease in brain weight at higher levels and so this brings into question the reliability of using protein for a baseline (Dhar and Banerjee 1983). To investigate this problem the DNA per gram wet weight of the brain was measured and this is presented in Table 4.5. A rise in DNA/gram wet weight is observed, however there is considerable overlap. When HPLC results and Crithidia results are compared it is seen that the HPLC results are considerably higher than Crithidia results (Table 4.7).

The addition of lead to the assay system causes a slight reduction in biopterin biosynthesis although not significantly (Table 4.8 and 4.9).

The inclusion of 5-methyl tetrahydrofolate causes an increase of 288% in biopterin biosynthesis. This is added in ascorbate. When ascorbate is added on its own no such rise is observed (Table 4.10).

Diethylstilboestrol, a synthetic oestrogen, had an inhibitory effect on synthesis reducing synthesis to between 17% and 8% of its original level. Dihydropteridine reductase activity rises (Table 4.12 and 4.14).

The addition of aluminium to one rat caused a rise in its capacity to synthesise biopterin.

TABLE

TABLE 4.1 Biopterin Biosynthesis (ng/hr/mg protein) and Dihydropteridine Reductase Activity (nmol/min mg protein) in the Striata of 30 Day Old Lead Dosed Rats.

Lead dose ppm	Synthesis ± SD	DHPR ± SD
0	1.18 ± 0.76	297 ± 61.5
300	1.66 ± 0.79	267.8 ± 32.2
1000	3.21 ± 0.63	289.4 ± 23.6

Synthesis = mean of 5 incubation. Each dose supernatent was prepared from 12 pooled striata. Results were by HPLC. DHPR's by Mr. Eggar.

TABLE

TABLE 4.2 Blood Lead Levels of the Litter Mates of 30 day Rats from which Striata were taken. Results mg lead/100ml Blood. Supplied by Dr. Winder.

Lead dose	ppm	mg lead / 100 ml blood ± SD
0	(12)	0.19 ± 0.15
300	(12)	29.5 ± 1.85
1000	(12)	36.3 ± 2.26

TABLE 4.3 Comparison of Biopterin Biosynthesis (ng/hr/mg protein) with Dihydropteridine Reductase (nmol/min/mg protein) for the Right Half of Brain in Lead Dosed Rats.

Lead dose ppm	Synthesis ± SD	DHPR
0	0.53 ± 0.3	237.2
300	0.73 ± 0.2	223.1
1000	1.3± 0.86	220.4
2000	0.69 ± 0.47	221.9

DHPR results supplied by Mr. C. Eggar.

Means are of 5 replicate assays on 1 sample for biosynthesis.

TABLE

TABLE 4.4 Comparison of Biopterin Biosynthesis (ng/hr/mg protein) with Dihydropteridine Reductase (nmol/min/mg protein) for the Left Half of Brain in Lead Dosed Rats.

Results by Crithida

Lead dose ppm	Synthesis ± SD	DHPR
0	0.41 ± 0.13	224.8
300	0.66 ± 0.36	179.9
1000	0.0	177.4
2000	0.0	199.9

DHPR results supplied by Mr. C. Eggar.

Means are of 5 replicate assays on 1 sample for biopterin biosynthesis.

TABLE

TABLE 4.5 DNA/g Wet Weight on Rat Brains Right Half Dosed with Lead.

Lead dose ppm		DNA µg/g ± 2σ
0	(12)	154.5 ± 24
300	(12)	219.5 ± 30
1000	(10)	227 ± 43.2
2000	(10)	194.5 ± 35

TABLE

TABLE 4.6 Change in Weight of Rat Brains with Lead Dosing.

Lead dose ppm		Weight in gms.
0	(6)	1.43 ± 0.05
300	(6)	1.43 ± 0.05
1000	(6)	1.25 ± 0.05
2000	(6)	1.28 ± 0.09

The reduction in 1000 and 2000 are both significant at the 1% level using Wilcoxon's sum of ranks test.

TABLE

TABLE 4.7 Comparison of Results Obtained by HPLC and Crithidia on Rat Brain Synthesis (ng/hr/mg protein) on Lead Dosed Rats Right Half Brain.

Lead ppm	Crithidia ±SD	HPLC ±SD
0	0.036 ± 0.09	0.53 ± 0.3
300	0.15 ± 0.08	0.73 ± 0.2
1000	0.11 ± 0.06	1.3 ± 0.86
2000	0.08 ± 0.1	0.69 ± 0.47

TABLE

TABLE 4.8 Effect of Adding Lead (5×10^{-5} molar) to Rat Brain right 1/2 Incubation on Biopterin Biosynthesis (ng/hr/mg protein) on Lead Dosed Rats and Controls.

Lead dose ppm	Synthesis ±SD	Synthesis + Pb ±SD
0	0.53 ± 0·3	0.5 ± 0.21
300	0.73 ± 0.2	0.78 ± 0.25
1000	1.3 ± 0.86	1.01 ± 1.0
2000	0.69 ± 0.47	0.489 ± 0.5

Means are of 5 replicate on 1 sample.

TABLE

TABLE 4.9 Effect of Adding Lead (5×10^{-5} molar) to Rat Brain left 1/2 Incubation on Biopterin Biosynthesis (ng/hr/mg protein) on Lead Dosed Rats and Controls. Results by Crithidia

Lead dose ppm	biopterin ±SD	biopterin + Pb ±SD
0	0.41 ± 0.13	0.37 ± 0.05
300	0.66 ± 0.36	0.47 ± 0.21
1000	0.0	0.16 ± 0.11
2000	0.0	0.0

Means are of 5 replicate assays on 1 sample.

TABLE

TABLE 4.10 Effect of Ascorbic Acid (5×10^{-3} molar) and 5-Methyltetrahydrofolate (1.1×10^{-4} molar) on Biopterin Biosynthesis (ng/hr/mg protein) in Rat Brains.

		biosynthesis ± SD
Control	(6)	1.38 ± 1.3
Ascorbate	(6)	1.33 ± 1.1
5 Methyl THF + ascorbate	(6)	3.98 ± 2.0

Effect of 5-methyl tetrahydrofolic acid is a 288% increase in biosynthesis by Wilcoxon's sum of ranks the rise is significant at better than the 5% level.

TABLE

TABLE 4.11 Effect of Ascorbic Acid (5×10^{-3} molar) and 5-Methyltetrahydrofolate (1.1×10^{-4} molar) on Biopterin Biosynthesis (ng / hr / mg protein) in Rat Brains.

Rat	Inc ^{n}	Inc ^{n} + Ascorbate	Inc ^{n} + 5me THF
1	0.56	0.8	3.13
2	0.64	1.2	4.94
3	1.5	0.46	2.3
4	3.9	3.55	7.6
5	0.74	0.96	2.47
6	0.97	1.02	3.4

TABLE

TABLE 4.12 Effect of Diethylstilboestrol on Biopterin Biosynthesis (ng/hr/mg protein) in Female Weaning Rats.

		biopterin biosynthesis ±SD	D HPR ±SD
Control	(6)	2.02 ± 1.5	NOT DONE
DES	(6)	0.17 ± 0.3	235.9 ± 31.2

Significant at the 1% level using Wilcoxon's sum of ranks.

TABLE

TABLE 4.13 Effect of Diethylstilboestrol on Biopterin Biosynthesis (ng/hr/mg protein) on female Weaning Rats.

Control	Biosynthesis
1	2.1
2	0.44
3	4.5
4	1.68
5	0.68
6	2.76

DES DOSED	
1	0.82
2	0.08
3	0.00
4	0.00
5	0.00
6	0.12

TABLE

TABLE 4.14 Effect of Diethylstilboestrol on Biopterin Biosynthesis (ng/hr/mg protein) and Dihydropteridine Reductase Activity (nmol/min/mg protein) in Weaning Rats'

		Biopterin Synthesis ± SD	DHPR ±SD
Control	(5)	5.86 ± 4.0	131 ± 50.39
DES	(4)	1.01 ± 0.39	.251 ± 127.8

The synthesis results are significant at the 5% level using Students t test.

DHPR by F. Al Salihi

TABLE

TABLE 4.15 Effect of Diethylstilboestrol on Rat Brain Biopterin Biosynthesis (ng/hr/mg protein)

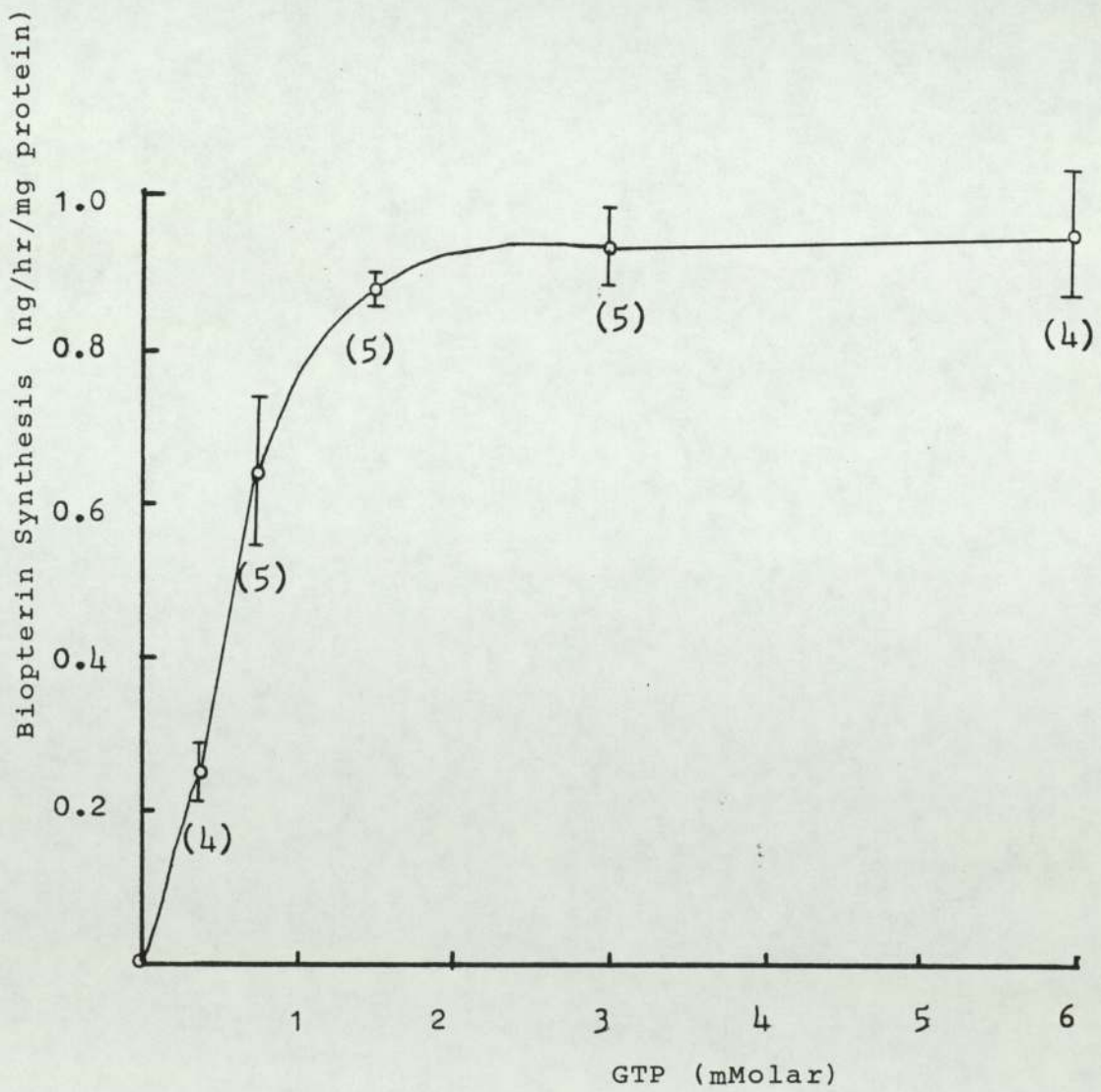
Control	Biosynthesis
1	5.45
2	9.0
3	10.6
4	3.54
5	0.74
DES DOSED	
1	0.62
2	1.56
3	0.94
4	0.94

