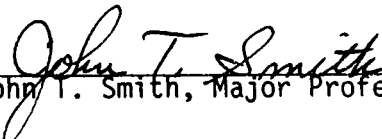
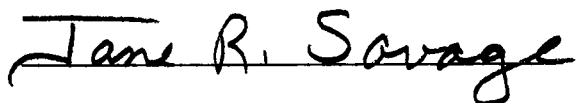


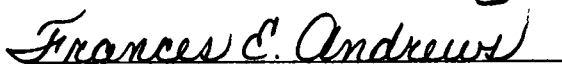
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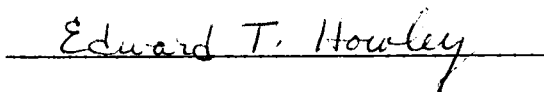
I am submitting herewith a dissertation written by Robert V. Acuff entitled "The Influence of Dietary Sulfur Upon Polyamine Biosynthesis in the Rat." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Home Economics


John T. Smith, Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the Council:


Vice Chancellor
Graduate Studies and Research

THE INFLUENCE OF DIETARY SULFUR UPON
POLYAMINE BIOSYNTHESIS IN THE RAT

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Robert V. Acuff

August 1982

3063178

To my parents, Bettye and Bob Acuff, who valued my education at all costs to themselves. To them I owe and dedicate this work.

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ABSTRACT

Thirty female and male Sprague-Dawley rats (175-200g) were randomly divided into six dietary groups of five animals per group and fed diets containing 15% of casein supplemented with cysteine (0.505%) or methionine (0.620%) and varying in the level of inorganic sulfate (0.0002, 0.02 and 0.42%) for a period of 17 days. The polyamines (putrescine, spermine and spermidine) and the enzymes controlling their biosyntheses were determined in tissue homogenates from the animals in each dietary group.

Rats fed the optimal level of inorganic sulfate (0.02% and supplemented with cysteine had increased L-ornithine decarboxylase (OD) activity in liver, kidney, and brain tissues and a decreased S-adenosyl-L-methionine decarboxylase (SAMD) activity in liver and brain tissues from male rats. Spermidine and spermine concentrations increased in the liver tissues of male rats fed the diet supplemented with cysteine.

S-adenosyl-L-methionine concentrations in the livers of cysteine supplemented animals fed the diets containing 0.02% of sulfate increased. But a decreased incorporation of ^{14}C -radioactivity from (methyl- ^{14}C) methionine into liver tRNA from female animals was observed.

The effect of dietary inorganic sulfate on ODC activity was inconclusive with either cysteine or methionine supplementation. Decreased or increased dietary inorganic sulfate increased S-adenosyl-L-methionine decarboxylase activity in liver, kidney, and brain tissue from male rats fed diets supplemented with methionine when compared to

those fed diets supplemented with methionine and containing the optimal (0.02%) level of inorganic sulfate. In a similar manner decreased or increased levels of inorganic sulfate increased spermine or spermidine concentrations in the liver of male animals fed diets supplemented with methionine. In male animals fed diets supplemented with cysteine, increasing inorganic sulfate levels increased incorporation of the methyl group from methyl ^{14}C -methionine into tRNA. Animals fed methionine supplemented diets had an increase in liver S-adenosyl methionine as dietary sulfate was increased.

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CHAPTER I

INTRODUCTION

The diet is probably the major factor in the delicate interplay between metabolic and biosynthetic processes of the body. The concept of dietary influences upon an organism has changed drastically in recent years; never before have scientists linked so many health and disease problems with dietary factors. This view has been a dramatic influence in the growing awareness of the physiological importance of diet. The identification of important dietary constituents to an animal is just beginning to be recognized as making an impact upon physiological processes at the cellular level. Among the dietary factors that influence physiological processes at the cellular level is sulfur nutrition. Unfortunately, the research related to the area of sulfur nutrition has not considered the importance of inorganic sulfate to an animal. Because the sulfur-containing amino acids, cysteine and methionine, can eventually contribute their respective sulfur moiety to the production of endogenous inorganic sulfate the importance of dietary inorganic sulfate has received little attention. Although data in this laboratory have, over a period of years, been collected to establish an optimal percentage (0.02%) of inorganic sulfate in the diet of a rat, the importance of dietary inorganic sulfate in the sparing of the sulfur-containing amino acids has been difficult to evaluate because the two sources of sulfur (inorganic and neutral) are interrelated.

One area of research that has been neglected in the evaluation of dietary sulfur is the role of methionine, cysteine, and inorganic sulfate in the formation and biosynthesis of the polyamines (putrescine, spermidine, and spermine). This area seems to have been overlooked with the one exception that methionine is essential for the formation of S-adenosyl-L-methionine (SAM) and that the decarboxylation of SAM supplies the propylamine groups for the biosynthesis of the polyamines--spermidine and spermine. In the absence of dietary cysteine or inorganic sulfate in the diet of an animal, methionine must supply all of the sulfur required by the animal. Because methionine is required for protein synthesis and, when activated, is the methyl donor in almost all transmethylation reactions, the omission or decrease of dietary cysteine or inorganic sulfate could increase the metabolism of methionine sulfur to meet the demands of an animal for other significant derivatives such as glutathione, mercapturic acids, and taurine. Investigations in this laboratory have documented the sparing effect of an optimal level of dietary inorganic sulfate upon the metabolism of methionine. Further investigations have revealed that the source of dietary sulfur could affect a number of enzymes involved in carbohydrate and lipid metabolism. The inequivalency of methionine and cysteine as dietary supplements was further substantiated.

If the alteration of dietary methionine decreased the formation of SAM or increased the metabolism of the sulfur moiety of methionine, the formation and biosynthesis of the polyamines could be altered. This possible alteration by diet is important to consider because the

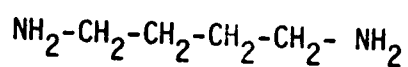
polyamines have been identified as stimulating growth in eukaryote cells, influencing protein biosynthesis, and having a potent physiological effect upon the metabolic processes of an organism. Therefore, the present investigation was designed to determine if the formation and biosynthesis of the polyamines could be altered through dietary manipulation of sulfur (neutral and inorganic) in the diet of a rat.

CHAPTER II

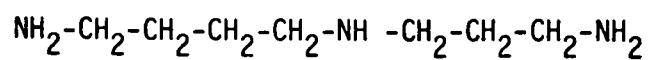
REVIEW OF LITERATURE

The polyamines (Fig. 1), putrescine, spermidine, and spermine are ubiquitous in nature. The polyamines are simple aliphatic amines that are polycationic in physiological fluids. Antoni van Lewenhoeck (1) discovered crystals of spermine phosphate in human semen in 1678. Spermine's structure was first elucidated in 1926 and remained a biological curiosity until efforts reopened the field of polyamine metabolism (1). Approximately 1500 papers had appeared from the time of of Lewenhoeck's initial report to 1970 dealing with the polyamines; in 1980 this number exceeded 3000 (2). Because of the diversity of research in the area of polyamine metabolism and their effects upon cellular function, the reader is referred to several sources for an in-depth review (1,3-5).

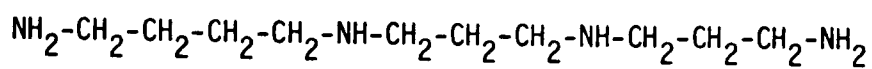
Interest in the regulation of polyamine biosynthesis in animal cells was stimulated in the 1960's when the polyamines were found to be associated with growth processes (6). The polyamines were first identified as growth factors in microbial organisms (7) followed by their identity in several animal tissues (8). The concentration of putrescine is lower in eukaryotes but spermine and spermidine are higher in concentration in eukaryotes in comparison to prokaryotes (9). The modification of tRNA by methylation is a well known control mechanism of protein synthesis. Spermidine has been found to elevate methylase activity and transfer methyl groups from



putrescine



spermidine



spermine

Figure 1. The chemical structures of the polyamines (putrescine, spermidine, and spermine).

S-adenosyl-L-methionine to tRNA molecules (10). The polyamines have a high affinity for nucleic acids (5) and interact stereo-specifically with DNA (11,12). In addition, the concentration of these amines and their biosynthetic enzymes are increased in proliferating tissues, usually preceding increases in DNA, RNA, and protein (5). There are four enzymes involved in polyamine biosynthesis: two decarboxylases and two synthases (Fig. 2).

Ornithine decarboxylase

The biosynthetic pathway for the polyamines has been determined (Fig. 2). In animal tissues, putrescine is derived by the decarboxylation of ornithine by ornithine decarboxylase (E.C.4.1.1.17). Ornithine decarboxylase (ODC) is the rate limiting enzyme in the formation of the polyamines (13) and has been identified as a key regulatory enzyme in the growth process (14). The enzyme is rapidly induced in response to a variety of growth stimulating factors such as trophic hormones, mitogens, partial hepatectomy, etc. (1,5,15).

The relationship of ODC activity to the conformation of its active site has not been defined probably due to its short half-life (10-20 min.); likewise the subunit structure and amino acid sequence need to be defined (4). The K_m of ODC, as prepared from Clostridium septicum is 4×10^{-3} M (16). Maudsley (17) has reported that the K_m of ODC for ornithine is typically 0.1 mM, whereas ornithine levels are 1.0 mM; therefore, it is unlikely that ornithine availability is a limiting factor in the formation of polyamines (17). ODC has an absolute requirement for pyridoxal-5-phosphate (17).

