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Age, gender, vitamin A status, and folate status modulate retinoid-mediated disruptions of methyl group metabolism in rats

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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10-formylTHF	10-formyltetrahydrofolate
5-CH_3THF	5-methyltetrahydrofolate
5,10-CH_2THF	5,10-methylenetetrahydrofolate
ANOVA	analysis of variance
BHMT	betaine-homocysteine methyltransferase
CBS	cystathionine β-synthase
CRA	13-*cis*-retinoic acid
DFE	dietary folate equivalents
dTMP	deoxythymidine monophosphate
dUMP	deoxyuracil monophosphate
GNMT	glycine *N*-methyltransferase
MAT	methionine adenosyltransferase
MS	methionine synthase
MTHFR	methylenetetrahydrofolate reductase
NTD	neural tube defect
RA	all-*trans*-retinoic acid
RAG	retinoyl β-glucuronide
RDA	recommended dietary allowance
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
THF	tetrahydrofolate
UL	upper level
VAD	vitamin A deficient
VAS	vitamin A sufficient

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CHAPTER 1
GENERAL INTRODUCTION

Thesis Organization

Chapter 1 of this thesis is a short introductory chapter that describes the research problems addressed by the author. Chapter 2 includes a comprehensive review of literature that explores literature relevant to folate and methyl group metabolism. Chapters 3, 4, and 5 discuss three separate research studies conducted by the author. Each of these chapters contains an introduction, materials and methods, results, and conclusions section that is pertinent to the individual study. Following Chapter 5 is a comprehensive list of literature cited for this thesis.

Description of the Research Questions

Methyl groups, provided from the folate-dependent one-carbon pool through the remethylation of homocysteine or from the diet as methionine or choline, are important for transmethylation reactions that occur throughout the body. After the conversion of methionine to S-adenosylmethionine (SAM), methyl groups are activated and available to participate in transmethylation reactions. SAM-dependent transmethylation reactions lead to the modification and/or synthesis of several biological compounds, including phospholipids, proteins, and nucleic acids.

Previous work in our laboratory has demonstrated that administration of the vitamin A derivatives 13-*cis*-retinoic acid (CRA) and all-*trans*-retinoic acid (RA) induced glycine *N*-methyltransferase (GNMT) activity and protein abundance in rats and cell culture (Rowling *et al.*, 2002; Rowling and Schalinske 2001; Rowling and Schalinske 2003). GNMT is a key

cytosolic protein that serves to regulate methyl group metabolism by producing sarcosine from glycine when methyl group supply is abundant. However, inappropriate activation of GNMT, as occurs with retinoid administration, can have adverse effects, such as diminishing the supply of methyl groups available for important transmethylation reactions. In fact, our laboratory has shown that GNMT up-regulation by retinoid compounds led to DNA hypomethylation (Rowling *et al.*, 2002).

Our previous findings of GNMT up-regulation by RA and CRA led us to investigate the effects of the glucuronic acid derivative of retinoic acid, retinoyl β-glucuronide (RAG) on methyl group metabolism. RAG has been shown to be less toxic than RA in animal and cell culture studies (Becker *et al.*, 1998; Gunning *et al.*, 1993); however, the effect of RAG on GNMT activity had not been studied previously. Therefore, we conducted the study presented in Chapter 3 of this thesis to determine if the oral administration of RAG affected GNMT in a similar fashion to RA, and if vitamin A status altered any effects of RA or RAG on GNMT activity.

Age and gender represent factors that alter methyl group metabolism. Previously our laboratory has shown that compared to female rats, male rats are more sensitive to retinoid-induced disruptions of methyl group metabolism (McMullen *et al.*, 2002). Additionally, Mays et al. (1973) showed that GNMT activity was increased in 12-month compared to 3-month old rats. Due to these previous findings, we conducted the study presented in Chapter 4 of this thesis, in which the differential effect of RA administration on methyl group and homocysteine metabolism in 4- and 10-wk old male and female rats was investigated.

Finally, our laboratory has demonstrated that RA induced GNMT activity in a dose-dependent manner, with significant induction beginning at doses of 5 μmol/kg body weight, and maximal induction at 30 μmol/kg body weight (Ozias and Schalinske 2003). These findings in addition to previous findings from our laboratory prompted the study described in Chapter 5, in which we examined the effects of RA in doses of 5 μmol/kg body weight and 30 μmol/kg body weight on methyl group metabolism in folate deficient, folate sufficient, and folate supplemented rats.

CHAPTER 2
LITERATURE REVIEW

Folate Metabolism

Chemical Structure and Properties of Folate

Folate, an essential water-soluble B-vitamin, has more biologically active forms than any other vitamin. Pure folate, known as pteroylmonoglutamic acid, has three distinct parts: glutamic acid, ρ-aminobenzoic acid, and a pteridine nucleus (**Figure 1**).

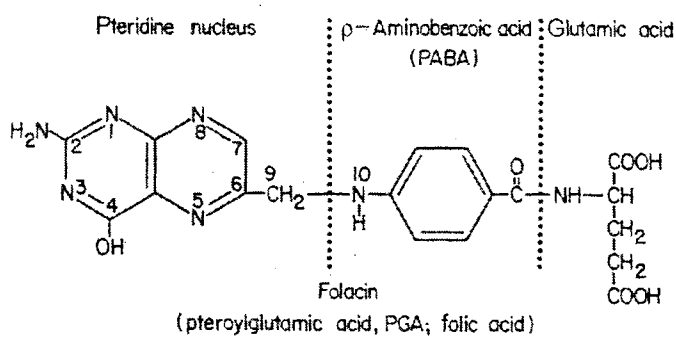

Figure 1. Folate structure (from McDowell 2000).

Naturally occurring folate is polyglutamated due to conjugation with a varying number of glutamic acid residues, which creates a large family of pteroylpolyglutamates. Folate moieties are added to the family of pteroylpolyglutamates through additional modifications of the parent molecule, including a change in reduction state of the pteridine moiety and/or addition of one-carbon units. Synthetic folate, or folic acid, produced for food fortification and vitamin supplements, is in the monoglutamate form. The pteridine ring of synthetic folate is not reduced, making it very resistant to chemical oxidation, and thus more stable than natural folate (Scott 1999). Biologically active folate compounds have a formyl (~CHO) or methyl (~CH_3) group attached to N5 or N10, or a methylene (~CH_2) group between N5

and N10 of the fully reduced form of folate, tetrahydrofolate (THF). THF is the main coenzyme form of folate, and it serves to donate the one-carbon units in one-carbon transfer reactions (McDowell 2000).

Digestion, Absorption, Transport, Storage, and Excretion

Prior to transport across the intestinal mucosa, polyglutamated folates are hydrolyzed to pteroylmonoglutamates by pteroylglutamate hydrolase, a conjugase located in the intestinal brush border. Because zinc serves as a cofactor for the activation of pteroylglutamate hydrolase, zinc deficiency can inhibit the intestinal hydrolysis of folate polyglutamates. Other factors known to interfere with folate absorption include chronic alcohol intake and foods, such as orange juice, tomatoes, and lima beans that contain naturally occurring inhibitors, which also decrease the activity of pteroylglutamate hydrolase (McDowell 2000). The process of removing the polyglutamate chain by intestinal conjugase delays the absorption of natural food folates, leading to a 25-50% decrease in bioavailability, whereas synthetic folate is believed to be 100% bioavailable in its monoglutamate form (Scott 1999).

Pteroylmonoglutamates are absorbed primarily in the jejunum by a sodium-coupled carrier mediated process, as well as by passive diffusion. Following intestinal absorption and cleavage of glutamate residues, dietary folates are transported to the liver where the monoglutamate derivatives are converted mainly to 5-methyltetrahydrofolate (5-CH_3THF) and 10-formyltetrahydrofolate (10-formylTHF) before transport to peripheral tissues. In cells, 5-CH_3THF and 10-formylTHF are converted back to their pteroylpolyglutamate form by folate polyglutamate synthetase. Polyglutamation traps folate inside of cells, as only

monoglutamated forms can be transported across cell membranes, and 5-CH_3THF serves as the primary storage form of folate in the body (McDowell 2000).

Humans normally have 5-10 mg of folate stored, with approximately 50% stored in the liver. These stores are adequate to meet requirements for 4-5 months. Folates are excreted through urine and bile, with biliary excretions being predominant (McDowell 2000).

Dietary Requirements

Folate requirements are designated as dietary folate equivalents (DFE) due to the difference in bioavailability of naturally occurring versus synthetic folate compounds. One DFE is equivalent to: 1 μg food folate; 0.6 μg folate from fortified food or as a supplement consumed with food; or 0.5 μg of supplemental folate taken on an empty stomach. For both males and females over the age of 14, the recommended dietary allowance (RDA) is 400 DFE, and the upper level (UL) is 800-1000 DFE. The UL applies to synthetic forms of folate from fortified foods or dietary supplements. The folate requirement is increased for pregnant and lactating women to 600 DFE and 500 DFE, respectively (Food and Nutrition Board 2000). Natural sources of folate include green leafy vegetables, potatoes, oranges, beans, yeast, and liver (van der Put *et al.*, 2001). In 1996 the U.S. Food and Drug Administration authorized the addition of folic acid to enriched grain products, and compliance with this law was mandatory by 1998. Cereal grains are fortified with 140 μg of pteroylmonoglutamic acid per 100 g of grain (Food Standards 1996), which has contributed to a significant reduction in folate deficiency-related diseases in the U.S (Honein *et al.*, 2001).

Biological Functions

Folate is involved in multiple metabolic pathways in which its primary role is to accept and donate one-carbon units (Bailey and Gregory 1999). The one-carbon units that folate accepts or donates can be formyl, formimino, methylene, or methyl groups. A majority of one-carbon units associated with folate are derived from serine; however, they may also originate from glycine, sarcosine, formate, and histidine. (Pasternack *et al.*, 1996). Specific reactions that involve the transfer of one-carbon units by folate compounds include: thymidylate and purine biosynthesis, serine and glycine interconversion, histidine and formate metabolism, and methionine synthesis via homocysteine remethylation (van der Put 2001) (**Figure 2**).

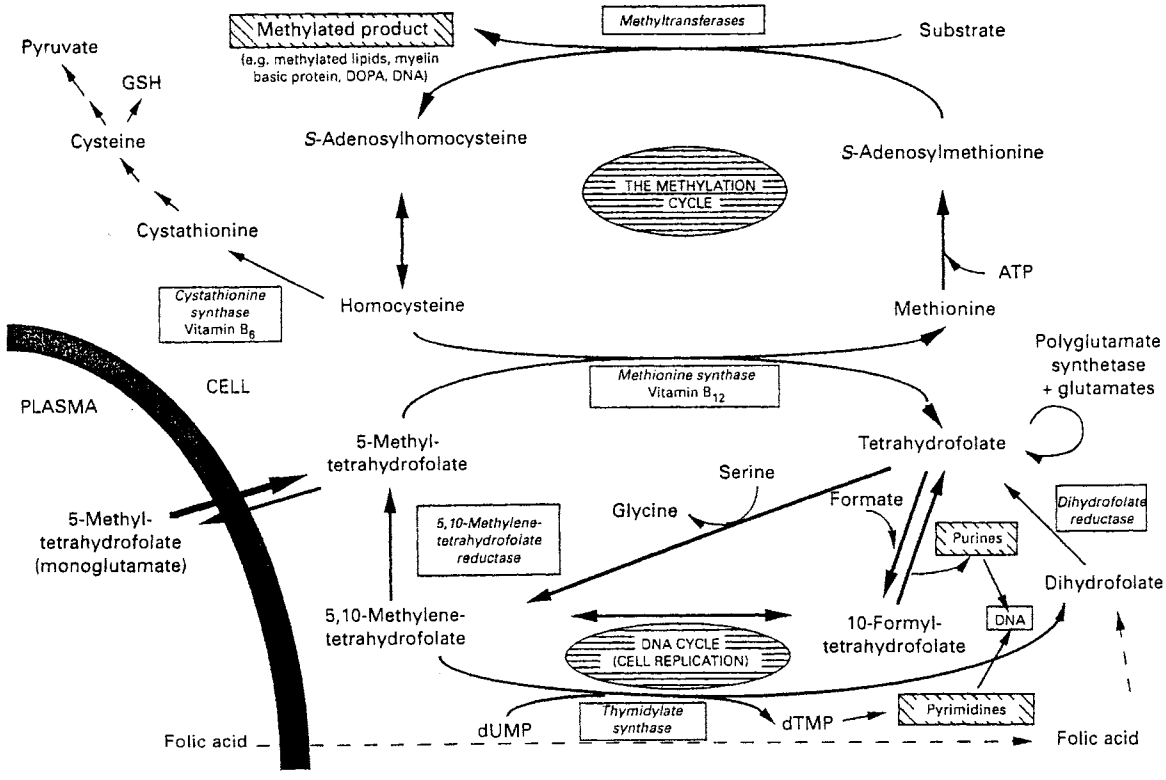

Figure 2. The biological functions of folate (from Scott 1999).

Folate Deficiency

Folate plays an integral role in the synthesis and modification of a number of important biological compounds; therefore, folate deficiency has the potential to disturb various biological functions required for optimum health. Folate is required for DNA synthesis and cell replication. A severe folate deficiency is manifested as megaloblastic anemia, which is caused by abnormal division of red blood cell precursors (Lindenbaum and Allen 1995). Additionally, a decrease in cell division leads to a diminished number of white blood cells and platelets. Generally there is impairment of overall cell division in tissues that are rapidly turning over, such as cells of the hematopoietic system and gastrointestinal tract, due to the important role that folate plays in nucleic acid synthesis (Lindenbaum and Allen 1995).

