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Modulation of the S-Adenosylmethionine to S-Adensoyhomocyseine Ratio: Effect on Transmethylation

George Loo
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by George Loo entitled "Modulation of the S-Adenosylmethionine to S-Adensoyhomocyseine Ratio: Effect on Transmethylation." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Jane R. Savage, Dileep S. Sachan, Robert H. Feinbert

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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MODULATION OF THE S-ADENOSYLMETHIONINE TO S-ADENOSYLHOMOCYSTEINE RATIO: EFFECT ON TRANSMETHYLATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

George Loo

June 1986

ACKNOWLEDGEMENTS

The author would like to express his sincere gratitude to Dr. John T. Smith, who has served as major professor and mentor for the entire graduate study curriculum. His overwhelming presence has certainly provided the spark needed to generate the research presented herein.

Deepest appreciation is also given to Drs. Jane R. Savage, Dileep S. Sachan, and Robert H. Feinberg for their willingness to serve as committee members. They are indeed a select group of faculty.

In addition, the author is thankful for the assistance of the undergraduate nutrition students, Patti J. Goodman and Kelli A. Hill, as well as the small animal laboratory supervisor, Deborah L. Miller, in facilitating the research.

Lastly, special thanks is extended to the Department of Nutrition and Food Sciences and Agricultural Experiment Station for providing financial support via graduate teaching and research assistantships.

ABSTRACT

Regulation of transmethylation by lowering the molar ratio of S-adenosylmethionine to S-adenosylhomocysteine (SAM:SAH ratio) was studied. Reduction of the ratio *in vitro* caused a decrease in the activities of rat liver thiopurine methyltransferase (TPMT), thiol methyltransferase (TMT), and protein(lysine)methyltransferase (PLMT). Furthermore, rats injected with D,L-homocysteine thiolactone had a reduction in the hepatic SAM:SAH ratio, as well as a decrease in the activities of TPMT and PLMT, but not TMT, in liver.

Reduction of the hepatic SAM:SAH ratio in rats fed a pyridoxine-deficient diet resulted in a decrease in the activity of PLMT, but not TPMT and TMT, in liver. Moreover, the levels of total acid-soluble carnitine (ASCNE) were lowered in skeletal muscle and heart of these rats, but palmitate oxidation *in vitro* was not impaired in either tissue. On the other hand, the rate of palmitate oxidation in skeletal muscle decreased in rats subjected to an apparent feed restriction, despite normal levels of tissue ASCNE and elevated cytochrome oxidase activity.

Phospholipid methylation was impaired during pyridoxine deficiency, above and beyond that seen during feed restriction. A reduction in the hepatic microsomal level of phosphatidylcholine and an elevation of

phosphatidylethanolamine was found, incongruous with an increase in the activity of phosphatidylethanolamine methyltransferase.

Pyridoxine-deficient rats displayed no significant change in the activity of guanidoacetate methyltransferase in liver, but a decrease in arginine-glycine transamidinase activity of kidney. The concentrations of creatine in liver and skeletal muscle of these animals increased, once again above and beyond that observed in feed-restricted rats alone. No change in urinary creatinine excretion was demonstrated.

The data indicate that under certain conditions, the SAM:SAH ratio may regulate some biochemical processes related to transmethylation. Cellular compartmentalization and nutritional state may perhaps determine if control is possible in vivo.

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CHAPTER I

INTRODUCTION

The importance of transmethylation, with S-adenosylmethionine serving as the methyl group donor, can only be fully realized when consideration is given to some of the crucial biochemical processes regulated by the group of enzymes known as the methyltransferases. For example, it is generally recognized that transmethylation is directly implicated in the biosynthesis of phospholipid and creatine, although its role in the formation of carnitine may not be as readily apparent. Yet, perhaps even more inconspicuous is the involvement of transmethylation in xenobiotic metabolism. All of these processes are of immediate concern herein, but by no means are they intended to be inclusive of all transmethylation reactions needed for maintenance of normal physiological function.

In view of their important functions, the regulation of the methyltransferases has attracted an enormous amount of attention. An important, putative regulator of the methyltransferases in general is the absolute molar ratio of S-adenosylmethionine to S-adenosylhomocysteine (SAM:SAH ratio). Theoretically, an increase in the SAM:SAH ratio would tend to stimulate transmethylation, whereas a decrease in the ratio would tend to inhibit this reaction. In turn, modulation of the SAM:SAH ratio *in vivo* might be

expected to influence biochemical processes regulated by the methyltransferases.

In the present work, the SAM:SAH ratio was altered both *in vitro* and *in vivo*. The latter condition was achieved in the rat via both pharmacological and dietary means. The effect of these alterations on various transmethylation reactions and related processes was studied.

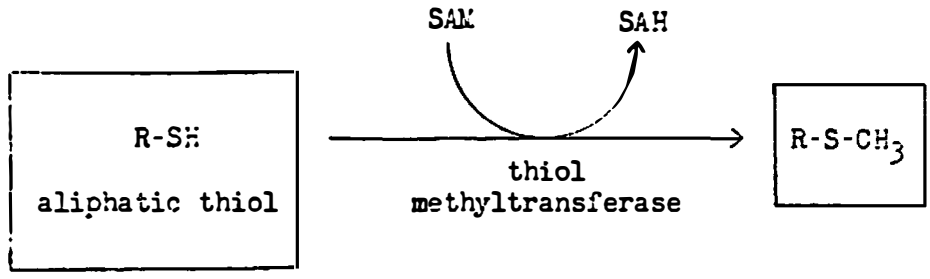
CHAPTER II

REVIEW OF LITERATURE

The Sulfhydryl Xenobiotic Transmethylases and Xenobiotic Metabolism

Two distinct sulfhydryl xenobiotic transmethylases are presently known to S-methylate a variety of sulfhydryl compounds of exogenous origin. They have been designated separately as thiol methyltransferase (TMT, EC 2.1.1.9) and thiopurine methyltransferase (TPMT, EC 2.1.1.67). The reactions catalyzed by TMT and TPMT are depicted in Figure 1, and apparently each enzyme possesses a low specificity for their respective substrates. As can be seen, transmethylation is achieved using S-adenosylmethionine (SAM) as the methyl group donor, which generates S-adenosylhomocysteine (SAH) as the product of the reaction.

With respect to subcellular localization and tissue distribution, TMT was first reported as a microsomal enzyme (1). More recently, noticeable enzymatic activity has also been reported in other subcellular particulate fractions (2,3,4). Using rat tissue homogenates, liver, lung, and kidney have all revealed relatively high levels of TMT activity, although of all the tissues examined, the cecal and colonic mucosa has displayed the highest concentration of the enzyme (5). On the other hand, TPMT is essentially



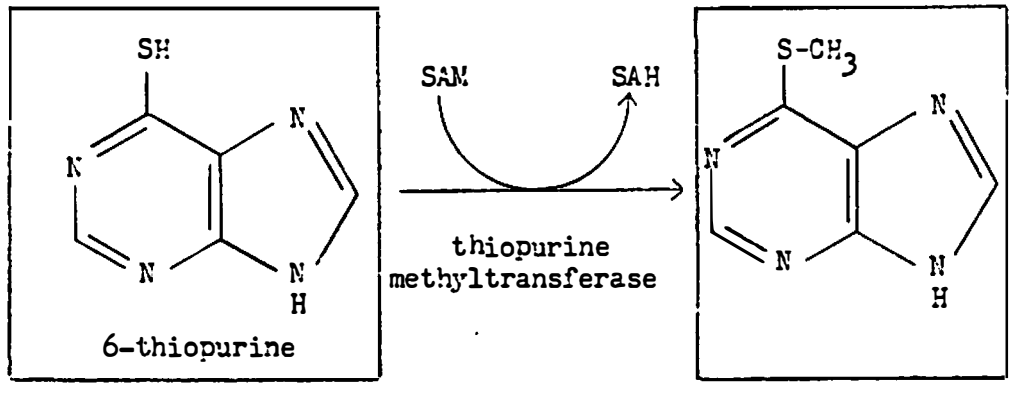


Figure 1. Reactions catalyzed by the sulfhydryl xenobiotic transmethylases

a cytosolic enzyme (3,6), with the greatest activity appearing to be situated in kidney (6).

Recent work has allowed the characterization of TMT. Using rat liver microsomes, the enzyme has been solubilized with Triton X-100 and partially purified, thus permitting the determination of some physicochemical properties of TMT (2). No metal requirement for the enzyme could be demonstrated, either by addition of various metals in an attempt to stimulate TMT activity, or EDTA to decrease the activity in the reaction mixture. The employment of sulfhydryl reagents, which caused inactivation of the enzyme, suggested the presence of free sulfhydryl groups on the enzyme essential for catalysis. Using 2-mercapto-ethanol as the substrate in the standard assay, maximum enzyme activity was observed within a pH range of 7.8-9.0. The K_m for 2-mercaptoethanol and SAM was 2.37 mM and 106 µM, respectively. Moreover, SAH inhibited TMT with a K_i of 144 µM. On the other hand, a non-detergent method has also been described for the purification and subsequent characterization of homogeneous rat liver microsomal TMT (7,8). From this work, TMT has been determined to be a single subunit enzyme with an estimated molecular weight of 28,000. Using 2-thioacetanilide as the substrate for TMT in the standard assay, the pH optimum for the enzyme was assessed at 7.5. The apparent K_m for 2-thioacetanilide was determined to be 43 µM, while for SAM it was 1 µM at 37^{o}

with lower values obtained as the temperature was reduced. Once again, TMT was inhibited by SAH, but with a K_i of 0.2 μM. It has been suggested (7,8) that the incongruous kinetic data that have been reported for rat liver TMT may be due to the two different types of enzyme preparations used to study this protein, i.e., a detergent-solubilized, partially purified (2) vs. a non-detergent-solubilized, homogeneous (7,8) source.

Likewise, the purification and characterization of TPMT has also been described, with respect to the enzyme in human kidney (3). Gel-permeation column chromatography yielded an estimated molecular weight of 36,000 daltons for the protein. Like its counterpart, TPMT appears to be a sulfhydryl-containing enzyme. Using 6-thiopurine as the substrate in the standard assay, a pH optimum of 6.7 was observed. K_m values for 6-thiopurine and SAM were determined to be 0.30 mM and 2.7 μM, respectively. SAH is also able to inhibit TPMT with a K_i of 0.75 μM.

With the exception of their participation in xenobiotic metabolism, the physiological roles of TMT and TPMT remain quite obscure. Attempts to identify endogenous substrates for each enzyme have been unsuccessful. Among the physiological sulfhydryl compounds tested, neither glutathione, cysteine, nor homocysteine appear to be methyl-accepting substrates in the presence of SAM and TMT (1,2,7). On the other hand, it has been shown that all

three of these endogenous thiols, but not methionine, can each inhibit TPMT (6). This has raised the possibility that these particular compounds may be endogenous substrates for TPMT, although no research has been presented to either confirm or refute this possibility until just recently. Work has been presented (9) that glutathione inhibits TPMT via changing the reaction mixture pH away from the optimal range. Moreover, chromatographic evidence indicated that glutathione is not an endogenous substrate for TPMT. As such, one can only speculate as to the identity of endogenous substrates for TPMT. In slight contrast, it has been proposed that TMT may function in the detoxication of "endogenous" hydrogen sulfide generated by the gut flora, in view of the high concentration of TMT in gut mucosa (5). In addition, recent research has suggested a novel role for rat lung TMT in the indirect methylation of fatty acids (10,11,12), although the physiological significance of this finding is not clear.

It is certain that many foreign compounds can be metabolized either directly or indirectly by the sulfhydryl xenobiotic transmethylases. Like other xenobiotic-metabolizing enzyme systems, both TMT and TPMT exhibit a specificity for a wide range of substrates. It is likely that S-methylation provides a mechanism for either bioactivation or detoxication. In some cases, however, it

is not known as to which one of the two processes is achieved as a result of catalysis by TMT or TPMT.

TPMT is involved in the metabolism of a number of important drugs, perhaps the most important being 6-mercaptopurine (6-MP). The pharmacotherapeutic potential of this thiopurine has been known for many years. While also possessing, immunosuppressive activity (13), 6-MP is perhaps better recognized as a potent antineoplastic drug, especially in the treatment of leukemia (14). The efficacy and/or toxicity of 6-MP is apparently regulated by its biotransformation to a highly active anabolite, via the catalytic action of TPMT. More specifically, research has shown that tumor cells have the capacity to convert 6-MP and also 6-MP ribonucleoside to 6-methyl-MP ribonucleotide (15,16). This product is a very powerful inhibitor of phosphoribosyl pyrophosphate (PRPP) amidotransferase (EC 2.4.2.14) (17,18), considered to be the rate-limiting enzyme in purine biosynthesis and a primary target site of 6-MP in disrupting nucleic acid biosynthesis. Furthermore, the direct enzymatic methylation of the thiopurine nucleotides has also been documented, as exemplified by the finding that 6-MP ribonucleotide can readily accept the methyl group from SAM in the presence of TPMT from mammalian cells (19). Most importantly, 6-methyl-MP ribonucleotide is much more potent as an inhibitor of PRPP-amidotransferase than its non-methylated form (17,18).

Therefore, S-methylation of 6-MP, or its nucleotide derivative, by TPMT appears to be very influential in determining the potency of this thiopurine as a chemotherapeutic agent.

TPMT is also implicated in the metabolism of the renal transplant immunosuppressant, azathioprine, since this particular drug is converted to 6-MP during its metabolism. In addition, TPMT is responsible for the S-methylation of some other related drugs, including the antithyroid and antineoplastic agents, 2-thiouracil and 6-thioguanine, respectively (6). It has been reported that methylation of the ribonucleotide derivative of 6-thioguanine reduces its potential to inhibit PRPP-amidotransferase, however (20). Recently, it has been observed that TPMT can S-methylate some other types of xenobiotics, such as the aromatic thiols, including thiosalicylic acid, thiophenol, and mercaptoacetanilide (21).

A diversity of exogenous substrates, but essentially aliphatic thiols, also exists for TMT. Some of the compounds S-methylated by TMT include hydrogen sulfide and methanethiol (1), as well as some other alkane thiols (22). It is probable that methylation of particularly H_2S, as well as CH_3SH, causes a reduction in the toxicity of these noxious compounds, as they are ultimately transformed to dimethyl sulfide, perhaps to a large extent in the intestinal mucosa (5). As a matter of fact, the generation

of H_2S and some alkane thiols beyond the metabolic capacity to detoxicate these substrates for TMT in liver may be responsible for methionine toxicity (23), and also some of the clinical manifestations seen in liver cirrhosis (24). For example, it has been suggested that CH_3SH may be a contributing factor in the etiology of hepatic coma, by virtue of its anesthetic-like effects on membranes (25). In addition to β-mercaptoethanol and 2,3-dimercaptopropanol (British Anti-Lewisite, BAL) (1,7), disulfiram can also serve as a substrate for TMT (26,27). Dithiothreitol does not appear to be a suitable substrate for rat liver TMT (7), in contrast to the enzyme in brain (28). Recently, the antihypertensive drug, captopril, was demonstrated to undergo S-methylation in the presence of TMT, both *in vitro* (29) and *in vivo* (30).

Due to the low specificities of both TPMT and TMT for substrate, it is possible that they may also share a few common substrates with one another. For instance, TMT has been reported to S-methylate 2-mercaptoacetanilide, 4-chlorothiophenol, phenyl sulfide, and 4-nitrothiophenol (7). These compounds are essentially aromatic thiols. Recently, however, it was reported that TPMT can also S-methylate some aromatic thiols (21), including the first and third compounds listed above. In addition, β-mercaptoethanol is a substrate for TMT (1,2,7) and at high concentrations for TPMT (31). Nevertheless, it has

been asserted that TPMT and TMT are distinct enzymes with different subcellular localizations (3). Other research (32) seems to support this contention.

It would appear that many xenobiotics capable of being conjugated to glutathione during detoxication may also have the potential of being indirectly methylated by the sulfhydryl xenobiotic transmethylases during further metabolism. The scheme depicting this possibility (33) is shown in Figure 2. As can be seen, once the appropriate xenobiotic is conjugated to glutathione, it is enzymatically cleaved to a cysteine conjugate. This product can then be N-acetylated to generate a mercapturic acid derivative that is more readily excretable. Alternatively, the cysteine conjugate may be metabolized through the so-called thiomethyl shunt (34), entailing the catalytic actions of a cytosolic cysteine conjugate β-lyase (35,36) and TMT. Essentially, the former enzyme produces a xenobiotic metabolite containing a free sulfhydryl group, which can be subsequently methylated by TMT. For example, work *in vitro* has demonstrated that the cysteine conjugate of 2,4-dinitrobenzene is converted to 2,4-dinitrobenzene thiol, pyruvic acid, and ammonia in the presence of partially purified cysteine conjugate β-lyase (35). Moreover, the cysteine conjugate of 2,4-dinitrobenzene and p-bromobenzene are converted to thiomethyl-containing metabolites in the presence of cysteine conjugate β-lyase

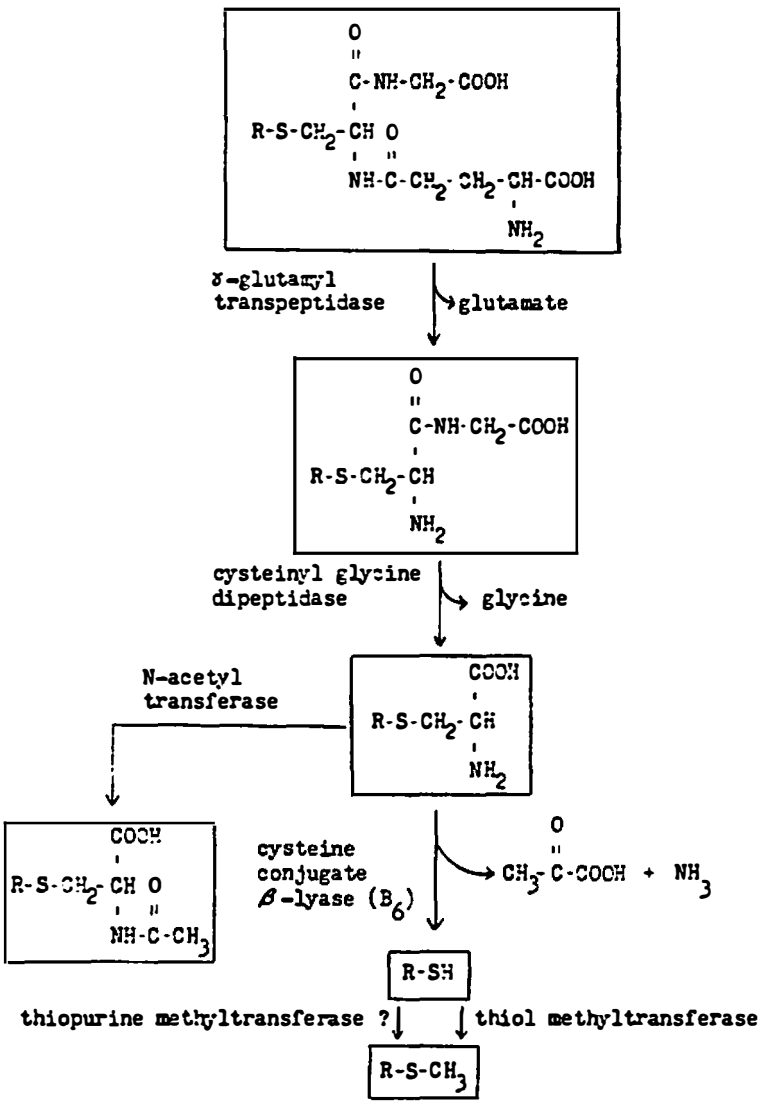


Figure 2. Role of sulfhydryl xenobiotic transmethylases and the thiomethyl shunt in the detoxication of xenobiotics via glutathione conjugation

and hepatic microsomes containing TMT activity (35). In addition to these aromatic conjugates, aliphatic derivatives, such as S-1,2-dichlorovinyl-L-cysteine, can also serve as substrates for cysteine conjugate β-lyase (36). Although TMT is clearly implicated in the S-methylation of aromatic thiols in the thiomethyl shunt (35), it is possible that TPMT may function similarly in this novel pathway, considering that TPMT can S-methylate the aromatic thiols as well (21).

The full significance of the thiomethyl shunt is not known. However, it is apparent that cysteine conjugates, as well as glutathione conjugates, are nephrotoxic (33). Then too, it has been said that cysteine conjugate β-lyase could generate sulfhydryl-containing products which would also be expected to be toxic (34). Therefore, once conjugation of a suitable foreign compound with glutathione has occurred, the thiomethyl shunt may provide an auxillary pathway to negate the potential toxicity of metabolites that may escape mercapturic acid formation, especially after xenobiotic overexposure. As such, S-methylation of sulfhydryl metabolites by TMT (TPMT?) would complete the final step in what would appear to be a detoxicative role for the shunt. Indeed, thiomethylated metabolites of phenacetin (37), propranolol (38), and naphthalene (39) have been identified as urinary products after administration of these aromatic xenobiotics.