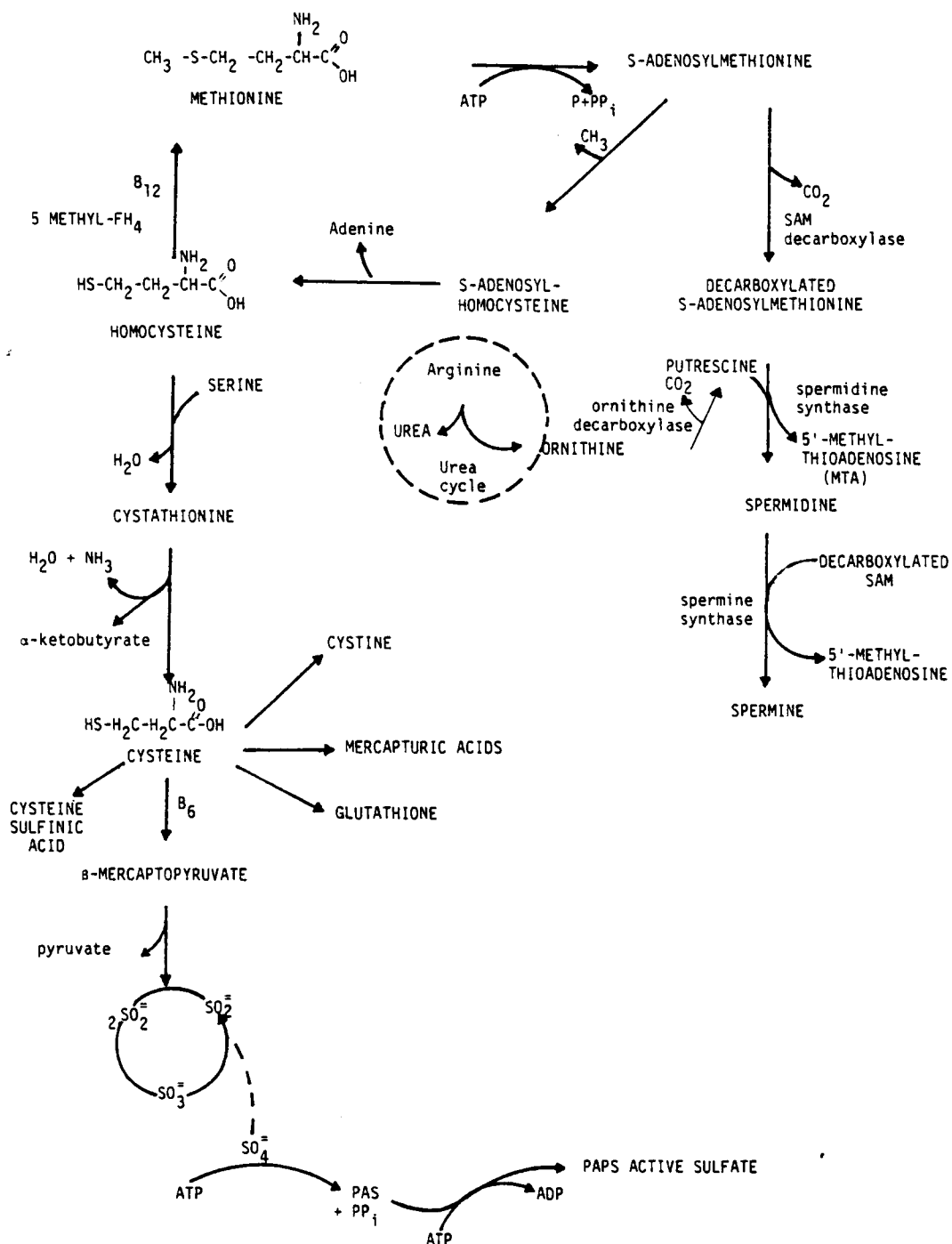


Figure 2. The metabolism of selected sulfur compounds and the biosynthesis of the polyamines.

There is substantial evidence in both normal and transformed cells, as well as tissues, that a major route of induction of ODC is via a cyclic-AMP-mediated cascade of events (18,19). This cascade includes the activation of cyclic-AMP-dependent protein kinase (E.C.2.7.1.37) and the phosphorylation of nonhistone chromosomal proteins related to the synthesis of new messenger RNA specific for ODC (19).

Although basal ODC activity is low in most tissues, marked increases occur with rapid tissue growth. Russell and Snyder (20) reported a "dramatic increase" in liver ODC activity after partial hepatectomy in rats which was found to be specific for the decarboxylation of ornithine. They found that ODC enzyme activity triples 1 hour after partial hepatectomy and was 25 times control levels (sham operated animals) after 16 hours. Russell et al. (21) reported the induction of ODC in adult and weanling rat liver in response to growth hormone. Other hormones that can induce ODC activity have been identified by Russell (22).

Fuller et al. (14), using Chinese hamster ovary cells, showed an increase in ODC activity in the G_1 phase (pre-DNA synthesis) of the cell cycle and a decreased activity as the cell progressed into the S phase (period of DNA replication), therefore indicating that ODC is a marker of G_1 progression. The inhibition of ODC activity is associated with an inhibition of cell replication (20).

Increases in ODC activity, and consequently polyamine biosynthesis, have been shown to be important factors in intestinal mucosal maturation and the recovery from a chemotherapeutical injury to the mucosa in rats (23). However, Bartolome et al. (24)

have shown that the inhibition of cardiac ODC by α -difluormethylornithine (DFMO) a potent, selective enzyme activated, irreversible inhibitor of ODC, did not prevent normal cardiac growth in mature rats. Therefore ODC activity may not be essential for normal growth of the heart in the adult (24).

Farwell et al. (25) and Russell (22), have indicated that the stimulation of ODC in liver and possibly other organs might have serious implications in the promotion of systemic tumors after exposure to environmental carcinogens. Russell (26) reported that 3,4-benzpyrene causes ODC induction in rat liver. Other carcinogens and drugs that induce ODC activity have been summarized by Russell (22). Farwell et al. (27) reported that dietary habits could cause a nutritional alteration of ODC activity in the liver and kidneys of experimental animals exposed to severe protein restriction for short periods followed by the ingestion of a meal high in protein. Feeding rats a cyclical regimen of protein-free and protein-containing (20%) diets provided a daily activation of ODC in response to the feeding of protein after 3 days feeding of a protein-free diet. Rozovski et al. (27) also investigated the ODC activity in rat liver during malnutrition (6% casein) and re-feeding of a 26% casein based diet. ODC activity increased above control levels in those animals that had been restricted, agreeing with the data of Farwell (25).

ODC is also stimulated by a variety of sulfhydryl compounds, notably dithiothreitol (29). Other thiol compounds that have an effect

on ODC activity included 2-mercaptoethanol, dihydrolipoic acid, 2,3-dimercapto-1-propanol, and reduced glutathione.

S-adenosyl-L-methionine decarboxylase and S-adenosyl-L-methionine

The second decarboxylase in the biosynthesis of the polyamines (Fig. 2) is S-adenosyl-L-methionine decarboxylase (E.C.4.1.1.50). As a key enzyme in the biosynthesis pathway of the polyamines, S-adenosyl-L-methionine decarboxylase (SAMD) acts upon S-adenosyl-L-methionine to form S-adenosyl-5'-deoxy-(5')-3-methylthiopropylamine (MTA), the donor of propylamino-groups for the conversion of putrescine into spermidine (29). Although this reaction occurs in bacteria (30), this review will be confined to the mammalian enzyme.

Tabor et al. (31) reported the incorporation of radioactive putrescine into spermidine in minced rat prostate, indicating that the polyamines are synthesized from putrescine in animal tissues. The mammalian enzyme system, like its bacterial counterpart, synthesizes S-adenosyl-L-methionine (SAM) from ATP and methionine as the first step in the formation of the polyamines spermine and spermidine (32). Putrescine, in contrast to the bacterial enzyme system, must be present for the decarboxylation of SAM by SAMD (29,33). Further, SAMD has been reported to require Mg^{2+} and has pyruvate as a prosthetic group (34).

Zappia et al. (35) have reported that varying the concentration of the substrate can affect the reaction rate in mammalian tissue. A maximal rate of decarboxylation by SAMD has been observed with 0.15 mM SAM, and $4 \times 10^{-5} M$ has been calculated for the K_m (35). The SAM concentration in various tissues has been estimated using isotopic dilution (36,37).

Since its discovery in 1952 by Cantoni (38), SAM has been reported to be the only biological methyl-donor in all methyl-transfer reactions, other than those yielding methionine and thymidine. The importance of SAM as the principal methyl-donor cannot be underestimated. The number of enzymatic reactions in which SAM functions either as substrate or modulator has been summarized (39). However, the main interest of this review is the formation of the "active propylamino-group" that results from the decarboxylation of SAM. The decarboxylation of SAM, as reported above, not only plays an important role in the formation of spermidine and spermine, but the two polyamines formed exhibit a profound influence upon certain biological methyl-group transfers, for which SAM is substrate (40).

SAMD has not received the same emphasis in the literature as has ODC. As reported above (34), pyruvate acts as the prosthetic group in SAMD and further differs from ODC in that putrescine is stimulatory upon the enzyme by lowering the K_m for the substrate and by partially protecting it against inactivation (33,41). Stoichiometric formation of spermidine, CO_2 , and MTA from putrescine and SAM have been reported (29,33). Although several species of bacteria accumulate MTA, this compound is not found in mammalian tissues indicating the presence of an enzyme that catalyzes the degradation of MTA (1). Pegg and Williams-Ashman (42) confirmed the existence of 5'-methylthioribase in liver and prostate homogenates. The reaction of 5'-methylthioribase upon MTA yields the formation of adenine which can be re-utilized in cellular processes.

Finkelstein et al. (37) have reported the effects of dietary protein upon hepatic SAM. Using either a 3.6 or 58% of casein based diet,

SAM liver concentrations increased in male rats in response to increasing dietary casein levels. Likewise, animals fed increasing amounts of casein had increased levels of liver methionine. The effects of fasting on liver metabolites of these animals showed decreased SAM with methionine unchanged. When methionine was injected (1 mmol/kg. body wt.) into animals receiving 3.6% or 58% of casein based diets, liver methionine showed a marked increase. SAM increased in response to the methionine injection. Finkelstein et al. (37) suggested that SAM concentrations in the liver reflect the availability of methionine and that a constant ratio of SAM: methionine may be characteristic of a steady-state situation.

The polyamines (putrescine, spermidine, and spermine)

The polyamines are products of the above reported decarboxylases and two synthases (Fig. 2). The polyamines have been identified as growth factors, as mentioned above (14), are polycationic and can therefore associate with phospholipids (1) and nucleic acids (5) to provide conformational stability. The decarboxylation of ornithine in animal tissues provides the first polyamine, 1,4-diaminobutane (putrescine). The half-life of putrescine is approximately 3 hr. (43). Putrescine is combined with the propylamine group from the decarboxylation of SAM to form spermidine. The inhibition of ODC by putrescine, as well as spermidine and spermine has been reported (1,3,4,17). SAMD is stimulated by putrescine and similar diamines in higher organisms (33). Putrescine induces the decarboxylation of SAM at a maximal activation of 0.7 mM; increasing the diamine concentration

up to 2.5mM maintains a constant reaction rate. Above a concentration of 70 mM putrescine no appreciable effect results (35). The mechanism by which putrescine stimulates SAMD is not known (35).