As mentioned previously, one of the major functions of folate is the donation of methyl groups to homocysteine during the de novo synthesis of methionine. Before this reaction can occur however, 5,10-methylenetetrahydrofolate (5,10-CH_2THF) must be reduced to 5-CH_3THF by an irreversible riboflavin-dependent reaction catalyzed by methylenetetrahyrdrofolate reductase (MTHFR). Following this reaction, 5-CH_3THF can then donate its methyl group to homocysteine and regenerate methionine in a vitamin B_{12}-dependent reaction catalyzed by methionine synthase (MS). Thus, folate or vitamin B_{12} deficiencies have obvious implications for the accumulation of homocysteine. Hyperhomocysteinemia has been implicated in a number of pathologies and it is now recognized as an independent risk factor for cardiovascular disease (Clarke *et al.*, 1991; Kang *et al.*, 1986; Pancharuniti *et al.*, 1994). Because the folate-dependent remethylation of

homocysteine is required to regenerate methionine for use in methyl group metabolism, it is not surprising that folate deficiency eventually can lead to a lack of methyl groups available for important methyltransferase reactions (Rampersaud *et al.*, 2000; Fournier *et al.*, 2002) during which biological compounds, such as nucleic acids, phospholipids, and neurotransmitters are methylated (Scott 1999).

The reactions described above illustrate the importance of maintaining the relationship between folate-dependent one-carbon metabolism and methyl group metabolism. Manifestations of folate deficiency and subsequent alterations of methyl group metabolism will be discussed in further detail in upcoming sections.

Methyl Group Metabolism

Transmethylation, Remethylation, and Transsulfuration

The conversion of methionine to S-adenosylmethionine (SAM) is catalyzed by the enzyme methionine adenosyltransferase (MAT). In this reaction, an adenosine group from ATP is transferred to methionine, thereby activating methyl groups for use in transmethylation reactions (Mudd 1963). Among the hundreds of SAM-dependent transmethylation reactions are the modification or synthesis of molecules such as nucleic acids, phospholipids, proteins, and neurotransmitters (Mason 2003) (**Figure 3**). Such methylation reactions are very dependent on the supply of SAM. When SAM is incubated with liver extracts, 99% of the SAM is utilized for the donation of methyl groups in transmethylation reactions (Eloranta and Kajander 1984).

Following the donation of methyl groups, SAM is converted to S-adenosylhomocysteine (SAH). Because methyltransferase reactions are inhibited by SAH,

the ratio of SAM:SAH is considered to be an index of transmethylation potential (Kerr 1972; Cantoni and Chiang 1980). Therefore, the removal of SAH is essential in the maintenance of normal methyl group metabolism. SAH is converted to homocysteine via a single pathway in a reaction catalyzed by SAH hydrolase. Because this reaction is not kinetically favored, the reaction is allowed to proceed in the direction of homocysteine only when the products of the reaction, adenosine and homocysteine, can be efficiently removed (Cantoni and Chiang 1980).

The removal of homocysteine can be achieved by two different mechanisms. Homocysteine can either be remethylated to regenerate methionine or further catabolized via the transsulfuration pathway. Homocysteine can be remethylated to generate methionine by either 5-CH_3THF or betaine. The remethylation reactions are catalyzed by MS and betaine-homocysteine methyltransferase (BHMT), respectively. The remethylation of homocysteine by MS occurs in nearly all mammalian tissues except for the small intestine, while remethylation by BHMT has been shown to occur only in mammalian liver and primate kidney (McKeever *et al.*, 1991; Finkelstein *et al.*, 1971). If remethylation of homocysteine does not occur, then further catabolism of homocysteine occurs via the transsulfuration pathway. The initial step in this pathway is the irreversible conversion of homocysteine to cystathionine by cystathionine β-synthase (CBS), a vitamin B_6-dependent enzyme. Cystathionine is catabolized by γ-cystathionase (also a vitamin B_6-dependent enzyme) to cysteine. The catabolism of homocysteine via the transsulfuration pathway generates several important metabolic products, including glutathione, taurine, pyruvate, and sulfates. The transsulfuration pathway has limited distribution in mammalian tissues. Both CBS and

γ-cystathionase are found primarily in the liver, kidney, small intestine, and pancreas, while the brain contains only CBS (Finkelstein and Martin 2000).

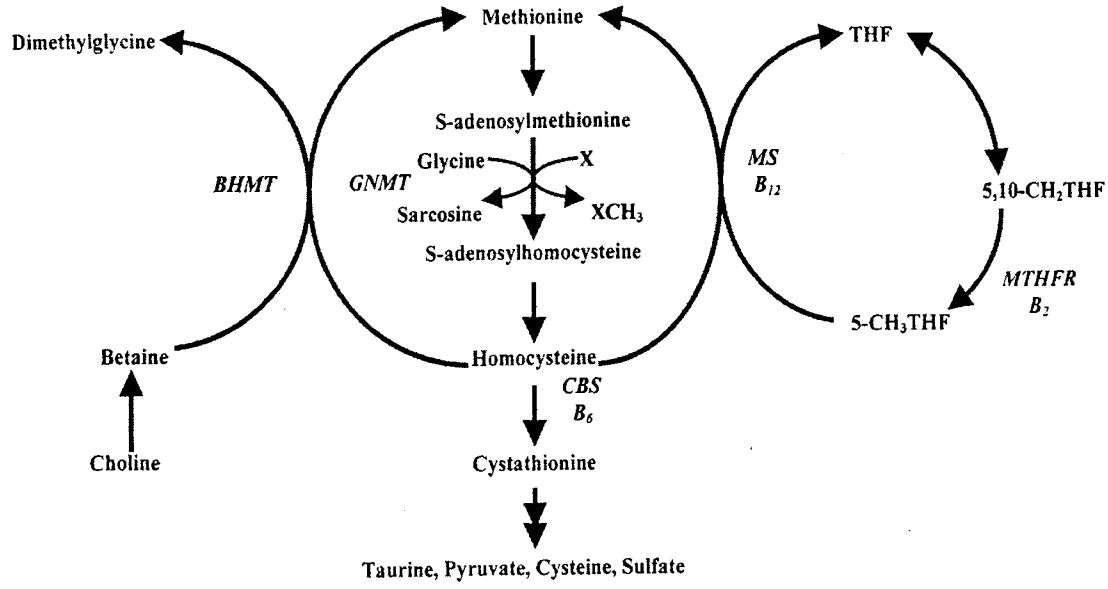

Figure 3. Methyl group metabolism.

Regulation of Methyl Group Metabolism

Effects of Dietary Methionine Content

There are several mechanisms that serve to regulate the transmethylation, remethylation, and transsulfuration pathways. In cases of excess dietary methionine and increased SAM concentrations regulatory mechanisms are in place to normalize their concentration. SAM inhibits BHMT and MTHFR, and activates CBS, which serves to inactivate the remethylation pathway and activate the transsulfuration pathway (Finkelstein and Martin 1984a; Finkelstein and Martin 1984b; Kutzbach and Stokstad 1967). MTHFR and BHMT are responsible for the remethylation of homocysteine, while CBS irreversibly commits homocysteine to the transsulfuration pathway. The inhibition of MTHFR by SAM leads to a decrease in 5-CH_3THF concentration. 5-CH_3THF is an allosteric inhibitor of

glycine *N*-methyltransferase (GNMT), an enzyme that functions to remove excess methyl groups through the SAM-dependent methylation of glycine to form sarcosine, a compound with no known physiological function (Wagner 1995; Cook and Wagner 1984; Selhub and Miller 1992). Hence, removal of GNMT inhibition due to a decrease in 5-CH_3THF concentrations leads to increased GNMT activity, which serves to maintain the SAM:SAH ratio by facilitating the catabolism of excess SAM (Wagner 1995).

Decreased concentrations of SAM within the cell leads to uninhibited synthesis of 5-CH_3THF and suppression of CBS activity. These regulatory mechanisms ensure that SAM concentrations can be restored by increased remethylation of homocysteine. Taken together, the ability of the cell to coordinate the remethylation and transsulfuration pathways indicates that the adaptation to varying levels of dietary methionine is of major biological significance (Selhub 1999).

Effects of Age and Gender

Previous work showed that compared to men, women have an increased flux through both the remethylation and transsulfuration pathways, indicating that women may have sex-related differences in MS and/or BHMT activity (Fukagawa *et al.*, 2000). In contrast, the activity of MS in the liver of female rats was 73% of the activity that was seen in male rats (Finkelstein *et al.*, 1971), indicating that the evidence is conflicting regarding gender differences in remethylation rates.

Aged rats weighing ~280 g have been shown to exhibit decreased liver MS activity, while hepatic CBS and γ-cystathionase activity has been shown to increase compared to younger rats (Finkelstein *et al.*, 1971). Moreover, these animals exhibited decreased MS

activity in kidney and brain, whereas pancreatic MS activity was increased. These data suggests that a transition from methionine conservation to catabolism occurs with aging (Finkelstein *et al.*, 1971). Mays et al. (1973) also found that GNMT activity was elevated by 30% in liver and 40% in kidney in12-month old rats compared to 3-month old rats. Total tRNA methylation was decreased by 35% in 12-month old rats, which indicates that there was the potential for tRNA hypomethylation and diminished tRNA functional fidelity in these animals.

Hormonal Regulation

Finkelstein et al. (1971) showed that several hormones have the ability to alter the function of enzymes involved in methyl group metabolism. Rats treated with hydrocortisone exhibited a 3-fold increase in hepatic BHMT activity, while thyroxine treatment significantly reduced the activity of this enzyme. MS activity increased with estrogens, while growth hormone had the opposite effects in the kidney.

It was recently demonstrated that dexamethasone, a synthetic glucocorticoid, induces GNMT activity and abundance in the liver of rats, independent of adrenal function (Rowling & Schalinske 2003). Furthermore, rats without intact adrenal function also exhibited increased homocysteine levels, which were reduced to normal concentrations following dexamethasone treatment (Rowling & Schalinske 2003). These findings indicate that the transsulfuration and/or remethylation pathways can be enhanced by treatment with glucocorticoids (Rowling & Schalinske 2003). Similarly, Jacobs et al. (2001) found that when treated with glucagon, rats exhibited a 30% decrease in total plasma homocysteine and increased hepatic GNMT, CBS, and γ-cystathionase activity. The fact that CBS activity was

increased indicates that an increased flux through the transsulfuration pathway occurred in these animals. Hepatic CBS activity was also elevated in streptozotocin-induced diabetic rats, leading to decreased homocysteine levels. CBS activation was prevented by insulin treatment, suggesting that the transsulfuration pathway within the liver is regulated by insulin in these animals (Ratnam *et al.*, 2002). Plasma homocysteine concentrations are known to be altered in diabetic patients. Homocysteine levels are increased in diabetic patients with renal insufficiency due to the role of the kidney in homocysteine metabolism; however, patients with insulin-dependent diabetes mellitus without clinical signs of renal insufficiency have lower than average levels of homocysteine due to insufficient levels of insulin and increased levels of glucagon and glucocorticoids (Hofmann *et al.*, 1997; Robillon *et al.*, 1994; Bostom *et al.*, 1995; Jacobs *et al.*, 1998).

Disruptions of Methyl Group Metabolism

Because several nutritional factors are involved in the metabolism of methionine and homocysteine, deficiencies of these factors, which include methyl groups, vitamin B_6, vitamin B_{12}, or folate can potentially lead to disruptions in methyl group metabolism. Additionally, congenital disorders resulting in genetic polymorphisms in enzymes involved in remethylation or transsulfuration reactions, or use of pharmacological agents for therapeutic purposes can also lead to alterations in methyl group metabolism. Disturbances to normal methyl group metabolism have obvious implications for the development of a number of pathologies. In fact, abnormal methyl group metabolism has been associated with an increased risk of developing neural tube defects (NTD), vascular disease, and certain types of cancer.

Nutrient Deficiencies and Vascular Disease, NTDs, and Cancer

Low folate intake has been associated with increased homocysteine levels in healthy men, middle-aged adults, elderly populations, and postmenopausal women (Jacob *et al.*, 1994; Shimakawa *et al.*, 1997; Selhub *et al.*, 1996; Jacob *et al.*, 1998). Additional nutrient deficiencies that could hypothetically lead to hyperhomocysteinemia, include vitamin B_{12} (MS cofactor) and vitamin B_6 (CBS cofactor). A review examining the role of nutrient deficiencies in the development of hyperhomocysteinemia suggested that vitamin B_{12} and folate deficiencies are associated with increased plasma homocysteine levels; however, the evidence for vitamin B_6 was less convincing (Selhub and Miller 1992).

Vascular Disease

As mentioned previously, several have proposed that hyperhomocysteinemia is an independent risk factor for vascular disease (Clarke *et al.*, 1991; Kang *et al.*, 1986; Pancharuniti *et al.*, 1994). It has been suggested that homocysteine may promote cardiovascular disease by inducing endothelial dysfunction, inhibiting endothelial cell regeneration, and by direct activation of quiescent vascular smooth muscle cells (Lee and Wang 1999).

In vitro studies have shown that homocysteine is an aggressive oxidizing agent because it increases hydrogen peroxide production in endothelial cells, which negatively affects antioxidant systems and promotes lipid peroxidation (Starkebaum *et al.*, 1986; Blundell *et al.*, 1996; Jones *et al.*, 1994). Huang et al. (2001) demonstrated that folate depletion led to compromised hepatic antioxidant enzymatic defense systems as evidenced by decreased glutathione peroxidase and superoxide dismutase in rats. These researchers

concluded that this was due to increased plasma homocysteine levels, and that folate depletion and increased homocysteine levels lead to hepatic oxidative stress in rats. In a study done among older adults (males and females 50-75 years old) with high plasma homocysteine concentrations, researchers found that a daily supplement with 400 μg folic acid decreased homocysteine levels by ~22%, while a supplement with 50-100 μg decreased levels ~10% in 12 weeks (van Dort *et al.*, 2003).

