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Nutritional and hormonal modulation of glycine N-methyltransferase: implications for aberrant methyl group metabolism

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Nutritional and hormonal modulation of glycine *N*-methyltransferase: Implications for aberrant methyl group metabolism

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For the Major Program

TABLE OF CONTENTS

LIST OF FIGURES

REVIEW OF LITERATURE

RETINOID COMPOUNDS ACTIVATE AND INDUCE HEPATIC GLYCINE *N*-METHYLTRANSFERASE IN RATS

VITAMIN A AND ITS DERIVATIVES INDUCE HEPATIC GLYCINE *N*-METHYLTRANSFERASE AND HYPOMETHYLATION OF DNA IN RATS

RETINOIC ACID AND GLUCOCORTICOID TREATMENT INDUCE HEPATIC GLYCINE *N*-METHYLTRANSFERASE AND LOWER PLASMA HOMOCYSTEINE LEVELS IN RATS

LIST OF ABBREVIATIONS

AAP	American Academy of Pediatrics
AI	adequate intake
AICAR	5-amino-4-imidazole carboxamide ribonucleotide
BHMT	betaine:homocysteine methyltransferase
cAMP	cyclic adenosinemonophosphate
CβS	cystathionine β-synthase
CDC	Centers for Disease Control and Prevention
CRA	13-*cis*-retinoic acid; isotretinoin
CVD	cardiovascular disease
DENA	diethylnitrosamine
DFE	dietary folate equivalent
DRI	dietary reference intakes
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
GAR	glycinamide ribonucleotide
GNMT	glycine *N*-methyltransferase
IU	international units
LDL	low density lipoprotein
MAT	methionine adenosyltransferase
MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
NTD	neural tube defect
PEPCK	phosphoenolpyruvate carboxykinase
RDA	recommended daily allowance
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SHMT	serine hydroxymethyltransferase
THF	tetrahydrofolate
UL	upper limit
USFDA	United States Food and Drug Association
USPHS	United States Public Health Services

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation consists of a general introductory chapter that includes a short description of the research problem investigated by the author. The second chapter is a comprehensive review of the literature relevant to the research area explored by the author. The third, fourth, and fifth chapters are manuscripts published in the Journal of Nutrition that document research completed by the author of this dissertation that investigated the role of retinoids and glucocorticoids in the modulation of various aspects of folate and methyl group metabolism. The final chapter contains general conclusions regarding the significance of the research findings of the author as well as future directions that should be explored. Following the general conclusions chapter of this dissertation, a comprehensive list of references for the introduction, review of literature, and general conclusions is included. Each manuscript has its own list of references.

Description of the Research Problem

Folate, homocysteine, and methyl group metabolism are interrelated pathways collectively referred to as one-carbon metabolism that are important in health and disease. Through the interaction between these pathways, methyl groups supplied from dietary methionine or from the folate-dependent remethylation of homocysteine are activated to *S*-adenosylmethionine (SAM), which then serves as a donor of methyl groups in a number of transmethylation reactions. SAM-dependent reactions are required for the synthesis and modification of numerous molecules, including polyamines, phosphatidylcholine, and neurotransmitters, as well as the methylation of DNA. Therefore, down regulation of one-carbon metabolism may result in pathological consequences. Previous work in our laboratory demonstrated that the administration of the vitamin A derivative, 13-*cis*-retinoic acid produced conditions indicative of elevated methyl group catabolism (Schalinske and Steele 1991). Based in part on these findings, I hypothesized that the loss of methyl groups due to retinoid treatment was mediated by the inappropriate activation of glycine *N*-methyltransferase (GNMT), a key cytosolic enzyme found primarily in the liver, kidney, and

pancreas that regulates the supply of SAM available for transmethylation reactions by disposing of excess methyl groups (Ogawa *et al.* 1998) and regulating the supply of methyl groups from the one-carbon pool (Wagner 1985). Moreover, I postulated that the loss of methyl groups through the activation of GNMT would lead to the down regulation of other SAM-dependent transmethylation reactions.

In Chapter 3 of this dissertation, I showed for the first time that retinoids can up-regulate hepatic GNMT activity and protein abundance in rats. To determine the physiological significance of these findings, I then conducted a series of experiments that examined the impact of GNMT up-regulation on other SAM-dependent transmethylation reactions. In Chapter 4, I illustrated that retinoid-mediated induction of GNMT leads to the down-regulation of SAM-dependent methylation of hepatic DNA, suggesting that GNMT up-regulation can lead to conditions consistent with methyl group deficiency.

GNMT activity has been shown to be elevated during uncontrolled diabetes (Xue and Snoswell 1985; Jacobs *et al.* 1998), a disease state associated with abnormal circulating concentrations of counter-regulatory hormones (i.e., glucagon and glucocorticoids) and increased expression of gluconeogenic enzymes. Furthermore, GNMT is found primarily in gluconeogenic tissues (e.g., liver, kidney, and pancreas) (Yeo and Wagner 1994). Therefore, I hypothesized that gluconeogenesis plays a critical role in regulating the expression of GNMT. In Chapter 5, I demonstrated that, like retinoids, glucocorticoids possess the ability to increase the enzymatic activity and protein abundance of GNMT. In addition, I provide strong evidence that an interaction exists between retinoids and gluconeogenesis in the increased up-regulation of GNMT. Taken together, these findings implicate increased GNMT expression as a potential complication of diabetes and/or use of retinoids for therapeutic purposes.

REVIEW OF LITERATURE

Folate Metabolism

Biological Role of Folate Compounds

Folate, otherwise known as pteroylmonoglutamate, is a generic term for a family of water soluble B-vitamins that function to accept and distribute one-carbon units to where they can be utilized in biosynthetic reactions that are required for maintenance of normal health. When its pteridine moeity is in its fully reduced form, folate becomes the physiologically functional molecule tetrahydrofolate (THF) **(Figure 1)**. Within the cell, THF derivatives coexist in metabolic equilibrium in the cytosol and mitochondria, cellular compartments where folate-dependent one-carbon transfer reactions, collectively referred to as one-carbon metabolism, readily occur. Critical biological processes that require folate dependent one-carbon transfer reactions include the methylation of DNA and the synthesis of purines, pyrimidines, neurotransmitters, phospholipids, methionine, and polyamines. Therefore, aberrations of folate metabolism due to conditions such as folate deficiency and polymorphisms of folate-dependent enzymes have the ability to produce a number of pathological conditions.

Absorption and Distribution of Folate Compounds

Natural folate compounds are acquired from the diet mainly by consuming dark green, leafy vegetables and citrus fruits. Folate in its natural form consists primarily of polyglutamated molecules, which are hydrolyzed by γ-glutamylhydrolase to the monoglutamated form in the gut prior to absorption across the intestinal mucosa (Shane 1995). After its intestinal absorption, the monoglutamated folate molecule is bound by folate-binding proteins and then transported via portal circulation to the liver, where it is absorbed and metabolized to various polyglutamated derivatives, or released into the blood or bile primarily as 5-methyltetrahydrofolate (5-methyl-THF) (Shane 1995). After hepatic release of 5-methyl-THF into the blood, the folate derivative is bound by folate-binding proteins or albumins and transported to various tissues for use. Uptake of 5-methyl-THF into

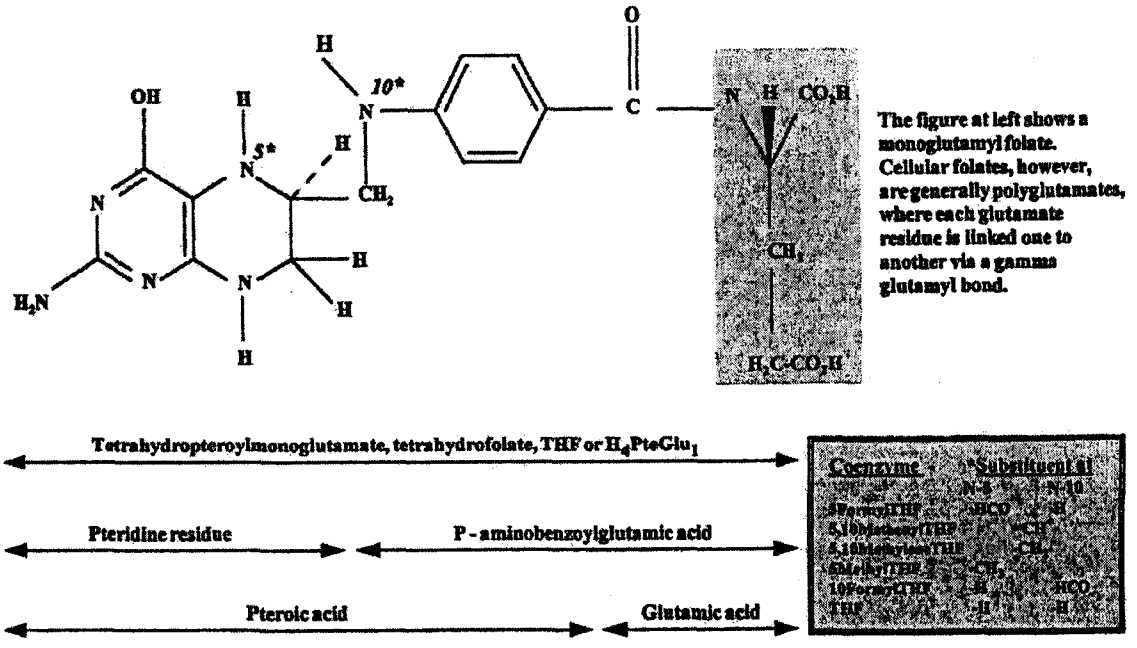

Figure 1. Chemical structure of tetrahydrofolate and its various derivatives ***(reprinted from Molecular Genetics and Metabolism, Vol 71, Lucock, M., 121-138, 2000, with permission from Elsevier).***

the cell is followed first by the removal of a methyl group by methionine synthase (MS) to generate THF, then the addition of several glutamate residues to the folate molecule by folylpolyglutamate synthase. This action serves to trap folate within the cell, where it is compartmentalized in the cytosol and mitochondria and readily converted to other folate derivatives. The addition of glutamate residues is also important for increasing the avidity of folate compounds for folate-dependent enzymes (Lucock 2000). Though trapped folate coenzymes and folate-dependent enzymes do not move freely between the two compartments within the cell, their metabolic products readily move between compartments as well as in and out of the cell (Bailey and Gregory 1999).

The Folate-dependent One-carbon Pool

Cellular folate coenzymes existing in metabolic equilibrium that have the ability to carry one-carbon units are collectively referred to as the folate-dependent one-carbon pool

(MacKenzie 1984). One-carbon units originating from the pool can undergo several redox reactions during folate metabolism **(Figure 2)**. The oxidation-state of one-carbon units dictates both the position of the THF molecule to which they can bind as well as their biological function. Formate for example, binds to THF at the 10-position to form 10-formyltetrahydrofolate (10-formyl-THF), which donates its one-carbon unit during the folate-dependent *de novo* biosynthesis of the purine ring (Shane and Stokstad 1985). Conversely,

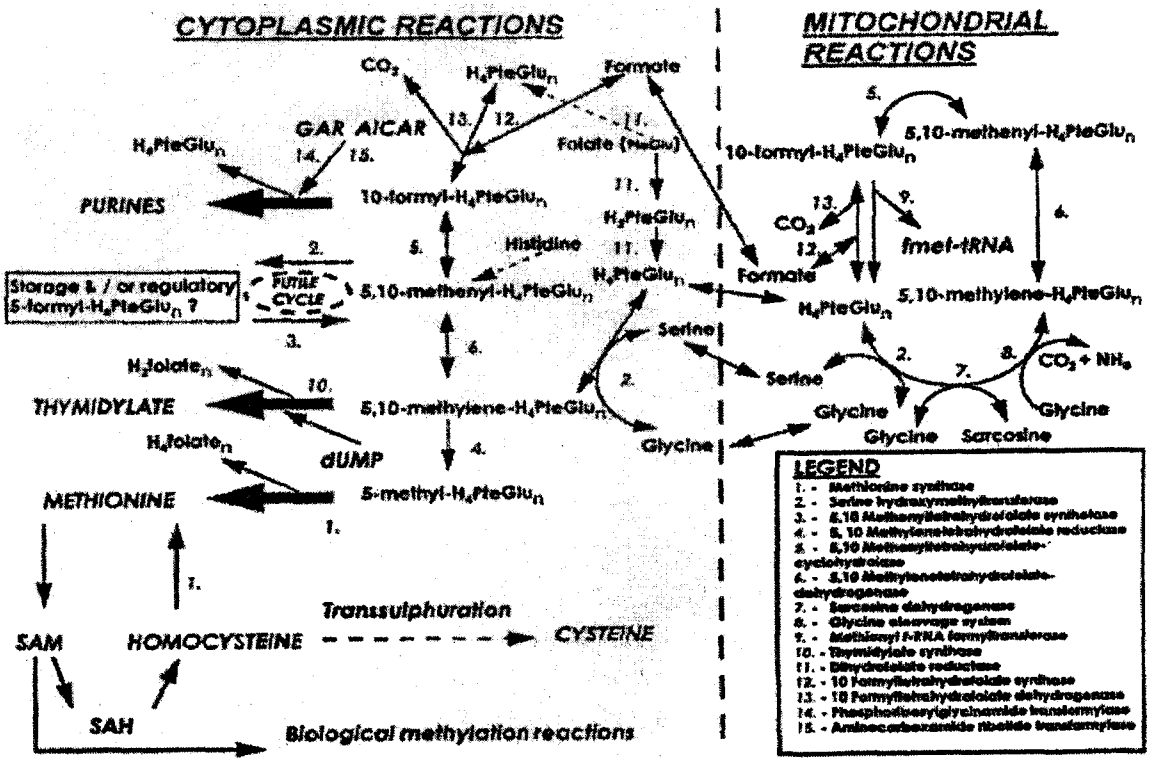

Figure 2. Folate-dependent one-carbon metabolism (*reprinted from Molecular Genetics and Metabolism, Vol 71, Lucock, M., 121-138, 2000, with permission from Elsevier*).

a reduced derivative of formate, formaldehyde, binds to THF at both the 5- and 10-positions of THF to generate 5,10-methylene-THF. 5,10-methylene-THF can then donate carbon units for three different purposes that include 1) the conversion of the pyrimidine uridylate to thymidylate by thymidylate synthase, a rate-limiting step in DNA synthesis, 2) reduction by 5,10-methylenetetrahydrofolate reductase (MTHFR) to generate 5-methyl-THF, a folate coenzyme that is critical for the regulation of methyl group metabolism (Wagner *et al.* 1985) and the donation of methyl groups for the synthesis of methionine, or 3) oxidation by 5,10-methylene-THF dehydrogenase to generate 5,10-methenyl-THF.

The following section describes the many other reactions that comprise folate-dependent one-carbon metabolism in more detail and how one-carbon units enter the folate-dependent one-carbon pool.

Entrance of One-carbon Units Into the Folate-dependent One-carbon Pool

There are five major folate-dependent one-carbon transfer reactions that occur in the mammalian cell: 1) conversion of serine to glycine, 2) catabolism of histidine, the synthesis of 3) thymidylate, 4) methionine, and 5) purine (Lucock 2000). Figure 2 illustrates the entrance of one-carbon units into the folate-dependent, one-carbon pool and the reactions that readily occur in the folate-dependent, one-carbon metabolism pathway.

Amino acids are the primary source of one-carbon units that enter the folate-dependent one-carbon pool with the major sources being serine and histidine (Wagner 1995). Serine enters the pool by transferring its formate group to THF to generate 5,10-methylene-THF in a vitamin B_6-dependent reaction catalyzed by serine hydroxymethyltransferase (SHMT). The carbon unit from 5,10-methylene-THF is then used for the synthesis of thymidylate, oxidized to generate 5,10-methenyl-THF, or reduced to 5-methyl-THF. The methyl group from 5-methyl-THF is a particularly important substrate during methyl group metabolism because it is used for the remethylation of homocysteine in a B_{12}-dependent reaction catalyzed by methionine synthase (MS) to generate methionine, which serves as a major source of methyl groups in transmethylation reactions. Histidine, another amino acid, donates one-carbon units to the folate-dependent one-carbon pool that are generated from its catabolism. The histidine breakdown product formiminoglutamate enters the pool by binding to THF at the 5-position to form 5-formyltetrahydrofolate (5-formyl-THF), which can be reduced to 5,10-methenyl-THF by 5,10-methenyl-THF reductase.

In addition to being donated by serine, formate can enter directly into the one-carbon pool as formic acid through a reaction catalyzed by 10-formyl-THF synthase, which attaches formic acid to THF at the 10-position, generating 10-formyl-THF in the process. The carbon group from 10-formyl-THF is used as a substrate during the synthesis of purines in reactions catalyzed by glycinamide ribonucleotide (GAR) transformylase and 5-amino-4-imidazole carboxamide ribonucleotide (AICAR) transformylase, respectively, or readily converted to 5,10-methenyl-THF in a reversible reaction catalyzed by methenyl-THF cyclohydrolase.

During conditions where adequate one-carbon units are available in the one-carbon pool, the formate group of 10-formyl-THF is disposed of as CO_2, in a reaction catalyzed by 10-formyl-THF dehydrogenase (Kutzbach and Stokstad 1968).

Clearly, the interrelated pathways of one-carbon metabolism are extremely complex and require a number of substrates and enzymes to function properly. The importance of these pathways with respect to methyl group metabolism will be focused on in greater detail below and will be one of the focal points of this thesis.

Methyl Group Metabolism

General Overview of Methyl Group Metabolism

Methyl group metabolism refers to a group of tightly coordinated reactions that supply methyl groups for use in over 100 transmethylation reactions. Enzymes involved in methyl group metabolism are found in most organs, but all are prevalent in the liver, where the majority of methyl group metabolism regulation is believed to occur (Finkelstein 1990). Methionine, which is synthesized *de novo* or provided from the diet, serves as the source of methyl groups for transmethylation reactions. In a reaction catalyzed by methionine adenosyltransferase (MAT), an adenosine molecule is attached to methionine to generate SAM, thereby activating methyl groups for use in transmethylation reactions **(Figure 3)** (Cantoni 1982; Cantoni and Chiang 1980; Mudd 1963). SAM-dependent transmethylation reactions, approximately 85% of which are believed to occur in the liver (Mato *et al.* 1994), are required for the biosynthesis of macromolecules such as phospholipids, neurotransmitters, and polyamines, as well as for the methylation of proteins and nucleic acids (Cantoni 1982; Cantoni and Chiang 1980). Therefore, acquiring an adequate dietary supply of methyl groups is a critical aspect of maintaining optimal health.

Following its donation of a methyl group for SAM-dependent transmethylation reactions, SAM is converted to S-adenosylhomocysteine (SAH), which is catabolized to generate homocysteine and adenosine by SAH hydrolase. The formation of homocysteine represents a metabolic branch point in the pathway because homocysteine is either remethylated to regenerate methionine or committed to the transsulfuration pathway for its catabolism.

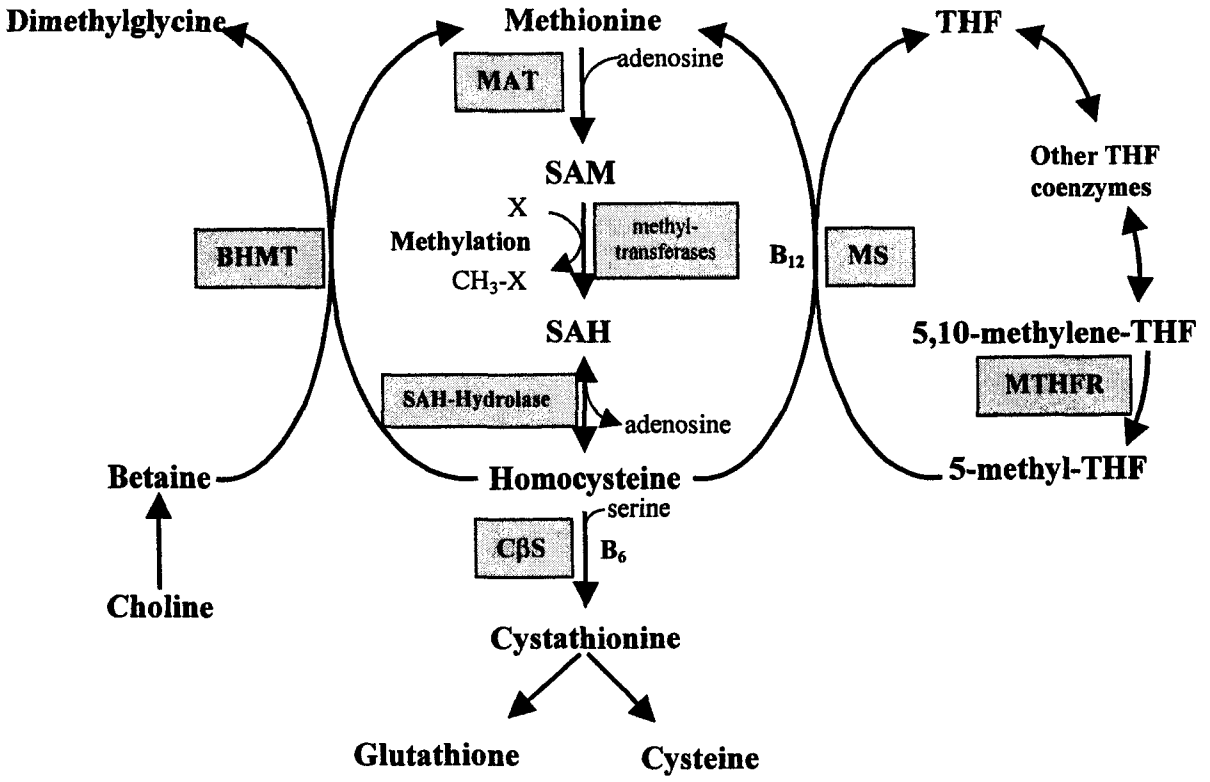

Figure 3. Methyl group metabolism.

During the remethylation process, methyl groups are utilized to remethylate homocysteine and generate methionine *de novo* by one of two methods: 1) addition of a methyl group from 5-methyl-THF to homocysteine in a vitamin B_{12}-dependent reaction catalyzed by MS or 2) the addition of a methyl group by the choline derivative, betaine by betaine:homocysteine methyltransferase (BHMT). Finkelstein and Martin (1984) reported that MS and BHMT, the latter being found primarily in the liver and kidney (Ueland and Refsum 1989), are equally important for the remethylation of homocysteine. The remethylation process ensures that the carbon chain of methionine is recycled for use in SAM-dependent transmethylation reactions multiple times (Eloranta *et al.* 1990).

If homocysteine is not remethylated, it is committed to the transsulfuration pathway by cystathionine β-synthase (CβS) for its catabolism in a vitamin B_6-dependent reaction that condenses homocysteine with serine to generate cystathionine (Finkelstein and Chalmers 1970), a molecule that is required for the synthesis of the essential compounds cysteine and

glutathione. The removal of homocysteine through transsulfuration by CβS is critical for maintaining normal methyl group metabolism; this is because the conversion of homocysteine back to SAH is kinetically favored (Cantoni and Chiang 1980), and SAH inhibits not only transmethylation reactions (Kerr 1972), but also the remethylation of homocysteine (Finkelstein *et al.* 1974).

Regulation of Methyl Group Metabolism

Allosteric regulation

The methyl group- and folate-dependent one-carbon metabolism pathways interact with each other to ensure an adequate supply of methyl groups is available for transmethylation reactions. In order to optimize the supply of methyl groups, a number of regulatory mechanisms exist between the two pathways that serve to normalize the methyl group supply when it fluctuates **(Figure 4)**. During conditions of elevated intracellular methionine concentrations for example, an increase in the supply of SAM occurs (Henning

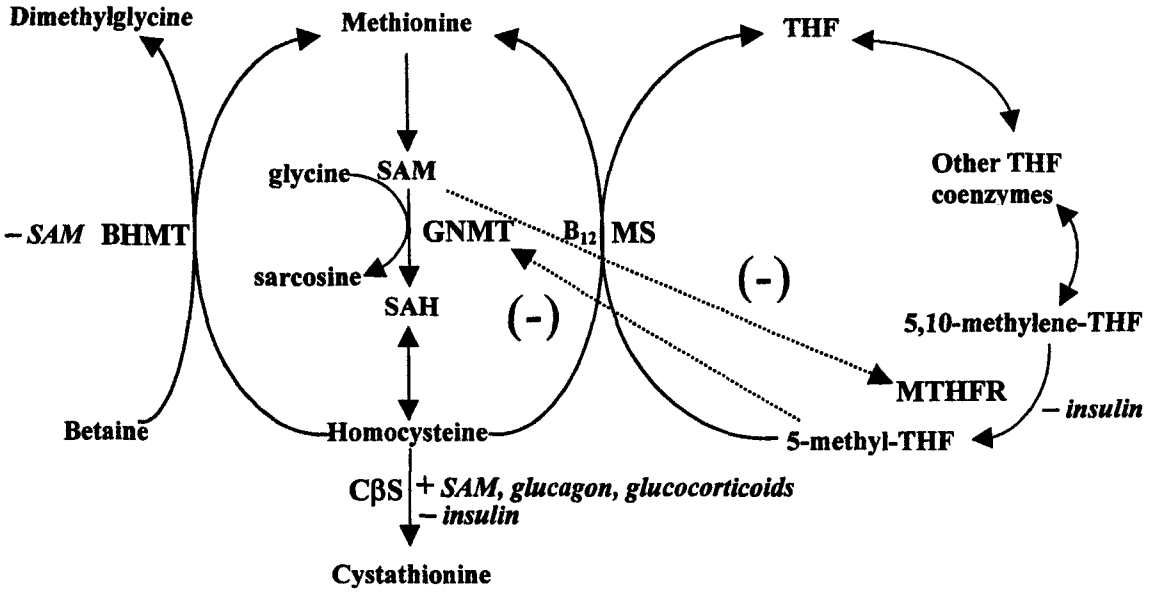

Figure 4. Regulation of methyl group metabolism.

et al. 1989). The elevation of SAM concentrations produces numerous regulatory consequences because SAM acts as an allosteric effector of several enzymes that catalyze critical methyl group- and folate-dependent reactions. The modulation of enzymes in the pathway during conditions of increased SAM levels serves two important purposes that include, 1) the inhibition of the flow of unneeded methyl groups into the pathway from both *de novo* sources, betaine (Finkelstein and Martin 1984) and the folate-dependent one-carbon pool (Kutzbach and Stokstad 1971) and 2) the normalization of the supply of SAM by facilitating its catabolism (Ogawa and Fujioka 1982). The SAM-mediated inhibition of both BHMT activity (Finkelstein and Martin 1984) and the conversion of 5,10-methylene-THF to 5-methyl-THF by MTHFR (Kutzbach and Stokstad 1967; 1971) diverts one-carbon units to where they can serve other important functions when there is an adequate supply of intracellular SAM for transmethylation reactions. With decreased 5-methyl-THF concentrations due to SAM-mediated inhibition of MTHFR, significant changes occur elsewhere in the methyl group metabolism pathway because 5-methyl-THF is an inhibitory ligand for glycine *N*-methyltransferase (GNMT) (Wagner *et al.* 1985), a key cytosolic protein that regulates the SAM/SAH ratio by disposing of excess SAM as sarcosine (Kerr 1972), a molecule with no known metabolic function. Therefore, when cellular 5-methyl-THF levels are decreased, GNMT becomes more active (Balaghi *et al.* 1993) and serves to normalize SAM concentrations and the SAM/SAH ratio by facilitating the catabolism of SAM. The regulation of the SAM/SAH ratio by GNMT is critical because this ratio is considered an index of transmethylation potential due to the ability of SAH to be a potent inhibitor of most methyltransferases (Kerr 1972). Elevated SAM concentrations also allosterically activate CβS (Finkelstein and Martin 1986), which assists in the catabolism of SAM by committing the increased volume of SAM degradation products to the transsulfuration pathway, as previously described.

Under conditions where SAM levels are decreased, highly coordinated mechanisms are in place to restore them. A decrease in SAM concentration releases MTHFR inhibition (Kutzbach and Stokstad 1967), which then facilitates the increased production of 5-methyl-THF. Consequently, the folate-dependent remethylation of homocysteine and subsequent flow of methyl groups from the one-carbon pool into the methyl group metabolism pathway

for production of SAM increase. The rise in 5-methyl-THF concentrations also allosterically inhibits GNMT activity, which serves to slow the disposal of SAM in order to conserve it for other transmethylation reactions (Wagner *et al.* 1985). Additionally, BHMT inhibition is released as SAM concentrations decline (Finkelstein and Martin 1984), which can then aid in the regeneration of methionine through the remethylation of homocysteine.

Hormonal modulation of methyl group metabolism

Several hormones are known to regulate the expression of key enzymes involved in methyl group metabolism. Hormones known to modulate methyl group metabolism include glucagon, glucocorticoids, insulin, thyroxine (thyroid hormone), and growth hormone. Although many of the general mechanisms by which these hormones exert their actions are known, the physiological consequences of their actions with respect to methyl group metabolism are poorly understood. Recent evidence suggests that the modulation of methyl group metabolism by hormones occurs as a result of alterations of glucose metabolism (Jacobs *et al.* 1998; 2001; Ratnam *et al.* 2002; Xue and Snoswell 1985).

Glucagon, glucocorticoids, and insulin. Hormones secreted at increased rates during a catabolic state (i.e. gluconeogenesis) such as glucagon and glucocorticoids (i.e. counter-regulatory hormones), appear to enhance the catabolism of methionine (Gil *et al.* 1997; Jacobs *et al.* 1998; 2001; Ratnam *et al.* 2002). In tissue culture and rat studies, treatment with glucocorticoids hormones elevated the gene expression of MAT 3-fold (Gil *et al.* 1997), whereas glucocorticoid (Ratnam *et al.* 2002) and glucagon (Jacobs *et al.* 2001) treatment increased CβS gene expression 1.5- and 2-fold, respectively. In all the described cases, treatment with insulin was effective in blocking the hormone-mediated induction of these enzymes. Additionally, CβS expression was elevated in streptozotocin-diabetic rats, animals that exhibit conditions consistent with elevated levels of counter-regulatory hormones, whereas insulin treatment was effective in preventing this occurrence (Ratnam *et al.* 2002). These findings suggest that the physiological consequences of up-regulated methionine metabolism may have significance during uncontrolled diabetes, during which insulin utilization is compromised and an increased circulating concentration of counter-

regulatory hormones is unchecked. Because methionine serves as an important source of methyl groups, its increased catabolism could have a detrimental effect on SAM-dependent transmethylation reactions. Xue and Snoswell (1985) found that the disposal of methyl groups by GNMT was increased 65-fold in alloxan-diabetic sheep, suggesting that diabetic conditions do in fact lead to unwarranted loss of methyl groups; however, it has yet to be investigated whether the increased disposal of methyl groups GNMT compromises other SAM-dependent transmethylation reactions.

Growth hormone and thyroxine. Associations between both thyroid (thyroxine) and growth hormones and the modulation of methyl group metabolism clearly exist, but are not well understood and contradictory. In animal studies, it appears that both hormones play an inhibitory role in the catabolism of methionine and its products, whereas the opposite effect usually occurs in humans. Animal experiments have demonstrated that the expression GNMT mRNA is decreased in growth hormone-treated mice (Aida *et al.* 1997). Moreover, Ames dwarf mice, animals that are deficient in growth hormone, prolactin, and thyroid stimulating hormone, exhibited symptoms consistent with elevated methionine catabolism including, increased activities of MAT (203%), CβS (50%), cystathionine γ-lyase (83%), and GNMT (91%) (Uthus and Brown-Borg 2003). Similarly, hypothyroid rats have been shown to exhibit decreased plasma homocysteine levels, which appear to be normalized by treatment with thyroxine (Jacobs *et al.* 2000). Collectively, these reports clearly indicate that thyroid and growth hormones play an integral role in modulating methionine metabolism, although that role is unclear. Interestingly, thyroxine administration to rats has been shown to increase the activity of hepatic BHMT (Finkelstein *et al.* 1971), demonstrating that in addition to modulating the activity of enzymes involved in the transsulfuration pathway, thyroxine may modulate homocysteine levels through its effect on the remethylation pathway.

In contrast to animal studies, human studies have demonstrated that growth hormone deficiency and hypothyroidism are conditions that appear to inhibit homocysteine catabolism (Sesmilo *et al.* 2001). Hussein et al. (1999) found that individuals suffering from hypothyroidism exhibited hyperhomocysteinemia, which was normalized by treatment with

thyroxine. Similarly, treatment with growth hormone has been shown to reduce elevated plasma homocysteine levels in patients with growth hormone deficiency (Sesmilo *et al.* 2001). Because these findings contradict those from animal studies, it appears that animal models are not a useful tool for studying the role of growth hormone and thyroxine in the modulation of methyl group metabolism in humans.

Dietary Requirements

Folate Requirements

Dietary Reference Intakes

In 2000, the latest dietary reference intakes (DRIs) reported by the Food and Nutrition Board based folate recommendations on data that was collected from controlled metabolic studies where blood folate concentrations were measured, and from data collected in population-based studies [Food and Nutrition Board (FNB) 2000]. DRI values for folate are expressed as dietary folate equivalents (DFE), where 1 DFE = 1 μg food folate = 0.6 μg of folic acid from fortified food or as a supplement consumed with food = 0.5 μg of a folic acid supplement taken on an empty stomach (FNB 2000). Folic acid refers to the synthetic derivative of folate, which has been shown to be more bioavailable than naturally occurring food folate, presumably due to the greater instability of food folate (O'Broin, et al 1975). Folate DRIs include a recommended daily allowance (RDA) that advises a minimum daily intake DFE for all age groups [except for infants (0-12 months)], including pregnant and/or lactating adults. Regardless of gender, once individuals reach age 14 years old, the RDA (400 DFE per day) remains constant in males and non-pregnant females. For pregnant and lactating adults, the RDA for folate is 600 and 500 DFE, respectively. Infants' daily requirements are expressed as adequate intakes (AI), which equals the DFE of folate an infant must consume daily in order to meet the requirements of the individuals in a particular infant age group. For infants 0-6 months of age, the AI of folate is 65 DFE, which increases to 80 DFE as infants reach the 7-12-month age group (FNB 2000). In addition to RDA and AI values, the DRIs for folate include an upper limit (UL), which is defined as the maximal daily nutrient intake that is likely to pose no risk of adverse effects (FNB 2000). In the case

of folate, an UL is listed because excessive consumption can mask symptoms of vitamin B_{12} (i.e. cobalamin) deficiency while neurological damage due to vitamin B_{12} deficiency is allowed to progress undetected (FNB 2000). With pregnancy and lactation being exceptions, the RDA and UL for folate increases with age (300-800 DFE) then becomes stable (1000 DFE) when individuals reach 19 years of age.

