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Title

**FOLIC ACID FORTIFICATION OF FLOUR: DETERMINATION OF THE
LEVEL THAT GIVES RISE TO UNMETABOLISED FOLIC ACID IN
SERUM**

A thesis presented for the degree of Doctor of Philosophy (Ph.D.) at the University of
Dublin, Trinity College.

By

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Department of Clinical Medicine

Trinity College, Dublin

September, 2000

1111

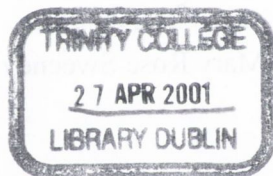
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A thesis presented for the degree of Doctor of Philosophy (Ph.D.) at the University of

Trinity College

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Thesis
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To my husband Gerry

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Declaration

I certify that none of the work in this thesis has been submitted for any degree or diploma at this, or any other, University and that all the work described in this thesis is entirely my own, except where indicated in the materials and methods section in Chapter 2. I hereby give my permission that this thesis can be borrowed from the library for the purpose of study.

Mary Rose Sweeney

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Summary

To prevent neural tube defects the Food and Drug Administration (FDA) implemented mandatory folic acid fortification of all flour milled in the USA at a level 140µg/100g. The Centers for Disease Control (CDC) have proposed that this level be increased to 350µg and even 700µg/100g. However previous research carried out in this laboratory has shown that doses of folic acid as low as 266µg administered acutely saturates the normal intestinal absorption mechanisms and results in unmetabolised folic acid in serum post-prandially. This may present a problem in the early diagnosis of pernicious anaemia. In addition the efficacy of anti-folate agents used in the treatment of various conditions such as epilepsy, rheumatoid arthritis and inflammatory intestinal disorders may be affected by the presence of unmetabolised folic acid in the circulation. Finally toxic effects as a result of large doses of folic acid in the circulation such as teratogenic defects and certain cancers have not been investigated.

This research investigated the effects of long-term consumption of fortified flour at the current level of fortification in practise in the USA and the two higher levels of fortification advocated by the CDC on unmetabolised folic acid in serum. A healthy adult population group was recruited. Subjects were pre-saturated and subsequently administered the three test doses of bread. Serum samples were collected pre and post prandially. Samples were assayed initially for unmetabolised folic acid in serum by a combined HPLC/microbiological assay. This methodology was unable to detect levels below 1ng/ml. Hence this thesis describes an improved methodology involving solid phase extraction which improved the lower limit of detection by six-fold. The new methodology was subsequently used to re-assay the samples collected. In addition acute studies were carried out investigating the validity of the FDA's safe upper daily limit for folic acid (1mg) in terms of folic acid appearance in serum post-prandially. Healthy adult subjects were recruited. No pre-saturation was given. A total of 1mg of folic acid in fortified bread was administered in five different combinations over an 8-10 hour period on five different study days. Serum was collected pre and post prandially.

This thesis also investigated the appearance of unmetabolised folic acid in cord blood of new-born infants and the effects of subsequent consumption of folic acid fortified infant formula feeds. A number of ancillary studies were also undertaken to investigate the stability of red cell folates over time, under different storage conditions.

The long-term study showed that the current level of fortification implemented in the USA (140µg/100g) will not lead to unmetabolised folic acid appearance in serum. Increasing the level to (350µg/100g) also will not lead to folic acid appearance. However, the highest level (700µg/100g) is likely to expose a large section of the population to unmetabolised folic acid in serum.

The acute studies showed that slices of folic acid fortified bread (containing 200µg) consumed repeatedly at ninety minute intervals over eight hours give rise to cumulative levels of folic acid in serum post-prandially. This cumulative elevation was not witnessed at the higher test doses of 333µg, and 500µg which, were administered four and two and a half-hours apart respectively. These results illustrate that if the time of t_{max} is exceeded between slices then a cumulative effect is not seen. These results indicate that renal clearance is more effective at getting rid of larger doses of unmetabolised folic acid delivered to the serum at intervals than small doses delivered often.

In the infants group we demonstrated folic acid in cord blood. It is noteworthy that the mothers of these infants had not been consuming folic acid supplements at the time of recruitment. Therefore these levels arose as a result of recent dietary intake of commercially available folic acid fortified foods. In addition consumption of folic acid fortified formula feeds gave rise to unmetabolised folic acid in the serum of these infants after only 4 days.

Red cell folate samples were prepared at different time points and stored under different conditions. Laboratory analysis was by *Lactobacillus casei* microbiological assay. The findings showed that red cell folates stored as whole blood are stable at room temperature for up to 72 hours on a laboratory bench but samples in constant motion on a shaker incubator (simulating transportation conditions) at room temperature may not be stable for 72 hours.

LIST OF ABBREVIATIONS

H ₄ PteGlu	Tetrahydrofolate
5-CH ₃ -H ₄ PteGlu	5-Methyltetrahydrofolate
10-CHO-H ₄ PteGlu	10-formlytetrahydrofolate
5-CHO-H ₄ PteGlu	5-formyltetrahydrofolate
5-CHNH-H ₄ PteGlu	5-formininotetrahydrofolate
5,10CH-H ₄ PteGlu	5,10-methenyltetrahydrofolate
μg	microgram
μmol	micromole
pg	picogram
ng	nanogram
ANOVA	Analysis of variance
CBL	Cobalamin
CDC	Centers for Disease Control
CNS	Central Nervous System
COMA	Commission on Medical Aspects of Food Policy
DHF	Dihydrofolate
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
DoH	Department of Health
DPM	Decays per minute
DRV's	Dietary Reference Values
EAR	Estimated Average Requirement

EUROCAT	European Registration of Congenital Anomalies and Twins
FDA	Food and Drugs Administration
HC	Haptocorrins
HPLC	High Performance Liquid Chromatography
IF	Intrinsic Factor
INDI	Irish Nutrition & Dietetic Institute
<i>L.casei</i>	<i>Lactobacillus casei</i>
LRNI	Lower Reference Nutrient Intake
MCV	Mean Cell Volume
MMA	Methylmalonic Acid
MRC	Medical Research Council
MTHFR	Methylenetetrahydrofolate Reductase
NO	Nitrous Oxide
NTDs	Neural Tube Defects
P	Probability
PA	Pernicious Anaemia
PLP	Pyridoxal-5-phosphate
RA	Rheumatoid Arthritis
RCF	Red Cell Folate
RDA	Recommended Daily Allowance
RNI	Reference Nutrient Intake
RPM	Revolutions per minute
SACD	Sub Acute Combined Degeneration of the Spinal Cord
SAH	S-adenosylhomocysteine

SAM	S-adenosylmethionine
SHMT	Serine hydroxymethyl transferase
TC-2	Transcobalamin
TNF- α	Tumour Necrosis Factor
v/v	volume over volume
w/v	weight per volume
WHO	World Health Organisation

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Chapter one

Introduction

1.1 History of folate

In Bombay in the 1920s an English physician Lucy Wills identified an anaemia seen frequently in late pregnancy which responded to treatment with a yeast extract called marmite (Wills, 1931). This anaemia was very similar to pernicious anaemia hence she named it “pernicious anaemia of pregnancy”. However unlike pernicious anaemia this anaemia of pregnancy redressed when the pregnancy ended. The active ingredient in marmite was subsequently identified as folic acid/folate by Mitchell (Mitchell *et al*, 1941) who was the first to purify it from spinach leaves and other foliage, hence the name folate. Later various groups including (Stockstad, 1979) carried on the work of isolating and determining the structure of what we now know to be folic acid. Its synthesis in 1946 (Aungier *et al*, 1946) provided final proof of its structure. Folic acid derivatives, which exist in vivo in a reduced state, have since been characterised. These include tetrahydrofolate ($H_4PteGlu$), 5-methyltetrahydrofolate ($5-CH_3-H_4PteGlu$), 5,10-methylenetetrahydrofolate ($5,10-CH_2-H_4PteGlu$), 10-formyltetrahydrofolate ($10-CHO-H_4PteGlu$), 5-formyltetrahydrofolate ($5-CHO-H_4PteGlu$), 5-formiminotetrahydrofolate ($5-CHNH-H_4PteGlu$) and 5,10-methenyltetrahydrofolate ($5,10CH-H_4PteGlu$) (Kisluk *et al*, 1984).

1.2 Structure and nomenclature

The term “folate” includes the entire group of folate vitamin forms, i.e. the naturally occurring folypolyglutamates found in food, and folic acid (pteroylglutamic acid), the synthetic form of the vitamin which is not found in nature but is commercially available in

a metabolically inactive form and is therefore termed a provitamin. However it is readily converted in vivo to the reduced form which is metabolically active. Folic acid is more stable than the majority of reduced folates (O'Brien *et al*, 1975; Temple & Montgomery, 1984).

Folate chemistry and metabolism have been reviewed comprehensively by Shane (1995). The basic structure of folic acid is shown in figure 1.1. It consists of three moieties, a 2-amino-4-hydroxy pteridine (pterin) moiety linked via a methylene group at the C-6 position to a *p*-aminobenzoyl-glutamate moiety. Mammals lack the ability to synthesise folates de novo and require preformed folates in the diet. The folates that occur naturally in food are largely 5,6,7,8-tetrahydrofolates, in which the pteridine ring is reduced. These exist in the form of unsubstituted tetrahydrofolates, as well as having one-carbon substituents. These naturally occurring folates generally have a polyglutamate side chain of typically less than seven glutamate residues in γ -peptide linkage (Gregory, 1997). Reduced folates are relatively unstable in foods and large losses can occur during food preparation and cooking. Oxidation during cooking can result in cleavage products, which lack vitamin activity (Shane, 1995).

Folate metabolism involves the reduction of the pyrazine ring of the pterin moiety to the co-enzymatically active tetrahydro form, the elongation of the glutamate chain by the addition of glutamate residues in γ -peptide linkage, and the acquisition and oxidation or reduction at the N-5 and/or N-10 positions (Shane, 1995). Folate coenzymes function as acceptors and donors of one-carbon moieties in reactions involving nucleoside and amino acid metabolism. The active coenzymes species used on these reactions are the

folypolyglutamates, which are more effective substrates and inhibitors than pteroylmonoglutamates (Shane, 1995). This role in one-carbon metabolism will be discussed later in the introduction.

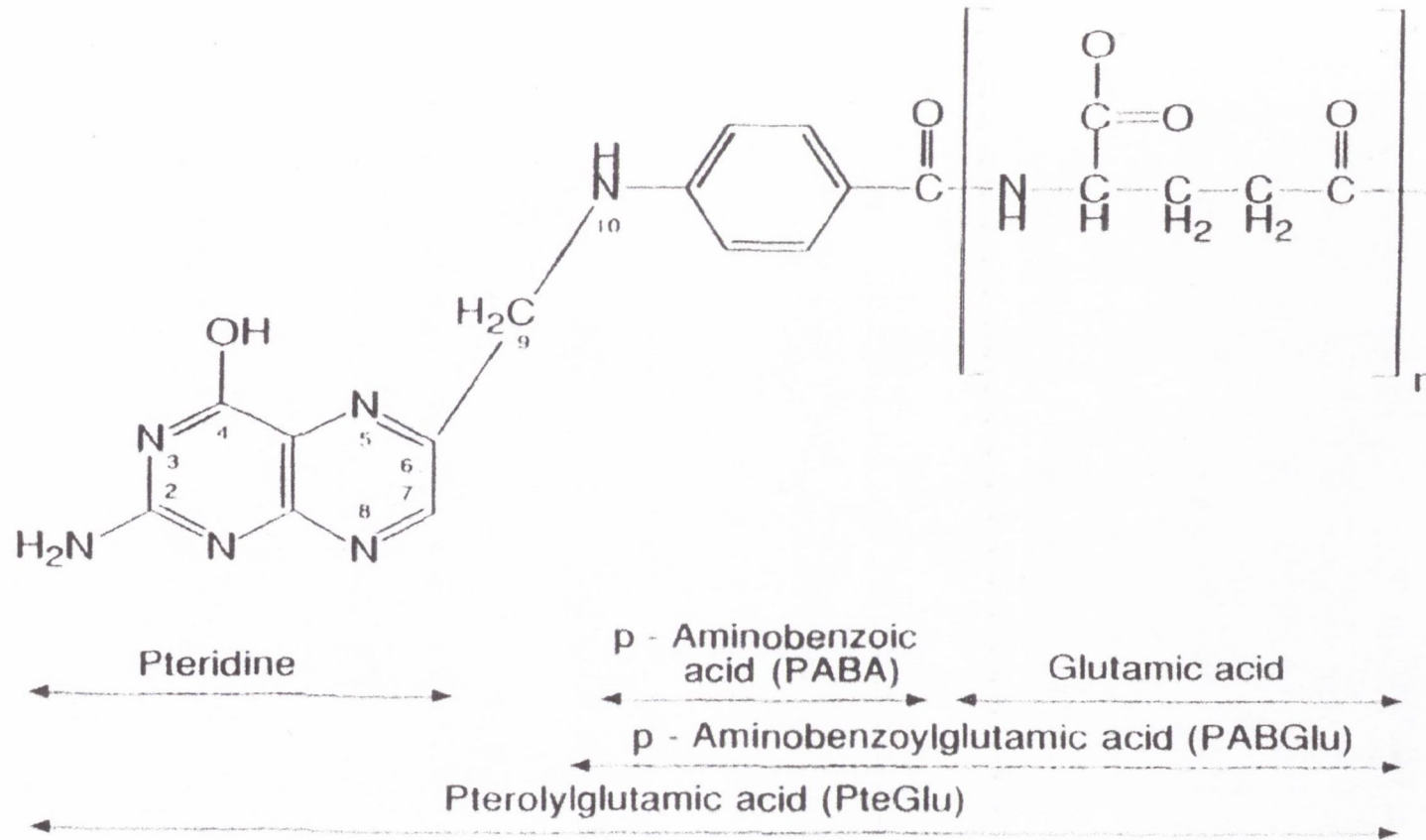


Figure 1.1. Structure of folic acid (pteroylmonoglutamate)

(Shane, 1995)

1.3 Sources

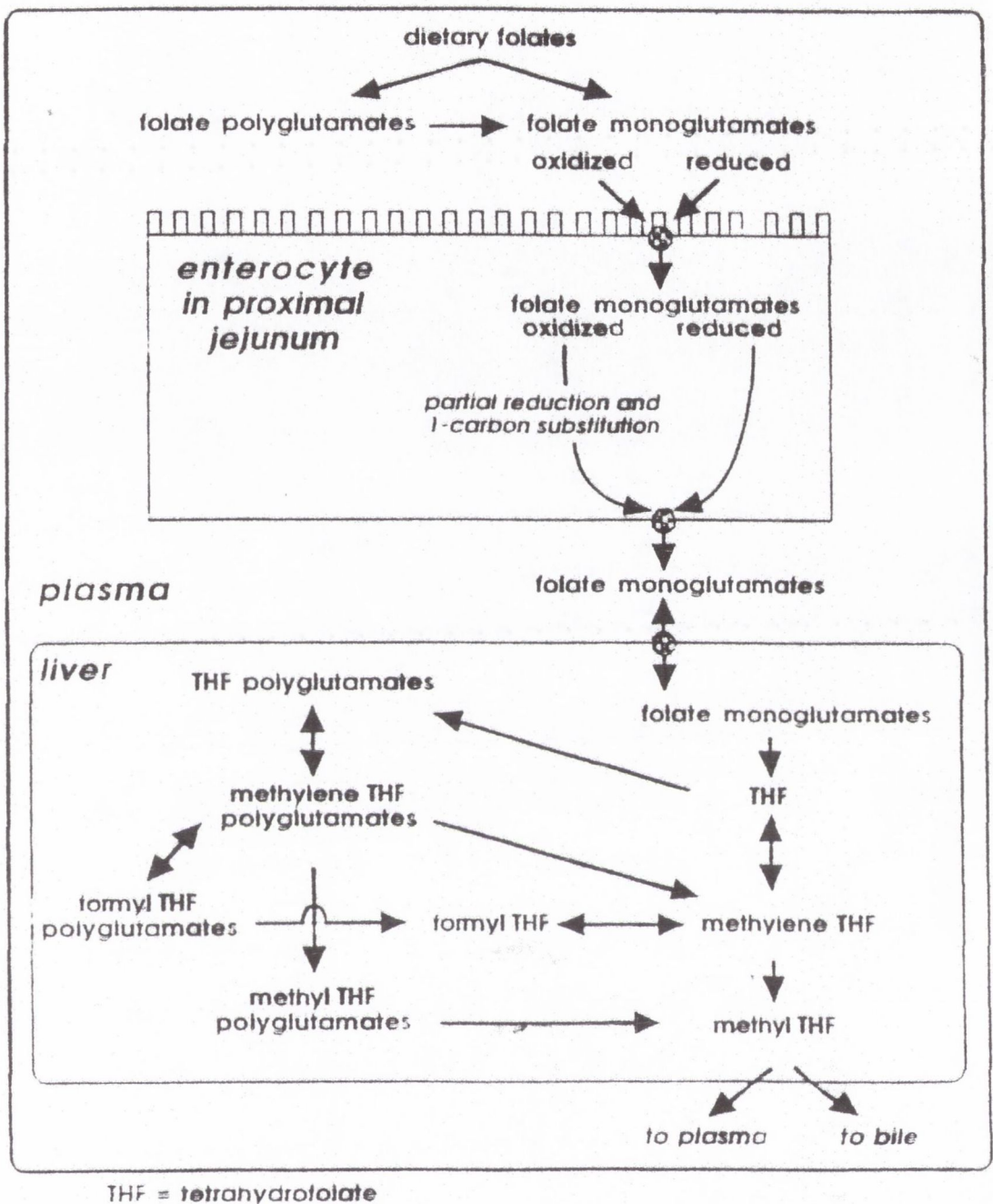
Folate is synthesised by microorganisms and higher plants. Folate synthesising bacteria in the gut of mammals contribute only minimally to folate status, however, making folate an essential nutrient. Folates are ubiquitous in nature being present in nearly all natural foods (Herbert & Kshitish, 1994). Rich natural food sources include liver, yeast extract, green leafy vegetables, legumes and oranges (DRVs, 1991). Synthetically added folic acid is increasingly being added to foodstuffs such as breakfast cereals, breads and milk. In the USA all flour milled is now fortified with folic acid at 140µg/100g. Synthetic folic acid is more stable than the natural form of the vitamin and appears to be more bioavailable (Cuskelly *et al*, 1994).

1.4 Absorption

Food folate is absorbed primarily from the proximal third of the small intestine, although it can be absorbed from the entire length of the small bowel (Rosenberg, 1976). Approximately 80% of dietary folates are polyglutamate derivatives and the remainder are in the 10-formyl form. Polyglutamates are not absorbed across the gut (Gregory, 1995). These must firstly be hydrolysed to monoglutamates in the gut prior to absorption across the intestinal mucosa. The hydrolysis of folypolyglutamates is catalysed by γ -glutamylhydrolase. This conjugase enzyme has been found on the luminal brush border membrane in the human jejunum and has equal affinity for polyglutamates of varying chain length (Chandler *et al*, 1986) but its physiological role or exact location has not been fully

determined. It has a pH optimum of 7.0. A second form of this enzyme has also been shown in intestinal fluid, which is of pancreatic origin (Bhandari *et al*, 1990) and has a pH optimum of 4.5. The hydrolytic deconjugation of polyglutamyl folates is an essential step in folate absorption but further study is needed to clarify the physiological roles of the various conjugases in folate digestion and absorption (Rosenberg, 1989; Halsted, 1990).

The second step in the absorption mechanism is luminal transport. The exact mechanism has not been fully elucidated but a number of theories have been postulated. It appears that there are two separate systems for folate absorption across the intestinal lumen (Dahr *et al*, 1977). One of these appears to be saturable, Na^+ , pH, and glucose dependent and an active carrier system is involved. The other system appears to be non-saturable, independent of energy, pH, temperature, or concentration and predominates at higher folate intakes. During the absorption process folate also undergoes reduction and methylation (Rosenberg, 1975). Methyl-THF monoglutamates are absorbed rapidly and remain virtually unchanged. Other monoglutamates are converted by the mucosal cell to formyl and methyl THF monoglutamates (Pratt & Cooper, 1971). Folates emerge in the portal circulation as 5-methyl-THF and are transported to the liver and peripheral tissue where the polyglutamal coenzyme is resynthesized. These polyglutamate derivatives are the regulators of one-carbon metabolism and are required for cellular retention of folates (Shane, 1990). The intercellular re-synthesis of folates to polyglutamates is of major importance in regulating folate homeostasis. However at a threshold oral dose of 266 μg , unmetabolised folic acid has been shown to appear in serum post-prandially (Kelly *et al*, 1997). The mechanism of intestinal absorption is shown diagrammatically in Figure 1.2.



(Journal of Nutritional Biochemistry, 1990)

Figure 1.2. Diagrammatic illustration of Intestinal absorption of folates

1.5 Bioavailability

Folate bioavailability can be defined as the amount, which is available for utilisation at a cellular level after digestive processes. The topic of folate bioavailability has been studied extensively and reviewed (Bailey *et al*, 1988; Gregory, 1995). Despite this however, our understanding of folate bioavailability is incomplete. The digestive/absorptive factors thought to play a role in bioavailability include a) binding or entrapment in the food matrix b) altered pH at the jejunal mucosal surface which could affect the conjugase action and/or folate transport c) inhibition of jejunal brush border folate conjugase and/or transport by food components (conjugase action may be specifically inhibited by food factors described in yeast and legumes although the evidence is conflicting) and d) effects on intestinal transit time (Gregory, 1989). Food folates are estimated to be only 50% bioavailable whereas the synthetic form of the vitamin folic acid is thought to be 100% bioavailable. The bioavailability of ingested folate monoglutamates is significantly greater than that of folate polyglutamates in humans presumably because of the requirement for hydrolysis of the latter (Herbert, 1999).

1.6 Transport in serum, cell uptake and retention

Folate transport by mammalian tissues has been the subject of numerous publications including Ratnam (1990), Henderson (1986) and Shane (1995). 5-methyl-THF is the main circulating form of folates in the circulation (Shane, 1995). This is therefore the principal form by which tissues are supplied with folate (Shane & Stockstad, 1983). They can be transported free or can be non-specifically bound to serum proteins such as albumin

(Shane, 1995). The role of serum binders remains unclear but it is thought that they may be used to control folate distribution and excretion during deficiency. Folates are rapidly cleaved from serum by tissues including the liver via specific cell surface binding proteins (Wagner *et al*, 1982). Intercellular metabolism to folypolyglutamates is required for the retention and concentration of transported folates (Shane, 1995). Folypolyglutamates are the active coenzyme species required for normal one-carbon metabolism. Once folate is inside the cell it is polyglutamated in order that it can be retained and utilised for cellular functions. 5-Methyl THF is a very poor substrate for polyglutamation; therefore it must firstly be demethylated by the action of the vitamin B₁₂ dependent enzyme methionine synthase to THF monoglutamate. This form is then readily polyglutamated (Shane, 1995). This enzyme is the only known enzyme capable of demethylating 5-methyl THF and is therefore an essential enzyme in the cell's supply of folate (Van Aerts, 1995). In vitamin B₁₂ deficiency the capacity to demethylate 5-methyl THF is diminished. This is of crucial importance to this thesis and this will be discussed later in the introduction. This enzyme also links folate to the methylation cycle. Synthetic folic acid emerges in the portal circulation after ingestion as 5-methyl THF and becomes available as a coenzyme form. However above certain oral doses previously shown to be 266µg in fortified foods (Kelly *et al*, 1997) folic acid can appear unmetabolised in the serum. Unmetabolised folic acid does not depend on methionine synthase for its uptake into cells. It is converted from the metabolically inactive provitamin format to dihydrofolate via the enzyme dihydrofolate reductase, which further reduces it to tetrahydrofolate. Tetrahydrofolate is readily polyglutamated and hence becomes metabolically active. This enzyme is also of vital importance to this project and reference will be made to it later in this chapter.

Small amounts of other one-carbon substituted folates (10-formyl-tetrahydrofolate) also circulate and will enter cells by the same carrier-mediated process. Under normal conditions the predominant folates in liver are pentaglutamates, with small amounts of tetra- and hexaglutamates. Plasma folate concentrations are a good indicator of status but tend to fluctuate with recent dietary intakes. Red blood cells accumulate several hundred-fold higher concentrations of folate than plasma. Red cell stores tend to be retained during their life span; hence they are a better indice of long term status (Shane, 1995).

1.7 Turnover and Excretion

The liver accounts for almost half of the estimated 7.5 ± 2.5 mg body pool of folate (Herbert, 1987). Dietary folate that is not accumulated by the liver is released primarily as 5-methyl-THF, as this is the major cytosolic form of the vitamin. Folate is concentrated in bile and the enterohepatic recirculation from the intestine accounts for reabsorption and reutilization of approximately 100 µg per day of folate (Steinberg, 1984). Faecal folates mostly arise through gut microflora biosynthesis of the vitamin with only a small contribution from unabsorbed dietary folate (Krumdieck *et al*, 1978). Urinary excretion of intact folates account for only a small fraction of ingested folates under normal circumstances. The greater amount of excretion in urine is accounted for by products of an intracellular scission of the folate molecule at the C9-N10 bond, consisting of one or more pteridines and *p*-acetamidobenzoylglutamate. The major route of whole body folate turnover therefore appears to be via catabolism to cleaved products. Any condition that increases oxidative cleavage of folate and consequently catabolism would be expected to increase folate requirements. Catabolism of folate has been shown to be markedly increased

in pregnancy (Mc Partlin *et al*, 1993). Folate turnover and excretion have been summarised by Shane (1995) and Herbert (1999).

1.8 Role in metabolism

The role of folate in metabolism has been reviewed and summarised by many authors including Wagner (1995) Scott & Weir (1998) and Bailey & Gregory (1999). The most important of these are summarised in this section. The principal function of folate is in its coenzymes role, which carries one-carbon units and thereby acts as a cofactor to specific enzymes allowing them to carry out their specific function or functions. One-carbon units are carried by folates only when the coenzyme is in the fully reduced tetrahydro form (Wagner, 1995). This reduction is carried out by a single enzyme, dihydrofolate reductase (DHFR) which reduces folic acid to dihydrofolate (DHF) and reduces dihydrofolate to tetrahydrofolate (THF) (Wagner, 1995). These one-carbon units are collectively called one-carbon groups and they can exist as formyl (-CHO), methylene (-CH₂-) and methyl (-CH₃) groups. One-carbon reactions include those involved in purine and pyrimidine synthesis, amino acid metabolism and the formation of the primary methylating agent S-adenosylmethionine (SAM).

One-carbon units at the oxidation level of formate can enter directly into the folate pool as formate in a reaction catalysed by 10-formyl-THF synthase (Wagner, 1995). Entry at the formate level of oxidation can also take place via a catabolic product of histidine, formimino-glutamic acid. The third entry mode of entry at the formate level of oxidation involves the formation of 5-formyl-THF from 5,10-methenyl-THF by the enzyme serine

hydroxymethyl transferase (SHMT) (Wagner, 1995). The 5-formyl-THF may be rapidly converted to other forms of folate. A final mode of entry arises from carbon-1 of the ribose moiety of methylthioribose, a by-product of the conversion of S-adenosyl-methionine to polyamines. The enzyme SHMT is involved in the entry of one-carbon units at the formaldehyde level of oxidation by catalysing the transfer of the β -carbon of serine to form glycine and 5,10-methylene-THF. Other sources of one-carbon entry include the glycine cleavage system and the choline-dependent pathway (Wagner, 1995). Figure 1. 3 shows an overview of these main metabolic functions and the most important reactions are discussed briefly. Single carbon units can be removed from folates by a number of important reactions including purine and pyrimidine biosynthesis and amino acid metabolism. One-carbon units from 10-formyl-THF are used in the biosynthesis of purines. Tetrahydrofolate is regenerated after the one-carbon group is transferred. The one-carbon transfer from 5,10-methylene-THF to deoxyuridylate forms thymidylic acid, a precursor of DNA. This is of crucial importance to cells. During pyrimidine biosynthesis, dihydrofolate is generated, which recycles back to tetrahydrofolate by dihydrofolate reductase (DHFR).

Histidine is a glucogenic amino acid and is catabolised primarily to glutamate in mammalian cells. N-formaminoglutamic acid is an intermediary in this pathway. Tetrahydrofolate has a role in the conversion of serine to glycine with the formation of 5,10-methylenetetrahydrofolate. Serine can be produced in glycolysis hence this pathway is a means by which carbon units present in carbohydrates can be incorporated into DNA.

The enzyme 5,10-methylenetetrahydrofolate reductase can channel one-carbon groups into the "methylation cycle". This cycle's function is to provide methyl groups in the form of

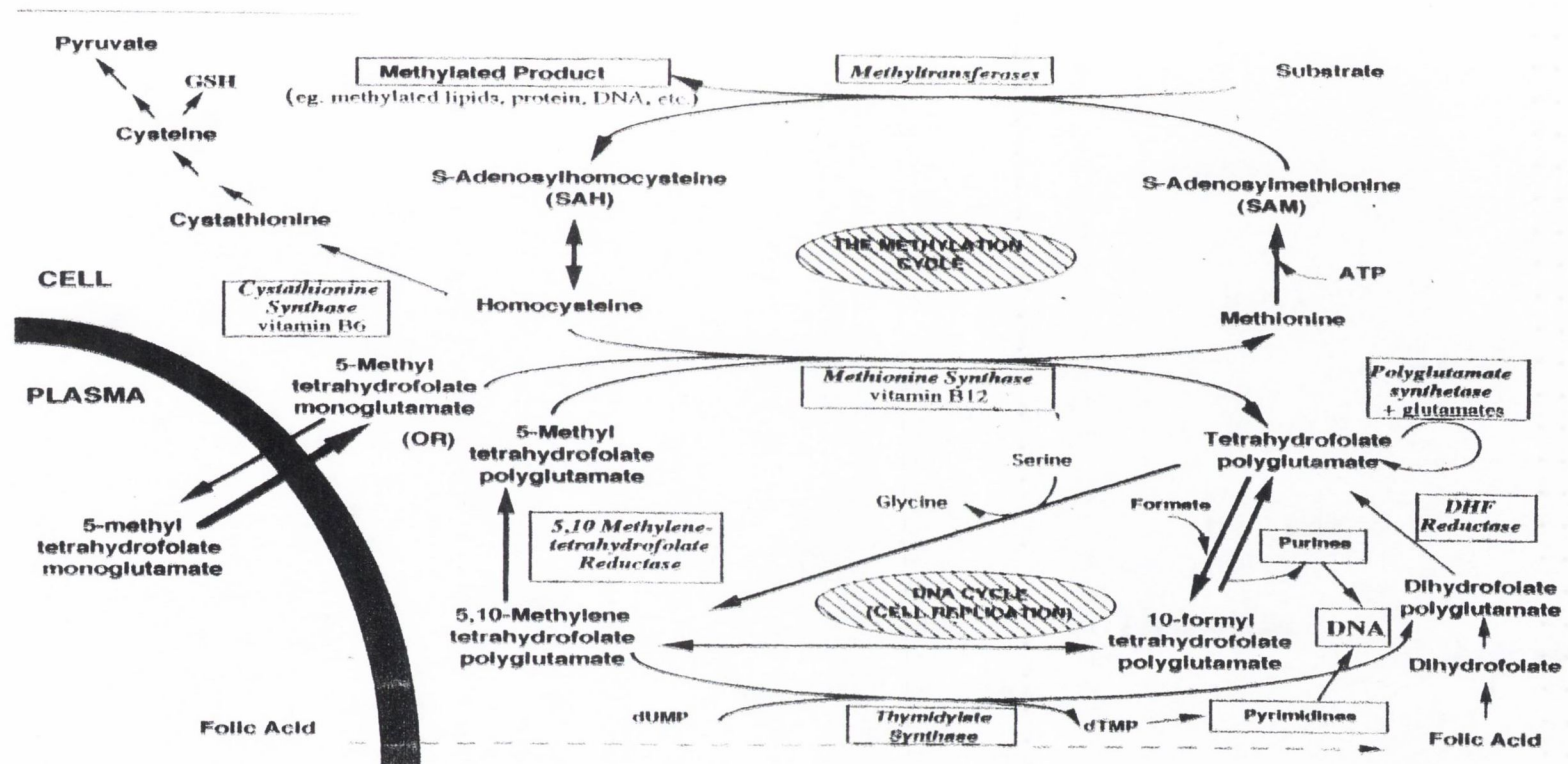
high-energy derivatives of methionine called S-adenosyl methionine (SAM), which acts as a cofactor for many methyltransferases present in all cells (Scott & Weir, 1998). These methyltransferase enzymes use SAM and donate methyl groups to a wide range of structures including phospholipids, proteins, hormones and DNA. The product of the transmethylation reactions, S-adenosylhomocysteine (SAH), is hydrolysed to adenosine and homocysteine through the action of SAH hydrolase. SAH hydrolase is a reversible enzyme, with the direction of SAH synthesis being favoured thermodynamically. The rapid metabolism of adenosine and homocysteine ensures that the direction of SAH hydrolysis predominates *in vivo*. This is a critical regulatory point as SAH is a potent inhibitor of transmethyloses. It is therefore imperative that homocysteine is shunted away from SAH synthesis. This is accomplished through the action of two pathways 1) remethylation back to methionine and b) transsulfuration; a pathway committed to cysteine formation. The regulation of homocysteine has been summarised by many authors including Selub (1999), House *et al* (1999) and Scott & Weir (1998).

Remethylation involves the transfer of the methyl group from 5-methyl-THF to homocysteine to form methionine and THF. This reaction is catalysed by the enzyme methionine synthase and requires vitamin B₁₂ in the form of methylcobalamin as a cofactor. A considerable amount of methionine is activated by ATP to form S-adenosylmethionine. S-adenosylmethionine serves as a universal methyl donor to a variety of acceptors. S-adenosylhomocysteine, the by-product of these methylations, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer. Inhibition of methionine synthase due to B₁₂ deficiency produces a megaloblastic anaemia-to be discussed later in the introduction. A

second enzyme involved in the remethylation pathway is methylenetetrahydrofolate reductase (MTHFR). This enzyme is responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which then acts as co-substrate for the B12-dependent remethylation of homocysteine to methionine. Riboflavin in the form of FAD is required as a prosthetic group for MTHFR (Bates & Fuller, 1986). Betaine: homocysteine methyltransferase catalyses a similar reaction, however it is independent of the folate pool. It utilises betaine, a metabolite of choline metabolism, as a methyl donor for the synthesis of methionine. The catabolism of the carbon and sulfur groups of methionine/homocysteine occurs via its oxidation through the transsulfuration pathway. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in a reversible reaction catalysed by the pyridoxal-5-phosphate (PLP)-containing enzyme, cystathionine β -synthase (Mudd *et al*, 1995). Cystathionine is hydrolysed by a second PLP-containing enzyme, γ -cystathionase, to form cysteine and α -ketobutyrate. Excess cysteine is oxidised to taurine or inorganic sulfates or is excreted in the urine. Thus in addition to the synthesis of cysteine, the transsulfuration pathway effectively catabolizes homocysteine, which is not required for methyl transfer.

Many of the enzymes involved in these reactions are multi-functional and are capable of channelling substrates and one-carbon units from one reaction to reaction within a protein matrix. Another feature of intracellular folate metabolism is the compartmentation of folate coenzymes between the cytosol and the mitochondria: 5-methyl-THF is associated with the cytosolic fraction of the cell whereas most of the 10-formyl-THF is located in the mitochondria (Wagner, 1995). Metabolic products of folate-dependent reactions such as

serine and glycine are readily transported between both locations but the folate coenzymes are not.



(Scott, 1999)

Figure 1.3. Pathways involved in folate metabolism

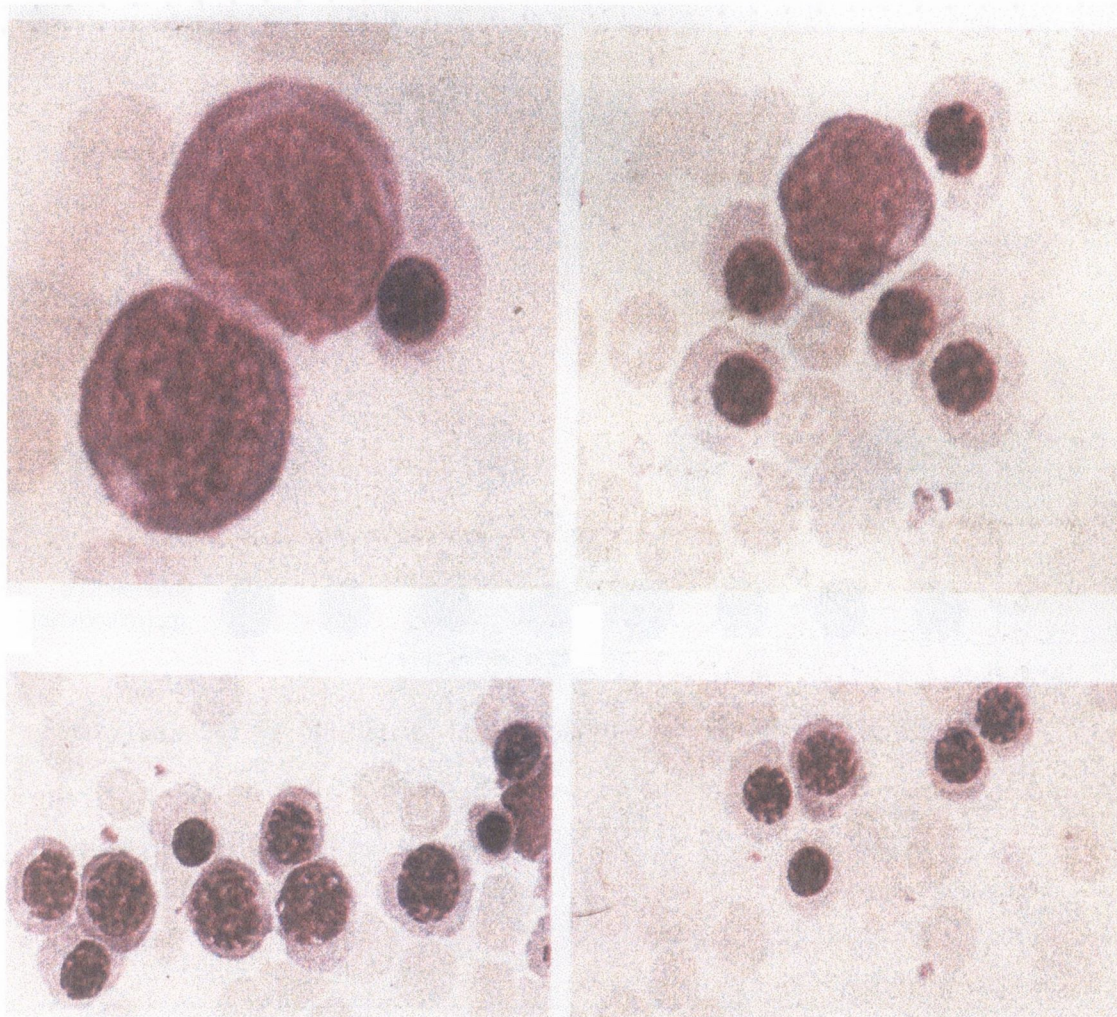
1.9 Folate Deficiency

Folate deficiency can be due to a poor dietary intake of folate alone or in combination with a condition of increased utilisation, requirement or malabsorption (Lindenbaum & Allen, 1995). Excess turnover of any type, including pregnancy can precipitate deficiency states. Certain drugs such as anticonvulsants can also cause deficiency although the mechanism of action is still a mystery. Alcohol, sulphasalazine and other drugs have been associated with impaired folate metabolism. In folate deficiency all the reactions of the one-carbon cycle will be compromised to varying degrees depending on the relative affinities of the enzymes for the respective folate molecules involved (Bailey & Gregory, 1999). Since folate co-factors are essential for both purine and pyrimidine biosynthesis, their deficiency compromises the cells ability to synthesise DNA and RNA and thus to divide. This reduced rate of cell division ultimately affects all cells, but initially affects rapidly dividing cells, such as those of the bone marrow or the intestinal mucosa (Chanarin, 1979).

a) Megaloblastic Anaemia in folate deficiency

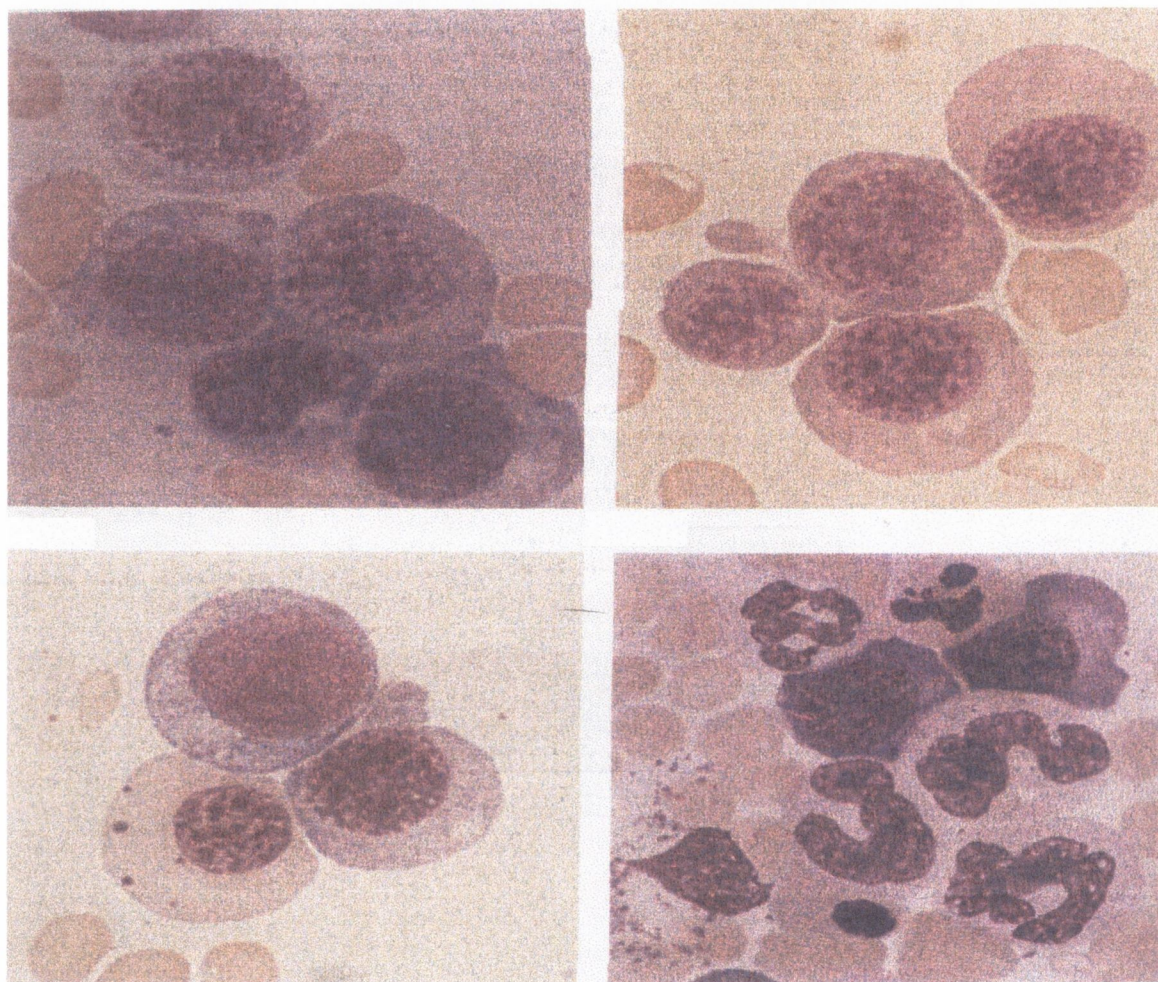
Clinically folate deficiency produces a macrocytic anaemia. Folate deficiency causes megaloblastic anaemia by inhibiting thymidylate synthesis, a rate-limiting step in DNA synthesis in which thymine, one of the two pyrimidine bases, is synthesised as its nucleotide-derivative thymidine monophosphate from deoxyuridine monophosphate (Herbert, 1985). This reaction needs 5,10-methylene THF as a coenzyme. The red cell precursors are megaloblastic before they leave the bone marrow and, in contrast to the usual normoblastic precursors they have a large diffuse nucleus, which results from the

slower rate of cell division (Herbert, 1985). Figure 1.4 shows normoblastic erythroblasts on the usual Romanowsky stain. Megaloblastic cells are demonstrated in Figure 1.5 (Hoffbrand & Pettit, 1995). The sequence of hematologic events of megaloblastic anaemia are gradually progressive and generally commence with a decrease in serum folate levels which can take up to two months to occur. This sequence of events was described by Herbert & Kshitish (1994) and subsequently by Herbert (1999). As folate stores begin to be depleted, red cell folate levels fall to subnormal over a period of months. Serum homocysteine levels start to increase at this time also. Megaloblastic changes then start to occur in the bone marrow followed by subtle changes in the peripheral blood. As the depletion progresses further, the mean cell volume (MCV) becomes increased. Neutrophil hypersegmentation is typically seen in the blood. Macrocytic anaemia develops and will be evident in depression of the red blood count. Haemoglobin and hematocrit levels eventually become depressed. At this point the patient can remain asymptomatic. As the anaemia progresses to severe symptoms such as weakness, fatigue, irritability, headache, palpitations, and shortness of breath begin to manifest and other systems may become involved such as the gastrointestinal tract. Glossitis is present in about a quarter of patients at this stage of anaemia (Savage *et al*, 1994). Nausea and vomiting can occur. Neuropsychiatric abnormalities of folate deficiency have been reported to include peripheral neuropathy, dementia, depression and spinal cord damage but folate deficiency as the definitive causative agent remains to be established (Lindenbaum *et al*, 1995).



(Hoffbrand & Pettit, 1995)

Figure 1.4. Normoblastic red blood cells.



(Hoffbrand & Pettit, 1995)

Figure 1.5. Megaloblastic red blood cells.

b) Folate deficiency and elevated Homocysteine

Various substrates and metabolic intermediates accumulate as a result of folate deficiency and these may have adverse effects (Bailey & Gregory, 1999). Of particular significance from a public health perspective is the relationship between folate status and homocysteine levels, which is an inverse one. Hence elevated plasma homocysteine can arise as a result of a low folate status. Hyperhomocysteinaemia can arise either from enhanced production or reduced rates of metabolism of homocysteine due to a defect of homocysteine, cobalamin or folate metabolism or a deficiency of one or all of the vitamins, B₁₂, folate, and B₆ (Weir & Scott, 1998). The severity of the resultant hyperhomocysteinaemia is dependent on the extent to which the particular disturbance affects the co-ordination of the two pathways of homocysteine metabolism.

In 1969 Mc Cully made the clinical observation linking elevated homocysteine and vascular disease. He reported extensive arterial thrombosis and atherosclerosis in a child with an inborn error of cobalamin metabolism who had elevated plasma homocysteine and homocystinuria. Subsequently Mc Cully and Wilson (1975) proposed that elevated levels of homocysteine might be pathogenically associated with atheroma of the vasculature. Many studies have since been carried out investigating this relationship. The findings from the most comprehensive of these were recently reviewed in an excellent paper by Mc Kinley (2000). Elevated plasma homocysteine has now been established as an independent risk for coronary heart disease (Boushey *et al*, 1995; Dalery *et al*, 1995; Hopkins *et al*, 1995; Yoo *et al*, 1997) and stroke (Markus *et al*, 1997; Lindgren *et al*, 1995; Brattstrom *et al*, 1984). Mild homocysteinaemia has also been associated with peripheral occlusive

arterial disease (Brattstrom *et al*, 1990; Aronson *et al*, 1994). Hyperhomocysteinaemia is also a risk factor for hyperlipaemic patients (Glueck *et al*, 1995). While a consensus on precise cut-off values has yet to be established, there is general agreement that increases in plasma homocysteine concentrations yield a significant, graded-risk for the development of cardiovascular disease (House *et al*, 1999). The precise pathological mechanisms whereby homocysteine may cause vascular lesions are currently unknown but several mechanisms have been proposed including vascular endothelial dysfunction (Wang *et al*, 1992), vascular smooth muscle proliferation (Tsai *et al*, 1994) and coagulation abnormalities (Fryer *et al*, 1993).

In a recent study, Ward *et al*, (1997) showed that plasma homocysteine levels can be lowered by consuming physiological doses of supplemental folic acid. This lowering effect was optimal at a level of 200µg even though 400µg was also tested. A recent publication (Peterson & Spena, 1998) showed regression of atheromatous plaques when patients were treated with cobalamin, folate and PLP. In the USA mandatory folic acid fortification of all flour milled at a level of 140µg/100g commenced in January 1998. Jacques *et al*, (1999) reported on the effects of this after one year. The authors showed that plasma folate levels increased by 117% and that the prevalence of low folate concentrations decreased by 92%. Fasting total homocysteine concentrations decreased by 7% and the prevalence of high homocysteine concentrations decreased by 48%. Hence fortification or supplementation is likely to reduce mortality and morbidity from coronary heart disease and stroke. It has been estimated that up to 50,000 lives could be saved annually (Boushey *et al*, 1995). The current mandatory fortification programme in place in the USA is likely to provide evidence in support of this. This will only become evident over time.

c) Folate deficiency-Possible Role in Cancer Development

Inadequate folate intake has also been implicated in the development of certain types of cancer. The mechanisms by which folate deficiency increases the risk of cancer are not known: however folic acid is involved in both methyl metabolism and in DNA synthesis and repair. The proposed role of folate in carcinogenesis is hypothesised to relate to DNA structure, stability and transcriptional regulation; this includes increased susceptibility of DNA to strand breakage, uracil misincorporation into DNA and hypomethylation of DNA. Misincorporation of uracil into DNA is thought to stress the mechanism of DNA repair and thereby results in subsequent increases in strand breakage and chromosomal instability. Uracil misincorporation was shown to be markedly reduced after folate supplementation, which restored thymidylate synthesis (Blount *et al*, 1997). Duthie & Hawdon, (1998) reported that DNA instability which included strand breakage, uracil misincorporation and defective repair was increased in lymphocyte cells when they were depleted of folic acid over a period of time in an *in vitro* experiment. The authors concluded that folic acid maintains DNA stability under these conditions.

