THE EFFECT OF A DIMINISHED FOLATE STATUS ON COLORECTAL CARCINOGENESIS

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by

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ERRATA

Page 15, lines 1 and 2 should read "small intestine. Therefore malabsorption syndromes such as gluten-induced enteropathy (celiac or tropical sprue) will ...."

Page 16, 1.3.1 line 12: insert the sentence "Deficiencies of the vitamins folate, vitamin B12 and vitamin B6 can potentially lead to increased plasma homocysteine levels (Selhub and Miller, 1992)."

Page 22, line 20: replace the word "was" with "were".

Page 25, last line: replace "This data" with "These data".

Page 72, 4.3.3 line 3: the word "fed" should be removed.

Page 77, 4.3.5 line 1: replace "Table 4.5" with "Figure 4.5".

Page 82, line 10 should read "the degree of folate depletion as the amount of folate contained in different protein sources can vary quite substantially."

Page 121, last line: omit the first "by".

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for photocopy or loan.

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ABSTRACT

The aim of the work described in this thesis was to investigate the relationship between folate status and colorectal cancer risk. To do this, the rat AOM intestinal cancer model was used for investigations. The AOM-induced intestinal cancer model in rats is a well established assay for investigation of chemopreventive or chemopromotive compounds on intestinal carcinogenesis and more specifically colorectal carcinogenesis. It has been suggested that a lowered folate status may increase the risk of developing colorectal cancer (Cravo *et al.* 1992). To date, experimental animal evidence examining folate status and carcinogenesis have been inconsistent. However, in terms of colorectal cancer risk only a few studies have examined the relationship to folate status and the results are also inconsistent.

In the first study, different protocols for modulating folate status in male Sprague-Dawley rats were investigated. Three short term feeding experiments were performed, with blood folate concentration, plasma homocysteine levels, haematocrit and body weight growth used as a biomarkers to assess folate status. The basal diet used throughout all studies in this thesis was a semi-purified AIN-93 diet (Reeves *et al.* 1993) that was modified to contain 12% protein and 20% fat. In the short term experiment there was no change in folate status by feeding rats any of the following probiotic bacteria strains: Lactobacillus rhamnosus GG, Lactobacillus casei Shirota, Lactobacillus acidophilus, Bifidobacterium longum or a combination of Lactobacillus acidophilus and Bifidobacterium longum. In the second experiment by omitting folate from the basal diet, folate status was lowered in the rat evidenced by lower blood folate and elevated plasma homocysteine concentrations. The level of folate deficiency created would be described only as moderate, as there were no signs of anaemia or growth retardation. By further including the sulfonamide drug,

succinylsulfathiazole at a level of 1% combined with a folate deficient diet, severe folate deficiency could be achieved. After 4 weeks of inclusion of the sulfonamide with a folate deficient diet significant growth retardation appeared, with severe growth retardation at 8 weeks. No alteration in folate status was observed in rats when only a diet deficient in choline was consumed. Potassium nitrite was fed to rats at a level of 0.5% in the diet which resulted in significant anaemia and growth retardation however, folate status was not altered. In the third short term feeding study, dietary methionine at 0%, 0.3% and 0.6% had no effect on folate status in the rat although a diet deficient in methionine significantly limited the growth of the rats. It was concluded that omission from the test diet of folate was the best protocol for creating experimental folate deficiency. The level of folate deficiency achieved with this protocol is moderate deficiency and can be achieved within 4 weeks of consuming such a diet in young rats. To further enhance the level of experimental folate deficiency the inclusion of a sulfonamide is necessary.

In a second rat study, the protein sources casein and soy protein isolate (SPI) were examined for their impact on folate status and DNA methylation status in rats. Also examined was the effect of feeding a folate deficient diet alone and with choline and methionine deficiencies superimposed. Significant depletion of blood folate concentrations could be achieved after 4 weeks of feeding folate deficient diets only. The level of folate depletion achieved was significantly greater in the casein fed rats consuming methyl deficient diets than the corresponding SPI fed rats. This most likely reflects the higher amount of endogenous folate present within the soy protein isolate (1.28 mg/kg compared with 0.045 mg/kg for casein). Homocysteine concentrations in the plasma were significantly elevated in the casein treatments (1.5 fold elevation) with deficient levels of folate, as well as in the treatments deficient in folate/choline (5 fold elevation) and folate/choline/methionine (7 fold elevation). Interestingly, homocysteine levels were not

increased in any of the SPI treatments. Hypomethylation of hepatic DNA was observed in casein fed rats when choline and methionine deficits were superimposed on a folate deficit. A folate deficit from the diet alone did not alter DNA methylation within the time frame studied. This decrease in methylation was not observed in the corresponding SPI treated rats.

In a third rat study, the effect of moderate and severe folate deficiency on the development of colonic pre-neoplastic aberrant crypt foci (ACF) induced by azoxymethane (AOM) in male Sprague-Dawley rats was studied. Further objectives were to test if DNA methylation was altered in the folate deplete treatments and to examine the quantitative effects of folate deficiency upon lymphocyte sub-populations in the thymus, spleen and mesenteric lymph nodes. There was a significant decrease in the formation of total colon aberrant crypts after 12 weeks of feeding experimental diets within the animals which were displaying severe folate deficiency. A significant decrease in the frequency of foci with single aberrant crypts was observed in all folate deplete treatments when compared to the folate adequate treatment. No significant differences were seen between the treatment groups in frequency of foci containing 2 or ≥3 aberrant crypts. There were no significant differences observed among the folate deplete and folate adequate treatments in the level of DNA methylation within either the liver or the colonic mucosa, despite the marked folate deficiency observed in certain treatment groups. No alteration in natural killer cells was found within the thymus, spleen or mesenteric lymph nodes with moderate or severe folate deficiency. When examining other subsets of lymphocytes the only change was a small increase in the CD8 expression within the spleen of the most severe folate deficient rats. It was concluded from this experiment that the findings of the present study do not support the concept that either a moderate or severe folate depletion will increase the risk of developing colorectal cancer of rats exposed experimentally to the chemical carcinogen AOM, but in so

far as ACF act as meaningful predictors of colorectal cancer, folate deficiency may actually reduce the risk. Also folate depletion does not appear to alter DNA methylation status in the colonic mucosa.

In a fourth study, the effect of a diminished folate status on intestinal cancer formation was examined. After 26 weeks of feeding experimental diets intestinal tumours were appraised. Most of the tumours observed were in the large intestine of all treatment groups. Overall there was a significant decrease ($p<0.01$) in total intestinal tumour incidence associated with the folate deplete treatments. The large intestinal tumour incidence showed a similar pattern to that of the total tumour incidence. A lower incidence of colon was observed in the rats which were folate deficient. However, these differences were not significant. The analyses of the small intestine tumour incidence data indicated that the folate deplete treatments were significantly lower ($p<0.05$) than the folate adequate treatments. Animals fed the folate deficient diets exhibited a significant decrease ($p<0.01$) in the total number of intestinal tumours when compared to animals consuming a folate adequate diet. There was a 32% fall in the number of colon tumours in the folate deplete treatments when compared to the folate adequate treatments, however this observation fell short of significance. Small intestinal tumour numbers significantly decreased ($p<0.05$) in the animals maintained on folate deplete diets. Tumour mass index ($\log_{10}$) data for large intestinal tumours also showed a significant reduction ($p<0.05$) in the animals maintained on folate deplete diets when compared to those maintained on folate adequate diets. Histopathological appraisal of colon tumour type showed the animals fed the folate deplete diets developed significantly less ($p<0.01$) adenocarcinomas than the folate adequate treatment groups. There was a 71% fall in the incidence of malignant tumours in the folate depleted treatment groups. Blood folate and colonic folate levels were significantly depleted in the folate deficient treatment groups when compared with the folate adequate groups.

There were no significant differences observed among the treatment groups in the level of unmethylated CpG within either the liver or the colonic mucosa after 26 weeks of consuming either an adequate folate diet or folate deficient diet. The results of the present study demonstrate that folate deficiency prior and during post tumour induction reduces the risk of intestinal cancer in AOM-treated rats. It is likely that the lower tissue folate levels present in the folate deficient animals may have had an inhibiting effect on the promotion/progression events of tumours.

Folate status can play an important role in modulating colorectal carcinogenesis. The results from the studies performed in this thesis show that folate deficiency appears to reduce the risk of developing colorectal cancer in rats, whereby folate deficiency most likely exerts its effect during the middle to later stages of tumourigenesis. DNA methylation status was shown not be altered in the rat with folate deficiency. An association of folate supplementation with enhanced cancer induction has been suggested previously (Herbert, 1985) and may be an effect of a demand of folate for tumour growth. Some anticancer drugs such as methotrexate, act by inhibiting enzymes that convert folate to the active form (Kamen, 1997), thereby blocking proliferation of rapidly dividing cells including or especially cancer cells. Therefore folate deficiency may well be reducing tumourigenesis by inhibiting cell proliferation.

PUBLICATIONS ARISING FROM THIS THESIS

Le Leu, RK., Young, GP., & McIntosh, G.H. (2000) Folate deficiency diminishes the occurrence of ACF in the rat colon but does not alter global DNA methylation status. *J. Gastroenterol. Hepatol.* (In press)

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Le Leu, RK., Young, GP., & McIntosh, GH. (1999). Effect of folate deficiency on formation of azoxymethane-induced aberrant crypt foci in the rat colon. *Gastroenterol*ogy **116** Suppl., A449. **Awarded DDW poster of distinction.**

Le Leu, RK., McIntosh, GH., & Young, GP. (1998). Ability of endogenous folate from soy protein isolate to maintain plasma homocysteine and hepatic DNA methylation during methyl group depletion in rats. *J. Nutr. Sci. Vitaminol.* **44**, 457-464.

Le Leu, RK.,Young, GP., & McIntosh, GH. (1998). Effect of folate deficiency on formation of azoxymethane-induced aberrant crypt foci in the rat colon. *Proc. Nutr. Soc. Aust.* **22**: pp 275.

Le Leu, RK., McIntosh, GH., & Young, GP. (1997). Dietary methyl depletion and folate status in male rats fed casein or soy diets *Proc. Nutr. Soc. Aust.* **21**: pp 73.

LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
AIN	American Institute of Nutrition
ala	alanine
AOM	azoxymethane
BW	body weight
CI	confidence intervals
C	cytosine
CpG	cytosine-phospho-guanine
CRC	colorectal cancer
°C	degrees, Celsius
CV	coefficient of variation
DMH	dimethylhydrazine
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetra-acetic acid
FD	folate deplete
h	hours
g	grams
HPLC	high performance liquid chromatography
mg	milligrams
mm	millimetres
min	minutes
KNO_2	potassium nitrite
NaCl	sodium chloride

kg	kilograms
LI	large intestine
MTHF	methyltetrahydrofolate
μg	micrograms
μl	microlitres
ηg	nanograms
OR	odds ratio
PBS	phosphate buffered saline
RBC	red blood cell
rpm	revolutions per minute
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SI	small intestine
SPI	soy protein isolate
THF	tetrahydrofolate
T	thyminine
val	valanine
λ	wavelength
WBC	white blood cell

Chapter 1

Introduction

1.1 Colorectal cancer

The incidence of colorectal cancer in western industrialized countries is high and is the second most common cause of cancer related deaths (after lung cancer) (Landis *et al.* 1998; Cassidy *et al.* 1994). Within Australia, there are over 9500 new cases and approximately 4500 deaths annually (Australian Health Technology Advisory Committee, 1997). Colorectal cancer occurs with approximately equal frequency in men and women (McMichael and Potter, 1980) and the incidence increases in the general population with age. Colorectal carcinogenesis is a complex multistep process involving both genetic and environmental factors. Cancer is thought to result from an accumulation of multiple genetic changes resulting in a transformed phenotype and eventual progression of cells to cancer (Vogelstein *et al.* 1988). Therefore agents capable of causing DNA structural damage may be potentially carcinogenic. A model for colorectal development is depicted in figure 1.1. There are two main processes by which a cell becomes an invasive cancer cell (carcinogenesis), namely initiation and promotion. It is often quite difficult due to the multi-step process of carcinogenesis to separate initiation and promotion. Initiation occurs as a result of DNA damage and this may lead to mutations that are more likely to proceed along the multistep pathway of carcinogenesis. The process of initiation is essential for the formation of cancer, as carcinogenesis does not proceed without damage to DNA and mutations occurring. A number of different initiating agents have been identified to cause colon cancer, although many are unknown. Some of the agents known include chemical mutagens, dietary contaminants, irradiation, pathogenic bacteria and viruses. Not all mutations necessarily lead to the development of cancer. Often other factors are needed to create an environment for the mutated cell to further multiply. These factors are known as promotive and unlike initiating factors do not directly induce changes in DNA (Kleibeuker

et al. 1996). Factors influencing promotion may also be involved in the initiatory process. During promotion there is a marked increase in the proliferation rate of the epithelial cells (Deschner, 1982; Terpstra *et al.* 1987). As a result of the increase in proliferation DNA replication is also increased. This may also enhance the risk of mutations occurring and if cells are already mutated the development into neoplastic lesions may also be enhanced. Agents which come in contact with the epithelial cell surface or reach the cells via the blood stream may directly stimulate proliferation. A variety of agents have been proposed to alter the promotion process of colon carcinogenesis and these include dietary fat, bile acids, diacylglycerol, and prostaglandins (PGE_2). Other agents such as calcium, dietary non starch polysaccharides, certain vitamins and antioxidants may reduce the promotion process of colon carcinogenesis.

There are two main inherited predisposition syndromes identified within the genetic component of colorectal cancer: familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) (Cannon-Albright *et al.* 1988). FAP is a rare autosomal syndrome which occurs as a result of an inherited mutation in the tumour suppressor gene (apc gene), and accounts for approximately 1% of all cases of colorectal cancer (Foulkes, 1995). HNPCC is an inherited autosomal dominant syndrome, and may account for approximately 2% of patients with colorectal cancer (Evans *et al.* 1997, Aaltonen *et al.* 1994).

Environmental and dietary factors are considered to be responsible for 85-90% of all cases of colorectal cancer known as sporadic colorectal cancers. Incidence rates have been shown to vary by as much as 20-fold around the world, with much higher incidences seen in developed countries, thereby suggesting that much of this variation can be attributed to environmental factors and more specifically, dietary factors (Armstrong and Doll, 1975; Potter, 1996). Evidence from epidemiological and laboratory animal studies suggest that

diets high in meat and fat increase the risk of colorectal cancer (Armstrong and Doll, 1975; Potter, 1996; MacLennan, 1997), whereas the intake of fibre rich foods such as cereals, fruits and vegetables may be protective (Howe *et al.* 1992, Giovannucci and Willett, 1994). This protective effect may not just be attributable to fibre alone, but may be due to selected micronutrients such as carotenoids, genistein, vitamins C and D and E and folate (Tseng *et al.* 1996).

1.1.1 Animal model for colorectal cancer

The study of the mechanisms of colorectal carcinogenesis in humans is quite difficult, with ethical and legalistic considerations often limiting the scope of experimentation. In order to conduct a comprehensive evaluation of the causative effects and mechanistic properties of colorectal carcinogenesis, animal models provide an ideal tool in carrying out these evaluations. Animal models allow for therapeutic intervention to be carried out in a controlled manner as well as the development of early markers or therapies for colon carcinogenesis.

1.1.2 Chemical induction of colon carcinogenesis

Experimental models for colorectal carcinogenesis were used as early as 1963. Laqueur *et al.* (1963) observed that cycasin, found in the Cycadaceae plant, is a potent carcinogen in the rat colon. Intestinal tumours have since been produced in strains of rats, mice, guinea pigs and hamsters by compounds related to the natural plant product cycasin or methylazoxymethanol β-glucoside, namely methylazoxymethanol acetate, azoxymethane (AOM), and 1,2-dimethylhydrazine (DMH) (Weisburger *et al.* 1977). The most widely used carcinogens are AOM and DMH, both of which require metabolic degradation to the active electrophilic compound. Following activation the electrophilic species react with nucleophilic centres on the DNA molecule. DNA adducts may be created leading to a

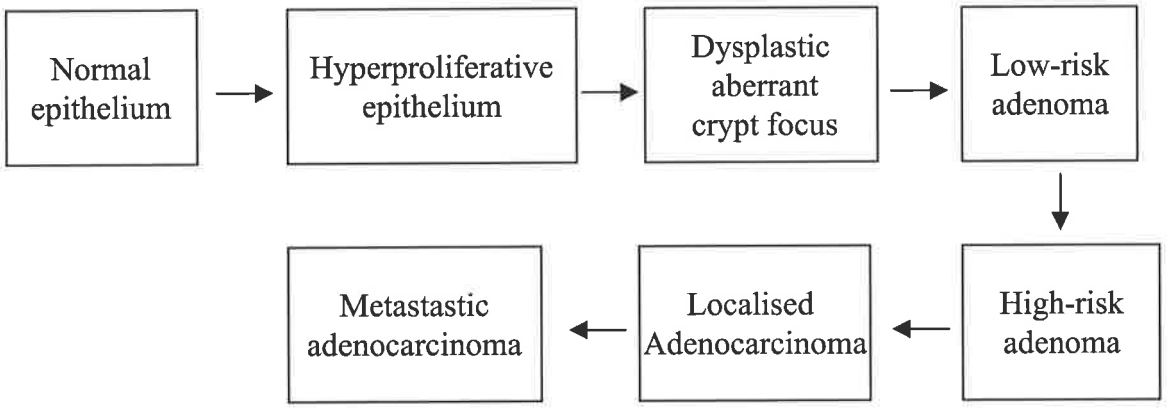


Figure 1.1 Model for development of colorectal cancer

mutation that makes the cell prone to develop along the multistep pathway (Figure 1.1) to cancer. In the commonest model, DMH or AOM is given to male Sprague-Dawley rats by weekly subcutaneous injections of 15 mg/kg (Hill, 1989; Goldin, 1988). Intestinal tumours appear about 6 months after the initial injection. Generally the higher the total dose administered, the higher the tumour incidence and the shorter the latency period. Figure 1.2 shows the metabolism and activation reactions required for these compounds.

There are many similarities of the process of intestinal tumourigenesis produced by AOM and DMH in rats to those arising in humans. AOM and DMH lead to an increase in cellular proliferation, which is critical to the process of carcinogenesis (Ryser, 1971; McGaririty *et al.* 1988). Crypts become aberrant and elongated and numerous aberrations occur and are thought to represent early preneoplastic change (McLennan and Bird, 1988). More recently ACFs have been identified in human colons and thought to be precursors of adenoma and cancer (Takayama *et al.* 1998). Alterations in DNA methylation patterns represent the early stages of carcinogenesis (Vogelstein *et al.* 1988). There are also many genetic changes that occur in carcinogen-induced animal tumours. These include amplication and elevation of the expression of the *myc* oncogene (Yander *et al.* 1985). Overexpression of the Ha-*ras* oncogene has also been observed in carcinogen-induced rat colon tumours (Yasui *et al.* 1987), while have also been mutational activation of ras protooncogenes have also been observed in AOM treated rats (Singh *et al.* 1994). The histopathological features of the AOM/DMH-induced colon tumours are similar to those seen in the human disease (Goldin, 1988). Rats developing intestinal tumours are also accompanied by anorexia, weight loss, bloody stools, bowel obstruction and occasional intersusception, all characteristic to the human disease.

The DMH/AOM model of colorectal cancer in rodents serves as an ideal model for the evaluation of different compounds including dietary components on colorectal carcinogenesis.

1.1.3 Biomarker of colorectal cancer: Aberrant crypt foci

Aberrant crypt foci (ACF) are considered to be precursor lesions of colonic adenomas and carcinomas (Figure 1.3) and are often used as an intermediate biomarker for colorectal tumourigenesis. ACF were first identified by Bird (1987) in the colons of carcinogen treated rodents and have also been shown to be present in humans who have a greater risk of developing colon cancer (Pretlow *et al.* 1992; Roncuucci *et al.* 1998). ACF in experimental animals can be observed as early as 2 weeks following a single injection of the carcinogen AOM (McLellan and Bird, 1988). Aberrant crypts can be easily distinguished under a light microscope after staining with methylene blue from surrounding normal crypts by a number of factors: enlarged diameter, thicker epithelial lining, darker staining appearance, increased pericryptal zone, and altered mucin pattern. Biological characteristics of ACF observed include: increased cell proliferative activity, dysplasia, altered histochemical state, with a decrease in mucin and goblet cell content, frequent mutations in *ras* oncogene and the *APC* tumour suppressor gene have also been characterised (Yamashita *et al.* 1994; Vivona *et al.* 1993; Pretlow *et al.* 1994). ACF may also harbour resistance to induction of apoptosis (normal or programmed cell death) (Magnuson *et al.* 1994).

It must be recognised that not all ACF that are formed progress through the multistep process leading to the formation of microadenomas and so on to adenomas and adenocarcinomas, only a select number progress (Bird, 1995). Investigators have used the ACF as an end point for a predictor of colon cancer incidence when evaluating chemopreventive agents (Wargovich *et al.* 1995; Pereira *et al.* 1991; Jenab and Thompson,

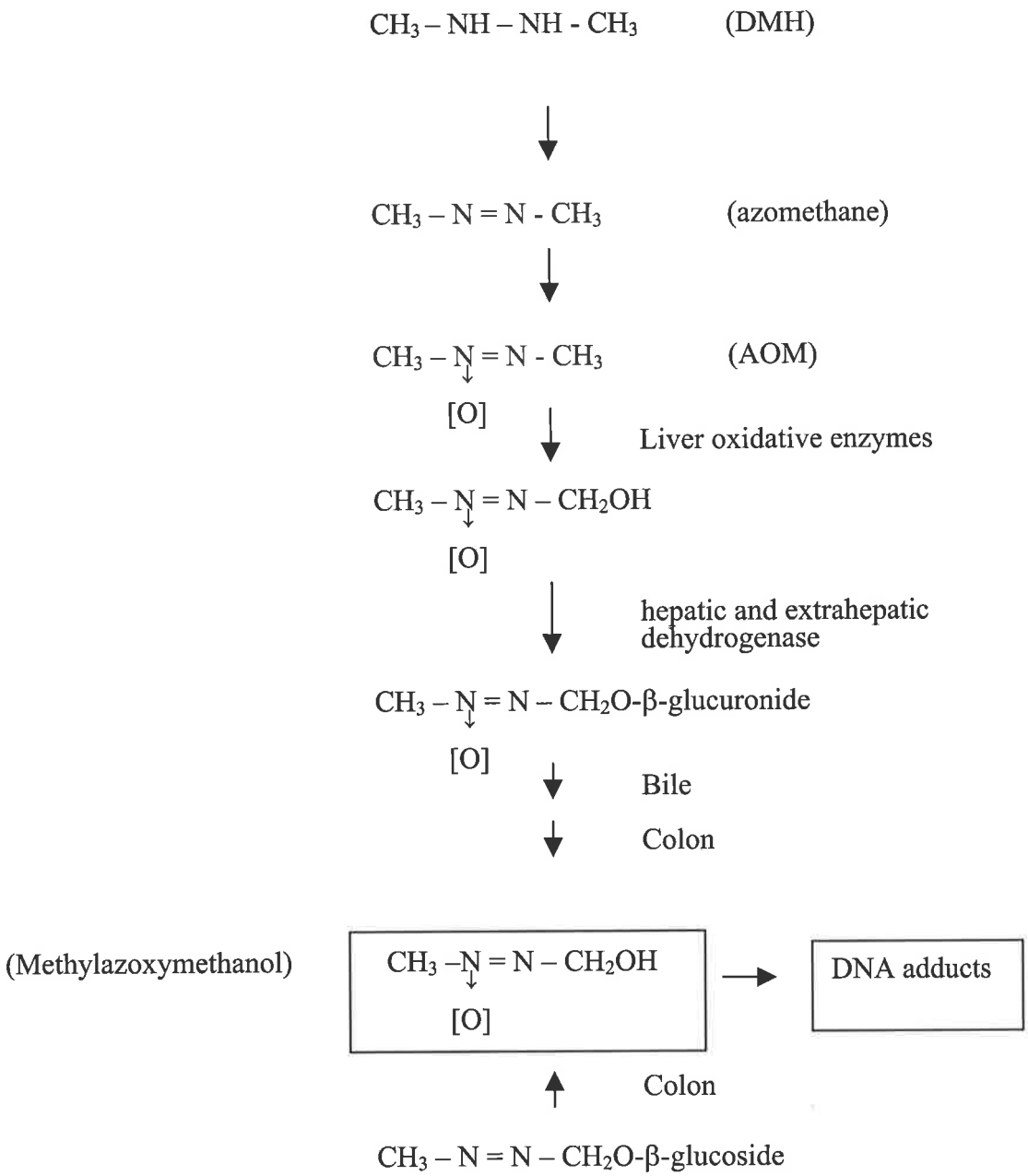


Figure 1.2 Metabolism and activation reactions for DMH and AOM. (Weisburger, 1971)

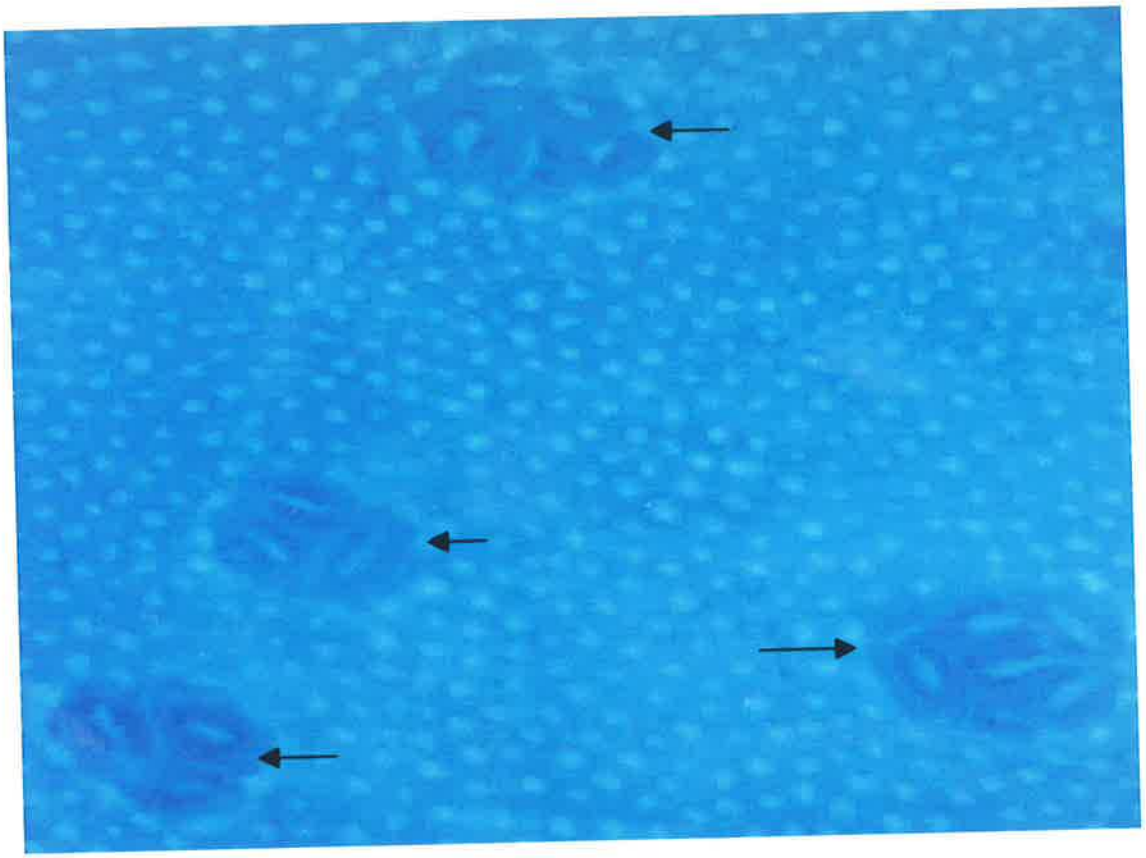


Figure 1.3 Section of rat colon which has been fixed in formalin and stained with haematoxlin and eosin. Aberrant crypt foci are visualised at 9x magnification under a light microscope. The arrows points out aberrant crypt foci.

1998). However, not all studies suggest that number of ACF present in the colon act as a reliable predictor of the incidence of tumours (Hardman *et al.* 1991) and care must be taken when extrapolating between ACF and colonic tumours.