TABLE

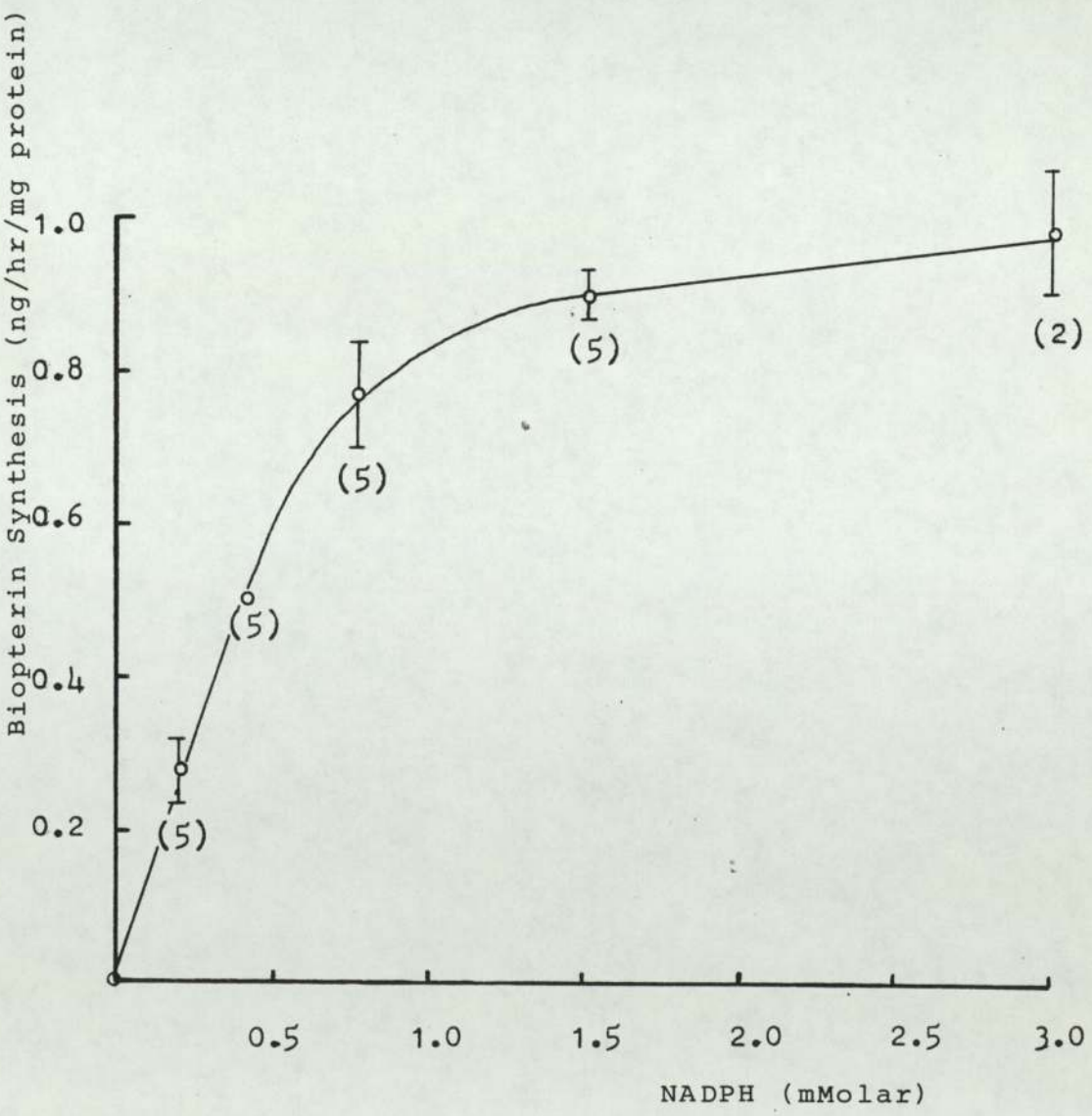
TABLE 4.16 Effect of Aluminium on Biopterin Biosynthesis (ng/hr/mg protein) and Dihydropteridine Reductase Activity (nmol/min/mg protein) on the Brain of One Rat.

		Biosynthesis ±SD	DHPR ±SD
Control	(5)	0.90 ± 0.37	
Al	(1)	3.45	455.2

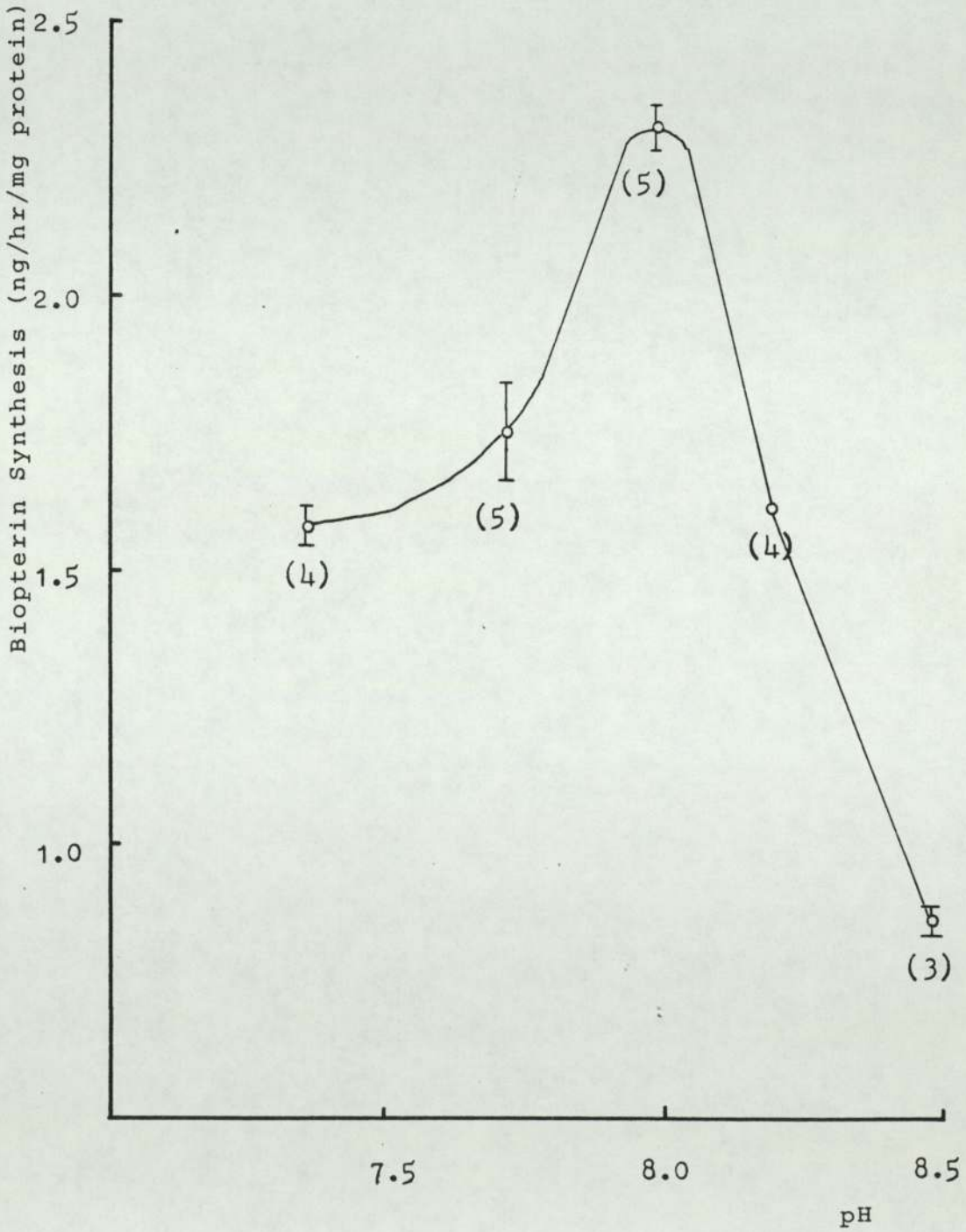
Graph 4.1 Effect Of GTP On Biopterin Biosynthesis In Rat Brain Preparation (measured by HPLC)



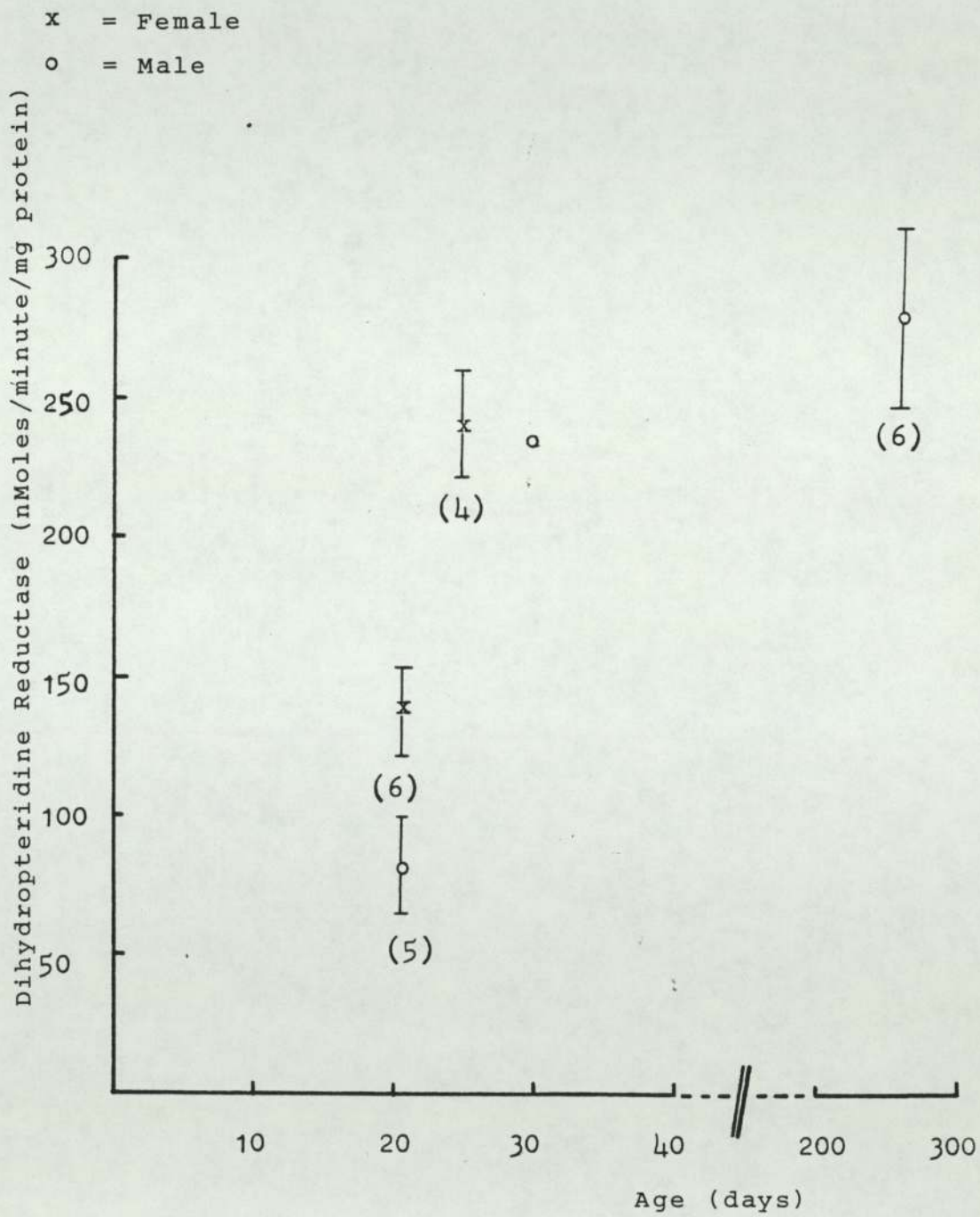
Graph 4.2 Effect Of NADPH On Biopterin Biosynthesis In Rat Brain Preparation (measured by HPLC)



