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I am submitting herewith a dissertation written by Mei Fung Chen entitled "The Effect of Dietary Sulfur on the Metabolism of Long Chain Polyunsaturated Fatty Acids." I have examined the final 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.

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THE EFFECT OF DIETARY SULFUR ON THE METABOLISM OF LONG CHAIN POLYUNSATURATED FATTY ACIDS

A Dissertation

presented for the

Doctor of Philosophy

Degree

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Mei Fung Chen

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ABSTRACT

The effect of methionine-supplemented diet on the metabolism of polyunsaturated fatty acids was studied by comparing results of control and cysteine-supplemented diets in rats. Adult Sprague-Dawley rats (250-300 g) were fed for a period of 17 days one of the following diets: control, cysteine-supplemented (0.505%), and/or methionine-supplemented (0.625%). On the last day of the feeding period, the rats were administered either [1-**14**C] arachidonic acid (AA) or [1-**14**C] eicosapentaenoic acid (EPA) by stomach tube five hours before sacrificing. The cyclooxygenase activity in liver microsomes, the apparent Km of the fatty acyl-CoA synthetase in liver and brain homogenates, and the incorporation of polyunsaturated fatty acids into the phosphatidylinositol fraction of brain, heart, lung, spleen, and kidney tissues were analyzed.

The cyclooxygenase activity of liver microsomes in rats fed the methionine-supplemented diets was statistically significant low compared with that in rats fed either the control or the cysteine-supplemented diets. Based upon previous observations and literature reports, this was presumed to be due to the increased liver concentration of S-Adenosyl-methionine (SAM) in

response to the methionine-supplemented dietary regimen. However, the incorporation of polyunsaturated fatty acids into phosphatidylinositol fraction in tissues investigated was not significantly different among rats fed the three different diets.

The differential incorporation of [1-**14**C] arachidonic acid or [1-**14**C] eicosapentaenoic acid into various tissues was partially explained by the observation of the apparent Kms of fatty acyl-CoA synthetase in brain and liver for AA and EPA. The approximate five-fold increase in the apparent Kms for these fatty acids in liver tissue compared to brain tissue explained the decrease in specific activity of liver phosphatidylinositol compared to brain phosphatidylinositol. The apparent Km for AA in the brain was about 1.5 times higher than that for EPA, thus the brain fatty acyl-CoA synthetase preferred EPA as the substrate. Whereas in the liver, the apparent Km for AA was about 0.6 of that for EPA. Therefore, liver incorporated about 1.5 times more of AA into phosphatidylinositol fraction.

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CHAPTER I

INTRODUCTION

S-Adenosylmethionine (SAM) has been shown to have analgesic and anti-inflammatory properties without damaging the gastrointestinal mucosa in experimental animals. The analgesic and anti-inflammatory properties of SAM have been explained by its action as an inhibitor of cyclooxygenase, an enzyme required for the conversion of the long chain polyunsaturated fatty acids into a precursor of the biologically active prostaglandins, prostacyclins and thromboxanes. Aspirin is also an inhibitor of the cyclooxygenase enzyme. Low doses of aspirin have been prescribed for patients with unstable angina, or those who are stroke-prone, and/or those who are considered at risk for coronary occulusion.

Loo and Smith (1) have shown that rats fed diets supplemented with methionine had a two-fold increase in liver SAM compared to those fed diets supplemented with cysteine. It is conceivable that methionine-supplemented diet might raise the tissue SAM concentrations to a sufficient level to inhibit cyclooxygenase. If supplemental methionine inhibited cyclooxygenase, then supplemental methionine could mimic the effect of aspirin upon the integrity of the cardiovascular systems. This might have implications for the protein

intake, specifically the methionine intake, of the unstable angina, stroke-prone and/or cardiac patient.

Therefore, the null hypothesis (H_o) of this investigation is that supplemental methionine has no effect on the metabolism of polyunsaturated fatty acids through the cyclooxygenase enzyme pathway or the incorporation of fatty acids into tissue phospholipid. The test hypothesis (H_a) is that supplemental methionine has an effect on the metabolism of polyunsaturated fatty acids through the cyclooxygenase enzyme pathway or the incorporation of fatty acids into tissue phospholipid.

CHAPTER II

REVIEW OF LITERATURE

I. BIOCHEMICAL AND PHARMACOLOGICAL ROLES OF S-ADENOSYL-METHIONINE

Biosynthesis of S-Adenosyl-L-methionine

S-Adenosyl-L-methionine (SAM, Figure 1) is synthesized by all living cells, and the enzyme, ATP:L-Methionine S-Adenosyltransferase, catalyzes the adenosylation of methionine at the expense of adenosine triphosphate (ATP). This activation is a pre-requisite for the transfer of a methyl group to suitable methyl acceptors. The stoichiometry and major features of the enzymatic synthesis were clarified by Cantoni, Mudd, and their colleagues (2,3,4)

$$\text{L-Methionine} + \text{ATP} \xrightarrow[\text{ATP:L-Methionine S-Adenosyltransferase}]{Mg^{2+},\ K^{+}} (-)\ \text{S-Adenosyl-L-methionine} + PPi + Pi$$

Maximum activity requires rather high concentrations of monovalent and divalent cations. The reaction involves complete dephosphorylation of ATP and the nucleophilic transfer of the 5'-deoxyadenosyl moiety of ATP to one of the free pairs of electrons of the thioether sulfur of L-methionine. The final product is one of the two

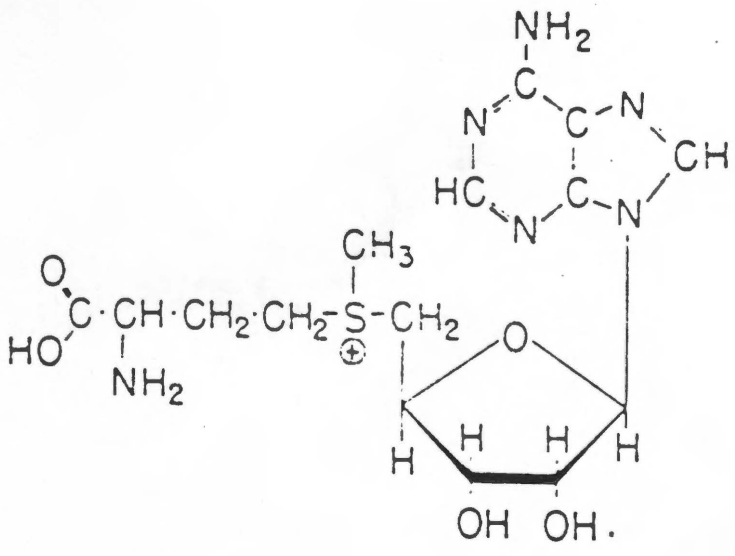


Figure 1. S-Adenosylmethionine (SAM, AdoMet, S(5'-deoxyadenosyl-5')-L-methionine) is the principal methyl donor in transmethylations. Adapted from: Cantoni, G. L., 1953 (2).

diastereoisomeric sulfonium compounds ((±)-S-Adenosyl-L-methionine). The two innermost phosphate groups of ATP give rise to inorganic pyrophosphate (PPi), whereas the terminal group is the source of inorganic phosphate (Pi).

Both yeast and liver adenosyltransferase preparations possess tripolyphosphatase activity for externally added tripolyphosphate (PPPi), and this activity is markedly and specifically stimulated by very low concentrations of SAM (5). One simplified formulation of the reaction steps is the following : (6)

```
            E + ATP <===> E ... ATP                (a)
  Met + E ... ATP <===> Met ... E ... ATP          (b)
Met ... E ... ATP <===> SAM ... E ... PPPi         (c)
SAM ... E ... PPPi<===> SAM + E + PPi + Pi         (d)
-------------------------------------------------------
          Met + ATP <===> SAM + PPi + Pi
```

Where Met and E designate L-methionine and the enzyme, respectively.

A systematic study (7) of the tissue distribution of adenosyltransferase indicates that this enzyme is most active in the liver of various mammalian species. This study also shows that with the exception of kidney and pancreas, which display relatively high activities, the supernatant fluids of homogenates of other tissues have only 5-10 percent of the specific enzyme activity of the liver.

SAM plays a dual role in the control of its synthesis, both as an allosteric effector at low concentrations and as an inhibitor at higher concentrations (5,8). The effect of hormone and diet on the activity of adenosyltransferase also has been investigated by several researchers. Natori (9) explained on the basis of ethionine studies that the adenosyltransferase activities of the livers of female rats are about twice as high as those of males. The administration of androgen to castrated male rats induces a fall in enzyme level. This finding is in complete disagreement with other reports. Investigations of the influences of other hormonal factors controlling transferase activity indicate that the levels of liver transferase in rats decrease slightly following adrenolectomy, and that administration of glucocorticosteroids (but not predominantly mineralocortcosteroids) increases the transferase activity to about 1.5-2-fold of the control levels in both intact and adrenalectomized animals (10).

The influence of diet on adenosyltransferase activity appears to be complex. In vivo, the concentration of methionine is probably the limiting factor for the synthesis of SAM. Normal tissue levels of L-methionine range between 50 and 80 nmoles/g wet tissue weight (8). Baldessarini (11) and

Salvatore et al. (12) have shown that SAM concentration in tissues increases after administration of methionine. Lombardini et al. (5) demonstrated that the rate of formation of SAM bears a sigmoidal relation to the L-methionine concentrations when ATP is saturating. Moreover, the liver tissue concentration of SAM in rats fed methionine-supplemented diets is about two-fold that of rats fed diets supplemented with cysteine (1). The decrease in liver SAM in rats fed the cysteine-supplemented diet appears to reflect a non-competitive inhibition of methionine adenosyltransferase by cysteine.

Metabolic Functions of SAM

SAM is a high energy sulfonium compound. Cantoni (13) has pointed out that in fact all of the three constituents on the sulfonium pole of SAM are energetically equivalent, and these properties give the SAM molecule its unique versatility. Recognition of the number of enzymatic reactions in which it functions either as a substrate or as a modulator has significantly increased in recent years. The important biological roles of SAM include transmethylation reactions and polyamines biosynthesis.

Transmethylation Reactions

Transmethylation is considered to be the earliest example of biochemical group transfer and represents by far the largest and most widely studied group of alkyl transfer reactions (14). The chemical variety of acceptors of a methyl group from SAM is unusually high. The attachment of the methyl group to the nitrogen atom is perhaps the most extensively studied reactions, but sulfur, oxygen and carbon are also other acceptors with binding sites.

Bacterial chemotaxis is related to methyl esterification of membrane protein, which, in turn, affects response to chemoattractants (15,16). In mammalian systems, such as neutrophils which possess chemotactic responsiveness, methyl esterification plays a critical role as a signal for leukocyte motion (17). The enzymatic esterification by methylation neutralizes the negative charge of the carboxyl groups of protein. Such neutralization of charge can alter the conformation of membrane proteins and thus initiate events leading to chemotaxis of leukocytes. Hirata and Axelrod (18) demonstrated the methylation of membrane phospholipids to elicit receptor-mediated signals through the membranes of a variety of cells. As the membrane phospholipids are successively methylated, they are translocated from the inside to the outside of the

membrane, resulting in the reduction of membrane viscosity. This is the mechanism and an initial common pathway by which specific biochemical messages, such as neurotransmitters, peptide hormones, lectin, and immunoglobulin ligands, are recognized by and bound to specific receptor macromolecules on the outer surface of cell membranes (18).

Methylation of nucleic acids at various base and sugar moieties appears to be involved in a variety of biological regulatory mechanisms. Kawarada and Okahara (19) demonstrated that 7-methyl guanine residues of a single-stranded structure of methylated DNA, which is immunogenic or antigenic, play an important role in forming a characteristic structure required for the manifestation of the specific antigenicity.

In vitro, the binding of a carcinogen, 3-N,N-acetoxyacetyl amino-4,6-dimethyldipyrido-[1,2-a:3',2'-d] imidazole, to guanine residues of a native DNA may block the movement of DNA-(cytosine-5-) methyltransferase along the helix and thereby cause hypomethylation (20). This event could have significance in gene activity, cellular differentiation, and cancer induction.

Polyamines Biosynthesis and Unusual Biochemical Roles of SAM

Recognition of the central role of SAM as a precursor of polyamines was provided by the pioneering studies of Tabor, Rosenthal and Tabor (21). In the biosynthesis of polyamines recognized so far, decarboxylated SAM is the only biological donor of the propylamine moiety. The connections between the metabolic pathways involving SAM in mammalian tissues is demonstrated in Figure 2 (22). Putrescine plays a central role in the overall regulation, since the diamine activates SAM decarboxylase and in turn inhibits ornithine decarboxylase and spermine synthetase (22). The inhibitory effect of decarboxylated SAM on methyl transfer reactions suggests that in cellular metabolism SAM decarboxylase not only competes with methyltransferases for the same substrate, but also indirectly inhibits this class of reactions.