In epidemiological studies, folate deficiency has been associated with an increased risk of several malignancies including cancer of the cervix, lung, colon and rectum (Glynn & Albanes, 1994). The Nurses' Health Study (Giovannucci *et al*, 1998) showed that long-term use of multi-vitamin supplements may decrease the risk of colon cancer by about 75%. The authors hypothesised that the risk reduction was associated with folic acid contained in the multi-vitamins. Supplementation with folic acid has also been reported to reduce the incidence of premalignant lesions in the cervix (Butterworth *et al*, 1982), bronchial

dysplasia in smokers (Heimberger *et al*, 1988) and the incidence of cancer development in ulcerative colitis patients (Lashner *et al*, 1989, 1997). Interestingly these effects seem to occur with relatively modest alterations in folate status and may even be present when vitamin status is altered within the range of what is presently considered to be normal. The mechanisms by which this may occur are diverse and under intense investigation.

d) Folate deficiency-Interruption of the methylation cycle

A vital function of folate is in supplying methyl groups to the methylation cycle. These methyl groups are necessary for the activation of a wide variety of methyltransferases, which regulate many vital processes of the central nervous system such as the maintenance of myelin. Interruption of the methylation cycle was achieved experimentally in monkeys by inactivating methionine synthase (Scott *et al*, 1981) with nitrous oxide. This was shown to produce a neuropathy identical to that seen in pernicious anaemia. It is thought that that inability to methylate myelin basic structure causes this structure to become unstable and to degenerate causing the neuropathy. This type of neuropathy is only seen in severe prolonged folate deficiency as neural tissue is preferentially supplied with folate at the expense of other tissues.

e) Sub-optimal folate status and Neural Tube Defects in early pregnancy

Neural tube defects (NTDs) are severe congenital malformations of the central nervous system which occur more frequently in Ireland than in most other countries (see Table 1.1) with an estimated current prevalence of 1.5 per 1000 births (Report to the Minister for

Health and Children 1997). The incidence in the US is estimated to be 0.6 per 1000 births and an unknown number of foetus are aborted (CDC, 1992). Failure of the embryonal neural tube to close normally within the first 4 weeks post conception can result in a group of major congenital malformations known as neural tube defects. These defects include spina bifida (51%), anencephalus (40%), encephalocoele (8%) and iniencephaly (1%). Anencephalus and iniencephaly are universally lethal conditions with death occurring either in utero or shortly after birth. Table 1.2 shows the definitions and variations of each of these conditions. About 80% of babies born with spina bifida and encephalocoele survive the new-born period but the vast majority (85%) of these children have lifelong moderate or severe handicap including paralysis and varying degrees of bladder and bowel incontinence.

Table 1.1. Prevalence of Neural Tube Defects in Europe.

Prevalence rates (per 10,000 births) of neural tube defects in EUROCAT registries, 1990 - 1994.

<i>Registry</i>	<i>Rate</i>
Dublin	14.2
Galway	14.7
Belfast*	10.0
Glasgow*	15.9
France (Paris)*	12.1
Netherlands (Groningen)*	11.5
Spain (Basque Country)*	11.0
Belgium (Antwerp)*	10.0
Denmark (Odense)*	9.1
Italy (Tuscany)*	6.8
Switzerland*	5.6

Source: EUROCAT Central Registry, Brussels

Table 1.2. Definition and variation of the various neural tube related birth defects.

Condition	Definition	Variants	Synonyms
Anencephalus and similar anomalies Acephalus	Absence of head		Acrania, acephalic monster, acephalia, (absence of brain), (agenesis, aplasia of brain)
Anencephalus	Partial absence of brain tissue and of cranial vault; eyes and face present	Incomplete	Hemianencephaly, hemicrania, hemicephaly, meroacrania
		Complete	Total dysraphism, (amyelencephalus)
		Cranioarachnischisis	
Iniencephalus	Anomaly of foramen magnum and cervical vertebrae with retroflexion of head and absence of neck groove	May be associated with anencephaly, spina bifida, microcephaly, hydrocephalus and encephalocoele	Closed and open iniencephaly
Spina bifida Spina bifida occulta	Only one vertebral arch involved More than one arch involved with widening of spinal canal, with or without dermal sinus, lipoma, dermatological or neurological lesion	Uncomplicated Complicated	
Spina bifida cystica	Cystic extension of meninges, with or without neural tissue, outside the vertebral canal		Spinal hernia, (imperfect closure), (hydrocoele spinal)
		Meningocoele (only meninges)	Hydromeningocoele, congenital hernia dura mater
		Myelocoele (meninges and neural tissue involved)	Spina bifida aperta, (hydromyelocoele), rachischisis, meningomyelocoele, myelomeningocoele, myelocystocoele, myeloschisis
Cranial meningocoele.	Cystic expansion of meninges outside the cranium; does not contain brain tissue		Cerebral hernia, imperfect closure skull, cranium bifidum cystica (pericranial sinus), cerebral meningocoele, (sinus/fistula pericranii)
Encephalocoele	Cystic expansion of meninges outside the cranium, containing brain tissue		Hydroencephalocoele, hydroencephalomeningocoele, cephalocoele, encephalomeningocoele, encephalomyelocoele, congenital hernia brain, congenital hernia of foramina, congenital cerebral hernia, hernia cerebral endaural, meningoencephalocoele

(Elwood *et al*, 1992)

Anencephalus and spina bifida are known to have occurred in ancient times. A mummified anencephalic human foetus discovered in a sarcophagus at Hermopolis on the Nile was demonstrated at a meeting of the Academy of Sciences in Paris by Etienne Saint-Hilaire in 1826. Figure 1. 6 shows an artist's depiction of the anencephalic mummy. This specimen was acquired by the King of Prussia and formed part of the collection of the Berlin Museum, which was later destroyed by one of the Allied Force's bombing during the Second World War (Elwood *et al*, 1992). Earlier reports of neural tube defects date as far back as 1557 and 1642, which were included in a register of a collection of specimens of "monstrosity", assembled by Aldrovandus. Pre-historic NTD affected skeletal remains have also been found in California, Denver and Alaska. The National Museum of Ireland holds 111 complete (13) or partial (98) vertebral columns derived from various archaeological excavations. Four out of 50 of these adequately preserved sacra exhibit spina bifida. In those ancient times various explanations for neural tube defects were postulated. Both in Egypt and India, the belief that union between members of different species could be fertile and produce monstrous offspring was widely held. Another explanation for anencephaly arose because of the alleged similarity between monkeys and anencephalic infants, which give rise to the theory that it was dangerous for a pregnant woman to look at a monkey. These theories abound in other cultures also and the practise of infanticide was widespread throughout Europe as well as in the Far East.



(Elwood *et al*, 1992)

Figure 1.6. Artists depiction of an encephalic mummy

Modern day knowledge of NTDs

More recently scientific researchers proposed various hypothetical causes of neural tube defects. These included fertility drug use (Cornel *et al* 1989; Vollset, 1990), the use of anti-epileptic drugs during pregnancy (Robert & Guibaud, 1982; Jones *et al*, 1989), and a possible nutrient deficiency due to crop failure as a result of a hurricane in Jamaica in September 1988, where the incidence of NTDs increased by 2.5 per 1000 births (Duff *et al*, 1991) in the 10-18 months after the hurricane. Interestingly it is now known that all of these factors could have contributed to or precipitated a folate deficiency, which is now known to be involved in neural tube defect causation.

Around the same time several groups suspected that NTDs were caused by genetic factors. This theory arose because of the strong genetic predisposition to having a birth affected by an NTD i.e. having one affected birth increased the risk of having a subsequent affected birth. In addition some ethnic groups have a high prevalence of NTD births (e.g. those of Celtic origin) while other groups (e.g. African Americans) have low prevalence. The condition also showed variation with geography, time, season and socio-economic status suggesting a nutritional component. These considerations suggested that there was a genetic-nutrient interaction at play in NTDs.

The role of folate in NTDs was implicated from an early stage because of its critical role in cell division and growth. Early studies by Hibbard (1964) suggested that folate deficiency, as well as leading to maternal anaemia and placental abruption, might also cause congenital birth defects. Smithells followed this by doing an intervention trial with a multivitamin

preparation that contained folic acid in an attempt to investigate if they could prevent recurrence of NTDs in women who already had a previously affected pregnancy. In a study that followed (Smithells *et al*, 1980) a dramatic reduction in prevalence was achieved in women consuming the multivitamin peri-conceptionally. Mothers with a previously affected pregnancy who were planning a further pregnancy were given the multi-vitamin preparation Pregnavite Forte F (containing vitamins A, B, C, thiamine, riboflavin, pyridoxil, niacin, calcium, phosphorous, and 360µg folic acid). The subjects were instructed to take the vitamins for at least two months prior to conception and to continue for the first two months into the pregnancy. Mothers who were pregnant when referred to the study or who declined to take part made up the control group. In the first report (Smithells *et al*, 1980) there was 1 recurrence in 178 infants in the supplemented group and 13 out of 260 in the unsupplemented group. In a subsequent report (Smithells *et al*, 1983) recurrence rates were 0.7% for 454 supplemented mothers and 4.7% for 519 unsupplemented mothers. While this study provided very impressive reductions in recurrence rates the study design was criticised by many expert groups. This was largely due to lack of comparability between the supplemented and unsupplemented group. There were marked differences between groups in terms of social classes, and previous obstetric history, factors known to influence recurrence. It became apparent that a randomised double blind trial was the way forward.

This task was undertaken by the British Medical Research Council and the MRC vitamin study commenced in 1983. Its objective was to obtain information on the outcome of at least 2000 pregnancies unless a sufficiently clear result emerged sooner. By April 1991, after 1995 pregnancies, the results were sufficiently conclusive and the study ended early.

This trial consisted of a double-blind prevention trial with a factorial design conducted in 33 centres. A total of 1817 women at high risk of a recurrent NTD affected pregnancy were allocated to one of four groups – folic acid, other vitamins, both or neither. The results showed conclusively that 72% of NTDs could be prevented by the peri-conceptional ingestion of 4mg of folic acid in supplemental form. The study also showed that “other vitamins” did not reduce the incidence of recurrence.

Around the same time other centres were conducting similar studies. Czeizel & Dudas (1992) demonstrated that folic acid supplementation at 800µg/day prevented the occurrence of NTDs in 2104 women while NTDs occurred in 6 women out of 2052 not receiving supplementation. These findings suggested that a lower dose of folic acid than the 4mg dose used in the MRC study might be effective in preventing NTDs. In the USA and Australia several other observational studies of the effect of taking supplements containing folic acid had also been conducted (Milunsky *et al*, 1989; Mulinare *et al*, 1988; Bower & Stanley, 1992; Werler *et al*, 1993; Mills *et al*, 1989). 4 of these studies showed that women who happened to be taking folic acid supplements before and during pregnancy had a significantly lower rate of NTD affected pregnancies than women who were not taking supplements. One of these studies (Mills *et al*, 1989) did not demonstrate an effect of folic acid. A satisfactory explanation for this has not been found. The main studies that were carried out are summarised in the Tables 1.3 and 1.4. (CDC, 1992) The amount of folic acid taken in these positive effect studies varied from 4mg to 360µg. In the Smithells *et al* trial (1981) the effects of consuming 360µg were as great as in any of the other trials. These results lead to a consensus that 400µg would afford optimum protection.

This led to the UK Department of Health and the Centers for Disease Control (CDC), Atlanta, to recommend that women of child-bearing age should take a 400 μ g folic acid supplement daily peri-conceptionally for at least 1 month and for 12 weeks post-conceptionally to prevent an NTD occurrence. They further recommended that 4mg should be taken daily to prevent a recurrence of an NTD pregnancy. In light of this, mandatory folic acid fortification has been implemented in the USA to order to increase the average daily total folate population intake. The process through which this decision was reached will be discussed separately under a section entitled fortification. While it is now clear that folic acid taken peri-conceptionally confers a 72% protective effect the mechanism by which folic acid confers this effect are very much in the investigative stage. Recent evidence suggested that cobalamin status may also be an independent risk factor for NTDs (Kirke *et al*, 1993). This makes it highly probable that the primary cause of NTDs is a defective enzyme namely methionine synthase, as this is the only point in metabolism at which folate and cobalamin interact. Intensive research is ongoing at present.

Table 1.3. Observational studies involving folic acid

Study	Design	Subjects	Exposure	Results	Comments
Laurence, et al, 1981 (1)	Randomized controlled trial in Wales	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 4 mg of folic acid daily. Unsupplemented mothers took a placebo.	Supplemented women were given 4 mg of folic acid or placebo daily at least 1 month before conception through the 1st trimester.	2 NTD-pregnancies among 60 supplemented women. 4 NTD-pregnancies among 51 placebo-treated women. Relative risk = 0.40, not statistically significant.	60% reduction in risk.
UK MRC study, 1991 (2)	Randomized controlled multicenter trial in UK and Hungary	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 4 mg of folic acid daily. Unsupplemented mothers took a placebo.	Women given 4 mg of folic acid or placebo daily at least 1 month before conception through the 1st trimester.	6 NTD-pregnancies among 593 supplemented women. 21 NTD-pregnancies among 602 unsupplemented women. Relative risk = 0.28, $p < 0.05$.	72% reduction in risk.
Smithells, et al, 1983 (3)	Nonrandomized controlled multicenter trial in UK	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 0.36 mg of folic acid + multivitamins daily. Unsupplemented mothers took nothing.	Women given 0.36 mg of folic acid + multivitamins or reported no use from 1 month before conception through the 1st trimester.	3 NTD-pregnancies among 454 supplemented women. 24 NTD-pregnancies among 519 unsupplemented women. Relative risk = 0.14, $p < 0.05$.	86% reduction in risk.
Vergel, et al, 1990 (4)	Nonrandomized controlled trial in Cuba	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 5 mg of folic acid daily. Unsupplemented mothers took nothing.	Women given 5 mg of folic acid or reported no use from 1 month before conception through the 1st trimester.	0 NTD-pregnancies among 81 supplemented women. 4 NTD-pregnancies among 114 untreated women. Indeterminant protective effect, not statistically significant.	Complete protective effect.

(CDC, 1992)

Table 1.4. Intervention trials involving folic acid

Study	Design	Subjects	Exposure	Results	Comments
Laurence, et al, 1981 (1)	Randomized controlled trial in Wales	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 4 mg of folic acid daily. Unsupplemented mothers took a placebo.	Supplemented women were given 4 mg of folic acid or placebo daily at least 1 month before conception through the 1st trimester.	2 NTD-pregnancies among 60 supplemented women. 4 NTD-pregnancies among 51 placebo-treated women. Relative risk = 0.40, not statistically significant.	60% reduction in risk.
UK MRC study, 1991 (2)	Randomized controlled multicenter trial in UK and Hungary	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 4 mg of folic acid daily. Unsupplemented mothers took a placebo.	Women given 4 mg of folic acid or placebo daily at least 1 month before conception through the 1st trimester.	6 NTD-pregnancies among 593 supplemented women. 21 NTD-pregnancies among 602 unsupplemented women. Relative risk = 0.28, $p < 0.05$.	72% reduction in risk.
Smithells, et al, 1983 (3)	Nonrandomized controlled multicenter trial in UK	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 0.36 mg of folic acid + multivitamins daily. Unsupplemented mothers took nothing.	Women given 0.36 mg of folic acid + multivitamins or reported no use from 1 month before conception through the 1st trimester.	3 NTD-pregnancies among 454 supplemented women. 24 NTD-pregnancies among 519 unsupplemented women. Relative risk = 0.14, $p < 0.05$.	86% reduction in risk.
Vergel, et al, 1990 (4)	Nonrandomized controlled trial in Cuba	Pregnant women with prior NTD-affected pregnancy: Supplemented mothers took 5 mg of folic acid daily. Unsupplemented mothers took nothing.	Women given 5 mg of folic acid or reported no use from 1 month before conception through the 1st trimester.	0 NTD-pregnancies among 81 supplemented women. 4 NTD-pregnancies among 114 untreated women. Indeterminant protective effect, not statistically significant.	Complete protective effect.

(CDC, 1992)

5,10-Methylenetetrahydrofolate Reductase

Much of this research has focussed on the genes which code for enzymes involved in folate metabolism. This line of thinking developed because it became apparent that folic acid does not correct a simple nutritional deficiency. This became evident from studies which showed that most pregnant women carrying an affected foetus have folate concentrations within what has been traditionally defined as the “normal reference ranges” (Molloy *et al*, 1985; Kirke *et al*, 1993). Thus a metabolic abnormality was suspected. Progress in the elucidation of this defect was made with the identification of a common 667C—T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. This mutation reduces the activity and causes thermolability of the reductase enzyme (Frosst *et al*, 1995). Van der Put *et al* (1995) and Whitehead *et al* (1995) were the first to show that NTDs are associated with this thermolabile variant of the gene. In an Irish population group Whitehead *et al* reported an increased frequency of 677C—T homozygosity among NTD affected children. Van der put *et al* reported an increase in the frequency of 677C—T homozygosity in spina bifida of affected children and their parents in a Dutch population group. MTHFR catalyses the reduction of 5,10-methylene THF to 5-methyl THF, which is the point of entry of folate coenzymes in the remethylation of homocysteine to methionine; this reaction increases the availability of the major methyl donor, S-adenosylmethionine. Thus the production of S-adenosylmethionine would be reduced compromising the many SAM-dependent methyltransferase reactions. Critical functions that might potentially be affected include DNA methylation, phospholipid biosynthesis and methylation of proteins. Homocysteine or its derivatives would also become elevated and may be potentially toxic to the developing neural tube (Whitehead *et al*, 1995).

Since 5,10-methylene THF is a cofactor for this reaction, the mutation increases dependence on folic acid for adequate remethylation, because of the reduced enzyme activity. Additional folic acid would increase the substrate availability for the thermolabile reductase. This would result in an increase of the rate of conversion of homocysteine to methionine. Those affected may have to increase their folate intake to maintain normal red cell and plasma folate concentrations and normal levels of remethylation especially at times of rapid growth such as in early pregnancy (Fowler, 1997).

Molloy *et al* (1997) genotyped pregnant and non-pregnant women and reported that red cell and plasma folate concentrations were significantly lower in TT homozygous (thermolabile variant) women than homozygous CC (wildtype/normal) women. The authors concluded from this that the thermolabile variant creates an additional requirement for folate. This may have particular consequences in pregnancy where it has been demonstrated that decreased risk of NTDs is related to increased folate status. Daly *et al* (1995) showed that maternal red cell folate concentrations $< 150\mu\text{g/l}$ posed a risk for NTDs of 6.6 per 1000 births while red cell folates greater than $400\mu\text{g}$ posed a risk of 0.8 per 1000.

The frequency of this 677C—T polymorphism of MTHFR varies among racial and ethnic groups. Caucasian and Asian populations typically show rates of 12% for homozygous (TT) and up to 50% for heterozygous (CT). African-Americans exhibit very low incidence of TT genotypes whereas, European Caucasians exhibit substantial variation between 5-15%. (Bailey & Gregory, 1999). It is estimated that this mutation is responsible for approximately 13% of NTDs in an Irish population (Whitehead *et al*, 1995). Other genetic

defects involved in folate metabolism are likely to be identified in the future. Research is ongoing.

f) Other effects of folate deficiency on pregnancy

Many women in late pregnancy have overt signs of folate deficiency due to the extra demands on the mother's folate stores as a result of increased requirements for DNA synthesis and growth during this period, which occur as a result of expansion of blood volume, growth of the uterus, placenta and foetus (Chanarin, 1979). Foetal blood folate concentrations appear to be maintained at maternal expense as evidenced by several fold higher blood folate levels in new-born compared to maternal levels (Landon & Oxley, 1971). Megaloblastic anaemia is a common finding in pregnancy (Rodriquez, 1980). Adolescent pregnancies are a particular risk group because of their own increased requirement for growth coupled with the demands of pregnancy. Low folate status in pregnancy has been associated with low infant birth weight (O'Scholl *et al*, 1996) and cleft palates (Peer *et al*, 1958). An increased incidence of obstetric complications such as accidental haemorrhage, foetal malformation, and spontaneous abortions have also been reported in patients who developed folate deficiency in pregnancy (Chanarin, 1979).

1.10 Diagnosis of folate deficiency

In diagnosing folate deficiency a clinician must consider a wide range of parameters including clinical, morphological, and biochemical to achieve an accurate diagnosis as no test in isolation can provide specific or sensitive information sufficient to make a definitive

diagnosis (Lindenbaum & Allen, 1995). The process though which folate deficiency can be diagnosed was covered comprehensively by Lindenbaum & Allen (1995). This is summarised briefly in the section below.

Serum folate concentration-This is clinically useful but should not be used in isolation to make a diagnosis as patients with clear-cut megaloblastic anaemia due to folate deficiency can present with only slightly decreased serum folate levels or can even fall into normal reference ranges. Also recent dietary folate intake can cause levels to fluctuate.

Erythrocyte folate concentration-This is a better marker of true folate status as it provides a direct measurement of tissue coenzyme concentrations in cells produced by the bone marrow (Lindenbaum & Allen, 1995). Erythrocyte folate is low in the majority of patients with megaloblastic anaemia and the degree of depression correlates with the severity of the anaemia (Hoffbrand *et al*, 1966). Unlike serum folate levels this provides information on long-term status as the life span of erythrocytes is approximately 120 days.

Serum homocysteine concentration- Total serum homocysteine levels are a functional test of folate coenzyme activity, which reflects tissue deficiency of methyltetrahydrofolate as a substrate in the methionine synthase reaction (Allen *et al*, 1990; Stabler *et al*, 1988). The sensitivity of serum homocysteine concentration in the diagnosis of patients with proven megaloblastic anaemia due to folate deficiency was in the region of 90% in a study carried out by Savage *et al* (1994). Serum homocysteine levels are also elevated in cobalamin deficiency so care must be taken to make a correct diagnosis.

Deoxyuridine suppression test-The ability of added deoxyuridine to suppress the incorporation of tritiated thymidine into DNA by bone marrow cells is tested. This technique is costly and cumbersome, hence is only suitable in a research setting.

Cobalamin deficiency tests- Due to the similar presentation of the anaemias caused by folate and cobalamin deficiency, care must be taken to rule out the possibility of cobalamin deficiency, as treatment with folate supplementation would not be appropriate. Serum cobalamin and serum methylmalonic acid used in combination are useful diagnostic tools of cobalamin deficiency.

Megaloblastic bone marrow-Megaloblastic changes in the bone marrow are seen in folate deficiency although a definitive diagnosis cannot be made on this alone as a cobalamin deficiency or an anaemia of a chronic disease could also cause a similar presentation.

Neutrophil Hypersegmentation-is often an early sign of dysfunctional granulopoiesis due to folate deficiency. Again this is not specific to folate deficiency and is frequently witnessed in cobalamin deficiency, as well as post anti-cancer treatments.

Macroovalocytosis-can be seen in peripheral blood films in severe anaemia due to folate deficiency. Again this presentation is not specific to folate deficiency.

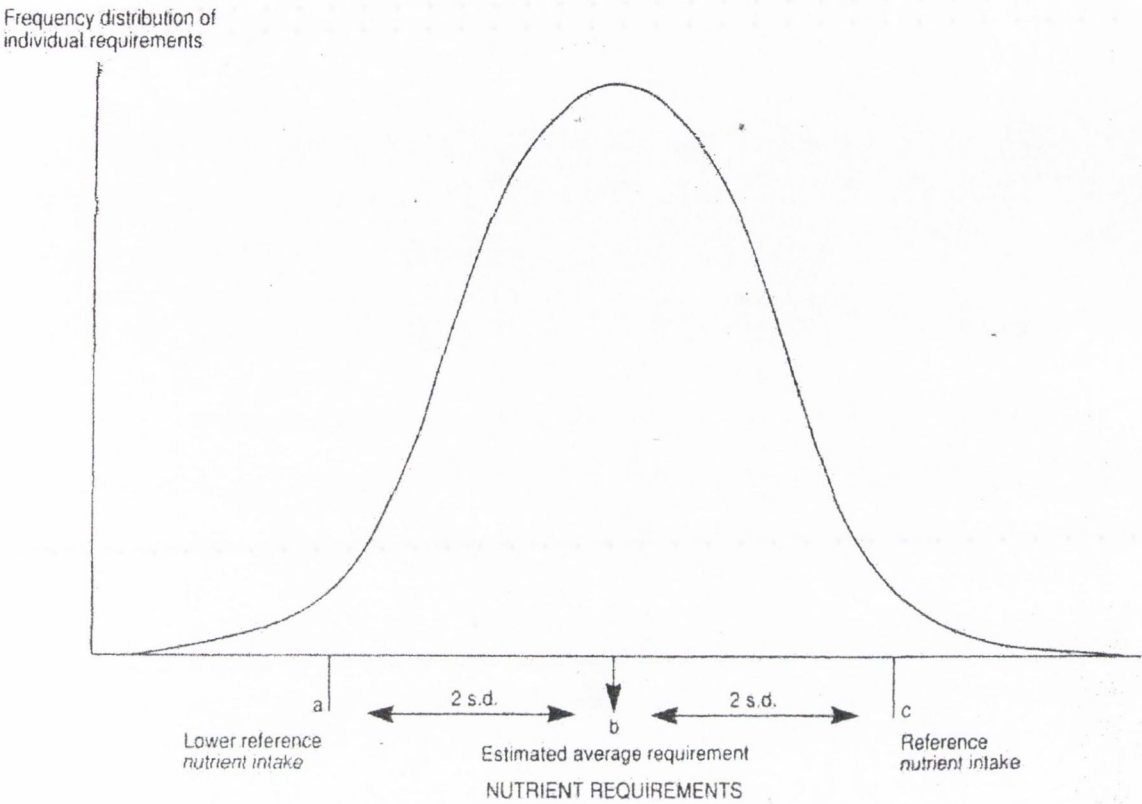
Hence, all of the above diagnostic tests have limitations in terms of sensitivity and specificity. However a thorough history, physical examination and combined use of the appropriate diagnostic tests mentioned should provide a correct diagnosis.

1.11 Folate Requirements and recommendations

Requirements for a nutrient differ from one individual to another. Classically the requirement for a nutrient has been described as the amount of that nutrient needed to prevent clinical signs of deficiency. More recently thinking has progressed to providing for additional needs so that demands can be met during periods of low intake or increased demand without detriment to health status. Hence emphasis has shifted to the role of nutrition in maintaining optimal health. The most recent amendment to recommended dietary intakes in the UK were published in the COMA (Commission on Medical Aspects of Food Policy) report of 1991. The existing nomenclature was changed to a new working title, which collectively comprised the "Dietary Reference Values". The Dietary Reference Values are diagrammatically illustrated in Figure 1.7 for ease of explanation. The Estimated Average Requirement (EAR) represents the mean requirement for a given nutrient. The Reference Nutrient Intake (RNI) falls 2 standard deviations above the EAR. Intakes above this will almost certainly be adequate. The Lower Reference Nutrient Intake (LRNI) falls 2 standard deviations below the EAR. This point represents the lowest intakes, which will meet the needs of some individuals in the group. Intakes below this will almost certainly be inadequate for most individuals within that group. This distribution curve was included here because of its direct relevancy to this project. This will be referred to later in the introduction. In the COMA report dietary reference values for folate were calculated individually for adults, infants, children, pregnant women, lactating mothers and the elderly because of the diversity of requirements. These values were arrived at via various means including experimental studies referred to as depletion-repletion studies. This is undertaken by removing all sources of folate from the diet and observing for the clinical and

haematological changes associated with deficiency. After the depletion period folic acid is introduced back into the diet in varying doses and the regression of the symptoms are observed. One of the most famous of these studies was the depletion study carried out by Herbert (1962) in which he placed himself on a folate deficient diet. Other means by which DRVs were devised for folate are based on average population dietary intakes and a follow-up study of the resultant folate status for that population to observe for deficiencies. One such type of study was carried out in Canada. This was described by Hoppner & Lampi (1980). Traditionally pregnancy has been associated with megaloblastic anaemia (Wills, 1931) and in more recent times before fortified foods were widely available in well-nourished western societies this condition was widespread. In addition findings by Mc Partlin *et al*, (1993) in human subjects and Mc Nulty *et al*, (1993) in rats showed that folate catabolism is increased during pregnancy. This provided further evidence of an increased requirement for folate during this time. The COMA report was drafted before the MRC (1991) results were available and the recommended intake during pregnancy was an additional 100µg/day. The DoH now recommends 400µg/d to prevent NTD occurrence and 4mg/d to prevent NTD recurrence. During lactation folate secreted in breast milk averages about 40µg/d, hence the additional requirement for lactating mothers was estimated to be 60µg to allow for incomplete absorption and utilisation of folates from the diet. In the elderly folate deficiency occurs frequently, however there is no evidence of an increased requirement, hence DRVs for folate were estimated to be the same as the other adult age groups for males and females. Table 1.5 shows these DRVs for folate from the COMA report of 1991. Similar dietary folate recommendations have been published in the USA, Canada and by the World Health Organisation (WHO). An overview of these is presented in Table 1.6. The Irish and UK values have been included for comparative purposes. The

section on "high intakes " in the COMA publication consists of 6 lines only, which state that high intakes of folate may reduce zinc absorption and obscure a diagnosis of pernicious anaemia. It further states that the danger of toxicity seems slight. This is an under-representation of the issues relating to safety. In the USA a safe upper limit of 1mg/day has been set by the Food and Drugs Administration (FDA) due to potentially unsafe effects of consuming large doses of folic acid. This issue of safety at high intakes of folic acid is the main focus of this research study because of the role of folic acid in obscuring the early diagnosis of pernicious anaemia. In order to begin to understand this well documented problem it is necessary to discuss the relationship between B₁₂ and folate/folic acid in cellular metabolism.



(DRVS, 1991)

Figure 1.7. Distribution curve for the estimation of dietary reference values.

Figure 1.5. UK Dietary Reference Values for Folate ($\mu\text{g/L}$).

Age	Lower Reference Nutrient Intake	Estimated Average Requirement	Reference Nutrient Intake
0-3 months	30	40	50
4-6 months	30	40	50
7-9 months	30	40	50
10-12 months	30	40	50
1-3 years	35	50	70
4-6 years	50	75	100
7-10 years	75	110	150
<i>Males</i>			
11-14 years	100	150	200
15-18 years	100	150	200
19-50 years	100	150	200
50+ years	100	150	200
<i>Females</i>			
11-14 years	100	150	200
15-18 years	100	150	200
19-50 years	100	150	200
50+ years	100	150	200
Pregnancy			+ 100
Lactation:			
0-4 months			+ 60
4+ months			+ 60

(DRVS, 1990)

Figure 1.6. Recommended dietary folate ($\mu\text{g/L}$) intakes for USA, UK, Canada and the WHO.

Age/Sex category	Recommended Dietary Allowance, 1980 [2]	Recommended Dietary Allowance, 1989 [1]	United Kingdom Dietary Reference Value [3]	Canadian Recommended Nutrient Intake [4]	WHO/FAO Safe Level of Intake [5]
Infants (months)					
0.0-0.5	30	25	50	50	16-24
0.5-1.0	45	35	50	50	32
Children (yr)					
1-3	100	50	70	65-80	50
4-6	200	75	100	90	50
7-10	300	100	150	125	102
Males (yr)					
11-14	400	150	200	170	170
15-18	400	200	200	150-185	200
19-24	400	200	200	210	200
25-50	400	200	200	220	200
51+	400	200	200	220-205	200
Women (yr)					
11-14	400	150	200	180	170
15-18	400	180	200	145-160	170
19-24	400	180	200	175	170
25-50	400	180	200	175	170
51+	400	180	200	190	170
Pregnancy	800	400	+ 100	300	370-470
Lactation					
1st 6 months	500	280	+ 60	+ 100	270
2nd 6 months	500	260	+ 60	+ 100	270

(Bailey, 1995)

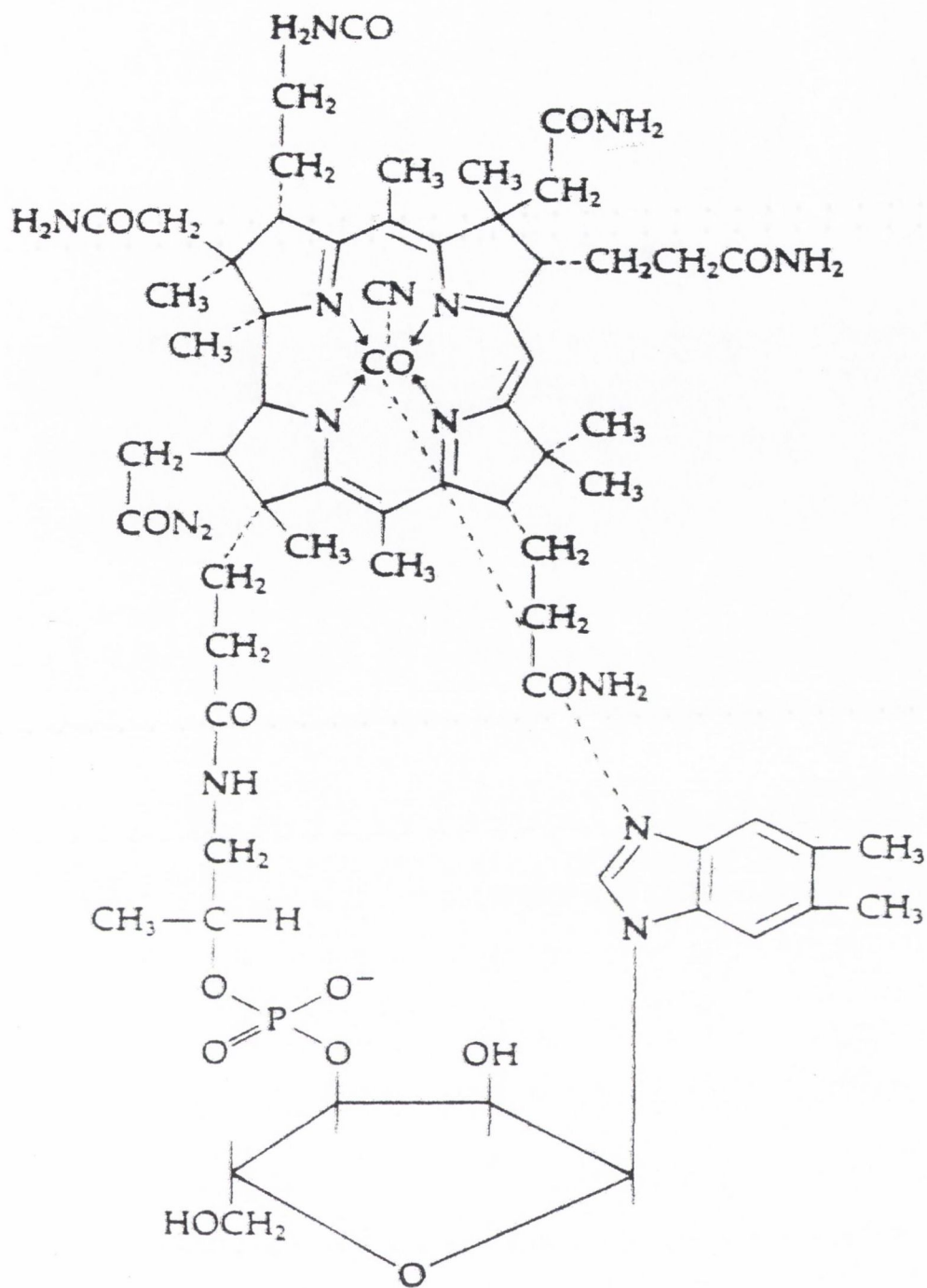
Vitamin B₁₂ (Cobalamin)

1.12 Structure and nomenclature

Vitamin B₁₂ (cobalamin) was first isolated as cyanocobalamin in 1948 (Rickes *et al*, 1948; Smith & Parker, 1948) and its structure was elucidated by x-ray crystallography in 1956 (Hodgkin *et al*, 1956). Vitamin B₁₂ is a member of a family of related compounds called corrinoids, a term used to describe all compounds that contain a corrin nucleus made up of a tetrapyrrolic ring structure. The centre of the tetrapyrrole contains a cobalt ion that can be variously attached to methyl, deoxyadenosyl, hydroxy, or cyano groups. The former two are the naturally active forms; the latter two are converted into them *in vivo*. The methyl form attaches to methionine synthase (Poston & Stadman, 1975) and the adenosyl form attaches to methylmalonyl CoA mutase (Babior, 1975) in order to perform their coenzyme functions. Figure 1.8 demonstrates the structure of vitamin B₁₂.

1.13 Sources

Vitamin B₁₂ is synthesised by bacteria, which is its only source. It is present in all forms of animal tissues, which acquire the vitamin indirectly from bacteria (Chanarin, 1979). Thus vitamin B₁₂ is not present in vegetables or fruit hence deficiency often presents in vegans. The highest levels of dietary folate are found in animal liver as approximately 50% of the body's stores of vitamin B₁₂ are in the liver (Chanarin, 1979). Dietary meat and fish are also good sources (DRVs). UK dietary reference values are shown in table 1.7.



(Mervyn, 1971).

Figure 1.8. Structure of cobalamin.

Figure 1.7. UK Dietary Reference Values for Vitamin B-12.

Age	Lower Reference Nutrient Intake	Estimated Average Requirement	Reference Nutrient Intake
0-3 months	0.1	0.25	0.3
4-6 months	0.1	0.25	0.3
7-9 months	0.25	0.35	0.4
10-12 months	0.25	0.35	0.4
1-3 years	0.3	0.4	0.5
4-6 years	0.5	0.7	0.8
7-10 years	0.6	0.8	1.0
<i>Males</i>			
11-14 years	0.8	1.0	1.2
15-18 years	1.0	1.25	1.5
19-50 years	1.0	1.25	1.5
50+ years	1.0	1.25	1.5
<i>Females</i>			
11-14 years	0.8	1.0	1.2
15-18 years	1.0	1.25	1.5
19-50 years	1.0	1.25	1.5
50+ years	1.0	1.25	1.5
Pregnancy	No increment		
<i>Lactation:</i>			
0-4 months			+0.5
4+ months			+0.5

(DRVs, 1991)

1.14 Absorption

The process of vitamin B₁₂ absorption has been reviewed and summarised by Herbert (1985 and 1994), Baik & Russell (1999), Weir & Scott (1999) and Scott (1997 and 1999). The main stages are described in the section below. Intestinal vitamin B₁₂ absorption occurs in several phases. After ingestion vitamin B₁₂ is released from dietary protein by a combination of the action of mastication and the presence of gastric acid (Cooper & Castle, 1960) and pepsin (Kittang & Schjonsby, 1987). The next phase of absorption were first elucidated by Allen *et al* (1987). They suggested that after its release from food proteins vitamin B₁₂ is not bound to intrinsic factor (IF) as previously thought but becomes bound to so-called R binders, a range of glycoproteins secreted in saliva. These glycoproteins include transcobalamin 2 (TC-2) and haptocorrins (HC). The vitamin B₁₂ remains attached to the R binders in the alkaline environment of the intestine until the R binders are degraded by pancreatic enzymes. Vitamin B₁₂ is then transferred exclusively to IF which has a high affinity for it at this pH. The IF is a glycoprotein produced by the parietal cells of the stomach. The vitamin B₁₂-IF complex then passes down to the ileum where it is absorbed by phagocytosis after attachment to specific membrane receptors. This attachment has a high degree of specificity and will not attach vitamin B₁₂ binding proteins other than IF. The attachment is calcium dependent and has a limited capacity, estimated to be approximately 1.5-2.0µg/meal (approximstely 1% of total). The system takes a number of hours to recover. Crystalline vitamin B₁₂ exhibits similar absorption capacity (Mollin 1959; Chanarin 1990). Vitamin B₁₂ can also be absorbed by passive diffusion. This was illustrated by Chanarin (1969, 1979) who showed that after saturation of the IF system with a meal containing approximately 1.5-2.0µg additional vitamin B₁₂ is absorbed by passive

diffusion. There is a large enterohepatic circulation of vitamin B₁₂ with intestinal re-absorption which is dependent on IF (Chanarin, 1979). The relevance of this enterohepatic circulation to pernicious anaemia will be dealt with in a later section (1.18). This mechanism of vitamin B₁₂ absorption is illustrated diagrammatically in Figure 1. 9.

Absorption of vitamin B₁₂ in man

Ingested food - vitamin B₁₂ bound to food proteins

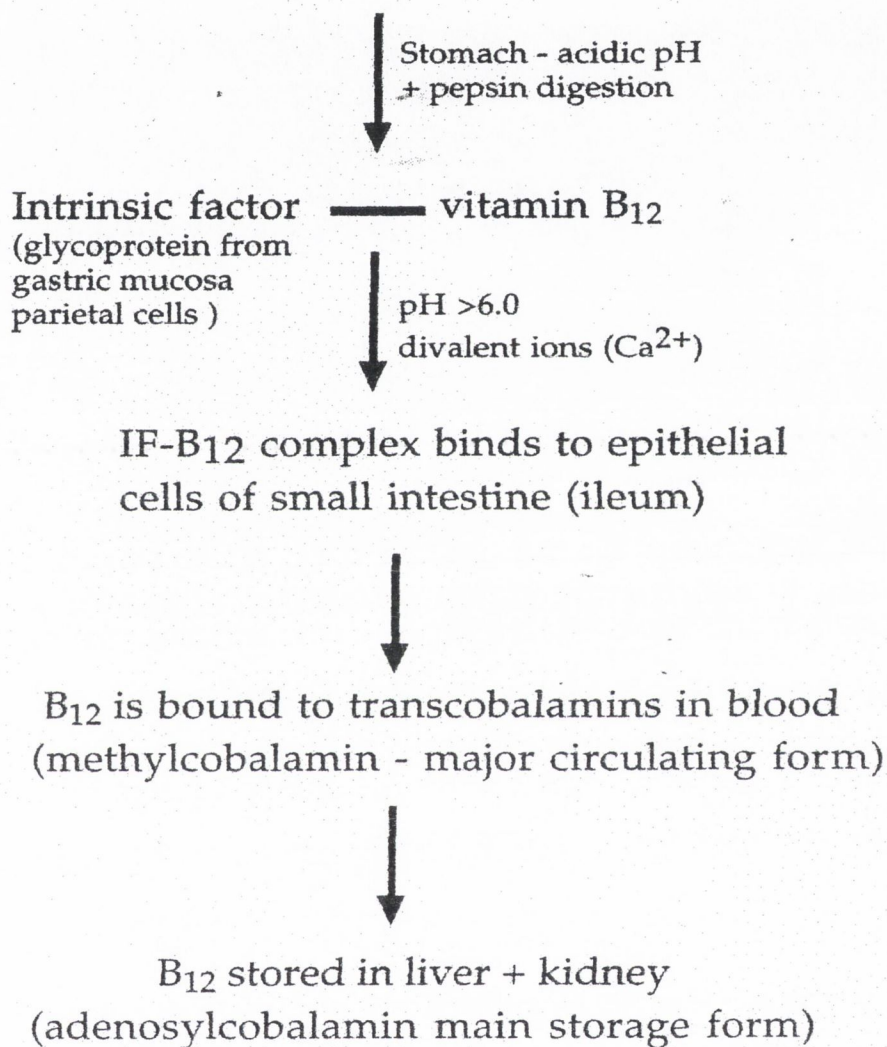


Figure 1.9. Schematic diagram of cobalamin absorption

1.15 Transport

The absorbed vitamin B₁₂ is converted to methyl cobalamin and adenosylcobalamin. This is thought to occur within the mitochondria of the ileal cell and appears in the portal blood stream bound to TC-2 approximately 3 hours after absorption. TC-2 is essential for the transport of cobalamins (CBL) into all cells of the body (Chanarin, 1990) where it is endocytosed by specific cell wall receptors.

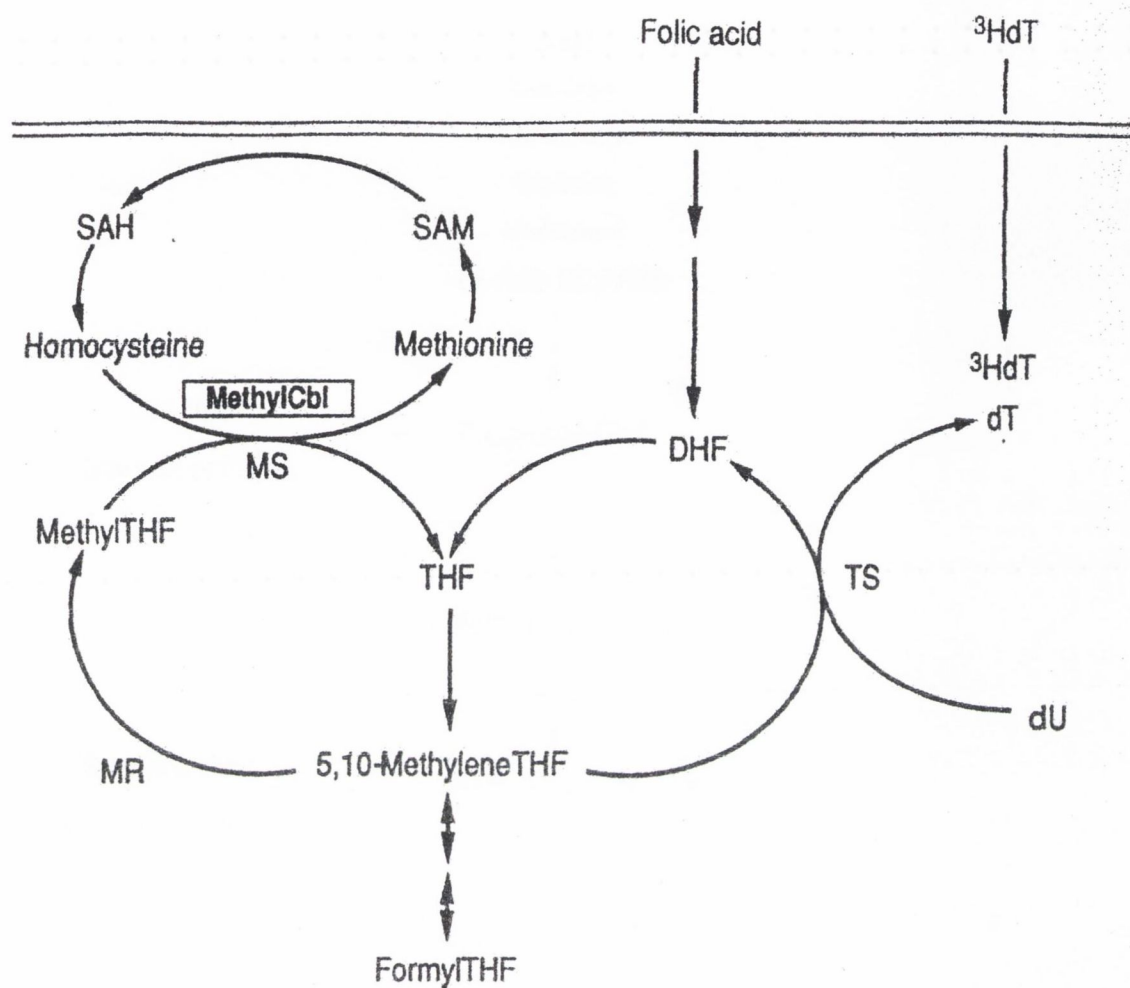
1.16 Metabolism

The TC-2-CBL complex is internalised by adsorptive endocytosis using a specific high-affinity receptor (Fenton & Rosenberg, 1995). TC-2 is then degraded by lysosomal proteases, releasing the cobalamin, which exits from the lysosome and is converted to either methyl CBL in the cytosol, which binds to methionine synthase or to adenosyl CBL in the mitochondria, which binds with methylmalonyl-CoA (Weir & Scott, 1999). Plasma cobalamin is mainly methyl CBL (60-80%). The remainder is OH-CBL, and adoCBL. Excretion of cobalamin occurs via cellular apoptosis into the gastrointestinal tract, kidney and skin. Daily losses are estimated to be in the region of 0.1% of total body stores (Scott, 1997).

1.17 Metabolic interactions between cobalamin and folate/folic acid

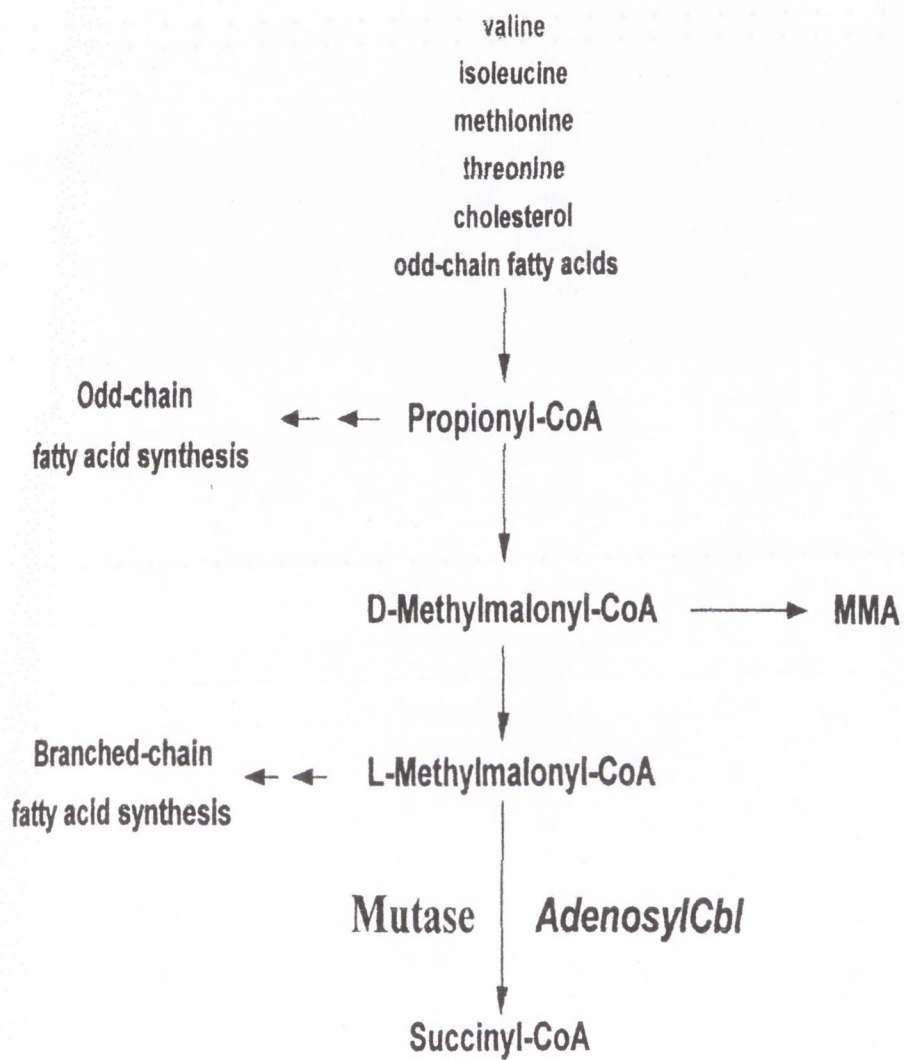
Cobalamin is known to be required for two metabolic reactions in humans (Reviewed by Savage & Lindenbaum, 1995). In the L-methylmalonyl-coenzyme A (CoA) mutase

reaction (figure 1.10), cobalamin serves as a coenzyme in the form of adenosylCBL for the isomerization of L-methylmalonyl-CoA to succinyl-CoA, the final step in the conversion of propionic acid to succinyl-CoA. Folate has no role in this reaction. Both folate and cobalamin are required for the second reaction, which is the methyl transfer reaction catalysed by methionine synthase (figure 1.11). In this reaction cobalamin serves as a cofactor (in the form of methylCBL) for the recycling of homocysteine to methionine and the demethylation of 5-methyltetrahydrofolate to tetrahydrofolate. This pathway is the only metabolic pathway in which cobalamin and folate are directly interrelated. Tetrahydrofolate serves as a source of active folate coenzyme forms for a host of biochemical reactions, including as previously mentioned purine, pyrimidine and amino acid synthesis.