NTDs

NTDs encompass a group of malformations of the embryonic brain and/or spinal cord that are characterized by incomplete development of the central nervous system and its related surrounding structures (van der Put *et al.*, 2001). The prevalence of NTDs in the United States is 1.3-2.0 per 1000 live births (Shoob *et al.*, 2001).

Both folate and methionine have been implicated in the etiology of NTDs. It has been demonstrated that methionine supplementation led to a 55% decrease in NTD risk in women who averaged a daily intake of methionine that was >2830 mg/day and a 30% decrease among women who averaged >1580 mg/day. Although these data indicate a decrease in NTD risk with increased maternal dietary intake of methionine 3-months pre- and post-conception, the researchers cautioned against assuming that methionine alone was responsible for the decreased NTD risk since methionine may affect folate, vitamin B_6, and vitamin B_{12} status (Shoob *et al.*, 2001).

The link between folate and NTDs was first suggested by Hibbard (1964), who performed a retrospective study of women who had NTD-affected pregnancies, and discovered that these women had an increased incidence of aberrant folate metabolism.

Similarly, Smithells et al. (1976) measured blood vitamin levels in women who experienced previous NTD-affected pregnancies and observed that RBC folate and leukocyte ascorbic acid levels were significantly decreased compared to mothers without NTD-affected pregnancies. Because these data suggested that folate stores are decreased in women who have suffered NTD-affected pregnancies, researchers hypothesized that periconceptual vitamin supplementation would reduce the incidence of NTDs.

Although the exact mechanism by which folate supplementation prevents NTDs is still not clear, data from several studies indicates that folic acid supplementation has decreased the rate of NTD-affected pregnancies (MRC Vitamin Study Research Group 1991; Czeizel 2000; Berry *et al.*, 1999). The U.S. Public Health Service recommends that women without a previous history of a NTD-affected pregnancy take 400 μg of folic acid/day in addition to DFE consumed in the diet before conception and through the first trimester, whereas women who have had a previous NTD-affected pregnancy are encouraged to take 4000 μg of folic acid daily before and during early pregnancy (Centers for Disease Control and Prevention 1992). Folate supplementation has led to a 50% decrease in NTDs among women without a history of NTD-affected pregnancy, and a 71% decrease in the recurrence of NTDs among women who had previous NTD-affected pregnancies (Am Academy of Pediatrics Committee on Genetics 1999).

There have been several proposed mechanisms through which folate prevents NTDs. It is well understood that adequate folate is required for DNA synthesis and cell division during fetal development; however, in recent years there has been a focus on studying the role of folate in NTD prevention in further detail to determine if there are additional underlying

mechanisms that folate works through to prevent NTDs. Researchers have hypothesized that the effect of folate on NTDs may be due to an increased folate requirement in women who are genetically predisposed to have a NTD-affected pregnancy (Scorah *et al.*, 1993), or that folic acid may overcome metabolic disturbances of the folate-related metabolic pathway, such as inefficient functioning of folate-dependent enzymes within the pathway (Habibzadeh *et al.*, 1993). Although the exact mechanism for prevention of NTDs by folate remains unclear, NTDs have consistently been associated with mildly elevated plasma homocysteine concentrations (Lucock and Daskalakis 2000). Because folate is required to maintain normal homocysteine concentrations elevations in homocysteine suggest that the preventative effect of folate may be due to its role in the remethylation pathway.

Cancer

Due to the role of folate in the de novo synthesis of methionine, folate deficiency can lead to an insufficient supply of methyl groups available for important methyltransferase reactions, such as the methylation of DNA. Several types of premalignant conditions and cancer have been associated with folate status, including premalignant cervical dysplasia, colon cancer, and breast cancer (Butterworth 1993; Slattery *et al.*, 1999; Zhang *et al.*, 1999). Folate deficiency has been associated with hypomethylated DNA, including specific sites on promoter regions controlling the expression of oncogenes. Methylation of these regions is believed to suppress their expression; however, the lack of methylation in these sites is believed to cause alterations in the normal control of proto-oncogene expression, and inappropriate gene expression may occur (Hug *et al.*, 1996).

Rodent studies have demonstrated that a combined methionine, choline, folate and vitamin B_{12} deficiency or a severe folate deficiency alone causes genomic DNA hypomethylation (Wainfan *et al.*, 1989; Balaghi and Wagner 1993). Additionally, folate deficiency leads to uracil misincorporation into DNA, which leads to chromosome instability and breakages (Bailey and Gregory 1999). This occurs primarily when 5,10-CH_2THF levels are low because it is required to for the reaction in which deoxyuracil monophosphate (dUMP) is converted to deoxythymidine monophosphate (dTMP) (Duthie and Hawdon 1998).

Genetic Polymorphisms

Genetic polymorphisms of enzymes involved in the remethylation or transsulfuration pathways can also contribute to the development of vascular disease, NTDs, and cancer.

C677T MTHFR Polymorphism

The most common mutation in the MTHFR enzyme is a C→T substitution at base pair 677, which leads to a substitution of valine for alanine in the functional enzyme (Frosst *et al.*, 1995). This mutation is an autosomal recessive mutation, and frequency is variable among racial and ethnic groups. Caucasian and Asian populations have mutation rates of ~12% for homozygous and up to 50% for heterozygous, while African-Americans have a very low incidence of the homozygous mutation (Frosst *et al.*, 1995; Austin *et al.*, 1997).

Studies have demonstrated that individuals who are homozygous for the MTHFR polymorphism are more prone to mild elevations in plasma total homocysteine levels compared to people who are heterozygous for the mutation or homozygous wild-type (Brattstrom *et al.*, 1998; Gudnason *et al.*, 1998). However, the relationship between

increased plasma homocysteine levels exists mainly in individuals with poor folate status. When folate status is adequate, plasma homocysteine levels are usually normal, independent of genotype (Jacques *et al.*, 1996; Kluijtmans *et al.*, 2003). It has been hypothesized that the amount of folate necessary to normalize homocysteine may be increased in individuals who are homozygous for the MTHFR polymorphism, and that these people may have a higher folate requirement (Rosenberg and Rosenberg 1998).

C677T MTHFR Polymorphism and Vascular Disease. Whether or not the C677T MTHFR polymorphism causes an increased risk for vascular disease is still a topic for debate. Although the polymorphism has been associated with elevations in homocysteine, studies have not made a direct association between the C677T MTHFR polymorphism and cardiovascular disease. In a meta-analysis done by Brattstrom et al. (1998), it was concluded that those who are homozygous for the mutation do not have an increased risk for cardiovascular disease. Moreover, others have found that individuals with the mutation are relatively equally distributed among participants with cardiovascular disease and controls (Verhoef *et al.*, 1997). In contrast, a meta-analysis performed by Klerk et al. (2002), concluded that there was a 16% increase in cardiovascular disease among C677T homozygotes.

C677T MTHFR Polymorphism and NTDs. Multiple studies have shown that homozygotes for the C677T MTHFR polymorphism are at increased risk for experiencing a NTD-affected pregnancy (Ou *et al.*, 1996; van der Put *et al.*, 1995; van der Put *et al.*, 1997; Whitehead *et al.*, 1995). In a 1997 meta-analysis performed by van der Put et al., it was concluded that ~15% of all NTD cases were attributed to the C677T MTHFR polymorphism.

Despite these findings, other researchers have concluded that the risk for NTDs associated with the MTHFR polymorphism is minimal if plasma homocysteine and folate status are normal. Molloy et al. (1998) found that participants who were C677T homozygotes had a marked reduction in blood folate concentrations compared to both heterozygous and homozygous wild-type participants. These findings suggest an indirect association between the C677T MTHFR polymorphism and NTDs exists.

C677T MTHFR Polymorphism and Cancer. Chen et al. (1996, 1998) demonstrated that there is an inverse relationship between the frequency of the homozygotic C677T MTHFR genotype and the risk for colorectal cancer in case-control studies. Similarly, data from the Physicians' Health Study indicated that individuals with normal folate levels and the homozygous C677T genotype have a 50% reduction in colon cancer risk compared to normal genotypes (Ma *et al.*, 1997); however, the researchers concluded that the reduction in risk is diminished in those who are C677T homozygotes and have low folate status. The decrease in colon cancer risk in those who are homozygous for the C677T MTHFR polymorphism may be due to increased availability of 5,10-CH_2THF, which is required for normal DNA synthesis and cell division. Chen et al. (1996) hypothesized that homozygotes for the C677T polymorphism who have normal folate status are also less likely to have insufficiencies in the pools of nucleotide precursors required for DNA synthesis.

CBS Enzyme Deficiency

Deficiency of the vitamin B_6-dependent CBS enzyme is an autosomal recessive inborn mutation that leads to disruptions in homocysteine metabolism. CBS deficiency has been associated with elevated plasma and urine homocysteine levels, as well as increased

methionine levels in the blood and tissues. This may be due to the diversion of homocysteine toward the remethylation pathway (Yaghmai *et al.*, 2002). With increased methionine synthesis via remethylation, SAM concentrations increase until the level is sufficient to inhibit the action of MTHFR. At this point, severe hyperhomocysteinemia occurs (to a greater extent in CBS deficient homozygotes) due to impairment of both pathways of homocysteine metabolism (Selhub 1999). Diseases such as osteoporosis, arteriosclerosis, and thromboembolic disease are associated with CBS deficiency, and 50% of patients who are untreated are mentally retarded. The exact mechanism through which these diseases linked with CBS deficiency develop remains unclear; however, hyperhomocysteinemia has been implicated in these disorders (Yaghmai *et al.*, 2002).

Vitamin B_6 therapy is often used to treat CBS deficiency; however, not all of these patients respond to B_6 treatment. In these cases, homocysteine levels can be normalized by a methionine-restricted diet, or through folate and vitamin B_{12} supplementation (Wilcken and Wilcken 1997). Betaine supplementation has also been an effective strategy in lowering plasma homocysteine levels in individuals with CBS deficiency, and several studies have shown that therapeutic betaine is effective in vitamin B_6 nonresponders (Smolin *et al.*, 1981; Wilcken *et al.*, 1983; Walter *et al.*, 1998)

MS Polymorphism

MS deficiency is an inborn error resulting from an A→G substitution at base pair 2756. This mutation leads to the substitution of glycine for aspartic acid in the amino acid sequence of the enzyme. It is not known whether or not this mutation causes changes in MS

enzyme activity levels; however, it has been hypothesized that it may increase homocysteine levels, which in turn would increase cardiovascular disease risk (Leclerc *et al.*, 1996).

In a case-control study of a Dutch population done by Klerk et al. (2003), there was not a significant difference in homocysteine concentrations in individuals with the 2756A→G polymorphism versus the control subjects. However, those who were homozygous for the MS polymorphism had serum vitamin B_{12} levels that were ~30% lower than heterozygotes and controls. There was no significant difference in red blood cell folate levels among the three groups. Overall there was a four-fold increase in cardiovascular disease risk among homozygotes for MS deficiency compared to heterozygotes and controls when adjustment was made for cardiovascular disease risk factors, such as total cholesterol, blood pressure, and current smoking and alcohol use. These results conflict with findings of similar studies investigating the association between the MS polymorphism and vascular disease. In fact, Chen et al. (2001) found that there was no significant association between the MS polymorphism and increased risk of myocardial infarction. Furthermore, there was a non-significant 49% reduction in risk for myocardial infarction in those who were homozygous for the MS polymorphism. The myocardial infarction risk reduction was believed to be due to decreased levels of plasma homocysteine in these individuals. Additional studies have also demonstrated that individuals with the MS polymorphism have a trend toward decreased circulating homocysteine levels and no increased risk for vascular disease, suggesting that this polymorphism may provide a genetic benefit (Ma *et al.*, 1999; Morita *et al.*, 1999; Tsai *et al.*, 1999).

Retinoids

Retinoid compounds, such as 13-*cis*-retinoic acid (CRA; Isotretinoin or Accutane) and all-*trans*-retinoic acid (RA; Vesanoid), are used in the treatment of several medical conditions. CRA is the primary treatment for cystic acne, while RA is used in the treatment of acute promyelocytic leukemia (Bershad 2001; Gollnick *et al.*, 2003; Ohno *et al.*, 2003). Additionally, thousands of individuals take vitamin supplements containing high doses of the parent compound of these retinoids, vitamin A. There was a large increase in prescriptions of isotretinoin dispensed in the United States from 1992 to 2000. From 1983 to 1993 there were ~800,000 new isotretinoin prescriptions dispensed each year, while in 2000 there were nearly 2 million dispensed (Wysowski *et al.*, 2002). Although effective for the treatment of certain medical conditions, individuals receiving therapeutic retinoids should be monitored due to toxic side effects associated with their use, including increased methionine catabolism, which has been demonstrated in animal studies (Schalinske and Steele 1991; Rowling *et al.*, 2002; Rowling and Schalinske 2001). These side effects are of concern due to the rise in usage of these compounds, and the potential for pathological consequences due to impaired methyl group metabolism.

Effects of Retinoids on Methyl Group and Folate Metabolism

Fell and Steele (1986) demonstrated that feeding rats excess retinol altered coenzymes in the folate-dependent one-carbon pool, and in a second study these researchers discovered that this led to decreased levels of SAM in the liver and fewer methyl groups available for important transmethylation reactions (Fell and Steele 1987). It has also been shown that patients on isotretinoin therapy for cystic acne have elevated plasma homocysteine levels;

however, concentrations of folic acid, vitamin B_6 and vitamin B_{12}, cofactors required for homocysteine metabolism, were not affected by isotretinoin treatment (Schulpis *et al.*, 2001). These findings suggest that isotretinoin inappropriately affects methyl group metabolism, and that the increased homocysteine concentrations are possibly due to inhibition of the CBS enzyme by the drug (Schulpis *et al.*, 2001).