Folate requirements during pregnancy

A strong correlation between low folate status and an elevated incidence of neural tube defects (NTDs) has been well documented (Mulinare *et al.* 1988; Werler *et al.* 1993; Milunsky *et al.* 1989). Vital biological processes that occur during fetal development requiring folate-dependent reactions include cell division, neural tube closure of the developing fetus during pregnancy, and a number of reactions requiring methyl groups. Out of an estimated 4000 pregnancies in the U.S. that are affected with NTDs each year, it is currently believed that 50% or more can be prevented by consuming a folic acid-containing supplement before and during the early weeks of pregnancy in addition to meeting dietary folate requirements [American Academy of Pediatrics (AAP) Committee on Genetics 1999]. In order to decrease the incidence of NTDs in women who have no history of a previous NTD-affected pregnancy as well as those women who have had a child with a NTD, new recommendations for folate consumption were developed by the US Public Health Service (USPHS) and Centers for Disease Control and Prevention (CDC) (CDC 1991; 1992). The USPHS recommendation for folate intake by women with no prior NTD-affected pregnancy who are of childbearing age is 400 μg of folic acid per day in addition to the dietary requirement (RDA) of 400 DFE from food (CDC 1991; 1992). These recommendations have been endorsed by the AAP (AAP Committee on Genetics 1999). For those women who have had NTD-affected pregnancies, the recommendation is a 4000 μg daily dosage of folic acid, which should not be consumed through the taking of over-the-counter folic acid-containing prescription multivitamins due to toxicity issues involving other nutrients (CDC 1991; 1992). The AAP recommended that these women should be offered prophylaxis (4000 μg DFE) one month before the time they plan to become pregnant and throughout the first 3 months of pregnancy (AAP Committee on Genetics 1999). However, these new recommendations may

reflect a need to review and perhaps redesign the current DRIs for pregnancy because the requirements endorsed by the AAP obviously differ from the current RDA of 600 DFE per day.

Methyl Group Requirements

Methyl groups that are utilized for SAM-dependent transmethylation reactions originate from the folate-dependent one-carbon pool or from essentially two dietary sources, methionine and choline. Methionine, an abundant essential amino acid in protein, is very toxic if consumed excessively (Benevenga and Steele 1984) and no dietary recommendations are currently in place for its consumption. However, as with all of the other essential amino acids, it is a reasonable assumption that daily protein requirements should be met to ensure that an adequate supply of methionine is consumed. An AI for choline is included in the current DRIs, although there are few data to assess whether a dietary supply is needed at all stages of the life cycle (FNB 2000). The AI for choline is 125-150 mg/day for infants and increases throughout childhood (200-250 mg/day) into adulthood, where the requirement is greater for males than for females (FNB 2000). According to the latest DRIs, the AI for adult males is 550 mg/day, whereas three separate AI classifications for female requirements exist. These classification groups include women that are non-pregnant and non-lactating, pregnant, or lactating (FNB 2000). The adult AI for non-lactating, non-pregnant women is 425 mg/day (FNB 2000). During pregnancy the AI is increased to 450 mg/day, whereas lactation increases the requirement to 550 mg/day (FNB 2000).

One-Carbon Metabolism in Health and Disease

General Overview

Diseases related to folate and/or methyl group deficiency can be catastrophic. As discussed previously, methyl group- and folate-dependent one-carbon metabolism are interrelated pathways that involve a large number of enzymes and metabolites that play major roles in sustaining life processes. The synthesis of nucleic acids, phospholipids, neurotransmitters, and polyamines, along with functions such as the methylation of DNA and

neural tube closure in the fetus, all require folate and methyl groups in some capacity. In addition to a lack of folate and sources of methyl groups in the diet, deficiencies of vitamin B_{12} or vitamin B_6, a number of drugs, such as anticonvulsants, oral contraceptives, retinoid-containing acne medications, and numerous environmental toxins can all perturb one-carbon metabolism reactions. Moreover, several genetic polymorphisms of enzymes and metabolic diseases, such as diabetes mellitus, have been implicated as factors that have a major impact on folate and/or methyl group requirements.

Neural Tube Defects

The role of folate and methyl group metabolism during fetal development is important for developing dietary recommendations for the prevention of birth defects, namely NTDs (Coelho *et al.* 1989; Coelho and Klein 1990; Lau and Li 1995; Kirke *et al.* 1993). NTDs, caused by congenital defects such as spina bifida, anencephalus (approximately 50% and 40% of cases, respectively), encephalocele, and iniencephaly (Scott *et al.* 1990), make up approximately 1 of 1000 births in the United States (AAP Committee on Genetics 1999) and are influenced by a number of nutritional factors, especially folate- and methyl group metabolism. Poor nutrition can lead to the perturbation of one-carbon metabolism, which, in turn, may lead to the inhibition of essential processes required for fetal development such as DNA synthesis and methylation, polyamine and phospholipid synthesis, and the post-translational modification of myelin basic protein, a protein required for neural tube closure (Benjamins *et al.* 1984; Chiang, *et al.* 1996).

The identification of folate as a dietary factor that clearly influences the outcome of pregnancies has been known for over 50 years. In 1949, it was first observed that rats kept on diets deficient in folic acid prior to gestation exhibited 100% resorption of their fetuses (Nelson and Evans 1949). Soon after, similar findings were reported in human studies, where spontaneous abortions occurred in individuals that unknowingly consumed folic acid antagonists prior to gestation (Thiersch 1952; Goetsch 1962). Findings such as these have helped spark intense research to determine the importance of folic acid supplementation during pregnancy. Data collected from case-control (Mulinare *et al.* 1988; Werler *et al.* 1993) and prospective (Milunsky *et al.* 1989) studies have provided strong evidence that folic acid

supplementation during periconception, a six-month period including three months prior through three months after conception, results in a significant decline in the incidence of NTD-affected pregnancies. These findings contributed to the 1992 decision of the USPHS to recommend that all reproductive-aged women capable of becoming pregnant should consume a daily dose of 400 μg of folic acid (CDC 1992) in addition to DFE consumed in the diet. However, this recommendation proved to be a failure. In fact, by 1998 only 29% of women in the United States were following the USPHS recommendation for folic acid consumption (CDC 1999). As a result, the United States Food and Drug Administration (USFDA) authorized mandatory folic acid fortification of grain products in the United States effective 1998, which is believed to have led to a 19% reduction in the incidence of NTDs (Honein *et al.* 2001).

Although the association between folate deficiency and alterations of embryogenesis is almost indisputable, the mechanistic link between folate deficiency and the increased incidence of NTDs is a topic of ongoing debate. It is well established that folate-dependent reactions are required for the synthesis of DNA and cell division during fetal development; however, current evidence suggests that abnormalities of other aspects of one-carbon metabolism associated with folate deficiency, such as hyperhomocysteinemia (Steegers-Theunissen 1994), play a significant role in the etiology of NTDs. Because folate is required for maintaining normal homocysteine concentrations (Brattstrom *et al.* 1988), it is possible that folate supplementation reduces the incidence of NTDs through its role in the remethylation pathway. Interestingly, a deficiency of vitamin B_{12}, a nutrient also required for the remethylation of homocysteine, has been implicated as an independent risk factor for NTDs (Daly *et al.* 1995). Additionally, genetic polymorphisms that produce deficiencies of CβS and MTHFR, enzymes that play central roles in homocysteine metabolism, have been strongly associated with an increased incidence of NTDs (Engbersen *et al.* 1995; Mills *et al.* 1995; Christensen *et al.* 1999). Thus, monitoring plasma homocysteine and screening for genetic polymorphisms during pregnancy may be useful strategies for decreasing the incidence of NTDs in addition to dietary intervention with folic acid (Brattstrom *et al.* 1988), vitamin B_{12} (Kirke *et al.* 1993), and vitamin B_6 supplementation (Cuskelly *et al.* 2001).

The inverse relationship between plasma homocysteine levels and the incidence of NTDs may also be related to the maintenance of normal methionine concentrations by folate-dependent remethylation (Eskes 1998). *In vitro* studies using embryo cultures and *in vivo* models have demonstrated that methionine is essential for neural tube closure (Coelho *et al.* 1989; Essien and Wannberg 1993). Likewise, additional methionine in the diet has been shown to decrease the incidence of NTDs in mice administered retinoid compounds (Lau and Li 1995), which are known to be teratogenic with respect to fetal development (Lammer *et al.* 1985) perhaps because of their ability to enhance the catabolism of methionine (Peng and Evenson 1982; Schalinske and Steele 1991) and alter the distribution of folate coenzymes in the folate-dependent one-carbon pool (Fell and Steele 1986). As discussed previously, methyl groups provided by methionine and the folate-dependent one-carbon pool are activated to SAM for use in transmethylation reactions, many of which are required during fetal development, including polyamine and phospholipid synthesis (Chiang *et al.* 1996), as well as the post-translational modification of myelin basic protein (Benjamins *et al.* 1984; Chiang *et al.* 1996). Because adequate folate status is essential for the maintenance of normal SAM concentrations (Balaghi *et al.* 1993), a lack of folate coenzymes that are capable of transferring methyl groups during the *de novo* synthesis of SAM may therefore be responsible, at least in part, for the increased incidence of NTDs due to folate and folate-dependent enzyme deficiencies.

Hyperhomocysteinemia and Cardiovascular Disease

Hyperhomocysteinemia as a risk factor for cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of mortality in the United States according to the latest report by the CDC using data collected from 2001 (United States Department of Health and Human Services 2003). Hyperhomocysteinemia has recently been added to the list of other commonly known risk factors for CVD such as hypertension, elevated low-density lipoprotein (LDL) concentrations, and smoking (Clarke *et al.* 1991). Research that led to the identification of hyperhomocysteinemia as a risk factor for CVD began over 30 years ago when homocysteine was in question as a possible underlying factor in the development of vascular lesions in an infant with abnormal vitamin B_{12} metabolism

and homocystinuria (McCully 1969). Since then, growing evidence has strongly associated hyperhomocysteinemia with vascular lesions and premature development of CVD (McCully and Wilson 1975; Brattstrom *et al.* 1992; Kang *et al.* 1986; Clarke *et al.* 1991). In 1991, Clarke et al. (1991) examined patients who were diagnosed with vascular disease before age 55 and found that homocysteine levels were 33% higher in those with vascular disease than in control subjects following a standard methionine-loading test.

Strong evidence exists that implicates homocysteine as an atherogenic compound. For example, direct administration of homocysteine thiolactone produces severe arteriosclerosis in rabbits (McCully and Ragsdale 1970), but the exact mechanism by which the molecule causes vascular dysfunction is not known. In a recent review, Haynes (2003) concluded that increased plasma homocysteine levels have the potential to mediate several atherogenic effects, including increased oxidative stress, impaired endothelial function, stimulation of mitogenesis, and induction of thrombosis.

Regulation of homocysteine metabolism

In order to maintain normal concentrations of plasma homocysteine, a highly coordinated system for homocysteine removal is in place. As previously mentioned, homocysteine is metabolized via three separate reactions: 1) vitamin B_6-dependent conversion of homocysteine to cystathionine by CβS followed by catabolism through the transsulfuration pathway, 2) folate- and vitamin B_{12}-dependent remethylation by MS, and 3) betaine-dependent remethylation by BHMT, a reaction confined primarily to the liver and kidney (Finkelstein 1990). It has been reported that under normal conditions, approximately 46% of homocysteine is committed to the transsulfuration pathway by CβS, with the rest being remethylated by MS and BHMT (approximately 27% and 27%, respectively) (Finkelstein and Martin 1984). A recent study demonstrated that homocysteine levels are also inversely related to the activity of phosphatidylethanolamine *N*-methyltransferase (Noga, *et al.* 2003), an enzyme responsible for the conversion of phosphatidylethanolamine to phosphatidylcholine, a source of choline that can serve as a source of three methyl groups for the betaine-dependent remethylation of homocysteine via BHMT.

Although CβS is responsible for removing the majority of circulating homocysteine compared to MS and BHMT, coordination between all three of these enzymes is crucial for the maintenance of normal homocysteine metabolism. In certain cases, a deficiency of one of the enzymes needed for the removal of homocysteine results in homocysteinemia, indicating that all three must function optimally to maintain normal homocysteine levels and compensation needed for the removal of excess homocysteine by complementary enzymes is limited.

Enzyme deficiencies due to polymorphisms of CβS and MTHFR, as well as nutritional deficiencies or defects in the transport of folate, vitamin B_6 (i.e. pyridoxal phosphate), and/or vitamin B_{12}, drugs, and metabolic diseases are among the conditions that have been associated with elevated homocysteine concentrations (Green and Jacobsen 1995). Deficiencies of folate, vitamin B_6 (i.e. pyridoxal phosphate), and/or vitamin B_{12} can also exacerbate abnormal methyl group metabolism caused by preexisting genetic conditions (Kluitmans *et al.* 2003). Taken together, the complexity of coordinating the activity of a number of enzymes and maintaining adequate nutritional status imparts a large number of potential causes for perturbation of homocysteine metabolism. In the following section, the causes and treatments of hyperhomocysteinemia with respect to metabolic disorders and nutrition will be reviewed in greater detail.

Etiology of Hyperhomocysteinemia

Enzyme deficiencies

Cystathionine β-synthase (CβS) polymorphisms. As discussed previously, CβS catalyzes the condensation of homocysteine and serine in a vitamin B_6-dependent reaction that produces cystathionine, committing homocysteine to the transsulfuration pathway for its catabolism in the process. A deficiency of CβS, usually caused by a 68-bp insertion between nucleotide 844 and 845 of the CβS gene (Sebastio *et al.* 1995), is the most common inborn error of methionine metabolism, occurring in 1 in 200,000 of the general population worldwide (Selhub and Miller 1992). This particular CβS mutation has been called a "classical congenital disorder" because it is inherited as an autosomal recessive trait

(Kluitjmans *et al.* 1999) and results in the blocking of the transsulfuration pathway (Finkelstein *et al.* 1964).

The importance of CβS during the removal of excess homocysteine has been examined by Finkelstein and Martin (1984), who demonstrated i*n vitro* that compared to BHMT and MS, CβS plays a much larger role in the removal of homocysteine when the flux through the methyl group metabolism pathway is increased. Therefore, it is not surprising that individuals with CβS deficiency are at an increased risk for developing severe hyperhomocysteinemia (Boddie *et al.* 1998; Silaste *et al.* 2001), cardiovascular disease (Ueland and Refsum 1989), and vascular dysfunction (Lentz *et al.* 2000). Likewise, individuals with CβS deficiency are more likely to suffer from other conditions that are consistent with hyperhomocysteinemia including, skeletal anomalies, mental retardation, and dislocation of the optic lens (Ueland and Refsum 1989).

Complications associated with CβS deficiency are exacerbated when combined with folate deficiency (Lentz 2000), a condition that simultaneously down-regulates the remethylation system and contributes to elevated plasma homocysteine concentrations (Jacob *et al.* 1994; Cuskelly *et al.* 2001). Folate deficiency may also contribute to homocysteinemia during CβS deficiency by indirectly leading to the enhanced catabolism of dietary methionine. A decline in the supply of 5-methyl-THF releases its inhibition of GNMT and as a result, increases the catabolism of SAM (Wagner *et al.* 1985). With the resulting elevation of GNMT activity, an increased production of methionine degradation products occurs and homocysteine levels are allowed to build up uncontrollably because defective CβS cannot commit homocysteine to the transsulfuration pathway and folate is unavailable for its remethylation (Selhub 1999). Similarly, when there is an adequate supply of folate, CβS deficiency still can cause hyperhomocysteinemia, but folate-dependent remethylation helps alleviate the buildup of homocysteine due to CβS deficiency because homocysteine can be diverted toward the remethylation pathway when homocysteine concentrations build up (Selhub 1999). However, the diversion of homocysteine toward the folate-dependent remethylation pathway during CβS deficiency is only transient. With CβS deficiency, the inhibition of the transsulfuration pathway leads to increased SAM concentrations (Selhub 1999). As SAM concentrations reach the level where it is able to cause feedback inhibition

of MTHFR, inhibition of the remethylation system occurs and hyperhomocysteinemia is the end result.

Methylenetetrahydrofolate reductase (MTHFR) polymorphisms. Hyperhomocysteinemia has been implicated as the underlying cause of extensive abnormalities in the arterial bed (Harmon *et al.* 1996), vascular thrombosis (Kanwar *et al.* 1976), and coronary artery disease (Kang *et al.* 1988) in cardiovascular patients carrying a mutation of the MTHFR gene. The two most common mutations of the MTHFR gene include base pair substitutions at either bp 677, which is associated with hyperhomocysteinemia (Goyette *et al.* 1995), or bp 1298, which apparently has little or no influence on homocysteine concentrations (Friedman, *et al.* 1999). The mutation of the MTHFR gene at bp 677 resulting from a C→T substitution (C677T) is commonly referred to as "thermolabile" MTHFR, due to its instability at increased temperatures. Like CβS deficiency, the C677T mutation of MTHFR is autosomal recessive and has been associated with homocysteinemia (Engbersen, *et al.* 1995) and cardiovascular disease (Kang, *et al.* 1988; Engbersen, *et al.* 1995) in individuals with the homozygotic genotype. The C6777T mutation of MTHFR is more prevalent than CβS deficiency however, with the homozygotic genotype (T/T) occurring in ~12% of caucasian and asian populations (Brattstrom *et al.* 1988; Frosst *et al.* 1995). Individuals carrying the homozygotic genotype appear to be more prone to elevated homocysteine levels than individuals carrying the heterozygotic (C/T) genotype for the C677T mutation (Silaste *et al.* 2001; Kluijtmans *et al.* 2003).

Unlike CβS deficiency where the cause of homocysteinemia has been established, the mechanism by which MTHFR deficiency causes hyperhomocysteinemia is not well understood. Recent evidence suggests that low folate status in combination with MTHFR deficiency leads to hyperhomocysteinemia (Bostom, *et al.* 1996; Meleady, *et al.* 2003). Likewise, researchers have discovered that individuals with the T/T mutation are more likely to exhibit significantly lower baseline plasma and red cell folate levels than individuals carrying the T/C or C/C genotypes (Kluijtmans *et al.* 2003; Meleady, *et al.* 2003). Furthermore, Nelen *et al.* (1998) and Silaste *et al.* (2001) found that folate supplementation was not as effective in improving folate status in T/T individuals when compared to the T/C

and C/C genotypes, but was effective in normalizing their homocysteine concentrations. These findings suggest that individuals suffering from MTHFR deficiency may need to consume supplemental folate in order to maintain adequate MTHFR activity. In a recent review, Bailey and Gregory (1999) proposed that when folate status is adequate, normal plasma homocysteine levels should be maintained, independent of genotype. Therefore, individuals with thermolabile MTHFR may require folate supplementation in order to 1) supply 5-methyl-THF for remethylation of homocysteine by bypassing the MTHFR reaction and/or 2) to provide excess folate substrates to ensure that a dysfunctional MTHFR enzyme is able to produce an adequate supply of 5-methyl-THF.

Methionine synthase (MS) and betaine:homocysteine methyltransferase (BHMT) polymorphisms. Recent studies that have examined the correlation between MS and BHMT function and homocysteine metabolism have provided convincing evidence that suggests genetic mutations of neither MS (Jacques *et al.* 2003; Harmon *et al.* 1999; Chen *et al.* 2001; Hyndman *et al.* 2000) nor BHMT (Weisberg *et al.* 2003) are associated with hyperhomocysteinemia and cardiovascular disease. In fact, the most common mutation of MS, which is a result of the substitution of a glycine residue for an aspartic acid residue at base pair 2756 (A2756G), has been paradoxically associated with a decrease in plasma homocysteine concentrations (Chen *et al.* 2001). Additionally, it has been reported that carriers of the 2756 A→G mutation are less likely to suffer from cardiovascular disease than individuals with the normal genotype (Hyndman *et al.* 2000).

Although no underlying mechanism has been elucidated concerning the correlation between MS deficiency and reduced homocysteine concentrations, it has been demonstrated that individuals carrying the A2756G mutation of MS exhibited elevated cystathionine levels when compared to the wild-type controls (Geisel *et al.* 2001) indicating that the removal of homocysteine by CβS and the transsulfuration pathway is increased during MS deficiency. One possible explanation for this occurrence is that a deficiency of MS results in a substantial build up of 5-methyl-THF levels due to the inability of MS to remethylate homocysteine. Because the conversion of 5,10-methylene-THF to 5-methyl-THF by MTHFR is an irreversible reaction (Kutzbach and Stokstad 1967; 1971), a deficiency of MS

could cause folate to be "trapped" as 5-methyl-THF. As 5-methyl-THF concentrations rise, it becomes readily available to allosterically inhibit the GNMT-dependent conversion of SAM to SAH, increasing SAM concentrations in the process. Therefore, homocysteine levels would be expected to decline considerably because increased levels of SAM, which is an allosteric activator of CβS, facilitate the catabolism of existing homocysteine through the transsulfuration pathway (Finkelstein and Martin 1986).

Nutrient deficiency and environmental factors

Nutrient deficiency. Deficiencies of folate, vitamin B_{12}, and/or vitamin B_6 have significant implications for the development of hyperhomocysteinemia. Folate and vitamin B_{12} are nutrients that are required for remethylation of homocysteine, and a deficiency of these nutrients has been associated with increased plasma concentrations of homocysteine and an increased risk for developing coronary artery disease, presumably due to insufficient remethylation (Kang *et al.* 1987; Jacob *et al.* 1994; Bostom *et al.* 1996; Selhub *et al.* 1993; Pancharuniti *et al.* 1994; Voutilainen *et al.* 2000). Likewise, vitamin B_6 deficiency has also been associated with moderate hyperhomocysteinemia (Cuskelly *et al.* 2001). Because vitamin B_6 is a cofactor for CβS and cystathionine γ-lyase during the catabolism of homocysteine through the transsulfuration pathway, it seems likely that vitamin B_6-deficiency would perturb the enzymatic function of CβS and cystathionine γ-lyase, and produce a concomitant rise in homocysteine levels. Interestingly, vitamin B_6-deficiency has been recently identified as an independent risk factor for stroke and transient ischemic attack, independent of its role in the transsulfuration pathway (Kelly *et al.* 2003).

Because a number of B vitamins are required for homocysteine metabolism, it seems plausible that a supplement containing a B vitamin complex including folate, vitamin B_{12}, and vitamin B_6 would be useful in the treatment of hyperhomocysteinemia. Supplementation with these vitamins has been effective in normalizing plasma homocysteine concentrations when they were given alone (Brattstrom *et al.* 1988; Shimakawa *et al.* 1997). Another relatively effective strategy for reducing homocysteine levels has been supplementation with

betaine, which has been shown to reduce homocysteine concentrations as long as the compound is continuously administered (Steenge *et al.* 2003).

Pharmacological agents. A number of medications are known to produce increased plasma homocysteine levels. Drugs that are known to impair folate metabolism, such as antiepileptics (Schawninger *et al.* 1999), oral contraceptives (Steegers-Theunissen *et al.* 1992), and the antifolate drug methotrexate (Refsum *et al.* 1989) have been linked to elevated plasma homocysteine concentrations. Additionally, Schulpis et al. (2001) recently discovered that the retinoid-containing oral acne medication, isotretinoin (13-*cis*-retinoic acid) elevated plasma homocysteine concentrations in patients treated for cystic acne, an occurrence that the authors suggested may have been caused by an inhibition of CβS activity resulting from liver dysfunction, which is a common side effect of the drug.

Diabetes mellitus. The number one complication of diabetes is cardiovascular disease (Anderson 1999). Long-term exposure to hyperglycemia associated with uncontrolled diabetes results in enhanced glycosylation and modification of proteins, thereby playing a role in atherosclerosis and microvascular complications such as nephropathy and retinopathy. Hyperhomocysteinemia is also believed to contribute to heart disease in diabetics (Munshi *et al.* 1996). Evidence suggests that hyperhomocysteinemia is prevalent in diabetics with compromised renal function (Poirier *et al.* 2001; Robillon *et al.* 1994). Because renal tubular epithelial tissue is believed to play a major role in the metabolism of homocysteine (Foreman *et al.* 1982), damage to this tissue may inhibit the removal of homocysteine from the blood (Robillon *et al.* 1994). Recent evidence suggests that hyperhomocysteinemia due to renal failure may also be due to altered remethylation of homocysteine due to decreased MTHFR activity in the kidney (Poirier *et al.* 2001).

The importance of the kidney in homocysteine metabolism is further explained in diabetics who are *not* suffering from nephropathy. The opposite effect (i.e., enhanced homocysteine metabolism) commonly occurs in these diabetics, and homocysteine levels are reduced when compared with non-diabetic subjects (Cronin *et al.* 1998). It has been postulated that the enhanced metabolism of plasma homocysteine in these diabetics is due to

renal hyperfusion and increased homocysteine catabolism in the renal tubular epithelium (Robillon *et al.* 1994).

Growing evidence suggests that the hormonal up-regulation of the transsulfuration pathway by counter-regulatory hormones (i.e., glucagon and glucocorticoids) also plays a major role in reducing circulating homocysteine levels during diabetes (Jacobs *et al.* Ratnam *et al.* 2002). Variations of circulating insulin levels as well as surging counter-regulatory hormone concentrations in the plasma are common complications associated with uncontrolled diabetes. During uncontrolled diabetes, counter-regulatory hormones are released into the bloodstream as a result of insufficient glucose uptake and cellular utilization caused by either poor insulin production by the pancreas or insulin insensitivity. The ability of counter-regulatory hormones to produce a catabolic state contributes to the complications of diabetes by causing substrates, such as glucose and fatty acids, to be released into the bloodstream in an effort to compensate for insufficient cellular glucose utilization. Recent studies have demonstrated that increased counter-regulatory hormone levels also have the ability to up-regulate homocysteine catabolism by increasing gene expression of key enzymes involved in the transsulfuration pathway (Jacobs *et al.* 1998; Jacobs *et al.* 2001; Ratnam *et al.* 2002). Jacobs *et al.* (2001) reported that glucagon administration to rats led to a 30% decrease in plasma homocysteine levels and a concomitant 75 and 29% increase in hepatic CβS and cystathionine γ-lyase activity, respectively. The increase in CβS activity in these animals was reflected by increased abundance of both CβS mRNA and protein, occurrences that were prevented by insulin treatment, indicating that modulation of CβS by glucagon occurs, at least in part, at the transcriptional level. In an expansion of the experiments with glucagon, Ratnam et al. (2002) demonstrated that glucocorticoid-treated H4IIE rat hepatoma cells and livers of streptozotocin-diabetic rats also exhibited an increase of CβS protein and mRNA abundance. Similar to the studies with glucagon-treated rats, induction of CβS protein and mRNA abundance in rats and hepatoma cells by glucocorticoids was prevented by insulin treatment. Moreover, treatment of Hep G2 human hepatoma cells with insulin also reduced CβS mRNA levels. Taken together, these findings clearly indicate that CβS expression can be modulated at the transcriptional level by hormonal factors that are central to glucose metabolism.

In addition to inhibiting the removal of homocysteine through the transsulfuration pathway, insulin appears to have a similar effect on the remethylation system. Dicker-Brown et al. (2001) demonstrated in Hep G2 cells that insulin inhibited both CβS and MTHFR activity in a similar fashion, making it apparent that insulin inhibits the two major pathways of homocysteine metabolism. Therefore, it is possible that diabetics with renal failure who are on insulin therapy may be at an increased risk for extreme cases of hyperhomocysteinemia.

Carcinogenesis

Hypomethylated DNA

An adequate dietary supply of folate and methyl groups has long been known to be essential for the prevention of cancer (Newberne and Rogers 1986). Diets that are deficient in methyl groups have been shown to lead to the spontaneous development of hepatocarcinogenesis in rats (Salmon and Copeland 1954; Ghoshal and Farber 1984). Although it is not totally clear how methyl deficiency induces carcinogenesis, several studies have discovered a strong correlation between impaired methylation of DNA and increased cancer incidence in methyl-deficient animals (Wainfan *et al.* 1989; Bhave *et al.* 1988; Wainfan and Poirier 1992). The methylation of DNA is believed to have a significant role in the prevention of neoplastic development through its ability to control gene expression (Jones and Takai 2001) and stabilize chromosomes (Baylin, *et al.* 1998). Bhave et al. (1988) examined the importance of DNA methylation in the prevention of hepatocarcinogenesis and discovered an increased incidence of hypomethylated oncogenes in methyl deficient rats. Oncogenes represent regions of DNA where increased gene expression is associated with uncontrolled cell replication and tumor development, and hypomethylation of these regions has been positively correlated with their expression in rats (Wainfan and Poirier 1992).

SAH and DNA hypomethylation

Hypomethylated DNA due to a deficiency of activated methyl groups (i.e., SAM) has been postulated to be partially responsible for tumor formation in liver tissue (Pascale *et al.*

1992); however, recent evidence argues that the intracellular concentration of SAH may be the most sensitive biomarker for cellular methylation status (Caudill *et al.* 2001). It is known that a deficiency of methyl groups results in a decrease of hepatic SAM concentrations and the SAM/SAH ratio, and a concomitant rise in hepatic SAH levels (Shivapurkar and Poirier 1983). Because SAH inhibits most methyl transfer reactions (Kerr 1972), increased SAH levels have obvious implications for the inhibition of DNA methylation. In fact, it has recently been reported that CβS heterozygous mice with elevated hepatic SAH concentrations exhibit global DNA hypomethylation in the liver (Caudill *et al.* 2001). The conversion of homocysteine back to SAH is kinetically favored, therefore; inhibition of DNA methylation could be an indirect consequence of increased homocysteine concentrations. Hypomethylated DNA has also been discovered in folate-deficient rats (Balaghi and Wagner 1993), animals that may have experienced increased homocysteine levels due to decreased folate-dependent remethylation, a common occurrence during folate deficiency (Kang 1987). Hence, increased homocysteine levels and the simultaneous decrease of *de novo* methionine synthesis due to folate deficiency may inhibit DNA methylation by 1) decreasing the supply of available methyl groups for the methylation of DNA and/or by 2) increasing SAH levels.

DNA strand breaks

It has been hypothesized that DNA strand breaks, which are known to occur as a result of genomic instability (Piyathilake and Johanning 2002), lead to the induction of carcinogenesis during methyl group- and/or folate deficiency (Blount *et al.* 1997). Evidence suggests that this occurrence may be related to structural changes of DNA resulting from a dUTP substitution for dTTP during DNA synthesis (Duthie *et al.* 2002; Kim *et al.* 1997; Blount *et al.* 1997) or from cytosine deamination to dUTP in existing DNA (Lindahl 1993). As described earlier, the conversion of uridylate to thymidylate requires the donation of a methylene group from the folate derivative 5,10-methylene-THF. Thus, a deficiency of this folate coenzyme may cause imbalances in the DNA precursor pool and lead to an increased incidence of dUTP incorporation into DNA and subsequent strand breakage (Blount *et al.* 1997).

The regions of DNA where strand breaks occur appears to be a significant factor that determines whether or not they contribute to neoplastic development. For instance, it is known that strand breaks occurring at the region of DNA encoding the *p53* tumor suppressor gene readily occur during folate deficiency (Kim *et al.* 1997). Strand breaks at the *p53* gene have been linked to neoplastic development in the colon and esophagus (Hollstein *et al.* 1991). Moreover, strand breaks and uracil misincorporation into the *p53* gene of hepatic DNA have been shown to be prevalent in folate/methyl-deficient rats exhibiting preneoplastic hepatic lesions (Pogribny *et al.* 1997).

Hepatocarcinogenesis and methyl group deficiency

The liver possesses high levels of enzymes involved in one-carbon metabolism (Finkelstein 1990). Therefore, it seems likely that if certain cancers were caused by a perturbation of one-carbon metabolism, they would be present in the liver. Aberrant one-carbon metabolism due to malnutrition may be a major contributor to hepatocarcinogenesis in humans (Giovannucci *et al.* 1993). Additionally, epidemiological and clinical evidence suggests that the consumption of foods contaminated with carcinogens, such as mycotoxins, some of which are known to perturb one-carbon metabolism (Ueno *et al.* 1997), leads to the high prevalence of hepatocarcinogenesis in regions where food-borne fungi are more prevalent (Ueno *et al.* 1997). Thus, examining the relationship between certain carcinogens and one-carbon metabolism has been a useful tool in determining the role of methyl groups in preventing hepatocarcinogenesis. Newberne *et al.* (1990) demonstrated that supplemental methionine and choline reduced the incidence of liver cancers in mice given a carcinogenic dose of aflatoxin B_1, a mycotoxin commonly found in contaminated grain. Similar results were found in studies where rats were exposed to diethylnitrosamine (DENA), a cancer-initiating agent (Pascale *et al.* 1992; Porta *et al.* 1985). In addition, Hoover et al. (1984) discovered that DENA-induced hepatocarcinogenesis was exacerbated in methyl-deficient rats. Taken together, these findings suggest supplemental methyl groups and/or folate could be useful in reducing the incidence of diet-related cancers.