(Savage & Lindenbaum, 1995)

Figure 1.10. Methyl transfer reaction



(Savage & Lindenbaum, 1995)

Figure 1.11. L-methylmalonyl-coenzyme A (CoA) mutase reaction..

1.18 Cobalamin Deficiency

Cobalamin deficiency is rare in the general population due to the body's huge reserves and the high content of cobalamin found in a normal diet. Certain sub-groups of the population are at risk however of cobalamin deficiency due to insufficient dietary intake of the vitamin namely vegans because of their animal product free diet or individuals with extreme animal protein deficiency (Weir & Scott, 1999). Malabsorption of cobalamin in the intestine as a result of various medical conditions can also lead to cobalamin deficiency. By far the most important of these is the auto-immune disease pernicious anaemia (Chanarin 1979). In most cases of pernicious anaemia auto-antibodies are produced against the parietal cells causing them to atrophy. The cells then lose their ability to produce intrinsic factor and also to secrete Hydrochloric acid (HCL). Pernicious anaemia thereby causes malabsorption of dietary cobalamin and also results in an inability to reabsorb the cobalamin that is secreted daily in the bile (usually calculated to be between 0.3-0.5 μ g/day). Interruption of this enterohepatic-circulation of cobalamin causes the body to go into a significant negative balance of the vitamin (Chanarin 1979). Once a negative balance is established anaemia presents, which is morphologically indistinguishable from that of folate deficiency. If the anaemia is not quickly corrected with cobalamin replacement a subsequent neuropathy often termed sub acute degeneration of the spinal cord (SACD), progresses quickly. This neuropathy does not present in folate deficiency except in very rare and extreme cases. The neuropathy presents as tingling of the fingers, ataxia, loss of memory in the early stages with eventual progression to paralysis and death if untreated. The nerve damage is untreatable and irreversible once it reaches a certain point. The incidence of pernicious anaemia increases markedly with age with the incidence at 6 months of age being one per

million whereas at 70 years of age it is approximately one per two hundred people (Herbert, 1985).

A more common problem (estimated to be present in one-quarter of the elderly population, Carmel, 1995) implicated in cobalamin deficiency is that of achlorhydria which is often associated with *Helicobacter pylori* infection (Baik & Russell, 1999). This results in a progressive reduction with age in the ability of the parietal cells to secrete HCL. This prevents the release of food-bound cobalamin but does not interfere with the absorption of free cobalamin such as in fortified foods or supplements. Furthermore the re-absorption of biliary cobalamin is not interfered with and therefore the negative balance seen in pernicious anaemia does not occur. However a long-term continuous reduction in the amount of cobalamin absorbed from dietary sources will result in overt deficiency. This area is covered comprehensively by Scott (1999) and Herbert (1985).

Other less common causes of cobalamin deficiency include intestinal malabsorption of cobalamin due to diseases such as diverticulitis, tropical sprue, an infectious disease of unknown causative agent endemic in the Caribbean and Southeast Asia (Herbert 1985) and intestinal infestation with fish tape-worm. In addition congenital defects in Transcobalamin 2 may interfere with cobalamin binding (Weir & Scott 1999) and pancreatic insufficiency with resultant reduction in pancreatic enzymes can lead to impaired digestion of HC and elevation of intestinal pH, both of which are necessary for the transfer of cobalamin from HC binders to IF. Finally certain drugs such as metformin used in the treatment of diabetes can also lead to cobalamin malabsorption which can result in megaloblastic changes (Scott & Weir, 1980).

1.19 Cobalamin deficiency and the “methyl trap hypothesis”

Deficiency of cobalamin or folate leads to the same metabolic defect: a lack of tetrahydrofolate and folate coenzymes required for the synthesis of purines and pyrimidines, including thymidylate, which is incorporated into DNA (Reviewed by Savage and Lindenbaum, 1995). Failure of DNA synthesis causes megaloblastic haematopoiesis, hence both deficiencies result in a similar morphological abnormality. MethylTHF must be converted to THF in order that other coenzymes can be formed. In folate deficiency methylTHF is not available for conversion to THF. In cobalamin deficiency, methylTHF is available but cannot be demethylated to THF because of the diminished activity of methionine synthase. This is commonly referred to as the “methylfolate trap hypothesis” which, postulates that impaired methionine synthase activity prevents the conversion of methyl THF to THF and results in a trapping of methylTHF. This deprives the cell of active folate coenzyme (Herbert & Zalusky, 1962). In addition methionine cannot be formed from homocysteine and S-adenosylmethionine production is diminished. This promotes the activity of 5,10-methyleneTHF reductase, which catalyses the conversion of 5,10-methyleneTHF to methylTHF. This reduction step is irreversible (Kutzbach & Stockstad, 1967) and leads to further “trapping” of methylTHF.

An additional problem arises in the retention of folates by cells. In order for folates to be retained by cells a polyglutamate chain must be attached by the enzyme folate polyglutamate synthase (Shane, 1995). This enzyme has negligible activity towards methylTHF (Savage & Lindenbaum, 1995). In cobalamin deficiency, most of the folate is

methylTHF which, cannot be converted by methionine synthase and hence cannot be retained by cells.

There are still a number of questions over the validity of the methyl trap hypothesis and several other theories have been put forward. Research by Scott *et al* (1981) reported that SCD is due to a methionine deficiency as a result of diminished methionine synthase activity in the face of cobalamin deficiency. This conclusion was reached from studies carried out on monkeys maintained in an environment of (nitrous oxide) N_2O , which was previously shown to inhibit methionine synthase in rats (Deacon *et al*, 1978) and cause a neuropathy in dentists using it as an anaesthetic agent (Layzer, 1978). One group of monkeys were supplemented with methionine and the other group remained unsupplemented. The supplemented monkey group did not develop any neurological abnormalities. The unsupplemented group developed spinal cord damage within weeks consistent with patients who had died from SCD after being deficient for many years. This led to the suggestion that the primary lesion in SCD is an inability to synthesise methionine as a result of the reduced methionine synthase activity. The hypothesis is that N_2O completely inhibits methionine synthase, thereby preventing the rapidly dividing cells from incorporating plasma 5-methyl THF into the intracellular pool by polyglutamate biosynthesis, which is necessary for DNA biosynthesis. The absence and disorganisation of intracellular folate produces the megaloblastic change (Scott & Weir, 1983). The SCD is thought to be caused by a deficiency of SAM-controlled methylation reactions occurring as a result of methionine synthase deficiency, which in turn blocks endogenous methionine synthesis and hence the methylation reactions necessary to maintain myelin. This theory is supported clinically by the fact that children born with 5,10-methyleneTHF reductase

deficiency, associated with diminished methionine and SAM production exhibit demyelination (Erbe, 1986).

Several other theories accounting for the defect have also been put forward by other groups. One such theory suggested that a defect in the cobalamin dependent L-methylmalonyl-CoA mutase reaction in which adenosylCBL serves as a cofactor for the conversion of L-methylmalonyl-CoA to succinyl-CoA may be responsible for the neuropathy. In this hypothesis, blockage of the mutase pathway causes accumulation of methylmalonyl-CoA, and propionyl-CoA, which substitute for malonyl-CoA and acetyl-CoA, respectively in fatty acid synthesis. This leads to the incorporation of defective odd-chain (Frenkel, 1973) and branched-chain fatty acids in myelin (Cardinale *et al*, 1970). This argument does not hold up however as patients with inherited defects of L-methylmalonylCoA-mutase do not develop the neurological complications of cobalamin deficiency (Metz *et al*, 1992) and patients born with in-born errors of cobalamin metabolism affecting the methionine synthase pathway but not impairing the mutase reaction have presented with nervous system disorders (Watkins & Rosenblatt, 1989; Hall, 1990).

More recently Buccellata *et al*, (1999) hypothesised that tumour necrosis factor (TNF- α) (a cytokine produced by the central nervous system) is involved in the pathogenesis of sub acute combined degeneration (SCAD) like lesions in the brain of totally gastrectomized rats. The authors showed that TNF- α is over-expressed in the spinal cord of B₁₂ deficient totally gastrectomized rats. Treatment with B₁₂ subsequently substantially reduced the levels of TNF- α . In addition normal rats were treated with TNF- α . They developed lesions

very similar to those seen in the totally gastrectomized rats, strongly suggesting that TNF- α has a physiological role to play in SACD. However the sequence of molecular events that triggers this response awaits investigation (Buccellata *et al*, 1999).

1.20 Diagnosis of cobalamin deficiency

As is the case with folate deficiency (see section 1.10) early diagnosis of cobalamin deficiency remains very challenging. The subject is comprehensively reviewed by Savage & Lindenbaum (1995). Megaloblastic changes present in the bone marrow of patients with anaemia due to cobalamin or folate deficiency cannot be differentiated morphologically and routine tests lack sensitivity and specificity. However a number of standard techniques are used.

Serum cobalamin assay

In most laboratories serum cobalamin remains the standard method for diagnosing deficiency, defined as less than 150ng/L. However significant deficiency may occur in the face of low normal, or only marginally reduced, levels of serum folate especially in the elderly (Weir & Scott, 1999). Evidence of deficiency has been found in patients, whose serum cobalamin ranged, from 200-300ng/l. Measurement of the substrates of the two cobalamin-dependent enzymes methylmalonic Acid (MMA) and homocysteine is a relatively new and more accurate method of assessing the presence of intracellular cobalamin deficiency.

Methylmalonic Acid

Plasma MMA levels rise in cobalamin deficiency but not in folate deficiency (Weir & Scott, 1999), therefore is a sensitive marker of absolute or functional cobalamin deficiency. In addition elevated MMA has been observed to precede a drop in serum cobalamin thereby allowing earlier diagnosis. MMA can also be measured in urine as its excretion correlates well with plasma levels.

Homocysteine

Plasma homocysteine levels can become elevated in a) deficiency states of folate, cobalamin and pyridoxine b) inborn errors of the enzymes associated with homocysteine metabolism namely cystathionine synthase, methylene reductase, and methionine synthase, and c) defects associated with the synthesis of the cobalamin coenzymes required for the normal function of methionine synthase.

Hence, all of the above diagnostic tests have limitations in terms of sensitivity and specificity. A clear-cut response to cobalamin-therapy, if demonstrable, is the most reliable evidence that a haematological or neurological abnormality is due to cobalamin deficiency.

Schillings test

After a diagnosis of vitamin B₁₂ deficiency has been established the schillings test is useful in differentiating between disorders associated with malabsorption of vitamin B₁₂. In this test, patients are first treated with parenteral vitamin B₁₂, to saturate body stores and are then given radiolabeled vitamin B₁₂ by mouth. Thereafter, urine is collected and analysed for radioactive B₁₂. Failure of vitamin B₁₂ to appear in the urine indicates impaired

absorption of the vitamin. During a repeat test, intrinsic factor is administered orally along with the labelled vitamin B₁₂. If radioactive B₁₂ now appears in the urine, this indicates that the patient lacks intrinsic factor and must therefore have a disease of the gastric fundal mucosa. In contrast if radioactive B₁₂ does not appear in the urine, this suggests the presence of another pathologic process, such as chronic pancreatitis, disease of the terminal ileum, or overgrowth of bacteria in the small intestine (Heuman *et al*, 1997).

1.21 Treatment of cobalamin deficiency and masking effects of folic acid.

Folate deficiency is responsive to treatment with folic acid supplementation, which replenishes the supply of folate coenzymes. Cobalamin deficiency must be treated with intramuscular injections of cobalamin in the form of cyanocobalamin or hydroxycobalamin, which corrects the anaemia and treats the neuropathy, as oral doses will not be adequately absorbed. This is in the form of monthly injections for an entire lifetime. The anaemia associated with cobalamin deficiency is also responsive to treatment with folic acid under certain conditions. Under normal conditions folic acid ingested orally emerges from the intestine into the portal circulation as 5-methyl THF. This form will not be retained by marrow cells, as it requires methionine synthase to convert it to tetrahydrofolate. However folic acid above certain threshold doses, shown to be 266µg or greater (Kelly *et al*, 1997) will saturate the normal absorptive mechanism and instead of appearing in the circulation as the normal metabolite 5-methylTHF, it presents as unmetabolised folic acid. Unmetabolised folic acid can gain direct entry into marrow and other cells by its reduction to DHF and subsequently to THF by the enzyme DHFR. This process is not dependent on cobalamin. The THF is then readily polyglutamated and retained by cells and can be used

in DNA division correcting the anaemia. This treatment will not treat the neuropathy however as it cannot restart the methylation cycle caused by the cobalamin deficiency. Thus the neuropathy can progress undetected due to the correction of the haematological manifestation. This is commonly referred to in the literature as “masking”. The extent to which this process occurs depends on the amount of unmetabolised folic acid available and the frequency with which it is present.

Several experimental studies have demonstrated the uptake of folic acid in cobalamin deficiency. Tisman & Herbert (1973) reported that bone marrow cells from B₁₂ deficient patients took up much less 5-methylTHF than normal cells but showed no significant reduction in folic acid uptake. The authors further showed that cell uptake of 5-methyl THF is B₁₂ dependent so in B₁₂ deficiency 5-methyl is not taken up. Hence 5-methylTHF cannot be used to precipitate a haematological response in the absence of vitamin B₁₂ but folic acid can. Metz *et al* (1968) showed that the defective incorporation of deoxyuridine into thymine DNA in B₁₂ deficient cells was corrected by the addition of folic acid but not by 5-MethylTHF. This was later backed up by Zittoun *et al* (1978) and Ganeshaguru & Hoffbrand (1978). These studies illustrate that the haematological symptoms of megaloblastosis from vitamin B₁₂ deficiency can be masked if unmetabolised folic acid is present in the circulation. This problem does not present if cells are exposed to the natural form of the vitamin 5-methylTHF, as this form will remain trapped in the cell due to the reduced methionine synthase activity. Thus unmetabolised folic acid which presents in the circulation after ingestion of threshold doses of supplements or in fortified food has the potential to cause potentially lethal effects. This is substantiated in a number of studies, which addressed this issue (see next section).

1.22 Clinical evidence of “masking effect” of folic acid.

After the synthesis of folic acid by Angier *et al* (1945) folic acid was shown to be effective in treating patients with pernicious anaemia diagnosed by the presence of morphological abnormalities. Some 60 patients were treated with folic acid orally in doses varying from 10-40mg daily (Vilter *et al*, 1954; Doan *et al*, 1946; Wilkinson *et al*, 1946) but some received up to 400mg/day (Spies, 1946). The results were very impressive. An improvement in the haematological response and general well being was achieved. However this early enthusiasm soon subsided with reports that treatment with folic acid was unable to treat the neuropsychiatric abnormalities. Meyer (1947) was the first to report that folic acid reversed the haematological complications of pernicious anaemia but was unable to correct or prevent the neuropathy. This has since been reported and confirmed in many, many publications some of which are listed here, Heinle & Walsh, 1947; Bethal & Surgis, 1948, Ross *et al*, 1948; Chodos & Ross, 1951; Vilter *et al*, 1950; Spies & Stone, 1947; Wagley, 1948; Davidson & Girdwood, 1947; Davidson, 1948; Wilkinson, 1948. In many of these reports neurological findings that were present before the folic acid therapy commenced persisted or progressed on treatment. In others the neuropathy developed after the treatment had begun. The neuropsychiatric abnormalities seen in these folic acid treated patients were identical to those seen in classical pernicious anaemia. Neuropathy also occurred in patients receiving folic acid given to treat cobalamin deficiency due to other causes including post gastrectomy, and crohns disease. Furthermore several studies reported that folic acid supplementation actually exacerbated the neurological damage. Data from several case reports and some case studies suggest that neurologic complications of cobalamin deficiency worsen when folic acid is administered (Dept. of Health and

Human Services, 1989; Savage & Lindenbaum, 1995; Chanarin, 1979; Bethel & Sturgis, 1948; Challenger & Korst, 1960; Hall & Watkins, 1947; Heinle & Walsh, 1947; Kosik *et al*, 1980; Schwartz *et al* 1950). In contradiction patients with known cobalamin deficiency have been treated with high doses of folic acid for years without the development of neurological complications (Dept. Of Health and Human Services, 1989; Savage & Lindenbaum, 1995; Schwartz *et al*, 1950). These conflicting reports are difficult to interpret and the lack of a randomised, controlled trial makes it impossible to determine if the neurological functioning was different from that expected if folic acid hadn't been administered.

1.23 Folate toxicity

In addition to the masking effects of unmetabolised folic acid on pernicious anaemia, folic acid has been reported to have several other potentially adverse effects for other subgroups of the population. These have been discussed in the literature and experimental work has been carried out. Controversy still exists however as to the validity of these reports. One such series of reports has focussed on the anti-epileptic drug, phenytoin which, acts as an anti-folate agent. It has been reported that folic acid supplementation may interfere with the effectiveness of phenytoin and lead to poorer control of seizures in this population group. Studies examining the effects of folic acid on seizure activity in epileptics taking anti-convulsant therapy have produced conflicting results. Chanarin *et al* (1960) first described the possibility that folic acid supplements in high doses might reverse the effectiveness of anticonvulsant therapy. This was later backed up by Reynolds (1967) who reported that epileptic patients on anti-convulsant therapy had an increased frequency and severity of

seizures. Ralston however (1970) found no significant change in fit severity or frequency in a double-blind trial involving long-stay epileptic patients consuming folic acid supplements over 3 months. More recently the literature regarding the effect of folic acid supplementation on anti-convulsant drug efficacy was reviewed by Campbell (1996). His review showed that in animal studies folic acid is epileptogenic. In human studies seizures occurred in a poorly controlled epileptic subject after intravenous administration of 14.2 and 19.2mg of folic acid. However further studies in 6 patients with epilepsy administered 75mg of folic acid intravenously showed that seizure control was not affected. In more long-term studies, several case reports and an uncontrolled study involving 26 subjects with epilepsy, who received 5mg of folic acid 3 times a day for 1 to 3 years showed that seizure incidence increased. However other studies involving more than 300 patients concluded that no change in seizure frequency with folic acid supplementation. These studies used folic acid supplements at doses of up to 15mg/d for 1 to 6 months. The matter remains unresolved but it is generally accepted that patients on anti-convulsants drugs consuming folic acid supplements should be have their serum drug levels monitored regularly.

Other anti-folate agents such as methotrexate (used widely in the treatment of rheumatoid arthritis (RA), sulfasalazine (used to treat inflammatory bowel disease) and trimethoprim (used in the treatment of bacterial infections) also interfere with folate metabolism. There is a theoretical concern that wide spread folic acid fortification of the general food supply which would lead to very large intakes of folic acid by certain sectors of the population could interfere with disease control by individuals on these drugs. Norman (1996) reviewed the literature regarding the effect of folic acid supplementation on methotrexate in the control of RA. The literature shows that high doses of an active folate (folinic acid) reduces

the efficacy of methotrexate in RA patients. However a study of folic acid supplementation in 111 patients with RA on methotrexate demonstrated reduced drug toxicity but not efficacy. The review concluded that there is no scientific evidence that shows that folic acid of <27mg/wk interferes with methotrexate efficacy.

During the past number of years an interest has developed in the relationship between zinc and folic acid. A number of conflicting reports have been published regarding this relationship. Milne *et al* (1984) reported that ingestion of a 400µg folic acid supplement on alternate days in addition to dietary folate intakes of 150-180µg/d was associated with an increased faecal zinc loss. Mukherjee *et al* (1984) postulated that folic acid supplementation may interfere with intestinal absorption of zinc,

Folate antagonists have been used in the treatment of cancer for almost half a century. One such agent methotrexate discovered prior to 1950 remains the most widely used chemotherapy drug used today. Methotrexate is a tight binding inhibitor of DHFR. Inhibition of DHFR will interfere with both utilisation of dietary folates and the recycling of intracellular dihydro-foyl-polyglutamates thereby decreasing folate cofactors available for cell biosynthesis. The success of this drug in itself raises questions about the safety of consuming large doses of folic acid. Since the mode of action of these drugs is to limit the amount of folic acid available to tumour cells will chronic exposure over a lifetime to large doses of this pro-vitamin lead tumour formation to be accelerated? Early studies using animal and human models gives rise to concern. Farber *et al* (1948) described acceleration of the leukaemic processes by folic acid conjugates to a degree not encountered in other children with acute leukaemia. Heinle & Welch (1948) reported that administration of folic

acid to three patients with myeloid leukaemia was followed by rapid haematologic and clinical relapse in each case. In one of these patients haematologic improvement accompanied the withdrawal of folic acid on two occasions. Studies in animal models subsequently showed that dietary restriction of folic acid inhibited the growth of tumours. Rosen & Nichol (1962) reported that when rats were placed on a folate-deficient diet 2 weeks before transplantation of Walker carcinosarcoma 256, tumour growth was inhibited by 95% on the 28th day after transplantation. The tumour resumed rapid growth when folate was added to the diet. Potter & Briggs (1962) also found that dietary folate deficiency in mice retarded the growth of ascites tumour cells of lymphocytic neoplasms. An attempt to extend these animal studies to humans by placing 7 patients with advanced malignancies on a folate-deficient diet for periods ranging from 25-140 days was unsuccessful, as no reduction in tumour growth was demonstrated (Gailani *et al*, 1970). The authors concluded that folate depletion by dietary means is an inappropriate approach to the treatment of human cancer. Hence clinicians remain uncertain about the management of folic acid deficiency in patients with cancer but to date are generally reluctant to administer folic acid to deficient cancer patients in case the vitamin might accelerate tumour growth (Branda *et al*, 1998).

Finally in relation to folate toxicity the effects of increasing folate intakes in young children and the physiological significance of circulating free folic acid in the blood of pregnant women are unknown. Available literature to date suggests that free folic acid is not normally found in the circulation but accumulates in foetal tissue (Federal Register, 1996).

1.24 Fortification- the process thus far.

Mandatory folic acid fortification of all flour was implemented by the FDA in the US in January 1998 after a long protracted process. Other countries are soon likely to follow this action. This has become the focus of attention of many expert groups and has divided the scientific community. Many publications have focussed on this issue with some experts advocating the higher levels of fortification (Oakley *et al*, 1996; Bentley *et al*, 1996) while others are urging caution and further research (Gaull *et al*, 1996; Tucker *et al*, 1996). It has become the subject of official government reports (Federal Register 1996) and nutrition symposiums (Nutrition Symposium 1996, Fortifying Policy with Science - The Case for Folate). The purpose of this final section in the introduction is to summarise the main research findings that have lead to the point that we have now reached in the fortification process.

Early in embryonic life, 24 - 28 days post conception the neural plate closes to form the spinal cord and skull. If the mother's folate status is not optimal at this time (MRC 1991) there is an increased risk that spina bifida or other neural tube defects may result. Daly *et al* (1995) reported that increasing maternal red cell folate concentrations from 150µg/L to greater than 400µg/L would reduce the risk of NTDs by 60% from 6.6 to 0.8 per 1000 births. The official recommendation in all westernised countries on the consumption of folic acid supplements to prevent NTDs is that all women who are capable of becoming pregnant should consume a 400µg supplement daily.

Given that 50% of pregnancies are unplanned (Grimes, 1986; DoH 1992) it seems that compliance with this recommendation would be very difficult to achieve. In a recent study 300 women attending their first antenatal appointment in three Dublin Hospitals were interviewed. 76% of respondents had heard of folic acid but only 16% had taken it peri-conceptionally at a time when it is protective (McDonnell *et al*, 1999). Similar compliance rates have been reported in other countries including the US. These poor compliance rates are thought to be as a result of a combination of forgetfulness, lack of motivation, perceived risk being low and a lack of credibility of nutrition scientists.

A low folate status is also associated with an increased risk for occlusive cardiovascular disease and stroke because of its role in homocysteine metabolism (Boushey *et al*, 1995). Supplementation with folic acid has been shown to reduce total plasma homocysteine concentrations (Ward *et al*, 1997).

Research by Cuskelly *et al* (1996) showed that the consumption of extra natural food folate is ineffective in optimising folate status. However an optimal folate status was achieved by subjects who took folic acid fortified foods or supplements. As the latter option is considered impractical the consumption of folic acid fortified foods appears to be the most suitable way of maintaining an optimal peri-conceptual folate status for a sustained duration of time.

These findings led the FDA to implement mandatory fortification of all flour milled in the USA. This was commenced in January 1998. This action was taken in the knowledge that a fortification strategy will provide folic acid in a continuous and passive manner. In addition

mass fortification of a food staple by legislation would prove to be an inexpensive and relatively easy method of increasing the daily consumption of folic acid.

The move to fortify was taken after intense deliberation, as no specific food is exclusive to women of childbearing age. Hence other sectors of the population would also be targeted. In addition much controversy surrounded the amount with which to fortify. In determining the appropriate levels of fortification the US Department of Agriculture's 1987-1988 national food consumption data was used to predict daily intakes of folic acid in the target population, as well as in the general population based on proposed fortification levels. The public health agency estimated the effects of three levels 70µg, 140µg and 350µg/100g in cereal grain products. The analysis showed that at the two lower levels intakes of folic acid would remain at less than 1mg/day. However at the highest level daily intakes could reach 1220µg/day. This exceeds the recommended safe upper limit. In addition the analysis showed that consumers who follow the DHHS/USDA food pyramid guide even at the lowest level of fortification proposed could consume 420µg or more without supplement use. This level would double and triple if the other proposed levels are implemented. If the fortification strategy were to be extended to include fruit juices and dairy products then even the lowest level proposed could result in intakes of several milligrams/day. Hence the FDA decided to limit fortification to cereal-grains only and tentatively decided that the appropriate fortification level for cereal-grains was 140µg/100g. It was estimated that this level of fortification would result in intakes of 840-860µg in adults of 51+years at the 95th percentile even with supplement use. The FDA also proposed a limit of 100µg on the addition of folic acid to ready to eat breakfast cereals. Hence the level of fortification was set at 140µg/100g. This was implemented in January 1998. Flour was chosen because it is a

very widely consumed food staple in the USA with over 90% of the population consuming flour of some description. The FDA estimated that this level of fortification would supply on average an extra 100µg to the daily US folate dietary intake. However people at the top end of the distribution curve (referred to earlier) will ultimately receive more than is necessary and people at the lower end of the distribution curve may still not get adequate to prevent NTDs. Therein lies a major public health problem-how do we target consumers at the lower end of the distribution curve without exposing high consumers to even more folic acid? The FDA set this level as a preliminary level in the knowledge that while it might not be the optimal level some improvement in population status, particularly in low consumers will occur. Therefore the FDA actively encourages the consumption of ready to eat fortified cereals and other fortified foods in order to achieve additional needs.

The Centers for Disease Control (CDC) Folic Acid Working Group (1992) Atlanta, GA supported the implementation of fortification but feel that levels need be increased. The CDC are now advocating an increment to 350µg and even 700µg/100g which they have estimated will supply an additional 200µg and 400µg respectively to the US daily intake. Caution is being exercised by the FDA about further increases in the level due to various public health safety issues.

Their primary concern is that a mass fortification scheme could result in potentially unsafe intakes of the vitamin by large sectors of the population. Crane *et al* (1996) reported that any attempts at increasing folic acid intakes in people with low folate intakes by mass fortification would concurrently result in significantly increased intakes of folic acid in people who are already high consumers of the vitamin. Folic acid is generally not thought

to be toxic but oral doses as low as 400µg in supplemental forms and 266µg in fortified foods have been demonstrated to saturate the normal absorption mechanisms. This resulted in both the normal metabolite 5-methyltetrahydrofolate and unmetabolised folic acid appearing in the serum (Kelly *et al*, 1997). High consumers of these fortified foods could then risk being exposed to this pro vitamin for an entire lifetime.

The chronic presence of unmetabolised folic acid in the circulation presents a problem in the early diagnosis of pernicious anaemia. If folic acid use becomes more widespread in an attempt to lower Coronary Heart Disease and Stroke as well as prevent NTDs this will be of particular concern in the elderly population. The FDA have estimated that the incidence of pernicious anaemia is approximately 9.5-16.7 per 100,000 persons. Even with the low fortification levels 5-10% of these patients may receive enough folic acid to mask pernicious anaemia. Based on these assumptions the FDA have estimated that approximately 500 people per year would develop neurologic disease as result of the fortification strategy (Federal Register, 1996).

At present no scientific evidence exists to ensure that adverse effects do not occur at levels less than 1mg/day. Hence the FDA have set an upper safe daily limit of 1mg and have stated that intakes above this must be medically supervised. It is apparent however that the two higher levels of fortification being advocated by the CDC could lead to intakes of greater than 1mg per day for some subgroups of the population i.e. those who consume a lot of flour products or those who already consume a lot of the vitamin.

Mass fortification of the food supply has other safety implications in other non-targeted sectors of the population, those taking medications which are based on anti-folates such as phenytoin used to control epilepsy, sulfasalazine used to treat inflammatory bowel disease and methotrexate used to treat rheumatoid arthritis. Large doses of folic acid in the food supply may lead to poorer control of these conditions but virtually nothing is known about this theory at present. As previously mentioned the success of anti-folate drugs used in cancer treatment also give rise to safety concerns.

Hence the level of fortification to date has been maintained at 140µg/100g flour. It is likely to remain at this level until scientific evidence becomes available to justify that it will be safe to increase it further. However a further complication has come to the attention of expert groups working in this area recently i.e. the problem of overage. It is widely believed though not yet proven that many food companies are adding more than the 140µg/100g specified by the FDA because food companies can be sued if their products do not contain enough folic acid. However this does not apply if products are over fortified. Recent reports have suggested that many food companies are adding double the amount specified due to fears that losses may be incurred during food processing or that losses may occur in products with a long shelf-life. This issue needs to be addressed by legislation and similar penalties need to be imposed on food companies guilty of over-fortifying their products as for those guilty of under-fortifying.

1.25 Aims of the thesis

- 1) The first aim of this thesis (chapter 2) was to reproduce the combined HPLC/microbiological methodology set-up by Kelly *et al* (1996) to measure unmetabolised folic acid in serum.
- 2) The second aim (also chapter 2) involved some preliminary work investigating the effects of baking on the stability of folic acid added to flour.
- 3) The effects of long-term consumption of folic acid fortified foods on unmetabolised folic acid in serum have never been investigated. This main aim of this research study (chapter 3) was to investigate if chronic consumption of the current level of fortification in the USA and the two higher levels under consideration by the CDC would give rise to unmetabolised folic acid in the serum of a normal healthy population group. Healthy adult subjects were recruited, pre-saturated and fed bread at three different levels of fortification. Serum samples were collected pre and post prandially. Samples were assayed for unmetabolised folic acid initially by the combined HPLC/microbiological methodology described in chapter 2. Unmetabolised folic acid was not detected at any of the test doses. This led us to reassess the sensitivity of the assay.
- 4) Hence the next aim of this research (chapter 4) arose as a result of aim 3. The existing technology was not sensitive enough to measure levels of unmetabolised folic acid in serum below 1ng/ml. The aim of chapter 4 was to improve the lower limit of detection of the existing methodology. Chapter 4 therefore describes an improved methodology for the

measurement of unmetabolised folic acid in serum which improved the lower limit of detection by six-fold.

5) The aim of chapter 5 was to re-assay the samples collected in the long-term study (chapter 3) by the improved methodology.

6) In the absence of scientific evidence to suggest otherwise the FDA have set a safe upper limit for folic acid of 1mg daily provided that consumption is well spread out. In order to investigate if this is a safe upper limit in terms of unmetabolised folic acid appearance in serum a series of short studies were planned (chapter 6). The aim of trials 2,3,4,5 and 6 was to investigate the effect of consuming different combinations of doses all totalling 1mg over a day on unmetabolised appearance in serum post-prandially.

7) The aim of chapter 7 was to investigate the appearance of unmetabolised folic acid in cord blood of full-term and premature new born infants immediately after birth. In addition we examined the appearance of unmetabolised folic acid after the consumption of folic acid fortified formula milk for 4 days.

8) The aim of the “red cell folate stability study” (Chapter 8) was to investigate the stability of red cell folates under different handling conditions over different time points.

Chapter Two

Materials and Methods

2.1 Equipment

2.1.1 Pipettes

Stepper semi-automated pipette (Socorex, Renens, Switzerland)

Automatic 5-50µl Pipette (Socorex, Renens, Switzerland)

Automatic 50-200µl Pipette (Socorex, Renens, Switzerland)

Automatic 200-1000µl Pipette (Socorex, Renens, Switzerland)

Micropipette Tips Ref No. 70.760.002 (Sarstedt, Wexford, Ireland)

Micropipette Tips Ref No. 70.760.002 (Sarstedt, Wexford, Ireland)

Tigerteck Digital Multichannel Pipette 50-250µl (Flow Laboratories, Ayreshire, Scotland)

2.1.2 Tubes

1.5ml Epindorph Tubes Ref No. 72.690 (Sarstedt, Wexford, Ireland)

75*12 mm Test Tubes Ref No 30916 (Sarstedt, Wexford, Ireland)

95* 16 mm Test Tubes Ref No 55.468 (Sarstedt, Wexford, Ireland)

2.1.3 Equipment for Blood Collection

Precision Glide Vacutainor Needles Ref No 607213 (Becton Dickinson, Plymouth, UK)

Serum Vacutainor Tubes Ref No 606430 (Becton Dickinson, Plymouth, UK)

EDTA Vacutainor Tubes Ref No 367652 (Becton Dickinson, Plymouth, UK)

Sterets Pre Injection Alcohol Wipes (Senton Prebbles Ltd. Oldham, UK)

Abbocath Catheters Ref No. G-719 (Abbott Laboratories, Queensborough, Kent, UK)

5ml Serum Containers Ref No 63.542.709 (Sarstedt, Wexford, Ireland)

Vacutainer barrells (SIMS Portex Inc. NH, USA)

2.1.4 Equipment for HPLC

Waters Associates Chromatography Pump 510 (Waters, Milford, MA, USA)

Waters Z-Module Radial Compression System (Waters, Milford, MA, USA)

Waters RCSS Guard Pak (Waters, Milford, MA, USA)

Fraction Collector FRAC-100 (Pharmacia Fine Chemicals)

Tri-CARB 1500 Liquid Scintillation Counter (Canberra Packard, Redding, UK)

Scintillation Vials Ref No. 75.680 & 75.662 (Sarstedt, Wexford, Ireland)

Classic C18 Sep-Paks (Waters, Milford, MA, USA)

200µl Hamilton syringe (SGE Incorporated, USA)

2.1.5 Equipment for microbiological assay

Beckman spectrophotometer 35 UV/VIS

Titrek Multiscan Plus Microtitre Plate Reader (Flow laboratories, Ayresshire, Scotland)

Microtitre Plate (Flat Bottom) 96 well Ref No. 82.1581 (Sarstedt, Wexford, Ireland)

Triteck Plate Sealer Ref No. 77.400.05 (ICN Biomedicals, Ayresshire, Scotland)

Shimadzu Electronic Top Pan Balance Ref No 321-33557 (Kyoto, Japan)

2.1.6 Other Equipment

WIFUG Lab Minifuge (med Labs)

Sanyo MSC Mistral 3000I Centrifuge

Hitachi U 2000 Spectrophotometer

Shaker Incubator – Stuart Scientific

2.2 Chemicals

2.2.1 Chemicals used in microbiological assay

Ascorbic acid (Sodium salt) C_6H_7Na (Sigma Chemical Company, London, UK)

Ascorbic acid (Sigma, London, UK)

Chloramphenicol (Parkes Daves and Co. Pontypool, Gwent, Wales)

Tween 80 (Sigma Chemical Company, London, UK)

Vitamin Folic Acid Assay Base Broth Ref No 11990 (Merck, Darmstadt, Germany)

Lactobacillus casei (NCIB 19463) (Torrey Research Station, Aberdeen, Scotland)

2.2.2 Chemicals used in HPLC

Pteroylmonoglutamate [$3'$, $5'$, $7'$, $9 - ^3H$] PteGlu (TRK212, sp act 46Ci/mmol) (Moravek Biochemicals, Brea, CA, USA)

Ecolite Scintillation Fluid Ref No. 882470 (ICN Biochemicals, Costa Mesa, CA, USA)

HPLC Grade Methanol (Scharlau Chemie S.A., Barcelona, Spain)

2.3 Consumables

Pteroylglutamic acid Ref No F.7876 (Sigma chemical Company, London, England)

Fresh yeast – Superquinn bakeries, Sundrive Road, Kimmage, Dublin, Ireland

Odlums Cream Flour- Odlum Group Limited, Alexandra Road, Dublin, Ireland

Folic acid tablets 400 μ g & 200 μ g Clonmel Health Care, Clonmel, Ireland

2.4 Buffers

2.4.1 0.5% Sodium Ascorbate Buffer

0.5g sodium ascorbate powder was added to 100ml of distilled H_2O .

2.4.2 1% Ascorbic Acid Buffer

1g of ascorbic acid powder was added to 100ml of distilled H₂O.

2.4.3 10X Citrate Phosphate Buffer

177.47g Na₂HPO₄ were added to 1460ml of distilled water in a conical flask and dissolved using a hot plate/stirrer. 210.14g citric acid crystals were then added and dissolved using a hot plate/stirrer. The pH of the dissolved buffer was checked and adjusted to pH 4.0. The concentrate was diluted 1:10 with distilled H₂O before use.

2.4.4 1 molar NaOH

10g NaOH was added to 250ml distilled H₂O in a conical flask. The mixture was inverted 9 times to ensure adequate mixing.

2.4.5 6 Molar Potassium Hydroxide

33.6g of KOH were added to 20ml of distilled water in a conical flask. The solution was placed on a hot plate/stirrer to mix and dissolve the powder. The solution was allowed to cool and was then made up to 100ml with the addition of distilled water. The mixture was inverted 9 times to ensure adequate mixing.

2.5 Total serum folate assay

2.5.1 Principles

The assay involves the addition of a chloramphenicol resistant organism *Lactobacillus casei* (*L. casei*) (an organism which needs folic acid or its derivatives for growth) to folate deficient media. Aliquots of this media are then added to test samples or to standards in a 96 well microtitre plate. The growth of the organism is directly proportional to the amount of folate present in the wells of the plate. This growth is quantified by scanning the plates using a densitometer @ 590nm after incubation @ 37°C for 42 hours. A standard curve is set up with each assay and the test results are calculated from the curve. Quality control samples were prepared by the laboratory technicians as described by Molloy & Scott (1997) and these are run with each assay.

2.5.2 Collection of samples

Blood for serum folate analysis was collected by venopuncture into non-heparinised serum separator tubes. Blood for red cell folate analysis was collected into tubes lined with ethylenediaminetetra-acetic acid (EDTA) (anticoagulant).

2.5.2 Preparation of samples

Serum was separated from whole blood by centrifuging at 2500rpms for 12 minutes. The serum was collected and stored in 1.5ml plastic tubes at -20°C until time of analysis. 50µl of each test serum was added to 950ml of 0.5% sodium ascorbate buffer in duplicate polypropylene tubes (1 in 20 dilution). A higher dilution was used when the concentration of folic acid in the test samples were too high to read off the standard curve at this dilution i.e. after subjects had been supplemented with folic acid. These were vortexed to ensure that the sample was mixed well with the buffer. Each test sample was then added to 4 wells of a microtitre plate as follows: 100µl to each of two wells and 50µl to the other two wells. This was repeated for the second tube. Thus 8 replicates were made of the starting sample. 50µl of 0.5 % sodium ascorbate buffer was added to the wells with the 50µl volume to bring the final volume up to 100 µl/well.

2.5.4 Preparation of standard curve

A working standard solution of folic acid (500pg/ml) was made up by the addition of 500µl of a stock standard to 0.5% sodium ascorbate buffer, which was brought up to a final volume of 100ml. A standard curve was constructed in the region of 0-50pg/well (0-100µl) using a 96 well microtitre plate. 12 columns with 8 replicates for each standard were prepared containing folic acid pg/well as shown below

0	0	2.5	5	7.5	10	12.5	15	20	30	40	50
---	---	-----	---	-----	----	------	----	----	----	----	----

All wells were brought to a volume of 100 μ l by the addition of 0.5% sodium ascorbate buffer.

2.5.5 Preparation of assay medium (to make 100ml, enough for 4 plates).

5.7g of folic acid assay broth

3.0mg of chloramphenicol

30 μ l of tween

75mg ascorbic acid

100ml H₂O

The folic acid assay broth, chloramphenicol, and water were mixed together and heated. The tween was added and the mixture was heated to boiling point. After cooling 75mg of ascorbic acid was added. The cryopreserved *L. casei* was made up by the laboratory technicians as described by Molloy *et al* (1997). This was added to the media at a concentration of 200 μ l/100ml and was mixed thoroughly using a magnetic stirrer.

2.5.6 Addition of media, incubation and reading of results

Media (200 μ l) was added to all wells of the microtitre plate using a multi-channel pipette. The plates were sealed using a plate sealer. The plates were incubated @ 37°C for 42 hours in a non-humidified warm air incubator. The plates were then mixed thoroughly by inversion, the plate sealers were removed and the plates were read by densitometry @

590nm. The test results were calculated from the standard curve by a computer package (Elisa Plus version 3.01.06 copyright 198- 1991 Meddata Incorporated, USA) to give results in $\mu\text{g/L}$. Figure 2.1 is an example of a typical standard curve.

2.6. Red cell folate assay

2.6.1 Principles

Lysates of red cell folates were prepared by dilution of the whole blood in a 1 in 10 dilution with freshly prepared 1-% ascorbic acid buffer. The samples were mixed thoroughly and placed at room temperature for 30 minutes to allow serum conjugase to convert folate polyglutamates release from the lysed erythrocytes to the assayable monoglutamate forms.

2.6.2 Sample preparation

The lysates were stored at $-20\text{ }^{\circ}\text{C}$ until time of analysis. A fresh solution of 0.5% sodium ascorbate buffer was prepared and used to dilute red cell lysates 1 in 40 (25 μl up to 1ml). The diluted lysates were mixed and added to 4 wells - 100 μl to each of two wells and 50 μl to each of the two remaining wells. These wells were brought to a final volume of 100 μl by the addition of 0.5 % sodium ascorbate buffer.

2.6.3 Preparation of packed cell volume

This was determined from the whole blood sample by obtaining a haematocrit reading using a microcapillary tube. The tube was spun at 2500 rpm's for 6 minutes and the result was read off a haematocrit reader. The value obtained from the microbiological red cell folate lysate was then divided by the haematocrit value obtained to give the final red cell folate result in $\mu\text{g/l}$.

2.6.4 Preparation of standard curve,

See preparation of standard curve for total serum folate described above.

2.6.5 Addition of media, incubation and reading of results

See above

Raw data values (detailed) - Scan 1 :												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.062	0.062	0.134	0.201	0.256	0.299	0.345	0.386	0.466	0.573	0.669	0.770
B	0.063	0.065	0.144	0.177	0.253	0.309	0.346	0.392	0.456	0.571	0.677	0.760
C	0.063	0.063	0.149	0.209	0.273	0.313	0.366	0.369	0.466	0.596	0.686	0.764
D	0.066	0.092	0.145	0.208	0.275	0.316	0.349	0.432	0.488	0.598	0.691	0.771
E	0.067	0.101	0.149	0.210	0.269	0.321	0.374	0.384	0.472	0.604	0.684	0.753
F	0.087	0.099	0.160	0.221	0.259	0.360	0.390	0.391	0.488	0.584	0.672	0.756
G	0.086	0.095	0.151	0.208	0.260	0.313	0.370	0.407	0.487	0.582	0.669	0.763
H	0.091	0.067	0.154	0.225	0.286	0.324	0.369	0.395	0.473	0.565	0.672	0.762

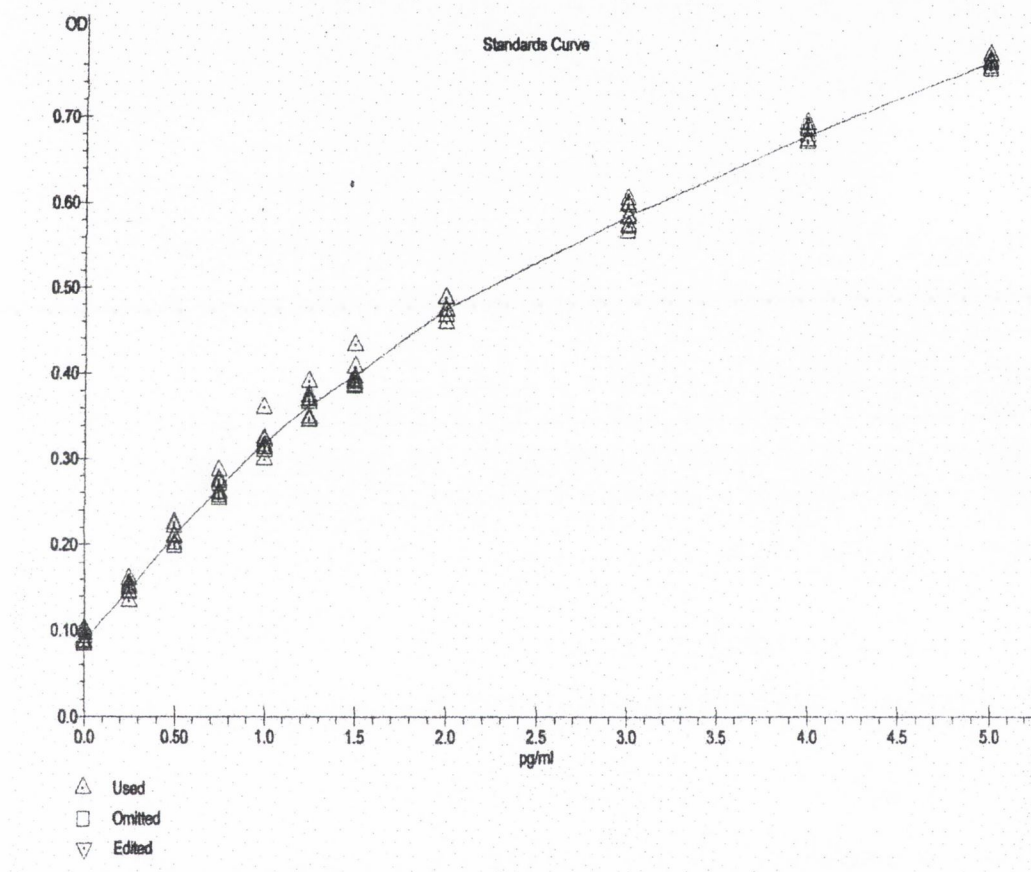


Figure 2.1. Typical Standard Curve.

2.7 Methodology to measure unmetabolised folic acid in serum

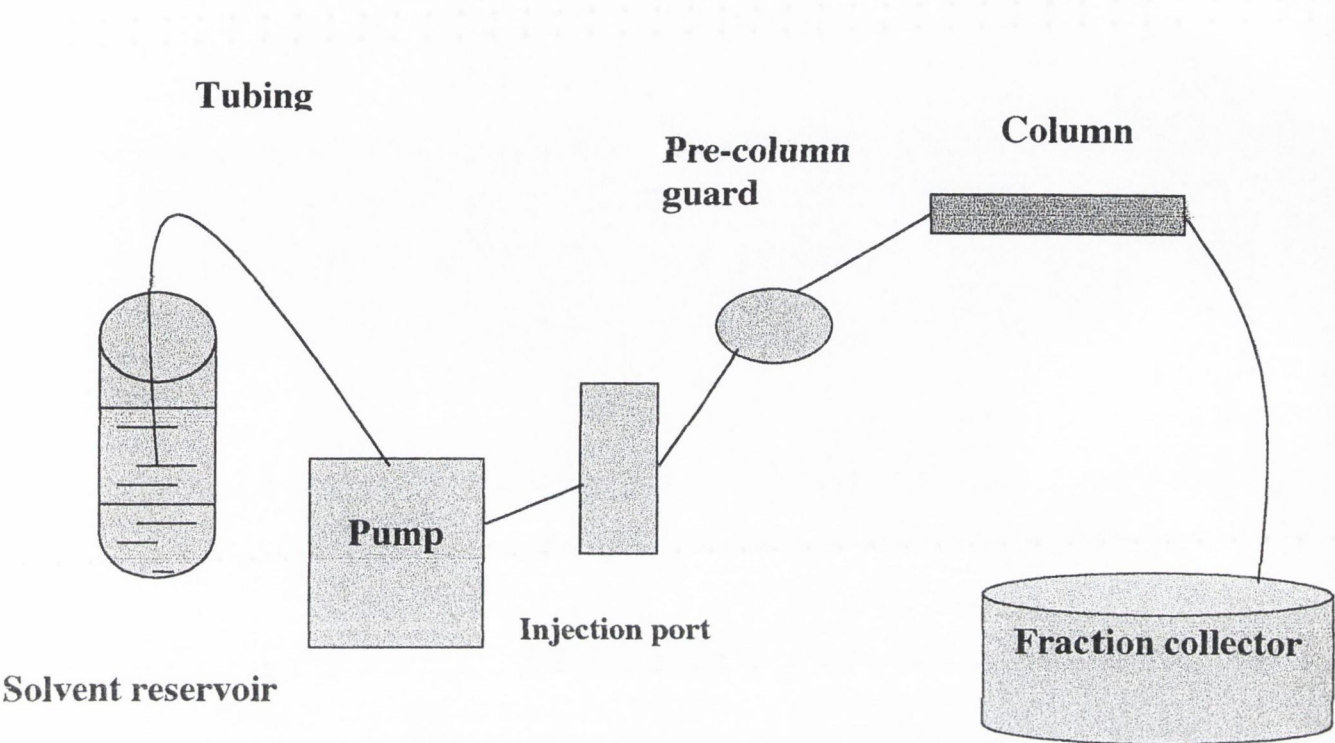
2.7.1 General principles of High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a commonly used method of separation of vitamins or other nutrients from serum or foodstuffs. HPLC refers to chromatography performed in closed, pressurised columns, which have small particles acting as a stationary phase material. The material to be separated is dissolved in a mobile phase, which is pumped into and through the column under pressure of a non-pulsating pneumatic pump. In this methodology adsorption reverse phase chromatography was used. Hence the stationary phase was less polar than the mobile phase. Hence substances which are most polar will emerge first while the non-polar substances emerge later.