1.2 Folate

The micronutrient 'folate', (a member of the B complex group of vitamins) is the generic term that refers to all compounds that exhibit vitamin activity similar to folic acid (pteroylmonoglutamate). The folate molecule is shown in figure 1.4 and consists of a pteridine ring linked via a methylene group to p-aminobenzoic acid, which is attached by a peptide linkage to glutamic acid. The water-soluble vitamin folate is found in good supply in foods such as green leafy vegetables, fruits, cereals and legumes. There are various forms of folate that occur naturally in food. However most dietary folates are polyglutamate derivatives whose bioavailabilty is dependent on deconjugation to the monoglutamyl form. Absorption of folates takes place in the jejunum of the small intestine after deconjugation by folate conjugase (pteroylpolyglutamate hydrolase) which is an intestinal enzyme found in the brush border. This process is optimal at a pH around neutrality. Folate is concentrated in bile and enterohepatic recirculation from the intestine allows for considerable reabsorption and reutilisation of folate (Bailey, 1995). Folate derived from the diet is the major source for humans, as they are unable to directly synthesize folate. However, many bacterial species (Camilo *et al.* 1996), including the intestinal microflora of the gut are capable of de novo synthesis of folate (Lascelles and Woods 1952; Miller and Luckay 1963, Rong *et al.* 1991), where folate can be indirectly synthesized and may then be incorporated into stores of the host tissue. Mammalian tissues accumulate almost entirely polyglutamate derivatives, whereas pteroylmonoglutamates are the forms which are found in the plasma and urine (Shane, 1995).

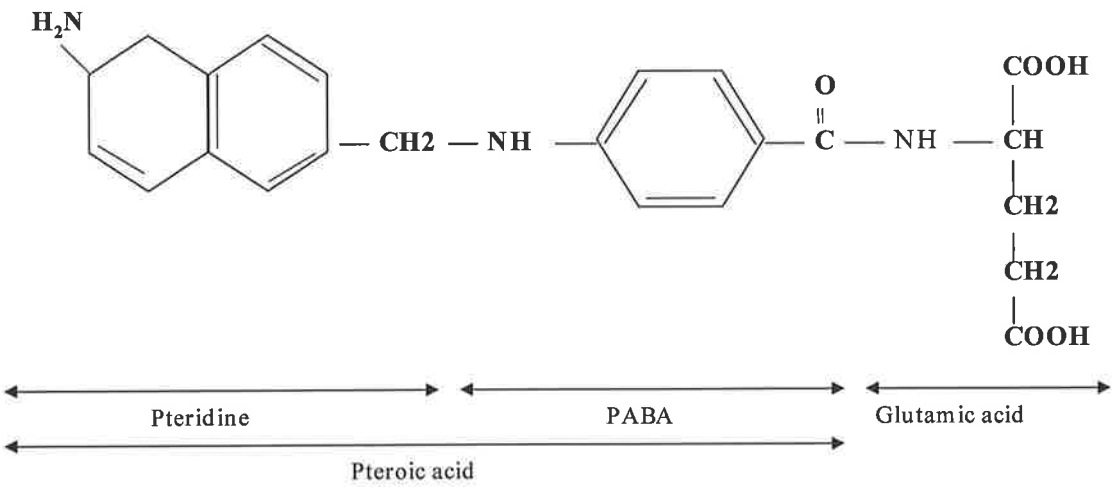


Figure 1.4 Structure of folic acid (pteroyl-glutamic acid)

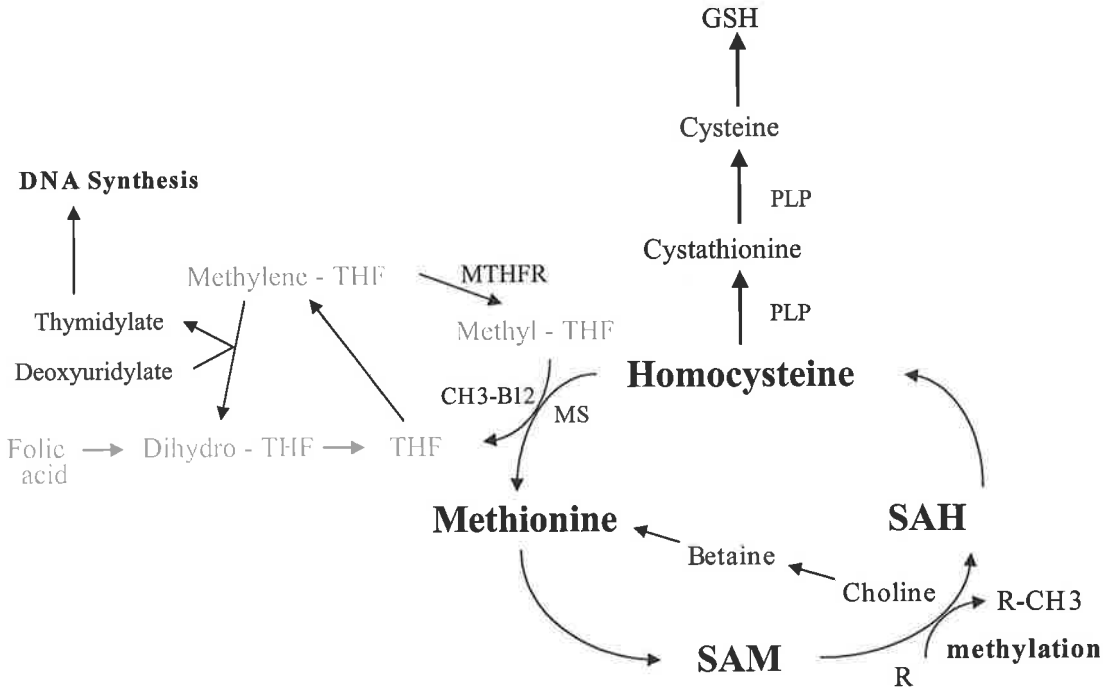


Figure 1.5 Representation of the role of folate and co-enzymes in the regulation of S-adenosylmethionine (SAM).
(THF - tetrahydrofolate, GSH - glutathione, SAH - S-adenosylhomocysteine, MTHFR – methylenetetrahydrofolate reductase, PLP – pyridoxal phosphate)

Folate functions in the transfer of single carbon units from donor compounds to biosynthetic pathways and is shown in figure 1.5 (Wagner, 1995). Folate plays a key role in nucleic acid biosynthesis, and is essential for normal cell proliferation and function. Folates also play an important function in amino-acid metabolism where they participate in the interconversion of serine and glycine. Coenzymes of folate are required for the regeneration of methionine from homocysteine, which is important in the production of S-adenosylmethionine (SAM), the proximal methyl donor for most biological transmethylation reactions, including that of DNA (Selhub and Miller, 1992). Consequently folate status has an important impact on the growth and development of new cells.

1.2.1 Measurement of folate status

Either serum folate levels or red cell folate usually reflects folate biochemical status. Red cell folate is able to better discriminate between adequate and deficient tissue stores (van den Berg *et al.* 1994). A number of different procedures are used to measure folate levels. These include microbiological assays, radioassay procedures, enzyme protein-binding assays and high-performance liquid chromatographic (HPLC) methods. Microbiological procedures which utilise Lactobacillus casei as the test organism use a value of <300 nmol/l as cut-off points for folate deficiency in humans (Herbert, 1991). However, this technique due to its long and tedious approach has been replaced over the last decade by enzyme protein-binding assays and commercial radioprotein-binding assay kits. HPLC methods of late have also been adopted for the analysis of folates in clinical as well as dietary samples (Wigertz and Jagerstad, 1995; Selhub, 1989; Lucock *et al.* 1995). In this thesis, the HPLC technique of Wigertz and Jagerstad (1995) has been adopted for the measurements of blood folates, while the microbiological procedure of Davis *et al.* (1970) will be used for measurement of food and tissue folates.

Homocysteine measurements also serve a sensitive marker of folate coenzyme activity, as homocysteine levels reflect tissue deficiency of methyltetrahydrofolate as a substrate in the methionine synthase reaction (Stabler *et al.* 1988). A better assessment of folate status can be determined by measuring folate concentrations in the blood along with plasma homocysteine levels.

1.2.2 Food sources of folate

The food sources that contain the highest levels of folate include liver, yeast extract, green leafy vegetables, legumes certain fruits and cereals. Although some foods contain very high amounts of folate they do not necessary contribute the greatest amount in terms of overall intakes of folate in the general population. Liver for example, contains high levels of folate, though it is not consumed by a significant proportion of the population, and therefore does not make any significant contribution to total dietary folate intakes. Conversely, cereal products along with orange juice contain lower levels of folate, but because they are consumed on a daily basis they contribute a much higher percentage of the daily intake of folate (Subar *et al.* 1989). Cereal products are now also being fortified with folic acid and therefore their consumption will only further increase the intake of folate.

Legumes are also a rich source of folate and with the inclusion of many soy protein based foods in the market place it is likely that they also may contribute a high level of the daily intake of folate. Soy protein, which contains high levels of folate, has been shown in experimental animal studies to inhibit various types of cancer (Hawrylewicz *et al.* 1995), however it is unclear whether this inhibition may be attributed to folate, phytate or isoflavonoids (phytoestrogens).

1.3 Folate deficiency

Folate deficiency is one of the most prevalent vitamin deficiencies occurring in populations all over the world (Hercberg and Galan, 1992; Joosten *et al.* 1993). Folate deficiency in humans can develop within a few months of reducing the intake of folate (Herbert, 1987), whereas in a animals folate deficiency may develop within a few weeks of consuming a folate deficient diet (Walzem and Clifford, 1983). The main groups at risk are those with the greatest need for increased folate intake, which includes pregnant women and the elderly, as well as those people under mental and physical stress and alcoholics (Bailey, 1995).

Clinical manifestations of folate deficiency include a specific type of anaemia, megaloblastic anaemia. In humans, there is an accumulation of megaloblasts during folate deficiency. These abnormal cells are large and nucleated, accumulate in the bone marrow and are precursors of erythrocytes (Chanarin, 1986). Decreased numbers of white cells and platelets are also observed. Generally there is an impairment of cell division occurring and this may be more apparent in tissues which turn over rapidly, such as the hematopoietic system and the gastrointestinal epithelial cells (Wagner, 1995). Other clinical manifestations of folate deficiency include hyperhomocysteinemia (Malinow, 1996).

There are a number of different ways in which folate status may be compromised: this may occur through nutritional and/or genetic factors. Nutritional deficiency of folate may be due to a number of factors: (1) An inadequate ingestion of folate - this may be due to a poor diet. Folates are quite unstable, as heating or exposure to air or ultraviolet light are known to inactivate the vitamin. Also overcooking leads to lower levels being present in the food and hence less folates being available for ingestion (Gregory, 1989). (2) Inadequate absorption of folate - most of the folates are absorbed in the upper third of the

small intestine. Therefore malabsorption syndromes such as gluten-induced enteropathy, celiac or tropical sprue will interfere with folate absorption and lower folate status (Pare *et al.* 1988). Specific drugs such as anticonvulsants, barbiturates, ethanol and sulfur drugs may also interfere with absorption of folates (Halsted *et al.* 1967; Walzem and Clifford, 1988). (3) Inadequate availability of folate - folate antagonists, which are used in chemotherapy (ie. methotrexate) inhibit dihydrofolate reductase thereby blocking the production of folate metabolites (Barford *et al.* 1980). Also sulfonamide drugs such as succinylsulfathiazole which can act by inhibiting microbial synthesis of folate in the colon (Wright *et al.* 1945; Walzem and Clifford, 1988). (4) Increased requirement of folate - during the development of the fetus there is an increased demand of folate (Bailey, 1990). Also during disorders such as cancer (ie. lymphoproliferative) the malignant tissue has an increased demand for folate (Herbert, 1986). During infancy the requirement of folate is also increased and if these requirements are not met by the diet, folate deficiency may prevail. (5) Increased excretion of folate - during vitamin B_{12} deficiency folate may be excreted in the urine and bile (Brody *et al.* 1984). Liver diseases and kidney dialysis may also increase the excretion of folates and contribute to folate deficiency. (6) Methylenetetrahydrofolate reductase polymorphism: A mutation in the methylene tetrahydrofolate gene causes reduced activity in the methylene tetrahydrofolate reductase (MTHFR) enzyme (Jacques *et al.* 1996; Ma *et al.* 1997). This enzyme is required for the generation of methyltetrahydrofolate. This mutation leads to lower levels of circulating 5-methyltetrahydrofolate as well as accumulation of 5,10-methylenetetrahydrofolate and a rise in plasma homocysteine.

By far the most common cause of folate deficiency is insufficient dietary intake of folate. An inadequate folate intake or a lowered folate status has been associated with increased risk of neural tube defects (Butterworth and Bendich, 1996) and vascular disease

(Malinow, 1996; Verhoef, 1996), and may well increase the risk of certain cancers (Glynn and Albanes, 1994) including that of colorectal cancer.

1.3.1 Marker of folate deficiency: hyperhomocysteinemia

Homocysteine is a sulfur-containing amino acid which is an intermediary product of methionine metabolism (Finkelstein, 1998). The metabolism of homocysteine is dependent on two main pathways: one via remethylation, to methionine; the other, via irreversible transulfuration, to cysteine via cystathionine β-synthase (Figure 1.6). Remethylation may occur through two alternative routes. The predominant route requires folate and its co-enzymes with vitamin B_{12} containing 5-methyl-tetrahydrofolate: homocysteine methyltransferase catalysing the reaction (Selhub and Miller, 1992). The other route in which homocysteine is remethylated utilizes choline and is confined mainly to the liver. Here choline is oxidized to betaine which serves as the methyl donor in a reaction catalysed by betaine:homocysteine methyltransferase (Finkelstein *et al.* 1982). A disruption of one or both of these pathways may result in hyperhomocysteinemia (sustained elevation of plasma homocysteine).

Plasma homocysteine levels have been shown to be inversely correlated with intracellular folate due to the requirement of folate and its co-enzymes. It thereby serves as a sensitive marker of folate deficiency (Selhub *et al.* 1993). Elevated levels of homocysteine in the serum and/or plasma have been reported in several studies on patients with folate deficiency (Kang *et al.* 1987; Stabler *et al.* 1988). There is now a considerable body of evidence that

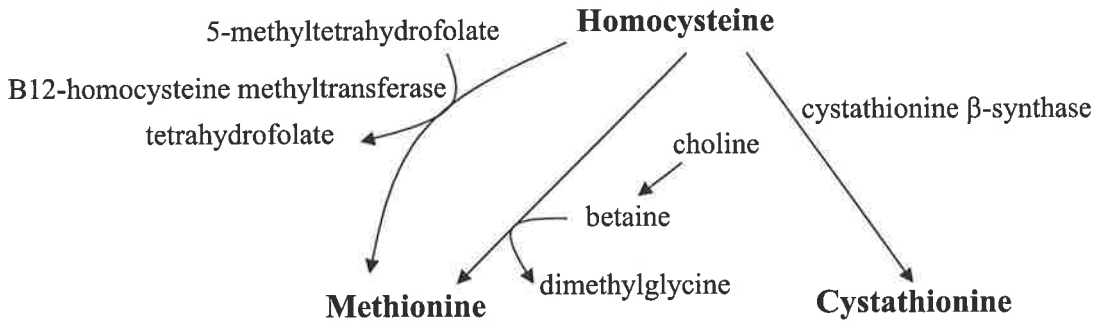


Figure 1.6 Metabolism of the sulfur containing amino-acid homocysteine

hyperhomocysteinemia is associated with an increased risk of coronary, cerebral and/or peripheral arterial diseases (Selhub *et al.* 1995; Robinson *et al.* 1995; Malinow, 1996).

1.3.2 Rat model for experimental folate deficiency

A variety of protocols have been proposed for the production of experimental folate deficiency in rats. The ideal protocol to produce folate deficiency in rats is to feed a folate-free diet that otherwise supports maximum growth. Test diets used to study folate deficiency include semi-purified diets with casein (Tagbo and Hill, 1977) or gelatin (Bachevalier and Botez, 1978) as the protein source and even a combination of both of these protein sources (Howard *et al.* 1974; Richardson *et al.* 1979) along with the omission of folate from the vitamin mixture. These particular intact protein based diets have been criticised by some investigators (Walzem and Clifford, 1988) when used to create experimental folate deficiency in rats. The main criticism was that although the intact proteins (casein, gelatin) are purified they still contain small amounts of the vitamin folate. Therefore in order to minimise the folate levels in these particular diets the protein levels tend to be lowered which can further disrupt the growth of the animals. However, supplementation with methionine of these lowered intact protein based diets is certainly an appropriate means of maintaining adequate growth of the animals thereby overcoming this criticism. Specific amino acid diets (high in glutamate) deplete in folate are an alternative to semi-purified diets (Walzem *et al.* 1983), however these diets are very artificial and often make experimental interpretation of results relative to humans quite difficult. Often sulfonamides (such as succinylsulfathiazole) are included with folate-deficient diets to minimise intestinal synthesis of folate and further enhance the level of folate deficiency (Wright *et al.* 1945; Walzem and Clifford, 1988). However, the inclusion of sulfonamides with a folate-deficient diet when fed to rats usually results in severe folate deficiency, and if

fed for a prolonged period of time can cause significant falls in bodyweight and premature death (Clifford *et al.* 1989). Therefore such a protocol becomes unsuitable if the experimental protocol are to be conducted over a long period of time, such as in AOM-induced rat colorectal cancer models. Folate antagonists such as methotrexate in the diet are another alternative protocol for disrupting folate metabolism and creating folate deficiency (Selhub *et al.* 1991). Test diets deficient in lipotropes choline and methionine can also increase the requirement of intracellular folate derived methyl groups and thereby lower intracellular folate levels (Horne *et al.* 1989). Chronic ethanol consumption has also been suggested to lead to the development of folate deficiency in humans (Sullivan and Herbert, 1964) as well as in rats (McMartin *et al.* 1989). Probiotics may also be a means of modulating folate status. Substantial quantities of folate may be produced by microorganisms in the colon (Cooperstock and Zedd, 1990; Denko *et al.* 1946). Studies in rats have demonstrated the importance of the intestinal environment in the biosynthesis of folate (Rong *et al.* 1991) and in fact this newly synthesised folate can be absorbed across the colon and be utilised by the host. Bifidobacteria strains have been shown to increase folate status in cultures (Deguchi *et al.* 1985) and in the rat by increasing folate synthesis (Krause *et al.* 1996). Other probiotic strains such as Streptococcus thermophilus and Lactobacillus acidophilus have been shown to increase folate concentrations by more than 200% during fermentation of skim milk in culture (Littlefield *et al.* 1996), however some strains such as Lactobacillus bulgaricus can utilise folate and reduce folate to negligible levels (Rao *et al.* 1984). It remains to be seen if these observations in culture can be reproduced in intact animals by feeding certain probiotic strains

In terms of selecting an appropriate protocol for creating folate deficiency in the rat for the use in the AOM-induced colorectal cancer model, a semi-purified casein based diet (American Institute of Nutrition style diet) with the omission of folate from the vitamin

mixture is appropriate. The American Institute of Nutrition (AIN-93) style diet (Reeves *et al.* 1993) is usually the diet of choice when investigating chemopreventive agents in the rat AOM-induced colorectal cancer model (Rao, 1988). This style of diet closely resembles that of a typical diet that is consumed by Western populations, thereby making it relevant for comparisons to the human population. Although the specific amino-acid based diet has been shown to be successful in creating experimental folate deficiency, it is a very artificial based diet, and not likely to be consumed by the normal human population.

1.4 Folate status and colorectal cancer (CRC)

Data collected from experimental animal studies, clinical and epidemiological studies suggest that alterations in folate status may modulate colorectal carcinogenesis. Animal studies conducted to date have provide conflicting results, with some studies suggesting protection with folate deprivation, while others indicate that deficiency may lead to an increased risk of colorectal cancer. Clinical observations and epidemiological studies tend to suggest an association between a diminished folate status and an increased risk of colorectal cancer.

1.4.1 Experimental studies: Folate and CRC

The experimental animal evidence examining the effects of folate status on colorectal carcinogenesis is sparse and contradictory. The conflicting results may well be due to variations in experimental design, or to differing animal models used by researchers.

There are two experimental animal studies that indicate folate deficiency may enhance colorectal carcinogenesis (Cravo *et al.* 1992; Kim *et al.* 1996). Cravo and colleagues (1992) using the dimethylhydrazine rat model of colonic carcinogenesis

examined the influence of a folate deficient diet compared with a diet containing adequate folate (8 mg/kg). In this study the basal diet was that of a high glutamate amino acid defined diet based on that of Walzem and Clifford (1988). Rats after consuming their defined diet for 5 weeks received weekly subcutaneous injections of DMH at a dosage of 20 mg/kg bodyweight for 20 weeks. They observed that moderate folate deficiency enhanced the development of colonic dysplasia and cancer. In this study, 100% of male Sprague-Dawley rats fed a folate depleted diet developed microscopic colorectal neoplasms 20 weeks after initiation of diets, compared with only 29% of the rats fed a control diet. There was also a 43% increase in the incidence of macroscopic colorectal neoplasms in the folate deficient groups. It was concluded from this study that although folate deficiency itself is not carcinogenic, it increases the risk of carcinogenesis. Also folate deficiency seemed to be affecting an early phase of colorectal carcinogenesis.

Kim *et al.* (1996) in a similar study utilizing the same DMH rodent model of colorectal cancer and high glutamate amino acid defined diet based (Walzem and Clifford, 1988), observed a reduction in the number of dimethyhydrazine induced macroscopic neoplasms from microscopic foci in rats by increasing the level of dietary folate up to four times the basal folate requirement. It was also shown in this study that increasing the level of dietary folate beyond four times the dietary requirement did not result in any further benefit in terms of tumour reduction. Also by increasing the level to twenty times the risk of developing colorectal tumours may even increase.

Three other experimental animal studies do not support a protective role of dietary folate against colorectal carcinogenesis (Shivapurkar *et al.* 1995; Reddy *et al.* 1996; Wargovich *et al.* 1996). Shivapurkar *et al.* (1995) examined the effect of a semi-purified diet that was high in fat and low in fibre with folate supplementation (3 mg/kg) on the incidence of aberrant crypt foci and colon tumours induced by azoxymethane (AOM), a

metabolite of DMH. Aberrant crypt foci (ACF) are putative preneoplastic lesions and are well-established intermediate biomarkers of colorectal cancer (Bird, 1995). Folate supplementation was found to have no influence on ACF, nor any effect on colon tumour incidence or tumour multiplicity. Wargovich and colleagues (1996) examined the effect of folate on AOM-induced aberrant crypt foci (ACF) in male F344 rats. Folate supplementation at 2.5 g/kg significantly increased the development of ACF when compared to a control diet (AIN-76A, 2mg/kg). Further supplementation with folate to 5 g/kg diet was found to have no effect on the number of ACF when compared to the control diet. It must be emphasized that the folate levels used in this study exceeded physiologic levels by 1000-fold. In another study by Reddy *et al.* (1996), the effect of folic acid (2 g/kg diet) on colon carcinogenesis was investigated in male F344 rats. The results of this investigation showed that folic acid had no effect on AOM-induced colon tumour incidence, however tumour size and multiplicity were increased when compared to a control diet.

In a study using transgenic mice that carry the bacterial *lac*Z gene as a mutational target the effect of maternal folate levels on the mutation rate in the developing colon was examined (Trentin *et al.* 1998). Somatic mutations are thought to play a critical role in carcinogenesis. A standard AIN-93G rodent diet (Reeves *et al.* 1993) was used as the basal diet. The sulfonamide succinylsulfathiazole and glycine were added to the folate-free AIN-93G diet, while folate was mixed in at a concentration of 0.5, 1, 2, 20 or 200 mg/kg of the diet for the other treatments. Results from this study showed that there was no significant differences in the frequency of *lac*Z mutations within mice that consumed either low-folate or folate supplemented diets. Song *et al.* (1999) using a genetic murine model of intestinal tumourigenesis found that the timing of dietary intervention with folate is important in modulating tumourigenesis. Folate supplementation prevented the progression of intestinal polyps if administered prior to the development of adenomas in the intestine. However,

mice receiving a folate-depleted diet during the development of intestinal polyps had a significantly lower incidence of tumours.

Only two experimental rat studies to date have found an increased risk of developing colorectal carcinogenesis with folate deficiency or conversely a protective effect with folate supplementation. Interestingly, both of these studies from the same institution and used a specific high glutamate amino acid defined diet as the basal diet. All other rat studies have used modified forms of the American Institute of Nutrition (AIN) form of diet and found either no effect or an increased risk of developing colorectal cancer with folate supplementation.

1.4.2 Epidemiological studies: Folate and CRC

Examination of the epidemiological studies to date relating folate with colorectal cancer risk shows mixed results (reviewed by Kim, 1998).

Lashner *et al.* (1989) in a case-control study first investigated the role of folate deficiency and colorectal cancer risk in 99 chronic ulcerative colitis patients. Ulcerative colitis is a disease that is associated with a greater risk of developing colorectal dysplasia and cancer when compared with the normal population (Isbell and Levin, 1988). The most commonly used medication in ulcerative colitis at the time of this study was sulfasalazine, a drug that impairs folate absorption (Swinson *et al.* 1981). Lashner *et al.* (1989) reported that chronic administration of sulfasalazine was associated with a 50% increase in the risk of dysplasia (OR = 1.50; 95% CI, 0.43-5.19) and that folate supplementation was associated with a 62% reduction (non-significant) in neoplasia compared with individuals not receiving the folate supplementation (OR = 0.38; 95% CI, 0.12-1.20). However, these results reported by Lashner *et al.* (1989) were not significant and suggest only an association of folate supplementation with colorectal cancer risk. In another case-control study, Lashner (1993)

measured dietary, serum and RBC folate in 67 patients with ulcerative colitis and dysplasia or cancer. There was a 13% non-significant reduction in RBC concentration in those with colonic neoplasia compared with controls, however the lowered RBC folate concentration was not in the deficient range but still in a range regarded as normal. The above two studies are only suggestive of a provocative association between folate deficiency and an increased risk of colorectal cancer. The provocative association between folate deficiency and an increased risk of colorectal cancer remains questionable as other investigators have reported opposite results to that of by Lashner *et al.* (1989,93). Fiedler *et al.* (1993) in a case-control study involving 67 ulcerative colitis patients with colonic dysplasia or cancer and 68 without, found that folate supplementation was not significantly associated with a decreased risk of developing colorectal neoplasia. Pinczowksi *et al* (1994) in another case-control study involving 102 ulcerative colitis patients with neoplasia and 196 matched controls reported that the folate antagonist sulfasalazine was associated with a 62% reduction in the risk of developing colonic neoplasia (RR = 0.38, 95% CI, 0.20-0.69).

