STUDIES IN THE HANDLING AND ABSORPTION OF BIOPTERIN DERIVATIVES IN MAN

A THESIS

submitted for the degree of

Master of Philosophy

by

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in

The University of Aston in Birmingham

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SUMMARY

The Crithidia fasciculata assay of pterins in biological fluids has been developed and the parameters which affect it assessed. The effects of various pteridines on the assay demonstrated sufficient specificity to allow qualitative deductions from results. Reproducibility and recovery experiments confirmed that it was usable for quantitative analysis. The narrow range of Crithidia active pterins in man with values for serum ranging from 0.4 ng/cm^3 to 3.6 ng/cm^3 and for urine 0.34 $\mu g/cm^3$ to 6.9 $\mu g/cm^3$ suggest homeostatic maintainance of normal serum concentrations. Urinary excretion was around 1.6 mg/day and all evidence supported an endogenous source.

Tetrahydrobiopterin was poorly absorbed in man and biopterin, was better absorbed.

Concentrations of Crithidia factor in different areas of brain from different cadavers ranged from 20 ng/g to 500 ng/g wet weight and in liver from 41 ng/g to 163 ng/g. Brain levels probably reflect the role of tetrahydrobiopterin in the hydroxylation of tyrosine to dopa and tryptophan to 5-hydroxytryptophan.

Serum Crithidia factor levels in phenylketonuria were elevated as a result of hyperphenylalaninaemia. This raised level could arise either from increased synthesis or altered tissue/body fluid partition. In kidney disease, where urinary concentration is impaired, urinary levels were extremely low and serum levels significantly elevated.

Low serum values were found in leukaemia, pernicious anaemia, psoriasis, myeloma, regional enteritis and rheumatoid arthritis, all diseases in which there is alteration in the pattern of cell division. The other disease investigated in which there were low serum levels was schizophrenia.

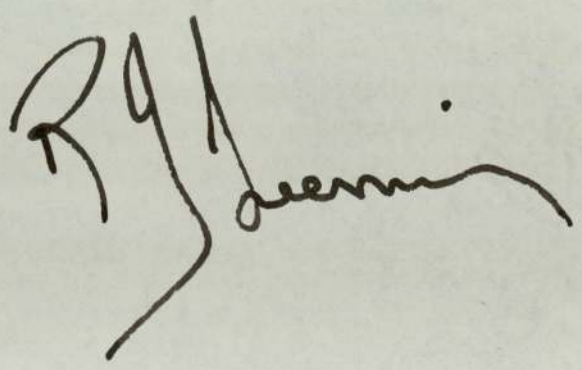
Serum values not significantly at variance with controls were found in cirrhosis, epileptics on anticonvulsants, treated Parkinsons

disease and carcinoma. The last group was small in number.

Significantly low urine levels were found in rheumatoid arthritis and controlled epilepsy but not in schizophrenia. Urinary concentrations of Crithidia factor are dependant on urinary flow which could mask small changes in output of biopterin derivatives unless timed and measured collections are made.

Raised serum levels of biopterin derivatives in man following oral and intravenous methotrexate suggest that the pteridine ring could be derived from folate if normal metabolism is blocked. Confirmation of this was given by the increase in serum Crithidia factor following folic acid and 5-formyltetrahydrofolic acid in patients on methotrexate, which contrasted to the total absence of response to folates in unmedicated subjects.

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



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CHAPTER ONE

HISTORICAL INTRODUCTION

The name pterin has been suggested for derivatives of 2-amino-4-hydroxypteridine (1) (Pfleiderer 1964) and it is in this way

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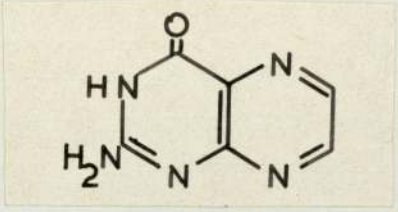

that the term is used throughout this thesis. The nomenclature used for folic acid and related folates is that given by the IUPAC-IUB Commission on biochemical nomenclature (1966). The first pterin to be isolated in a pure state was xanthopterin (2) in 1925 by Wieland and

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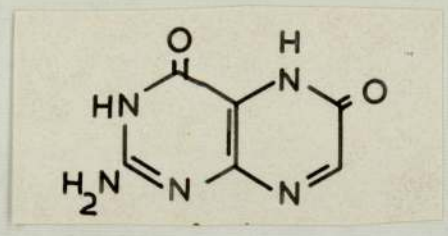

Schopf although its structure was not determined until 1940 (Purrmann). It is interesting to note that the structure of folic acid (pteroyl-L-monoglutamic acid (3) was not described until six years later (Angier,

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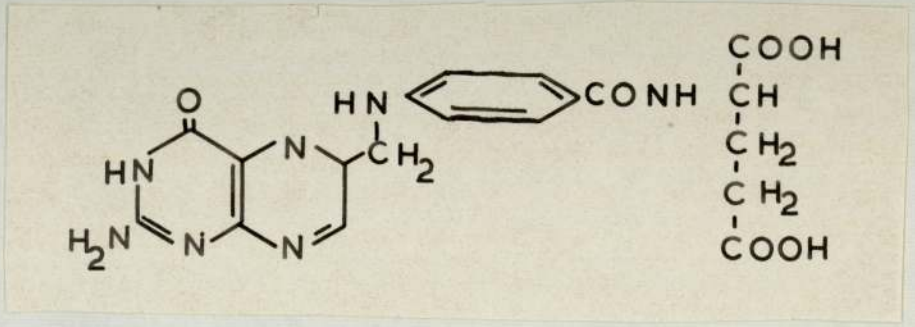

Boothe, Hutchings et al, 1946).

A great deal of work on pterins has concerned the isolation and identification of compounds from butterflies, moths and other insects (Ziegler and Harmsen, 1969). The pteridines in amphibia and fish have also excited the interest of biochemists and have been reviewed (Hama, 1963).

Biopterin (4) itself is not the commonest pterin to have been

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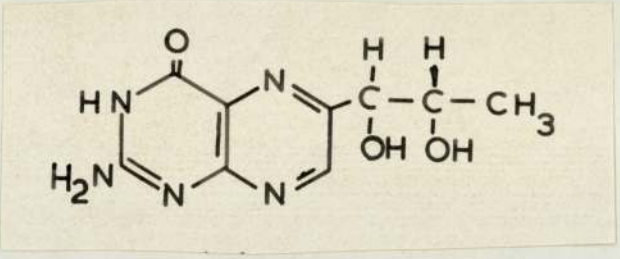

found in insects, xanthopterin (2) probably has that distinction. However, identification of pterins from natural sources presents difficulties in that many of these compounds are labile and the materials isolated could be analytical artifacts, a point made with regard to xanthopterin (2) (Koschara, 1936) and isoxanthopterin (5) (Blair, 1958)

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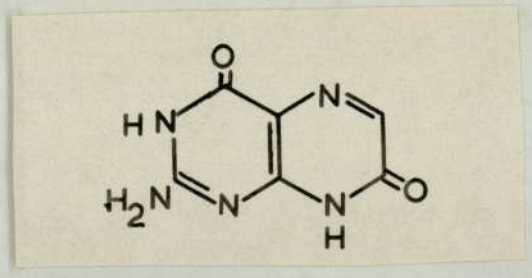

in human urine and more lately to xanthopterin (2) in Drosophila eyes by Zeigler (1961). The function of pterins in insects is not clear apart from their capacity as pigments but it has been suggested (Hama and Huruichi, 1958) that in amphibia the photolabile pterins in the retinal pigments may have a role associated with vision. The skins of fish, reptiles and amphibia contain pterins including, in some cases, biopterin (4) (Hama, 1963).

Removal and isolation of pterins from their natural sources can be achieved by a variety of methods depending on the lability and solubility of the species in question. Biopterin (4), and neopterin (6)

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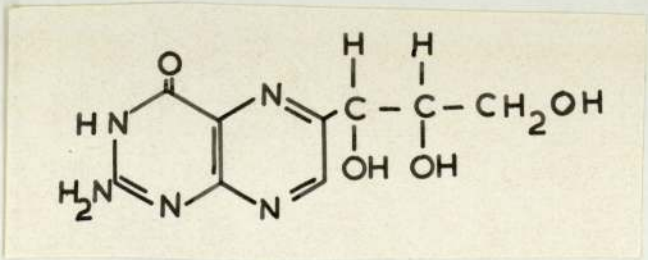

and sepiapterin (7) are readily extracted by trichloracetic acid and

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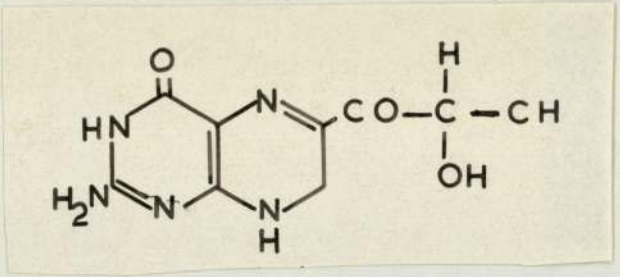

labile hydrogenated forms are stabilized under the acidic condition. Acid extraction cannot be used for isoxanthopterin (5) and leucopterin (8), if these are complexed with protein then basic solvents can be

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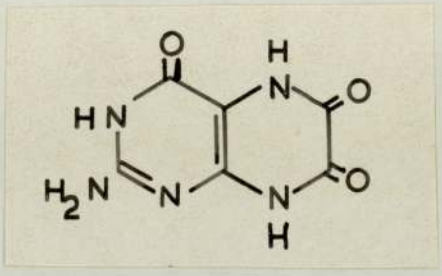

used with the addition of mercaptoethanol to prevent degradation (Lagowski and Forrest, 1967). Identification can be carried out using mass spectroscopy, nuclear magnetic resonance, ultra-violet absorption spectra, chromatography and Crithidia growth tests (Dewey and Kidder, 1971).

Pterins have been isolated from human urine (Patterson, Von Saltza and Stokstad, 1956) and measured in mammalian blood and serum (Frank and Baker, 1963), human urine (Fukushima and Shiota, 1972) in rat urine (Pabst and Rembold, 1966) and in human and rat fluids and tissues (Baker, Frank, Bacchi and Hutner, 1974). One of the biochemical characteristics of pteridines is their presence in tetrahydro and dihydro forms, these derivatives are active as specific reductants and participate in electron transfer in mitochondria (Rembold and Buff, 1972). Because these reduced forms are so reactive (Blair and Pearson, 1974) it is difficult to identify materials in tissues with precision, any extraction technique has to combine efficiency of extraction with protection of labile and reactive compounds. Biopterin identified in mammalian tissues and body fluids most probably had its origins in

tetrahydro and dihydro forms as the oxidation of tetrahydrobiopterin (9)

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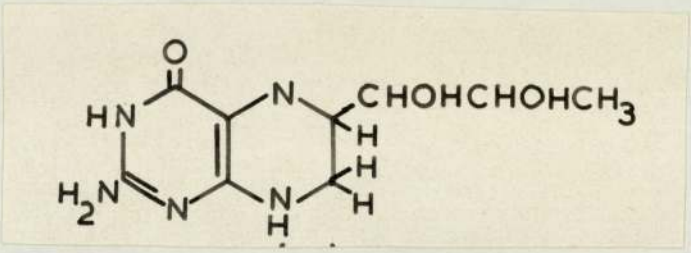

proceeds rapidly to 7,8,dihydrobiopterin (10) and then to biopterin (4)

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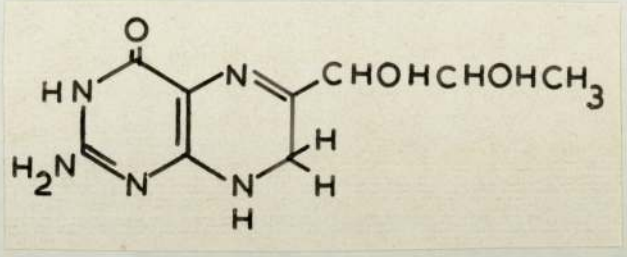

(Rembold, Metzger and Gutensohn, 1971; Blair and Pearson, 1974). A role has been given for tetrahydrobiopterin as cofactor in the enzymatic hydroxylation of phenylalanine (11) to tyrosine (12) (Kaufman, 1963)

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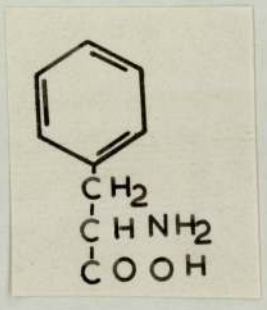

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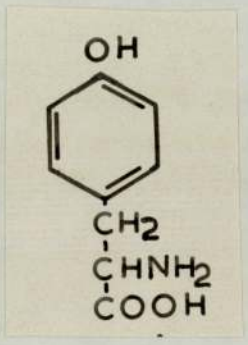

and the further hydroxylation of tyrosine (12) to dopa (13), this

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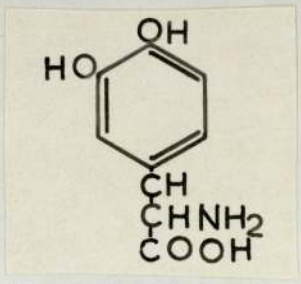
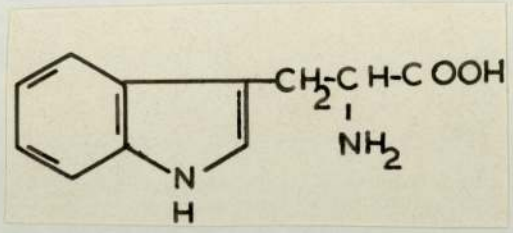


latter is the rate limiting step in catecholamine biosynthesis (Levitt, Spector, Sjoerdsma and Udenfriend, 1965). Tetrahydrobiopterin (9) has been shown to enhance tyrosine (12) hydroxylation in rat striatum (Kettler, Bartholini and Pletscher, 1974) which suggests that cofactor concentration at the enzyme site could be limiting. These same workers showed that intravenous administration of tetrahydrobiopterin did not

give a rise in striatal tetrahydrobiopterin which suggests rapid assimilation to some other site or an effective blood-brain barrier. Rembold and Metzger (1967) showed that orally administered 8a-^{14}C-biopterin was absorbed and retained in the tissues of rats.

A less well documented reaction, apparently requiring reduced pteridine cofactor is tryptophan (14) to 5-hydroxytryptophan (15)



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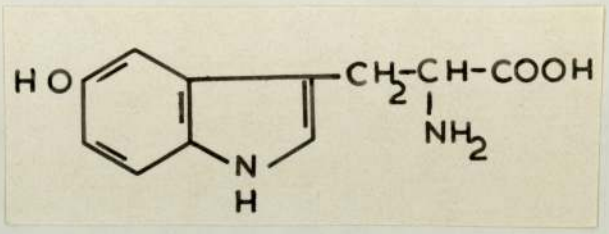

(15)

(Hosoda and Glick, 1966). In view of reports that tetrahydropterins can act as nonspecific hydroxylation agents for aromatic amino acids (Coulson, Powers and Japson, 1970; Woolf, Jakobovic and Chan-Henry, 1971) and that reduced pteridines can protect enzymes from inactivation and therefore indirectly catalyze reactions (Zamoni, Brown and La Du, 1963) it would be wise to await validation of less well characterized reactions.

The dietary sources of folates and their absorption have been the subject of a great deal of research. Butterworth, Santini and Frommeyer (1963) analysed a number of diets and found that 50 per cent was 10-formylfolic acid (16) 34 per cent 5-formyltetrahydrofolic acid (17)

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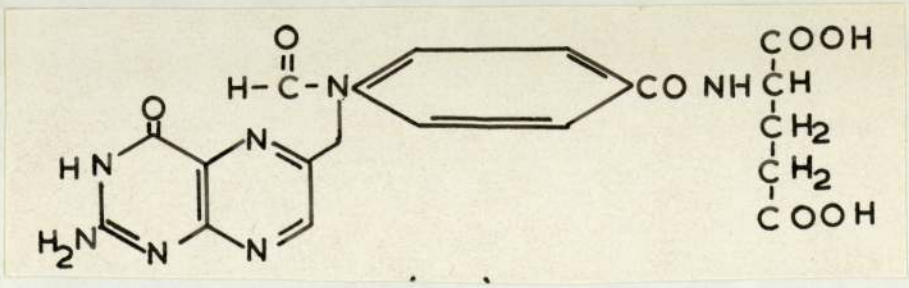

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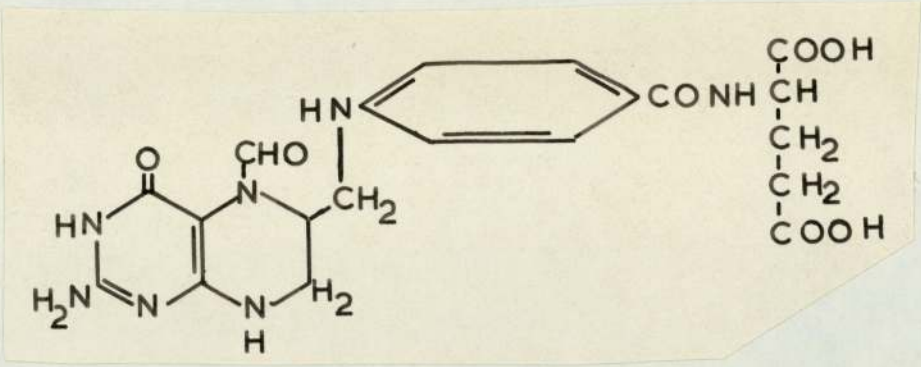

and 11 per cent pteroylglutamic acid (3). Chanarin, Rothman, Perry and Stratfull (1968) carried out differential microbiological assays with Streptococcus faecalis and Lactobacillus casei on diets collected into ascorbate buffer to prevent oxidation and added the refinement of treatment with chicken pancreas conugase. Their conclusions, based on the non-availability to microbiological analysis of 75 per cent of food folate without pre-treatment with enzyme, was that the larger part of food folate was heptaglutamate (18). The absorption of folates has

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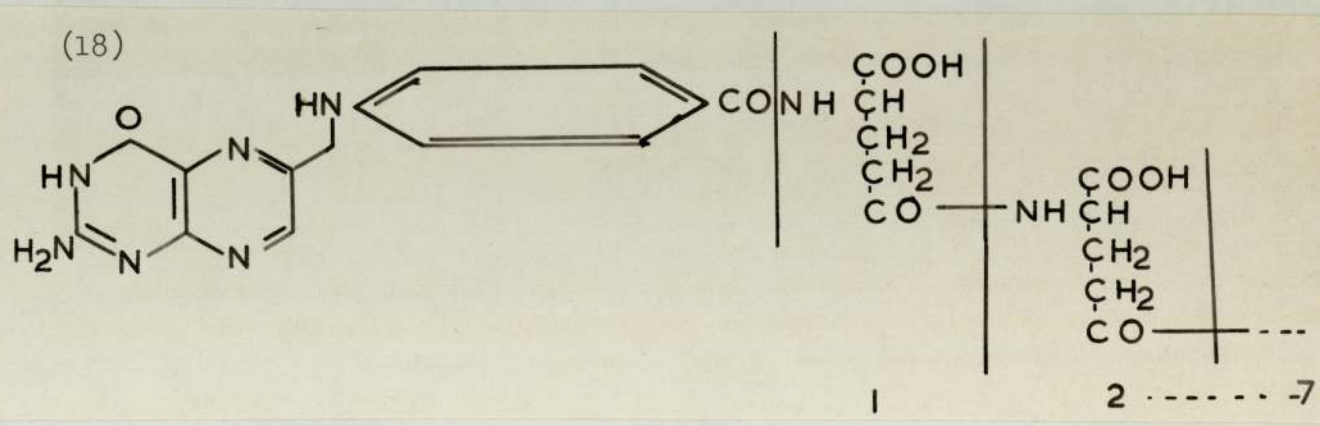


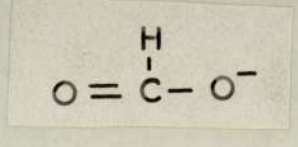
been fully reviewed (Blair and Matty, 1974) but there is still divergence of opinion about the mechanism.

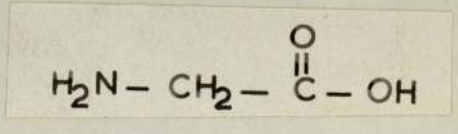
The dietary sources of pterins are potentially great, but experiments in have shown tetrahydrobiopterin to be poorly absorbed in man (Blair, Ratanasthien and Leeming, 1974) and rats although if administered parentally it is retained (Stone, 1974). Biopterin is more readily absorbed and bound to tissue (Rembold and Metzger, 1967)

but only the reduced forms are known to have metabolic activity. The remaining possible source of reduced pterins is through biosynthesis, either from other pteridines (derived from the diet) or from substances structurally at a greater distance.

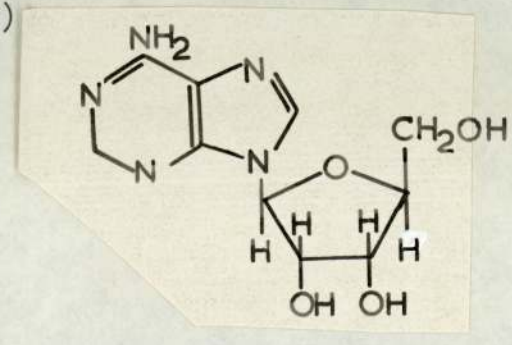
Pabst and Rembold (1966) established that biopterin (4) excreted in the urine of the rat was constant at about 30 μg per day even when several generations were fed on a biopterin free diet. This is fairly conclusive evidence of biopterin formation in a mammal which is incapable of forming folic acid and is therefore a likely model for human biopterin biosynthesis, although a note of caution must be added when transposing data from one species to another. As well as the more familiar physiological differences in the gastro-intestinal tract, rats have enzymatic differences - for example sepiapterin reductase in both erythrocytes and liver is much greater than in several other species (Katoh, Arai, Taketani and Yamanda, 1974).

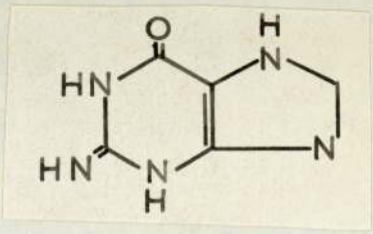
The injection or feeding of purine precursors such as formate (19) and glycine (20) or possible pteridine precursors such as

(19) 

(20) 

adenosine (21), guanine (22), guanosine (23 or guanylic acid (24)

(21) 

(22) 

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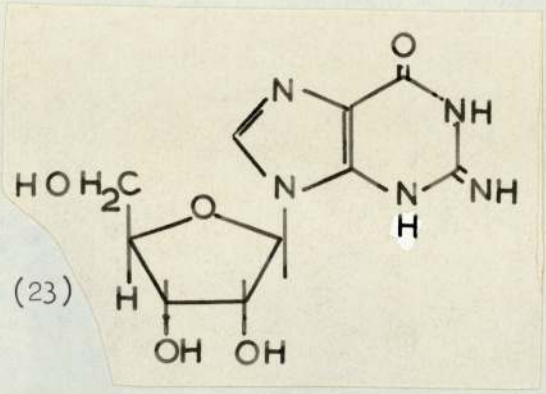

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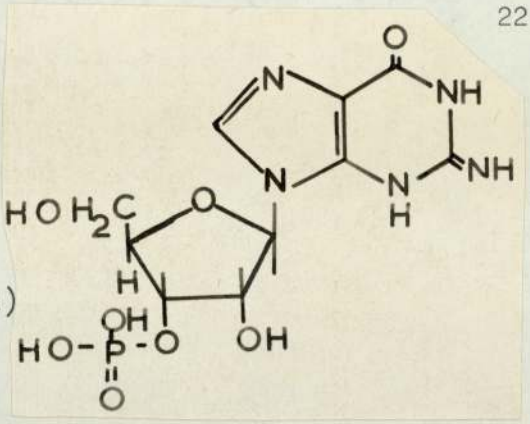

produced only slight rises in biopterin (4) in the rat. The formation of biopterin was somewhat increased when nucleic acid synthesis was inhibited by an injection of actinomycin D and the accompanying flow of purine precursors into nucleic acid biosynthesis was decreased (Rembold and Gyure, 1972).

When Fukishima (1970) injected radioactive dihydroneopterin into tadpoles there was a very low level of incorporation of the label into biopterin. However, Rembold, Chandrashekar and Sudershan (1971) could not detect any radioactivity in excreted biopterin within four days of injecting radioactive neopterin (6) and tetrahydroneopterin (25)

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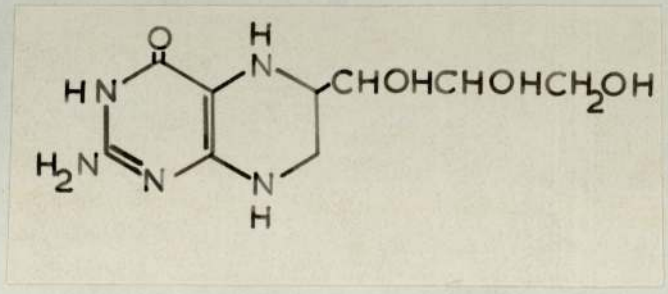

into rats.

If folates were the source of biopterin one would expect low folate status to be accompanied by reduced tissue levels and excretion of biopterin and its derivatives. By the same argument administration of folate should increase biopterin levels unless there is a homeostatic mechanism which maintains a constant level. It has already been established that oral administration of large amounts of folic acid do not effect urinary pterin concentrations (Fukishima and Shiota, 1972) but there is no information on serum and tissue levels.

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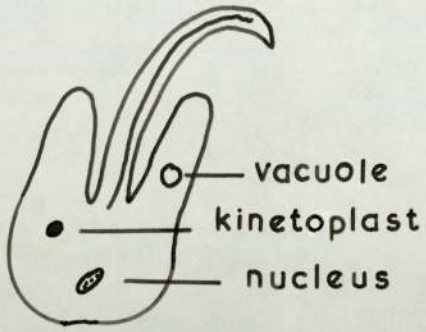
CHAPTER TWO

MICROBIOLOGICAL ASSAY OF BIOPTERIN DERIVATIVES

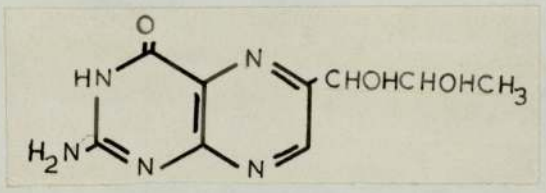
INTRODUCTION.

Members of the genus Crithidia were first described by Leger (1902) and are monoxenous insect parasites ranging from 4 to 10 μm in length.



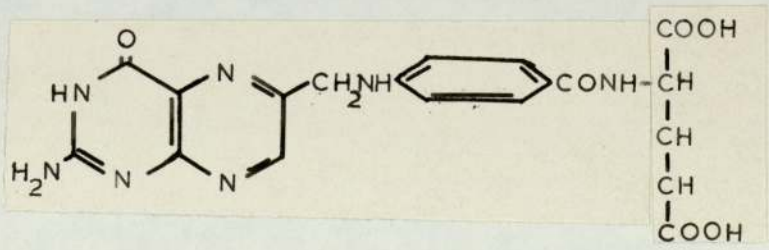
The first chemically defined medium for Crithidia fasciculata was given by Cowperthwaite, Weber, Packer and Hutner (1953), who described the extremely high folic acid requirement. Subsequently this requirement was shown to be spared by biopterin (1) (Patterson, Broquist,

(1)



Albrecht, Von Saltza and Stokstad, 1955). Folic acid (2) gives enhanced

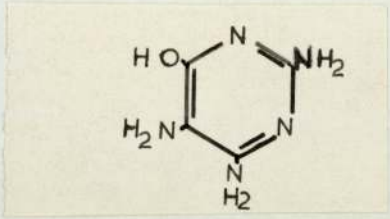
(2)



growth even in the presence of biopterin and in media in which folic acid is the sole source of pteridine Crithidia factor is produced from folic acid (Guttman and Wallace 1964). Under some circumstances C. fasciculata will synthesise Crithidia factor in a medium containing a

carbohydrate substrate such as sorbitol and 2,4,5-triamino-6-hydroxy-pyrimidine (3) together with either L-rhamnose, D-mannose, D-galactose,

(3)



gluconolactose, sucrose or glucose (Nathan and Funk 1959). Crithidia fasciculata will grow well at pH 3.8 - 6.3 in a defined medium with the precipitation of haemin not appearing to hinder growth (Tamburro and Hutner 1971). However, measurement of growth using turbidometric methods is not easy when there is a background of precipitated haemin. The addition of a carbohydrate permits growth up to pH 8.2 (Nathan and Cowperthwaite 1955), therefore, the medium was prepared at pH 7.5 and buffered as described later.

CULTURE MEDIA

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, autoclavedat 120°C for 15 minutes and storedin the refrigerator at 4°C.

Stock Assay Medium

Part A.

L-arginine hydrochloride	5.0	grams
L-glutamic acid	10.0	
L-histinine 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 ($Mn\ SO_4 H_2O$)	1.4	
Magnesium sulphate ($Mg\ SO_4 7H_2O$)	6.5	
Tri-potassium phosphate ($K_3\ PO_4$)	1.5	
Zinc sulphate ($2_2SO_4 7H_1O$)	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.

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 $4^{\circ}C$ in the dark.

Part C.

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

Part D.

Folic acid 100 ng/ml - freshly prepared.

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.

METHODS AND RESULTS

Maintenance of Culture and Preparation of Inoculum

A stock culture of Crithidia fasciculata - American Type Culture Collection No. 12857 was obtained from Dr. S. H. Hutner of Haskins Laboratory, New York. This was subcultured every week into maintenance medium, incubated for two days at 29°C in the dark and then placed in the refrigerator. The addition of 1 mg/ml ampicillin when inoculating assisted in preventing contamination without affecting the growth of the flagellate. Maintenance medium containing 8% glycerol was also inoculated and, after growth had occurred, placed at -20°C, cultures stored in this way remained viable for up to six months.

The inoculum was prepared by adding 1 drop of a two day culture aseptically to 15 ml of single strength assay medium and incubating at 29°C for four days to exhaust the pteridines in the flagellates (Dewey and Kidder 1971). 0.2 ml of the resultant growth was added to 20 ml of single strength assay medium and this constituted the inoculum. One drop of the inoculum was added to each assay tube using a sterile Pasteur pipette, the presence of 500 mg ampicillin in the 20 ml inoculum was found to be advantageous.

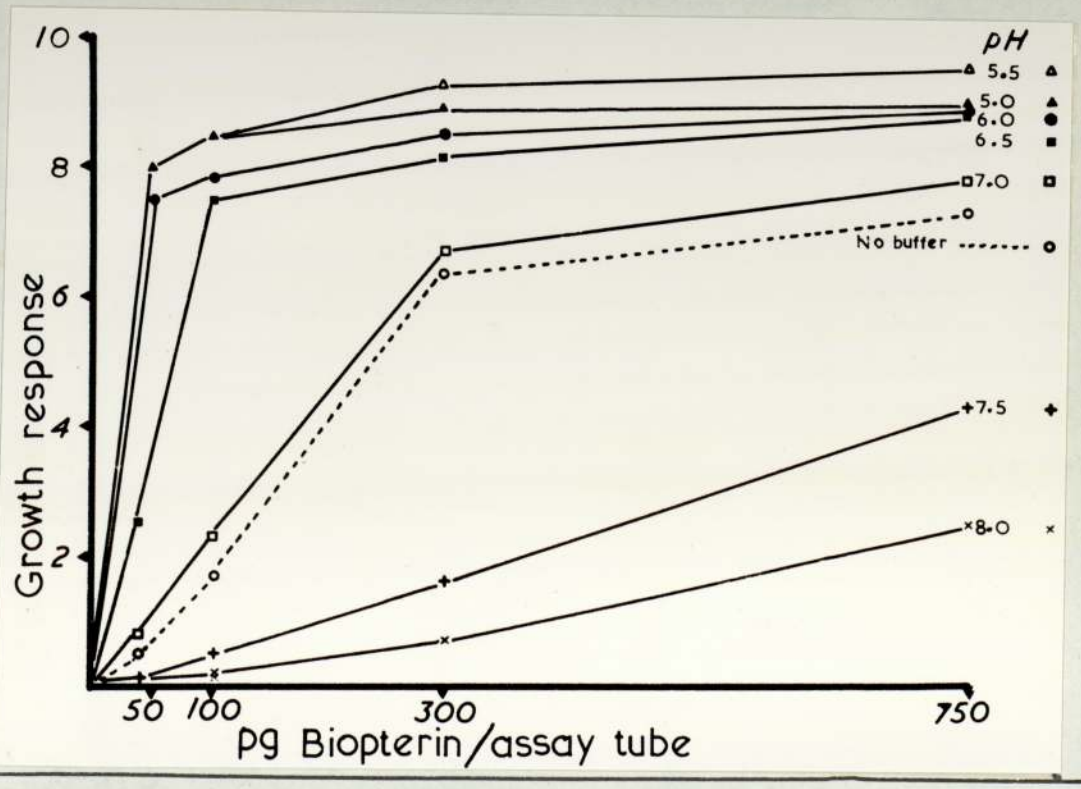
The Effects of Buffers on Growth Response.

The wide range of pH values at which Crithidia fasciculata grows prompted the optimising of the initial pH of assay medium and buffering the acidity produced to prevent precipitation of haemin.

0.2 M phosphate buffers (Sorenson's) were prepared ranging from pH 5.0 to pH 8.0 at 0.5 intervals. 0.5 ml amounts were made to 4.0 ml with double strength assay medium, distilled water and increasing concentrations of biopterin. The resultant growth curves, compared with the total absence of added buffer, are given in Figure 2-1. There was little difference between growth responses in the presence of buffers of pH 5.0 and pH 5.5 over the most sensitive part of the curve (below 0.1 mg biopterin/4.0 ml), whilst increasing the pH decreased growth to where buffers of greater than pH 7.0 were inhibitory when compared with distilled water.

Fig. 2-1

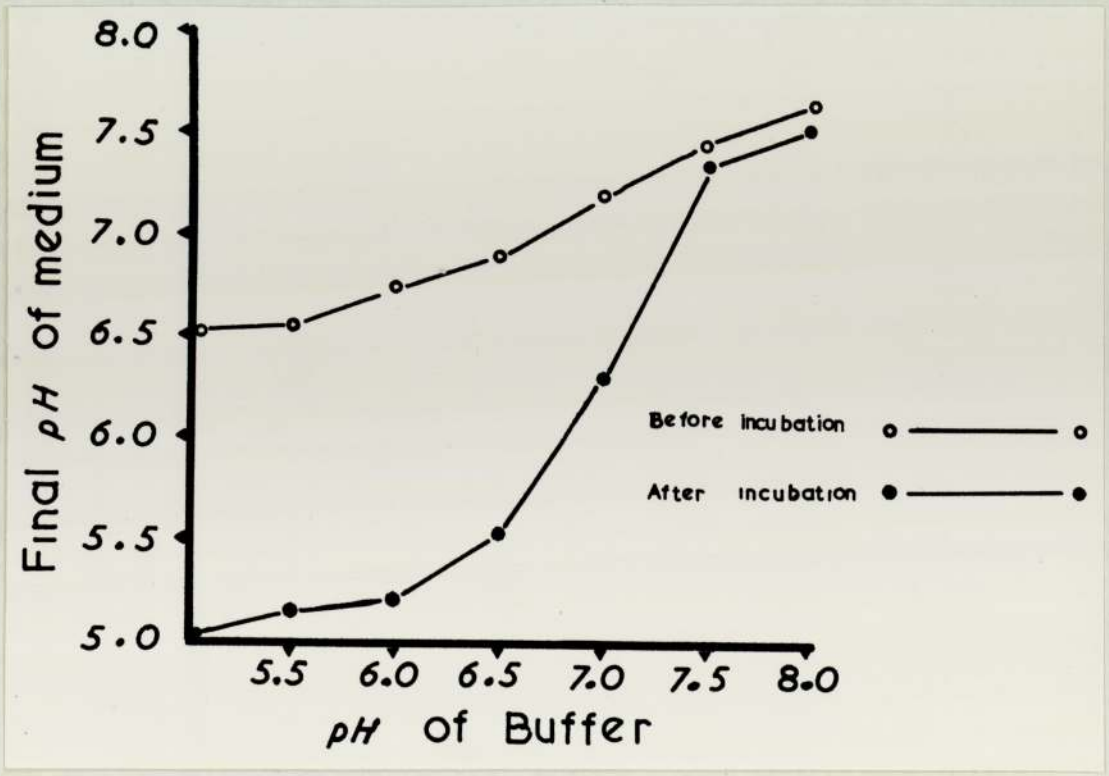
Growth response of Crithidia fasciculata to biopterin in the presence of 0.5 cm^3 0.2 M phosphate buffer/4 cm^3 assay medium.
Buffers from pH 5.0 to pH 8.0 were used.