Chadwick et al. (44) measured ODC activity and putrescine levels in female albino rats following a deprivation of lysine, methionine, or tryptophan for 7 days. The hepatic levels of ODC and putrescine were enhanced in the methionine deprived rats. Because of the suggested relationships that the altered levels of putrescine and the polyamines may have upon cell multiplication (14,20), Chadwick et al. (44) have emphasized that this relationship is not clear-cut. However, their results seem to indicate that changes in the level of putrescine and the polyamines are not essential for the entry of the cell into the S phase (period of DNA replication) but may be the result of disturbances in the metabolism of critical amino acids that influence cell growth and multiplications.

Other specific functions have been outlined for putrescine (1,4, 5,17). Some of these functions remain unclear as to mechanisms and regulatory manipulations.

Spermidine and spermine are the products of the addition of one and two propylamine groups, respectively, to putrescine (Fig. 2). Two different transferases have been partially identified in mammalian tissue (5); one enzyme acts with putrescine to form spermidine, the second reacts with spermidine to yield spermine (4,5). The maximal velocity of SAMD is decreased by spermine at physiological concentrations (17).

The concentration and distribution of spermidine and spermine vary in organ tissue from species to species (1). The primary area

of research involving spermidine and spermine has been the relationship of these amines with protein biosynthesis. Raina et al. (45) found these amines to be present in increased concentrations as a response to rapidly growing tissue and RNA concentrations in regenerating liver. Cohen (46) was the first to develop a method to examine the relationship of spermidine to the tRNA of *E. coli*. Since their initial endeavors, literally hundreds of papers have contributed to this area of polyamine research (47). The findings of Cohen (47) indicate that putrescine binds slightly to tRNA, spermine binds tightly, and spermidine complexes 10 to 11 molecules per mole of tRNA. Cohen further suggests (47) that spermidine may stabilize the helical region and arm of tRNA, and that both Mg^{2+} and spermidine can act synergistically upon the tertiary structure of tRNA in facilitating folding of tRNA in the helical region. This is consistent with the suggestion of Leboy (48) in the role that Mg^{2+} and the polyamines play in the methylation of tRNA.

It is necessary to associate each amino acid with a molecule that can recognize the nucleotide code for a specific amino acid in order to build up a long polypeptide chain utilizing twenty different amino acids. The tRNA's (one tRNA for each amino acid) act as recognition molecules and must be present for the biosynthesis of any protein molecule (49). The tRNA methyltransferases modify the structure of pre-formed tRNA by adding methyl groups. The transferases require an intact tRNA molecule that is base-, sequence-, and conformation-specific in order for recognition of that molecule (49). Any alteration of the conformation of tRNA can modify the specificity of

recognition; therefore, there is a tendency for under-methylated tRNA's to be both less active and less specific (49).

Other functions of spermidine and spermine may include the suppression of mitochondrial metabolism (50). Spermidine has been shown to regulate the rate of O_2 utilization in animals with metabolically altered states.

Jamdar (51) has indicated that the polyamines (spermidine and spermine) act through a common mechanism in stimulating glycerolipid formation in adipose tissue homogenates and microsomal fractions from the epididymal fat pads of male Sprague-Dawley rats.

Seiler and Deckardt (52) have reported the uptake of ornithine by synaptosomes of rat brain cortex and the occurrence of ODC and SAMD activity in nerve endings. Their observation strongly supports the possibility of the polyamines functioning as neurotransmitters. Shaw (53) has summarized the function of the polyamines in the central nervous system. He suggests that although it may be premature to draw any conclusions with respect to the role of polyamines in neurotransmitter functions. If the polyamines have a neurotransmitter function the most likely candidate for transmitter function would be spermine due to the extensive transport of this amine into the intact nerve. Ingoglia and Sturman (54) have indicated the same possible function for spermine but also have reported an increase in the axonal transport of putrescine and spermidine in regenerating nerves, suggesting a role for these two amines during regeneration.

The clinical studies have increased in number that implicate changes in polyamine levels in humans as factors influencing health and

disease states. Because of the volume of literature, the reader is referred to several summaries (22,57).

Inorganic and organic sulfur in the diet

In the schematic representation of the biosynthesis of the polyamines (Fig. 2) it should be obvious that the sulfur-containing amino acid, methionine, has a decisive role in the biosynthesis of the polyamines. Greenberg (58) reported an "interesting" utilization of methionine for the biosynthesis of spermidine and spermine. As reported above, methionine is activated to SAM, decarboxylated by SAMD which yields MTA, and the aminopropyl group formed from methionine is transferred to putrescine to form spermidine.

Greenberg devoted only a limited amount of space to the role of methionine in the biosynthesis of the polyamines and no research reports have discussed the possible effect that dietary methionine may have upon the biosynthesis of spermidine and spermine. Sturman and Gaull (59) indicated that methionine, transsulfuration, transmethylation and polyamine biosynthesis are interrelated in the developing human and rhesus monkey, but have not implicated diet as a possible factor for manipulation of the polyamine levels in tissues.

The sulfur-containing amino acids, cysteine and methionine, have been recognized as supplying the required sulfur nutrition for an animal (60). Theoretically, in consideration of the metabolic fate of the sulfur atom on methionine to inorganic sulfate, the sulfate requirement of an animal could be entirely supplied by methionine (60). Finkelstein and Mudd (61) have reported the metabolic inequivalency of methionine when compared to cysteine as a dietary constituent. Their

findings indicate that cysteine has a sparing effect upon methionine. If inorganic sulfate is omitted from the diet, an animal is forced to satisfy its sulfate requirement by the means stated above; that is, the oxidation of amino acid sulfur to sulfate. However, the oxidation of cysteine sulfur to sulfate is a wasteful process which uses excess sulfur-containing amino acids. Whittle and Smith (62) have reported that rats fed diets containing low levels of inorganic sulfate excrete two times the amount of cysteine sulfur as taurine sulfur when compared to animals fed a diet containing 0.1% of inorganic sulfate. This finding is in agreement with Michels and Smith (1963) that diets low in inorganic sulfate appear to raise the methionine requirement of a rat. Other metabolic alterations in response to diets low in inorganic sulfate include a decreased aorta strength (64), and an increased glycocholic: taurocholic acid ratio (65), and an increase in the mobilization of tissue sulfur following malathion intoxication (66). Brown et al. (67) reported a decrease in the ability of sulfate-deprived animals to synthesize urea. In order to avoid metabolic alterations in an animal, Smith (68) determined that the optimal level of inorganic sulfate in the diet of a rat was 0.02%.

Rose et al. (69) reported that the methionine requirement of man must be supplied by dietary sources; however, the cysteine requirement of man could be met by biosynthesis. As mentioned above (61), cysteine has a sparing effect upon methionine. The data presented by Barnes et al. (70) and Kwong and Barnes (71) have shown a dietary requirement for cysteine that cannot be met by methionine supplementation when a rat consumes a diet containing raw soy-protein and coprophagy is prevented.

Byington et al. (72) and Byington and Howe (73) have suggested that the ratio of organic sulfur from methionine to organic sulfur from cysteine should be 70:30 in the diet of a rat. This would support the previous data (70,71) in the necessity of dietary cysteine for the rat. Based on the functional cysteine deficiency reported by Feland et al. (65) and the data recently presented by Kurzynske and Smith (74) that 15% of a casein based diet supplemented with 0.626% of methionine resulted in a decreased ratio of urinary glucuronide-salicylamide: sulfate salicylamide when compared to supplementation with 0.325% of methionine, it could appear that a diet containing 15% of casein must be supplemented with sulfur-containing amino acids equivalent to 0.325-0.625% of methionine.

Recently, data have been collected in this laboratory to show that the storage of energy in rats fed diets containing 15% of casein supplemented with cysteine (0.505%) is different from the storage of energy in rats fed the same diet but supplemented with equal molar amounts of methionine (75). The observed effect of methionine upon the enzymes of carbohydrate biosynthesis and lipogenesis is one of suppression and stimulation, respectively. It appears that methionine decreases gluconeogenesis by decreasing the activity of phosphoenolpyruvate carboxykinase and increasing the activity of glucose-6- PO_4 dehydrogenase, malic enzyme and citrate cleavage enzyme. Cysteine supplementation on the other hand influences, as a result of enzyme control, the storage of energy as glycogen while methionine supplementation influences an animal to store energy as lipid.

In view of the data presented above, the importance of sulfur nutrition in the monogastric animal should not be underestimated. Because the data collected in this laboratory have indicated not only the optimal level of inorganic sulfate for an animal but a difference in the metabolic response obtained when either cysteine or methionine are supplemented at varying levels of inorganic sulfate, it seemed reasonable to assume that sulfur nutrition could influence the identified growth factors, the polyamines. Further, because of the central role of methionine in the biosynthesis of the polyamines, spermidine and spermine, differences in the cysteine and methionine as well as the inorganic sulfate content of the diet could influence the biosynthesis of these amines. Therefore, the following experiments were conducted to evaluate the effect of dietary sulfur (neutral and inorganic) upon the biosynthesis of the polyamines in the rat.

CHAPTER III

EXPERIMENTAL PROCEDURES

Animals and experimental diets

Female and male rats (175-200g) of the Sprague-Dawley strain were randomly assigned 5 animals to a group and housed in galvanized steel cages. The animals were fed ad libitum one of 6 dietary regimens (Tables 1 and 2) varying in inorganic sulfate and supplemented with either L-cysteine (0.505%) or DL-methionine (0.620%). Distilled water was available for the 17 day dietary period. Following the dietary period, the animals were stunned by a blow to the back of the head and decapitated.