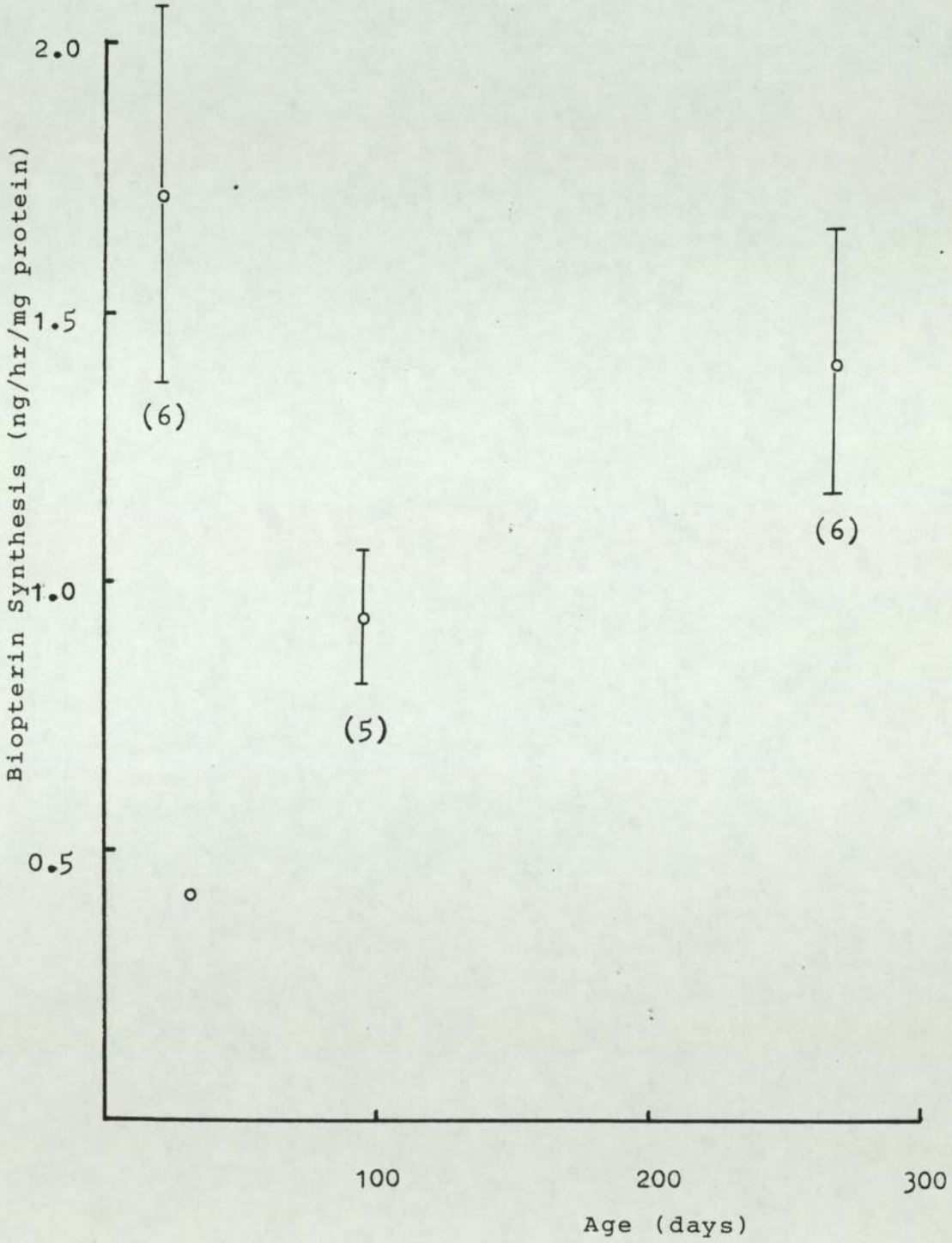
Graph 4.3 Effect of pH On Biopterin Biosynthesis In Rat Brain Preparation (measured by HPLC)



Graph 4.4 Effect Of Age On Dihydropteridine Reductase Activity In Rats



Graph 4.5 Effect Of Age On Biopterin Biosynthesis In Male Rats (measured by HPLC)



SUMMARY

a) Optimal substrate concentrations are 3m molar for both GTP and NADPH. An optimum pH is 8.0.

b) Lead dosing to rats causes a rise in biopterin synthesis with a fall off of synthesis at high doses. There is a loss of brain weight with higher lead doses but DNA levels appear relatively unaffected. The addition of lead to the assay causes a slight decrease in biopterin synthesis.

c) HPLC reads higher than Crithidia.

d) 5-methyl tetrahydrofolate stimulates biopterin synthesis. The addition of ascorbic acid has no effect.

e) Diethylstilboestrol inhibits biopterin biosynthesis and increases dihydropteridine reductase activity.

f) Aluminium increased biopterin biosynthesis in one sample.

g) Dihydropteridine reductase activity rises with age in juvenile rats and reaches a plateau. The biosynthesis of biopterin comes from a peak to a possible trough followed by a rise.

CHAPTER 5.

BIOPTERIN DERIVATIVES IN NEOPLASIA AND WHOLE BLOOD

INTRODUCTION

The levels of several pterins in urine, blood and cerebral spinal fluid in normal and diseased subjects have been reported. Rokos et al (1980) reported that urinary levels of neopterin, biopterin, xanthopterin and pterin in patients with neoplasia. They noted that biopterin levels were elevated in some subjects. Neopterin levels have frequently been found to be elevated in cancer patients (Hetzel et al 1983, Aulitzky et al 1983). Rokos et al (1980) have also reported pterin to be usually but not always lowered. Stea et al (1981) made similar observations but found biopterin only slightly raised but not significantly.

Leeming et al (1976, 1980, 1980) investigated the level of biopterin derivatives in body fluids, principally serum. They discovered the level of biopterin derivatives to be altered in many conditions. An increase in biopterin derivatives was noted with increased age and to be higher than adults in foetal cord blood. A depression of serum biopterin occurred in malignant carcinoid disease, senile dementia, mentally disabled patients, schizophrenia and pernicious anaemia.

Leeming et al obtained their results using the Crithidia assay which has two drawbacks. Firstly that it is a long assay, about 4-5 days, and second that it does not differentiate between pterins.

The result presented in this chapter are for biopterin biosynthesis in tumour and control tissue, the estimation of whole blood biopterin by

HPLC to obtain values for the oxidised and reduced forms and a comparison of results obtained by Crithidia and HPLC.

MATERIALS AND METHODS

As outlined in Chapter 2.

RESULTS

The biosynthesis of biopterin derivatives in neoplastic and control tissue was examined. The tissue was of the same type and subject for control and tumour. Tumours were from the breast and large bowel and the results are presented in Table 5.1 and 5.2. No significant difference was found between tumour and control in breast. No correlation was found with oestrogen receptor levels or menopausal state. In the bowel the tumour always had higher synthetic capacity than the control tissue.

When Crithidia results for two tumours and controls were compared with results obtained by HPLC it is found that Crithidia reads higher although not significantly. (Table 5.3).

Biopterin derivatives have been measured in whole blood samples by HPLC without oxidation indicating the level of oxidised biopterin present, after acid iodine oxidation, reading the total level of biopterin, dihydrobiopterin and tetrahydrobiopterin, and after alkaline iodine oxidation, reading biopterin and dihydrobiopterin together. The same has been done using the Crithidia assay. (Table 5.4 and 5.5). A breakdown of these results into components is presented in Table 5.6.

In a comparison of unoxidised Crithidia and HPLC, acid iodine Crithidia and acid iodine HPLC, and alkaline iodine Crithidia with alkaline and iodine HPLC the Crithidia always reads higher (Table 5.8).

Using HPLC biopterin derivatives can be measured as fully oxidised, dihydro and tetrahydro derivatives. Unoxidised HPLC gives a measure of fully oxidised biopterin, acid oxidation gives a value of total biopterin and alkaline oxidation gives a value of dihydrobiopterin. Results are presented in Table 5.9.

The effect of an oral dose of phenylalanine on human blood biopterin is presented in Table 5.10 and the effect of tyrosine Table 5.11.

Table 5.12 shows the level of biopterin in a cancer patient, a B12 deficient patient and a group of normals.

TABLE

TABLE 5.1 Biopterin Derivatives (ng/mg protein) Synthesised by Human Breast Tissue with Tumour and Control Tissue without Tumour. Results were Obtained by Crithidia . Oestrogen Receptor Levels (Fmol/mg protein) and Menopausal State are Reported.

Patient	Biopterin Tumour	Deriv ISD Control	Student's t	Oestrogen receptor	Menopause State
M	1.15^{+} 0.06 (6)	$2.96^{\pm}$0.11(6)	1% c>t	94.5	Post
R	1.88^{+}0.07(6)	$1.96^{\pm}$0.08(6)	10% c>t	20.4	Pre
ST	1.54^{+}0.4 (5)	$0.96^{\pm}$0.18(6)	1% t>c	22.4	Post
SH	Low	Low		197.5	Post
C	0.69^{+}0.2 (6)	Low	t>c	19.6	Pre
A	Low	Low		67.9	Post
DS	0.42^{+}0.14 (6)	Low	t>c	98.3	Post
BH	Low	Low	1% t>c	34.9	Pre
QE	0.61^{+}0.12(2)	$0.12^{\pm}$0.01(3)	1% t>c	34.9	Pre
GM	0.32^{+}0.11(2)	Low	t>c	87.9	Post
VT	0.66^{+}0.09(6)	$0.95^{\pm}$0.06(6)	1% t>c	37.4	Post
NB	1.82^{+}0.23(4)	$0.96^{\pm}$0.13(5)	1% t>c	77.8	N/A
DB	0.38^{+}0.15(4)	$0.46^{\pm}$002(4)	10% c>t	114.5	N/A
MB	1.64^{+}0.6(5)	$0.09^{\pm}$0.36(4)	NS c>t	N/A	N/A
BT	1.44^{+}0.05(5)	$1.31^{\pm}$0.06(5)	1% t>c	N/A	N/A
JH	1.5^{+}0.12(5)	$1.17^{\pm}$0.08(5)	0.1% t>c	N/A	N/A
RH	1.22^{+}0.04(5)	$1.66^{\pm}$0.02(5)	10% t>c	N/A	N/A

Numbers in brackets indicates N° of replicate assays

TABLE

TABLE 5.2 Biopterin Derivatives (ng/mg protein) Synthesised by Human Colon and Rectal Tissue by Crithidia.

Subject		Biopterin Derivatives ±SD Tumour	Control	Significance
15-1-82	Colon	0.89 ±0.06 (5)	0.23 Max (5)	T > C
MR	Rectum	0.61 ± 0.03 (2)	0.23 ± 0.02 (5)	T > C
TG	Rectum	0.64± 0.08 (5)	0.34± 0.11 (5)	1% T > C
JC	Caecum	1.97 ± 1.2 (5)	0.98 ± 0.2 (5)	1% T > C
AH	Colon	1.54 Min (6)	0.51 ± 0.9 (6)	1% T > C
HS	Colon	0.75 ± 0.16 (5)	0.68 ± 0.15 (5)	ns T > C
CJ	Colon	3.9 ± 0.48 (5)	0.38 ± 0.08 (5)	1% T > C
EM	Colon	3.52± 0.57 (5)	0.82± 9.22 (5)	1% T > C
BL	Rectum	1,21± 0.49 (5)	1.12± 0.38 (5)	ns T > C
AT	Rectum	1.5 Max (5)	0.89 ± 0.09 (5)	T > C

Statistic's Wilcoxon's sum of ranks

Max refers to the upper limit the value could be since the concentration at the dilution used was too low for the Crithidia to read.

TABLE

TABLE 5.3 Comparison of HPLC with Acid Iodine Oxidation and Crithidia between two Tumours Obtained from Human Gut and Two Portions of Control Tissue Obtained from the same Patient Incubated as 5-Replicates.

		HPLC		
	Crithidia	neopterin	biopterin	total n+B
Tumour 1	3.94 ± 0.47	1.87 ± 0.4	1.44 ± 0.5	3.32 ± 0.8
Control 1	0.37 ± 0.08	0.0	0.0	0.0
Tumour 2	between 0.1-0.5	0.0	1.08 ± 0.5	1.08 ± 0.5
Control 2	0.86 ± 0.9	0.0	0.56 ± 0.31	0.56 ± 0.3

Crithidia biopterin derivatives ng/mgprotein

neopterin + biopterin ng / mg protein by HPLC.

TABLE

TABLE 5.4 Whole Blood Biopterin (ng biopterin/ml blood) by Acid Iodine HPLC, Crithidia and by Unoxidised Crithidia for Normal Subjects.

Subject	HPLC/I_2	Crithidia/I_2	Crithidia
Barford	2.8		3.4
Heaven	2.8		4.4
Ray	3.7		
Mansell	4.1	5.0	1.6
Bennet	4.1	5.4	2.0
Control 1	2.5	4.0	0.8
control 2	3.6	4.4	1.2
Control 3	2.6	5.2	1.6
Eggar	3.18	2.0	2.5
Morar	3.5	4.0	2.0
Potter	0.29	3.8	1.6
Partridge	1.79	3.3	2.0
Hamon	1.04	7.4	3.0
Abbot	1.28	3.0	2.5
Whitburn	0.85	2.6	3.3
Mean $\pm$ SD	2.54 ± 1.22	4.1 ± 1.4	2.2 ± 0.9

Paired t test

HPLC / I_2	vss	Crithidia / I_2	$t < 1\%$
HPLC / I_2	vss	Crithidia	NS
Crithidia / I_2	vss	Crithidia	$t < 0.2\%$

Blood was prepared as described in Chapter 2.