The effect of the addition of buffers on the initial pH of the assay medium is shown in Figure 2–2. It can be seen that the final pH produced by Crithidia fasciculata in the unbuffered situation was lower than that produced even with the use of pH 5.0 phosphate buffer although its growth was shown to be less (Figure 2–1). It must be assumed that enhanced growth, where it did occur, was achieved by restricting the pH movement to an optimum range during the incubation period.

Fig. 2–2

The effects of adding 0.2 M phosphate buffers of given pH to assay medium on the pH values before and after incubation with Crithidia fasciculata.



0.2 M acetate buffers (Figure 2–3) of pH 5.0 and 5.5 did not approach the growth enhancing effect of phosphate buffers with the same degree of acidity. Buffers of pH 3.5 and pH 4.0 completely inhibited growth, this inhibition was probably due to incompatibility of buffer

and medium or buffer and test organism as Crithidia fasciculata will grow well at pH 3.8 in a defined medium (Tamburro and Hutner 1971).

Fig. 2-3

Growth response of Crithidia fasciculata to biopterin in the presence of 0.5 ml 0.2 M acetate buffer/4 cm^3 assay medium.

Buffers from pH 3.5 to 5.5 were used.

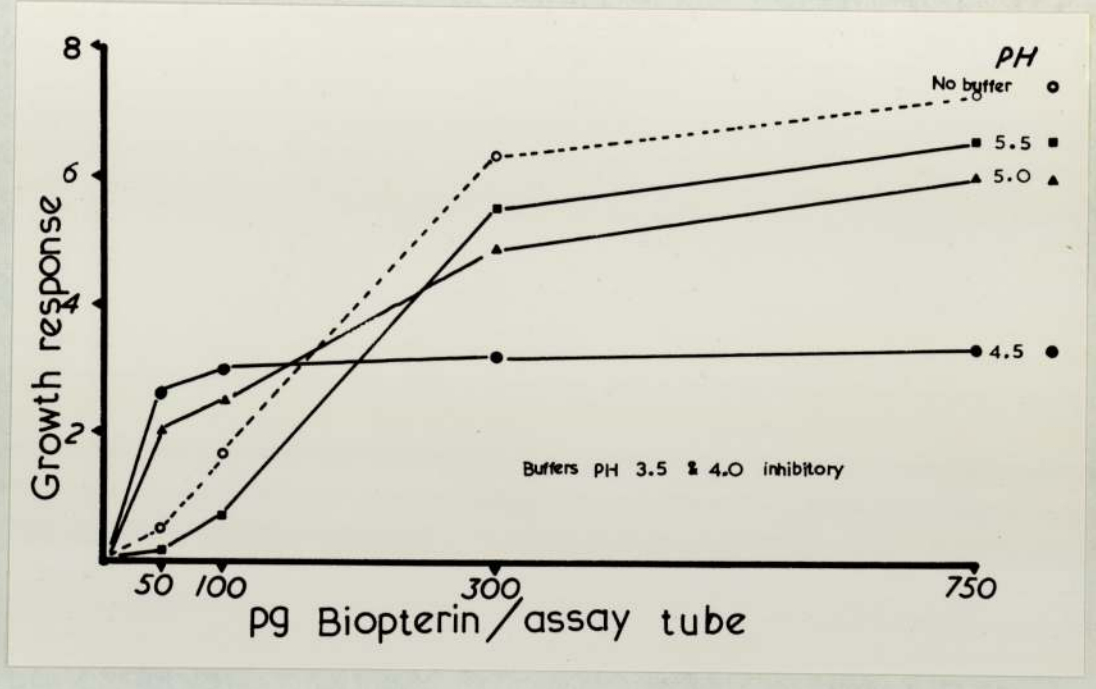
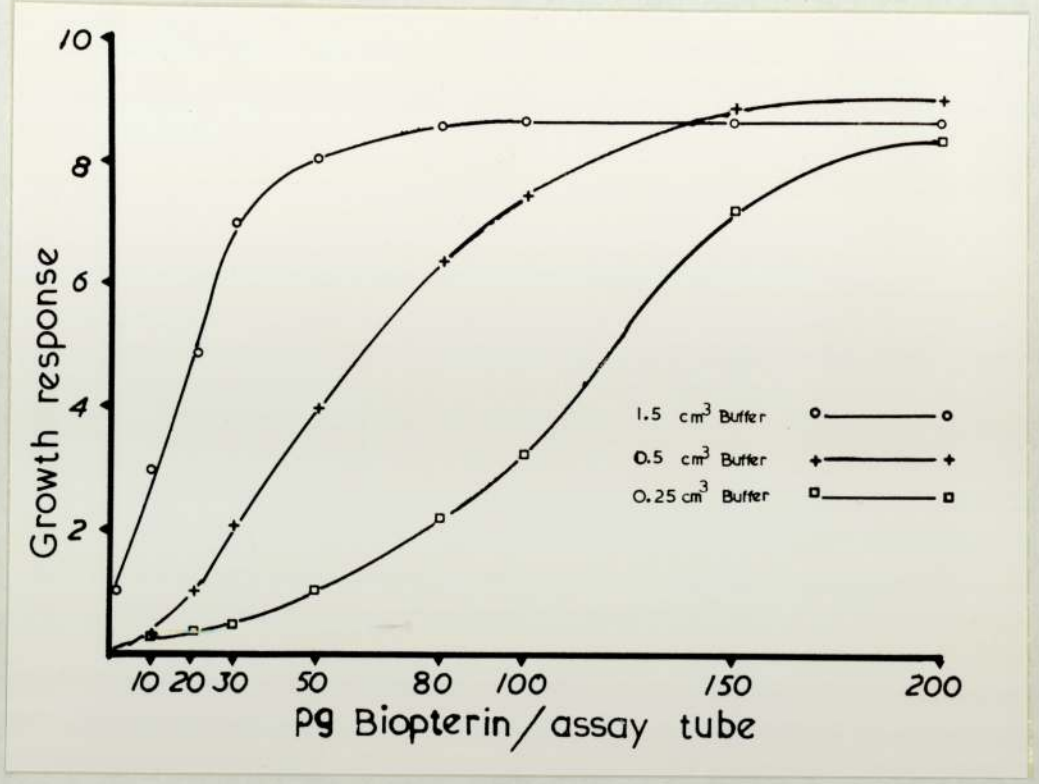


Figure 2-4 shows the growth response enhanced by increasing concentrations of 0.2 M phosphate buffer pH 5.0. The baseline was raised with 1.5 ml, because of this and the fact that 0.5 ml gave a wider effective range whilst retaining sensitivity at the lower end, the latter was chosen as a standard amount for adding to the assay tubes. The initial pH of the medium now became pH 6.5, at this level there was little or no interference from haemin in measuring growth response turbidometrically.

Fig. 2-4

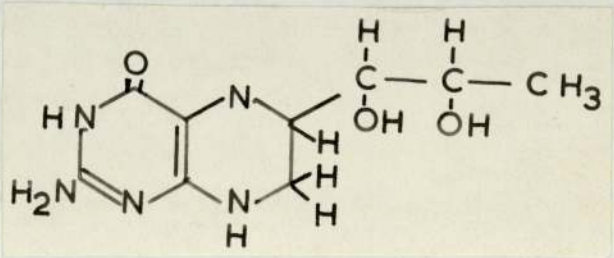
Growth response of Crithidia fasciculata to biopterin in the presence of differing amounts of 0.2 M phosphate buffer pH 5.0.



The Effects of Phosphate Buffer on Response to Tetrahydrobiopterin

Assuming that biopterin isolated from biological materials was in many cases an artifact arising from reduced forms, the effects of the buffer to be used for preparative work on tissue and biological fluids were of great importance. Tetrahydrobiopterin (4) was tested

(4)



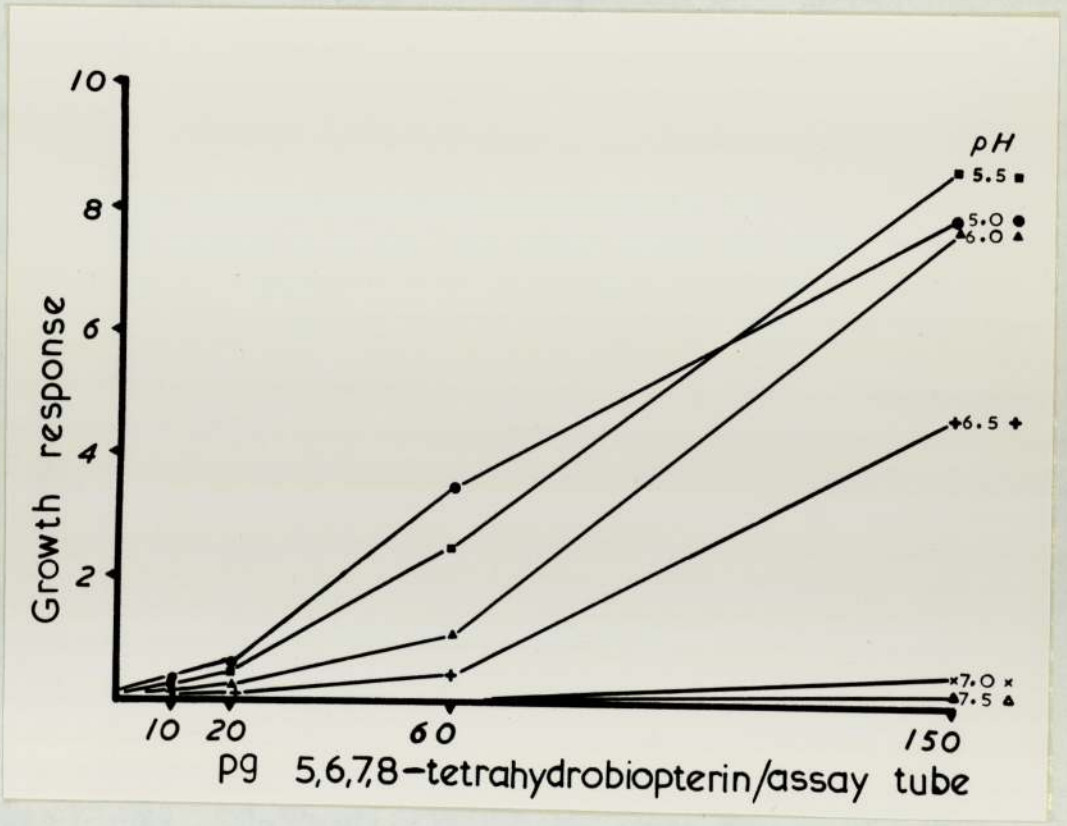
in a similar way to that described (above) for biopterin using 0.2 M phosphate buffers ranging from pH 5.0 to pH 8.0. The resultant growth curves are shown in Figure 2-5. Maximum response was obtained with

buffers pH 5.0 and pH 5.5, it was decided to uniformly use 0.2 M phosphate buffer of pH 5.0 at a concentration of 0.5 cm^3/4 cm^3 total volume when assaying any material.

Fig. 2-5

Growth response of Crithidia fasciculata to 5,6,7,8-tetrahydrobiopterin in the presence of 0.5 cm^3 0.2 M phosphate buffer/4 cm^3 assay medium. Buffers from pH 5.0 to 8.0 were used.

FOOT NOTE: Buffer pH 8.0 inhibited growth.



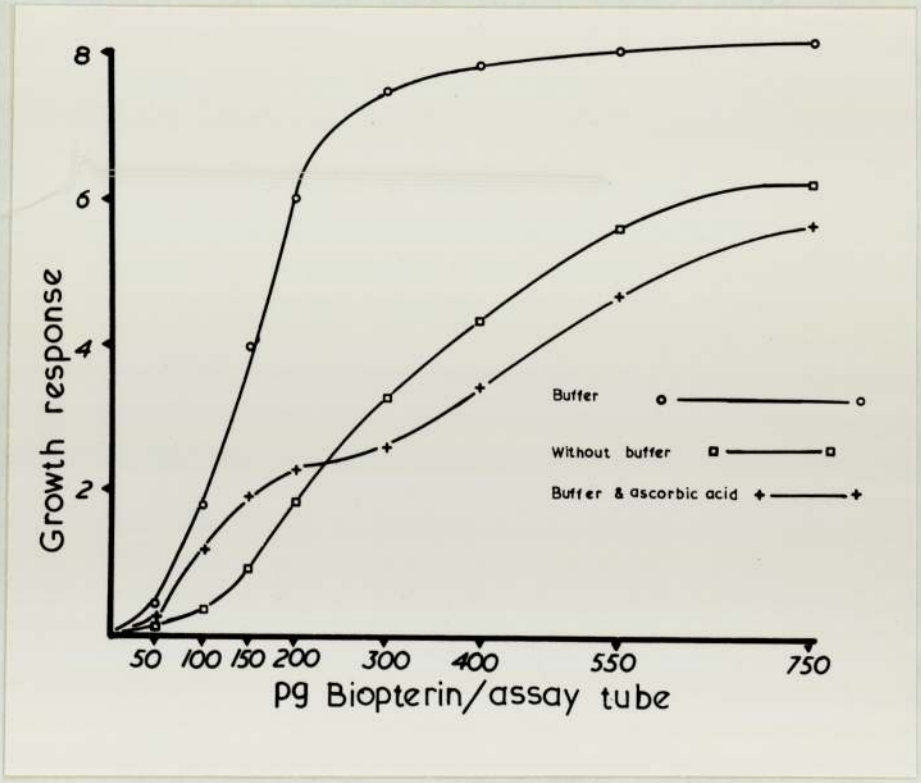
Ascorbic Acid and Growth Response

Ascorbic acid is commonly used as an antioxidant when measuring folates by microbiological assay (Spray 1964; Temperley and Horner 1966) and it was considered for use in the assay of reduced biopterin derivatives with Crithidia fasciculata. Figure 2-6 shows the depression of growth by ascorbic acid at a final concentration of 0.25 g/litre, there was competition for available oxygen between Crithidia

fasciculata and ascorbic acid. Crithidia fasciculata has an abnormally high rate of oxygen consumption (Cosgrove 1959). Therefore its avidity for oxygen may in turn protect labile materials being assayed from oxidation.

Fig. 2-6

Growth response of Crithidia fasciculata to biopterin with 0.5 cm^3 2.0 M phosphate buffer, pH 6.0 and 0.5 M phosphate buffer containing 0.2% ascorbic acid/4 cm^3 assay medium.

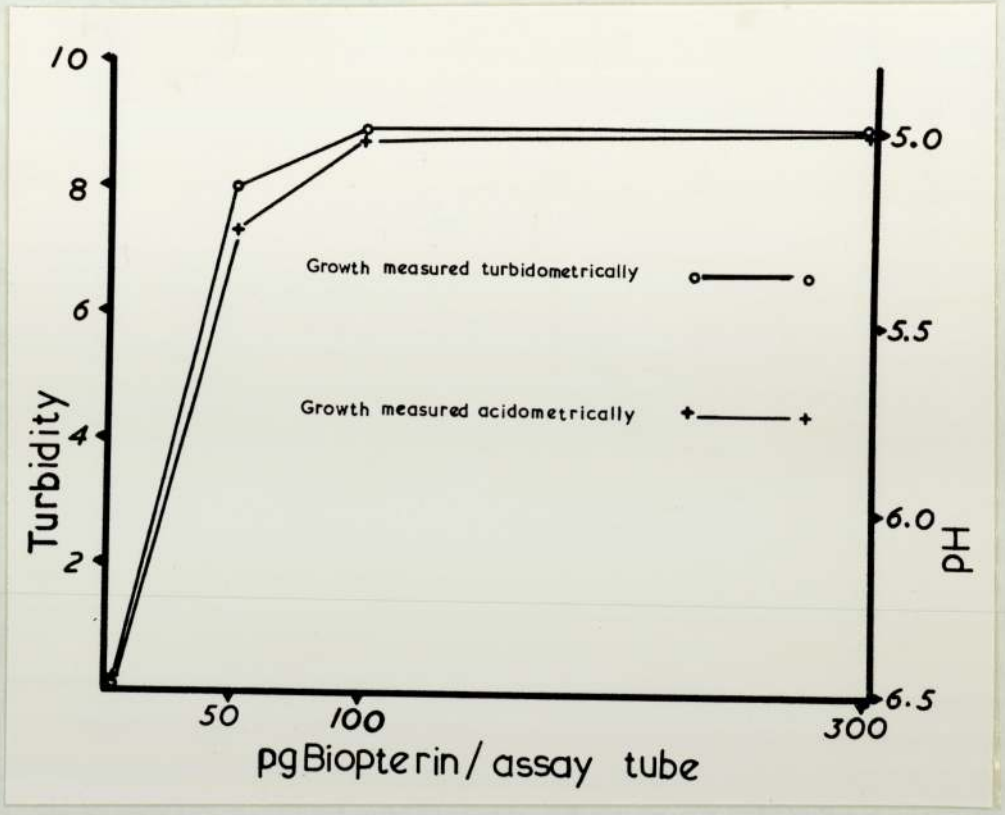


The Measurement of Growth by Acid Production

The pH after growth was proportionate to the growth as determined turbidometrically (Fig. 2-7) and so could be used in its place. However, as a semi-automated system (Leeming and Portman-Graham 1973) existed coupled to a spectrophotometer it would have been unnecessarily time consuming to use a pH meter. All assays were read as absorption at a wavelength of 590 nm against uninoculated medium.

Fig. 2-7

Growth response to biopterin measured turbidometrically and acidometrically.



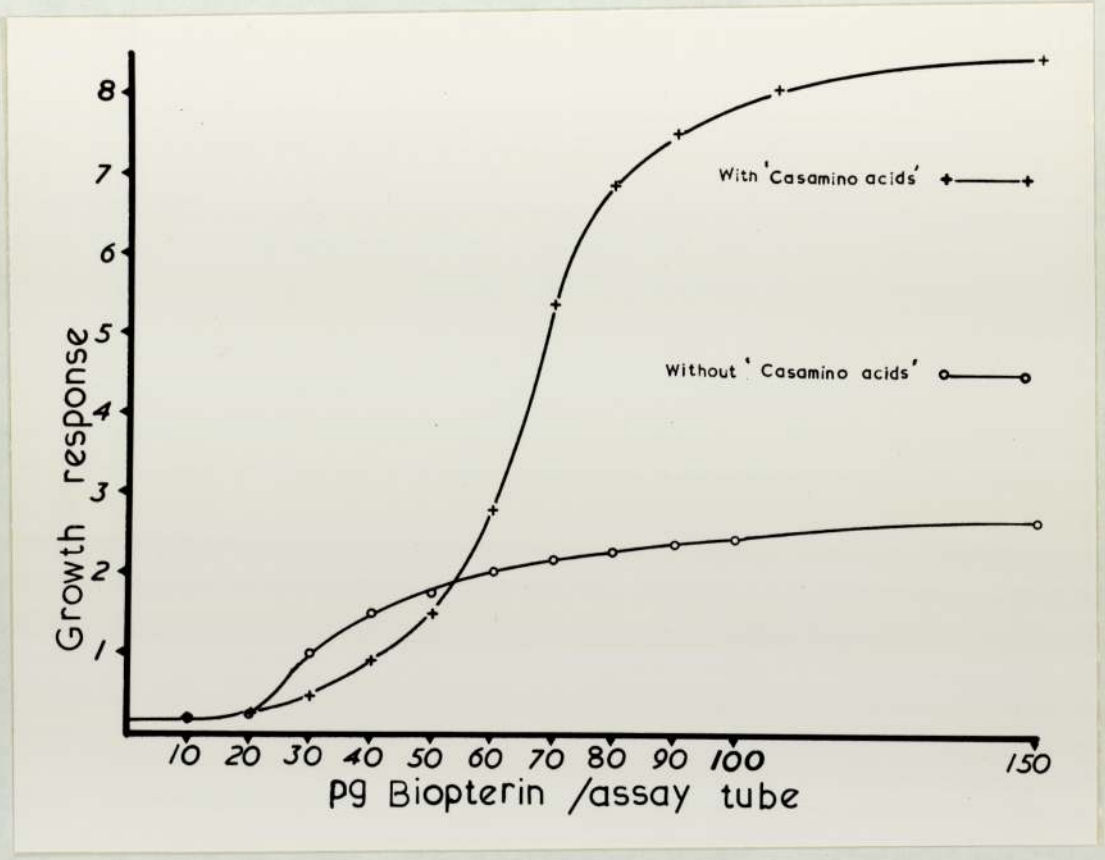
The Inclusion of 'Casamino acids' in the Assay Medium

Use of 'Casamino acids' (Difco) to supplement the defined medium has previously been described (Iwai, Kobashi and Fujisawa, 1970) although these workers claimed to find it contaminated with Crithidia factor and filtered it through charcoal to make it suitable. Figure 2-8 shows the standard growth curve with 1% 'Casamino acids' added compared to identical medium without it. The results give ample reason for its use and demonstrate the lack of growth response when no biopterin was added. When drawing conclusions about growth factors, they must always be considered in the total context of experimental detail and attention has to be drawn to the altered requirements of the Crithidia fasciculata at different temperatures (Guttman 1963) and pH

(Tamburro and Hutner 1971). This experiment was carried out at 29°C and at a commencing pH of 6.5.

Fig. 2-8

Growth response of Crithidia fasciculata to biopterin with and without added 1% 'Casamino acids'.



DISCUSSION

The medium described here does not differ substantially from the one outlined by Cowperthwaite, Weber, Packer and Hutner (1953), although the nutritional requirements of Crithidia fasciculata have been more closely defined (Patterson, Broquist, Albrecht, Von Saltza and Stokstad, 1955), (Tamburro and Hutner 1971), (Nathan and Cowperthwaite 1955), (Baker, Frank, Bacchi and Hutner 1974). It is interesting that other workers (Baker et al 1974) also arrived at a commencing pH of 6.5 for the assay medium although they did not use buffer except for extracting biopterin derivatives from biological materials. The work in this chapter was carried out prior to their publication.

The effects of buffering the medium were greater than merely adjusting the initial pH and the difference between the effects of phosphate and acetate buffers emphasize the inconclusiveness of this observation.

The enhancement of growth response by the addition of 'Casamino acids' is quite clear from Figure 2-8. Care must be taken in using such commercial preparations as others have found this particular product contaminated with Crithidia active material (Iwai et al 1970) and found it necessary to filter it through charcoal. Each batch should therefore be tested before use.

SUMMARY

A method for the assay of Crithidia factor has been described and various parameters examined for their effects on the growth of Crithidia fasciculata.

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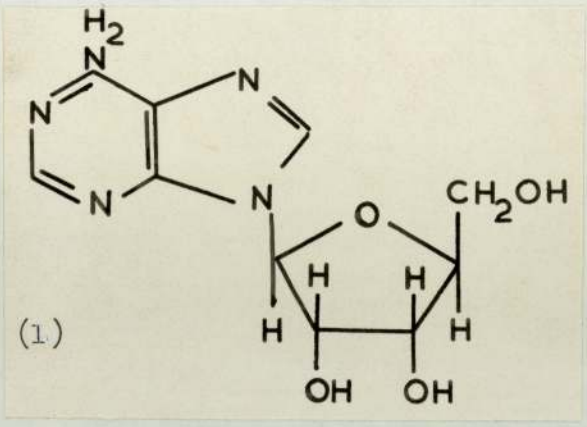
CHAPTER THREE

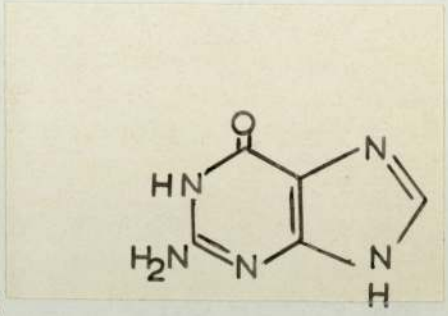
COMPOUNDS ACTIVE FOR CRITHIDIA FASCICULATA

INTRODUCTION

When the first defined medium for Crithidia fasciculata was described by Cowperthwaite, Weber, Packer and Hutner (1953) the hyper-physiological amount of folic acid required for growth was apparently spared by guanine (1), adenosine (2) and certain other nucleosides.



(1)

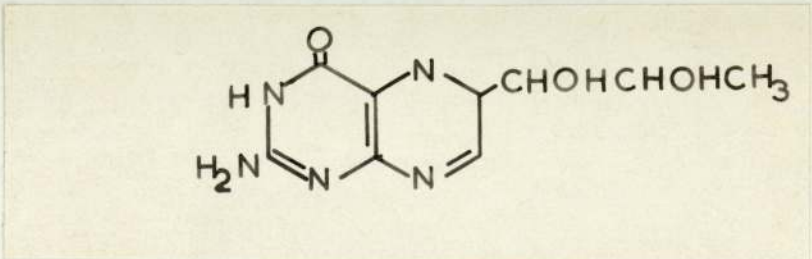


(2)

It was later discovered that these commercially 'purified' compounds were contaminated with Crithidia factor. When guanine was re-crystallised from hot water, the mother liquor, after chromatographic separation was found to contain a 2-amino-4-hydroxypteridine with an aliphatic substituent in the 6-position (Nathan and Cowperthwaite, 1955).

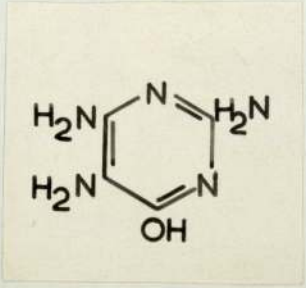
There are several potent, naturally occurring materials capable of supplying the pteridine requirements of Crithidia fasciculata; liver fractions and human urine are principal amongst these together with extracts of Ochromonas malhamensis cultures and various bacteria (Iwai, Kobashi and Fujisawa, 1970). The compound in human urine has been isolated and identified as biopterin (3)

(3)



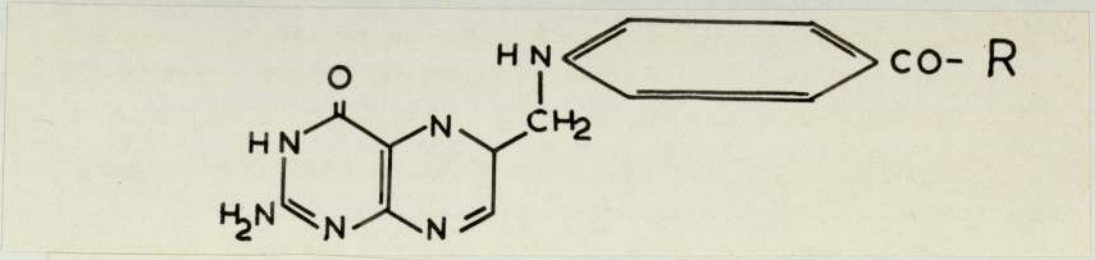
(Patterson, Broquist, Albrecht, Von Saltza and Stokstad, 1955) (Patterson, Von Saltza and Stokstad, 1956) and later synthesised from 5-deoxy-L-arabinose with 2,5,6-triamino-4-hydroxypyrimidine (4)

(4)



(Patterson, Milstrey and Stokstad, 1956).

A number of other pterins active in supporting Crithidia fasciculata were prepared shortly after using other carbohydrates as substrates (Patterson, Milstrey and Stokstad, 1958). The several unconjugated 2-amino-4-hydroxypteridines which reduce the folic acid requirement of Crithidia fasciculata from 1.0 $\mu g/cm^3$ to 0.001 $\mu g/cm^3$ are characterised by an aliphatic substituent in the 6-position at least 2 carbons long which bears at least two hydroxy groups. Other materials comparable to folic acid (5) in their ability to permit growth if present in large amounts are pteroyl-L-aspartic acid (6) pteroyl-glycine (7) and pteroyl-alanine (8) (Broquist and Albrecht, 1955).



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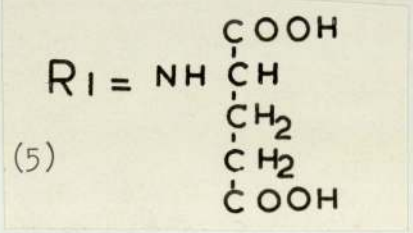

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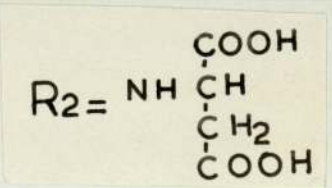

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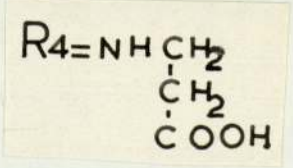
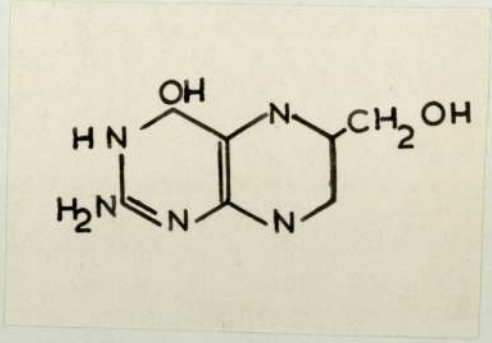


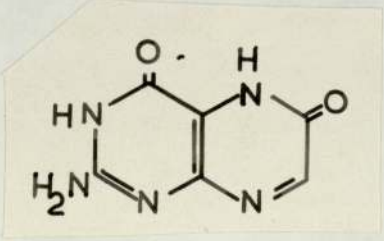
This was confirmed by Kidder, Dewey and Rembold, (1967) who also used folic acid -2-^{14}C as a sole source of pteridine to demonstrate the production of labelled biopterin and 2-amino-4-hydroxy-6-hydroxymethyl-pteridine (9) which they claimed to be derived from the pteridine moiety of folic acid and the primary intermediate in the

(9)



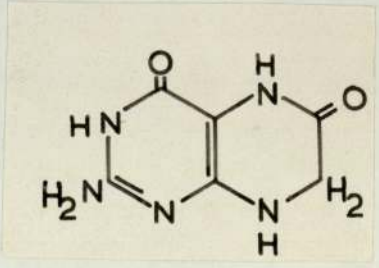
synthesis of biopterin.

Experiments are to be described later in which pteridines, amino acids and metabolites of amino acids were used. The effect of these materials on the growth response of Crithidia fasciculata was therefore of importance and tests were made of their ability to enhance growth either alone or in some cases in combinations with biopterin. The chemical structures of compounds tested, with the exception of those already stated in this chapter, are as follows.



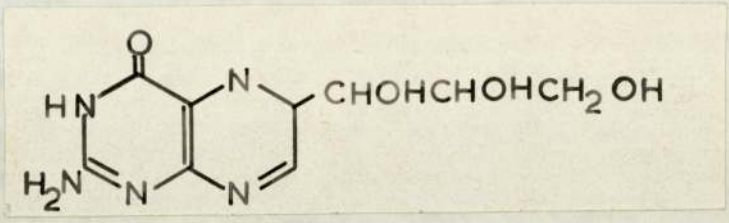
Xanthopterin

(10)

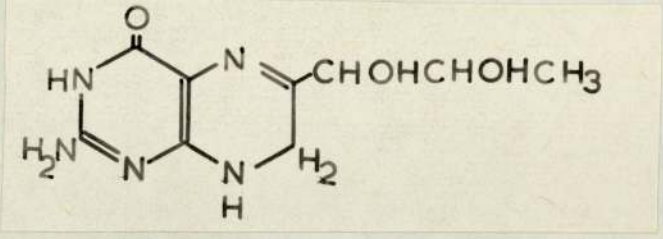


7,8-Dihydroxanthopterin

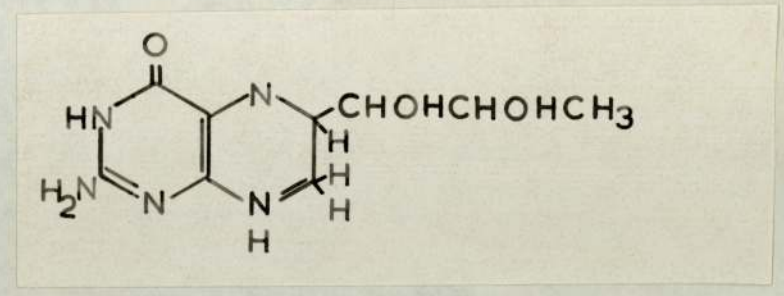
(11)



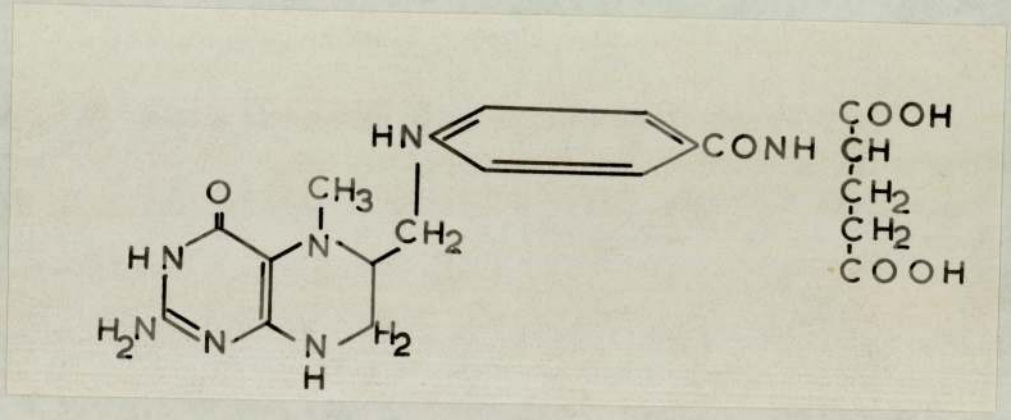
Neopterin (12)



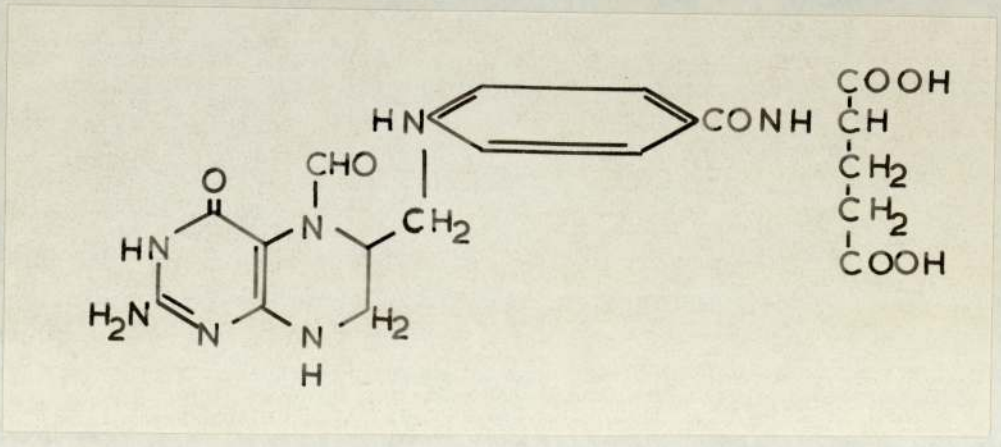
7,8-Dihydrobiopterin (13)



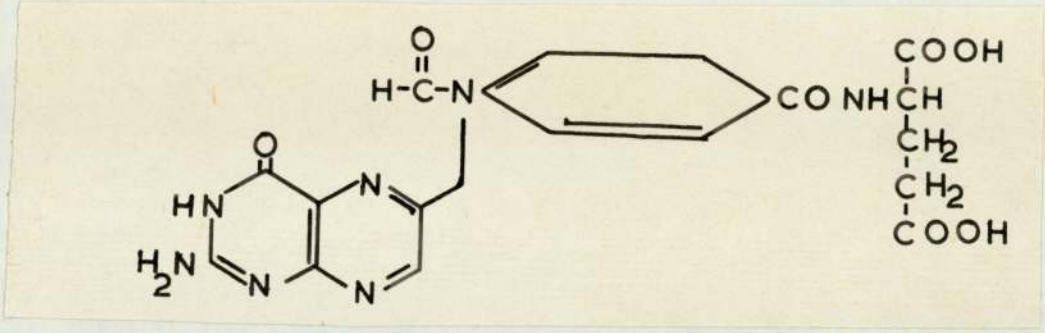
5,6,7,8-Tetrahydrobiopterin (14)



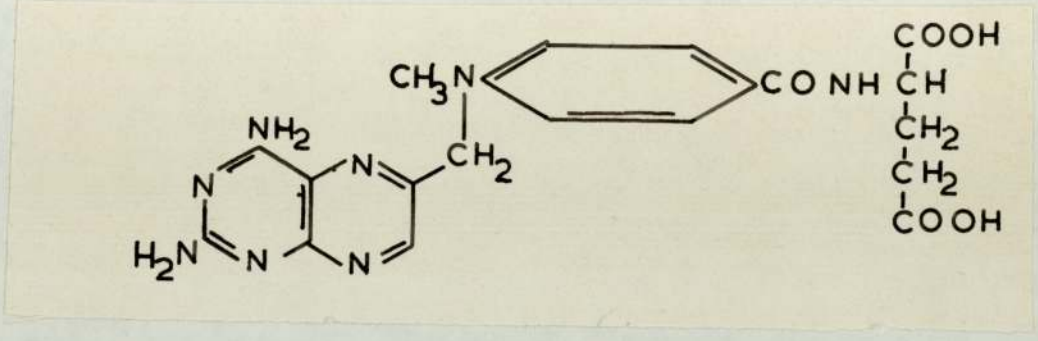
5-Methyltetrahydrofolic acid (15)



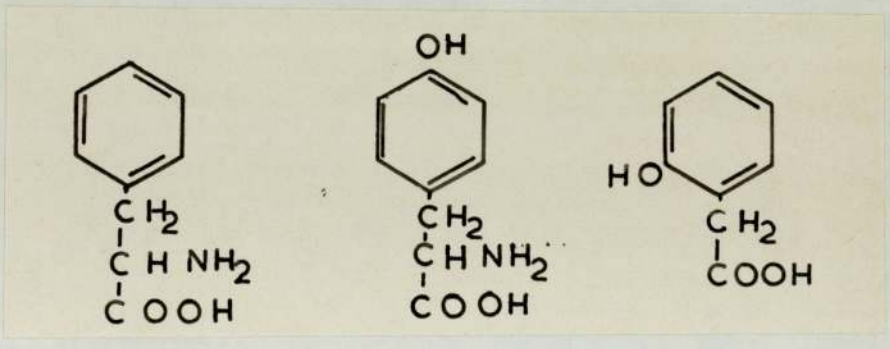
5-Formyltetrahydrofolic acid (16)



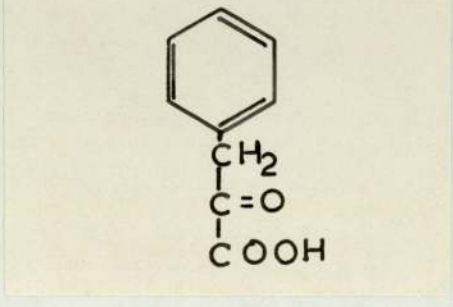
10-Formylfolic acid (17)



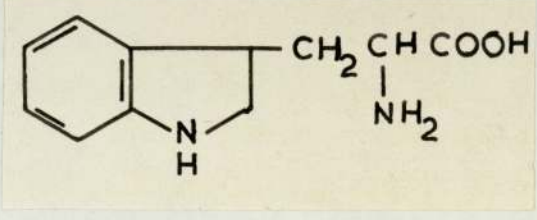
Amethopterin (Methotrexate) (18)



Phenylalanine(19) Tyrosine(20) Ortho-hydroxyphenylacetic acid (21)



Phenylpyruvic acid (23)



Tryptophan (22)

METHODS

Serial dilutions were made of L-neopterin*, D-neopterin*, tetrahydrobiopterin, xanthopterin, dihydroxanthopterin, 5-methyltetrahydrofolic acid, 5-formyltetrahydrofolic acid, 10-formylfolic acid and pteroyl-L-monoglutamic acid. These were added to culture medium, inoculated and their relative activities in stimulating the growth of Crithidia fasciculata were compared (weight/weight with biopterin).