The regulation of the sulfhydryl xenobiotic transmethylases has not been investigated to an appreciable extent. Nevertheless, some progress has been made at identifying factors which can control the activities of TPMT and TMT. During a 14-week study involving male Sprague-Dawley rats, the activity of kidney TPMT increased from birth, reaching a peak at about 10 weeks of age (40). Moreover, it has been observed that male rats display higher TPMT activity in kidney than their age-matched female counterparts, which is believed to be due to the regulation of TPMT by testosterone (41). Finally, the activity levels of TPMT and TMT may be under hereditary control, which has been suggested to explain the individual variation in the ability to metabolize sulfhydryl drugs (42). Needless to say, more research is needed on the regulation of the sulfhydryl xenobiotic transmethylases.

Protein(Lysine)Methyltransferase, Carnitine Biosynthesis and Fatty Acid Oxidation

Transmethylation is involved in the post-translational modification of protein macromolecules. Three types of SAM-dependent protein methyltransferases have been distinguished (43), each capable of transferring methyl groups from SAM to amino acid side chains. Of immediate interest here is protein(lysine)methyltransferase (PLMT, EC 2.1.1.43), i.e., protein methylase III, which catalyzes the

methylation of the ε-amino group of lysine residues, especially in histone (44,45). PLMT is a nuclear enzyme with greatest activity localized in the thymus and spleen, with intermediate levels of enzyme activity in liver, and low amounts in heart and muscle of the rat (44). The overall role of PLMT in protein methylation and its full biological significance have been described elsewhere (43).

One very important function of PLMT is to support the formation of carnitine. The biosynthesis of carnitine has been fully reviewed (46). As illustrated in Figure 3, carnitine biosynthesis in mammals is initiated by PLMT, which catalyzes the methylation of lysine residues present in protein macromolecules such as histone, myosin, and actin. It is unknown whether the entire methylation sequence is augmented by a single methyltransferase. On the other hand, SAM-6-N-L-lysine methyltransferase present in microbes can catalyze all three methylation reactions to produce ε-N-trimethyl-L-lysine from free lysine during carnitine biosynthesis. In any event, protein-bound ε-N-trimethyl-L-lysyl residues can be liberated via lysosomal action to allow carnitine biosynthesis to proceed in animals.

Once carnitine has been synthesized, with the last reaction in the biosynthetic pathway occurring almost exclusively in liver (47,48,49), it enters the bloodstream from where it is taken up by various tissues, especially

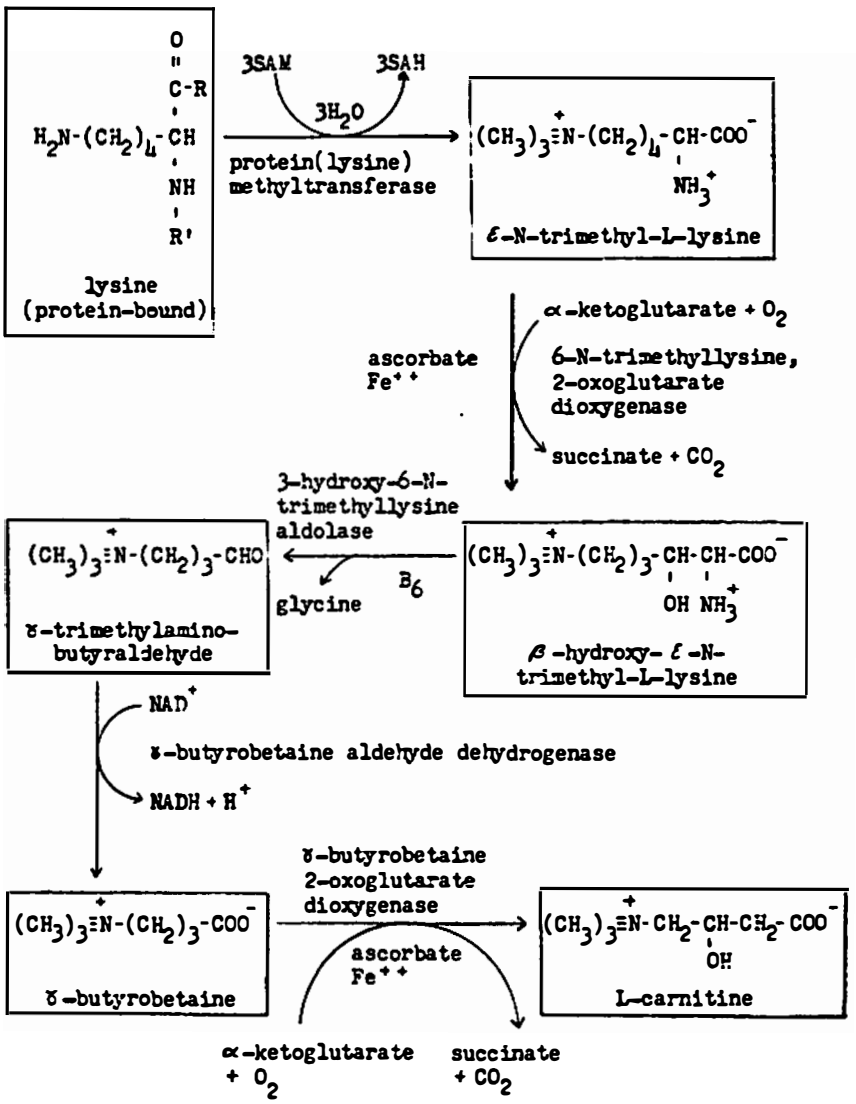


Figure 3. Biosynthesis of carnitine in mammals

skeletal muscle and heart. The uptake of carnitine into skeletal muscle is believed to be consistent with an energy-dependent active transport mechanism, which has a high affinity for carnitine with rate of transport saturated at high substrate concentration (50). Similarly, carnitine is also actively transported into heart cells with the rate dependent on inducible protein carriers (51).

The biochemical function of carnitine in facilitating the ß-oxidation of fatty acids is well established (52,53,54). This essential compound participates in the metabolic scheme depicted in Figure 4. In essence, by a carnitine-dependent process, the fatty acyl groups are initially transformed to acylcarnitine derivatives, a conversion occurring at the outer face of the inner mitochondrial membrane under the influence of carnitine:acyl CoA transferase I. Next, the acylcarnitines are transported inwards by a carnitine-acylcarnitine translocase system (55) through the inner mitochondrial membrane, thus reaching the mitochondrial matrix border. Finally, the acyl groups are liberated into the matrix through carnitine:acyl CoA transferase II for oxidation and entry into the citric acid cycle, while the carnitine fractions are exported outwards by the translocase system to function again in the fatty acid transport cycle.

It should be evident that maintenance of sufficient carnitine levels may be an important factor in allowing

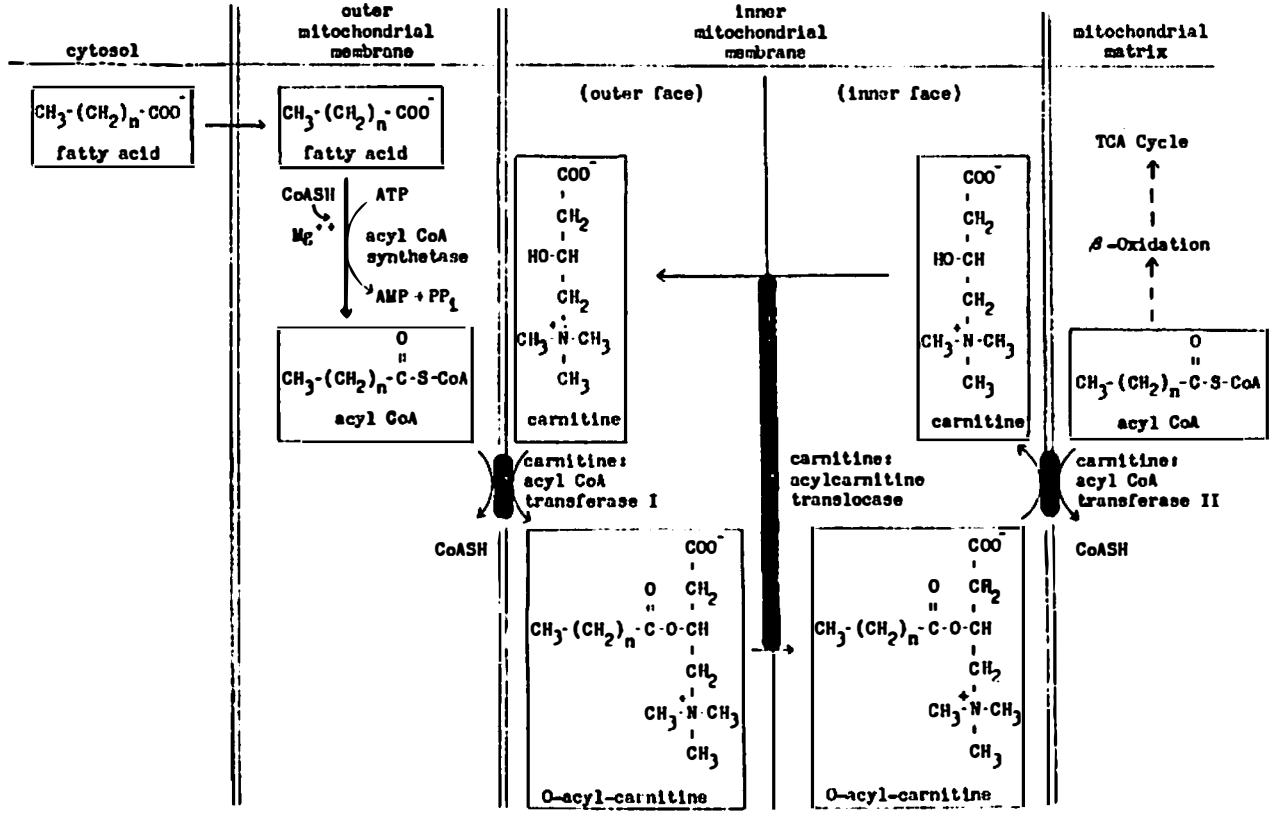


Figure 4. Facilitation of oxidation of fatty acids by carnitine

fatty acid oxidation to proceed at a normal rate to meet the physiological energy demands. Since PLMT catalyzes the first step in the biosynthetic pathway of carnitine, it is possible that modulating the activity of this enzyme may influence cellular levels of carnitine. Research is needed on the regulation of PLMT, since little if any work has been done in this area.

Phosphatidylethanolamine Methyltransferase, Phospholipid Biosynthesis and Biomembrane Function

There are two major pathways for the biosynthesis of phosphatidylcholine (PC), an important structural component of the endoplasmic reticulum and other membrane-containing parts of the cell. The first route utilizes choline and the cytidine coenzymes (56), while the other pathway (57) involves the SAM-dependent methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine methyltransferase (PEMT, EC 2.1.1.17). Although the rate of PC biosynthesis via the former route is considerably higher than that of the methylation pathway (58), the latter pathway is of concern here and is portrayed in Figure 5.

In rat liver microsomes, it is believed that two methyltransferases may be involved in the conversion of PE to PC (59). PEMT I catalyzes the initial methylation, and it has a high affinity for SAM (K_m < 1 μM), pH optimum of 8 and is activated by Mg^{++}. On the other hand, PEMT II

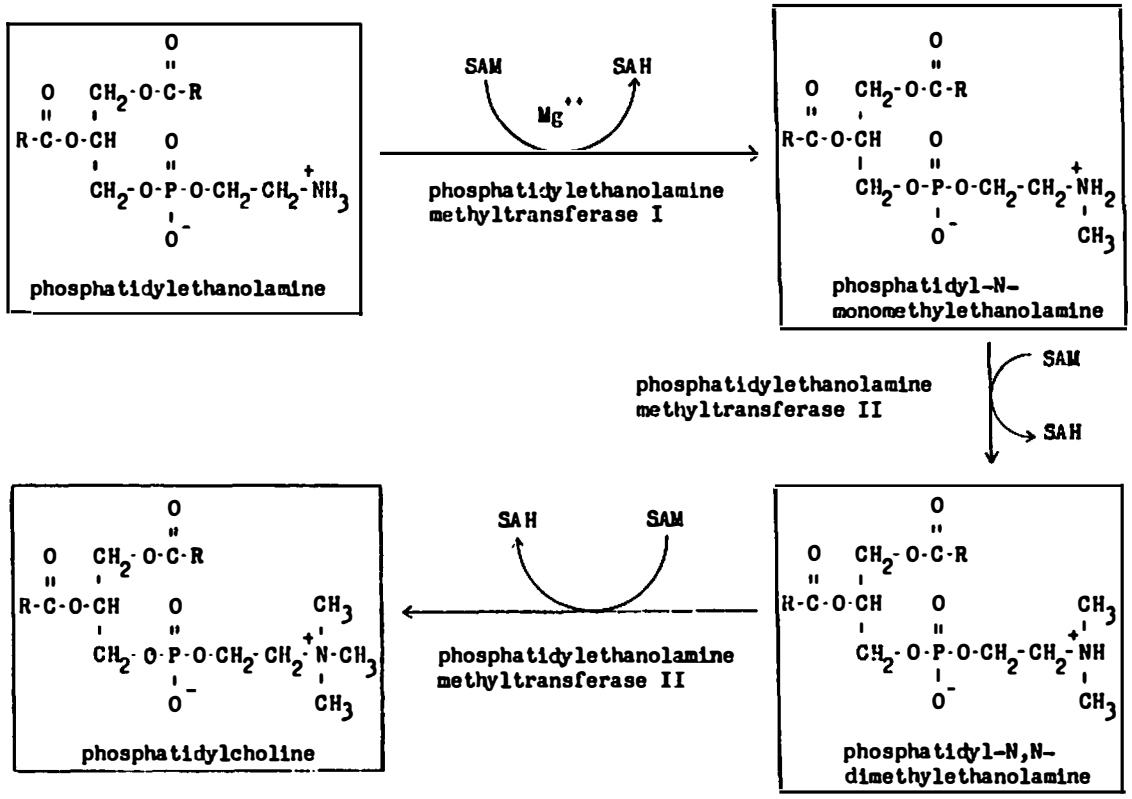


Figure 5. Formation of phosphatidylcholine from phosphatidylethanolamine via transmethylation

catalyzes the second and third methylation reactions, and it displays a low affinity for SAM (K_m ~67 μM) and a pH optimum of 10. As such, the conversion of PE to its monomethyl derivative is the rate limiting step in the formation of PC (60) via the methylation pathway. Moreover, SAH is a competitive inhibitor with respect to SAM of PEMT (60,61,62,63).

The most widely accepted model for the structure of cell membranes is the "fluid-mosaic" concept (64,65,66), which depicts a dynamic system consisting essentially of functional, globular proteins immersed in a fluidic phospholipid bilayer matrix. Using microsomal fragments from the hepatic endoplasmic reticulum, a comparative study has revealed that the phospholipid composition of this membrane fraction is similar in humans and rats (67). PC and PE are the major components, and comprise roughly 50 and 25%, respectively, of the total phospholipid.

The membrane phospholipid may assume significance in the function or associated activity of the endoplasmic reticulum. For example, with respect to xenobiotic metabolism, administration of phenobarbital to rats caused an increase in phospholipid content, especially PC, along with protein in liver microsomes (68). The increase in PC was associated with increases in the *in vivo* incorporation of radiolabeled methyl groups into PC and in the specific activity of PEMT. Thus, these findings indicate that PC

may support xenobiotic metabolism by facilitating the catalytic effects of cytochrome P-450 and NADPH-cytochrome c reductase, since PC is required for substrate hydroxylation by these two membrane-bound protein components of the mixed-function oxidase system (69,70). The importance of phospholipid in regulating the activities of membrane-bound enzymic proteins has been further reviewed (71).

Phospholipid methylation has also been implicated in the function of certain receptor proteins of the plasma membrane. In this regard, perhaps the most studied has been the β-adrenergic receptor site. Current belief (72,73,74) envisions this membrane-bound protein positioned to the exterior surface of the plasma membrane. Binding of an appropriate hormone or agonist to the receptor causes activation of PEMT, which proceeds to catalyze phospholipid methylation. The generated phospholipid species apparently lowers the microviscosity of the bilayer. The increase in membrane fluidity allows the receptor to move laterally within the bilayer matrix, enabling it to couple with the adenylate cyclase protein embedded to the interior surface of the plasma membrane. Coupling activates adenylate cyclase, which then catalyzes the production of c-AMP. Thus, this scheme of events is proposed to be prerequisite for elicitation of the physiological response by the hormone or agonist.

While it appears that the β-adrenergic receptor may regulate phospholipid methylation, one can argue that phospholipid methylation may regulate the β-adrenergic receptor. Regardless, it seems that modulation of the plasma membrane phospholipid content by PEMT is of prime importance. This further appears to be the case with respect to membrane-bound transport systems, such as the one involved in the aldosterone-stimulated transport of sodium (75). Therefore, more knowledge is needed on the regulation of PEMT, since PC biosynthesis via the methylation pathway may contribute to biomembrane integrity and function.

Guanidoacetate Methyltransferase and Creatine Metabolism

The complete pathway for the formation of creatine in the rat was depicted nearly four and a half decades ago (76). Biosynthesis of this compound initially requires the generation of guanidoacetate in kidney from arginine and glycine in the presence of arginine-glycine transamidinase (AGTA, EC 2.1.4.1). Guanidoacetate is then methylated in the liver by guanidoacetate methyltransferase (GAMT, EC 2.1.1.2) to produce creatine, with SAM serving as the methyl group donor. Once synthesized, creatine presumably exits the liver to be taken up and apparently concentrated particularly by muscle tissue. The movement of creatine from the bloodstream into muscle tissue is believed to be facilitated by a saturable, energy-requiring system, which

is capable of functioning against a concentration gradient (77). It has been reported that the concentration of creatine in resting muscle is about 12-fold higher than that of blood plasma (78).

Once uptake has occurred, creatine functions in an energy shuttle (78). It is phosphorylated in the presence of ATP and creatine phosphokinase (CPK, EC 2.7.3.2), and the phosphocreatine formed serves as a reservoir of high energy. When conditions favor it, e.g., muscle contraction, CPK works in reverse for the conversion of ADP to ATP. Thus, energy is readily available to muscle and other tissues due to the presence of phosphocreatine. On the other hand, the spontaneous dephosphorylation of phosphocreatine in muscle also occurs, which generates creatinine. Since it has no function, creatinine is excreted as a waste product by the kidneys. Figure 6 summarizes the biosynthesis of creatine and its metabolism.

GAMT apparently has the capacity to consume a sizable amount of SAM. It has been said that creatine metabolism is responsible for utilizing about 75% of the net methionine turnover (79), as originally concluded (80). Since the daily disposal of creatinine represents a loss of methyl groups, this conclusion may indeed be valid if replenishment of creatine is to be achieved.

It is evident that GAMT is undoubtedly the key enzyme in the biosynthesis of creatine. This methyltransferase

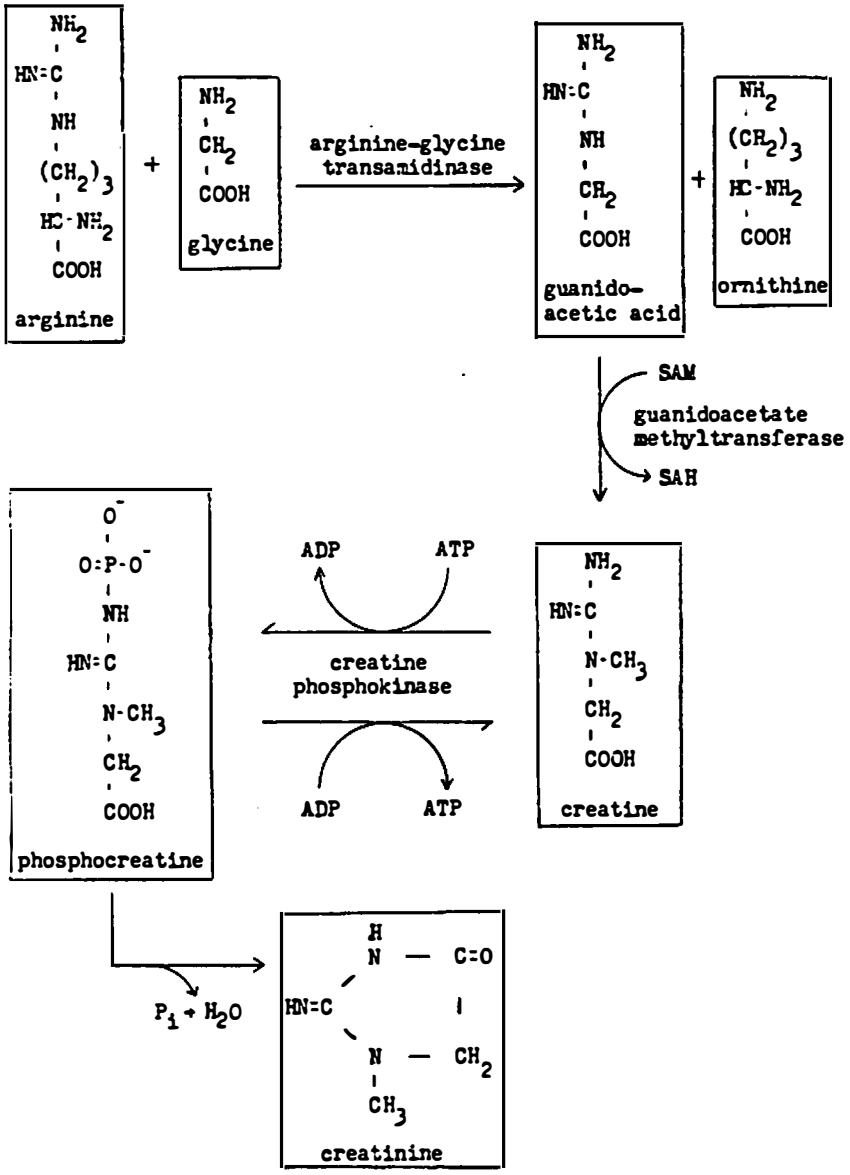


Figure 6. Biosynthesis of creatine and its metabolism

has been purified from both rat (81) and pig (79) livers. The rat enzyme appears to be a monomeric protein with an estimated molecular weight of 26,000. A similar value has been obtained for the pig enzyme. GAMT has been shown to require free sulfhydryl groups for activity; the oxidized enzyme shows no affinity for SAM (81). Once again, SAH is a strong regulator of GAMT (79,82), acting as a competitive inhibitor with SAM. A deeper probe into the nature of this inhibition with respect to GAMT, as well as the other methyltransferases, follows.