2.7.2 Summary of methodology to measure unmetabolised folic acid

The method to be described in this section was set up by Kelly *et al* (1996) to measure serum unmetabolised folic acid. In the methodology, a 100 x 8-mm Radial Pak C18 cartridge column was used as the stationary phase. A pre-column module containing a disposable C18 insert was attached between the injection port and the column. This was used to prevent any impurities from damaging the radial Pak column. This insert was changed regularly to prolong the lifetime of the main column. A Waters 510 pump was used to pump a polar mobile phase containing acetic acid and methanol. Separated samples were collected in a Frac-100 fraction collector (Pharmacia, Upsala) at a rate of flow of 3ml/min. Figure 2.2 shows a schematic diagram of a HPLC unit.

Figure 2.2. Schematic diagram of HPLC unit



All folates are soluble in methanol but 5-methyl THF is more soluble than 5-Formyl THF, which is more soluble than Folic acid. Hence 5-methyl THF elutes first from the column, followed by 10-formyl THF and then folic acid, which is, retained the longest. This methodology incorporates the combined use of HPLC and a microbiological assay as the microbiological assay for total folate on its own does not distinguish unmetabolised folic acid from other circulating serum folates, namely 5-methyl THF and 10-formyl THF. In other words the microbiological assay will measure all folates present in the microtitre plate. Folic acid must therefore be separated from other folates.

In summary the principle of the combined methodology is based therefore on the addition of an internal standard, radiolabelled Pteroylmonoglutamate [3', 5', 7', 9 -³H] (³H-folic acid) to a test sample, its separation by HPLC and identification by scintillation counting. The radioactive peak is then collected and pooled. A microbiological assay for serum folic acid is then performed and a quantitative value is obtained for folic acid (µg/L), corrected for percentage recovery of internal standard.

2.8 Method Description

2.8.1 Calibration of scintillation counter

The scintillation counter was calibrated to count ³H-folic acid for 5 minutes counting a single label radionuclide. See copy of print out from counter - figure 2.3. The quench curve was corrected at regular intervals - see figure 2.4.

Protocol #=	3	Integer: (1-10)
Copy Protocol=	0	Integer: No(0) (1-10)
Cycles=	1	Integer: Infinite(0) (1-9)
Time=	5.00	Range: (.10-999.99) minutes
# Counts/Vial=	1	Integer: (1-99)
# Vials/Sample=	1	Integer: (1-9)
# Vials/Standard=	1	Integer: (1-9)
% Reference=	0	No(0) Yes(1)
Data Mode=	3	CPM(1) SPC(2) DPM(3) FS DPM(4)
Background=	0	None(0) FV(1) Manual(2)
Label=	1	Single(1) Dual(2)
Single Label Radionuclide=	1	H3(1) C14(2) P32(3) I125(4) Manual(5)
Region A: 2 Sigma %=	.00	Range: Off(0) (.10-10)
Region A: Lo Cnt Reject	0	Range: No(0) (1-99999) CPM
Region A: Divide Factor=	1.00	Range: (.001-9999)
Region B: 2 Sigma %=	.00	Range: Off(0) (.10-10)
Region B: Lo Cnt Reject	0	Range: No(0) (1-99999) CPM
Region B: Divide Factor=	1.00	Range: (.001-9999)
tSIE=	1	No(0) Yes(1)
Colored Samples=	0	No(0) Yes(1)
AEC=	0	No(0) Yes(1)
Heterogeneity Monitor=	0	No(0) Yes(1)
Count Standards=	0	No(0) Yes(1)
Constant Quench=	0	No(0) Yes(1)
Luminescence Detection=	0	No(0) Yes(1)
Half life=	.00	Range: Off(0) (.01-999999) Hours
Printer Format=	1	Short(1) Long(2) Programmed(3)

Figure 2.3. Print out from scintillation counter calibrated to count ^3H folic acid for 5 minutes.

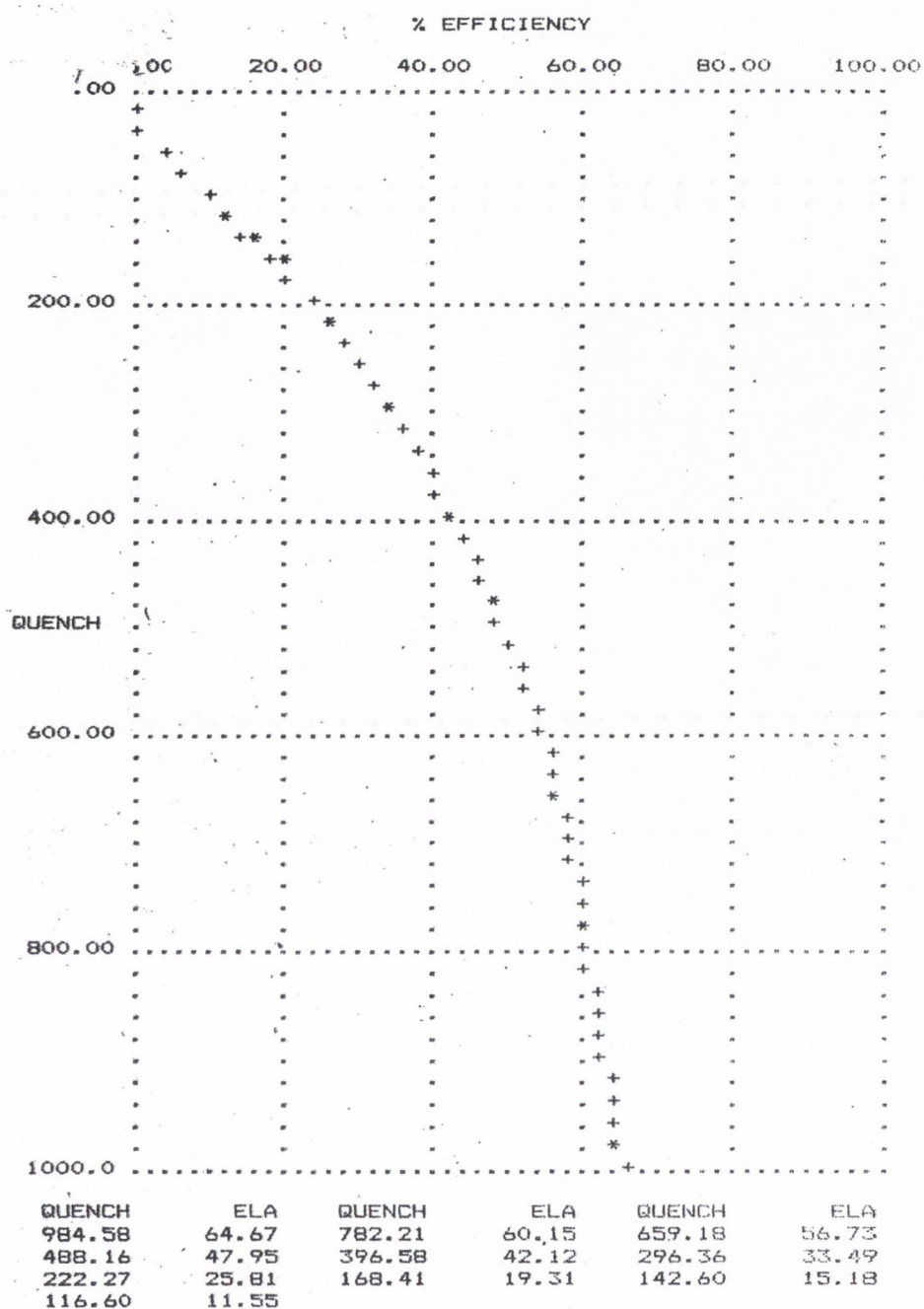


Figure 2.4. Quench curve corrected with internal standards.

2.8.2 Preparation of mobile phase (made up to 1L) and ^3H folic acid internal standard

430ml 1x citric phosphate buffer (see section 2.4.3)

420ml 1% acetic acid

150ml HPLC grade methanol

The mixture was filtered and degassed using the solvent clarification apparatus as air bubbles in the column can lead to serious damage. ^3H -folic acid was used as the internal standard. This radioisotope is generally stable but can break down to ^3H Pteridine and ^3H PabGlu. Hence it was necessary to check for purity at regular intervals. This was done by fractionating the ^3H folic acid by HPLC. The elution time of the ^3H folic acid was also determined by this procedure as follows. The column was eluted isocratically @ 3ml/min with mobile phase for 15 minutes. 50 μl (100,000 dpm) of ^3H folic acid was injected on and the fractions were collected at one minute intervals. 18 3ml fractions were collected. A 1ml sample from each fraction was placed in a 25ml scintillation vial with 9ml of ecolyte scintillation fluid. The vials were counted by scintillation counting for 5 minutes each. A radioactive peak was identified in the fractions collected at 8 and 9 minutes. There were no other peaks present. Figure 2.5 illustrates this. These two fractions were then pooled and counted by scintillation counting. The total radioactivity of the pooled sample accounted for approximately 90% of the starting counts. The purified radiolabelled ^3H -folic acid was then used as the internal standard. In order to prolong the elution time however, so as to allow more effective separation of the folic acid from the 5-methyl THF it was decided to make up the mobile phase with 12.5% methanol as follows.

2.8.3 Mobile phase (made up to 1L)

430 ml 1X citric acid buffer

445ml 1% acetic acid

125ml methanol.

The buffer was filtered and degassed as previously described. A sample was spiked with 50000 decays per minute (dpm) ^3H -folic acid and injected, eluted and collected as described previously. The elution time was then found to occur around 11 minutes - see figure 2.6. This concentration of methanol was used for all subsequent HPLC buffers.

2.8.4. Preparation of test sample for HPLC.

A 60% perchlorate solution for protein precipitation was prepared by the addition of 60ml of perchlorate to 40ml of distilled H_2O . A serum sample was collected from an individual known not to have consumed folic acid in the previous twenty-four hours. 14,000 dpm of ^3H -folic acid was added to 0.5ml of the test serum in a 1.5ml microfuge tube. This sample was prepared in duplicate to investigate if this amount of radioactivity used as an internal standard would be sufficient to determine the elution time of folic acid by a radioactive peak and also to investigate if this amount would contaminate the microbiological assay for serum folic acid. Kelly *et al* (1996) found that this amount of radioactivity did not augment the background absorbance reading of the assay. After mixing thoroughly 50 μl of 60% perchlorate was added to the serum. This method of precipitation removes serum proteins

from the sample, which could otherwise damage the column. (Leeming *et al*, 1990). The sample was vortexed and frozen at - 18°C. After thawing each tube was centrifuged at 3000rpm and the supernatant was removed. 50µl 6M KOH was added to neutralise the supernatant to pH 7.0.

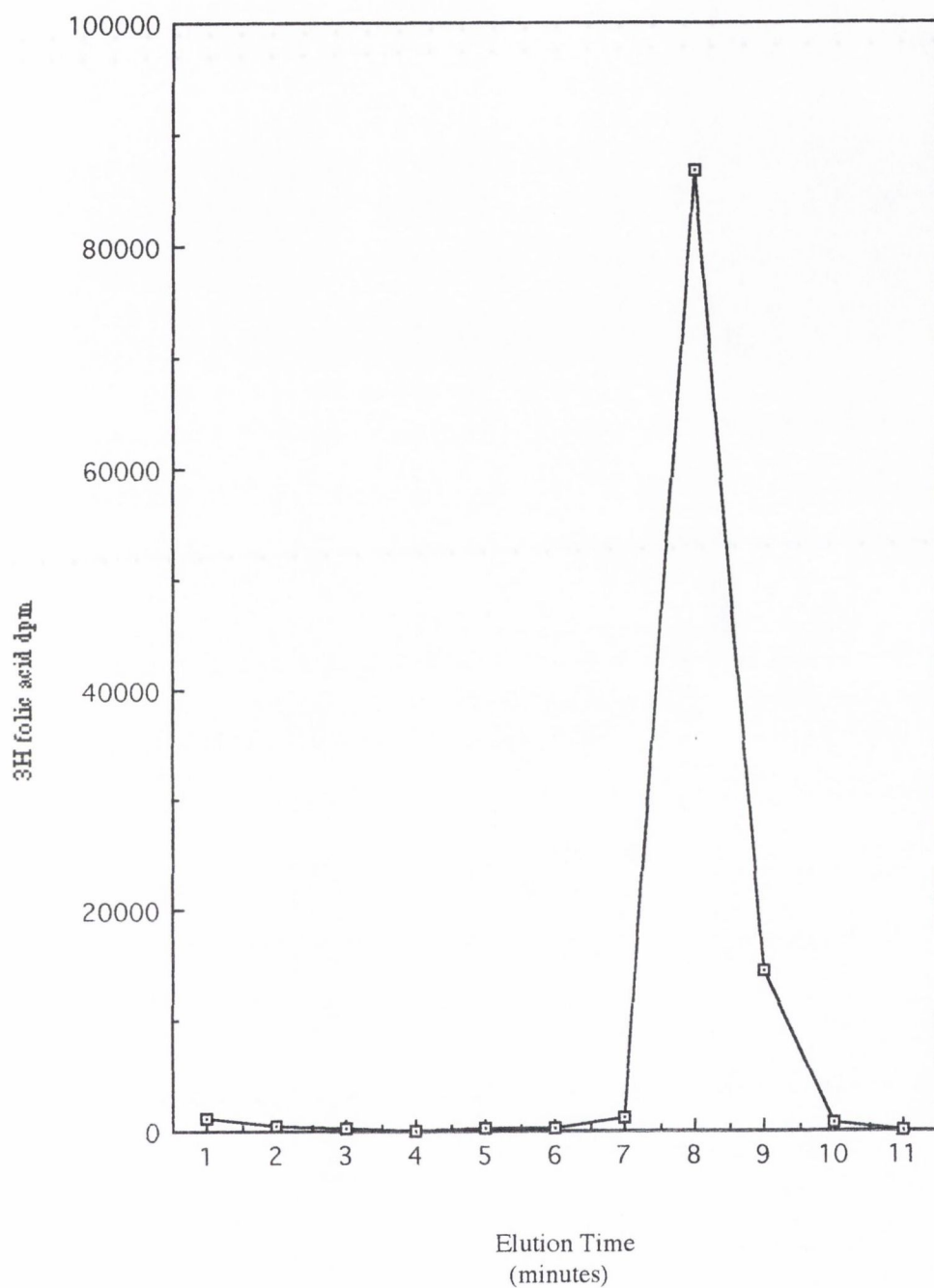


Figure 2.5. HPLC elution of 3H folic acid in 15% methanol HPLC buffer

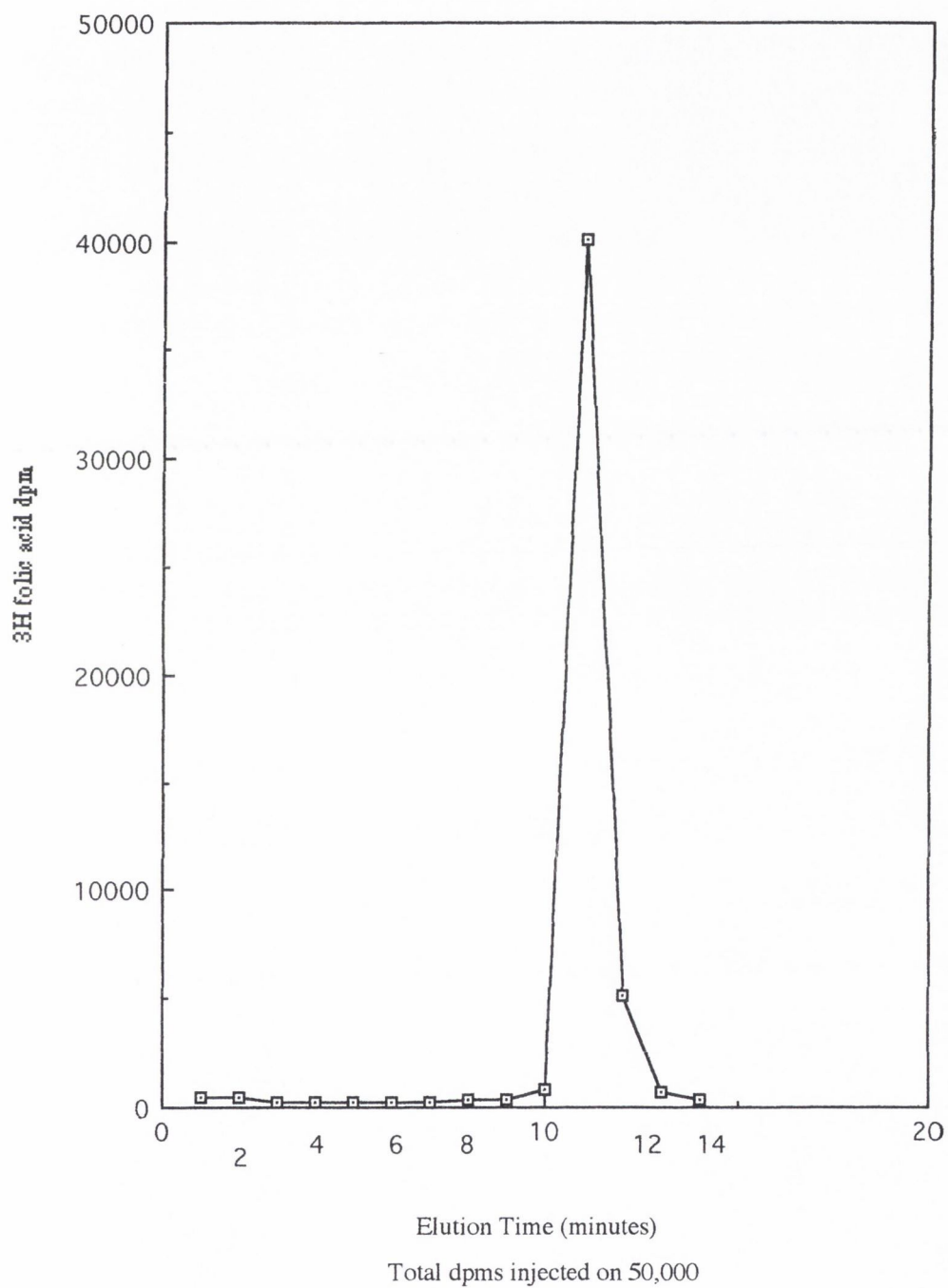


Figure 2.6. Elution time of 3H folic acid in 12.5% methanol HPLC buffer.

2.8.5 Injection of sample onto HPLC, fractionation of folic acid and collection of peak.

The supernatant (200 μ l) was injected onto the injection port of the HPLC. The column was eluted isocratically at 3ml/min for 20 minutes and six fractions known to be in the region of the retention time were collected in 5ml polypropylene tubes. The six fractions were assayed for radioactivity by counting 2ml of each in 9ml of ecolyte scintillation fluid for 1 minute. The remaining 1ml volume from the two fractions containing the radioactive peak were then pooled and this pooled fraction was saved for the microbiological assay. The counts were recorded in dpm and the percentage recovery was calculated to estimate the final folic acid concentration present. This was calculated after the microbiological assay for serum folic acid was completed.

2.8.6 Experiment to examine the effect of mobile phase on growth of *L. casei*.

All of the pooled samples collected after HPLC separation were in mobile phase, therefore it was necessary to prepare the standard curve using mobile phase also to ensure that both the test samples and the standard were prepared using the same diluent. Hence it was necessary to investigate whether the mobile phase would interfere with or inhibit the normal growth of the *L. casei*. This was investigated by preparing two separate standard curves. The first standard solution of folic acid was prepared by dilution of a stock standard in 0.5% sodium ascorbate buffer. The second curve was prepared in mobile phase diluted 1:1 with 0.5 % sodium ascorbate. Both curves were constructed in the ranges 0 - 12.5 pg/well. Both growth responses were found to be similar from 0 -12.5 pg/well. This

indicates that the mobile phase buffer does not interfere with the growth response of the *L. casei* up to 12.5 pg/well. Figures 2.7 and 2.8 illustrate this. This growth curve was used for all subsequent microbiological assays for serum folic acid determination.

Data File.....: MRS20

Data Reduction Template: FOL

Plate #:.....: 1

Plate Read: 12/18/96 10:58:31

Plate ID.....:

Comment 1.....:

Comment 2.....:

Measuring Wavelength: 590 nm Reference Wavelength: No nm Blank: Air

Plate 1 Standard Curve 1

ab.

Linear Interpolation

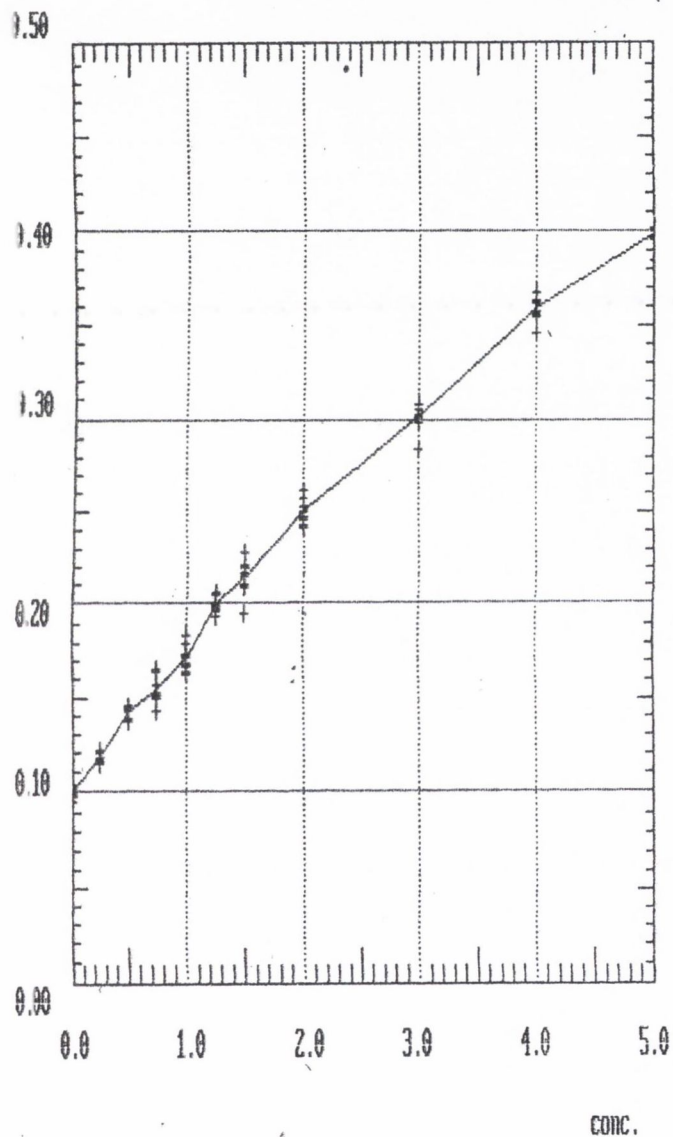


Figure 2.7. Standard curve A diluted with 0.5% sodium ascorbate buffer.

Data File.....: MRS21

Data Reduction Template: FOL

Plate #:.....: 1

Plate Read....: 12/18/96 11:02:11

Plate ID.....:

Comment 1.....:

Comment 2.....:

Measuring Wavelength: 590 nm Reference Wavelength: No nm Blank: Air

Plate 1 Standard Curve 1

abs. Linear Interpolation

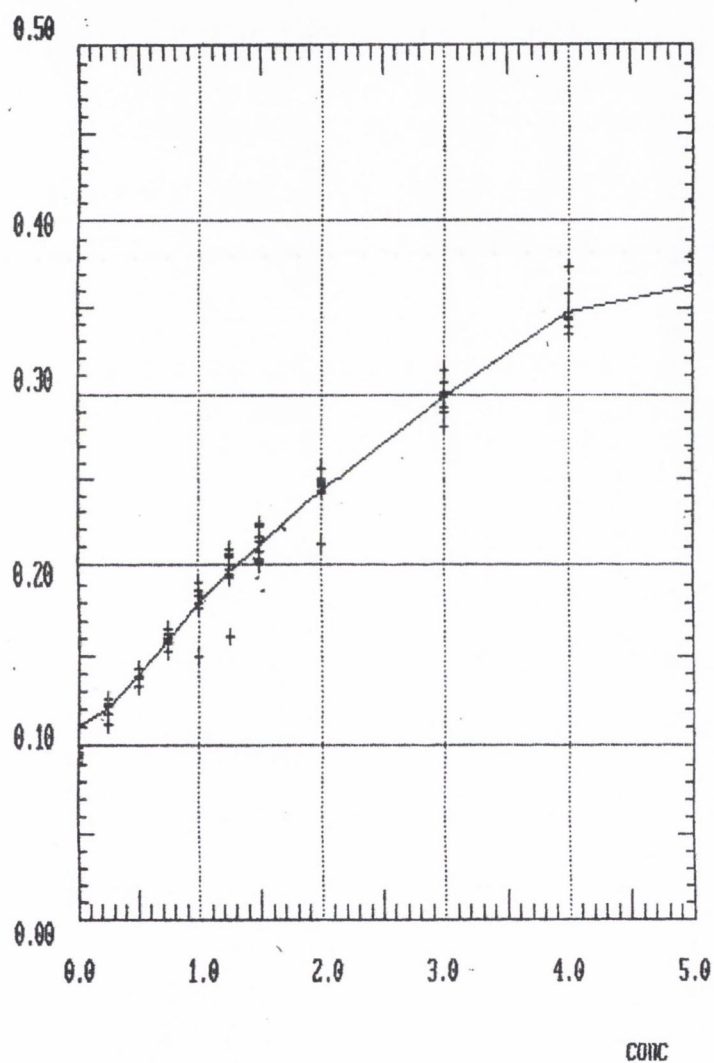


Figure 2.8. Standard curve B diluted 1:1 with mobile phase and 0.5% sodium ascorbate

2.9 Microbiological assay of samples post HPLC.

2.9.1 Dilution of samples for assay

200µl of test sample (folic acid peak pooled) was diluted with 25µl 1M NaOH and 175µl of 0.5 % of sodium ascorbate. Each test sample was then added to 4 wells of a microtitre plate as follows: 100µl to each of two wells and 50µl to each of the other two wells. 50µl of mobile phase diluted 1:1 with 0.5 % sodium ascorbate was added to the latter two wells to bring the final volume up to 100 µl/well.

2.9.2 Standard curve preparation

A standard curve was constructed in the range of 0-12.5pg/well using a stock solution of folic acid (125pg/ml) diluted as above in mobile phase 1:1 with 0.5 % sodium ascorbate buffer. It was hoped that this standard curve would improve the sensitivity of the assay as all the results would be read off this linear part of the curve. All wells were brought to a final volume of 100µl with mobile phase diluted 1:1 with 0.5 % sodium ascorbate buffer.

2.9.3 Preparation of Media.

200 µl of media was added to all wells as per total serum folate assay and the plates were incubated and read in the same way.

2.9.4 Lower limit of detection of the standard curve

The lowest limit of detection of the standard curve was taken to be 0.5pg/well. This was calculated as the mean absorbance of the 16 zero wells plus 2 standard deviations.

2.9.5 Calculation of [FA] post combined assay

$$= (c \times d \times 10 \times 4) / R$$

Where c is the standard curve value

d is the dilution factor of mobile phase with ascorbate: 2

a factor of 10 adjusts the curve value to pg/ml.

4 refers to the radioassayed volume of 4ml of the initial fractionated serum sample

R is the % recovery of the ^3H internal standard, approximately 8 - 10% of the initial amount added.

2.9.6 Lower limit of detection of the combined assay.

$$0.5 \times 2 \times 10 \times 4 \times 10 = 0.4\text{ng/ml.}$$

0.5 refers to the lower limit of detection of the microbiological assay

2 refers to the dilution factor of mobile phase and ascorbate

10 adjusts the curve value to pg/ml

4 refers to the volume of the pooled radioactivity counted

10 refers to the average percentage recovery of the internal standard from the 4ml counted

Therefore this methodology improved the lower limit of detection by two-fold. The reasons for the improved lower limit detection included the use of a different standard curve and the fact that our peak eluted in 2 fractions whereas the peak used by Kelly *et al* (1996) eluted over 4 fractions, the outer two of which were discarded. Only the 2 in the middle were pooled. Hence some of the samples was discarded.

2.9.7 Preparation of negative and positive controls for unaltered folic acid assay.

Serum was collected from 10 individuals after an overnight fast from folic acid. 1 sample from each subject was prepared and assayed for serum folic acid. Unmetabolised folic acid was not detected in any of the samples. 1 sample from each subject was then spiked with folic acid at the following concentrations 1ng/ml, 3ng/ml and 5ng/ml. The samples were assayed by the combined HPLC/microbiological assay to obtain mean, standard deviation and coefficient of variation (Intra-assay). The 10 serum samples were then pooled into three batches of 1ng/ml, 3ng/ml and 5ng/ml and assayed for unmetabolised folic acid (Inter-assay). The values obtained are shown in Table 2.1. A negative or unspiked control and one of each positive controls was ran with all subsequent assays as a quality control measure.

2.9.8 Purification of folic acid

Throughout the duration of the study it was necessary to check the ^3H -folic acid for purity as it can break down to pteridine and pABGlu. This was checked at regular intervals prior to spiking the samples as follows. A serum sample was spiked with 5000dpms ^3H -folic

acid. The sample was treated, injected, collected and counted as previously described. When the folic acid was intact only one peak of radioactivity occurred and a recovery of between 85 - 90 % was obtained at around 12 minutes. However, on occasions the ^3H -folic acid was found to have broken down as two radioactive peaks were identified as shown in figure 2.9. It was then necessary to purify the ^3H -folic acid before adding it to any test samples. This was carried out as follows. A 10 μl sample of the stock solution of ^3H -folic acid was added to 1ml of water. 5 μl was taken off and added to 5ml of scintillation fluid. This was counted by scintillation counting for 1 minute to obtain total counts. A classic Sep-Pak was then activated with 5ml of methanol and washed with 10ml of 10X citrate phosphate. The 1ml sample was then eluted slowly at 1ml/min through the Sep-Pak into a 1.5ml eppendorf tube. The Sep -Pak was then washed with 1ml of 10X citrate phosphate buffer and air dried with a 10ml syringe into a second eppendorf tube. The Sep-Pak was then flushed with 1ml methanol into a third eppendorf tube. A further 1ml of methanol was then eluted through the Sep-Pak into a fourth eppendorf tube. 10 μl was then taken from each of the eppendorf tubes and placed in 4 scintillation vials. 5ml of scintillation fluid was added and the vials were counted for 1 minute each. A very large radioactive peak was present in the scintillation vial with the first methanol elution. No radioactive peak was identified in the scintillation vial after the second methanol wash indicating that all the pure folic acid had eluted after the first methanol wash. A sample from the folic acid peak (11,000 dpm) was injected onto HPLC, separated, collected and counted as described. Only one radioactive peak was identified and 88.8% recovery was obtained - see figure 2.10. The purified ^3H -folic acid was then suitable for use as an internal standard.

Table 2.1 Illustrating values obtained from spiked serum samples.

Run number	0ng/ml	1ng/ml (Intra- assay)	1ng/ml (Inter- assay)	3ng/ml (Intra- assay)	3ng/ml (Inter- assay)	5ng/ml [Intra- assay]	5ng/ml [Inter- assay]
1	0.00	0.89	1.06	2.89	2.86	4.92	5.11
2	0.00	0.95	1.08	3.05	2.95	4.95	5.09
3	0.00	1.05	1.05	3.01	2.99	5.07	4.80
4	0.00	1.02	0.87	2.94	3.02	5.10	4.92
5	0.00	1.08	0.91	2.95	2.96	5.15	4.89
6	0.00	0.97	0.93	3.07	3.07	5.04	5.13
7	0.00	1.10	1.12	2.98	3.09	4.96	5.15
8	0.00	1.06	1.09	2.95	3.13	5.06	5.10
9	0.00	1.06	1.05	3.05	3.02	5.10	4.85
10	0.00	0.95	1.02	3.09	2.98	4.93	4.89
mean		1.013	1.018	2.998	3.007	5.028	4.993
s.d.		0.069	0.085	0.066	0.078	0.082	0.134
% c.v.		6.8	8.3	2.19	2.58	1.62	2.68

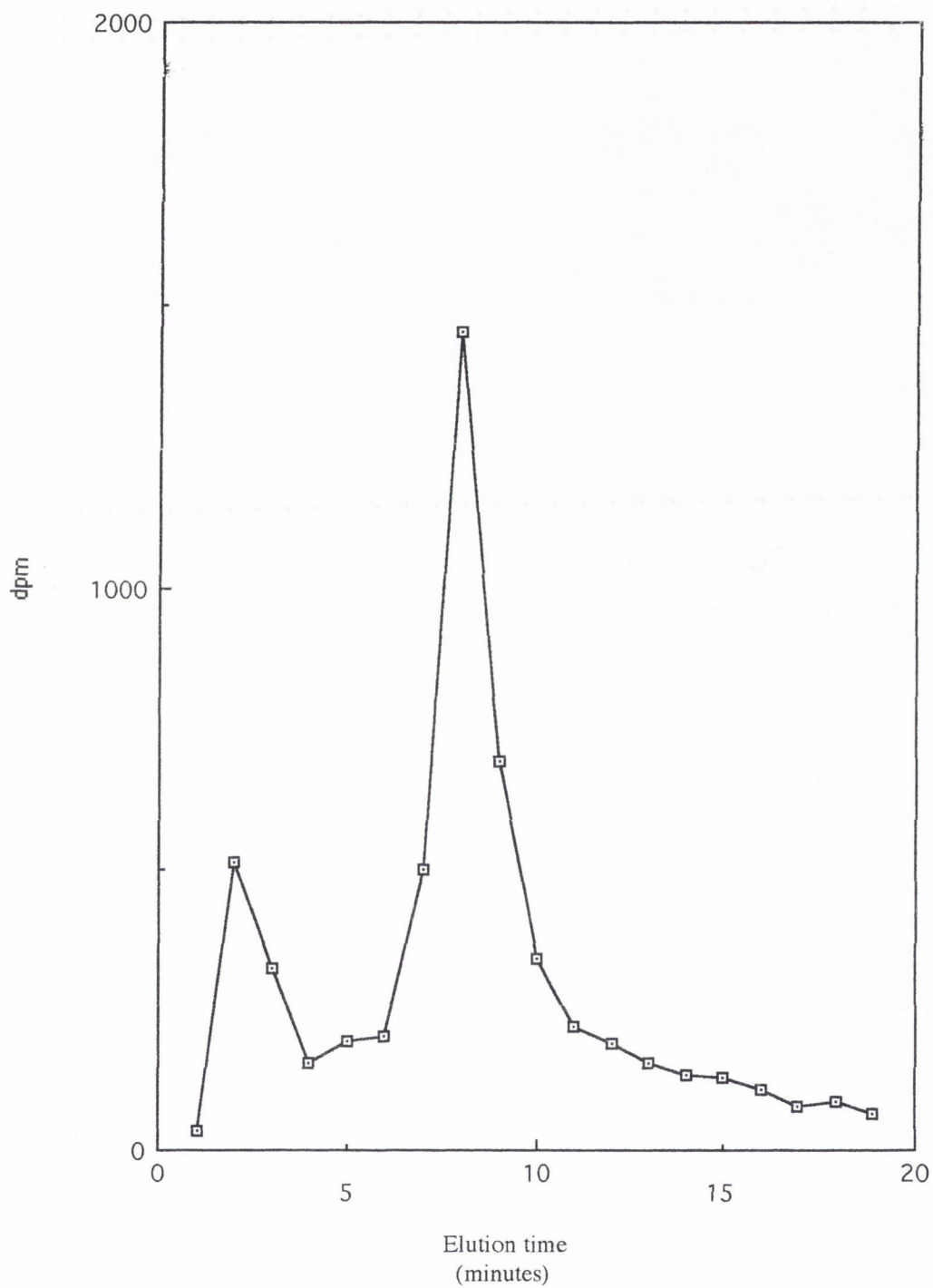


Figure 2.9. Elution of 3H folic acid before purification

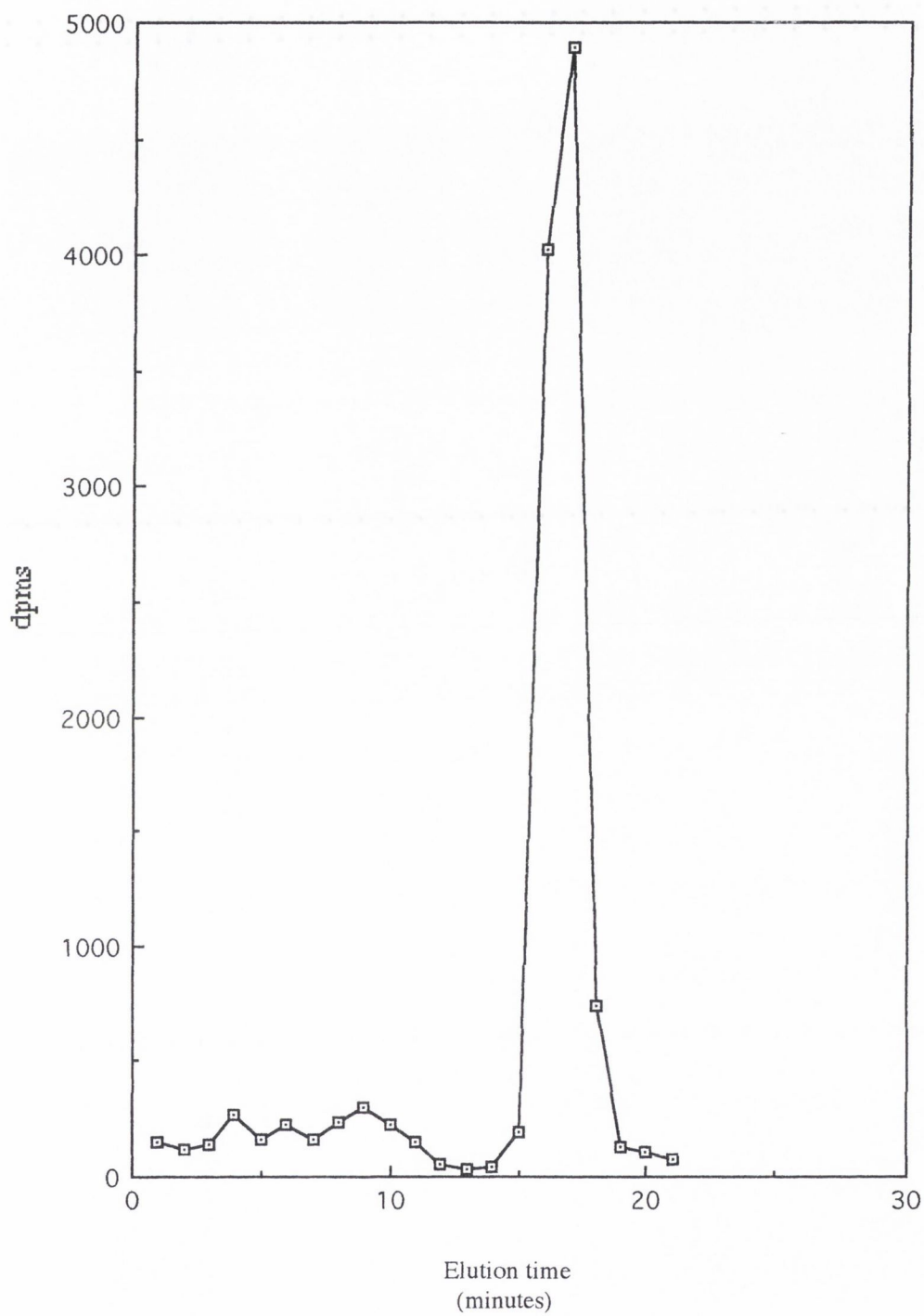


Figure 2.10. Elution of 3H folic acid after purification

2.10 Pilot study to determine the reproducibility of the combined assay.

This pilot study was carried out to investigate the reproducibility of the combined assay set up by Kelly *et al* (1996) who reported that unaltered folic acid was detected in the sera of subjects at a $T_{\max}$ of 80 minutes after ingestion of folic acid supplements of 400µg or greater.

2.10.1 Recruitment

8 healthy adult subjects (6 females, 2 males) from the laboratory staff were recruited by voluntary participation. Ethical approval was obtained from the Federated Dublin Voluntary Hospitals and consent forms were completed. A medical history was obtained and all subjects were free from vascular, hepatic, renal or gastrointestinal disease. Oral contraceptive pill users were excluded from participating, as were those taking anti-convulsant therapy or multi-vitamin preparations. Baseline bloods were collected and assayed for serum and red cell folate to establish folate status. All subjects were found to be folate replete

2.10.2 Subject Participation

Subjects were asked to omit folic acid fortified foods on the morning prior to the blood collection. A baseline serum sample was collected. Each subject then consumed a 200µg folic acid supplement. Serum samples were collected from all subjects' 80 minutes post-prandially. To prevent carry-over of the vitamin between test doses the intervention trials

were conducted one week apart. After one week the trial was repeated using 400µg supplements. After a further week the trial was repeated using 600µg supplements. Serum samples were separated and frozen until time of analysis. All samples were assayed in duplicate and in the same assay batch to eliminate inter-assay variation. The results are illustrated in tables 2.2 and 2.3.

Table 2.2 (a) Baseline red cell and serum folate ($\mu\text{g/L}$) concentrations

	Serum Folate	Red Cell Folate
Subject 1	6.6	31
Subject 2	21.3	577
Subject 3	18.2	402
Subject 4	22.3	600
Subject 5	11.2	338
Subject 6	12.9	361
Subject 7	3.3	320
Subject 8	19.1	323

Table 2.2 (b) Pre and post-prandial serum folate concentrations ($\mu\text{g/L}$) at 200 μg test dose

	Pre-prandial	Post-prandial
Subject 1	5.9	7.7
Subject 2	20	27.4
Subject 3	19.3	29
Subject 4	21.8	26.7
Subject 5	9.4	10.7
Subject 6	13	16.9
Subject 7	3.5	4.9
Subject 8	20	22.1

Table 2.2 (c) Pre and post-prandial serum folate concentrations ($\mu\text{g/L}$) at 400 μg test dose

	Pre-prandial	Post-prandial
Subject 1	4.4	5.2
Subject 2	19.3	27
Subject 3	24.3	31.6
Subject 4	15.2	18.6
Subject 5	9.7	13
Subject 6	12	19
Subject 7	3.9	8.4
Subject 8	15.3	20.8

Table 2.2 (d) Pre and post-prandial serum folate concentrations ($\mu\text{g/L}$) at 600 μg test dose

	Pre - prandial	Post - prandial
Subject 1	3.1	8.0
Subject 2	25.7	52.6
Subject 3	25.1	36.4
Subject 4	29.6	59.2
Subject 5	10.9	25.2
Subject 6	15.9	30.6
Subject 7	4.1	12
Subject 8	10	15.4

Table 2.3 Unmetabolised Folic Acid ($\mu\text{g/l}$): pre and post supplementation.

200μg	Pre-prandial	Post-prandial
Subject 1	0.0	0.0
Subject 2	0.0	0.0
Subject 3	0.0	0.42
Subject 4	0.0	0.0
Subject 5	0.0	0.0
Subject 6	0.0	0.0
Subject 7	0.0	0.0
Subject 8	0.0	0.0
<u>Duplicates at 200μg</u>		
Subject 1	0.0	0.0
Subject 2	0.0	0.0
Subject 3	0.0	0.48
Subject 4	0.0	0.0
Subject 5	0.0	0.0
Subject 6	0.0	0.0
Subject 7	0.0	0.0
Subject 8	0.0	0.0
<u>At 400μg</u>		
Subject 1	0.0	0.0
Subject 2	0.0	0.58
Subject 3	0.0	1.173
Subject 4	0.0	0.0
Subject 5	0.0	0.0
Subject 6	0.0	0.448
Subject 7	0.0	0.0
Subject 8	0.0	0.414
<u>Duplicates at 400μg</u>		
Subject 1	0.0	0.0
Subject 2	0.0	0.68
Subject 3	0.0	1.2
Subject 4	0.0	0.0
Subject 5	0.0	0.0
Subject 6	0.0	0.5
Subject 7	0.0	0.0
Subject 8	0.0	0.410
<u>At 600 μg</u>		
Subject 1	0.0	0.0
Subject 2	0.0	1.701
Subject 3	0.0	1.70
Subject 4	0.0	1.64
Subject 5	0.0	1.192
Subject 6	0.0	0.688
Subject 7	0.0	0.0
Subject 8	0.0	0.738
<u>Duplicates at 600μg</u>		
Subject 1	0.0	0.0
Subject 2	0.0	1.773
Subject 3	0.0	1.74
Subject 4	0.0	1.4
Subject 5	0.0	1.17
Subject 6	0.0	0.738
Subject 7	0.0	0.0
Subject 8	0.0	0.7130

2.11 Discussion of results

2.11.1 Elution time of folic acid

Figure 2.5 shows the elution time of ^3H folic acid in mobile phase with 15% methanol which, occurs at 8-9 minutes. This retention time was considered to be too short in view of the possibility that the folic acid might overlap with the 5-methyl THF and could possibly cause contamination of unaltered folic acid in the microbiological assay. A fresh mobile phase buffer was made up using a lower concentration of methanol (12.5%). It was shown that this increases the retention time of the ^3H -folic acid standard by 2-3 minutes as illustrated in Figure 2.6. The elution time was shown to occur around 11-12 minutes. This was considered to be a satisfactory retention time as the folic acid is unlikely to overlap with the 5-methyl THF. This concentration of methanol was used in all subsequent mobile phase buffers.

2.11.2 Effect of mobile phase on the growth of *L. casei*.

Figure 2.7 shows a standard curve prepared with 0.5% sodium ascorbate buffer made up in the range of 0-12.5 pg/well. Figure 2.8 shows a standard curve prepared with 0.5% sodium ascorbate and mobile phase buffer (1:1) made up in the range of 0 - 12.5 pg/well. Both curves were found to be identical demonstrating that the mobile phase does not affect the growth of the *L.casei* up to 12.5pg/well. All future standard curves for the microbiological assay for serum folic acid were made within the range 0-12.5pg/well in diluent consisting

of mobile phase and 0.5% sodium ascorbate buffer (1:1). Test samples were also diluted in mobile phase and 0.5% sodium ascorbate (1:1) buffer in wells where 50µl of test sample had to be diluted to a final volume of 100µl.

2.11.3 Purification of ^3H folic acid

Examination of figure 2.19 and 2.10 demonstrates the importance of checking the ^3H -folic acid standard for purity at regular intervals before use. Figure 2.9 shows that the radioactivity had broken down and would therefore gave a very poor percentage recovery if it were used in a test sample. This would then lead to inaccuracies in the final calculation of the folic acid from the microtitre plate. Throughout the entire study the radioactivity was checked for purity at regular intervals (approximately 4-6 monthly)

2.11.4 Reproducibility of pilot study results.

A comparison of table 2.1 and 2.2 show that the assay gives good reproducibility as the results appear to correlate well with each other. However the sample number was too small to show this statistically. The finding also back up those reported by Kelly *et al* (1996) except in the case of subject 1 where folic acid appeared after the ingestion of 200µg folic acid. Kelly *et al* (1996) reported the appearance of folic acid in the serum only when supplements of 400µg or greater were consumed. An explanation for this finding lies in the fact that our column gave a sharper peak resulting in the peak eluting over 2 fractions. In the methodology described by Kelly *et al* (1996) the peak fell over 4 fractions. 2 of these

were then collected and pooled to estimate percentage recovery usually about 8%. The percentage recovery obtained in this methodology was usually in the region of 10% as the 2 fractions containing the entire peak were pooled. Therefore none of the peak was lost. Also the lower limit of the detection of the microbiological assay described in this methodology is 0.5pg/well whereas Kelly *et al*, (1996) reported a detection limit of 1pg/well. An alternative explanation could be that this subject ingested another source of folic acid and that they were unaware that they had done this. This subject was questioned about this and reported that to the best of their knowledge they had not ingested any other sources of the vitamin.

2.12 Principles of Sep-Pak separation.

Sep-Paks are small C18 radial pak cartridges useful for solid phase extractions. The principle of the Sep-Pak is that intact ^3H -folic acid will be retained by the Sep-Pak using the mobile phase described for the HPLC previously. Under these conditions cleaved species, including Pteridine and *para*-aminobenzoylglutamate (p-ABGlu), will not. These species have been demonstrated to auto-elute during the sample addition to the Sep-Pak or in the initial wash with aqueous buffer (Geoghan *et al*, 1995). Hence ^3H -folic acid is retained longest.

2.12.1 Pilot study to examine the stability of folic acid in bread.

In January 1998 the FDA implemented mandatory folic acid fortification of all flour milled in the USA at a level of $140\mu\text{g}/100\text{g}$ of flour. Many food companies in Ireland and the UK are already adding vitamins to their produce. While this has been effective in raising public awareness about the importance of consuming more folic acid peri-conceptionally a more scientific approach is warranted and some fundamental issues need to be addressed. One such issue which, arises is the stability of folic acid when it is exposed to high temperatures i.e. during the baking process. Do high temperatures cause folic acid to break down to pABGlu and pteridine? How much folic acid is actually available to the consumer at the end of food processing? This pilot study was carried out to investigate the stability of folic acid throughout the various food processing and preparation stages involved in bread making.

2.12.2 Recipe

500g flour

8.85g salt

8.8g margarine

414.95ml water

14.17mg yeast

2.12.3 Method

The flour and salt were sieved into a bowl. The margarine was rubbed in. 700 μ g folic acid (140 μ g/100g of flour) was added to the water in a jug and mixed thoroughly. 4.6 million dpm 3 H-folic acid were added to the liquid mixture. The liquid was mixed thoroughly. A 2ml sample from the liquid mixture was taken off. 1ml was stored at -20°C until time of analysis for total folate (Table 2.2.a). The other 1ml was added to 9ml scintillation fluid in a 25ml scintillation vial and counted for 5 minutes (Table 2.2.a). The yeast was then added to the liquid mixture and the mixture was stirred until all of the yeast had dissolved. This liquid mixture was then added to the dry ingredients in the bowl. Dough was formed by mixing the all the ingredients together with a wooden spoon for 10 minutes to ensure that the folic acid was evenly distributed. This dough was then kneaded for 10 minutes on a floured surface. It was then placed in a plastic bag beside a hot oven for one hour to allow the dough to improve. After this time the dough had doubled in size. The dough was then kneaded for a further 10 minutes and placed in a loaf tin in a refrigerator for 1 $\frac{1}{2}$ hours until

the dough had risen up over the sides of the tin. The bread was then placed in an oven and baked for 35 minutes at 230°C until golden brown. A duplicate loaf was prepared in the same way.