L-β-phenylalanine, L-tyrosine, L-tryptophan, phenylpyruvic acid and ortho-hydroxyphenylacetic acid were tested for antagonistic and synergistic reactions with biopterin as well as their ability to spare Crithidia fasciculata's need for biopterin. Five tenfold dilutions were made to give final concentrations from 125 $\mu g/cm^3$ to 1.25 ng/cm^3 contained in 4.0 cm^3 of medium. A parallel series additionally contained 0.05 ng biopterin/tube.

Tenfold serial dilutions of Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) were made and added to 4.0 cm^3 of culture medium to give final concentrations from 62.5 $\mu g/cm^3$ to 62.5 fg/cm^3. An identical series also contained 0.075 ng of biopterin/4.0 cm^3.

FOOT NOTE: * Stereoisomers of neopterin.

RESULTS

The relative activities of various pteridines are given in Table 3-1.

L-β-phenylalanine, L-tryptophan and phenylpyruvic acid did not spare Crithidia fasciculata's need for biopterin or have any synergistic or antagonistic effect at the concentrations used.

Tyrosine at 125 $\mu g/cm^3$ did not support growth but at this concentration showed some synergism with biopterin, increasing the reading of 0.05 ng biopterin to 0.09 ng when compared with appropriate controls. Ortho-hydroxyphenylacetic acid at 125 $\mu g/cm^3$ supported the growth of Crithidia fasciculata and was equivalent to 0.003 ng biopterin, synergism with biopterin was not detected. 'Methotrexate' alone failed to support growth and at a concentration of 62.5 ng/cm^3 inhibited Crithidia fasciculata's response to biopterin. The inhibitory effect was diluted out at 625 pg/cm^3.

TABLE 3-1

Relative activity of Crithidia fasciculata for pteridines measured against biopterin.

Biopterin	100 %
5,6,7,8-Tetrahydrobiopterin	50 - 80 % (On different occasions)
7,8-Dihydrobiopterin	50 - 100%
D-Neopterin	0.22 %
L-Neopterin	56 %
Xanthopterin	0.0004 %
Dihydroxanthopterin	0.0006 %
5-Methyltetrahydrofolic acid	0.005 %
5-Formyltetrahydrofolic acid	0.008 %
10-Formylfolic acid	0.008 %
Pteroyl-L-monoglutamic acid	0.07 %

DISCUSSION

Only 5,6,7,8-tetrahydrobiopterin, 7,8-dihydrobiopterin and L-neopterin showed a degree of activity for Crithidia fasciculata which approached the response produced by biopterin. Of the other pteridines tested, the most active was pteroyl-L-monoglutamic acid (folic acid), but this was in the non-physiological range originally reported (Cowperthwaite et al, 1953). Amethopterin was frankly inhibitory but in the serum assay described later, a concentration of 100 ng/cm^3 serum would be necessary before the assay would be affected.

The weak synergism between tyrosine and biopterin is difficult to explain as is the slight response to ortho-hydroxyphenylacetic acid. A cautious interpretation is required for results which show exceptionally high concentrations of materials which apparently spare the need for biopterin as the purity of compounds could be suspect. Many amino acids contain bacterial spores (personal observation) and have previously been reported as containing pterins (Nathan and Cowperthwaite, 1955). Even if the purity of compounds was not in question it would be difficult to imagine these high concentrations occurring in biological fluids. Taking into account the dilution factors involved in the microbiological assay technique, any effect is unlikely to be detectable.

The assay appeared to be very selective in its response to pteridines and from this standpoint, promised to be a useful tool in the measurement of active materials in biological fluids.

SUMMARY

Most pteridines proved unable to spare Crithidia fasciculata's need for biopterin, folic acid was the most active of the folate analogues but at a concentration above normal physiological levels. 5,6,7,8-tetrahydrobiopterin, 7,8-dihydrobiopterin and L-neopterin had approximately 50% of the activity of biopterin. Amethopterin was inhibitory. Tyrosine, phenylalanine and metabolites of phenylalanine were unable to replace biopterin or act synergistically with it in the defined medium, except at exceptionally high concentrations.

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CHAPTER FOUR

CRITHIDIA FACTOR IN NORMAL SERUM

INTRODUCTION

There was little in the literature to indicate levels of Crithidia factor in serum. Frank and Baker (1963) found a level of 27 ng/cm^3 in normal pooled serum and Guttman (1964) reported values of 11.2 ng/cm^3 to 38.4 ng/cm^3 in 16 subjects. Baker, Frank, Bacchi and Hutner (1974) found values in 31 normal subjects ranged from 1.1 ng/cm^3 to 2.8 ng/cm^3 (mean 1.9 ± 0.8 S.D) in whole blood and from 0.7 ng/cm^3 to 1.7 ng/cm^3 in plasma (mean 0.9 ± 0.2 S.D).

METHODS

Blood samples were collected from 42 males and 73 females. Only those who were not on any form of medication other than oral contraceptives were considered acceptable. The decision to include those taking oral contraceptives was prompted by the reluctance of volunteers to disclose their contraceptive status because of social considerations. After separation, sera were stored at $-20^{\circ}C$ until assayed. Assays were almost invariably carried out within a week of collection.

Sera were diluted one in twenty with 0.2 M phosphate buffer pH 5.0 and autoclaved for 3 minutes at $115^{\circ}C$ to effect deproteinisation. After centrifugation 0.5 cm^3 of supernatant was added to each of three culture tubes containing 1.5 cm^3 distilled water and 2.0 cm^3 of double strength assay medium. When the sample had previously proved to be high or was from a patient whose condition led to the anticipation of an elevated result, the one in twenty extract was further diluted to one in one hundred with buffer. Inoculation, incubation and reading were carried out as already described but with the colorimeter blanked on uninoculated medium containing the appropriate concentration of serum extract. The standard contained 12 tubes with 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10 and 0.15 ng of biopterin in 0.5 cm^3 quantities of 0.2 M phosphate buffer pH 5.0, added to 1.5 cm^3 of distilled water and 2.0 cm^3 of double strength medium.

Thirty six samples of serum were divided into two and distributed in pairs between 8 separate batches of assays. The pairs were distributed in random fashion within each batch so that they could not readily be identified. Fifteen more sera were aliquoted and assayed on a minimum of 5 and a maximum of 16 separate occasions, making a total of 128 determinations.

Twelve sera were aliquoted, six were stored at $-20^{\circ}C$, six at $-70^{\circ}C$, and assayed on successive occasions for up to nine weeks. Three freshly taken samples were separated and assayed immediately the clots had retracted, the residual serum was frozen at $-20^{\circ}C$ and assayed on four successive occasions at weekly intervals. Additionally, 30 sera were diluted 1/20 with 0.2 M phosphate buffer and heated in the normal way described for serum estimations. After the routine assays had been carried out, the extracts were stored at $4^{\circ}C$ in the dark for four days and reassayed.

To determine the effect of haemolysis on serum levels, Crithidia fasciculata was used to measure biopterin derivatives in whole blood. Ten samples were lysed by freezing and thawing and assays carried out as for serum but with further dilutions, red cell concentrations were calculated using the formula

$$\text{Red cell concentration} = \frac{\text{Whole blood concentration} \; \frac{(1-PCV)}{100}}{\frac{PCV}{100}}$$

in a similar way to the calculation of red cell folates (Hoffbrand, Newcombe and Mollin, 1966).

Five fasting subjects had blood samples taken at hourly intervals commencing at 8.30 to 09.00 hours. Three of these had final specimens 8 hours after commencement, and the other two after 9 hours.

Solutions of biopterin and tetrahydrobiopterin were prepared at concentrations which when diluted 1 in 20 in pooled serum gave 10, 5, 3, 2 and 1 ng/cm^3 of added material. A control was included which had distilled water in place of pterin. Crithidia factor assays were carried out on the sera and on solutions prepared by adding identical amounts of biopterin and tetrahydrobiopterin to 0.5 M phosphate buffer pH 5.0 instead of serum, the solutions were not heated but were otherwise diluted and assayed exactly as the sera.

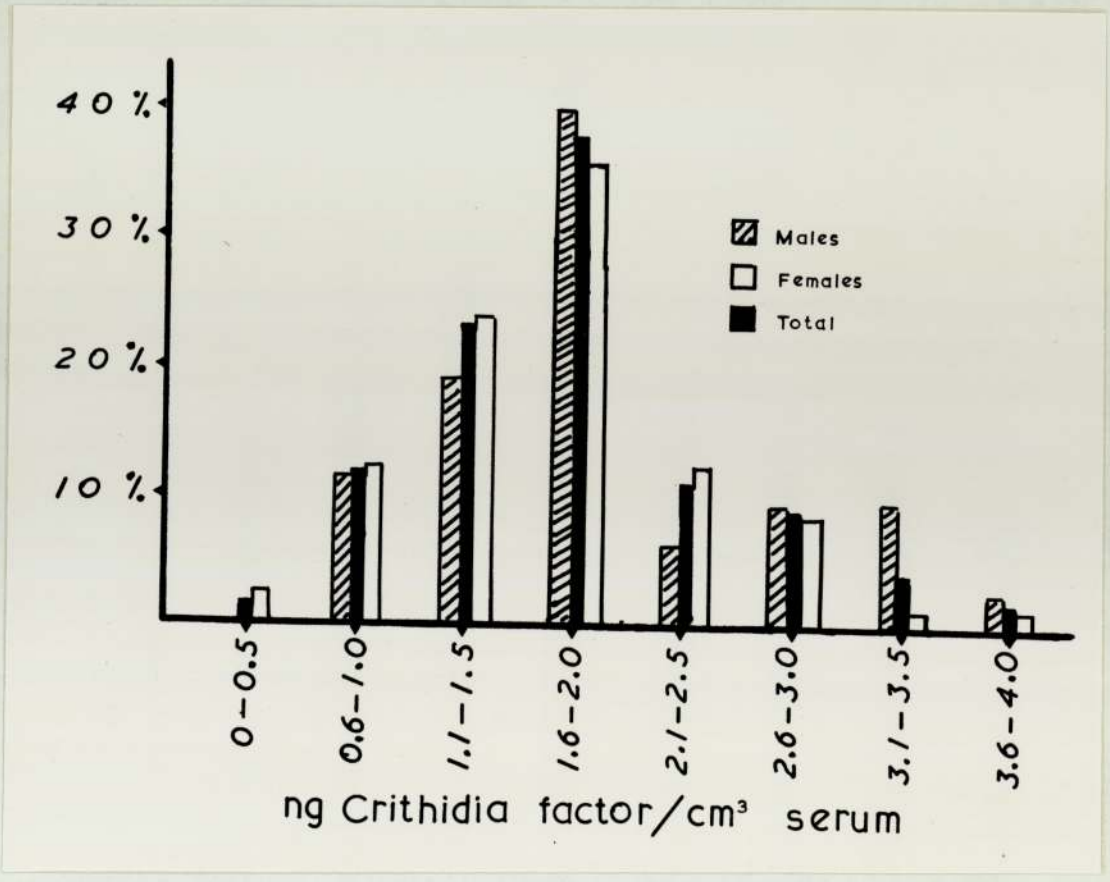
Streaked thin layer cellulose chromatograms of normal serum, serum with added biopterin and serum with normal urine were run in three solvent systems. (3% ammonium chloride, 5% acetic acid and 0.5% sodium carbonate) to 15 cms at room temperature in the dark. The plates were cut horizontally into ten strips 1.5 cms wide and eluted with phosphate buffer pH 5.0. The eluates were centrifuged and the clear supernatants assayed using Crithidia fasciculata.

RESULTS

The mean level for males was 1.9 ± 0.12 ng/cm^3 and for females 1.75 ± 0.07 ng/cm^3. A Student's 't' test showed the difference between these two values as not significant. (p = 0.2 to 0.3). The mean for total male and female sera was 1.81 ± 0.06 ng/cm^3. The distribution of values is shown in Figure 4-1. The distribution of values between age

Fig. 4-1

Distribution of serum Crithidia factor levels in 42 normal adult males and 72 normal adult females, expressed as a percentage of the total number.



groups and sex is given in Table 4-1. The group aged less than 20 years was tested by Student's 't' against those in the third and fourth decades (p = 0.1 - 0.2 and p = 0.05 - 0.1 respectively). The difference between those below 20 years of age and those above fifty was more significant although the numbers were smaller, p = 0.02 - 0.05 for the females alone as well as for both sexes together. However, the males did not contribute

TABLE 4-1

Mean Serum Crithidia Factor Levels in Normal Subjects

Grouped According to Age and Sex

Age in Years	< 20	21 - 30	31 - 40	41 - 50	51 - 60
	(7)	(18)	(10)	(5)	(2)
Males	1.63 ± 0.36	1.99 ± 0.22	1.88 ± 0.18	1.92 ± 0.42	1.65 ± 0.15
	(12)	(29)	(14)	(13)	(5)
Females	1.42 ± 0.12	1.62 ± 0.11	2.00 ± 0.18	1.81 ± 0.12	2.36 ± 0.35
	(19)	(47)	(24)	(18)	(7)
Total	1.49 ± 0.14	1.76 ± 0.11	1.97 ± 0.14	1.84 ± 0.14	2.16 ± 0.28

Females known to be taking oral contraceptives

(8)

1.72 ± 0.24

Results are given in ng/cm^3 with standard errors of means. Figures in brackets are numbers of subjects in groups.

greatly to this difference (p = 0.3 - 0.4). There were eight females who were known to be taking oral contraceptives, they did not show any significant difference from the total female sample (p = > 0.9).

The results from specimens paired within batches showed the within batch coefficient of variation to be 7.9% ± 0.66% around a mean of 1.9 ng/cm^3. This was better than the coefficient of variation for between batch variation, 20.52% ± 1.28% around a mean of 1.59 ng/cm^3.

There was no significant difference between results obtained from freshly assayed samples and those from specimens which had been stored, the results are shown in Table 4-2. In the experiment where extracts were stored the means were 1.77 ng/cm^3 ± 0.15 and 1.68 ng/cm^3 ± 0.16 respectively. Whole blood levels together with serum values and red cell volumes in ten patients are given in Table 4-3.

Figure 4-2 gives the serum level in five subjects over an eight

Fig. 4-2

Variations in serum Crithidia factor levels in 5 subjects over 8 hours. Results given are means with standard errors of means.

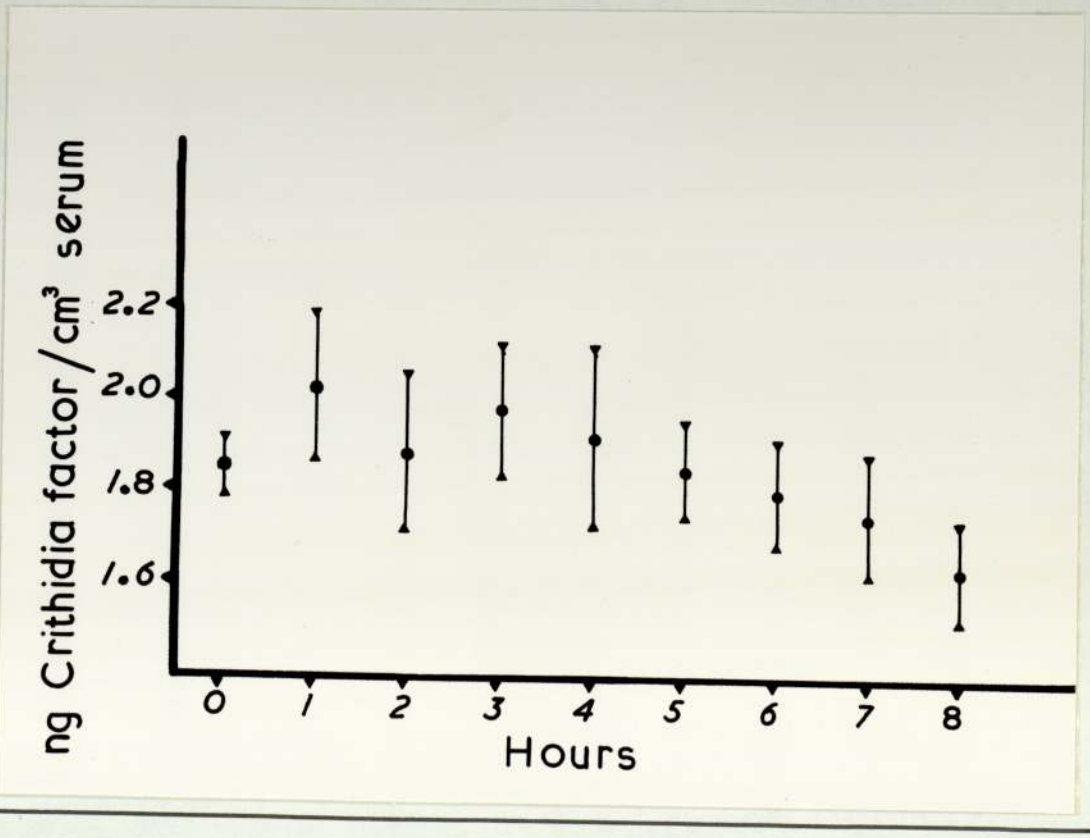


TABLE 4-2

Storage of Sera

Results are means given as ng/cm^3 with standard errors of means.

	I 6 samples at $-20^{\circ}C$	II 3 samples at $-20^{\circ}C$	III 6 samples at $-70^{\circ}C$
Immediate Assay	-	1.55 ± 0.03	-
Stored 1 week(s)	1.57 ± 0.40	1.57 ± 0.07	2.08 ± 0.29
2 "	1.48 ± 0.24	2.0 ± 0.12	-
3 "	1.48 ± 0.19	1.23 ± 0.07	1.58 ± 0.25
4 "	1.56 ± 0.24	1.53 ± 0.07	-
5 "	1.34 ± 0.34		1.55 ± 0.17
6 "	1.2 ± 0.22		1.61 ± 0.19
7 "	2.02 ± 0.18		1.79 ± 0.19
8 "	2.05 ± 0.20		
9 "	1.63 ± 0.21		

TABLE 4-3

Red Cell and Whole Blood Crithidia Factor levels in 10 Subjects

Subject No.	Packed Red Cell Volume	Serum Level	Whole Blood Level	Red Cell Level
1	43.5	1.5	3.9	7.1
2	43.9	1.8	3.9	6.6
3	40.1	1.2	3.4	6.7
4	28.4	1.2	4.1	8.9
5	42.3	1.3	4.5	11.1
6	44.2	1.7	3.7	6.3
7	42.2	1.2	3.8	7.1
8	47.5	1.8	5.2	9.1
9	43.9	1.3	3.2	5.7
10	35.6	1.4	4.3	9.6
Mean levels with Standard Errors of means	42.2 ± 1.1	1.4 ± 0.1	4.0 ± 0.2	7.8 ± 0.6

hour fasting period. Although there was a distinct downward drift, the greatest difference between samples at hours 1 and 8, did not stand up to testing by Student's 't' at the 5% level ($p = 0.05 - 0.10$).

The material in serum proved to be chromatographically distinct from biopterin but co-chromatographed with the major material in urine, 7,8-dihydrobiopterin (Leeming and Blair, 1974).

The recoveries of added biopterin and tetrahydrobiopterin from sera are shown in Table 4-4.

TABLE 4-4

The Recovery of Added Biopterin and Tetrahydrobiopterin from Pooled Serum

Added Biopterin in ng/cm^3 serum	Assay of Biopterin in buffer	Assay of Biopterin in serum	Assay of Pooled serum
10	> 6.0	> 6.0	1.4
5	5.0	> 6.0	1.4
3	2.8	4.9	1.4
2	1.6	4.1	1.4
1	0.8	2.2	1.4

Added Tetrahydrobiopterin in ng/cm^3 serum	Assay of Tetrahydrobiopterin in buffer	Assay of Tetrahydrobiopterin in serum	Assay of Pooled serum
10	4.8	6.0	1.4
5	2.7	4.0	1.4
3	1.6	2.8	1.4
2	1.2	2.5	1.4
1	0.6	2.0	1.4

DISCUSSION

Baker, Frank, Pasher, Sobotka and Hutner (1961) found the range of unconjugated pteridine in the sera of twenty eight subjects to be 11 ng/cm^3 to 43 ng/cm^3 and in whole blood 27 ng/cm^3 to 70 ng/cm^3. Frank and Baker (1963) further reported levels of 27 ng/cm^3 in pooled human serum and 48 ng/cm^3 in pooled whole blood and compared these with levels in animals. Guttman (1963) found levels of 11.2 ng/cm^3 to 38.4 ng/cm^3 in sixteen subjects. These values are considerably higher than those reported in this thesis.

One would expect the purity of the standard and preparation of the serum for assay to be the most important factors in determining reported levels.

Neither Baker et al (1961) nor Frank and Baker (1963) gave any purity data on their standard or detailed the preparation. Guttman (1964) used biopterin extracted from Drosophila melanogaster or Anacystis nidulans and separated it chromatographically. Guttman (1964) autoclaved sera in phosphate buffer of pH 6.0 which contained 0.05% ascorbic acid as an antioxidant, her relatively elevated results were therefore unlikely to have been caused by the extraction process, in thislaboratory ascorbic acid has been found to hinder the growth of Crithidia fasciculata. A partially inactive standard would give the impression of raised results but in the absence of comparative trials no firm conclusion is possible. The biopterin used in the work presented here was a gift from Roche Products Ltd. Recent work using biopterin from the same source (Baker, Frank, Bacchi and Hutner, 1974) showed results much closer to those given (0.9 ng/cm^3 in plasma). The remarkably narrow distribution suggests that serum concentrations are controlled by a homeostatic mechanism. This has already been suggested for another pteridine, 10-formyltetrahydrofolic acid (Blair, Ratanasthien,

Leeming, Melikian and Cooke, 1974). The distribution was slightly positively skewed, the arithmetic mean was 1.9 ng/cm^3 in contrast to the median of 1.75 ng/cm^3.

Variation between assays was too great to allow small shifts to be significant, but it was hoped that the narrow spread of normal values would, to some extent, counter this deficiency of the technique. Storage of specimens, whether as unprocessed sera or as extracts did not appear to affect the results substantially and from the results of the red cell assays it would seem that a slight degree of haemolysis would also have little impact. It can be seen from the mean values in Table 4-3 that lysis of all the red cells, when the haematocrit was normal, would approximately double the Crithidia factor level. Frank and Baker (1963) also found that the level in human whole blood was double that in serum although their values for each of these was considerably greater. No significant variation in serum levels was detected in the five subjects over nine hours during the day, although there appeared to be a steady downward shift again lending weight to the observation that levels are remarkably stable.

The identity of the material in normal serum was probably 7,8-dihydrobiopterin as elicited from the results of the chromatography although if it was 5,6,7,8-tetrahydrobiopterin, this would rapidly be re-arranged to the dihydro form during the analytical process, the kinetics of this oxidation have been well documented (Blair and Pearson, 1974). Protection from oxidation is feasable using ascorbic acid. However, Crithidia fasciculata is an obligatory aerobe and its growth is inhibited by antioxidants.

When biopterin and tetrahydrobiopterin were added to sera, the extraction technique permitted a good recovery of both materials (Table 4-4).

SUMMARY

A technique has been described for the measurement of Crithidia factor in serum. There was a narrow distribution of normal values without significant differences between males and females or divisions according to age. The effects of storage of specimens and reproducability of results have been given in detail and added biopterin and tetrahydrobiopterin have been recovered in full by the extraction procedure when tested against appropriate standards. The isolated material active for Crithidia fasciculata was probably 7,8-dihydrobiopterin.

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CHAPTER FIVE

CRITHIDIA FACTORS IN NORMAL URINE

INTRODUCTION

Biopterin has been isolated from human urine (Patterson, Von Saltza and Stokstad, 1956) but little attention has been given to the labile reduced pterins although Fukushima and Shiota (1972) deduced that these were the principal components of the urinary pterin pool, identified 7,8-dihydrobiopterin and concluded that the daily pterin output was 1.4 mg/day. In this chapter an attempt will be made to establish normal urinary levels and the reproducibility of the assay will be examined. Variation in excretion both diurnally and over a protracted period will be discussed. Chromatographic evidence of the structure of urinary Crithidia factors will be presented.

METHODS

Urines collected from thirty normal males and thirty normal females were frozen at $-20^{\circ}C$ and subsequently assayed for Crithidia factor. The urines were diluted 1 in 500 with 0.2 M phosphate buffer pH 5.0 and three further serial 1 in 5 dilutions made, 0.5 cm^3 volumes each of these last three dilutions were assayed in triplicate.

In order to test the reproducibility of the assay from batch to batch and within each batch, six samples of urine were divided into ten aliquots, frozen at $-20^{\circ}C$ until the day of the test and distributed between three batches of assays.

Variation in excretion was measured in a healthy male adult, four hour urine collections were made daily over a period of ten days. Random untimed collections were made from the same subject over a period of two years nine months. Diurnal variation was assessed in five subjects who collected urine during an eight hour sleeping period at night and for a similar waking period during the day.

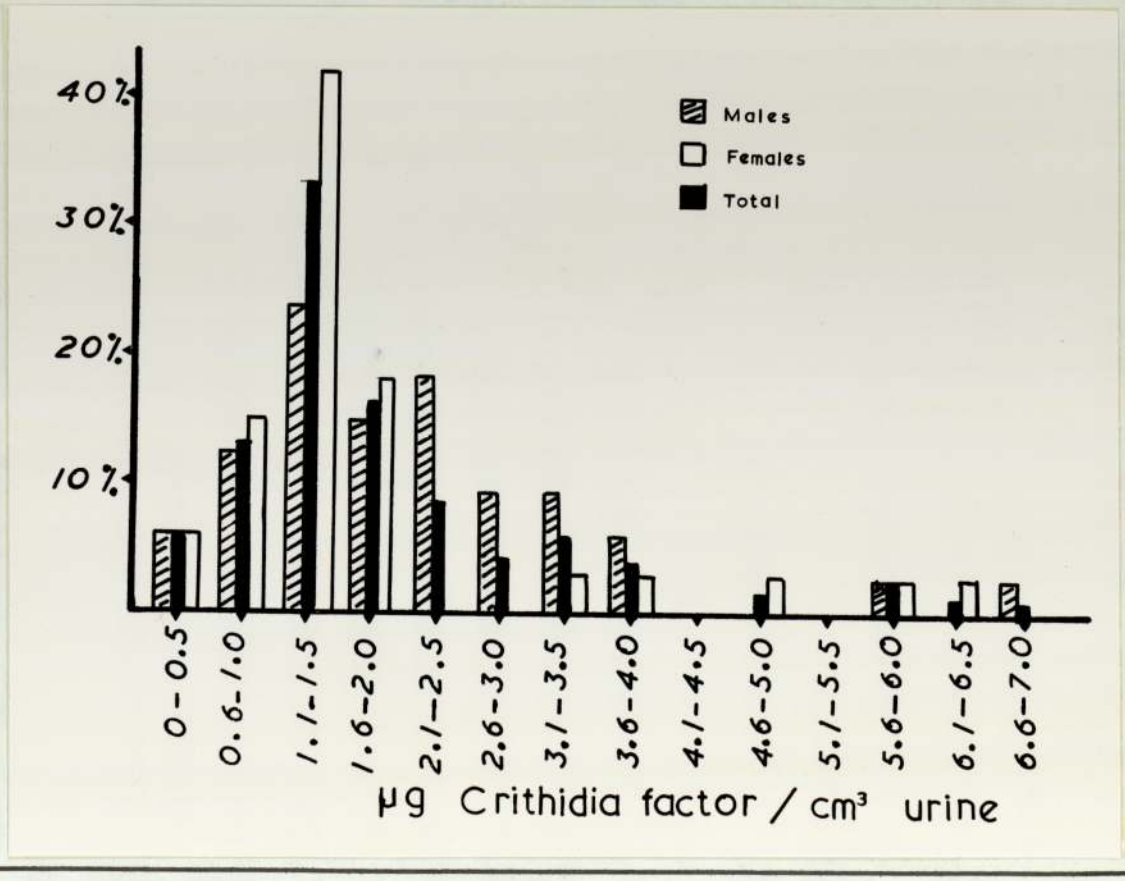
Chromatographic identification of materials in urine was attempted by spotting cellulose thin layer plates and developing in eight solvents, 3% aqueous ammonium chloride, 0.1 M phosphate buffers of pH 6.0 and pH 7.0, 0.5% aqueous sodium carbonate, 5% aqueous acetic acid, 5% aqueous acetic acid with 1% ascorbic acid added, propanol/1% aqueous ammonia (2:1 v/v) and butanol/acetic acid/water (4:1:1 v/v).

RESULTS

The mean level for both males and females was 2.1 $\mu g/cm^3$ with standard errors of 0.39 $\mu g/cm^3$ and 0.37 $\mu g/cm^3$ respectively. Distribution of values according to age and sex are shown in Table 5-1. No significant difference was shown by any division of the groups. The distribution of all the values obtained is given in Figure 5-1, the median was 1.6 $\mu g/cm^3$ and the distribution positively skewed. The concentrations varied considerably from serum levels but again showed a narrow distribution, the highest and lowest values obtained were 0.34 $\mu g/cm^3$ and 6.9 $\mu g/cm^3$.

Fig. 5-1

Distribution of urinary Crithidia factor levels in 30 normal adult males and 30 normal adult females, expressed as a percentage of the total number.



The variation between samples assayed on separate days gave a coefficient of 15.49% ± 2.58% around a mean of 2.3 $\mu g/cm^3$.

TABLE 5-1

Mean levels of Crithidia factor in the urine of

60 normal subjects distributed according to age and sex

Age in years	Males	Females	Total
21 - 30	(19) 1.90 ± 0.29	(16) 2.00 ± 0.44	(35) 1.97 ± 0.25
31 - 40	(7) 1.78 ± 0.41	(8) 1.85 ± 0.45	(15) 1.82 ± 0.30
41 - 50	(4) 2.20 ± 0.71	(6) 2.55 ± 1.07	(10) 2.40 ± 0.62

Results are given in ng/cm^3 with standard errors of means.

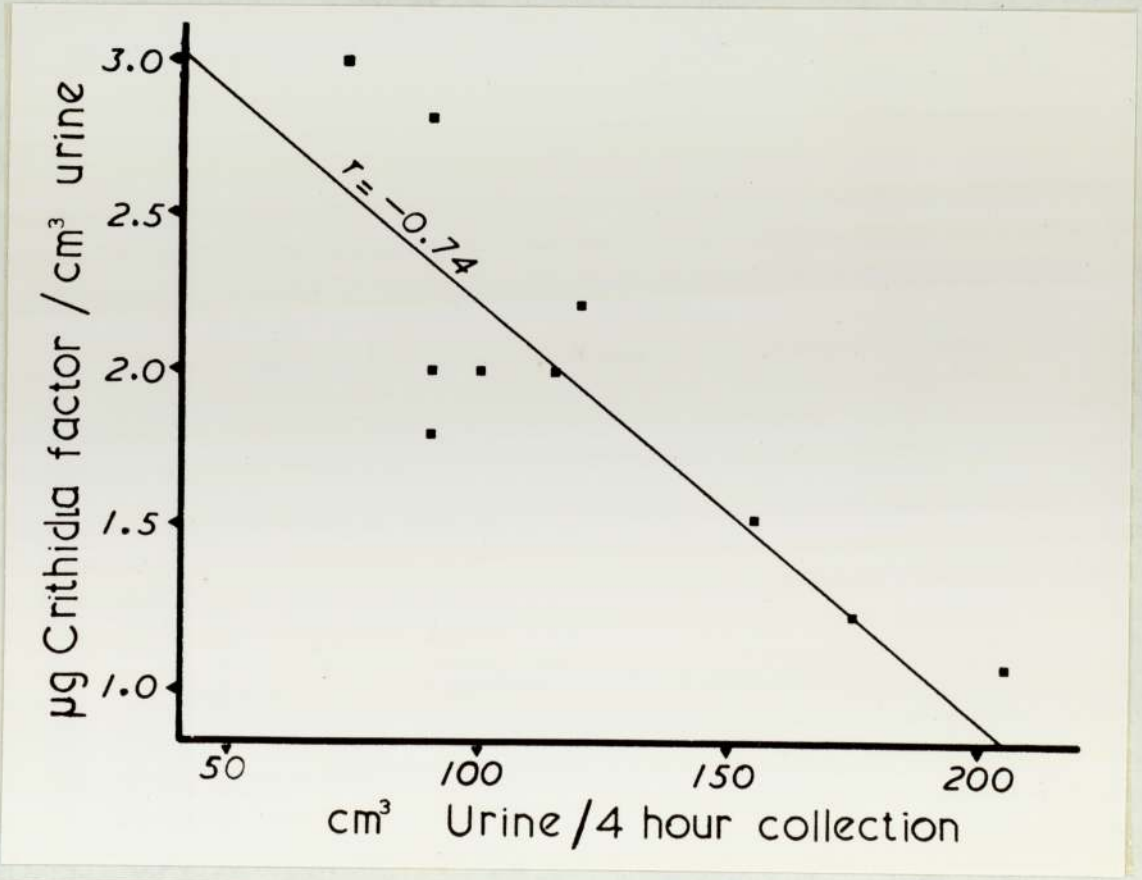
Figures in brackets are numbers of subjects in group.