Regulation of Transmethylation by S-Adenosylhomocysteine and Related Compounds

The metabolism of methionine via the general pathway depicted below in Figure 7 offers a perspective for discussing possible control of the methyltransferases by SAH. As can be seen, once active methionine (SAM) has been formed, this compound can function as the methyl group donor in numerous transmethylation reactions. SAH is produced after methyl group transfer. In turn, S-adenosyl-homocysteine hydrolase (EC 3.3.1.1) is able to catalyze the reversible cleavage of SAH to homocysteine and adenosine, although the equilibrium of the reaction favors the condensation of homocysteine and adenosine to form SAH (83). The metabolism of SAH may be critical for the control of transmethylation.

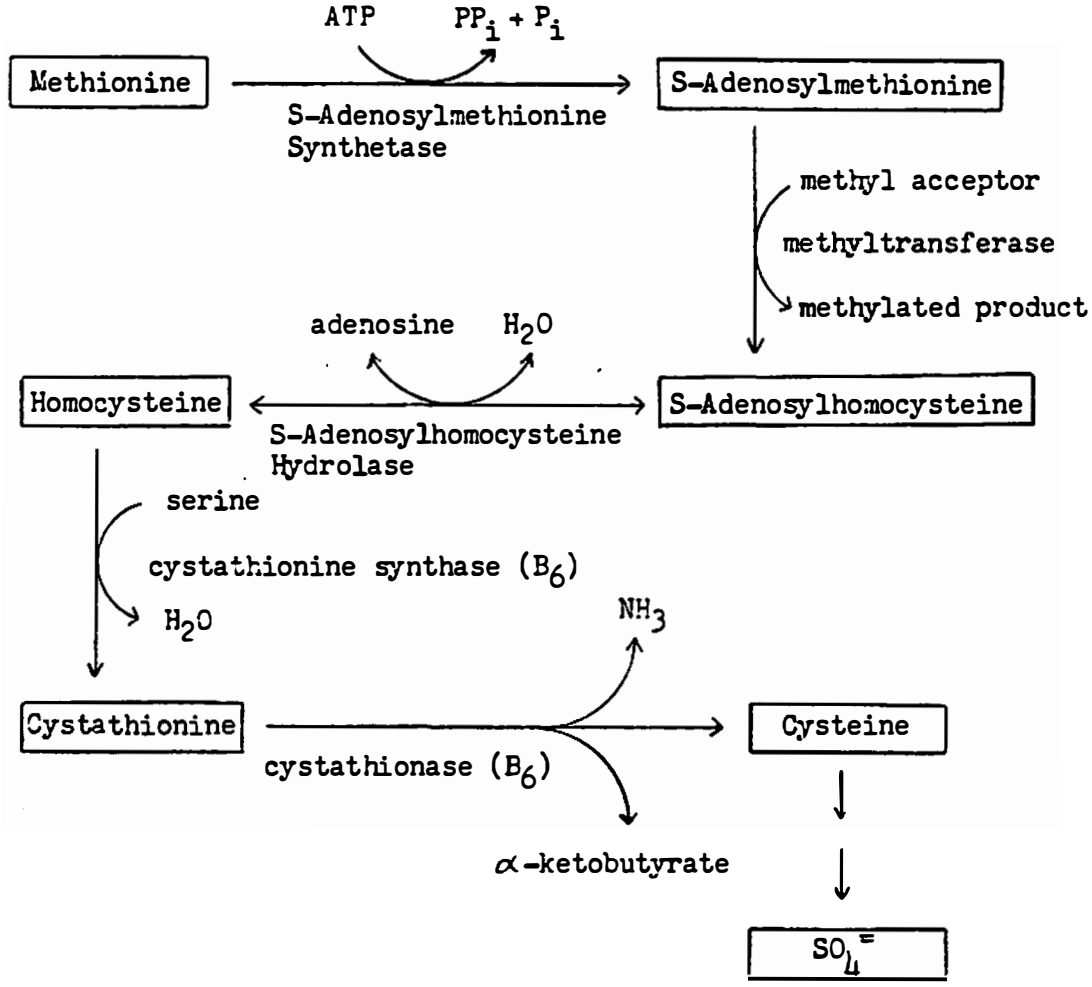


Figure 7. General metabolism of methionine

Using cell-free enzyme preparations, SAH has been demonstrated to be a potent, competitive inhibitor of many SAM-dependent transmethylation reactions. The methyltransferases of interest in the present review are all subject to inhibition by SAH, as reported previously for TMT (2,7,8), TPMT (3), PLMT (84), PEMT (60,61,62,63), and GAMT (79,82). Some other enzymes inhibited by SAH *in vitro* include catechol-O-methyltransferase (EC 2.1.1.6) and phenylethanolamine-N-methyltransferase (EC 2.1.1.28) (85), histamine-N-methyltransferase (EC 2.1.1.8) (86), protein carboxyl-O-methyltransferase (EC 2.1.1.24) (87), DNA (cytosine-5-)-methyltransferase (EC 2.1.1.37) (88), and tRNA(adenine-1-)-methyltransferase (EC 2.1.1.36) and tRNA(guanine-2-)-methyltransferase (EC 2.1.1.32) (89). Table 1 shows some kinetic data obtained for these methyltransferases with respect to their affinities for SAM and SAH. The K_i is lower than the K_m for the majority of these enzymes. In other words, SAH has a greater affinity than SAM for certain methyltransferases, including TMT, TPMT, PLMT, PEMT, and GAMT. Thus, it is a distinct possibility that the absolute molar ratio of SAM to SAH (SAM:SAH ratio) may regulate transmethylation. Elevation of the ratio should stimulate the reaction, whereas reduction of the ratio should inhibit methyl group transfer. For instance, decreasing the SAM:SAH ratio *in*

TABLE 1

Affinities of Some Methyltransferases for S-Adenosylmethionine and S-Adenosylhomocysteine

Enzyme	Source	K_m	K_i
		μM	
Thiol Methyltransferase (7,8)[1]	Rat Liver	1	0.2
Thiopurine Methyltransferase (3)	Human Kidney	2.7	0.75
Protein(Lysine)Methyltransferase (84)	Rat Brain	11.5	5.5
Phosphatidylethanolamine Methyltransferase I (63)	Rat Liver	18	3.8
Guanidoacetate Methyltransferase (79)	Pig Liver	49	16
Catechol-O-Methyltransferase (85)	Rat Liver	14	4.4
Phenylethanolamine-N-Methyltransferase (85)	Rabbit Adrenal	10	1.4
Histamine-N-Methyltransferase (86)	Guinea Pig Liver	6	5
Protein Carboxyl-O-Methyltransferase (87)	Human RBC	1.9	1.6
DNA (Cytosine-5-)-Methyltransferase (88)	Rat Liver	1.3	21
tRNA(adenine-1-)-Methyltransferase (89)	Rat Liver	1.5	2.4
tRNA(guanine-2-)-Methyltransferase I (89)	Rat Liver	2.0	8.0
tRNA(guanine-2-)-Methyltransferase II (89)	Rat Liver	2.0	0.3

[1]Numbers in parentheses indicate references.

vitro has been shown to inhibit PEMT, even when the concentration of SAM was varied over a wide range (62,63).

In view of the rather impermeable nature of SAH to biomembranes (90), it has become necessary to study the effect of SAH on transmethylation *in vivo* using a different approach than merely treating isolated whole cells with exogenous SAH, or administering this purine nucleoside to animals. For this reason, the precursors of SAH, i.e., adenosine and L-homocysteine, offer the potential to delineate the regulation of transmethylation by SAH *in vivo*. Presumably, exogenous adenosine and L-homocysteine are able to permeate the cell membrane, and once inside the cell can react in the presence of SAH hydrolase to form SAH. Indeed, the equilibrium of the reaction catalyzed by this enzyme favors condensation (83). Moreover, when the adenosine and L-homocysteine are not removed at sufficient rates, e.g., by adenosine deaminase and the remethylation pathway or cystathionine formation, respectively, the hydrolysis of SAH may be prevented (83). In support of this concept, when isolated lymphoblasts were incubated in the presence of added adenosine and homocysteine, cellular levels of SAH increased markedly with only slight elevation of SAM (91). Consequently, the fall in the cellular SAM:SAH ratio was accompanied by inhibition of DNA and RNA methylation. Using the perfused rat liver (92), similar results have been obtained. Lowering the SAM:SAH ratio *in*

vivo by the addition of adenosine and homocysteine to the perfusate caused a decrease in the activities of the enzymes involved in the methylation of not only DNA, but also histone and phospholipid.

The intraperitoneal co-administration of adenosine and homocysteine to animals has also been attempted with positive results. After receiving an equal dose by weight of adenosine and homocysteine, tissue levels of SAH in the mouse were elevated markedly, with a resultant decline in the SAM:SAH ratio (93,94). The lowered SAM:SAH ratio was accompanied by a decrease in the activities of brain catechol-O-methyltransferase and histamine-N-methyltransferase as measured *in vitro* (93), and also by inhibition of protein and phospholipid methylation *in vivo* (94). However, these pharmacological effects may have been due to homocysteine alone, without the involvement of exogenous adenosine, in the injections used (95). It is known that homocysteine, per se, is an inhibitor of SAH hydrolase (83), and thus could promote SAH accumulation when administered alone. The possibility also exists that the supply of endogenous adenosine is more than adequate to condense with the exogenous homocysteine to form SAH.

Several adenosine analogues, illustrated in Figure 8 with adenosine, may competitively inhibit SAH hydrolase, allowing the accumulation of endogenous SAH, in addition to the formation of their respective SAH analogues. Thus,

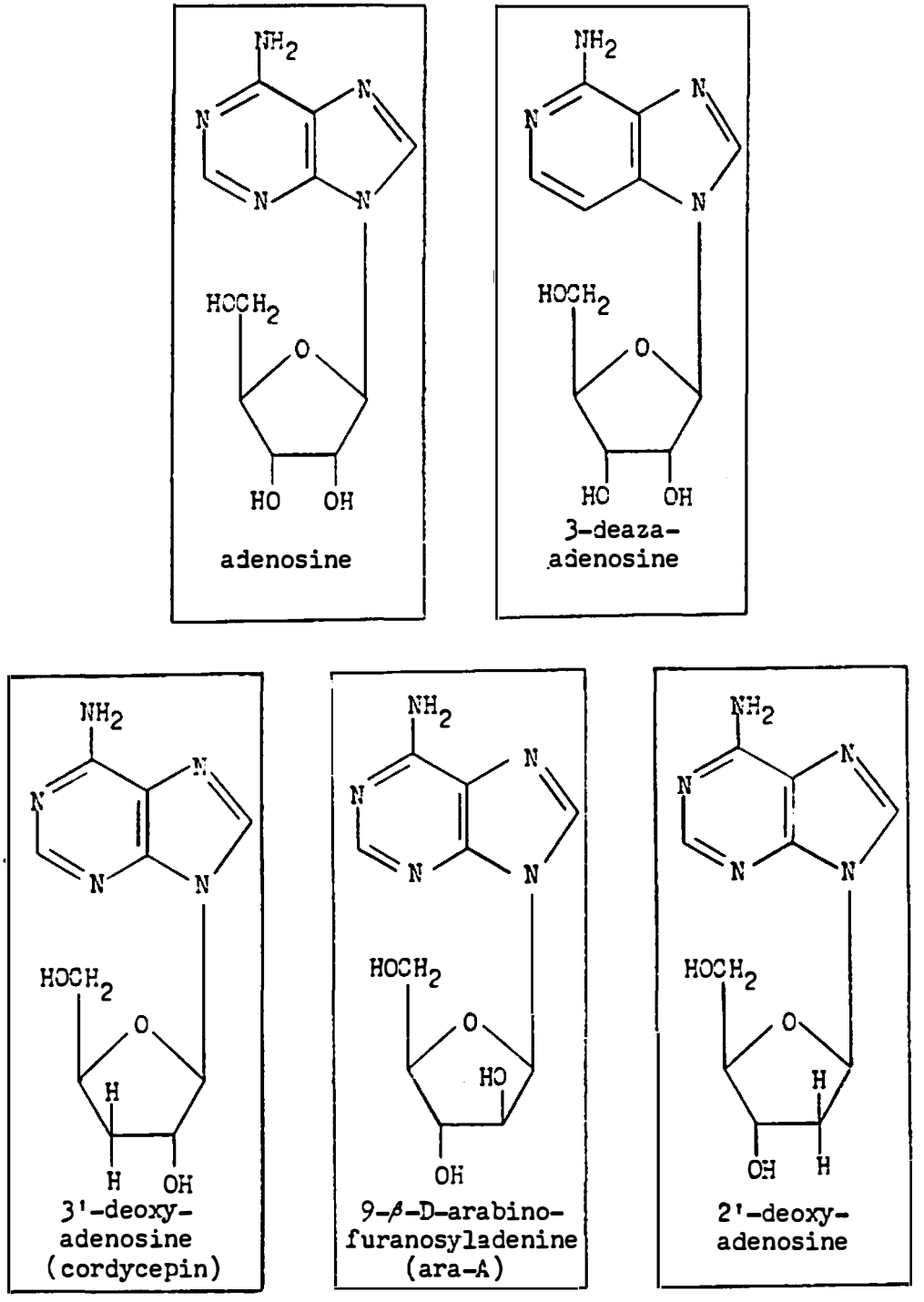


Figure 8. Adenosine and some of its analogues

adenosine analogues have also been employed to study the regulation of transmethylation by SAH. For instance, 3-deazaadenosine promoted an accumulation of SAH and inhibited phospholipid methylation in isolated hepatocytes (96). Moreover, rats administered injections of 3-deazaadenosine formed 3-deazaadenosylhomocysteine and showed a reduction in the SAM:SAH ratio (97). When transmethylation was evaluated in these animals, there were reduced levels of liver creatine and methylated lipids, as well as a decrease in the urinary levels of 3-methoxy-4-hydroxymandelic acid (VMA), suggesting an inhibition of the methylation of the catecholamines. Decreased phospholipid methylation in livers of rodents treated with 3-deazaadenosine has also been reported (98).

Mice receiving repeated injections of ara-A (Figure 8) along with the adenosine deaminase inhibitor, 2'-deoxycoformycin, displayed a marked reduction in the SAM:SAH ratio in several tissues (99), although the effect on transmethylation was not evaluated. However, ara-A has been shown to inhibit phospholipid methylation in isolated hepatocytes (96). Furthermore, 2'-deoxyadenosine has been shown to inhibit SAH hydrolase, which was associated with an elevation of SAH and inhibition of nucleic acid methylation in lymphoblasts (91). Cordycepin can also inhibit nucleic acid methylation, although this effect may not be due to changes in the levels of SAH per se (91).

Some synthetic analogues of SAH, shown in Figure 9 with SAH, have provided yet another means to delve into the control of transmethylation. The effect of these chemicals on phospholipid methylation varied according to whether cell-free or whole cell preparations were used (96). In the former condition, c^7AdoHcy (Figure 9) and sinefungin were inhibitory, but SIBA (Figure 9) was not. Using intact cells, c^7AdoHcy was once again effective along with SIBA, but sinefungin was without effect. Incidentally, SAH was inhibitory using broken cells, but not when using intact, isolated hepatocytes.

It is apparent that modulation of the cellular SAM:SAH ratio, and accordingly the activity of the methyltransferases, may have far-reaching physiological ramifications. Therefore, factors altering the ratio *in vivo* merit further investigation.

Methionine Metabolism During Pyridoxine Deficiency

A closer examination of Figure 7 will reveal that pyridoxine is involved in the metabolism of methionine to cysteine. As can be seen, once the intermediary metabolite, homocysteine, has been produced, this product can react with serine in the presence of cystathionine synthase (EC 4.2.1.22) to form cystathionine. Subsequently, cleavage of cystathionine by cystathionase (EC 4.4.1.1) generates cysteine. Evidence has been

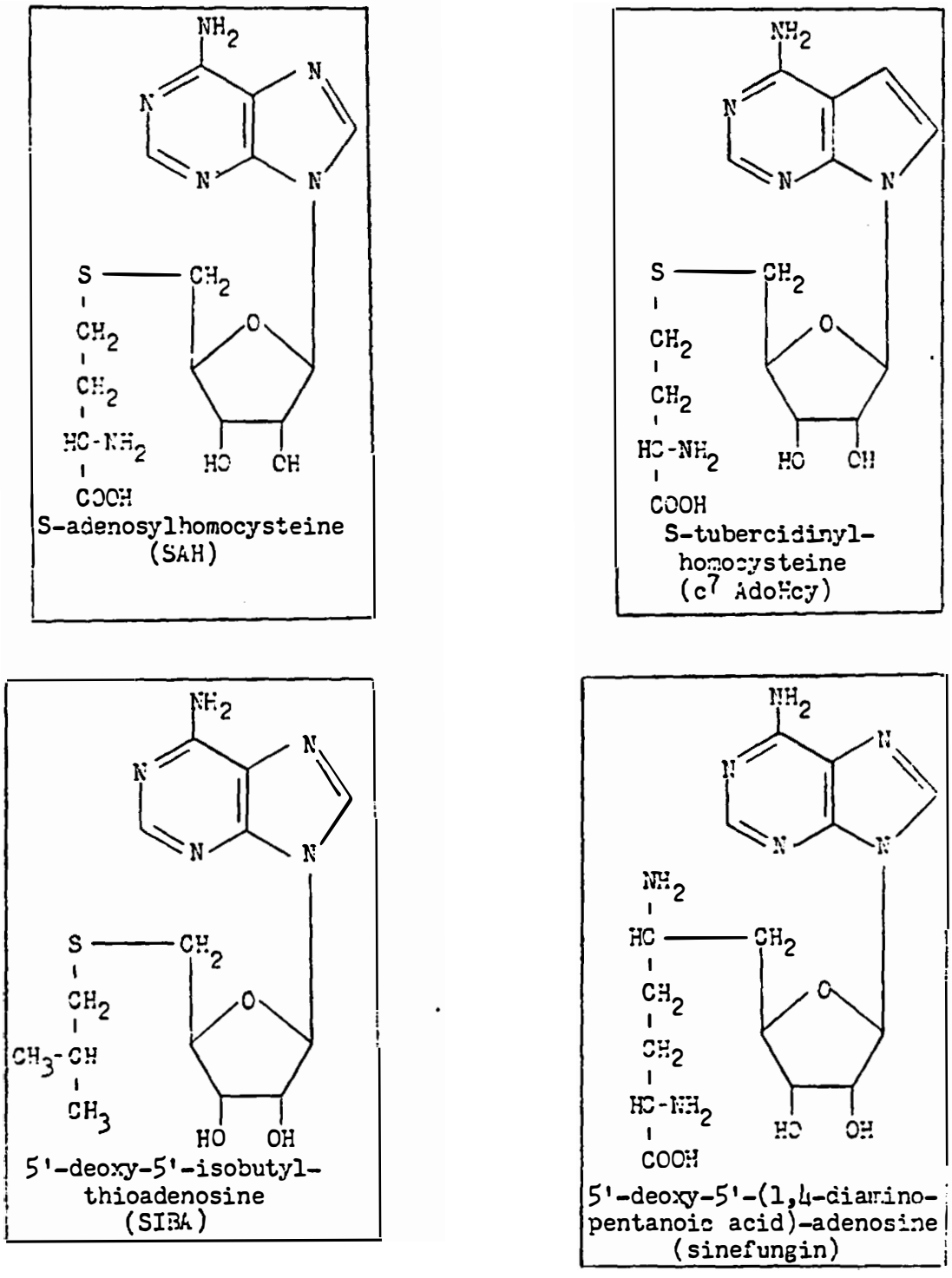


Figure 9. S-Adenosylhomocysteine and some of its analogues

presented that cystathionine synthase and cystathionase each require pyridoxal phosphate as a cofactor. Rats fed a pyridoxine-deficient diet for 5 weeks displayed a decrease in the activity of liver cystathionase, and when this enzyme from these animals was assayed in the presence of added pyridoxal phosphate, a noticeable increase in activity was observed (100, 101). Moreover, pyridoxine-deprived animals repleted with injections of pyridoxine hydrochloride also showed a restoration of cystathionase activity (101). On the other hand, rats fed a pyridoxine-deficient diet for 5 weeks did not show any significant decrease in the specific activity of liver cystathionine synthase, even when assayed with added pyridoxal phosphate (100). However, rats fed a pyridoxine-deficient diet for 8 weeks did show a decrease in liver cystathionine synthase activity, which could be stimulated by added pyridoxal phosphate (101). Thus, it would appear that cystathionase is more sensitive to pyridoxine depletion. This would explain why pyridoxine deficiency produces an increase in tissue cystathionine levels (100). Furthermore, it is known that pyridoxine deficiency induces the urinary excretion of cystathionine (102).