TABLE

TABLE 5.5 A Comparison of Results for whole blood Biopterin by Crithidia, Crithidia with Acid iodine Oxidation, HPLC and HPLC Acid iodine oxidation. Crithidia Results are ng/ml blood. HPLC results are ng biopterin / ml blood.

Subject	HPLC	Crithidia	HPLC/I_2	Crithidia /I_2.
A		1.6	4.1	5.0
B	0.88	2.0	4.1	5.4
C	1.88	1.2	3.6	4.4
E	0.46	1.6	2.6	5.2
Eggar		2.5	3.18	2.0
Hamon	3.18	3.7		3.0
Morar	3.17	2.0	3.5	4.0
Surdar	3.7	2.0		4.2
Potter	0.24	1.6	0.29	3.8
Godfrey	0.41	1.6		1.7

Blood was obtained as described in Chapter 2.

Using a paired t test

HPLC	vss	HPLC / I_2	1%
HPLC	vss	Crithidia	N.S
HPLC	vss	Crithidia / I_2	better than 5% C> HPLC
Crithidia	vss	Crithidia / I_2	0.2%
Crithidia	vss	HPLC / I_2	better than 5%

TABLE

TABLE 5.6 A Comparison of Results for Whole Blood Biopterin by Crithidia, Crithidia Acid Iodine, HPLC, and HPLC Acid Iodine in Subjects Having Received Oral Doses of Phenylalanine or Tyrosine. Crithidia results are biopterin derivatives ng/ml blood and HPLC results are ng biopterin / ml blood.

Subject	HPLC	Crithidia	HPLC/I_2	Crithidia /I_2.	
Eggar	3.6	5.0	9.0	9.7	P
Hamon	4.5	6.4	5.9	10.7	P
Morar	2.3	2.0	2.3	3.4	T
Surdar	1.54	2.2	2.8	3.8	T
Godfrey	0.4	1.7	0.97	1.7	T
Partridge	0.76	2.0	1.9	1.7	starved 12hrT

P received 7.0 g phenylalanine read after 2 hrs.

T received 7.0 g tyrosine read after two hours.

Blood was obtained as outlined in Chapter 2.

TABLE

TABLE 5.7 Whole Blood Components Obtained by joint HPLC and Crithidia (ng/ml blood)

Subject	Biopterin	BH_2 & BH_4	Other Crithidia actives	
Sargent	0	1.29	0.31	
Potter	0.24	0.05	1.41	
Godfrey	0.41	0.62	0.66	T
Partridge	0.41	1.38	0.21	T
Partridge	0.69	1.11	0.20	T
C	1.88	1.72	0.8	
D	1.88	1.72	0.8	
E	0.46	2.1	2.6	
B	0.88	3.2	1.3	
Morar 0	3.1	0.45	0.6	
Morar 2	2.3	0.2	1.05	T
Surdar 2	1.25	1.59	0.76	T

Biopterin = HPLC pre, acid oxidation

BH_2 & BH_4 = HPLC post acid oxidation - HPLC pre oxidation

Other = difference btween Crithidia / I_2 and HPLC/I_2

T = 7.0 g tyrosine taken orally.

Blood was obtained as described in Chapter 2.

TABLE 5.8 Whole Blood Results by HPLC and Crithidia Without Oxidation, After Acid Iodine Oxidation and After Alkaline Iodine Oxidation.

Subject	CF	HPLC	CF/H^+I_2	$HPLC/H^+I_2$	$CF/0H^-I_2$	$HPLC/0H^-I_2$
JDB	2.6	0.7	4.8	4.2	2.0	1.2
Sue	2.8	0.21	2.6	0.85	4.6	0
Jane	2.5	0.21	3.0	1.28	5.2	0
Chris	3.0	0.21	7.4	1.04	7.1	0
Pat	2.6	0.34	4.4	0	5.2	0
DL	2.6	0.82	4.6	2.96	1.8	1.18

results ng biopterin / ml blood for HPLC

ng biopterin derivatives / ml blood for Crithidia

Blood was obtained as described in Chapter 2.

TABLE 5.9 Reduced forms of Biopterin Calculated from HPLC Results of Table 5.8

	B	BH_2	BH_4	Total
JDB	0.7	2.55	1.95	42
Sue	0.21	0	0.64	0.85
Jane	0.21	0	1.07	1.28
Chris	0.21	0	0.83	1.04
Pat	0.34	0		
DL	0.8	0.36	1.8	2.96

B = Pre acid oxidation

Total = Post acid oxidation

BH_2 = Alkaline oxidation

BH_4 = Total less B and BH_2

results ng / ml blood

TABLE

TABLE 5.10 Whole Blood Biopterin by Crithidia and, After Acid Oxidation, by HPLC in Subjects Having Received 7.0g Phenylalanine.

Values Before and 2 hrs. After.

Subject	time	Crithidia	HPLC acid oxid
Whitburn	0	1.6	0.0
Whitburn	2	9.5	4.4
Hamon	0	1.8	0.0
Hamon	2	10.0	5.0
Eggar	0	3.2	0.0
Eggar	2	5.2	0.0
Marshall	0	4.8	7.1
Marshall	2	7.0	19.4
Barford	0	3.4	2.8
Barford	2	4.7	8.0
Heaven	0	4.4	2.8
Heaven	2	7.2	0.0
Eggar	0	2.5	3.18
Eggar	2	5.0	9.0
Hamon	0	3.7	
Hamon	2	6.4	5.9

results ng biopterin / ml blood for HPLC

ng biopterin derivatives / ml blood for Crithidia

No significant difference using a paired t test

Blood was obtained as outlined in Chapter 2.

TABLE

TABLE 5.11 Whole Blood Biopterin by Crithidia and, After Acid Oxidation, by HPLC in Subjects Having Received 7.0g Tyrosine. Values before and 2 hrs. after.

Subject	Time	Crithidia	HPLC
Morar	0	2.0	3.5
Morar	2	2.0	2.3
Surdar	0	2.0	
Surdar	2	1.6	2.8
Godfrey	0	1.6	
Godfrey	2	1.7	0.97
Partridge	0	2.0	1.79
Partridge	2	2.0	1.8

Results ng biopterin / ml blood for HPLC

ng biopterin derivatives / ml blood for Crithidia

Blood obtained as described in Chapter 2.

TABLE

TABLE 5.12 Whole Blood Biopterin (ng biopterin / ml blood) by Acid Iodine Oxidation and HPLC for Normals, One Methotrexate and One B_{12} Deficient Patient.

	Biopterin	Biopterin / I_2
Normal ± SD (15)		2.54 ± 1.22
MG pre Mix	6.6	14.2
MG Post Mix	10.6	17.6
B_{12} Deficient		9.8

Blood was obtained as described in Chapter 2.

SUMMARY

a) Biopterin synthesis in breast tumours is unaffected compared to controls.

b) Biopterin synthesis in bowel tumours is elevated compared to controls.

c) Results for biopterin biosynthesis obtained by HPLC and Crithidia for bowel tumour and control tissue are similar.

d) Crithidia after acid/iodine oxidation reads higher than HPLC after acid/iodine oxidation P = 5% and both are higher than unoxidised Crithidia.

e) HPLC and Crithidia can give a breakdown of the various states of biopterin but Crithidia is also reading other pterins at the same time.

f) Tetrahydrobiopterin seems to be the principal component of whole blood.

g) Phenylalanine causes a rise in whole blood biopterin but tyrosine has no effect.

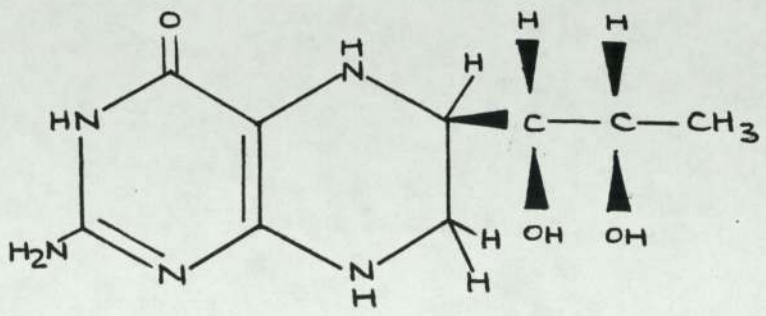
h) Methotrexate causes a rise in blood biopterin.

i) B12 deficiency causes a rise in blood biopterin.

CHAPTER 6 DISCUSSION

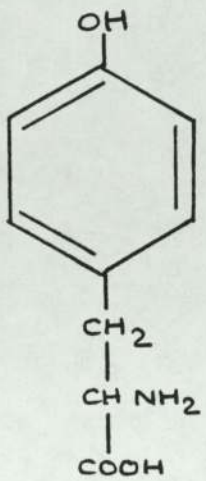
Tetrahydrobiopterin (1) is widely distributed in tissues (Rembold and Gyure 1972) and its role as cofactor in phenylalanine, tyrosine and tryptophan hydroxylases firmly established (Kaufman 1963, Lloyd and Weiner 1971, Friedman et al 1972). Tyrosine hydroxylase converts tyrosine (2) to L-dopa (3) and is rate-limiting for dopamine (4) and noradrenalin biosynthesis (5) (Levitt et al 1964, Nagatsu et al 1964). Tryptophan hydroxylase is responsible for the conversion of tryptophan (6) to 5-hydroxytryptophan (7) and is the rate-limiting step for the biosynthesis of serotonin (8) (Hosoda and Glick 1966, Friedman et al 1972). Cellular tetrahydrobiopterin levels are rate-limiting for tyrosine hydroxylase (Kettler et al 1974) and are also rate-limited for tryptophan hydroxylase although the concentration of tryptophan is the most common rate determining factor in this case (Costa and Neek 1974). Tetrahydrobiopterin concentrations have a significant effect on the production of the catecholamine neurotransmitters but could also have an effect on the entry of reducing factors into the mitochondria, (Rembold and Buff 1972) or the α-oxidation of fatty acids and consequently myelination (Stumpf 1969).

Tetrahydrobiopterin levels are maintained by two pathways. The de novo biosynthetic pathway from guanosine-5'-triphosphate (9). This has not been fully elucidated and the various proposals are laid out in the introductory chapter and by Curtius et al (1983). All are in agreement that the first step is the formation of neopterin triphosphate (10) from GTP. The second pathway is a salvage pathway regenerating tetrahydrobiopterin (1) from quinonoid dihydrobiopterin (11) formed as a result of cofactor activity in the hydroxylase reactions.



(I)

(2)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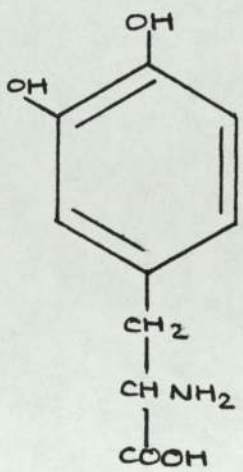
(3)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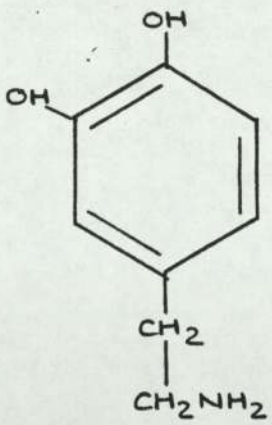
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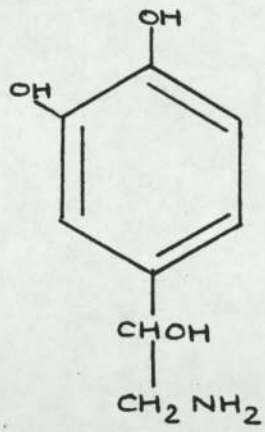
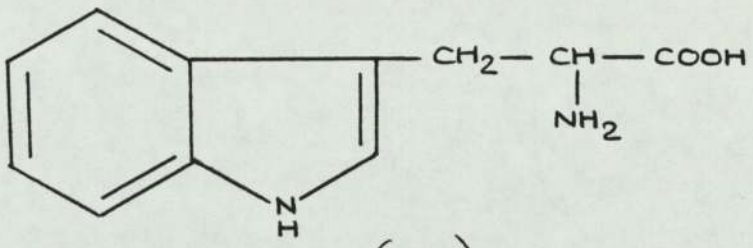
OH
CH2
CH2 NH2

(4)


OH
OH
CHOH
CH2 NH2

(5)