The values obtained for each sample in a batch were meaned and the coefficient of variation calculated for all sixty samples as 12.26% ± 1.12%. This value was predictably better than that obtained for between batch variation.

Urinary excretion was fairly constant in the subject producing four hour urine collections for ten days (216 µg ± 11µg). There was some indication of urinary concentration when samples were smaller in volume (Figure 5-2) and this effect may have been a contributory factor in variations observed in the long term experiment

Fig. 5-2

Volumes of 4 hour urine collections plotted against concentrations of Crithidia factor.



(Table 5-2) in which random samples were used, over the period two years nine months considerable variation occurred in the absence of overt disease. The mean level for excretion during eight hour night-

TABLE 5-2

Variation in Crithidia factor levels in random urine collections from one person

Date	Crithidia factor level $\mu g/cm^3$
5.12.71	1.75
3.1.72	2.5
15.11.72	1.4
30.11.72	1.4
12.1.73	0.24
9.2.73	0.72
23.2.73	0.8
9.3.73	1.2
21.5.73	0.94
6.6.73	1.8
26.6.73	0.4
29.6.73	0.94
6.7.73	0.4
3.8.73	0.95
31.8.73	1.1
21.9.73	0.75
28.9.73	0.7
1.2.74	0.3
20.6.74	0.9
6.7.74	0.65
24.8.74	1.0

time collections was 1.32 $\mu g/cm^3$ ± 0.77 $\mu g/cm^3$ and 1.09 $\mu g/cm^3$ ± 0.55 $\mu g/cm^3$ for the day time collection with mean excretory rates of 62.2 µg/hour ± 18.8 µg/hour and 71.4 µg/hour ± 10.9 µg/hour respectively. The difference between these levels was not significant but allowed a calculation of the approximate daily excretion at 1.6 mg.

The Rf values for Crithidia factor materials from eluted thin layer chromatograms of urine are shown in Table 5-3. The major material was 7,8-dihydrobiopterin, bioautograms run after oxidation of sterile urine give a major material of which 80% chromatographed with biopterin and did not co-chromatograph with L-neopterin which excludes 7,8-dihydroneopterin as a candidate. The trace material could have been tetrahydrobiopterin, biopterin, D-neopterin or L-neopterin. However, it consistently could not be detected in phosphate buffer solvent systems which would have clearly revealed it if it had been biopterin, D-neopterin or L-neopterin. Tetrahydrobiopterin was readily oxidised in phosphate buffers to dihydrobiopterin (Blair and Pearson, 1974) and the trace amounts could have been oxidised during development of the chromatograms. The minor component was therefore most likely to have been tetrahydrobiopterin.

TABLE 5-3

R_f values of urine constituents and known pteridines

Solvent	Major urine component	Minor urine component	Dihydro-biopterin	Tetrahydro-biopterin	Biopterin	D-neopterin	L-neopterin
3% aq. ammonium chloride	0.3	0.5	0.3	0.55	0.5	0.5	0.5
0.1 M phosphate buffer pH 6.0	0.3	*	0.3	0.55	0.55	0.55	0.5
0.1 M phosphate buffer pH 7.0	0.3	*	0.3	0.55	0.55	0.55	0.5
0.5% aq. sodium carbonate	0.5	0.7	0.5	0.7	0.7	0.7	0.7
5% aq. acetic acid	0.5	0.6	+	0.6	0.6	0.6	0.6
5% aq. acetic acid + 1% ascorbate	0.5	0.6	+	+	+	+	+
Propanol - 1% aq. ammonia solution (2:1 v/v)	0.2	*	+	0.2	0.2	0.1	0.1
Butanol - acetic acid - water (4:1:1 v/v)	0.2	*	+	0.2	0.2	0.1	0.2

* = Only major component seen as single spot

+ = Not recorded

DISCUSSION

The level of urinary Crithidia factor was much higher than that found in serum and suggested an active excretion of about 1.6 mg/day. This is very close to the value given by Fukushima and Shiota (1974) who concluded that the daily output of pterin was approximately 1.4 mg/day. Baker, Frank, Bacchi and Hutner (1974) found a range of 1.5 - 2.4 mg/24 hours in 19 subjects. Pabst and Rembold (1966) established that the daily biopterin excretion in rats was constant at about 30 µg even when a biopterin free diet was given for several generations. Taking into account relative body weights, a close approximation of the rates of excretion in man and rat is suggested by these figures.

The material identified in urine by Patterson, Broquist, Albrecht, Von Saltza and Stokstad (1955) and later isolated and characterised as biopterin (Patterson, Von Saltza and Stokstad, 1956) was probably an artifact. From 1000 litres of urine they only isolated 26 mg which could have only been a fraction of the amount originally present, presumably the major portion of biopterin derivative contained in their samples were degraded during the isolation procedures. Fukushima and Shiota (1972) deduced that the principal components of the urinary pterin pool was in labile reduced forms and identified 7,8-dihydro-biopterin. The evidence produced here agrees with the findings of these workers and further suggests that a minor material exists which is also a labile pterin, probably 5,6,7,8-tetrahydrobiopterin.

SUMMARY

A method of measurement of Crithidia factor in urine has been described. The daily output was approximately 1.6 mg/day of which the major portion was 7,8-dihydrobiopterin and a minor component was 5,6,7,8-tetrahydrobiopterin. Distribution of levels was greater than that found in serum and this was probably due to dilution effects.

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CHAPTER SIX

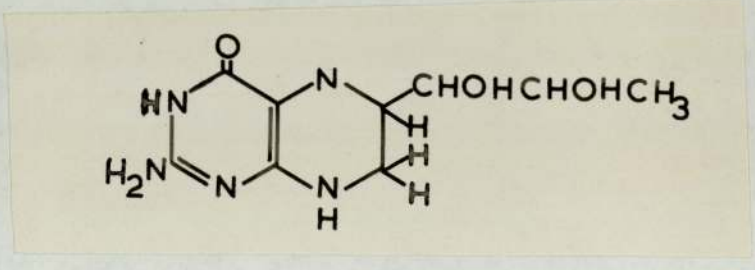
CRITHIDIA FACTOR IN HUMAN CEREBROSPINAL FLUID, LIVER, BRAIN AND MILK

INTRODUCTION

Levels of biopterin derivatives in cerebrospinal fluid and tissue have been reported (Baker, Frank, Bacchi and Hutner, 1974). Liver taken at autopsy or by needle biopsy was shown to contain 0.36 - 0.9 μg/g (mean 0.52 μg/g) in 26 subjects and brain in 19 subjects contained 0.13 - 0.52 μg/g (mean 0.34 μg/g). Both these tissues were lyophilized to dryness before assay. Cerebrospinal fluid contained 0.25 - 0.7 ng/cm^3 (mean 0.4 ng/cm^3). Folate in cerebrospinal fluid, in patients without neurological disease is considerably higher than the serum folate. Wells and Casey (1967) found a mean total folate of 33.6 ng/cm^3 in thirty cerebrospinal fluids ranging from 12.6 ng/cm^3 to 67 ng/cm^3. Hepatic folates from various sources have been compared (Chanarin, 1969) and subjects with normal serum folate levels had a mean of 7.1 μg/g and ranged from 4.4 μg/g to 10.3 μg/g.

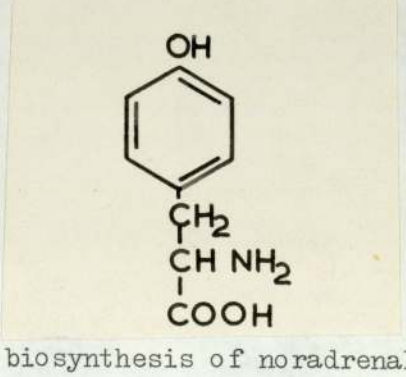
Tetrahydrobiopterin (1) has already been shown to have a

(1)



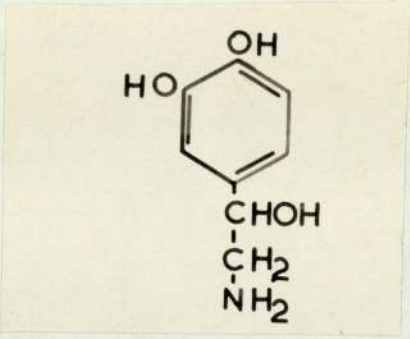
function in the brains of rats (Kettler, Bartholini and Pletscher, 1974) in the hydroxylation of tyrosine (2). Tyrosine hydroxylation is an

(2)



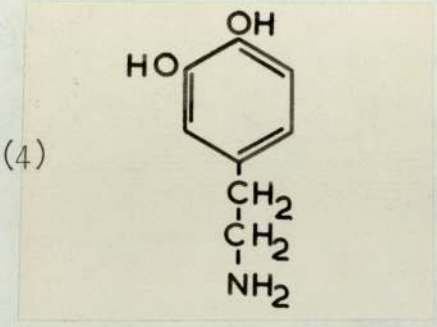
initial step in the biosynthesis of noradrenaline (3) (Nagatsu, Levitt

(3)

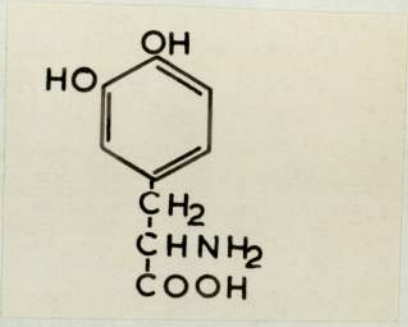


and Udenfriend, 1964) with such biologically important intermediates as dopamine (4) and dopa (5). The concentration of active cofactor in the

(4)



(5)



brain must be sufficient for adequate hydroxylation even if it is not rate determining. Distribution of cofactor in different parts of the brain could vary with the local need for enzymatic activity.

METHODS

Nineteen cerebrospinal fluids without abnormal cell counts were assayed for Crithidia factor in an identical way to sera except that deproteinisation was not necessary. Small pieces of human brain and liver obtained as freshly as possible at postmortem examinations, were frozen, finely sliced and homogenized in a laboratory blender to give a concentration of 5% tissue (wet weight) in 0.2 M phosphate buffer pH 5.0. These suspensions were kept frozen at $-20^{\circ}C$ until the day of the test, when they were thawed, autoclaved at $115^{\circ}C$ for three minutes, cooled, centrifuged and the clear supernatants serially diluted in phosphate buffer for assay in a similar way to sera. One piece each of brain and liver were blended in phosphate buffer pH 7.0 and incubated for two hours with 0.1 cm^3 of chicken pancreas conjugase (Perry and Chanarin, 1968) before being heated and serially diluted in buffer of pH 5.0. Some of the samples of tissue were freeze-dried in vacuo and their dried weight compared with the wet weight of the original tissue.

Eight samples of expressed human milk were centrifuged at 40,000 R.C.F. for 30 minutes at $2^{\circ}C$, the fat and deposited casein were discarded (Ford, 1974). A 1 in 20 dilution was made of the aqueous phase followed by four fivefold dilutions. 0.5 cm^3 of all dilutions were assayed in triplicate for Crithidia factor. Total folate was also measured with L.casei. Some of the defatted milk was filtered through 8/32" 'Visking' tubing under negative pressure in the dark at $2^{\circ}C$. 0.5 cm^3 of six of the specimens were incubated with 0.1 cm^3 of chicken pancreas conjugase in 9.4 cm^3 0.1 M phosphate buffer pH 7.0 for two hours before filtration. The potency of the conjugase was tested with yeast extract under identical conditions. A sample of goats milk was obtained by courtesy of the National Institute for Research in dairying and put through the same procedure as the human samples.

RESULTS

The Crithidia factor levels in cerebrospinal fluids were much the same as those in sera, a mean of 1.9 ng/cm^3 with low and high results of 1.2 ng/cm^3 and 3.4 ng/cm^3 respectively.

The tissue concentrations of Crithidia factor varied enormously, the lowest and highest levels were both found in brain (Table 5-1). The liver samples examined contained between 41 ng/g and 144 ng/g. In some cases dry weights were compared with weights of fresh specimens and these values are also given. Serum levels of biopterin derivatives (Chapter 4) and levels in liver are much closer than folate levels, which suggests that storage in the liver does not exist to any great extent. The conjugase treatment of brain and liver did not alter the observed values for untreated tissues, but it has to be stressed that heating was carried out which may have masked any effect of conjugase. Table 5-2 gives the levels found in human milk and a sample of goats milk. The difference between the means of unfiltered milk and filtered milk was highly significant when samples 3 and 4 were deleted ($p = < 0.001$) and the difference between conjugase treated and untreated milk after filtration was significant at the 5% level ($p = 0.02 - 0.05$). The total folate (L.casei) level, was 5.5 ng/cm^3 $\pm$ 1.0 ng/cm^3 in unfiltered human milk. The values for the goat's milk were very similar to those from humans.

TABLE 6-1

Biopterin derivatives in human cerebrospinal fluid, brain and liver

Brain	White matter		Grey matter		Substantia nigra	
	ng/fresh g.	$\frac{\text{dry wt.}}{\text{fresh wt.}} \times 100$	ng/fresh g.	$\frac{\text{dry wt.}}{\text{fresh wt.}} \times 100$	ng/fresh g.	$\frac{\text{dry wt.}}{\text{fresh wt.}} \times 100$
1.	25	-	25	-	53	-
2.	20	27%	20	16%	500	11%
3.	112	-	22	-	175	-
4.	28	-	35	17%	175	24%
Liver						
1.	41	23%				
2.	100	27%				
3.	163	24%				
4.	144	-				

C.S.F. (19 samples) 1.9 ng/cm^3 ± 0.13

TABLE 6-2

Concentration of biopterin derivatives in human milk

Sample	Unfiltered Milk	(Total folate) L.casei	% age of total after passage through 'Visking' tubing	% age of total after Conjugase treatment and passage through 'Visking' tubing
1.	85 ng/cm^3	(6.0 ng/cm^3)	71%	80%
2.	100 "	(4.4 ")	63%	70%
3.	175 "	(2.8 ")	86%	-
4.	50 "	(4.0 ")	100%	-
5.	163 "	(5.2 ")	46%	57%
6.	163 "	(6.0 ")	48%	77%
7.	150 "	(12.0 ")	75%	93%
8.	125 "	(3.6 ")	60%	87%
Mean with Standard error of mean	126 ± 16	(5.5 ± 1.0)	69 ± 7	77 ± 5
Goats milk	138 ng/cm^3	(6.0 ng/cm^3)	62%	68%

DISCUSSION

Crithidia factor levels in solid tissues were very much higher than those found in serum and cerebrospinal fluid, this could be the result of intracellular concentration or indicate sites of biosynthesis. The contrast between cerebrospinal fluid and brain was particularly interesting as this area represents a relatively closed system with its own fluid quite distinct from the general circulation. The suggestion of biosynthesis in tissue is strengthened by the difference found between different parts of the brain. Even if the dried weight of tissue is taken into account - although this could be complex taking into consideration cell size and available cell fluid for saturation - there was a marked concentration gradient between selected parts of the brain. The hydroxylation of tyrosine to dopa has a rate limiting effect on catecholamine biosynthesis (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) and requires a reduced pteridine cofactor for this process. Dopamine which is produced by the decarboxylation of dopa is present in high concentration in the basal ganglia but is much reduced in Parkinsonism (Hornykiewicz, 1966). If concentrations of dopamine are related to the activity (and health) of a particular site, then it is conceivable that the cofactor level will also be affected. Variations between similar tissues from different cadavers were also considerable, but a note of caution must be entered here as the freshness of tissue obtained postmortem adds another variable as does the cause of death. Tissues contain enzymes known to degrade hydrogenated cofactors like tetrahydrofolic acid and tetrahydrobiopterin (Rembold and Gutensohn, 1968).

Kettler, Bartholini and Plescher (1974) showed that tyrosine hydroxylation was enhanced by injecting tetrahydrobiopterin directly into the lateral brain ventricle of a rat but not by administration of

the same material intravenously, because the latter route did not produce a significant rise in the striatal cofactor level. This observation is again in accord with synthesis in the brain rather than local concentration.

Levels in human milk were more like those found in tissue than serum although total folate levels measured by L.casei were very similar to those in sera (5.5 $ng/cm^3 \pm 1.0$ ng/cm^3). The significance of the difference between Crithidia factor in unfiltered and protein free milk compares with the findings with folates which are strongly bound to minor whey proteins (Ford, 1974). The function, if any, of Crithidia factor in milk is difficult to decide on the evidence presented. If the material is tetrahydrobiopterin it is poorly absorbed in adults although this may not be the case with neonates whose intestinal pH could differ for physiological and dietary reasons. Ford, Knaggs, Salter and Scott (1972) showed that folate-protein complex was absorbed intact for a brief neonatal period into the kid's circulation from goat colostrum and this absorptive pattern could hold good for other materials.

SUMMARY

Levels of Crithidia factor in human cerebrospinal fluid, brain, liver and milk have been measured. Cerebrospinal fluids showed a level and distribution very similar to those given previously for sera. Brain and liver showed considerable variation. There were differences between different parts of the brain and individual cadavers, which could be due to postmortem changes or reflect the specific activity of the brain sample measured. It has been proposed that biosynthesis takes place in the intracellular position. Concentrations in milk are discussed and contrasted with folates.

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CHAPTER SEVEN

INTESTINAL ABSORPTION OF TETRAHYDROBIOPTERIN AND BIOPTERIN

INTRODUCTION

The fate of orally administered pteroyl-L-monoglutamic acid has been described by many workers in the fields of medicine and pteridine chemistry (Butterworth, Nadel, Perez-Santiago and Gardner, 1957; Chanarin, Anderson and Mollin, 1958; Dormandy, Waters and Mollin, 1963; Melikian, Paton, Leeming and Portman-Graham, 1971; Leeming, Portman-Graham and Blair, 1972; Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974). The absorption of folate analogues has also been described (Baker, Frank, Feingold, Ziffer, Gellene, Leevey and Sobotka, 1965; Perry and Chanarin, 1970; Nixon and Bertino, 1972; Brown, Scott, Foster and Weir, 1973; Ratanasthien et al., 1974). There is little information on the bodies handling of biopterin and tetrahydrobiopterin although work carried out on the rat has shown that intraperitoneally injected biopterin was excreted rapidly and unchanged in the urine whereas tetrahydrobiopterin was retained almost completely (Rembold, 1970). However, when biopterin was administered via gastric intubation it was completely absorbed and only appeared in the urine in insignificant amounts (Rembold and Metzger, 1967).

Transport across the small intestine may be by passive diffusion or require some active mechanism. Metabolism may also occur during the absorptive process, this has been suggested for folic acid (Perry and Chanarin, 1970; Baker, Frank, Feingold, Ziffer, Gellene, Leevey and Sobotka, 1965) although it has been shown that folic acid crosses the small intestine unaltered (Leeming, Portman-Graham and Blair, 1972). pH has an effect on the rate of transport of folic acid in the rat (Smith, Matty and Blair, 1970), it is likely that this holds good for other folates and other mammalian species. Again in the rat, the rate of folic acid absorption has a saturation value of between 10^{-6}M and 10^{-5}M. (Burgen and Goldberg, 1962; Smith, Matty and Blair, 1970). The

rate of folic acid absorption is demonstrably slower in the ileum.

Biopterin and tetrahydrobiopterin have a close structural resemblance to folic acid and it would seem likely that if a specific permease existed for transporting the pteridine moiety (Weir, Brown, Freedman and Scott, 1973) then it would operate for all species having the necessary configuration. If, however, absorption is dependant on physiochemical characteristics of the intestinal wall (Blair and Matty, 1974) then much greater variation must be expected between different pteridines.

The aim of this section is to study the absorption of oral doses of tetrahydrobiopterin and biopterin in man.

TOXICOLOGY OF TETRAHYDROBIOPTERIN IN RATS

SUMMARY OF A REPORT BY ROCHE PRODUCTS LTD.

Groups of four dogs were kept in cages and received food and water freely. Each group had a different dose level of tetrahydrobiopterin administered intravenously five times a week for four weeks. The doses were 1, 5 and 20mg/kg body weight. A control group had 1.0 cm^3 of physiological saline/kg body weight in lieu of tetrahydrobiopterin.

TOXICOLOGY

Blood specimens at two and four weeks were compared with samples taken before the experimental period.

The following examinations were carried out:-

Haemoglobin	Plasma glutamate-oxalacetic transaminase
Red cell count	Plasma glutamate-pyruvate transaminase
Packed cell volume	Alkaline phosphatase
Reticulocyte count	Plasma total bilirubin
White cell count	Plasma urea
Differential cell count	Plasma glucose
Platelet count	Serum sodium
Prothrombin time	Serum potassium
Blood sedimentation rate	Plasma cholesterol
	Serum total protein
	Serum electrophoresis

Bromsulpthalein clearance was carried out at the beginning of the experiment and at week four.

Urines were examined at week four and compared with samples at commencement for the following:-

Colour	Protein
Specific gravity	Bilirubin
pH	Glucose

A microscopical examination of urine deposits was also carried out.

The following organs were weighed:-

Thyroid, heart, liver, spleen, kidneys, adrenals, testes, prostate, ovaries, uterus, brain and pituitary. Histological examinations were performed.

TOXICOLOGY

RESULTS

The compound was tolerated well by all dogs. Weekly weights of the experimental groups were comparable to controls and there were no premature deaths.

Blood and urine samples showed no significant differences in biochemical or haematological parameters and organ function tests remained normal.

The heart weights of two dogs in the experimental group were significantly lower than those of the other dogs and the weight of testes and prostates were less in the treated groups.

The most significant finding was that there was light to medium kidney damage in two of the most highly dosed animals although not sufficient to affect kidney function. This damage took the form of atrophy and enlargement of the tubules.

METHODS

A number of volunteers were recruited, none of whom had known absorptive defects or were taking medication. They were all carefully apprised of the nature and as far as this was known, the danger inherent in the experiment. Five took 10 mg of tetrahydrobiopterin orally together with 2 - 3 grams of ascorbic acid as antioxidant, dissolved in about 10 cm^3 of tap water. Blood samples were collected before dose and for up to eight hours thereafter. Urine collections were made which spanned parts of the experimental period. One volunteer took 10 mg of tetrahydrobiopterin without ascorbic acid, three had a similar dose but had simultaneous sodium bicarbonate, two had 5 g each and the other 15 g, blood and urine samples were again collected. A patient who was having hepatic vein catheterisation for therapeutic purposes also consented to take part in the investigation and had 10 mg of tetrahydrobiopterin orally, blood samples were collected from the antecubital and hepatic veins at intervals for two hours. Five further subjects each had 5 mg of biopterin orally, blood samples were collected at 0, $\frac{1}{2}$, 1, 2, 3 and 4 hours.

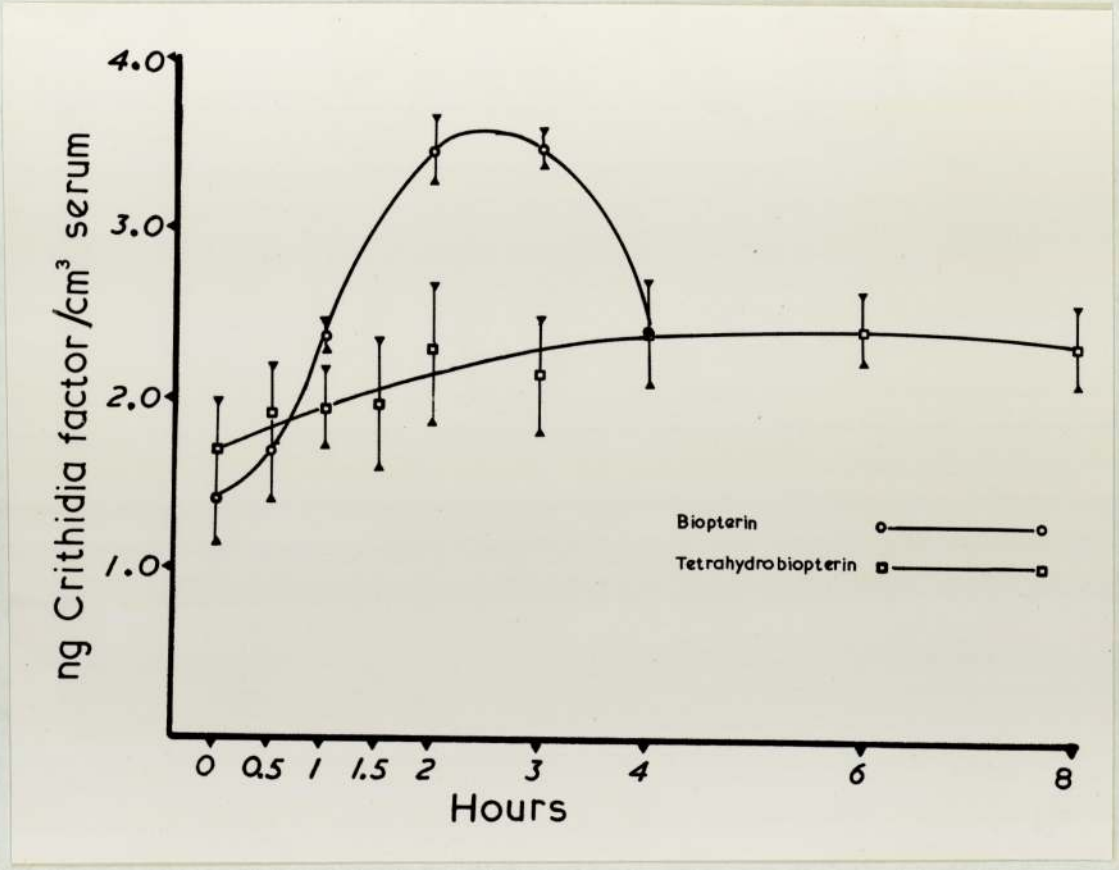
Crithidia factor assays were carried out on all samples, plasma phenylalanine and tyrosine levels were measured in two subjects taking biopterin and in the subject with hepatic vein catheterisation. Bioautography was carried out on serum after oral doses of both materials.

RESULTS

Mean serum levels of Crithidia factor at stated time intervals after oral biopterin and tetrahydrobiopterin are given in Figure 7-1.

Fig. 7-1

Mean serum Crithidia factor levels with standard errors of means following 10 mg/tetrahydrobiopterin (taken with 2 grams ascorbic acid) and 5 mg biopterin orally (5 subjects each).



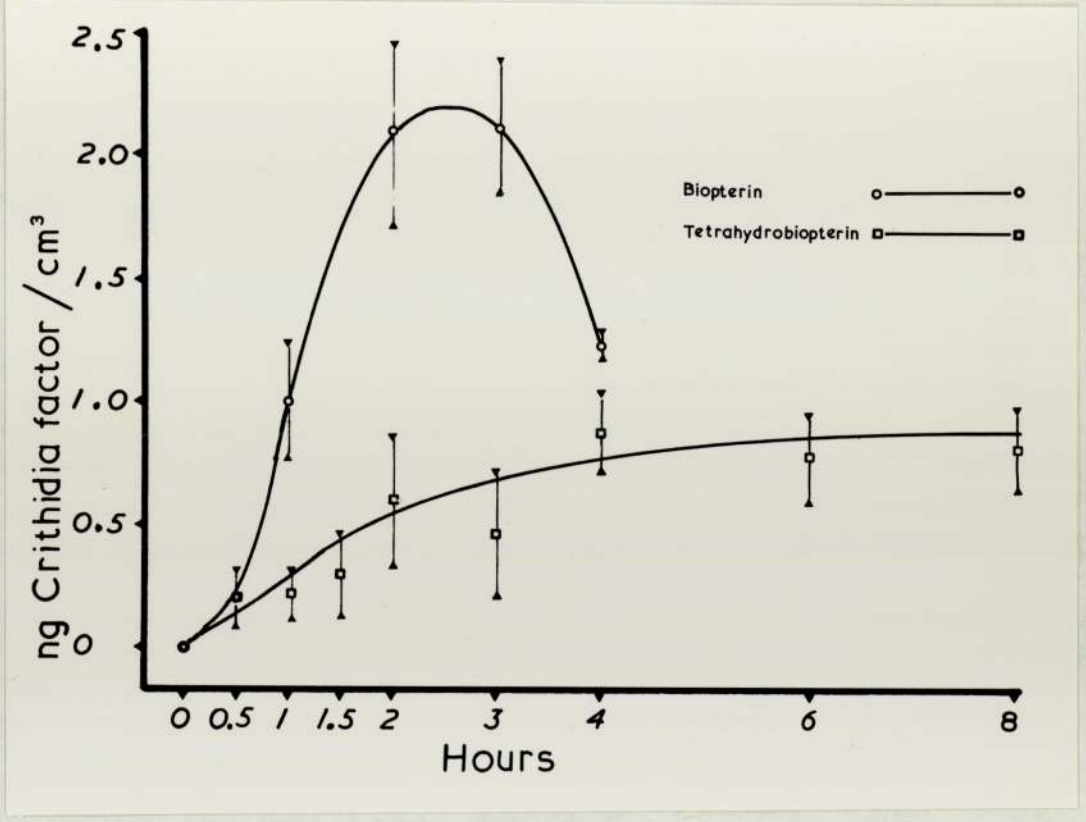
The serum value after tetrahydrobiopterin reached a level of significance when tested by Student's 't' at the 5% level only at six and eight hours following the oral dose. Biopterin produced a significant increase after one hour ($p = 0.001$ to 0.01) and this level of significance was maintained ($p = < 0.001$ for second and third hour specimens) until after the last specimen was taken at four hours although the serum concentration was dropping markedly at this interval of time

Significances of differences between serum increments are

shown in Table 7-1, also included are mean rises obtained by subtracting fasting levels as these did not coincide in the two groups and could have masked changes in the relatively small variations (Figure 7-2).

Fig. 7-2

Serum levels of Crithidia factor following 5 mg biopterin and 10 mg tetrahydrobiopterin (5 subjects each) results are expressed as mean rises above the fasting levels, standard errors of means are given.



At two and three hours after the oral doses Crithidia factor concentrations in the two groups were significantly different only when tested at the 5% level, the mean rises however were significantly different at the 2% level at one, two and three hours.

The curve drawn through serum concentrations following 10 mg of tetrahydrobiopterin without ascorbic acid to protect it from oxidation (Figure 7-3) was similar to the curve resulting from oral tetrahydrobiopterin with ascorbic acid. The mean rise following tetrahydrobiopterin

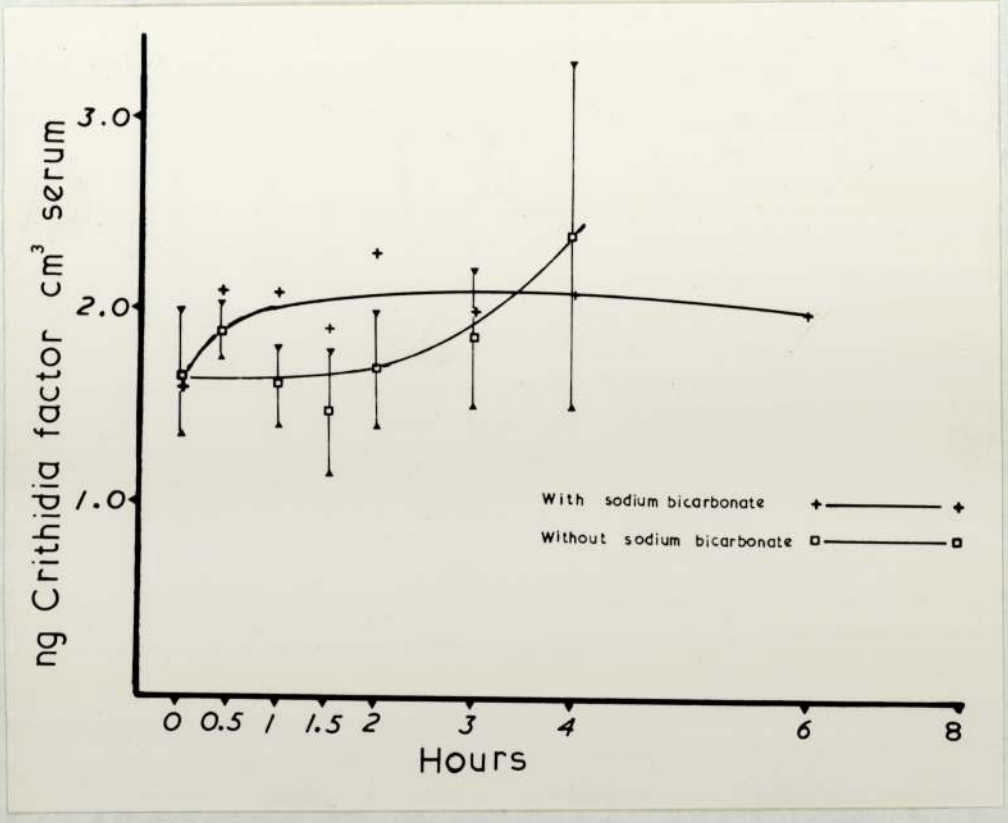
TABLE 7-1

Significance by Student's 't' test of the difference from the fasting level of Crithidia factor at timed intervals following oral biopterin and tetrahydrobiopterin.

Hours	5 mg Biopterin		10 mg Tetrahydrobiopterin	
	Mean level	Mean rise	Mean level	Mean rise
½	p = 0.5 – 0.6	p = 0.1 – 0.2	p = 0.6 – 0.7	p = 0.1 – 0.2
1	p = 0.001 – 0.01	p = 0.01 – 0.02	p = 0.5 – 0.6	p = 0.05 – 0.1
1½	–	–	p = 0.5 – 0.6	p = 0.1 – 0.2
2	p = < 0.001	p = 0.001 – 0.1	p = 0.2 – 0.3	p = 0.05 – 0.1
3	p = < 0.001	p = < 0.001	p = 0.3 – 0.4	p = 0.1 – 0.2
4	p = 0.001 – 0.01	p = 0.001 – 0.1	p = 0.1 – 0.2	p = 0.05 – 0.1
6	–	–	p = 0.02 – 0.05	p = 0.01 – 0.02
7	–	–	p = 0.1 – 0.2	p = 0.02 – 0.05

Fig. 7-3

Serum levels of Crithidia factor following 10 mg of 5,6,7,8-tetrahydrobiopterin orally taken alone (2 subjects) and with 5 g sodium bicarbonate (1 subject)



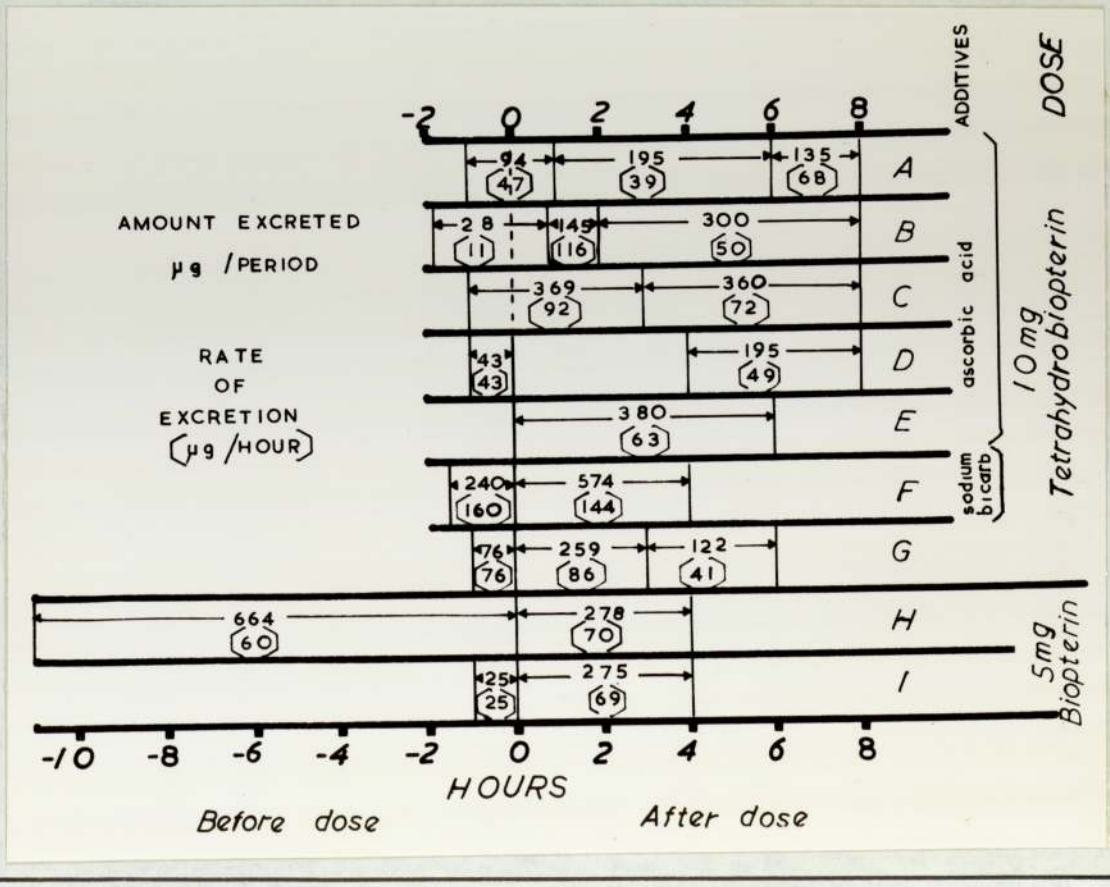
taken with sodium bicarbonate in three subjects appeared to be delayed and although these values would not bear a close statistical scrutiny the shape of the curve suggests that the absorption, such as there was, had a distinctive pattern when compared with the same material taken with ascorbic acid.