Previous research has also shown that a pyridoxine-deficient diet can produce a marked elevation of SAH in rat liver, with a slight rise in the level of SAM (103). It is likely that this alteration results from a reduction in the

activity of cystathionase, and perhaps cystathionine synthase, which would lead ultimately to the accumulation of homocysteine. With the remethylation pathway unable to function at a sufficient rate to remove excess homocysteine, coupled with the presence of adequate levels of adenosine, the formation and elevation of SAH to abnormal levels would ensue. The accumulation of SAH would occur because the condensation of homocysteine and adenosine would be favored (83). In addition, homocysteine may inhibit the hydrolysis of SAH by SAH hydrolase (83) and cause an elevation of SAH in this manner. In any event, the full ramifications of a lowered SAM:SAH ratio in liver brought on by a pyridoxine deficiency are not known.

As reviewed up to this point, SAH is a competitive inhibitor of various SAM-dependent, transmethylation reactions. For this reason, it is hypothesized that modulation of the SAM:SAH ratio may modify the activity of some methyltransferases and the related biochemical processes facilitated by these enzymes.

CHAPTER III

EXPERIMENTAL PROCEDURE

General Outline of Research

In order to study the effect of modulating the SAM:SAH ratio on the activity of some methyltransferases and transmethylation-related processes, three phases of investigation were essentially conducted and involved both *in vitro* and *in vivo* alteration of the ratio. In the initial phase, the SAM:SAH ratio was modulated *in vitro*, and the effect of this manipulation on the activities of rat liver TPMT, TMT, and PLMT was evaluated. Next, alteration of the hepatic SAM:SAH ratio was achieved *in vivo* by pharmacological means, and this procedure was used to study the regulation of TPMT, TMT, and PLMT as well. Finally, the hepatic SAM:SAH ratio was also altered *in vivo* by diet, i.e., pyridoxine deficiency, to study its effect on the activities of TPMT, TMT, PLMT, PEMT, and GAMT in liver, along with some of the biochemical processes facilitated by these enzymes. With respect to PLMT, cardiac and skeletal muscle carnitine levels were determined, as well as the capacity of heart and skeletal muscle to carry out fatty acid oxidation *in vitro*. Cytochrome oxidase activity was also assayed in these two muscle tissues, as a parameter of mitochondria content and/or activity. In the case of PEMT, the phospholipid content (PC and PE) of hepatic

microsomes was determined. Assay of GAMT necessitated the determination of creatine levels in liver and skeletal muscle, the activity of AGTA in kidney, and the excretion of urinary creatinine.

Preparative and Analytical Instrumentation

Tissue homogenization was carried out on ice using a Thomas glass tissue homogenizer (Arthur H. Thomas Co., Philadelphia, PA) with a motor-driven teflon pestle for liver and kidney. A Duall #23 ground-glass tissue homogenizer (Kontes Scientific Glassware, Vineland, NJ) or a Brinkmann polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) was employed for preparing skeletal muscle and heart homogenates. Subcellular fractionation at 2^{o} was accomplished using a Beckman L5-50 ultracentrifuge with either a type 30 aluminum alloy or a type 50 titanium fixed-angle rotor (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). Otherwise, an International model V, size 2 centrifuge with a type 241 rotor (International Equipment Co., Boston, MA) was used for general, low-speed (<1000 x g) centrifugation at room temperature.

Enzyme assays were generally performed in a controlled-temperature, water bath shaker (Eberbach Corp., Ann Arbor, MI). Samples requiring sonication were processed with a model BP1 Biosonik Sonicator (Bronwill Scientific, Rochester, NY). A tube block heater (Precision

Scientific Group, Chicago, IL) was employed to digest samples at high temperature.

Radiochemical assays were facilitated by a Beckman LS-100C Liquid Scintillation System (Beckman Instruments, Inc., Irvine, CA) with quenching monitored by the external standard ratio. Generally, samples were counted to yield a 2% error in a standard scintillation cocktail consisting of 10 ml 2,5-diphenyloxazole (PPO) in toluene (12 g/L) and 6 ml ethylene glycol monoethyl ether (cellosolve). Spectrophotometric determinations were conducted using Beckman models 34 and DU-30 vis-uv spectrophotometers (Beckman Instruments, Inc., Fullerton, CA). Colorimetric assays near the infra-red wavelength spectrum were made possible using a Bausch and Lomb Spectronic 88 spectrophotometer (Bausch and Lomb, Rochester, NY).

Modulation of the S-Adenosylmethionine to S-Adenosylhomocysteine Ratio In Vitro

The SAM:SAH ratio was modulated *in vitro* by merely adjusting the levels of exogenously-added SAM and SAH in the standard enzyme assay reaction mixtures for TPMT, TMT, and PLMT.

Pharmacological Alteration of the Hepatic S-Adenosylmethionine to S-Adenosylhomocysteine Ratio

The hepatic SAM:SAH ratio of male Sprague-Dawley rats (350-425 g bwt) from Taconic Farms, Germantown, NY, was

altered *in vivo* by the intraperitoneal injection of either 500 or 1000 mg D,L-homocysteine thiolactone/2 ml of 0.9% NaCl/kg bwt. Control animals were injected with the saline solution. These rodents were used for the study of the sulfhydryl xenobiotic transmethylases and were fed standard chow and water *ad libitum* up to the time of drug treatment. PLMT was studied separately using slightly smaller rats (225-300 g bwt). About 30-40 minutes after injection, rats were sacrificed and processed as described below (p. 43). The lower limit of the time range (30 minutes) was more often selected, since the drug elicited signs of convulsion in a few animals allowed to sit the additional minutes.

Dietary Alteration of the Hepatic S-Adenosylmethionine to S-Adenosylhomocysteine Ratio

Three groups of 15 weaning-age (45-60 g bwt), male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were fed their respective diets for 6-7 weeks. One group of animals was fed *ad libitum* a pyridoxine-deficient diet (104), slightly modified as shown in Table 2, while a second group was pair-fed the same diet supplemented with pyridoxine hydrochloride (22 mg/kg prepared diet). The third group was fed the latter diet *ad libitum* and served as the control. Water was freely available to all animals, and their feeding bowls were changed daily with clean counterparts containing fresh food. Feed intake was

TABLE 2

Pyridoxine-Deficient Diet

Component	g/kg
Vitamin-Free Casein[1]	293.5
Sucrose	274.0
Corn Starch	274.0
Corn Oil[2]	19.6
Hydrogenated Vegetable Oil[3]	58.7
Alphacel[1]	19.6
Vitamin Diet Fortification Mixture Without Pyridoxine Hydrochloride	21.5
Hawk Oser Salt Mixture[1,5]	39.1

[1]ICN Nutritional Biochemicals, Cleveland, Ohio.

[2]Mazola (Best Foods, Englewood Cliffs, New Jersey).

[3]Crisco (Procter and Gamble, Cincinnati, Ohio).

[4]Composition: Vitamin A Acetate (500,000 IU/g), 1.8 g/kg; Vitamin D_2 (850,000 IU/g), 0.125 g/kg; Alpha Tocopherol Acetate (250 IU/g), 22.0 g/kg; Ascorbic Acid, 45.0 g/kg; Inositol, 5.0 g/kg; Choline Chloride, 75.0 g/kg; Menadione, 2.25 g/kg; p-Aminobenzoic Acid, 5.0 g/kg; Niacin, 4.25 g/kg; Riboflavin, 1.0 g/kg; Thiamine Hydrochloride, 1.0 g/kg; Calcium Pantothenate, 3.0 g/kg; Biotin, 0.02 g/kg; Folic Acid, 0.09 g/kg; Vitamin B_{12}, 0.00135 g/kg.

[5]Composition: Calcium Carbonate, 6.860%; Calcium Citrate, 30.830%; Calcium Phosphate, Monobasic, 11.280%; Ferric Citrate, 1.532%; Magnesium Carbonate, 3.520%; Magnesium Sulfate, Anhydrous, 3.830%; Manganous Sulfate, 0.020%; Potassium Aluminum Sulfate, 0.009%; Potassium Chloride, 12.470%; Potassium Iodide, 0.004%; Potassium Phosphate, Dibasic, 21.880%; Sodium Chloride, 7.710%; Sodium Fluoride, 0.051%.

recorded each day, and the rats were weighed weekly. Three days before animal sacrifice, 5 rats from each of the 3 groups were placed individually in metabolic cages for collection of the 24-hour urinary output. The urine was collected for each rat in a 40 ml conical centrifuge tube containing 1 ml of 0.1 N HCl for 3 consecutive days. The urine was then processed for creatinine determination as described later (p. 69). Otherwise, all animals were housed individually in wire-bottom cages with an automated light cycle from 6:00 AM to 6:00 PM.

Animal Sacrifice, Tissue Preparation and Subcellular Fractionation

Rats were stunned by a blow to the back of the head and decapitated with shears. Selected tissues were rapidly excised, and if not used immediately, placed in ice-cold 5 mM potassium phosphate buffer, pH 7.5, for storage at -80°. Only liver was removed from the drug-treated animals, while liver, kidney, heart, and muscle tissue from the rear of the thigh of the hind legs were taken from rats on the experimental diets. Immediately after excision of the liver, approximately 4-5 g of the tissue was quickly placed in ice-cold 10% (w/v) TCA for mincing. Within a few minutes, a 1:5 (w/v) homogenate was prepared, which was then centrifuged at 10,000 x g for 15 minutes at 2°. The tissue extract, i.e., TCA supernatant, was stored at -20°

if not used immediately for quantitative analysis of SAM and SAH, as detailed below (p. 45). It should be noted that direct homogenization of hepatic tissue in acid is necessary to prevent enzymatic degradation of endogenous SAM to SAH (105).

Differential centrifugation was used to obtain the microsomal and cytoplasmic fractions from liver tissue. In the case of kidney, only the cytoplasmic fraction was isolated. A 1:9 (w/v) liver homogenate was prepared in ice-cold 5 mM potassium phosphate buffer, pH 7.5. The same tissue homogenate concentration was also applied to kidney, but in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. The homogenates were centrifuged at 10,000 x g for 10 minutes at 2^{o}. Next, the supernatant was centrifuged at 100,000 x g for 60 minutes. The high-speed supernatant, i.e., cytoplasmic fraction, from liver was used for the assay of TPMT and GAMT. On the other hand, the cytoplasmic fraction from kidney was utilized for the assay of AGTA. The liver microsomal pellet was rinsed and resuspended in the homogenizing buffer to give a protein concentration of 1-2 mg/ml. This microsomal suspension was used for the assay of TMT, PEMT, and phospholipid concentration.

Quantitative Analysis of Tissue Levels of S-Adenosyl-methionine and S-Adenosylhomocysteine in Liver

In order to determine the concentrations of SAM and SAH present in the liver TCA supernatant prepared previously (pp. 43-44) using a coupled radiochromatographic-spectrophotometric method (103), radiolabeled SAM and SAH are needed as internal standards. While the former compound is commercially available as [^{14}C-methyl]-SAM, [8-^{14}C]-SAH must be synthesized and purified (103).

Initially, L-homocysteine was prepared (106) by incubating 0.1 mmole of L-homocysteine thiolactone·HCl with 0.3 ml of 5 N NaOH for 5 minutes at room temperature. The mixture was immediately neutralized with 1.5 ml of 1 M KH_2PO_4 and diluted to a total volume of 5.33 ml. A theoretical yield of about 37 µmoles/ml has been determined (106). Next, [8-^{14}C]-SAH was synthesized by incubating the following mixture, in a total volume of 0.25 ml, at 37^{o} for 12 minutes: 0.025 ml of 1 M potassium phosphate buffer, pH 7.3, 0.5 µmole dithiothreitol, 0.006 µmole of erythro-9-(2-hydroxyl-3-nonyl)adenine·HCl (courtesy of Burrough Wellcome Co., Research Triangle Park, NC), 0.75 µmole of L-homocysteine from above, 0.31 µmole of [8-^{14}C]-adenosine (sp. act. 52 mCi/mmole) and rat liver cytosol (0.5 mg protein). The reaction was terminated by addition of 0.025 ml of 30% (w/v) $HClO_4$. The precipitate was pelleted by centrifuging at 900 x g for 10 minutes.

Purification of the radiolabeled product was performed using cation-exchange chromatography. The acid supernatant was applied to a 1 X 6 cm column of phosphocellulose (Cellex P, H^+ form from Bio-Rad, Richmond, CA). The column was washed with 100 ml of 1 mM HCl, and then 10 mM HCl until no more radioactivity was detected in the effluent. The resin is able to remove unreacted [8-^{14}C]-adenosine and other contaminants, while binding the [8-^{14}C]-SAH formed. Detection of radioactivity was carried out by periodically taking small samples of the effluent (ca. 1 ml) coming off the column and assaying them by liquid scintillation counting (p. 40). The [8-^{14}C]-SAH was eluted from the column with 100 mM HCl. The effluent was collected in 2.5 ml fractions. The radioactive fractions were pooled and adjusted to pH 9 with 10 M NaOH and stored at -20°. The radiochemical purity of the product, as similarly prepared, has been claimed to be about 98% (103). In any event, the final preparation provided about 3.5 X 10^4 cpm/0.05 ml. Since [8-^{14}C]-SAH was to be used only as an internal "detection" standard, this value was deemed adequate. Otherwise, the radioactive solution can be concentrated by lyophilization and resuspension.

Finally, the liver TCA supernatant was processed for measurement of SAM and SAH. First, 10 ml of this tissue extract were extracted 3 times with an equal volume of ethyl ether in a glass-stoppered, conical centrifuge tube.

After centrifugation at 600 x g for 5 minutes each time, the organic phase was removed and discarded by aspiration. The final ether-extracted tissue extract was then filtered through a glass funnel lined with Whatman #1 filter paper. To 5 ml of the filtrate (corresponding to 0.83 g wet liver tissue weight) was added 0.05 ml each of [8-^{14}C]-SAH (3.5 X 10^4 cpm) and [^{14}C-methyl]-SAM (4.5 X 10^4 cpm). Incidentally, the specific activities of these two radiochemicals are not given, since they were used strictly as detectors. The radiolabeled mixture was quantitatively transferred to a 1 X 7 cm phosphocellulose column (Cellex P, H^+ form from Bio-Rad, Richmond, CA) for cation-exchange chromatography. The column was washed with 100 ml of 1 mM HCl, then 100 ml of 10 mM HCl to remove U.V.-absorbing contaminants. SAH was eluted from the column with 80 ml of 50 mM HCl, followed by SAM with 60 ml of 500 mM HCl. In each case, only the first five, 10 ml fractions were collected, and the presence of SAH and SAM was detected by counting 1 ml portions from the fractions for radioactivity (p. 40).

The actual quantitation of SAM and SAH was made spectrophotometrically on the radioactive fractions. This was done by measuring the absorbance of the samples at 257 nm and using a molar extinction coefficient of 15,000 liter$\cdot$mol$^{-1}\cdot$cm^{-1} for both substances. Therefore, the concentrations of SAM and SAH can be derived by

substituting $A=(a_m)(C)(L)$. The results were expressed as nanomoles/g wet tissue weight.

Assay of Thiopurine Methyltransferase

The assay procedure for TPMT (40) is based on the transfer of the methyl group from [^{14}C-methyl]-SAM to 6-thiopurine. The radiolabeled product, 6-[^{14}C-methyl]-thiopurine, is isolated by organic solvent extraction, and its radioactivity measured by liquid scintillation spectrometry.

The liver cytoplasmic fraction (p. 44) was diluted with 5 mM potassium phosphate buffer, pH 7.5, containing 0.25% bovine serum albumin (2:7, v/v). To 9 volumes of this preparation was added 1 volume of a 50% suspension in water of Chelex-100 chelating resin (Bio-Rad, Richmond, CA). This mixture, inside a sealed tube, was then allowed to rotate end-over-end for 1 hour at 4^{o} using a fabricated rotary mixer. The speed of rotation was about 15 rpm. This chelating process is said to remove magnesium, therefore inhibiting hypoxanthine guanine phosphoribosyl-transferase activity (3). The resin was separated by centrifuging at 5,000 x g for 10 minutes at 2^{o}. The supernatant was used as the enzyme preparation for the assay procedure.

Into a 15-ml conical glass centrifuge tube were added 0.025 ml of 0.4 M potassium phosphate buffer (pH 6.2), 0.01

ml of 6-thiopurine in dimethyl sulfoxide (DMSO) (23.6 mg/ml), and 0.1 ml of the enzyme preparation (~0.4 mg protein). The reaction was initiated by adding 0.025 ml of the following mixture: 0.02 mmole of dithiothreitol, 0.077 µmole of allopurinol (to inhibit xanthine oxidase), and 0.044 µmole of [^{14}C-methyl]-SAM (sp. act. 16.4 X 10^6 cpm/ µmole) in a total volume of 0.3 ml. The blank contained all of the components in the reaction mixture, including DMSO, but without 6-thiopurine. Concentrations of SAM and 6-thiopurine in the reaction mixture were 23 µM and 9.7 mM, respectively, in a total incubation volume of 0.16 ml. Accordingly, 6.0 X 10^4 cpm as [^{14}C-methyl]-SAM were used per enzyme assay tube. Incubation was for 30 minutes at 37^{o}.

The reaction was terminated by adding 0.5 ml of 0.5 M sodium borate buffer, pH 10. After addition of 2.5 ml of 20% (v/v) isoamyl alcohol in toluene, the tube was stoppered and vortexed for 10 seconds. The mixture was then centrifuged at 700 x g for 10 minutes. Finally, a portion of the top organic phase containing the radiolabeled product was removed for liquid scintillation counting (p. 40). With respect to the drug-treated animals, 1.5 ml of the organic phase was counted. In the case of animals fed the experimental diets, 2.0 ml of the organic phase were counted. The specific activity of TPMT was expressed as CPM/mg protein/30 minutes.

Assay of Thiol Methyltransferase

The principle for the assay of TMT (2) is similar to that described above for TPMT, but methylation of β-mercaptoethanol in the presence of the liver microsomal suspension (p. 44) occurs instead. Into a 15-ml conical glass centrifuge tube were added the following components in a total volume of 0.25 ml: 0.05 ml of 0.5 M potassium phosphate buffer (pH 7.9), 2.4 μmoles of β-mercaptoethanol, 0.25 μmole of [^{14}C-methyl]-SAM (sp. act. 4.1 X 10^5 cpm/μmole), and 0.1 ml of the microsomal suspension (~ 0.2 mg protein). The blank contained all of these components with the exception of β-mercaptoethanol. The reaction was initiated by the enzyme preparation. Thus, concentrations of SAM and β-mercaptoethanol in the assay were 1.1 and 9.6 mM, respectively. About 1.0 x 10^5 cpm as [^{14}C-methyl]-SAM were present in the reaction mixture. The reaction was allowed to run for 30 minutes at 37°.

The reaction was stopped by addition of 0.5 ml of 0.5 M sodium borate buffer, pH 10. Three ml of toluene/isoamyl alcohol (3:2, v/v) were then added. The tube was stoppered and vortexed for 10 seconds. Following centrifugation at 900 x g for 10 minutes, 2 ml of the organic phase on top were removed and counted for radioactivity via liquid scintillation spectrometry (p. 40). The specific activity of TMT was expressed as CPM/mg protein/30 minutes.