Determination of ornithine decarboxylase and S-adenosylmethionine decarboxylase in rat tissues

Brain, kidney, and liver tissue was excised to pre-weighted beakers containing a cold 0.025 M sodium phosphate buffer (pH 7.2), containing 0.005 M dithiothreitol, and 0.0001 M ethylenediaminetetraacetic acid (EDTA). The tissues were homogenized with 3 volumes of the cold buffer solution. The homogenates were centrifuged in an ultracentrifuge¹ at 25,000xg for 20 minutes. The 25,000xg supernatant was then centrifuged at 45,000xg for 90 minutes to obtain a supernatant fluid for enzyme assay.

¹Model L5-50, Beckman Instruments, P.O. Box C-1960, Irvine, California, 92801.

TABLE 1
Composition of the basal diet

Dietary constituent	g/100g
Casein ¹	15.00
Sucrose	30.00
Cornstarch	30.00
Cod liver oil	2.00
Vegetable shortening ²	6.00
Vitamin mix ³	2.00
Basic salt mix ⁴	1.34
Variable compounds	See Table 2

¹ICN Biochemicals, Inc., Cleveland, Ohio, 44128.

²Crisco--Proctor and Gamble, Cincinnati, Ohio, 45202.

³Nutritional Biochemicals Corp. Cleveland, Ohio, 44128,
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 panto-
thenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45, inositol
510, choline chloride 75, menadione 2.25, biotin 0.020, folic acid
0.090, vitamin B₁₂ 0.00135, α -D-tocopherol 5.0, vitamin A 9×10^5
units, vitamin D 1×10^5 units, and sufficient glucose to make 1000 g.

⁴Formulated to supply the following amounts of minerals:
(g/445.5 g salt mixture) magnesium carbonate 30.6, sodium chloride
69.0, potassium chloride 112, potassium monobasic phosphate 212, ferric
phosphate 20.5, potassium iodide 0.08, sodium fluoride 0.10, manganese
chloride 0.4, aluminum potassium sulfate 0.1, and cupric acetate 0.72.

TABLE 2
Variable components for the basal diet

Dietary inorganic sulfate %	CaSO ₄ ·2H ₂ O	CaCO ₃	DL-methionine ¹	L-cysteine ¹	Non-nutritive bulk ²
			g/100g diet		
0.0002	0.00	1.34	0.000	0.505	11.79
0.0002	0.00	1.34	0.620	0.000	11.67
0.02	0.04	1.32	0.000	0.505	11.80
0.02	0.04	1.32	0.620	0.000	11.68
0.42	0.75	0.91	0.000	0.505	11.50
0.42	0.75	0.91	0.620	0.000	11.38

¹ICN Biochemicals, Inc., Cleveland, Ohio, 44128.

²Alphacel, ICN Biochemicals, Inc., Cleveland, Ohio, 44128.

The enzyme activity of ODC was measured by determining the liberation of $^{14}\text{CO}_2$ from carboxyl-labeled substrate, according to the method of Russell and Snyder (20). Incubations were carried out in 10-ml, one-armed, glass reaction flasks² equipped with a rubber stopper supporting a disposable polypropylene cup that contained 0.1 ml of Soluene-100.³ The incubation mixture consisted of 0.1 μmole pyridoxal-5-phosphate, 0.3 ml of enzyme preparation, 2.5 μmoles of dithiothreitol, 0.5 μmoles of L-ornithine, 0.4 μCi of DL-ornithine-1- ^{14}C , and 50 μmoles of glycylglycine buffer, pH 7.2, to make a final volume of 0.5 ml. Duplicate samples of each tissue supernatant fluid and a blank (boiled preparation) were incubated in a shaking water bath⁴ at 37° for 1 hour. The reaction was stopped by injecting 0.5 ml trichloroacetic acid (40% w/v) through the stopper fitted to the side-arm of the flask. The flasks were allowed to incubate 20 min at 25° to insure the release of dissolved $^{14}\text{CO}_2$ from the acidified medium. The polypropylene cup and its contents were removed, placed in a low K scintillation vial containing 10 ml of a solution of 2,5-diphenyloxazole (PPO, 12 g/l) in toluene, and assayed for radioactivity in a liquid scintillation spectrometer.⁵ An external standard was used to evaluate

²Kontes Glass Company, 1916 Greenleaf Street, P.O. Box 30, Evanston, Illinois, 60204.

³Soluene-100, Packard Instrument Company, Inc., 2200 Warrenville Road, Downers Grove, Illinois, 60515.

⁴Eberbach Corporation, P.O. Box 1024, Ann Arbor, Michigan, 48106.

⁵Model LS-100C, Beckman Instrument Company, P.O. Box C-1960, Irvine, California, 92801.

quenching. The enzyme activity of SAMD was determined by the method of Jänne and Williams-Ashman (76). The liberation of $^{14}\text{CO}_2$ from carboxyl-labeled substrate was carried out as outlined above. The incubation mixture consisted of 1.25 μmoles of putrescine, 0.1 μmoles of S-adenosyl-L-methionine, 0.2 μCi of S-adenosyl-L-carboxyl- ^{14}C methionine 0.2 ml of enzyme preparation, 2.5 μmoles of sodium phosphate buffer, pH 7.2, to make a final volume of 0.5 ml. Radioactivity was assayed as described above.

Determination of the polyamines (putrescine, spermine, and spermidine) in rat tissues

Tissue samples (liver, kidney and brain) were excised to cold 0.2 N perchloric acid (PCA) and stored in labeled vials at -20° until polyamine determinations could be performed.

The method of Seiler and Wiechman (77) as adapted by Herbst and Dion (78) and Fleisher and Russell (79) was used to measure the fluorescence intensity of dansyl-(5-dimethylaminonaphthalene sulfonyl chloride) derivatives of putrescine, spermine and spermidine on thin layer chromatography (TLC) plates. The amines were extracted by homogenation of the tissue with 0.2 N PCA so that 0.2 ml of the extract contained approximately 50 mg of tissue (wet weight). A 0.2 ml sample was reacted with dansyl-chloride in acetone (2.7 mg/ml). This sample was incubated overnight in the dark and at room temperature. In order to remove any excess dansyl-chloride, 200 μl of L-Proline (200 mg/ml) was added to the sample and stored in the dark at room

temperature for 20 minutes. One ml of benzene was used for extraction of the dansyl-derivative by vortex shaking of the sample for 1 minute. Then 0.8 ml of the sample was transferred to a small polypropylene vial and dried in a convection oven. Known concentrations of the polyamines were prepared in 0.2 N PCA and treated in the manner stated above.

Benzene in the volume of 200 μ l, 100 μ l, or 50 μ l, was used to reconstitute the samples and standards. After activation of Silica Gel-G plates for 1 hour at 100° the samples and standards were applied in μ l-quantities onto the plates. Because of variation two concentrations of the polyamines were used on each plate.

After applying the samples and standards, the plates were developed in triethylamine: chloroform (1:5). The solvent was allowed to ascend approximately 14 cm from the origin. Following the development of each plate, they were air-dried and sprayed with triethanolamine: isopropanol (2:8) to enhance and stabilize the fluorescence. The plates were dried at room temperature, observed under a UV-lamp and scored with a grease pencil to identify the location of each band containing the standards and samples.

Measurement of fluorescence intensity was performed with a spectrophotofluorimeter equipped with a TLC scanner and XY-recorder using wavelengths of 365 and 500 nm for activation and emission, respectively. The proportionality between the peak heights of the dansylated derivatives of the polyamine standards and the unknown

samples was used for evaluation of the polyamine content of each unknown.

Quantitative analysis of S-adenosyl-L-methionine in rat liver tissue

Liver tissues were excised to cold 1.5 N PCA. The estimation of S-adenosyl-L-methionine (SAM) was achieved by the method of Shapiro and Ehninger (80) and Salvatore et al. (36). The extraction of SAM from the above excised tissues was carried out by preparing a tissue homogenate of 1.5 N PCA (1:4 w/v) to which 1-5 μ moles of S-adenosyl-L-methionine with a specific radioactivity ranging from 1×10^5 to 5×10^5 cpm/ μ mole had been added. The homogenate was centrifuged at 9000xg for 20 min. The precipitate was discarded and the supernatant solution was neutralized to pH 6 with 2 M KHCO_3 in the presence of Thomas Antifoam spray.⁶ The mixture was centrifuged for 15 minutes at 9000xg. Pure SAM was isolated from the supernatant fluid by column chromatography on Dowex 50 ($-\text{Na}^+$ form). The eluting agents were 0.1 M NaCl and 3 M H_2SO_4 .

Elution was carried out with 0.1 M NaCl until the absorbance at 256 nm was less than 0.05. A DU spectrophotometer⁷ was used to measure ultraviolet absorption and radioactivity was determined by a liquid scintillation spectrometer. Duplicate 3 ml samples were

⁶Arthur H. Thomas, Philadelphia, Pennsylvania, 19140.

⁷Beckman Instrument Company, P. O. Box C-1960, Irvine, California, 92801. Beckman Quartz Spectrophotometer, Model DU, modified to solid state configuration by Update Instruments, Inc., P.O. Box 5177, Madison, Wisconsin, 53705.

taken from the 3 M H_2SO_4 eluant to evaluate SAM concentrations at 256 nm; a molar absorbance of 14,700 was used to calculate SAM. A 3 ml standard of a solution containing the pure compound was also evaluated. A 0.5 ml sample of the eluant was taken for the assay of radioactivity. The sample was pipetted into a low-K scintillation vial to which 10 ml toluene containing PPO (12 g/l) and 5 ml ethylcelosolve had been added. The mixture was evaluated for radioactivity by a liquid scintillation spectrometer. An external standard was used to evaluate quenching. Because the dilution of specific radioactivity is proportional to the content of the unlabeled compound originally present in the tissue, SAM could be measured with accuracy when using a solution containing the pure compound (36).

Determination of the ^{14}C -methylation of tRNA in rat liver tissue

Sixty adult, female and male rats were randomly divided by gender into 6 groups of 5 animals of each gender per group. The animals were housed in galvanized steel cages for a 17 day dietary period. Each group of animals received one of the experimental diets (Tables 1 and 2, pages 19 and 20) and distilled water ad libitum. At the end of the experimental period the animals were given an intraperitoneal injection of 0.5 ml of an isotonic saline solution containing 12 mg of ^{14}C -methionine (specific activity 9.0×10^4 cpm/mg) and sacrificed after two hours.