Neurological Disorders

SAM is utilized in the brain for the synthesis of neurotransmitters such as dopamine, serotonin, and norepinephrine. Recent evidence suggests that the role of SAM during the synthesis of neurotransmitters is important for the prevention of symptoms related to depression (Mishoulon and Fava 2002). In fact, evidence collected from trial studies suggests that the efficacy of oral SAM administration in treating depression is comparable to that of tricyclic antidepressants (Bressa 1994). Based in part on such findings, SAM is now marketed as an alternative to prescription anti-depressant medications and is available as an over-the-counter dietary supplement (Mischoulon and Fava 2002).

Folate (Bottiglieri *et al.* 1992; Reynolds *et al.* 1970) and vitamin B_{12} deficiency (Botez *et al.* 1982) have also been linked to depression; however, the mechanistic basis of these findings is yet to be determined. Since vitamin B_{12} and folate are required for the *de novo* synthesis of SAM, it is possible that depression associated with a deficiency of these nutrients is due, at least in part, to a decline in the synthesis of neurotransmitters.

In addition to depression, a number of other neurological disorders have been associated with folate deficiency including, polyneuropathy (Botez *et al.* 1978; Grant *et al.* 1965), Alzheimer's disease, and Parkinson's disease (Mattson and Shea 2003). These disorders are a currently a topic of intense research. Evidence suggests that a disruption of homocysteine metabolism due to folate deficiency may contribute to the progression of such diseases by leading to decreased function and atrophy of neurotransmitter-producing neurons (Mattson 2003).

Megaloblastic Anemia and the "Methyl Trap" Hypothesis

The relationship between folate and vitamin B_{12} is critical during the MS-dependent remethylation of homocysteine by 5-methyl-THF, the only reaction known to require both folate and vitamin B_{12} (Hatch *et al.* 1961). Because the conversion of 5,10-methylene-THF to 5-methyl-THF by MTHFR is physiologically irreversible (Kutzbach and Stokstad 1971), it is therefore essential that an adequate supply of vitamin B_{12} is available to ensure that normal functioning of MS is maintained and to avoid what is known as the "methyl trap". During vitamin B_{12} deficiency, a "functional folate deficiency" occurs where the supply of folate

becomes trapped as 5-methyl-THF (Shane and Stokstad 1985), and tissue concentrations of other folate coenzymes, such as those used for the synthesis of DNA precursors, declines. A pathological condition that commonly results from a deficiency of either folate or vitamin B_{12} is megaloblastic anemia. Megaloblastic anemia is characterized by the appearance of large undifferentiated erythroblasts in circulation and a reduction in the number of circulating erythrocytes, which are believed to be due to decreased DNA synthesis and poor cell division (Hoffbrand *et al.* 1976). Depending on their respective nutrient deficiency, individuals suffering from megaloblastic anemia should be provided with supplemental folate or vitamin B_{12}. It has been hypothesized that supplementary methyl groups may also help alleviate symptoms of megaloblastic anemia through their ability to spare folate coenzymes from being trapped as 5-methyl-THF through SAM-mediated inhibition of MTHFR (Shane and Stokstad 1985).

Impaired Pancreatic Function

Folate deficiency has been shown to inhibit pancreatic function (Balaghi and Wagner 1992; 1995; Elseweidy and Singh 1984), which appears to be due to the inhibition of SAM-dependent methylation of proteins that are responsible for the secretion of enzymes such as amylase (Capdevila *et al.* 1997). These findings obviously have serious implications for the disruption of normal digestion in folate-deficient individuals, alcoholics, and those using drugs that produce side effects that alter folate and methyl group metabolism.

Drugs and Toxins

As previously mentioned, pharmacological agents and environmental toxins can have a major impact on one-carbon metabolism,. Summarized below are some important findings regarding the effect of various toxicants on methyl group and/or folate-dependent, one-carbon metabolism.

Retinoids

Vitamin A compounds, collectively referred to as retinoids, are utilized as therapeutic agents in the treatment of a number of conditions, including cystic acne and leukemia.

However, commonly prescribed in medications containing pharmacological doses of retinoid compounds have been shown to produce side effects that may be related to a disruption of one-carbon metabolism (Schulpis *et al.* 2001). Retinoid therapy is contraindicated with respect to pregnancy because of its association with an increased incidence of neural tube defects and other congenital malformations (Lammer *et al.* 1985), conditions associated with abnormal one-carbon metabolism. Studies that examined the ability of retinoids to alter the distribution of folate coenzymes in the one-carbon pool showed that the activity of hepatic MTHFR was inhibited in rats treated with pharmacological doses (1,000 IU/g diet) of retinol (vitamin A), which in turn resulted in decreased 5-methyl-THF synthesis (Fell and Steele 1986). Furthermore, the oxidation rate of formate to CO_2 was increased in these animals, indicating that retinol administration increased the catabolism of one-carbon units in the folate-dependent one-carbon pool. Retinol-treated rats also exhibited a reduction of hepatic SAM levels and the SAM/SAH ratio (Fell and Steele 1986). Similarly, Peng and Evenson (1982) found that retinol treatment increased the oxidation rate of the methyl group from methionine to CO_2 in rats.

An inhibition of SAM-dependent transmethylation reactions has been determined to be a consequence of retinoid-mediated depletion of SAM and a decreased SAM/SAH ratio (Fell and Steele 1987). In an extension of their earlier work (Fell and Steele 1986), Fell and Steele (1987) demonstrated that decreased hepatic levels of SAM due to retinol administration resulted in a reduction of the SAM-dependent conversion of phosphatidyl ethanolamine to phosphatidyl choline in rats, indicating that high intakes of retinoid compounds do in fact have the potential to impair SAM-dependent transmethylation reactions. Moreover, treatment with 13-*cis*-retinoic acid [isotretinoin (CRA)], a synthetic retinoid commonly used for treatment of cystic acne, reduced the hepatic concentration of SAM and the SAM/SAH ratio in rats (Schalinske and Steele 1991). Such findings linking retinoids to perturbations of one carbon metabolism clearly have importance for individuals receiving therapeutic retinoids for treatment of medical conditions. Interestingly, depression has been mentioned as a potential side effect of retinoid-containing acne medication (Enders and Enders 2003), which may be due, at least in part, to a decrease in the SAM-dependent synthesis of neurotransmitters. Other side effects of retinoid administration associated with

altered one-carbon metabolism include elevated plasma homocysteine levels (Schulpis *et al.* 2001) and hepatic steatosis (Schalinske and Steele 1993), a condition that may be related to the decreased SAM-dependent synthesis of phosphatidyl choline, a phospholipid utilized for membrane synthesis of apolipoproteins and excretion of hepatic lipid via LDL.

Retinoid compounds typically exert their actions at the transcriptional level via retinoid response elements; however, the mechanism through which retinoids alter the methyl group supply remains unclear. There is currently no evidence that indicates that retinoid response elements exist on the promoter regions of genes that encode key enzymes involved in methyl group metabolism. This suggests that the action of retinoids on the methyl group supply may occur, at least in part, at the translational and/or posttranslational level. Interestingly, increased catabolism of methionine caused by retinol treatment was prevented in adrenalectomized rats (Peng and Evenson 1982), indicating that the secretion of hormones, such as glucocorticoids from the adrenal glands, may play a mediating role between retinoids and the increased catabolism of methyl groups. Glucocorticoids and retinoids (Singh *et al.* 1976) are both known to enhance the gene expression of gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK) (Singh *et al.* 1976; Pan *et al.* 1990; Shin and McGrane 1997), the rate-limiting enzyme of gluconeogenesis. Because methionine is catabolized to pyruvate during gluconeogenesis, it is possible that methionine catabolism is increased during retinoid treatment through the induction of a gluconeogenic state.

Ethanol

Folate deficiency is a common clinical sign of alcoholism (Halsted *et al.* 2002). A number of animal studies have demonstrated that in addition to inadequate dietary intake of folate, folate deficiency may be caused by increased excretion as well as elevated catabolism of folate coenzymes in alcoholics. Studies examining the effect of chronic ethanol exposure on folate metabolism demonstrated that ethanol 1) increased the catabolism of folate in the kidney (Eisenga *et al.* 1989), 2) increased urinary folate excretion in a dose- and time-dependent manner (McMartin *et al.* 1986; 1986) through the inhibition of folate reabsorption by the renal tubules (McMartin *et al.* 1989), and 3) inhibited intestinal absorption of folate (Halsted *et al.* 1967; Romero *et al.* 1981).

In addition to the disruption of one-carbon metabolism through its effect on folate, exposure to ethanol also appears to enhance the catabolism of methionine (Trimble *et al.* 1993). Trimble et al. (1993) reported that rats fed 42% of their calories as ethanol exhibited symptoms associated with an increased oxidation rate of methyl groups. When compared to control rats, ethanol-treated rats exhibited a 2.9-fold increase in the oxidation of the methyl group from methionine in the liver, a ~4-fold increased turnover of methyl groups from choline, and a 69% reduction of hepatic SAM levels. Likewise, Halsted et al. (2002) reported that ethanol-fed micropigs exhibited a decreased SAM/SAH ratio, an increased prevalence of DNA strand breaks, and elevated plasma homocysteine levels even when they were provided with supplemental folate.

Clearly, the effect of chronic ethanol exposure on increased catabolism of methyl groups has the potential for producing pathological consequences; however, the mechanistic relationship between ethanol and methyl group metabolism is not well understood. Trimble et al. (1993) suggested that ethanol-mediated catabolism of methyl groups occurs in order to provide the carbon skeleton of methionine for its conversion in the transsulfuration pathway to glutathione, a natural antioxidant that appears to be depleted as a result of ethanol-induced oxidative damage to the liver.

Anticonvulsants

Long-term use of anticonvulsants, which has been associated folate deficiency (Dansky *et al.* 1987) and aberrant one-carbon metabolism (Alonso-Aperte *et al.* 1999) has also been associated with megaloblastic anemia (Hawkins and Meynell 1958) and congenital malformations during pregnancy (Dansky *et al.* 1987). Anticonvulsants, including the drugs phenytoin, phenobarbitone, phenobarbital, primidone, carbamazepine, and valproate, are thought to exert their actions on folate-dependent one-carbon metabolism by interfering with intestinal folate absorption (Meynell 1966; Hoffbrand and Necheles 1968) and/or inducing hepatic enzymes involved in the degradation of folate (Labadarios *et al.* 1978; Kishi *et al.* 1997). Valproate in particular, has been shown to be a potent disrupter of methyl group metabolism, which appears to be due, at least in part, to its ability to alter the methyl group supply (Alonso-Aperte *et al.* 1999; Carl 1986).

Oral contraceptives

The use of oral contraceptives has been associated with reduced serum folate levels (Pietarinen *et al*, 1977) and megaloblastic anemia (Baroné *et al*, 1979), conditions that are consistent with both increased plasma clearance of folate and elevated urinary folate excretion (Shojania *et al.* 1975). Sütterlin et al. (2003) recently reported that oral contraceptives also lower vitamin B_{12} levels, which may put individuals using these medications at even more of an increased risk for developing megaloblastic anemia and other conditions associated with impaired folate metabolism.

Fumonisins

Fumonisins are mycotoxins found primarily in contaminated corn that have been shown to produce alterations of folate metabolism (Carratú *et al*, 2003). Fumonisins are thought to disrupt folate metabolism by blocking folate transport into the cell by inhibiting the synthesis of sphingolipids (Carratú *et al*, 2003), molecules required for cell membrane synthesis and cell signaling. Exposure to fumonisin B_1 has been implicated in a number of pathologies commonly observed during folate deficiency, such as neural tube defects (Hibbard 1993) and liver cancer (Ueno *et al.* 1997). Therefore, it is not surprising that folic acid supplementation has been shown to be protective against these fumonisin-related pathologies (Sadler *et al.* 2002).

Glycine *N*-methyltransferase (GNMT)

Glycine *N*-methyltransferase (GNMT) is a cytosolic protein found primarily in the liver, pancreas, and kidney (Yeo and Wagner 1994) that functions to regulate the methyl group supply by disposing of excess methyl groups and controlling of the SAM/SAH ratio. GNMT is an especially abundant protein, constituting 1-3% of total cytosolic protein in the liver (Heady and Kerr 1975), suggesting that its role in regulating the methyl group supply is of major biological significance. The physiological importance of GNMT has been illustrated in clinical and metabolic studies (Mudd *et al.* 2001; Luka *et al.* 2002), where it was discovered that mild liver disease, persistent hypermethioninaemia, and elevated SAM

concentrations were present in siblings carrying a rare mutation that causes GNMT deficiency.

The importance of GNMT in one-carbon metabolism is a relatively new research area. Hence, it is currently unknown how GNMT deficiency or perturbations of GNMT affect other SAM-dependent transmethylation reactions. It is also unclear how physiological factors can alter GNMT expression. It is understood that enzymatically active GNMT (132 kDa), a tetrameric protein comprised of 4 identical subunits (Ogawa and Fujioka 1982), can be modulated at the posttranslational level through the phosphorylation of each GNMT subunit by cAMP-dependent protein kinases, which serves to increase the enzymatic activity of the protein (Wagner *et al.* 1989). Additionally, GNMT's role as a folate-binding protein allows it to be allosterically inhibited by 5-methyl-THF, as discussed earlier. It is unclear, however, whether GNMT activity is modulated at the transcriptional or translational levels. GNMT activity is elevated during diabetes (Xue and Snoswell 1985), a condition that is associated with increased levels of glucagon and glucocorticoids, counterregulatory hormones that induce gluconeogenesis. As previously described, these hormones have been shown to increase the expression of other key enzymes in methyl group metabolism at the transcriptional level. Because the presence of GNMT is essentially limited to gluconeogenic tissues (Yeo and Wagner 1994), it is possible that counterregulatory hormones also play a role in regulating GNMT gene expression.

In our laboratory, I have examined the role of GNMT in mediating the perturbation of methyl group metabolism by retinoid compounds and have illustrated for the first time that the modulation of GNMT is regulated, at least in part, at the transcriptional/translational levels by vitamin A compounds and glucocorticoids. I have also reported that retinoid-mediated GNMT induction leads to impaired SAM-dependent transmethylation reactions (i.e. DNA methylation). Furthermore, I have shown that homocysteine metabolism is altered by retinoids and glucocorticoids. In the following chapters, original research articles that have been published in peer-reviewed journals discuss our observations in greater detail.

RETINOID COMPOUNDS ACTIVATE AND INDUCE HEPATIC GLYCINE *N*-METHYLTRANSFERASE IN RATS [1,2]

A paper published in the Journal of Nutrition[3]

Matthew J. Rowling[4] and Kevin L. Schalinske [4,5]

Abstract

Glycine *N*-methyltransferase (GNMT) functions to regulate S-adenosylmethionine (SAM) levels and the ratio of SAM/ S-adenosylhomocysteine (SAH). SAM is a universal methyl group donor and up-regulation of GNMT may lead to wastage of methyl groups required for transmethylation reactions. Previously, we demonstrated that dietary treatment of rats with 13-*cis*-retinoic acid (CRA) decreased the hepatic concentration of SAM and the SAM/SAH ratio. Here, we examined the ability of CRA, as well as all-*trans*-retinoic acid (ATRA), to regulate hepatic GNMT as a potential basis for our earlier observations. Rats were fed either a control (10% casein + 0.3% L-methionine) diet or a control diet supplemented with L-methionine (MS, 10 g/kg diet). Animals from each group were orally given either ATRA, CRA (both @ 30 μmol/kg body weight), or vehicle daily for 7 d. For control rats, administration of both CRA and ATRA elevated the hepatic GNMT activity 49 and 34%, respectively, compared to the control group. Similar results were exhibited by rats fed the MS diet. Moreover, the retinoid-induced elevations in enzymatic activity were reflected in the abundance of GNMT protein. To our knowledge, this is the first report of a nutritional compound that induces GNMT activity at the transcriptional and/or translational level.

Key Words: retinoic acid • S-adenosylmethionine • transmethylation • glycine N-methyltransferase • rats

Footnotes

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[2]A preliminary report of this manuscript was presented at Experimental Biology 2001, Orlando, FL [Rowling, M.J. & Schalinske, K.L. (2001) Glycine N-methyltransferase is up-regulated by all-*trans*- and 13-*cis*-retinoic acid in rats. FASEB J. 15: A602 (abs.)].

[3]Reprinted with permission of J. Nutr., 2001, 131, 1914-1917.

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[6]Abbreviations used: ATRA, all-*trans*-retinoic acid; BSA, bovine serum albumin; CRA, 13-*cis*-retinoic acid; GAR-HRP, goat anti-rabbit horseradish peroxidase; GNMT, glycine N-methyltransferase; MS, methionine-supplemented; MTHFR, 5,10-methylene-tetrahydrofolate reductase; PMSF, phenylmethylsulfonylflouride; PEPCK, phosphoenolpyruvate carboxykinase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TTBS, tween-tris-buffered saline.

Introduction

Methyl groups supplied from dietary methionine or choline are required for the synthesis of S-adenosylmethionine (SAM[6]), a vital compound that is required as a substrate in SAM-dependent transmethylation reactions (1,2) (**Figure 1**). SAM-dependent transmethylation reactions include the posttranslational modification of nucleic acids and proteins (3), and the synthesis of important molecules such as neurotransmitters, phospholipids, and nucleic acids (1,2). Therefore, a lack of dietary methyl groups from the diet or the folate-dependent one-carbon pool can result in pathological conditions. A deficiency of dietary methyl groups leads to hepatocarcinogenesis (4,5), a condition that may be due to hypomethylated DNA (6). Additionally, folate deficiency down-regulates SAM-dependent transmethylation reactions (7) and has been implicated in a number of related pathologies (8).

Glycine *N*-methyltransferase (GNMT) is a key cytosolic protein that controls the intracellular supply of available methyl groups for SAM-dependent transmethylation reactions (9). GNMT is known to dispose of excess SAM as sarcosine, a product that has no known metabolic function, thus controlling the SAM/S-adenosylhomocysteine (SAH) ratio, an index of transmethylation potential due to the ability of SAH to inhibit the majority of methyltransferases (9). The activity of GNMT is controlled by a number of regulatory mechanisms that are dependent on methyl group supply. For instance, when an abundant intracellular supply of SAM exists, SAM serves to allosterically inhibit 5,10-methylenetetrahydrofolate reductase (MTHFR) (10,11), an enzyme that catalyzes the irreversible conversion of 5,10-methylene-tetrahydrofolate (5-MTHFR) to 5-methyl-tetrahydrofolate (5-methyl-THF). Because GNMT serves as a folate-binding protein that is inhibited by 5-methyl-THF, decreased synthesis of 5-methyl-THF facilitates the disposal of excess SAM by GNMT. In contrast, a lack of SAM results in the increased synthesis of 5-methyl-THF, which in turn inhibits GNMT (12) and thus allows SAM to serve as a substrate in important transmethylation reactions. Collectively, these regulatory mechanisms serve to optimize the methyl group supply; therefore, factors that can disturb the function of GNMT may have pathological consequences with respect to folate and methyl group metabolism.

Previous work in our laboratory has demonstrated that the administration of 13-*cis*-retinoic acid (CRA), a retinoid compound commonly used therapeutically for the treatment of cystic acne, alters methyl group metabolism by reducing hepatic SAM concentrations and the SAM/SAH in rats treated with CRA (13). In an effort to understand the role of retinoids in the increased catabolism of methyl groups, we focused on the activation of GNMT as a potential mechanism. Additionally, we examined the ability all-*trans*-retinoic acid (ATRA), the biologically active form of vitamin A commonly prescribed for treatment of certain types of leukemia, to perturb methyl group metabolism.

Materials and Methods

Chemicals and Reagents.

Reagents were obtained from the following: *S*-adenosyl-L-[*methyl*-^{3}H]methionine, New England Nuclear (Boston, MA); phenylmethylsulfonylflouride (PMSF), Calbiochem (La Jolla, CA); goat anti-rabbit IgG horseradish peroxidase (GAR-HRP), Southern Biotechnology (Birmingham, AL); ECL™ Western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ); and *S*-adenosyl-L-methionine, Sigma Chemical Co. (St. Louis, MO). GNMT antibody was kindly provided by Conrad Wagner, Vanderbilt University. ATRA and CRA were provided courtesy of Hoffmann-LaRoche (Nutley, NJ). All other chemicals were of analytical grade.

Animals and Diets.

All animal experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. Male Sprague Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats were housed in suspended wire-mesh cages in a room with a 12-h light: dark cycle and given free access to food and water. The composition of the control diet was the same as previously described (13). The methionine-supplemented (MS) diet contained additional L-methionine (10 g/kg diet) at the expense of glucose monohydrate.

Following an 11-d acclimation period during which rats were adapted to both the control diet and oral administration of corn oil, they were divided into six treatment groups consisting of five rats per group. Rats were fed one of the two diets (control or MS) and

were orally administered either vehicle (corn oil, 1 μL/g body weight), vehicle containing CRA, or vehicle containing ATRA daily. Both retinoids were administered at a level of 30 μmol/kg body weight. Following the 7-d treatment period, rats were anesthetized and liver samples were rapidly removed for analysis.

Measurement of GNMT activity.

The enzymatic activity of GNMT was assayed as described by Cook and Wagner (14) with minor modifications. Portions of liver were homogenized in 3 volumes of ice-cold phosphate buffered (10 mmol/L, pH 7.0) sucrose (0.25 mol/L) containing 1 mmol/L EDTA, 1 mmol/L sodium azide, and 0.1 mmol/L PMSF. Following centrifugation at 20,000 × *g* for 30 min, the resulting supernatant was removed and 2-mercaptoethanol was added to a final concentration of 10 mmol/L. The assay mixture consisted of 0.1 mol/L Tris buffer (pH 9.0), 5 mmol/L dithiothreitol, 1 mmol/L glycine, and 1 mmol/L *S*-adenosyl-L-[methyl-^{3}H]methionine (4.77 x 10^6 Bq/mmol). The reaction was initiated upon addition of 250 μg of sample protein. The assay was linear with respect to time and protein concentration. For the determination of total soluble protein in the tissue extract, a commercial kit (Coomassie Plus, Pierce, Rockford, IL) based on the method of Bradford (15) was used with bovine serum albumin (BSA) as a standard.

Measurement of GNMT Protein.

For the determination of the abundance of GNMT protein, immunoblotting with chemiluminescence detection was employed. SDS-polyacrylamide gel electrophoresis was performed using a 10-20% gradient gel and 75 μg sample protein per lane. Following separation, the proteins were electrophoretically transferred to nitrocellulose and the membrane was incubated at room temperature in a blocking solution containing nonfat dry milk (5 g/100 mL) in TTBS buffer consisting of 20 mmol/L Tris, (pH 7.5) and 500 μl/L Tween 20. Affinity-purified polyclonal GNMT antibody in BSA (1 g/100 mL) - TTBS (1:1000) was added and the blot was incubated at 4°C overnight. The blot was incubated for 1 h at room temperature with GAR-HRP in TTBS (1:5000), followed by a 1-min incubation

in Western blot chemiluminescent detection reagents prior to multiple exposures to autoradiography film. Densitometric analysis was performed using the NIH Image software.

Statistical Analysis.

The means of each treatment group were subjected to a two-way ANOVA. When the ANOVA proved to be significant ($P < 0.05$), the means were compared using the Fisher LSD procedure (16).

Results

Retinoid treatment did not alter rat growth rates.

Body weights were measured during both the acclimation phase (data not shown) and retinoid-treatment period of the study (**Fig. 2**). All rat groups exhibited similar growth patterns regardless of treatment and no significant differences were detected in either the initial (81 ± 2 g) or final (181 ± 3 g) body weights. As indicated in **Fig. 2**, no significant differences were observed in the cumulative weight gain across the treatment groups, indicating that neither the MS diet nor the retinoids were overtly toxic to the animals. We have shown previously that administration of CRA does have the ability to induce hepatic steatosis in rats fed a similar diet (13). This was also found to be the case in the present study for both CRA and ATRA (data not shown).

Retinoids increased the enzymatic activity of hepatic GNMT.

As shown in **Fig. 3**, administration of CRA and ATRA consistently elevated the enzymatic activity of hepatic GNMT. For rats receiving the control diet, CRA administration increased GNMT activity 49%. ATRA also increased enzyme activity (34%), however, this change was not statistically significant (P=0.081). In contrast, both CRA and ATRA significantly induced GNMT activity 41 and 45%, respectively, in rats receiving the MS diet. For the MS diet, the addition of L-methionine alone (10 g/kg diet) was without significant effect. Previous studies have demonstrated that induction of GNMT activity by dietary methionine requires closer to 20 g methionine/kg diet (17).

Both CRA and ATRA induce GNMT protein abundance.

Using immunoblotting with a GNMT antibody, we determined whether the retinoid-induced increase in GNMT activity was due to changes in the production of the enzyme (Fig. 4). The bar graph reflects the mean result from all of the experimental animals, whereas the immunoblot shown above is a representative example with the relative fold induction shown for each lane. Both CRA and ATRA markedly induced GNMT protein abundance in control rats and animals receiving the MS diet. The induction of GNMT protein by retinoids across treatment groups ranged from approximately 8- to 16-fold. Similar to the enzyme activity data, the MS diet alone did not significantly alter GNMT protein levels based on mean values. However, it does appear to have some effect as can be seen in the representative blot (3.1-fold induction). There was no difference in actin protein abundance across the treatment groups. Interestingly, in both the bar graph and representative immunoblot, the effect of the MS diet and retinoids on GNMT protein induction appears to be additive. As suggested by others (17), we have directly confirmed that the addition of graded levels of L-methionine to the diet and subsequent induction of GNMT enzyme activity is reflected in an increase in GNMT protein abundance (Rowling and Schalinske, unpublished data).

Discussion

GNMT serves a crucial role in methyl group- and folate-dependent one-carbon metabolism by regulating the methyl group supply and the SAM/SAH ratio (9). Hence, factors that can disrupt the function of this enzyme may produce deleterious physiological consequences. We have demonstrated that the vitamin A derivatives have the ability to activate GNMT, which was reflected by the abundance of the protein. Thus, we have demonstrated for the first time, at least to our knowledge, that GNMT can be modulated at the transcriptional/translational level. Furthermore, we have clearly shown that ATRA, the active form of vitamin A, has a similar ability to CRA in altering methyl group metabolism.

Our observations with CRA and ATRA have clear implications for those individuals receiving therapeutic retinoids for medical conditions such as skin disorders and leukemia. Although we administered these compounds to our animals in pharmacological doses (30 μmol/kg) that are far above the clinical dosage normally prescribed to humans receiving retinoid therapy (2-6 μmol/kg), we have discovered that doses as low as 1 μmol/kg have a

similar ability to activate and induce GNMT protein in rats (Rowling and Schalinske, unpublished observations). Additionally, rats are known to be less sensitive to CRA than humans due to their increased ability to eliminate the drug (18), indicating that doses used in our studies may be more effective in perturbing methyl group metabolism in humans. Thus, humans receiving retinoids for therapeutic use may be at an increased risk for developing complications related to methyl group metabolism even when given low doses of retinoid compounds.

A number of factors have been shown to increase the activity of GNMT at the posttranslational level, including folate deficiency (7), ethanol administration (19), and phosphorylation of the protein by cyclic adenosine 5'-monophosphate (cAMP)-dependent kinases (20). The present study raises a number of questions concerning the regulation of GNMT protein synthesis by retinoids. Because there is no known retinoid response element in the promoter region of the GNMT gene, it is possible that the apparent induction of GNMT in our studies was a result of either increased translation or stabilization of the protein. It is known that in addition to its enzymatic function as a tetramer, the dimeric form of GNMT serves as a polycyclic aromatic hydrocarbon binding protein (21). Hence, the possibility exists that the activation and induction of GNMT could be achieved by separate mechanisms.

Our laboratory is currently investigating the possible role of gluconeogenesis in mediating the induction of GNMT by retinoids because GNMT is found primarily in gluconeogenic tissues, which include the liver, kidney, and pancreas (22). Retinoids have been shown to exacerbate symptoms of diabetes (23), a condition that is associated with elevations in circulating counter regulatory hormones (i.e. glucagon and glucocorticoids) and increased gluconeogenesis. Moreover, Xue and Snoswell (24) reported that GNMT activity was elevated 65-fold in alloxan-diabetic sheep. Interestingly, glucagon and glucocorticoids also play an integral role in the induction of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis, which also possesses a retinoid response element (25,26). Likewise, GNMT activity is up regulated as a result of phosphorylation by cAMP-dependent kinases (20), which have also been shown to increase PEPCK gene

expression (25). Therefore, it is possible that a number of signals may play a mediating role in retinoid-induced GNMT expression.

Undoubtedly, a number of potential mechanisms exist that may be responsible for the induction of GNMT by retinoids, and the action of retinoid compounds on methyl group metabolism may be mediated by other factors. We are currently exploring these research areas and the effect of GNMT up-regulation on other aspects of folate and methyl group metabolism. These research topics will be important for developing future dietary recommendations and the evaluation of other retinoid compounds for clinical use.

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Literature Cited

1. Cantoni, G. L. (1982) *S*-adenosyl amino acids thirty years later: 1951-1981. In: *The Biochemistry of S-adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., and Creveling, C. R., eds.) pp. 3-10. Macmillan Press, London, England.

2. Cantoni, G. L. and Chiang, P. K. (1980) The role of *S*-adenosylhomocysteine hydrolase in the control of biological methylation reactions. In: *Natural Sulfur Compounds* (Cavallini, D., Gaull, G. E., and Zappia, V., eds.) pp. 67-80. Plenum Press, New York, NY.

3. Benjamins, J. A., Morell, P., Hartman, B. K., and Agrawal, H. C. (1984) Central nervous system myelin. In: *Handbook of Neurochemistry,* 2nd edition (Lajtha, A., ed.) Plenum Press, New York, pp. 361-415.

4. Ghoshal, A. K. and Farber, E. (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis.* 5(10): 1367-1370.

5. Salmon, W. D. and Copeland, D. H. (1954) Liver carcinoma and related lesions in chronic choline deficiency. *Ann. NY. Acad. Sci.* 57: 664-677.

6. Balaghi, M. and Wagner, C. (1993) DNA methylation in folate deficiency: use of CpG methylase. *Biochem. Biophys. Res. Comm.* 193(3): 1184-1190.

7. Balaghi, M., Horne, D. W., and Wagner, C. (1993) Hepatic one-carbon metabolism ion early folate deficiency in rats. *Biochem. J.* 291: 145-149.

8. Pogribny, I. P., Basnakian, A. G., Miller, B. J., Lopatina, N. G., Poirier, L. A., and James, S. J. (1995) Breaks in genomic DNA and within the *p53* gene are associated with hypomethylation in livers of folate/methyl-deficient rats. *Cancer Res.* 55: 1894-1901.

9. Kerr, S. J. (1972) Competing methyltransferase systems. *J. Biol. Chem.* 247: 4248-4252.

10. Kutzbach, C. and Stokstad, E. L. R. (1967) Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by *S*-adenosylmethionine. *Biochim. Biophys. Acta.* 139: 217-220.

11. Kutzbach, C. and Stokstad, E. L. R. (1971) Mammalian methylenetetrahydrofolate reductase. Partial purification, properties, and inhibition by *S*-adenosylmethionine. *Biochim. Biophys. Acta.* 250: 459-477.

12. Wagner, C., Briggs, W. T., and Cook, R. B. (1985) Inhibition of glycine *N*-methyltransferase activity by folate derivatives: Implications for regulation of methyl group metabolism. *Biochem. Biophys. Res. Commun.* 127(3) 746-752.

13. Schalinske, K. L. and Steele, R. D. (1991) 13-*cis*-retinoic alters methionine metabolism in rats. *J. Nutr.* 121: 1714-1719.

14. Cook, R. J. and Wagner, C. (1984) Glycine *N*-methyltransferase is a folate binding protein of rat liver cytosol. *Proc. Natl. Acad. Sci. U.S.A.* 81: 3631-3634.

15. Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* 72: 248-254.

16. Snedecor, G. W. and Cochran, W. G. (1980) Statistical Methods, 7th ed. Iowa State University Press, Ames, IA.

17. Ogawa, H. and Fujioka, M. (1982) Induction of rat liver glycine methyltransferase by high methionine diet. *Biochem. Biophys. Res. Commun.* 108: 227-232.

18. Nau, H. (2001) Teratogenecity of isotretinoin revisited: species variation and the role of all-*trans*-retinoic acid. *J. Am. Acad. Dermatol.* 45(5): S189-187.

19. Trimble, K. C., Molloy, A. M., Scott, J. M., and Weir, D. G. (1993) The effect of ethanol on one-carbon metabolism: increased methionine catabolism and lipotrope methyl-group wastage. *Hepatology*: 18(4): 984-989.

20. Wagner, C., Decha-Unphai, W., and Corbin, J. (1989) Phosphorylation modulates the activity of glycine *N*-methyltransferase, a folate binding protein. *J. Biol. Chem.* 264(16): 9638-9642.

21. Raha, A., Wagner, C., MacDonald, R. G., and Bresnick, E. (1994) Rat liver cytosolic 4 S polycyclic aromatic hydrocarbon-binding protein is glycine *N*-methyltransferase. *J. Biol. Chem.* 269: 5750-5756.

22. Yeo, E-J. and Wagner, C. (1994) Tissue distribution of glycine *N*-methyltransferase, a major folate-binding protein of liver. *Proc. Natl. Acad. Sci. USA.* 91: 210-214.

23. Driscoll, H. K., Chertow, B. S., Jelic, T. M., Baltaro, R. J., Chandor, S. B., Walker, E. M., Dadgari, J. M., and Pofahl, A. B. (1996) Vitamin A status affects the development of diabetes and insulitis in BB rats. *Metabolism.* 45: 248-253.

24. Xue, G-P. and Snoswell, A. M. (1985) Disturbance of methyl group metabolism in alloxan-diabetic sheep. *Biochem. Int.* 10(6): 897-905.

25. Pan, C-J., Hoeppner, W., and Yang Chou, J. (1990) Induction of phosphoenolypyruvate carboxykinase gene expression by retinoic acid in an adult rat hepatocye line. *Biochemistry.* 29(49): 10883-10888.

26. Shin, D-J., McGrane, M. M. (1997) Vitamin A regulates genes involved in hepatic gluconeogenesis in mice: phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J. Nutr.* 127: 1274-1278.