The transfer of the unmodified amino acid side chain of SAM to the uridine nitrogen of tRNA represents one of the proposed nucleoside hypermodifications occurring in the maturation of tRNA (23). The modified uridine, i.e., 3-(3-amino-3-carboxypropyl)-uridine, has been detected in bacterial and mammalian tRNA, and

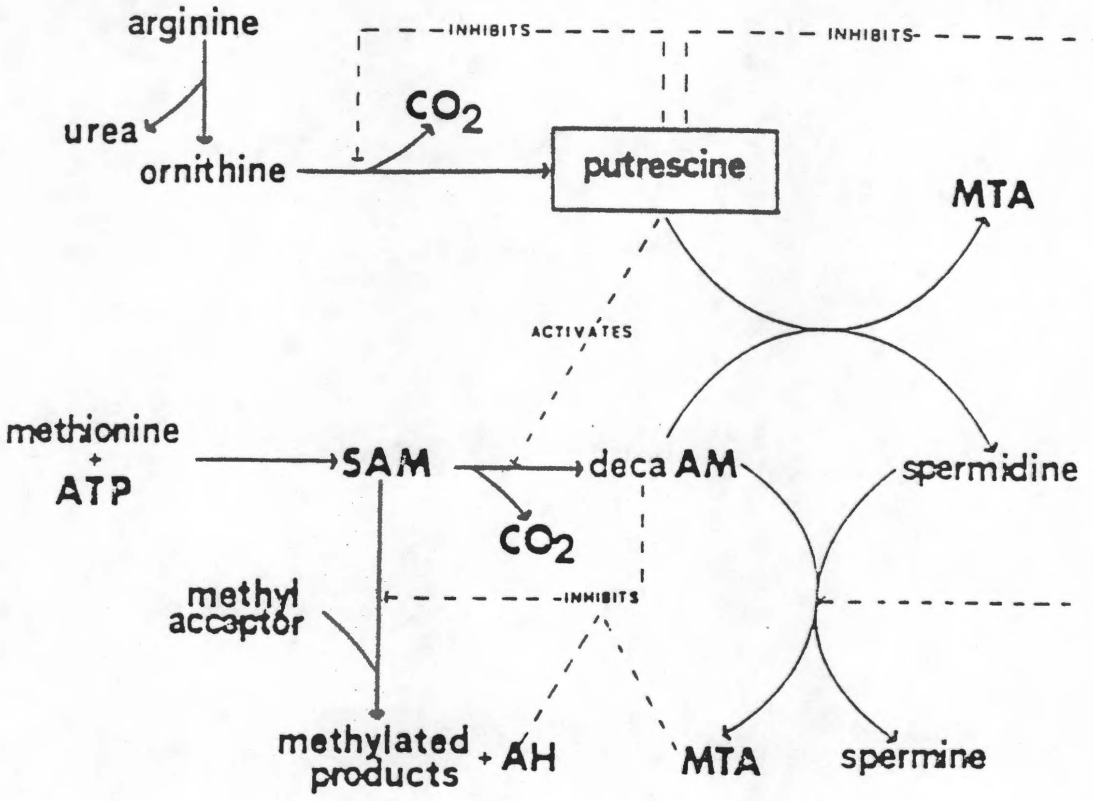


Figure 2. Biosynthesis of polyamines and S-adenosylmethionine metabolism in mammalian tissues. decaAM, S-adenosyl-(5')-3-methylthiopropylamine; MTA, 5'-methylthio-adenosine; AH, S-adenosylhomocysteine. Adapted from: Zappia, V., et al., 1977, (22).

its biological significance is probably related to the stabilization of the three-dimensional structure of the polynucleotide (24).

In E. coli SAM participates in a transamination reaction in the biotin biosynthesis pathway (25,26). The reaction, catalyzed by diaminopelargonic acid aminotransferase, is illustrated in Figure 3. The enzyme requires the sulfonium ion of SAM for activity but not for binding to the enzyme and appears to be highly specific for SAM. The analogs of SAM are unable to transaminate with 7-keto-8-aminopelargonic acid (26).

Recognition of the number of reactions that SAM exerts a regulatory role, either as an activator or as an inhibitor, has significantly increased in the past few years. SAM is an activator in vitamin B_{12}-dependent methionine biosynthesis in microbial systems (homocysteine remethylation in mammalian systems) (27). Inactive B_{12}-dependent N^5-methyltetrahydrofolate-homocysteine methyltransferase is first primed or activated by B_{12}-cobalt methylation. This priming requires both SAM and reduced flavoprotein. Once the enzyme has been converted to one of its functional forms by SAM methylation, it should catalyze the reaction which remethylates L-homocysteine to L-methionine (27).

Conversion of the inactive form of pyruvate formate-lyase (PFL) in the anaerobic metabolism of

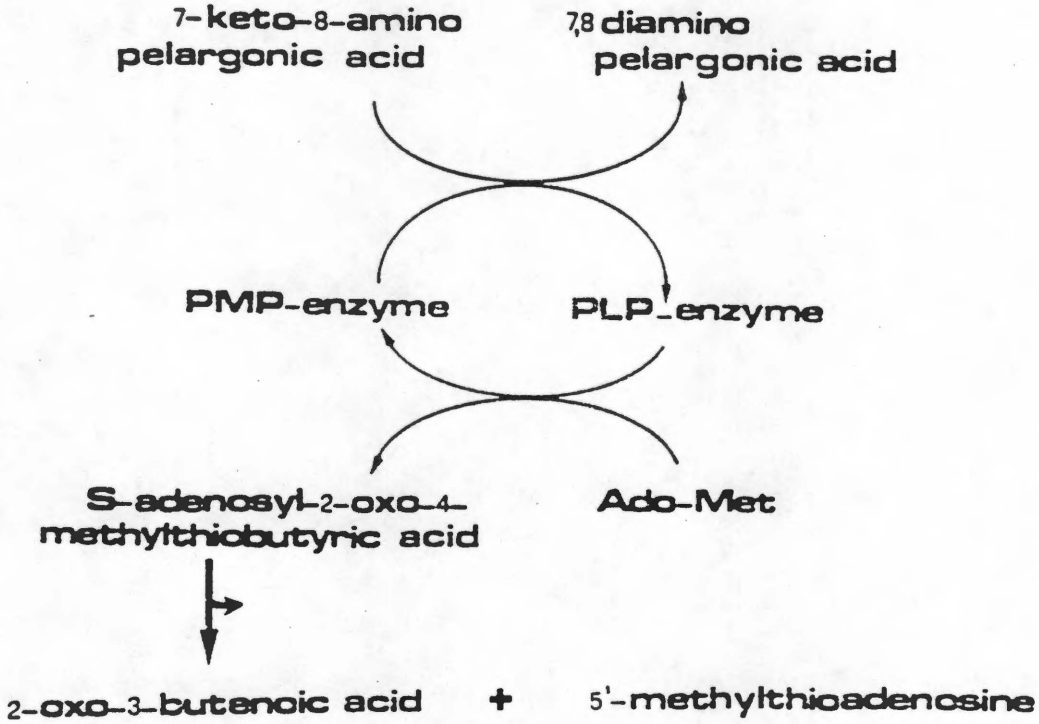


Figure 3. Role of SAM in a transamination reaction in the pathway of biotin biosynthesis. Adapted from: Stoner & Eisenberg, 1975 (26).

E. Coli and other bacteria also requires SAM, reduced flavodoxin, and the Fe-dependent protein (28). It is found that SAM is reductively processed during activation of PFL to yield methionine, adenine and 5'-deoxyribose. Researchers (28) propose that rearrangements in the protein structure of PFL are involved in the interconversion of the two enzyme forms. In the active form of PFL, the protein might be under conformational strain which would provide a rationale for the apparent spontaneity of its conversion to the inactive form of this enzyme. Using SAM to redistribute a disulfide bond and SH-group(s), for example, would be a feasible rearrangement process.

Jencks and Matthews (29) demonstrated that SAM allosterically inhibits pig liver methylene tetrahydrofolate reductase. They suggest there is more than one binding site for SAM at each subunit of the reductase, and NADPH counteracts the inhibition by SAM. The same mechanism has been proposed to explain the irreversible inactivation of calf thymus ribonuclease H exerted by SAM (30).

The biological roles of this compound are summarized as follows:

* Donor of methyl groups in a wide variety of transmethylation reactions (14);

* Required for bacterial chemotaxis, leukocyte motion, and receptor-mediated signals (15,16,17,18);
* Required in the restriction and modification system of DNA in prokaryotes and eukaryotes (19,20);
* Donor of propylamine moiety, after decarboxylation, in the biosynthesis of spermidine, sym-nor-spermidine, spermine and sym-nor-spermine (21,22);
* Donor of the aminobutyryl side chain to tRNA (23,24);
* Donor of the side chain amino group in biotin biosynthesis (25,26);
* Activator of N5-methyltetrahydrofolate-homocysteine methyltransferase and pyruvate-formate-lyase (27,28); and
* Inhibitor of methylene tetrahydrofolate reductase and ribonuclease H (29,30).

Metabolic Pathways Related to SAM

The metabolic pathways which involve SAM are shown in Figure 4, i.e., transmethylation, transsulfuration and the folate cycle. Via transmethylation, SAM is converted into S-adenosyl-L-homocysteine (SAH), which is a potent inhibitor of most methyltransfering enzymes

METABOLIC PATHWAYS RELATED TO ADENOSINE-SULFUR COMPOUNDS

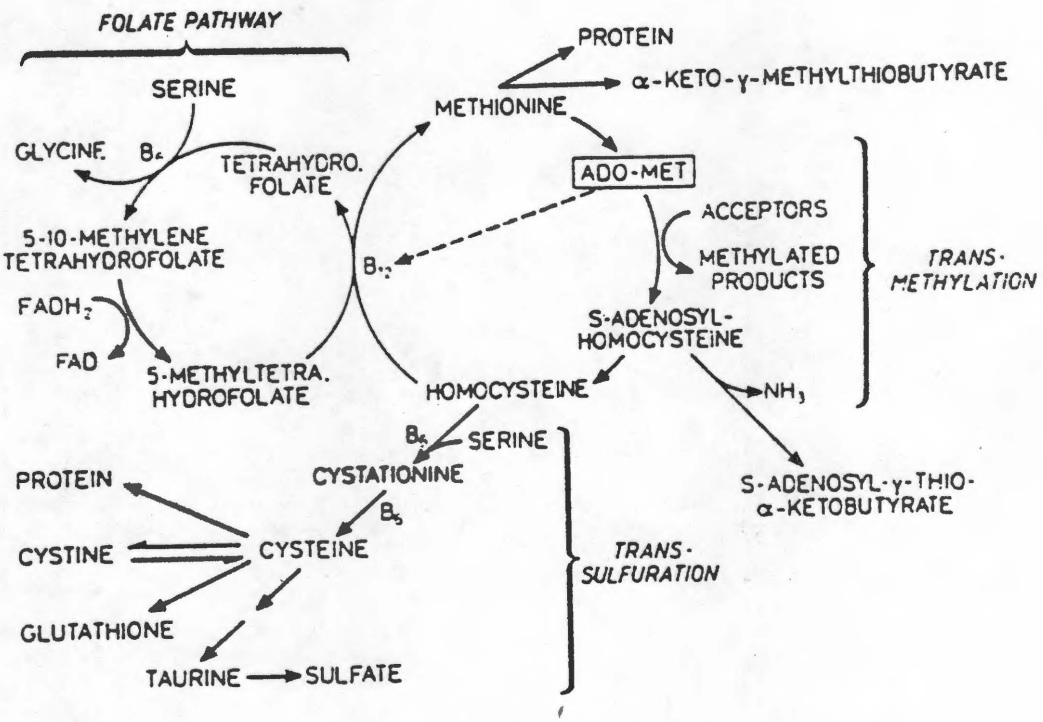


Figure 4. Metabolic relationships among transmethylation, transsulfuration and the folate cycle. Adapted from: Mudd & Cantoni, 1964 (6).

(31). SAH is further cleaved to adenosine and L-homocysteine by a specific hydrolase (8). Adenosine is effectively deaminated and further metabolized to uric acid (32), and homocysteine is metabolized by two competing systems. These are the trans-sulfuration pathways leading to cysteine, the major route for methionine catabolism, and the proposed "sulfur conservation cycle" and connecting with the folate pathway. Mammalian livers contain a sensitive regulatory mechanism which allows either conservation or catabolism of methionine as a function of the dietary content of this nutrient (33).

Pharmacological Effects of SAM

SAM has been the subject of many experimental and clinical investigations because of its diverse functions. The pharmacological properties of SAM most extensively studied in recent years include: (a) effects of SAM on the central nervous system function; (b) effects of SAM as a detoxicant and cytoprotective agent in the treatment of liver disease; and (c) effects of SAM as an anti-inflammatory agent.