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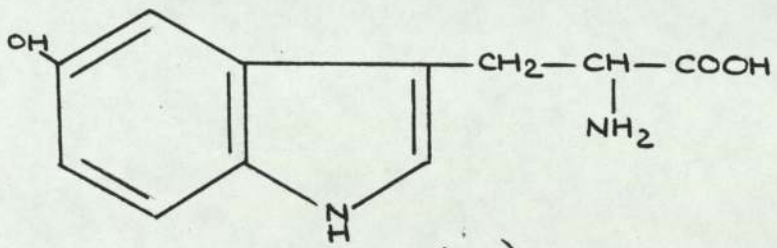

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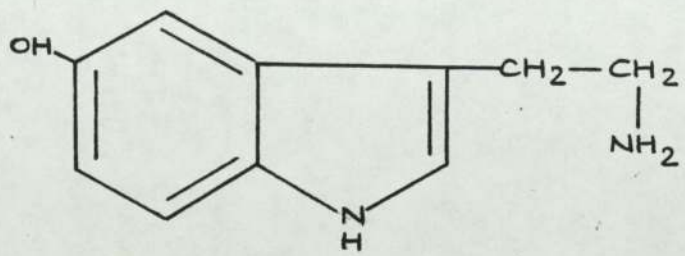

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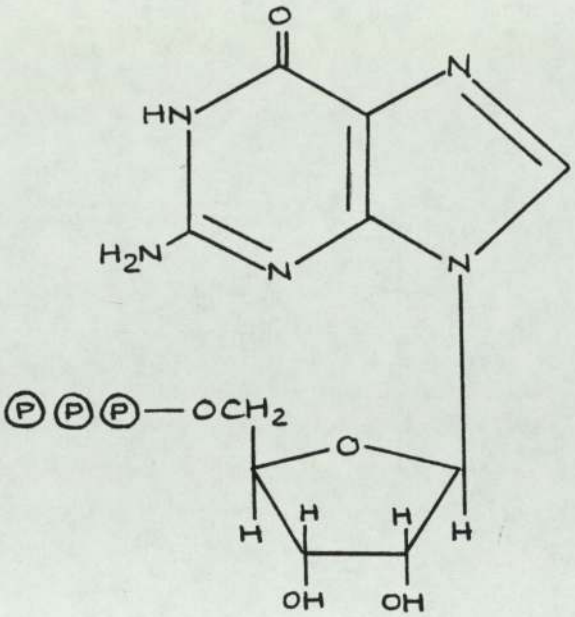

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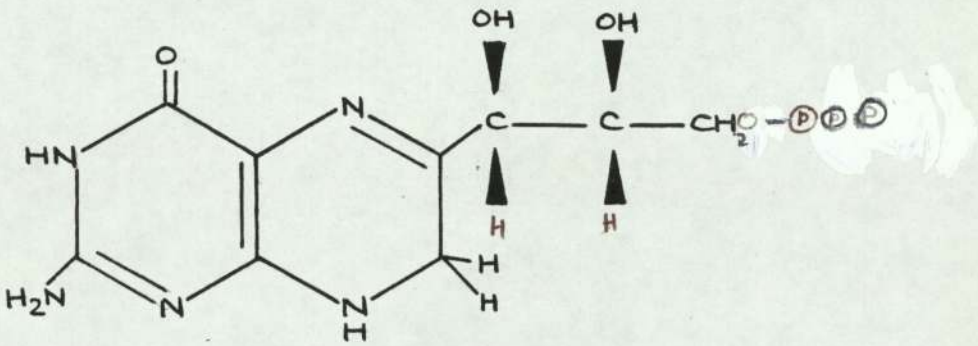
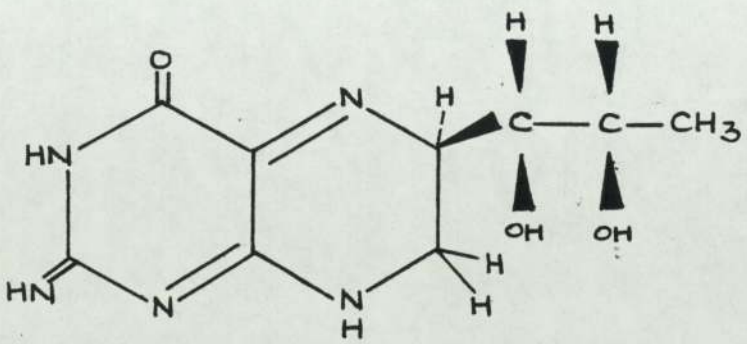




(11)

The importance of tetrahydrobiopterin is demonstrated by the severe neurological consequences which occur in malignant hyperphenylalanaemia of which two forms exist, a failure in tetrahydrobiopterin biosynthesis (Kaufman et al 1978, Leeming et al 1976) and a failure in the salvage of quinonoid dihydrobiopterin (Kaufman et al 1975).

The aim of the work presented in this thesis was to evaluate two methods for biopterin analysis, to develop an analytical techniqe for whole blood biopterin using HPLC and to investigate biopterin biosynthesis in human and rat brains, The results are compared with other published data to see if in vitro and in vivo observations could be correlated.

During the period of study two analytical techniques were used Crithida fasciculata and high performance liquid chromatography (HPLC). Both techniques have advantages. Crithidia is able to measure very low concentrations of biopterin directly and is convenient for large numbers of samples through automation. Its principal disadvantage is the destruction of tetrahydrobiopterins during the autoclaving procedure to pterin which has an almost zero growth factor activity for Crithidia (Blair et al 1963, Milstein 1983). It is also a lengthy assay. HPLC is a more rapid assay and can differentiate neopterin, biopterin, sepiapterin, dihydro and tetrahydrobiopterin in one sample where Crithidia would read them all as one. However HPLC is a much less sensitive technique. Without prior oxidation biopterin only is measured since HPLC with spectrofluorimetric detection cannot detect reduced biopterins which are non-fluorescent. Reduced biopterins are destroyed by autoclaving in the Crithidia assay. Under acid iodine conditions

reduced biopterins are oxidised to biopterin and read by both techniques. Under acid iodine conditions dihydrobiopterin is oxidised but tetrahydrobiopterin is converted to pterin.

The conversion of reduced biopterin in the autoclave in the Crithidia assay is dependent on the pH. Under acid conditions the conversion is totally to biopterin and under alkaline totally to pterin. At pH 's between these two extremes the division between the two is not clear cut and because of the inability of exact pH measurements it is not possible to estimate what proportion of tetrahydrobiopterin is converted to biopterin and apply a correction factor.

Whole blood biopterins were measured by HPLC and Crithidia. On examination it is found that Crithidia measurement on a sample which has undergone acid oxidation reads higher than an unoxidised Crithidia assay and this significant at the 0.2% level (Table 6.1). Crithidia reads higher when compared to HPLC after both have undergone acid/iodine oxidation. These observations can possibly be Crithidia reading several growth-active compounds whilst HPLC reads only one. Another contributing factor could be the purity of the two different biopterin standards used. That for Crithidia being only about 86% pure whilst the HPLC standard was of a higher purity. No significant difference is observed when Crithidia and HPLC are compared when they have not undergone oxidation (Table 6.1). The observation that with oxidised samples Crithidia reads higher than with unoxidised samples confirms the observation of Milstein (1983) that reduced biopterin is destroyed in the autoclave and during the assay.

In results obtained for whole blood possibly the best indication for the levels of total biopterin derivatives is HPLC after sample oxidation,

since this reads only biopterin and is not likely to give erroneously high results like prior oxidised samples by Crithidia. The differential oxidation of biopterin derivatives coupled with the HPLC assay is able to give differential levels of various reduced biopterins in a sample. Under acid iodine oxidation tetrahydrobiopterin and dihydrobiopterin are oxidised to biopterin whilst under alkaline oxidation, dihydrobiopterin is oxidised to biopterin whilst tetrahydrobiopterin is converted to pterin.

If table 6.1 is taken and broken down to table 6.2, reduced and oxidised biopterin, it can be seen that in most cases the majority of the biopterin in the sample is the reduced form. If the acid HPLC result is subtracted from acid oxidised Crithidia result we can obtain a value for the other pterin which Crithidia is reading, but it must be remembered that different pterins have different growth effects.

In comparison of Crithidia with HPLC under all oxidation conditions Crithidia always reads higher. (Table 6.3). The results can be broken down into the various reduced pterins by HPLC. (Table 6.4). It can be seen that the majority of the pterin is in the tetrahydrobiopterin except with JDB where dihydrobiopterin is high. In many cases dihydrobiopterin is absent. This type of calculation cannot be done with the Crithidia results since they suggest that there is more dihydrobiopterin present than total biopterin. Possibly alkaline oxidation is having an effect on other pterins as well as biopterin.

Because of the precise way that HPLC could be used to determine the level of reduced biopterin it was decided that this was the better detection technique to use. The technique is also better since it can distinguish between several pterins.

Because of the differences in results obtained by the two techniques it was decided to investigate the effect of foreign compounds on blood

TABLE 6.1 A comparison of results for whole blood biopterin by Crithidia and HPLC without oxidation and after acid iodine oxidation. Crithidia results are biopterin derivatives ng / ml blood. HPLC results are biopterin ng / ml blood.

Subject	HPLC	Crithidia	HPLC/I_2	Crithidia/I_2
A		1.6	4.1	5.0
B	0.88	2.0	4.1	5.4
C	0.7	0.8	2.5	4.0
D	1.88	1.2	3.6	4.4
E	0.46	1.6	2.6	5.2
Eggar		2.5	3.18	2.0
Hamon	3.18	3.7		3.0
Morar	3.17	2.0	3.5	4.0
Surdar	3.7	2.0		4.2
Potter	0.24	1.6	0.29	3.8
Godfrey	0.41	1.6		1.7

Blood was obtained as described in Chapter 2. Precautions were taken against oxidation. Using a paired t test.

HPLC vss Crithidia ns

HPLC vss HPLC/I_2 % (HPLC/I_2)

HPLC/I_2 vss Crithidia/I_2 better than 5% (Crithidia/I_2 > HPLC/I_2)

Crithidia vss Crithidia/I_2 0.2% Crithidia/I_2 > (Crithidia/I_2)

Crithidia vss HPIC/I_2 Better than 5% (HPLC/I_2 > Crithidia)

TABLE 6.2 Whole blood components obtained by joint HPLC and Crithidia (ng/ml blood)

Subject	Biopterin	$BH_2 + BH_4$	Other Crithidia active
C	0.7	1.8	1.5
D	1.88	1.72	0.8
E	0.46	2.1	2.6
B	0.88	3.2	1.3
Morar	3.1	0.45	0.6
Potter	0.24	0.05	1.41

Biopterin = HPLC pre acid oxidation

$BH_4 + BH_2$ = HPLC post oxid - HPLC pre oxid

Other = difference btween Crithidia / I_2 and HPLC/I_2

Blood was obtained as described in Chapter 2.

TABLE

TABLE 6.3 Whole blood results by HPLC and Crithidia without oxidation, after acid oxidation alkaline oxidation.

Subject	CF	HPLC	CF/H^+I_2	$HPLC/H^+I_2$	$CF/0H^-I_2$	$HPLC/0H^-I_2$
JDB	2.6	0.7	4.8	4.2	2.0	1.2
Sue	2.8	0.21	2.6	0.85	4.6	0
Jane	2.5	0.21	3.0	1.28	5.2	0
Chris	3.0	0.21	7.4	1.04	7.1	0
Pat	2.6	0.34	4.4	0.0	5.2	0
DL	2.6	0.82	4.6	2.96	1.8	1.18

results ng biopterin /ml blood for HPLC

ng biopterin derivatives /ml blood for Crithidia

Blood was obtained as described in Chapter 2.

TABLE 6.4 Reduced forms of biopterin calculated from HPLC results Table 6.3

	B	BH_2	BH_4	Total
JDB	0.7	2.25	1.95	4.2
Sue	0.21	0	0.64	0.85
Jane	0.21	0	1.07	1.28
Chris	0.21	0	0.83	1.04
Pat	0.34	0		
DL	0.8	0.36	1.8	2.96

B = pre acid oxidation

Total = acid oxidation

BH_2 = Alkaline oxidation

BH_4 = Total less B and BH_2

results ng/ml blood

biopterin to see if the HPLC and Crithidia results are comparable.

When a 7.0g oral dose of phenylalanine is administered to a normal adult there is a rise in biopterin derivatives as measured by Crithidia (Leeming et al 1976) when this was re-examined using the Crithidia assay without prior oxidation and total biopterin by HPLC it was found that a rise in biopterin level was the general case. Crithidia tended to give higher values but this is probably due to the presence of other Crithidia active substances which HPLC, with fluorimetric detection, does not read (Table 6.5). The rise in biopterin after phenylalanine, measured by HPLC, is significant at the 5% level using a paired t test.