Urine levels obtained during the course of these observations are set out in Figure 7-4. Chromatography with bioautographic development in fasting serum and following tetrahydrobiopterin showed only one peak corresponding to 7,8-dihydrobiopterin. After biopterin a second peak occurred which co-chromatographed with biopterin and tetrahydrobiopterin. The 7,8-dihydrobiopterin peak at 2 hours after

5 mg biopterin was little altered from its fasting level. The new peak increased the total detected by a factor of 1.5 when checked against a standard growth curve. This factor supports the evidence from assay results, that biopterin was absorbed and caused the increase in serum Crithidia factor. Results from hepatic and antecubital vein samples did not differ substantially from each other or from results of the other similar absorption experiments with tetrahydrobiopterin. After oral biopterin and tetrahydrobiopterin no alteration in serum phenylalanine or tyrosine concentration was detected.

Fig. 7-4

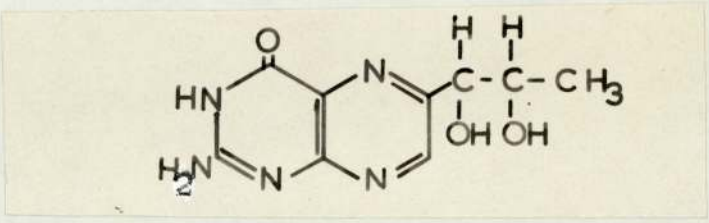
Urinary excretion of Crithidia factor following oral biopterin and tetrahydrobiopterin.



DISCUSSION

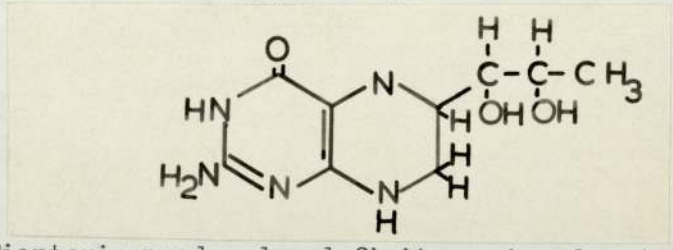
The patterns of intestinal absorption for both biopterin (1)

(1)



and tetrahydrobiopterin (2) were quite distinct from folate absorption

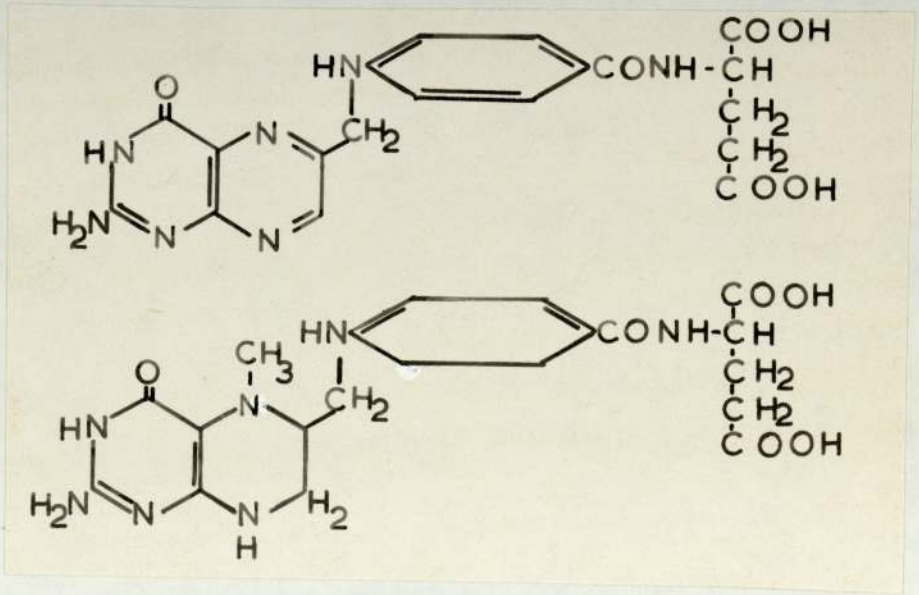
(2)



curves. Biopterin produced a definite peak value in serum at about 2 hours whereas tetrahydrobiopterin gave a slow rise without a definite peak, suggesting that absorption was continuing into the large bowel. The peak increase following oral biopterin slightly more than doubled the fasting level of serum Crithidia factor and from chromatographic evidence the compound causing this increase was biopterin or if metabolism occurred during transport (Rembold and Metzger, 1967) tetrahydrobiopterin. The increase following tetrahydrobiopterin was less, at about 50% above the fasting level and tetrahydrobiopterin was not detected in the serum by chromatography. Results following similar oral doses of folic acid (3) and 5-methyltetrahydrofolic acid(4)

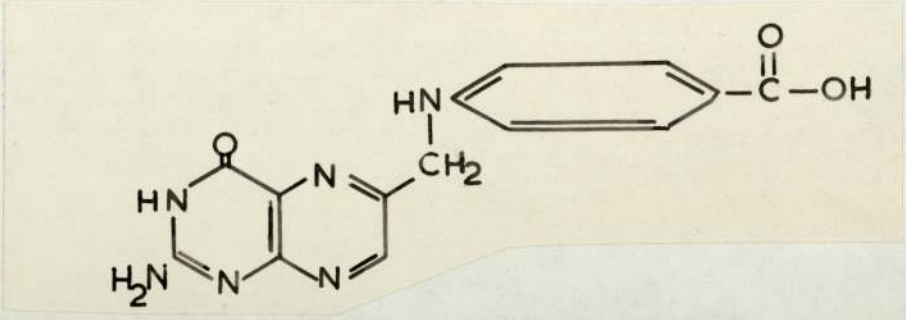
(3)

(4)



produced peak levels of 200 ng/cm^3 from a fasting level of around 5.0 ng/cm^3 (Ratanasthien et al, 1974). The difference between these two pterins and their variance from folate absorption generally with the exception of pteroic acid (5) (Brown et al, 1973), which is poorly

(5)



absorbed, suggest that there is no specific carrier system for the pterin ring and throw doubt on the proposal for a specific permease for the tetrahydropterin ring affected by the stereo chemistry at carbon 6 (Weir, Brown, Freedman and Scott, 1973)

Another suggestion that pteridines may be transported through the lipoid membrane of the small bowel as the neutral species (Blair, Johnson and Matty, 1974; Blair and Matty, 1974) in an acid microclimate of pH 3.5 could reasonably account for the poor absorption of tetrahydrobiopterin which would largely be protonated at this pH having a pKa of 5.0. This hypothesis would also explain the slow rise in serum biopterin derivative levels following oral tetrahydrobiopterin, if the pH at the surface of the intestine increases towards the ileum then there would be more unionised material available for absorption. Adenosine triphosphate decreases in concentration down the intestine (Hanninen, Hartiala and Nurmikko, 1964) and as this enzyme is concerned with hydrogen ion production (Blair, Lucas and Matty, 1972, 1973) then the acidity of the proposed microclimate will also decrease (Blair and Matty, 1974). This theory holds good for folic acid which is largely undissociated at a lower pH and which is absorbed at a slower rate from the ileum (Hepner, 1969).

The reduction of adenosine triphosphate in coeliac disease (Riekan, Stewart, Booth and Pearse, 1966) would make this group of patients an admirable model for testing the hypothesis by absorption studies with tetrahydrobiopterin and biopterin. The jejunal pH in coeliacs is greater than 7.0 as opposed to the normal pH of 6.5 and folate absorption is decreased (Benn, Swan, Cooke, Blair, Matty and Smith, 1971; Dormandy, Waters and Mollin, 1963). Following dietary treatment the adenosine triphosphate returns to normal (Riekan et al, 1966), folate absorption improves and the lumen pH returns to 6.5 (Benn et al, 1971). The poor absorption of oral pteroic acid (Brown et al, 1973; Blair, Ratanasthien and Leeming, 1974) could also be accounted for by the same reasoning. The somewhat better absorption of biopterin may be used as an extension of this logic although some other mechanism would have to be involved in view of the large discrepancy between peak serum values of biopterin and other folates after similar oral doses. Rapid urinary excretion is ruled out by the lack of increase in urinary concentration following oral doses. This leaves the alternative of binding to tissues and tissue fluids, there is some evidence to support this in Rembold and Metzgers' work on the rat (1967) when 90% of the oral dose remained within the rat and was distributed throughout the organs, although intraperitoneal administration resulted in rapid resorption and excretion. When tetrahydrobiopterin is given parentally it is also distributed throughout the organs as measured enzymatically (Stone, 1974).

Neither biopterin nor tetrahydrobiopterin appeared to affect the balance of phenylalanine and tyrosine in serum, if either material was absorbed in sufficient quantity it was either metabolically inactive or non-rate determining.

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FOR

CHAPTER SEVEN

SUMMARY

Intestinal absorption of tetrahydrobiopterin and biopterin were distinct from each other and from the absorption of other pteridines. It is suggested that an acid micro climate at the surface of the small intestine could be a primary cause of the poor absorption of tetrahydrobiopterin and rapid binding to tissues is proposed as a reason for low serum levels of oral biopterin.

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CHAPTER EIGHT

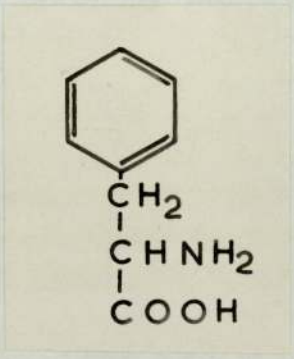
AMINO ACIDS AND BIOPTERIN DERIVATIVES

INTRODUCTION

Loading doses of phenylalanine have been used in the diagnosis of variant forms of hyperphenylalaninaemia, such challenges have been intravenous (Lambert, Vrailhet, Monot, Lepaire, Baradel, Nabet, Martin and Pierson, 1973; Woolf, Cranston and Goodwin, 1967) or oral (Hsai and Driscoll, 1956; Jervis, 1960) and have been well described. Tyrosine tolerance tests have also been carried out on normal subjects and phenylketonurics (Yo, Adams and O'Halloran, 1970) using much the same technique as the oral phenylalanine tolerance test.

Raised levels of Crithidia factor in patients with phenylketonuria (Chapter 9) prompted the examination of serum Crithidia factor following loading doses of phenylalanine (1) and tyrosine (2).

(1)



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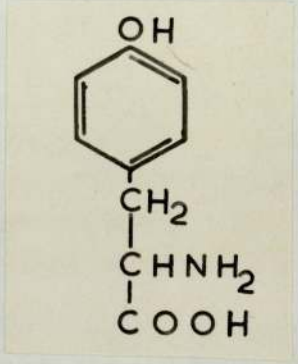
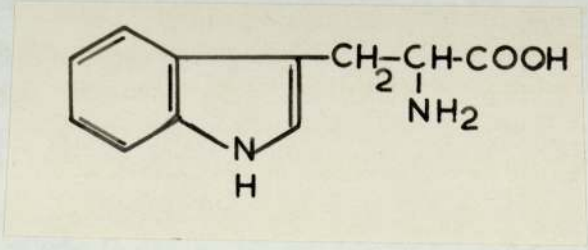


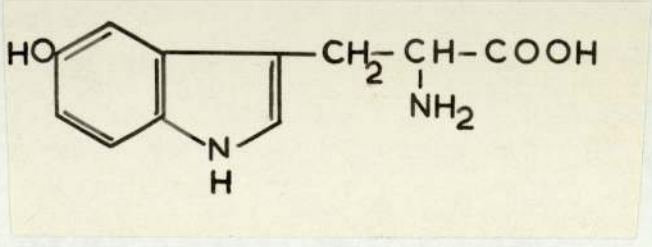
The role of reduced pteridines in the hydroxylation of tryptophan (3)

(3)



to 5-hydroxytryptophan (4) (Hosoda and Glick, 1966) suggested that

(4)



tryptophan absorption could be profitably examined in relationship to Crithidia factor levels.

METHODS

Seven volunteers took 7 g of L-β-phenylalanine orally, this quantity was determined from the standard loading dose of the phenylalanine tolerance test (Hsai and Driscoll, 1960) at 100 mg/kg body weight. Two of these volunteers had blood samples taken at 0, 15, 30 and 60 minutes and the other five at 0, 1, 2, 3 and 4 hours. Three further subjects had 1 g, 2 g and 4 g of phenylalanine with blood samples at hourly intervals for 4 hours. Two others had 7 g of tryptophan and 7 g of tyrosine with similar blood sampling. Bioautography was carried out on sera taken before and after oral phenylalanine. Crithidia factor, phenylalanine and tyrosine were measured in all serum samples.

Two subjects who had 7 g of phenylalanine produced timed and measured urine samples before and after the oral dose, only Crithidia factor was measured in urine specimens.

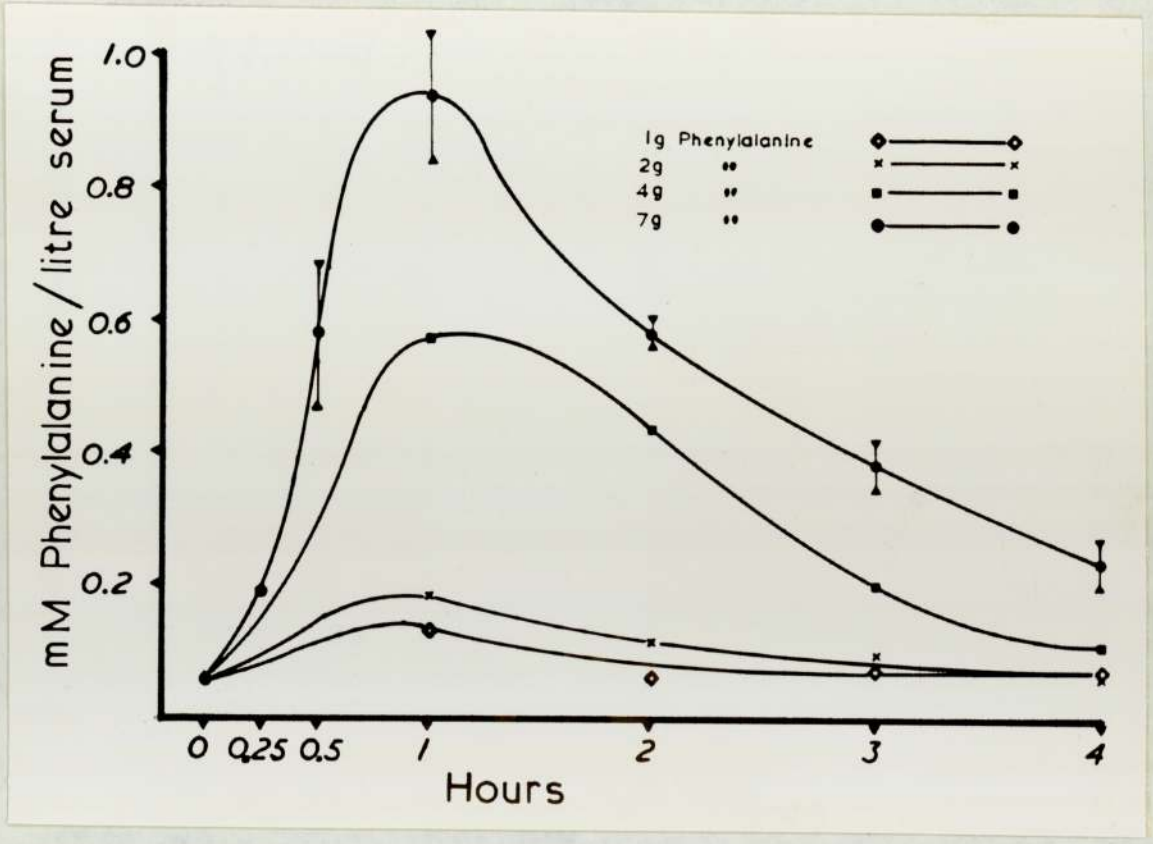
One volunteer had a phenylalanine free diet for 10 days with daily blood samples and a four hour urine collection every morning from 7.00 a.m. to 11.00 a.m.

RESULTS

Oral doses of phenylalanine produced rises in serum phenylalanine peaking at approximately 1 hour. The response in serum level increased with increase in dose, 1 g which was the smallest quantity given, produced a barely perceptible serum response (Figure 8-1).

Fig. 8-1

Mean serum levels of phenylalanine following oral doses of 1, 2, 4 and 7 grams of phenylalanine.



Serum tyrosine levels, in these same subjects, were also raised but the increases in this metabolite of phenylalanine did not bear the same proportional relationship to dose (Figure 8-2). Figure 8-3 shows the increase in serum Crithidia factor and again the levels did not have the same dose related elevation as the serum phenylalanine levels. The levels of Crithidia factor following these different oral doses did not follow the pattern of tyrosine response. These differences were further

Fig. 8-2

Mean levels of tyrosine following oral doses of 1, 2, 4 and 7 grams of phenylalanine.

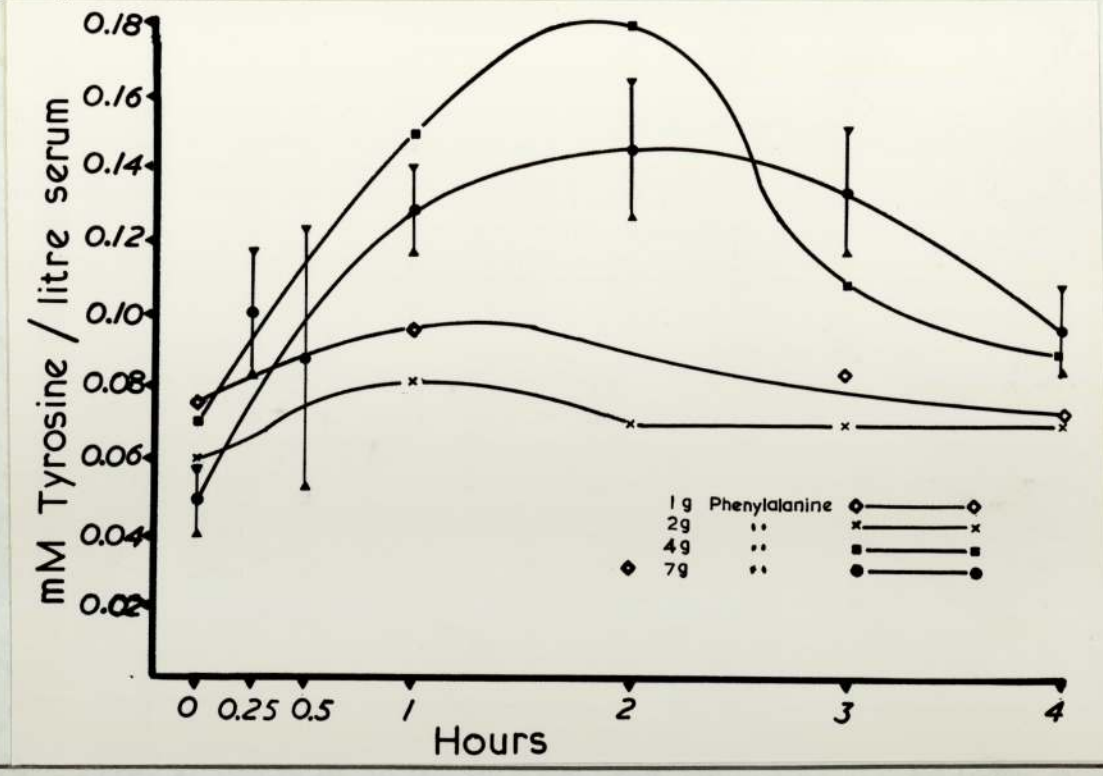
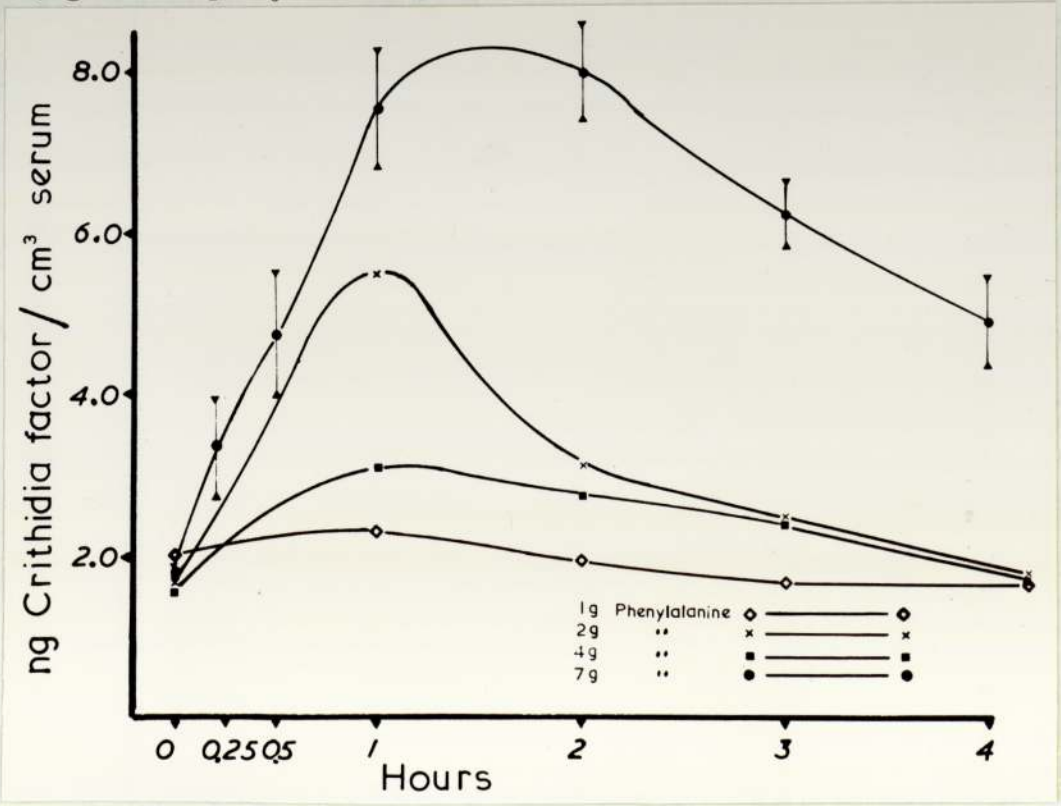


Fig. 8-3

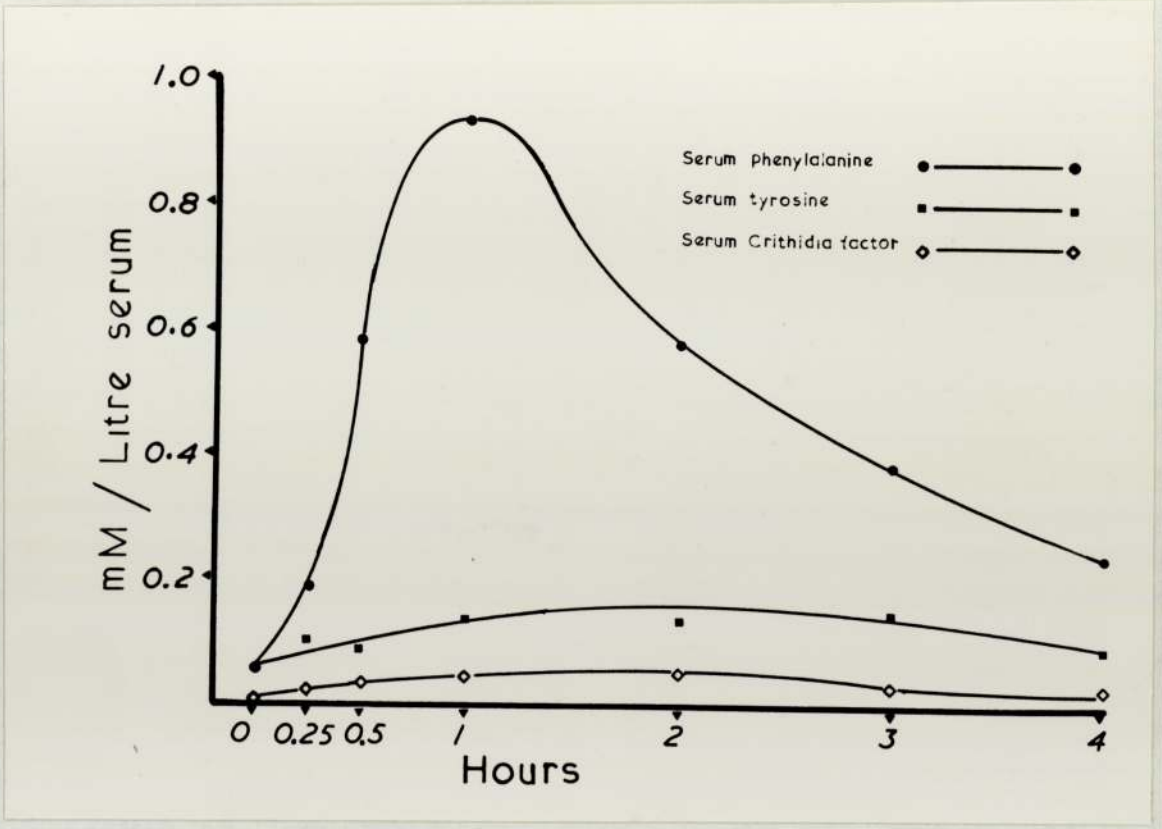
Mean serum levels of Crithidia factor following oral doses of 1, 2, 4 and 7 grams of phenylalanine.



underlined by comparison of the areas under the absorption curves which, corrected to give a value of 7 for the 7 g dose of phenylalanine, are given in Table 8-1. Figure 8-4 expresses all the values following 7 g

Fig. 8-4

Serum phenylalanine, tyrosine and Crithidia factor levels in 5 subjects following 7 grams of phenylalanine orally. All results are expressed in mM/litre to show relative values.



of phenylalanine in mM/litre and it can be seen that the phenylalanine response was far in excess (weight/weight) of the increase in either tyrosine or Crithidia factor. Of the two subjects who had timed and measured urine samples, the first was excreting Crithidia factor at 75 µg/hour before the experiment and the average for the four hour period after oral phenylalanine was 220 µg/hour. The values for the second subject were 30 µg/hour and 50 µg/hour respectively.

Figure 8-5 gives the levels of Crithidia factor, tyrosine and

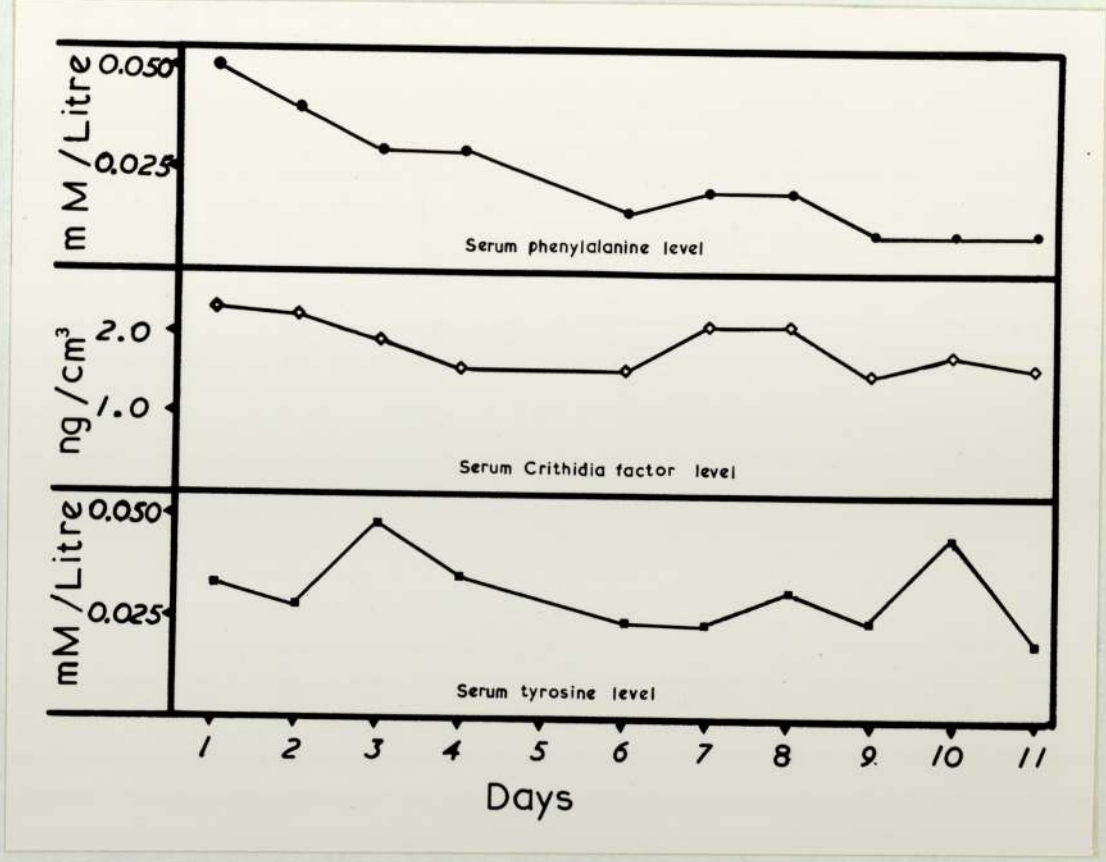
TABLE 8-1

Serum concentrations of phenylalanine, tyrosine and Crithidia factor compared as areas under absorption curves following 7, 4, 2 and 1 g of phenylalanine all corrected to give a value of 7 following the 7 g dose

	Corrected serum values		
Phenylalanine dose	Phenylalanine	Tyrosine	Crithidia factor
7 g	7	7	7
4 g	3.98	7.74	1.49
2 g	0.76	1.21	2.40
1 g	0.38	1.42	0.55

Fig. 8-5

Serum phenylalanine, tyrosine and Crithidia factor levels over 11 days in a normal adult male on a phenylalanine free diet.



phenylalanine in the serum of the subject on a low phenylalanine diet. The downward movement of the phenylalanine level over the period was not reflected to any certain degree in the concentration of tyrosine although alterations in the Crithidia factor, at some points appeared to be similar and when serum phenylalanine and Crithidia factor levels were plotted against each other there was a significant degree of correlation $p = 0.01 - 0.02$, see Figure 8-6. Figure 8-7 gives the pattern of urinary excretion in the same subject, the excretion rate was fairly constant except at the beginning and end of the experiment, these variations could easily be accounted for by daily variation or compounded sampling and experimental errors.

The elevation of serum tyrosine following oral tyrosine did not

Fig. 8-6

Serum phenylalanine plotted against serum Crithidia factor levels in a normal adult who was on a phenylalanine free diet for 11 days.

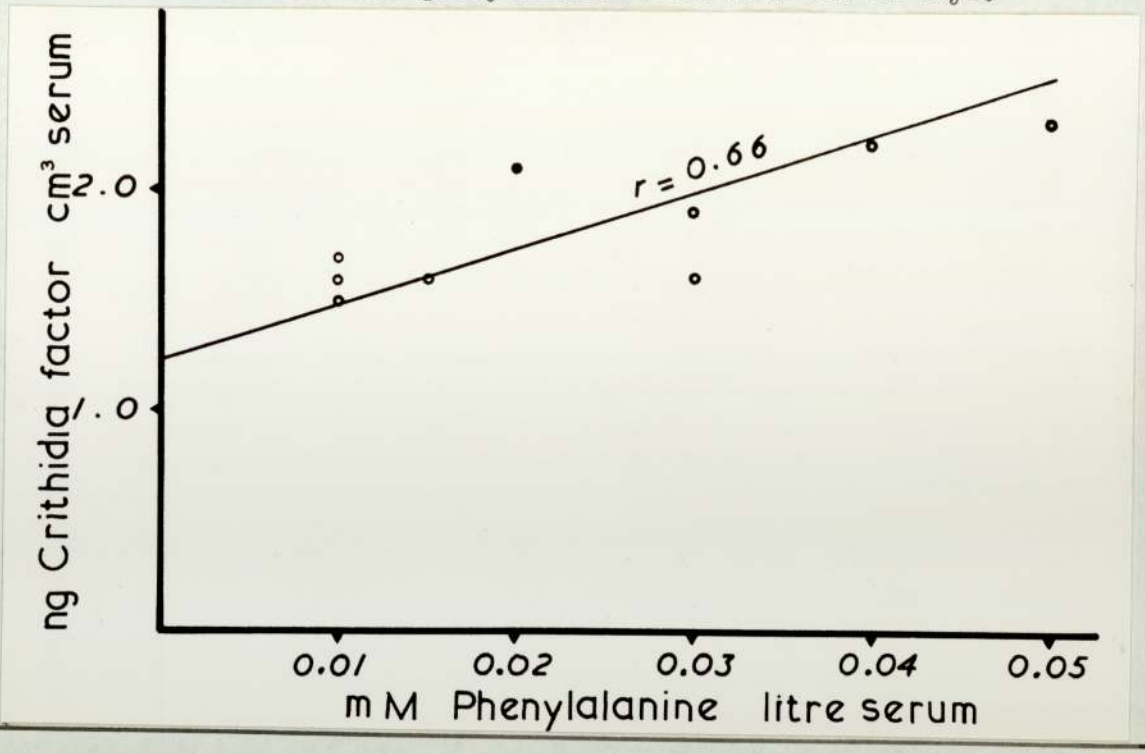
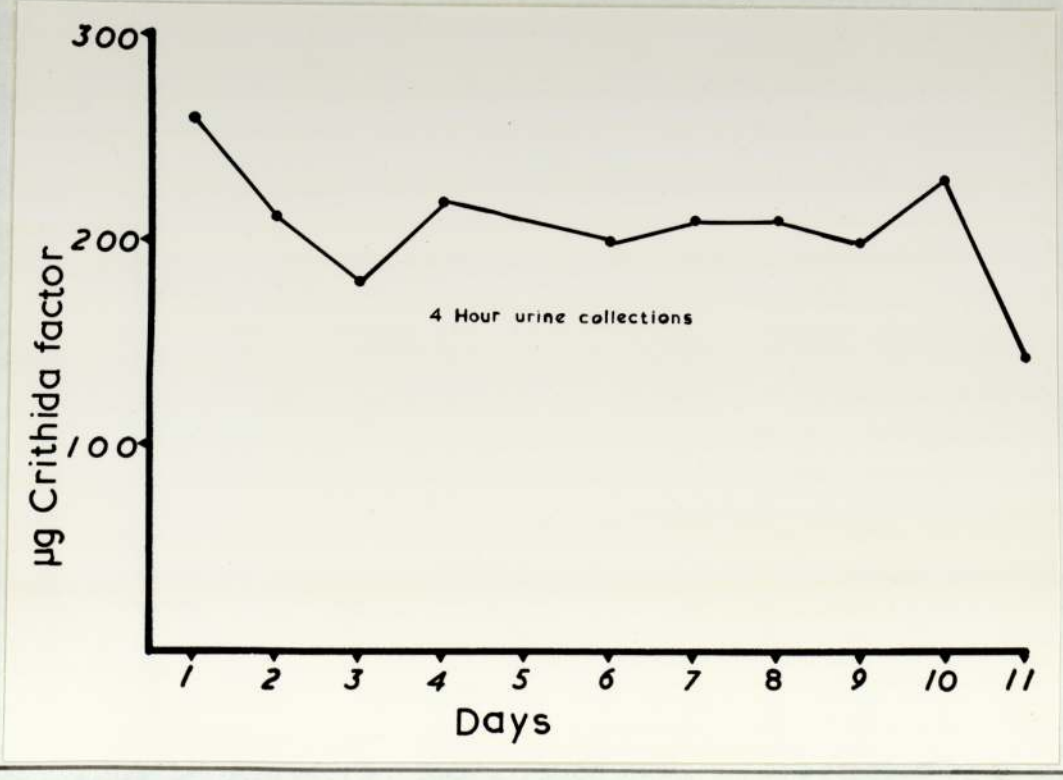


Fig. 8-7

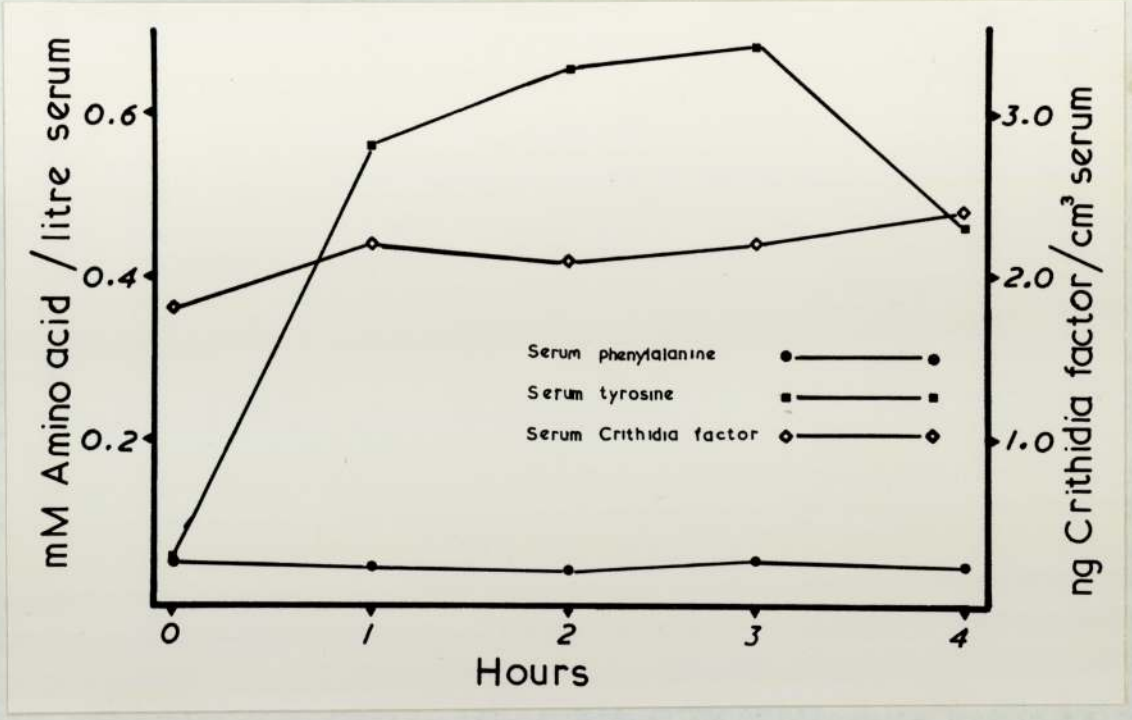
Crithidia factor excretion in the urine of a normal adult on a phenylalanine free diet over a period of 11 days.



affect Crithidia factor or phenylalanine concentrations significantly (Figure 8-8).

