METABOLISM AND HANDLING OF FOLATES IN THE MAMMAL ESPECIALLY MAN.

A THESIS

submitted for the degree of

Doctor of Philosophy

by

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at

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Summary.

The metabolism of oral administered folates and their handling has been studied in humans. Aseptic addition methods of microbiological assay with L.casei,S.faecalis,P.cerevisiae and bioautographic techniques were employed in investigations of serum and urine folates.

Studies of normal human serum and urine showed them to contain four folates,5-methyltetrahydropteroylglutamic acid as the major folate,5-methyl-5,6-dihydropteroylglutamic acid, 10-formyltetrahydropteroylglutamic acid and 10-formylpteroylglutamic acid as minor folates. 10-formyltetrahydropteroylglutamic acid was maintained at a constant level by a homeostatic mechanism and 5-methyltetrahydropteroylglutamic acid acted as a storage form. Studies in rats and man showed that there was a diurnal variation of serum folates and folates in urine excretion. In some diseases eventhough total serum folates assayed with L.casei were in the normal range the three minor folates seemed to show abnormalities.

In normal human volunteers there was rapid and almost complete metabolism in the serum to 5-methyltetrahydropteroylglutamic acid after oral administration of 7,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid,5-formyltetrahydropteroylglutamic acid and 5,10-methenyltetrahydropteroylglutamic acid. Oral administration of pteroylglutamic acid itself gave a partial metabolism to 5-methyltetrahydropteroylglutamic acid whilst 10-formylpteroylglutamic acid remained unchanged. Oral administration of the oxidation products of 5-methyltetrahydropteroylglutamic acid showed that 5-methyl-5,6-dihydropteroylglutamic acid is partially utilized but

mainly underwent rearrangement to microbiologically inactive 5-methyl-5,8-dihydropteroylglutamic acid in the stomach acid. Neutralization of stomach acid of normal humans with oral sodiumbicarbonate enhanced the availability of 5-methyl-5,6-dihydropteroylglutamic acid and also in cases of achlorhydria of pernicious anaemia patients. This latter observation provides an alternative explanation of the elevated serum folate level found in pernicious anaemia. Other folates,5-methyl-5,8-dihydropteroylglutamic acid and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid did not enter folate metabolic pool. Application of urine from these subjects to a Sephadex chromatograms suggested that they may be absorbed.

It is established that the metabolism of oral folate was altered in some diseases. Malabsorption of 5-methyltetrahydropteroylglutamic acid was demonstrated in adult coeliac disease and leukemias. Oral folate metabolism in the presence of a dihydrofolate reductase inhibitor (methotrexate) administered orally 24 hour before the test was studied.No evidence could be found for a build up of dihydrofolic acid.

The work described in this thesis was carried out from October 1972 to August 1975 in the Department of Chemistry in the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.

K. Ratnasthien.

To my parents

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Chapter 1

1.1.1 Historical summary.

The initiation of studies concerning compounds later known as folic acid date back to the end of the nineteenth century when Hopkins (1889) isolated a pigment from butterfly wings, a compound now known as leucopterin. Little progress in this field was made until Wieland and Schopf re-investigated the structure of the pigments. They isolated and established the structures of compounds xanthopterin and leucopterin (Wieland and Schopf, 1925; Schopf and Wieland. 1926). From India Wills and her co-workers reported a factor from yeast or liver extracts that cured anaemia in pregnant women (Wills, 1931). Later it was shown that in monkeys with restricted diet, the blood picture became normal after treatment with liver or yeast extract (Wills and Bilmoria, 1932). A few years later Day et al (1935) also showed experimentally an induced deficiency condition in monkeys which had caused anaemia which they called nutritional cytopenia. The condition in these monkeys also responded to the oral treatment with liver or yeast extract and they termed the compound in the extracts as 'Vitamin M'. Nutritional cytopenia was also shown in chicks on a restricted diet, described in this case as macrocytic anaemia (Stokstad and Manning, 1938; Hogan and Parrot, 1939; 1940). The active factor was called Factor U by Stokstad and Manning (1938). The term vitamin B_c was used by Hogan and Parrot (1939). The response of chicks to the vitamin was then used as a tool for the detection of the compound and the first biological assay method using the chick was described by O'Dell and Hogan (1943).

The method was not widely used because it was time consuming and expensive as well as having very high variation as to be expected in the animal assays. Snell and Peterson (1940) and Stokstad (1943) demonstrated that there are growth substances in liver and yeast extracts which are essential for the lactic bacterium,Lactobacillus casei. A factor needed for the growth of Streptococcus lactis R later known as Streptococcus faecalis had also been extracted from spinach leaves by Mitchell,Snell and Williams (1941;1944) and they termed the material extracted from leaves'Folic acid'.A factor required for the growth of Leuconostoc citrovorum or later known as Pediococcus cerevisiae (Felton and Niven,1953) was isolated from liver (Sauberlich and Baumann,1948).

These many factors were later shown to belong to the same family of compounds. Pfiffner et al (1943) isolated the crystalline form of the material from liver.This was followed by the synthesis of a pteridine identical in structure and biological function by Angier and his co-workers (Angier et al 1945;Subbarow et al 1946).The essential requirement of folic acid by microorganisms led to many useful methods of detecting of these compounds and will be discussed in a greater detail in Chapter 2.

1.1.2 Structure,Nomenclature and Abbreviation.

Purrman (1940) elucidated the structure of pteridines isolated from butterfly wings.He showed that these compounds are derivatives of the bicyclic nitrogenous system,pyrimido (4,5-b)pyrazine.This is shown in Figure 1.1 and it is known simply as 'pteridine'.

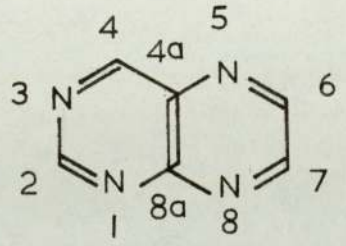


Figure 1.1 Structure and numbering of pteridine.

The term 'pterin' has been used as a general term for pigments of insects in the past but in more modern literature the term is restricted to derivatives of 2-amino-4-hydroxypteridine as shown in Figure 1.2,the keto-form is recognised as a stable form of these compounds (Blakley,1969).

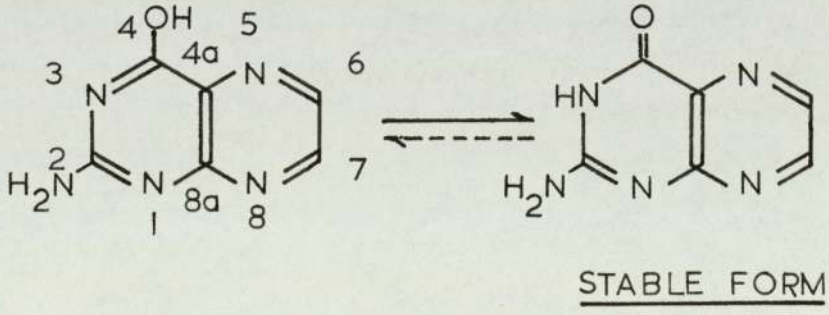


Figure 1.2 Structure and numbering of pterin.

The structures of xanthopterin,isoxanthopterin and leucopterin are shown in Figures 1.3,1.4 and 1.5,respectively.

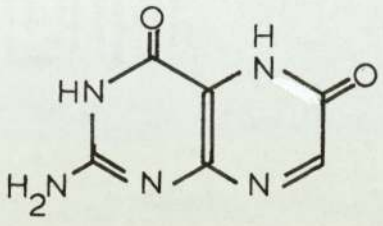


Figure 1.3 Structure of xanthopterin.

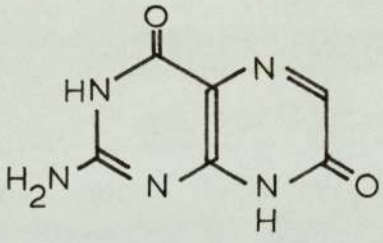


Figure 1.4 Structure of isoxanthopterin.

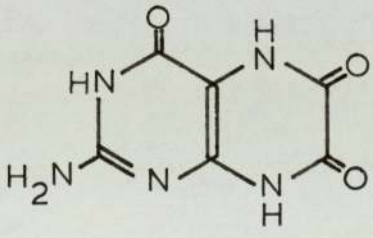


Figure 1.5 Structure of leucopterin.

Folic acid was shown to be a 6-alkylpterin by Angier et al 1945 and the term 'pteroylglutamic acid' was proposed by them.The term 'pteroylglutamic acid'was given on the basis that it is the derivative of 'pteroic acid' a compound shown in Figure 1.6 and 'pteroylglutamic acid' or 'Folic acid' was shown in Figure 1.7.

Figure 1.6 Structure and numbering of pteroic acid.

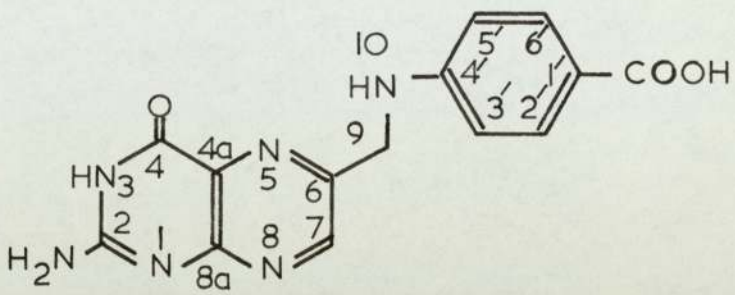


Figure 1.7 Structure and numbering of pteroylglutamic acid or folic acid.

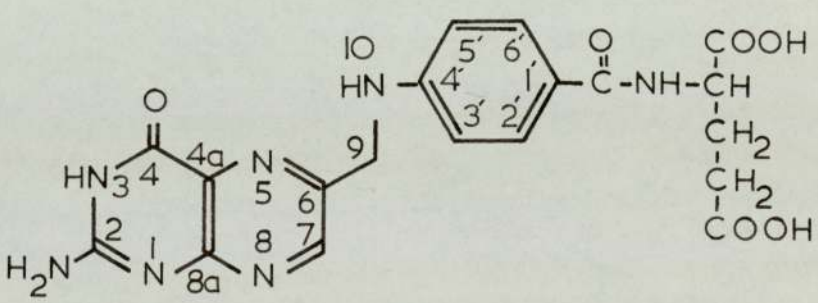
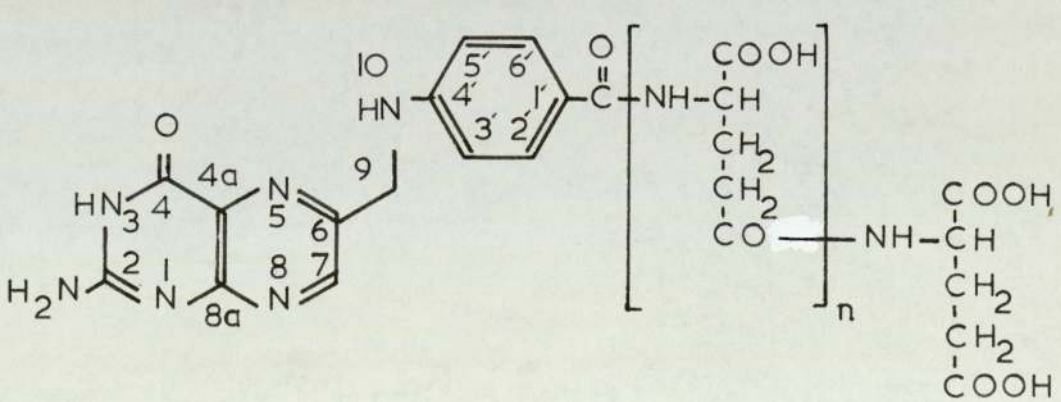


Figure 1.8 Structure and numbering of pteroylpolyglutamic acid.



n = 0 to 6

Pteroylpolyglutamic acids were found with various numbers of the conjugated glutamate residues(Blinkley et al 1944;Pfiffner,1946) and the general form of their structures is shown in Figure 1.8.

After many years of confusion in naming these compounds, the Commission on Biochemical Nomenclature (IUPAC-IUB Commission,1966) set out rules for naming these compounds in 'Folic acid' family but many scientists are prefer to use the old term 'Folic acid' for 'pteroylglutamic acid'. To avoid confusion the convention in this thesis is summarized as following:-

1) The term 'folic acid' is used synonymously with pteroyl-L-glutamic acid usually termed as pteroylglutamic acid. The structure of this compound was shown in Figure 1.7 and will be abbreviated as PteGlu.

2) The term 'folate' is used as a general term for all compounds known as 'folic acid' and its derivatives and the list of folates and their abbreviation were presented in Table 1.1.2.1.

3) The polyglutamates of folates have a general form as shown in Figure 1.8 and a specific compound will be indicated by its specified name together with number of glutamate residues. The abbreviation is similar to those of 2) and its appropriate suffix i.e. $PteGlu_3$ for pteroyltriglutamic acid or others as also summarized in Table 1.1.2.1.

4) The term tetrahydropteroylglutamic acid represented 5,6,7,8-tetrahydropteroylglutamic acid or else otherwise specified.

Table 1.1.2.1 The list of folates and other compounds concerning with this study and their abbreviations.

Compound	Abbreviation
Biopterin	-
Tetrahydrobiopterin	-
Pteroic acid	Pte
Pteroylglutamic acid	PteGlu
10-formylpteroylglutamic acid	10-CHO PteGlu
7,8-dihydropteroylglutamic acid	7,8-H_2PteGlu
Tetrahydropteroylglutamic acid	H_4PteGlu
5-formyltetrahydropteroylglutamic acid	5-CHO H_4PteGlu
10-formyltetrahydropteroylglutamic acid	10-CHO H_4PteGlu
5,10-methylenetetrahydropteroylglutamic acid	5,10-CH_2H_4PteGlu
5,10-methenyltetrahydropteroylglutamic acid	5,10-CH=H_4PteGlu
5-methyl-5,6-dihydropteroylglutamic acid	5-CH_3-5,6-H_2PteGlu
5-methyltetrahydropteroylglutamic acid	5-CH_3H_4PteGlu
4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid.	4a(OH)-5-CH_3H_4PteGlu
Methotrexate. 4-amino-4-deoxy-10-methylpteroylglutamic acid.	MTX.
Pteroyltriglutamic acid	$PteGlu_3$
Pteroylheptaglutamic acid	$PteGlu_7$
5-methyltetrahydropteroyltriglutamic acid	5-$CH_3H_4PteGlu_3$
10-formylpteroylheptaglutamic acid	10-CHO $PteGlu_7$
10-formyltetrahydropteroylheptaglutamic acid	10-CHO $H_4PteGlu_7$

Many folates present in Nature are known and can be synthesized chemically (Blakley,1969).Structures and the nomenclature of those folates concerning with this study are shown here.

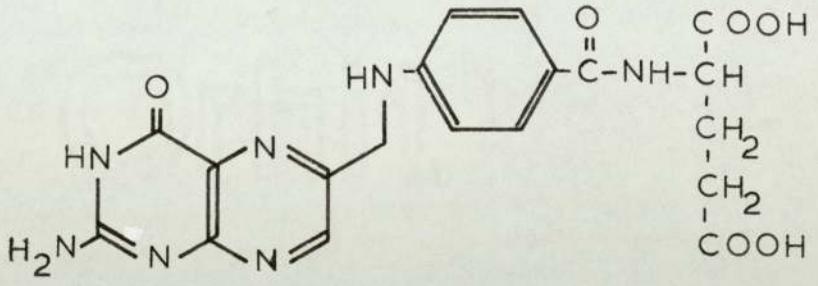


Figure 1.9 Pteroyl-D-glutamic acid

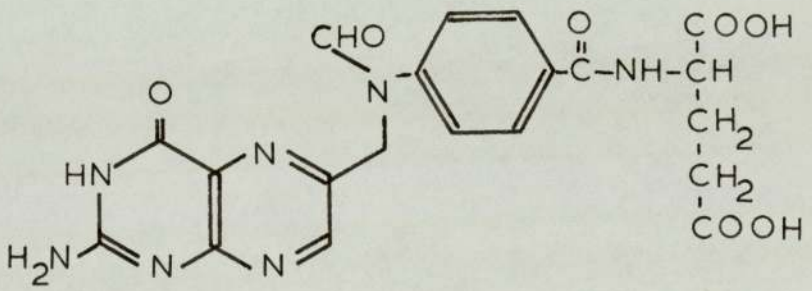


Figure 1.10 Structure of 10-formylpteroylglutamic acid

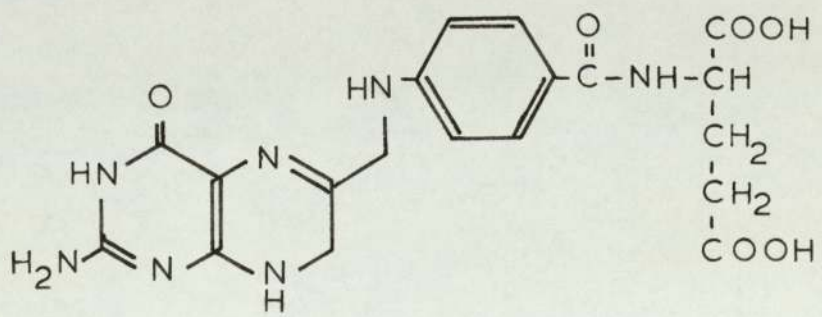


Figure 1.11 Structure of 7,8-dihydropteroylglutamic acid

Figure 1.12 Structure of 5-methyl-5,6-dihydropteroylglutamic acid.

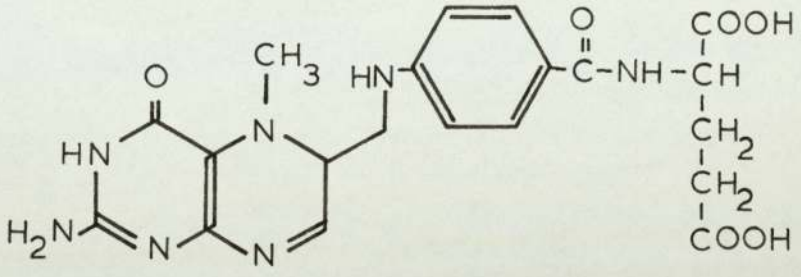


Figure 1.13 Structure of 5-methyl-5,8-dihydropteroylglutamic acid.

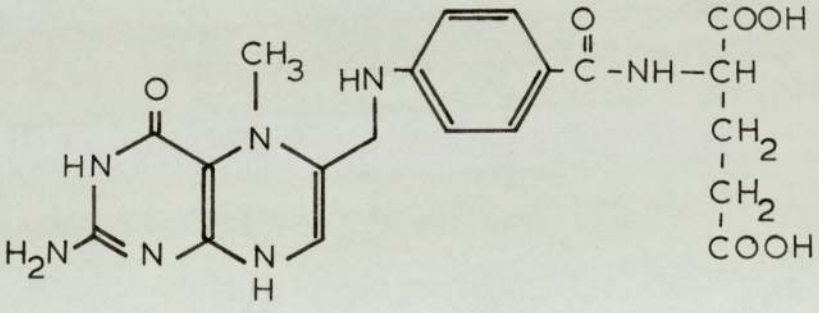


Figure 1.14 Structure of 5-methyltetrahydropteroylglutamic acid.

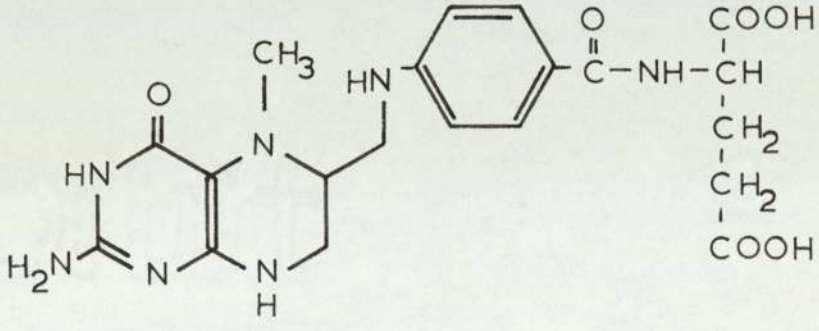


Figure 1.15 Structure of 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid.

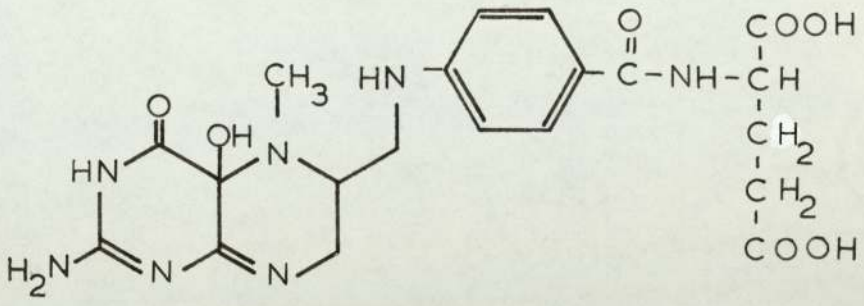


Figure 1.16 Structure of 5-formyltetrahydropteroylglutamic acid.

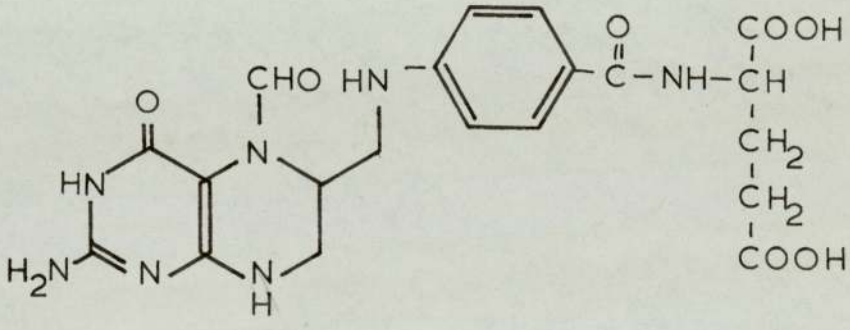


Figure 1.17 Structure of 10-formyltetrahydropteroylglutamic acid.

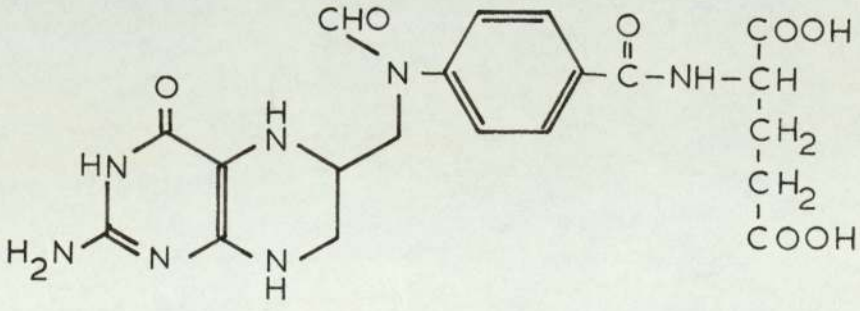


Figure 1.18 Structure of 5,10-methylenetetrahydropteroyl-glutamic acid.

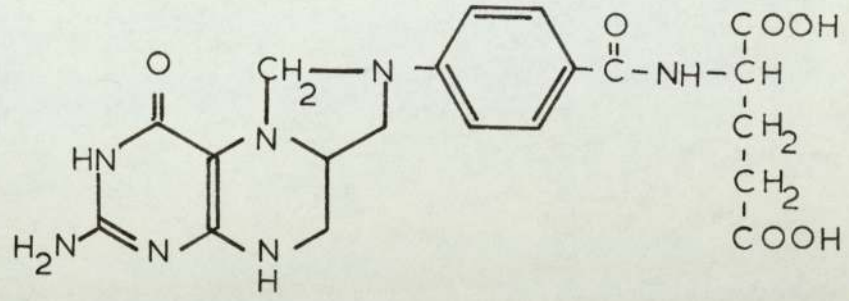


Figure 1.19 Structure of 5,10-methenyltetrahydropteroyl-glutamic acid.

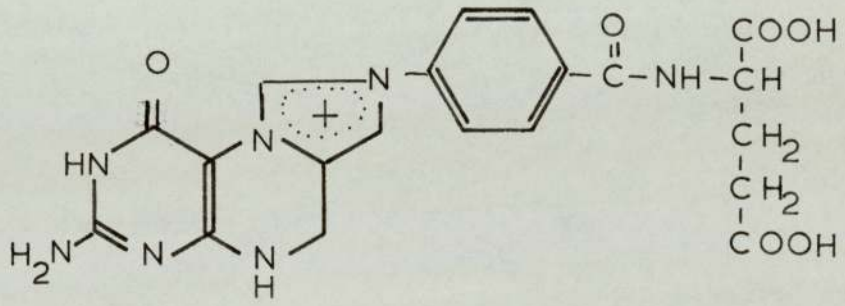


Figure 1.20 Structure of 5-formiminotetrahydropteroyl-glutamic acid.

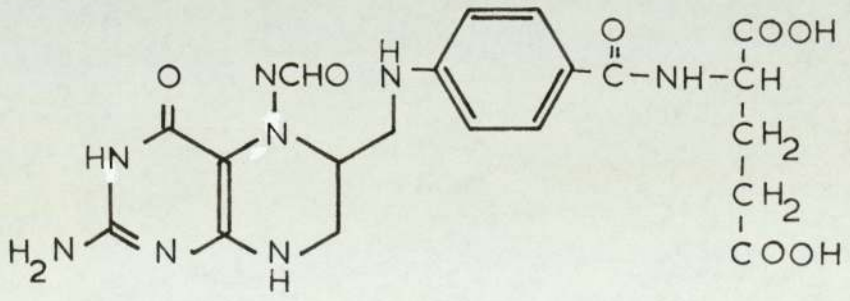
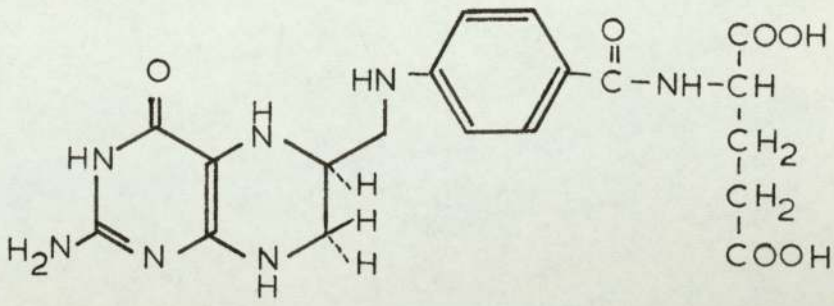


Figure 1.21 Structure of tetrahydropteroylglutamic acid.



Folic acid antagonists have been synthesized and were known as antimetabolite of folates and used in treatment of certain neoplastic diseases (Farber et al 1948).Aminopterin is 4-amino-4-deoxypteroylglutamic acid and the structure is shown in Figure 1.22. The other anti-metabolite known in a commercial name as methotrexate is 4-amino-4-deoxy-10-methyl-pteroylglutamic acid,Figure 1.23,is known to be the most effective in treatment of neoplastic diseases (Werkheiser,1961; Hertz et al 1961).

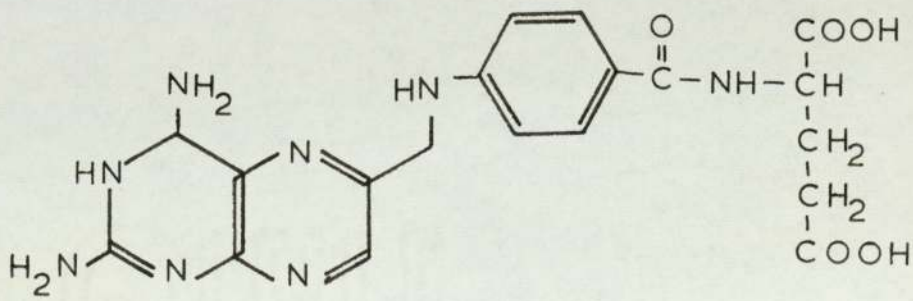
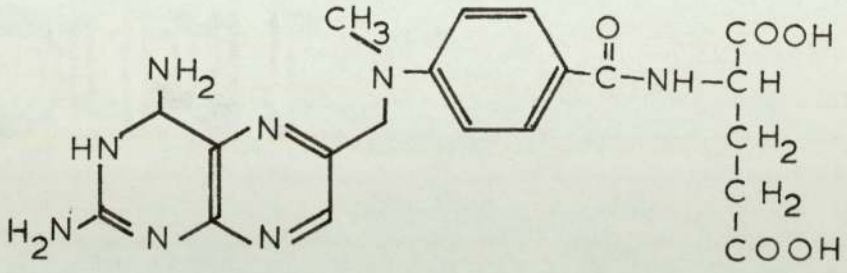


Figure 1.22 Structure of aminopterin or 4-amino-4-deoxy-pteroylglutamic acid.

Figure 1.23 Structure of methotrexate or 4-amino-4-deoxy-10-methylpteroylglutamic acid.



1.2 Biosynthesis and degradation of folate.

It is generally accepted that folic acid is built up stepwise (Goodwin,1963) as shown in reactions (1) and (2).

Pteridine + p-aminobenzoylglutamate ⟶ Folic acid ....(1)

Pteroic acid + glutamate ⟶ Folic acid ....(2)

The first and second reactions are the two possible routes of formation of folic acid and were mainly studied in microorganisms.The first reaction involving condensation of pteridine moiety and p-aminobenzoylglutamic acid whilst the second reaction are involving condensation of pteroic acid and glutamate residue.The formation of pteroic acid from pteridine and p-aminobenzoic acid in reaction (2) were shown to be a step antagonized by sulphonamides (Woods,1960;Brown,1961;1962). The conjugated forms of folate were shown to have been formed by further addition of single glutamic acid residues (Krumdieck et al 1966;Stokstad and Koch,1967).The breakdown of folic acid are involving both enzymatic and oxidation with split at the 9,10-position in general (Stokstad et al 1946) and will be discussed later on in greater detail.

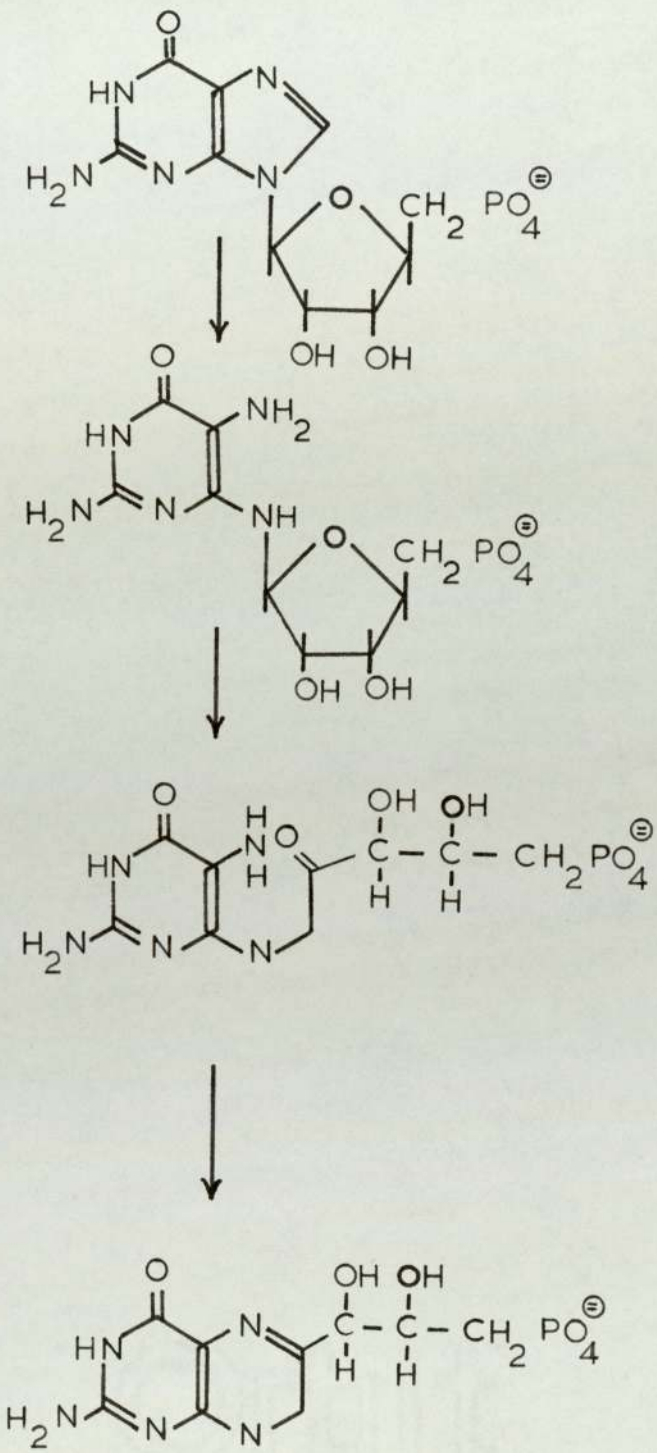
1.2.1 Biosynthesis of 2-amino-4-hydroxy-6-substituted pteridines

Because of structural similarities purines and pyrimidines were early considered as potential starting materials for pteridine biosynthesis.

Weygand and Waldschmidt (1955) found that ^{14}C-formate, $^{14}CO_2$ and ^{14}C-glycine were incorporated by butterflies into positions 2,4 and 4a and 8a, respectively, of xanthopterin and leucopterin. The pattern of labelling is the same as that observed with purines and riboflavin. Riboflavin is synthesized via purines as well reviewed by Goodwin (1963). In case of pteridines it has been shown that 2-^{14}C-adenine is incorporated into the pteroylglutamic acid by Corynebacterium (Viera and Shaw, 1961). The biosynthetic pathway of pteridine by Corynebacteria is similar to those proposed by Brenner-Holzach and Leuthard (1959,1961) and shown in Figure 1.24 and 1.25, respectively. Reynolds and Brown (1962) showed that by using cell-free extracts of Escherichiacoli guanine is utilized in significant amounts for the formation of folates if ribose or its phosphorylated derivative is present. When guanine-8-^{14}C was used as substrate, an unlabelled dihydropteroylglutamic acid was produced.

More evidences about biosynthesis of pteridines has been obtained during the past ten years. Krumdieck et al 1965 have shown that by using uniformly labelled ^{14}C-cytidine to prefer-entially label the ribosyl moiety of purine nucleotides in growing cultures of Corynebacterium species, the entire 2-amino-4-hydroxy-6-substituted pteridine extracted from the growing cultures exhibited specific radioactivity at carbon atoms 6,7 and 9 as shown to be originated from carbon atoms of ribosyl moiety at positions 2',1' and 3', respectively. The enzyme capable of catalysing the conversion of guanosine--5'-triphosphate into D-erythro-7,8-dihydroneopterin-3'-triphosphate had been isolated from E.coli (Brown, G.M., 1971).

Figure 1.24 The Brenner-Holzach and Leuthard biosynthetic pathway of pteridines.



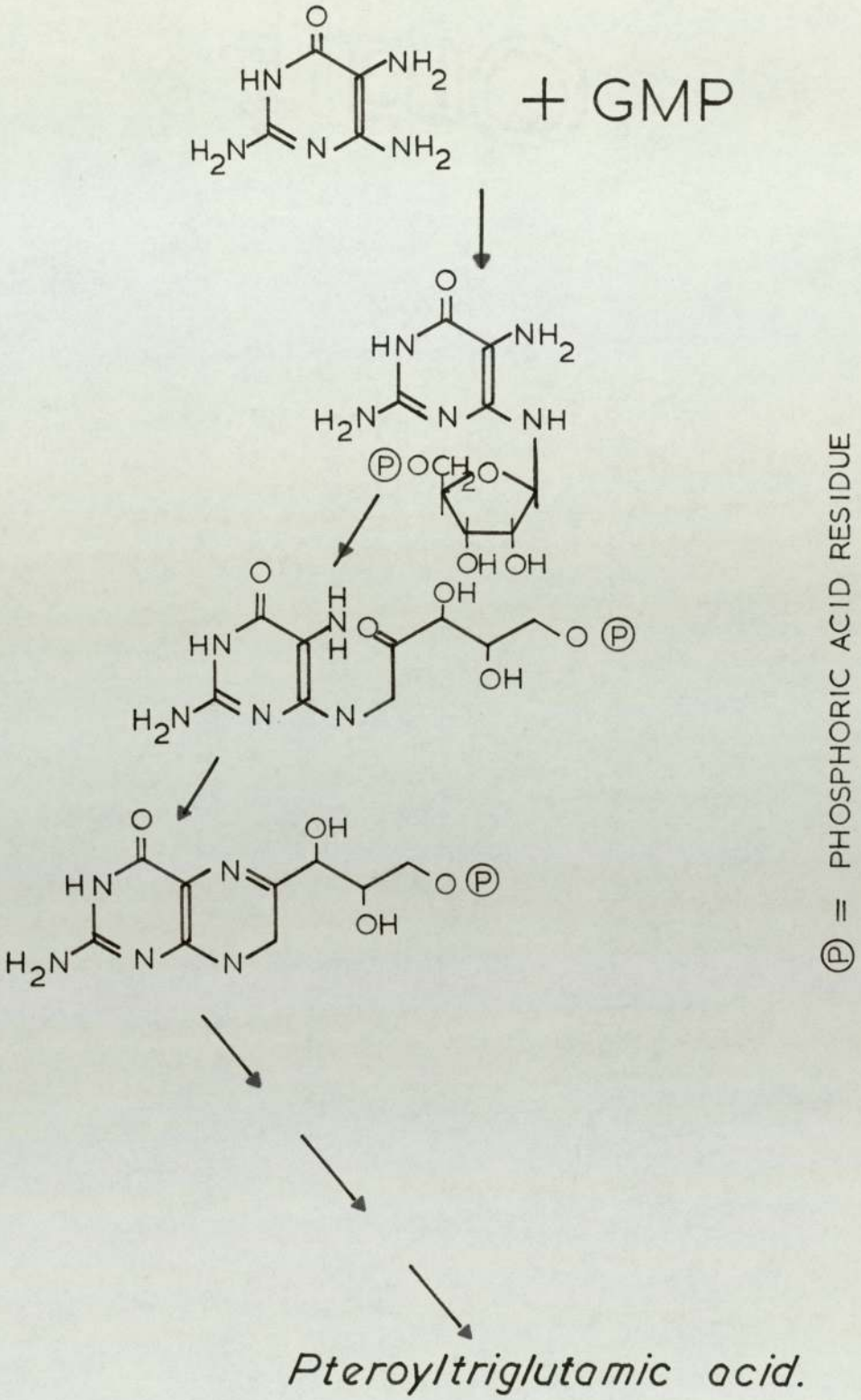


Figure 1.25 : Pteridines biosynthetic pathway in Corynebact.spp.

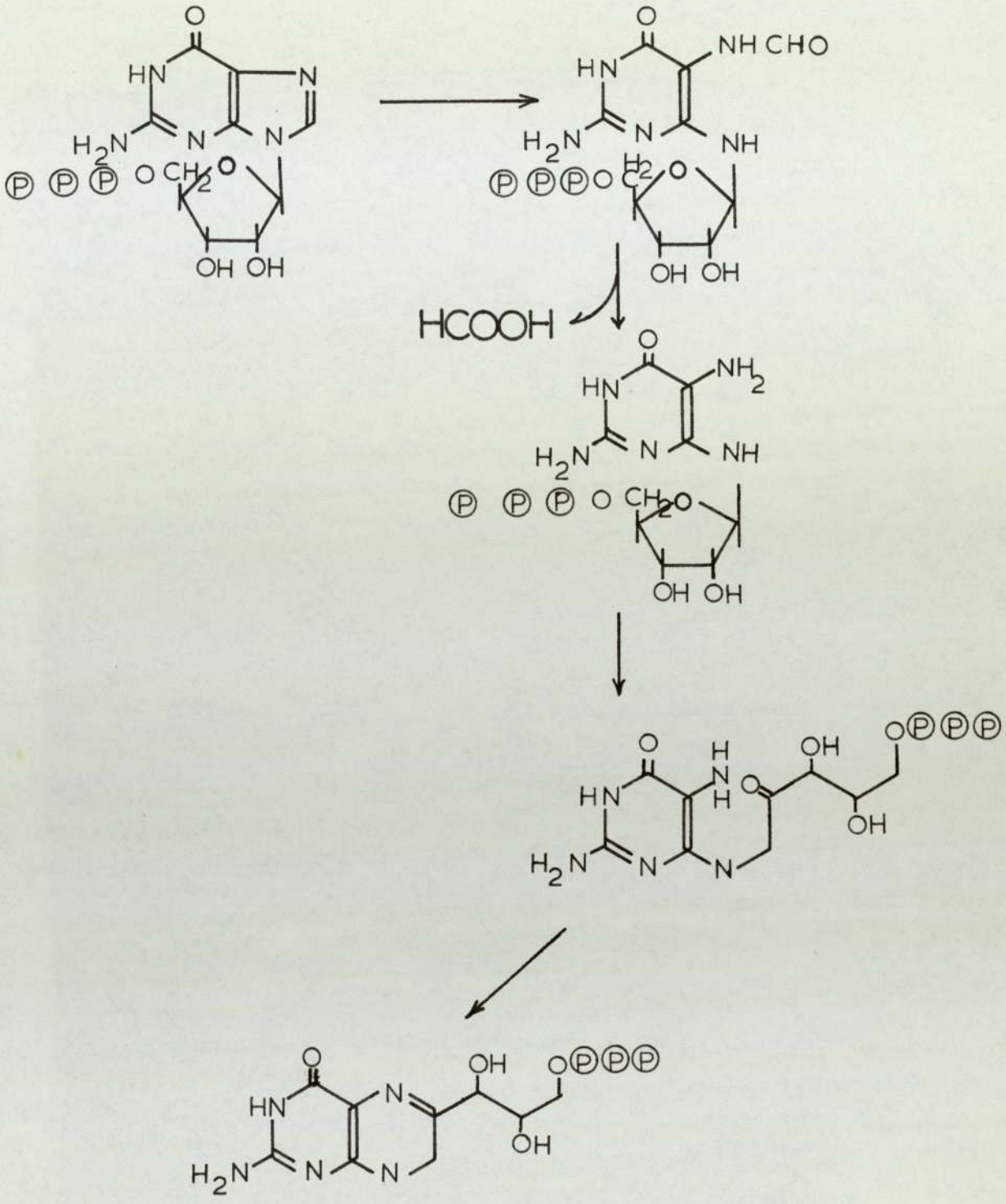


Figure 1.26 : Biosynthesis pathway of pteridine (Brown-Shiota pathway).

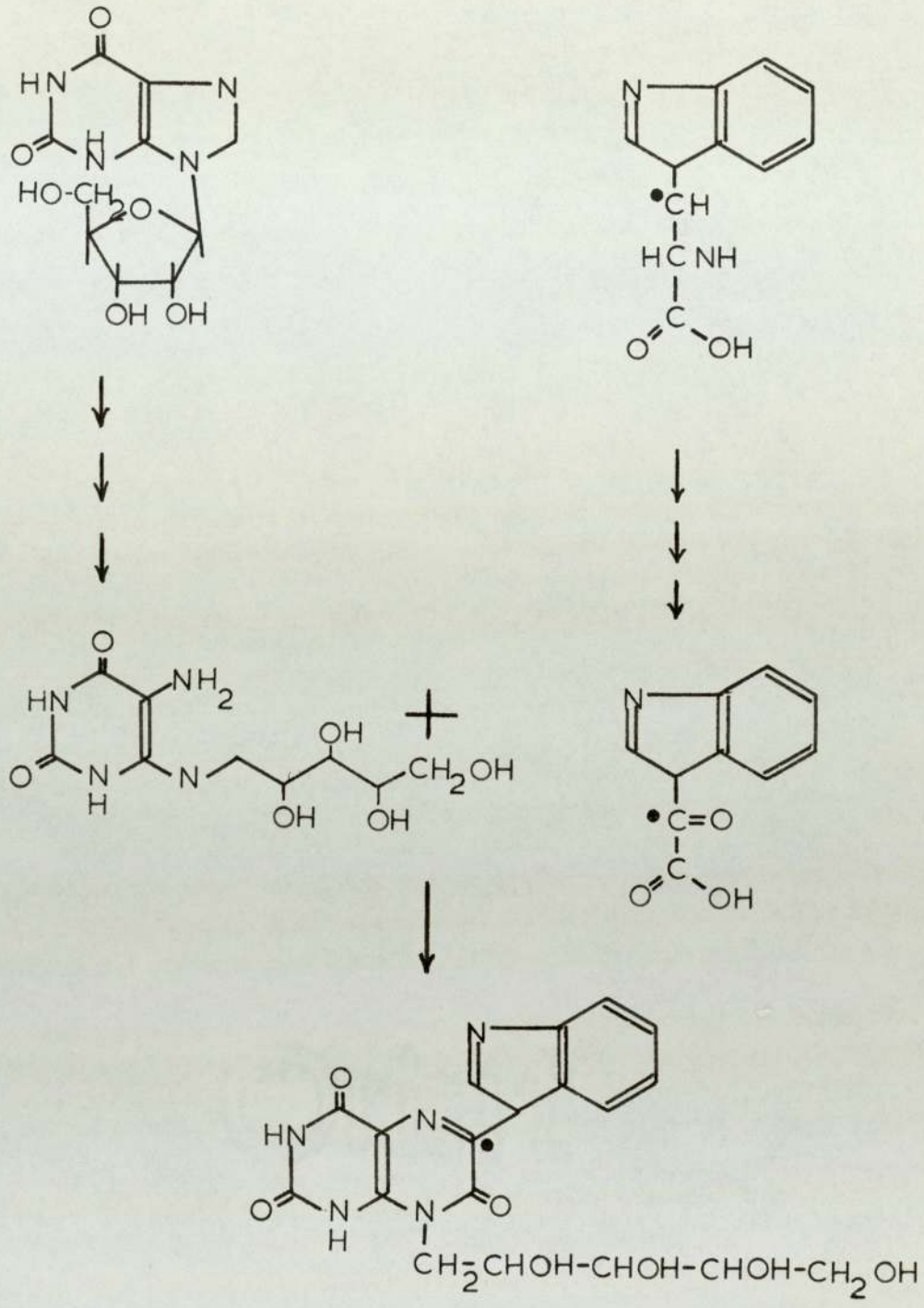


Figure 1.27 : Biosynthesis of pteridine in Achromobacter petrophilum (Takeda et al 1970).

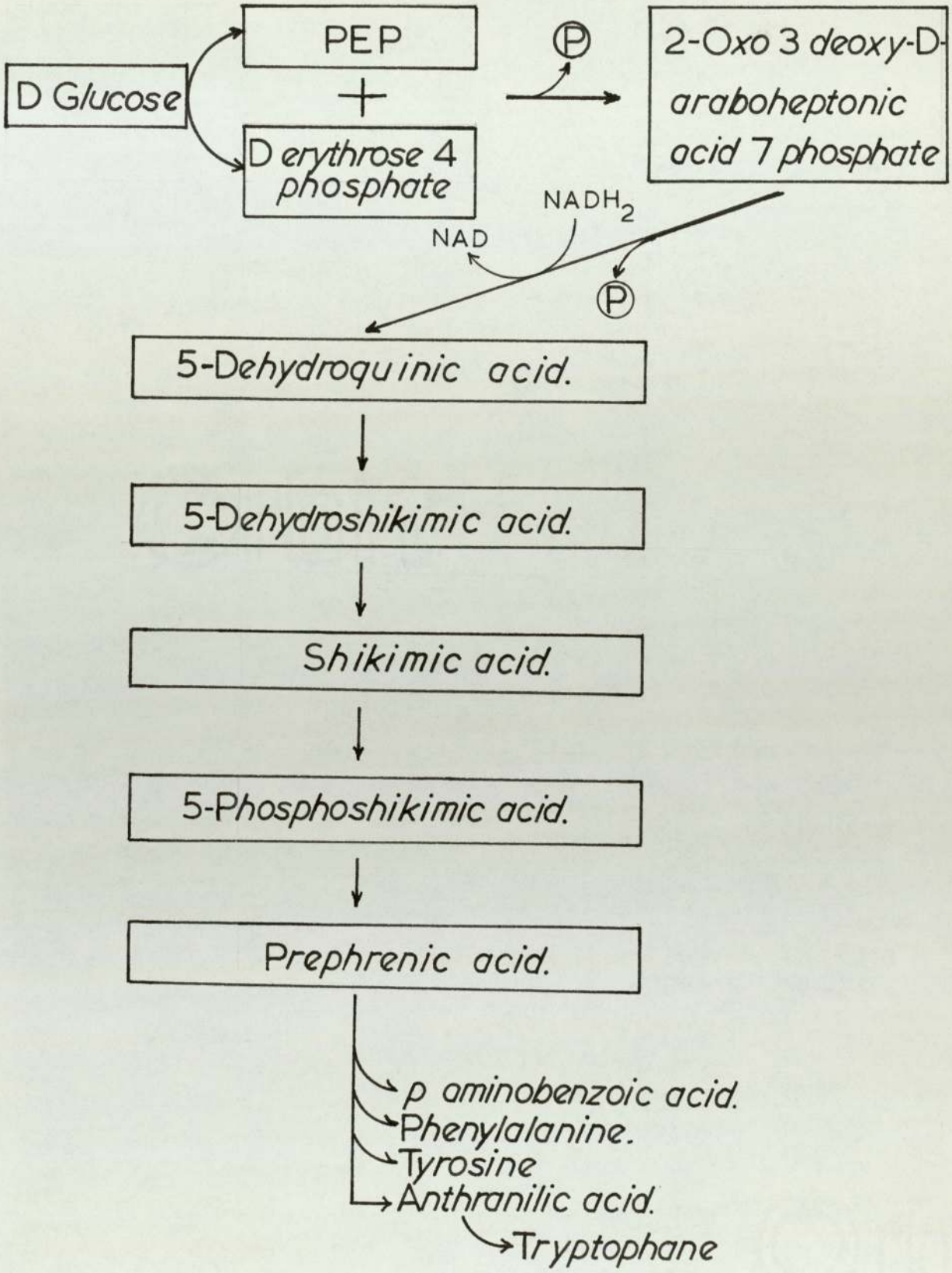


Figure 1.28 : Biosynthesis of p-aminobenzoic acid (Goodwin,1963).

They proposed the biosynthesis of pteridine in their studies as shown in Figure 1.26. Shiota and his co-workers had also isolated a similar enzyme from Lactobacillus plantarum and their works had been reviewed (Shiota ,T., 1971). Cone and Guroff (1971) have shown that a cyclic dihydroneopterin phosphate is the end product of GTP-conversion in Comamonas.Forrest and Baalen (1970) have also discussed other possible pathways for pteridine biosynthesis and a route via a pyrazine compound was proposed. Takeda et al (1970) in their studies on the formation of indolyl- and hydroxyphenylpteridines by Achromobacter petrophilum,found that GTP and xanthine derivatives were utilized in the formation of pteridines but radioactivity was incorporated into the ring system when 3-^{14}C-tryptophan added into the culture media. The possible pathway was summarized as shown in Figure 1.27. Studies in the rat have established that the biopterin excretion is constant at about 30 ug/day even when 2 generations were fed on a biopterin-free diet (Pabst et al 1966).This finding indicates biopterin formation in a mammal known to be incapable of forming folic acid.They also found that when purine precursors such as formate or glycine or those pteridine precursors i.e. adenine,guanine,guanosine, guanylic acid or GTP were injected or fed to rats only trace amounts were converted to biopterin.Increased formation of biopterin was noted when nucleic acid synthesis was inhibited by injection of actinomycin D and the accompanying flow of purine precursors into nucleic acid biosynthesis was decreased (Rembold and Gyure,1972).

It has also been shown that injection of radioactive neopterin or tetrahydroneopterin did not result in incorporation of label into the biopterin excreted in the next four days(Rembold et al 1971).

1.2.2 Biosynthesis of p-aminobenzoic acid and p-aminobenzoyl-glutamic acid.

The microbiological investigations which led to the identification of shikimic acid as the intermediate in the biosynthesis of aromatic aminoacids and p-aminobenzoic acid had been extensively discussed by Goodwin (1963).The scheme is summarized as shown in Figure 1.28.

Studies with cell-free extracts of Mycobacterium avium have led to the formation of p-aminobenzoylglutamic acid in system containing p-aminobenzoic acid,glutamate,ATP,Mg^{+2} and CoA (Katunuma et al 1956,1957). If glutamate were omitted from the reaction mixture a positive hydroxamic reaction was obtained suggesting the formation of coenzyme A derivative of p-aminobenzoic acid and the involving reactions are summarized in Figure 1.29.

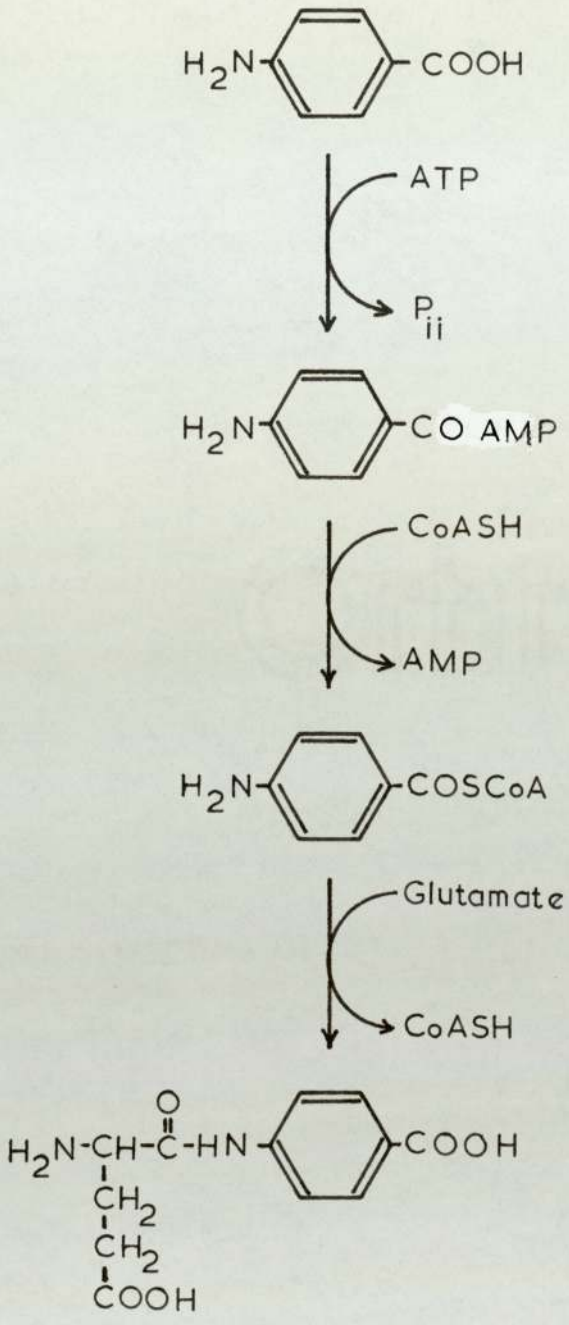


Figure 1.29 : Biosynthesis of p-aminobenzoylglutamic acid (Katunuma et al 1956,1957).

1.2.3 Biosynthesis of glutamic acid.

Glutamic acid is a key compound in nitrogen metabolism in both plants and animals and the basic route of formation of glutamic acid is shown in Figure 1.30.

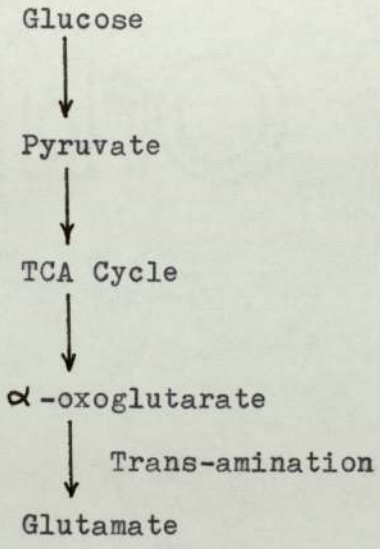


Figure 1.30 Basic route of formation of glutamate

There are many other pathways which give rise to the glutamic acid formation including the carboxylation of γ-aminobutyrate and deamination of glutamine and many more as summarized in the metabolic pathways (Nicholson,1972).The catabolism of histidine,a compound known to be formed from glutamate,is considered as one of a major source of glutamic acid and the view is well doccumented (Goodwin,1963) as it is summarized in Figure 1.31.

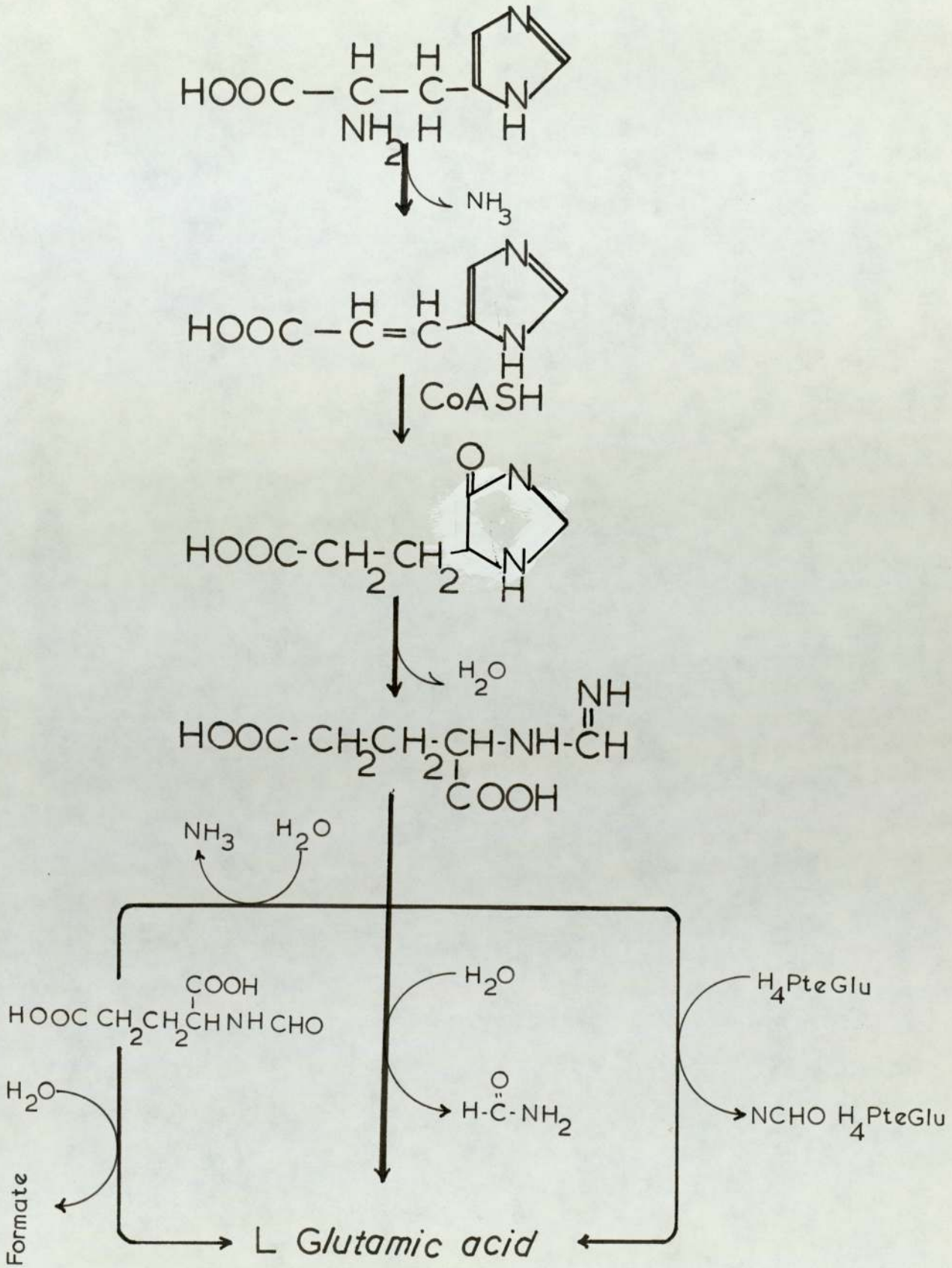


Figure 1.31 : Catabolism of histidine.

1.2.4 Condensation of pteridine with p-aminobenzoylglutamic acid or pteroic acid with glutamic acid and pteridine with p-aminobenzoic acid.

The inhibition effect of sulphonamides on bacterial utilization of p-aminobenzoic acid is known to be the competitive action (Woods,1940). The concept of antimetabolic action of sulphonamides were also seen in the biosynthesis of folic acid (Woods,1960;Brown,1961,1962).Folic acid and p-aminobenzoic acid were shown to be synthesized by staphylococci resistant to sulphonamides but not by those of sulphonamides sensitive bacteria. The condensation of pteridine with p-aminobenzoic acid or p-aminobenzoylglutamic acid is a step require Mg^{+2} and ATP (Brown et al 1959,1961;Shiota et al 1959, 1961). The propose action of sulphonamides on the biosynthesis of folic acid is shown in Figure 1.32.

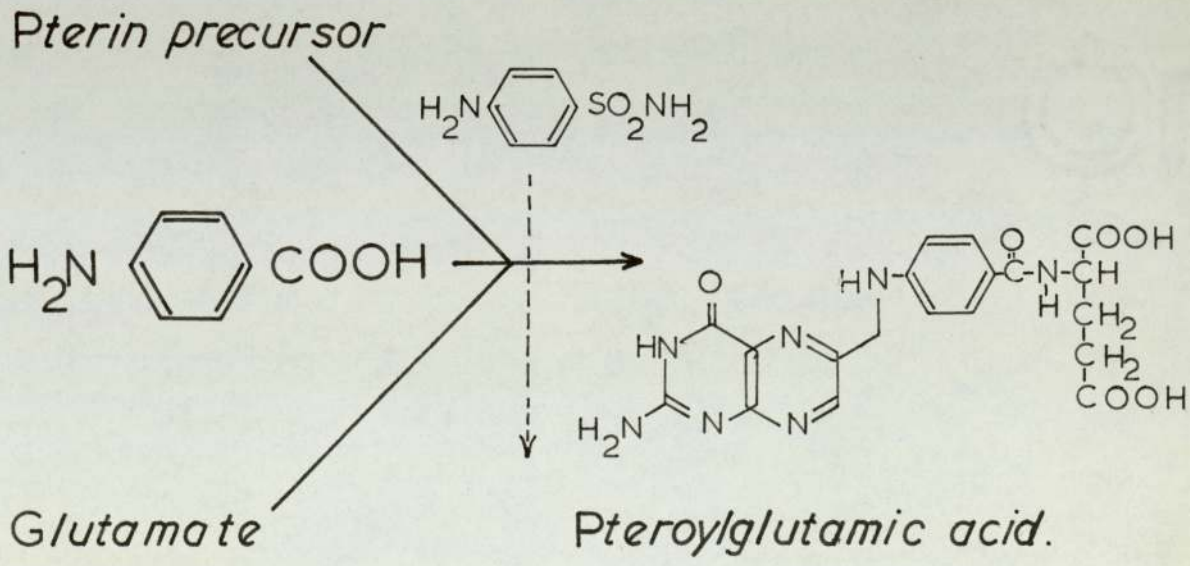
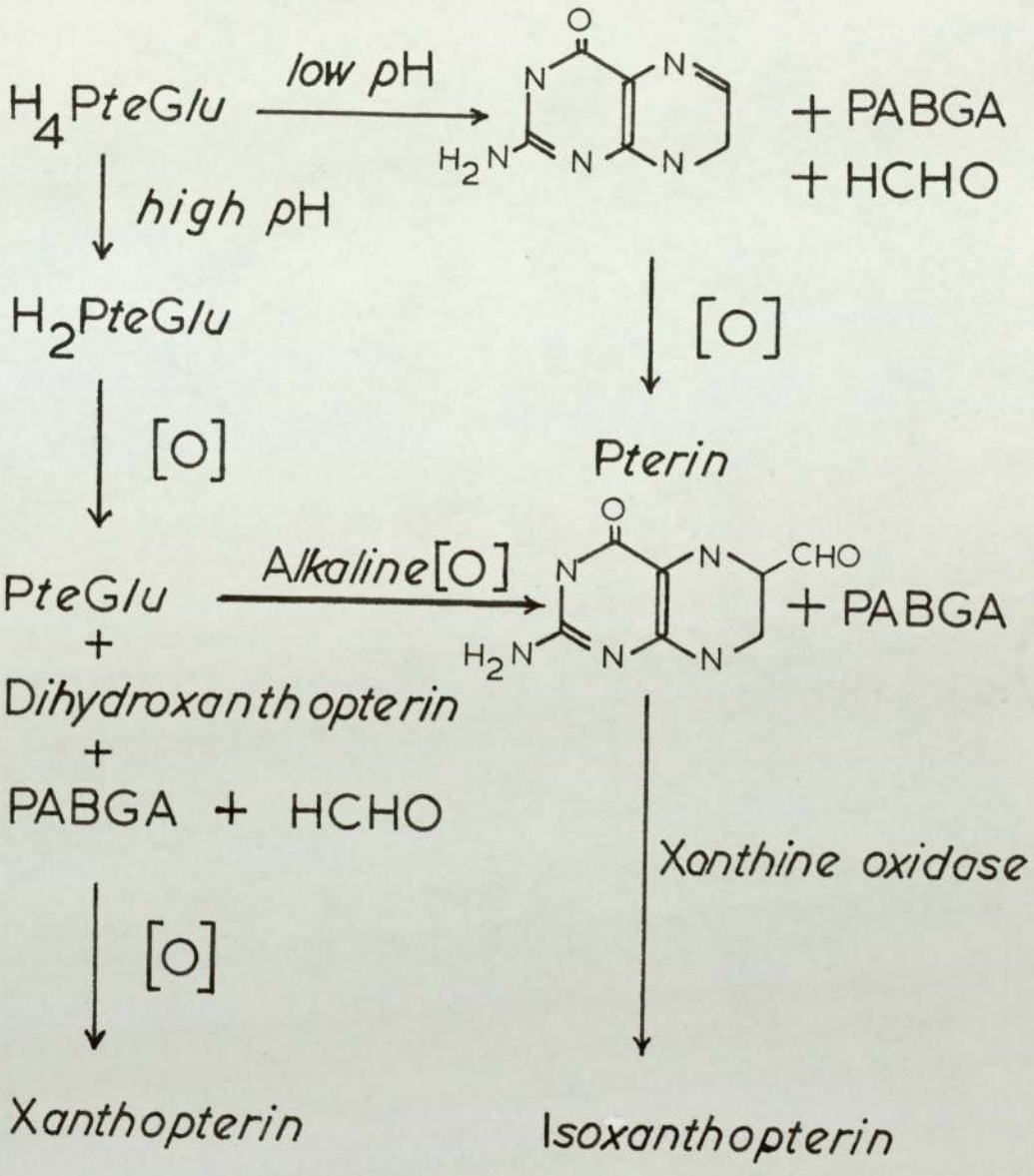


Figure 1.32 Sulphonamides inhibition of folate biosynthesis.

1.2.5 Degradation of folic acid its derivatives.

The oxidative hydrolysis of pteroylglutamic acid in alkaline medium is followed by splitting at 9-10 position (Stokstad et al 1946). Isoxanthopterin is a pteridine portion of folic acid isolated from fresh human urine (Blair,1958). In human red cells it has been shown that there is an enzyme capable of splitting folate and pteridine (Braganca et al 1957). Enzyme splitting 5-formyltetrahydropteroylglutamic acid had been isolated and purified from hog liver (Silverman et al 1957).The degradation of folic acid by the enzyme peroxidase from Cicer arietinum (Chanekar and Braganca,1960) and the reaction involved deamination,amide cleavage of folic acid had been reported (Mcnutt,1963).The autoxidation of folates and tetrahydrobiopterin had been extensively studied by Blair and his co-workers (Blair and Pearson,1974;Pearson,1974; Blair,Pearson and Robb,1975).10-formylpteroylglutamic acid at a low dose given to the rat has been shown to be metabolised to simple pteridines (Beavon,1973). Xanthopterin was shown to be one of the oxidation products of tetrahydropteroylglutamic acid (Stocks-Wilson,1971).Photodecomposition of folates and folates decomposition after autoclaving for 15 minute at 110^{o}C had been shown to give some pteridines in the products, xanthopterin,2-amino-4-hydroxypteridine,2-amino-4-hydroxy-6-formylpteridine,2-amino-4-hydroxypteridine-6-carboxylic acid are those detected pteridines (Beavon,1973).

These evidences suggested that folate degradation can occur both enzymic and non-enzymic reactions as summarized in Figure 1.33.



PABGA = *p*-aminobenzoylglutamic acid.

Figure 1.33 : The degradation of folates.

1.3 Occurrence and biological activities of folates and related pteridines.

Folates and related pteridines are widely distributed in Nature.They were found distributed in varying amounts and forms in insects,amphibia,crustacea,reptiles,fishes,mammals, microorganisms and plants (Blakley,1969). Mainly mammals will be dealt with a great detail in this study.

The origin of pteridines in mammals is not clearly understood. It has been demonstrated that biopterin excretion in rats is constant at about 30 ug/day eventhough these animals were fed on a biopterin-free diet for 2 generations (Pabst,W., and Rembold,H.,1966).This evidence suggested that biopterin is synthesized in the mammal. Isoxanthopterin had been isolated from fresh human urine (Blair,1958).Unidentified pterins active for the growth of Crithidia fasciculata in serum and blood of man,cattle,sheep,horse,rabbit and chicken had been reported (Frank,Baker and Sobotka,1963).In human urine the pterins active for the growth of Crithidia fasciculata has recently been identified as 7,8-dihydrobiopterin and probably 5,6,7,8-tetrahydrobiopterin (Leeming and Blair,1974).Biopterin is a compound shown in Figure 1.34 and its reduced form, tetrahydrobiopterin is shown in Figure 1.35.

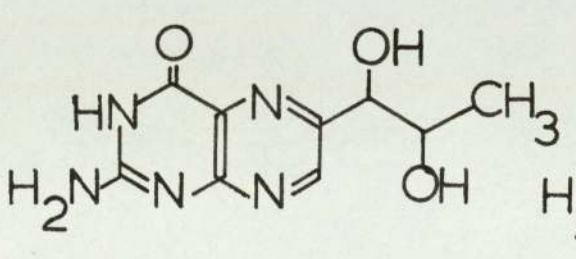


Figure 1.34 Biopterin

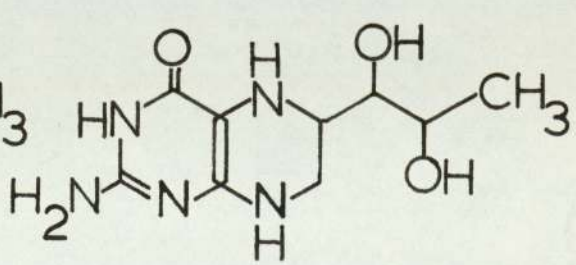


Figure 1.35 Tetrahydrobiopterin

5,6,7,8 - Tetrahydrobiopterin is a cofactor of many important biological oxidations involving the use of molecular oxygen. The hydroxylation of phenylalanine to tyrosine in the enzymic systems being the most closely studied,and the overall reaction was suggested by Kaufman (1957) is shown here:-

$$\text{Phenylalanine} + \text{NADPH} + H^+ + O_2 \longrightarrow \text{NADH}^+ + H_2O + \text{Tyrosine} \quad ..(3)$$

The above reaction was desrived from the studies of enzymes from rat and sheep livers.After a further 100-fold purification these enzymes exhibited an absolute requirement of a co-factor. The known co-factors are tetrahydro-6-methylpterin and tetrahydro-6,7-dimethylpterin (Kaufman,1958).The stepwise reactions of the phenylalanine hydroxylation to tyrosine were also ruled out (Kaufman,1959) and are shown in reaction (4) and (5).

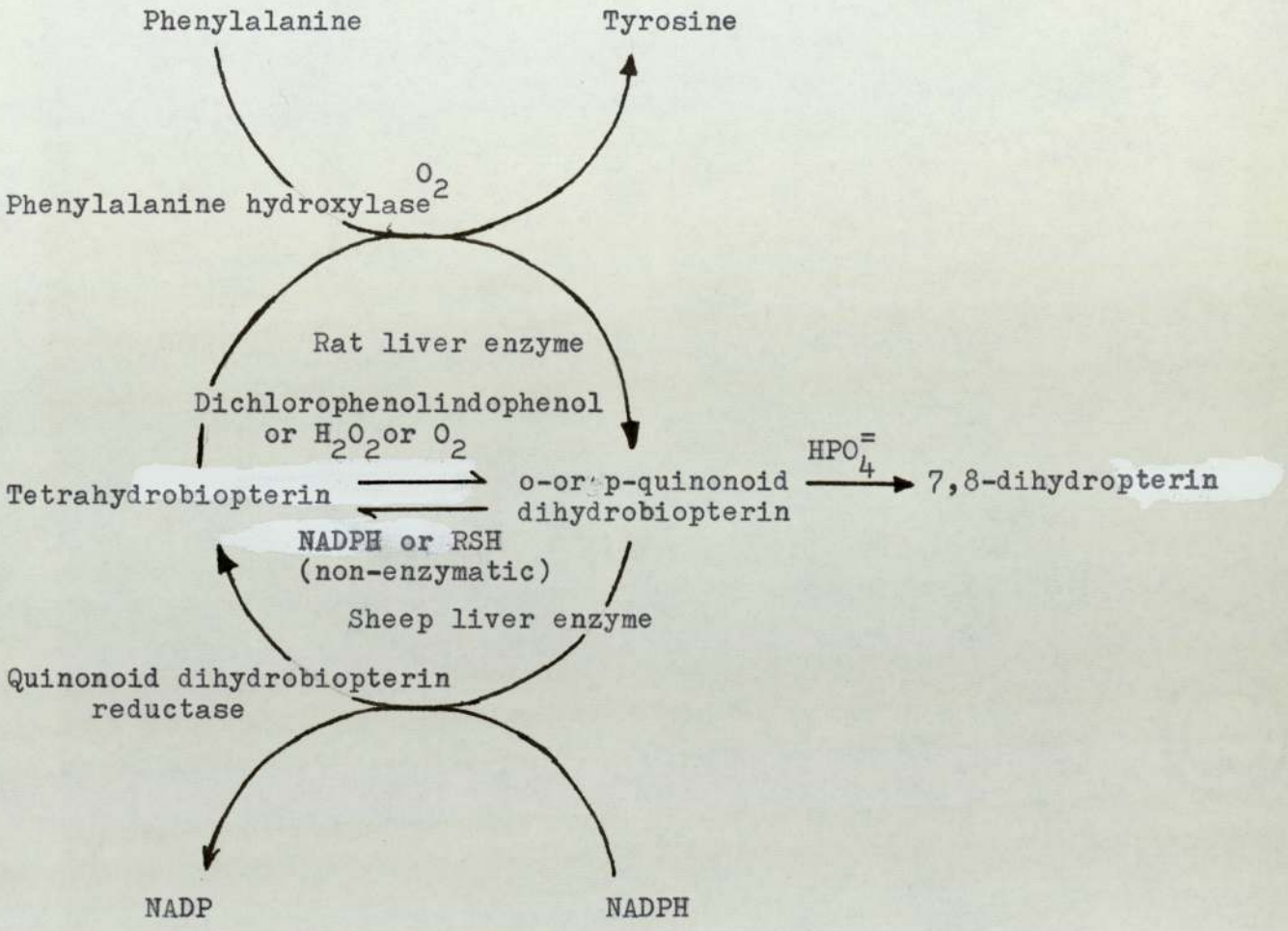
$$\text{Phenylalanine} + \text{Tetrahydrobiopterin} + O_2 \downarrow$$

$$\text{Tyrosine} + H_2O + \text{Dihydrobiopterin} \quad .....(4)$$

$$\text{Dihydrobiopterin} + \text{NADPH} + H^+ \longrightarrow \text{Tetrahydrobiopterin} + \text{NADH}^+ \quad ..(5)$$

He showed that in reaction (4) the rate of hydroxylation will be substantially boosted by catalyses of the liver enzyme and NADPH of reaction (5) would served as reducing reagent converting the inactive dihydrobiopterin back to the active tetrahydrobiopterin and thus maintain the process.Without NADPH the reaction is not changed but soon terminated.A scheme summarized the process was shown in Figure 1.36.

Figure 1.36 A scheme summarized the reactions of the dihydrobiopterin intermediate of phenylalanine hydroxylation from Blakley (1969).



Investigations into folate content of various tissues of mammals indicated that liver contains the highest amount of folates. Baker et al (1964) estimated that human liver contains 7 mg of folates. The total folate content of human body had been shown to be 12-15 mg (Today's drugs,1964).Horse liver folate has been islated and identified to be mainly 5-methyl-tetrahydropteroylglutamic acid (Donaldson and Keresztesy,1959). Liver folates of sheep with vitamin B_{12} deficiency is 0.03-0.14 ug/g liver as compared with 0.19-1.87 ug/g liver in normal animals and the low liver folates of these vitamin B_{12} deficiency animals became normal after treatment with vitamin B_{12} indicated that vitamin B_{12} playing some part in the metabolism of folate (Dawbarn et al 1958).

Post-mortem specimens of liver were shown to have folate of 0.6-12 ug/g liver (Romine,1960). Liver folates of liver biopsy specimens from subjects with folate deficiency had been shown to have a mean value of about 2 ug/g liver and the highest value is 3.9 ug/g liver (Waters,1963).Liver biopsy specimens from patients with carcinoma and leukemia were shown to have liver folate of 4.0 ug/g and 2.9 ug/g liver, respectively (Leevy et al 1965).Liver specimens taken at laparotomy contain folates 0.7-17 ug/g liver (Chanarin et al 1966).The folates in liver are 5-methyltetrahydropteroylglutamic acid,5-formyltetrahydropteroylglutamic acid,10-formyltetrahydropteroylglutamic acid and their polyglutamate forms were demonstrated (Wang et al 1967;Whitehead,1971).Similar folates were also present in rat liver with values from 7-15 ug/g liver (Bennett et al 1964),9-15 ug/g liver (Grossowicz et al 1963,1964) and with a mean value of 20 ug/g (Bird et al 1965).