• The method of Allen et al. (81) was used to isolate tRNA. The liver of each animal was excised to a cold Waring

Blender⁸ containing 20 ml extraction buffer and 15 ml phenol at 0-10°. Blending of each liver was within 1 sec. The extraction buffer contained 0.36% sodium dodecyl sulfate, 0.17% naphalene disulfonic acid, 2 mM EDTA, 0.01 M magnesium acetate, 0.2 M sodium acetate (pH 5) and 5% methanol. All steps, except where noted, were carried out at 0-2°. The homogenates were centrifuged at 9000xg for 20 min. The aqueous supernatant solutions were transferred to a mixture of 10 ml phenol and 2 ml chloroform. The mixture was shaken and centrifuged for 20 min at 9000xg. The supernatants were mixed with 2.5 vol of ethanol (100%) and stored at -20° overnight. The resulting precipitates were collected by centrifugation for 20 min at 9000xg. Twenty-nine ml of a 0.1 M sodium acetate (pH 4) solution containing 0.01 M magnesium acetate were added to the resulting pellet, mixed and centrifuged at 100,000xg to remove glycogen. The supernatants were mixed with 0.05 vol 2M sodium acetate and 2.5 vol ethanol and stored at -20° for 2 hr. The precipitates were collected by centrifugation for 20 min at 9000xg. The pellets were dissolved in 2 ml 0.1 M sodium acetate (pH 4) containing 0.01 M magnesium acetate. A duplicate aliquoit of 0.5 ml was taken to evaluate ¹⁴C-activity by liquid scintillation spectrometry. The remaining 1 ml was used to determine the concentration of RNA according to the orcinol method (82).

In order to express selected data in terms of mg of protein, protein was determined by the Folin-Ciocalteu method, as described

⁸Eberback Corporation, P.O. Box 1024, Ann Arbor, Michigan, 48106.

by Lowry et al. (83) with bovine serum as a reference standard.

Statistical significance of the data was evaluated using the method of paired comparisons.

CHAPTER IV

RESULTS

The purpose of this investigation was to determine the effect of dietary sulfur (neutral and inorganic) upon the biosynthesis and tissue accumulation of the polyamines in the rat. In order to determine if the supplementation of diets containing 15% of casein and varying in inorganic sulfate with the sulfur-containing amino acids would alter the potential for polyamine biosynthesis, the activity of ornithine decarboxylase (ODC) in rat tissues was determined.

Ornithine decarboxylase activity in rat tissues

The activity of ODC in the liver tissue (Table 3) obtained from animals fed the experimental diets was significantly different ($p < 0.05$) at the 0.02% level of inorganic sulfate when comparing cysteine and methionine supplementation in both male and female rats. Cysteine supplementation at the 0.02% level of inorganic sulfate significantly stimulated the liver ODC activity when compared to the ODC activity in the liver of animals supplemented with methionine ($p < 0.05$).

In female animals a sulfate effect upon liver ODC activity was observed in comparing the 0.02% level of inorganic sulfate to the 0.0002% level of dietary inorganic sulfate. No statistically significant differences existed in the ODC liver activity in female rats fed a diet containing 0.42% of inorganic sulfate when compared to the ODC liver activity in those animals fed the optimal level (0.02%) of inorganic sulfate.

TABLE 3

L-ornithine decarboxylase activity in the liver of animals
fed different levels of inorganic sulfate (%)
supplemented with cysteine or methionine

S-containing amino acid	Dietary inorganic sulfate (%)		
	pmoles CO ₂ /mg protein/hr		
	0.0002	0.02	0.42
Cysteine (0.505%)	26.5 ± 4.9 ^{1a2}	42.0 ± 10.0 ^d	35.0 ± 9.0 ^d
♀ Methionine (0.620%)	41.0 ± 15.0 ^a	0.7 ± 0.6 ^b	0.5 ± 0.4 ^b
Cysteine (0.505%)	512.0 ± 19.0 ^b	157.0 ± 23.0 ^e	128.0 ± 3.0 ^e
♂ Methionine (0.620%)	4.0 ± 1.0 ^c	6.0 ± 2.0 ^c	241.0 ± 25.0 ^a

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or a row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

In male rats, cysteine supplementation allowed dietary sulfate to express differences in the activity of liver ODC in animals fed the 0.0002% level of inorganic sulfate. This difference disappeared when the level of inorganic sulfate was increased above the optimal percentage of inorganic sulfate.

The ODC activity in the kidneys of animals fed the experimental diets (Table 4) shows that cysteine supplementation at the 0.02% and the 0.42% level of dietary inorganic sulfate significantly ($p < 0.05$) stimulates ODC activity in both male and female animals. Methionine supplementation allowed dietary sulfate to express differences in ODC in comparison to supplementation with cysteine in which the sulfate effect disappeared when the optimal level was reached. The sulfate effect with methionine supplementation was different for females and males. In female rats supplemented with methionine, the ODC activity decreased with increasing dietary inorganic sulfate; however, with male animals, a low inflection point occurred in those animals fed the diet containing 0.02% of inorganic sulfate.

Brain activity of ODC in animals fed the experimental diets (Table 5) has a different enzyme response in comparing the gender of the animals. However, at the 0.0002% + 0.02% level of dietary inorganic sulfate both sexes and at the 0.42% of inorganic sulfate females responded to cysteine supplementation with a significant ($p < 0.05$) stimulation of brain ODC activity when compared to those animals supplemented with methionine.

In both male and female animals there was a non-significant average increase in brain ODC activity when dietary inorganic sulfate

TABLE 4

L-ornithine decarboxylase activity in the kidneys of
animals fed different levels of inorganic sulfate (%)
supplemented with cysteine or methionine

S-containing amino acid	Dietary inorganic sulfate (%)		
	pmoles CO ₂ /mg protein/hr		
	0.0002	0.02	0.42
Cysteine (0.505%)	2410 ± 66.9 ^{1a2}	3316 ± 106.4 ^b	3364 ± 64.9 ^b
♀ Methionine (0.620%)	1895 ± 59.8 ^a	266 ± 17.2 ^c	1.0 ± 1.0 ^d
Cysteine (0.505%)	394 ± 34.0 ^b	1473 ± 328.0 ^a	1295 ± 338.0 ^a
♂ Methionine (0.620%)	874 ± 99.0 ^c	441 ± 39.0 ^d	1957 ± 348.0 ^e

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or a row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

TABLE 5

L-orthinine decarboxylase activity in the brain of animals
fed different levels of inorganic sulfate (%)
supplemented with cysteine or methionine

S-containing amino acid	Dietary inorganic sulfate (%)		
	pmoles CO ₂ /mg protein/hr		
	0.0002	0.02	0.42
Cysteine (0.505%) ♀	879 ± 219.0 ^{1a2}	1045 ± 457.0 ^a	1402 ± 664 ^a
Methionine (0.620%)	416 ± 216.0 ^b	47 ± 4.6 ^c	520 ± 276 ^b
Cysteine (0.505%) ♂	58 ± 2.0 ^c	365 ± 43.0 ^b	402 ± 33 ^b
Methionine (0.620%)	4 ± 0.3 ^d	2 ± 0.4 ^d	1151 ± 177 ^a

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly (p<0.05) by the method of paired comparisons.

was increased with cysteine supplementation. In female rats, the supplementation of methionine allowed an increase in ODC activity at the lowest and highest levels of dietary inorganic sulfate when compared to the 0.02% level of inorganic sulfate. There was a sulfate effect in male rats on brain ODC activity in those rats fed the 0.42% level of dietary inorganic sulfate and supplemented with methionine compared to those fed the 0.02 or 0.0002% level of inorganic sulfate.

S-adenosyl-L-methionine decarboxylase activity in rat tissues

The activity of S-adenosyl-L-methionine decarboxylase (SAMD) in the liver of animals receiving the experimental diets (Table 6) indicates gender difference between these animals. The SAMD activity at the 0.02% level of dietary inorganic sulfate exhibits the same response in each gender when considering the supplementation of cysteine versus methionine; cysteine supplementation significantly decreases ($p < 0.05$) the liver activity of SAMD. In both male and female animals, there was a response in the activity of liver SAMD to dietary inorganic sulfate in animals fed the diets supplemented with methionine. In male animals SAMD activity increased at both low (0.0002%) and high (0.42%) of dietary inorganic sulfate, while in female animals a decreased activity was associated with both high and low levels of sulfate.

SAMD activity in the kidneys of animals receiving the experimental diets (Table 7) responded in approximately the same manner as did the liver SAMD enzyme activity. In comparing the effects of the sulfur-containing amino acid supplemented at the 0.02% level of dietary inorganic sulfate, there was a slight decrease in the SAMD activity of those animals supplemented with cysteine compared to those

TABLE 6

Activity of S-adenosyl-L-methionine decarboxylase in the liver
of animals fed different levels of inorganic sulfate (%)
supplemented with cysteine or methionine

S-containing amino acid	Dietary inorganic sulfate (%)		
	pmoles CO ₂ /mg protein/hr		
	0.0002	0.02	0.42
Cysteine (0.505%)	346 ± 21 ^{1a2}	253 ± 18 ^b	286 ± 23 ^b
♀ Methionine (0.620%)	106 ± 16 ^c	320 ± 32 ^a	123 ± 21 ^c
Cysteine (0.505%)	97 ± 10 ^e	65 ± 9 ^e	115 ± 23 ^a
♂ Methionine (0.620%)	214 ± 35 ^d	74 ± 21 ^c	191 ± 17 ^d

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

TABLE 7

S-adenosyl-L-methionine decarboxylase activity in the kidneys of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid	Dietary inorganic sulfate (%)		
	pmoles CO ₂ /mg protein/hr		
	0.0002	0.02	0.42
Cysteine (0.505%) ♀	154 ± 10 ^{1a2}	171 ± 13 ^a	202 ± 20 ^b
Methionine (0.620%)	41 ± 3 ^b	204 ± 28 ^a	16 ± 5 ^c
Cysteine (0.505%) ♂	103 ± 9 ^c	127 ± 9 ^b	394 ± 34 ^a
Methionine (0.620%)	322 ± 35 ^d	138 ± 11 ^b	556 ± 10 ^e

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or a row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

supplemented with methionine. This difference was not statistically significant in either female or male animals. In female and male animals fed diets supplemented with cysteine, there was a sulfate effect that tended to show an increase in SAMD activity with increasing dietary sulfate. A statistically significant ($p < 0.05$) decrease in SAMD activity was observed at either side of the optimal level (0.02%) of inorganic sulfate in female animals fed diets supplemented with methionine. In contrast, the response in male animals was a significant ($p < 0.05$) increase in SAMD activity in rats fed diets containing sulfur levels on either side of the 0.02% level.