Effects on Central Nervous System.

In the central nervous system, SAM-dependent methylation processes are of particular importance in the biosynthesis and inactivation of neurotransmitters

and hormonally active biogenic amines. For example, both the formation of melatonin and the inactivation of catecholamines involve O-methylation processes, whereas the synthesis of adrenaline and the inactivation of histamine involve N-methylation reactions. In all cases SAM is the methyl donor.

For many years, there has been considerable interest in the chemistry and pharmacology of biological transmethylation in the field of neuropsychiatry (34). This interest was largely stimulated by the fact that many natural or synthetic psychotropic substances are methylated amines. A hallucinogen, dimethyltryptamine (DMT), is synthesized by a "non-specific N-methyl transferase" in the brain and several other tissues; tryptophan, tryptamine, or N-methyltryptamine (NMT) could serve as a substrate for this methylating enzyme (35). In one study (35) the ability of human platelets to form DMT from NMT enzymatically tended to be highest in schizophrenic subjects, compared to non-schizophrenic subjects. This elevated enzyme activity was due to a reduced amount of normally occurring enzyme inhibitor in schizophrenics. In addition, Andreoli and Maffei (36) reported that blood SAM levels are decreased in acute schizophrenia, but not in chronic schizophrenia.

The hypothesis that depression is due to a metabolic disorder involving catecholamines, serotonin

or, most likely, their functional balance, supports the use of a variety of therapeutic agents, such as SAM. It has been observed that depressed patients have reduced catechol O-methyltransferase (COMT) activity in red cells (37). This might indicate a decrease of the concentration of SAM as a methyl donor.

Stramentinoli et al. (38) administered SAM to male Sprague-Dawley rats and determined the level of 5-hydroxytryptamine and 5-hydroxyindolacetic acid (metabolites of serotonin) in the brainstem and forebrain. The results (38) indicated that SAM administration induces a significant increase in the levels of both metabolites in the forebrain, but only a slight increase (not a statistically significant increase) in the brainstem. These results were confirmed by additional experiments using depressed patients (39). Another clinical trial with a small number of patients was also performed by Scarzella (40). Preliminary data indicate that SAM exerts an antidepressant effect which is detectable after the first week, and no side effects have been observed except for occasional and mild symptoms of anxiety.

The effects of SAM on dopaminergic and B-adrenergic receptor systems were studied in aging rats (41). Earlier investigations demonstrated that membrane fluidity and receptor properties could be altered by the

degree of methylation of the membrane phospholipid (42). The viscosity of the striatal membranes in aging rats was significantly higher than in young rats, thereby affecting the receptor binding activity and the signal transmission for intracellular responses. The decrease in B-adrenergic binding sites induced by aging was antagonized by administration of SAM, suggesting that a deficit in this brain area of aging animals can be reversed by episodic treatment with this particular drug.

SAM concentration may be depleted by some drugs, such as L-dopa. L-dopa is used to treat Parkinson's disease and acts as methyl group acceptor (43). Chalmers et al. (44) demonstrated that acute L-dopa administration to rats intracisternally injected with [3H] norepinephrine caused a depletion of brain SAM as well as a reduction in O-methylated [3H] norepinephrine derivatives. This could directly or indirectly influence the normal functioning of certain neuronal systems. Stramentinoli and his associates (45) have shown that exogenous administration of SAM with L-dopa was not enough to compensate completely for the reduced concentration of SAM in the brain, but exogenous SAM could reverse the effect of L-dopa by a 63 percent increase of endogenous SAM.

Treatment of Liver Diseases with SAM

SAM has proved to be a methyl donor as well as a precursor of endogenous sulfurated products, and in particular of glutathione (GSH). Owing to these properties, SAM has been found to be a valuable therapeutic tool in the treatment of liver diseases as an antisteatosic, antinecrotic, detoxicant, and cytoprotective agent.

The antisteatosic effect of SAM has been demonstrated in both ethanol-intoxicated mice and rats (46,47) and in humans with alcoholic liver diseases (48,49). Although the mechanism by which SAM antagonizes alcoholic fatty liver degeneration is still unknown, several lines of evidence have been reported.

An acute ethanol-intoxicted mice, the administration of SAM reversed the effect of ethanol on hepatic GSH concentration and r-aminolevulinic acid dehydratase (ALA-D) activity. ALA-D has been demonstrated to require reduced sulfhydryl groups for its activity (46). SAM, via transsulfuration reactions, is a better precursor of GSH than methionine, has an extremely low toxicity, and is easily available as a pharmaceutical preparation (51).

Feo et al. (47) demonstrated that SAM's protective effect in liver steatosis, caused by chronic ethanol ingestion, was through inhibition of alcohol

dehydrogenase activity. Approximately 30 percent of the dehydrogenase activity was inhibited in both normal rats and those subjected to acute ethanol toxicity. This decrease in activity was not observed in the microsomal ethanol oxidizing systems of normal or intoxicated rats. Moreover, SAM treatment almost completely reconstituted the liver GSH pool and caused a large decrease in the liver and blood acetaldehyde contents. The Feo group (47) proposed that SAM's antisteatosic effect largely depended on its role as a modulator of the GSH liver content.

A preliminary clinical trial with SAM was conducted by Feaccadori et al. (48). This trial evaluated effects of long-term (six months) administration of SAM on eleven out-patients with alcoholic liver disease. Liver biopsy specimens of these patients showed a significant regression of steatosis after treatment with SAM. They reported that the antagonistic action of SAM against alcoholic fatty liver could be related to its capability to methylate phospholipids involved in lipoprotein metabolism.

Furthermore, Micali et al. (49) investigated the administration of SAM in chronic liver diseases in a double blind controlled study. Patients treated with SAM showed statistically significant improvement in Bromosulfophthalein (BSP) clearance, liver biopsy, and

other biochemical parameters. These results (49) suggest that SAM possesses a membrane-stabilizing action secondary to its primary role in the phospholipid metabolism of membranes. This stabilizing action accounts for an improvement in the capture and/or conjugation of the dye-stuff (BSP). Also, the formation of endogenous SAM is influenced by the availability of ATP, which is reduced in steatosis (52). Exogenous SAM helps spare energy for the formation of SAM. Thus, hepatocyte lipid deposition could be removed by SAM intervening in the phospholipid methylation in the endoplasmic reticulum of the liver cell (49). Histological findings (49) indicate that steatosis is noticeably influenced by SAM through methylation of phosphatidylethanolamine to increase phosphatidylcholine synthesis. This phospholipid is capable of enhancing the formation of lipoproteins which could remove the excessive triglyceride, thereby preventing fatty liver from becoming extended as large fat droplets (49). Micali et al. also found that treatment with SAM could affect the metabolism of neurotransmitters, thereby causing significant improvements in the symptom complex of liver disease patients with hyperammonemia encephalopathy.

SAM has been shown to protect the liver of mice from injuries produced by hepatotoxic agents, such as

large doses of acetaminophen (54). The hepatotoxicity of acetaminophen is related to its P**450** mediated metabolism to a reactive intermediate (55). Glutathione, through an attack of its nucleophilic sulfur atom, binds to reactive metabolite giving a readily eliminative conjugate (55). After doses of acetaminophen that exceed the available glutathione, binding of this reactive intermediate to liver proteins occurs, and is accompanied by liver necrosis. In one study (54) SAM treatment did not prevent the fall of liver glutathione concentrations, caused by large doses of acetaminophen. Histologic observations revealed that the hepatocellular damage was confined to a small area. The possibility was considered that SAM may influence protein binding of the metabolite. Actually, decreased binding was observed in animals given SAM in addition to 3H-acetaminophen (54). On the other hand, SAM failed to act on binding in *in vitro* experiments at a concentration at which cysteamine almost completely inhibited protein binding (54). The researchers (54) suggested that the decreased protein binding observed *in vivo* might depend on the formation of a nucleophilic metabolite of SAM, which was capable of binding to the reactive acetaminophen derivative and thus favored its elimination.

SAM is also beneficial in the treatment of lead intoxication (51,56). The effect of SAM administration on rats that received both acute and chronic lead exposure was demonstrated by Paredes et al. (51). Reduced GSH concentrations in blood and liver of intoxicated animals were reversed after SAM administration, presumably due to the role of SAM as a precursor of GSH (51). Moreover, reduced sulfhydryl groups are essential for the activity of ALA-D, a zinc metalloenzyme. Zinc also prevents oxidation of those sulfhydryl residues (50). Lead easily displaces zinc and blocks the active site by bridging two vicinal SH groups, inhibiting the enzyme. In one study (51) a significant recovery of tissue ALA-D activity, initially reduced in poisoned animals, was clearly produced after SAM treatment. This result supported the axiom that an enhancement of thiol content as a result of SAM administration facilitates the detoxication process and lead removal, consequently reversing the inactivation of the enzyme. Because of the important function of SAM in the central nervous system and its direct involvement in catecholamine metabolism (22), the authors suggested that effects of SAM, unrelated to GSH, on lead neuropathy can not be excluded (51).

Prevention of ethynylestradiol (EE)-induced cholestasis by SAM was demonstrated in both rats and

humans (57,58,59,60). Ethynylestradiol, an estrogenic factor of many "combination" oral contraceptives, has been demonstrated to impair bile secretion and decrease liver plasma membrane fluidity in several animal species including humans, so that bile becomes supersaturated with cholesterol and its flow is reduced (61). Results of experimental investigations (57,58,62,63) indicate that SAM can: (a) reverse both cholestasis and related abnormalities of biliary lipids; (b) modify the pattern but not the total amount of biliary EE excretion; and (c) reduce the irreversible binding of estrogen to rat liver microsomes, probably favoring EE biotransformation into its methyl-derivatives in the presence of catechol-O-methyltransferase (62,63).

Schreiber et al. (58) suggested that another explanation for SAM's anti-lithogenic effect may be its stimulation of phosphatidylcholine synthesis, resulting in an increased hepatic Na+/K+ ATPase activity and liver plasma membrane fluidity (18). The regulation of phosphatidylcholine turnover by SAM may be useful in hepatic cytoprotection. Clinical investigation (59) with non-obese subjects showed that SAM prevents EE-induced bile lipid changes without interfering with EE kinetics. The researchers concluded that SAM can be considered an adjuvant to chronic estrogen therapy in subjects at the risk of developing lithogenic bile.

Frezza et al. (60) further investigated SAM-induced reversal of cholestasis of hypersensitive pregnant patients due to the striking estrogen increase in late pregnancy. The result (60) suggested that SAM decreases some markers of cholestasis elevated in pregnancy without affecting placental growth.

Anti-inflammatory Effect of SAM

The anti-inflammatory and analgesic properties of SAM in experimental animals (64,65) and its therapeutic action on symptoms of degenerative arthropathies (66,67) were reported by Italian researchers.

Gualano et al. (64) evaluated the effect of SAM on carrageenin-induced edema in rats and writhings provoked in mice by phenylquinone and acetic acid. The data indicated that SAM prevents the swelling process induced by the above treatments in a dose-dependent manner. These researchers (64) also demonstrated that SAM inhibits the formation of prostaglandin E_2-like material in the exudate produced by subcutaneous implantation of sterile sponges in the rat. When the ability of histamine to activate arachidonic acid metabolism in perfused guinea-pig lungs treated with SAM were studied, the appearance in the pulmonary effluent of TXA_2-like material was significantly impaired (64). These results implied that SAM affects, by the inhibition of

cyclooxygenase, the activation of the endogenous eicosanoid system promoted by histamine. This experiment with arachidonic acid suggests that, as in the case of Indomethacin, SAM may affect pulmonary cyclo-oxygenase activity or other steps involved in TXA2-like material formation.

Gualano and his associates (65) further demonstrated that oral and injected SAM in rats had similar effects upon the production of prostaglandin-like material in sponge exudates and by rat peritoneal leukocytes during bacterial phagocytosis. The authors confirmed that SAM is able to interfere with arachidonic acid metabolism through the cyclooxygenase metabolite pathway (38,65).

The therapeutic effectiveness and tolerance of SAM given orally to two groups of patients with hip, knee and vertebral arthroses and osteoarthritis were studied, respectively, by Capretto et al. (66) and Marcolongl et al. (67). Results were compared with those obtained in a similar number of patients treated with Ibuprofen, a non-steroidal anti-inflammatory agent largely used for the symptomatic treatment of inflammatory conditions. Both studies (66,67) confirmed the effectiveness of SAM in the treatment of degenerative joint diseases. SAM is not only specifically capable of relieving painful

joint conditions, but also excellently tolerated, particularly in the gastro-intestinal tract.