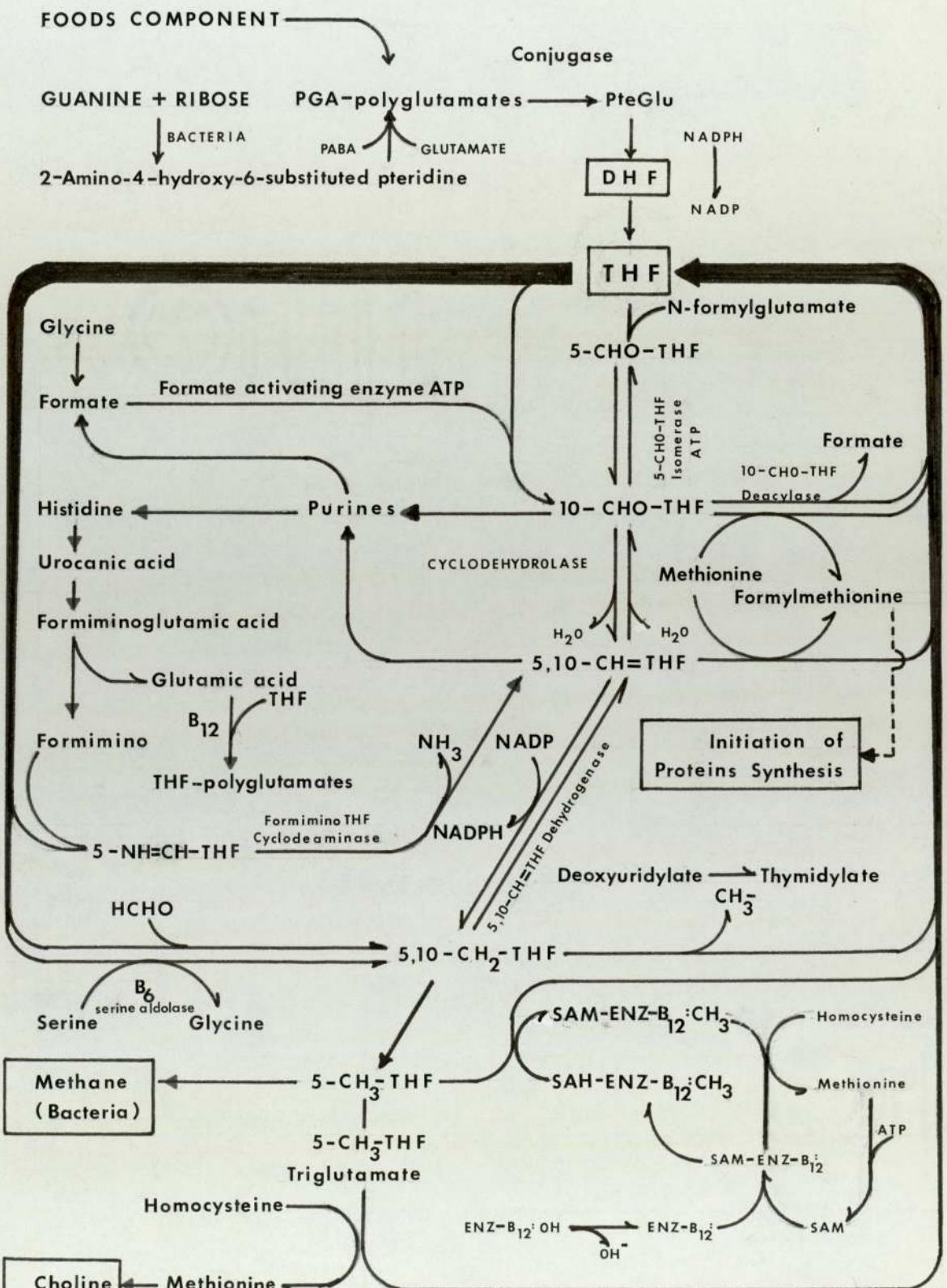
Human serum or plasma contains folates 3-20 ng/ml and the packed cells contains 105-407 ng/ml (Chanarin,1969). Values of normal total folates as assayed with L.casei are usually known to be positive skewed about the mean according to a dependence on dietary folates (Ratanasthien et al 1974). 5-methyltetrahydropteroylglutamic acid is a major component of serum folates and may be present as peptides in the redcells (Noronha and Aboobaker,1963;Iwai et al 1964).A minor folate active for supporting the growth of P.cerevisiae had been shown in both red cells (Iwai et al 1964) and serum of human (Ratanasthien et al 1974;Blair et al 1974).Folates in other tissues had been studied,rat kidney contains 1.6 ug/g of tissue and intestine and spleen contained 0.6 ug/g tissue (Grossowicz et al 1964). Folates of human marrow cells were 95-726 ng/ml (Cooke et al 1959) and 64 ng/10^9cells with standard deviation of 42.5 (James and Hart,1966).

Human breast milk contains folates, a range of 0.9-1.8 ng/ml when microbiologically assayed with S.faecalis (Collins et al 1951).Matoth et al (1965) reported ranges from 7.4-61 ng/ml and 2.4-17.6 ng/ml when microbiologically assayed with L.casei and P.cerevisiae,respectively.

The cerebrospinal fluid has been studied for the folate content.Sobotka et al (1960) reported values of 1-5 ng/ml and 10-30 ng/ml when assayed with P.cerevisiae and L.casei, respectively. A mean value of 23.6 ng/ml of cerebrospinal fluid L.casei level had also been reported (Wells and Casey,1967). Human bile contains folates 8-65 ng/ml with a mean value of 33 ng/ml (Baker et al 1965).

Folates in saliva ranging from 2-165 ng/ml with a mean of 41 ng/ml have been reported(Markkanen and Makila,1965). The main biological function of folates is a source of single carbon supplier,the units were known at methane,methanol, formaldehyde levels of oxidation. The details covering references and discussion are well documented(Blakley,1969). The biological reactions of folates were summarized as shown in Figure 1.37.

Figure 1.37 The biological reactions of folates (From Baker and Frank, 1968; Blakley, 1969). DHF = H_2PteGlu and THF = H_4PteGlu.



1.4 Scope of the present study.

A lot of work has been done in this field and they were carried out both in vivo and in vitro in experimental animals with varying amount of detail . The administration of radioactive ^{14}C and ^{3}H folates have been widely used in the experimental animals. In this study the metabolism and handling of folates in the mammal especially man were studied. Since the radioactive ^{14}C and ^{3}H labelled folate is not approved to be safe for human administration only non-radioactive folates were used in this study.

Various folates were prepared and purified mainly according to the established methods.Their microbiological properties were studied using microbiological assays with L.casei,S.faecalis,P.cerevisiae and bioautographic techniques. The microbiological techniques were discussed in details, aseptic addition methods were employed for these microbiological assays (Herbert,1966). Microbiological assays with Crithidia fasciculata were used for determination of biopterin and its derivatives (Leeming and Blair,1974). Attempts were made to verify folates constituted in serum and urine of both normal human and some diseases.

Oral administration of various folates at both physiological and pharmacological doses were studied mainly in normal human. Oral administration of some specific folates were studied in some patients mainly at pharmacological doses. Serum and urine folates from these subjects were microbiologically assayed and identified.

Metabolism of folates in subjects on treatment with dihydrofolate reductase inhibitor drugs especially methotrexate were also studied. The inhibition effect of methotrexate on microbiological assays with L.case,S.faecalis and P.cerevisiae were studied.

The relationship of folates and biopterin derivatives were studied in some details. Seasonal and diurnal variations of serum and urine folates were studied in both man and rat. In addition metabolism of some folates were studied in normal subjects with temporary achlorhydria after oral administration of 3 to 5 gm of sodiumbicarbonate.

These results were discussed in the light of those results cited from literature.Pharmacokinetics of these folates were also studied.The other clinical parameters were also discussed for their relationship to the obtained results.

Chapter 2.

Measurement and Identification of folates.

2.1 Physical and Chemical methods of measurement and identification of folates.

2.2 Biological and Microbiological assays.

2.3 The combination of methods of 2.1 and 2.2.

Measurement and identification of folates.

Due to the clinical importance of folates there have been extensive investigations of these compounds.The minute amount of these compounds usually present in Nature has made their measurement and identification difficult.There have been many attempts to find satisfactory methods for the measurement and identification of folates with various degrees of success. Many techniques are employed in the measurement and identification of folates and these techniques can be classified into three groups. The first group is based on the biological activity of the folates and include biological assays,microbiological assays and enzymatic methods. The second group of techniques is based on the physical and chemical properties of folates and include chromatographic techniques,spectroscopic techniques,chemical methods and radioisotopic techniques. The third group consists of those methods employing the first and second principles and these include bioautographic techniques,combination of column chromatography and microbiological assays and application of radioactive folates with chromatography and microbiological assays.These methods will be dealt with in a greater detail separately below.The discussion will lay stress on the methods used in this study.

2.1 Physical and Chemical methods of measurement and identification of folates.

Due to minute amounts and great interference by other compounds these methods are not of great value in the determination of folates in natural substances.These methods are useful in limited ways.Chemical methods may be used in determining folic acid in pharmaceutical preparations.This method is accomplished by digesting the drug in mildly alkaline buffer,oxidation with permanganate and diazotization of the resulting amine.Colorimetry was used to determine the colour produced after coupling the diazotized product with N-(1-naphthyl)ethylenediamine using isobutanol as solvent and the absorbance determined at wavelength 550 nm.This method is used in U.S. as an official method for pharmaceutical preparation (U.S.Pharmacopoeia,1965).

There are many physical methods available.Ultraviolet spectroscopy has been widely used for both qualitative and quantitative analyses of pure compounds.The qualitative analyses were done by comparing the unknown spectra with those of identified spectra in various pH buffers as summarized by Blakley (1969).This method has only a certain degree of success because of its low specificity.Quantitative analysis of folates with ultraviolet spectroscopy can be done using the following equation :-

$$A = C.E.d$$

Where A = absorbance; C = molar concentration; E = extinction coefficient of the folate and d = length in cm of the light path. Extinction coefficient of folates are summarized in Blakley(1969).

Mass spectroscopy has been used with some degree of success.Foxall (1967) described the mass spectra of simple pterins. Acetylated derivatives (Yamakami,Sakurai and Goto,1967) and trimethylsilyl derivatives of simple pteridines (Haug,1970; Lloyd,Markey and Weiner,1971) also been obtained.No folates have been shown to have a satisfactory spectra.

Nuclear magnetic resonance spectroscopy has also been used in this field.Studies have been mainly on synthetic pterins or folates (Viscontini et al 1963;Viscontini et al 1964; Viscontini and Okada,1967;Gapski,Whiteley and Huennekens,1971). NMR studies use a relatively large amount of folate which has reduces its application to natural materials and is principally used for structure analysis.

Chromatography on column and thin layer are widely used as techniques for separation of a mixture of folates. Thin layer chromatography in various solvent systems on MN 300 F 254 cellulose plate gives a good separation and folates were mainly characterized by this method. Table 2.1.1 shows Rf values of folates on MN 300 F 254 in various solvent systems.

A certain amount of folates can be bound to the folate binder in milk (Ghitis,1967) .Application of radioactive ^{14}C or ^{3}H labelled folate and folate binder in milk have led to another method of estimation of folates in natural materials.Known quantities of radioactive labelled folate and unlabelled folate were added into a mixture containing certain amount of folate binder and standard curve was obtained by varying the amount of unlabelled folate.The ratio of the bound and unbound radioactivities as a function against the quantity of the unlabelled folate were plotted to obtain a standard curve and similarly for unknown.

Various methods using this principle have been described by many authors (Waxman,Schreiber and Herbert,1971;Rothenberg, daCosta and Rosenberg,1972;Archibald,Mincey and Morrison,1972; Tajuddin and Gardyna,1973).Radioassay has so far only one advantage over microbiological assays in that it can be done quicker but it has not been widely used.

TABLE 2.1.1

R_f values of pteroates and folates. MN 300 UV plates.(Beavon,1973)

Compound	Appearance under 254* or 365 nm UV light.	R_f values in solvent 1	3	2	4
Pteroic acid	Absorbing*	0.15	0.00	0.28	0.00
10-formylpteroic acid	Blue	0.78	0.68	0.24	-
Pteroylglutamic acid	Absorbing*	0.50	0.00	0.12	0.05
10-formylpteroylglutamic acid	Blue	0.92	0.65	0.30	0.78
7,8-dihydropteroylglutamic acid	Light blue	0.39	0.55	0.30	0.15
Tetrahydropteroylglutamic acid	Absorbing*	0.78	0.43	0.35	0.64
5-formyl-H_4PteGlu	Absorbing*	0.87	0.69	0.32	0.71
5,10-CH=H_4PteGlu	Bluish-White	0.65	0.38	decomposed	0.42
5,10-CH_2H_4PteGlu	Absorbing*	0.92	0.85	0.28	0.61
5-CH_3H_4PteGlu	Absorbing*	0.85	0.68	0.55	0.78
10-formyl-H_4PteGlu	Absorbing*	0.82	0.43	0.52	-
Teropterin($PteGlu_3$)	Absorbing*	0.94	0.30	0.08	-

These solvents are shown in page 78.

2.2 Biological and Microbiological assays.

The chick assay was the first biological technique to determine folates and described by O'Dell and Hogan (1943). Chicks were first placed on the basal diet until they became anaemic.The supplement of folate and test materials were then administered and the growth response was measured.This method is time-consuming and expensive.Therefore it has not been widely used and passed out of use when microbiological assays were developed.

Microbiological assays are the most widely used at present.Throughout this work folates were microbiologically assayed with L.casei,S.faecalis and P.cerevisiae by using aseptic addition techniques (Herbert,1966). Crithidia fasciculata is specific for biopterin and neopterin and their reduced derivatives.The microbiological assay with Crithidia fasciculata was employed (Leeming and Blair,1974).Response of L.casei, S.faecalis and P.cerevisiae to various folates and some other compounds was summarized in Table 2.2.1. The principle of microbiological assays is based on the ability of folates to support the growth of these microorganisms.Thus growing these microorganisms in the media containing different known amount of folate could be used as standard curves.Turbidity of the growing microorganisms media can be measured by using colourimeter at wavelength 590 nm.

The protozoan Tetrahymena geleii W has been suggested and the assay procedure described(Kidder and Dewey,1949; Jukes,1955).A thermophilic bacteria,Bacillus coagulans has also been used for microbiological assay of folates(Baker et al 1955).

Materials and Equipments.

The microorganisms,L.casei (8010),S.faecalis (8123) and P.cerevisiae (7838) were obtained from the National Collection of Industrial Bacteria,Torry Research Station, P.O.Box31,135 Abbey Road,Aberdeen AB9 8DS,Scotland.These microorganisms were maintained on liquid medium prepared from Bacto-Micro inoculum broth (Difco Laboratories,Detroit Michigan,U.S.A.). Crithidia fasciculata culture (ATCC 12857) was supplied by Dr.S.H.Hutner of Haskins Laboratories, Pace College,New York,U.S.A. The culture was maintained and microbiological assay methods were performed as described by Leeming and Blair (1974).Assay media for microbiological assays of folates were commercial products recommended for each appropriate microorganism and purchased from Difco Laboratories Detroit Michigan U.S.A. The basal media for these microbiological assays were summarized in Tables 2.2.2,2.2.3,2.2.4 for L.casei, S.faecalis and P.cerevisiae,respectively.The basal media for microbiological assay with Crithidia fasciculata was prepared as summarized in Table 2.2.5. The composition of micro inoculum broth is shown in Table 2.2.6.

All chemicals were of analytical grade and purchased from BDH Chemicals Ltd.,Poole,England.

The spectrophotometer (micro-sample spectrophotometer 300 with direct digital concentration read-out,Gilford Instrument) was equiped with sample changer and chart recorder for automatic readout of the growth media as described by Leeming and Portman-Graham (1973). Automatic pipettes were used for transferring assay media into the assay tubes.Sterilized

graduated pipettes were used for adding samples. An incubator maintained at 37°C by ventilated air was used for incubating the growing cultures of L.casei,S.faecalis and P.cerevisiae. Crithidia fasciculata was grown in incubator maintained at 29°C. Laboratory non-refrigerated centrifuge was used when centrifugation required. The steam pressure autoclave was used for sterilization of assay media at 115°C and 5 minutes for those of microbiological assays of folates and Crithidia factors.

Table 2.2.1 Response of folates on various microbiological assays.

Compound	L.casei	S.faecalis	P.cerevisiae
Pte	-	+	-
5-CHO H_4Pte	-	+	-
10-CHO H_4Pte	-	+	-
PteGlu	+	+	-
$PteGlu_2$	+	+	-
$PteGlu_3$	+	-	-
$PteGlu_7$	-	-	-
10-CHO PteGlu	+	+	-
10-CHO $PteGlu_3$	+	-	-
H_2PteGlu	+	+	-
10-CHO H_2PteGlu	+	+	-
5-CH_3-5,6-H_2PteGlu	(±) +	-	-
5-CH_3-5,8-H_2PteGlu	-	-	-
H_4PteGlu	+	+	+
5-CHO H_4PteGlu	+	+	+
5-CHO $H_4PteGlu_2$	+	+	+
5-CHO $H_4PteGlu_3$	+	-	+
5-NCHO H_4PteGlu	+	+	+
10-CHO H_4PteGlu	+	+	+
5,10-CH_2-H_4PteGlu	+	+	+
5,10-CH=H_4PteGlu	+	+	+
5-CH_3H_4PteGlu	+	-	-
4a-OH-5-CH_3-4a,5,6,7-H_4PteGlu	-	-	-

(±) indicates results are negative when ascorbic acid was omitted and + indicates activity of higher than 50 % of maximum value, - indicates response less than 5 % of the maximum value.

Table 2.2.2 Bacto-Folic Acid Casei Medium (Flynn et al 1951; Baker et al 1959;Waters and Mollin,1961).

Charcoal Treated Casitone	10 g
Bacto-Dextrose	40 g
Sodium Acetate	40 g
Dipotassium Phosphate	1 g
Monopotassium Phosphate	1 g
dl-Tryptophane	0.2 g
L-Asparagine	0.6 g
L-Cysteine HCl	0.5 g
Adenine Sulfate	10 mg
Guanine Hydrochloride	10 mg
Uracil	10 mg
Xanthine	20 mg
Tween 80	100 mg
Glutathione (reduced)	5 mg
Magnesium Sulfate	400 mg
Sodium Chloride U.S.P.	20 mg
Ferrous Sulfate	20 mg
Manganese Sulfate	15 mg
Riboflavin	1 mg
p-Aminobenzoic Acid	2 mg
Pyridoxine Hydrochloride	4 mg
Thiamine Hydrochloride	0.4 mg
Calcium Pantothenate	0.8 mg
Nicotinic Acid	0.8 mg
Biotin	0.02 mg

Table 2.2.3 Bacto-Folic T E Medium (Teply and Elvehjem,1945).

Bacto-Vitamin Free Casamino Acids	10	g
dl-Alanine	0.4	g
L-Cystine	0.4	g
Tryptophane	0.4	g
Bacto-Dextrose	40	g
Adenine Sulfate	20	mg
Guanine Hydrochloride	20	mg
Uracil	20	mg
Xanthine	20	mg
Thiamine Hydrochloride	0.4	mg
Riboflavin	0.4	mg
Nicotinic acid	1.2	mg
Pyridoxine Hydrochloride	2.4	mg
Calcium Pantothenate	0.8	mg
Biotin	800	ng
p-Aminobenzoic Acid	0.02	mg
Sodium Citrate	50	g
Charcoal Treated Peptone	0.6	g
Asparagine	0.2	g
Magnesium Sulfate	0.4	g
Sodium Chloride	20	mg
Ferrous Sulfate	20	mg
Manganese Sulfate	14	mg
Dipotassium Phosphate	5	g

Table 2.2.4 Bacto-CF Assay Medium (Sauberlich and Baumann,1948).

Bacto-Vitamin Free Casamino acids	10	g
Bacto-Dextrose	50	g
Sodium Acetate	40	g
Ammonium Chloride	6	g
L-Cystine	0.2	g
L-Cysteine	0.02	g
dl-Tryptophane	0.2	g
dl-Alanine	0.2	g
Glycine	0.2	g
Adenine Sulfate	20	mg
Guanine Hydrochloride	20	mg
Uracil	20	mg
Xanthine	20	mg
Thiamine Hydrochloride	1	mg
Pyridoxine Hydrochloride	2	mg
Pyridoxamine Hydrochloride	6	mg
Pyridoxal Hydrochloride	0.6	mg
Calcium Pantothenate	1	mg
Riboflavin	1	mg
Nicotinic Acid	2	mg
p-Aminobenzoic Acid	0.2	mg
Biotin	2000	ng
Folic Acid	0.02	mg
Monopotassium Phosphate	1.2	g
Dipotassium Phosphate	1.2	g
Magnesium Sulfate	0.4	g
Sodium Chloride	20	mg
Ferrous Sulfate	20	mg
Manganese Sulfate	40	mg

Table 2.2.5 Culture media for microbiological assay with Crithidia fasciculata.

Maintenance Medium (Hutner,1971).

Yeast extract (Oxoid)	0.30	g
Trypticase	0.30	g
Sucrose	0.25	g
Liver fraction L (Nutr.Biochem.Co.)	0.01	g
Haemin (5 mg/ml in 50% triethanolamine)	0.50	ml
Distilled water	100.00	ml

Adjusted to pH 6.8 to 7.6 and autoclaved at 120°C for 15 minutes and stored in the refrigerator at 4°C.

Stock Assay Medium.

Part A.

L-arginine hydrochloride	5.00	g
L-glutamic acid	10.00	g
L-histidine hydrochloride	3.00	g
DL-isoleucine	1.00	g
Dl-leucine	1.00	g
L-lysine hydrochloride	4.00	g
DL-methionine	1.00	g
DL-phenylalanine	0.60	g
DL-tryptophane	0.80	g
L-tyrosine	0.60	g
DL-valine	0.50	g
Ethylene diamine tetra-acetic acid	6.00	g
Boric acid	5.00	mg
Calcium chloride	5.00	mg
Cobalt sulphate	25.00	mg
Copper sulphate	25.00	mg
Ferric ammonium sulphate	10.00	mg
Manganese sulphate	1.40	g
Magnesium sulphate	6.50	g
Potassium phosphate	1.50	g
Zinc sulphate	0.50	g
Sucrose	150.00	g
Distilled water	1000.00	ml

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

Part B.

Adenine	1.00	g
Biotin	1.00	mg
Calcium pantothenate	0.30	g
Nicotinic acid	0.30	g
Pyridoxamine dihydrochloride	0.10	g
Riboflavin	0.06	g
Thiamine hydrochloride	0.60	g

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

Part C.

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

Part D.

Folic acid 100 ng/ml, freshly prepared.

Double strength assay media (100 ml).

Distilled water	78.00	ml
Assay medium part A	20.00	ml
Assay medium part B	4.80	ml
Vitamin free casamino acids (Difco)	2.00	g
Triethanolamine (added before part C)	0.50	ml
Assay medium part C	1.00	ml
Assay medium part D	0.50	ml

Adjusted to pH 7.5 with sulphuric acid.

Table 2.2.6 Bacto-Micro Inoculum Broth.

The dehydrated Bacto-Micro Inoculum Broth contains:-

Bacto-Yeast Extract	20 g
Proteose Peptone No.3 (Difco)	5 g
Bacto-Dextrose	10 g
Monopotassium Phosphate	2 g
Sorbitan Monooleate Complex	0.1 g

The Bacto-Micro Inoculum Broth was rehydrated by dissolve 37 grams of the dehydrated broth in 1000 ml of distilled water.Distribute in 10 ml quantities in 25 ml universal containers and autoclave for 15 minute at 121°. The final reaction of the medium will be pH 6.7 at 25°C and were used for the subculturing of L.casei,S.faecalis and P.cerevisiae.

Methods and Discussion.

Appropriate microorganisms were subcultured weekly or not more than three weekly by maintaining them in liquid Bacto-Micro Inoculum Broth. The duplicated subcultures were grown a day before the assays were done,one was used for each assay and the others were stored in refrigerator at 4-6°C for the next assay.The culture used for the assays was washed 3-5 times with sterilized single strength assay media and diluted 1 in 20 or 1 in 10 before inoculated into the tests. The innoculated tests for all assays with L.casei,S.faecalis and P.cerevisiae were incubated at 37°C for a period of 18-22 hours before they were read out. The microbiological assay with Crithidia fasciculata were incubated at 29°C for a period of 72 to 90 hours.

Setting up the assays was done so that about 50 to 70 samples could be done at the same time for all three test microorganisms.In case of Crithidia fasciculata up to 180 samples could be done at the same time.The appropriate commercial assay media was rehydrated to a double strength solution and dispensed in triplicate into sterilized rimless 12 x 75 mm test tubes to make a combination as shown in Table 2.2.7.

Table 2.2.7 The composition of assay media for microbiological assays with L.casei,S.faecalis and P.cerevisiae.

Double strength media (ml)	2 g/l Ascorbate water (ml)	Total volume (ml)
2.0	1.9	3.9

The dispensed assay media were capped and autoclaved at 115°C for 5 minute and left to cool to room temperature in shaded area. To these media standard folates with varying amount as shown in Table 2.2.8 were added aseptically at 0.1 ml for each tube. Similarly 0.1 ml of unknown is used and both standard and unknown were done in triplicate.

Table 2.2.8 Standard solutions for the standard curve of microbiological assays with L.casei,S.faecalis and P.cerevisiae.

Test organism	Concentration of standard (ng/ml)											
	0	0.5	1.0	1.5	2.0	3.0	4.0	5.0	10.0	15.0	20.0	30.0
L.casei	x		x		x			x	x	x	x	x
S.faecalis	x	x	x	x	x	x	x	x				
P.cerevisiae	x	x	x	x	x	x	x	x				

Pteroylglutamic acid is used for the preparation of standard solution in L.casei and S.faecalis microbiological assays. 5-formyltetrahydropteroylglutamic acid was used for the standard solution in P.cerevisiae microbiological assay.

After all standard and samples were aseptically added into the assay media two of each triplicate were inoculated with appropriate culture previously washed and diluted.The third tube was left uninoculated and used as blank for each sample. The 18 to 22 hour grown cultures were read out as described by Leeming and Portman-Graham (1973).

The microbiological assay with Crithidia fasciculata has been described elsewhere (Leeming and Blair,1974).The use of aseptic addition was to avoid the destruction of the heat labile folates in the analyzed samples (Herbert,1966;Beavon and Blair,1972;O'Broin et al 1973).

On using the aseptic technique caution must be taken so that there is no contamination from any source of material used.All glass-ware must be clean and sterilized.Water is also an important source of contamination so distilled water always be used for making the de-ionized water. Samples were also collected in such a manner that they were sterilized as much as possible.The reliability of the assay have to be considered.

Since it is not easy to know if the assay is of reliable or not there must be some kind of quality control. The addition of samples previously assayed into each test is one of the best mean of quality control methods.The variation of values assayed this way within the order of not more than 20 per cent is acceptable,provided that antioxidant were presented and samples were kept frozen. The other means of quality control used in our laboratories is by doing 'recovery test' every now and then. The recovery was done by adding known amount of folate i.e. from standard to the samples and the percentage of recovery should be high.These methods were satisfactorily employed through all assays.The recovery test were shown in Table 2.2.9.

Standard curves for microbiological assays with L.casei,S.faecalis,P.cerevisiae and Crithidia fasciculata were shown in Figures 2.2.1,2.2.2,2.2.3 and 2.2.4,respectively.

Table 2.2.9 The recovery test.

Recovery sample		Detected value	Recovery (%)
Serum sample	Known standard		
A	-	A	-
A	B	C	D

Recovery ; $D = \frac{C-A}{B} \times 100\ \%$

Where, A = detected value of serum folate used in the test.

B = known amount of folate added to the recovery test.

C = detected amount of folate in the recovery test.

Figure 2.2.1 Standard curve of microbiological assay with L.casei.

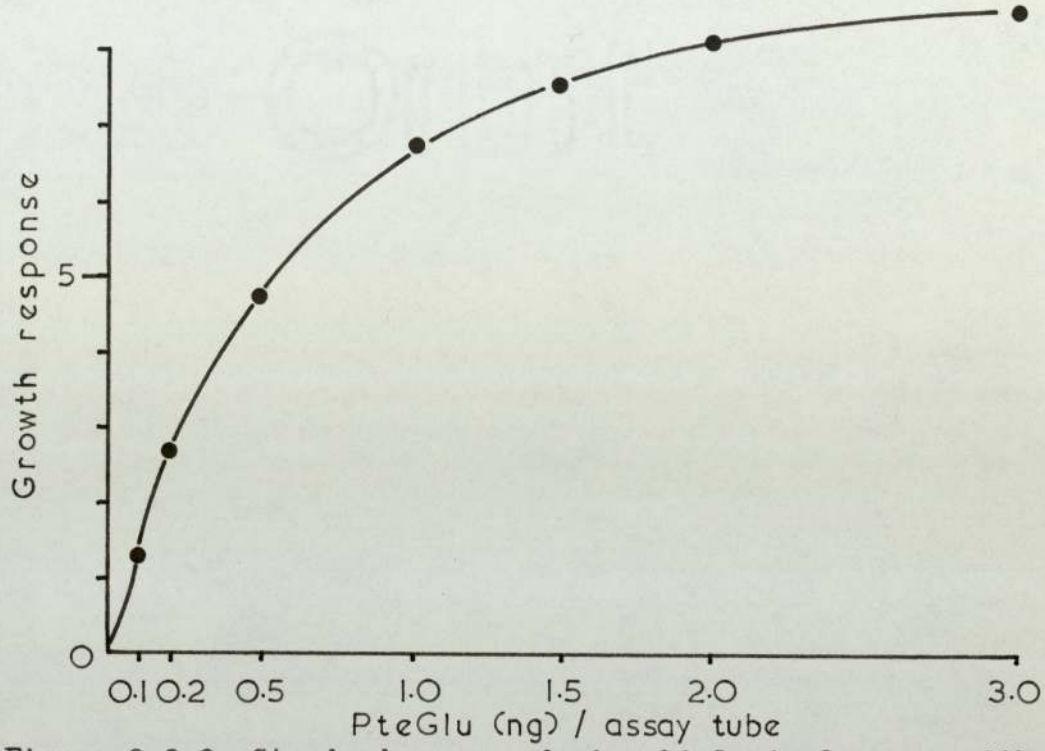


Figure 2.2.2 Standard curve of microbiological assay with S.faecalis.

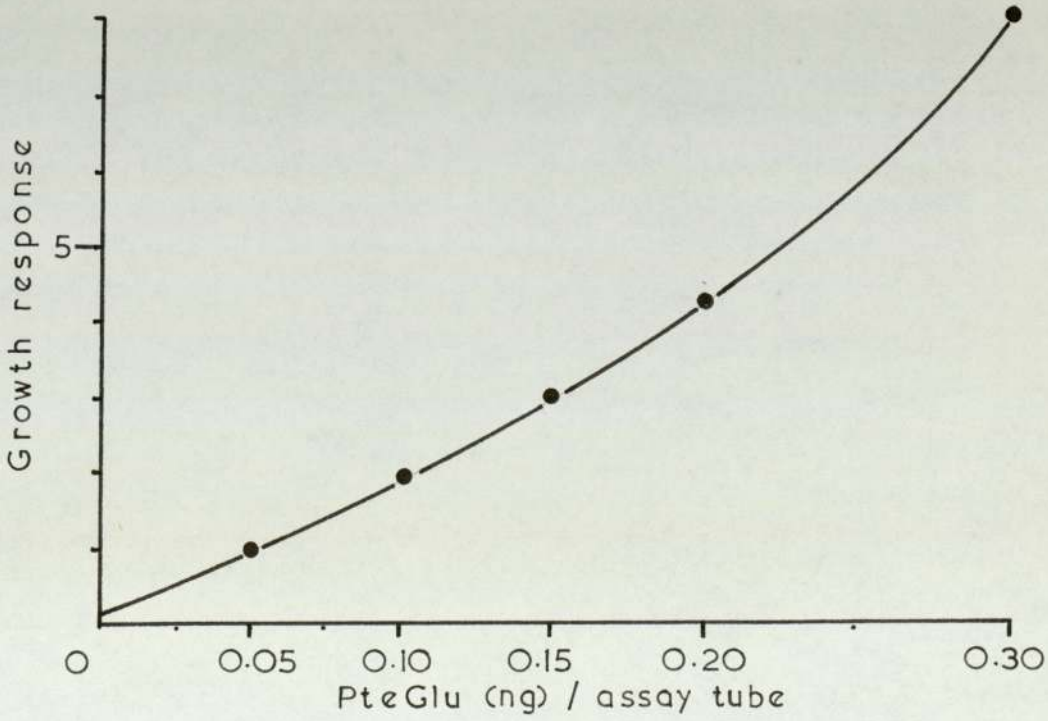


Figure 2.2.3 Standard curve of microbiological assay with P.cerevisiae.

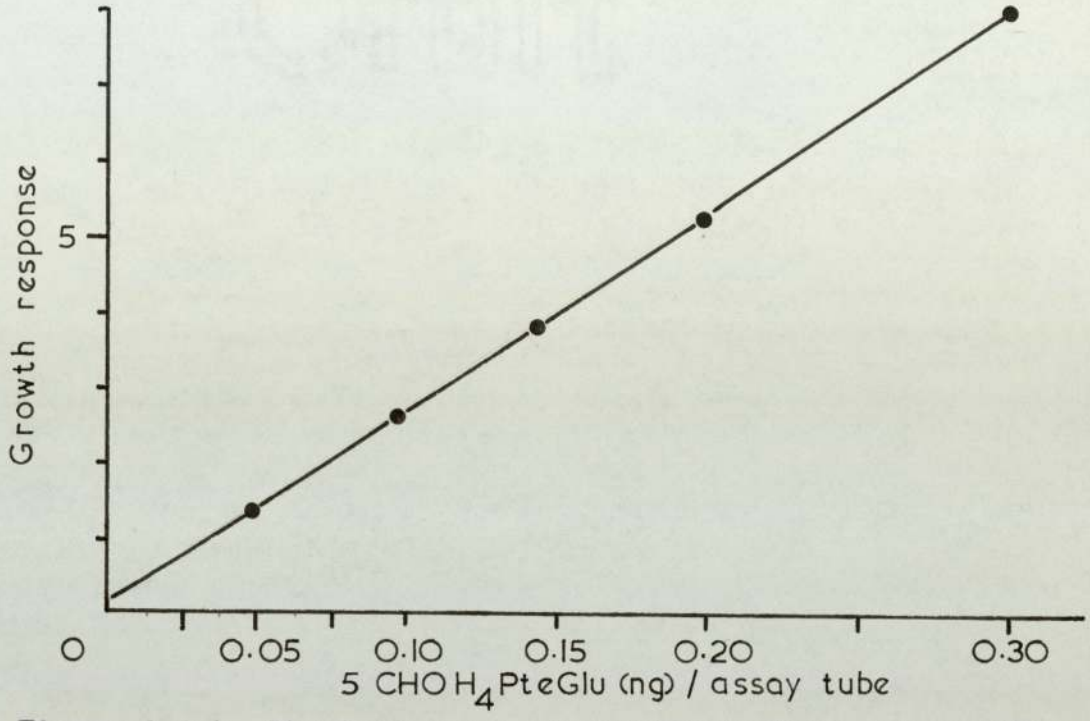
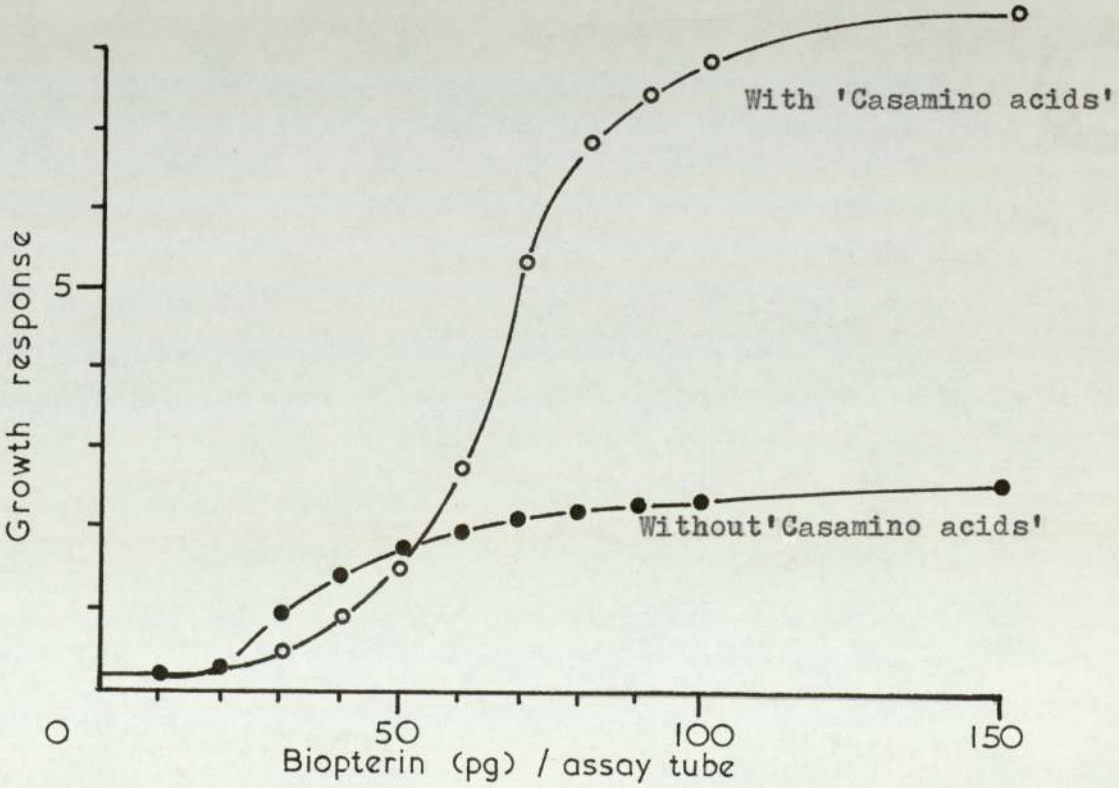


Figure 2.2.4 Standard curve of microbiological assay with Crithidia fasciculata.



2.3 Combination of physical and microbiological methods.

The microbiological assay of chromatographic fractions with L.casei,S.faecalis and P.cerevisiae can be used for the detection and identification of folates in natural materials (Silverman et al 1961;Butterworth,Santini and Frommeyer,1963; Santini,Brewster and Butterworth,1964).Tetrazolium bioautography developed by Usdin et al (1954) had been used for the identification of folates in natural materials (Iwai,Nakagawa and Okinaka,1959; Leeming et al 1970;Brown,Davidson and Scott,1973;Ratanasthien, Blair,Leeming,Cooke and Melikian,1974).The aseptic differential microbiological assays with L.casei,S.faecalis and P.cerevisiae together with their tetrazolium bioautography techniques were used for identification of folates in serum and urine throughout this work.Since folates can be satisfactorily separated on chromatographic plates using a suitable solvent system as shown in Table 2.1.1 and folates are responded to these test microorganisms in different ways as shown in Table 2.2.1.Thus using bioautography with these microorganisms led to a specific identification of various folates.

Materials and Methods.

Triphenyltetrazoliumchloride was purchased from BDH Chemicals Ltd.,Poole,England.Folates were obtained by preparations as described in Chapter 3.Thin layer chromatographic plates were Art.5716 DC-Fertigplatten cellulose (pre-coated TLC plates without fluorescent indicator) dimension 0.1 mm x 20 cm x 20 cm and purchased from Merk (U.K.).

After 2 to 5 ul of samples were applied onto the thin layer chromatographic plates with sterilized micropipettes they were developed mainly with 3% aqeous ammonium chloride containing 1% ascorbic acid (W/V). The distance from the application points to the solvent front is adjusted for 15 cm. The developed plates were left to half dried in refrigerator at 6°C before putting on the tray of settled sterilized plain agar (Oxoid Ionagar No. 2 purchased from BDH Chemicals Ltd., Poole,England.).

The plain agar was prepared by dissolving 3 to 5 g of the Oxoid Ionagar No.2 in 250 ml distilled water and sterilized by autoclaving at 121°C for 15 minutes. The agar medium was prepared by adding appropriate assay media,5 gm Ionagar No.2, 0.5 gm ascorbic acid,30 ml 0.1M phosphate buffer pH 6.1,5 ml of 2 % sterilized tetrazolium solution (W/V),10 ml of diluted appropriate microorganism in the final volume of 300ml adjusted by adding distilled water. The agar media were prepared in such a manner that the media was sterilized by autoclaving at 121°C for 15 minutes and cooled to 45°C before the tetrazolium and diluted washed microorganism solutions were added.The cooled agar media (45°C) were poured to cover the developed thin layer chromatographic plates and let settle before covering with thin film of plastic sheet and covered.The prepared plates were incubated in the incubator at 37°C for 24 hours.The red spots developed at various Rf values of various folates can be visualized and identified as shown in Table 2.3.1.Photographs of developed plates were shown in Plate 2.3.1.

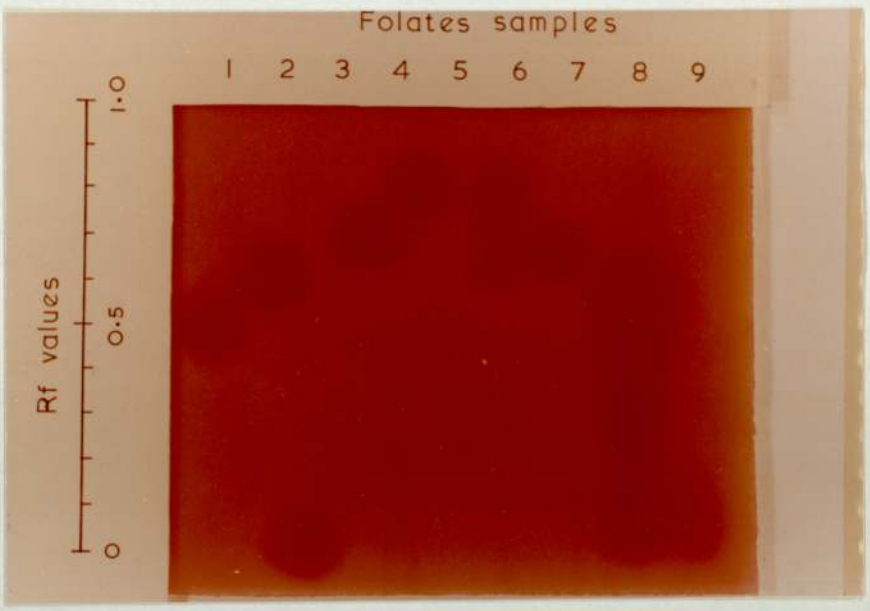


Plate 2.2.1 Bioautography of some folates with L.casei.

1 = 5-CHO H_4PteGlu
2 = 10-CHO PteGlu
3 = PteGlu
4 = 5-CH_3H_4PteGlu
5 = 5-CH_3-5,6-H_2PteGlu
6 = 5-CH_3H_4PteGlu + 5-CH_3-5,6-H_2PteGlu
7 = 5-CH_3H_4PteGlu
8 = H_4PteGlu
9 = H_2PteGlu

This plate was developed in 3 % aqeous ammonium chloride without ascorbic acid. All samples except numbers 5 and 6 containing 5 mg/ml ascorbic acid.

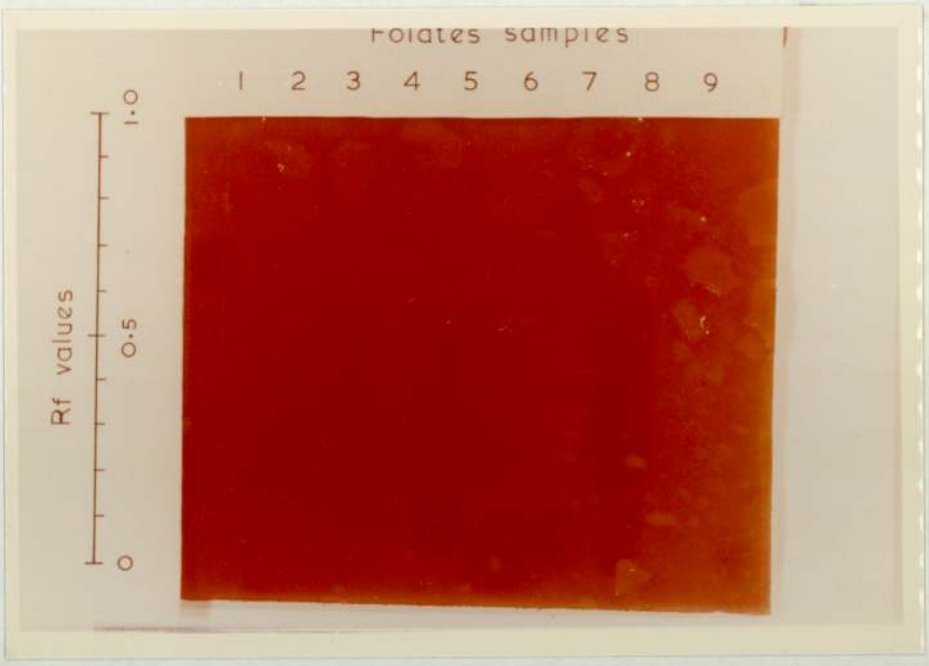


Plate 2.2.2 Bioautography of some folates with S.faecalis.

1 = 10-CHO PteGlu
2 = 5-CHO H_4PteGlu
3 = Subject after 25-hour MTX and 5 mg 5-CH_3H_4PteGlu(1 hour)
4 = H_2PteGlu
5 = PteGlu
6 = Subject 25-hour after MTX and 5 mg of H_2PteGlu(1 hour)
7 = H_4PteGlu
8 = Similar to number 3.
9 = Subject 25-hour after MTX and 5 mg of PteGlu(1 hour)

Table 2.3.1 Rf values of some folates developed with 3 % aqeuos ammoniumchloride containing 1 % ascorbic acid on the Art.5716 DC-Fertigplatten cellulose (pre-coated TLC plates without fluorescent indicator) plates.

Compounds	Rf values
Pte	0.00
PteGlu	0.05
10-CHO PteGlu	0.60
7,8-H_2PteGlu	0.20
5-CH_3-5,6-H_2PteGlu	0.75*
H_4PteGlu	0.40 (t)
5-CH_3H_4PteGlu	0.65
5-CHO H_4PteGlu	0.50
10-CHO H_4PteGlu	0.45 (t)
5,10-CH_2-H_4PteGlu	0.60
5,10-CH=H_4PteGlu	0.50 (t)

* indicates results when plates were developed without ascorbic acid in the solvent system, (t) indicates results with no clear single spot but tailed with intensed area indicated.

Preparation of TLC plate for using with microbiological assays.

A t.l.c. plate (pre-coated t.l.c. plates without fluorescent indicator) was stroked with sample horizontally along the application origin. Up to 300 ul can be applied in a plate. After the plate was developed to a desired solvent front it was let dried in refrigerator at 6°C and cut horizontally into 10 equal strips.The coated cellulose from each strip was transferred into a clean test tube and 3 ml of 2 g/l ascorbic acid added.After shaking for a few minutes the cellulose was discarded by centrifugation.The eluates were then assayed by using various microorganisms and the results can be verified by comparring with those of Table 2.3.1 and 2.2.1.

By using this technique only one sample can be used in a plate but it can be used for the study of samples containing antibiotics inhibited the growth of assay microorganisms.This technique is successful providing that the Rf value of the inhibitor is different from the detecting compounds.

Chapter 3.

Preparation and some properties of the materials used in this study.

Preparation and some properties of materials used in this study.

Biopterin and tetrahydrobiopterin were gifts from Roch Products Ltd.Pteroic acid was a gift from Professor I.H.Rosenberg,Department of Medicine,University of Chicago, Chicago,U.S.A. It was prepared and purified by microbiological degradation of pteroylglutamic acid (Houlihan,Boyle and Scott,1972). Pteroylglutamic acid was purchased from Koch-Light Laboratories, Cornbrook,Buckinghamshire.Folic acid tablets were puechased from Roche Products Ltd,London.Some physical and microbiological properties of these materials will be shown later on.

7,8-dihydropteroylglutamic acid was prepared by the method modified from Futterman (1963).

N KOH solution was slowly added to a suspension of 200 mg of the potassium salt of folic acid in 20 ml water until solution was completed.The solution was then carefully adjusted to pH 6.0 by adding 2N HCl after 50 ml of 10 % (W/V) potassium ascorbate was added to the solution.To the pH 6.0 solution 2 g of sodium dithionite was added.Reaction was let continue for 10 minutes at room temperature in the dark and then the mixture was adjusted to pH 2.8 with 2N HCl. The creamy-white precipitate was collected by centrifugation,the supernatant was discarded and the precipitate redissolved in 50 ml of 10 % (W/V) potassium ascorbate at pH 6.0.Reprecipitation was done by adjusting to pH 2.8 with 2N HCl and centrifuged.The supernatant was discarded and the solid washed with 3 x 20 ml pH 2.8 ascorbic acid solution (1 %,W/V).The creamy-white product of 7,8-dihydropteroylglutamic acid was stored frozen as a suspension in pH 4.0 solution of 10 % (W/V) ascorbic acid.The product can be obtained free of ascorbic acid by washing with very diluted

hydrochloric acid followed by acetone and lyophilisation or drying under vaccuum.

The creamy-white crystalline solid of 7,8-dihydropteroylglutamic acid is readily soluble in water but it is sparingly soluble in solution of pH 5.0 or lower.The ultraviolet spectra of compound obtained free of ascorbic acid are used to characterize the compound. At pH 7.0 it has a λ_{max}(nm) of 227 and 282 and at pH 13.0 a λ_{max}(nm) 221 and 285.Ascending t.l.c. on cellulose plate (MN 300 F 254) was also used for checking it purity and single spot developed in various solvent systems (Table 3.1) is summarized in Table 3.2.The microbiological assays with L.casei and S.faecalis are fully active and showed no activity with P.cerevisiae.

5,6,7,8-tetrahydropteroylglutamic acid was purchased from Sigma Products,U.K. and is used without further purification. It is used as a reference standard in the bioautographic studies shown in the previous Chapter 2.Rf values of this product in various solvent systems (Table 3.1) is summarized in Table 3.2.

10-formylpteroylglutamic acid was prepared from pteroylglutamic acid (Blakley,1959).

Pteroylglutamic acid (4 g) was dissolved in 160 ml of 98 % (W/V) formic acid and left standing in the dark at room temperature for 3 days.The mixture of mainly 10-formylpteroylglutamic acid was poured into a 500 ml solution of cold dry diethyl ether and the precipitate collected on a Buchner funnel. The very pale yellow product was washed with ether and sucked dry on the funnel and finally in vacuo to yield about 95 %.

The ultraviolet spectra of the product showed a λ_{max} (nm)252 and 322 at pH 1.0 , λ_{max} (nm) 245 and 270 and 350 at pH 7.0 , λ_{max} (nm) 256 and 367 at pH 13.0.Microbiological assays with L.casei and S.faecalis are fully active and no activity is seen with P.cerevisiae.The product is also used as a reference standard in the bioautography.Thin layer chromatography of the product in various solvent systems (Table 3.1) showed a single spot and its Rf values is summarized in Table 3.2.

5-formyltetrahydropteroylglutamic acid is a calcium salt of leucovorin and was a gift from Lederle Laboratories. The product is used without further purification.It is used as a reference standard in bioautography and as standard in the microbiological assay with P.cerevisiae.Rf values of this product in various solvent systems (Table 3.1) is summarized in Table 3.2.

5,10-methenyltetrahydropteroylglutamic acid was prepared by acidic cyclisation of 5-formyltetrahydropteroyl-glutamic acid (Roth et al 1952).

500 mg of the calcium salt of 5-formyltetrahydropteroyl-glutamic acid was dissolved in the least amount of distilled water containing 0.02 molar 2-mercaptoethanol.The solution was adjusted to pH 1.5 with 0.2 M HCl and left standing in the dark at room temperature with occasional shaking for 3 hours. The cloudy yellow solution was filterred through a Buchner funnel with Watman No.1 filter paper.The crude product of crystalline 5,10-methenyltetrahydropteroylglutamic acid was

recrystallized from 0.2 M HCl at room temperature.The crystalline product was filterred and washed with ethanol and dried in vaccuo overnight.The yield of this method was about 95 % as judged by ultraviolet spectroscopy.Microbiological assays with L.casei, S.faecalis and P.cerevisiae showed activity about 95 %.Rf values of the product on MN 300 F 254 cellulose plate in various solvent systems (Table 3.1) is summarized in Table 3.2.

10-formyltetrahydropteroylglutamic acid was prepared from 5,10-methenyltetrahydropteroylglutamic acid according to the method (Beavon and Blair,1972).

A solution of 5,10-methenyltetrahydropteroylglutamic acid was made to a desired concentration in water or buffer pH 7.0 and left standing at room temperature in the dark for 30 to 60 minutes and use immediately,the solution containing 5 mg/ml ascorbic acid.Rf values of this product in various solvent systems (Table 3.1) is summarized in Table 3.2.The fresh product showed fully microbiological activities with L.casei,S.faecalis and P.cerevisiae.

5-methyltetrahydropteroylglutamic acid was prepared from folic acid according to the method of Blair and Saunders, 1970.

In a 500 ml five necked round bottom flask 6 g of pteroylglutamic acid was stirred in 200 ml of 0.066 M Trisbuffer pH 7.8 under slow steam of nitrogen at room temperature.
50 ml of a solution containing 6 g sodiumborohydride was slowly added and the mixture was left stirring for a further 15 minutes.

The excess sodiumborohydride was destroyed by adding 5N acetic acid to bring the pH down to the original pH 7.8,8 ml of 37 % (W/V) formaldehyde solution was added and after a few minutes 100 ml of 12 % (W/V) solution of sodiumborohydride was added.The mixture was incubated at 45°C for 1 hour and cooled to room temperature and 1.5 ml of 2-mercaptoethanol was added and the mixture pH was adjusted by adding 5N acetic acid to pH 7.0 and diluted to final volume of 500 ml.The pale yellow crude product was then chromatographed through a 3 x 90 cm column of DEAE-Cellulose (DE 52) previously equilibrated with 2 to 3 litres of 0.13M pH 7.0 ammonium acetate containing 0.2M 2-mercaptoethanol. The chromatography was operated by ascending application using peristaltic pressure at a rate of 3 ml/minute.The column was eluted with a gradient of ammonium acetate (0.13M;0.4M,pH 7.0:2 litres each) and the eluate was collected with automatic fraction collector at 18 ml per tube.Every fifth tubes of eluate were checked with the ultraviolet spectrophotometer and those tubes with λ_{max} 290 nm and λ_{min} 250 nm in pH 7.0 solution with ratio of $\lambda_{max}/\lambda_{min}$ better than 2 were pooled and lyophilized to yield a product of 6 g of 90 % purity as judged by the spectroscopic techniques at pH 1,7 and 13.

The calcium salt was prepared by dissolving the 6 g product in 100 ml dearated distilled water containing 1.2 g sodium chloride and adjusting to pH 7.0 with N sodium hydroxide, adding 30 ml of 10 %(W/V) $CaCl_2.6H_2O$,2-mercaptoethanol to a final concentration of 0.01 molar and then 500 ml absolute ethanol before cooling the mixture in refrigerator overnight. The yellowish-white precipitate was filtered at 5°C through a

pre-cooled Buchner funnel,washed 3 to 5 times with 20 ml of 75 % (V/V) aqeous ethanol followed by several washes with 20 ml absolute ethanol and dried in vacuo.The product was checked for identity and purity by various means.Ultraviolet spectroscopy at pH 1.0 showed λ_{max} at 270 nm and 294 nm,at pH 7.0 and 13.0 the λ_{max} of both at 290 nm.The purity by this method is 95 %. A single spot was seen on t.l.c. in various solvent systems (Table 3.1) is summarized in Table 3.2.The microbiological assay with **L**.casei showed better than 90 % purity and no activities were seen on microbiological assays with S.faecalis and P.cerevisiae.

5-methyl-5,6-dihydropteroylglutamic acid was prepared by oxidation of 5-methyltetrahydropteroylglutamic acid with molecular oxygen as described below.

A sample of 400 mg of calcium salt of 5-methyltetrahydropteroylglutamic acid was dissolved in 200 ml of distilled water and 5 mg $CuSO_4$ was added and the mixture was stirred under slow steam of oxygen for 60 to 90 minutes.The ultraviolet spectra were used for timing the reaction as the maximum ratio of absorption peaks at 250/290 nm wavelengths in solution at pH 7.0. The solution of 5-methyl-5,6-dihydropteroylglutamic acid was filtered through a Watman No.1 filter paper and the filtrate lyophilized.The product at this state showed purity of 90 to 95 % as judged by ultravilet spectroscopy using extinction coefficient of 31.2×10^3 $(mole/liter)^{-1}$ from Gupta and Huennekens (1967).Microbiological assays showed similar results to those of 5-methyltetrahydropteroylglutamic acid but no activity in all assays when ascorbic acid was omitted

from the assay media.The product can be further purified by column chromatography similar to those for the purification of 5-methyltetrahydropteroylglutamic acid previously described but 2-mercaptoethanol must be omitted at all steps.Thin layer chromatography of the product in various solvent systems (Table 3.1) showed single spot with Rf values in Table3.2.

5-methyl-5,8-dihydropteroylglutamic acid was prepared by acidic rearrangement of 5-methyl-5,6-dihydropteroylglutamic acid previously noted (Blair et al 1974;Robb,1975).

A desired concentration or 1 mg/ml of 5-methyl-5,6-dihydropteroylglutamic acid was dissolved in 0.1 molar HCl for 30 to 60 minutes and adjusted to pH 7.0 with diluted NaOH. The product was prepared just before use to avoid further changes.The product prepared by this way showed no microbiological activity in all assays with L.casei,S.faecalis and P.cerevisiae. Ultraviolet spectra at pH 1 and 7 showed λ_{max} 260 nm and λ_{max} 278 nm, respectively. Thin layer chromatography of the product in various solvent systems (Table 3.1) showed a single spot in each test and Rf values are summarized in Table 3.2.

4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid was prepared by oxidation of 5-methyltetrahydropteroylglutamic acid or 5-methyl-5,6-dihydropteroylglutamic acid as described (Gapski,Whiteley and Huennekens,1971).

5-methyltetrahydropteroylglutamic acid (1.5 g) was dissolved in 50 ml of 0.1M ammonium acetate pH 6.0 and added to a mixture of 50 ml of 0.1M ammonium acetate pH 6.0 and 5 ml of 20 volume hydrogenperoxide (H_2O_2). The mixture was also adjusted

to pH 6.0 with 0.1N acetic acid and after the reaction had been allow to proceed for 1 hour at room temperature, the solution was lyophilized. The residue was dissolved in 25 ml distilled water and chromatographed on a 3 x 60 cm DEAE-Cellulose (DE 52) column previously equilibrated with 0.1M ammonium acetate. The column was washed with 500 ml of water and then followed by eluation with 0.1M ammonium acetate and eluate collected by using automatic fraction collector at 18 ml/tube. Ultraviolet spectra of the eluate were made at pH 7.0 and those tubes (30-75) exhibited λ_{max} 278 to 280 were combined and lyophilized. The yellow crystalline product of 400 to 500 mg was obtained with purity of better than 90 % using extinction coefficients of 21.3×10^3 $M^{-1}cm^{-1}$ at λ_{max} 275 pH 1.0 or 17.8×10^3 $M^{-1}cm^{-1}$ at λ_{max} 278 pH 7.0 or 18.0×10^3 $M^{-1}cm^{-1}$ at λ_{max} 278 pH 13.0. The product showed no microbiological activity with L.casei, S.faecalis and P.cerevisiae. Thin layer chromatography of the product in various solvent systems (Table 3.1) showed single spot with Rf values summarized in Table 3.2.

<u>γ-glutamyl carboxypeptidase or conjugase</u> was prepared as dsecribed below and was modified from Laskowski, Mims and Day (1945).

A 10 gm portion of chicken pancreas was dissolved in 300 ml of 0.1M phosphate buffer pH 7.0 and covered with toluene and incubated at 37°C overnight. The mixture was centrifuged at 1000 x g for 15 minutes. The residue was discarded and the supernatant was mixed 1:1 with 0.1M tricalcium phosphate gel in ice bath for 30 minutes. The mixture was then centrifuged at 1000g for 15 minutes in a refrigerated centrifuge (0°C).

The lower layer was discarded and the upper layer was mixed vigorously 1:1 with ice cold ethanol and let stand in a refrigerator (6°C) overnight.The mixture was then centrifuged in a refrigerated centrifuge (0°C) at 1000 x g for 15 minutes. The supernatant was discarded and the residue was dissolved in 25 ml ice cold 0.1M phosphate buffer pH 7.0 with 10 % (W/V) Dowex 1x8 (Chloride) or ambulite IR 400 added and allowed to stand at 0°C for 1 hour.The mixture was then centrifuged in a refrigerated centrifuge (0°C) at 1000 x g for 15 minutes. The clear colourless solution of -glutamyl carboxypeptidase or conjugase was then kept frozen (-20°C) in small quantities in screw cap tubes.

The activity of conjugase was checked by microbiological assay with L.casei of yeast extract solution after treatment with the enzyme.Microbiological assay with L.casei of the enzyme preparation showed activity lower than 1.0 ng/ml.

Table 3.1

Chromatography solvents used in the work of Chapter 3.

Solvent system	Composition
1	0.1M phosphate buffer, pH 7.0.
2	n-Propanol/ammonium hydroxide (s.g. 0.88) /water , 200/1/99 (V/V). Made immediately before use.
3	n-Butanol/acetic acid/water, 4/1/5, upper phase. Equilibrated for 18 to 24 hour at room temperature before use.
4	3% (W/V) aqeous ammonium chloride.

All chromatography solvent systems contained 1%(V/V) 2-mercaptoethanol as anti-oxidant unless otherwise specified.

Table 3.2

Rf values of folates and some other compounds on MN 300 F 254 plates.

Compound	Appearance under 254 or 365* nm UV light.	Rf values			
		1	2	3	4
Biopterin	Light blue*	0.63	0.49	0.34	0.70
H_4-biopterin	Absorbing	0.68	0.49	0.47	0.70
Pteroic acid	Absorbing	0.15	0.28	0.00	0.00
Folic acid	Absorbing	0.50	0.28	0.00	0.00
10-CHO PteGlu	Blue*	0.92	0.30	0.65	0.75
7,8-H_2PteGlu	Light blue*	0.35	0.25	0.50	0.15
H_4PteGlu	Absorbing	0.75	0.35	0.40	0.60
5-CHO H_4PteGlu	Absorbing	0.86	0.30	0.70	0.70
5,10-CH_2H_4PteGlu	Absorbing	0.92	0.28	0.85	0.61
5,10-$CH{=}H_4$PteGlu	Bluish-White*	0.65	decomp.	0.35	0.50
10-CHO H_4PteGlu	Absorbing	0.82	0.50	0.40	0.65
5-CH_3H_4PteGlu	Absorbing	0.87	0.55	0.65	0.75
5-CH_3-5,6-H_2PteGlu@	Absorbing	0.95	0.60	0.40	0.80
5-CH_3-5,8-H_2PteGlu	Absorbing	0.85	0.50	0.30	0.60
4a-OH-5-CH_3H_4PteGlu	"	0.95	0.43	0.40	0.70

@ indicates results obtained from solvent systems without anti-oxidant and * indicates results from corresponding source.

Some microbiological properties of folates.

Microbiological activities of folates are now known as summarized (Blakley,1969;Chanarin,1969).It is possible to determine the purity of some folates with microbiological assay methods. Inthis experiment the microbiological assays with L.casei,S.faecalis and P.cerevisiae are used for the study of the synthesized folates.The effect of storage on the microbiological activities of folates are also studied.

Materials and Methods.

Folates obtained as described in the early part of this chapter were used in this study. Each compound was dissolved in 2 g/l ascorbate water excepted 5-methyl-5,6-dihydropteroylglutamic acid dissolved in distilled water to the final concentrations of 10 ng/ml and 100 ng/ml.These solutions were aseptic microbiologicallyassayed with L.casei,S.faecalis and P.cerevisiae as described in Chapter 2. 5-methyl-5,6-dihydropteroylglutamic acid is easily reduced and acts as 5-methyltetrahydropteroylglutamic acid in the present of ascorbic acid (Donaldson and Keresztesy,1962).Thus this compound was microbiologically assayed with and without ascorbic acid added into the assay media.

Studies of the effect of storage on folate activities were done by incubation of 5-methyltetrahydropteroylglutamic acid,10-formylpteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid at 37°C. The incubation schedule is shown in Table 3.4. After incubation to the required periods they were kept frozen at -20°C and all are microbiologically assayed with L.casei,S.faecalis and P.cerevisiae.

Table 3.3 Microbiological activities of folates synthesized, used in this experiment. Results are expressed in percentage of the expecting values from the weighed samples.

Compound tested	L.casei@ (activity,%)	S.faecalis@ (activity,%)	P.cerevisiae@@ (activity,%)
10-CHO PteGlu	95 - 100	95 - 100	0 - 5
7,8-H_2PteGlu *	95 - 100	95 - 100	0 - 10
5-CH_3-5,6-H_2PteGlu**	0 - 10	0	0
5-CH_3-5,6-H_2PteGlu	95 - 100	0	0
5-CH_3-5,8-H_2PteGlu	0	0	0
5-CH_3H_4PteGlu	95 - 100	0 - 5	0
4a(OH)-5-CH_3H_4PteGlu	0	0	0
5,10-CH=H_4PteGlu	95 - 100	95 - 100	95 - 100
Pte-D-Glu	0	0 - 15	0
Pte	0	60 - 80	0

* spectrophotometric concentration used instead of weighing.

** results obtained when ascorbic acid was omitted from the assay.

@ pteroylglutamic acid was used as standard.

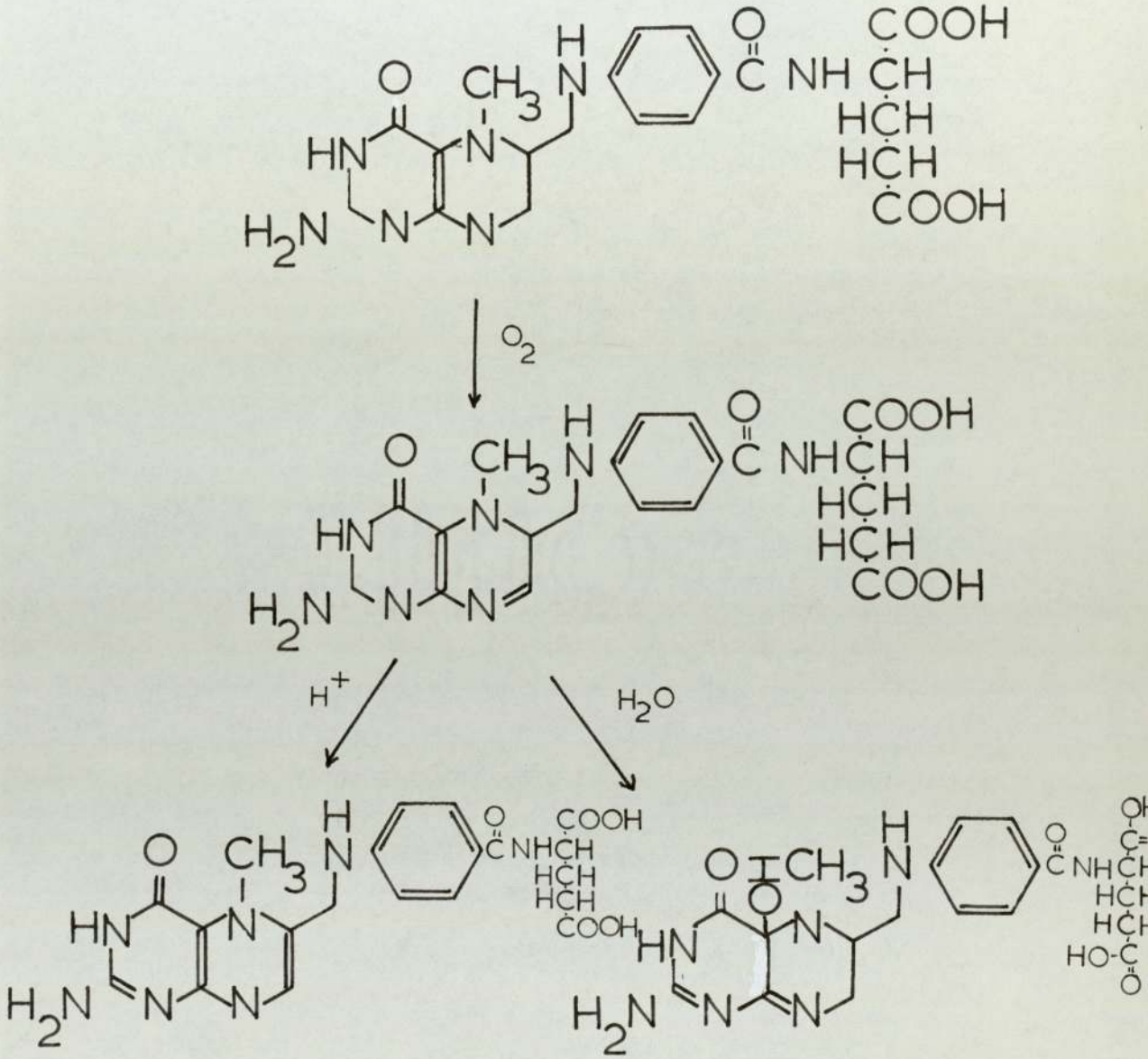
@@ 5-formylpteroylglutamic acid was used as standard.

Table 3.4 The effect of incubation (37°C) on microbiological activities of folates. Results are mean (ng/ml) ± S.E.M. of three samples.

Compound tested	$T_{0(h)}$	$T_{\frac{1}{2}(h)}$	$T_{1(h)}$	$T_{2(h)}$	$T_{3(h)}$	$T_{5(h)}$	T_{24}
5-CHO H_4PteGlu*	12.8±1	12.8±1	12.6±1	12.8±1	12.6±1	12.2±1	10.5±1
5-CHO H_4PteGlu**	13.0±1	12.9±1	13.0±1	12.0±1	12.2±1	12.0±1	11.0±1
5-CHO H_4PteGlu***	12.5±1	12.6±1	12.8±1	12.0±1	12.0±1	12.1±1	10.9±1
(All samples are in ascorbate water,2 g/l;pH 4.5 to 4.7)							
10-CHO PteGlu*	12.0±1	12.2±1	12.0±1	12.0±1	12.1±1	12.0±1	11.2±1
10-CHO PteGlu**	12.2±1	12.0±1	12.1±1	12.0±1	12.0±1	12.0±1	10.8±1
10-CHO PteGlu***	0	0	0	0	0	0	0
(All samples are in ascorbate water,2 g/l;pH 4.5 to 4.7)							
5-CH_3H_4PteGlu****							
in water pH 7	15.0		12.5		12.0	5.0	2.0
in water + 0.1 ml of conjugase pH 7	15.0		15.0		16.0	12.5	10.0
in phosphate buffer pH 7.0	15.0		15.0		10.0	7.0	2.0
in phosphate buffer pH 7.0 + conjugase	15.0		15.0		14.6	12.0	4.0
in ascorbate water pH 4.5 to 4.7	12.0±1	12.0±1	10.0±1	9.0±1	8.5±1	5.0±1	2.0±1
in ascorbate water + conjugase pH 6.7	15.0		13.0		16.0	13.0	15.0
in phosphate buffer +ascorbic acid pH 7	15.0		14.0		12.0	9.0	3.0
in phosphate buffer +ascorbate+conjugase pH 7.0	15.0		15.0		15.0	12.0	8.0

* L.casei,** S.faecalis,*** P.cerevisiae,**** results from L.casei; with S.faecalis and P.cerevisiae results are zero. All ascorbate water containing ascorbic acid (2 g/l),all phosphate buffers are 0.1M and 0.1 ml of conjugase was used in 10 ml incubation mixture. PteGlu was used as standards for L.casei and S.faecalis;5-CHO H_4PteGlu was used for the standards of P.cerevisiae. All folates in each test are equal. Results without S.E.M. are single samples.

Figure 3.1 The scheme of folate conversion judging by microbiological assays.



Results.

Microbiological activities of various folates assayed with L.casei,S.faecalis and P.cerevisiae are summarized in Table 3.3. The results are expressed in term of percentage of the expected values. The effect of incubation of some folates are studied and the microbiological activities of these folates before and after incubation are summarized in Table 3.4.Figure 3.1 showed the possible scheme of the loss in microbiological activities of folates i.e. 5-methyltetrahydropteroylglutamic acid.

Discussion.

The microbiological activities of various folates are summarized in Table 3.3. 5-methyl-5,6-dihydropteroylglutamic acid in the conventional microbiological methods is reduced and acts as 5-methyltetrahydropteroylglutamic acid by the ascorbic acid present (Donaldson and Keresztesy,1962).Microbiological assay of 5-methyl-5,6-dihydropteroylglutamic acid in the media without ascorbic acid added showed much less activities for L.casei,S.faecalis and P.cerevisiae.Therefore this compound is not microbiologically active for these microorganisms and the small amount of activities may due to some impurities. 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid showed no microbiological activity with L.casei,S.faecalis and P.cerevisiae. 5-methyl-5,8-dihydropteroylglutamic acid showed no microbiological activity in the assays with and without ascorbic acid using L.casei,S.faecalis and P.cerevisiae. Thus ascorbic acid did not reduce 5-methyl-5,8-dihydropteroylglutamic acid to 5-methyltetrahydropteroylglutamic acid as it did with

5-methyl-5,6-dihydropteroylglutamic acid (Donaldson and Keresztesy, 1962). It is also possible that 5-methyl-5,8-dihydropteroyl-glutamic acid as an acid rearrangement product of 5-methyl-5,6-dihydropteroylglutamic acid (Blair et al 1974;Robb,1975) may be a degradation product of this compound of which the p-aminobenzoylglutamate moiety of the parent compound had been liberated (Deits et al 1975).Pteroyl-D-glutamic acid showed no microbiological activity with L.casei and P.cerevisiae but about 15 % of microbiological activity was seen when assayed with S.faecalis.Pteroic acid also showed no microbiological activity with L.casei and P.cerevisiae but fully active with S.faecalis.The observation that pteroyl-D-glutamic acid showed microbiological similarity with pteroic acid may derived from pteroic acid impurity , it is used as a starting material for the preparation of pteroyl-D-glutamic acid. All other folates exhibited similar microbiological activities to those reported (Blakley,1969;Chanarin,1969). Incubation of these folates with conjugase showed no enhancement of the microbiological activity. The rate of loss in the microbiological activities of various folates are partially protected by ascorbic acid from 2 mg/ml upwards.The presence of conjugase also protected the loss in the microbiological activity but both ascorbic acid and conjugase can give a better protection during the twenty four hour incubations at 37°C in aqueous medium. Aqueous solutions of 5-methyltetrahydropteroylglutamic acid showed a very quick loss in the microbiological activity with L.casei but there is no alteration in the microbiological activities of S.faecalis and P.cerevisiae (Table 3.4).The L.casei activity was reduced to about 20 percent of the starting activity within 6 to 8 hours

of incubation (starting activity = 7 to 15 ng/ml).

These microbiological assays showed that the loss in microbiological activity of 5-methyltetrahydropteroylglutamic acid was due to autoxidation to the non-microbiologically active compounds as may be shown in the Figure 3.1 similar to Blair et al (1975). With other folates,5-formyltetrahydropteroyl-glutamic acid and 10-formylpteroylglutamic acid showed slight decreases in their microbiological activities after incubation for 24 hours at 37°C. At room temperature these compounds are quite microbiologically stable except 5-methyltetrahydropteroyl-glutamic acid and tetrahydropteroylglutamic acid(O'Broin et al 1975).

Summary.

Compounds synthesized in this experiment showed high microbiological purity. 5-methyl-5,6-dihydropteroylglutamic acid has no microbiological activity with L.casei,S.faecalis and P.cerevisiae but in the presence of ascorbic acid in the conventional microbiological methods it is reduced and acts as 5-methyltetrahydropteroylglutamic acid. 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid and the acid rearrangement product of 5-methyl-5,6-dihydropteroylglutamic acid(probably 5-methyl-5,8-dihydropteroylglutamic acid) showed no microbiological activity with all three test microorganisms. 5-methyltetrahydropteroylglutamic acid in aqeous solution lost its microbiological activity via autoxidation to microbiologically inactive compounds. 5-formyltetrahydropteroylglutamic acid and 10-formylpteroylglutamic acid lost their microbiological activities slowly.The lost in microbiological activities of folates at 37°C can be partially protected by ascorbic acid 2 mg/ml upwards or conjugase. A mixture of ascorbic acid and conjugase exhibited a very high ability to protect the microbiological activity lost of folates.

Chapter 4.

4.1 Serum folates and urine folates excretion in normal humans.

4.2 Constituents of folate in serum and urine of normal humans.

4.3 Seasonal and diurnal variation of folate in man and rat.

4.4 Folate binding proteins.

4.1 Serum folates and urine folates excretion in normal humans.

Folate was known to cure various kinds of anaemia as described previously in Chapter 1.Due to lack of specific method of detection of folate derivatives,serum and urine folates were mainly measured by microbiological assay with L.casei (Blakley,1969;Chanarin,1969).In this study serum folates and folates in urine excretion were studied using aseptic microbiological assay with L.casei,S.faecalis and P.cerevisiae.

Materials and Methods.

Normal human subjects in this study are those people who have no known clinical abnormality.Where applied their haematological and clinical chemistry tests showed normal result as shown in Table 4.1 and 4.2.Those who had defective eye-sight are not regard as abnormal and in cases of women volunteers those taking contraceptive pill were also used.

Venous blood samples were taken from each volunteer. A sample of 10 ml of venous blood was drawn into a sterilized plastic syringe and quickly transferred into a sterilized plain glass sample tube with screw cap and allowed to clot during one-half to two hours.The clotted blood was centrifuged and sera transferred carefully into clean and sterilized screw cap bottles with sterilized Pasteur pipettes.Ascorbic acid (2 to 5 mg/ml) was added and samples were stored frozen at $-20^{\circ}C$ until assayed.

Urine samples were collected 24 hourly in bulk and in periods of 3 to 6 hours.Urine samples were also collected in 3 hours period during 9.00 a.m. to 12.00 noon in subjects on normal diet and on starvation 15 hours before and during the

collecting period.The 24 hour urine samples were collected directly into 500 ml bottles containing 1 g ascorbic acid and these samples were stored at room temperature during the collection period.At the end of 24 hours period urine volumes were measured and some 10 to 20 ml samples were kept frozen at -20°C until assayed.Those 24 hour urine samples collected in periods of 3 to 6 hours were also collected at room temperature during the collecting periods and ascorbic acid 2 g/l added to the known volume samples and some 10 to 20 ml of each samples were kept frozen at -20°C until assayed.Those urine samples collected during 9.00 a.m. to 12.00 noon were measured for their volumes and ascorbic acid (2 mg/ml) added and kept frozen at -20°C until assayed.

Blood samples were collected from normal subjects after normal lunch and from subjects previously starved overnight and proceeded as previously described. Some blood samples from normal subjects were collected and separated at various different times.

Microbiological assays with L.casei,S.faecalis and P.cerevisiae were performed aseptically as previously described in Chapter 2. Sera from these normal human volunteers were some treated with conjugase before microbiological assay with L.casei. Some samples were used for comparing the effect of heating before aseptically added to the assay with L.casei.The incubation with conjugase were done as shown in Table 4.3 before 1 ml of the incubated mixture aseptically added to the test assay.

Table 4.1 Normal haematological values.

Test	Normal result	
Red cells		
Erythrocytes (Male)	5.0 to 6.4	millions/mm^3
" (Female)	4.2 to 6.0	"
Reticulocytes (of total red cells)	0.2 to 2.0	%
Haemoglobin (Male)	14.0 to 17.0	g %
(Female)	12.0 to 15.5	g %
PCV (haematocrit) Male	40.0 to 54.0	%
Female	37.0 to 47.0	%
MCV (mean corpuscular volume)	78.0 to 94.0	u^3
MCH (mean " haemoglobin)	27.0 to 32.0	pg.
MCHC(MCH concentration)	32.0 to 38.0	%
MCD (mean corpuscular diameter)	6.7 to 7.7	u
MCF (meancorpuscularfragility)	0.4 to 0.45	%NaCl
ESR (erythrocyte sedimentation rate)		
:Male (Wintrobe)	0.0 to 9.0	mm
:Female(Wintrobe)	0.0 to 15.0	mm
:Male (Westergren)	1.0 to 5.0	mm
:Female(Westergren)	4.0 to 7.0	mm
White cells		
Total leucocytes	4000 to 10000	mm^{-1}
Differential count:		
Neutrophils	60 to 70	%
Eosinophils	1 to 4	%
Basophils	0 to 1	%
Lymphocytes	25 to 30	%
Monocytes	5 to 10	%
Platelets	150000 to 500000	mm^{-1}

Table 4.2 Normal biochemical values.