Effect of Retinoids on GNMT. GNMT is a 32 kd cytosolic protein that composes 1-3% of total cytosolic protein in the liver (Heady and Kerr 1975; Suzuki and Wagner 1980), making it one of the most abundant cytosolic proteins. GNMT is distributed in the periportal region of the liver, proximal convoluted tubules of the kidney, acinar cells of the pancreas, and epithelial cells lining the villi of the jejunum in rats (Yeo and Wagner 1994). Due to its limited tissue distribution in gluconeogenic tissues (liver, kidney, and pancreas), it has been suggested that GNMT plays a role in gluconeogenesis by functioning to convert methionine to pyruvate, an end-product of the transsulfuration pathway (Yeo and Wagner 1994).

Studies have demonstrated that GNMT is potently inhibited by 5-CH_3THF, suggesting that GNMT plays a regulatory role in methyl group availability (Wagner *et al.*, 1985). As described previously, GNMT is active when there is an abundant supply of methyl groups due to the lack of inhibition by 5-CH_3THF, and is inactive when SAM concentration is low. This indicates that the enzyme functions to regulate the SAM:SAH ratio and helps to ensure that there is an adequate supply of methyl groups for the methylation of DNA, RNA, phospholipids, and other biologically important compounds. In addition to allosteric inhibition by 5-CH_3THF, GNMT can be regulated by phosphorylation. GNMT is stimulated by covalent phosphorylation by protein kinases, which leads to a ~2-fold increase in GNMT

enzyme activity. Regardless of whether GNMT is phosphorylated or unphosphorylated, its activity remains inhibited by 5-CH_3THF, and when it is in its unphosphorylated state, 5-CH_3THF decreases GNMT effectiveness as a protein kinase substrate (Wagner *et al.*, 1989).

It has been demonstrated that retinoid compounds are responsible for activating and inducing GNMT in rat tissues in a tissue- and gender-specific manner, thereby leading to the down-regulation of SAM-dependent transmethylation reactions. Thus, inappropriate activation of GNMT could lead to pathological consequences. McMullen et al. (2002) showed that GNMT activity and abundance was increased ~2-fold in male rats treated with RA. Female rats also demonstrated an increase in hepatic GNMT activity, but to a lesser extent than male rats. Retinoid treatment was without effect on pancreatic and renal GNMT (McMullen *et al.*, 2002).

The next three chapters in this thesis will examine the effects of retinoids on methyl group metabolism in male and female rats of different ages, as well as in male rats with altered vitamin A and folate status.

CHAPTER 3
RETINOYL β-GLUCURONIDE ALTERS GLYCINE *N*-METHYLTRANSFERASE ACTIVITY TO A LESSER DEGREE THAN ALL-*TRANS*-RETINOIC ACID IN VITAMIN A SUFFICIENT AND VITAMIN A DEFICIENT RATS

Introduction

Methyl groups, supplied from the diet (i.e. as methionine and/or choline) or folate-dependent one-carbon pool are required for the hundreds of transmethylation reactions that occur throughout the body. Methionine is converted to S-adenosylmethionine (SAM), which activates methyl groups for transmethylation reactions. Included in transmethylation reactions are SAM-dependent reactions that are required for the modification and/or synthesis of biological compounds, such as nucleic acids, neurotransmitters, and phospholipids. Therefore, without an adequate supply of methyl groups, pathological conditions may result. In fact, several studies have linked an inadequate supply of methyl groups with hepatocarcinogenesis (Ghoshal and Farber 1984; Salmon and Copeland 1954).

Upon donating methyl groups, SAM is converted to S-adenosylhomocysteine (SAH), a compound that inhibits most transmethylation reactions. The ratio of SAM:SAH is therefore considered an index of transmethylation potential (Kerr 1972; Cantoni and Chiang 1980), and efficient removal of SAH must be achieved to ensure normal methyl group metabolism. After catabolism of SAH to homocysteine and adenosine, homocysteine is removed via two different mechanisms: remethylation or transsulfuration. Homocysteine can be remethylated by either 5-methyltetrahydrofolate (5-CH_3THF) or betaine to generate methionine, in reactions catalyzed by methionine synthase (MS) and betaine-homocysteine methyltransferase (BHMT), respectively. If remethylation does not occur, homocysteine is

further catabolized through the transsulfuration pathway. In the first step of this pathway homocysteine is irreversibly converted to cystathionine by a vitamin B_6-dependent enzyme, cystathionine β-synthase (CBS). Further down the transsulfuration pathway several important metabolic products are generated, including glutathione, taurine, and pyruvate.

Regulation of the methyl group supply is dependent upon several factors, including the intracellular concentration of SAM. For example, when SAM concentrations are high, excess methyl groups are disposed of by glycine *N*-methyltransferase (GNMT), an abundant protein that composes 1-3% of the total cytosolic protein in the liver (Heady and Kerr 1975; Suzuki and Wagner 1985). GNMT regulates the concentration of SAM by using excess methyl groups to convert glycine to sarcosine, a compound that has no known physiological role. During times of high SAM concentrations, 5-CH_3THF production is inhibited due to SAM-mediated allosteric inhibition of methylenetetrahydrofolate reductase (MTHFR), the enzyme responsible for converting 5,10-methylenetetrahydrofolate (5,10-CH_2THF) to 5-CH_3THF (Wagner 1995; Cook and Wagner 1984; Selhub and Miller 1992). The inhibition of MTHFR by SAM reduces the concentration of 5-CH_3THF, which in turn allows GNMT to be fully active to normalize the methyl group supply by facilitating the catabolism of excess SAM. In contrast, when SAM concentrations are low, allosteric inhibition of MTHFR by SAM is lifted, thus allowing for 5-CH_3THF production and the remethylation of homocysteine to readily occur. Because GNMT activity is potently inhibited by the folate coenzyme 5-CH_3THF an increased flux of methyl groups from the one-carbon pool inhibits the disposal of methyl groups when the supply of SAM is diminished. Collectively these

actions restore SAM concentrations and ensure that an adequate supply of methyl groups is available for important SAM-dependent transmethylation reactions.

Previous work in our laboratory has demonstrated that the retinoids 13-*cis*-retinoic acid (CRA) and all-*trans*-retinoic acid (RA) induce GNMT activity and protein abundance in rats and cell culture (Rowling *et al.*, 2002; Rowling and Schalinske 2001; Rowling and Schalinske 2003). Furthermore, our laboratory has shown that GNMT up-regulation by retinoid compounds can have adverse effects on other SAM-dependent transmethylation reactions, such as the methylation of DNA (Rowling *et al.*, 2002). Clearly, the modulation of methyl group metabolism by retinoids is of significance due to the increasing number of retinoid-containing prescription medications dispensed for the treatment of various conditions, such as cystic acne (Wysowski *et al.*, 2002).

A retinoid compound of interest is retinoyl β-glucuronide (RAG), the glucuronic acid derivative of retinoic acid that has been shown to be less toxic than RA in animal and cell culture studies (Becker *et al.*, 1998; Gunning *et al.*, 1993). It has been postulated that RAG has less toxicity than RA due to its structural difference (no free carboxyl group conjugated with a polyene chain), and its inability to bind with cellular retinoid binding proteins or nuclear transcription factors for retinoids (Mehta *et al.*, 1992; Sani *et al.*, 1992). Additionally, it is unknown if RAG must be hydrolyzed to RA before it can become biologically active. The intestinal absorption of orally dosed RAG has been studied, and the results from these studies indicate that the absorption and hydrolysis of RAG to RA in vitamin A sufficient rats and humans is either very slow or does not occur to any appreciable extent, whereas, RAG absorption and hydrolysis to RA in vitamin A deficient rats occurs

more rapidly and to a measurable extent (Barua *et al.*, 1998; Gunning *et al.*, 1993; Barua and Sidell 2004).

The effect of RAG on GNMT activity has not been studied; therefore, this study was conducted to determine if oral dosing with RAG affected GNMT activity in a similar fashion to RA, and if vitamin A status altered any effects of these retinoids on GNMT activity. Additionally, the effect of RAG and RA on homocysteine concentrations was determined.

Materials and Methods

Chemicals and Reagents

Reagents were obtained from the following: S-adenosyl-L-[*methyl*-^{3}H] methionine, Perkin-Elmer Life Sciences, Inc. (Boston, MA); RA, Calbiochem (La Jolla, CA); tributylphosphine, Aldrich (Milwaukee, WI); dimethylformamide, Fisher (Fair Lawn, NJ); phenylmethylsufonylfluoride, Calbiochem (La Jolla, CA); N-acetylcysteine, Sigma (St. Louis, MO). RAG was kindly provided by Dr. Arun Barua, Iowa State University. All other chemicals were of analytical grade.

Animals and Diets

This animal experiment was approved by and conducted according to guidelines established by the Iowa State University Laboratory Animal Resource Committee. Male Sprague-Dawley rats (55-74 g; Harlan Sprague-Dawley, Indianapolis, IN) were housed in individual plastic cages in a room with a 12-hour light:dark cycle, and were given free access to food and water. Composition of the vitamin A sufficient (VAS) control diet (AIN-93G) has been previously described (Rowling and Schalinske 2003). Vitamin A deficient (VAD)

diet had the same composition of the control diet, except a vitamin A-free vitamin mix (Harlan Teklad, Madison, WI) was utilized in the VAD diet.

After a 2-day acclimation period to the control diet, rats were randomly assigned to one of six groups (n=6, VAS diet/Control, VAS diet/RA, VAS diet/RAG, VAD diet/Control, VAD diet/RA, VAD diet/RAG). Following a 46-day period on the VAS or VAD diet, rats were acclimated to oral dosing with vehicle (corn oil). Beginning on day 48, rats were orally administered vehicle (corn oil, 1 μl/g body weight), RA (30 μmol/kg body weight), or RAG (30 μmol/kg body weight) daily for 7 days. At the end of the treatment period, rats were anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg body weight), after which blood was collected via cardiac puncture using heparinized syringes, and portions of liver were removed for analysis.

Analysis of Hepatic GNMT Activity

GNMT enzyme activity was measured employing the methods of Cook and Wagner (1984) with minor modifications (Rowling *et al.*, 2002). Portions of liver were homogenized in 4 volumes of ice-cold phosphate buffer [10 mM sodium phosphate (pH 7.0), 0.25 M sucrose, 1 mM EDTA, 1 mM sodium azide, 0.1 mM phenylmethylsufonylfluoride] and centrifuged (20,000 x *g*) at 4° C for 30 minutes. The resulting supernatant was removed, and β-mercaptoethanol was added to a final concentration of 10 mM. The assay mixture contained 0.1 mM Tris buffer (pH 9.0), 50 mM dithiotreitol, 1 mM glycine, and 1 mM S-adenosyl-L-[*methyl*-^{3}H] methionine (129 μCi). A commercial kit (Coomassie Plus; Pierce; Rockford, IL) based on the method of Bradford (1976) was used to determine the amount of total soluble protein in the liver extract with bovine serum albumin as the assay standard.

The addition of 250 μg of soluble protein initiated the reaction, and incubation of the assay mixture was carried out at 25° C for 30 minutes. Trichloroacetic acid (10%) was used to terminate the reaction, and activated charcoal was added to remove unreacted radiolabeled SAM. Following centrifugation at 14,000 x *g* for 5 minutes, a 200 μl aliquot of the resulting supernatant was removed for liquid scintillation counting. All GNMT assays were performed in triplicate.

Homocysteine Concentration Analysis

Heparinized blood samples were centrifuged at 2940 x *g* for 5 minutes, after which plasma was removed and stored at -20° C for subsequent analysis of homocysteine concentrations. Equal volumes of tributylphosphine (10%) in dimethylformamide and 1 mmol N-acetylcysteine internal standard were added to 300 μl of plasma, followed by incubation at 4° C for 30 minutes and addition of 300 μl ice-cold 10% trichloroacetic acid with 1 mM EDTA. Following centrifugation at 1000 x *g* for 5 minutes, 100 μl of the resulting supernatant was added to a mixture of 20 μl sodium hydroxide (1.55 M), 250 μl borate buffer (0.125 M, pH 9.5 containing 4 mmol K_2EDTA) and 100 μl ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (1 mg/ml). Samples were incubated for 1 hour at 60° C, which was followed by HPLC analysis of total (free + protein-bound) plasma homocysteine concentration using a μBondapak C_{18} Radial-Pak column (Waters, Milford, MA) and fluorometric detection. The mobile phase consisted of 40 ml/L acetonitrile in 0.1 M potassium phosphate (pH 2.1) buffer, as previously described (Rowling and Schalinske 2003).

Statistical Analysis

The means across treatment groups (diet x, retinoic acid x, and interactions) were analyzed by 2-way analysis of variance (ANOVA). If the ANOVA was significant ($p<0.05$), the means were compared using Fisher's least significant difference procedure (Snedecor and Cochran 1980). SigmaStat software (SPSS, Chicago, IL) was used for all statistical analyses.

Results

Vitamin A Deficiency and Cumulative Weight Gain

The cumulative weight gain between VAS and VAD rats did not differ across treatment groups. Regardless of retinoid treatment, both VAS and VAD rats displayed similar weight gains (242 ± 6 for VAS rats and 240 ± 6 for VAD rats). These results indicate that a moderate vitamin A deficiency rather than a severe deficiency may have been achieved. Severe vitamin A deficiency is characterized by a number of health problems, including substantial weight loss (Collins and Mao 1999).

Effect of Retinoids on Hepatic GNMT Activity

There were significant differences across treatment groups with respect to GNMT activity. Hepatic GNMT activity of both VAS and VAD RA-treated rats was elevated ~230% compared to their respective controls. GNMT activity of VAS RAG-treated rats was elevated by 107%, whereas RAG was without effect in VAD rats (**Figure 1**).