2.12.4 Preparation of bread extracts for estimation of recovery and stability

The finished product weighed 1kg. 100g of the loaf was cut off and soaked to saturation in 500ml of 0.5% ascorbic acid buffer. Folic acid is known to leach readily into ascorbic acid buffer. 2 x 20ml aliquots of the saturated bread were removed and placed in 25ml universal containers. These were centrifuged at 2500rpms for 10 minutes. The supernatants were removed and these were further centrifuged at 10,000gs for 10 minutes in 1.5ml eppendorf tubes. 1ml of supernatant was frozen at - 20°C prior to analysis for total folate (Table 2.4.b). Another 1ml was added to 9ml of scintillation fluid in a 25ml scintillation vial and counted for 5 minutes (Table 2.4.b).

2.12.5 Sep-Pak separation of starting counts (before baking).

In order to assess the stability of the ^3H folic acid at the end of the baking process it was necessary to assess its purity before baking. This was carried out as follows. A classic C18 waters Sep-Pak was activated sequentially with 1ml of methanol and 1ml of H_2O through the Sep-Pak at 1ml/min. A 1ml sample of the starting ^3H -folic acid was injected straight into a 25ml scintillation vial. 9ml of scintillation fluid were added and dpm were counted for 5 minutes. Another 1ml of starting ^3H -folic acid was eluted through the Sep-Pak and

washed with 1ml of 0.5 % ascorbic acid buffer at 1ml/min into a scintillation vial and air-dried with a syringe. The ^3H -folic acid was then eluted from the Sep-Pak at 1ml/ minute with 1ml of methanol and air dried as before into a scintillation vial. This step was repeated into another scintillation vial. One vial was left blank to act as a negative control. 9ml of scintillation fluid was added to all the vials. These were shaken vigorously and the dpm were counted for 5 minutes by scintillation counting (Table 2.4.c) This exercise was repeated for the duplicate loaf (bread 2) (Table 2.5.c).

2.12.6 Sep - Pak separation of end counts (after baking).

A 1ml sample from the supernatant obtained at the end of the baking process was pipetted directly into a scintillation vial. 9ml of ecolite scintillation fluid was added and the sample was counted for 5 minutes. This was used as a reference for total counts in 1ml. A Sep-Pak was activated as previously described. Another 1ml sample from the supernatant was eluted slowly at 1ml/min via the Sep-Pak into a scintillation vial and air dried as described. The Sep-Pak was then washed with 1ml of 0.5% ascorbic acid buffer. The Sep-Pak was then eluted with 1ml methanol and air-dried into a scintillation vial. This step was repeated. Scintillation counting was carried out as before for 5 minutes (Table 2.4.d). This exercise was repeated for the duplicate loaf (bread 2) (Table 2.5.d).

Table 2.4. Folic acid ($\mu\text{g/L}$) and dpm obtained pre and post bread making.

<i>Bread 1</i>	
<i>a) Before Baking</i>	
dpm = 10310.7	
microbiological assay value 1.635 $\mu\text{g/L}$	
<i>b) After Baking</i>	
dpm = 8925.2 (86.5% recovery)	
microbiological assay value 1.664 $\mu\text{g/L}$	
<i>c) Counts before baking</i>	
Total counts injected on 18855 dpm	
counts recovered	vial 1 124.00 (sample injected on)
	vial 2 15.12 (after 0.5% Ascorbic acid wash)
	vial 3 8109.8 (after 1 st methanol wash)
	vial 4 6803.5 (after 2 nd methanol wash)
	vial 5 11.9 (blank)
	(79% recovery)
<i>d) Counts after baking</i>	
Total counts injected on 472.9 dpm	
counts recovered	vial 1 22.27 (sample injected on)
	vial 2 18.61 (after 0.5% Ascorbic acid wash)
	vial 3 48.14 (after 1 st methanol wash)
	vial 4 337.3 (after 2 nd methanol wash)
	vial 5 17.73 (blank)
	(81% recovery)

Table 2.5 Folic acid ($\mu\text{g/L}$) and dpm obtained pre and post bread making.

<i>Bread 2</i>											
<i>a) Before Baking</i>											
dpm = 10077.5											
microbiological assay value 1.682 $\mu\text{g/L}$											
<i>b) After Baking</i>											
dpm = 9129.2 (90.5% recovery)											
microbiological assay value 1.728 $\mu\text{g/L}$											
<i>c) Counts before baking</i>											
Total counts injected on 21746.2 dpm											
counts recovered	<table><tr><td>vial 1</td><td>36.95 (sample injected on)</td></tr><tr><td>vial 2</td><td>51.25 (after 0.5% Ascorbic acid wash)</td></tr><tr><td>vial 3</td><td>19458.6 (after 1st methanol wash)</td></tr><tr><td>vial 4</td><td>270.74 (after 2nd methanol wash)</td></tr><tr><td>vial 5</td><td>35.53 (blank)</td></tr></table>	vial 1	36.95 (sample injected on)	vial 2	51.25 (after 0.5% Ascorbic acid wash)	vial 3	19458.6 (after 1 st methanol wash)	vial 4	270.74 (after 2 nd methanol wash)	vial 5	35.53 (blank)
vial 1	36.95 (sample injected on)										
vial 2	51.25 (after 0.5% Ascorbic acid wash)										
vial 3	19458.6 (after 1 st methanol wash)										
vial 4	270.74 (after 2 nd methanol wash)										
vial 5	35.53 (blank)										
(91% recovery)											
<i>d) Counts after baking</i>											
Total counts injected on 604.5 dpm											
counts recovered	<table><tr><td>vial 1</td><td>22.63 (sample injected on)</td></tr><tr><td>vial 2</td><td>18.38 (after 0.5% Ascorbic acid wash)</td></tr><tr><td>vial 3</td><td>439.9 (after 1st methanol wash)</td></tr><tr><td>vial 4</td><td>138.4 (after 2nd methanol wash)</td></tr><tr><td>vial 5</td><td>29.84 (blank)</td></tr></table>	vial 1	22.63 (sample injected on)	vial 2	18.38 (after 0.5% Ascorbic acid wash)	vial 3	439.9 (after 1 st methanol wash)	vial 4	138.4 (after 2 nd methanol wash)	vial 5	29.84 (blank)
vial 1	22.63 (sample injected on)										
vial 2	18.38 (after 0.5% Ascorbic acid wash)										
vial 3	439.9 (after 1 st methanol wash)										
vial 4	138.4 (after 2 nd methanol wash)										
vial 5	29.84 (blank)										
(95% recovery)											

2.13 Discussion of results

2.13.1 Stability of ^3H folic acid during the bread making process

Before baking

It was considered important to determine experimentally if the ^3H -folic acid to be added to the bread was pure as it would be futile to investigate the integrity of the radioactivity after baking if purity hadn't been established at the beginning of the experiment. In both experiments *Bread 1* and *Bread 2*, the results before baking (Table 2.4 and 2.5) demonstrate that the ^3H -folic acid was pure as no radioactivity was present in the ascorbic acid wash obtained from the Sep -Pak prior to the methanol wash. In the first experiment 79% of the radioactivity was recovered and in experiment 2 approximately 91% recovery was obtained after the methanol flush indicating that the radioactivity was pure.

After baking

Tables 2.4 and 2.5 show the percentage intact folic acid recovered from the folic acid fortified bread after baking. The results show that no degradation products of folic acid occurred during the baking process as only a background level of counts were found in the eluent recovered from the Sep - Pak after the ascorbic acid wash. After the methanol wash in both experiments a very large percentage recovery of intact folic acid was obtained demonstrating that folic acid added to bread did not degrade when exposed to temperatures

up to 230°C for 35 minutes. In *Bread 1* an 81% recovery was obtained and in *Bread 2* the results show that a 95 % recovery was obtained. Thus it would appear that the folic acid added to the bread remains intact during the baking process. This recovery indicates relatively high stability of folic acid during the baking process indicating that the amount of folic acid added to fortified flour is the same amount that is available to the consumer in the finished product.

2.13.2 Results of the microbiological assay pre and post baking

The results from both experiments illustrate that the folic acid was well dispersed throughout the bread as the theoretical recovery ($\mu\text{g/L}$) obtained from the microbiological assay was equal to the actual recovery even though the dough was mixed by hand in the laboratory. An industrial mixer would provide a much more vigorous mixing process. Therefore it seems that there would be minimal risk of large quantities of folic acid being clumped together in sections of the bread.

Chapter Three

Intervention trial 1

**Folic acid fortification of flour:
Determination of the level of unmetabolised
folic acid in serum. Folic acid analysis by
HPLC/microbiological assay**

3.1 Introduction

A mass folic acid fortification programme gives rise to concern about a number of public health safety issues chief of which is that folic acid has the potential to mask the early haematological symptoms of pernicious anaemia. In addition other potentially toxic effects such as interference with the efficacy of certain drugs, and potential cancer induction in the population after chronic ingestion are theoretical concerns. These concerns principally relate to long-term consumption of folic acid and in particular the presence of free folic acid in the circulation. However no previous research has been carried out investigating the appearance of unmetabolised folic acid in serum after long-term consumption of folic acid fortified products.

The present folic acid fortification programme currently in practise in the US is estimated to supply an additional 100 μ g to the average US daily folate intake. At present no evidence exists to verify that chronic consumption of this level will be safe in terms of unmetabolised folic acid appearance in serum. The only evidence showing threshold doses likely to cause unmetabolised folic acid appearance in serum are those demonstrated in the studies carried out by Kelly *et al* (1997). These findings provided the first evidence that physiological doses of folic acid may have potential for harm in that the synthetic provitamin not normally presented to human cells, will be encountered by a wide range of cells, and organs in the body. All of the Kelly *et al*, (1997) studies were very acute studies with the longest pre-saturation period being only 18 days. In addition folic acid was merely applied and dried onto the surface of foodstuffs. Hence a matrix effect of the food was not taken into consideration. The results therefore cannot be extrapolated to provide

information on the effects of long-term consumption of folic acid fortified foods on the presence or absence of unmetabolised folic acid in serum.

This study undertook the task of determining the level of folic acid fortified flour consumed on a long-term basis by a general population group that would lead to unmetabolised folic acid appearance in serum post-prandially. The effect of chronic consumption of the level of flour fortification currently in practice in the USA (140µg/100g) was chosen to be investigated as other countries internationally including the UK are likely to fortify at similar levels in the future. The study also investigated the effects of the two higher levels being advocated by the CDC (350µg and 700µg/100g) on unmetabolised folic acid in serum.

Subjects were pre-saturated for 14 weeks prior to commencing the study thereby mimicking serum folate levels after long-term consumption. This is sufficient time for serum folate to plateau at this level of supplementation as demonstrated by Ward *et al* (1997). It is also sufficient time for complete red cell turnover in the body. In addition the bread used in the trial was fortified under industrial conditions prior to baking thereby creating realistic conditions of mandatory fortification and incorporating a matrix effect of the bread into the study.

Folic acid fortified plain white bread was chosen as the vehicle of administration to be tested as it is a very widely consumed food staple with 99% of the Irish population consuming bread of some description (Irish National Nutrition Survey 1990). Flour is also the vehicle of fortification currently in use in the US.

3.2 Methods

3.2.1 Recruitment

20 healthy adult subjects, 10 females and 10 males, aged 20-40 years were recruited by voluntary participation from the student and staff population of the Central Pathology Laboratory, St. James's Hospital and from the general population. Ethical approval was obtained from the Ethics Committee of the Federated Dublin Voluntary Hospital, James's Street, Dublin 8. Written consent and a medical history was obtained and all subjects were found to be free from vascular, hepatic, renal or gastrointestinal disease. Oral contraceptive pill users were excluded from participating (as the contraceptive pill is thought to interfere with folate metabolism), as were those taking multi-vitamin preparations or anti-convulsant therapy. One subject dropped out of the study for personal reasons. 19 subjects completed the study. All subjects were folate replete upon recruitment as determined by serum and red cell folate concentrations at baseline. A baseline sample was collected and assayed for unmetabolised folic acid to establish pre-intervention levels.

3.2.2 Pre-saturation

Subjects ($n = 19$) were placed on a regime of 400 μ g folic acid supplements daily for 14 weeks as a pre-saturation phase. Serum samples were collected at week 10 for total folate (to monitor compliance with the supplementation regime) and unmetabolised folic acid to see if an increase had occurred from baseline levels. This sample was collected six hours after the vitamin had last been consumed to allow time for an acute post-prandial folic acid

serum response to have cleared from the serum. This would allow us to determine if any accumulation of the vitamin had occurred as a result of the supplementation phase. At week 14 blood samples were collected and analysed for red cell folate, serum folate and unmetabolised folic acid. This was again collected six hours after the vitamin had last been consumed to investigate whether or not any accumulation of unmetabolised folic acid had occurred.

3.2.3 Fortification of bread

After this initial supplementation phase, subjects consumed bread that was fortified with folic acid at three different levels over three weeks. The bread was specially prepared in three batches by Superquinn Bakeries, Kimmage, Dublin, under the supervision of the principal investigator. The folic acid was weighed out in the laboratory on the evening before the bread was baked and was placed in three sterile universal containers. These containers were stored in a refrigerator overnight. The folic acid was added to water, which was then mixed with the flour and other dry ingredients. Dough was formed by thorough mixing with an industrial mixer. The bread was then baked under normal conditions.

3.2.4 Intervention phases

Bread consumed at week 1 (*Regime C*) supplied a total of 400µg of folic acid in 2 slices. The 1st slice was consumed each day at breakfast time and the 2nd slice was consumed 4 hours later at lunchtime. The subjects then proceeded onto a further 7-day regime of 400µg folic acid supplements. This was administered to re-saturate the subjects after the bread

phase prior to commencing week 2 bread. After this 7-day supplementation phase the subjects consumed week 2 (*regime B*) bread for 7 days. Week 2 bread supplied 100µg in each slice. In keeping with week 1 bread subjects were asked to consume one slice of bread each morning and one each lunchtime. At the end of week 2 bread the subjects were again asked to consume 400µg supplements daily for a further 7 days to re-saturate their folate status before commencing the last bread regime. After 7 days on the supplements the subjects consumed week 3 bread (*regime A*) for 7 days. Week 3 bread supplied 50µg in each slice. One slice was consumed each morning and one slice was consumed each lunchtime. All other folic acid fortified foods were omitted for the entire duration of the study and a comprehensive list of all commercially fortified foods was issued to each subject. In addition a list suggesting alternative non-fortified products to substitute these fortified foods was supplied. These products were supplied free of charge to the subjects. Subjects were contacted by telephone or in person every 2 weeks to ensure compliance with the study protocol and to help motivate and encourage them.

3.2.5 Blood collection

On day 7 of each bread regime the subjects were asked to come to the laboratory at 8am having consumed their last slice of folic acid fortified bread the previous lunchtime at 1pm. Hence approximately 20 hours has lapsed since their last intake of folic acid. An intravenous cannula was inserted and a pre-prandial serum sample (A) was collected to determine unmetabolised folic acid as well as serum folate for each subject. This sample was collected in order to ascertain if any folic acid was present as a result of an accumulation from the fortified bread and also to establish a pre-prandial baseline. The

first slice of bread was then administered. Serum samples were then collected every hour for four hours (samples B, C, D and E respectively). The second slice of bread was then administered and serum samples were collected every hour for three hours (samples F, G and H respectively). It was thought that this pattern would give a good overall picture of the appearance (if present) of unmetabolised folic acid and of the duration of its presence.

Figure 3.1 illustrates schematically the intervention phases of the study and sample collection.

3.2.6 Urine collection

Urine samples were collected for unmetabolised folic acid from two out of the nineteen subjects on day 7 of each bread regime. Pre-prandial urine samples were collected upon their arrival into the laboratory. All urine passed post prandially was collected over the 8-hour time frame. The urine samples were placed in universal containers, in 1g of ascorbic acid and frozen at -20°C until time of analysis.

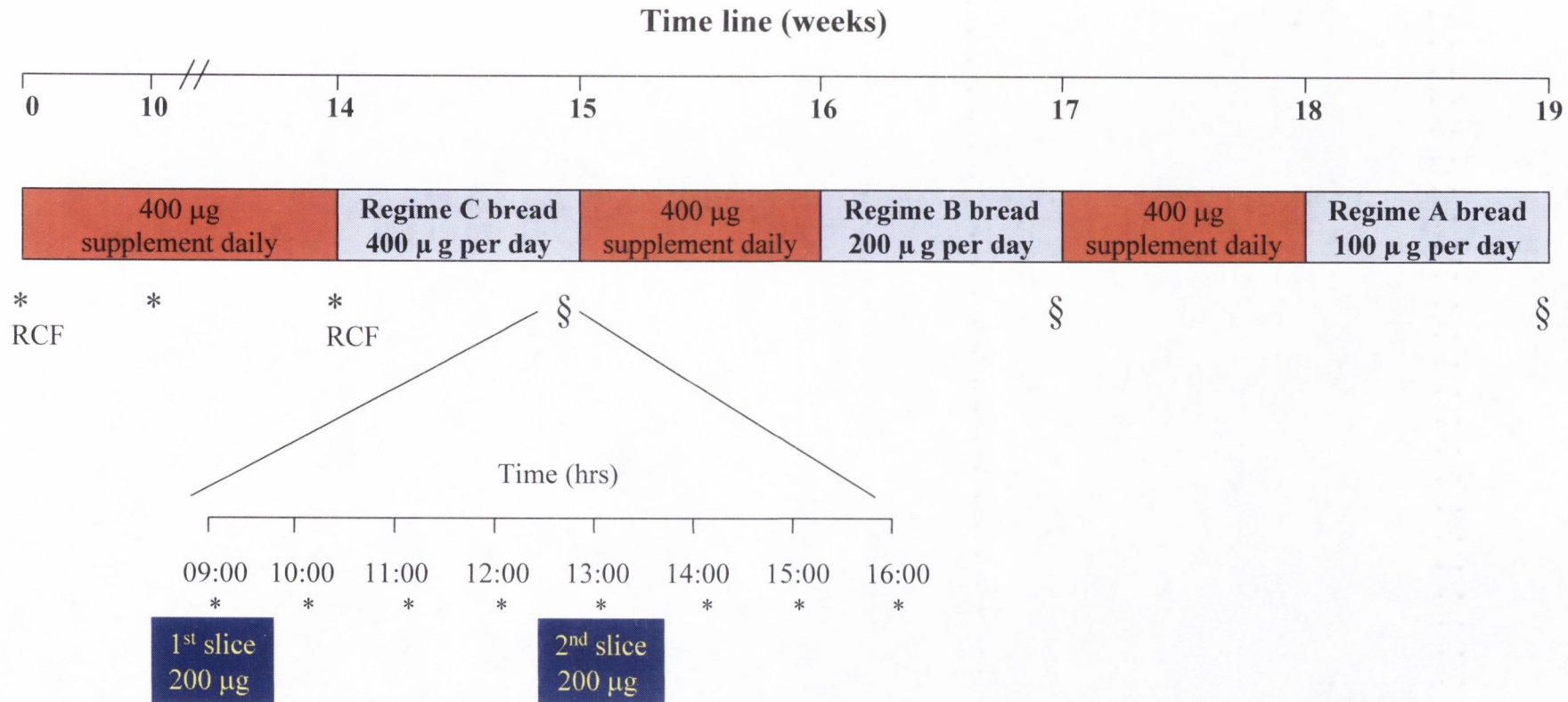
3.2.7 Laboratory analysis

All samples were assayed for total serum and red cell folates by the *L. casei* microbiological assay described by Molloy & Scott (1997). Analysis of samples for unmetabolised folic acid was carried out by combined HPLC/microbiological assay described by Kelly *et al* (1996).

3.2.8 Statistical analysis

Statistical analysis was performed using the computer software package Data Desk 5.0.

Paired t-tests, Anova and Post hoc Scheffe tests were used to analyse the data.



Legend

* = Folate and Folic Acid blood sample

RCF = Red cell folate blood sample

§ = Day 7, trial of bread regimes (400 µg, 200 µg, 100 µg)

Fig. 3.1 Schematic summary of intervention phases and sample collection

3.3 Results

Baseline serum (figure 3.2) and red cell folate (figure 3.3) concentrations were within normal reference ranges in all subjects. Serum folate concentrations increased significantly from week 0 to week 14, after the supplementation regime ($p=0.000097$) (figure 3.2) as did red cell folate concentrations ($p=0.0001$) (figure 3.3).

Table 3.1 illustrates that unmetabolised folic acid was not detected in any of the subject's samples collected at week 0. At week 10 and week 14 six hours after consumption of the 400 μ g supplement unmetabolised folic acid was not present in the sera of any subjects ($n=19$).

Unmetabolised folic acid was not detected in the sera of any of the subjects on day 7 of *regime C*, *regime B* or *A* bread pre or post prandially (see tables 3.2, 3.3 & 3.4 respectively).

Total serum folate concentrations did not change significantly over the 8 hours after *regime C* ($p=0.8941$), *regime B* ($p=0.9820$) or *regime A* breads ($p=0.8829$), see figure 3.4.

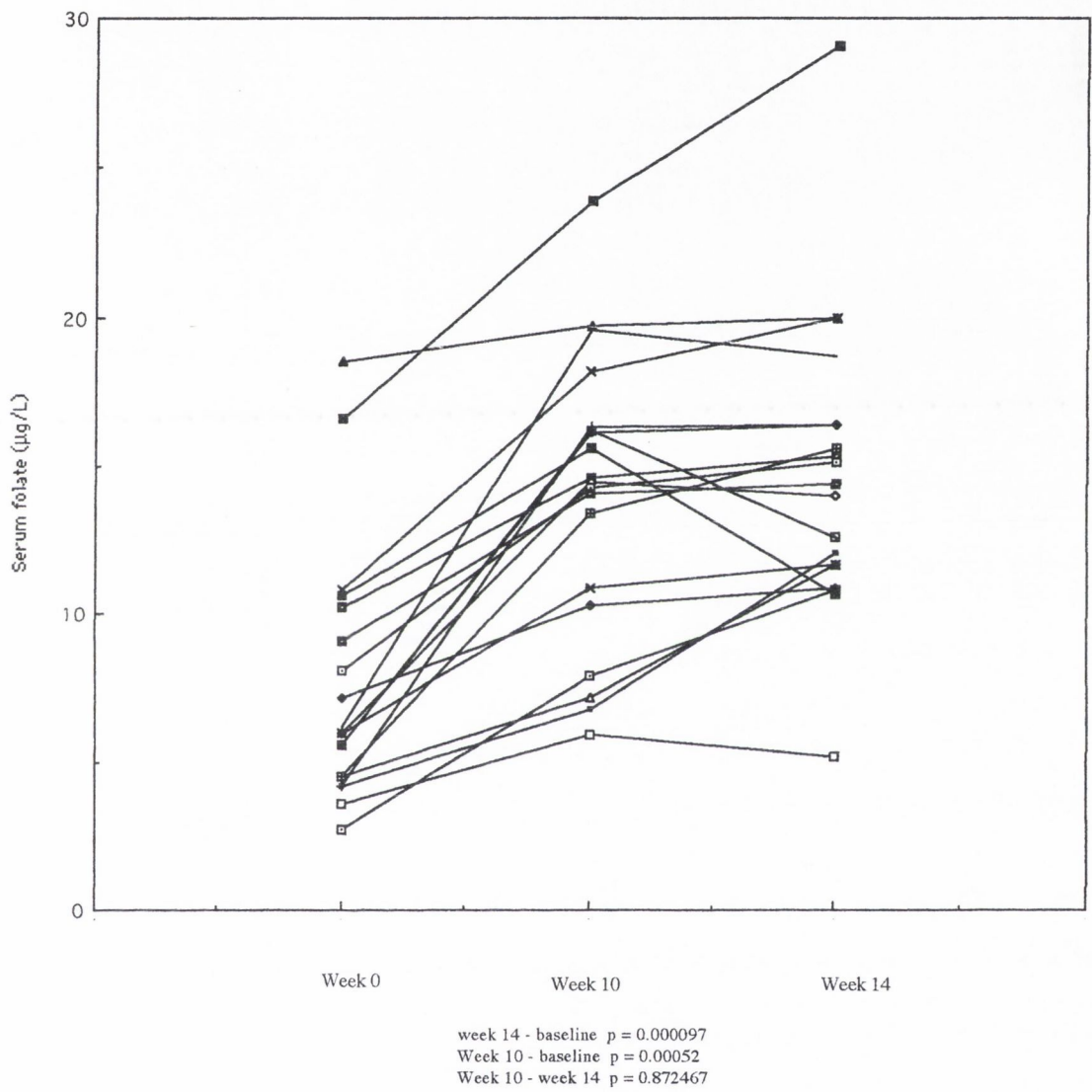


Figure 3.2. Serum folate concentrations (µg/L) at week 0, week 10 and week 14

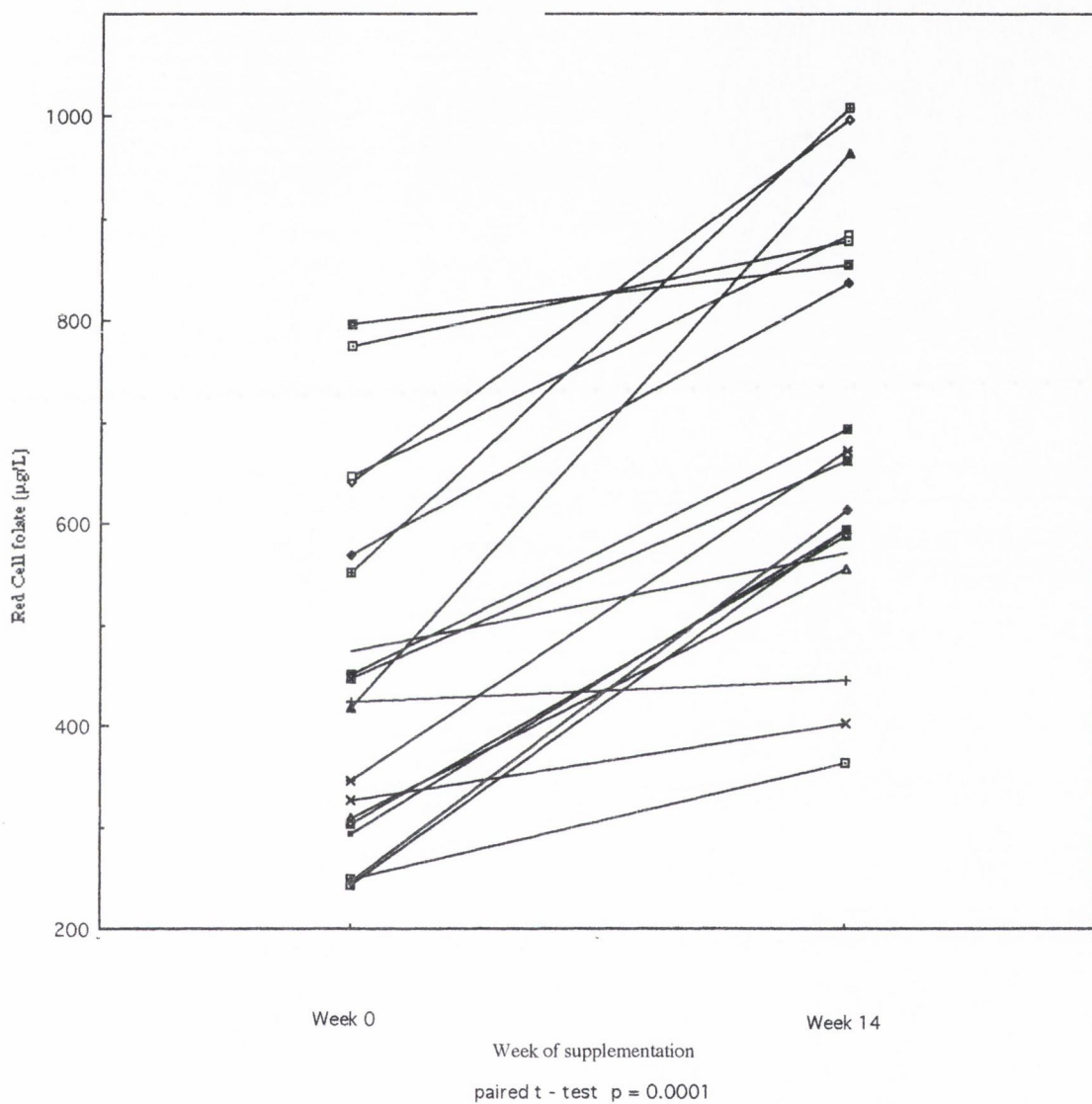


Figure 3.3. Red cell folate concentrations ($\mu\text{g/L}$) at week 0 and week 14

Table 3.1 Serum folates and serum folic acid ($\mu\text{g/L}$)

Samples collected at week 0, week 10 and 14 of folic acid supplementation regime (6 hours after the last vitamin had been consumed).

	Subjects	Baseline	Week 10 (6 hrs post)	Week 14 (6 hrs post)
folate	1	2.800	7.900	10.800
folic acid		0.0	0.0	0.0
folate	2	7.200	10.300	10.900
folic acid		0.0	0.0	0.0
folate	3	10.250	14.600	15.300
folic acid		0.0	0.0	0.0
folate	4	6.000	14.500	14.000
folic acid		0.0	0.0	0.0
folate	5	10.600	15.600	10.700
folic acid		0.0	0.0	0.0
folate	6	3.600	5.900	5.200
folic acid		0.0	0.0	0.0
folate	7	18.500	19.700	20.000
folic acid		0.0	0.0	0.0
folate	8	4.500	7.200	11.700
folic acid		0.0	0.0	0.0
folate	9	16.600	23.900	29.100
folic acid		0.0	0.0	0.0
folate	10	4.200	16.300	16.400
folic acid		0.0	0.0	0.0
folate	11	4.500	13.400	15.600
folic acid		0.0	0.0	0.0
folate	12	10.800	18.200	20.000
folic acid		0.0	0.0	0.0
folate	13	6.000	10.900	11.700
folic acid		0.0	0.0	0.0
folate	14	5.600	16.200	12.600
folic acid		0.0	0.0	0.0
folate	15	4.200	6.800	12.100
folic acid		0.0	0.0	0.0
folate	16	6.200	19.600	18.700
folic acid		0.0	0.0	0.0
folate	17	8.100	14.300	15.100
folic acid		0.0	0.0	0.0
folate	18	5.600	16.100	16.400
folic acid		0.0	0.0	0.0
folate	19	9.100	14.100	14.400
folic acid		0.0	0.0	0.0

Table 3.2 Serum folate and serum folic acid (µg/L)

Samples collected on day 7 of *regime C bread* (200µg in each slice) pre and post prandially

		Pre 1 st slice 200µg	1 hour post	2 hours post	1 hour post 2 nd slice 200µg	2 hours post
Subjects	Samples	A	B	C	F	G
1	folate	10.6	12.4	15.6	14.9	19.8
	folic acid	0.0	0.0	0.0	0.0	0.0
2	folate	14.2	15.2	13.5	11.3	10.1
	folic acid	0.0	0.0	0.0	0.0	0.0
3	folate	23.3	24.6	23.2	19.2	19.6
	folic acid	0.0	0.00	0.0	0.0	0.0
4	folate	18.3	17	15.1	12.3	13.3
	folic acid	0.0	0.0	0.0	0.0	0.0
5	folate	12.4	13.8	15.3	14.8	15
	folic acid	0.0	0.0	0.0	0.0	0.0
6	folate	8.3	8.7	12.2	10.2	10.2
	folic acid	0.0	0.0	0.0	0.0	0.0
7	folate	23.5	24.8	23.9	20	21.7
	folic acid	0.0	0.0	0.0	0.0	0.0
8	folate	12	11.9	12.1	18.4	16.3
	folic acid	0.0	0.0	0.0	0.0	0.0
9	folate	30.5	25.6	21.5	29.2	22.1
	folic acid	0.0	0.0	0.0	0.0	0.0
10	folate	16.4	19.8	20.4	22.5	21.5
	folic acid	0.0	0.0	0.0	0.0	0.0
11	folate	8	12	13.7	16.1	17.2
	folic acid	0.0	0.0	0.0	0.0	0.0
12	folate	17.3	20.4	24.8	26.7	30.5
	folic acid	0.0	0.0	0.0	0.0	0.0
13	folate	7.8	9.7	10.9	8.5	8.3
	folic acid	0.0	0.0	0.0	0.0	0.0
14	folate	9.9	10.3	10.6	12	10.6
	folic acid	0.0	0.0	0.0	0.0	0.0
15	folate	5.9	5.9	6.2	9	12.4
	folic acid	0.0	0.0	0.0	0.0	0.0
16	folate	21.2	23	26.7	23.6	25.9
	folic acid	0.0	0.0	0.0	0.0	0.0
17	folate	19.4	10	11.8	12.4	12.7
	folic acid	0.0	0.0	0.0	0.0	0.0
18	folate	21.8	19.6	25	21.5	28.4
	folic acid	0.0	0.0	0.0	0.0	0.0
19	folate	16	19.9	24.1	20	21.4
	folic acid	0.0	0.0	0.0	0.0	0.0

Table 3.3 Serum folate and serum folic acid (µg/l)

Samples collected on day 7 of *regime B bread* (100µg in each slice) pre and post prandially.

		pre 1 st slice	1 hour post	2 hrs post	3 hrs post	4 hrs post	1 hour post 2 nd slice	2 hrs post	3 hrs post
Subject	samples	A	B	C	D	E	F	G	H
1	folate	14.4	14.7	14.6	15.2	13.1	13.3	10.7	9.4
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	folate	22.7	20.6	20.6	21.6	18.9	13.6	13.6	18
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	folate	18.6	21.3	17.0	16.6	11.9	16.5	18.3	18.8
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	folate	16.9	17.4	15	14	10.9	15.6	19.6	16.1
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	folate	13.6	12.6	13.4	10.7	10.1	11	12.7	12.7
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	folate	6.8	8.3	9.9	10.6	11.4	10.5	10.2	11.4
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	folate	14.1	13.2	15.5	16.6	14.2	13	13.3	12.6
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	folate	10.2	10.6	12.5	12.1	15	14.9	12.7	11.8
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	folate	13.6	15.7	17.6	21.1	20.1	19.9	18.9	17.1
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	-----	-----
10	folate	16.6	18.8	20	19.7	20.2	27	-----	-----
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	-----	-----
11	folate	8.1	9.9	11.4	10.7	9.7	9.1	9.5	9.2
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	folate	14.5	12.9	14.7	13.9	15.8	15.4	15.6	13.7
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	folate	9.4	8.6	10.6	7.6	7.2	7.3	7.4	7.5
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	folate	8.5	8.9	8.5	7.1	7.2	6.8	6.7	5.5
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	folate	9	8.7	8.5	8.7	8.1	8.4	8.1	8.5
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	folate	23.7	23.2	22.6	22	23.7	18	18.4	18.4
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17	folate	8.5	8.5	10.5	8.8	9.1	8.2	8.2	7.9
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	folate	22.6	26.1	26.9	23.6	27.2	24.8	24.8	27.2
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19	folate	16.3	19.3	18.2	19.1	20	20.9	20.4	-----
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-----

Table 3.4 Serum folate and folic acid ($\mu\text{g/L}$)

Samples collected on day 7 of *regime A bread* (50 μg in each slice) pre and post prandially.

		pre 50 μg	1 hour post	2 hrs post	3 hrs post	4 hrs post	1 hour post 2 nd slice	2 hrs post	3 hrs post
Subject	samples	A	B	C	D	E	F	G	H
1	folate	21.2	18.2	16.9	22.7	20.3	18	15.1	14.1
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	folate	9.8	8.7	11.1	11.3	9.3	8.4	8.2	8.3
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	folate	17.6	15.7	18.1	17.5	16	15.7	14.4	16.8
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	folate	25.5	19.9	19	17.6	18	15.9	15	15
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	folate	12.8	11.1	14.5	12.6	13.3	11.6	10.7	11
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	folate	9.1	8.9	10.4	9.5	13.3	10.6	10.7	10.1
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	folate	26.4	27	28.1	27.6	24.9	19.8	23.1	19.2
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	folate	11.2	11.1	11.2	11.4	11	10.3	9.9	9.5
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	folate	27.5	25.4	27.8	26.9	36.1	30	30.1	27.1
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	folate	14.9	13.3	12.6	13	12.3	13.8	14.8	14
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11	folate	12.2	11.2	12.6	12.4	14.4	13	11.8	12.2
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	folate	25.8	27.6	26.8	24.0	27.4	23.2	22.2	20.2
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	folate	12.3	12.7	18	14.6	15.3	12.1	11.4	11.9
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	folate	14.1	13.8	11.5	10.5	12.6	13.4	14.4	14.7
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	folate	11.4	10.4	9.7	10.2	10.5	9.9	11	10.8
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	folate	20.9	25	24.9	24.8	21.2	19.3	20.2	16.2
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17	folate	8.8	9.1	9.4	11.5	11.7	10.1	9.2	9.4
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	folate	25	24.3	23.3	23.4	27.2	24.9	25.2	25.5
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19	folate	14.7	16.3	16.9	19.2	15.8	15.7	15.2	15.6
	folic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.	0.0

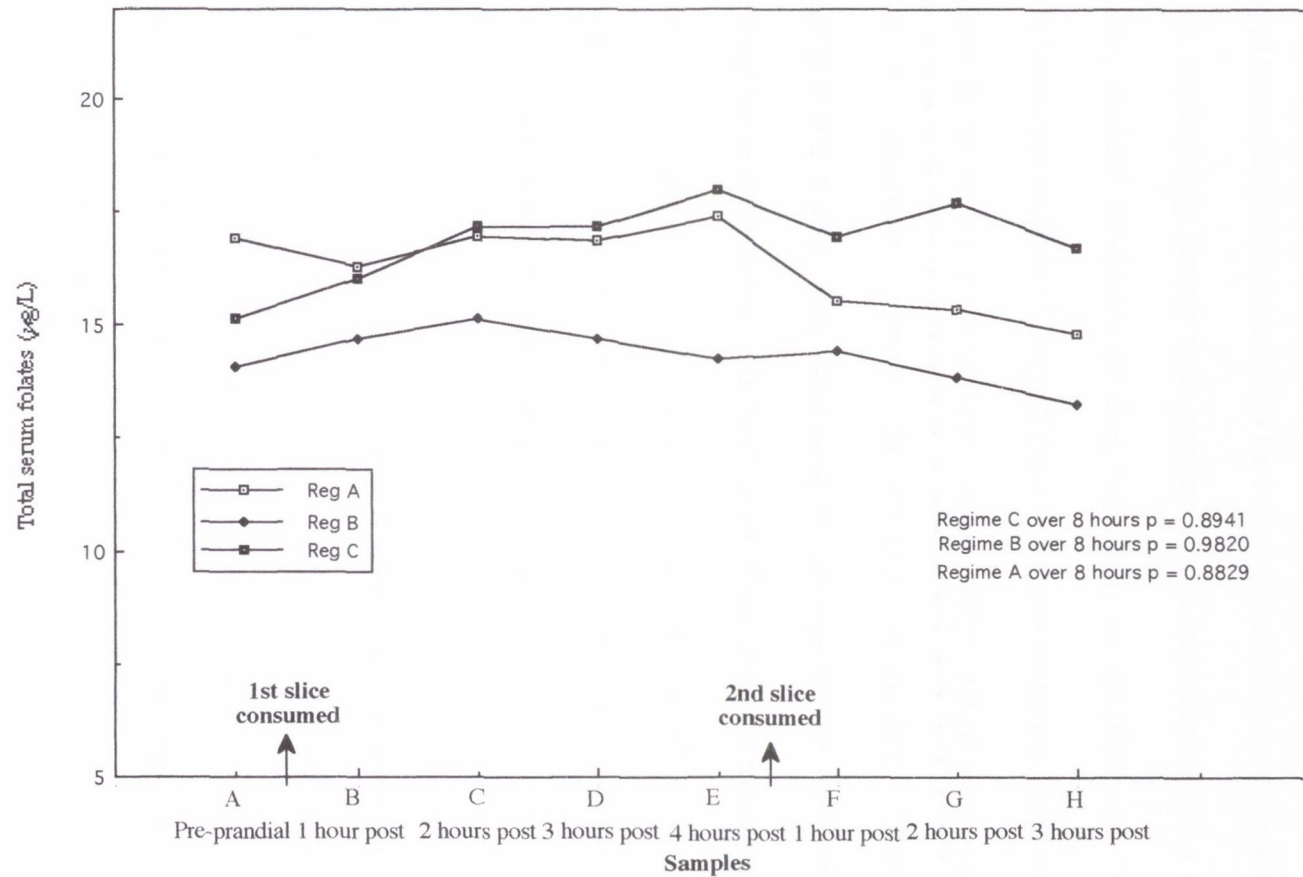


Figure 3.4. Total serum folate ($\mu\text{g/L}$) concentrations pre and post-regime A, B & C breads

3.3 Discussion

This research study was undertaken to look at the effect of chronic consumption of the current level of fortified flour in practice in the USA and of two higher levels under consideration on unmetabolised folic acid in serum post-prandially. Subjects were carefully selected at recruitment to ensure that they were representative of the normal population and that no confounding factors were present. Hence individuals with a folate deficiency at baseline, medical conditions or drug use likely to interfere with folate absorption or metabolism were excluded from participating. A pre-saturation phase was incorporated into the study design such that serum folate concentrations would be consistent with those of a free-living population exposed to chronic fortification. Compliance with the supplementation regime was monitored by regular contact with the subjects and by measuring folate status markers at baseline and throughout the intervention. The bread used was specially prepared for the trial and the folic acid was added to the bread before it was baked ensuring that a matrix effect would be present. In addition the entire bread making process was supervised by the principal researcher.

The results showed that serum and red cell folate concentrations increased significantly from baseline after the supplementation phase indicating good compliance. By week 10 subjects had reached a serum folate plateau as statistical analysis ($p = 0.00052$) revealed that concentrations increased significantly from week 0 to week 10 but did not show any further significant increases from week 10 to week 14 ($p = 0.87246$).

In addition the results revealed that serum unmetabolised folic acid was not detectable at week 0, week 10 or week 14. It is worth reiterating that the samples collected at week 10 and week 14 were collected 6 hours after the vitamin had last been consumed. Previous findings from the materials and methods chapter of this thesis and findings reported by Kelly *et al* (1997) show that unmetabolised folic acid does appear in serum approximately 80 minutes after the ingestion of 400µg of folic acid in supplemental form. Our findings suggest that 6 hours is sufficient time for this acute post-prandial response to clear from the serum. Furthermore we ascertained that this level of supplementation even after chronic pre-saturation did not lead to a persistently elevated or accumulating concentration of folic acid in serum. This may have implications from a public health perspective as the principal concern about folic acid masking the haematological symptoms of vitamin B₁₂ deficiency is thought to relate to cells being repeatedly exposed to unmetabolised folic acid in serum. The evidence from this work demonstrates that cells will be exposed to folic acid in serum after the consumption of a 400µg supplement (Kelly *et al*, 1997, Chapter 2 of thesis) but that this exposure will be for a duration of less than 6 hours.

Unmetabolised folic acid was not detected in serum pre or post-prandially at any of the three different levels of fortification of flour tested. These findings are perhaps of more significance than those discussed in the previous paragraph, as fortification is likely to be the intervention choice in most countries eventually. Therefore unmetabolised folic acid appearance in serum after supplementation may not be an important issue. In this study the maximum dose of folic acid delivered was 400µg in two equal divided doses over 4 hours. The highest level of fortification advocated is 700µg/100g of flour (Oakley *et al*, 1996), which is estimated to supply an extra 400µg daily. This model assumes that consumption

of this extra 400µg will be spread out over a day and will not be consumed in a single dose. The findings support the findings of Kelly *et al* (1997) who reported that folic acid was not detectable in the sera of any of the subjects post ingestion of foodstuffs containing < 266µg of folic acid.

From these results it appears that the current level of flour fortification in place in the USA will not cause unmetabolised folic acid to appear in serum. Furthermore the incremental levels recommended by some are unlikely to cause folic acid appearance in serum.

From these negative results we concluded that 1) these levels of fortification genuinely do not give rise to folic acid in serum post-prandially or 2) that the methodology developed by Kelly *et al* (1996) was not sensitive enough to detect very low concentrations present in the samples. In the next chapter (chapter 4) our aim was to determine which of these conclusions was the correct one.

Chapter Four

Improved methodology for the measurement of unmetabolised folic acid in serum

4.1 Introduction

Kelly *et al* (1996) developed a novel methodology to measure unmetabolised folic acid in serum using HPLC to separate the folic acid from other folate metabolites and then quantifying the amount present by the *L.casei* microbiological assay described by Molloy & Scott (1997). The lower limit of detection of folic acid using this methodology was estimated to be approximately 1ng/ml. No methodology exists which is able to detect levels lower than 1ng/ml. Findings from intervention trial 1 using the Kelly *et al* (1996) methodology described in the previous chapter reported negative results only in terms of folic acid appearance. As a result of this we decided to look more closely at the methodology and see whether we could improve the lower limit of detection of the assay. By re-examining the results obtained in intervention trial 1 we discovered that all the test samples collected post prandially gave absorbance values which were similar to the absorbance values obtained from the zero wells of the standard curve (figure 2.8) and similar to the baseline samples collected at week 0 but some of them appeared to give slightly higher readings. However when these were tested statistically no differences were demonstrated. This made it difficult to say with certainty whether the values obtained from the test samples were true negative values. Hence we set out to improve the lower limit of detection of the assay. This chapter describes an additional separation step in the methodology to measure unmetabolised which improved the lower limit of detection of the assay by concentrating the volume of liquid in the pooled peak while at the same time retaining the amount of solute present. Hence we ended up with a much more concentrated solution. This was achieved using solid phase extraction. The method development is described in the next section.

4.2 Methods

4.2.1 Sample collection and preparation

Sample preparation was carried out as per the methodology described by Kelly *et al* (1996). ^3H -Folic acid was purified and its retention time was determined by isocratic HPLC elution and fractionation. Folic acid was found to elute at 12-14 minutes. Serum was centrifuged at 2500rpms for 12 minutes and aliquoted into 500 μl samples in 1.5ml microfuge tubes. 1.0×10^4 dpm of ^3H -folic acid was added to each serum sample to act as an internal standard. The samples were de-proteinized by the addition of 50 μl of perchloric acid (60% v/v). Samples were then vortexed, frozen, thawed and the supernatant was removed. 50 μl KOH (6 m) was added to the supernatant to bring the samples to pH 7.0.

4.2.2 HPLC separation

HPLC separation was carried out as per the methodology described by Kelly *et al* (1996). 200 μl of each sample were injected manually onto a 100 x 8-mm C_{18} μ Bondapak cartridge column which was contained in a Z-Module. The column was eluted isocratically in mobile phase made up with citric phosphate buffer (0.1 m, pH 4.0): acetic acid (1%v/v): methanol (43:42:15) at 3ml/min for 20 minutes. 1ml was taken from each of 6 fractions in the region of the retention time and was added to 5ml of ecolite scintillation fluid. (Note that 2mls from each fraction were counted by scintillation using the methodology described by Kelly *et al* (1996). Each sample was counted by scintillation counting to identify the radioactive peak. The peak was found to occur over 2 fractions. The remaining 2mls from each of these fractions containing the peak were then pooled to give a total volume of 4ml.

4.2.3 Solid phase extraction

1x citrate phosphate buffer (4ml) (from concentrate above) was added to the 4ml pooled peak to dilute the methanol and prevent auto-elution of folic acid from the Sep-Pak. A Waters's classic C18 Sep-Pak was activated by eluting with 5ml of methanol followed by 5ml of 1x-citrate phosphate buffer to remove any methanol retained by the Sep-Pak. The 8ml sample was then eluted slowly via the Sep-Pak and the Sep-Pak was air-dried with a syringe. The eluent was added to 5ml scintillation fluid and dpm were counted for 5 minutes. There were no counts present. 1ml of methanol was then eluted slowly at 1ml/min via the Sep-Pak and the eluent was collected in a 1.5ml eppendorf tube. The Sep-Pak was then air-dried into another eppendorf tube. The methanol was dried off using a speedy vacuum dryer until all the liquid had evaporated. The retained folic acid solute was re-suspended in 1150µl of 0.5% sodium ascorbate buffer. A 500µl aliquot was added to 5ml of scintillation fluid in a 25ml plastic scintillation vial and was counted to estimate recovery. Recovery was found to be 80% of the amount injected onto the Sep-Pak. This is approximately 4-5% of the starting material.

4.2.4 Microbiological assay for serum folic acid

An amended version of *L.casei* microbiological assay for folic acid (described by Molloy & Scott, 1997) was carried out using the remainder of the sample. A 9-point standard curve was constructed in the range of 0–12.5pg/well in order to improve the sensitivity of the curve used by Kelly *et al* (1996). 100µl of each re-suspended sample were plated out in triplicate onto a 96 well microtitre plate. The samples were not diluted further in sodium

ascorbic buffer or mobile phase as per the method described in section 2.9. as they were already in an ideal buffer having been resuspended in 0.5% sodium ascorbate buffer. The method development reduced the volume of liquid from 4ml to 1.15ml while at the same time retaining all of the solute. 200µl of *L.casei* (Merck, Darmstadt, Germany) inoculated assay media was added to all sample wells. The plates were sealed with a plate sealer, incubated at 37°C for 42 hours. The samples were read on a 96-well microplate reader (Titertek, Multiscan Plus, Flow Laboratories, Ayrshire, Scotland) at a wavelength of 590nm. The results were read from a standard curve as described by Molloy & Scott (1997).

4.2.5 *In vivo* investigation to compare directly the lower limit of detection of the old and the new methodologies.

19 healthy adult subjects were recruited by voluntary participation from the general public with ethical approval from the Federated Dublin Voluntary Hospitals. Consent forms were completed by all subjects. The subjects were males and females aged 20 – 30 years. All were folate replete at recruitment as determined by total serum and red cell folate levels. Serum was collected for unmetabolised folic acid to establish baseline levels (sample A). Subjects were administered 1 slice of bread fortified with 200µg folic acid. Serum samples were collected one (sample B) and two (samples c) hours post-prandially. A second slice of bread fortified with 200µg was then administered to all subjects. Serum was again collected one (samples F) and two hours (samples G) post - prandially.

4.2.6 Statistical analysis

Statistical analysis was performed using Data Desk 5.0. Paired t-tests, Anova and Post hoc Scheefe tests were used.