Assay of Protein(Lysine)Methyltransferase

The radiochemical procedure is based on the methylation of histone in the presence of [^{14}C-methyl]-SAM (44). Initially, a 1:9 (w/v) whole liver tissue homogenate was prepared in ice-cold 0.25 M sucrose containing 3 mM $CaCl_2$. The homogenate was passed through a double layer of cheese cloth. The filtrate served as the enzyme preparation, while a filtrate heated for 5 minutes at 100^{o} was used in the blank.

The following mixture was incubated in a test tube at 37^{o} for 5 minutes: 0.1 ml of 0.5 M Tris·HCl buffer (pH 9.0), 1 mg of histone (type II-A from Sigma Chemical Co., St. Louis, MO), 4.78 nanomoles of [^{14}C-methyl]-SAM (sp. act. 5.6 x 10^4 cpm/nanomole), and 0.1 ml of enzyme preparation (~ 1.5 mg protein) in a total volume of 0.5 ml. The blank contained the heated enzyme preparation.

The reaction was terminated with 0.5 ml of 30% (w/v) TCA, which was also added to the blank. Next, 9.0 ml of 10% TCA were added to the mixture, followed by centrifugation at 900 x g for 10 minutes. The supernatant was then aspirated off. The residue was washed 3 times with 10 ml of 10% TCA, by breaking up the pellet with a glass stirring rod, centrifuging and aspirating the supernatant off each time as before. After washing, the residue was resuspended in 10 ml of 10% TCA and heated at 90^{o} for 20 minutes. After cooling, the mixture was again centrifuged at 900 x g for 10 minutes,

and the nucleic acid-containing supernatant aspirated off. The residue was washed once more with 10 ml of 10% TCA, the tube centrifuged, and the supernatant suctioned off.

To remove lipids, the precipitate was washed with 10 ml of hot (65°) 95% (v/v) ethanol. The mixture was centrifuged at 900 x g for 10 minutes, and the supernatant aspirated off. Next, the precipitate was washed twice with 10 ml of a warm (35°) ethanol/ethyl ether/chloroform mixture (2:2:1, v/v). Each time, the mixture was centrifuged and the supernatant aspirated off as before. Final washing was with 10 ml of ethyl ether, followed by centrifugation and aspiration of the solvent.

After the precipitate was allowed to dry at room temperature, it was resuspended in 1.0 ml of 0.2 N NaOH and heated at 100° for 2 hours without cover in the tube block heater (p. 39). After cooling, 0.4 ml of 0.5 N HCl was added to the mixture to neutralize the NaOH. Finally, this preparation was quantitatively transferred to a liquid scintillation counting vial and processed for counting (p. 40). The specific activity of PLMT was expressed as picomoles of [^{14}C-methyl]-SAM utilized/minute/mg protein.

Determination of Tissue Levels of Total Acid-Soluble Carnitine

Tissue extracts were initially prepared from heart and hind leg skeletal muscle (107). Prior to processing,

remaining connective and fatty tissues attached to the heart were removed. Residual fluid inside the organ was allowed to drain off, while the tissue was minced and blotted on tissue paper. In the case of skeletal muscle, an attempt was made to process tissue free of visible fat and collagen. For both tissues, one gram (wet weight) was homogenized. A 1:5 (w/v) tissue homogenate was prepared in 3% (w/v), i.e., 0.3 M, $HClO_4$. Three 2-ml portions of additional acid were used to facilitate complete transfer of the homogenate from the homogenizing vessel to a centrifuge tube. The tube was centrifuged at 900 x g for 15 minutes. To 4 ml of the supernatant were added 2.4 ml of 1 N KOH. The mixture was incubated at 40° for 30 minutes for alkaline hydrolysis of short chain fatty acids. Neutralization of the mixture was then performed by addition of 2 ml of 6% (w/v), i.e., 0.6 M, $HClO_4$ and allowing the combination to chill on ice for 30 minutes. The mixture was then centrifuged again at 900 x g for 15 minutes to obtain the supernatant used for determination of total acid-soluble carnitine (ASCNE).

The carnitine present in the supernatant was assayed by an enzymatic, spectrophotometric procedure (108), as described elsewhere as the DTNB method (109). In this procedure, the carnitine is allowed to react with acetyl-CoA in the presence of carnitine acetyltransferase (EC 2.3.1.7). The CoASH formed is then allowed to react with

5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) to form as a product, 5-thio-2-nitrobenzoate. This compound absorbs light at 412 nm.

To the sample cuvet were added 0.2 ml of 1 M Tris·HCl (pH 8.0), 0.05 ml of 50 mM EDTA (neutralized), 0.025 ml of 10 mM DTNB, 0.025 ml of 15 mM acetyl-CoA, 1.0 ml of tissue extract, and 0.69 ml of water. A standard cuvet was also prepared, containing 80 nanomoles of L-carnitine in place of the tissue extract. In addition, a reference cuvet was prepared, containing all of the components except the carnitine-containing solution(s). The initial absorbance (E_I) was obtained for all three cuvets at 412 nm against water. The reaction sequence was initiated by adding 0.01 ml of carnitine acetyltransferase suspension (sp. act. 94 units/mg protein, 5 mg protein/ml) to all cuvets with gentle mixing. The final absorbance (E_F) was read after 20 minutes. The change in absorbance (ΔE) for all three cuvets was obtained by subtracting E_I from E_F. The net ΔE for the sample and standard cuvets was obtained by subtracting the ΔE obtained for the reference cuvet.

Using a molar extinction coefficient of 13,600 liter·$mol^{-1}\cdot cm^{-1}$ for 5-thio-2-nitrobenzoate under conditions of the assay, the presence of 1 nanomole of carnitine should cause an increase in absorbance of 0.0068. Therefore, the carnitine concentration can be derived by either noting the net ΔE of the sample cuvet, or by comparison with

the standard. The tissue levels of carnitine, i.e., ASCNE, were expressed as nanomoles/g wet tissue weight.

Determination of Rate of Palmitate Oxidation

The capacity of heart and hind leg skeletal muscle to carry out fatty acid oxidation *in vitro* was measured radiochemically as described elsewhere (110). The method takes into account both the $^{14}CO_2$ and ^{14}C-labeled acid-soluble products generated from [1-^{14}C]-palmitate using whole tissue homogenates.

Before running the assay, [1-^{14}C]-palmitate bound to bovine serum albumin must be prepared, as essentially described elsewhere (111). To a vial was added 5 µmoles of palmitic acid, followed by 2.5 µmoles of [1-^{14}C]-palmitic acid (sp. act. 4.1 mCi/mmole). After evaporation of the stock solvent for the radionuclide with N_2 gas, 6.24 ml of 0.9% (w/v) NaCl were added to the vial, followed by a drop of 5 N NaOH to produce a pH of 8-9. The mixture was gently warmed over a flame until the palmitic acid was dissolved completely. It was then allowed to cool at room temperature until the solution reverted back to a white, opague suspension (10-15 minutes). At this critical point, 104 mg of fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) were slowly added to the vial and allowed to stand. During this period, the albumin binds the radiolabeled palmitate. This process can be

further facilitated by gently shaking the vial after a few minutes. The final clear solution yields a complex of [1-^{14}C]-palmitate bound to bovine serum albumin in a molar ratio of 5:1, assuming a molecular weight for the protein of 69,000 (112). Adjustment of the solution to physiological pH may be needed. The apparent specific activity was 2.9 x 10^6 cpm/μmole of [1-^{14}C]-palmitate.

Next, 1:12 and 1:36 (w/v) homogenates were prepared of skeletal muscle and heart, respectively, in ice-cold buffer containing 0.25 M sucrose, 2 mM EDTA, and 10 mM Tris·HCl, pH 7.4. The homogenates were filtered through double-layered cheese cloth, and the filtrates used as the enzyme preparations. The following components, whose concentrations are based on a total incubation volume of 0.5 ml, were incubated at 37° for 30 minutes: 75 mM Tris·HCl (pH 7.4), 10 mM K_2HPO_4, 1 mM EDTA, 25 mM sucrose, 5 mM $MgCl_2$, 1 mM NAD^+, 5 mM ATP (neutralized), 25 μM cytochrome c, 0.1 mM CoA, 0.5 mM L-malate, 120 μM [1-^{14}C]-palmitate (sp. act. 2.9 x 10^6 cpm/μmole) bound to bovine serum albumin in a molar ratio of 5:1, 0.5 mM L-carnitine, and 0.05 ml of the filtered whole tissue homogenate (~ 0.6 and 0.2 mg protein for skeletal muscle and heart, respectively). The reaction was also carried out in the absence of L-carnitine. A blank was processed by adding 0.4 ml of 3 M $HClO_4$ before addition of the tissue preparation to initiate the reaction. The reaction mixture was contained in a test

tube fitted with a rubber septum and plastic center well assembly (Kontes Scientific Glassware, Vineland, NJ). Inside the well was a strip of filter paper wetted with 0.2 ml of tissue solubilizer (Soluene 100 from Packard Instrument Co., Downers Grove, IL) to trap $^{14}CO_2$.

After incubation, the reaction was stopped by immersing the bottom of the reaction tubes into a slurry of crushed ice, followed immediately by injection via a syringe of 0.4 ml of 3 M $HClO_4$ through the rubber septum down into the tubes. Next, the tubes were returned to the incubation bath for an additional 90 minutes to allow the soluene 100 to absorb $^{14}CO_2$ in the plastic well. Afterwards, the well with its contents was snipped off with scissors directly into a liquid scintillation counting vial containing the standard scintillation cocktail (p. 40). After capping and vigorous shaking of the vial, it was placed in the dark and counted the next day. Meanwhile, the incubation tubes were centrifuged at 900 x g for 10 minutes. Then, 0.2 ml of the supernatant was counted for radioactivity. The rate of palmitate oxidation was expressed as nanomoles oxidized/minute/mg protein, and was calculated from the summation of radioactivity as $^{14}CO_2$ and ^{14}C-labeled acid-soluble products produced from [1-^{14}C]-palmitate. Radiolabeled CO_2 accounted for less than 4% of the total radioactivity detected.

Assay of Cytochrome Oxidase

The activity of cytochrome oxidase was measured according to a spectrophotometric method (113). The assay was performed on the heart and hind leg skeletal muscle whole tissue homogenates that were prepared for determination of the rate of palmitate oxidation, just described above. In addition, the activity of the enzyme was measured on these same homogenates after undergoing freeze-thawing and sonication (p. 39) for 15 seconds on ice. The assay is based on the rate of oxidation of reduced cytochrome c (ferrocytochrome c), which is measured by following the decrease in the absorbancy of its α-band at 550 nm.

Initially, a 1% (w/v) solution of ferrocytochrome c was prepared as follows. One hundred mg of horse heart cytochrome c were dissolved in 10 mM potassium phosphate buffer, pH 7.0. About 10-25 mg of ascorbic acid were then added to the mixture. After dissolving, the solution was transferred to a length of dialysis tubing (size 8), and the excess ascorbic acid removed by dialyzing against 10 mM potassium phosphate buffer, pH 7.0, for about 24 hours with three changes of the buffer. Even though the stability of the prepared ferrocytochrome c solution is suppose to be for several months, a fresh batch was prepared the day before the enzyme assay.

To a cuvet were added 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.0), 1.66 ml of water, and 0.14 ml of 1% ferrocytochrome c solution. A blank was prepared by adding 0.02 ml of 0.1 M potassium ferricyanide to a cuvet containing all of the components above to oxidize the ferrocytochrome c. After temperature equilibration to 25°, 0.02 ml of the whole tissue homogenate (~0.2 and 0.1 mg protein for skeletal muscle and heart, respectively) was added to the sample cuvet and gently mixed. The decrease in absorbance was measured at 550 nm at 15-second intervals. The activity of the enzyme was derived from $V = k[S]$, where $k = 2.3 \log \frac{A(\text{time}_o)}{A(\text{time}_o + 1 \text{ min})} \text{ min}^{-1}$ and S = concentration of ferrocytochrome c in the reaction mixture. The specific activity of cytochrome oxidase was expressed as nanomoles ferrocytochrome c oxidized/minute/mg protein.

Assay of Phosphatidylethanolamine Methyltransferase

The activity of PEMT was measured using a radiochemical method (114), but without exogenous phospholipid (PC) and detergent (deoxycholate) in the reaction mixture. The assay is based upon the methylation of endogenous PE present in microsomes to form PC with [^{14}C-methyl]-SAM as the methyl donor. The hepatic microsomal suspension (p. 44) was assayed.

The following components, whose concentrations are based upon a total incubation volume of 1.4 ml, were

incubated at 37° for 15 minutes: 0.3 M Tris·HCl (pH 8.6), 0.2 mM [^{14}C-methyl]-SAM (sp. act. 2.8 x 10^5 cpm/μmole) and 1.0 ml of the microsomal suspension (~ 2 mg protein) to initiate the reaction. A blank was prepared by adding 0.14 ml of concentrated HCl before addition of microsomes. This same volume of acid was used to terminate the reaction in the sample tube.

After stopping the reaction, the radiolabeled product (PC) was extracted from the reaction mixture. To the tube were added 4.25 ml of methanol/chloroform (2.5:1, v/v). After vortexing, 1.5 ml of water and 1.5 ml of chloroform were added and again mixed. After centrifugation at 800 x g, a biphasic mixture was obtained. The upper, aqueous-methanolic phase was suctioned off, and 1.0 ml of the chloroform phase was processed for liquid scintillation counting (p. 40). The specific activity of PEMT was expressed as CPM/mg protein/15 minutes.

Determination of Microsomal Phospholipid

The level of PE and PC in the hepatic microsomes was determined using the microsomal suspension (p. 44). Initially, extraction and purification of lipid from the microsomes was performed (115). To 3.2 ml of the microsomal suspension (~6 mg protein) was mixed 12.0 ml of methanol/chloroform (2:1, v/v). After mixing in 4.0 ml each of chloroform and water, the mixture was filtered

through a glass-sintered crucible with mechanical vacuum directly into a 40-ml conical glass centrifuge tube. After stoppering, the tube was centrifuged at 800 x g for 10 minutes. The top, aqueous-methanolic layer was suctioned off, along with the thin, denatured protein layer. Three ml of the lipid-containing chloroform phase were removed into a small vial and evaporated to dryness with N_2 gas. The residue was resuspended in 0.1 ml of chloroform. Quantitative analysis of PE and PC in the concentrated lipid extract was performed by thin layer chromatography and determination of inorganic phosphorus (116).

A silica gel G TLC plate (20 x 20 cm, thickness of gel-250 µm) was first scraped so that a 1 cm clear glass border free of silica gel was produced along the sides and top of the plate. Seven 2.5 cm wide lanes were then made, parallel to the sides of the plate. The plate was activated by placing it in an oven at 120^{o} for 30-60 minutes. After cooling somewhat, 0.03 ml of the concentrated lipid extract was spotted in duplicate about 2.5 cm from the bottom of the plate. On occasion, authentic PE and PC standards were also spotted for reference, with respect to migration during ascending development. The spotting was done with a Hamilton microliter syringe (Alltech Associates, Inc., Deerfield, IL) as a series of horizontal, overlapping spots. Drying was promoted with a hair dryer set on the cold-air setting.

After application and drying of all samples, a 1 cm thick 3-sided cardboard spacer (20 x 20 x 0.5 cm) was placed on the scraped glass borders of the plate. Next, a 20 x 20 cm clear glass cover plate faced with a similarly sized piece of Whatman #1 filter paper was placed against the cardboard spacer, so that the filter paper was positioned between the cover plate and spacer. Two large paper clamps were used to hold this saturation chamber in place. They were clamped at the top of the assembly. An illustration of this setup can be seen elsewhere (116).

Once the sandwiched plate assembly was secured, it was immediately positioned, bottom end first, in a closed, glass developing tank (27 x 7 x 27 cm, inside dimensions) containing chloroform/methanol/glacial acetic acid/ water (175:105:28:14, v/v in ml). The developing tank was allowed to equilibrate for at least 4 hours before plate insertion. The polarity of the mobile phase may have to be adjusted by manipulation of the water content to give satisfactory migration of PE and PC. The mobile phase was allowed to migrate to about 0.5 cm of the top of the plate, at which time it was removed from the tank and allowed to dry.

PE and PC were identified against the reference standards using iodine vapor as a detector. The plate was placed in a closed chamber containing a few crystals of iodine. After exposure to the vapor for about 15-30 minutes, the presence of phospholipid was revealed as

yellowish-brown spots. PC displayed a lower migration than PE under the conditions of development. After removal of the plate from the iodine chamber, the colored spots representing the unknown samples were immediately encircled using a needle point. After emission of the iodine vapor from the spots, each encircled spot was scraped directly into an acid-washed test tube for phospholipid digestion and oxidation. Adjacent areas of blank silica gel corresponding in size and position to the areas containing PE and PC were also scraped into tubes. After adding 0.5 ml of concentrated sulfuric acid to all tubes, they were placed in the tube block heater (p. 39) at 250° for three hours. After about two hours of digestion, all tubes were swirled gently to resuspend the silica gel. Following this digestion period, the tubes were allowed to cool, and 2-3 drops of 30% (w/w) H_2O_2 were added to each tube. The tubes were then placed in a 160° oven for an additional hour, with a glass marble cover.

After cooling, the digested and oxidized samples were processed for the colorimetric determination of phospholipid phosphorus. Initially, the Fiske and SubbaRow reagent must be prepared as described elsewhere (117). To 200 ml of freshly prepared 15% (w/v) sodium bisulfite (anhydrous) was added 0.5 g of 1-amino-2-naphthol-4-sulfonic acid ($\geq$ 98% purity, Eastman Kodak Co., Rochester, NY) with mechanical stirring. This was followed by the

addition of 1.0 g of sodium sulfite (anhydrous). The mixture was filtered, and the filtrate stored in a dark bottle. The reagent was prepared fresh weekly.

To the silica gel-containing test tubes (samples and blanks) were added 9.1 ml of 0.26% (w/v) ammonium molybdate solution. To other acid-washed tubes containing an inorganic phosphorus standard (2.5 µg P/1.0 ml, i.e., 11.0 µg KH_2PO_4/1.0 ml) in a total volume of 3.6 ml, inclusive of 0.5 ml concentrated H_2SO_4, were added 6 ml of 0.4% (w/v) ammonium molybdate. A blank for the phosphorus standards was also prepared, containing only 3.1 ml deionized water, 0.5 ml of concentrated H_2SO_4, and the molybdate solution. All of the tubes were then mixed thoroughly by vortexing. Next, 0.4 ml of the Fiske and SubbaRow reagent was added to each tube and again mixed. The tubes were placed in a boiling water bath for 10 minutes for color development. Alternatively, they were placed in a 120° oven for 30-45 minutes with a glass marble cover.

At the end of color development, the tubes were vortexed once more and allowed to cool completely. Silica gel-containing tubes were then centrifuged for 10 minutes at 800 x g. The absorbance of the supernatant fluid, as well as the phosphorus standards, was measured spectrophotometrically at 820 nm against the blank prepared for the phosphorus standards. The values obtained for the silica gel-containing tubes were appropriately corrected due to

the small absorbance contributed by the silica gel. Therefore, the levels of hepatic microsomal PE and PC were expressed as μg phospholipid phosphorus/mg protein.

Assay of Guanidoacetate Methyltransferase

The activity of GAMT in the hepatic cytoplasmic fraction (p. 44) was assayed by determining the rate of creatine formation from guanidoacetate in the presence of SAM. The substrate concentration of the components in the reaction mixture was the same as that described elsewhere (79,82). After termination of the reaction, the creatine formed was measured with an enzymatic, spectrophotometric method (118). This coupled procedure has been employed similarly by others (119).

The following components, whose concentrations are based on a total incubation volume of 1.0 ml, were incubated at 37° for 60 minutes: 50 mM Tris·HCl (pH 7.4), 2 mM guanidoacetic acid, 2 mM dithiothreitol, 0.5 mM SAM, and 0.4 ml of the hepatic cytoplasmic fraction (~5 mg protein). The reaction was terminated with 0.5 ml of 10% (w/v) $HClO_4$. A blank was run for each individual sample of cytoplasmic fraction to correct for the pre-formed creatine present before the reaction. This was done by adding acid to the reaction mixture prior to addition of the enzyme preparation.

After termination of the reaction, 2.0 ml of a triethanolamine·HCl/K_2CO_3 buffer solution (2:1.9, w/w in

grams, dissolved in water to a volume of 25 ml and yielding a pH of 9) were added to each tube. The tubes were then placed in an ice bath for 30 minutes to precipitate $KClO_4$. After centrifuging at 900 x g for 10 minutes, the supernatant was assayed for creatine after equilibrating to room temperature.