A number of studies have been conducted which examined dietary folate intake and the risk of developing colorectal cancer. These studies assessed dietary folate intake by the use of various food frequency questionaires. In a population-based case-control study in Majorca, Benito *et al.* (1991) found a significant protective effect of dietary folate against colorectal cancer (relative risk (RR) of 0.61). The major sources of dietary folate were vegetables, particularly the cruciferae, however also in this study, fibre was shown to be protective, with legumes the major contributor. Legumes are an excellent source of folate and along with vegetables also contain many other phytochemicals which may in fact be offering this protection against colorectal cancer. In another case-control study conducted in Majorca by Benito *et al.* (1993) a protective effect was found for fibre from fruits and vegetables against colorectal adenomas. Analyses by nutrients identified folate as one of

the protective factors from fruits and vegetables, with an odds ratio (OR) of 0.27, when comparing the highest with the lowest quartile after adjusting for total calories, age, sex, physical activity and rural residence. It must be emphasized that it was the fruits and vegetables that offered protection against colorectal adenomas. Although folate was identified as one of the nutrients supplied through fruits and vegetables, it still only suggests that folate may be offering protection. Freudenheim *et al.* (1991) in a case-control study found that folate intake was associated with a reduced risk for rectal cancer (OR = 0.31, 95% confidence interval (CI) 0.16–0.59). However, there was no association of risk of colon cancer with folate intake. Giovannucci *et al.* (1993) using data collected from the Nurse's Health Study (121, 700 U.S female nurses) and Health Professional Follow-Up Study (51, 529 U.S male health professionals) examined the associations between intake of folate and the risk of colorectal adenoma. Dietary intake was assessed in 15, 984 women and 9490 men who had undergone endoscopy. The authors found that high dietary folate intake was inversely associated with risk of colorectal adenoma in women (P trend = 0.04)(RR=0.66, 95% CI 0.46-0.95) and in men (P trend = 0.03) (RR=0.63, 95% CI 0.41-0.98) after adjusting for confounding factors. In another study by Giovannucci *et al.* (1995) using data from 47, 931 U.S male health professionals, a weak non-significant inverse correlation with dietary folate and colon cancer risk was observed in male subjects. However, when high folate intake was combined with low alcohol and high methionine intake there was a significant reduction in relative risk of developing colon cancer (RR=0.3, 95% CI 0.14-0.63). However in this study when other factors were combined with the low folate intake such as high alcohol-low methionine and compared to high folate-low alcohol-low methionine there was a 70% reduction in risk of developing colorectal cancer. This study highlights the point that other factors may be involved in developing colorectal cancer and not just a low dietary folate intake. This data also suggests that factors that reduce methyl group content of diets

may increase the risk of colorectal cancer. Tseng *et al.* (1996) found a protective effect with dietary folate against the incidence of colorectal adenomas in subjects that had undergone colonoscopy. This study was based on 236 cases with adenomatous polyps or cancer and 409 controls. Here the protection appeared to be strongest in women (OR=0.39, 95% CI 0.15-1.01), however the results were not significant (P trend = 0.08). Baron *et al.* (1998) in a prospective study found a significant protective association of dietary folate with the risk of recurrence of colon adenoma (P for trend = 0.04). However, the association of adenoma risk with dietary intake of folate was substantially attenuated after adjustment for dietary fibre, thereby suggesting that substances other than folate in fruits and vegetables may explain at least some of the inverse association with risk of adenoma.

Not all the epidemiological studies have shown a reduction in the risk of developing colorectal cancer. Boutron-Rualt *et al.* (1996) in a case-control study found a no significant reduction with folate intake in reducing the risk of adenomas (OR=0.5, 95% CI 0.3-1). Ferraroni *et al.* (1994) examined the intake of selected micronutrients and the risk of colorectal cancer in 1324 patients with colorectal cancer and 2024 controls, using data from a case-control study conducted in northern Italy. For folate there was a trend of a protective effect with increasing consumption. However, this was found to be non-significant and therefore this data should only be regarded as suggestive. Meyer and White (1993) in a population-based case-control study conducted in western Washington assessed a variety of nutrients in relation to colon cancer in 424 cases and 414 controls, found no association in either men or women between colon cancer and dietary folate. In a cohort study in United States, Ford *et al.* (1998) examined serum folate levels and associations with chronic disease risk. No associations with colorectal cancer or any other forms of cancer were observed.

In summary, the majority of epidemiological studies published to date, evaluating dietary folate intake and colorectal cancer risk suggest a protective effect with dietary folate.

However, it must be emphasised that all of these studies only show associations and do not demonstrate a cause and effect of colorectal cancer. None of these studies relate actual folate deficiency with increased colorectal cancer risk. If there is a lower intake of folate this may well be related to lower intakes of other phytochemicals which may also alter colorectal cancer risk.

1.4.3 Folate levels: Folate and CRC

Results from prospectively conducted clinical studies indicate that serum folate concentrations do not differ significantly between subjects with and without colonic adenoma or cancer (Paspatis *et al.* 1995; Meenan *et al.* 1997; Kim *et al.* 1998). However, in the study conducted by Paspatis *et al.*, (1995) a significant reduction (28%) was found in the RBC folate concentration in patients with colorectal adenoma compared to normal control subjects. This reduction in RBC folate concentration was still within the normal range. The authors suggested that the depressed red blood cell folate levels were associated with development of colonic adenomas. However, this statement may be over interpreting the results, as another possibility is that the demand for folate by tumour growth may have lowered the folate levels in the blood of these individuals (Herbert, 1986). Bird *et al.*, (1995) conducted a case-control study of the relationship among folate status (red cell folate, plasma folate), folate intake, and adenomatous polyps. There was a significant fall in red cell folate levels in males with colorectal polyps. In this study the authors attributed a protective effect of red cell folate concentration against the development of colorectal polyps at least in men (OR=0.53, 95% CI 0.32-0.87). However, as with the study conducted by Paspatis *et al.*, (1995) the reduction in RBC folate concentration was still considered to be in the normal range and may just be an effect of an increased requirement for folate by the colorectal polyps. Conversely, Glynn *et al.*, (1996) in another case-control study found

no difference in serum folate concentrations between subjects with and without colorectal cancer.

The above studies relating folate status with colorectal cancer risk are conflicting. Whether lower folate levels in the blood are associated with development of colorectal cancer or just an effect of an increased requirement for folate by the colorectal tumours remain speculative and unproven.

1.4.4 Methylenetetrahydrafolate reductase (MTHFR) polymorphism

MTHFR is a critical enzyme which catalyses the biologically irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (figure 1.7), the main form of folate in plasma. 5-methyltetrahydrofolate is required as a methyl donor for methionine synthesis from homocysteine. A common mutation $^{677}C \rightarrow T$ (Ala $\rightarrow$ Val) of the gene was found to reduce the enzyme activity (Frosst *et al.* 1995). This leads to lower levels of 5-methyltetrahydrofolate in the plasma and elevated levels of plasma homocysteine. This mutation has shown to be linked with a reduction in the risk of developing colorectal cancer.

In a study published by Chen *et al.*, (1996) a 40% reduction in the risk of developing colorectal cancer was found in individuals with the homozygous (val/val) genotype when compared with the MTHFR heterozygous (val/ala) or normal (ala/ala) genotypes. In another study published by the same investigators (Ma *et al.* 1997) 202 colorectal cancer subjects were compared with 326 normal subjects. A 50% reduction in the risk of developing colorectal cancer was found in male subjects who had the homozygous MTHFR mutation when compared with heterozygous or normal genotypes. Overall they observed a marginal increased risk of colorectal cancer among those whose plasma folate levels indicated deficiency compared with men with adequate folate levels. Among men with adequate folate levels there was a 3-fold decrease in risk observed with men with the

homozygous mutation compared with those with the normal or heterozygous genotypes. However, this protection due to the mutation disappeared in men with folate deficiency. The authors hypothesized that the increased 5,10-methylenetetrahydrofolate levels available for DNA synthesis may be reducing the colon cancer risk.

1.5 Speculative mechanisms for effect of folate status on CRC

The mechanism(s) by which folate deficiency or a low folate status increases the risk of developing colorectal cancer remains speculative. However, Mason (1994) has proposed a number of possible mechanisms:

- Alterations in DNA methylation
- Misincorporation of uridylate for thymidylate synthesis
- Chromosome damage and impaired DNA repair
- Effects on the immune system

1.5.1 Alteration in DNA methylation

DNA methylation is a naturally occurring modification of DNA that occurs in many prokaryotes and eukaryotes. Within mammals it occurs at the 5′ position of cytosine © at the cytosine-guanine (CG) dinucleotide and it is estimated that about 70% of the cytosines are methylated in normal, differentiated cells (Razin and Szyf, 1984). Alteration in DNA methylation patterns has been suggested by some researchers to be play a crucial role in carcinogenesis (Baylin *et al.* 1991; Laird and Jaenisch, 1994). Changes in genomic methylation are one of the most consistent findings in human cancers (Gama-Sosa *et al.* 1983; Feinberg *et al.* 1988; Vertino *et al.* 1993) including that of colorectal cancer (Feinberg and Vogelstein, 1983; Issa *et al.* 1993). Changes in global genomic DNA methylation and

methylation patterns has led to the hypothesis of an epigenetic mechanism for cancer development, whereby changes in methylation interfere with expression of proto-oncogenes and tumour suppressor genes (Jones and Buckley, 1990). Hypomethylation of several proto-oncogenes including *ras* (Bhave *et al.* 1988; Feinberg and Vogelstein, 1983), c-*myc* (Munzel *et al.* 1991), *bcl*-2 (Hanada *et al.* 1983) have been reported in various cancerous tissues. While expression of several tumour-suppressor genes may be turned off by methylation (Gonzalez-Zulueta *et al.* 1995). However, it must be noted that the time at which alterations in DNA methylation takes place during tumourigenesis remain unclear.

A possible direct role for DNA hypomethylation in the carcinogenesis process stems from experimental data in animals. Studies have demonstrated that dietary restriction of methyl groups leads to a significant fall in SAM, and induction of DNA hypomethylation within in the liver. This hypomethylation was associated with tumour development (Christman *et al.* 1993; Pogribny *et al.* 1995; Wainfan *et al.* 1989). A role for folate deficiency in affecting colonic DNA methylation may act through a similar mechanism, as global hypomethylation has been observed in the colons of people with adenomas and adenocarcinomas (Feinberg *et al.* 1988). Folate is needed for the synthesis of S-adenosylmethionine (SAM), which is the proximal methyl donor for many DNA methylation reactions (figure 1.4). In regulating intracellular SAM levels, folate in the form of 5-methyl-THF provides a methyl group to homocysteine for the synthesis of methionine, which is the precursor to SAM. It is thought that depletion of SAM pools or a reduction in the ratio of SAM to S-adenosylhomocysteine (SAH) concentrations (the methylation ratio, which determines the degree to which methylation reactions will occur) may result in DNA being undermethylated (hypomethylation), thereby increasing colorectal cancer risk.

It is not established to date whether folate deficiency can perturb DNA methylation patterns in the colonic mucosa or if folate deficiency is in fact related to colorectal carcinogenesis.

1.5.2 Misincorporation of uridylate into DNA, chromosome breakage and impaired DNA repair

Misincorporation of uridylate for thymidylate into DNA, chromosome breakage and impaired DNA repair are all hypothesised mechanisms for folate deficiency and colorectal carcinogenesis. Folate is required for transferring one-carbon units in the synthesis of thymidylate, which is the rate limiting nucleotide in the synthesis of DNA (Wagner, 1995). During folate deficiency there is less N^5, N^{10}-methylene THF available for methylation in thymidylate synthesis, thereby increasing the cellular ratio of uridylate to thymidylate and subsequently resulting in uracil misincorporation into DNA (Das and Herbert, 1989). When uracil levels are increased, the likelihood of two adjacent uracil residues on opposite strands being repaired simultaneously is markedly increased, thereby resulting in a double-strand DNA breaks (Blount and Ames, 1995). It is hypothesised that these unrepaired double-strand breaks decrease genetic stability and therefore increase the risk of cancer (Rosin and Ochs, 1986, Weinberg, 1988, Blount *et al.* 1997). There is accumulating evidence from human, animal and *in vitro* studies that supports a role of folate deficiency with DNA damage (Libbus *et al.* 1990; Pogribny *et al.* 1997; Blount *et al.* 1997; Everson *et al.* 1988). Defective repair of DNA breaks has been implicated in colorectal cacinogenesis (Fishel *et al.* 1993, Ionov *et al.* 1993), and also may be a mechanism for folate deficiency involvement in colorectal cancer. Folate deficiency induced experimental animal has been shown to impair DNA excision repair in the colonic mucosa (Choi *et al.* 1998), further adding support for a role in colorectal carcinogenesis.

1.5.3 Reduced immune function

Resistance to infections is decreased in folate-deficient individuals and animals (Nauss *et al.* 1982; Jacobson *et al.* 1987). A decrease in the immune function through folate deficiency may also increase the risk of colorectal carcinogenesis. Folate deficiency in some animal studies has been shown to suppress the functions of natural killer cells (Trinchieri, 1989). It is hypothesized that a decreased folate status may impair the ability of natural killer cells to destroy dysplastic or cancerous cells (Mason, 1994).

1.6 Specific aims and objectives of thesis

The aim of this research was to gain an insight into whether and how folate status regulates colorectal tumourigenesis.

To investigate this aim the following studies were undertaken using a rodent animal model:

1) Evaluate different models for modulating folate status. Here the effect of probiotics, sulfonamides, folate and choline as well as methionine deficiency and supplementation on folate status was examined.
2) Determine the effect of different protein sources (casein versus soy protein) on folate status and parameters relating to folate status.
3) Examine the relationship between folate deficiency and colorectal cancer risk using the ACF as a biomarker of risk.
4) Determine the effect of moderate and severe folate deficiency as well as adequate folate status on intestinal tumourigenesis.

Other objectives of the present study were to determine the effect of folate status on a number of related parameters including:

- S-adenosylmethionine and S-adenosylhomocysteine levels
- DNA methylation status
- Plasma homocysteine
- Immune parameters in the spleen, thymus and mesenteric lymph nodes.

Chapter 2

Materials and Methods

2.1 Animals and Diets

Weanling male Sprague-Dawley rats were purchased from Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C with a 12 hour light : 12 hour dark cycle. Rats, at the age of 4 weeks were randomly sorted into experimental groups of even bodyweight and housed 5 per cage and fed *ad libitum* experimental powdered diets. Animals were given free access to deionised water throughout the duration of the experiment. Body weights were recorded weekly.

The experimental diets were modified forms of the AIN-93 (Reeves *et al.* 1993) semi-purified diet. The rodent diet of Reeves *et al.* (1993) is shown in Table 2.1.

Table 2.1. AIN-93G diet[1]

Ingredient	(g/100g)
Casein	20.0
Fibre (Solko-floc)	5.0
Cornstarch	39.7
Dextrinized cornstarch	13.2
Sucrose	10.0
Fat (Soybean oil)	7.0
Vitamin mix (AIN-93G-VX)[1]	1.0
Mineral mix (AIN-93G-MX)[1]	3.5
L-Cystine	0.3
Choline	0.25
Tert-butylhydroquinone	0.0014

1 The vitamin and mineral mixtures were prepared according to the AIN-93G-MX and AIN-93G-VX formula (Reeves *et al.* 1993) and supplied folic acid equivalent to 2 mg/kg diet.

The base experimental diet used in this thesis is shown in Table 2.2. The protein level was modified to 12% supplied as casein. Fat was modified to a level of 20%, and this was suppled as a mixture (1:1) of lard and sunflowerseed oil. The fibre was supplied as α-cellulose equivalent to 5%. No cystine was added to this diet, however methionine at 0.3% was added. The sucrose level was at 20%. The diets were prepared at the CSIRO, Health Sciences and Nutrition in a powdered form.

Table 2.2. Base experimental diet[1]

Ingredient	(g/100g)
Casein	14.3
α-Cellulose	5.0
Cornstarch	35.7
Sucrose	20.0
Fat (50% sunflowerseed oil: 50% lard)	20.0
AIN-vitamin mix[1,2]	1.0
AIN-mineral mix[1]	3.5
L-methionine	0.3
Choline	0.2

1 The vitamin and mineral mixtures were prepared according to the AIN-76 formula (AIN 76, 1977) but contained folic acid equivalent to 8 mg/kg diet.

2.1.1 Probiotic bacteria used in experimental diets

The following stains of bacteria were used in experimental diet preparation: Lactobacillus acidophilus (L.A)(DD910), Lactobacillus rhamnosus (L. GG)(A60418), Bifidobacterium longum (B.L)(DD920), Lactobacillus casei Shirota (L.Sh)(DD930).

2.1.2 Tumour induction protocol

After 4 weeks on experimental diets, animals were injected subcutaneously with AOM (Sigma Chemical, St Louis, MO) dissolved in normal saline at a dosage of 15 mg/kg body weight once weekly for 3 weeks. Animals were killed after 12 weeks of consuming experimental diets for ACF identification and after 26 weeks for tumour evaluation.

2.2 General Methods

2.2.1 Blood folate concentration

Whole blood folate was determined by HPLC with fluorescence detection. Standard concentrations of 5-methyltetrahydrofolate (5 MTHF)(barium salt) and tetrahydrofolate (THF)(Sigma, St Louis, MO) were prepared in 50 mmol/L sodium borate with 0.4% (v/v) 2-mercaptethanol. The standard curve was prepared by diluting (5 MTHF) and THF in 1 % (w/v) sodium ascorbate. Folates were extracted from whole blood using a modified method of Wigertz and Jagerstad, (1995). Briefly, 1 ml of whole blood diluted 1:1 in 1% sodium ascorbate solution was mixed with 7 ml of water to ensure effective haemolysis. Thereafter, 2 ml of 250 mmol/l potassium phosphate buffer (pH 6.1, containing 0.3% sodium ascorbate) was added. The samples were incubated at 37°C for 90 min. After spinning the samples (15,000 rpm for 10 min) the supernatant was loaded onto a 100 mg disposable strong anion-exchange column that had been preconditioned with 1 ml each of methanol and water. A vacuum manifold (VacMaster, Activon) capable of holding up to 10 disposable columns was used. The solution was pulled through under vacuum and the eluate was discarded. The columns were further washed with 2 ml of water and the folates eluted with 2 ml of 10 % sodium chloride solution containing freshly added 1 % sodium ascorbate. The mobile

phase (85 % 50 mmol/L potassium phosphate buffer, pH 3.5 and 15 % methanol) was pumped through a spherisorb ODS-2, 5u (25 cm x 4.6 mm) column at a flow rate of 0.8 ml/min. Folates were detected by measuring absorbance at fluorescent wavelength of 295 nm and emission wavelength of 365 nm following injection of 20-40 ul. The retention times of 5-MTHF and THF were 17.4 min and 11.2 min, respectively. Total folate concentration was determined by the addition of individual folates in the blood. The intra- and interassay CV for the folate standards was 4.0% and 5.0% repectively.

The HPLC system used was from Shimadzu (Shimadzu Corporation, Kyoto, Japan). The system was equipped with a pump (Shimadzu, LC10AT), spectrofluorometer (Shimadzu, RF-10A) and an autoinjector (Shimadzu, SIL-10A). The analysis of HPLC chromatographs was performed with Shimadzu Class-LC10 software.

2.2.2 Folate concentration in tissue

Folate was measured in colonic tissue by a microbiological technique utilising its effect upon growth of a chloramphenicol resistant strain of Lactobacillus casei according to the method of Davis *et al.* (1970). Briefly, colonic tissue was homogenised in phosphate buffer using a micro-blender, following this, tissue samples were treated with chicken pancreas conjugase. The growth of Lactobacillus casei was then measured turbidimetrically. Reduced folate results in reduced bacterial growth. The intra- and interassay CV for the folate measurements was 7.0%.

2.2.3 Haematocrit

Haematocrit values were determined in the EDTA collected whole blood. Briefly, approximately 100 ul of blood was drawn up into a micro-haematocrit tube (Hirschmann Laborgerate, Germany), sealed and centrifuged in an Adam micro-haematocrit centrifuge

(Clay-adams, inc. New York) for 5 min. The % of packed red cells (haematocrit) was read using an Adams micro-haematocrit reader (Clay-Adams, Inc., New York).

2.2.4 Plasma homocysteine

Homocysteine concentration in the plasma was determined using HPLC and fluorometric detection according to a modified method of Vester and Rasmussen (1991). Homocysteine standard (Sigma, St Louis, MO) was prepared by dissolving in milli-Q water to give a stock concentration of 2 mmol/l. Mercaptopropionylglycine (0.2 mmol/l) in 0.1 mol/l potassium borate, pH 9.5, containing 2 mmol/l EDTA was used as the internal standard. Briefly, samples of plasma or calibration material (60 ul) were mixed with 20 ul of internal standard. Eight microlitres of 10% (v/v) tri-n-butyl phosphine in dimethylformamide were added, and samples cooled at 4°C for 30 min. Samples were then mixed with 50 ul of 0.6 mol/l perchloric acid, containing 1 mmol/l EDTA, left at room temperature for 10 min, then centrifuged at 10,000 rpm for 10 min. Forty microlitres of supernatant was taken and mixed with 80 ul of 2 mol/l potassium borate, pH 10.5, containing 5 mmol/l EDTA. Ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate solution (40 ul)(1.0 g/l of 2 mol/l potassium borate, pH 9.5) were added and the mixture incubated at 60°C for 60 min. After cooling in an icebath, 20 ul of sample was used for injection into HPLC. The mobile phase consisted of buffer A: 0.1 mol/l acetate buffer, pH 4.0, containing 40 ml/l methanol, buffer B: 0.1 mol/l acetate buffer, containing 150 ml/l methanol. A linear gradient was run from solvent A to solvent B over 7 min (then 10 min B, a 2 min gradient back to A, and 4 min A before the next injection) at a flow rate of 1.0 ml/min. A spherisorb ODS-2, 5u (25 cm x 4.6 mm) column was used in the analysis. Homocysteine was detected by measuring absorbance at excitation wavelength of 385 nm and emission wavelength of 515 nm following injection of 20-40 ul. Total homocysteine

concentration was determined from a four point calibration line obtained from duplicate analysis of a plasma pool. Spiked plasma with 5, 10, 20 and 40 umol/l homocysteine were used to construct a linear regression line, and the ratio between the area of the homocysteine peak and the mercaptopropionylglycine peak as the ordinate, was calculated to determine the slope of the calibration line. The concentration of homocysteine in the samples was then determined by dividing the ratio between the area of the homocysteine peak and the mercaptopropionylglycine peak by the slope of the calibration line. The retention times of homocysteine and internal standard were 5.7 min and 17.4 min, respectively. The coefficient of variation (CV) was 3.0% for inter-day assay was 2.9%, while the intra-day assay was 2.3%.

The HPLC system used was from Shimadzu (Shimadzu Corporation, Kyoto, Japan). The system was equipped with a pump (Shimadzu, LC10AT), spectrofluorometer (Shimadzu, RF-10A) and an autoinjector (Shimadzu, SIL-10A). The analysis of HPLC chromatographs was performed with Shimadzu Class-LC10 software.

2.2.5 S-adenosyl-methionine and S-adenosyl-homocysteine

S-adenosyl-methionine (SAM) and S-adenosyl-homocysteine (SAH) concentrations were determined in tissue samples according to the method of She *et al.* (1994). Standard solutions of SAM and SAH (Sigma, St Louis, MO) were prepared in Milli-Q water at a concentration of 1 mmol/l with dilution's made with 0.4 mol/l perchloric acid to final concentration used during HPLC analysis. For SAM and SAH analysis, briefly, rat tissues were weighed and homogenised in 3 ml of 0.4 mol/l perchloric acid and centrifuged at 10,000 g for 20 min. The supernatant was filtered through a Millipore membrane (0.45 um) and 20 ul applied directly to the HPLC. The mobile phase consisted of 40 mmol/l ammonium dihydrogen phosphate, 8 mmol/l heptanesulfonic acid and 18% (v/v) methanol, pH 3.0. An isocratic flow of 0.8 ml/min was pumped through a 10 x 4.6 mm, 3 u, ODS2

spherisorb column and the absorbance monitored at 254 nm. The retention times of SAM and SAH were 6.2 min and 11.6 min, respectively. The intra- and inter CV for the assay were 6.2% and 8% respectively.

The HPLC system used was from Shimadzu (Shimadzu Corporation, Kyoto, Japan). The system was equipped with a pump (Shimadzu, LC10AT), diode array detector (Shimadzu, SPD-MAVP) and an autoinjector (Shimadzu, SIL-10A). The analysis of HPLC chromatographs was performed with Shimadzu Class-LC10 software.

2.2.6 Short chain fatty acid analysis

Short chain fatty acids in the faeces were measured by gas chromatography. Briefly, faeces were homogenized in 3 ml of water and a 200 ul aliquot was mixed with 20 ul of 0.05 mol/l 4-methyl-n-valeric acid (internal standard). The mixture was then centrifuged at 10,000 rpm at room temperature for 5 min, and 0.5 ul of the clear supernatant injected directly into the gas chromatograph. Gas chromatography was performed on a Shimadzu GC-17a (Shimadzu Corporation, Kyoto, Japan) using a BPX-21 megapore capillary column (25 m x 0.5 mm) (SGE, Victoria, Australia) with hydrogen flame ionization detection. Gas chromatograph conditions were as follows: injector temperature of 180°C, detector temperature of 220°C, column temperature of 110°C, and helium as the carrier gas at a flow rate of 30 ml/min. A standard SCFA mixture containing acetate, butyrate, and propionate was used for calculation and the results were expressed as umol/g faeces. The intra-assay CV for measurement of SCFA was 4.2%.

2.2.7 Differential leukocyte analysis

A single drop of blood was placed on the end of a microscope slide and smeared across creating a thin film of blood. The slide was then allowed to dry for 10 min. Following drying, the slide was then fixed and stained using Diff-Quik staining set (Lab

Aids Pty Ltd, Narrabeen, Australia). To obtain a differential leukocyte count, one hundred cells which were not overlapping were counted under a microscope at 10x power using oil immersion lens and each of the 5 types of WBCs (neutrophil, eosinophil, basophil, lymphocyte and monocyte) were scored. Total differential cell counts were calculated by multiplying the percentage of nucleated cells as assessed by the differential count on the blood smear by the total cell concentration obtained from coulter counter analysis (see 2.2.9).

2.2.8 T cell isolation

Thymus, spleen and/or lymph nodes (pooled from mesenteric site) were removed from anaesthetised animals and placed in a small volume of PBS containing 1% (v/v) fetal calf serum (FCS) (MultiSer^{TM}, Cytosystems, Castle Hill, NSW, Australia) which had been heat inactivated previously by incubating at 56°C for 45 minutes. The tissue was chopped finely with scissors and gently homogenised. The suspension was filtered through cotton wool and Ficoll-paque (2 ml) was overlayed with 5 ml of the homogenate and then centrifuged at 400 g for 20 min. The fluffy coat layer at the ficoll/media interface was removed and 10 ml of wash buffer added and the mixture was centrifuged at 180 x g for 5 minutes. The supernatant was discarded and the pellet resuspended in 10 ml of fresh wash buffer. After centrifugation (180 g, 5 min) the cells were finally resuspended in 1 ml of wash buffer.

2.2.9 Coulter counter analysis

Total cell concentration of leukocytes was determined by counting on Becton Dickinson FAC-Scan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, California). For cell suspensions or blood, 15 µl was diluted with 15 ml of

isoton (Coulter electronics limited) and 5 drops of Zap-oglobin (Coulter electronics limited) was added. The threshold was set to exclude cells with a volume of less than 120 μm^3.

2.2.9.1 Antibodies for labelling of cells for flow cytometric analyses

The following anti-rat mouse monoclonal antibodies were used for flow cytometry analysis: R-73 (anti-αβ T cell receptor), W3/25 (anti-CD4), OX-8 (anti-CD8), OX-33 (anti-CD45, B cell form), NKR-P1A (anti-natural killer cells). Dr Graham Mayrhofer (University of Adelaide, South Australia) kindly provided the antibodies.

The secondary antibody used for indirect immunofluorescent labelling of cells was Ig FITC-conjugate (PharMingen, USA, Cat No. 12064D).

2.2.9.2 Immunofluorescence staining and analysis

For single colour immunofluorescence, 1 x 10^6 cells were incubated in 0.05 ml of primary antibody in 10% normal rat serum for 60 min, with frequent mixing. The cells were washed twice in chilled wash buffer, resuspended in 0.05 ml of the secondary FITC-conjugated antibody diluted in wash buffer (1 in 100 with 10% normal rat serum, wash buffer) and incubated for 45 min, with frequent mixing. Cells were washed twice, resuspended in 1% paraformaldehyde in PBS, and stored in the dark at 4°C until analysed.

Cells were analysed on a Becton Dickinson FAC-Scan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, California) and antigen expression was measured using the Consort 30 data management and analysis program (Becton Dickinson), drawing light scatter gates around lymphocyte cell populations.

2.3 General DNA methods

2.3.1 Isolation of DNA

The DNA from liver and colonic mucosal scrapings was isolated using a High Pure PCR Template preparation kit (Boehringer Mannheim) followed by treatment with 10 mg/ml solution of RNAse that had been preheated to 100°C for 15 min and cooled slowly to room temperature. A Wizard Genomic DNA Purification kit (Promega) was also used for isolation of DNA.

2.3.2 Quantification of DNA

DNA samples were quantified using a Hoefer TKO 100 fluorometer. Briefly, a 4 point calibration curve was constructed using 2 ul aliquots of calf thymus DNA (Sigma, St Louis, MO) and dissolved in working buffer solution (1 mol/l NaCl, 100 mmol/l Tris.Cl pH 8, 10mmol/l EDTA, 0.1 ug/ml Hoecst dye). Samples were read in the fluorometer in 2 ml of working solution and the DNA concentration obtained by reading the sample absorbance from the 4 point DNA calf thymus calibration line.

