Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

THE FOLATE CATABOLIC PROCESS,

ITS IMPLICATIONS FOR FOLATE REQUIREMENT IN HUMANS

AND ITS

ANTIBACTERIAL DRUG FUNCTION

by

EOIN P. QUINLIVAN

Ph.D. Thesis

University of Dublin

Trinity College

"So many ships on the high sea..."

David Vincent

To my parents, Patricia & Eugene,

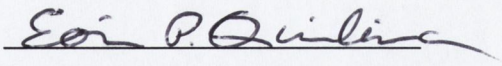
with all my love.

DECLARATION

This thesis is submitted by the undersigned to the University of Dublin for examination for the degree of Doctor of Philosophy. It has not been submitted as an exercise for a degree to any other university. With the exception of the subjects recruitment and sample collection for the human studies, Chapters 2 to 6, or where work is attributed to Dr Joseph McPartlin [Appendix A], the work herein is entirely my own.

This thesis may be made available for consultation within the University library, and may be photocopied or loaned for the purposes of consultation.

SIGNED:



Eoin P. Quinlivan

October 2000

ACKNOWLEDGEMENTS

I wish to thank Doctor Joseph McPartlin for his advice, his invaluable help in writing this thesis and general wit. My thanks also to all who work in Sir Patrick Dunn's Research Laboratories, where I conducted my work, for their support, encouragement, and camaraderie; which made my doing this thesis so enjoyable (in a that-which-does-not-destroy-us-makes-us-stronger sort of way). Especial thanks to Celine Herra for her invaluable advise in conducting the bacterial studies, and for her monologues on shopping, and to Zana Kelly for teaching me the folate and vitamin B_{12} assays.

To Miss McHugh and Eoin McConville, my teachers in primary school, and Brendan Henehan, in secondary school, for instilling a love of, and a thirst for, knowledge.

My brothers, David and Brendan and my friends, Robbie, James, Daragh, James and Paul, for keeping me sane through the whole process:. To my team-mates for providing an oasis from the stresses of life.

Is maith liom cur buíochas ar Christina, mar bhí sí ann.

Finally, Profs. John Scott and Donald Weir.

PUBLICATIONS BY THE AUTHOR

• E.P. Quinlivan, J. McPartlin, D.G. Weir and J. Scott (2000). Mechanism of the Antimicrobial Drug Trimethoprim Revisited; FASEB J. (In Press; December)

• J.R.J. Higgins, E.P. Quinlivan, J. McPartlin, J.M. Scott, D.G. Weir and M.R.N. Darling (2000). The Relationship Between Folate Catabolism and Increased Requirement for Folate in Pregnancy; Br J Obstet. Gynaecol. **9**; 1149-54

• E.P. Quinlivan, J. McPartlin, D.G. Weir and J.M. Scott (2000). Adaptations to Folate Requirement in Pregnancy. Proc. Nutr. Soc. **59**; 81A

• E.P. Quinlivan, J. McPartlin, D.G. Weir and J.M. Scott (2000). Decreased Serum Homocysteine in Pregnancy: Possible Role in Methylation Cycle Regulation. Proc. Nutr. Soc. **59**; 96A

ABSTRACT

The one-carbon cycle, of which folate is the predominant constituent, is a major carrier of one-carbon units for biochemical synthesis, and is thought to be the sole mechanism of methyl-group transfer. It is involved both in purine and pyrimidine biosynthesis, in the regulation of transcription and epigenics, and in the synthesis of important biological compounds.

In man folate is subject to a continuous catabolic process. By measuring the rate of excretion of the products of folate catabolism, *para*-aminobenzoylglutamate (pABGlu) and its acetamido derivative (apABGlu), we determined the requirement for folate in humans. We also show that this requirement increases during pregnancy, due to an increase in the rate of excretion of apABGlu. This increase in requirement may explain the prevalence of folate deficiency during pregnancy. We provide evidence to suggest that only apABGlu is a true catabolic product of folate, while pABGlu appears to be the product of a non-specific cleavage process. From this data we derive recommendations for the folate requirement for men, and for both pregnant and non-pregnant women.

One of the major metabolic functions of folate is the methylation of homocysteine (Hcy), to produce methionine, a reaction which uses vitamin B_{12} as co-enzyme. Elevated serum Hcy concentration has been linked to the pathologies of several diseases, and is associated with an increased risk of cardiovascular disease. As a facet of public health policy the use of folic acid fortification has been advocated to lower Hcy. However, we show that increased intake of folic acid is only partially effective at lowering Hcy, as above a certain threshold folate status is no longer limiting homocysteine methylation as vitamin B_{12} concentrations become limiting.

Hcy is a potent undermethylating agent. Thus, its accumulation *in vivo* during pregnancy is potentially detrimental to foetal development. However, we show that, despite the increased rate of folate catabolism observed during pregnancy, Hcy concentrations fell during this period. We propose that the observed decrease in Hcy may be an adaptive mechanism to protect methyl-transfer at a time of increased risk of folate deficiency.

We devised a bacterial model, whereby the folate pools of representative microorganisms are radiolabelled, using either the folate precursor [^{3}H]-*p*-aminobenzoic acid or tritiated forms of folate. Using this model we showed that the antibacterial drug trimethoprim (Tmp), an inhibitor of the bacterial enzyme dihydrofolate reductase (DHFR), caused the catabilic loss of folate in two representitive microorganisms, *Eschericia coli* and *Lactobacillus casei*.

A further consequence of Tmp inhibition of *E.coli* was the accumulation of folic acid, which upon removal of inhibition, was shown to be metabolically active. After 4 hours incubation in the presence of Tmp folic acid accounted for over 45% of the radiolabel in the cell, with the rest resulting from catabolic products of folate. This is the first time such extensive accumulation of folic acid has been reported *in vivo*.

As the synergy observed between Tmp and sulfamethoxazol (SMX), a potent inhibitor of folate biosynthesis in bacteria, has been attributed to the possibility that SMX is also an inhibitor of DHFR we set out to test this hypothesis. However, while we were unable to show any such inhibition of DHFR by SMX, we did show that SMX caused the metabolic inhibition of the one-carbon cycle, resulting in depletion of concentrations of 10-formyltetrahydrofolate, the folate co-enzyme involved in purine biosynthesis. This disruption to the purine biosynthetic pathway, along with the induction of folate catabolism and inhibition of *de novo* folate biosynthesis caused by Tmp may in part explain the synergy observed between both drugs.

In developing a tissue culture model we hoped to demonstrate attrition on mammalian folate store similar to that observed in bacteria when faced with inhibition of DHFR. However, while a minor increase in catabolism was observed, the predominant effect of inhibition was the accumulation of dihydrofolate (DHF). As a proportion of the cellular folates were shown to be protein bound we suggest that a factor in the increased stability of the DHF in mammalian cells may be due to the stabilising influences of such binding.

LIST OF ABBREVIATIONS

A. Reduced Folate Co-Enzymes

DHF	dihydrofolate
THF	tetrahydrofolate
5-CH_3-THF	5-methyltetrahydrofolate
5,10-CH=THF	5-methenyltetrahydrofolate
5,10-CH_2-THF	5,10-methylenetetrahydrofolate
5-CHO-THF	5-formyltetrahydrofolate
10-CHO-THF	10-formyltetrahydrofolate

B. Folate Precursors

pABA	*para*-aminobenzoic acid
pABGlu	*para*-aminobenzoyl-γ-glutamic acid
$pABGlu_n$	*para*-aminobenzoylpolyglutamic acid (n = chain length)
GTP	guanosine 5'-triphosphate
DHNpt	dihydroneopterin
DHpt-CH_2OH	dihydroterin-6-hydroxymethyl
DHpt-CHO	dihydroterin-6-carboxyaldehyde

C. Antifolate drugs

MTX	methotrexate
SMX	sulfamethoxazol
Tmp	trimethoprim
FdU	5-fluorodeoxyuridine

D. Folate Utilising Enzymes

DHFR	dihydrofolate reductase
MTHFR	methylenetetrahydrofolate reductase

E. Intermeduries in the One-Carbon Cycle

Hcy	homocysteine
tHcy	total homocysteine concentration
SAH	s-adenosylhomocysteine
SAM	s-adenosylmethionine

F. General Chemical Abbreviations

ATP	adenosine 5'-triphosphate
DNA	deoxyribonucleic acid
NAD(P)+	β-nicotinamide adenine dinucleotide (3'-phosphate)
NAD(P)H	nicotinamide adenine dinucleotide (3'-phosphate), reduced
Pi	inorganic phosphate (PO_4)

G. Statistical Terms

ANOVA	one way analysis of variance
p (-value)	significance level
r	correlation co-efficient
SD	standard deviation

H. General Abbreviations

CHD	coronary heart disease
NTD	neural tube defect
HPLC	high pressure (a.k.a, performance) liquid chromatography

TABEL OF CONTENTS

PART II: STUDIES IN BACTERIA AND CELL CULTURES

CHAPTER 7

TRIMETHOPRIM INHIBITION OF FOLATE METABOLISM AND BIOSYNTHESIS IN *ESCHERICIA COLI*

CHAPTER 11

DISCUSSION

CHAPTER 1

INTRODUCTION

1. Preamble

In the 1930's Lucy Wills published two papers which showed that an unidentified component from yeast could cure (Wills, 1931) or prevent (Wills and Stewart, 1935) anaemia caused by nutritional deficiency. It was for this initial discovery that Watson and Castle (1946) named the compound "Wills' factor" when they demonstrated that this "new haemopoietic" compound was contained in fractions of liver extract distinct from those which were curative for pernicious anaemia. Final evidence that the vitamin was not vitamin B_{12} came with its synthesis by Angier *et al.* in 1946. We now more commonly know the vitamin as folate or folic acid.

In the intervening years our understanding of the vitamin has extended not just to its use in the prevention and cure of diseases of nutritional deficiency, but to our understanding of vitamin sufficiency, how folate concentrations which were once considered adequate in the prevention of the nutritional anaemias may be inadequate in the prevention of cancer, birth defects and vascular disease. However, until recently over 40% of the US adult population had a folate intake below the recommended daily allowance (RDA) (Wilson *et al.*, 1997), with more than 8% having a folate intake less than half the RDA; over 20% had plasma folate concentrations deemed to be deficient (Jacques *et al.*, 1999) resulting in elevated concentrations of total plasma homocysteine (tHcy). We discuss below many of the factors

which give rise to folate deficiency, particularly the loss of folate due to increased catabolism at times of rapid cell proliferation. As mentioned already, folate deficiency gives rise to increased tHcy. Elevation of tHcy has been associated with increased incidence of coronary heart disease (CHD) and birth defects. We review the public health strategies which have been introduced to increase folate status and decrease tHcy. Both outcomes would be anticipated to improve public health and decrease the rate of neural tube defects (NTDs) and CHD occurrence.

Because of the importance of folate in DNA biosynthesis antifolate drugs have proved to be extremely effective in the treatment of cancer (Jackman, 1999) and bacterial infection (de Ferranti *et al.*, 1998). In particular we describe how the antibacterial drug trimethoprim (Tmp) exerts its effect on folate depletion. Furthermore, we shed new light on the synergism that exists between Tmp and the folate synthesis inhibitor sulphamethoxazol (SMX).

But before discussing these matters we describe in detail the structure, biogenesis and physiology of folate.

1.1. Occurrence

1.1.1. Folic Acid [Fig. 1.1] shows the structure of folic acid. Folic acid is thought not to occur in nature to any great extent as it is not a product of either the biosynthetic or metabolic folate pathways. Instead its occurrence is dependent on the chemical oxidation of reduced folates or from commercial synthesis for use in supplements and in food fortification. Folic acid is regarded as the most stable form of the vitamin.

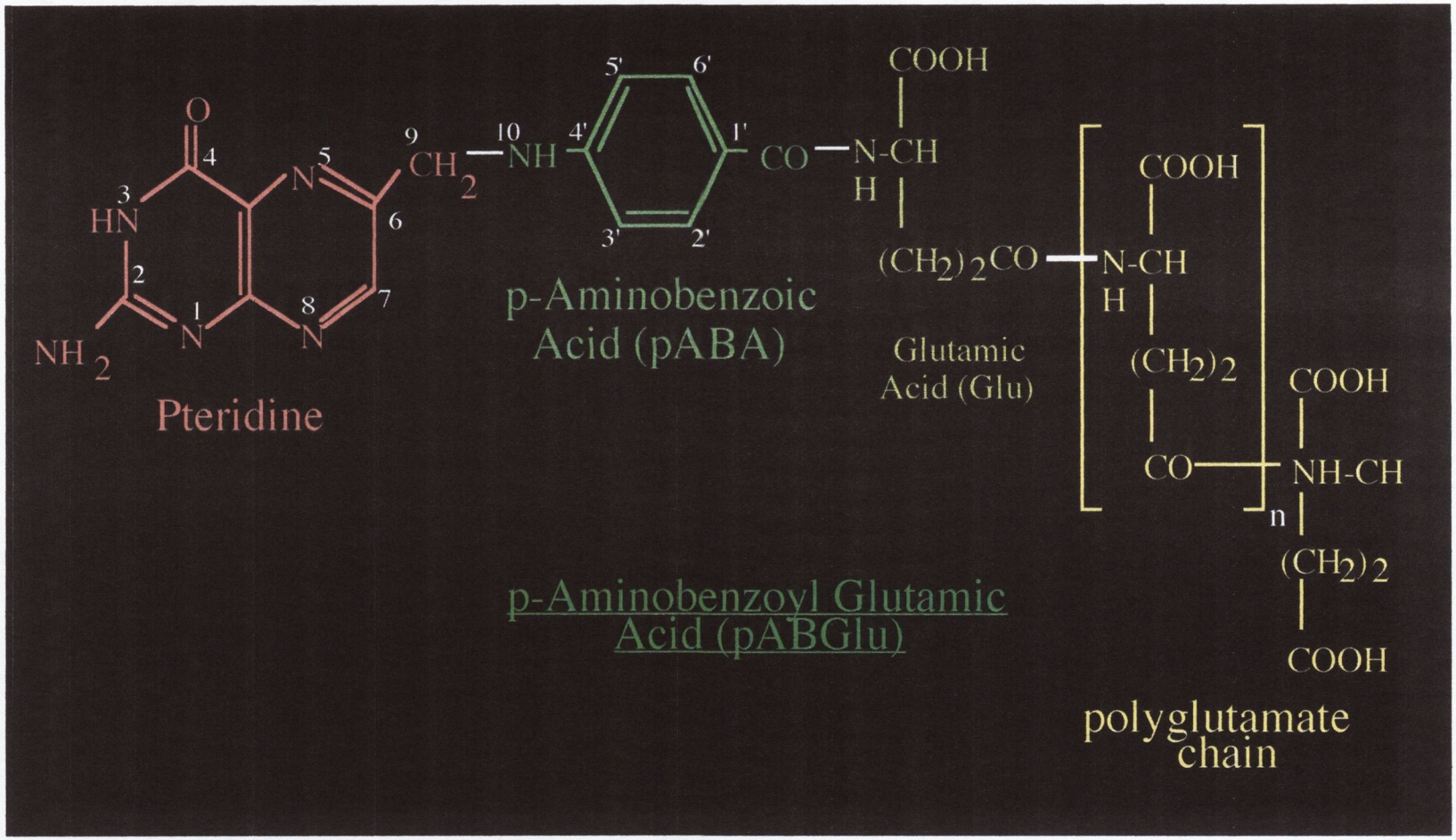


Fig 1.1. Basic structure of folate showing the numbering system for the pteridine and aminobenzoate rings. Reduction of the pteridine ring occurs at C7 and N8 (dihydro-) or at N5, C6, C7 and N8 (tetrahydro-). One carbon substitution occurs on, or bridging, N5 and N10.

1.1.2. Reduced Folates The reduced folates are naturally occurring and, because of their role as one-carbon donors for a number of biological reactions, are ubiquitous to all known living cells, where they are found in various states of N-5 and N-10 substitution [Fig 1.1]. Reduced folates are synthesised by most plants and micro-organisms, which accounts for their natural abundance in nature. However, as the folate biosynthetic pathway is absent in mammals and most higher organisms (Kishore *et al.*; 1988, Haslam, 1993) their nutritional requirement for folate must be derived from the diet. This may be through direct consumption of plants and micro-organisms, or through ingestion of material from higher organisms, which have obtained their folate from primary dietary sources [Table 1.1]. Because of the inability of mammals, and in particular humans, to synthesise folate, folate is termed a vitamin. The term "vitamine" was coined by Funk (1912) to describe a group of "vital amines" which were required for proper metabolic function. The term vitamin, minus the 'e' to remove the implication that all such compounds are amines, has since been used to describe a compound that cannot be synthesised by a given organism and must therefore be supplied exogenously.

1.2. Nomenclature

1.2.1. General Comments The independent discovery of an unknown vitamin required for cell proliferation spawned a plethora of names for the as yet unidentified compound: folate, folic acid, pteroylglutamic acid, liver *L.casei* factor, vitamin Bc, vitamin M, Folsäure (Merck Index, 1996a), *Streptococcus lactus* R-Stimulating Factor (Laskowski *et al.*, 1945) and Wills' factor (Watson and Castle1946). Matters were further complicated by the partial characterisation of compounds whose physiological properties differed from those of folate due to either one carbon substitution (*i.e.* 5-formyltetrahydrofolate has been

Food Source	Folate (μg/ 100g)		Free folate as a % of Total folate
	Free	Total	
Grains:			
Oat meal	32.0	34.0	94.1
Rice (boiled / milled)	8.9	11.0	81.0
Wheat (whole)	14.2	36.6	38.8
Puleses and legumes:			
Peas (dry)	4.6	7.5	61.3
Soyabean	8.7	100	8.7
Chick Peas	34.0	186.0	18.3
Mung bean	24.5	140.0	17.5
Leafy Vegetables:			
Cabbage	13.3	23.0	57.9
Spinach	51.0	123.0	41.5
Roots and tubers:			
Carrot	5.0	15.0	33.3
Onion	1.5	6.0	25.0
Potato	3.0	7.0	42.9
Other Vegetables:			
Cucumber	12.6	14.7	85.8
Pumpkin	3.0	13.0	23.1
Meat:			
Chicken	3.2	6.8	47.4
Liver (Sheep)	65.6	188.0	65.1
Mutton	1.0	5.8	17.3
Sundry:			
Whole egg (Chicken)	70.3	78.3	89.8
Milk (Cow)	5.6	8.5	65.9
Yeast (dried)	150.0	1640.0	9.2

Table 1.1. Folate composition of some typical foodstuff. Free Folate: Relative activity of crude extracts in sustaining *L.casei* growth. Total Folate: Relative activity of extracts upon conjugase treatment [see Section 1.4] (from Lakshmaiah and Ramasastri , 1975c)

called folinic acid, citrovorum factor, leucovorin (Merck Index, 1996b)) or due to their polyglutamylated nature (*i.e.* fermentation *L.casei* factor (Laskowski *et al.*, 1945; Hutchings *et al.*, 1948) [Fig 1.1]. It was therefore not until the chemical structure of these compounds was determined that the common locus for the names was established.

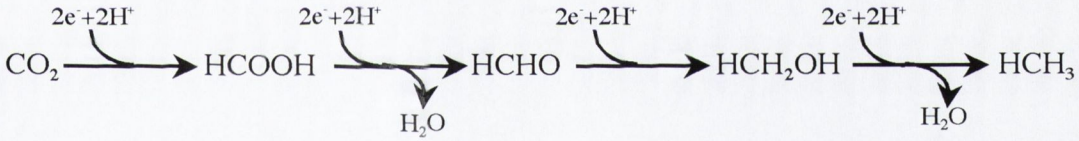
While the IUPAC-IUB Commission name for the vitamin is pteroylglutamic acid (PteGlu) (Blakely and Benkovic, 1984), this has proved unwieldy and cumbersome, thus confusion over the name has continued with the more common use of the names folic acid and folate to describe the vitamin. The name folic acid, and thence folate, was first coined in 1941 by Mitchell *et al.* (1941) after the 4 tons of spinach leaves from which it was purified (Latin, *folium* – leaf).

1.2.2. Substitution and Reduction The carbon and nitrogen atoms of folate are numbered as shown in [Fig 1.1] (Blakely, 1969). The naturally occurring folates differ from folic acid by the degree of reduction, di- or tetrahydro-, of the pteridine ring and by substitution of one-carbon sub-units on or bridging nitrogens 5 and 10. The naming convention for the vitamin is: [(atom number(s))-Substituent group]-[(atom numbers)-Number of hydrogens]-folate / pteroylglutamate. Using this convention the term "folate" has in the past been used to describe folic acid/ pteroylglutamic acid, again causing confusion. In recent years, therefore, use of the name folic acid has increased when referring to pteroylglutamic acid. Unfortunately this has lead to complications of its own, with the term folic acid now becoming synonymous with the vitamin in the mind of the public, due to health promotional campaigns.

1.2.3. Polyglutamates As the term folate/folic acid refers to compounds containing one glutamate residue [see Fig 1.1] this has lead to a further awkwardness in naming compounds with more than one glutamate. When discussing poly-glutamates all residues are counted including the glutamate attached to the pABA. This confusion does not arise when using the IUPAC nomenclature pteroyl-polyglutamate ($PteGlu_n$): e.g. is pteroyl-triglutamate ($PteGlu_3$) = folate-triglutamate.

1.3. Folate Biochemistry

The major metabolic function of folate *in vivo* is as a carrier and donor of one carbon units at various levels of oxidation. The successive two electron reduction of one carbon is as follows:



The one carbon unit of carbon dioxide is sequentially reduced to formic acid, formaldehyde, methanol and methane with two protons accepted at each stage, water is release during the conversion of formic acid to formaldehyde and of methanol to methane.

1.3.1. Source of One-Carbon Units One carbon units at the level of formate can directly enter the one-carbon cycle as formic acid in a reaction mediated by 10-CHO-THF synthase [Fig 1.2; Enzyme 8] (Smith *et al.*, 1980; Caperelli *et al.*, 1980), or as a consequence of the catabolism of histidine by glutamate formiminotransferase [Fig 1.2; Enzyme 15] (Tabor and Wyngarden, 1959; Mackenzie and Baugh, 1980). Entry at the level of formaldehyde occurs through the synthesis of 5,10-CH_2-THF via β-carbon transfer from serine in the

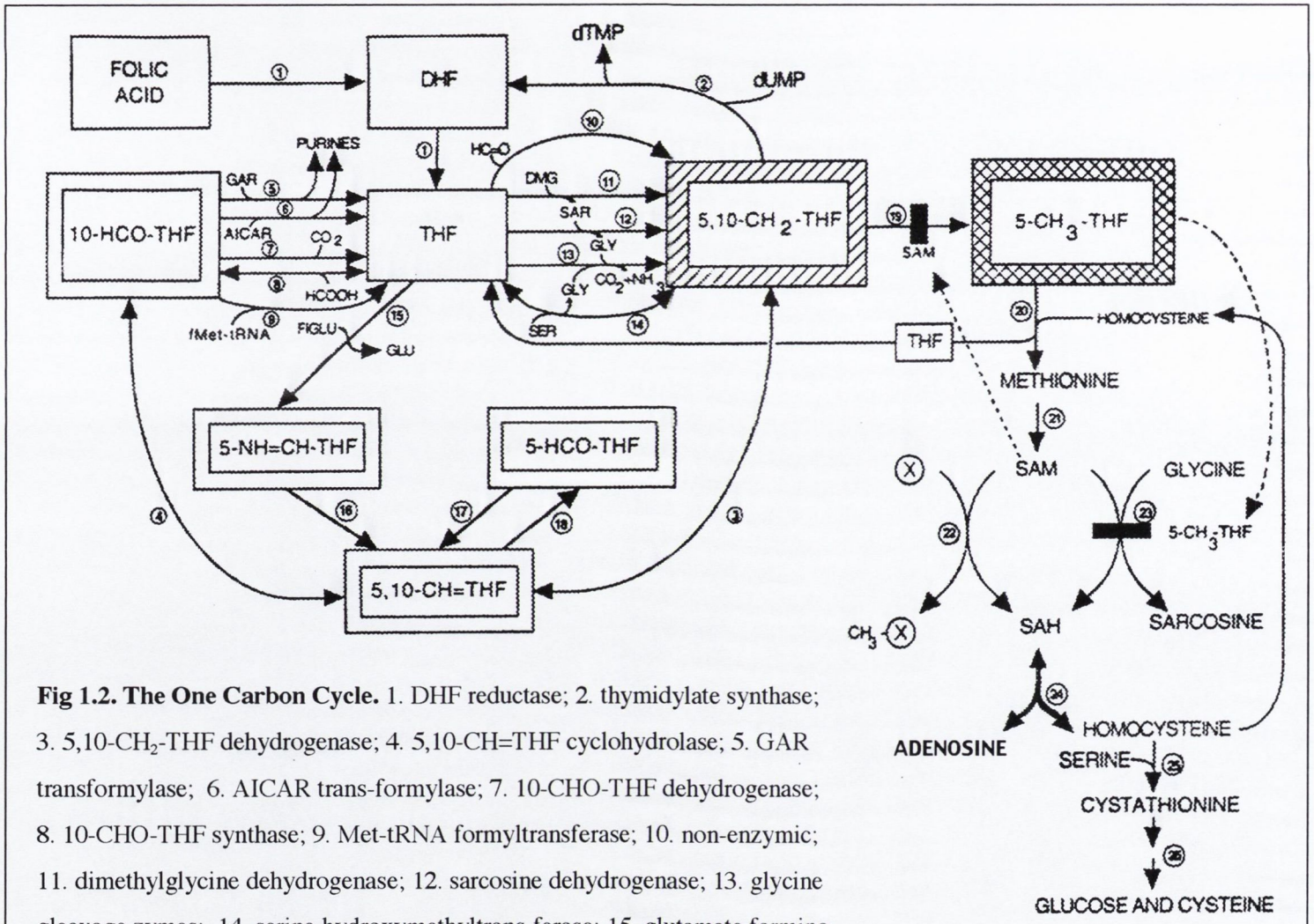


Fig 1.2. The One Carbon Cycle. 1. DHF reductase; 2. thymidylate synthase; 3. 5,10-CH_2-THF dehydrogenase; 4. 5,10-CH=THF cyclohydrolase; 5. GAR transformylase; 6. AICAR trans-formylase; 7. 10-CHO-THF dehydrogenase; 8. 10-CHO-THF synthase; 9. Met-tRNA formyltransferase; 10. non-enzymic; 11. dimethylglycine dehydrogenase; 12. sarcosine dehydrogenase; 13. glycine gleavage zymes; 14. serine hydroxymethyltrans-ferase; 15. glutamate formino-transferase; 16. 5-NH=CH-THF cyclodeaminase; 17. 5,10-CH=THF synthase; 18. catalysed by serine hydroxymethyl-transferase; 19. 5,10-CH_2-THF reductase; 20. methionine synthase; 21. SAM synthase; 22. variety of methyltransferases; 23. glycine *N*-methyltransferase; 24. SAH hydrolase; 25. cystathionine-β synthase; 26. pathway leading to glucose

cytoplasm and mitochondria [Fig 1.2; Enzyme 14] (Smith *et al.*, 1980; Caperelli *et al.*, 1980) or via dimethylglycine dehydrogenase [Fig 1.2; Enzyme 11] (Wittwer and Wagner, 1980) sarcosine dehydrogenase [Fig 1.2; Enzyme 12] (Wittwer and Wagner, 1980) and glycine cleavage [Fig 1.2; Enzyme 13] (Motokawa and Kikuchi, 1971; Hirage *et al.*, 1972) in the mitochondria. Enzymatic one-carbon unit inter-conversion occurs as shown in [Fig 1.2].

1.3.2. Utilisation of One-Carbon Units [Table 1.2] summarises the metabolic role of one-carbon units in the production of functional cellular components, while a more detailed discussion of these reactions is given in the following sections.

Pyrimidine Synthesis The relationship between folate derivatives and thymidine was first determined by the ability of folate to replace thymidine or thymine as bacterial growth factors (Stokes, 1944). We now know that the folate dependent reaction in the synthesis of thymidine is the methylation of deoxyuridine monophosphate (dUMP) to produce thymidine monophosphate (dTMP). The dUMP biosynthetic pathway is shown in [Fig 1.3] (Mathews and van Holde, 1990). While the one-carbon unit transferred to dUMP is at the methanol state of oxidation the one-carbon unit of 5,10-CH_2-THF is at the formaldehyde level of oxidation, the additional reducing equivalents are thus provided by donation of two protons from the pteridine ring of THF. This reaction is the only metabolic reaction whereby net oxidation of THF occurs, and is one of only two metabolic reactions whereby there is a net change in the redox state of the pteridine ring of folate, the other being reduction of DHF to THF.

Reaction	Enzyme [Fig 1.2]	One Carbon Donor	Oxidative State
Pyrimidine Synthesis:			
dUMP → dTMP	2	5,10-CH_2-THF	Formaldehyde
De novo purine synthesis:			
Formylation of GAR	5	10-CHO-THF	Formate
Formylation of AICAR	6	10-CHO-THF	Formate
Methylation Cycle:			
Methionine Synthesis	20	5-CH_3-THF	Methanol
Methyltransferases	22	SAM	Methanol
Translation Initiation:			
fMet-tRNA^{Met} Formation	9	10-CHO-THF	Formate

Table 1.2. Metabolic function of one carbon donors.

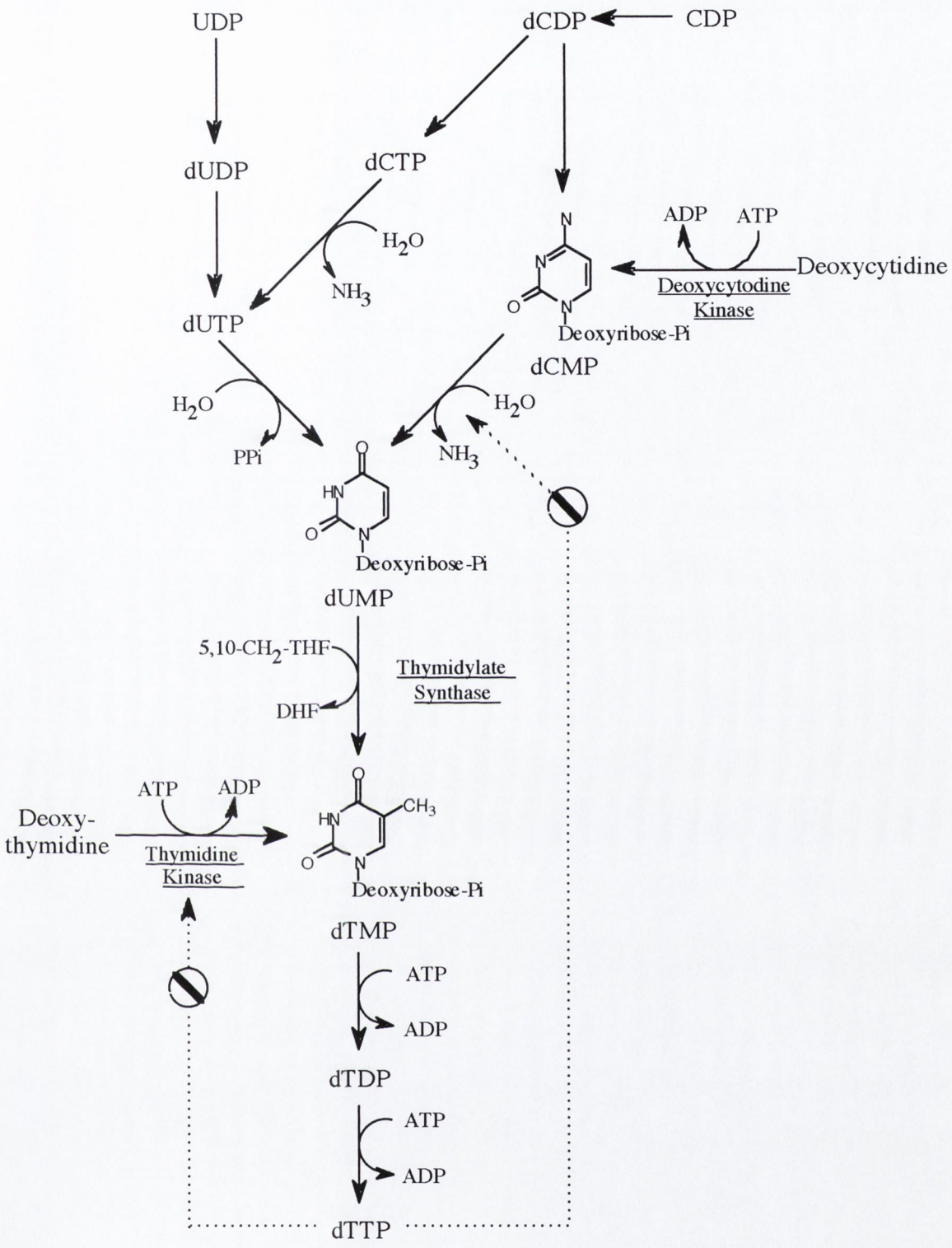


Fig 1.3. The thymidylate biosynthetic pathway. Both deoxythymidine salvage and *de novo* synthesis from deoxycytidinemonophosphate (dCMP) are inhibited by deoxythymidylate-triphosphate (dTTP)

De Novo Purine Synthesis The metabolic origin of the atoms of the purine ring is shown in [Fig 1.4]. As can be seen two of the purine carbon atoms are derived from the one carbon units of folate. The two folate dependent reactions involve the transfer of one carbon units at the formate level of oxidation to phosphoribosylglycimate (GAR; Fig 1.5, Reaction 3) (Warren and Buchanan, 1957; Caperelli *et al.*, 1980) and phosphoribosylaminoimidazolcarboxamide (AICAR; Fig 1.5, Reaction 9) (Flaks *et al.*, 1957). AICAR transformylase activity is contained on a bifunctional enzyme which also has IMP cyclohydrolase activity, the final reaction in purine synthesis (Akira *et al.*, 1997; Ni *et al.*, 1991; Yamauchi *et al.*, 1995). The rat AICAR transformylase exhibits a high degree of sequence homology with the human (91%) and chicken (83%) transformylase but considerably less homology with the *E.coli* (37%) (Akira *et al.*, 1997). While 5,10-CH=THF and 10-CHO-THF were traditionally thought to be the respective folate donors for the formyltransferases (Hartman and Buchanan, 1959) it is now considered likely that 10-CHO-THF is the sole carbon donor in both cases. This initial confusion may derive from the apparent co-localisation of the formyltransferases on an enzyme complex containing trifunctional enzyme activity (10-CHO-THF synthase, 5,10-CH=THF cyclohydrolase and 5,10-CH_2-THF dehydrogenase), as well as serine hydroxymethyltransferase (Smith *et al.*, 1980; Caperelli *et al.*, 1980). Therefore while 5,10-CH=THF is an apparent substrate for GAR transformylase, removal of the trifunctional enzyme prevented the use of 5,10-CH=THF (Smith *et al.*, 1980; Smith *et al.*, 1981; Dev and Harvey,1978a). In a similar vein, 10-formyl analogues of folate, chemically engineered so as to be unable to form 5,10-bridges, were still substrates for GAR transformylase (Smith *et al.*, 1980). [6R]-10-CHO-THF, the natural chiral substrate for the enzyme (Km = 6.8μM), may not have been identified as such in initial studies due to contamination of the sample with its stereoisomer [6S]-10-CHO-THF (Ki = 0.75μM)

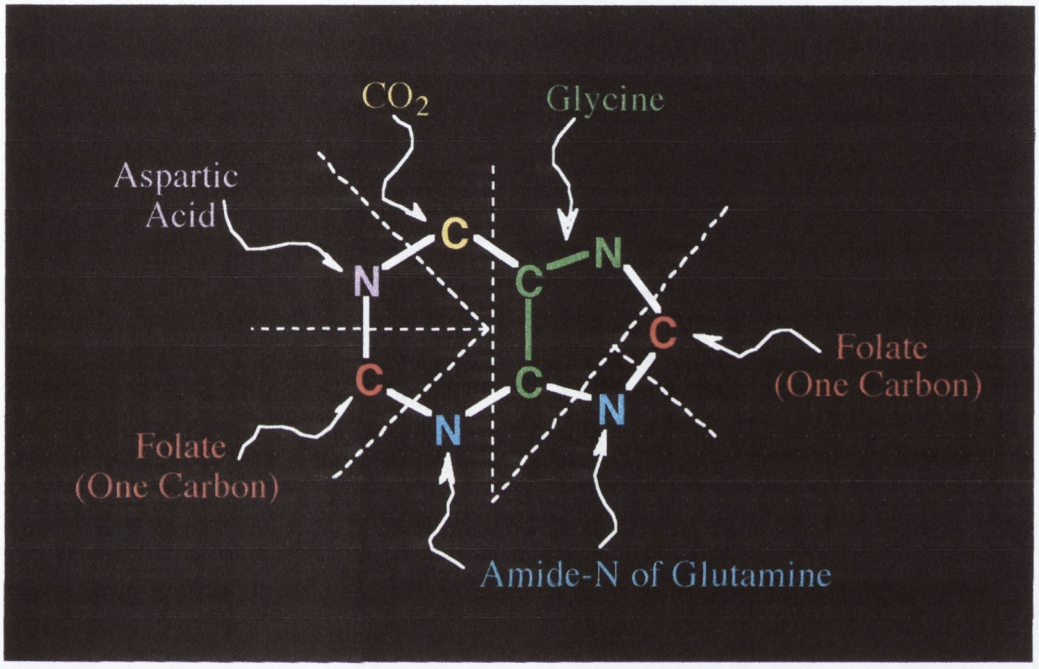


Fig 1.4. Origin of the carbon atoms of the purine base (Rowe, 1984).

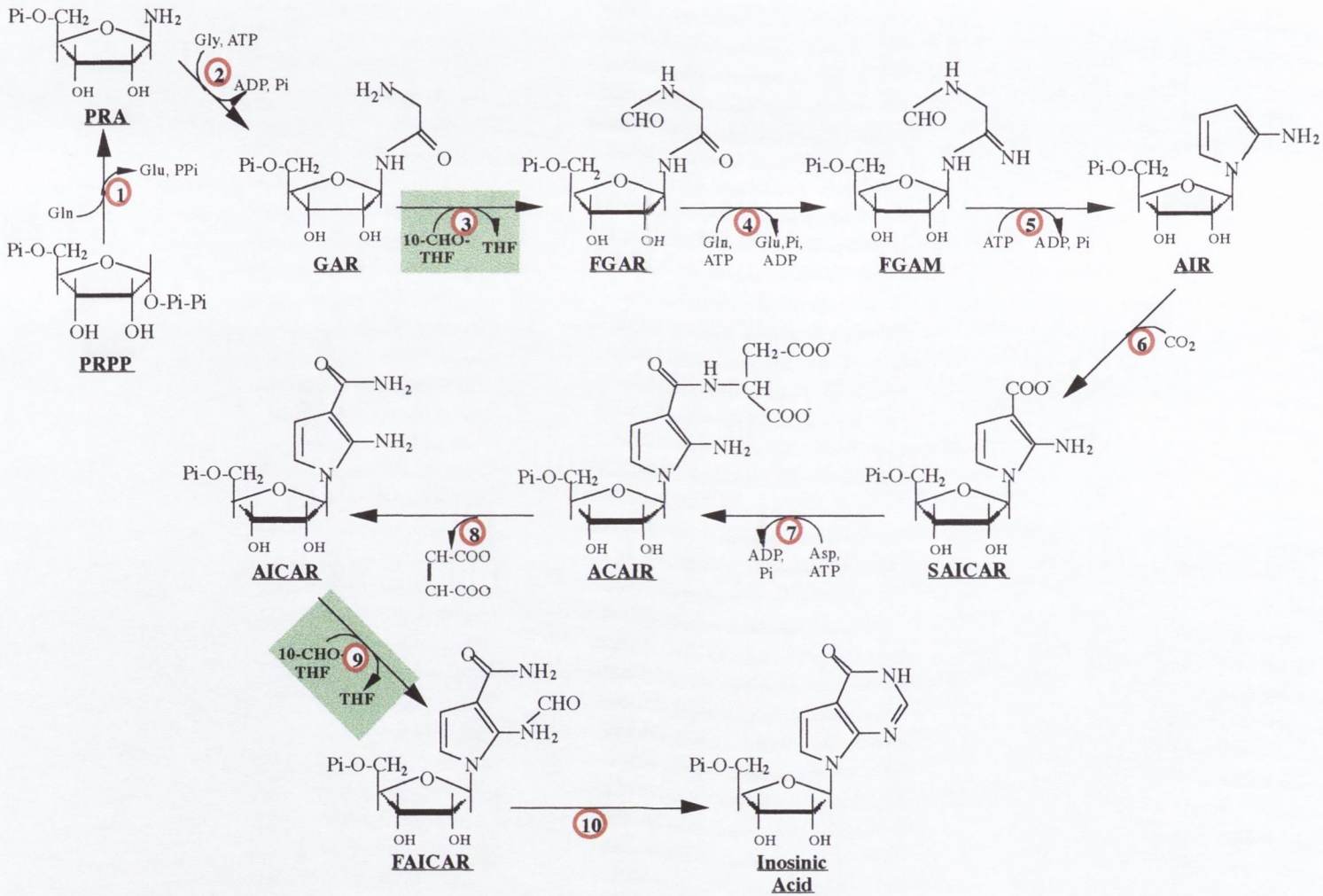


Fig 1.5. The purine biosynthetic pathway. The green boxes show the two formyltransferase reactions in which 10-CHO-THF is used as substrate.

(Smith, 1981). This type of stereoisomer inhibition is unprecedented in one-carbon metabolism.

Methylation Cycle Entry of one carbon groups into the methylation cycle is regulated by the allosteric inhibition of MTHFR by SAM (Jencks and Matthews, 1987; Kutzbach and Stokstad, 1971) or more likely, as SAH competes in a non-inhibitory manner for the SAM binding site, by a ratio of SAM to SAH. The synthesis of 5-CH_3-THF from 5,10-CH_2-THF is practically irreversible ($K_{eq} = 10^7$) (Kutzbach and Stokstad, 1971). Thus cycling of 5-CH_3-THF is dependent on the methyl-transfer from 5-CH_3-THF to methionine via the B_{12} dependent methionine synthase (MS) enzyme (Rüdiger and Jaenicke, 1969). The folate moiety is then free to enter the one carbon cycle as THF, while the methionine condenses with ATP to produce SAM, phosphate and pyrophosphate (Kobt and Kredich, 1985 & 1990). As SAM synthase has a K_i for SAM close to the physiological concentration of SAM (Oden and Clarke, 1983) enzyme activity is regulated by fluctuations in cellular SAM concentrations. In this case the reverse reaction is strongly prohibited due to the energy required to reform the phospho-ester and phospho-anhydride bonds of ATP (Kobt and Kredich, 1990). SAM is the universal one-carbon donor, forming SAH upon donation of its methyl-group. The enzymatic hydrolysis of SAH is an ordered Uni-Bi reaction which favours the reverse reaction (Porter and Boyd, 1991), with only the removal of products driving the reaction forward. As SAH is inhibitory to the SAM dependent methyltransferases, with K_i values close to physiological concentrations [Table 1.3 & 1.4], methyltransferase activity is dependent on the rate at which homocysteine (Hcy) and adenosine are removed. Hcy is removed by its remethylation to methionine, thus restarting the methylation cycle, or by its catabolism via the transsulphuration pathway. Adenosine is removed by its metabolism by adenosine kinase and adenosine deaminase (Chagoya De Sánchez *et al.*, 1991; Chagoya De Sánchez *et al.*, 1995).

Methyl-Transferase	K_M SAM (μM)	K_i SAH (μM)
Epinephrine-O	570	15
Phenylethanolamine-N	10	1.4
Acetylserotonin	14	2
Glycine	100	35
Guanidoacetate	47	17
Histone	14	5.5
Gua-7	1.4	1
Phosphatidylethanolamine	1.6	1.4
Proteine	3	7

Table 1.3. Binding and Inhibition constants for SAM dependent methyltransferases (Reproduced from Cantoni *et al.*., 1978)

Tissue	[SAM] μM (±SD)	[SAH] μM (±SD)
Liver	23.5 (3.1)	100.9 (40.8)
Kidney	8.4 (2.5)	41.4 (6.7)
Heart	5.8 (3.2)	41.6 (12.1)
Muscle	5.1 (2.5)	23.6 (12.8)
Cortex	4.9 (2.0)	35.6 (13.2)
Cerebellum	3.8 (1.4)	49.0 (10.6)
Striatum	3.8 (0.7)	45.3 (10.6)
Spinal Chord	3.4 (1.3)	34.1 (9.9)

Table 1.4. SAM and SAH concentrations in pig tissue. (Molloy *et al.*., 1990).

The feedback regulation and kinetics of the methylation cycle mean that regulation of the methyltransferases is not due to SAM concentration alone but also to cellular SAH concentration (Ingrosso *et al.*, 1997) and the rate of SAH metabolism (Kramer *et al.*, 1990). These are in turn dependent on the concentrations of homocysteine and adenosine (DeCabo *et al.*, 1995), and the rate at which they in turn are metabolised. Such regulation can come in the form of circadian variation in the activity of SAH hydrolase and the enzymes which metabolise adenosine (Chagoya De Sánchez *et al.*, 1991; Chagoya De Sánchez *et al.*, 1995) or in the form of cell cycle specific variation in SAH hydrolase activity (Ichikawa *et al.*, 1985; Chiba *et al.*, 1984).

The methylation cycle, therefore, not only provides a source of methionine for use in protein synthesis but it also regulats the availability of reactive methyl-groups. Provision of reactive methyl-groups by the methylation cycle occurs in two ways: Firstly the reactivity of the methyl-group for "soft" polarizable nucleophilic substrates [see Fig 1.2] is increased by transferring the methyl-group from the relatively inactive methyl-ammonium centre of 5-methyltetrahydrofolate to the 1000-fold greater reactivity of the sulfonium centre of the homocysteine moiety of SAM (Cantoni , 1975). The unreactive nature of 5-CH_3-THF require the nucleophilic properties of vitamin B_{12} as co-enzyme to enable the methyltransfer. Secondly the methylation cycle regulates transmethylation activity by regulating methyl-groups synthesis, via allosteric inhibition of MTHFR and cycle kinetics.

Initiation of Translation Two forms of methionyl-tRNA (Met-tRNA) exist in *E.coli*, a formylatable form, Met-$tRNA_f^{Met}$, which is used for the initiation of transcription and an unformylatable form, Met-$tRNA_m^{Met}$, which can not be used for initiation but is instead

used for peptide elongation (review by Lengyel and Söll, 1969; Lucas-Lenard and Lipmann, 1971). Unformylated Met-$tRNA_f^{Met}$ can be misappropriated for use in peptide elongation (Guillon *et al.*, 1996) however, such misappropriation is limited by formylation to formyl-Met-$tRNA_f^{Met}$ (fMet-$tRNA_f^{Met}$) which increases affinity for initiation factor (IF2) while reducing affinity for elongation factor Tu (EF-Tu) (Guillon *et al.*, 1993). The Met-$tRNA_f^{Met}$ is therefore directed towards transcription initiation and away from peptide elongation by its formylation.

In the mammalian mitochondria a single gene encodes the $tRNA^{Met}$ used in both peptide initiation and elongation (Anderson *et al.*, 1982), but how the resulting gene product can perform both functions is unknown. One proposal is that competition between EF-Tu and the formyltransferase may regulate the ratio of fMet-$tRNA^{Met}$ to Met-$tRNA^{Met}$ (Takeuchi *et al.*, 1998). This proposal seems likely as fMet-$tRNA^{Met}$ is required for the $IF2_{mt}$-dependent initiation of transcription, while the unformylated form is required for EF-Tu chain elongation in mammalian mitochondria (Lioa and Spremulli 1990; Schwartzbach and Spremulli, 1989). Formylation of $tRNA^{Met}$ in mammalian mitochondria could therefore play a more important role in the mammalian mitochondria than in bacteria as it may be the sole means of differentiating between initiation and elongation.

The formyl donor to $tRNA^{Met}$ in bacteria (Adams and Capecchi, 1966; Marcker, 1965) and in the mammalian mitochondria (Takeuchi *et al.*, 1998) is 10-CHO-THF. Met-$tRNA_f^{Met}$ formyltransferases (FMT) from *E.coli* has been isolated (Dickerman *et al.*, 1967), cloned (Guillon *et al.*, 1992) and its crystal structure determined (Schmitt *et al.*, 1998), while the mammalian mitochondria FMT has been partially purified and characterised (Takeuchi *et al.*, 1998). The requirement for FMT in regulating cellular function is apparent from the

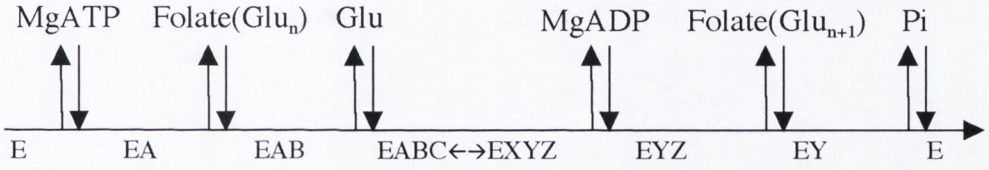
retarded growth observed in *E.coli* constructs with aberrant FMT enzyme activity (Guillon *et al.*, 1992).

1.4. Folate-Polyglutamates

The discovery of polyglutamated forms of folate stemmed from the finding that crude folate preparations contained biologically inactive compounds which "differ in biological activities and physical properties" (Stokstad *et al.*, 1948) from folate. Incubating the "'potential' folic acid" (Mimms. 1944) from a variety of sources with crude organ extracts (Wright and Welch, 1943; Bird *et al.*, 1946; Totter *et al.* 1944; Laskowski *et al.*, 1945; Binkley *et al.*, 1944) or under acid or alkali conditions (Briggs *et al.*, 1944) increased the ability of these extracts to support the growth of folate-requiring bacteria, due to the hydrolytic cleavage of the folate polyglutamate side chain, as folates with short glutamate chained are better able to support the growth of *L.casei* (Tamura *et al.*, 1972; Goli and Vanderslice, 1992). Folate polyglutamates were once thought to be storage forms of the vitamin because of this apparent biological inactivity (Shane, 1989). We now know this inactivity is due to their low bioavailability due to decreased cell permeability. *In vivo* glutamate chain length is regulated by the enzymatic addition or removal of glutamate residues by folate-poly-γ-glutamate synthases and folate conjugases, respectively. Glutamate chain length regulates folate transport across the intestine and cell membrane and folate storage while folate polyglutamates are better substrates for most folate dependent enzymes.

1.4.1. Polyglutamate synthesis and Cleavage

Folate poly-γ-glutamate synthase The kinetic mechanism of mammalian (Chichowicz and Shane, 1987) and bacterial (Shane, 1980b; Bogner and Shane, 1983) folate-polyglutamate synthase is ordered Ter-Ter:



The order of substrate binding and product release precludes sequential addition of glutamate residues to enzyme bound folate, resulting in homologous elongation of the glutamate chain *in vivo* (Thompson and Krumdieck, 1977; Baugh *et al.*, 1975). The mammalian enzyme exhibits a high degree of folate co-enzyme specificity [Table 1.5], with co-enzyme specificity increasing significantly for chain lengths of diglutamate or longer. Enzyme kinetics favour pentaglutamates formation [Table 1.6], the form predominantly found in tissue, however enzyme-substrate interaction can limit chain elongation (Chichowicz and Shane, 1987; Cook *et al.*,1987), resulting in retardation of chain elongation (Cassady *et al.*, 1980; Varela-Moreiras and Selhub, 1992).

Folate Conjugase / Hydrolase (γ-glutamylcarboxypeptidases) Enzymes that deconjugate folate polyglutamates were first identified by Wright and Welch (1943). Conjugase activity has been detected in a number of different mammalian sources (Lakshmaiah and Ramasa, 1975a) where they cleave the γ-carboxyl bonds of glutamate to remove the polyglutamate side chain. In humans this results in the formation of folate-monoglutamate

n	THF	5-CH_3-THF	Folic Acid	10-CHO-THF	5,10-CH_2-THF
1	100	20	17	31	20
2	63	1.7	45	13	18
3	15	0	≈5	1.7	4
4	7	ND	ND	0	3.5
5	2.2	ND	ND	0	1.5
6	1.3	ND	ND	0	0
7	0.3	ND	ND	0	0

Table 1.5: Relative activity of the folate mono- and polyglutamate cofactors for purified hog liver folate polyglutamate synthase. Reaction mixture contained 40μM folate substrate (naturally occurring diastereoisomer) and activity expressed relative to THF (Chichowicz and Shane, 1987).

Substrate	Mono- and Polylutamate Chain (n) Distribution Post Incubation						
	1	2	3	4	5	6	7
$H_4PteGlu_1$	**24**	3.8	2.4	10.2	50	9.8	0
$H_4PteGlu_2$		**30**	5	8.4	48	8	0
$H_4PteGlu_3$			**38**	10.2	44	7.2	0
$H_4PteGlu_4$				**34**	56	9.2	0
$H_4PteGlu_5$					**90**	10	0
$H_4PteGlu_6$						**98.4**	1.6
$5CH_3$-$H_4PteGlu_1$	**32**	60	7.4	0.5	0.2	0	0
$5CH_3$-$H_4PteGlu_2$		**85.6**	12.1	0.8	0.1	0	0
$PteGlu_1$	**48**	10.6	13	28	1.4	0	0

Table 1.6: Glutamate chain length distribution of folate mono- and polyglutamate substrates post 24 hour incubation with hog liver folate polyglutamate synthase. Percentages in **Bold** is the percentage of unreacted substrate recovered post incubation. (Cook *et al.*., 1987)

products while chicken conjugases produce folate-diglutamate (Goli and Vanderslice, 1991). In humans there would appear to be at least two forms of the enzyme: a serum conjugase, which is probably derived, like the rat enzyme (Lakshmaiah and Ramasa, 1975b), from the liver and a jejunal conjugase. The jejunal conjugase has a lower apparent molecular weight by gel filtration and a much higher thermostability (Lavoie *et al.*, 1975). Both human conjugases have a pH optimum of pH 4.5 (Goli and Vanderslice, 1991; Lavoie *et al.*, 1975; Lakshmaiah and Ramasa, 1975a), in contrast to the pH optimum of about pH 7 found for conjugases from rat plasma (Tamura, 1998) and chicken pancreas (Goli and Vanderslice, 1991).

1.4.2. Function of Folate-Polyglutamate Synthase and Conjugase

Folate Transport Natural folates (all except folic acid) are actively transported across the intestine, with kinetics governed by glutamate side-chain length, shorter side-chains having a higher bio-availability (Rosenberg, 1981). This specificity is in part a result of the increased α-carboxyl charge of each additional glutamate residue, but may also be due to transporter specificity. Conjugases in the jejunum therefore play a major role in folate bio-availability (Bandari and Gregory 1990; Hoffbrand and Peters 1969).

Storage Dietary folate enters the serum in the monoglutamate form, while folate polyglutamates, which might result from cell necrosis or apoptosis, are hydrolysed by conjugases in the plasma. Folates are therefore present exclusively as monoglutamates in serum. Within the cell, however, folates are predominantly found in polyglutamlyated forms as elongation of the glutamate chain is the principle means by which cellular storage occurs: polyglutamation increases folate binder affinity (Shane, 1989) while the increased charge due to the α-carboxyl prevents free diffusion of the polyglutamats from the cell.

Chain elongation may also prevent active transport by decreasing affinity for the membrane transporter. In this manner a concentration gradient is maintained across the cell membrane, requiring only the energy expenditure for polyglutamate formation (one ATP converted to ADP per glutamate residue added).

The consequences of the inability to form folate-polyglutamates has been investigate in Chinese Hamster Ovary "knock-out" cells which lack synthetase activity (AUX B1) (McBurney and Whitmore, 1974). While these cells were still able to transport and utilise folate their cellular concentrations were decreased due to decreased folate retention. Cells transfected with *E.coli* folate-polyglutamase synthase (AUX B1-*folC*) were able to synthesise folate-triglutamates and appeared to have similar folate retention and function as wild type cells (Osborne *et al.*, 1993; Lowe *et al.*, 1993) suggesting that formation of folate-triglutamates was the critical step in folate retention.

Compartmentalisation of folate within the cell is thought to occur via a similar mechanism. Sarcosine dehydrogenase activity is confined to the mitocondria, thus while AUXB1-*folC* cells had normal folate retention and function they required an exogenous source of glycine (Lin *et al.*, 1993) as their folate polyglutamate synthase activity was confined to the cytoplasm. However targeting folate polyglutamate synthase to the mitochondria, by genetically encoding a mitochondrial presequence, resulted in mitocondrial folate polyglutamate synthesis and removed the requirement for exogenous glycine (Appling, 1991).

Clinically, the consequences of retarded folate-polyglutamate synthesis are well documented in the case of B_{12} deficiency. 5-CH_3-THF is the predominant form of folate found in plasma (Perry and Chanarin, 1970), however as 5-CH_3-THF is a poor substrate

for folate polyglutamate synthase (Chichowicz and Shane, 1987; George *et al.*, 1987; Cook *et al.*,1987) [see also Table 1.5] it must be converted to THF in the cell before it can be polyglutamated. As the demethylation reaction is B_{12} dependent, a deficiency in this co-enzyme can retard cycling to THF. B_{12} deficiency is therefore associated with reduced folate uptake (Lavoie *et al.*, 1974; Davidson *et al.*, 1975; Shane *et al.*, 1977) and cellular concentrations (Gutstein *et al.*, 1973; Perry *et al.*, 1976). Moreover, the decrease in folate concentration is greatest (Perry *et al.*, 1976) for long chain ($n \geq 3$) folates, the more biologically active form of folate. A similar disruption of polyglutamate formation is observed with N_2O inactivation of B_{12} (McGing *et al.*, 1978).

Regulation of Enzyme Activity by Polyglutamate Chain Length Most of the enzymes in the one carbon cycle exhibit a greater affinity with increasing polyglutamate chain length ($3 \leq n \leq 6$) [Table 1.7]. However, changes in V_{max} are minor (2-3 fold at most). *Saccharomyces cerevisiae* mutant lacking FPGS are auxotrophic for methionine and growth is impaired in the absence of adenosine (Cherest *et al.*, 2000). Of special importance is the preference of methionine synthase for 5-CH_3-THF polyglutamates as they inhibit demethylation of 5-CH_3-THF-monoglutamate at physiological concentrations (Matthews *et al.*, 1987) and are thus likely to inhibit the rate of exogenous folate incorporation into the cell.

Folate polyglutamate synthesis has also been implicated in substrate channeling within multifunctional complexes. In the formiminotransferase-cyclodeaminase complex [Fig 1.2; Reactions 15 &16] use of short chain THF ($n \leq 3$) results in accumulation of the intermediary, 5-formimino-THF (Mackenzie and Baugh, 1980). However, use of the pentaglutamate substrate results in neither intermediary release nor lag in 5,10-CH=THF formation. The methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate

Enzyme	Source	Substrate	K_M Values for Folate Substrate						
			n = 1	n = 2	n = 3	n = 4	n = 5	n = 6	n = 7
Serine Hydroxymethyl-transferase	Pig Liver	THF	56	3.9	1.7				0.07
MTHF Reductase	Pig Liver	5,10-CH_2-THF	7.1	5.2	1.7	0.62	0.26	0.10	0.51
MTHF Dehydrogenase†	Pig Liver	5,10-CH_2-THF	25		6.8		3		
10-CHO-THF Synthase	Chicken Liver	THF	67		4.1				
MTHF Cyclohydrolase	Chicken Liver	5,10-CH=THF	20		4.3				
Thymidylate Synthase*	Fetal Pig Liver	5,10-CH_2-THF	5.2	2	1.9	1.9	1.6	1.6	2.1
AICAR Transformylase	Chicken Liver	10-CHO-THF	84		0.72				
Methionine Synthase*		5-CH_3-THF	6					0.05	

Table 1.7: Polyglutamate substrate specificity of folate utilising enzymes (from McGuire & Coward, 1984; †Mackenzie and Baugh, 1980; *Shane, 1989).

cyclohydrolase function [Fig 1.2; Reactions 3 & 4] of C_1 synthase has a common folate binding site which exhibits substrate channeling. The channeling observed increase marginally with glutamate chain length (Mackenzie and Baugh, 1980).

Speculation has arisen as to whether regulation of enzyme activity, and therefore metabolic flux, can occur due to modulation of the polyglutamate distribution (Krum. *et al.* '87). While changes in polyglutamate synthase activity (*i.e.* decreased during B_{12} inactivation by nitrous oxide or during folate depletion, increased during liver regeneration) are unlikely to effect folate storage they may effect the chain length distribution. Folate conjugase activity has been shown to modulate during the menstrual cycle in rats (Krumdieck *et al.*, 1975 & 1976) and with growth rate and phase of tumour growth, while polyglutamate chain elongation occurs during folate deficiency. While it is unlikely that slight modulation of polyglutamate chain length would significantly change the substrate specificity [cf. Table 1.7] it may result in the partitioning of folate coenzymes between the one carbon metabolic pathways.

Catabolic Products Conjugase activity may play a role in cell "housekeeping" as the folate catabolites pABGlu and apABGlu are excreted as monoglutamates (Gregory *et al.*, 1995; McPartlin *et al.*, 1992; McPartlin *et al.*, 1993). While deconjugation may occur in the plasma, post-clearance from the cell, it is probable that apABpolyGlu and pABpolyGlu are as unlikely to cross the cell membrane as folate-polyglutamates.

1.5. Folate Deficiency

1.5.1. Causes of Folate Deficiency

The causes of folate deficiency can be classified under a number of arbitrary headings.

Increased Folate Loss

Increased Folate Catabolism By far the greatest cause of folate loss is through its catabolism (McPartlin *et al.*, 1993; Geoghegan *et al.*, 1995). Folate catabolism increases during pregnancy (McPartlin *et al.* , 1993; McNulty *et al.*, 1992) and may also be increased by use of anticonvulsant and antifolate drugs and possibly during chronic alcohol consumption. Folate catabolism is one of the main topics of this thesis and is discussed in greater detail in the introductions to [Chapters 3 & 5].

Increased Excretion While calculated values for the folate content of human milk vary from 50μg/l to 82-99μg/l (Keizer *et al.*, 1995; Mackey and Picciano, 1998; respectively) they none the less represent a drain on maternal folate stores. Chronic haemodialysis has also been shown to increased folate loss (Arnadottir *et al.*, 2000; Sunder-Plassmann *et al.*, 2000; Bostom *et al.*, 2000; Kimura *et al.*, 2000) while acute alcohol consumption increases urinary excretion in rats (McMartin, 1984).

Decreased Folate Intake

As humans are unable to synthesis folate (Kishore *et al.*; 1988, Haslam, 1993) the folate lost due to folate catabolism and excretion of intact folate must be replaced. Thus, even

under conditions of normal folate loss, a decrease in folate intake to below the level required to replace this loss will result in folate deficiency.

Dietary Deficiency A major cause of folate deficiency is poverty, where groups on low income are unable to afford, or are unaware of the benefits of, a diet sufficiently high in folate rich foods, *i.e.* fresh fruit and vegetables (Rogers *et al.*, 1998). This problem may be further exacerbated by seasonal variability in the availability and price of fresh produce. Poor food preparation techniques may result in destruction of folate by prolonged heating, or by over refining grains; folate loss may also occur by leaching of the folates into the cooking water (Herbert, 1961). While folate consumption is generally not effected by ethical constraints, unlike say, vitamin B_{12} in vegan diets, the folate composition of the diet may be deficient for aesthetic reasons, dislike of vegetables, preference for boiled foods. Psychological disorders such as bulimia or anorexia result in nutritional deficiency. Chronic alcoholism or drug dependence can suppress appetite, cause lethargy or may divert money from purchasing food.

Malabsorption: Medical disorders resulting in inflammation of the intestine (*i.e.* coeliac disease (Kemppainen *et al.*, 1998), tropical sprue, Crohne's disease; surgical removal of part of the gastrointestinal tract, *i.e.* jejunal resection; decreased GIT pH, *i.e.* atrophic gastritis, pancreatic insufficiency; mechanical blockage of folate transport by bacterial overgrowth and congenital disorders resulting in poor folate transport. Chronic alcoholics also have a decreased rate of absorption of water and electrolytes (Krasner *et al.*, 1976) which may also effect folate absorption.

Other Nutritional Factors The nature of the food matrix or food composition can effect intestinal pteroylpolyglutamate hydrolase susceptibility and folate bioavailability (Tamura,

1998; Seyoum and Selhub, 1998; Wei and Gregory; 1998). Vitamin B_{12} deficiency can cause folate deficiency, probably due to the "methyl-trap" hypothesis whereby 5-CH_3-THF entering the tissue is trapped in this form due to ineffective methionine synthase activity and thus can not be polyglutamated for storage [see Section 1.4].

Other Factors Which May Cause Folate Deficiency

Increased Utilisation At times of increased cell proliferation, as in pregnancy and some forms of cancer, the folate requirement for DNA synthesis increases. Sequestration of folate by the placenta and embryo also represents a drain on maternal folate stores (Ek, 1980).

Drug Interaction The contraceptive pills is known to effect folate metabolism (Whitehead *et al.*, 1973; Durand *et al.*, 1997, Lewis *et al.*, 1998) possibly by effecting conjugase activity (Krumdieck *et al.*, 1974; Krumdieck *et al.*, 1975) while there is a mutual antagonism between folate and anticonvulsant drugs (Meynell, 1966; Hishida and Nau, 1998; Lewis *et al.*, 1998). Cyclosporine may also effect folate metabolism as it has been shown to positively correlate with homocysteine concentrations in post transplant subjects (Cole*et al.*, 1998). Chronic alcohol consumption causes altered folate metabolism (Weir *et al.*, 1985). Cigarette smokers have decreased red cell and serum folate concentrations relative to non smokers (Mansoor *et al.*, 1997, Lewis *et al.*, 1998). Antifolate drugs used in cancer therapy and as antibiotics can, by their very nature, alter folate co-enzyme distribution and co-enzyme flux through the one carbon cycle.

1.5.2. Classic Folate Deficiency

Chronology The earliest sign of possible folate deficiency is a decrease in serum folate concentrations (Herbert, 1987), resulting from a negative balance between the rate at which folate is absorbed and the rate at which it is taken up by the tissue, excreted or catabolised. Thus within 2-3 weeks of negative folate balance serum folate concentrations can fall to levels normally considered to be deficient (< 3 ng/ml). However such depleted concentrations of serum folate are only reflective of the folate balance at the time of sampling and need not be indicative of cellular folate deficiency, either current or impending. However, if folate utilisation continues to exceed folate absorption concentrations of both red cell and tissue folate would begin to fall.

A truer indicator of tissue folate concentrations is the red cell folate (RCF) concentration. During prolonged dietary inadequacy as tissue folate concentrations fall so also do RCF concentrations. Thus, RCF concentrations of less than 160 ng/ml are indicative of a underlying deficient in tissue folate reserves: while reserves are sufficient to satisfy current requirements, as determined by normal biochemical and clinical function, they may not be sufficient if folate utilisation increases, as during pregnancy, or if the folate inadequacy is prolonged.

It is only as RCF concentrations fall below approx. 120 ng/ml that the first biochemical ("subclinical") markers of folate deficiency become apparent, characterised by increased thymidine salvage and incorporation into the DNA, relative to *de novo* synthesis from deoxyuridine [See below]. As rapidly dividing cells are most sensitive to folate deficiency, signs of "subclinical deficiency" first appear in bone marrow cells, appearing soon afterwards in peripheral lymphocytes.

The final stage of folate deficiency, as RCF concentrations fall below 100 ng/ml, is associated with the development of megaloblastic anaemia and decreased hemoglobin levels. Folate deficiency can also cause neurodegenerative effects, but as folate concentrations are relatively high within the brain and cerebrospinal fluid (Herbert and Zalusky, 1961; Varela-Moreiras and Selhub, 1992), this neuropathy may only occur in the case of prolonged or severe folate deficiency.

Pathology

Deoxyuridine Suppression Under normal metabolic conditions the thymidine salvage pathway is suppressed by feed back inhibition of thymidine kinase by thymidine triphosphate (dTTP) (Pelliniemi and W. Beck, 1980; Ellims and Van der Weyden, 1981; Sasvári Székely *et al.*, 1985; Just *et al.*, 1975; Lee and Cheng, 1976). dTTP is therefore preferentially synthesised, *de novo*, from deoxyuridine monophosphate (dUMP), or its precursors (*i.e.* deoxyuridine (dU) [See Fig 1.3]. However at times of decreased TS activity, as during folate deficiency (Waxman *et al.*, 1969), the ratio of thymidine salvage to *de novo* synthesis increases as synthesis from dUMP is retarded, decreasing dTTP concentrations and reducing feedback inhibition of thymidine kinase. Thus "subclinical" folate deficiency, and consequently more severe folate deficiency, can be diagnosed by the inability of exogenous dU to suppress "salvage" of exogenous radioactive thymidine, resulting in increased incorporation of radiolabel into the DNA (Pelliniemi and W. Beck, 1980). This is the basis for the functional test of folate deficiency, the "dU suppression test" (Metz, 1984).

Megaloblastic Anaemia Cells in the G_0 and G_1 phases of cell growth contain only one set of DNA (2d), however during the S-phase of cell growth the nucleus enlarges as its undergoes DNA replication in preparation for mitosis (2d → 4d). The majority of cells in a

population are normally in the resting state (2d) with only a small percentage in the S-phase. However depletion of 5,10-CH_2-THF, as occurs during folate or B_{12} deficiency, cause a decrease in the rate of dTTP synthesis, retarding the rate of DNA replication and passage of cells through the S-phase. As entry into the S-phase is uninhibited the number of cells in the S-phase increases, resulting in a cell population that is apparently rapidly proliferating, but whose replication is actually retarded. The resulting megaloblastic anaemia (Greek: *megas* = large, *blaste* = germ) is characterised by a proliferation of enlarged cells. Cell enlargement is due to both hypersegmentation of the nucleii and cytoplasmic enlargement due to nuclear-cycoplasmic asynchrony (RNA synthesis continues unimpeded despite the slowing of DNA synthesis).

Complications of Pregnancy The first association between megaloblastic anaemia and nutritional deficiency was reported by Lucy Wills in 1931, this "pernicious anaemia of pregnancy" was as a result of women, whose folate status would normally be borderline or even "sub-clinically" deficient, becoming clinically folate deficient due to the increase in folate requirement in pregnancy.

Vollset *et al.* (2000) showed that risk of pre-eclampsia, prematurity and very low birth weight was greater in mothers with the highest quartile of tHcy relative to those in the lowest quartile and that there was a correlation between tHcy and still-birth and a significant association with development of NTDs. Elevated tHcy are also associated with increased risk of spontaneous abortion (Steegers-Theunissen *et al.*, 1992; Wouters *et al.*, 1993). Folate deficiency is associated with increased risk of low birth weight, due to retarded fetal growth and premature birth. A literature review by Scholl and Johnson (2000), showed that of 34 studies involving women, 24 showed that (i) there was an associated between low folate concentrations or high homocysteine concentrations (pre or

post methionine loading) and low birth weight, spontaneous abortion or premature birth, or (ii) that folate supplementation decreased the risk of these deleterious events occurring. Two of these studies reported adverse effects of folate supplementation. However, one of these studies showed no significant adverse effect when multiple pregnancies were excluded. The other study recorded a 1.8% increase in spontaneous abortions (from 11.2%) with supplementation. However, as the number of diagnosed pregnancies also increased (up 3.4% from 67.1%) this effect may have resulted from the survival, to detection, of zygotes which would normally have aborted pre-detection. The 7 remaining studies showed no significant effect of either intervention or nutritional status on outcome of pregnancy.

Neuropathy Neurodegenerative effects of folate deficiency, though less common than those of B_{12} deficiency, have been reported (Pincus *et al.*, 1972; Reynolds *et al.*, 1973; Manzoor and Runcie, 1976). The etiology is thought to be similar in both deficiencies: hypomethylation of the neural myelin sheath resulting in myelin instability. The rarity of neuropathy due to folate deficiency, relative to that of other diseases of folate deficiency, is thought to be due to preferential retention of folate within the brain and cerebrospinal fluid (Herbert and Zalusky, 1961; Varela-Moreiras and Selhub, 1992), perhaps in part due to an efficient blood-brain barrier. An outcome of folate deficiency in tissue is elongation of the polyglutamate chain (Cassady *et al.*, 1980; Varela-Moreiras and Selhub, 1992). Thus, while feeding rats a folate deficient diet causes a 20-25% elongation of the mean folate polyglutamate chain length in the liver, kidney and spleen, the increase in chain length in the brain was less than 6% (Varela-Moreiras and Selhub, 1992).

1.5.3 New Paradigm of Folate Deficiency

Cancer Several reports have linked deficient nutritional intake or status of folate and/or methionine with the onset or development of colorectal cancer (Giovannucci *et al.*, 1993, 1995 and 1998), pancreatic cancer (Stolzenberg-Solomon *et al.*, 1999), breast cancer (Zhang *et al.*, 1999; Rohan *et al.*, 2000), cancer in ulcerative colitis (Lashner, 1993) and Wilms tumors (Qu *et al.*, 1999) in humans, and liver cancer in rats (Pogribny *et al.*, 1997a and 1997b) and mice (Tao *et al.*, 2000). An association, while not significant, has also been shown between folate supplementation and protection against cancer associated with ulcerative colitis (Lashner *et al.*, 1989 and 1997). The etiology of carcinogenesis resulting from deficiency is likely to be multifactorial. However, understanding of at least two mechanisms, induction of DNA strand breaks and cytosine hypomethylation, is increasing.