FIGURE LEGENDS

FIGURE 1 Interrelationship between methyl group and folate metabolism. Hepatic S-adenosylmethionine (SAM), a methyl group donor in a number of transmethylation reactions, is produced from methionine, via the diet and from the remethylation of homocysteine in a B_{12}-dependent reaction that utilizes the folate-dependent one-carbon pool as a methyl group source (i.e., 5-methyl-tetrahydrofolate, 5-methyl-THF). S-adenosylhomocysteine (SAH), the product of SAM-dependent transmethylation reactions, is a potent inhibitor of most methyltransferases and thus the ratio of SAM/SAH is an index of transmethylation potential. SAM is also an allosteric inhibitor of 5,10-methylene-THF reductase (MTHFR), the enzyme that catalyzes the irreversible reduction of 5,10-methylene-THF to 5-methyl-THF. In turn, 5-methyl-THF is an inhibitor of glycine N-methyltransferase (GNMT), a key protein involved in the regulation of transmethylation by controlling the ratio of SAM/SAH. Both of these inhibitory relationships are indicated by the *dashed arrows*.

FIGURE 2 Administration of 13-*cis* -retinoic acid (CRA) and all-*trans*-retinoic acid (ATRA) to rats for 7 d did not alter their growth rates. Data are means (n=5) ± SEM and were compared across treatment groups (P=0.05) at each time point. Following an adaptation period, an equal number of rats in both the control and L-methionine-supplemented (MS, 10 g/kg diet) groups were treated with either vehicle (corn oil), CRA (30 μmol/kg body weight), or ATRA (30 μmol/kg body weight) daily. For all rats (n=30), mean (± SEM) body weights at treatment d=0 and d=7 were 146 ± 3 g and 181 ± 3 g, respectively. N.S. indicates not statistically significant.

FIGURE 3 Administration of 13-*cis*-retinoic acid (CRA) and all-*trans*-retinoic acid (ATRA) to rats increased the hepatic activity of glycine N-methyltransferase (GNMT) in both control and L-methionine-supplemented (MS, 10 g/kg diet) rats. Data are means (n=5) ± SEM. Bars denoted by different letters are significantly different (P<0.05). All assays were performed in triplicate.

FIGURE 4 Administration of 13-*cis*-retinoic acid (CRA) and all-*trans*-retinoic acid (ATRA) to rats increased the hepatic abundance of glycine N-methyltransferase (GNMT) in both control and L-methionine-supplemented (MS, 10 g/kg diet) rats. Data are means (n=5) ± SEM. Bars denoted by different letters are significantly different ($P<0.05$). Above the bar graph is a representative immunoblot with the relative fold-induction provided below each lane.

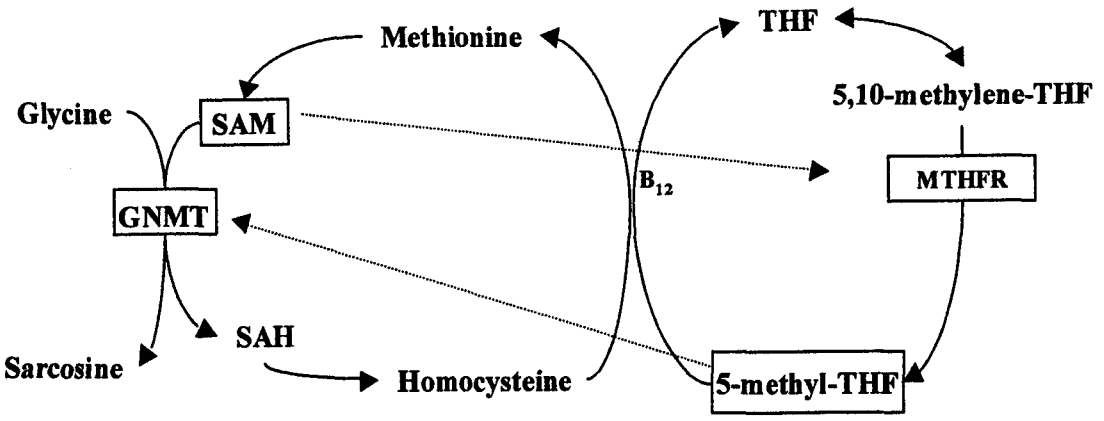
Methionine
THF
5,10-methylene-THF
Glycine
SAM
MTHFR
B_{12}
GNMT
SAH
Sarcosine
Homocysteine
5-methyl-THF

Figure 1

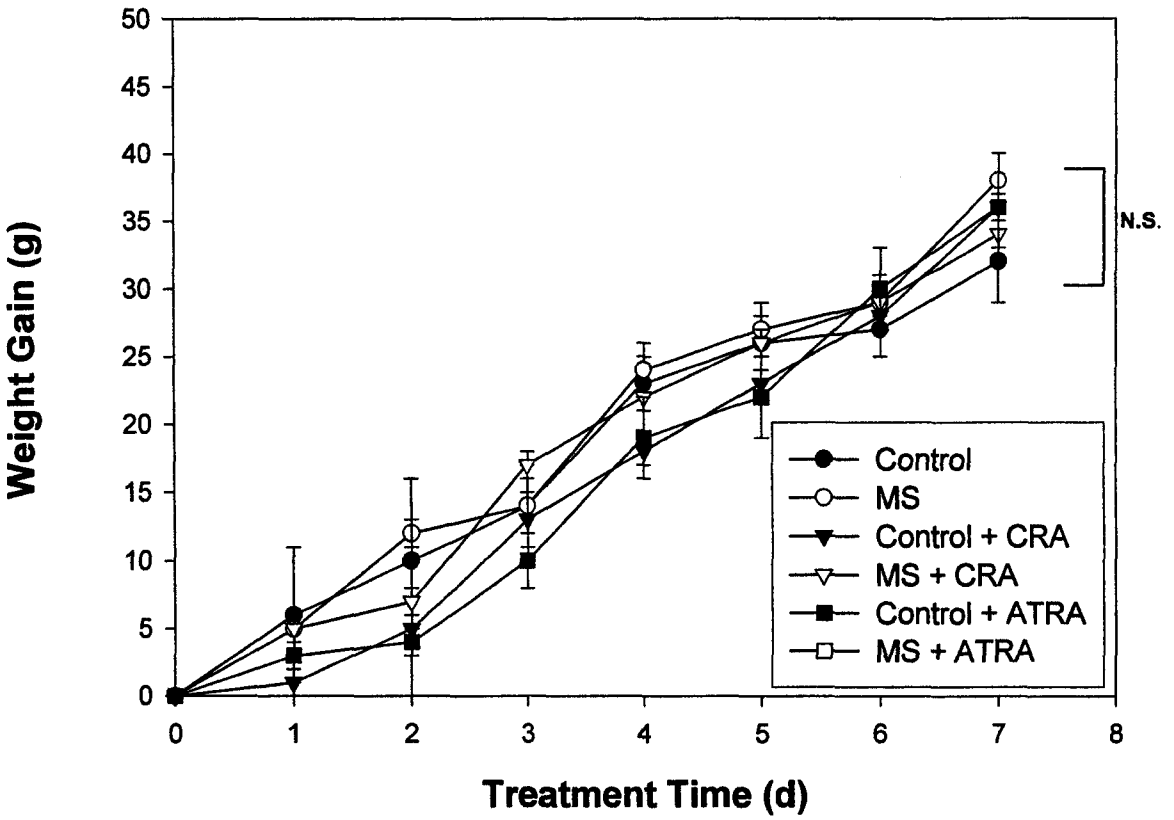
Weight Gain (g)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Treatment Time (d)
N.S.
Control
MS
Control + CRA
MS + CRA
Control + ATRA
MS + ATRA

Figure 2

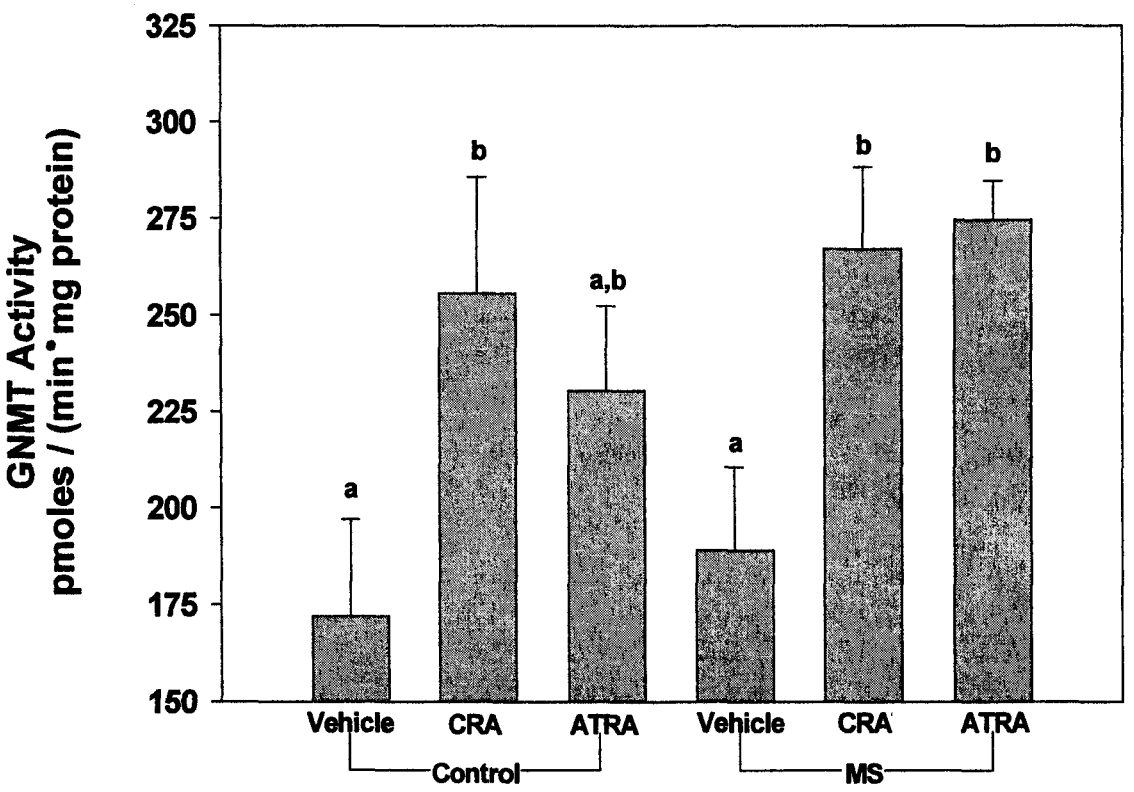
GNMT Activity
pmoles / (min*mg protein)
325
300
275
250
225
200
175
150
a
b
a,b
a
b
b
Vehicle
CRA
ATRA
Vehicle
CRA
ATRA
Control
MS

Figure 3

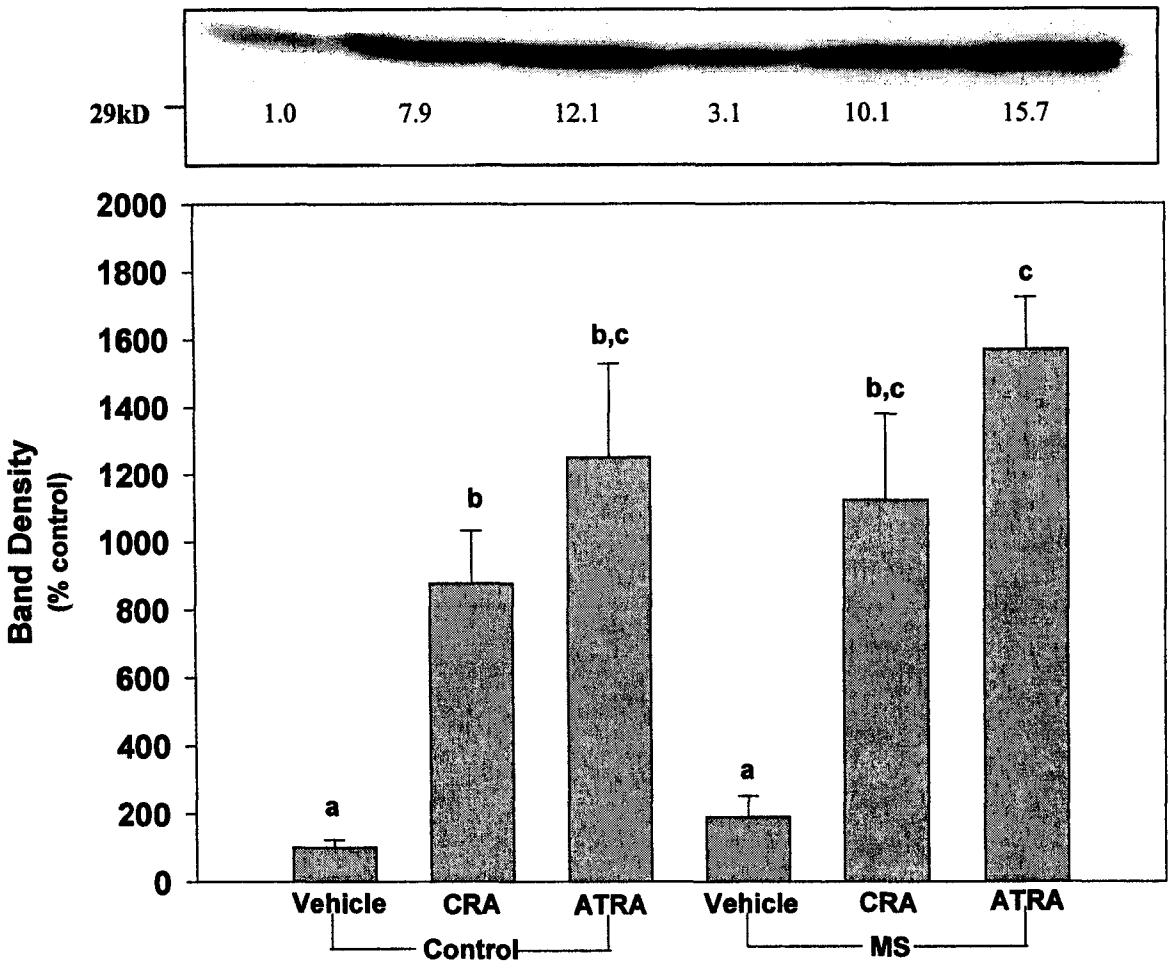
29kD
1.0
7.9
12.1
3.1
10.1
15.7
Band Density
(% control)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
b,c
a
b,c
c
Vehicle
CRA
ATRA
Vehicle
CRA
ATRA
Control
MS

Figure 4

VITAMIN A AND ITS DERIVATIVES INDUCE HEPATIC GLYCINE *N*-METHYLTRANSFERASE AND HYPOMETHYLATION OF DNA IN RATS[1]

A paper published in the Journal of Nutrition[2]

Matthew J. Rowling[3], Mary H. McMullen[3], & Kevin L. Schalinske[3,4]

Abstract

Regulation of S-adenosylmethionine (SAM) and the SAM/S-adenosylhomocysteine (SAH) ratio by the key cytosolic enzyme glycine *N*-methyltransferase (GNMT) is essential in optimizing methyl group supply and subsequent functioning of methyltransferase enzymes. Therefore, inappropriate activation of GNMT may lead to the loss of methyl groups vital for many SAM-dependent transmethylation reactions. Previously, we demonstrated that the retinoid derivatives 13-*cis*- (CRA) and all-*trans*-retinoic acid (ATRA) mediated both the activity of GNMT and its abundance. The present study was conducted to determine if vitamin A had a similar ability to up-regulate GNMT as well as to assess the biological significance of GNMT modulation by examining both the transmethylation and transsulfuration pathways following retinoid treatment. Rats were fed a control (10% casein + 0.3% L-methionine) diet and orally given retinyl palmitate (RP), CRA, ATRA, or vehicle daily for 10 d. RP, CRA, and ATRA elevated hepatic GNMT activity 32, 74, and 124% respectively, compared to the control group. Moreover, the retinoid-mediated changes in GNMT activity were reflected in GNMT abundance (38, 89, and 107% increase for RP-, CRA-, and ATRA-treated rats, respectively). In addition, hepatic DNA, a substrate for SAM-dependent transmethylation, was hypomethylated (~100%) following ATRA treatment compared to the control group. In contrast, the transsulfuration product glutathione was unaffected by retinoid treatment. These results provide evidence that (i) vitamin A, like its retinoic acid derivatives, can induce enzymatically active GNMT; and (ii) inappropriate induction of GNMT can lead to a biologically significant loss of methyl groups and the subsequent impairment of essential transmethylation processes.

Key words: • *S-adenosylmethionine* • *glycine N-methyltransferase* • *vitamin A* • *hypomethylation* • *rats*

Footnotes

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[5] Abbreviations used: ATRA, all-*trans*-retinoic acid; CRA, 13-*cis*-retinoic acid; DMSO, dimethylsulfoxide; DSS, disuccinimidyl suberate; GNMT, glycine *N*-methyltransferase; MTHFR, 5,10-methylenetetrahydrofolate reductase; RP, retinyl palmitate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

Introduction

S-adenosylmethionine (SAM)[5] is a product of methionine metabolism that serves as a donor of methyl groups for transmethylation reactions that are required for the maintenance of optimum health and the prevention of disease (1,2). SAM-dependent transmethylation reactions include the synthesis of phospholipids, neurotransmitters, and the posttranslational modification of nucleic acids and proteins (1,2). Hence, an adequate supply of methyl groups provided by the diet in the form of choline and methionine or from the folate-dependent one-carbon pool must be maintained to ensure these reactions function optimally. If an adequate methyl group supply is not maintained, pathological conditions such as hepatocarcinogenesis (3,4) and hepatic steatosis (5) may occur.

Among the end products of SAM-dependent transmethylation reactions is *S*-adenosylhomocysteine (SAH), a molecule that is a potent inhibitor of most methyltransferase reactions (6). Therefore, control of the SAM/SAH ratio, which is considered an index of transmethylation potential (1,2), is a critical aspect of one-carbon metabolism. In order to optimize the SAM/SAH ratio, the cytosolic enzyme glycine *N*-methyltransferase (GNMT) functions in conjunction with folate to regulate the methyl group supply. The enzymatic activity of GNMT serves to dispose of excess methyl groups through the methylation of glycine and subsequent formation sarcosine, a molecule with no known metabolic function **(Fig. 1)**. When there is an excess supply of SAM (i.e. methyl groups), SAM allosterically inhibits the folate-dependent enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) (7,8). This process serves to inhibit the formation of 5-methyltetrahydrofolate (5-methyl-THF), which in turn, slows the flow of methyl groups from the one-carbon pool into the methyl group metabolism pathway and diverts one-carbon units to where they are needed. Decreased 5-methyl-THF synthesis also results in elevated GNMT activity and the increased catabolism of SAM because GNMT is allosterically inhibited by 5-methyl-THF (9,10). In contrast, a compromised supply of SAM releases the inhibition of MTHFR, resulting in increased 5-methyl-THF formation and GNMT inhibition. This helps to ensure that methyl groups are available for other transmethylation reactions. Therefore, inappropriate activation of GNMT during conditions when the methyl group supply is compromised may result in a

down-regulation of important transmethylation reactions and subsequent pathological conditions.

Besides transmethylation, activation of GNMT may have important consequences on the metabolism of homocysteine, a subsequent product of SAH hydrolysis. After SAH is converted to homocysteine, a metabolic branch point exists where homocysteine is either remethylated to generate methionine or irreversibly committed to the transsulfuration pathway where it can serve as a precursor to biologically significant compounds such as cysteine and glutathione (Fig. 1). Currently, no evidence exists that associates activation of GNMT and alterations in components of the transsulfuration pathway such as glutathione or homocysteine.

Previously, we showed that methionine catabolism appears to be enhanced in rats given the retinoid compound 13-*cis*-retinoic acid (CRA) (11). Recently, we reported that a potential mechanism for the retinoid-induced increase in methionine/SAM catabolism was the activation and induction of hepatic GNMT mediated by both CRA and all-*trans*-retinoic acid (ATRA) (12). Based on these findings two obvious questions emerged: 1) does vitamin A modulate hepatic GNMT? and 2) does the induction of enzymatically active GNMT by retinoids deplete the methyl group supply to the point where hepatic transmethylation and/or transsulfuration pathways are compromised? In the present study, we present data that indicates that vitamin A does in fact mediate an increase in GNMT, which in turn leads to an inability to maintain SAM-dependent methylation of DNA. Furthermore, the increase in GNMT abundance by retinoid administration produces GNMT protein that exists primarily in its enzymatically active tetrameric state.

Materials and Methods

Chemicals and reagents.

Reagents were obtained from the following: S-adenosyl-L-[*methyl*-^{3}H]methionine for GNMT activity, New England Nuclear (Boston, MA);, S-adenosyl-L-[*methyl*-^{3}H]methionine for DNA methylation assay and ECL Western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ); phenylmethylsulfonylflouride and dimethylsulfoxide (DMSO),

Calbiochem (La Jolla, CA); disuccinimidyl suberate (DSS), Pierce Chemical (Rockford, IL); goat anti-rabbit immunoglobulin G horseradish peroxidase, Southern Biotechnology (Birmingham, AL); S-adenosyl-L-methionine and glutathione reductase, Sigma Chemical (St. Louis, MO); and *Sss I* methylase, New England Biolabs (Beverly, MA). GNMT antibodies were kindly provided by Conrad Wagner, Vanderbilt University. Retinyl palmitate (RP), 13-*cis*-retinoic acid (CRA), and all-*trans*-retinoic acid (ATRA) were provided courtesy of Hoffmann-LaRoche (Nutley, NJ). All other chemicals were of analytical grade.

Animals and diets.

All animal experiments were approved and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. Male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats were housed in plastic cages with a 12-h light:dark cycle and given free access to food and water. The control diet was the same as described previously (11). After a 7-d acclimation period during which rats were adapted to both the diet and oral administration of corn oil, rats were divided into four treatment groups (five rats per group) and were orally given either vehicle (corn oil, 1 μL/g body weight), RP, CRA, or ATRA on a daily basis for 10 days. Retinoids were prepared in corn oil and administered at a level of 30 μmol/kg body weight. Although pharmacological in magnitude, this dosage was similar to levels used previously (11,12), thus maintaining the continuity of this research and allowing comparisons to be drawn. However, current research using more physiological levels of retinoids (~1 μmol/kg body weight) is of significant importance as well. Following the 10-d treatment period, rats were anesthetized and portions of liver were removed for analysis of GNMT activity, GNMT protein abundance, DNA methylation, and total glutathione concentration.

Measurement of GNMT activity and protein abundance.

The enzymatic activity of GNMT was measured using the method of Cook and Wagner (13) with minor modifications. Portions of liver were homogenized in three volumes of ice-cold phosphate buffered (10 mmol/L, pH 7.0) sucrose (0.25 mol/L) containing 1mmol/L EDTA, 1 mmol/L sodium azide, and 0.1 mmol/L phenylmethylsulfonylflouride.

After centrifugation at 20,000 × *g* for 30 min, the resulting supernatant was removed and 2-mercaptoethanol was added to a final concentration of 10 mmol/L. The assay reaction mixture (100 µl) contained 0.2 mol/L Tris buffer (pH 9.0), 5 mmol/L dithiothreitol, 2 mmol/L glycine, and 0.2 mmol/L *S*-adenosyl-*L*-[*methyl*-^{3}H]methionine (47.7 kBq/µmol). The reaction was initiated upon the addition of 250 µg of sample protein and was performed in triplicate. GNMT protein abundance was measured using immunoblotting followed by chemiluminescence detection as described previously (12). Samples (75 µg total protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10-20% gradient gel and were electrophoretically transferred to a nitrocellulose membrane. For the chemiluminescent detection of GNMT, the membrane was incubated with affinity-purified polyclonal GNMT antibody followed by goat anti-rabbit horseradish peroxidase. Densitometric analysis was performed using the National Institutes of Health Image software. For the determination of total soluble protein concentration in liver extracts, a commercial kit (Coomassie Plus, Pierce, Rockford, IL) based on the method of Bradford (14) was used with bovine serum albumin as the standard.

Chemical cross-linking of endogenous GNMT.

For determining the oligomeric state of GNMT following retinoid treatment, liver extracts were incubated with 1.5 mmol/L DSS in DMSO for 30 min at room temperature followed by the addition of 6 µL ethanolamine to terminate the cross-linking reaction (15). Samples were subjected to SDS-PAGE followed by immunoblotting and chemiluminescence detection as described above.

DNA methylation.

To determine whether retinoids induced hypomethylation of hepatic DNA (i.e., altered transmethylation), an assay to measure the *in vitro* incorporation of methyl groups into DNA was used (16) with minor modifications. DNA was purified from liver samples based on the method of Miller *et al.* (17) using a commercial kit (Promega, Madison, WI). All isolated DNA samples exhibited an $A_{260/280}$ ratio > 1.8 and consisted of DNA > 20 kb as determined by agarose gel electrophoresis and ethidium bromide staining. The assay mixture

consisted of 1.0 μg DNA, 2.7 μmol/L S-adenosyl-L-[*methyl*^{3}H]methionine (555 GBq/mmol) and reaction buffer [10 mmol/L Tris buffer (pH 7.9), 50 mmol/L NaCl, 10 mmol/L EDTA, 1 mmol/L dithiothreitol] in a total volume of 50 μL. The reaction was initiated upon the addition of *Sss I* methylase (4 units) and was carried out at 30°C for 1 h. After the reaction was stopped by heating the samples at 65°C for 20 min, the mixture was applied to Whatman DE-81 ion exchange filters fixed on a suction apparatus and washed successively with 20 mL 500 mmol/L sodium phosphate buffer (pH 7.0), 2 mL 70% ethanol, and 2 mL absolute ethanol. The filters were allowed to air dry and subjected to liquid scintillation counting.

Total liver glutathione.

For the measurement of total hepatic glutathione concentrations, portions of liver were homogenized in 2 volumes of 0.4 mol/L perchloric acid followed by centrifugation at 10,000 × *g* for 10 min at 4°C. The resulting supernatant was diluted 100-fold with 125 mmol/L sodium phosphate buffer (pH 7.5) containing 6.3 mmol/L EDTA and 30 μL was used to spectrophometrically measure total glutathione concentrations as described by Tietze (18).

Statistical analysis.

The means of each treatment group were subjected to a one-way ANOVA ($P \leq 0.05$) and compared using Fisher's least significant difference procedure (19). For the GNMT activity data in Fig. 2A, the mean of each retinoid-treated group was also individually compared to the control group using Dunnet's Test. This second post-hoc test determines significant differences between a given treatment group and the control group only, and does not compare mean values across treatment groups. For Fig. 3, an association between GNMT activity and protein abundance across treatment groups was determined using the Pearson Correlation procedure. All statistical analysis was performed using SigmaStat (SPSS Inc., Chicago, IL).

Results

Retinoids did not alter the growth rate or relative liver size of rats.

As we have previously reported (12), all rat groups exhibited similar growth patterns (data not shown) and no significant differences in initial (65 $\pm$ 1 g) nor final (157 $\pm$ 2 g) body weights were observed across the treatment groups. Likewise, relative liver size was not affected by retinoid administration (data not shown).

Retinoids activate hepatic GNMT.

The ability of RP, CRA, and ATRA to activate GNMT is shown in **Fig. 2A**. Both CRA and ATRA significantly elevated the activity of GNMT 74% and 124%, respectively, compared to controls. Likewise, RP administration increased GNMT activity (32%), however this alteration was not statistically significant (P=0.052). The increase in GNMT activity due to RP administration was significant (P=0.020) when the retinoid treatment groups were compared to the control group alone (denoted by the asterisk).

Retinoid compounds, including vitamin A, induce GNMT abundance.

Fig. 2B illustrates the ability of RP, CRA, and ATRA to significantly induce hepatic production of GNMT protein. The bar graph reflects the mean from all experimental animals, whereas the immunoblot above is a representative example with the relative fold induction located under each lane. All three retinoids, including RP, significantly induced GNMT protein abundance. The mean induction of GNMT protein due to retinoid treatment ranged from 38 to 107%. Interestingly, it appears that all of the retinoid-induced synthesis of GNMT resulted in enzymatically active protein. As shown in **Fig. 3**, GNMT protein abundance was positively correlated with the increase in GNMT activity ($r=0.772$, $P < 0.001$). The ability of retinoids to induce hepatic GNMT in its enzymatically active tetrameric state is further illustrated in **Fig. 4**. Using DSS to cross-link subunits, we determined the oligomeric state of GNMT following retinoid treatment. Similar to Fig. 2B, all three retinoids induced GNMT monomer (32kD) synthesis (*lanes 1-4*). The same samples were also analyzed following incubation with the cross-linking reagent DSS (*lanes 5-8*).

DSS-treatment of both control and retinoid samples demonstrated that (i) little, if any, of the newly synthesized GNMT protein remains as a monomer; and (ii) the majority of the GNMT protein was in its tetrameric (128 kD) enzymatically active form. No GNMT was detected in the dimeric (64 kD) form (data not shown).

Retinoid treatment induced hepatic DNA hypomethylation, but failed to alter hepatic glutathione levels.

The effects of RP, CRA, and ATRA on hepatic DNA methylation status are shown in **Fig. 5**. Compared to control rats, hepatic DNA isolated from ATRA-treated rats exhibited a greater (~100%) ability to incorporate methyl groups from SAM into hepatic DNA, indicating a significant reduction in endogenous methylation status was present following retinoid treatment. The mean level of DNA methylation exhibited by RP- and CRA-treated rats was not significantly different from control values. To determine whether the disruption in the transmethylation pathway due to retinoid administration had a potential effect on specific components of the transsulfuration pathway, the hepatic concentration of total glutathione was assessed. No significant differences in glutathione levels or the total glutathione content of the liver were observed across the treatment groups (data not shown).

Discussion

The regulation of the SAM/SAH ratio by GNMT is critical for maintaining an optimum supply of methyl groups for transmethylation reactions. Therefore, disruption of GNMT function may lead to the unwarranted loss of methyl groups and compromised transmethylation reactions. GNMT function has been shown to be altered during diabetic conditions (20), ethanol administration (21), and folate deficiency (22); however, the physiological consequences of aberrant GNMT function were not explored in these studies. Recently, we demonstrated that retinoid compounds have a similar ability to increase GNMT activity, an occurrence that was reflected in abundance of the protein (12). In the present study, we extending these findings by illustrating that like CRA and ATRA, vitamin A, though to a lesser degree, has a similar ability to activate and induce the active tetrameric form of GNMT, as was illustrated in our cross-linking studies. Because vitamin A needs to

be converted to its active retinoic acid derivatives before it can function physiologically, the reduced sensitivity of methyl group metabolism to retinyl palmitate is not surprising.

Determining the mechanism by which retinoids exert their actions on GNMT remains a major focus of ongoing research in our laboratory. It has been documented that GNMT activity is regulated at the posttranslational level by phosphorylation of the subunits that constitute the active GNMT tetramer (10) and by allosteric inhibition by folate coenzymes (9). Therefore, we certainly have not ruled such posttranslational modification out as a potential mechanism for retinoid-mediated GNMT up-regulation. However, we are intrigued by the findings of the present study illustrating that GNMT activity was directly proportional to abundance of the protein following treatment with all three retinoids. These findings clearly indicate that vitamin A compounds modulate GNMT activity at the transcriptional/translational level however, because the promoter region for GNMT does not contain a retinoid response element, retinoid-mediated GNMT induction may be due to increased stability of GNMT protein and/or messenger RNA. We are currently exploring these research areas as well as the possibility that the effect of retinoids on GNMT is mediated by other factors such as gluconeogenesis. GNMT is found primarily in gluconeogenic tissues and is up-regulated during conditions consistent with gluconeogenesis such as diabetes (20). Moreover, retinoids, along with hormones such as glucocorticoids and glucagon, are required for the expression of phosphoenolpyruvate carboxykinase (PEPCK) (23), the rate-limiting enzyme for gluconeogenesis. Hence, it seems logical that the stimulation of gluconeogenesis could play a role in mediating the effects of retinoids on GNMT. Peng and Evenson (24) showed that alleviation of methionine toxicity by retinol pretreatment was prevented after rats were adrenalectomized, therefore; glucocorticoids may be required for vitamin A to exert its actions on the catabolism of methionine.

Our findings also indicate that up-regulation of GNMT results in aberrant methyl group metabolism. GNMT constitutes 1-3% of cytosolic protein in the liver (6), therefore; this finding is not surprising. The degree of GNMT activation by retinoid compounds appears to be directly related to their ability to compromise other SAM-dependent transmethylation reactions. This is supported by the fact that ATRA, the most biologically active of the retinoid compounds utilized in the present study was the most effective in

producing hypomethylated DNA. Such findings clearly indicate that retinoid administration may produce a functional methyl group deficiency. The potential for retinoid treatment to compromise SAM-dependent transmethylation is also supported by the observation that rats treated with ATRA exhibited a significant reduction in creatinine synthesis (McMullen, M.H., Rowling, M.J., Ozias, M.K. & Schalinske, K.L., unpublished observations). Because creatinine synthesis is one of the major depots for methyl groups from SAM, this further stresses the physiological significance of retinoid-mediated alterations in GNMT. Retinoid-mediated hypomethylation of DNA has significant implications due to the link between DNA methylation and a number of important processes, such as gene expression and development (25-28).

During the course of these and other studies, it was evident that rats within a treatment group exhibited a significant degree of variability with respect to methylation status of DNA. Our treatment period (10 d) was fairly rapid compared to other reports on hypomethylation of DNA that typically employ treatment periods ranging from 1-4 weeks using methyl- and/or folate-deficient diets (16,28,29). We are currently conducting time course studies examining retinoid-mediated changes in methyl group metabolism, which we expect will aid in explaining this variability. Although only ATRA-treated rats exhibited a statistically significant increase in hypomethylation of DNA, it is important to note that we found a positive correlation ($r=0.667$, $P=0.0013$) between GNMT activity and DNA methylation (data not shown). In contrast to SAM-dependent transmethylation, retinoid-treated rats failed to exhibit significant changes in hepatic concentrations of total glutathione, indicating that the transsulfuration pathway remains partially intact, at least with respect to maintaining normal glutathione levels. Earlier work demonstrated that increased methionine catabolism by dietary CRA resulted in a significant increase in hepatic taurine concentrations that was achieved, in part, at the expense of reduced inorganic sulfate excretion and diminished hepatic glutathione levels (5,11).