2.3.3 CpG methylation measurement in DNA

Global colonic DNA methylation status at the CpG dinucleotides was assayed using a modification of the method described by Balaghi and Wagner (1993). Briefly, 2 ug of lyophilized DNA were incubated with 7.5 μCi S-adenosyl-[methyl ^{3}H]-methionine (15 Ci/mmol. Amersham) and 6 U Sss1 Methylase (CpG) (New England Biolabs) in a final volume of 30 μl in a reaction buffer containing 10 MM Tris-HCl, 120 mmol/l NaCl, 10 mmol/l EDTA and 1 mmol/l DTT, pH 7.9, for 4 h at 37°C. The reactions were performed in duplicate and sampled in duplicate onto Whatman DE-81 ion exchange cellulose filters

which were subsequently washed in 5% NaH_2PO_4 buffer for 1 hour dried, placed in a vial with 5 ml of aqueous scintillant and assayed. A higher degree of incorporation of [^{3}H] methyl groups reflects a lower degree of DNA methylation at CpG sites. The intra-assay CV for measurement of CpG was 6.1%.

2.3.4 HPLC measurement of DNA methylation

The DNA methylation status (% of 5-methylcytosine) was analysed by HPLC according to the method of Corvetta *et al.* (1991). Briefly, 100 ul of 90% (v/v) formic acid was added to 20 ug of isolated DNA in a glass culture tube. The capped tubes were heated at 180°C for 25 min. After cooling the samples were evaporated to dryness under nitrogen and the residue dissolved in sterile Milli-Q water. Twenty microlitres was injected into the HPLC. The mobile phase was 20 mmol/l Potassium phosphate (pH 4), 20 mmol/l hexanesulfonic acid, methanol, tetrahydrofuran (39:55:3:1.5:1.5), the column was a Hypersil ODS 250 mm x 4 mm and the flow rate was 0.8 ml/min. The diode detector was set at a wavelength of 280 nm. A standard mix of 5-methylcytosine and cytosine (Sigma, St Louis, MO) were used and the percentage of methylation was calculated from the content of 5-methylcytosine over 5-methylcytosine plus cytosine content multiplied by 100. The intra-assay CV for measurement of methylation was 10.5%.

The HPLC system used was from Shimadzu (Shimadzu Corporation, Kyoto, Japan). The system was equipped with a pump (Shimadzu, LC10AT), diode array detector (Shimadzu, SPD-MAVP) and an autoinjector (Shimadzu, SIL-10A). The analysis of HPLC chromatographs was performed with Shimadzu Class-LC10 software.

2.4 Histological methods

2.4.1 Aberrant crypt foci assay

ACF formation was assayed according to the method of McLellan and Bird (1988). Briefly, the colons were removed and flushed with PBS (pH 7.4) from the caecal end, expanded with 10% formalin-phosphate buffered saline for a few minutes, slit open longitudinally and fixed flat between filter papers in the same buffer. The colons were then stained briefly with 0.2% methylene blue and examined mucosal surface up under a light microscope at 40x magnification. The aberrant crypts were analysed between the distal Peyers patch and the beginning of the Herring bone musculature. Aberrant crypts were identified by alterations in size, shape of luminal opening and stain intensity according to the method of Bird (1987). Only crypts that met the above criteria were counted. Aberrant crypts were counted and scored as containing 1, 2 and ≥3 crypts/focus.

2.4.2 Classification of tumours

Intestinal tissues with attached tumours were dissected and placed into 10% formalin-phosphate buffered saline for storing. These tissues were subsequently processed (dehydrated, embedded in paraffin blocks, sectioned (4μm) stained with haematoxlin and eosin) for microscopic examination. Tumours were identified as to type and graded with regard to malignancy and penetration according to Duke's classification (Young *et al.* 1996) and classified as either adenoma or adenocarcinoma. Tumour incidence of a treatment group was the percentage of rats in that group which contained at least one tumour. Tumour burden was the number of tumours per treatment group. The size and growth of the colon tumours (tumour mass index) was measured according the following formula:

$$\text{Tumour mass index} = \log 10 \left[\sum_{i}^{n} \pi \left(D1 + D2 / 2 \right)2\right]$$

where D_1 and D_2 are the diameters of the tumour.

Chapter 3

Examination of Different Protocols to Modulate Folate Status

3.1 Introduction

Alterations in folate status can have an important impact on public health. An inadequate folate status during pregnancy, particularly around conception has been shown to increase the risk of miscarriage (Pietrzik and Bronstrup, 1997) and increase neural tube defects (Czeizel and Dudas, 1992). Folate supplementation may improve the folate status, which also decreases elevated total plasma homocysteine concentration, which has been shown to be an independent risk factor for cardiovascular disease (Boushey *et al.* 1995). Alterations in the folate status may also be important in carcinogenesis (Glynn and Albanes, 1994).

A variety of protocols have been proposed for the production of experimental folate deficiency in rats. Test diets used to study folate deficiency include semi-purified diets with casein (Tagbo and Hill, 1976) or gelatin (Bachevalier and Botez, 1978) as the protein sources or a combination of both of these protein sources (Richardson *et al.* 1979) along with the omission of folate from the vitamin mixture. Specific amino acid diets deplete in folate are an alternative to semi-purified diets (Walzem *et al.* 1983). Often sulfonamides are included with these diets to minimize intestinal synthesis of folate and further enhance the level of folate deficiency (Wright *et al.* 1945; Walzem and Clifford, 1988). Folate antagonists such as methotrexate in the diet are another protocol for disrupting folate metabolism and creating folate deficiency (Selhub *et al.* 1991). Test diets deficient in lipotropes choline and methionine can also increase the requirement of intracellular folate derived methyl groups and thereby lower intracellular folate levels (Horne *et al.* 1989). Chronic ethanol consumption has also been suggested to lead to the development of folate deficiency in humans (Sullivan and Herbert, 1964) as well as in rats (McMartin *et al.* 1989). Studies in rats have demonstrated the importance of the intestinal environment in the

biosynthesis of folate (Rong *et al.* 1991) as newly synthesised folate has been shown to be absorbed across the colon and utilized by the host. Studies also have demonstrated the importance of controlling coprophagy in the rat, as faecal folates may significantly contribute to the folate status of the rat (Abad and Gergory, 1987). Probiotics may also be a means of modulating folate status. Substantial quantities of folate may be produced by microorganisms in the colon (Cooperstock and Zedd, 1990; Denko *et al.* 1946). Bifidobacteria strains have been shown to increase folate status in cultures (Deguchi *et al.* 1985) and in the rat by increasing folate synthesis (Krause *et al.* 1996). Other probiotic strains such as Streptococcus thermophilus and Lactobacillus acidophilus have been shown to increase folate concentrations by more than 200% during fermentation of skim milk in culture (Littlefield *et al.* 1996). However some strains such as L. bulgaricus can utilise folate and reduce folate to negligible levels (Rao *et al.* 1984). It remains to be seen if these observations in culture can be reproduced in intact animals.

The objectives of this chapter were to investigate different protocols in modulating folate status in rats using a semi-purified basal diet consisting of 12% protein. Three feeding experiments with rats were used to assess alterations in folate status. In the first experiment a variety of probiotic bacteria were evaluated for there influence on folate status. In the second experiment, the effect of a sulfonamide (succinylsulfathiazole) which is known to interfere with folate metabolism was included in a folate deficient diet and fed to rats. Also in this experiment the effect of feeding potassium nitrite to rats was evaluated as a novel means of creating folate depletion. The third experiment of this chapter evaluated the effect of feeding a folate deficient diet which was also deficient or supplemented with methionine. A variety of markers were measured and these included haematocrit, blood folate concentrations, plasma homocysteine levels and animal growth.

3.2 Experimental design

3.2.1 Experiment 1: Probiotics

A total of 30 male Sprague-Dawley rats (70g body-weight) were purchased from the Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C ± 2 with a 12 h light: 12 h dark cycle. The animals aged 4 weeks old were randomly sorted into four dietary treatment groups of equivalent weight and fed *ad libitum* experimental powdered diets and given free access to distilled water for a period of eight weeks. Body weights were recorded weekly. The semi-purified experimental diets were modified forms of the AIN-93 (American Institute of Nutrition, 1993) diet and are shown in Table 3.1. Treatment 1 (Control) contained adequate folate at 2 mg/kg diet, treatment 2 (L.a) was identical to control with addition of 1% L.acidophilus. Treatment 3 (L.a/B.l) was identical to control with addition of 1% L.acidophilus and 1% Bifidobacterium longum. Treatment 4 (L.Rh) was identical to control with addition of 1% Lactobacillus Rhamnosus. Treatment 5 (L.Sh) was identical to control with addition of 1% L.casei Shirota. The probiotic bacteria were supplied by Dr Martin Playne, Dairy Research Laboratory, Highett, Victoria, Australia.

3.2.2 Experiment 2: Sulfonamides/Potassium nitrite

A total of forty-eight male Sprague-Dawley rats (70g) were purchased from the Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C ± 2 with a 12 h light: 12 h dark cycle. The animals aged 4 weeks old were randomly divided evenly into six dietary treatment groups and fed *ad libitum* experimental powdered diets and

given free access to distilled water for a period of twelve weeks. Body weights were recorded weekly. The semi-purified experimental diets were modified forms of the AIN-93 (American Institute of Nutrition, 1993) diet (Table 3.2). Treatment 1 (Control) contained adequate folate at 2 mg folate/kg diet. Treatment 2 (FD) contained no added folate. Treatment 3 (FD-1%S) contained no added folate and 1% succinylsulfathiazole (Sigma Chemical, St Louis, MO). Treatment 4 (CD) was choline deplete and contained adequate folate at 2 mg folate/kg diet. Treatment 5 (KNO_2) contained adequate folate at 2 mg/kg diet and potassium nitrite at 0.5% of diet. Treatment 6 (FD/KNO_2) was folate deplete and contained potassium nitrite at 0.5% of diet.

3.2.3 Experiment 3: Methionine and folate deplete/supplementation

A total of forty male Sprague-Dawley rats (70g) were purchased from the Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C ± 2 with a 12 h light: 12 h dark cycle. The animals aged 6 weeks old were randomly divided evenly into four dietary treatment groups and fed *ad libitum* experimental powdered diets and given free access to distilled water for a period of twelve weeks. Body weights were recorded weekly. The semi-purified experimental diets were modified forms of the AIN-93 diet (American Institute of Nutrition, 1993) (Table 3.3). Treatment 1 (Control) contained adequate folate at 2 mg/kg diet and 0.3% methionine, treatment 2 (FMD) contained no added folate or methionine, treatment 3 (FD) contained no added folate and 0.3% methionine, treatment 4 (FDMS) contained no added folate and supplemented with 0.6% methionine.

Table 3.1. Composition of experimental diets for experiment 1

Diet					
Ingredient (g/100g)	**Control**	**L.a**	**L.a/B.l**	**L. Rh**	**L.Sh**
Casein	14.3	14.3	14.3	14.3	14.3
Cellulose	5	5	5	5	5
Cornstarch	35.4	34.4	33.4	34.4	34.4
Sucrose	20	20	20	20	20
Fat (50% sunflowerseed oil: 50% lard)	20	20	20	20	20
AIN-vitamin mix[1]	1	1	1	1	1
Folic acid[2]	0.2	0.2	0.2	0.2	0.2
AIN-mineral mix[1]	3.5	3.5	3.5	3.5	3.5
L-methionine	0.3	0.3	0.3	0.3	0.3
Choline	0.2	0.2	0.2	0.2	0.2
Lactobacillus acidophilus	-	1	1	-	-
Bifidobacterium longum	-	-	1	-	-
Lactobacillus Rhamnosus	-	-	-	1	-
Lactobacillus casei Shirota	-	-	-	-	1

[1] The AIN vitamin and mineral mixture was based on the AIN-76 formula (AIN, 1977)

[2] Folic acid was prepared within the vitamin mixture at a level of 2 mg/kg diet.

Table 3.2 Composition of experimental diets for experiment 2

Diet						
Ingredient (g/100g)	**Control**	**FD**	**FD-1%S**	**CD**	**$KN0_2$**	**FD/$KN0_2$**
Casein	14.3	14.3	14.3	14.3	14.3	14.3
Cellulose	5	5	5	5	5	5
Cornstarch	35.4	35.4	34.4	35.4	34.9	34.9
Sucrose	20	20	20	20	20	20
Fat (sunflowerseed oil)	20	20	20	20	20	20
AIN-vitamin mix[1]	1	1	1	1	1	1
Folic acid[2]	0.2	-	-	0.2	0.2	-
AIN-mineral mix[1]	3.5	3.5	3.5	3.5	3.5	3.5
L-methionine	0.3	0.3	0.3	0.3	0.3	0.3
Choline	0.2	0.2	0.2	-	0.2	0.2
Succinylsulfathiazole	-	-	1	-	-	-
Potassium Nitrite	-	-	-	-	0.5	0.5

1 The AIN vitamin and mineral mixture was based on the AIN-76 formula (AIN, 1977)

2 Folic acid was prepared within the vitamin mixture at a level of 2 mg/kg diet.

Table 3.3 Composition of experimental diets for experiment 3

	Diet			
Ingredient (g/100g)	**Control**	**FMD**	**FD**	**FDMS**
Casein	14.3	14.3	14.3	14.3
Cellulose	5.0	5.0	5.0	5.0
Cornstarch	35.4	35.4	35.4	35.4
Sucrose	20.0	20.0	20.0	20.0
Sunflowerseed oil	20.0	20.0	20.0	20.0
AIN-vitamin mix[1]	1.0	1.0	1.0	1.0
AIN-mineral mix	3.5	3.5	3.5	3.5
L-methionine	0.3	0.0	0.3	0.6
Choline	0.2	0.2	0.2	0.2

[1] The vitamin and mineral mixtures were prepared according to the AIN-76 formula. The control diets contained 8 mg/kg folate, supplied through the vitamin mix.

3.2.4 Sample collection

The rats were killed by exsanguination while under halothane anesthesia after 12 weeks on experimental diets, blood was collected into EDTA treated tubes, haematocrits were determined, a portion was diluted with 1% ascorbic acid and stored at -80°C for subsequent folate analyses. The remaining blood was centrifuged at 1000 g for 10 minutes, plasma was collected and stored at -80°C for subsequent homocysteine measurements.

3.2.5 Statistical evaluation

The GraphPad Instat software package (version 2; San Diego, CA, USA) was used for statistical analysis of experimental data. Statistical significance of the mean values was tested by one way analysis of variance with the criterion for significance taken at $p<0.05$. Differences between the means were then subjected to the Tukey-Kramer multiple comparison tests with significance obtained at $p<0.05$.

3.3 Results

3.3.1 Growth of animals

In experiment 1, there was no significant difference between groups in the bodyweights of the animals (Figure 3.1). In experiment 2, the KNO_2 fed rats and the rats consuming succinylsulfathiazole (FD-1%S) weighed significantly less ($p<0.001$) than the other groups after 8 weeks on the experimental diets (Figure 3.2). In experiment 3, after 12 weeks on experimental diets, treatment 2 (FMD) gained significantly less weight ($p<0.05$) than the other treatment groups (Figure 3.3).

3.3.2 Haematocrit

The haematocrit values are shown in Table 3.4. No significant differences were observed between the treatment groups in experiment 1 and 3. Haematocrit values were significantly depleted ($p<0.001$) within the KNO_2 fed rats and the rats consuming succinylsulfathiazole (FD-1%S).

3.3.3 Blood folate concentration

The blood folate concentrations for experiments 1,2 and 3 are depicted in Table 3.5. There was no change in blood folate concentration between the dietary treatment groups in experiment 1. Blood folate concentration were significantly depleted ($p<0.001$) within the KNO_2 fed rats and the rats consuming succinylsulfathiazole (FD-1%S) showed the greatest depletion. In experiment 3, blood folate concentrations were significantly depleted in the three treatment groups that were depleted of folate, methionine depletion or supplementation did not further modulate blood folate levels.

3.3.4 Plasma homocysteine concentration

Plasma homocysteine concentrations are shown in Table 3.6. There was no change in the plasma homocysteine concentration in the treatment groups within experiment 1. In experiment 2, plasma homocysteine concentrations increased significantly ($p<0.01$) when folate was excluded from the diet (FD and FD/KNO_2). When succinylsulfathiazole at a level of 1% was also incorporated into a folate deficient diet (FD-1%S) there was a four-fold increase in plasma homocysteine concentration ($p<0.001$).

3.4 Discussion

In this chapter, different protocols for modulating folate status in rats have been examined. The basal diet used was a modified form of the semi-purified AIN-93 diet Reeves *et al.* 1993). This diet was modified to contain a lower level of protein (12%) supplied from vitamin free casein and a high level of fat (20%). The casein however, still contained a low level of endogenous folate (0.045ug/g). By modifying the protein level to 12%, the amount of folate supplied was minimized and by further supplementing the diet with 0.3% methionine the rats still maintained adequate growth when compared to rats consuming an AIN-93 diet (McIntosh *et al.* 1995) It is important to maintain a maximum growth rate and have a minimal dietary folate content in the protocol for producing experimental folate deficiency (Walzem and Clifford, 1988).

3.4.1 Experiment 1

In experiment 1, different probiotic bacteria strains were examined for their influence on folate status. There was no change in folate status with any of the probiotic bacterial strains. Studies by other researchers have demonstrated that folate status may be enhanced in rats with bifidobacteria (Krause *et al.* 1996) while other studies have shown that folate levels in the blood may be decreased with the consumption of different probiotic bacteria (McIntosh *et al.* 1999). In the present study, the probiotics were fed to the rats for a period of 8 weeks and this time frame may not have been long enough to observe any changes in folate status. Also folate at a level of 2 mg/kg diet was included in all of the diets and this level was able to maintain adequate folate status in all of the rats.

3.4.2 Experiment 2

In experiment 2, a folate deplete diet and a folate deplete diet with the inclusion of a sulfonamide were examined for their effects on folate status. Also examined were a diet deficient of choline and a diet containing potassium nitrite with and without folate.

By omitting folate from the basal diet, folate status was lowered in the rat evidenced by lower blood folate and elevated plasma homocysteine. The degree of folate deficiency created was only moderate, as there were no signs of anemia or any growth retardation. This moderate folate deficiency is in a similar range to that created by other researchers (Cravo *et al.* 1992; Kim *et al.* 1996) who have used specific amino acids deficient in folate as their protocol for creating experimental folate deficiency. By further including the sulfonamide drug, succinylsulfathiazole at a level of 1% combined with a folate deficient diet, severe folate deficiency could be achieved. Succinylsulfathiazole is a sulfonamide antibiotic drug, that acts by inhibiting the production of folate by the intestinal bacteria. After 4 weeks of inclusion of the sulfonamide with a folate deficient diet, significant growth retardation was observed with severe growth retardation after 8 weeks. This result is similar to that achieved by Walzem and Clifford (1988) with a specific amino acid diet deficient in folate and with 1% succinylsulfathiazole included. However, Walzem and Clifford (1988) were unable to demonstrate this observation when using a diet with a 10% or 20% casein diet deficient in folate with 1% succinylsulfathiazole included.

When a combined deficient diet (choline and folate) was fed to rats folate status was further compromised when compared to just consuming a folate deficient. However, no alteration in folate status was observed in rats when only a diet deficient in choline was consumed.

Nitrite ingestion has been shown to result in deficiency of certain vitamins (Phillips, 1966) and may effect folate bioavailability (Abu Khaled *et al.* 1986; Hoppner and Lampi,

1992). In this study, potassium nitrite was fed to rats at a level of 0.5% in the diet. Although there was significant anemia and growth retardation in rats associated with the consumption of potassium nitrite, folate status was not altered.

3.4.3 Experiment 3

The lowered folate status in this experiment was a result of the omission of folate from the diet. Dietary methionine at 0%, 0.3% and 0.6% had no effect on folate status in the rat.

A dietary deficiency of methionine significantly decreased the growth of the rats. This result was expected as it is well established that methionine through its role in protein synthesis is essential for the growth of animals. Studies by Yoshida *et al.* (1990) have demonstrated that the sulfur amino acid requirement of growing rats is 0.6%. The fall in bodyweight of the animals' fed the methionine deficient diet was accentuated by the low level of protein (12% protein) used in the test diet and sulfur amino acids not meeting the requirement.

3.5 Summary of experiments

Omission from the test diet of folate results in a reduction of folate status. The level of folate deficiency achieved with this protocol is a moderate deficiency and can be achieved within 4 weeks of consuming such a diet in young rats. To further enhance the level of experimental folate deficiency the inclusion of a sulfonamide is necessary to reduce colonic microflora production of folate. However the negative aspect associated with this sulfonamide inclusion is that after a period of more than 4 weeks, marked growth retardation occurs. Therefore if succinylsulfathiazole at a level of 1% combined with a folate deficient diet is to be fed to rats in longer term experiments, such as carcinogen

initiated intestinal cancer experiments that require a time frame around 24-30 weeks the maximum time for this inclusion should be 4 weeks. The ideal protocol from the current experiments for creating experimental folate deficiency is a modified AIN-93 diet consisting of 12% protein (casein based), 20% fat, 0.3% methionine and no added folate. This diet when fed to rats ensures adequate growth and sustains a moderate level of folate deficiency.

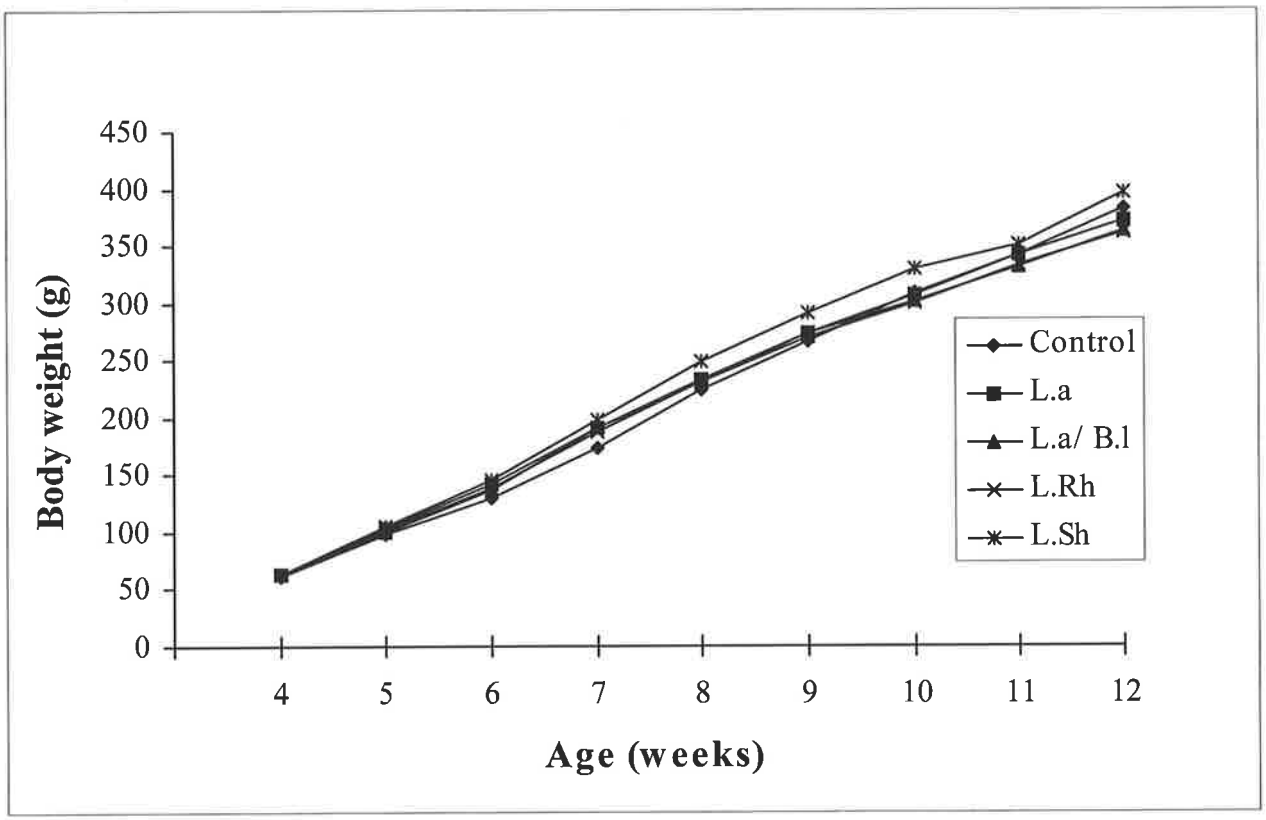


Figure 3.1 Growth curves of rats fed the different experimental diets

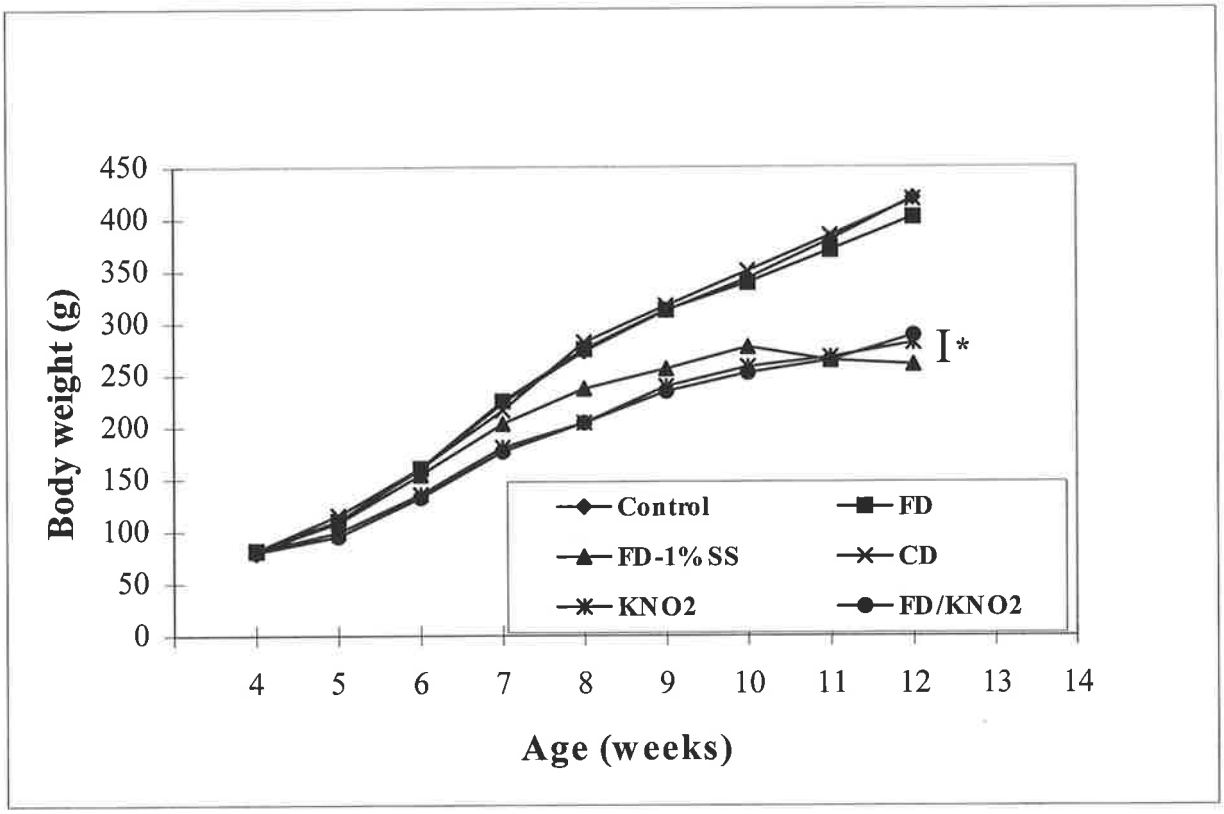


Figure 3.2 Growth curves of rats fed the different experimental diets

* FD-1% SS, KNO_2 and FD/ KNO_2 significantly different from Control, FD and CD.