DNA Strand Breaks Folate deficiency has been shown to induce DNA strand breaks both *in vivo* (Kim *et al.*, 1997; Blount *et al.*, 1997; Pogribny *et al.*, 1997b) and *in vitro* (Duthie and Hawdon, 1998), as well as delaying DNA repair (Choi *et al.*, 1998; Duthie and Hawdon, 1998). In humans a correlation has been observed between plasma homocysteine concentration and the severity of DNA strand breakage in older men (60.6±1.0 years) (Fenech *et al.*, 1997), and in the young men and women (25.5±0.6 years) with the most DNA damage (top 50%) (Fenech *et al.*, 1998). The rate of DNA strand breakage decreased in the young men and women when supplemented with folic acid and B_{12} (Fenech *et al.*, 1998).

One of the main causes of DNA instability is thought to be the aberrant incorporation of uracil into DNA. Uracil is a RNA base, however it may also appear in DNA due to deamination of the cytosine base pair *in situ*, at a rate of 100-500 uracil bases per day (data

derived from Lindahl, 1993), or misincorporation of dUTP, in place of dTTP, during DNA replication or repair. Where uracil mismatches do occur they are removed by uracil-DNA glycosylase which flips the uracil base from the DNA major groove and cleaves it from the deoxyribose backbone (Slupphaug *et al.*, 1996), the DNA backbone is then cleaved by apurinic/apyrimidinic (AP) endonuclease (Mol *et al.*, 1995) and replaced with the correct nucleotide by DNA polymerases (Lindahl, 1993). As the DNA polymerases are unable to distinguish dUTP from dTTP during DNA replication/ repair the cellular concentrations of dUTP are kept low by the action of dUTP phosphorylase to prevent misincorporation (Bertani *et al.*, 1963). However, under conditions of one-carbon deficiency the risk of dUTP misincorporation increases as the ratio dUTP : dTTP increases (James *et al.*, 1997), while the rate of cytosine deamination may be increased due to cytosine hypomethylation. The resulting uracil accumulation in the DNA is then compounded by defective excision repair (Duthie and Hawdon, 1998; Choi *et al.*, 1998; Duthie and McMillan, 1997). This increases the chance of two uracil excision repairs occurring within 12 base pairs of each other on opposing DNA strands, a distance shown to be critical in the formation of double strand breaks (Dianov *et al.*, 1991), partly accounting for the increased DNA instability and strand breakage observed (Duthie and Hawdon, 1998; Blount *et al.*, 1997; Pogribny *et al.*, 1997b).

From these results it would appear that induction of DNA instability by one-carbon deficiency is dependent on dUTP accumulation. However while dUTP accumulation probably plays a part, DNA hypomethylation alone can cause DNA instability, as is observed in genetic constructs lacking the DNA methyltransferase gene *Dnmt1* (Chen *et al.*, 1998).

Cytosine Hypomethylation: CpG islands are 0.5-5 kd 5'-CG-3' rich regions of DNA. In mammalian cells these regions are initiation sites for DNA replication (Delgado, 1998). Methylation of the CpG islands is involved in the epigenic regulation, *i.e.* inheritance of information on the basis of gene expression as opposed to inheritance on the basis of gene sequence (genetics), and differential regulation of gene transcription. Methylation of the CpG promoter regions of a gene can regulate transcription directly by inhibiting transcription factor binding (Tao *et al.*, 2000 Steiner *et al.*, 1982; Vardimon *et al.*, 1982; Wolf and Migeon, 1985) or indirectly by initiating chromatin formation (see below). In the case of transcription factor binding, the extent of transcriptional repression is an exponential function of CpG methylation density (Hsieh, 1994). However repression can be overcome by increasing promoter concentration. Regulation of CpG island methylation may play a part in regulating gene transcription and cell differentiation, as the extent of DNA methylation is tissue specific (Gama-Sosa *et al.*, 1983; Ehrlich *et al.*, 1982) and changes during tissue maturation (Gama-Sosa *et al.*, 1983) in mammalian cells.

One form of epigenic regulation of gene transcription (gene silencing, genomic imprinting) is stable chromatin formation. Chromatin formation is initiated by the binding of methyl-C binding proteins (MeCP1 and MeCP2) to the methylated CpG motif (Nan *et al.*, 1998; Cameron *et al.*, 1999, reviewed in Razin, 1998), this initiates recruitment of the transcription repression complex containing the corepressor protein, mSin3A, and the histone deacetylases HDAC1 and HDAC2 (Nan *et al.*, 1998; Cameron *et al.*, 1999), these two deacetylases stabilise the chromatin by deacetylating histones H3 and H4. During DNA replication methyltransferases (possibly Dnmt3a and Dnmt3b (Okano *et al.*, 1998)) methylate the nascent DNA strand within the replication fork (Gruenbaum *et al.*, 1983), this is thought to initiate chromatin activation, causing gene inactivation in the region (Razin, 1998). Chromatin formation has been cited as one possible locus of epigenics,

where the vast majority of the mammalian genomic sequence is silenced by recruitment of chromatin to the clonally inherited methylated CpG regions. Examples of epigenic gene regulation include genomic imprinting in autosomal genes inheritance, where one parental copy is silenced (Wuta *et al.*, 1997; reviewed by Bartolomei and Tilghman, 1997), and X-chromosome inactivation in mammals (reviewed by Heard *et al.*, 1997). The importance of chromatin in regulating gene transcription is apparent from the high degree of homology and the low level of genetic drift observed between H3 and H4 from pea seedling and calf thymus (DeLang *et al.*, 1969). The unit evolutionary periods for these two organisms, *i.e.* the time it takes for sequence homology to diverge by 1%, for H3 and H4 were calculated to be 300 and 600 million years, respectively.

CpG hypomethylation has been associated with a number of carcinoma types including Wilms tumors (Qu *et al.*, 1999) (typified by frequent chromosome 1 and chromosome 16 pericentromeric rearrangements), ovarian epithelial carcinoma (Qu *et al.*, 1999) and breast cancer (Bernardino *et al.*, 1997), while the pericentromeric rearrangements observed in the rare genetic disease ICF (immunodeficiency, centromeric heterochromatin instability, and facial anomalies) is thought to be due to the associated hypomethylation of chromosome 1 (Gi *et al.* 1997) as similar hypomethylation and pericentromeric rearrangement of chromosome 1 are observed with DNA methyltransferase inhibition.

Thus, nutritional one carbon deficiency can cause DNA hypomethylation and increasing risk of carcinogenisis. Mice on methyl-deficient (low methionine and betain) diets exhibit DNA hypomethylation and *h-ras*, *c-myc* and *c-fos* protooncogene activation (Henning and Swendseid 1996; Wainfan and Poirier 1992) while inhibition of the methylation cycle also activates gene expression (Chiang *et al.*, 1992). The carcinogens trichloroethylene, dichloroacetic acid and trichloroacetic acid cause hypomethylation of the CCGG promoter

region of *c-jun* and *c-myc*, up-regulating gene expression (Tao *et al.*, 2000), however concurrent methionine supplementation prevented demethylation, blocking mRNA and proteins expression.

The role of DNA methylation in cancer development is further complicated by the finding that regional hypermethylation in the CpG promoter region of several tumour suppressor genes can occur despite global DNA hypomethylation (Baylin *et al.*, 1995). Similar results have been demonstrated in folate / methyl-deficiency where hypermethylation of the p53 promoter region was observed in tumour cells (Pogribny *et al.*, 1997). This methylation of the gene sequence must have occurred *de novo* as the corresponding region in preneoplastic nodules was hypomethylated. Thus, a further etiology of tumour development may be selective (re)methylation of the promoter region of tumour suppressor genes, resulting in repressed transcription of the tumour suppressors. A review of the role of tumour suppresser gene inactivation in cancer epigenetics is given by Jones and Laird (1999).

Neural Tube Defects Neural tube defects (NTDs) are malformations of the spinal column resulting from the non-closure of the neural plate in the developing embryo. Incomplete closure of the spinal column results in spina bifida, the severity of which can vary, depending upon the size of the aperture left open and the degree of hydrocephalus, from clinically undetectable to paralysis of the lower limbs and bladder and mental retardation. In severe cases anencephaly occurs, whereby the cerebral hemispheres are malformed, a deformity inimical to life. The first report showing a prophylactic effect of folic acid supplementation in preventing neural tube defects was published in 1980 (Smithells *et al.*, 1980) and followed up in 1983 (Smithells *et al.*, 1983). However, as a requirement for attaining ethical approval for the study Smithells's group were required to supplement all

subjects presenting with folate deficiency (Editorial: Eur. J. Obstet. Gynecol. Reprod. Biol., 1999), as a consequence both studies were unable to use a randomised placebo group as controls and instead used the normal (non-recruited) hospital birth population for statistical comparison. Thus the link between supplementation and decreased risk was not satisfactorily proven. This proof awaited a stringent placebo control randomised study (MRC Vitamin Research Group, 1991) which showed that nearly three quarters of NTDs could be prevented by periconceptional folic acid supplementation. As a result of this finding the Irish Department of Health (Food Safety Advisory Board, 1997), the UK Department of Health (Expert Advisory Group, 1992) and the U.S. Public Health Service (Public Health Service, 1992) recommended that women who are pregnant, or are cosidering becoming pregnant, should take 400 μg folic acid per day to reduce the risk of neural tube defects.

Etiology The etiology of NTD has still not been determined, and thus the role folic acid supplementation plays in its prevention remains unclear. However the finding that folate supplementation can reduce the incidence of NTDs, that a folate enzyme polymorphic genotype is over-represented in the mothers of NTD affected children [see below] and that an elevation in tHcy is associated with an increases risk of NTD (Vollset *et al.*, 2000), suggests that aberrant folate metabolism, possibly resulting in a generalised one-carbon deficiency, may be a significant factor in the development of nearly 70% of NTD (MRC Vitamin Research Group, 1991).

One possible role of one-carbon deficiency in NTD development may be disruption of methyltransferase activity. This may retard synthesis of products, such as myelin and phospholipid, required for neural tube development. Alternatively NTDs may be caused by disruption to gene transcription, or by DNA instability and chromosomal rearrangement in

a manner similar to that in carcinogenesis. The suggestion that homocysteine may itself be teratogenic has arisen from the finding that it can induce neural tube and cardiovascular defects in avian embryos (Rosenquist *et al.*, 1996). However, it is not clear whether this was as a result of methyltransferases inhibition (DeCabo *et al.*, 1995) or some other physiological effect.

Of relevance to the etiology of NTDs was the finding that in the *splotch* mouse embryo model for NTD the homozygous mice displayed a greater requirement for exogenous thymidine than wild-type or heterozygous mice (Fleming and Copp, 1998), suggesting that thymidine synthesis was defective as both folic acid and thymidine were protective against NTDs *in vitro* and *in utero* and suppressed [^{3}H]-thymidine incorporation. Surprisingly, however, it was found that methionine supplementation increased [^{3}H]-thymidine salvage and caused NTDs in 47% of the heterozygous mice, mice which did not normally develop NTDs. These results suggest that NTD occurrence in *splotch* mice is due to thymidine deficiency, a perturbance that may be exacerbated by an altered folate distribution due to feedback inhibition of MTHFR by SAM. A further confounding factor in our understanding of how the one-carbon cycle may be involved in the ethiology of NTD is the fact that *sploch* mice are not mutated in genes related to folate metabolism, instead they are mutated in the *Pax3* gene. The *Pax3* transcription factor is thought to regulate the *N-CAM, N-cadherin, c-met, MyoD, Myf-5* and versican genes. The relevancy of the *sploch* model of NTD development to the human disease is not clear, but suggest that genetic causes of the disease may not be reserved solely to mutations of the one-carbon cycle.

Down Syndrome Down syndrome is a neurological and dysmorphic disorder resulting from abnormal chromosomal segregation during meiosis (meiotic nondisjunction), causing

the transfer of an extra copy of chromosome 21 (trisomy 21) and expression of 3 copies of the genes located on chromosome 21 (reviewed by Epstein, 1995).

As the nondisjunction event is maternal in about 95% of cases it is noteworthy to find that mothers of Down syndrome babies are metabolically compromised in their ability to metabolise folate (James *et al.*, 1999), having significantly higher plasma homocysteine concentrations and greater susceptibility to perturbations in folate metabolism. The higher homocysteine and lower methionine concentrations observed in mothers with Down syndrome mean they have a two fold higher homocysteine to methionine ratio in plasma than non-affected mothers. It is therefore possible that the pericentromeric rearrangements observed may have a similar etiology to carcinogenesis.

Other Birth Defects Mothers who took multivitamins, containing folic acid, for ≥1 month pre- and ≥ 2 months post conception had a reduced risk of delivering a child with limb defects and, when adjusted for confounding factors, a reduced risk of conotruncal heart defects (Shaw *et al.*, 1995). However, this association cannot separate the potential protective role of folic acid from that of the other vitamins in the multivitamin. The overall incidence of those congenital malformations classified as occurring in the first 5-6 weeks of gestation, *i.e.* NTD's, microcephalus, anomalies of the eye or ear, cleft lip, *etc.*, decreased significantly when mothers began supplementation before or within the first 5 weeks of pregnancy, relative to mothers who began supplementation after this period (Ulrich *et al.*, 1999)

Cardiovascular Disease The first report connecting elevated tHcy and risk of arteriosclerosis was published in 1969 (McCully, 1969). Since then numerous studies have been published showing an association between homocysteine concentration and increased

risk of cardiovascular disease (Graham *et al.*, 1997; Clarke *et al.*, 1991), including extracranial carotid-artery stenosis (Selhub *et al.*, 1995), ischemic heart disease (Wald *et al.*, 1998) and coronary artery disease (CAD) (Pancharuniti *et al.*, 1994). Meta-analysis of the results from 27 studies relating homocysteine to artheriosclerotic vascular disease (Boushey *et al.*, 1995) showed that tHcy was an independent graded risk and that the increase in risk of CAD due to a 5 μmol/l increase in tHcy was comparable to that from a 0.5 mmol/l increase in cholesterol. The overall conclusion of this review was that, assuming a causal link between CAD and tHcy, the fortification of food with folic acid had the potential to reduce deaths from CAD by 13,500 to 50,000 annually in the United States. A more recent review of the literature (Eikelboom *et al.*, 1999) has shown the risk from tHcy to be independent of the classic cardiovascular risk factors. However, they also highlight the lack of evidence to show a causal relationship between tHcy and cardiovascular risk. Studies looking at nutritional folate intake have shown an inverse association between folate intake and risk of carotid-artery stenosis (Selhub *et al.*, 1995), while men (Rimm *et al.*, 1996) and women (Rimm *et al.*, 1998) who consumed the least (by quintite) folate or vitamin B_6 were at greater risk from coronary heart disease than those in the highest quartiles and that women who took multi-vitamin supplements were at less risk from coronary heart disease than those who did not (Rimm *et al.*, 1998). While these three studies go some way towards proving a link we must await the results of randomised clinical trials for a definitive answer.

Other Disorders Associated With Folate Deficiency

Depression: A low folate (Fava *et al.*, 1997) or vitamin B_{12} (Pennix *et al.*, 2000) status has been associated with an increased risk of severe depression.

Alzheimer's Disease An inverse correlation was observed between the severity of atrophy of the neocortex and serum folate status in subjects with Alzheimer disease (Snowdon *et al.*, 2000).

Amnesia Methotrexate-induced folate deficiency has been shown to disrupt memory function, resulting in amnesia, in young chicks (Crowe and Ross, 1997).

Cataracts People in the lowest quintile of folate intake were shown to be more at risk of developing cataracts than those in the highest (Tavani *et al.*, 1996).

Hearing Loss An inverse correlation was observed between red cell folate and serum vitamin B_{12} concentrations and severity of age related auditory dysfunction in elderly women (60-71y) (Houston *et al.*, 1999). Likewise the incidence of serum B_{12} deficiency was higher in subjects with chronic tinnitus than in subjects with either noise-induced hearing loss or controls (Shemesh *et al.*, 1993).

1.5.4. Enzyme Polymorphism and Folate Requirement

Up to this point folate sufficiency has been discussed in relation to an empirical measure of folate concentration. However the discovery of an association between a maternal homozygous mutation (677C→T) of MTHFR and risk of having a child affected by NTDs has generated interest in gene-nutrition interactions, that is the role of enzyme polymorphism and nutritional requirement. In an Irish study group (case: n = 82; control: n = 99) mothers with children affected by NTDs had three times the prevalence of the 677TT mutation (18.3%) as a matched control group (6.1%) (Whitehead *et al.*, 1995). A similar result was found for mothers of Down syndrome (DS) children (James *et al.*, 1999)

where the 677TT variant in DS mothers (14%) was nearly twice that in the control mothers (8%). This result was not significant ($p < 0.1$) due to the small number of subjects, however, the combined mutant frequency (677CT + 677TT) was significantly ($p < 0.03$) higher in DS mothers (73.6%) than in control mothers (52%). The C677T mutation results in substitution of valine for alanine in the transcription product, resulting in 35% and 70% decreases in specific activity, respectively, for the heterozygous and homozygous enzymes, relative to wild type enzyme (Frosst *et al.*, 1995).

The possible involvement of other one-carbon utilising enzymes in the etiology of DS was suggested by the finding (James *et al.*, 1999) that DS mothers homozygous for the wild type (677CC) enzyme has twice the homocysteine to methionine ratio of control mothers ($p < 0.001$) while lymphocytes from DS mothers were more sensitive to methotrexate than control mothers, even when selected by 677CT genotype ($p < 0.02$). The suggestion that Down syndrome in 677CT mothers may be due to its combination with another genetic polymorphism(s) deserves attention both in the case DS and in the case of NTDs. One target for investigation might be another mutation of MTHFR, 1298A→C as heterozygotes for the combined mutation (A1298C and C677T) have lower MTHFR enzyme activity than subjects with the homozygous C677T variant. As an increasing number of genes involved in folate metabolism are cloned it is likely that further genetic-environmental interactions will be unmasked (Rosenblatt, 1999). Increased incidence of the homozygous C677T polymorph has also been reported in subjects with ulcerative colitis (17.5%) and Crohn's disease (16.8%) relative to control subjects (7.3%) (Mahmud *et al.*, 1999).

It is apparent that such obviously deleterious mutants such as the C677T MTHFR polymorphism must confer some evolutionary advantage to persist at such a high frequency. Two cases where MTHFR polymorphism may confer benefits are acute

lymphocytic leukemia (ALL) (Skibola *et al.*, 1999) and colorectal cancer (Chen *et al.*, 1996; Ma *et al.*, 1997). The risk of having ALL was reduced 4.3 fold (CI =1.2-16.7) in subjects homozygous for 677TT and 3.0 fold (CI = 1.4-6.7) for those with MTHFR 1298AC. When adjusted for age and smoking habits, C677T homozygous subjects had a 2-fold decrease in risk (OR, 0.50; 95% CI, 0.27-0.92) from colorectal cancer relative to subjects with the wild type or hetrozygous enzyme (Ma *et al.*, 1997).

One proposed mechanism whereby mutations of MTHFR may protect against ALL is by altering the distribution of folate co-enzymes *in vivo*. Skibola *et al.* (1999) proposed that the decrease in MTHFR activity due to the polymorphic mutation may alter the folate distribution *in vitro*, so that 5,10-CH_2-THF concentrations might be higher in the subjects with the homozygous or heterozygous mutation than subjects with the wild type enzyme. This theory is consistent with the finding that 30% of folate found in the red cells of homozygous 677TT subjects was found as 10-CHO-THF unlike the 100% found as 5-CH_3-THF in subjects with the wild genotype (677CC) (Bagley and Selhub, 1998). While not 5,10-CH_2-THF as predicted by Skibola *et al.* the 10-CHO-THF is readily converted to 5,10-CH_2-THF without dependence on MTHFR [Fig 1.2]. Furthermore, any 10-CHO-THF circulating in the plasma would be readily polyglutamated upon entry into the cell [Table 1.6], and thus would be retained by tissue without prior removal of the one-carbon unit.

The occurrence of the homozygous C677T mutation is lower in Irish and Dutch population than in most other populations assessed [Table 1.8], yet the Irish (Eurocat Working Group, 1991) and the Dutch (anecdotal evidence) have two of the highest reported incidence of NTDs. It would be interesting to speculate that other factors, genetic or environmental, may have outweighed the benefits of having the 677TT mutation, thus suppressing its prevalence, factors that are reflected in the high incidence of NTDs in these groups.

Nationality	Controls (n)	Controls[A] %TT
Irish	99[B]	6.1
	242[C]	8.3
	273[D]	7.3
Dutch	207[E]	4.8%
Italian	289[F]	16.3%
Australian	235[G]	10.7%
British	199[H]	12%
French Canadian	114[I]	12%
Japanese	674[J]	14.5%

Table 1.8: Distribution of the homozygous MTHFR 677TT genotype among several different nationalities. [A]Distribution of the TT genotype in the normal healthy population. (from [B]Whitehead *et al.*, 1995; [C]Molloy *et al.*, 199?; [D]Mahmud *et al.*, 1999; [E]van der Put *et al.*, 1995; [F]de Franchis *et al.*, 1995; [G]Wilcken and Wang, 1996; [H]Papapetrou *et al.*, 1996; [I]Frosst *et al.*, 1995; [J]Kimura *et al.*, 2000).

1.6. Biogenesis of Folate

While the folate biosynthetic pathway are similar in both plants and bacteria this review is confined mostly to the discussion of bacterial folate synthesis. The review will firstly deal with the independent synthesis of the pteridine and pABA moieties of folate before discussing their condensation and conversion to folate.

1.6.1. Pteridine Biosynthesis [Fig 1.6]

GTP Cyclohydrolase GTP cyclohydrolase is a homodecameric enzyme (Nar *et al.*, 1995), five diamer subunits forming a ring structure. The enzyme catalyses the conversion of GTP to formate, through the expulsion of the 8-carbon of the guanine ring (Vieira and Shaw, 1961; Levy, 1964; Reynolds and Brown, 1964; Shiota and Palumbo, 1965; Levenberg and Kaczmarek; 1966; Burg and Brown, 1966; Yim and Brown, 1976; Guroff and Strenkoski, 1966), and dihydroneopterin-triphosphate (DHNpt-Pi_3) (Burg and Brown, 1968; Yim and Brown, 1976;Bracher *et al.*, 1998; Howell and White, 1997; Cone *et al.*, 1974; Plowman *et al.*, 1974).

The enzyme has been purified to apparent homology from *E.coli* (Burg and Brown, 1968; Yim and Brown, 1976; Bracher *et al.*, 1999; Nar *et al.*, 1995) and *Comamonas Sp.* (Cone *et al.*, 1974), while two isoforms of the enzyme have been identified in *Lactobacillus plantarum* (Jackson and Shiota, 1971). This enzyme is the committal step in the synthesis of pteridines from purines in both plants and bacteria. While a structurally and functionally similar GTP cyclohydrolase enzyme is present in humans (Fischer *et al.*, 1997; Hasler and Niederweiser, 1986; Nagatsu *et al.*, 1986; Ichinose *et al.*, 1995), mice (Hasler and Niederweiser, 1986; Duch *et al.*, 1986; Ichinose *et al.*, 1995), rats (Hirayama and Kapatos,

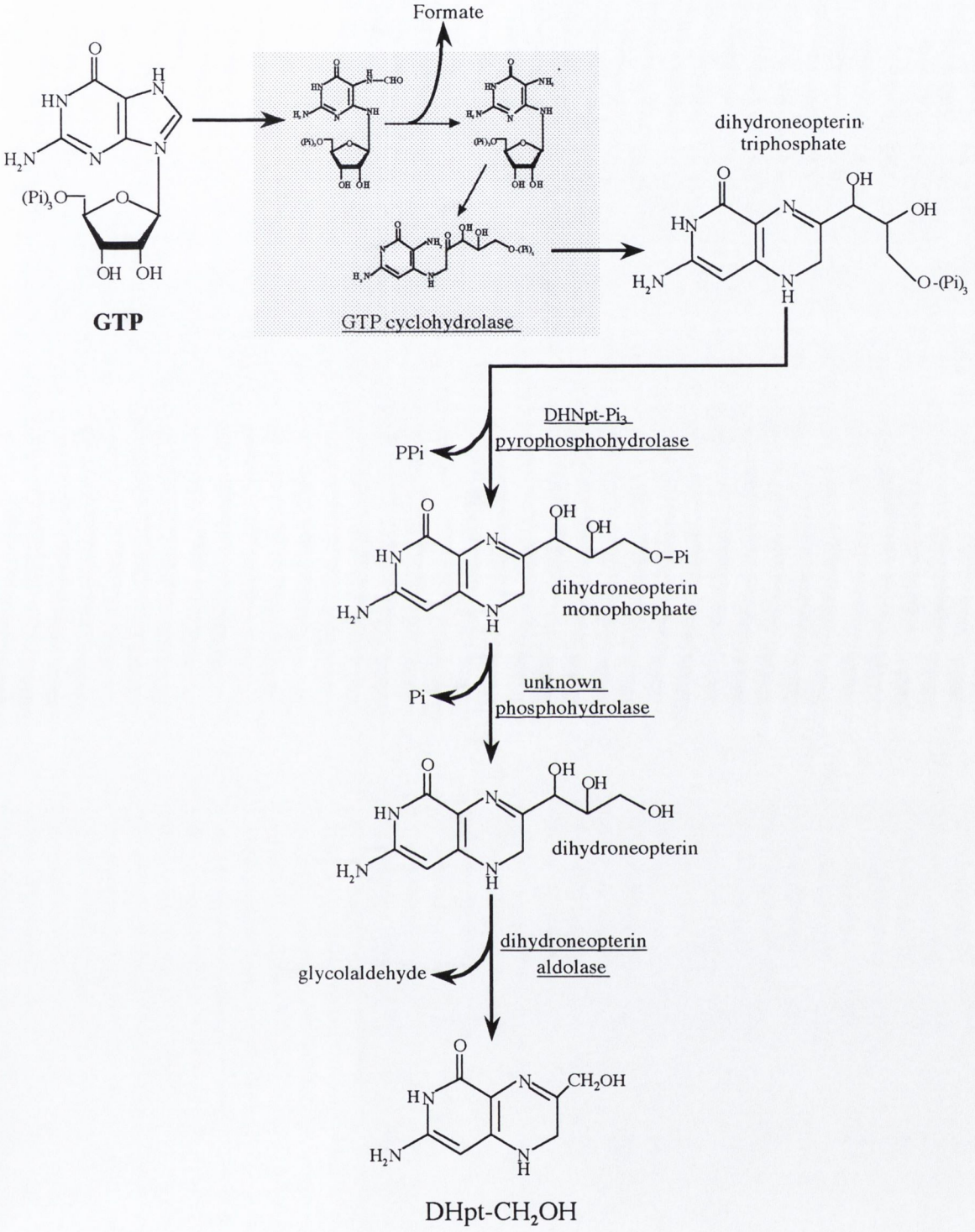


Fig 1.6. The biosynthetic pathway for 6-hydroxy-dihydropterin (DHpt-CH_2OH) from guanosine triphosphate (GTP).

1997; Hasler and Niederweiser, 1986; Duch *et al.*, 1986), chickens (Gütlich *et al.*, 1997; Hasler and Niederweiser, 1986) and other vertebrates (Hasler and Niederweiser, 1986), these higher organisms are unable to synthesise folate (Kishore and Shah, 1988; Haslam, 1993), instead it acts as the committal step in the biosynthesis (Hasler and Niederweiser, 1986) of other functional pteridines such as biopterin (Dhondt, 1986).

Dephosphorylation of Dihydroneopterin-Triphosphate DHNpt-Pi_3 dephosphorylation occurs in two steps: The β-γ-phosphate groups of DHNpt-Pi_3 are first cleaved by dihydroneopterin-triphosphate pyrophosphohydrolase (Suzuki and Brown, 1974), the α-phosphate is then removed by an unidentified phosphatase. The dephosphorylate end product, dihydroneopterin (DHNpt), is a better substrate for folate synthesis than DHNpt-Pi_3, whose incorporation into folate could be inhibited by the phosphatase inhibitor arsenate (Burg and Brown, 1968).

Cleavage of the Dihydroneopterin Side-Chain It has been long known (Shiota *et al.*, 1964; Shiota, 1959; Brown *et al.*, 1961; Shiota and Disraely, 1961; Weisman and Brown, 1964; Reynolds and Brown, 1964) that the pteridine precursors of folate are one-carbon substituents of carbon-6, and not three-carbon substituents like DHNpt, so it was assumed that the dihydroxypropyl chain must be cleaved. The enzyme that cleaves the C1'-C2' bond of DHNpt, dihydroneopterin aldolase, has been identified (Jones and Brown, 1967), purified (Mathis and Brown, 1970; Haußmann *et al.*, 1998) and over-expressed (Haußmann *et al.*, 1997) in *E.coli*. The products of the enzyme are glycolaldehyde (Mathis and Brown, 1970; Brown, 1970) and 6-hydroxymethyl-dihydropterin (DHpt-CH_2OH).

1.6.2. *para*-Aminobenzoic Acid Biosynthesis [Fig 1.7]

Biosynthesis of Shikimic Acid from Glucose The initiating reaction (Srinivansan *et al.*, 1955) in the biosynthesis of shikimic acid is the condensation of erythrose-4-phosphate (E-4-P) with phosphoenolpyruvate (PEP) to produce 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) (Srinivansan *et al.*, 1963; Srinivansan *et al.*, 1959; Srinivansan and Sprinson, 1959; DeLeo *et al.*, 1973; Simpson and Davidson, 1976; Schoner and Herrman, 1976) which is then dephosphorylated by 3-dehydroquinate synthase to produce 3-dehydroquinate (DHQ) and free phosphate (Srinivansan *et al.*, 1963; Sprinson *et al.*, 1962; Maitra and Sprinson, 1978; Berlyn and Giles, 1969; Mitsuhashi and Davis, 1954). The DHQ is dehydrated by 3-dehydroquinate dehydratase to produce dehydroshikimate (DHS), introducing the first double bond into the aromatic ring. The final step in the synthesis of shikimic acid is the NADPH-dependent reduction of dehydroshikimate to shikimic acid (Berlyn and Giles, 1969;Chaudhuri and Coggins, 1985; Dansette and Azerad, 1974), however as the initial catalytic studies were conducted in the reverse direction the enzyme was named shikimate dehydrogenase (Yaniv and Gilvarg, 1955).

Conversion of Shikimic Acid to Chorismic Acid Shikimic acid is first phosphorylated by shikimic acid kinase and the resulting shikimate-5-phosphate (Fewster, 1962) condensed with phosphoenolpyruvate to produce 3-enolpyruvateshikimate-5-phosphate (ESP) (Srinivansan and Rivera, 1963; Rivera and Srinivansan, 1963; Levin and Sprinson, 1964; Du *et al.*, 2000; Majumder *et al.*, 1995; Anderson and Johnson, 1990; Huynh *et al.*, 1988) and free phosphate (Levin and Sprinson, 1964), with glutamine providing one possible source of enolpyruvate (Rivera and Srinivansan, 1963). The ESP is then dephosphorylated by 3-enolpyruvateshikimate-5-phosphate phosphatase, producing phosphate and 3-enolpyruvateshikimate (Levin and Sprinson, 1964). This latter product has been named

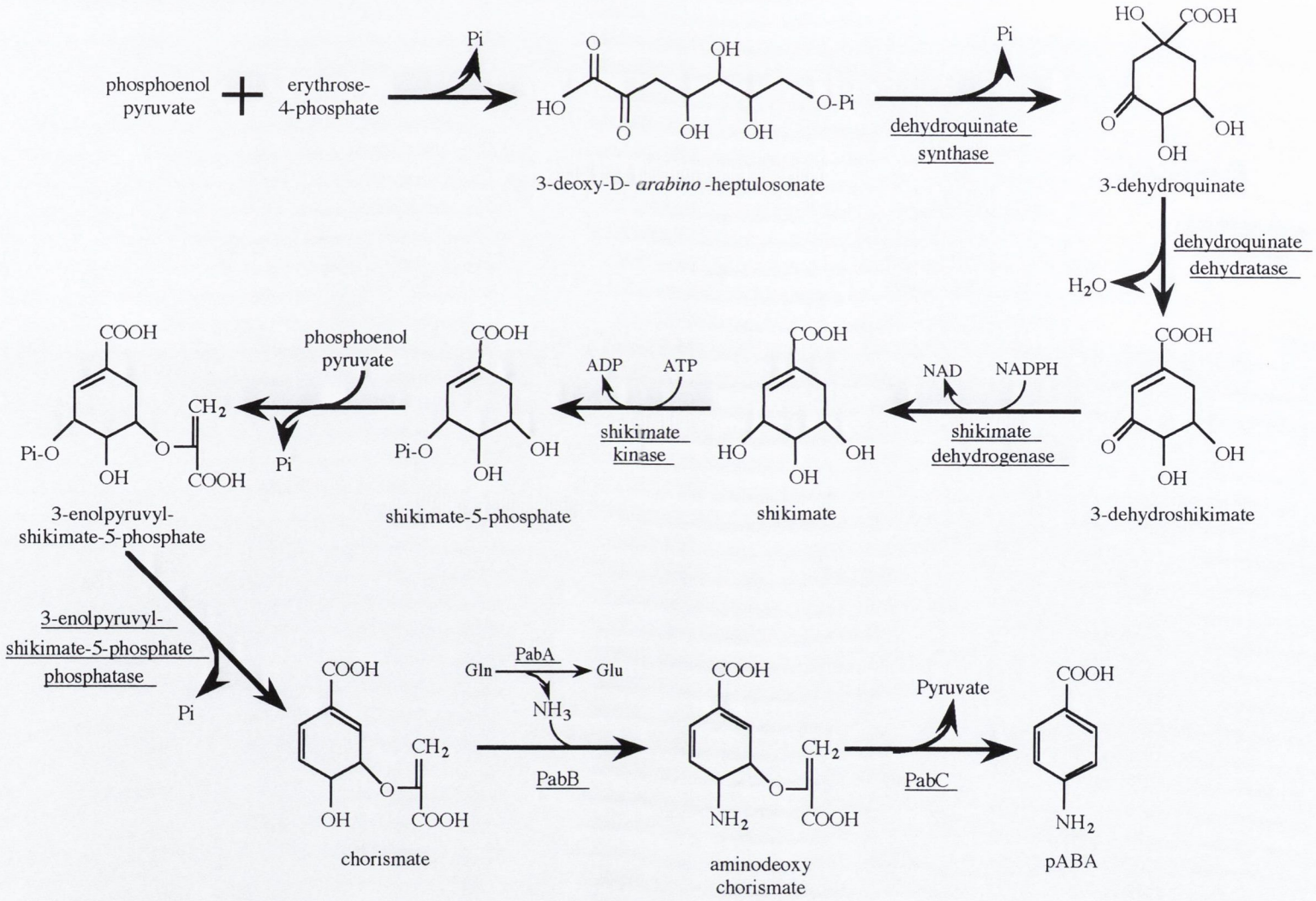


Fig 1.7. The *para*-aminobenzoic acid (pABA) biosynthetic pathway.

chorismic acid (Gibson and Gibson, 1964) as it occupies the branching point [*chorismic* = separating] in the biosynthesis pathway of several aromatic compounds [Fig 1.8].

Conversion of Chorismic Acid to pABA It was originally thought that a single bi-enzyme complex (PabA/PabB) was sufficient for the conversion of chorismic acid to pABA. This erroneous conclusion may have been reached because of the similarity in molecular weight (≈ 51kDa) between PabB and the PabC homodiamer (Ye *et al.*, 1990): apparently homogeneous preparations of PabB may have been contaminated with PabC. While the PabB (aminodeoxychorismate synthase) was shown to use ammonia in the synthesis of aminodeoxychorismate (ADC) the PabA/PabB complex exhibits increased activity when glutamine is used as an amino-source. This increase in activity may be due to a number of reasons: (i) increased amino-group availability due to the glutamase function of PabA (Ye *et al.*, 1990; Viswanathan *et al.*, 1995), (ii) stabilisation of PabB due to formation of the PabA/PabB complex (Viswanathan *et al.*, 1995), (iii) PabA may channel free ammonia in solution to PabB (Viswanathan *et al.*, 1995). Kinetic characteristics of the enzyme complex suggest an ordered Bi-Bi mechanism, with chorismate binding prior to glutamine (Viswanathan *et al.*, 1995).

ADC is converted to pABA by PabC (aminodeoxychorismate lyase) with the exclusion of pyruvate and aromatizaton of the benzene ring (Nichols *et al.*, 1989; Green and Nichols, 1991; Green *et al.*, 1992). The functionally similar anthranilate synthase enzyme (TrpE) aminates chorismate (Knöchel *et al.*, 1999; Poulsen *et al.*, 1993; Morollo *et al.*, 1993) but, unlike PabB, aromatizes the benzene ring via pyruvate elimination prior to release. The rationale behind the free diffusion of ADC from the PabA/PabB complex is unclear. One possible reason is that ADC may be a biosynthetic precursor in multiple pathways, *i.e.* synthesise of chloramphenicol (Teng *et al.*, 1985).

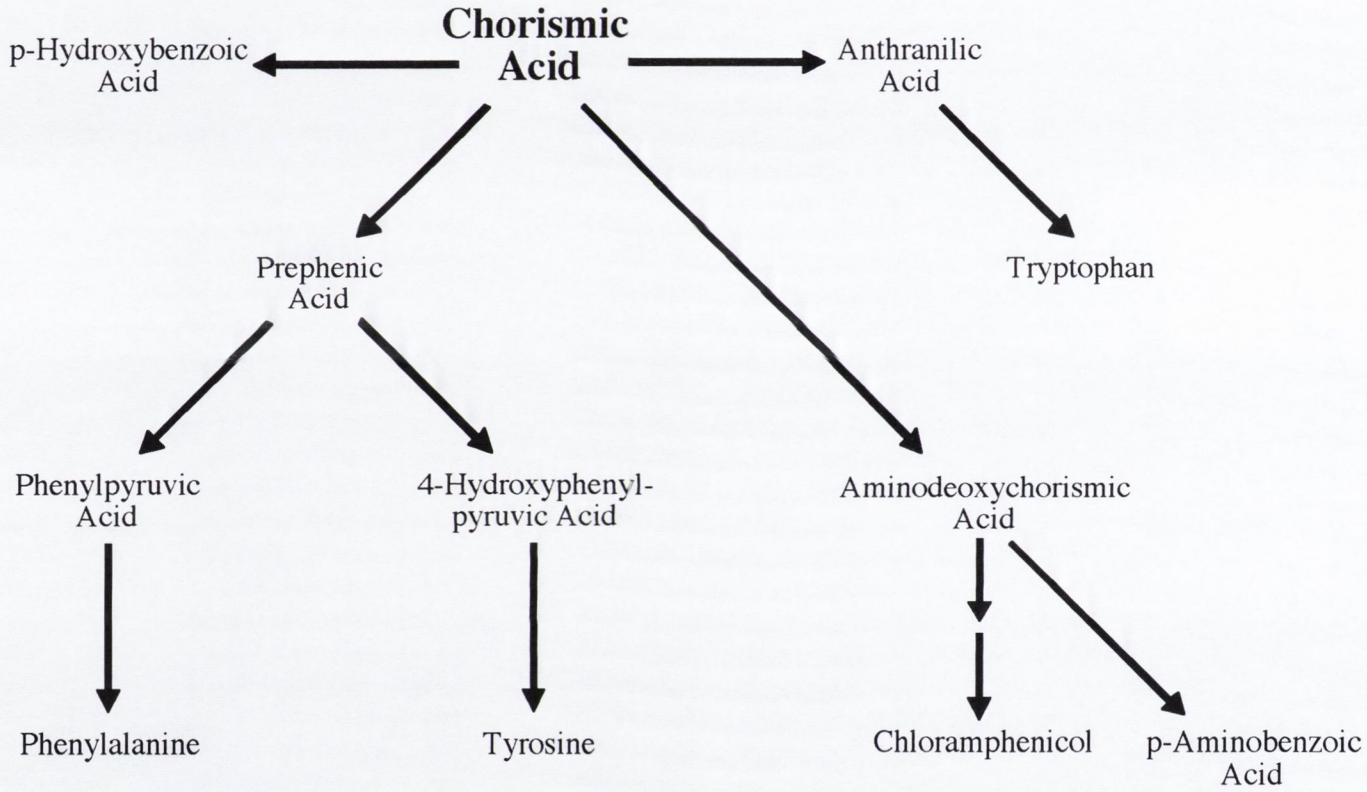


Fig 1.8. The biosynthesis of aromatic products from chorismic acid (from Gibson and Gibson, 1964; Gibson, 1964).

1.6.3. Dihydrofolate Biosynthesis from *para*-Aminobenzoic Acid and 6-Hydroxymethyl-Dihydropteridine [Fig 1.9]

Phosphorylation of the Hydroxymethyl-Dihydropterin A phosphorylated intermediary in the condensation of DHpt-CH_2OH with pABA/ pABGlu was suggested by the finding that dihydropterin synthesis had an absolute requirement for ATP (Shiota *et al.*, 1964; Shiota, 1959; Brown *et al.*, 1961; Shiota and Disraely, 1961; Weisman and Brown, 1964; Reynolds and Brown, 1964). Synthetic pyrophosphoryl derivatives of DHpt-CH_2OH were substrates for folate synthesis (Shiota *et al.*, 1964; Weisman and Brown, 1964) and only the DHpt-CH_2OH-utilising fraction of *E.coli* extracts required ATP (Weisman and Brown, 1964). Formation of the pyrophosphate ester is thought to occur through a single step, rather than through sequential phosphorylation, as the phosphate ester is inactive even in the presence of ATP (Shiota *et al.*, 1964; Weisman and Brown, 1964) while the reaction products have been identified as DHpt-CH_2O-PPi and AMP (Richey and Brown, 1969).

Synthesis of Dihydropterin 7,8-dihydropterin synthase catalyses the ordered binding of DHpt-CH_2O-PPi and pABA and the subsequent release of pyrophosphate and dihydropteroate (Ferone and Webb, 1975). The reaction products, pyrophosphate and dihydropteroate, have been identified by directs product analysis (Richey and Brown, 1969) and by the finding that PPi, but not Pi, inhibited the reaction (Ortiz and Hotchkiss, 1966; Ferone and Webb, 1975). The enzyme is also inhibited, in *S.aureus*, by pharmacological concentrations of pABA (Hampele *et al.*, 1997), suggesting that the homodimeric enzyme normally operates a half site reactivity with binding of a second pABA molecule resulting in complex inactivation.

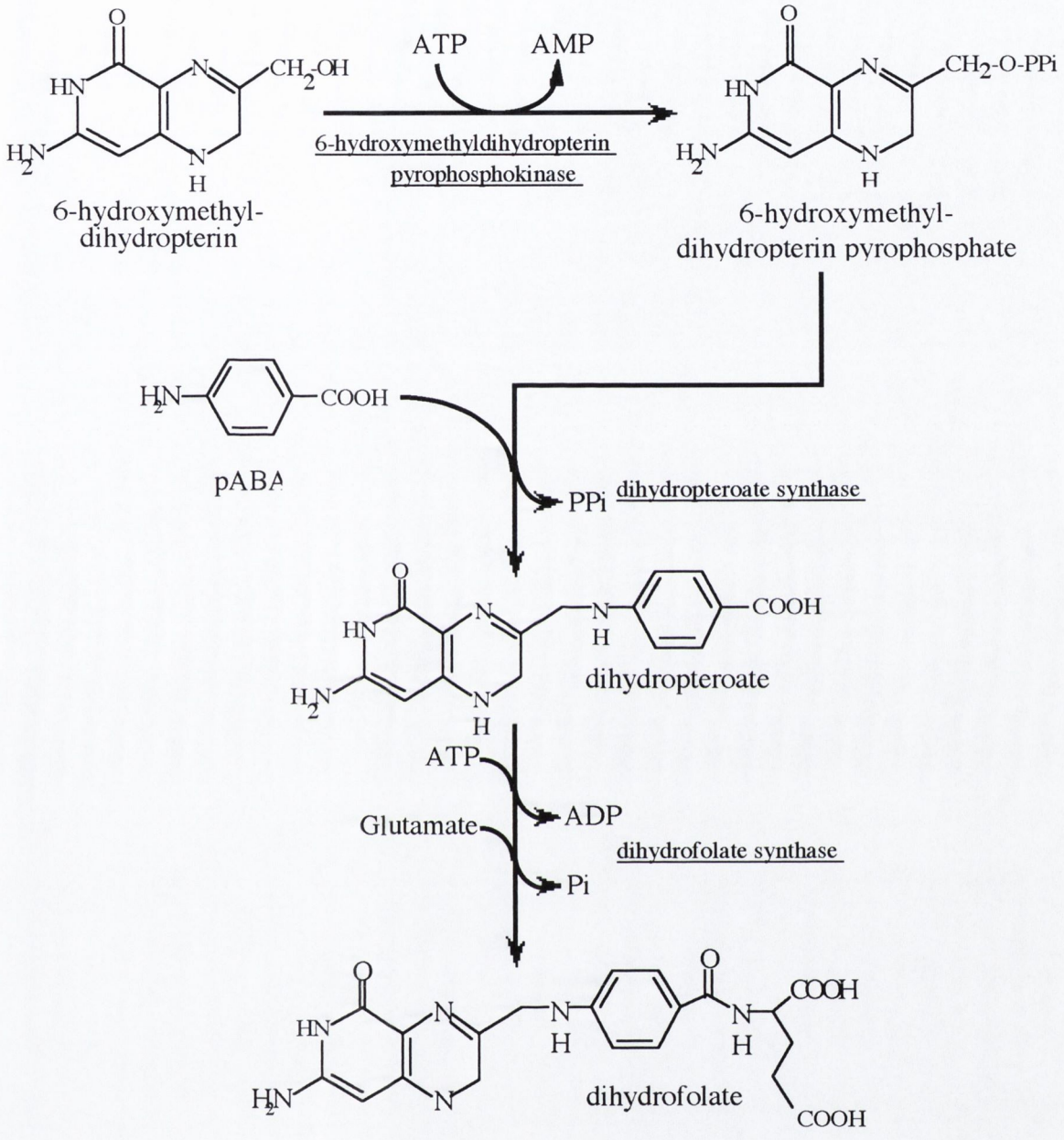


Fig 1.9. Biosynthesis of dihydrofolate from 6-hydroxymethyl-dihydropterin and *para*-aminobenzoic acid (pABA).

Aminobenzoic Substrate Specificity for Dihydropterin Synthase As both pABA and pABGlu are both substrates for DHPS, forming 7,8-Dihydropterin and 7,8-Dihydrofolate respectively, debate arose over which is the natural substrate for the enzyme: The identity of pABA as the physiological substrate was suggested by the kinetic data, as pABA has lower K_M values than pABGlu (Lampen and Jones, 1946; Shiota, 1959; Shiota and Disraely, 1961; Reynolds and Brown, 1964;Swedberg *et al.*, 1979; Hampele *et al.*, 1997; Roland *et al.*, 1979; Talarico *et al.*, 1991; Ortiz and Hotchkiss, 1966; Ortiz 1970). Apart from pABGlu being a product of folate catabolism, no mechanism for pABGlu synthesis has been identified.

Synthesis of Dihydrofolate from Dihydropterin Dihydrofolate is synthesised from dihydropterin by its condensation with glutamate. As with many substrates in the synthesis of folate a phosphorylated intermediary is predicted by a requirement for ATP in the conversion of dihydropterin to DHF (Griffin and Brown, 1964; Brown *et al.*, 1961). However, in this case no phosphorylated pterin intermediary is released as condensation of the phospho-ester with glutamate is catalysed by the same enzyme, dihydrofolate synthase (DHFS). Moreover, the phosphorylated intermediary of this enzyme is probably a monophosphate and not a pyrophosphate. This is apparent from the ability of ADT to substitute for ATP (Griffin and Brown, 1964) and from DHFS's apparent mechanistic homology to folate polyglutamate synthase (FPGS) and by the identification of ADP and phosphate as reaction products (Iwai and Kobashi. 1975). By inhibiting dihydrofolate reductase the product of the reaction was shown to be DHF (Brown *et al.*, 1961).

Conflicting Evidence for a Single, Bifunctional, Dihydrofolate Synthase / Folate Polyglutamate Synthase Semi-purified extracts from *E.coli* exhibit both DHFS and FPGS activity (Griffin and Brown, 1964) while apparently homologous FPGS from *E.coli*

(Ferone *et al.*, 1983; Ferone and Warskow, 1983) or *Corynebacterium* (Shane, 1980a & 1980b) also exhibits DHFS activity. Over expression of the folC gene in *E.coli* (Bogner *et al.*, 1985) resulted in 400-fold amplification of an enzyme which, when purified to homology, exhibited both DHF synthase and folate polyglutamate synthase activity. However *Saccharomyces cerevisiae* appears to have distinct DHFS and FPGS enzymes encoded by separate genes, *fol3* and *fol7*. Knock-out mutants of *fol3* exhibiting no DHFS activity while the FPGS activity appears to be coded for by *fol7* (Cherest *et al.*, 2000).

1.7. Food Fortification and Supplementation:

While the role of folate supplementation has long been recognised in the prevention of "pernicious anaemia of pregnancy" (Wills, 1931) and other diseases of outward nutritional deficiency (Wills and Stewart, 1935) it is only recently that a wider possible role in disease prevention has been recognised [see previous section]. While folic acid fortification has been employed for a number of years, it has been on a haphazard basis, with some foods being fortified while others have not, even within the same food type, *i.e.* some breads but not others. In 1992 the U.S. Public Health Service (Public Health Service, 1992) recommended that, in order to reduce the occurrence of NTDs, women of childbearing years should consume 400μg folic acid per day. To partly achieve this goal the U.S. Food and Drug Administration (FDA) issued regulations requiring the fortification of all grain products as a level of 140μg/100g (Food and Drug Administration, 1996), a value estimated to increase average folate consumption by 100μg/day. This decision was probably influenced by the lack of awareness in the general population of the benefits of increasing folate consumption and by the finding that most ($\approx$80%) pregnant women did not take folic acid supplements preconceptually (Wild, 1996; Roberts, 1996; Kloeblen,

1999; Floyd *et al.*, 2000). Early indicators have shown that the current level of fortification has increased folate intake by 85μg/day in children ($\leq$ 9 yrs of age) (Food Surveys Research Group, 1998). Values for adult have not yet been reported, though the number of subjects with serum deficiency (< 3ng/ml) in one sample group has decreased from 22% to less than 2% (Jacques *et al.*, 1999). There have already been calls for fortification level to be increased (Oakley, 1997 & 1999).

1.7.1. Use of Folic Acid

Historically folate fortification and supplementation has been in the form of folic acid. The reasons for choosing this form of the vitamin were probably its greater stability, ease of manufacture and the fact that there are no substituent groups on the pteridine moiety to introduce chirality. However the consequences of this choice are still not clear.

Cost Benefit Analysis of Folic Acid Use I. Benefit: While studies have shown folic acid to have a higher bio-availability than food folate (Cuskelly *et al.*, 1996) this is probably due to the polyglutamate nature of food folate. Most folate monoglutamates appear to have similar, if not greater, bio-availability to folic acid (Brown *et al.*, 1973; Perry and Chanarin, 1970). However, the biochemical characteristics of folic acid may make it particularly appropriate for use in fortification: it is more stable than the reduced folates; it is a monoglutamate, making it highly bio-available; it is transported independently of folate, therefore its transport is not inhibited by factors which inhibit folate uptake (Niethammer and Jackson, 1975; Huennekens and Henderson; 1975 Rader *et al.*, 1974); at current fortification levels in the United States a proportion of the folic acid enters the blood unaltered (S.W. Bailey, personal communication), it therefore does not require demethylation prior to polyglutamation and thus avoids the "methyl-trap" [see Section

1.4.2]. Another form of "methyl-trap" may be decreased enzyme activity of the homozygous or heterozygous C677T MTHFR polymorphs (Frosst *et al.*, 1995). This may, in part, explain the observation that pregnant women homozygous for the C677T genotype had lower red cell and plasma folate (Molloy *et al.*, 1997) and why homozygous men (40-84 yrs) had lower plasma folate (Ma *et al.*, 1997), than heterozygous or wild type subjects.

Cost Benefit Analysis of Folic Acid Use II. Cost: While one of the benefits of using folic acid supplementation is its ability to contravene the "methyl-trap" this has been one of the reasons most often cited why folic acid should not be used in fortification: one of the first clinical indicators of B_{12} deficiency is megalobastic anaemia, caused by a concurrent decrease in folate concentration (Gutstein *et al.*, 1973; Perry *et al.*, 1976). However, as folic acid can enter, and be retained, by the cell in a vitamin B_{12} independent manner it could potentially prevent folate deficiency, thus masking the underlying vitamin B_{12} deficiency (Lachance, 1998). The undiagnosed vitamin B_{12} deficiency may then progress to a stage where irreparable neurological damage may occur. Other concerns about the use of folic acid supplementation are the unknown effects it may have on tumour growth and the antagonistic effects it may have on anticonvulsants and the antifolates used in cancer and antibacterial treatment. Concern about folic acid supplementation in the treatment of cancer may be unfounded as a recent study by Branda *et al.* (1998) found that, while tumour growth was greater in folic acid supplemented rats than in folate deficient rats, supplementation had no significant effect on tumour growth in replete rats. Furthermore, post-chemotherapy survival was found to be higher in the supplemented rats than in folate deficient rats, while the anti-tumour drugs were apparently more effective in replete or supplemented rats compared to deficient rats.

Of further concern are possible teragenic effects unmetabolised folic acid may have on the body. Fortification doses as low as 200μg have been shown to cause the accumulation of unmetabolised folic acid in serum (Kelly *et al.*, 1997; Sweeney, 2000) while the current fortification regime used in the United States has been shown to causes the appearance of folic acid in plasma (S.W. Bailey, personal communication). Furthermore, amounts as low as 100μg, taken within an hour of each other, can cause accumulation of folic acid in the serum (Sweeney, 2000).

Achón *et al.* demonstrated that high dose (40 mg/kg body weight) folic acid supplementation decreased the birth weight and size of rats (Achón *et al.*, 1999; Achón *et al.*, 2000), while also effecting protein metabolism in pregnant and non-pregnant rats (Achón *et al.*, 1999). While it has not been demonstrated that these effects are deleterious, they still represent an effect of high dose supplementation. Although such large doses might appear excessive, at 20 times the folate requirement of rats, they are comparable to the level recommended by the CDC for the prevention of recurrent NTDs (Center for Disease Control and Prevention, 1991) and may be comparable to the potential exposure of high consumers of fortified food. Current fortification levels are estimated to increase mean folic acid consumption by 100μg/day while ensuring the majority (95%) of the people consume less than 1mg total folate per day; thus up to 5% of the U.S. population could be exposed to greater than 1mg/day folic acid. The implementation of a five fold increase in fortification (Oakley, 1997) could result in a large minority of people being exposed to folic acid concentrations greater than 5 mg/day. At present children of nine years and younger have a mean exposure of 4.3 times the RDA for folate [Table 1.9] while three year olds are exposed to 5.7 times the RDA. A five fold increase in supplementation would thus result in under 9y olds being exposure to 8.9 times the RDA and three year olds being exposed to 12 times the RDA. As these are mean intake values some children

Age Group (yrs of age)	Consumption Fortified Mean (%RDA)[A]	Increased intake Fortified Mean (%RDA)[B]	Increased intake Fortified x 5 Mean (%RDA)[C]	Consumption Fortified x 5 Mean (%RDA)[D]
1-2	242 (485)	64 (131)	320 (655)	497 (1009)
3	286 (572)	79 (158)	395 (790)	602 (1204)
3-5	311 (479)	96 (147)	480 (735)	695 (1067)
6-9	343 (370)	94 (100)	470 (500)	719 (770)
≤ 9	299 (431)	85 (115)	425 (575)	639 (891)

Table 1.9: [A]Mean folate consumption of U.S. children after the introduction of food fortification and [B]mean increase in folate intake upon introduction of fortification (adapted from Food Surveys Research Group, 1998). Calculated [C]increase in folate intake and [D]total folate intake if folate fortification were to increase five fold. %RDA: Increase in folate intake or total folate intake calculated as a percent of RDA for each age group.

would be exposed far higher amounts than these. The majority of this exposure would be in the form of folic acid.

Cost Benefit Analysis of Folic Acid Use III. Conclusions People can not easily "opt out" of such wide scale food fortification. Thus a balance must be struck between providing the maximum benefit to the majority while minimising risk to the minority. However, as knowledge about folic acid increases it may then be decided whether it is safe to increase fortification levels. However, attempts at increasing folate consumption by potential mothers will always be confounded by the finding that this group consume the least fortified food [Fig 1.10].

1.8. Research Aims:

The aims of the thesis were as follows:

Part I. Human Studies

- To determine the rate of folate loss in humans due to the urinary excretion of intact folate and of its catabolic products. As this is the main route by which folate is loss from the body, this data could then be used as a basis for determining an RDA value for humans.

- To examine the effect of the combined oral contraceptive pill (COCP) on the rate of folate excretion and catabolism.

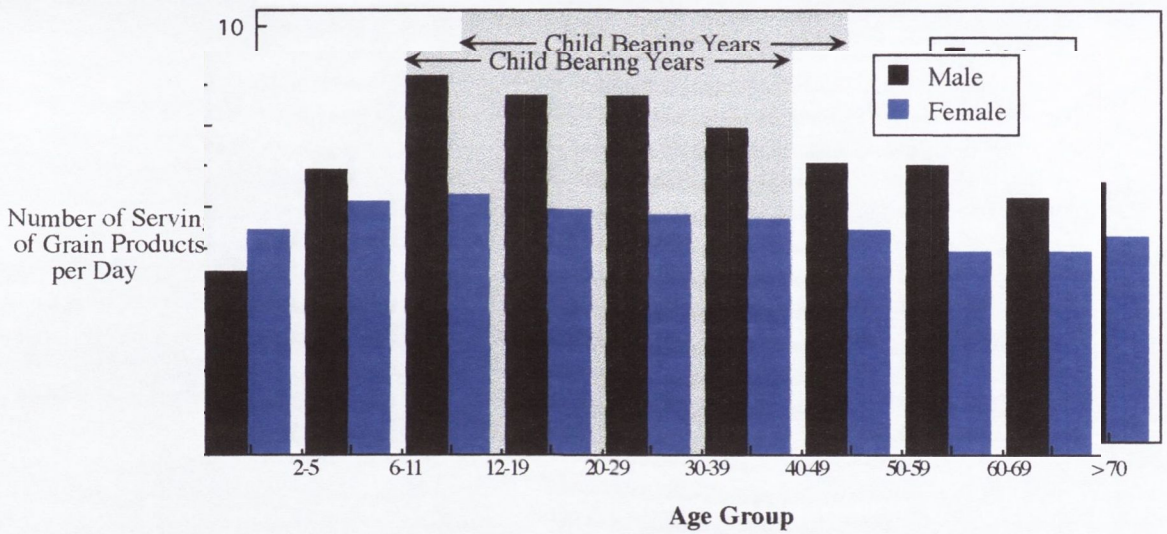


Fig 1.10: Average consumption of grain products in the United States by age group between 1994-6. One serving had a grain content equivalent to approximately one standard slice of bread. These are the foods which are currently being fortified in the U.S. Adapted from (Food Survey Research Group, 1999)

• To examine the relationship between folate status and tHcy, and the effects of folic acid supplementation on this relationship. As the one carbon cycle is dependent on B_{12} for the transfer of methyl-groups to homocysteine, we wished to extend this analysis to also look at the effect of B_{12} status on tHcy.

• To expand upon the results of an earlier pilot study conducted by this lab (McPartlin *et al.*, 1993) which showed that folate catabolism increased during pregnancy. To quantify the extent of this increase, thereby demonstrating the cause of increased folate requirement during pregnancy, and to quantify an empirical value for the increase.

• To confirm earlier findings (Andersson *et al.*, 1992 and Kang *et al.*, 1986) that tHcy decreases during pregnancy. To examine the effect folate and B12 status may have on tHcy under these circumstances.

Part II. Bacteria and Cell Cultures Studies

• To design an *in vivo* bacterial model for examining the effect of different classes of anti-folate drugs on folate co-enzyme distribution, thereby deriving a better understanding of how these drugs work and interact synergistically or antagonistically. Furthermore, by determining the effects of SMX and pABA on the folate distribution, and comparing the results to those of a known DHFR inhibitor, we wished to test the hypothesis that both these compounds are DHFR inhibitors.

• To design a mammalian cell line model for examining the effect of the anti-folate drug MTX on folate distribution.

CHAPTER 2

METHODS

PART I: STUDIES IN HUMANS

2.1. Human Subjects and Sample Collection

Ethics Subjects gave written informed consent. The female studies were approved by the Rotunda Hospital Ethics Committee, Rotunda Hospital, Dublin, Ireland. Ethical approval was not required for the male study due to its non-invasive (collection of urine) nature.

Subjects and Sample Collection Healthy men (n = 23) and women (n = 28) were recruited. Specific exclusion criteria for the study included a history of anaemia, inflammatory bowel disease, coeliac disease, epilepsy or renal disease. Women with a personal or family history of neural tube defect affected children were excluded. Women (n = 4) taking the combined oral contraceptive pill (COCP) were also excluded from the main study group. Subjects were placed on a nutritionally-complete, folate catabolite free, liquid enteral feed (Fortisip® , Cow & Gate) for 16hrs before, and during, sample collection to ensure that the folate catabolites found in the urine were derived from endogenous catabolism and not from catabolites in their diet. From 09.00 hours on day two to 09.00 hours on day three total urinary output from each subject was collected in a five-litre plastic containers containing two grams of ascorbic acid. Blood was collected from the women on the morning of day two. After sample collection the women were commenced on

iron/multivitamin supplementation (Givitol®, Galen Pharmaceutical Limited, ferrous fumarate 305mg, folic acid 500 μg, riboflavin 2mg, pyridoxine hydrochloride 4mg, thiamine mononitrate 2mg, nicotinamide 10mg and ascorbic acid) and sample collection was repeated four months later.

Pregnant Subjects Healthy gravidae (n = 31) were recruited from the Rotunda Hospital to undergo measurement of the daily rate of folate catabolism on three occasions during pregnancy (12-16 weeks, 26-30 weeks and 34+ weeks) and on day three postpartum. Specific exclusion criteria for the study included a history of anaemia, inflammatory bowel disease, coeliac disease, epilepsy or renal disease, as was a personal or family history of neural tube defect affected children. The women were admitted in small groups to a specially supervised metabolic ward for a period of approximately 40 hours. On arrival on the ward, at 17.00 hours on day one, subjects were placed on Fortisip®. After the initial sixteen hours "wash-out" period a 24-hour urine collection was performed between 09.00 hours on day two and 09.00 hours on day three. Samples were collected at first trimester (12-16 weeks), second trimester (26-30 weeks), third trimester (34+ weeks) and on day three postpartum. As part of the Rotunda Hospital's policy to combat the occurrence of NTD's, and consequently a requirement for attaining ethical approval, subjects were prescribed Givitol®, containing 500μg folic acid, upon presentation at their first antinatal clinic, in all cases this corresponded to the start of the first collection period (first trimester). On each admission blood samples were taken on the morning of day two for haemoglobin, red cell folate, and serum folate, vitamin B_{12} and total homocysteine.

Urine Treatment and Storage The volume of each 24hr urine collection was noted before the total volume of each sample was made up to 2L with H_2O. 300ml aliquots of sample were stored at -20°C until required for urinary folate, pABGlu and apABGlu analysis.

Dietary analysis Dietary folate intake (μg/day) was measured in the pregnant (second trimester) and non-pregnant women by a qualified dietitian using an interview assisted food frequency questionnaire and intake calculated by a food-nutrient conversion package (Foodbase, 1992, The Institute of Brain Chemistry and Human Nutrition).

Statistical analysis The catabolite data were shown to be normally distributed. ANOVA analysis (Datadesk 4.0, Data Description Inc.) was used to assess the effect of gestation on catabolite levels within the pregnant group and the effect of supplementation in the non-pregnant control group. Scheffe post hoc tests were used to compare different gestational sub-groups and as p-values were calculated to allow for the effect of repeated measurement $p < 0.05$ was taken as significant. To compare the non-pregnant and pregnant sub-groups non-paired t-tests were used. Pearson product-moment correlation was used to assess correlation between clinical markers.

2.2. Analysis of Urinary pABGlu and apABGlu - Daily Catabolic Loss

2.2.1. Preparation of [3,5-^{3}H]-pABGlu and [3,5-^{3}H]-apABGlu Standards

Synthesis of [3,5-^{3}H]-pABGlu To 100μl [^{3}H]-PteGlu was added 50μl HCl (6M) and 25μl zinc dust. The mixture was vortex-mixed intermittently for 20min and centrifuged to remove excess zinc. The supernatant was removed and the [^{3}H]-pABGlu purified by HPLC.

Purification of [3,5-^{3}H]-pABGlu The zinc cleaved folic acid sample was chromatographed on a Radial Pak C_{18} (100 x 8mm) cartridge column (Waters) using a RCM radial compression system. A RCSS Guard Pak C_{18} cartridge(Waters), fitted in a pre-column module, was attached between a U6K injector (Waters) and the column. The column was eluted isocratically at 2ml/min with citrate-phosphate buffer (0.1M, pH 4): acetic acid (1% v/v): methanol:: 80: 14: 4. Fractions were collected around the retention time of cold pABGlu standard and fractions containing the greatest radioactivity were pooled. Part of the purified [^{3}H]-pABGlu was retained for use in the pABGlu assay while the remainder was used in the synthesis of [^{3}H]-apABGlu.

Synthesis and Purification of [3,5-^{3}H]-apABGlu The 5-10ml [^{3}H]-pABGlu was diazotized by adding 100μl HCl (6M) and 100μl sodium nitrite (1% w/v). The mixture was allowed stand for 5min before 100μl ammonium sulfamate (5% w/v) was added. After 5min 100μl N-1-naphthylethylenediamine (1% w/v) was added. The sample was incubated for 30min before the sample was loaded onto an activated (3ml methanol followed by 5ml water) C_{18} Sep-Pak (Waters). The Sep-Pak was washed with 10ml HCl (0.05M) and the diazotized material eluted with 3ml methanol. The [^{3}H]-pABGlu was regenerated by the addition of 50μl HCl and zinc dust, shaken intermittently for 5min and centrifuged to remove excess zinc. The supernatant was transferred to a conical glass tube and evaporated to dryness in a water bath at 37°C under nitrogen.

The acetamido-derivative of pABGlu was prepared by resuspending the dried residue in 34μl acetic acid (50% v/v) and 5μl acetic anhydride and incubating for 24hrs at 22°C. The [^{3}H]-apABGlu was purified by HPLC by resuspending the reaction mixture in 100μl H_2O and chromatographing on the system described for the purification of [^{3}H]-pABGlu. Fractions were collected around the retention time of cold apABGlu standard and fractions

containing the greatest radioactivity were pooled and retained for use in the assay of apABGlu.

2.2.2. Assay for Urinary pABGlu

Preparation of ion-exchange column A glass column (200 x 15mm with a 200ml reservoir) plugged with glass wool was filled with Dowex 50W (Sigma 50X8-400) cation-exchange resin (7g in 10ml H_2O). The column was washed with 50ml HCl (6M) and 50ml H_2O to equilibrate.

Fractionation of Urine by Ion-Exchange Chromatography To duplicate 25ml aliquots of urine was added 100 kdpm [^{3}H]-pABGlu and HCl (6M) to give a final concentration of 0.1M. The urine sample was then applied to the ion-exchange column, the column was washed with 50ml HCl (0.3M) and the pABGlu eluted with 100ml HCl (0.6M).

Diazotization and Solid Phase Extraction The samples were diazotized as described above except 1ml of each reagent was used, the mixture was allowed stand 30min between additions and the final reaction mixture was left overnight. The resulting diazotized material was loaded onto an activated Sep-Pak under reduced pressure, washed with 10ml HCl (0.05M) and 3ml methanol (25% v/v) and the diazatized material eluted with pure methanol

Regeneration of pABGlu The solution was evaporated to dryness in a water bath (60°C) before it was resuspended in 300μl HCl (1M) to which was added zinc dust. After 10min the suspension was transferred to a microfuge tube and centrifuged to clarify.

Chromatographic Quantitation of pABGlu 100μl of supernatant was chromatographed using the HPLC system described above and the peak corresponding to pABGlu manually collected for determination of radioactivity, and consequently percentage recovery. The pABGlu content of the injected sample was determined by recording the change in absorbance at 280nm, measured using an SPD-6A on-line UV-spectrophotometer (Shimadzu, Kyoto, Japan) and relating its peak height to a standard plot (10-200ng) of injected pABGlu standard. The pABGlu content of each sample was corrected for percentage recovery before adjusting for total urinary volume:

$$[\text{pABGlu}]\ (\text{ng}/25\text{ml}) = \frac{\text{HPLC peak (ng)} \times [^3\text{H}]\text{-Standard added (dpm)}}{[^3\text{H}]\text{-pABGlu peak from HPLC (dpm)}} \qquad \text{Eqn. 2.1}$$

$$[\text{pABGlu}]\ (\text{ng/day}) = \frac{[\text{pABGlu}]\ (\text{ng}/\ 25\text{ml}) \times \text{Total Urine Volume (ml)}}{\text{Sample Volume (25ml)}} \qquad \text{Eqn. 2.2}$$

2.2.3. Assay for Urinary apABGlu

Ion Exchange Method 25ml aliquots of urine were prepared and loaded onto an equilibrated cation exchange column as described for pABGlu except that [^{3}H]-apABGlu (100 kdpm) was added in place of [^{3}H]-pABGlu. apABGlu eluted from the cation exchange column in the sample volume and a further 50ml HCl (0.1M). Both fractions were pooled and sufficient HCl (6M) was added to the resulting 75ml sample to made it 0.2M. The sample was boiled in a water bath for 60min to deacetylate the apABGlu before it was re-loaded onto the washed cation exchange column (50ml HCl (6M)

followed by 50ml H_2O). The column was washed with 50ml HCl (0.3M) and eluted with 100ml HCl (0.6M). The resulting sample was diazotized and extracted, regenerated to pABGlu and quantified by HPLC as previously described.

Solid Phase Extraction Method To 25ml of urine was added [^{3}H]-apABGlu and the pH adjusted to pH 4.0 with citric acid (2M). The sample was loaded onto an activated (3ml methanol followed by 5ml citrate-phosphate buffer (0.1 mol/L, pH 4)) C_{18} Sep-Pak (Waters) under reduced pressure. The SepPak was washed with 5ml methanol (2% v/v in citrate-phosphate buffer (0.1 mol/L, pH 4)) and eluted into a glass bottle with 10ml methanol (10% v/v in citrate-phosphate buffer (0.1 mol/L, pH 4)). 0.66ml HCl (6M) was added to the sample, the bottle was plugged with non-absorbent cotton wool and boiled for 60min in a water bath. The sample was cooled, diazotized, extracted and regenerated to pABGlu before quantified by HPLC as previously described, except the running buffer was adjusted to pH 3.5 with citric acid (2M).

Comparison of methods A comparison of the results for samples assayed blind using both methods for the determination of apABGlu concentrations is contained in Appendix A.

The solid phase extraction method was preferentially used in the quantification of apABGlu as the solid phase extraction method was: (i) Faster. Less than 2.5hrs (+Overnight evaporation of the diazotised material) compared to the 1.5 days (+Overnight evaporation of the diazotised material) required for the ion exchange method; (ii) Less labour intensive. Use of the Dowex column involved several stages where the column was washed or where sample was added to the column. This process was labour intensive and took most of the day. Sample preparation by solid phase extraction took less than 60min with a one hour boil; (iii) Required smaller elution and reagent volumes; (iv) Far less

odour: Boiling the samples from the Dowex column, especially when several were being treated together, resulted in a pungent and unpleasant smell of urea and other compounds. Due to the smaller volume and the retention of compounds on the Sep-Pak, the samples prepared by solid phase extraction were far less pungent when boiled; (v) "Standardised" and more "transportable" : The glass columns were bulky and fragile and had to be specially made for the assay. The Sep-Pak are universally available.

Samples prepared by solid phase extraction were not "cleaned up" as much as those prepared by the ion exchange method. This was not a significant factor for any samples except for a few of the postpartum samples which were too contaminated after solid phase extraction. Postpartum samples were therefore assayed using the ion exchange method. Ten postpartum samples were analysed using both the solid phase extraction and ion exchange methods; the mean difference between the samples was less than 10%.

2.3. Blood Analysis for Folate, Vitamin B_{12} and Total Homocysteine

2.3.1. Blood Preparation

Serum Folate Blood was collected in a Vacutainer Serum tube. The tube was centrifuged at 1,500g for 15min, the serum removed and stored at -20°C.

Red Cell Folate Blood was collected in a Vacutainer K_2EDTA tube. The blood was mixed and diluted 1 in 10 in fresh ascorbic acid (1% w/v). The sample was incubated on a roller for 30min at room temperature complete cell lysis and to deconjugate folate polyglutamates. Lysates were then stored at -20°C. Packed Cell Volumes (PCV) were

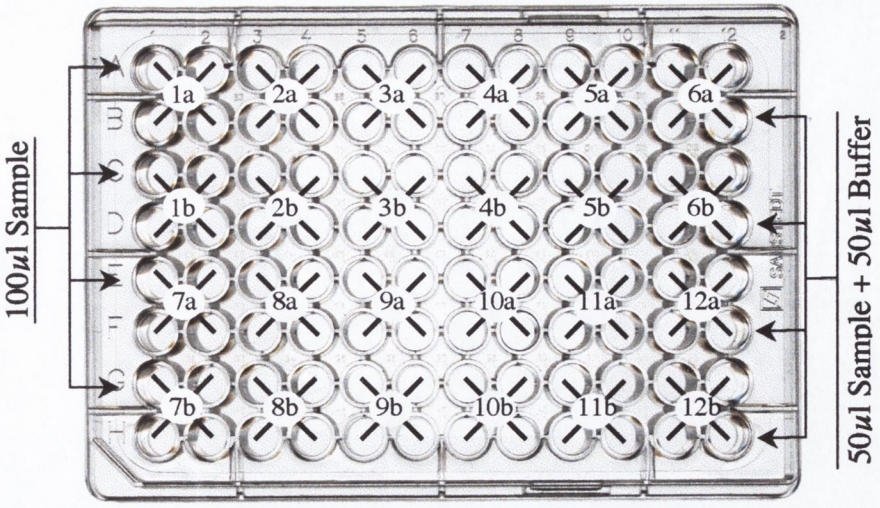


Fig 2.1. Diagram showing the layout of the sample plate for the folate assay. Samples were assayed in duplicate (a & b) with each duplicate plated in duplicate at two dilutions (100µl sample and 50µl sample plus 50µl sodium ascorbate (0.5%, w/v)) as shown. Folate content was then assayed as described in the methods [Section 2.3].