Effect of Retinoids on Plasma Homocysteine Concentrations

RA and RAG did not appear to alter plasma homocysteine concentrations in VAS or VAD rats. However, plasma homocysteine concentrations were reduced (24%) in VAS RA-treated rats compared to VAS RAG-treated rats (**Figure 2**).

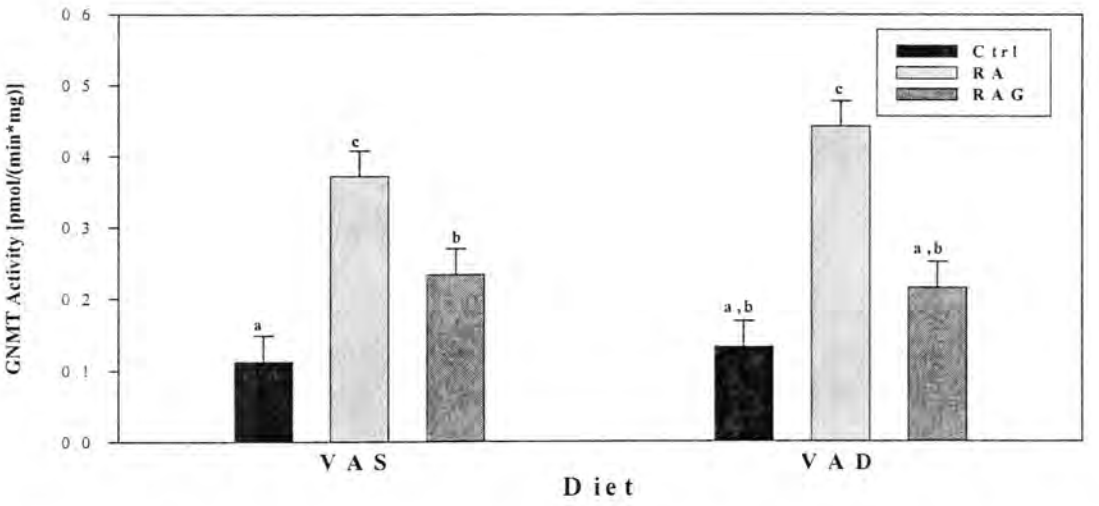

Figure 1. All-*trans*-retinoic acid (RA) increased hepatic glycine *N*-methyltransferase (GNMT) activity in rats on either a vitamin A sufficient (VAS) or vitamin A deficient (VAD) diet. Retinoyl β-glucuronide (RAG) increased GNMT activity in VAS rats only. Data are means ± SEM, n=6. Bars without a common letter are statistically different ($p \leq 0.05$).

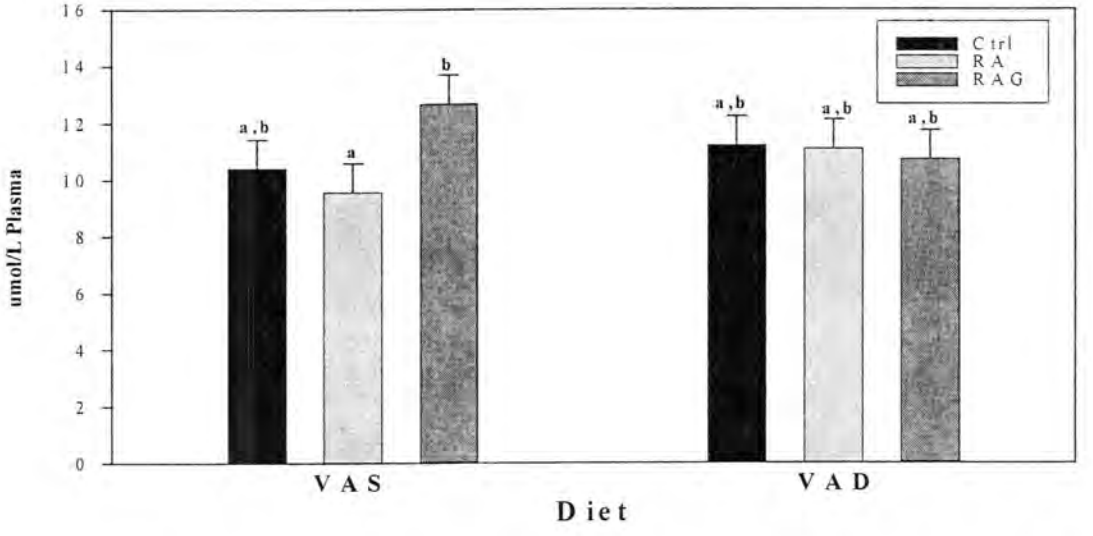

Figure 2. All-*trans*-retinoic acid (RA) and retinoyl β-glucuronide (RAG) did not affect plasma homocysteine concentrations in vitamin A sufficient (VAS) or vitamin A deficient (VAD) rats. Data are means ± SEM, n=6. Bars without a common letter are statistically different ($p \leq 0.05$).

Discussion

GNMT plays an important role in regulating the methyl group supply for SAM-dependent transmethylation reactions. Thus, factors that can alter the function of GNMT may lead to pathological conditions. We have previously shown that the retinoid compounds RA and CRA inappropriately activate GNMT in rats (Rowling *et al.*, 2002; Rowling and Schalinske 2001), compromising the availability of methyl groups necessary for the methylation of DNA.

In addition to inappropriately activating GNMT, retinoids, such as RA have also been shown to be teratogenic (Gunning *et al.*, 1993; Mulder *et al.*, 2000; Collins and Mao 1999). Interestingly, others have reported that the retinoic acid derivative RAG is less toxic than RA both in vitro and in vivo, thus leading to its identification as a potential alternative retinoid compound to be used for therapeutic purposes (Becker *et al.*, 1998; Gunning *et al.*, 1993). However, when dosed orally, the absorption of RAG and hydrolysis of RAG to RA may be dependent on vitamin A status (Barua *et al.*, 1998; Gunning *et al.*, 1993; Barua and Sidell 2004). It has been hypothesized that RAG is less toxic because it does not interact with cellular retinoid binding proteins or nuclear transcription factors for retinoids, and it is structurally different than retinoic acid (Mehta *et al.*, 1992; Sani *et al.*, 1992). Because the promoter region of DNA encoding GNMT does not have a known retinoic acid response element, the mechanism through which RA increases the expression of GNMT protein is unclear. Additionally, to our knowledge there is no previous research indicating that RAG has an ability similar to that of RA to increase GNMT activity.

Evidence regarding the absorption of RAG and hydrolysis of RAG to RA when given orally to VAS animals and humans is conflicting. Gunning et al. (1993) concluded that RAG was absorbed and hydrolyzed to RA slowly when given orally to pregnant Sprague-Dawley rats. In a separate study, it was concluded that undetectable blood levels of RAG and RA were present in rats orally administered RAG, whereas VAD rats had detectable levels of RA formed from RAG in the blood (Barua *et al.*, 1998). Barua and Sidell (2004) reported that humans with normal vitamin A status who were given oral RAG had no detectable plasma RAG or RA. Additionally, Kaul and Olson (1998) reported that the conversion of RAG to RA was enhanced in VAD rats, with the relative rates of hydrolysis of RAG to RA higher in organelles of liver, kidney, and intestine in VAD rats compared to VAS rats (Kaul and Olson 1998).

The results from the current study indicate that RAG was not as effective as RA with respect to activating GNMT, regardless of vitamin A status. Although VAS rats exhibited increased GNMT activity when treated with RAG, the VAD rats did not display a significant elevation. Moreover, the increase in GNMT activity in VAS RAG-treated rats was much less pronounced compared to VAS and VAD RA-treated rats. Both VAS and VAD rats experienced a ~230% increase in GNMT activity when treated with RA, whereas VAS rats treated with RAG displayed a 107% increase in activity. These results suggest that RAG was readily absorbed and hydrolyzed to RA to some degree in VAS rats when orally administered.

Regardless of vitamin A status administration of RAG or RA did not alter plasma homocysteine concentrations. However, homocysteine levels were 24% lower in VAS RA-

treated rats compared to VAS RAG-treated rats, indicating that RA enhanced the metabolism of homocysteine through the remethylation and/or transsulfuration pathways, whereas RAG did not share this ability of RA.

The results from this study clearly indicate that RAG does not alter GNMT activity to the same degree as RA. In addition, the findings show that when rats are orally dosed with RAG alone, the compound appears to be absorbed and hydrolyzed to RA to some extent regardless of vitamin A status. However, the amount of RA generated through the hydrolysis of RAG may be insufficient to activate GNMT compared to RA. Taken together, the results from this study indicate that RAG, unlike other retinoid compounds, does not up-regulate GNMT activity. Up-regulation of GNMT has proven to have adverse effects on SAM-dependent transmethylation reactions, such as the methylation of DNA; therefore, RAG may be a safer therapeutic alternative for treating medical conditions in which retinoids are utilized, such as cystic acne or acute promyelocytic leukemia.

CHAPTER 4
AGE- AND GENDER-SPECIFIC DISRUPTION OF METHYL GROUP METABOLISM BY ALL-*TRANS*-RETINOIC ACID

Introduction

An adequate supply of methyl groups is necessary for ensuring optimal health. Methyl groups, provided from the folate-dependent one-carbon pool or from the diet (i.e., as methionine and/or choline), are involved in many important transmethylation reactions. A lack of methyl groups has been shown to result in hepatocarcinogenesis (Ghoshal and Farber 1984; Salmon and Copeland 1954), which may be due in part to hypomethylation of DNA. Methyl groups are activated to S-adenosylmethionine (SAM), which is the substrate for a number of transmethylation reactions. The product of transmethylation reactions, S-adenosylhomocysteine (SAH), inhibits a majority of SAM-dependent methyltransferases (Kerr 1972). SAH is further catabolized to homocysteine, which can be remethylated using 5-methyltetrahydrofolate (5-CH_3THF) or betaine as methyl group donors, to regenerate methionine. Homocysteine can also be further degraded via the transsulfuration pathway to generate catabolites such as taurine, glutathione, and pyruvate.

Glycine N-methyltransferase (GNMT), a key cytosolic enzyme, serves to optimize transmethylation reactions by regulating the SAM:SAH ratio. If the supply of methyl groups is abundant, GNMT forms the inactive metabolite sarcosine from glycine, which serves as a disposal mechanism for excess methyl groups. SAM reduces the supply of methyl groups from the one-carbon pool by allosterically inhibiting 5,10-methylenetetrahydrofolate reductase (MTHFR) (Jencks and Matthews 1987; Kutzbach and Stokstad 1967), the enzyme responsible for 5-CH_3THF synthesis. In turn, 5-CH_3THF also functions as a ligand to inhibit

GNMT activity. When methyl group supply is diminished, MTHFR is active, leading to an increase in 5-CH_3THF concentration and thus an increase in remethylation, and a decrease in GNMT activity, thereby allowing methyl groups to be conserved for important transmethylation reactions.

Age and gender represent factors that significantly alter the metabolism of methyl groups and homocysteine. Women have lower fasting homocysteine concentrations compared to men, which may be associated with the lower risk of cardiovascular disease in premenopausal women (Boers *et al.*, 1983). Fukagawa et al. (2000) found that the lower homocysteine concentration displayed in women may be due in part to increased homocysteine remethylation. Also, Mays et al. (1973) showed that 12-month old rats displayed increased GNMT activity compared to 3-month old rats, which could lead to alterations in protein synthesis.

Previously, our laboratory has shown that all-*trans*-retinoic acid (RA) induces the abundance and activity of GNMT, thereby compromising transmethylation reactions and the metabolism of homocysteine (McMullen *et al.* 2002). It has also been demonstrated that male rats, compared to female rats are more sensitive to the retinoid-mediated induction of GNMT (McMullen *et al.* 2002). To further understand these findings, the aim of our current study was to determine the differential effect of retinoid administration on methyl group and homocysteine metabolism in 4- and 10-wk old male and female rats.

Materials and Methods

Chemicals and Reagents

Reagents were obtained from the following: S-adenosyl-L-[*methyl*-^{3}H] methionine, Perkin-Elmer Life Sciences, Inc. (Boston, MA); phenylmethylsufonylfluoride, Calbiochem (La Jolla, CA); RA, Calbiochem (La Jolla, CA); N-acetylcysteine, Sigma (St. Louis, MO); tributylphosphine, Aldrich (Milwaukee, WI); dimethylformamide, Fisher (Fair Lawn, NJ). All other chemicals were of analytical grade.

Animals and Diets

This animal experiment was performed in compliance with the Iowa State University Laboratory Animal Resources Guidelines. The study was conducted using male (4-wk, 50-74 g, n=10 and 10-wk, 250-274 g, n=10) and female (4-wk, 50-74 g, n=10 and 10-wk, 175-199 g, n=10) Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN). Rats were housed in individual plastic cages in a room with a 12-hour light:dark cycle and consumed water and control diet (Rowling and Schalinske 2003) ad libitum. Rats were adapted to the control diet and oral administration of corn oil during a 6-day acclimation period. Rats were randomly assigned to receive either RA (30 μmol/kg body weight) or vehicle (corn oil; 1 μl/g body weight) daily for 9 days after the acclimation period (n=5). At the end of the 9-day treatment period, all rats were anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg body weight). Portions of liver were removed for analysis, and blood was collected via cardiac puncture using heparinized syringes.

Analysis of Hepatic GNMT Activity

GNMT enzyme activity was measured utilizing the methods of Cook and Wagner (1984) with minor modifications (Rowling *et al.*, 2002). A detailed account of this method is outlined in Chapter 3 of this thesis.

Homocysteine Concentration Analysis

Heparinized blood samples were centrifuged at 2940 x *g* for 10 min, and the plasma was removed and stored at -20° C for subsequent for analysis of homocysteine concentrations. A detailed summary of homocysteine sample derivatization and analysis is outlined in Chapter 3 of this thesis.