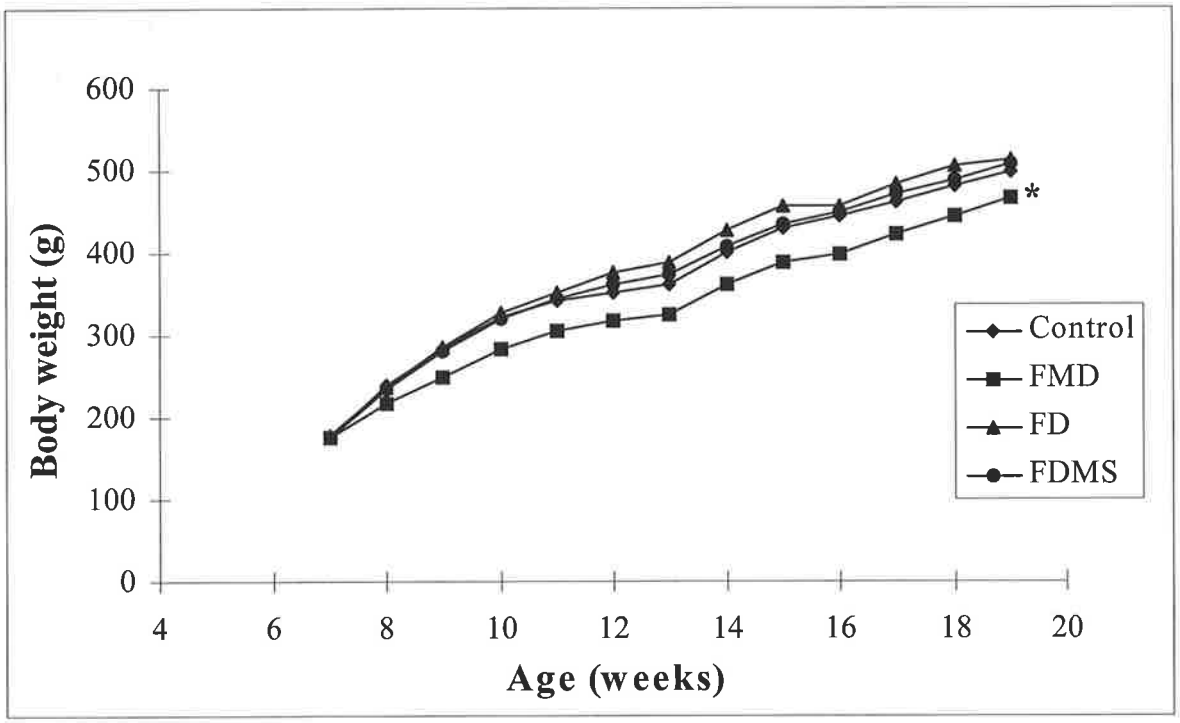


Figure 3.3 Growth curves of rats fed the different experimental diets

* FMD significantly different from all other groups ($p<0.05$).

Table 3.4 Haematocrit values of the rats fed different dietary treatments in experiment 1, 2 and 3.

Experiments	Dietary treatment	Haematocrit (%)
Experiment 1 (n=6)	Control	38.0 ± 1.0
	L.a	37.8 ± 0.9
	L.a/B.l	38.3 ± 0.7
	L.Rh	37.6 ± 0.6
	L.Sh	37.9 ± 0.6
Experiment 2 (n=8)	Control	39.5 ± 0.8
	FD	39.9 ± 1.1
	FD-1%S	27.4 ± 2.7**
	CD	39.1 ± 0.8
	KNO_2	34.9 ± 2.4*
	FD/KNO_2	34.0 ± 1.9*
Experiment 3 (n=10)	Control	37.3 ± 0.8
	FMD	37.3 ± 1.3
	FD	37.1 ± 0.9
	FDMS	37.0 ± 1.6

Values are mean ± SD, * $p<0.01$, ** $p<0.001$ from control.

Table 3.5 Blood folate concentrations of the rats fed different dietary treatments in experiment 1, 2 and 3.

Experiments	Dietary treatment	Blood folate concentration (ηg/ml)
Experiment 1 (n=6)	Control	783 ± 247
	L.a	796 ± 92
	L.a/B.l	789 ± 117
	L.Rh	692 ± 191
	L.Sh	880 ± 62
Experiment 2 (n=6)	Control	659 ± 133
	FD	161 ± 31**
	FD-1%S	92 ± 18**
	CD	713 ± 115
	KNO_2	681 ± 128
	FD/KNO_2	106 ± 17**
Experiment 3 (n=10)	Control	865 ± 116
	FMD	196 ± 35**
	FD	154 ± 27**
	FDMS	136 ± 20**

Values are mean ± SD, * $p<0.01$, ** $p<0.001$ from control.

Table 3.6 Plasma homocysteine concentrations of the rats fed different dietary treatments in experiment 1, 2 and 3.

Experiments	Dietary treatment	Plasma homocysteine concentration (μM)
Experiment 1 (n=6)	Control	2.0 ± 0.5
	L.a	2.2 ± 0.3
	L.a/B.l	2.3 ± 0.5
	L.Rh	2.4 ± 0.6
	L.Sh	2.0 ± 0.7
Experiment 2 (n=8)	Control	2.3 ± 0.4
	FD	3.9 ± 0.4*
	FD-1%S	9.0 ± 0.6**
	CD	2.0 ± 0.3
	KNO_2	1.4 ± 0.2
	FD/KNO_2	3.8 ± 0.5
Experiment 3 (n=10)	Control	1.9 ± 0.3
	FMD	4.2 ± 1.1**
	FD	3.6 ± 1.0*
	FDMS	4.2 ± 1.1**

Values are mean ± SD, * $p<0.01$, ** $p<0.001$ from control.

Chapter 4

Effect of Dietary Choline and Methionine Deficiencies Superimposed on Dietary Folate Deficiency on Folate Status in Rats Fed Proteins Based on Casein and Soy

4.1 Introduction

Folates are present in nearly all natural foods. However, the folate content can vary quite substantially between food sources. Foods with the highest folate content include yeast, liver, fresh green vegetables, fresh fruits and legumes. Soy protein which is consumed in large amounts by certain populations contains a quite substantial amount of folate (~128 μg/g). Conversely, casein which is a major component of milk, contains very low amounts of folate (~ 4.5 μg/g). Therefore the consumption of these food components is likely to impact on folate status quite differently.

The dietary component 'folate' functions in the transfer of methyl groups from donor compounds to biosynthetic pathways (Wagner, 1995). Other components such as choline, methionine, betaine and vitamin B_{12} (some of these are donors, some catalyse the effect) also play a central role in cellular metabolism through their regulation of the transfer and utilisation of methyl groups (Newberne and Rogers, 1986). A dietary deficiency of choline and methionine results in methyl group deficiency and if fed to rats for a substantial period of time may initiate hepatic carcinogenesis (Rogers, 1993) without any exogenous carcinogen introduction. Experimental animal evidence from Wilson *et al.* (1984) and Newberne and Rogers (1986) pointed toward hypomethylation of DNA as a mechanism involved in liver carcinogenesis. A dietary deficiency of folate has previously been shown to impact on folate status in the rat (See Chapter 3; Walzem and Clifford, 1988), however results from experimental studies involving folate deficiency and altered DNA methylation status are inconsistent (Kim *et al.* 1996; Wilson *et al.* 1984). The severity of the methyl group depletion may be crucial in order to induce changes in DNA methylation status.

The aim of the present study was firstly to compare the protein source casein with that of soy protein isolate. The impact of feeding these diets with and without folate added

on folate status was achieved by measuring blood folate levels and plasma homocysteine concentrations. A secondary objective was to examine the impact of methyl group deficiency on folate status and methylation status. To carry out this second aim, rats were fed diets which were progressively depleted of methyl groups (folate, choline and methionine) in both a casein and soy protein based diet. Methylation status was assessed by measuring S-adenosylmethionine and S-adenosylhomocysteine levels in the liver and colon as well as DNA methylation levels in the liver.

4.2 Experimental Design

4.2.1 Animals and diets

A total of forty-eight weanling male Sprague-Dawley rats (70g) were purchased from Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C with a 12 hour light : 12 hour dark cycle. Rats, at the age of 4 weeks were randomly divided into eight groups of six animals and fed experimental powdered diets and given *ad libitum* access to water for a period of eight weeks. Body weights were recorded weekly. Each rat was placed in a metabolic cage for 48 hours during the seventh week of the study. Food intake, faecal and urine outputs were recorded.

The experimental diets are shown in Table 4.1, and were modified forms of the AIN-93 (Reeves, 1993) semi-purified diet. They consisted of 12% protein, either casein (84% protein, Murray Goulburn, CO-OP, CO LTD, Sydney, Australia) or soy protein isolate (90% protein, Protein Technologies International, St. Louis, MO), 5% alpha-cellulose as dietary fibre, 35% starch, 20% sunflower seed oil as the fat source, 20% sucrose, 3.5% AIN-76

minerals, 1% AIN-76 vitamins. The nutrient replete diets contained 2 mg/kg folic acid, 0.2% choline and 0.4% methionine.

The amino acid composition of casein and soy protein isolate were measured using an AminoQuant II amino acid analyzer (Hewlett-Packard) and results are shown in Table 4.2. Casein provided 38% more sulfur amino acids than SPI, mainly as methionine. The casein protein contained 0.045 mg/kg endogenous folate and the soy protein contained 1.28 mg/kg endogenous folate, when analysed by the L.casei assay, (Chemistry Centre, WA).

This protocol was approved by the Animal Care and Ethics Committee in the CSIRO, Division of Human Nutrition.

4.2.2 Sample collection

After 4 weeks on experimental diets blood samples were collected via the tail vein under ether anaesthesia. Blood was collected into EDTA tubes and immediately diluted 1:1 with fresh 1% ascorbic acid and stored at -80°C for folate determinations.

After 8 weeks on experimental diets the rats were killed by exsanguination under ether anaesthesia. Blood was collected into EDTA tubes, a portion was diluted with 1% ascorbic acid for folate analyses, the remaining blood was centrifuged at 1000 g for 10 minutes, plasma was collected and stored at -80°C for subsequent homocysteine measurements. Livers were excised, weighed and frozen in liquid nitrogen and stored at -80°C. A portion of liver and colonic mucosal scrapings was homogenized in 0.4% perchloric acid, centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was collected and stored at -80°C for subsequent SAM and SAH analysis.

Table 4.1. Composition of experimental diets

Diet								
Ingredient (g/100g)	Casein - control	cas-FD	cas-FCD	cas-FCMD	SPI-control	SPI-FD	SPI-FCD	SPI-FCMD
Casein	14.3	14.3	14.3	14.3	-	-	-	-
Soy protein isolate	-	-	-	-	13.3	13.3	13.3	13.3
Cellulose	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cornstarch	35.4	35.4	35.6	36.0	36.4	36.4	36.6	37.0
Sucrose	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sunflowerseed oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
AIN-vitamin mix[1,2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
AIN-mineral mix[1]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
L-methionine	0.4	0.4	0.4	-	0.4	0.4	0.4	-
Choline	0.2	0.2	-	-	0.2	0.2	-	-
L-cystine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

[1] The vitamin and mineral mixtures were prepared according to the AIN-76 formula. The control diets contained 2mg/kg folate, supplied through the vitamin mix.

[2] Control diets casein and soy protein isolate (SPI) contained folic acid only.

FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

Table 4.2. Amino acid composition of casein and soy protein isolate

Amino acid (AA)	Casein (gAA/100g sample)	Soy protein isolate (g/100g sample)
Alanine	2.10	2.21
Arginine	2.97	4.51
Aspartic acid	5.27	6.44
Cysteine	0.30	0.73
Glutamic acid	16.61	10.65
Glycine	1.22	2.07
Histidine	2.32	1.45
Isoleucine	4.05	2.80
Leucine	7.21	4.55
Lysine	5.88	3.36
Methionine	2.25	0.85
Phenylalanine	4.03	3.17
Proline	10.63	3.71
Serine	4.08	2.88
Threonine	3.32	2.25
Tyrosine	4.59	2.33
Valine	4.95	2.79

4.2.3 Statistical evaluation

The GraphPad Instat software package (version 2; San Diego, CA, USA) was used for statistical analysis of experimental data. Statistical significance of the mean values was tested by one way analysis of variance with the criterion for significance taken at $p<0.05$. Differences between the means were then subjected to the Tukey-Kramer multiple comparison test with significance obtained at $p<0.05$.

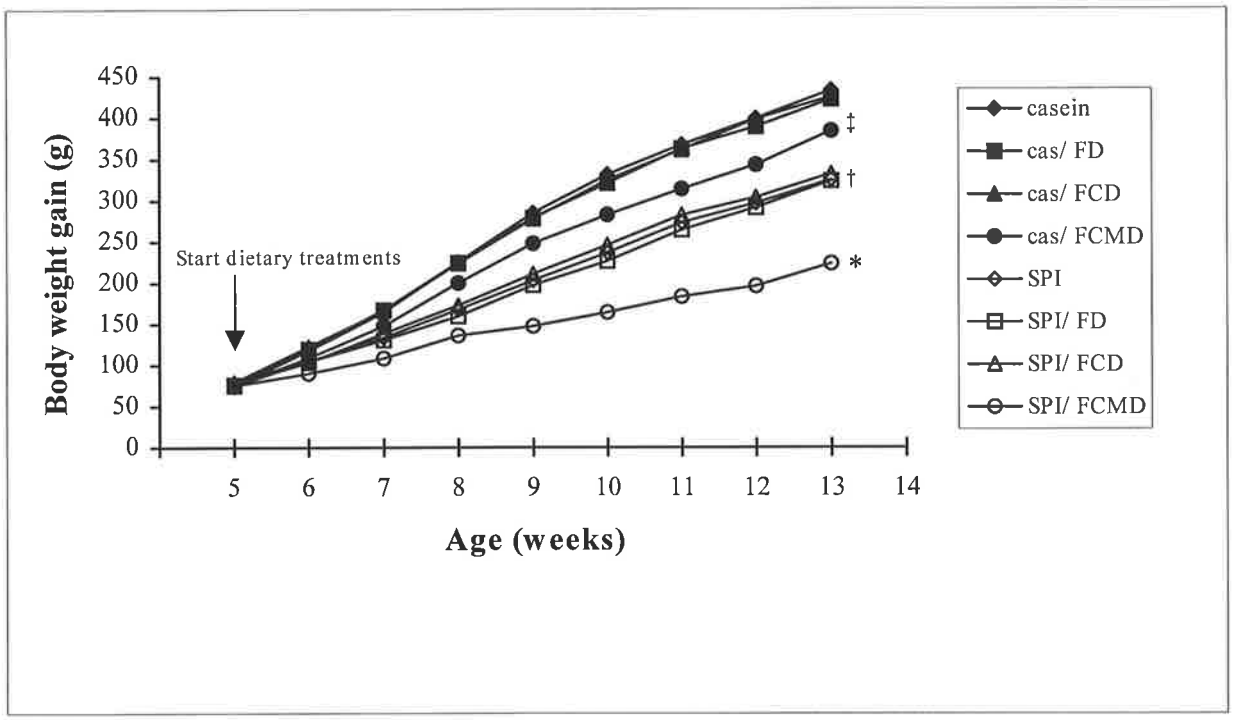
4.3 Results

4.3.1 Food consumption and growth

The mean daily food consumption of the animals fed the different dietary treatments were as follows (mean ± sd): casein- 17.4 g ± 1.8, casein/FD- 16.2 g ± 2.3, casein/FCD- 17.8 g ± 2.2, casein/FCMD- 18.0 g ± 1.1, SPI- 14.5g ± 2.2, SPI/FD- 15.5 g ± 1.0, SPI/FCD- 14.1 g ± 1.3, SPI/FCMD- 14.0 g ± 2.0. Depleting the experimental diets of folate, choline and methionine had no significant effect on the food intake of the animals. However, the rats fed the casein background diets consumed significantly more food per day (3 g/day) ($p<0.001$).

Dietary lack of folate and choline had no influence on the growth of the experimental animals (Figure 4.1). Lack of dietary methionine resulted in significantly reduced weight gains in both casein and SPI fed animals with this being more evident with the SPI treatment (33% fall) compared to the casein treatments (10% fall). The final body weights of the casein fed rats were 25% heavier than the SPI fed rats ($p<0.001$) this result being independent of the methionine effect.

Figure 4.1. Body weight growth curves of rats fed experimental diets



Average bodyweights of 6 rats. * SPI/FCMD significantly different from other groups ($p<0.001$), † SPI, SPI/FD and SPI/FCD significantly different from all casein groups ($p<0.001$), ‡ cas/FCMD significantly different from casein, cas/FD and cas/FCD.

SPI-soy protein isolate, FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

4.3.2 Folate status and haematocrit

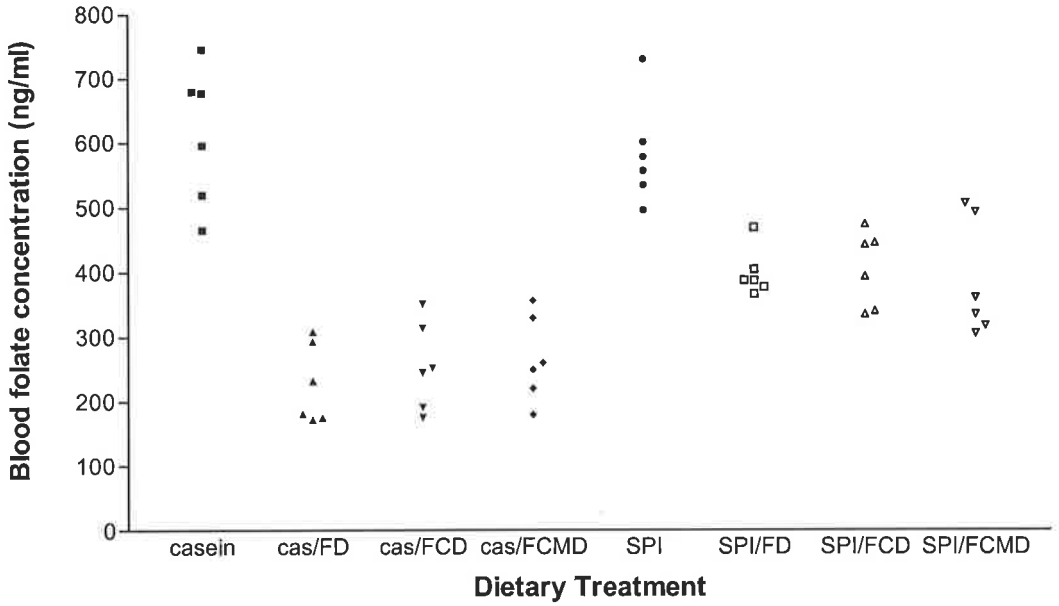
Figure 4.2 shows the effect of feeding rats folate deficient experimental diets on whole blood folate concentration after 4 and 8 weeks. As observed, those dietary treatments not receiving folate had significantly lower concentrations of folate in their whole blood. After 4 weeks, significant depletion ($p<0.01$) of folate concentrations was evident. In the casein fed animals folate concentrations fell by 60%, while in the SPI fed animals a fall of 32% was observed when compared to their control treatments. Feeding the diets for a further 4 weeks (total of 8 weeks) resulted in only minor additional reductions in folate concentrations. Depleting the diets progressively with choline and methionine on top of the folate deficit seemed to have no additional effect on the folate status in the whole blood.

Haematocrit values (Figure 4.3) measured after 8 weeks of feeding, showed that rats fed the casein/FCMD diet had a 8% lower ($p<0.05$) haematocrit than those fed all other diets except the SPI/FD diet. Overall, despite halving of folate levels, changes in haematocrit were small.

4.3.3 Plasma homocysteine levels

The mean plasma homocysteine concentrations are shown in Figure 4.4. Plasma homocysteine concentrations were significantly elevated ($p<0.0001$) in the casein fed rats fed which consumed the methyl group deficient treatments. A significant negative correlation was observed in the casein fed rats between plasma homocysteine concentration and decreasing methyl content of the diet ($r^2=0.978$, $p=0.002$). Neither a significant correlation nor an elevation of plasma homocysteine was observed with the corresponding SPI treatments.

After 4 weeks



After 8 weeks

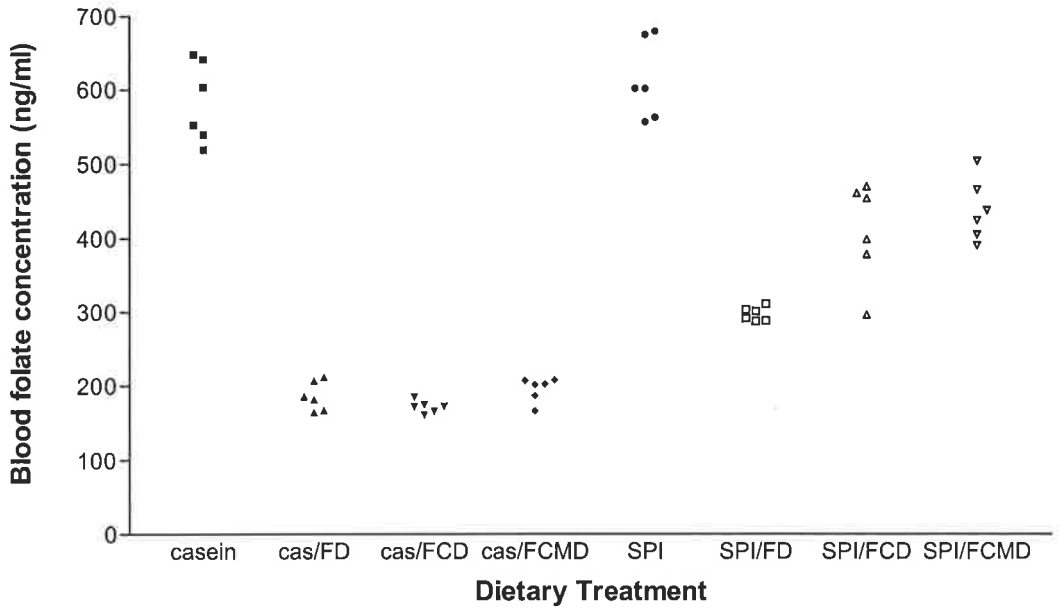


Figure 4.2 Effect of folate, choline and methionine deficiencies on whole blood folate concentration after 4 and 8 weeks in rats fed casein or SPI diets deficient in folate, choline and methionine. SPI-soy protein isolate, FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

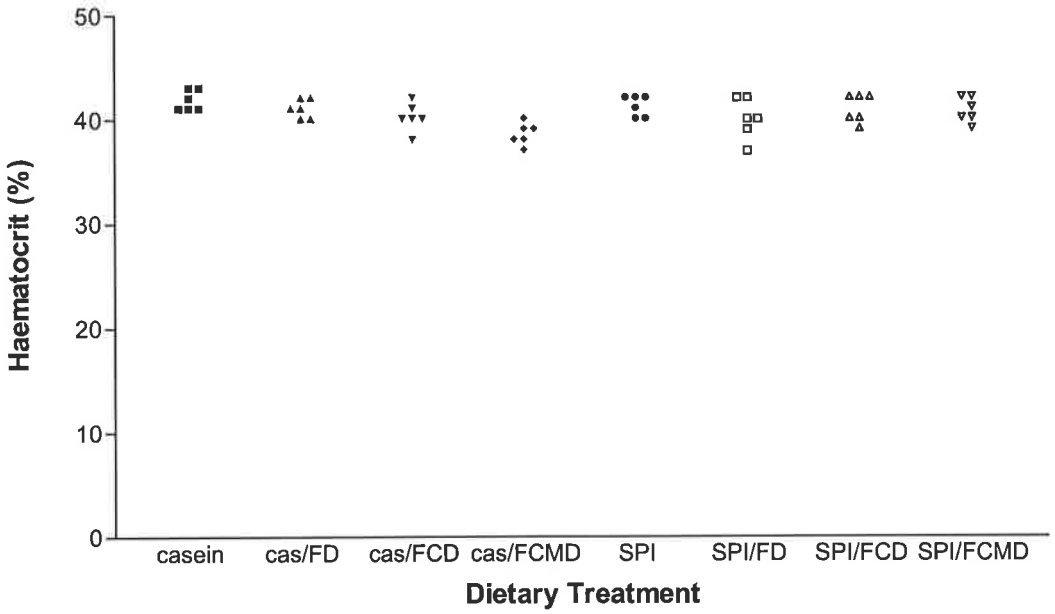


Figure 4.3 Effect of folate, choline and methionine deficiencies on haematocrit values after 8 weeks in rats fed casein or SPI diets deficient in folate, choline and methionine. SPI-soy protein isolate, FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

Table 4.3 Concentrations of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in hepatic and colon tissue*

Group	Hepatic SAM	Hepatic SAH	Hepatic SAM/SAH	Colon SAM	Colon SAH	Colon SAM/SAH
	nmol/g liver			nmol/g colon		
casein (cas)	83.8 ± 19.9	19.0 ± 4.5	4.5 ± 0.1^{ab}	34.0 ± 2.9	3.5 ± 0.4	9.1 ± 1.6
cas / FD	61.2 ± 15.5	19.2 ± 4.1	3.1 ± 0.2^{b}	35.8 ± 6.1	4.2 ± 0.8	8.5 ± 0.4
cas / FCD	71.3 ± 15.1	35.1 ± 7.7	2.2 ± 0.2^{c}	36.1 ± 2.9	7.3 ± 0.4	5.2 ± 0.8
cas / FCMD	41.4 ± 6.1	24.7 ± 4.9	1.8 ± 0.1^{c}	39.4 ± 4.5	5.9 ± 0.4	7.1 ± 0.8
SPI	135.2 ± 24.9	29.6 ± 5.7	4.7 ± 0.5^{a}	39.3 ± 4.9	7.4 ± 1.9	6.3 ± 1.6
SPI / FD	89.0 ± 24.9	24.0 ± 9.4	4.4 ± 0.5^{ab}	32.5 ± 4.1	4.5 ± 0.4	7.9 ± 1.6
SPI / FCD	91.5 ± 18.4	26.2 ± 4.1	3.4 ± 0.4^{bc}	26.6 ± 2.9	5.1 ± 0.8	5.5 ± 0.4
SPI / FCMD	66.0 ± 17.9	26.4 ± 6.9	2.4 ± 0.2^{c}	41.9 ± 6.5	4.6 ± 0.8	9.5 ± 2.0

*Values are means ± SEM of six rats. Values in the same column not sharing a common superscript letter are significantly different. FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

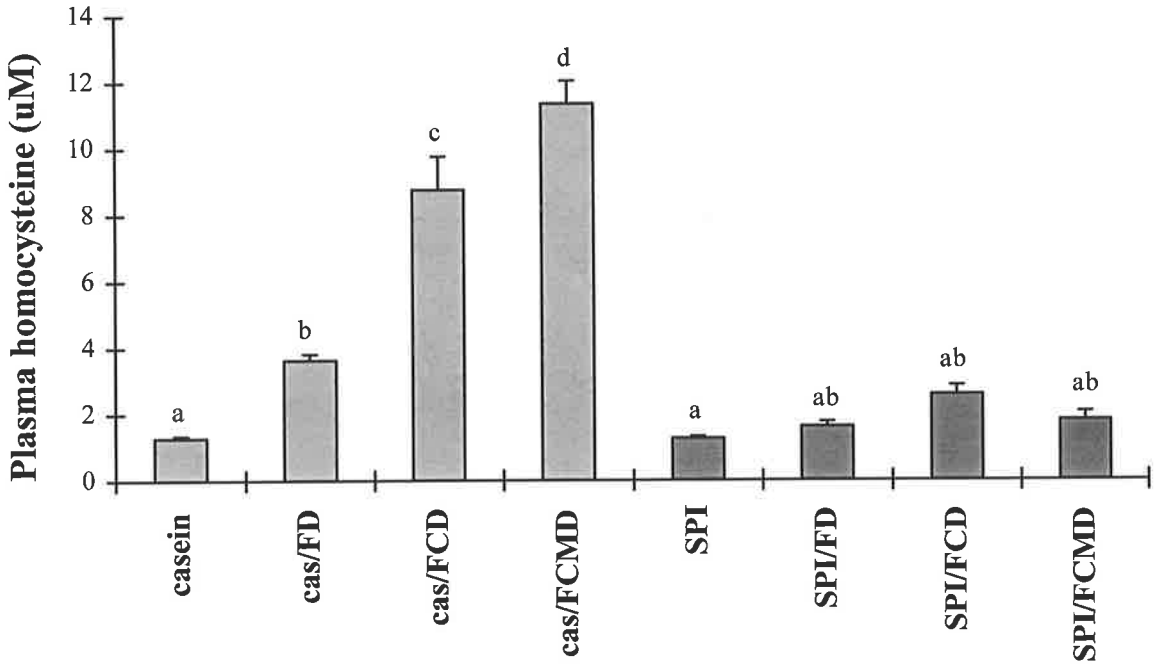


Figure 4.4 Plasma homocysteine concentrations after 8 weeks in rats fed casein or SPI diets deficient in folate, choline and methionine. Values are means ± SEM of six rats. Values within each column with different superscript letters are significantly different ($p<0.05$). SPI-soy protein isolate, FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

4.3.4 SAM/SAH levels

Table 4.3 shows the effect of depleting the diets progressively of folate, choline and methionine on mean hepatic and colonic SAM and SAH concentrations including the SAM: SAH ratio. In the liver there was a consistent pattern observed within the casein and SPI fed rats with SAM levels being the highest in the control treatments and lowest in the treatments depleted of folate, choline and methionine. The ratio of SAM to SAH (suggested to be a reliable indicator of the methylation capacity of cells)(Hoffman *et al.* 1980) fell progressively in the liver from 4.5 in the control treatment to about 2 in the treatment groups depleted of folate, choline and methionine. There were no significant changes observed in SAM or SAH or the ratio in the colonic mucosa.