In summary, it is clear that retinoid compounds, as vitamin A or as a retinoic acid derivative, represent a potent group of compounds capable of perturbing methyl group metabolism. Although the doses we utilized in these studies are pharmacological in magnitude, our preliminary work suggests that modulation of GNMT can be achieved using

more physiological levels (~1 μmol/kg body weight) as well (data not shown). Thus, future work is being directed at this issue by performing both dose-response and time-course studies. Moreover, the potential impact of retinoid administration on perturbation of the folate-dependent one-carbon pool and subsequent remethylation of homocysteine is a component of these future studies. This research direction is supported by previous studies demonstrating that vitamin A status influences the one-carbon pool (30,31) as well as a recent report that plasma homocysteine levels were elevated in patients receiving CRA therapy (32).

Literature Cited

1. Cantoni, G. L. (1982) *S*-adenosyl amino acids thirty years later: 1951-1981. In: *The Biochemistry of S-adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., and Creveling, C. R., eds.) pp. 3-10. Macmillan Press, London, England.

2. Cantoni, G. L. and Chiang, P. K. (1980) The role of *S*-adenosylhomocysteine hydrolase in the control of biological methylation reactions. In: *Natural Sulfur Compounds* (Cavallini, D., Gaull, G. E., and Zappia, V., eds.) pp. 67-80. Plenum Press, New York, NY.

3. Ghoshal, A. K. and Farber, E. (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis.* 5(10): 1367-1370.

4. Salmon, W. D. and Copeland, D. H. (1954) Liver carcinoma and related lesions in chronic choline deficiency. *Ann. NY. Acad. Sci.* 57: 664-677.

5. Schalinske, K.L. & Steele, R.D. (1993) 13-*cis*-Retinoic acid and hepatic steatosis in rats. *Biochem. Pharmacol.* 46: 319-325.

6. Kerr, S. J. (1972) Competing methyltransferase systems. *J. Biol. Chem.* 247: 4248-4252.

7. Kutzbach, C. and Stokstad, E. L. R. (1967) Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by *S*-adenosylmethionine. *Biochim. Biophys. Acta.* 139: 217-220.

8. Kutzbach, C. and Stokstad, E. L. R. (1971) Mammalian methylenetetrahydrofolate reductase. Partial purification, properties, and inhibition by *S*-adenosylmethionine. *Biochim. Biophys. Acta.* 250: 459-477.

9. Wagner, C., Briggs, W. T., and Cook, R. B. (1985) Inhibition of glycine *N*-methyltransferase activity by folate derivatives: Implications for regulation of methyl group metabolism. *Biochem. Biophys. Res. Commun.* 127(3) 746-752.

10. Wagner, C., Decha-Unphai, W., and Corbin, J. (1989) Phosphorylation modulates the activity of glycine *N*-methyltransferase, a folate binding protein. *J. Biol. Chem.* 264(16): 9638-9642.

11. Schalinske, K. L. and Steele, R. D. (1991) 13-*cis*-retinoic alters methionine metabolism in rats. *J. Nutr.* 121: 1714-1719.

12. Rowling, M.J. & Schalinske, K.L. (2001) Retinoid compounds activate and induce hepatic glycine N-methyltransferase in rats. *J. Nutr.* 131: 1914-1917.

13. Cook, R. J. and Wagner, C. (1984) Glycine *N*-methyltransferase is a folate binding protein of rat liver cytosol. *Proc. Natl. Acad. Sci. U.S.A.* 81: 3631-3634.

14. Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* 72: 248-254.

15. Bhat, R., Wagner, C. & Bresnick, E. (1997) The homodimeric form of glycine N-methyltransferase acts as a polycyclic aromatic hydrocarbon-binding receptor. *Biochemistry* 36: 9906-9910.

16. Balaghi, M. and Wagner, C. (1993) DNA methylation in folate deficiency: use of CpG methylase. *Biochem. Biophys. Res. Comm.* 193(3): 1184-1190.

17. Miller, S.A., Dykes, D.D. & Polesky, H.F. (1983) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res.* 16: 1215.

18. Tietze, F. (1969) Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27: 502-522.

19. Snedecor, G. W. and Cochran, W. G. (1980) Statistical Methods, 7th ed. Iowa State University Press, Ames, IA.

20. Xue, G-P. and Snoswell, A. M. (1985) Disturbance of methyl group metabolism in alloxan-diabetic sheep. *Biochem. Int.* 10(6): 897-905.

21. Trimble, K. C., Molloy, A. M., Scott, J. M., and Weir, D. G. (1993) The effect of ethanol on one-carbon metabolism: increased methionine catabolism and lipotrope methyl-group wastage. *Hepatology*: 18(4): 984-989.

22. Balaghi, M., Horne, D. W., and Wagner, C. (1993) Hepatic one-carbon metabolism ion early folate deficiency in rats. *Biochem. J.* 291: 145-149.

23. Pan, C-J., Hoeppner, W., and Yang Chou, J. (1990) Induction of phosphoenolypyruvate carboxykinase gene expression by retinoic acid in an adult rat hepatocye line. *Biochemistry.* 29(49): 10883-10888.

24. Peng, Y-S. and Evenson, J. K. (1982) Alleviation of methionine toxicity in rats by retinol-pretreatment: *in vivo* methionine catabolism and role of adrenal glands. *Nutr. Reports Int.* 25(1): 139-144.

25. Hoffman, R.M. (1984) Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. *Biochim. Biophys. Acta* 738: 49-87.

26. Hoffman, R.M. (1990) Unbalanced transmethylation and the perturbation of the differentiated state leading to cancer. *BioEssays.* 12: 163-166.

27. Razin, A. & Shemer, R. (1995) DNA methylation in early development. *Human Mol. Genet.* 4: 1751-1755.

28. Wainfan, E. & Poirier, L.A. (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res.* 52: 2071S-2077S.

29. Wainfan, E., Dizik, M., Stender, M. & Christman, J.K. (1989) Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. *Cancer Res.* 49: 4094-4097.

30. Fell, D. & Steele, R.D. (1985) The effects of vitamin A deficiency on hepatic folate metabolism in rats. *Arch. Biochem. Biophys.* 240: 843-850.

31. Fell, D. & Steele, R.D. (1986) Modification of hepatic folate metabolism in rats fed excess retinol. *Life Sci.* 38: 1959-1965.

32. Schulpis, K. H., Karikas, G. A., Georgala, S., Michas, T., and Tsakiris, S. (2001) Elevated plasma homocysteine levels in patients on isotretinoin therapy for cystic acne. *Int. J. Dermatol.* 40: 33-36.

FIGURE LEGENDS

FIGURE 1 Methyl group metabolism. Methyl groups, as methionine, can be converted to SAM and function as a substrate in numerous SAM-dependent transmethylation reactions. GNMT regulates the transmethylation potential by controlling the SAM/SAH ratio. Following the hydrolysis of SAH, a branch point exists where homocysteine can either be remethylated by 5-methyl-THF to generate methionine, or used for the synthesis of biologically significant metabolites such as cysteine and glutathione via the transsulfuration pathway. Abbreviations used: GNMT; glycine *N*-methyltransferase, SAH; S-adenosylhomocysteine, SAM; S-adenosylmethionine, THF; tetrahydrofolate.

FIGURE 2 Retinoid administration activated hepatic GNMT in rats. *Panel A*, elevation of hepatic GNMT activity mediated by RP, CRA, and ATRA. All assays were performed in triplicate. *Panel B*, induction of GNMT protein mediated by RP, CRA, and ATRA. The immunoblot above the bar graph (Fig. 2B) is a representative example with the relative fold induction located below each lane. Data are means $\pm$ SEM, $n = 5$. Bars without a common letter are significantly different ($P \leq 0.05$). Bars with an asterisk are significantly different ($P \leq 0.05$) when compared to the control group alone.

FIGURE 3 Correlation between GNMT activity and abundance across retinoid treatment groups. Symbols represent individual values within each group. Results of a Pearson correlation test are indicated by the solid line ($r = 0.772$, $P = 0.00007$).

FIGURE 4 The enzymatically active tetrameric form of GNMT was mediated by retinoid treatment. Cytosolic extracts were subjected to the cross-linking reagent disuccinimidyl suberate (DSS) prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using a polyclonal GNMT antibody. As indicated by the arrows, bands at ~32 kD represent the monomeric form of GNMT, whereas the tetrameric form is shown at a molecular weight of ~128 kD monomer. No immunoreactive bands were discernible at 64 kD, representing the dimeric form of the protein (data not shown). Hepatic

extracts from control, RP-, CRA-, and ATRA-treated rats in the absence of DSS treatment are shown in lanes 1-4, respectively, whereas the same samples subjected to DSS cross-linking prior to SDS-PAGE are shown in lanes 5-8.

FIGURE 5 Administration of ATRA to rats resulted in hepatic DNA hypomethylation. In this assay, the ability to serve as an acceptor for methyl groups is inversely proportional to endogenous methylation status. Data are means $\pm$ SEM, $n = 5$. Bars without a common letter are significantly different ($P \leq 0.05$).

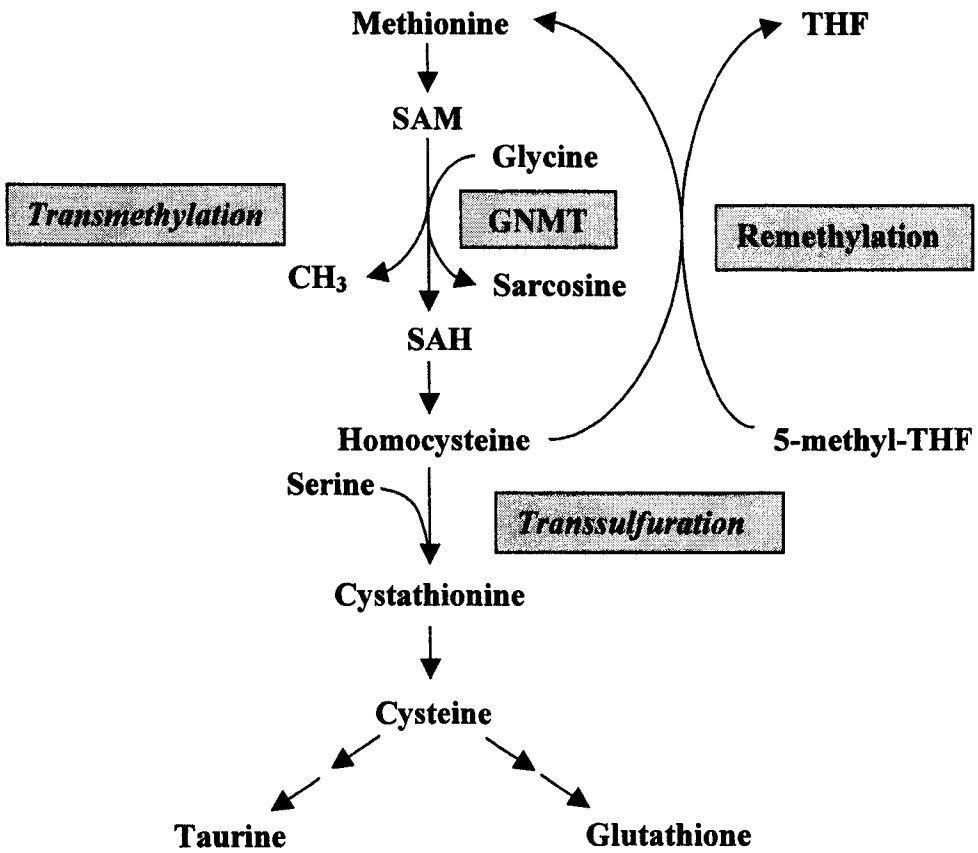
Methionine
THF
SAM
Glycine
Transmethylation
GNMT
Remethylation
CH3
Sarcosine
SAH
Homocysteine
5-methyl-THF
Serine
Transsulfuration
Cystathionine
Cysteine
Taurine
Glutathione

Figure 1

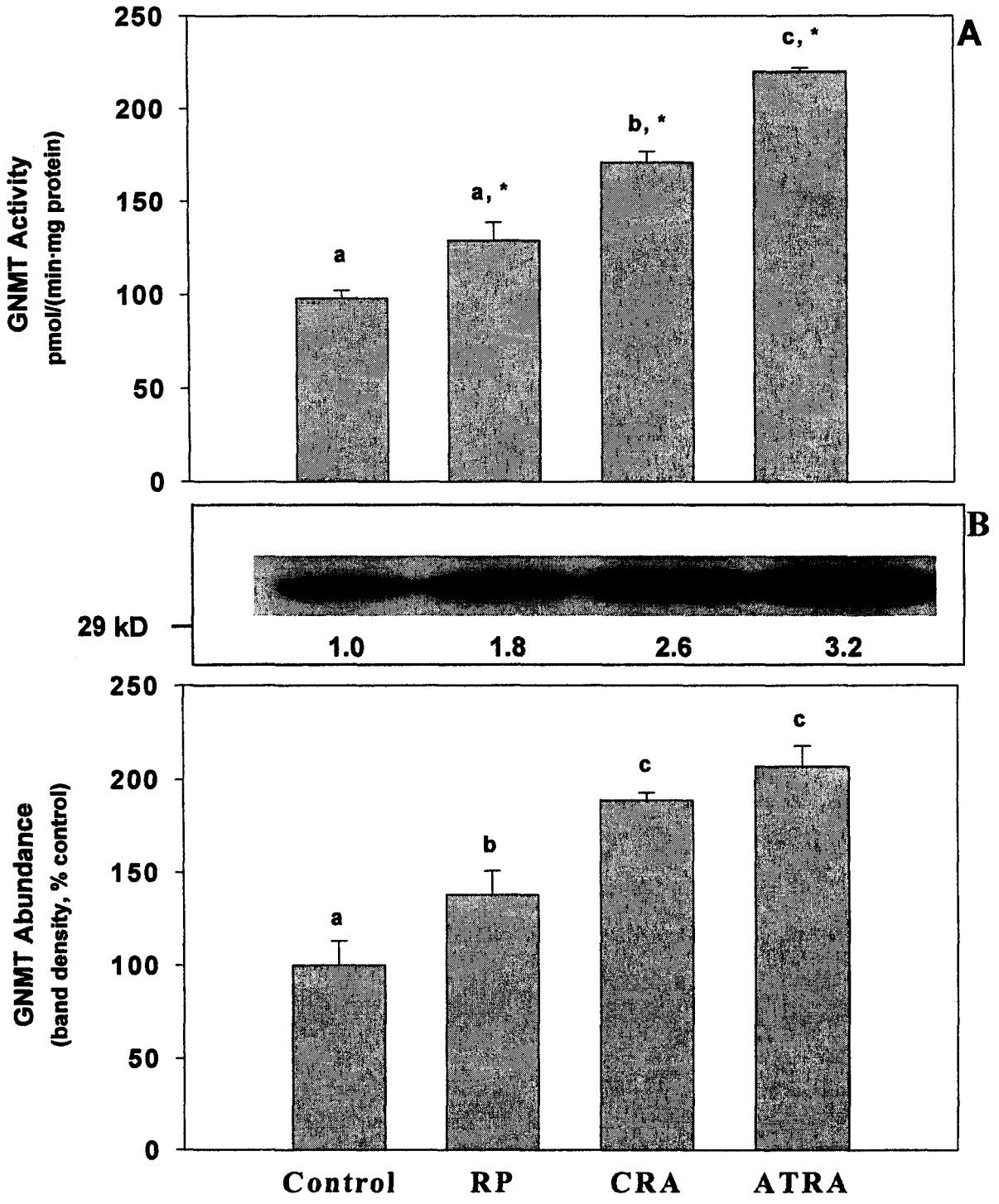
A
GNMT Activity
pmol/(min·mg protein)
250
200
150
100
50
0
a
a, *
b, *
c, *
B
29 kD
1.0
1.8
2.6
3.2
GNMT Abundance
(band density, % control)
250
200
150
100
50
0
a
b
c
c
Control
RP
CRA
ATRA

Figure 2

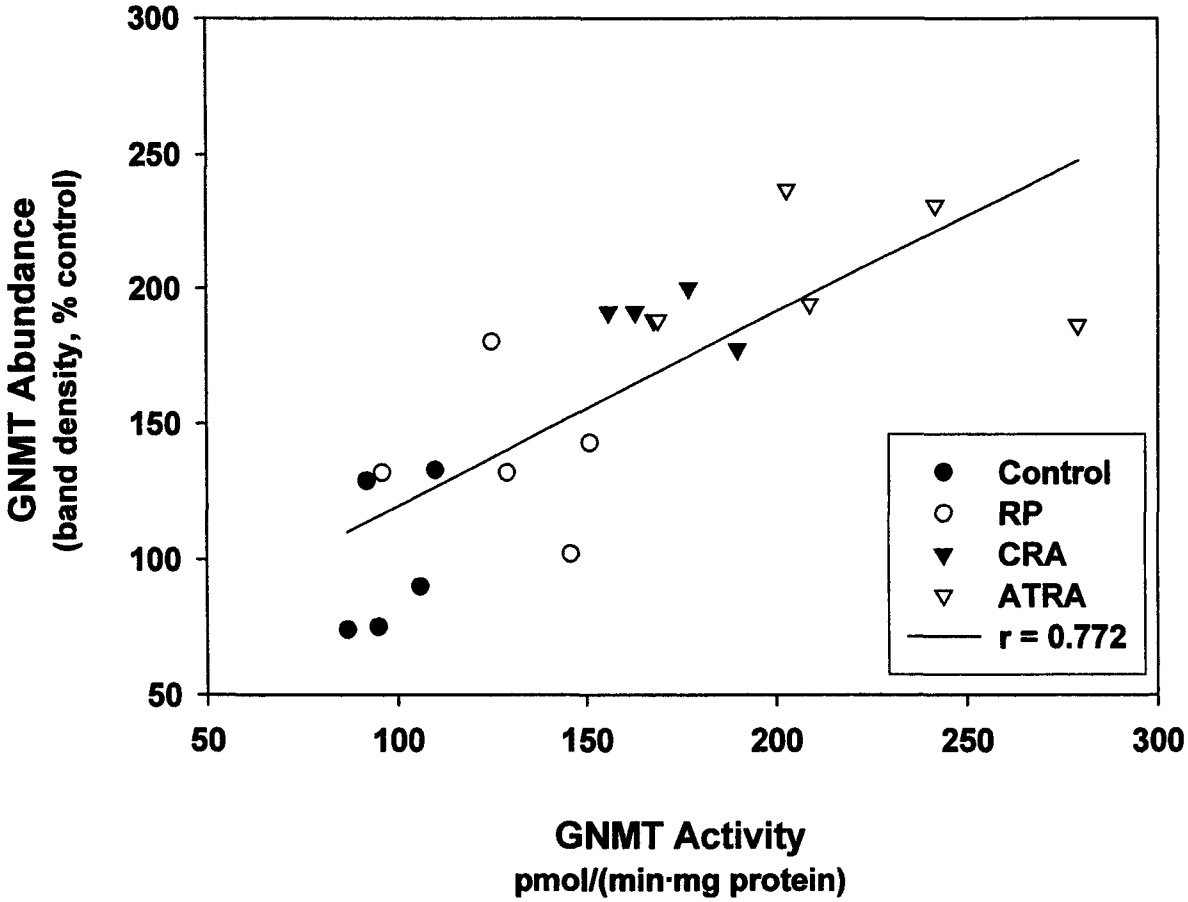
GNMT Abundance
(band density, % control)
300
250
200
150
100
50
50
100
150
200
250
300
GNMT Activity
pmol/(min·mg protein)
Control
RP
CRA
ATRA
r = 0.772

Figure 3

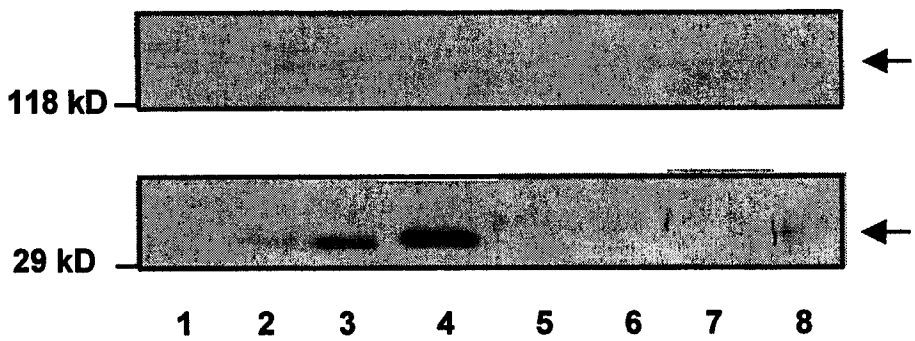

Figure 4

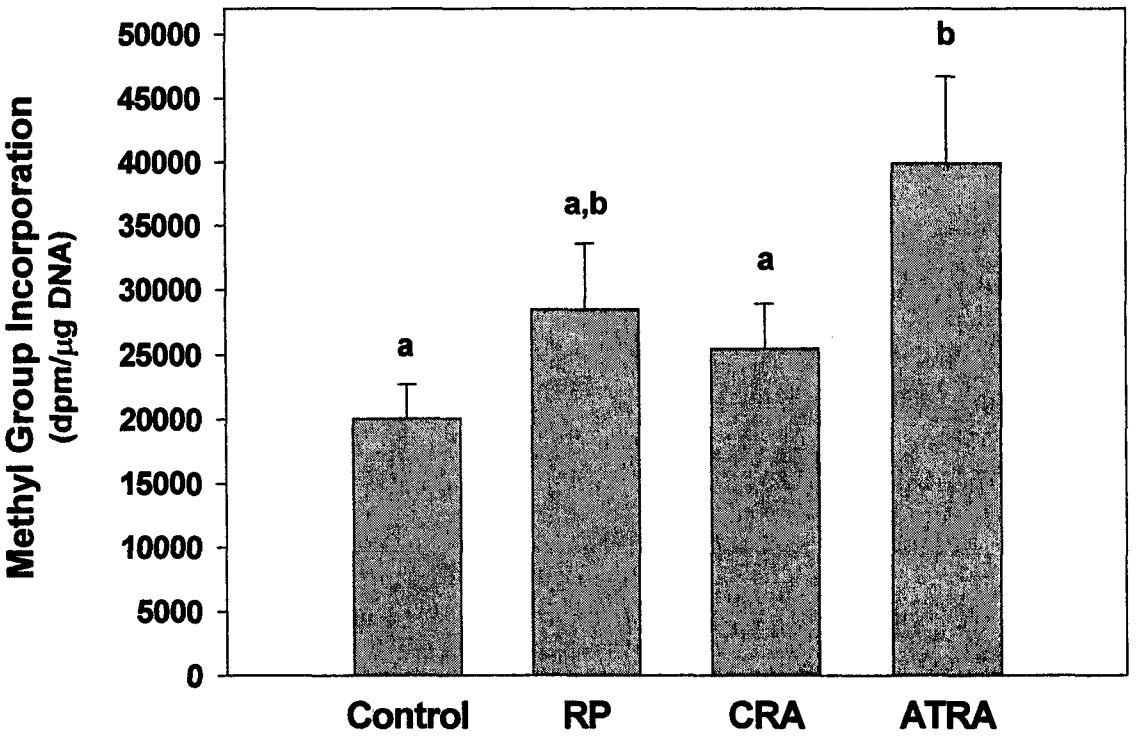
Methyl Group Incorporation
(dpm/μg DNA)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
a
a,b
a
b
Control
RP
CRA
ATRA

Figure 5

RETINOIC ACID AND GLUCOCORTICOID TREATMENT INDUCE HEPATIC GLYCINE *N*-METHYLTRANSFERASE AND LOWER PLASMA HOMOCYSTEINE CONCENTRATIONS IN RATS AND RAT HEPATOMA CELLS[1]

A paper published in the Journal of Nutrition[2,3]

Matthew J. Rowling[4] and Kevin L. Schalinske[4,5]

Abstract

Perturbation of folate and methyl group metabolism is associated with a number of pathological conditions, including cardiovascular disease and neoplastic development. Glycine *N*-methyltransferase (GNMT) is a key protein that functions to regulate the supply and utilization of methyl groups for S-adenosylmethionine (SAM)-dependent transmethylation reactions. Factors or conditions that have the ability to regulate GNMT and the generation of homocysteine, a product of transmethylation, have significant implications in the potential perturbation of methyl group metabolism. We have shown that retinoid compounds induce active hepatic GNMT, resulting in compromised transmethylation processes. Because retinoids can stimulate gluconeogenesis, a condition known to alter methyl group and homocysteine metabolism, the current study was undertaken to determine the relationship between all-*trans*-retinoic acid (RA) and gluconeogenic hormones on these metabolic pathways. It was found that intact adrenal function was not required for RA to induce and activate hepatic GNMT; however, treatment of rats with dexamethasone (DEX) was as effective as RA to induce GNMT in rat liver. The marked increase in plasma total homocysteine levels observed in adrenalectomized rats was reduced to normal levels by treatment with either RA or DEX, indicating that the transsulfuration and/or remethylation pathways may be enhanced. Moreover, co-administration of RA and DEX resulted in an additive effect on GNMT induction. Similar findings were also observed in a rat hepatoma cell culture model using H4IIE cells. Taken together, these results demonstrate that both RA

and DEX independently induce GNMT, thereby having significant implications for the potential interaction of retinoid administration with diabetes.

Key words: • *glycine N-methyltransferase* • *methyl groups* • *homocysteine* • *retinoic acid* • *dexamethasone*

Footnotes

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[2] Presented in part at Experimental Biology 2003, San Diego, CA [Rowling, M.J. & Schalinske, K.L. (2003) Gluconeogenic modulation of retinoid-mediated perturbation of methyl group metabolism. FASEB J. 17: A310 (abs.)].

[3] Reprinted with permission of J. Nutr., 2003, 133: 3392-3398.

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[6]The abbreviations used are: ADX, adrenalectomized; Bt_2cAMP, dibutyryl-adenosine-3,5-cyclic monophosphate; CBS, cystathionine β-synthase; DEX, dexamethasone; GNMT, glycine N-methyltransferase; Me_2SO, dimethylsulfoxide;.MTHFR, 5,10-methylenetetrahydrofolate reductase; 5-CH_3-THF, 5-methyltetrahydrofolate; PC, phosphatidylcholine; PEPCK, phosphoenolpyruvate carboxykinase; RA, all-*trans*-retinoic acid; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

[7] Rowling, M.J. and Schalinske, K.L., unpublished data.

Introduction

Glycine *N*-methyltransferase (GNMT) [6] is a key cytosolic protein in the regulation of methyl group and folate metabolism that controls the supply of available methyl groups for *S*-adenosylmethionine (SAM)-dependent transmethylation reactions (1). By disposing of excess methyl groups as sarcosine, a molecule that has no known physiological function, GNMT controls the SAM/*S*-adenosylhomocysteine (SAH) ratio (2), which is considered an important index of transmethylation potential because of the ability of SAH to inhibit the majority of SAM-dependent transmethylation reactions (3). Interestingly, GNMT does not appear to be among the methyltransferases that are sensitive to such SAH inhibition (4). Over 100 critical transmethylation reactions readily occur in the mammalian cell, including the methylation of DNA and methylation reactions required during the synthesis of vital biological compounds such as neurotransmitters, phospholipids, and nucleic acids (3,4). Therefore, maintaining an optimum supply of activated methyl groups (i.e. SAM) is critical for the proper functioning of a vast number of enzymes that catalyze methyl transfer reactions that are essential for the prevention of disease.

A number of interactions exist between the methyl group- and folate-dependent one-carbon metabolism pathways to ensure that methyl groups are readily available for use in SAM-dependent transmethylation reactions **(Fig. 1)**. SAM serves as an inhibitory ligand for 5,10-methylenetetrahydrofolate reductase (MTHFR) (5), therefore; an elevated intracellular supply of SAM decreases the synthesis of 5-methyltetrahydrofolate (5-methyl-THF). Additionally, 5-methyl-THF is an allosteric inhibitor of GNMT (6,7), hence; an elevated concentration of SAM indirectly leads to its own catabolism by increasing GNMT activity through the inhibition of 5-methyl-THF synthesis. GNMT can then dispose of the excess methyl groups supplied by SAM as sarcosine. In contrast, decreased SAM levels facilitate the production of 5-methyl-THF and subsequent folate-dependent remethylation of homocysteine by 5-methyl-THF to regenerate methionine. The rise in 5-methyl-THF concentrations also inhibits GNMT activity, which in turn conserves the supply of activated methyl groups for important SAM-dependent transmethylation reactions. Following transmethylation, SAH is hydrolyzed to generate homocysteine and adenosine. Homocysteine can then be remethylated by either methionine synthase (MS) or

betaine:homocysteine methyltransferase (BHMT), processes that require 5-methyl-THF or betaine, respectively, or committed to the transsulfuration pathway by cystathionine β-synthase (CβS) for its catabolism. Besides the removal of homocysteine, transsulfuration is essential for the production of important molecules such as cysteine and glutathione. Because of the central role of GNMT in the regulation of methyl group and folate metabolism, a disturbance of GNMT function may alter the synthesis of such compounds and thus result in a number of pathological conditions (8-10).

A number of nutritional components and hormones have recently been identified as factors that modulate the expression of key enzymes that play critical roles in methyl group and folate metabolism. We showed that the administration of retinoid compounds activated and induced GNMT expression in rats, which resulted in compromised methylation of hepatic DNA (11). Others have shown in animal and cell culture studies that conditions associated with a lack of insulin (e.g. diabetes) and administration of the counterregulatory hormones, glucagon and glucocorticoids increased the expression of critical enzymes central to methyl group and folate metabolism, including CβS and MTHFR (12-14), which are required for the removal of homocysteine. Moreover, Xue and Snoswell (15) discovered that GNMT activity was elevated 65-fold in diabetic sheep. Although it has not been determined whether gluconeogenic conditions prevalent during uncontrolled diabetes increase the expression of GNMT, it is known that GNMT resides primarily in the liver, kidney, and pancreas (16), organs that play significant roles during gluconeogenesis. Therefore, it is conceivable that GNMT can be regulated by gluconeogenic factors such as increased counterregulatory hormone levels, an occurrence that in turn, may affect other critical aspects of methyl group and folate metabolism such as the metabolism of homocysteine.

Hence, we postulated that the ability of retinoids to modulate GNMT was due to an association between retinoids and gluconeogenesis. All-*trans*-retinoic acid has been shown to increase the gene expression of phosphoenolpyruvate carboxykinase (PEPCK) (17,18), the rate-limiting enzyme of gluconeogenesis. However, unlike PEPCK, no retinoic acid response element has been found in the promoter region for GNMT. Thus, it seems likely that the affect of retinoids on GNMT expression is secondary to other events, such as elevated counterregulatory hormone levels. Peng and Evenson (19-21) showed that

methionine toxicity was alleviated in rats pretreated with retinol, an occurrence that was reversed when rats were adrenalectomized, indicating that the adrenal glands could play a key mediating role in the induction of GNMT by retinoid compounds. In the present study, we examined the role of the adrenals and dexamethasone (DEX) in the regulation of GNMT, alone and in combination with RA using both an *in vivo* rat model and cell culture studies. Furthermore, we determined whether the modulation of GNMT due to the various treatments was associated with alterations of plasma homocysteine levels.

Materials and Methods

Chemicals

Materials were obtained form the following sources: tissue culture reagents, Life Technologies, Inc.(Rockville, MD); S-adenosyl-L-[*methyl*-^{3}H]methionine, PerkinElmer (Boston, MA); goat anti-rabbit immunoglobulin G horseradish peroxidase, Southern Biotechnology (Birmingham, AL); ECL Western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ); S-adenosyl-L-methionine and dexamethasone, Sigma Chemical (St. Louis, MO); and protease inhibitors and RA, Calbiochem (La Jolla, CA). All other reagents were of analytical grade.

Animal experiments

All animal experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. Adrenalectomized (ADX) and sham-operated male Sprague Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats (125-149 g) were housed in plastic cages and given free access to food and water in a room with a 12-h light: dark cycle. ADX rats received saline (10 g/L NaCl) as drinking water. The composition of the control diet was the same as previously described (22) except the AIN-93G formulation of vitamin and mineral mix was utilized. After rats were acclimated to both the control diet and oral administration of corn oil, they were randomly assigned to various treatment groups (five rats per group) and given a daily oral dose of either vehicle (corn oil) or vehicle containing RA (30 μmol/kg BW). Although this represents a pharmacological dose of RA and we have shown that levels as low as 5 μmol/kg BW effectively elevated

GNMT activity, the maximal saturating effect of RA on GNMT induction was achieved using the higher concentrations [7]. For glucocorticoid studies, rats were given a daily intraperitoneal injection of DEX (1 mg/kg BW) that was dissolved in a vehicle containing propylene glycol: absolute ethanol: glacial acetic acid: ascorbic acid (95:5:0.6:0.01, v/v/v/w). In preliminary studies, we found that this level of DEX administration was equally effective as higher doses (i.e., saturating) and allowed maintenance of normal body weight. After a 5-7 d treatment period with RA and/or DEX, rats were anesthetized with a intraperitoneal injection of ketamine: xylazine (90:10 mg/kg BW) and blood samples were collected via cardiac puncture in heparinized syringes, centrifuged at 4,000 × *g* for 8 min, and stored at -20°C until analysis. Liver and pancreatic tissue samples were rapidly removed and homogenized in ice-cold buffer containing: 10 mmol/L sodium phosphate (pH 7.0), 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/LM sodium azide, 0.1 mmol/L phenylmethylsulfonyl fluoride. Following centrifugation at 20,000 × *g* for 30 min, β-mercaptoethanol was added to the resulting supernatants to a final concentration of 10 mmol/L and they were stored at -70°C for subsequent analysis of GNMT activity and abundance as previously described (11,23).