Test	Normal result			
Serum electrolytes				
Sodium	141	to	152	mEq/litre
Potassium	4.6	to	5.4	"
Calcium	4.5	to	5.5	"
Chloride	96	to	107	"
Serum bilirubin, total				
Indirect method	0.1	to	0.5	mg %
Direct method	0.0	to	0.25	mg %
Bicarbonate (CO_2)	24	to	31	mEq/litre
Liver function				
Thymol turbidity	0.0	to	2.5	units
Serum transaminase				
Glutamate-oxalacetate transaminase	1	to	40	units
Glutamate-pyruvate transaminase	1	to	45	units

Table 4.3 Incubation mixture in the study of conjugase activity.

Sample No.	Sample Vol. (ml)	0.1M phosphate buffer, pH 7.0 (ml)	Conjugase (ml)	Ascorbate Water(2 g/l) (ml)
1 A	1.0	5.0	-	4.0
1 B	1.0	5.0	0.1	3.9

The final volume is 10 ml and for conjugase blank sample was replaced by equal volume of ascorbate water (2 g/l).

Results.

Microbiological assays with L.casei,S.faecalis and P.cerevisiae of serum folates from subjects after normal lunch are summarized in Table 4.4 and from those subjects fasted overnight are summarized in Table 4.5.

Table 4.4 Serum folates in normal human volunteers after normal lunch.

No. of subjects	L.casei (ng/ml) ±S.E.M.	S.faecalis (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
52	5.6 ± 0.3	0.78 ± 0.03	0.57 ± 0.03

Table 4.5 Serum folates in morning samples from subjects fasted overnight.

No. of subjects	L.casei (ng/ml) ±S.E.M.	S.faecalis (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
70	6.5 ± 0.4	0.70 ± 0.04	0.52 ± 0.02

The effect of heating samples before as assayed by aseptic addition with L.casei were summarized as shown in Table 4.6 and their correlation is shown in Figure 4.1.

Table 4.6 Effect of heating samples before aseptic addition into microbiological assay with L.casei.

No. of sample	Heated samples* (ng/ml)±S.E.M.	Non-heated samples (ng/ml)± S.E.M.	Student 't'test
35	3.3 ± 0.3	3.6 ± 0.3	N.S.

* These samples were diluted 1 in 10 with ascorbate water (2g/l) before heated by autoclaving at 115°C for 3 minutes and proteins discarded by centrifugation. One ml of 1 in 10 dilution of both heated and non-heated samples were used in the assay.

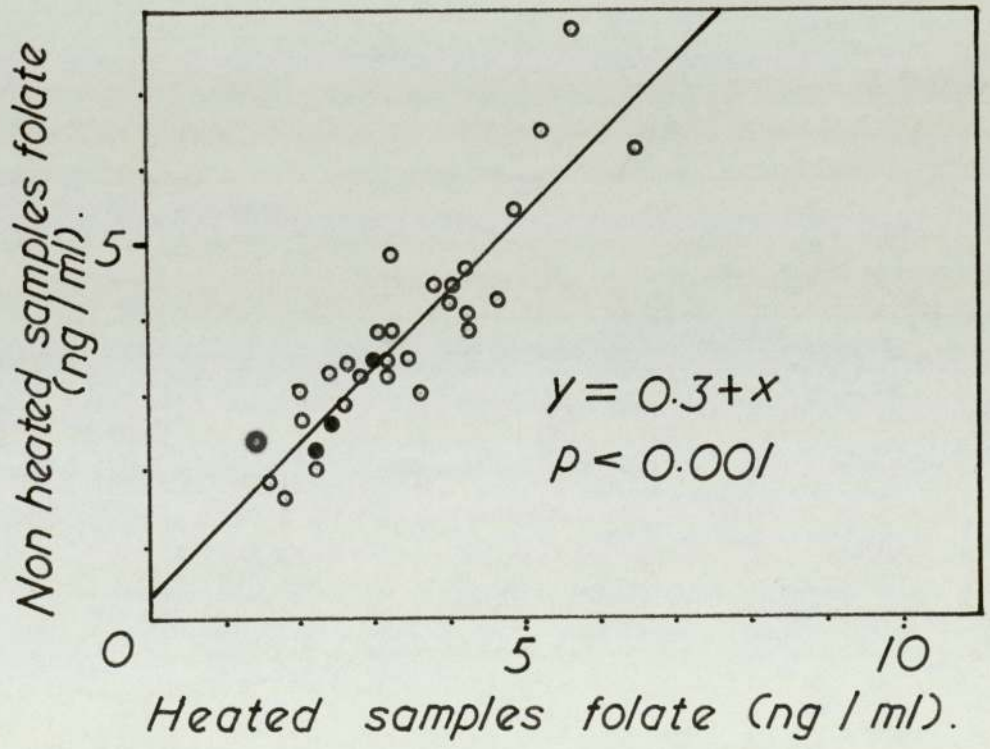


Figure 4.1 The correlationship of serum folates from heated and non-heated samples summarized in Table 4.6.

● = more than one results.

Twenty four hour urine samples from those collected in bulk were microbiologically assayed with L.casei, S.faecalis and P.cerevisiae. The amount of folates assayed by these methods were summarized as shown in Table 4.7. Those urine folates collected in 3 to 6 hours periods were similarly analysed and summarized in Table 4.8.

Table 4.7 Twenty four hour urine folates of normal human subjects.

No. of subjects	L.casei (ug) ±S.E.M.	S.faecalis (ug) ±S.E.M.	P.cerevisiae (ug) ±S.E.M.
6	7.6 ± 0.7	2.8 ± 0.2	0.3 ± 0.1

Table 4.8 Twenty four hour urine folates collected in 3 to 6 hour periods.

Collection period	No. of subjects	L.casei (ug) ±S.E.M.	S.faecalis (ug) ±S.E.M.	P.cerevisiae (ug) ±S.E.M.
0.00 - 6.00a.m.	2	2.2 ± 0.2	1.3 ± 0.3	0.1 ± 0.0
6.00 - 9.00a.m.	2	3.8 ± 0.1	1.4 ± 0.1	0.1 ± 0.0
9.00 - noon	2	1.8 ± 0.1	0.7 ± 0.0	0.0 ± 0.0
noon - 6.00p.m.	2	1.0 ± 0.1	0.8 ± 0.0	0.0 ± 0.0
6.00 - 9.00p.m.	2	1.0 ± 0.2	1.0 ± 0.2	0.0 ± 0.0
9.00 - midnight	2	1.9 ± 0.1	1.6 ± 0.2	0.1 ± 0.1
Twenty four hour	2	11.7 ± 0.7	6.8 ± 0.7	0.3 ± 0.05

Three hours folate excretion during hour 9.00 a.m. to noon in subjects on normal daily diet is summarized in Table 4.9 and from those subjects fasted overnight and during the collection period is summarized in Table 4.10.

Table 4.9 Urine folates excreted during 9.00 a.m. to noon in normal human volunteers on normal daily diet.

No. of subject	L.casei (ug) ±S.E.M.	S.faecalis (ug) ±S.E.M.	P.cerevisiae (ug) ±S.E.M.
4	2.0±0.2	0.7±0.1	0.05±0.01

Table 4.10 Urine folates excreted during 9.00 a.m. to noon in normal human volunteers fasted overnight and during the urine collection period.

No. of subject	L.casei (ug) ±S.E.M.	S.faecalis (ug) ±S.E.M.	P.cerevisiae (ug) ±S.E.M.
14	1.41 ± 0.2	0.43 ± 0.05	0.05 ± 0.01

Microbiological assay with L.casei of samples with and without treatment with conjugase is summarized in Table 4.11.

Table 4.11 Serum folates of normal human and rat before and after treatment with 'conjugase'.

Serum sample (no.of sample)	Before'conjugase' L.casei (ng/ml) ± S.E.M.	After'conjugase' L.casei (ng/ml) ± S.E.M.	Student 't'test
Human (15)	5.5 ± 0.6	5.7 ± 0.7	N.S.
Rat (6)*	69.0 ± 7.0	70.0 ± 7.0	N.S.

* Samples were pooled from those in the diurnal variation studies described later on.

The effect of clotting time on serum folates had been studied by microbiological assays of samples separated at various clotting times and the results is summarized in Table 4.12.

Table 4.12 The effect of clotting time on serum folates of the average of 3 samples.

Test microorganism	Serum folates(ng/ml)±S.E.M. at time within(hour)								
	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	$T_{2\frac{1}{2}}$	T_3	T_4	T_6	T_8
L.casei	7.0	7.0	6.8	7.0	7.2	7.0	6.7	6.7	6.8
(S.E.M.)	0.7	0.7	0.7	0.7	0.8	0.7	0.8	0.8	0.8
S.faecalis	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
(S.E.M.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
P.cerevisiae	0.80	0.7	0.65	0.60	0.65	0.6	0.6	0.6	0.5
(S.E.M.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

4.2 Constituent of folates in serum and urine of normal humans.

Evidences from previous studies indicated that human serum folates are predominated by 5-methyltetrahydropteroylglutamic acid and no conjugated form of folate was found (Herbert,Larrabee and Buchanan,1962;Noronha and Aboobaker,1963; Bird,McGlohon and Vaitkus,1965,Chanarin,1969;Ratanasthien et al 1974).Eventhough folate activity for L.casei in serum exceeds S.faecalis by five to sixteen fold,little is known about the minor folate in serum (Herbert et al 1962;Grossowicz,Mandelbaum-Shavit,Davidoff,Aronovitch,1962;Santini,Perez-Santiago,Wheby and Butterworth,1966;Ratanasthien et al 1974).10-formylpteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid had been microbiologically identified as minor folates found in both normal human and in diseases (Ratanasthien et al 1974; Blair et al 1974a).

In this study the attempts were made to identify serum folates and folates excreted in normal human urine.

Materials and Methods.

Those normal human sera collected as previously used in the in the early part of this Chapter were also used for this study.

5-methyl-5,6-dihydropteroylglutamic acid is active in conventional microbiological assay with L.casei but gave no microbiological activity when ascorbic acid was omitted (Blair et al 1974).In acid media at pH 4.5 or lower 5-methyl-5,6-dihydropteroylglutamic acid is rearranged to microbiologically inactive material 5-methyl-5,8-dihydropteroylglutamic acid (Blair et al 1974).

These properties were used used to see if 5-methyl--tetrahydropteroylglutamic acid measured as a major folate in the serum and urine were derived from 5-methyl-5,6-dihydropteroylglutamic acid. Blood samples from normal subjects were collected and separated into two equal amounts,the first portion of samples were immediately added 2 to 5 mg/ml ascorbic acid and the second portion of those corresponding samples were acidified with small amount of concentrated hydrochloric acid to pH 3.0 and both set of samples were let standing at room temperature for 30 minutes.Then the second portion of samples were adjusted to the starting pH 7.4 with 20 % (W/V) sodium hydroxide solution and ascorbic acid (2 to 5 mg/ml) added. To the first portion of samples added water amount equal to acid and base used in the second portion of samples.These samples were then kept frozen at -20°C until assayed.

Fresh void urine from normal human was collected into sterilized plastic bottle and separated into two portions and proceeded similar to those of serum samples.

Serum and urine samples were also eluted from thin layer chromatography as previously described in Chapter 2. A 300 ul of each saple was used in each plate and the eluates microbiological assayed with L.casei and S.faecalis.

Plasma quickly separated from heparinized blood samples were used to see if 10-formylpteroylglutamic acid was the oxidation product of 10-formyltetrahydropteroylglutamic acid. Venous blood samples were drawn and transferred into heparinized samples tubes and plasma separated,ascorbic acid (2 to 5 mg/ml) added and kept frozen at -20°C until assayed and this process was done within 15 minutes after blood drawn.

Microbiological assays and bioautography with L.casei,S.faecalis and P.cerevisiae were done as previously described in Chapter 2.

Results.

L.casei is active for all folates in serum whilst S.faecalis is active for all except 5-methyltetrahydropteroyl-glutamic acid (Johns and Bertino,1965);therefore,the difference in activity of those two test organisms is a measure of 5-methyltetrahydropteroylglutamic acid.P.cerevisiae is active for 10-formyltetrahydropteroylglutamic acid,5-formyltetrahydro-pteroylglutamic acid but not for 10-formylpteroylglutamic acid and pteroylglutamic acid whereas S.faecalis is active all four compounds.Therefore the difference between the S.faecalis and P.cerevisiae assays measures the amount of folic acid and 10-formylpteroylglutamic acid present.Microbiological assays of samples eluted from thin layer chromatography showed no activity of pteroylglutamic acid as shown in Table 4.2.1. Therefore the difference between S.faecalis and P.cerevisiae assays of serum measures the amount of 10-formylpteroylglutamic acid.The individual serum folates of normal subjects is summarized in Table 4.2.2. The correlation of total serum folates and serum 5-methyltetrahydropteroylglutamic acid is shown in Figure 4.2.1. Plasma folates from those samples quickly separated is summarized in Table 4.2.3. 5-methyl-5,6-dihydropteroylglutamic acid in serum and urine are shown in Table 4.2.4. Various folates in urine are summarized in Table 4.2.5.

Table 4.2.1 Serum folates of one normal human from eluates of t.l.c. developed in 3% aqeous ammoniumchloride containing 1% (W/V) ascorbic acid.

Rf values (ranges)	L.casei (ng/ml)±S.E.M.	S.faecalis (ng/ml)±S.E.M.	No.of samples
0 to.1	0	0	3
.1 to.2	0	0	3
.2 to.3	0	0	3
.3 to.4	0	0	3
.4 to.5	0.8 ± 0.1	0.8 ± 0.1	3
.5 to.6	0	0	3
.6 to.7	3.5 ± 0.5	0	3
.7 to.8	2.0 ± 0.3	0	3
.8 to.9	0.8 ± 0.1	0	3
.9 to 1.0	0.8 ± 0.1	0	3

Table 4.2.2 Serum folates of normal human subjects by aseptic differential microbiological assays with L.casei, S.faecalis and P.cerevisiae.

Sample from (no.of subject)	5-CH_3H_4PteGlu (ng/ml)±S.E.M.	10-CHO PteGlu (ng/ml)±S.E.M.	10-CHO H_4PteGlu (ng/ml)±S.E.M.
Morning samples (70)	5.8 ± 0.5	0.18 ± 0.02	0.52 ± 0.02
After lunch " (52)	4.8 ± 0.3	0.21 ± 0.03	0.57 ± 0.03

Table 4.2.3 Serum folates from samples quickly separated (within 30 minutes after blood drawn) from normal humans.

No.of subjects	5-CH_3H_4PteGlu* (ng/ml)±S.E.M.	10-CHO PteGlu (ng/ml)±S.E.M.	10-CHO H_4PteGlu (ng/ml)±S.E.M.
20**	5.0±0.4	0.05±0.01	0.75±0.10
12***	5.2±0.4	0.05±0.01	0.75±0.09
8****	4.7±0.4	0.05±0.01	0.75±0.11

* indicates results are the summation of true 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu activities,** whole group,*** heparinized and **** serum from plain glass tubes.

Table 4.2.4 Serum and urine 5-CH_3-5,6-H_2PteGlu.

Sample (no.of samples) in parentheses.	Unacidified L.casei(A) (ng/ml)±S.E.M.	Acidified L.casei(B) (ng/ml)±S.E.M.	5-CH_3-5,6-H_2PteGlu (A) - (B) (ng/ml)±S.E.M.
Serum (30)*	5.75±0.48	5.00±0.63	0.75±0.15*
Urine (16)**	11.2 ± 2.0	10.0 ± 1.5	1.2 ± 0.5

* sera were quickly separated within 30 minutes after blood samples were drawn and total 5-methyltetrahydropteroylglutamic acid activities are 4.95±0.4 ng/ml, ** indicates fresh void urine samples were used in acidification treatment before ascorbic acid 5 ng/ml were added.

Table 4.2.5 Urine folates of normal humans daily excretion.

No.of subjects	5-CH_3H_4PteGlu (ug) ± S.E.M.	10-CHO PteGlu (ug) ± S.E.M.	10-CHO H_4PteGlu (ug) ± S.E.M.
6*	4.8 ± 0.7	2.5 ± 0.2	0.3 ± 0.1

* samples collected into 500 ml plastic bottles each bottle containing 2.5 g of ascorbic acid and samples were kept at room temperature or refrigerated during collection periods.

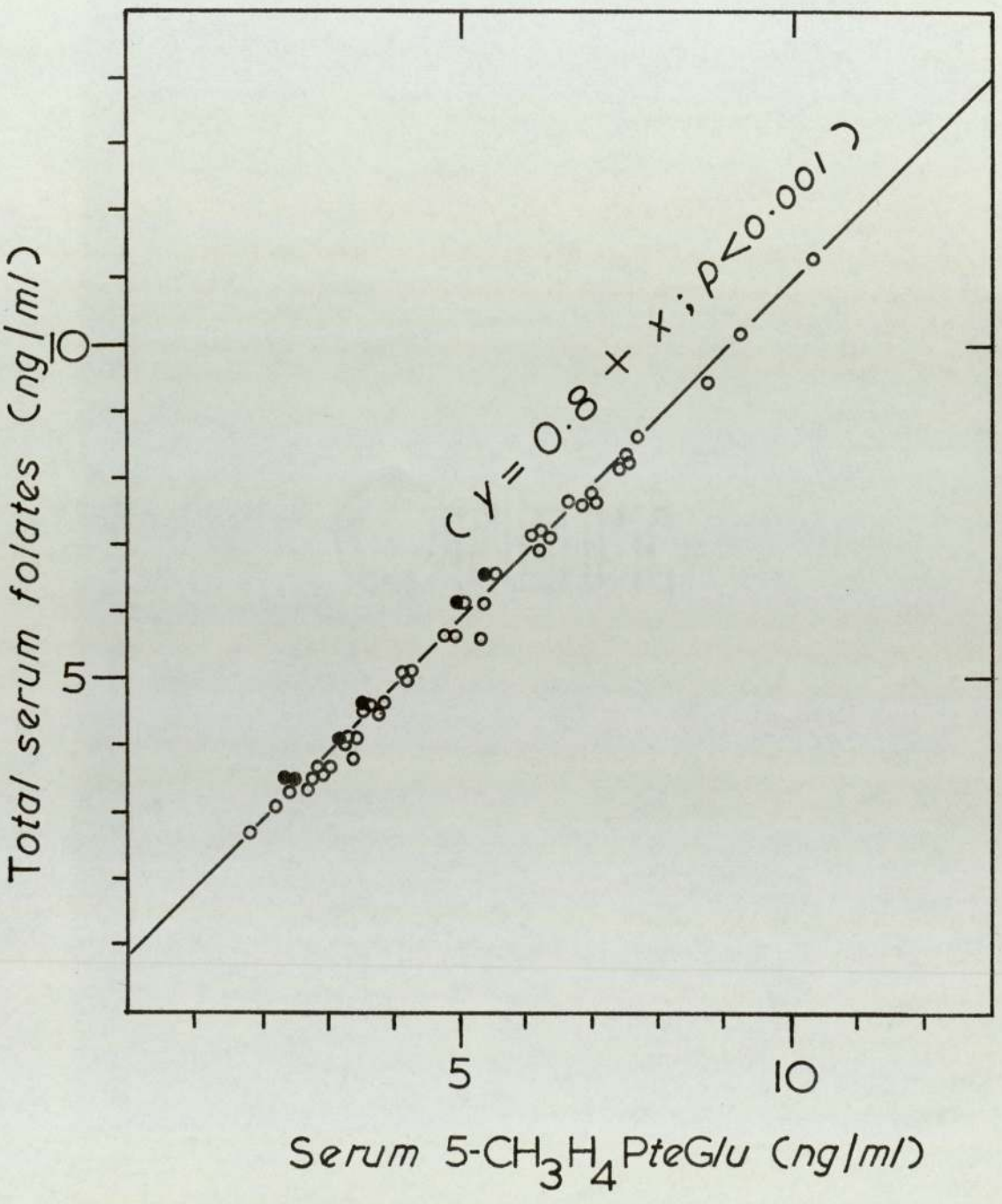


Figure 4.2.1 Correlation of total folate and 5-methyl-tetrahydropteroylglutamic acid level in 52 normal subjects.

4.3 Seasonal and diurnal variations of folate in man and rat.

There is no direct information about diurnal and seasonal variations of folate in man available in the literature. DNA synthesis have been demonstrated to have diurnal variation in experimental animals (Barbiroli and Potter,1971). Diurnal variation of liver folate metabolism in rats had been studied. Barbiroli et al (1974) reported daily rhythms of enzyme activities involved in folate metabolism as shown in Figure 4.3.1.

In this study the attempts were made to the study of serum and urine folate daily rhythms using both man and rat in the experimental studies.

Materials and Methods.

Serum folate of those patients who had come to the General Hospital in Birmingham over the past three years were used for the seasonal variation study.The weekly results of about 100 to 120 samples were microbiological assayed with L.casei (Leeming and Portman-Graham,1973).

Twenty four hour urine samples collected in periods of 3 to 6 hours similarly proceeded as those in the study of urinary folate excretion.

Serum samples collected hourly from subjects fasted overnight and during the period of sample collection (9.00 a.m. to 5.00 p.m.). These samples were collected as described for the collection of single samples in the early part of this Chapter.

Diurnal variation studies were mainly done in the rat. Seven-week-old male Wistar albino rats weighing 200 to 250 g were caged in group of 6 animals with Heygates Diet 41B supplied

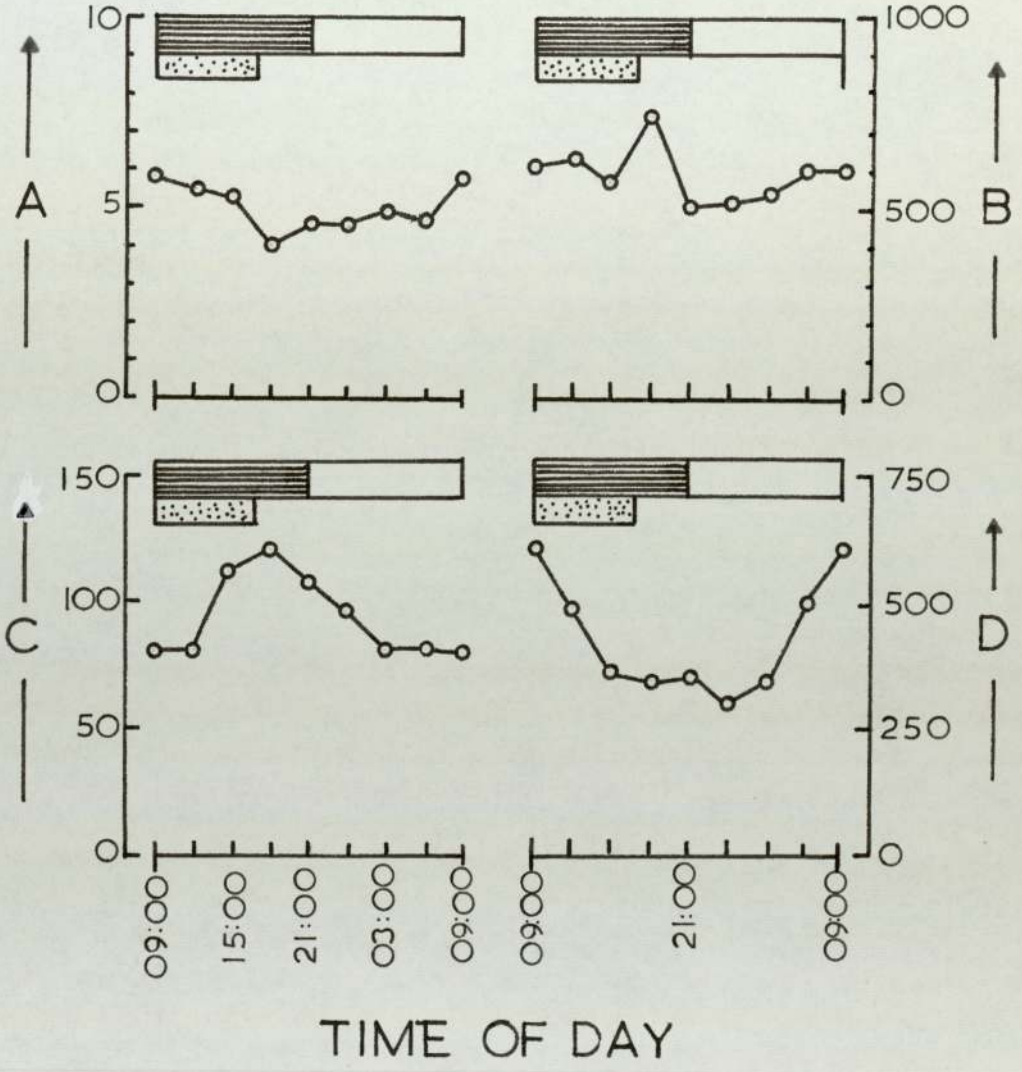
ad lib.The lights in the animal house or experimental area were on from 8.00 a.m. to 8.00 p.m. in a 24 hour cycle. On experimental day 48 rats were brought into laboratory at 9.30 a.m. and the first experiment started at 10.00 a.m. A pair of rats were anaesthetized and bled to death at the start of experiment and then hourly for the next 24 hours. Blood samples were collected into heparinized test tubes and plasma decanted into clean sample tubes or screw cap bottles with ascorbic acid 5 mg/ml added and these samples were kept frozen at -20°C until assayed.

Similar experiments were performed on rats previously starved for 24 hours and during the experimental period.

These samples were microbiologically assayed with L.casei,S.faecalis and P.cerevisiae using aseptic addition as described in Chapter 2. Bioautography of some samples with these microorganisms were also used for confirmation of folate as described in Chapter 2.

Results.

Average of weekly results of those patients who had come to the General Hospital in Birmingham were plotted against the time of year as shown in Figure 4.3.2. Normal human serum folate of hourly samples were summarized as shown in Table 4.3.1 and Figure 4.3.3. Urinary folates were summarized as shown in Table 4.3.2 and Figure 4.3.4. Serum folates of rats on normal daily diet is summarized as shown in Table 4.3.3 and Figure 4.3.5. Those results of starving animals are shown in Table 4.3.4 and Figure 4.3.6.



A = H_4PteGlu reductase activity(n mole of H_2PteGlu reduced/min.)
B = Serine hydroxymethyl transferase activity(n moleHCOH used per 20 minutes per mg protein).
C = 5,10-CH H_4PteGlu dehydrogenase activity(n mole of 5,10-CH=H_4PteGlu formed/20 minutes/mg protein).
D = 10-CHO H PteGlu synthetase activity(n mole of 5,10-CH=H_4 PteGlu formed/20 minutes/mg protein).
= Feeding periods.

Figure 4.3.1 Hepatic enzyme activities of folate metabolism as a function of time of day (A) H_4PteGlu dehydrogenase; (B) serine hydroxymethyl transferase; (C) 5,10-CH_2H_4PteGlu dehydrogenase; (D) 10-CHO H_4PteGlu synthetase.From Barbiroli et al 1974.

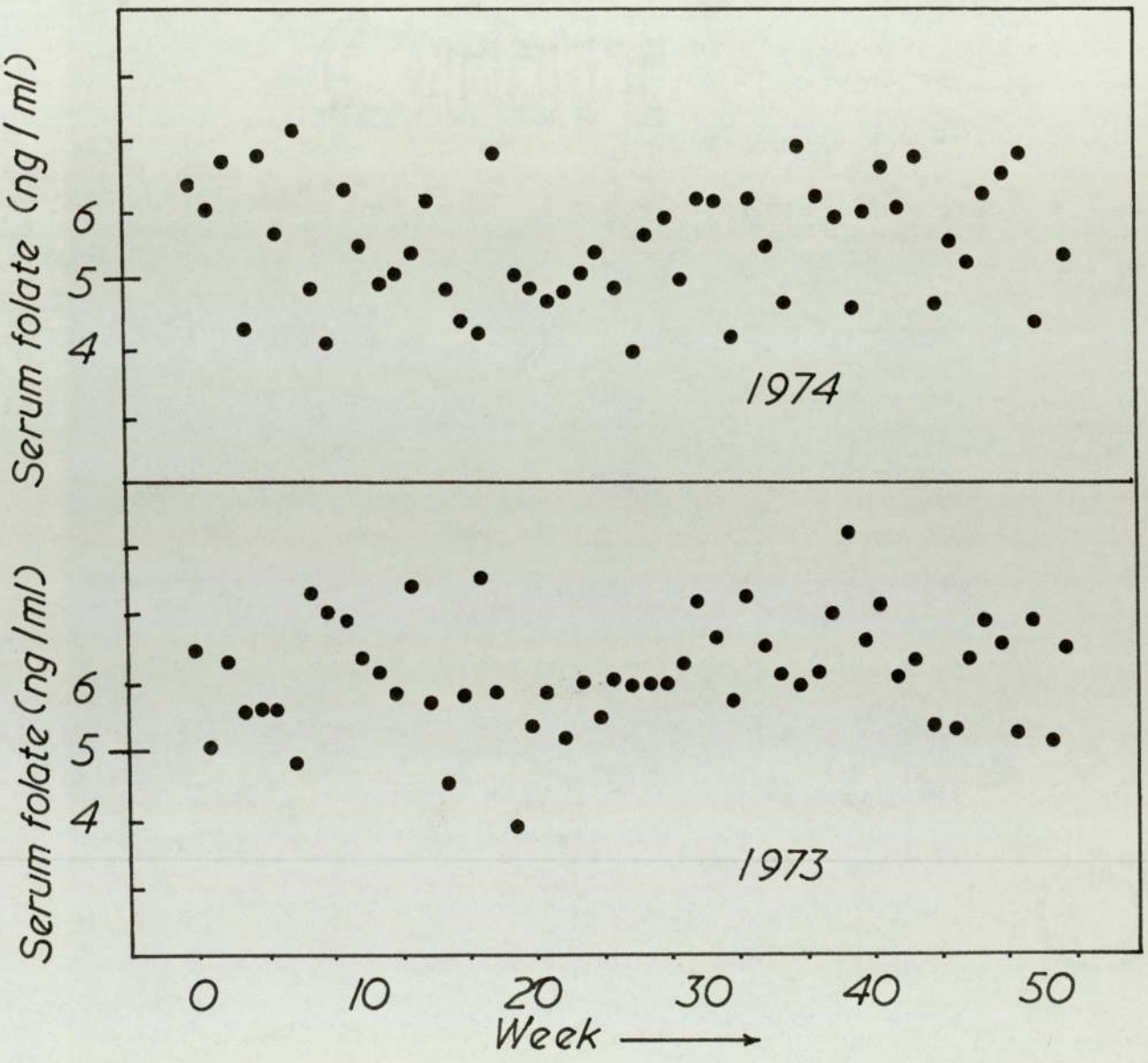
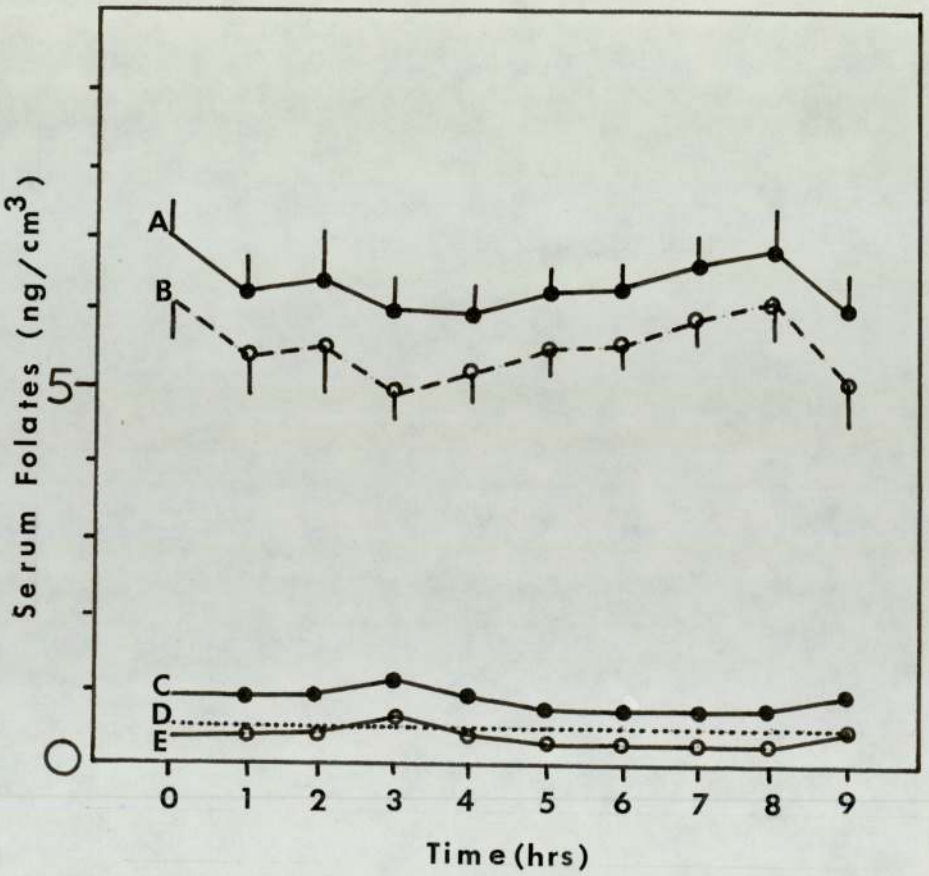


Figure 4.3.2 Average results of serum folate analysed weekly as a function of time of year. Each point represent the mean ± S.E.M. of 100 to 120 samples.

Figure 4.3.3 Normal human serum folate during day time after overnight and throughout collection period starvation.Each point represent a mean ± S.E.M. of 5 samples,except the last point for only two samples. Collection period from 9.00 a.m.to 6.00 p.m.,hourly.



A = Total serum folate(L.casei)

B = Serum 5-methyltetrahydropteroylglutamic acid.

C = S.faecalis levels.

D = 10-CHO H_4PteGlu (P.cerevisiae).

E = 10-CHO PteGlu (S.faecalis - P.cerevisiae).

B = L.casei - S.faecalis.

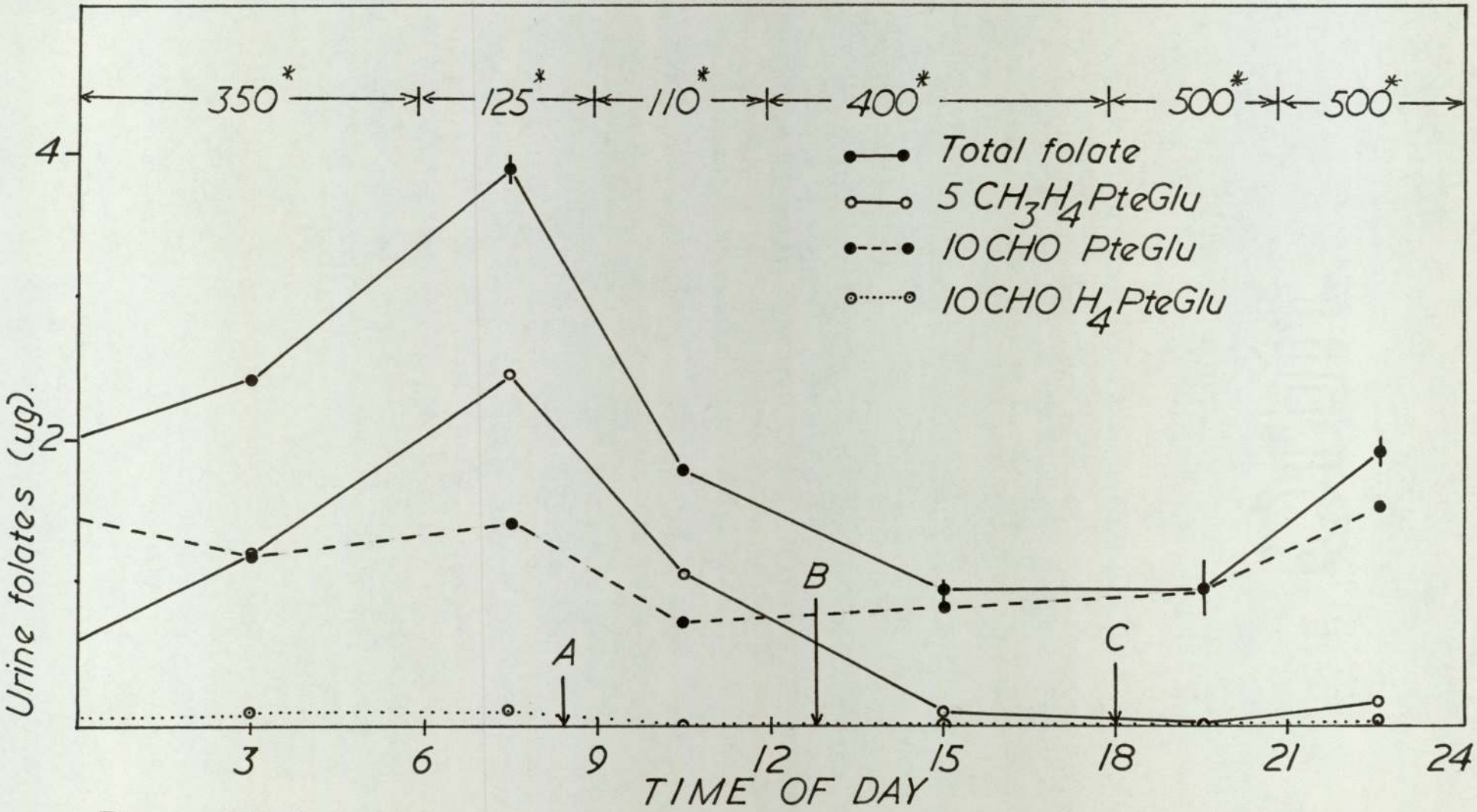


Figure 4.3.4 Urine folates of normal humans on normal daily diet.
A= brekfast ; B= lunch ; C = dinner ; *Urine volume(ml).

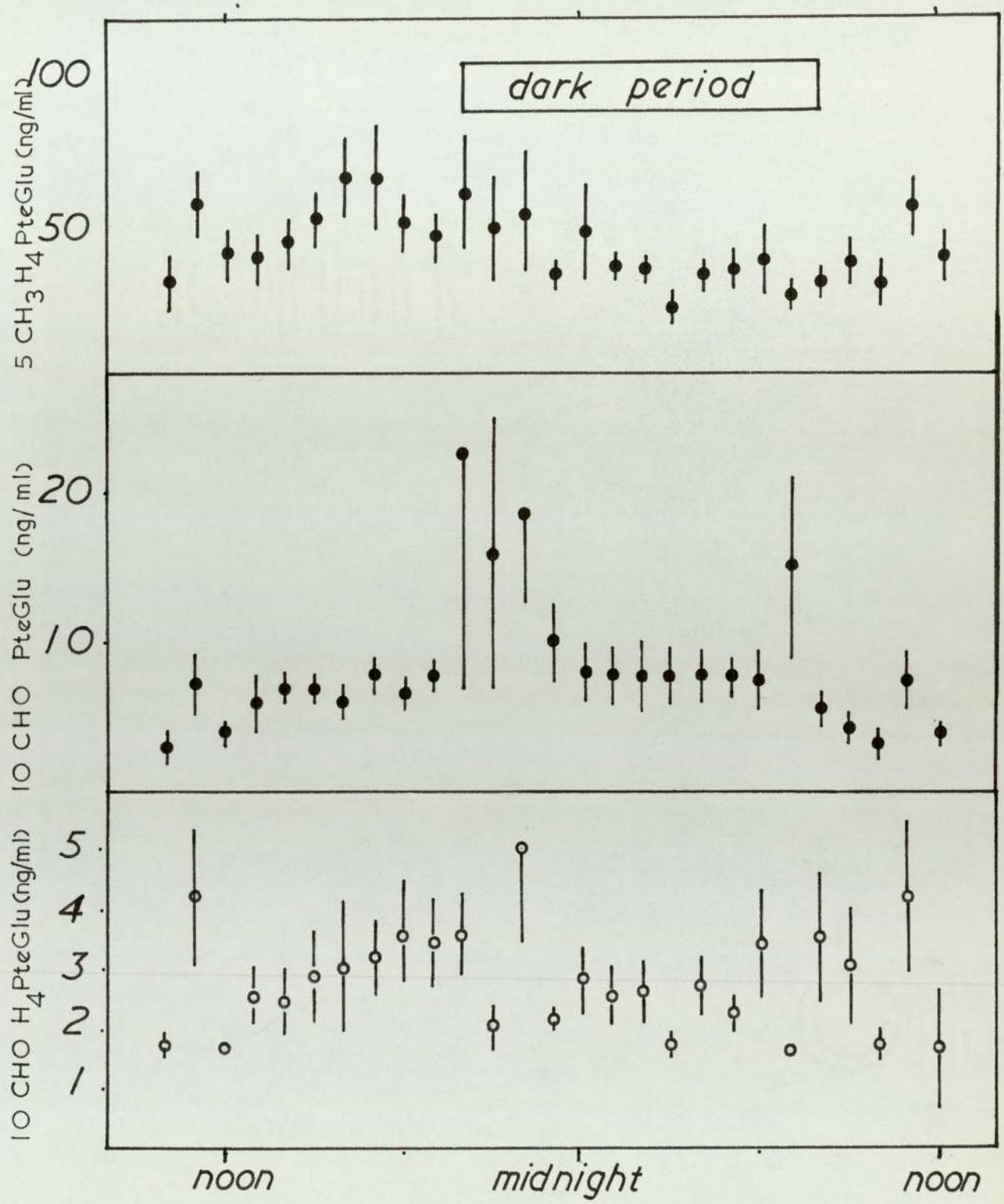


Figure 4.3.5 Serum folate of rats maintained on normal daily diet as a function of time of day. Each point represent a mean of 6 animals ± S.E.M.

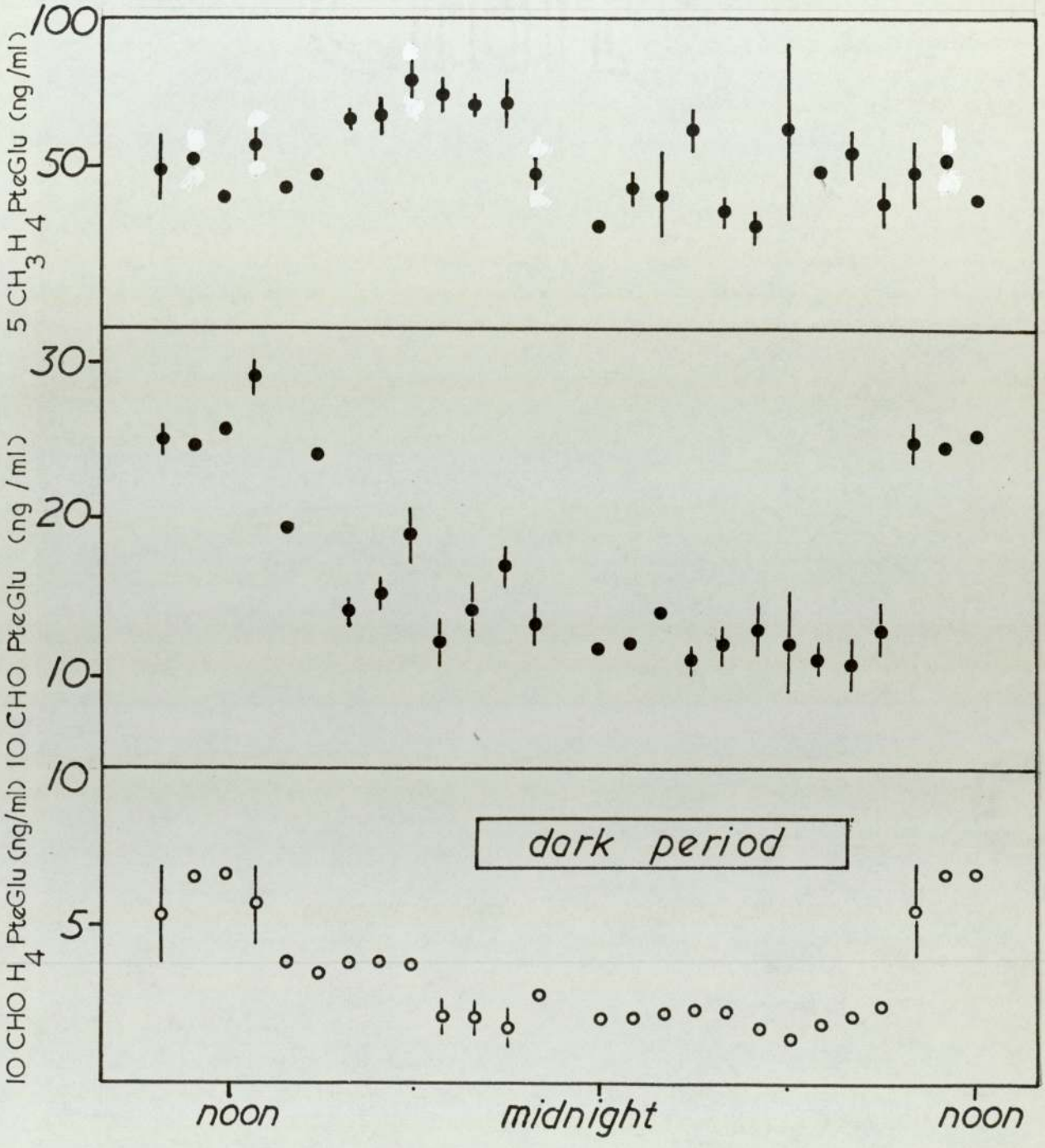


Figure 4.3.6 Serum folate of rats maintained on starvation 24 hours before and throughout the experimental period as a function of time of day. Each point represent a mean ± S.E.M. of 2 samples.

Table 4.3.1 Normal human serum folate as a function of time of day.Each result is an average of 5 samples.

Time of day.	Total Folate (ng/ml)±S.E.M.	5-CH_3H_4PteGlu (ng/ml)±S.E.M.	10-CHO PteGlu (ng/ml)±S.E.M.	10-CHO H_4PteGlu (ng/ml)±S.E.M.
09.00 a.m.	7.1 ± 0.4	6.2 ± 0.4	0.3 ± 0.1	0.6 ± 0.1
10.00 a.m.	6.3 ± 0.4	5.4 ± 0.5	0.4 ± 0.1	0.5 ± 0.1
11.00 a.m.	6.5 ± 0.6	5.5 ± 0.6	0.5 ± 0.1	0.5 ± 0.1
12.00 noon	6.0 ± 0.4	4.9 ± 0.5	0.7 ± 0.2	0.5 ± 0.1
01.00 p.m.	6.0 ± 0.3	5.2 ± 0.4	0.4 ± 0.1	0.4 ± 0.1
02.00 p.m.	6.2 ± 0.3	5.5 ± 0.3	0.2 ± 0.1	0.5 ± 0.1
03.00 p.m.	6.3 ± 0.2	5.5 ± 0.2	0.3 ± 0.1	0.5 ± 0.1
04.00 p.m.	6.6 ± 0.4	5.8 ± 0.4	0.3 ± 0.1	0.5 ± 0.1
05.00 p.m.	6.8 ± 0.5	6.2 ± 0.5	0.2 ± 0.1	0.4 ± 0.1

Table 4.3.2 Urinary folate of normal subjects (2) on normal daily diet and collected in 3 to 6 hours periods.

Sample Period (From To)	Total folate (ug) ± S.E.M.	5-CH_3H_4PteGlu (ug)±S.E.M.	10-CHOPteGlu (ug)±S.E.M.	10-$CHOH_4$PteGlu (ug)±S.E.M.
00.00 to 06.00 a.m.	2.2 ± 0.2	0.9 ± 0.2	1.2 ± 0.3	0.1 ± 0.0
06.00 to 09.00 a.m.	3.8 ± 0.1	2.4 ± 0.1	1.3 ± 0.1	0.1 ± 0.0
09.00 to 12.00 noon	1.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.0	0.0 ± 0.0
noon to 06.00 p.m.	1.0 ± 0.1	0.2 ± 0.1	0.8 ± 0.0	0.0 ± 0.0
06.00 to 09.00 p.m.	1.0 ± 0.2	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0
09.00 to midnight.	1.9 ± 0.1	0.3 ± 0.1	1.5 ± 0.2	0.1 ± 0.1
Total 24 hour urine	11.7 ± 0.7	4.9 ± 0.5	6.5 ± 0.7	0.3 ± 0.05

Table 4.3.3 Serum folate of rats on normal daily diet as a function of time of day. Each result represent a mean of 6 animals.

Sample at time	Total folate (ng/ml)±S.E.M.	5-CH_3H_4PteGlu (ng/ml)±S.E.M.	10-CHO PteGlu (ng/ml)±S.E.M.	10-CHO H_4PteGlu (ng/ml)±S.E.M.
10.00 a.m.	35.0 ± 6.8	31.3 ± 6.7	3.0 ± 0.5	1.7 ± 0.2
11.00 a.m.	68.0 ±16.8	56.7 ±15.0	7.1 ± 1.9	4.2 ± 1.3
12.00 noon	45.0 ± 6.0	39.9 ± 6.0	3.5 ± 1.0	1.6 ± 0.1
01.00 p.m.	47.0 ± 8.5	38.5 ± 8.0	5.9 ± 2.1	2.6 ± 0.5
02.00 p.m.	52.0 ± 5.8	42.7 ± 5.0	6.8 ± 1.0	2.5 ± 0.7
03.00 p.m.	61.0 ±10.5	50.9 ± 8.7	7.2 ± 1.0	2.9 ± 0.8
04.00 p.m.	74.0 ±15.6	65.4 ±13.2	5.6 ± 1.2	3.0 ± 1.2
05.00 p.m.	75.0 ±20.6	64.1 ±18.6	7.7 ± 1.2	3.2 ± 0.8
06.00 p.m.	60.0 ± 9.7	50.0 ± 7.6	6.4 ± 0.8	3.6 ± 0.8
07.00 p.m.	57.0 ± 9.4	45.9 ± 7.3	7.7 ± 1.4	3.4 ± 0.7
08.00 p.m.	86.0 ±22.5	65.9 ±18.5	23.0 ±17.6	3.5 ± 0.8
09.00 p.m.	66.0 ±21.0	47.9 ±15.8	16.0 ± 9.0	2.1 ± 0.4
10.00 p.m.	77.0 ±23.4	52.7 ±15.2	19.1 ± 6.6	5.2 ± 1.6
11.00 p.m.	44.0 ± 4.7	31.9 ± 4.1	9.9 ± 3.0	2.2 ± 0.2
midnight.	58.0 ±19.8	46.3 ±16.3	8.9 ± 2.9	2.8 ± 0.6
01.00 a.m.	46.0 ±11.3	35.0 ± 7.3	8.4 ± 3.5	2.6 ± 0.5
02.00 a.m.	45.0 ± 6.4	34.9 ± 4.0	8.5 ± 2.3	2.6 ± 0.5
03.00 a.m.	32.0 ± 4.5	21.3 ± 3.6	8.0 ± 2.6	1.7 ± 0.1
04.00 a.m.	44.0 ± 4.9	33.0 ± 3.5	8.3 ± 1.5	2.7 ± 0.5
05.00 a.m.	45.0 ± 3.2	34.9 ± 2.7	7.9 ± 1.8	2.2 ± 0.3
06.00 a.m.	49.0 ±10.6	37.4 ± 8.6	8.1 ± 1.0	3.5 ± 0.9
07.00 a.m.	35.0 ± 4.5	35.9 ± 4.0	7.5 ± 1.9	1.6 ± 0.1
08.00 a.m.	50.0 ±13.3	30.6 ±10.2	15.9 ± 7.0	3.5 ± 1.3
09.00 a.m.	46.0 ± 4.5	37.9 ± 3.3	5.1 ± 1.1	3.0 ± 1.0

Table 4.3.4 Serum folate of rats on starvation 24 hours before and during the experimental period.Each result represent mean of 2 samples.

Sample at time	Total folate (ng/ml)±S.E.M.	5-CH_3H_4PteGlu (ng/ml)±S.E.M.	10-CHO PteGlu (ng/ml)±S.E.M.	10-CHO H_4PteGlu (ng/ml)±S.E.M
10.00 a.m.	79.0 ± 16.0	48.0 ± 10.0	25.6 ± 2.0	5.4 ± 1.6
11.00 a.m.	82.0 ± 0.0	52.0 ± 0.0	23.5 ± 0.0	6.5 ± 0.0
12.00 noon	72.0 ± 0.0	40.0 ± 0.0	25.4 ± 0.0	6.6 ± 0.0
01.00 p.m.	92.0 ± 12.0	57.0 ± 3.0	29.3 ± 3.8	5.7 ± 1.3
02.00 p.m.	66.0 ± 0.0	43.0 ± 0.0	19.2 ± 0.0	3.8 ± 0.0
03.00 p.m.	75.0 ± 0.0	47.0 ± 0.0	24.5 ± 0.0	3.5 ± 0.0
04.00 p.m.	83.0 ± 3.0	65.0 ± 2.0	14.2 ± 1.0	3.8 ± 0.0
05.00 p.m.	86.0 ± 6.0	67.0 ± 4.0	15.2 ± 1.0	3.8 ± 0.0
06.00 p.m.	101.0 ± 7.0	78.0 ± 5.0	19.2 ± 2.0	3.8 ± 0.0
07.00 p.m.	87.0 ± 7.0	73.0 ± 5.0	12.0 ± 1.4	2.0 ± 0.4
08.00 p.m.	85.0 ± 5.0	70.0 ± 2.0	12.9 ± 2.0	2.1 ± 0.5
09.00 p.m.	88.0 ± 14.0	70.0 ± 8.0	16.4 ± 4.0	1.6 ± 0.6
10.00 p.m.	64.0 ± 6.0	47.0 ± 5.0	17.0 ± 3.0	2.8 ± 0.0
midnight.**	45.0 ± 0.0	31.0 ± 0.0	12.0 ± 0.0	2.0 ± 0.0
01.00 a.m.	58.0 ± 3.0	44.0 ± 4.0	12.0 ± 0.0	2.0 ± 0.0
02.00 a.m.	57.0 ± 13.0	41.0 ± 13.0	13.8 ± 0.0	2.2 ± 0.2
03.00 a.m.	75.0 ± 5.0	61.0 ± 5.0	11.7 ± 1.0	2.3 ± 0.3
04.00 a.m.	50.0 ± 5.0	36.0 ± 5.0	11.8 ± 2.0	2.2 ± 0.1
05.00 a.m.	45.0 ± 5.0	31.0 ± 4.0	12.4 ± 2.4	1.6 ± 0.2
06.00 a.m.	71.0 ± 29.0	63.0 ± 34.0	6.7 ± 4.7	1.3 ± 0.3
07.00 a.m.	60.0 ± 0.0	47.0 ± 1.0	11.2 ± 0.8	1.8 ± 0.2
08.00 a.m.	67.5 ± 7.5	55.0 ± 5.0	10.5 ± 2.0	2.0 ± 0.2
09.00 a.m.	53.0 ± 3.0	38.0 ± 5.0	12.7 ± 1.7	2.3 ± 0.3

** 11.00 p.m. sample haemolyzed.

4.4 Folate binding proteins.

Following a report of there was a specific folate binding protein in cow's milk (Ghitis,1967), a minor whey protein in the beta lactoglobulin fraction of cow's milk was shown to have the ability to bind with folates (Ford,Salter and Scott,1969).Metz et al (1968) reported the presence of folate binding proteins in serum and milk of human.Folate binding was also found in other tissues.Leslie and Rowe (1972) reported folate binding by brush border membrane proteins of small intestinal epithelial cells.Intracellular folate binding protein has been reported in the studies of patients with chronic myelogenous leukemia (Rothenberg,1970;Rothenberg and daCosta,1971).They found that leucocyte lysates from thier patients containing a high molecular weight protein which bound (^{3}H)pteroylglutamic acid.

Folate binding protein was shown to spread throughout the body fluids (Markkanen and Peltola,1970;Markkanen and Peltola,1971;Markkanen,Pajula;Virtanin and Himanen,1972).

In this experiment the attempts were made to study of protein binding folates by using simple methods.The application of t.l.c. combining with differential microbiological assays with L.casei,S.faecalis and P.cerevisiae was used.Proteins were simply detected by using thin layer chromatography and ninhydrin spray.

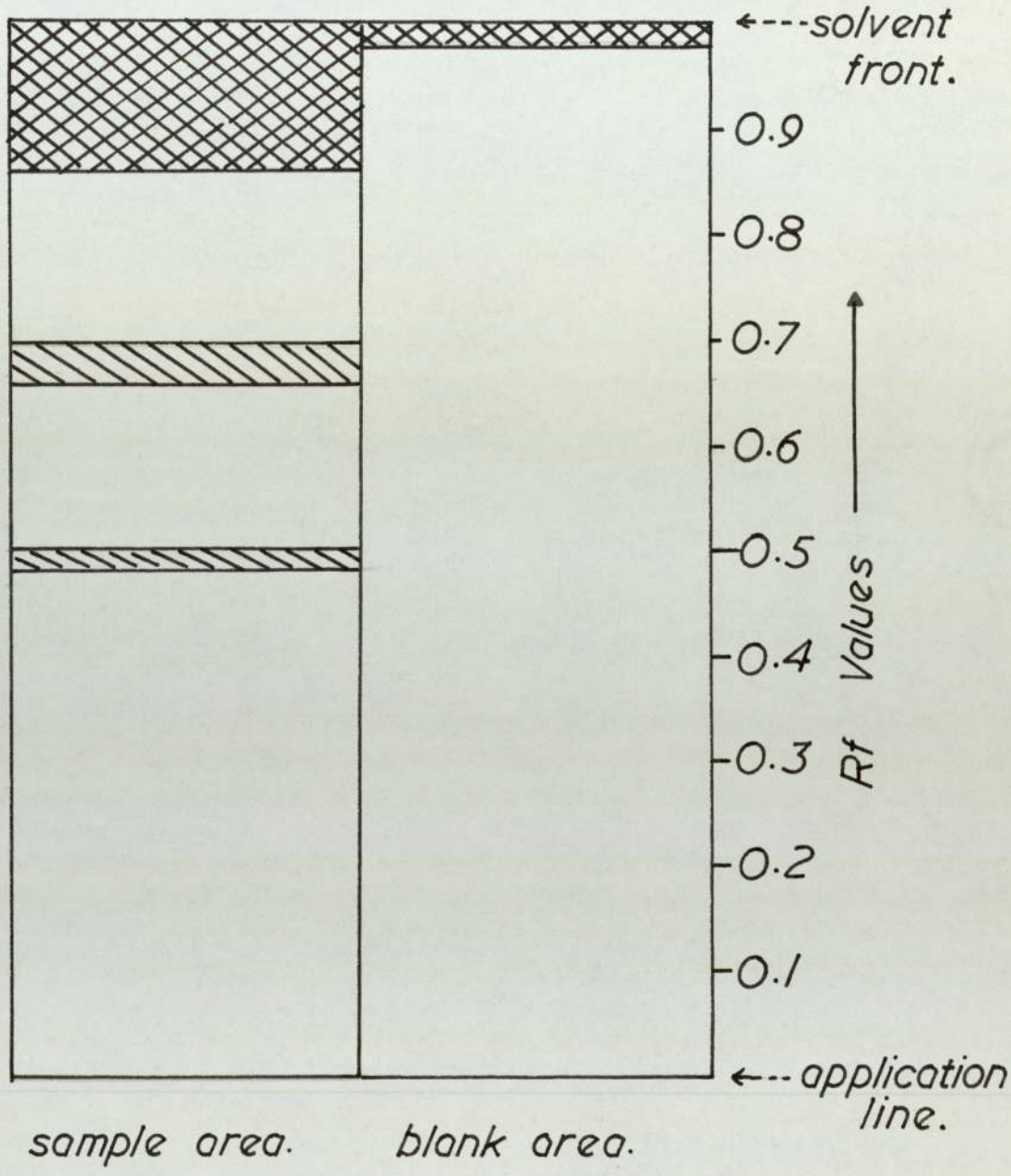
Materials and Methods.

Normal human sera collected as used in the study of serum folate were used in this study.Samples were applied as

a horizontal line along the origin of a pre-coated cellulose plate (without fluorescent indicator) and developed to a desired length.The application of samples were done in two ways.The first one using neat samples and the second one using deproteinized corresponding samples.The deproteinized samples were prepared by adding a few drops of concentrated HCl into sample and proteins discarded after centrifugation.The supernatant was neutralized with 20 % (W/V) sodiumhydroxide solution to the starting pH 7.4.In all cases 300 ul of samples were used and the ascending thin layer chromatography developed in 3%(W/V) aqeous ammonium chloride containing 1%(W/V) ascorbic acid.The developed plates were cut into 10 equal strips and the coated cellulose transfered from each strip into test tube contained 3 ml of 2 g/l ascorbate water.The mixture was shaked for a few minutes and the centrifuge at about 1000 g and one ml of the supernatant was used in the microbiological assay tube.Aseptic addition microbiological assays with L.casei and S.faecalis were used as described in Chapter 2.

Results.

The proteins distribution on t.l.c. plate developed in 3%(W/V) aqeous ammonium chloride containing 1%(W/V) ascorbic acid after ninhydrin spray was shown in Figure 4.4.1.Serum folate from eluates of neat samples is summarized in Table 4.4.1 and from eluates of deproteinized samples is summarized in Table 4.4.2.



= Intensive purple area after ninhydrin spray.

= Light purple area after ninhydrin spray.

Figure 4.4.1 Proteins distribution on t.l.c. plate developed in 3%(W/V) aqeous ammonium chloride containing 1%(W/V) ascorbic acid after ninhydrin spray.

Table 4.4.1 Serum folate of eluates when neat samples were used on t.l.c. plate and developed in 3%(W/V) aqeous ammonium chloride containing 1%(W/V) ascorbic acid. Samples from one subject.

Rf values (ranges)	L.casei (ng/ml)±S.E.M.	S.faecalis (ng/ml)±S.E.M.	No. of samples
0.0 to 0.1	0	0	3
0.1 to 0.2	0	0	3
0.2 to 0.3	0	0	3
0.3 to 0.4	0	0	3
0.4 to 0.5	0.8 ± 0.1	0.8 ± 0.1	3
0.5 to 0.6	0	0	3
0.6 to 0.7	3.5 ± 0.5	0	3
0.7 to 0.8	2.0 ± 0.3	0	3
0.8 to 0.9	0.8 ± 0.1	0	3
0.9 to 1.0	0.8 ± 0.1	0	3
Total	7.9	0.8	3

Table 4.4.2 Serum folate of eluates when deproteinized samples were used on t.l.c. plate and developed in 3%(W/V) aqeous ammonium chloride containing 1%(W/V) ascorbic acid.

Rf values (ranges)	L.casei (ng/ml)±S.E.M.	S.faecalis (ng/ml)±S.E.M.	No. of samples*
0.0 to 0.1	0	0	3
0.1 to 0.2	0	0	3
0.2 to 0.3	0	0	3
0.3 to 0.4	0	0	3
0.4 to 0.5	0.8 ± 0.1	0.8 ± 0.1	3
0.5 to 0.6	0	0	3
0.6 to 0.7	3.5 ± 0.5	0	3
0.7 to 0.8	2.7 ± 0.3	0	3
0.8 to 0.9	0	0	3
0.9 to 1.0	0	0	3
Total	7.0	0.8	3

* samples obtained from one subject only.

Discussion.

Serum folates detected with L.casei are much higher than those assayed with S.faecalis and P.cerevisiae in both samples from subjects after normal lunch and those on starvation overnight (Table 4.4 and Table 4.5). Three folates can be detected in normal human sera (Ratanasthien et al 1974). 5-methyltetrahydropteroylglutamic acid is a major folate and 10-formylpteroylglutamic acid and 10-formyltetrahydropteroyl-glutamic acid the two minor folates. Additional studies of 5-methyltetrahydropteroylglutamic acid by microbiological assay with L.casei of serum and urine samples before and after acid treatment (to destroy the 5-methyl-5,6-dihydropteroyl-glutamic acid) indicated that small amount of 5-methyl-5,6-dihydropteroylglutamic acid was present in normal human serum and urine (Table 4.2.4).

Studies on the effect of conjugase on serum folate levels showed that both human and rat sera have no conjugated folate as there is no enhancement of the serum folate levels after conjugase treatment (Tables 4.3 and 4.11). This observation is in agreement with others (Herbert et al 1962;Noronha and Aboobaker,1963;Bird et al 1965) but not with those demonstrated by Banerjee and Chatterjea (1966). Heating samples at 115°C for three minutes in the presence of 2 mg/ml of ascorbic acid before aseptically addition into microbiological assay with L.casei (Herbert,1966) showed some lost in the microbiological activity (Table 4.6 and Figure 4.1). Studies on the effect of clotting time showed that there is little effect on the level of L.casei and none on the level of S.faecalis but marked effect

was seen on the level of P.cerevisiae (Table 4.12). This observation indicated that 10-formyltetrahydropteroylglutamic was oxidised to 10-formylpteroylglutamic acid which gave no effect on the S.faecalis activity (Chapter 2). The observation that 10-formylpteroylglutamic acid is an oxidation product of 10-formyltetrahydropteroylglutamic acid (Blair and Pearson,1974) was confirmed by the study of samples quickly separated which thus reduced the level of 10-formylpteroylglutamic acid to nearly zero (Table 4.2.3).

Studies of urinary folates indicated that in normal human daily excretion of folate are varied with the intake folate (Chanarin,1969). Our results showed that on normal daily diet urinary folates are about 4 to 13 ug/day (Table 4.7). Urine samples collected during 9.00 a.m. to 12.00 noon from subjects with and without overnight starvation and during collection showed little differences,those on normal diet excreted more than those on starvation (Table 4.9 and Table 4.10). This observation idicated the presence of basal urinary folate excretion. Analysis of urinary folates of normal human showed that 5-methylterahydropteroylglutamic acid is a major folate with 10-formylpteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid as minor folates (Table 4.2.5). The presence of 10-formyltetrahydropteroylglutamic acid was known (Albrecht and Broquist,1956). The detection of 10-formylpteroylglutamic acid in the urine is likely to be analytical artefact since the urine samples were collected in the presence of ascorbic acid but kept at room temperature or refrigerated during the collection period. The presence of 5-methyl-5,6-

dihydropteroylglutamic acid which is readily reduced *in vitro* to 5-methyltetrahydropteroylglutamic acid by various means i.e. ascorbic acid (Donaldson and Keresztesy,1962) indicated that this compound is not quickly reduced in human probably due to the presence of significant amounts of copper and oxygen in the body (Diem and Lentner,1971) a condition in which favoured the oxidation of 5-methyltetrahydropteroylglutamic acid to dihydropteroylglutamic acid as used for the preparation of this latter compound (Chapter 3).

Studies on the identification of serum and urine folates can be confirmed by the microbiological assay of the eluates from thin-layer chromatography (Table 4.2.1). This latter study indicates that there are atleast two distinguishable folates which are corresponding to 5-methyltetrahydropteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid and is also confirmed by the relationship of 5-methyltetrahydropteroylglutamic acid to the total folates (Figure 4.2.1). 5-methyltetrahydropteroylglutamic acid activity was seen at the Rf values much higher than usual at the proteins zone (Figure 4.4.1). The presence of 5-methyltetrahydropteroylglutamic acid in this zone indicates that it is binding with proteins (Ghitis,1967; Ford et al 1969;Ghitis et al 1969;Leslie and Rowe,1972;Metz et al 1968;Markkanen and Peltola,1971;Markkanen and Peltola,1970; Markkanen et al 1972;Waxman,1975). This is confirmed by the disappearance of the activity when deproteinized samples were used in comparing with neat samples (Table 4.4.1 and Table 4.4.2). The thin-layer chromatography of serum folate developed in 3 % aqeous ammonium chloride in the presence of 1 % ascorbic

acid showed 3 bands of purple colour of ninhydrin complex. The two minor bands are probably due to the ninhydrin complexes of folates which also contained primary aminogroups or due to the proteins-folate-ninhydrin complex (Mahler and Cordes,1969). From these studies it is possible to conclude that those folates of human serum and urine (Table 4.2.2 and Table 4.2.5) identified by aseptic addition methods of differential microbiological assays with L.casei,S.faecalis and P.cerevisiae are that 5-methyltetrahydropteroylglutamic acid is the combination of activities of true 5-methyltetrahydropteroylglutamic acid and that derived from 5-methyl-5,6-dihydropteroylglutamic acid and S.faecalis represents the true levels of 10-formyltetrahydropteroylglutamic acid.

Diurnal variation of liver enzyme activities concerning with folic acid and its derivatives metabolism was clearly demonstrated in the rat (Figure 4.3.1). Studies of serum folates analysed hourly showed a pattern of diurnal variation (Figures 4.3.5 and 4.3.6;Table 4.3.3 andTable 4.3.4). In man serum folates analysed hourly showed little variation (Figure 4.3.3) which may be due to the low serum level as compared with the rat. Average of results of total serum folates from more than 100 samples analysed weekly for the past years showed no seasonal variation (Figure 4.3.2). Studies of urinary folates excreted daily in two normal human subjects on similar diet showed a pattern of diurnal variation with 5-methyltetrahydropteroylglutamic acid excreted at a peak level in the morning (6.00 to to 9.00 a.m.) and 10-formyltetrahydropteroylglutamic acid showed little variation. The peak urinary 5-methyltetrahydropteroyl-

glutamic acid in man is corresponded with the high serum level of 5-methyltetrahydropteroylglutamic acid of the rat at the latter hours of resting peroid.

Diurnal variation of serum folate detected with L.casei of baboons were recently reported (Boots,Cornwell,and Beck,1975).These results showed that serum folate varied significantly over a 24-hour period with the mean level at 1800 hours significantly higher than at all other times. These results are in agreement with our studies with rats described here and humans.

Summary.

Four derivatives of folic acid can be detected in normal serum and urine, 5-methyltetrahydropteroylglutamic acid as the major folate, 5-methyl-5,6-dihydropteroylglutamic acid, 10-formyltetrahydropteroylglutamic acid and 10-formylpteroylglutamic acid as minor folates. 10-formyltetrahydropteroylglutamic acid was maintained at a constant level by a homeostatic mechanism and 5-methyltetrahydropteroylglutamic acid acted as a storage form. Studies in rats and man showed that there was a diurnal variation of serum folates and folates in the urine excretion. 10-formylpteroylglutamic acid is likely to be an analytical artefact and thus the level of 10-formyltetrahydropteroylglutamic acid is best represented by the level of the microbiological assay with S.faecalis. Urinary folates excreted daily ranges from 4.0 to 12.7 ug per day.

Chapter 5.

Oral folates

5.1 Metabolism of oral folates.

5.2 Stability of Pediococcus cerevisiae activity.

5.3 Pharmacokinetics analyses of serum folates after oral doses.

5.4 Dietary folates and folate requirement by man.

5.5 Evidence of storage forms.

5.1 Metabolism of oral folates in man.

Folate deficiency can caused by many reasons (Herbert, 1972).Laboratory methods are available for the diagnosis of folate deficiency before the onset of anaemia.Microbiological assays of serum and red cell folate with L.casei are widely used with precautions taken to protect the labile folate with ascorbic acid during assay (Toennies,Usdin and Philips,1956; Baker,Herbert,Frank,Pasher,Hutner,Wasserman and Sobotka,1959; Waters and Mollin,1961).These techniques are the extraction method using deproteinized serum (Waters and Mollin,1961,1963), and an aseptic addition technique (Herbert,,1961,1966).Histidine loading test (FIGLU excretion) is also widely used(Brit.Med.J., 1969).The production of folate deficiency can be easily produced in experimental animals simply by maintaining the animals on a predetermined low folate diet for a period of time and after 20 days folate in various tissues,serum,liver,kidney were reduced to about 20 percent of the control animals (Grossowicz et al 1964).The production of folate deficiency from normal human can also be done similarly to those of experimental animals. Herbert (1962) showed that on maintaining a healthy human subject on a diet from which folate content had been extracted by boiling in a large amount of water down to 5 ug/day.The level of serum folate fell to about 1 ng/ml after 3 weeks on diet and after 7 weeks blood pictures showed hypersegmentation of nuclei of polymorphs,and FIGLU excretion was raised after oral histidine loading test.After 4 months on the diet red cell folate was decreased and a week later the megaloblastic of bone marrow cells developed and the anaemic condition confirmed a few days later.

There are some other methods used for the assessment of folate defficiency.Chanarin et al (1958a,1958b) used the rate of plasma clearance for the determination of folate defficiency.They found that after injection of 15 ug/kg body weight of folic acid the clearance rate in patients with megaloblastic anaemia of pregnancy,leukemia and idiopathic steatorrhoea were faster than in normal subjects.The determination of leucocyte folate of patients with sub-normal serum folate levels were shown to be lower than of normal subjects at ranges 7 to 109 with mean value of 46 and 60 to 123 with mean value 92 ng/ml of packed cells,respectively (Hoffbrand and Newcombe,1967). Many other methods used in the detection of folate defficiency were well reviewed (Blakley,1969;Chanarin,1969).

Eventhough many methods are available they can not give any better understanding of the folate deficiency. In order to understand more about folate deficiency the metabolism of each individual oral folate should be well understood.The experimental studies in this chapter were set out for the metabolism of oral folates in normal humans.

Materials and Methods.

Folates were obtained from various sources as previously described in Chapter 3.Pteroylglutamic acid and its derivatives were used in these experiments. They were 10-formylpteroylglutamic acid,7,8-dihydropteroylglutamic acid,5-methyl-5,6-dihydropteroylglutamic acid,5-methyl-5,8-dihydropteroylglutamic acid, 5-methyltetrahydropteroylglutamic acid,4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid,5-formyltetrahydropteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid as 5,10-methenyltetrahydropteroylglutamic acid.These folates were checked for their purities by various means i.e. (a) by using microbiological assays with L.casei,S.faecalis,P.cerevisiae including their bioautography,(b) a study of ultraviolet spectra of these folates at pH 1.0,7.0 and 13.0 and (c) by using thin-layer chromatography in atleast 3 solvent systems (Table 3.1). Purities of folates used in this study were higher than 90 % and details were given in Chapter 3.

Normal human volunteers were fasted overnight and throughout the experimental periods.Before doses of 5 mg of microbiologically active materials or their equivalent were given these volunteers emptied their bladders.Venous blood samples were taken immediately before doses and $\frac{1}{2}$,1,$1\frac{1}{2}$,2,$2\frac{1}{2}$,3 and in some cases up to 6 hours.Blood specimens were drawn into a plastic syringe and immediately transferred into sterilized screw cap glass specimen containers and stood at room temperature for one-half to one hour.After centrifugation the sera were decanted into clean sample bottles,ascorbic acid (5 mg/ml) added and stored frozen at -20^{o}C until assayed.

Some samples were also used for the determination of 5-methyl-5,6-dihydropteroylglutamic acid and thus treated similarly to those samples in Chapter 4.

Urine samples were collected at the end of test periods and some were collected between the test periods and to 24 hours after doses.The volume of urine samples collected was noted and 5 mg/ml ascorbic acid added before they were kept frozen at -20°C until assayed.

Microbiological assays with L.casei,S.faecalis and P.cerevisiae were carried out within a week after samples collected. These microbiological assays were performed as previously described in Chapter 2 and bioautography with these microorganisms were used for the identification of folates in these samples as also described in Chapter 2.

Results.

After doses of 5 mg oral pteroylglutamic acid serum folate in 5 volunteers was microbiologically assayed and summarized in Table 5.1.1. In 4 normal human volunteers after 5 mg of 7,8-dihydropteroylglutamic acid serum folate is summarized in Table 5.1.2 and Table 5.1.3 is the summary of serum folate from 6 subjects after 5 mg of 10-formylpteroylglutamic acid. Serum folate of 6 subjects after 5 mg of 5-formyltetrahydropteroyl-glutamic acid is summarized in Table 5.1.4 and in 6 subjects after 5 mg of 5,10-methenyltetrahydropteroylglutamic acid is summarized in Table 5.1.5. After 5 mg of 5-methyltetrahydropteroyl-glutamic acid in 6 normal human volunteers is summarized in Table 5.1.6. In 5 normal human volunteers after 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid serum folate is summarized

in Table 5.1.7 and serum 5-methyl-5,6-dihydropteroylglutamic acid is summarized in Table 5.1.8. Serum folate of 3 normal human volunteers after 5 mg of 5-methyl-5,8-dihydropteroyl-glutamic acid is summarized in Table 5.1.9. and 3 subjects after 5 mg of 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroyl-glutamic acid,serum folate is summarized in Table 5.1.10. Serum folate of 4 normal human subjects taking 3 g sodium bicarbonate (10 to 20 minutes) before 5 mg 5-methyltetrahydro-pteroylglutamic acid is summarized in Table 5.1.11 and serum folate of 5 subjects taken 5 g sodium bicarbonate (10 to 20 minutes) before 5 mg 5-methyltetrahydropteroylglutamic acid is summarized in Table 5.1.12. Serum folate of 3 normal human subjects taking 3 g sodium bicarbonate (10 to 20 minutes) before 5 mg 5-methyl-5,6-dihydropteroylglutamic acid is summarized in Table 5.1.13. Three hours urine folate from these subjects taking various folate is summarized in Table 5.1.14.Urine folate from subjects taking 5 mg 5-methyltetrahydropteroylglutamic acid collected in intervals up to 6 hours is summarized in Table 5.1.15 and urine folate from subjects taking 5 mg 7,8-dihydropteroyl-glutamic acid collected in intervals up to twenty four hour after oral doses is summarized in Table 5.1.16.

Serum 5-methyl-5,6-dihydropteroylglutamic acid of subjects after 5 mg of 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydro-pteroylglutamic acid is shown in Table 5.1.17 and Table 5.1.18 is the summary of 5-methyl-5,6-dihydropteroylglutamic acid after 5 mg of 7,8-dihydropteroylglutamic acid orally.Table 5.1.19 and Table 5.1.21 are the serum 5-methyl-5,6-dihydropteroylglutamic acid after 3 g sodium bicarbonate (10 to 20 minutes) and 5 mg of 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydro-

pteroylglutamic acid,respectively.Serum 5-methyl-5,6-dihydro-pteroylglutamic acid of subjects taking 5 g sodium bicarbonate (10 to 20 minutes) before taking 5 mg of 5-methyltetrahydro-pteroylglutamic acid is summarized in Table 5.1.20.

The distribution of urinary folates of 2 subjects taking 5 mg of 7,8-dihydropteroylglutamic acid throughout the twenty four hour after the test dose is shown in Figure 5.1.1. Serum 5-methyl-5,6-dihydropteroylglutamic acid from subjects taking various folates are summarized in Table 5.1.22.