Standard (µl / well)	0	0	5	10	15	20	25	30	40	60	80	100
Folate (pg / well)	0	0	2.5	5	7.5	10	12.5	15	20	30	40	50
Folate (fmol / well)	0	0	5.7	11.3	17	23	28	34	45	68	91	113

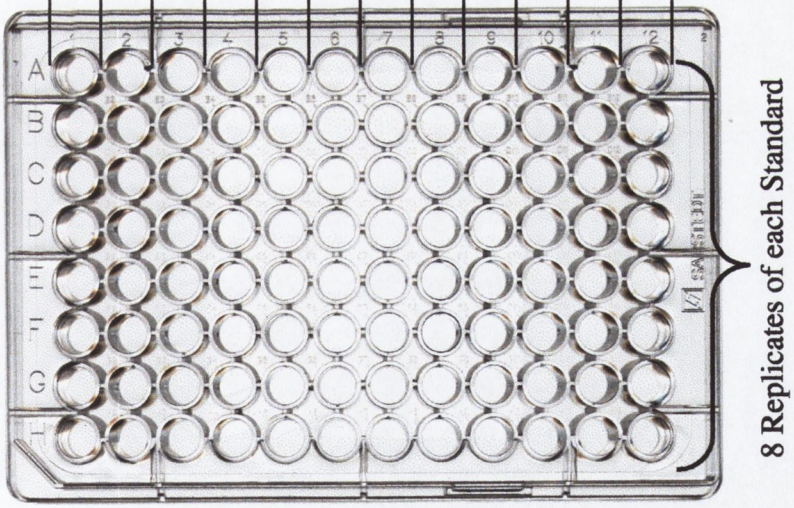


Fig 2.2. Diagram showing the layout of the standard plate for the folate assay. The volume shown (µl/well) is the volume of folic acid standard added to each well in the column. The well volumes were then made up to 100µl with sodium ascorbate (0.5%, w/v) and the plate assayed as described in the methods [Section 2.3].

measured by filling a capillary tube with blood and sealing the end. The tube was centrifuged and the PCV read using a micro-haematocrit reader (Hawksley, London, UK).

2.3.2. Microbiological Assay for Serum, Red Cell and Urinary Folate A modification of the methods of Molloy and Scott (1997) was used for the determination of serum, red cell and urinary folate.

Preparation of Folate Assay Media 5.7g folic acid broth (Merck) and 3mg chloramphenicol were desolve in 100ml H_2O and the mixture heated. Just before bp was reached 30μl Tween 30 was added. The mixture was brought to the boil after which the mixture was covered and cooled to room temperature when 75mg ascorbic acid and 200μl of the cryopreserved *L.casei* suspension were added.

Preparation of Cryopreserved L.casei Suspension 200ml of folate assay media was prepared containing 7.6g folic acid broth, 40mg chloramphenicol, 80μl Tween, 50mg ascorbic acid and 50ng folic acid. This was then stored in 20ml aliquots at -20°C until required.

A lyophilised *L.casei* (NCIMB 10463) vial was used to inoculate 20ml of thawed media and the culture incubated for 42hrs at 37°C. 10μl of this first growth are then used to inoculate a further 20ml of thawed media which was incubated a further 24hrs. This process was repeated a further two times by which time the bacteria were fully in log phase. 1ml of the last growth was then used to inoculate a further 20ml of media, the tube incubated for a further 7hrs before 20ml autoclaved (10min at 121°C) glycerol (80% v/v) was added, mixed, aliquoted and stored at -70°C.

Preparation of Controls Pools of serum and whole blood, representing low, medium and high folate concentrations, were prepared by the routine lab. The mean and standard deviation (SD) for each pool were determined by assaying 6 aliquots of each on 3 separate occasions. Duplicate sets of the respective controls (serum or whole blood) were run with each assay (serum controls were used for the urinary folate assay). If all three standards were not within 2SD of the previously determined mean the assay was rejected.

Dilution and Plating of Samples Assays for each analyte (serum, red cell or urinary folate) were conducted separately. Serum or whole blood lysates were diluted 1 in 20 in sodium ascorbate (0.5% w/v) while urine samples were diluted 1 in 10. Duplicate 100μl or 50μl amounts of each sample were pippetted into respective wells of a 96-well plate [Fig 2.1]. 50μl sodium ascorbate (0.5% w/v) was added to those wells containing only 50μl of sample (to give a further 1 in 2 dilution).

Preparation of Standard Curve 20.00mg folic acid was dissolved in 4ml NaOH (0.1M) and diluted to 1L with H_2O. This was divided into 20ml amounts and stored at 4°C. Fresh stock solutions were prepared every 8-12 weeks and their standard curves compared to those of the existing stock solutions. To minimise cross contamination stock solutions were prepared in a separate lab to the assay lab.

To prepare a standard curve for use in the folate assay 50μl of the stock folic acid solution was made up to 100ml with sodium ascorbate (0.5% w/v). 5ml of this first dilution was then diluted to 100ml with sodium ascorbate (0.5% w/v) giving a final solution of 0.5ng/ml. The required volume of this final dilution were dispenses into a 96-well plate [Fig 2.2] and the volume of each well was made up to 100μl with sodium ascorbate (0.5%

w/v). Standard curves were prepared independently of the assay to minimise possible cross contamination.

Incubation and Determination of Folate Concentrations 200μl of the *L.casei* inoculated assay media was added to each well of the sample and standard curve plates and the plates sealed with Linbro adhesive plate sealer (ICN Biomedicals Inc., Aurora, Ohio). Plates were incubated at 37°C for 42hrs, the plate inverted several times to suspend the bacteria before the plate sealer was removed. The plates were read at 590nm using a Multiscan Plus (Labsystems, Helsinki, Finland) plate reader connected to a PC. The folate concentrations per well (pg) and thus for each sample (serum folate (μg/L), red cell folate (μg/L) and urinary folate (μg/2L)) were calculated by the computer package (ELISA+, Meddata Inc. New York, NY) by comparing the well's optical density to those of the standard curve, and from the dilution factors used. Samples giving concentrations greater than those of the highest standard well (50pg/well) were assayed at a higher dilution.

2.3.3. Microbiological Assay for Serum Vitamin B_{12} A modification of the methods of Kelleher and O'Broin (1991) was used for the determination of serum vitamin B_{12}.

Preparation of Vitamin B_{12} Assay Media 6.2g Vitamin B_{12} assay broth base (Merck) and 150μl Tween 80 were dissolved in 100ml distilled water and boiled. Upon cooling 11mg colistin sulphate and 200μl of thawed cryopreserved *L.leichmannii* suspension were added.

Preparation of Cryopreserved L. leichmannii Suspension 200ml of B_{12} assay media was prepared containing16.6g vitamin B_{12} assay broth base (Merck), 400μl Tween 80, 50ng cyanocobalamin (added before boiling) and 100mg colistin sulphate. This was then stored in 20ml aliquots at -20°C until required.

A lyophilised *L.leichmannii* (NCIMB 12519) vial was used to inoculate 20ml of thawed media and the culture incubated for 24hrs at 37°C. 10μl of this first growth are then used to inoculate a further 20ml of thawed media which was incubated a further 24hrs. This process was repeated once more by which time the bacteria were fully in log phase. 2ml of the last growth was then used to inoculate a further 20ml of media, the tube incubated for a further 8hrs before 20ml autoclaved (10min at 121°C) glycerol (80% v/v) was added, mixed, aliquoted and stored at -70°C.

Preparation of Controls Pools of serum and whole blood, representing low, medium and high vitamin B_{12} concentrations, were prepared by the routine lab. The mean and standard deviation (SD) for each pool were determined by assaying 6 aliquots of each on 3 separate occasions. Duplicate sets of the controls were run with each assay. If all three standards were not within 2SD of the previously determined mean the assay was rejected.

Preparation and Plating of Samples Serum was diluted 1 in 10 with extraction buffer (sodium hydroxide (8.3mM), acetic acid (20.7mM), sodium cyanide (0.45mM), pH4.5). Samples were autoclaved at 115°C for 10min, vortexed to remove precipitate from the tube sides and centrifuged at 1,000g for 10min. The supernatant removed and 100μl or 50μl amounts of each sample were added to duplicate wells in a 96-well plate [Fig 2.3]. 50μl of extraction buffer was added to those wells containing only 50μl of sample (to give a further 1 in 2 dilution).

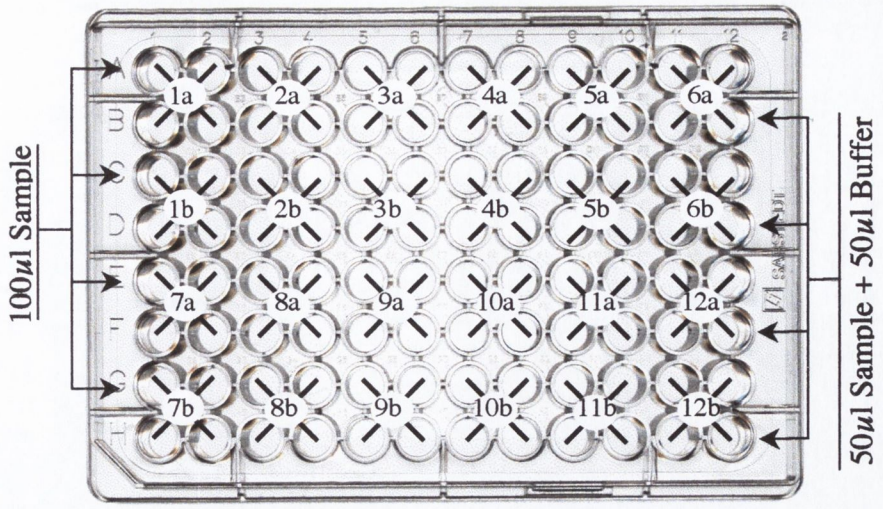


Fig 2.3. Diagram showing the layout of the sample plate for the vitamin B_{12} assay. Samples were assayed in duplicate (a & b) with each duplicate plated in duplicate at two dilutions (100μl sample and 50μl sample plus 50μl extraction buffer) as shown. Vitamin B_{12} content was then assayed as described in the methods [Section 2.3].

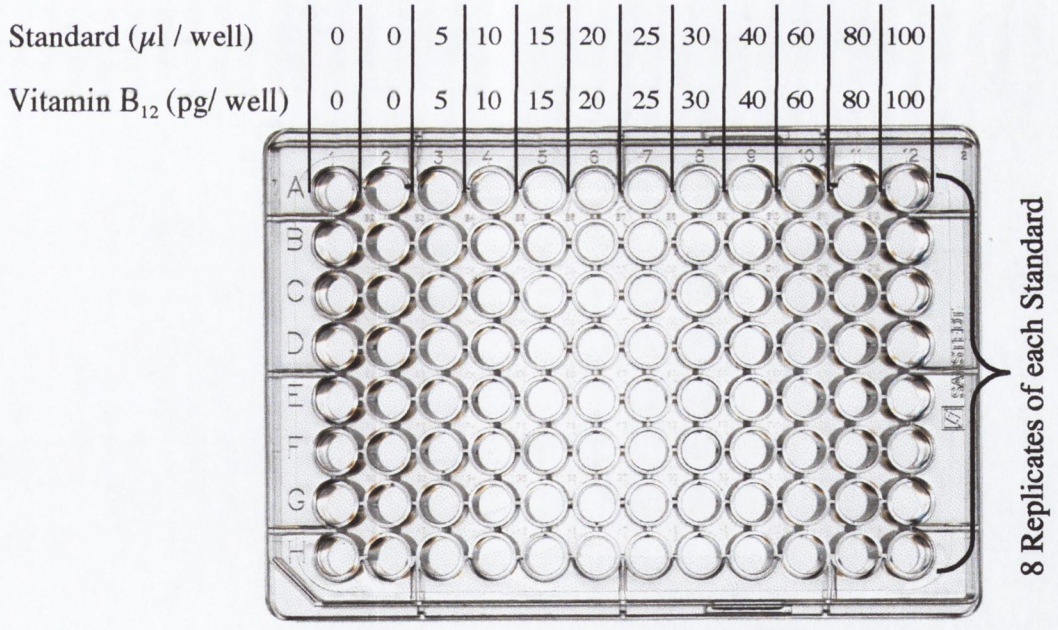


Fig 2.4. Diagram showing the layout of the standard plate for the vitamin B_{12} assay. The volume shown (μl/well) is the volume of vitamin B_{12} standard added to each well in the column. The well volumes were then made up to 100μl with extraction buffer and the plate assayed as described in the methods [Section 2.3].

Preparation of Standard Curve 200μl of Cytamen 1000 was diluted to 4.00ml, the solution mixed and the vitamin B_{12} concentration determined using the following equation:

$$\text{Vitamin } B_{12} \text{ concentration} = (48.545 \times Abs_{361nm}) \ \mu g/ml$$

This value was multiplied by 20 to give the concentration of the Cytamen 1000 stock solution. The verified Cytamen 1000 solution was diluted to give a 10μg/ml solution. As the Cytamen 1000 normally has a concentration of 1000μg/ml this usually involved a 1 in 100 dilution. After mixing the standard B_{12} solution was stored in 1.2ml volumes at -20°C.

Standard B_{12} solutions were thawed as required and diluted 1 in 100 with distilled water. This initial dilution was mixed and diluted 1 in 100 in extraction buffer to give the working solution. Volumes of working solution were added to a 96-well plate as shown [Fig 2.4] and the volume of each well was made up to 100μl with extraction buffer.

Incubation and Determination of Vitamin B_{12} Concentrations 200μl of the *L.leichmannii* inoculated assay media was added to each well of the sample and standard plates and the plates sealed using Linbro adhesive plate sealer. Plate were incubated at 37°C for 42hrs, the plate inverted several times to suspend the bacteria and the plate sealer removed. The plates were read at 590nm using a Multiscan Plus (Labsystems, Helsinki, Finland) plate reader connected to a PC. The vitamin B_{12} concentrations per well (pg) and thus for each sample (ng/l) was calculated by the computer package (ELISA+, Meddata Inc. New York, NY) by comparing the well's optical density to those of the standard curve, and from the dilution factors used. Samples giving concentrations greater than those of the highest standard well were assayed at a higher dilution.

2.3.4. HPLC Assay for Total Serum Homocysteine The methods of Ubbink *et al.* (1991) were employed for the determination of total serum homocysteine concentrations (tHcy).

Serum Homocysteine Derivatisation To 300μl of serum was added 30μl tri-*n*-butylphosphine (10% in dimethylformamide). After incubating for 30min at 4°C to reduce the homocysteine and mixed disulphide and to release protein bound homocysteine, 300μl trichloroacetic acid (10%), EDTA (1mM) was added. The sample was centrifuged and 100μl of supernatant added to 20μl NaOH (1.55M), 250μl borate buffer (125mM, pH9.5) cotaining EDTA (4mM) and 100μl ammonium 7-flourobenzo-2-oxa-1,3-diazole-4-sulphonate (SBF-F, 1mg/ml in borate buffer). The sample was incubated at 60°C for 60min to derivatise the thiol species.

Chromatographic Quantification of Total Homocysteine Sample were injected onto a Supelco LC-18-DB analytical column (150 x 4.6mm) using a 710B WISP (Waters) automatic sample injector. A reverse phase GuardPak C_{18} (Waters) column was fitted between the injector and the column. The column was eluted with potassium dihydrogenphosphate buffer (100mM, pH2.1 with ortophosphoric acid) : acetonitrile :: 96 : 4 at a flow rate of 2ml/min. The derivatised homocysteine peak was quantified by measuring its fluorescence (ex. 385nm / em. 515nm) using a RF535 fluresence detector (Shimadzu) and comparing the result to a standard curve of known homocysteine concentrations.

PART II: STUDIES IN BACTERIA AND CELL CULTURES

2.4. Inhibition of Folate Metabolism in *E.coli*

2.4.1. Basic Techniques

Preparation of Growth Media Glucose media (8) was composed of (g/l): NaCl, 2.94; NH_4Cl, 2.66; KH_2PO_4, 3.4; CaCl, $7.4x10^{-3}$; $MgSO_4$, 0.25; Glucose, 2.0; pH7.2. Columbia agar (Lab M, Bury, UK) supplemented with 7% horse blood was prepared by the Microbiology Department, St James's Hospital, Ireland.

Preparation of E. coli stocks on cryo-preserve beads The lyophilised *E.coli* was suspended in 400μl glucose media and 200μl was used to inoculate a Columbia blood agar incubated at 37°C. The resulting culture was stored at -70°C on Protect beads (Technical Services Consultants Ltd., Lancashire, UK). Bacterial cultures were reconstituted from –70°C, sub-cultured on Columbia blood agar as required and checked for purity.

Determination of bacterial turbidity The turbidity of 300μl of samples in a 96-well plate was measured at 590nm using a Multiskan Plus plate-reader (Lab Systems, Helsinki, Finland).

Determination of bacterial density Bacterial colonies from a Columbia blood agar were suspended in glucose media to give a stock suspension with a turbidity of 0.642AU. From this a dilution series was prepared from 0 to 100% of the stock suspension in 10 equal intervals and the turbidity was measured. The viable cell number in the stock suspension was determined by making 1 in 10^4, 1 in 10^6 and 1 in 10^8 dilutions of the initial bacterial suspension in glucose media. 100μl of each dilution was used to inoculate duplicate blood agar plates. Plates were incubated overnight, colonies were counted and the bacterial density (cells/ml) of the initial suspension calculated. The bacteria density of each standard dilution was calculated and a plot of bacterial density versus turbidity was used to calculate the second order polynomial equation which related absorbance (at 590nm) to bacterial density:

$$\text{Bacterial Density } (10^9 \text{ Cells/ml}) = 2.1270 \times Abs^2 + 3.5928 \times Abs - 0.0195 \quad [r = 1.000]$$

2.4.2. Optimisation of Growth Conditions

Optimisation of incubation periods for each growth For each growth plots of incubation time versus bacterial density were prepared to determine when the bacteria was in log phase growth. All experimental procedures were performed on bacteria in log phase growth. Approximately 50 *E.coli* colonies from a Columbia blood agar were suspended in 500μl of glucose media and diluted so that 300μl had a turbidity of 1.00 A.U. A 100μl aliquot of this suspension was inoculated into 5ml of glucose media and incubated at 37°C in an orbital incubator at 80rpm. After incubating for 11h, 250μl (460×10^6 ($\pm 20 \times 10^6$) cells) was inoculated into 5ml of glucose media containing [^{3}H]-pABA. After 9h the bacteria was harvested by centrifugation at 4,500rcf for 8min, the pellet resuspended in 2ml glucose media and repelleted. The washed pellet was resuspended in 1ml glucose

media and 200μl ($1.8x10^9$ ($\pm 0.1x10^9$) cells) inoculated into 5ml of glucose media containing the required concentration of Tmp.

Determination of optimum [^{3}H]-pABA concentration The first growth was incubated for 11h before 250μl was inoculated into tubes containing 5ml glucose media and 100, 200, 300 or 400 pmole [^{3}H]-pABA. After incubating for 9h the *E.coli* was harvested and the radioactive content of each pellet expressed as a percentage of total [^{3}H]-pABA added. Concentrations of 100-300pmole/5ml [^{3}H]-pABA gave roughly 42% incorporation while 400pmole/5ml gave only 37% incorporation. Therefore 300 pmole/5ml was the concentration used.

2.4.3. Optimisation of Inhibitor Concentrations

Determination of the Tmp concentration to be used A 200μl aliquot of the washed second growth pellet was added to a duplicate series of tubes containing Tmp serially-diluted from 200μg/ml to 200ng/ml. The tubes were incubated for 4h when bacterial density was determined and plotted against Tmp concentration. From this, the minimum concentration of Tmp giving maximum inhibition was determined.

Determination of the sulfamethoxazol concentration to be used A 200μl aliquot of the washed second growth pellet was added to a duplicate series of tubes containing sulfamethoxazol (SMX) serially-diluted from 2mg/ml to 200ng/ml. The tubes were incubated for 4h when bacterial density was determined and plotted against SMX concentration.

Determination of the inhibitory concentration of pABA A stock solution of containing 8mg pABA per ml was made up in glucose media and the pH adjusted to pH 7.2 with K_2HPO_4. This stock solution was diluted appropriately with glucose media to give a duplicate dilution series from 8mg/ml to 0.8mg/ml. Tubes were inoculated with 200μl of the washed second growth pellet and incubated for 4h before bacterial density was determined.

2.4.4. Comparison of the Extraction and Deconjugation Methods

Human Serum Conjugase The washed pellet was resuspended in 500μl ascorbic acid (2% w/v), 10mM mercaptoethanol (pH 4.5) and the cells lysed by sonicating on ice for 20sec x 3 (with 30sec break between cycles). Debris and intact cells were removed by centrifuging at 10,000g for 2min. The supernatant was deconjugated by adding 100μl freshly prepared human serum and incubating for 60min at 37°C.

Rat Plasma Conjugase The washed pellet was resuspended in 500μl HEPES buffer (100mM), ascorbic acid (2%), mercaptoetanol (100mM) and the cells lysed by sonicating on ice for 20sec x 3 (with 30sec intervals between cycles). Debris was removed by centrifuging at 10,000g for 2min. The supernatant was deconjugated by adding 100μl EDTA rat plasma (stored at –70°C) and incubating for 45min at 37°C.

Determination of dihydrofolate stability during extraction and deconjugation After washing the *E.coli* pellet was spiked with a known quantities of cold DHF before it was resuspended in buffer, lysed and deconjugated. Samples were chromatographed and DHF stability determined by relating DHF peak heights to a standard plot. DHF was found to be 87±2% (n = 3) stable under the conditions used for rat plasma deconjugation while it

was approximately 30% stable under the conditions used for human serum deconjugation. The DHF was almost totally destroyed (< 5% recovery) when stored at -70°C in ascorbic acid buffer.

2.4.5. Sample Preparation and Chromatographic Analysis of Cell Extracts:

Several chromatographic methods for the separation of folate monoglutamates were tested. The two methods used here were found to give best peak resolution (results not shown).

Harvesting of bacteria, sample preparation and HPLC analysis The bacteria was harvested at timed incubation periods and the pellet resuspended in 500μl HEPES (100mM, pH 7.3) containing β-mercaptoethanol (100mM),and sodium ascorbate (2% w/v). The bacteria were lysed by sonication on ice for 20sec x 3 (Sonifier 450, Branson Ultrasonics, Danbury, CT) fitted with a microtip.

Samples were deconjugated by incubating for 45min at 37°C with 100μl rat plasma and chromatographed on a 3μ Kingsorb (4.6x150mm) ODS-2 column (Phenomenex). A SecurityGuard pre-column module (Phenomenex) containing a disposable ODS insert (3x4mm, Phenomenex) was attached between the injection port and the column. The column was eluted isocratically at 1ml/min with 5mM Pic A (Waters, Milford, MA) in 0.1M sodium acetate (pH 6.0), containing methanol at 16% (v/v). Fractions were collected at 0.5min intervals using a Frac-100 fraction collector (Pharmacia Biotech, Uppsala, Sweden), dissolved in EcoLite scintillation fluid (ICN, Costa Mesa, CA) and counted on a 1500 Tricarb scintillation counter (Packard Canberra, Reading, UK). The magnitude of each peak was expressed as a percentage of total radioactivity injected onto the column

and the retention times compared to those of folate standards detected at 280nm on a SPD-6A UV spectrophotometer detector (Shimadzu, Kyoto, Japan).

Chromatographic separation of THF and 10-CHO-THF On occasion growth conditions resulted in the accumulation of THF and/ or 10-CHO-THF, resulting in their co-elution from the Kingsorb column. In this case deconjugated extracts were also chromatographed on a 10μ μBondapak (3.9 x 300mm) C_{18} column (Waters) eluted with citrate-phosphate buffer. A GuardPak pre-column module (Waters) containing a disposable C_{18} insert (Waters) was attached between the injection port and the column. The column was eluted isocratically at 2ml/min with citrate-phosphate (50μM, pH 4.0) : acetic acid (1.5%, v/v) : methanol :: 80: 8: 12. Fractions were collected and counted as before.

A schematic flow diagram of the experimental procedure is shown in [Fig. 2.5]

2.4.6. Sundry Techniques Employed

Identification of metabolically-produced folic acid Extracts from Tmp treated samples (24h) were prepared and chromatographed as described and the fractions corresponding to the folic acid peak were retained and pooled. To 100μl of this pool was added 40μl HEPES buffer (1M), 80μl sodium ascorbate (10% w/v), 40μl β-mercaptoethanol (1M) and 40μl NADPH (1M). The volume was made up to 400μl with H_2O and divided in two. To one half was added 2μl bovine liver dihydrofolate reductase (1 unit/ 20μl, Sigma), the other was left untreated Both samples were incubated for 15min at 25°C and chromatographed by HPLC. [^{3}H]-Folic acid was identified by its co-elution with an authentic standard and through enzymatic conversion by bovine liver dihydrofolate

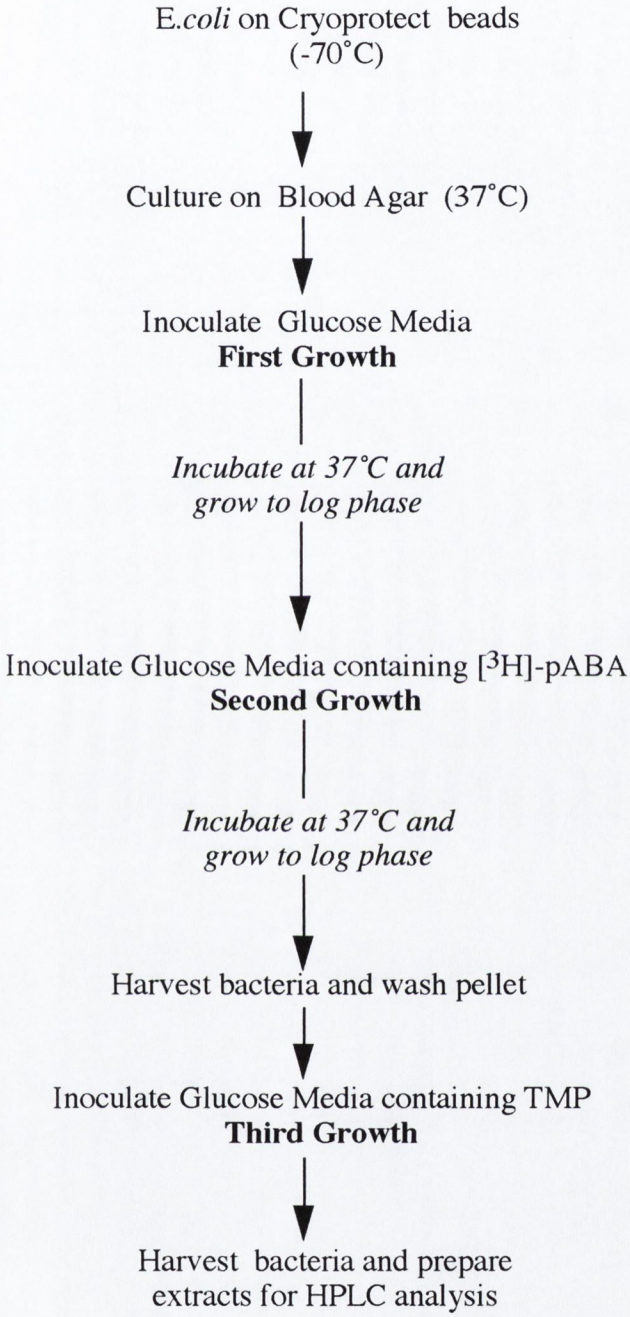


Fig 2.5. Schematic diagram of the experimental proceedure.

reductase to tetrahydrofolate and co-elution with authentic standard on HPLC. The chromatographic profile of the untreated sample remained unchanged.

Metabolic re-utilisation of folic acid upon removal of inhibitor Two tubes each containing 5ml of glucose media and Tmp were inoculated with 200μl of the second growth pellet. After 2h incubation both cultures were mixed and re-divided before harvesting. One pellet was prepared for HPLC analysis while the other was resuspended in 8ml glucose media and centrifuged. The washed pellet was resuspended in 5ml glucose media and incubated for a further 4h, then harvested and extracted for HPLC analysis.

Identification of folic acid and pABGlu as polyglutamates *Tmp*-treated cultures were harvested at 4h and extracted for folic acid and pABGlu. Without conjugase treatment, this extract was divided in two. A 200μl aliquot of one half was incubated for 10min with 20μl HCl (6M) and 10mg zinc dust. The other half was left untreated. Both samples were chromatographed on a μBondapak (3.9mm x 30cm) C_{18} column (Waters). A pre-column module containing a disposable C_{18} insert (RCSS Guard Pak, Waters) was attached between the injection port and the column. The column was eluted isocratically at 2ml / min with citrate-phosphate (0.1mM, pH 4.0): acetic acid (1%): methanol (80: 14: 6). Fractions were collected at 0.5min intervals and assayed for radioactivity by scintillation counting. Authentic pABA-polyglutamates (n=1, 3, 5, 7) and folate monoglutamate standards were chromatographed to establish their elution position on the chromatogram.

Determination of total folate in E.coli Bacterial cultures were harvested, the cell pellets were resuspended in 500μl ascorbic acid (1%w/v), ß-mercaptoethanol (10mM) and lysed by sonication. Extracts were deconjugated by incubation with fresh human serum (100μl) for 1h at 37°C. Serial dilutions in triplicate (from 1 in 4 to 1 in 4^6) were made of the

deconjugated sample in ascorbic acid (1%) and assayed by microbiological assay [see Section 2.3.2]. The serum folate concentration was determined and subtracted to give bacterial folate values.

2.5. Inhibition of *De Novo* Folate Biosynthesis in *E.coli.*

Inhibition of exogenous [^{3}H]-pABA incorporation by Tmp The first *E.coli* growth was grown to log phase as usual. 500μl of this first growth was used to inoculate 10ml of glucose media containing 100pmol [^{3}H]-pABA and either 0, 6ng/ml or 600ng/ml Tmp. At timed intervals the bacterial density of each growth was determined. At the same time 600μl of the bacterial suspension was removed, the bacteria pelleted by centrifugation at 4,500rpm for 12min. The radioactivity of 500μl of supernatant measured and the percentage radiolabel incorporated into the bacteria calculated.

Identification of the radiolabelled species in the supernatant After either 6.25h (0 or 6ng/ml Tmp) or 11.5h (60ng/ml Tmp) incubation, 600μl of the *E.coli* growth was removed and the bacteria pelleted (as above). The supernatant was chromatographed on a μBondapak (4.6x300mm) C_{18} column (Waters) using the HPLC system described in Section 2.4. A SecurityGuard pre-column module (Phenomenex) containing a disposable ODS insert (3x4mm, Phenomenex) was attached between the injection port and the column. The column was eluted isocratically at 2ml/min with 80:14: 6:: citrate-phosphate buffer (50μM, pH4.0): acetic acid (1% v/v): methanol. Fractions were collected at 0.25min intervals and counted on a scintillation counter.

2.6. Inhibition of Folate Metabolism in *L.casei*

2.6.1. Basic Techniques

Preparation of growth media The growth media used was a modification of the Folate Assay Media as 7g/100ml Folic Acid *Casei* Medium (Difco) was substituted for the Folic Acid Broth (Merck).

Determination of bacterial turbidity The turbidity of 300μl of samples in a 96-well plate was measured at 590nm using a Multiskan Plus plate-reader (Lab Systems, Helsinki, Finland).

2.6.2. Optimisation of Growth Conditions

Optimisation of incubation period for each growth A plot of incubation time versus bacterial density was drawn for each growth, from this the incubation period when the *L.casei* was in log phase growth were determined. All experimental procedures were performed on bacteria in log phase growth.

50μl of cryopreserved *L.casei* suspension was used to inoculate 10ml *L.casei* growth media containing 2ng/ml folic acid. The first growth was incubated for 33hrs at 37°C before 20μl was used to inoculate 10ml *L.casei* growth media containing 10μl (130ng) [^{3}H]-folic acid. The second growth was harvested after 22hrs incubation by centrifugation at 3,000g for 10min. The pellet was resuspended in 40ml *L.casei* growth media containing 2ng/ml folic acid and 10ml pipetted into each incubation tube. The required amount of TMP or MTX was then added to each tube and the tubes incubated for the required period

before they were harvested and prepared for HPLC using a modification of the method employed for the *E.coli*, whereby cells were sonicated for 30sec each time.

2.6.3. Optimisation of Inhibitor Concentrations

Determining the minimum inhibitory concentration of Tmp or MTX A duplicate series of tubes containing 10ml of inoculated *L.casei* growth media and Tmp or MTX serially-diluted from 200μg/ml to 200ng/ml was prepared. The tubes were incubated for 12h when bacterial density was determined and plotted against inhibitor concentration. From this, the minimum concentration of Tmp and MTX giving maximum inhibition were determined.

Effects of duel inhibition (FDU and Tmp) on L.casei growth An inhibition series was prepared in triplicate containing 2ng/ml-200μg/ml 5-FDU ± 1μg/ml Tmp. Each 10ml of *L.casei* growth media was then inoculated with half the normal inoculate (i.e. the pellet from each tube was resuspended in 4ml of growth media and 0.5ml used to inoculate each growth tube). Growth turbidity was measured after 12 hrs incubation and plotted against 5-FDU concentration.

Effects of thymine supplementation on growth inhibition Inoculated tubes were prepared ± 200μg/ml thymine. The tubes were then incubated for 30min before the following additions were made to quadruplicate tubes:

Tube Number	**1**	**2**	**3**	**4**
TMP (1μg/ml)	−	+	+	−
5-FDU (20μg/ml)	−	−	+	+

Bacterial turbidity was measured at timed intervals and plotted against incubation time.

2.7. Inhibition of Folate Metabolism in Mouse Lymphocytes (L1210 Cells)

2.7.1. Basic Techniques

Maintenance of cell cultures L1210 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1mg/L folic acid incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were maintained by passaging 1 in 10 in fresh media every two days.

Folate depletion of cells 0.5ml of maintained growth ($8x10^5$ cells/ml) was passaged into 4.5ml of folate free DMEM and incubated for 48h, 0.5ml was then passaged into 4.5ml folate free DMEM and again incubated for 48h.

Labelling of cells 0.5ml of the folate depleted cells were passaged into 4.5ml of DMEM containing 15μl [^{3}H]-5-CHO-THF and grown for 60Hrs. Cells were harvested by centrifugation at 1,000rpm for 5min, the pellet was washed by resuspending in 2ml DMEM and repelleted.

2.7.2. Treatment of Cells and Chromatographic Analysis of Extracts

MTX inhibition of cells The pellet was resuspended in 20ml DMEM containing 1mg/l folic acid and 10% conditioned media (supernatant from maintained culture, grown for 48hr). Cells were plate in 5ml amounts and the required amount of MTX added. Cell suspensions were incubated for timed intervals before the cells were harvested by centrifugation at 1,000rpm for 5min., the pellet washed in 2ml DMEM and pelleted.

Folate extraction and treatment The pellet was resuspended in 500μl of extraction buffer (HEPES (100mM), sodium ascorbate (2% w/v), mercaptoethanol (100mM)) and sonicated on ice for 15sec. Protein was digested by adding 0.5units proteinase K-agarose (Sigma) and incubating at 37°C for 30min. The protease was removed by centrifuging at 13,000rcf for 4min. 400μl of supernatant was carefully removed and 100μl rat serum added (stored at –70°C), this was incubated for 45min at 37°C to deconjugate folates. Samples were chromatographed by HPLC as described previously.

Determination of bound radiolabel Sephadex G-50 (Sigma) was pre-swollen in HEPES (100mM), sodium ascorbate (2% w/v), mercaptoethanol (100mM) and used to fill a glass column (300 x 8mm) to a height of 12cm. The column was bedded down using 30ml of the same buffer. 200μl of protease-treated or untreated L1210 lysate or *E.coli* lysate (deconjugase untreated) were mixed with 100μl of a blue dextran (Mr = 66kDa) solution and loaded onto the column. The column was eluted with the same HEPES / ascorbate buffer, 3ml fractions were collected and counted for [^{3}H]. The elution volume of blue dextran was determined by its blue colour.

PART I

STUDIES IN HUMANS

[S]tructure, since it survives nearly intact when a cell or organism dies, is clearly not life, although a high degree of structure is probably required for life. Disruption of the…chemical activities of an organism is death; by the same token, these chemical activities, collectively, are life.

Daniel E. Atkinson (1977)

Cellular Energy Metabolism and Its Regulation

(Academic Press , New York)

CHAPTER 3

FOLATE CATABOLISM AND EXCRETION IN (NON-PREGNANT) HUMANS

3.1. Introduction

It is well established that the daily requirement for folate is far greater than that lost due to excretion of intact folate, suggesting that the utilisation of folate is associated with a regular catabolic process (Jukes *et al.*, 1947). Early studies by this laboratory in rats (Murphy and Scott, 1976; Murphy *et al.*, 1976a & 1976b) and by Krumdieck (1978) in man using [^{3}H] or [^{14}C]-labelled PteGlu have shown that folate catabolism proceeds by cleavage of the C9-N10 bond with release of pteridins and p-aminobenzoglutamate (pABGlu). In mammals the pABGlu is acetylated to p-acetamidobenzoylglutamate (apABGlu), the major catabolite found in urine (McPartlin *et al.*, 1992 and 1993). While radio-labelling studies have proved invaluable in providing data about the mechanism (Murphy and Scott, 1976; Murphy *et al.*, 1976a & 1976b; Krumdieck, 1978) and kinetics (Geoghegan *et al.*, 1995) of folate catabolism they have not provided quantitative information about the extent of folate catabolism. Thus an assay for determining the excretion levels of pABGlu and apABGlu was developed by this laboratory (McPartlin *et al.*, 1992). This method utilises the fact that while several forms of pteridine may be formed during folate cleavage pABGlu and apABGlu are the sole cleavage products formed and excreted on the N10 side of the bond (Geoghegan *et al.*, 1995). By quantifying the extent of their excretion into the urine over a 24h period the rate of folate

catabolism can be determined. We therefore set out to assess the rate of folate catabolism in men and non-pregnant women, by measuring pABGlu and apABGlu concentrations in 24 hour urine samples, and thereby determine folate loss by this route. As the female subjects also served as a control (non-pregnant) cohort for our pregnancy group [Chapters 5 and 6] we were in a position to examine the effect of supplementation on the rate of folate catabolism. This was considered important in light of the suggestion (Wang *et al.*, 1994; Sauberlich *et al.*, 1987; McNulty *et al.* 1993a) that folate status or intake may influence such catabolism.

We also investigated whether the combined oral contraceptive pill (COCP) increased the rate folate catabolism, as it has long been recognised that the use of the COCP can cause folate deficiency (Lambie and Johnson 1985).

3.2. Results

3.2.1. Folate Loss in Men The urine from twenty three men was assayed for pABGlu, apABGlu and folate as described in [Chapter 2]. The results for these samples are shown in [Table 3.1]. No correlation observed between the daily rate of excretion of apABGlu, pABGlu or urinary folate with the basic clinical characteristics of weight or age.

Age (Years)	Weight (kg)	apABGlu* (μg/day)	pABGlu* (μg/day)	Urinary folate (μg/day)
28.0 (14.1)	71 (14)	65.2 (26.3)	12.6 (4.3)	6.1 (3.4)

Table 3.1: Basic clinical characteristics (mean (SD)) of male subjects (n = 23). *Both apABGlu and pABGlu are expresses as folate equivalents, on the basis that pABGlu (Mr = 266.3) is 0.6 times the molecular weight of folic acid (Mr = 441).

3.2.2. Folate Loss in Women In a similar manner to the men, 24 hour urine samples were collected from non-pregnant women (n = 24) and assayed for pABGlu, apABGlu and folate. In addition to the urine, bloods were collected for full blood count, serum folate, total serum homocysteine, serum vitamin B_{12} and red cell folate analysis [Table 3.2]. A dietary questionnaire was taken to assess folate intake. Subsequently the women were supplemented with 500μg folic acid per day for 4 months after which period urine and blood were again collected for analysis. At 4 months supplementation there was a significant increase in levels of haemoglobin ($p= 0.02$), red cell folate ($p \leq 0.001$), serum folate ($p = 0.001$) and serum B_{12} ($p = 0.009$) [Table 3.2]. Urine analysis showed that the mean rate of total folate loss significantly increased with supplementation ($p \leq 0.001$), however this was driven by an increase in the rate of pABGlu ($p \leq 0.001$) and folate ($p = 0.014$) excretion [Table 3.2] as apABGlu excretion had not significantly changed ($p = 0.0785$). There was no correlation between the rate of excretion of apABGlu, pABGlu or urinary folate with the basic clinical characteristics of dietary folate intake, weight, age or height.

In examining the relationship between catabolism and folate status we showed a significant correlation ($p < 0.05$) between pABGlu and serum folate concentrations post supplementation and between apABGlu and serum folate concentrations at baseline and post supplementation.

Since there was a significant correlation between pABGlu and apABGlu with serum folate concentration we sought to determine how this relationship related to the change in folate status observed with supplementation. In doing so we hoped to examine the divergence between the two elements, pABGlu and apABGlu, in their relationship to the change in

	Control group (n = 24)		COCP group (n = 4)
	Baseline	**Supplemented**	
Age (years)	24.6 (1.5)	N.D.	25 (0.8)
Weight (kg)	65.6 (6.6)	N.D.	64.2 (4.5)
Haemoglobin (gm/dl)	13.1 (0.8)	13.8 (0.8)*	13.0 (1.1)
Red cell folate (ng/ml)	256 (85.6)	427.4 (197.1)†	232 (55.4)
Serum Folate (ng/ml)	8.8 (6.1)	16.0 (8.4)	7.5 (3.0)
Serum B_{12} (pg/ml)	624 (192)	710 (237)†	N.D.
Dietary folate intake (μg/d)	318 (121)	N.D.	442.3 (256)
apABGlu (μg/day)	43.1 (23.3)	53.3 (24.3)	81.7 (28.0)†
pABGlu (μg/day)	12.8 (5.1)	17.3 (7.0)†	16.2 (2.7)
Urinary folate (μg/day)	5.8 (4.0)	12.2 (10.5)*	9.2 (10.9)

Table 3.2: Basic clinical characteristics (mean (SD)) of female subjects. Both apABGlu and pABGlu are expresses as folate equivalents, on the basis that pABGlu (Mr = 266.3) is 0.6 times the molecular weight of folic acid (Mr = 441). Significantly different to control (Baseline) group: *$p \leq 0.05$; $^{\dagger}p \leq 0.005$.

folate status. While there was a significant correlation between the change in pABGlu excretion and change in serum folate concentration with supplementation ($p = 0.015$; $r = 0.50$) [Fig 3.1a] there was none between change in apABGlu excretion and change in serum folate ($p = 0.221$; $r = 0.25$) [Fig 3.1b].

3.2.3. Folate Loss in Women using the Combined Oral Contraceptive Pill: Four of the twenty eight female subjects recruited for the study were taking the combined oral contraceptive pill (COCP). As use of hormonal contraceptives is known to interfere with folate metabolism they were excluded from our main control group and were instead assessed separately. Subjects taking the COCP has a significant higher rate of apABGlu excretion ($p = 0.0049$, pooled t-test) compared to the control (baseline) group [Table 3.2], but were not significantly different ($p > 0.05$) with respect to any of the other clinical characteristics measured.

3.3. Discussion

Our findings show that folate supplementation significantly increased serum folate and red cell folate concentrations over the experimental period. At the same time, pABGlu concentrations also increased. The mode of pABGlu production is as yet unclear but may be the result of chemical or bacterial folate breakdown in the bloodstream, kidney or bladder, or in the case of orally administered folate, in the intestine. Definitive evidence for pABGlu breakdown in the blood would have been available with direct measurement of pABGlu in the serum. However this was not technically feasible. The possibility that

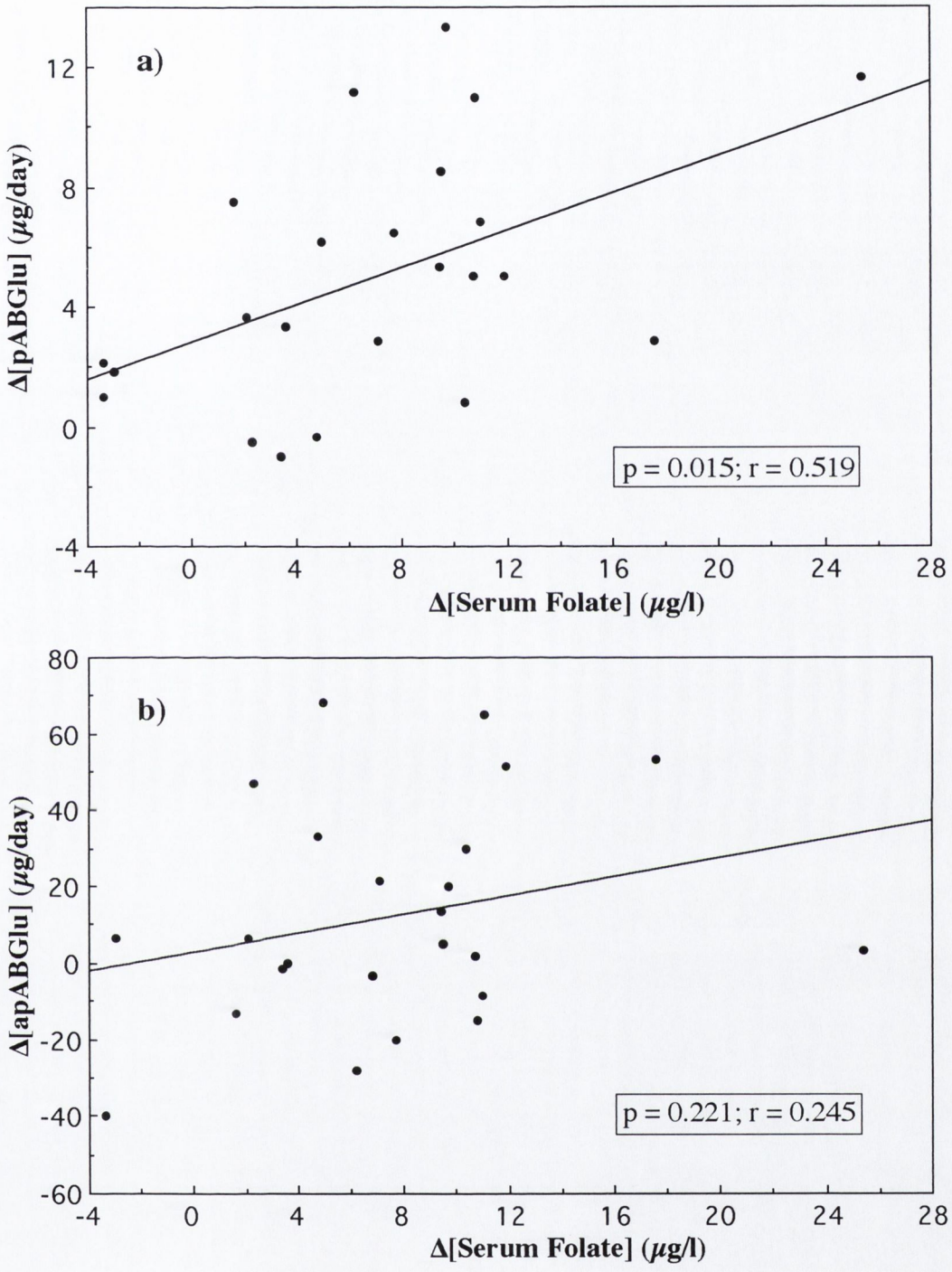


Fig 3.1. Correlation between change in serum folate concentration and a) change in pABGlu excretion or b) change in apABGlu excretion with supplemntation.

pABGlu is a surrogate of urinary folate is substantiated by the research by Anderson *et al.* (1960) and Johns *et al.* (1961) who showed that in acute experiments the rate of radiolabel excretion was a function of the amount of [^{3}H]-folic acid administered. In the present study we show that the rate of folate excretion is also increased in the urine by prolonged supplementation.

While there was a slight increase in apABGlu excretion over the supplementation period this increase, in contrast to pABGlu excretion, was not significant. By expressing the data as a correlation between the change in serum folate concentration and catabolite concentrations at baseline and post supplementation it was possible to demonstrate the critical metabolic difference between pABGlu and apABGlu. On the one hand, the change in pABGlu excretion during supplementation appears to be driven by change in serum folate [Fig 3.1a] providing further evidence in support of Geoghegan *et al.* (1995) who showed that pABGlu is the product of non-catabolic folate breakdown. On the other hand, the change in apABGlu appears to be independent of change in serum folate [Fig 3.1b]. At the cellular level this may indicate a threshold folate concentration required for optimum function, above which additional co-enzyme is superfluous. This corroborates the findings of McNulty *et al.* (1993a) that pABGlu excretion decreased in rats when switched from a folate sufficient diet to a folate deficient diet while apABGlu excretion remained unchanged, and is consistent with the finding of Geoghegan *et al.* (1995) that apABGlu represents metabolic utilisation and catabolism of folate [see also Chapter 6].

In the case of subjects who were taking the combined oral contraceptive pill (COCP) we demonstrated a significant increase in the rate of apABGlu excretion. This data may explain the folate deficiency often associated with use of the COCP. The increased rate of

folate catabolism with use of the COCP may reflect a hormonally induced changes in redox state or in enzyme activity or concentrations and that a similar mechanism pertains in pregnancy. For instance activity of another folate metabolising enzyme, folate conjugase, can be hormonally stimulated (Krumdieck, 1975), as are enzymes in the methylation cycle (M.J. van der Mooren *et al.*, 1994 & 1995; J. Silberberg *et al.*, 1995). This finding further highlights the particular importance of periconceptional folic acid supplementation in women who have been using COCP.

While pABGlu excretion may not be a product of metabolic folate catabolism, it still represents an inescapable folate loss, much like urinary folate. Therefore, concentrations of all three urinary products, intact folate and both catabolic products are used to determine the rate of folate loss. In calculating these values the non-supplemented baseline urinary concentrations are used as they represent the loss accruing to a normal replete population. Thus the total urinary folate (folate plus catabolites) loss in normal healthy men and non-pregnant women was 84 (30) μg/day and 62 (26) μg/day, respectively. The implicating of these findings in determining a Recommended Daily Allowance (RDA) are discussed in [Section 11.1].

CHAPTER 4

RESPONSE OF TOTAL HOMOCYSTEINE TO FOLIC ACID SUPPLEMENTATION, AND ITS CHANGING RELATIONSHIP WITH SERUM FOLATE AND VITAMIN B_{12}

4.1. Introduction

A 5 μmol/l increase in tHcy is thought to increase in risk of CAD by a comparable level to a 0.5 mmol/l increase in cholesterol (Boushey *et al.*, 1995). Thus assuming a causal link between CAD and tHcy, the potential decrease in tHcy due to food fortification with folic acid had the potential to reduce deaths from CAD by 13,500 to 50,000 annually in the United States.

A number of reports have shown that folic acid supplementation and fortification are effective in increasing serum and red cell folate concentrations and lowering tHcy (Ward *et al.*, 1997, Cuskelly *et al.*, 1996, Jacques *et al.*, 1999). However, the maximum effective dose in lowering tHcy was found to be 200μg folic acid per day (Ward *et al.*, 1997). While higher levels of supplementation increased serum folate concentrations they had no additional effect in lowering tHcy. We therefore set out to examine the affect high dose (>200μg/day) folic acid supplementation might have on the relationship between tHcy and two of the co-enzymes involved in its metabolism, folate and vitamin B_{12}.

4.2. Results

As two of the prime candidates for the regulation of tHcy are folate and vitamin B_{12}, both these vitamins were assayed, as well as tHcy, in the serum of the 24 student midwives [see Chapter 3]. After the initial sample collection subjects took 500μg/day folic acid for four months, at which time blood was again taken for analysis. Supplementation resulted in a significant increase in serum concentrations of both vitamin B_{12} and folate while serum tHcy concentrations were significantly lower in comparison to baseline concentrations ($p < 0.05$) [Table 4.1].

	Baseline	Supplemented
Serum Folate (μg/l)	8.6 (6.1)	15.5 (8.5)*
Serum B_{12} (ng/l)	616 (192)	690 (220)*
Serum tHcy (μmol/l)	11.0 (3.6)	7.4 (2.3)*

Table 4.1. Mean (SD) serum folate, serum vitamin B_{12} and serum tHcy concentrations at baseline and post supplementation with 500μg/d folic acid for 4 months. Significantly different from baseline: *$p \leq 0.001$

Further analysis showed that at baseline (pre-supplementation) there was an inverse correlation between tHcy and serum folate concentrations [Fig 4.1a], but no correlation between tHcy and vitamin B_{12} concentrations [Fig 4.1b]. However, post supplementation the correlation between tHcy and serum folate concentrations was absent [Fig 4.1c] as there was now an inverse correlation between tHcy and vitamin B_{12} concentrations [Fig 4.1d].

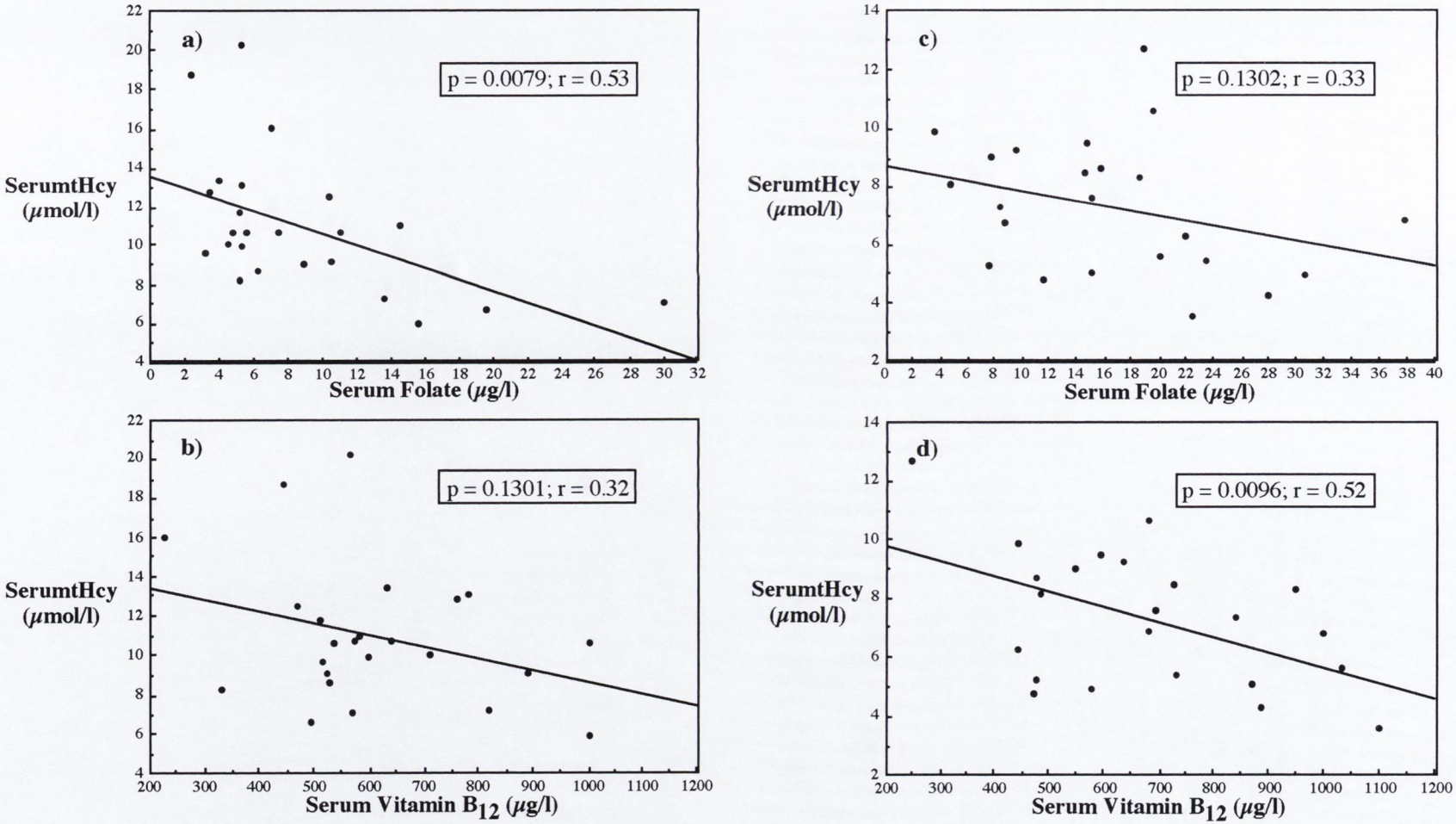


Fig 4.1. At baseline there was a) an inverse correlation between serum folate and serum tHcy but b) none between serum B_{12} and serum tHcy. However, after subjects took 500μg/d folic acid supplementation for four months c) there was no correlation between serum folate and serum tHcy but d) there was between serum B_{12} and serum tHcy.

4.3. Discussion

Here we confirm the previous finding (Ward *et al.*, 1997; Cuskelly *et al.*, 1996; Jacques *et al.*, 1999) that folic acid, when given to subjects normally considered to be replete for the vitamin, was effective in lowering tHcy. On further analysis, we show that tHcy concentrations in these subjects were dependent on the serum folate concentrations, suggesting that in these subjects folate availability is the rate limiting factor in the cycling of homocysteine. However, upon folic acid supplementation this dependence on folate concentration disappears. This suggested that folate concentrations had increased sufficiently so as to be no longer rate limiting. However, as a correlation was now observed between vitamin B_{12} concentrations and tHcy it would appear that availability of vitamin B_{12} coenzyme was now the rate limiting factor in the homocysteine remethylation.

We have shown elsewhere (Quinlivan *et al.*, unpublished data) that this shift in regulation of tHcy concentration from a dependence on folate concentration to a dependence on vitamin B_{12} concentration is gradual and dependent on the level of folic acid supplementation. As supplementation concentrations increase the correlation between serum folate and tHcy decreases until, at about 200μg/day there was no significant correlation between them. Finally, at folic acid concentrations above 200μg/day the correlation between vitamin B_{12} and tHcy increases, becoming significant at supplementation levels of 400μg/day. In this study subjects were replete for vitamin B_{12}. Thus the threshold effect, where folate supplementation has no additional effect in lowering tHcy, could be lower in subjects who are less replete for vitamin B_{12}.

Thus, the finding (Ward *et al.*, 1997) that increasing folic acid supplementation from 200μg/day to 400μg/day had no significant effect on plasma Hcy concentrations may be explained by the fact that supplementation with 200μg/day optimised folate status, resulting in a folate status which was no longer limiting the rate of homocysteine methylation. Therefore, additional supplementation had no further effect on tHcy. However, as vitamin B_{12} would now appeared to be limiting the rate at which homocysteine was recycled. This suggests that vitamin B_{12}, when taken in conjunction with continued folic acid supplementation, may be of further benefit in lowering tHcy relative to folic acid supplementation alone.

CHAPTER 5

FOLATE CATABOLISM AND EXCRETION IN PREGNANT HUMANS

5.1. Introduction

The earliest reported cases of clinical folate deficiency was the "pernicious anaemia of pregnancy" reported by Wills in 1931. As discussed in [Section 1.5] a number of studies have reported an association between inadequate folate intake or status and low birth weight, spontaneous abortion and premature birth. The role of folic acid supplementation in preventing dietary folate deficiency is probably best demonstrated by the study by Baumslag *et al.* (1970) in South Africa. They recruited two population groups, a Caucasian group who's normal diet was folate replete and a Bantu group who consumed a low folate diet. In the Caucasian group folate supplementation had no effect on the already small number of low birth weight children, as the mothers' diet was presumably already replete for folate. However, in the Bantu group folic acid supplementation reduced the incidence of children with birth weight of less than 2.27kg from 30% (19/63) to 6% (4/65), a rate comparable to that of the Caucasian research group. But even in mothers normally considered to be replete, as judged by their plasma and red cell folate concentrations, a positive correlation has been observed between maternal red cell folate concentration and birth weight (Ek, 1982). This increase in birth weight may in part be due to an increased gestational age, as folic acid supplementation has been shown to prolong the mean duration of pregnancy by a week in replete subjects (Blot *et al.*, 1981). This addition

gestational period may be important to the development of the child as term infants have significantly higher red cell folate concentrations than infants of lower gestational age (Ek, 1980). This increase in red cell folate concentrations at term is thought to occur through the increased transfer of folate from the mother to the foetus in the last weeks of pregnancy. The importance of taking folic acid supplements preconceptually or within the first few weeks of pregnancy was demonstrated by the finding that women who begun supplement before their last menstruation period had a significantly higher birth-weight and a lower incidence of children with low birth weight (below 2.5kg) than women who only began supplementation during the first 19 weeks of pregnancy (Rolschau *et al.*, 1999). Meanwhile, the incidence of those congenital malformations classified as occurring in the first 5-6 weeks of gestation (*i.e.* NTD's, microcephalus, anomalies of the eye or ear, cleft lip) when compared as a whole, were significantly decreased when supplementation began before or within the first 5 weeks of pregnancy, compared to mothers who began supplementation after this period (Ulrich *et al.*, 1999). Thus, while in many cases dietary consumption of folate is adequate to sustain normal metabolic function during pregnancy, where there is an underlying folate deficiency, or where folate intake is inadequate to meet demand, this inadequacy may be exacerbated by pregnancy.

Due to complications of pregnancy caused by folate deficiency and because of its historical significance, the means by which folate requirement increases during pregnancy has become a subject for investigation. As the predominant mode of folate loss is through catabolism [see Chapter 3], the role of this mechanism in causing folate deficiency in pregnancy has been investigated. The rate of folate catabolism was shown to increase significantly in rats during pregnancy (McNulty *et al.*, 1993b), while a pilot study (n = 6) conducted in this laboratory (McPartlin *et al.*, 1993) demonstrated a similar increase in

humans. We therefore set out to repeat this earlier human study using a larger number of subjects and employing a more stringent collection protocol.

6.2. Results

Of 31 women recruited five were excluded because they could not tolerate the strict dietary regimen, one woman had a spontaneous abortion at 20 weeks gestation and another was excluded as she developed pre-eclampsia. Of the 24 women included in the final analysis 22 completed all four phases while the remaining two women completed the three antenatal phases but not the postpartum phase [Table 5.1].

Age (years)	Height (cm)	Folate intake (μg/day)*	Birth Weight (gm)
28.4 (5.0)	160.1 (5.8)	365 (148)	3557 (291)
Placental Weight (gm)	Gestation at Birth (weeks)	Nulliparity	Caesarean Section
540 (56)	39.7 (1.2)	12 (50%)	2 (8%)

Table 5.1: Basic clinical characteristics of the pregnant subjects. Results expressed as mean (SD) or n (%) where appropriate. *Measured at second trimester.

Analysis by ANOVA revealed a significant effect of gestation on the mean rate of total folate loss ($p \leq 0.001$), mainly driven by the change in the rate of apABGlu excretion [Fig 5.1]. Mean apABGlu excretion was significantly higher in the third trimester than in the first ($p<0.001$) and second ($p=0.03$) trimesters and postpartum ($p<0.001$). There was no effect of gestation on haemoglobin, red cell folate or serum vitamin B_{12} nor, upon

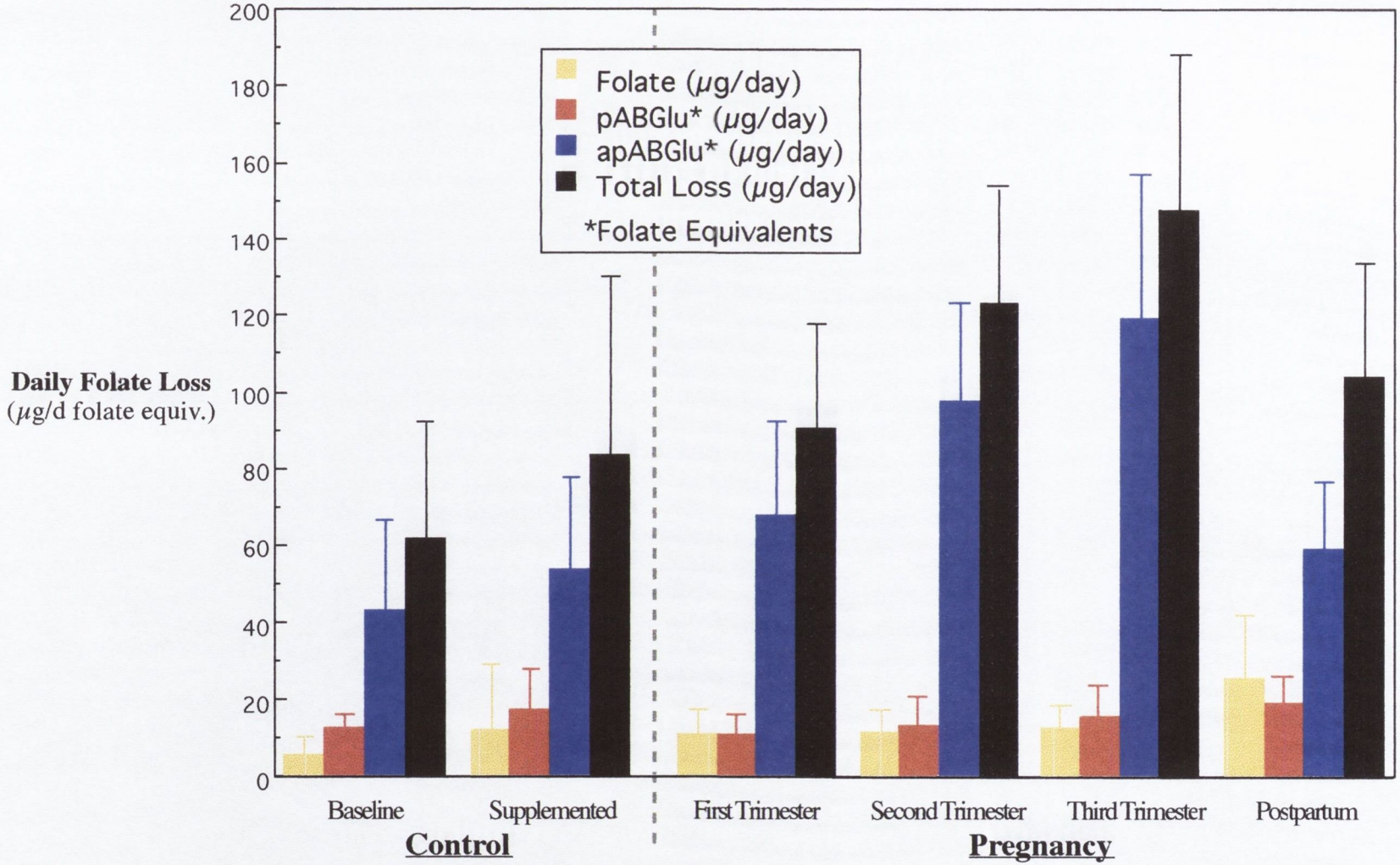


Fig 5.1. Excretion of folate and its catabolites, pABGlu and apABGlu, by non-pregnant controls and at each trimester of pregnancy.

exclusion of postpartum changes, on serum folate [Table 5.2] nor on the rate of excretion of intact folate [Fig 5.1]. The increased folate excretion postpartum ($p \leq 0.001$) must be interpreted carefully due to possible contamination with lochia but correspond to a significant decrease in serum folate concentrations ($p \leq 0.001$).

	1st Trimester	2nd Trimester	3rd Trimester	Postpartum
Haemoglobin (mg/dl)	12.1 (0.9)	11.7 (0.8)	11.9 (1.2)	11.3 (1.4)
Red Cell Folate (ng/ml)	450 (235)	575 (245)	504 (181)	496 (231)
Serum Folate (ng/ml)	11.6 (5.5)	15.3 (8.0)	14.7 (5.9)	5.0 (2.6)
Serum B_{12} (pg/ml)	522 (173)	480 (179)	433 (168)	516 (175)

Table 5.2. Haematological effects of pregnancy. Results expressed as mean (SD).

There was no correlation between the excretion rate of apABGlu, pABGlu or folate with maternal age, maternal weight, maternal dietary folate intake (second trimester), birth weight or placental weight. Parity had no effect on the rate of excretion of folate and its catabolites. However, there was a significant correlation between serum folate concentration and rate of apABGlu excretion at each trimester ($p < 0.01$) but not postpartum. There was also correlation between Δ[serum folate] and Δ[apABGlu] between first and second trimester ($p = 0.002$; $r = 0.656$) and between third trimester and postpartum ($p = 0.002$; $r = 0.646$) but not between second and third trimester, where a bi-phase relationship was observed [Fig 5.2].

The mean rate of total folate loss, i.e. the sum of pABGlu, apABGlu and intact folate, was significantly greater at each stage of pregnancy than the baseline controls [Fig 5.1] and the rate of apABGlu excretion greater than either the non-pregnant baseline or post supplemented controls [see Table 3.2] The dietary folate intake (mean ±SD) in the

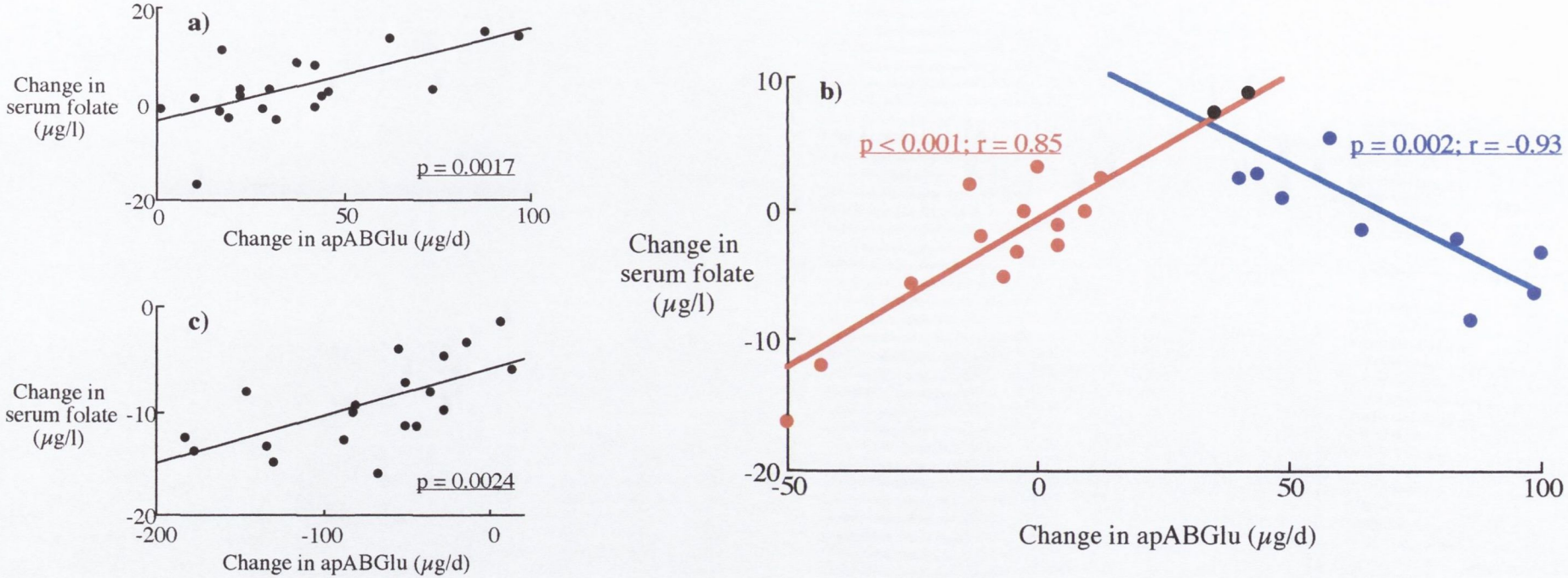


Fig 5.2. Graphs showing the correlation between change in serum folate and change in apABGlu excretion between (a) first and second trimester, (b) second and third trimester, and (c) third trimester and postpartum. The two points in black in (b) are points common to both lines. Points in red and blue are common to their respective lines.

pregnant group (365μg±148) was not significantly different from the non-pregnant group (318μg±120).

5.3. Discussion

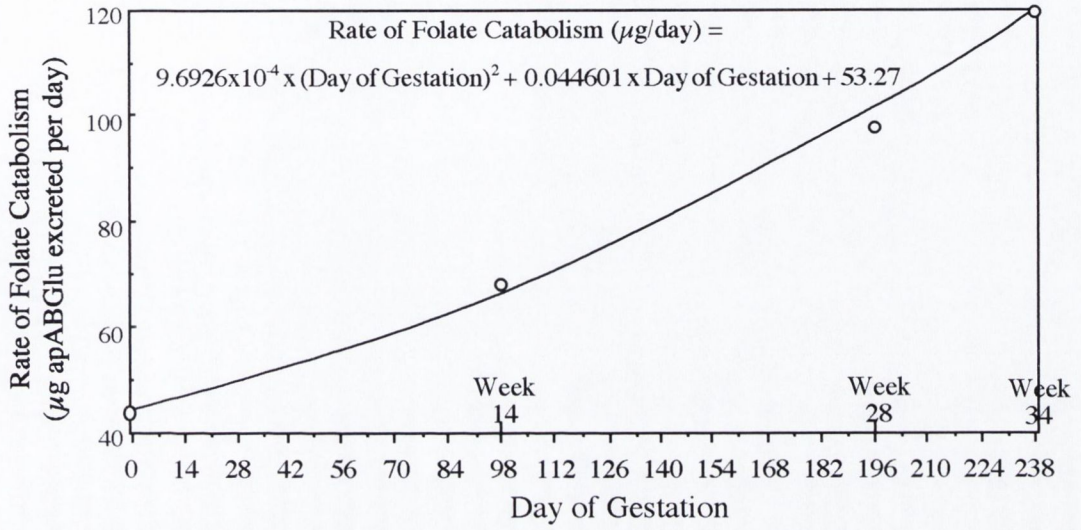
We show that the rate of total folate loss increased significantly during pregnancy [Fig 5.1]. This increased folate loss was driven solely by an increase in the rate of folate catabolism, as represented by the increase in apABGlu excretion. As with non-pregnant subjects, apABGlu excretion accounted for the majority of folate lost during pregnancy.