4.3.5 DNA methylation

The percentage of 5-methylcytosine content in hepatic DNA is shown in Table 4.5. Hypomethylation of DNA, that is a decrease in the level of 5-methylcytosine was observed in the casein groups with lowered folate status. This observation was significant ($p<0.05$) in the casein treatments depleted of FCD and FCMD, with a greater than 20% fall in methylation. There was no significant change in methylation relative to control in the SPI fed rats.

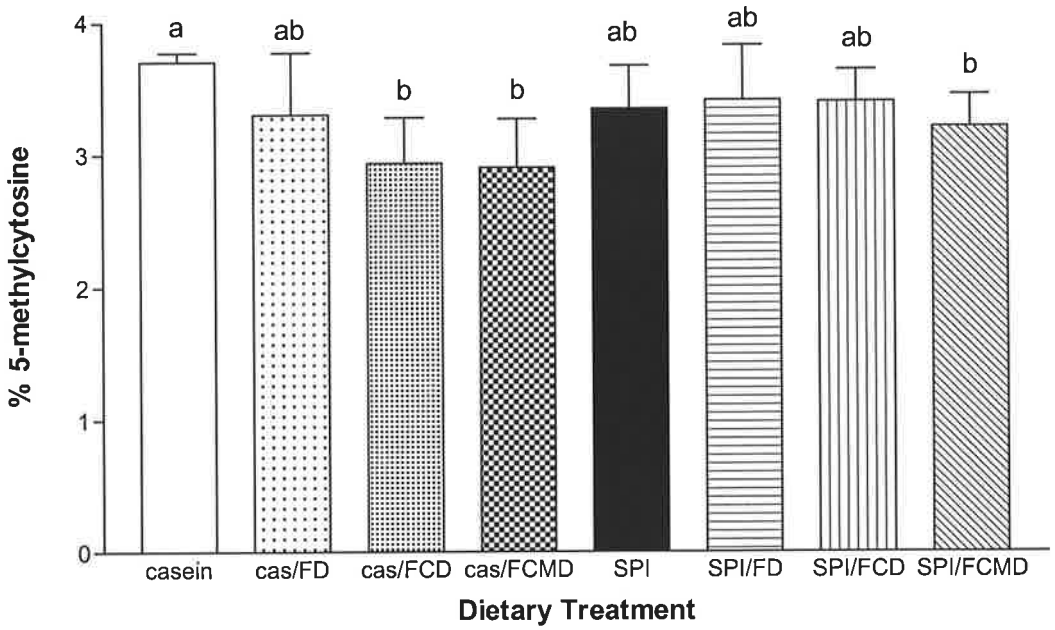


Figure 4.5 Percentage of 5-methylcytosine in hepatic DNA*

*Values are means ± SD of six rats. Values in the same column not sharing a common superscript letter are significantly different. SPI-soy protein isolate, FD-folate deficient, FCD-folate, choline deficient, FCMD-folate, choline, methionine deficient.

4.4 Discussion

The results from the experiment reported in this chapter show that blood folate concentrations in rats can be significantly reduced after only four weeks of feeding a semi-purified diet with no added folic acid. The degree of folate depletion achieved with the casein fed rats is within the range reported by other researchers (Cravo *et al.* 1992, Kim *et al.* 1996) and would be regarded as a moderate folate depletion, as haematocrits were only marginally affected and body weight gain of the animals was not compromised. It should be noted that the present study achieved this depletion in rats using a diet deficient in folate and with casein as the protein source, whereas other studies (Cravo *et al.* 1992, Kim *et al.* 1996, Miller *et al.* 1994) have created the folate depletion using a specific amino acid defined diet deficient of folate. The failure to produce significant hematological change is consistent with the knowledge that the rat is resistant to such changes, relative to other species studied (Friedrich, 1988).

The level of folate depletion achieved was significantly greater in the casein fed rats consuming methyl deficient diets than the corresponding SPI fed rats. This most likely reflects the much higher amount of endogenous folate present within the soy protein isolate (1.28 mg/kg compared with 0.045 mg/kg for casein). The higher body weight gain of the casein fed rats may also have contributed to the slight difference in folate depletion levels. Although the sulfur amino acid content of the diets were balanced, the casein fed rats had a much greater body weight growth over the 8 week period compared to the SPI fed rats. This may be partly explained by the slightly increased food consumption of the casein fed rats and may also indicate that casein is a superior quality protein to SPI for rat growth. As casein has been shown to contain a higher level of methionine but also a higher content of

other essential amino acids, isoleucine and threonine which are inadequate in SPI (Yoshida, 1990).

Homocysteine concentrations in the plasma were significantly elevated in the casein treatments with deficient levels of folate, as well as in the treatments deficient in choline and methionine. Interestingly, homocysteine levels were not increased in any of the SPI treatments. Plasma homocysteine levels have been shown by other researchers to be inversely correlated with intracellular folate due to the requirement of folate and its co-enzymes (Kim *et al.* 1996). The remethylation of homocysteine to methionine is dependent on two main pathways. The first pathway requires folate and its co-enzymes with vitamin B-12 containing 5-methyl-tetrahydrofolate: homocysteine methyltransferase catalysing the reaction (Selhub and Miller, 1992). The other pathway in which homocysteine is remethylated utilizes choline. Here choline is oxidised to betaine which serves as the methyl donor in a reaction catalysed by betaine:homocysteine methyltransferase (Finkelstein *et al.* 1982). The occurrence of hyperhomocysteinemia in the casein fed rats depleted of dietary methyl groups suggests a functional or biochemical deficiency is present within those treatments and that a disruption in one or both of the above pathways has occurred. Since plasma homocysteine levels were not significantly elevated in the SPI treatments fed depleted methyl group diets despite the fact that blood folate levels were depleted, it suggests that there are endogenous factor(s) within SPI that are preventing a functional deficiency of folate. The most obvious factor is folate, as this was present in SPI at over 28 times the concentration as in the casein. The elevation of plasma homocysteine observed in the casein fed rats depleted of folate, choline and methionine may also be of importance in terms of risk of cardiovascular disease. Elevated levels of plasma homocysteine have been identified as being an independent risk factor for premature vascular disease (Kang *et al.* 1992).

Our results showed that the ratio of SAM to SAH (known as the methylation ratio) in the liver was modified by these diets. The methylation ratio decreased from the control values as the methyl content of the dietary treatments decreased. This suggests that a progressive disruption of the transmethylation cycle had taken place (Cravo *et al.* 1996). As expected, the lowest value occurred within the casein treatment depleted of folate, choline and methionine (cas/FCMD). This group also had the highest plasma homocysteine concentration suggesting that the greatest stress on the transmethylation cycle occurred as a result of this combined deficiency treatment. There was no significant effect of feeding methyl deficient diets on the methylation ratio within the colon mucosa. This result is consistent with other researchers (Kim *et al.* 1995, Kim *et al.* 1996), who also found no significant regional or peripheral effect of moderate folate deficiency on SAM or the methylation ratio. In the present study the methyl deficient diets were only fed for a period of 8 weeks and this may have been insufficient for significant changes to take place within the colonic tissue. Another possibility is that there may be local production of folate taking place associated with large intestinal bacterial fermentation, as it has been demonstrated by other researchers (Rong *et al.* 1991) that local intestinal production of folate may incorporated back into the host's tissue stores of folate. If this is so, then combining a folate deficiency regime with an antibiotic agent capable of inhibiting such gut flora would have a more profound effect.

A disruption of the transmethylation cycle in the liver was observed in the casein fed rats, as indicated by the hypomethylation of hepatic DNA. This decrease in methylation was not observed in the corresponding SPI treated rats that also had a lesser degree of folate depletion. Altered DNA methylation has been proposed to be an early step in the multistep process of tumourigenesis, as changes in DNA methylation can lead to altered regulation of gene expression (Fearon and Vogelstein, 1990). Induction of DNA hypomethylation could

then be a mechanism by which diminished folate status acts to enhance carcinogenesis (Mason, 1994). Hypomethylation of hepatic DNA has been observed in rats fed methyl-deficient diets (Wilson *et al.* 1984; Locker *et al.* 1986; Wainfan *et al.* 1989). The data presented here confirm that a folate deficit from the diet alone was unable to significantly alter DNA methylation within the time frame studied. However, when choline and methionine deficits from the diet were superimposed on a folate deficit, hypomethylation of hepatic DNA was observed.

In summary, the present study has demonstrated that feeding diets depleted of folate can lower folate status. The source of protein fed to the animals is important in influencing the degree of folate depletion. As the amount of folate contained in different protein sources can vary quite substantially. Casein as a protein source contains very low levels of folate and when fed to rats as part of an AIN diet preparation with no folate added a much greater level depletion of folate status can be achieved when compared to feeding SPI as the protein source. Deficiencies of choline and methionine superimposed on a dietary folate deficiency can further enhance the level of functional folate deficiency. This was evident in the casein fed animals, where increased levels of homocysteine in the plasma were observed, an observation not seen in SPI fed animals. Feeding a SPI diet to the rats allowed an adequate level of DNA methylation to be maintained during methyl group depletion. A folate deficit in the diet alone is unable to induce liver DNA hypomethylation, however if choline and/or methionine deficiencies are superimposed hepatic hypomethylation may be observed, but only in casein fed rats. Hypomethylation of DNA seems to occur only when a marked level of folate deficiency occurs or a more severe methyl group depletion. The source of dietary protein or its purity is important in influencing these metabolic parameters, which is a reflection of the amount of endogenous folate present within the protein source.

Chapter 5

Effect of Moderate and Severe Folate Depletion on the Formation of Colonic Precancer Lesions and DNA Methylation

5.1 Introduction

The experimental animal evidence examining the effect of folate status on carcinogenesis is conflicting. These conflicting results may be due to variations in the animal model used and/or experimental design. The evidence surrounding colorectal cancer and folate status is sparse and also contradictory. Studies examining the effect of dietary folate on the development of precancerous aberrant crypt foci suggest either a promotional lesions (Wargovich *et al.* 1992) or no effect (Shivapurkar *et al.* 1995) . Reddy *et al.* (1996) showed that folate supplementation above normal dietary levels had no effect on AOM-induced colon tumour incidence in male F344 rats, however tumour size and multiplicity was increased. While other researchers suggest a chemopreventive role of dietary folate adequacy against the development of colonic dysplasia and cancer relative to folate deficiency(Cravo *et al.* 1992; Kim *et al.* 1996). A number of potential mechanisms have been proposed by which a diminished folate status may enhance colorectal carcinogenesis (Mason, 1994), a possible candidate mechanism is induction of hypomethylation of DNA. Modulation of the immune system may also be another mechanism by which folate deficiency alters carcinogenesis (Mason, 1994).

The purpose of this study was to determine the effect of a diminished folate status on the development of colonic pre-neoplastic aberrant crypt foci (ACF) induced by azoxymethane (AOM) in male Sprague-Dawley rats. To achieve this aim, rats were fed modified AIN semi-purified diets that were designed to alter folate status to either a moderate or a severe level of folate deficiency. Succinylsulfathiazole (a non-absorbable antibiotic drug) was employed in some dietary treatments to further enhance the level of folate depletion. The protocol for creating folate deficiency was based on that established in Chapter 3. The degree of folate deficiency was enhanced either prior to initiation of

carcinogenesis in one treatment group and also during the last four weeks of the study, while in another treatment a moderate level of folate deficiency was maintained throughout the study. Further objectives were to test if DNA methylation status was altered as a result of folate depletion and to examine the quantitative effects of folate deficiency upon lymphocyte sub-populations in the thymus, spleen and mesenteric lymph nodes.

5.2 Experimental design

5.2.1 Animals and diets

A total of forty-eight weanling male Sprague-Dawley rats (70g) were purchased from the Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C ± 2 with a 12 h light: 12 h dark cycle. Rats, at the age of 4 weeks were randomly divided into four dietary treatment groups of twelve animals and fed *ad libitum* experimental powdered diets and given free access to distilled water for a period of twelve weeks. Body weights were recorded weekly. After 4 weeks on experimental diets, animals were injected with AOM (Sigma Chemical, St Louis, MO) dissolved in normal saline at a dosage of 15 mg/kg body weight once weekly for 3 weeks. The rats were euthanased 8 weeks after the first AOM injection. The experimental diets were modified forms of the AIN-93 (Reeves *et al.* 1993) semi-purified diet (Table 5.1). Treatment 1 (control) contained 8 mg folate/kg diet. Treatment 2 (FD) contained no added dietary. Treatment 3 (FD1) consumed FD as well as 1% succinylsulfathiazole (Sigma Chemical, St Louis, MO) for the first 4 weeks of the study. Treatment 4 (FD2) consumed FD as well as 1% succinylsulfathiazole for the last 4 weeks of the study. The experimental protocol is shown

in Figure 5.1. The rationale for the different treatment group is as follows. Treatment 1 was the control group for the study representing an adequate folate status. Treatment 2 (FD) will diminish folate status in the rats to a moderate folate deficiency level. Treatment 3 (FD1) will create a more extreme level of folate deficiency prior to induction of carcinogenesis with AOM, the animals will then revert back to a moderate level of folate deficiency. Treatment 4 (FD2) will allow examination of the effect of a more severe level of folate deficiency during the later 4 weeks of the study, while a moderate decrease in folate status will be maintained throughout the early stages of the study (ie. similar to FD).

The experimental protocol and use of rats was approved by the CSIRO, Human Nutrition, Animal Experimentation and Ethics Committee prior to commencing.

5.2.2 Sample collection

The rats were killed by exsanguination while under halothane anesthesia after 12 weeks on experimental diets, blood was collected into EDTA treated tubes, a portion was diluted with 1% ascorbic acid and stored at -80°C for subsequent folate analyses. The remaining blood was centrifuged at 1000 g for 10 minutes, plasma was collected and stored at -80°C for subsequent homocysteine measurements. A small portion of colon (approximate length of 4 cm) (distal to the Peyers Patch) and liver (approximate weight of 0.5 g) were frozen in liquid nitrogen and stored at -80°C for subsequent DNA extraction and DNA methylation analysis. The remaining length of colon was fixed and stored in 10% formalin-phosphate buffered saline prior to ACF counting.

Table 5.1. Composition of experimental diets

Ingredient (g/100g)	Control diet	Folate deplete (FD) diet
Casein	14.3	14.3
Cellulose	5	5
Cornstarch	35.4	35.4
Sucrose	20	20
Fat (50% sunflowerseed oil: 50% lard)	20	20
AIN-vitamin mix*	1	1
AIN-mineral mix	3.5	3.5
L-methionine	0.3	0.3
Choline	0.2	0.2
1% succinylsulfathiazole	-	-

* The Control treatment contained folate at 8 mg/kg diet. The FD, FD1 and FD2 treatments contained no added folate in their vitamin mix. Treatment FD1 also contained 1% succinylsulfathiazole only for the first 4 weeks of the study, FD2 contained 1% succinylsulfathiazole only for the last 4 weeks of the study.

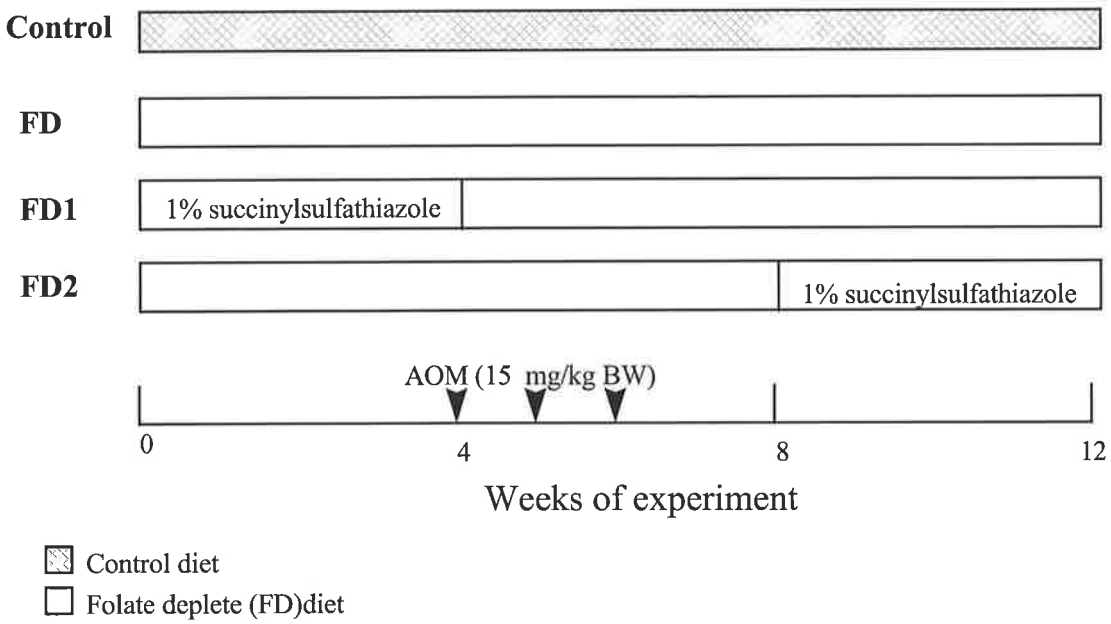


Figure. 5.1. Diagrammatic representation of experimental protocol. FD1, contained 1% succinylsulfathiazole only for the first 4 weeks of the study, FD2 contained 1% succinylsulfathiazole only for the last 4 weeks of the study.

5.2.3 Statistical analysis

The GraphPad Prism software package (version 2; San Diego, CA, USA) was used for statistical analysis of experimental data. Differences between the control and treatment groups were analysed by ANOVA. Differences were considered statistically significant at $p<0.05$.

5.3 Results

5.3.1 Body Weight

At the time of death the animals fed the folate deficient diet plus the 1% succinylsulfathiazole for the last 4 weeks of the study (FD2) had a significantly lower body weight than the other three treatments ($p<0.05$). The final body weights (g) were as follows: (mean ± SEM); Control (494±13), FD (473±12), FD1 (478±13), FD2 (411±10).

5.3.2 Folate status, hematological parameters, plasma homocysteine and hepatic S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations

Table 5.2 shows that feeding folate deficient diets significantly lowered the folate concentrations in the whole blood of the animals ($p<0.001$). Administration of 1% succinylsulfathiazole for the final 4 weeks of the study (FD2-treatment) resulted in the greatest decrease in folate whole blood concentration. Hematocrit values and total leukocyte counts (Table 5.2) within the FD2 treated rats were significantly reduced ($p<0.001$) when compared to the other treatment groups. Plasma homocysteine concentrations (Table 5.2) were significantly elevated ($p<0.001$) in the animals fed the

Table 5.2. Effect of folate depletion and repletion on folate status, hematological parameters and plasma homocysteine and hepatic S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) concentrations, hepatic and colonic DNA methylation status [1]

Parameter	Control	FD	FD1	FD2
Hematocrit (%)	37.8 ± 0.7^{a}	37.2 ± 0.5^{a}	38.0 ± 0.7^{a}	33.4 ± 0.9^{b}
Leukocyte (wbc x 10^6/ml blood)	6.8 ± 0.9^{a}	6.7 ± 0.2^{a}	7.1 ± 1.2^{a}	2.8 ± 0.3^{b}
Whole blood folate (ng/ml)	657 ± 17.90^{a}	125 ± 7.50^{bc}	114 ± 17.04^{c}	61 ± 4.6^{d}
Plasma homocysteine (μM)	1.1 ± 0.1^{c}	3.0 ± 0.2^{b}	3.0 ± 0.4^{b}	5.3 ± 0.4^{a}
Hepatic SAM (umol/g liver)	92.0 ± 6.2^{a}	48.6 ± 5.2^{b}	37.1 ± 4.7^{bc}	24.0 ± 2.7^{c}
Hepatic SAH (umol/g liver)	25.8 ± 1.1^{b}	$22.8^{b} \pm 1.5$	$22.7^{b} \pm 1.3$	$32.2^{a} \pm 2.0$
Hepatic SAM:SAH ratio	3.6 ± 0.3^{a}	2.1 ± 0.2^{b}	1.6 ± 0.2^{b}	0.75 ± 0.1^{c}
Hepatic DNA methylation (d.p.m x 10^3 per ug DNA)	12.8 ± 0.6	11.9 ± 1.8	12.0 ± 1.3	12.2 ± 1.4
Colonic DNA methylation (d.p.m x 10^3 per ug DNA)	19.9 ± 1.4	19.5 ± 1.4	17.6 ± 2.7	18.6 ± 1.0

[1] Values are mean ± SEM with 12 rats/group. Means with different superscript letters in each row are significantly different, $p<0.05$.

folate deplete diets, with the highest plasma homocysteine concentrations observed in the most severely folate depleted treatment group (FD2).

S-adenosylmethionine concentrations in the liver (Table 5.2) were significantly decreased in the folate deficient treatments ($p<0.001$), while the S-adenosylhomocysteine concentration was elevated only in the FD2 treatment group.

5.3.3 Aberrant crypt formation

There was a significant decrease in the number of total colonic aberrant crypts in the FD2 treatment when compared to the control and FD treatment ($p<0.05$) (Figure 2). A significant decrease in the frequency of foci with single aberrant crypts ($p<0.05$) was observed in all folate deplete treatments when compared to the control treatment. No significant differences were seen between the treatment groups in frequency of foci containing 2 or ≥3 aberrant crypts in the colons, although there was a trend to lower numbers with FD1 and FD2 treatments.

5.3.4 DNA methylation status

The results of DNA methylation status in the liver and colonic mucosa are shown in Table 5.2. There were no significant differences observed among the four groups in the level of unmethylated CpG within either the liver or the colonic mucosa, despite the marked folate deficiency observed in the FD, FD1 and FD2 treatments.

5.3.5 Lymphocyte sub-populations

The lymphocyte cell subsets in the thymus, mesenteric lymph nodes and spleen of the rats fed the folate replete and deficient diets are shown in table 5.3. There were no

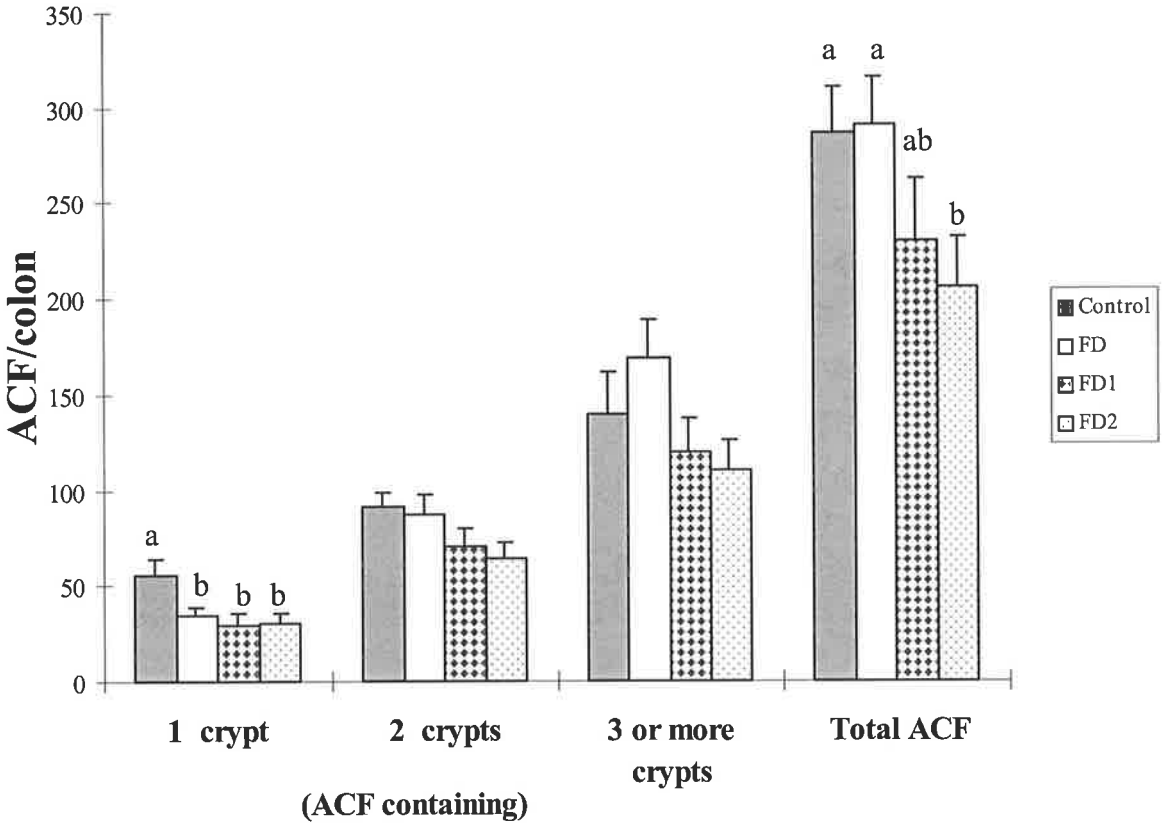


Fig. 5.2. Effect of folate depletion and repletion on foci of aberrant crypts induced by AOM in the rat colon. Values are mean ± SEM with 12 rats/group. Means with different superscript letters in each column are significantly different ($p<0.05$).

Table 5.3 Lymphocyte cell subsets in thymus, mesenteric lymph nodes and spleen of rats fed folate replete and folate deficient diets.

Treatment group				
Cell subset	**Control**	**FD**	**FD1**	**FD2**
Thymus				
α/β T cells	80.3 ± 3.1	79.5 ± 1.0	82.0 ± 2.5	82.6 ± 4.3
CD4	85.1 ± 2.7	82.0 ± 4.8	87.0 ± 1.0	86.5 ± 4.8
CD8	81.4 ± 8.1	79.9 ± 6.3	79.3 ± 1.6	77.5 ± 5.4
B cells	3.5 ± 2.6	4.5 ± 3.8	2.9 ± 2.4	4.2 ± 0.5
Natural killer cells	1.1 ± 0.7	2.1 ± 1.8	1.1 ± 0.1	2.0 ± 0.8
Mesenteric lymph nodes				
α/β T cells	52.6 ± 1.3	58.0 ± 3.1	60.1 ± 6.4	63.3 ± 1.0
CD4	31.9 ± 4.1	38.8 ± 3.0	41.5 ± 9.6	38.7 ± 3.0
CD8	16.4 ± 1.1	18.4 ± 2.0	15.7 ± 1.1	20.8 ± 3.1
B cells	23.4 ± 7.3	13.6 ± 2.3	18.6 ± 12.4	17.1 ± 9.1
Natural killer cells	1.3 ± 0.1	1.4 ± 0.3	1.5 ± 0.4	2.3 ± 0.2
Spleen				
α/β T cells	21.5 ± 5.2	23.8 ± 5.6	23.3 ± 2.3	27.8 ± 11.8
CD4	13.8 ± 2.7	13.8 ± 2.3	13.5 ± 1.0	13.6 ± 8.6
CD8	13.8 ± 1.8	12.5 ± 0.5	12.6 ± 1.0	14.6 ± 4.5
B cells	36.6 ± 9.0	33.3 ± 2.3	38.5 ± 6.23	30.0 ± 3.0
Natural killer cells	10.8 ± 1.3	9.5 ± 1.0	10.1 ± 1.0	9.4 ± 1.0

Values are mean ± SD with 3 rats/group.

changes in cell populations in either the thymus, spleen or the mesenteric lymph nodes.