Table 5.1.1 Serum folates of 5 normal human subjects (mean±S.E.M.) after 5 mg of pteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis) *** 10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	8.5±1.60	7.3±1.60	0.40±00.10	0.80± 0.10
½	44.0±20.0	16.0±4.50	27.12±14.90*	0.88± 0.10
1	133.0±31.0	39.0±10.0	93.04±34.00*	0.96± 0.10
1½	180.0±35.0	50.0± 8.5	129.04±40.00*	0.96± 0.10
2	200.0±50.0	60.0±26.0	139.12±39.90*	0.88± 0.10
3	226.0±43.0	68.0±20.0	157.02±35.70*	0.98± 0.10

* indicates sample is pteroylglutamic acid when confirmed with bioautography with L.casei,S.faecalis and P.cerevisiae.

** indicates summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

*** S.faecalis = 10-CHO PteGlu + P.cerevisiae.

Table 5.1.2 Serum folates (mean±S.E.M.) of 4 normal human subjects after 5 mg of 7,8-dihydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu ** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis) *	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
1	5.9 ± 0.7	5.2 ± 0.7	0.10 ± 0.03	0.60 ± 0.03
½	132.5 ±18.8	129.9 ±18.2	1.10 ± 0.10	1.50 ± 0.09
1	200.0 ±28.6	195.6 ±26.7	2.60 ± 0.30	1.80 ± 0.21
1½	260.0 ±27.4	257.0 ±27.2	1.20 ± 0.10	1.80 ± 0.15
2	210.0 ±22.7	207.0 ±23.4	1.50 ± 0.10	1.50 ± 0.23
3	122.5 ±20.9	121.3 ±21.0	0.10 ± 0.10	1.10 ± 0.19

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.3 Serum folates (mean ± S.E.M.) of 6 normal human subjects after 5 mg of 10-formylpteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu*** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)**	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	5.2 ± 0.3	4.4 ± 0.4	0.2 ± 0.08	0.6 ± 0.08
½	115.0 ± 35.1	-1.0 ± 3.3	115.0 ± 33.15@	1.0 ± 0.07
1	182.0 ± 37.0	-4.7 ± 4.4	185.5 ± 34.28@	1.2 ± 0.08
1½	185.0 ± 15.0	3.6 ± 13.2	180.0 ± 6.63@	1.4 ± 0.08
2	118.0 ± 16.0	13.6 ± 9.9	103.3 ± 12.06@	1.1 ± 0.10
3	71.0 ± 9.2	9.0 ± 6.2	60.7 ± 5.04@	1.3 ± 0.20
4*	46.0 ± 0.0	4.9 ± 0.0	40.2 ± 0.00@	0.9 ± 0.00
5*	40.0 ± 0.0	4.0 ± 0.0	35.1 ± 0.00@	0.9 ± 0.00

* indicates result of only one subject in the test.

** S.faecalis = 10-CHO PteGlu + P.cerevisiae.

*** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

@ confirmed as 10-CHO PteGlu by bioautography.

Table 5.1.4 Serum folates (mean ± S.E.M.) of 6 normal human subjects after 5 mg of 5-formyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	10.4 ± 2.4	9.6 ± 2.5	0.2 ± 0.05	0.6 ± 0.10
½	96.3 ± 23.9	90.0 ± 21.0	4.4 ± 1.00	1.9 ± 0.60
1	187.2 ± 26.4	182.0 ± 25.3	3.2 ± 0.60	2.0 ± 0.65
1½	287.0 ± 39.1	282.0 ± 39.0	3.2 ± 0.45	1.8 ± 0.50
2	199.0 ± 16.4	194.0 ± 16.0	3.3 ± 0.50	1.7 ± 0.24
3	135.0 ± 8.7	132.0 ± 10.0	1.7 ± 0.30	1.3 ± 0.14

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.5 Serum folates (mean ± S.E.M.) of 6 normal human subjects after 5 mg of 5,10-methenyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	6.7 ± 1.5	5.9 ± 1.5	0.2 ± 0.05	0.6 ± 0.20
½	74.0 ± 11.9	61.0 ± 10.7	12.0 ± 2.31	1.0 ± 0.30
1	194.0 ± 17.3	175.0 ± 16.8	17.9 ± 4.26	1.1 ± 0.18
1½	292.0 ± 28.4	270.0 ± 27.1	20.9 ± 5.06	1.1 ± 0.20
2	325.0 ± 45.0	313.0 ± 37.5	10.9 ± 6.55	1.1 ± 0.20
3	214.0 ± 38.9	200.0 ± 38.1	13.0 ± 1.90	1.0 ± 0.20
4	130.0 ± 19.7	122.5 ± 18.4	6.6 ± 1.09	0.9 ± 0.19
5	114.0 ± 11.7	107.4 ± 11.0	5.6 ± 0.62	1.0 ± 0.30

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.6 Serum folates (mean ± S.E.M.) of 6 normal human subjects after 5 mg of 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu*** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)**	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	9.1 ± 2.1	8.3 ± 2.3	0.2 ± 0.05	0.6 ± 0.10
½	106.0 ± 15.2	103.1 ± 16.0	2.4 ± 0.50	0.5 ± 0.10
1	166.0 ± 21.0	162.2 ± 21.6	3.1 ± 0.50	0.7 ± 0.10
1½	188.0 ± 19.0	184.3 ± 21.0	3.1 ± 0.50	0.6 ± 0.10
2	180.0 ± 14.7	175.4 ± 14.7	3.8 ± 0.49	0.8 ± 0.09
3	110.0 ± 9.4	106.3 ± 9.4	3.0 ± 0.45	0.7 ± 0.09
4	81.0 ± 9.8	77.5 ± 10.6	2.8 ± 0.45	0.7 ± 0.10
5	55.8 ± 5.2	52.4 ± 5.8	2.7 ± 0.50	0.7 ± 0.10
6*	41.0 ± 0.0	37.8 ± 0.0	2.5 ± 0.00	0.7 ± 0.00

* indicates result derived from only one subject.

** S.faecalis = 10-CHO PteGlu + P.cerevisiae.

*** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.7 Serum folates (mean ± S.E.M.) of 5 normal human subjects after 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu*** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)**	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	6.2 ± 1.3	5.5 ± 1.3	0.1 ± 0.05	0.6 ± 0.2
½	37.6 ± 5.9	36.6 ± 5.9	0.1 ± 0.06	0.9 ± 0.2
1	32.8 ± 4.5	31.6 ± 4.6	0.2 ± 0.10	1.0 ± 0.2
1½	27.8 ± 2.0	26.8 ± 2.0	0.1 ± 0.05	0.9 ± 0.2
2	23.6 ± 0.9	22.7 ± 0.9	0.1 ± 0.05	0.8 ± 0.2
3	21.8 ± 0.8	21.1 ± 0.2	0.1 ± 0.02	0.6 ± 0.1
4*	21.0 ± 0.0	20.0 ± 0.0	0.0 ± 0.00	1.0 ± 0.0
6*	20.0 ± 0.0	19.0 ± 0.0	0.0 ± 0.00	1.0 ± 0.0

* indicates result derived from only one subject.

** S.faecalis = 10-CHO PteGlu + P.cerevisiae.

*** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.8 Serum 5-methyl-5,6-dihydropteroylglutamic acid in a normal human subject after taking 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid orally.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		$5\text{-}CH_3\text{-}5,6\text{-}H_2PteGlu$
	Before acid and base treatment(A)	After acid and base treatment(B)	(A)-(B) (ng/ml ± S.E.M.)
0	10.0	10.0	0.0
$\frac{1}{2}$	32.0	30.0	2.0
1	25.0	24.0	1.0
$1\frac{1}{2}$	23.0	22.0	1.0
2	22.0	21.0	1.0
3	22.0	21.0	1.0
4	21.0	20.5	0.5
6	20.0	19.5	0.5

Table 5.1.9 Serum folates (mean ± S.E.M.) of 3 normal human subjects after 5 mg of 5-methyl-5,8-dihydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	7.3 ± 3.0	6.8 ± 3.0	0.0 ± 0.00	0.5 ± 0.1
½	7.7 ± 2.7	7.0 ± 2.8	0.1 ± 0.00	0.6 ± 0.1
1	8.2 ± 2.7	7.3 ± 2.8	0.3 ± 0.10	0.6 ± 0.1
1½	8.3 ± 2.8	7.5 ± 2.7	0.3 ± 0.10	0.5 ± 0.1
2	7.9 ± 3.1	7.3 ± 3.0	0.1 ± 0.10	0.5 ± 0.1
3	7.2 ± 3.6	6.6 ± 3.5	0.1 ± 0.10	0.5 ± 0.1

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.10 Serum folates (mean ± S.E.M.) of 3 normal human subjects after 5 mg of 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	11.8 ± 6.7	11.2 ± 6.7	0.0 ± 0.0	0.6 ± 0.0
½	11.5 ± 6.6	10.8 ± 6.7	0.1 ± 0.1	0.6 ± 0.0
1	10.8 ± 6.0	10.1 ± 6.0	0.1 ± 0.1	0.6 ± 0.0
1½	10.6 ± 7.0	10.0 ± 7.0	0.0 ± 0.0	0.6 ± 0.0
2	10.8 ± 7.1	10.2 ± 7.1	0.0 ± 0.0	0.6 ± 0.0
2½	11.8 ± 7.3	11.2 ± 7.3	0.0 ± 0.0	0.6 ± 0.0
3	11.8 ± 7.3	11.2 ± 7.3	0.0 ± 0.0	0.6 ± 0.0

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.11 Serum folates (mean ± S.E.M.) of 4 normal human subjects after 3 g sodium bicarbonate for 10 to 20 minutes and 5 mg of 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)**	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	7.5 ± 2.2	7.0 ± 2.0	0.0 ± 0.0	0.5 ± 0.1
½	75.0 ± 7.6	72.2 ± 7.9	1.8 ± 1.6	1.0 ± 0.3
1	155.0 ± 21.8	149.6 ± 22.0	3.9 ± 2.0	1.5 ± 0.3
1½	176.7 ± 24.8	170.0 ± 25.0	5.0 ± 2.0	1.7 ± 0.4
2	147.5 ± 28.7	141.0 ± 29.0	5.0 ± 2.9	1.5 ± 0.5
3	106.0 ± 29.0	101.5 ± 29.9	3.1 ± 1.9	1.4 ± 0.5

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

** S.faecalis = 10-CHO PteGlu + P.cerevisiae.

Table 5.1.12 Serum folates (mean ± S.E.M.) of 5 normal human subjects after 5 g sodium bicarbonate (10 to 20 minutes) and 5 mg of 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	8.0 ± 3.0	7.3 ± 3.1	0.05 ± 0.0	0.65 ± 0.05
½	172.0 ± 18.8	170.1 ± 19.0	0.44 ± 0.1	1.46 ± 0.46
1	246.0 ± 19.4	242.8 ± 19.7	1.00 ± 0.2	2.20 ± 0.53
1½	259.0 ± 18.5	255.3 ± 18.8	1.80 ± 0.2	1.90 ± 0.54
2	220.0 ± 22.6	217.3 ± 22.9	1.14 ± 0.2	1.56 ± 0.36
3	145.0 ± 23.2	143.4 ± 23.5	0.50 ± 0.1	1.10 ± 0.22

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.13 Serum folates (mean ± S.E.M.) of 3 normal human subjects after 3 g of sodium bicarbonate (10 to 20 minutes) and 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu** (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)@	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	8.8 ± 3.0	8.0 ± 3.0	0.1 ± 0.0	0.7 ± 0.1
½	19.0 ± 1.0	18.2 ± 1.0	0.0 ± 0.0	0.8 ± 0.1
1	51.6 ± 3.4	50.2 ± 4.0	0.4 ± 0.2	1.0 ± 0.1
1½	112.3 ± 10.0	109.9 ± 10.2	1.1 ± 0.1	1.3 ± 0.2
2	99.3 ± 12.0	96.4 ± 12.4	1.4 ± 0.3	1.5 ± 0.4
3	51.3 ± 6.0	48.8 ± 6.4	1.3 ± 1.0	1.2 ± 0.5
4½*	45.0 ± 0.0	43.5 ± 0.0	0.5 ± 0.0	1.0 ± 0.0
6*	32.0 ± 0.0	30.8 ± 0.0	0.3 ± 0.0	0.9 ± 0.0

* indicates result derived from only one subject.

** indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

@ S.faecalis = 10-CHO PteGlu + P.cerevisiae.

Table 5.1.14 Three hour urine folates (mean ± S.E.M.) of normal human subjects after oral administration of 5 mg doses of various folates.

Administered folates	No. of subjects	Urine folates (ug ± S.E.M.)		
		5-CH_3H_4PteGlu	10-CHO PteGlu	10-$CHOH_4$PteGlu
Fasted subjects	14	0.98± 0.2	0.38± 0.05	0.05± 0.01
PteGlu	4	100.00± 30.0	533.20±240.0*	16.80±10.32
10-CHO PteGlu	3	53.10± 21.4	259.30± 41.4@	10.60± 2.09
7,8-H_2PteGlu	2	1202.50±182.0	46.90± 5.00@	38.60± 7.95
5-CH_3-5,6-H_2PteGlu	3	50.30± 10.8	10.10± 4.10@	1.60± 1.02
5-CH_3-5,8-H_2PteGlu	3	1.84± 0.4	0.22± 0.03	0.04± 0.00
5-CH_3H_4PteGlu	2	222.00± 22.5	24.00±10.35@	12.00± 0.05
5-CHO H_4PteGlu	6**	282.30± 48.4	85.87±23.59@	39.00±14.07
5,10-CH=H_4PteGlu	5	706.86± 79.3	107.02±20.00@	45.52± 6.66
4a(OH)-5-CH_3H_4PteGlu	3	1.35± 0.5	0.38± 0.04	0.10± 0.00
5-CH_3-5,6-H_2PteGlu	2***	295.00± 40.5	13.68± 5.30@	1.32± 1.08
5-CH_3H_4PteGlu	3***	656.00± 73.4	26.80±13.20@	5.20± 3.08

* indicates results confirmed by bioautography as pteroylglutamic acid,

** indicates urine samples collected for two hours only and

*** indicates results from subjects taken 3 g sodium bicarbonate 10 to 20 minutes before administration of test folate.

@ results confirmed by bioautography as 10-CHO PteGlu.

Table 5.1.15 Urine folates (mean ± S.E.M.) of 2 normal human subjects collected in periods up to 6 hours after doses of 5 mg of 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ug) ±S.E.M.	5-CH_3H_4PteGlu** (ug) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ug) ±S.E.M.	P.cerevisiae (ug) ±S.E.M.
0 to 1	55.0 ± 4.9	49.5 ± 3.3	3.0 ± 0.95	2.5 ± 0.48
1 to 2	75.0 ± 0.9	67.0 ± 2.9	5.0 ± 0.95	3.0 ± 0.95
2 to 3	127.5 ±26.5	104.0 ±22.0	16.0 ± 7.60	6.5 ± 1.40
3 to 6	140.0 ±56.0	122.7 ±53.0	8.8 ± 0.72	5.6 ± 4.68

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.16 Urine folates (mean ± S.E.M.) collected from 2 subjects taking 5 mg of 7,8-dihydropteroylglutamic acid in periods until 24 hour after doses.

Time after doses (hour)	Total folate (ug) ±S.E.M.	5-CH_3H_4PteGlu** (ug) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)*	
			10-CHO PteGlu (ug) ±S.E.M.	P.cerevisiae (ug) ±S.E.M.
0 to 1½	660 ± 60.0	619.5 ± 61.1	20.7 ± 9.4	19.8 ± 4.0
1½ to 3	628 ±116.0	583.0 ±111.0	26.2 ± 1.2	18.8 ± 3.8
3 to 6	254 ± 47.5	248.2 ± 44.0	5.5 ± 0.9	0.3 ± 0.0
6 to 9	100 ± 3.8	95.8 ± 5.5	3.9 ± 1.5	0.3 ± 0.0
9 to 13	61 ± 1.0	60.4 ± 1.0	0.6 ± 0.0	0.0 ± 0.0
13 to 24	5.5 ± 0.5	5.3 ± 0.5	0.0 ± 0.0	0.2 ± 0.0

* S.faecalis = 10-CHO PteGlu + P.cerevisiae.

** indicates the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 5.1.17 Serum 5-methyl-5,6-dihydropteroylglutamic acid in 2 normal human subjects after taking 5 mg 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid orally.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		$5\text{-}CH_3\text{-}5,6\text{-}H_2PteGlu$
	Before acid and base treatment(A)	After acid and base treatment(B)	(A) - (B) (ng/ml ± S.E.M.)
0	9.2 ± 5.7	8.2 ± 5.7	1.0 ± 0.0
½	9.2 ± 5.7	6.0 ± 3.5	3.2 ± 2.2
1	8.8 ± 5.3	6.0 ± 3.5	2.8 ± 1.8
1½	6.8 ± 3.3	5.8 ± 3.3	1.0 ± 0.0
2	6.8 ± 3.3	6.2 ± 3.8	0.6 ± 0.5
3	7.8 ± 4.2	7.2 ± 4.8	0.6 ± 0.5

Table 5.1.18 Serum 5-methyl-5,6-dihydropteroylglutamic acid in 2 normal human subjects after taking 5 mg of 7,8-dihydropteroyl-glutamic acid orally.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		$5\text{-}CH_3\text{-}5,6\text{-}H_2PteGlu$
	Before acid and base treatment(A)	After acid and base treatment(B)	(A) - (B) (ng/ml ± S.E.M.)
0	6.7 ± 1.3	6.7 ± 1.3	0
½	110.0 ± 30.0	110.0 ± 30.0	0
1	175.0 ± 55.0	175.0 ± 55.0	0
1½	280.0 ± 10.0	280.0 ± 10.0	0
2	245.0 ± 25.0	245.0 ± 25.0	0
3	145.0 ± 5.0	145.0 ± 5.0	0

Table 5.1.19 Serum 5-methyl-5,6-dihydropteroylglutamic acid in 4 normal human subjects after taking 3 g sodium bicarbonate (10 to 20 minutes) before taking 5 mg of 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		$5\text{-}CH_3\text{-}5,6\text{-}H_2PteGlu$
	Before acid and base treatment(A)	After acid and base treatment(B)	(A) - (B) (ng/ml ± S.E.M.)
0	7.5 ± 2.2	4.5 ± 4.3	3.0 ± 2.1
½	75.0 ± 7.6	68.0 ± 10.3	17.0 ± 2.7
1	155.0 ± 21.8	107.0 ± 39.0	48.0 ± 17.3
1½	176.7 ± 24.8	102.7 ± 64.8	74.0 ± 40.0
2	147.5 ± 28.7	82.5 ± 60.2	65.0 ± 31.5
3	106.0 ± 29.0	56.0 ± 12.3	50.0 ± 16.7

These results are set out in the following table.

Subject number	Serum $5\text{-methyl-}5,6\text{-}H_2PteGlu$ (ng/ml)					
	$T_{0(h)}$	$T_{\frac{1}{2}(h)}$	$T_{1(h)}$	$T_{1\frac{1}{2}(h)}$	$T_{2(h)}$	$T_{3(h)}$
1	9.0	22.0	35.0	38.0	37.0	33.0
2	0.0	10.0	30.0	74.0	65.0	55.0
3	0.0	15.0	28.0	28.0	30.0	18.0
4	3.0	20.0	100.0	155.0	128.0	95.0

Table 5.1.20 Serum 5-methyl-5,6-dihydropteroylglutamic acid in 5 normal human subjects after taking 5 g sodium bicarbonate (10 to 20 minutes) before taking 5 mg of 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		5-CH_3-5,6-H_2PteGlu
	Before acid and base treatment(A)	After acid and base treatment(B)	(A) - (B) (ng/ml±S.E.M.)
0	8.0 ± 3.0	7.1 ± 3.3	0.9 ± 0.3
½	172.0 ± 18.8	102.0 ± 47.6	70.0 ± 28.8
1	246.0 ± 19.4	160.0 ± 60.1	86.0 ± 40.7
1½	259.0 ± 18.5	143.6 ± 61.5	115.4 ± 43.0
2	220.0 ± 22.6	145.0 ± 54.3	75.0 ± 31.7
3	145.0 ± 23.2	91.0 ± 51.4	54.0 ± 28.2

These results are set out in the following table.

Subject number	Serum 5-methyl-5,6-H_2PteGlu(ng/ml)					
	$T_{0(h)}$	$T_{\frac{1}{2}(h)}$	$T_{1(h)}$	$T_{1\frac{1}{2}(h)}$	$T_{2(h)}$	$T_{3(h)}$
1	1.0	110.0	180.0	200.0	195.0	68.0
2	1.0	160.0	190.0	220.0	60.0	0.0
3	0.4	48.0	5.0	20.0	40.0	155.0
4	0.0	0.0	30.0	120.0	70.0	40.0
5	2.0	32.0	25.0	17.0	10.0	5.0

Table 5.1.21 Serum 5-methyl-5,6-dihydropteroylglutamic acid in a normal human subject after taking 3 g sodium bicarbonate (10 to 20 minutes) before taking 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid orally.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		$5\text{-}CH_3\text{-}5,6\text{-}H_2PteGlu$
	Before acid and base treatment(A)	After acid and base treatment(B)	(A) - (B) (ng/ml ± S.E.M.)
0	5.0	4.5	0.5
½	18.0	9.0	11.0
1	55.0	37.0	18.0
1½	117.0	84.0	33.0
2	114.0	88.0	26.0
3	60.0	50.0	10.0

Table 5.1.22

Serum 5-methyl-5,6-dihydropteroylglutamic acid of subjects taking 5 mg doses of various folates orally. Those results with * and ** were derived from subjects taking 3 and 5 g sodium bicarbonate (10 to 20 minutes) before the test folate.

Administered folate	No. of Subjects	5-CH_3-5,6-H_2PteGlu(ng/ml ± S.E.M.) at time (h)					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
5-CH_3-5,6-H_2PteGlu	1	0.0±0.0	2.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
5-CH_3-5,6-H_2PteGlu	1*	0.5±0.0	11.0±0.0	18.0±0.0	33.0±0.0	26.0±0.0	10.0±0.0
4a(OH)-5-CH_3H_4PteGlu	2	1.0±0.0	3.2±2.2	2.8±1.8	1.0±0.0	0.6±0.5	0.6±0.5
7,8-H_2PteGlu	2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5-CH_3H_4PteGlu	4*	3.0±2.1	17.0±2.7	48.0±17.3	74.0±40.0	65.0±31.5	50.0±16.7
5-CH_3H_4PteGlu	5**	0.9±0.3	70.0±28.8	86.0±40.7	115.4±43.0	75.0±31.7	54.0±28.2

Table 5.1.23

Serum folate (mean ± S.E.M.) of normal human subjects after 5 mg oral doses of various folates as detected by P.cerevisiae.

Administered folate	No. of Subjects	P.cerevisiae (ng/ml) at time (h) after doses					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
PteGlu	5	0.8±0.1	0.88±0.10	0.96±0.10	0.96±0.10	0.88±0.10	0.98±0.10
10-CHO PteGlu	6	0.6±0.08	1.00±0.07	1.20±0.08	1.40±0.08	1.10±0.10	1.30±0.20
7,8-H_2PteGlu	4	0.6±0.03	1.50±0.09	1.80±0.21	1.80±0.15	1.50±0.23	1.10±0.19
5-CHO H_4PteGlu	6	0.6±0.1	1.90±0.6	2.00±0.65	1.80±0.50	1.70±0.24	1.30±0.14
5,10-CH=H_4PteGlu	6	0.6±0.2	1.00±0.30	1.10±0.18	1.10±0.20	1.10±0.20	1.00±0.20
5-CH_3H_4PteGlu	6	0.6±0.1	0.50±0.10	0.70±0.10	0.60±0.10	0.80±0.09	0.70±0.09
5-CH_3-5,6-H_2PteGlu	5	0.6±0.2	0.90±0.20	1.00±0.20	0.90±0.20	0.80±0.20	0.60±0.10
5-CH_3-5,8-H_2PteGlu	3	0.5±0.1	0.60±0.10	0.60±0.10	0.50±0.10	0.50±0.10	0.50±0.10
4a(OH)-5-CH_3H_4PteGlu	3	0.6±0.0	0.60±0.00	0.60±0.00	0.60±0.00	0.60±0.00	0.60±0.00
Average	9	0.6±0.02	1.00±0.15	1.10±0.17	1.10±0.17	1.00±0.13	0.90±0.10

Table 5.1.24

Serum folate (mean ± S.E.M.) of normal human subjects after 5mg oral doses of various folates as detected by S.faecalis.

Administered folate	No. of Subjects	S.faecalis (ng/ml) at time (h) after doses					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
PteGlu	5	1.2±0.2	28.0±15.0 ●	94.0±34.1 ●	130.0±40.1 ●	140.0±40.0 ●	158.0±35.8 ●
10-CHO PteGlu	6	0.8±0.1	116.0±33.2 o	186.7±34.4 o	181.4± 6.7 o	104.4±12.2 o	62.0± 5.2 o
7,8-H_2PteGlu	4	0.7±0.1	2.6± 0.2	4.4± 0.5	3.0± 0.2	3.3± 0.3	1.2± 0.3
5-CHO H_4PteGlu	6	0.8±0.1	6.3± 1.6	5.2± 1.2	5.0± 0.9	5.0± 0.7	3.0± 0.4
5,10-CH=H_4PteGlu	6	0.8±0.2	13.0± 2.6	19.0± 4.4	22.0± 5.3	12.0± 6.8	14.0± 2.1
5-CH_3H_4PteGlu	6	0.8±0.1	2.9± 0.6	3.8± 0.6	3.7± 0.6	4.6± 0.6	3.7± 0.5
5-CH_3-5,6-H_2PteGlu	5	0.7±0.2	1.0± 0.3	1.2± 0.3	1.0± 0.2	0.9± 0.2	0.7± 0.1
5-CH_3-5,8-H_2PteGlu	3	0.5±0.1	0.7± 0.1	0.9± 0.2	0.8± 0.2	0.6± 0.2	0.6± 0.2
4a(OH)-5-CH_3H_4PteGlu	3	0.6±0.0	0.7± 0.1	0.7± 0.1	0.6± 0.0	0.6± 0.0	0.6± 0.0

● = PteGlu and o = 10 CHO PteGlu as confirmed by bioautography.

Discussion.

In normal humans oral administration of folates is readily absorbed and metabolised (Butterworth et al 1957; Johns and Bertino,1965;Perry and Chanarin,1970;Brown et al 1973a; Ratanasthien et al 1974).Oral administration of 5 mg of 5-methyltetrahydropteroylglutamic acid to normal humans is well absorbed and enters into blood levels mainly as 5-methyltetrahydropteroylglutamic acid itself (Table 5.1.6).There are relatively small changes in levels of 10-formylpteroylglutamic acid and practically stable in the levels of 10-formyltetrahydropteroylglutamic acid after oral administration of 5 mg 5-methyltetrahydropteroylglutamic acid.Studies in experimental animals indicated that intestinal absorption of 5-($^{14}CH_3$)-tetrahydropteroylglutamic acid were followed by metabolism of the compound.The methyl group being rapidly lost and a small amount exhaled as $^{14}CO_2$ (Beavon,1973).Eventhough the methyl group was rapidly lost the major folate in blood is 5-methyltetrahydropteroylglutamic acid and this evidence suggested that the administered compound is being metabolised.In normal human 5-methyltetrahydropteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid are in equilibration by means of homeostatic balance (Blair et al 1974a;Ratanasthien et al 1974) as also discussed in Chapter 4. The presence of 10-formylpteroylglutamic acid after oral administration of 5-methyltetrahydropteroylglutamic acid may infact derived from the administered compound itself according to the homeostatic mechanism in Scheme 4.1 by oxidation of 10-formyltetrahydropteroylglutamic acid (Blair and Pearson,1974). The levels of 10-formyltetrahydropteroylglutamic acid were represented by the levels of P.cerevisiae microbiological assays

but the true levels before oxidation may be best represented by the results from S.faecalis assays.The use of S.faecalis levels for 10-formyltetrahydropteroylglutamic acid must be used with great caution since the difference between S.faecalis and P.cerevisiae may also indicate the presence of pteroylglutamic acid as seen in cases of serum samples from subjects taken pteroylglutamic acid orally which will be discussed later on.

5-methyl-5,6-dihydropteroylglutamic acid is converted to 5-methyltetrahydropteroylglutamic acid in conventional microbiological assay and thus active for supporting only the growth of L.casei and not S.faecalis and P.cerevisiae.Omitting ascorbic acid in the assay media 5-methyl-5,6-dihydropteroylglutamic acid showed no activity in all microbiological assays as demonstrated in Chapter 3. Oral administration of 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid is readily absorbed and entered blood levels mainly identified as 5-methyltetrahydropteroylglutamic acid (Table 5.1.7).A much lower peak level is seen when compared with the level after 5-methyltetrahydropteroylglutamic acid and this is simply explained by the acidic conversion of 5-methyl-5,6-dihydropteroylglutamic acid by acid in stomach to 5-methyl-5,8-dihydropteroylglutamic acid which does not enter folate pool as seen in Table 5.1.9.(Blair et al 1974).

Studies of oral administrations of 5 mg doses of 5-formyltetrahydropteroylglutamic acid and 5,10-methenyltetrahydropteroylglutamic acid showed that they are well absorbed and entered serum levels mainly as 5-methyltetrahydropteroylglutamic acid (Table 5.1.4 and Table 5.1.5,respectively).Eventhough 5-formyltetrahydropteroylglutamic acid and 5,10-methenyltetrahydropteroylglutamic acid were both microbiologically active for

all three test microorganisms they clearly entered blood levels with the loss in the ability to support the growth of S.faecalis and P.cerevisiae.Higher levels of 10-formylpteroylglutamic acid seen in subjects taken 5 mg of 5,10-methenyltetrahydropteroylglutamic acid were by oxidation of 10-formyltetrahydropteroylglutamic acid in the jejunum before absorption (Beavon and Blair,1972;Blair and Pearson,1974;Ratanasthien et al 1974).The effective absorption and utilization of formyltetrahydropteroylglutamic acid had been well demonstrated (Perry and Chanarin,1970;Nixon and Bertino,1972;Brown et al 1973a; Ratanasthien et al 1974).

4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid does not support the growth of the three test microorganisms (Chapter 3).Oral administration of 5 mg of this compound did not enter folate pool as seen in Table 5.1.10.It is not clear if this compound were absorbed but using column chromatography and ultraviolet spectroscopy in dicates that some of the administered 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid was present in urine collected 3 hours after the dose (Figure 5.1).

Oral administration of pteroylglutamic acid is followed by partial metabolism(Table 5.1.1)whilst after oral administration 7,8-dihydropteroylglutamic acid is quickly metabolised and enters blood levels mainly as 5-methyltetrahydropteroylglutamic acid (5.1.2).Both pteroylglutamic acid and 7,8-dihydropteroylglutamic acid are substrates in the dihydrofolate reductase reaction (Tzortzatou and Hayhoe,1974) but the latter is involved in a much faster reaction.The reaction of dihydrofolate reductase and folates are shown below.

$$PteGlu + NADPH^{+} \xrightarrow{PteGlu\ redutase} H_2PteGlu + NADP^{+} \quad \text{(Step 1)}$$

This is a very slow reaction.

$$H_2PteGlu + NADPH^{+} \xrightarrow{H_2PteGlu\ reductase} H_4PteGlu + NADP^{+} \quad \text{(Step 2)}$$

This is a fast reaction.

From this evidence it is certain that folate was firstly reduced before any further metabolism.Methylation of the reduced folate is the product of metabolism of the oral folates and only 5-methyltetrahydropteroylglutamic acid had been known (Baker et al 1965; Chanarin and Perry,1969; Perry and Chanarin,1970;Pratt and Cooper,1971;Nixon and Bertino, 1972;Ratanasthien et al 1974).The mechanism and site of the methylation are not clearly understood.The formylation of folate had been claimed after incubation of pteroylglutamic acid in the mucosal side of everted sac of rat gut (Perry and Chanarin, 1973).They showed that in the serosal side after incubation the everted sac of rat gut with pteroylglutamic acid,10-formyl-pteroylglutamic acid,5-methyltetrahydropteroylglutamic acid and pteroylglutamic acid were found.They concluded that 10-formyl-pteroylglutamic acid or formylation of folate is the step before further reduction to the methylfolate and this is because of their failure to recognise the tissue folates as pointed out by Blair and his co-workers (Blair and Beavon,1973;Blair et al 1973). The formylfolate is infact demonstrated to be derived from the tissue folates by (Blair et al 1973).

Studies of oral administration of 5 mg of 10-formyl-pteroylglutamic acid indicated that it is readily absorbed but entered blood level unchanged as shown in Table 5.1.3.

Studies of metabolism of oral folates after subjects taking 3 to 5 g sodium bicarbonate (10 to 20 minutes before the test were performed.On those subjects taking 3 g sodium bicarbonate and 5 mg of 5-methyl-5,6-dihydropteroylglutamic acid serum folate was enhanced up to about 4 times of the same dose of folate without sodium bicarbonate (Table 5.1.13).These results confirmed that 5-methyl-5,6-dihydropteroylglutamic acid is readily absorbed and the low peak levels are caused by rearrangement to inactive folate (5-methyl-5,8-dihydropteroylglutamic acid) in normal human stomach. In subjects taking 3 g sodium bicarbonate and 5 mg 5-methyltetrahydropteroylglutamic acid serum folate (Table 5.1.11) did not significantly change from Table 5.1.6 but when 5 g of sodium bicarbonate was used serum folate (Table 5.1.12) was significantly increased.The enhancement of serum folates in the later test may be derived from the fact that the administered folate is more available at higher pH.

In the previous Chapter serum 5-methyltetrahydropteroylglutamic acid is partially derived from 5-methyl-5,6-dihydropteroylglutamic acid and a small quantity of 5-methyl-5,6-dihydropteroylglutamic acid (0.75 ± 0.15 ng/ml) is seen in normal human.Serum 5-methyl-5,6-dihydropteroylglutamic acid from subjects taken various folates were determined as shown in the results section or as summarized in Table 5.1.22.Serum 5-methyl-5,6-dihydropteroylglutamic acid in subjects taken sodium bicarbonate before folate administration orally were significantly raised.The increase in serum 5-methyl-5,6-dihydropteroylglutamic acid in these subjects indicated that the metabolism of folates took place at the gut and the enzymic system or systems at low pH is in favour of producing

5-methyltetrahydropteroylglutamic acid and at higher pH it is in favour of producing 5-methyl-5,6-dihydropteroylglutamic acid.

The metabolism of these folates were also confirmed by microbiologically analysis of the urinary folates as summarized in Table 5.1.14.

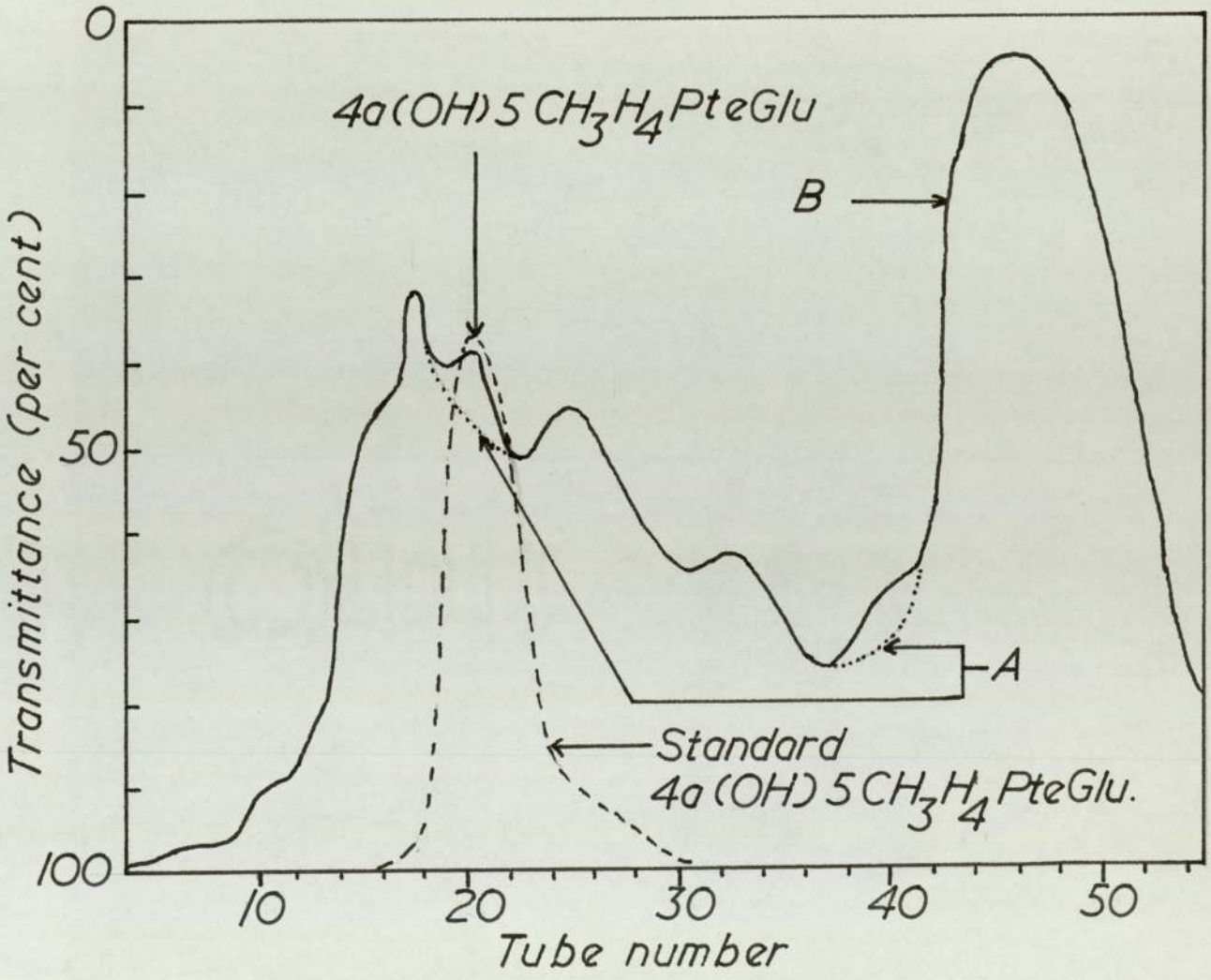


Figure 5.1 Urine sample from subject taken 10 mg of 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid orally on a Sephadex chromatograms (eluted by 0.1M phosphate buffer pH 7.0) detected at 280 nm. A = before dose and B = after dose.

5.2 Stability of P.cerevisiae activity.

Few reduced folates are responsible for supporting the growth of P.cerevisiae (Table 2.2.1).Human serum folate is variable with the dietary folate or folate derivative taken. The experiments in the early part of this chapter indicated that only serum folate supporting the growth of L.casei and S.faecalis can be easily increased by oral administration of folate derivatives.Drastic increase in serum folate supporting the growth of L.casei in most of the administered folates but only pteroylglutamic acid and 10-formylpteroylglutamic acid can cause drastic increase in serum S.faecalis activity. Other folates gives only slight increases in the S.faecalis activity in human blood.P.cerevisiae activity in human sera is of very high stability and does not vary with the administration of large doses of various folates as summarized in Table 5.1.23.

Oral administration of 5 mg of pteroylglutamic acid is known to enter blood levels as unchanged pteroylglutamic acid and 5-methyltetrahydropteroylglutamic acid (Ratanasthien et al 1974).There is little increase in P.cerevisiae which may be derived from dihydrofolate reductase reaction and or with enzymatic reaction (Mathews and Huennekens,1960) as shown (3)

$$\text{Formate} + \text{ATP} + H_4\text{PteGlu} \xrightarrow{Mg^{++},\ K^{+}} 10\text{-CHO } H_4\text{PteGlu} + \text{ATP} + P \quad (3)$$

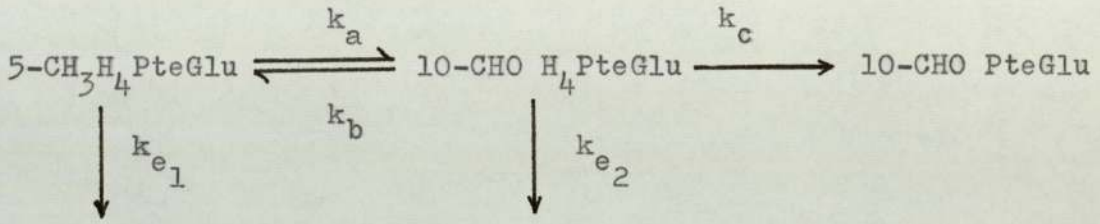
The evidence suggested that as soon as pteroylglutamic acid is reduced to tetrahydropteroylglutamic acid it is quickly methylated and thus only 5-methyltetrahydropteroylglutamic acid and pteroylglutamic acid were detected in big quantities as seen in Table 5.1.1.

Oral administration of 7,8-dihydropteroylglutamic acid at doses of 5 mg only 5-methyltetrahydropteroylglutamic acid is seen drastically increased and little increase in P.cerevisiae and S.faecalis activities were seen in serum folates (Table 5.1.2). The increase in P.cerevisiae activity in this experiment is more than that of pteroylglutamic acid administration and would be becaause of 7,8-dihydropteroylglutamic acid is quicker reduced by dihydrofolate reductase in tissue and plasma (Tzortzatou and Hayhoe,1974).

Oral administration of 10-formylpteroylglutamic acid at 5 mg doses is readily absorbed and entered the blood unchanged. 10-formylpteroylglutamic acid can be chemically reduced in the presence of Platinum and acetic acid to 10-formyltetrahydropteroylglutamic acid (May et al 1951;Flynn et al 1951 and Roth et al 1952).The presence of only 10-formylpteroylglutamic acid after the test dose indicated that in normal human there is no mechanism or enzyme system to reduce 10-formylpteroylglutamic acid to 10-formyltetrahydropteroylglutamic acid.Since if there was a reduction system the oral administration of this folate would be similarly metabolised as seen in cases of oral administration of 5-formyltetrahydropteroylglutamic acid, 10-formyltetrahydropteroylglutamic acid or 5,10-methenyltetrahydropteroylglutamic acid.The later three formylfolates are microbiologically active for P.cerevisiae (Table 2.2.1) but after oral administration of 5 mg doses only slight increase in P.cerevisiae activity was seen in the serum folate (Table 5.1.23). This evidence indicated that reduced formylfolates are rapidly metabolised to 5-methyltetrahydropteroylglutamic acid.

Serum folates were known to in balance by homeostatic mechanism (Blair et al 1974a;Ratanasthien et al 1974).The overall mechanisms is shown in Scheme 5.2.1.

Scheme 5.2.1.



Where k_{e_1} = Rate of elimination of 5-CH_3H_4PteGlu from blood.

k_{e_2} = Rate of elimination of 10-CHO H_4PteGlu from blood.

k_a & k_b = Rate of conversion from 5-CH_3H_4PteGlu to 10-CHO H_4PteGlu and vis versa.

k_c = Rate of oxidation of 10-CHO H_4PteGlu or utilization to the end product as 10-CHO PteGlu.

In normal human the equilibration of 5-methyltetrahydropteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid represents the existence of 'two-pool'system. One of which 5-methyltetrahydropteroylglutamic acid is represented the 'storage pool' and is variable to the intake dietary folates. The other pool is of 10-formyltetrahydropteroylglutamic acid represented the 'active folate pool' and is not variable to the intake dietary folates.This view is well supported by the

experimental results from the early part of this chapter.

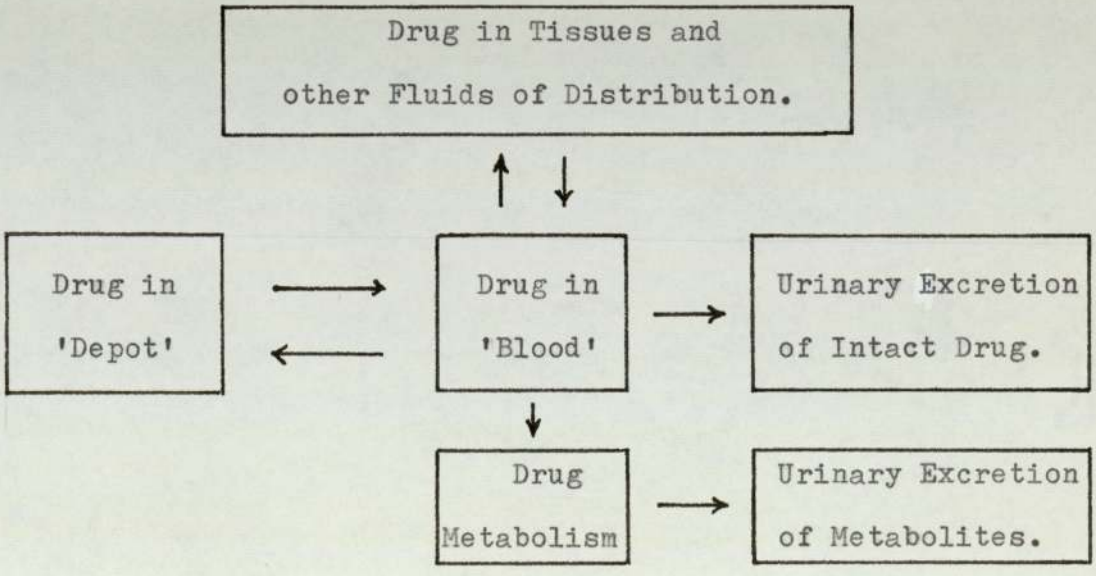
The oral administration of 5-methyltetrahydropteroylglutamic acid and its oxidation products 5-methyl-5,6-dihydropteroylglutamic acid,5-methyl-5,8-dihydropteroylglutamic acid and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid to normal human indicates that they do not raise the level of 10-formyltetrahydropteroylglutamic acid as seen in Tables 5.1.23 and 5.1.24. The use of S.faecalis activity for the activity of 10-formyltetrahydropteroylglutamic acid is justified as previously discussed in Chapter 4.

5.3 Pharmacokinetics of oral folates.

Pharmacokinetics is concerned with quantitatively accounting for the kinetics of drugs and in this case folates after they had been introduced into the body.The analysis of pharmacokinetics parameters are not easy and sacrifice can be done only on experimental animals.

In man analyses were carried out in those of assessible fluids and using kinetics to make deductions regarding the quantity of folates or their metabolites in the non-assessible regions.Blood and urine are the assessible regions and are widely used for assessing the pharmacokinetics of various drugs (Notari,1971).The model used for the analysis of pharmacokinetics of drugs is shown in the following scheme:

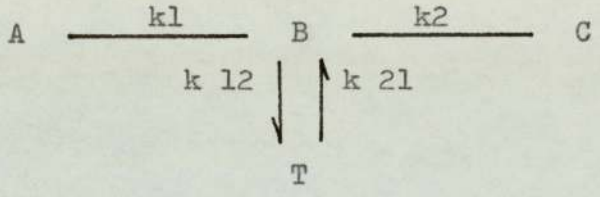
Scheme 5.3.1.



In case of using experimental animals all variables may be determined by the sacrifice of the animals.In man analyses

of pharmacokinetic parameters were not easy and only two compartments could be analysed.These two components are the drug in blood and excretion of drug in urine.Pharmacokinetics of oral folates could also be studied using the mentioned scheme.The scheme is simply rewritten as shown in Scheme 5.3.2.

Scheme 5.3.2.



Where A = Folate in stomach or intestine

B = Folate and its metabolites in blood.

C = Folate and its metabolites in urine.

T = Folate and its metabolites in other tissues and fluids.

k1 = Rate of folate availability for transport into blood.

k2 = Rate of elimination of folates via urine excretion.

k12 = Rate of transfer of folates from blood to tissues and other fluids.

k21 = Rate of transfer of folates from tissues and other fluids to blood.

Analyses of blood levels and urine excretions of folates can easily be done.Microbiological assays with L.casei, S.faecalis and P.cerevisiae and their bioautographic techniques were used as previously described in Chapter 2.

The pharmacokinetics of oral folates can simply be calculated from those results of the early part of this Chapter. The availability of oral folates can be assessed by comparing the blood level curves.Serum folate and time profiles of each dose is a net result of the dose.The comparison of serum folate and time profiles of various doses were shown and the availability of each folate is summarized in Table 5.3.1.

Analysed urinary folates after each dose can also be used for determination of the availability of the administered doses but to get an accurate result the urinary excretion must be completed.Studies on subjects after 5 mg of 7,8-dihydropteroylglutamic acid indicated that the urinary excretion of the administered dose would be completed within 24 hour after the dose.At 9 to 13 hours after the test dose more than 99 % of the urinary folates were excreted (Table 5.1.16).

Determination of other pharmacokinetic parameters.

Peak time and peak level of serum folate after dose of folate are the two pharmacokinetic parameters easily determined. These parameters are widely used in the study of folate metabolism (Chanarin,1969).The clearance of serum folate is also one of the kinetics used in the study of metabolism of oral and intravenously administered folate.When considering the clearance of folates from plasma with respect to the folates presented in urine the following equations and graphical methods may be applied.

$$A = k(B)^n \quad \text{.....(5.3.1)}$$

Where A = The amount of folates cleared from serum.

B = The amount of folates cleared into urine.

k & n = Rate constant and order reaction,respectively.

The above equation may derived the following equations:

$A = k$ (n=0), for zero order rate reaction with respect to B.

$A = kB$ (n=1), for first order rate reaction with respect to B.

$A = kB^2$ (n=2), for second order reaction with respect to B.

These equations may also be represented graphically as shown in Figure 5.3.2.

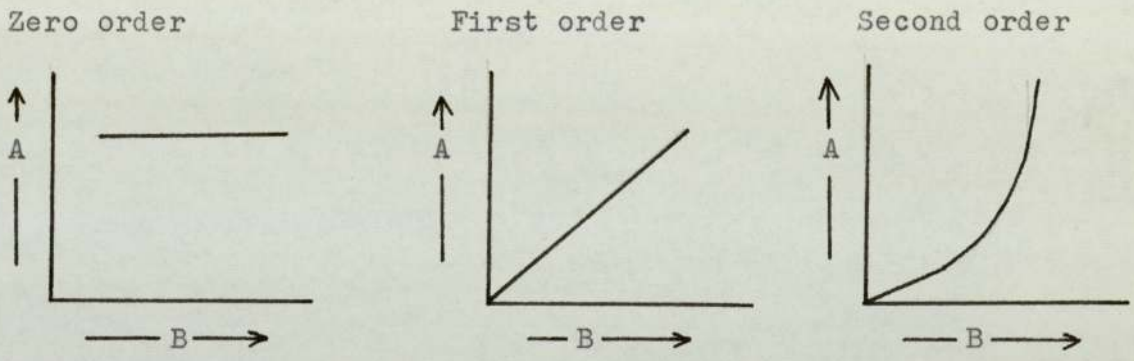


Figure 5.3.2 The graphical representation of serum folate clearance with respect to urine folate.

The equation (5.3.1) may also be considered against time since k is the rate constant dependent on time. If consider the amount cleared from blood (A) with respect to the amount left in blood (B) by mathematical treatment (integration) the above equations may be rewriten as following equations:

Zero order, $A = kt$

First order, $\log\frac{(A+B)}{B} = \frac{kt}{2.303}$

Second order, $\frac{A}{B(B+A)} = kt$

These equations can also be represented graphically as shown in Figure 5.3.3.

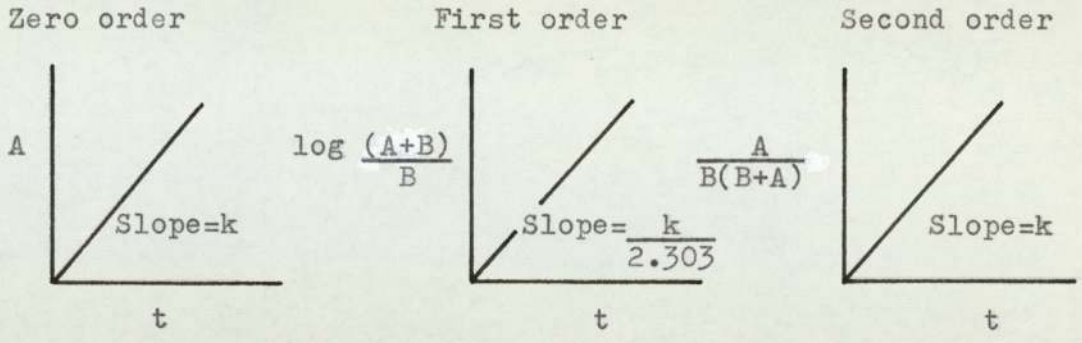


Figure 5.3.3 Straight line plots of kinetic equations for zero, first and second order reactions.From the slope of these straight line plots the values of the respective rate constants can be obtained.

Those results of oral folate metabolism were used for these rate order studies and straight line plots were obtained by plotting log serum folate against time.These straight line graphs of various folates oral administrations are shown in Figure 5.3.4 to Figure 5.3.15 and the $t_{\frac{1}{2}}$ (hour) for each test is summarized in Table 5.3.1.

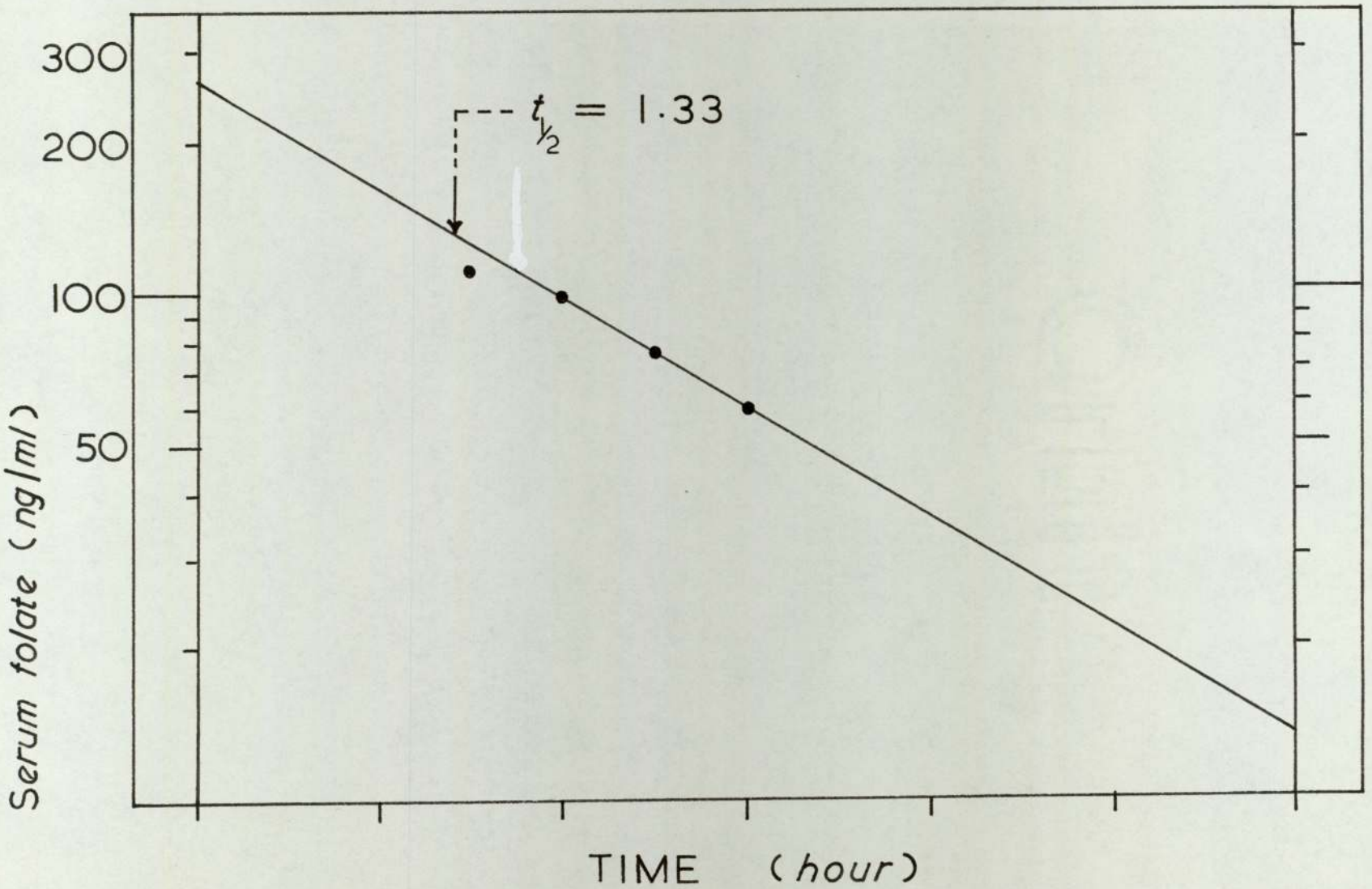


Figure 5.3.4 First order rate of serum folate after oral (5mg) PteGlu.

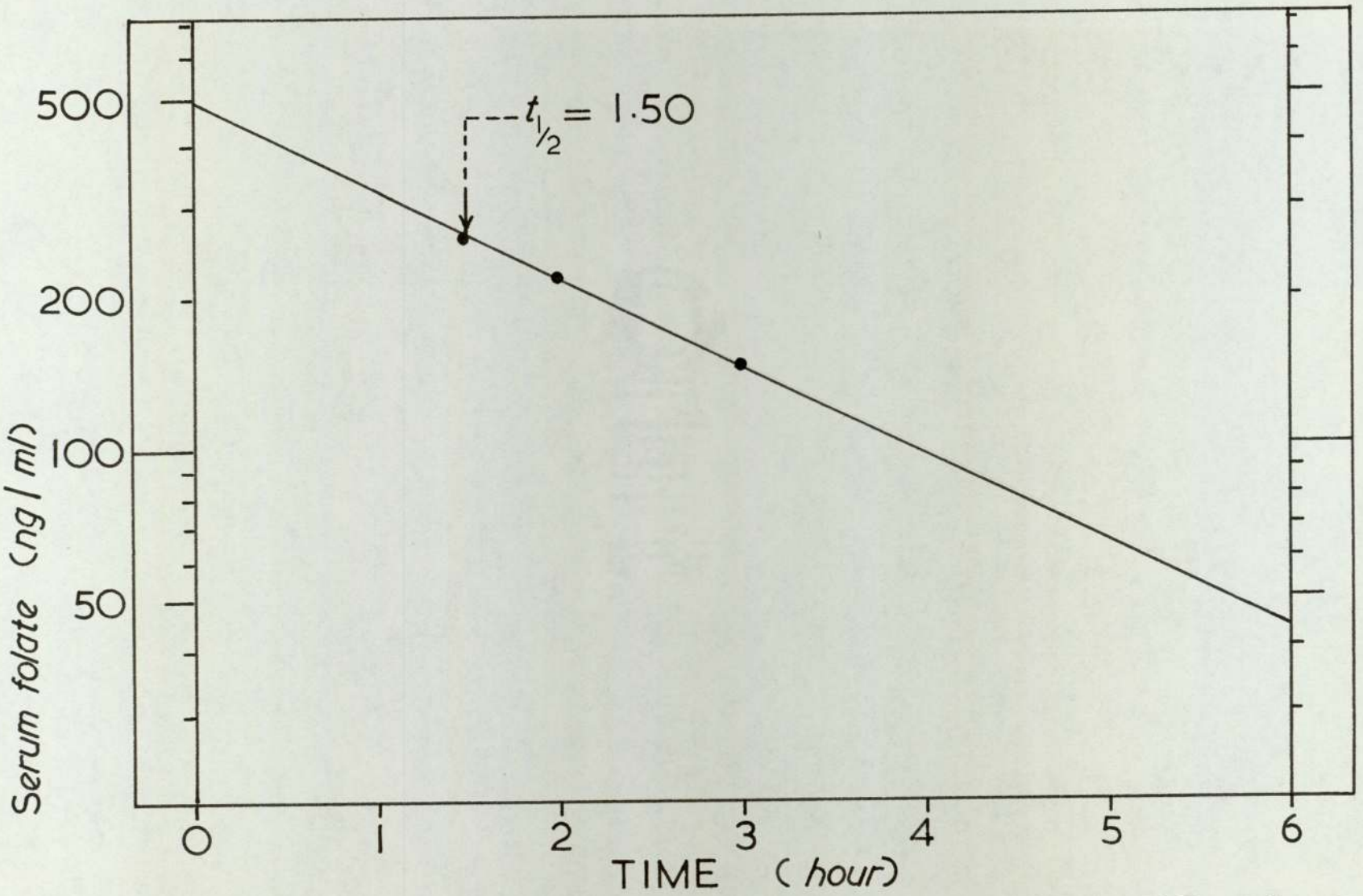


Figure 5·3·5 First order rate of serum folate after oral (5 mg) of H_2PteGlu.

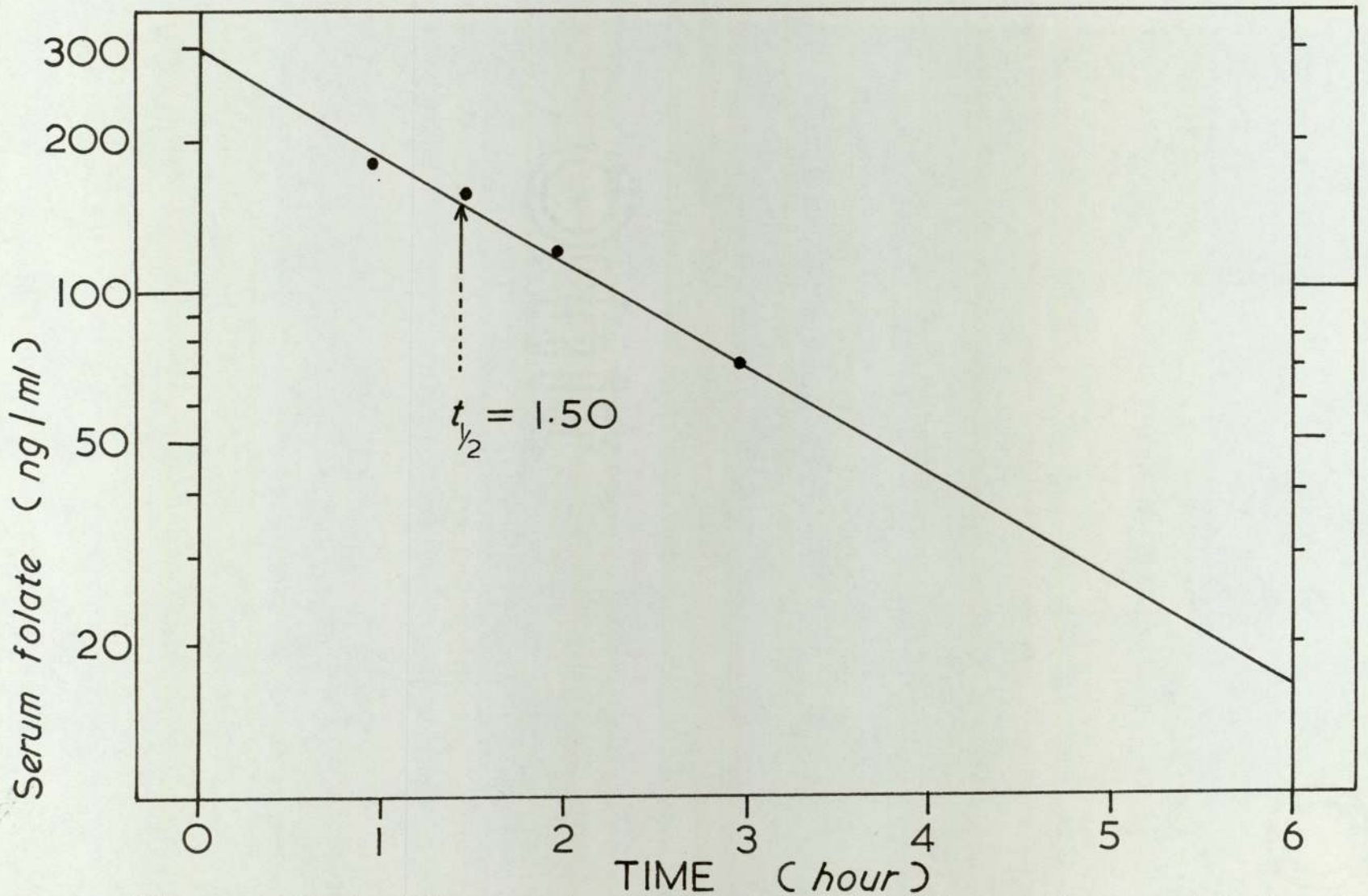


Figure 5.3.6 First order rate of serum folate after oral (5mg) 10-CHO PteGlu.

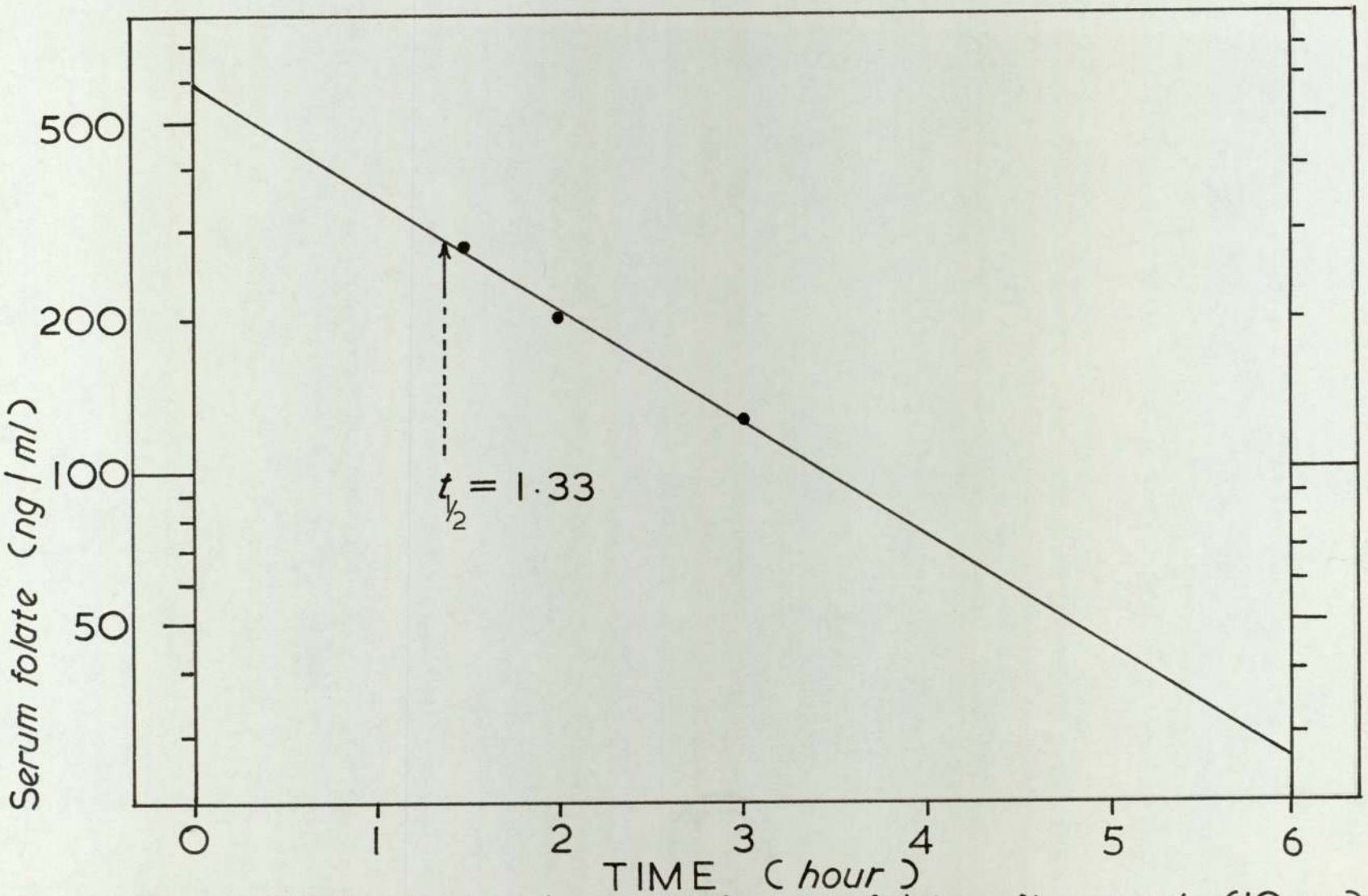


Figure 5.3.7 First order rate of serum folate after oral (10 mg) (±) 5-CHO H$_4$ PteGlu.

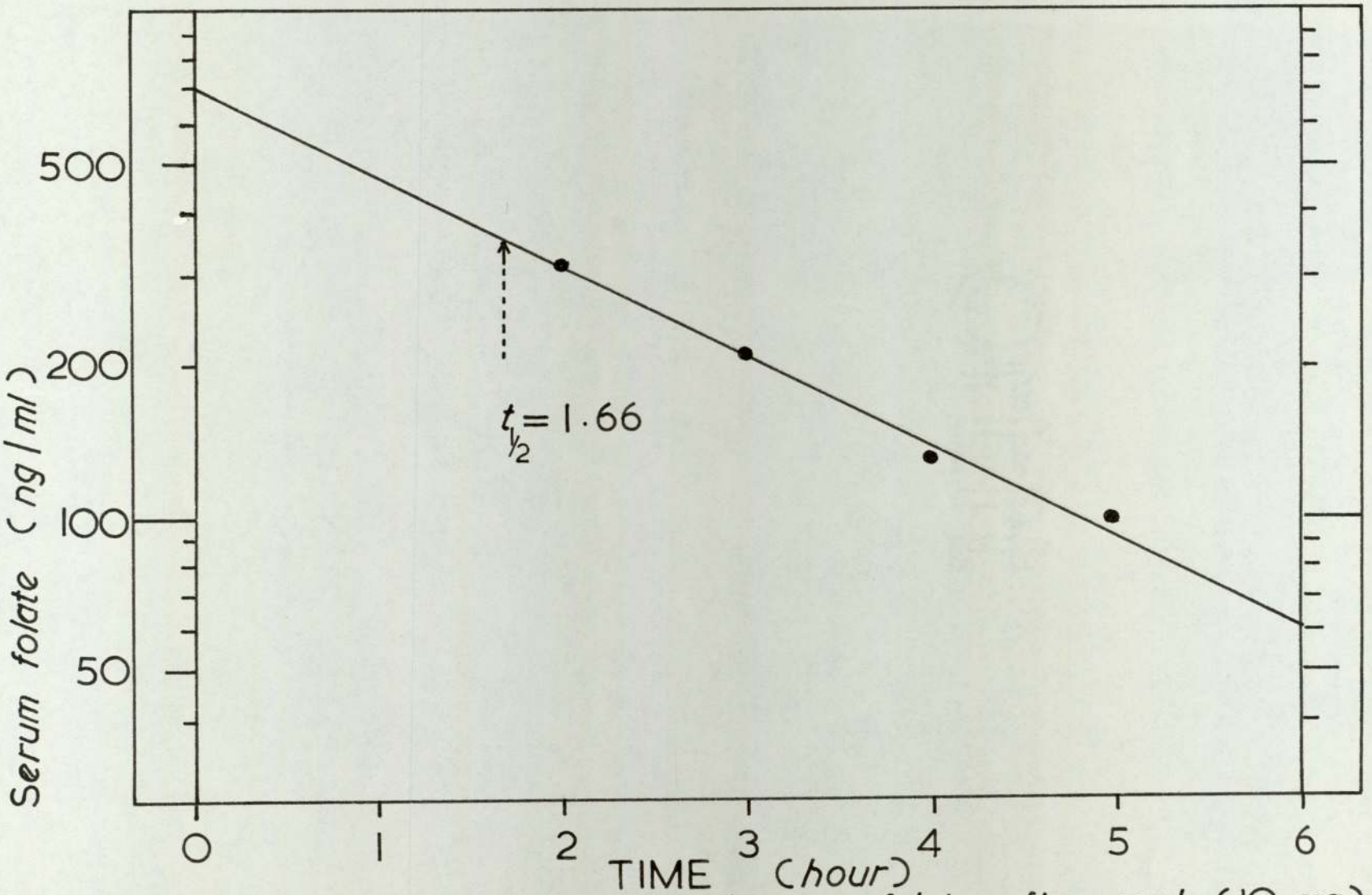


Figure 5.3.8 First order rate of serum folate after oral (10 mg) (±) 5,10-CH=H_4PteGlu

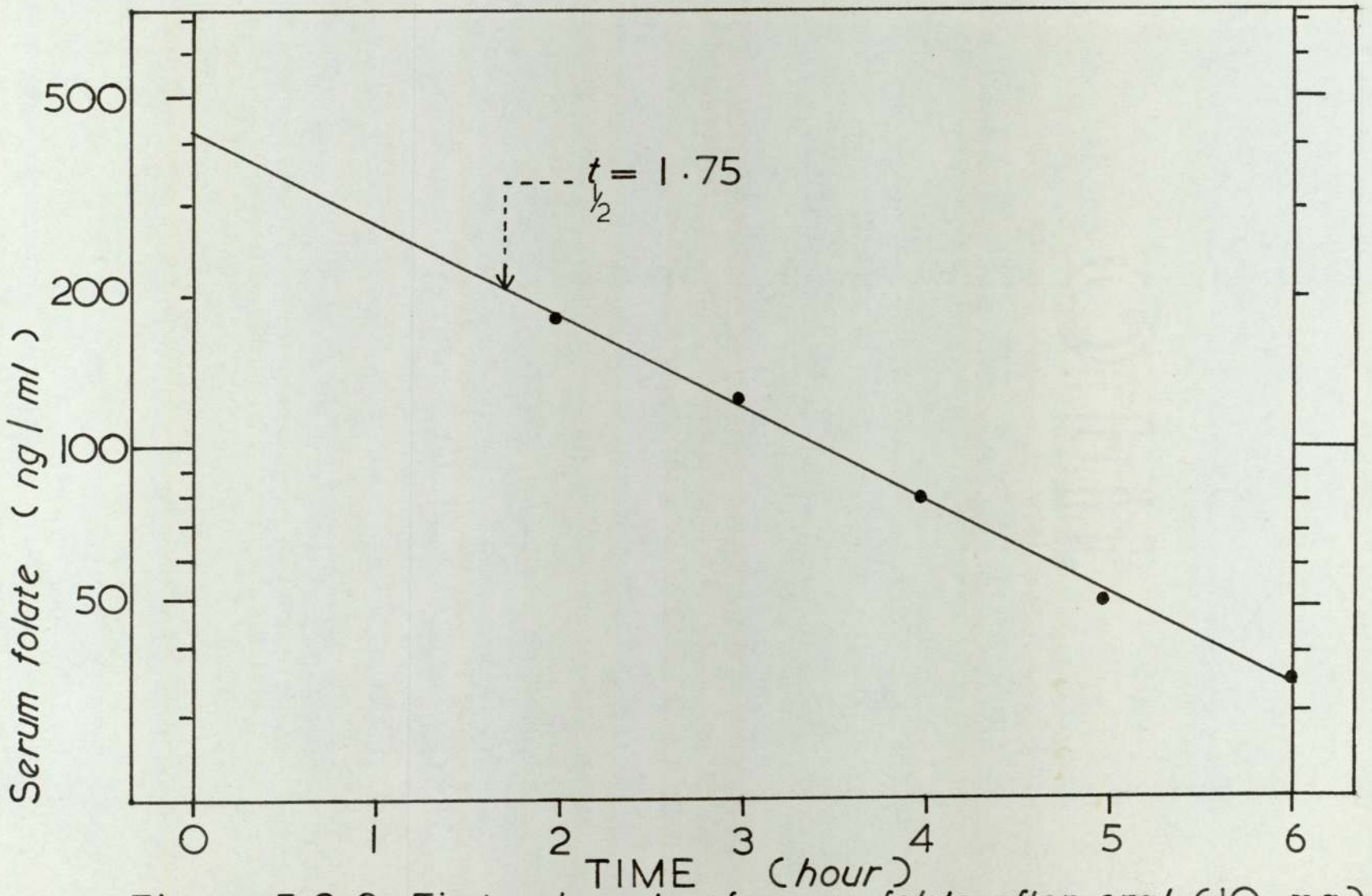


Figure 5.3.9 First order rate of serum folate after oral (10 mg) (±) 5 CH H PteGlu
3 4

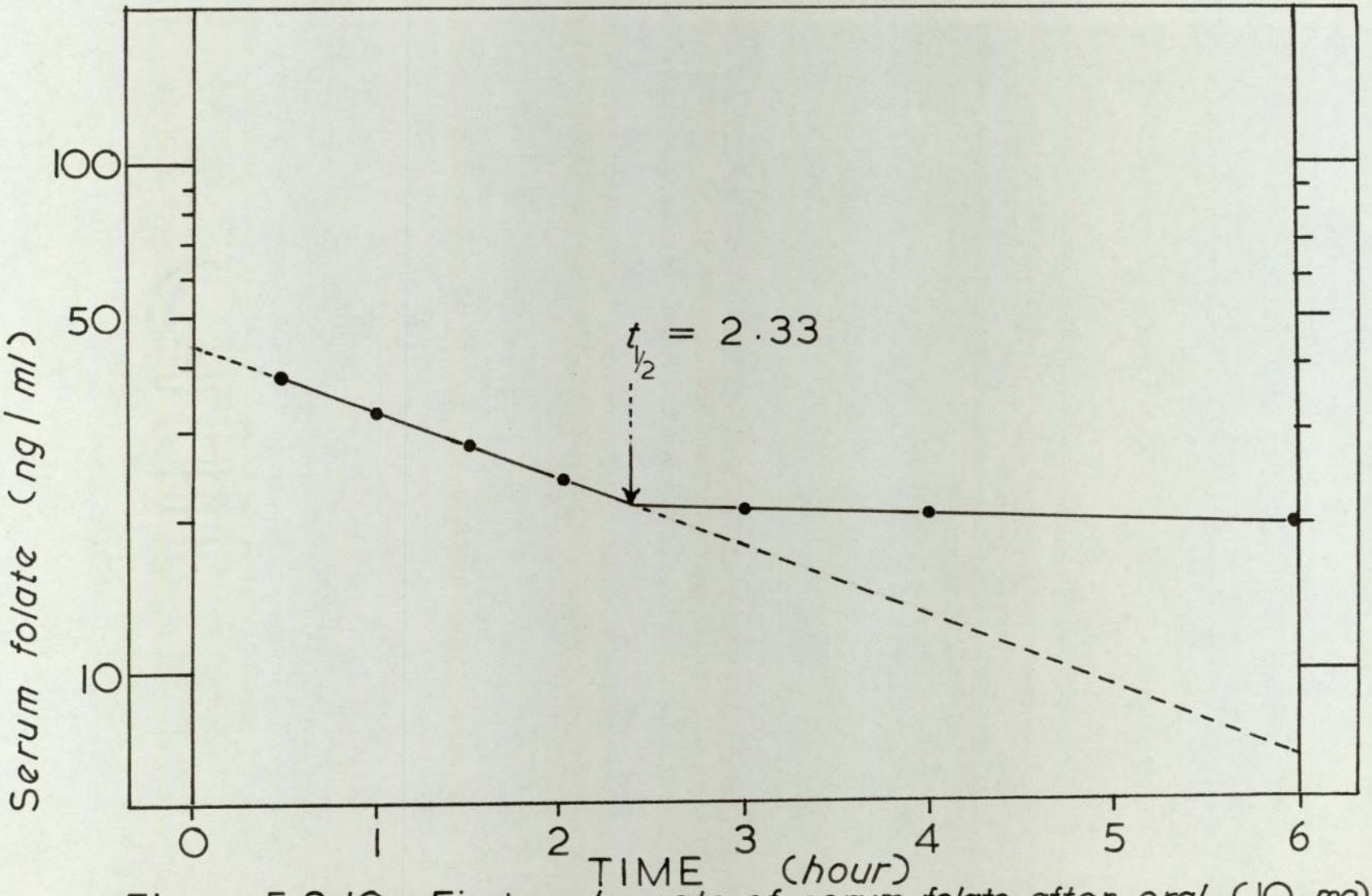


Figure 5.3.10 First order rate of serum folate after oral (10 mg) (±) 5-CH_3-5,6-H_2 PteGlu

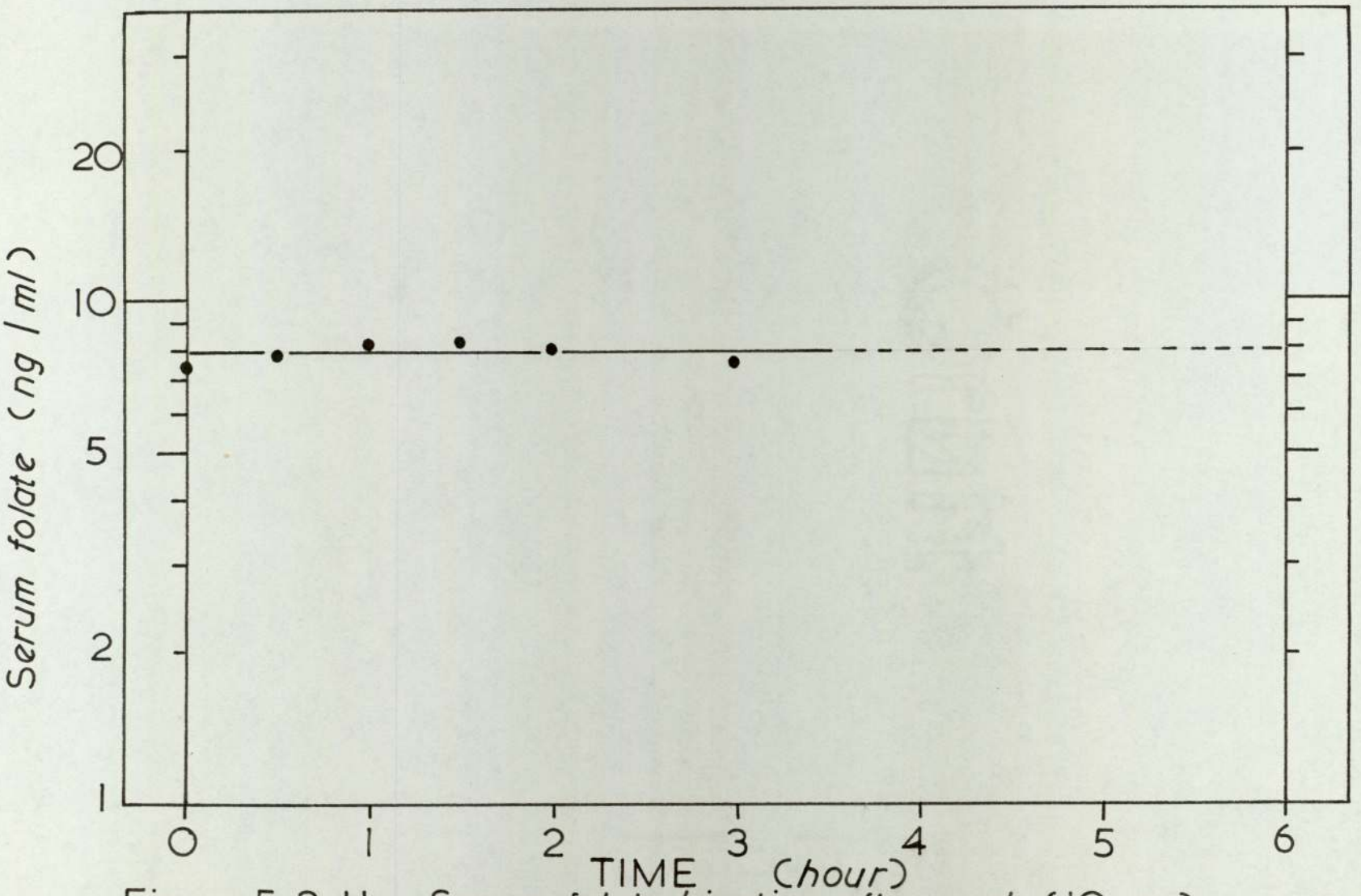


Figure 5.3.11 Serum folate kinetics after oral (10 mg) 5-CH_3-5,8-H_2PteGlu.