Folate and pABGlu excretion increased significantly with supplementation in non-pregnant subjects, corresponding to an almost doubling of serum and red cell folate concentrations [Table 3.2]. Pregnant subjects were similarly supplemented between the start of the second trimester and several weeks postpartum. However, there was no significant increase in folate or pABGlu excretion during pregnancy (apart for the increase in folate postpartum) [Fig 5.1]. But neither was there an increase in serum and red cell folate concentrations [Table 5.2]. It is therefore apparent that the increase in pABGlu and folate excretion in non-pregnant subjects was not as a result of folic acid supplementation, *per se*, but rather on the improvement in folate status resulting from supplementation. While folate (Anderson *et al.*, 1960; Johns *et al.*, 1961) and pABGlu excretion (our group, unpublished data) increase post folate consumption, our use of a 16 hour washout period and restricted diet negated the acute effect of dietary folate on this non-metabolic loss of folate.

In contrast, the increase in folate catabolism during pregnancy, as represented by apABGlu, is likely to be metabolic in nature, resulting from hypertrophic and hyperplastic events, such as foetal-placental growth, breast development and expansion of blood volume. By graphing the rate of apABGlu excretion against mean time of gestation and integrating the resulting curve [Box 5.1] we show that by the 34th week of pregnancy a mean of 18.1mg of folate had been catabolised to apABGlu. This represented an increase in folate catabolism due to pregnancy of 7.8mg when compared to the folate catabolised by a non-pregnant subject over the same period (10.3mg). This increase in catabolism represents a considerable drain on the body's total folate reserve, representing a loss equivalent to between 35% and 100% of the body's folate stores (estimates for the body's total folate stores range from 7.5± 2.5mg (Herbert, 1987) to 22±7mg [value derived from Hoppner and Lampi, 1980]). While such an increase in folate catabolism should be adequately provided for by the 500μg/day of folic acid supplement taken by our subjects, it might be expected that in the absence of supplementation pregnant women would suffer severe depletion of folate stores, resulting in a decrease in both serum and red cell folate concentrations. Yet Ek and Magnus (1981) show that in non-supplemented subjects plasma folate concentrations had only fallen 30% by the 34th week of gestation, while red cell folate concentrations increase during pregnancy [Fig 5.3]. This is contrary to the result expected in the absence of supplementation, and points to the possibility of an unknown adaptive mechanism, resulting in increased folate bioavailability in pregnancy, presumably either at the level of the gastro-intestinal tract or at the tissue level. In light of this finding we further analysed our data for evidence in support of this hypothesis.

In both pregnant and non-pregnant subjects a correlation is observed between the rate of folate catabolism (apABGlu excretion) and folate status (serum folate concentrations). It would be easy to assume that the reciprocity was due to folate availability regulating folate

Box 5.1: A graph of rate of folate catabolism (μg apABGlu excretion per day, expressed as folate equivalents) versus mean day of gestation was drawn:



By fitting a second order polynomial equation (r = 1.000):

(1) Catabolism = 5.6925×10^{-4} x (Day)^2 + 1.7639×10^{-1} x (Day) + 43.75

and integrating the equation:

(2) $\int y.dDay = 1.975 \times 10^{-4}$ x (Day)^3 + 8.8195×10^{-2} x (Day)^2 + 43.7 x (Day)

the extent of folate catabolism up to the 34th week (238th day) of pregnancy was calculated:

(3) Iff Day = 0 to 238 days $\therefore \int_0^{238} y.dDay = 18{,}070\mu g \approx 18.1mg$

Over the corresponding period the amount of folate catabolised by a non-pregnant subject was calculated as:

(4) Catabolism per day x Number of days = 43.3μg/d x 34 x 7 = 10.3mg

$\therefore$ The increase in folate catabolism over 34 weeks of pregnancy is therefore:

(5) Pregnancy - Control = 18.1mg - 10.3mg = 7.8mg

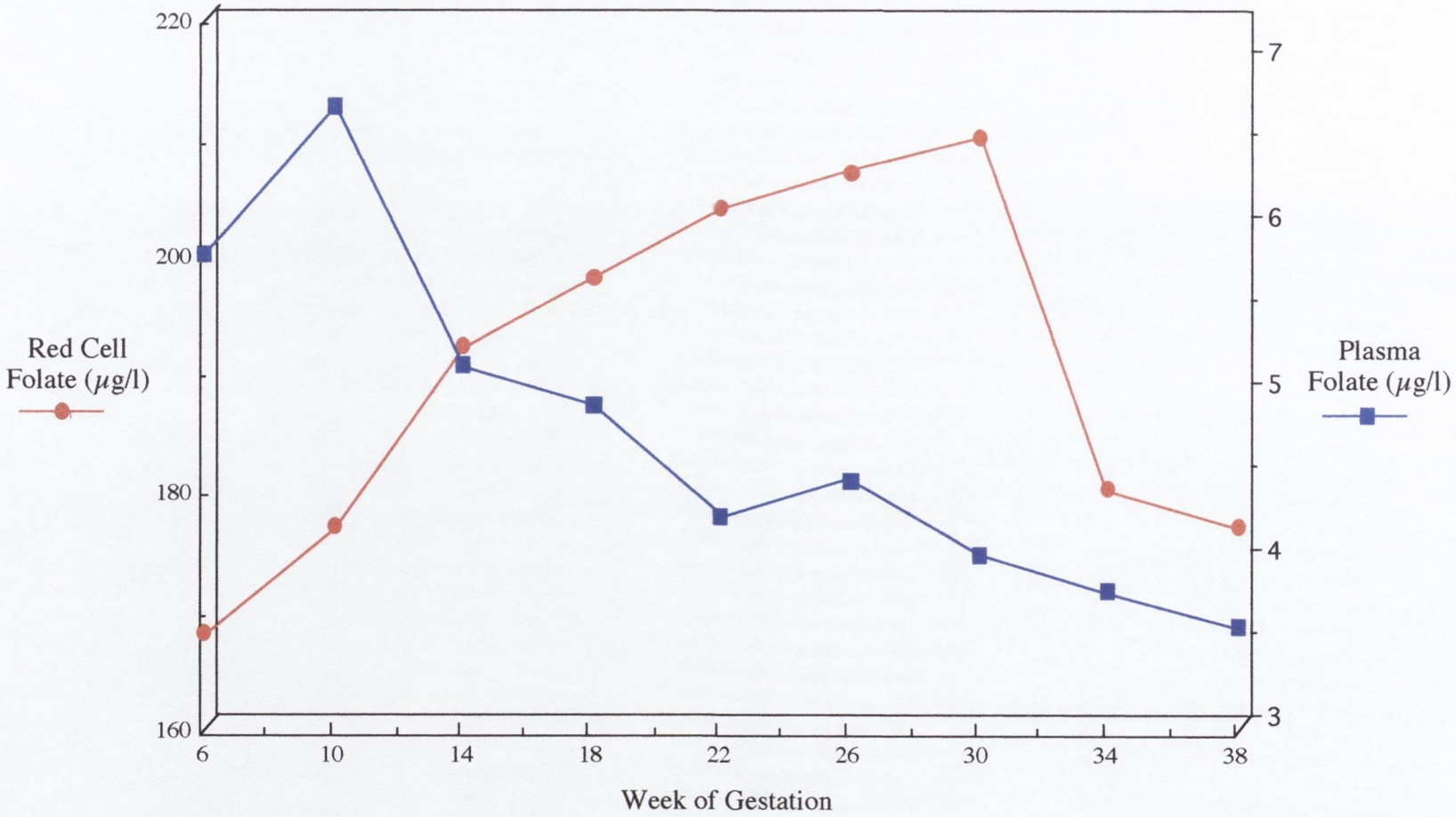


Fig 5.3. Normal changes in red cell folate and plasma folate during pregnancy in non-supplemented subject (Ek and Magnus, 1981).

metabolism. However, analysis of the data does not support such a causal relationship. If serum folate concentrations were regulating apABGlu production it would be expected that upon supplementation, as serum folate concentrations increase, apABGlu concentrations would also increase. However, in non-pregnant subjects apABGlu was not significantly increased by supplementation [see Table 3.2], nor was there a correlation between the change in apABGlu and increase in serum folate [see Fig 3.1b]. It is only during pregnancy that the rate of apABGlu excretion increases, an increase probably due to the acceleration of folate metabolism in pregnancy. This increase in apABGlu excretion is apparent even by the first trimester of pregnancy before supplementation began.

A further illustration that this relationship is driven by apABGlu and not folate status is provided by examining the relationship between the change in serum folate and changes in apABGlu excretion between the trimesters of pregnancy and postpartum. While there was a significantly and positively correlation between the first and second trimesters [Fig 5.2a], and between the third trimester and postpartum [Fig 5.2c], a biphasic relationship was found between the second and third trimester [Fig 5.2b], *i.e.* the stage of pregnancy at which folate catabolism was greatest [Fig 5.1]. We show that between the second and third trimesters, for an increase in apABGlu excretion less than 30μg/day, there is a positive correlation between the change in apABGlu and change in serum folate concentration. However, when the increase in apABGlu is greater than 30μg/day a negative correlation was observed. Thus the rate of apABGlu excretion continues to rise despite the relative decrease in serum folate concentration.

This suggests a mechanism whereby folate status (serum folate) is adaptive to requirement (apABGlu) [Fig 3.1b & Fig 5.2]. It is apparent that this mechanism somehow results from an increase in serum folate concentrations with increasing apABGlu concentrations (Fig

5.2a, and left-hand part of Fig 5.2b). However, while this adaptive mechanism appears useful in maintaining a folate status commensurate to requirement it would appear to be only partially effective as large increases in folate catabolism, such as occur between the second and third trimester, result in a relative decrease in serum folate (right-hand side of [Fig 5.2b]). However, large (>30μg/day apABGlu) increases in catabolism are observed at other stages of pregnancy, as between the first and second trimester of pregnancy, yet a biphasic relationship is only apparent between the second and third trimesters. A likely explanation for this observation is that during this period catabolism reaches a maximum. This, coupled to the earlier attrition of pregnancy (during the first and second trimesters), may result in a situation whereby the body was unable to adapt to large increases in requirement, resulting in the biphasic correlation. As the folate requirement (apABGlu) decreases between the third trimester and postpartum, in part due to cessation of the foetal-placental drain and corresponding maternal physiological changes, a corresponding decrease in serum folate concentration was observed [Fig 5.2c].

A possible mechanism whereby modulation of folate bioavailability may occur is discussed below while a discussion as to why folate status may be matched to requirement is discussed later [Section 11.1].

It is apparent from our data that serum folate concentration and apABGlu excretion are related. However, it is also apparent that this relationship is not driven by a dependence of apABGlu excretion on serum folate concentration, but rather the opposite, whereby serum folate concentrations are dependent on the rate at which apABGlu is produced and excreted. But rather than suggesting apABGlu's production and consequent excretion was directly regulating folate status, a more likely explanation is that the factors, hormonal or otherwise, which regulate catabolism also exert an influence on folate status. However,

above a certain requirement threshold the body is unable to compensate for the increased loss of folate and serum folate concentrations no longer truly reflect requirement.

Possible Site of Modulation Increasing dietary folate consumption, even by 200μg/day, does not result in elevated serum or red cell folate concentrations (Cuskelley *et al.*, 1996), so it is unlikely that the folate status is maintained by increased folate consumption during pregnancy. While far higher (418μg/d) increase in folate consumption did elevate folate status (Riddell *et al.*, 2000) such a doubling of folate intake is unlikely to have physiological relevence in pregnancy. The resulting increase in serum folate concentration was a fourth of that observed with comparable (437μg/d) folic acid supplementation and may thus be a result of increased consumption of "free" folates within the diet [Table 1.1].

The use of folic acid supplementation or fortification has been shown to be effective at elevating serum and red cell folate concentrations, while the increased consumption of natural folates was ineffective at increasing folate status (Cuskelley *et al.*, 1996; Riddell *et al.*, 2000). This suggests that there may be a difference in the manner which the body handles reduced folates and folic acid.

Amounts of folic acid less than 200μg, while effective at elevating serum folate concentrations (Ward *et al.*, 1997), are readily reduced as they pass through the intestinal wall (Sweeny, personal communication). Thus the only manner in which folic acid differs from reduced dietary folate is the manner of its transport across the gut and its ability to increase folate status. This suggests that the possible site of regulation is at the level of the intestine.

Reduced dietary folates are actively transported across the intestine by reduced folate transporters. These reduced folate transporters have a higher affinity for folates with a

short polyglutamate chain. However, as the majority of dietary folates are in the polyglutamate form, with chain lengths predominantly of 4-8 residues, they must first be deconjugated before they are efficiently transported. Dietary folate bioavailability is thus dependent on deconjugase activity in the lumen, with dietary polyglutamates roughly 50% bio-available (Bhandari and J.F. Gregory,1990; Rosenberg, 1981) and monoglutamates 90-100% available (Rosenberg, 1981).

Folic acid, in contrast, is manufactured in the monoglutamate form and is thus not dependent on deconjugase activity for its uptake. In addition folic acid can either be transported by the same carriers as folate or can defuse freely across the lumen wall.

Regulation may be due either to modulation of conjugase activity in the lumen, or may be due to changes in the folate transporter activity. A precedence for such modulation of conjugase activity is the endocrinal regulation of conjugase activity observed by Krumdieck *et al.* (1975 & 1976). Conjugase activity fluctuates in the rat uterus during the menstrual cycle (Krumdieck *et al.*, 1976), reaching a maximum during proestrus, when estrogen secretion is highest, and resulting in elevated uterine folate concentrations. In a similar manner administration of estrogen, in the form of estradiol-17β, resulted in elevated conjugase activity in the uterus of ovariectomised rats (Krumdieck *et al.*, 1975). During pregnancy estrogen concentration increase [Fig 5.4] in much the same manner as apABGlu excretion increases, concentrations of both rising during gestation before falling postpartum.

Stover *et al.* (2000) report that folate is catabolised enzymatically . As the activity of this catabolic enzyme (Stover, personal communication) increases during pregnancy it may be that the factors governing its activity also regulate lumen conjugase activity or trans-intestinal folate transport.

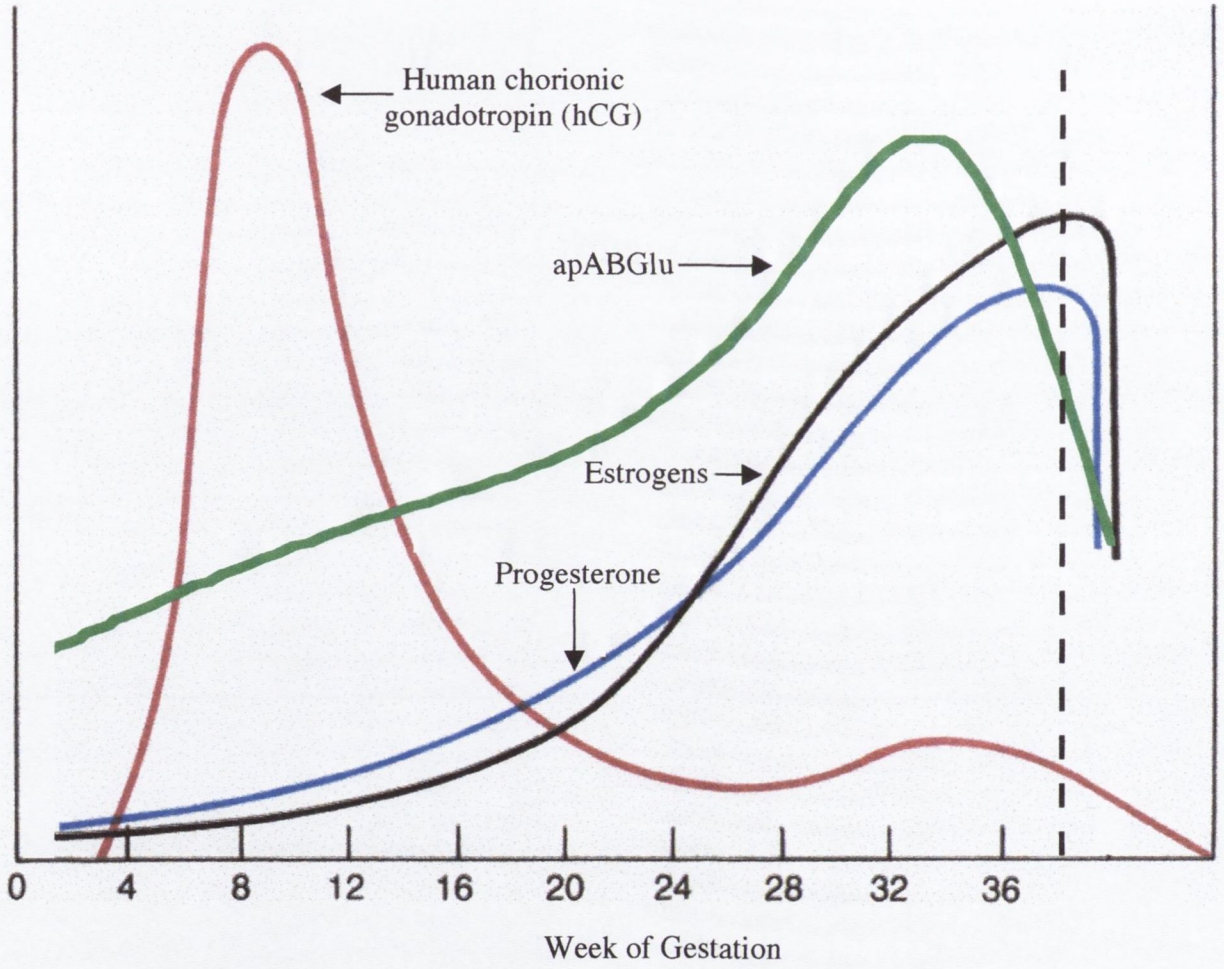


Fig 5.4. Change in sex hormone concentrations (Tortora and Grabowski, 1993) and folate catabolism (apABGlu excretion) during pregnancy and postpartum.

CHAPTER 6

THE EFFECT OF PREGNANCY ON TOTAL SERUM CONCENTRATIONS OF HOMOCYSTEINE

6.1. Introduction

We have already discussed the association between folate deficiency and birth defects and complications of pregnancy in [Section 1.5] and in the introduction to [Chapter 5]. One of the ways in which folate deficiency can manifest itself is by disrupting the methylation cycle, which results in increased tHcy concentrations [Chapter 4]. However, disruption to the methylation cycle can also result from defective enzyme function, *i.e.* thermolabile MTHFR [Section 1.5], or from vitamin B_{12} deficiency. Thus, tHcy may be a better marker of methylation cycle dysfunction than folate status alone. Conditions where a direct associated has been shown between elevated serum tHcy and increased risk include placental abruption, low birth weight, spontaneous abortion (Burke *et al.*, 1992; Wouters *et al.*, 1993; Dekker *et al.*, 1995; Mills *et al.*, 1995;Goddijn-Wessel *et al.*, 1996; Rajkovic *et al.*, 1997) and coronary-heart disease (CHD) (Boushey *et al.*, 1995). However, while it is possible that the association between serum tHcy and risk of CHD results from direct haematological interaction, for instance activation of blood clotting (Rodgers and Conn, 1990; Rodgers and Kane, 1986; Lentz and Sadler, 1991; Hayashi *et al.*, 1992; Hajjar, 1993) or endothelial dysfunction (Chambers *et al.*, 1997), it is not immediately clear how serum tHcy would cause complications during pregnancy. It appears more likely that serum tHcy is a secondary marker of cellular conditions, and in particular cellular SAH

concentrations (Dayal *et al.*, 2000). Thus elevated serum tHcy concentration would be indicative of elevated cellular SAH concentrations. Accumulation of SAH in the cell would result from, and result in, aberrant function of the methylation cycle. Defective entry of methyl-groups into the cycle, as may occur during folate deficiency, retards Hcy methylation which in turn increases SAH concentrations and consequently increase product inhibition of the methyltransferases (Cantoni *et al.*, 1978). Studies *in vitro* show that SAH is an effective DNA hypomethylating agent (DeCabo *et al.*, 1995), while adenosine and homocysteine in combination inhibit protein methylation (Ingrosso *et al.*, 1997). Thus serum tHcy is likely to be good indicator of methylation cycle function. Further evidence to suggest that serum tHcy is a true indicator of cellular methylation is suggested by the inverse correlation observed between the methyl-group donors, *i.e.* folate and vitamin B_{12}, and serum tHcy [see Chapter 4].

As we have shown in [Chapter 5] folate utilisation is increased during pregnancy. Where folate intake is insufficient to replace this increased loss this could result in a depletion of folate concentrations, manifesting itself as clinical deficiency (Wills, 1930). This, coupled with the decrease in vitamin B_{12} observed in pregnancy [see Chapter 5] should theoretically affect the methylation cycle's ability to maintain adequate methyl-transfer. As methyl-groups are required in the synthesis of numerous compounds required for foetal development [Table 6.1] disruption of the methylation cycle may account for the complications of pregnancy observed. We therefore set out to determine what mechanism may exist to prevent hypomethylation. When our study was originally initiated only two papers had been published looking at the effects of pregnancy on tHcy (Kang *et al.*, 1986; Andersson *et al.*, 1992). While both showed that maternal tHcy decreased during pregnancy, neither study looked at the effect of pregnancy on serum folate and vitamin B_{12}, or at the relationship of serum tHcy to these two co-enzymes during pregnancy.

Product	Function
DNA methylation	Epigenic gene regulation[1], regulation of transcription[2] and tissue differentiation[3]
Histone[4]	Epigenic gene regulation and regulation of transcription[5]
RNA methylation[6]	Regulation of transcription and RNA maturation
Myelin production[7]	Nerve development
Phosphatidylcholine[8]	Development of the cell membranes and nerves
Neurochemicals[9]	Neural function
Creatine[10]	Muscle development
Protein	Activation of heat shock proteins[11]. Signal Transduction[12]

Table 6.1. List of some of the end products of pathways involving SAM dependent methyltransferases. ([1]Bartolomei and Tilghman, 1997; Heard *et al.*, 1997; Wutz *et al.*, 1997; Okano, 1998; Gruenbaum *et al.*, 1983. [2]Tao *et al.*, 2000; Wolf and Migeon, 1985; Nan *et al.*, 1998; Hsieh, 1994; Razin, 1998. [3]Gama-Sosa *et al.*, 1983; Ehrlich *et al.*, 1982. [4]Cantoni *et al.*, 1979. [5]Cameon *et al.*, 1999; Razin, 1998; Nan *et al.*, 1998. [6]Cantoni *et al.*, 1979; Long *et al.*, 1983. [7]Cantoni *et al.*, 1979. [8]Prasad and Edwards, 1981; Crews *et al.*, 1980. [9]Cantoni *et al.*, 1979. [10]Cantoni *et al.*, 1979. [11]Ladino and O'Connor, 1992; Wang *et al.*, 1992. [12]Hrycyna and Clarke, 1993; Yamane and Fung, 1993).

6.2. Results

We show tHcy concentrations were significantly ($n = 24$; $p<0.02$) lower during the second trimester than at any other stage of pregnancy [Table 6.2]. Furthermore, tHcy concentrations during all 3 trimester of pregnancy were significantly lower than the postpartum ($p < 0.001$) or baseline control ($p < 0.001$) concentrations. The second and third trimester concentrations were also lower ($p < 0.01$) than the supplemented control concentrations [Table 6.2]. Postpartum tHcy concentrations increased to a level which was comparable ($p > 0.05$) to that of the baseline or supplemented control groups. This postpartum rise in tHCy corresponded to a 60% decrease in serum folate concentrations compared to the 3rd trimester [Table 6.2].

As stated previously [Chapter 5] there was no significant change ($p > 0.05$) in serum or red cell folate concentrations during pregnancy except for the decrease in serum folate observed postpartum [Table 5.2]. B_{12} concentrations were lower during the third trimester than at any other stage of pregnancy or postpartum, however, this difference was only significant ($p < 0.005$) when compared to first trimester or postpartum concentrations.

6.3. Discussion

The design of this study was constrained by the Ethical Committee's requirement that all pregnant subjects had to be supplemented with folic acid. However, the observed decrease in tHcy concentration during pregnancy could not be accounted for by this intervention as serum tHcy concentrations were considerably lower at all stages of pregnancy than even the supplemented control group despite both groups having comparable concentrations of

	baseline controls	supplemented controls	first trimester	second trimester	third trimester	postpartum
serum homocysteine (μmol/l)	$11.0\ (3.6)_{A}$	$7.4\ (2.3)_{A}$	$6.0\ (3.6)^{1}{}_{a,C}$	$4.2\ (1.5)^{1,2}{}_{b,D}$	$5.6\ (1.6)^{1,2}{}_{E}$	$9.0\ (3.4)^{2}{}_{C,D,E}$
serum folate (μg/l)	$8.6\ (6.1)_{A}$	$15.5\ (8.5)_{A}$	$11.6\ (5.5)_{B}$	$14.8\ (8.0)^{1}{}_{C}$	$14.7\ (5.9)^{1}{}_{D}$	$5.9\ (4.9)^{2}{}_{B,C,D}$
serum vitamin B_{12} (ng/l)	$616\ (192)_{a}$	$690\ (220)_{a}$	$522\ (172)^{2}$	$480\ (178)^{1,2}$	$433\ (168)^{1,2}$	$516\ (175)^{2}$

Table 6.2. Serum homocysteine, folate and vitamin B_{12} concentrations [mean (±SD)] for blood taken from controls, at each trimester of pregnancy and postpartum. Values with the same subscript are significantly different: Uppercase ($p< 0.001$; LSD), Lowercase ($p<0.05$; LSD). Superscripts: significantly different ($p<0.05$; pooled t-test) from [1]baseline controls; [2]supplemented controls.

serum folate, red cell folate and vitamin B_{12} (red cell folate was higher only in the third trimester of pregnancy than the supplemented controls). Moreover, by the first trimester of pregnancy, before supplementation began, serum tHcy concentrations were significantly lower than either the baseline or supplemented controls. Also, concentrations of tHcy increased to non-pregnant control levels postpartum despite continued supplementation. However, of greatest relevance in showing that the decrease in tHcy observed in pregnancy was not the result of supplementation are those studies where supplements were not taken (Andersson *et al.*, 1992) or where comparisons were made between those taking and not taking supplements (Walker *et al.*, 1999). The results from both these studies appear comparable to ours [Fig 6.1].

None of the papers published thus far (Andersson *et al.*, 1992; Walker *et al.*, 1999; Bonnette *et al.*, 1998) have suggested a suitable explanation to explain why tHcy falls during pregnancy. The decrease in tHcy is of too great a magnitude and occurred to early in pregnancy to be caused by haemodilution. Likewise, the decrease in tHcy occurs too early in foetal development to be due to the transference of Hcy or its metabolites (SAM, SAH or Methionine) from the mother to the embryo. By the 10^{th} week of gestation maternal tHcy had fallen more than 30% relative to non-pregnant subjects (Andersson *et al.*, 1992), yet the foetus weighs only 1g. We propose that the decrease in tHcy during pregnancy may be the result of an adaptive mechanism invoked to protect the methylation function of the one carbon cycle and thus foetal development. We further propose that this mechanism may be hormonally regulated.

SAH is a competitive (product) inhibitor for the majority of methyltransferase reactions for which SAM is substrate. Under physiological conditions methyltransferase activities are regulated not only by concentration of SAM but also of SAH [Table 6.3 and 6.4]. The

		Liver	Kidney	Muscle	Heart	Cortex	Spinal Cord
SAM (μM)	Air[1]	101 (40)	41 (7)	24 (13)	42 (12)	36 (13)	34 (10)
	N_20[2]	50 (23)	39 (10)	25 (13)	34 (13)	27 (9)	19 (16)
Mann-Whitney		$p < 0.02$	NS	NS	NS	NS	NS
SAH (μM)	Air[1]	24 (3)	8 (3)	5 (3)	6 (3)	5 (2)	3 (1)
	N_20[2]	60 (34)	48 (16)	26 (24)	14 (7)	41 (18)	17 (3)
Mann-Whitney		NS	$p < 0.01$	$p < 0.005$	$p < 0.02$	$p < 0.005$	$p < 0.005$

Table 6.3. Concentration of SAM and SAH in pig tissue [1]before and [2]after inhalation of N_20 for seven days to inhibit methionine synthase. NS: No significant difference (from Molloy *et al.*, 1990).

	K_m SAM (μM)	**K_i SAH (μM)**	**%Inhibition in Tissue***			
			Liver	Kidney	Cortex	Spinal Chord
epinephrine-O-MT	570	15	57	34	24	18
Phenylethanolamine-MT	10	1.4	60	54	43	36
acetylserotonin-MT	14	2	59	52	41	33
glycine-MT	100	35	25	15	9	7
guanidoacetate-MT	47	17	31	21	14	10
histone-MT	14	5.5	34	28	20	15
(Gua-7)-MT	14	1	74	68	58	49
phosphatidylethanolamine-MT	1.6	1.4	21	18	13	10
protein-MT	3	7	9	8	5	4

Table 6.4. Kinetic constants, K_m and K_i, for a selection of methyltransferase (MT) enzymes (from Cantoni *et al.*, 1978). *Table also shows the percentage enzyme inhibition due to product inhibition by SAH as predicted by Michaelis-Menten kinetics, tissue concentrations for SAM and SAH are from [Table 6.3].

potential of the methylation cycle to transfer methyl-groups is often, if somewhat erroneously [Box 6.1], referred to as the "SAM:SAH ratio", the ratio of substrate to inhibitor. *In vivo* this ratio is acutely sensitive to changes in methionine synthase (MS) activity (Molloy *et al.*, 1990 and 1992).

However, both SAM synthase (Oden and Clarke, 1983; Kotb and Kredich, 1985 and 1990) and MTHFR (Kutzbach and Stokstad, 1971; Jencks and Matthews, 1987; Green *et al.*, 1988) are inhibited by physiological concentrations of SAM. Thus, a fall in SAM concentrations would decrease both feedback inhibition of SAM synthase and allosteric inhibition of MTHFR, increasing SAM production and stabilising SAM concentrations. Therefore, the decrease in SAM: SAH ratio is due predominantly to elevated SAH concentrations, rather than decreased SAM concentrations [Table 6.3 (Molloy *et al.*, 1990)]. This increase in cellular SAH concentrations would be reflected in a corresponding increase in serum tHcy concentrations (Dayal *et al.*, 2000).

In pregnancy the entry of methyl-groups into the methylation cycle would be expected to decrease due to lower B_{12} concentrations [Table 5.2] and the prevalence of folate deficiency (Wills, 1930). As outlined above, such disruption of the methylation cycle would be expected to increase SAH concentrations and, consequently, serum tHcy concentrations. During pregnancy tHcy concentrations would be expected to rise or at best, if the perturbation to the cycle was not severe, perhaps remain unchanged. On the contrary, we found a significant decreases in serum tHcy concentrations in pregnancy suggesting an adaptive mechanism in pregnancy.

The importance of such an adaptive mechanism in pregnancy is obvious. Even a mild decrease in folate or B_{12} concentrations, as can occur in pregnancy, could decrease the

Box 6.1 Kinetics of Inhibition.

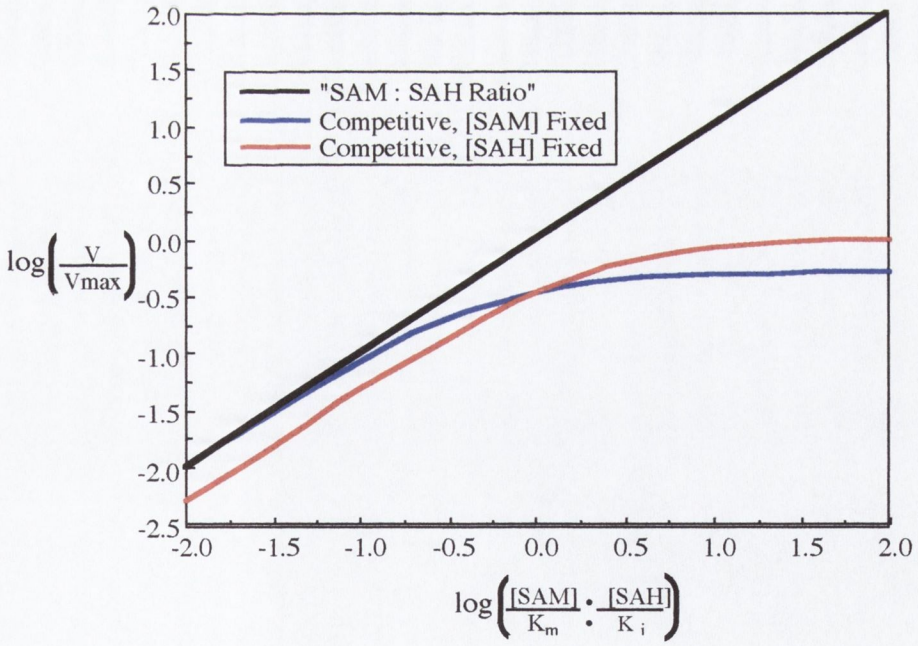
If methyltransferase (MTase) activity was regulated by a simply SAM: SAH ratio the Michaelis-Menten equation for the reaction could be written as:

$$V = \text{Vmax} \frac{[SAM] / K_M}{[SAH] / K_i}$$

However, the Michaelis-Menten equation describing the kinetic behaviour of an enzyme in the presence of a competitive inhibitor, such as the SAH inhibition of MTase activity with SAM as substrate, is given by:

$$V = \text{Vmax} \frac{[SAM] / K_M}{1 + [SAM] / K_M + [SAH] / K_i}$$

It is obvious therefore, as there is an additional factor $(1 + [SAM] / K_M)$ in the denominator, that the MTases do not conform to simple kinetics defined by the SAM : SAH ratio. Using arbitrary values to plot the ratio of $[SAM] / K_M$ to $[SAH] / K_i$ against $V / Vmax$ it becomes apparent that the actual reaction rate is lower than that predicted by the SAM: SAH ratio:



As the concentration of inhibitor, [SAH], increases the contribution of the factor $(1 + [SAM] / K_M)$ to the denominator diminishes, such that when $[SAH] / K_i$ is 100 times $(1 + [SAM] / K_M)$ there is only about a 1% variation from "SAM: SAH ratio" kinetics. However, as can be seen from [Table 6.3 & 6.4], such a physiological state is unlikely to be reached.

efficiency of the methylation cycle and increase SAH concentrations. The methyl-transfer function of the cycle would thus be compromised not only because of decreased influx of methyl-groups into the cycle, due to decreased co-enzyme concentrations, but also because of the increased inhibition of the methyltransferases by SAH. As many of the products of the methyltransferases are important for embryonic development [Table 6.1] such hypomethylation could be detrimental to foetal development. However, by lowering SAH concentration *in vivo* the risk of hypomethylation occurring is also decreased.

Regulation of SAH metabolism SAH hydrolase exhibits considerable (3-4 fold) variation in activity, being influenced diurnally (Chagoya De Sánchez *et al.*, 1991 and 1996; Chagoya De Sánchez, 1994) as well as by changes during the cell cycle (Ichikawa *et al.*, 1985; Chiba *et al.*, 1984). During cell replication (S- and G_2-phases) SAH hydrolase activity increases three-fold relative to the resting cell (G_1-phase). Thus, as the number of replicating cells increases, for instance during pregnancy, SAH hydrolase activity also increases. However, as the equilibrium constant for SAH hydrolase is strongly displaced towards SAH synthesis (Døskeland and Ueland, 1982; Hershfield *et al.*, 1985; Porter and Boyd, 1991; Ueland, 1982) this activation of SAH hydrolase would not automatically result in a decreased SAH concentration. For this to happen further metabolism of the two reactions products, adenosine and Hcy, would be required.

Regulation of adenosine metabolism As with SAH concentrations, adenosine also exhibits considerable diurnal variation (Chagoya De Sánchez *et al.*, 1991 and 1996; Chagoya De Sánchez, 1994). This in turn is due to a 3-4 fold circadian variation in the activity of the enzymes which synthesise (5'-nucleotidase and SAH hydrolase) and catabolise (adenosine deaminase and adenosine kinase) adenosine. This circadian modulation of all four adenosine metabolising enzymes would appear to contributes to variation in the SAM:

SAH ratio (Chagoya De Sánchez *et al.*, 1991). One physiological consequence of this variation in methylation function is apparent in the circadian fluctuation in the phosphatidylcholine (PC): phosphatidylethanolamine (PE) ratio (Chagoya De Sánchez, 1994): As three molecules of SAM are required to convert PE to PC (Crews *et al.*, 1980; Prasad and Edwards, 1981) this reaction would be sensitive to fluctuation in the SAM: SAH ratio. This may have implications for the critical development of central nervous system (CNS) for the developing foetus.

Regulation of homocysteine metabolism It is likely that Hcy metabolism is hormonally regulated, as postmenopausal increases in tHcy are lowered by hormone replacement therapy (M.J. van der Mooren *et al.*, 1994 & 1995; J. Silberberg *et al.*, 1995). Further evidence for endocrine regulation of Hcy metabolism comes from Jacobs *et al.* (personal communication) who show that glucagon can decrease tHcy concentrations by 30% due to a two-fold increase in cysthationine-β-synthase (CβS) concentrations and that a similar induction of CβS concentrations can be achieved using the synthetic glucocorticoid triamcinolone. Fig 6.2 shows some of the hormonal changes which occur in pregnancy.

Both SAH (DeCabo *et al.*, 1995) and a combination of adenosine and homocysteine (Ingrosso *et al.*, 1997) are potent undermethylating agent. Likewise, inhibition of SAH hydrolase, resulting in SAH accumulation, causes DNA and RNA hypomethylation and inhibition of cell proliferation (Kramer *et al.*, 1990). Thus, accumulation of Hcy or SAH during pregnancy could have detrimental effects on embryonic development. However, it is also apparent that most of the enzymes which regulate SAH metabolism exhibit considerable variation in activity due endocrinal, circadian and cell cycle influences. These regulators may thus contribute to the control of Hcy/ SAH metabolism in pregnancy, preventing Hcy/ SAM accumulation and thus preventing hypomethylation.

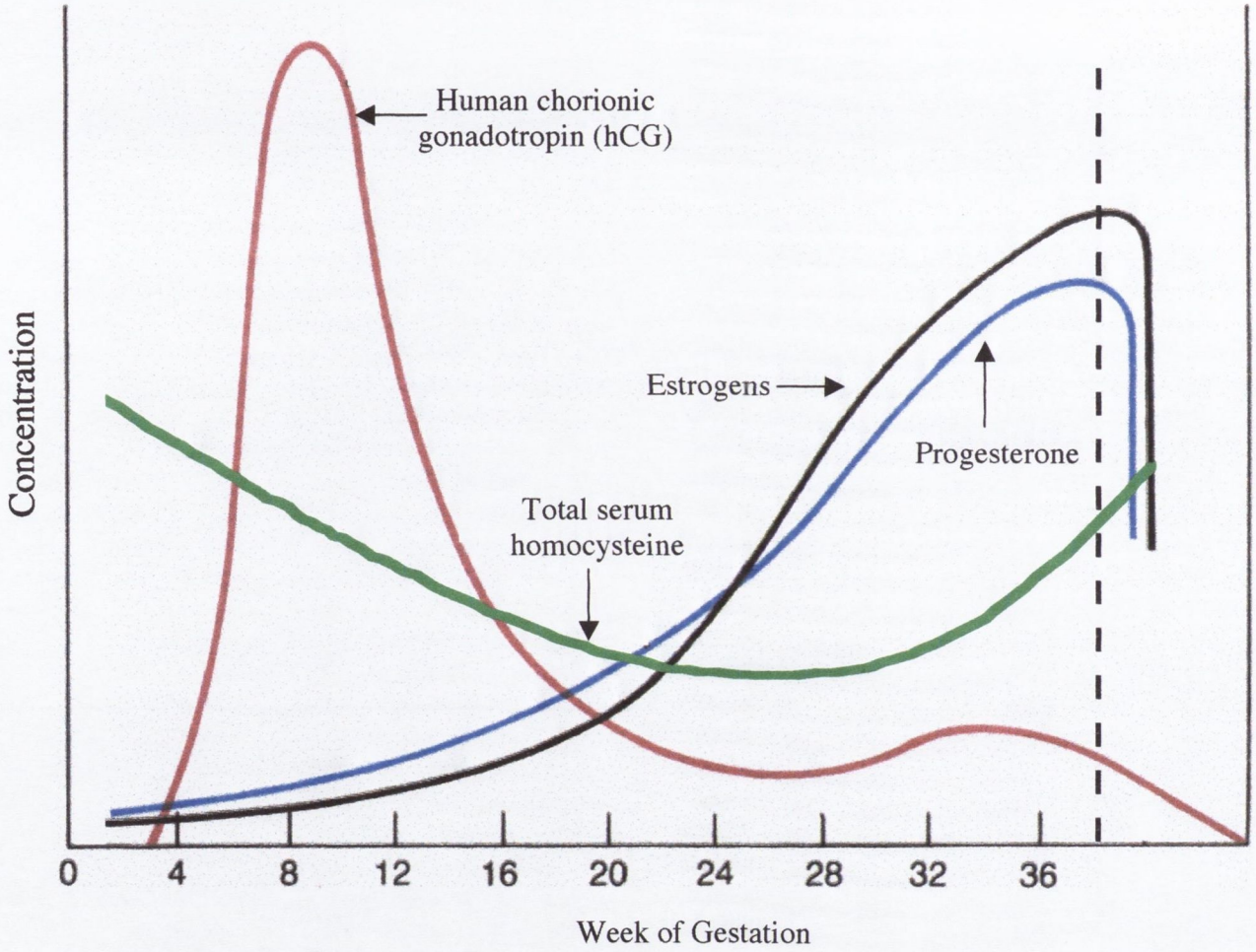


Fig 6.2. Hormone concentrations (Tortora and Grabowski, 1993) and total serum homocysteine concentrations during pregnancy and postpartum.

PART II

STUDIES IN BACTERIA AND CELL CULTURES

Let's set the existence-of-god issue aside for a later volume, and just stipulate that in some way, self-replicating organisms came into existence on this planet and immediately began trying to get rid of each other, either by spamming their environments with rough copies of themselves, or by more direct means which hardly need to be belabored. Most of them failed, and their genetic legacy was erased from the universe forever, but a few found some way to survive and to propagate.

Neal Stephenson (1999)

Cryptonomicon

(Avon Books, New York)

CHAPTER 7

TRIMETHOPRIM INHIBITION OF FOLATE METABOLISM AND BIOSYNTHESIS IN *ESCHERICIA COLI*

7.1. Introduction

Folate coenzymes are required for the transfer of one-carbon units in the biosynthesis of purines and pyrimidines, in amino acid interconversions and for the provision of methyl groups [See Section 1.3]. The critical function of dihydrofolate reductase (DHFR) in restoring tetrahydrofolate (THF) concentrations following thymidylate biosynthesis established the basis for antifolate drug therapy not only in infection but also in cancer, inflammatory and autoimmune conditions (Brumfitt and Hamilton Miller, 1980). Though the consequences of THF depletion include the cessation in biosynthesis of purines and protein, the metabolic block of greatest severity in animals and bacteria is thought to be the interruption of thymidylate synthesis , the so-called 'thymine-less death' (Blakley, 1969).

The 2,4-diaminopyrimidine derivative Tmp is the preferred inhibitor of bacterial dihydrofolate reductase in clinical and veterinary use. This is because of its broad spectrum of antibacterial effects, its high degree of selectivity for the bacterial enzyme and its suitable pharmacodynamic and pharmacokinetic properties (Bushby and Hitchings, 1968). It was for their work in this sphere that Hitchings and Eilion were awarded the Nobel Prize for Medicine in 1988 (Hitchings, 1989). In combination with sulfonamides such as sulphamethoxazole, which inhibit folate biosynthesis, the antibacterial spectrum of

Tmp was extended, greater potency was achievable with lower doses resulting in fewer side effects, and bactericidal power was increased compared to the more bacteriostatic activity of the single components. With or without a sulphonamide, however, a feature of antifolate action is the cessation of nucleotide biosynthesis coincident with disruption of folate metabolism.

The purpose of this study was to examine the hypothesis that antifolates such as Tmp cause their effect through accumulation of dihydrofolate (DHF) and the consequent diminution of the tetrahydrofolate pool that occurs with inhibition of dihydrofolate reductase.

7.2. Results

7.2.1. Inhibition of Folate Metabolism by Trimethoprim in *E.coli*

Optimisation of growth conditions The first aim of the methods was to ensure that the labelling and treatment phases were conducted during log-phase growth [Table 7.1]. After these arbitrary incubation periods the culture was prepared for inoculation of the subsequent growth. Harvesting and washing the labelled *E.coli* by centrifugation had little effect on their subsequent growth [Fig 7.1].

Preparation of inhibition curves Treatment with 20pg/ml Tmp resulted in a 2% inhibition of growth [Fig 7.2]. Half maximum growth, after 4h incubation, was achieved using approx. 60ng/ml Tmp, while 2μg/ml was the lowest concentration of Tmp to give maximum (approx. 98%) growth inhibition.

Growth Period	Approx. Start Log-Phase Growth	Approx. End Log-Phase Growth	Bacteria Harvested
Pre-labelling	2-3 h	13-14 h	11 h
Labelling	0 h	11-12 h	9 h
Treatment	0 h	5-6 h	0 to 4 h

Table 7.1. Periods of log phase growth in *E.coli* under the growth conditions used.

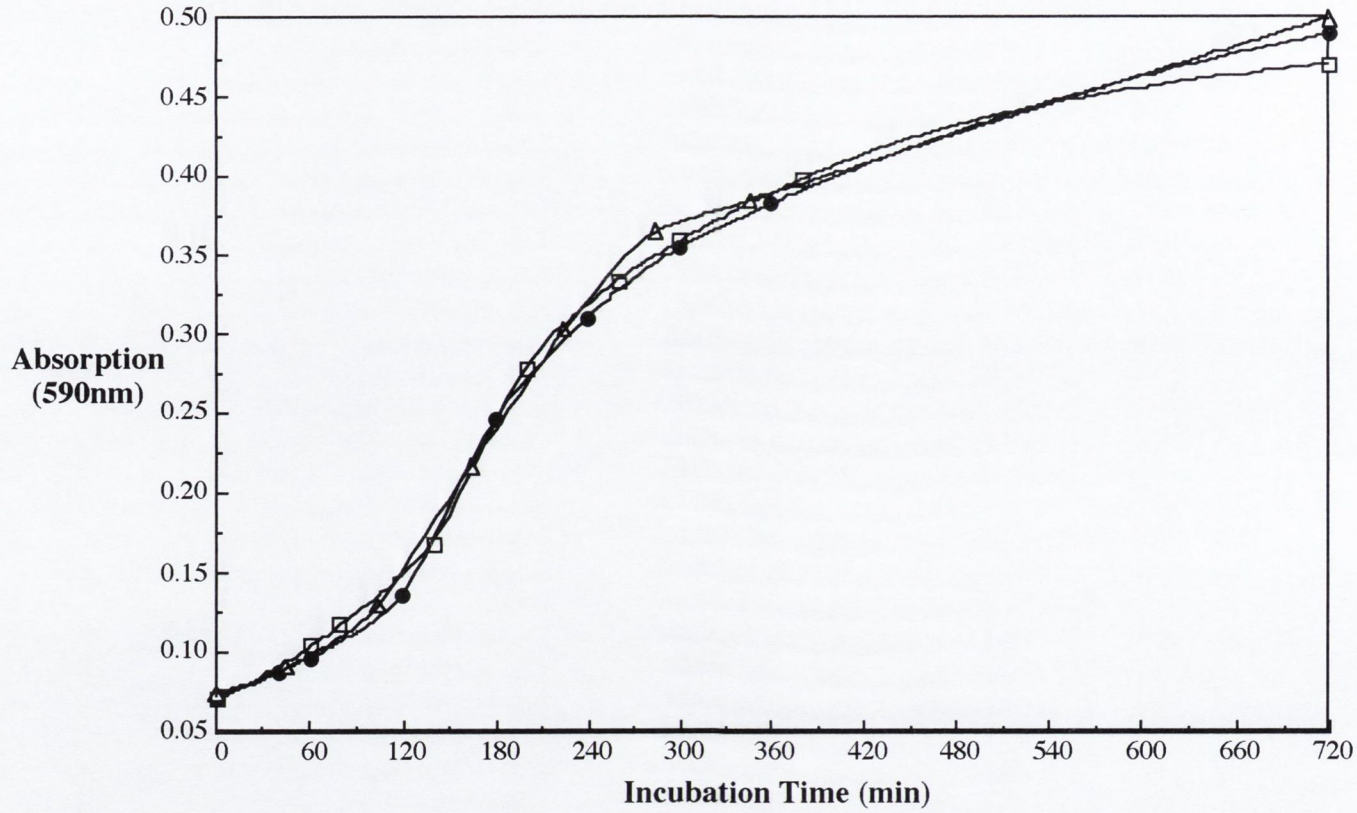


Fig 7.1. *E.coli* growth curve for the third (treatment) incubation period. The second incubation growth was grown to log phase before □ 1ml of the suspension, ● 1/5th of the unwashed pellet (harvested by centrifugation) or Δ 1/5th of the washed pellet (resuspended in growth media and repelleted) was used to inoculate the third growth.

Effect of optimum Tmp treatment on folate distribution At time zero or after 4h incubation the folates in the untreated *E.coli* were present exclusively as THF co-enzymes [Table 7.2]. Treatment with 2μg/ml Tmp, however, resulted in the rapid but transient accumulation of dihydrofolate. Thus, within 10min of Tmp treatment the radiolabeled THF co-factor content was reduced by two thirds, half the folate was in the form of dihydrofolate and a significant fraction had catabolised to pABGlu. Within 4h of treatment, however, the distribution of intracellular radiolabel stabilised, with cells depleted not only of THF co-enzymes but also of dihydrofolate. Assay of total cellular folate by microbiological assay [Table 7.3] showed a substantial reduction in folate in Tmp-cells at 4h (to 25% of control folate/ cell).

	Folate Content (ng/1x10^9 Cells)	Folate Content % Control (4h)	% Change in Growth (ΔAbs_{590})
Control[1]	144.8	96.0	0.0
Control[2]	150.7	100	100
60ng/ml Tmp[2]	51.1	33.9	55.7
2μg/ml Tmp[2]	37.5	24.9	2.7

Table 7.3. Folate content of trimethoprim treated or untreated (control) *E.coli* at the [1]start of incubation or [2]after 4h incubation

Authentication of folic acid The folic acid component of the Tmp treated cellular extract was authenticated by its co-elution with folic acid standards from a Kingsorb column, eluted with PicA/ acetate buffer, or from a μBondapak column, eluted with citrate-phosphate buffer [see Chapter 2]. The folic acid peak from the Kingsorb column was further authenticated by its enzymatic conversion by bovine liver DHFR to THF. While there was no change in elution time of the radiolabel peak from the control sample,

Incubation Time	*THF coenzymes*	*DHF*[A]	*pABGlu*	*Folic Acid*[B]
Control[C]	100	0	0	0
10 min.[D]	30	48	17	5
25 min.[E]	12	49	23	16
60 min.	7	33	33	27
120 min.	6	23	39	32
240 min.	0	2	58	40
24 Hrs	0	0	55	45

Table 7.2. Results of a representative experiment showing the effect of Tmp inhibition on the percentage [^{3}H]-folate distribution in *E.coli* . Control samples were harvested at 0 or 240 minutes incubation. Trimethoprim treated (2μg/ml) samples were harvested [D]as soon as the Tmp was added, after [E]15 min. incubation (harvesting and extraction took 10 min.) or after the incubation time shown. [A]Under the extraction and deconjugation conditions used dihydrofolate standard was found to be greater than 89% (±2%; n= 4) stable. [B][^{3}H]-Folic acid was authenticated as described in [Section 2.4.6].

incubated in the absence of DHFR, the labelled folate from the DHFR treated sample co-eluted with an authentic THF standard.

Effect of sub-optimum concentrations of Tmp on folate distribution While the previous experiments contrasted the effect of maximum inhibition to that of uninhibited conditions we also wished to determine the effects of sub-optimum inhibitory conditions on folate catabolism. As the number of samples was too large to chromatograph at once all the samples were stored at –70°C pre-analysis. This resulted in the complete catabolism of any residual DHF to pABGlu (and pteridine). "catabolism" in [Fig 7.2] therefore represents the sum of DHF and pABGlu in the cell. At 2μg Tmp per ml this would represent total *in vivo* cleavage to pABGlu, however, at lower Tmp concentrations an indeterminate ratio of pABGlu to DHF may exist.

Tmp concentrations between 20pg/ml and 2μg/ml resulted in the dose dependent inhibition of growth and increase in "catabolism" [Fig 7.2], resulting in an inverse correlation between "catabolism" and cell proliferation [Fig 7.2, inset].

Characterisation of the pABGlu product of Tmp inhibition To characterise further the pABGlu product of Tmp inhibition [Table 7.1] we determined its polyglutamate distribution. Chromatographic conditions utilised for the separation of $pABGlu_n$ [Chapter 2] adequately separated the polyglu derivatives according to their chain length, expect for those of n = 1 to 3, which co-eluted. The first folate monoglutamate eluted after 29min, subsequent to $pABGlu_8$ but before $pABGlu_9$. Chromatography of cell extracts untreated with conjugase [Fig 7.3] showed that the pABGlu portion, *i.e.* 57.1% of total radiolabel [Fig 7.3, inset], consisted mainly of $pABA\text{-}polyglutamates_{(5\text{-}8)}$.

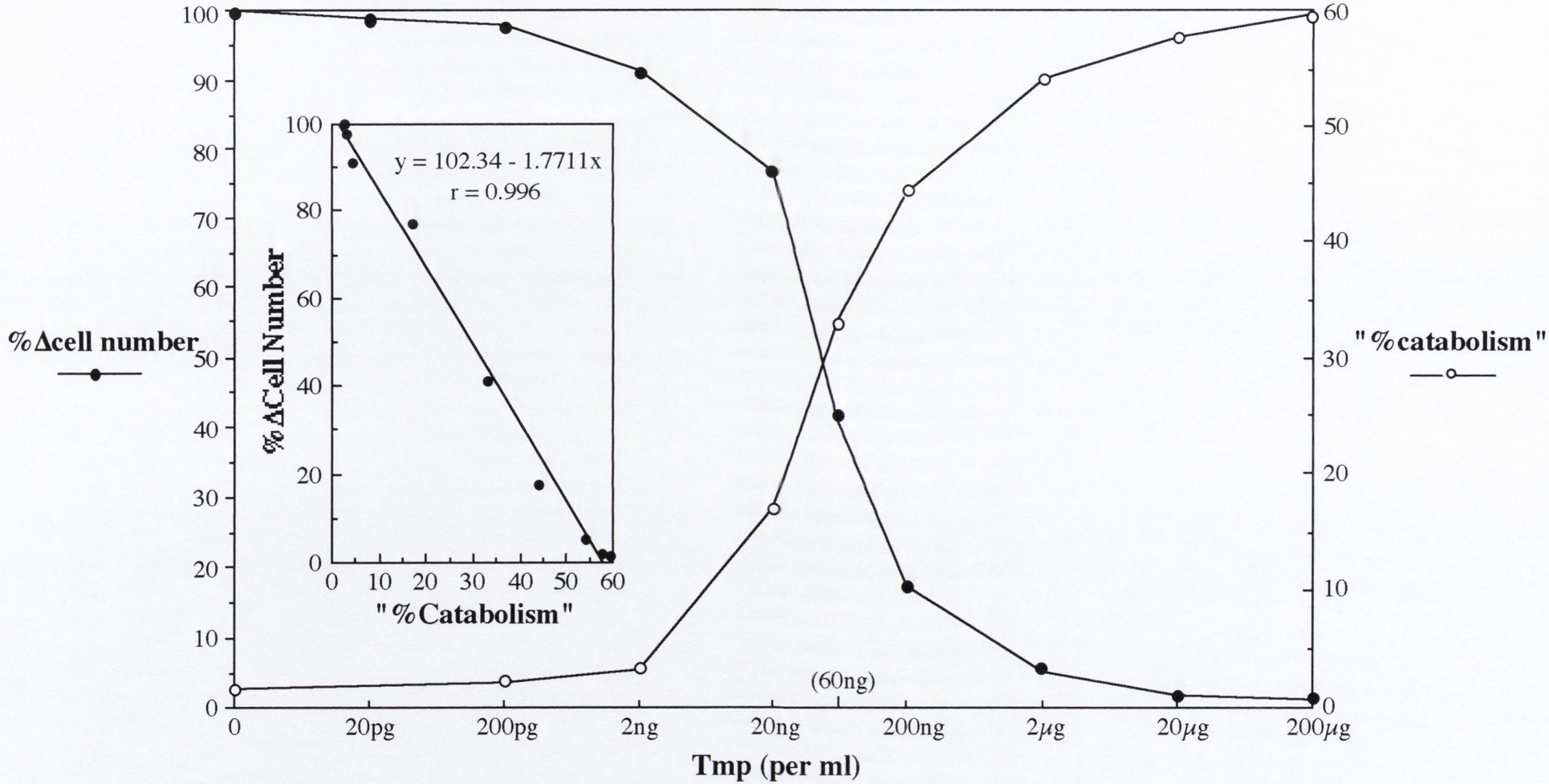


Fig 7.2. Effect of increasing the concentration of Tmp on *E.coli* proliferation (%Δcell number) and on the accumulation of pABGlu and DHF ("%catabolism").

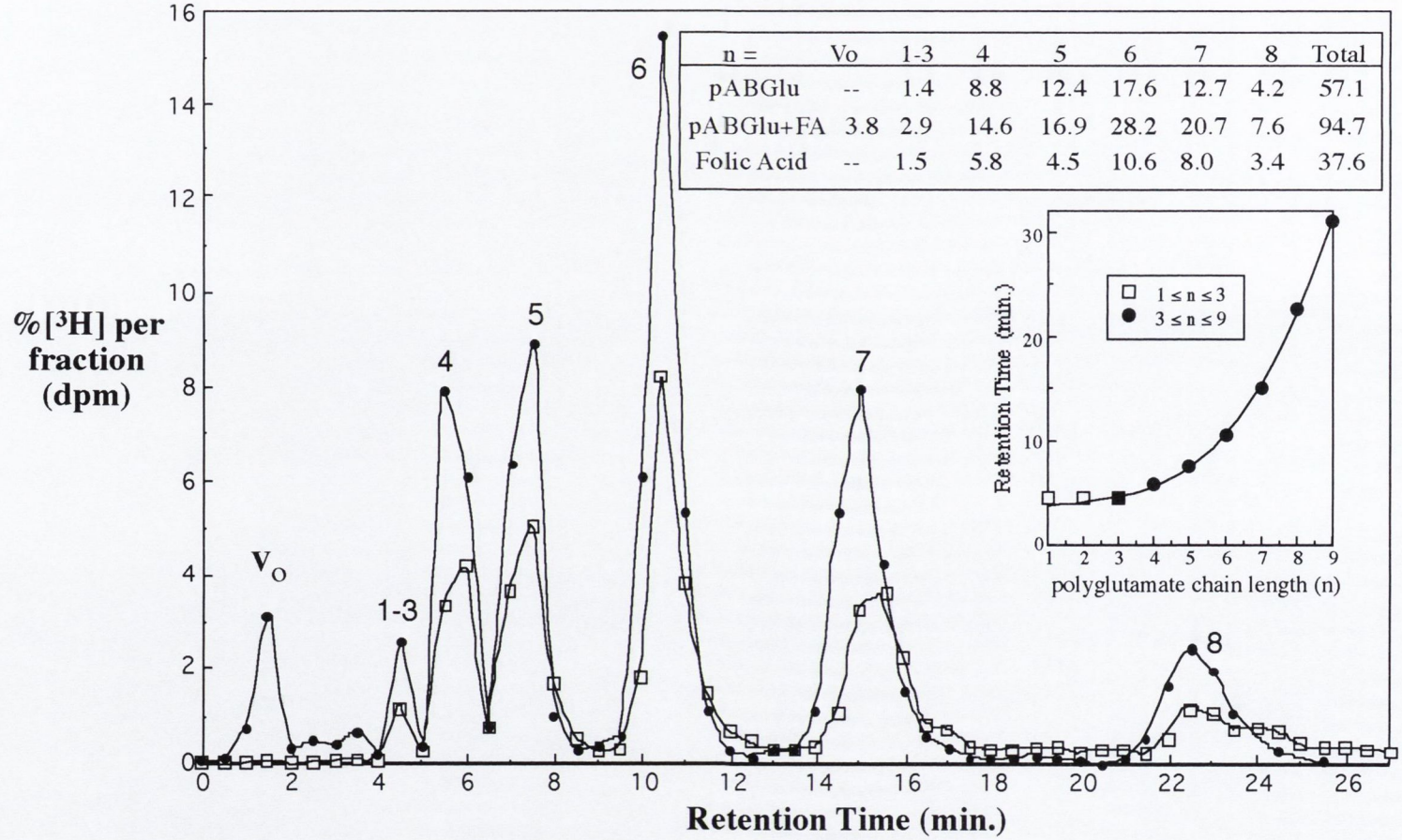
Inset, correlation between the percentage accumulation of pABGlu and DHF ("%catabolism") and the rate of *E.coli* proliferation.

Characterisation of the folic acid product of Tmp inhibition The reductive cleavage of folic acid results in the formation of pABGlu (and a pteridine) (McPartlin *et al.*, 1992). As the magnitude of the polyglutamate peaks due to pABGlu was known it was thus possible, by re-chromatographing the sample after Zn/HCl treatment and calculating the increase in magnitude of each peak, to show that the cellular folic acid had a similar polyglutamate distribution to pABGlu [Fig 7.3].

Metabolic re-utilisation of folic acid upon removal of inhibitor Re-colonisation of the host can occur when antibacterial treatment is terminated before the infection is fully cleared. Thus the sequestering of 40% of cellular folate as folic acid may represent a major metabolic advantage to the bacteria by providing a pre-formed folate source [see Section 1.6]. We therefore set out to test whether, upon removal of inhibitor, the folic acid could be recycled by the cell. After two hours inhibition folic acid represented 31% of the radiolabel in the cell. [Fig 7.4, inset]. Upon removal of Tmp cell division resumed and after a further 4h incubation the folic acid content of the cells was reduced to one-third the Tmp-treatment level. The redistributed radiolabel was found mainly in chromatography peaks corresponding to THF and 10-CHO-THF. There was no change in the proportion of radiolabel accruing to either pABGlu or DHF.

7.2.2 Inhibition of Folate Biosynthesis by Trimethoprim in *E.coli*

As DHF is the first folate synthesised during *de novo* folate biosynthesis [Section 1.6] we examined the effect inhibition of DHFR would have on folate synthesis. As a surrogate for folate synthesis we measured the rate of incorporation of labelled pABA into cellular folates. Growth curves [Fig 7.5] show that a concentration of 6ng/ml Tmp was insufficient to cause growth inhibition, while 60ng/ml Tmp caused considerable growth inhibition.



n =	Vo	1-3	4	5	6	7	8	Total
pABGlu	--	1.4	8.8	12.4	17.6	12.7	4.2	57.1
pABGlu+FA	3.8	2.9	14.6	16.9	28.2	20.7	7.6	94.7
Folic Acid	--	1.5	5.8	4.5	10.6	8.0	3.4	37.6

Fig 7.3. Polyglutamate distribution of □ intact and ● Zn / HCl-cleaved extracts of TMP-treated (2μg/ml) E.*coli* incubated for 4h. Cell extracts were prepared and chromatographed as described in [Section 2.4]. **Inset:** Graph of retention time versus polyglutamate chain length (n). For $n \geq 3$ a third order polynomial ($y = 3.0298 + 0.90179x - 0.31548x^2 + 6.2500e\text{-}2x^3$) gave best fit ($r = 1.00$). **Table:** Tabulation of the polyglutamate distribution as a percentage of the total radiolabel chromatographed for pABGlu (uncleaved extract) and pABGlu + FA (Zn-cleaved extract). The percentage accruing to folic acid was found by subtraction. Peak numbers in main diagram and numbers (n) in inset table refer to polyglutamate chain length, V_o= column void volume.

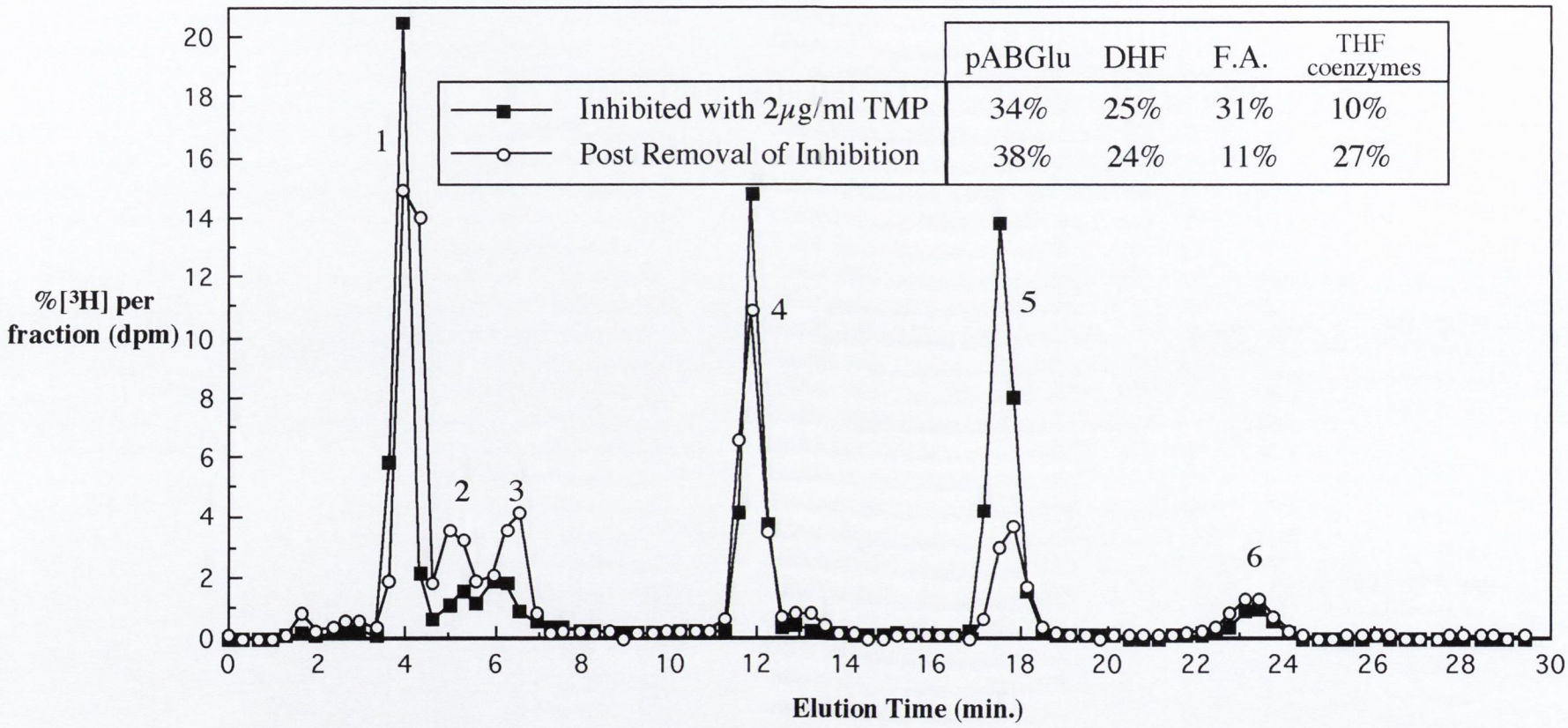


Fig 7.4. Representative chromatography of *E.coli* cell extracts during and after removal of Tmp treatment. Graph shows the HPLC elution of radiolabel, expressed as a percentage of total extract. Chromatographic peaks: 1, pABGlu; 2, 10-CHO-THF; 3, THF; 4, DHF; folic acid; 6, 5-CH_3-THF. **Inset** summarises the percentage distribution of radiolabelled species after 2h Tmp treatment and after 4h incubation post removal of Tmp.

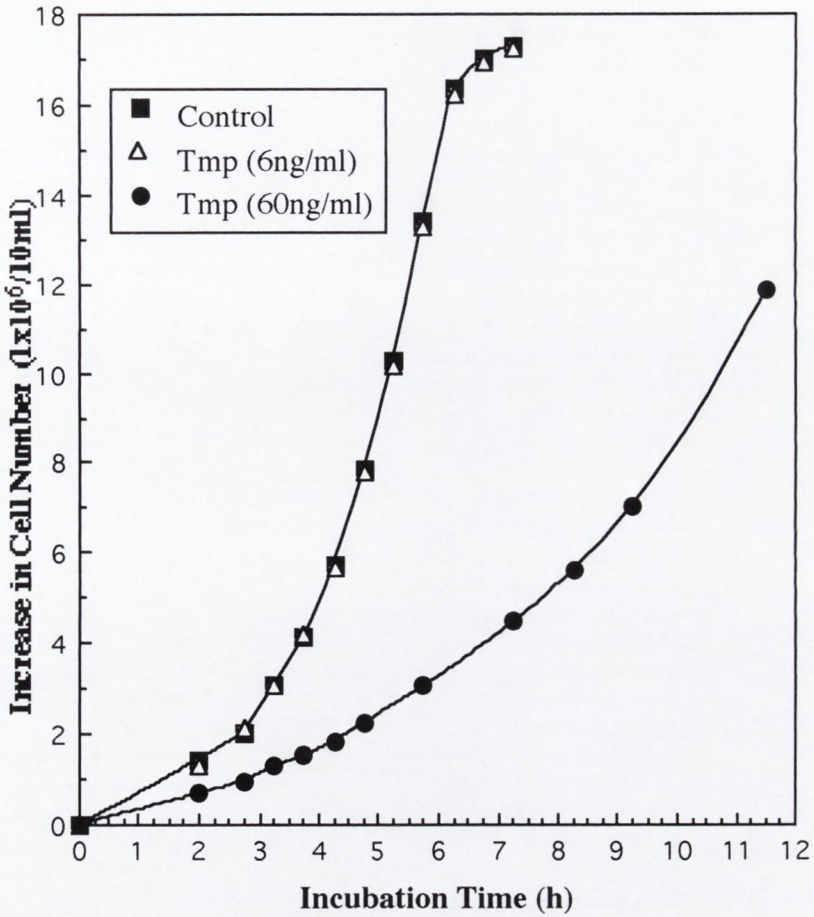


Fig 7.5. Uptake of [^{3}H]-pABA by *E.coli* as a function of incubation time in the presence (Δ, ●) or absence (■) of Tmp.

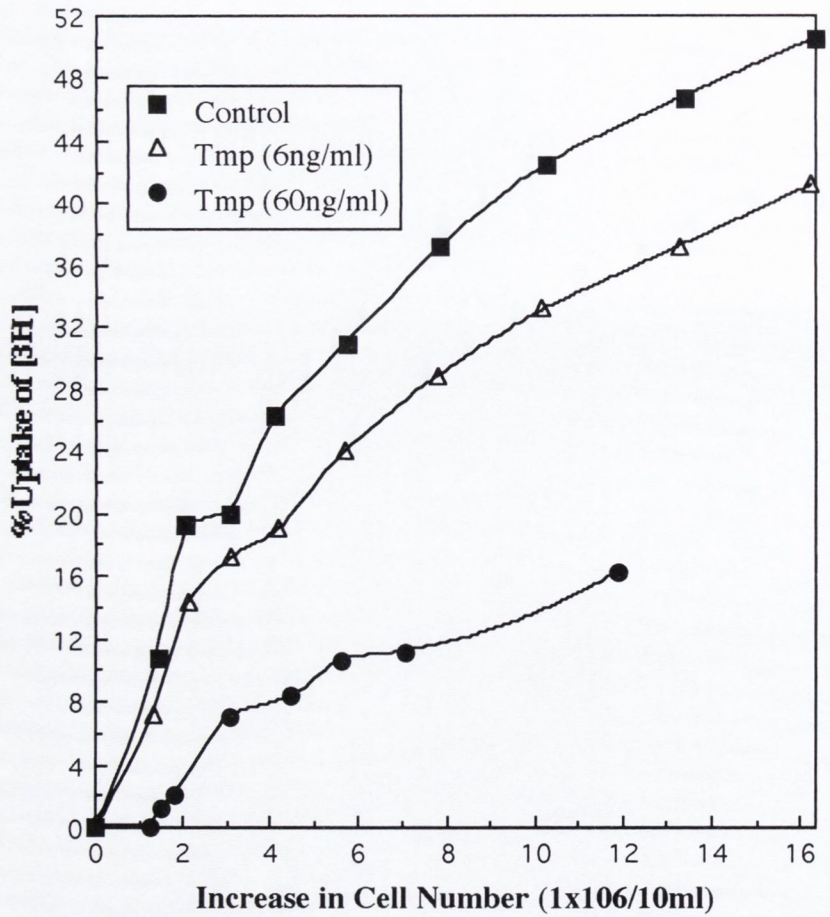


Fig 7.6. Uptake of [^{3}H]-pABA by *E.coli* as a function of bacterial proliferation in the presence (Δ, ●) or absence (■) of Tmp.