Cell culture

Rat hepatoma H4IIE cells (American Type Culture Collection, Manassas, VA) were grown in 150-cm^2 flasks to 70-75% confluence under 5% CO_2 in a humidified incubator at 37°C in Dulbecco's modified Eagles medium containing 100 mL/L fetal bovine serum, penicillin (100,000 units/L), and streptomycin (100 mg/L). Cells were given fresh media immediately prior to treatments. Cells were either treated alone, or with various combinations of DEX (0.1 μmol/L), dibutyryl-adenosine-3,5-cyclic monophosphate (Bt_2cAMP, 0.5 mmol/L), glucagon (0.5 μmol/L) and/or RA (10 μmol/L). Preliminary dose-response studies established these levels as optimal for modulating methyl group metabolism. Following a 72- h incubation period in the presence of the various treatment reagents, cells were detached with 2.5 g/L trypsin/ 1 mmol/L EDTA, washed twice in Hanks' balanced salt solution, and lysed on ice in a buffer containing: 10 mmol/L Hepes (pH 7.4), 10 mmol/L

sodium pyrophosphate, 50 mmol/L sodium fluoride, 50 mmol/L β-glycerophosphate, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 2 mmol/L benzamidine, 100 mg/L leupeptin and pepstatin, 250 mg/L soybean trypsin inhibitor, 0.2 mmol/L phenylmethylsulfonylflouride, 24 mg/L *p*-nitroguanidinobenzoate, and 5 mL/L Nonidet P-40. Lysates were centrifuged at 16,000 × *g* for 8 min after which supernatants were removed and stored at -70°C for subsequent analysis of GNMT protein abundance.

Measurement of GNMT activity

The enzymatic activity of GNMT was determined in tissue supernatants as described by Cook and Wagner (1984) with minor modifications (11). The assay reaction mixture (100 μL) consisted of 200 mmol/L Tris buffer (pH 9.0), 5 mmol/L dithiothreitol, 2 mmol/L glycine, and 2 mmol/L [*methyl*-^{3}H]SAM (47.7 kBq/μmol). The reaction was initiated with 250 μg cytosolic protein and incubation of the assay mixture was carried out at 25°C for 30 min. The reaction was terminated by the addition of trichloroacetic acid and activated charcoal was used to remove unreacted radiolabeled SAM by centrifugation. An aliquot of the resulting supernatant containing the radiolabeled product was removed for liquid scintillation counting and GNMT activity was expressed as pmoles sarcosine formed/ (min·mg protein). All GNMT assays were performed in triplicate.

Analysis of GNMT protein abundance

The abundance of GNMT protein in tissue and cell culture extracts was determined using immunoblotting techniques as previously described (11,23,25). Briefly, a 10-20% gradient gel was cast for SDS-polyacrylamide gel electrophoresis for determination of the abundance of the 32 kDa monomer subunit of GNMT, which functions enzymatically in its homotetrameric form. Following separation, proteins were electrophoretically transferred to a nitrocellulose membrane, followed by incubation with either an affinity-purified polyclonal GNMT antibody (kindly provided by C. Wagner, Vanderbilt University) or a monoclonal GNMT antibody (26) that recognizes a specific peptide sequence within the protein (kindly provided by Y-M.A. Chen, National Yang-Ming University, Taipei, Taiwan). For either

primary antibody, blots were incubated overnight at 4°C followed by a 1-h incubation at room temperature with goat anti-rabbit horseradish peroxidase secondary antibody. GNMT abundance was assessed by chemiluminescence detection and, following multiple exposures to Kodak X-Omat AR film, densitometric analysis was performed using SigmaGel Software (SPSS, Chicago, Il). For both enzyme activity measurements and Western blot analysis, total soluble protein concentration in liver and cell extracts was determined by the method of Bradford (27) using a commercial kit (Coomassie Plus, Pierce Chemical, Rockford, IL) and bovine serum albumin as a standard.

Determination of plasma homocysteine concentrations

Total (free + protein bound) homocysteine concentrations were determined using high-performance liquid chromatography with fluorescence detection (28). Plasma samples were derivatized with 7-flouro-2-oxa-1,3-diazole-4-sulfonate and a 100 μL sample containing the thiol adducts was injected onto a μBondapak C_{18} Radial-Pak column (Waters Corporation, Milford, MA) using a mobile phase consisting of 40 mL/L acetonitrile in 0.1 mol/L potassium phosphate (pH 2.1) buffer. N-acetylcysteine was added to plasma samples prior to derivatization as an internal standard.

Statistical analysis

The mean values from each treatment group were subjected to a one- or two-way ANOVA (29). If the ANOVA was significant ($P < 0.05$), the means were compared using Fisher's least significant difference procedure. All statistical analysis was performed using SigmaStat software (SPSS, Chicago, IL).

Results

Intact adrenal function was not required for the induction of hepatic GNMT by RA

As shown in **Fig. 2**, the activity and abundance of GNMT was elevated following administration of RA to adrenalectomized rats. As we have shown previously, RA increased

both the activity (**Fig. 2*A***) and abundance (**Fig. 2*B***) of hepatic GNMT ~2.0-fold in sham-operated control rats. The immunoblot above the bar graph in **Fig. 2*B*** is a representative sample from each treatment group with the relative fold-induction indicated under each lane. Removal of the adrenal glands did not alter the basal activity or the abundance of GNMT; administration of RA was equally effective at elevating GNMT activity (1.9-fold) and abundance (2.0-fold) in ADX rats compared to their respective untreated controls. These findings indicate that RA regulates methyl group metabolism in a manner that is independent of adrenal function.

Hepatic GNMT was activated and induced by both RA and glucocorticoid treatment

Although it did not appear that adrenal function was required for GNMT regulation by RA, a primary objective was to determine whether glucocorticoids, alone or in combination with RA, could also stimulate GNMT expression (**Fig. 3**). For sham-operated rats, treatment with DEX increased the activity of GNMT in a manner similar to that exhibited by RA-treated rats (1.6- and 1.9-fold, respectively). Similar results were observed following treatment of ADX rats with RA and DEX; hepatic GNMT activity was elevated 2.0- and 2.4-fold, respectively. Moreover, when administered together, RA and DEX clearly appeared to have an additive effect on activating GNMT in both sham-operated (2.6-fold) and ADX (3.6-fold) rats compared to control values. The activation of hepatic GNMT by RA and DEX, alone or in combination, was reflected in the abundance of the protein, as shown by the Western blot in **Fig. 4*A***. In contrast, pancreatic GNMT protein abundance was not responsive to either RA, as we have previously reported (25), or DEX treatment in sham-operated (**Fig. 4*B***) or ADX rats (data not shown). The activity of GNMT in the pancreas also remained constant regardless of RA or DEX administration in both sham-operated and ADX rats (data not shown). These results indicate that in addition to RA, the glucocorticoid DEX was equally effective at regulating the expression of GNMT in both sham-operated and ADX rats in a tissue-specific manner; the additive effect of co-administration of RA and DEX suggest that the basis for their action, at least in part, is mechanistically distinct from each other.

Homocysteine levels were reduced by RA and DEX treatment of adrenalectomized rats

Recent work has shown that a diabetic state (i.e., the absence of insulin) or an equivalent elevation in circulating counter-regulatory hormones (i.e., glucagon and/or glucocorticoids) leads to a decrease in plasma homocysteine concentrations in rats due to an increase in homocysteine catabolism through the transsulfuration pathway (12-14). We explored the possibility that RA had a similar ability to reduce homocysteine levels, indicating that the excessive production of homocysteine due to GNMT induction resulted in an increase in its metabolism. **Fig. 5** demonstrates that plasma homocysteine levels were elevated greater than 2-fold as a result of adrenalectomy. Moreover, both RA and DEX were effective in reducing homocysteine levels (41 and 74%, respectively) in ADX rats; no further decrease was observed when RA and DEX were provided simultaneously. In contrast, homocysteine concentrations were not significantly reduced by either RA and/or DEX treatment in sham-operated rats.

Induction of GNMT in rat hepatoma cells

To assess the ability of RA and hormones to directly induce expression of GNMT protein, we utilized a cell culture system consisting of a rat hepatoma cell line. GNMT is not expressed to a significant degree in most cell lines, including human HepG2 cells; however, in preliminary experiments we found that H4IIE cells did express discernible levels of GNMT and the regulation of GNMT in this cell line was sensitive to factors shown to be effective *in vivo*. As shown in **Fig. 6**, GNMT abundance in H4IIE cells was subject to regulation by RA (*lanes 1-4*). This regulation of GNMT protein abundance by RA in H4IIE cells was similar to what was observed in rat liver (*lanes 5-8*). For comparative purposes, **Fig. 6** also demonstrates that the level of expressed GNMT protein, relative to a given amount of total cellular protein, was significantly less in H4IIE cells compared to rat liver. Further comparison of GNMT regulation in H4IIE cells to rat liver is shown in **Fig. 7**. Similar to the rat liver data shown earlier in **Fig. 3**, RA and DEX treatment of H4IIE cells increased GNMT abundance 2.6- and 3.6-fold, respectively. Moreover, co-administration with both compounds resulted in an additive 4.8-fold stimulation of GNMT expression. These results using a cell culture model demonstrate that both RA and DEX regulate methyl group metabolism directly, in the absence of other physiological factors. Moreover,

pretreatment of H4IIE cells with actinomycin D abolished the ability of both RA and DEX to increase GNMT abundance, indicating transcriptional mechanisms were involved (data not shown).

In addition to DEX, a number of other components have also been shown to play a potential role in the regulation of GNMT, methyl group and homocysteine metabolism. Thus, H4IIE cells were treated with various combinations of RA, DEX, Bt_2cAMP, and glucagon (**Fig. 8**). As shown earlier in **Fig. 7**, the immunoblot shown in **Fig. 8*A*** demonstrates that exposure of H4IIE cells to RA or DEX (*lanes 2* and *3*, respectively, compared to *lane 1*) resulted in the induction of GNMT protein (2.1- and 1.8-fold, respectively) and cells incubated with both RA and DEX (*lane 6*) exhibited an elevation of GNMT protein abundance that appeared to be additive (4.8-fold increase). In contrast, incubation with Bt_2cAMP alone did not induce GNMT compared to controls (compare *lanes 4* and *lane 1*). Moreover, the addition of Bt_2cAMP appeared to attenuate GNMT induction in cell cultures treated with RA and/or DEX (compare *lanes 5* and *2*; *lanes 7* and *3*; *lanes 8* and *6*). As shown in **Fig. 8*B***, experiments with glucagon-treated H4IIE cells (*lane 3*) resulted in similar findings as Bt_2cAMP (*lane 4*) in that no induction was discernible compared to untreated cells (*lane 1*), in contrast to the 4.5-fold increase exhibited by cells treated with both RA and DEX (*lane 2*).

Discussion

GNMT regulates the supply of methyl groups available for transmethylation reactions through its control of the SAM/SAH ratio (1). Because GNMT constitutes 1-3% of total cytosolic protein in the liver (2), and its enzymatic activity is strongly inhibited by folate coenzymes (6,7), the physiological role of GNMT appears to be of major significance. A number of conditions are known to produce alterations in GNMT function and subsequent methyl group metabolism including various nutritional and/or metabolic disorders such as folate deficiency (30), methyl group excess (31,32), ethanol administration (33), diabetes (15), genetic disorders (34,35) as well as gender (25), and age (36). Because an adequate supply of methyl groups must be maintained to ensure that important SAM-dependent

transmethylation reactions (e.g. DNA methylation) can readily occur, a perturbation of GNMT may lead to a number of pathological conditions, such as carcinogenesis.

Here, and in our earlier work (11,23,25), we showed that retinoid compound represent a family of compounds that have the ability to modulate GNMT and methyl group metabolism. In addition, the results of the present study clearly indicate that GNMT is modulated by RA at the transcriptional/translational level in H4IIE rat hepatoma cells. This is a novel regulatory aspect because previous work in tissue culture has focused on posttranslational modification of GNMT via phosphorylation or allosteric binding by 5-methyl-THF as mechanisms by which GNMT can be modulated (6,7). Although we certainly have not ruled such posttranslational modification of GNMT out as an explanation of our findings, a more likely scenario for the retinoid-mediated increase of GNMT protein abundance observed in the present study may be increased stability of the protein and/or GNMT mRNA.

In contrast to previous work that showed adrenal function was required to mediate the catabolic events of vitamin A compounds on methionine metabolism (19-21), our results indicate that RA exerted its effects on GNMT independent of intact adrenal function and increased secretion of glucocorticoids. We demonstrated for the first time, however, that treatment with exogenous glucocorticoids (DEX) was as effective as RA in inducing active GNMT in hepatic tissue of both intact and ADX rats. Furthermore, when DEX and RA were co-administered to rats and H4IIE cells, the induction of active GNMT appeared to be additive, suggesting that these compounds may exert their actions by two distinct cellular mechanisms. In contrast, it was clear that GNMT was not sensitive to treatment with glucagon or Bt_2cAMP treatment. Because no response elements for RA, glucocorticoids, or cAMP are known to exist in the promoter region of GNMT, it is likely that the effects of these compounds on GNMT is secondary to other effects.

Based on earlier studies that showed that the catabolism of methionine was enhanced in rats pre-treated with retinol (21), we hypothesized that increased production of methionine degradation products (i.e. homocysteine) would occur as a result of GNMT activation. However, our results do not indicate that GNMT induction by either RA or DEX treatment is consistent with increased homocysteine concentrations. In fact, both RA and DEX

treatments essentially normalized elevated homocysteine concentrations present in ADX rats, indicating that the catabolism of homocysteine through the transsulfuration pathway may have been enhanced following treatment with these compounds. This is consistent with earlier findings that showed diabetic conditions (i.e. gluconeogenesis) and treatment with glucocorticoids facilitated the removal of homocysteine through the transsulfuration pathway (12,14,15). Thus, our findings with RA-treated rats indicate that in addition to glucocorticoids (14), retinoids may represent a group of compounds that increase the expression of enzymes central to the transsulfuration pathway. We are intrigued by the fact that homocysteine levels were elevated in ADX rats before treatment with RA and/or DEX despite GNMT activity remaining unaltered compared to the intact control animals. The latter indicates that the basal rate of methionine catabolism may not be dependent on adrenal function. Likewise, the induction of active GNMT by RA and DEX was similar in both intact and ADX rats. Therefore, it is possible the increased homocysteine levels in ADX rats resulted from the down-regulation of the remethylation process, which may be related to decreased glucocorticoid synthesis by the adrenals. This is supported by previous studies examining the hormonal regulation of MTHFR that showed glucocorticoids enhance its enzymatic activity (37). Recent data in our laboratory indicates that retinoids may have a similar ability to enhance the folate-dependent remethylation of homocysteine in intact rats by increasing methionine synthase (MS) activity (38). In the present study, administration of RA and/or DEX did not reduce plasma homocysteine levels in intact rats; however, we have consistently found that younger rats treated with RA for longer time periods (8-10 d) had a significant (~50%) reduction in the concentration of plasma homocysteine (38).

A number of other possible explanations for the reduction of homocysteine levels must be considered in addition to enhanced transsulfuration. Although it is logical to presume that the increased catabolism of methionine following GNMT induction would produce elevated homocysteine concentrations, the subsequent down-regulation of other SAM-dependent transmethylation reactions may be responsible for reducing homocysteine levels. Previous work in our laboratory has demonstrated retinoid-mediated GNMT induction is consistent with the perturbation of a number of SAM-dependent transmethylation reactions, including the methylation of DNA and the synthesis of creatinine

(25), and may be indirectly responsible for the development hepatic steatosis (39), presumably due to decreased phosphatidylcholine (PC) synthesis (40). Interestingly, a recent study showed that phosphatidylethanolamine *N*-methyltransferase, the SAM-dependent enzyme required for PC synthesis, plays a major role in the regulation of homocysteine metabolism (41). Creatinine and PC synthesis are SAM-dependent transmethylation reactions that require a significant supply of methyl groups, therefore; it seems plausible that if either reaction were perturbed, a reduction of plasma homocysteine concentrations would occur.

We have found an association between glucocorticoid and retinoid administration with alterations of methyl group/homocysteine metabolism. Thus, our findings have significant implications in a number of different areas related to health and disease. Our findings add to the increasing evidence that implicates gluconeogenic conditions, such as uncontrolled diabetes, in the alteration of methyl group and homocysteine metabolism. Diabetes and administration of glucocorticoids and/or glucagon are known to modulate homocysteine metabolism and the activity of GNMT (12-15). Similarly, the results in the present study indicate that administration of RA can modulate methyl group metabolism, which may be related to retinoid-mediated gluconeogenic conditions. This is supported by preliminary data in our lab that showed diabetic rats were significantly more sensitive to increases in blood glucose levels and GNMT induction by treatment with RA (Rowling, M. J. and Schalinske, K. L., unpublished data). These findings suggest that diabetic individuals receiving retinoid therapy for conditions such as cystic acne and various forms of leukemia may require alternative therapies for their conditions or need to be closely monitored. The sensitivity of humans to perturbation of methyl group metabolism by retinoid compounds may also have significance with respect to individuals carrying common mutations of key enzymes involved in folate, and methyl group/homocysteine metabolism, such as CβS and MTHFR polymorphisms. With the increasing prevalence of retinoids being prescribed for therapeutic purposes (~800,000 new cases each year) (42), the need for monitoring these individuals may become more apparent.

Literature Cited

1. Wagner, C. (1995) Biochemical role of folate in cellular metabolism. In: *Folate in Health and Disease* (Bailey, L. B., ed.), pp. 23-42, Marcel Dekker, New York, NY.

2. Kerr, S. J. (1972) Competing methyltransferase systems. *J. Biol. Chem.* 247: 4248-4252.

3. Cantoni, G. L. and Chiang, P. K. (1980) The role of *S*-adenosylhomocysteine hydrolase in the control of biological methylation reactions. In: *Natural Sulfur Compounds* (Cavallini, D., Gaull, G. E., and Zappia, V., eds.) pp. 67-80. Plenum Press, New York, NY.

4. Cantoni, G. L. (1982) *S*-adenosyl amino acids thirty years later: 1951-1981. In: *The Biochemistry of S-adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., and Creveling, C. R., eds.) pp. 3-10. Macmillan Press, London, England.

5. Jencks, D. A. and Matthews, R. G. (1987) Allosteric inhibition of methylenetetrahydrofolate reductase by adenosylmethionine. *J. Biol. Chem.* 262: 2485-2493.

6. Wagner, C., Briggs, W. T., and Cook, R. B. (1985) Inhibition of glycine *N*-methyltransferase activity by folate derivatives: Implications for regulation of methyl group metabolism. *Biochem. Biophys. Res. Commun.* 127(3) 746-752.

7. Wagner, C., Decha-Unphai, W., and Corbin, J. (1989) Phosphorylation modulates the activity of glycine *N*-methyltransferase, a folate binding protein. *J. Biol. Chem.* 264(16): 9638-9642.

8. Steegers-Theunissen, R. P. M., Boers, G. H. J., Trijbels, F. J. M., Finkelsteine, J. D., Blom, H. J., Thomas, C. M. G., Borm, G. F., Wouters, M. G. A. J., and Eskes, T. K. A. B. (1994) Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? *Metabolism.* 43(12): 1475-1480.

9. McCully, K. S. and Wilson, R. B. (1975) Homocysteine theory of arteriosclerosis. *Atherosclerosis.* 22: 215-227.

10. Newberne, P. M. and Rogers, A. E. (1986) Labile methyl groups and the promotion of cancer. *Ann. Rev. Nutr.* 6: 407-432.

11. Rowling, M. J., McMullen, M. H., and Schalinske, K. L. (2002) Vitamin A and its derivatives activate and induce glycine *N*-methyltransferase and DNA hypomethylation in rats. *J. Nutr.* 132: 365-369.

12. Jacobs, R. L., House, J. D., Brosnan, M. E., and Brosnan, J. T. (1998) Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat. *Diabetes.* 47(12): 1967-70.

13. Jacobs, R. L., Stead, L. M., Brosnan, M. E., and Brosnan, J. T. (2001) Hyperglucagonemia in rats results in decreased plasma homocysteine and increased flux through the transsulfuration pathway. *J. Biol. Chem.* 276(47): 43740-43747.

14. Ratnam, S., Maclean, K. N., Jacobs, R. L., Brosnan, M. E., Kraus, J. P., and Brosnan, J. T. (2002) Hormonal regulation of cystathionine β-synthase expression in liver. *J. Biol. Chem.* 277(45) 42912-42918.

15. Xue, G-P. and Snoswell, A. M. (1985) Disturbance of methyl group metabolism in alloxan-diabetic sheep. *Biochem. Int.* 10(6): 897-905.

16. Yeo, E-J. and Wagner, C. (1994) Tissue distribution of pancreatic glycine *N*-methyltransferase, a major folate-binding protein of liver. *Proc. Natl. Acad. Sci. USA.* 91: 210-214.

17. Pan, C-J., Hoeppner, W., and Yang Chou, J. (1990) Induction of phosphoenolypyruvate carboxykinase gene expression by retinoic acid in an adult rat hepatocye line. *Biochemistry.* 29(49): 10883-10888.

18. Shin, D. J. and McGrane, M. M. (1987) Vitamin A regulates genes involved in hepatic gluconeogenesis in mice: phophoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J. Nutr.* 127: 1274-1278.

19. Peng, Y-S. & Evenson, J.K. (1979) Alleviation of methionine toxicity in young male rats fed high levels of retinol. *J. Nutr.* 109: 281-290.

20. Peng, Y-S., Russell, D.H. & Evenson, J.K. (1981) Alleviation of methionine toxicity by glycine and serine in rats pretreated with excess retinol. *Nutr. Rep. Inter.* 23: 303-311.

21. Peng, Y-S. and Evenson, J. K. (1982) Alleviation of methionine toxicity in rats by retinol-pretreatment: *in vivo* methionine catabolism and role of adrenal glands. *Nutr. Reports Int.* 25(1): 139-144.

22. Schalinske, K. L. and Steele, R. D. (1991) 13-*cis*-retinoic alters methionine metabolism in rats. *J. Nutr.* 121: 1714-1719.

23. Rowling, M.J. & Schalinske, K.L. (2001) Retinoid compounds activate and induce hepatic glycine N-methyltransferase in rats. *J. Nutr.* 131: 1914-1917.

24. Cook, R. J. and Wagner, C. (1984) Glycine *N*-methyltransferase is a folate binding protein of rat liver cytosol. *Proc. Natl. Acad. Sci. U.S.A.* 81: 3631-3634.

25. McMullen, M. H., Rowling, M. J., Ozias, M. K., and Schalinske, K. L. (2002) Activation and induction of glycine *N*-methyltransferase by retinoids are tissue- and gender-specific. *Arch. Biochem. Biophys.* 401: 73-80.

26. Liu, H.-H., Chen, K.-H., Shih, Y.-P., Lui, W.-Y., Wong, F.-H., and Chen, Y.-M. A. (2003) Characterization of reduced expression of glycine *N*-methyltransferase in cancerous hepatic tissues using two newly developed monoclonal antibodies. *J. Biomed. Sci.* 10: 87-97.

27. Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* 72: 248-254.

28. Araki, A. and Sato, Y. (1987) Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with flourescence detection. *J. Chromatogr.* 422: 43-52.

29. Snedecor, G. W. and Cochran, W. G. (1980) Statistical Methods, 7th ed. Iowa State University Press, Ames, IA.

30. Balaghi, M., Horne, D. W., and Wagner, C. (1993) Hepatic one-carbon metabolism ion early folate deficiency in rats. *Biochem. J.* 291: 145-149.

31. Rowling, M. J., McMullen, M. H., Chipman, D. C., and Schalinske, K. L. (2002) Hepatic glycine *N*-methyltransferase is up-regulated by excess dietary methionine in rats. *J. Nutr.* 132: 2545-2549.

32. Ogawa, H. and Fujioka, M. (1982) Induction of rat liver glycine methyltransferase by high methionine diet. *Biochem. Biophys. Res. Commun.* 108: 227-232.

33. Trimble, K. C., Molloy, A. M., Scott, J. M., and Weir, D. G. (1993) The effect of ethanol on one-carbon metabolism: increased methionine catabolism and lipotrope methyl-group wastage. *Hepatology*: 18(4): 984-989.

34. Luka, Z., Cerone, R., Phillips III, J. A., Mudd, S. H., and Wagner, C. (2002) Mutations in human glycine *N*-methyltransferase give insights into its role in methionine metabolism. *Hum. Genet.* 110: 68-74.

35. Mudd, S. H., Cerone, R., Schiaffino, M. C., Fantasia, A. R., Minniti, G., Caruso, U., Lorini, R., Watkins, D., Matiaszuk, N., Rosenblatt, D. S., Schwahn, B., Rozen, R., LeGros, L., Kotb, M., Capdevila, A., Luka, Z., Finkelstein, J. D., Tangerman, A., Stabler, S. P., Allen, R. H., and Wagner, C. (2001) Glycine *N*-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninemia. *J. Inherit. Metab. Dis.* 24: 448-464.

36. Mays, L.L., Borek, E. & Finch, C.E. (1973) Glycine N-methyltransferase is a regulatory enzyme which increases in ageing animals. *Nature* 243: 411-413.

37. Finkelstein, J.D., Martin, J.J., Kyle, W.E. & Harris, B.J. (1978) Methionine metabolism in mammals: regulation of methylenetetrahydrofolate reductase content of rat tissues. *Arch. Biochem. Biophys.* 191: 153-160.

38. Ozias, M. K. and Schalinske, K. L. (2003) All-*trans*-retinoic acid rapidly induces glycine *N*-methyltransferase in a dose-dependent manner and reduces circulating methionine and homocysteine levels in rats. *J. Nutr.* 133: in press.

39. Schalinske, K.L. & Steele, R.D. (1993) 13-*cis*-Retinoic acid and hepatic steatosis in rats. *Biochem. Pharmacol.* 46: 319-325.

40. Fell, D. and Steele, R. D. (1987) Effect of retinol toxicity on hepatic *S*-adenosylmethionine-dependent transmethylation in rats. *Drug Nutr. Interact.* 5: 1-7.

41. Noga, A. A., Stead, L. M., Zhao, Y., Brosnan, M. E., Brosnan, J. T., and Vance, D. E. (2003) Plasma homocysteine is regulated by phospholipid methylation. *J. Biol. Chem.* 278(8): 5952-5955.

42. Wysowski, D.K., Swann, J. & Vega, A. (2002) Use of isotretinoin (Accutane) in the United States: rapid increase from 1992 through 2000. *J. Am. Acad. Dermatol.* 46: 505-509.

FIGURE LEGENDS

FIGURE 1 Folate, methyl group, and homocysteine metabolism. Glycine *N*-methyltransferase (GNMT) regulates the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH), thereby optimizing transmethylation reactions ($X \rightarrow X\text{-}CH_3$), such as the methylation of DNA, RNA, proteins, and phospholipids. SAM is an allosteric inhibitor of 5,10-methylenetetrahydrofolate reductase (MTHFR) and positive modulator of cystathionine β-synthase (CBS), whereas 5-methyltetrahydrofolate ($5\text{-}CH_3\text{-}THF$) is an inhibitory ligand for GNMT. Other abbreviations used: BHMT, betaine homocysteine methyltransferase; MS, methionine synthase; THF, tetrahydrofolate.

FIGURE 2 Induction of hepatic glycine N-methyltransferase (GNMT) by retinoic acid (RA) in sham-operated and adrenalectomized (ADX) rats. Sham-operated and ADX male Sprague Dawley rats were treated with a daily dose of RA (30 μmol/kg BW) for 7 d. Liver samples were removed and analyzed for GNMT activity and abundance as described under "Materials and Methods." Data are expressed as means ± SEM (n=5) and bars denoted with different letters are significantly different ($p < 0.05$). *A*, GNMT enzyme activity in sham-operated and ADX rats following administration of RA or corn oil. *B*, Western blot analysis of GNMT abundance in sham-operated and ADX rats following administration of RA or corn oil. A polyclonal IgG-purified antibody against rat liver GNMT was used and a representative immunoblot is shown with the relative fold induction indicated under each lane.

FIGURE 3 Induction of hepatic glycine N-methyltransferase (GNMT) by retinoic acid (RA), dexamethasone (DEX) in sham-operated and adrenalectomized (ADX) rats. Sham-operated and ADX male Sprague Dawley rats were treated with either a daily dose of RA (30 μmol/kg BW), DEX (1 mg/kg BW), or both. After 5 d, liver samples were removed and GNMT activity was determined as described under "Materials and Methods". Data are means ± SEM (n=5) and bars denoted by a different letter are significantly different ($p < 0.05$).

FIGURE 4 Regulation of hepatic and pancreatic glycine N-methyltransferase (GNMT) abundance by retinoic acid (RA) and dexamethasone (DEX) in sham-operated and adrenalectomized (ADX) rats. Tissue samples from the same rats as described for Fig. 3 were used for the determination of GNMT abundance by Western blot analysis as described under "Materials and Methods". *A*, representative immunoblot of hepatic GNMT abundance in sham-operated and ADX rats treated with RA, DEX, or both compounds. The relative fold-induction is indicated under each lane. *B*, representative immunoblot of pancreatic GNMT abundance in sham-operated rats treated with RA, DEX, or both compounds. All blots are representative examples from individual rat tissue samples.

FIGURE 5 Plasma homocysteine concentrations in sham-operated and adrenalectomized (ADX) rats following treatment with retinoic acid (RA), dexamethasone (DEX), or both. Plasma samples from the same rats as described for Fig. 3 were obtained for the assessment of total homocysteine concentrations by high-performance liquid chromatography as described under "Materials and Methods". Data are means ± SEM (n=5) and bars denoted by a different letter are significantly different ($p < 0.05$).

FIGURE 6 Comparison of retinoic acid (RA) regulation of glycine N-methyltransferase (GNMT) abundance in rat hepatoma cells and rat liver. H4IIE rat hepatoma cells (*lanes 1-4*) were treated with 10 μmol/L RA dissolved in Me_2SO (*lanes 2* and *4*) or an equivalent volume of Me_2SO (100 μL/L final concentration) alone (*lanes 1* and *3*). Following a 72-h incubation period, cells were lysed and Western blot analysis was performed as described under "Materials and Methods". Male Sprague Dawley rats were administered a single dose of RA (30 μmol/kg BW) or corn oil daily for a total of 7 d. Liver samples were removed for determination of GNMT abundance by Western blot analysis as described under "Materials and Methods". For comparison purposes, 75 (*lanes 1, 2, 5* and *6*) and 150 μg of total protein (*lanes 3,4,7* and *8*) were analyzed for both cell and tissue lysates.

FIGURE 7 Regulation of glycine N-methyltransferase (GNMT) by retinoic acid (RA) and dexamethasone (DEX) in rat hepatoma cells. H4IIE rat hepatoma cells were treated with

either DEX (0.1 μmol/L), RA (10 μmol/L), or both. After a 72-h incubation period, cells were lysed and GNMT abundance was determined as described under "Materials and Methods". The representative immunoblot and the densitometric analysis shown in the bar graph were generated from three independent experiments.

FIGURE 8 Regulation of glycine N-methyltransferase (GNMT) abundance by various combinations of retinoic acid (RA), dexamethasone (DEX), dibutyryl-cAMP (Bt_2cAMP), and glucagon in rat hepatoma cells. H4IIE rat hepatoma cells were treated alone or with various combinations of DEX, RA, Bt_2cAMP, or glucagon. After a 72-h incubation period, cells were lysed and GNMT abundance was determined as described under "Materials and Methods". *A, lane 1*, Me_2SO-treated control; *lane 2*, RA (10 μmol/L); *lane 3*, DEX (0.1 μmol/L); *lane 4*, Bt_2cAMP (0.5 mmol/L); *lane 5*, RA + Bt_2cAMP; *lane 6*, RA + DEX; *lane 7*, DEX + Bt_2cAMP; and *lane 8*, RA + DEX + Bt_2cAMP. *B, lane 1*, Me_2SO-treated control; *lane 2*, RA + DEX; *lane 3*, glucagon (0.5 μmol/L); *lane 4*, Bt_2cAMP; and *lane 5*, rat liver (RA-treated) positive control.