The rise in biopterin in the blood after an oral dose of phenylalanine could be due to one of two things. It could be due to increased phenylalanine increasing phenylalanine hydroxylase activity with a consequent increase in biopterin biosynthesis or by phenylalanine or one of its metabolites inhibiting dihydropteridine reductase. The latter is the more likely since both phenylalanine and phenylpyruvate have been shown to be inhibitors of dihydropteridine reductase (Purdy and Blair 1980). It has also been found that the administration of phenylalanine causes no change in the level of total biopterin in rat brain but causes a shift from tetrahydro to dihydrobiopterin which is consistent with dihydropteridine reductase inhibition (Blair et al 1984).

Leeming et al (1976) had previously reported that a 7.0g dose of tyrosine has no effect on blood biopterin levels. Recently (Yamaguchi et al 1983) reported that a tyrosine load did have an effect on biopterin levels. This was repeated using both Crithidia and HPLC assays and no rise was observed with either (Table 6.6).

TABLE

TABLE 6.5 Whole blood biopterins by unoxidised Crithidia and HPLC after acid oxidation in subjects having received a 7.0g oral dose of phenylalanine. Values before and 2hrs after.

Subject	time	Crithidia	HPLC acid oxid
Whitburn	0	1.6	0.0
Whitburn	2	9.6	4.4
Hamon	0	1.8	0.0
Hamon	2	10.0	5.0
Eggar	2	3.2	0.0
Eggar	2	5.2	0.0
Marshall	0	4.8	7.1
Marshall	2	7.0	19.4
Barford	0	3.4	2.8
Barford	2	4.7	8.0
Heaven	0	4.4	2.8
Heaven	2	7.2	0.0
Eggar	0	2.5	3.18
Eggar	2	5.0	9.0
Hamon	0	3.7	N/A
Hamon	2	6.4	5.9

results ng biopterin / ml blood for HPLC

ng biopterin derivation / ml blood for Crithidia

Blood was obtained as outlined in Chapter 2.

TABLE

TABLE 6.6 Whole blood biopterins by Crithidia and HPLC after acid oxidation in subjects having received a 7.0g oral dose of tyrosine. Values before and 2hrs after.

Subject	time	Crithidia	HPLC
Morar	0	2.0	3.5.
Morar	2	2.0	2.3
Surdar	0	2.0	
Surdar	2	2.2	2.8
Godfrey	0	1.6	
Godfrey	2	1.7	0.97
Partridge	0	2.0	1.79
Partridge	2	2.0	1.8

Results ng biopterin / ml blood for HPLC

ng biopterin derivatives / ml blood for Crithidia

Blood obtained as described in Chapter 2.

The whole blood biopterin level in a patient on methotrexate therapy was found to be elevated 14.2 ng biopterin / ml blood compared to 2.54 ± 1.22 ng / ml blood (n=15) and after an administration of methotrexate this rose further to 17.6 ng biopterin / ml blood. This is in keeping with the findings of Leeming and Blair (1980) and Leeming (personal communication) that a dose of methotrexate causes a rise in biopterin and subsequent doses a further rise. Stea et al (1981) and Rokos et al (1980) have also observed this rise.

The level of biopterin was found to be elevated in a B_{12} deficient subject 9.8 ng/ml blood compared to a norm of 2.54 ± 1.22 ng/ml blood (n=15). This is contrary to the findings of Leeming et al 1976 who found that serum biopterin was depressed in pernicious anaemia.

The biosynthesis of tetrahydrobiopterin in breast tumour tissue showed no significant difference from control tissue (taken from the same breast) and no correlation could be found with oestrogen receptors or menopausal state. The level of dihydropteridine reductase was investigated in these breast tumours by Mr. Eggar and found to be elevated over control tissue (Eggar et al 1983). The level of tetrahydrobiopterin biosynthesis in bowel tumours was elevated over controls but no change in dihydropteridine reductase activity was observed. The results for bowel tumours fit in with the observations of Baker et al (1981) that the levels of biopterin were significantly raised in these tumours (Table 5.2). They suggested that it was possible that primary site tumours require extra vitamins since they also noted an elevation in eight other vitamins. This extra requirement for biopterin could be met by enhanced synthesis as in colonic tumours or by enhanced salvage as in breast tumours. This could also explain why biopterin levels are sometimes but not always elevated

in the serum of patients with neoplastic disease. With enhanced synthesis we might expect to see a rise in serum biopterin but with enhanced salvage a fall.

The urinary neopterin in patients with breast cancer has not been found to be significantly different from controls whilst in gastrointestinal cancer urinary neopterin was elevated (Rokos and Rokos 1983).

The assay conditions used for tetrahydrobiopterin biosynthesis were those of Brown (1981). The concentrations of 3m molar GTP and NADPH were found to be optimal for both rat and human brain. A pH of 8.0 was found to be optimal for rat brain but the optimum in human brain was pH 7.5. This finding has been confirmed (Anderson personal communication). The pH used throughout was 8.0, since because there was a good rate of synthesis at at pH 8.0 and for comparability of results it was thought unnecessary to change. The biosynthesis in human brain tissue was found to be linear over the whole three hour assay. The enzyme fraction was isolated in the supernatant.

Biopterin biosynthesis was examined in several regions of the brain of one subject with a wide range of synthesis being observed. The highest synthesis was found in the frontal cortex and no synthesis found in the hippocampus, putamen and globus pallidus (Table 6.7) The level of synthesis in the hippocampus could be dependent on the area taken since it is a relatively large differentiated structure (Meencke et al 1983) with several areas and synthesis in all areas may not be necessary. Synthesis may not be necessary for the hippocampus since it is not an area of serotonergic or dopaminergic origin. The hippocampus is known to be innervated by noradrenrgic neurones (Iverson 1982) and must consequently

TABLE 6.7 Biopterin biosynthesis (ng / hr / mg protein) and DHPR activity (nmol / min / mg protein) in differing brain areas (GF)

	Biosynthesis	DHPR
Temporal Cortex	1.3	328.1
frontal cortex	2.3	427.6
Hippocampus	0.0	1011.0
Putamen	0.0	538.0
Globus pallidus	0.0	863.1
Locus coeruleus	0.49	1044.0
Pineal	0.24	
Amygdala	0.35	

have noradrenergic axons within it so a level of biopterin biosynthesis may be expected. The locus coeruleus has a relativly high level of biosynthesis and this is understandable since it is a centre of noradrenergic origin (Iverson 1982). The pineal gland has been reported to synthesis biopterin in rats (Kapatos et al 1983) and this has also been found to be the case in humans.

The regional distribution of dihydropteridine reductase activity in human brain (Table 6.7) showed similarities to that of rat brain (Bullard et al 1978) with the exception of the human hippocampus which has a much higher activity. The levels in humans are, in all cases, at least tenfold higher.

The temporal lobe was chosen for investigation partly because it was fairly easily obtained and also because that it shows the most pronounced neuropathological changes in senile dementia of the Alzheimer type (Deakan 1983).

The biopterin biosynthesis in control tissue from Brodmann areas 20 and 21, which are ajacent areas of the temporal lobe, was investigated. To these can be added the results from Brodmann area 9, an area of the frontal cortex. (Anderson personal communication) (Table 6.8). The synthesis in these is fairly similar although it must be remembered that in BA 9 pH 7.5 was used and this is the optimal pH.

Control samples were also obtained from human foetus age 19-24 weeks gestation and from young children age 5 days to 7 months. These plus the samples from adult control brains made it possible to assess age effect on biopterin biosynthesis and salvage. An increase in biosynthesis

TABLE

TABLE 6.8 A comparison of biopterin biosynthesis in BA 9, 20 and 21 for human control brains (biopterin ng / hr / mg protein)

Patient	BA 9	BA 20	BA 21
C240		0.11	
C241		0.28	
C242		0.47	
C244		0.52	
C234		1.05	
C248		1.52	
C236		0.0	
C238		1.008	
C253		0.33	
C254	0.28		0.16
C256	0.43		0.24
C273	0.58		0.84
C257	0.51		
C258	0.57		
C267	0.32		
C278	0.28		
Mean $\pm$SD	0.42 $\pm$ 0.1	0.58 $\pm$ 0.5	0.41 $\pm$ 0.37

B.A9 (Anderson personal communication) pH 7.5.

with age occurred until a maximum was observed after which it tailed off. The position of the maximum and whether it has a plateau is unknown due to a shortage of samples in the mid-age group. Leeming and Blair (1980) found that serum biopterin levels increased with age, 16 yrs - 66 yrs, and were possibly looking at the rise towards a maximum. Dihydropteridine reductase was found to reach a plateau with age and apparently not decline. It was found that biopterin biosynthesis was absent in foetal brains although Leeming and Blair (1980) found biopterin to be present in foetal brain and liver age 16-20 weeks gestation at levels not dissimilar to the levels found in adults. This suggests that biopterin in the foetus is synthesised in parts of the foetus other than the brain and distributed to the brain or exported to the foetus from the mother. Serum biopterin in foetal blood from the umbilicus was found, by Leeming and Blair (1980) to be significantly higher than adult levels.

Leeming and Blair were perhaps measuring a sepiapterin derivative. Sepiapterin reads at the same level as biopterin with Crithidia and a sepiapterin derivatives occurs on the biosynthetic pathway. It is possible that in foetal brains biopterin synthesis is complete as far as a sepiapterin intermediate and that some later step is incomplete. Because of the unavailability of foetus's of an age greater than twenty four weeks the point at which foetal brain biopterin biosynthesis starts could not be determined. It has been found that tyrosine hydroxylase activity has not appeared at week 33 (Bessman et al 1977) and so the cofactor would not be needed for this. However it may be required for other functions before 33 weeks. It is interesting to note that a development of the electroencephalogram occurs with age (Rose 1976). Before twenty eight weeks the patterns are very simple with a change towards adult patterns at twenty eight weeks gestation. By birth the

normal pattern becomes well developed and the EEG pattern continues to approach that of the adult until the adult pattern is reached between eleven and fourteen years of age (Rose 1976).

In rats a similar biopterin development pattern is observed to that in humans with dihydropteridine reductase activity rising to a plateau in juvenile rats in agreement with Algeri et al (1977). There is a discrepancy in biopterin biosynthesis results suggesting it falls from a peak around birth. Kapatos et al 1983 report a peak two days prior to birth. They also found GTP cyclohydrolase, the enzyme required for the first step in biopterin biosynthesis, had two peaks of activity at two days prior to birth and ten to fifteen days after birth. Why this pattern should occur is unknown.

The effect of sex and time to postmortem was investigated to see if it had any effect on biosynthesis or reductase activity but none was found.

The addition of 5-methyltetrahydrofolate to rat brain preparation was found to enhance biopterin biosynthesis when measured by Crithidia (Leeming et al 1982). This was confirmed using HPLC and a 288% rise in synthesis was observed. The addition of 5-methyltetrahydrofolate to human control brains caused an enhancement in two out of the four samples but overall the enhancement was not significant (Table 6.9).

Because of the possible involvement of folate and because folate is known to function in conjunction with vitamin B_{12} it was thought that B_{12} might have a role in biopterin biosynthesis. It has also been reported that vitamin B_{12} deficiency can lead to neurological deficiencies (Hoey et al 1982). When 5-deoxyadenosyl cobalamin (Coenzyme

TABLE

TABLE 6.9 Effect of 5-methyltetrahydrofolate on biopterin biosynthesis. (ng/ hr / mg protein.

Brain	Age	Synthesis	Synthesis + 5MeTHF
CFC 58	5 day	0.23	0.36
CFC 96	7 month	0.21	0.44
CFC 99	6 month	0.50	0.44
C253	74 yr	0.33	0.29
Mean ± SD		0.317 ± 0.13	0.38 ± 0.07

B_{12}) was included in the assay no increase in biopterin biosynthesis was observed in two out of three samples for which results were obtained (Table 6.10). There are several possibilities as to why no effect was seen. It may be that it is not required, or that there is already sufficient vitamin in the assay system or that the form of the vitamin being supplied is wrong and the systems necessary for interconversion are absent.