Hepatic Lipid Analysis

Liver samples were frozen in liquid nitrogen for later analysis of lipids. To determine lipid concentration, samples were homogenized in ice-cold choloroform:methanol (2:1) and filtered into graduated cylinders. Chloroform:methanol (2:1) was added to bring the volume to 20 ml, and 4 ml 0.05% calcium chloride was added before allowing covered cylinders to sit overnight. An assay mixture consisting of chloroform, methanol, 0.04% calcium chloride (in a ratio of 8:4:3) was added to each sample, followed by the addition of methanol to remove the interface. Volume was brought to 20 ml with chloroform:methanol (2:1), and 5 ml of each sample (in triplicate) was placed into pre-weighed aluminum cups. After a 4-hour evaporation period the aluminum cups were re-weighed, and the difference was used to determine the liver lipid concentration.

Statistical Analysis

The means across treatment groups (age x, gender x, retinoic acid x, and interactions) were analyzed by a one-tailed 3-way analysis of variance (ANOVA). If the ANOVA was significant ($p<0.05$), the means were compared using Fisher's least significant difference procedure (Snedecor and Cochran 1980). SigmaStat software (SPSS, Chicago, IL) was used for all statistical analyses.

Results

Cumulative Weight Gain

Rats did not display any overt signs of toxicity from RA treatment. However, 4-wk old female and 10-wk old male RA-treated rats displayed a significant decrease in growth (16.4% and 29.7% respectively) compared to their respective controls (**Table 1**).

Table 1. Cumulative weight gain after 9 days of treatment was decreased by all-*trans*-retinoic acid (RA) in 4-wk old female and 10-wk old male rats.[a]

		Initial wt (g)	Final wt (g)	Cumulative wt gain (g) after 9 d treatment
Female	**4-wk**			
	Ctrl	68	133	$43 \pm 3^{***}$
	+RA	64	121	$36 \pm 3^{**}$
	10-wk			
	Ctrl	186	219	$17 \pm 3^{*}$
	+RA	187	215	$17 \pm 2^{*}$
Male	**4-wk**			
	Ctrl	50	119	$49 \pm 3^{****}$
	+RA	49	111	$44 \pm 1^{***,****}$
	10-wk			
	Ctrl	269	352	$50 \pm 3^{****}$
	+RA	262	326	$35 \pm 2^{**}$

[a] Data are means ± SEM, n=5. Values without a common number of asterisks are significantly different ($p \leq 0.05$).

Effect of Retinoids on GNMT Activity

GNMT activity was significantly elevated in both 4- and 10-wk old male RA-treated rats compared to their respective controls (**Figure 1**). RA increased enzyme activity ~2-fold in 4-wk and ~6-fold in 10-wk old male rats. 10-wk old female RA-treated rats also experienced a significant ~2-fold increase in GNMT activity. The increase in GNMT activity in 10-wk compared to 4-wk old RA-treated rats was significant in male rats only.

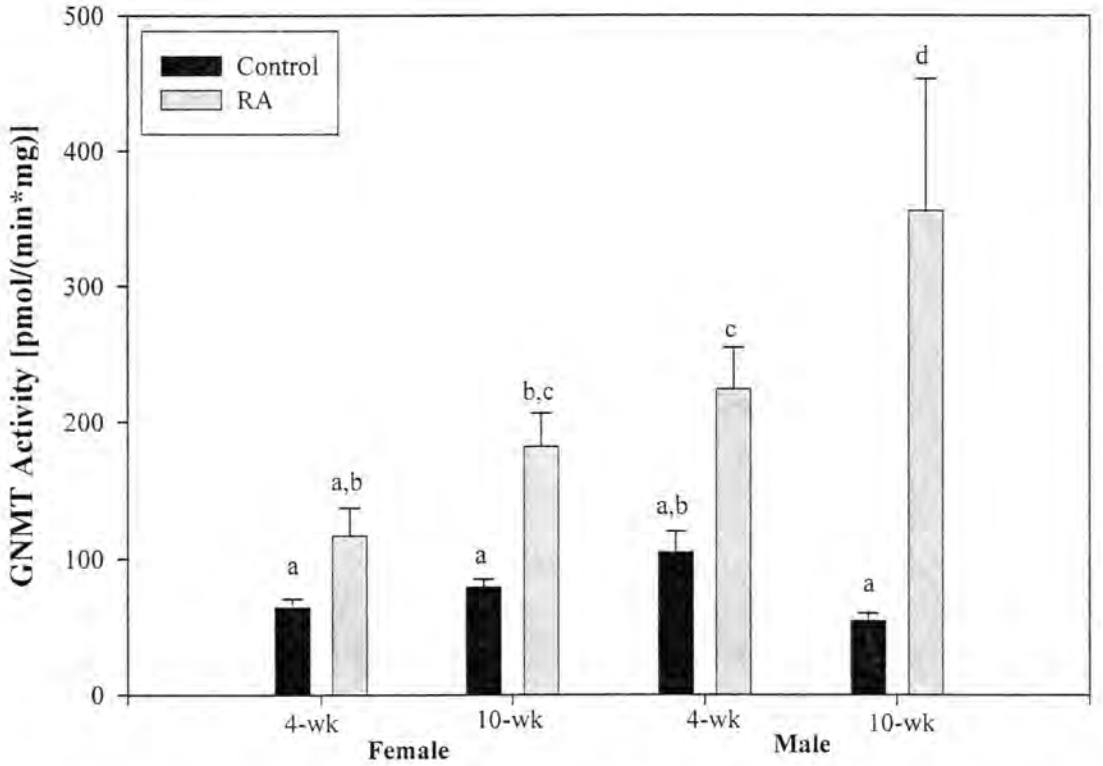

Figure 1. Hepatic glycine *N*-methyltransferase (GNMT) activity was increased by all-*trans*-retinoic acid (RA) in 4-wk old male rats and 10-wk old male and female rats. Data are means ± SEM, n=5. Bars without a common letter are significantly different ($p \leq 0.05$).

Effect of Retinoids on Plasma Homocysteine Concentrations

The homocysteine concentration in 10-wk old male RA-treated rats was significantly reduced by ~36% compared to controls (**Figure 2**). In contrast, RA was without effect in both male and female 4-wk old RA-treated rats and 10-wk old female RA-treated rats.

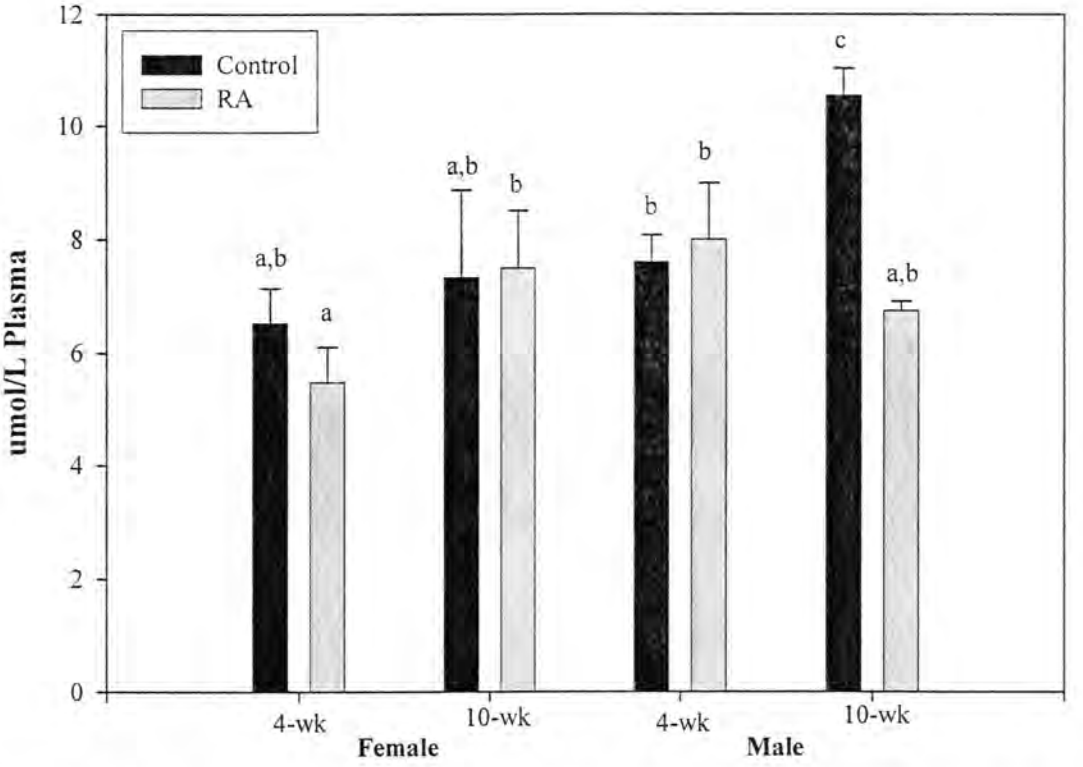

Figure 2. Plasma total homocysteine concentrations were significantly reduced in 10-wk old male rats treated with all-*trans*-retinoic acid (RA). Data are means ± SEM, n=5. Bars without a common letter are significantly different ($p \leq 0.05$).

Effect of Retinoids on Hepatic Lipid Concentration

Treatment with RA induced a significant increase in hepatic lipid concentration in 4-wk old male and female rats. Male and female 4-wk old RA-treated rats experienced a significant 78 and 48% increase in liver lipid concentration respectively. Male and female

10-wk old rats treated with RA did not display significant increases in liver lipid concentration.

Discussion

GNMT is a key regulatory protein in methionine metabolism because it functions to control the supply of methyl groups available for SAM-dependent transmethylation reactions. However, if GNMT activity is increased above the level mandated by nutrition and/or physiologic conditions, a loss of methyl groups may ensue. Methyl group wastage can lead to adverse effects, such as DNA hypomethylation (Friso and Choi 2002). Previously our lab has demonstrated that the administration of retinoid compounds, such as RA and 13-*cis*-retinoic acid (CRA) significantly induce GNMT activity and abundance in 4-wk old rats (Rowling *et al.*, 2002; Rowling and Schalinske 2001; McMullen *et al.*, 2002). The current study was done with both 4- and 10-wk old male and female rats to determine the degree of metabolic alteration displayed in methyl group and homocysteine metabolism with RA administration. This study demonstrates that the degree of metabolic alteration is age- and gender-dependent.

With regard to GNMT activity, 10-wk old male rats were more sensitive to RA treatment than 4-wk old male rats. In addition, 4- and 10-wk old male rats treated with RA were more sensitive than 4- and 10-wk old RA-treated female rats. Although RA led to elevations in GNMT activity in both genders, RA enhanced methionine metabolism to a greater extent in male rats compared to female rats, which could lead to methyl group insufficiency. Mays et al. (1973) found that GNMT activity was increased by 30% in 12-month old male Sprague-Dawley rats compared to 3-month old rats, and that the total capacity for tRNA methylation was decreased by 35% in the 12-month old rats. This

indicates that aging animals may experience progressive hypomethylation of tRNA, leading to altered protein synthesis (Mays *et al.*, 1973). The results from our current study indicate that this increase in GNMT activity occurs at an earlier age and to a greater extent with RA treatment.

Decreased plasma homocysteine concentrations in 10-wk old male RA-treated rats compared to 10-wk old male control rats indicates an increased sensitivity to RA treatment in 10-wk old male rats compared to 10-wk old female and 4-wk old male and female rats. However, these results must be interpreted carefully if extrapolating these data from rats to humans, as it has been shown that homocysteine levels are significantly increased in people treated with retinoids for dermatological disorders (Schulpis *et al.*, 2001).

Fukagawa et al. (2000) demonstrated that compared to men, women have an increased flux through both the remethylation and transsulfuration pathways. This provides a potential explanation for the finding of significantly lower homocysteine levels in both 4- and 10-wk old female control and RA-treated rats compared to 10-wk old male control rats. However, only 10-wk male RA-treated rats had significant decreases in homocysteine concentrations compared to 10-wk old male controls, indicating that RA may enhance homocysteine metabolism by increasing flux through the remethylation and/or transsulfuration pathways.

This study provides evidence that pharmacologic doses of RA have the ability to alter methionine and homocysteine metabolism in rats in an age- and gender-specific fashion, with 10-wk old male rats displaying the highest degree of sensitivity to retinoid administration.

Further studies will be conducted to determine the effects of physiologic and pharmacologic doses of retinoids on methyl group metabolism in rats that are greater than 10 weeks old.

CHAPTER 5
FOLATE STATUS ATTENUATES THE DISRUPTION OF METHYL GROUP METABOLISM BY ALL-*TRANS*-RETINOIC ACID

Introduction

The water-soluble B vitamin folate plays the integral role of accepting and donating one-carbon units in multiple metabolic pathways (Bailey and Gregory 1999), including pathways involved in the synthesis of nucleic acids and methionine. Folate deficiency has been implicated in the development of several pathological conditions, including megaloblastic anemia, neural tube defects (NTD) (Hibbard 1964; Smithells *et al.*, 1976), and hyperhomocysteinemia, a condition that has been identified as an independent risk factor for cardiovascular disease (Clarke *et al.*, 1991; Kang *et al.*, 1986; Pancharuniti *et al.*, 1994).

The interrelationship between the folate-dependent one-carbon pool and methyl group metabolism is important for the maintenance of multiple metabolic pathways. During methyl group metabolism, methionine is converted to S-adenosylmethionine (SAM), which activates methyl groups for transmethylation reactions. The modification and/or synthesis of biological compounds, such as nucleic acids, phospholipids, and proteins are included in these SAM-dependent transmethylation reactions. S-adenosylhomocysteine (SAH) is the product of transmethylation reactions, and this compound inhibits most SAM-dependent methyltransferases (Kerr 1972). Therefore, further catabolism of SAH to homocysteine is important for ensuring normal methyl group metabolism, as the ratio of SAM:SAH is considered an index of transmethylation potential (Kerr 1972; Cantoni and Chiang 1980).