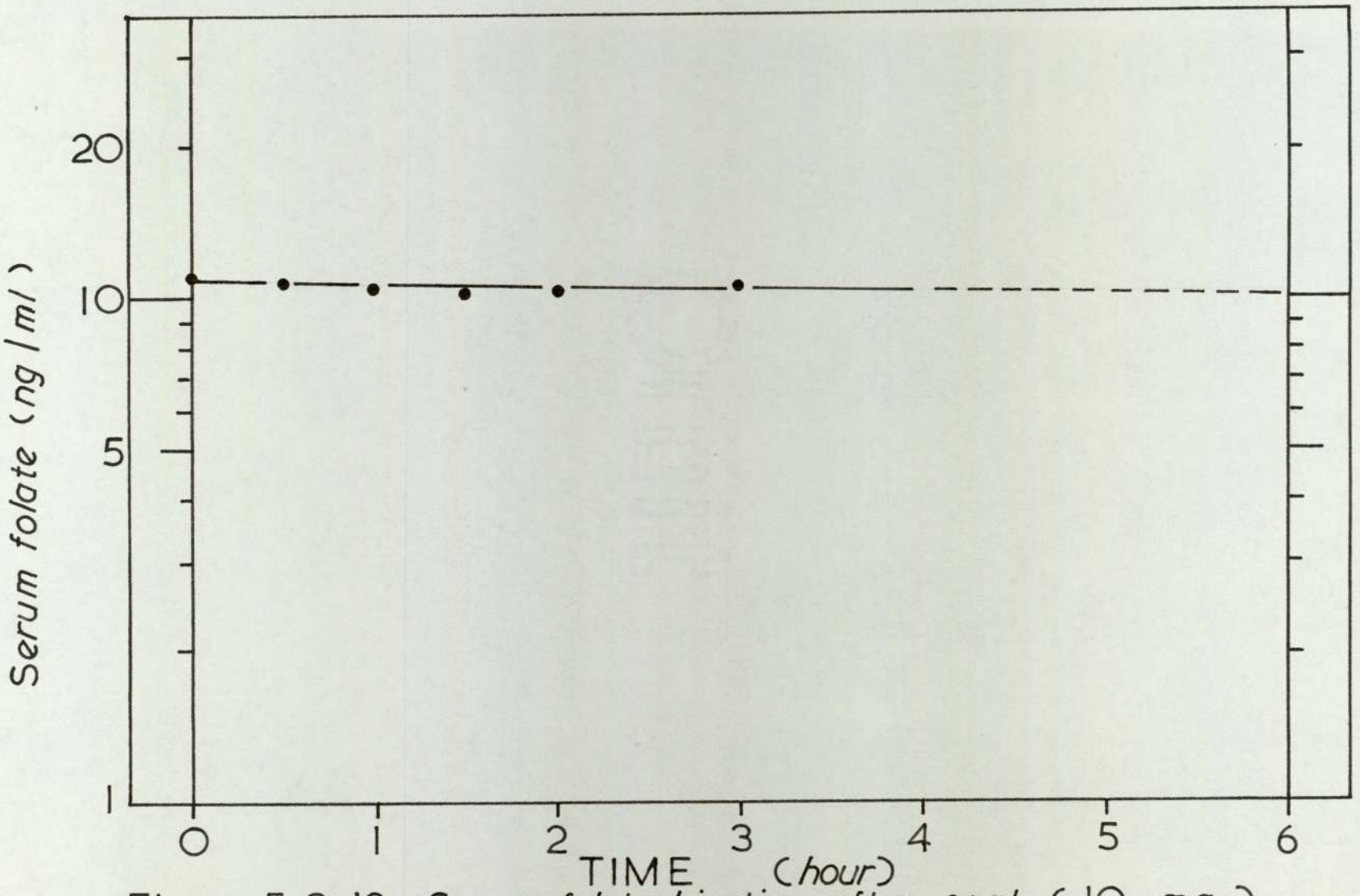


Figure 5.3.12 Serum folate kinetics after oral (10 mg) 4a(OH)-5-CH_3-4a,5,6,7-H_4 PteGlu

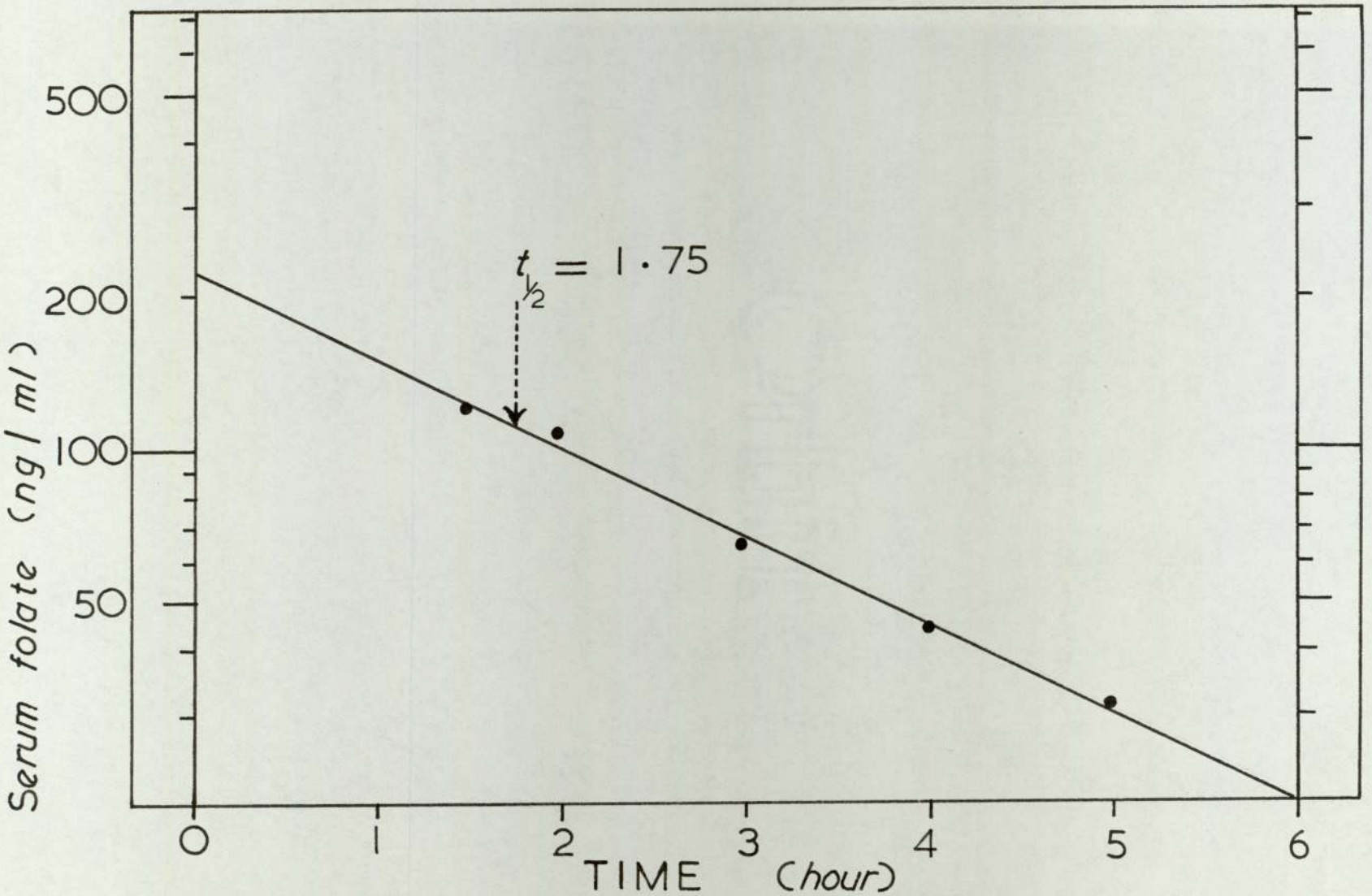


Figure 5·3·13 First order rate of serum folate after oral $NaHCO_3$ (3 g) and 5·CH_3·5,6·H_2PteGlu (10 mg).

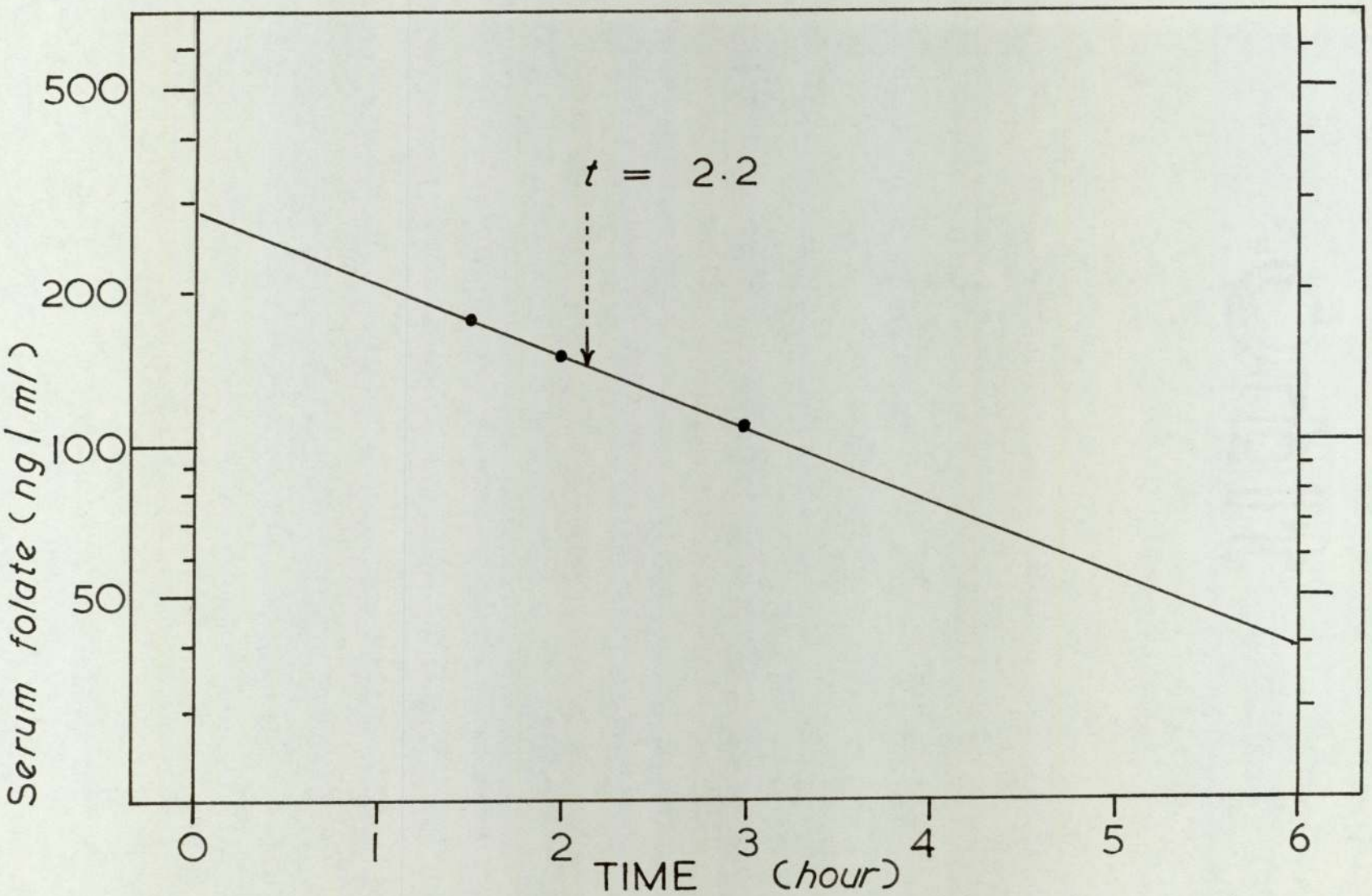


Figure 5·3·14 *First order rate of serum folate after oral* $NaHCO_3$ *(3 g) and ±5-*CH_3H_4*PteGlu (10 mg).*

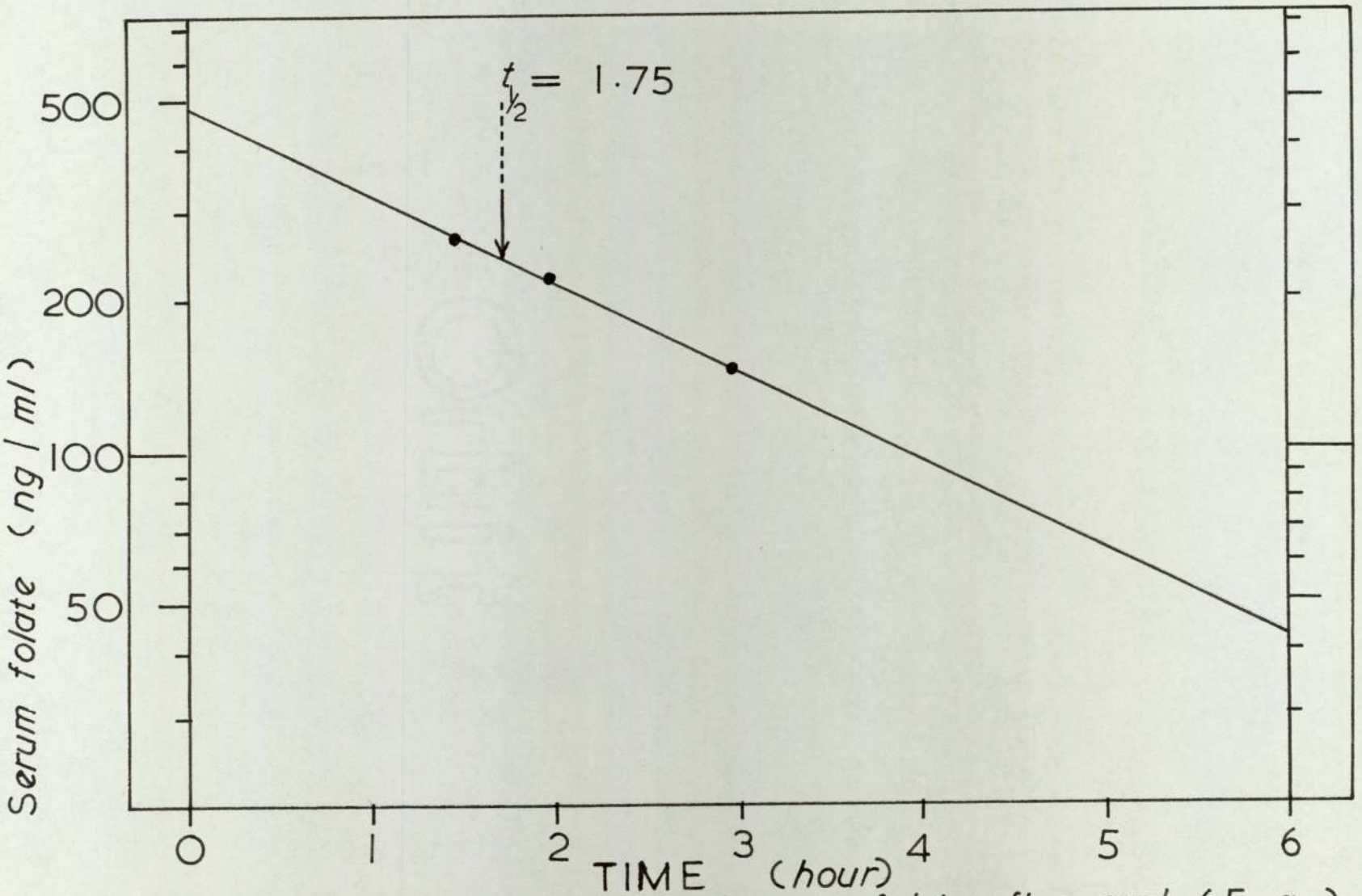


Figure 5.3.15 First order rate of serum folate after oral (5 g) $NaHCO_3$ and 10 mg (±) 5-CH_3H_4PteGlu

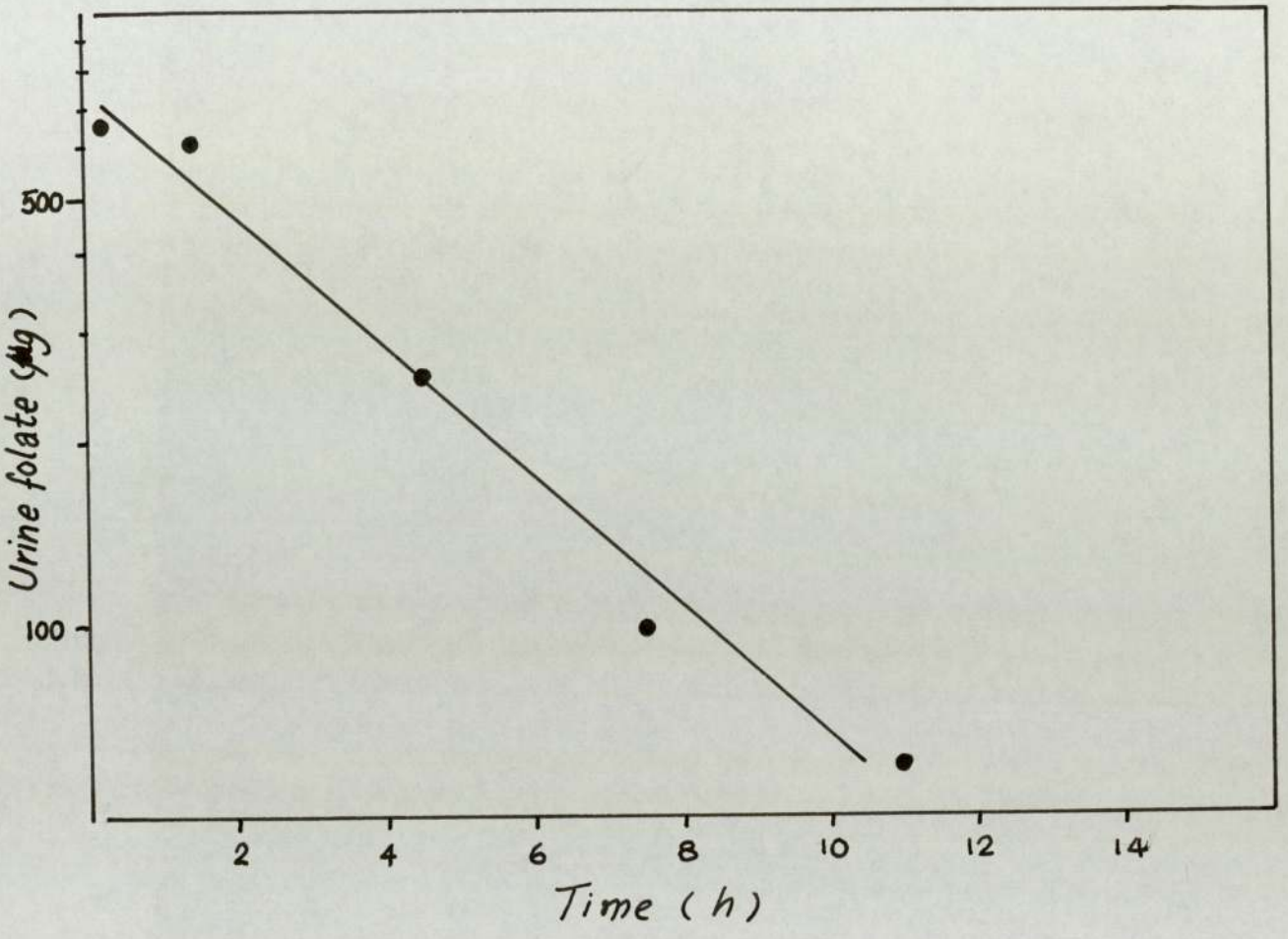


Figure 5.3.1 Urine folate excretion after 7,8-H_2 PteGlu (Table 5.1.16)

Table 5.3.1 Pharmacokinetics analyses of serum folate after oral administration of various folates.The summarized results from Figure 5.3.4 to Figure 5.3.15.

Administered folate.	Availability at time 0 hour (ng/ml)	Order of clearance.	Rate constant $t_{\frac{1}{2}}$ (hour).
PteGlu	265.0 (1)	1 st.	1.33
7,8-H_2PteGlu	490.0 (4)	1 st.	1.50
10-CHO PteGlu	300.0 (6)	1 st.	1.50
5-CHO H_4PteGlu	590.0 (6)	1 st.	1.33
5,10-CH=H_4PteGlu	700.0 (6)	1 st.	1.66
5-CH_3H_4PteGlu	430.0 (6)	1 st.	1.75
5-CH_3-5,6-H_2PteGlu	44.0 (5)	1 st.	2.33
5-CH_3-5,8-H_2PteGlu	0.0 (3)	-	-
4a(OH)-5-CH_3H_4PteGlu	0.0 (3)	-	-
5-CH_3-5,6-H_2PteGlu*	220.0 (3)	1 st.	1.75
5-CH_3H_4PteGlu*	290.0 (4)	1 st.	2.20
5-CH_3H_4PteGlu**	480.0 (5)	1 st.	1.75

* and ** indicate subjects taken 3 and 5 g of sodium bicarbonate (respectively) before oral folate administration and - indicates the administered folate did not enter folate pool. No of subjects in parentheses.

The handling of oral folates absorption in normal human by pharmacokinetic studies of serum and urine folates indicated that the clearance rate is first order reaction. The first order rate reaction is seen as far as the serum folate of higher than 22 ng/ml and when serum folate become lower than 22 ng/ml the $t_{\frac{1}{2}}$ of clearance increased significantly as seen in Figure 5.3.10.The increase in $t_{\frac{1}{2}}$ of the clearance rate may be the effect of the reabsorption of folate by renal tubules (Condit and Grob,1958;Johns et al 1961;Goresky et al 1963). Thus from this study the renal reabsorption is negligible at serum folate of 22 ng/ml or higher.

Eventhough the body folate is in the order of 12 to 15 mg (Today's drugs,Brit.Med.J.,1964),studies of Herbert (1962) indicated that not all of body folate can be used for maintaining the body requirement.Since on maintaining a normal healthy human on low folate diet (5ug/day) only 3 weeks later serum folate was decreased to 1 ng/ml a value well below normal level. Folate excretion in normal human is from 3 to 15 ug/day as previously discussed in Chapter 4.In patients with renal disease the folate excretion into urine is around 52 ug/day in those with severe renal failure (Sidiqui et al 1970). This evidence indicated that there are reabsorption of folate before excretion.

5.4 Dietary folates,their metabolism and requirement by man.

Pteroylglutamic acid and its derivatives are widely distributed in the Nature.Toepfer et al (1951) reported folate content of about 400 items of food but no detail about individual folate components was known. Folate in green leaves of a number of higher plants have been investigated (Iwai and Nakagawa,1958; 1959).They found that most of the folate is presented as labile reduced form which are converted to 5-formyltetrahydropteroyl-glutamic acid on heating under argon in the presence of ascorbic acid at alkaline pH. No information about the presence of 5-methyltetrahydropteroylglutamic acid was given since micro-biological assays employed only S.faecalis and P.cerevisiae which do not respond to 5-methyltetrahydropteroylglutamic acid (Table 2.2.1).Chung et al 1961 have analysed folates in American diets and found that folates other than 5-methyltetrahydro-pteroylglutamic acid and its conjugated forms as assessed by microbiological assays with S.faecalis ranged from 27 to 346 ug/day. Of diets analysed,high cost diets ranged from 160 to 346 ug/day with a mean of 193 ug/day; low cost diets ranged from 105 to 310 ug/day with a mean of 157 ug/day and poor diets ranged from 27 to 65 ug/day with a mean of 47 ug/day. Butterworth et al 1963 studied the folate content of foods and found that folate content before and after treatment with conjugase were 157 and 689 ug/day, respectively. Santini et al (1964) using column chromatography with DEAE-Cellulose for separating the folates of food extracts and microbiological assays with L.casei and S.faecalis found 10-formylpteroylglutamic acid,5-formyltetrahydropteroylglutamic acid and pteroylglutamic acid.The reported values were 10-formyl-pteroylglutamic acid from 30 to 88 % with a mean of 55 %,

5-formyltetrahydropteroylglutamic acid from 12 to 59 % with a mean of 34 % and pteroylglutamic acid ranged from 0 to 30 % with a mean of 11 %.Unprocessed liver when analysed with protected against oxidation contains 5-methyltetrahydropteroyl-glutamic acid as a major folate (Donaldson and Keresztesy,1959; Silverman et al 1961;Noronha and Silverman,1962;Bird et al,1965; and Chanarin et al 1966).These evidences have led to the conclusion that folates present in food before degradation as 5-methyltetrahydropteroylglutamic acid and 10-formyltetra-hydropteroylglutamic acid (Blair et al 1974).

Daily requirement of folate and dietary folate by man.

Studies in various kinds of patients for the smallest amount of folate required to restore the haematological response were used for the determination of daily requirement of folate by man. Studies on daily requirements of folic acid for remission of megaloblastic dysplasia seen in malnourished children showed that oral folate of 20 ug/day (in some cases 10 or even 5 ug/day) can produce a remission in the megaloblastic bone marrow (Velez et al 1963). In patients with tropical sprue oral administration of folic acid (25 ug/day) can produced a remission of the megaloblastic anaemia.The diets fed these patients contained 500 to 1500 ug/day of folacin as measured by micro-biological assay with L.casei (Perez-Santiago et al 1960). Izak et al (1963) showed that 1000 ug of folate is required per day in oral administration for the cure of megaloblastic anaemias and 10 ug/day intramascularly is not enough to produce a haematological response.A value of 25 ug/day had been reported (Sheehy et al 1961;Druskin et al 1962).Some responded to higher

doses,100 ug/day (Sheehy,1961),200 ug/day (Hansen and Weinfeld, 1962;Herbert,1962). Studies in protein-calorie malnourished children indicated that in addition to other anti-anemia nutrients folic acid needed in all cases were around 11.2 ug/kg body weight per day (Kamel et al 1972).In cases of pregnancy there are increasing requirement of folate to 500 to 600 ug/day of food folate or 200 to 300 ug of folic acid per day (Cooper et al 1970).Summarized from these data would come to ranges as shown in Table 5.4.1.

Table 5.4.1. Daily requirement of folate in humans.

Subject	PteGlu (ug/day)		Dietary folate (ug/day)
Children	5 to 50	or	50 to 500
Adults	50 to 200	or	50 to 500
Pregnancy	200 to 300	or	500 to 600
Megaloblastosis	25 to 200	and	600 to1500

Metabolism of dietary folates.

It is justified to use pteroylglutamic acid and its derivatives as a model for the study of the corresponding dietary folate metabolism.Eventhough pteroylpolyglutamic acid and its derivatives are represented the major component of the dietary folates they are well absorbed and enter blood levels as unconjugated folate (Rosenberg et al 1969).The deconjugation of pteroylpolyglutamic acid and other polyglutamate forms by conjugase is known to occur before their absorption by mucosal

conjugase (Rosenberg et al 1969;Perry and Chanarin,1968; Butterworth,Baugh and Krumdieck,1969).As previously discussed it is clear that folates present in food before degradation are 5-methyltetrahydropteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid.These folates are readily oxidised and the rate of oxidation increasing with increasing temperature and increasing pH (Blair et al 1974).10-formyltetrahydropteroylglutamic acid is oxidised to 10-formylpteroylglutamic acid and 5-methyltetrahydropteroylglutamic acid is oxidised to 5-methyl-5,6-dihydropteroylglutamic acid and this may rearrange to 5-methyl-5,8-dihydropteroylglutamic acid very readily at pH 4.5 or lower or add a molecule of water to form 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid.

Both 5-methyl-5,8-dihydropteroylglutamic acid and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid are inactive for all three microorganisms used in the microbiological assays as shown in Chapter 3.Oral administration of these two folates did not enter folate pool in man (Table 5.1.9 and 5.1.10).5-methyl-5,6-dihydropteroylglutamic acid is inactive for L.casei when ascorbic acid was omitted from the assay media as also shown in Chapter 3. Oral administration of 5-methyl-5,6-dihydropteroylglutamic acid is readily absorbed but small amount reached blood level as 5-methyltetrahydropteroylglutamic acid because of the rearrangement of the administered folate by stomach acid to inactive material before absorption (Table 5.1.7). The neutralisation of acid stomach by sodium bicarbonate enhanced the availability of 5-methyl-5,6-dihydropteroylglutamic acid as seen in Table 5.1.13.

Oral administration of 10-formylpteroylglutamic acid is readily absorbed but reached blood levels unchanged as shown in Table 5.1.3 and also in previous report (Ratanasthien et al 1974).Since 10-formylpteroylglutamic acid did not metabolised by man and animal it is possible that this compound may not nutritionally important (Beavon and Blair,1975).Thus diets containing adequate folate for human nutrition as judged by microbiological assay with L.casei may not infact be so.

In determination of availability of dietary folates by using synthetic materials for oral administration studies it is clear that condition of folate absorption by this method is different from those of folate absorption from diet. In foods folates are strongly bound to the insoluble component of diets (mainly cellulose) and thus may not available for absorption (Luther et al 1965). The changes in condition of absorption site by oral administration of sodium bicarbonate has also been demonstrated to produce changes in metabolism of folates as seen in Table 5.1.19 to Table 5.1.21 in comparing with those of Table 5.1.6 and Table 5.1.7.

5-methyltetrahydropteroylglutamic acid is a metabolite of various folate metabolism in normal human and this is changed in favour of 5-methyl-5,6-dihydropteroylglutamic acid as a metabolite when stomach and intestinal pH increased.Thus it is possible that an increased serum level of 5-methyl-5,6-dihydropteroylglutamic acid is an abnormal condition.Traces amount of copper in the solution of 5-methyltetrahydropteroylglutamic catalysed the oxidation of this compound.(Blair et al 1975)

5.5 Evidence of storage forms.

Liver had been shown to have contained very high amount of folates at 0.6 to 15 ug/g of liver (Grossowicz et al 1963,1964;Romine,1960;Bennett et al 1964;Chanarin et al 1966; Wang et al 1967).Many forms of folate are found in livers of various species of mammals.Rat liver contains a large pool of 5-methyltetrahydropteroylpolyglutamates (Bird et al 1965). Horse liver contains 10-formyltetrahydropteroylglutamic acid and its derivatives as high as 90 % of liver folate (Silverman et al 1954).Folate in liver of human contained 5-methyltetrahydropteroylglutamic acid as a major component (Romine,1960; Chanarin et al 1966).

Folate binding proteins had been demonstrated in many parts of the body.Folate binding proteins in milk is widely studied (Ghitis,1967;Ford et al 1969;Metz et al 1968). Eventhough folate binding proteins are distributed throughout body fluids and tissues as previously discussed in Chapter 4 there is no clear role of these folate binding proteins. There are possible roles of folate binding proteins one of which is being a folate carrier in the circulating folate.The binding of folates to those folate binding proteins in various tiisues may also served as a storage site.The presence of folate binding proteins in plasma would infact retained some folates from passing through the glomerular filtration.There are evidences indicating the presence of folate re-absorption in the renal tubules after glomerular filtration (Condit and Grob,1958; Johns et al 1961;Goresky et al 1963).These evidences suggested that folates may be stored as both monoglutamate and polyglutamate

forms. If considered a storage folate as form or forms of folates variable to dietary folates only 5-methyltetrahydropteroyl-glutamic acid is the storage form of human folate stored in blood circulating system.5-methyltetrahydropteroylglutamic acid is slowly metabolised to 10-formyltetrahydropteroylglutamic acid and thus serves as a storage form (Blair et al 1974b).

Studies with everted sacs of rat intestine indicated that folates are stored in the intestinal wall and readily available as seen in increased serosal side folates after incubation for 60 minutes in the standard incubation medium without added mucosal folate (Blair et al 1973).

Summary.

In normal humans there was rapid and almost complete metabolism in the serum to 5-methyltetrahydropteroylglutamic acid after oral administration of 7,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid,5-formyltetrahydropteroylglutamic acid and 5,10-methenyltetrahydropteroylglutamic acid. Oral administration of pteroylglutamic acid itself gave a partial metabolism to 5-methyltetrahydropteroylglutamic acid whilst 10-formylpteroylglutamic acid remained unchanged. Oral administration of the oxidation products of 5-methyltetrahydropteroylglutamic acid showed that 5-methyl-5,6-dihydropteroylglutamic acid is partially utilized but mainly underwent rearrangement to microbiologically inactive 5-methyl-5,8-dihydropteroylglutamic acid in the stomach acid. Neutralization of stomach acid of normal humans with oral sodiumbicarbonate enhanced the availability of 5-methyl-5,6-dihydropteroylglutamic acid. Other folates,5-methyl-5,8-dihydropteroylglutamic acid and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid did not enter folate metabolic pool. Application of urine from these subjects to a Sephadex chromatograms suggested that they may be absorbed.

Chapter 6.

Relationship of folates, biopterin and tetrahydrobiopterin.

6.1 Crithidia factor in human serum and urine.

6.2 Effect of folate absorption on Crithidia factor levels.

6.3 Biopterin, tetrahydrobiopterin absorptions and their effects on serum folate levels.

Relationship of folates to biopterin and tetrahydrobiopterin.

It is generally accepted that mammals are not able to synthesize folate (Blakley,1969).There is a group of compounds excreted in urine of mammals and known to be essential for the growth of trypanosomid flagellate Crithidia fasciculata (Patterson et al 1955;Kraut et al 1963;Pabst and Rembold,1966). It has been shown that biopterin and its related compounds were responsible for Crithidia factors in urine of mammals (Pabst and Rembold,1966;Leeming and Blair,1974).

There is no clear evidence about the origin of biopterin and its related compounds in mammals.Oral administration of biopterin is absorbed but does not appear to be utilised by rats (Kraut et al 1963).The possibility that biopterin and its related compounds were derived from diets proved not to be the major source of serum Crithidia factors (Kraut et al 1963;Pabst and Rembold,1966).They showed that on maintaining rats on biopterin free diet the urinary biopterin excretion did not chage from those of animals on normal diet.There are possibilities that biopterin and its related compounds were synthesized by mammals and many possible routes of synthesis were postulated as summarized in Scheme 6.1.

In these experiments the attempts were made to study the relationship of folates to biopterin and tetrahydrobiopterin in humans. All microbiological assay with Crithidia fasciculata were done by Mr.R.J.Leeming.

Materials and Methods.

Folates used in this experiment were obtained as described in Chapter 3. Biopterin and tetrahydrobiopterin were gift from Roche products.Pteroic acid was also used in this experiment.Folates used in this experiment were,pteroylglutamic acid,10-formylpteroylglutamic acid,7,8-dihydropteroylglutamic acid,5-methyl-5,6-dihydropteroylglutamic acid,5-methyl-5,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid.

Plasma or sera from normal human subjects were collected similarly to those used in the folate studies but kept frozen at -20^{o}C without antioxidant added until microbiological assay with Crithidia fasciculata as described in Chapter 2. Morning urine samples were also similarly collected and microbiological assayed with Crithidia fasciculata.

Oral absorption studies were performed similarly to those in Chapter 5 but samples were collected in two different ways for each test.One part of samples were used for microbiological assay with Crithidia fasciculata and thus collected without added antioxidant.The other portion of samples were used for microbiological assays with L.casei,S.faecalis and P.cerevisiae and collected as those used in Chapter 5.All those compounds which were diastereoisomer mixtures were used at 10 mg doses and the rest were used at 5 mg doses.Microbiological assay with Crithidia fasciculata were done by Mr.R.J.Leeming.

Results.

Crithidia factor levels in normal human serum and urine excretion were summarized in Table 6.1. and Crithidia factor levels in normal humans after various folates were summarized in Table 6.2. Crithidia factors of normal human serum after oral administration of biopterin (5mg) and tetrahydrobiopterin (10 mg) were summarized in Table 6.3.

Serum folate of subjects corresponding to those in Table 6.2 were microbiologically assayed with L.casei as summarized in Table 6.4,with S.faecalis in Table 6.5 and with P.cerevisiae in Table 6.6. Serum folates of subjects taken 5 mg of biopterin is summarized in Table 6.7 and Table 6.8 is the summarized serum folates from subjects taken 10 mg of tetrahydrobiopterin orally.

The relationship of serum folates and serum Crithidia factor from subjects taking biopterin is shown in Figure 6.1 and from those subjects taking tetrahydrobiopterin is shown in Figure 6.2.The relationship of serum folate and serum Crithidia factor after pteroic acid is shown in Figure 6.3,after pteroylglutamic acid (Figure 6.4),after 10-formylpteroylglutamic acid (Figure 6.5), after 7,8-dihydropteroylglutamic acid (Figure 6.6),after 5-methyl-5,6-dihydropteroylglutamic acid (Figure 6.7),after 5-methyl-5,8-dihydropteroylglutamic acid (Figure 6.8),after 5-methyltetrahydropteroylglutamic acid (Figure 6.9),after 5-formyltetrahydropteroylglutamic acid (Figure 6.10).

Table 6.1 Serum and urine Crithidia factors of normal humans.

Sample	No.of subject	Crithidia factor (mean ± S.E.M.)
Normal human serum	114	1.81 ± 0.06 (ng/ml)
Normal human urine	60	2.10 ± 0.39 (ug/ml)

Table 6.2 Serum Crithidia factor (mean±S.E.M.) from subjects taken various folates (5mg of microbiologically active or 10 mg of diastereoisomers).

Administered folate	No.of subject	Serum Crithidia factors(ng/ml) at time(h) T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Pte	2	1.6±0.1	1.5±0.2	1.5±0.1	1.5±0.1	1.6±0.1	1.6±0.4
PteGlu	3	1.2±0.2	1.2±0.1	1.3±0.2	1.3±0.1	1.4±0.2	1.3±0.1
10-CHO PteGlu	2	0.6±0.1	0.5±0.2	0.6±0.2	0.5±0.3	0.7±0.0	0.8±0.0
7,8-H_2PteGlu	4	2.2±0.5	1.9±0.3	1.8±0.2	1.8±0.2	1.8±0.2	1.9±0.2
5-CH_3-5,6-H_2PteGlu	5	2.4±0.6	2.4±0.6	2.3±0.5	2.2±0.6	2.0±0.4	2.0±0.4
5-CH_3-5,8-H_2PteGlu	3	1.2±0.2	1.3±0.3	1.3±0.4	1.2±0.4	1.5±0.3	1.3±0.4
5-CH_3H_4PteGlu	5	2.1±0.1	1.9±0.1	1.7±0.1	1.8±0.1	1.8±0.1	1.8±0.2
5-CHO H_4PteGlu	4	1.6±0.3	1.7±0.2	1.4±0.2	1.2±0.3	1.2±0.1	1.2±0.2

Table 6.3 Serum Crithidia factor levels after 5 mg of biopterin and after 10 mg of tetrahydrobiopterin orally (5 subjects each).

Time after doses (hour) After:	Serum Crithidia factor (ng/ml ± S.E.M.) Biopterin	Tetrahydrobiopterin
0	1.40 ± 0.23	1.67 ± 0.28
½	1.66 ± 0.25	1.89 ± 0.31
1	2.42 ± 0.12	1.90 ± 0.30
1½	3.00 ± 0.10	1.93 ± 0.35
2	3.50 ± 0.20	2.26 ± 0.41
3	3.52 ± 0.12	2.14 ± 0.35
4	2.43 ± 0.19	2.38 ± 0.32
6	-	2.43 ± 0.17
8	-	2.37 ± 0.29

Table 6.4 Serum folate of subjects taken 5 mg (or 10 mg of diastereo-isomers) of various folate orally.

Administered folate	No.of subjects	Serum L.casei(ng/ml ± S.E.M.)at time (hour)					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Pte	2	6.6±2.3	7.1±1.8	7.0±2.0	6.6±2.1	7.3±1.6	7.7±2.2
PteGlu	5	8.5±1.6	44.0± 20	133.0± 31	180.0±35	200 ± 50	226± 43
10-CHO PteGlu	6	5.2±0.3	115.0± 35	182.0± 37	185.0±15	118 ± 16	71±9.2
7,8-H_2PteGlu	4	5.9±0.7	132.5± 19	200.0± 29	260.0±27	210 ± 23	123± 21
5-CH_3-5,6-H_2PteGlu	5	6.2±1.3	37.6±5.9	32.8±4.5	27.8±2.0	24 ±0.9	21.8±0.8
5-CH_3-5,8-H_2PteGlu	3	7.3±3.0	7.7±2.7	8.2±2.7	8.3±2.8	7.9±3.1	7.2±3.6
5-CH_3H_4PteGlu	6	9.1±2.1	106.0± 15	166.0± 21	188.0±19	180 ± 15	110±9.4
5-CHO H_4PteGlu	6	10.4±2.4	96.3± 24	187.2± 26	287.0±39	199 ± 16	135±8.7

Table 6.5 Serum folate of subjects taken 5 mg (or 10 mg of diastereoisomers) of various folate orally.

Administered folate	No.of subject	Serum S.faecalis(ng/ml±S.E.M.) at time (h)					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Pte	2	0.6±0.2	0.6±0.2	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
PteGlu*	5	1.2±0.2	28.0± 15	94.0± 34	130 ± 40	140.0± 40	158± 36
10-CHO PteGlu**	6	0.8±0.1	116.0± 32	186.7± 34	181 ±6.7	104.4± 12	62.0±5.2
7,8-H_2PteGlu	4	0.7±0.1	2.6±0.2	4.4±0.5	3.0±0.2	3.3±0.3	1.2±0.3
5-CH_3-5,6-H_2PteGlu	5	0.7±0.2	1.0±0.3	1.2±0.3	1.0±0.2	0.9±0.2	0.7±0.1
5-CH_3-5,8-H_2PteGlu	3	0.5±0.1	0.7±0.1	0.9±0.2	0.8±0.2	0.6±0.2	0.6±0.2
5-CH_3H_4PteGlu	6	0.8±0.1	2.9±0.6	3.8±0.6	3.7±0.6	4.6±0.6	3.7±0.5
5-CHO H_4PteGlu	6	0.8±0.1	6.3±1.6	5.2±1.2	5.0±0.9	5.0±0.7	3.0±0.4

* the increased levels identified by bioautography as PteGlu.

** the increased levels identified by bioautography as 10-CHO PteGlu.

Table 6.6 Serum folate of subjects taken 5 mg (or 10 mg of diastereoisomers) of various folate orally.

Administered folate	No.of subject	Serum P.cerevisiae(ng/ml±S.E.M)at time (h)					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Pte	2	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
PteGlu	5	0.8±0.1	0.9±0.1	1.0±0.1	1.0±0.1	0.9±0.1	0.9±0.1
10-CHO PteGlu	6	0.6±0.1	1.0±0.1	1.2±0.1	1.4±0.1	1.1±0.1	1.3±0.1
7,8-H_2PteGlu	4	0.6±0.0	1.5±0.1	1.8±0.2	1.8±0.2	1.5±0.1	1.1±0.1
5-CH_3-5,6-H_2PteGlu	5	0.6±0.2	0.9±0.2	1.0±0.2	0.9±0.2	0.8±0.2	0.7±0.1
5-CH_3-5,8-H_2PteGlu	3	0.5±0.1	0.6±0.1	0.6±0.1	0.5±0.1	0.5±0.1	0.5±0.1
5-CH_3H_4PteGlu	6	0.6±0.1	0.5±0.1	0.7±0.1	0.6±0.1	0.8±0.1	0.7±0.1
5-CHO H_4PteGlu	6	0.6±0.1	1.9±0.6	2.0±0.6	1.8±0.5	1.7±0.2	1.3±0.1

Table 6.7 Serum folates of normal human (5 subjects) after oral administration of 5 mg of biopterin.

Time after doses (h)	L.casei (ng/ml±S.E.M.)	S.faecalis (ng/ml±S.E.M.)	P.cerevisiae (ng/ml±S.E.M.)
0	4.6 ± 0.5	0.8 ± 0.1	0.7 ± 0.1
½	4.7 ± 0.5	0.8 ± 0.1	0.6 ± 0.1
1	4.6 ± 0.5	0.7 ± 0.1	0.6 ± 0.1
1½	4.7 ± 0.5	0.6 ± 0.1	0.5 ± 0.1
2	4.9 ± 0.5	0.6 ± 0.1	0.5 ± 0.1
3	4.9 ± 0.5	0.7 ± 0.1	0.5 ± 0.1
4	4.6 ± 0.5	0.6 ± 0.1	0.5 ± 0.1

Table 6.8 Serum folates of normal human (5 subjects) after oral administration of 10 mg of tetrahydrobiopterin.

Time after doses (h)	L.casei (ng/ml±S.E.M.)	S.faecalis (ng/ml ± S.E.M.)	P.cerevisiae (ng/ml±S.E.M.)
0	7.0 ± 1.9	0.80± 0.08	0.52± 0.05
½	7.3 ± 1.9	0.76± 0.08	0.54± 0.07
1	8.3 ± 2.1	0.76± 0.07	0.52± 0.05
1½	8.8 ± 2.2	0.75± 0.10	0.60± 0.10
2	9.3 ± 2.5	0.74± 0.09	0.54± 0.05
3	9.4 ± 2.4	0.66± 0.09	0.54± 0.05
4	9.0 ± 2.0	0.68± 0.09	0.55± 0.05
6	7.9 ± 2.0	0.72± 0.09	0.56± 0.05
8	7.0 ± 2.0	0.72± 0.09	0.56± 0.05

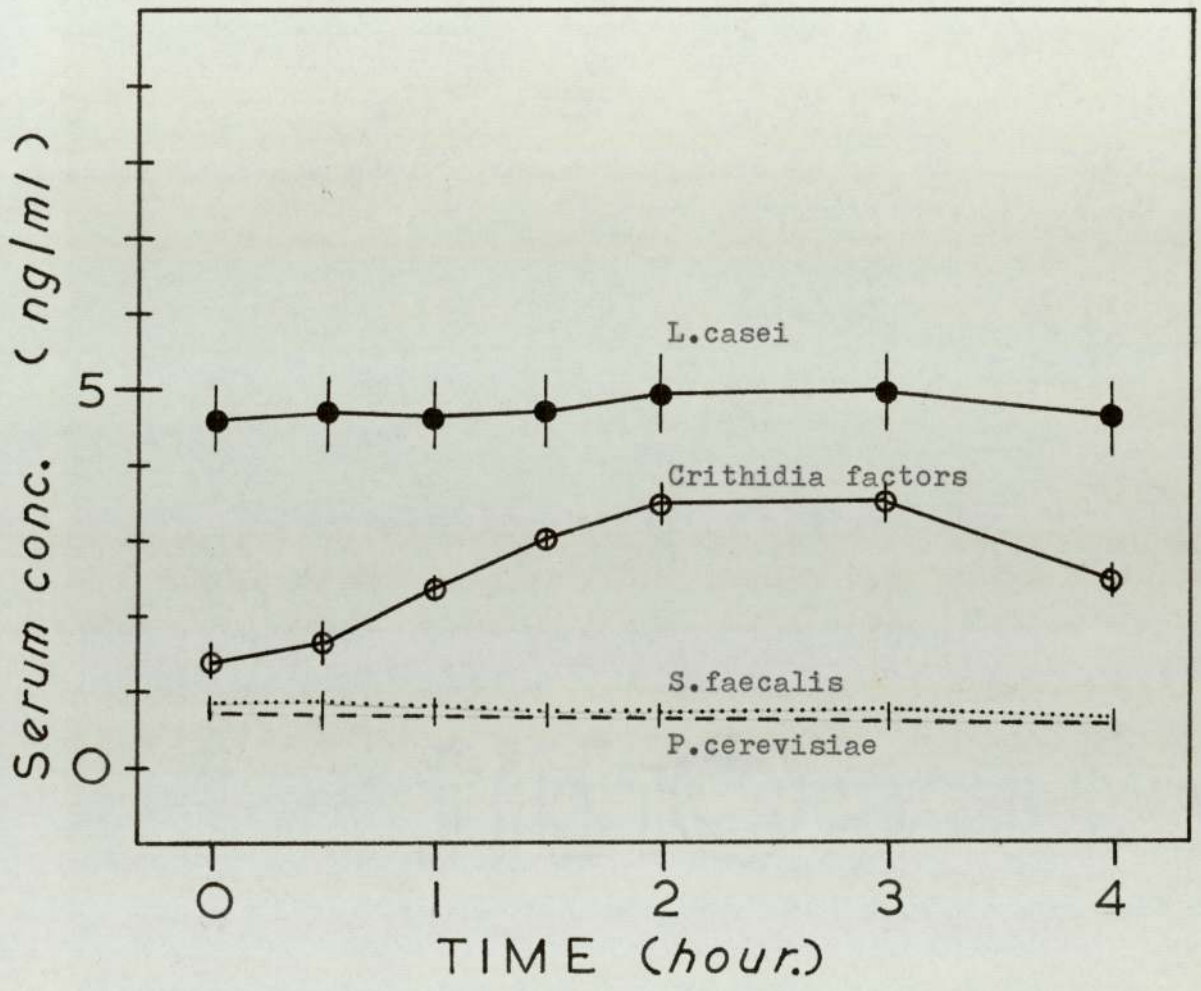


Figure 6.1 Relationship of serum folates and Crithidia factors in 5 normal human subjects taking 5 mg of biopterin orally.

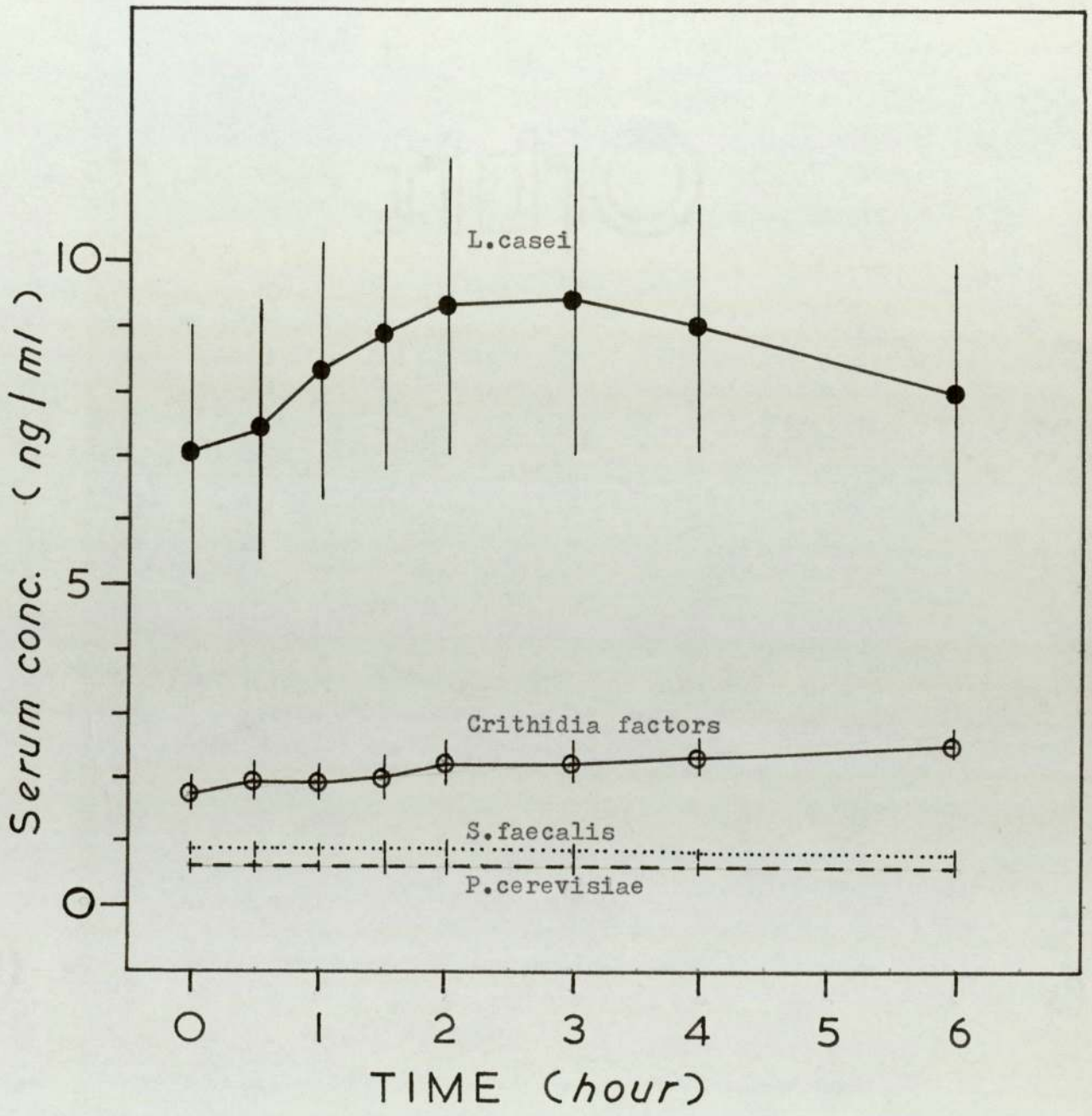


Figure 6.2 Relationship of serum folates and Crithidia factors in 5 normal human subjects taking 10 mg tetrahydrobiopterin orally.

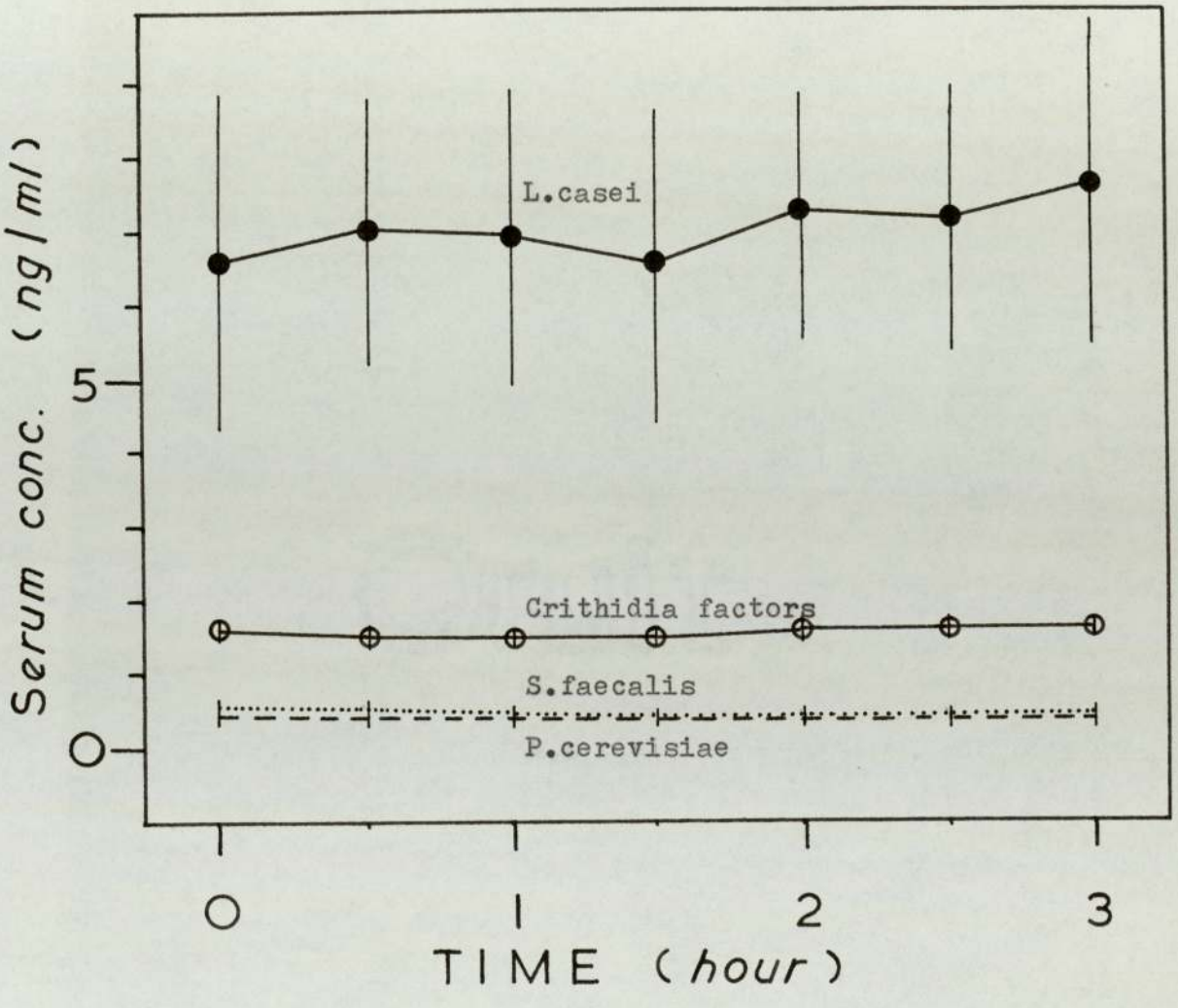


Figure 6.3 Relationship of serum folates and Crithidia factors in 2 normal human subjects taking 5 mg pteroic acid orally.

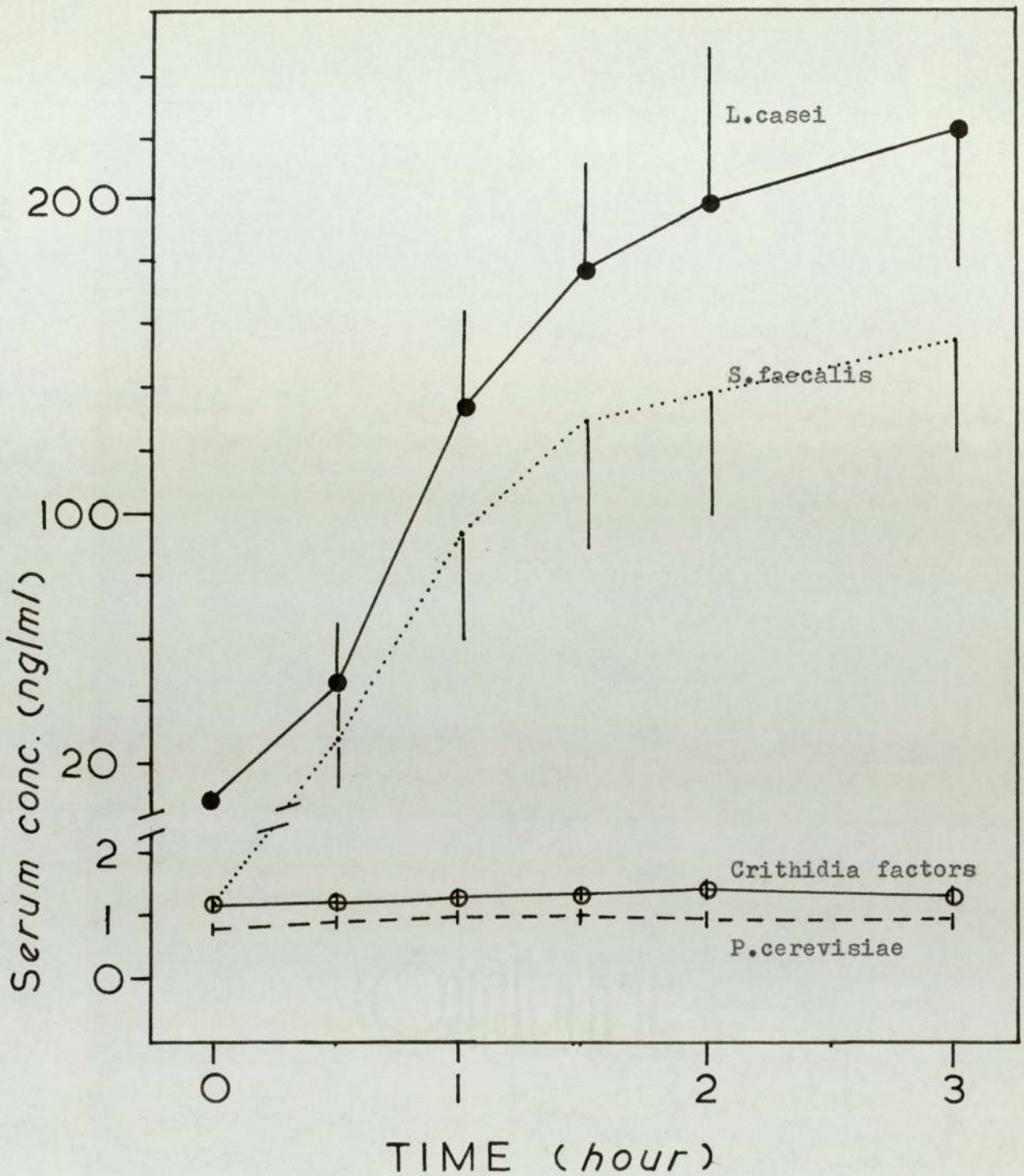


Figure 6.4 Relationship of serum folates and Crithidia factors of 3 normal human subjects taking 5 mg pteroylglutamic acid orally.

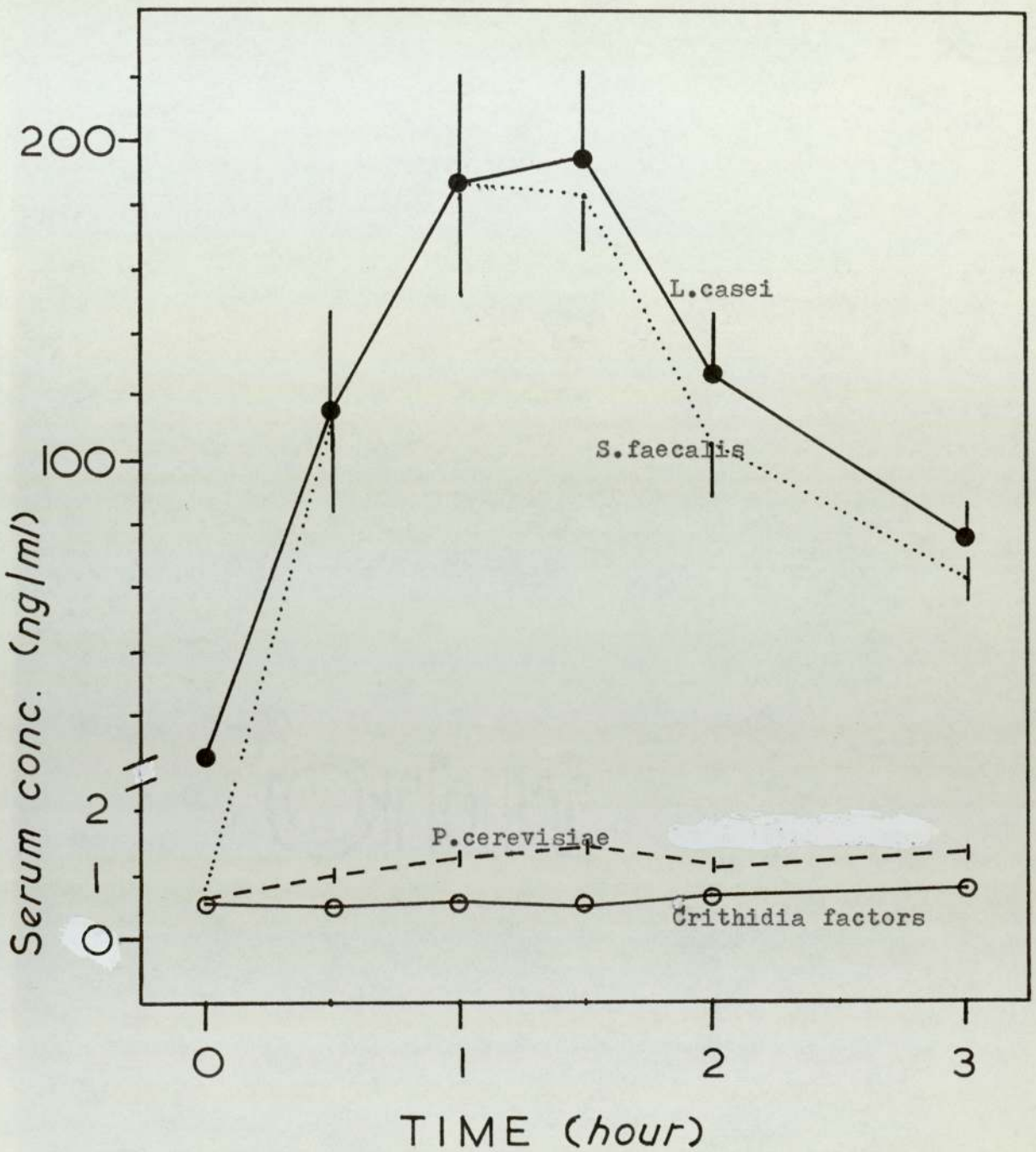


Figure 6.5 Relationship ofserum folates and Crithidia factors in 2 normal human subjects taking 5 mg of 10-formylpteroyl-glutamic acid orally.

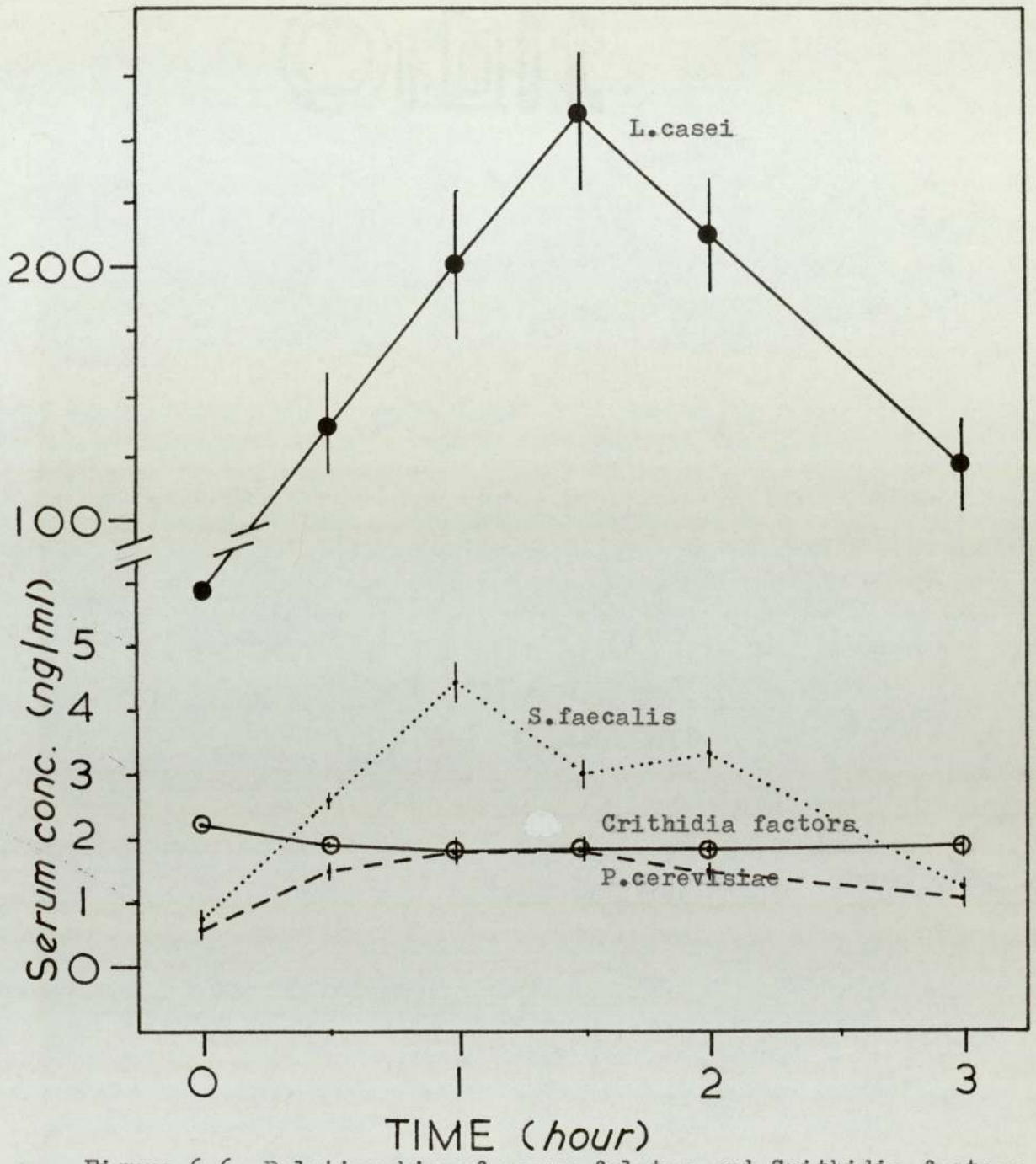


Figure 6.6 Relationship of serum folates and Crithidia factors in 4 normal human subjects taking 5 mg 7,8-dihydropteroylglutamic acid orally.

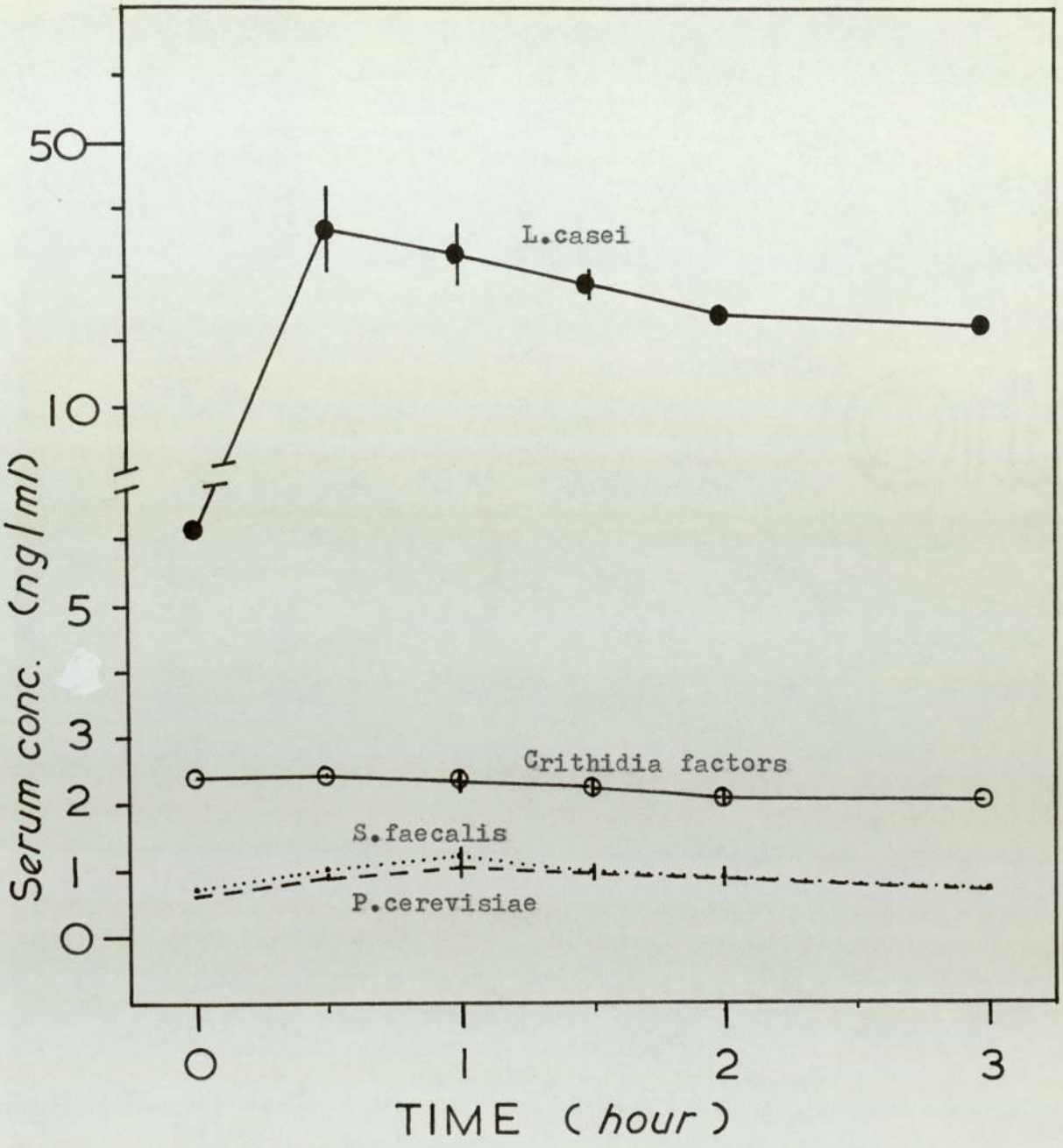


Figure 6.7 Relationship of serum folates and Crithidia factors in 5 normal human subjects taking 10 mg of 5-methyl-5,6-dihydropteroylglutamic acid orally.

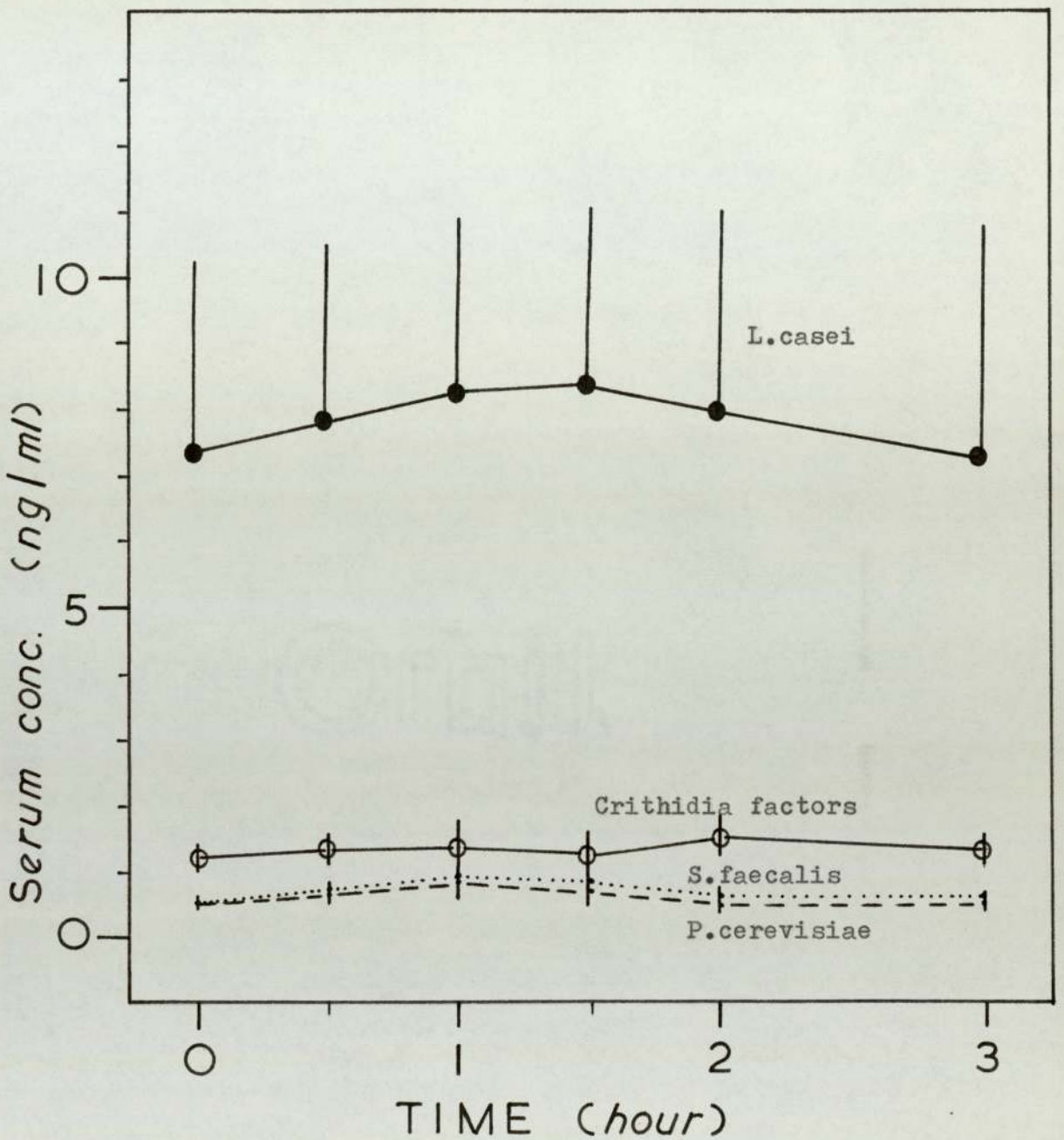


Figure 6.8 Relationship of serum folates and Crithidia factors in 3 normal human subjects taking 10 mg of 5-methyl-5,8-dihydropteroylglutamic acid orally.