II. METABOLISM OF LONG CHAIN POLYUNSATURATED FATTY ACIDS

It was not until 1929 that Burr and Burr (68) discovered that polyunsaturated fatty acids are essential for the growth and normal function of all tissues. These fatty acids can be classified into three categories on the basis of their biosynthetic precursors: (1) the oleate (ω-9), (2) the linoleate (ω-6), and (3) the linolenate (ω-3) series (69). These parent essential fatty acids undergo chain elongation and desaturation to obtain various long chain polyunsaturated fatty acids, such as arachidonic acid (c20:4), eicosapentaenoic acid (c20:5), and docosahexaenoic acid (c22:5). In mammalian tissues, the double bond nearest the methyl end of the chain is not altered when new double bonds are inserted toward the carboxyl terminal, and the various fatty acids in the three categories are not interchangeable metabolically . Some of the polyunsaturated fatty acids may be converted to physiologically potent metabolites, but others are not (70,71); some fatty acids may even inhibit the conversion of precursor acids to corresponding metabolites (72,73). Consequently, the relative

proportions of precursor and inhibitor acids at the 2-position of phospholipids may have an important influence upon cellular physiology.

The Incorporation of Polyunsaturated Fatty Acids into Cellular Phospholipids.

The incorporation of polyunsaturated fatty acids into the 2-position of phospholipids is catalyzed sequentially by acyl CoA synthetase, then by acyltransferase systems into various forms of phospholipids. The selectivities of the acyltransferase systems have been determined in the presence of mixtures of acyl donors and suboptimal concentrations of acceptors (74,75,76). Lands et al. (77) demonstrated that rat liver has a high selectivity for polyunsaturated acids, but does not discriminate among the polyunsaturated acids of the ω-6 and ω-3 series. The phospholipid composition in fully functional membrane is highly specific and complex. The acyl groups in phospholipids could affect membrane function in one or more of four ways: (1) effects on lipid fluidity; (2) effects on membrane thickness; (3) effects on lipid phase; and (4) effects of specific interaction with membrane proteins (78).

Arachidonic acid (AA), rich in the Western diet, is preferentially incorporated into diacyl-

glycerophosphoinositols (GPI) and diacyl-glycerophosphocholines (GPC) (79). These membrane phospholipids act as a depository of arachidonic acid for eicosanoid synthesis, and phosphatidylinositols are important for signalling specific cellular activity (78). In general, turnover of arachidonoyl-GPI in the brain membranes is more rapid than other types of phosphoglycerides (80). This phenomenon is probably due to the presence of enzymes specific for metabolizing the glycerophosphoinositol. In vitro experiments (81) have shown that two types of cyclic events are related to metabolism of brain fatty acids: (1) the ATP-dependent activation of fatty acids to their acyl CoA via the acyl CoA synthetase, and subsequent hydrolysis of acyl CoA by acyl CoA hydrolase(s); and (2) the release of polyunsaturated fatty acids from membrane phosphoglycerides via phospholipase A2 and subsequent reacylation of the lysophosphoglycerides by the acyltransferases. The polyunsaturated fatty acid level in brain may be regulated by enzymes responsible for both cyclic events (Figure 5).

Eicosapentaenoic acid (c20:5, EPA) is a ω-3 polyunsaturated fatty acid found in marine foods. In vivo EPA is readily incorporated into tissue lipids (82) and, at the same time, there is a reduction in their AA content. Philbrick et al. fed rats a diet rich in ω-3

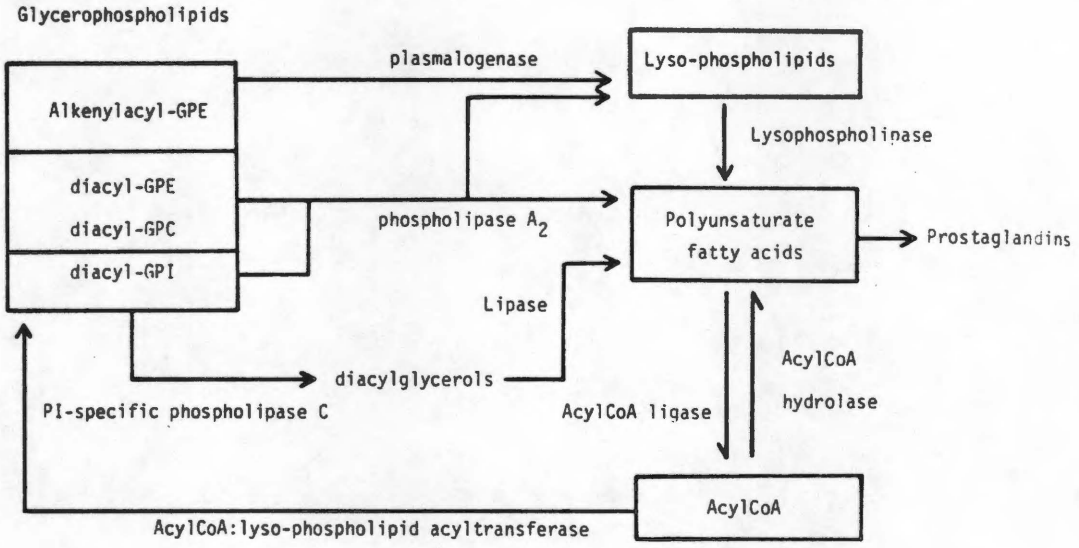


Figure 5. A scheme showing the metabolic relationship between polyunsaturated fatty acids and membrane phospholipids in brain. Adapted from: Sun, G. Y., et al., 1979 (81).

fatty acids and demonstrated a reciprocal replacement of the ω-6 by the ω-3 fatty acids in brain phospholipids (83). This finding is in agreement with reports of altered fatty acid composition in membrane phospholipids following changes in dietary fat source and changes in ω-6/ω-3 fatty acid ratios in the postweanling rat (84-87) . It also provides further evidence that fatty acid composition of membrane phospholipids can be altered by dietary fatty acid levels after the major period of brain growth and development in rats.

The consumption by human subjects of diets containing fish or supplemented with fish oil for one to 11 weeks produces an elevation in the percentage of EPA in fatty acids of platelet phospholipids (82,88-92). However, the biochemical mechanisms by which dietary fish oil and a derived ω-3 fatty acid concentrate produce elevations in ω-3 polyunsaturated fatty acid and suppress ω-6 polyunsaturated fatty acid in individual phospholipids remains unknown. The dietary effects on fatty acid desaturation, availability of various acyl coenzyme A esters in the cellular pool, and involvement of deacylation-reacylation reactions are the possible mechanisms.

Biologically Active Metabolites of Polyunsaturated Fatty Acids.

One of the roles of polyunsaturated fatty acids is to act as precursors of prostaglandins and related substances, generally referred to as prostanoids (93). Prostanoids are made from fatty acid precursors such as arachidonic acid and are a family of chemically related compounds comprised of prostaglandins, thromboxanes, and prostacyclins. Mammalian tissues are able to synthesize up to 20 separate prostanoids, each with subtly different biological activity. Leukotrienes, another family of biologically active substances, are synthesized from the same precursors as prostanoids, but are structurally different from prostanoids. The collective name for prostanoids and leukotrienes is eicosanoids.

Arachidonic acid and the other prostaglandin precursors are not normally found in the free state in cells, but occur bound at 2-position of phospholipids. Lands and Samuelsson (94) demonstrated that the release of precursor acids from phospholipids must occur before the formation of eicosanoids. This is the primary rate-limiting step in the synthesis of these active metabolites.

Phosphatidylcholine and phosphatidylethanolamine have been known to incorporate polyunsaturated fatty

acids at c-2 of the glycerol moiety, and are favored as sources of prostaglandin precursors through the action of a phospholipase A2 (93). However, the long-known "Inositol effect," i.e., the rapid turnover of phosphatidylinositides in cells and tissues after a variety of stimuli (95,96), called attention to phosphoinosites as a possible source of the polyunsaturated fatty acid used in prostaglandin biosynthesis (97).

In response to various stimuli (mechanical, hormonal, and coagulation factors) arachidonic acid is released from cell phospholipids by the action of phospholipases (Figure 6) (98). It is subsequently reacylated back into the phospholipids or is metabolized via the cyclooxygenase and/or lipoxygenases pathways to the eicosanoids characteristic of the given type of cell and biological response (98). The first pathway involves the enzyme cyclooxygenase, which oxygenates arachidonic acid to the prostaglandin endoperoxides--PGG2 and PGH2. These endoperoxides are then converted to the various prostaglandins, thromboxanes, and prostacyclins.

Cells like platelets contain both cyclooxygenase and 12-and 15-lipoxygenases. The most important platelet cyclooxygenase product, derived from AA, is thromboxane A2 (TXA2) (Figure 6). Thromboxane synthetase in platelets converts PGH2 to TXA2, which is a powerful

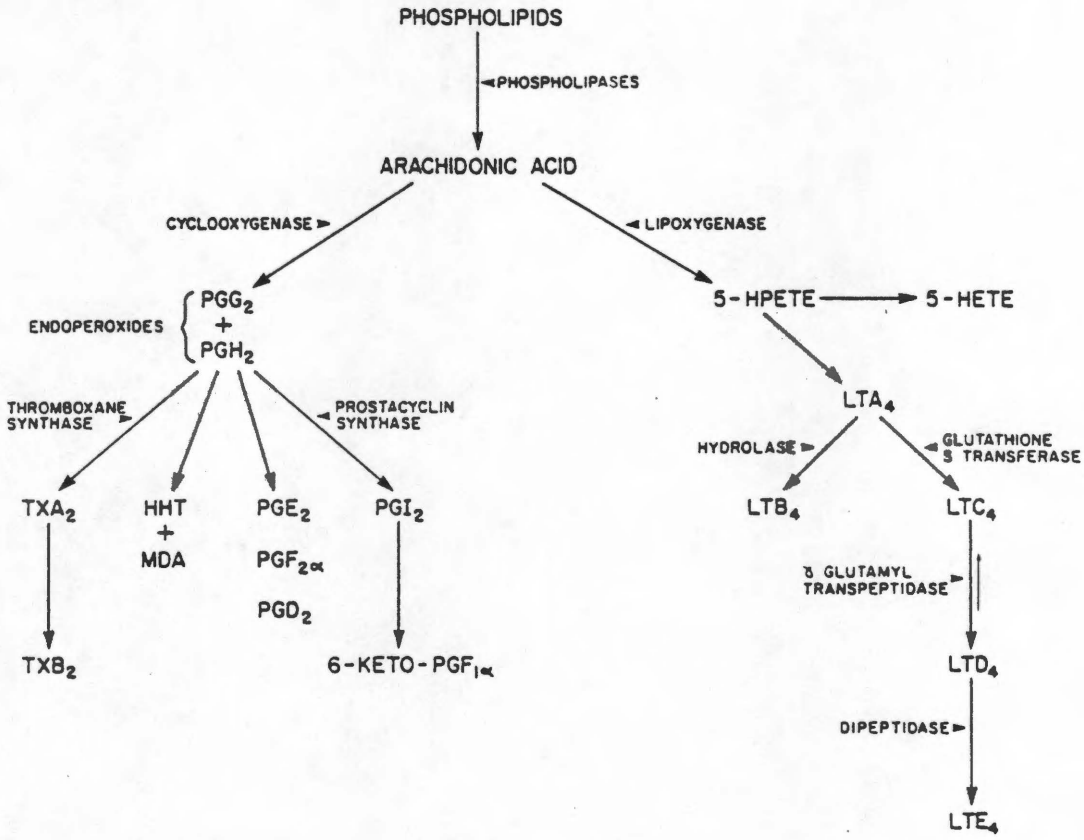


Figure 6. Pathways of arachidonic acid metabolism. Adapted from: Cannon, P. J., 1984 (98).

vasoconstrictor. TXA2 also promotes platelet activation and release of their granular contents: adenosine diphosphate, serotonin, and platelet derived growth factor. However, thromboxane A3 (TXA3), which is synthesized from EPA, is far less proaggregatory than TXA2 and is a weak vasoconstrictor (98).

Vascular endothelial cells synthesize a series of vasodilatory prostaglandins, particularly prostacyclin (PGI2) (99). These cells have receptors for angiotensin II, bradykinin, histamine, and thrombin. All of the above substances activate phospholipases and release arachidonate to cyclooxygenase for conversion to the endoperoxides. PGH2 is further converted in endothelial cells to PGI2 by prostacyclin synthetase (Figure 6). PGI2, the major arachidonic acid product of endothelial cells, is a potent vasodilator of the coronary and other vascular beds; it also inhibits platelet aggregation by stimulating adenylate cyclase and elevating platelet levels of cyclic adenosine monophosphate (cAMP) (98). Dietary EPA is transformed *in vivo* to prostacyclin I3 (PGI3), which is as active as the vasodilatory and antiaggregatory PGI2 (100,101) . Thus its actions are opposite to those of TXA2. Endothelial cells synthesize lesser amounts of PGE2, also a vasodilator, and PGF2x, which promotes venoconstriction (98).