4.2.7 Laboratory analysis

The serum samples were left at room temperature for 30 minutes to allow time to clot and were then centrifuged at 2500rpms for 12 minutes. To investigate if the new step in the methodology actually improved the lower limit of detection we assayed all samples by the existing HPLC/microbiological assay (Kelly *et al*, 1996) and also by the HPLC/Sep-Pak/microbiological described in this chapter. All samples were assayed for total serum folate and red cell folate by the *L. casei* microbiological assay described by Molloy & Scott (1997).

The sensitivity of the Kelly methodology was 1ng/ml. The extra separation step improved the limit of detection 6-fold with a new lower limit of detection of 0.16ng/ml.

4.3.3 Assay Precision

Intra-assay

Serum samples were collected from 10 individuals known not to have consumed folic acid in the previous 24 hours. A sample from each subject was then spiked with folic acid at known concentrations of 1ng/ml, 3ng/ml and 5ng/ml. A further sample from each subject was left unspiked. The samples were assayed for unmetabolised folic acid as previously described. See table 4.1 for results

Inter-assay

The serum samples from the different individuals were then pooled together into four batches of 0µg/ml, 1ng/ml, 3ng/ml and 5ng/ml. Analysis for unmetabolised folic acid was carried out as previously described. These results are illustrated in table 4.1.

Table 4.1 Serum folic acid assay performance

Run number	0ng/ml [Intra & Inter assay]	1ng/ml (Intra assay)	1ng/ml (Inter assay)	3ng/ml (Intra assay)	3ng/ml (Inter assay)	5ng/ml [Intra assay]	5ng/ml [Inter assay]
1	0.00	1.03	1.02	3.11	2.94	5.15	5.20
2	0.00	1.09	0.88	3.02	2.85	5.12	5.12
3	0.00	1.05	0.89	2.87	2.96	5.16	5.15
4	0.00	0.90	1.06	2.92	3.05	4.90	5.06
5	0.00	0.95	1.15	3.07	3.09	4.95	4.80
6	0.00	0.97	1.12	2.90	3.16	5.06	4.85
7	0.00	1.09	1.05	2.95	3.05	4.90	4.86
8	0.00	1.13	0.95	3.14	2.90	4.91	5.17
9	0.00	1.11	0.89	2.99	2.95	5.21	4.91
10	0.00	0.94	1.11	2.96	3.02	4.95	4.90
mean		1.026	1.012	2.993	2.997	5.031	5.002
S.D.		0.081	0.103	0.091	0.094	0.122	0.152
% C.V.		7.8	10.15	3.03	3.12	2.42	3.04

4.3.4 Test sample results

The sample results are shown in Table 4.2. The new methodology did not detect a statistically higher level of folic acid in the pre-prandial samples (A) than the old method (paired t-test $p = 0.2006$) indicating that this level of folic acid was in fact a true background level of the assay.

Statistical analysis (Anova) showed that when samples were assayed by the old methodology there were no significant differences between samples A and B, A and C, A and F or A and G ($p = 0.4680$).

Statistical analysis (Post-hoc Scheffe tests) showed that samples assayed by the new methodology showed statistical differences between A and B ($p = 0.000001$), A and C ($p = 0.000000$), A and F ($p = 0.000000$) and A and G ($p = 0.000000$).

The results shown in table 4.2 clearly illustrate that the new methodology can detect lower levels of folic acid than the existing methodology. The improvement in detection levels using the new method was shown to be statistically significant between old B and new B ($p = 0.0001$), old C and new C ($p = 0.001$), old F and new F ($p = 0.001$) and old G and new G ($p = 0.001$). Mean values are illustrated in Figure 4.1.

4.3.5 Comparison of results

Table 4.2. Absorbance values obtained from the HPLC/microbiological assay (old method) and from the HPLS/Sep-Pak/microbiological assay (new method).

Sample	A	A	B	B	C	C	F	F	G	G
Subjects	Old method	New method	Old method	New method	Old method	New method	Old method	New method	Old method	New method
1	0.110	0.123	0.102	0.181	0.100	0.190	0.102	0.253	0.106	0.202
2	0.124	0.120	0.114	0.176	0.102	0.190	0.112	0.181	0.103	0.220
3	0.107	0.098	0.116	0.165	0.102	0.156	0.100	0.208	0.108	0.199
4	0.107	0.096	0.109	0.307	0.104	0.244	0.114	0.200	0.109	0.224
5	0.106	0.084	0.105	0.170	0.112	0.167	0.106	0.223	0.114	0.216
6	0.109	0.100	0.103	0.154	0.106	0.326	0.106	0.166	0.104	0.138
7	0.112	0.100	0.108	0.164	0.112	0.197	0.102	0.165	0.109	0.197
8	0.107	0.094	0.120	0.179	0.109	0.182	0.122	0.266	0.117	0.203
9	0.112	0.134	0.102	0.160	0.104	0.141	0.100	0.166	0.104	0.171
10	0.114	0.120	0.113	0.217	0.109	0.202	0.109	0.191	0.108	0.171
11	0.104	0.105	0.109	0.130	0.108	0.151	0.116	0.244	0.119	0.238
12	0.109	0.109	0.104	0.199	0.113	0.215	0.115	0.268	0.113	0.241
13	0.108	0.110	0.104	-----	0.106	0.173	0.100	0.190	0.108	0.187
14	0.112	0.101	0.110	0.183	0.110	0.170	0.107	0.165	0.104	0.150
15	0.107	0.112	0.099	0.152	0.108	0.166	0.101	0.171	0.107	0.179
16	0.104	0.085	0.105	0.153	0.107	0.186	0.106	0.207	0.118	0.212
17	0.111	0.088	0.103	0.211	0.115	0.194	0.107	0.186	0.114	0.200
18	0.110	0.111	0.104	0.179	0.097	0.172	0.100	0.172	0.104	0.250
19	0.115	0.127	0.105	0.197	0.121	0.255	0.105	0.177	0.109	0.250

Footnote: (A= pre-prandial samples, B & C= samples collected 1 & 2 hours post 1st slice, F & G= samples collected 1 & 2 hours post 2nd slice).

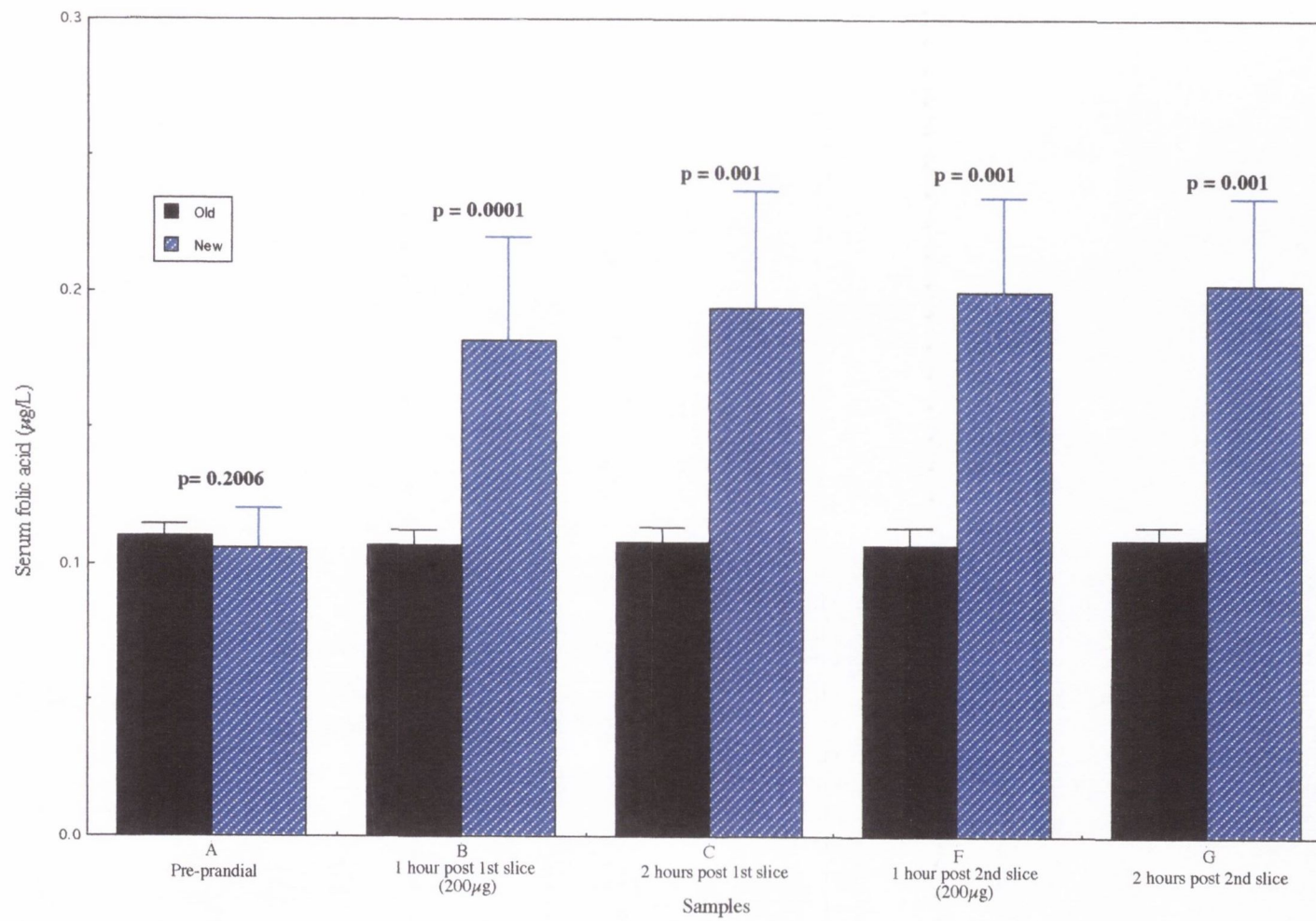


Figure 4.1. Histogram illustrating the detection limits of the old and the new methodology

4.4 Discussion

This chapter set out to improve the lower limit of detection of the combined HPLC/microbiological assay described by Kelly *et al* (1996). This objective was achieved by adding an extra separation step onto the assay after separating the samples by HPLC. The liquid from this concentrated sample was then completely dried off and the solute was re-suspended in the minimum amount of buffer necessary for the remaining analysis. This newly developed step in the methodology yielded a six-fold improvement in the lower limit of detection of the assay.

Using the old methodology it was difficult to differentiate between absorbance values obtained from a negative result and a positive result. All samples tested in this experiment using the old methodology gave similar absorbance values and post-prandial samples (B, C, F and G) were not found to be statistically different from those collected at baseline (A) falsely indicating that folic acid was not present in any of these samples.

On the other hand the newly developed methodology allows us to clearly differentiate between a positive and a negative value. Looking at the values obtained pre-prandially (samples A) it is clear that no folic acid was present as both the old and the new methodology give background absorbance readings only. This is not the case in the post-prandial samples (B, C, F and G) where it is very clearly illustrated by the new method that folic acid is present in these samples as the absorbance values are much higher and demonstrate statistical differences from those assayed by the old method.

The methodology developed by Kelly *et al* (1996) was unable to detect folic acid in serum below concentrations of 1ng/ml. The additional separation step improved the lower limit of detection by approximately six times demonstrating a lower limit of detection of 0.16ng/ml $\pm$ 2.s.d. Furthermore the old methodology was unable to detect folic acid at oral doses of less than 266 μ g. The new methodology detected unmetabolised folic acid in serum after oral doses of 200 μ g.

As described in the introduction (chapter 1) the presence of free folic acid in the circulation could have very significant public health implications in terms of pernicious anaemia masking and other as yet unknown toxic effects. The FDA's upper safe limit (Federal Register, 1996) for folic acid intake is currently set at five times the amount that we have now shown gives rise to folic acid in the circulation. (Federal Register, 1996). The next chapter (chapter 5) describes the re-assaying of the samples collected (chapter 3) investigating the effects of long-term consumption of the levels of fortification a) currently in practice in the US and b) the higher levels under consideration by the CDC on unmetabolised folic acid in serum.

Chapter Five

Intervention Trial 1. Re-assaying of samples by new methodology

5.1 Introduction

In Intervention Trial 1 (chapter 3) we set out to investigate the effects of long-term consumption of folic acid fortified flour at a) the current level of fortification in practice in the US ($140\mu\text{g}/100\text{g}$) and b) the two higher levels under consideration ($350\mu\text{g}$ and $700\mu\text{g}/100\text{g}$) by the CDC on unmetabolised folic acid in serum in a healthy population group. Subjects were pre-saturated with $400\mu\text{g}$ folic acid supplements daily for 14 weeks. After this time-frame subjects had reached a serum folate plateau. The subjects then consumed bread that was fortified with folic acid at three different levels supplying $400\mu\text{g}$, $200\mu\text{g}$ and $100\mu\text{g}$ each day in 2 equal doses respectively over 3 weeks. Each level was consumed for a period of 7 days commencing with the highest level first. Between week 1 and week 2 breads all subjects consumed a $400\mu\text{g}$ supplement daily for 7 days. Similarly between week 2 and week 3 breads subjects consumed a $400\mu\text{g}$ supplement daily.

Blood samples were collected for unmetabolised folic acid at week 0, week 10 and week 14 of the supplementation phase. Blood samples were also collected on day 7 of each bread regime pre and post-prandially over an 8-hour period. Samples collected at week 0, week 10, week 14 and pre and post the fortified bread were assayed for unmetabolised folic acid by the methodology described in chapter 2. Unmetabolised folic acid was not detected in any of these samples. Chapter 4 describes a method development, which improved the lower limit of detection of the assay to measure folic acid in serum by approximately six-fold. In this chapter samples from Intervention Trial 1 were re-assayed by the newly developed methodology (described in chapter 4) to investigate if the more sensitive methodology would detect folic acid in any of the samples that had already been assayed

by the existing methodology but were found not to have detectable levels of unmetabolised folic acid.

5.2 Methodology

The methodology set up by Kelly *et al* (1996) was unable to detect folic acid in serum at levels lower than 1ng/ml. The newly developed methodology improved the limit of detection of the assay by six-fold. In intervention Trial 1 described previously a large number of test samples were assayed by the methodology described by Kelly *et al* (1996). These yielded negative results. Hence we decided to re-assay a selection of the samples from this Intervention Trial using the new methodology to investigate if the methodology had given rise to false negative values.

5.2.1 Repeat analysis of test samples for unmetabolised folic acid.

A selection of samples that were thought to be most likely to have detectable folic acid present were selected to be re-assayed first. Therefore, samples collected immediately post-prandially after *regime C bread* were re-assayed initially. This analysis yielded positive results (see results section). Hence it was necessary to re-assay the pre-prandial samples collected on day 7 of *regime C bread* to investigate how much of the folic acid was due to an acute post-prandial response from the fortified bread. The pre-prandial samples also yielded positive results. This meant that it was necessary to re-assay samples collected at week 14 so that we could investigate if this was due to an accumulation from the supplemental phase of the study or due to an accumulative effect of the bread which had

consumed over the previous 6 days. Hence all samples collected at week 14 were re-assayed. These also gave positive results. This (see results section) indicated that it was necessary to go back to week 0 samples and repeat the baseline analysis. Folic acid was not detected in the baseline (week 0) samples. Samples collected on day 7 of *regime B bread* pre and post prandially were also re-assayed. These yielded negative results (see results section), hence it was not necessary to re-assay samples collected at *regime A*. Refer to the results section (5.3) for a thorough report of the results and statistical analysis.

Because the serum samples collected pre and post prandially on day 7 of *regime C bread* were positive for unmetabolised folic acid we decided that we should also measure the urine samples collected from the 2 subjects to see if unmetabolised folic acid had been excreted in the urine.

5.2.2 Laboratory analysis of urine samples for unmetabolised folic acid

Urine samples were defrosted and the samples were prepared and assayed as per the methodology described for serum samples. Perchloric acid was not added however as urine samples do not need to undergo protein precipitation.

5.2.3 Statistical Analysis

Statistical analysis was performed using the computer software package Data Desk 5.0. Paired t-tests, Anova and Post hoc Scheefe tests were used to analyse the data.

5.3 Results

5.3.1 Serum samples

Table 5.1 shows that unmetabolised folic acid was not detected in any of the subject's samples collected at week 0. At week 14 six hours after consumption of the 400µg supplement unmetabolised folic acid was present in the sera of 18 subjects (n=19). 7 of these 18 subjects demonstrated only slightly elevated folic acid levels, which were calculated to be below the level of that which is accurately quantifiable. Nonetheless the difference between week 0 and week 14 was statistically significant ($p = 0.0001$) demonstrating that the supplementation phase did give rise to an elevated folic acid level which was still detectable six hours after the last supplement had been consumed.

Table 5.2 shows that unmetabolised folic acid was present in the sera of 17 subjects (n=19) on day 7 of regime C bread pre-prandially (sample A). All of these were above the lowest limit of quantification and were statistically higher than week 14 levels ($p=0.0041$).

Unmetabolised folic acid levels increased post-prandially in all subjects after consumption of the first slice of bread containing 200µg of folic acid ($p= 0.000021$). It was also present in the sera of all subjects after the 2nd slice containing a further 200µg ($p=0.000000$). Statistical analysis showed that differences in serum folic acid were significant between samples A and samples B (0.000021), samples A and samples C ($p=0.00001$), samples A and samples F ($p=0.000000$) and samples A and samples G ($p=0.000000$). An accumulative elevation of unmetabolised folic acid was demonstrated in 12 out of 19 subjects after the

2nd slice. Mean serum folic acid results for all time points from week 0 through to regime C bread are illustrated in figure 5.1. A schematic summary (Figure 5.2) is included in the results section to re-cap the interventions stages, the sample collection and the main findings in this chapter. Unmetabolised folic acid was not detected in the sera of any of the subjects on day 7 of *regime B* or *A* breads pre or post prandially.

5.3.2 Urine samples

Unmetabolised folic acid was not detected in the urine of either of the two subjects at any of the time points tested.

Table 5.1 Serum folic acid ($\mu\text{g/l}$).

Samples collected at week 0 and at week 14 of folic acid supplementation regime (6 hours after the last vitamin had been consumed).

Subjects	Week 0	Week 14
1	0.0	0.32
2	0.0	0.06
3	0.0	0.35
4	0.0	0.18
5	0.0	0.21
6	0.0	0.16
7	0.0	0.50
8	0.0	0.20
9	0.0	0.23
10	0.0	0.14
11	0.0	0.32
12	0.0	0.10
13	0.0	0.0
14	0.0	0.09
15	0.0	0.01
16	0.0	0.36
17	0.0	0.02
18	0.0	0.07
19	0.0	0.58

Table 5.2 Serum folic acid ($\mu\text{g/l}$).

Samples collected on day 7 of *regime C bread* pre and post prandially.

	Pre 1 st slice	1 hour post 1 st slice	2 hrs post 1 st slice	1 hour post 2 nd slice	2 hrs post 2 nd slice
Subjects	A	B	C	F	G
1	0.27	1.22	1.34	2.21*	1.48
2	0.59	1.45	1.66	0.84	2.17*
3	0.54	1.27	1.12	1.97*	1.81
4	0.32	3.95	2.75	2.27	1.35
5	0.55	1.59	1.53	2.54*	2.41
6	0.54	0.92	1.69	1.11	0.35
7	0.28	0.40	1.07	0.75	1.06
8	0.45	1.47	1.51	2.91*	1.84
9	0.46	0.82	0.58	0.90	0.98*
10	0.37	1.75	1.52	1.34	1.05
11	0.38	0.24	0.56	3.47*	3.31
12	0.36	1.42	1.65	2.48*	2.04
13	0.42	-----	1.24	1.49*	1.43
14	0.35	1.04	1.24	0.96	0.85
15	0.50	1.74	2.04	2.19*	1.86
16	0.23	0.60	1.06	1.35	1.42*
17	0.00	1.15	0.95	0.86	1.01
18	0.00	1.19	1.08	1.09	2.31*
19	0.19	1.90	2.93	1.58	1.94

Samples marked with * demonstrated an accumulative effect.

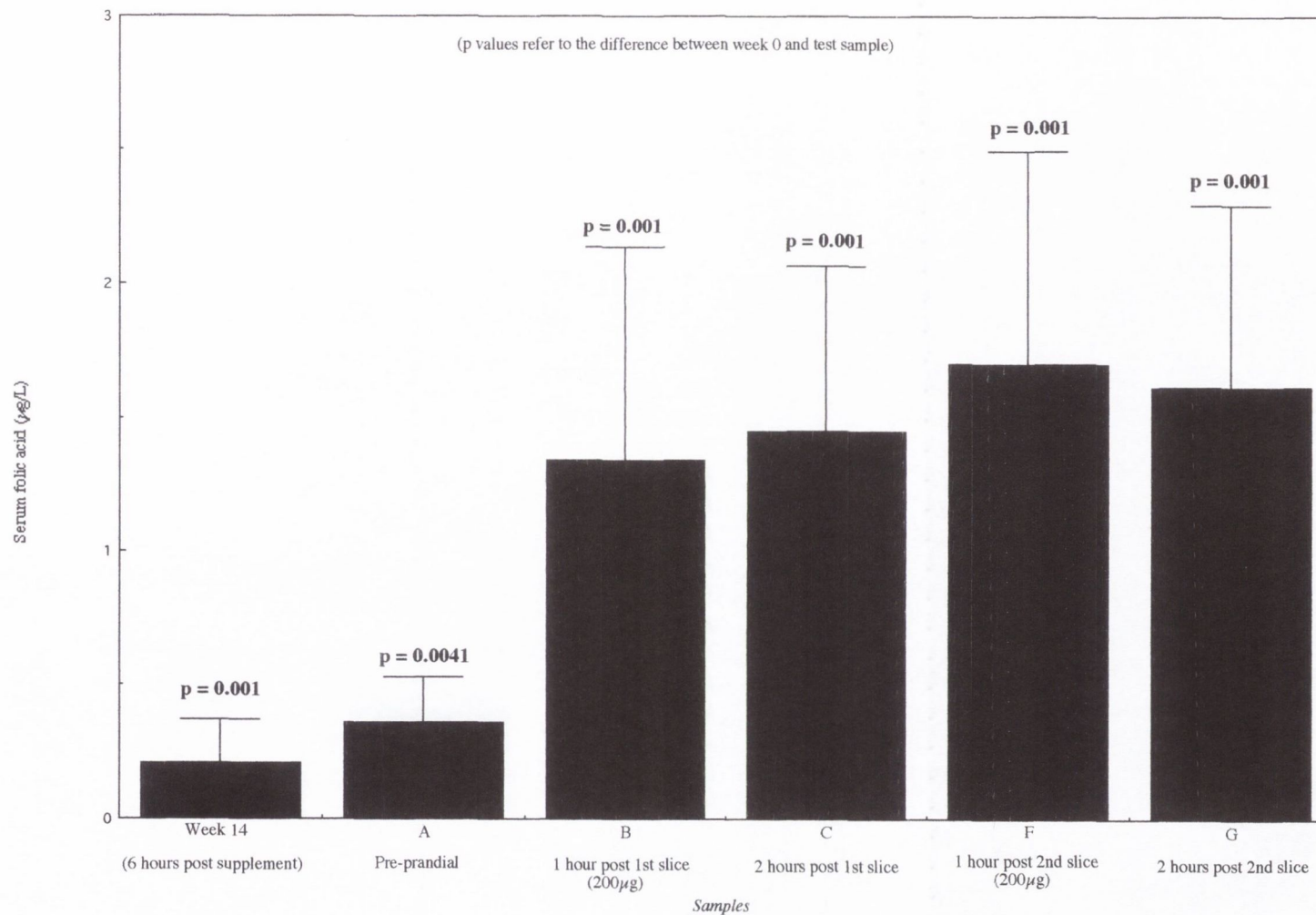


Figure 5.2. Serum unmetabolised folic acid ($\mu\text{g/L}$) at week 14, and pre and post-prandially at regime C bread (samples A, B, C, F & G).

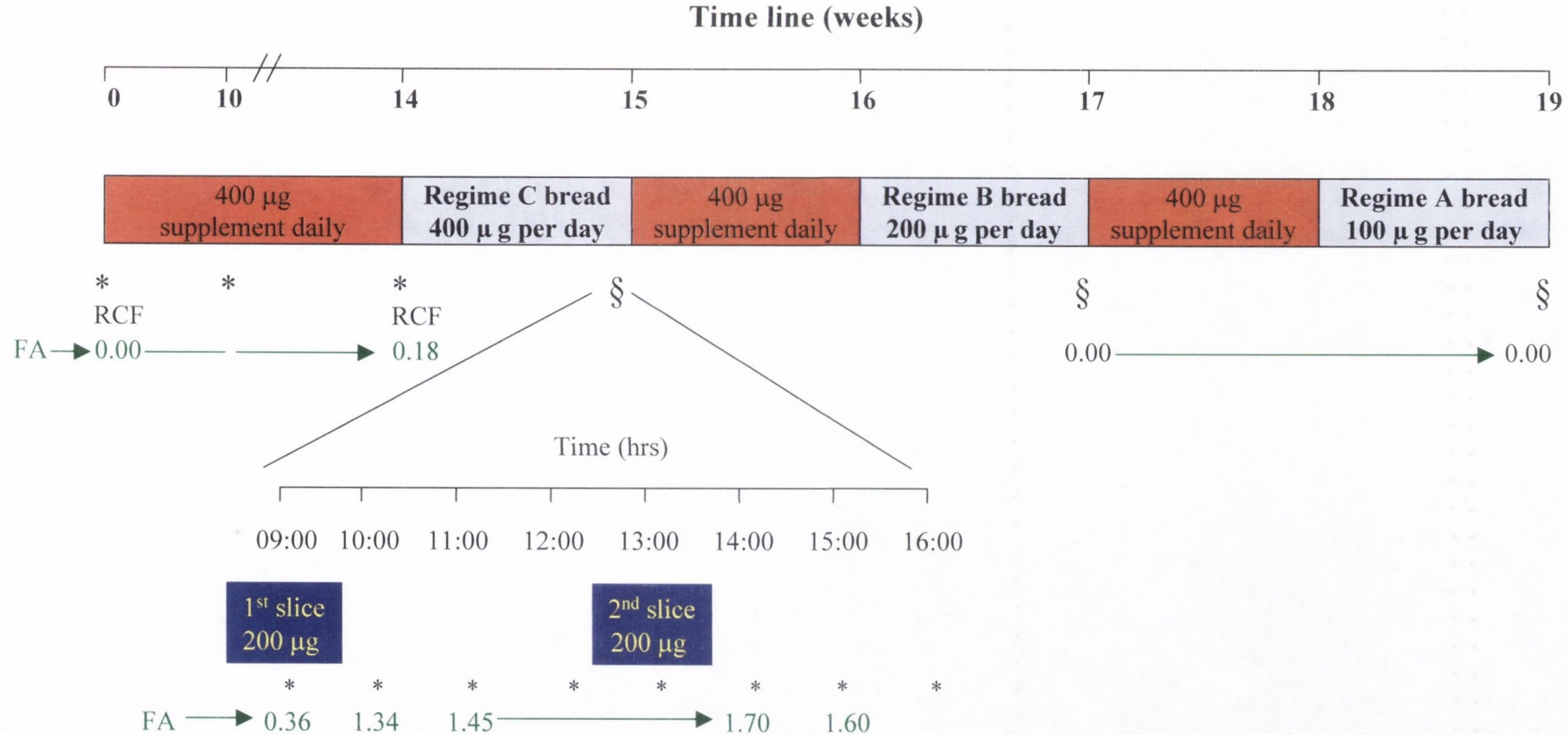


Fig. 5.2 Schematic summary of intervention phases, sample collection and mean serum folic acid concentrations

Legend

- * = Folate and Folic Acid blood sample
- FA = Mean serum folic acid concentration (µg/l)
- RCF = Red cell folate blood sample
- § = Day 7, trial of bread regimes (400 µg, 200 µg, 100 µg)

5.4 Discussion

Unmetabolised folic acid was detected in serum of subjects after the consumption of 400µg folic acid supplements daily for 14 weeks. 14 weeks is a sufficient time frame for serum folate concentrations to reach a plateau at this level of supplementation as demonstrated by Ward *et al* (1997). These samples were collected 6 hours after the vitamin supplement had last been consumed. This illustrates that it takes at least 6 hours for folic acid to clear from the serum after supplementation at this level, for this duration of time. This implies that the current DoH recommendation that childbearing women should consume a 400µg supplement daily for their entire childbearing years holds potential for harm as this supplementation regime will expose these individuals to unmetabolised folic acid in serum for at least six out of twenty-four hours per day. Therefore if this recommendation is retained in addition to the current US fortification programme then the likelihood of chronic exposure to unmetabolised folic acid in serum is greatly exacerbated. The potentially adverse effects of circulating folic acid have already been discussed in the introduction of this thesis.

Other results which, emerged from this study warrant pause for thought particularly the cumulative effect of small doses of oral folic acid consumed repeatedly in fortified bread. Two slices of fortified bread containing 200µg folic acid each were consumed 4 hours apart for a period of 6 days. Unmetabolised folic acid was detected in serum on day 7 even though 20 hours had lapsed between consumption of the last slice of bread on day 6 and blood collection on day 7. Furthermore the levels of folic acid detected after consuming the fortified bread (400µg in two equal doses) for only 6 days were higher than those detected

after consuming 400 μ g supplements daily for 14 weeks. These results would indicate that renal clearance is more effective at getting rid of larger doses of unmetabolised folic acid in the serum than small doses delivered frequently. This previously unreported cumulative effect as a result of repeated consumption of physiological doses of folic acid adds further difficulties and considerations for those involved in the planning of a fortification strategy.

From a positive viewpoint however our data shows that unmetabolised folic acid does not appear in serum after the consumption of 100 μ g of folic acid in fortified bread twice daily for 6 days, as the pre-prandial samples collected on day 7 (20 hours after the last slice on day 6) did not contain unmetabolised folic acid. These results indicate that in pre-saturated healthy subjects the threshold dose above which unmetabolised folic acid appears in serum is between 100 μ g and 200 μ g. In addition unmetabolised folic acid did not present after the first slice of bread (100 μ g) on day 7 and did not present or accumulate in the circulation after the 2nd slice of bread (100 μ g) which was consumed four hours later. These results indicate that if the time of $t_{\max}$ is exceeded between slices then an accumulative effect is not seen.

This study was carried out in a 20-40 age group. However the subject of unmetabolised folic acid in serum has most relevance to the elderly population. This study would have been logistically difficult to carry out in an elderly group but nonetheless would be an important study to do. Hence we need to extrapolate the results to have relevance in an elderly group. Obviously gastrointestinal functioning in the elderly will be somewhat diminished due to reduced acid secretion, gastric atrophy, microvilli flattening and reduced conjugase activity causing folic acid to be absorbed and metabolised less efficiently or

effectively in this group. Elderly persons are also likely to have reduced appetite. Therefore their dietary intake of folic acid is likely to be reduced. Therefore if folic acid did not appear in serum after *regime A* or *regime B* bread in our middle aged healthy subjects then it is even less likely that it would occur in elderly individuals. However *regime C* bread is likely to cause unmetabolised folic acid appearance in an elderly age group. This scenario may be confounded by the fact that renal function may not be as efficient in an elderly group hence folic acid may not be cleared from the circulation as quickly. Hence unmetabolised folic acid may be present for longer periods in an elderly person at this level of fortification.

These results indicate that it would be potentially unsafe, with respect to the presence of unmetabolised folic acid in serum to implement the highest level of fortification tested in this study (700µg/100g). However it appears that implementing the middle level tested (350µg/100g) would be safe, provided that consumption is well spread out. It seems fortuitous that the middle level would not lead to folic acid appearance in serum as several other researchers have estimated that this may be the optimal level of fortification. Daly *et al* (1997) estimated that the three levels of fortification under consideration in the USA supplying 400µg, 200µg and 100µg to the daily folate intake would yield reductions in NTD incidence by 47%, 41% and 22% respectively. The authors concluded that the addition of 200µg extra would be very beneficial but the addition of 400µg would give little extra protection over and above this, only 6% in the difference.

Jacques *et al* (1999) reported on the effects the mandatory folic acid fortification of enriched grain products implemented in the USA in January 1998 on plasma folate and

total homocysteine concentrations. The authors reported that plasma folate concentrations increased by 117% after the introduction of fortification at this level and that the prevalence of low folate concentrations reduced by 92%. Fasting total homocysteine concentrations decreased by 7% and the prevalence of high homocysteine concentrations decreased by 48%. These results demonstrate that the first year of fortification at the lowest level tested in this present study has been very effective in improving folate status. However as discussed in the introduction (chapter 1) it is reported that many food companies are adding more folic acid to their products than the amount specified by the FDA. It is believed that this may be in the region of double the amount specified. This problem of overage may have contributed greatly to the success of the fortification to date in terms of homocysteine lowering and improvements in population folate status.

Previous findings by Ward *et al* (1997) can be extrapolated to suggest that increasing the level of fortification from the current 140µg/L to 350µg/100g would result in an improvement of these parameters but above this the effects may not be linear. Ward *et al* (1997) administered folic acid supplements to healthy subjects in increasing doses from 100µg (for 6 weeks), to 200µg (further 6 weeks), to 400µg (further 14 weeks). The results showed in terms of homocysteine lowering that 200µg of folic acid appeared to be most effective of the three doses studied, as increasing the dose to 400µg for a further 14 weeks showed no further significant homocysteine lowering.

Based on the above observations and taking into account our findings from this study it seems that increasing the level to 350µg/100g would improve overall population folate status, reduce homocysteine concentrations and decrease NTD incidence by 41% while at

the same time posing minimal or no risk of adverse effects to sub-groups of the population susceptible to pernicious anaemia masking. Increasing the level to 700µg/100g will only achieve a 6% improvement in NTD incidence but could put a substantial proportion of the population at risk of over fortification.

We conclude that the CDC (1992) and DoH (1992) recommendation to consume a 400µg supplement peri-conceptionally on an on-going basis will need to be reviewed. The upper safe limit of 1mg/day set by the FDA will also need to be reviewed on the basis of these findings. Consideration should now be given to increasing the level of fortification in the USA to 350µg/100g and the highest level of fortification advocated by the CDC (700µg/100g) should not be implemented.

Chapter Six

Intervention Trial 2: Examining the effects of 1mg of folic acid administered in a single dose-over a 4 hour period.

Intervention Trial 3: Examining the effects of 1mg of folic acid administered in 2 equal doses of 500µg + 500µg - over an 8 hour period.

Intervention Trial 4: Examining the effects of 1mg of folic acid administered in 3 equal doses of 333µg - over an 8 hour period.

Intervention Trial 5: Examining the effects of 1mg of folic acid administered in 5 equal doses of 200µg - over an 8 hour period.

Intervention Trial 6: Examining the effects of 1mg of folic acid administered in 10 equal doses of 100µg - over a 10 hour period.

6.1 Introduction

In the absence of evidence to suggest otherwise the FDA have stated that it is safe to consume up to 1mg/day of folic acid as a combination of fortified foods and supplemental forms provided that consumption is well spread out over the day. This recommendation raises a significant public health safety issue as Kelly *et al* (1997) detected folic acid in serum after oral doses of only 266 μ g which, is almost four times less than the FDA's safe upper limit. The presence of free folic acid in the circulation has safety implications chief of which, is the potential masking of the early haematological symptoms of pernicious anaemia. In addition other potentially toxic effects are unknown (see section 1.23). The new methodology described in this thesis has demonstrated that folic acid occurs at doses lower than this. We have shown that folic acid is present after consumption of oral doses between 100 μ g and 200 μ g. In addition folic acid in serum accumulated in 12 subjects (n=19) when 200 μ g was administered twice within a four hour time frame after a pre-saturation phase.

The aim of the acute studies described in the next section was to investigate if consumption of the FDA's 1mg upper limit in any combination of doses, i.e. as a single bolus dose or as smaller doses spread out over a day, would not lead to unmetabolised folic acid appearance in serum. Based on our findings from the previous chapter, which showed that folic acid did not appear in serum at oral doses of 100 μ g or less we anticipated that small doses administered frequently would not hold as much potential for harm as large doses administered on a once-off basis.

In order to test this hypothesis we set up a number of acute studies. In these studies we looked at the effects of consuming 1mg of folic acid as a single dose, 1mg as 2 divided doses, 1mg as 3 divided doses, 1mg as 5 divided doses and 1mg as 10 divided doses on unmetabolised folic acid in serum. Serum was collected hourly for 4 hours after the consumption of 1mg as a single dose (Intervention trial 2) as the known t-max of folic acid in serum from fortified foods is approximately 2.5 hours (Kelly *et al*, 1997). Intervention trials 2 - 5 were conducted over an 8 hour period to attempt to mimic a 'normal day'. Intervention trial 6 was conducted over 10 hours to allow a one hour interval between slices. All studies were conducted two weeks apart to prevent the likelihood that unmetabolised folic acid could be carried over in the circulation between studies. The bread was specially prepared for each of the studies under the supervision of the principal researcher and folic acid was added to the flour prior to baking to ensure that a matrix effect would be present.

6.2 Methods

6.2.1 Recruitment

4 subjects were recruited with ethical approval from the Dublin Federated Voluntary Hospitals. Written consent was obtained from all subjects. All were healthy and free from hepatic or renal disease. None of the subjects had been consuming vitamin supplements and none of them were oral contraceptive pill users. Baseline total serum and red cell folates were collected and assayed from all subjects. All subjects were found to be folate replete. Subjects were advised of the various folic acid fortified food products available on the Irish

market prior to commencement of the study and a list of alternative products available was issued.

6.2.2 Intervention Trial 2: Examining the effects of 1mg of folic acid consumed as a single dose in 1 slice of bread.

This study looked at the effects of consuming 1mg of folic acid as a single bolus dose in fortified bread. Subjects were asked to come to the laboratory at 9am having omitted folic acid fortified food products for 24 hours prior to sample collection. An intravenous cannula was inserted into each subject. A pre-prandial serum sample was then collected from all subjects. Subjects were asked to consume one slice of bread, which contained 1mg of folic acid. Serum samples were then collected 1 hour, 2 hours, 2¹/₂ hours, 3 hours and 4 hours post-prandially.

6.2.3 Intervention Trial 3: Examining the effects of 1mg of folic acid given in 2 equal doses of 500µg + 500µg over an 8-hour period.

This study examined the effects of consuming 1mg of folic acid in fortified bread in 2 equal doses of 500µg on the appearance of unmetabolised folic acid in serum over an 8-hour period. Again the subjects came to the laboratory at 9am having been asked to omit folic acid fortified foods for 24 hours prior to their arrival in the laboratory. An intravenous cannula was inserted into each subject. Pre - prandial serum samples were collected. All subjects consumed 1 slice of bread fortified with 500µg folic acid. Serum samples were

collected 1 hour, 2 hours, 2 ¹/₂ hours, 3 hours and 4 hours post-prandially. A 2nd slice of bread fortified with 500µg was then consumed by all subjects. Serum was collected 1 hour, 2 hours, 2 ¹/₂ hours and 3 hours post-prandially.

6.2.4 Intervention Trial 4: Examining the effects of 1mg of folic acid given in 3 equal doses of 333µg over an 8-hour period.

Intervention trial 4 investigated unmetabolised folic acid appearance in serum after a total of 1mg, which was consumed in 3 equal doses of 333µg over an 8-hour period. The four subjects came to the laboratory at 9am having been asked to omit folic acid fortified foods for 24 hours prior to their arrival in the laboratory. An intravenous cannula was inserted. Pre-prandial serum samples were collected. All subjects consumed 1 slice of bread fortified with 333µg folic acid. Serum samples were collected at 1 hour, 2 hours and 2 ¹/₂ hours post-prandially. This was repeated twice throughout the day until 3 slices containing 333µg had been consumed.

6.2.5 Intervention Trial 5: Examining the effects of 1mg of folic acid given in 5 equal doses of 200µg over an 8-hour period.

This study looked at the effects of consuming 5 equal doses of 200µg of folic acid in fortified bread over an 8-hour period on unmetabolised folic acid in serum post-prandially. The subjects came to the laboratory at 9am as before having fasted from folic acid fortified foods or supplements for the previous 24 hours. An intravenous cannula was inserted into

each subject. Pre-prandial serum samples were collected. The 1st slice of bread fortified with 200µg of folic acid was then consumed by each subject. A serum sample was collected from each subject 45 and 90 minutes later. The 2nd slice of bread was then given and serum samples were collected again 45 and 90 minutes post-prandially. This pattern continued until all 5 slices had been consumed.

6.2.6 Intervention Trial 6: Examining the effects of 1mg of folic acid given in 10 equal doses of 100µg over a 10-hour period.

This study was performed in order to see if 100µg of folic acid in fortified flour consumed repeatedly in 10 equal doses over a ten-hour time frame would give rise to the appearance of unmetabolised folic acid in serum post-prandially. Subjects came to the laboratory at 9am having fasted from folic acid fortified foods or supplements for the previous 24 hours. An intravenous cannula was inserted into all subjects. Pre-prandial samples were collected from all subjects. The 1st slice of bread containing 100µg was then administered to all subjects. Post-prandial samples were collected one hour later. This pattern was repeated 9 times until a total of 1mg had been consumed.

6.2.7 Laboratory analysis.

All samples were assayed for total serum and red cell folates by the *L. casei* microbiological assay described by Molloy & Scott (1997). Analysis of samples for unmetabolised folic acid was carried out by the HPLC/Sep-Pak/microbiological assay described in chapter 4 of this thesis.

6.2.8 Statistical analysis

Statistical analysis was performed using the statistics package Data Desk 5.0. Results were analysed using, Anova and post hoc Scheffe tests.

For ease of explanation the interventions and sample collection for each of the trials 2-6 are illustrated schematically in figure 6.1.

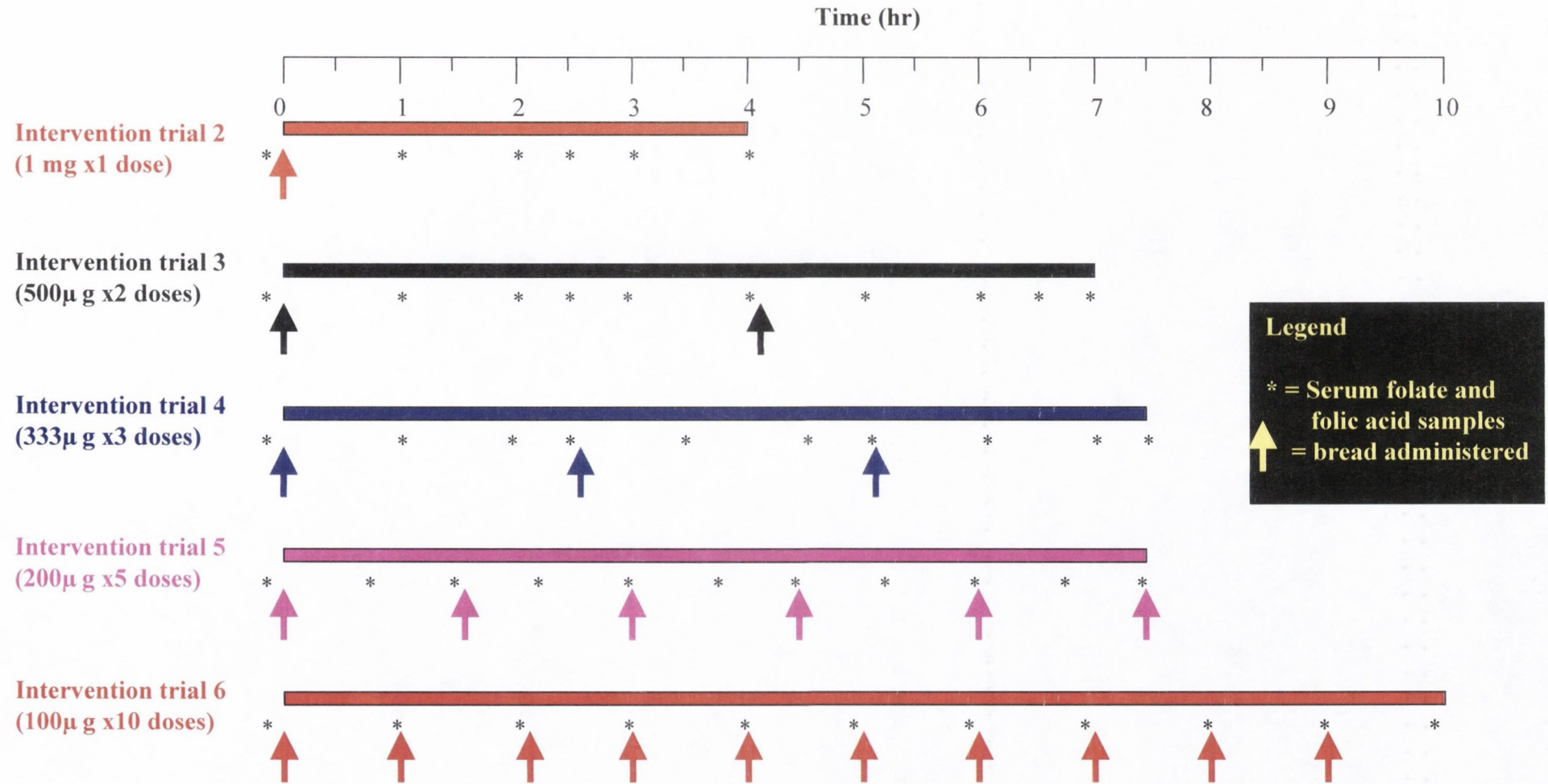


Fig. 6.1 Schematic diagram of intervention trials 2-6 illustrating interventions and sample collection

6.3 Results

Table 6.1 illustrates baseline red cell and serum folate concentrations.

Table 6.1 Baseline red cell and serum folate (µg/L) concentrations

	Subject 1	Subject 2	Subject 3	Subject 4	mean
Red cell folate	424	436	448	221	383
Serum folate	20.2	26.1	28.9	8.3	20.8

6.3.1 Intervention Trial 2

Unmetabolised Folic acid was not detected in the sera of any of the 4 subjects pre-prandially. After ingestion of 1 slice of bread containing 1mg of oral folic acid, unmetabolised folic acid was detected in the sera of all 4 subjects at 1 hour, 2 hours, 2½ hours, 3 hours and 4 hours post-prandially. Statistical analysis (Anova) showed that the post-prandial samples were significantly different from pre-prandial samples (p = 0.0168). Total serum folate concentrations increased significantly from baseline (p = 0.0006) indicating compliance with the intervention (see table 6.2).

Table 6.2 Serum folate and serum folic acid (µg/l): Intervention Trial 2.

Subjects	1	1	2	2	3	3	4	4	mean	mean
	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid
Pre 1mg	25	0.0	15.3	0.0	28.4	0.0	8.8	0.0	19.4	0.00
1mg given										
1 hour post	44.8	1.78	25.5	1.68	39.7	0.76	16.2	0.7	31.5	1.23
2 hours post	49.2	1.78	38.8	1.98	51.8	1.77	29	1.08	42.2	1.65
2.5 hours post	48.4	2.11	35.1	0.62	48.4	1.77	27.3	0.58	39.8	1.27
3 hours post	48.2	2.22	36	1.98	50.6	1.05	26.7	0.65	40.4	1.47
4 hours post	38.2	2.22	37	1.36	47.2	0.79	19.5	0.45	35.5	1.21

6.3.2 Intervention Trial 3

Unmetabolised folic acid was not present in any of the pre-prandial samples collected from the 4 subjects. Folic acid was detected in the sera of all subjects after the 1st and 2nd slice of bread. However the levels were not statistically different from baseline. Perhaps the sample size was too small to demonstrate statistical differences. No accumulative effect was demonstrated when the second slice was consumed indicating that the first dose had cleared from the serum by the time the second dose was administered. Total serum folate concentrations increased significantly from baseline (p = 0.0001) indicating compliance with the intervention (see table 6.3).

Table 6.3 Serum folate and serum folic acid ($\mu\text{g/l}$): Intervention Trial 3.

Subjects	1	1	2	2	3	3	4	4	mean	mean
	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid
Pre 500 μg 500 μg (1 ST)	20.8	0.00	26.3	0.00	34.2	0.00	8.7	0.00	22.5	0.00
1 hour post	25.2	0.10	45.8	1.07	48.2	0.35	13.2	0.04	33.1	0.39
2 hours post	29.8	0.09	38.1	0.15	44.0	0.30	13.5	0.18	31.3	0.18
2.5 hours post	31.0	0.36	36.0	0.00	36.2	1.11	15.1	0.26	29.5	0.43
3 hours post	35.8	0.26	30.1	0.00	57.6	1.49	17.4	0.00	35.2	0.44
4 hours post	33.8	0.00	29.3	0.00	40.6	0.25	15.8	0.00	29.9	0.06
500 μg (2 ND)										
1 hour post	28.1	0.00	34.7	0.00	41.4	0.00	16.2	0.00	30.1	0.00
2 hours post	31.4	0.95	38.8	0.43	43.5	0.05	20.6	0.71	33.5	0.53
2.5 hours post	35.5	0.99	35.5	0.10	45.4	0.10	30.9	0.26	36.8	0.36
3 hours post	35.1	0.97	33.2	0.11	40.2	0.49	26.9	0.11	33.8	0.42

6.3.3 Intervention Trial 4

Unmetabolised folic acid was not detected in any of the 4 subjects pre-prandially. Folic acid was detected in all 4 subjects after consumption of the 1st, 2nd and 3rd slices of bread. However the levels were not statistically different from the pre-prandial levels perhaps indicating that the sample size was too small. No accumulation of serum folic acid was demonstrated even after the third slice. Total serum folate concentrations increased significantly from baseline ($p = 0.0001$) indicating compliance with the intervention (see table 6.4).

Table 6.4 Serum folate and serum folic acid ($\mu\text{g/l}$): Intervention Trial 4.