Fig. 8-8

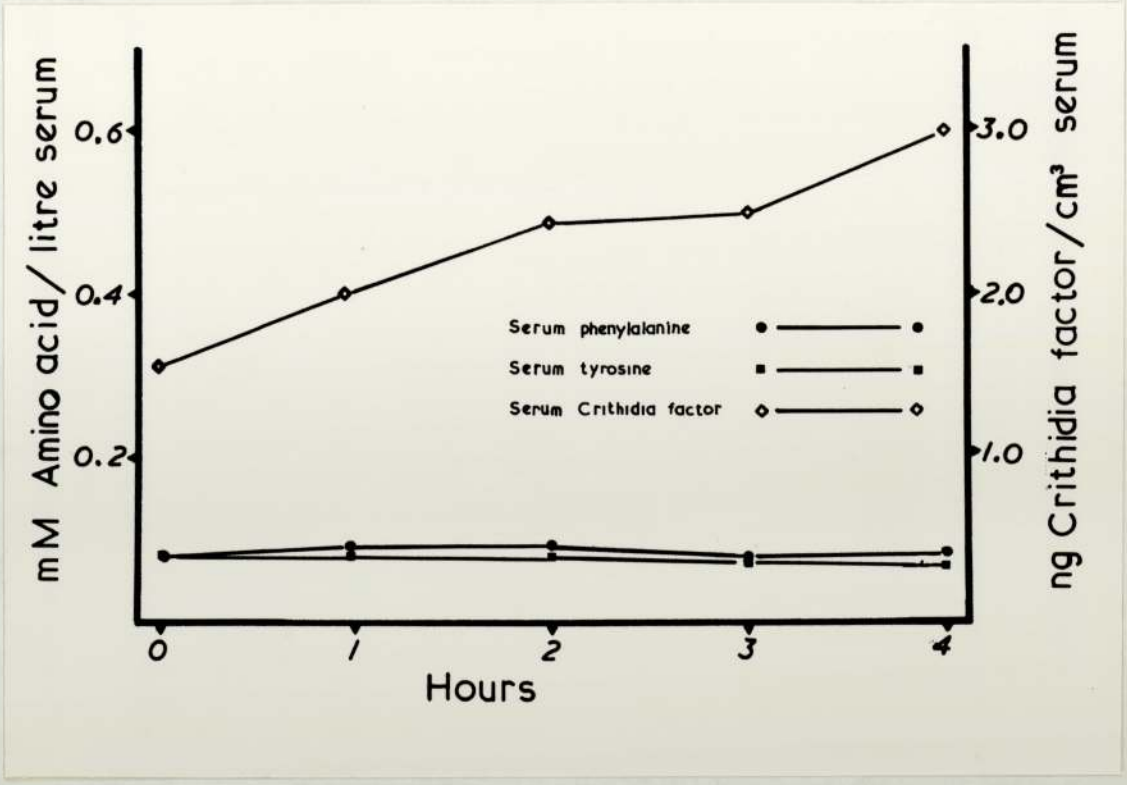
Serum levels of phenylalanine, tyrosine, Crithidia factor in 1 subject following 7 grams of oral tyrosine.



Crithidia factor was increased in the serum of the subject who took tryptophan (Figure 8-9) but not to the high levels following equal doses of phenylalanine. Unfortunately, it was not possible to obtain measurements of serum tryptophan.

Fig. 8-9

Serum phenylalanine, tyrosine and Crithidia factor levels in 1 subject following 7 grams of oral tryptophan.



DISCUSSION

The peak heights and absorption of phenylalanine were of the same order as the given doses. One would have expected this because these levels would be dependant only on absorption and metabolism which should be related to dose, providing there is no saturation effect. Tyrosine levels did not follow the same pattern of response and there was an additional factor produced by the seeming delay in reaching peak values after the higher two doses. Amino acids in tissues do not simply parallel those in serum (McKean and Peterson, 1970) and high concentrations of phenylalanine can inhibit the uptake of tyrosine by brain cells (McKean, Schanberg and Giarman, 1962), if the uptake by other tissues is also affected then the rises after varying amounts of substrates would be dependant both in rate and quantity on this variable as well as on enzyme and cofactor availability. Rises in Crithidia factor were again not strictly related to dose although the highest and lowest doses produced rises roughly in proportion to serum phenylalanine response. As tissue concentrations (Chapter 6) are vastly greater than serum concentrations of Crithidia factor (Chapter 4), one must assume either an accumulatory process or intracellular biosynthesis, either of which would make serum levels dependant on another variable. Idiosyncratic effects must also be taken into consideration, particularly in a long chain of events from rate of absorption through the triggering of metabolic processes to the utilisation of products, which have individual functions as well as supplying material for further essential metabolites. Note must be taken of the significant ($p = 0.01 - 0.02$) relationship between serum phenylalanine and Crithidia factor in the subject on a phenylalanine free diet (Figure 8-6).

The graphs (Figures 8-2, 8-3 and 8-4) show that peak levels for the three materials were at fifty minutes for phenylalanine, ninety

minutes for Crithidia factor and a hundred and ninety minutes for tyrosine. This distribution in time would appear compatible with either phenylalanine, or some metabolite of phenylalanine being the trigger for cofactor biosynthesis and the subsequent hydroxylation of phenylalanine to tyrosine.

Results following oral tyrosine were predictable, one would not expect a rise in precursor following the administration of any material. The failure of the cofactor to increase shows tyrosine is not the primary trigger in the biosynthesis of the Crithidia factor although the role of reduced pteridines in the hydroxylation of tyrosine to dopamine (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) is not disputed. The slow rise in Crithidia factor following tryptophan suggests a weaker biosynthetic stimulus unrelated to the level of phenylalanine. However, the limited data from this experiment provides insufficient information on which to base firm conclusions.

SUMMARY

Serum phenylalanine, tyrosine and Crithidia factor have been measured following oral phenylalanine, tyrosine and tryptophan. Phenylalanine produced large increases in serum phenylalanine followed by lower amounts of Crithidia factor and tyrosine. Tyrosine produced only a rise in serum tyrosine whilst tryptophan caused a small rise in the Crithidia factor level.

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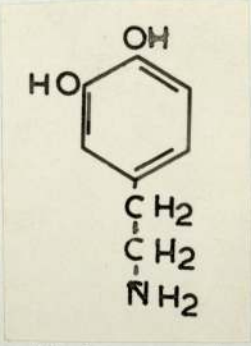
CHAPTER NINE

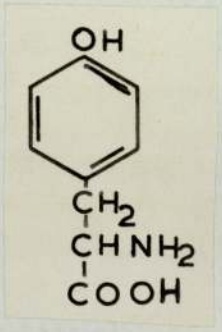
BIOPTERIN DERIVATIVES IN DISEASE

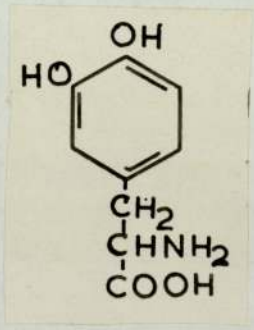
INTRODUCTION

Phenylketonuria seemed an obvious disease to study and will be considered in detail in Chapter 10. Parkinson's disease in which there is a defect in dopamine (1) production also seemed worthy of

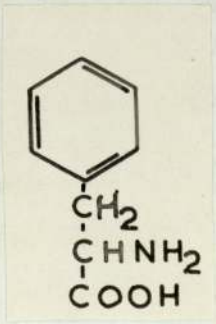
(1) 

consideration. Normally high concentrations of dopamine in basal ganglia are much reduced (Hornykiewicz, 1966). Hydroxylation of tyrosine (2) to dopa (3) is considered to be the rate limiting step in

(2) 

(3) 

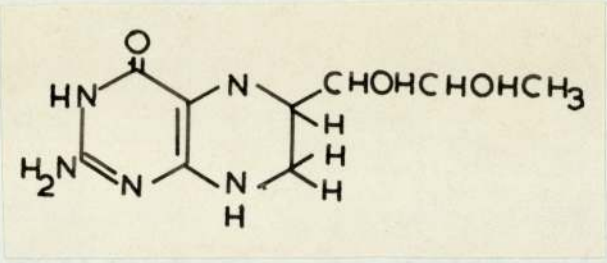
catecholamine biosynthesis (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) although from their work on guinea pig tissue it is not clear which component functions as a limiting factor. As the tyrosine level has a limited dependancy on the hydroxylation of phenylalanine (4) and

(4) 

tetrahydrobiopterin (5) is a cofactor for both these processes, Parkinson's disease could be a source of altered cofactor levels.

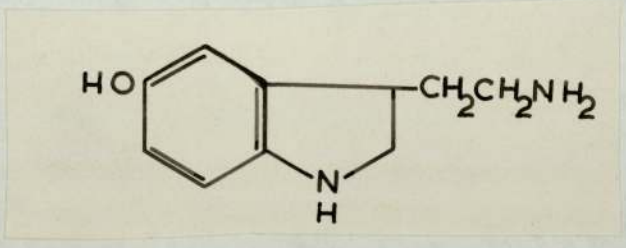
It has been proposed that there is a genetic basis for

(5)



Schizophrenia (Kallman, 1952) and further suggested that an endotoxin is produced which gives rise to psychotic behaviour. One of the normal metabolic routes for inactivation of catecholamines is by methylation of one of their hydroxyls and many compounds which can induce psychosis (as distinct from the delirium produced by such agents as atropine) are methylated derivatives of central neurotransmitters, serotonin (6) and

(6)



noradrenaline (7) (Antun, Eccleston and Smythies, 1971).

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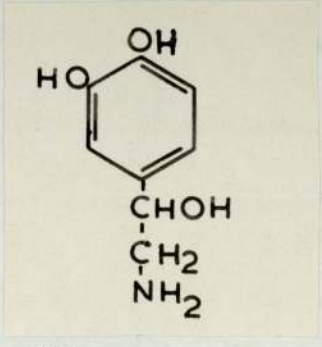
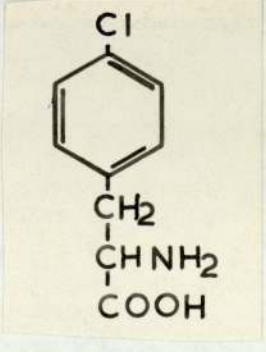


p-Chlorophenylalanine (8) is an example of a drug which specifically

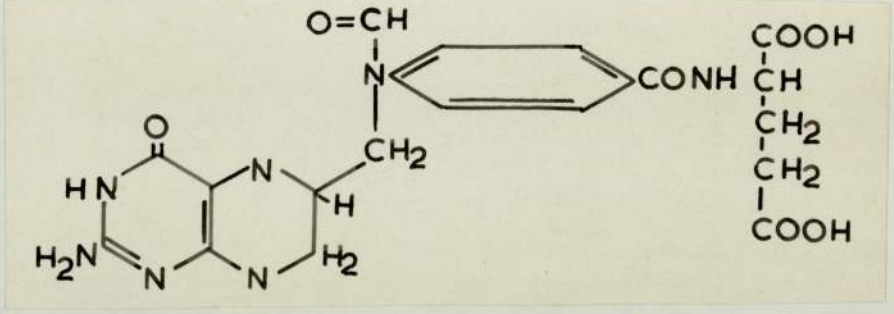
(8)



blocks formation of serotonin - cats treated with this drug show gross disturbances of behaviour and some electroencephalographic similarities to schizophrenia. Any interference with the biosynthesis of catecholamines could be reflected in the enzymatic precursor stages.

Leukaemia and carcinomatosis were chosen as examples of abnormal proliferation of cells and could be compared with psoriasis which is a non-malignant proliferative disorder. Raised rates of cellular replication have already been shown to alter the 10-formyltetrahydrofolate (9) level in rats (Sotobayashi, Rosen and

(9)



Nichol, 1966) and humans (Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974).

Pernicious anaemia with its shortened red cell survival, death of cells in marrow and altered folate metabolism, together with cirrhosis in which the degenerative state of the liver alters its storage and enzymatic capabilities seemed possible sources of pterin abnormalities.

Rheumatoid arthritis is known to be associated with folate deficiency (Carter, Ardeman, Winocour, Perry and Chanarin, 1968). It was a useful example of an inflammatory disease with an immunity component, the one disadvantage of studying this group was the impractability of finding untreated cases, most (if not all) are treated empirically before being referred to a consultant rheumatologist by the general practitioner.

With the very high gradient existing between serum and urine levels of Crithidia factor, the role of the kidney could be of considerable importance, examination in states of kidney dysfunction would supply information in this case. Protein binding of folates has already been established (Ford, 1974) and as a few specimens were available from patients with myelomatosis it was considered worthwhile examining these to see if the abnormal protein was associated with altered levels of Crithidia factor.

Epileptics on anticonvulsants are known to have lowered folate absorption (Meynell, 1966) and there is evidence of reduction in the brain barrier system in epileptics (Bakay, 1972). Both these facts could have some effect on pterin levels, firstly the source of the pterin ring (if it arises from folates) might be reduced and secondly increased permeability of the brain could release some of the biopterin derivatives which are present in high concentration in this organ.

METHODS

Biopterin derivatives were measured in serum and urine samples from patients with a variety of diseases. These patients are categorised in Table 9-1 and 9-2. The psoriatics were not in receipt of systemic treatment. The rheumatoid arthritics from whom urine samples were obtained were only taking aspirin, the others were having a variety of drugs. The schizophrenics who supplied the blood samples were taking 'Modecate' the rest were on multi-drug therapy. Patients with Parkinson's were, with one known exception, taking L-dopa. The epileptics were taking phenytoin and mostly, additionally, phenobarbitone. The phenylketonurics had varying degrees of dietary control. Patients with kidney dysfunction were bled immediately before dialysis so that the cumulative effects of persistant uraemia would be at their highest. Pernicious anaemia and regional enteritis patients were newly diagnosed, one of the myelomatous patients was bled before therapy started, the others were being controlled with cytotoxic drugs as were the other cases of neoplastic disease. The cirrhotics were in varying stages of liver damage and disease activity which conditioned their therapy.

Chromatography was carried out on urines from rheumatoid and epileptic patients and on sera from patients with kidney dysfunction, phenylketonuria and normals. Eluates were assayed for biopterin derivatives.

RESULTS

The mean serum biopterin derivative levels and standard errors are given in Table 9-1. The patients with cirrhosis, Parkinson's disease and epilepsy did not show any significant difference from the normal group. Those with proliferative disorders were all low although carcinoma patients were not significantly lower (only six samples were tested however). Patients with pernicious anaemia and those with schizophrenia also had low serum values as did the rheumatoid arthritics and patients with regional enteritis. Phenylketonurics and candidates for renal dialysis both had significantly raised results. The hospital control group of children showed very similar results to the normal adult group. Urine levels were low in controlled epilepsy but only at a 5% level of significance by Students 't' test. If, however, the number of results below 1.0 $\mu g/cm^3$ was compared with the number of normals below 1.0 $\mu g/cm^3$ the value obtained by applying the X^2 test was more significant ($p = < 0.01$). The figure for the urines of rheumatoid arthritics was significantly low and that in kidney dysfunction the lowest of all. Schizophrenics gave values which although raised were not statistically elevated, the standard error reflects the greater variation in results from this group.

Chromatography revealed the material in serum of patients with kidney disease and phenylketonuria to be the same as in normal serum (probably 7,8-dihydrobiopterin) although increased in quantity. One patient (not included in Table 9-1) with untreated myeloma had a very raised level of Crithidia activity (70 ng/cm^3) and chromatography exposed a second material which co-chromatographed with biopterin and tetrahydrobiopterin. The urine of epileptics and rheumatoid arthritics showed two peaks identical to those found in normal urine, probably tetrahydrobiopterin and 7,8-dihydrobiopterin.

TABLE 9-1

Serum levels of biopterin derivatives in disease and the significance of their variation from normal

Disease	No. of observations	Means with standard error	Significance by Students 't'
1A	No statistically significant difference from normal		
Normal adults	114	1.81 ± 0.06 ng/cm^3	Control
Children (Hospital control) and peer group for phenylketonuria	10	1.78 ± 0.25 ng/cm^3	Control
Carcinomatosis	6	1.42 ± 0.30 ng/cm^3	0.2 - 0.3
Cirrhosis	20	1.63 ± 0.10 ng/cm^3	0.1 - 0.2
Controlled epilepsy	18	1.93 ± 0.14 ng/cm^3	N.S.
Parkinsons	25	2.05 ± 0.17 ng/cm^3	0.2 - 0.3

continued over....

....continued

1B		Higher than normal	
Kidney dysfunction	4	8.75 ± 0.98 ng/cm^3	< 0.001
Phenylketonuria	30	4.86 ± 0.51 ng/cm^3	< 0.001
1C		Lower than normal	
Leukaemia	25	1.30 ± 0.12 ng/cm^3	< 0.001
Pernicious anaemia	4	1.0 ± 0.22 ng/cm^3	< 0.001
Psoriasis	9	1.43 ± 0.08 ng/cm^3	< 0.001
Schizophrenia	17	1.51 ± 0.07 ng/cm^3	0.001 - 0.01
Regional enteritis	5	0.94 ± 0.21 ng/cm^3	< 0.001
Rheumatoid arthritis	55	1.46 ± 0.11 ng/cm^3	0.001 - 0.01
Myelomatosis	3	1.17 ± 0.20 ng/cm^3	0.001 - 0.01

TABLE 9-2

Urine levels of biopterin derivatives in disease and the significance of their variation from normal

Disease	No. of observations	Mean with standard error	Significance by Students 't'
Normal	60	2.1 ± 0.19 $\mu g/cm^3$	Control
Rheumatoid arthritis	17	0.75 ± 0.18 $\mu g/cm^3$	< 0.001
Controlled epilepsy	16	1.41 ± 0.35 $\mu g/cm^3$	0.05 - 0.1
Kidney dysfunction	4	0.077 ± 0.048 $\mu g/cm^3$	< 0.001
Schizophrenia	20	3.55 ± 1.09 $\mu g/cm^3$	0.1 - 0.2

DISCUSSION

The levels found in kidney disease were much further from the normal ranges in serum and urine than any other group. Normally a very restricted range is found in both serum and urine although the urinary concentration is to some extent dependant on urinary flow (Chapter 5). This block in excretion suggests that the kidney plays a vital role in maintaining the serum level within narrow limits, this view is supported by the extremely low concentrations found in the urine of these patients. It must be accepted however, that other constituents of the body and its fluids are in some disarray in the uraemic condition and physiological function may be impaired to the extent of affecting pathways to biopterin synthesis or homeostatic mechanisms which could act concurrently with reduced excretion. Bioautography of serum from these patients showed a raised level of the Crithidia active constituent present in normal serum.

The proliferative disorders all showed low serum levels (the small number from patients with carcinoma not being significantly so), suggesting that increased metabolic activity drains the cofactor. It would be interesting to estimate Crithidia factor during periods of intense activity and in anxiety states. The low levels in the few patients with pernicious anaemia can be compared with the proliferative disorders in terms of cellular turnover in the bone marrow affecting the tissue levels of biopterin derivatives. Increase in biopterin synthesis has been demonstrated in the rat following inhibition of nucleic acid synthesis (Rembold and Gyure, 1972).

The patients with schizophrenia showed significantly depressed serum levels which were unlikely to have been caused by the 'Modecate' acting in brain tissue if, as the manufacturer states, the material does

not pass the blood brain barrier. Altered metabolism could be involved and if an abnormal metabolite occurs in schizophrenia which inactivates catecholamines (Antun, Eccleston and Smythies, 1971) then this in turn could depress the rate of synthesis of pteridine cofactor.

In Parkinson's disease the mean serum level was slightly above that for normals but not significantly so ($p = 0.2 - 0.3$). The main difficulty with this group of patients lay in finding some who were unmedicated, most were being treated with L-dopa. It may be significant that the one man who was certainly not medicated had a serum level of 5.2 ng/cm^3 which was considerably in excess of the highest value for a normal subject (3.8 ng/cm^3). Others have pointed out the possible value of obtaining biopterin levels in Parkinsonism (Baker, Frank, Bacchi and Hutner, 1974) and of course any changes may not necessarily be reflected solely, if at all, by alterations in serum levels if the blood brain barrier is intact. In rats tetrahydrobiopterin has difficulty in penetrating this barrier in the opposite direction (Kettler, Bartholini and Pletscher, 1974).

The cirrhotics did not have significantly different levels from the normals in contrast to the findings of Baker et al (1974) who found raised levels in patients with alcoholic liver disease which they assumed to reflect impairment of biopterin catabolism accompanied by lesser impairment in biopterin synthesis, leading to biopterin accumulation. This view is not in accord with the narrow normal range which would make such an accumulation an unlikely occurrence dependant on the disturbance of a very strictly maintained balance. The epileptics on anticonvulsants were not at variance with normal subjects. The 'Extra Pharmocopoeia' (Martindale, 1972) lists the multifocal side-effects of diphenylhydantoin which is hydroxylated in the liver (Butler, 1957). Diphenylhydantoin also increases sodium transport from cells

(Woodbury, 1955) thus decreasing intracellular sodium concentration which in turn implies altered uptake and discharge of metabolites by cells. Other complications of diphenylhydantoin therapy include leucopenia, thrombocytopenia and erythroid aplasia (Sparberg, 1963). The lowered results found in the urine of these patients could be explained by a partial block in biopterin synthesis which might have been balanced by a reduced urinary output to maintain the serum level. If this was so it would reinforce the role of the kidney in homeostatic control.

That three of the four myelomatous patients had significantly lower serum levels than normal is in agreement with the other malignant diseases. However one patient had a very raised level (70 ng/cm^3) in plasma taken off for replacement with protein free substrate prior to maintainance on cytotoxic drugs. Protein binders for folate are currently of interest to haematologists (Waxman, 1975) and nutritionists (Ford, 1974) alike and the possibility suggested by this finding is that there is a protein with an affinity for biopterin or its derivatives and in this particular patient it was stimulated to over production. Some reservation is indicated as the patient had access to drugs and may have had unrecorded treatment. Chromatography in 3% ammonium chloride, 5% acetic acid and 0.5% sodium carbonate showed a sharp increase in the normally undetectable (in serum) component of Crithidia factor which co-chromatographed with biopterin and tetrahydrobiopterin. The other material normally found in serum and previously described as 7,8-dihydrobiopterin was also raised. Whether this is purely a quantitative observation or a more fundamental departure from the normal is unclear from the data available.

As the Crithidia assay showed a low level of growth in the absence of added biopterin, any inhibitory substance would have been

apparent in chromatography. Therefore inhibition by drugs or abnormal metabolites can be ruled out as causing low results in those cases where chromatography was carried out.

SUMMARY

Biopterin levels have been measured in a variety of disease states. Malignant disease, psoriasis, pernicious anaemia, rheumatoid arthritis, regional enteritis and schizophrenia gave low serum levels whilst kidney dysfunction and phenylketonuria gave raised results.

Low urinary levels were found in controlled epilepsy, rheumatoid arthritis and kidney dysfunction. The possible origins of these variations from normality have been discussed.

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CHAPTER TEN

BIOPTERIN DERIVATIVES IN PHENYLKETONURIA

INTRODUCTION

Phenylketonuria is characterised clinically by mental retardation and convulsive seizures, even in the presence of severe psychological symptoms, neurological symptoms are not noticeable. Fölling (1934) was the first to demonstrate phenylpyruvic acid in the urine of imbeciles and to draw attention to the association. Akert (1966) simulated the disease in rats with high doses of L-phenylalanine and produced faults in orientation and timing but no striking changes in brain tissue. However, electron microscopy did reveal consistant changes in glial cells in the cerebral and cerebellar cortex, astrocyte mitochondria were enlarged and granules were present in the matrix. Granulation increased as the disease progressed. Anderson, Rowe and Guroff (1971) treated baby rats with high doses of phenylalanine and demonstrated that the weights of their brains did not show the increase of controls. Rats which were allowed to reach the adult state gave poor performances in problem solving even long after cessation of treatment. Geller and Yuwiler (1969) had already shown that dietary phenylalanine was more effective in producing experimental phenylketonuria in juvenile rats than in adults. Prensky, Fishman and Daftar (1974) demonstrated that hyperphenylalanaemic insult with 4 mg/g/day phenylalanine on the eighth to twelth days of life permanently affected the cell replication and growth of rat cerebellum but not of the cerebrum.

The disease arises from an inability to metabolise phenylalanine to tyrosine, phenylalanine hydroxylase is not the only enzyme to act on phenylalanine but reactions other than conversion to tyrosine are relatively slow. Abnormal metabolites in the serum include ortho-hydroxyphenylacetic acid and phenylacetic acid which is conjugated with glutamine and excreted in the urine as phenylacetylglutamine (Woolf, 1951), although urinary phenylpyruvic acid is usually the first

material to identify the existance of the disease in neonates. About 1g of phenylpyruvic acid is excreted each day although this varies with diet (Penrose, 1955); oral phenyllactic acid, phenylpyruvic acid and phenylalanine all effect the excretory rate although tyrosine and alanine do not have any influence on urinary phenylpyruvic acid (Cowie, 1951). 15 g of phenylalanine can cause normal human subjects to excrete noticeable quantities of phenylpyruvic acid (Penrose and Quastel, 1937). Although, in phenylketonuria, the serum level of phenylalanine is invariably raised there is no clear evidence of a linear relationship between daily phenylalanine intake and brain damage (B.M.J. 1971). Transmission of the disease is via a recessive gene and the propositus is always homozygous.

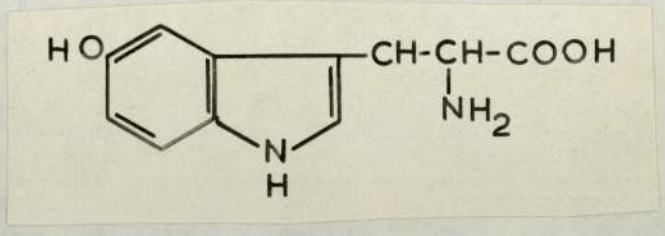
The suggestion by Woolf and Vulliamy (1951) that the mental abnormalities were the result of intoxication by phenylalanine or its abnormal metabolites stimulated the use of dietary treatment which has proved beneficial particularly if initiated in the first year of life when myelin is being laid down at the maximum rate and fundamental learning is occurring. However, if the level of phenylalanine in the diet is too low, wasting and death occur (Moncrieff and Wilkinson, 1961).

Cowie, (1951), and Woolf (1963) are among those who have described 'atypical phenylketonurics' of normal intelligence and without history of epilepsy who were able to convert phenylalanine to tyrosine at a rate higher than typical phenylketonurics though much lower than normal individuals. Transient hyperphenylalaninaemia has also been described (Scriver, 1967) and evidence has been presented (Woolf, Cranston and Goodwin, 1967) of a variant phenylalanine hydroxylase which loses its activity at high concentrations of phenylalanine.

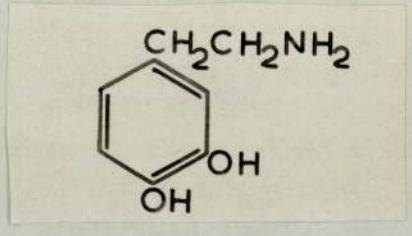
A further atypical phenylketonuria has been described which was accompanied by severe progressive neurological illness unresponsive

to dietary treatment (Smith and Lloyd, 1974; Smith, 1975). All three reported children lost their social awareness by 18 months and died at ages 2, 6 and 7 years of bronchopneumonia. It was postulated that there was a defect in the metabolism of biopterin derivatives, possibly by an abnormality in dihydropteridine reductase.

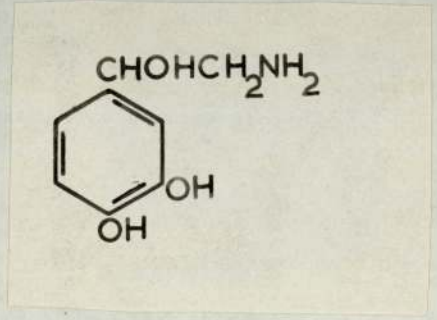
Phenylalanine in high concentration inhibits the uptake of tyrosine and 5-hydroxytryptophan (1) by brain cells (McKean, Schanberg

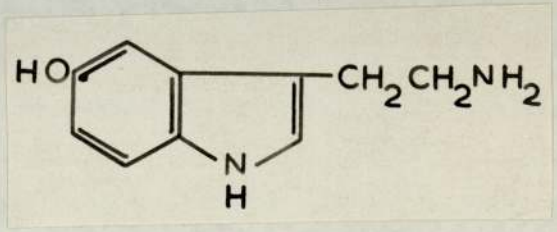
(1) 

and Giarman, 1962). These amino acids are precursors of dopamine (2),

(2) 

noradrenaline (3) and serotonin (4) and restriction of their entry into

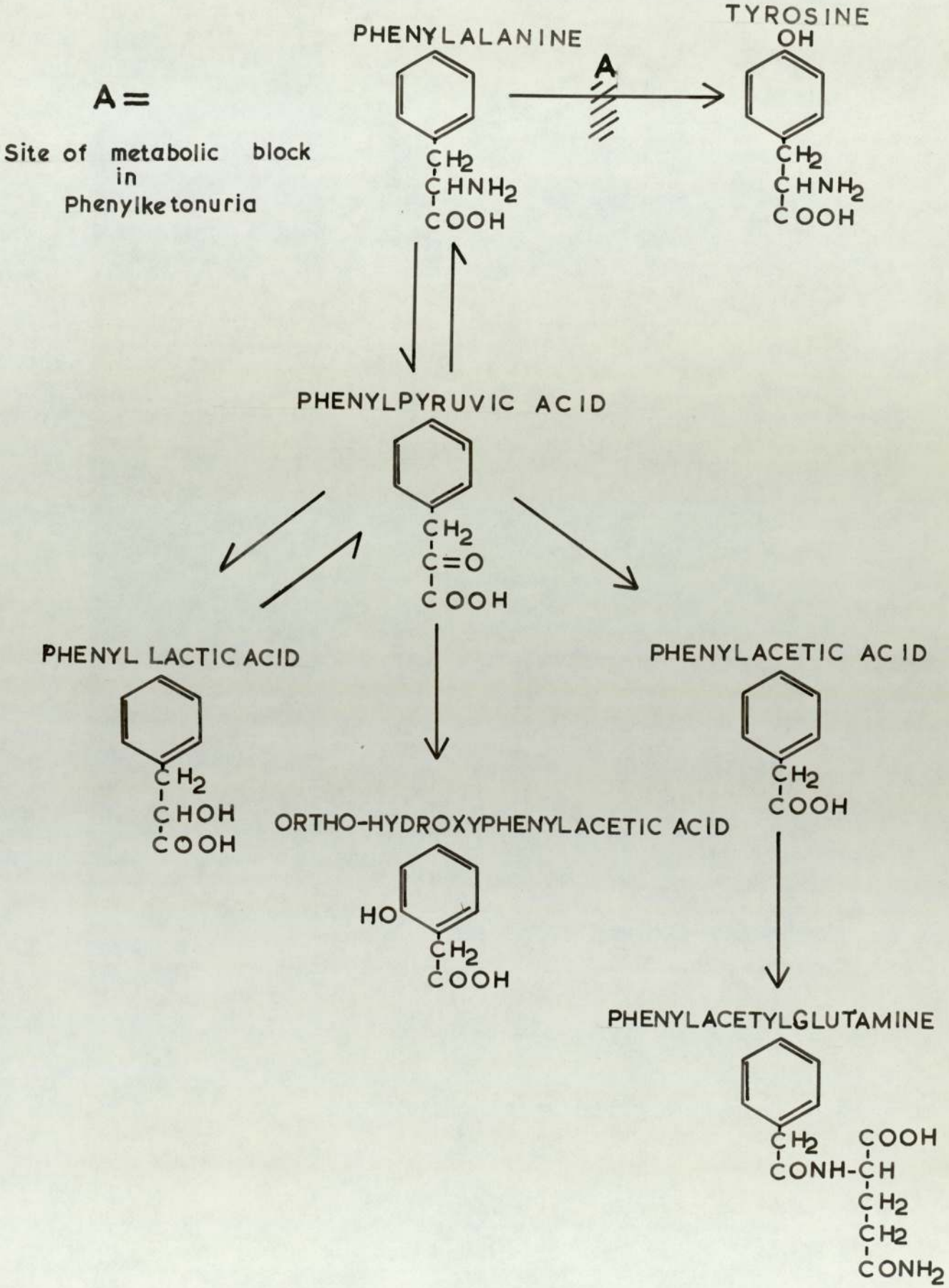
(3) 

(4) 

neurones could reduce the production of neuro-hormones. This phenomenon could account for the reversible behavioural component of phenylketonuria, the irreversible component being structural damage to the brain during the first year of life. (Woolf, Griffiths, Moncrieff, Coates and Dillistone, 1958; Crome, Tymms and Woolf, 1962).