To investigate vitamin B_{12} function further it was decided to see if there was any effect with the known B_{12} inhibitor nitrous oxide (Amess et al 1978, Skacell et al 1983) which functions, by oxidising Co^{I} and Co^{II} to Co^{III} (Banks et al 1968).

$$Co^{I} \text{ and } Co^{II} + N_2O \longrightarrow Co^{III} + N_2$$

It was found that when incubated under a nitrous oxide atmosphere biopterin biosynthesis ceased. It was assumed that the cobalt in vitamin B_{12} was being kept as Co^{III} by nitrous oxide. When nitrite was added to human brain assays, biopterin biosynthesis was also found to cease. The effect of nitrite on vitamin B_{12} was investigated spectrophotometrically and it was found that nitrite was capable of oxidising vitamin B_{12} and making it inactive. It is possible that vitamin B_{12} functions with 5-methyltetrahydrofolate in the acceptance of one carbon fragments.

Dementia is a largely age related phenomenon and it has been estimated that about six percent of the population aged sixty five years and over suffer from the condition. The condition is more common in females 75 years and over. (Swash 1983). Dementia is a syndrome of global impairement of mental function occurring in a patient in whom conciousness is unimpaired. Senile dementia of the Alzheimer type has

TABLE

TABLE 6.10 The effect of adenosyl cobalamin on biopterin biosynthesis (ng / hr / mg protein)

Subject	biosynthesis	biosynthesis + B_{12}
TC 99	0.5	0.5
C 254	0.16	0.24
C 256	0.24	0.11

been defined in relation to pathological rather than clinical features. The pathology of the disease is characterised by a reduction in brain weight and volume, a loss of neurones in the cortex and also in certain centrally situated nuclei, such as the basal nucleus of Meynert. Cell loss is particularly marked in the cortex of the temporal and frontal lobes. Two histological features are a high density of senile plaques and interneuronal neurofibrillary tangles. Senile plaques represent proliferated axons derived from the projecting system deep in the control nuclei of the brain and consists of multiple regenerating axons attempting to form synaptic connections with cortical neurones (Swash 1983). The plaques are particularly prominent in the hippocampus and temporal lobes. Neurones of the hippocampus and other parts of the cortex shows tangles of the neurofilaments (Swash 1983).

Two innervative systems have been reported to be disrupted in Alzheimer's disease. The cholinergic system from the nucleus basalis of Meynert and the nordrenergic system from the locus coeruleus. Wilcock et al (1983) have observed that there is a significant loss of neurones in the nucleus basalis of Meynert in Alzheimer's diseas and that this accounts for the cholinergic deficits in the cerebral cortex. Bondareff et al (1982) and Mann (1983) have reported a reduction in the number of neurones in the locus coeruleus which could result in a loss of noradrenalin in the brain.

Tetrahydrobiopterin biosynthesis was investigated in subjects with senile dementia and found to be significantly lower P=0.2% in demented brains (0.07ng biopterin/mg protein/hr n=17 against 0.66 ng/hr/mg protein). Clinically these subjects did not all present with the same dementias. Twelve were senile dementia of the Alzheimer type with characteristics plaques

and tangles two had dementia of vascular origin and three had a medium to high dementia score but no evidence of plaques and tangles (Table 6.11). Dihydropteridine reductase activity in BA 20 of all dements was not significantly different from controls. Results obtained on a smaller number of these brains are presented in a paper by Barford et al (1984). Attempts were made to correlate the results from the dements and controls with known information about age, plaques, sex, postmortem delay and neronal fallout, by Dr. Mountjoy (Addenbrooks Hospital). He found that the only correlation occurred with neuronal fallout. As the number of neurones decreased the biopterin biosynthesis increased. (Pearson correlation - 0.369 P = 0.011).

When 5-methyltetrahydrofolate was included in the assay with demented brains four out of the five assayed showed a rise in synthesis to the lower end of the control value (Table 6.12). No further effect was noted when adenosyl cobalamin was included in the assay. The observation with folate fits in with those of Sneath et al (1973) who found that mean red cell folate was low in patients with dementia. Shaw et al (1971) administered folic acid to dements with low serum folate but found no effect. Investigations of plasma and red blood cell folate indicated adequate intestinal absorption but the cerebral spinal fluid folate level rose very slowly.

The biosynthesis of biopterin in brain area BA 9. (Frontal lobe) of dements showed no reduction when compared to controls (Dements 0.74 $\pm$ 0.5 ng/hr/mg protein vss control 0.46 $\pm$ 0.12 ng/hr/mg protein (Anderson personal communication). However it was found that in dementia brain (BA 20) samples total biopterin is lower than that of the controls, and neopterin is higher. All these results indicate

TABLE

TABLE 6.11 Biopterin biosynthesis (ng / hr / mg protein) in control and demented subjects

Subject	Biopterin Synthesis ±SD
Senile dementia of Alzheimer type (n=12)	0.09 ± 0.14
Vascular dementia (n=2)	0.04 ± 0.05
Medium - high dementia score without plaques and tangles (n=3)	0.0
Control (n=8)	0.66 ± 0.48
Wilcoxon's sum of ranks	
Control vss SDAT	P = 0.2%

TABLE 6.12 Effect of 5-methyltetrahydrofolate with adenosyl-cobalamin on demented brains (BA 20) ng/ hr / mg protein.

	Incubation	Incubation + 5-MeTHF	Incubation + 5MeTHF + Vitamin B12
D50	0.02	0.37	0.26
D51	0.29	0.42	0.38
D58	0.0	0.57	0.51
D60	0.0	0.0	0.0
D61	0.0	0.64	0.57
Mean ±SD	0.06 ± 0.12	0.4 ± 0.24	0.34 ± 0.2
Control	0.66 ± 0.48		

Incubation without folate and with is not significant with Wilcoxon's sum of ranks but becomes significant at 5% when D60 is omitted.

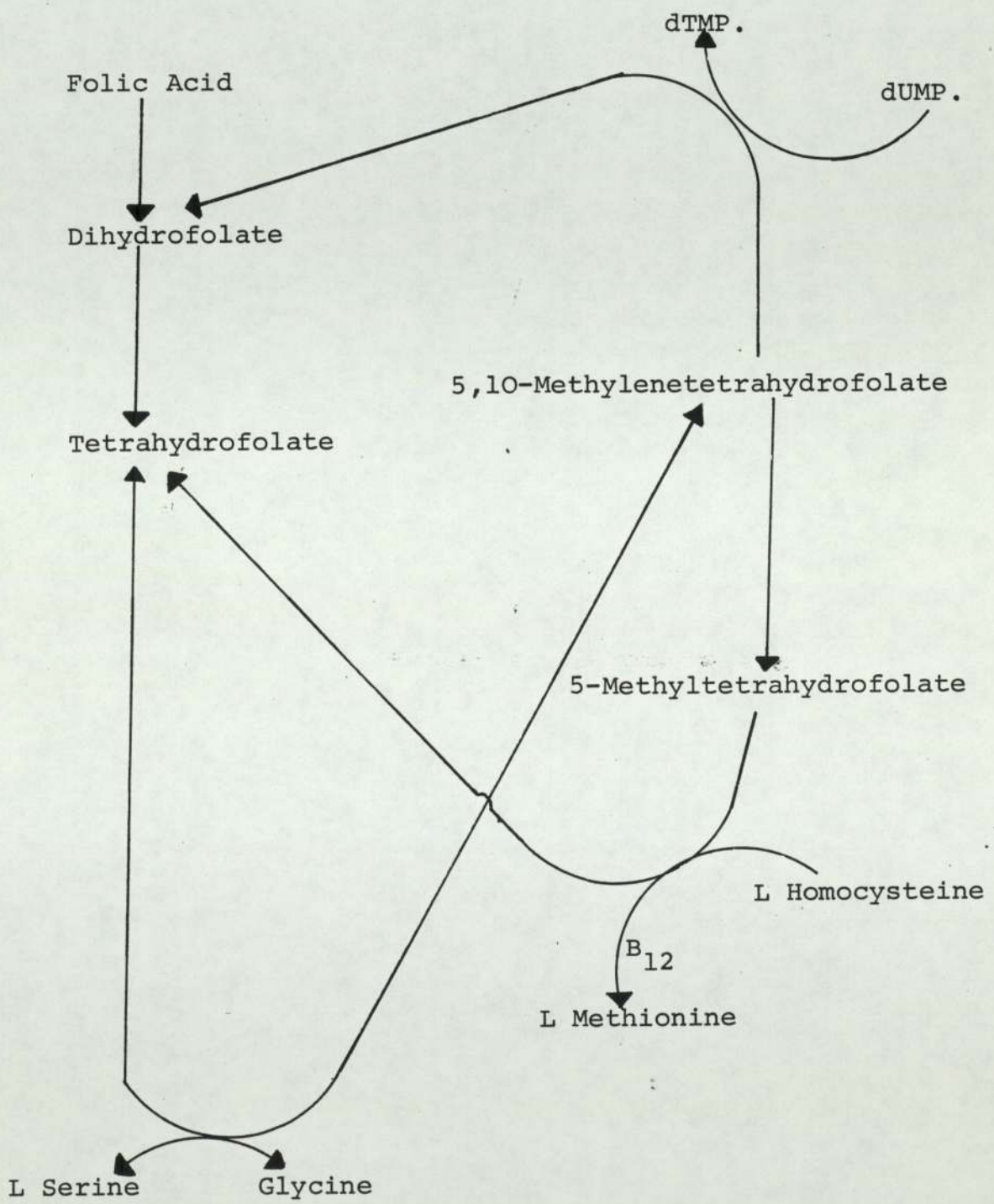
that biopterin deficiency in dementia temporal lobes is due to a failure in biopterin synthesis at some point between neopterin and biopterin and that there is no failure in dihydropteridine reductase activity. These results are in good agreement with Morar et al (1983) who have shown that biopterin in the cerebral spinal fluid of dements obtained at autopsy is significantly lower than controls.

In three subjects dying with Down's Syndrome it was found that like dements the biosynthesis of tetrahydrobiopterin (1) was vastly reduced (Table 6.13) and was significant at the 5% level. The dementia of Down's syndrome is considered to be a varient of senile dementia of the Alzheimer type with the occurrence of senile plaques and neurofibrillary tangles, and changes in the hippocampus are characteristic in both. Senile dementia, presenile dementia and Down's syndrome all have a characteristic immunosuppression with deviations from age matched normal controls (Torack and Gebel 1983).

One subject studied had methylene tetrahydrofolate reductase deficiency. This is a disorder of the folate cycle (fig.6.1) where 5,10 methylenetetrahydrofolate (12) is not converted to 5-methyltetrahydrofolate (13) due to a methylene tetrahydrofolate reductase deficiency through genetic inheritance. Clinically the subject showed mental retardation and deterioration of locomotor activity. When the biosynthesis of biopterin was examined in the temporal cortex no synthesis was found to be present. The synthesis returned to the normal range when 5-methyltetrahydrofolate was added to the assay (Table 6.14).

The recent observations that folate improves biopterin biosynthesis in rats, dements and a folate deficient child coupled with those of

Figure 6.1 The Interconversion Of 5-Methyl tetrahydrofolate, Tetrahydrofolate And 5,10-Methylenetetrahydrofolate.