However, treatment with either concentration of Tmp resulted in a significant decrease in [^{3}H]-pABA incorporation into *E.coli* [Fig 7.6]. The vast majority (approx. 99%) of radiolabel in the supernatant, prepared after 6.25hrs (0 and 6ng/ml Tmp) or after 11.5hrs (60ng/ml Tmp) incubation, when chromatographed eluted in the peak corresponding to pABA, the remaining radiolabel (approx. 1%) eluted in the void volume.

7.3. Discussion

7.3.1. Effect of Tmp Inhibition in *E.coli*

Inhibition of Folate Metabolism and Induction of Catabolism The purpose of prelabelling *E.coli* with [^{3}H]-pABA was to distinguish the metabolic effects of Tmp from the folate biosynthetic effects, since both processes lead to the formation of dihydrofolate. Thus the current study was designed to investigate the metabolic consequences of Tmp inhibition. Given the central role of folates in DNA replication the disruption of folate metabolism is apparent in the cessation of cell division upon treatment with Tmp. The greater the requirement for thymidylate the more rapid the effect on the depletion of the active tetrahydrofolate pools.

Hitherto, the disruption of folate metabolism due to DHFR inhibition has been postulated to be due to the accumulation of DHF (Fig 7.7b), the folate cofactor dependent on reduction to THF for its further participation in the one-carbon cycle. In this study we provide evidence that the commonly-observed attrition of the total folate pool (Blakley, 1969) upon antifolate treatment is explained by catabolism of folate. Catabolism and depletion of folate has been demonstrated to occur in rats (Murphy *et al.*, 1976; McNulty

et al., 1992 & 1995) and in humans (McPartlin *et al.*, 1992; Chapter 3) and is accelerated under condition of rapid cell division [McPartlin *et al.*, 1992; McNulty *et al.*, 1995; Chapter 6]. Ineluctable losses of folate in this fashion may reflect the requirement for constant replenishment of folate in those organisms which cannot synthesise their own. In this study, DHF was shown to accumulate acutely upon inhibition of DHFR but to diminish to undetectable levels with time as the concentration of pABGlu, the product of C9-N10 bond cleavage, correspondingly increased. Catabolism has been postulated to occur enzymatically (P.Stover, personal communication) but it is also possible that the parallel effects of antifolates on disruption of the cell redox state and decrease in intracellular pH (Oliveira *et al.*, 1989; Lukienko *et al.*, 1985) may contribute to the spontaneous cleavage of labile folates such as DHF in the cell. As DHF accumulates it opposes the competitive inhibition by Tmp (Harvey, 1982), thus at first there would be no decrease in DHFR activity. Computer modelling (Jackson and Harrap, 1973) of the one carbon cycle of L1210 cells suggest that, because of this elevation in substrate concentration, greater than 95% inhibition of the reductase must occur before growth rates are effected. Thus by removing DHF through scission to inactive catabolites and by its oxidation to folic acid the effects of Tmp are enhanced by decreasing competition for DHFR binding. In addition, the accumulation of pABGlu may itself cause secondary inhibition of DHFR (Ki = 2.6mM (Bowden*et al.*, 1989)). Meanwhile, reactivation of the folic acid by DHFR [Scheme 7.1] would be more sensitive to Tmp inhibition than DHF due to the higher K_M and lower V_{max}.

Folic Acid ----------> Dihydrofolate ----------> Tetrahydrofolate
Km = 16μM; Vmax=0.001 Km = 3.2; Vmax = 1

Scheme 7.1. Reduction of folic acid to THF by *E.coli* DHFR. V_{max} are relative to the rate of reduction of DHF (Baccanari *et al.*, 1975).

These results call into question the proposed 'thymine-less death' model (Blakley, 1969) of antifolate action which suggests that the folate cofactor most vulnerable would be that involved in thymine synthesis, that is 5,10-methylenetetrahydrofolate. This is in contrast to our finding in *E.coli* of a more generalised loss of folate, suggesting disruption of all folate dependent pathways. This was reflected not only by the radiolabel loss in treated cells but also in decreased total folate concentrations, as shown in [Table 7.3].

Characterisation of the pABGlu product of Tmp inhibition From chromatography of conjugase untreated cell extracts we demonstrated that folate scission caused polyglutamated pABA of up to eight glutamate residues to accumulate in the cell. The greater efficiency of polyglutamate forms of folate in metabolic reactions is believed to relate to enhanced binding to folate-related enzymes compared to folate monoglutamates [Table 1.7], suggesting that polyglutamated catabolites present an additional disruption to folate-dependent reactions. [Fig. 7.3] also demonstrates that the folic acid produced in Tmp-treated cells is present in polglutamate forms.

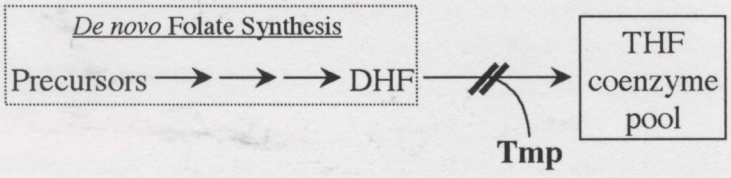
Authentication of folic acid The microorganism used in these studies, E. *coli* (NCIMB 8879) displayed a further characteristic response to inhibitor insult, that is, the ability to store folate in its most stable chemical form, namely folic acid, such that within 4h of Tmp treatment it constituted the only intact folate species in the cell.

Metabolic re-utilisation of folic acid upon removal of inhibitor But upon removal of the inhibitor, it was shown that the radiolabel accruing to folic acid was distributed among other reduced folate coenzymes. This was coincident with a resumption of cell

proliferation. Folic acid has previously been shown to occur to a minor extent in nature due to non-specific chemical oxidation of reduced folates during the extraction process (Mitchell *et al.*, 1941). Here, however, we show that not only does it occur extensively, but that it also exhibits metabolic activity through re-conversion to reduced folates. The accumulation of folic acid may have physiological consequences for bacterial survival and re-colonisation upon premature termination of treatment of infection. This duel effect of trimethoprim may reflect just one part of a spectrum of microbial response, from total catabolism on the one hand to complete sequestration of folates, as folic acid, on the other. The relative degree of catabolism or oxidation to folic acid might vary depending on such factors as species of bacteria, the redox state of the cell or on the therapeutic regime of antifolate. These considerations may even determine the response to be either bacteriostatic through oxidation of DHF to folic acid, or bactericidal through folate catabolism. [Fig 7.7] illustrates schematically the contrast between the established postulate of antifolate action whereby the disruption of folate metabolism is due to DHF accumulation and the model of catabolism and/or oxidation to folic acid which we describe here.

7.3.2 Inhibition of Folate Biosynthesis by Trimethoprim in *E.coli*

As DHF is the first folate formed during *de novo* folate biosynthesis in bacteria a further consequence of using Tmp may be the inhibition of this pathway:



However, attempts to assess the effect of Tmp on the incorporation of [^{3}H]-pABA into folate, by analysing the cellular folate distribution by HPLC, were frustrated by the finding

that Tmp concentrations sufficient to inhibited bacterial growth also inhibited [^{3}H]-pABA incorporation. While [^{3}H]-pABA incorporation was achieved using lower Tmp concentrations, the effects of DHFR inhibition on *de novo* synthesis was indistinguishable from its effect on metabolic function. However, confining our research to examining the effect of Tmp on the gross incorporation of [^{3}H]-pABA we were able to show that Tmp significantly retarded the incorporation of radiolabel into the bacteria. As further analysis showed that the radiolabelled species present in the supernatant was almost exclusively [^{3}H]-pABA, we propose that use of Tmp inhibits the incorporation of exogenous pABA into bacterial folate rather than causing the egress of radiolabelled folates from the *E.coli*. Such a finding is consistent with the proposal that, in addition to its effect on metabolic activity, Tmp inhibits *de novo* folate synthesis.

However, despite the apparent inhibition of folate synthesis with 6ng/ml Tmp, there was no inhibition of bacterial growth. A possible explanation for this observation is that under normal growth conditions folate availability was not growth limiting. That is, the *E.coli* has sufficient folate reserves or excess biosynthetic capacity, such that some inhibition of synthesis or depletion of reserves can occur without affecting growth.

CHAPTER 8

SULFONAMIDE AND *p*-AMINOBENZOATE INHIBITION OF FOLATE METABOLISM IN *ESCHERICIA COLI*

8.1. Introduction

The majority of bacteria satisfy their folate requirement by synthesising folate *de novo*. As this pathway is absent in mammalian cells (Kishore *et al.*; 1988, Haslam, 1993), the folate biosynthetic enzymes have become widely explored targets for the specific inhibition of bacterial growth. To this end, dihydropterin synthase has been target for the development of antibiotics (Woods, 1940; Lampen and Jones 1946; Swedberg *et al.*, 1979; Roland *et al.*, 1979; Brown, 1962; van Miert, 1994), while the shikimate / chorismate biosynthetic pathway has been targeted for the development of antibiotics (Bornemann *et al.*, 1995; Balasubramanian *et al.*, 1991; McConkey, 1999; Davies *et al.*,1994; Ewart *et al.*, 1995), antiparasitics (Ridley, 1998; Roberts *et al.*, 1998), herbicides (Huynh, 1988; Smart *et al.*, 1985; Steinerucken and Amrhein, 1984) and fungicides.

One such family of antibiotics which inhibit dihydropterin synthase are the sulfonamides. The antibacterial use of sulfonamides predates the discovery of their role in inhibiting folate synthesis by more than a decade (van Miert, 1994) and it was knowledge of their molecular structure that contributed to the elucidation of a structure for folate (Woods, 1940; Lampen and Jones 1946; Angier *et al.*, 1946). The sulfonamides inhibit dihydropterin synthase by acting as alternate substrates for the enzyme (Swedberg *et al.*,

1979; Roland *et al.*, 1979; Brown, 1962), blocking p-aminobenzoate binding and diminishing cellular concentrations of the pteridine substrate. These sulfonamide-pteridine analogues are in themselves inhibitors of dihydropterin synthase (Roland *et al.*, 1979) and, to a lesser extent, are inhibitors of other enzymes in the biosynthetic pathway.

A further proposed site of sulfonamide inhibition is DHFR. Both Poe (1976) and Richards *et al.* (1996) suggest that this additional site of inhibition contributes to the synergy between the sulfonamides and Tmp. Similar, it has been proposed that pABA also inhibits DHFR (Richards *et al.*, 1996). In support of this theory Richards *et al.* cite their observation that pharmacological concentrations of pABA inhibit *E.coli* growth, that pABA is an antagonist of Tmp and that pABA causes similar cytoplasmic membrane damage to that caused by Tmp. Futhermore, they propose that if the sulfonamides are inhibitors of DHFR it would not be surprising to find that their structural analogue, pABA, was also a DHFR inhibitor.

In [Chapter 7] we show that Tmp, a known DHFR inhibitor, causes the accumulation and subsequent catabolism of DHF in *E.coli*. Furthermore, we showed that the accumulation of pABGlu and/or DHF was commensurate to the growth inhibition observed. Therefore, by extension of this concept, it would be expected that all inhibitors of DHFR would cause pABGlu and/or DHF accumulation. Using this test for DHFR inhibition, we set out to determine if either sulfamathoxazol, a member of the sulfonamide family of drugs, or pABA were inhibitors of DHFR.

8.2. Results

8.2.1. Effect of sulfamethoxazol inhibition

Concentration of SMX above 200ng/ml were found to inhibit *E.coli* growth. However, a plateau of inhibition, where an increases in SMX concentration resulted in no further inhibition of growth, was observed between 20μg and 200μg/ml sulfamethoxazol [Fig 8.1] This plateau corresponded to approximately 17% growth inhibition. Growth inhibition increased at SMX above 200μg/ml. As the drug was insoluble at concentrations greater than 2mg/ml this was the highest concentration of inhibitor tested, this corresponded to approximately 75% growth inhibition.

Chromatographic analysis of the deconjugated cellular extracts revealed that treatment with 20μg/ml or 200μg/ml SMX resulted in THF accumulation and depletion of 10-CHO-THF and/ or 5,10-CH=THF [Fig 8.2]. However, as there was considerable interconversion of these two folate species during the extraction and deconjugation process, due to a pH dependent chemical rearrangement of the one-carbon subunit (Tabor and Wyngarden, 1959), the exact nature of the depletion was indeterminable. Increasing inhibitor concentration to 2mg/ml increased growth inhibition to 75% [Fig 8.1] but had no effect on folate distribution relative to that observed with 20-200μg/ml sulfamethoxazol, apart from a minor increase in 5-CH_3-THF, from 9.8% to 13.4% (not shown). There was no accumulation of pABGlu or DHF at any of the concentrations of SMX used.

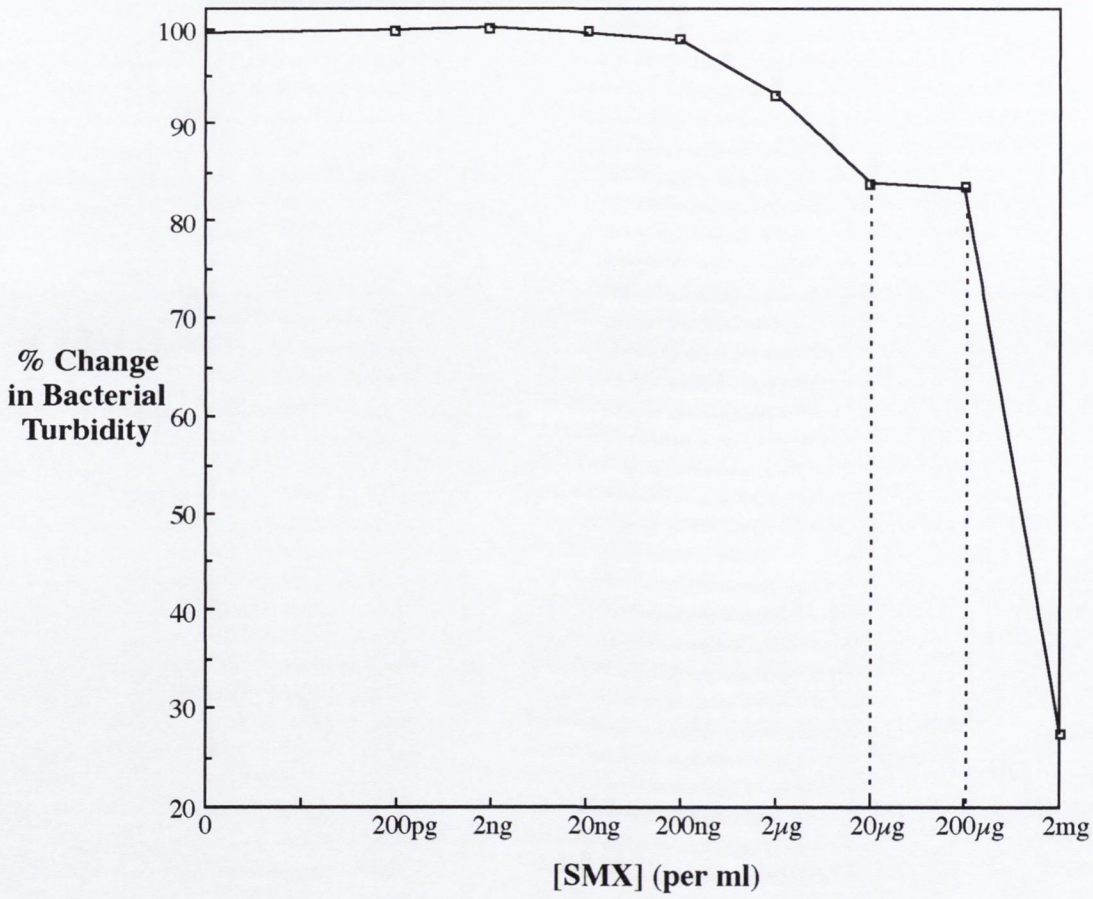


Fig 8.1. Sulfamethoxazol (SMX) inhibition curve. Percentage growth, relative to control, of *E.coli* in the presence of increasing concentrations of SMX.

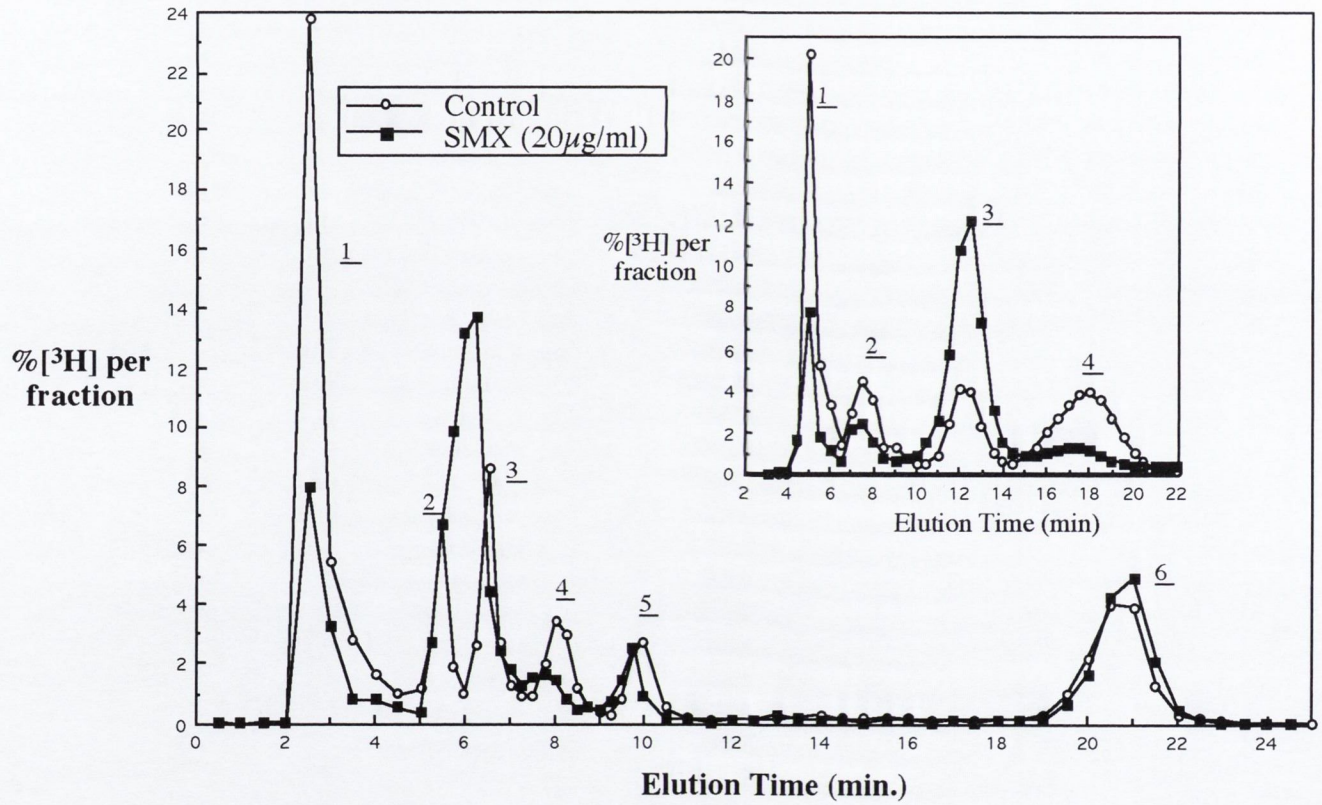


Fig 8.2. Folate distribution in *E.coli* NCIMB 8879 grown in the presence and absence 20μg/ml SMX. Samples were chromatographed on Kingsorb C_{18} column. **Inset,** samples were chromatographed on a μBondapak C_{18} column to resolve the co-eluting peaks at ≈6min. Peaks: 1) 5,10-CH=THF; 2) 10-CHO-THF; 3) THF 4) 5-CHO-THF; 5) 5,10-CH2-THF; 6) 5-CH3-THF. pABGu eluted between peak 1) and 2).

8.2.2. Effect of *para*-Aminobenzoic Acid inhibition

Concentrations of pABA of 0.8mg/ml or greater inhibited *E.coli* growth. A linear relationship was observed between pABA concentration and growth inhibition at pABA concentration between 0 and 4mg/ml, while maximum growth inhibition (97%) was achieved with 8mg/ml pABA [Fig 8.3; Table 8.1]. Chromatographic analysis of the deconjugated cellular extracts revealed that increasing the inhibitory concentrations of pABA resulted in the progressive accumulation of 5-CH_3-THF, at the expense of the other THF co-enzymes [Table 8.1]. No accumulation of pABGlu or DHF was observed, even at concentrations of pABA giving maximum growth inhibition [Fig 8.4].

[PABA]	% Growth Inhibition	% 5-CH_3-THF	% Other THF Coenzymes*
0 mg/ml	0	9.9	90.1
1 mg/ml	18	15.8	84.2
2 mg/ml	34	13.7	86.3
4 mg/ml	68	19.2	80.8
8 mg/ml	97	22.0	78.0

Table 8.1: Effect of pharmacological concentrations of pABA on *E.coli* growth and cellular THF co-enzyme distribution. *Reduced folate coenzymes apart from 5-CH_3-THF.

When *E.coli* were treated with Tmp (2μg/ml) in conjunction with pABA (8mg/ml) a redistribution of [^{3}H] was observed characteristic of that observed with Tmp inhibition alone, *i.e.* accumulation of both pABGlu and folic acid at the expense of the THF co-enzyme pool [Fig 8.5].

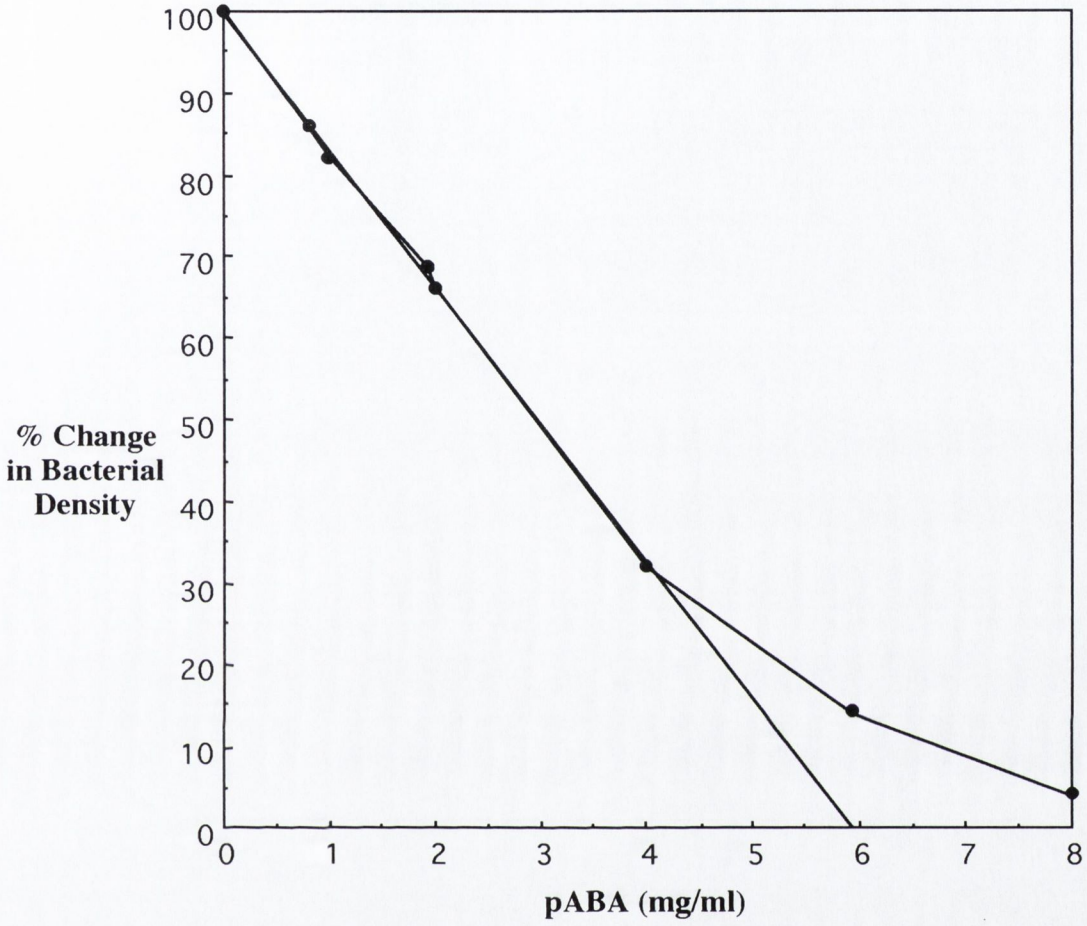


Fig 8.3. *para*-Aminobenzoate (pABA) inhibition curve. Percentage growth, relative to control, of *E.coli* in the presence of increasing concentrations of pABA.

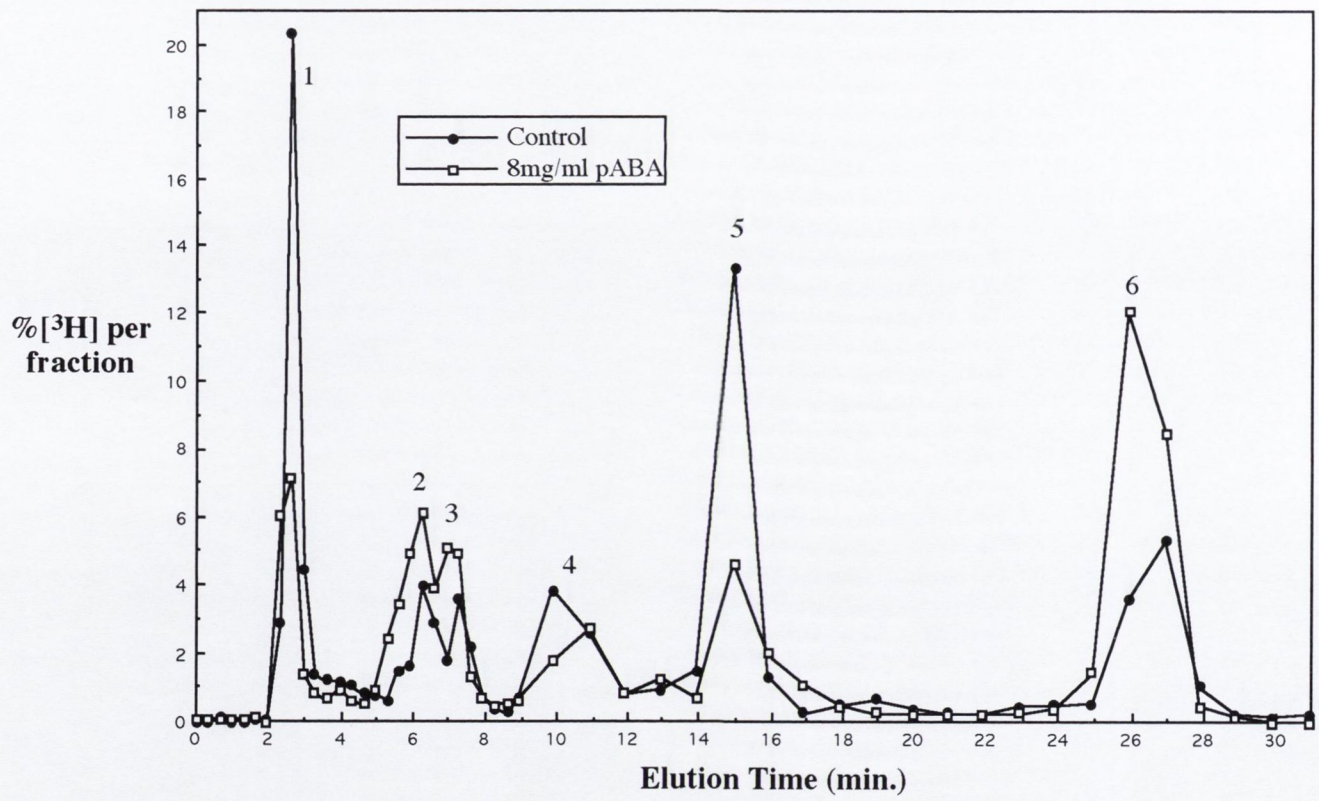


Fig 8.4. Folate distribution in *E.coli* NCIMB 8879 grown in the presence and absence 8mg/ml pABA. Samples were chromatographed on Kingsorb C_{18} column. Peaks: 1) 5,10-CH=THF; 2) 10-CHO-THF; 3) THF 4) 5-CHO-THF; 5) 5,10-CH2-THF; 6) 5-CH3-THF. pABGu standards eluted between peak 1) and 2).

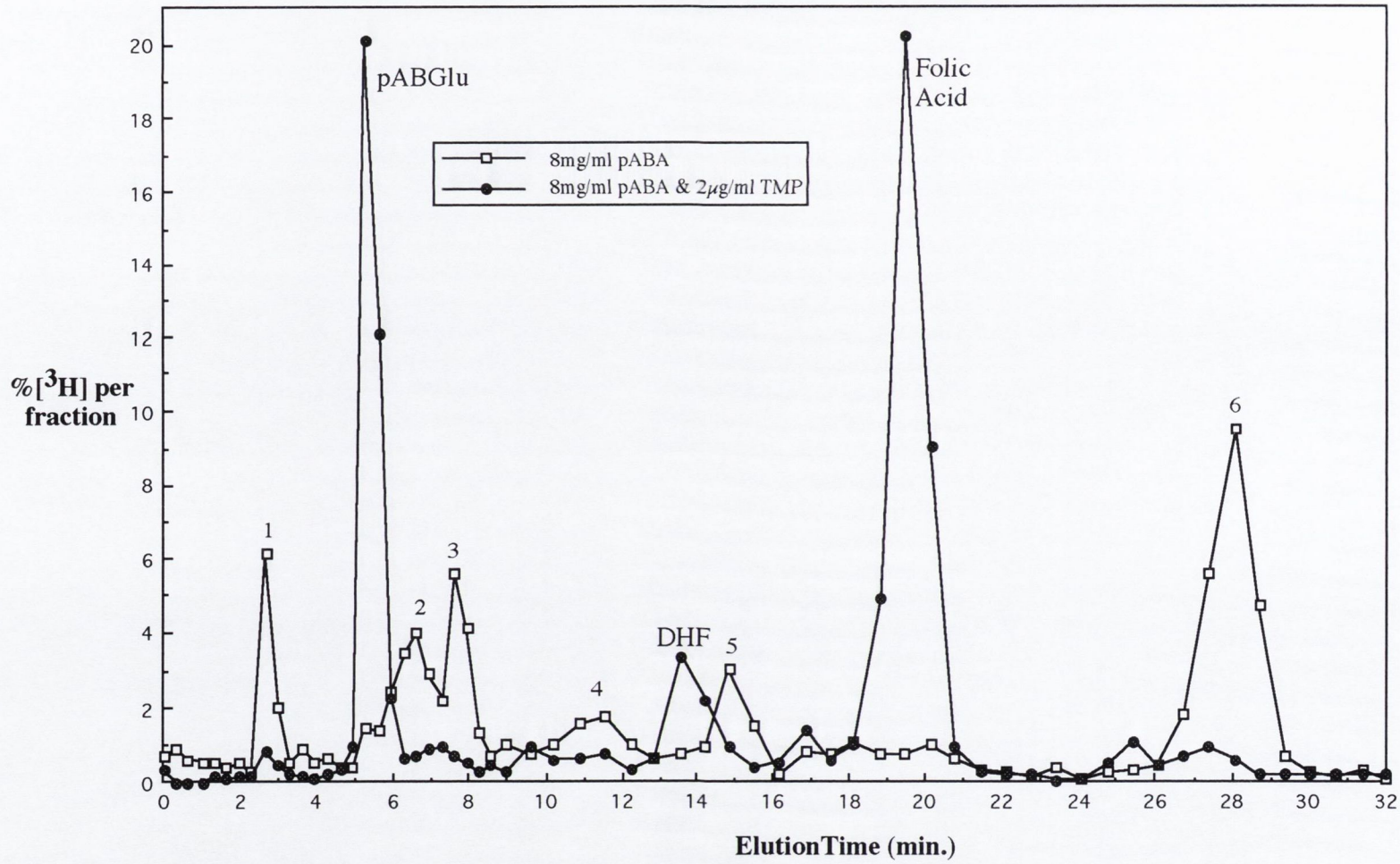


Fig 8.5. Folate distribution in *E.coli* NCIMB 8879 treated with 8mg/ml pABA or 8mg/ml pABA plus 2μg/ml Tmp. Samples were chromatographed as in [Fig 8.4] and labelled accordingly, except for the pABGlu, DHF and folic acid peaks which were not present in that chromatograph.

8.3. Discussion

8.3.1. Effect of sulfamethoxazol inhibition

These results show that inhibition of *E.coli* with sub-MIC concentrations of SMX result in the redistribution of folate co-factors *in vivo*. Whether this altered distribution was due to inhibition of folate one carbon metabolism or a redistribution of co-factors due to folate deficiency is unclear, but appears to correspond to a 17% inhibition of growth.

If the redistribution of folate co-factors were due to enzyme inhibition the most likely target is 10-CHO-THF synthase [Fig 1.2, Enzyme 5,] as there was accumulation of co-factor (THF) before this enzyme in the cycle, while folates (10-CHO-THF / 5,10-CH=THF) beyond this enzyme were depleted. The limited inhibition of growth (17%) due to such inhibition may result from the availability of alternative pathways by which 10-CHO-THF can be synthesised within the one carbon cycle [Fig 8.6]. Inhibition of bacterial growth at concentrations of SMX greater than 200μg/ml was probably the result of folate biosynthesis inhibition as there was minimal redistribution of the pre-labelled folates,

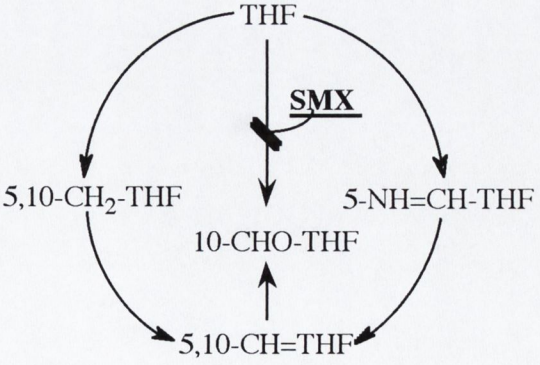


Fig 8.6. Pathway by which 10-CHO-THF is synthesised, from THF, *in vivo*.

relative to that observed at 20μg/ml SMX .

The hypothesis that the sulfonamide family of drugs are inhibitors of DHFR was not

substantiated by our findings, at least in the case of SMX. While Tmp caused pABGlu and/or DHF accumulation over its full inhibitory concentration range [Fig 7.2] SMX did not cause accumulation of either pABGlu or DHF, even at inhibitory concentrations sufficient to cause 75% inhibition of growth. It must be assumed therefore that inhibition of DHFR does not appear to play a role in antibacterial action SMX. Such a finding is consistent with the observation (Baccanari and Joyner, 1981) that concentrations of sulfamethoxazol as high as 6mM were not inhibitory to DHFR, even in the presence of Tmp.

8.2.2. Effect of para-Aminobenzoic Acid inhibition

We confirmed the finding that pharmacological concentrations of pABA are inhibitory to *E.coli* growth (Richards *et al.*, 1995a; Richards *et al.*, 1996). However, we are unable to substantiate the theory that this antibacterial effect was due to DHFR inhibition by pABA, as the chronic accumulation of pABGlu and/or DHF expected of a DHFR inhibitor did not occur in the face of inhibition with pABA.

Analysis of the THF co-enzyme distribution post pABA inhibition suggests that pABA's mode of antibacterial action is by inhibition of methionine synthase, as the relative distribution of 5-CH_3-THF had more than doubled [Table 8.1]. However, an alternative explanation is that pABA, at pharmacological concentrations, is an inhibitor of folate synthesis. Such a conclusion is suggested by the finding that pharmacological concentrations of pABA are inhibitory to dihydropteroate synthase from S.aureus (Hampele *et al.*, 1997). The resulting folate depletion, accruing from the inhibition of *de novo* synthesis, could conceivably result in the redistribution of folate co-enzymes *in vivo*. Similar accumulation of 5-CH_3-THF was observed with 2mg/ml SMX, but to a lesser

extent, as for pABA. However, in the case of SMX growth inhibition was due to inhibition of both folate metabolism and *de novo* synthesis, the degree 5-CH_3-THF accumulation relative to the extent of growth inhibition are not directly comparable. Furthermore, the metabolic inhibition observed with SMX may have retard the rate at which 5-CH_3-THF accumulates. While Tmp also causes folate depletion *in vivo*, the attrition on pre-labelled folates would be expected to prevent 5-CH_3-THF accumulation.

Whether the growth retardation observed with pharmacological concentrations of pABA was due to inhibition of methionine synthase or folate biosynthesis is still not clear. However either mechanism would likely result in defective peptidoglycan production due to the ensuing disruption of the methylation cycle. This may explain the cycoplasmic membrane damage associated with pABA inhibition (Richards *et al.*, 1995a) and account for its similar to the damage observed with Tmp or sulfonamide inhibition (Richards *et al.*, 1995b).

Richards *et al.* (1995a) suggested that the antagonism of sub-inhibitory concentrations of pABA towards Tmp resulted from pABA displacing Tmp from its binding sight on DHFR. However, we showed no apparent antagonism of pABA towards Tmp in its inhibition of DHFR, as determined by the propensity of Tmp to cause pABGlu and folic acid accumulation. An alternative explanation for the antagonism of pABA towards Tmp is the possible induction of *de novo* folate synthesis by pABA (Hampele *et al.*, 1997; Nimmo-Smith *et al.*, 1948).

The pteridine moiety required for folate synthesis can be provided by 2-amino-4-hydroxy-dihydropteridine-6-carboxyaldehyde (DHpt-CHO) (Shiota, 1959; Brown *et al.*, 1961), the pteridine product of DHF oxidative cleavage (Blair, 1957; Waller *et al.*, 1950). However,

pABGlu, the other product of DHF cleavage, is a poor substrate for folate biosynthesis (Shiota, 1984) especially in the polyglutamate form found *in vivo* [Chapter 8]. Thus the provision of exogenous pABA would provide a ready source of preformed substrate, while alleviating the metabolic drain required for *de novo* folate biosynthesis. This, when coupled with the high concentrations of endogenous DHpt-CHO present due to Tmp inhibition, may aid in the replenishment of folate stocks while reducing the metabolic cost of such replenishment.

CHAPTER 9

TRIMETHOPRIM AND METHOTREXATE INHIBITION OF FOLATE METABOLISM IN *LACTOBCILLUS CASEI*

ANTAGONISM BY PURINE AND PYRIMIDINE PRECURSORS AND BY THYMIDYLATE SYNTHASE INHIBITORS

9.1. Introduction

Antifolates are commonly classified under one of two heading, those which are substrates for folate-polyglutamate synthase and can thus form polyglutamate chains (often referred to as "classical" antifolates) and those which can not be polyglutamated ("non-classical" antifolates).

"Classical" antifolates While as diverse in their enzyme targets as the "non-classical" antifolates [Table 9.1] the "classic" antifolates tend to be closer structural analogues to folate [Fig 9.1] since they must also act substrates for folate-polyglutamate synthase. The resulting polyglutamated antifolate behave similarly to folate-polyglutamates [Section 1.4] in that they are preferentially retained within the cell, creating a concentration gradient of inhibitor across the cell membrane (Rosenblatt *et al.*, 1978a & 1978b), and in that they have a higher enzyme affinity (Kisliuk *et al.*, 1985). In some cases polyglutamation may also increase the range of enzyme inhibited by the drug (Kisliuk *et al.*, 1985).

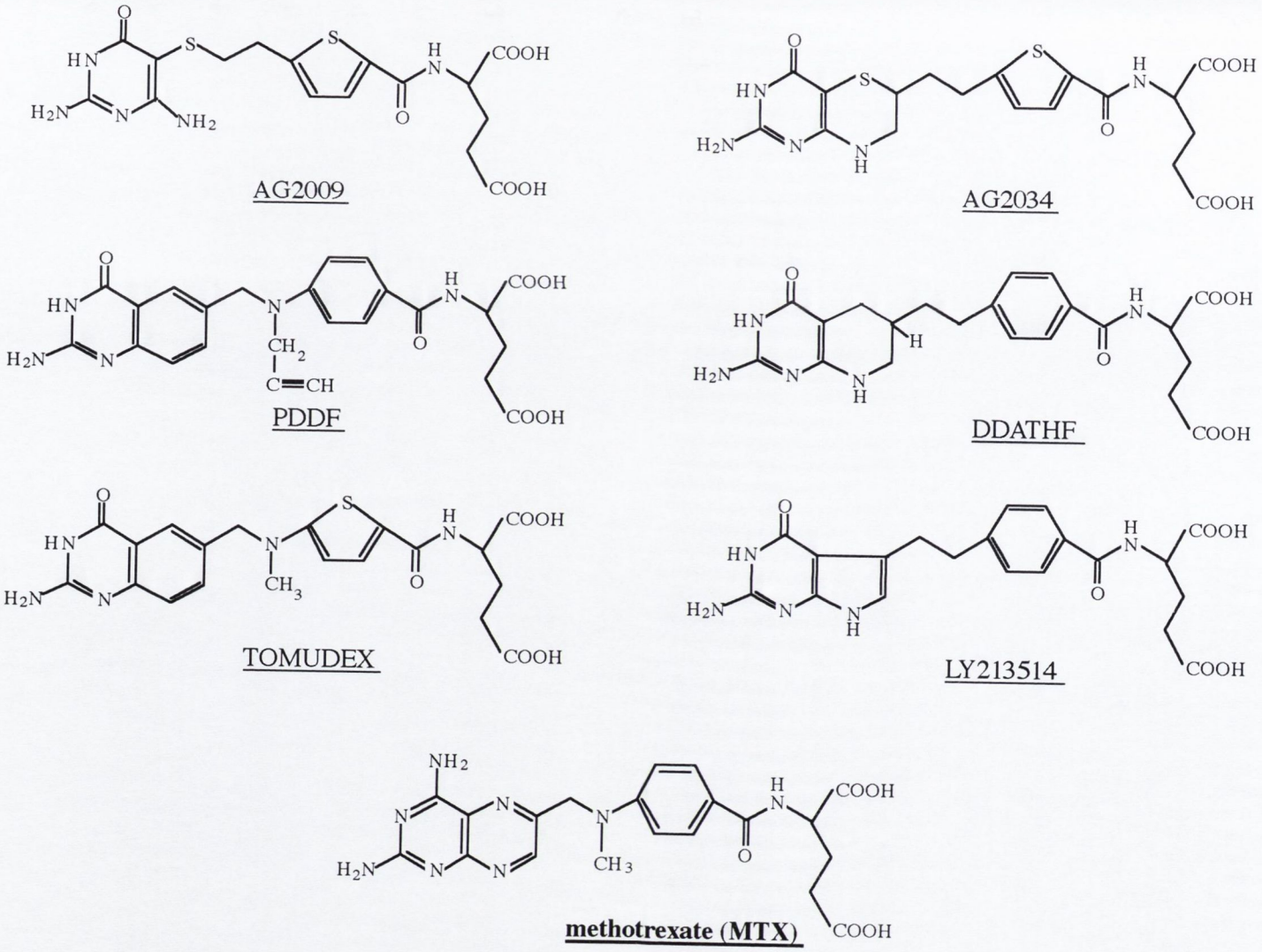


Fig 9.1. "Classic" antifolates, inhibitors of folate utilising enzymes which are polyglutamated *in vivo*.

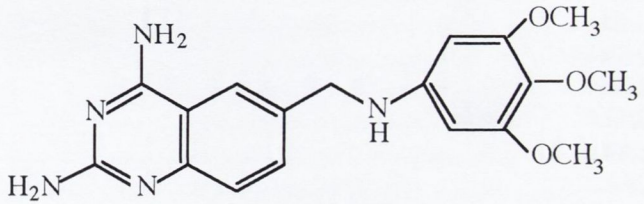
"Non-classical" antifolates This classification of antifolates has come not only come to represent drugs which are analogues of folate but also drugs (such as the fluorouracil derivatives) which, while not competitive to folate, inhibit folate metabolism [Table 9.1]. Thus the structure [Fig 9.2] of "non-classical" antifolates range from folate analogues (*i.e.* ZD9331), to substituted analogues of pABA (*i.e.* the sulfonamides) or pteridine (*i.e.* AG337), to compounds such as FdU which bear little resemblance to folate.

Target Enzyme	**"Classic" Inhibitor**	**"Non-classic" Inhibitor**
dihydrofolate reductase	MTX	Tmp, TMTX
thymidylate synthase	PDDF, Tomudex	AG337, ZD9331, FdU
GAR transformylase	DDATHF, AG2034	LL95509
dihydropterin synthase		sulfonamides
multi-target	LY231514	

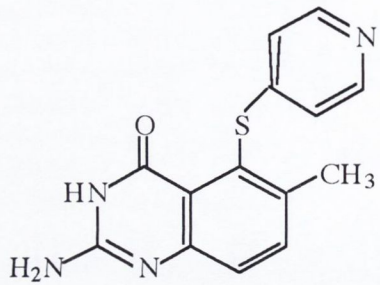
Table 9.1. "Classic" and "non-classic" inhibitors of folate metabolism and their target enzymes [see also Fig 9.1 & 9.2].

Two of the most popular antifolate drugs in current use are the dihydrofolate reductase (DHFR) inhibitors trimethoprim (Tmp) and methotrexate (MTX). Tmp is a "non-classical" drug, which as we discussed in [Chapter 7], is extensively used as an antibiotic. MTX, on the other hand, is a "classic" antifolate whose efficacy is greatly potentiated *in vivo* by polyglutamation. MTX is commonly used both as an anti-inflammatory and in the treatment of cancer. It was for the discovery of both these drugs that Hitchings and Elion were awarded the Nobel Prize for Medicine in 1998 (Hitchings, 1989).

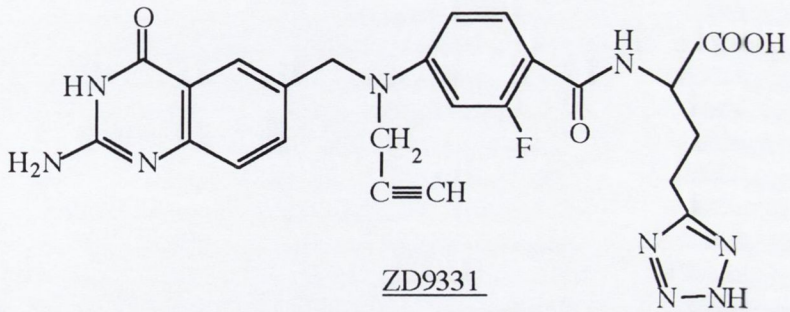
In looking at the effect of these two drugs on folate metabolism in a strain of *L.casei* (NCIMB 10463) we hoped to determine if there was a difference in the way in which



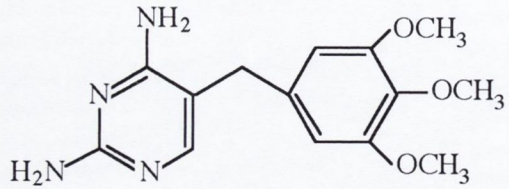
TMTX



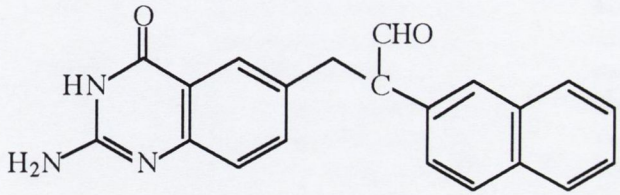
AG337



ZD9331



Trimethoprim (Tmp)



LL95509

Fig 9.2. Some "non-classical" antifolates. Inhibitors of folate utilising enzymes which are not polyglutamated *in vivo*.

"classical" and "non-classical" inhibitors of DHFR affected folate metabolism *in vivo*. We also hoped to use this *L.casei* model of DHFR inhibition to examine its effect on folate metabolism and to compare it to the effect observed in *E.coli* [Chapter 7]. The methods used are as described in [Section 2.5].

9.2. Results

9.2.1. Inhibition of Folate Metabolism by Trimethoprim or Methotrexate

Radiolabel distribution on pteridine and pABGlu moieties of folic acid The percentage distribution of tritium on the commercial [^{3}H]-folic acid used was reported to be pteridine ([7, 9-^{3}H])= 52.6%, pABGlu ([3', 5'-^{3}H]) = 47.4% (Moravek Biochemicals Customer Services, personal communication). This was consistent with the ratio determined by treating a portion of the [^{3}H]-folic acid with Zn/ HCl and chromatographing the cleavage products: pteridine (void volume) = 54.3%, pABGlu = 45.7%.

Effect of Tmp or MTX treatment on bacterial growth and folate distribution Both Tmp and MTX inhibited *L.casei* growth [Fig 9.3]. However, *L.casei* displayed considerable proliferation, approx. 30% of control growth, even at inhibitor concentrations giving maximum growth inhibition. This was in contrast to the almost complete (>98%) growth inhibition observed in *E.coli*,

As the magnitude of growth inhibition was similar for both Tmp and MTX, and as both drugs caused similar attrition on THF co-enzyme concentrations at concentrations of 20μg/ml Tmp or 0.7μg/ml MTX [Fig 9.4], all further experiments were conducted using

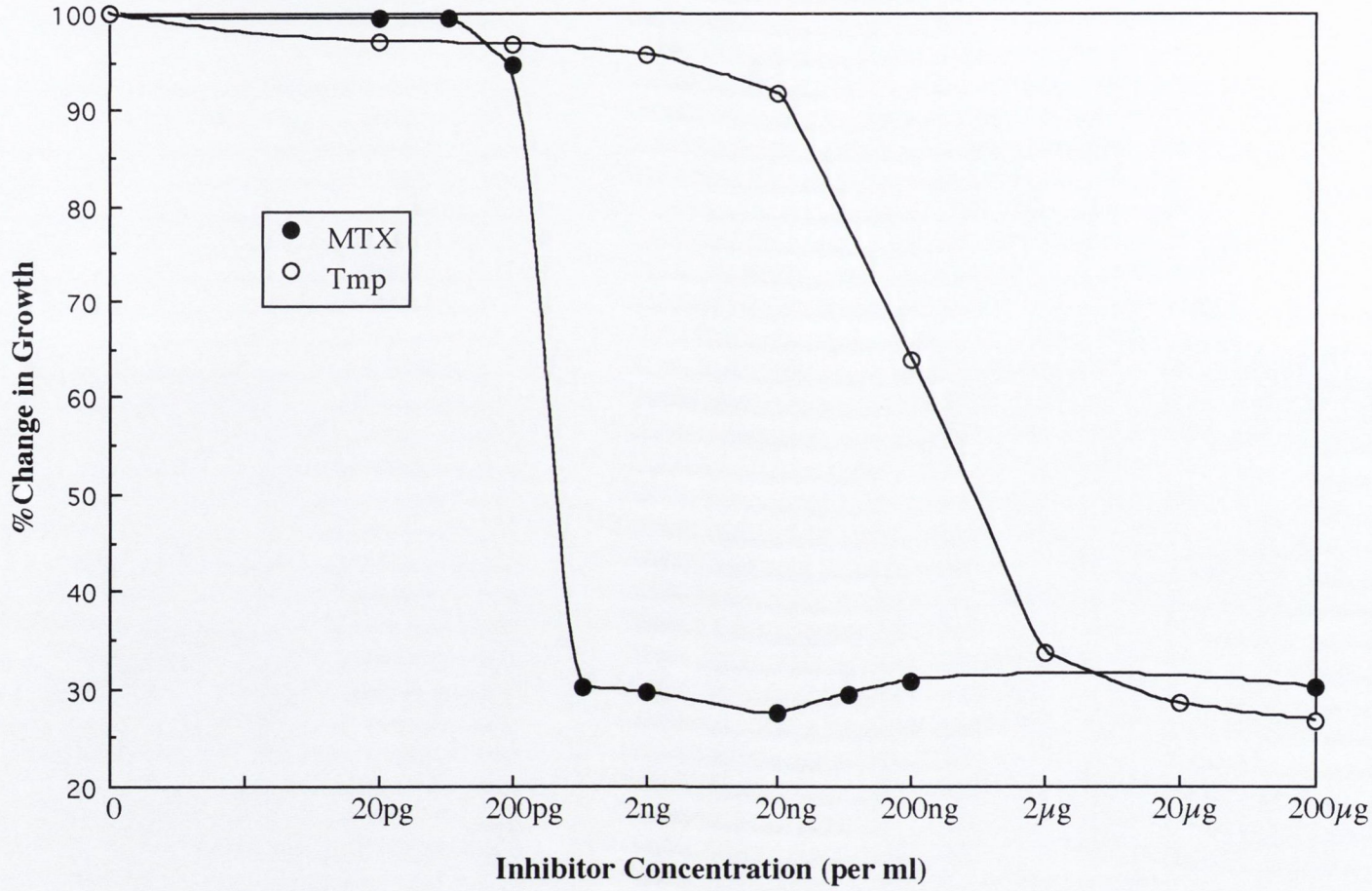


Figure 9.3. Inhibition of *L.casei* growth by different concentrations of ● MTX or ○ Tmp.

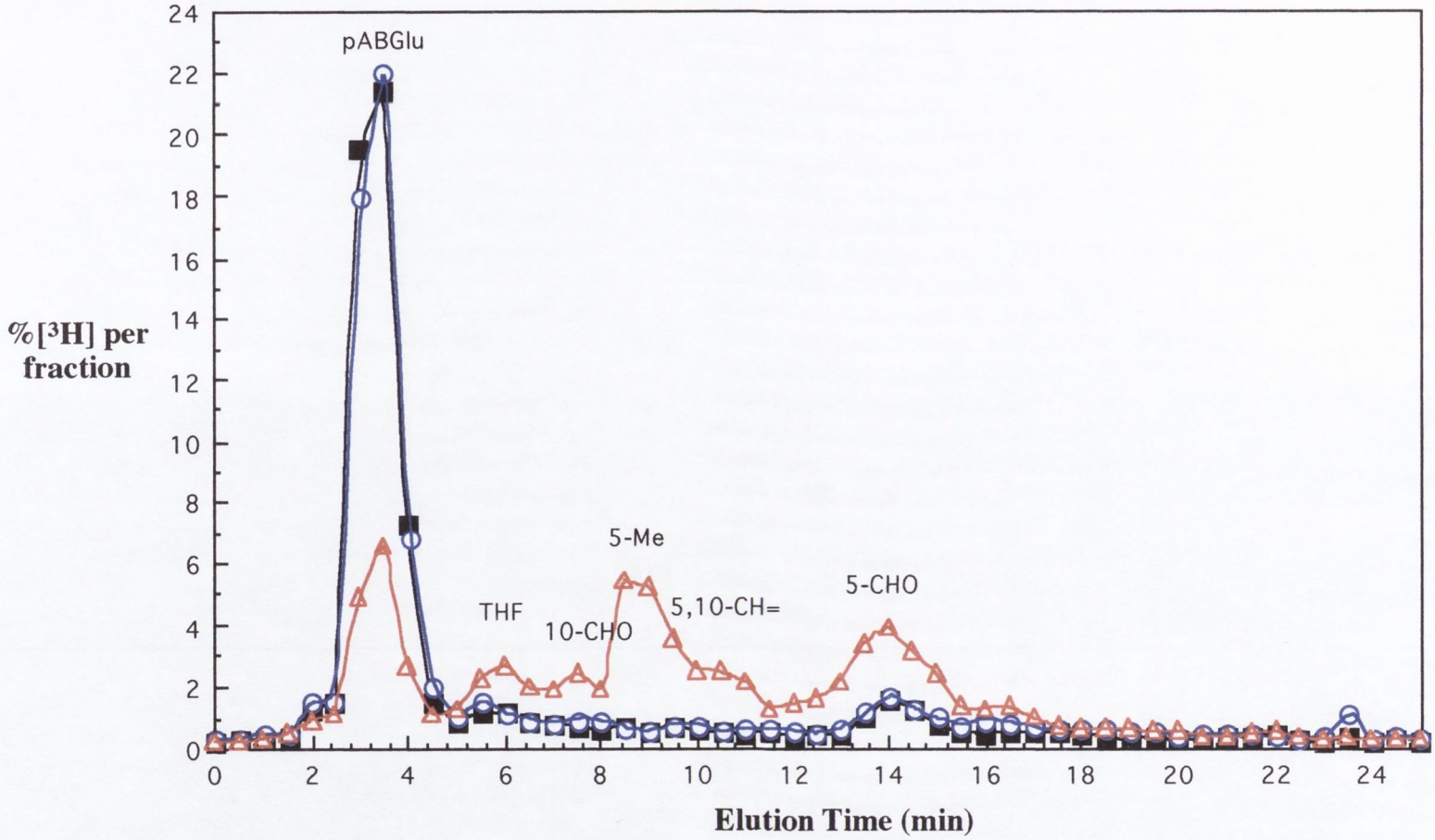


Fig 9.4. Representative chromatography of *L.casei* cell extracts Δ untreated, or treated with either ■ 20μg/ml Tmp or ○ 0.7μg/ml MTX.

Extracts were chromatographed on a μBondapak C_{18} column eluted with citrate-phosphate buffer.

only Tmp. The reason for this choice was twofold: Tmp is a "non-classical" inhibitor and thus, unlike MTX, is not polyglutamylated. Secondly, Tmp is a specific inhibitor of DHFR, however, MTX may also inhibit other folate utilising enzymes. Thus the use of Tmp in allows a direct comparison of its effect in *L.casei* and *E.coli.*

There was evidence of catabolism, (presence of pABGlu) in the untreated *L.casei* at time zero (T_0) and after a further 4h incubation [Fig 9.5]. While treatment with Tmp increased the rate of catabolism, in further contrast to the response observed in *E.coli*, there was no detectable accumulation of DHF prior to cleavage. However, this could not be attributed to cleavage of the DHF during sample preparation as the magnitude of the pteridine chromatographic peak was much smaller than that of the pABGlu peak. This is further corroboration that catabolism occurred *in vivo*, as the pteridine has been lost to the media before harvesting.

Calculating the extent of folate catabolism As pABGlu had only 47% the specific activity of the intact folate molecule, this was take this into account when calculating the extent of folate catabolism. The pteridine contribution to sample [^{3}H]-activity should also be taken into account when calculating catabolism. However, as its contribution to the [^{3}H]-total was minor it could be ignored without introducing a significant error. Knowing the total radioactivity of the sample (Total), the activity of pABGlu in the sample (pABGlu) and the relative specific activity of the pABGlu (47%) we were able to calculate the percentage pABGlu (%pABGlu (adj.)) on a molar basis (derivation of equation is in [Appendix C]):

$$\%\text{pABGlu (adj.)} = \frac{\text{pABGlu} \div 47\%}{\text{Total} - \text{pABGlu} + \text{pABGlu} \div 47\%} \qquad \text{Eqn. 9.1}$$

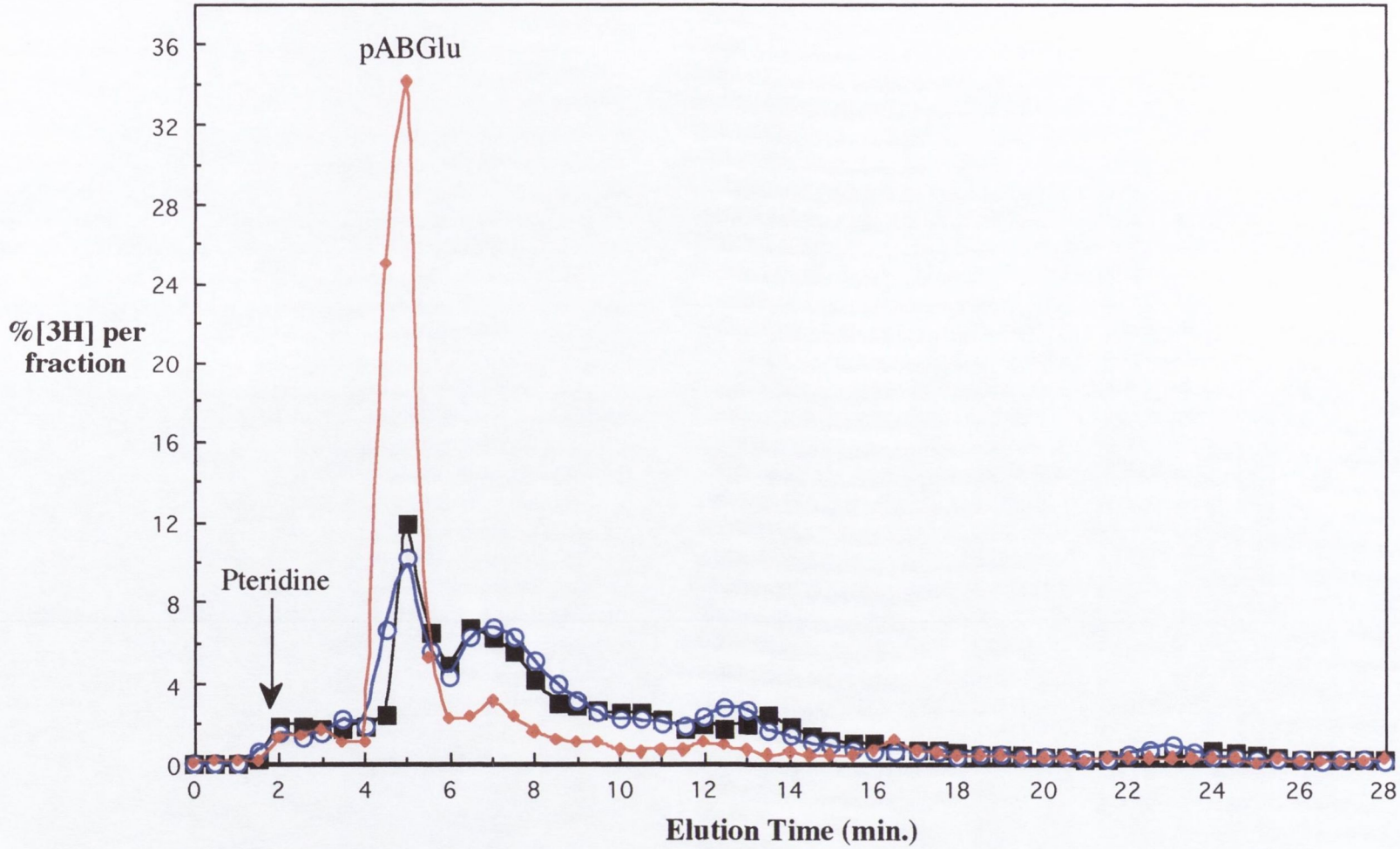


Fig 9.5. Representative chromatography of *L.casei* cell extracts prepared ■ prior to incubation (T_0) or after 4h incubation in the ◆ presence or ○ absence of 20μg/ml Tmp. Chromatography was on a Kingsorb ODS column eluted with PicA buffer.

Some catabolism had occurred during the labelling growth (T_0). This had to be accounted for when calculating catabolism due to Tmp inhibition. Thus, knowing the percentage pABGlu (adj.) in the pellet at the start of the treatment growth (pABGlu (T_0)) and after each of the incubation periods (pABGlu (T_{INC})) we were able to calculate the percentage increase in catabolism (ΔCatabolism) for each incubation period (the derivation of this formula is in [Appendix C]):

$$\Delta\text{Catabolism} = \frac{\text{pABGlu } (T_{INC}) - \text{pABGlu } (T_0)}{\text{Total (adj.)} - \text{pABGlu } (T_0)} \qquad \text{Eqn. 9.2}$$

Table 9.1, therefore, gives the percentage catabolism (%pABGlu(adj.) and the percentage increase in catabolism (ΔCatabolism) for the incubation times tested. Thus, within 60min of treatment with Tmp 42% of the folate present at T_0 had catabolised to pABGlu. [Fig 9.6] shows that by 4h incubation the rate of catabolism had slowed dramatically, by this time only 67% of the starting radiolabelled folate had catabolised.. Over the corresponding period only 5% catabolism had occurred in the untreated sample.

9.2.2. Antagonism of Trimethoprim Inhibition

Interaction Purine and Pyrimidine Precursors with Tmp Both thymine and its deoxyribose derivative, thymidine, were antagonistic towards Tmp [Fig 9.7]. However, thymine was a more effective antagonist than thymidine, eliciting greater bacterial growth at a 10-fold lower concentration than thymidine. Inosine, the purine base precursor, was mildly antagonistic towards Tmp, but required high concentrations (200μg/ml) to achieve a modest (3-5%) increase in growth.

	%[^{3}H]-pABGlu in pellet	%pABGlu (Adj,)[1]	ΔCatabolism (%)[2]
Untreated (0min)	14.4	26.4	--
20μg/ml TMP (60 min)	38.3	56.9	41.5
20μg/ml TMP (120 min)	53.5	71.0	60.6
20μg/ml TMP (240 min)	59.5	75.8	67.1
Untreated (240min)	16.8	30.1	5.0

Table 9.2. Effect of Tmp inhibition on folate catabolism in *L.casei*. [1]Adjusted for the lower specific activity of pABGlu relative to intact folate [see Eqn. 9.1]. [2]Percentage increase in catabolism after the incubation time shown [see Eqn. 9.2].

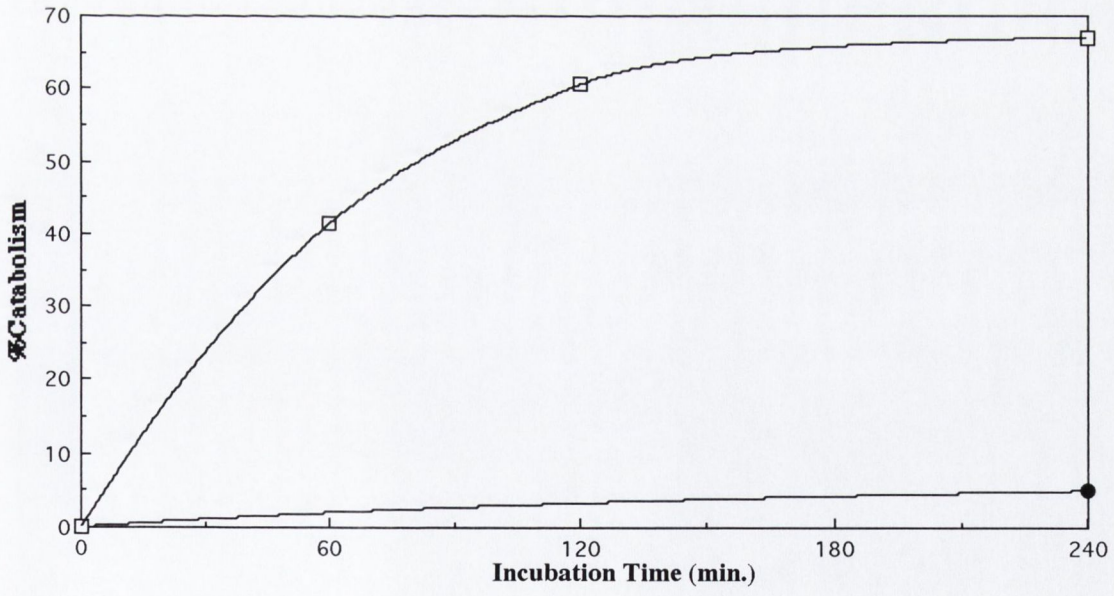


Fig 9.6. Increase in folate catabolism with incubation time in □ control (no inhibitor) or in ● trimethoprim treated (20μg/ml) *L.casei*.

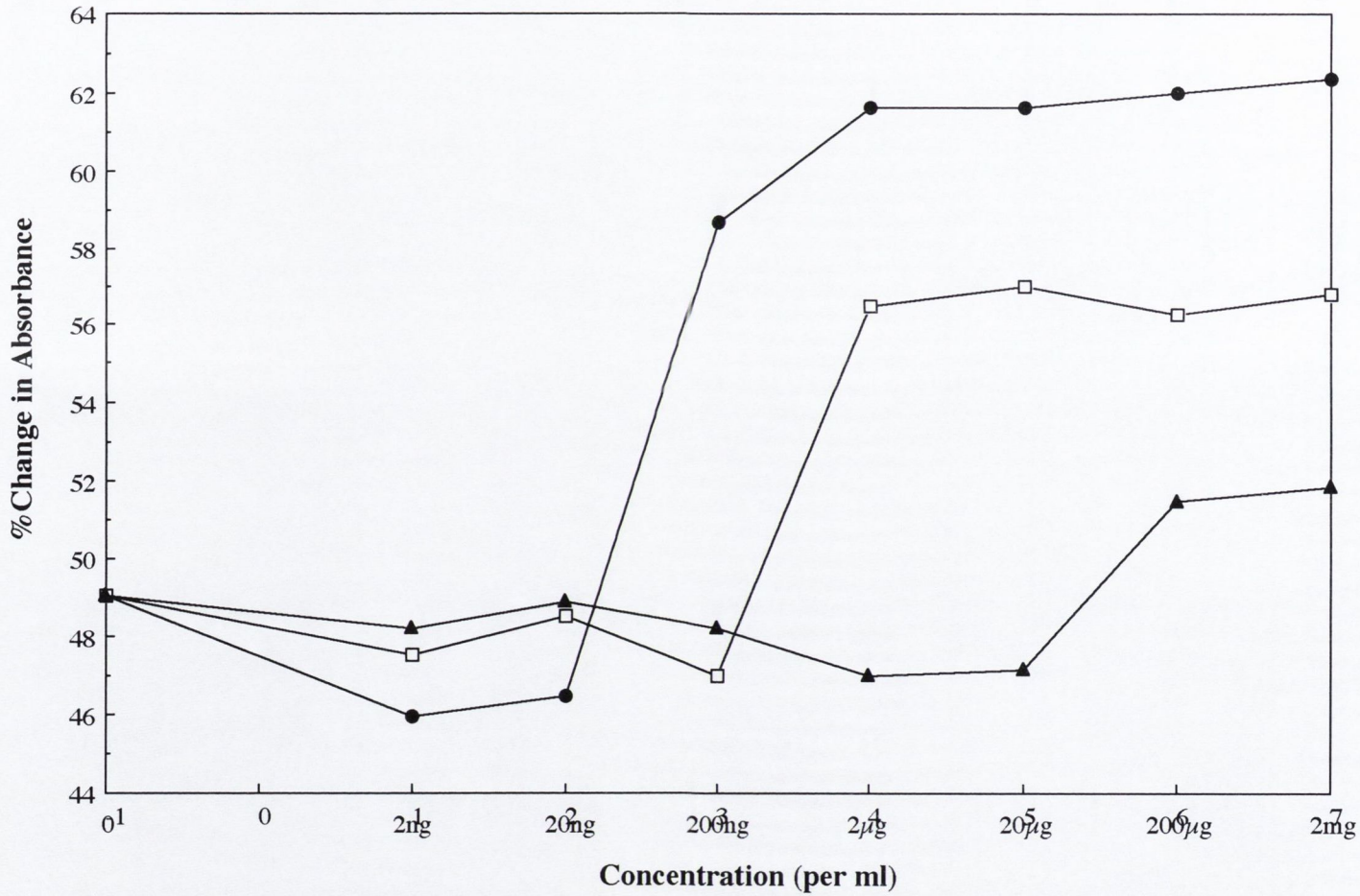


Fig 9.7. Antagonism to Tmp (20μg/ml) inhibition of *L.casei* growth by increasing concentrations of ● thymine, □ thymidine or ▲ inosine.

Interaction of FdU with Tmp When the *L.casei* inoculation density was halved 1μg/ml Tmp caused 70% growth inhibition [Fig 9.8]. However, such inhibition was antagonised by concentration of FdU in the range 2μg to 2mg per ml.

Interaction of FdU and Thymine with Tmp Both FdU and thymine were antagonistic towards Tmp, either alone or in combination [Fig 9.9a]. As expected thymine behaved as an agonist towards FdU, significantly increasing the rate at which *L.casei* grew [Fig 9.9b]. However, Tmp was also found to be mildly antagonistic towards FdU, marginally increasing the rate of growth.