The measured activity of SAMD brain (Table 8) shows a decreased activity with cysteine supplementation at the 0.02% level of inorganic sulfate in male rats and a non-significant trend in female rats. In both male and female animals fed diets supplemented with methionine a significant but opposite sulfate effect was obtained when decreasing or increasing levels of dietary inorganic sulfate compared to when the optimal levels of inorganic sulfate were fed. In female rats low or high levels of sulfate decreased SAMD activity but in male rats low and high levels of inorganic sulfate increased levels of SAMD. In female rats fed diets supplemented with cysteine increasing sulfate decreased SAMD, while in male rats both decreased and increased levels of inorganic sulfate increased SAMD activity.

Polyamine concentrations in rat tissues

The polyamine concentrations are reported in μ moles per gram wet tissue weight and total μ moles per wet tissue organ. The concentration of putrescine in the liver of animals fed varying levels of inorganic

TABLE 8

S-adenosyl-L-methionine decarboxylase activity in the brain of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid	Dietary inorganic sulfate (%)		
	pmoles CO ₂ /mg protein/hr		
	0.0002	0.02	0.42
Cysteine (0.505%)	834 ± 37 ^{1a2}	688 ± 70 ^b	348 ± 13 ^d
♀ Methionine (0.620%)	187 ± 16 ^c	692 ± 67 ^b	170 ± 41 ^c
Cysteine (0.505%)	505 ± 17 ^d	373 ± 15 ^e	2689 ± 272 ^b
♂ Methionine (0.620%)	2574 ± 60 ^b	411 ± 10 ^c	3065 ± 311 ^a

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or a row followed by the same superscript number do not differ significantly (p<0.05) by the method of paired comparisons.

sulfate and supplemented with cysteine or methionine (Table 9) did not differ significantly in a response to amino acid supplementation.

A statistically significant ($p < 0.05$) sulfate effect upon the formation of liver putrescine was observed in male rats. In male rats, the sulfate effect was observed at the 0.42% level of inorganic sulfate in diets supplemented with either cysteine or methionine. There was an increased putrescine formation when rats fed 0.42% of sulfate were compared to those fed 0.02% of sulfate.

Spermidine concentrations in the liver of animals consuming graded levels of inorganic sulfate and supplemented with cysteine or methionine (Table 10) show that in female animals no differences exist at the 0.02% level of inorganic sulfate; however, in male rats cysteine supplementation significantly increased the formation of liver spermidine when compared to methionine supplementation at the optimal level of inorganic sulfate.

In female animals there was a non-significant trend towards increased liver spermidine concentrations with increases in dietary inorganic sulfate. This trend was not observed in male rats.

The concentration of spermine in the liver of animals consuming graded amounts of inorganic sulfate and supplemented with cysteine or methionine (Table 11) shows that cysteine has a stimulatory effect upon the formation of spermine in liver of male rats fed the optimal (0.02%) and 0.42% level of inorganic sulfate. This stimulatory effect of cysteine was statistically significant ($p < 0.05$) when compared to those animals receiving the 0.02% level of inorganic sulfate and supplemented with methionine. In female rats, there was an average

TABLE 9
Putrescine liver concentrations in animals fed different levels of inorganic sulfate (%)
and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	μ moles putrescine/wet wt					
	0.0002		0.02		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%) ♀	11.4 $\pm$ 3 ^{1a2}	92.2 $\pm$ 24 ^a	14.3 $\pm$ 4 ^a	121.7 $\pm$ 37 ^a	15.2 $\pm$ 4 ^a	142.4 $\pm$ 41 ^a
Methionine (0.620%)	16.2 $\pm$ 4 ^b	134.6 $\pm$ 36 ^b	14.6 $\pm$ 2 ^{ab}	125.0 $\pm$ 21 ^{ab}	9.7 $\pm$ 2 ^{ab}	91.4 $\pm$ 21 ^{ab}
Cysteine (0.505%) ♂	22.9 $\pm$ 6 ^c	291.3 $\pm$ 74 ^c	23.5 $\pm$ 11 ^c	293.9 $\pm$ 134 ^c	31.2 $\pm$ 7 ^d	440.8 $\pm$ 104 ^d
Methionine (0.620%)	26.0 $\pm$ 5 ^c	267.5 $\pm$ 56 ^c	16.8 $\pm$ 8 ^c	200.3 $\pm$ 93 ^c	38.3 $\pm$ 8 ^d	463.6 $\pm$ 97 ^d

¹Values are means $\pm$ SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

TABLE 10
Spermidine liver concentrations in animals fed different levels of inorganic sulfate (%)
and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	μ moles spermidine/wet wt					
	0.0002		0.02		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%) ^{1a2}	0.13 $\pm$ 0.03	1.1 $\pm$ 0.2 ^a	0.20 $\pm$ 0.06 ^b	1.7 $\pm$ 0.5 ^b	0.23 $\pm$ 0.02 ^b	2.1 $\pm$ 0.2 ^b
Methionine (0.620%) ^{1a2}	0.19 $\pm$ 0.06 ^a	1.5 $\pm$ 0.5 ^a	0.20 $\pm$ 0.05 ^{ab}	1.7 $\pm$ 0.5 ^{ab}	0.20 $\pm$ 0.07 ^{ab}	1.9 $\pm$ 0.7 ^{ab}
Cysteine (0.505%) ^{1a2}	0.44 $\pm$ 0.07 ^c	5.6 $\pm$ 0.9 ^c	0.55 $\pm$ 0.12 ^c	6.9 $\pm$ 1.5 ^c	0.42 $\pm$ 0.12 ^c	6.0 $\pm$ 1.6 ^c
Methionine (0.620%) ^{1a2}	0.65 $\pm$ 0.17 ^c	6.6 $\pm$ 1.8 ^c	0.29 $\pm$ 0.09 ^d	3.5 $\pm$ 1.1 ^d	0.44 $\pm$ 0.07 ^c	5.3 $\pm$ 0.9 ^c

¹Values are means $\pm$ SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

TABLE 11
Spermine liver concentrations in animals fed different levels of inorganic sulfate (%)
and supplemented with cysteine or methionine

S-contained amino acid added	Dietary inorganic sulfate (%)					
	0.0002			0.02		
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%)	0.14 ± 0.05 ^{1a2}	1.2 ± 0.4 ^a	0.18 ± 0.04 ^a	1.5 ± 0.4 ^a	0.15 ± 0.03 ^a	1.4 ± 0.3 ^a
Methionine (0.620%) _♀	0.17 ± 0.05 ^a	1.4 ± 0.4 ^a	0.14 ± 0.02 ^a	1.2 ± 0.3 ^a	0.15 ± 0.05 ^a	1.4 ± 0.5 ^a
Cysteine (0.505%) _♂	0.39 ± 0.09 ^b	5.0 ± 1.2 ^b	0.34 ± 0.04 ^b	4.2 ± 0.5 ^b	0.41 ± 0.07 ^b	5.8 ± 0.9 ^b
Methionine (0.620%)	0.54 ± 0.15 ^b	5.5 ± 1.6 ^b	0.23 ± 0.05 ^c	2.7 ± 0.6 ^c	0.36 ± 0.11 ^d	4.4 ± 1.2 ^d

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly (p<0.05) by the method of paired comparisons.

increase in the formation of liver spermine when cysteine versus methionine was supplemented at the optimal level of inorganic sulfate; however, this average increase was not statistically significant. In female rats, no sulfate effect was observed. However, in male rats fed diets supplemented with methionine the formation of spermine in liver tissue of rats fed the lowest and highest levels of inorganic sulfate was increased when compared to those rats fed the optimal level of dietary inorganic sulfate.

Putrescine concentrations in the kidneys of animals fed the experimental diets (Table 12) show a stimulatory effect upon the formation of kidney putrescine in female rats fed the optimal (0.02%) and the low (0.0002%) level of inorganic sulfate by cysteine when compared to those rats fed diets supplemented with methionine. This difference was statistically significant ($p < 0.05$). This difference was also observed in male animals fed diets containing 0.42% of sulfate but did not exist in male animals fed the optimal level of inorganic sulfate. In male animals fed the low (0.0002%) level of sulfate, cysteine supplementation decreased putrescine formation. In female animals there were decreasing putrescine concentrations in those rats fed 0.42% of sulfate. This trend was not observed in male animals.

Spermidine concentrations in the kidney (Table 13) indicate that neither cysteine nor methionine supplementation showed statistically significant differences at the 0.02% level of dietary inorganic sulfate in female and male animals. However, in female animals there was a non-significant average in increased spermidine concentrations

TABLE 12

Putrescine concentrations in the kidneys of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	$\mu\text{moles putrescine/wet wt}$					
	0.0002		0.2		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%)	16.6 ± 2^{1a2}	34.1 ± 4^a	16.9 ± 4^a	33.4 ± 8^a	12.8 ± 2^b	23.2 ± 4^b
Methionine (0.620%) ♀	12.5 ± 3^b	25.3 ± 6^b	11.7 ± 1^b	22.8 ± 3^b	10.8 ± 2^b	20.3 ± 4^b
Cysteine (0.505%) ♂	9.1 ± 1^b	23.7 ± 2^b	12.8 ± 1^a	32.0 ± 2^a	16.0 ± 3^a	33.7 ± 7^a
Methionine (0.620%)	16.2 ± 2^a	37.9 ± 5^a	16.8 ± 2^a	37.5 ± 4^a	12.7 ± 3^b	26.8 ± 3^b

¹Values are means $\pm$ SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

TABLE 13

Spermidine concentrations in the kidneys of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	0.0002		0.02		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%) ♀	0.34 ± 0.13 ^{1c2}	0.72 ± 0.29 ^c	0.20 ± 0.13 ^b	0.39 ± 0.26 ^b	0.16 ± 0.06 ^b	0.30 ± 0.11 ^b
Methionine (0.620%)	0.11 ± 0.05 ^a	0.23 ± 0.11 ^a	0.15 ± 0.05 ^{ab}	0.30 ± 0.05 ^{ab}	0.06 ± 0.02 ^c	0.11 ± 0.03 ^c
Cysteine (0.505%) ♂	0.11 ± 0.01 ^a	0.28 ± 0.03 ^a	0.15 ± 0.03 ^{ab}	0.38 ± 0.07 ^{ab}	0.21 ± 0.04 ^a	0.44 ± 0.08 ^a
Methionine (0.620%)	0.15 ± 0.03 ^a	0.36 ± 0.08 ^a	0.15 ± 0.03 ^{ab}	0.34 ± 0.07 ^{ab}	0.12 ± 0.03 ^b	0.25 ± 0.07 ^b

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly (p<0.05) by the method of paired comparisons.

in the kidney of rats fed diets containing the optimal level of dietary inorganic sulfate when cysteine was added to the diet compared to the addition of methionine. In female animals, there was an increased formation of kidney spermidine in those fed the lowest level of inorganic sulfate when compared to those rats fed the optimal level of dietary inorganic sulfate.