The second pathway of arachidonic acid metabolism involves lipoxygenases that attack the various double bonds and form several unstable compounds known as hydroperoxy-arachidonic acids. Recently, Samuelsson (102) identified the products of arachidonate metabolism by studying the activity of lipoxygenase enzymes in polymorphonuclear leukocytes, macrophages, eosinophils, and mast cells.

The enzyme, 5-lipoxygenase, converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acid, which then is converted to the corresponding 5-hydroxyacid or to a family of compounds containing a conjugated triene structure, the leukotrienes: LTA4, LTB4, LTC4, LTD4 and LTE4. LTB4 appears to be important in inflammatory conditions; it is a powerful chemoattractant of polymorphonuclear white blood cells and promotes the transvenular movement of white cells and fluid (102,103). LTB4 also mobilizes calcium in polymorphonuclear leukocytes and induces the discharge of lysosomal contents and the formation of superoxides. The peptide-containing leukotrienes LTC4, LTD4, and LTE4 collectively account for the biological activity of slow-reacting anaphylaxic substances and are major mediators of the bronchial contraction observed in allergen response (102,103).

Eicosanoids are not stored in cells, but are released immediately after biosynthesis (94). Many eicosanoids are biologically active and act locally in the immediate microenvironment as cellular messengers. Frequently, they function to amplify or inhibit other biological responses, such as the action of a hormone (94). The eicosanoids also appear to be physiologic and pathologic mediators in a variety of clinical conditions, such as inflammation, immune responses, thrombosis, control of tissue perfusion, and atherosclerotic cardiovascular disease (94,102,103).

Pharmacological and Dietary Modulations on the Biosynthesis of Eicosanoids

It has been apparent for many years that numerous clinically important diseases, such as cardiovascular disease, are associated with severe alterations in eicosanoid metabolism (98). Because the active eicosanoids are rapidly inactivated by subsequent metabolism, the speed of the rate-limiting biosynthetic reaction can easily influence the steady-state level of active eicosanoid and its pathophysiological impact (99). Many successful therapeutic (Figure 7) and dietary interventions in eicosanoid metabolism have been established during the past three decades (104).

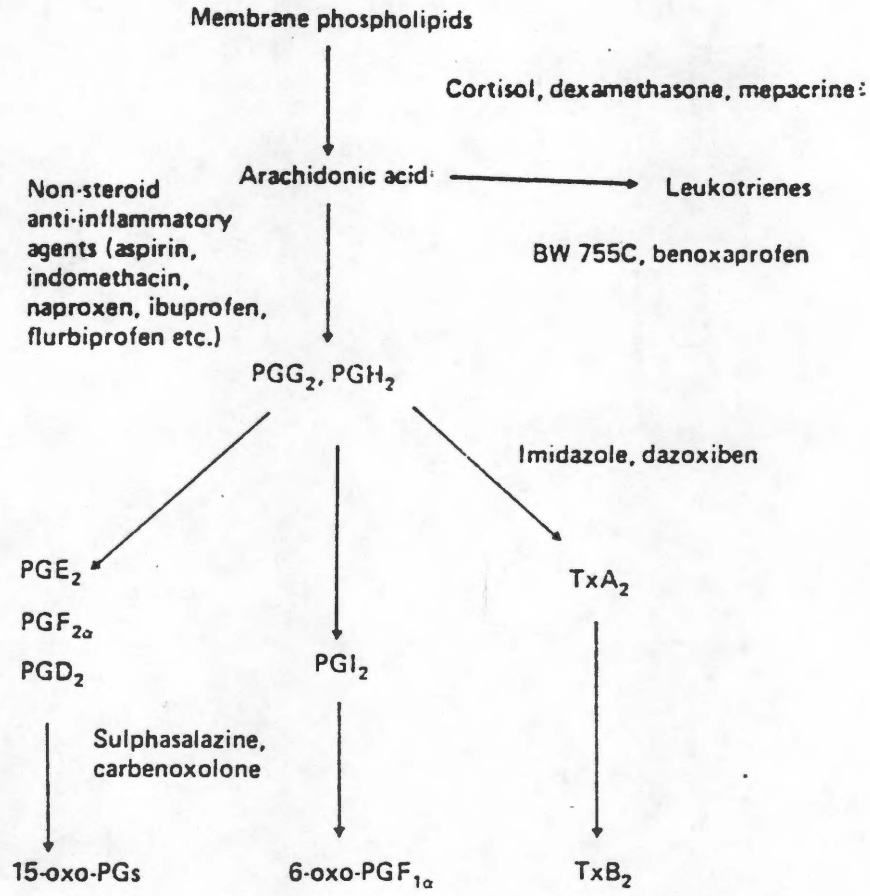


Figure 7. Effect of drugs on eicosanoid (prostanoid and leukotriene) biosynthesis. Adapted from: Moore, P. K., 1985 (104).

Pharmacological Modulation

The most important group of drugs which prevent phospholipase A2 activity are the corticosteroids (Figure 7). They are extremely valuable drugs, but have several harmful side effects, including peptic ulcer, osteoporosis and a generalized increase in susceptibility to infection (104). Corticosteroids exert most of their anti-inflammatory action by indirectly preventing phospholipase activity, as the general action of all steroids, and thereby stop the formation of inflammatory prostanoids and leukotrienes (104). Three groups of investigators (105-107) demonstrated that glucocorticoids induce synthesis and release of antiphospholipase proteins (macrocortin, lipomodulin, renocortin), thereby inhibiting the phospholipase A2 activity.

Drugs which inhibit cyclooxygenase enzyme activity are usually referred to as non-steroidal anti-inflammatory drugs (NSAID) (Figure 7). Aspirin (acetylsalicylate) is the prototype of all NSAID, and was reported by the Reverend Edmund Stone (104) of the Royal Society in 1763. The elegant experiments of Roth and Siok (108) elucidated how aspirin covalently acetylates and inactivates a serine at the active site of cyclooxygenase in all type of cells. Another often used NSAID, acetaminophen (paracetamol), is a potent

antipyretic and analgesic agent, but it is more active on brain cyclooxygenase than on peripheral tissues (109).

Aspirin has been used in clinical trials to treat patients with thrombotic disorders. In two randomized trials of large doses of aspirin in patients with transient cerebral ischemic attacks, aspirin significantly lowered the incidence of stroke or death by 42 and 31 percent, respectively (110). In another study (111) of 1266 veterans with unstable angina, aspirin reduced by 51 percent the rate of infarction or cardiac death in treated patients. The most frequently observed side effects of NSAID are ulceration and bleeding from the gut, renal damage and bronchoconstriction (104). Since prostanoids formed naturally in the body prevent formation of gastrointestinal ulcers, dilate the renal vasculature (thus protecting the kidney from being damaged by ischemia) and dilate the airways, it has been proposed that all of these side effects are due to inhibition of prostanoid biosynthesis (112,113).

Dietary Modulation

The discovery that Greenland Eskimos have a low incidence of myocardial infarction and ischemic heart disease prompted several studies of the effect of

dietary fat on polyenoic acid metabolism and the beneficial effects of such diets on cardiovascular physiology. Several investigations suggested that subjects whose diet consisted of fish as the major source of nutrients had a low risk of cardiovascular disease (82,114-116).

Fish fats are rich in docosahexaenoic acid (DHA, 22:6ω3), docosapentaenoic acid (DPA, 22:5ω3), and EPA (117). These polyenoic acids, added exogenously or released *in vivo* at the cellular level may induce inhibition of fatty acid metabolism or offer some beneficial effect against the risks of cardiovascular disease in several ways. First, they may exert their effect through vasoactive metabolites generated by cyclooxygenase and lipoxygenase pathways. For instance, formation of a potent prostacyclin (PGI3) and not so potent thromboxane (TXA3) by conversion of EPA could provide antithrombotic effects (117).

A second mechanism to explain the beneficial effect of polyenoic acids is the generation of cyclooxygenase inhibitors through conversion of the substrate by lipoxygenase. DHA, for example, has been shown to form two different hydroxy acids by platelet lipoxygenase (118). Several recent studies have suggested that the hydroperoxy acids of polyenoic acids are potent inhibitors of platelet function (119-121). If the

hydroperoxy acids of EPA, DHA and DPA are potent inhibitors of platelet function, then the release and metabolism of these fatty acids in sufficient quantity may offer protection against the contribution of platelets to thrombotic disease.

Finally, all of these fatty acids may co-exist in membrane phospholipids. Simultaneous release of EPA, DHA and DPA may inhibit significantly the conversion of AA, since all four fatty acids compete for the available heme sites of cyclooxygenase instead of just one, even though some of these fatty acids are poor substrates for the enzyme. In conclusion, if sufficient quantities of these fatty acids are incorporated, released and metabolized by the cells, the fatty acids by themselves or through their metabolites may exert inhibitory effects and thereby alter AA metabolism.

Supplemental methionine has been shown to raise SAM concentration in the liver of rats (1). Since SAM has been proved to be an analgesia and a cyclooxygenase inhibitor, it would therefore mimic the action of aspirin in lowering the prostanoid formation and preventing cardiovascular diseases in humans. Thus, this investigation set forth to elucidate the effect of supplemental methionine upon the cyclooxygenase activity

and upon the incorporation of long chain polyunsaturated fatty acids into tissue phospholipid.

CHAPTER III

EXPERIMENTAL PROCEDURE

General Outline of Research

Based on previous investigations and the literature evidence cited earlier, this study was initiated to assess the effect of methionine-supplemented versus cysteine-supplemented diets upon the catabolism and uptake of long chain polyunsaturated fatty acids--arachidonic acid (AA) and eicosapentaenoic acid (EPA). Two separate experiments were conducted. In one, [1-**14**C] AA in corn oil was administered by stomach tube to each animal before sacrificing. In the second experiment, [1-**14**C] EPA was used instead of [1-**14**C] AA. In both experiments, cyclooxygenase enzyme activity in rat liver microsomes was measured. Radioactive **14**C-phosphatidylinositol fractions in various tissue lipids were also quantitated by measuring radioactivity using Beckman LS-100C Liquid Scintillation system (Beckman Instruments, Inc., Irvine, CA).

Finally, to elucidate the finding of organ specificity from preliminary study with respect to the uptake of AA and EPA into phosphatidylinositol fraction, the activities of brain and liver fatty acyl-CoA synthetases were measured to obtain apparent Michaelis-Menten Constants (Km) for AA and EPA.

Dietary Modulation

For each of the two experiments, three groups of 15 male Sprague-Dawley rats were randomly assigned and housed individually in wire bottom stainless steel cages. The animals were fed one of three prepared diets for 17 days. Whittle and Smith (122) demonstrated that metabolic alterations would be detected within a period of two weeks when rats were fed the diets shown in Table 1.

All diets contained 15 percent casein and 0.02 percent inorganic sulfate. One group of animals was fed a diet supplemented with 0.505 percent cysteine, while a second group was fed a diet supplemented with 0.625 percent methionine. A control group was fed unsupplemented diets. Diet was given *ad libitum* and tap water were freely available to all animals.

The rats used to study the apparent Km for AA and EPA of tissue fatty acyl-CoA synthetases were fed standard chow and tap water *ad libitum* until the day of sacrifice.

TABLE 1

COMPOSITION OF DIETS

Component	Supplemented with		Control
	Methionine	Cysteine	
	g/100g		
Casein[1]	15	15	15
Sucrose	30	30	30
Cornstarch	30	30	30
Vegetable shortening[2]	6	6	6
Corn oil[3]	2	2	2
Basic salt mix[4]	1.34	1.34	1.34
$CaCO_3$	1.32	1.32	1.32
$CaSO_4.2H_2O$	0.04	0.04	0.04
Methionine	0.625	---	---
Cysteine	---	0.505	---
Vitamin mix[5]	2	2	2
Nonnutritive bulk[6]	11.68	11.80	12.3

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2]Crisco, Procter and Gamble, Cincinnati, Ohio.

[3]Mazola, Best Foods, Englewood Cliffs, New Jersey.

[4]1.34g equals in mg: 92.8 $MgCO_3$, 207.0 NaCl, 336.0 KCl, 636.0 KH_2PO_4, 61.5 $FePO_4.2H_2O$, 0.2 KI, 0.3 NaF, 1.8 $AlK(SO_4)_2.12H_2O$, 2.1 $Cu(C_2H_3O_2).H_2O$, 1.2 $MnCl_2.6H_2O$.

[5]Nutritional Biochemicals Corporation, Cleveland, Ohio. Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamin hydrochloride 1.0, riboflavin 1.0, niacin 4.5, p-amino-benzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B_{12} 0.00135, -tocopherol 5.0, vitamin A 9 x 10^5 units, vitamin D 1 x 10^5 units, and sufficient glucose to make 1 kg.