9.3 Discussion

9.3.1. Inhibition of Folate Metabolism by Trimethoprim or Methotrexate

Effect of Tmp and MTX inhibition With *L.casei* we see a further possible outcome to DHFR inhibition. While the response observed in *E.coli* was a sudden and almost total (98%) inhibition of bacterial growth, the *L.casei* continued to grow but at a slower rate than the controls. This may be due to the slower accumulation/catabolism of DHF, resulting in partial conservation of the THF co-enzyme pool. While in *E.coli* concentrations of THF co-enzymes had fallen to 12% of controls within 25min, in *L.casei* the reduced folate pool still represented nearly 60% of labelled species after 1h inhibition. Whether this is due to the slower proliferation rate of the *L.casei* (approx. 7hr doubling time) relative to *E.coli* (approx. 90min doubling time) or due to lower TS activity. A low TS activity would prevent the rapid accumulation of DHF, thus slowing the rate at which the cellular folate became trapped as DHF. This is consistent with the finding that cells can

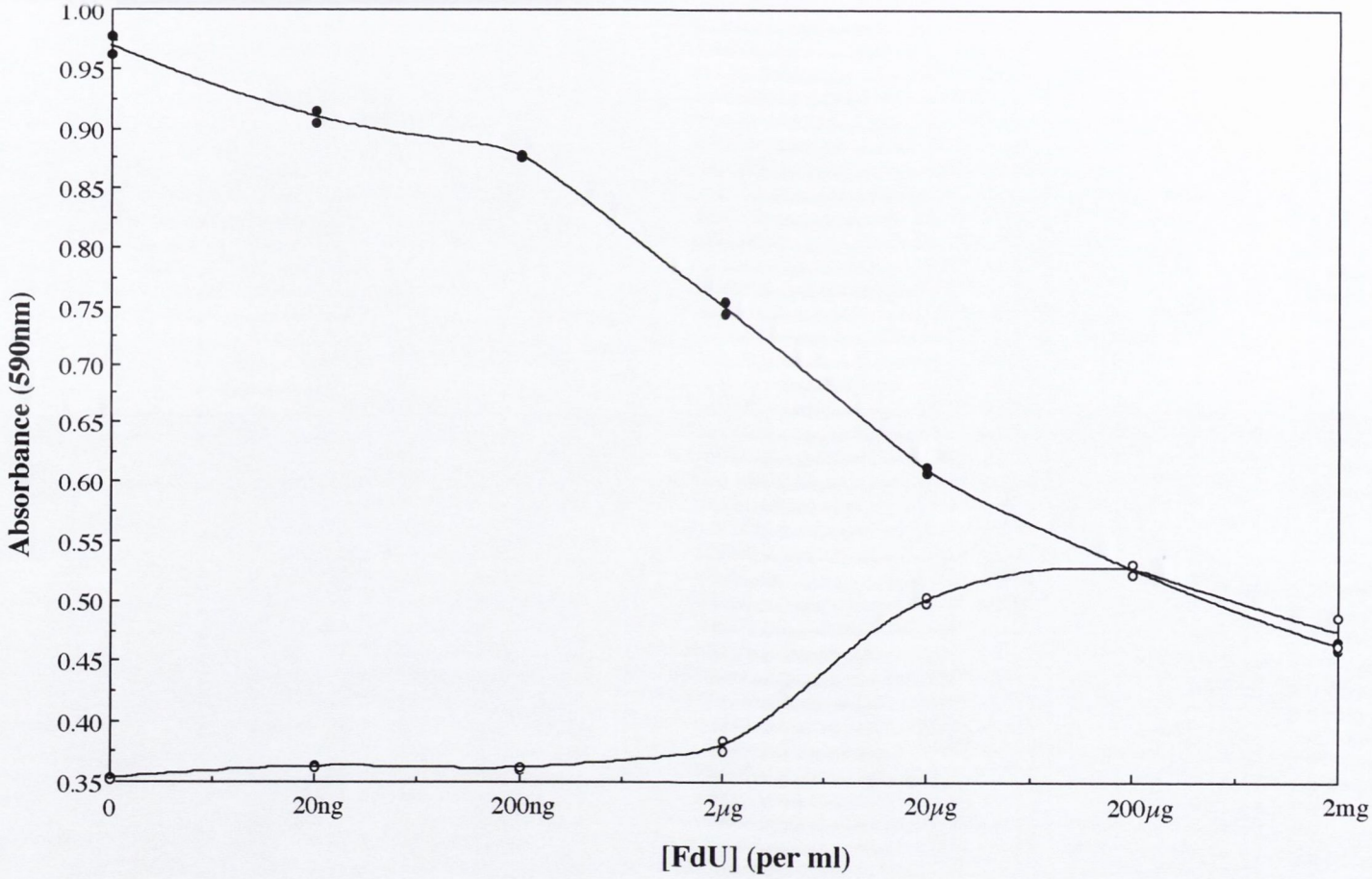


Fig 9.8. Antagonism of FdU towards Tmp inhibition of *L.casei* growth. The bactria were incubated in the presence of increasing concentrations of FdU and in the ○ presence or ● absence of Tmp (1μg/ml)

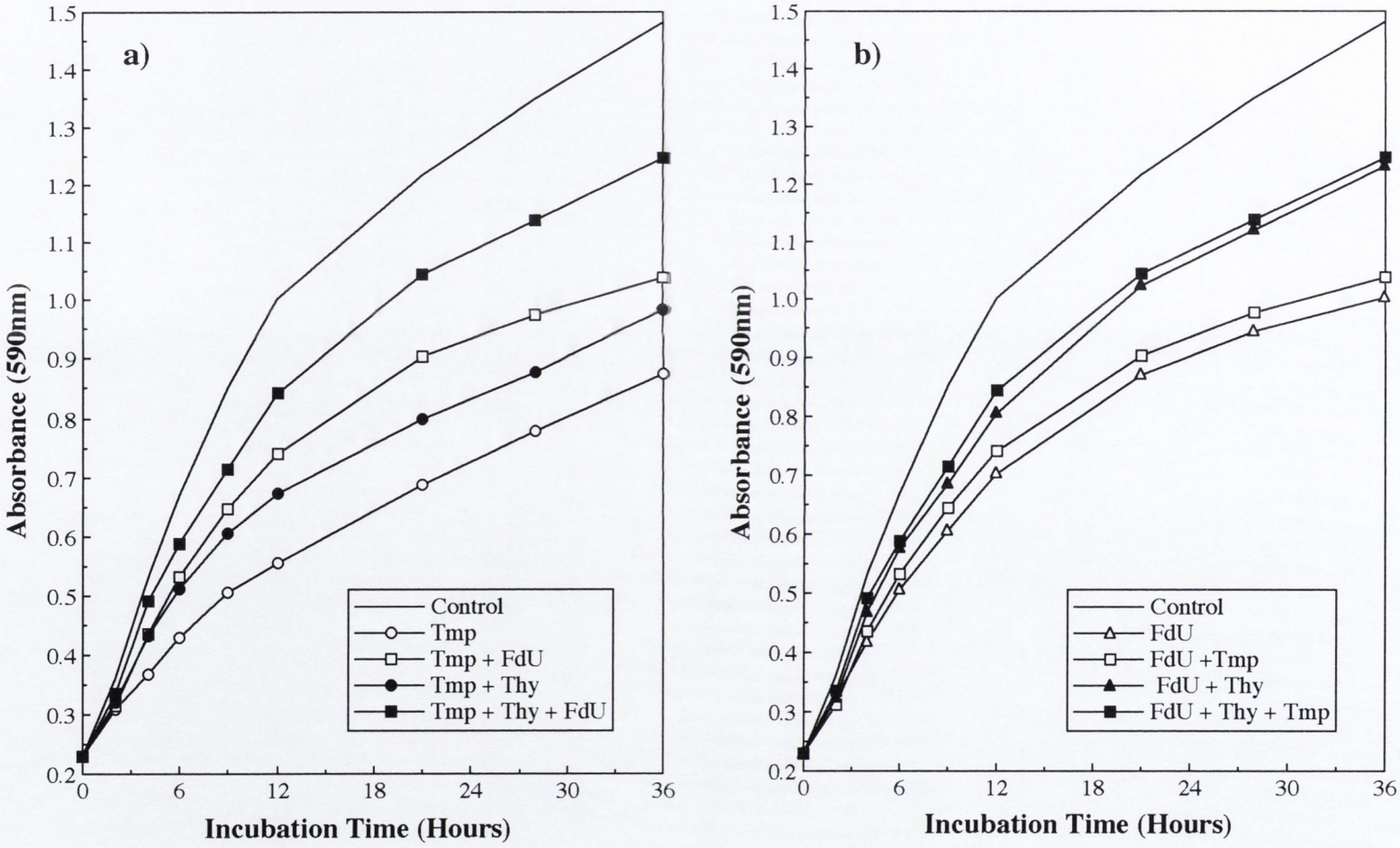


Fig 9.9. Growth response of *L.casei* to various inhibitors and stimuli. a) Antagonism to Tmp (1μg/ml) inhibition by FdU (20μg/ml) and/or thymine (Thy; 100μg/ml) . b) Antagonism to FdU (20μg/ml) inhibition by Tmp (1μg/ml) and/or Thy (100μg/ml).

naturally acquire MTX resistance by suppressing TS activity (White and Goldman, 1981), while *in vitro* inhibition of TS slowed the rate at which DHF accumulated (Seither *et al.*, 1991 & 1992; Moran *et al.*, 1979; present study).

The dependence of DHF accumulation, in the presence of inhibitors of DHFR, on the rate of cell proliferation is well documented in mammalian cells (Trent *et al.*, 1991a). During most of the cell cycle TS activity is low. It is only during the S-phase of the cell cycle that TS becomes active (Rode *et al.*, 1978). Thus in confluent cells DHF accumulation is slow as relatively few cells are in the S-phase.

Another observation in *L.casei* was that even in the absence of DHFR inhibition folate catabolism and pABGlu accumulation was observed. Thus even at the start of incubation roughly 25% of the folic acid used to label the bacteria had catabolised to pABGlu. During the 4h treatment phase this had increased to approx, 30% in the untreated sample. This attrition on folate stores in the absence of inhibition may indicate that conditions are conductive to catabolismis in *L.casei*. It may also reflect the total absence of a folate biosynthetic, and consequently a pABGlu salvage, pathway.

9.3.2. Antagonism of Tmp Inhibition

Interaction of Purine and Pyrimidine Precursors with Tmp It had been postulated that Tmp causes "thymine-less death", whereby disruption of the one-carbon cycle results in retardation of thymidylate synthesis. In support of this theory Then and Angehrn (1973) demonstrated that thymine was an antagonist of Tmp inhibition in *E.coli*. They suggested that the provision of an alternative means of dTMP synthesis, via the salvage pathway [Fig 1.3], satisfied the requirement for thymidylate. Our finding that thymine was also an

antagonist of Tmp in *L.casei* would appear to support this theory. However, the antagonism may have an alternative explanation. By providing an alternative substrate for dTMP synthesis the thymine may increase cellular concentrations of dTMP and thus feedback inhibition of TS (Daron and Aull , 1978), thus suppressing enzyme activity. Thus, by inhibiting DHF synthesis, the THF co-enzymes were diverted back into the one carbon cycle, conserving THF co-enzyme concentrations and sustaining one-carbon transfer in the rest of the cycle. Therefore thymine was probably more antagonistic towards Tmp than inosine not because it better fulfils a metabolic requirement, but because it alone prevents folate catabolism. This theory suggests that other TS inhibitors would be Tmp antagonists. To test this hypothesis we studied the interaction of FdU with Tmp.

Interaction of FdU with Tmp In support of this hypothesis we showed that FdU was also antagonistic towards Tmp. At concentrations of FdU between 200ng/ml and 20μg/ml intermediary inhibition was observed, *i.e.* growth inhibition was greater than that observed with FdU alone but was not as great as that observed with Tmp alone, however, at higher concentrations of FdU ($\geq$20μg/ml) the inhibition appeared identical to that achieved with FdU alone. We propose that, in the face of Tmp inhibition: (i) concentrations of FdU below 200ng/ml did not sufficiently inhibit DHF production so as to prevent its catabolism, (ii) concentrations of FdU above 200ng/ml caused significant inhibition of TS, thus diverting folate away from the DHFR block and consequent catabolism, (iii) however, concentrations of FdU $\geq$20μg/ml caused sufficient inhibition of dTMP synthesis to inhibit growth. This suggest that, in the presence of Tmp, *L.casei* is able to withstand some degree of TS inhibition before growth was affected, thus suggesting that "thymine-less death" is not the principle means by which Tmp inhibitions *L.casei* growth.

NOTE In examining the antagonism observed between DHFR inhibitors and TS inhibitors a distinction must be drawn between "classic" antifolate inhibitors such as PDDF, Tomudex, MTX, TMTX and DDATHF (Kisliuk, 2000), the activity of which is increased by polyglutamylation, and "non-classical" inhibitors such as FdU, which can not form polyglutamyl-chains. These "classic" antifolates compete with cellular folates for folate polyglutamate synthase (FPGS). Thus the decrease in THF co-enzyme concentrations due to DHFR inhibition decreases competition for FPGS. The resulting increase in concentrations of polyglutamylated antifolate enhances the anti-proliferative activity of the inhibitors as polyglutamylation increases the cellular retention of the drugs and enzyme affinity. Thus the synergy observed between the "classical" TS inhibitors, such as PDDF and Tomudex, and inhibitors of DHFR (Kisliuk *et al.*, 1990; Kisliuk, 2000; Galivan *et al.*, 1989) is probably due increased inhibitor polyglutamylation.

Interaction of Tmp with FdU The antagonism of Tmp towards FdU was slight [Fig 9.9] and may be explained by one or more possible mechanisms: (i) FdUMP is a "quasi substrate" for TS meaning it forms a semi-stable TS•FdUMP•5,10-CH_2-THF ternary reaction intermediate (Santi and Danenberg,, 1984). The decrease in THF co-enzyme concentrations may decrease ternary complex formation; (ii) 5,10-CH_2-THF is a substrate inhibitor of TS in the presence of BrdUMP (Santi and Danenberg, 1984), thus the Tmp induced decrease in 5,10-CH_2-THF concentrations would lower such inhibition; (iii) Tmp inhibition, by disrupting the one-carbon cycle, may increase concentrations of dUMP and thus competition with FdUMP for TS binding.

CHAPTER 10

METHOTREXATE INHIBITION OF FOLATE METABOLISM IN MAMMALIAN (L1210 CELL) CULTURES

10.1. Introduction

As discussed in the last chapter both trimethoprim (Tmp) and methotrexate (MTX) are inhibitors of dihydrofolate reductase (DHFR). Thus their chemotherapeutic specificity is not as a result of their targeting different enzymes in the one-carbon cycle. Instead their pharmaceutical specificity is derived from their exploitation of interspecies variations in DHFR structure, or more specifically, differences between the folate binding site of the mammalian and bacterial DHFR.

One of the first major breakthroughs in the design of metabolic inhibitors specifically designed as antibiotics came with the discovery of the sulfonamides. Up until the mid-1930's it was widely believed that all the key biochemical pathways were common to both prokaryotes and eukaryotes (Florkin, 1974). However, it soon became clear that some pathways, such as the folate biosynthetic pathway, were in fact specific to bacteria and could thus be inhibited (Woods, 1940; Lampen and Jones 1946; Swedberg *et al.*, 1979; Roland *et al.*, 1979; Brown, 1962; van Miert, 1994) without affecting the host.

It was not until the 1948, with the discovery of the diaminopyrimidines by Hitchings and co-workers (Hitchings *et al.*, 1948), that it became possible to selectively inhibit enzymes of the one-carbon cycle common to both prokaryotes and eukaryotes. This specificity is as a result of the high degree of heterogeneity (70%) in amino acid sequence between prokaryotes and eukaryotes, or even between bacterial species, where there is still only about 30% homology (Hitchings, 1989). It is only among the higher species that sequence is conserved, with up to 90% homology. Thus, with careful synthesis and testing of diaminopyrimidines analogues Hitchings and co-workers (Then, 1993) increased the drug specificity for bacterial DHFR [Table 10.1]. It was for this work that Hitchings and Elion were awarded the Nobel Prize for Medicine in 1998 (Hitchings, 1989).

Inhibitor	*E.coli*	*P.berghei*	Rat Liver
methotrexate	5	0.7	2
trimethoprim	1	7	30,000

Table 10.1. Selectivity (IC_{50} in nmol/l) of methotrexate and trimethoprim against bacterial and mammalian dihydrofolate reductase (Burchall, 1979).

The selectivity of Tmp is as a result of differences between mammalian and bacterial DHFR. Specifically, the folate binding pocket is smaller in *E.coli* than in both chicken (Matthews *et al.*, 1985) and mouse lymphocyte (Stammer *et al.*, 1987) enzymes. This makes Tmp binding to the vertebrate enzyme less energetically favourable and probably distorts its conformation within the binding pocket (Matthews *et al.*, 1985). This may account for the loss of co-operativety of binding between Tmp and NADPH [Table 10.2]. Likewise, binding of MTX to the vertebrate DHFR•NADPH complex causes a conformational change in the enzyme, increasing the binding "tightness" (Appleman,

1988; Stone and Morrison, 1986). No such increase in binding "tightness" was observed when Tmp bound to the chicken DHFR (Stone and Morrison, 1986).

DHFR Source	*E.coli*	*S.faecium*	*L.casei*	Mouse	Bovine
Co-operativity	41	55	450	4.7	17

Table 10.2. Co-operativity of binding between trimethoprim and NADPH to bacterial and mammalian dihydrofolate reductase (adapted from Baccanari *et al.*, 1987).

Thus, one of the major differences between bacterial and mammalian DHFR is their susceptibility to different inhibitory drugs. However, we wished to see whether there were any other differences between mammalian cells and bacterial cells in their response to DHFR inhibition. To this end, we designed a mouse lymphocytes (L1210 cells) model to examine the effects of MTX inhibition on folate metabolism in mammalian cells. The effects of such inhibition on folate distribution and catabolism could then be compare to the response observed in *E.coli* [Chapter 7] and *L.casei* [Chapter 9]. The methods used are as described in [Section 2.6].

10.2. Results

Qualitative Identification of Protein Bound Folate Chromatography of deconjugated extracts from L1210 cells, in the absence of protease treatment, resulted in poor chromatographic traces with numerous "ghost" peaks [Fig 10.1], i.e. peaks which did not elute with known folate monoglutamate standards, but instead eluted after the large authenticated peaks. Prolonged incubation periods of up to 90min (twice the normal

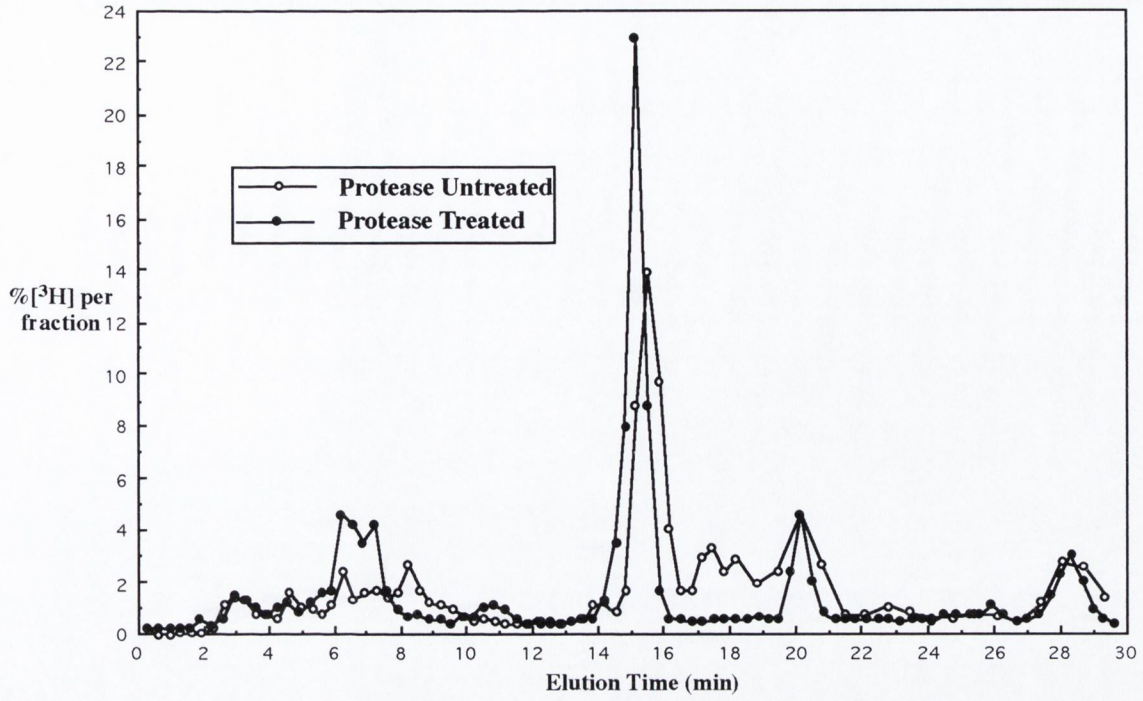


Fig 10.1. Radiolabel elution profile of L1210 cells extracts (treated for 12h with MTX) chromatographed on a Kingsorb C_{18} column ○ before and ● after protease treatment.

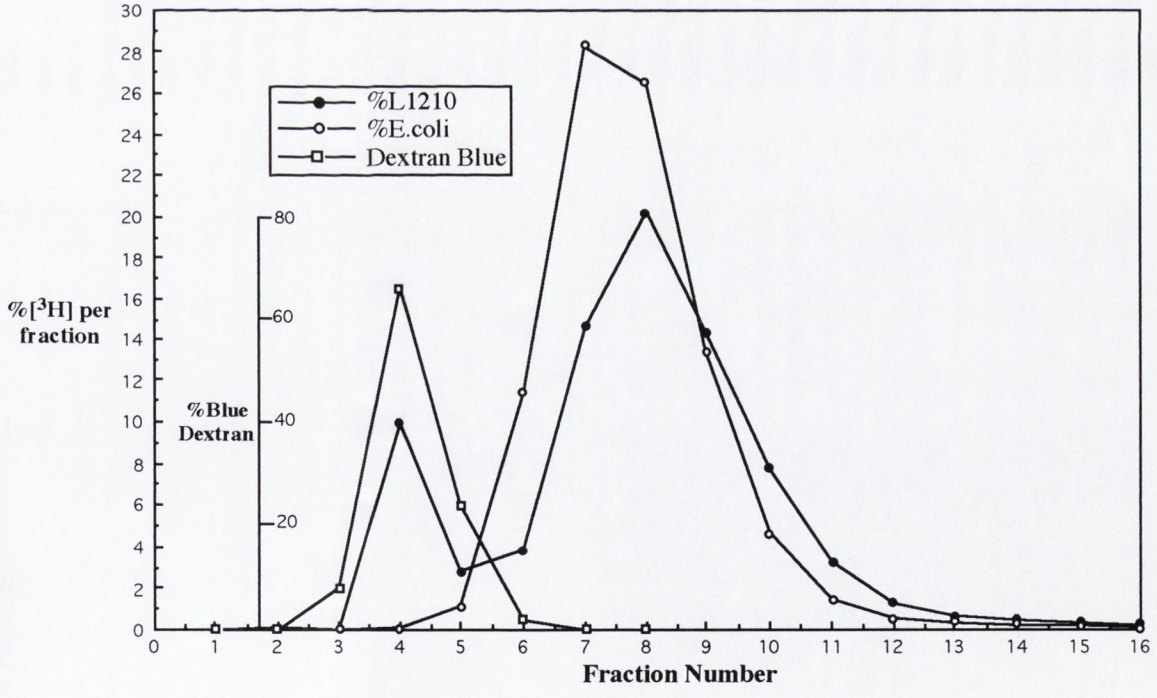


Fig 10.2. Radiolabel elution profile of ● L1210 cell extracts and ○ *E.coli* extracts chromatographed on a Sephadex G-50 gel filtration column. □ Blue dextran (M_r = 66kDa) was used to mark the void volume.

incubation period) had little effect in removing these "ghost" peaks (results not shown). Gel filtration chromatography of untreated L1210 cell extracts resulted in co-eluting of approx. 10% of the radiolabel with the blue dextran marker in the void volume [Fig 10.2]. All the radiolabel from *E.coli* extracts was retarded on the Sephadex column when chromatographed using the same elution conditions. Treatment of L1210 extracts with protease resulted in 100% retardation of radiolabel on the gel filtration column (results not shown) and the elimination of the "ghost" peaks from chromatograms [Fig 10.1].

Effect of MTX Inhibition on Folate Distribution At time zero or after 12h incubation the folates in the untreated *E.coli* were present exclusively as THF co-enzymes. However, with time the cellular 5-CH_3-THF content increased with time at the expense of the other co-enzymes [Table 10.3]. Treatment with 1μM MTX resulted in the accumulation of DHF. The DHF appeared relatively stable over the time period examined as there was only minor oxidation to folic acid and cleavage to pABGlu. There was a slight decrease in the pABGlu content of the cells between 6 and 12h incubation. However, it had increased again by 24h incubation.

10.3. Discussion

Incomplete deconjugation of folates In determining the optimum deconjugation period for *E.coli* extracts it was noted that chromatograms of samples incubated for sub-optimum periods exhibited "ghost" peaks similar to those in the protease untreated L1210 extracts. As re-incubation of the *E.coli* sample resulted in the disappearance of these peaks it was concluded that these "ghost" peaks were probably polyglutamate forms of the folate monoglutamate after which they eluted.

	THF coenzymes	5-CH_3-THF	DHF	Folic Acid	pABGlu
Control (T_0)	95.4	(13.9)	1.5	ND	3.1
MTX (6h)	53.9	(28.4)	35.7	4.2	6.2
Control (12h)	97.4	(49.1)	0.8	ND	1.8
MTX (12h)	42.2	(10.3)	47.8	9.3	2.7
MTX (24h)	24.1	(3.7)	55.4	12.4	8.1

Table 10.3. Folate distribution in L1210 cells after the indicated incubation periods in the presence (MTX) or absence (Control) of 1μM MTX. THF co-enzymes represent total reduced folates (including 5-CH_3-THF).

A possible reason for incomplete deconjugation is that large percentage of intracellular folates in L1210 cells are protein bound (Matherly and Muench, 1990) and may not be readily deconjugated. To test this hypothesis we passed L1210 extracts (pre and post protease treatment) down a Sephadex G-50 column. As the Sephadex G-50 has an exclusion size of 10,000 for dextrans (30,000 for protein) it must be assumed that the radiolabel which eluted in the void volume (= elution volume of blue dextran (M_r = 66kDa)) had a complex weight of ≥ 10,000. Such an apparent molecular weights could not be accounted for by polyglutamation as even folate-decaglutamates would have a molecular weight of less than 1,800. This suggests that the protease digestion freed the bound folate from its protein complex, resulting in its retardation of the G-50 column. As the protease K enzyme was attached to a agrose bead it was easily removed by centrifugation and therefore did not interfere with the later folate deconjugation step.

Effect of MTX Inhibition on Folate Distribution These results confirm the finding (Trent *et al.*, 1991a & 1991b; Seither *et al.*, 1991; Matherly and Muench, 1990) that, unlike the rapid and complete accumulation of DHF in *E.coli*, inhibition of DHFR in L1210 cells resulted in a slow and incomplete accumulation of DHF, even after 24h growth there was not complete conversion of the THF coenzymes to DHF. This slow and incomplete accumulation of DHF was attributed to the fact that only cells in the S-phase of the cell cycle were producing DHF (Trent *et al.*, 1991a) and that, due to partitioning of thymidylate synthase activity, only folate in the cytoplasm, and not in the mitocondria, is be converted to DHF (Trent *et al.*, 1991b). Such a conclusion is consistent with the fact that DHF is a by-product of thymidylate synthesis, and as such would only be produced at times of DNA replication, as during the S-phase of the cell cycle. In this manner Rode *et al.* (1979) demonstrated that thymidylate synthase activity was strictly regulated *in vivo*, increasing only during the S-phase. However, TS activity in cell free extracts was constant

whichever phase of the cell cycle the cells were harvested in. This suggest that the sight of TS regulation is through *in vivo* modulation of enzyme activity rather than through modulation of enzyme concentration.

Under the conditions used MTX inhibition of L1210 cells represent another possible extreme of DHFR inhibition, namely DHF stability. While MTX inhibition of L1210 cells causes DHF accumulation, in contrast to both *E.coli* and *L.casei*, relatively little catabolism was observed. Thus the stability of DHF in mammalian (L1210) cells is at variance with that observed bacteria. This may be due to a number of factors: (i) mammalian cells may be better able to regulate their own redox state and internal pH; (ii) alternatively, a large proportion of intra-cellular folate in L1210 cells is protein bound. During folate deficiency there may be little free folate with the vast majority being folate bound (Matherly and Muench, 1990). As the cellular folate concentration increases the percentage of bound folate can drop below 50%. While experimentally we show that approximately 10% of the folate was protein bound [Fig 10.2] the methods were used were only qualitative, no attempt was made to prevent the free defusion of protein bound folate during extraction. This protein binding may stabilised the C9-N10 bond of DHF via protein-folate interaction, *i.e.* ionic bonds or van de Waals forces. The protein bulk may also shield the C9-N10 bond from attack by reactive elements. Alternatively, the hydrophobic microclimate of the binding pocket may protect the DHF, preventing attack by oxidising elements and providing its own redox state and pH.

Accumulation of 5-CH_3-THF in control cells It is unlikely that the accumulation of 5-CH_3-THF was due to "methyl-trapping" [see Section 1.4] as there should have been sufficient vitamin B_{12} (680μg/L) in the media to meet requirement. However, there are several other explanations that may explain the 5-CH_3-THF accumulation: (i) Accumulation may be due

to folate "partitioning" by polyglutamate chain length. Elongation of the polyglutamate chain is time dependent. Therefore, as the cells were pulse labelled the polyglutamate distribution of the radiolabelled folate would not be representative of the total folate polyglutamate distribution, having longer glutamate chains than folate added subsequently. Thus, "mature" [^{3}H]-folate may preferentially accumulate in the methylation cycle as 5-CH_3-THF. (ii) The cells may still be folate deficient after the labelling process. The resulting disruption to the one carbon cycle may result in 5-CH_3-THF accumulation. (iii) The high concentration of methionine in the growth media may cause feedback inhibit of methionine synthase, thus preventing the demethylation of 5-CH_3-THF and causing its accumulation. (iv) The folic acid in the media may have provided an alternate source of THF, decreasing the need to recycle 5-CH_3-THF.

CHAPTER 11

DISCUSSION

"Necessity is the mother of invention"

Anon.

11.1. Calculating the Recommended Daily Allowance for Folate

We have calculated the daily rate of folate loss in men and (non-pregnant) women [Chapter 3] and in pregnant women [Chapter 5], they are presented in [Table 11.1]. This represents the amount of folate lost by the body each day and thus the amount of folate which has to be replaced to maintain folate homeostasis. However, dietary folate is only about 50% bio-available (Bhiandari and Gregory, 1990; Rosenberg, 1981). The average requirement (AR) for folate, the amount of folate that must be consumed by an "average" person to replace loss, is therefore twice the daily folate loss. The Recommended Daily Allowance (RDA) for folate is calculated as the AR plus two standard deviations (SD), statistically this satisfies the requirement of 97.5% of a normally distributed population (National Research Council, 1989).

		Daily loss (μg/d)	AR ±SD (μg/d)	RDA (μg/d)
	Men	84 (30)	168 (60)	290
Women	Non-pregnant	62 (26)	124 (52)	230
	First trimester	90 (28)	180 (56)	290
	Second trimester	124 (30)	247 (60)	370
	Third timester	147 (41)	295 (82)	460
	Postpartum	103 (30)	207 (60)	330

Table 11.1. The daily loss, average requirements (AR) and recommended daily allowance (RDA) for folate for men and women and at each trimester of pregnancy [Mean (± SD)].

11.2. Evolutionary Design and Deletion

11.2.1. Metabolic Cost (ATP Equivalents) of Folate Biogenesis

In recognition of the almost ubiquitous nature of the adenine nucleotide pool (AMP, ADP and ATP) in cellular metabolism, Atkinson (1977) uses the ATP equivalent as the basis for calculating the metabolic cost of a reaction [Table 11.2]. By extending this analogy we can calculate a compound's biosynthetic cost by adding the metabolic cost of each coupling reaction involved in that compound's synthesis. However as this may involve the sum of several converging pathways, or involve repetitive calculations, we can first calculate the metabolic cost of *de novo* biosynthesis for the more common biochemical intermediaries [Table 11.3]. From these initial "building blocks" the metabolic cost of each product can then be calculated by adding the cost of the intermediaries and of the connecting reactions [Table 11.4].

11.2.2. Evolutionary Design and Deletion

It has been postulated that the large metabolic cost of the chorismate biosynthetic pathway may have been a factor in its deletion during mammalian evolution (Kishore *et al.*, 1988; Haslam, 1993). The absence of this pathway in mammals is the reason why the three aromatic amino acids are essential amino acids or, in the case of tyrosine, are synthesised from essential amino acids. In fact the 10 essential amino acids, plus tyrosine, make up all but one of the 12 most metabolically expensive amino acids [Table 11.5]. Thus the absolute requirement for folate in mammals is due in part to an inability to synthesise chorismate. However this may not the full explanation: mammals can synthesise both dihydroneopterin (DHNpt) (Fischer *et al.*, 1997; Hasler and Niederweiser, 1986; Nagatsu *et al.*, 1986; Ichinose *et al.*, 1995; Duch *et al.*, 1986; Hirayama and Kapatos, 1997;

Coupling Agent	ATP equivalent
Primary	
ATP → ADP	1
ATP → AMP	2
NADPH → NADP	4
Secondary	
NADH → NAD	3
$FADH_2$ → FAD	2

Table 11.2. Metabolic Cost of Coupling Agents: Prices are expressed as ATP equivalents. Adapted from Atkinson (1977).

Compound	ATP equivalent
Erythrose-4-phosphate	26
Phosphoenolpyruvate (PEP)	16
Pyruvate	15
Ribose-5-phosphate	33
α-Ketogluterate	25

Table 11.3. Metabolic Price of Biosynthetic Starting Materials: Selection of biosynthetic starting material (building blocks). Since intermediates of glycolysis and the citrate cycle are common starting materials in many biosynthetic sequences they are ideal building blocks for calculating subsequent metabolic costs. The compounds listed were chosen because of their association with the folate synthesis pathway. Adapted from Atkinson (1977).

Compound	Starting Material	Conversion Requirement	ATP equivalent
Amino Acids:			
Glutamate	α-Ketogluterate (25)	NADPH (4); ATP (1)	30
Glutamine	Glutamate (30)	ATP (1)	31
Chorismate	2 x PEP (32) + Erythrose 4-P (26)	NAD → NADH (-3); NADPH (4); ATP (1)	60
Phenylalinine	Chorismate (60)	trans-NH_2 (5)	65
Tyrosine	Chorismate (60)	NAD → NADH (-3); trans-NH_2 (5)	62
Tryptophan	Chorismate (60) + P-ribosyl-PP (35) - Pyruvate (-15)	Gln → Glu (1)	78
Nucletides:			
IMP	P-ribose-PP (35), Glycine (12)	2 Gln → 2 Glu (2); 4 ATP → 4 ADP (4); 2 5-CHO-THF → 2 THF (4) aspartate → fumerate (2)	59
GMP	IMP (59)	NAD → NADH (-3); ATP → AMP (2); Gln → Glu (1)	59
GDP	GMP	NADPH (4); ATP (1)	64
GTP	GDP	ATP (1)	65

Table 11.4. Metabolic Price of Biochemical Compounds: Selection of biochemical compounds and the metabolic cost of their synthesis. The compounds listed were chosen because of their association with the folate synthesis pathway. Values in parentheses are the cost of starting material or the cost of regenerating the coupling agents used. Compounds in **Bold** are intermediaries used for further synthesis. (Adapted from Atkinson, 1977).

Rank	Amino Acid	ATP cost	Rank	Amino Acid	ATP cost
1	**tryptophan**	78	=	proline	39
2	**phenylalanine**	65	12	**threonine**	31
3	tyrosine*	62	=	glutamine	31
4	**isoleucine**	55	14	glutamic acid	30
5	**lysine**	50	15	asparagine	22
6	**leucine**	47	16	aspartic acid	21
7	**arginine**	44	17	alanine	20
=	**methionine**	44	18	cysteine	19
9	**histidine**	42	19	serine	18
10	**valine**	39	20	glycine	12

Table 11.5. List of amino acids, ranked in order of their metabolic cost (ATP equilalents) (Adapted from Atkinson, 1977). Amino acids in **bold** are essential amino acids (from Weller and Wells, 1990). *Tyrosine is synthesised from phenylalanine, an essential amino acids.

Gütlich *et al.*, 1997) and glutamate and should therefore be able to synthesise folate from exogenous pABA. Yet they are unable to do so.

Reasons for this additional loss of function may be: i) exogenous pABA concentrations may not be sufficient to sustain folate synthesis, ii) the savings in metabolic cost may have been sufficient to out-weight the risk of folate deficiency which results from an inability to synthesise the vitamin. In any event, by eliminating the folate biosynthetic pathway and instead using preformed folate a saving of approximately 132 ATP equivalents is achievable [Fig 11.1]. As we have already calculated the daily folate loss for men and women [Chapter 3] and have shown how this increases during pregnancy [Chapter 5] we can therefore calculate the daily metabolic savings achieved by using the preformed form of the vitamin, rather than synthesising it *de novo* [Table 11.6]. Even just concentrating on the folate loss due to catabolism (apABGlu excretion) the metabolic saving achieved by the evolutionary deletion of the folate biosynthetic pathway are considerable [Table 11.7]. It is anecdotally suggested that incidence of folate deficiency is the highest of all vitamins and results from the dependency arising from this inability of humans to synthesise folate. Thus, the metabolic saving, resulting from utilisation of intact folate (folate salvage), must have been sufficient to counterbalance this risk of folate deficiency.

As we have described, folate deficiency is frequently seen in pregnancy. The consequence of this deficiency is often seen in the degree of megaloblastic anaemia often associated with pregnancy particularly in poorer countries (Wills, 1930). Thus, we have determined the increase in folate loss during pregnancy and can thus calculate the corresponding increase in ATP that would be required to replace it [Table 11.6 & 11.7]. As we have shown in Chapter 5 folate loss increased by 7.8 mg during the first 34 weeks of pregnancy. To replace this folate loss *de novo* would cost 2.33 mmoles of ATP or 1.29 grams of ATP (M_r ATP = 551). This additional metabolic saving must again have been sufficient to

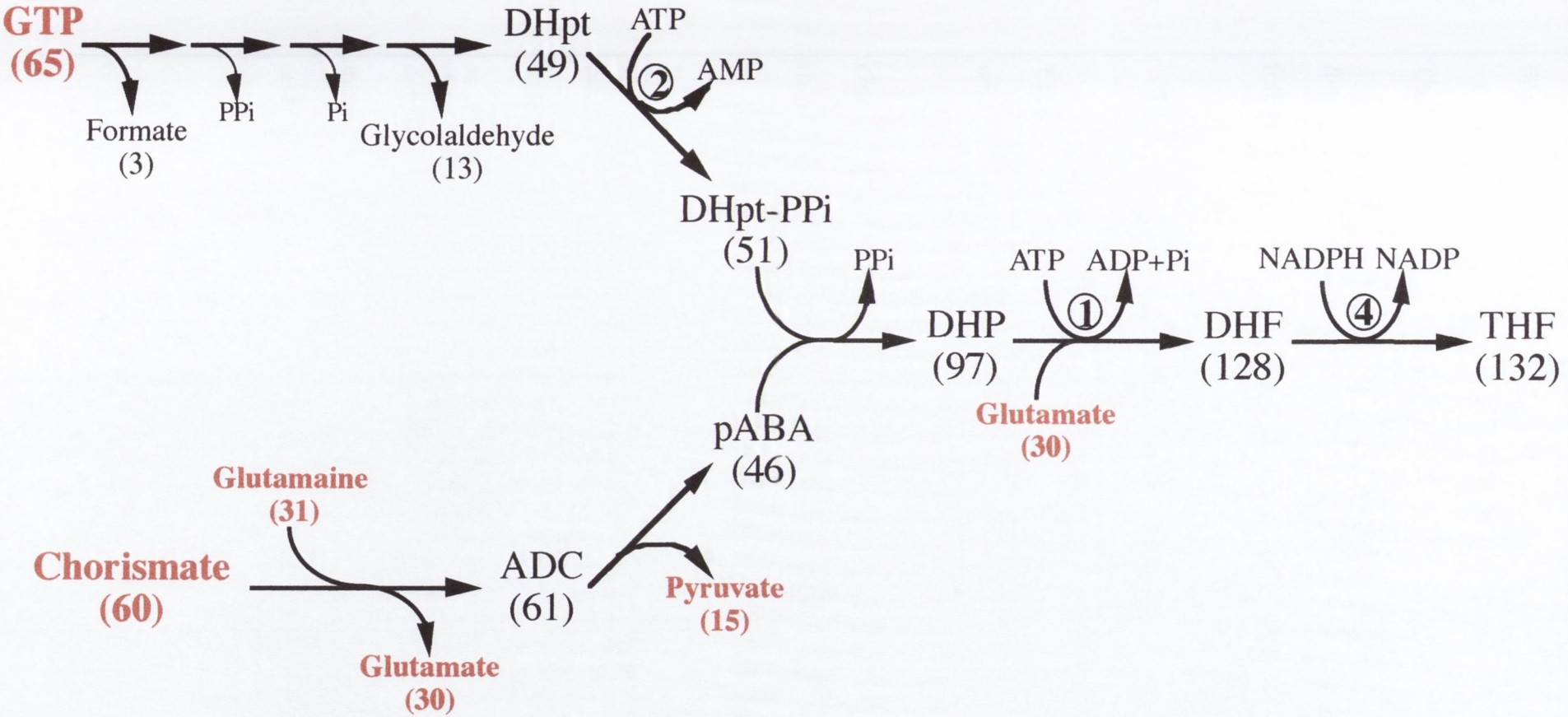
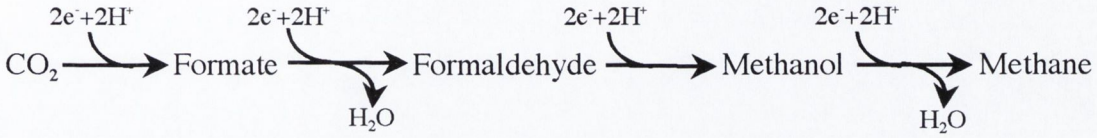


Fig 11.1. Metabolic cost (ATP equivalents) of *de novo* folate synthesis. Diagram shows the biosynthetic pathway for folate. Also shown is the metabolic cost (in brackets) of each of the substrates and by-products of the pathway and the metabolic cost (circled) of the coupling reactions. The metabolic cost of compounds in red are from Atkinson, 1977. The metabolic cost of formate and glycolaldehyde are calculated in [Box 11.1] and [Box 11.2] respectively.

Box 11.1. Metabolic cost of formate and formaldehyde synthesis

There are two methods for calculating the metabolic cost of formate and formaldehyde.

Reduction of carbon-dioxide The successive two electron reduction of one carbon is as follows:



Thus, using NADH as reducing agent, the metabolic cost of formate is 3 ATP equivalents while that of formaldehyde is 6 ATP equivalents.

One-carbon cycle Alternatively, the metabolic cost of formaldehyde can be calculated from the one carbon cycle:

Serine (18 ATP) + THF → Glycine (12 ATP) + 5,10-CH_2-THF Eqn 11.1

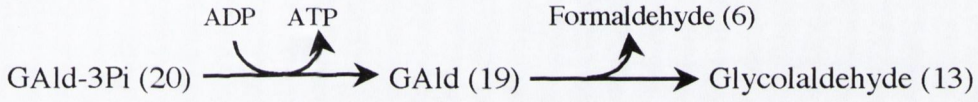
The one-carbon unit of 5,10-CH_2-THF is at the formaldehyde level of oxidation and, balancing Eqn. 11.1, costs 6 ATP equivalents. Oxidation of 5,10-CH_2-THF to 10-CHO-THF (formate level of oxidation) gives a metabolic cost of formate of 2 ATP equivalents as NADP is used as co-factor. However, metabolicly, as with formaldehyde dehydrogenase (Stirling and Dalton, 1978; Blakely and Benkovic, 1984), NAD is probably more commonly used as co-factor. Therefore, the metabolic cost of formate is again given as 3 ATP equivalents.

Conclusion The metabolic cost of formate is 3 ATP equivalents. That of formaldehyde is 6 ATP equivalents.

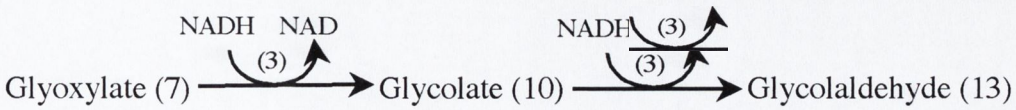
Box 11.2. Metabolic cost of glycolaldehyde synthesis

As when calculating the metabolic cost of formate and formaldehyde there are a number of ways of calculating the metabolic cost of glycolaldehyde. Here we chose two pathways in which glycolaldehyde or its metabolites may participate, glycolysis and the glycolate methabolic pathway.

Glycolysis One of the metabolic pathways for the biosynthesis of glyceraldehyde (GAld) is the condensation of glycolaldehyde and formaldehyde (Yaylayan *et al*, 2000). GAld can be phosphorylated to glyceraldehyde-3-phosphate (GAld-3Pi) by triokinase at the expense of 1 ATP → 1 ADP. GAld-3Pi is a participant in the glycolysis pathway whose metabolic cost has been calculated as 20 ATP equivalents (Atkinson, 1977). Therefore, calculating back from GAld-3Pi:



Glycolate metabolic pathway Glycolaldehyde can be converted to glyoxylate by two successively oxidation reactions. Firstly by glycolaldehyde dehydrogenase : glycolate reductase (Boronat *et al*, 1983; Tani *et al*, 1984) or non-specific dehydrogenases (Baldomá and Aguilar, 1987; Ting and Crabbe, 1983; Stirling and Dalton, 1978) to form glycolate, The glycolate is thence converted to glyoxylate by glyoxylate reductase (van Schaftingen *et al*, 1989; Suzuki *et al*, 1973; Kleczkowski *et al*, 1986). As the metabolic cost of glyoxylate is 7 ATP equivalents (Atkinson, 1977). Again we can calculate the metabolic cost of glycolaldehyde by following the reverse reaction:



Conclusion From both these reaction pathways it is apparent that the metabolic cost of glycolaldehyde is 13 ATP equivalents.

	Folate Loss (μg/d)[1]	**Folate Loss (nmole/d)[2]**	**ATP Cost (μmole/d)[3]**
Men	84 (30)	190 (68)	25.1 (9.0)
Women (N.P.)[4]	62 (26)	140 (59)	18.6 (7.8)
3rd Trimester[5]	148 (29)	335 (93)	44.3 (8.7)

Table 11.6. Metabolic cost of total folate loss. [1]Folate loss was calculated as the total folate loss due to the urinary excretion of intact folate and its catabolites (apABGlu and pABGlu, both expressed as folate equivalents). [2]Calculated on the basis that folic acid has a molecular weight of 441. [3]Calculated on the basis that 132 moles of ATP are required to synthesise 1 mole of DHF (see Fig 11.1). [4]Non-pregnant women (baseline). [5]Pregnant women, third trimester.

	Folate Loss (μg/d)[1]	Folate Loss (nmole/d)[2]	ATP Cost (μmole/d)[3]
Men	65 (26)	148 (60)	19.5 (7.8)
Women (N.P.)[4]	43 (23)	98 (53)	12.9 (6.9)
3rd Trimester[5]	119 (38)	27 (86)	33.6 (11.4)

Table 11.7. Metabolic cost of total folate catabolism (apABGlu excretion). [1]Folate loss due to catabolism to apABGlu (expressed as folate equivalents). [2]Calculated on the basis that folic acid has a molecular weight of 441. [3]Calculated on the basis that 132 moles of ATP are required to synthesise 1 mole of DHF (see Fig 11.1). [4]Non-pregnant women (baseline). [5]Pregnant women, third trimester.

compensate for the complications of pregnancy caused by folate deficiency (Wills, 1930) thus making folate salvage evolutionary advantageous from an evolutionary standpoint.

11.2.3. Cost of Folate Storage (Polyglutamate Synthase)

At first glance the cost of folate storage appears high, one glutamate (30 ATP equivalents) plus one ATP per glutamate added, a total of 31 ATP equivalents. The apparent minimum length required for folate storage is triglutamate (Osborne *et al.*, 1993; Lowe *et al.*, 1993). As folate is already a monoglutamate the production of the triglutamate would required the addition of two glutamates to the folate, at a cost of 62 ATP equivalents. In a similar manner, the average cellular polyglutamate length is approximately pentaglutamate (Thompson and Krumdieck, 1977; Brown *et al.*, 1974, Baugh *et al.*, 1975) at an additional cost of 124 ATP equivalents. However, as folate and its catabolites (apABGlu and pABGlu) are excreted in the urine as monoglutamates, the additional glutamates are probably salvaged. Chain elongation therefore only costs one ATP per glutamate added, or an average of 4 ATP equivalents per cellular folate.

11.2.4. Cost of "activating" folic acid

The majority of folates in the diet are present as the fully reduced active form of the co-enzyme, *i.e.* they are present as either substituted or unsubstituted species at the tetrahydro-level of oxidation. However, folic acid in the diet is in a fully oxidised inactive form of the vitamin. Thus it must be reduced by DHFR, first to DHF and thence to THF, to be activated. As each cycle of DHFR requires the oxidation of one molecule of NADPH, and folic acid must be reduced twice, the metabolic cost of folic acid activation is 8 ATP equivalents.

11.3. Regulation of Folate Homeostasis

11.3.1. Protection of methyl-transfer (vitamin B_{12} regulation of the one-carbon cycle)

Vitamin B_{12} deficiency does not cause the megaloblastosis associated with megalobastic anaemia.

Rather it is the folate deficiency, due to either the accumulation of 5-CH_3-THF at the expense of other functional THF co-enzymes or depletion of total folate concentrations (Lavoie *et al.*, 1974; Davidson *et al.*, 1975; Shane *et al.*, 1977; Gutstein *et al.*, 1973; Perry *et al.*, 1976), that causes megalobastic anaemia. Thus the question arises, why should the deficiency of one vitamin (vitamin B_{12}) result in the functional or absolute deficiency of another vitamin (folate)? To answer this question we must look more closely at the one carbon cycle.

Conceptually, at least, the one carbon cycle can be divided into two distinct parts, the methylation cycle and the rest of the one-carbon cycle [Fig 11.2]. In reality such a simplistic division of the one-carbon cycle is not possible as each is interdependent. The methylation cycle is dependent on the rest of the cycle for the generation of "active" methyl-groups in the form of 5-CH_3-THF while the rest of the cycle is dependent on the methylation cycle for the regeneration of THF from 5-CH_3-THF [Fig 1.2]. There is probably also some degree of inter-regulation of metabolic flux between the two parts, *i.e.* the allosteric regulation of MTHFR by SAM. However, if we look at our two halves of the one-carbon cycle not only do we see two distinct halves but we also see two distinct functional roles, the "proliferative" function of the cycle outside methylation and the "developmental" function of the methylation cycle[Table 11.8; Fig 11.2]. In this manner

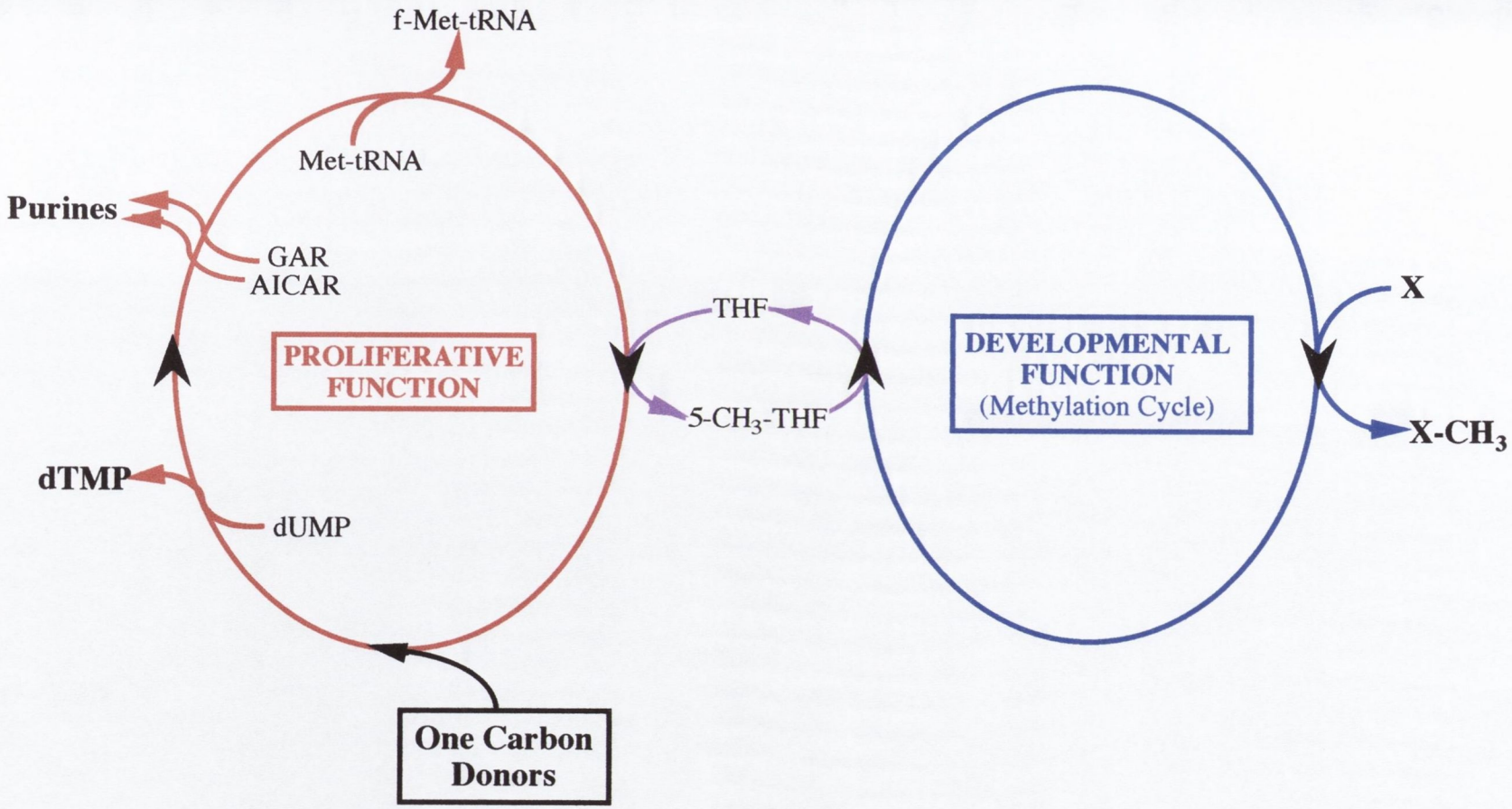


Fig 11.2. Diagrammatic representation of the one-carbon cycle showing its "proliferative" and "developmental" functions.

the "proliferative" function regulates the rate at which cells divide and at which protein is synthesised, thus governing the rate at which the other cellular components must be produced for the cell to function correctly. Through its involvement in the manufacture of some of the components required for cellular function the methylation cycle determines the rate at which these products can actually be produced.

Proliferative function	**Developmental function**
Purine biosynthesis:	Regulation of epogenics:
formylation of GAR and AICAR	methylation of cytosine in DNA
Thymidylate biosynthesis:	Methylation of substrates:
dUMP → dTMP	*i.e.* biosynthesis of myelin,
Initiation of protein synthesis:	neurochemical, creatine,
formylation of Met-tRNA	phosphatidylcholine.

Table 11.8. The distinct proliferative and developmental functions of the one-carbon cycle.

Note While the one-carbon cycle has other functional role other than the two outlined above, *i.e.* conversion of serine (Smith *et al.*, 1980; Caperelli *et al.*, 1980) or sarcosine (Wittwer and Wagner, 1980) to glycine, their main function is probably to generate one-carbon units for use by the rest of the cycle.

Thus, the consequences of an aberrant methylation cycle is cellular dysfunction, *i.e.* disruption to epigenic gene transcription, neuro-degenerative disease and possibly also cardiovascular disease. The consequences of an aberrant "proliferative" function are retarded cell proliferation rates due a decreased rate of DNA synthesis and suppression of protein synthesis initiation. While severe inhibition of cell proliferation would be detrimental disruption to the methylation cycle would be more likely to cause acute disorders.

The regulation of folate homeostasis by the methylation cycle [Fig 11.3], therefore, is probably a evolutionary development to regulate the rate at which cells proliferate, thus controlling the requirement for "active" methyl-groups. In the case of vitamin B_{12} deficiency, and possibly also in the case of folate deficiency [Chapters 7 & 10], accumulation of 5-CH_3-THF is likely to occur within the cell at the expense of the other co-enzymes. This has the effect of increasing methylation cycle substrate concentrations while reducing (by retarding cell proliferation) demand for methylation cycle products.

Such a role in the regulation of folate influx and retention by the cell appears to be the most logical hypothesis to explanation the "methyl-trap". Folate in the serum is in the form of 5-CH_3-THF (Perry and Chanarin, 1970). This is the only form of the vitamin that can not be polyglutamated and retained by the cell (Chichowicz and Shane, 1987; George *et al.*, 1987; Cook *et al.*, 1987) without prior de-methylation to THF, but this conversion to THF is greatly retarded in the face of vitamin B_{12} deficiency [cf. Shane, 1983].

11.3.2. Matching of folate status to requirement

In nutritionally replete subjects, and despite attempts to increase folate status using folic acid, the body appears to have a natural level of folate homeostasis to which it returns upon cessation of supplementation. Thus within 10-12 weeks of ceasing supplementation or fortification serum folate (Ward *et al.*, 1997), red cell folate (Cuskelly *et al.*, 1999) and plasma tHcy (Ubbink *et al.*, 1993) concentrations had returned to levels approaching baseline (non-supplemented). Such a timeframe for the clearance of folate from the body is consistent with the known life-time of the red blood cell, thus suggesting that this 10-12 week delay in returning to baseline status is a function of the clearance of the elevated folate concentrations from the red blood cells.

The question therefore arises as to why, despite the beneficial role an elevated folate status may play in the prevention of cardiovascular and other diseases [Section 1.5], suppression of folate status may occur? As we have shown in [Chapters 3 and 5] folate status, at least in the form of serum folate, appears to be matched to the (catabolic) requirement for folate which in itself appears to be linked to a metabolic requirement for folate [Chapter 5]. Thus, folate status appears to adapted to requirement. But why should such linkage occur?

One possibility is that folate status is suppressed so as to maintain tHcy concentrations. While such a concept is an anathema to current thinking regarding health promotion, especially with the association between cardiovascular disease and elevated tHcy (Boushey, 1995), this mechanism of metabolic regulation did not evolve in accordance with our current sedentary lifestyle.

Instead the regulation of folate status and tHcy probably evolved, at the latest, during early *homo sapien* evolution, but it is more likely that it developed even before then, when there was greater physical activity within a shorter lifespan. Thus, the risk of cardiovascular disease would have been greatly attenuated as would the risk of neuropathy and neural degeneration. The risk of folate deficiency during pregnancy would still have been a problem, yet systems may have adapted to compensate for this and to protect the methylation transfer function of the one carbon cycle during embryonic development [Chapter 6].

But what are the benefits of having elevated tHcy? One benefit may be in regulating blood clotting. By stimulating blood clotting (Rodgers and Kane, 1986; Rodgers and Conn, 1990; Lentz and Sadler, 1991; Hayashi *et al.*, 1992; Hajjar, 1993) Hcy may have played a

functional role in trauma survival. While today trauma care reduces the risks from injury in our "primordial" ancestral past even a slight advantage attained with faster blood clotting may have been the difference between life and death. This may in part explain why tHcy increases so quickly postpartum [Chapter 6]: the requirement to protect the methylation cycle ends with the delivery of the baby, it then becomes more important to lessen risk of postpartum haemorrhage and bleeding.

Thus the regulation of folate status may be a careful balance between satisfying the requirement for folate one the one hand and maintaining tHcy concentrations to help prevent excessive bleeding and consequently help in survival, on the other.

11.3.3. MTHFR polymorphism

This involvement of Hcy in aiding blood clotting may provide an explanation, along with those offered in [Section 1.5.4], as to why both the MTHFR polymorphs (C677T and A1298C) persist. However, as three variants of the enzyme exist they are still under constant evolutionary pressure. Thus, current ambient environmental conditions do not confer sufficient advantage to any one genetic polymorph to cause the deletion of the other variants, although the frequency of both the C677T and A1298C polymorphs is suppressed relative to the "wild type" variant. [In cases where only one form of an enzyme still exists, due to the deletion of its genetic variant, we can only tell that conditions existed at one stage to make the surviving gene dominant. Conditions may have changed since, possibly even making the altered function of the surviving gene a liability.] The advantages, much like the disadvantages, of having the either of the MTHFR polymorphisms (C677T or A1298C) are probably multifactorial but our understanding of the polymorphic interactions is increasing.

11.4. Possible Reasons for Increased Folate Catabolism During Pregnancy

11.4.1. Catabolic loss due to increased metabolic activity

Dihydrofolate (DHF) is the most labile form of the vitamin. In humans DHF is only produced during thymidylate synthesis [Fig 1.2]. Thus, at times of rapid cell proliferation, as during pregnancy, thymidine production, and consequently DHF production increases.

Increase in thymidylate and dihydrofolate synthesis during pregnancy Each human cell contains approximately 12 x 10^9 DNA nucleotides (derived from Cooper, 1996) approximately one fourth (3 x 10^9) of which are thymidine. Meanwhile, there are between 75-100 trillion (10^{12}) cells in the human adult body, this would correspond to about 4-5 trillion cells in a new-born baby. Thus, during the development of the human foetus approximately 12 x 10^{21} [(3 x 10^9)(4 x 10^{12})] thymidine nucleotides would have had to be synthesised, with each molecule of thymidine synthesised resulting in the generation of one molecule of DHF.

$$\text{dUMP} + 5,10\text{-}CH_2\text{-THF} \xrightarrow{\textit{thymidylate synthase}} \text{dTMP} + \text{DHF}$$

Provisos A number of assumptions have been made in calculating both the number of thymidine molecules in a human cell and in calculating the number of cells in a new born baby. (i) The major assumption in calculating thymidine per cell is that there are equal numbers of each of the 4 nucleotides in DNA. If nucleotide distribution was random such an assumption would not be unwarranted. However, genetic and evolutionary constraints have resulted in deviation from such a random distribution, *i.e.* the 80% suppression of the cytosine-guanine (CpG) islands in DNA (Jones and Gonzalgo, 1997)[see also Section 1.5.3]. (ii) The assumption made in calculating number of cells in a new born baby is that a 7lb baby has 1/20th the number of cells of a 140lb adult just because they are 1/20th the weight of the adult, again these weights are arbitrarily chosen but are probably conservative. (iii) No consideration is made for the increase in thymidylate synthase activity required for placental growth, for cells lost due to apoptotic sculpting during foetal development, nor for physiological changes in the mother, *i.e.* breast development

Increase in folate catabolism during pregnancy During pregnancy we estimated the increase in folate catabolism to be 7.8 mg [Chapter 5] or 17.7μmol of folate (M_r = 441). This means that 10.6 x 10^{18} catabolic events must have occurred to generate this amount of apABGlu (Avogadro's number (N) = 6.022 x 10^{23} molecules/ mol).

Increase in folate catabolism as a function of increased DFH synthesis Thus, we roughly know by how much thymidine (and thus DHF) synthesis increased during pregnancy. We also have a fairly accurate calculate for how much folate catabolism increased over the same period. From these two values, and with the provisos discussed above, we can estimate the rate at which DHF breakdown would have to occur to account for the increase in folate catabolism observed:

10.6 x 10^{18} molecules of apABGlu produced per 12 x 10^{21} molecules of DHF synthesised

= 1 catabolic event per 1,132 molecules of DHF produced

This represents less than one catabolic even (breakdown of DHF to pABGlu and pteridine) per thousand thymidylate synthase cycles. As we have shown in [Chapters 7 & 9] DHF is the most labile form of the folate in bacteria, and while not as labile in mammalian cells [Chapter 10] its accumulation in cell cultures did appear to cause increased folate catabolism. Thus it is possible that the increase in folate catabolism observed during pregnancy was a result of the increased requirement for thymidylate synthesis during this period.

11.4.2. Induction of folate catabolising enzymes

There is evidence for the existence of at least two folate catabolising enzymes, a non-specific enzyme (Stover, 2000) and one that appears to utilise only 10-CHO-THF as substrate (Stover, personal communication). The activity of the former enzyme has been shown to increase during pregnancy (Stover, personal communication). The reason for such possible induction of folate catabolism during pregnancy are unclear but (i) could be as a result of increased requirement for catabolic products, or (ii) could be as a means of regulating folate homeostasis and the rate of cell replication.

Requirement for catabolic products It appears unlikely that pABGlu or apABGlu, the catabolic products of folate breakdown [Chapters 3 & 5], are used as functional groups during pregnancy as they are freely excreated in the urine. However, it is possible that the pteridine moiety of folate may have a functional role during pregnancy or in foetal development. A functionally similar enzyme to bacterial GTP cyclohydrolase [Section 1.6] has been identified in humans (Fischer *et al.*, 1997; Hasler and Niederweiser, 1986; Nagatsu *et al.*, 1986; Ichinose *et al.*, 1995), but while the main function of the enzyme in bacteria is in folate biosynthesis, its function in humans is in the biosynthesis (Hasler and Niederweiser, 1986) of other functional pteridines such as biopterin (Dhondt, 1986). It is therefore possible that folate catabolism during pregnancy may be part of a salvage pathway for the production of functional pteridines.

Regulation folate homeostasis and the rate of cell replication As discussed in the [Section 11.3.1] one possible role folate homeostasis may be to protect the methylation function of the one-carbon cycle. One means of doing so is by vitamin B_{12} regulation of folate entry and retention by the cell [Fig 11.3]. Thus the role of the folate catabolising enzymes during

pregnancy may be to lower cellular concentrations of folate. The cell must therefore replenish this loss from exogenous sources in a vitamin B_{12} dependent manner. Such replenishment would thus be dependent on adequate function of the methylation cycle. In cases where the methylation cycle was not functioning adequatly, *i.e.* due to vitamin B_{12} deficiency cell proliferation would be retarded, easing the burden on the methylation cycle [Section 11.3.1].

11.5. Possible Source of the Folic Acid in *E.coli* and Mammalian Cell Cultures

The means by which folic acid accumulation occurs in *E.coli*, and to a lesser extent in L1210 cells, is unclear. While spontaneous production of folic acid can occur due to the chemical oxidation of reduced folates, it is possible that the reduction to folic acid is enzymatic. Blakley and Cocco (1984) show that DHFR can mediate a dismutase reaction whereby the shuttling of NADP(H) from reduced to oxidised state produces a molecule of THF and folic acid per cycle [Fig 11.4].

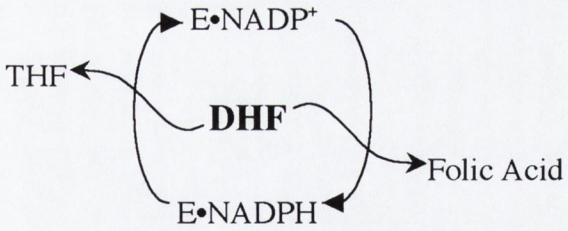


Fig 11.4. Dismutation of DHF by DHFR

The DHFR•$NADP^+$ complex can form either by oxidation of bound NADPH during normal enzyme function or by binding of $NADP^+$ to the enzyme. Due to enzyme kinetics and the high concentrations of NADPH and low concentrations of DHF the predominant reaction carried out by the enzyme is reduction of DHF, with any folic acid produced reduced back to DHF (and thence to THF). However, by inhibiting only the forward

(reduction of DHF) reaction and increasing the concentration of DHF it may be possible to reverse the direction of the reaction, resulting in the oxidation of DHF. This would cause folic acid to accumulate, as inhibition of the forward reaction would prevent reduction of folic acid.

Such selective inhibition of DHFR, preventing folate reduction but not oxidation, may occur during Tmp inhibition of the enzyme [Box 11.3]. Rather than act as a reductase the enzyme would instead function as an oxidase.

While the binding of DHF to the *E.coli* DHFR•$NADP^+$ complex is marginally more favourable than to DHFR•NADPH complex ($K_{E•NADP,DHF}$ / $K_{E•NADPH,DHF}$ = 5) this is due to binding to both the *in* and *out* conformations of DHFR•$NADP^+$ [see Box 11.3] However, if the affinity of DHF for the DHFR•$NADP^+$(*in*) complex is decreased the resulting increase in K_M would probably be overcome by the increase in DHF concentration observed. Likewise, even though the oxidation of DHF is thought to be kinetically unfavourable it it has been shown to occur (Blakley and Cocco, 1984). In fact Tmp may make folic acid accumulation more favourable since substrate concentrations are increased and reduction back to DHF is inhibited.

It is possible that a similar mechanism may account for the accumulation of folic acid observed in L1210 cells. An explanation as to why we don't see a similar accumulation of folic acid in *L.casei* is that DHF was not observed to accumulate in this bacteria, consequently concentrations of DHF may not be sufficient to drive the reverse reaction. In addition, the ratio of $K_{E, NADPH}$ to $K_{E, NADP}$ of *L.casei* DHFR is more than ten times that of *E. coli* DHFR [Table 11.9], consequently there may be insufficient free DHFR•$NADP^+$ to catalyse the oxidative reaction.

Box 11.3. Inhibition of the DHFR•NADP(H) binary complex by Tmp

In *L.casei* the binding of Tmp to DHFR is greatly enhanced by the prior binding of NADPH to the enzyme ($K_{E•NADPH,TMP} / K_{E,TMP} = 135$) (Birdsall et al, 1980a). This co-operativety is due to conformational changes in enzyme structure and stabilisation of the binding pocket due to the overlap of the nicotinamide ring of NADPH with Tmp (Blakley, 1984) .

While Tmp exhibits some co-operativity when binding to the DHFR•$NADP^+$ complex ($K_{E•NADP,TMP} / K_{E,TMP} = 2$) it is much smaller than that for the DHFR•NADPH complex (see above) and is due to co-operative binding to the inactive "out" conformation of $NADP^+$ (Polshakov *et al*, 1999). The DHFR•$NADP^+$ complex has two conformations, the inactive "out" conformation where the nicotinamide ring of $NADP^+$ is facing *out* of the binding cleft ($K_{E•NADP(out),TMP} / K_{E,TMP} = 5$) or the active "in" conformation where the nicotinamide ring is buried *in* the binding pocket ($K_{E•NADP(in),TMP} / K_{E,TMP} = 0.8$). This loss of co-operativity observed with the *in* conformation is due in part to the interaction of the positively charged nicotinamide ring with the hydrophobic binding pocket of DHFR and with the methoxy group of Tmp.

E.coli DHFR exhibits a higher degree of co-operativety of binding between Tmp and NADPH ($K_{E•NADPH,TMP} / K_{E,TMP} = 230$) (Baccanari *et al*, 1982) than the *L.casei* enzyme. Data concerning the interaction of TMP with the DHFR•$NADP^+$ complex is limited. However, if the interaction is similar to the *L.casei* enzyme, co-operativity is likely to be negligible. In the absence of co-operativety the K_D for TMP binding to DHFR•$NADP^+$ would be 230 times its K_D for DHFR•NADPH.

	$K_{E, NADPH}$ (μM)	$K_{E, NADP}$ (μM)	$K_{E, NADPH}$ / $K_{E, NADP}$
E.coli	5.7	0.043	130
L.casei	187	0.12	1,560

Table 11.9. Kinetic constants for NADPH and NADP+ binding to DHFR from *E.coli* and *L.casei*.

11.6. Mechanism of Synergy Between Trimethoprim and Sulfamethoxazol

In the light of our new findings on the role of Tmp [Chapters 7 & 9] and SMX [Chapter 8] in the inhibition of folate biosynthesis, metabolism and catabolism we propose the following modifications to the hypothesis explaining the synergy when both drugs are used in combination.

11.6.1. Synergy in bacterial strains sensitive to both inhibitors

Increased cell permeability and drug uptake Inhibition by either Tmp or SMX results in the increased permeability of the cell membrane, resulting in increased uptake of the drugs into the cell (Richards *et al.*, 1996). This is possibly due to the observed aberrant peptiglycan production (Richards and Xing, 1994a & 1994b), presumably resulting from disruption of the one-carbon cycle.

Depletion of cellular folate concentrations By causing the catabolic loss of cellular folate [Chapter 7]Tmp increases the requirement for *de novo* folate synthesis (Fig 11.4; **1**). However, SMX inhibits such *de novo* folate synthesis, as well as the folate synthesis required to maintain folate concentrations during normal cellular replication (Fig 11.4; **2**). Depletion of cellular folate may be potentiated by the inhibition of *de novo* folate synthesis by Tmp (Fig 11.4; **3**), which occurs at concentrations of inhibitor lower than those required for growth inhibition [Chapter 7].

Depletion of 10-CHO-THF concentrations As a function of their ability to cause generalised folate depletion both Tmp [Chapter 7] and SMX inhibition results in depletion of 10-CHO-THF concentrations. However, SMX also causes the specific depletion of 10-

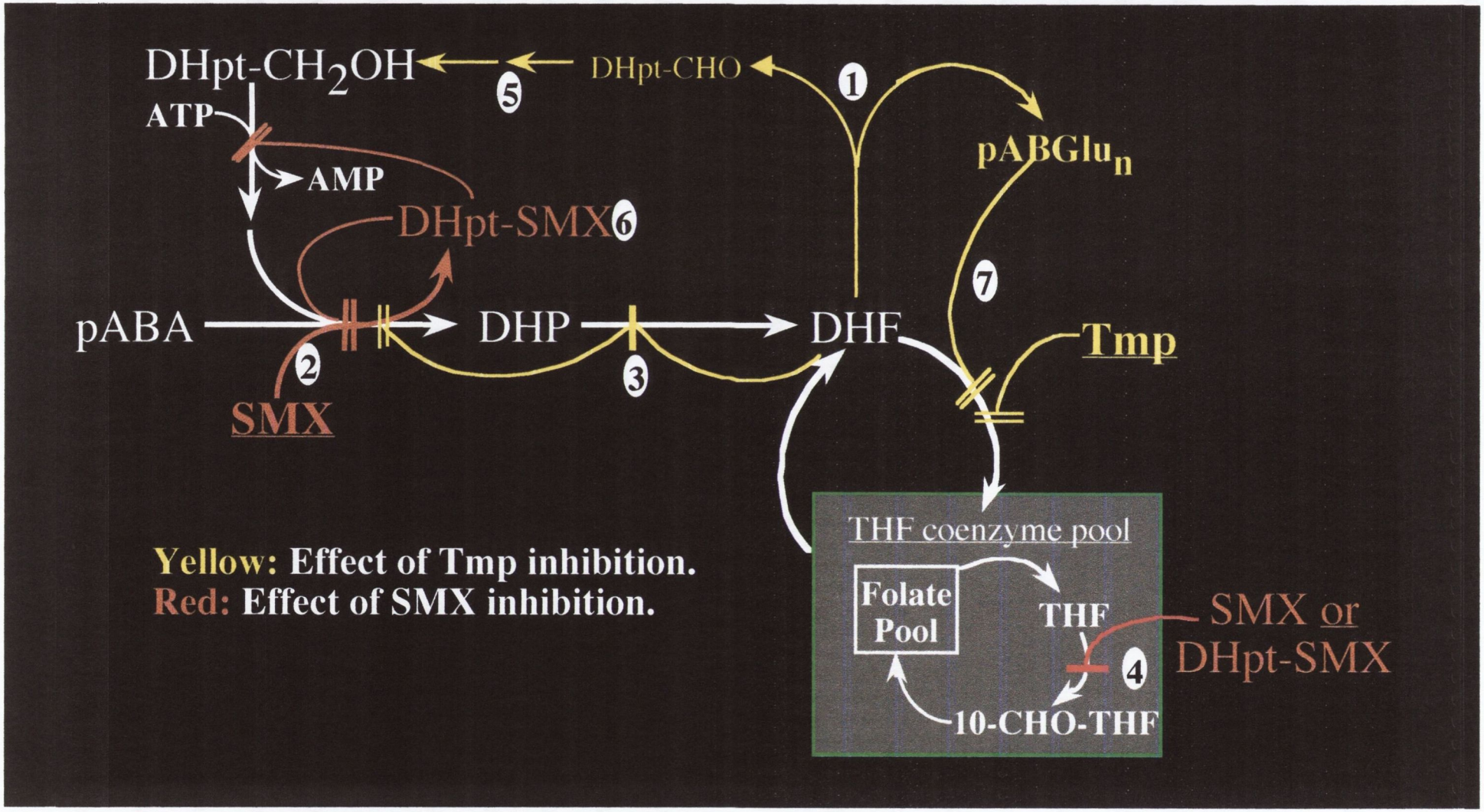


Fig 11.4. Some of the interactions of Tmp and SMX inhibition resulting in potentiation of growth inhibition.