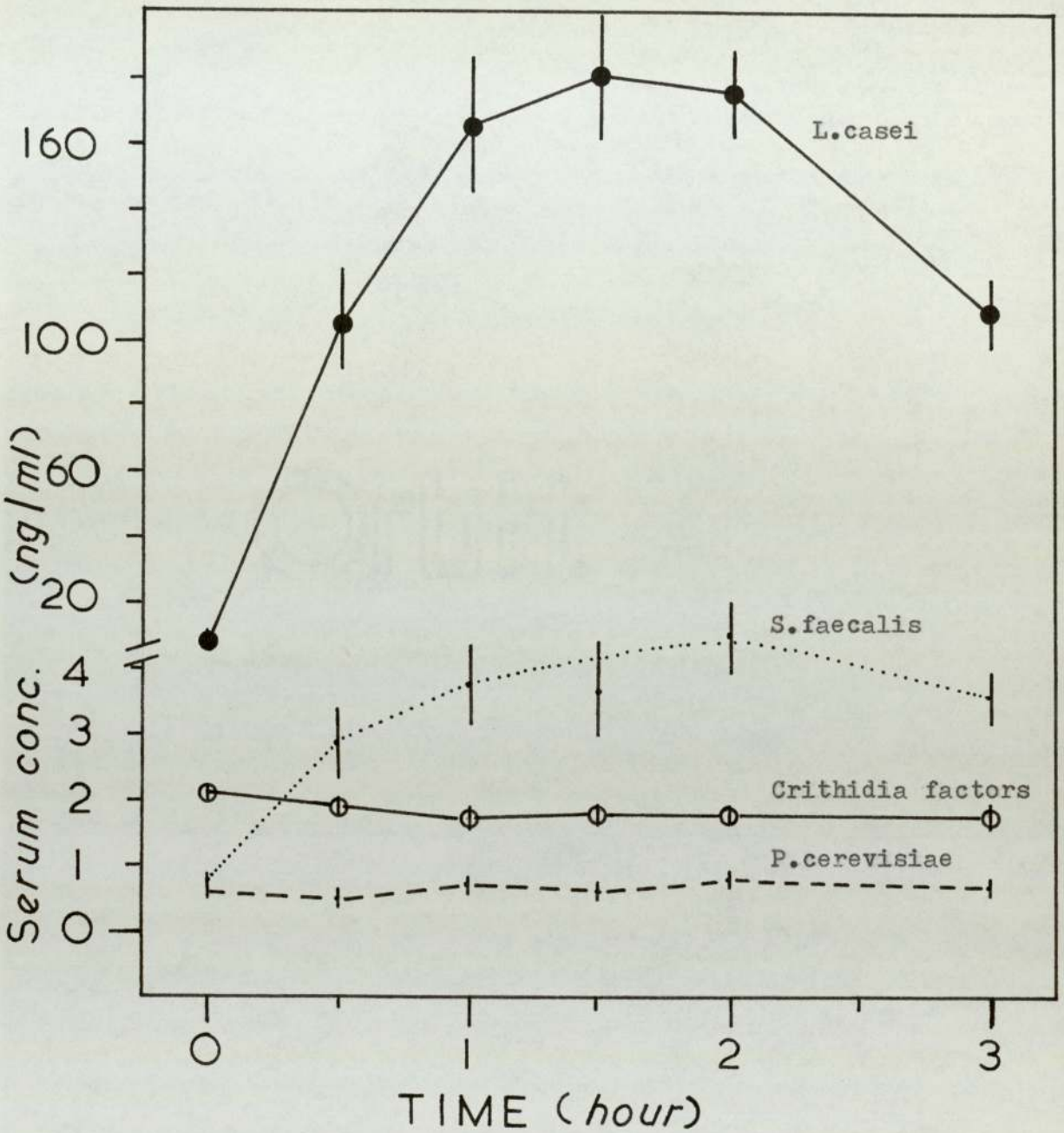


Figure 6.9 Relationship of serum folates and Crithidia factors in 5 normal human subjects taking 10 mg of 5-methyltetrahydropteroylglutamic acid orally.

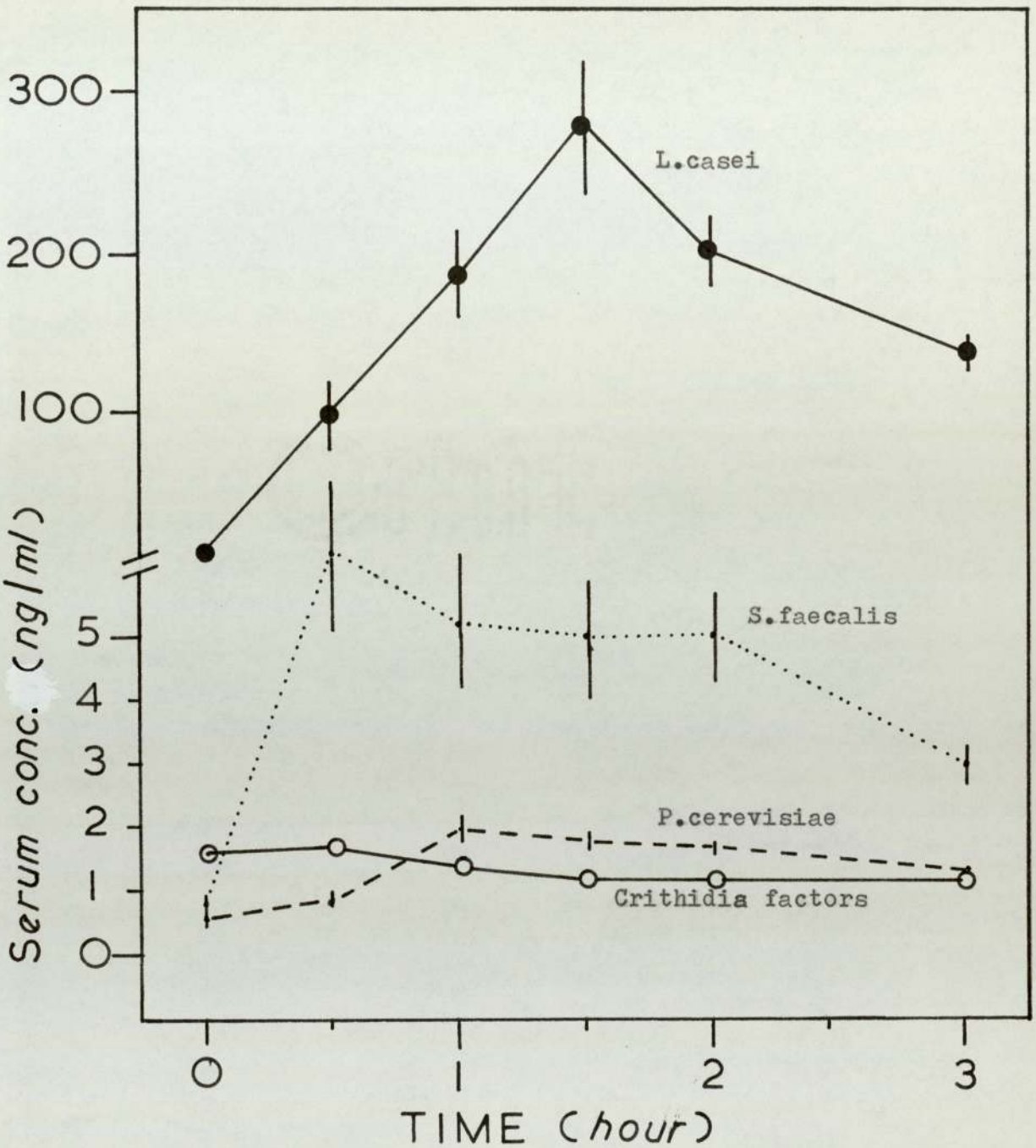


Figure 6.10 Relationship of serum folates and Crithidia factors in 4 normal human subjects taking 10 mg of 5-formyltetrahydropteroylglutamic acid orally.

Table 6.9 Serum (L.casei) folate of subjects after 10 mg of tetrahydrobiopterin in subjects A = starting serum folate of 6.0 ng/ml or higher, B = starting serum folate lower than 6.0 ng/ml and C = starting after 5 g of sodiumbicarbonate, all tests are done by oral administration. No of subjects in each group in parenthesis.

Time after doses (hour)	L.casei (ng/ml ± S.E.M.)		
	A(3)	B(2)	C(3)
0	9.6 ± 1.8	3.0 ± 0.0	3.4 ± 0.8
½	10.0 ± 1.5	3.2 ± 0.2	3.3 ± 0.8
1	11.7 ± 1.0	3.2 ± 0.2	3.6 ± 1.3
1½	12.7 ± 1.3	3.2 ± 0.2	3.4 ± 1.3
2	13.3 ± 0.7	3.2 ± 0.2	3.3 ± 1.0
3	13.3 ± 0.7	3.5 ± 0.5	3.3 ± 1.0
4	-	-	4.1 ± 1.5
6	11.0 ± 1.0	3.2 ± 0.2	-

Discussion.

The origin of biopterin and its derivatives in human are not clearly known. Small amount of orally administered doses of biopterin and tetrahydrobiopterin reached the blood circulation as seen by microbiological assay with Crithidia fasciculata. After 5 mg of biopterin and 10 mg of tetrahydrobiopterin the serum levels of Crithidia factors are summarized in Table 6.1 and Table 6.2,respectively. These results indicated that biopterin and tetrahydrobiopterin in human were not mainly derived from dietary sources. It was established that in the rat eventhough they were fed on the biopterin free diets for two generations there still be a constant excretion of urine Crithidia factors at about 30 ug/day (Pabst and Rembold,1966). Studies similar to those performed with the rat are not always be done easily in man. Eventhough small amount of biopterin or tetrahydrobiopterin were absorbed after oral doses as previously discussed the microbiological assay of human urine for the Crithidia factors showed that in normal human the daily excretion of the Crithidia factors were around 0.5 to 2.0 mg (Patterson et al 1956; Fugushima and Shiota,1972;Leeming,1975).

Microbiological assays with L.casei,S.faecalis and P.cerevisiae of the from subjects after oral doses of 5 mg of biopterin and 10 mg biopterin were done. Assays of those samples from subjects taken 5 mg of biopterin showed no change in their serum folate levels as shown in Table 6.7 or figure 6.1 but a raised serum folate (L.casei) was seen amongst some of those subjects taken 10 mg of tetrahydrobiopterin orally. The raised serum folate (L.casei) was found only amongst those subjects with the starting serum folate of 6.0 ng/ml or higher and oral

administration of 5 g of sodiumbicarbonate before the doses of tetrahydrobiopterin did not change these observations and they are shown in Table 6.9. The raised serum (L.casei) folate found amongst these subjects may have been derived from the displacement (Johns and Plenderleith,1963)of the tissue folate i.e. at the gut wall which is readily diffused (Blair et al 1973;Elsborg, 1974). The zero effect or less effect on those subjects with serum folate (L.casei) lower than 6.0 ng/ml may be derived from the lack of or unsaturated tissue folates. These observations may be serve as a useful tool for the early detection of folate deficiency.

After oral administration of folates the results of their serum folates and Crithidia factors are summarized in Tables 6.2,6.4,6.5,6.6 or in Figures 6.3 to 6.10. In all cases the serum levels of Crithidia factors were not significantly changed. This view had also been shown with folic acid (Fugushima and Shiota,1972). It was shown that urine excretion of Crithidia factors were altered in some diseases (Leeming and Blair,1974). Whilst oral administration of folic acid derivatives, biopterin and tetrahydrobiopterin have nearly no effect on the serum Crithidia factors the altered excretion of urine Crithidia factors were known to be raised and decreased (Leeming and Blair, 1974). There is no correlation between Crithidia factors and folates seen in normal human. The folate is independent of Crithidia factors and the very small serum changes after oral administration of the Crithidia factors (biopterin and tetrahydrobiopterin) suggested that they might have their own synthetic pathways in man. Pabst and Rembold (1966) had proposed a de novo

synthesis of biopterin in the rat based on their observations that the biopterin excretion was not altered by feeding biopterin free diet for two generations. They also observed that varying amounts of folic acid or riboflavin were not effect the biopterin excretion. Biopterin excretion in man were reported varied from 0.5 to 2.0 mg daily as previously mentioned. Eventhough there is a little relationship between tetrahydrobiopterin and serum folate little is known about biopterin derivatives and folate metabolism relationship. The experiments for the studies of folate metabolism and their relationship to Crithidia factors in some abnormal conditions were studied as will be described latter on in Chapter 7.

Summary.

Oral administration of various folates have no effect on serum Crithidia factors. Oral administration of biopterin is absorbed a little more than tetrahydrobiopterin and the former has no effect on serum (L.casei) levels. Tetrahydrobiopterin raised serum folate (L.casei) of subjects with the starting serum (L.casei) folate of 6.0 ng/ml or higher but there is no effect on those with starting serum folate of less than 6.0 ng/ml.

Chapter 7.

Studies on other pteridines and their effects.

Studies of pteroic acid and pteroylglutamic acid.

Pteroic acid is a compound which with its L-glutamic acid amide derivatives forms a family of folates. Studies of its microbiological activity shows that pteroic acid supports the growth of S.faecalis but not L.casei and P.cerevisiae (Chapter 3).

The amide of pteroic acid with D-glutamic acid is available by chemical synthesis and a study of the microbiological activity of pteroyl-D-glutamic acid showed it to be inactive for supporting the growth of L.casei,S.faecalis and P.cerevisiae (Chapter 3).

Pteroic acid is a degradation product of pteroylglutamic acid in microbiological system and this is used for the preparation of the compound (Houlihan,Boyle and Scott,1972).It is also known to be the precursor of folates biosynthesis in the microbiological system as discussed in Chapter 1. There is no known biochemical role of pteroic acid and pteroyl-D-glutamic acid in human.

In the experiments to be described an attempt was made to study these compounds in normal humans.The oral administration of pteroic acid and pteroyl-D-glutamic acid were studied to compare with folate metabolism and handling in man after oral administration.

Studies of folate antagonist and dihydrofolate reductase inhibitors.

The folic acid antagonists are known to have anti-leukemia activity (Farber et al 1948). They were shown to have a biochemical role as dihydrofolate reductase inhibitors and this property is used as a method for their detection (Bertino and Fischer,1964). Investigations into the anti-microbial actions of 2,4-diaminopyrimidines showed them to have anti-folate activity (Hitchings and Burchall,1965). Ferone and his co-workers showed

that pyrimethamine (Figure 7.1) and trimethoprim (Figure 7.2) acted as dihydrofolate reductase inhibitors.They found that dihydrofolate reductase from various sources exhibit differences in sensitivity to the inhibition by those diaminopyrimidines (Ferone,Burchall and Hitchings,1969).

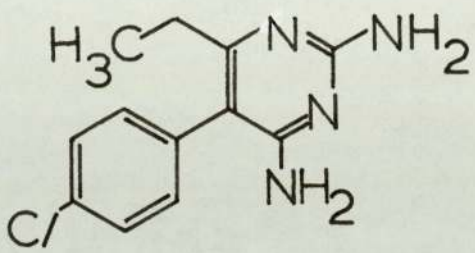


Figure 7.1 Structure of pyrimethamine.

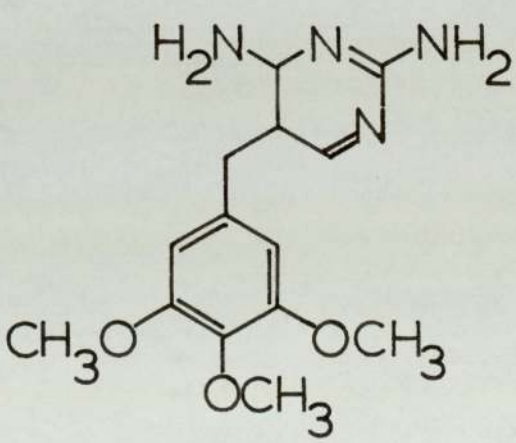


Figure 7.2 Structure of trimethoprim.

The inhibition of dihydrofolate reductases in various sources by pyrimethamine and trimethoprim were modified from Ferone et al (1969) and shown in Table 7.1. 10-methyl-4-amino-pteroylglutamic acid (methotrexate) one of the folate antagonists has been shown to have the most effective curing effect in cancer chemotherapy and known to have a biochemical role as dihydrofolate reductase inhibitor (Condit,1960;Condit and Eliel,1960;

Sullivan et al 1959;Wright et al 1960;Hertz et al 1961;Hustu et al 1973;Frei et al 1975).

Table 7.1

Inhibition of dihydrofolate reductases from various sources by pyrimethamine and trimethoprim (Ferone et al 1969).

Inhibitor	Concentration (x 10^{-8}M) for 50% inhibition		
	Mammalian (rat liver)	Bacterial (E.coli)	Protozoal (P.berghei)
Pyrimethamine	70	250.0	0.05
Trimethoprim	26 000	0.5	7.00

Pharmacokinetics of various routes of methotrexate after oral,intravenous and intrathecal administrations had been studied (Condit,1960;Henderson et al 1965;Huffman et al 1973; Jacobs et al 1975;Evans et al 1964).There is no information on metabolism of folates following methotrexate chemotherapy other than the rescue effect of 5-formyltetrahydropteroyl-glutamic acid (Duff et al 1961;Sullivan et al 1959;Goldin et al 1953) or with pteroylglutamic acid (Goldin et al 1953).Experiments on the rat showed that 5-methyltetrahydropteroylglutamic acid can protect against the lethal effects of repeated injections of methotrexate (Blair and Searle,1970).

In these experiments the metabolism of oral methotrexate have been studied.The metabolism of oral folates after previous oral doses of 10 mg of methotrexate have also been studied.The inhibition of growth of the microorganisms,L.casei,S.faecalis and

P.cerevisiae by methotrexate has also been studied.Studies of inhibition effect of methotrexate in the bioautography of L.casei have led to a method of detection of methotrexate and folate in samples containing methotrexate.

Materials and Methods.

4-amino-10-methylpteroylglutamic acid (methotrexate) tablets or sodium parenteral were obtained from Lederle laboratories division,American Cyanamid Company.They were used for oral absorption studies and also used for the inhibition studies.Folates used in these studies were pteroylglutamic acid, 7,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid.

Pteroylglutamic acid was purchased from Koch-Light Laboratories,Cornbrook,Buckinghamshire.5-formyltetrahydropteroylglutamic acid was a gift from Lederle Laboratories. 5-formyltetrahydropteroylglutamic acid 5 mg doses for intravenous injection were obtained from Lederle Laboratories.Other folates were prepared as described in Chapter 3.Pteroic acid was a gift from Professor I.H.Rosenberg and pteroyl-D-glutamic acid was a gift from Dr.Yeadon of Lederle Laboratories Division,American Cyanamide. Metabolism of oral pteroic acid and pteroyl-D-glutamic acid in normal human were performed similar to those of other folates used in Chapter 5.

Patients with psoriasis on treatment with methotrexate were used in the studies of folate metabolism.Blood samples were collected just before oral doses (10 mg) of methotrexate were given and then at 1,2,3,4 and 24 hours.The metabolism of folates were studied using subjects who had had an oral dose of

10 mg of methotrexate 24 hours prior to the test. Oral folates studies in this manner were pteroylglutamic acid,7,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid was studied both orally and intravenously.

Table 7.2

Schedule of the studies of inhibition of methotrexate on microbiological assay of folates with L.casei,S.faecalis and P.cerevisiae.

Concentration of methotrexate(ng/ml)	Concentration of added folate*(ng/ml)					
	1	2	3	4	5	6
0	0	2	5	10	20	30
0.5	0	2	5	10	20	30
5.0	0	2	5	10	20	30
50.0	0	2	5	10	20	30
500.0	0	2	5	10	20	30

* indicates pteroylglutamic acid,7,8-dihydropteroylglutamic acid, 5-methyltetrahydropteroylglutamic acid,10-formylpteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid were used in the studies with L.casei. Pteroylglutamic acid,7,8-dihydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid were used with S.faecalis and only 5-formyltetrahydropteroylglutamic acid was used with P.cerevisiae. Concentration of the appropiate folates in each assay media were similar to those of the assay media without methotrexate and as shown in the results section.

Blood samples from these absorption studies were collected at time 0,1,2,3,4,24 hours for oral absorption studies and at time 0,0.5,1,2,3,4, and 24 hours for intravenous injection studies. Blood samples were separated,sera were collected with

and without 2 to 5 mg/ml ascorbic acid added before kept frozen at -20°C until assayed.

Studies of the inhibition effects of methotrexate on microbiological assay of folates with L.casei,S.faecalis and P.cerevisiae were done by adding various amounts of methotrexate into the assay media. The schedule of the studies were set out as shown in Table 7.2.

Blood samples from patients on treatment with dihydrofolate reductase inhibitor drugs and from these absorption studies were microbiologically assayed with L.casei, S.faecalis and P.cerevisiae. Microbiological assay with Crithidia fasciculata was done in some samples. Bioautography with L.casei,S.faecalis and P.cerevisiae were carried out for those samples from subjects after oral methotrexate with and without following folates. Approximated determinations of methotrexate concentration were done simply by using its inhibition property on L.casei. Tetrazolium bioautography of folates with L.casei (Chapter 2) gives intensed red spots of reduced tetrazolium compounds at the folate supported growth zones. Without folate there is normally a pale background of red colour due to the slow growth of microorganisms,possibly by using their stored folates. The colour of the background may be increased by using a higher amount of microorganisms or by using microorganisms without washing. Employing these ideas a method of determination of methotrexate and its related compounds is possible.

Qualitative and quantitative analysis can be done by using known amounts and concentrations of methotrexate on a precoated thin-layer plate as standard. Similar methods can be

applied with sera or urine samples for determination of methotrexate. Plates of methotrexate standards and samples can be prepared by two methods. The first is by running a thin-layer chromatogram in the solvent systems as usually employed for folate bioautography (Leeming et al 1973;Brown et al 1974). The second method is by application of spots similar to the previous method and instead of chromatographing the spots to let them diffuse for equal times.This is achieved by wetting the applied plates with water or by application of samples to a pre-wetted plate or plates.The prepared plates were then treated similarly to the bioautography of folates with L.casei and in some with S.faecalis and P.cerevisiae (Chapter 2),double amount of microorganisms were used.

Results.

The inhibition effects of methotrexate were studied with growing cultures of L.casei,S.faecalis and P.cerevisiae. Various degrees of inhibition effects were seen in all three cultures.With L.casei total inhibition was seen at a concentration of methotrexate as low as 0.5 ng/ml of the added sample. The results are summarized in Table 7.3. With S.faecalis the inhibition effect of methotrexate was less than that with L.casei. Total inhibition of S.faecalis was found at a concentration of methotrexate higher than 50 ng/ml of the added sample and results are summarized in Table 7.4 when pteroylglutamic acid is used in the test and Table 7.5 and Table 7.6 are the results when 7,8-dihydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid are used,respectively.Results of studies with P.cerevisiae are summarized in Table 7.7.

Bioautography of methotrexate standards at various concentrations are shown in Plate 7.1 and Plate 7.2 for thin-layer chromatogram and simple diffusion bioautography with L.casei,respectively. Serum samples from subjects after oral methotrexate were microbiologically assayed with L.casei,S.faecalis and P.cerevisiae are summarized together with the serum methotrexate concentrations in Table 7.8. Bioautography of serum methotrexate are shown in Plate 7.2 and Plate 7.3.

Serum samples from subjects orally administered with various folates 24 hours after oral methotrexate were also assayed microbiologically and bioautographically.Analysis of samples after 5 mg oral pteroylglutamic acid showed that there is an increase in folate level detected only with S.faecalis.The results are confirmed by bioautography as pteroylglutamic acid and summarized in Table 7.9. After oral administration of 7,8-dihydropteroyl-glutamic acid there is an increase in folate level detected only by S.faecalis (Table 7.10).5-methyltetrahydropteroylglutamic acid given orally to these subjects give very little increase in serum folate as detected by S.faecalis (Table 7.11). 5-formyl-tetrahydropteroylglutamic acid given (5 mg) intravenously to subjects post 24 hour oral methotrexate gave increased folate levels as detected by both S.faecalis and P.cerevisiae (Table 7.12). 5-formyltetrahydropteroylglutamic acid was also given orally to a subject post 24 hour methotrexate and microbiological assays showed small increase in P.cerevisiae activity (Table 7.13).

Serum folate of some patients on treatment with dihydrofolate reductase inhibitor drugs were microbiologically assayed and are summarized in Table 7.14.

Microbiological assay with Crithidia fasciculata of samples after various absorption studies are summarized in Table 7.15.

Studies of serum folates after oral administration of 5 mg pteroic acid in two normal human subjects are summarized in Table 7.16.After oral administration of 5 mg pteroyl-D-glutamic acid there is no effect on serum folates of a normal human (Table 7.17).

Table 7.3. The inhibition of methotrexate on the microbiological assay of folates with L.casei.

Concentration of methotrexate(ng/ml)	Concentration of folate * (ng/ml)					
	1	2	3	4	5	6
0.00	0	2	5	10	20	30
0.50	0	0	0	0	0	0
5.00	0	0	0	0	0	0
50.00	0	0	0	0	0	0
500.00	0	0	0	0	0	0

* indicates same results obtained when pteroylglutamic acid, 7,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid,10-formylpteroylglutamic acid and 5-formyltetrahydropteroyl-glutamic acid were used in the tests.

The relationship of serum folates , Crithidia factor and methotrexate is shown in Figure 7.3. Figure 7.4 showed the first order plot of serum methotrexate clearance.

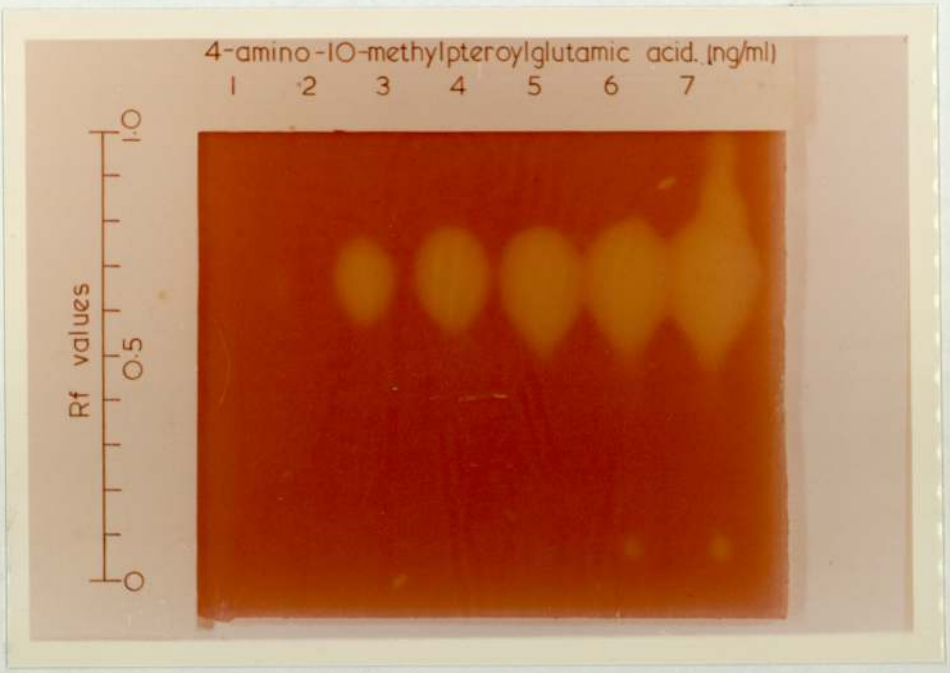


Plate 7.1 Bioautography of methotrexate with L.casei

1 = 0 ng/ml
2 = 5 ng/ml
3 = 50 ng/ml
4 = 100 ng/ml
5 = 200 ng/ml
6 = 300 ng/ml
7 = 500 ng/ml

Table 7.4 The inhibition of methotrexate on the microbiological assay of pteroylglutamic acid with S.faecalis.

Concentration of methotrexate(ng/ml)	Concentration of PteGlu * (ng/ml)					
	1	2	3	4	5	6
0.0	0	2.0±0.0	5.0±0.0	10.0±0	20.0±0	30.0±0
0.5	0	1.9±0.1	4.8±0.2	9.6±0.5	19±1.0	27±2.0
5.0	0	1.0±0.1	3.2±0.3	6.0±1.0	11±1.5	20±1.0
50.0	0	0	0	0	1.0±0	2.0±0
500.0	0	0	0	0	0	0

* indicates results obtained from samples contained folic acid equal to those without methotrexate in each column (average of 3 results ± S.E.M.).

Table 7.5 The inhibition of methotrexate on the microbiological assay of 7,8-dihydropteroylglutamic acid with S.faecalis.

Concentration of methotrexate(ng/ml)	Concentration of 7,8-H_2PteGlu*(ng/ml)					
	1	2	3	4	5	6
0.0	0	2.0±0.0	3.0±0.0	5.0±0.0	8.0±0.0	10.0±0.0
0.5	0	1.8±0.1	2.8±0.2	4.8±0.2	7.5±0.5	9.7±0.3
5.0	0	1.0±0.2	2.0±0.3	3.0±0.5	5.0±0.5	6.0±1.0
50.0	0	0	0	0	0	0
500.0	0	0	0	0	0	0

* indicates average of 3 results ± S.E.M. from samples contained 7,8-dihydropteroylglutamic acid equal to those without methotrexate in each column.

Table 7.6 The inhibition of methotrexate on the microbiological assay of 5-formyltetrahydropteroylglutamic acid with S.faecalis.

Concentration of methotrexate(ng/ml)	Concentration of 5-CHO H_4PteGlu*(ng/ml)					
	1	2	3	4	5	6
0.0	0	1.5±0.1	3.0±0.1	5.0±0.5	8.0±1.0	13.0±1.0
0.5	0	1.5±0.1	3.0±0.1	4.9±0.5	7.9±1.0	12.8±1.0
5.0	0	1.5±0.1	3.0±0.1	4.5±0.5	6.0±1.0	11.0±1.0
50.0	0	0	0	0	2.5±0.5	5.0±0.5
500.0	0	0	0	0	0	0

* indicates results as average of 3 results±S.E.M. from samples contained 5-CHO H_4PteGlu equal to those without methotrexate in each column.

Table 7.7 The inhibition of methotrexate on the microbiological assay of 5-formyltetrahydropteroylglutamic acid with P.cerevisiae.

Concentration of methotrexate(ng/ml)	Concentration of 5-CHO H_4PteGlu*(ng/ml)					
	1	2	3	4	5	6
0.0	0	1.0±0.0	3.0±0.5	5.0±0.5	8.0±0.5	13.0±0.5
0.5	0	1.0±0.0	3.0±0.5	5.0±0.5	8.0±0.5	13.0±0.5
5.0	0	1.0±0.0	3.0±0.0	4.8±0.5	6.5±0.5	11.0±0.5
50.0	0	0.9±0.1	2.9±0.1	4.8±0.5	7.0±1.0	8.5±0.5
500.0	0	0.5±0.0	2.6±0.2	4.2±0.5	5.0±0.5	5.7±0.2

* indicates results as average of 3 results±S.E.M. from samples contained 5-CHO H_4PteGlu equal to those without methotrexate in each column.

Table 7.8 Analysis of sera from subjects taken 10 mg of methotrexate orally.

Serum constituent (ng/ml ± S.E.M.)	No.of subjects	Serum concentration at time (h)after dose					
		T_0	T_1	T_2	T_3	T_4	T_{24}
Methotrexate	2	0	200±25	100±0.0	70±5.0	50±0.0	20±5.0
L.casei	2	4.5±1.5	0	0	0	0	0
S.faecalis	2	0.9±0.1	0	0.1±0.1	0.1±0.1	0.2±0.2	0.5±0
P.cerevisiae	2	0.6±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.6±0.0
Crithidia factors	2	1.5±0.5	2.0±0.2	2.8±0.2	3.7±0.1	4.0±0.1	6.0±1.0

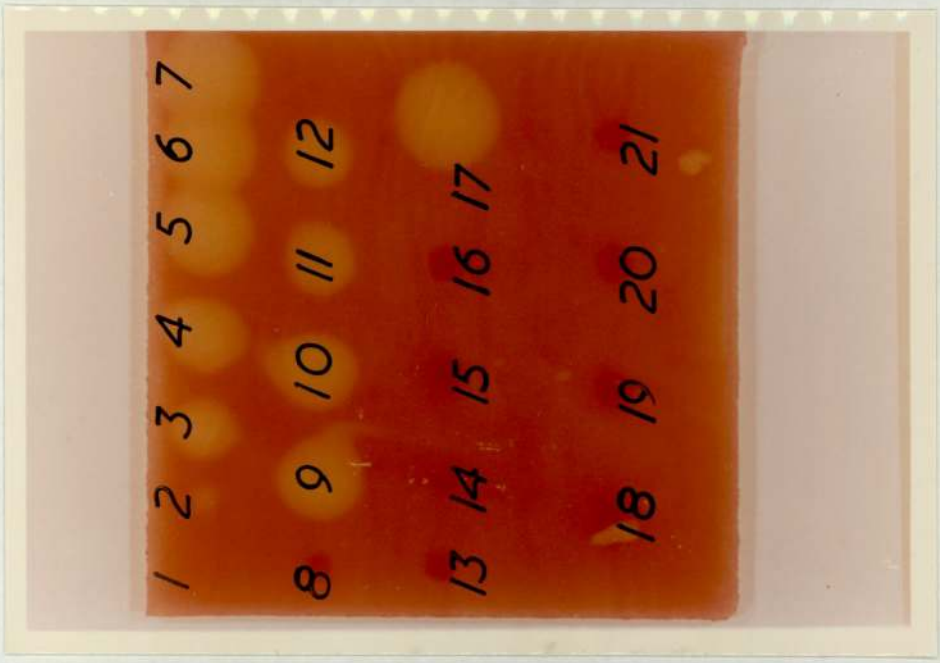


Plate 7.2 Bioautography of diffused spots of methotrexate and sera of subjects taking MTX 10 mg orally. Numbers 1 to 7 are similar to those of plate 7.1 and number 7 = number 17. Numbers 8 to 12 are sera from a subject taking MTX at time 0,1,2,3 and 4 hours respectively. Numbers 13 to 16 are sera from subjects 48 hours after MTX and numbers 18 to 21 are sera from normal humans.

Table 7.9 Serum folates after oral administration of 5 mg of pteroylglutamic acid post (10 mg) 24 hour oral methotrexate.

Time (hour) after dose	No.of subjects	Serum folates (ng/ml)±S.E.M.		
		L.casei	S.faecalis	P.cerevisiae
0	2	0	0	0.2±0.0
1	2	0	30±0.0	0.2±0.0
2	2	0	80±10.0	0.2±0.0
3	2	0	100±10.0	0.2±0.0
4	2	0	120±5.0	0.2±0.0
24	2	0	6±0.0	0.2±0.0

Table 7.10 Serum folates after oral administration of 5 mg of 7,8-dihydropteroylglutamic acid post (10 mg) 24 hour oral methotrexate.

Time (hour) after dose	No.of subjects	Serum folates (ng/ml)±S.E.M.		
		L.casei	S.faecalis	P.cerevisiae
0	1	0	0.0	0.2
1	1	0	1.0	0.2
2	1	0	4.5	0.2
3	1	0	4.5	0.2
4	1	0	4.5	0.2
24	1	0	0.0	0.2

Table 7.11 Serum folates of patients after oral administration of 5 mg of microbiological active 5-methyltetrahydropteroyl-glutamic acid (10 mg of diastereoisomers) post 24 hour methotrexate (10 mg).

Time (hour) after dose	No.of subjects	Serum folates (ng/ml) ± S.E.M.		
		L.casei	S.faecalis	P.cerevisiae
0	2	0	0	0.4±0.2
1	2	0	2.0±0.0	0.4±0.2
2	2	0	1.4±0.1	0.5±0.2
3	2	0	1.1±0.1	0.5±0.2
4	2	0	0.7±0.1	0.5±0.3
24	1	2	0.6	0.2

Table 7.12 Serum folates after intravenous administration of 5 mg of microbiologically active 5-formyltetrahydropteroylglutamic acid post 24 hour of oral (10 mg) methotrexate.

Time (hour) after dose	No.of subjects	Serum folates (ng/ml) ± S.E.M.		
		L.casei	S.faecalis	P.cerevisiae
0	1	0	0.0	0.2
1	1	0	200	200
2	1	0	25	25
3	1	0	15	16
4	1	0	1.0	1.8
24	1	0	0.0	0.2

Table 7.13 Serum folates of a patient after oral administration of 5 mg 5-formyltetrahydropteroylglutamic acid post 24 hour oral 10 mg of methotrexate.

Time (hour) after dose	No.of subjects	Serum folates (ng/ml) ± S.E.M.		
		L.casei	S.faecalis	P.cerevisiae
0	1	0	0.0	0.2
1	1	0	0.1	2.4
2	1	0	0.1	1.0
3	1	0	0.0	0.5
4	1	0	0.0	0.3
24	1	0	0.0	0.2

Table 7.14 Serum folates of patients on treatment with dihydrofolate reductase inhibitor drugs.

Microbiological assay with	Psoriatics(6)* (ng/ml)±S.E.M.	Psoriatics(4)** (ng/ml)±S.E.M.	Psoriatics(4)*** (ng/ml)±S.E.M.
L.casei	0	2.3±0.5 (2.0-3.6)	1.0±0.5 (0.5-1.5)
S.faecalis	0.73±0.2 (0.00-1.0)	1.0±0.2 (0.7-1.3)	0.3±0.3 (0.0-0.6)
P.cerevisiae	0.63±0.2 (0.30-0.8)	0.8±0.1 (0.7-1.0)	0.4±0.4 (0.0-0.9)

* Receiving methotrexate 5 to 25 mg weekly.

**Receiving azothioprine 2 to 5 mg/kg body weight daily.

***Receiving septrin 960 mg twice daily.

( ) Number of subjects.

Table 7.15 Serum Crithidia factors after various folates at doses of 5 mg of microbiologically active materials (10 mg of diastereoisomers) post 24 hour oral administration of 10 mg methotrexate.

Administered folates	No.of subjects	Crithidia factors (ng/ml)±S.E.M.at time(h)					
		T_0	T_1	T_2	T_3	T_4	T_{24}
PteGlu	2	7.3±1.2	10.0±2	11.5±2.5	11.5±1.5	11.5±1	8.5±0.0
7,8-H_2PteGlu	1	6.5	6.5	6.0	6.5	8.5	6.5
5-CH_3H_4PteGlu	2	4.7±0.1	5.1±0.4	5.0±0	4.9±0.1	4.9±0.1	4.9±0.1
5-CHO H_4PteGlu*	1	7.5	7.5	8.5	9.5	10.5	8.5

* indicates folate given intravenously whilst others are given orally.

Table 7.16 Study of pteroic acid: Serum folates of two normal human subjects taken 5 mg pteroic acid orally.

Time after doses(hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(ng/ml) ±S.E.M.	
			10-CHO PteGlu	P.cerevisiae
0	6.6±2.0	6.0±2.0	0.1±0.1	0.5±0.1
½	7.1±2.0	6.5±2.0	0.1±0.1	0.5±0.1
1	7.0±2.0	6.5±2.0	0	0.5±0.1
1½	6.6±2.0	6.1±2.0	0	0.5±0.1
2	7.2±2.0	6.5±2.0	0	0.5±0.1
3	7.7±2.0	7.2±2.0	0	0.5±0.1

* indicates sumation of activities of 5-methyltetrahydropteroylglutamic acid and of 5-methyl-5,6-dihydropteroylglutamic acid.

Table 7.17 Study of pteroyl-D-glutamic acid: Serum folates of one normal human subject taken 5 mg pteroyl-D-glutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(ng/ml) ±S.E.M.	
			10-CHO PteGlu	P.cerevisiae
0	9.0	8.2	0	0.8
½	9.0	8.2	0	0.8
1	9.0	8.2	0	0.8
2	9.0	8.3	0	0.7
3	8.4	7.7	0	0.7
4	8.6	7.8	0	0.8

* indicates summation of activities of 5-methyltetrahydropteroyl-glutamic acid and of 5-methyl-5,6-dihydropteroylglutamic acid.

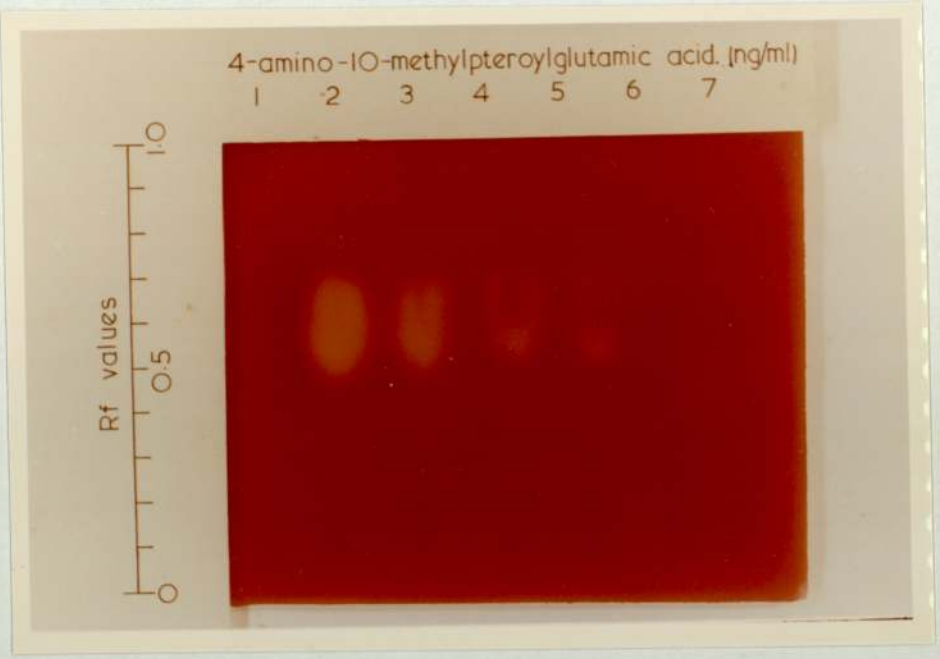


Plate 7.3a Bioautography of sera from subjects taking 10 mg of methotrexate orally. Numbers 1 to 7 for times 0,1,2,3,4,48 and 48 hours after doses respectively.

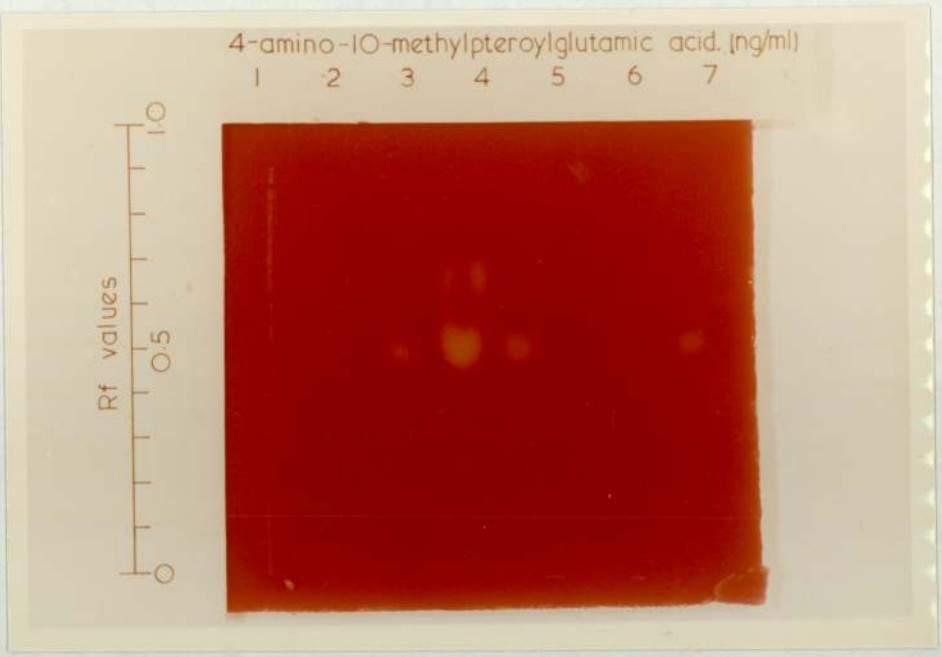


Plate 7.3b Bioautography of sera from subjects taking 10 mg of methotrexate orally. Numbers 1 and 2 samples before doses; numbers 3,4,5 and 7 are samples 24 hours after doses and number 6 is 48 hours after doses.

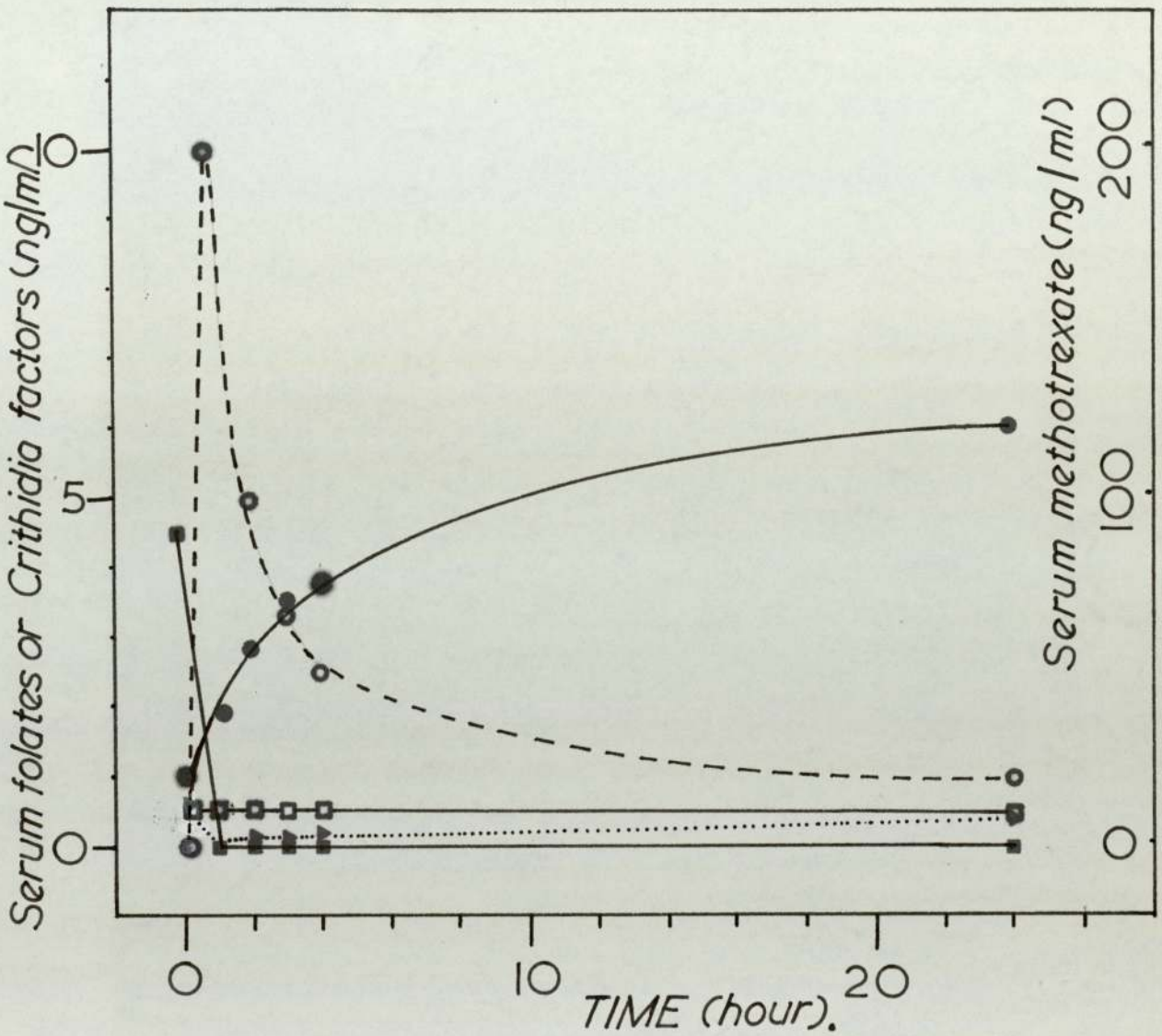


Figure 7.3 The relationship of measured serum folates, Crithidia factors and methotrexate after 10 mg of methotrexate, orally.

= serum L.casei

= serum S.faecalis

= serum P.cerevisiae

= serum Crithidia factors

= serum methotrexate

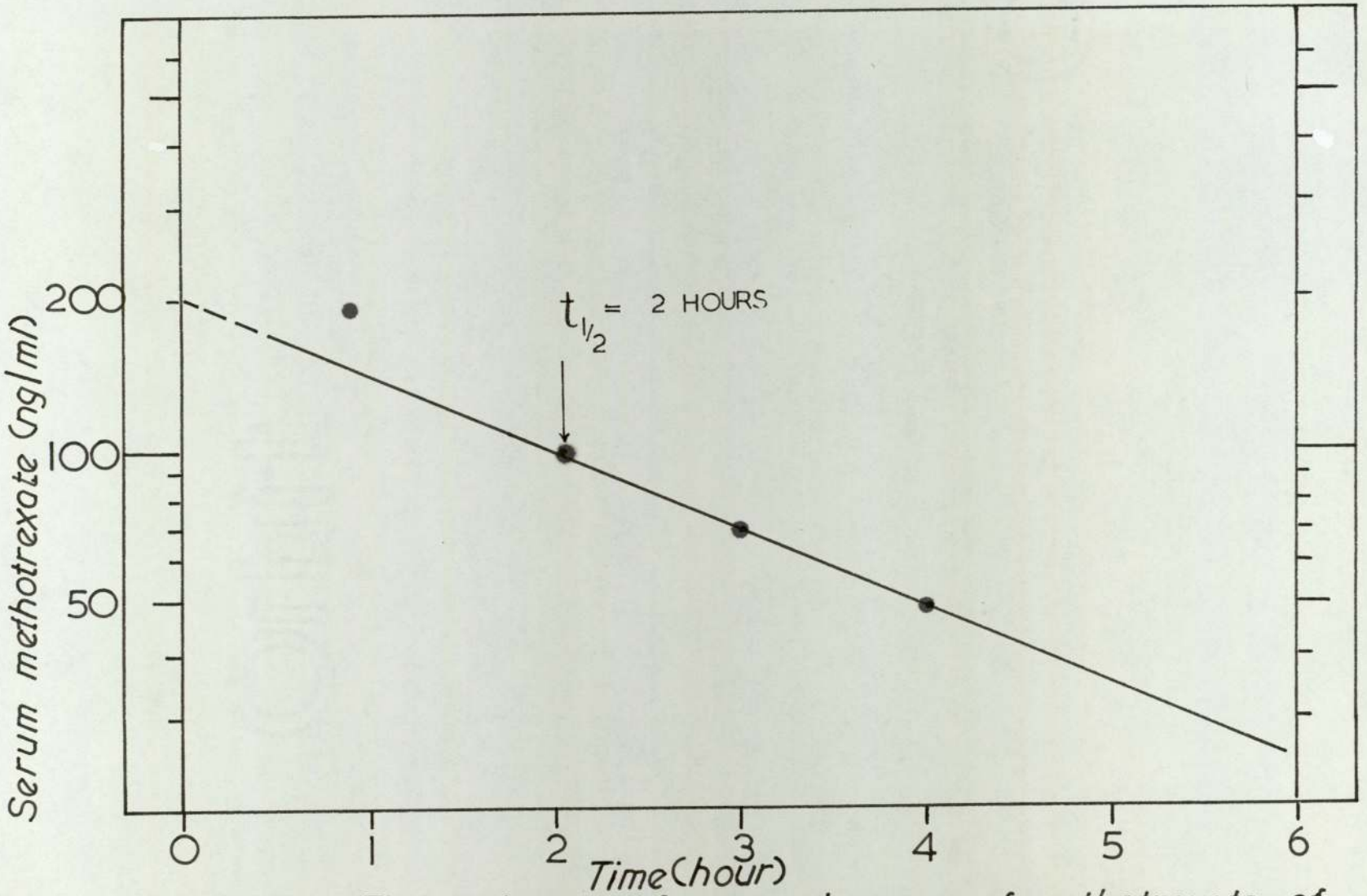


Figure 7.4 First order rate of serum clearance of methotrexate of 2 subjects after 10 mg of methotrexate orally.

Discussion.

Our data show that oral administration of 10 mg of 4-amino-4-deoxy-10-methylpteroylglutamic acid is quickly absorbed and reached a peak level within 1 hour after the dose (Table 7.8). Other studies have shown that methotrexate is readily absorbed and excreted in urine unchanged (Goodman and Gilman,1970;Henderson et al 1965;Huffman et al 1973). This was confirmed in this experiment by seeing a single spot of inhibition zone of methotrexate at Rf value similar to the administered methotrexate as seen in Plate 7.1 to Plate 7.3. Pharmacokinetic analysis of oral doses of methotrexate show a first order excretion rate from plasma with a half-life ($t_{\frac{1}{2}}$) of 2.0 hours (Figure 7.4) and is similar to those 1.9 hours (Henderson et al 1965;Huffman et al 1973). Microbiological assays of these samples with L.casei,S.faecalis,P.cerevisiae and Crithidia fasciculata are shown in Table 7.8 and their relationship is shown in Figure 7.3. In all cases assaying with L.casei showed negative values according to the inhibition of methotrexate on the growth of the test microorganisms. Amounts of methotrexate as low as 0.5 ng/ml have been shown to have a total inhibition effect on the growth of L.casei (Table 7.3). Microbiological assay with S.faecalis showed values decreased after oral methotrexate and increased to about normal level after twenty-four hours of oral methotrexate (Table 7.8). The decreased S.faecalis levels are due to the inhibition of methotrexate to the microbiological assay test microorganism (Table 7.4,7.5 and 7.6). For microbiological assay with P.cerevisiae the inhibition effect of methotrexate was less (Table 7.7)and thus after oral

methotrexate microbiological assay with P.cerevisiae was only slightly decreased (Table 7.8). Microbiological assay with Crithidia factors showed increase values after oral methotrexate (Table 7.8).

Twenty-four hours after oral administration of 10 mg of methotrexate the folate metabolism was studied by both oral and intravenous administrations. Oral administration of most folates in the absence of methotrexate was followed by rapid metabolism to mainly 5-methyltetrahydropteroylglutamic acid except 10-formylpteroylglutamic acid which was unmetabolized and folic acid which was slowly metabolized (Ratanasthien et al 1974;Beavon and Blair,1975). Oral administration of folic acid twenty-four hours after oral methotrexate showed it to be readily absorbed as expected,since methotrexate has been shown to have no effect on intestinal transmural transport (Olinger et al 1973). Bioautography and microbiological assays of these samples indicated that the absorbed folic acid entered blood levels with metabolism(Tables5.1.1 cf 7.9 and Plate 7.3). It is known that 'dihydrofolate reductase' is also able to reduce pteroylglutamic acid to dihydropteroylglutamic acid as well as reduction of dihydropteroylglutamic acid to tetrahydropteroylglutamic acid the latter is a fast step whilst the former is a slow step; (Tzortzatou and Hayhoe,1974). Thus the presence of methotrexate (a dihydrofolate reductase inhibitor) would inhibit the reduction and methylation of pteroylglutamic acid as observed. This evidence strongly suggests that metabolism of pteroylglutamic acid mainly involves the action of 'dihydrofolate reductase' for the reduction of the absorbed pteroylglutamic acid before its methylation. The slow metabolism of folic acid confirmed the

slow rate of reduction of pteroylglutamic acid to dihydropteroylglutamic acid. Oral administration of 7,8-dihydropteroylglutamic acid to subjects post twenty-four hours of oral methotrexate showed no increase in serum S.faecalis and P.cerevisiae and only a small increase was detected when the inhibition effect of methotrexate was accounted for. Bioautography of samples from these subjects showed small amount of serum folate is pteroylglutamic acid probably derived from the oxidation (Blair and Pearson,1974) of 7,8-dihydropteroylglutamic acid.The evidence from bioautography and microbiological assays indicated that 7,8-dihydropteroylglutamic acid orally administered twenty-four hours after oral methotrexate was metabolised to 5-methyltetrahydropteroylglutamic acid and may not be partially inhibited by methotrexate. Since both pteroylglutamic acid and 7,8-dihydropteroylglutamic acid are readily absorbed in subjects after 24 hours of oral methotrexate as previously discussed it is unlikely that this is not so with other folates. Thus oral administration of 5-methyltetrahydropteroylglutamic acid would have been absorbed. Microbiological assays and bioautography of these samples showed no accumulation of dihydrofolate which is expected if methotrexate inhibited the metabolism of this compound. After oral administration of methotrexate for 24 hours 5-formyltetrahydropteroylglutamic acid orally administered showed small increases in S.faecalis and P.cerevisiae when the inhibition effects were accounted for. The bioautography and microbiological assays indicated that 5-formyltetrahydropteroylglutamic acid was readily metabolized when administered orally to subjects post 24 hours methotrexate (10 mg orally).

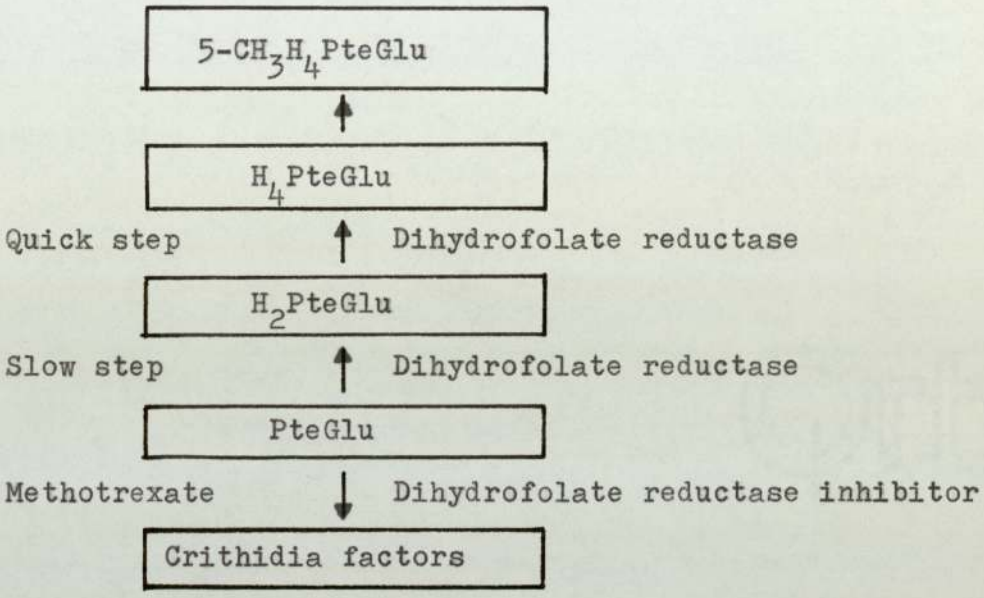
Intravenously administered 5-formyltetrahydropteroyl-glutamic acid to subjects post 24 hours of oral methotrexate showed that doses of 10 mg of 5-formyltetrahydropteroylglutamic acid were quickly distributed. The quick loss in microbiological activities with S.faecalis and P.cerevisiae (Table 7.12) indicated that 5-formyltetrahydropteroylglutamic acid was metabolized to 5-methyltetrahydropteroylglutamic acid which is not microbiologically active for these microorganisms.Bioautography of serum sample at time 2 hours after dose of 5-formyltetrahydropteroylglutamic acid intravenously showed the presence of 5-methyltetrahydropteroylglutamic acid (Plate 7.3). From these observations it is possible to say that the pharmaceutical effects of folates in rescue process after methotrexate treatment is by the conversion to 5-methyltetrahydropteroylglutamic acid (Blair and Searle,1970).

Microbiological assay with Crithidia fasciculata detects biopterin and its derivatives as discussed in Chapter 6. There seems to be no relationship between folates and Crithidia factors in normal humans (Chapter 6). Pabst and Rembold (1966) showed that biopterin levels in the rat does not vary with folate intake. However Rembold observed that the increased biopterin formation was seen when nucleic acid synthesis was inhibited by injection with actinomycin D (Rembold and Guyre,1972). Folate antagonists especially methotrexate are known to inhibit the synthesis of nucleic acid at concentration as low as 10^{-8}M (Chabner and Young,1973). Microbiological assay of sera from subjects taken 10 mg of methotrexate orally showed that the Crithidia factors were markedly increased (Figure 7.3). Oral biopterin gives only

a small increase in the sera Crithidia factors in normal humans (Chapter 6). In the subject taking methotrexate 24 hour before the oral absorption test of biopterin, a striking increase in Crithidia factors was seen (Leeming,1975). Oral administration of various folates to subjects taking methotrexate 24 hours before the test had little effect on the Crithidia factor levels but pteroylglutamic acid gave some increase in the Crithidia factor levels (Table 7.9).From these observations it may not be right to conclude that there is no relationship between folates and Crithidia factors. There are possibilities that biopterin or Crithidia factors can be synthesized from pteroylglutamic acid in human in some special conditions. From this studies it may be that methotrexate showed the special conditions underwhich biopterin can be derived from pteroylglutamic acid. It is also possible that the production of biopterin from pteroylglutamic acid in normal human is controlled by some enzymic system or systems and these systems were inhibited by methotrexate. Since methotrexate is known as a dihydrofolate reductase inhibitor it is reasonable to propose 'dihydrofolate reductase' as one of the enzymes regulate the production of Crithidia factors from pteroylglutamic acid. That is in the normal condition very small or slowly or even none of Crithidia factors can be synthesized from pteroylglutamic acid in the presence of 'dihydrofolate reductase'. Pteroylglutamic acid is normally reduced by the enzyme 'dihydrofolate reductase' slowly to dihydropteroylglutamic acid and tetrahydropteroylglutamic acid which then methylated to 5-methyltetrahydropteroylglutamic acid as shown in Chapter 5. Thus as we saw when this dihydrofolate reductase was inhibited

by the oral administration of methotrexate pteroylglutamic acid is then can caused the increase in Crithidia factor levels. The increase in Crithidia factors after oral administration of biopterin to subjects taking methotrexate 24 hours before the test suggested that the Crithidia factors may be usually bound to the enzyme i.e. dihydrofolate reductase as it is widely spread throughout the body tissues and fluids.The relationship of these factors may be written in scheme as shown in the metabolic pathways of pteroylglutamic acid in Scheme 7.1.

Studies of pteroic acid and pteroyl-D-glutamic acid gave no effect on serum folate levels. Microbiological assay with S.faecalis can be used for detection of pteroic acid as shown in Chapter 2. Results in Table 7.16 and Table 7.17 indicated that both compounds can not be converted into folate metabolic pool.Pteroic acid is poorly absorbed as previously pointed out (Brown et al 1973;Blair,Ratanasthien,Leeming,1974) but it is not clear if pteroyl-D-glutamic acid is absorbed since it has no microbiological activities with these microorganisms used in this study (Chapter 3).



Scheme 7.1 Metabolic pathways of pteroylglutamic acid metabolism as deduction from folate metabolism and effect of oral administration of methotrexate and post methotrexate oral folate metabolism on the serum folates and Crithidia factors.

Summary.

Eventhough there is no clear relationship between folates and Crithidia factors as shown in the previous Chapter, it is established that at some special condition in this case after oral administration of methotrexate, Crithidia factors can be derived from pteroylglutamic acid (Scheme 7.1). After oral administration of methotrexate for 24 hours metabolism of oral folates were changed from those of normal humans shown in Chapter 5. Pteroylglutamic acid absorption was not inhibited by methotrexate but its subsequent metabolism was completely inhibited. There is no inhibition of 5-methyltetrahydropteroyl-glutamic acid as there is no accumulation of S.faecalis and P.cerevisiae activities. Post methotrexate oral administration of 7,8-dihydropteroylglutamic acid and 5-formyltetrahydropteroyl-glutamic acid are well absorbed and metabolized.

Chapter 8.

Serum folates in disease.

Serum folates in disease.

Serum folate levels of normal human subjects have been widely studied but mainly as total folate levels obtained by microbiological assay with L.casei (Blakley,1969;Chanarin,1969). There is little information about the individual folates of the total normal human serum folate.A recent report showed that atleast two folates can be detected in normal human serum, 5-methyltetrahydropteroylglutamic acid as a major component and 10-formyltetrahydropteroylglutamic acid as a minor component (Ratanasthien et al 1974;Blair et al 1974). 10-formylpteroyl-glutamic acid may be present as a third component but this may be an analytical artefact.A detailed study of normal human serum folates was discussed in Chapter 4.

There are many reports of folate abnormalities in various diseases and most of which were reported as folate deficiency.The earliest reports were by Wills and her co-workers (Wills,1931;Wills and Bilmoria,1932;Wills,Clutterbuck and Evans, 1937).They reported that the anaemia of pregnancy later shown to be caused by folate deficiencies was cured by supplementation using yeast or liver extracts.Following those reports there were many other reports of folate deficiencies in disease.Many reports were published on the folate deficiencies of patients with intest-inal diseases and patients with higher or increased metabolic rate(Blakley,1969;Chanarin,1969).Low serum folate and folate deficiency may be caused by many factors(Herbert,1972).

In the experiments to be described attempts were made to identify and determine the individual serum folates in various kinds of patients.Studies were made of both patients with normal L.casei serum folates and those with low serum folates.

Materials and Methods.

Patients with various diseases were used in these studies.Those patients with adult coeliac disease,pernicious anaemia,regional enteritis or Crohn's disease and ulcerative colitis were amonst patients diagnosed at the Genaral Hospital in Birmingham,Birmingham,U.K.These patients were under the care of Dr.W.T.Cooke and his collegues.

Rheumatoid arthritis patients were those who had come for the attention at the out-patient department,Dudley Road Hospital.These patients were diagnosed by and were under the care of Dr.B.McConkey and Dr.M.Shadforth.

Patients with various types of leukemias were amongst those diagnosed at the Dudley Road Hospital.These patients were under the care of Dr.V.Melikian and his collegues.

10 ml of venous blood samples were taken aseptically from those patients attending the after noon clinics during 2.00 p.m. to 4.00 p.m. The blood samples were quickly transfered into sterile plain glass sample tubes and left standing to clot at room temperature for a period of 1 to 2 hours.After centrifugation sera were decanted into sterile sample bottles,5 mg/ml of ascorbic acid were added into the sera and kept frozen at -20^{o}C until assayed.

Samples for the determination of 5-methyl-5,6-dihydropteroylglutamic acid were collected by different methods.To blood samples to be determined for the 5-methyl-5,6-dihydropteroylglutamic acid,10 ml venous blood samples were also taken and allowed to clot in sterile plain glass tubes for not more than half an hour to minimize the oxidation of folates in the blood samples.After centrifugation these sera were separated into two

portions and the first portion after addition of ascorbic acid (5 mg/ml) was kept frozen at -20°C until assayed.To the second portion of about 1.5 to 2.0 ml sera,concentrated HCl was added to adjust the pH of these samples to 3.0 and they were left to stand at room temperature for 15 to 20 minutes.These samples were then neutralized to pH 7.4 with 20 %(W/V) sodiumhydroxide solution,5 mg/ml ascorbic acid was added and samples were stored frozen at -20°C until assayed.The volumes of concentrated HCl and 20 % sodiumhydroxide added were noted and they were between 0.1 to 0.2 ml for all cases.The added volumes were used as a correction factor by calculation or by adding equal amount of water to the first portion of corresponding samples.

All samples were microbiologically assayed by aseptic addition with L.casei,S.faecalis and P.cerevisiae according to methods described in Chapter 2.Samples were kept frozen at -20°C and thawed just before adding to the tests of each assay.

Some samples were collected into heparinized test tubes and sera were quickly separated within 15 minutes and they were used for confirmation of 10-formylpteroylglutamic acid as an oxidation product of 10-formyltetrahydropteroylglutamic acid.

Results.

Samples collected from the different groups of patients were microbiologically assayed with L.casei,S.faecalis and P.cerevisiae.Differences between L.casei and S.faecalis assays were shown to be the amount of 5-methyltetrahydropteroylglutamic acid (Johns and Bertino,1965;Ratanasthien et al 1974).But this could also include 5-methyl-5,6-dihydropteroylglutamic acid as

previously discussed in Chapters 3 and 4.Thus the differences between L.casei and S.faecalis are the summation of true activity 5-methyltetrahydropteroylglutamic acid and of 5-methyltetrahydropteroylglutamic acid by reduction of 5-methyl-5,6-dihydropteroylglutamic acid with the added ascorbic acid. Differences between S.faecalis and P.cerevisiae are due to 10-formylpteroylglutamic acid or pteroylglutamic acid(Ratanasthien et al 1974).P.cerevisiae is supported by tetrahydrofolates except 5-methyltetrahydropteroylglutamic acid (Chapter 2).In the analysed sera or plasma and urine of mammals i.e. rats and man the P.cerevisiae response is due to 10-formyltetrahydropteroylglutamic acid(Nixon and Bertino, 1972;Ratanasthien et al 1974;Albrecht and Broquist,1956;Silverman, Ebough and Gardiner,1956). 5-methyl-5,6-dihydropteroylglutamic acid was determined by the differences between L.casei of the same sample with and without acidification as previously discussed in Chapter 3 and Chapter 4.

Serum folates assayed with L.casei,S.faecalis and P.cerevisiae in various diseases are summarized in Table 8.1 for the whole group,Table 8.2 for L.casei of 2.6 to 20 ng/ml and Table 8.3 for L.casei values lower than 2.6 ng/ml.Serum folates expressed in chemical compounds for these three tables are summarized in Tables8.4,8.5 and 8.6 for the corresponding Tables 8.1,8.2 and 8.3,respectively.Serum folate detected as 5-methyl-5,6-dihydropteroylglutamic acid are compared with the level of normal human volunteers as shown in Table 8.7.Distribution of serum folates for normal subjects and various disease subjects are shown in Figure 8.1 to Figure 8.9 for normal subjects,leukemia subjects,pernicious anaemia subjects,schizophrenia subjects, adult coeliac disease on gluten free diet,adult coeliac disease

on normal diet,regional enteritis patients,rheumatoid arthritis patients and psoriasis patients,respectively.

Discussion.

Serum folate of normal humans as microbiologically assayed with L.casei has been reported varying from 2.6 to 45.6 ng/ml (Chanarin,1969;Blakley,1969).The microbiological assay with L.casei for low serum folate was used as indication for early detection of folate deficiency (Herbert,1962).As previously shown there are very high degrees of variation in serum folates of normal humans,thus the use of low serum folates is uncertain in the clinical point of view.

The association of low serum folates with adult coeliac disease has been established (Cooke et al 1963; Dormandy et al 1963).The low serum folates of leukemia,Hodgkins disease,multiple myeloma and other neoplastic diseases of the lymphorecticular system has also been established (Rao et al 1963;Rao et al 1965;Kershaw and Girdwood,1964;Hoogstraten et al 1965;Rose,1966).In untreated pernicious anaemia cases around 20 to 33% of studied cases have low serum folates (Hansen and Weinfeld,1962;Herbert and Zalusky,1962;Chanarin,1964).Patients with untreated pernicious anaemia have also been recorded with raised serum folates (Herbert et al 1960;Herbert and Zalusky,1962). Higher mean serum folates amongst pernicious anaemia patients as comparing with normal subjects have been well recognised (Waters and Mollin,1961;Kohn et al 1961;Waters and Mollin,1963; Cooper and Lowenstein,1964).Patients with other kinds of intestinal abnormalities had also been shown to have high incidence of low serum folates e.g. regional enteritis or Crohn's disease

(Cox et al 1958;Chanarin and Bennett,1962;Hoffbrand et al 1968), and ulcerative colitis (Franklin and Rosenberg,1973). Patients with rheumatoid arthritis were also reported to have low serum folates (Doig et al 1957;Partridge and Duthie,1963;Gough et al 1964;Deller et al 1966).Although low serum folates have been established in these diseases,it gave no clear solution of the pathogenesis of the relationship of folates and diseases.

In the recent reports by J.A.Blair and his co-workers established the present of two folates in equilibrium in normal human,one of which is 10-formyltetrahydropteroylglutamic acid at a constant level of 0.78 ng/ml and the other,5-methyltetrahydropteroylglutamic acid is a dietary variable and acts as a storage form.It was also reported that when considering serum folates from adult coeliac and leukemic patients with serum folates in the normal range,there were increases in the level of 10-formyltetrahydropteroylglutamic acid (Blair et al 1974a; Ratanasthien et al 1974),and decrease in the level of 5-methyltetrahydropteroylglutamic acid compared with normals.

The level of 5-methyl-5,6-dihydropteroylglutamic acid was determined using its rearrangement to a microbiologically inactive 5-methyl-5,8-dihydropteroylglutamic acid in the acid media (Blair et al 1974;Robb,1975).Results from various patients are shown in Table 8.7.Only patients with pernicious anaemia and leukemia showed raised serum level of 5-methyl-5,6-dihydropteroylglutamic acid.The increase in serum 5-methyl-5,6-dihydropteroylglutamic acid in these pernicious anaemia patients may have been derived from the fact that these patients have high stomach pH. It has been demonstrated that by increasing stomach pH of normal humans by oral administration of sodiumbicarbonate the serum

level of folates from oral 5-methyl-5,6-dihydropteroylglutamic acid is much increased (Chapter 5).The observation that the additional increment of total serum folate in pernicious anaemia over the normal total serum folate is due to 5-methyl-5,6-dihydropteroylglutamic acid supports the hypothesis that the increased serum folate in pernicious anaemia is due to superior absorption of this compound.

The relationships of serum 5-methyltetrahydropteroylglutamic acid and total serum folate from these patients are shown in Tables 8.1 to 8.6. These studies indicated that patients showed raised levels of 10-formyltetrahydropteroylglutamic acid even when total folate levels are lowered and 5-methyltetrahydropteroylglutamic acid are lowered.

Correlations between pernicious anaemia and other kinds of diseases are known.The combination of pernicious anaemia and various kinds of leukemias are not uncommon.The correlations of pernicious anaemia and various kinds of disease is documented (Chanarin,1969).

Table 8.1 Serum folates as microbiologically assayed with L.casei, S.faecalis and P.cerevisiae for the whole group of each disease.

Diagnosis of subjects	No.of subjects	L.casei (ng/ml) ±S.E.M.	S.faecalis (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
Normal human	52	5.57±0.28	0.78±0.03	0.57±0.03
Adult coeliac disease	104	5.06±0.35	0.98±0.05	0.62±0.04
Psoriasis	11	3.16±0.63	1.04±0.11	0.82±0.09
Leukemias	110	4.46±0.26	1.50±0.09	0.70±0.04
Pernicious anaemia*	20	6.40±0.60	0.88±0.10	0.70±0.08
Rheumatoid arthritis	47	3.49±0.24	1.58±0.18	1.30±0.12
Regional enteritis	234	3.90±0.18	0.67±0.03	0.51±0.02
Ulcerative colitis	6	10.10±4.16	0.71±0.30	0.50±0.13
Schizophrenia	17	2.59±0.20	0.88±0.05	0.70±0.05

Compared with normal	:	't' Test	p values (better than)	
Normal human	52	-	-	-
Adult coeliac disease	104	N.S.	0.01	N.S.
Psoriasis	11	0.001	0.005	0.010
Leukemias	110	0.050	0.001	0.025
Pernicious anaemia*	20	N.S.	0.200	0.100
Rheumatoid arthritis	47	0.001	0.001	0.001
Regional enteritis	234	0.001	0.100	N.S.
Schizophrenia	17	0.001	0.200	N.S.
Ulcerative colitis	6	0.001	N.S.	N.S.

* indicates subjects on vitamin B_{12} therapy.N.S.=not significant.

Table 8.2 Serum folates as microbiologically assayed with L.casei, S.faecalis and P.cerevisiae of those patients with L.casei of 2.6 ng/ml to 20 ng/ml.

Diagnosis of subjects	No.of subjects	L.casei (ng/ml) ±S.E.M.	S.faecalis (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
Normal human	52	5.57±0.28	0.78±0.03	0.57±0.03
Adult coeliac disease	74	6.10±0.40	1.00±0.06	0.58±0.03
Psoriasis	6	4.73±0.50	0.95±0.14	0.73±0.12
Leukemias	79	5.56±0.28	1.65±0.10	0.76±0.04
Pernicious anaemia*	18	6.50±0.70	0.89±0.10	0.70±0.08
Rheumatoid arthritis	25	4.72±0.36	1.85±0.30	1.42±0.18
Regional enteritis	144	5.29±0.22	0.84±0.03	0.63±0.03
Ulcerative colitis	4	14.58±4.80	1.27±0.42	0.70±0.10
Schizophrenia	7	3.94±0.40	0.93±0.10	0.70±0.08
Compared with normal	:	't' Test p values (better than)		
Normal human	52	-	-	-
Adult coeliac disease	74	N.S.	0.005	N.S.
Psoriasis	6	N.S.	0.100	N.S.
Leukemias	79	N.S.	0.001	0.005
Pernicious anaemia	18	N.S.	N.S.	0.100
Rheumatoid arthritis	25	0.100	0.001	0.001
Regional enteritis	144	N.S.	N.S.	N.S.
Ulcerative colitis	4	0.001	0.001	0.100
Schizophrenia	7	0.005	0.050	N.S.

* receiving vitamin B_{12} therapy and N.S. = not significant.

Table 8.3 Serum folates as microbiologically assayed with L.casei, S.faecalis and P.cerevisiae of those patients with L.casei serum folate lower than 2.6 ng/ml.