[6]Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

Preparation and Administration of Radioactive Test Solutions

A mixture of 1.18 uCi [1-**14**C] AA and 20 mg AA (or 0.625 uCi [1-**14**C] EPA and 25mg EPA) in 1/2 ml corn oil for each animal was prepared 30 minutes before using in each experiment. The test solution was mixed thoroughly and administered to the animals by stomach tube five hours before sacrificing. Respective diets and tap water were provided throughout the five hour period.

Animal Sacrifice and Tissue Preparation

Rats were stunned by a blow to the back of the head and decapitated with sharp shears. The brain, heart, lung, kidney and spleen were removed quickly and frozen until analysis of the phosphatidylinositol fraction was performed. Immediately after excision of the liver, the whole tissue were placed in ice-cold 1.15% KCl buffer solution for homogenizing. A 1:4 (w/v) liver homogenate was carried out on ice using a Thomas glass tissue homogenizer (Arthur H. Thomas Co., Philadelphia, PA) with a motor-driven teflon pestle.

Preparation of Liver Microsome

Cyclooxygenase has been purified from bovine vesicular gland microsomes by Miyamoto et al. (123) and described as a microsomal complex of enzymes. Therefore,

a preparation of liver microsomal fraction is necessary for assay of this enzyme. Liver homogenate was centrifuged at 10,000 x g for 25 minutes at 4°, using a 30 type rotor in a L5-50 Beckman centrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). The resultant supernatant fluid was subsequently centrifuged at 100,000 x g for 60 minutes at 4°, using a 50-Ti type rotor in a L5-50 Beckman centrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA), to obtain the liver microsomal fraction. The mitochondria and nuclei were transferred into labeled vial and frozen until the phosphatidylinositol fraction analysis.

The liver microsomal pellet was resuspended in 2ml of cold 100mM-Tris/HCl buffer to give a final protein concentration of 0.1-1 mg/ml in the enzyme assay mixture.

Determination of Cyclooxygenase Enzyme Activity

Cyclooxygenase activity was measured by the thiobarbituric acid (TBA) color reaction of malonaldehyde (MDA) formed from the cyclooxygenase pathway (124). PGG_2 and PGH_2 (or PGG_3 and PGH_3), the products of the reaction from AA (or EPA), give rise to MDA in the presence of heme and reduced glutathione (GSH) (125,126). The indirect measurement of MDA has been widely used to reflect the cyclooxygenase activity

in biological materials such as stimulated platelets (127). The TBA analysis has the advantage that the principal reactant, MDA, is a water-soluble substance formed or released upon heating the sample in an acid medium, and the intensity of color of the red pigment formed in the TBA reaction is so great that the method offers great sensitivity.

The assay procedure was performed according to Boopathy and Balasubramanian (128) with slight modification. The assay mixture contained 1 ml of 200 mM-Tris/HCl, pH 8.0, 0.2 ml of 50 mM-GSH, 0.2 ml of 50 uM-hemoglobin, 3 ul of 0.5 mM-AA, 0.497 ml of distilled H2O and 0.1 ml of enzyme. The reaction was started by the addition of AA and terminated after one minute of incubation at 27□ by the addition of 0.4 ml of 100% (w/v) trichloroacetic acid (TCA) in 1 M-HCl. After thorough mixing, 0.4 ml of 1% (w/v)-TBA solution was added and the mixture was heated in a boiling-water bath for 20 minutes. After cooling to room temperature, the final assay mixture was transferred to a centrifuge tube and briefly centrifuged at low speed (<1000 x g), using an International Model V, size 2 centrifuge with 241 rotor (International Equipment Co., Boston, MA). The absorbance of the clear supernatant fluid was measured in a Beckman spectrophotometer at 532 nm. In the control reaction mixture, the cofactor hemoglobin was omitted at

the time of incubation and added after the addition of TCA. Alternative controls were run when AA or enzyme was added at the end of incubation period.

One unit of enzyme activity was defined as one nmole of MDA formed/min. The amount of MDA formed was calculated from a standard curve of MDA obtained by periodate oxidation of 2-deoxyribose (129).

The amount of protein in the liver microsome preparation was determined by a simplified method of quantitating protein using the biuret and phenol reagents as described by Ohnishi and Barr (130). The Biuret reagent was prepared as follows: 1.5 g of $CuSO_4 \cdot 5H_2O$ and 6.0 g of $NaKC_4H_4O_6 \cdot 4H_2O$ were dissolved in approximate 500 ml of water. Then, 300 ml of freshly prepared, carbonate-free 10% (w/v) NaOH (30 g NaOH dissolved in 300 ml of pre-boiled water) was added to the first mixture with constant stirring. The final mixture was diluted to one liter, which was called the "Biuret reagent". The "protein reagent" was further prepared by mixing the Biuret reagent with 2.3% (w/v) Na_2CO_3 (1:7, v/v).

One-half ml of the liver microsomal protein was diluted with 4.5 ml of 0.5 N NaOH to obtain a range of 0.1-0.5 mg protein in the sample. To 0.8 ml of the sample was added 3.2 ml of the protein reagent. The mixture was quickly vortexed and allowed to stand at

room temperature for 10 minutes. Then, 0.1 ml of 2 N Folin-Ciocatleau phenol reagent was added while the mixture was vortexed again. After the final mixture was set at room temperature for 30 minutes, the absorbance was measured at 750 nm against a blank. Bovine serum albumin (139.2 ug/0.8 ml) was used as the standard. The amount of protein present in the samples was calculated by comparing it with known amount of protein in the standard. The enzyme activity was expressed as unit/mg protein/minute.

Tissue Lipid Extraction

Lipids from brain, heart, lung, spleen, kidney, and liver mitochondria and nuclei fractions were extracted and purified by the method of Folch et al. (131).

The excess fat and other materials of frozen tissue sample were removed completely. Tissue was weighed and recorded before extraction of lipids began. The tissue or tissue fraction (liver mitochondria and nuclei) was homogenized with 2:1 chloroform-methanol mixture (v/v) to a final dilution five-fold of the weight of tissue sample to obtain a maximal lipid extraction (131). For brain or tissues of similar consistency, a Bronwill Biosonik Sonicator (Bronwill Scientific, Rochester, NY) set at maximum power and maximum transducence was used to homogenize the tissue with the proper amount of

chloroform-methanol mixture. Tougher tissues such as heart were cut up with shears before sonication.

The homogenate was filtered through Whatman No. 1 filter paper into a labeled vial and washed thoroughly with 5 ml chloroform-methanol. The upper layer was removed by aspiration and a small volume of the chloroform layer was removed also to ensure complete removal of the top layer. Subsequently, the vial was set under a fume hood until the chloroform was completely evaporated. The residue was resuspended in 0.1 ml of chloroform. Quantitative analysis of radioactive phosphatidylinositol (PI) in the concentrated lipid extract was performed by thin layer chromatography (TLC) (132).

Analysis of Radioactive Phosphatidylinositol Fraction

A silica gel G TLC plate (20 x 20 cm, thickness of gel-250 um) was first scraped so that a 1 cm clear margin of plate was produced along the sides and top of the plate. Two 2.5 cm wide lanes on both sides were then made parallel to the sides of the plate. The plate was activated in an oven at 120° for one hour. As soon as the plate was completely cool, 0.01 ml of standard L-PI from bovine liver (Sigma Chemical Co., St. Louis, MO) was spotted about 1.5 cm from the bottom of the plate on both side lanes for reference, with

respect to migration of phosphatidylinositol fraction during ascending chromatography. Thereafter, 0.03 ml of the concentrated lipid extract was spotted as a narrow streak in the middle section and 1.5 cm from the bottom of the plate. The lipid was applied with a Hamilton microliter syringe (Alltech Associates, Inc., Deerfield, IL).

Once six spotted plates were dried, they were immediately and carefully positioned, bottom end first, in a multiplate rack (American Scientific Products, Stone Mountain, GA). The rack was then placed in a closed, glass developing tank (27 x 7 x 27 cm) containing chloroform/methanol/glacial acetic acid/water (175:105:28:14, v/v in ml). The developing tank was saturated with the developing mixture vapor by inserting two pieces of Whatman No. 1 filter paper before rack insertion. The solvent was allowed to migrate to about 0.5 cm of the top of the plate. The time required for plate development was usually two hours. The plate was then removed from the tank and allowed to dry under a fume hood at room temperature.

The plate was placed in a closed chamber containing crystals of iodine. Using iodine vapor as a detector, PI was identified against the reference standard. After about 20 minutes exposure to the vapor, narrow bands of phospholipids appeared as yellow stripes. After the

plate was taken out of the iodine chamber, the colored stripe representing the PI was scraped directly into a labelled vial. As soon as 10 ml of 2,5-diphenyloxazole (PPO) in toluene (12 g/L) was added and thoroughly mixed, the vial was placed in the liquid scintillation spectrometer for radioactive counting. The amount of radioactive PI was expressed as CPM/g wet tissue weight.

Assay of Long Chain Fatty Acyl-CoA Synthetase

The assay of long chain acyl-CoA synthetase was measured by a modification of the procedure of Tanaka et al. (133), which is based upon an isotopic assay. The rate of [1-**14**C] arachidonoyl (or [1-**14**C] eicosapentaenoyl) CoA formation from [1-**14**C] AA (or [1-**14**C] EPA) was determined by measuring radioactivity in the aqueous phase after extraction of the unreacted [1-**14**C] AA (or [1-**14**C] EPA) from the assay mixture.

Brain and liver homogenates were obtained by homogenizing tissues with four volumes of a buffer solution containing 0.5 M Tris/HCl, pH 8.0, 20 mM monobasic phosphate, and 5 mM 2-mercaptoethanol. The homogenate was subjected to centrifugation at 10,000 x g for 25 minutes at 4°, using a 30 type rotor in a L5-50 Beckman centrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). The supernatant fluid

was saved and kept in ice until the assay procedure began.

A 0.4 ml assay mixture contained the following reagents: 0.08 ml of 0.5 M Tris-HCl, pH 8.0; 0.004 ml of 0.5 M dithiothreitol; 0.03 ml of 2 M KCl; 0.04 ml of 0.15 M $MgCl_2$; 0.04 ml of 0.1 M adenosine triphosphate (ATP); 0.02 ml of 20 mM coenzyme A (CoA); 0.04 ml of 10 mM potassium [1-^{14}C] AA (or [1-^{14}C] EPA) (0.2 Ci/mol), in 16 mM Triton X-100 (Sigma Chemical Co., St. Louis, MO); and 0.146 ml of enzyme (brain or liver supernatant). A mixture (0.38 ml) containing all ingredients except CoA was preincubated at 35° in a water bath (Eberbach Corp., Ann Arbor, MI) for one minute. The reaction was started by adding 0.02 ml of 20 mM CoA. After incubation at 35° for two minutes, the reaction was terminated by adding 2.5 ml of a mixture containing isopropanol, n-heptane, and 1 M H_2SO_4 (40/10/1, v/v/v). Then, 1.5 ml of n-heptane and 1 ml of H_2O were added and thoroughly mixed; the mixture was then transferred into a centrifuge tube for subsequent centrifugation at 1,000 x g for 10 minutes at room temperature. The upper layer, containing the unreacted [1-^{14}C] AA (or [1-^{14}C] EPA), was removed by suction, while the lower layer (aqueous phase) was washed twice with 2 ml of n-heptane, each time discarding the upper layer. Then, a 1 ml sample of the lower layer was

assayed for radioactivity by scintillation counting with 10 ml 2,5-diphenyloxazole (PPO) in toluene (12 g/L) and 6 ml ethylene glycol monoethyl ether.

One unit of acyl-CoA synthetase activity was defined as the amount which catalyzed the formation of one uM of [1-**14**C] arachidonoyl CoA (or [1-**14**C] eicosapentaenoyl CoA) per minute under the assay conditions used. Enzyme protein was determined by the method of Ohnishi and Barr (130) with bovine serum albumin as a standard. The specific activity was expressed as uM/mg protein/minute.

In order to calculate acyl-CoA synthetase apparent Km for AA or EPA, a series of diluted concentrations of the substrates was prepared by diluting the original substrate concentration (0.2 uM) with Triton X-100 to 0.1, 0.04, 0.02 and 0.01 uM. A double reciprocal plot of activity versus substrate concentration for brain and liver acyl-CoA synthetases was plotted to obtain the apparent Km.

Statistics

Experimental data were analyzed by using analysis of variance (ANOVA). When F-tests were significant ($p<0.05$), comparisons were made using Duncan's new multiple range test to determine which pairs of means were significantly different ($p<0.05$) (134).