CHO-THF (Fig 11.4; **4**), probably due to inhibition of 10-CHO-THF synthase, and at concentrations of SMX one thousand times lower than the minimum inhibitory concentration [Chapter 8]. Thus, both inhibitors effect purine synthesis by depleting substrate concentrations of 10-CHO-THF.

Decreased competition for binding to dihydrofolate reductase Tmp is a competitive inhibitor of DHFR with respect to DHF as they share a common binding site(Williams *et al.*, 1980; Stone and Morrison, 1986; Polshakov er al, 1999). Thus, while the accumulation of labelled DHF observed in *E.coli* was transient, the accumulation of *de novo* synthsised DHF would be ongoing. This would represent an increase in competition for DHFR binding that Tmp would have to overcome. However, by inhibiting the rate of DHF synthesis (Fig 11.4; **2**)SMX would diminish the extent of DHF accumulation and thus competition with Tmp for binding to DHFR.

Depletion of substrates for folate biosynthesis The catabolic products of folate cleavage resulting from Tmp inhibition are pABpolyGlu [Chapter 7] and 2-amino-4-hydroxy-dihydropteridine-6-carboxyaldehyde (DHpt-CHO) (Blair, 1957; Waller *et al.*, 1950). While pABGlu is a poor substrate for folate synthesis (Lampen and Jones, 1946; Shiota, 1959; Shiota and Disraely, 1961; Reynolds and Brown, 1964;Swedberg *et al.*, 1979; Hampele *et al.*, 1997; Roland *et al.*, 1979; Talarico *et al.*, 1991; Ortiz and Hotchkiss, 1966; Ortiz 1970), especially in this polyglutamated form, DHpt-CHO has been shown to be a pteridine precursor for folate synthesis (Shiota, 1959; Brown *et al.*, 1961). By salvaging the DHpt-CHO for use in folate synthesis not only is there provision of a ready source of the preformed pteridine (Fig 11.4; **5**) there is also a saving of the metabolic cost involved [Fig 11.1]. However, by acting as an alternative substrate for dihydropteroate synthase SMX not only inhibits the enzyme utilisation of the salvaged pteridine but it also

diminishes pteridine concentrations by forming the SMX-pteridine analogue (Fig 11.4; **6**). Thus SMX may inhibit the re-utilisation of the DHpt-CHO resulting from Tmp inhibition while increasing secondary inhibition (see below).

Secondary inhibition by the SMX-pteridine analogue The SMX-pteridine analogue, formed by the condensation of SMX with DHpt-CH_2OH (Swedberg *et al.*, 1979; Roland *et al.*, 1979; Brown, 1962), inhibits both folate biosynthesis and metabolism (Roland *et al.*, 1979). Thus, by causing the accumulation of DHpt-CHO Tmp may increase the rate at which this analogue is formed and thus increase the extent of secondary inhibition (Fig 11.4; 6).

11.6.2. Synergy in bacterial strains resistant to either drug

Resistance to either drug can result from a number of different mechanisms, *i.e.* in the case of sulfonamide resistance can result from modification of cellular transport (Brown, 1962; Hwang *et al.*, 1989; Pato and Brown, 1963), increased competition due to overproduction of pABA (Green *et al.*, 1996; Bruce *et al.*, 1984) or enzyme mutations resulting in an increase in the ratio of Ki (sulfonamide) to K_M (pABA) (Green *et al.*, 1996; Dallas, *et al.*, 1992; Swedberg et l, 1979 & 1998). However, in light of our further understanding of how Tmp and SMX interact we a offer further explanation of how synergy may occur where one of the drugs has no apparent effect on its own.

Modification of cellular transport In this case drug resistance results from a decrease or loss in ability of the bacteria to transport the inhibitor. In the case of sulfonamides this may results in a concommittant decrease in the ability of the bacteria to transport exogenous pABA as both inhibitor and substrate are transported by the same carrier. However, by one

inhibitor causing the increased permeability of the cell membrane (Richards *et al.*, 1996) the requirement of the other inhibitor to be transported decreases as the inhibitors more readily defuses across the cell membrane and into the bacteria.

Increased substrate competition As discussed above SMX inhibits the rate at which DHF is synthesised and thus diminishes competition between DHF and Tmp.

While Tmp does not preventing pABA synthesis, and can not therefore prevent pABA overproduction, it can, by creating a futile cycle of *de novo* folate synthesis and catabolism (Fig 11.4; **1**), prevent pABA accumulation. Such overproduction of pABA could be detrimental to the bacteria by causing the rapid accumulation of catabolic pABGlu (Fig 11.4; **7**), which is itself inhibitory to DHFR (Bowden *et al.*, 1989; Birdsall *et al.*, 1980; Roberts, 1969; Roberts *et al.*, 1974), and by depleting cellular concentrations of glutamate. A further consequence may be the accumulation of less metabolicly active short polyglutamate chain folates [see Section 1.4]. This increased substrate competition would be further overcome by the increase in cellular concentrations of both drugs due to the increase in cell permeability (Richards *et al.*, 1996).

Enzyme mutation resulting in an increase in the K_i : K_M ratio While an increases in K_i value of SMX for dihydropteroate synthase would decrease its efficacy against that enzyme, it would not effect the way in which it inhibits folate metabolism. Thus, the synergy of effect resulting from depletion of 10-CHO-THF concentrations would not be effected by such mutations (Fig 11.4; 4). The increased in the K_i : K_M ratio would be further overcome by the increase in cellular concentration of inhibitor due to the increase in cell permeability (Richards *et al.*, 1996).

APPENDIX A

VALIDATION OF THE SOLID PHASE (SEPPAK) ASSAY FOR FOLATE CATABOLITES IN URINE

A.1 Reproducibility

Four of the second trimester urine samples were randomly chosen. A proportion of each sample was pooled and used to determine the reproducibility of the method. The sample was assayed 6 times for apABGlu to determine the intra assay variation [Table A.1]. The pooled sample was also spiked with 80μg/2L apABGlu and assayed in triplicate to determine recovery.

	[apAGlu] μg/2L mean (±SD)	Coefficient of Variation (CV)
Pooled Sample (n =6)	99 (4.2)	4.2%
Spiked (80μg/2l) (n = 3)	167 (6.4)	3.8%
recovery (spiked - unspiked)	68μg/2L	--

Table A.1. Intra assay variation (CV) and reproducibility of the solid phase (SepPak) method for assaying urinary apABGlu. The percentage recovery of the spiked sample was 85% (Recovery ÷ Spike = 68μg/2L ÷ 80μg/2L).

A.2. Comparison of Methods. I

Two baseline control samples were randomly chosen (the only selection process being that both samples were of approximately the same colour so as to avoid easy identification). Two pools of each sample were made, one of which was subsequently spiked with 500ng/20ml apABGlu. Each pool (± spike) was then divided into six 35ml lots, the samples were then divided into 3 sets of duplicate tubes, *i.e.* each set had duplicate of each sample ± spike. Each sample in each set was then blinded, *i.e.* the samples in each set was randomly labelled and the key retained, the samples were then randomly numbered by a second person who prepared a key before removing the first identifying number. Neither person knew the key used by the other person until after the sample codes were at the end of the experiment. No two corresponding samples in separate sets were identically numbered (unless such numbering occurred by chance). [Fig A.1] diagrammatically shows how the samples were prepared and randomised.

One set of samples was assayed using the Dowex column method by Dr. Joseph McPartlin, the originator of this method (McPartlin *et al.*, 1992). Both the remaining sets were assayed by me, one using Dowex columns, the other by solid phase extraction. The concentration of apABGlu in each sample was calculate before the sample code was finally broken. [Table A.1] gives a summary of the results as well as the percentage recovery of the apABGlu spike, the results are also graphed in [Fig A.2].

Note: These urine samples were not diluted to 2L before assaying. Thus, apABGlu concentrations are not comparable to the values reported for daily apABGlu excretion.

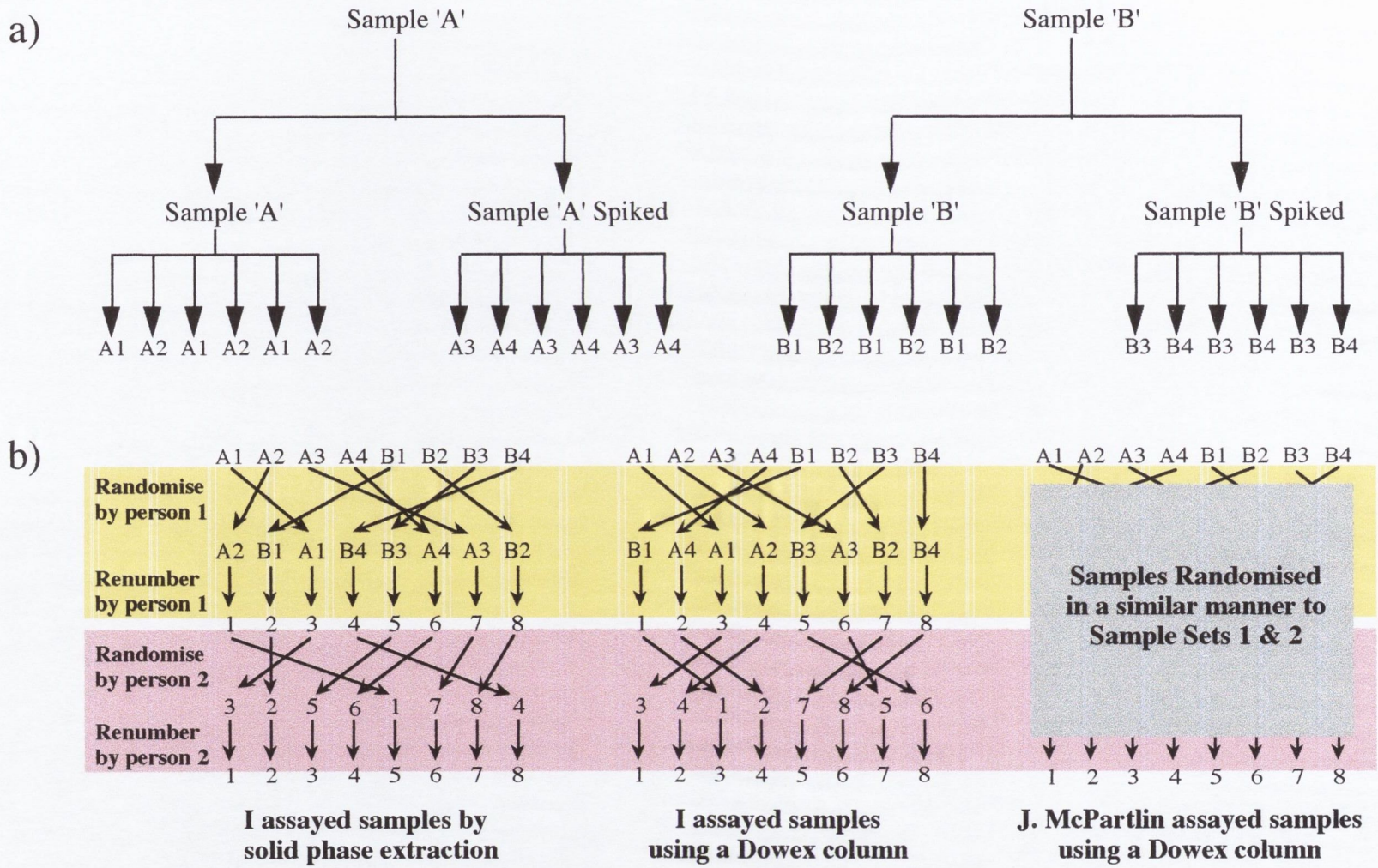


Fig A.1. The a) preparation and b) randomised blinding of samples for use in comparison of assay methods.

	Dowex[1]	SepPak[1]	Dowex[2]
Sample 'A'	2334 (14)	2248 (74)	2395 (53)
Sample 'A' (spiked)	2843 (171)	2813 (140)	2848 (103)
%Recovery	102%	113%	90%
Sample 'B'	1236 (76)	1191 (58)	1137 (64)
Sample 'B' (spiked)	1594 (41)	1606 (51)	1574 (62)
%Recovery	72%	83%	87%

Table A.1. Concentration (ng/20ml) of apABGlu in urine samples [mean (±SD); n = 2]. Samples were also spiked with 500ng/20ml apABGlu and the percentage recovery calculated. Samples were assayed [1]by me using a Dowex ion exchange column or a SepPak C_{18} solid phase extraction cartridge, or [2]by Dr. Joseph McPartlin using a Dowex column.

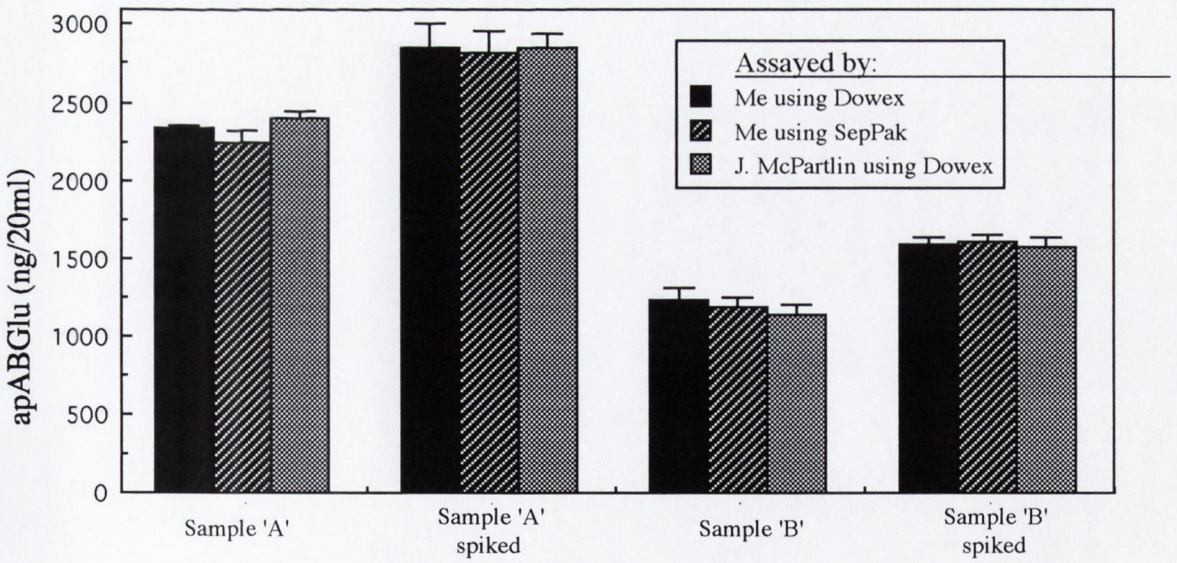


Fig A.2. Graph of data from [Table A.1]

A.2. Comparison of Methods. II

Eight of the pregnant subjects were randomly selected. The urine from all three trimesters, as well as from 6 of the baseline control subjects, were thawed before duplicate 30ml volumes were removed. The samples were then blinded as in A.2 except that, due to the large number of samples involved, the corresponding samples in each set were given the same code. One set of samples was assayed by me using the SepPak method while the other was independently assayed by Dr. Joseph McPartlin using the Dowex column method. Upon completion of both assays the sample code was broken and the results compared [Fig A.3]. The results from both sets of data exhibited the same trend as observed in [Chapter 5], apABGlu concentrations were predominantly higher in pregnancy than in the baseline controls and increased with the progression of pregnancy.

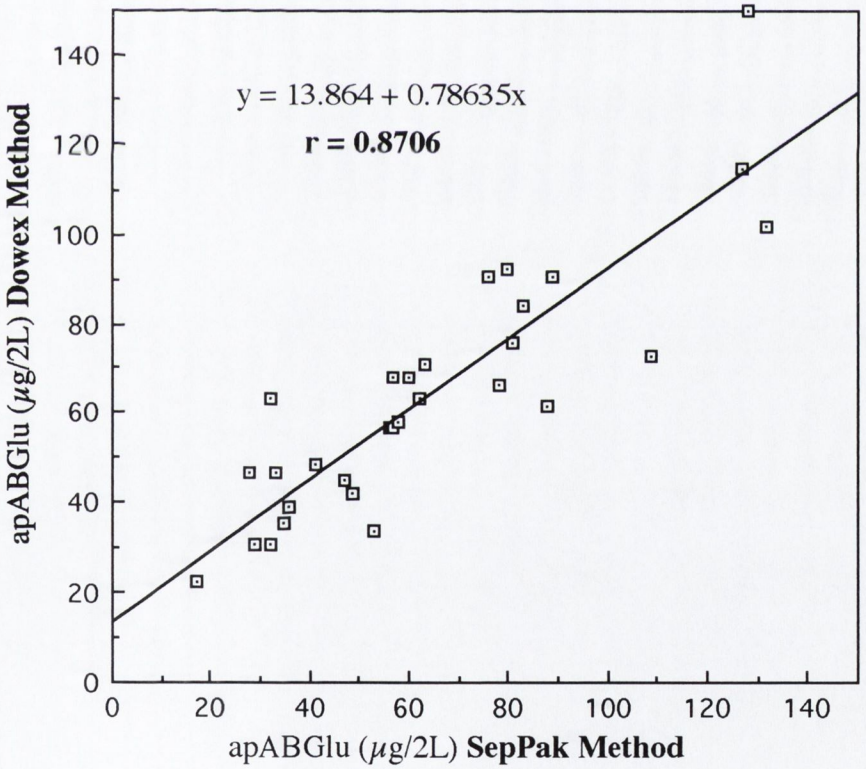


Fig A.3. Comparison of results for apABGlu concentration determined using the SepPak method or the Dowex column method.

APPENDIX B

PRINCIPLES OF INTEGRATION

A graph is prepared using the experimentally derived data, a curve giving best fit is plotted using the data points and the equation of the curve calculated [Fig B.1]. We now wish to find the area under the curve [Fig B.2]. One way of doing this is by filling the area with rectangles of uniform width, dX, and of height Y_n [Fig B.3]. The height of each rectangle, Y_n, can then be calculated by substituting [dX • (n-1)] for X in the equation of the curve, *i.e.* in the case of a second order polynomial equation ($y = a.X^2 + b.X + c$):

$$Y_n = a \bullet [dX \bullet (n-1)]^2 + b \bullet [dX \bullet (n-1)] + c \qquad \text{[Eqn. B.1]}$$

The area under the curve can then be approximated by adding the areas of each rectangle:

$$\text{Area Under Curve} \approx \sum dX \bullet Y_1 + dX \bullet Y_2 + \ldots + dX \bullet Y_N \qquad \text{[Eqn. B.2]}$$

However, as the area between the curve and the rectangles (coloured red in Fig B.3) is not determined the resulting area is underestimated. A more accurate determination can be made by decreasing the width, dX, of each rectangle, as the area above the rectangles (red) becomes smaller [Fig B.4]. Making the width of the rectangles sufficiently small, *i.e.* δX, the area above the rectangle becomes negligible, as does the error in calculating the area under the curve. A mathematical means of doing this is by integration. Each factor in the equation is transformed using defined mathematical principles. A few simple integration transformations are given in [Table B.1].

Fig B.1

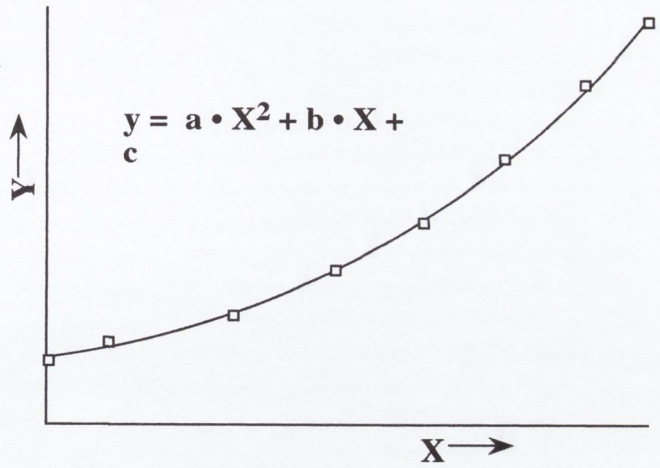


Fig B.2

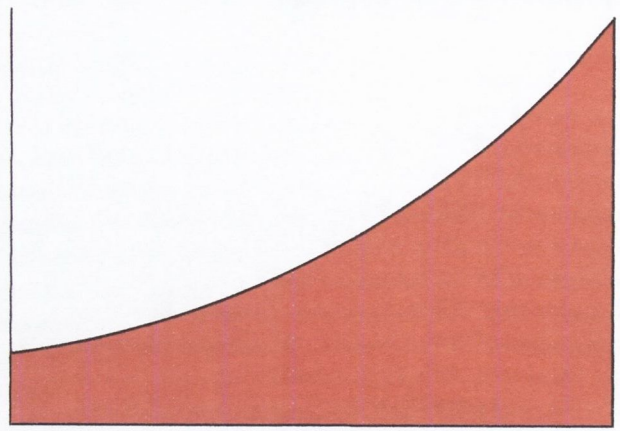


Fig B.3

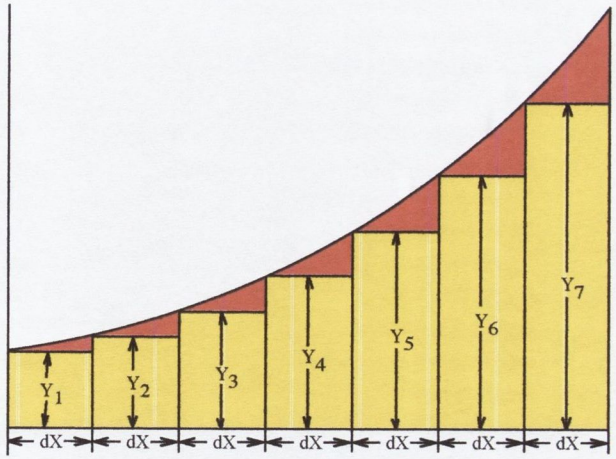
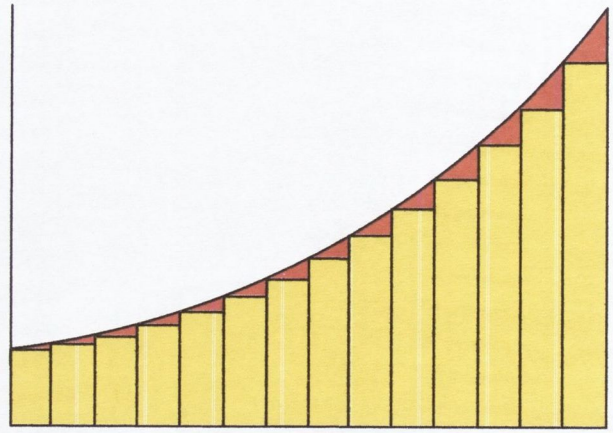


Fig B.4



$y =$	$\int y.dX$
X^Z	$X^{Z+1} \div Z+1$
c (constant) $= c \bullet X^0$	$c \bullet X^1$

Table B.1: Integration of common mathematical functions.

Thus integrating the polymonial equation,

$$y = a.X^2 + b.X + c$$

we derive the equation,

$$\int y.dX = a \bullet X^3 \div 3 + b \bullet X^2 \div 2 + c \bullet X \qquad \text{[Eqn B.3]}$$

The equation can then be solved by substituting known value of X into the equation and finding the product.

APPENDIX C

CALCULATING THE EXTENT OF FOLATE CATABOLISM IN *L.CASEI*

Introduction

When examining the effect of Tmp inhibition of DHFR on folate distribution in *E.coli* we were able to calculate the ratio of pABGlu to intact folate(s) on a one to one basis as each had the same specific activity, *e.g.* the same radioactivity (dpm) per mole. This resulted from the fact that, as we used [^{3}H]-pABA as a precursor for folate biosynthesis, the radiolabel was present exclusively on the pABGlu moiety of folate, with none on the pteridine.

However, the strain of *L.casei* used were unable to synthesis folate and thus had to be labelled using pre-formed folates. This folate, in the form of folic acid, was labelled on both the pABGlu and pteridine moieties. While this had an advantage, in the fact that the fate of both the pABGlu and pteridine cleavage products could be determined, it meant that the specific activity of each was less than that of the intact folate. When calculating folate catabolism this fact had to be taken into account.

In preparing such calculations the contribution of both pABGlu and pteridine to sample radioactivity would normally be taken into account. However, as there was relatively little

pteridine present in the samples, equations excluding a pteridine factor could be derived without introducing appreciable error.

C.1. Derivation of [Eqn 9.1]

The radioactivity in the pellet is due to [^{3}H]-pABGlu (pABGlu) and [^{3}H]-folate (Folate), thus:

$$\text{Total radiolabel in pellet} = \text{Total} = \text{pABGlu} + \text{Folate} \qquad \text{Eqn. C.1}$$

Thus the percentage pABGlu in the pellet (%pABGlu) is equal to:

$$\%\text{pABGlu} = \frac{\text{pABGlu}}{\text{Total}} \qquad \text{Eqn. C.2}$$

However, pABGlu had only 47% the specific activity of intact folate. Therefore, adjusting for relative molar concentrations:

$$\text{pABGlu (Adj.)} = \text{pABGlu} \div 47\% \qquad \text{Eqn. C.3}$$

But, by adjusting for the specific activity of pABGlu, we change the total "apparent" activity of the pellet (Total (adj.)):

$$\text{Total (adj.)} = \text{pABGlu (adj.)} + \text{Folate} \qquad \text{Eqn. C.4}$$

Re-writing [Eqn. C.1] we get:

$$\text{Folate} = \text{Total} - \text{pABGlu} \qquad \text{Eqn C.5}$$

Substituting this into [Eqn. C.4] we get:

$$\text{Total (adj.)} = \text{pABGlu (adj.)} + \text{Total} - \text{pABGlu} \qquad \text{Eqn. C.6}$$

Therefore the percentage pABGlu (adj.) in the pellet (%pABGlu (adj.)) was equal to:

$$\%\text{pABGlu (adj.)} = \frac{\text{pABGlu (adj.)}}{\text{Total (adj.)}} \qquad \text{Eqn. C.7}$$

Or, substituting [Eqn. C.6] into [Eqn. C.7] we get:

$$\%\text{pABGlu (adj.)} = \frac{\text{pABGlu (adj.)}}{\text{Total} - \text{pABGlu} + \text{pABGlu (adj.)}} \qquad \text{Eqn. C.8}$$

Therefore, substituting [Eqn. C.3] into [Eqn. C.8] we get the percentage catabolism (%pABGlu (adj.)) in the bacteria:

$$\%\text{pABGlu (adj.)} = \frac{\text{pABGlu} \div 47\%}{\text{Total} - \text{pABGlu} + \text{pABGlu} \div 47\%} \qquad \text{Eqn. C.9}$$

C.2. Derivation of [Eqn 9.2]

[Eqn. C.8] gives the total percentage pABGlu (adj.) in the bacteria. However, some of this pABGlu was present before incubation began. Therefore, knowing the value for pABGlu (adj.) at the start of the incubation period (pABGlu (T_0)) and after each incubation period (pABGlu (T_{INC})), we are able to calculate the change in catabolism which occurred during the treatment phase (ΔpABGlu):

$$\Delta pABGlu = pABGlu\ (T_{INC}) - pABGlu\ (T_0) \qquad \text{Eqn. C.10}$$

However, we must also subtract the pABGlu present at the start of the incubation (pABGlu (T_0)) from the total :

$$Total_{INC} = Total\ (adj.) - pABGlu\ (T_0) \qquad \text{Eqn. C.11}$$

Therefore the percentage change in catabolism (ΔCatabolism) during the treatment phase was:

$$\Delta Catabolism = \frac{\Delta pABGlu}{Total_{INC}} \qquad \text{Eqn. C.12}$$

Substituting [Eqn. C.10] and [Eqn. C.11] into [Eqn. C.12] the percentage increase in catabolism which occurred during the treatment growth was:

$$\Delta Catabolism = \frac{pABGlu\ (T_{INC}) - pABGlu\ (T_0)}{Total\ (adj.) - pABGlu\ (T_0)} \qquad \text{Eqn. C.12}$$

REFERENCES

M. Achón, E. Alonso-Aperte, L. Reyes, N. Úbeda and G. Varela-Moreiras; High Dietary Folic Acid Supplementation in Rats: Effects on Gestation and the Methionine Cycle (2000) Br. J. Nutr. **83**; 177-83

M. Achón, L. Reyes, E. Alonso-Aperte, N. Úbeda and G. Varela-Moreiras; High Dietary Folate Supplementation Affects Gestational Development and Dietary Protein Utilization in Rats (1999) J. Nutr. **129**; 1204-8

J.M. Adams and M.R. Capecchi; N-formylmethiomyl-sRNA as the initiator of protein synthesis (1966) Proc. Natl. Acad. Sci. USA **55**; 147-55

T. Akira, M. Komatsu, R. Nango, A. Tomooka, K. Konaka, M. Yamauchi, Y. Kitamura, S. Nomura and I. Tsukamoto; Molecular Cloning and Expression of a Rat cDNA Encoding 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase/IMP cyclohydrolase (1997) Gene **197**; 289-93

K.S. Anderson and KA Johnson; "Kinetic competence" of the 5-enolpyruvoylshikimate-3-phosphate synthase tetrahedral intermediate (1990) J. Biol. Chem., **265**: 10; 5567-72

B. Anderson, E.H. Belcher, I. Chanarin and D.L. Mollin (1960) Br. J. Haematol. **6**; 439

S. Anderson, M.H. de Bruijn, A.R. Coulson, I.C. Eperon, F. Sanger and I.G. Young; Complete Sequence of Bovine DNA. Conserved Features of the Mammalian Mitocondrial Genome (1982) J. Mol. Biol. **156**; 683-717

R.B. Angier, J.H. Booth, B.L. Hutchings, J.H. Mowat, J. Semb, E.L.R. Stodstad, Y. SubbaRow and C.W. Waller; The Structure and Synthesis of the Liver *L.casei* Factor (1946) **103**; 667-9

J.R. Appleman, N. Prendergast, T.J. Delcamp, J.H. Freisheim and R.L. Blakley; Kinetics of the Formation and Isomerisation of Methotrexate Complexes of Recombinant Human Dihydrofolate Reductase (1988) J. Biol. Chem. **263**; 10304-13

D.R. Appling; Compartmentation of Folate-Mediated One-Carbon Metabolism in Eukaryotes (1991) FASEB J. **5**; 2645-51

M. Arnadotti, V. Gudnason and B. Hultberg; Treatment With Different Doses of Folic Acid in Haemodialysis Patients: Effects on Folate Distribution and Aminothiol Concentrations (2000) Nephrol. Dial. Transplant **15**; 524-8

D.E. Atkinson; Cellular Energy Metabolism and Its Regulation (1977) Academic Press , New York

D. Baccanari, A. Phillips, S. Smith, D. Sinski and J. Burchall; Purification and Properties of *Escherichia coli* Dihydrofolate Reductase (1975) Biochemistry **14**: 24; 5267-73

D. Baccanari, S. Daluge and R.W. King; Inhibition of Dihydrofolate Reductase: Effect of Reduced Nicotinamide Adenine Dinucleotide Phosphate on the Selectivity and Affinity of Diaminobenzylpyrimidines (1982) Biochemistry **21**: 5068-75

D.P. Baccanari and S.S. Joyner; Dihydrofolate Reductase Hysteresis and Its Effects on Inhibitor Binding Analysis (1981) Biochemistry **20**; 1710-6

D.P. Baccanari, R.L. Tansik and G.H. Hitchings; Substrate-Inhibitor Cooperative Interactions with Microbial Dihydrofolate Reductase (1987) Adv. Enzyme Regul. **26**; 3-15

P.J. Bagley and J. Selhub; A Common Mutation in the Methylenetetrahydrofolate Reductse Gene is Associated with an Accumulation of Formylated Tetrahydrofolates in Red Blood Cells (1998) Proc. Natl. Acad. Sci. USA **95**; 13217-20

S. Balasubramanian, G.M. Davies, R.J. Coggins, C. Abell; (1991) J. Am. Chem. Soc. **113**; 8945-6

L. Baldomá and J. Aguilar; Involvement of Lactaldehyde Dehydrogenase in Several Metabolic Pathways of *Eschericia coli* K12 (1987) J. Biol. Chem. **262**; 13991-6

S. Bandari and J. Gregory III; Inhibition by selected food compounds of human and porcine intestinal pteroylpolyglutamate hydrolase activity; (1990) Am. J. Clin. Nutr. **51**, 87-94

M.S. Bartolomei and S.M. Tilghman; Genomic Imprinting in Mammals (1997) Annu. Rev. Genet. **31**; 571-610

C.M. Baugh, E. Braverman and M.G. Nair; Poly-γ-Glutamyl Chain Lengths in Some Nautral Folates and Contributions of Folic Acid Synthesized by Intestinal Microflora to Rat Nutrition (1975) In: *Chemistry and Biology of Pterines* (W. Pfleiderer, ed.) Walter de Gruyter, Berlin pp 465-74

N. Baumslag, T. Edelstein and J. Metz; Reduction of Incidence of Prematurity by Folic Acid Supplementation in Pregnancy (1970) Br. Med. J. **1**; 16-17

S.B. Baylin, J.G. Herman, M.M. Wales, P. Vertino, J. Wu, S. Kuerbitz and J-P. Issa; Hypermethhylation of CpG Islands and Inactivation of Tumor Supprssor Genes (1995) Proc. Am. Assoc. Cancer Res. **36**; 691-2

A.W. Berg and G.M. Brown; The Biosynthesis of Folic Acid. VIII. Purification and Properties of the Enzyme that Catalyzes the Production of Formate from Carbon Atom 8 of Guanosine Triphosphate (1968) J. Biol. Chem. **243**; 2349-58

M.D. Berlyn and N.H. Giles; Organization of Enzymes in the Polyaromatic Synthetic Pathway: Seperability in Bacteria (1969) J. Bacteriol. **99**; 222-30

J. Bernardino, C. Roux, A. Almeida, N. Vogt, A. Gibaud, M. Gerbault-Seureau, H. Magdelenet, C.A. Bourgeois, B. Malfoy and B. Dutrillaux; DNA hypomethylation in Breast Cancer: An Independent Parameter of Tumor Progression (1997) Cancer Genet. Cytogenet. **97**; 83-9

E. Bertani, A. Häggmark and P. Reichard; Enzymatic Synthesis of Deoxyribonucleotides: II. Formation and Interconversion of Deoxyuridine Phosphate (1963) J. Biol. Chem. **238**; 3407-13

S.D. Bhandari and J.F. Gregory; Inhibition by Selected Food Components of Human and Porcine Intestinal Pteroylpolyglutamate Hydrolase Activity (1990) Am J. Clin. Nutr. **51**; 87-94

S. Binkley, O. Bird, E. Bloom, R. Brown, D. Calkins, C. Campbell, A. Emmet, J. Pfiffner; On the vitamin Bc conjugate in yeast; (1944) Science **100**, 37-8.

O. Bird, M Robbins, J. Vandenbelt and J. Pfiffner; Observations on Vitamin Bc conjugase from hog kidney; (1946) J. Biol. Chem. **163**, 649.

B. Birdsall, A.S.V. Burgen and G.C.K. Roberts; Binding of Coenzyme Analogues to *Lactobcillus casei* Dihydrofolate Reductase: Binary and Ternary Complexex (1980a) Biochemistry **19**; 3725-31

B. Birdsall, A.S.V. Burgen and G.C.K. Roberts; Effect of Coenzyme Analogues on the Binding of p-Aminobenzoyl-L-glutamate and 2,4 Diaminopyrimidine to *Lactobcillus casei* Dihydrofolate Reductase (1980) Biochemistry **19**; 3732-7

J.A. Blair; Some observations on the Oxidative Degradation of Pteroyl-L-glutamic acid (1957) Biochem. **65**; 209-11

R.L. Blakely; *The Biochemistry of Folic Acid and Related Pteridines* (1969) North-Holland, London.

R.L. Blakley; Dihydrofolate Reductase (1984) In*: Folates and Pteridines, Vol 1: Chemistry and Biochemistry of Folates* (R.L. Blakley and S.J. Benkovic, eds.) John Wiley and Sons, New York. Pp191-253

R.L. Blakely and S.J. Benkovic; In *Folates and Pteridines Vol 1 Chemistry and Biochemistry of Folates* (1984) John Wiley and Sons, New York pp xi-xiv

R.L. Blakley and L. Cocco; Dismutation of Dihydrofolate by Dihydrofolate Reductase (1984) Biochemistry **23**; 2377-83

I. Blot, E. Papiernik, J.P. Kaltwasser, E. Werner and G. Tchernia; Influence of Routine Administration of Folic Acid and Iron During Pregnancy (1981) Gynecol. Obstet. Invest. **12**; 294-304

B.C. Blount, M.M. Mack, C.M. Wehr,J.T. MacGregor, R.A. Hiatt, G. Wang, S.N. Wickramasinghe, R.B. Everson and B.N. Ames; Folate Deficiency Causes Uracil Misincorporation into Human DNA and Chromosome Breakage: Implications for Cancer and Neuronal Damage (1997) Proc. Natl. Acad. Sci. USA **94**; 3290-5

A.L. Bogner and B. Shane; Purification and Properties of *Lactobacillus casei* Folylpoly-gamma-Glutamate Synthetase. (1983) J. Biol. Chem. **258**; 12574-81

A.L. Bogner, C. Osborne, B. Shane, C.S. Singer and R. Ferone; Foly-Poly-γ-Glutamate Synthetase-Dihydrofolate Synthetase: Cloning and High Expression of the *Escherichia coli* folC Gene and Purification and Properties of the Gene Product (1985) J. Biol. Chem. **260**; 5625-30

S. Bornemann, M.K. Ramjee, S. Balasubramanian, C. Abell, R.J. Coggins, D.J. Lowe and R.N.F. Thorneley; *Eschericia coli* Chorismate Synthase Catalyzes the Conversion of (6S)-6-Fluoro-5-enolpyruvylshikimate-3-phosphate to 6-Fluorochorismate. Implications for the Enzyme Mechanism and the Antimicrobial Action of (6S)-6-Fluoroshikimate (1995) J. Biol. Chem **270**; 22811-5

A. Boronat, E. Caballero and J. Aguilar; Experimental Evolution of a Methabolic Pathway for Ethanol Glycol Utilization by *Eschericia coli* (1983) J. Bacteriol. **153**; 134-9

A.G. Bosom, D. Shemin, P. Bagley, Z.A. Massy, A. Zanabli, K. Christopher, P. Spiegel, P.F. Jacques, L. Dworkin and J. Selhub; Controlled Comparison of L-5-Methyltetrahydrofolate Versus Folic Acid for the Treatment of Hyperhomocysteinemia in Hemodialysis Patients (2000) Circulation **101**; 2829

C.J. Boushey, S.A.A. Beresford, G.S. Omenn and A.G. Motulsky; A Quantitative Assessment of Plasma Homocysteine as a Risk Factor for Vascular Disease: Probable Benefits of Increasing Folic Acid Intakes (1995) JAMA **274**; 1049-57

K. Bowden, A.D. Hall, B. Birdsall, J. Feeney and G.C.K. Roberts; Interaction between Inhibitors of Dihydrofolate Reductase (1989) Biochem J. **258**; 335-42

A. Bracher, M. Fischer, W. Eisenreich, H. Ritz, N. Schramek, P. Boyle, P. Gentili, R. Huber, H. Nar, G. Auerbach and A. Bacher; Histidine 179 Mutations of GTP Cyclohydrolase I Catalyze the Formation of 2-Amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone Triphosphate (1999) J. Biol. Chem. **274**; 16727-35

A. Bracher, W. Eisenreich, N. Schramek, H. Ritz, E. Götze, A. Heffermann, M. Gütlich and A. Bacher; Biosynthesis of Pteridines. NMR Studies on the Reaction Mechanism of GTP Cyclohydrolase I, Pyruvoyltetrahydropterin Synthase, and Sepiapterin Reductase (1998) J. Biol. Chem. **273**; 28132-41

R.F. Branda, E. Nigels, A.R. Lafayette and M. Hacker; Nutritional Folate Status Influences the Efficacy and Toxicity of Chemotherapy in Rats (1998) Blood **92**; 2471-6

G. Briggs, T. Lacey, C. Elvejem, B Hart; Studies on methods of increasing folic acid activity with liver fractions in yeast; (1994) J. Biol. Chem. **155**, 687-8

G.M. Brown; Biogenesis and Metabolism of Folic Acid (1970) In: Metabolic Pathways (3rd ed., Volume IV) (D.M. Greenberg, ed.) Academic Press, New York. pp383-410

G.M. Brown; The Biosynthesis of Folic Acid - II. Inhibition by Sulfonamides (1962) J. Biol. Chem. **237**: 2; 536-40

J.P. Brown, F. Dobbs, G.E. Davidson and J.M. Scott (1974) Microbial synthesis of folate polyglutamates from labelled precursors. *J. Gen. Microbiol* 84, 163-171.

J.P. Brown, J.M. Scott, F.G. Foster and D.G. Weir; Ingestion and Absorption of Naturally Occurring Pteroylmonoglutamates (Folates) in Man (1973) Gastroenterology **64**; 223-32

G.M. Brown, R.A. Weisman and D.A. Molner; The Biosynthesis of Folic Acid. I. Substrate and Co-factor Requirement for Enzymatic Synthesis by Cell Free Extracts of *Escherichia coli* (1961) J. Biol. Chem. **236**; 2534-43

I. Bruce, J. Hardy and K.A. Stacey; Potentiation by Purines of the Growth-Inhibitory Effects of Sulfonamides on *Eschericia coli* and the Location of the Gene Which Mediates this Effect (1984) J. Gen. Microbiol. **130**; 2489-95

W. Brumfitt and J. Hamilton Miller; Trimethoprim. (1980)Br. J. Hosp. Med. 281-288.

J.J. Burchall; The Development of the Diaminopyrimidines (1979) J. Antimicrob. Chemother. (Suppl. B) **5**; 3-14

A.W. Burg and G.M. Brown; The Biosynthesis of Folic Acid. VI. Enzymatic Conversion of Carbon Atom 8 of Guanosine Triphosphate to Formic Acid (1966) Biochim. Biophys. Acta **117**; 275-8

G. Burke, J. Robinson, H. Refsum, J. Drumm and I. Graham; Interuterine Growth Retardation, Perinatal Death and Maternal Homocysteine Levels (1992) N. Engl. J. Med. **326**; 69-70

S.R.M. Bushby and G.H. Hitchings; Trimethoprim, a sulphonamide potentiator (1968) Brit. J. Pharmacol **33**, 72-90.

E.E. Cameron, K.E. Bachman, S. Myöhänen, J.G. Herman and S.B. Baylin; Synergy of Demethylation and Histone Deacetylase Inhibition in the Re-expression of Genes Silenced in Cancer (1999) Nat. Genet. **21**; 103-7

G.L. Cantoni, H.H. Richards and P.K. Chiang; Inhibitors of S-Adenosylhomocysteine Hydrolase and Their Role in the Regulation of Biological Methylation (1978) In: Transmethylation: Proceedings of the Conference on Transmethylation (eds: E. Usdin, R.T. Borchardt and C.R. Creveling) Elsevier/ North-Holland, New York; pp155-64

G.L. Cantoni; Biological Methylation: Selected Aspects (1975) Annu. Rev. Biochem. **44**; 435-51

C.A. Caperelli, P.A. Benkovic, G. Chettur and S.J. Benkovic; Purification of a Complex Catalyzing Folate Cofactor Synthesis and Transformylation in De Novo Purine Synthesis (1980) J. Biol. Chem. **255**; 1885-90

I.A. Cassady M.M. Budges, M.J. Healy and P.F. Nixon; An Inverse Relationship of Rat Liver Folate Polyglutamate Chain Length to Nutritional Folate Sufficiency (1980) Biochim Biophys Acta **633**; 258-68

Center for Disease Control and Prevention; Use of Folic Acid for Prevention of Spina Bifida and Other Neural Tube Defects1983-1991 (1991) Morb. Mortal. Wkly Rep. **40**; 513-6

V. Chagoya De Sánchez, R. Hernández-Muñoz, J. Suárez, S. Vidrio, L. Yáñez et al; Temporal Variations of Adenosine Metabolism in Human Blood (1995) Chronobiol. Int. **13**; 163-177

V. Chagoya De Sánchez, R. Hernández-Muñoz, L. Sánchez, S. Vidrio, L. Yáñez and J. Suárez; Twenty-Four-Hour Changes of S-Adenosylmethionine, S-Adenosylhomocysteine, Adenosine and Their Metabolizing Enzymes in Rat Liver; Possible Physiological Significance in Phospholipid Methylation (1991) Int. J. Biochem. **23**; 1439-43

V. Chagoya De Sánchez; Circadian Variations of Adenosine and of Its Metabolism. Could Adenosine be a Molecular Oscillator for Circadian Rhythems (1994) Can. J. Physiol. Pharmacol. **73**; 339-55

J.C. Chambers, A. McGregor, J. Jean-Marie and J.S. Kooner; Acute Hyperhomocyseinaemia and Endothelial Dysfunction.(1997) Lancet **351**; 36-7

S. Chaudhuri and J.R. Coggins; The Purification of Shikimate Dehydrogenase from *Escherichia coli* (1985) Biochem. J. **226**; 217-23

J. Chen, E. Giovannucci, K. Kelsey, E.B. Rimm, M.J. Stampfer, G.A. Colditz, D. Spiegelman, W.C. Willet and D. Hunter; A Methylenetetrahydrofolate Reductase Polymorphism and the Risk of Colorectal Cancer (1996) Cancer Res. **56**; 4862-4

R.Z. Chen, U. Pettersson, C. Beard, L. Jackson-Grusby and R. Jaenisch; DNA Hypomethylation Leads to Elevated Mutation Rates (1998) Nature **395**; 89-93

H. Cherest, D. Thomas and Y. Surdin-Kerjan; Polyglutamylation of Folate Coenzymes Is Necessary for Methionine Biosynthesis and Maintenance of Intact Mitochondrial Genome in *Saccharomyces cerevisiae* (2000) J. Biol. Chem. **275**; 14056-63

P.K. Chiang, P.D. Burbelo, S.A. Brugh, R.K. Gordon, K. Fukuda and Y. Yamada; Activation of Collagen IV Gene Expression in F9 Teracarcinoma Cells by Deazaadenosine Analogues (1992) J. Biol. Chem. **267**; 4988-91

P. Chiba, A. Plas, J.M.C. Wessels and C.H.M.M. DeBruyn; S-Adenosylhomocysteine Hydrolase Activity During Differentiation of HL-60 Cells (1984) Biosci. Rep. **4**; 687-94

S-W. Choi, Y-I. Kim, J.N. Weitzel anf J.B. Mason; Folate Depletion Impair DNA Excision Repair in the Colon of the Rat (1998) Gut **48**; 93-9

D.J. Cichowicz and B. Shane; Mammalian Folypoly-γ-glutamate Synthetase. 2: Substrate Specificity and Kinetic Properties (1987) Biochemistry **26**; 513-21

R. Clarke, L.Daly, K.Robinson, E. Naughten, S. Cahalane, B. Fowler and I. Graham; Hyperhomocysteinemia: An Independent Risk Factor for Vascular Disease (1991) N. Engl. J. Med. **324**; 1149-55

D.E.C. Cole, H.J. Ross, J. Evrovski, L.J. Langman, S.E.S. Miner, P.A. Daly and P-Y. Wong; Correlation Between Total Homocysteine and Cyclosporine Concentrations in Cardiac Transplant Recipients (1998) Clin. Chem. **44**; 2307-2312

J.E. Cone, J. Plowman and G. Guroff; Purification and Properties of Guanosine Triphosphate Cyclohydrolase and a Stimulating Protein from *Comamonas* Specie (ATCC 11299a) (1974) J. Biol. Chem. **249**; 5551-8

J.D. Cook, D.J. Cichowicz, S. George, A. Lawler and B. Shane; Mammalian Folypoly-γ-glutamate Synthetase. 4: *In Vitro* and *Invivo* Metabolism of Folates and Analogues and Regulation of Folate Homostatasis (1987) Biochemistry **26**; 530-9

F.T. Crews, F. Hirata, J. Axelrod; Identification and Properties of Methyltransferases that Synthesize Phosphatidylcholine in Rat Brain Synaptosomes (1980) J. Neurochem. **36**: 6; 1491-8

S.F. Crowe and C.K. Ross; Effect of Folate Deficiency and Folate and B_{12} Excess on Memory Function in Young Chicks (1997) Pharmacol. Biochem. Behav. **56**; 189-197

G.J. Cuskelly, H. McNulty and J.M. Scott; Effect of Increasing Dietary Folate on Red Cell Folate: Implications for Prevention of Neural Tube Defects. (1996) Lancet **347**; 657-9

G.J. Cuskelly, H. McNulty and J.M. Scott; Fortification with Low Amounts of Folic Acid Makes a Significant Difference in Folate Status in Young Women: Implications for the Prevention of Neural Tube Defects (1999) Am. J. Clin. Nutr. **70**; 234-9

W.S. Dallas, J.E. Gowan, P.H. Ray, M.J. Cox and I.K. Dev; Cloning, Sequencing and Enhanced Expression of the Dihydropteroate Gene of *Eschericia coli* MC400 (1992) J. Bacteriol. **174**; 5961-70

R. de Franchis, G. Sebastio, C. Mandato, G. Andria and P. Mastroiacovo; Spina Bifida 677T→C Mutation, and Role of Folate (1995) Lancet **346**; 1703

P. Dansette and R. Azerad; The Shikimate Pathway. II. Steriospecificity of Hydrogen Transfer Catalysed by NADPH-Dehydroshikimate Reductase from *E.coli* (1974) Biochimie **56**;751-5

H.H. Daron and J.L. Aull; A Kinetic Study of Thymidylate Synthase from *Lactobcillus casei* (1978) J. Biol. Chem. **253**: 3; 940-5

G.E. Davidson, D.G. Weir and J. Scott; The Metabolic Consequences of Vitamin B12/Methionine Deficiency in Rats; (1975) Biochim. et Biophys. Acta **392**; 207-15

G.M. Davis, K.J. Barrett-Bee, D.A. Jude, M. Lehan, W.W. Nichols, P.E. Pinder, J.L. Thain, W.J. Watkins and R.G. Wilson; (6S)-6-Fluoroshikimic Acid, an Antibacterial Agent Acting on the Aromatic Biosynthetic Pathway (1994) Antimicrob. Agents Chemother. **38**; 403-6

S. Dayal, T. Bottiglieri, E. Arning, N. Maeda, M. R. Malinow, D. D. Heistad, F. M. Faraci and S. R. Lentz; Endothelial dysfunction and elevation of S-adenosylhomocysteine in hyperhomocysteinemic CBS-deficient mice (2000) Circulation **In Press**.

S.D. De Ferranti, J.P.A. Ioannidis, J. Lau, W.V. Anninger and M. Barza; Are Amoxycillin and Folate Inghibitors as Effective as Other Antibiotics for Acute Sinusitis? A Meta-Analysis (1998) BMJ **317**; 632-7

S.F. DeCabo, J. Santos and J. Fernandez-Piqueras; Molecular and Cytological Evidence of S-Adenosyl-L-homocysteine as an Innocuous Undermethylating Agent *In Vivo* (1995) Cytogenet. Cell Genet. **71**; 187-92

G.A. Dekker, J. de Vries, M.S. Doelitzsch et al; Underlying Disorders with Severe Early-Onset Preeclempsia (1995) Am. J. Obstet. Gynecol. **173**; 1042-8

R.J. DeLange, D.M. Fambrough, E.L. Smith and J. Bonner; Calf and Pea Histone IV. 3. Complete Amino Acid Sequence of Pea Seedling Histone IV; Comparison with the Homologous Calf Thymus Histone (1969) J. Biol. Chem. **244**; 5669-79

A.B. DeLeo, J. Dayan and D.B. Sprinson; Purification and Kinetics of tyrosine-sensitive 3-Deoxy-D-*Arabino*-Heptulosonic Acid 7-Phosphate Synthetase from *salmonella* (1973) J. Biol. Chem. **248**; 2344-2353

S. Delgado, M. Gómez, A. Bird and F. Antrquera; Initiation of DNA Replication at CpG Islands in Mammalian Chromosomes (1998) EMBO J. **17**; 2426-35

I.K. Dev and R.J. Harvey; N^{10}-Formyltetrahydrofolate Is the Formyl Donor for Glycinamide Ribotide Transformylase in Escherichia coli (1978) J. Biol. Chem. **253**; 4242-4

J-L. Dhondt; Tetrahydrobiopterin Deficiency. Analysis from an International Syrvey (1986) In: *Chemistry and Biology of Pteridines* (Eds: B.A. Cooper and V.M. Whitehead) Walter de Gruyter, Berlin. Pp385-90

G.L. Dianov, T.V. Timchenko, O.I. Sinitsina, A.V. Kuzminiv, O.A. Medvedev and R.I. Salganik; Repair of Uracil Residues Closely Spaced on the Pooosite Strands of Plasmid DNA Redults in Double-Strand Break and Deletion Formation (1991) Mol. Gen. Genet. **225**; 448-52

H.W. Dickerman, E. Steers, B.G. Redfield and H. Weissbach; Methionyl Soluble Ribonulleic Acid Transformylase. I. Purification and Partial Characterization (1967) J. Biol. Chem. **242**; 1522-5

S.O. Døskeland and P.M. Ueland; Comparison of some Physiological and Kinetic Properties S-Adenosylhomocysteine from Bovine Liver, Bovine Adrenal Cortex and Mouse Liver (1982) Biochim. Biophys. Acta **708**; 185-93

W. Du, N.G. Wallis, M.J. Mazzulla, A.F. Chalker, L. Zhang. W-S. Liu, H.Kallender and D.J. Payne; Characterization of *Streptococcus pneumoniae* 5- Enolpyruvylshikimate 3-Phosphate Synthase and its Activation by Univalent Cations (2000) FEBS J. **267**; 222-7

D.S. Duch, S.W. Bowers and C. A. Nichol; Role of the Pituitary in the Regulation of Tetrahydrobiopterine Biosynthesis in Non-Neural Tissues (1986) In: *Chemistry and Biology of Pteridines* (Eds: B.A. Cooper and V.M. Whitehead) Walter de Gruyter, Berlin. pp219-22

P. Durand, M. Prost and D. Blache; Folic Acid Deficiency Enhances Oral Contraceptive-Induced Platelet Hyperactivity (1997) Arteriosclerosis, Thrombosis, and Vascular Biology **17**; 1939-46

S.J. Duthie and A. Hawdon; DNA Instability (Strand Breaks, Uricil Misincorporation, and Defective Repair) is Increased by Folic Acid Depletion in Human Lymphocytes In Vitro (1998) FASEB J. **12**; 1491-7

S.J. Duthie and P. McMillan; Uracil Misincorporation in Human DNA Detected Using Single Cell Gel Electrophoresis (1997) Carcinogenesis **18**; 1709-14

Editorial (1999) Eur. J. Obstet. Gynecol. Reprod. Biol. **87**; 103-4

M. Ehrlich, M.A. Gama-Sosa, L-H. Huang, R.M. Midgett, K.C. Kuo, R.A. McCune and C. Gehrke; Amount and Distribution of 5-Methylcytosine in Human DNA from Different Types of Tissue or Cells (1982) Nucleic Acids Res. **10**; 2709-21

J.W. Eikelboom, E.Lonn, J. Gwnest, G. Hankey, and S. Yusef; Homocyst(e)ine and Cardiovascular Disease: A Critical Review of the Epidemiologic Evidence (1999) Ann. Intern. Med. **132**; 363-75

J.Ek and E.M. Magnus; Plasma and Red Blood Cell Folate During Normal Pregnancies (1981) Acta Obstet. Gynecol. Scanad. **60**; 247-51

J. Ek; Plasma and Red Cell Folate in Mothers and Infants in Normal Pregnancies. Relation to Birth Weight (1982) Acta Obstet. Gynecol. Scand. **61**; 17-20

J. Ek; Plasma and Red Cell Folate Values in Newborn Infants and Their Mothers in Relation to Gestational Age (1980) J. Pediatr. **97**; 288-92

P.H. Ellims and M.B. Van der Weyden; Kinetic mechanism and inhibition of human liver thymidine kinase(1981) Biochim. Biophys. Acta **660**: 2; 238-42

C.J. Epstein; Down Syndrome (Trisomy 21); In The Metabolic and Molecular Basis of Inherited Disease (Edited by: C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle) McGraw-Hill Inc., New York (1995) pp749-94

Eurocat Working Group; Prevalence of Neural Tube Defects in 20 Regions of Europe and the Impact of Prenatal Diagnosis 1980-1985 (1991) J. Epidemiol. Community Health **45**; 52-8

C.D.C. Ewart, D.A. Jude, J.L. Thain and W.W. Nichols; Frequency and Mechanism of Resistance to Antibacterial Action of ZM 240401, (6S)-6-Fluoro-Shikimic Acid (1995) Antimicrob. Agents Chemother. **39**; 87-93

Expert Advisory Group; Folic Acid and the Prevention of Neural Tube Defects (1992) Department of Health, London, UK

M. Fava, J.S. Borus, J.E. Alpert, A.A. Nierenberg, J.F. Rosenbaum and T. Bottiglieri; Folate, Vitamin B12, and Homocysteine in Major Depressive Disorders (1997) Am. J. Psychiatry. **154**; 426-8

M. Fenech, C. Aitken and J. Rinaldi; Folate, Vitamin B_{12}, Homocysteine Status and DNA Damage in Young Australian Adults. (1998) Carcinogenesis **19**; 1163-71

M.F. Fenech, I.E. Dreosti and J.R. Rinaldi; Folate, Vitamin B_{12}, Homocysteine Status and Chromosome Damage Rate in Lymphocytes of Older Men (1997) Carcinogenesis **18**; 1329-36

R. Ferone and A. Warskow; Co-Purification of Dihydrofolate Synthetase and N^{10}-formyltetrahydropteroyldiglutamate Synthase from *E.coli* (1983) In: *Folyl and Antifolyl Polyglutamates* (eds: I.D. Goldman, B.A. Chabner and J.R. Bertino) Plenum Press, New York. pp. 167-81

R. Ferone and S.R. Webb; Kinetic Studies of Escherichia coli Dihydropteroate Synthetase (1975) In: Chemistry and Biology of Pterines (W. Pfleiderer, ed.) Walter de Gruyter, Berlin pp61-71

R.S. Ferone, C. Singer, M.H. Hanlon and S. Roland; Isolation and Characterisation of an *E.coli* Mutant Effected in Dihydrofolate- and folypolyglutamate Synthetase (1983) In: *Chemistry and Biology of Pteridines* (ed: J. Blair) Walter de Gruyer, Berlin. pp. 585-9

J.A. Fewster; Phosphorylation of Shikimic Acid by Ultrasonic Extracts of Micro-Organisms (1962) Biochem. J. **85**; 388-93

M. Fischer, A. Bracher, R.Lutz, A. Herrmann, G. Auerbach et al; Heterologous Expression of Recombinant Human GTP Cyclohydrolase I (1997) In: *Chemistry and Biology of Pterine and Folate* (W. Pfleiderer and H. Rokos, eds.) Blackwell Science, Berlin pp611-4

J.G. Flaks, M.J. Erwin and J.M. Buchanan; Biosynthesis of the Purines: XVIII. 5-Amino-1-Imidazolecarboxamide 5'-Phosphate Transformylase and Inosinicase (1957) J. Biol. Chem. **229**; 603-12

A. Fleming and A.J. Copp; Embryonic Folate Metabolism and Mouse Neural Tube Defects (1998) Science **280**; 2107-9

M. Florkin; Concepts of Molecular Biosemiotics and of Molecular Evolution (1974) In *Comprehensive Biochemistry Vol. 29A* (M. Florkin and E.H. Stotz, eds)Elsevier Amsterdam. pp1-124

S.E. Floyd, P.J. Finn, S. Patterson ans M.A.T. Flynn; Food and Nutrient Intakes in Pregnant Irish Adolescents and Young Women (2000) Proc. Nutr. Soc. **59**; 81A

Food and Drug Administration; Food Standards: Amendment of the Standards of Identity for Enriched Grain Products to Require Addition of Folic Acid (1996) Fed. Regist. **61**; 1879-85

Food Safety Advisory Board; The Value of Folic Acid in the Prevention of Neural Tube Defects. Report to the Minister for Health and Children (1997) Food Safety Advisory Board, Dublin, Ireland

Food Surveys Research Group; Food and Nutrient Intake by Children 1994-96, 1998. Table Set 17 [Online] (1998) Available (under "Releases"): http://www.barc.usda.gov/bhnrc/foodsurvey/home.htm [accessed 2000, 21st June]

P. Frosst, H.J. Blom. R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, G.J.H. Boers, M. den Heijer, L.A.J. Kluijtmans, L.P. van denn Heuvel and R. Rozen; A Candidate Genetic Risk Factor for Vascular Disease: A Common Mutation in Methylenetetrahydrofolate Reductase (1995) Nature Genetics **10**; 111-113

C. Funk; Further Experimental Studies on Beri Beri the Action of Certain Purine and Pyrimidine Derivatives (1912) J. Physiol. **45**; 489-92

J. Galivan, M.S. Rhee, T.B. Johnson, R. Dilwith, M.G. Nair, M. Bunni and D.G. Priest; The Role of Cellular Folates in the Enhancement of Activity of the Thymidylate Synthase Inhibitor 10-propargyl-5,8-dideazafolate Against Hepatoma Cells *In Vitro* by Inhibitors of Dihydrofolate Reductase. (1989) J. Biol. Chem. **264**; 10685-92

M.A. Gama-Sosa, R.M. Midgett, V.A. Slager, S. Githens, K.C. Kuo, C.W. Gehrke and M. Ehrich; Tissue-specific Differences in DNA Methylation in Various Mammals. (1983) Biochim. Biophys. Acta **740**; 212-9

F. Geoghegan, J.M. McPartlin, D.G. Weir and J.M. Scott; para-Acetamibobenzoylglutamate is a suitable indicator of folate catabolism in rats; (1995) J. Nutr 125, 2563-70

S. George, D. Cichowicz and B. Shane; Mammalian Folypoly-γ-glutamate Synthase. 3. Specificity for folate analogues. (1987) Biochem. **26**;522-9

M.I. Gibson and F. Gibson; Preliminary Studies on the Isolation and Metabolism of an Intermediate in Aromatic Biosynthesis: Chorismic Acid (1964) Biochem. J. **90**; 248-56

F. Gibson; Chorismic Acid: Purification and Some Chemical and Phyical Studies (1964) Biochem. J. **90**; 256-61

E. Giovannucci, E.B. Rimm, A. Ascherio, M.J. Stampfer, G.A. Colditz and W.C. Willett; Alcohol. Low-Methionine, Low Folate Diets and Risk of Colon Cancer in Men (1995) J. Natl. Cancer Inst, **87**; 265-73

E. Giovannucci, M.J. Stampfer, G.A. Colditz, D.J. Hunter, C. Fuchs, B.A. Rosner, F.E. Speizer and W.C. Willett; Multivitamin Use, Folate, and Colon Cancer in Women in the Nurses' Health Study (1998) Ann. Intern. Med. **129**; 517-24

E. Giovannucci, M.J. Stampfer, G.A. Colditz, E.B. Rimm, D. Trichopoulos, B.A. Rosner, F.E. Speizer and W.C. Willett; Folate, Methionine, and Alcohol Intake and Risk of Colorectal Adenoma (1993) J. Natl. Cancer Inst, **85**; 875-84

T.A. Goddijn-Wessel, M.G. Wooters E.F. van de Molen et al; Hyperhomocysteinemia: A Risk Factor for Placental Abruption or Infarction (1996) Eur. J. Obstet. Gynecol. Reprod. Biol. **66**; 23-9

D.M. Goli and J.T. Vanderslice; Investigation of the Conjugase Treatment Procedure in the Microbiological Assay of Folate (1992) Food Chem. **43**; 57-64

I.M. Graham, L.E. Daly, H.M. Refsum, K. Robinson, L.E. Brattström, *et al.*; Plasma Homocysteine as a Risk Factor for Vascular Disease: The European Concerted Action Project (1997) JAMA **277**; 1775-81

J.M. Gree, B.P. Nichols and R.G. Matthews; Folate Biosynthesis, Reduction, and Polyglutamation (1996) In Eschericia coli *and* Salmonella. *Cellular and Molecular Biology* 2nd Edn. (F.C. Neidhardt et al, eds.) ASM Press, Washington, D.C. Vol. 1; pp 665-73

J.M. Green and B.P. Nichols; p-Aminobenzoate Biosynthesis in *Eschericia coli*. Purification of Aminodeoxychorismate Lyase and Cloning of pabC (1991) J. Biol. Chem. **266**; 12971-5

J.M. Green, D.P. Ballou and R.G. Matthews; Examination of the Role of Methylenetetrahydrofolate Reductase in Incorporation of Methyltetrahydrofolate into Cellular Metabolism (1988) FASEB J. **2**; 42-7

J.M. Green, W.K. Merkel and B.P. Nichols; Characterisation and Sequencing of *Eschericia coli* pabC, the Gene Encoding Aminodeoxychorismate Lyase, a Pyridoxal Phosphate-Containing Enzyme (1992) J. Bacteriol. **174**; 5317-23

M.J. Griffin and G.M. Brown; The Biosynthesis of Folic Acid. III: Enzymic Formation of Dihydrofolic Acid from Dihydropteroic Acid and of Tetrahydropteroylpolyglutamic Acid Compounds from Tetrahydrofolic Acid (1964) J. Biol. Chem. **239**: 1; 310-316

Y. Gruenbaum, M. Szyf, H. Cedar and A. Razin; Methylation of Replicating and Postreplicated Mouse L Cell DNA (1983) Proc. Natl. Acad. Sci. USA **80**; 4919-21.

J-M. Guillon, S. Heiss, J. Soutourina, Y. Mechulam, S. Laalamis, M. Grumberg-Manago and S. Blanquet; Interplay of Methionine tRNAs with Translation Elongation Factor Tu and Translation Initiation Factor 2 in Eschericia coli (1996) J. Biol. Chem.**217** 22321-5

J-M. Guillon, Y. Mechulam, J-M. Schmitter, S. Blanquet and G. Fayat; Disruption of the Gene for Met-tRNA$_f^{Met}$ Formyltransferase Severely Impairs Growth of Escherichia coli (1992) J. Bacteriol. **174**; 4294-301

J-M. Guillon, Y. Mechulam, S. Blanquet and G. Fayat; Importance of Formylability and Anticodon Stem Sequence to Give a tRNA^{Met} an Initiator Identity in *Escherichia coli* (1993) J. Bacteriol. **175**; 4507-14

G. Guroff and C.A. Strenkoski; Biosynthesis of Pteridines and of Phenylalanine Hydroxylase Cofactor in Cell Extracts of *Pseudomonas* Species (ATCC 11299a) (1966) J. Biol. Chem. **241**; 2220-7

M. Gütlich, M. Fischer, D. Cahill, A. Bacher, V. Holdengreber, M. Vechoropoulos, Y. BenShaul and K. Witter; GTP Cyclohydrolase I in Developing Chicken Embrionic Retina (1997) In: *Chemistry and Biology of Pterine and Folate* (W. Pfleiderer and H. Rokos, eds.) Blackwell Science, Berlin pp607-10

S. Gutstein, L. Bernsteine, L. Levy and G. Wagner; Failure of Response to N5-methyltetrahydrofolate in Combined Folate B12 Deficiency; (1973) Digestive Diseases **18**; 2:142-6

K.A. Hajjar; Homocysteine-induced Modulation of Tissue Plasminogen Activator Binding to its Endothelial Cell Membrane Receptor (1993) J. Clin. Invest. **91**; 2873-9

I.C. Hampele, A. D'Arcy, G.E. Dale, Dirk Kotrewa, J. Nielsen, C. Oefner, M.G.P. Page, H-J. Schönfeld, D. Stüber and R.L. Then; Structure and Function of the Dihydropteroate Synthase from *Staphlococcus aureus* (1997) J. Mol. Biol. **268**; 21-30

S.C. Hartman and J.M. Buchanan; Biosynthesis of Purines: XXVI. The Identification of the Formyl Donors of the Transformylation Reactions (1959) J. Biol. Chem. **234**; 1812-6

R.J. Harvey; Synergism in the Folate Pathway (1982) Rev. Infect. Dis. **4**: 2; 255-60

E. Haslam; *Shikimic Acid: Metabolism and Metabolites* (1993) John Wiley, Chichester

T. Hasler and A. Niederwieser; Tetrahydrobiopterin-Producing Enzyme Activities in Liver of Animals and Man (1986) In: *Chemistry and Biology of Pteridines* (Eds: B.A. Cooper and V.M. Whitehead) Walter de Gruyter, Berlin. pp319-22

C. Haußmann, F. Rohdich, E. Schmidt, A. Bacher and G. Richer; Biosynthesis of Pteridines in *Escherichia coli*. Structural and Mechanistic Similarity of Dihydroneopterin-Triphosphate Epimerase and Dihydroneopterin Aldolase (1998) J. Biol. Chem. **273**; 17418-24

C. Haußmann, G. Richter, F. Rohdich, A. Bacher, M. Gomez-Ortiz, S. Grüneberg, G. Auerbach, T. Ploom and R. Huber; Dihydroneopterin Aldolase: Properties and Preliminary Crystallographic Analysis (1997) In: *Chemistry and Biology of Pterine and Folate* (W. Pfleiderer and H. Rokos, eds.) Blackwell Science, Berlin pp651-4

T. Hayashi, G. Honda and K. Suzuki; An Atherogenic Stimulus Homocysteine Inhibits Cofactor Activation of Thrombomodulin and Enhances Thrombomodulin Expression in Human Umbilical Vein Endothelial Cells (1992) Blood **79**: 11; 2930-6

E. Heard, P. Clerc and P. Avner; X-Chromosome Inactivation in Mammals (1997) Annu. Rev. Genet. **31**; 571-610

S.M. Henning and M.E. Swendseid; The Role of Folate, Choline, and Methionine in Carcinogenesis Induced by Methyl-deficient Diets (1996) Adv. Exp. Med. Biol. **399**; 143-155

V. Herbert and R. Zalusky; Selective Concentration of Folic Acid Activity in Cerebo-Spinal Fluid (1961) Fed. Proc. **20**; 453

V. Herbert; Making Sense of Laboratory Tests of Folate Status: Folate Requirements to Sustain Normality (1987) Amer. J. Hematol. **26**; 199-207

V. Herbert; Recommended Dietary Intakes (RDI) of Folate in Humans (1987) Am. J. Clin. Nutr. **45**; 661-70

M.S. Hershfield, V.N. Aiyar, R. Premakumar and W.C. Smalls; S-Adenosylhomocysteine Hydrolase from Human Placenta: Affinity Purification and Characterization (1985) Biochem. J. **230**; 43-52

K. Hiraga, H. Kochi, Y. Motokawa and G. Kikuchi; Enzyme Complex Nature of the Reversible Glycine Cleavage System of Cock Liver Mitocondria (1972) J. Biochem. **72**; 1285-9

K. Hirayama and G. Kapatos; Immunohistochemical Localization and Quantification of GTP Cyclohydrolase I in Rat Brain Monoamine Neurons (1997) In: *Chemistry and Biology of Pterine and Folate* (W. Pfleiderer and H. Rokos, eds.) Blackwell Science, Berlin pp615-8

R. Hishida and H. Nau; VPA-Induced Neural Tube Defects in Mice. I. Altered Metabolism of Sulfur Amino Acids and Glutathione (1998) Teratog. Carcinog. Mutagen **18**; 49-61

G.H. Hitchings, G.B. Eilon, H. Vanderweff and E.H. Falco; Pyrimidine derivatives as antagonists of Pteroylglutamic acid (1948) J. Biol. Chem. **174**; 765-6

G.H. Hitchings; Nobel lecture in physiology or medicine - 1988. Selective inhibitors of dihydrofolate reductase (1989) In Vitro Cell. Develop. Biol. **25**; 303-310.