5.4 Discussion

In this study we examined the effect of a diminished folate status in rats on the risk of developing chemically-induced colon cancer in rats. We also examined the effect of folate depletion on colonic DNA methylation and immune status in these animals, as some researchers have proposed that these factors may be reduced and may provide mechanism(s) by which a lowered folate status mediates the enhancement of colorectal carcinogenesis (Mason, 1984; Giovannucci *et al.* 1995). Aberrant crypt foci (ACF) are precursor lesions for colon cancer in rodents and humans (Bird, 1995; Pretlow *et al.* 1992; Ronucci *et al.* 1998) and were used to evaluate the risk of colon carcinogenesis.

The rationale for adding 1% succinylsulfathiazole to a folate deplete diet was to further enhance the level of folate depletion achievable in the rats. Succinylsulfathiazole is a non-absorbable antibiotic drug which inhibits the *de novo* synthesis of folate by the intestinal microflora of the gut. This is important in facilitating folate deficiency, as some of the folate produced by the animals intestinal bacteria has been shown by researchers to be incorporated back into stores of host tissue folate (Rong *et al.* 1991; Camilo *et al.* 1996). A period of 4 weeks of inclusion of the antibiotic drug was employed to assess the effects of more severe folate depletion on both the pre-initiation and post-initiation phases of carcinogenesis.

Folate status was decreased significantly in the animals fed the folate deplete diets in this study. A moderate level of folate depletion was present in the FD and FD1 treatments, and was evident as a significant fall in whole blood folate concentration, a modest rise (3

fold) in plasma homocysteine and fall in hepatic SAM concentration. The folate deficiency reported in the FD and FD1 groups was similar to the moderate folate deficiency achieved by that of Cravo *et al.* (1992) and Kim *et al.* (1996). A more severe level of depletion was achieved with the rats receiving the FD2 treatment and this was evident not only as the lower blood folate and higher plasma homocysteine levels but also the reduced white cell and red cell counts in whole blood. Plasma homocysteine levels and hepatic SAM have been shown to be inversely correlated with folate status (Miller *et al.* 1994). The most severe folate depletion was present in the FD2 treated animals and was due to the incorporation of 1% succinylsulfathiazole in the folate deplete diet (Walzem and Clifford, 1988) for the last 4 weeks of the study. Folate concentration in the blood was significantly lower than all other treatments, as was hepatic SAM, while plasma homocysteine concentrations rose significantly higher than the other treatments. Further evidence of severe depletion was shown by a significant fall in body weight, lower hematocrit values and significantly decreased leukocyte counts in the blood of these rats, hematological markers which have been observed in rats previously (Friedrich, 1988). Accumulation of hepatic SAH usually occurs as a result of folate deficiency and a decreased ability to remethylate homocysteine (Balaghi *et al.* 1993; Kim *et al.* 1995). In this study there was accumulation of hepatic SAH in the FD2 treatment group. The lowest hepatic SAM:SAH ratio was also detected in these rats which were displaying folate deficiency. Other studies (Kim *et al.* 1995) have also observed substantially lower hepatic SAM concentrations and SAM:SAH ratio in rats rendered folate deficient and were not accompanied by any change in hepatic DNA methylation. Despite this presence of folate deficiency in the present study, the data failed to show any alterations in DNA methylation status within either the liver or colonic mucosa. This is in agreement with prior studies (Locker *et al.* 1986; Wainfan *et al.* 1989; Kim *et al.* 1995 ; Kim, Pogribny *et al.* 1996; Le Leu *et al.* 1998), that suggested that

multiple deficiencies of lipotropes were required to produce methylation changes at a genomic level.

The data of the present study show that folate deficiency decreased the formation of colonic ACF within the rats. Fewer numbers of ACF were actually observed in the colons of the rats with the most severely depleted folate status (ie. FD2 treatment) when compared to the folate replete animals. The above data indicates that folate depletion in rats does not increase the risk of developing colon cancer. It actually decreases risk, despite anaemia and hyperhomocysteinaemia. This fall in ACF may relate to the role of folate in cell multiplication with folate deficiency inhibiting proliferation of initiated cells. Only two other studies to date have examined the relationship of folate and ACF. Neither of these studies investigated the effect of folate deficiency on ACF. Wargovich and colleagues (1996) examined the effect of folate on AOM-induced aberrant crypt foci (ACF) in male F344 rats. Folate supplementation at 2.5 g/kg significantly increased the development of ACF when compared to a control diet (AIN-76A, 2mg/kg). Further supplementation with folate to 5 g/kg diet was found to have no further effect on the number of ACF when compared to the control diet. In the other study, Shivapurkar *et al.* (1995) examined the effect of a semi-purified diet that was high in fat and low in fibre with folate supplementation (3 mg/kg) on the incidence of aberrant crypt foci induced by AOM. Folate supplementation was found to have no influence on ACF.

The reliability of ACF as a marker of colorectal tumor risk still remains a matter of debate. Studies by some researchers (Magnuson *et al.* 1993; Corpet *et al.* 1990; Lafave *et al.* 1994; Caderni *et al.* 1995) using the ACF as a preneoplastic marker of colon carcinogenesis suggest that the total number of ACF combined with a high crypt multiplicity (number of crypts per focus) of the larger ACF is the more reliable predictor of tumor outcome. However, in the present study no differences in foci with multiple crypts

were observed between any of the treatment groups. A possible explanation for this is that evaluating ACF only 6 weeks after the last AOM injection may be too early to see effects on larger ACF. The reduction in total ACF in the FD2 treatment may be explained either by these animals being in a more severe folate deficient state and directly affecting the growth of the ACF by its influence on cell multiplication or by the effect of succinylsulfathiazole on the intestinal bacterial flora. Reddy *et al.* (1976) found a 73% reduction in colon tumor incidence in germ free animals compared with conventional animals with a normal microflora. Goldin (1988) also showed an inhibitory effect on the incidence of DMH-induced colon cancer in rats by suppressing the intestinal microflora by feeding certain antibiotics.

Experimental animal studies by Cravo and colleagues (1992) and Kim *et al.* (1996) reported an increased risk of colorectal cancer when folate deficiency was present. Their methodology however differed from ours in several ways. Dimethylhydrazine (DMH) was used as the carcinogen, not AOM, to induce colorectal carcinogenesis and the period and dosage used in induction was substantially longer (20 weeks for dosing protocol), thus causing continued mutational insults in the initiatory stage. These r`esearchers also used a specific amino acid defined diet as opposed to a semi-purified diet. Their studies also used cancer as the end point rather than ACF. Both studies concluded that consumption of a folate deficient diet was associated with a potentiation of benign and malignant neoplasia. DNA methylation was also examined as a possible mechanism by which a diminished folate status may enhance colorectal carcinogenesis in the study by Kim *et al.* (1996). However, like the present study they found no alteration in colonic DNA methylation status, in agreement with our colonic results, whether with moderate or severe folate depletion. The discrepancy in the purported role of folate and colorectal cancer risk between their studies

and the present one may be due to the contrasting experimental protocol (ie. experimental diets and cancer induction period). Further studies are needed to clarify this point.

The immune system may play an important role in the development of colorectal cancer. Studies by Pierre *et al.* (1999) have shown that T cell status influence colon tumour occurrence in APC ^{Min} mice. Folate deficient individuals have been shown to have a decreased resistance to infections, and this has been confirmed in animal experiments (Nauss *et al.* 1982; Jacobson *et al.* 1987). In folate deficiency animal experiments natural killer cells have been shown to be decreased, and this reduced immune function has been proposed by Mason (1984) to be a possible mechanism for folate deficiency increasing the risk for colorectal cancer. However, in the present study no alteration in natural killer cells was found within the thymus, spleen or mesenteric lymph nodes with moderate or severe folate deficiency. In a study in mice, folate deficiency was also shown not to alter lymphocyte subsets within the mesenteric lymph nodes, spleen or the thymus (Dhur *et al.* 1991).

Folate functions in the transfer of single carbon units from donor compounds to biosynthetic pathways as well as in the synthesis of DNA. Here folates act as cofactors in the synthesis of thymidylate and purines (Wagner, 1995). Therefore, tissues that are more active in cell proliferation are more likely to be vulnerable to folate deficiency. This could explain why white blood cells of the rat is affected by folate deficiency (Friedrich, 1988). The epithelial cells of the colon, due to their nature of rapid proliferation and turnover are likely to be influenced by folate deficiency. Some studies suggest that tumor growth may be arrested at specific sites by targeting with antimetabolites (Clarke *et al.* 1991). The use of folate antagonists in cancer chemotherapy which target specific enzymes critical for cell growth may explain the findings in this study, of how severe folate deficiency inhibited the growth of the aberrant crypt foci in the rat colon. There is a case for further careful

examination of the hypothesis that folate deficiency invokes an increased colorectal cancer risk based on evaluation of the results of the present study.

The findings of the present study do not support the concept that either a moderate or severe folate depletion will increase the development of precancer lesions, or alter DNA methylation status in the colonic mucosa of rats exposed experimentally to the chemical carcinogen AOM. In this context, severe folate deficiency which was without effect on colonic DNA methylation was found to decrease colorectal cancer risk, based on the occurrence of ACF.

Chapter 6

Effect of Moderate and Severe Folate Depletion on the Incidence of Intestinal Tumours and DNA Methylation

6.1 Introduction

Colorectal cancer is one of the most common cancers occurring in men and women in the Western world (Potter, 1999). Dietary factors are thought to play a predominant role in the causation of colorectal cancer. Diets high in fat and/or meat and low in fruits, cereals and/or vegetables have been found to be associated with a higher risk of colorectal cancer (Willet *et al.* 1990; Steinmetz and Potter, 1996). The reduced risk of colorectal cancer in association with consumption of fruits, cereals and vegetables may be explained by certain micronutrients (Tseng *et al.* 1996). A higher intake of the micronutrient 'folate' was first proposed by Fredenheim *et al.* (1991) to reduce the risk of colorectal cancer. Folate deficiency has been found to be one of the most prevalent vitamin deficiencies occurring in populations worldwide (Jossten *et al.* 1993). Since the case-control study conducted by Freudenheim *et al.* (1991), other epidemiological studies have tended to suggest an association between a diminished folate intake and an increased risk of developing colorectal cancer (Kim *et al.* 1999). However, none of these studies relate actual folate deficiency with increased colorectal cancer risk. If there is a lower intake of folate this may well be related to lower intakes of other phytochemicals which may also alter colorectal cancer risk. Animal studies performed under controlled conditions have directly examined the relationship between folate status and colorectal carcinogenesis. Studies examining the role of dietary folate adequacy and deficiency in laboratory animals have been somewhat inconsistent. Some studies indicate protection against colorectal cancer with dietary folate (Cravo *et al.* 1992; Kim *et al.* 1996), while others suggest an opposite effect (Reddy *et al.* 1996) or even no effect at all (Shivapurkar *et al.* 1995). The conflicting results between these experimental animal observations may well be due to variations in experimental design, or to differing animal models used by researchers. Previously in Chapter 5 of this

thesis the results demonstrated that total numbers of ACF in the rat colon may be altered by a severe degree of folate deficiency in the rat, however a moderate folate deficiency did not significantly alter total ACF numbers when compared to rats with an adequate folate status.

The purpose of this study was to pursue the findings in the previous chapter, thereby examining the effect of a diminished folate status past the development of pre-neoplastic lesions right through to intestinal cancer, as folate deficiency might be altering cancer development in a stage specific manner. To evaluate this aim, the same animal model of intestinal cancer used in Chapter 5 will again be used in this study, as will the same dietary treatment groups with the inclusion of an extra control group (discussed in experimental design). The influence of folate deficiency on DNA methylation status will again be examined, however unlike Chapter 5 where methylation status was measured after 12 weeks, the DNA methylation in the colon and liver will be measured after 26 weeks.

6.2 Experimental design

6.2.1 Animals and diets

A total of 125 weanling male Sprague-Dawley rats (70g) were purchased from the Animal Resource Centre, Murdoch University, Perth, Australia. Animals were housed in stainless steel wire bottom cages, maintained in an air-conditioned environment 23°C ± 2 with a 12 h light: 12 h dark cycle. At the age of 4 weeks they were sorted into equal bodyweights and divided into five dietary treatment groups of twenty-five animals. The animals were fed *ad libitum* experimental powdered diets and given free access to distilled water for a period of 26 weeks. Body weights were recorded weekly. After 4 weeks on experimental diets, animals were injected with AOM (Sigma Chemical, St Louis, MO)

dissolved in normal saline at a dosage of 15 mg/kg body weight once weekly for 3 weeks. The rats were euthanased 22 weeks after the first AOM injection. The experimental diets were based on modified AIN-93 (Reeves *et al.* 1993) semi-purified diet (Table 6.1). Treatment 1 (control) contained 8 mg folate/kg diet (folate replete). Treatment 2 (control + S) was control with 1% succinylsulfathiazole added (Sigma Chemical, St Louis, MO) for the first 4 weeks of the study. Treatment 3 had no added dietary folate added, (FD). Treatment 4 (FD1) consumed FD diet and 1% succinylsulfathiazole for the first 4 weeks of the study. Treatment 5 (FD2) consumed FD and 1% succinylsulfathiazole for 4 weeks between week 8 and 12 of the study. The experimental protocol for the study is shown in Figure 6.1.

The rationale for the different treatment groups are as follows. Treatment 1 acts as the control group for the study representing an adequate folate status. Treatment 2 (control + S) is also adequate folate and acts as a control group for the succinylsulfathiazole added treatments (with the inclusion of succinylsulfathiazole prior to AOM injections). Treatment 3 (FD) will diminish folate status in the rats to a moderate folate deficiency level throughout the study. Treatment 4 (FD1) will create a more extreme level of folate deficiency prior to induction of carcinogenesis with AOM (with the inclusion of succinylsulfathiazole for the first 4 weeks), the animals will then revert back to a moderate level of folate deficiency. Treatment 5 (FD2) will allow examination of the effect of a more severe level of folate deficiency during week 8 to 12 of the study (with the inclusion of succinylsulfathiazole between week 8 to 12), while a moderate decrease in folate status will be maintained throughout the early stages of the study (ie. similar to FD) and after week 12 to the end of the study. Prior work in this thesis (Chapter 3) has demonstrated that the inclusion of succinylsulfathiazole at a level of 1% in a folate deficient diet should not be fed to rats for a

Table 6.1 Composition of experimental diets[1]

	Diet				
Ingredient (g/100g)	**Control**	**Cont + S**	**FD**	**FD1**	**FD2**
Casein	14.3	14.3	14.3	14.3	14.3
Cellulose	5.0	5.0	5.0	5.0	5.0
Cornstarch	35.4	35.4	35.4	35.4	35.4
Sucrose	20.0	20.0	20.0	20.0	20.0
Fat (50% lard and 50% sunflowerseed oil)	20.0	20.0	20.0	20.0	20.0
AIN-vitamin mix[2]	1.0	1.0	1.0	1.0	1.0
Folate in vitamin mix	0.8	0.8	-	-	-
AIN-mineral mix	3.5	3.5	3.5	3.5	3.5
L-methionine	0.3	0.3	0.6	0.3	0.6
Choline	0.2	0.2	0.2	0.2	0.2
Succinylsulfathiazole[3]	-	1.0	-	1.0	1.0

1 The FD, FD1 and FD2 treatments contained 0 mg folate in their vitamin mix.

2 The vitamin and mineral mixtures were prepared according to the AIN-76 formula. The control diets contained 8mg/kg folate, supplied through the vitamin mix.

3 Cont + S, contained 1% succinylsulfathiazole only for the first 4 weeks of the study, FD1 also contained 1% succinylsulfathiazole only for the first 4 weeks of the study, FD2 contained 1% succinylsulfathiazole only between week 8-12 of the study.

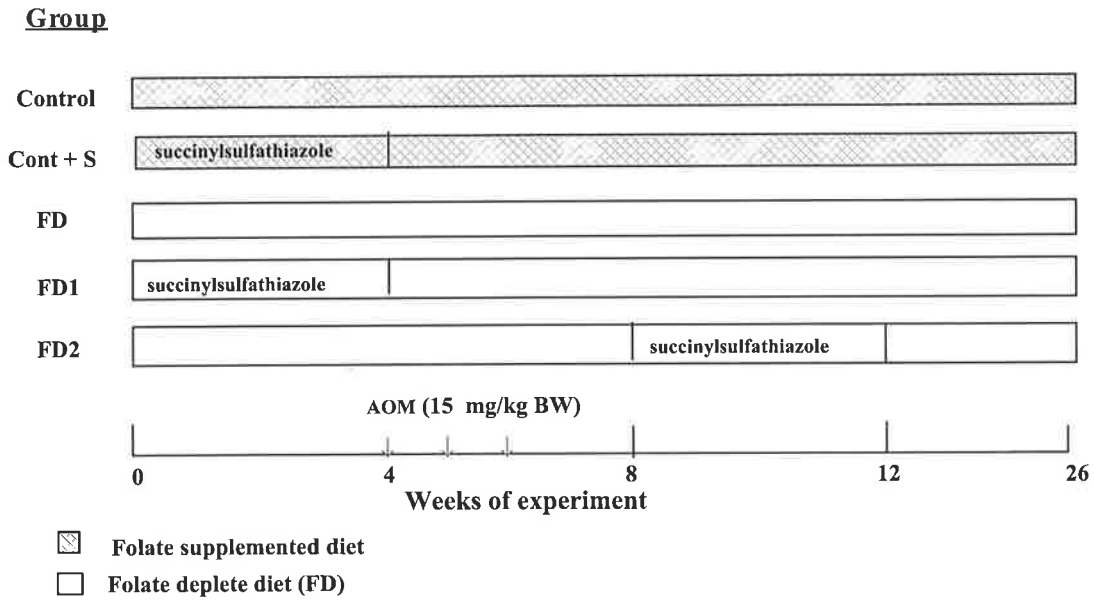


Figure 6.1. Diagrammatic representation of experimental protocol. Cont + S and FD1, contained 1% succinylsulfathiazole only for the first 4 weeks of the study, FD2 contained 1% succinylsulfathiazole only between week 8 and 12 of the study.

period any longer than 4 weeks otherwise significant bodyweight falls will result. A fall in bodyweight may impede interpretation results of this tumour study.

The experimental protocol and use of rats was approved by the CSIRO, Human Nutrition, Animal Experimentation and Ethics Committee prior to commencing the study.

6.2.2 Sample collection

Fresh faecal samples were collected from each rat after 4 weeks on experimental diets. Faeces were placed in 3 ml of deionised water, homogenized, pH read and then the samples stored at -20°C for SCFA measurements. The rats were killed by exsanguination while under halothane anesthesia after 26 weeks on experimental diets, blood was collected into EDTA treated tubes, a portion was diluted with 1% ascorbic acid and stored at -80°C for subsequent folate analyses. The remaining blood was centrifuged at 1000 g for 10 minutes, plasma was collected and stored at -80°C for subsequent homocysteine measurements. A small portion of colon (distal to the Peyers Patch) and liver were frozen in liquid nitrogen and stored at -80°C for subsequent DNA extraction and DNA methylation analysis. A section of the colon was also stored at -80°C for subsequent folate analysis. All tumours were fixed in 10% formalin in phosphate buffered saline for histopathological examination.

6.2.3 Statistical analysis

Group results of biochemical markers were compared using a one-way analysis of variance test followed by Tukey's Multiple Comparison test. Analysis of tumour incidence was carried out using Chi-squared test, and a generalized linear model with Poisson distribution of errors was used to analyze differences between treatments for tumour numbers in the large and small intestines. For tumour mass index, the data was

logarithmically transformed to allow for normality of data and then analyzed using a one-way analysis of variance (ANOVA). The Kruskall-Wallis posthoc test was used after ANOVA to determine group differences. The statistical analyses of tumour data were undertaken by CSIRO Mathematical and Information Services (Glen Osmond, South Australia). Differences between the treatment means for all tests were considered significant at $p<0.05$ unless otherwise stated.

6.3 Results

6.3.1 Body Weight

The growth of the animals fed the different experimental diets is shown in Figure 6.2. After 26 weeks on the experimental diets there was no significant difference in body weight between the experimental treatment groups.

6.3.2 Intestinal tumour data

The incidence, number, type and size of intestinal tumours are shown in Table 6.2. Most of the tumours observed in all treatment groups were in the large intestine. There was no effect of the added succinylsulfathiazole treatments on tumour parameters and therefore treatments groups were pooled into folate replete treatment or folate deplete treatments. The control groups (folate replete) demonstrated the highest number of tumour bearing animals, 100% for (control) and 88% for (cont + S) whereas the folate deplete treatments showed the lowest number of tumour bearing animals, 76% for (FD), 68% for (FD1) and 70% for (FD2). Overall there was a significant decrease ($p<0.01$) in total intestinal tumour incidence associated with the folate deplete treatments. The large intestinal tumour incidence showed a similar pattern to that of the total tumour incidence. Higher large

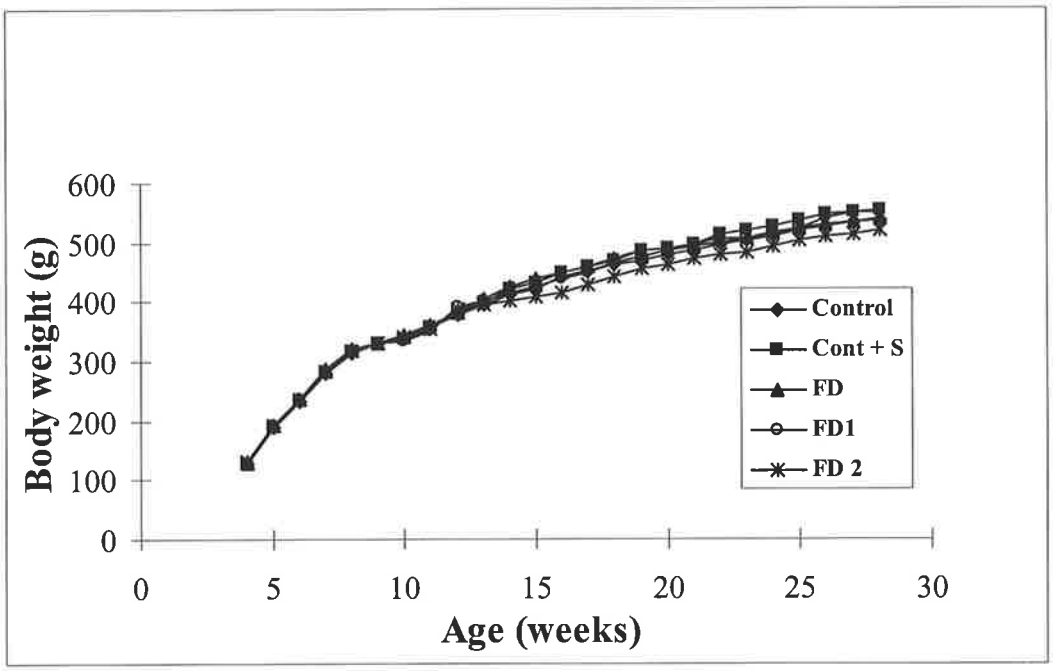


Figure 6.2 Body growth curves of the different experimental treatment groups

Table 6.2 Effect of folate deficiency on Indexes of AOM-induced small and large intestinal tumours in rats

	Control	Cont + S	FD	FD1	FD2	Folate adequate vs Folate deficient
Incidence (%)						
SI + LI	100	88	76	68	70	$p<0.01$
SI only	50	63	36	28	22	$p<0.05$
LI only	79	75	68	64	65	not significant
Tumour number						
SI + LI	58	54	37	31	31	$p<0.01$
SI only	16	21	10	7	6	$p<0.05$
LI only	42	33	27	24	25	not significant
Tumour mass index (log_{10})						
LI only	2.39	2.16	1.85	1.90	1.91	$p<0.05$
Tumour type (LI)						
adenomas	29	21	23	21	21	not significant
adenocarcinomas	13	12	4	3	4	$p<0.01$

Note: n=25 rats/treatment, LI, large intestine; SI, small intestine. Control and Cont + S are folate adequate treatments while FD, FD1 and FD2 are folate deficient treatments.

intestinal incidences were seen in the folate replete treatments (control: 79%; cont + S: 75%) when compared to the folate deplete treatments (FD: 68%; FD1: 64%; FD2: 65%). However, these differences were not significant.

The analyses of the small intestine tumour incidence data indicated that the folate deplete treatments were significantly lower ($p<0.05$) than that of the folate replete treatments.

Animals on the folate adequate diets exhibited a significant increase (41%) in the total number of intestinal tumours when compared to animals consuming folate deplete diets. There was a 32% fall in the number of colon tumours in the folate deplete treatments when compared to the folate adequate treatments, however this observation was not significant. Small intestinal tumour numbers were significantly decreased (59%) in the animals maintained on folate deplete diets.

Tumour mass index ($\log_{10}$) data for large intestinal tumours also showed a significant reduction ($p<0.05$) in the animals maintained on folate deplete diets when compared to those maintained on folate supplemented diets.

Histopathological appraisal of colon tumour type showed the animals fed the folate deplete diets developed significantly less ($p<0.01$) adenocarcinomas than the folate supplemented treatment groups. There was a 71% fall in the incidence of malignant tumours in the folate depleted treatment groups. Figure 6.3 illustrates a section of a colon adenoma (5μm) stained with haematoxlin and eosin at a magnification of 20x. Figure 6.4 illustrates a colon adenocarcinoma section been stained with haematoxlin and eosin at 20x magnification.