When it was found that serum Crithidia factor was increased in children with phenylketonuria it was assumed that this had a bearing on the metabolism of phenylalanine and a study was carried out into the relationships between phenyalanine, tyrosine and Crithidia factor.

PATHWAYS IN PHENYLALANINE METABOLISM



METHODS

For the purposes of this study, children were used who were attending hospital for diagnosis and dietary control. Altogether there were 21 patients and a total of 34 samples were analysed with up to 4 separate specimens from those who attended successive clinics for dietary assessment. The aim of the diet was to maintain the plasma phenylalanine between 0.8 mM/litre and 0.48 mM/litre. Plasma phenylalanine, tyrosine and Crithidia factor levels were measured in all cases. The determination of phenylalanine and tyrosine was carried out on a Technicon Multisample Analyser by a modification of the technique of Cooke and Raine (1970).

Sera from 10 children who were in hospital for other reasons were assayed for Crithidia factor.

Bioautography of serum was carried out by streaking across the width of 20 cm thin layer cellulose plates, running to 15 cms in the dark in 0.5% sodium carbonate, 3% ammonium chloride and 5% acetic acid, scraping at 1.5 cm intervals and eluting with 0.5 M phosphate buffer pH 5.0. The eluates were assayed with Crithidia fasciculata. Phenylalanine, tyrosine, phenylpyruvic acid and ortho-hydroxyphenylacetic acid were chromatographed in the same solvents. The amino acids were identified with 'ninhydrin' the phenylpyruvic acid with ferric chloride reagent (Smith, 1968) and the ortho-hydroxyphenylacetic acid by spraying the plates with 10% sodium carbonate followed by 0.9% sulphanilic acid in 9% hydrochloric acid 1:1 with 5% sodium nitrite (Smith, 1968).

It was found that oral phenylalanine in normals raised the serum concentration of Crithidia factor in addition to the anticipated increase in phenylalanine and tyrosine (Chapter 8). Specimens obtained between $\frac{1}{2}$ and 4 hours after 1 g to 7 g oral doses of phenylalanine were assayed for these three compounds and compared with results from

phenylketonurics. One child who presented as a probable phenylketonuric on preliminary screening was given a diagnostic loading dose of phenylalanine 100 mg/kg, this patient later proved to have transient hyperphenylalaninaemia.

RESULTS

The first finding was a significantly raised mean level of Crithidia factor in the group (4.86 ± 0.52 ng/cm^3) $P = <0.001$ by Student's 't' when compared with normal adults and non-phenylketonuric children (Table 10-1). In only one case was the plasma phenylalanine level of less than 0.48 mM/litre, accompanied by a Crithidia factor level of greater than 4.0 ng/cm^3. (0.45 mM/litre phenylalanine and 6.0 ng/cm^3 Crithidia factor). Where normal subjects were given phenylalanine, the levels of serum Crithidia factor were raised and of 33 estimations at different times during the experiment, 7 had phenylalanine levels greater than 4.0 ng/cm^3 with phenylalanine levels of less than 0.48 mM/litre (Figure 10-1). This is suggestive of ($P = 0.025$ to 0.05) by Xy^2 of a different relationship between the two parameters in the patients with phenylketonuria from normals who have serum Crithidia factor levels raised by stimulation of the biosynthetic process. Yates correction was applied to the standard x^2 test because the sample size was relatively small and the binomial distribution skewed.

In four children who were observed for up to 5 months, the serum Crithidia factor, with one exception, only dropped below 4.0 ng/cm^3 when dietary success was accomplished as indicated by a plasma phenylalanine of less than 0.48 mM/litre (Table 10-2).

Bioautography showed the material in sera with raised Crithidia factor concentrations from PKU patients to be chromographically identical in the three solvent systems to that obtained from normal subjects and to 7,8-dihydrobiopterin. Further chromatography showed that phenylalanine and the available normal and abnormal metabolites of phenylalanine had distinctive Rf values and these are given in Table 10-3.

TABLE 10-1

Phenylalanine, Tyrosine and Crithidia factor levels in phenylketonurics, non-phenylketonuric children, fasting normals and normals ½ to 4 hours after 7 g phenylalanine

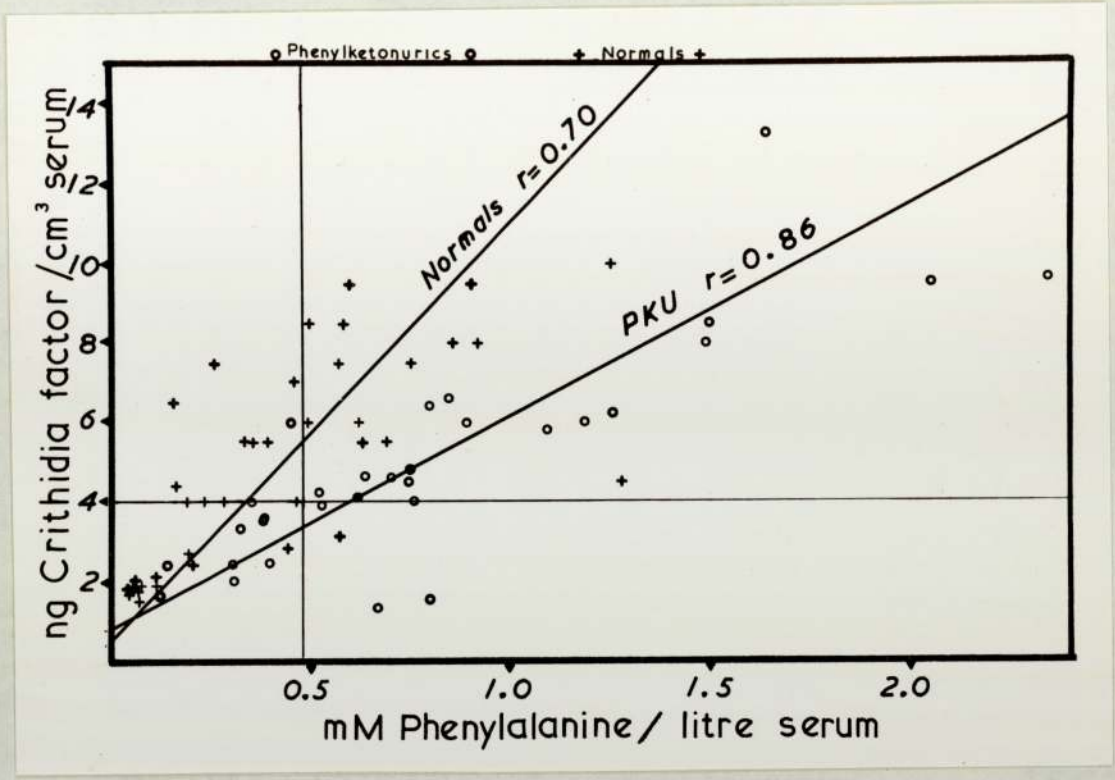
	Phenylketonuric children	Non-phenylketonuric children	Fasting normal adults	Normal adults following 7 g Phenylalanine
Serum phenylalanine mM/litre	(34) 0.771 ± 0.102	*	(10) 0.065 ± 0.009	(26) 0.542 ± 0.061
Serum Tyrosine mM/litre	(34) 0.068 ± 0.08	*	(10) 0.069 ± 0.011	(26) 0.122 ± 0.007
Serum Crithidia factor ng/cm^3	(34) 4.86 ± 0.52	(10) 1.78 ± 0.25	(10) 1.87 ± 0.06	(26) 6.35 ± 0.39
Urinary Crithidia factor $\mu g/cm^3$	(3) 2.46 ± 0.98	(5) 2.26 ± 0.18	(114)2.1 ± 0.19	(4) 4.0 ± 1.2

Figures in brackets are the number of samples assayed

* Test not carried out

Fig. 10-1

Distribution of Crithidia factor levels and phenylalanine levels in the serum of phenylketonurics compared with those from normal adults at varying times following oral phenylalanine.



Urinary levels, in the few cases where these are available, were well within the normal range even when serum levels were elevated (Table 10-1).

When the serum levels of tyrosine and Crithidia factor were plotted against each other (Figure 10-2) the lowest level of tyrosine in the normal group with Crithidia factor levels above 4.0 ng/cm^3, was 0.54 mM/litre. There were thirteen patients with phenylketonuria who had values for tyrosine below 0.54 mM/litre with Crithidia factors in this elevated range. Figure 10-3 shows the distribution of phenylalanine and tyrosine levels and includes two values from the patient with transient hyperphenylalaninaemia.

TABLE 10-2

Serum Crithidia factor, phenylalanine and tyrosine levels in four phenylketonuric children over periods of up to 5 months

Subject	Crithidia factor ng/cm^3	Phenylalanine mM/litre	Tyrosine mM/litre	Date
A	4.2	0.52	0.05	29th Jan.
	4.6	0.64	0.04	7th May
	6.0	0.89	0.04	6th Aug.
B	6.3	0.70	-	9th April
	6.6	0.85	0.05	7th May
	6.4	0.80	0.04	6th June
	6.0	1.19	0.05	6th Aug.
C	5.8	1.095	0.052	8th April
	2.4	0.396	0.09	24th April
	2.0	0.307	0.041	1st July
	3.5	0.378	0.073	14th Sept.
D	1.3	0.67	0.04	6th June
	9.6	2.36	0.07	13th July
	10.8	1.6	-	15th July
	3.6	0.378	0.073	24th Sept.

TABLE 10-3

Rf values of phenylalanine and some of its abnormal metabolites compared with biopterin, 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin

	Solvent Systems		
	5% Acetic acid	0.5% Sodium carbonate	3% Ammonium chloride
Phenylalanine	0.83	0.85	0.87
Tyrosine	0.75	0.83	0.76
Phenylpyruvic acid	0.95	0.93	0.91
Ortho-hydroxyphenylacetic acid	0.86	0.93	0.91
Biopterin	0.7	0.65	0.55
7,8-dihydrobiopterin	0.55	0.45	0.3
5,6,7,8-tetrahydrobiopterin	0.7	0.65	0.55

Fig. 10-2

Distribution of Crithidia factor levels and tyrosine levels in the serum of phenylketonurics compared with those from normal adults at varying times following oral phenylalanine.

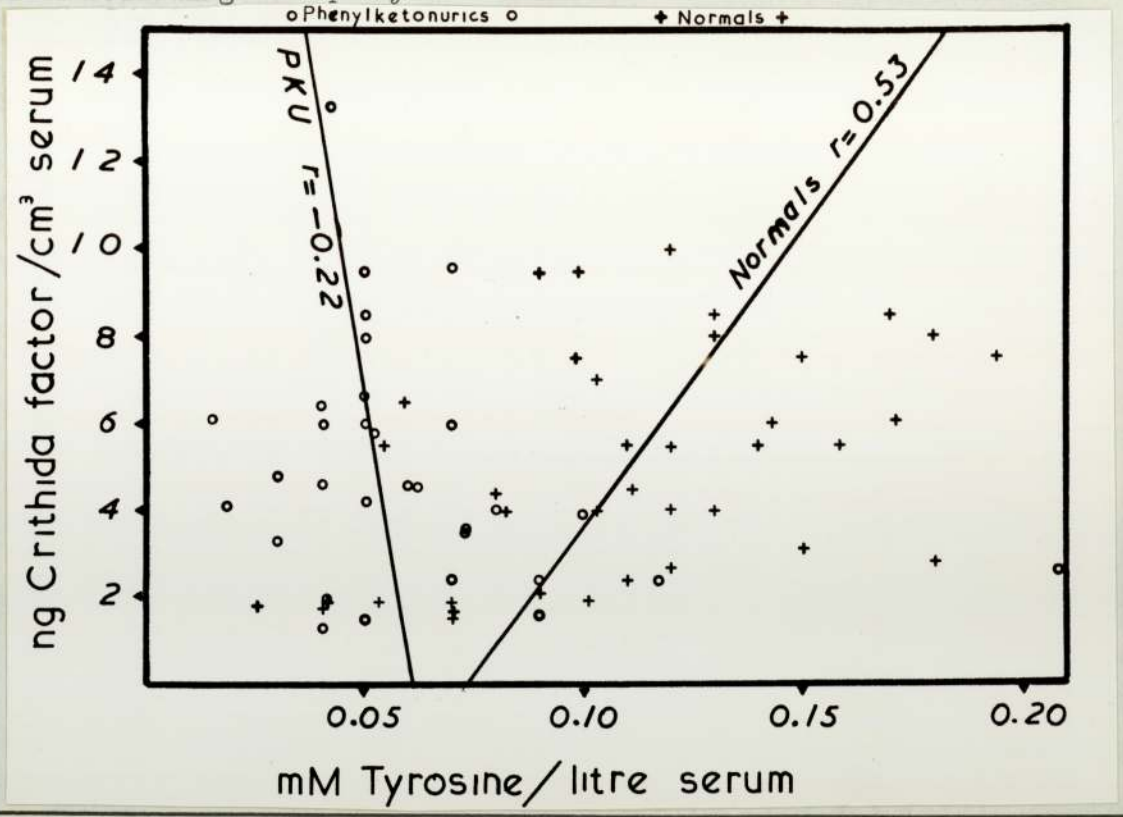
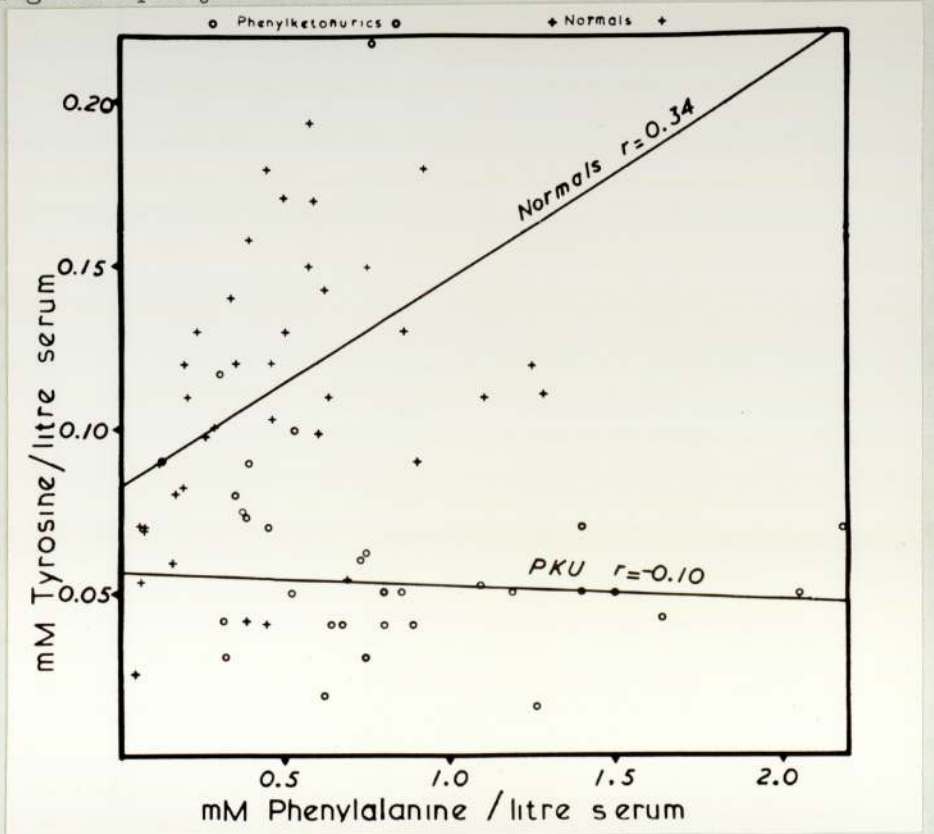


Fig. 10-3

Distribution of phenylalanine levels and tyrosine levels in the serum of phenylketonurics compared with those from normal adults at varying times following oral phenylalanine.

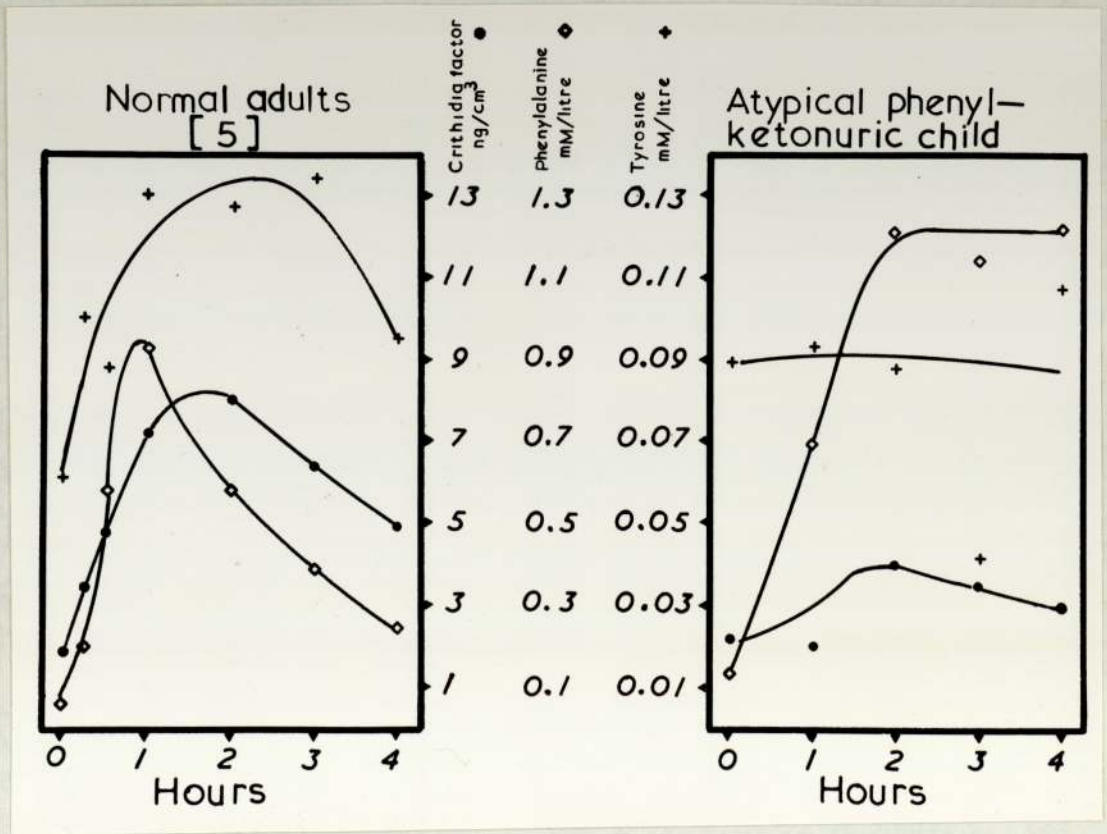


Mean phenylalanine, tyrosine and Crithidia factor values in phenylketonurics, fasting normals and normal subjects at various times from $\frac{1}{2}$ an hour to 4 hours after an oral dose of 7 g of phenylalanine are given in Table 10-1. Only those subjects for whom all parameters were available were used for this comparison. The tyrosine level for fasting normals was very similar to that from ketonurics, but after a loading dose of phenylalanine the normals produced a significantly raised level ($p - < 0.001$). The phenylalanine levels in normals after phenylalanine were similar to that found in phenylketonurics and both were quite distinct from fasting normals ($p = < 0.001$).

Figure 10-4 shows the effect of a loading dose of phenylalanine

Fig. 10-4

Serum levels of phenylalanine, Crithidia factor and tyrosine in a child with transient hyperphenylalaninaemia associated with tyrosinaemia following oral phenylalanine 100 mg/kg body weight compared with normal adults given 7 g phenylalanine orally.



on serum phenylalanine, tyrosine and Crithidia factor in five normal adults compared with a child with transient hyperphenylalaminaemia.

DISCUSSION

The suggestion that Crithidia factor levels in phenylketonuria are stimulated by serum phenylalanine was given impetus by the observation that the levels in five children observed for five months dropped below 4.0 ng/cm^3 only when the serum phenylalanine fell to less than 0.48 mM/litre. However, the patient with hyperphenylalaninaemia had a greater serum phenylalanine response but showed an unsubstantial increase in Crithidia factor indicating that phenylalanine alone is not the trigger for maximum biosynthesis. The persistance of the raised phenylalanine level in this child was also distinctive, in normal subjects the phenylalanine peak was much lower and preceded the peak value for Crithidia factor which was followed closely by that for tyrosine, a logical progression of events

The correlations between phenylalanine and Crithidia factor levels in both phenylketonurics and normals was highly significant ($p = < 0.001$). Correlation between Crithidia factor and tyrosine was significant in normals ($p = 0.001 - 0.01$) but not significant at the 5% level in phenylketonurics, this was predictable in view of the lesion in these patients. What is more interesting is that phenylalanine and tyrosine do not provide significant correlation in normals and thereby point to the complexity of rate determining factors in the metabolism of phenylalanine. Perhaps the poor response of some patients to a low phenylalanine diet may not be due to inadequate supervision of their eating habits. The serum levels of phenylalanine following oral phenylalanine were related to dose (Chapter 8) but tyrosine levels did not correspond as closely to the amounts of phenylalanine given (Figure 8-2).

The relationship between phenylalanine levels and Crithidia factor is emphasised by the fall in serum Crithidia factor level which

was significantly correlated to serum phenylalanine ($p = 0.01 - 0.02$) when phenylalanine was removed from the diet of a healthy adult (Figure 8-6).

Distribution of tyrosine and Crithidia factor levels (Figure 10-2) in the phenylketonurics was different from the normals and it can be inferred that if Crithidia factor has a role in the production of tyrosine then it ceases to function in the absence of phenylalanine hydroxylase. This observation was confirmed by the distribution of values plotted between phenylalanine and tyrosine concentrations (Figure 10-3) which showed phenylketonurics to have lower tyrosine levels even when the serum phenylalanine was raised, the exception was one patient for whom two values are given both of which are raised and who was later found to have transient hyperphenylalaninaemia.

The type of phenylketonuria unresponsive to dietary control, described by Smith and Lloyd (1974) would be interesting to investigate. If phenylalanine levels were raised and there was a defect in tetrahydrobiopterin reduction then, providing synthesis of biopterin derivatives remained normal, one might expect a rise in Crithidia factor levels in response to the phenylalanine in the diet. The constituents of this rise would depend on the rate of metabolism and excretion of each component. Should the lesion lie in faulty biosynthesis of biopterin derivatives then these levels would be low or show a metabolically inactive variant of the cofactor.

SUMMARY

Levels of Crithidia factor in phenylketonuria were raised and varied with phenylalanine levels. The material active for Crithidia fasciculata was chromatographically similar to that found in normal serum, probably 7,8-dihydrobiopterin. Levels of Crithidia factor provided an additional index of dietary success.

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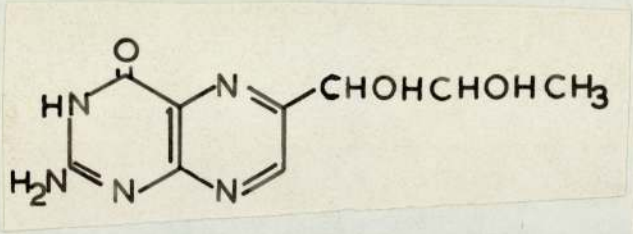
CHAPTER ELEVEN

THE EFFECTS OF FOLATES AND METHOTREXATE ON SERUM LEVELS OF BIOPTERIN DERIVATIVES

INTRODUCTION

The effects of oral biopterin (1) and tetrahydrobiopterin (2)



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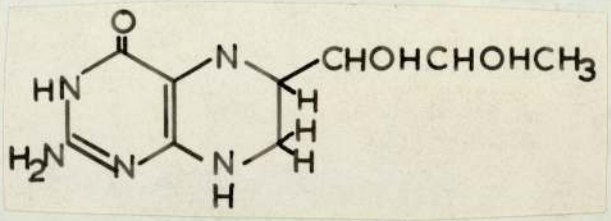

(2)

on serum levels of biopterin derivatives have been shown already and discussed in detail (Chapter 7). Folates in human serum have been studied using absorption experiments and by plotting the constituent folates appearing in serum against time and the remarkable stability of the 10-formyltetrahydrofolic acid (3) level has been demonstrated

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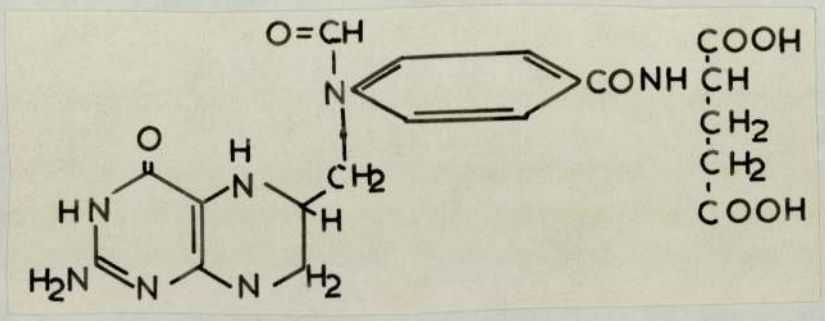

(Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974). The elevation of 10-formyltetrahydrofolate in conditions of heightened cellular activity has been noted (Sotobayashi, Rosen and Nichol, 1966; Ratanasthien et al, 1974). Barford and Blair (1975) described folic acid (4), 5-methyltetra-

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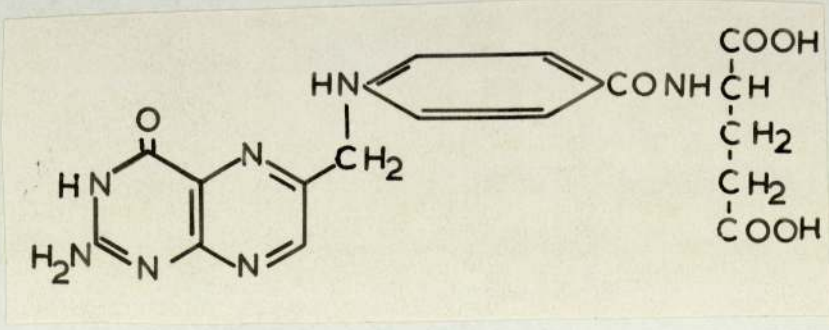
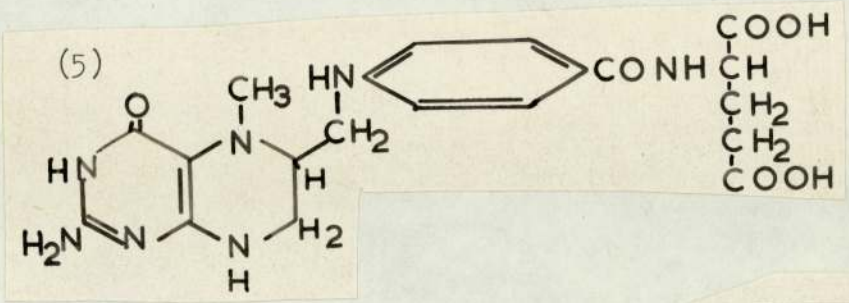
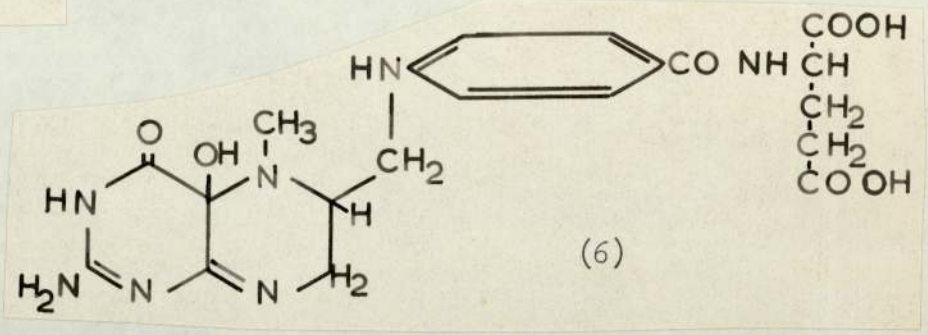


hydrofolic acid (5), 10-formyltetrahydrofolic acid and 4a-hydroxy-5-methyltetrahydrofolic acid (6) in urine of rats following oral ^{14}C folic

(5)





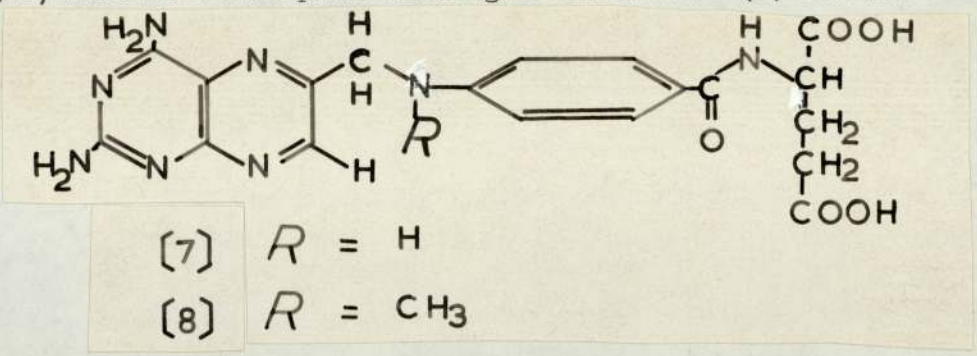
(6)

acid. Crithidia fasciculata has been shown to synthesize biopterin from folic acid, albeit in low yield (Kidder, Dewey and Rembold, 1967). This pathway does not appear to exist in mammals in which there is evidence of synthesis from purines .2 $^{-14}$C guanine and guanosine promote the inclusion of the radioactive label into biopterin in cultures of Chinese hamster ovary cells (Shiota, 1975). Biopterin derivatives in rat liver have been shown to be independent of biopterin in the diet (Pabst and Rembold, 1966) although they are slightly decreased in livers of folate deficient rats (Goodfriend and Kaufman, 1961). Biopterin levels in the rat have been reported as being increased by actinomycin D inhibition of nucleic acid synthesis (Rembold and Gyure, 1974).

The origin of biopterin and its reduced derivatives is of considerable importance and the effects of available folates, taken orally, on serum levels of these materials measured by Crithidia fasciculata, would indicate if the biosynthesis of biopterin could commence from an already formed pteridine administered in this way.

Folate analogues were the first antimetobolites to produce striking, although temporary, remissions in leukaemia (Farber, Diamond, Mercer, Sylvester and Wolff, 1948) and long-lasting remissions in chorio-carcinoma (Hertz, 1963). The first compound to be used was aminopterin (7) (4-amino-4-deoxyfolic acid) synthesised in 1947 by

Seeger, Smith and Hultquist although methotrexate (8) has now largely



(7) R = H

(8) R = CH_3

superceded aminopterin in clinical use because of its lower toxicity and the stability of its bond with dihydrofolate reductase. Methrotrexate is readily absorbed from the gastrointestinal tract at doses routinely employed in clinical practice (0.1 mg/kg), but large doses are incompletely absorbed. The drug is also absorbed from parental sites of injection (Goodman and Gilman, 1970). A direct relationship exists between dose and plasma levels. There is no difference in the pharmacokinetic behaviour of methotrexate when given intravenously or orally (Jacobs, Bleyer, Chabnes and Johns, 1975). The mean plasma half life of methotrexate varies from four hours to twenty four hours (Frei, Jaffe, Tattersall, Pitman and Parker, 1975) and approximately 50% of the drug is bound to plasma proteins although it may be displaced by a number of drugs including sulphonamides and salicylates. Methotrexate acts by binding to the dihydrofolate reductase which is thereby prevented from functioning (Goodman and Gilman, 1970). It has been shown to inhibit tyrosine metabolism in the rat (Kaufman and Levenberg, 1959). Methotrexate is very poorly transported across the blood brain barrier (Whiteside, Phillips, Dargeon and Burchenal, 1958) which characteristic it has in common with tetrahydrobiopterin (Kettler, Bartholini and Pletscher, 1974).

Folate antagonists are potent inhibitors of immune reactions and have been employed as such in organ transplantation (Hitchings and Elion, 1963), this topic has been reviewed by Bertino and Johns (1967).

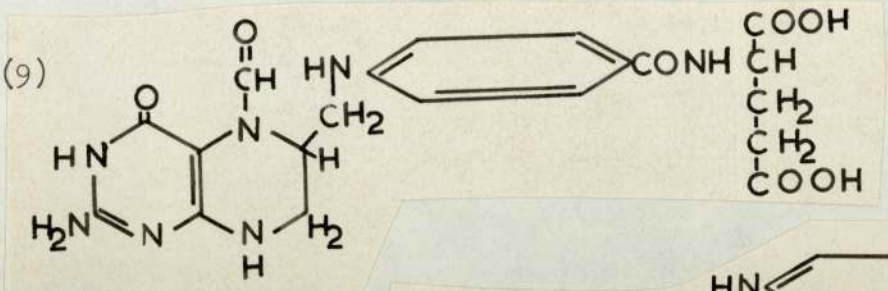
Methotrexate has been used in the therapy of psoriasis following the work of Van Scott, Auerbach and Weinstein (1964). This non-neoplastic disease of the skin is characterised by rapid proliferation of epidermal cells and is a difficult condition in which to produce a permanent remission. Crithidia factor levels are low in patients with this disorder (Chapter 9).

When examining the sera of psoriatics for Crithidia factor a group contrasted starkly with the others by having very raised levels. On further enquiry it was found that this group was being treated with oral and parental methotrexate. In view of the site of action of the drug it was considered that valuable information might be forthcoming on routes to the synthesis of biopterin derivatives if the nature of the material which constituted this raised level could be elucidated. Also oral and parental folates administered to patients on methotrexate might demonstrate minor pathways from the administered compound to biopterin derivatives when the route to the reduction of folate was blocked by the antimetabolite.

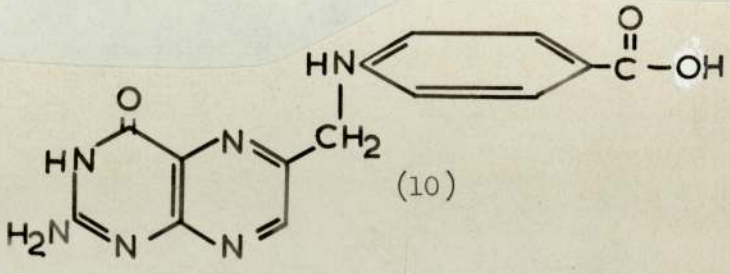
METHODS

Methotrexate and folic acid were commercial products. 5-formyltetrahydrofolic acid (9) was a gift from Lederle Laboratories Ltd. Biopterin was a gift from Roche Products Ltd. Pteroic acid (10)

(9)

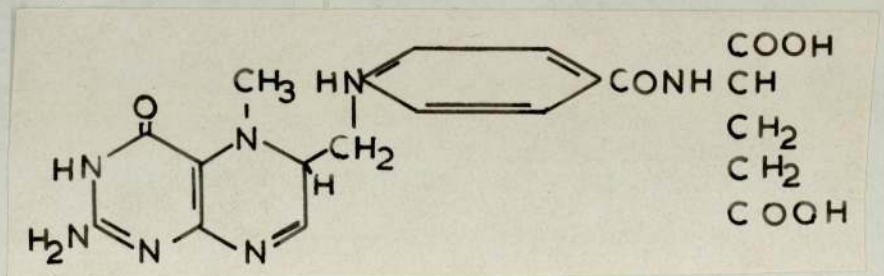


(10)



was a gift from Professor I. H. Rosenberg. 5-methyltetrahydrofolic acid, calcium salt, was prepared by the method of Blair and Saunders (1970). 5-methyl-5,6-dihydrofolic acid (11) was prepared by dissolving 400 mg of

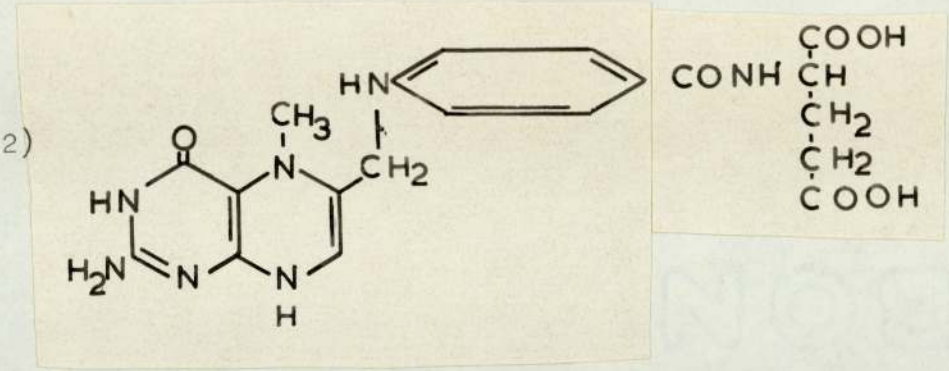
(11)



5-methyltetrahydrofolic acid in 200 cm^3 of distilled water containing 5 mg of copper sulphate. The mixture was stirred under a slow stream of oxygen for 60 to 100 minutes, the reaction was timed by testing for ultra-violet absorption maxima at 248 nm and 280 nm at pH 7.0 in 0.1 M phosphate buffer. Further purification was carried out according to Gapski, Whiteley and Huenneken (1971).