Homocysteine can be remethylated by either 5-methyltetrahydrofolate (5-CH_3THF) or betaine to generate methionine in reactions catalyzed by methionine synthase (MS) and

betaine-homocysteine methyltransferase (BHMT), respectively, or it can be further degraded via the transsulfuration pathway to generate important catabolites, such as taurine, glutathione, and pyruvate. An adequate supply of folate is necessary for the remethylation of homocysteine when SAM concentrations are diminished. The remethylation of homocysteine prevents accumulation of homocysteine, and ensures that there will be a sufficient supply of methyl groups available for important transmethylation reactions.

Glycine *N*-methyltransferase (GNMT), an abundant cytosolic protein in the liver, functions to optimize transmethylation reactions by regulating the SAM:SAH ratio. When methyl group supply is abundant, GNMT regulates SAM concentrations by using excess methyl groups to convert glycine to sarcosine, a compound with no known physiological role. SAM diminishes the supply of methyl groups from the folate-dependent one-carbon pool by allosterically inhibiting 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme responsible for converting 5,10-methylentetrahydrofolate (5,10-CH_2THF) to 5-CH_3THF (Jencks and Matthews 1987; Kutzbach and Stokstad 1967). 5-CH_3THF serves as a ligand to inhibit GNMT activity; therefore, diminished production of 5-CH_3THF allows GNMT to be fully active and dispose of excess methyl groups. When methyl group supply is limited, MTHFR is active, leading to an increased supply of 5-CH_3THF, which functions to inhibit GNMT activity, and remethylate homocysteine, thus ensuring an adequate supply of methyl groups for important transmethylation reactions.

Previously our laboratory has demonstrated that the retinoids 13-*cis*-retinoic acid (CRA) and all-*trans*-retinoic acid (RA) disrupt methyl group metabolism by elevating the activity and abundance of GNMT in rats and cell culture, leading to a loss of methyl groups

required for important transmethylation reactions, such as the methylation of DNA (Rowling *et al.*, 2002; Rowling and Schalinske 2001; Rowling and Schalinske 2003). Additionally, our laboratory has demonstrated that RA significantly induces GNMT activity in rats in doses of 5 μmol/kg body weight, with maximal induction at 30 μmol/kg body weight (Ozias and Schalinske 2003). However, the effect of RA on rats with altered folate status has not been determined. Therefore, the current study was conducted to determine the effects of retinoids on rats fed a folate deficient (0 ppm), folate sufficient (2 ppm), or folate supplemented (8 ppm) amino acid-defined diet.

Materials and Methods

Chemicals and Reagents

Reagents were obtained from the following: S-adenosyl-L-[*methyl*-^{3}H] methionine, Perkin-Elmer Life Sciences, Inc. (Boston, MA); 5-[*methyl*-^{14}C]-THF, Amersham Pharmacia (Piscataway, NJ); phenylmethylsufonylfluoride, Calbiochem (La Jolla, CA); RA, Calbiochem (La Jolla, CA); N-acetylcysteine, Sigma (St. Louis, MO); tributylphosphine, Aldrich (Milwaukee, WI); dimethylformamide, Fisher (Fair Lawn, NJ); goat anti-mouse horseradish peroxidase and goat anti-rabbit horseradish peroxidase, Southern Biotechnology (Birmingham, AL); ECL Western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ); GNMT antibody was kindly provided by Y.-M.A. Chen, National Yang-Ming University, Taipei, Taiwan. All other chemicals were of analytical grade.

Animals and Diets

This animal experiment was performed in compliance with the Iowa State University Laboratory Animal Resources Guidelines. The study was conducted using male Sprague-

Dawley rats (50-74 g; n=45; Harlan Sprague-Dawley, Indianapolis, IN). Rats were housed in individual plastic cages in a room with a 12-hour light:dark cycle and consumed water and food ad libitum. After an 8-day acclimation period rats were randomly assigned to receive an amino acid-defined diet (**Table 1**) containing 0 (deficient), 2 (sufficient), or 8 (supplemented) ppm folate (Harlan Teklad, Madison, WI) for 37 days. Rats in each dietary folate group were also randomly assigned to receive 0, 5, or 30 μmol RA/kg body weight for the last 7 days of the study.

Table 1. Amino acid profile of the amino acid-defined diet.

Amino Acid	Quantity (g/kg diet)
L-Alanine	3.5
L-Arginine HCl	12.1
L-Asparagine	6.0
L-Aspartic Acid	3.5
L-Cystine	3.5
L-Glutamic Acid	40.0
Glycine	23.3
L-Histidine	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18.0
L-Methionine	8.2
L-phenylalanine	7.5
L-Proline	3.5
L-Serine	3.5
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5.0
L-Valine	8.2

On days 28 and 29 rats were acclimated to oral dosing with vehicle (corn oil), followed by the 7-day treatment period in which rats were orally administered either vehicle (corn oil; 1 μl/g body weight) or RA (5 μmol/kg body weight or 30 μmol/kg body weight) daily. At the

end of the treatment period, all rats were anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg body weight). Portions of liver were removed for analysis, and blood was collected via cardiac puncture using heparinized syringes.

Analysis of Hepatic GNMT Activity

GNMT enzyme activity was measured utilizing the methods of Cook and Wagner (1984) with minor modifications (Rowling *et al.*, 2002). A detailed account of this method is outlined in Chapter 3 of this thesis.

Measurement of GNMT Protein Abundance

Immunoblotting with chemiluminescence detection was utilized for the determination of GNMT protein abundance. A commercial kit (Coomassie Plus; Pierce; Rockford, IL) based on the method of Bradford (1976) was used to determine the amount of total soluble protein in the liver extract with bovine serum albumin as the assay standard. SDS-polyacrylamide gel electrophoresis was carried out with a 10-20% gradient gel and 75 μg sample protein per lane. After separation, proteins were transferred by electrophoresis to a nitrocellulose membrane, which was incubated at room temperature in a blocking solution consisting of nonfat dry milk (5 g/100 ml) in TTBS buffer (20 mmol Tris, ph 7.5; 500 μml/L Tween 20). GNMT blots were incubated in a monoclonal mouse anti-GNMT primary antibody in BSA (1 g/100 ml)-TTBS (1:2500) at 4° C overnight, followed by incubation for 1 hour at room temperature with goat anti-mouse horseradish peroxidase secondary antibody in TTBS (1:5000). After a 1-minute incubation in Western blot chemiluminescent detection reagents (ECL Western blotting reagents, Amersham Pharmacia) GNMT protein abundance

was determined after exposure to Kodak X-Omat AR film. Densitometric analysis was performed using SigmaGel Software (SPSS, Chicago, IL).

Homocysteine Concentration Analysis

Heparinized blood samples were centrifuged at 2940 x *g* for 10 min, and the plasma was removed and stored at -20° C for subsequent for analysis of homocysteine concentrations. A detailed summary of homocysteine sample derivatization and analysis is outlined in Chapter 3 of this thesis.

Analysis of Hepatic MS Activity

MS activity was determined utilizing the method of Keating et al. (1985). Samples of liver supernatant in a reaction mixture containing sodium phosphate buffer (500 mmol, pH 7.5), cyanocobalamin (1.3 μmol), dithiothreitol (1 mol), SAM (10 mmol), β-mercaptoethanol (82.4 mmol), homocysteine (100 mmol) and 15 mmol 5-[*methyl*-^{14}C]-THF (6.44 kBq/μmol) were incubated for 1 hour in a 37° C water bath. The addition of ice-cold water stopped the reactions, and samples were immediately applied to AG 1-X8 (Cl form) resin columns (Bio-Rad, Hercules, CA). Flow through fractions (3 ml total) were collected for liquid scintillation counting.

Analysis of Hepatic Folate Coenzyme Concentrations

The method of Rubello (1987) was employed to determine 5-CH_3THF and tetrahydrofolate (THF) concentrations by HPLC and fluorometric detection, with minor modifications. Under a steady stream of nitrogen, portions of liver were homogenized in ice-cold sodium acetate buffer (0.1 μmol, pH 4.9) containing 5 ml/L ascorbate and 20 mmol β-mercaptoethanol, tightly capped, and stored at -70° C until analysis. Samples were incubated

in a boiling water bath for 1 hour, followed by centrifugation at 20,000 x *g* for 10 minutes. Rat serum conjugase was added to the resulting supernatant, and this mixture was incubated in a 37° C shaking water bath for 1 hour. Following activation of Sep-Pak NH_2 columns (Waters, Milford, MA) with acetonitrile and sodium acetate buffer (16 mmol, pH 4.5), samples were added and washed with acetate buffer, and were eluted off of the column with sodium phosphate buffer (0.1 M) containing 50 mmol β-mercaptoethanol. A Phenyl Radial-Pak column (Waters, Milford, MA) was utilized to separate folate conenzymes, and fluorometric detection (excitation wavelength, 300 nm; emission wavelength, 356 nm) was used to quantify the coenzymes. A gradient mobile phase operated at 2.0 ml/minute was composed of: 760 ml/L sodium phosphate (0.033 M, pH 2.3)-240 ml/L acetonitrile for 4 minutes; a linear gradient (2 minutes) to 500 ml/L and maintained from 6 to 10 minutes; a linear gradient (2 min) to 100 ml/L-900 ml/L and maintained from 12 to 16 minutes; and a linear gradient (2 minutes) back to initial conditions (760 ml-240 ml/L) for up to 20 minutes to reequilibrate the column.

Statistical Analysis

The means across treatment groups (diet x, retinoic acid x, and interactions) were analyzed by a 2-way analysis of variance (ANOVA). If the ANOVA was significant ($p<0.05$), the means were compared using Fisher's least significant difference procedure (Snedecor and Cochran 1980). SigmaStat software (SPSS, Chicago, IL) was used for all statistical analyses.

Results

Effect of Folate Concentration and Retinoids on Cumulative Weight Gain

Neither dietary folate concentration nor RA administration affected cumulative weight gain (**Table 2**). No overt signs of toxicity were displayed with RA treatment or supplemental folate, and rats fed 0 ppm folate did not display any overt signs of deficiency.

Table 2. Cumulative weight gain (g) was not affected by dietary folate concentration or all-*trans*-retinoic acid.[a]

Folate Conc. (ppm)	Gain During 30-d AA-Defined Diet Pd.	RA (μmol/kg BW)	Gain During 7-d RA Treatment Pd.	Total Cumulative Gain
0	149 ± 4*	0	20 ± 6*,**,***	163 ± 6*
		5	22 ± 3**,***	174 ± 9*
		30	21 ± 3*,**,***	172 ± 6*
2	153 ± 4*	0	16 ± 5*	165 ± 5*
		5	20 ± 3*,**,***	179 ± 4*
		30	19 ± 4*,**	170 ± 12*
8	155 ± 4*	0	21 ± 3*,**,***	177 ± 10*
		5	24 ± 5***	174 ± 6*
		30	20 ± 3*,**,***	179 ± 5*

[a] Data are means ± SEM, n=15 (column 2) and n=5 (columns 4 and 5). Values without a common number of asterisks within a column are statistically different, $p \leq 0.05$.

Effect of Folate Concentration and Retinoids on GNMT Activity

GNMT activity was elevated by retinoid treatment in all three dietary folate groups (**Figure 1**). Regardless of folate concentration in the diet, rats treated with 5 μmol RA exhibited a significant ~50% increase in GNMT activity compared to their respective controls. Although rats treated with 30 μmol RA displayed significant increases in GNMT activity, folate status attenuated the elevation. Rats in the 2 ppm/30 μmol RA and 8 ppm/30 μmol RA groups experienced a 79% and 104% elevation in GNMT activity compared to their respective controls, while rats in the 0 ppm/30 μmol RA group exhibited a 121% elevation in GNMT activity. The activity of GNMT in rats in the 0 ppm/30 μmol RA group was significantly higher than in either the 2 ppm/30 μmol RA or 8 ppm/30 μmol RA rats.

Effect of Folate Concentration and Retinoids on GNMT Protein Abundance

Despite elevations in GNMT activity in 0 ppm/5 μmol RA and 2 ppm/5 μmol RA rats, dietary folate concentration and retinoids did not significantly increase GNMT protein abundance in these groups compared to their respective controls (**Figure 2**). However, rats in the 8 ppm/5 μmol RA group experienced a 44% increase in GNMT protein abundance compared to controls. Additionally, rats in the 8 ppm/30 μmol RA group exhibited a 93% increase in GNMT protein abundance compared to controls, while rats in the 0 ppm/30 μmol RA and 2 ppm/30 μmol RA groups displayed a ~50% elevation in GNMT protein abundance compared to their respective controls.

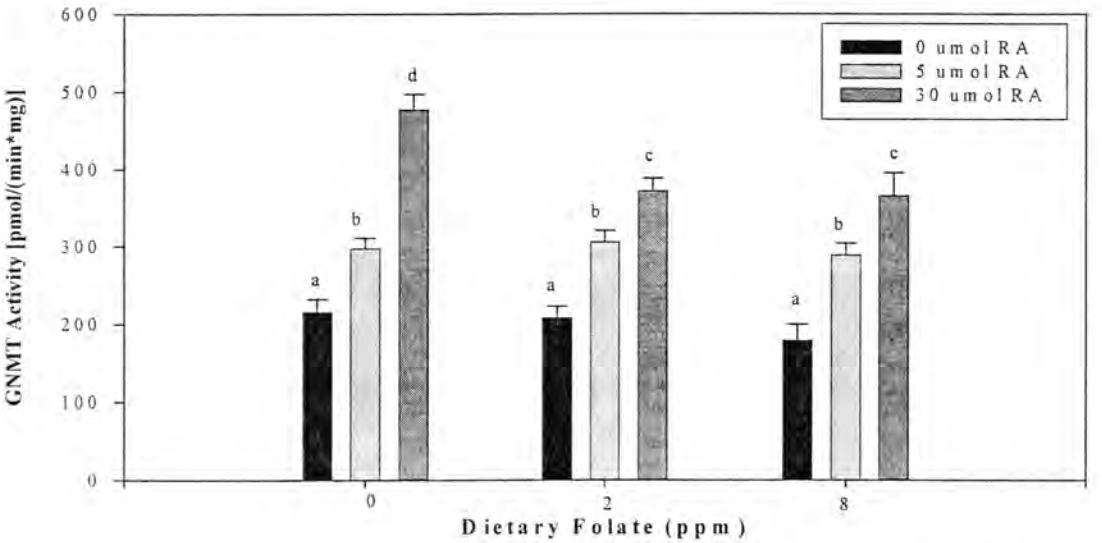

Figure 1. Hepatic glycine *N*-methyltransferase (GNMT) activity was elevated by treatment with all-*trans*-retinoic acid (RA), and GNMT activity was significantly higher in folate deficient (0 ppm) rats compared to folate sufficient (2 ppm) and folate supplemented (8 ppm) rats. Data are means ± SEM, n=5. Bars without a common letter are significantly different ($p \leq 0.05$).