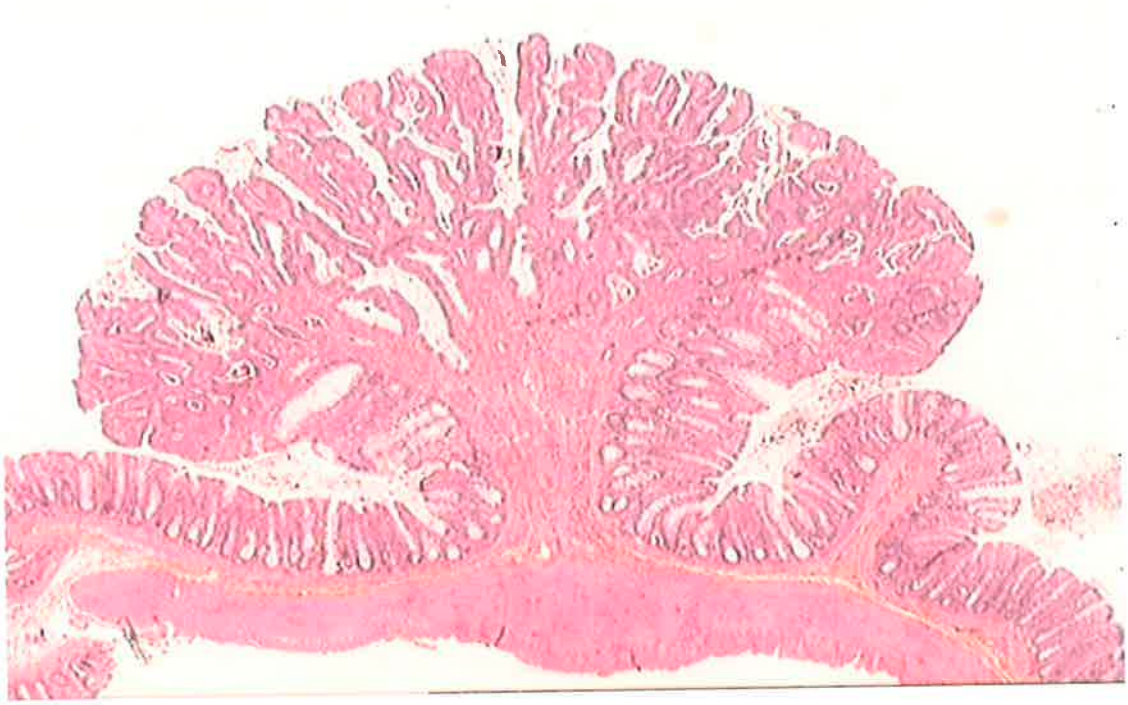


Figure 6.3 Colon adenoma section (5 μm thickness) stained with haematoxylin and eosin, viewed under 20x magnification

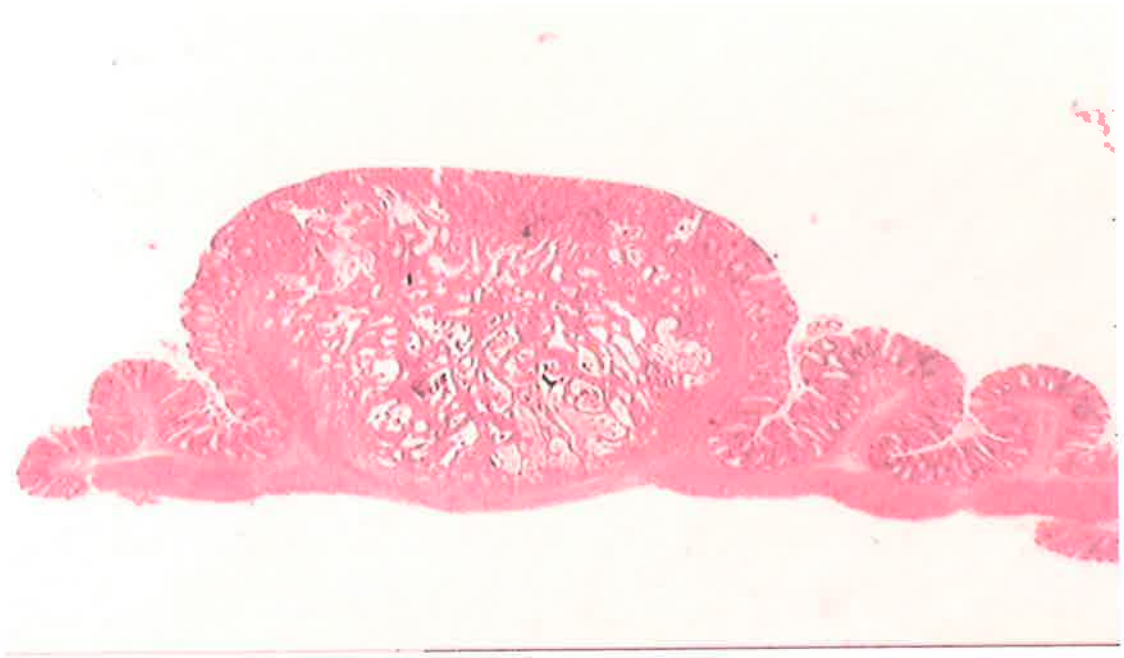


Figure 6.4 Colon adenocarcinoma section (5 μm thickness) stained with haematoxylin and eosin, viewed under 10x magnification

6.3.2.1 Folate status, haematocrit, plasma homocysteine

Folate concentrations in the blood and colonic mucosa are shown in Table 6.3. Whole blood folate concentrations were significantly reduced ($p<0.001$) in the treatment groups which consumed the folate deplete diets. Colonic mucosa folate levels also were significantly depleted ($p<0.01$) in the treatment groups which consumed the folate deplete diets. Haematocrit values did not differ between any of the treatment groups (Table 6.3). Plasma homocysteine concentrations (Table 6.3) were significantly elevated ($p<0.001$) in the animals fed the folate deplete diets.

6.3.4 S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations in liver and colon

SAM concentrations in the liver (Table 6.4) were significantly decreased in the folate deficient treatments ($p<0.001$). SAH concentrations in the liver were not significantly different. When expressed as a ratio (SAM: SAH) there was a significant decrease ($p<0.001$) in the folate deficient treatment groups. No significant differences were observed with SAM within the colonic mucosa for any of the treatment groups. SAH within the colonic mucosa was unable to be measured by HPLC due to interfering peaks.

6.3.5 DNA methylation status

The results of DNA methylation status in the liver and colonic mucosa are shown in Table 6.4. There were no significant differences observed among the four groups in the level of unmethylated CpG within the colonic mucosa, despite the marked folate deficiency observed in the FD, FD1 and FD2 treatments.

Table 6.3 Effect of folate depletion and repletion on folate status, hematological parameters and plasma homocysteine[1]

Parameter	Control	Cont + S	FD	FD1	FD2
Haematocrit (%)	38.0 ± 2.9	37.5 ± 2.9	37.5 ± 1.5	37.8 ± 3.5	37.0 ± 2.8
Leukocyte (wbc x 10^6 /ml blood)	6.2 ± 3.2	6.0 ± 4.4	5.2 ± 2.6	5.2 ± 3.2	4.8 ± 1.8
Whole blood folate (ng/ml)	684 ± 91^a	694 ± 86^a	99 ± 21^b	100 ± 25^b	96 ± 35^b
Colon folate (ug/mg tissue)	9.6 ± 1.8^a	8.0 ± 1.6^a	2.7 ± 1.4^b	3.1 ± 1.3^b	4.3 ± 2.4^b
Plasma homocysteine (μM)	1.8 ± 0.3^b	1.6 ± 0.5^b	4.5 ± 1.3^a	4.0 ± 1.3^a	4.1 ± 1.3^a

[1] Values are mean ± SD with 25 rats/group. Means with different superscript letters in each row are significantly different, $p<0.05$.

Table 6.4. Effect of folate depletion and repletion on folate status, hematological parameters and plasma homocysteine and hepatic S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) concentrations, hepatic and colonic DNA methylation status [1]

Parameter	Control	Cont + S	FD	FD1	FD2
Hepatic SAM (umol/g liver)	72.2 ± 7.9[a]	79.3 ± 15.1[a]	47.3 ± 7.9[b]	50.9 ± 10.1[b]	42.9 ± 7.1[b]
Hepatic SAH (umol/g liver)	10.6 ± 2.2	13.5 ± 4.4	10.7 ± 2.6	11.8 ± 3.3	10.5 ± 2.0
Hepatic SAM:SAH ratio	7.0 ± 1.5[a]	6.4 ± 2.2[a]	4.6 ± 1.1[b]	4.5 ± 1.3[b]	4.4 ± 1.1[b]
Colon SAM (umol/g colon)	33.1 ± 13	32.8 ± 16	30.0 ± 11	31.0 ± 9	39.6 ± 11
Colonic DNA methylation (d.p.m x 10^3 per ug DNA)	13.2 ± 2.4	14.0 ± 1.3	14.1 ± 0.9	13.5 ± 0.9	13.0 ± 2.9

[1] Values are mean ± SD with 25 rats/group. Means with different superscript letters in each row are significantly different, $p<0.05$.

Table 6.5. Effect of incorporation of 1% succinylsulfathiazole into diets on rat faecal SCFA concentrations and faecal pH [1,2]

Parameter	**Control**	**Cont + S**	**FD**	**FD1**
Acetate (μmol/g faeces)	80.8 ± 23.0	25.0 ± 3.9**	78.0 ± 22.3	17.0 ± 3.0**
Propionate (μmol/g faeces)	9.5 ± 2.1	6.8 ± 2.1	7.6 ± 2.2	6.4 ± 2.3
Butyrate (μmol/g faeces)	7.8 ± 1.5	4.7 ± 1.6*	7.2 ± 2.7	3.5 ± 1.6**
Total SCFA (μmol/g faeces)	98.1 ± 26.6	36.3 ± 7.6**	92.8 ± 27.2	26.9 ± 6.9**
Faecal pH	6.9 ± 0.2	6.4 ± 0.1**	6.9 ± 0.3	6.2 ± 0.1**

[1] Values are mean ± SD with 25 rats/group. Means with different superscript letters in each row are significantly different, $p<0.05$.

[2] Samples were collected after 4 weeks of consuming dietary treatments, Cont + S and FD1 contained 1% succinylsulfathiazole.

[3] * $p<0.01$, ** $p<0.001$ significantly different from control within each row.

6.3.6 SCFA concentrations and pH in faeces

SCFA concentrations (acetate, propionate and butyrate) are shown in Table 6.5. SCFA levels were significantly lower ($p<0.001$) in the rats consuming succinylsulfathiazole. There were no differences in SCFA concentrations between the folate-adequate rats and the folate-deplete rats. The pH of the faeces of the rats consuming succinylsulfathiazole was significantly lower ($p<0.01$) than those rats not consuming succinylsulfathiazole (Table 6.5).

6.4 Discussion

The main aim of the study presented in this chapter was to examine the effect of folate deficiency on intestinal tumourigenesis in the rat. The results clearly demonstrate that folate deficiency is associated with a reduction in the risk of intestinal tumourigenesis in rats. Intestinal tumour incidence and the total number of tumours were significantly reduced in the folate deficient rats relative to the folate adequate rats. Colorectal tumour risk was lowered as a result of significantly fewer adenocarcinomas being observed in the colon and rectum as well as a reduction in the tumour mass index in the folate deficient rats compared with the folate-supplemented rats.

The addition of succinylsulfathiazole was employed to further enhance the level of folate depletion achieved in the rats. Succinylsulfathiazole is a non-absorbable antibiotic drug, which inhibits the *de novo* synthesis of folate by the intestinal microflora of the gut. This is important in facilitating folate deficiency, as rats are known to be coprophagic, and folate produced by the intestinal bacteria may be incorporated back into stores of host tissue folate (Rong *et al.* 1991; Camilo *et al.* 1996). In the present study this inhibition of the intestinal microflora was evident after 4 weeks in the rats consuming succinylsulfathiazole by significant falls in faecal pH and in SCFA concentrations. SCFAs are produced when

the intestinal microflora break down non-starch polysaccharides and resistant starch (McIntyre *et al.* 1993). By inhibiting the intestinal microflora's production of folate, a more extreme level of folate deficiency may be attained at a particular time. However, the incorporation of succinylsulfathiazole into such treatments either before cancer initiation or after initiation did not further influence intestinal tumour outcomes in this study.

The reduced intestinal tumourigenesis as a result of folate deficiency in the present study was not entirely unexpected. Folate plays an important role in nucleic acid biosynthesis and processes involved in cell proliferation (Wagner, 1995). An association of adequate folate status with enhanced cancer induction has been suggested previously (Herbert, 1985) and may be an effect of a requirement of tumour growth for folate. Indeed interruption of folate metabolism is a basis for anti-tumour therapy (Calvert, 1999). Therefore folate deficiency may well be reducing tumourigenesis by inhibiting cell proliferation. In the present study, folate deficiency supports suppression of initiation as well as promotion/progression of intestinal cancer, as both the incidence and number of intestinal tumours were reduced. Conversely, folate supplementation may be stimulating initiation and/or promotion/progression of intestinal carcinogenesis.

Measurement of folate levels showed that blood concentrations and colonic mucosa levels were significantly depleted in the animals fed folate deficient diets. There was a modest rise in homocysteine only in the animals that exhibited decreased blood folate levels, adding further evidence of biochemically significant folate deficiency. The level of folate deficiency observed in rats in the present study can be regarded as moderate and is consistent with other studies (Cravo *et al.* 1992; Le Leu *et al.* 1998) which demonstrated moderate folate deficiency. This magnitude of folate depletion has previously been shown to be associated with a functionally significant degree of cellular folate depletion within the colonic mucosa (Cravo *et al.* 1991) and this has also been observed in the present study. To

further reinforce the notion of moderate folate deficiency, body growth of the animals were not affected nor was there any alteration in haematocrits or white cell levels.

Alterations in global methylation patterns are among the earliest abnormalities to occur during the development of colorectal cancer (Baylin *et al.* 1998), although whether it is biologically significant is uncertain. Folate plays a key role in the transfer of methyl units to donor compounds (Wagner, 1995) including many biological transmethylation reactions such as that of DNA (Selhub and Miller, 1992). Cravo *et al.* (1992) and Mason (1994) have proposed that altered DNA methylation as a result of changes in folate status may be a mechanism whereby colorectal tumourigenesis is enhanced. In the present study, folate supplementation or folate deficiency did not alter global DNA methylation in the colonic mucosa. Other studies have also demonstrated that genomic DNA methylation status in the colonic mucosa is not altered by folate supplementation or folate deficiency (Kim *et al.* 1995; Kim *et al.* 1996; see chapter 5). In the present study colonic mucosal SAM levels were also shown not to change as a result of folate deficiency. SAM is the proximal methyl donor for DNA methylation reactions (Selhub and Miller, 1992). The colon may well be resistant to changes in SAM levels and DNA methylation (Kim *et al.* 1995). No differences in hepatic DNA methylation were observed in the present study despite marked decreases in hepatic SAM concentrations and the ratio of SAM to SAH in the folate deficient animals. This is consistent with other studies (Kim *et al.* 1995) which also found that moderate folate deficiency does not alter global DNA methylation patterns in rats, despite changes in hepatic SAM and the hepatic ratio of SAM to SAH.

The results of the present study demonstrate that folate deficiency reduced the risk of progression of tumourigenesis right through to cancer in AOM-treated rats. This was emphasized by a reduction in intestinal tumour incidence and fewer intestinal tumours observed in the folate deficient animals. Colorectal risk was also decreased in the folate

deficient animals due to fewer malignant tumours arising and a reduction in the tumour mass index of the colonic tumours. Folate deficiency was also shown not to alter the global DNA methylation status in the colonic mucosa. It is likely that the lower tissue folate levels present in the folate deficient animals may have had an inhibiting effect on initiation or the promotion/progression events of tumourigenesis.

Chapter 7

General Discussion

7.1 General discussion

The water-soluble vitamin 'folate' has attracted much attention of late because of its purported role in the pathogenesis of neural tube defects (Butterworth and Bendich, 1996) and cardiovascular disease (Malinow, 1996; Verhoef, 1996). There is also some evidence suggesting that a deficiency of folate may play a role in carcinogenesis (Butterworth, 1991; Kim, 1999).

In this thesis, the role of folate status on intestinal carcinogenesis was investigated using the AOM-rat model of intestinal cancer. Also investigated was the effect of folate supplementation and moderate and severe folate deficiency on DNA methylation status, plasma homocysteine and immune parameters.

Different protocols for creating experimental folate deficiency in the rat were investigated (Chapter 3). The experimental diet of choice was that of the AIN-93 semi-purified formula (Reeves *et al.* 1993) and this diet was modified to contain 12% protein, supplied as casein and the fat was at a level of 20% and 0.3% added methionine. By withholding folate from the test diet a moderate level of folate deficiency can be achieved after 4 weeks of feeding such diets to young rats. To further enhance the level of folate deficiency the inclusion of a sulfonamide is necessary. It has been suggested by Walzem and Clifford (1988) that the most effective protocol to produce experimental folate deficiency in rats is to feed a folate-free diet that otherwise supports maximum growth in young animals. An amino-acid defined diet was the diet of choice to produce experimental folate deficiency according to Walzem and Clifford. Other researchers (Cravo *et al.* 1992; Kim *et al.* 1996) have adopted this approach when evaluating the effect of folate deficiency on colorectal carcinogenesis. However, this diet is artificial and difficult to interpret with regard to commonly ingested foods, whereas the AIN-93 diet with the fat content increased,

more closely reflects diets consumed in Western populations. In this thesis, a high-fat AIN-93 semi-purified diet deficient in folate when fed to rats was able to maintain a growth rate equivalent to that of other rat studies using the AIN-93 diet (McIntosh *et al.* 1995), but still achieved a significant decrease in folate status. The AIN rodent diets are usually the formula of choice when evaluating chemopreventive agents in rat cancer studies (Rao, 1988; McIntosh *et al.* 1996). Therefore the modified AIN-93 diet (12% protein, supplied as casein and 20% fat as sunflowerseed oil, 0.3% methionine) with no added folate has been used in this thesis, and appears to be more relevant for use in intestinal cancer studies than that of a specific amino acid defined diet deficient in folate. This diet when fed to rats is capable of inducing a moderate fall in folate status without the addition of any folate anatgonists or sulfonamide drugs.

When soy protein isolate was substituted for casein in the modified AIN-93 diet and fed to rats, folate status was unable to be perturbed. As discussed in Chapter 4, feeding rats a soy protein diet with no added folate, and with dietary choline and methionine deficits superimposed produced no alterations in plasma homocysteine (a sensitive marker of folate deficiency) or hepatic DNA methylation levels were observed. Conversely, rats consuming similar diet with casein as the protein source exhibited marked increases in plasma homocysteine as the severity of the methyl group depletion was increased. Also DNA hypomethylation was observed in the liver in the rats consuming the most severely methyl-depleted diet. A deficiency of folate alone was unable to induce hepatic DNA methylation. Other studies (Wilson *et al.* 1984; Newberne and Rogers, 1986) have demonstrated that a lipotrope deficient diet (deficient in choline and methionine) when fed to rats is capable of initiating the development of DNA hypomethylation. Studies from this thesis show that a diet deficient in folate and choline was able to induce hepatic DNA hypomethylation, however further depletion of the diet with methionine did not further alter DNA methylation

status. Diets deficient in choline and methionine (lipotrope deficient diet) besides inducing hepatic DNA hypomethylation have also been shown to be associated with hepatocellular carcinoma (Newberne, 1986). Whether altered DNA methylation is involved in the process of carcinogenesis is still a matter of debate. However, the present study has demonstrated that a deficiency of folate alone does not alter DNA methylation, and to alter hepatic DNA methylation further deficiency of choline and/or methionine is required.

The difference between the influence of feeding a diet with a protein source based on casein and one as soy protein isolate on folate status and DNA methylation was attributed to the different endogenous folate levels present in these protein sources. The casein was shown to contain 0.045 mg/kg of endogenous folate and soy protein isolate 1.28 mg/kg of endogenous folate. The above observations may be very relevant to certain disease states. High plasma homocysteine levels have been attributed to an increase in risk for cardiovascular disease (Boushey, 1991). On the other hand dietary methyl group deficiency has been linked to carcinogenesis, in particular liver carcinogenesis (Newberne and Rogers, 1986) where hypomethylation of hepatic DNA is suggested as a likely mechanism for the increased risk of carcinogenesis. In terms of creating experimental folate deficiency, then soy is not an ideal protein source. A further shortcoming of feeding soy protein is that bodyweight gain of the animals was markedly less than that of casein fed animals, despite supplementation with exogenous methionine. Other amino acid deficits eg. threonine and/or lysine have been identified to be lower in soy which may have contributed to the poorer weight gain of the soy fed animals.

The data in the literature examining the relationship between experimental folate status and colorectal carcinogenesis is sparse and at times contradictory. It has been suggested that a diminished folate status may enhance colorectal carcinogenesis and this may be by caused by DNA hypomethylation. Only a couple of studies have examined the

effect of folate status on colorectal carcinogenesis using ACF as a biomarker of risk. ACF are pre-neoplastic lesions which occur in the colons of experimentally induced colon carcinogenesis rodents as well as in the colonic mucosa of humans with colon cancer. It is hypothesised that ACF are precursors of colorectal cancer (Bird, 1987; Pretlow *et al.* 1992). Folate supplementation at high levels has been shown to increase ACF numbers in the colon of rats (Wargovich *et al.* 1996) or have no effect (Shivapurkar *et al.* 1995). The present study (Chapter 5) found that feeding a folate deficient diet to rats does not alter ACF multiplicity but reduced total ACF numbers. When severe folate deficiency was present in the rats a reduction in total ACF in the colon was observed. Also associated with the severe folate deficiency was a reduction in bodyweight, and anemia in the animals. This reduction in bodyweight may have contributed to the lower total ACF numbers. If this state of severe folate deficiency was extended for any length of time severe falls in bodyweight would have occurred, followed by premature death. An interesting observation in this study was that moderate folate deficiency did not alter the total ACF numbers. The animals which displayed only a moderate level of folate deficiency had no significant differences in total ACF numbers. However there were significantly fewer smaller foci (single AC/focus) in these animals. Severe folate deficiency decreases the risk of developing colorectal cancer by lowering ACF numbers, and thereby impacting on the developmental stages of colorectal cancer. Considering that folate antagonists are used in the treatment of certain cancers to reduce the cell growth, the lower ACF observed in the severe folate deficient animals probably is due to an effect of a requirement of folate for cancer growth. Because no differences occurred in ACF numbers between the rats exhibiting an adequate folate status and those with moderate folate deficiency, it does suggest that folate and/or folate deficiency is not exerting any significant effects during the early events of colon carcinogenesis (initiation and/or early promotion/progression).

The reliability of colonic ACF as a marker of predicting colorectal tumours remains a matter of debate. Whether total ACF numbers or ACF containing 4 or more aberrant crypts (ACF multiplicity) is the more reliable predictor of tumour outcome remains debatable (Magnuson *et al.* 1993; Corpet *et al.* 1990; Lafave *et al.* 1994; Calderni *et al.* 1995). Some studies even suggest that colonic ACF do not correlate with colorectal tumours (Hardman *et al.* 1991). Because ACF are only a biomarker of colorectal cancer risk care must be taken when extrapolating results of ACF studies to those of colorectal tumour studies.

Despite the moderate and severe folate deficiency observed in the animals in this study, there was no alteration in colonic DNA methylation status. Changes in DNA methylation, particularly DNA hypomethylation, have been suggested to be involved in colorectal cancer development and some researchers have suggested that folate deficiency may be linked to DNA hypomethylation. Considering that the treatment group which displayed a severe level of folate deficiency still did not alter DNA methylation status in the colonic mucosa, it suggests that other factors must be required to perturb DNA status. Alternatively the time frame of this particular experiment may not have been sufficient to alter DNA methylation status.

Only a handful of studies have directly examined the effect of folate status on intestinal tumour formation in rats. Reddy *et al.* 1996 using a semi-purified AIN diet observed an increase in colon tumour size and multiplicity when the rats consumed a very high level of folate (2 g/kg). Shivapurkar *et al.* (1995) examined the effect of a semi-purified diet that was high in fat and low in fibre with normal folate (3 mg/kg) on the incidence of colon tumours induced by azoxymethane and found that folate supplementation had no influence on colon tumour incidence or tumour multiplicity. In contrast, two experimental studies using a specific amino acid defined diet high in fat indicated that folate

deficiency would enhance colorectal carcinogenesis (Cravo *et al.* 1992; Kim *et al.* 1996). Folate at a level of 8 mg/kg was found to be protective in these studies when compared to a folate deficient diet. The present study using a high fat modified AIN-93 diet has shown that folate deficiency decreases the risk of intestinal tumour formation and tumour numbers as well as increasing the size and malignancy of colon tumours, when compared to a diet containing folate at 8 mg/kg. The lower intestinal cancer risk was associated with a moderate level of folate deficiency. Although the level of folate depletion was enhanced by use of a sulfonamide drug both before and shortly after initiation of cancer, the same degree of protection was observed in these groups as for the moderate folate deficient group. It appears then that a diminished folate status is affecting the development of colorectal cancer in the later stages. Moderate folate deficiency was reported in Chapter 5 to not alter ACF total numbers or ACF multiplicity in the colon of rats, when compared to rats with an adequate folate status. It does appear then that moderate folate deficiency is not affecting the early stages of colorectal cancer development by not altering aberrant crypt foci development. However moderate folate deficiency does significantly alter the later stages of cancer development by decreasing the intestinal tumour incidence and total number of tumours as well as reducing the size and malignancy of colorectal tumours. Certainly a deficiency of folate in the later stages of tumourigenesis reduces risk, that is post initiation and during promotion phase. The association of folate supplementation with enhanced cancer induction in humans has been suggested previously (Herbert, 1985). Therefore the concept of folate deficiency with decreased tumourigenesis does appear to be feasible.

The contrasting results of this thesis to that of Cravo *et al.* (1992) and Kim *et al.* (1996) may be related to a number of factors. Firstly, the different basal diets used in the evaluations. Cravo *et al.* (1992) and Kim *et al.* (1996) used a specific amino acid defined diet in their studies. That diet is certainly not in any sense similar to one that would be

consumed by a human population, other than maybe some hospitalized patients receiving elemental diets. The modified AIN-93 diet used in this study, is much closer to that consumed by some Western populations. Another major difference between the present study and that of Cravo and Kim was the initiation protocol and dosage. Cravo *et al.* (1992) and Kim *et al.* (1996) used a higher dosage of DMH for initiation (20 mg/kg bodyweight) and repeated this for 20 weeks (total dosage of 400 mg/kg bodyweight), while the present study used 3 weekly injections of AOM at a level of 15 mg/kg bodyweight (total dosage of 45 mg/kg bodyweight). Given the heavy induction aspect their study places an emphasis on initiatory events with the procarcinogen DMH. Whereas my study has a significant influence during promotion and proression of tumours.

Studies examining the role of dietary folate adequacy and deficiency in laboratory animals on carcinogenesis using other experimental models have been somewhat inconsistent. Bills *et al.* (1992) found that the onset of spontaneous tumours in *taxl* transgenic mice could be delayed by feeding the minimal requirement of folate. In a study of transgenic mice that carry the bacterial *lac*Z gene as a mutational target the effect of maternal folate levels on the mutation rate in the developing colon was examined (Trentin *et al.* 1998). Mice who were contained on a high-folate diet exhibited no significant difference in the frequency of *lac*Z mutations compared to mice on low-folate diets. Song *et al.* (1999) using a genetic murine model of intestinal tumourigenesis found that the timing of dietary intervention with folate is important in modulating tumourigenesis. Folate supplementation prevented the progression of intestinal polyps if administered early and prior to the development of adenomas in the intestine. However mice receiving a folate depleted diet during the development of intestinal polyps, had a significantly lower incidence of tumours. Baggot *et al.* (1992) examined the incidence of rat mammary tumours induced by methylnitrosurea and found that folate supplementation (20 and 40 mg/kg) enhanced the

incidence, while folate deficiency suppresses the incidence of tumours. The conflicting results between these experimental animal observations may well be due to variations in experimental design, or to differing animal models used by researchers.

DNA hypomethylation in the colonic mucosa has been proposed as a possible mechanism by which folate deficiency may increase colorectal carcinogenesis (Mason, 1994). Data from the present studies have demonstrated this is unlikely to apply. Folate deficiency was shown not to influence global DNA methylation status within the colonic mucosa when examined after a moderate time of folate deficiency (12 weeks), as well as over the more prolonged period of folate deficiency (26 weeks).

The epidemiological evidence published to date (reviewed by Kim, 1999) tends to suggest a protective effect with dietary folate against colorectal cancer. However, it must be emphasised that all of these studies only show associations and do not demonstrate a cause and effect on colorectal cancer. Unlike the studies presented in this thesis, none of these epidemiological studies relate actual folate deficiency with colorectal cancer risk. If there is a lower intake of folate there is also likely to be lower intakes of other phytochemicals which may also alter colorectal cancer risk. In one of the largest and probably best documented prospective study examining folate intake in humans (high compared with those with the lowest intake) and risk of developing colorectal cancer, Giovannucci *et al.* (1995) found a non-significant inverse dose-response relationship. However when other factors were combined with the low folate intake such as high alcohol-low methionine and compared to high folate-low alcohol-low methionine there was a 70% reduction in risk of developing colorectal cancer. Their study highlights the point that other factors maybe involved or a combination of these factors with folate are required to effect colorectal cancer risk. Many of the published epidemiological studies examining dietary folate intakes and risk of developing colorectal cancer cite animal studies to support their

findings. The rodent colorectal cancer study conducted by Cravo *et al.* (1992) is the major animal study cited to support their findings. Considering that the present findings are not in agreement with that of Cravo *et al.* (1992), in fact opposite conclusions being generated, concern must obviously arise. Further animal studies need to be conducted to establish whether a causal and relationship exists between folate deficiency and colorectal carcinogenesis.

The results presented in this thesis show that a diminished folate status in the rat intestinal cancer model is associated with a reduction in the risk of intestinal tumourigenesis. Folate deficiency appears to reduce intestinal tumourigenesis, most likely by exerting its effect during the middle to later stages of cancer development, an effect that is independent of any alteration in global DNA methylation status.

7.2 Future Work

This thesis has showed that folate deficiency reduces the risk of colorectal cancer. To assess at what stage of colorectal cancer development folate deficiency inhibits or whether folate is important prior to development of tumours, further experimental work would be required. An experiment could be designed where adequate folate is fed to the rats prior to initiation and shortly after initiation (ie. prior to tumour appearance) followed by a folate deficient diet during the middle and later stages of tumourigenesis and compared to a treatment group which consumes just a folate deficient diet. By including another treatment group that consumes a folate deficient diet prior to initiation and shortly after, followed by folate adequacy throughout the rest of the study, it would provide the necessary

data to examine any stage-specific effects of folate and/or deficiency on colorectal tumourigenesis.

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