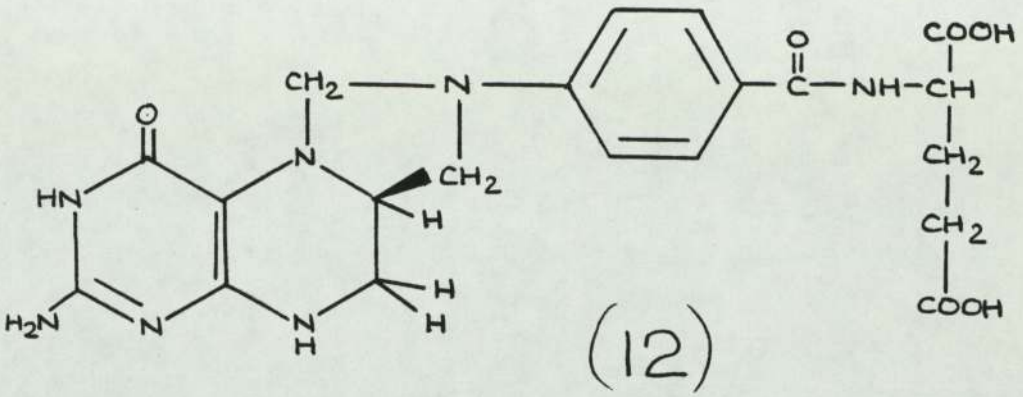
TABLE

TABLE 6.13 Biopterin biosynthesis (ng / hr / mg protein) in Down's syndrome and control patients

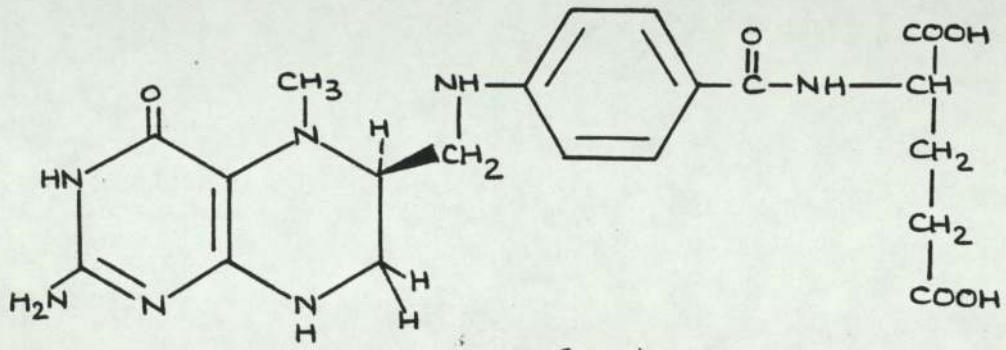
Patients	Synthesis
WH	0.11
ML	0.0
DT	0.0
Control	0.66 $\pm$ 0.48

TABLE 6.14 Synthesis of biopterin (ng / hr / mg protein) and dihydropteridine reductase activity (nmol / min / mg protein) in a patient with methylenetetrahydrofolate reductase deficiency

	Age	Biopterin synthesis	DHPR
LC	3	0.0	131.9
LC +5MeTHF		0.62	
Control	6wk-7mth	0.308 ± 0.11	90.2 ± 26.3 (n=6)
Control	44 - 89 yr	0.65 ±0 0.48	280 ± 148 (n=17)



(12)



(13)

Leeming et al (1982), who noted clinical improvement in dihydropteridine reductase deficient child receiving hydroxocobalamin and 5-methyltetrahydrofolate, suggests it is important for biopterin biosynthesis. Hase et al (1982) have reported that the administration of folic acid to a biopterin deficient subject reduced serum phenylalanine and raised serum tyrosine and serotonin. Serum biopterin activity increased four to six times after two or four weeks of folate administration.

A possible role for 5-methyltetrahydrofolate in biopterin biosynthesis could be that it is first converted to tetrahydrofolic acid (fig. 6.1) and this function as an acceptor of the 1 carbon fragment in the ring opening of guanosine-5'triphosphate during the formation of neopterin triphosphate (fig. 1.5). This does not fit in with the results of Barford et al (1984) who found the level of neopterin was elevated in the brains of dements. If the lesion were a folate requirement for the first step much lower neopterin levels might be expected. It is possible that the lesion is further on in the biosynthetic pathway and that folate is boosting the levels of synthesis is an attempt to compensate.

Depression is a neurological disorder associated with the disruption of tetrahydrobiopterin metabolism (Leeming 1979) and the prognosis of depression appears to improve as serum folate rises (Coppen and Abou-Saleh 1982). There is one reported case of improvement in endogenous depression following the administration of tetrahydrobiopterin (Levine et al 1983). A study of bioperin biosynthesis in human temporal lobe (BA 21) of depressives showed a marked reduction in biopterin biosynthesis relative to age matched controls. Dihydropteridine reductase activity seemed to be unaffected

(Table 6.15). In one of the four depressed brains the addition of 5-methyltetrahydrofolate put the synthesis into the same range as the controls whilst the addition of adenosyl cobalamin pushed the synthesis of biopterin in a second sample to the normal range. This suggests that some but not all cases of depression could be due to either a folate or vitamin B_{12} deficiency. These results also add further weight to the involvement of vitamin B_{12} and folate in biopterin biosynthesis.

Using data put forward in this thesis and coupled with our knowledge of amentia through biopterin deficiency it is possible to suggest a model for dementia. Absence of tetrahydropterin through either failure in dihydropteridine reductase or biopterin biosynthesis leads to amentia and this has been well documented (Danks et al 1978, Smith et al 1975, Kaufman et al 1978, Leeming et al 1976, Rey et al 1980). L.C. shows amentia with gross folate deficiency and this may well be a good example of amentia through lack of tetrahydrobiopterin by the failure of another cycle, in this case the folate cycle. Another example of amentia through the lack of another system is that of R.W who shows reduced blood dihydropteridine reductase activity with a genetic deficiency of cortisol and shows severe neurological impairment (Blair personal communication). Abou-Donia et al (1983) have reported the possibility of a relationship between tetrahydrobiopterin and steroid synthesis in the adrenal cortex of rats.

Dementia is possibly due to a failure of tetrahydrobiopterin metabolism causing a marked reduction in the level of noradrenalin. Mann (1983) has proposed a model for the failure of the locus coeruleus in dementia. Stimulation of the locus coeruleus chemically or electrically results in a reduction in cerebral blood flow, tissue deoxyglucose and water

TABLE 6.15 Biopterin biosynthesis (ng / hr / mg protein) in depressed patients (BA 21) with matched controls

	Synthesis	+ 5Me THF	+ 5Me THF + B_{12}
X5	0.0	0.0	0.0
X7	0.0	0.0	1.4
X8	0.03	0.21	0.12
X12	0.0	0.0	0.0
C254	0.16	0.19	0.24
C256	0.24	0.11	0.11
C273	0.84	0.28	

TABLE 6.16 The effect of diethylstilboestrol on biopterin biosynthesis (ng / hr / mg protein) and dihydropteridine reductase (n mol / min / mg protein) on two groups of weaning rats.

		Synthesis ±SD	DHPR ±SD
Control	(6)	2.02 ± 1.5	Not done
DES	(6)	0.17 ± 0.3	235.9 ± 31.2
Control	(5)	5.86 ± 4.0	131 ± 50.39
DES	(4)	1.01 ± 0.39	251 ± 127.8

TABLE 6.17 Total biopterin in oestrogen dosed and control rat brains (pmol / g wet weight)

	Control	Dosed	P
Total biopterin	289 ± 24.0	160 ± 19.5	< 0.01
% BH_4	95%	95%	

permeability of the cerebral vessels. Lesions of the noradrenalin system cause an increase in cerebral blood supply and an increase in deoxyglucose uptake. These noradrenergic cells act to maintain central nervous system homeostasis. With increasing age noradrenalin levels decrease. In Alzheimer's disease there is a loss of cells from the locus coeruleus causing loss of mediation within the noradrenergic pathway. Because of the noradrenergic contribution to brain homestasis this failure has widespread effects causing changes in the brain capilliaries which may effect the control of access of vital metabolic substances or cytotoxic substances.

It is possible that failure of the locus coeruleus in Alzheimer's disease could be due to a failure in biopterin biosynthesis giving rise to a failure in noradrenalin with resulting damage to the locus coeruleus.

Down's syndrome patients have severe neurological problems throughout life. These patients show the classical plaques and tangles of Alzheimer's disease and also reductions in noradrenalin. Although there is an overlap of pathology between the two disease states the neurological problems of Down's subjects make it difficult to assess whether they become more demented with age.

Because of the possible control of tetrahydrobiopterin in dementia agents disrupting its metabolism could have serious effects.

The administration of the synthetic oestrogen diethylstilboestrol to female weaning rats was found to significantly reduce biopterin biosynthesis compared to matched controls killed at the same time (Table 6.16). This was reflected in a fall in total brain biopterins in

some brains (Table 6.17) (Morar personal communication). In both groups 95% of the total biopterin was in reduced tetrahydro form indicating that the salvage of quinonoid dihydrobiopterin is not inhibited by oestrogens. Results on the reductase activity of some of those brains (Table 6.16) (Salihi personal communications) show a rise in activity. This is consistent with increased dihydropteridine reductase activity reported in the liver of diethylstilboestrol dosed rats (Eggar et al 1983). Decrease in total serum biopterin in menstruating women, women on the "pill" and in pregnancy (Leeming and Blair 198, Barford et al 1983) are probably explained by a reduction in tetrahydrobiopterin biosynthesis. Reduction of biopterin could well lead to a reduction of dopamine since tetrahydrobiopterin concentration is rate limiting for the tyrosine hydroxylase reaction (Lovenburg et al 1979). A reduction in dopamine levels is in keeping with the work of Bedard et al (1979) and Di Paulo et al (1982) who reported that oestrogens reduced brain dopamine levels.

The inhibition of tetrahydrobiopterin biosynthesis by diethylstilboestrol could account for the increased incidence of dementia in women in the age-groups 75-84 85-94. (Male : Female 8-36 and 3-23 per million respectively. Office of population censuses and surveys).

The effect of long term lead administration on rats gave surprising results. It was found that it increased biopterin biosynthesis in the brain. The reductase results obtained by Mr. Eggar for the samples showed a dose related decline. Possibly the biosynthesis increases to compensate for a failure of the reductase (Table 6.18) although the actual inhibition of the reductase appears to be slight. This is in keeping with the work of McIntosh et al (1984) who observed a rise in

TABLE

TABLE 6.18 Biopterin biosynthesis (ng / hr / mg protein) and dihydropteridine reductase activity (nmol / min / mg protein) in the striata of 30 days old lead dosed rats and the right 1/2 brain of lead dosed rats.

LEAD DOSE	STRIATA SYNTHESIS ± SD	STRIATA DHPR ± SD	RIGHT 1/2 SYNTHESIS ± SD	RIGHT 1/2 DHPR ± DP
0	1.18 ± 0.76	297 ± 61.5	053 ± 0.3	237.2
300	1.66 ± 0.79	267.8 ± 53.2	073 ± 0.2	231.1
1000	3.21 ± 0.63	289.4 ± 23.6	1.3 ± 0.86	220.4
2000			0.69 ± 0.47	221.9

total biopterin in the brain of rats exposed to lead in their drinking fluid for four to twelve weeks past weaning. This rise may be due to an increase in de novo synthesis or an increase in the salvage pathway. The addition of further lead to the biosynthetic assay caused a slight drop in biopterin biosynthesis and when lead is dosed orally at high levels the synthesis of biopterin is decreased. These results are an example of the non comparability of HPLC with Crithidia (Tables 4.3, 4.4 and 4.7). Reductions in blood dihydropteridine reductase has been noticed in samples taken from workers in lead industry (Blair et al 1984), and Purdy et al (1981) reported that lead was an irreversable inhibitor of dihydropteridine reductase and of biopterin biosynthesis. The fact that rats having been exposed to lead from before birth differed from rats having been exposed to lead later in life may be due to the age at which exposure occurred. It is possible that the presentation of the results are erroneous due to the use of protein as a baseline since Dhan and Banerjee (1983) reported a decrease in protein biosynthesis in the lead dosed rat although body weights were unaffected. They also found that the levels of DNA were unaffected although lead inhibited DNA activities and so the unaltered DNA level could be reflecting a decrease in DNA synthesis coupled with a decrease in DNA breakdown. Results on the concentration of DNA in lead rat brains showed an initial rise and then fell with increasing leaded dose (Table 6.19). The weight of the brains was found to decrease with increasing lead dose and this was significant (Table 6.20).

The addition of aluminium was found to increase biopterin biosynthesis in the one rat brain investigated. This may be in order to compensate for a decrease in dihydropteridine reductase activity reported by Leeming and Blair (1979).

TABLE

TABLE 6.19 DNA/g wet weight on rat brains right 1/2 dosed with lead

Dose ppm		DNA µg/g ± SD
0	(n=12)	154.5 ± 24
300	(n=12)	219.5 ± 30
1000	(n=10)	227 ± 43.2
2000	(n=10)	194.5 ± 35

TABLE 6.20 Changes in weight of rat brains with lead dosing.

Lead dose ppm		Weight in grams
0	(6)	1.43 ± 0.05
300	(6)	1.43 ± 0.05
1000	(6)	1.25 ± 0.05
2000	(6)	1.28 ± 0.09

1000 and 2000 are both significant at the 1% level with Wilcoxon's sum of ranks test

This thesis reports the development and use of an HPLC assay as a method for measuring biopterin in blood and synthesis mixtures. It has been compared with Crithidia fasciculata assay with reasonable correlation although further work needs to be done to confirm or eliminate the presence of other pterins which cause higher readings with the microbiological assay and are not detected by the HPLC method.

The results obtained from tumours are of interest in the different responses of the two main types of tumour and perhaps further tumour types should be examined.

The work with human brain has given an insight into its biopterin metabolism and shows it to be similar to that in rats. This needs to be taken further by looking at different brain areas for synthesis activity and also different neurological diseases. The involvement of folate and vitamin B_{12} needs to be examined more thoroughly to see where the two act on the synthetic path and also to see which are the most active forms of the cofactors. An idea of optimal concentrations is also needed. The work on human brain is in its early stages and must be carried much further to see if it can yield insight to brain functions and dysfunction with respect to biopterin metabolism.

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