The following components were pipetted successively into a quartz cuvet: 2.0 ml of the neutralized and buffered supernatant, 0.15 ml of a phosphoenolpyruvate (PEP)-magnesium chloride solution (14 mg PEP, tricyclohexylammonium salt, and 300 mg $MgCl_2$ in 3.0 ml water), 0.075 ml of a NADH-ATP solution (16 mg NADH and 30 mg ATP, disodium salt, in 2.0 ml of 5% (w/v) $NaHCO_3$), 0.04 ml of a lactate dehydrogenase (LDH, EC 1.1.1.27) pyruvate kinase (PK, EC 2.7.1.40) enzyme mixture (1 mg of each protein/ml of 2.1 M $(NH_4)_2SO_4$, pH 7; sp. act.: LDH-960 units/mg protein and 10.3 mg protein/ml, PK-515 units/mg protein and 18.3 mg protein/ml). After mixing with a thin glass rod and waiting 10 minutes, the initial absorbance (E_I) was recorded at 340 nm against water. Next, 0.05 ml of a freshly prepared enzyme solution of CPK (60 mg of the lyophilized protein, sp. act. 190 units/mg solid or 190 units/mg protein, per ml of a 1:10 dilution of 5% (w/v) $NaHCO_3$) was added and gently mixed to initiate the coupled sequence of reactions.

After 60 minutes, the final absorbance (E_F) was taken. The change in absorbance (ΔE) was obtained from $E_I - E_F$, and reflects the oxidation of NADH during the reaction sequence. Using a molar extinction coefficient of 6.22 x 10^3 for NADH at 340 nm and substituting $A = (a_m)(C)(L)$, a ΔE of 0.100 corresponds to 0.016 µmole of NADH/ml. Thus, with a total incubation volume of 2.315 ml, a ΔE of 0.100, as the reaction proceeds, should correspond to 0.037 µmole of NADH oxidized to NAD. As such, 0.037 µmole of creatine would have to be present to allow for the oxidation. In other words, a ΔE of 0.100 should indicate the presence of 0.037 µmole of creatine. Therefore, the amount of creatine generated during the enzyme assay can be assessed, and after conversion, the activity of GAMT determined. The specific activity of GAMT was expressed as nanomoles creatine formed/hr/mg protein.

Assay of Arginine-Glycine Transamidinase

The activity of AGTA was measured colorimetrically (120) in the kidney cytoplasmic fraction (p. 44). In the presence of arginine and hydroxylamine, the enzyme catalyzes the formation of ornithine and hydroxyguanidine. The latter product can be further reacted to form a chromogenic complex.

The following mixture was incubated for 90 minutes at 37°: 0.1 ml of 1 M potassium phosphate buffer (pH 7.4),

0.1 ml of 1 M L-arginine HCl, 0.3 ml of 2 M $NH_2OH \cdot HCl$ (neutralized), and 0.5 ml of kidney cytosol (~4 mg protein). A blank was prepared by substituting 10 mg of L-ornithine·HCl in place of the arginine. A standard contained 150 µg (0.563 µmole) of hydroxyguanidine sulfate in place of the enzyme preparation. The reaction was terminated by adding 0.4 ml of 30% (w/v) TCA and 1.0 ml water to the incubation mixture. After 10 minutes, the mixture was centrifuged at 900 x g for 10 minutes.

To a test tube was added 1.0 ml of the supernatant, followed by 0.5 ml of water, 2.0 ml of 1 M potassium phosphate buffer (pH 7.0), 0.3 ml of acetone, and 0.3 ml of a 1% (w/v) solution of trisodium pentacyanoamino ferrate. The mixture was thoroughly vortexed. After standing for 10 minutes, the absorbance was measured at 480 nm against the blank. The specific activity of AGTA was expressed as nanomoles hydroxyguanidine formed/hr/mg protein.

Determination of Tissue Levels of Creatine

With respect to liver, one gram of tissue (wet weight) was homogenized in 3 ml of 10% (w/v), i.e., 1 M, $HClO_4$. Quantitative transfer of the homogenate from the homogenizing vessel to a centrifuge tube was facilitated by using an additional 3 ml of the acid as the transfer medium. The tube was centrifuged at 900 x g for 10 minutes. Next, 5 ml of the supernatant were neutralized with 2.15 ml of 2

N KOH while in an ice bath for 15 minutes. This mixture was subsequently centrifuged again at 900 x g for 10 minutes. Finally, 1.5 ml of the supernatant was assayed for creatine in the same manner as before (pp. 66-67), after addition of 0.5 ml of the triethanolamine·HCl/K_2CO_3 buffer, pH 9, (pp. 65-66) to give a volume of 2.0 ml.

In the case of hind leg skeletal muscle, one gram of tissue was homogenized in 5 ml of 5% (w/v), i.e., 0.5 M, $HClO_4$. The homogenate was transferred to a centrifuge tube, using 6 ml more of the acid to facilitate complete transfer. After centrifugation at 900 x g for 10 minutes, 4 ml of the supernatant were neutralized in an ice bath with 1.8 ml of 1 N KOH. After 15 minutes, the mixture was again centrifuged at 900 x g for 10 minutes. Finally, 0.2 ml of the supernatant was used for the creatine assay (pp. 66-67), after adding 0.8 ml of water and 1 ml of the triethanolamine·HCl/K_2CO_3 buffer, pH 9, (pp. 65-66) to give a volume of 2.0 ml.

Results were expressed as µg creatine/g wet tissue weight.

Determination of Creatinine in 24-Hour Urine Sample

The urine collected in the centrifuge tubes for each animal in the metabolic cages (p. 43) was centrifuged at 900 x g for 15 minutes to remove any debris present. The supernatant was poured into a graduated cylinder through

filter paper. The volume was recorded for the 24-hour collection period.

Creatinine in the urine was measured colorimetrically (121). To a test tube were added 0.05 ml of the filtered urine, 3.95 ml of water, 1.0 ml of a picric acid solution (0.04 mole/L), and 1.0 ml of a NaOH solution (0.75 mole/L). A standard was also prepared by substituting 0.02 mg of creatinine in 0.05 ml of water in place of urine. Water was used instead of urine in the blank. After mixing the contents of the tubes and allowing to stand for 15 minutes, the absorbance was measured at 500 nm against the blank. The creatinine present in the sample was derived by comparison with the standard, and then quantitated for the 24-hour period. Results were expressed as mg creatinine excreted/24 hours or 100 g bwt.

Protein Determination

The amount of protein present in the tissue homogenates and subcellular fractions was quantitated using a spectrophotometric procedure (122). Initially, the Biuret reagent was prepared as follows: 1.5 g of $CuSO_4 \cdot 5 H_2O$ and 6.0 g of $NaKC_4H_4O_6 \cdot 4 H_2O$ were dissolved in about 500 ml of water. To this mixture was added with constant stirring 300 ml of freshly prepared, carbonate-free 10% (w/v) NaOH (30 g NaOH dissolved in 300 ml of pre-boiled water). This mixture in turn was diluted to 1 liter. The

"protein reagent" was prepared by mixing the Biuret reagent with 2.3% (w/v) Na_2CO_3 (1:7, v/v).

The tissue homogenates and subcellular fractions were appropriately diluted to yield 50-600 µg protein/ml. To 0.8 ml of the sample were added 3.2 ml of the "protein reagent". The mixture was vortexed and allowed to stand 10 minutes. Next, while the mixture was being vortexed again, 0.1 ml of undiluted 2N Folin-Ciocalteau phenol reagent was added. The mixture was allowed to stand for 30 minutes, and the absorbance was measured at 750 nm against a blank. A standard was also prepared using bovine serum albumin (160 µg/0.8 ml). The amount of protein in the samples was derived by comparison with this standard.

Statistical Analysis

Statistical differences were evaluated using analysis of variance (ANOVA) and Duncan's new multiple range test (123).

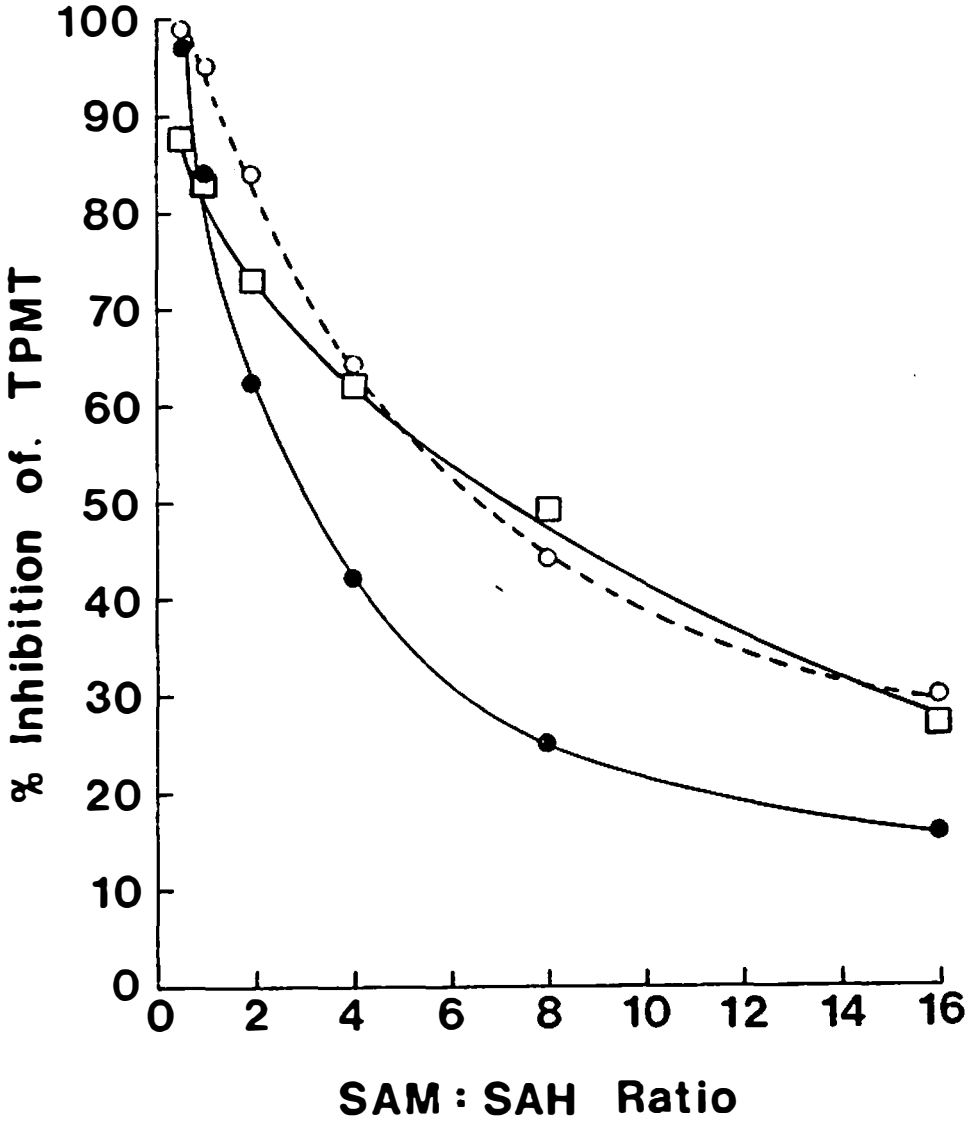
CHAPTER IV

RESULTS

Effect of Modulating the S-Adenosylmethionine to S-Adenosylhomocysteine Ratio In Vitro on the Activities of the Sulfhydryl Xenobiotic Transmethylases and Protein (Lysine) Methyltransferase in Rat Liver

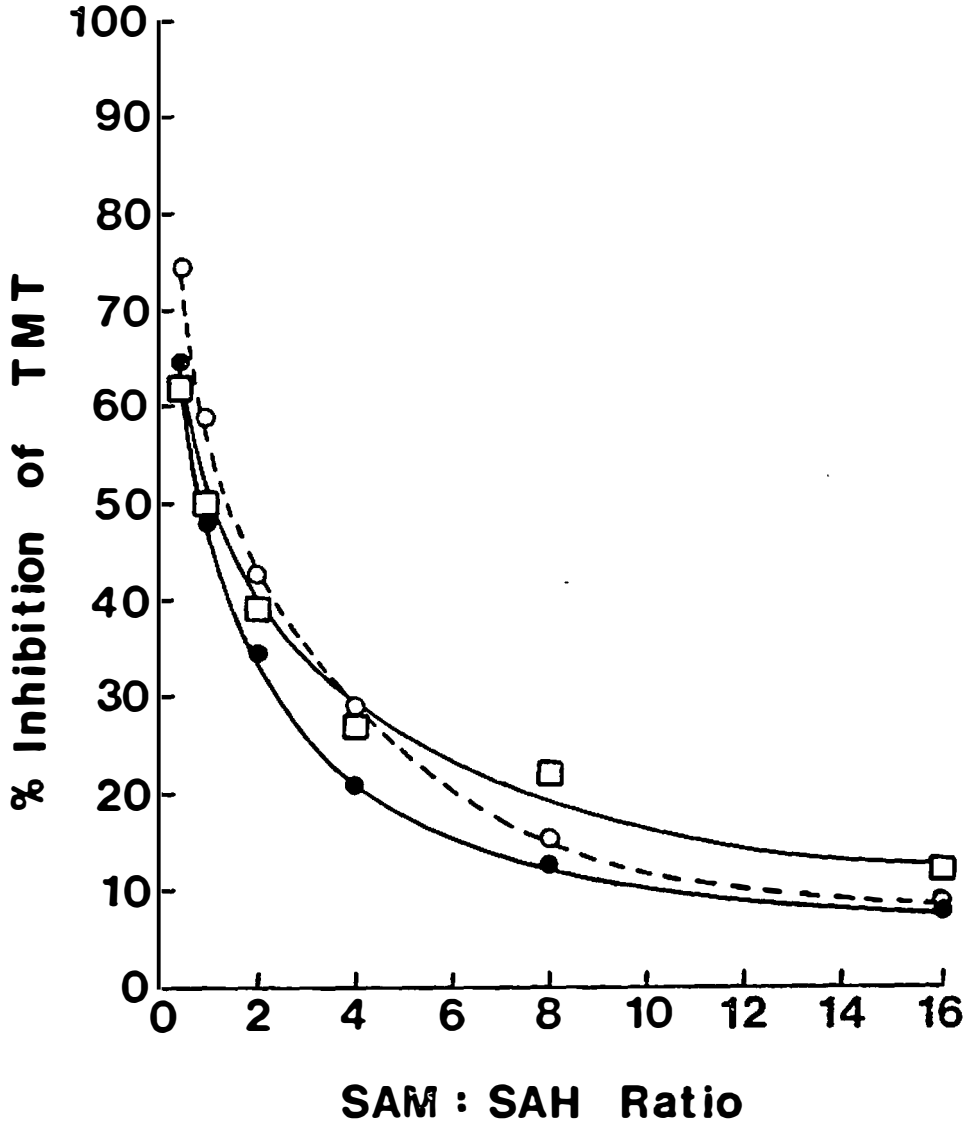
Using cell-free enzyme preparations from rat liver, both TPMT and TMT activities were influenced by modulation of the SAM:SAH ratio. As shown by Figure 10, decreasing the ratio over a broad range (16:1 - 0.5:1) caused a corresponding inhibition of TPMT activity. This inhibitory effect occurred at 40 and 120 μM SAM, as long as the concentration of SAH in the reaction mixture was appropriately adjusted to the desired absolute molar ratio. However, at 4 μM SAM, the effect was not as pronounced. With respect to TMT, the modulating effect of the SAM:SAH ratio was observed more clearly (Figure 11). Even though only one concentration of SAM was employed, PLMT activity also tended to be modified in the same manner (Table 3).

Effect of D,L-Homocysteine Thiolactone Administration on Rat Liver Tissue Levels of S-Adenosylmethionine and S-Adenosylhomocysteine and the Activities of the Hepatic Sulfhydryl Xenobiotic Transmethylases and Protein(Lysine)Methyltransferase



The livers from three animals were pooled to obtain the enzyme preparation used in these experiments. The assay procedure was performed with three different concentrations of SAM: 4 µm (●—●—●), 40 µm (○- -○- -○), and 120 µm (□—□—□) The concentration of SAH was appropriately adjusted in each case to obtain the desired SAM:SAH ratio in the reaction mixtures. Each point represents the average of three duplicate determinations. Each duplicate determination was conducted separately using a freshly-prepared, pooled tissue extract each time.

Figure 10. Modulation of rat liver thiopurine methyltransferase *in vitro* by the S-adenosylmethionine to S-adenosylhomocysteine ratio



The livers from three animals were pooled to obtain the enzyme preparation used in these experiments. The assay procedure was performed with three different concentrations of SAM: 10 µm (●——●——●), 100 µm (○- -○- -○), and 1000 µm (□——□——□) The concentration of SAH was appropriately adjusted in each case to obtain the desired SAM:SAH ratio in the reaction mixtures. Each point represents the average of three duplicate determinations. Each duplicate determination was conducted separately using a freshly-prepared, pooled tissue extract each time.

Figure 11. Modulation of rat liver thiol methyltransferase *in vitro* by the S-adenosylmethionine to S-adenosylhomocysteine ratio

TABLE 3

Effect of S-Adenosylhomocysteine on Rat Liver Protein(Lysine)Methyltransferase Activity *In Vitro*

SAM	SAH	SAM:SAH	Protein(Lysine) Methyltransferase	% Enzyme Inhibition by SAH
M	M		pmol [^{14}C-methyl]-SAM utilized min^{-1} mg protein $^{-1}$	
9.6	0.0	--	1.07	--
9.6	0.6	16.0	0.98	8
9.6	1.2	8.0	0.93	13
9.6	2.4	4.0	0.81	24
9.6	4.8	2.0	0.68	36
9.6	9.6	1.0	0.52	51
9.6	19.2	0.5	0.34	68

The standard enzyme assay was carried out using a 1:9 (w/v) tissue homogenate in 0.25 M sucrose containing 3 mM $CaCl_2$. SAH was added to the incubation mixture as indicated. Results are the average of 2 duplicate determinations.

As shown by the data in Table 4, administration of D,L-homocysteine thiolactone to rats produced a dose-related elevation in the hepatic tissue levels of both SAM and SAH *in vivo*. However, there was a substantially greater, relative elevation of SAH following drug treatment (500 or 1000 mg/kg bwt), as reflected by the decline in the SAM:SAH ratio. This in turn was associated with inhibition of only one of the hepatic sulfhydryl xenobiotic transmethylases, namely TPMT. Specifically, when the SAM:SAH ratio was lowered from 3.19 in the control group to 0.91 (500 mg/kg bwt) and 0.67 (1000 mg/kg bwt), the activity of TPMT was reduced by about 35 and 63%, respectively. Under the same conditions, however, there was no significant effect on the activity of TMT.

In other experiments, the activity of PLMT in liver was also shown to be subject to modulation, as the hepatic SAM:SAH ratio was lowered by injection of D,L-homocysteine thiolactone to animals (Table 5). At the highest dose utilized (1000 mg/kg bwt), the level of SAH increased 40-fold with about an 8-fold decrease in the SAM:SAH ratio. PLMT activity in liver of the test animals was inhibited by more than 80% compared to saline-treated control animals.

TABLE 4

Effect of D,L-Homocysteine Thiolactone Administration on Rat Liver Tissue Levels of S-Adenosylmethionine and S-Adenosylhomocysteine and the Activities of Thiopurine Methyltransferase and Thiol Methyltransferase

Treatment	SAM	SAH	SAM:SAH	TPMT	TMT
	nmol/g			cpm x 10^{-3}/30 min/mg Pro	
Saline (2 ml/kg bwt)	98 ± 4[a]	32 ± 2[a]	3.19 ± 0.33[a]	2.5 ± 0.1[a]	17.5 ± 2.1[a]
D,L-homocysteine thiolactone (500 mg/2 ml saline/kg bwt)	223 ± 45[b]	253 ± 31[b]	0.91 ± 0.20[b]	1.6 ± 0.2[b]	17.8 ± 3.4[a]
D,L-homocysteine thiolactone (1000 mg/2 ml saline/kg bwt)	360 ± 35[c]	560 ± 62[c]	0.67 ± 0.09[b]	0.9 ± 0.3[c]	15.7 ± 2.9[a]

Animals were injected intraperitoneally and sacrificed 40 minutes later. Values represent the average ± SEM for 5 rats. Data sharing a common superscript in a column are not significantly different ($p>0.05$).

TABLE 5

Effect of D,L-Homocysteine Thiolactone Administration on Rat Liver Tissue Levels of S-Adenosylmethionine and S-Adenosylhomocysteine and Protein(Lysine)Methyltransferase Activity

Treatment	SAM	SAH	SAM:SAH	Protein(Lysine) Methyltransferase
	nmol/g			pmol [^{14}C-methyl]-SAM utilized min^{-1} mg protein^{-1}
Saline (2 ml/kg bwt)	101 ± 4^{a}	24 ± 2^{a}	4.35 ± 0.49^{a}	1.12 ± 0.08^{a}
D,L-homocysteine thiolactone (500 mg/2 ml saline/kg bwt)	450 ± 72^{b}	377 ± 100^{b}	1.33 ± 0.18^{b}	0.42 ± 0.06^{b}
D,L-homocysteine thiolactone (1000 mg/2 ml saline/kg bwt)	525 ± 51^{b}	970 ± 126^{c}	0.56 ± 0.06^{b}	0.20 ± 0.02^{c}

Animals (225-300 g bwt) were injected intraperitoneally and sacrificed 30 minutes later. Values represent the average ± SEM for 5 rats. Data sharing a common superscript in a column are not significantly different ($p>0.05$).