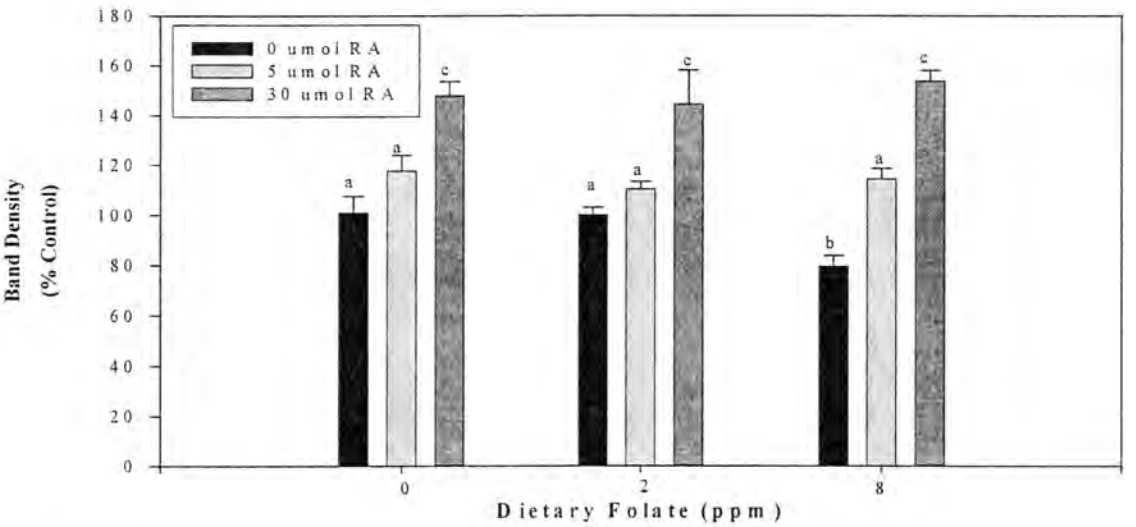

Figure 2. Glycine *N*-methyltransferase (GNMT) protein abundance was significantly elevated in folate deficient (0 ppm), folate sufficient (2 ppm), and folate supplemented (8 ppm) rats treated with 30 μmol/kg body weight all-*trans*-retinoic acid (RA), and folate supplemented rats treated with 5 μmol/kg body weight RA. Data are means ± SEM, n=5. Bars without a common letter are significantly different ($p \leq 0.05$).

Effect of Folate Concentration and Retinoids on Plasma Homocysteine Concentrations

Rats in the 0 ppm/0 μmol RA, 0 ppm/5 μmol RA and 0 ppm/30 μmol RA groups had significantly higher plasma homocysteine concentrations (~5-fold) than rats in comparative 2 ppm and 8 ppm groups, indicating that dietary folate concentration impacted plasma homocysteine concentrations (**Figure 3**). Retinoid treatment, regardless of concentration, was without effect in the 0 ppm, 2 ppm, and 8 ppm rats compared their respective controls.

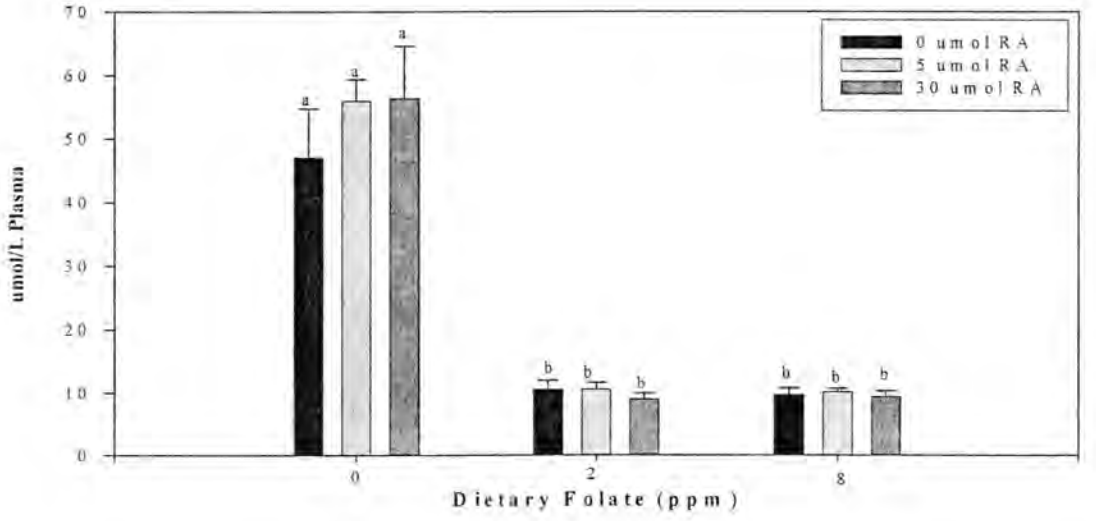

Figure 3. Plasma homocysteine concentrations were significantly higher in folate deficient (0 ppm) rats compared to folate sufficient (2 ppm) and folate supplemented (8 ppm) rats. Regardless of concentration, all-*trans*-retinoic acid (RA) was without effect in 0 ppm, 2 ppm, or 8 ppm rats compared to their respective controls. Data are means ± SEM, n=5. Bars without a common letter are significantly different ($p \leq 0.05$).

Effect of Folate Concentration and Retinoids on MS Activity

Except for a significant 37% decrease in MS activity in rats in the 2 ppm/5 μmol RA group compared to controls, RA and dietary folate concentration were without effect.

Effect of Folate Concentration and Retinoids on Hepatic Folate Coenzyme Concentrations

Rats fed a folate deficient diet had significantly lower THF and 5-CH_3THF concentrations compared to rats fed sufficient or supplemental folate (**Table 3**). Additionally, only 8 ppm/0 μmol RA rats had significantly higher THF and 5-CH_3THF compared with 2 ppm/μmol RA rats. There was no difference in THF or 5-CH_3THF concentrations in 8 ppm RA-treated rats compared to 2 ppm RA-treated rats.

Table 3. Hepatic folate coenzyme concentrations (nmol/g liver).[a]

	Dietary Folate (ppm)					
	0		2		8	
RA (μmol/kg)	THF	5-CH_3THF	THF	5-CH_3THF	THF	5-CH_3THF
0	0.71 ± 0.16*	2.07 ± 0.21*	4.16 ± 0.39**	7.06 ± 0.47**	5.47 ± 0.77***	11.51 ± 1.18***
5	0.78 ± 0.15*	2.27 ± 0.21*	4.15 ± 0.33**	11.26 ± 1.24***	4.83 ± 0.46**,***	11.12 ± 1.66***
30	0.78 ± 0.10*	2.43 ± 0.20*	4.61 ± 0.47**,***	9.49 ± 2.14**,***	4.30 ± 0.32**	11.77 ± 1.44***

[a] Data are means ± SEM, n=5. Values without a common number of asterisks in the THF columns are significantly different, and values without a common number of asterisks in the 5-CH_3THF columns are significantly different ($p \leq 0.05$).

Discussion

Methyl groups, supplied from the diet or folate-dependent one carbon pool are important for the modification and synthesis of multiple biological compounds, such as nucleic acids, phospholipids, and proteins. GNMT, a key regulatory protein in methionine

metabolism functions to control the supply of methyl groups available for SAM-dependent methylation reactions; however, our laboratory has demonstrated that the inappropriate activation of GNMT by retinoid compounds can have adverse effects on these SAM-dependent transmethylation reactions, such as DNA hypomethylation (Rowling *et al.*, 2002). In times of diminished methyl group supply, GNMT is inactive, and methyl group supply remains adequate due to the remethylation of homocysteine by 5-CH_3THF or betaine. The remethylation reaction involving 5-CH_3THF demonstrates the importance of adequate dietary folate intake, and the interrelationship between the folate-dependent one-carbon pool and methyl group metabolism. Homocysteine remethylation by 5-CH_3THF also aids in the prevention of hyperhomocysteinemia, a condition that is implicated in the development of several pathological conditions, including cardiovascular disease (Clarke *et al.*, 1991; Kang *et al.*, 1986; Pancharuniti *et al.*, 1994).

The current study demonstrates that GNMT activity was elevated by RA in all three dietary folate groups. However, this response was attenuated in the 2 ppm and 8 ppm folate groups. Rats receiving 0 ppm dietary folate and 30 μmol RA exhibited a greater increase in GNMT activity compared to rats in the 2 ppm/30 μmol RA or 8 ppm/30 μmol RA groups; however, this finding was not reflected in GNMT protein abundance. There was not a difference in abundance of GNMT protein in rats in the 0, 2, and 8 30 μmol RA groups indicating that the attenuation of GNMT activity in the 2 and 8 ppm groups was potentially caused by inhibition of the enzyme due to adequate folate. These results indicate that moderate folate deficiency results in increased sensitivity to RA-mediated disruption of methyl group metabolism, compared to adequate or supplemental dietary folate.

Results from a study by Balaghi et al. (1993) showed a significant increase in GNMT activity in folate deficient rats that were not treated with retinoids compared to control rats. The folate deficient rats were fed an amino acid-defined diet devoid of folate for 4 weeks; however, these rats were likely more folate deplete than the rats in our current study due to treatment with 1% succinylsulfathiazole, an antibiotic that facilitates the induction of folate deficiency by eliminating intestinal flora that have the ability to synthesize folate which can be incorporated into tissue folate of the host (Kim *et al.*, 2002). Our current study did not utilize succinylsulfathiozole, as our goal was not severe folate depletion, but instead moderate depletion. The difference in severity of folate deficiency between rats in our study and the study described provides a possible explanation for why there were no differences in GNMT activity between the 0 ppm/0 μmol RA, 2 ppm/0 μmol RA, and 8 ppm/0 μmol RA groups in our study.

Regardless of retinoid treatment, rats fed 0 ppm folate exhibited homocysteine concentrations that were significantly higher than rats receiving either 2 ppm or 8 ppm dietary folate, indicating that moderate folate deficiency led to an inability to remethylate homocysteine. However, treatment with RA (5 μmol or 30 μmol) did not lead to decreased homocysteine concentrations in rats fed 0 ppm, 2 ppm, or 8 ppm folate compared to their respective controls. This may in part be due to the length of retinoid administration (7 days), and the ~1:1 methionine:cysteine ratio of the amino acid-defined diet utilized for this study. Previously, our laboratory has demonstrated decreased plasma homocysteine concentrations with RA treatment (30 μmol/kg body weight for 10 days); however, a casein-based diet with a 14:1 methionine:cysteine ratio, which likely further increased flux through the methionine

pathway, was utilized in this study (Ozias and Schalinske 2003). The difference in the methionine:cysteine ratio and length of treatment period may provide an explanation for the discrepancy between the findings of the current and previous study.

Additionally, although GNMT was up-regulated by RA, indicating an increased flux through the methionine pathway, RA did not significantly affect MS activity, regardless of dietary folate concentration. In fact, rats in the 2 ppm/5 μmol RA group exhibited a significant 37% decrease in MS activity. In a representative Western blot of CBS activity, there were no significant differences in CBS protein abundance between RA-treated (5 μmol or 30 μmol) rats fed 0, 2, and 8 ppm folate and their respective controls, indicating that the transsulfuration pathway was likely not enhanced by retinoid treatment (unpublished observations, Knoblock and Schalinske) in this study. Despite GNMT up-regulation and increased flux through the methionine pathway, the lack of up-regulation of MS with retinoid treatment may lead to an inadequate supply of methyl groups for important SAM-dependent transmethylation reactions. Previous work from our laboratory has demonstrated an increase in MS activity with retinoid treatment (Ozias and Schalinske 2003; unpublished observations, Tanghe and Schalinske; unpublished observations, Nieman and Schalinske); however, these studies utilized a casein-based diet with a 14:1 methionine:cysteine ratio, which likely increased flux through the methionine pathway to a greater extent than the amino acid-defined diet with a ~1:1 methionine:cysteine ratio used in the current study.

This study clearly demonstrates that folate status is a significant determinant in modulating the metabolic response to retinoid administration. The findings presented in this chapter are of importance due to the rising number of retinoid compounds that are dispensed

for therapeutic purposes, such as cystic acne (Wysowski *et al.*, 2002). The lower dose of RA (5 μmol) that was utilized in this study is within the therapeutic range for humans (Bershad 2001), indicating that use of retinoid compounds by people with low folate status may have several adverse effects, including hyperhomocysteinemia and methyl group wastage due to GNMT up-regulation. Folate supplementation among individuals who are taking prescription retinoids may prove to be beneficial for combating disruptions in methyl group metabolism that occur with retinoid administration; however, further research must be done to determine an optimal dose of folic acid for supplementation.

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