Subjects	1	1	2	2	3	3	4	4	mean	mean
	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid	folate	Folic acid
Pre 333 μg	18.4	0.00	24.1	0.00	30.9	0.00	14.2	0.00	21.9	0.00
333μg (1st)										
1 hour post	21.7	0.15	30.4	0.29	34.4	0.50	16.4	0.07	25.7	0.25
2 hours post	24.5	0.00	29.7	0.30	48.4	0.89	22.8	0.30	31.4	0.37
2.5 hours post	26.1	0.00	29.6	0.00	41.1	1.20	23.2	0.35	30.0	0.39
333μg (2nd)										
1 hour post	37.8	1.83	30.4	0.74	45.0	1.10	26.6	0.43	34.9	1.02
2 hours post	31.4	0.33	29.8	0.09	51.6	1.67	31.1	1.15	35.9	0.81
2.5 hours post	24.2	0.08	30.5	0.09	36.2	1.72	28.5	0.54	29.8	0.61
333μg (3rd)										
1 hour post	25.5	0.41	27.5	0.20	38.6	0.91	29.5	0.50	30.3	0.50
2 hours post	28.3	0.85	32.7	0.43	41.6	0.96	32.7	1.08	33.8	0.83
2.5 hours post	28.7	0.77	39.0	0.25	40.1	0.97	33.8	0.46	35.4	0.61

6.3.4 Intervention Trial 5

Unmetabolised folic acid was not detected in the sera of any of the subjects pre-prandially. Unmetabolised folic acid was detected in the sera in 3 out of the four subjects after consumption of the 1st slice containing 200 μg of folic acid. Folic acid was detected in the sera of all 4 subjects after consumption of every subsequent slice. The results show that an accumulative elevation occurred over the eight-hour period with the highest level of folic acid being present after the 5th slice. Statistical analysis (Anova) showed that post-prandial folic acid concentrations were significantly increased from baseline ($p=0.0015$). Total serum folate concentrations increased significantly from baseline ($p = 0.0179$) indicating compliance with the intervention (see table 6.5).

Table 6.5 Serum folate and serum folic acid ($\mu\text{g/l}$): Intervention Trial 5.

Subjects	1	1	2	2	3	3	4	4	mean	mean
	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid
Pre 200 μg	45.9	0.00	20.2	0.00	29.4	0.00	16.5	0.00	28	0.00
200 μg (1 st)										
45 mins post	49.2	0.26	21.7	0.00	28.2	0.00	18.7	0.23	29.4	0.12
90 mins post	57.3	0.43	35.6	0.26	34.8	0.00	25.9	0.26	38.4	0.24
200 μg (2 nd)										
45 mins post	41.3	0.34	42.9	0.06	36.5	0.79	33.5	0.65	38.5	0.46
90 mins post	51.8	1.00	58.4	0.96	38.8	0.66	35.3	3.29	37.3	1.47
200 μg (3 rd)										
45 mins post	49.6	1.12	59.9	0.00	42.7	0.06	32.2	3.59	46.1	1.19
90 mins post	47.0	2.07	54.5	1.10	36.7	1.64	36.8	1.20	43.7	1.50
200 μg (4 th)										
45 mins post	38.4	0.92	50.6	0.9	40.7	1.74	34.2	2.84	40.9	1.60
90 mins post	44.1	1.04	56.3	1.15	40.3	3.37	39.3	2.10	45	1.91
200 μg (5 th)										
45 mins post	47.8	2.38	52.7	2.83	43.7	4.02	46.8	3.99	47.75	3.31
90 mins post	52.5	4.30	57.8	2.42	49.9	3.99	64.6	3.26	56.2	3.49

6.3.5 Intervention Trial 6

Folic acid was not present in the serum of any of the subjects pre-prandially. Folic acid was not present post-prandially after the 1st slice of bread in any of the subjects. Subsequent slices administered however caused folic acid appearance in serum with a cumulative effect evident in all 4 subjects after repeated administrations. However these increases in the post-prandial samples were not significantly different from the pre-prandial samples even though a trend towards a cumulative effect seemed to occur. Perhaps the sample size was too small to show statistical significance. Total serum folate concentrations increased significantly from baseline ($p = 0.001$) indicating compliance with the intervention.

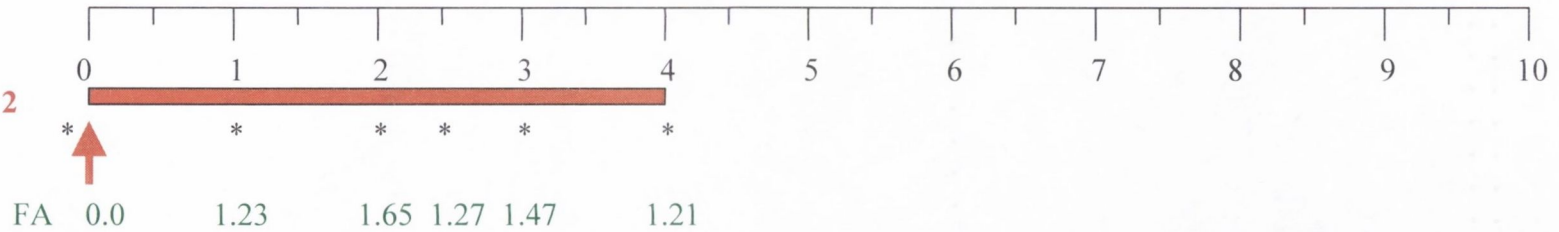
Table 6.6 Total serum folate and serum folic acid (µg/L): Intervention Trial 6.

Subjects	1	1	2	2	3	3	4	4	mean	mean
	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid	folate	folic acid
Pre 100µg	20.8	0.00	12.6	0.00	21.5	0.00	6.19	0.00	15.3	0.00
100µg (1st)										
1 hour post	21.9	0.00	14.8	0.00	24.2	0.00	7.89	0.00	17.2	0.00
100µg (2nd)										
1 hour post	23.1	0.83	16.2	0.18	23.5	0.12	9.18	0.06	17.9	0.29
100µg (3rd)										
1 hour post	25.9	1.57	17.5	0.02	27.6	0.53	10.8	0.36	20.4	0.62
100µg (4th)										
1 hour post	26.8	2.02	18.7	0.38	28.9	0.57	10.7	0.25	21.3	0.80
100µg (5th)										
1 hour post	30.9	3.81	21.7	0.63	30.1	0.72	11.7	0.39	23.6	1.38
100µg (6th)										
1 hour post	29.4	1.28	21.6	0.53	32.3	0.70	11.8	0.46	23.7	0.74
100µg (7th)										
1 hour post	30.4	2.98	21.7	0.42	24.8	0.34	12.9	0.84	22.4	1.14
100µg (8th)										
1 hour post	29.2	3.11	20.9	0.35	23.8	0.47	13.4	0.79	21.8	1.18
100µg (9th)										
1 hour post	28.5	4.13	21.9	0.87	22.8	0.88	14.3	1.19	21.9	1.76
100 (10th)										
1 hour post	32.0	4.95	21.7	0.76	22.5	0.96	15.1	1.93	22.8	2.15

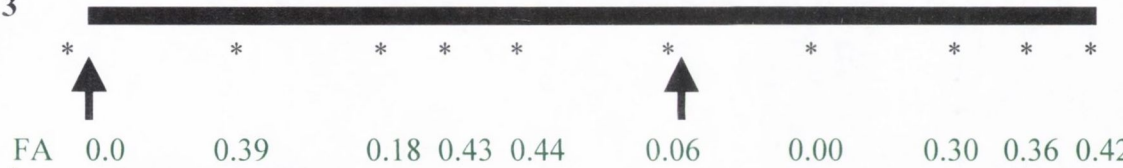
A schematic summary (figure 6.2) allows the intervention phases, sample collection and overall results obtained from the 5 Trials to be visualised together in one clear diagram.

Time (hr)

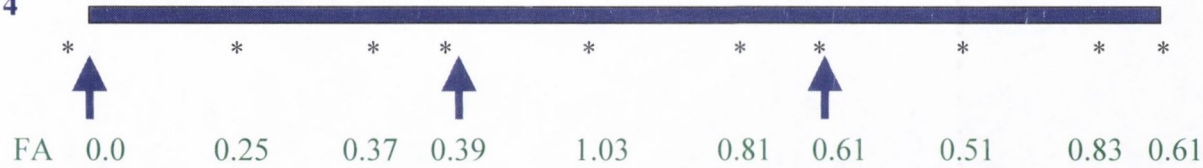
Intervention trial 2
(1 mg x1 dose)



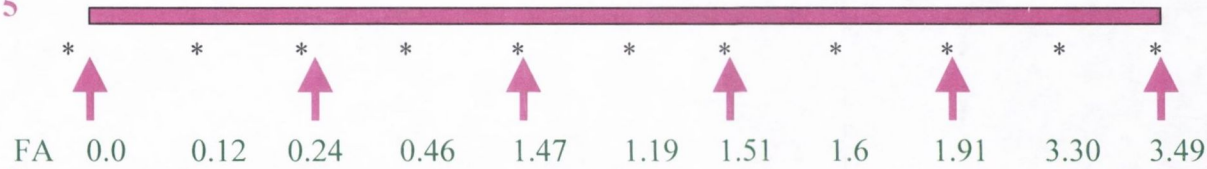
Intervention trial 3
(500 μ g x2 doses)



Intervention trial 4
(333 μ g x3 doses)



Intervention trial 5
(200 μ g x5 doses)



Intervention trial 6
(100 μ g x10 doses)



Legend

- * = Serum folate and folic acid samples
- FA = Serum folic acid (μ g/l)
- ↑ = bread administered

Fig. 6.2 Schematic diagram of intervention trials 2-6 illustrating interventions, sample collection and mean serum folic acid concentrations

6.4 Discussion

A safe upper limit is described as a level of a nutrient above which there is no risk of deficiency, and below a level where there are no undesirable effects. It is not intended as a toxic level and although exceeding this safe intake would not necessarily result in undesirable effects, equally there is no evidence for any benefits (DRVs 1991). On this occasion it is apparent that increasing the intake of folic acid would have beneficial effects in terms of NTS reduction, coronary heart disease and stroke reduction, and possible colorectal cancer prevention. On the other hand, however, at levels below the FDA's safe upper limit set for folic acid our findings indicate that undesirable effects could occur.

Findings from intervention trial 2 show that when 1mg of folic acid in fortified food was consumed in a single dose unmetabolised folic acid appeared in serum. This persisted for a least 4 hours. In Trial 3 a slice of bread fortified with 500 μ g of folic acid was administered. While there was an increase in unmetabolised folic acid post-prandially this was found to be non-significant statistically. A 2nd slice containing 500 μ g administered 4 hours later gave rise to folic acid appearance in serum, which was not statistically significant from the pre-prandial concentrations and did not cause a cumulative effect. Perhaps the sample size was too small to demonstrate significance. However lack of a cumulative effect indicates that if the 1st dose has sufficient time to clear from the serum before a 2nd dose is consumed a cumulative effect is not seen. Study 4 illustrates similar findings. 333 μ g of folic acid was administered in 3 different slices of bread spread out over 8 hours. All 3 slices caused folic acid appearance in serum but no evidence of an accumulative effect was shown after the 2nd or 3rd slices.

Conversely in Intervention Trials 5 and 6 the fortified bread was administered repeatedly in quick succession. In Trial 5 unmetabolised folic acid accumulated after repeated consumption of slices containing 200 μ g of folic acid. In Trial 6 unmetabolised folic acid did not appear in any of the subjects after the 1st slice of bread but did appear after the 2nd slice consumed 1 hour later. Subsequent slices lead to a cumulative increase of folic acid in serum, which were not statistically different from the pre-prandial concentrations. The increases were not as marked as those seen in Trial 5. The most likely explanation for the cumulative effect seen in both of these studies is that the repeat doses of folic acid were given around the time of known $T_{\max}$ (2 hours approximately, Kelly *et al*, 1997) for folic acid in serum of the preceding slice. This caused the cumulative increase demonstrated. It is noteworthy that in these acute studies no pre-saturation with folic acid was given to any of the subjects. Based on the fact that earlier findings from this thesis (Intervention Trial 1) showed that pre-saturation with folic acid fortified foods caused a cumulative effect of folic acid in serum it is likely that these findings underrepresented what would have occurred if subjects had been pre-saturated or indeed if they were consuming these doses on a regular basis i.e. as a result of mass fortification. See Table 6.7 for a summary of which doses caused folic acid appearance and subsequent accumulation.

These finding give rise to concern because they show that low doses of folic acid in fortified bread consumed frequently throughout the day may actually hold more potential for harm than larger dose consumed as a bolus. Generally it would have been an *a priori* assumption that the safest means of delivering folic acid to a population would be in smaller doses more often in preference to large doses as a bolus.

These studies back up the findings from Intervention Trial 1 and indicate that renal clearance is more effective in the face of large doses of folic acid given as a bolus rather than small doses released repeatedly into the circulation. This research highlights the fact that consumption of either 1mg as a single bolus dose or as separate 100 μ g amounts over 8-10 hours has the potential to produce circulating folic acid. These acute studies show however that if folic acid is given time to clear from the serum between doses a cumulative effect does not occur even at doses as high as 500 μ g. Bearing in mind that the 1st dose of 100 μ g (in Intervention Trial 6 and in Intervention Trial 1(Chapter 5) even after pre-saturation) did not cause folic acid appearance in serum post-prandially there are grounds for optimism that fortification can be undertaken without the possibility of toxic effects. It is likely therefore that it would be safe to consume a maximum dose of 100 μ g at one sitting and that a repeat dose should not be administered until this has had time to clear from the serum. Previous findings from Intervention Trial 1 of this thesis showed that four hours was sufficient for 100 μ g of folic acid to clear from the serum even after subjects were pre-saturated.

In conclusion therefore, the FDA's safe upper daily limit for folic acid should be reviewed such that a fortification strategy should ensure that a maximum of 100 μ g should be administered at one sitting and that consumption of subsequent doses should be at intervals of 4 hours or more. Such a strategy is unlikely to expose the general population to unmetabolised folic acid in the serum and hence toxic effects of folic acid are unlikely to occur.

Table 6.7 Illustrates a summary of the results obtained from these acute studies.

Levels of fortification tested	Unmetabolised folic acid in serum	Accumulation of folic acid in serum	Intervals between doses
100µg (single dose)	NO	–	1 hour or less
100µg (2 doses or more)	YES	YES	
200µg (single dose)	YES	–	
200µg (2 doses or more)	YES	YES	1 hour or less
333µg (single dose)	YES	–	2.5 hours or greater
333µg (2 doses or more)	YES	NO	
500µg (single dose)	YES	–	
500µg (2 doses or more)	YES	NO	4 hours or greater
1mg (single dose)	YES	–	

Chapter Seven

***In vivo* experiment**

**investigating the appearance of serum
unmetabolised folic acid in premature and
full term infants pre- and post-feeding
with formula milk**

7.1 Introduction

Oral folic acid above certain threshold doses saturates the normal absorptive mechanisms and results in the appearance of unmetabolised folic acid in serum. This has been demonstrated in a young adult population (Intervention trial 1) and also in the elderly (Kelly *et al*, 1997). To date no such studies have been carried in an infant population group. This study was carried out to investigate the effect of feeding standard commercially available folic acid fortified-formula milk on unmetabolised folic acid in serum in premature and full-term new born infants. This was considered an important study because the longer the duration of exposure to unmetabolised folic acid in serum the greater the potential for harm. In other words if it is present from shortly after birth as a result of formula feeding or even in utero as a result of the maternal dietary intake there is a possibility that the individual will be exposed to folic acid in serum for an entire lifetime.

7.2 Methods

7.2.1

Ethical approval was obtained from the Coombe Woman's Hospital, Master's Office. Consent forms and parent information leaflets were issued to parents who consented to their infant taking part in the study. Exclusion criteria included babies on parenteral feeds intravenously, breast-feeding or any infants with any metabolic or gut disorders which might have interfered with the uptake of folic acid. We set out to recruit full-term and premature babies so that we could compare the responses in both. A total of 11 infants

were recruited. One set of these was twins born at 32 weeks. The other premature babies were born at 30 and 34 weeks. All the remaining babies were born full-term.

7.2.2 Blood Collection

One half ml of serum was necessary to perform the laboratory analysis. We estimated that a 1ml blood sample would provide us with enough for the analysis. Blood was collected from the umbilical cord of each infant in the delivery room immediately after birth. It was hoped that this would provide a baseline serum folic acid level at birth. A second serum sample was collected four days after the birth date at the time of the heel-prick test. This was collected approximately 90 minutes after their last formula feed to anticipate the T-max of 90 mins (Kelly *et al*, 1997). Kelly et al (1997) showed that the T-max of folic acid dissolved in solution was approximately 90 minutes where as it was approximately 2.5 hours in fortified foods. All babies were on 3-4 hourly formula feeds. 11 baseline samples and only 9 follow up samples were collected due to parental refusal to allow the second sampling to take place after originally consenting to the study.

7.2.3 Dietary questionnaire

A questionnaire (see Appendix 3) was issued to the clinician collecting the blood samples from the infants. This included details on brand name of feed, number of feeds taken between birth and the second sampling, the amount taken at each feed and particularly the amount taken at the last feed prior to the second sampling. In addition the mothers of these

infants were asked about their use of folic acid supplements or other multi-vitamins at the time of cord-blood collection.

7.2.4 Formula feeds administered

3 standard formula feeds were given to the babies in this study at the discretion of the medical staff on the neonatal and post-natal wards. These included Cow and Gate Premium, which was fortified with folic acid at 10µg/100ml, SMA Gold Cap which, was fortified at 8µg/100mls and Optimal which, was fortified at 10µg/100ml. The type of feed consumed by each infant and the amount of folic acid consumed at their last feed prior to blood sampling are listed in table 7.1.

Table 7.1 Brand name of feed and amount of folic acid consumed in the last feed prior to blood sampling.

Subjects	Type of feed	Amount of folic acid consumed at last feed
Baby 1 (full term)	Cow & Gate Premium	8µg
Baby 2 (full term)	SMA Gold Cap	-----
Baby 3 (full term)	Cow and Gate Premium	4µg
Baby 4 (premature twin 1)	SMA Gold Cap	4µg
Baby 5 (premature twin 2)	SMA Gold Cap	4µg
Baby 6 (full term)	Cow & Gate Premium	8µg
Baby 7 (Premature)	Cow & Gate Premium	8.5µg
Baby 8 (full term)	Cow & Gate Premium	5µg
Baby 9 (full term)	SMA Gold Cap	6µg
Baby 10 (full term)	SMA Gold Cap	6µg
Baby 11 (full term)	Cow & Gate Premium	-----

7.2.5 Laboratory analysis

All samples were assayed for unmetabolised folic acid by the HPLC/Sep-Pak/microbiological assay described in chapter 4. Unfortunately we had insufficient sample remaining to perform total serum folate measurements.

7.2.6 Statistical analysis

Statistical analysis was performed using data desk 5.0. Results were analysed by Anova and post hoc Scheffe tests.

7.3 Results

Unmetabolised folic acid was detected in the cord blood of all babies collected immediately after birth (n=11). Unmetabolised folic acid was detected in 7 out of the 9 samples collected on day 4 of formula feeding. In 6 of these cases an increase in unmetabolised folic acid concentrations from baseline was demonstrated. In 3 of the babies unmetabolised folic acid concentrations decreased after formula feeding (see figure 7.2).

Table 7.2 Unmetabolised folic acid (µg/L) in serum of premature and full term infants pre and post formula feeding.

Subjects	Sample 1 (from cord)	Sample 2 (venopuncture)
Baby 1 (full term)	0.08	1.31*
Baby 2 (full term)	0.47	No sample
Baby 3 (full term)	0.22	0.0
Baby 4 (premature twin 1)	0.22	0.31*
Baby 5 (premature twin 2)	0.17	0.54*
Baby 6 (full term)	0.31	0.18
Baby 7 (Premature)	0.15	0.32*
Baby 8 (full term)	0.16	0.86*
Baby 9 (full term)	0.14	0.0
Baby 10 (full term)	0.22	0.70*
Baby 11 (full term)	0.43	No sample

* Denotes an increase in unmetabolised folic acid concentrations from baseline.

7.4 Discussion

All of the mothers recruited for this study were asked about supplement use at time of recruitment. None of the mothers were consuming folic acid supplements at this time. Therefore the levels of unmetabolised folic acid detected in the cord blood arose due to recent dietary intake of folic acid from commercially available foods. We detected unmetabolised folic acid in cord blood as high as $0.47\mu\text{g/L}$. This is almost 25% of the lower limit of normal reference ranges for total serum folate in adults (normal $2.7\text{--}20\mu\text{g/L}$). Unmetabolised folic acid had obviously passed from the maternal circulation through the placental barrier to the developing foetus.

It has been previously documented that many serum constituents including water-soluble vitamins measured at birth in maternal and cord blood show higher concentrations in the cord than in the mother's blood (Berger 1988). While unmetabolised folic acid has never previously been measured in cord blood (to our knowledge) the belief was held that it would not pass to the foetus unmetabolised. The presence of unmetabolised folic acid in cord blood may hold potential for harm in terms of adverse effect in the developing foetus particularly if large doses are able to pass over the placenta barrier to the foetus. Adverse effects of large doses of folic acid have previously been demonstrated in a rat model (Achon *et al*, 1999, 2000). The authors showed that long-term high levels ($40\mu\text{g}$) of folic acid supplementation resulted in lower birth weights and shorter vertex-coccyx length in rats. In addition the potentially toxic effects of folic acid supplementation discussed previously in section 1.23 of this thesis warrant consideration.

The increase in folic acid concentrations after formula feeding in some of the infants also gives rise to concern. The amounts of folic acid administered in the feeds were in the region of 4-10µg per feed. These were administered 3-5 hourly for only 4 days. Yet the levels of folic acid in the serum increased over this time. The premature babies did not exhibit different responses to the full-term babies.

These results indicate that the consumption of commercially available folic acid fortified foods by pregnant women does lead to the appearance of unmetabolised folic acid in cord blood. In addition consumption of commercially available folic acid fortified formula feeds by infants gives rise to the appearance of unmetabolised folic acid in serum. These results give rise to some concern as they suggest that the current levels of folic acid fortified foods consumed by pregnant women in Ireland cause exposure of the developing foetus to unmetabolised folic acid. The adverse effects of this are currently unknown but may have potential for harm particularly at high doses. The level of fortification of formula feeds currently in common use in maternity hospitals and in the general population may also have potential for harm. These levels may need to be reviewed.

Chapter Eight

Red Cell Folate Stability Studies

8.1 Introduction

Blood samples collected in a clinic or hospital setting often may not reach the laboratory for several hours afterwards due to limited portering services or prioritising of other services by hospital staff. In nutrition research studies blood samples are often collected off-site in order to facilitate the volunteers. Because of this samples are often stored at room temperature or are in transportation for long periods of time before they can be processed in an appropriate way. Recent research findings in the folate and neural tube defect field have highlighted the importance of establishing very precise, accurate measurements of folate status particularly in red cells (Daly *et al*, 1997) in terms of establishing NTD risk. In the past it was safe to assume that there was a very clear cut-off point in reference ranges between deficiency and replete levels and adverse or beneficial effects were not known to occur above these classically defined deficiency levels. Now we know that this is not the case as NTDs occur in pregnancies where the maternal red cell folate levels (while lower than non-NTD affected pregnancies) are not classically deficient (Molloy *et al*, 1985; Kirke *et al*, 1993) but instead risk is characterised as a continuum of folate status (Daly *et al*, 1995). Therefore in attempting to establish firm cut-off points in terms of risk in this manner for NTDs and other diseases such as cardiovascular disease (Wald *et al*, 1998) it is imperative that we are able to perform very accurate analytical measurements of red cell folate status. Hence, sample processing must be consistent from the moment of collection right through to the analytical process. As mentioned previously this is often not the case as some samples are processed immediately while, other samples are left lying around on hospital wards at room temperature, or are in transportation for long periods of time. This time delay is not taken into account by laboratories and a deterioration factor (if necessary)

is not accounted for. The purpose of this study was to establish optimum criteria for red cell folate storage prior to long-term freezing. In this chapter we investigated the stability of whole blood red cell folates and red cell folate lysates over time, under different storage and handling conditions. Two pilot studies are described initially.

8.2 Methods

8.2.1 Pilot Study 1. Stability of red cell folates stored as whole blood.

Four EDTA whole blood samples were collected by venopuncture from 4 volunteers. Four lysates samples were prepared (in the normal way for red cell folate analysis by a 1 in 10 dilution of sample in ascorbic acid buffer –see section 2.6.1) using one tube from each volunteer. These samples were frozen immediately at -20°C and used as a reference sample. A 2nd sample from each subject was left on a laboratory bench at room temperature (approximately 22°), a 3rd sample from each subject was left in a cold room at 4°C , and a 4th sample from each subject was left on a shaker incubator at room temperature at a speed of 100rpms. The shaker incubator was an attempt to mimic transportation conditions such as by bus or train. Lysates were prepared on day 3, 5, 7 and 11 from each of these whole blood samples, and then frozen at -20°C . Duplicate lysates were prepared at all time points. All samples were assayed for red cell folate by the microbiological assay described by Molloy & Scott (1997). All samples were assayed in the same assay batch to eliminate inter-assay variation. The results are shown in section 8.3.1. Figure 8.1 illustrates this pilot study schematically.

8.2.2 Pilot Study 2. Stability of lysates prepared immediately from whole blood.

One whole blood EDTA sample was collected from 4 subjects as before. Four lysates samples were prepared from each subject. Following deconjugation one lysate sample from each subject was frozen immediately at -20°C . This was used as a reference sample. A 2nd lysate sample from each subject was stored on a laboratory bench at room temperature (approximately 22°C), a 3rd lysate sample from each subject was stored in a cold room (4°C) and the 4th lysate sample was placed on a shaker incubator at room temperature at 100rpms. Samples ($200\mu\text{l}$ volume) were taken from each tube on day 2, 4, 6 and 10. These were frozen immediately at -20°C until time of analysis. All lysates were prepared in duplicate. The results are shown in the results section 8.3.2. Figure 8.2 illustrates this pilot study schematically.

8.2.3 Pilot Study 3. Red cell folate stability study.

The pilot studies above showed (refer to results section 8.3.1 and 8.3.2) that red cell folate stored as whole blood appears to be more stable than red cell folate stored as lysates under any of the conditions tested. In pilot study 1 above lysate samples were prepared from whole blood on day 0 and then on day 3, 5, 7 and 11. Statistical differences were not demonstrated between day 0 and day 3 under any of the any of the conditions tested. However this may have been because the sample size was too small. Hence we decided to look more closely at what was actually happening between day 0 and day 3. From these small studies we established that cold room storage is optimum regardless of whether

samples are stored as whole blood or as lysates. As this is not an option during transportation we set out to look at the effects of storage of whole blood on a laboratory bench at room temperature and storage of whole blood on a shaker incubator at room temperature. As most samples reach their laboratory destination between day 0 and day 3 we set up a study with shorter time points so that we could investigate losses incurred over a 4, 8, 24, 48 and 72 hour time frame. In addition we looked at a larger sample size.

In order to examine this 2 whole blood tubes (EDTA) were collected from 23 healthy volunteers. A lysate sample was prepared (in the normal way for red cell folate analysis by a 1 in 10 dilution of sample in ascorbic acid buffer – see section 2.6.1) from each sample immediately after collection (in duplicate). These were frozen at -20°C immediately after preparation and used as the reference sample. One whole blood tube was then stored on a laboratory bench at room temperature the 2nd tube was stored on a shaker incubator at room temperature. Further lysates were then prepared from each tube (in duplicate) at 4, 8, 24, 48 and 72 hours. All samples were assayed in batch by the microbiological assay described by Molloy & Scott (1997). The results are illustrated in the results section 8.3.3. Figure 8.3 illustrates this pilot study schematically.

8.2.4 Statistical Analysis

Statistical analysis was by Anova, and post hoc Scheefe tests using the computer package Data Desk 5.0.

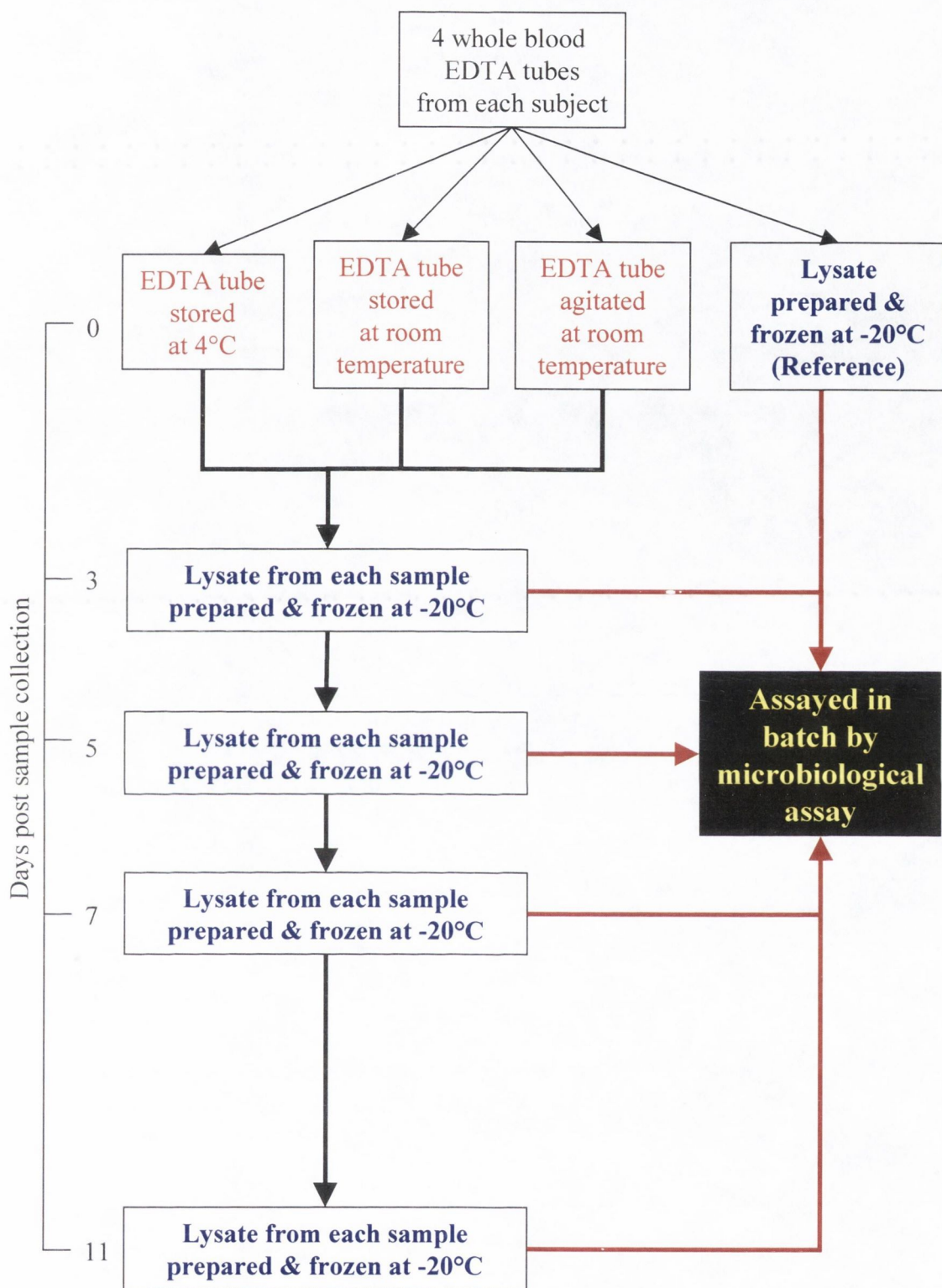


Figure 8.1. Whole blood red cell folate stability study. Pilot study 1.

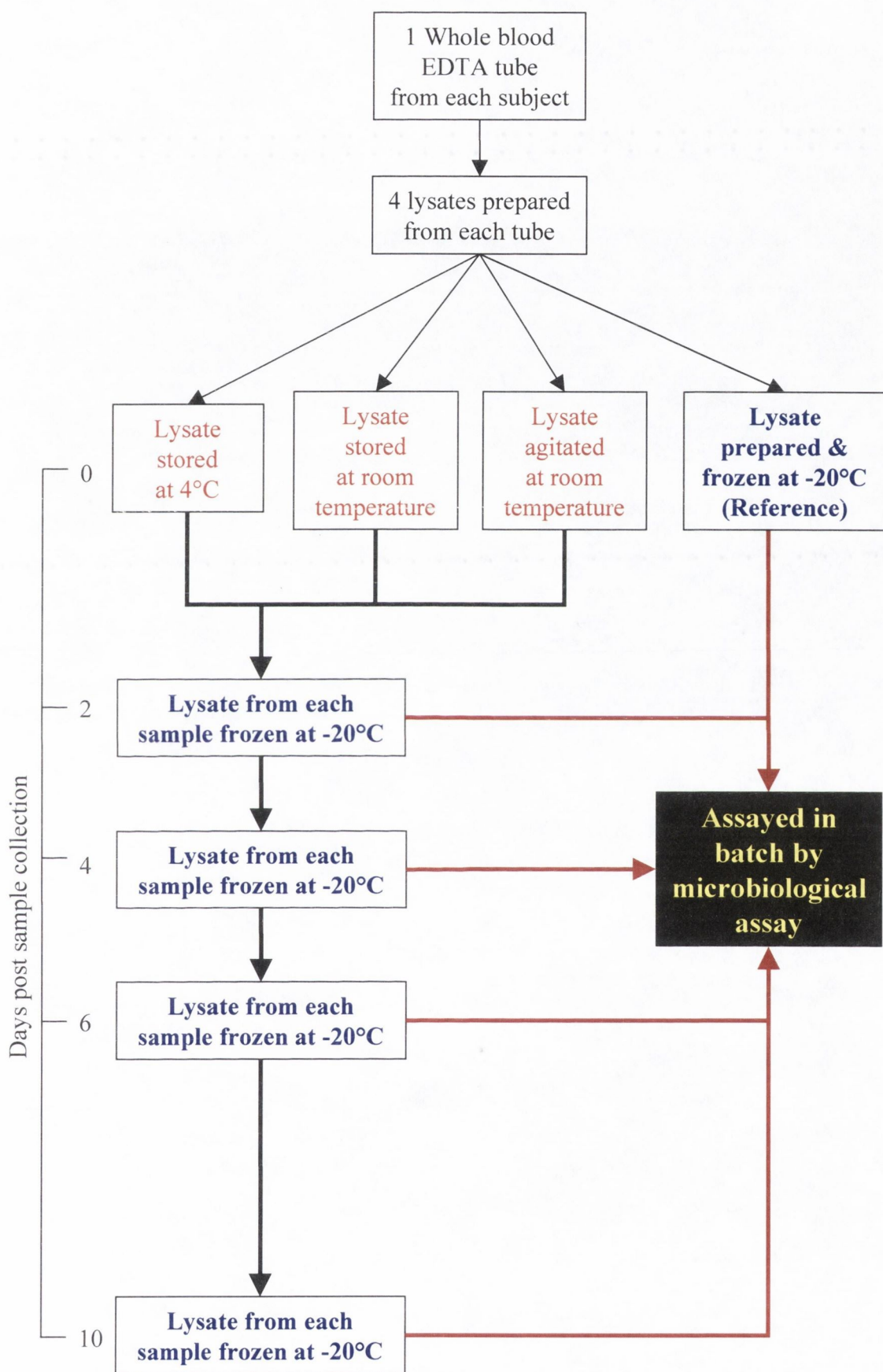


Figure 8.2. Red cell folate lysate stability study. Pilot study 2.

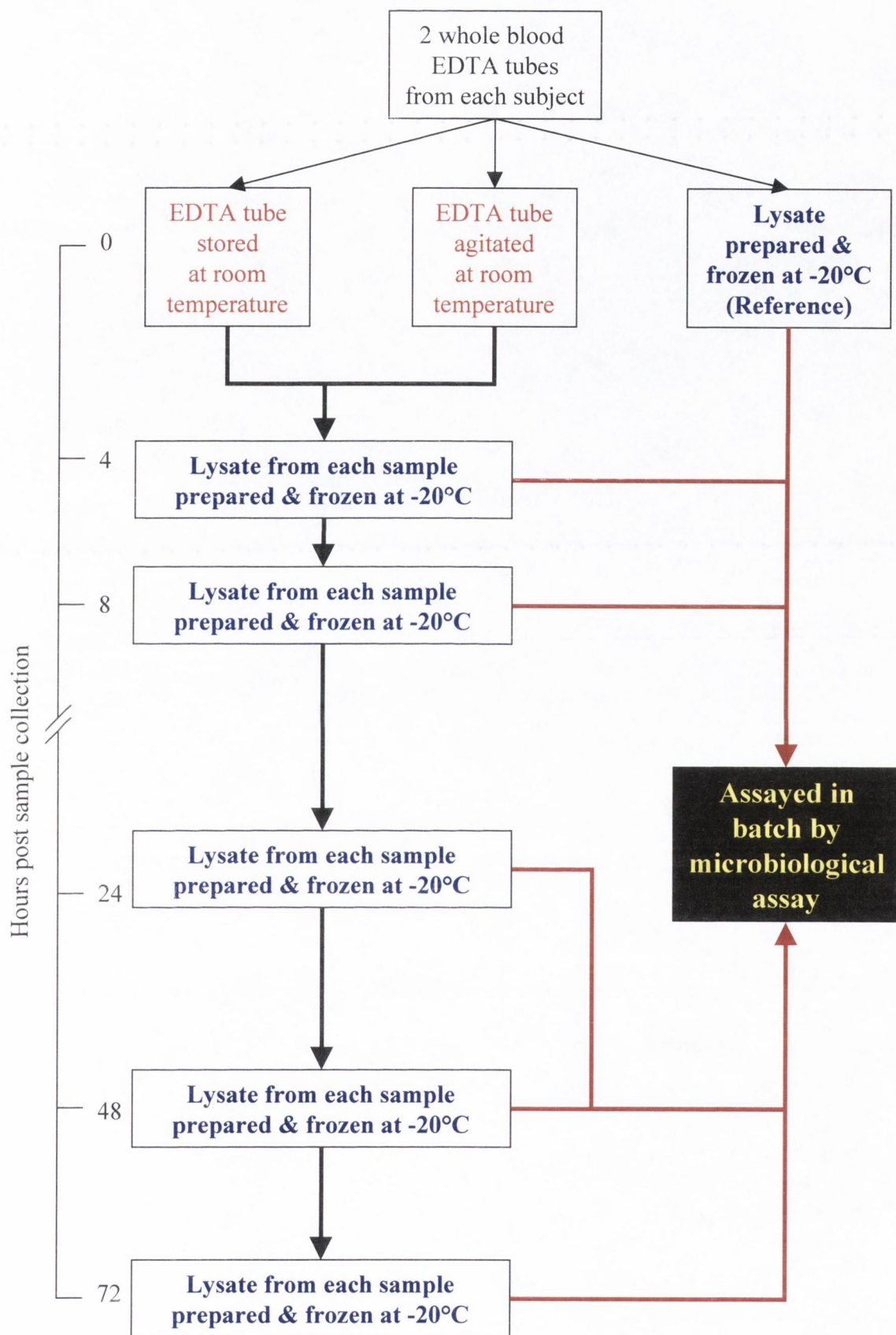


Figure 8.3. Whole blood red cell folate stability study. Pilot study 3.

8.3 Results

8.3.1 Pilot Study 1. Stability of red cell folates stored as whole blood.

The results obtained from Pilot Study 1 show that red cell folate lysates prepared from whole blood stored at room temperature on day 3, 5, 7 and 11 after collection were not statistically different from the reference samples ($p = 0.1657$). These results were reproduced in the duplicate samples tested (0.1608) also (see Table 8.1 and 8.2. and Figure 8.4).

The red cell folate lysates which were prepared from the whole blood stored in the cold room were not significantly different from the reference sample after 11 days ($p = 0.9798$). This was demonstrated in the duplicates samples also ($p = 0.9929$) (see Table 8.1 and 8.2. and Figure 8.4).

The red cell folate lysates, which were prepared from whole blood stored on a shaker incubator at room temperature, were least stable of all. From day 0 up to day 5 no losses occurred however lysates prepared on day 7 were statistically different from the reference sample ($p = 0.0005$). Duplicate samples demonstrated similar findings with statistical differences between the reference sample and day 7 samples ($p = 0.0003$) (see Table 8.1 and 8.2. and Figure 8.4).

Table 8.1. Pilot Study 1. Stability of red cell folates stored as whole blood.

Red cell folate ($\mu\text{g/L}$) values of reference, day 3, 5, 7 and 11 samples a) at room temperature, b) in cold room and c) on shaker incubator at room temperature.

	Subject 1	Subject 2	Subject 3	Subject 4	means
Reference sample	158.7	195.5	223.0	93.00	167.5
Day 3 Room temperature	143.2	152.0	182.0	88.00	141.31
Day 3 Cold room	150.2	187.0	191.2	96.25	156.18
Day 3 Incubator	147.0	166.7	216.7	95.5	163.25
Day 5 Room temperature	144.7	144.2	192.5	96.75	144.5
Day 5 Cold room	177.5	204.0	232.7	102.0	179.0
Day 5 Incubator	115.0	69.75	147.7	72.50	101.25
Day 7 Room temperature	126.5	104.2	205.2	77.75	128.4
Day 7 Cold room	150.5	219.2	200.2	101.0	167.75
Day 7 Incubator	71.75	47.00	94.00	50.50	65.81
Day 11 Room temperature	87.25	47.50	146.0	34.00	78.68
Day 11 Cold room	153.5	192.2	210.5	95.70	162.98
Day 11 Incubator	36.50	25.50	23.50	20.75	26.56

Table 8.2. Pilot Study 1. Stability of red cell folates stored as whole blood.

Duplicate red cell folate ($\mu\text{g/L}$) values of reference, day 3, 5, 7 and 11 samples a) at room temperature, b) in cold room and c) on shaker incubator at room temperature.

	Subject 1	Subject 2	Subject 3	Subject 4	mean
Reference sample	151.2	194.5	232.0	103.2	170.25
Day 3 Room temperature	140.5	153.7	177.5	92.75	141.12
Day 3 Cold room	155.7	178.7	204.5	99.00	159.5
Day 3 Incubator	168.0	163.5	216.2	89.75	159.37
Day 5 Room temperature	155.8	147.0	190.2	99.25	148.08
Day 5 Cold room	178.2	203.0	228.7	95.25	176.31
Day 5 Incubator	112.5	72.75	134.2	71.50	97.75
Day 7 Room temperature	126.7	102.5	200.5	81.00	127.68
Day 7 Cold room	145.5	205.5	210.7	98.25	165.0
Day 7 Incubator	73.25	43.75	93.2	54.50	66.18
Day 11 Room temperature	96.00	48.00	150.0	34.75	82.187
Day 11 Cold room	154.0	195.0	208.5	108.0	166.3
Day 11 Incubator	36.00	23.00	27.50	21.25	26.93

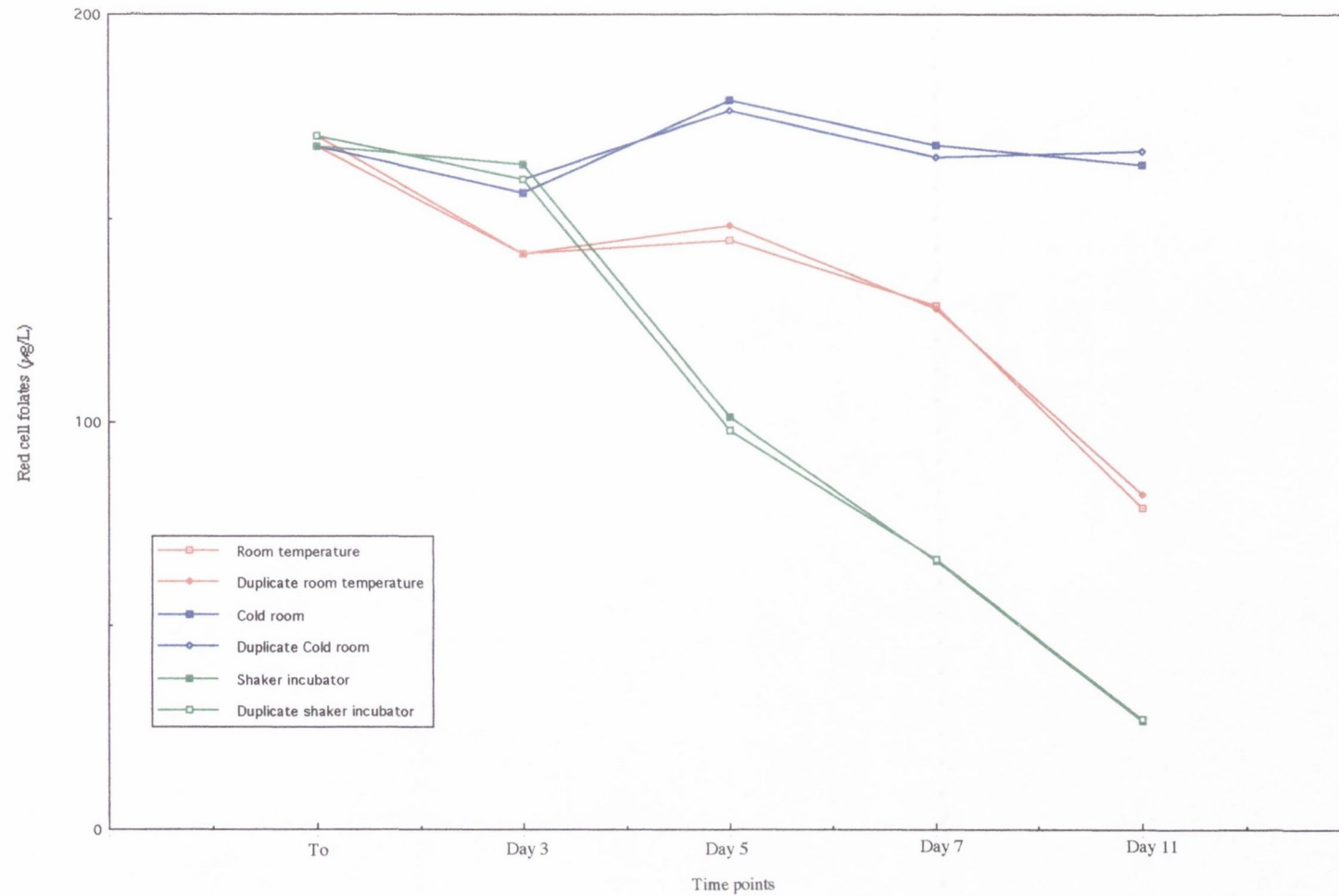


Figure 8.4. Pilot study 1. Whole blood red cell folate ($\mu\text{g/L}$) in duplicate at a) room temperature, b) in cold room and c) on shaker incubator

8.3.2. Pilot Study 2. Stability of lysates prepared immediately from whole blood.

Red cell folate lysates stored at room temperature were statistically different from the reference sample at day 2 ($p = 0.0001$). These finding were also demonstrated in the duplicate samples, statistical differences were reported between the reference sample and day 2 samples ($p=0.0001$) (see Tables 8.3. and 8.4. and Figure 8.5).

Lysates stored in the cold room did not degrade during the period tested ($p = 0.9939$). No statistical differences were demonstrated between any of the time points tested indicating that under this conditions red cell folates lysates are stable for up to 9 days. This was also demonstrated in the duplicate samples tested ($p = 0.9448$) (see Tables 8.3. and 8.4. and Figure 8.5).

Lysate samples stored on a shaker incubator at room temperature were stable for 6 days. By day 9 however losses had occurred ($p = 0.0087$). Duplicate results confirmed this also ($p = 0.0069$) (see Tables 8.3. and 8.4. and Figure 8.5).

Table 8.3. Pilot Study 2. Stability of lysates prepared immediately from whole blood.

Red cell folate ($\mu\text{g/L}$) values of the reference sample and samples prepared on day 2, 4, 6 and 9 a) at room temperature, b) in cold room and c) on shaker incubator at room temperature.

	Subject 1	Subject 2	Subject 3	Subject 4	mean
Reference sample	110.0	234.0	199.0	142.5	171.3
Day 2 Room temperature	17.50	21.25	19.25	17.25	18.81
Day 2 Cold room	108.5	222.5	191.2	153.3	168.9
Day 2 Incubator	117.0	218.0	195.5	160.0	172.9
Day 4 Room temperature	26.00	19.50	30.25	25.75	25.5
Day 4 Cold room	111.7	218.0	190.0	157.0	169.2
Day 4 Incubator	112.5	239.7	207	151.7	177.2
Day 6 Room temperature	19.25	26.25	25.70	23.25	23.61
Day 6 Cold room	117.7	178.6	191.7	158.2	161.5
Day 6 Incubator	104.2	197.0	175.0	142.2	154.6
Day 9 Room temperature	22.75	50.00	20.00	24.00	28.68
Day 9 Cold room	121.3	189.5	187.0	141.0	159.7
Day 9 Incubator	40.00	49.50	49.20	86.7	56.6

Table 8.4. Pilot Study 2. Stability of lysates prepared immediately from whole blood.

Duplicate red cell folate ($\mu\text{g/L}$) values of the reference sample and samples prepared on day 2, 4, 6 and 9 a) at room temperature, b) in cold room and c) on shaker incubator at room temperature.