Feed Consumption, Growth, and Symptomatology of Pyridoxine-Deficient Rats

Rats fed the pyridoxine-deficient diet for the 6-7 week period displayed symptoms of vitamin deficiency. In addition to an unkempt appearance, dermatosis of the paws and nasal area was evident. In a few isolated cases, i.e., 2 out of 15 rats, hematuria was observed. As shown in Table 6, these animals consumed only 45% of the total feed intake of animals in the control group receiving the same diet supplemented with pyridoxine. Moreover, pyridoxine-deficient and pair-fed rats had a total weight gain of only 25 and 35%, respectively, of that experienced by control animals. The significantly lower feed efficiency ratio ascertained for the pyridoxine-deficient diet confirms the inability of this diet to support animal growth to the same extent, as in the presence of added pyridoxine.

Effect of Pyridoxine Deficiency on the Hepatic Tissue Levels of S-Adenosylmethionine and S-Adenosylhomocysteine

As depicted in Figure 12, the tissue level of SAM in liver did not differ among the three groups of animals. However, pyridoxine-deficient rats had a concentration of SAH approximately 5-fold higher than that of either the pair-fed or control rats. In other words, the absolute molar ratio of SAM to SAH in liver dropped from about 4.4 in both the control and pair-fed rats to 0.9 in the

TABLE 6

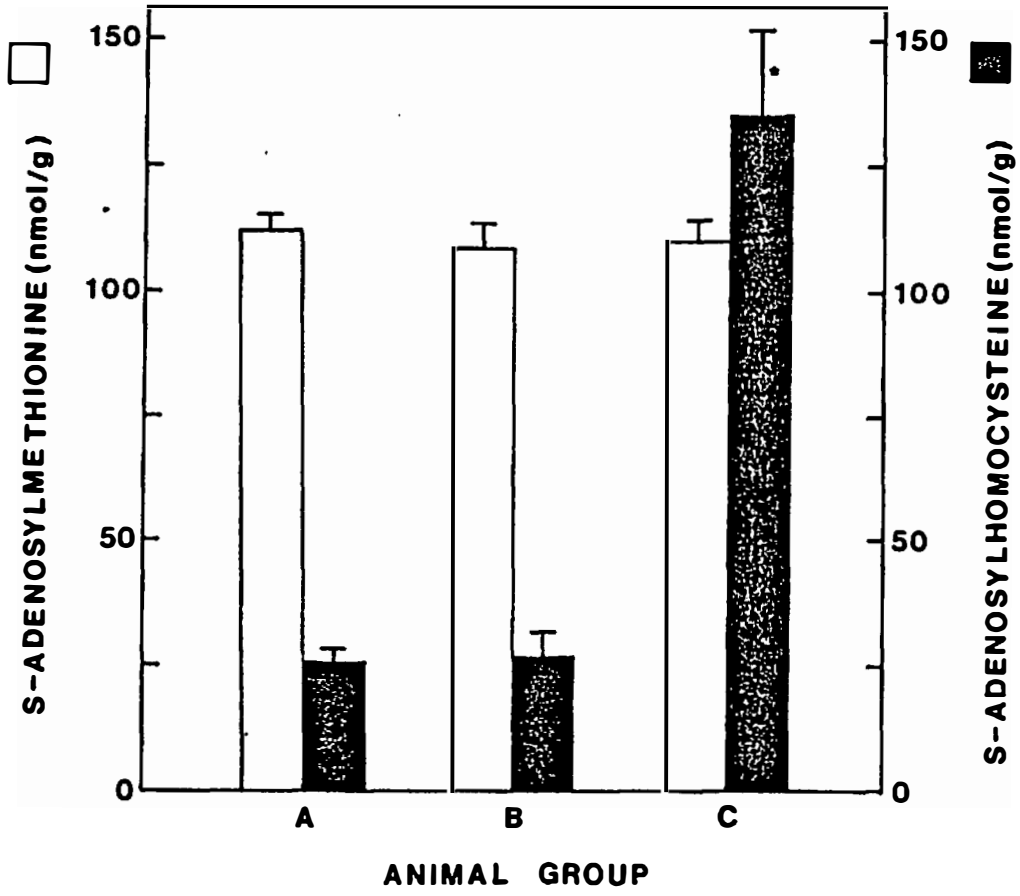
Total Feed Intake and Weight Gain of Control, Pair-Fed, and Pyridoxine-Deficient Rats Fed Dietary Regimes for 6-7 Weeks

Animal Group	Feed Intake	Weight Gain	Feed Efficiency Ratio of Diet[1]
	g	g	
Control	867.3 ± 12.7[2a3]	342 ± 8[a]	0.394 ± 0.005[a]
Pair-Fed	383.4 ± 11.8[b]	121 ± 8[b]	0.314 ± 0.011[b]
Pyridoxine-Deficient	392.1 ± 13.1[b]	87 ± 3[c]	0.222 ± 0.007[c]

[1] $\frac{\text{Total Weight Gain}}{\text{Total Food Intake}}$

[2] Values are the average ± SEM for 5 animals.

[3] Values in a column not followed by the same superscript letter are significantly different, $p<0.05$.



Values represent the average ± SEM for 5 rats in either the control (A), pair-fed (B), or pyridoxine-deficient (C) animal groups.

*Significantly different from control and pair-fed groups ($p<0.01$).

Figure 12. Rat liver tissue levels of S-adenosylmethionine and S-adenosylhomocysteine during pyridoxine deficiency

pyridoxine-deficient ones. This finding warranted the investigation of the effect of pyridoxine deficiency on transmethylation.

Effect of Pyridoxine Deficiency on the Activities of the Hepatic Sulfhydryl Xenobiotic Transmethylases

Despite a reduction of the hepatic SAM:SAH ratio in the pyridoxine-deficient rat, no significant effect on the activities of either TPMT or TMT in liver was observed (Table 7).

Effect of Pyridoxine Deficiency on Hepatic Protein(Lysine) Methyltransferase Activity and Carnitine Levels and Fatty Acid Oxidative Capacities of Skeletal Muscle and Heart

As shown in Table 8, the activity of PLMT in liver was reduced by about 26% in the pyridoxine-deficient rat. Therefore, tissue levels of total acid-soluble carnitine (ASCNE) in hind leg skeletal muscle and heart were examined (Figure 13). ASCNE concentrations in these two tissues were roughly 29 and 25% less, respectively, than the values obtained either for pair-fed or control rats. These data prompted determination of the capacity of the tissues to carry out fatty acid oxidation *in vitro*.

When the rate of total palmitate oxidation was evaluated in both skeletal muscle and heart tissues using whole homogenates (Table 9), pair-fed rats unexpectedly

TABLE 7

Activities of Rat Liver Sulfhydryl Xenobiotic Transmethylases During Pyridoxine Deficiency

Animal Group	TPMT	TMT
	cpm x 10^{-3}/30 min/mg Pro	
Control	3.5 ± 0.3[a]	18.8 ± 4.4[a]
Pair-Fed	3.4 ± 0.4[a]	14.8 ± 2.0[a]
Pyridoxine-Deficient	3.3 ± 0.4[a]	17.1 ± 3.5[a]

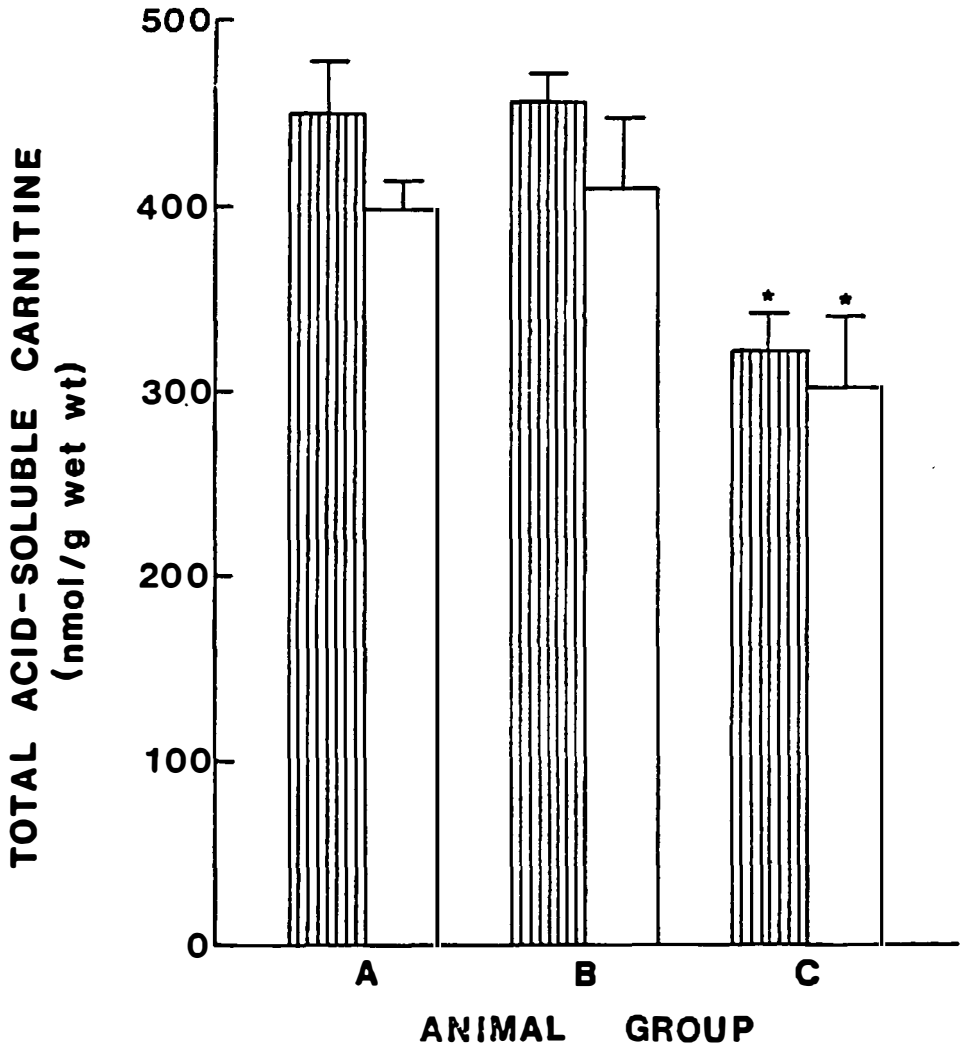
Values represent the average ± SEM for 5 rats. Data sharing a common superscript letter in a column are not significantly different ($p>0.05$).

TABLE 8

Effect of Pyridoxine Deficiency on the Activity of Protein(Lysine)Methyltransferase in Rat Liver

Animal Group	Protein(Lysine) Methyltransferase
	pmol [^{14}C-methyl]-SAM utilized min^{-1} mg $protein^{-1}$
Control	1.01 ± 0.02^{a}
Pair-Fed	0.98 ± 0.03^{a}
Pyridoxine-Deficient	0.73 ± 0.05^{b}

Values represent the average ± SEM for 5 rats. Data sharing a common superscript letter in a column are not significantly different ($p>0.05$).



Values represent the average ± SEM for 4 animals

*Significantly different from control and pair-fed groups ($p<0.05$).

Figure 13. Total acid-soluble carnitine in skeletal muscle ▨ and heart □ of control (A), pair-fed (B), and pyridoxine-deficient (C) rats

TABLE 9

Palmitate Oxidation in Skeletal Muscle and Heart Tissue of Control, Pair-Fed, and Pyridoxine-Deficient Rats

Animal Group	Palmitate Oxidation (pmol/min/mg protein)					
	Skeletal Muscle Tissue			Heart Tissue		
	without carnitine	with carnitine	% stimulation	without carnitine	with carnitine	% stimulation
Control	201 ± 28[a]	259 ± 46[a]	29	962 ± 128[a]	1082 ± 179[a]	12
Pair-Fed	111 ± 5[b]	148 ± 10[b]	33	720 ± 55[a]	841 ± 96[a]	17
Pyridoxine-Deficient	149 ± 23[ab]	198 ± 28[ab]	33	798 ± 38[a]	918 ± 52[a]	15

Total palmitate oxidation was determined using whole tissue homogenates and is represented by the summation of radioactivity as $^{14}CO_2$ and ^{14}C-labeled acid-soluble products formed in the absence and presence of exogenous L-carnitine (0.5 mM) using [1-^{14}C]palmitate as the substrate. Values are the average + SEM for 5 rats. Data sharing a common superscript in a column are not significantly different ($p>0.05$).

tended to have a slightly lower activity than pyridoxine-deficient rats in each case. However, no significant statistical differences were arrived at. Nevertheless, pair-fed rats, that were restricted to less than half the feed intake of control animals, had only 55% of the capacity to oxidize palmitate in skeletal muscle, in comparison to that of control rat tissue. A somewhat similar, but insignificant, decrease in the rate of palmitate oxidation was seen with respect to heart tissue from the feed-restricted (pair-fed) rat, relative to the control.

Since the mitochondria content and/or activity in tissue can conceivably influence fatty acid oxidation, a marker enzyme was measured. As can be seen in Table 10, cytochrome oxidase activity in freeze-thawed, sonicated homogenates from skeletal muscle and heart of pair-fed rats was more than 2-fold higher than that from control rats. Thus, the data suggest that the change in rate of mitochondrial palmitate oxidation in the feed-restricted rat cannot be explained by a reduction in the number and/or activity of mitochondria.

Effect of Pyridoxine Deficiency on Hepatic Microsomal Phosphatidylethanolamine Methyltransferase Activity and Phospholipid Levels

When hepatic microsomal PEMT was assayed *in vitro*, an unexpected increase in activity was observed using enzyme

TABLE 10

Cytochrome Oxidase Activity in Skeletal Muscle and Heart Tissue of Control, Pair-Fed, and Pyridoxine-Deficient Rats

Animal Group	Cytochrome Oxidase (nmol/min/mg protein)	
	Skeletal Muscle	Heart
Control	39 ± 7^{a}	104 ± 16^{a}
Pair-Fed	94 ± 7^{b}	280 ± 13^{b}
Pyridoxine-Deficient	53 ± 2^{ac}	219 ± 14^{c}

Enzyme activity was determined in freeze-thawed, sonicated whole tissue homogenates. Values represent the average $\pm$ SEM for 5 rats. Data not sharing a common superscript letter in a column are significantly different ($p<0.05$).

preparations from either pair-fed or pyridoxine-deficient rats (Table 11). Somewhat contradictory as shown in Table 12, the level of PC in microsomes obtained for these two particular groups was significantly lower than that determined for the control group of rodents. However, the level of PC was significantly lower in microsomes from pyridoxine-deficient rats than that from pair-fed rats. In addition, the level of PE in microsomes from pair-fed and pyridoxine-deficient animals was significantly higher than that from the control animals, further suggesting decreased methylation of substrate to product *in vivo*.

Effect of Pyridoxine Deficiency on Hepatic Guanidoacetate Methyltransferase Activity and Creatine Metabolism

As shown in Table 13, the activity of GAMT in liver did not differ significantly among the three groups of animals. Kidney AGTA activity was significantly lower in the pyridoxine-deficient rats in relation to pair-fed rats, as well as pair-fed rats relative to control rats. Nevertheless, as can be seen in Table 14, there were significant increases in the concentration of creatine in liver and skeletal muscle of pyridoxine-deficient rats, in comparison to their pair-fed counterparts. Moreover, pair-fed rats had a higher creatine level in the two tissues examined than control rats. Thus, elevation of tissue creatine did not conform to the measured activity of

TABLE 11

Rat Liver Microsomal Phosphatidylethanolamine Methyltransferase Activity in Control, Pair-Fed, and Pyridoxine-Deficient Rats

Animal Group	Phosphatidylethanolamine Methyltransferase
	cpm x 10^{-3}/15 min/mg Pro
Control	0.40 ± 0.04^{a}
Pair-Fed	0.55 ± 0.02^{b}
Pyridoxine-Deficient	0.58 ± 0.01^{b}

Values represent the average ± SEM for 5 rats. Data sharing a common superscript letter are not significantly different ($p>0.05$).

TABLE 12

Rat Liver Microsomal Phospholipid in Control, Pair-Fed, and Pyridoxine-Deficient Rats

Animal Group	Phosphatidyl-ethanolamine	Phosphatidyl-choline
	μg phospholipid phosphorus per mg protein	
Control	2.11 ± 0.11[a]	8.01 ± 0.23[a]
Pair-Fed	3.28 ± 0.07[b]	7.19 ± 0.12[b]
Pyridoxine-Deficient	2.90 ± 0.22[b]	6.25 ± 0.19[c]

Values represent the average ± SEM for 5 rats. Data sharing a common superscript letter in a column are not significantly different ($p>0.05$).

TABLE 13

Effect of Pyridoxine Deficiency on the Enzymes of Creatine Biosynthesis

Animal Group	Kidney Arginine-Glycine Transamidinase	Liver Guanidoacetate Methyltransferase
	nmol hydroxyguanidine formed hr^{-1} $mg\ protein^{-1}$	nmol creatine formed hr^{-1} $mg\ protein^{-1}$
Control	113 ± 6^{a}	8.24 ± 0.80^{a}
Pair-Fed	64 ± 7^{b}	5.45 ± 0.98^{a}
Pyridoxine-Deficient	39 ± 2^{c}	6.08 ± 0.83^{a}

Values represent the average ± SEM for 5 rats. Data sharing a common superscript letter in a column are not significantly different ($p>0.05$).

TABLE 14

Creatine Concentrations in Liver and Skeletal Muscle of Control, Pair-Fed, and Pyridoxine-Deficient Rats

Animal Group	Creatine	
	Liver	Skeletal Muscle
	µg/g wet weight	
Control	36 ± 2[a]	3,360 ± 80[a]
Pair-Fed	57 ± 3[b]	3,810 ± 110[b]
Pyridoxine-Deficient	67 ± 3[c]	4,110 ± 120[c]

Values represent the average ± SEM for 5 rats. Data not sharing a common superscript letter in a column are significantly different ($p<0.05$).

the enzymes involved in creatine biosynthesis. Lastly, creatinine excretion in urine per unit of bodyweight did not differ significantly among the three rat groups (Table 15).

TABLE 15

Urinary Excretion of Creatinine by Control, Pair-Fed, and Pyridoxine-Deficient Rats

Animal Group	Creatinine	
	mg/24 hrs	mg/100 g bwt
Control	6.61 ± 0.72[a]	2.64 ± 0.20[a]
Pair-Fed	4.22 ± 0.33[b]	2.95 ± 0.12[a]
Pyridoxine-Deficient	3.17 ± 0.35[b]	2.48 ± 0.09[a]

Values represent the average ± SEM for 4 rats. Data not sharing a common superscript letter in a column are significantly different ($p<0.05$).

CHAPTER V

DISCUSSION

In view of their similarities in chemical structure, it is not suprising that SAH is a competitive inhibitor of SAM. Kinetic data obtained for a number of these enzymes, including TPMT (3), TMT (7,8), PLMT (84), PEMT (63), and GAMT (79), reveal that SAH has an inhibition constant (K_i) significantly lower than the Michaelis constant (K_m) possessed by SAM, although this situation does not apply to all methyltransferases. In other words, a much greater concentration of SAM would be required to obtain maximal reaction rate in the presence of SAH. Therefore, it is conceivable that modulation of the SAM:SAH ratio may regulate these enzymes, and in turn the biochemical processes facilitated by them.

Using cell-free enzyme preparations, the SAM:SAH ratio appears to be more important than the level of SAH *per se* in regulating the activity of the methyltransferases. This view is supported by the fact that TPMT, TMT, and PLMT were each found to be subject to control by the SAM:SAH ratio *in vitro*, which is in accord with other research focusing on PEMT (62,63). Other studies have shown that altering the SAM:SAH ratio in the intact cell can regulate transmethylation as well (91,92,93,94,96,97). Thus, it is conceivable that factors which can change the SAM:SAH ratio *in vivo*

may similarly control the activities of the sulfhydryl xenobiotic transmethylases and PLMT as measured *in vitro*.

Upon administration of D,L-homocysteine thiolactone, this compound presumably permeates the rat hepatocyte membrane for the most part as L-homocysteine. In the presence of endogenous adenosine, the synthesis of SAH proceeds due to the catalytic activity of SAH hydrolase, a cytosolic enzyme (83). Thus, an elevation of SAH and secondarily of SAM, is achieved *in vivo*. The latter may be due to inhibition of transmethylation reactions, therefore decreasing the consumption of SAM and allowing its accumulation. Furthermore, the resultant fall in the SAM:SAH ratio most likely accounts for the inhibition of TPMT, also situated in the cytosol, after D,L-homocysteine thiolactone treatment. On the other hand, since D,L-homocysteine thiolactone would provide a mixture of the "D" and "L" isomers of homocysteine in aqueous solution for injection, one may argue that D-homocysteine could promote an elevation of SAH and/or inhibit TPMT activity. However, the fact that SAH is synthesized from L-homocysteine and adenosine in the presence of SAH hydrolase makes the former possibility somewhat remote. It is unknown whether or not D-homocysteine is a direct inhibitor of the methyltransferases.