The concentration of spermine in the kidneys of animals fed the experimental diets (Table 14) shows a stimulatory effect upon spermine formation by the addition of cysteine to the optimal level (0.02%) of dietary inorganic sulfate fed to male rats compared to those fed the methionine supplemented diet. The effect of sulfate upon the formation of kidney spermine was not as pronounced as the cysteine versus methionine effect at the 0.02% level of inorganic sulfate in male rats; however, methionine supplementation allowed dietary sulfate to express differences in male animals fed the 0.0002% and the 0.42% levels of inorganic sulfate.

The brain concentration of putrescine in animals fed the experimental diets (Table 15) indicates that no statistical differences existed between the effect of the addition of cysteine or methionine to the optimal level of dietary inorganic sulfate upon the concentration of brain putrescine. However, a sulfate effect was demonstrated in each gender when comparisons were made to the 0.02% (optimal) level of dietary inorganic sulfate. Methionine supplementation allowed dietary sulfate to express differences in both sexes fed the 0.0002% and 0.42% when compared to the 0.02% level of inorganic sulfate. Cysteine supplementation allowed dietary sulfate to express

TABLE 14

Spermine concentrations in the kidneys of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	0.0002			0.02		
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%)	0.11 ± 0.03 ^{1a,2}	0.23 ± 0.07 ^a	0.09 ± 0.01 ^a	0.17 ± 0.03 ^a	0.08 ± 0.01 ^b	0.15 ± 0.02 ^b
Methionine (0.620%)	0.08 ± 0.02 ^a	0.16 ± 0.03 ^a	0.08 ± 0.01 ^a	0.16 ± 0.02 ^a	0.06 ± 0.01 ^a	0.12 ± 0.03 ^a
Cysteine (0.505%)	0.20 ± 0.05 ^b	0.51 ± 0.13 ^b	0.32 ± 0.04 ^d	0.73 ± 0.10 ^d	0.35 ± 0.05 ^c	0.74 ± 0.11 ^c
Methionine (0.620%)	0.32 ± 0.11 ^b	0.74 ± 0.25 ^b	0.11 ± 0.07 ^c	0.26 ± 0.10 ^c	0.14 ± 0.03 ^d	0.29 ± 0.06 ^d

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly (p<0.05) by the method of paired comparisons.

TABLE 15
Brain putrescine concentrations in animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	μ moles putrescine/wet wt					
	0.0002		0.02		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%) ♀	13 $\pm$ 0.7 ^{1a2}	24 $\pm$ 1.3 ^a	11 $\pm$ 1.3 ^b	21 $\pm$ 2.5 ^b	11 $\pm$ 1.1 ^b	19 $\pm$ 2.0 ^b
Methionine (0.620%)	17 $\pm$ 1.3 ^c	32 $\pm$ 2.4 ^c	10 $\pm$ 1.1 ^b	18 $\pm$ 2.0 ^b	13 $\pm$ 1.0 ^d	24 $\pm$ 2.0 ^d
Cysteine (0.505%) ♂	8 $\pm$ 1.4 ^b	14 $\pm$ 2.6 ^b	4 $\pm$ 1.2 ^a	9 $\pm$ 2.0 ^a	6 $\pm$ 1.1 ^a	11 $\pm$ 2.1 ^a
Methionine (0.620%)	7 $\pm$ 1.2 ^b	13 $\pm$ 2.5 ^b	6 $\pm$ 0.8 ^a	12 $\pm$ 1.6 ^a	9 $\pm$ 0.7 ^c	16 $\pm$ 1.3 ^c

¹Values are means $\pm$ SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

differences in the concentration of putrescine in the brains of animals fed the 0.0002% level of dietary inorganic sulfate but not in those animals fed the 0.42% level.

Spermidine brain concentrations (Table 16) show that cysteine supplementation at the 0.02% level of inorganic sulfate was a statistically significant ($p < 0.05$) source of stimulation in the formation of this amine when compared to female rats supplemented with methionine and fed the optimal level of inorganic sulfate. This response was not demonstrated in male rats eating comparable diets. Cysteine supplementation allowed dietary sulfate to express differences only in female animals fed the 0.42% compared to the optimal percentage of 0.02% inorganic sulfate. Methionine supplementation allowed dietary sulfate to increase brain spermidine concentration in female animals fed the 0.0002% and 0.42% when compared to those fed the 0.02% level of inorganic sulfate.

The statistically significant ($p < 0.05$) increase in the spermine concentrations in the brain of female animals (Table 17) fed the 0.02% level of inorganic sulfate with added cysteine show that cysteine was stimulatory in the formation of this amine when compared to those female animals fed the same level of inorganic sulfate with added methionine. In male animals, however, only an average increase in concentrations of spermine in the brain of animals fed the optimal percentage of inorganic sulfate with added cysteine was observed. Female rats fed the high (0.42%) level of inorganic sulfate with methionine supplementation had an increased concentration of brain spermine compared to those fed methionine supplemented diets and 0.02% of inorganic sulfate.

TABLE 16
Brain spermidine concentration in animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	μ moles spermidine/wet wt					
	0.0002		0.02		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%) ♀	0.08 ± 0.01 ^{1a2}	0.16 ± 0.02 ^a	0.10 ± 0.01 ^a	0.20 ± 0.04 ^a	0.07 ± 0.01 ^b	0.11 ± 0.01 ^b
Methionine (0.620%)	0.09 ± 0.01 ^d	0.18 ± 0.02 ^d	0.06 ± 0.01 ^c	0.11 ± 0.05 ^c	0.10 ± 0.01 ^d	0.19 ± 0.02 ^d
Cysteine (0.505%) ♂	0.06 ± 0.01 ^b	0.12 ± 0.01 ^b	0.07 ± 0.01 ^b	0.14 ± 0.01 ^b	0.07 ± 0.01 ^b	0.14 ± 0.01 ^b
Methionine (0.620%)	0.07 ± 0.01 ^b	0.14 ± 0.02 ^b	0.08 ± 0.01 ^b	0.16 ± 0.02 ^b	0.07 ± 0.01 ^{bc}	0.13 ± 0.02 ^{bc}

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly (p<0.05) by the method of paired comparisons.

TABLE 17

Spermine brain concentrations in animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)					
	0.0002		0.02		0.42	
	per gram	total	per gram	total	per gram	total
Cysteine (0.505%) ^{1a2}	0.10 ± 0.01	0.20 ± 0.02 ^a	0.12 ± 0.02 ^a	0.23 ± 0.03 ^a	0.10 ± 0.01 ^a	0.18 ± 0.02 ^a
Methionine (0.620%) ^{1b}	0.08 ± 0.01	0.16 ± 0.02 ^b	0.08 ± 0.01 ^{bc}	0.15 ± 0.02 ^{bc}	0.11 ± 0.01 ^d	0.21 ± 0.02 ^d
Cysteine (0.505%) ^{2a}	0.05 ± 0.01	0.09 ± 0.01 ^c	0.06 ± 0.01 ^a	0.14 ± 0.02 ^a	0.05 ± 0.01 ^a	0.11 ± 0.01 ^c
Methionine (0.620%) ^{2b}	0.06 ± 0.01	0.11 ± 0.02 ^c	0.06 ± 0.01 ^{ac}	0.12 ± 0.01 ^{ac}	0.05 ± 0.01 ^{ac}	0.09 ± 0.01 ^b

¹Values are means ± SEM of duplicate determinations from five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly (p<0.05) by the method of paired comparisons.

S-adenosyl-L-methionine concentrations in rat liver

S-adenosyl-L-methionine (SAM) concentrations in the liver of animals fed the experimental diets and supplemented with equal molar cysteine or methionine (Table 18) indicate the same trend for both male and female animals. Animals supplemented with cysteine at the optimal level of dietary inorganic sulfate had a statistically non-significant average increase in the concentration of SAM in the liver when compared to animals fed the optimal percentage of inorganic sulfate supplemented with methionine. Both male and female rats fed the diets containing 0.42% of sulfate had increased liver S-adenosyl methionine irrespective of the amino acid supplemented, when compared to those rats fed either diets containing 0.0002 or 0.02% of inorganic sulfate.

^{14}C -methylation of tRNA in rat liver

The incorporation of the ^{14}C -methyl group from (methyl- ^{14}C) methionine in rats fed different levels of inorganic sulfate and supplemented with cysteine or methionine (Table 19) demonstrated that female animals supplemented with cysteine at the optimal percentage of dietary inorganic sulfate have a statistically significant ($p < 0.05$) decrease in the incorporation of carbon labeled methyl-group into tRNA compared to female animals supplemented with an equal molar amount of methionine. In male animals, there was an average decrease in the methylation of tRNA in cysteine versus methionine supplemented rats fed the optimal

TABLE 18

S-adenosyl-L-methionine (SAM) concentrations in the liver of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)		
	μ moles SAM/g liver		
	0.0002	0.02	0.42
Cysteine (0.505%)	61.8 $\pm$ 4.4 ^{1a2}	115.1 $\pm$ 28.8 ^b	95.2 $\pm$ 18.8 ^c
[♀] Methionine (0.620%)	91.0 $\pm$ 15.0 ^a	105.7 $\pm$ 22.8 ^{ab}	144.2 $\pm$ 33.8 ^d
Cysteine (0.505%)	70.8 $\pm$ 3.0 ^a	125.3 $\pm$ 21.0 ^b	85.6 $\pm$ 12.2 ^{ac}
[♂] Methionine (0.620%)	83.4 $\pm$ 11.0 ^a	106.2 $\pm$ 23.0 ^{ab}	135 $\pm$ 24.6 ^d

¹Values are means $\pm$ SEM of five animals per group.