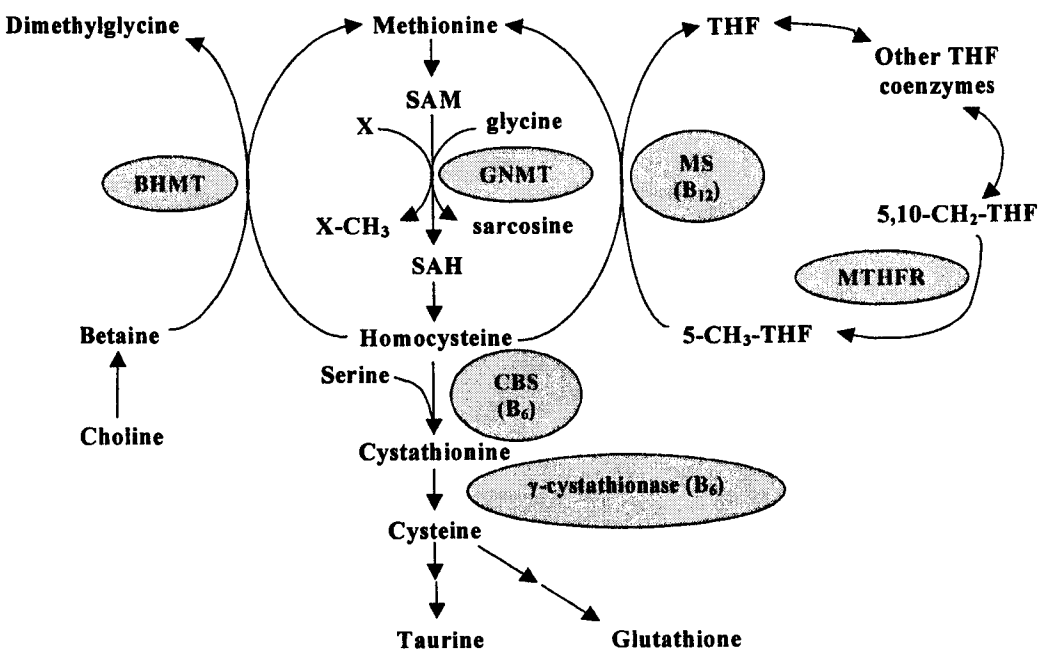
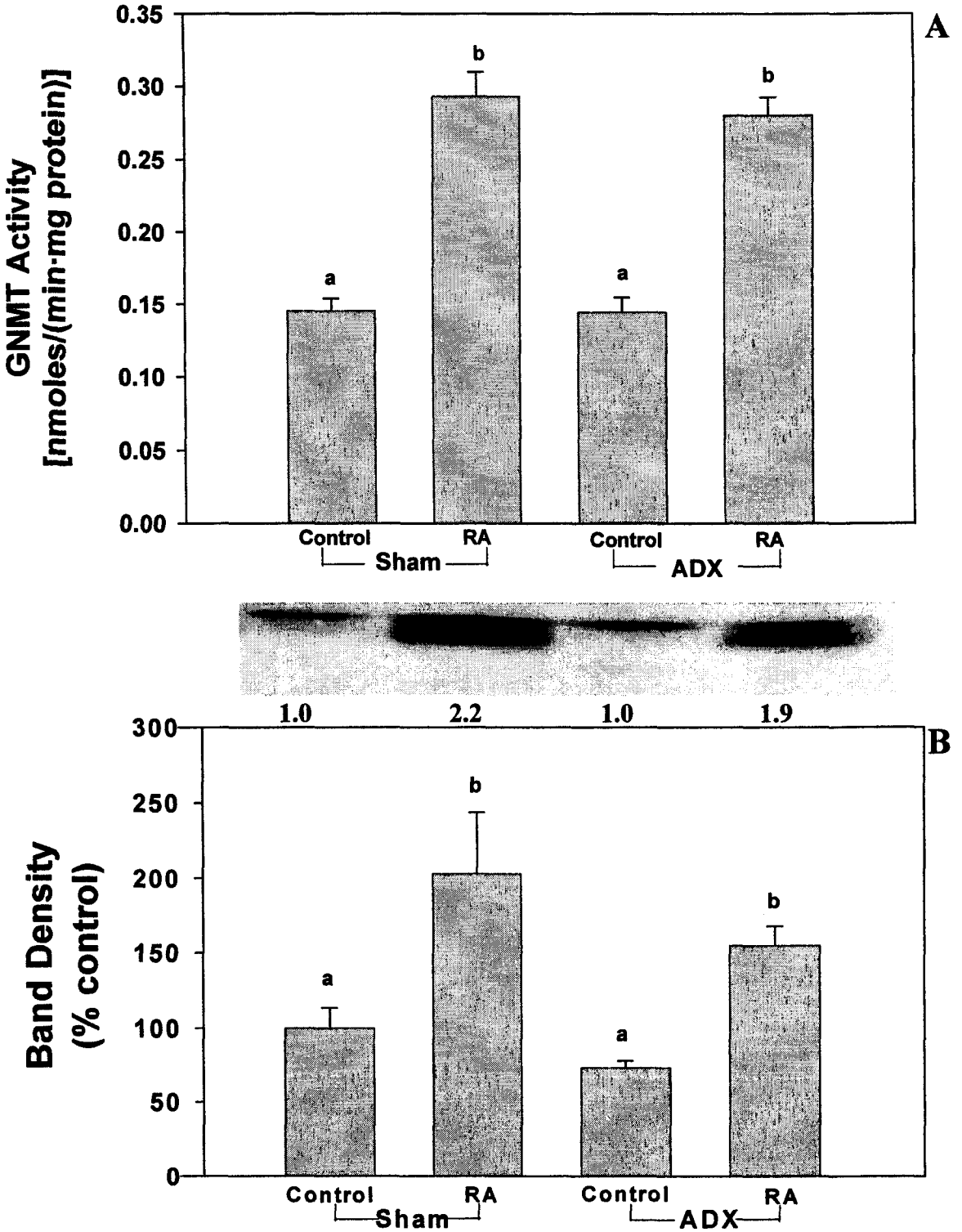

Dimethylglycine
Methionine
THF
Other THF coenzymes
SAM
X
glycine
BHMT
GNMT
MS (B_{12})
$X\text{-}CH_3$
sarcosine
$5,10\text{-}CH_2\text{-}THF$
SAH
MTHFR
Betaine
Homocysteine
$5\text{-}CH_3\text{-}THF$
Serine
CBS (B_6)
Choline
Cystathionine
γ-cystathionase (B_6)
Cysteine
Taurine
Glutathione

Figure 1


A
GNMT Activity
[nmoles/(min·mg protein)]
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
b
a
b
Control
RA
Control
RA
Sham
ADX
1.0
2.2
1.0
1.9
B
Band Density
(% control)
300
250
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150
100
50
0
a
b
a
b
Control
RA
Control
RA
Sham
ADX

Figure 2

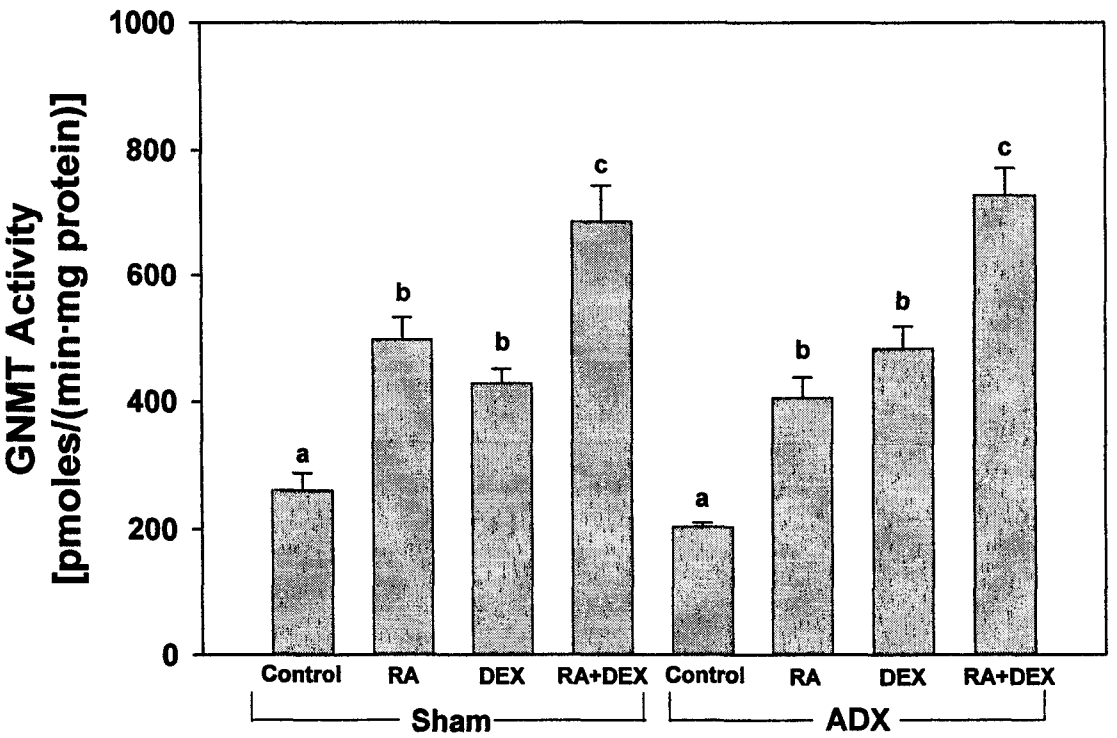
GNMT Activity
[pmoles/(min·mg protein)]
1000
800
600
400
200
0
a
b
b
c
a
b
b
c
Control
RA
DEX
RA+DEX
Control
RA
DEX
RA+DEX
Sham
ADX

Figure 3

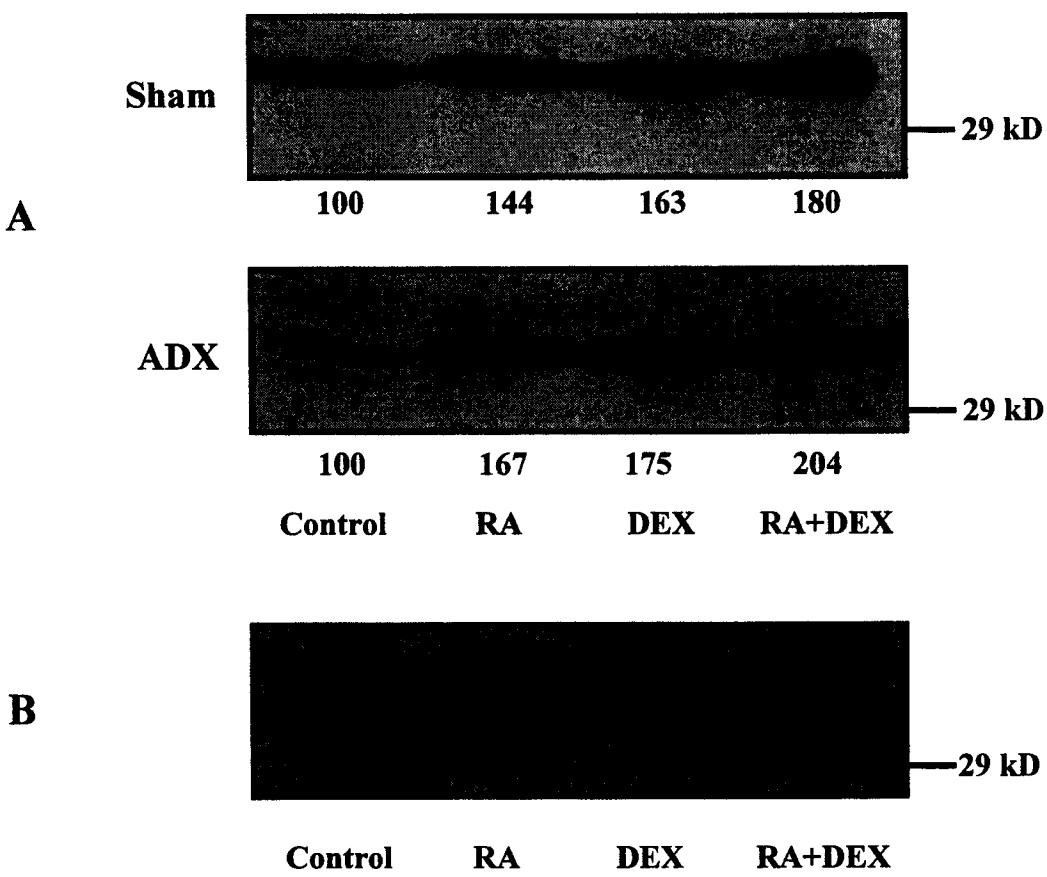
A
Sham
29 kD
100
144
163
180
ADX
29 kD
100
167
175
204
Control
RA
DEX
RA+DEX
B
29 kD
Control
RA
DEX
RA+DEX

Figure 4

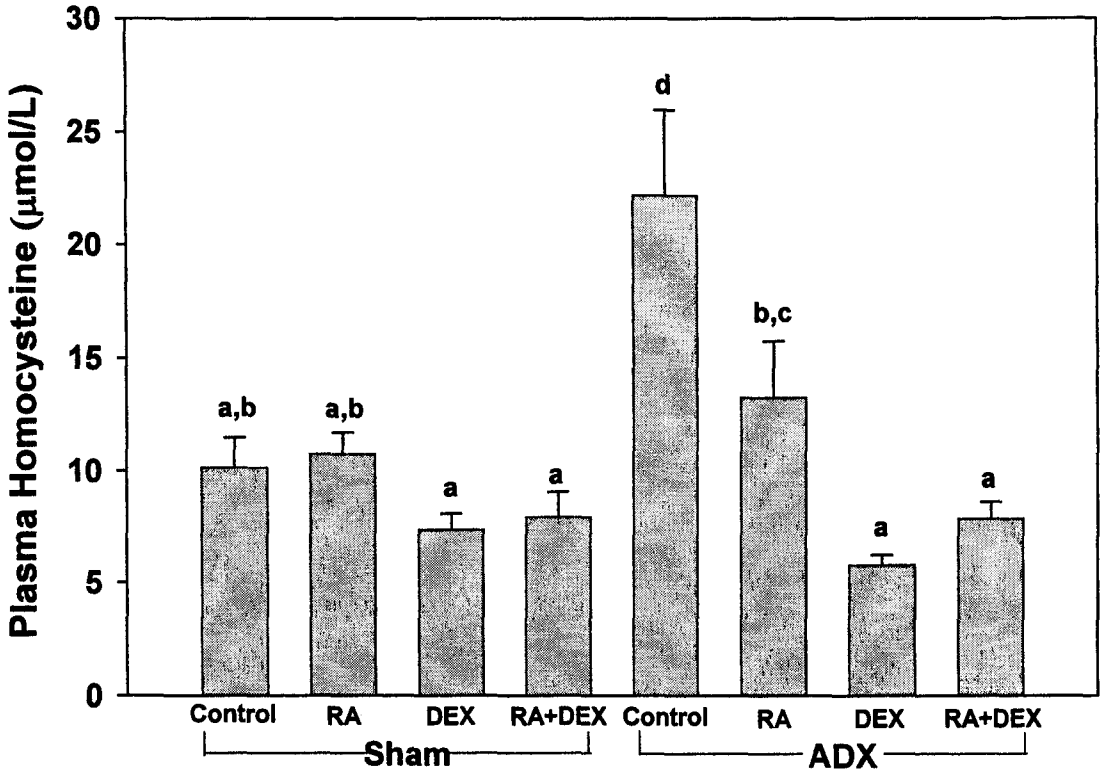
Plasma Homocysteine (µmol/L)
30
25
20
15
10
5
0
a,b
a,b
a
a
d
b,c
a
a
Control
RA
DEX
RA+DEX
Control
RA
DEX
RA+DEX
Sham
ADX

Figure 5

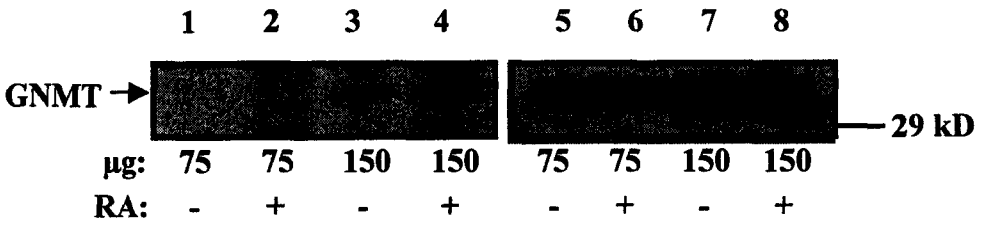

Figure 6

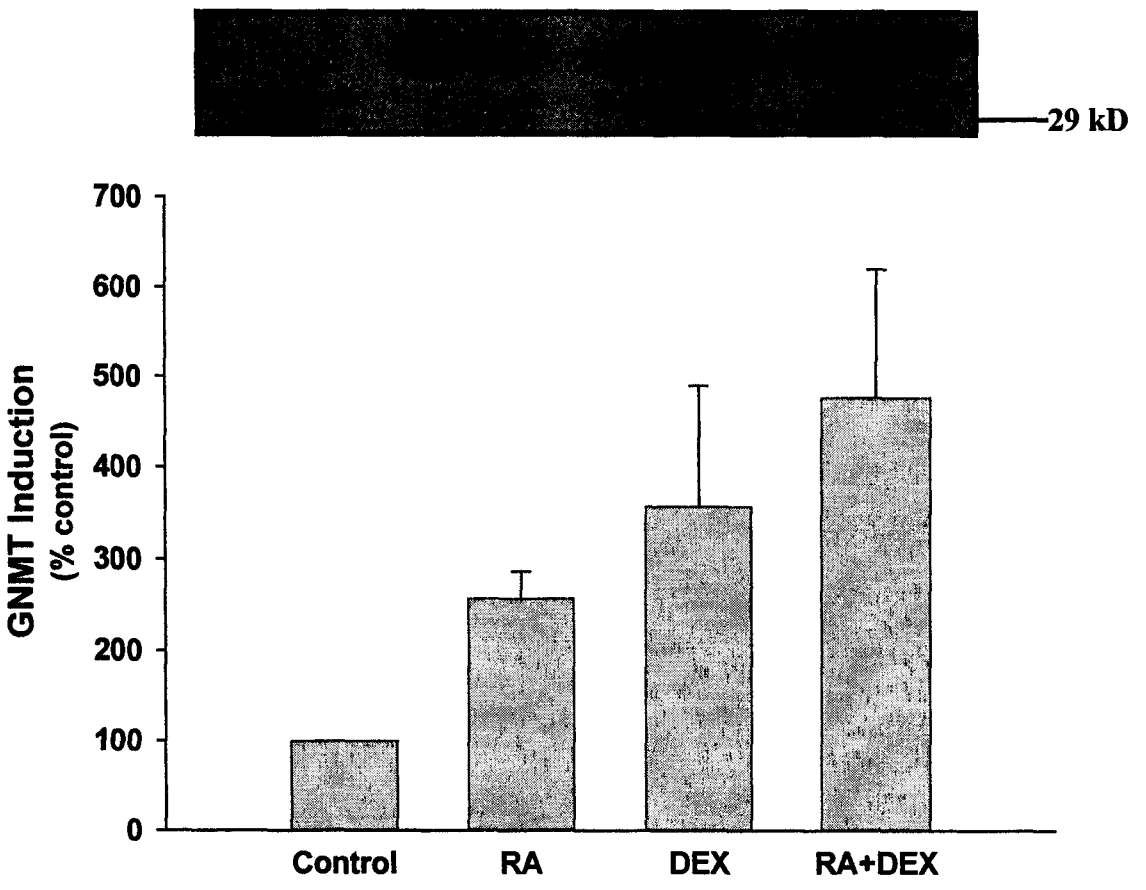
29 kD
GNMT Induction
(% control)
700
600
500
400
300
200
100
0
Control
RA
DEX
RA+DEX

Figure 7

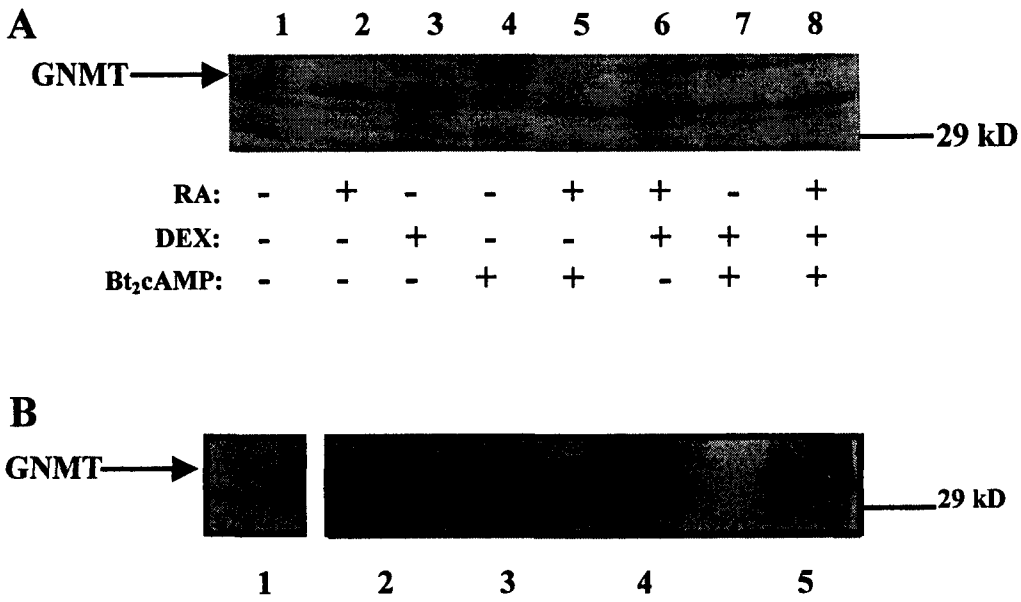

Figure 8

GENERAL CONCLUSIONS

GNMT is a key regulatory protein that controls the supply of methyl groups available for transmethylation reactions by facilitating the disposal of excess SAM. Therefore, inappropriate activation of GNMT may lead to conditions consistent with methyl group deficiency. In the work presented in this dissertation, I illustrated for the first time that retinoids represent a family of nutritional compounds that have the ability to induce the gene expression of hepatic GNMT, which in turn resulted in the increased catabolism of methyl groups and compromised transmethylation reactions, namely the methylation of hepatic DNA. These findings clearly indicate that GNMT up-regulation has possible implications for the development of pathological conditions that are associated with abnormal methyl group metabolism, such as cancer, neural tube defects, and heart disease (Salmon and Copeland 1954; Clarke *et al.* 1991; Haynes 2003).

Enzymatically active GNMT resides primarily in gluconeogenic tissues, such as the liver, kidney, and pancreas (Yeo and Wagner 1994), which suggests that GNMT has a role during gluconeogenesis. Additionally, GNMT has been shown to be up-regulated during conditions associated with gluconeogenesis, a process during which retinoic acid, along with hormones such as glucocorticoids and glucagon, are known to increase the gene expression of various gluconeogenic enzymes, such as PEPCK, the rate-limiting enzyme of gluconeogenesis (Pan *et al.* 1990; Shin and McGrane 1998). However, to date no retinoic acid response elements have been discovered in the promoter region of the GNMT gene, indicating RA may require alternative signals to exert its effect on GNMT. Previous studies showed that functional adrenal glands were required to alleviate the symptoms of methionine toxicity following treatment with retinol (Peng and Evenson 1982), indicating that glucocorticoid secretion from the adrenals may play a key role in the catabolism of methionine. Hence, we postulated that the effect of retinoids on GNMT expression is mediated by an induction of gluconeogenesis due to increased blood concentrations of counter-regulatory hormones, namely glucocorticoids secreted from the adrenals. However, since my studies showed that GNMT activity and abundance did not differ between ADX and sham control rats given DEX and/or RA, I have concluded that, although it is clear that

glucocorticoids have a similar ability to that of retinoids to induce enzymatically active GNMT, the induction of GNMT by retinoids does not require adrenal function. We believe that the discovery of glucocorticoids as hormonal factors that can modulate GNMT expression is a significant finding however, because it indicates that, like retinoids, elevated levels of glucocorticoids, which are commonplace during uncontrolled diabetes, may perturb methyl group metabolism in a fashion similar to that of RA.

The mechanism by which nutritional and hormonal factors increase GNMT expression remains unclear. Because our findings do not suggest a synergistic effect results from the co-administration of retinoids and glucocorticoids, but rather an additive effect on the induction of active GNMT protein in both animal and cell culture models, we believe it is likely that these two families of compounds modulate GNMT by two separate mechanisms. This is supported by our work that showed co-administration of RA and DEX at saturating concentrations produced an additive effect in increasing GNMT activity and abundance in rats. Likewise, GNMT abundance was elevated in H4IIE cells in a similar fashion upon direct administration of DEX and RA, which clearly suggests that the effect of these compounds on GNMT is mediated at the cellular level.

Elucidating the mechanism by which nutritional and hormonal factors influence GNMT gene expression remains an area of intense research in our laboratory. We have recently discovered that the effect of RA and DEX on GNMT was prevented in H4IIE cells pre-treated with actinomycin D (Rowling and Schalinske, unpublished observations), a potent inhibitor of transcription. This provides evidence that the effect of RA and DEX on GNMT may be mediated at the transcriptional level. To expand on these findings, we are currently conducting experiments that measure GNMT mRNA levels in rats and cells that have been treated with a number of nutritional and hormonal factors, including as RA and DEX. However, because RA- and DEX- mediated GNMT protein induction may not be due solely to increased transcription, but rather stabilization of GNMT mRNA, increased translation and/or GNMT protein stabilization, we are conducting sets of experiments that measure the synthesis and degradation rates of GNMT mRNA and GNMT protein. Taken together, we expect that these experiments will assist us in pinpointing the cellular mechanism that is being utilized to increase the expression of GNMT.

We are also in the process of planning and conducting experiments that utilize our diabetic rat and cell culture models to explore the possible mechanistic role of insulin in the prevention of abnormal methyl group metabolism as well as to discover interactions between diabetes and retinoid compounds as they relate to increased GNMT expression and alterations of other aspects of methyl group, folate, and homocysteine metabolism. Although we are still at the early stages of this research area, we have recently discovered that treatment of H4IIE hepatoma and AR42J pancreatic tumor cells with insulin prevented induction of GNMT protein abundance following RA and DEX treatment (Rowling and Schalinske, unpublished observations).

I expect that the research presented in this dissertation, as well as future research in Dr. Schalinske's laboratory, will help provide a basis for (i) establishing future dietary recommendations to prevent abnormal methyl group metabolism in diabetics and those using therapeutic retinoids, (ii) identifying individuals at risk for pathological conditions associated with abnormal methyl group metabolism, and (iii) evaluating interactions between increased GNMT expression and common inborn errors of methyl group metabolism.

LITERATURE CITED

Aida, K., Tawata, M., Negishi, M., and Onaya, T. (1997) Mouse glycine *N*-methyltransferase is sexually dimorphic and regulated by growth hormone. *Horm. Metab. Res.* 29: 646-649.

Alonso-Aperte, E., Ubeda, N., Achón, M., Pérez-Miguelsanz, J., and Varela-Moreiras, G. (1999) Impaired methionine synthesis and hypomethylation in rats exposed to valproate during gestation. *Neurology.* 52: 750-756.

American Academy of Pediatrics Committee on Genetics. (1999) Folic acid for the prevention of birth defects. *Pediatrics.* 104(2): 325-327.

Anderson, J. W. (1999) Nutritional management of diabetes mellitus. In: *Modern Nutrition in Health and Disease* (Shils, M. E., Olson, J. A., Shike, M., and Ross, A. C., eds.) pp. 1365-1418. Williams & Wilkins, Baltimore, USA.

Bailey, L. B. and Gregory, J. F. (1999) Folate metabolism and requirements. *J. Nutr.* 129: 779-782.

Balaghi, M., Horne, D. W., and Wagner, C. (1993) Hepatic one-carbon metabolism in early folate deficiency in rats. *Biochem. J.* 291: 145-149.

Balaghi, M. and Wagner, C. (1992) Methyl group metabolism in the pancreas of folate-deficient rats. *J. Nutr.* 122: 1391-1396.

Balaghi, M. and Wagner, C. (1993) DNA methylation in folate deficiency: use of CpG methylase. *Biochem. Biophys. Res. Comm.* 193(3): 1184-1190.

Balaghi, M. and Wagner, C. (1995) Folate deficiency inhibits pancreatic amylase secretion in rats. *Am. J. Clin. Nutr.* 61: 90-96.

Baroné, C., Bartoloni, C., Ghirlanda, G., and Gentiloni, N. (1979) Megaloblastic anemia due to folic acid deficiency after oral contraceptives. *Haematologica.* 64(2): 190-195.

Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv. Cancer Res.* 72: 141-196.

Benevenga, N. J. and Steele, R. D. (1984) Adverse effects of excessive consumption of amino acids. *Ann. Rev. Nutr.* 4: 157-181.

Benjamins, J. A., Morell, P., Hartman, B. K., and Agrawal, H. C. (1984) Central nervous system myelin. In: *Handbook of Neurochemistry,* 2nd edition (Lajtha, A., ed.) Plenum Press, New York, pp. 361-415.

Bhave, M. R., Wilson, M. J., and Poirier, L. A. (1988) c-H-*ras* and c-K-*ras* gene hypomethylation in the livers and hepatomas of rats fed methyl-deficient, amino acid-defined diets. *Carcinogenesis.* 9(3): 343-348.

Blount, B. C., Mack, M. M., Wehr, C. M., MacGregor, J. T., Hiatt, R. A., Wang, G., Wickramasinghe, S. N., Everson, R. B., and Ames, B. N. (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc. Natl. Acad. Sci. USA.* 94: 3290-3295.

Boddie, A. M., Steen, M. T., Sullivan, K. M., Pasquali, M., Dembure, P. P., Coates, R. J., and Elsas II, L. J. (1998) Cystathionine-β-synthase deficiency: detection of heterozygotes by the ratios of homocysteine to cysteine and folate. *Metabolism.* 47(2): 207-211.

Bostom, A. G., Shemin, D., Lapane, K. L., Nadeau, M. R., Sutherland, P., Chan, J., Rozen, R., Yoburn, D., Jacques, P. F., Selhub, J., and Rosenberg, I. H. (1996) Folate status is the major determinant of fasting total plasma homocysteine levels in maintenance dialysis patients. *Atherosclerosis.* 123: 193-202.

Botez, M. I., Peyronnard, J. M., Bachevalier, J., and Charron, L. (1978) Polyneuropathy and folate deficiency. *Arch. Neurology.* 35(9): 581-4.

Botez, M. I., Young, S. N., Bachevalier, J., and Gauthier, S. (1982) Effect of folic acid and vitamin B12 deficiencies on 5-hydroxyindoleacetic acid in human cerebrospinal fluid. *Ann. Neurol.* 12(5): 479-484.

Bottiglieri, T., Hyland, K., Laundy, M., Godfrey, P., Carney, M. W. P., Toone, B. K., and Reynolds, E. H. (1992) Folate deficiency, biopterin and monoamine metabolism in depression. *Psychol. Med.* 22: 871-876.

Brattström, L. E., Israelsson, B., Jeppson, J-O., and Hultberg, B. L. (1988) Folic acid-an innocuous means to reduce plasma homocysteine. *Scand. J. Clin. Lab. Invest.* 48: 215-221.

Brattström, L., Lindgren, A., Israelsson, B., Malinow, M. R., Norrving, B., Upson, B., and Hamfelt, A. (1992) Hyperhomocysteinemia in stroke: prevalence, cause, and relationships to type of stroke and stroke risk factors. *Eur. J. Clin. Invest.* 22: 214-221.

Bressa, G. M. (1994) S-adenosyl-l-methionine (SAMe) as antidepressant: meta-analysis of clinical studies. *Acta. Neurol. Scand. Suppl.* 154: 7-14.

Cantoni, G. L. (1982) *S*-adenosyl amino acids thirty years later: 1951-1981. In: *The Biochemistry of S-adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., and Creveling, C. R., eds.) pp. 3-10. Macmillan Press, London, England.

Cantoni, G. L. and Chiang, P. K. (1980) The role of *S*-adenosylhomocysteine hydrolase in the control of biological methylation reactions. In: *Natural Sulfur Compounds* (Cavallini, D., Gaull, G. E., and Zappia, V., eds.) pp. 67-80. Plenum Press, New York, NY.

Capdevila, A., Decha-Umphai, W., Song, K-H., Borchardt, R. T., and Wagner, C. (1997) Pancreatic exocrine secretion is blocked by inhibitors of methylation. *Arch. Biochem. Biophys.* 345(1): 47-55.

Carl, G. F. (1986) Effect of chronic valproate treatment on folate-dependent methyl biosynthesis in the rat. *Neurochem. Res.* 11(5): 671-685.

Carratú, M. R., Cassano, T., Coluccia, A., Borracci, P., and Cuomo, V. (2003) Antinutritional effects of fumonisin B1 and pathophysiological consequences. *Toxicol. Lett.* 140-141: 459-463.

Caudill, M. A., Wang, J. C., Melnyk, S., Pogribny, I. P., Jernigan, S., Collins, M. D., Santos-Guzman, J., Swendseid, M. E., Cogger, E. A., and James, S. J. (2001) Intracellular *S*-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine β-synthase heterozygous mice. *J. Nutr.* 131: 2811-2818.

Centers for Disease Control and Prevention. (1991) Use of folic acid for prevention of spina bifida and other neural tube defects: 1983-1991. *MMWR.* 40: 513-516.

Centers for Disease Control and Prevention. (1992) Recommendations for the use folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR.* 41: 1-8.

Centers for Disease Control and Prevention. (1999) Knowledge and use of folic acid by women of childbearing age-United States, 1995 and 1998. *MMWR.* 48: 325-327.

Chen, J., Stampfer, M. J., Ma, J., Selhub, J., Malinow, M. R., Hennekens, C. H., and Hunter, D. J. (2001) Influence of a methionine synthase (D919G) polymorphism on plasma homocysteine and folate levels and relation to risk of myocardial infarction. *Atherosclerosis.* 154: 667-672.

Chiang P. K, Gordon R. K., Tal J., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K., and McCann, P. P. (1996) S-Adenosylmethionine and methylation. *FASEB J.* 10(4): 471-80

Christensen, B., Arbour, L., Tran, P., Leclerc, D., Sabbaghian, N., Platt, R., Gilfix, B. M., Rosenblatt, D. S., Gravel, R. A., Forbes, P., and Rozen, R. (1999) Genetic polymorphisms in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. *Am. J. Med. Gen.* 84: 151-157.

Clarke, R., Daly, L., Robinson, K., Naughten, E., Cahalane, S., Fowler, B., and Graham, I. (1991) Hyperhomocysteinemia: an independent risk factor for vascular disease. *N. Engl. J. Med.* 324: 1149-1155.

Coelho, C. N. D., Weber, J. A., Klein, N. W., Daniels, W. G., and Hoagland, T. A. (1989) Whole rat embryos require methionine for neural tube closure when cultured on cow serum. *J. Nutr.* 119: 1716-1725.

Coelho, C. N. D. and Klein, N. W. (1990) Methionine and neural tube closure in cultured rat embryos: morphological and biochemical analysis. *Teratology.* 42: 437-451.

Cronin, C. C., McPartlin, J. M., Barry, D. G., Barry Ferris, J., Scott, J. M., Weir, D. G. (1998) Plasma homocysteine concentrations in patients with type 1 diabetes. *Diabetes Care.* 21: 1843-1847.