Diagnosis of subjects	No.of subjects	L.casei (ng/ml) ±S.E.M.	S.faecalis (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
Normal human	52	5.57±0.28	0.78±0.03	0.57±0.03
Adult coeliac disease	30	1.85±0.15	0.90±0.08	0.67±0.06
Psoriasis	5	1.72±0.18	1.14±0.18	0.92±0.13
Leukemias	31	1.67±0.14	1.03±0.10	0.55±0.07
Pernicious anaemia*	2	1.50±1.00	0.70±0.20	0.60±0.10
Rheumatoid arthritis	22	2.30±0.10	1.29±0.15	1.16±0.15
Regional enteritis	90	1.50±0.07	0.38±0.03	0.30±0.02
Ulcerative colitis	2	1.10±0.10	0.60±0.30	0.20±0.15
Schizophrenia	10	1.65±0.18	0.84±0.10	0.70±0.08
Compared with normal	:	't' Test p values (better than)		
Normal human	52	-	-	-
Adult coeliac disease	30	0.001	0.100	N.S.
Psoriasis	5	0.001	0.001	0.01
Leukemias	31	0.001	0.005	N.S.
Pernicious anaemia*	2	0.001	N.S.	N.S.
Rheumatoid arthritis	22	0.001	0.001	0.001
Regional enteritis	90	0.001	0.001	0.001
Ulcerative colitis	2	0.001	N.S.	0.001
Schizophrenia	10	0.001	N.S.	N.S.

* receiving vitamin B_{12} therapy and N.S. = not significant.

Table 8.4 Serum folates chemically expressed of results from those of Table 8.1. Results are (mean ± S.E.M.)

Diagnosis of subjects	No.of subjects	5-CH_3H_4-* PteGlu (ng/ml)	10-CHO H_4PteGlu 10-CHO PteGlu (ng/ml)	PteGlu(S.faecalis) P.cerevisiae (ng/ml)
Normal human	52	4.80±0.28	0.21±0.03	0.57±0.03
Adult coeliac disease	104	4.08±0.30	0.36±0.04	0.62±0.04
Psoriasis	11	2.12±0.52	0.22±0.03	0.82±0.09
Leukemias	110	2.94±0.20	0.80±0.05	0.70±0.04
Pernicious anaemia**	20	5.52±0.50	0.18±0.02	0.70±0.08
Rheumatoid arthritis	47	1.91±0.21	0.28±0.06	1.30±0.12
Regional enteritis	234	3.23±0.15	0.16±0.01	0.51±0.02
Ulcerative colitis	6	9.39±3.87	0.21±0.17	0.50±0.13
Schizophrenia	17	1.71±0.15	0.18±0.01	0.70±0.05

Compared with normal	:	't' Test	p values (better than)	
Normal human	52	-	-	-
Adult coeliac disease	104	0.05	0.01	N.S.
Psoriasis	11	0.001	N.S.	0.01
Leukemias	110	0.010	0.001	0.025
Pernicious anaemia**	20	N.S.	N.S.	0.100
Rheumatoid arthritis	47	0.001	0.05	0.001
Regional enteritis	234	0.001	0.10	N.S.
Ulcerative colitis	6	0.001	N.S.	N.S.
Schizophrenia	17	0.001	N.S.	N.S.

** receiving vitamin B_{12} therapy and * combination of true 5-CH_3H_4 PteGlu and 5-CH_3-5,6-H_2PteGlu activities.

Table 8.5 Serum folates chemically expressed of results from those of Table 8.2. Results are mean ± S.E.M.

Diagnosis of subjects	No.of subjects	5-CH_3H_4-* PteGlu (ng/ml)	10-CHO H_4PteGlu(S.faecalis) 10-CHO PteGlu (ng/ml)	P.cerevisiae (ng/ml)
Normal human	52	4.80±0.28	0.21±0.03	0.57±0.03
Adult coeliac disease	74	5.10±0.34	0.42±0.03	0.58±0.03
Psoriasis	6	3.68±0.36	0.22±0.02	0.73±0.12
Leukemias	79	3.91±0.18	0.89±0.06	0.76±0.04
Pernicious anaemia**	18	5.61±0.60	0.19±0.02	0.70±0.08
Rheumatoid arthritis	25	2.87±0.33	0.43±0.12	1.42±0.18
Ulcerative colitis	4	13.23±4.38	0.57±0.26	0.70±0.10
Regional enteritis	144	4.45±0.19	0.21±0.03	0.63±0.03
Schizophrenia	7	3.01±0.30	0.23±0.02	0.70±0.08
Compared with normal	:	't' Test	p values (better than)	
Normal human	52	-	-	-
Adult coeliac disease	74	N.S.	0.001	N.S.
Psoriasis	6	0.05	N.S.	N.S.
Leukemias	79	0.05	0.001	0.005
Pernicious anaemia**	18	N.S.	N.S.	0.100
Rheumatoid arthritis	25	0.001	0.001	0.001
Ulcerative colitis	4	0.001	0.001	0.100
Regional enteritis	144	N.S.	N.S.	N.S.
Schizophrenia	7	0.005	N.S.	0.100

** receiving vitamin B_{12} therapy and * combination of true 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu.

Table 8.6 Serum folates chemically expressed of results from those of Table 8.3. Results are mean ± S.E.M.

Diagnosis of subjects	No.of subjects	5-CH_3H_4-* PteGlu (ng/ml)	10-CHO H_4PteGlu(S.faecalis) 10-CHO PteGlu (ng/ml)	P.cerevisiae (ng/ml)
Normal human	52	4.80±0.28	0.21±0.03	0.57±0.03
Adult coeliac disease	30	0.95±0.07	0.23±0.02	0.67±0.06
Psoriasis	5	0.58±0.01	0.22±0.05	0.92±0.13
Leukemias	31	0.64±0.04	0.48±0.03	0.55±0.07
Pernicious anaemia	2	0.80±0.80	0.10±0.10	0.60±0.10
Rheumatoid arthritis	22	1.01±0.10	0.13±0.00	1.16±0.15
Regional enteritis	90	1.12±0.04	0.08±0.01	0.30±0.02
Schizophrenia	10	0.81±0.08	0.14±0.02	0.70±0.08
Ulcerative colitis	2	0.50±0.20	0.40±0.15	0.20±0.15
Compared with normal	:	't' Test p values (better than)		
Normal human	52	-	-	-
Adult coeliac disease	30	0.001	N.S.	N.S.
Psoriasis	5	0.001	N.S.	0.01
Leukemias	31	0.001	0.005	N.S.
Pernicious anaemia	2	0.001	0.05	N.S.
Rheumatoid arthritis	22	0.001	0.10	0.001
Regional enteritis	90	0.001	0.001	0.001
Schizophrenia	10	0.001	0.05	N.S.
Ulcerative colitis	2	0.001	0.05	0.001

** receiving vitamin B_{12} therapy and * summation of true 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu.

Table 8.7 Serum 5-methyl-5,6-dihydropteroylglutamic acid of various diseases (number of subjects in parenthesis).

Diagnosis of subjects	L.casei(ng/ml±S.E.M.) Before acid treatment	After acid treatment	$5\text{-}CH_3\text{-}5,6\text{-}H_2$ PteGlu (ng/ml±S.E.M.)	$5\text{-}CH_3H_4$-* PteGlu (ng/ml±S.E.M.)
Normal human(30)	5.75±0.48	5.00±0.63	0.75±0.15	4.95±0.40
Leukemias(5)	5.18±0.50	4.50±0.93	1.68±0.43	4.00±0.45
Regional enteritis(8)	4.78±0.38	4.47±0.63	0.31±0.25	4.00±0.35
Pernicious anaemia**(4)	9.50±4.30	5.50±2.90	4.00±1.41	8.70±4.20
Rheumatoid arthritis(30)	3.30±0.28	2.42±0.44	0.88±0.16	1.80±0.20
Adult coeliac disease(4)	6.40±1.00	5.55±0.50	0.85±0.50	5.40±0.90
Compared with normal subjects : 't' Test p values (better than)				
Normal human (30)	-	-	-	-***
Leukemias(5)	N.S.	N.S.	0.05	0.001
Regional enteritis(8)	N.S.	N.S.	N.S.	N.S.
Pernicious anaemia**(4)	0.001	N.S.	0.001	N.S.
Rheumatoid arthritis(30)	0.001	0.001	N.S.	0.001
Adult coeliac disease(4)	N.S.	N.S.	N.S.	N.S.

* summation of true $5\text{-}CH_3H_4$PteGlu (***) and $5\text{-}CH_3\text{-}5,6\text{-}H_2$PteGlu activities and ** receiving vitamin B_{12} treatment.

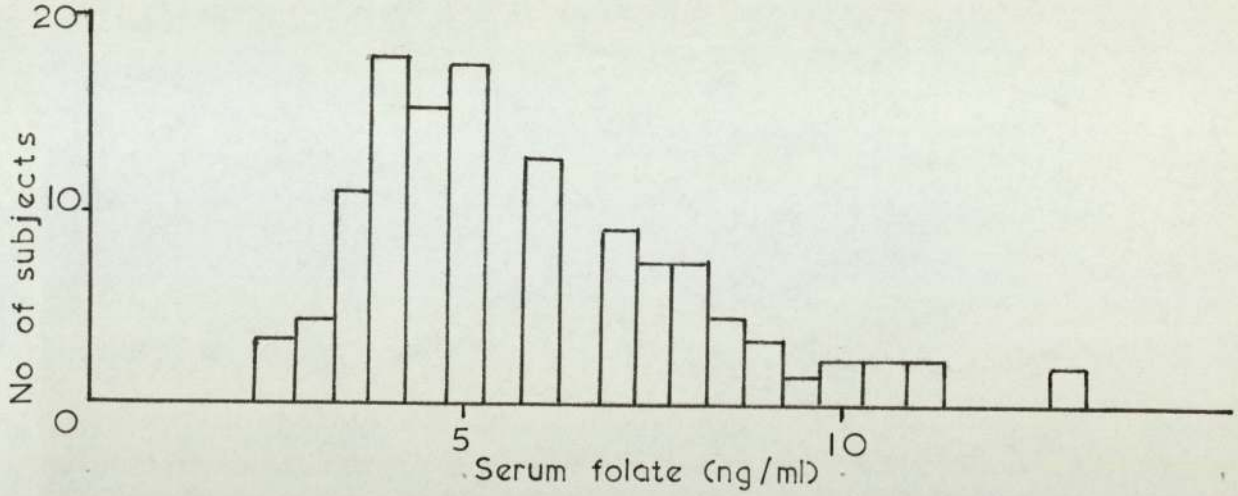


Figure 8.1 Distribution of serum folate in normal humans.

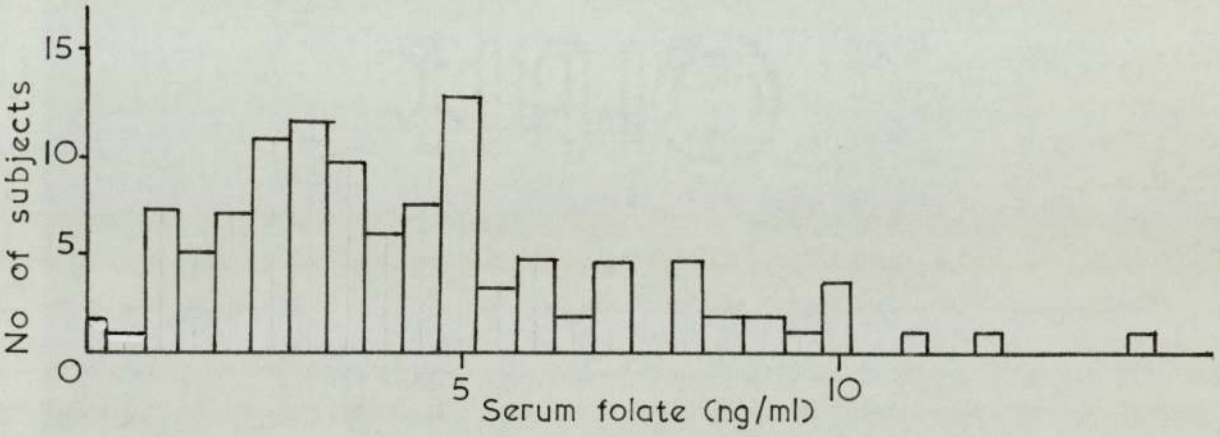


Figure 8.2 Distribution of serum folate in leukemia patients.

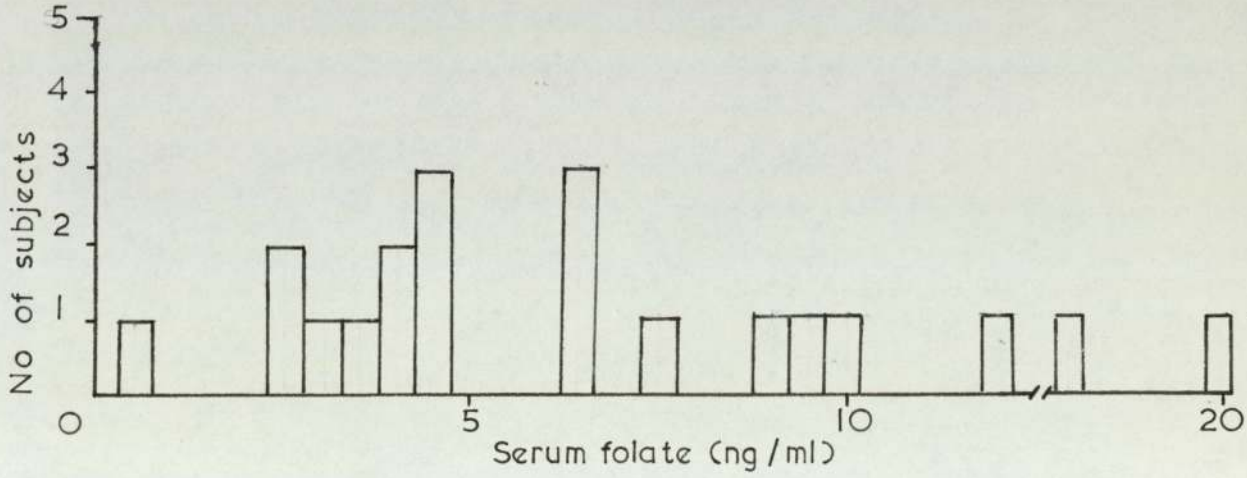


Figure 8.3 Distribution of serum folate in pernicious anaemia patients.

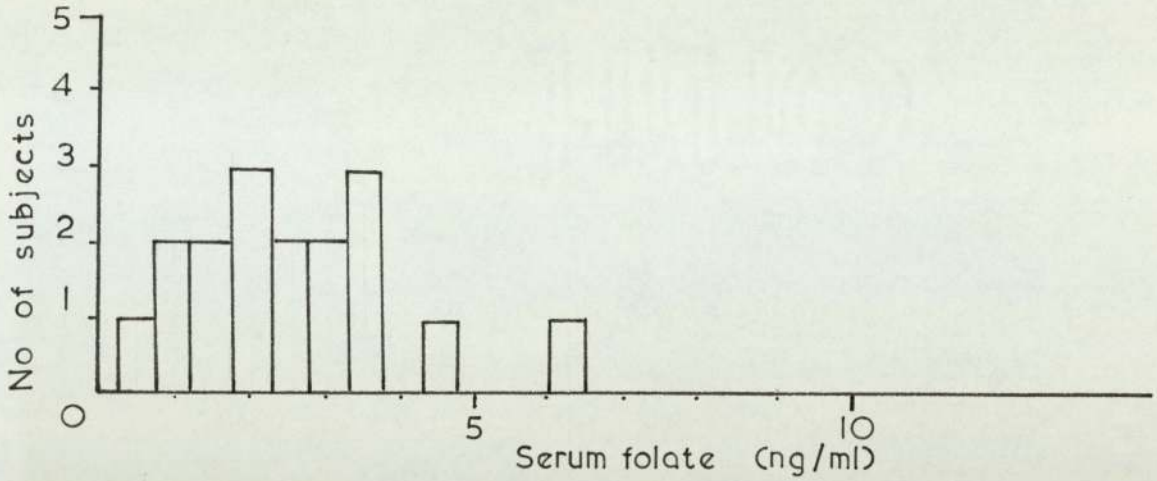


Figure 8.4 Distribution of serum folate in schizophrenia patients.

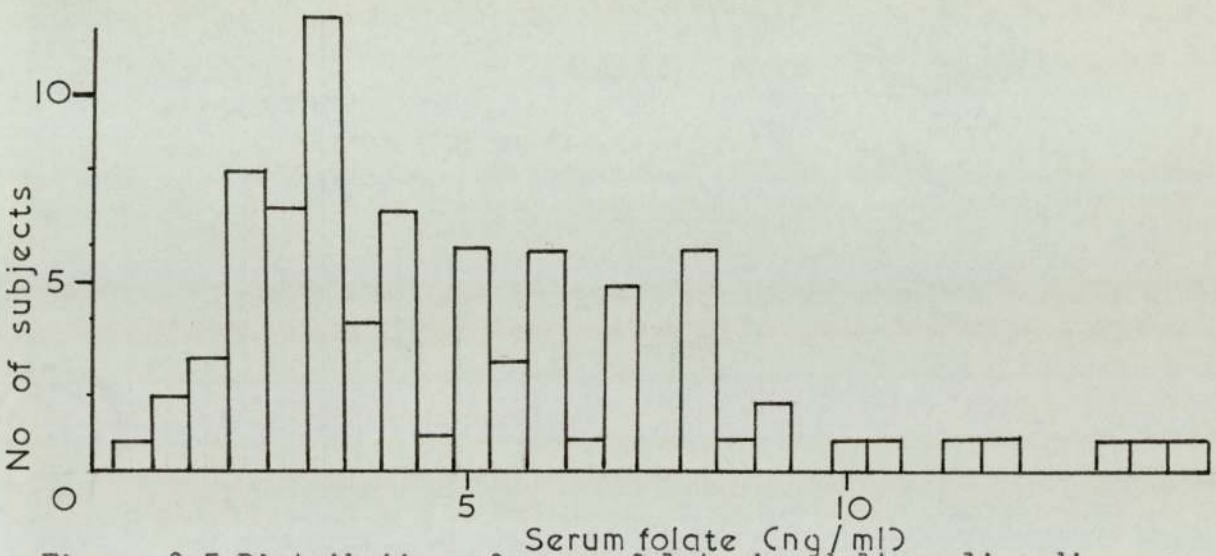


Figure 8.5 Distribution of serum folate in adult coeliac disease patients on gluten free diet.

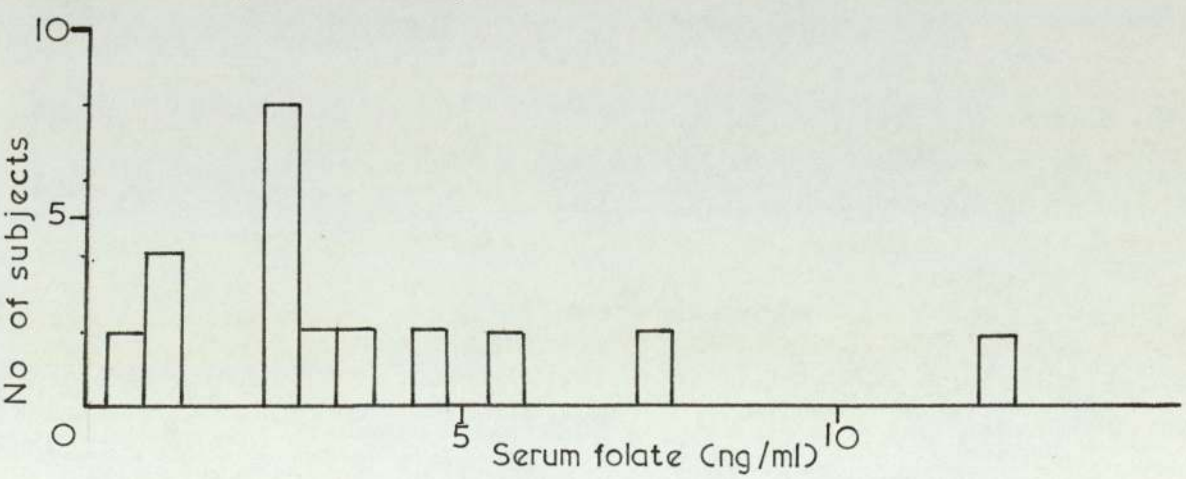


Figure 8.6 Distribution of serum folate in adult coeliac disease patients on normal diet.

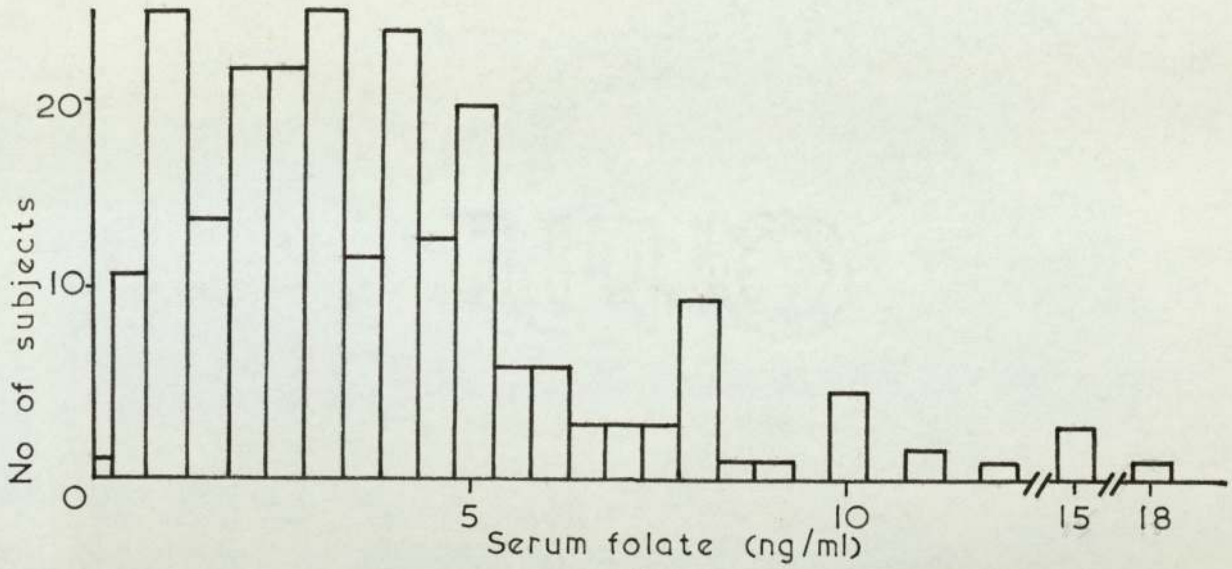


Figure 8.7 Distribution of serum folate in regional enteritis patients.

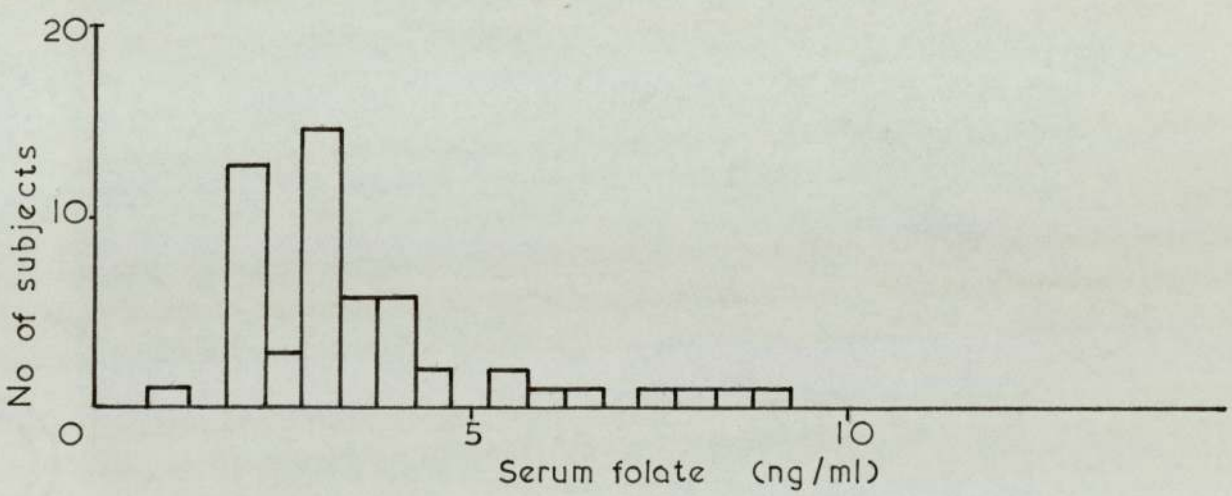


Figure 8.8 Distribution of serum folate in rheumatoid arthritis patients.

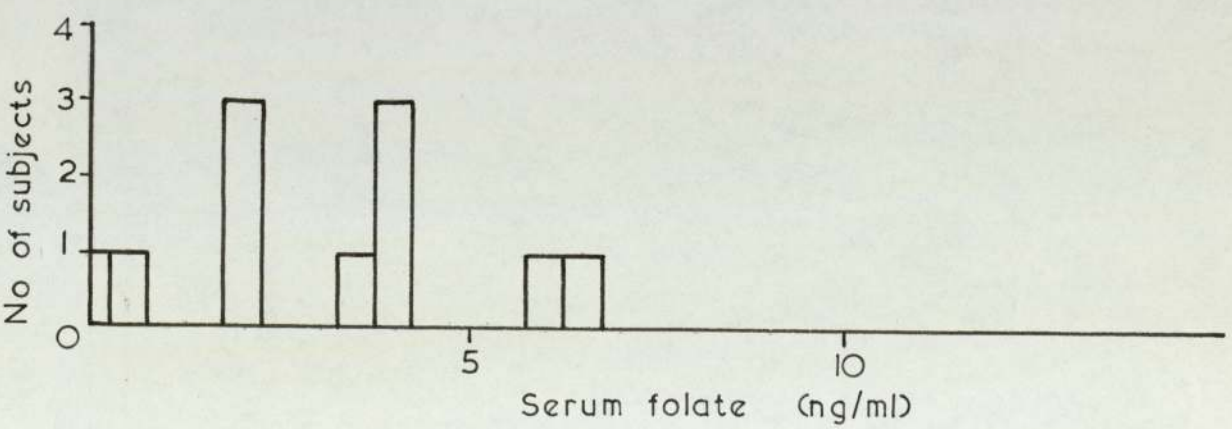


Figure 8.9 Distribution of serum folate in psoriasis patients.

Summary.

Studies in this Chapter had come to a conclusion that patients showed raised levels of 10-formyltetrahydropteroyl-glutamic acid even when total folate levels are lowered and serum 5-methyltetrahydropteroylglutamic acid are lowered. Serum 5-methyl-5,6-dihydropteroylglutamic acid levels are raised in patients with pernicious anaemia and leukemia are due to their superior absorption of the compound which is slowly meatbolised.

Chapter 9.

Longterm follow up of patients on folic acid treatment, effect of long term folic acid administration and a study of low serum folates.

Longterm follow up of the folate status of some patients.

Studies of the serum folates of various patients were described in the previous chapter.Most of them were single samples from many patients.Little is known about the folate status in patients over a period of time.Patients receiving folic acid treatment were not included in those studies.

There are other biochemical parameters used in the clinical studies of patients and many of them were used as a routine clinical tests as compared with normal clinical chemistry and haematology of blood previously mentioned in Chapter 4.

In these experiments attempts were made to measure the folate status of patients during follow up. Some patients on folic acid treatment were also studied.The relationship of folate status and other clinical parameters was also studied.Patients with low serum folate levels as measured with L.casei were also studied in details with other clinical parameters.

Patients and Methods.

There were three groups of patients involved in this study of longterm folate.Amongst the patients studied were patients diagnosed as suffering from adult coeliac disease.The second group of patients were those sufferring from rheumatoid arthritis.The third group of patients were those sufferring from various kinds of leukemias.These are including acute myeloid leukemia,chronic granulocytic leukemia,hypernephroma and carcinoma of various sties i.e. ovary and colon.

The patients involved in these studies had serum folate measured at frequent intervals but there was not a specific fixed

schedule.The treatment of each patient varied and thus will be incorporated in the results section.

Some patients having oral administration of 10 to 15 mg folic acid daily were also studied.

Classification of the patients was done by physicians concerned by using clinical conditions and other clinical tests for the purpose.The classification of these patients is thus incorporated in the results section.

Blood samples from these patients were taken,collected and stored in a similar fashion to that described in Chapter 8. The microbiological assays were employed L.casei,S.faecalis and P.cerevisiae as previously discussed in Chapter 2. Other clinical parameters employed as routine tests were made at the request of the physicians concerned.The tests were performed in the hospital clinical laboratories concerned and the detail of experimental methods will not be considered here.Only appropiate clinical results will be used in this studies.

Serum folates were also studied on patients with low L.casei levels. These patients were diagnosed as (a) folate deficiency and (b) not folate deficiency by using other clinical parameters i.e. by haematological studies.

Results.

The folate status during follow up of patients suffering from various kinds of leukemias are presented as case reports from cases 1 to 13.Their clinical condition as classified by the physicians concerned are also included.

Adult coeliac disease patients on 10 mg daily treatment with oral pteroylglutamic acid were studied for their folate

status. Serum folates from these patients as microbiologically assayed with L.casei,S.faecalis and P.cerevisiae are summarized in Table 9.1 and case reports of longterm follow up of patients with adult coeliac disease were shown in cases 14 to 20.Case reports are also given on those patients diagnosed as sufferring from rheumatoid arthritis,from case 21 to case 23.

Serum folates of patients with low serum folate but not identified as folic acid deficiency and those identified as folic acid deficiency are summarized in Table 9.2 and Table 9.3, respectively. Serum 10-formyltetrahydropteroylglutamic acid of adult coeliac disease patients on folic acid treatment were compared with those without treatment with folic acid and also with normal subjects (Table 9.4). Serum folates from those of Table 9.2 and 9.3 were compared with normal levels (Table 9.5). Serum folates from those subjects were compared with those patients with L.casei serum folates lower than 2.6 ng/ml (Table 9.6).

Case 1 : L.M. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
4/2/1974				4.4	2.0	0.0
19/2/1974	A	5000	0	8.0	1.4	0.6
6 /3/1974	A	8400	0	8.0	1.3	0.9
9 /4/1974	R	6000	0	3.0	3.0	0.9
		------Died------				

A = active disease; R = remission.

Case 2 : E.J. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
07/8/1973	-	-	-	4.2	3.2	0.8
08/8/1973	-	-	-	4.2	3.2	1.0
12/9/1973	-	-	-	2.5	1.0	0.0
28/1/1974	A	-	-	6.0	1.0	0.3
04/2/1974	A	-	-	7.0	1.3	0.0
12/2/1974	R	-	-	4.5	0.6	0.4
20/2/1974	A	-	-	5.0	3.0	0.6
09/4/1974	R	-	-	3.6	3.0	1.0
07/7/1974	R	-	-	1.3	1.0	1.0
		------Died------				

A = active disease; R = remission.

Case 3 : A.F. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
04/2/1974	-	-	-	0.0	0.0	0.0
06/3/1974	A	2200	16	2.0	1.2	0.6
02/4/1974	A	1200	10	1.5	1.0	0.9
		------Died------				

A = active disease; R = remission.

Case 4 : J.R. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
16/10/1973	-	-	-	4.3	4.0	1.6
25/11/1973	A	2300	30	5.0	1.0	0.6
04/02/1974	A	3500	5	6.0	1.4	0.0
12/02/1974	A	2800	5	5.0	1.5	0.5
12/03/1974	R	3500	6	10.0	3.0	1.2
02/04/1974	R	3300	4	1.0	0.8	0.1
09/04/1974	R	1700	3	4.5	1.3	1.0
07/07/1974	-	-	-	0.0	0.0	0.0
		------Died------				

A = active disease; R = remission.

Case 5 : L.G. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
28/01/1974	Relapse	2500	0	9.0	1.2	0.3
04/02/1974	Relapse	2300	3	5.0	2.0	0.0
13/02/1974	Relapse	1500	1	4.3	1.5	0.7
	------Died------					

Case 6 : M.S. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
16/10/1973	-	-	-	2.1	1.8	0.8
25/11/1973	A	5900	44	3.8	1.4	0.6
28/01/1974	A	1400	12	8.0	1.0	0.3
04/02/1974	A	-	-	5.4	1.0	0.3
12/02/1974	A	1800	8	5.0	0.6	0.6
19/02/1974	A	1800	8	7.0	1.0	0.6
05/03/1974	R	1700	0	4.5	1.3	0.8
12/03/1974	R	1500	5	3.5	2.3	0.8
	------Died------					

A = active disease; R = remission.

Case 7 : R.S. diagnosed as chronic granulocytic leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
20/02/1974	Relapse A	2400	0	2.0	1.2	0.8
07/07/1974	A	7100	2	1.5	0.9	0.0
		------Died------				

A = active disease.

Case 8 : H.B. diagnosed as hypernephroma.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
25/11/1973	-	-	-	2.8	1.0	0.6
13/02/1974	-	-	-	1.0	1.0	0.0
		------Died------				

Case 9 : F.B. diagnosed as chronic granulocytic leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
28/01/1974	A	151000	9	7.0	2.0	0.3
12/02/1974	A	105000	6	4.0	1.4	0.6
02/04/1974	R	20300	0	2.0	1.2	1.2
29/01/1975	-	-	-	5.5	0.8	0.8

A = active disease; R = remission.

Case 10 : M.B. diagnosed as chronic granulocytic leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
28/01/1974	-	-	-	5.0	3.0	0.6
05/02/1974	-	-	-	3.0	3.0	0.6
12/02/1974	A	17500	-	10.0	1.8	1.0
05/03/1974	A	7500	-	10.0	1.5	1.5
09/04/1974	R	8000	-	4.5	3.3	1.2
07/07/1974	R	10900	-	2.4	1.2	1.0
29/01/1975	-	-	-	7.0	0.7	0.7
05/02/1975	-	-	-	5.0	1.0	0.8
12/02/1975	-	-	-	11.0	1.3	1.1

A = active disease; R = remission.

Case 11 : H.E. diagnosed as chronic granulocytic leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
12/09/1973	-	-	-	3.5	2.0	0.6
29/01/1975	-	-	-	3.5	0.8	0.7
05/02/1975	-	-	-	3.0	0.8	0.8
12/02/1975	-	-	-	8.0	1.0	1.0

Case 12 : M.G. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
04/02/1974	A	-	-	2.0	1.3	0.0
20/02/1974	A	-	-	1.0	1.0	0.7
02/04/1974	A	-	-	2.4	0.5	0.0
		------Died------				

A = active disease.

Case 13 : H.B. diagnosed as acute myeloid leukemia.

Date of samples	Condition of patients	W.B.C. (mm^{-3})	Blast cell(%)	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml
06/03/1974	A	-	-	3.5	1.1	0.2
13/03/1974	A	-	-	2.5	1.0	1.0
02/04/1974	A	-	-	5.0	0.2	0.2

A = active disease.

From cases 1 to 13, W.B.C. = White blood count.

Case 14 : M.W. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g%	MCV (u^3)
07/02/1974	20.0	1.8	0.0	3.6	8.7	87
19/02/1974	2.5	0.4	0.2			
07/03/1974	16.0	1.0	0.8			
11/04/1974	14.0	0.6	0.5	4.3	12.7	89

Case 15 : W.B. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g %	MCV (u^3)
07/03/1974	7.0	1.4	0.9			
11/04/1974	17.0	1.2	1.2	4.7	14.3	92
10/06/1974	4.0	0.0	0.0			
22/07/1974	1.0	0.9	0.5			
15/02/1975	0.5	0.4	0.2			

Case 16 : E.S. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g %	MCV (u^3)
22/01/1974	2.3	0.9	0.9	4.2	13.8	99
07/02/1974	2.0	1.5	0.0	4.5	12.4	82
19/02/1974	5.0	0.7	0.4			
11/04/1974	0.0	0.0	0.0	3.9	12.8	103
02/07/1974	1.2	0.1	0.1			
17/07/1974	3.0	1.8	1.2			

Case 17 : J.P. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g %	MCV (u^3)
22/01/1974	5.7	0.7	0.6	12.2	15.3	33
01/02/1974	3.5	1.0	0.3			
08/04/1974	3.5	0.8	0.6			

Case 18 : E.F. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g %	MCV (u^3)
11/12/1973	2.5	1.5	0.6	4.0	16.0	86
11/02/1974	2.5	1.4	0.8	5.0	13.9	83
28/10/1974	1.5	0.6	0.1			

Case 19 : P.B. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g %	MCV (u^3)
19/02/1974	1.0	0.6	0.6	4.9	14.0	84
05/12/1974	3.5	0.5	0.4			
12/02/1975	2.5	0.3	0.2			

Case 20 : F.W. diagnosed as adult coeliac disease.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	R.B.C. ($x10^6$)	Haemoglobin g %	MCV (u^3)
04/06/1973	146.0	30.0	1.6			
20/11/1973	30.0	30.0	1.2			
07/02/1974	300.0	79.0	0.4	3.9	11.7	87
19/02/1974	30.0	2.2	0.7	Post mortem sample.		

Case reports 14 to 20 are adult coeliac disease patients and R.B.C. = red blood cell count;MCV = mean cell volume.

Case 21 : P.H. diagnosed as rheumatoid arthritis.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	Erythrocyte sedimentation rate (mm.Wintrobe).
05/11/1974	3.3	1.1	1.1	98
28/01/1975	3.5	0.7	0.7	

Case 22 : R.T. diagnosed as rheumatoid arthritis.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	Erythrocyte sedimentation rate (mm.Wintrobe).
05/11/1974	2.0	2.0	1.2	58
28/01/1975	2.8	0.8	0.8	

Case 23 : E.R. diagnosed as rheumatoid arthritis.

Date of samples	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	Erythrocyte sedimentation rate (mm.Wintrobe)
05/11/1974	2.0	1.8	1.5	115
03/12/1974	2.0	1.3	1.3	

Table 9.1 Serum folates of some adult coeliac patients on treatment with folic acid (10 mg orally) and samples collected at 6 to 8 hours after doses.

Patients	L.casei (ng/ml)	S.faecalis (ng/ml)	P.cerevisiae (ng/ml)
F.L.	70	5.3	-
D.M.	62	31.0	-
D.M.	62	31.0	-
D.M.	45	2.0	-
J.W.	76	31.0	-
J.W.	91	54.0	1.2
R.H.	220	220.0	-
E.C.	140	100.0	0.7
E.C.	*	0.7	0.5
D.B.	26	6.1	-
R.B.	23	2.0	-
P.T.	21	2.0	-
E.B.	270	270.0	-
K.B.	39	18.0	1.1
A.B.	24	3.0	0.7
G.W.	21	0.7	0.7
G.W.	*	**	0.5
N.G.	*	3.0	1.1
J.C.	*	1.3	0.0
F.W.	300	79.0	0.4
D.K.	*	**	1.0
E.E.	*	**	0.8

cont...

Patients	L.casei (ng/ml)	S.faecalis (ng/ml)	P.cerevisiae (ng/ml)
continue...			
C.G.	*	**	0.4
U.Z.	*	**	0.2
S.G.	*	**	1.2
M.P.	*	1.0	0.9
G.W.	*	1.0	1.0
J.H.	*	0.9	0.9
L.H.	*	**	0.4
B.T.	*	1.1	1.1
J.Ma.	50	24.0	0.3
J.Mu.	50	14.0	1.0
E.B.	300	185.0	0.5
D.M.	*	0.6	0.6
W.J.	*	**	0.2
G.L.	*	**	0.5
Average	98 ± 27	38.2 ± 12.9	0.69 ± 0.07

* indicates results are higher than 30 ng/ml and ** indicates results are higher than 10 ng/ml but no values are determined. All subjects are on gluten-free diet.

Table 9.2 : Serum folate of patients with low L.casei serum folates but not identified as folate deficient from other clinical data i.e.normal haematological values.

Subject	Haemoglobin g %	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	MCV (u^3)
E.R.	13.4	2.0	0.3	0.2	84
E.S.	13.5	2.2	0.1	0.1	87
P.C.	15.7	1.0	0.2	0.2	93
S.S.	12.7	2.4	0.2	0.2	89
D.B.	13.0	3.0	0.5	0.5	87
S.C.	12.3	0.8	0.1	0.1	93
G.W.	12.5	2.0	0.1	0.1	85
M.H.	14.0	2.0	0.1	0.1	89
K.P.	13.8	1.4	0.5	0.5	93
Y.T.	14.5	3.0	0.3	0.2	87
C.P.	12.3	1.0	0.2	0.2	87
J.Q.	13.8	1.2	0.5	0.5	88
M.C.	15.0	1.2	0.8	0.8	89
P.H.	13.2	2.2	0.5	0.5	90
D.H.	12.9	2.6	0.6	0.6	85
A.S.	12.5	2.4	0.5	0.5	83
J.G.	13.1	2.4	1.1	1.0	90
J.W.	12.7	3.0	0.4	0.1	85
F.H.	12.8	1.5	0.4	0.3	90
Average	13.3±0.2	2.0±0.2	0.4±0.06	0.35±0.06	88±0.7

MCV = mean cell volume.

Table 9.3 : Serum folate of patients with low serum folate(L.casei) and identified as folate deficient from other clinical test i.e. abnormal haematological values.

Subject	Haemoglobin g %	L.casei ng/ml	S.faecalis ng/ml	P.cerevisiae ng/ml	MCV (u^3)
W.J.	11.4	0.5	0.4	0.3	106
K.S.	13.6	2.4	0.1	0.1	109
P.As.	9.9	0.4	0.2	0.2	110
J.H.	12.2	1.0	0.1	0.0	107
M.H.	14.5	1.0	0.1	0.1	102
R.P.	3.9	0.8	0.2	0.2	116
F.R.	12.5	1.3	0.1	0.1	105
P.Au.	11.7	0.5	0.1	0.1	112
Average ±S.E.M.	11.2±1.2	1.0±0.2	0.16±0.04	0.13±0.03	108±1.5

MCV = mean cell volume.

Table 9.4 Comparison of 10-formyltetrahydropteroylglutamic acid of adult coeliac disease patients on treatment with oral folic acid (6 to 8 hours before blood samples were taken) with normal humans and other adult coeliac disease patients without folic acid treatment.No of subjects in parenthesis.

Subject (no.of subjects)	10-CHO H_4PteGlu (ng/ml±S.E.M.)			
	S.faecalis	t-test(p)	P.cerevisiae	t-test(p)
Adult coeliac disease on folic acid (27)	38.2±12.9	-	0.69±0.07	-
Normal (52)	0.78±0.03	0.001	0.57±0.03	0.05
Adult coeliac disease without folic acid treatment (104)	0.98±0.05	0.001	0.62±0.04	0.15

Table 9.5 Comparison of serum folates from Table 9.2 and Table 9.3 to those of normal serum folates.

Subjects (no.of)	L.casei (ng/ml)	t-test p	S.faecalis (ng/ml)	t-test p	P.cerevisiae (ng/ml)	't' test(p)
Normal(52)	5.6±0.3	-	0.78±0.03	-	0.57±0.03	-
Table 9.2*	2.0±0.2	0.001	0.40±0.06	0.050	0.35±0.06	0.050
Table 9.3**	1.0±0.2	0.001	0.16±0.04	0.001	0.13±0.03	0.001

* indicates results of 19 subjects and ** 8 subjects.

Table 9.6 Comparison of serum folate of Table 9.2 and those of patients with L.casei serum folates lower than 2.6 ng/ml.

Diagnosis of subjects	No.of subjects	L.casei (ng/ml) ±S.E.M.	S.faecalis (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
Normal human(Table 9.2)	19	2.00±0.20	0.40±0.06	0.35±0.06
Adult coeliac disease	30	1.85±0.15	0.90±0.08	0.67±0.06
Psoriasis	5	1.72±0.18	1.14±0.18	0.92±0.13
Leukemias	31	1.67±0.14	1.03±0.10	0.55±0.07
Pernicious anaemia*	2	1.50±1.00	0.70±0.20	0.60±0.10
Rheumatoid arthritis	22	2.30±0.10	1.29±0.15	1.16±0.15
Regional enteritis	90	1.50±0.07	0.38±0.03	0.30±0.02
Ulcerative colitis	2	1.10±0.10	0.60±0.30	0.20±0.15
Schizophrenia	10	1.65±0.18	0.84±0.10	0.70±0.08

Compared with normal	:	't' Test p values(better than)		
Normal human(Table 9.2)	19	-	-	-
Adult coeliac disease	30	N.S.	0.001	0.005
Psoriasis	5	N.S.	0.001	0.001
Leukemias	31	0.1	0.001	0.05
Pernicious anaemia*	2	N.S.	0.05	0.05
Rheumatoid arthritis	22	N.S.	0.001	0.001
Regional enteritis	90	0.05	N.S.	N.S.
Ulcerative colitis	2	0.001	N.S.	N.S.
Schizophrenia	10	0.10	0.001	0.001

* receiving vitamin B_{12} therapy.

Student 't' Test of serum 10-CHO H_4PteGlu with p values better than :-

A	-	5	0.1
B	5	-	5
C	0.1	5	-
	A	B	C

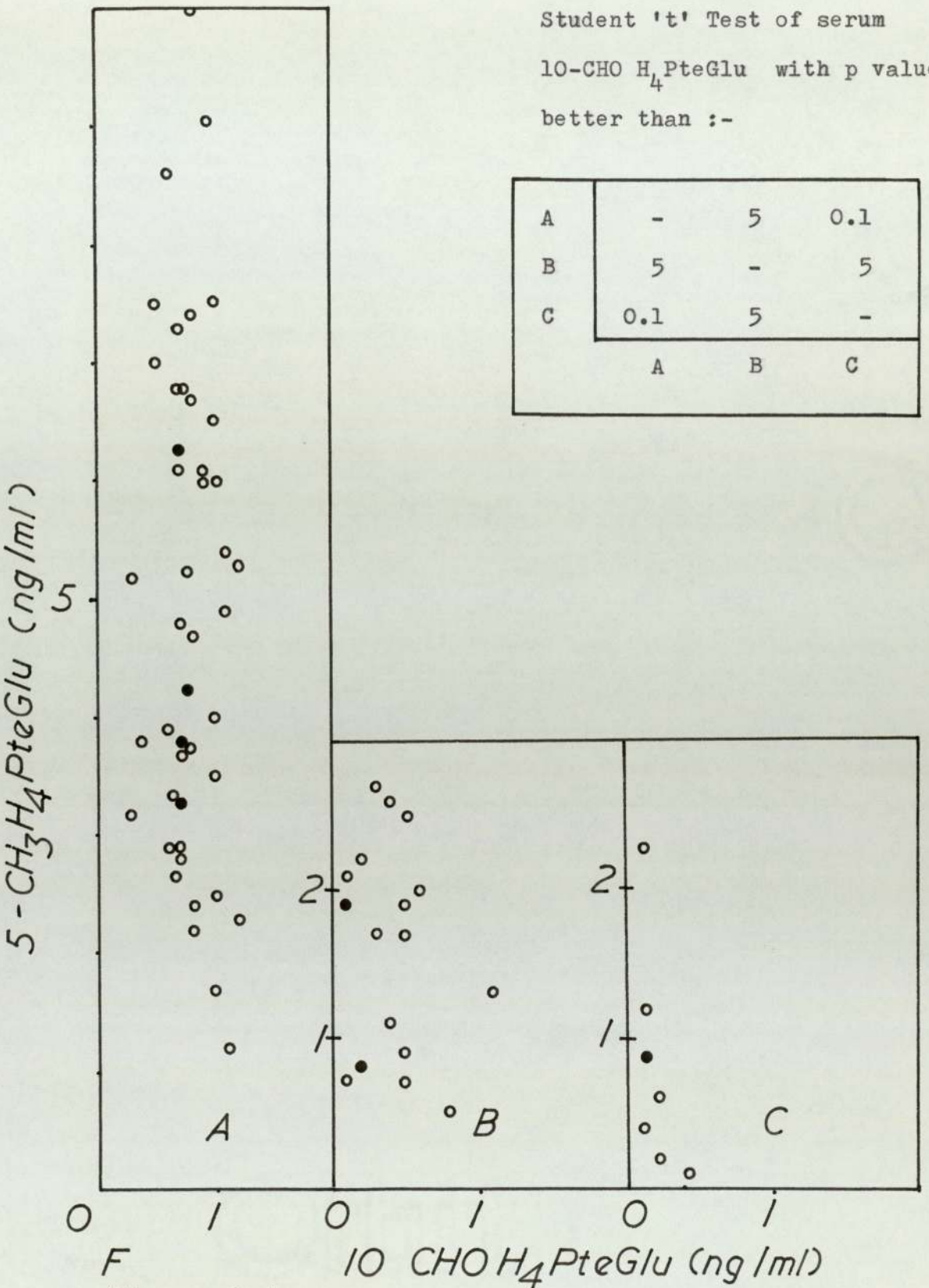


Figure 9.1 The relationship between 5-CH_3H_4PteGlu and 10-CHO-H_4PteGlu of A, normal humans, B, normal humans with low L.casei and C, megaloblastic patients.

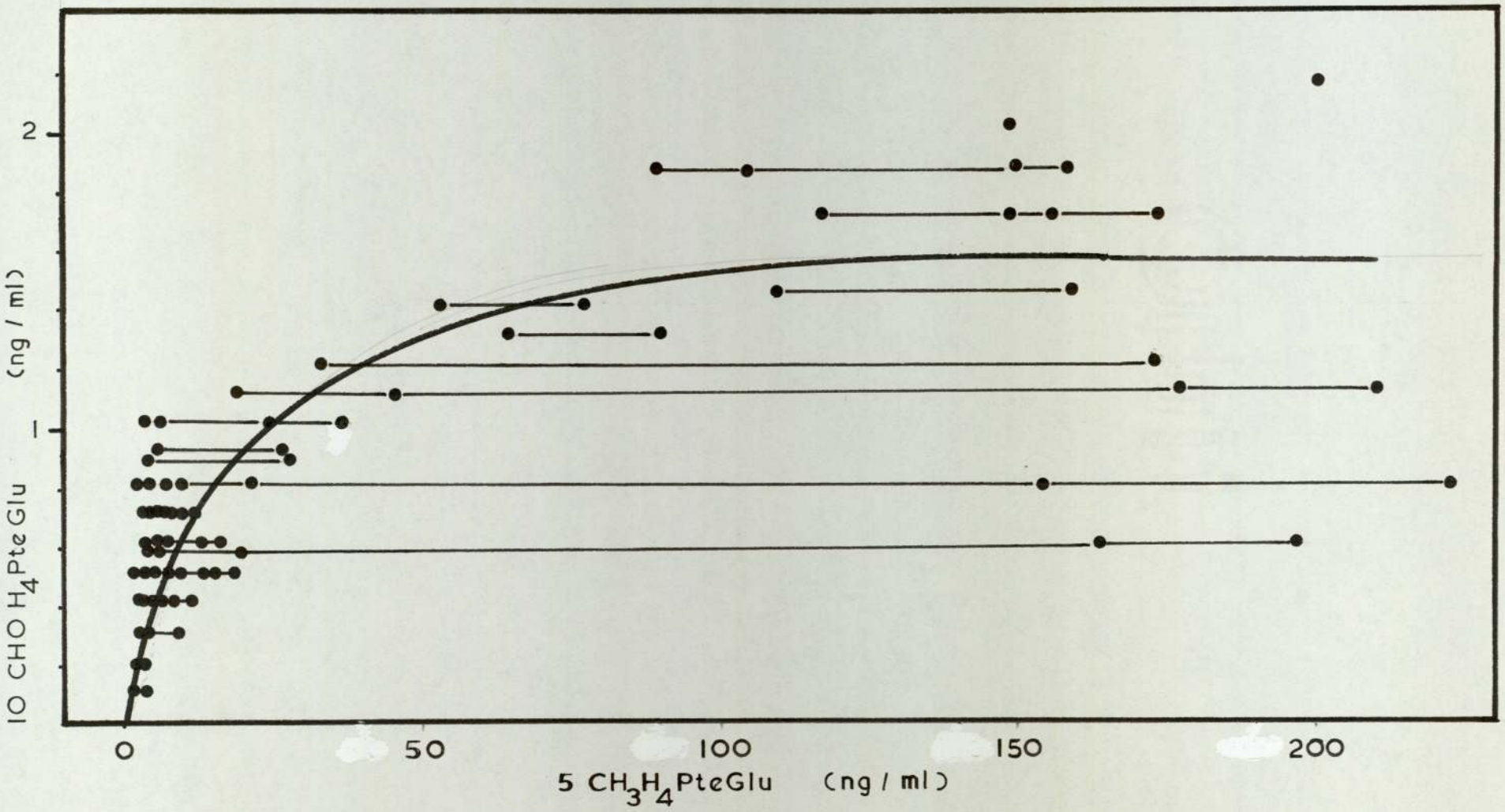


Figure 9.2 Michaelis Menten relationship of 10 CHO H_4 PteGlu and 5 CH_3H_4 PteGlu in normal human serum.

Discussion.

Because these adult coeliac patients were suffering from folate deficiency they were receiving 10 mg of folic acid per day. Analysis of their serum folate collected from the afternoon clinics (2.00 to 4.00 p.m.) are summarized in Table 9.1. In all cases folic acid is absorbed and in most cases are metabolised to 5-methyltetrahydropteroylglutamic acid as seen by the differences between L.casei and S.faecalis activities. Two cases showed only pteroylglutamic acid as a major folate. These two patients are to be discussed latter on. 10-formyltetrahydropteroylglutamic acid is responsible for the activity of serum folate measured with P.cerevisiae (Ratanasthien et al 1974).Comparing P.cerevisiae results from these patients with those of normal humans or other patients with adult coeliac disease but not receiving folic acid treatment showed them to be elevated but not higher than S.faecalis values of normal human serum,as shown in Table 9.4.

10-formyltetrahydropteroylglutamic acid is about ten time more easily to be oxidised than 5-methyltetrahydropteroylglutamic acid (Blair and Pearson 1974). 10-formylpteroylglutamic acid is an oxidation product of 10-formyltetrahydropteroylglutamic acid and it is not metabolized (Ratanasthien et al 1974;Beavon and Blair,1975;Beavon,1973).Thus a high level of 10-formyltetrahydropteroylglutamic acid would led to a higher rate of loss of serum folate.

Studies of leukemic patients for their serum folate status over a period of time showed that most of the time serum folate (10-formyltetrahydropteroylglutamic acid) is raised above

the normal. Serum folate as measured with L.casei fluctuated which indicated lack of intake folate or increase in the metabolic requirement. Blast cells are seen in both patients with low serum folate and some in those patients with total serum folate in normal range. Serum folate before the death of these patients were either high 10-formyltetrahydropteroyl-glutamic acid or low total serum folate and in some case nil.

Follow up studies on serum folates of patients with rheumatoid arthritis is done only for a few patients and all of them has only two tests done. Serum folates of these patients are not too low but lower than normal range in all tests. Serum 10-formyltetrahydropteroylglutamic acid is significantly elevated as compared with normals. These patients were on treatment schedule (Constable et al 1975). The erythrocyte sedimentation rate is used as one of indexes of the disease state. There is no good correlation amongst these factors apart from the raised 10-formyltetrahydropteroylglutamic acid and the raised erythrocyte sedimentation rate levels.

Follow up studies on serum folates of adult coeliac disease patients also shown a high degree of variation in total serum folate as seen in cases 14 to 20. On treatment with oral folic acid serum folate is higher than 30 ng/ml as long as 6 to 8 hours after the 10 mg doses (Table 9.1).Following the oral treatment with folic acid these patients responded well as seen in the improved blood pictures. The high degree of variation of serum folate amongst these patients may be because of the increase in requirement or because of their malabsorption (Cooke et al 1963).

Patients with total serum folate less than 3 ng/ml were studied in greater detail to see if any better definition of folate deficiency could be found. These patients are separated into two groups A and B and their serum folates are shown in Table 9.2 and Table 9.3, respectively. Amongst these patients, groupA was identified as being clinically normal by haematological studies and group B classified as folate deficiency from the haematological point of view. The classification is done by using blood pictures as judging factors. Statistical analysis show that total serum folate of both groups is significantly decreased. Amongst those of group A their serum folates measured with S.faecalis and P.cerevisiae are higher than those of group B (Table 9.5). Group A patients eventhough their total serum folate is very significantly lower than those of normal subjects at $p < 0.1$ % serum S.faecalis and P.cerevisiae levels are lower than those of normal humans at $p < 5$ % whilst in group B both S.faecalis and P.cerevisiae are very significantly lowered at $p < 0.1$ % when compared with normal values.

Comparison of these folates of group A and those of patients with serum folates lower than 2.6 ng/ml in Chapter 8 show clearly that in those patients eventhough L.casei is not significantly different their levels of 10-formyltetrahydropteroylglutamic acid are significantly increased and only those patients with regional enteritis gave no difference (Table 9.6).

From these studies it is possible to conclude that clinical folate deficiency is not only detected by low serum (L.casei) folate but also by low 10-formyltetrahydropteroylglutamic acid. Studies of the homeostatic mechanism of the

equilibrium between 5-methyltetrahydropteroylglutamic acid and 10-formyltetrahydropteroylglutamic acid were made from these observations.

$$5\text{-}CH_3H_4PteGlu \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 10\text{-}CHO\ H_4PteGlu$$

The above reaction mechanisms are possibly regulated by enzyme systems in normal human in which the reaction rate k_1 is a rate limiting step due to enzyme activity and varies a little with the increased serum 5-methyltetrahydropteroyl-glutamic acid levels as seen in the absorption experiments (Chapter 5). The rate of conversion from 10-formyltetrahydro-pteroylglutamic acid to 5-methyltetrahydropteroylglutamic acid (k_{-1}) is not variable with the serum levels of 5-methyltetra-hydropteroylglutamic acid as also seen in the absorption experiments (Chapter 5). In diseases with a high proliferation rate the enzyme systems regulating k_1 are disturbed and the reaction shifted to the right and thus seen as an increase in tissue or serum folate of 10-formyltetrahydropteroylglutamic acid(Sotobayashi,Rosen and Nichol,1966;Ratanasthien,Blair, Leeming,Cooke,and Melikian,1974;Silverman,Law and Kaufman,1961). Because of low 5-methyltetrahydropteroylglutamic acid in the clinically normal (group A) and the fact that the enzymes controlling k_1 are no longer rate limiting therefore the level of 10-formyltetrahydropteroylglutamic acid is lowered but in those of group B patients the 10-formyltetrahydropteroylglutamic acid is much relatively lower than that of group A and normal human. Comparison of 10-formyltetrahydropteroylglutamic acid amongst the normal subjects,group A and group B patients are

shown graphically in Figure 9.1a,b,c for normal,group A and group B,respectively. It is clearly seen that the levels of 10-formyltetrahydropteroylglutamic acid of group A and group B are lower than those of normal humans but those of group A are at the lower limit of the normal whilst those of group B are well outside the normal range. These observations indicated that blast cells of folate deficiency occurred in subjects with serum 10-formyltetrahydropteroylglutamic acid deficiency.

The relationship of serum 10-formyltetrahydropteroyl-glutamic acid and 5-methyltetrahydropteroylglutamic acid of normal humans (from group A,normal humans and normal humans after oral administration of 10 mg dl-5-methyltetrahydropteroyl-glutamic acid) followed a 'Michaelis-Menten'plot (Figure 9.2) confirmed the rate limiting step previously mentioned.

Summary.

Longterm follow up of folate status of patients with adult coeliac disease, rheumatoid arthritis and leukemia were studied. Studies of leukemic patients showed that most of the time their serum 10-formyltetrahydropteroylglutamic acid were raised above the normal levels and serum folate before the death of these patients were either high level of 10-formyltetrahydropteroylglutamic acid or low total serum folate and in some cases nil. Patients with rheumatoid arthritis also have low serum folate and with raised 10-formyltetrahydropteroylglutamic acid whilst patients with adult coeliac disease have both serum folate at normal and low levels with also raised 10-formyltetrahydropteroylglutamic acid. There is no clear correlation between serum folate status and other clinical parameters amongst these patients. Studies of adult coeliac disease patients on treatment with oral folic acid showed that the level of 10-formyltetrahydropteroylglutamic acid did not boost by this treatment. Patients with low serum (L.casei) folate but not identified as folate deficiency by other clinical factors have relatively higher serum 10-formyl-H_4PteGlu than those identified as folic acid deficiency. Thus folate deficiency is not only detected by low serum folate (L.casei) but also by low 10-formyltetrahydropteroylglutamic acid. These studies support the homeostasis of serum folates.

Chapter 10.

Absorption and metabolism of folates in disease.

1) Oral 5-methyltetrahydropteroylglutamic acid in adult coeliac disease patients.

2) Oral 5-methyltetrahydropteroylglutamic acid in leukemia patients.

3) Oral 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid in patients with pernicious anaemia.

Metabolism of folates in disease.

Although folates are known to be responsible for curing patients with anaemia using liver or yeast extracts,the clinical treatment of patients with folate deficiency employs synthetic pteroylglutamic acid (Chanarin,1969).Pteroylglutamic acid may be present in very small amount in the Nature.Many investigators believe that it be absent(Butterworth et al 1963;Santini et al 1964).Little pteroylglutamic acid is found in food stuffs and this may be an analytical artefact.Folates found in diets are reduced forms or non-reduced forms other than pteroylglutamic acid itself,5-methyltetrahydropteroylglutamic acid and 10-formyl-tetrahydropteroylglutamic acid and their derivatives are probably the major folates in food stuffs.

Because of the clinical use of pteroylglutamic acid in treatment of patients with folate deficiency there is a considerable amount of information about this compound available in various diseases (Blakley,1969;Chanarin,1969).Metabolism of some folates has been studied in some detail for normal humans and was discussed in Chapter 5.

In these experiments the metabolism of oral folates other than folic acid were studied in some patients and results were compared with normal humans and those of literature.Normal human volunteers with temporary achlorhydria caused by simultaneous administration of sodium bicarbonate were also used in these experiments.

Materials,Patients and Methods.

The folates used in these studies were described in greater detail in Chapter 3. 5-methyltetrahydropteroylglutamic acid was prepared and purified according to Blair and Saunders (1970). 5-methyl-5,6-dihydropteroylglutamic acid was prepared by oxidation of 5-methyltetrahydropteroylglutamic acid as described in Chapter 3.

Patients and normal human volunteers were used in the study of oral folate metabolism.Metabolism of oral folate in temporary achlorhydria are those discussed in Chapter 5.

Patients diagnosed as adult coeliac disease and leukemia patients were used for the studies of the oral metabolism of 5-methyltetrahydropteroylglutamic acid.Patients with pernicious anaemia receiving vitamin B_{12} treatment were used for the studies of oral metabolism of 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid. They were fasted overnight and emptied their bladders just before the test started. 10 ml of venous blood samples were aseptically taken just before the oral doses of 5 mg of microbiologically active materials or 10 mg of diastereoisomers of folate were given.After oral doses of the test folate blood samples were taken at time $\frac{1}{2}$,1,1$\frac{1}{2}$,2 and 3 hours.Blood samples were quickly transferred into sterile plain glass sample tubes and allowed to clot at room temperature for one half to two hours.After centrifugation at room temperature sera were decanted and separated into two portions when determination of 5-methyl-5,6-dihydropteroylglutamic acid was desired. Otherwise they were collected as a single sample.To the first portion of each sample 5 mg/ml of ascorbic acid were added and the sample stored at -20°C until assayed.Where used the second portion

of each sample was acidified with a small amount of concentrated HCl to pH 3.0 and neutralised to pH 7.4 after 10 to 20 minutes with 20 % (W/V) sodium hydroxide solution.To the neutralized samples 5 mg/ml of ascorbic acid were added and stored at -20°C until assayed.When the content of 5-methyl-5,6-dihydropteroyl-glutamic acid in serum samples was desired the clotting time was reduced to within half an hour to minimize the oxidation of 5-methyltetrahydropteroylglutamic acid in the samples.

At the end of the test period urine samples were collected and treated similarly to those of sera but the total volumes were noted.

Microbiological assays with L.casei,S.faecalis, P.cerevisiae and their bioautography as described in Chapter 2 were used for detection and identification of these samples.

Results.

The metabolism of oral 5-methyltetrahydropteroyl-glutamic acid was studied at a pharmacological doses in normal humans at various conditions and the results are summarized in Table 10.1 for serum 5-methyl-5,6-dihydropteroylglutamic acid after various folates,Table 10.2 for serum folates of normal humans after 10 mg of dl-5-methyltetrahydropteroylglutamic acid, Table 10.3 for serum folates of normal humans taking 3 g of sodium bicarbonate orally (10 to 20 minutes)before10 mg of dl-5-methyltetrahydropteroylglutamic acid and Table 10.4 for serum folates of normal humans taking 5 g of sodium bicarbonate (10 to 20 minutes) before 10 mg of dl-5-methyltetrahydropteroyl-glutamic acid orally.

The results from the studies of oral 5-methyltetrahydropteroyl-glutamic acid metabolism in adult coeliac desease,leukemia, and pernicious anaemia patients are summarized in Table 10.5, Table 10.6 and Table 10.7,respectively.

The metabolism of oral 5-methyl-5,6-dihydropteroyl-glutamic acid in pernicious anaemia patients are summarized in Table 10.8.

One patient with alleged pernicious anaemia had serum folates similar to normal subjects after oral administration of 5-methyl-5,6-dihydropteroylglutamic acid.This diagnosis was later repudiated by Dr.W.T.Cooke.The results of this study are summarized in Table 10.9 as compared with those of normal humans taken similar doses of 5-methyl-5,6-dihydropteroylglutamic acid (Table 10.10), and with sodium bicarbonate 3 g (Table 10.11).

Three hour urine excretion of these subjects taken various folates are summarized in Table 10.12.

Table 10.1

Serum 5-methyl-5,6-H_2PteGlu of normal human subjects taking 5 mg doses of various folates orally. Those results with * and ** were derived from subjects taking 3 and 5 g sodium bicarbonate (10 to 20 minutes) before the test folate.

Administered folate	No. of Subjects	5-CH_3-5,6-H_2PteGlu (ng/ml ± S.E.M.) at time (h)					
		T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
5-CH_3-5,6-H_2PteGlu	1	0.0	2.0	1.0	1.0	1.0	1.0
5-CH_3-5,6-H_2PteGlu	1*	0.5	11.0	18.0	33.0	26.0	10.0
4a(OH)-5-CH_3H_4PteGlu	2	1.0±0.0	3.2±2.2	2.8±1.8	1.0±0.0	0.6±0.5	0.6±0.5
7,8-H_2PteGlu	2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5-CH_3H_4PteGlu	4*	3.0±2.1	17.0±2.7	48.0±17.3	74.0±40.0	65.0±31.5	50.0±16.7
5-CH_3H_4PteGlu	5**	0.9±0.3	70.0±28.8	86.0±40.7	115.4±43.0	75.0±31.7	54.0±28.2

Table 10.2 Serum folates (mean±S.E.M.) of 6 normal human subjects after 5 mg (10 mg of diastereoisomers) of microbiologically active 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	9.1 ± 2.1	8.3± 2.3	0.2 ± 0.05	0.6 ± 0.10
½	106.0 ± 15.2	103.1± 16.0	2.4 ± 0.50	0.5 ± 0.10
1	166.0 ± 21.0	162.2± 21.6	3.1 ± 0.50	0.7 ± 0.10
1½	188.0 ± 19.0	184.3± 21.0	3.1 ± 0.50	0.6 ± 0.10
2	180.0 ± 14.7	175.4± 14.7	3.8 ± 0.49	0.8 ± 0.09
3	110.0 ± 9.4	106.3± 9.4	3.0 ± 0.45	0.7 ± 0.09
4	81.0 ± 9.8	77.5± 10.6	2.8 ± 0.45	0.7 ± 0.10
5	55.8 ± 5.2	52.4± 5.8	2.7 ± 0.50	0.7 ± 0.10

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 10.3 Serum folates (mean±S.E.M.) of 4 normal humans after 3 g of sodium bicarbonate for 10 to 20 minutes and 10 mg dl-5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	7.5 ± 2.2	7.0 ± 2.0	0.0 ± 0.0	0.5 ± 0.1
½	75.0 ± 7.6	72.2 ± 7.9	1.8 ± 1.6	1.0 ± 0.3
1	155.0 ±21.8	149.6 ± 22.0	3.9 ± 2.0	1.5 ± 0.3
1½	176.7 ±24.8	170.0 ± 25.0	5.0 ± 2.0	1.7 ± 0.4
2	147.5 ±28.7	141.0 ± 29.0	5.0 ± 2.9	1.5 ± 0.5
3	106.0 ±29.0	101.5 ± 29.9	3.1 ± 1.9	1.4 ± 0.5

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 10.4 Serum folates (mean ± S.E.M.) of 5 normal humans after 5 g of sodium bicarbonate (10 to 20 minutes) and 5 mg of microbiologically active 5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	8.0 ± 3.0	7.3 ± 3.1	0.05 ± 0.0	0.65 ± 0.05
½	172.0 ± 18.8	170.1 ± 19.0	0.44 ± 0.1	1.46 ± 0.46
1	246.0 ± 19.4	242.8 ± 19.7	1.00 ± 0.2	2.20 ± 0.53
1½	259.0 ± 18.5	255.3 ± 18.8	1.80 ± 0.2	1.90 ± 0.54
2	220.0 ± 22.6	217.3 ± 22.9	1.14 ± 0.2	1.56 ± 0.36
3	145.0 ± 23.2	143.4 ± 23.5	0.50 ± 0.1	1.10 ± 0.22

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 10.5 Serum folates (mean ± S.E.M.) of 6 adult coeliac disease patients after 10 mg of dl-5-methyltetrahydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4-* PteGlu (ng/ml) ±S.E.M.	5-CH_3H_2-** PteGlu (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(S.faecalis). 10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	4.0± 0.8	3.2± 0.7	0.85±0.5	0.2±0.03	0.6±0.13
½	65.4±23.9	61.4±22.1	16.6± 16	3.0±1.51	1.0±0.24
1	96.6±24.3	89.6±21.2	20.0± 15	5.6±2.60	1.4±0.51
1½	138.0±25.4	128.2±21.0	20.0± 15	8.0±3.50	1.8±0.88
2	132.0±15.7	121.8±10.5	33.3± 24	8.0±4.10	2.2±1.19
3	100.0±20.0	92.6±16.5	36.7± 20	6.2±2.70	1.2±0.78

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities and ** indicates results derived from only 3 subjects.

Subject number	Serum 5-methyl-5,6-H_2PteGlu (ng/ml)					
	$T_{0(h)}$	$T_{\frac{1}{2}(h)}$	$T_{1(h)}$	$T_{1\frac{1}{2}(h)}$	$T_{2(h)}$	$T_{3(hour)}$
1 J.Mu	1.4	2.0	2.0	10.0	80.0	40.0
2 J.Ma	0.9	47.0	49.0	49.0	20.0	70.0
3 L.B	0.3	1.0	9.0	1.0	0.0	0.0

Table 10.6 Serum folates (mean ± S.E.M.) of 8 leukemic patients after oral administration of 10 mg dl-5-methyltetrahydropteroyl-glutamic acid.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4-* PteGlu (ng/ml) ±S.E.M.	5-CH_3H_2-** PteGlu (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(S.faecalis) 10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	5.7± 1.1	4.8± 1.0	2.5±0.5	0.1±0.07	0.8±0.05
½	65.6±12.6	64.6±12.6	35.0±5.0	0.2±0.07	0.8±0.07
1	117.9±19.7	116.4±19.5	23.0±12	0.5±0.25	1.0±0.17
1½	105.3±13.8	103.8±13.8	27.0±17	0.5±0.31	1.0±0.13
2	94.8±12.7	93.0±12.7	45.0±25	0.8±0.39	1.0±0.16

* indicates results are the summation of true 5-methyltetrahydro-pteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities and ** indicates results derived from only two subjects.

Subject Number	Serum 5-CH_3-5,6-H_2PteGlu(ng/ml)				
	$T_{0(h)}$	$T_{\frac{1}{2}(h)}$	$T_{1(h)}$	$T_{1\frac{1}{2}(h)}$	$T_{2(h)}$
1(F.B.)	3.0	40.0	10.0	10.0	70.0
2(A.E.)	2.0	30.0	35.0	45.0	20.0

Table 10.7 Serum folates (mean ± S.E.M.) of 2 subjects with pernicious anaemia (on treatment with vitamin B_{12}) after oral administration of 10 mg of dl-5-methyltetrahydropteroylglutamic acid.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4-* PteGlu (ng/ml) ±S.E.M.	5-CH_3H_2-** PteGlu (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(S.faecalis) 10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	3.8±0.2	3.4±0.1	1.7	0.1±0.1	0.3±0.3
½	92.0± 28	91.5± 27	20.0	0.1±0.1	0.4±0.4
1	190.0± 40	188.6± 40	70.0	0.8±0.5	0.6±0.6
1½	240.0± 60	238.7± 60	120.0	0.8±0.3	0.5±0.5
2	220.0± 50	218.7± 50	70.0	0.4±0.1	0.9±0.0
3	160.0± 60	158.7± 60	20.0	0.5±0.3	0.8±0.0

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities and ** indicates results derived from only one subject.

Table 10.8 Serum folates (mean ± S.E.M.) of 5 pernicious anaemia patients on 10 mg of 5-methyl-5,6-dihydropteroylglutamic acid orally. These subjects were receiving vitamin B_{12} treatment.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4-* PteGlu (ng/ml) ±S.E.M.	5-CH_3H_2-** PteGlu (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(S.faecalis) 10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	8.6± 2.8	7.9± 2.8	4.6± 1.8	0.14±0.04	0.56±0.08
½	47.0± 6.8	45.8± 6.8	32.0±17.5	0.47±0.05	0.73±0.14
1	72.4±10.2	70.8±10.1	65.0±22.0	0.65±0.04	0.95±0.14
1½	96.8±14.1	95.1±13.9	79.0±34.0	0.84±0.13	0.86±0.12
2	97.4±15.0	95.7±14.7	70.0±26.2	0.85±0.22	0.85±0.06
3	84.8±15.9	83.6±15.9	47.0±16.1	0.47±0.10	0.73±0.09

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities and ** indicates results derived from 3 subjects:-

Subject number	Serum 5-methyl-5,6-H_2PteGlu (ng/ml) $T_{0(h)}$	$T_{\frac{1}{2}(h)}$	$T_{1(h)}$	$T_{1\frac{1}{2}(h)}$	$T_{2(h)}$	$T_{3(hour)}$
1 J.M.	8.0	59.0	105.0	140.0	120.0	70.0
2 Shaw	2.0	2.0	59.0	76.0	32.0	55.0
3 Martin	4.0	35.0	28.0	22.0	23.0	16.0

Table 10.9 Serum folates of one patient with alleged pernicious anaemia after oral administration of 5-methyl-5,6-dihydropteroyl-glutamic acid 10 mg orally.This diagnosis was later repudiated by Dr.W.T.Cooke.

Time after doses(h)	Total folate (ng/ml)	5-CH_3H_4PteGlu (ng/ml)*	5-CH_3-5,6-H_2PteGlu (ng/ml)	10-CHO H_4PteGlu(S.faecalis)	
				10-CHO PteGlu (ng/ml)	P.cerevisiae (ng/ml)
0	9.0	8.5	2.0	0.0	0.5
½	24.0	21.5	0.0	2.0	0.5
1	28.0	26.5	0.0	1.0	0.5
1½	30.0	29.1	0.0	0.4	0.5
2	26.0	25.3	3.0	0.2	0.5
3	26.0	25.4	8.0	0.1	0.5

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid.

Table 10.10 Serum folates (mean±S.E.M.) of 5 normal humans after 5 mg of microbiologically active 5-methyl-5,6-dihydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu(S.faecalis) 10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	6.2 ± 1.3	5.5 ± 1.3	0.1 ± 0.05	0.6 ± 0.2
½	37.6 ± 5.9	36.6 ± 5.9	0.1 ± 0.06	0.9 ± 0.2
1	32.8 ± 4.5	31.6 ± 4.6	0.2 ± 0.10	1.0 ± 0.2
1½	27.8 ± 2.0	26.8 ± 2.0	0.1 ± 0.05	0.9 ± 0.2
2	23.6 ± 0.9	22.7 ± 0.9	0.1 ± 0.05	0.8 ± 0.2
3	21.8 ± 0.8	21.1 ± 0.2	0.1 ± 0.02	0.6 ± 0.1

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

Table 10.11 Serum folates (mean ± S.E.M.) of 3 normal humans after 3 g of sodium bicarbonate (10 to 20 minutes) and 5 mg of microbiologically active 5-methyl-5,6-dihydropteroylglutamic acid orally.

Time after doses (hour)	Total folate (ng/ml) ±S.E.M.	5-CH_3H_4PteGlu* (ng/ml) ±S.E.M.	10-CHO H_4PteGlu (S.faecalis)	
			10-CHO PteGlu (ng/ml) ±S.E.M.	P.cerevisiae (ng/ml) ±S.E.M.
0	8.8 ± 3.0	8.0 ± 3.0	0.1 ± 0.0	0.7 ± 0.1
½	19.0 ± 1.0	18.2 ± 1.0	0.0 ± 0.0	0.8 ± 0.1
1	51.6 ± 3.4	50.2 ± 4.0	0.4 ± 0.2	1.0 ± 0.1
1½	112.3 ± 10.0	109.9 ± 10.2	1.1 ± 0.1	1.3 ± 0.2
2	99.3 ± 12.0	96.4 ± 12.4	1.4 ± 0.3	1.5 ± 0.4
3	51.3 ± 6.0	48.8 ± 6.4	1.3 ± 1.0	1.2 ± 0.5

* indicates results are the summation of true 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid activities.

TABLE 10.12 Urinary folates (mean ± S.E.M.) of subjects taken various folates at doses equivalent to 5 mg of microbiologically active materials , orally. Urine samples collected for 3 hours after doses.

Diagnosis of subjects	Administered doses	Total folates ug	5 CH_3H_4 PteGlu• ug	10 CHO PteGlu ug	10 CHO H_4 PteGlu ug	No. of subjects
Normal	5 CH_3H_4 PteGlu	258 ± 42	222 ± 23	24.0 ± 10	12.0 ± 0.05	2
Normal	5 CH_3 5,6 H_2 PteGlu	62 ± 12	50.3 ± 11	10.1 ± 4	1.6 ± 1.02	3
Normal	3g $NaHCO_3$ ———	310 ± 45	295 ± 40	13.7 ± 5	1.3 ± 1.08	2
Normal	——— 5 CH_3H_4 PteGlu	688 ± 75	656 ± 73	26.8 ± 13	5.2 ± 3.08	3
Pernicious anaemia	5 CH_3H_4 PteGlu	121	115.5	4.4	1.1	1
Pernicious anaemia	5 CH_3 5 6 H_2 PteGlu	223 ± 25	211 ± 23	10.6 ± 2	1.4 ± 0.15	5
Alleged P.A.	5 CH_3 5 6 H_2 PteGlu	21	5	15.1	0.9	1
Adult coeliac disease	5 CH_3H_4 PteGlu	287 ± 64	266 ± 58	17.7 ± 4	3.3 ± 2.10	2

• indicates results are the summation of true 5 CH_3H_4 PteGlu and 5 CH_3 5 6 H_2 PteGlu activities.

Table 10.13 Pharmacokinetics studies of serum folates after oral administration of various folates in normal human and in disease.