	Subject 1	Subject 2	Subject 3	Subject 4	mean
Reference sample	110.0	200.0	182.5	149.7	168.5
Day 2 Room temperature	18.50	23.25	17.75	18.75	19.56
Day 2 Cold room	115.2	222.5	194.0	159.7	172.8
Day 2 Incubator	115.2	210.0	195.5	150.0	166.7
Day 4 Room temperature	23.50	18.30	16.25	25.75	20.95
Day 4 Cold room	111.2	207.0	196.0	161.5	168.9
Day 4 Incubator	115.5	235.0	212.5	146.5	177.4
Day 6 Room temperature	16.50	22.50	20.00	25.50	21.13
Day 6 Cold room	119.0	176.5	158.0	156.2	152.4
Day 6 Incubator	104.2	203.0	178.0	136.0	122.3
Day 9 Room temperature	18.50	18.00	17.75	24.00	19.56
Day 9 Cold room	121.5	184.5	189.0	144.0	159.7
Day 9 Incubator	40.00	52.50	51.00	86.75	57.56

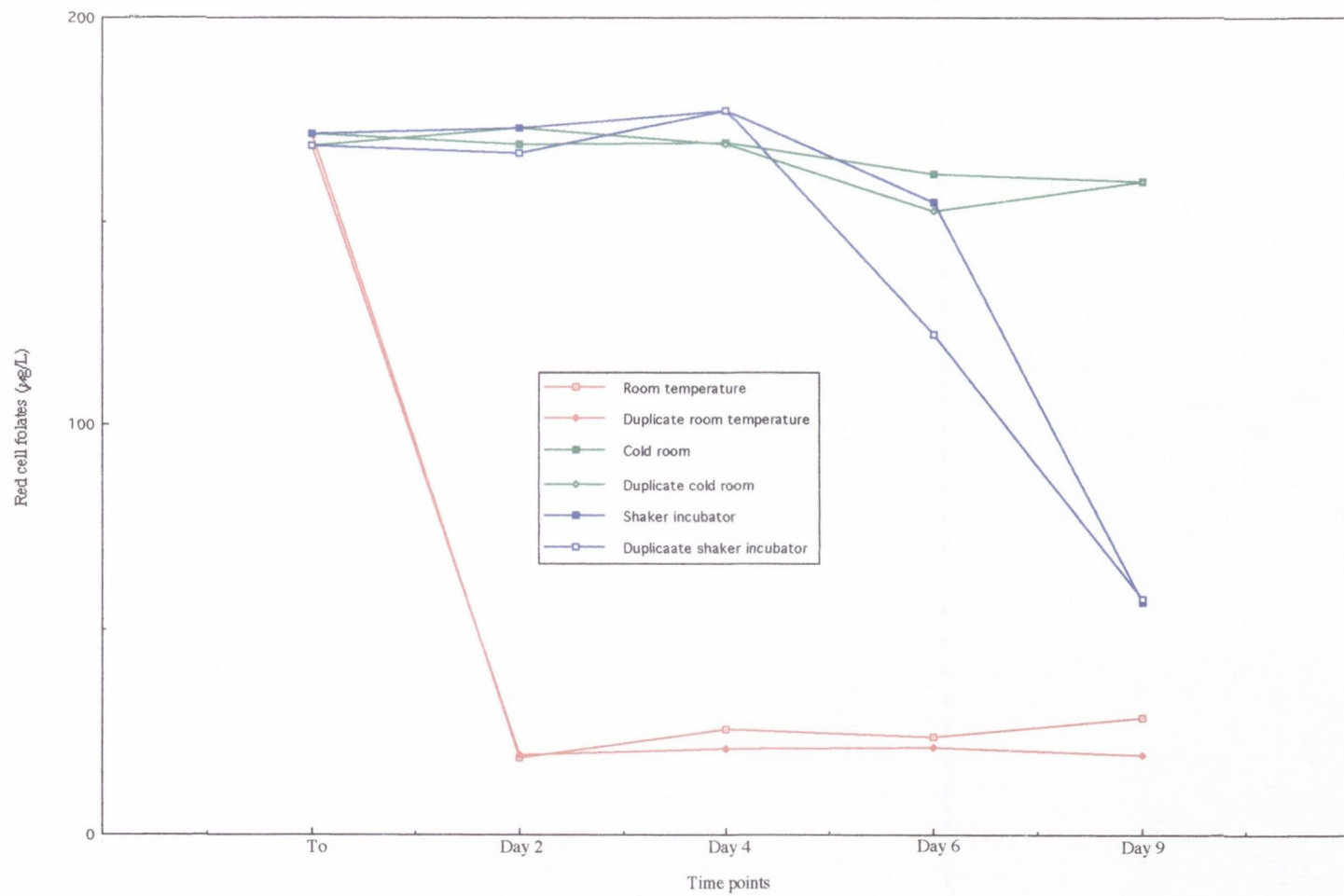


Figure 8.5. Pilot Study 2. Red cell folate lysates ($\mu\text{g/L}$) in duplicate at a) room temperature, b) in cold room and c) on shaker incubator.

8.8.3. Pilot Study 3. Red cell folate stability study.

The data did not appear to be normally distributed so we attempted a number of transformations of the data including natural logs and reciprocals of the data. Since no improvement resulted from this procedure the original data was used for statistical analysis.

Our results showed that red cell folate lysates prepared from whole blood stored at room temperature were not statistically different from the reference sample after 72 hours ($p=0.8778$ and duplicates $p=0.8250$) (See Table 8.5. and 8.6 and Figure 8.6)

Red cell folate lysates prepared from whole blood, which were in constant motion on a shaker incubator at room temperature did not show statistical differences from the reference sample up to 72 hours ($p=0.523$ and duplicates 0.1321). (See Tables 8.7. and 8.8. and Figure 8.7). However closer examination of the data showed that losses of approximately 20% were incurred after 72 hours.

Table 8.5. Pilot Study 3. Red cell folate stability study.

Red cell folates ($\mu\text{g/L}$) at room temperature over 72 hours.

Subjects	To	4 hours	8 hours	24 hours	48 hours	72 hours
1	125.840	120.010	111.870	111.240	113.130	101.570
2	196.290	179.480	150.660	149.490	165.270	148.779
3	193.230	197.870	177.730	174.150	178.660	169.590
4	124.400	121.410	121.080	126.050	120.180	123.730
5	245.830	227.210	221.320	221.320	34.970	224.340
6	110.900	120.600	129.130	141.730	128.380	114.890
7	114.040	117.240	108.760	118.280	113.680	92.194
8	133.510	126.160	104.330	124.170	116.450	103.700
9	129.980	113.480	117.990	114.330	115.680	103.170
10	266.100	267.750	247.590	242.440	240.210	189.040
11	160.080	151.740	151.220	140.300	143.750	121.760
12	75.370	75.093	78.525	61.936	60.780	50.631
13	388.380	386.250	385.330	375.520	386.520	366.080
14	119.260	124.430	111.680	113.740	121.110	93.569
15	201.710	176.780	196.420	185.700	218.290	185.240
16	71.982	80.843	69.097	65.527	69.955	53.814
17	124.020	117.160	122.060	118.400	107.830	104.330
18	197.050	201.72	211.210	216.920	216.260	191.190
19	140.2560	143.370	150.190	144.750	139.190	126.620
20	101.830	109.730	113.010	102.930	98.328	87.611
21	108.390	106.510	97.433	102.230	86.365	78.955
22	171.210	163.360	144.980	135.310	111.310	146.560
23	214.220	197.350	198.110	182.330	193.140	156.970
mean	161.486	157.623	153.032	150.817	151.280	136.275

Table 8.6. Pilot Study 3. Red cell folate stability study.

Duplicate red cell folate ($\mu\text{g/L}$) at room temperature over 72 hours.

Subjects	To	4 hours	8 hours	24 hours	48 hours	72 hours
1	126.780	130.670	123.870	98.270	107.760	126.420
2	173.230	183.630	162.420	160.690	158.700	129.870
3	206.990	183.490	191.430	162.410	178.100	162.010
4	123.930	132.850	142.640	130.910	119.370	104.480
5	226.790	231.480	195.590	239.070	245.540	227.560
6	127.530	157.6300	149.110	121.230	121.120	117.640
7	124.760	131.310	109.330	105.160	107.530	82.012
8	112.840	135.160	122.440	90.112	105.650	103.010
9	120.150	138.650	140.690	102.380	106.770	107.900
10	266.100	279.110	257.870	212.440	240.210	202.240
11	160.080	157.220	150.040	129.770	118.000	120.920
12	79.440	74.917	71.474	54.788	60.016	59.004
13	371.270	368.280	369.800	383.370	386.52	354.470
14	105.670	114.050	110.060	99.330	114.710	108.330
15	185.020	184.390	214.100	196.090	188.700	168.200
16	72.418	69.542	67.935	65.875	67.267	64.503
17	121.030	105.650	116.120	112.360	110.920	102.090
18	196.140	207.960	205.860	201.870	196.790	194.720
19	138.750	110.640	151.980	152.020	158.180	121.660
20	115.280	115.970	122.230	92.412	97.060	91.623
21	104.350	107.790	93.661	86.378	73.382	77.808
22	166.130	159.470	149.680	144.090	153.060	141.850
23	212.800	191.000	196.250	189.760	169.780	149.250
mean	154.455	157.065	154.139	1142.317	144.704	134.815

Table 8.7. Pilot Study 3. Red cell folate stability study.

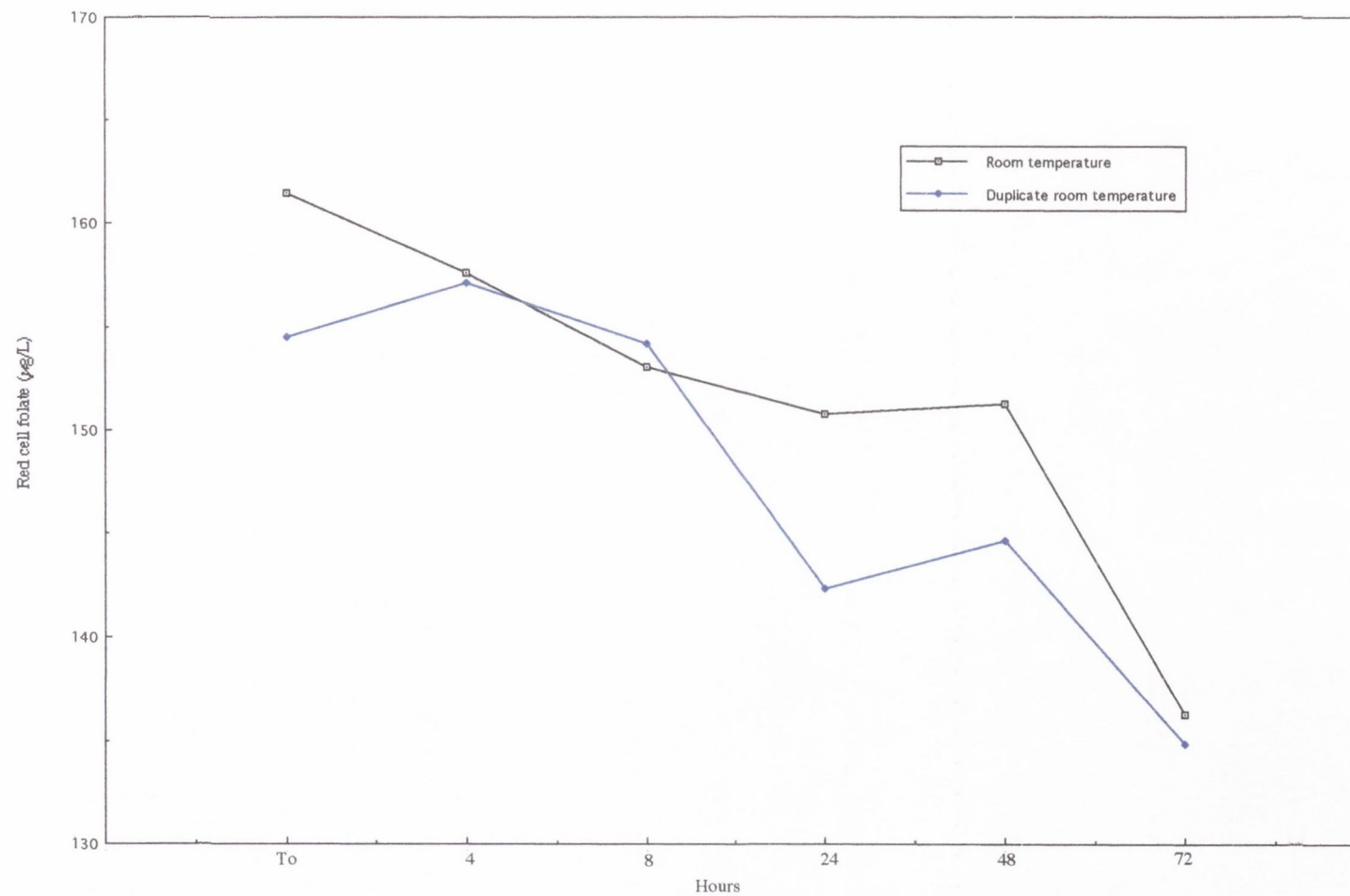
Whole blood red cell folate ($\mu\text{g/L}$) on shaker incubator at room temperature over 72 hours.

Subjects	To	4 hours	8 hours	24 hours	48 hours	72 hours
1	149.400	140.390	145.650	151.610	139.060	110.750
2	162.660	158.100	151.320	144.500	152.730	145.000
3	179.750	165.100	168.380	151.990	147.980	148.950
4	135.740	151.100	107.220	120.900	101.380	73.980
5	226.950	234.750	201.620	198.620	211.690	199.120
6	119.180	152.330	126.130	131.180	110.120	109.440
7	88.882	104.120	96.595	97.548	101.470	93.109
8	97.053	107.240	91.698	103.130	93.363	82.646
9	119.190	116.520	125.960	113.050	123.270	106.560
10	195.330	225.420	220.620	212.850	173.240	177.930
11	133.910	99.660	136.980	160.220	153.570	134.380
12	64.962	55.259	55.260	56.983	50.135	37.448
13	377.540	370.260	361.840	367.250	335.830	265.930
14	141.920	128.320	120.440	116.390	114.820	66.432
15	196.300	183.410	164.030	150.430	173.080	171.080
16	66.886	67.728	65.692	64.311	60.071	55.600
17	131.270	99.253	113.460	116.400	104.540	89.388
18	204.630	205.470	177.520	200.840	166.240	142.770
19	133.910	118.050	140.120	134.620	134.500	120.420
20	99.870	110.330	109.540	106.540	92.739	82.467
21	108.460	92.365	92.030	84.111	75.666	65.922
22	164.270	155.680	151.070	136.520	124.630	150.600
23	193.210	175.080	174.390	176.090	142.890	113.210
mean	151.838	148.606	143.505	143.482	134.261	119.527

Table 8.8. Study 3. Red cell folate stability study.

Duplicate red cell folate ($\mu\text{g/L}$) on shaker incubator at room temperature over 72 hours.

Subjects	To	4 hours	8 hours	24 hours	48 hours	72 hours
1	149.890	165.620	171.240	112.360	128.290	102.030
2	147.790	170.470	169.400	151.340	152.530	130.350
3	199.420	200.850	188.290	157.410	130.440	127.930
4	141.830	116.870	142.750	131.540	99.786	69.517
5	265.470	258.510	241.170	201.840	198.240	177.710
6	148.620	148.840	137.690	119.650	124.410	102.440
7	112.710	105.400	99.478	105.710	95.856	75.409
8	106.490	121.950	124.100	103.590	90.483	91.287
9	116.390	130.900	121.610	121.890	121.010	108.670
10	232.000	251.830	213.410	189.590	187.250	175.590
11	141.700	151.330	131.710	163.690	141.310	135.030
12	61.730	55.090	55.850	55.235	49.464	36.249
13	357.420	377.390	354.290	302.730	314.970	259.610
14	129.760	128.220	121.330	116.710	97.013	79.598
15	196.360	186.190	161.610	163.600	188.320	171.080
16	64.922	63.147	64.708	62.452	64.451	44.044
17	116.080	105.020	107.880	95.047	90.524	91.791
18	215.040	215.040	187.610	182.000	169.120	132.270
19	133.730	126.050	157.380	153.100	122.780	116.790
20	102.750	110.520	99.030	104.630	89.089	71.438
21	94.101	90.392	101.740	77.558	68.717	61.315
22	164.270	144.750	145.230	135.310	153.230	139.280
23	178.350	164.250	167.570	166.020	134.690	117.100
mean	155.558	156.114	150.786	138.131	131.173	114.023



Room temperature over 72 hours $p = 0.8878$, duplicate $p = 0.8250$

Figure 8.6. Pilot Study 3. Whole blood red cell folate (in duplicate) at room temperature over 72 hours

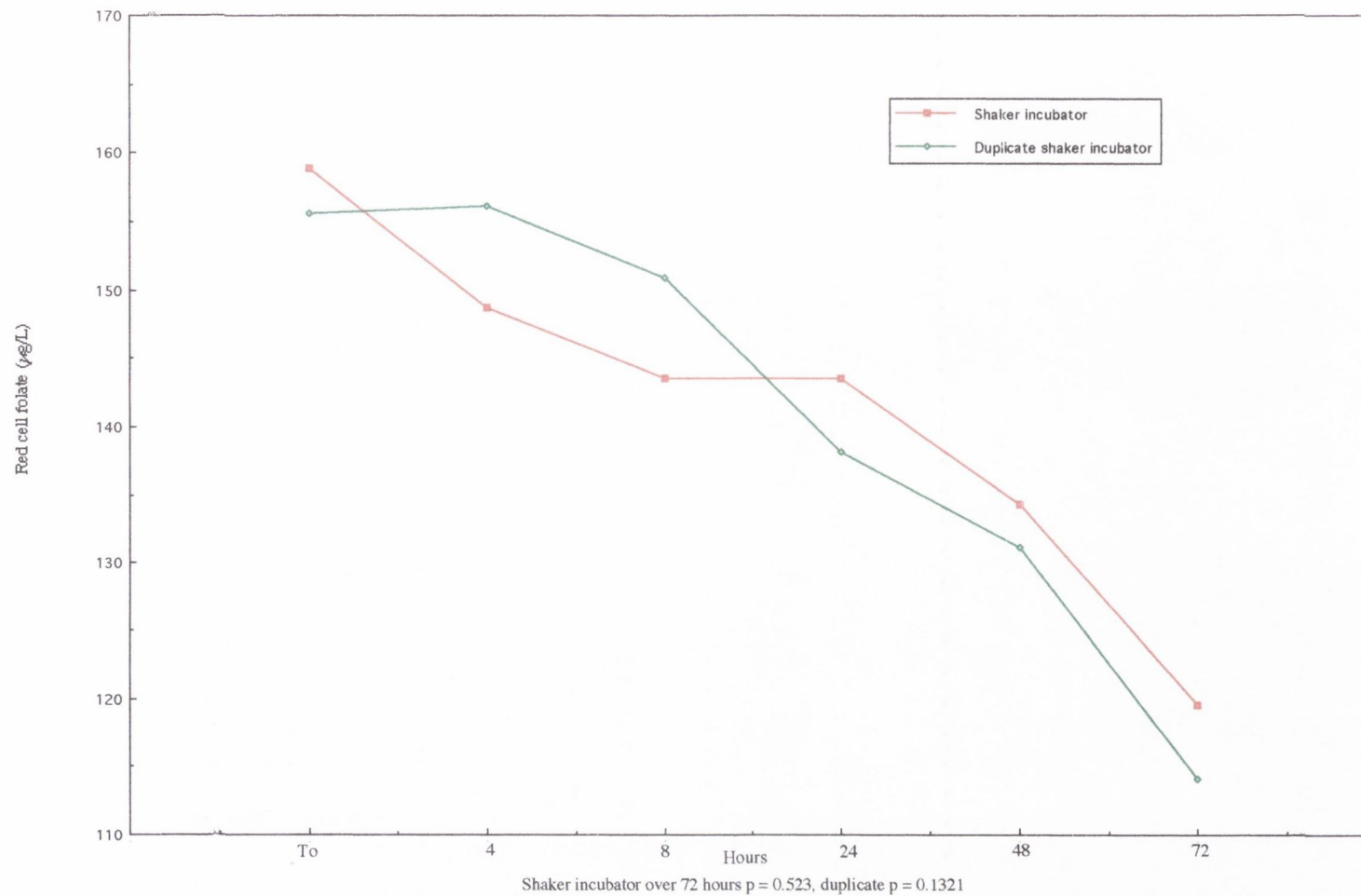


Figure 8.7. Pilot Study 3. Whole blood red cell folate (in duplicate) at room temperature on shaker incubator over 72 hours

8.4 Discussion

Statistical analysis of the results from the first study (Pilot study 1) in this chapter investigating the effects of time on the stability of red cell folate stored as whole blood at room temperature show that red cell folate levels of the group are stable for up to 7 days. However examination of individual results shows that under these conditions losses had occurred by day 3 indicating that the sample size may have been too small to show statistical differences. Similarly statistical analysis of the whole blood red cell folates in constant motion on a shaker incubator at room temperature showed deterioration only after 7 days. Again examination of the individual results showed that losses had occurred before this period. The levels may have deteriorated before this but the sample size was too small to show statistical significance. Cold room storage however appeared to be the optimum storage condition as statistical differences were not demonstrated between the reference samples and lysate samples prepared on day 11. Analysis of the individual results illustrated similar findings.

The second study (Pilot study 2) in this chapter investigated the stability of red cell folate lysates prepared immediately after collection from whole blood under conditions of storage at room temperature, storage in a cool room and storage on shaker incubator at room temperature. Statistical analysis showed that lysates stored at room temperature were stable up to day 6, however, after this deterioration occurred. Analysis of the individual results revealed similar findings.

Lysate samples stored in constant motion on the shaker incubator degraded very quickly and statistical differences were demonstrated after only 2 days. Analysis of the individual results clearly illustrates this also. Lysate samples stored in the cold room did not degrade over the period tested indicating that this is the optimum storage condition for red cell lysates.

Statistical analysis of our larger third pilot study in this chapter illustrated that whole blood red cell folates did not degrade up to 72 hours either at room temperature on a laboratory bench or in constant motion on the shaker incubator. Closer examination of our data however suggests that losses of approximately 20% did occur in the samples stored on the shaker incubator at room temperature. However we failed to show this statistically. A possible explanation for this may lie in the fact that there was marked variability between individual responses to the conditions tested. This made statistical analysis very difficult to interpret. Perhaps certain individuals exhibited greater oxidation activity due to smoking status, vitamin C status or drug use. Perhaps these results can help explain why poor agreement has been reported between different centres involved in red cell folate analysis.

In conclusion the results illustrate that storage of whole blood at room temperature will not result in losses in red cell folates up to 72 hours. Hence it appears that it is acceptable for samples to remain at room temperature provided they are not constantly moved around. In contrast it appears that conditions of constant motion such as by rail or bus would result in losses of up to 20% in red cell folates. Hence it appears that this would be an unsuitable

storage condition. It is worth bearing in mind that these studies were only pilot studies and that larger numbers are necessary to carry out a more thorough investigation.

Chapter Nine

General Discussion

First time occurrence of NTDs accounts for 95% of all NTD affected pregnancies. Therefore, it is in this group that a reduction in incidence would have the greatest impact on the total number of NTD affected children born each year. The MRC Vitamin Study Research Group (1991) showed conclusively that peri-conceptional supplementation with folic acid would prevent the recurrence of 72% of NTDs. A subsequent study (Ceizel & Dudas, 1992) showed that peri-conceptional supplementation also prevented the first-time occurrence of NTDs. Although the mechanism by which folic acid confers this protective effect is currently unknown this was a major public health advancement. Current research would indicate that a metabolic block rather than a nutritional deficiency is implicated in folate related NTDs since it has been shown that most pregnant women carrying an affected foetus have folate concentrations within what has been traditionally defined as the normal reference ranges (Molloy *et al* 1985; Kirke *et al* 1993; Daly *et al*, 1995).

Improving the folate status of the high-risk population is a formidable task as research by Cuskelly *et al* (1996) showed that the natural form of the vitamin is relatively ineffective in optimising folate status. Hence the synthetic form of the vitamin must be consumed either in supplemental form or in fortified food. In response to the MRC findings, several public health agencies internationally including the UK DoH and the CDC, USA recommended that all women capable of conceiving should consume a 400µg supplement daily. This presents a number of problems. Firstly compliance with this recommendation has been shown to be very poor (Clark & Fisk, 1994; Mc Donnell 1999) as approximately 50% of pregnancies are unplanned. Therefore a woman who is not planning a pregnancy is very unlikely to consume a vitamin pill every day for her entire childbearing years. Bearing in mind that the neural tube is formed in the first 21-28 days post conception a pregnancy may

only begin to be suspected when it is already too late for folic acid supplements to be protective. In addition forgetfulness, lack of awareness and perceived low risk are thought to contribute to poor compliance rates. Therefore promoting the peri-conceptional use of folic acid supplements is likely to be an ineffective public health measure.

A universal fortification strategy of a widely consumed food staple seemed the likeliest option, as it would target almost all of the general population supplying folic acid in a continuous and passive manner. In addition it would be relatively simple and inexpensive to implement. However choosing an appropriate food in itself proved problematic as no single food is exclusive to women of childbearing age. After much consideration flour was chosen, as it is a very widely consumed food staple, which is consumed by greater than 95% of the American population. In addition, known population intakes of flour products in the target group and in the general population allowed models to be produced by the FDA of daily folic acid intakes at the levels of fortification proposed. The FDA estimated that a fortification level of $140\mu\text{g}/100\text{g}$ flour would not result in intakes in excess of 1 mg by any population group even after allowing for daily intakes of a $400\mu\text{g}$ supplement. One mg is the current safe upper limit in the USA for total daily folate intakes, as adverse effects have not been reported to occur at intakes less than this. Therefore in January 1998 the US Government implemented mandatory folic acid fortification of all flour milled at a level of $140\mu\text{g}/100\text{g}$. This level of fortification is estimated to supply an additional $100\mu\text{g}$ to the average US daily folate intake. This is four times less than the current target figure. However some improvement in population folate status will certainly occur and many NTD affected pregnancies will be prevented as a result of this fortification strategy. Such a strategy is most likely to have the greatest impact in those with poor folate status. In

addition, it is documented that the protective effect of folic acid in preventing NTDs is not linear (Daly *et al* 1995) and that even moderate improvements in a poor folate status reduce the risk of NTD pregnancies by 7 per 1000 to 2 per 1000. Therefore this level of fortification has been maintained. In the meantime the consumption of additional ready-to-eat commercially available folic acid fortified foods and supplements is actively encouraged by the FDA. Furthermore as mentioned previously (in section 1.24.) it is believed that many food companies are already adding more folic acid to their products than the amount specified by the FDA. Hence consumers may already be getting extra folic acid.

In addition to reducing the occurrence and recurrence of neural tube defects a universal folic acid fortification strategy could benefit other sectors of the population. Folic acid deficiency has been implicated in coronary heart disease and cerebral vascular disease (Boushey *et al*, 1995), Alzheimers disease (Clarke *et al*, 1998) and certain cancers particularly colorectal (Giovannucci *et al*, 1998). The present fortification strategy in the USA will show if significant reductions in the incidence of these diseases are demonstrated over time. This will provide definitive evidence of the protective role of folic acid in these conditions.

The CDC supported the fortification strategy as a means to reduce the incidence of NTDs but advocated, in addition, that the level of fortification be increased up to 350 μ g and even 700 μ g/100g which, they have estimated will supply an additional 200 μ g and 400 μ g respectively to the average US daily folate intake. The highest level being advocated is 4 times greater than the current level of fortification. This raises a very important public

health issue, as fortification at this level would almost certainly over-expose individuals who already consume large amounts of the vitamin in an attempt to target low consumers but may even over-expose currently low consumers of the vitamin. Research to date suggests that the reduction of folic acid during its absorption in the intestinal mucosa is dose-dependent and oral folic acid above certain threshold doses has been shown to saturate the normal absorptive mechanisms. This resulted in unmetabolised folic acid appearance in serum as well as the normal metabolite 5-methyl-THF. A threshold dose of 266µg has previously been demonstrated (Kelly *et al* 1997). Unmetabolised folic acid in serum has the potential to mask the early haematological manifestations of pernicious anaemia. This has been demonstrated experimentally (Tisman *et al* 1973; Metz *et al* 1986; Zittoun *et al* 1978; Ganeshaguru & Hoffbrand 1978) and clinically (Heinle *et al* 1947; Bethal *et al* 1948; Ross *et al* 1948; Vilter *et al* 1950; Israels & Wilkinson 1948). End stage pernicious anaemia can result in an irreversible often, fatal neuropathy. However early diagnosis and treatment would completely reverse the deficiency and treat the neuropathy.

This is of particular concern in an elderly population as the incidence of pernicious anaemia in the over 70 age group has been reported to be approximately 1:200 (Herbert 1985). Pernicious anaemia leads to malabsorption of vitamin B₁₂. In most cases of pernicious anaemia auto-antibodies are produced against the parietal cells causing them to atrophy. The cells then lose their ability to produce intrinsic factor and also to secrete Hydrochloric acid (HCL). Pernicious anaemia thereby causes malabsorption of dietary cobalamin and also results in an inability to reabsorb the cobalamin that is secreted daily in the bile. Interruption of this enterohepatic-circulation of cobalamin causes the body to go into a

significant negative balance of the vitamin (Chanarin 1979). Once a negative balance is established anaemia presents. If the anaemia is not quickly corrected with cobalamin replacement a subsequent neuropathy often termed sub acute degeneration of the spinal cord, progresses quickly. The nerve damage is untreatable, irreversible and fatal once it reaches a certain point.

A more common problem (estimated to be present in one-quarter of the elderly population, Carmel, 1995) implicated in cobalamin deficiency is that of achlorhydria which is often associated with *Helicobacter pylori* infection (Baik & Russell, 1999). This results in a progressive reduction with age in the ability of the parietal cells to secrete HCL. This prevents the release of food-bound cobalamin but does not interfere with the absorption of free cobalamin such as in fortified foods or supplements. Furthermore the reabsorption of biliary cobalamin is not interfered with and therefore the negative balance seen in pernicious anaemia does not occur. However a long-term continuous reduction in the amount of cobalamin absorbed from dietary sources will result in overt deficiency. Therefore a mass fortification strategy while conferring benefits to one sector of population could potentially harm an even larger sector of the population.

A number of other potentially adverse effects of a folic acid fortification strategy also warrant consideration. Anti-folates are among the most effective anti-cancer drugs. One such drug methotrexate limits folate availability to the tumour cells to prevent proliferation. Hence the question arises could too much folic acid in the circulation over a lifetime actually promote malignant tumour growth? Early studies using animal and human models

gives rise to concern. Farber *et al* (1948) described acceleration of the leukaemic processes by folic acid supplementation in children with acute leukaemia. Heinle & Welch (1948) reported that administration of folic acid to three patients with myeloid leukaemia was followed by rapid haematologic and clinical relapse in each case. In one of these patients haematologic improvement accompanied the withdrawal of folic acid on two occasions. Studies in animal models subsequently showed that dietary restriction of folic acid inhibited the growth of tumours (Rosen & Nichol, 1962). The tumour resumed rapid growth when folate was added to the diet. Potter & Briggs (1962) also found that dietary folate deficiency in mice retarded the growth of ascites tumour cells of lymphocytic neoplasms. These early studies involving human subjects have not since been corroborated but nonetheless give rise to concern about the risk of exposing the general population to large doses of folic acid on an ongoing basis.

Another consideration includes the use of these anti-folate drugs by a subset of the population to control a variety of medical conditions including, epilepsy, crohns disease, rheumatoid arthritis and inflammatory skin disorders. A fortification strategy which supplies large doses of folic acid could on the face of it be counter-intuitive to anti-folate action and thereby inadvertently affect drug efficacy in these individuals.

Folic acid is thought to interfere with the absorption of zinc. Zinc is present in all tissues of the body and is directly or indirectly involved in the major pathways contributing to the metabolism of protein, carbohydrate, energy, nucleic acids and lipids (DRV's 1991).

Safety considerations regarding the masking of pernicious anaemia by large doses of folic acid in the circulation however are the main reasons why the FDA has not yet made the decision to increase the current level of fortification. In the absence of evidence to suggest otherwise the FDA have set a safe upper daily limit for folic acid intakes at 1mg/day and they have stated that intakes above this must be medically supervised. From mathematic modelling systems it is clear however that implementing the highest level of fortification (700µg/100g) would result in intakes in the general population well in excess of the 1mg safe upper limit (Federal Register, 1996).

At present the only evidence showing threshold doses likely to cause unmetabolised folic acid appearance in serum are those demonstrated in the studies carried out by Kelly *et al* (1997). These findings provided the first evidence that physiological doses of folic acid may have potential for harm. However the studies were very acute and folic acid was merely applied onto the surface of foodstuffs. Hence a matrix effect of the flour was not taken into consideration. Therefore the results cannot be extrapolated to provide information on the effects of chronic consumption of fortified foods on unmetabolised folic acid in serum.

This study undertook the task of determining the level of folic acid fortified flour consumed on a chronic basis by a general population group that would lead to unmetabolised folic acid appearance post prandially. This objective was satisfactorily achieved. The effects of the level of flour fortification currently in practice in the USA (140µg/100g) and the two

higher levels under consideration (350µg and 700µg/100g) on unmetabolised folic acid in serum were investigated.

Healthy adult male and female human subjects were recruited from the general population. In this intervention subjects were pre-saturated with 400µg folic acid supplements daily for 14 weeks to allow total serum folate levels to plateau (previously demonstrated by Ward *et al*, 1997). Folic acid fortified bread was prepared and baked under industrial conditions using fortified flour. Three different batches were prepared. The highest level was fortified at 700µg/100g of flour. The next highest level was fortified at 350µg/100g of flour and the lowest level was fortified at 140µg/100g of flour. Serum samples were collected at week 0, week 10, week 14 and pre and post prandially at each level of fortification.

A series of acute trials were also carried out. These trials were set up to investigate the validity of the FDA's upper safe limit for daily folic acid intakes. The effects of consuming a total of 1mg of folic acid in fortified foods in 5 varying combinations of doses on unmetabolised folic acid were investigated. Serum samples were collected pre and post-prandially. In these trials subjects were not pre-saturated.

In addition serum was collected from premature and full term new-born infants immediately after birth and four days later during the consumption of formula feeds containing folic acid to investigate the appearance of unmetabolised folic acid.

Throughout the course of this investigation it became apparent that the existing HPLC/microbiological methodology described by Kelly *et al* (1996) to measure folic acid

in serum was not sensitive enough to detect levels of folic acid in serum below 1ng/ml. Hence the methodology was improved to include an additional separation step using solid phase extraction which concentrated the sample sufficiently so that the amount of folic acid present was detectable by the microbiological assay. This step improved the lower limit of detection of the assay described by Kelly *et al* (1996) by six-fold. Using this new methodology these intervention trials established new threshold levels of oral folic acid in fortified foods above which unmetabolised folic acid appears in serum. These levels would not have been detectable by the Kelly *et al* (1996) methodology.

Unmetabolised folic acid was detected in serum of subjects after the consumption of 400µg folic acid supplements daily for 14 weeks. These samples were collected six hours after the vitamin supplement had last been consumed. This illustrates that it takes at least six hours for folic acid to clear from the serum after supplementation at this level for this duration of time. This implies that the current DOH recommendation that childbearing women should consume a 400µg supplement daily for their entire childbearing years holds potential for harm as this supplementation regime will expose these individuals to unmetabolised folic acid in serum for at least six out of twenty-four hours per day. Therefore if this recommendation is retained in addition to the current US fortification programme then the likelihood of chronic exposure to unmetabolised folic acid in serum is greatly exacerbated.

Other results, which emerged from this study, warrant pause for thought particularly the cumulative effect of small doses of oral folic acid in fortified bread consumed repeatedly. Two slices of fortified bread (consumed 4 hours apart) containing 200µg folic acid each

were consumed for a period of six days. Unmetabolised folic acid was present on day seven even though twenty hours had lapsed between consumption of the last slice of bread on day six and blood collection on day seven. Furthermore the levels of folic acid detected 20 hours after consuming the fortified bread (total of 400µg daily in two doses) for only six days were higher than those detected 6 hours after consuming folic acid supplements for 14 weeks (400µg daily in a single dose). These results would indicate that renal clearance is more effective at getting rid of larger doses of unmetabolised folic acid in the serum than small doses delivered frequently.

In terms of cut off points above which unmetabolised folic acid appear in serum our acute and chronic studies demonstrated that 200µg consumed in 1 slice of bread lead to unmetabolised folic acid in serum post-prandially. In the chronic study where subjects were pre-saturated a second slice containing 200µg consumed four hours later not only lead to folic acid appearance but actually demonstrated a cumulative elevation. Also cause for concern, in the acute studies folic acid in serum accumulated after every subsequent slice containing 200µg.

In addition our acute studies demonstrated that slices of bread fortified at a level of 100µg consumed within 1 hour of each other lead to the acute appearance of unmetabolised folic acid in serum, which accumulated as subsequent slices were consumed. While these results were not statistically significant a trend was demonstrated. This occurred in the absence of pre-saturation. It is reasonable to extrapolate from this that chronic consumption of these levels of fortified foods would exacerbate this. These results show that even doses as low as 100µg have the potential to introduce synthetic folic acid into the circulation but this

may even occur at lower doses. This previously unreported cumulative effect of folic acid in serum after the consumption of small amount of oral folic acid at regular intervals introduces further complexity and considerations for those planning a fortification strategy. From a positive viewpoint our chronic data shows that even after chronic pre-saturation unmetabolised folic acid does not appear in serum when two slices of bread fortified at 100µg are consumed four hours apart. These results indicate that if the time of $t_{\max}$ is exceeded between slices then an accumulative effect is not seen. This was also demonstrated in the acute studies at doses as high as 500µg because repeat doses administered four hours apart did not lead to an accumulation of unmetabolised folic acid after the 2nd slice. The acute studies investigated many possible permutations of folic acid consumption patterns totalling 1mg. In reality it is unlikely that an individual would actually consume 10 meals at hourly intervals. A more likely scenario is that meals are eaten 3 times daily at 3-4 hourly intervals. However, reliable information regarding meal consumption patterns in Ireland is not available at this time. The Irish Nutrition and Dietetics Institute (INDI) are currently analysing data, which looked at food consumption patterns. This will be available in 2001.

As regards the consumption of folic acid by infants, we demonstrated folic acid in cord blood of premature and full term new-born infants. This was demonstrated in a group of infants whose mothers were not taking folic acid supplements. Therefore the levels of unmetabolised folic acid in serum were generated by the consumption of commercially available folic acid fortified foods currently available in Ireland. This gives rise to concern, as it was previously believed that unmetabolised folic acid would not pass the placental barrier over to the foetus's circulation. The presence of unmetabolised folic acid in cord

blood may hold potential for harm in terms of adverse effects in the developing foetus particularly if large doses are able to pass over the placenta barrier to the foetus. . However little is known about this maternal-foetal risk at present. Adverse effects of large doses of folic acid have previously been demonstrated in a rat model (Achon *et al*, 1999, 2000). The authors showed that long-term high levels of folic acid supplementation resulted in lower birth weights and shorter vertex-coccyx length in rats. These previously unreported results indicate that careful consideration should be given to this effect on the foetal circulation before a final fortification strategy is agreed on.

In addition folic acid fortified formula feeds gave rise to elevated levels of unmetabolised folic acid in the serum of these infants post-prandially after only 4 days. Unfortunately it was not possible to collect subsequent blood samples from these infants. However from our results demonstrated previously in the adult group consuming fortified foods on a chronic basis it is likely that a cumulative effect of unmetabolised folic acid could occur in this infant group also. Hence exposure of the general public to folic acid in serum as a result of fortification could be for an entire lifetime.

Finally we investigated the stability of red cell folates after collection under different handling conditions. Data from our red cell folate stability studies demonstrated that red cell folates stored as whole blood do not degrade before 72 hours on a laboratory bench at room temperature. Whole blood red cell folate samples in constant motion at room temperature appeared to degrade by approximately 20%. Results from the latter revealed marked differences between individual responses making statistical analysis very difficult

to interpret. Further studies may be necessary using larger numbers to investigate this matter further.

In conclusion it appears that Public Health Agencies should review the recommendation that women of childbearing age should consume a 400 μ g supplement daily on an ongoing basis. Assuming that the problem of overage can be addressed by legislation it appears that chronic consumption of the current level of folic acid fortification of flour (140 μ g/100g) supplying an extra 100 μ g to the average US daily folate intake will not cause unmetabolised folic acid appearance in serum post-prandially. The incremental level under consideration (350 μ g/100g) supplying an extra 200 μ g to the average US daily folate intake is also unlikely to lead to the appearance of unmetabolised folic acid in serum. This strategy should be planned such that consumption of fortified foods must be spread out such that a maximum of 100 μ g is consumed at one sitting and that at least four hours lapses between doses.

The highest level of fortification under consideration by the FDA (700 μ g/100g) supplying on average an extra 400 μ g to the US daily folate intake will most certainly cause unmetabolised folic acid to appear in serum post prandially. Unless doses are split into 100 μ g and spaced out such that four hours is allowed between each dose it appears that this regime will not be safe in terms of unmetabolised folic acid appearance. This would a very difficult strategy to plan because of the wide range of foods which will be targeted by flour fortification e.g. Breads, cereals, cakes, pasta, biscuits, pastries. We have shown that at average intakes at of this level unmetabolised folic acid will present in serum. This is not to speak of individuals who consume more than average or indeed individuals at the 95th

percentile. This level of fortification is therefore unlikely to be safe in terms of unmetabolised folic acid appearance. Finally in light of our findings the current FDA 1mg upper safe limit should be revised downwards.

Suggested future work

Folic acid fortification of flour was implemented in January 1998 at a level of 140µg/100g. This thesis was commenced in 1996 two years previous to this. Therefore the only way in which to test the effects of this level and the two higher levels under consideration was to set up an intervention trial exposing healthy individuals to these levels on an-ongoing basis. Our findings are only applicable to individuals at or below the 50th percentile as we used the FDA modelling system based on average intakes. The results can be extrapolated to apply to individuals who consume less than average intakes of folic acid but may not be representative of individuals who consume greater than this. Our positive results show that the highest level of fortification tested in this study (700µg/100g) can be extrapolated to conclude that intakes greater than average i.e. at the 95th percentile will also cause unmetabolised folic acid appearance but our negative findings may not mirror what would happen at the 95th percentile. While we felt that this study was representative of a real-life scenario an improved means by which they can be tested now exists. Jacques *et al* (1999) measured plasma homocysteine and folate concentrations in a population group in the USA pre and post fortification. These samples should also be analysed for unmetabolised folic acid. This will provide an absolutely fool-proof means of determining whether folic acid levels have increased in any subset of the population at the current level of fortification. This should be across the board and include samples from the elderly, middle aged, teenagers, children, pregnant and lactating women and new-born infants. A definitive answer to the question of unmetabolised folic acid in serum would then be established beyond all doubt. In the mean time the role of the recently commercially available 5-methyl THF should now be urgently investigated. Studies to include bioavailability,

efficacy and stability of this agent such be implemented immediately as this agent if shown to be as functionally effective as folic acid may in the future replace folic acid with no risk of pernicious anaemia masking or other adverse side effects.

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Appendix 1

Intervention Trial 1

Consent Form

Folic acid fortification of flour consumed under normal dietary conditions

This study and this consent form have been explained to me. All questions on my part have been answered satisfactorily. I understand what will happen if I agree to be part of the study. I have read and I understand this consent form. I freely and voluntarily agree to be part of this research study.

Participant's Name:

Participant's Signature:

Date:

Statement of investigator's responsibility: I have explained the nature, purpose and procedures of this research study. I have offered to answer any question and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Researcher's signature:

Date:

Patient Information Leaflet

1. Title of study:

Folic acid fortification of flour consumed under normal dietary conditions.

2. Introduction:

Folate is a B complex vitamin. We cannot make it ourselves and we must ingest it in our food. Alternatively we can take it in its synthetic form - folic acid, in fortified food or in supplements. Recently it has been shown that a high intake of folic acid in the early stages of pregnancy can greatly reduce the prevalence of neural tube defects.

It has also been established that high levels of folic acid may be effective in lowering the risk of Coronary Heart Disease by its action on homocysteine.

The purpose of this study is to look at the response of healthy human subjects to folic acid fortified foodstuffs taken under normal dietary conditions.

3. Procedures:

Healthy adult subjects will be recruited for the study.

The subjects will be involved in the study for a period of 19 weeks. Blood will be taken initially to establish folate status. For the first 14 weeks of the study subjects will be asked to consume a 400 μ g folic acid supplement every morning and to keep a record of this. At week 10 a blood sample will be taken in the afternoon approximately 6 hours after consumption of that morning's supplement. After the 14week period another blood sample will be taken.

Subjects will then proceed to stage 2 of the study. This will be for 7 days. During this time the subjects will be asked to consume 2 slices of specially prepared folic acid fortified bread daily. The subjects will consume 1 slice in the morning and 1 slice at lunchtime. On day 7 of the bread consumption subjects will be asked to come to the laboratory at 9am and bloods will be taken by intravenous cannulation at hourly intervals for 8 hours. Each subject will be asked to keep a daily record of the times and amount of fortified bread eaten during the previous week.

The subjects will then take folic acid supplements again for a period of 7 days.

After this 7 days fortified bread will again be consumed as previously described for 7 days and bloods will be taken as described.

Supplements will then be consumed for a further 7 days. Bread will be consumed as previously described for 7 days and blood will be taken as described.

For the entire duration of the study subjects will be asked to omit all other folic acid fortified foods

Medical history questionnaire

Name:

Age:

Weight:

Height:

Are you on any medications?

Are you using an oral contraceptive pill?

DO you suffer from any gastrointestinal disorders?

Do you take any multivitamin preparations?

Are you on any special diets?

Please not all information will be treated as strictly confidential.

Appendix 2

Intervention Trials 2-6.

Consent Form

Folic acid fortification of flour : Determination of the level that gives rise to unmetabolised folic acid in serum.

This study and this consent form have been explained to me. All questions on my part have been answered satisfactorily. I understand what will happen if I agree to be part of the study. I have read and I understand this consent form. I freely and voluntarily agree to be part of this research study.

Participant's Name:

Participant's Signature:

Date:

Statement of investigator's responsibility: I have explained the nature, purpose and procedures of this research study. I have offered to answer any question and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Researcher's signature:

Date:

Patient Information Leaflet

1. Title of study:

Folic acid fortification of flour: Determination of the level that gives rise to unmetabolised folic acid in serum.

2. Introduction:

Folate is a B complex vitamin. We cannot make it ourselves and we must ingest it in our food. Alternatively we can take it in its synthetic form - folic acid, in fortified food or in supplements. Recently it has been shown that a high intake of folic acid in the early stages of pregnancy can greatly reduce the prevalence of neural tube defects.

It has also been established that high levels of folic acid may be effective in lowering the risk of Coronary Heart Disease by its action on homocysteine.

The purpose of this study is to look at the response of healthy human subjects to folic acid fortified foodstuffs taken under normal dietary conditions.

3. Procedures:

Healthy adult subjects will be recruited for the study. The subjects will be involved in the study for a period of 5 days. Each of these 5 days will be separated by a period of two weeks. Hence the study will take approximately 9 weeks to complete. Blood will be taken initially to establish folate status.

On the first study day subjects will be asked to come to the laboratory at 9am having omitted folic acid fortified foods or supplements for at least 24 hours. An intravenous cannula will be inserted and a pre-prandial sample will be collected. The subjects will then be asked to consume 1 slice of bread containing 1mg of folic acid. Blood samples will be collected hourly for 4 hours from the cannula.

On the second study day subjects will be asked to come to the laboratory at 9am having omitted folic acid fortified foods or supplements for at least 24 hours. An intravenous cannula will be inserted and a pre-prandial sample will be collected. The subjects will then be asked to consume 1 slice of bread containing 500 μ g of folic acid. Blood samples will be collected hourly at 1, 2, 2 $\frac{1}{2}$, 3 and 4 hours post-prandially. A second slice of bread containing 500 μ g of folic acid will then be administered and blood will be collected at 1, 2, 2 $\frac{1}{2}$, and 3 hours post-prandially.

On the third study day subjects will be asked to come to the laboratory at 9am having omitted folic acid fortified foods or supplements for at least 24 hours. An intravenous cannula will be inserted and a pre-prandial sample will be collected. The subjects will then be asked to consume 1 slice of bread containing 333 μ g of folic acid. Blood samples will be collected at 1, 2 and 2 $\frac{1}{2}$ hours post-prandially. This will be repeated three over the remainder of the day.

On the fourth study day subjects will be asked to come to the laboratory at 9am having omitted folic acid fortified foods or supplements for at least 24 hours. An intravenous cannula will be inserted and a pre-prandial sample will be collected. The subjects will then be asked to consume 1 slice of bread containing 200 μ g of folic acid. Blood samples will be collected 45 and 90 minutes post-prandially. This will be repeated 5 over the remainder of the day.

On the fifth study day subjects will be asked to come to the laboratory at 9am having omitted folic acid fortified foods or supplements for at least 24 hours. An intravenous cannula will be inserted and a pre-prandial sample will be collected. The subjects will then be asked to consume 1 slice of bread containing 100 μ g of folic acid. Blood samples 1 hour later. This will be repeated 10 over the remainder of the day.

For the entire duration of the study subjects will be asked to omit all other folic acid fortified foods

4. Benefits:

There will be no direct benefit to the individual subjects in the study. However the study will help determine safe levels of fortification which may be used in future fortification programmes. In this way the study will be of benefit to the population at large.

5. Risks:

There are no risks to the subjects in this study.

6. Exclusion from participation:

Subjects must be folate replete at the beginning of the study. Exclusion criteria would exclude those taking multi vitamins or anti convulsant medications, contraceptive pill users, persons on special diets or those with a medical condition known to affect folate absorption or metabolism eg - crohns disease or ulcerative collitis.

7. Confidentiality:

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the hospital.

8. Voluntary Participation:

You have volunteered to participate in the study. You may quit at any time.

9. Permission:

The study has received ethical approval from the St. James's Hospital Ethical Committee .

10. Further information:

You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Mary Rose Sweeney who can be telephoned at 4964310 home or work 6082113.

Medical history questionnaire

Name:

Age:

Weight:

Height:

Are you on any medications?

Are you using an oral contraceptive pill?

DO you suffer from any gastrointestinal disorders?

Do you take any multivitamin preparations?

Are you on any special diets?

Please not all information will be treated as strictly confidential.

Appendix 3

Coombe Study

Consent Form

Folic acid fortified formula feeds: Effect of consumption on unmetabolised folic acid in the serum of premature and full term infants.

This study and this consent form have been explained to me. All questions on my part have been answered satisfactorily. I understand what will happen if I agree to my child taking part in the study. I have read and I understand this consent form. I freely and voluntarily agree for my child to be part of this research study.

Parent's Name:

Parent's Signature:

Date:

Statement of investigator's responsibility: I have explained the nature, purpose and procedures of this research study. I have offered to answer any question and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Researcher's signature:

Date:

Information leaflet for Parent:

Title of study:

Folic acid fortified formula feeds: Effect of consumption on unmetabolised folic acid in the serum of premature and full term infants.

Introduction:

Folate is a B complex vitamin. We cannot make it ourselves and we must ingest it in our food. Alternatively we can take it in its synthetic form - folic acid, in fortified food or in supplements. Recently it has been shown that a high intake of folic acid in the early stages of pregnancy can greatly reduce the prevalence of spina bifida.

The purpose of this study is to look at the response of premature and full term infants to folic acid fortified formula feeds.

3. Procedures:

A Doctor will collect 2 blood samples from your baby. The first sample will be collected before your baby has had his/her first feed. The second sample will be collected when your baby is 4 days old. Each blood sample will be approximately the size of a $\frac{1}{2}$ teaspoon of liquid.

Benefits:

There will be no direct benefit to the individual babies in the study. However the study will help determine safe levels of fortification which may be used in the future. In this way the study will be of benefit to the population at large.

Risks:

There are no risks involved in this study.

Confidentiality:

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the hospital.

Voluntary Participation:

Participation is completely voluntarily.

Permission:

The study has received ethical approval from the St. James's Hospital Ethical Committee.

Further information:

You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Mary Rose Sweeney who can be telephoned at work 6082113/4.

Questionnaire for Doctor collecting samples from infants.

Baby's Name

Date of Birth

Full term/Premature please tick

Date first sample collected

Date second sample collected

Type of feed-brand name

Number of feeds consumed before second sample collected

Amount taken at last feed

Time of last feed prior to second sample