Cuskelly, G. J., Stacpoole, P. W., Williamson, J., Baumgartner, T. G., and Gregory III, J. F. (2001) Deficiencies of folate and vitamin B_6 exert distinct effects on homocysteine, serine, and methionine kinetics. *Am. J. Physiol. Endocrinol. Metab.* 281: E1182-E1190.

Dansky, L. V., Andermann, E., Rosenblatt, D., Sherwin, A. L., and Andermann, F. (1987) Anticonvulsants, folate levels, and pregnancy outcome: a prospective study. *Ann. Neurol.* 21: 176-182.

Dicker-Brown, A., Fonseca, V. A., Fink, L. M., and Kern, P. A. (2001) The effect of glucose and insulin on the activity of methylene tetrahydrofolate reductase and cystathionine-β-synthase: studies in hepatocytes. *Atherosclerosis.* 158: 297-301.

Duthie, S. J., Narayanan, S., Brand, G. M., Pirie, L., and Grant, G. (2002) Impact of folate deficiency on DNA stability. *J. Nutr.* 132: 2444S-2449S.

Eisenga, B. H., Collins, T. D., and McMartin, K. E. (1989) Effects of acute ethanol on urinary excretion of 5-methyltetrahydrofolic acid and folate derivatives in the rat. *J. Nutr.* 119: 1498-1505.

Eloranta, T. O., Martikainen, V., and Smith, T. K. (1990) Adaptation of adenosylmethionine metabolism and methionine recycling to variations in dietary methionine in the rat. *Exp. Biol. Med.* 194: 364-371.

Elseweidy, M. and Singh, M. (1984) Folate deficiency and pancreatic acinar cell function. *Proc. Soc. Exp. Biol. Med.* 177: 247-252.

Enders, S. J. and Enders, J. M. (2003) Isotretinoin and Psychiatric Illness in Adolescents and Young Adults. *Ann. Pharmacother.* 37(7): 1124–1127.

Engbersen, A. M. T., Franken, D. G., Boers, G. H. J., Stevens, E. M. B., Trijbels, F. J. M., and Blom, H. J. (1995) Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. *Am. J. Hum. Genet.* 56: 142-150.

Eskes, T. K. A. B. (1998) Neural tube defects, vitamins and homocysteine. *Eur. J. Pediatr.* 157: S139-S141.

Essien, F. B. and Wannberg, S. L. (1993) Methionine but not folinic acid or vitamin B-12 alters the frequency of neural tube defects in *Axd* mutant mice. *J. Nutr.* 123: 27-34.

Fell, D. and Steele, R. D. (1986) Modification of hepatic folate metabolism in rats fed excess retinol. *Life Sciences.* 38: 1959-1965.

Fell, D. and Steele, R. D. (1987) Effect of retinol toxicity on hepatic *S*-adenosylmethionine-dependent transmethylation in rats. *Drug Nutr. Interact.* 5: 1-7.

Finkelstein, J. D., Mudd, S. H., Irreverre, F., and Laster, L. (1964) Homocystinuria due to cystathionine synthetase deficiency: the mode of inheritance. *Science.* 146: 785-787.

Finkelstein, J. D. and Chalmers, F. T. (1970) Pyridoxine effects on cystathionine synthase in rat liver. *J. Nutr.* 100: 467-469.

Finkelstein, J. D., Kyle, W. E., and Harris, B. J. (1971) Methionine metabolism in mammals: Regulation of homocysteine methyltransferases in rat tissue. *Arch. Biochem. Biophys.* 146: 84-92.

Finkelstein, J. D., Kyle, W. E., and Harris, B. J. (1974) Methionine metabolism in mammals: Regulatory effects of *S*-adenosylhomocysteine. *Arch. Biochem. Biophys.* 165: 774-779.

Finkelstein, J. D. and Martin, J. J. (1984) Methionine metabolism in mammals: Distribution of homocysteine between competing pathways. *J. Biol. Chem.* 259(15) 9508-9513.

Finkelstein, J. D. and Martin, J. J. (1986) Methionine metabolism in mammals: adaptation to methionine excess. *J. Biol. Chem.* 261(4) 1582-1587.

Finkelstein, J. D. (1990) Methionine metabolism in mammals. *J. Nutr. Biochem.* 1: 228-237.

Food and Nutrition Board. (2000) Dietary reference intakes for thiamin, riboflavin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline. pp. 196-305. National Academy Press. Washington, DC.

Foreman J. W., Wald, H., Blumberg, G., Pepe, L. M., and Segal, S. (1982) Homocystine uptake in isolated rat renal cortical tubules. *Metabolism.* 31(6): 613-9

Friedman, G., Goldschmidt, N., Friedlander, Y., Ben-Yehuda, A., Selhub, J., Babaey, S., Mendel, M., Kidron, M., and Bar-On. (1999) A common mutation A1298C in human methylenetetrahydrofolate reductase gene: assocation with plasma total homocysteine and folate concentrations. *J. Nutr.* 129: 1656-1661.

Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J. H., den Heijer, M., Kluijtmans, L. A. J., van den Heuvel, L. P., and Rozen, R. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.* 10: 111-113.

Geisel, J., Zimbelman, I., Schorr, H., Knapp, J-P., Bodis, M., Hübner, U., and Herrmann, W. (2001) Genetic defects as important factors for moderate hyperhomocysteinemia. *Clin. Chem. Lab. Med.* 39(8): 698-704.

Ghoshal, A. K. and Farber, E. (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis.* 5(10): 1367-1370.

Gil, B., Pajares, M. A., Mato, J. E., and Alvarez, L. (1997) Glucocorticoid regulation of hepatic *S*-adenosylmethionine synthetase gene expression. *Endocrinology.* 138(9): 1251-1258.

Giovannucci, E., Stampfer, M. J., Colditz, G. A., Rimm, E. B., Trichopoulos, D., Rosner, B. A., Speizer, F. E., and Willett, W. C. (1993) Folate, methionine, and alcohol intake and risk of colorectal adenoma. *J. Natl. Cancer Inst.* 85: 875-884.

Goetsch, C. (1962) An evaluation of aminopterin as an abortifacient. *Am. J. Obst. & Gynec.* 83(11): 1474-1477.

Goyette, P., Frosst, P., Rosenblatt, D. S., and Rozen, R. (1995) Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. *Am. J. Hum. Gen.* 56: 1052-1059.

Grant, H. C., Hoffbrand, A.V., Wells D. G. (1965) Folate deficiency and neurological disease. *Lancet.* 2(7416): 763-7.

Green, R. and Jacobsen, D. W. (1995) Biochemical role of folate in cellular metabolism. In: *Folates in Health and Disease* (Bailey, L. B., ed.) pp. 75-122. Marcel Dekker, Inc., New York, NY.

Halsted, C. H., Griggs, R. C., and Harris, J. W. (1967) The effect of alcoholism on the absorption of folic acid (H^3-PGA) evaluated by plasma levels and urine excretion. *J. Lab. Clin. Med.* 69(1): 116-131.

Halsted, C. H., Villanueva, J. A., Devlin, A. M., Niemelä, O., Parkkila, S., Garrow, T. A., Wallock, L. M., Shigenaga, M. K., Melnyk, S., and James, S. J. (2002) Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. *Proc. Nat. Acad. Sci.* 99(15): 10072-10077.

Halsted, C. H., Villanueva, J. A., Devlin, A. M., and Chandler, C. J. (2002) Metabolic interactions of alcohol and folate. *J. Nutr.* 132: 2367S-2372S.

Harmon, D. L., Woodside, J. V., Yarnell, J. W. G., McMaster, D., Young, I. S., McCrum, E. E., Gey, K. F., Whitehead, A. S., and Evans, A. E. (1996) The common 'thermolabile' variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinemia. *Q. J. Med.* 89: 571-577.

Harmon, D. L., Shields, D. C., Woodside, J. V., McMaster, D., Yarnell, J. W. G., Young, I. S., Peng, K., Shane, B., Evans, A. E., and Whitehead, A. S. (1999) Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations. *Genet. Epidemiol.* 17: 298-309.

Hatch, F. T., Larabee, A. R., Cathou, R. E., Buchanan, J. M. (1961) Enzymatic synthesis of the methyl group of methionine. Identification of the enzymes and cofactors involved in the system isolated from *Escherichia coli. J. Biol. Chem.* 236: 1095-1101.

Hawkins C, Meynell M. (1958) Macrocytosis and macrocytic anemia caused by anticonvulsant drugs. *Am. J. Med.* 27: 45-63.

Haynes, W. G. (2003) Hyperhomocysteinemia: vascular function and atherosclerosis: effects of vitamins. *Cardio. Drugs Ther.* 16: 391-399.

Heady, J. E. and Kerr, S. J. (1975) Alteration of glycine *N*-methyltransferase activity in fetal, adult, and tumor tissues. *Cancer Res.* 35: 640-643.

Henning, S. M., McKee, R. W., and Swendseid, M. E. (1989) Hepatic content of *S*-adenosylmethionine, *S*-adenosylhomocysteine and glutathione in rats receiving treatments modulating methyl donor availability. *J. Nutr.* 119: 1478-1482.

Hibbard, B. M. (1993) Folates and fetal development. *Br. J. Obstet. Gynaecol.* 100: 307-309.

Hoffbrand, A. V. and Necheles, T. F. (1968) Mechanism of folate deficiency in patients receiving phenytoin. *Lancet* 2(7567): 528-30.

Hoffbrand, A. V., Ganeshaguru, K., Hooton, J. W. L., and Tripp, E. (1976) Megaloblastic anemia: Initiation of DNA synthesis in excess of DNA chain elongation as the underlying mechanism. *Clin. Haematol.* 5: 727-745.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) p53 mutations in human cancers. *Science*. 253(5015): 49-53.

Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, J. D., and Wong, L-Y. C. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA*. 285(23): 2981-2986.

Hoover, K. L., Lynch, P. H., and Poirier, L. A. (1984) Profound postinitiation enhancement by short-term severe methionine, choline, vitamin B_{12}, and folate deficiency of hepatocarcinogenesis in F344 rats given a single low-dose diethylnitrosamine injection. *J. Natl. Cancer Inst.* 73: 1327-1336.

Hussein, W. I., Green, R., Jacobsen, D. W., and Faiman, C. (1999) Normalization of hyperhomocysteinemia with L-thyroxine in hypothyroidism. *Ann. Intern. Med.* 131: 348-351.

Hyndman, M. E., Bridge, P. J., Warnica, J. W., Fick, G., and Parsons, H. G. (2000) Effect of heterozygosity for the methionine synthase gene 2756 A→G mutation on the risk for recurrent cardiovascular events. *Am. J. Cardiol.* 86: 1144-1145.

Jacob, R. A., Wu, M-M., Henning, S. M., and Swendseid, M. E. (1994) Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction. *J. Nutr.* 124: 1072-1080.

Jacobs, R. L., House, J. D., Brosnan, M. E., and Brosnan, J. T. (1998) Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat. *Diabetes*. 47(12): 1967-70.

Jacobs R. L., Stead L. M., Brosnan, M. E., Brosnan, M. E., and Brosnan, J. T. (2000) Plasma homocysteine is decreased in the hypothyroid rat. *Can. J. Physiol. Pharmacol.* 78(7): 565-70.

Jacobs, R. L., Stead, L. M., Brosnan, M. E., and Brosnan, J. T. (2001) Hyperglucagonemia in rats results in decreased plasma homocysteine and increased flux through the transsulfuration pathway. *J. Biol. Chem.* 276(47): 43740-43747.

Jacques, P. F., Bostom, A. G., Selhub, J., Rich, S., Ellison, R. C., Eckfeldt, J. H., Gravel, R. A., and Rozen, R. (2003) Effects of polymorphisms of methionine synthase and methionine synthase reductase on total plasma homocysteine in the NHLBI Family Heart Study. *Atherosclerosis.* 166: 49-55.

Jones, P. A. and Takai, D. (2001) The role of DNA methylation in mammalian epigenetics. *Science.* 293(5532): 1068-70.

Kang, S-S., Wong, P. W. K., Cook, H. Y., Norusis, M., and Messer, J. V. (1986) Protein-bound homocyst(e)ine: a possible risk factor for coronary artery disease. *J. Clin. Invest.* 77: 1482-1486.

Kang, S-S., Wong, P. W. K., and Norusis, M. (1987) Homocysteinemia due to folate deficiency. *Metabolism.* 36(5): 458-462.

Kang, S-S., Wong, P. W. K., Zhou, J., Sora, J., Lessick, M., Ruggie, N., and Grcevich, G. (1988) Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. *Metabolism.* 37(7): 611-613.

Kelly, P. J., Shih, V. E., Kistler, J. P., Barron, M., Lee, H., Mandell, R., and Furie, K. L. (2003) Low vitamin B6 but not homocyst(e)ine is associated with increased risk of stroke and transient ischemic attack in the era of folic acid grain fortification. *Stroke.* 34: e51-e54.

Kerr, S. J. (1972) Competing methyltransferase systems. *J. Biol. Chem.* 247: 4248-4252.

Kim, Y-I., Pogribny, I. P., Basnakian, A. G., Miller, J. W., Selhub, J., James, S. J., and Mason, J. B. (1997) Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am. J. Clin. Nutr.* 65: 46-52.

Kirke, P. N., Molloy, A. M., Daly, L. E., Burke, H., Weir, D. G., and Scott, J. M. (1993) Maternal plasma folate and vitamin B_{12} are independent risk factors for neural tube defects. *Q. J. Med.* 86: 703-708.

Kishi, T., Fujita, N., Eguchi, T., and Ueda, K. (1997) Mechanism for reduction of serum folate by antiepileptic drugs during prolonged therapy. *J. Neurol. Sci.* 145: 109-112.

Kluijtmans, L. A. J., Boers, G. H. J., Kraus, J. P., van den Heuvel, L. P. W. J., Cruysberg, J. R. M., Trijbels, F. J. M., and Blom, H. J. (1999) The molecular basis of cystathionine β-synthase deficiency in Dutch patients with homocystinuria: effect of CβS genotype on biochemical and clinical phenotype and on response to treatment. *Am. J. Hum. Genet.* 65: 59-67.

Kluijtmans, L. A. J., Young, I. S., Boreham, C. A., Murray, L., McMaster, D., McNulty, H., Strain, J. J., McPartlin, J., Scott, J. M., and Whitehead, A. S. (2003) Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. *Blood.* 101: 2483-2488.

Kutzbach, C. and Stokstad, E. L. R. (1967) Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by *S*-adenosylmethionine. *Biochim. Biophys. Acta.* 139: 217-220.

Kutzbach, C. and Stokstad, E. L. R. (1968) Partial purification of a 10-formyltetrahydrofolate: NADP oxidoreductase from mammalian liver. *Biochem. Biophys. Res. Commun.* 30: 111-117.

Kutzbach, C. and Stokstad, E. L. R. (1971) Mammalian methylenetetrahydrofolate reductase. Partial purification, properties, and inhibition by *S*-adenosylmethionine. *Biochim. Biophys. Acta.* 250: 459-477.

Labadarios, D., Dickerson, J. W. T., Parke, D. V., Lucas, E. G., and Obuwa, G. H. (1978) The effects of chronic drug administration on enzyme induction and folate metabolism. *Br. J. Clin. Pharmac.* 5: 167-173.

Lammer, E. J., Chen, D. T., Hoar, R. M., Agnish, N. D., Benke, M. D., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W., Lott, I. T., Richard, J. M., and Sun, S. C. (1985) Retinoic acid embryopathy. *N. Engl. J. Med.* 313: 837-841.

Lau, E. C. and Li, Z-Q. (1995) Protection of mice form teratogen-induced cleft palate by exogenous methionine. *Proc. Soc. Exp. Biol. Med.* 209: 141-145.

Lentz, S. R., Erger, R. A., Dayal, S., Maeda, N., Malinow, M. R., Heistad, D. D., and Faraci, F. M. (2000) Folate dependence of hyperhomocysteinemia and vascular dysfunction in cystathionine β-synthase-deficiency mice. *Am. J. Physiol. Heart. Circ. Physiol.* 279: H970-H975.

Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature.* 362: 709-715.

Lucock, M. (2000) Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol. Genet. Metab.* 71: 121-138.

Luka, Z., Cerone, R., Phillips III, J. A., Mudd, S. H., and Wagner, C. (2002) Mutations in human glycine *N*-methyltransferase give insights into its role in methionine metabolism. *Hum. Genet.* 110: 68-74.

MacKenzie, R. E. (1984) Biogenesis and interconversion of substituted tetrahydrofolates. In: *Folates and Pterins. Vol. 1. Chemistry and biochemistry of folates* (Blakley, R. L. and Benkovic, S. J., eds.) pp. 255-306. John Wiley and Sons Inc., New York.

Mato, J. M., Alvarez, L., Corrales, F., and Pajares, M. A. (1994) In: *The Liver; Biology and Pathobiology* (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D. A., and Shafritz, D. A., eds) 3rd Ed., pp. 461-470. Raven Press, Ltd., New York, NY.

Mattson, M. P. (2003) Gene-Diet Interactions in Brain Aging and Neurodegenerative Disorders. *Ann. Intern. Med.* 139(5): 441-444.

Mattson, M. P. and Shea, T. B. (2003) Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. *Trends Neurosci.* 26(3): 137-46.

McCully, K. S. (1969) Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am. J. Pathol.* 56: 111-128.

McCully, K. S. and Ragsdale, B. D. (1970) Production of arteriosclerosis by homocysteinemia. *Am. J. Pathol.* 61: 1-11.

McCully, K. S. and Wilson, R. B. (1975) Homocysteine theory of arteriosclerosis. *Atherosclerosis.* 22: 215-227.

McMartin, K. E., Collins, T. D., Bairnsfather, L. (1986) Cumulative excess urinary excretion of folate in rats after repeated ethanol treatment. *J. Nutr.* 116: 1316-1325.

McMartin, K. E., Collins, T. D., Eisenga, B. H., Fortney, T., Bates, W. R., Bairnsfather, L. (1989) Effects of chronic ethanol feeding and diet treatment on urinary folate excretion and development of folate deficiency in the rat. *J. Nutr.* 119: 1490-1497.

Meleady, R., Ueland, P. M., Blom, H., Whitehead, A. S., Refsum, H., Daly, L. E., Vollset, S. E., Donohue, C., Giesendorf, B., Graham, I. M., Ulvik, A., Zhang, Y., Bjorke Monsen, A-L. (2003) Thermolabile methylenetetrahydrofolate reductase, homocysteine, and cardiovascular disease risk: the European Concerted Action Project. *Am. J. Clin. Nutr.* 77: 63-70.

Meynell, M. J. (1966) Megaloblastic anaemia in anticonvulsant therapy. *Lancet.* 1(7435): 487.

Mills, J. L., McPartlin, J. M., Kirke, P. N., Lee, Y. J., Conley, M. R., Weir, D. G., and Scott, J. M. (1995) Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet.* 345: 149-151.

Milunsky, A., Jick, H., Jick, S. S., Bruell, C. L., MacLaughlin, D. S., Rothman, K. J., and Willett, W. (1989) Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. *JAMA.* 262(20): 2847-2852.

Mudd, S. H. (1963) Activation of methionine for transmethylation. *J. Biol. Chem.* 238(6) 2156-2163.

Mudd, S. H., Cerone, R., Schiaffino, M. C., Fantasia, A. R., Minniti, G., Caruso, U., Lorini, R., Watkins, D., Matiaszuk, N., Rosenblatt, D. S., Schwahn, B., Rozen, R., LeGros, L., Kotb, M., Capdevila, A., Luka, Z., Finkelstein, J. D., Tangerman, A., Stabler, S. P., Allen, R. H., and Wagner, C. (2001) Glycine *N*-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninemia. *J. Inherit. Metab. Dis.* 24: 448-464.

Mulinare, J., Cordero, J. F., Erickson, J. D., and Berry, R. J. (1988) Periconceptional use of multivitamins and the occurrence of neural tube defects. *JAMA.* 260(21): 3141-3145.

Munshi, M. N., Stone, A., Fink, L., and Fonseca, V. (1996) Hyperhomocysteinemia following a methionine load in patients with non-insulin-dependent diabetes mellitus and macrovascular disease. *Metabolism.* 45(1): 133-135.

Mischoulon, D. and Fava, M. (2002) Role of *S*-adenosyl-L-methionine in the treatment of depression: a review of the evidence. *Am. J. Clin. Nutr.* 76: 1158S-1161S.

Morin, I., Platt, R., Weisberg, I., Sabbaghian, N., Wu, Q., Garrow, T. A., and Rozen, R. (2003) Common variant in betaine-homocysteine methyltransferase (BHMT) and risk for spina bifida. *Am. J. Med. Gen.* 119A: 172-176.

National Vital Statistics System. (2003) Deaths: final data for 2001. *National Vital Statistics Reports.* 52(3): 1-116.

Nelen, W. L. D. M., Blom, H. J., Thomas, C. M. G., Steegers, E. A. P., Boers, G. H. J., and Eskes, T. K. A. B. (1998) Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting form low dose folic acid supplementation in women with unexplained recurrent miscarriages. *J. Nutr.* 128: 1336-1341.

Nelson, M. M. and Evans, H. M. (1949) Folic acid and reproduction in the rat. *J. Nutr.* 38: 11-24.

Newberne, P. M. and Rogers, A. E. (1986) Labile methyl groups and the promotion of cancer. *Ann. Rev. Nutr.* 6: 407-432.

Newberne, P. M., Suphiphat, V., Locniskar, M., and de Camargo, J. L. V. (1990) Inhibition of hepatocarcinogenesis in mice by dietary methyl donors methionine and choline. *Nutr. Cancer.* 14: 175-181.

Noga, A. A., Stead, L. M., Zhao, Y., Brosnan, M. E., Brosnan, J. T., and Vance, D. E. (2003) Plasma homocysteine is regulated by phospholipid methylation. *J. Biol. Chem.* 278(8): 5952-5955.

O'Broin, J. D., Temperley, I. J., Brown, J. P., and Scott, J. M. (1975) Nutritional stability of various naturally occuring monoglutamate derivatives of folic acid. *Am. J. Clin. Nutr.* 28: 438-444.

Ogawa, H. and Fujioka, M. (1982) Induction of rat liver glycine methyltransferase by high methionine diet. *Biochem. Biophys. Res. Commun.* 108: 227-232.

Pan, C-J., Hoeppner, W., and Yang Chou, J. (1990) Induction of phosphoenolypyruvate carboxykinase gene expression by retinoic acid in an adult rat hepatocye line. *Biochemistry.* 29(49): 10883-10888.

Pancharuniti, N., Lewis, C. A., Sauberlich, H. E., Perkins, L. L., Go, R. C. P., Alvarez, J. O., Macaluso, M., Acton, R. T., Copeland, R. B., Cousins, A. L., Gore, T. B., Cornwell, P. E., and Roseman, J. M. (1994) Plasma homocyst(e)ine, folate, and vitamin B-12 concentrations and risk for early-onset coronary artery disease. *Am. J. Clin. Nutr.* 59: 940-948.

Pascale, R. M., Marras, V., Simile, M. M., Daino, L., Pinna, G., Bennati, S., Carta, M., Seddaiu, M. A., Masserelli, G., and Feo, F. (1992) Chemoprevention of rat liver carcinogenesis by *S*-adenosyl-L-methionine: a long term study. *Cancer Res.* 52: 4979-4986.

Peng, Y-S. and Evenson, J. K. (1982) Alleviation of methionine toxicity in rats by retinol-pretreatment: *in vivo* methionine catabolism and role of adrenal glands. *Nutr. Reports Int.* 25(1): 139-144.

Pietarinen, G. J., Leichter, J., and Pratt, R. F. (1977) Dietary folate intake and concentration of folate in serum and erythrocytes in women using oral contraceptives. *Am. J. Clin. Nutr.* 30: 375-380.

Piyathilake, C. J. and Johanning, G. L. (2002) Cellular vitamins, DNA methylation, and cancer risk. *J. Nutr.* 132: 2340S-2344S.

Pogribny, I. P., Muskhelishvili, L., Miller, B. J., and James, S. J. (1997) Presence and consequence of uracil in preneoplastic DNA from folate/methyl-deficient rats. *Carcinogenesis.* 18(11): 2071-2076.

Poirier, L. A., Brown, A. T., Fink, L. M., Wise, C. K., Randolph, C. J., Delongchamp, R. R., and Fonseca, V. A. (2001) Blood S-adenosylmethionine and lymphocyte methylenetetrahydrofolate reductase activity in diabetes mellitus and diabetic nephropathy. *Metabolism.* 50(9): 1014-1018.

Porta, E. A., Markell, N., and Dorado, R. D. (1985) Chronic alcoholism enhances hepatocarcinogenicity of diethylnitrosamine in rats fed a marginally methyl-deficient diet. *Hepatology.* 5(6): 1120-1125.

Ratnam, S., Maclean, K. N., Jacobs, R. L., Brosnan, M. E., Kraus, J. P., and Brosnan, J. T. (2002) Hormonal regulation of cystathionine β-synthase expression in liver. *J. Biol. Chem.* 277(45) 42912-42918.

Refsum, H., Gelland, S., and Ueland, P. M. (1989) Fasting plasma homocysteine as a sensitive parameter to antifolate effect. A study on psoriasis patients receiving low-dose methotrexate treatment. *Clin. Pharmacol. Ther.* 46(5): 510-20.

Reynolds E. H., Preece, J. M., Bailey, J., and Coppen, A. (1970) Folate deficiency in depressive illness. *Br. J. Psych.* 117(538): 287-92.

Robillon, J. F., Canivet, B., Candito, M., Sadoul, J. L., Jullien, D., Morand, P., Chambon, P., and Freychet, P. (1994) Type 1 diabetes mellitus and homocyst(e)ine. *Diabete Metab.* 20(5): 494-6.

Sadler, T. W., Merrill, A. H., Stevens, V. L., Cameron Sullards, M., Wang, E., and Wang, P. (2002) Prevention of fumonisin B1-induced neural tube defects by folic acid. *Teratology.* 66: 169-176.

Salmon, W. D. and Copeland, D. H. (1954) Liver carcinoma and related lesions in chronic choline deficiency. *Ann. NY. Acad. Sci.* 57: 664-677.

Schalinske, K. L. and Steele, R. D. (1991) 13-*cis*-retinoic alters methionine metabolism in rats. *J. Nutr.* 121: 1714-1719.

Schalinske, K. L. and Steele, R. D. (1993) 13-*cis*-retinoic acid and hepatic steatosis in rats. *Biochem. Pharmacol.* 46(2): 319-325.

Schulpis, K. H., Karikas, G. A., Georgala, S., Michas, T., and Tsakiris, S. (2001) Elevated plasma homocysteine levels in patients on isotretinoin therapy for cystic acne. *Int. J. Dermatol.* 40: 33-36.

Schwaninger, M., Ringleb, P., Winter, R., Kohl, B., Fiehn, W., Rieser, P. A., and Walter-Sack, I. (1999) Elevated plasma concentrations of homocysteine in antiepileptic drug treatment. *Epilepsia.* 40(3): 345-350.

Scott, J. M., Kirke, P. N., and Weir, D. G. (1990) The role of nutrition in neural tube defects. *Annu. Rev. Nutr.* 10: 277-295.

Sebastio, G., Sperandeo, M. P., Panico, M., and de Franchis, R., Kraus, J. P., and Andria, G. (1995) The molecular basis of homocystinuria due to cystathionine beta-synthase deficiency in Italian families, and report of four novel mutations. *Am. J. Hum. Genet.* 56(6): 1324-33.

Selhub, J. and Miller, J. W. (1992) The pathogenesis of homocysteinemia: interruption of the coordinate regulation by *S*-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am. J. Clin. Nutr.* 55: 131-138.

Selhub, J. (1999) Homocysteine metabolism. *Annu. Rev. Nutr.* 19: 217-246.

Sesmilo G., Biller, B. M., Llevadot J., Hayden, D., Hanson, G., Rifai, N., and Klibanske, A. (2001) Effects of growth hormone (GH) administration on homocyst(e)ine levels in men with GH deficiency: a randomized controlled trial. *J. Clin. Endocrinol. Metab.* 86(4): 1518-24

Shane, B. (1989) Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vit. Horm.* 45: 263-335.

Shane, B. (1995) Folate chemistry and metabolism. In: *Folates in Health and Disease* (Bailey, L. B., ed.) pp. 1-22. Marcel Dekker, Inc., New York, NY.

Shane, B. and Stokstad, E. L. R. (1985) Vitamin B_{12}-folate interrelationships. *Ann. Rev. Nutr.* 5: 115-141.

Shimakawa T., Nieto F. J. Malinow, M. R., Chambless, L. E., Schreiner, P. J., and Szklo, M. (1997) Vitamin intake: a possible determinant of plasma homocyst(e)ine among middle-aged adults. *Ann. Epidemiol.* 7(4): 285-93.

Shin, D-J., McGrane, M. M. (1997) Vitamin A regulates genes involved in hepatic gluconeogenesis in mice: phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J. Nutr.* 127: 1274-1278.

Shivapurkar, N. and Poirier, L. A. (1983) Tissue levels of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks. *Carcinogenesis.* 4(8): 1051-1057.

Shojania, A. M., Hornady, G. J., and Scalletta, D. (1975) The effect of oral contraceptives on folate metabolism. III. Plasma clearance and urinary folate excretion. *J. Lab. Clin. Med.* 85(2):185-190.

Silaste, M-L., Rantala, M., Sämpi, M., Alfthan, G., Aro, A., and Kesäniemi, Y. A. (2001) Polymorphisms of key enzymes in homocysteine metabolism affect diet responsiveness of plasma homocysteine in healthy women. *J. Nutr.* 131: 2643-2647.

Singh, M., Singh, V. N., and Venkitasubramanian, T. A. (1976) Early effects of excessive retinol intake on gluconeogenesis: involvement of adrenals in the increased activities of gluconeogenic enzymes of rat liver. *Arch. Biochem. Biophys.* 173: 82-92.

Steegers-Theunissen R. P., Boers, G. H., Steegers, E. A., Trijbels, F. J., Thomas, C. M., and Eskes, T. K. (1992) Effects of sub-50 oral contraceptives on homocysteine metabolism: a preliminary study. *Contraception.* 45(2): 129-39.

Steegers-Theunissen, R. P. M., Boers, G. H. J., Trijbels, F. J. M., Finkelsteine, J. D., Blom, H. J., Thomas, C. M. G., Borm, G. F., Wouters, M. G. A. J., and Eskes, T. K. A. B. (1994) Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? *Metabolism.* 43(12): 1475-1480.

Steenge, G. R., Verhoef, P., and Katan, M. B. (2003) Betaine supplementation lowers plasma homocysteine in healthy men and women. *J. Nutr.* 133: 1291-1295.

Sütterlin, M. W., Bussen, S. S., Rieger, L., Dietl, J., and Steck, T. (2003) Serum folate and vitamin B12 levels in women using modern oral contraceptives (OC) containing 20 µg ethinyl estradiol. *Eur. J. Obstet. Gynecol. Repr. Biol.* 107: 57-61.

Thiersch, J. B. (1952) Therapeutic abortions with a folic acid antagonist, 4-aminopteroylglutamic acid (4-amino P. G. A.) administered by the oral route. *Am. J. Obst. & Gynec.* 63(6): 1298-1304.

Trimble, K. C., Molloy, A. M., Scott, J. M., and Weir, D. G. (1993) The effect of ethanol on one-carbon metabolism: increased methionine catabolism and lipotrope methyl-group wastage. *Hepatology*: 18(4): 984-989.

Ueland, P. M. and Refsum, H. (1989) Plasma homocysteine, a risk factor for vascular disease: Plasma levels in health, disease, and drug therapy. *J. Clin. Lab. Med.* 114: 473-501.

Ueno, Y., Iijima, K., Wang, S-D., Sugiura, Y., Sekijima, M., Tanaka, T., Chen, C., and Yu, S-Z. (1997) Fumonisins as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. *Food Chem. Toxicol.* 35: 1143-1150.

Uthus, E. O. and Brown-Borg, H. M. (2003) Altered methionine metabolism in long living Ames dwarf mice. *Exp. Gerontol.* 38: 491-498.

Voutilainen, S., Lakka, T. A., Porkkala-Sarataho, E., Rissanen, T., Kaplan, G. A., and Salonen, J. T. (2000) Low serum folate concentrations are associated with an excess incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Eur. J. Clin. Nutr.* 54(5): 424-8.

Wagner, C., Briggs, W. T., and Cook, R. B. (1985) Inhibition of glycine *N*-methyltransferase activity by folate derivatives: Implications for regulation of methyl group metabolism. *Biochem. Biophys. Res. Commun.* 127(3) 746-752.

Wagner, C., Decha-Unphai, W., and Corbin, J. (1989) Phosphorylation modulates the activity of glycine *N*-methyltransferase, a folate binding protein. *J. Biol. Chem.* 264(16): 9638-9642.

Wagner, C. (1995) Biochemical role of folate in cellular metabolism. In *Folates in Health and Disease* (Bailey, L. B., ed.) pp. 1-22. Marcel Dekker, Inc., New York, NY.

Wainfan, E., Dizik, M., Stender, M., and Christman, J. K. (1989) Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. *Cancer Res.* 49: 4094-4097.

Wainfan, E. and Poirier, L. A. (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res.* 52: 2071s-2077s.

Weisberg, I. S., Park, E., Ballman, K. V., Berger, P., Nunn, M., Suh, D. S., Breksa III, A. P., Garrow, T. A., and Rozen, R. (2003) Investigations of a common genetic variant in betaine-homocysteine methyltransferase (BHMT) in coronary artery disease. *Atherosclerosis.* 167: 205-214.

Werler, M. M., Shapiro, S., and Mitchell, A. A. (1993) Periconceptional folic acid exposure and risk of occurrent neural tube defects. *JAMA.* 269(10): 1257-1261.

Xue, G-P. and Snoswell, A. M. (1985) Disturbance of methyl group metabolism in alloxan-diabetic sheep. *Biochem. Int.* 10(6): 897-905.

Yeo, E-J. and Wagner, C. (1994) Tissue distribution of pancreatic glycine *N*-methyltransferase, a major folate-binding protein of liver. *Proc. Natl. Acad. Sci. USA.* 91: 210-214.