CHAPTER IV

RESULTS

Effect of Methionine-Supplemented Versus Cysteine-Supplemented Diets on Cyclooxygenase Enzyme Activity in Rat Liver Microsomes

The data in Table 2 show the units of cyclooxygenase activity of liver microsomes of rats fed the three diets. These data present a significant decrease in enzyme activity in the liver microsomes from rats fed the methionine-supplemented diet. The decrease in enzyme activity is significant when compared with that of rats fed the cysteine-supplemented diet, but it is not significant when compared with that of rats fed the control diet. There was a statistically significant difference in enzyme activity between cysteine-supplemented and control groups with respect to the administration of [1-**14**C] AA. However, no statistically significant difference was observed in the enzyme activity between these two groups upon the administration of [1-**14**C] EPA. Furthermore, there was no significant difference in the the cyclooxygenase activity within any dietary group of animals with respect to the treatment of [1-**14**C] AA or [1-**14**C] EPA.

TABLE 2

Cyclooxygenase Activities in Rat Liver Microsomes of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-14C] AA[1]	[1-14C] EPA[1]
	unit/min/mg protein[2]	
Control	85.9 ± 16.3 (5)[a]	102.1 ± 24.0 (5)[3ab]
Cysteine-Supplemented	119.0 ± 26.2 (5)[b]	114.7 ± 22.5 (5)[b]
Methionine-Supplemented	75.9 ± 19.9 (5)[a]	75.2 ± 6.1 (5)[a]

[1]All animals were administered [1-14C] AA or [1-14C] EPA in corn oil by stomach tube five hours prior to sacrifice.

[2]Data are averages ± SEM expressed as unit of enzyme activity/min/mg of microsomal protein.

[3]Numbers in parentheses indicate number of animals per treatment.

[4]Values in a column not followed by the same superscript are significantly different ($p<0.05$).

Effect of Methionine-Supplemented Versus Cysteine-Supplemented Diets upon the Incorporation of [1-**14**C] AA or [1-**14**C] EPA into the Phosphatidylinositol Fraction of Various Tissues

The data presented in Tables 3 through 6 show no statistically significant differences due to the diet regimen in the incorporation of [1-**14**C] AA or [1-**14**C] EPA into the PI fraction of heart, lung, spleen and liver mitochondria and nuclei. The data in Tables 7 and 8 demonstrate a significant difference of [1-**14**C] EPA uptake into the PI fraction of brain and kidney between animals fed the methionine-supplemented diet and those fed the control diet.

However, a statistically significant difference ($p<0.005$) was observed with respect to the incorporation of either [1-**14**C] AA or [1-**14**C] EPA into the PI fraction of various tissues. The PI of heart tissue incorporated approximately three times more [1-**14**C] EPA than [1-**14**C] AA, whereas PI of spleen, brain, and kidney tissues took up nearly two times more [1-**14**C] EPA than [1-**14**C] AA. On the contrary, PI fraction of liver mitochondria and nuclei incorporated about 1.5-fold more [1-**14**C] AA than [1-**14**C] EPA.

TABLE 3

The Incorporation of [1-14C] AA and [1-14C] EPA into Phosphatidylinositol Fraction of Heart Tissue of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-14C] AA[1]	[1-14C] EPA[1]
	cpm/g of wet weight[2]	
Control	540.5 ± 165.7 (5)[3a]	1707.3 ± 385.0 (5)[a]
Cysteine-Supplemented	520.4 ± 100.7 (5)[a]	1653.2 ± 314.0 (4)[a]
Methionine-Supplemented	632.2 ± 110.5 (5)[a]	1846.0 ± 518.0 (5)[a]

[1]All animals were administered [1-14C] AA or [1-14C] EPA in corn oil by stomach tube five hours prior to sacrifice.

[2]Data are averages ± SEM expressed as cpm/g of wet tissue weight.

[3]Numbers in parentheses indicate number of animals per treatment.

[4]Values in a column not followed by the same superscript are significantly different ($p<0.05$).

TABLE 4

The Incorporation of [1-14C] AA and [1-14C] EPA into Phosphatidylinositol Fraction of Lung Tissue of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-14C] AA[1]	[1-14C] EPA[1]
	cpm/g of wet weight[2]	
Control	468.6 ± 159.0 (5)[3]a	1121.1 ± 295.2 (5)a
Cysteine-Supplemented	418.1 ± 172.5 (5)a	1130.0 ± 294.5 (4)a
Methionine-Supplemented	400.9 ± 50.6 (5)a	984.9 ± 294.4 (5)a

[1]All animals were administered [1-14C] AA (or [1-14C] EPA in corn oil by stomach tube five hours prior to sacrifice.

[2]Data are averages ± SEM expressed as cpm/g of wet tissue weight.

[3]Numbers in parentheses indicate number of animals per treatment.

[4]Values in a column not followed by the same superscript are significantly different ($p<0.05$).

TABLE 5

The Incorporation of [1-14C] AA and [1-14C] EPA into Phosphatidylinositol Fraction of Spleen Tissue of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-14C] AA[1]	[1-14C] EPA[1]
	cpm/g of wet weight[2]	
Control	1309.0 ± 124.0 (5)[3]a	2961.0 ± 636.0 (5)a
Cysteine-Supplemented	1252.0 ± 135.0 (5)a	2378.0 ± 584.0 (4)a
Methionine-Supplemented	1458.0 ± 298.0 (5)a	2904.0 ± 306.0 (5)a

[1]All animals were administered [1-14C] AA or [1-14C] EPA in corn oil by stomach tube five hours prior to sacrifice.

[2]Data are averages ± SEM expressed as cpm/g of wet tissue weight.

[3]Numbers in parentheses indicate number of animals per treatment.

[4]Values in a column not followed by the same superscript are significantly different ($p<0.05$).

TABLE 6

The Incorporation of [1-**14**C] AA and [1-**14**C] EPA into Phosphatidylinositol Fraction of Liver Mitochondria and Nuclei of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-**14**C] AA**1**	[1-**14**C] EPA**1**
	cpm/g of wet weight**2**	
Control	417.0 ± 84.5 (5)**3a**	241.4 ± 54.0 (5)**a**
Cysteine-Supplemented	348.8 ± 81.9 (5)**a**	253.3 ± 33.8 (4)**a**
Methionine-Supplemented	349.4 ± 55.8 (5)**a**	240.0 ± 48.7 (5)**a**

1All animals were administered [1-**14**C] AA or [1-**14**C] EPA in corn oil by stomach tube five hours prior to sacrifice.

2Data are averages ± SEM expressed as cpm/g of wet tissue weight.

3Numbers in parentheses indicate number of animals per treatment.

4Values in a column not followed by the same superscript are significantly different ($p<0.05$).

TABLE 7

The Incorporation of [1-**14**C] AA and [1-**14**C] EPA into Phosphatidylinositol Fraction of Brain Tissue of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-**14**C] AA**1**	[1-**14**C] EPA**1**
	cpm/g of wet weight**2**	
Control	597.0 ± 50.0 (5)**3a**	1285.4 ± 138.3 (5)**a**
Cysteine-Supplemented	579.0 ± 133.0 (5)**a**	1178.4 ± 81.2 (4)**ab**
Methionine-Supplemented	559.4 ± 74.1 (5)**a**	1054.2 ± 177.0 (5)**b**

1All animals were administered [1-**14**C] AA or [1-**14**C] EPA in corn oil by stomach tube five hours prior to sacrifice.

2Data are averages ± SEM expressed as cpm/g of wet tissue weight.

3Numbers in parentheses indicate number of animals per treatment.

4Values in a column not followed by the same superscript are significantly different ($p<0.05$).

TABLE 8

The Incorporation of [1-**14**C] AA and [1-**14**C] EPA into Phosphatidylinositol Fraction of Kidney Tissue of Control, Cysteine-Supplemented, and Methionine-Supplemented Rats

Animal Group	[1-**14**C] AA**1**	[1-**14**C] EPA**1**
	cpm/g of wet weight**2**	
Control	365.0 ± 53.7 (5)**3a**	1172.6 ± 187.5 (5)**a**
Cysteine-Supplemented	370.8 ± 109.4 (5)**a**	888.0 ± 71.7 (4)**b**
Methionine-Supplemented	429.6 ± 62.0 (5)**a**	801.4 ± 113.5 (5)**b**

1All animals were administered [1-**14**C] AA or [1-**14**C] EPA in corn oil by stomach tube five hours prior to sacrifice.

2Data are averages ± SEM expressed as cpm/g of wet tissue weight.

3Numbers in parentheses indicate number of animals per treatment.

4Values in a column not followed by the same superscript are significantly different ($p<0.05$).

The Apparent Km Constants of Brain and Liver Acyl-CoA Synthetases for Substrates--[1-**14**C] AA and [1-**14**C] EPA

Lineweaver-Burk plots in Figures 8 and 9 were prepared under standard assay conditions to obtain the maximal acyl-CoA synthetase activity. The apparent Km values for the reaction using either [1-**14**C] AA or [1-**14**C] EPA as substrates were calculated from the graphs of Figures 8 and 9. In the reaction using [1-**14**C] AA as a substrate, the apparent Km of brain and liver acyl-CoA synthetases for this substrate are approximately 4×10^{-8} M and 2×10^{-7} M, respectively. In the case of [1-**14**C] EPA, the respective apparent Km of brain and liver acyl-CoA synthetases are about 2.8×10^{-8} M and 3.3×10^{-7} M.

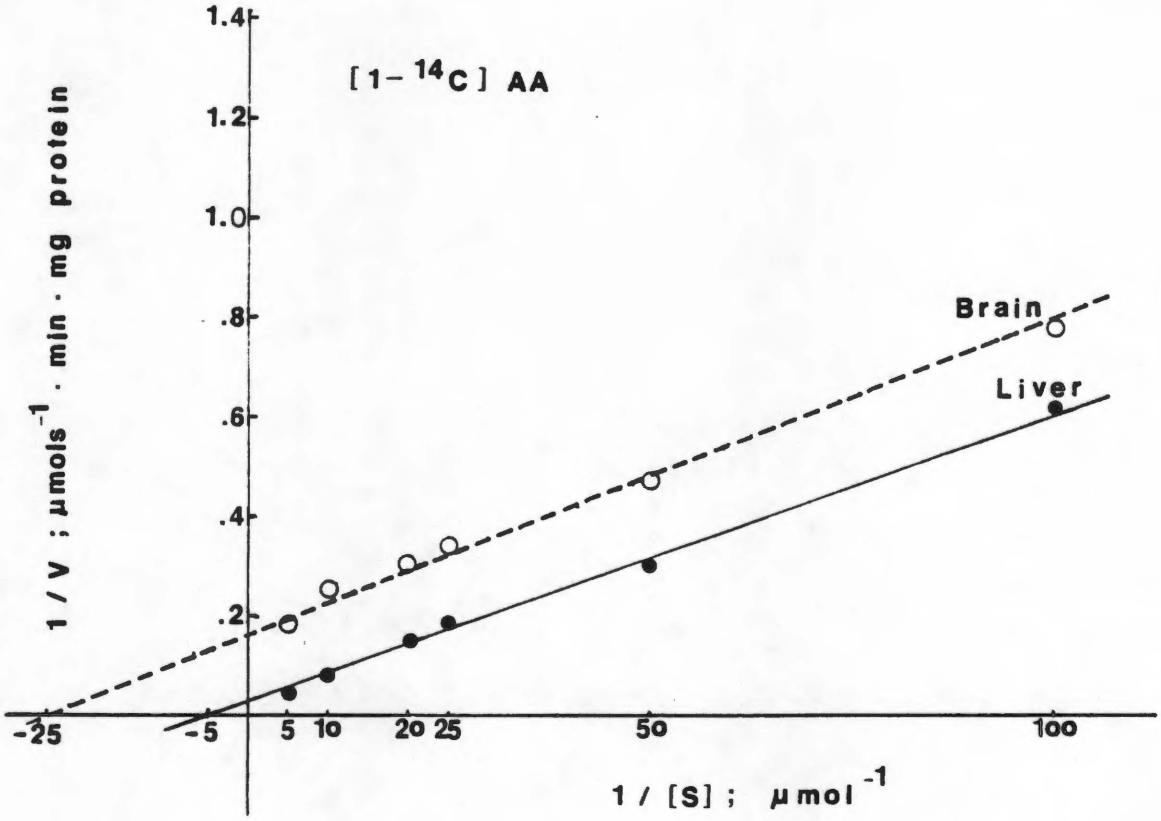


Figure 8. Typical Lineweaver-Burk (double-reciprocal) plots of acyl-CoA synthetase activity versus substrate ([1-^{14}C] AA) concentration for the brain and liver . Acyl-CoA synthetase in the supernatant of tissue homogenate was incubated for 1 minute with optimal concentrations of ATP and CoA, and various concentrations of [1-^{14}C] AA as indicated. Data points represent quadruplicate analyses of two animals.