TMT localized within the endoplasmic reticulum was not significantly affected under these same experimental

conditions. This observation may perhaps be explained by the rather impermeable nature of SAH to fully intact membranes (90), such as the endoplasmic reticulum, once this purine nucleoside has been rapidly synthesized in the cytosol after D,L-homocysteine thiolactone treatment. In other words, the generated SAH may not have been available to interact with the SAH-binding site on TMT due to compartmentalization. This would especially be true if TMT is deeply embedded within the membrane bilayer. In addition, it is known that there may be competition for SAH among the methyltransferases (124), which could also limit the availability of SAH for binding on TMT.

It has been demonstrated that raising the hepatic tissue level of SAH *in vivo* can inhibit the activity of microsomal PEMT (125), an enzyme sharing the same subcellular localization as TMT, and can also decrease phospholipid methylation *in vivo* (92,97). The endoplasmic reticulum contains SAH-binding sites which may be identical to the SAH-binding sites of the SAM-dependent methyltransferases (126), such as PEMT. Work with microsomal vesicles (127) has indicated that PEMT is situated to the external (cytosolic) side of the endoplasmic reticulum membrane, in essence making this particular enzyme more accessible to SAH in the cytosol. Hence, although the topography of TMT within the microsomes has not been delineated, it is reasonable to speculate that

TMT may be positioned in the interior of the endoplasmic reticulum, i.e., to the luminal side of the microsome, essentially separated from the SAH in the cytosol. Thus, even though SAH can inhibit TMT using microsomal preparations, it is possible that isolation of the microsomal fraction may have induced morphological changes to the endoplasmic reticulum (128), resulting in the formation of "leaky" or semi-sealed microsomes. This in turn could allow the binding of SAH to TMT and therefore inhibit the enzyme *in vitro*.

On the other hand, it is possible that the elevation of the hepatic SAH concentration *in vivo* after D,L-homocysteine thiolactone treatment was diluted out *in vitro* during cell fractionation and enzyme preparation. Such processing of the tissue could conceivably mask any inhibitory effect of SAH on transmethylation reactions as measured *in vitro* with $^{14}CH_3$-SAM.

It has been implied from other research (7) that the two sulfhydryl xenobiotic transmethylase activities examined in the present work represent the same enzymic entity associated with the microsomal fraction. However, the data seem to support the argument that TMT and TPMT have different subcellular localizations (3), since the SAH generated after D,L-homocysteine thiolactone treatment inhibited only the cytosolic, but not the microsomal enzyme.

On the other hand, since TMT was not influenced by D,L-homocysteine thiolactone treatment, one might have expected PLMT in the nucleus to be also unaffected by the generation of pharmacological levels of SAH in liver. This expectation would be due to the presence of the nuclear membrane. However, the membrane of the nucleus is somewhat distinct from that of the endoplasmic reticulum. It is actually a double membrane. The individual membranes are apparently fused at various points around the circumference of the nucleus, thus creating membrane pores. These openings evidently allow the exit of RNA molecules, made in the nucleus, into the cytosol. Hence, it may well be that the SAH generated after D,L-homocysteine thiolactone treatment was able to enter the nucleus via these same membrane pores to bind to PLMT. This would explain the inhibition of PLMT activity by D,L-homocysteine thiolactone administration to rats.

Previous research has shown that a pyridoxine deficiency induced by a vitamin-deficient diet can interfere with the metabolism of methionine (103). An accumulation of SAH and an overall decrease in the SAM:SAH ratio in liver were found to be the most noticeable effects. Presumably, degradation of SAH is somewhat thwarted during pyridoxine deficiency due to a reduction in the activity of cystathionine synthase and/or cystathionase, both pyridoxal phosphate-requiring enzymes

in the transsulfuration pathway. As such, some transmethylation reactions would be expected to be impeded during pyridoxine deficiency.

The interference of methionine metabolism by pyridoxine deficiency has been confirmed in the present study. A 5-fold increase in the hepatic tissue level of SAH, without a concurrent change in SAM, was obtained in pyridoxine-deficient rats. Although marked reduction of the hepatic SAM:SAH ratio was observed in weanling rats fed the pyridoxine-deficient diet for a period of 6-7 weeks in the present study, a similar effect has been achieved only after 3 1/2 weeks on the diet (103). Thus, any regulating role(s) of pyridoxine deficiency on transmethylation may possibly be expressed during the earlier stages of vitamin depletion. As pointed out previously (p. 99), however, elevation of SAH *in vivo* may not be expressed *in vitro* with respect to modifying the activity of the methyltransferases.

Pyridoxine deficiency did not seem to regulate the sulfhydryl xenobiotic transmethylases. It is difficult to address the full significance of this finding, since the endogenous roles of TMT and TPMT are presently unknown. However, one may conclude that sulfhydryl xenobiotic metabolism via transmethylation may not be affected by either pyridoxine deficiency or feed restriction.

On the other hand, it appears that hepatic PLMT is subject to control under the same vitamin-deficient state.

Reduction of the SAM:SAH ratio during pyridoxine deficiency causes a decrease in the activity of the enzyme. This finding suggests that modification of protein structure by methylation of specific amino acid residues, in the presence of PLMT, may be curtailed during pyridoxine deficiency. For example, methylation of the ε-amino group of lysine residues present in histone by PLMT may be important in supplying eventually the initial substrate for the biosynthesis of carnitine (44,46). Hence, vitamin deficiency may influence the formation of carnitine.

The effect of vitamin deficiency on tissue carnitine levels has been investigated previously, but with ascorbic acid being evaluated on the premise that it is needed for the hydroxylating enzymes in the formation of carnitine. Scorbutic guinea-pigs have been reported to have a reduction in carnitine levels in muscle (129) and liver (130). Moreover, underfeeding these animals with an ascorbic acid-deficient diet, while giving them adequate ascorbic acid subcutaneously, was shown to decrease the muscle (131) and hepatic (130,131) carnitine concentrations. However, larger doses of ascorbic acid administered to these same underfed animals prevented depression of tissue carnitine (130). Thus, feed restriction per se does not appear to influence the tissue carnitine levels, which has been corroborated by the current study.

The notable decrease in the concentration of carnitine, i.e., ASCNE, in skeletal muscle and heart tissues of the pyridoxine-deficient rat may perhaps be explained by impairment in the biosynthesis of its precursors. There are two reactions in the biosynthetic pathway for carnitine that can be implicated. The most obvious is the reaction catalyzed by 3-hydroxy-6-N-trimethyllysine aldolase, which cleaves its substrate to butyrobetaine aldehyde and glycine (46). Evidence indicates that this enzyme requires pyridoxal phosphate (132,133), and hence could conceivably be regulated in a negative manner during pyridoxine deficiency. The other reaction is catalyzed by PLMT, whose activity in rat liver decreases during pyridoxine deficiency. The activity of PLMT in liver is about 21 and 2 1/2 times higher than that of skeletal muscle and heart, respectively, in the normal rat (44). Even though the reaction catalyzed by PLMT occurs before the reaction catalyzed by the aldolase in the pathway, it is not known whether the former reaction may assume more importance in influencing tissue carnitine levels during pyridoxine deficiency.

Even though a significant reduction in the level of carnitine in both skeletal muscle and heart was found in pyridoxine-deficient rats, the rate of total palmitate oxidation in these tissues was not any lower than that of pair-fed rats, whose tissue carnitine levels were similar

to that of control rats. As a matter of fact, the reaction rate tended to be slightly lower in the two muscle tissues of the pair-fed rats than that of their pyridoxine-deficient counterparts, although statistical differences were not obtained. In any event, it seems that carnitine is not limiting in facilitating fatty acid oxidation during pyridoxine deficiency.

Previously, it was reported that feed restriction tended to increase palmitate oxidation in heart (134). Rats were underfed for a 10-week period, and the reaction rate was based on the production of $^{14}CO_2$ from [1-^{14}C]-palmitate. In the present study, rats were restricted in food intake for 6-7 weeks, and total palmitate oxidation was based on the production of $^{14}CO_2$ and also ^{14}C-labeled acid-soluble products from [1-^{14}C]palmitate. This particular assay procedure is believed to be a more sensitive and accurate method for assessing palmitate oxidation than from $^{14}CO_2$ production alone (135,136). In view of these conditions, a tendency for feed restriction to reduce the rate of palmitate oxidation in heart was observed, although this propensity was not of significance. On the other hand, feed restriction caused a marked decrease in the rate of palmitate oxidation in skeletal muscle. Simple starvation for 66 hours has also been found to decrease palmitate oxidation in skeletal muscle, using whole tissue homogenates and the assay procedure used in the present study (136).

Since skeletal muscle and heart carnitine levels were similar in both pair-fed and control rats, other factors, besides carnitine, may be responsible for limiting the rate of fatty acid oxidation in the tissues of the feed-restricted animal. During food deprivation, hormonal stimulation of fatty acid release from adipose tissue depots would be expected, in response to meeting the physiological energy demands when the caloric/energy balance is not maintained by the diet. This situation would be particularly applicable to the pair-fed rats, as they appeared more physically active than either the pyridoxine-deficient or control rats. Therefore, based on cytochrome oxidase activity, it is possible that the mitochondria content and/or activity of skeletal muscle and heart increased in pair-fed rats. Such a change could accommodate endogenously-mobilized fatty acids destined for β-oxidation and liberation of needed energy.

On the other hand, increased mitochondrial content and/or activity would not necessarily indicate that the rate of fatty acid oxidation would conform. Since fatty acid oxidation can be considered a multiple-step process, it follows that regulation can occur at several sites not exclusively restricted to inside the mitochondria. For example, there is some indirect evidence (137) that feed restriction may decrease the rate of palmitate oxidation in heart by inhibiting and/or repressing palmitoyl CoA

synthetase (EC 6.2.1.3), an extramitochondrial enzyme required for fatty acid activation, but unaffecting carnitine palmitoyltransferase (CPT, EC 2.3.1.21). The ability of exogenous carnitine in the present study to similarly stimulate palmitate oxidation in both skeletal muscle and heart tissue homogenates of both control and pair-fed rats tends to confirm that CPT is not influenced by feed restriction.

As previously mentioned, reduction of the hepatic SAM:SAH ratio *in vivo* can result in decreases in the activity of PEMT (125) and in the methylation of phospholipid (92,97). The marked reduction of the hepatic SAM:SAH ratio in pyridoxine-deficient rats was not accompanied by any inhibition of PEMT in liver microsomes. Instead, an increase in activity was observed for the enzyme, relative to control, which was also the case using liver microsomes from pair-fed animal counterparts. The tissue level of SAH, as well as SAM, in liver was not altered in pair-fed rats in relation to values obtained for control animals. On the other hand, decreased methylation of phospholipid was indicated by an accumulation of PE and a reduction of PC in liver microsomes from both pyridoxine-deficient and pair-fed animals. A similar disparity between PEMT activity *in vitro* and phospholipid levels in rat liver microsomes during depression of the SAM:SAH ratio after feeding rats a methyl-deficient diet has been

reported by Hoffman and coworkers (138). It was suggested that endogenous accumulation of PE may stimulate PEMT activity *in vitro*. Since hepatic microsomal phospholipid was not measured in the study reporting a reduction of PEMT activity in the presence of a lowered SAM:SAH ratio (125), this condition may have presented itself without an elevation of PE.

The possibility exists, also, that PEMT is being induced during the stressful dietary conditions, but full enzyme activity is not expressed in either case *in vivo*, due to elevated levels of SAH acting as a successful competitive inhibitor with SAM. Hence, when the enzyme is measured *in vitro* with much higher concentrations of exogenous SAM than present endogenously, SAH may be displaced from the binding site by SAM to allow expression of more enzyme activity. In any event, when Hoffman et al. (138) assessed methylation of PE *in vivo* by incorporation of [1,2-^{14}C]ethanolamine into PC, methyl-deficient rat liver microsomes displayed a reduction in the level of radiolabeled PC. Thus, it seems that disturbed methionine metabolism during pyridoxine deficiency may alter phospholipid metabolism in the endoplasmic reticulum of rat liver in a manner similar to that obtained with a methyldeficient diet.

While pyridoxine deficiency can negatively influence phospholipid methylation, it appears that chronic feed

restriction is also capable of exerting the same effect to a significant extent. However, this finding is not readily explainable. The effect seems to occur in the absence of disturbed methionine metabolism, since SAM and SAH concentrations are not altered in pair-fed rat livers when compared to those values seen in control rat livers. While this would suggest that the level of protein in the limited feed intake of the pair-fed group, as well as the pyridoxine-deficient group, is able to maintain normal SAM levels, one would also expect tissue protein catabolism to occur during the caloric/energy deprivation, which could also contribute to the total availability of active methionine. Since there may be competition of SAH among the methyltransferases (124), it is reasonable to suspect that the same situation exists with respect to SAM. Hence, during dietary stress, certain transmethylation reactions may possibly take precedence over others in an attempt to maintain normal physiological function.

As pointed out before, GAMT is one enzyme with a heavy demand on the supply of SAM (79,80). As a result, it may be that methylation of PE to PC is somewhat constrained, since this process requires the equivalent of three moles of SAM. In other words, it is suggested that SAM must be used conservatively during dietary stress to support other biological transmethylation processes, such as creatine formation. As a matter of fact, there was a significant

increase in liver creatine in pair-fed, and also pyridoxine-deficient rats, when compared to control animals. Yet, it is rather interesting that the alternative pathway for the synthesis of PC involving choline phosphotransferase (EC 2.7.8.2) was apparently unable to compensate for the reduction of PC in liver microsomes from the pair-fed, as well as the pyridoxine-deficient animals. Although the activity of rat liver choline phosphotransferase was not measured in the present study, apparently the activity of the enzyme decreases during methyl group deficiency (61,138). However, it has also been reported that the formation of PC via this alternative pathway is inhibited (98), but after drug treatment. Nevertheless, it would appear that pyridoxine deficiency decreases phospholipid methylation in the endoplasmic reticulum above and beyond that produced by feed restriction alone.

Significant elevation of creatine concentrations in liver and skeletal muscle of pyridoxine-deficient rats in relation to pair-fed rats, as well as pair-fed rats relative to control rats, are not immediately explainable in retrospect to their kidney AGTA activities. However, previous dietary studies have indicated that creatine formation is not hindered under certain types of nutritional stress. Even though there was an 85% decrease in the activity of kidney AGTA in weanling rats fed a

protein-free diet for 16 days, the amount of total body creatine or phosphocreatine in these animals did not differ from that of rats fed an adequate diet, suggesting that sufficient guanidoacetate can be synthesized in the presence of substantially reduced kidney AGTA activity to maintain normal creatine (phosphocreatine) levels (139). Accordingly, this would imply that sufficient arginine, glycine, and active methionine, along with an appropriate amount of AGTA activity, would have to be present to allow for the complete pathway of creatine biosynthesis to proceed under the adversity of dietary protein abstinence. Presumably, tissue protein catabolism would facilitate this process by liberation of free amino acids, which could also be employed for enzyme synthesis. On the other hand, it may be that kidney AGTA is relatively insignificant under these conditions. Other rat tissues, besides kidney and pancreas, also possess significant AGTA activity when their summation is taken into account which is not subject to regulation by a protein-free diet (140). Hence, it would appear that AGTA activity is usually in excess of that amount needed for the formation of guanidoacetate for creatine biosynthesis. Likewise, liver GAMT activity does not appear to be rate-limiting either in the formation of creatine. This is further supported by the practice of using guanidoacetate as a methyl group-depleting agent (61,138). Moreover, as in the case of AGTA, it is possible

that GAMT activity, undetectable by current methodology, may exist in non-hepatic tissue which may be physiologically significant when considered cumulatively.

In view of the above observations, it becomes conceivable that increased creatine biosynthesis in liver of pair-fed and pyridoxine-deficient rats my occur, in spite of marked reduction of AGTA activity in kidney and a slight but insignificant decrease of GAMT activity in liver. If in fact the elevation of creatine in liver represents increased synthesis, the flow of more creatine out of the liver into the blood stream, eventually for increased uptake by skeletal muscle, could explain the reduced AGTA activity in kidney. It has been suggested that creatine serves to repress kidney AGTA synthesis, since creatine does not inhibit the enzyme in vitro (141,142). Although pyridoxine-deficient rats displayed kidney AGTA activity lower than pair-fed rats, pyridoxine does not appear to be a cofactor for the enzyme. Addition of pyridoxal phosphate in vitro to kidney enzyme preparations from pyridoxine-deficient rats failed to increase activity. Therefore, the indirect effect of pyridoxine deficiency on kidney AGTA is similar to that observed in vitamin E deficiency (143), where creatinuria was present. It has been proposed that the physiological significance of creatine repression of AGTA is to conserve arginine, glycine, and methionine for

protein synthesis (142). Even though casein comprised 29% of the dietary regimes, amino acid conservation may be important in the pyridoxine-deficient and pair-fed rats since these animals consumed only about half of that by control rats.

It is not known why rats subjected to an apparent feed restriction or pyridoxine deficiency would show increases of creatine in liver and skeletal muscle from either a biochemical or physiological standpoint. Lowering the hepatic SAM:SAH ratio in rats, by feeding the pyridoxine-deficient diet, did not promote a significant reduction in GAMT activity or creatine levels. This is in somewhat of a contrast to a previous study (97), where the SAM:SAH ratio was acutely lowered by pharmacological means and a reduction in liver creatine observed. In any event, it is further suggested that pyridoxine-deficient and pair-fed rats may conserve creatine during nutritional stress, since creatine formation requires a substantial input of amino acids. This is supported by their increased levels of creatine in liver and skeletal muscle and also similar excretion of creatinine in urine with the control rats. Pyridoxine deficiency may possibly reduce creatine turnover further, due to the vitamin's role in amino acid metabolism.

In conclusion, the results generated by the present research generally support the concept that the SAM:SAH

ratio is an important regulator of the methyltransferases. Factors which can lower the ratio *in vivo* may have a significant impact on certain biological processes related to transmethylation. This possibility may have practical significance. For instance, transmethylation is undoubtedly an essential biochemical process. As such, certain methyltransferases have been considered as suitable targets for chemotherapy (144). Accordingly, SAH and related compounds, as reviewed here and elsewhere (145), may offer potential for the treatment of disease.

CHAPTER IV

SUMMARY

The effect of modulating the absolute molar ratio of S-adenosylmethionine to S-adenosylhomocysteine (SAM:SAH ratio), both *in vitro* and *in vivo*, upon various transmethylation reactions was studied. Using cell-free enzyme preparations from rat liver, reduction of the SAM:SAH ratio over a broad range caused a corresponding decrease in the specific activities of thiopurine methyltransferase (TPMT), thiol methyltransferase (TMT), and protein(lysine)methyltransferase (PLMT). Similar regulation of TPMT and PLMT was found when the hepatic SAM:SAH ratio was lowered *in vivo* by administration of D,L-homocysteine thiolactone to animals. TMT was not affected after drug treatment, suggesting a compartmentalization of SAH in the intact hepatocyte.

Feeding rats a pyridoxine-deficient diet for 6-7 weeks also caused a reduction in the SAM:SAH ratio in liver. Unexpectedly, the specific activities of TPMT and TMT were not influenced under these conditions. On the other hand, a significant decrease in the specific activity of PLMT was observed during pyridoxine deficiency, along with tissue levels of total acid-soluble carnitine in skeletal muscle and heart. However, the capacity of these two tissues from the pyridoxine-deficient rat to carry out fatty acid

oxidation *in vitro* was not affected. Yet, feed restriction tended to decrease the rate of fatty acid oxidation in skeletal muscle, in the presence of normal carnitine levels and elevated cytochrome oxidase activity.

In parallel experiments, dietary pyridoxine deficiency caused an apparent decrease in phospholipid methylation in rat liver microsomes, above and beyond that produced by feed restriction alone, even though the specific activity of phosphatidylethanolamine methyltransferase as measured *in vitro* did not conform. Furthermore, despite no change in specific activity of hepatic guanidoacetate methyltransferase and a decrease of kidney arginine-glycine transamidinase during pyridoxine deficiency, both liver and skeletal muscle creatine concentrations were elevated, once again above and beyond that caused by feed restriction alone. Since urinary creatinine excretion remained unchanged, conservation of creatine may possibly transpire during the imposed nutritional stress.

The results obtained in this study provide further evidence that the SAM:SAH ratio is an important regulator of the methyltransferases. In other words, factors modulating the SAM:SAH ratio may in turn regulate transmethylation.

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VITA

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