A.V. Hoffbrand and T.J. Peters; The Subcellular Localization of Pteroyl Polyglutamate Hydrolase and Folate in Guinea Pig Intestinal Mucosa (1969) Biochim. Biophys. Acta **192**; 479-85

K. Hoppner and B. Lampi; Folate Levels in Human Liver from Authopsies in Canada (1980) Am. J. Clin. Nutr. **33**; 862-4

D.K. Houston, M.J. Johnson, R.J. Nozza, E.W. Gunter, K.J. Shea, G.M. Cutler and J.T. Edmonds; Age-Related Hearing Loss, Vitamin B-12, and Folate in Elderly Women (1999) Am. J. Clin. Nutr. **69**; 564-71

D.M. Howell and R.H. White; D-*erythro*-Neopterin Biosynthesis in the Methanogenic Archaea *Methanococcus thermophila* and *Methanobacterium thermoautophicum* ΔH (1997) J. Bacteriol. **179**; 5165-70

C.A. Hrycyna and S. Clarke; Modification of Eukaryotic Signaling Proteins by C-Terminal Methylation Reactions (1993) Pharmacol. Ther. **59**; 281-300

C-L. Hsieh; Dependence of Transcriptional Repression on CpG Methylation Density (1994) Mol. Cell. Biol. **14**; 5487-94

F.M. Huennekens and G.B. Henderson; Transport of Folate Compounds into Mammalian and Bacterial Cells (1975) In *Chemostry and Biology of Pteridines* (W. Pfleidered, ed.) Walter de Gruyter, Berlin pp177-196

B.L. Hutchings, E.L.R. Stokstad, J.H. Mowat, J.H. Booth, C.W. Waller, R.B. Angier, J. Semb and Y SubbaRow; The Degradation of the Fermentation Lactobacillus casei Factor II (1948) J. Am. Chem. Soc. **70**; 10-13

Q.K. Huynh, SC Bauer, GS Bild, GM Kishore and JR Borgmeyer; Site-directed mutagenesis of Petunia hybrida 5-enolpyruvylshikimate-3-phosphate synthase: Lys-23 is essential for substrate binding (1988) J. Biol. Chem., **263**: 24; 11636-9

Q.K. Huynh; Evidence for a Reactive gamma-Carboxyl Group (Glu-418) at the Herbicide Glyphosate Binding Sight of 5-Enolpyruvylshikimate-3-Phosphate Synthase from Escherichia coli (1988) J. Biol. Chem. **263**; 11631-5

S.Y Hwang, D.A. Berges, J.J. Taggart and C. Gilverg; Portage Transport of Sulfonamides and Sulfanilic Acid (1989) J. Med. Chem. **32**; 694-8

A. Ichikawa, S. Sato and T. Tomita; Purification and Characterization of S-Adenosylhomocysteine Hydrolase from Mouse Mastocytoma P-815 Cells: Evidence for Cell Cycle-Specific Fluctuation of the Enzyme Activity (1985) J. Biochem. **97**; 189-97

H. Ichinose, T. Ohye, Y. Matsuda, T. Hori, N. Blau et al; Characterisation of Mouse and Human Cyclohydrolase I Genes. Mutations in Patients with GTP Cyclohydrolase I Deficiency (1995) J. Biol. Chem. **270**; 10062-71

D. Ingrosso, M.G. Cottichelli, S. D'Angelo, M.D. Buro, V. Zappia and P. Galletti; Influence of Osmotic Stress on Protein Methylation in Resealed Eryhrocytes (1997) Eur. J. Biochem. **244**; 918-22

K. Iwai and M. Kobashi; The Biosynthesis of Folic Acid and Pteridine Cofactor(s) and its Regulation (1975) In: Chemistry and Biology of Pterines (W. Pfleiderer, ed.) Walter de Gruyter, Berlin pp341-357

A.L. Jackman; *Antifolate Drugs in Cancer Therapy* (1999) Humana Press, Totowa, New Jersey.

R.C. Jackson and K.R. Harrap; Studies with a Mathematical Model of Folate Metabolism (1973) Arch. Biochem. Biophys. **158**; 827-41

R.J. Jackson and T. Shiota; Identification of the Isomer of Dihydroneopterin Triphosphate Synthesised by Two Enzyme Fractions from *Lactobacillus plantarum* (1971) J. Biol. Chem. **246**; 7454-9

P.F. Jacques, J. Selhub, A.G. Bostom, P.F.W. Wilson and I.H. Rosenberg; The Effect of Folic Acid Fortification on Plasma Folate and Total Homocysteine Concentrations (1999) N. Engl. J. Med. **340**; 1449-54

S.J. James, B.J. Miller, A.G. Basnakian, I.P. Pogribney, M. Pogribna and L. Muskhelshvili; Apoptosis and Proliferation Under Conditions of Deoxynucleotide Pool Imbalance in Liver of Folate / Methyl Deficient Rats (1997) Carcinogenesis **18**; 287-93

S.J. James, M. Pogribna, I.G. Pogribny, S. Melnyk, R.J. Hine, J.B. Gibson, P.Yi, D.L. Tafoya, D.H. Swenson, V.L. Wilson and D.W. Gaynor; Abnormal Folate Metabolism and Mutation in the Methyltetrahydrofolate Reductase Gene May beMaternal Risk Factors for Down Syndrome (1999) Am. J. Clin. Nutr. **70**; 495-501

D.A. Jencks and R.G. Matthews; Allosteric Inhibition of Methylenetetrahydrofolate Reductase by Adenosylmethionine: Effects of Adenosylmethionine and NADPH on the Equilibrium Between Active and Inactive Forms of the Enzyme and on the Kinetics of Approach to Equilibrium (1987) J. Biol. Chem. **262**: 6; 2485-93

W. Ji, R. Hernandez, X-Y. Zhang, G. Qu, A. Frady, M. Varela and M. Ehrlich; DNA Demethylation and Pericentromeric Rearrangements of Chromosome 1 (1997) Mut. Res. **379**; 33-41

D.G. Johns, S. Sperti and A.S.V. Burger, (1961) J. Clin. Invest. **40**; 1684

P.A. Jones and M.L. Gonzalgo; Altered DNA Methylation and Genome Instability: A New Pathway to Cancer? (1997) Proc. Natl. Acad. Sci. USA **94**; 2103-5

P.A. Jones and P.W. Laird; Cancer Epigenetics Comes of Age (1999) Nature Genet. **21**; 163-7

T.H.D. Jones and G.M. Brown; The Biosynthesis of Folic Acid. VII. Enzymatic Synthesis of Pteridines from Guanosine Triphosphate (1967) **242**; 3989-97

T.H. Jukes, A.L. Franklin, E.L.R. Stokstad, J.W. Boehne; The urinary excretion of pteroglutamic acid and certain related compounds (1947) J. Lab. Clin. Med. **32**; 1350-5.

I. Just, S. Dundaroff, D. Faulk and H.U. Wolf; Regulation of Thymidine Monophosphate and Other Nucleotides of Thymidine Kinase Activity in Extracts From Primary Rabbit Kidney Cells Infected With HSV Type 1 and 2 (1975) J. Gen. Virol. **29**; 69-80

S-S. Kang, P.W.K. Wong, J. Zhou and H.Y. Cook; Total Homocysteine in Plasma and Amniotic Fluid of Pregnant Women (1986) Metabolism **35**; 889-91

S.E. Keizer, R.S. Gibson and D.L. O'Connor; Postpartum Folic Acid Supplementation of Adolescents: Impact on Maternal Folate and Zinc Status and Milk Composition (1995) Am. J. Clin. Nutr. **62**; 377-84

B.P. Kelleher and S.D. O'Broin; Microbiological Assay for Vitamin B_{12} Performed in 96-well Microtiter Plates (1991) J. Clin. Pathol. **44**; 592-5

P. Kelly, J. McPartlin, M. Goggins, D.G. Weir and J.M. Scott; Unmetabolized Folic Acid Iin Serum: Acute Studies in Subjects Consuming Fortified Food and Supplements (1997) Am. J. Clin. Nutr. **65**; 1790-5

T.A. Kemppainen, V.M. Kosma, E.K. Janatuinen, R.J. Julkunen, P.H. Pikkarainen and M.I. Uusitupa; Nutritional Status of Newly Diagnosed Celiac Disease Patients Before and After the Institutionof a Celiac Diseae Diet--Association with the Grade of Mucosal Villous Atrophy (1998) Am. J. Clin. Nutr. **67**; 482-7

Y-I. Kim, I.P. Pogribny, A.G. Basnakian, J.W. Miller, J. Selhub, S.J. James and J.B. Mason; Folate Deficiency in Rats Induces DNA Strand Breaks and Hypomethylaton Within the p53 Tumor Suppressor Gene (1997) Am. J. Clin. Nutr. **65**; 46-52

H. Kimura, F. Gejyo, S. Suzuki and R. Miyazaki; The C677T Methylenetetrahydrofolate Reductase Gene Mutation in Hemodialysis Patients (2000) J. Am Soc. Nephrol. **11**; 885-93

G.M. Kishore and D.M. Shah; Amino Acid Biosynthesis Inhibitors as Herbicides (1988) Annu. Rev. Biochem. **57**; 627-63

R.L. Kisliuk, Y. Gaumont, J.F. Powers, J. Thorndike, M.G. Nair and J.R. Piper; Synergistic Growth Inhibition by Combination of Antifolates (1990) In: *Folic Acid in Heath and Disease* (Ed: M. Picciano) Wiley-Liss, Inc., New York pp79-89

R.L. Kisliuk,Y. Gaumont, P. Kumar, M. Coutts M.G. Nair, N.T. Nanavati and T.I. Kalman; The Effect of Polyglutamylation on the Inhibition Activity of Folate Analogues (1985) In: *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates* (ed: I.D. Goldman) Praegen, New York, pp319-28

R.L. Kisliuk; Synergistic Interactions among Antifolates (2000) Pharmacology and Therapeutics **85**; 183-90

L.A. Kleczkowski, D.D. Randall and D.G. Blevins; Purification and Characterization of a Novel NADPH (NADH)-Dependent Glyoxyate Reductase from Spinach Leaves. Comparison of Immunological Propertie of Leaf Glyoxylate Reductase and Hydroxypyruvate Reductase (1986) Biochem J. **239**; 653-9

A.S. Kloeblen; Folate Knowledge, Intake from Fortified Grain Products, and Periconceptional Supplementation Patterns of a Sample of Low-Income Pregnant Women According to the Health Belief Model (1999) J. Am. Diet Ass. **99**; 33-8

T. Knöchel, A. Ivens, G.Hester, A.Gonzalez, R. Bauerle, M. Wilmanns, K. Kirschner, and J.N. Jansonius; The crystal structure of anthranilate synthase from Sulfolobus solfataricus: Functional implications (1999)PNAS 96: 9479-9484.

M. Kobt and N.M. Kredich; Regulation of Human Lymphocyte S-Adenosylmethionine Synthtase by Product Inhibition (1990) Biochim. Biophys. Acta **1039**; 253-60

M. Kobt and N.M. Kredich; S-Adenosylmethionine Synthase from Human Lymphocytes: Purification and Characterization (1985) J. Biol. Chem. **260**: 7; 3923-30

D.L. Kramer, C.W. Porter, R.T. Borchardt and J.R. Sufrin; Combined Modulation of S-Adenosylmethionine Biosynthesis and S-Adenosylhomocysteine Metabolism Enhances Inhibition of Nucleic Acid Methylation and L1210 Cell Growth (1990) Cancer Res. **50**; 3838-42

N. Krasner, K.M. Cochran, R.I. Russell, H.A. Carmicheal and G.G. Thompson; Alcohol and Absorption from the Small Intestine. 1. Impairment of Absorption from the Small Intestine in Alcoholics (1976) Gut **17**; 245-8

C. Krumdieck, K. Fukushima, T. Fukushima, T. Shiola and C. Butterworth; A Long-Term Study of the Excretion of Folate and Pterins in a Human Subject After Ingestion of 14C Folic Acid, With Observations on the Effect of Diphenylhydantoin Administration. (1978) Am. J. Clin. Nutr. 31, 88-93

C.L. Krumdieck, L.R. Boots, P.E. Cornwell and C.E. Butterworth; Cyclic variations in Folate Composition and Pteroylpolyglutamate Hydrolase (Conjugase) Activity of the Rat Uterus (1976) Am. J. Clin. Nutr. **29**; 288-94

C.L. Krumdieck, L.R. Boots, P.E. Cornwell and C.E. Butterworth; Estrogen Stimulation of Conjugase Activity in the Uterus of Ovariectomized Rats (1975) Am. J. Clin. Nutr. **28**; 530-4

C. Kutzbach and E.L.R. Stokstad; Mammalian Methylenetetrahydrofolate Reductae: Partial Purification, Properties and Inhibition by S-Adenosylmethionine (1971) Biochim. Biophys. Acta **250**; 459-77

A.P. Lachance; Overview of Key Nutrients: Micronutrient Aspects (1998) Nutr. Rev. **56**; 34S-39S

C.A. Ladino and C.M. O'Connor; Methylation of Atypical Protein Aspartyl Residues During the Stress Response of HeLa Cells (1992) J. Cell. Physiol. **153**; 297-304

N. Lakshmaiah and B.V. Ramasastri; Folic Acid Conjugase from Plasma. I: Partial Purification and Properties (1975a) Internat. J. Vit. Nutr. Res. **45**; 183-93

N. Lakshmaiah and B.V. Ramasastri; Folic Acid Conjugase from Plasma. II: Studies on the Souirce of the Enzyme in Blood(1975b) Internat. J. Vit. Nutr. Res. **45**; 194-200

N. Lakshmaiah and B.V. Ramasastri; Folic Acid Conjugase from Plasma. III: Use of the Enzyme in the Estimation of Folate Activity in Foods (1975c) Internat. J. Vit. Nutr. Res. **45**; 262-72

D.G. Lambie, Johnson RH; Drugs and folate metabolism. (1985) Drugs **30**; 145-55

J.O. Lampen and M.J. Jones; The Antagonism of Sulfonamide Inhibition of Certain *Lactobacilli* And *Enterococci* by Pteroylglutamic Acid and Related Compounds (1946) J. Biol. Chem. **166**; 435-48

B.A. Lashner, K.S. Provencher, D.L. Seidner, A. Knesebeck and A. Brzezinski; The effect of folate Supplementation on the Risk for Cancer or Dysplasia in Ulcerative Colitis (1997) Gasteroenterology **112**; 29-32

B.A. Lashner, P.A. Heidenreich, G.L. Su, S.V. Kane and S.B. Hanauer; Effect of Folate Supplementation on the Incidence of Dysplasia ad Cancer in Chronic Ulcerative Colitis. A Case-Control Study (1989) Gasteroenterology **97**; 255-9

B.A. Lashner; Red Blood Cell Folate is Associated with the Development of Dysplasia and Cancer in Ulcerative Colitis (1993) J. Cancer Res. Clin. Oncol. **119**; 549-54

M. Laskowski, V. Mims and P. Day; Studies on the Enzyme Which Produces the Streptococcus Lactus R-Stimulating Factor From Inactive Precursor Substance in Yeast (1945) J. Biol. Chem. **157**; 731-9

A. Lavoie, E. Tripp and A.V. Hoffbrand; Sephadex-Gel Filtration and Heat Stability of Human Jejunal and Serum Pteroylpolyglutamate Hydrolase (Folate Conjugase): Evidence for Two Different Forms (1975) **13**; 1-6

A. Lavoie, E. Tripp and A.V. Hoffbrand; The Effect of Vitamin B12 Deficiency on Methylfolate Metabolism and Pteroylpolyglutamate Synthesis in Human Cells; (1974) Clin. Sci. Mol. Biol. **47**;617-30

L.S. Lee and Y. Cheng; Human Deoxythymidine Kinase II: Substrate Specificity and Kinetic Behavior of the Cytoplasmic and Miticondrial Isozymes Derived From Blast Cells of Acute Myelocytic Leukemic (1976) Biochemistry **15**; 3686-90

P. Lengyel and D. Söll; Mechanism of Protein Biosynthesis (1969) Bacteriol. Rev. **33**; 264-301

S.R. Lentz and J.E. Sadler; Inhibition of Thrombomodulin Surface Expression and Proteine C Activation by the Thrombogenic Agent Homocysteine (1991) J. Clin. Invest. **88**; 1906-14

B. Levenberg and D.K. Kaczmarek; Enzymatic Release of Carbon Atom 8 from Guanosine Triphosphate, an Early Reaction in the Conversion of Purines to Pteridines (1966) Biochim. Biophys. Acta **117**; 272-5

J.G. Levin and D.B. Sprinson; The Enzymatic Formation and Isolation of 3- Enolpyruvylshikimate 5-Phosphate (1964) J. Biol. Chem. **239**; 1142-50

C.C. Levy; Pteridine Metabolism in the Skin of the Tadpole *Rana catesbeiana* (1964) J. Biol. Chem. **239**; 560-6

D.P. Lewis, D.C. Van Dyke, P.J. Stumbo and M.J. Berg; Drug and Environmental Factors Associated with Adverse Pregnancy Outcomes. Part I: Antiepileptic Drugs, Contraceptives, Smoking, and Folate (1998) Ann. Pharmacother. **32**; 802-17

H.X. Liao and L.L. Spremulli; Identification and Initial Characterization of Translational Initiation Factor 2 from Bovine Mitochondria (1990) J. Biol. Chem. **265**; 13618-22

B-F. Lin, R-F syu Huang and B. Shane; Regulation of Folate and One-Carbon Metabolism in Mammalian Cells. 3: Role of Folypoly-γ-glutamate Synthetase ; (1993) J. Biol Chem. **268**: 29; 21674-79

T. Lindahl; Instability and Decay of the Primary Structure of DNA (1993) Nature **362**; 709-15

T.W. Long, H. teraoka and K. Tsukada; Partial Purification of a Ribosomal Ribonucleic Acid Methylase from Rat Liver Nuclei and Methylation of Undermethylated Nuclear Ribonucleic Acid from Regenerating Liver of Ethionine-Treated Rats (1983) Biochim. Biophys. Acta **740**; 29-37

K.E. Lowe, C.B. Osborne, B-F. Lin, J-S. Kim, J-C. Hsu and B. Shane; Regulation of Folate and One-Carbon Metabolism in Mammalian Cells. 2: Effects of Folypoly-γ-glutamate Synthetase Substrate Specificity and Level on Folate Metabolism and Folypoly-γ-glutamate Specificity of Metabolic Cycles of One-Carbon Metabolism; (1993) J. Biol Chem. **268**: 29; 21665-73

J. Lucas-Lenard and F. Lipmann; Protein Biosynthesis (1971) Annu. Rev. Biochem. **40**; 409-48

P.I. Lukienko. M.I. Bushma, L.F. Legon'kova and G.Z. Abakimov; Effect of Folic Acid and Methotrexate on the Function of the Hydroxylating System and the Cholesterol and Phospholipid Content in the Liver Microsomes of Rats (1985) Farmokol. Toksikol. **48**; 53-5

J. Ma, M.J. Stampfer, E. Giovannucci, C. Artigas, D.J. Hunter, C. Fuchs, W.C. Willett, J. Selhub, C.H. Hennekens and R. Rozen; Methylenetetrahydrofolate Reductase Polymorphism, Dietary Interactions, and Risk of Colorectal Cancer (1997) Cancer Res. **57**; 1098-102

R.E. Mackenzie and C.M. Baugh; Tetrahydropteroylpolyglutamate Derivatives as Substrates of Two Multifunctional Proteins with Folate-Dependent Enzyme Activities (1980) Biochim. Biophys. Acta **611**; 187-95

A.D. Mackey and M.F. Picciano; Maternal Folate Status During Extended Lactation and the Effect of Supplemental Folic Acid (1999) Am. J. Clin. Nutr. **69**; 285-92

N. Mahmud, A. Molloy, J. McPartlin, R. Corbally, A.S. Whitehead, J.M. Scott and D.G. Weir; Increased Prevalence of Methylenetetrahydrofolate Reductase C677T Variant in Patients with Inflammatory Bowel Disease, and its Clinical Implications (1999) Gut **45**; 389-94

U.S. Maitra and D.B. Sprinson; 5-Dehydro-3-Deoxy-D-*Arabino*-Heptulosonic Acid 7-Phosphate. An Intermediate in the 3-Dehydroquinate Synthase Reaction (1978) J. Biol. Chem. **253**; 5426-5430

K. Majumder, A Selvapandiyan, FA Fattah, N Arora, S Ahmad, and RK Bhatnagar ; 5-Enolpyruvylshikimate-3-phosphate synthase of Bacillus subtilis is an allosteric enzyme. Analysis of Arg24→Asp, Pro105→Ser and His385→Lys mutations suggests a hidden phosphoenolpyruvate-binding site (1995) Eur. J. Biochem. **229**: 99-106

M.A. Mansoor, O. Kristensen, T. Hervig, P.A. Drabløs, J.A. Strakkestad, L. Woie, Ø. Hetland and A. Osland; Low Concentrations of Folate in Serum and Erythrocytes of Smokers: Methionine Loading Decreases Folate Concentrations in Serum of Smokers and Nonsmokers (1997) Clin. Chem. **43**; 2192-4

M. Manzoor and J. Runcie; Folae-Responsive Neuropathy: Reports of 10 Cases (1976) BMJ **1**; 1176-8

K. Marcker; The Formation of N-formyl-methionyl-sRNA (1965) J. Mol. Biol. **14**; 63-70

L.H. Matherly and S.P. Muench; Evidence for a Localized Conversion of Endogenous Tetrahydrofolate Cofactors to Dihydrofolate as an Important Element in Antifolate Action in Murine Leukemia Cells (1990) Biochem. Pharmacol. **39**: 12; 2005-14

C.K. Mathews and K.E. van Holde; Chapter 22: Nucleotide Metabolism. Deoxyribonuclotide Biosynthesis and Metabolism; In: *Biochemistry* (1990) Benjamin / Cummings Pub. Co. Redwood City, Ca.

J.B. Mathis and G.M. Brown; The Biosynthesis of Folic Acid. XI. Purification and Properties of Dihydroneopterin Aldolase (1970) J. Biol. Chem. **245**; 3015-25

R.G. Matthews, C. Ghose, J.M. Green, K.D. Matthews and R.B. Dunlap; Folypolyglutamates as Substrates and Inhibitors of Folate-Dependent Enzymes (1987) Adv. Enzyme Regul. **26**; 157-71

D.A. Matthews, J.T. Bolin, J.M. Burridge, D.J. Filman, K.W. Volz and J. Kraut; Dihydrofolate reductase. The stereochemistry of inhibitor selectivity. (1985) J. Biol. Chem. **260**; 292-9

M.W. McBurney and G.F. Withmore; Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells (1974) Cell **2**; 173-182

G.A. McConkey; Targeting the Shikimate Pathway in the Malaria Parasite (1999) Antimicrob. Agents Chemother. **43**; 175-7

K.S. McCully; Vascular Pathology of Homocysteine: Implications for the Pathogenesis of Artheriosclerosis (1969) Am. J. Pathol. **56**; 111-28

P. McGing, B. Reed, D.G. Weir and J.M. Scott; The Effect of Vitamin B12 Inhibition *In Vivo*: Impaired Folate Polyglutamate Biosynthesis Indicating that 5-Methyltetrahydropteroylglutamate is not is usual Substrate. (1978) Biochem. Biophys. Res. Commun. **82**; 2: 540-6

J.J. McGuire and J.K. Coward; Pteroylpolyglutamates: Biosynthesis, Degradation, and Function. In: Folates and Pteridines (R.L. Blakley and S.J. Benkovic, Editors) John Wiley and Sons, New York (1984) Vol. 1, pp135-190

K. McMartin; Increased Urinary Folate Excreation and Decreased Plasma Folate Levels in the Rat After Acute Ethanol Treatment (1984) Alcohol. Clin. Exp. Res. **8**; 172-8

H. McNulty, J. McPartlin, D. Weir and J. Scott; Reverse phase HPLC method for the quantitation of endogenous folatecatabolites in rat urine (1993a) J. Chromatography **614**; 59-66

H. McNulty, J.M. McPartlin, D.G. Weir and J.M. Scott; Folate Catabolism is Increased During Pregnancy in Rats (1993b) J. Nutr. **123**; 1089-93

H. McNulty, J.M. McPartlin, D.G. Weir and J.M. Scott; Folate Catabolism is Related to Growth Rate in Weanling Rats (1995) J. Nutr. **125**; 99-103

J.M. McPartlin, G. Cortney, H. McNulty, D.G. Weir and J.M. Scott; The quantitative analysis of endogenous folate catabolites in human urine; (1992) Analytical Biochem. 206, 256-61

J.M. McPartlin, A. Halligan,J.M. Scott, M. Darling and D.G. Weir; Accelerated folate breakdown in pregnancy; (1993) Lancet **341**; 148-9

Merck Index, 12th Edition; Monograph No. 4253 "Folic Acid"; S.Budavari, Ed. ; Merck Research Labs., New Jersey.

Merck Index, 12th Edition; Monograph No. 4254 "Folinic Acid"; S.Budavari, Ed. ; Merck Research Labs., New Jersey.

J. Metz; The Deoxyuridine Suppression Test (1984) CRC Crit. Rev. Clin. Lab. Sci, **20**; 205

M.J. Meynell; Megaloblastic Anæmia in Anticonvulsant Therapy (1966) Lancet **1**; 487

J.L. Mills, J.M. McPartlin, P.N. Kirke et al; Homocysteine Metabolism in Birhs Complicated by Neural Tube Defects (1995) Lancet **345**; 149-51

V. Mims, J.R. Totter and P. Day; A method for the Determination of Substances Enzymattically Convertible to the Factor Stimulating Streptococcus Lactus R (1944) J. Biol. Chem. **155**; 401-405

H.K. Mitchell, E.E. Snell and R.J. Williams; The Concentration of "Folic Acid"; Journal of the American Chemical Society (1941) **63**; 2284

S. Mitsuhashi and B.D. Davis; Aromatic Biosynthesis. XII. Conversion of 5-dehydroquinic Acid to 5-Dehydroshikimic Acid by 5-Dehydroquinase (1954) Biochim. Biophys. Acta **15**; 54-61

C.D. Mol, C-F. Kuo, M.M. Thayer, R.P. Cunningham and J.A. Tainer; Structure and Function of the Multifunctional DNA-Repair Enzyme Exonuclease III (1995) Nature **374**; 381-6

A.M. Molloy, D.G. Weir, D.G. Kennedy, S. Kennedy, and J.M. Scott; A New High Performance Liquid Chromatographic Method for the Simultaneous Measurement of S-Adenosylmethionine and S-Adenosylhomocysteine: Concentrations in Pig Tissue after Inactivation of Methionine Synthase by Nitrous Oxide (1990) Biomed. Chromatog. **4**: 6; 257-9

A.M. Molloy and J.M. Scott; Microbiological Assay for Serum, Plasma and Red Cell Folate Using Cryopreserved, Microtiter Plate Method (1997) Methods in Enzymology **281**; 43-53

A.M. Molloy, D.G. Weir, D.G. Kennedy, S. Kennedy, and J.M. Scott; A New High Performance Liquid Chromatographic Method for the Simultaneous Measurement of S-Adenosylmethionine and S-Adenosylhomocysteine: Concentrations in Pig Tissue after Inactivation of Methionine Synthase by Nitrous Oxide (1990) Biomed. Chromatog. **4**: 6; 257-9

A.M. Molloy, B. Orsi, D.G. Kennedy, S. Kennedy, D.G. Weir and J.M. Scott; The Relationship between the Activity of Methionine Synthase and the Ratio of S-Adenosylmethionine to S-Adenosylhomocysteine in the Brain and other Tissues of the Pig (1992) Biochem Pharmacol **44**: 7; 1349-55

A.M. Molloy, D. Ramsbottom, J. McPartlin, A.S. Whitehead, D.G. Weir, J.M. Scott, *et al*; 5,10-Methylenetetrahydrofolate Reductase C677T Genotypes and Folate Related Risk Factors for Neural Tube Defects (1997) In: *Chemistry and Biology of Pteridines and Folate* (W. Pfleiderer and H. Rokos, eds) Blackwell Science, Berlin pp291-6

R.G. Moran, M. Mulkins and C. Heidelberger; Role of Thymidylate Synthetase Activity in Development of Methotrexate Cytotoxicity (1979) Proc. Nalt. Acad. Sci. **76**: 11; 5924-8

A.A. Morollo, and R Bauerle; Characterization of Composite Aminodeoxyisochorismate Synthase and Aminodeoxyisochorismate Lyase Activities of Anthranilate Synthase (1993) PNAS **90**: 9983-9987.

Y. Motokawa and G. Kikuchi; Glycine Metabolism in Rat Liver Mitocondria. V. Intramitocondrial Localization of the Reversible Glycine Cleavage System and Serine Hydroxymethyltransferase (1971) Arch. Biochem. Biophys. **146**; 461-4

MRC Vitamin Research Group; Prevention of Neural Tube Defects: Results of the Medical Research Council Vitamin Study (1991) **338**; 131-7

M. Murphy and J.M. Scott; The Turnover, Catabolism and Excretion of Folate Administered at Physiological Concentrations in the Rat (1976) Biochem. Biophys. Acta 583,535-9

M. Murphy, M. Keating, P. Boyle, D.G. Weir, J.M. Scott; The Elucidation of the Mechanism of Folate Catabolism in the Rat (1976a) Biochem. Biophys. Res. Commun. 71, 1017-24

M. Murphy, P. Boyle, D.G. Weir and J.M. Scott; The Identification of the Products of Folate Catabolism in Rats (1976b) Br. J. Haematol. **84**; 211-8

T. Nagatsu, M. Sawada, M. Akino, M.Masada, T. Sugimoto and S. Matsuura; Distribution of GTP Cyclohydrolase I, Neopterine, and Biopterin in the Human Brain (1986) In: *Chemistry and Biology of Pteridines* (Eds: B.A. Cooper and V.M. Whitehead) Walter de Gruyter, Berlin. Pp223-6

X. Nan, H-H. Ng, C.A. Johnson, C.D. Laherty, B.M. Turner, R.N. Eisenman and A. Bird; Transcriptional Repression by the Methyl-CpG-Binding Protein MeCP2 Involves a Histone Deacetylation Complex (1998) Nature **393**; 386-9

H. Nar, R. Huber, G. AuerbachM. Fischer, C. Hösl, H. Ritz, A. Bracher, W. Meining, S. Eberhardt and A. Bacher; Active Sight Topology and Reaction Sight Mechanism of GTP Cyclohydrolase I (1995) Proc. Natl. Acad. Sci. USA **92**; 12120-5

National Research Council; Recommended Dietary Allowances (1989) National Acadamy Press, Washington D.C.

L. Ni, K. Guan, H. Zalkin, J.E. Dixon; De Novo Purine Nucleotide biosynthesis: Cloning, Sequencing and Expression of Chicken *PurH* cDNA Encoding 5-Aminoimidazole-4-Carboxamide Ribonucleotide Transformylase-IMP cyclohydrolase (1991) Gene **106**; 197-205

B.P. Nichols, A.M. Seibold and S.Z. Doktor; para-Aminobenzoate Synthesis from Chorismate Occurs in Two Steps (1989) J. Biol. Chem. **264**; 8597-601

D. Niethammer and R.C. Jackson; Transport of Folate Compounds Through the Membrane of Lymphoblastoid Cells (1975) In *Chemostry and Biology of Pteridines* (W. Pfleidered, ed.) Walter de Gruyter, Berlin pp197-207

R.H. Nimmo-Smith, J. Lascelles and D.D. Woods; The Synthesis of "Folic Acid" by Streptobacterium Planatarum and its Inhibition by Sulphonamides (1948) Br. J. Exp. Pathol. **29**; 264-81

G.P. Oakley; Let's Increase Folic Acid Fortification and Include Vitamin B-12 (1997) Am. J. Clin. Nutr. **65**; 1889-90

G.P. Oakley; Folic Acid Fortification (1999) N. Engl. J. Med. **341**; 922-4

K.L. Oden and S. Clarke; S-Adenosyl-L-methionine Synthase from Human Erythrocytes: Role in the Regulation of Cellular S-Adenosylmethionine Levels (1983) Biochemistry **22**: 12; 2978-86

M. Okano, S. Xie and E. Li; Cloning and Characterization of a Family of Novel Mammalian DNA (Cysteine-5) methyltransferases (1998) Nature Genet. **19**; 219-20.

M.B. Oliveira, A.P. Campello and W.L. Kloppel; Methotrexate: Studies on Cellular Metabolism. III> Effect on the Transplasma-Membrane Redox Activity and On Ferricyanide-Induced Proton Extrusion by HeLa Cell (1989) Cell. Biochem. Funct. **7**; 135-7

P.J. Ortiz ; Dihydrofolate and Dihydropteroate Synthesis by Partially Purified Enzymes from Wild-Type and Sulfonamide-Resistant *Pneumococcus* (1970) **9**: 2; 355-61

P.J. Ortiz and R.D. Hotchkiss; The Enzymic Synthesis of Dihydrofolate and Dihydropteroate in Cell-Free Preperations From Wild-Type and Sulfonamide-Resistant *Pneumococcus* (1966) Biochemistry **5**: 1; 67-73

C.B. Osborne, K.E, Lowe and B. Shane; Regulation of Folate Metabolism and One-carbon Metabolism in Mammalia Cells. (1993) J. Biol. Chem. **268**; 29: 21657-64

N. Pancharuniti, C.A. Lewis, H.E. Sauberlich, L.L. Perkins, R.C.P. Go, *et al.*; Plasma Homocyst(e)ine, Folate, and Vitamin B-12 Concentrations and Risk from Early-Onset Coronary Artery Disease (1994) Am. J. Clin. Nutr. **59**; 940-8

C. Papapetrou, S.A. Lynch, J. Burns and Y.H. Edwards; Methylenetetrahydrofolate Reductase and Neural Tube Defects (1996) Lancet **348**; 58

M.L. Pato and G.M. Brown; Mechanism of Resistance of *Eschericia coli* to Sulfonamides (1963) Arch. Biochem. Biophys. **103**; 443-8

T-T. Pelliniemi and W. Beck; Biochemical Mechanisms in the Kellmann Experiment: Critique of the Deoxyuridine Suppression Test (1980) J. Clin. Invest. **65**; 449-60

B.W.J. H. Penninx, J.M. Guralnik, L. Ferrucci, L.P. Fried, R.H. Allen and S.P. Stabler; Vitamin B_{12} Deficiency and Depression in Physically Disabled Older Women: Epidemiologic Evidence From the Women's Health and Aging Study (2000) Am. J. Psychiatry **157**; 715-21

J. Perry and I. Chanarin; Intestinal Absorption of Reduced Folate Compounds in Man; (1970) Br. J. Haematology **18**; 329-39

J. Perry, M. Lamb, M. Laundy E. Reynolds and I. Chanarin; Role of Vitamin B12 in Folate Coenzyme Synthesis; (1976) Br. J. Haematol. **32**; 243-8

J.H. Pincus, E.H. Reynolds and G.H. Glasner; Subacute Combined System Degeneration With Folate Deficiency (1972) JAMA **221**; 496-7

J. Plowman, J.E. Cone and G. Guroff; Identification of D-*erythro*-Neopterin Triphosphate, the First Product of Pteridine Biosynthesis in *Comamonas* Specie (ATCC 11299a) (1974) J. Biol. Chem. **249**; 5559-64

M. Poe; Antibacterial Synergism: A proposal for Chemotherapeutic Potentiation Between Trimethoprim and Sulfamethoxazole (1976) Science **194**; 533-5

I.P. Pogribny, B. Miller and S.J. James; Alterations in Hepatic p53 Gene Methylation Patters During Tumor Progression with Folate/ Methyl Deficiency in the Rat (1997a) Cancer Letters **115**; 31-38

I.P. Pogribny, L. Muskhelishvili, B. Miller and S.J. James; Presence and Consequence of Uricil in Preneoplastic DNA from Folate/Methyl-Deficient Rats (1997b) Carcinogenesis **18**; 2071-6

V.I. Polshakov, B. Birdsall and J. Feeney; Characterisation of Rates of Ring-Flipping in Trimethoprim in Its Ternary Complexes with *Lactobacillus casei* Dihydrofolate Reductase and Co-enzyme Analogues (1999) Biochemistry **38**; 15962-9

D.J.T. Porter and F.L. Boyd; Mechanism of Liver S-Adenosylhomocysteine Hydrolase: Steady-State and Pre-Steady-State Kinetic Analysis (1991) J. Biol. Chem. **266**: 32; 21616-25

C. Poulsen, RJ Bongaerts, and R Verpoorte; Purification and characterization of anthranilate synthase from Catharanthus roseus (1993) Eur. J. Biochem. **212**: 431-440

C. Prasad and R.M. Edwards; Synthesis of Phosphatidylcholine from Phosphatidylethanolamine by at Least Two Methyltransferases in Rat Pituitary Extracts (1981) **256**: 24; 13000-3

Public Health Service; Recommendations for Use of Folic Acid to Reduce the Number of Cases of Spina Bifida and Other Neural Tube Defects (1992) Morb. Mortal. Wkly. Rep. **41**: (RR-141); 1-7

G. Qu, P.E. Grundy, A. Narayan and M. Ehrlich; Frequent Hypomethylation in Wilms Tumors of Pericentromeric DNA in Chromosome 1 and 16 (1999) Cancer Genet. Cytogenet. **109**; 34-9

J.I. Rader, D. Neithammer and F.M. Huennekens; Effects of Sulphydryl inhibitors upon Transport of Folate Compounds into L1210 Cells (1974) Biochem Pharmacol. **23**; 2057-9

A. Rajkovic, P.M. Catalano and M.R. Manilow; Elevated Homocysteine Levels with Preeclempsia (1997) Obstet. Gynecol. **90**; 168-71

A. Razin; CpG Methylation, Chromatin Structure and Gene Silencing - a Three-Way Connection (1998) EMBO J. **17**; 4905-8

J.J. Reynolds and G.M. Brown; The Biosynthesis of Folic Acid. IV. Enzymatic Synthesis of Dihydrofolic Acid from Guanine and Ribose Compounds (1964) J. Biol. Chem. **239**; 317-25

E.H. Reynolds, P. Rothfeld and J.H. Pincus; Neurological Disease Associated With Folate Deficiency (1973) BMJ **2**; 398-400

R.M.E. Richards and D.K.L. Xing; Separation and Quantification of Murein and Precursors from *Enterobacter cloacae* After Treatment with Trimethoprim and Sulphadiazine (1994a) J. Pharm. Pharmicol. **46**; 690-6

R.M.E. Richards and D.K.L. Xing; Capillary Zone Electrophoresis Assay of the Uridine Diphosphate N-Acetylmuramyl Peptide Precursors and Disaccharide Pentapeptide Derivative of Bacterial Cell Wall Peptidoglycan. (1994b) J. Pharm. Biomed. Anal. **3**; 305-10

R.M.E. Richards, D.K.L. Xing and T.P. King; Activity of p-Aminobenzoic Acid Compared with Other Organic Acids Against Selected Bacteria (1995a) J. Applied Bacteriol. **78**; 209-15

R.M.E. Richards, J.Z. Xing, D.W. Gregory and D. Marshall; Mechanism of Sulphadiazine Enhancement of Trimethoprim Activity Against Sulphadiazine-Resistant Enterococcus faecalis (1995b) J. Antimicrob. Chemother. **36**; 607-18

R.M.E. Richards, R.B. Taylor and Z.Y. Zhu; Mechanism for Synergism between Sulfonamides and Trimethoprim Clarified (1996) J. Pharm. Pharmacol. **48**; 981-4

D.P. Richey and G.M. Brown; The Biosynthesis of Folic Acid. IX. Purification and Properties of the Enzyme Required for the Formation of Dihydropteroic Acid (1969) J. Biol, Chem. **244**; 1582-92

L.J. Riddell, A. Chisholm, S. Williams and J.I. Mann; Dietary Strategies for Lowering Homocysteine Concentrations (2000) Am. J. Clin. Nutr. **71**; 1448-54

R.G. Ridley; Planting New Targets for Antiparasitic Drugs (1998) Nature Medicine **4**; 894-5

E.B. Rimm, M.J. Stampfer, A. Ascherio, E. Giovannucci and W.C. Willett; Dietary Folate, Vitamin B6, and Vitamin B12 Intake and Risk of CHD Among a Large Population of US Men (1996) Circulation **93**; 625

E.B. Rimm, W.C. Willett, F.B. Hu, L. Sampson, G.A. Colditz, J.E. Mason, C. Hennekens and M.J. Sampfer; Folate and Vitamin B_6 From Diet and Supplements in Relation to Risk of Coronary Heart Disease Among Women (1998) JAMA **279**; 359-64

A. Rivera and P.R. Srinivansan; The Role of 3-Enolpyruvylshikimate 5-Phosphate in the Biosynthesis of Anthranilate(1963) Biochemistry **2**; 1063-9

F. Roberts, C.W. Roberts, J.J. Johnson, D.E. Kyle, T. Krell, et al; Evidence for the Shikimate Pathway in Apicomplexan Parasites (1998) Nature **393**; 801-5

G.C.K. Roberts, J. Feeney, A.S.V. Burgen, V. Yuferov, J.G. Dann and R. Bjur; Nuclear Magnetic Resonance Studies of the Binding of Substrate Analogues and Coenzyme to Dihydrofolate Reductase from *Lactobcillus casei* (1974) Biochemistry **13**:26; 5351-7

L.J. Roberts; Knowledge and Use of Periconceptual folic Acid Supplements by British Forces Germany Presonnel and Dependents (1996) J. R. Army Med.Corps **142**; 116-9

W. Rode, K.J. Sanlon and J.R. Bertino; thymidylate Synthase (E.C. 2.1.1.45) from Mouse Leukemia Cells – Cell Cycle Pattersn and Affinity Chromatography Purification (1979) In *Chemistry and Biology of Pteridines* (R.L. Kisliuk and G.M. Brown, eds.) Elsevier / North-Holland, New York pp489-94

G.M. Rodgers and M.T. Conn; Homocysteine, an Atherogenic Stimulus, Reduces Proteine C Activation by Artherial and Venous Endothelial Cells (1990) Blood **75**: 4; 895-901

G.M. Rodgers and W.H. Kane; Activation of Endogenous Factor V by a Homocysteine-induced Vascular Endothelial Cell Activator (1986) J. Clin. Invest. **77**; 1909-16

I. Rogers, P. Emmett, D. Barker, J. Golding; Financial Difficulties, Smoking Habits, Composition of the Diet and Birthweight in a Population of Pregnant Women in the South West of England. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood (1998) Eur. J. Clin. Nutr. **52**; 251-60

T.E. Rohan, M.G. Jain, G.H. Howe and A.B. Miller; Dietary Folate Consumption and Breast Cancer Risk (2000) J. Natl. Cancer Inst. **92**; 266-9

S. Roland, R. Ferone, R.J. Harvey, V.L. Styles and R.W. Morrison; The Characteristics and Significance of Sulfonamides as Substrates for *Escherichia coli* Dihydropteroate Synthase (1979) J. Biol. Chem. **254**: 20; 10337-45

J. Rolschau, K. Kristoffersen, M. Ulrich, P. Grinsted, E. Schaumberg and N. Foger; The Influence of Folic Acid Supplementation on the Outcome of Pregnancies in the County of Funen in Denmark. Part I (1999) Eur. J. Obstet. Gynecol. Reprod. Biol. **87**; 105-10

I.R. Rosenberg; Intestinal Absorption of Folate; (1981) In: *Physiology of the Gastrointestinal Tract* (L.R. Johnson, ed) Raven Press, New York pp 12211-30

D.S. Rosenblatt, V.M. Whitehead, M.M. Dupont, M-J. Vuchich and N. Vera; Sythesis of Methotrexate Polyglutamates in Cultured Human Cells (1978a) Mol. Pharmacol. **14**; 210-4

D.S. Rosenblatt, V.M. Whitehead, N. Vera, A. Pottier, M.M. Dupont and M-J. Vuchich; Prolonged Inhibition of DNA Synthesis Associated with the Accumulation of Methotrexate Polyglutamates by Cultured Human Cells (1978b) Mol. Pharmacol. **14**; 1143-7

D.S. Rosenblatt; Folate and Homocysteine Metabolism and Gene Polymorphism in the Ethiology of Down Syndrome (1999) Am. J. Clin. Nutr. **70**; 429-30

P.B. Rowe; Folates in the Biosynthesis and Degradation of Purines (1984) In *Folates and Pteridines Vol 1 Chemistry and Biochemistry of Folates* (R.L. Blakely and S.J. Benkovic, eds.) John Wiley and Sons, New York pp 329-44

H. Rüdiger and L. Jaenicke; Methionine Synthesis: Demonstration of the Reversibility of the Reaction (1969) FEBS Letters **4**: 4; 316-8

D.V. Santi and P.V. Danenberg; Folates in Pyrimidine Nucleotide Biosynthesis (1984) In: *Folates and Pteridines, Vol 1: Chemistry and Biochemistry of Folates* (R.L. Blakley and S.J. Benkovic, eds.) John Wiley and Sons, New York. Pp345-98

M. Sasvári Székely , M. Staub, A. Guttmann, V. Törcsvári and F. Antoni ; Pyrimidine Salvage Enzymes in Human Tonsil Lymphocytes. I. Seperation and Characterisation of Thymidine Kinase Isoenzymes (1985) Acta Biochim. Biophys. Acad. Sci. Hung. **20**; 163-72

H.E. Sauberlich, M.J. Kretsch, J.H. Skala, H.L. Johnson and P.C. Taylor; Folate Requirement and Metabolism in Nonpregnant Women (1987) Am. J. Clin. Nutr. **46**; 1016-28

E. Schmitt, M. Panvert, S. Blanquet and Y. Mechulam; Crystal Structure of Methionyl-$tRNA_f^{Met}$ Transformylase Complexed With the Initiator Formyl-Methionyl-$tRNA_f^{Met}$ (1998) EMBO J. **17**; 6819-26

T.O. Scholl and W.G. Johnson; Folic Acid: Influence on the Outcome of Pregnancy (2000) Am. J. Clin. Nutr. **71** (suppl.); 1295S-303S

R. Schoner and K.M. Herrman; 3-Deoxy-D-*Arabino*-Heptulosonic 7-Phosphate Synthetase (1976) J. Biol. Chem. **251**; 5440-7

C.J. Schwartzbach and L.L. Spremulli; Bovine Mitocondrial Protein Synthesis Elongation Factors. Identification and Initial Characterisation of an Elongation Factor Tu-Elongation Factor TS Complex (1989) J. Biol. Chem. **264**; 19125-31

R.L. Seither, D.F. Trent, D.C. Mikulecky, T.J. Rape and I.D. Goldman; Effect of Direct Suppression of Thymidylate Synthase at the 5,10-Methylenetetrahydrofolate Binding Site on the Interconversion of Tetrahydrofolate Cofactors to Dihydrofolate by Antifolates: Influence of Degree of Dihydrofolate Reductase Inhibition (1991) **266**: 7; 4112-8

R.L. Seither, T.J. Rape and I.D. Goldman; Interconversion of Tetrahydrofolate Cofactors to Dihydrofolate Induced by Trimetrexate after Suppression of Thymidylate Synthase by Fluorodeoxyuridine in L1210 Leukemia Cells (1992) Biochem. Pharmacol. **43**: 12; 2647-54

J. Selhub, P.F. Jacques, A.G. Bostom, R.B. D'Agostino, P.W.F. Wilson, A.J. Belanger, D.H. O'Leary, P.A. Wolf, E.J. Schaefer and I.H. Rosenberg; Association Between Plasma Homocysteine Concentrations and Extracranial Carotid-Artery Stenosis (1995) New Engl. J. Med. **332**; 286-91

E. Seyoum and J. Selhub; Properties of Food Folates Determined by Stability and Susceptibility to Intestinal Pteroylpolyglutamate Hydrolase Action (1998) J. Nutr. 128; 1956-60

B. Shane; Pteroylpoly(γ-glutamate) Synthesis by *Corynebaterium* Species: Purification and Properties of Folylpoly(γ-glutamate) Synthetase; (1980a) J. Biol. Chem. **255**: 12; 5655-62

B. Shane; Pteroylpoly((γ-glutamate) Synthesis by *Corynebacterium* Species. Studies on the Mechanism of Folypoly((γ-Glutamate) Synthetase. (1980b) J. Biol. Chem. **255**; 5663-7

B. Shane; Folypolyglutamate Synthesis and Role in the Regulation of One-Carbon Metabolism (1989) Vit. Hormones **45**; 263-335

B. Shane, J. Watson and E. Stokstad; Uptake and Metabolism of [^{3}H]Folate by Normal and by Vitamin B12 and Methionine-Deficient Rats; (1977) Biochim. et Biophys. Acta **497**; 241-52

G.M. Shaw, C.D. O'Malley, C.R. Wasserman, M.M. Tolarova and E.J. Lammer; Maternal Periconceptional Use of Multivitamins and Reduced Risk for Conotruncal Heart Defects and Limb Deficiencies Among Offspring (1995) Am J. Med. Genet. **59**; 536-45

Z. Shemesh, J. Attias, M. Ornan, N. Shapira and A. Shahar; Vitamin B12 Deficiency in Patients with Chronic-Tinnitus and Noise-Induced Hearing Loss (1993) Am. J. Otolaryngol. **14**; 94-9

T. Shiota and M.N. Disraely; The Enzymic Synthesis of Dihydrofolate from 2-Amino-4-Hydroxymethyldihydropterine and *p*-Aminobenzoylglutamate by Extracts of *Lactobacillus plantarum* (1961) Biochim. Biophys. Acta **52**; 455-66

T. Shiota; Enzymic Synthesis of Folic Acid-Like Compounds by Cell Free Extracts of *Lactobacillus arabinosus* (1959) Arch. Biochem. Biophys. **80**; 155-61

T. Shiota; Biosynthesis of Folate from Pterin Precursors (1984) In: *Folates and Pteridines, Vol 1: Chemistry and Biochemistry of Folates* (R.L. Blakely and S.J. Benkovic, eds.) John Wiley and Sons, New York. pp121-34

T. Shiota, M.N. Disraely and M.P. McCann; The Enzymatic Synthesis of Folate-Like Compounds from Hydroxymethyldihydropteridine Pyrophosphate (1964) J. Biol. Chem. **239**; 2259-66

T. Shiota and M.P. Palumbo; Enzymatic Synthesis of the Pteridine Moiety of Dihydrofolate from Guanine Nucleotides (1965) J. Biol. Chem. **240**; 4449-53

J. Silberberg, R, Crooks, J. Fryer, C. Ray, X. Guo, L. Xie and N. Dudman; Age, Homocyst(e)ine and Hormone Replacement Therepy: The Elite Seniors Study (1995) Irish J. Medical Science **164**: 15S; 18

R.J. Simpson and B.E. Davidson; Studies on 3-Deoxy-D-*Arabino*-Heptulosonic-7-Phosphate Synthetase (phe) from *Escherichia coli* K-12 (1976) Eur. J. Biochem. **70**; 501-7

C.F. Skibola, M.T. Smith, E. Kane, E. Roman, S. Rollinson, R.A. Cartwright and G. Morgan; Polymorphisms in the Methylenetetrahydrofolate Reductase Gene are Associated with Susceptibility to Acute Leukemia in Adults (1999) Proc. Natl. Acad. Sci. USA **96**; 12810-5

G. Slupphaug, C.D. Mol, B. Kavli, A.S. Arvai, H.E. Krokan and J.A. Tainer; A Nuceotide-Flipping Mechanism from the Structure of Human Uracil-DNA Glycosylase Bound to DNA (1996) Nature **384**; 87-92

C.C. Smart, D. Johanning, G. Muller and N. Amrhein; Selective Overproduction of 5-Enolpyruvylshikimic Acid 3-Phosphate Synthase in a Plant Cell Culture Which Tolerates High Doses of the Herbicide Glyphosate (1985) J. Biol. Chem **260**; 16338-46

G.K. Smith, P.A. Benkovic and S.J. Benkovic; L(-)-10-Formytetrahydrofolate Is the Cofactor for Glycinamide Ribonucleotide Transformylase from Chicken Liver (1981) Biochemistry **20**; 4034-6

G.K. Smith, W.T. Mueller, G.F. Wasserman, W.D. Taylor and S.J. Benkovic; Characterization of the Enzyme Complex Involving the Folate-Requiring Enzymes of de Novo Purine Biosynthesis (1980) **19**; 4313-21

G.K. Smith, W.T. Mueller, P.A. Benkovic and S.J. Benkovic; On the Cofactor Specificity of Glycinamide Ribonucleotide and 5-Aminoimidazole-4-Carboxamide Ribonucleotide Transformylase from Chicken Liver (1981) Biochemistry **20**; 1241-5

R.W. Smithells, N.C. Nevin, M.J. Seller, S. Sheppard, A.P. Reid, D.W. Field, S. Walker, C.J. Schorah and J. Wild; Further Expericence of Vitamin Supplementation for Prevention of Neural Tube Defect Recurrences (1983) Lancet **1**: 8332; 1027-31

R.W. Smithells, S. Sheppard, C.J. Shorah, M.J. Seller, N.C. Nevin R. Harris, A.P. Reid and D.W. Field; Possibe Prevention of Neural-Tube Defects by Periconceptional Vitamin Supplementation (1980) Lancet **1**: 8164; 339-40

D.A. Snowdon, C.L. Tully, C.D. Smith, K.P. Riley and W.R. Markesbery; Serum Folate and the Severity of Atrophy of the Neocortex in Alzheimer Disease: Findings From the Nun Study (2000) Am. J. Clin. Nutr. **71**; 993-8

D.B. Sprinson, P.R. Srinivansan and J. Rothschild; 5-Dehydroquinate Synthase (1962) Methods in Enz. **5**; 398-402

P.R. Srinivansan and D.B. Sprinson; 2-Keto-3-deoxy-D-*arabino*-heptonic acid 7-Phosphate Synthetase (1959) J. Biol. Chem. **234**; 716-22

P.R. Srinivansan and A. Rivera; The Enzymatic Synthesis of Anthranilate from Shikimate 5-Phosphate and l-Glutamine (1963) Biochemistry **2**; 1059-62

P.R. Srinivansan, M. Katagiri and D.B. Sprinson; The Enzymatic Synthesis of Shikimic Acid from D-Erythrose-4-Phosphate and Phosphoenolpyruvate (1955) J. Am. Chem. Soc. **77**; 4943-4

P.R. Srinivansan, M. Katagiri and D.B. Sprinson; The Conversion of Phosphoenolpyruvic Acid and D-Eryhtrose 4-phosphate to 5-Dehydroquinic Acid. (1959) J. Biol. Chem **234**; 713-5

P.R. Srinivansan, J. Rothschild and D.B. Sprinson; The Enzymic Conversion of 3-Deoxy-D-*Arabino*-Heptulosonic Acid 7-Phosphate to 5-Dehydroquinate (1963) J. Biol. Chem. **238**; 3176-82

D.K. Stammers, J.N. Champness, C.R. Beddell, J.G. Dann, E. Eliopoulos, A.J. Geddes, D. Ogg and A.C.T. North; The Structure of Mouse L1210 Dihydrofolate Reductase-Drug Complexes and the Construction of a Model of Human Enzyme (1987) FEBS Letters **218**: 1; 178-84

R. Steegers-Theunissen, G.H. Boers, H.J. Blom, F.J. Trijbels, T.K Eskes; Hyperhomocysteinaemia and recurrent spontaneous abortion or abruption of placentae (1992) Lancet 339:1122-3

R. Stein, A. Razin and H.Cedar; In Vitro Methylation of the Hamster Adenosine Phosphoribosyltransferase Gene Inhibits Its Expression in Mouse L Cells (1982) Proc. Natl. Acad. Sci. USA **79**; 3418-22

H.C. Steinrucken and N. Amrhein; 5-Enolpyruvylshikimate-3-Phosphate Synthase of Klebsiella pneumoniae 2: Inhibition by Glyphosate [N-(phosphonomethyl)glycine] (1984) Eur. J. Biochem. **143**; 351-7

D.I. Stirling and H. Dalton; Purification and Properties of an $NAD(P)^+$-Linked Formaldehyde Dehydrogenase from Methylococcus Capsulatus (Bath) (1978) J. Gen. Microbiol. **107**; 19-29

J.L. Stokes (1944) J. Bacteriol. **48**; 201-6

E.L.R. Stokstad, B.L. Hutchings, J.H. Mowat, J.H. Booth, C.W. Waller, R.B. Angier, J. Semb and Y SubbaRow; The Degradation of the Fermentation Lactobacillus casei Factor I (1948) J. Am. Chem. Soc. **70**; 5-9

R.Z. Stolzenberg-Solomon, D. Albanes, F.J. Nieto, T.J. Hartman, J.A. Tangrea, M. Rautalahti, J. Selhub, J. Virtamo, P.R. Taylor; Pancreatic Cancer Risk and Nutrition-Related Methyl-Group Availability Indicators in Male Smokers (1999) J. Natl. Cancer Inst. **91**; 535-41

S.R. Stone and J.F. Morrison; Mechanism of Inhibition of Dihydrofolate Reductase from Bacterial and Vertebrate Sources by Various Classes of Folate Analogues (1986) Biochim. Biophys. Acta **869**; 275-85

G. Sunder-Plassmann, M. Födinger, H. Buchmayer, M. Papagiannopoulos, J. Wojcik, et al; Effect of High Dose Folic Acid Therapy on Hyperhomocysteinemia in Hemodialysis Patients: Results of the Vienna Multicenter Study (2000) J. Am Soc. Nephrol. **11**; 1106-16

Y. Suzuki and G.M. Brown; The Biosynthesis of Folic Acid. XII. Purification and Properties of Dihydroneopterine Triphosphate Pyrophosphohydrolase (1974) J. Biol. Chem. **249**; 2405-10

S. Suzuki, T. Suga and Niinobe; Studies on Peroxisomes. IV. Intracellular Localization of $NADH_2$-Glyoxylate Reductase in Rat Liver (1973) J. Biochem. (Tokyo) **73**; 1033-8

G. Swedberg, S. Castensson and O. Sköld; Characterization of Mutationally Altered Dihydropteroate Synthase and Its Ability to Form a Sulfonamide Containing Dihydrofolate Analog; Jnl. Bacteriol. (1979) **137**: 1; 129-36

G. Swedberg, S. Ringertz and O. Scöld; Sulfonamide Resistance in *Streptococcus pyogenes* Is Associate with Differences in the Amini Acid Sequence of Its Chromosomal Dihydropteroate Synthase (1998) Antimicrob. Agents Chemother. **42**; 1062-7

M.R. Sweeney; PhD. Thesis (2000) University of Dublin, Dublin

H. Tabor and L. Wyngarden; The Enzymatic Formation of Formiminotetrahydrofolic Acid, 5,10-Methylenyltetrahydrofolic Acid, and 10-Formyltetrahydrofolic Acid in the Metabolism of Formiminoglutamic Acid (1959) J. Biol. Chem. **234**; 1831-46

N. Takeuchi, M. Kawakami, A. Omori, T. Ueda, L.L. Spremulli and K. Watanabe; Mammalian Mitochondrial Methionyl-tRNA Transformylase from Bovine Liver. Purification, Characterisation, and Gene Structure (1998) J. Biol. Chem. **273**; 15085-90

T.L. Talarico, I.J. Dev, W.S. Dallas, R. Ferone and P.H. Ray; Purification and Partial Characterization of 7,8-Dihydro-6-Hydroxymethylpterine-Pyrophosphokinase and Dihydropteroate Synthase from *Escherichia coli* MC4100 (1991) J. Bacteriol. **173**: 21; 7029-32

T. Tamura, Y.S. Shin, M.A. williams and E.L. Stokstad; Lactobacillus casei Response to Pteroylpolyglutamates; (1972) Analy. Biochem. **49**, 517-21

T. Tamura; Determination of Food Folates (1998) Nutritional Biochem **9**; 285-93

Y. Tani, H. Nishise, H. Morita and H. Yamada; Vitamin B_6 Biosynthesis and Glycolate Reductase in Flavobacterium sp. 238-7 (1984) J. Nutr. Sci. Vitaminol. (Tokyo) **30**; 415-20

L. Tao, S. Yang, M. Xi, P.M. Kramer and M.A. Pereira; Effect of Trichloroethylene and Its Metabolites, Dichloroacetic Acid and Trichloroacetic Acid, on the Methylation and Expression of c-Jun and c-Myc Protooncogenes in Mouse Liver: Prevention by Methionine (2000) Toxicol. Sci. **54**; 399-407

A. Tavani, E. Negri, C. La Vecchia; Food and Nutrient Intake and Risk of Cataract (1996) Ann. Epidemiol. **6**; 41-6

C-Y. Teng, B. Ganem, S.Z. Doktor, B.P. Nichols, R.K. Bhatnagar and L.C. Vining; (1985) J. Am. Chem. Soc. **107**; 5008-9

R. Then and P. Angehrn; Nature of the Batericidal Action of Sulfonamides and Trimethoprim, Alone and in Combination (1973) J. Infect. Diseases **128**; S498-501

R.L. Then; History and Future of Antimicrobial Diaminopyrimidines (1993) J. Chemother. **5**: 6;361-8

R.W. Thompson and C.L. Krumdieck; Time Course Study of the In Vitro Synthesis of Avian Liver Pteroylpoly-γ-glutamates; (1977) Am. J. Clin. Nutr. **30;** 1576-82

H.H. Ting and M.J. Crabbe; Bovine Lens Aldehyde Dehydrogenae. Kinetics and Mechanism. (1983) Biochem. J. **215**; 361-8

G.J. Tortora and S.R. Grabowski; "Principles of Anatomy and Physiology (7th Ed.)" (1993), HarperCollins College Publishers, New York.

J. Totter, V. Mims, P. Day; The possible existance of a microbial inactive "folic acid" like material possessing vitamin activity in rats; (1944) Science **100**, 153.

D.F. Trent, R.L. Seither and I.D. Goldman; Rate and Extent of Interconversion of Tetrahydrofolate Cofactors to Dihydrofolate after Cessation of Dihydrofolate Reductase Activity in Stationary *Versus* Log Phase L1210 Leukemia Cells (1991a) J. Biol. Chem **266**: 9; 5445-9

D.F. Trent, R.L. Seither and I.D. Goldman; Compartmentation of Intracellular Folates: Failure to Interconvert of Tetrahydrofolate Cofactors to Dihydrofolate in Mitocondria of L1210 Leukemia Cells Treated with Trimetrexate (1991b) Biochem. Pharmacol. **42**: 5; 1015-9

J.B. Ubbink, A. van der Merwe, W.J. Vermaak and R. Delport; Hyperhomocysteinemia and the Response to Vitamin Supplementation (1993) Clin. Investig. **71**; 993-8

J.B. Ubbink, W.J.H. Vermaak and S. Bissbort; Rapid High-Performance Chromatographic Assay for Total Homocysteine Levels in Human Serum (1991) J. Chromatog. **565**; 441-6

P.M. Ueland; Pharmacological and Biochemical Aspects of S-Adenosylhomocysteine and S-Adenosylhomocysteine Hydrolase (1982) Pharmacol. Rev. **34**: 3; 223-53

M. Ulrich, K. Kristoffersen, J. Rolschau, P. Grinsted, E. Schaumberg and N. Foger; The Influence of Folic Acid Supplementation on the Outcome of Pregnancies in the County of Funen in Denmark. Part III. Congenital Anomalies. An Observational Study (1999) Eur. J. Obstet. Gynecol. Reprod. Biol. **87**; 115-18

M.J. van der Mooren, M.G. Wouters, H.J. Blom, L.A. Schellekens, T.K. Eskes and R. Rolland; Hormone Replacement Therapy May Reduce High Serum Homocysteine in Postmenopausal Women (1994) Eur. J. Clin. Invest. **24**; 733-6

M.J. van der Mooren, M.G. Wouters, H.J. Blom, L.A. Schellekens, T.K. Eskes and R. Rolland; Homocysteine Concentrations May DecreaseDuring Postmenopausal Hormone Replacement Therapy (1995) Irish J. Medical Science **164**: 15S; 21

A.S.J. van Miert; The Sulfonamide-Diaminopyrimidine Story (1994) J. Vet. Pharmacol. Therap. **17**; 309-16

N.M.J. van der Put, R.P.M. Steegers-Theunissen, P. Frosst, F.J.M. Trijbel, T.K.A.B. Eskes, L.P. van den Heuvel, E.C.M. Mariman, M. den Heyer, R.Rozen and H.J. Blom; Mutated Methylenetetrahydrofolate Reductase as a Risk Factor for Spina Bifida (1995) Lancet **346**; 1070-1

E. van Schaftingen, J.P. Draye and F. van Hoof; Coenzyme Specificity of Mammalian Liver D-Glycerate Dehydrogenase (1989) Eur. J. Biochem. **186**; 355-9

L. Vardimon, A. Kressmann, H. Cedar, M. Maechler and Walter Doerfler; Expression of a Cloned Adenovirus Gene is Inhibited by In Vitro Methylation (1982) Proc. Natl. Acad. Sci. USA **79**; 1073-7

G. Varela-Moreiras and J. Selhub; Long-Term Folate Deficiency Alters Folate Content and Distribution Differently in Rat Tissues (1992) J. Nutr. **122**; 986-91

E. Vieira and E. Shaw; Th Utilization of Purines in the Biosynthesis of Folic Acid (1961) J. Biol. Chem. **236**; 2507-10

V.K. Viswanathan, J.M. Brown and B.P. Nichols; Kinetic Characterisation of 4-Amino 4-Deoxychorismate Synthase from *Eschericia coli* (1995) J. Bacteriol. **177**; 5918-23

S.E. Vollset, H. Refsum, L.M. irgens, B.M. Emblem, A. Tverdal, H.K. Gjessing, L.B.J. Monsen and P.M. Ueland; Plasma Total Homocysteine, Pregnancy Complecations, and Adverse Pregnancy Outcome: the Hordaland Homocysteine Study (2000) **71**; 962-8

E. Wainfan and L.A. Poirier; Methyl Groups in Carcinogenesis: Effects on DNA Methylation and Gene Expression (1992) Cancer Res. **52**; 2071S-7S

N.J. Wald, H.C. Watt, M.R. Law, D.G. Weir, J. McPartlin and J.M. Scott; Homocysteine and Ischemic Heart Disease (1998) Arch. Intern. Med. **158**; 862-7

C. Waller, A.A. Goldman, R.B. Angier, J.H. Boothe, B.L. Hutchings, J.H. Mowat and J. Semb; 2-amino-4-hydroxy-6-pteridinecarboxyaldehyde (1950) J. Am. Chem. Soc. **72**; 4630-3

C. Wang; J-M. Lin and E. Lazarides; Methylation 0f 70,000 Heat Shock Proteins in 3T3 Cells: Alterations by Arsenite Treatment, by Different Stages of Growth and by Virus Transformation (1992) Arch. Biochem. Biophys. **297**; 169-75

C. Wang, S. Song, L.B. Bailey and J.F. Gregory; Relationship Between Excretion of p-Aminobenzoylglutamate and Folate Status of Growing Rats (1994) Nutritional Res. **14**: 6; 875-84

M. Ward,H. McNulty, J. McPartlin, J.J. Strain, D.G. Weir and J.M. Scott; Plasma Homocysteine, a Risk Factor for Cardiovascular Disease, is Lowered by Physiological Doses of Folic Acid (1997) Q. J. Med. **90**; 519-24

L. Warren and J.M. Buchanan; Biosynthesis of the Purines: XIX. 2-Amino-*N*-Ribosylacemide 5'-Phosphate (Glycinamide Riboside) Transformylase (1957) J. Biol. Chem. **229**; 613-26

J. Watson and W.B. Castle; Nutritional Macrocytic Anaemia, Especially in Pregnancy. Response to a Substance in Liver Other than that Effective in Pernicious Anaemia (1946) Am. J. Med. Sci. **211**; 513-30

S. Waxman, J. Metz and V. Herbert; Defective DNA Synthesis in Human Megaloblastic Bone Marrow: Effects of Homocysteine and Methionine (1969) J. Clin. Invest. **48**; 284-9

M-M. Wei and J.F. Gregory; Organic Acids in Selected Foods Inhibit Intestinal Brush Border Pteroylpolyglutamate Hydrolase In Vitro: Potential Mechanism Affecting the Bioavailability of Dietary Polyglutamyl Folate (1998) J. Agric. Food Chem **46**; 211-219

D.G. Weir, P.G. McGing and J.M. Scott; Folate Metabolism, The Enterohipatic Circulation and Alcohol (1985) Biochem. Pharmacol. **34**; 1-7

R.A. Weisman and G.M. Brown; The Biosynthesis of Folic Acid. V. Characterisation of the Enzyme System that Catalyzes the Synthesis of Dihydropteroic Acid (1964) J. Biol. Chem. **239**; 326-31

B.F. Weller and R.J. Wells; Baillière's Nurses' Dictionary (1990) Baillière Trindall Ltd., London

J.C. White and I.D. Goldman; Methotrexate Resistance in an L1210 Cell Line Resulting from Increased Dihydrofolate Reductase, Decreased Thymidylate Synthase Activity, and Normal Membrane Transport (1981) J. Biol. Chem. **256**: 11; 5722-7

A.S. Whiltehead, P. Gallagher, J.L. Mills, P.N. Kirke, H. Burke, A.M. Molloy, D.G. Weir, D.C. Shields and J.M. Scott; A Genetic Defect in 5,10 Methylenetetrahydrofolate Reductase in Neural Tube Defects (1995) Q. J. Med **88**; 763-6

N. Whitehead, F. Reyner and J. Lindenbaum; Megaloblastic Changes in the Cervical Epithelium. Association With Oral Contraceptive Therapy and Reversal With Folic Acid (1973) JAMA **226**; 1421-4

D.E.L. Wilcken, X.L. Wang; Relevance to Spina Bifida of Mutated Methylenetetrahydrofolate Reductase (1996) Lancet **347**; 340

J. Wild, C.J. Schorah, K. Maude and M.I. Levene; Folate Intake in Young Women and Their Knowledge of Preconceptional Folate Supplementation to Prevent Neural Tube Defects (1996) Eur. J. Obstet. Gynecol. Reprod. Biol. **70**; 185-9

J.W. Williams, R.G. Duggleby, R. Cutler and J.F. Morrison; The Inhibition of Dihydrofolate Reductase by Folate Analogues: Structural Requirements for Slow- and Tight-Binding Inhibiton (1980) Biochem. Pharmacol. **29**; 589-95

L. Wills and A. Stewart; Experimental Anaemia in Monkeys with Special Reference to Macrocytic Nutritional Anemia (1935) **16**; 444

L. Wills; Treatment of "Pernicious anaemia of Pregnancy" and "Tropical Nemia" (1931) Br. Med. J. **1**; 1059

J.W. Wilson, C. W. Enns, J.D. Goldman, K.S. Tippett, S.J. Mickle, L.E. Cleveland and P.S. Chahil; Data Taables: Combined Results from USDA's 1994 and 1995 Continuing Survey of Food Intakes by Individuals and 1994 and 1995 Diet and Health Knowledge Survey. Table Set 5 [Online] (1997) ARS Food Survays Research Group. Available (under "Releases"): http://www.barc.usda.gov/bhnrc/foodsurvey/home.htm [accessed 2000, 21st June]

A.J. Wittwer and C. Wagner; Identification of the Folate Binding Protein of Mitocondria as Dimethylglycine Dehydrogenase (1980) Proc. Natl. Acad. Sci. USA **77**; 4484-8

S.F. Wolf and B.R. Migeon; Clusters of CpG Dinucleotides Implicated by Nuclease Hypersensitivity as Control Elements of Housekeeping Genes (1985) Nature **314**; 467-9

M.G. Wouters, C.M. Thomas, G.H. Boers et al; Hyperhomocysteinemia: A Risk Factor in Women with Unexplained Recurrent Early Pregnancy Loss (1993) Fertil. Steril. **60**; 820-5

L. Wright and A. Welch; The production of folic acid by rat liver in vitro; (1943) Science N.Y. 98,179

A. Wuta, O.W. Smrzka, N. Schweifer, K. Schellander, E.F. Wagner and D.P. Barlow; Imprinted Expression of the Igf2r Gene Depends on the Intronic CpG Island (1997) Nature **389**; 745-9

H.K. Yamane and B. K-K. Fung; Covalent Modification of G-Proteins (1993) Ann. Rev. Pharmacol. Toxicol. **32**; 201-41

M. Yamauchi, N. Seki, K. Mit, T. Saito, S. Tsuji, E. Hongo, M. Morimyo, T. Shiomi, H. Koyama, D. Ayusawa and T. Hori; Isolation of Human *pur*H Gene Expressed in the Rodent Transformant Cells by Subtractive Enrichment of 3'-Untranslated Region of Human Transcript (1995) DNA Res. **2**; 269-75

H. Yaniv and C. Gilvarg; Aromatic Synthesis. XIV. 5-Dehydroshikimic Reductase (1955) J. Biol. Chem. **213**; 787-95

V.A. Yaylayan, A. Keyhani and A. Wnorowski; Formation of Sugar-Specific Reactive Intermediates from (13)C-Labeled L-Serines (2000) J. Agric. Food Chem. **48**; 636-41

Q-Z. Ye, J. Liu and C.T. Walsh; p-Aminobenzoate Synthesis in *Escherichia coli*: Purification and Characterisation of PabB as Aminodeoxychorismate Synthase and Enzyme X as Aminodeoxychorismate Lyase (1990) Proc. Natl. Acad. Sci. USA **87**; 9391-5

J.J. Yim and G.M. Brown; Characteristics of Guanosine Triphosphate Cyclohydrolase I Purified from *Escherichia coli* (1976) J. Biol. Chem. **251**; 5087-94

S. Zhang, D.J. Hunter, S.E. Hankinson, E.L. Giovannucci, B.A. Rosner, G.A. Colditz, F.E. Speizer and W.C. Willett; A Prospective Study of Folate Intake and the Risk of Breast Cancer (1999) JAMA **281**; 1632-7