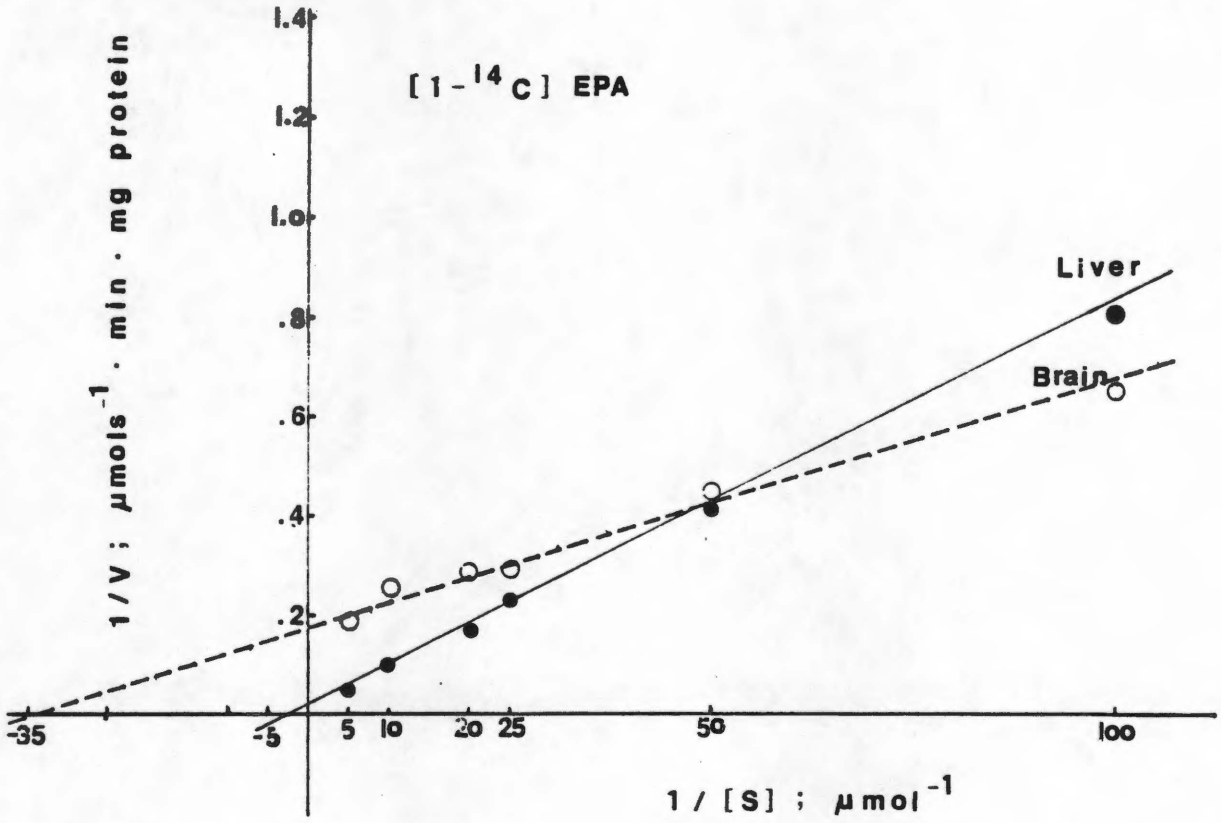


Figure 9. Typical Lineweaver-Burk (double-reciprocal) plots of acyl-CoA synthetase activity versus substrate ([1-^{14}C] EPA) concentration for the brain and liver . Acyl-CoA synthetase in the supernatant of tissue homogenate was incubated for 1 minute with optimal concentrations of ATP and CoA, and various concentrations of [1-^{14}C] EPA as indicated. Data points represent quadruplicate analyses of two animals.

CHAPTER V

DISCUSSION

Cyclooxygenase activity (Table 2) was significantly decreased ($p<0.05$) in the liver microsomes from rats fed the methionine-supplemented diet. There was a 34 percent decrease in cyclooxygenase activity with methionine supplementation compared to cysteine supplementation. S-Adenosyl-methionine (SAM) has been shown to inhibit cyclooxygenase activity (64). Also, previous investigations in this laboratory (1) have shown that rats fed diets supplemented with methionine have a two-fold increase in their liver concentrations of SAM compared to those rats fed a diet supplemented with cysteine. It is reasonable to assume, therefore, that the inhibition of cyclooxygenase activity reported in Table 2 is a reflection of the inhibition of cyclooxygenase activity, due to an increase in the tissue concentration of SAM.

SAM has been demonstrated to have analgesic and anti-inflammatory properties because of its action as an inhibitor of cyclooxygenase. Cyclooxygenase is a key enzyme required for the conversion of the long chain polyunsaturated fatty acids into the biologically active prostaglandins, prostacyclins and thromboxanes in all tissues.

Inhibition of cyclooxygenase activity is the basis for the anti-inflammatory action of most non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin. Because eicosanoids have been shown to have vascular and hemostatic actions, many studies have examined this possibility. In platelets, thromboxane A_2 is a major cyclooxygenase product synthesized from arachidonic acid. Thromboxane A_2 has highly potent vasoconstricting and platelet-aggregating effects--actions that are associated with diseases of the cardiovascular systems. Low doses of aspirin have been prescribed for patients with unstable angina, those who are stroke prone or those who are considered at risk for coronary occulsion.

This investigation demonstrated that 0.625 percent supplementation of dietary methionine fed to rats did raise the liver tissue concentrations of SAM to a sufficient level to inhibit cyclooxygenase in rat liver microsomes, thereby possibly mimicing the action of low dose of aspirin upon the integrity of the cardiovascular system. Therefore, it is attractive to speculate that supplemental methionine might be beneficial to unstable angina, stroke-prone and/or cardiac patients. Due to the analgesic properties of SAM (64), it is also attractive to speculate that a diet high in methionine might reduce symptoms in the chronic

pain patient and/or the muscle pain associated with a fever.

The incorporation of long chain polyunsaturated fatty acids into phospholipids occurs rapidly and is maintained under a dynamic equilibrium. This was evidenced from the data obtained (Tables 3-8) following the administration of [1-**14**C] AA and [1-**14**C] EPA by stomach tube to rats that were sacrificed 5 hours later. The radiolabeled long chain polyunsaturated fatty acids are metabolized through a sequence of events. First, [1-**14**C] AA or [1-**14**C] EPA is incorporated more rapidly into the 2-position of phospholipids than into the 1-position of phospholipids or triglycerides during fatty acid incorporation into chylomicrons in the intestinal mucosa (135). Then, the labeled phospholipids are removed from the hepato-circulation by selected regions of cells and become the metabolic pool for the synthesis of tissue phospholipids.

From the findings of this study, rat spleen tissue incorporated the greatest amount of [1-**14**C] AA or [1-**14**C] EPA into the phosphatidylinositol fraction (Table 5) compared to all the other tissue investigated. Heart tissue (Table 3) incorporated the second most, whereas phosphatidylinositol of the liver mitochondria and nuclei (Table 6) incorporated the last. The spleen removes products of red-cell degradation and other

foreign matter through phagocytosis by macrophages; [1-**14**C] AA or [1-**14**C] EPA might be recognized as a foreign substance by macrophages of the spleen. Consequently the spleen would have the highest quantity of the radiolabeled phosphatidylinositol.

The heart has been reported as one of the most responsive tissues to changes in dietary polyunsaturated fatty acids, especially those of 20 and 22 carbon chain lengths (136). Results of this study could probably explain the relative high uptake of AA and EPA into the phosphatidylinositol fraction of heart tissue lipid.

The liver is the primary organ for degradation of all substances. The removal and reutilization of the remaining labeled long chain fatty acids would result in the least amount of labeled phosphatidylinositol in liver mitochondria and nuclei fractions.

Based upon the cyclooxygenase data, it would be reasonable to assume that, if SAM inhibits cyclooxygenase and if phosphatidylinositol is the storage form of the C-20 polyunsaturated fatty acids, the phosphatidylinositol fraction from rats fed the methionine-supplemented diets would contain the highest level of radioactivity. However, in heart, lung, spleen and particulate fractions of liver evaluated for phosphatidylinositol radioactivity (Tables 3-6), there was no difference in the specific activity of

phosphatidylinositol among rats fed the control, cysteine-supplemented or methionine-supplemented diets. Furthermore, the observations of phosphatidylinositol radioactivity of brain and kidney tissues of rats administered [1-**14**C] EPA (Tables 7 and 8) demonstrated different phenomena. The specific activity of brain phosphatidylinositol of rats fed control diet was significantly higher than that of rats fed methionine-supplemented diet and was not significantly different from that of rats fed cysteine-supplemented diet (Table 7). On the other hand, phosphatidylinositol radioactivity in kidney tissue of rats fed control diet (Table 8) was significantly higher than those of rats fed both cysteine-supplemented and methionine-supplemented diets. The reason for these phenomena remains to be elucidated.

Moreover, there are other enzymes governing the release and metabolism of long chain fatty acids in tissue phospholipids that need to be considered. These include phospholipase A**2**, fatty acyl-CoA synthetase, acyl-CoA: lysophosphatide acyl transferase, acyl-CoA hydrolase and lysophospholipase.

A comparison of data in Tables 3-8 indicates that the heart tissue incorporated approximately three times as much [1-**14**C] EPA into the phosphatidylinositol fraction as [1-**14**C] AA. A lesser amount was incorporated

by the brain, lung, kidney and spleen tissues. In contrast, however, the particulate fraction of liver incorporated almost twice as much [1-**14**C] AA into its phosphatidylinositol as it did [1-**14**C] EPA.

In order to provide a partial enzymatic explanation for differences in the incorporation of [1-**14**C] EPA and [1-**14**C] AA into phosphatidylinositol in liver and in other tissues, the apparent Km of fatty acyl-CoA synthetase was determined for both [1-**14**C] AA and [1-**14**C] EPA in liver and brain. The results, in Figures 8 and 9, demonstrate that the apparent Km for AA is 4x10-**8** M in the brain and 2x10-**7** M in the liver, while the apparent Km for EPA is 2.8x10-**8** M in the brain and 3.3x10-**7** M in the liver. The approximate five-fold increase in the apparent Kms for EPA and AA in liver tissue compared to brain tissue would explain the decrease in specific activity of liver phosphatidylinositol compared to brain phosphatidylinositol. In addition, the ratio between the apparent Kms for EPA and AA is 0.7 in the brain and 1.65 in the liver. Thus the difference in Km between EPA and AA in the liver and brain tissues could explain the preferential incorporation of EPA into brain tissue phosphatidylinositol and AA into liver tissue phosphatidylinositol. However, the observation that the Vmax of acyl-CoA synthetase, Figures 8 and 9, for AA and

EPA is approximately five-fold higher in the liver tissue than in the brain tissue would tend to offset the effect of the higher apparent Km in the liver tissue, if both tissues contained saturating levels of the fatty acids. Thus it appears that the physiological concentration of long chain polyunsaturated fatty acids is not sufficient to saturate the fatty acyl-CoA synthetase and that the acylation and subsequent incorporation of these fatty acids into phosphatidylinositol is regulated by the Kms of the enzymes.

CHAPTER VI

SUMMARY

The test hypothesis of this investigation was that methionine-supplemented diets could possibly interfere with the metabolism of polyunsaturated fatty acids, thereby altering the biosynthesis of the active metabolites, prostanoids, and the profiles of tissue phospholipids. The postulated mechanism of action for this dietary regimen would be to increase the tissue concentration of the active methyl donor, S-Adenosyl-methionine (SAM) which is also called an activated methionine. SAM is a potent inhibitor of the cyclooxygenase enzyme which is important for the synthesis of biologically active prostanoids.

The results demonstrated that liver microsomes from rats fed the methionine-supplemented diet had a statistically significant decrease in cyclooxygenase activity. However, metabolic regulation of the polyunsaturated fatty acids metabolism is controlled by a host of enzymes and in a dynamic equilibrium state. Measurement of the incorporation of eicosapentaenoic and arachidonic acids into the phosphatidylinositol fraction of rat tissues demonstrated no difference among the rats fed the control, cysteine-supplemented, and methionine-supplemented diets.

The differential incorporation of polyunsaturated fatty acids into phosphatidylinositol fraction by various tissues was partially explained by the apparent Kms for both arachidonic and eicosapentaenoic acids of fatty acyl-CoA synthetase in liver and brain tissues. Many other factors and enzymes which regulate the metabolism of phospholipids and the mechanism of action of SAM need to be elucidated in the future research on the beneficial effect of a methionine-supplemented diet.

Therefore, one part of the null hypothesis is rejected and restated that supplemental methionine has an effect on the metabolism of polyunsaturated fatty acids through the cyclooxygenase enzyme pathway. The other part of the null hypothesis that supplemental methionine has no effect on the incorporation of polyunsaturated fatty acids into tissue phospholipids remains to be further investigated.

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