5-Methyl-5,8-dihydrofolic acid (12) was prepared by dissolving 10 mg of 5-methyl-5,6-dihydrofolic acid in 0.01 M hydrochloric acid

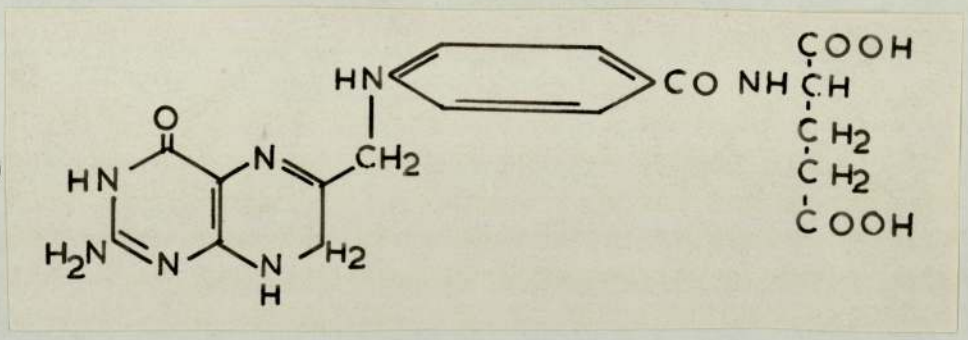
(12)



30 minutes before administration, neutralisation with dilute sodium hydroxide was carried out immediately before use.

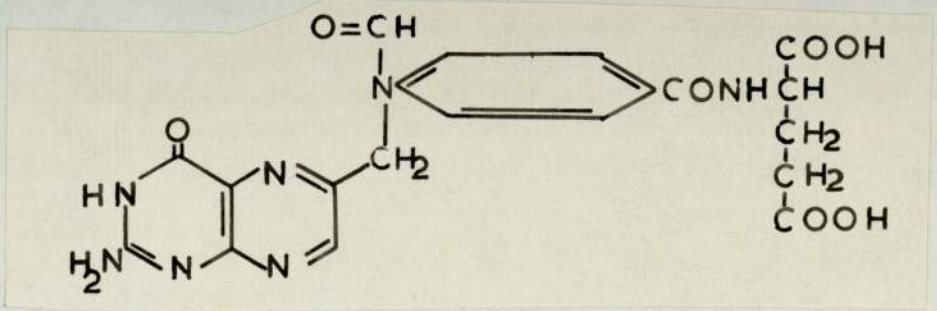
7,8-dihydrofolic acid (13) was prepared according to the method of Futterman (1963).

(13)



10-formylfolic acid (14) was prepared according to the method

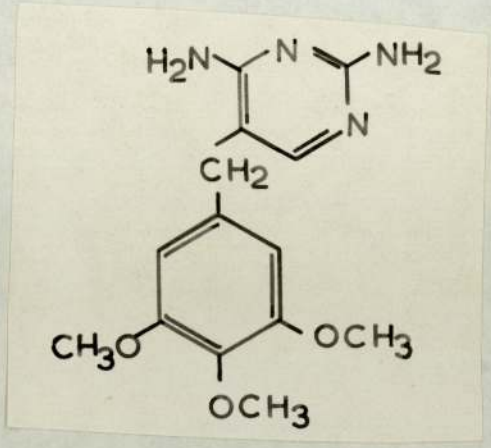
(14)



of Blakeley (1959). All the diastereoisomers were given as 10 mg oral doses and the rest as 5 mg. A number of normal volunteers, hospital and university staff and students were used together with hospital patients who agreed to take part in the investigation. Biopterin derivatives in sera following oral doses were measured with Crithidia fasciculata.

Fourteen patients with psoriasis who had had methotrexate from 5 to 25 mg orally or parentally within the previous six days had serum Crithidia factor levels measured. These were compared with nine patients without therapy and four patients on azothioprine, twelve children with leukaemia who had had methotrexate from 1 to 14 days before sampling, three patients on 'Septrin' (trimethoprim (15) with sulphamethoxazole)

(15)



and normals.

Blood samples were taken at 0, 1, 2, 3 and 6 days from a patient who had received 25 mg of methotrexate intravenously for extensive erythrodermic psoriasis and at 0, 1, 2, 3, 4 and 24 hours from two other psoriatics with severe lesions who had had 5 mg of methotrexate orally. Seven patients had 10 mg of methotrexate orally, followed 24 hours later by different pteridines. Two of these had 5 mg of oral folic acid, two had 10 mg of 5-methyltetrahydrofolic acid orally, two had 5 mg of 5-formyltetrahydrofolic acid intravenously and one had 5 mg of biopterin orally. Blood samples were taken before methotrexate was given and then immediately before the pteridine and at 1, 2, 3, 4 and 24 hours after for Crithidia factor assay.

A sample of serum from a patient on methotrexate therapy was chromatographed in 0.5% sodium carbonate, 5% acetic acid and 3% ammonium chloride. Three psoriatics on methotrexate and three without systemic treatment had serum phenylalanine and tyrosine estimated.

RESULTS

The levels of serum Crithidia factor after oral folates are given in Table 11-1. On no occasion was the serum concentration raised significantly above the fasting level. Table 11-2 gives the levels of serum Crithidia factor in sera of treated and untreated psoriatics, leukaemic children on methotrexate and patients on 'Septrin'. The patients on folate antagonists all had clearly raised levels.

Fig. 11-1

Crithidia factor levels in the serum of twelve leukaemic children following discontinuation of chemotherapy with methotrexate.

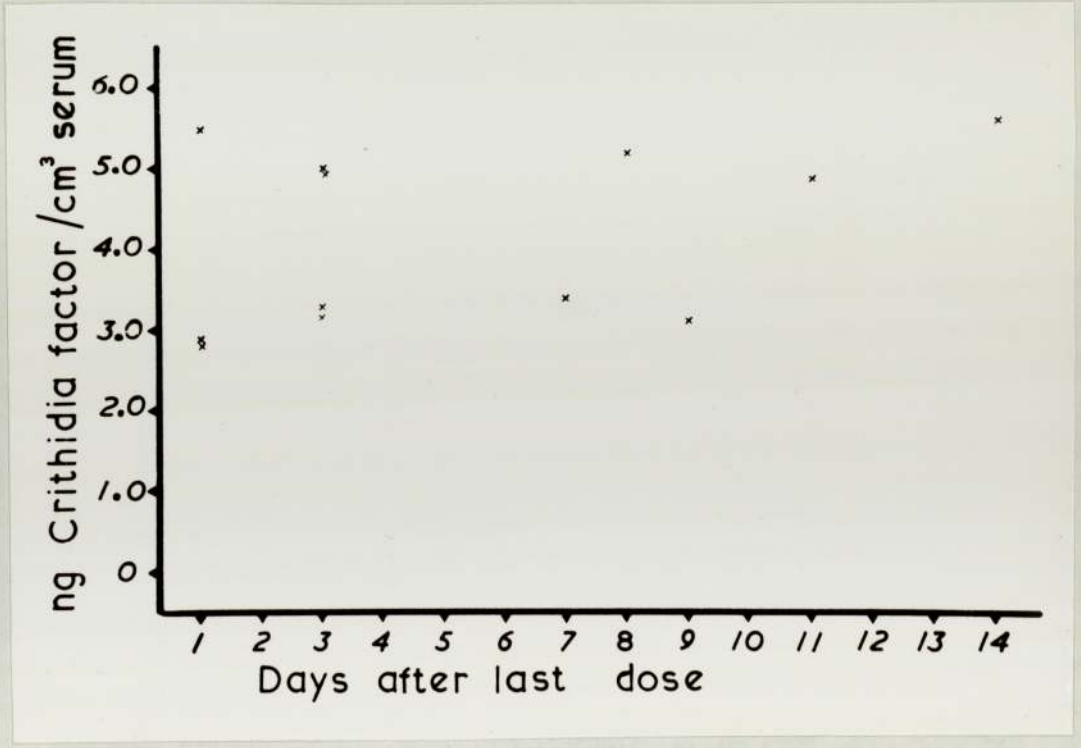


Figure 11-1 shows the distribution of Crithidia factor levels in the twelve children on methotrexate therapy for leukaemia plotted in time against their last dose. Figures 11-2 and 11-3 show the rise in serum Crithidia factor in three subjects following varying doses of

TABLE 11-1

Serum Crithidia factor levels in ng/cm^3 following oral folates in fasting subjects

		5-methyltetrahydrofolic acid	Folic acid	Pteroic acid	5-formyltetrahydrofolic acid	10-formylfolic acid	5-methyl-5,6-dihydrofolic acid	5-methyl-5,8-dihydrofolic acid	7,8-dihydrofolic acid
No. of subjects		5	3	2	4	2	5	3	4
HOURS	0	2.07 ± 0.13	1.17 ± 0.17	1.63 ± 0.12	1.63 ± 0.03	0.55 ± 0.05	2.36 ± 0.62	1.20 ± 0.23	2.15 ± 0.48
	½	1.88 ± 0.11	1.23 ± 0.09	1.50 ± 0.20	1.70 ± 0.24	0.53 ± 0.17	2.42 ± 0.55	1.30 ± 0.26	1.9 ± 0.26
	1	1.73 ± 0.09	1.27 ± 0.20	1.50 ± 0.10	1.43 ± 0.21	0.58 ± 0.22	2.28 ± 0.53	1.33 ± 0.35	1.78 ± 0.24
	1½	1.80 ± 0.09	1.30 ± 0.12	1.53 ± 0.02	1.20 ± 0.33	0.50 ± 0.30	2.24 ± 0.57	1.17 ± 0.37	1.83 ± 0.24
	2	1.80 ± 0.09	1.38 ± 0.16	1.55 ± 0.05	1.25 ± 0.13	0.60 ± 0.02	1.94 ± 0.36	1.53 ± 0.32	1.78 ± 0.22
	2½	-	-	-	-	-	-	-	-
	3	1.85 ± 0.15	1.3 ± 0.09	1.65 ± 0.45	1.18 ± 0.25	0.80 ± -	1.96 ± 0.36	1.30 ± 0.35	1.93 ± 0.23

TABLE 11-2

Biopterin derivative levels in treated and untreated psoriatics compared with other patients on dihydrofolate reductase inhibiting drugs

	Normals (Chapter 4)	Untreated psoriatics	Psoriatics on azothioprine*	Psoriatics on methotrexate**	Leukaemic children on methotrexate***	Patients on septrin****
No. of samples	114	9	4	14	12	4
Mean serum level (ng/cm^3) with standard error of means.	1.81 ± 0.06	1.43 ± 0.08	1.18 ± 0.23	15.24 ± 4.94	4.3 ± 0.31	7.35 ± 2.49
Range (ng/cms)	0.4 - 3.8	1.0 - 1.8	0.7 - 1.7	3.0 - 60.0	2.8 - 5.6	1.4 - 13.5

* 2-5 mg/kg body weight daily

** 5 mg to 25 mg weekly

*** 5 mg or 7.5 mg at last dose

**** 960 mg twice daily

Fig. 11-2

Serum Crithidia levels in two patients following oral methotrexate.

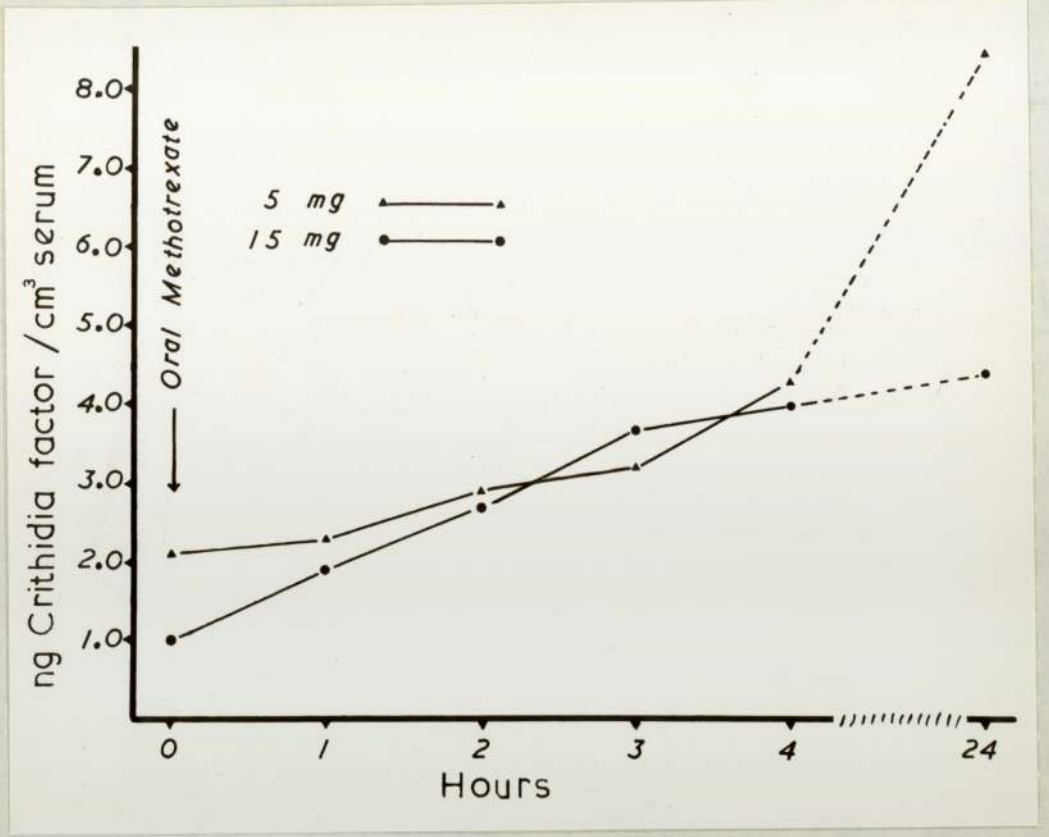
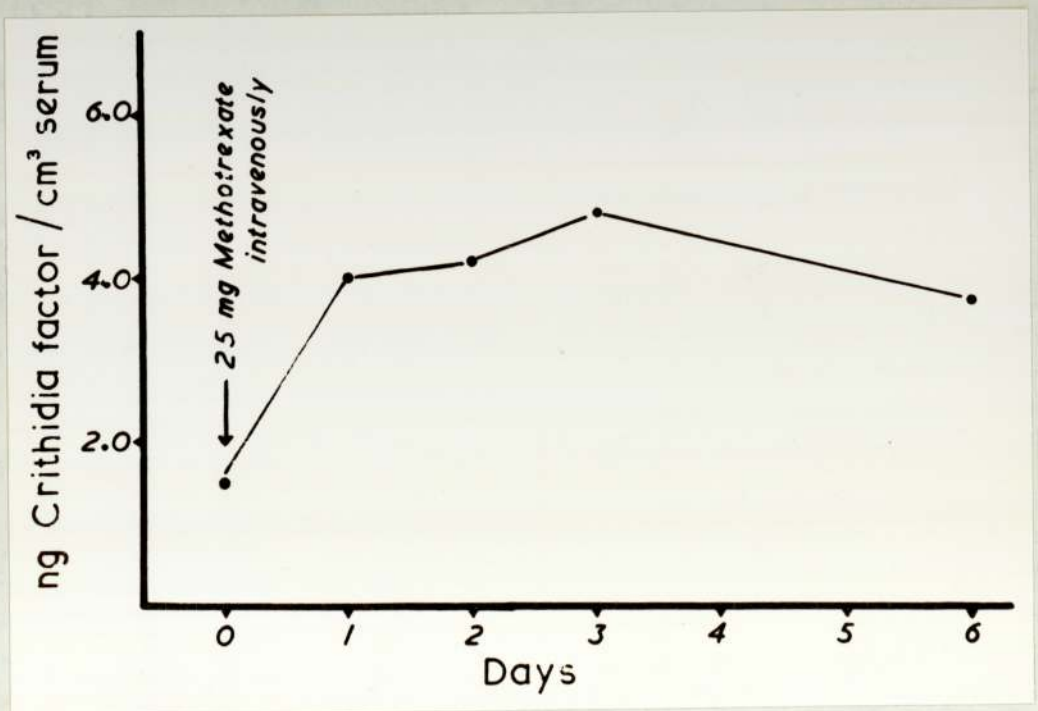


Fig. 11-3

Serum Crithidia factor levels over six days in a patient following intravenous methotrexate.



methotrexate. The rate of increase appeared at its greatest during the first 24 hours, thereafter the concentration remained fairly constant. Figure 11-4 shows the effects of 10 mg oral 5-methyltetrahydrofolic acid, 5 mg folic acid orally and 5 mg 5-formyltetrahydrofolic acid intravenously, 24 hours after 10 mg of methotrexate orally.

Fig. 11-4

Serum Crithidia factor levels when folates were given 24 hours after 10 mg methotrexate.

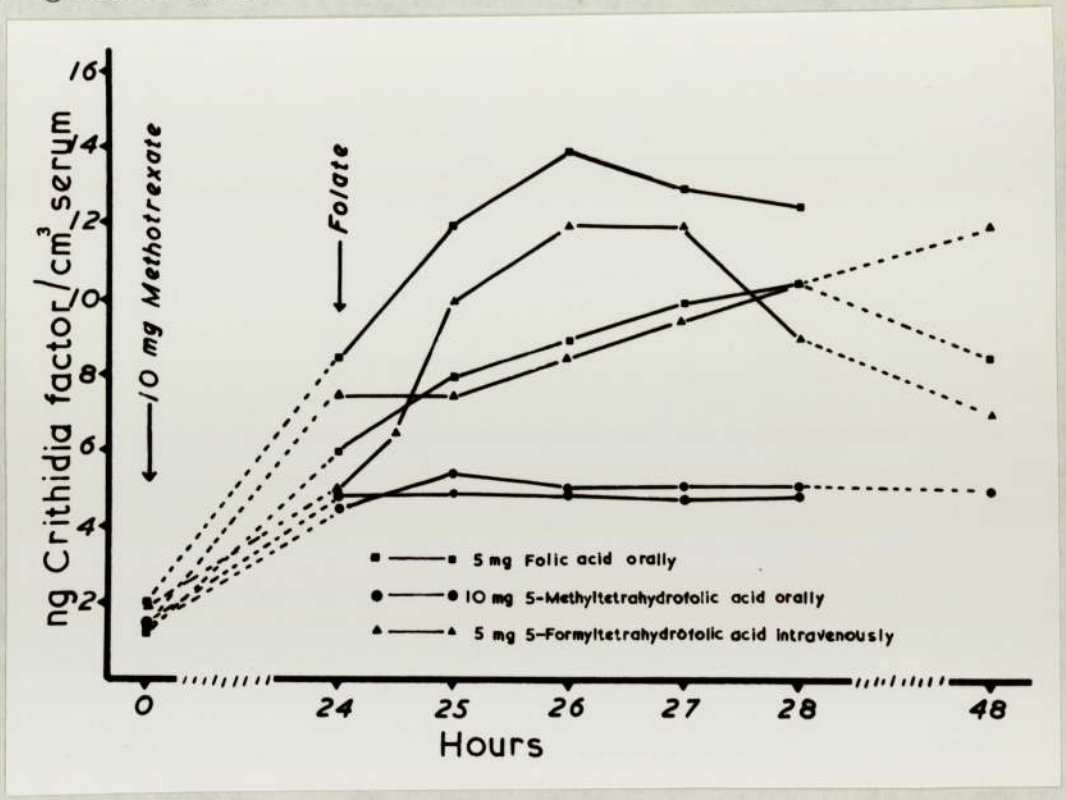
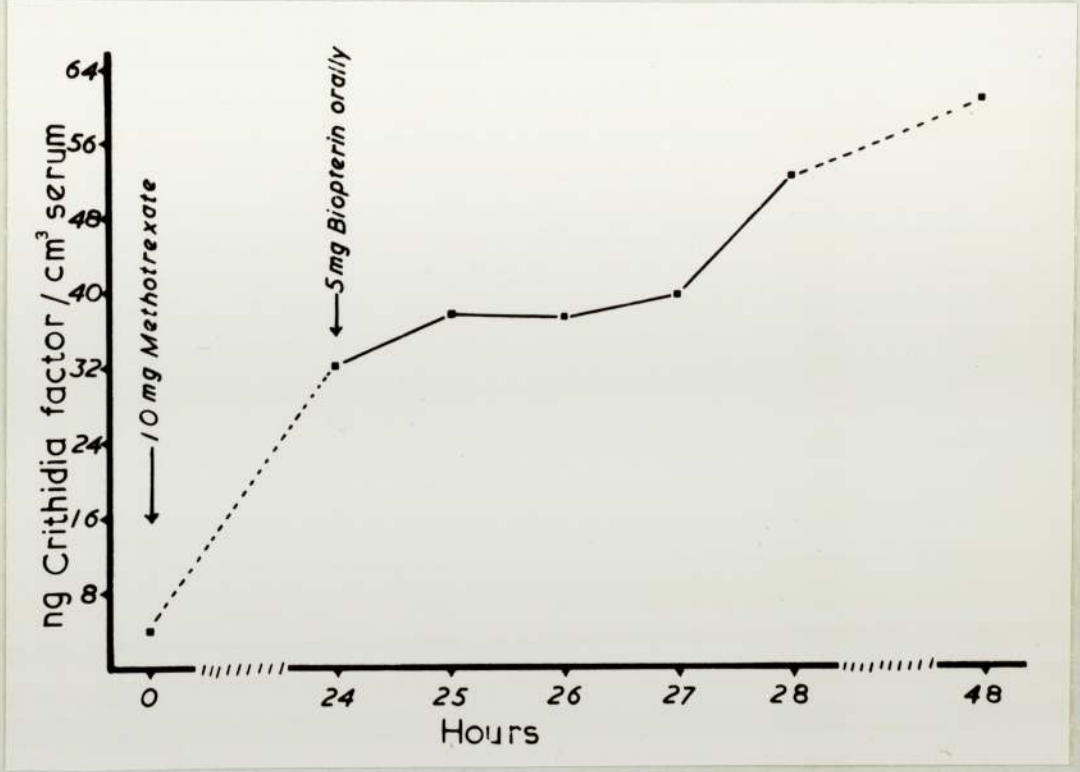


Figure 11-5 shows the rise in serum Crithidia factor level in a patient who had 5 mg of biopterin orally 24 hours after 10 mg of oral methotrexate. Folic acid and 5-formyltetrahydrofolic acid raised the serum level of Crithidia factor which then dropped within 24 hours to approximately the level it had been before the folate was given. 5-methyltetrahydrofolic acid failed to have any impact on the Crithidia factor level. Biopterin produced a massive rise in a patient who was later found to have a raised

fasting level (4.0 ng/cm^3).

Fig. 11-5

Serum Crithidia factor levels when biopterin was given 24 hours after 10 mg methotrexate.



Chromatography of serum following methotrexate revealed 7,8-dihydrobiopterin as the major material and a second material which co-chromatographed with biopterin and 5,6,7,8-tetrahydrobiopterin. Serum phenylalanine and tyrosine levels were normal in all six patients with psoriasis including the three patients undergoing methotrexate therapy.

DISCUSSION

There was a complete lack of significant response in serum Crithidia factor following oral folates in agreement with the work of Fukushima and Shiota (1972) who did not get a rise following oral folic acid. The serum folates themselves are subject to large increases (Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974; Brown, Scott, Foster and Weir, 1973; Leeming, Portman-Graham and Blair, 1972) in contrast to oral pteroic acid which is poorly absorbed (Brown et al, 1973; Blair, Ratanasthien and Leeming, 1974). These results show that, under normal circumstances, folates administered orally do not make a significant contribution to the pool of biopterin derivatives in the body. It is unlikely that they enter the body and are rapidly disposed to sites outside the vascular system or excreted; raised serum levels of biopterin derivatives following methotrexate and phenylalanine (Chapter 8) are sustained for an appreciable period of time following the dose. The level following oral biopterin is also in agreement (Chapter 7) even though it is known to have a great affinity for tissue binding sites (Rembold and Metzger, 1967).

The raised levels of Crithidia factor following oral or parental methotrexate could be caused by a block in folate metabolism feeding a minor pathway, or by simple displacement. Methotrexate itself does not support the growth of Crithidia fasciculata nor does it inhibit it at the concentrations achieved by therapeutic doses (Chapter 3). Alternatively if the biosynthetic process was unduly stimulated by a lowered rate of cofactor reduction then overproduction and accumulation of unreduced cofactor might result. The second material demonstrated in the serum from a patient on methotrexate could have been either biopterin or tetrahydrobiopterin. Metabolised biopterin is bound to tissue (Rembold and Metzger, 1967) and one would expect a time to be reached when

saturation would occur. Excretion or feedback to regulate the synthesis must be consequent on this otherwise the serum level would continue to rise, which it most clearly did not (Figures 11-1 and 11-2).

Folates given to patients on methotrexate produced an interesting array of results. Firstly, biopterin gave rise to a greater increase in serum Crithidia factor than any of the others but this patient had a raised level before methotrexate was started (4.0 ng/cm^3). The rise in serum Crithidia factor following methotrexate was also much higher than in the others and it was found that there was a slight uraemia in this patient which could account for the steep increase in serum Crithidia factor (blood urea 55 mg%). It has already been shown that uraemic patients have raised serum levels of biopterin derivatives (Baker, Frank, Bacchi and Hutner, 1974). The serum concentration of Crithidia factor in this patient also was unlike those obtained following other pteridines in that the high level was substantially increased 24 hours after the biopterin had been given. Results like these could easily be idiosyncratic but any greater effect than an additive one would have to arise from displacement of tissue held Crithidia active compounds or from the metabolism of the administered biopterin triggering biosynthesis in some way. Chromatography of the sample taken 48 hours after methotrexate and 24 hours after biopterin showed an increase in 7,8-dihydrobiopterin as well as a considerable quantity of a material which chromatographed with biopterin and tetrahydrobiopterin.

The rises in serum levels following oral folic acid and intravenous 5-formyltetrahydrofolic acid in patients on methotrexate were quite distinct from results obtained following administration of folates without prior treatment with methotrexate. The slow rise in serum Crithidia factor levels in these patients was different from the pattern following oral biopterin (Chapter 7, Figure 7-1) and contrasted

to the serum Crithidia factor levels following oral phenylalanine (Chapter 8, Figure 8-3). In both those cases the levels were falling at three hours. The serum levels of biopterin derivatives following oral folates in 25 subjects on methotrexate were still rising at four hours, long after the folates would have been absorbed. This is compatible with slow metabolism of the folates to a pool from which the Crithidia active compounds could be derived. Oral 5-methyltetrahydrofolic acid did not alter the serum Crithidia factor level and therefore did not enter such a pool. Before accepting de novo synthesis of biopterin derivatives, the possibility of folates with methotrexate affecting renal function would need consideration. Uraemia has a substantial effect on serum levels of Crithidia active pterins (this thesis, Chapter 9; Baker, Frank, Bacchi and Hutner, 1974).

ADDENDUM

Because of the difference in handling oral 5-methyltetrahydrofolic acid and intravenous 5-formyltetrahydrofolic acid (Fig. 11-4), 10 mg of 5-formyltetrahydrofolic acid were administered to a subject 24 hours after oral methotrexate and Crithidia factor levels measured at timed intervals. In normal, unmedicated subjects oral 5-formyltetrahydrofolic acid would appear rapidly in the serum as 5-methyltetrahydrofolic acid (Ratanasthien, Blair, Leeming, Cooke and Melikian, 1974). It would be absorbed unaltered if the patient was achlorhydric, there was no clinical indication of this in the patient described here but hypertension and uraemia (70 mg%) were known to exist. The serum Crithidia factor was raised initially (7.5 ng/cm^3), reached 8.8 ng/cm^3 24 hours after 10 mg methotrexate and 37.5 ng/cm^3 24 hours after oral 5-formyltetrahydrofolic acid. One would have expected a lack of response comparable to oral 5-methyltetrahydrofolic acid. In the absence of further clinical details it is impossible to do more than record this observation.

SUMMARY

Serum Crithidia factor levels in psoriatic patients on methotrexate were raised. This was shown to be caused by the methotrexate and was maintained for up to two weeks following discontinuation of the drug. Septrin had a similar effect. Oral folic acid and intravenous 5-formyltetrahydrofolic acid caused the serum level of biopterin derivatives to rise in patients on methotrexate in contrast to the total absence of response to oral folates without prior methotrexate. 5-methyltetrahydrofolic acid did not increase the serum Crithidia factor either with or without prior methotrexate. Oral biopterin, in one patient (who may have been atypical) on methotrexate, produced a rise in serum Crithidia factor in excess of and more persistant than, the increase produced by biopterin alone.

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CHAPTER TWELVE

GENERAL COMMENTS AND SUGGESTIONS FOR FURTHER WORK

The process of biosynthesis of biopterin derivatives is not known. The purines seem likely substrates and studies with radioactively labelled guanine and guanosine in Chinese hamster ovary cells and neuroblastoma have produced labelled biopterin (Shiota, 1975). The pathway from folic acid to Crithidia active pterin has only been shown to exist in Crithidia fasciculata and even here the yield was low (Kidder, Dewey and Rembold, 1967). In man folic acid is not normally converted into, nor does it cause an increase in, urinary biopterin derivatives (Fukushima and Shiota, 1972). This is in agreement with the findings detailed in this thesis. Oral folic acid, 5-formyltetrahydrofolic acid, 10-formylfolic acid, pteroic acid, 5-methyltetrahydrofolic acid, 5-methyl-5,6-dihydrofolic acid, 5-methyl-5,8-dihydrofolic acid and 7,8-dihydrofolic acid did not produce an increase in serum Crithidia factor in fasting subjects (Chapter 11).

The pathways from folate following the administration of methotrexate are difficult to interpret, particularly the rise in serum Crithidia factor following intravenous 5-formyltetrahydrofolic acid which contrasts with the lack of response to oral 5-methyltetrahydrofolic acid. Both these folates are in reduced forms and both share transport sites with methotrexate (Goldman, 1971). These differences in behaviour of closely related folates could be of value if they shed more light on the mammalian metabolism of methotrexate and indicate more subtle ways to control therapy and increase the specificity of the dose for the malignant clone. Although methotrexate is an effective drug with proven usefulness, particularly in the treatment of leukaemia in children, choriocarcinoma and Burkitt's lymphoma, its therapeutic level is near its level of toxicity (Goodman and Gilman, 1970). Following large doses of methotrexate

parental administration of 5-formyltetrahydrofolic acid is used to 'rescue' normal tissues. One reason for the success of this treatment in osteogenic sarcoma has been given as the differential cell membrane transport of folates (Frei, Jaffe, Tattersall and Parker, 1975). Normal doses of methotrexate do not penetrate the malignant tissue in sufficient concentration to affect the course of the disease but high doses enter the cells and attain therapeutic concentrations there. Frei et al (1975) further postulate that 5-formyltetrahydrofolic acid will enter and rescue normal tissue cells, which are susceptible to normal concentrations of methotrexate but will not enter and rescue osteogenic sarcoma cells which lack transport sites. This is difficult to align with the increased need of tumour cells for 10-formyltetrahydrofolic acid (Sotobayashi, Rosen and Nichol, 1966) which is readily formed from 5-formyltetrahydrofolic acid.

The metabolism of folates and the synthesis of biopterin derivatives in patients on methotrexate might be followed using radioactive tracers. However, such experiments in humans would be unlikely to satisfy stringent ethical requirements and would have to be performed on experimental animals. Alternatively, stable nuclides of carbon (^{13}C) and hydrogen (^{2}H) could be introduced into the pteridine ring. Tritium (^{3}H) as a label is worthy of consideration providing note is taken of the possibility of interchange with nonradioactive hydrogen.

Oral biopterin is absorbed by the rat, is slowly converted to tetrahydrobiopterin and binds to tissues throughout the body (Rembold and Metzger, 1967). The same workers showed that biopterin administered peritoneally was rapidly excreted unchanged in the urine. Their deduction was that reduction of biopterin took place during

transport through the bowel, findings given earlier (Chapter 7) from chromatography of serum following oral biopterin do not conflict with this. Biopterin is absorbed from the small bowel following an oral dose although serum levels do not attain the high levels of folates after similar oral doses. Tetrahydrobiopterin is poorly absorbed without a peak serum level becoming apparent at approximately two hours as is found with both folates and biopterin (Chapter 7). Rembold (1970) showed that intraperitoneally injected tetrahydrobiopterin was retained almost completely in contrast to the rapid excretion of unchanged biopterin when administered via the same route. Again the use of labelled chemical species is indicated. Autoradiography could be useful in identifying sites of metabolism and absorption.

Synthesis of biopterin derivatives following oral phenylalanine was followed by a rise in urinary biopterin derivatives, but not a sufficiently high rise to indicate rapid and total excretion. Also, chromatography showed the material in serum to be 7,8-dihydrobiopterin and not biopterin (Chapter 8). Measurement of the various metabolites of phenylalanine as well as Crithidia factor and phenylalanine in serum and urine following oral phenylalanine would produce evidence of the pharmacokinetics of the biosynthetic process. Concurrent measurement of the rate of hydroxylation of tyrosine would allow a comparison with the effects of direct parental administration of tetrahydrobiopterin in experimental animals. The enhancement of tyrosine hydroxylation in rat striatum by the intraventricular administration of tetrahydrobiopterin has already been demonstrated (Kettler, Bartholini and Pletscher, 1974). There was poor penetration of cofactor into the brain following intravenous administration of tetrahydrobiopterin in the same group of experiments.

Raised levels of Crithidia factor in phenylketonuria (Chapter 10) are interesting from several points of view, not least because theye appear to be dependent on the phenylalanine content of the body. In classical phenylketonuria phenylalanine hydroxylase is absent. In other cases there is evidence of an enzyme giving a slower rate of hydroxylation (Woolf, Cranston and Goodwin, 1967). Phenylketonuria has recently been described in which the phenylalaninaemia is corrected by a low phenylalanine diet but in which the progressive neurological symptoms are unresponsive (Smith, Clayton and Wolff, 1975). It has been suggested that enzymatic reduction of the cofactor is not operating. Measurement and identification of the cofactor in such cases, both with a phenylalanine free diet, a normal diet and possibly following oral phenylalanine would elucidate at least part of the defect. The prognosis of these unfortunate infants might be influenced if the role of tetrahydrobiopterin in the foetal brain was known. If there was a total dependancy on maternal cofactor then its ability to pass both the placenta and the foetal blood-brain barrier would be vital. Hyperphenylalaninaemia in juvenile rats is more effective than in adult rats in producing phenylketonuria (Geller and Yuwiler, 1969) and there is adequate evidence for parallel results in humans.

Crithidia active pterins have been demonstrated in mitochondria from rat liver. Tetrahydropterins activate mitochondrial respiration (Rembold and Buff, 1972a) and have been shown to have a role in cytochrome reduction although concentrations required are really too high to have a normal physiological role. Added ^{14}C labelled tetrahydrobiopterin results in the radioactivity being incorporated into inact mitochondria. Submitochondrial particles obtained by sonifying intact rat liver mitochondria in aqueous

suspension were found to increase their oxygen consumption in the presence of tetrahydrobiopterin (Rembold and Buff, 1972b). The affinity of tissues for tetrahydrobiopterin (Rembold and Metzger, 1967; Rembold, 1970) and the wide distribution of tetrahydrobiopterin in tissues and body fluids (Baker, Frank, Bacchi and Hutner, 1974; this thesis, Chapter 6) point to the general importance of the electron transfer chain (Rembold and Buff, 1972b) and suggest that there may be medically important information to be gained from further exploration of this field.

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