Diagnosis	5 mg doses (orally)	No.of subjects	First order $t_{\frac{1}{2}}$ (hour)	Availability in ng/ml at time 0 h.
Normal	PteGlu	1	1.33	260
Normal	H_2PteGlu	4	1.50	500
Normal	5-CH_3-5,6-H_2PteGlu	5	2.33	44
Normal	5-CH_3-5,6-H_2PteGlu	3*	1.75	220
Normal	5-CH_3H_4PteGlu	6	1.75	420
Normal	5-CH_3H_4PteGlu	4*	2.20	290
Normal	5-CH_3H_4PteGlu	5**	1.75	480
Alleged P.A	5-CH_3-5,6-H_2PteGlu	1	2.50	45
P.A.	5-CH_3-5,6-H_2PteGlu	5	4.80	130
P.A.	5-CH_3H_4PteGlu	2	2.30	390
A.C.D.	5-CH_3H_4PteGlu	6	3.00	205
Leukemia	5-CH_3H_4PteGlu	8	3.00	150

* indicates subjects taken 3 g of sodiumbicarbonate just before the test doses and ** 5 g of sodiumbicarbonate was taken;P.A. is pernicious anaemia patients on vitamin B_{12} treatment and A.C.D. is adult coeliac disease patients.

Table 10.14 Serum folates of normal human and disease (peak time and peak levels).

Administered doses of oral folate(5 mg)	No.of subjects	Peak Time (hour)	Peak Levels(L.casei) (ng/ml)
Normal subjects:			
PteGlu	5	3	226.0±43.0
H_2PteGlu	4	1½	260.0±27.4
10-CHO PteGlu	6	1½	185.0±15.0
5-CHO H_4PteGlu	6	1½	287.0±39.1
5,10-CH=H_4PteGlu	6	2	325.0±45.0
5-CH_3H_4PteGlu	6	1½	188.0±14.7
5-CH_3-5,6-H_2PteGlu	5	½	37.6± 5.9
5-CH_3H_4PteGlu	4*	1½	176.7±24.8
5-CH_3H_4PteGlu	5**	1½	259.0±18.5
5-CH_3-5,6-H_2PteGlu	3*	1½	112.3±10.0
Alleged pernicious anaemia (latter repudiated)			
5-CH_3-5,6-H_2PteGlu	1	1½	30.0
Pernicious anaemia			
5-CH_3-5,6-H_2PteGlu	5	2	97.4±15.0
5-CH_3H_4PteGlu	2	1½	240.0±60.0
Adult coeliac disease			
5-CH_3H_4PteGlu	6	1½	138.0±25.4
Leukemia			
5-CH_3H_4PteGlu	8	1	117.9±19.7

* indicates subjects taken 3 g of sodiumbicarbonate before the test and ** indicates subjects taken 5 g of sodiumbicarbonate orally before the test.

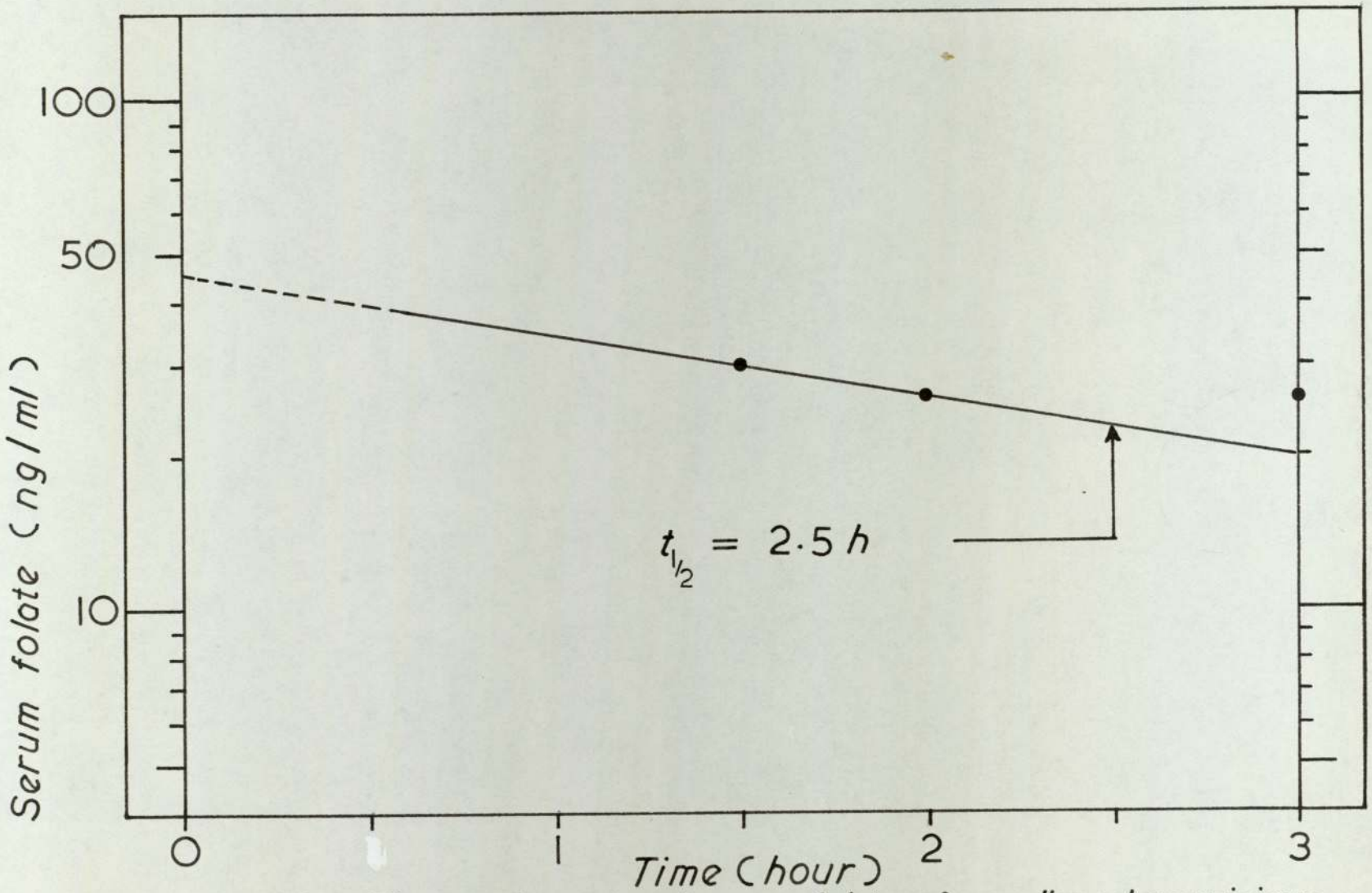


Figure 10·1 First order rate of serum folate of an alleged pernicious anaemia patient after 10 mg of 5 CH_3 5,6 H_2 Pte Glu, orally.

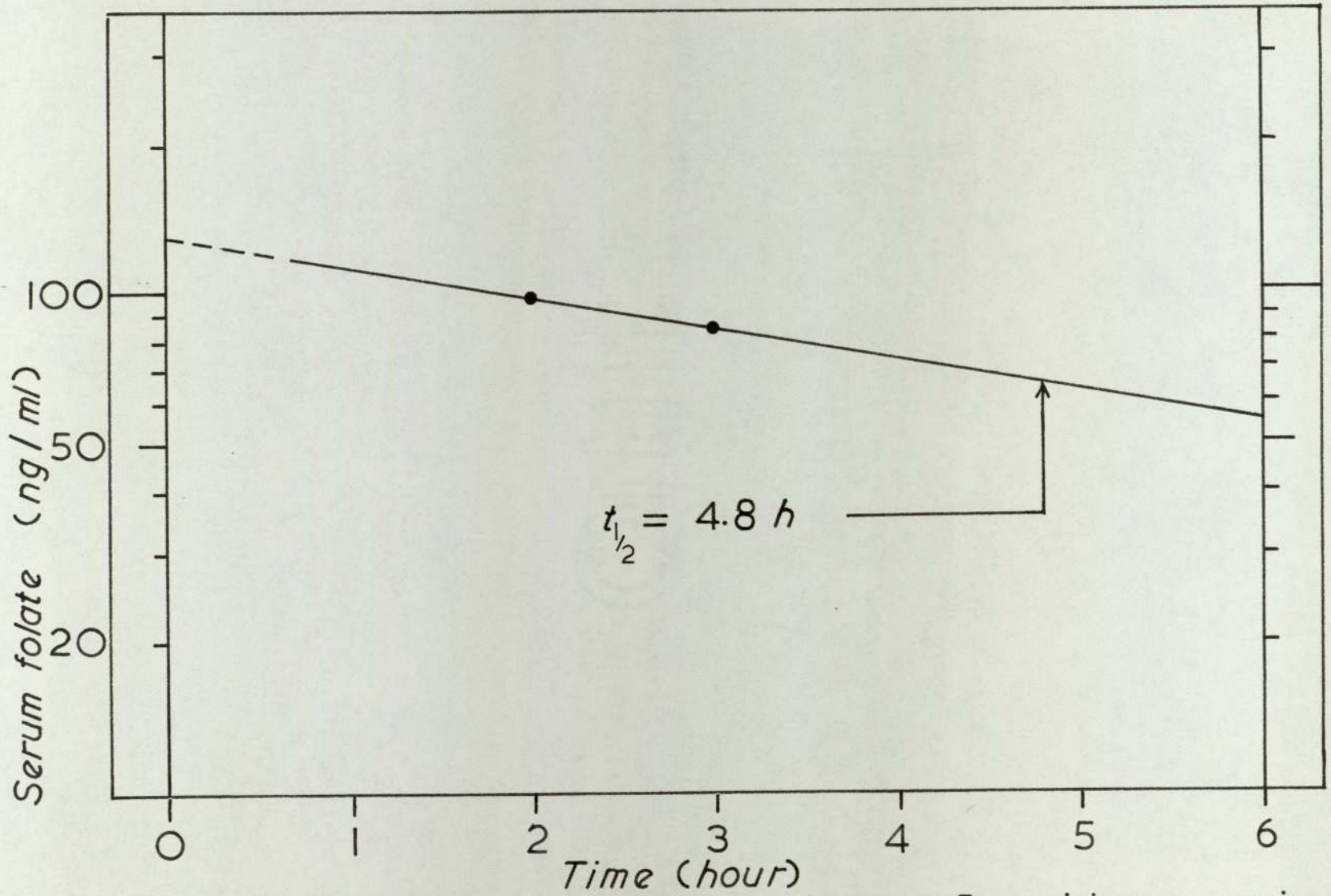


Figure 10.2 First order rate of serum folate of 5 pernicious anaemia patients after 10 mg of $5CH_3 5,6H_2$ PteGlu , orally.

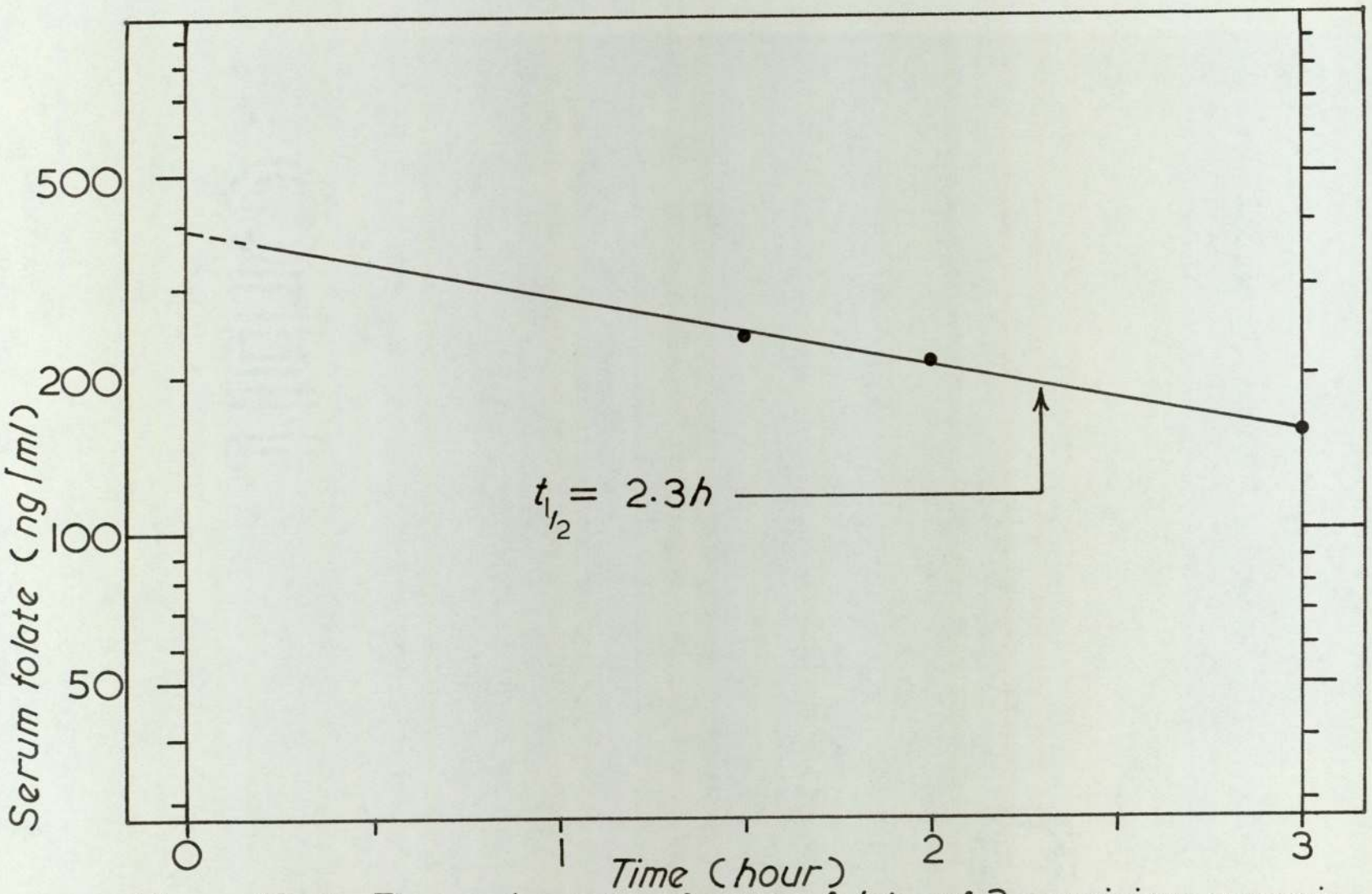


Figure 10.3 First order rate of serum folate of 2 pernicious anaemia patients after 10 mg of dl 5 CH_3H_4 Pte Glu, orally.

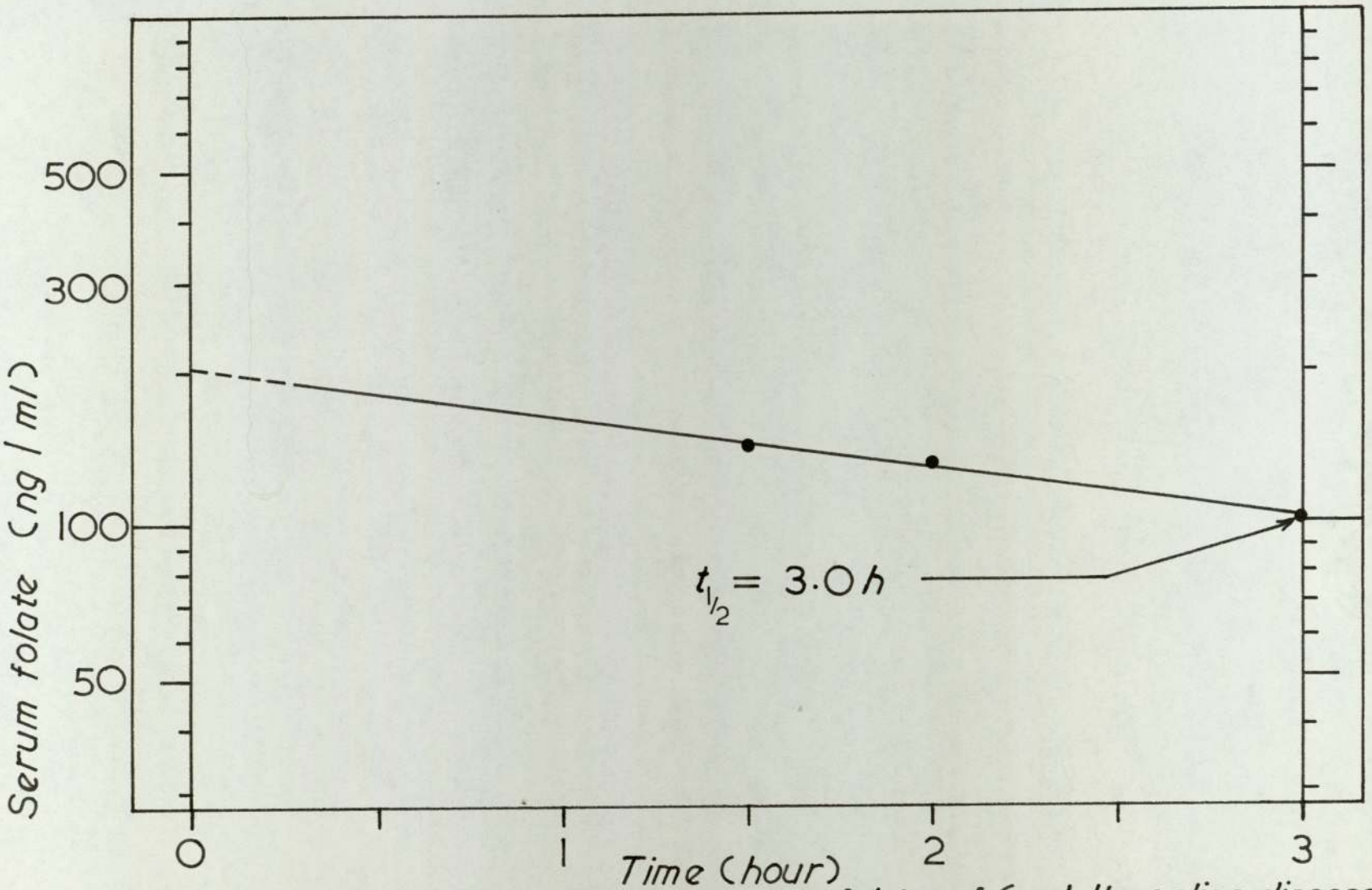


Figure 10.4 First order rate of serum folate of 6 adult coeliac disease patients after 10 mg of dl $5CH_3H_4$ PteGlu, orally.

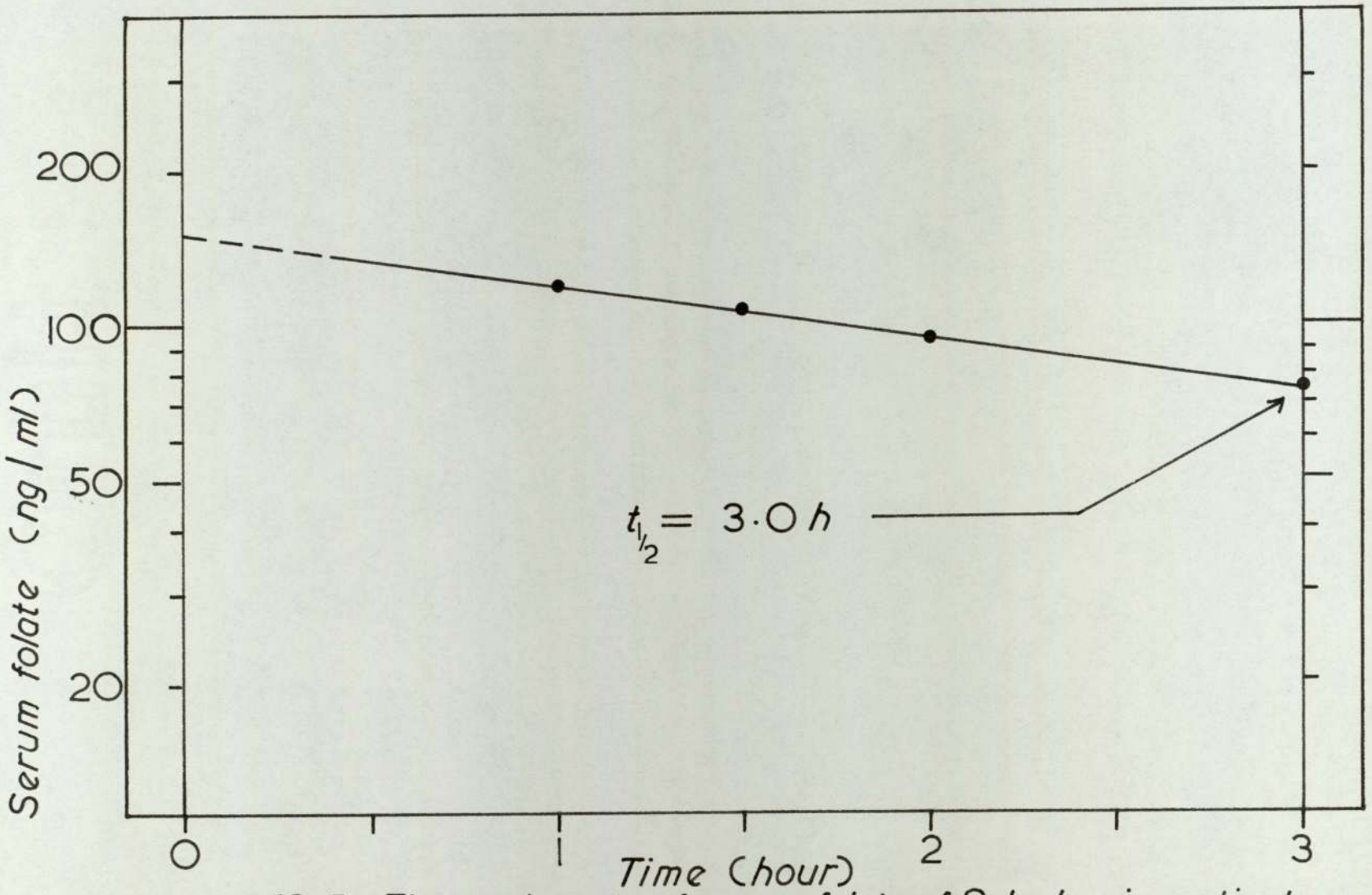


Figure 10.5 First order rate of serum folate of 8 leukemic potients after 10 mg of dl 5 CH_3H_4 Pte Glu, orally.

Discussion.

Malabsorption of folic acid in patients with adult coeliac disease have led to anaemia of folate deficiency amongst these patients (Cooke et al 1963). Studies of oral absorption and metabolism of 5-methyltetrahydropteroylglutamic acid (Table 10.5) showed them to be much lower than those of normal humans at similar doses. Fair amounts of 5-methyl-5,6-dihydropteroylglutamic acid is found in serum of these patients after oral absorption of 10 mg dl-5-methyltetrahydropteroylglutamic acid. This may be because of similarity of microclimate pH or the jejunum pH of these patients and those of pernicious anaemia patients (Benn and Cooke,1971;Blair,Lucas and Cooke unpublished observations). Impaired folic acid absorption in leukemia is known (Uchino et al 1965). This is confirmed also on the absorption of 5-methyltetrahydropteroylglutamic acid as seen in the Table 10.6 when compared with normal humans at similar doses (Table 10.2). Although subnormal absorption of folic acid in some patients with pernicious anaemia had been reported (Spray and Witts,1952;Uchino et al 1965) most other investigators have found that folic acid absorption in patients with pernicious anaemia is normal (Cox et al 1958b;Anderson et al 1960;Klipstein 1963;Girdwood,1953). In this study the oral absorption of 5-methyltetrahydropteroylglutamic acid in patients with pernicious anaemia is similar to those of normal humans. Oral 5-methyl-5,6-dihydropteroylglutamic acid is better absorbed by patients with pernicious anaemia than normal humans. The superior absorption by patients with pernicious anaemia of 5-methyl-5,6-dihydropteroylglutamic acid is because of their lack of stomach acid. 5-methyl-5,6-dihydropteroylglutamic acid

undergoes rearrangement to the microbiologically inactive 5-methyl-5,8-dihydropteroylglutamic acid (Blair et al 1974; Robb,1975)in acid solution. In normal human small amount of 5-methyl-5,6-dihydropteroylglutamic acid is utilized and reached blood level mainly as 5-methyltetrahydropteroylglutamic acid. The availability of 5-methyl-5,6-dihydropteroylglutamic acid to normal human is possibly explained by the competitive action of the acidic rearrangement and reduction of 5-methyl-5,6-dihydropteroylglutamic acid to 5-methyltetrahydropteroylglutamic acid by stomach ascorbic acid (Diem and Lentner,1971). This effect is easily demonstrated by neutralization of normal human stomach with oral sodiumbicarbonate before oral absorption of 5-methyl-5,6-dihydropteroylglutamic acid (Table 10.11). Oral administration of sodiumbicarbonate reduced the serum level of serum folate after oral administration with folic acid (Benn et al 1971). This is not so in cases of 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid as demonstrated in Chapter 5. The superiority of achlorhydria subjects to absorb more 5-methyltetrahydropteroylglutamic acid or its oxidation product 5-methyl-5,6-dihydropteroylglutamic acid have made it possible that these subjects may have high serum folate. Thus the methyl-trap hypothesis proposed by Herbert and Zalusky (1962) can be simply explained by this phenomena.

Pharmacokinetics studies of serum folate status from these patients after oral doses of 5-methyltetrahydropteroylglutamic acid indicated that the availability of the compound to patients with pernicious anaemia is similar to the normal humans with similar doses but the availabilities are much lower

in other patients (Table 10.13 and Table 10.14). Oral absorption of 5-methyl-5,6-dihydropteroylglutamic acid to patients with pernicious anaemia showed a much enhanced in the availability to a level nearly as high as those of normal humans after 3 g of sodiumbicarbonate and similar doses of 5-methyl-5,6-dihydropteroyl-glutamic acid (Table 10.13 and Table 10.14).

Studies of clearance rate of these folates from serum of various groups of patients are summarized in Table 10.13. Eventhough they were all followed first order excretion rate of clearance the rate constants(half-life, $t_{\frac{1}{2}}$) of various conditions are changed in most cases the half-life (time) are increased after oral absorption of 5-methyltetrahydropteroylglutamic acid and patients with pernicious anaemia showed similar half-life to that of normal human after 3 g of sodiumbicarbonate before a similar test dose. The half-life of serum folate after oral doses of 5-methyl-5,6-dihydropteroylglutamic acid in normal human is a little longer than that of 5-methyltetrahydropteroylglutamic acid (Table 10.13). Patients with pernicious anaemia have a half-life $t_{\frac{1}{2}}$ = 4.8 hours a value of more than double of normal humans. The increased half-life of serum folate after oral 5-methyl-5,6-dihydropteroylglutamic acid might also have been responsible for the high serum folate found amongst these patients (Herbert et al 1960;Waters and Mollin,1961;Kohn et al 1961;Herbert and Zalusky, 1962;Waters and Mollin,1963;Cooper and Lowenstein,1964). The longer half-life ($t_{\frac{1}{2}}$) of serum folate of pernicious anaemia patients after 5-methyl-5,6-dihydropteroylglutamic acid is nearly three times that of normal humans after oral 3 g sodiumbicarbonate and similar doses of 5-methyl-5,6-dihydropteroylglutamic acid.

Studies of other folate in blood (10-formylpteroyl-glutamic acid and 10-formyltetrahydropteroylglutamic acid) as detected by microbiological assays with S.faecalis and P.cerevisiae showed them to be similar to those of normal humans or with some increases. The increase in blood levels of 10-formyltetrahydro-pteroylglutamic acid in a whole group of adult coeliac diasease patients is summarized (Table 10.5). One patient with widespread lymphoma at progressive disease state showed an increase of levels of S.faecalis at 30 ng/ml and P.cerevisiae at 8 ng/ml after oral 5-methyltetrahydropteroylglutamic acid (5 mg of microbiologically active material). These values were obtained at peak levels two hours after dose,they are much higher than those of normal subjects on similar doses and smaller is found in other subjects.From these studies it is clear that metabolism of oral folates are depended on the stomach and intestinal conditions of each individual.

Studies of serum 5-methyl-5,6-dihydropteroylglutamic acid of subjects taken various folates showed that in normal humans after 3 and 5 g of sodiumbicarbonate oral absorption of 10 mg dl-5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid showed increase serum folate with 5-methyl-5,6-dihydropteroylglutamic acid as on of the major folates (Table 10.1) and this is also true with pernicious anaemia patients. The increase in serum 5-methyl-5,6-dihydro-pteroylglutamic acid in these subjects may be because of the oxidation of the administered compound with trace amount of copper in stomach or gastric juice as catalyst (Blair et al 1974; Diem and Lentner,1971).The presence of 5-methyl-5,6-dihydro-pteroylglutamic acid in these conditions indicated that

5-methyl-5,6-dihydropteroylglutamic acid is slowly metabolized. Thus subjects with high stomach pH i.e.achlorhydria of pernicious anaemia may have high serum folates but with altered levels of 5-methyltetrahydropteroylglutamic acid partially substituted by 5-methyl-5,6-dihydropteroylglutamic acid.

Summary.

Malabsorption of 5-methyltetrahydropteroylglutamic acid is demonstrated amongst patients with adult coeliac disease and leukemia but not with pernicious anaemia as judged with the peak levels and pharmacokinetics availability at time 0 hour. After oral 5-methyltetrahydropteroylglutamic acid the serum 5-methyl-5,6-dihydropteroylglutamic acid levels are increased by the high stomach and intestinal pH with oral sodiumbicarbonate or in pernicious anaemia patients. 5-methyl-5,6-dihydropteroyl-glutamic acid is little available to normal human but increased stomach pH by oral sodiumbicarbonate enhanced the availability and is also true in the achlorhydria of pernicious anaemia patients. 5-methyl-5,6-dihydropteroylglutamic acid is slowly metabolized as seen mainly as 5-methyl-5,6-dihydropteroylglutamic acid in the serum after doses of 5-methyl-5,6-dihydropteroyl-glutamic acid orally. 5-methyl-5,6-dihydropteroylglutamic acid may be partially reduced by stomach ascorbic acid before absorption to 5-methyltetrahydropteroylglutamic acid.The enhanced of serum folate of pernicious anaemia patients after oral 5-methyl-5,6-dihydropteroylglutamic acid provides an alternative explanation of the elevated serum folate found in these patients. The raised 10-formyltetrahydropteroylglutamic acid of one patient with widespread lymphoma after oral 5-methyltetrahydro-pteroylglutamic acid supported the observations of increased serum 10-formyltetrahydropteroylglutamic acid amongst those patients with high proliferation rate.

Chapter 11.

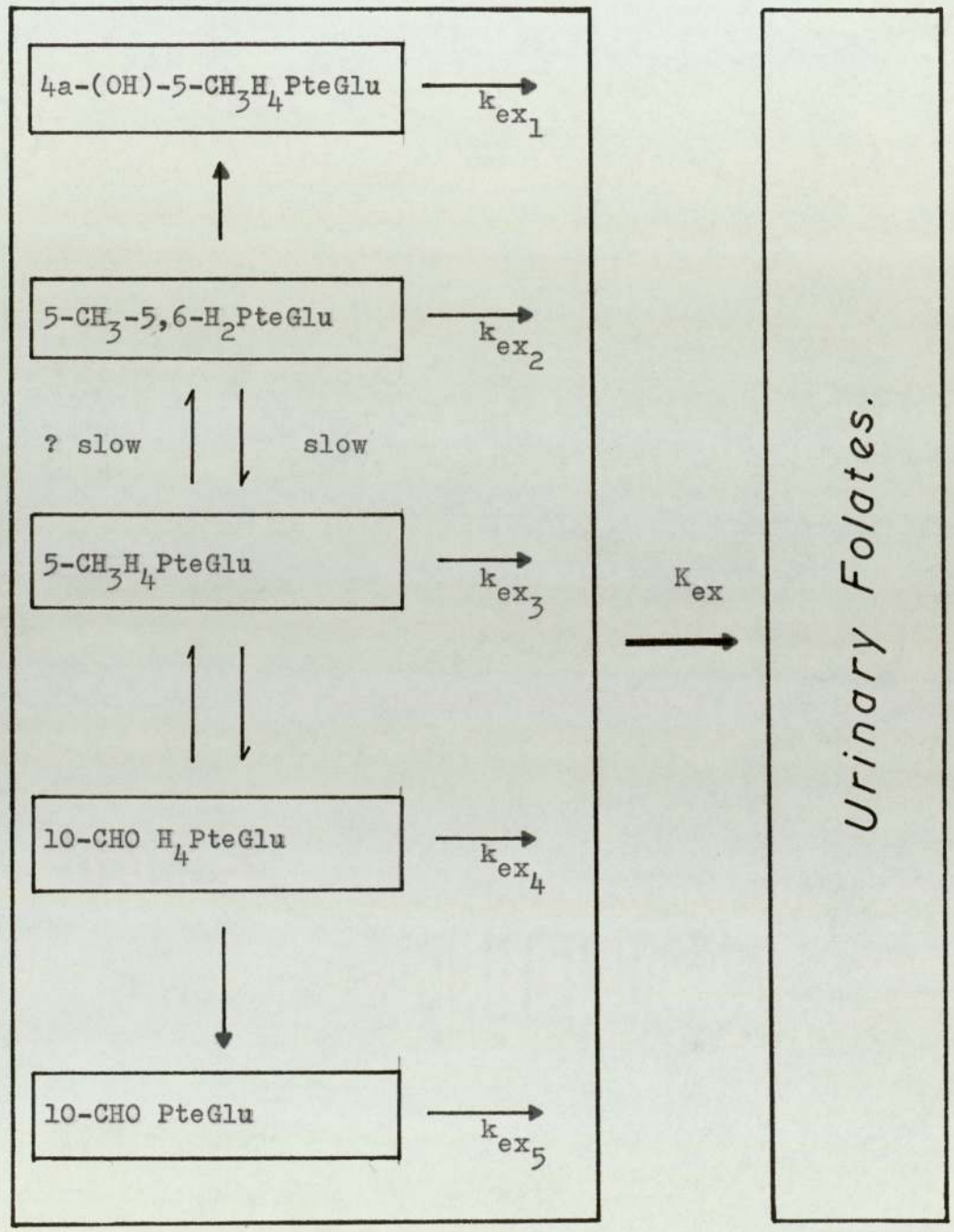
General discussion, conclusion and suggestions for further work.

General discussion,conclusion and suggestions for further work.

Folic acid and its derivatives are known to be synthesized by microorganisms and plants (Goodwin,1963). There is no evidence to suggest that folates are synthesized by mammals and it is clearly known that folates play an important role in major metabolic pathways of the mammal (Blakley,1969;Chanarin,1969). Since folates can not be synthesized by man,their availability from dietary sources is of great significance. Eventhough folate metabolism has been studied since their discovery it is still not clearly understood. This study represents yet another attempt to extend our knowledge of these compounds.

From our studies we have concluded that various folates are present in human blood. Serum folates and their excretion in urine in normal humans were studied as described in Chapter 4. It has been shown that these folates are in equilibrium with each other with a homeostatic mechanism controlling the level of 10-formyltetrahydropteroylglutamic acid. The relationship of these folates may be represented by the following scheme 11.1. If there were no other interfering action on the handling of folates other than metabolism and urinary excretion,it would be reasonable to speculate that folate excreted daily in urine represented the daily requirement of folate by man. It has been clearly demonstrated that there is a reabsorption process of circulating folates before excretion by renal tubules (Condit and Grob,1958;Johns et al 1961;Goresky et al 1963). These authors showed that around 30 ng of circulating folates were reabsorbed per minute by the renal tubules. Thus,the daily folates reabsorbed before

Scheme 11.1 The relationship of serum folates and urinary excretion.



$K_{ex} = k_{ex_1} + k_{ex_2} + k_{ex_3} + k_{ex_4} + k_{ex_5} + \ldots\ldots$

$= K_{elimination - reabsorption}$

= Rate of excretion into urine and other sources.

their excretion would be around 43 ug. The combination of the reabsorbed folates and those excreted in urine daily would be a reasonable value for the daily folate requirement. This is around 50 to 70 ug per day,when urinary folates as previously discussed in Chapter 4 are around 4 to 17 ug/day. Consideration of urinary folates as a folate requirement may be confirmed by the analysis of urinary folates of those subjects with a severe renal failure (Sidiqui et al 1970). They showed that patients with severe renal failure excreted folates at about 52 ug for a period of 12 hours. Analysis of daily requirement of folate by these means would assumes that it is the amount that can maintain the serum folate level. There are other means of estimating the daily requirement of folates by man i.e. studies of patients with folate deficiency on treatment with small amount of folic acid. The smallest amount of folic acid that can give a haematological response was used to determine minimum folate requirement as discussed in Chapter 5. The latter method gave much lower results and a value as low as 5 ug/day has been reported (Kamel et al 1972) but many investigators reported values of higher than 50 ug/day. From these observations it would be reasonable to conclude that daily folate requirement in man is around 50 to 100 ug. Studies of serum folates pattern in man throughout the 24 hour of the day is not easily performed and those results of studies during the day time showed little variation as discussed in Chapter 4. Studies of urinary folates throughout the 24 hour of the day were performed in two subjects on similar daily diet. Eventhough studied in two subjects it showed a very similar results which indicated some variation of folates during the

day. 5-methyltetrahydropteroylglutamic acid is the major urinary folate and represented around 70 % of the excreted folates. 10-formyltetrahydropteroylglutamic acid and 10-formylpteroylglutamic acid represented the rest of urinary folates. The latter may infact derived from 10-formyltetrahydropteroylglutamic acid as previously discussed in Chapter 4. Amongst these folates excreted in urine only 5-methyltetrahydropteroylglutamic acid showed a very significant variation reaching a peak at the early morning and remained at some low level throughout the day as shown in Figure 4.3.4.

Eventhough studies of serum folates of normal human showed little variation it may be simply explained because of change can be controlled by supplementation of folates from other storage sites. Studies of serum folates diurnal variation in rat which have a much higher serum folates showed them to have clearly show diurnal variation. The variation of these folates are justified as discussed in Chapter 4. Daily rhythm of liver folate metabolism have been demonstrated in the rat (Barbiroli et al 1974). Assumption can be made that these variations are occurring in humans but it still needs to be experimentally proved.

Studies of oral folate metabolism at doses of 5 mg of microbiologically active materials were discussed in Chapter 5 for normal human. Microbiological assays of serum folates after oral administration of various folates were made, these serum and urine folates were also identified by using the bioautographic techniques. After oral administration of 5 mg pteroylglutamic acid it is clearly shown that it is well absorbed and 5-methyltetrahydropteroylglutamic acid is slowly

increased whilst the major folate in the blood is the administered pteroylglutamic acid itself. The presence of 5-methyltetrahydropteroylglutamic acid indicates the metabolism of the administered pteroylglutamic acid. Oral administration of 7,8-dihydropteroylglutamic acid showed quick metabolism being detected in serum mainly as 5-methyltetrahydropteroylglutamic acid. 5-methyl-5,6-dihydropteroylglutamic acid is a compound which is microbiologically inactive. Oral administration of 5 mg of microbiologically active equivalent of 5-methyl-5,6-dihydropteroylglutamic acid is well absorbed. It is detected in serum mainly as 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid. Eventhough this 5-methyl-5,6-dihydropteroylglutamic acid is quickly absorbed to reach a peak level within half an hour its availability is much lower than other microbiologically active folates as seen in Chapter 5. Its low availability is mainly due to the rapid rearrangement to microbiologically inactive 5-methyl-5,8-dihydropteroylglutamic acid in the presence of stomach acid. The neutralization of stomach acid with oral sodiumbicarbonate indeed enhanced the availability of 5-methyl-5,6-dihydropteroylglutamic acid as shown in Chapter 5. Oral administration of 5 mg of microbiologically active 5-methyltetrahydropteroylglutamic acid is well absorbed to reach a peak level at about one and a half hour and detected in the serum as 5-methyltetrahydropteroylglutamic acid and 5-methyl-5,6-dihydropteroylglutamic acid as shown in Chapter 5. 5-formyltetrahydropteroylglutamic acid and 5,10-methenyltetrahydropteroylglutamic acid at doses of 5 mg of microbiologically active materials were well absorbed and metabolized to

5-methyltetrahydropteroylglutamic acid as shown in Chapter 5. 10-formylpteroylglutamic acid at a dose of 5 mg of microbiologically active material was well absorbed but not metabolized and thus seen in the serum as the administered compound as shown in Chapter 5. The folates,5-methyl-5,8-dihydropteroylglutamic acid and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydropteroylglutamic acid,are microbiologically inactive for L.casei,S.faecalis and P.cerevisiae. Oral administration of these compounds at doses similar to the above folates did not enter serum folate metabolic pool as also summarized in Chapter 5.

Studies of the relationship of folates to the serum Crithidia factors in normal human showed no positive result as shown in Chapter 6 eventhough it has been reported that in vitro pteroylglutamic acid is active for the protozoon Crithidia fasciculata when a catalytic amount of biopterin was also present (Broquist and Albrecht,1955).Further studies in Chapter 7 showed that oral administration of methotrexate, a compound known as a dihydrofolate reductase inhibitor,caused a raised serum Crithidia factors level. Oral administration of various folates post 24 hour oral methotrexate showed that pteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid can give a further increase in serum Crithidia factors. 5-formyltetrahydropteroylglutamic acid given intravenously also raised serum Crithidia factors level on subjects post 24 hour of oral methotrexate. Bioautography of samples from subjects given methotrexate showed an increase in serum pteroylglutamic acid which is not detected in normal human serum as shown in Chapter 7. Therefore the increase in serum Crithidia factor level may be due to the pteroylglutamic acid as shown by Broquist and

Albrecht (1955). Thus the increase in Crithidia factor levels in subjects taken dihydrofolate reductase inhibitor drugs as shown in Chapter 7 may not be represented the true Crithidia factors as mainly identified as biopterin or its derivatives (Leeming and Blair 1974). Studies on the metabolism of folates on subjects taken dihydrofolate reductase inhibitor drugs showed that serum folates of these subjects are lower than normal level as shown in Chapter 7 in Table 7.14. Folates given orally at doses of 5 mg of microbiologically active materials have been studied. Microbiological assays of serum from subjects taken pteroylglutamic acid post 24 hour oral methotrexate showed that the major folate is pteroylglutamic acid itself and this is confirmed by the bioautography as shown in Plate 7.1 to Plate 7.3. 7,8-dihydropteroylglutamic acid given orally to subjects taking methotrexate orally 24 hour before the test showed that the small amount of pteroylglutamic acid was detected in the serum as also shown in Chapter 7. Oral administration of 5-formyltetrahydropteroylglutamic acid to a subject post 24 hour oral methotrexate showed that small amount of folates were detected by S.faecalis and P.cerevisiae as summarized in Table 7.13. Intravenous injection of 5 mg of microbiologically active 5-formyltetrahydropteroylglutamic acid was detected mainly as the administered compound by S.faecalis and P.cerevisiae summarized in Table 7.12.Bioautography of serum folates from these subjects suggested that 5-methyltetrahydropteroylglutamic acid is a major folate amongst those who has low S.faecalis and P.cerevisiae.

Major dietary folates before degradation are 5-methyl-tetrahydropteroylglutamic acid and 10-formyltetrahydropteroyl-

glutamic acid as previously discussed in Chapter 5. After cooking their oxidation products are prebably the major folates. 5-methyltetrahydropteroylglutamic acid is shown to be very labile to oxidation and converted to a microbiologically inactive material as shown in Chapter 3. Without antioxidant 5-methyltetrahydropteroylglutamic acid loses its microbiological activity to about 20 % within 12 hour at 24°C. Eventhough antioxidant is present at a considerable amount the activity of 5-methyltetrahydropteroylglutamic acid is still lost at a considerable amount as discussed in Chapter 3. This observation was also confirmed by the studies at room temperature (O'Broin et al 1975). 10-formyltetrahydropteroylglutamic acid oxidises similarly to the tetrahydropteroylglutamic acid as shown by Blair and Pearson(1974). Thus eventhough a considerable amount of dietary folates as detected by L.casei microbiological assay amount of around 500 ug/daily diet had been reported as discussed in Chapter 5. It has been clearly shown that 10-formylpteroyl-glutamic acid is well absorbed but never reachs the folate pool as 5-methyltetrahydropteroylglutamic acid therefore it may not important or useful nutritionally (Ratanasthien et al 1974; Beavon and Blair,1975). Thus dietary folates as detected by using L.casei microbiological assay would infact represent a much smaller amount of usable folates and therefore the available of dietary folates would need to be used efficiently.

The disturbance of dietary folates utilization in many cases led to a folate deficiency i.e. on treatment with dihydrofolate reductase inhibitor drugs as shown in Chapter 7. The malabsorption syndrome is another aspect led to folates deficiency as seen in those patients with intestinal diseases

discussed in Chapter 8 and Chapter 10. Increased in folates requirement in some diseases would also led to folates deficiency as also discussed in Chapter 8 and Chapter 10.

Altered serum folates were seen in various kinds of patients studied in Chapter 8 as compared to those of normal humans studied in Chapter 4 and Chapter 5. 5-methyltetrahydropteroylglutamic acid is a major folate of serum and urine of normal human and these activities of 5-methyltetrahydropteroylglutamic acid is infact partially derived from 5-methyl-5,6-dihydropteroylglutamic acid as previously discussed in Chapter 4 in a greater detail. 10-formyltetrahydropteroylglutamic acid is a minor folate in serum and urine and unlike 5-methyltetrahydropteroylglutamic acid it is not variable with dietary folates. Level of 10-formyltetrahydropteroylglutamic acid were shown to be raised in patients with adult coeliac disease,leukemias, rheumatoid arthritis,psoriasis,schizophrenia and regional enteritis. Patients with pernicous anaemia under vitamin B_{12} treatment and ulcerative colitis showed a normal 10-formyltetrahydropteroylglutamic acid level. Analysis of serum 5-methyl-5,6-dihydropteroylglutamic acid only patients with pernicious anaemia showed a very significant increase in this folate as shown in Chapter 8 and Chapter 10. The increase in the level of 10-formyltetrahydropteroylglutamic acid amongst those patients mentioned above may infact indicate the increase in folate requirement of these subjects or the disturbance of the enzyme or enzymes system regulating the homeostatic mechanism of serum folates and these are discussed in a greater detail in Chapter 8.

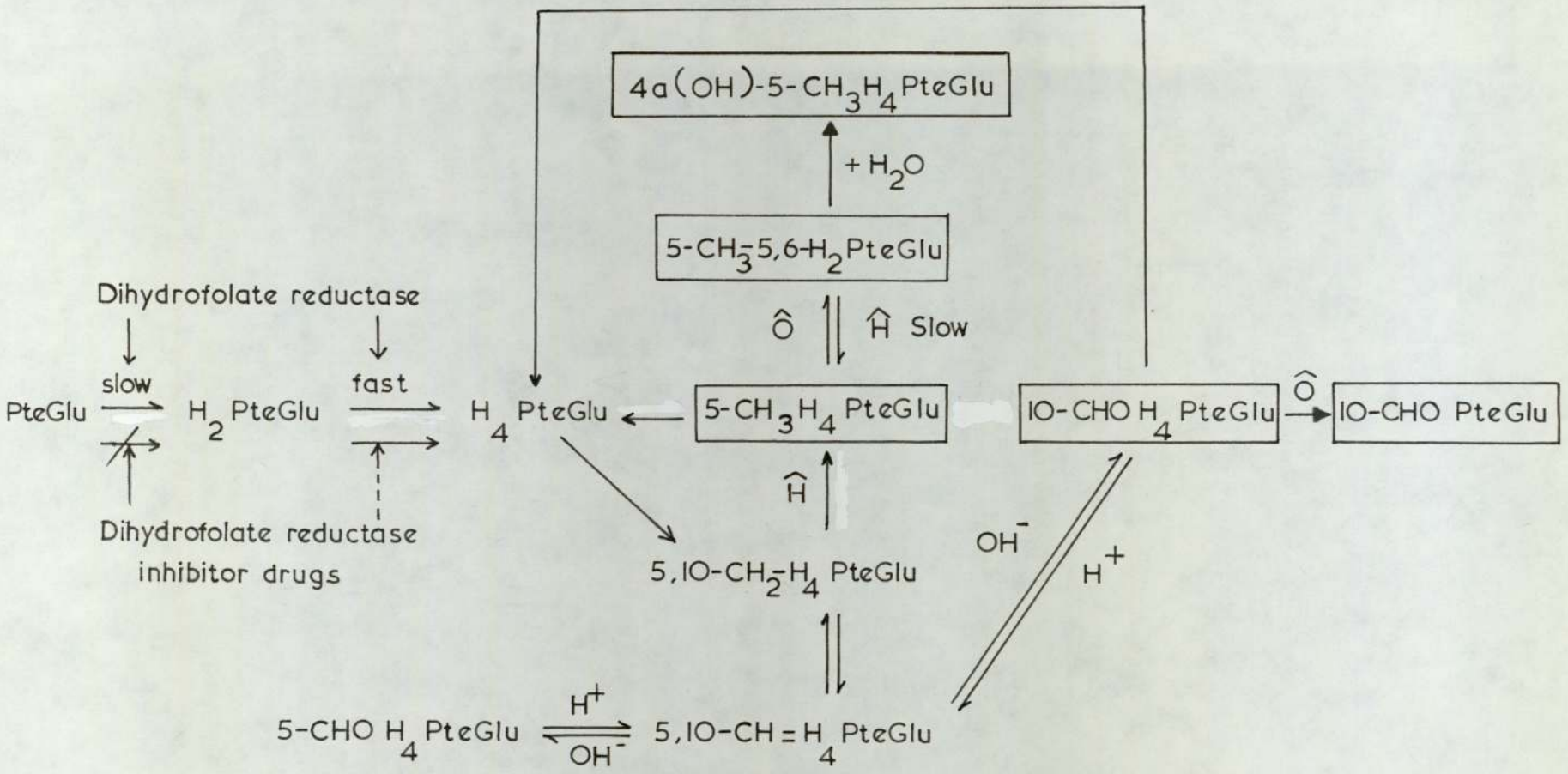
The metabolism of oral folates had been studied in some patients as shown in details in Chapter 10. Oral administration of 5-methyltetrahydropteroylglutamic acid to pernicious anaemia patients on treatment with vitamin B_{12} showed them to exhibit a normal absorption of folate. Studies of these pernicious anaemia patients on oral absorption of 5-methyl-5,6-dihydropteroylglutamic acid showed them to absorb more than in the normal subjects and it was shown to be because of their lack of stomach acid. The neutralization of stomach acid with oral sodiumbicarbonate enhanced the availability of 5-methyl-5,6-dihydropteroylglutamic acid in normal humans to a similar level to that of pernicious anaemia patients. The superiority of pernicious anaemia patients to absorb the oxidation product of 5-methyltetrahydropteroyl-glutamic acid may infact responsible for the raised serum folates found amongst these patients. Eventhough patients with pernicious anaemia absorbed more 5-methyl-5,6-dihydro-pteroylglutamic acid than normal human or similar to those normal human with temporary achlorhydria,5-methyl-5,6-dihydropteroylglutamic acid entered folate pool of these patients unchanged or slowly metabolised and thus the availability of high serum folate of these patients is doubtful (Chapter 8 and Chapter 10).

The longterm treatment with pteroylglutamic acid of adult coeliac patients did not give much effect on the level of 10-formyltetrahydropteroylglutamic acid as shown in Chapter 9. The follow up of folate status amongst those patients with leukemia showed that those who died had had

two different kinds of folate status. The first group are amongst those whose serum folates approached to zero and the second group were amongst those who had a raised level of 10-formyltetrahydropteroylglutamic acid.

Studies of oral administration of pteroic acid and pteroyl-D-glutamic acid showed them did not enter the folate metabolic pool as seen with no change in the serum folate levels. Oral administration of 10 mg of methotrexate is quickly absorbed without metabolism, seen as a single spot of inhibition zone on the bioautography with L.casei discussed in Chapter 7, and reached a peak level of about 200 ng/ml at 1 hour after the dose. The serum methotrexate levels were cleared to a range of 0 to 30 ng/ml within twenty four hour after the dose.

Oral administration of biopterin did not show any effect on serum folate and urine folate levels. Studies of the oral administration of tetrahydrobiopterin showed peculiar results. There is no effect of tetrahydrobiopterin absorption on serum folates from those subjects with the starting serum folates assayed with L.casei lower than 6.0 ng/ml but there are some increases in serum folates as 5-methyltetrahydropteroylglutamic acid amongst those subjects with the starting serum folates (L.casei) of 6.0 ng/ml or higher (Chapter 6). It is possible that the increased 5-methyltetrahydropteroylglutamic acid is derived from replacement by tetrahydrobiopterin in the tissues. The zero effect on those subjects with serum folates lower than 6.0 ng/ml may due to the lack of or low tissue folates. Whether this is true or not remains to be seen and if this is so it may serve as a useful tool for



Scheme 11.2 The role of metabolism of oral folate in man. Those in frames are the folates of serum and urine. ---→ indicates the competitive step.

determination of folate deficiency.

From these studies the role of metabolism of oral folates may be summarized as shown in Scheme 11.2. The handling of serum folates shown them to be of first order rate process. The reabsorption of folates by renal tubules is possibly of significant at serum folates of 20 ng/ml or lower as discussed in Chapter 5. Folates may be presented binding with proteins as discussed in Chapter 4. The major folates cleared into urine within 6 to 8 hours after the oral doses.

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ADDENDUM :Two subjects,on starvation overnight and throughout the test period, took 10 mg of microbiological active 5-methyltetrahydropteroylglutamic acid(20 mg of dl-5-methyltetrahydropteroylglutamic acid) in 200 ml water containing 1 g of ascorbic acid.Blood samples were collected at time 0,$\frac{1}{2}$,1,1$\frac{1}{2}$,2 and 3 hours after doses.These samples were collected into plain glass tubes and let clott at room temperature for 10 to 15 minutes and after centrifugation sera from each sample were separated into two portions.One portion of each sample was acidified to pH 3 for 10 minutes with concentrated HCl while sample shaking and after 10 minutes these samples were neutralized with 20 % sodiumhydroxide solution.The amount of added acid and alkali were used as the correction factor(dilution factor); in all cases 0.1 to 0.2 ml of acid and alkaline were used per 1 to 2 ml of serum.

Fresh void urine was collected in one subject (K.R.) after the end of 3 hour period,this subject was emptied his bladder at the beginning of the test. Sujbect number 2 (P.A.S.) had 2 cups of coffee with coffee-mate just before the test. Urine sample collected from subject 1 (K.R.) was also acidified and neutralized similar to those of serum samples.

These samples were microbiologically assayed with L.casei,S.faecalis and P.cerevisiae on the day performing the experiment,using the aseptic addition methods as described in Chapter 2.Results of the microbiological assays are summarized for subject 1 (K.R.) and subject 2 (P.A.S.).

Subject 1 (K.R.)

Time after doses	L.casei (ng/ml) A	L.casei (ng/ml) B	S.faecalis(ng/ml) A	S.faecalis(ng/ml) B	P.cerevisiae(ng/ml) A	P.cerevisiae(ng/ml) B
0	10	10	0.7	0.7	0.6	0.6
½	300	260	4.0	4.0	1.0	1.0
1	500	440	5.0	5.0	2.6	1.3
1½	700	650	8.0	8.0	3.0	2.3
2	600	580	7.0	10.0	4.4	2.0
3	420	400	5.5	5.5	4.2	2.0
Urine	800*	736*	72.0*	80.0	48.0*	32.0*

* indicates results in ug of three-hour urine.

Subject 2 (P.A.S.)

Time after doses	L.casei(ng/ml) A	L.casei(ng/ml) B	S.faecalis(ng/ml) A	S.faecalis(ng/ml) B	P.cerevisiae(ng/ml) A	P.cerevisiae(ng/ml) B
0	8.0	1.0	0.7	0.0	0.4	0.0
½	240.0	20.0	5.0	4.0	1.2	1.2
1	400.0	240.0	4.5	4.0	2.7	2.3
1½	700.0	300.0	6.0	6.0	3.0	3.0
2	600.0	280.0	6.0	6.5	3.8	2.6
3	500.0	260.0	7.0	7.0	3.0	2.4
Urine not collected						

A = Samples before acid treatment.

B = Samples after acid treatment.

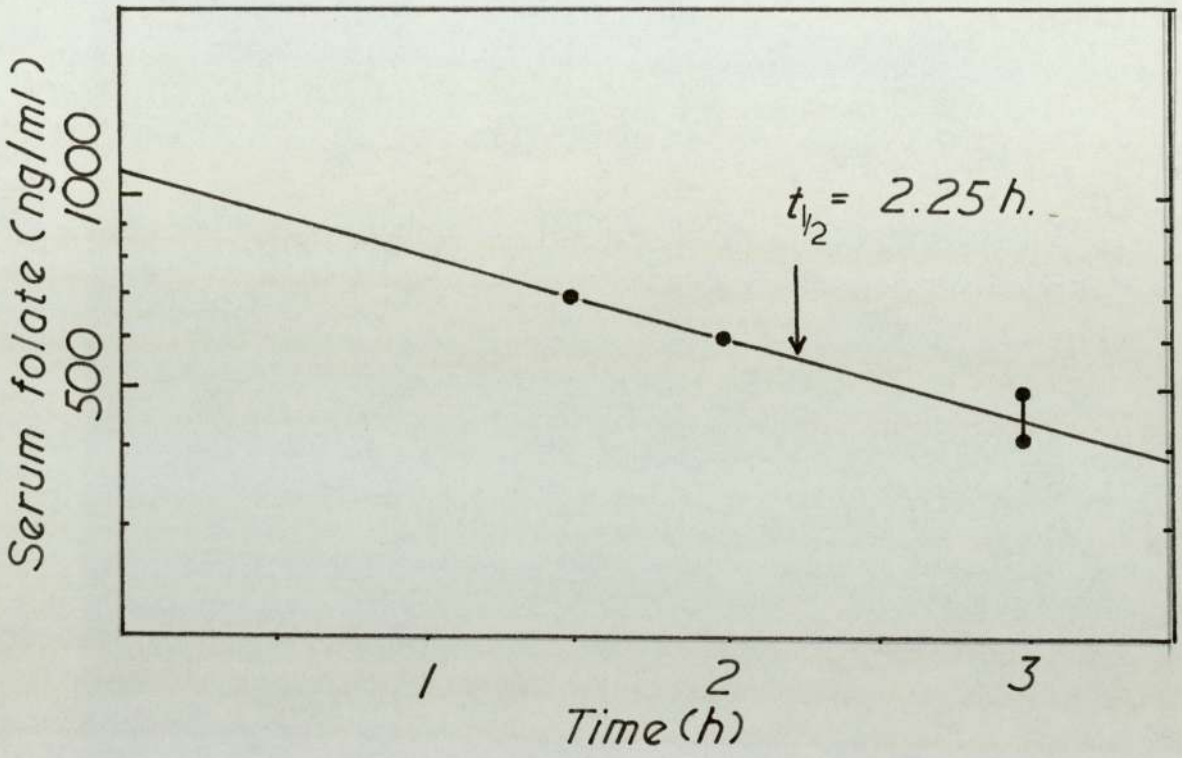

Figure First order rate of serum folate clearance of two subjects taking 10 mg of microbiologically active 5-methyl-tetrahydropteroylglutamic acid in 200 ml of water containing 1 gm of ascorbic acid, orally.

Discussion

In both subjects addition of ascorbic acid enhanced the availability of the administered 5-methyltetrahydropteroylglutamic acid as compared with those subjects taking 5 mg of microbiologically active 5-methyltetrahydropteroylglutamic acid orally (Table 5.1.6). The enhancement is about three times when only doubling the doses. In subject 1 (K.R.) serum 5-methyl-5,6-dihydropteroylglutamic acid was relatively lower than in normal humans taking 3 g to 5 g of sodiumbicarbonate before 5 mg of microbiologically active 5-methyltetrahydropteroylglutamic acid orally (Table 5.1.22) but in subject number 2 (P.A.S.) the serum 5-methyl-5,6-dihydropteroylglutamic acid is significantly increased.Pharmacokinetics of serum folate from these two subjects also showed first order rate of clearance with the availability at time 0 hour of 1100 ng/ml in serum and with $t_{\frac{1}{2}}$ = 2.25 hour.

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Folates in human serum

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Folates in human serum

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SYNOPSIS Constituent serum folates have been shown to be altered and in some cases raised in diseases in which total folate levels are known to be frequently decreased. Absorption experiments showed that orally administered folate analogues affect the folate pool in different ways. The stability of the 10-formyltetrahydrofolate level in normal subjects is demonstrated.

The association of low serum folate levels with adult coeliac disease (Cooke, Fone, Cox, Meynell, and Gaddie, 1963; Dormandy, Waters, and Mollin, 1963) and leukaemia (Rama Rao, Lagerlof, Einhorn, and Reizenstein, 1965) is established although the pathogenesis of the relationship is less well understood. The fate of orally administered pteroyl-L-monoglutamic acid is well documented (Dormandy *et al*, 1963; Butterworth, Nadel, Perez-Santiago, Santini, Rafael, and Gardner, 1957; Chanarin, Anderson, and Mollin, 1958; Melikian, Paton, Leeming, and Portman-Graham, 1971; Leeming, Portman-Graham, and Blair, 1972). There is information on the absorption of the folate analogues (Baker, Frank, Feingold, Ziffer, Gellene, Leevy, and Sobotka, 1965; Perry and Chanarin, 1970; Nixon and Bertino, 1972; Brown, Scott, Foster, and Weir, 1973) but less on their impact on the components of the total folate pool.

This study describes relative levels of 5-methyltetrahydrofolic acid (5Me-THF), 10-formyltetrahydrofolic acid (10-CHO-THF), and 10-formylfolic acid (10-CHO-FA) in normal subjects and in patients with adult coeliac disease and leukaemia.

The effects of orally administered folates on individual serum constituents are demonstrated.

Materials and Methods

Pteroyl-L-monoglutamic acid was a commercial product. The calcium salt of 5-formyltetrahydrofolic acid (5-CHO-THF) was a gift from Lederle Laboratories Ltd; 5-Me-THF-Ca salt was prepared according to Blair and Saunders (1970), 10-CHO-FA according to Blakley (1959), and 5,10-methenyl-THF by the method of Roth, Hultqvist, Fahrenbach, Cosulich, Broquist, Brockman, Smith, Parker, Stokstad, and Jukes (1952). All products were checked for purity and identity by (a) microbiological assay with *L. casei*, *S. faecalis*, and *P. cerevisiae*, (*b*) uv spectra in water at pH 1, 7, and 13, and (*c*) thin-layer chromatography in three solvent systems, namely, 0·1 M phosphate buffer pH 7·0, butanol: acetic acid:water (4:1:5 v/v, upper phase), and propanol:1% ammonia (2:1 v/v).

Five mg doses of biologically active materials were prepared for absorption experiments. The subjects fasted overnight and during the collection of blood samples. Blood specimens were taken aseptically immediately before the administration of folates and then at 30, 60, 90, 120, 180 and, in some instances, at 240, 300, and 360 minutes. They were immediately decanted into glass specimen containers and stood at room temperature for half to one hour. After centrifugation the sera were stored at -20° C with the addition of 5 mg/ml ascorbic acid and thawed immediately before adding to assay tubes. Assays were carried out as soon as possible after the collection of specimens and always within seven days.

Single samples were taken after a normal lunch.

L. casei assays were carried out using an aseptic technique (Herbert, 1966). *Streptococcus faecalis* and *P. cerevisiae* assays were also performed aseptically. Bioautography (Leeming, Portman-Graham, Swan, and Blair, 1970) with the three test organisms was used to confirm the identity of the serum folates after absorption.

The normal subjects were hospital staff and medical students.

Results

The individual serum folates in normal subjects and in the diseased states are given in table I.

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Subjects	No. of Subjects	5 Me-THF (ng/cm³)		10 CHO-THF (ng/cm³)		10 CHO-FA (ng/cm³)		S. faecalis (ng/cm³)	
		Mean ± SEM	t Test (%)	Mean ± SEM	t Test (%)	Mean ± SEM	t Test (%)	Mean ± SEM	t Test (%)
Normal	51	4·80 ± 0·28	—	0·57 ± 0·03	—	0·21 ± 0·03	—	0·78 ± 0·03	—
Leukaemia	41	3·70 ± 0·37	<1	0·50 ± 0·03	NS	0·70 ± 0·08	<0·05	1·23 ± 0·07	<0·05
Adult coeliac disease	56	4·59 ± 0·46	NS	0·56 ± 0·03	NS	0·54 ± 0·07	<0·05	1·1 ± 0·06	<0·05

Table I *Mean levels of folate analogues in normal controls and patients with leukaemia and adult coeliac disease*

Folate Analogues	Rf Values	L. casei	S. faecalis	P. cerevisiae
Folic acid	0·05	+	+	−
10 CHO-FA	0·60	+	+	−
10 CHO-THF	0·45 (t)[1]	+	+	+
5 CHO-THF	0·50	+	+	+
5 Me-THF	0·65	+	−	−

Table II *Bioautographic Rf values and microbiological activity of folates on cellulose TLC developed in 3% ammonium chloride*

[1](t) = tail

The Rf values of the folates are given in table II, together with their activity for the three test organisms.

L. casei is active for all folates in serum whilst *Strep. faecalis* is active for all except 5-methyltetrahydrofolic acid (Johns and Bertino, 1965); therefore, the difference in activity of these two test organisms is a measure of 5-methyltetrahydrofolic acid. *P. cerevisiae* is active for 10 formyl-tetrahydrofolic acid 5-formyltetrahydrofolic acid but not for 10-formylfolic acid or folic acid whereas *Streptococcus faecalis* is active for all four compounds. Therefore the difference between the *S. faecalis* and *P. cerevisiae* assays measures the amount of folic acid and 10-formylfolic acid present.

Bioautography of serum and urine samples showed that except after folic acid administration the folate responsible for the *S. faecalis-P. cerevisiae* difference is 10-formylfolic acid. Of the folates active for *P. cerevisiae*, only 10-CHOTHF is found in serum (Nixon and Bertino, 1972). Individual folates were further identified by bioautography in 3% aqueous ammonium chloride, a solvent system in which all commonly occurring folate monoglutamates are clearly separated (Leeming *et al*, 1970; Brown, Davidson, and Scott, 1973). Additional precision in identification was obtained by carrying out bioautography with *L. casei*, *S. faecalis*, and *P. cerevisiae* as developing microorganisms.

The correlation of total folate and 5-Me-THF is shown in figs 1 and figs 2-6 show serum folate constituents after oral administration of folate analogues. In 10 patients with adult coeliac disease on long-term treatment with folic acid, the mean values of the total folate, *Streptococcus faecalis*, and *P. cerevisiae* (10-CHOTHF) levels were > 20·0, > 3·0, and 0·56 ng/cm³ respectively.

Discussion

Three folates can be assayed in normal human blood serum. The means of our values were 5-methyltetrahydrofolic acid, 4·80 ng/cm³, 10-formyltetrahydrofolic acid, 0·57 ng/cm³, and 10-formylfolic acid 0·21 ng/cm³ (table I). As 10-formyltetrahydrofolic acid is very readily oxidized to 10-formylfolic acid (similar

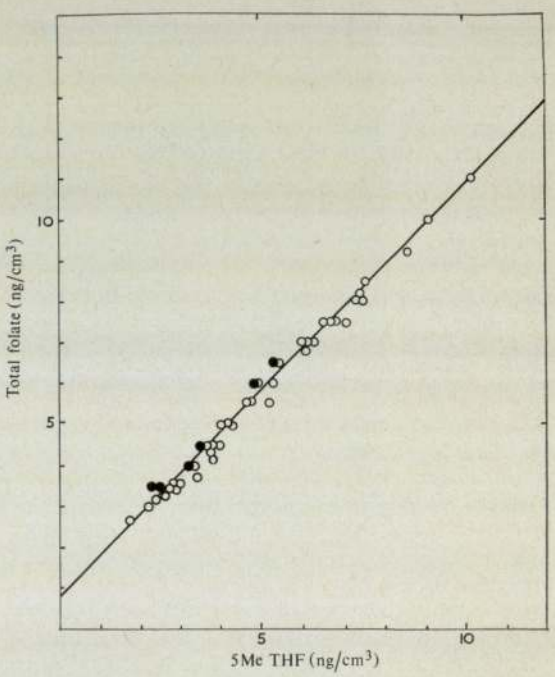

Fig 1 *Correlation of total folate and 5-methyltetrahydrofolate levels in 51 normal subjects.*

Symbols for figs 2-6 together with standard errors of means.

●—● *L. casei (total folate)*

●----● *5-Me THF*

○—○ *10-CHO FA*

■—■ *10-CHO THF*

✳—✳ *Pteroyl-L-monoglutamic acid*

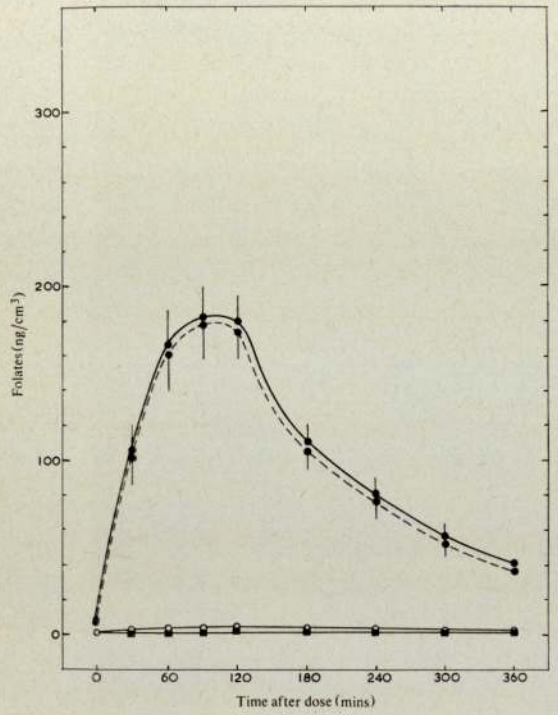

Fig 2 *The effect of orally administered 5-Me-THF (10 mg) on the mean levels of folate analogues in six subjects.*

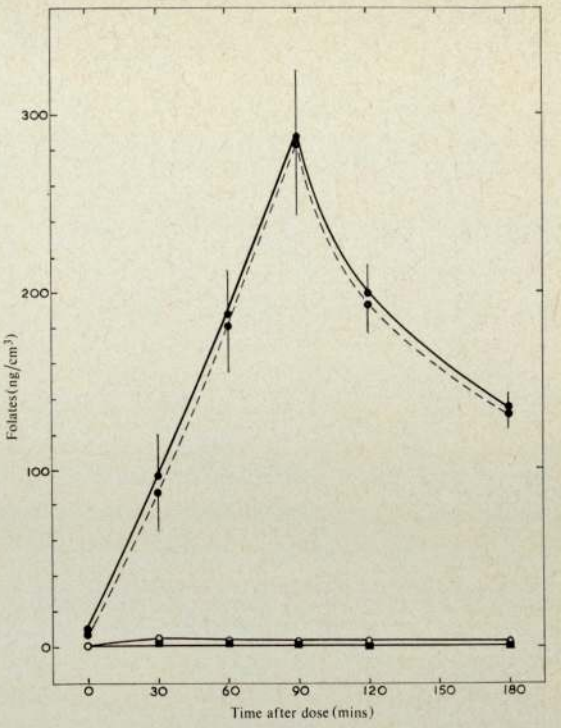

Fig 3 *The effect of orally administered 5-CHO THF (10 mg) on the mean levels of folate analogues in six subjects.*

to tetrahydrofolic acid (Blair and Pearson, 1974), the 10-formylfolic acid is very likely an analytical artifact. When serum samples were taken and analysed under carefully controlled conditions no 10-formylfolic acid could be identified. The 10-formyltetrahydrofolic acid level in serum is thus best represented by the *S. faecalis* level. Normal serum folates are therefore 5-methyltetrahydrofolic acid 4·80 ng/cm³ and 10-formyltetrahydrofolic acid 0·78 ng/cm³.

In man 5-formyl and 5-methyltetrahydrofolic acid and folic acid are converted into 10-formyltetrahydrofolic acid (Albrecht and Broquist, 1956; Silverman, Ebaugh, and Gardiner, 1956; Nixon and Bertino, 1972) and 10-formyltetrahydrofolic acid administered as 5,10-methenyltetrahydrofolic acid is converted into 5-methyltetrahydrofolic acid (fig 4). Thus the two serum folates are in dynamic metabolic equilibrium.

When the total serum folates are plotted against the serum 5-methyltetrahydrofolic acid, an excellent straight line fit with an intercept on the total folate axis at 0·8 ng/cm³ is obtained (fig 1). Therefore, normal serum folates consist of a constant level of 10-formyltetrahydrofolic acid maintained by some homeostatic mechanism and a variable level of 5-methyltetrahydrofolic acid acting as storage form. Statistical analysis of the *S. faecalis* and *P. cerevisiae* values show them to be normally distributed about the mean as might be expected for the analysis of a serum component maintained at a constant level. Similar analysis of the *L. casei* values show them to have a skewed distribution about the mean as might be expected from a summation of serum components, one of which varies with dietary intake.

Further confirmation of the homeostatic control of 10-formyltetrahydrofolic acid levels and the role of 5-methyltetrahydrofolic acid as a storage form is shown by the changes in serum folates after oral

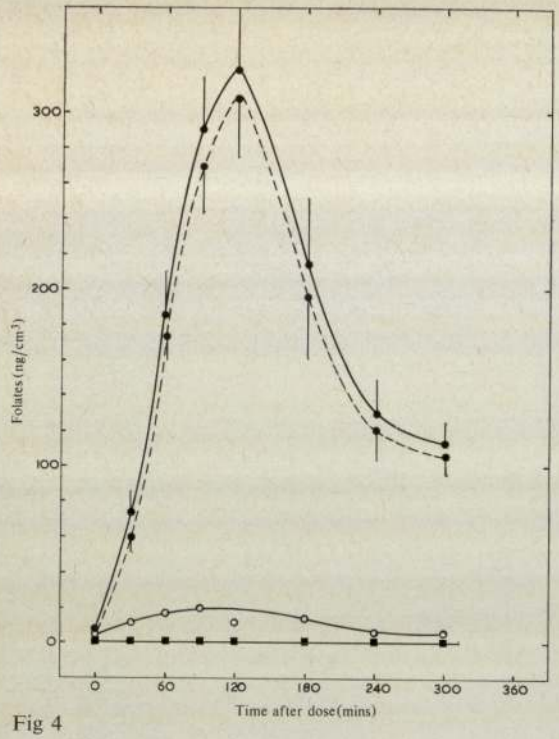

Fig 4

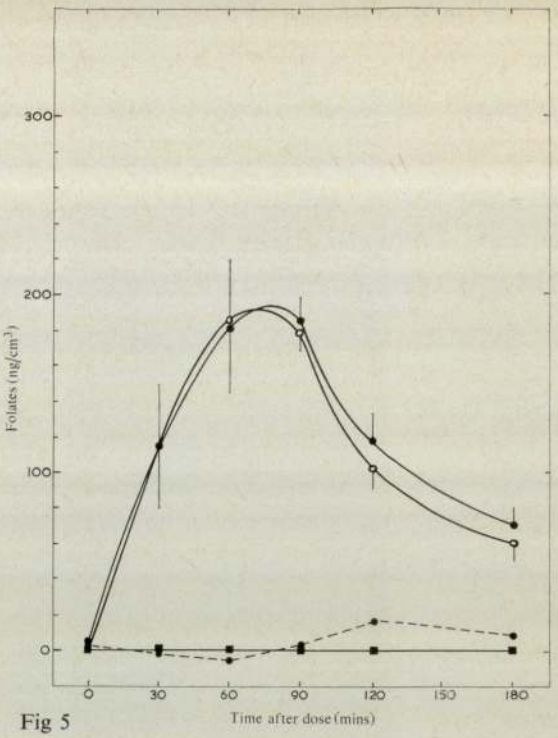

Fig 5

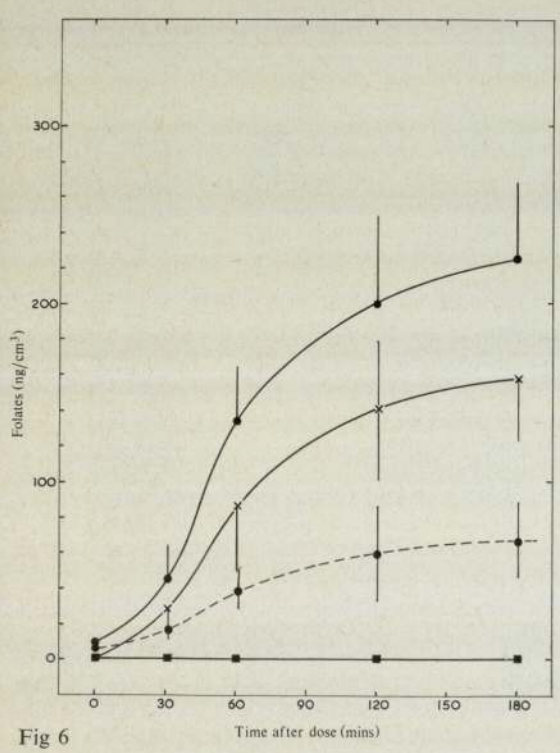

Fig 6

Fig 4 *The effect of orally administered 5,10 methenyl THF (10 mg) on the mean levels of folate analogues in six subjects.*

Fig 5 *The effect of orally administered 10-CHO FA (5 mg) on the mean levels of folate analogues in six subjects.*

Fig 6 *The effect of orally administered pteroyl-L-monoglutamic acid (5 mg) on the mean levels of folate analogues in six subjects.*

doses of folates. After oral doses of folic acid, 10-formylfolic acid, 5-formyltetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, and 5-methyltetrahydrofolic acid total serum folates rise rapidly to high levels while the 10-formyltetrahydrofolic acid remains practically constant (figures 2-6). Long-term administration of folic acid also fails to raise the 10-formyltetrahydrofolic acid level. The 10-formylfolic acid is not converted to 5-methyltetrahydrofolic acid (figure 5). As previously noted (Leeming *et al*, 1972), folic acid is slowly converted to 5-methyltetrahydrofolic acid (figure 6). The 5,10-methenyltetrahydrofolic acid also gives a small amount of serum 10-formylfolic acid probably derived by oxidation of 10-formyltetrahydrofolic acid in the jejunum before absorption (figure 4) (Beavon and Blair, 1972; Blair and Pearson, 1974). The persistence of these small amounts of 10-formylfolic acid in the serum again shows that this compound is not converted to 5-methyltetrahydrofolic acid.

The relative peak serum levels obtained after administration of folic acid and the tetrahydrofolates, particularly the high level after 5,10-methenyl-THF, was similar to that shown by Brown *et al* (1973) although these authors did not use differential microbiological assays or separative techniques to identify individual serum constituents.

Measurement of the individual serum folates in adult coeliac disease and leukaemia shows a significant increase over normals of the 10-formylfolic acid level (table I). However, if as in normal subjects this is an analytical artefact derived from the oxidation of 10-formyltetrahydrofolic acid then in both adult coeliac disease and leukaemia there are significantly increased levels of serum 10-formyltetrahydrofolic acid. In the leukaemic subjects both total folate and 5-methyltetrahydrofolic acid are significantly reduced (table I).

Sotobayashi, Rosen, and Nichol (1965) showed that tissues with high cell replication rates had a much smaller proportion of folate as 5-methyltetrahydrofolic acid and a much higher proportion as 10-formyltetrahydrofolic acid (or derived compounds) than tissues with slow cell replication rates. Thus the increased serum levels of 10-formyltetrahydrofolic acid in adult coeliac disease and leukaemia may be due to the increased cell replication rate in these diseases.

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