²Mean values in a column or row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

TABLE 19

The incorporation of $^{14}\text{CH}_3$ from (methyl- ^{14}C) methionine into liver tRNA of animals fed different levels of inorganic sulfate (%) and supplemented with cysteine or methionine

S-containing amino acid added	Dietary inorganic sulfate (%)		
	cpm x 10^2 /mg RNA		
	0.0002	0.02	0.42
Cysteine (0.505%)	1.02 ± 0.17^{2a3}	0.93 ± 0.13^a	1.16 ± 0.23^b
♀ Methionine (0.620%)	1.04 ± 0.05^a	1.25 ± 0.20^b	1.65 ± 0.46^b
Cysteine (0.505%)	1.61 ± 0.63^b	1.89 ± 0.38^c	2.08 ± 0.34^a
♂ Methionine (0.620%)	2.33 ± 0.47^c	1.99 ± 0.21^c	2.93 ± 0.62^c

¹Values are means $\pm$ SEM of five animals per group.

²Mean values in a column or a row followed by the same superscript letter do not differ significantly ($p < 0.05$) by the method of paired comparisons.

percentage (0.02) of dietary inorganic sulfate, but this decrease was not statistically significant.

In both male and female animals, cysteine supplementation allowed dietary sulfate to stimulate increased methylation of tRNA in the liver of male and female animals fed the 0.42% level of inorganic sulfate. In male animals, there was a decrease in the methylation of tRNA when comparing the values for rats fed the 0.0002% to the optimal level of dietary inorganic sulfate when cysteine was added to the diet versus methionine.

Methionine supplementation in female rats allowed dietary sulfate to decrease the methylation of liver tRNA in animals fed the 0.0002% level of inorganic sulfate. There were no differences observed in male animals as to a sulfate effect when methionine, at a constant level, was supplemented at varying levels of dietary inorganic sulfate.

CHAPTER V

DISCUSSION

The physiological significance of the polyamines in biological processes has increased the understanding and application of these amines in both basic and clinical sciences (1,3,9,22,55). These physiologically potent compounds have been shown to influence physiological processes and metabolic alterations in an organism (1,5). Because of the effect the polyamines have upon an organism, it was appropriate to evaluate the effect of diet upon these amines and their biosynthesis in an animal. One of the sulfur-containing amino acids, methionine, because of its importance in the biosynthesis of the polyamines (27,39), was added at a constant level to diets containing varying levels of inorganic sulfate, including the optimal level reported by Smith (68). Cysteine has been reported to have a sparing effect upon methionine (61); therefore, another dietary variable was the substitution of equal molar amounts of cysteine for methionine in diets containing the same varying levels of inorganic sulfate as those to which methionine was added.

Data have been collected which show differences in enzyme activity, polyamine biosynthesis, tRNA methylation and S-adenosyl-L-methionine concentrations in female compared to male rats. These differences were anticipated and this study is not the first to indicate opposing responses to similar treatments in the different genders of an animal. Therefore, the gender related differences will

not be discussed but the agreement across dietary groups will be pointed out when the responses are similar in both genders.

The effect of dietary sulfur upon ornithine decarboxylase activity in rat tissue

Cysteine supplementation of diets containing the optimal level of dietary inorganic sulfate (0.02%) had a stimulating effect upon ODC activity in the liver, kidney, and brain tissues of male and female rats when these data were compared with those obtained from animals fed methionine supplemented diets at the same level of inorganic sulfate (Tables 3, 4, 5, pages 31, 33, 34). This increase in activity may be partially explained by comparing the structural similarities of cysteine to dimercaprol (2,3-dimercapto-1-propanol). As reported (29) dimercaprol has a stimulatory effect upon the decarboxylation of L-ornithine by ODC purified from rat prostate tissue.

Although these investigators observed that, in general, dithiols, as opposed to monothiol compounds, stimulated the enzyme activity of ODC, L-cysteine added to the enzyme preparation caused a decolorization of the reaction mixture due to the interaction of cysteine with the pyridoxal-5-phosphate added to the preparation (29). Therefore, the interpretation of their data was difficult. It is possible that increases of tissue cysteine may stimulate ODC activity and would provide an explanation for this observation.

A second consideration may also account for this increased activity. Reduced glutathione was evaluated by the above investigators (29) and was found to stimulate ODC activity in vitro, but to a lesser

extent than either dithiothreitol or dimercaprol. Smith (75) has reported liver and kidney glutathione levels to be slightly, but significantly increased in both the reduced and total form in rats fed diets containing equal molar levels of cysteine supplemented in the place of methionine to a 15% of casein based diet containing the optimal percentage (0.02%) of dietary inorganic sulfate. This observation can therefore contribute to the explanation of increased ODC activity in the tissues of animals receiving a diet optimal in sulfate and supplemented with an equal molar amount of cysteine versus methionine.

The inhibition of ODC by the polyamines (putrescine, spermidine and spermine), as well as other related amines has been reported but the mechanisms for their actions are not apparent (17). Because the activity of ODC in cultured eukaryote cells has been shown to be inhibited by low concentrations of the polyamines as well as higher concentrations, Canellakis et al. (4) have suggested that the extracellular concentrations of the polyamines, in low concentrations, may be acting on specific cell membrane sites to mediate ODC activity. In the data presented here, putrescine, spermidine, and spermine tissue levels are consistently higher in the liver of male rats supplemented with cysteine versus methionine supplemented rats at the 0.02% level of dietary inorganic sulfate. It would be presumptuous to speculate with respect to the difference in the concentrations of extra- or intra-cellular levels of the polyamines in the data collected from the above tissues; however, an extra-cellular spermidine level in concentrations of 10^{-7} M in vitro has been shown to be a potent inhibitor of ODC activity (4).

Spermidine concentrations in the liver tissues of male animals and in the kidney and brain tissues of female animals supplemented with cysteine are greater than in those animals supplemented with methionine at the optimal percentage of dietary inorganic sulfate. The ratio of intra- to extra-cellular spermidine levels could possibly explain decreased ODC activity in methionine supplemented animals if this amine could be found in higher concentrations, extra-cellularly, in these animals.

The sulfate effect upon the activity of ODC in male and female animals is not as clear-cut as the comparison for cysteine versus methionine at the optimal percentage of dietary inorganic sulfate. In female animals, the supplementation with cysteine allowed dietary inorganic sulfate to express differences in the activity of ODC in all tissues of animals fed the 0.0002% level of inorganic sulfate. One explanation for the decreased ODC activity may be a decrease in free coenzyme A in the liver, kidney, and brain of animals fed the 0.0002% level when compared to the 0.02% level of dietary inorganic sulfate. Smith (75) has reported a decreased amount of free coenzyme A in the liver of animals consuming a diet low in sulfate. Although it has been reported (29) that coenzyme A, as a thiol compound is inert in the catalytic activity of ODC, the investigators point out that coenzyme A may possibly activate other forms of ODC in tissue. This may be the response in female rats fed the diets low in sulfate. This explanation becomes more firm when examining the data for ODC activity in female rats fed the low level of dietary inorganic sulfate

with added methionine. In liver, kidney and brain tissues, methionine supplementation allowed dietary inorganic sulfate to have a stimulatory effect upon the activity of ODC in female animals fed the 0.0002% level of inorganic sulfate. If a rat is forced to satisfy its sulfate requirement by endogenous synthesis, a methionine deficit would be created (61). Omission of methionine from the diet of a rat has been shown to increase hepatic ODC activity (44); this would be consistent with the data presented here.

The effect of dietary sulfur upon S-adenosyl-L-methionine decarboxylase activity in rat tissue

A decrease in SAMD activity in male and female rats fed a diet containing 0.02% of inorganic sulfate and supplemented with cysteine when compared to animals receiving a methionine supplemented diet at the 0.02% level of inorganic sulfate was observed. Because spermine inhibits SAMD at physiological concentrations (17) the spermine concentrations observed should be considered also at this point (Tables 11, 14, 17, pages 43, 48, 52). The data obtained from liver and kidney tissues in male rats demonstrated that dietary cysteine supplementation at the optimal level of inorganic sulfate had a stimulating effect upon the production of spermine when compared to the tissue concentrations of animals fed an equal molar methionine supplemented diet.

This response (increased spermine, decreased SAMD activity) is in agreement with the suggested interrelationship between the activity of SAMD and the tissue concentration of spermine. Those animals receiving a diet of added cysteine at the 0.02% level of inorganic

sulfate had an inhibition of SAMD that can be attributed to the increase in spermine tissue concentrations in these animals. Likewise, animals fed a cysteine supplemented diet at the 0.02% level of inorganic sulfate had decreased SAMD activity that was not inhibited by the increased production of spermine in the tissue. This can be further substantiated when the SAM concentrations for liver are observed (Table 18, page 54). The increase in SAM average in the liver tissues of animals fed the 0.02% level of inorganic sulfate concentration and supplemented with cysteine compared to methionine supplemented rats would be consistent with a decreased enzyme activity and increased liver spermine levels in cysteine supplemented rats (17). Although S-adenosyl-homocysteine (SAH) and MTA were not measured, these data seem to indicate that the parameters measured do show an influence of cysteine and methionine upon SAM, SAMD, and spermine interactions. Another possible explanation may be the sparing effect that cysteine has upon methionine (61). This can be seen in the concentration of SAM in the liver of animals fed the cysteine supplemented diet, when increases of SAM concentrations were observed.

The sulfate effect upon SAMD activity is again variable between female animals and male animals. However, in comparing the tissues from the animals studied, in most cases the increased SAMD activity in the tissue of an animal consuming a particular diet is consistent with decreased spermine concentrations in that tissue. Further decreases in tissue SAMD activity usually are indicative of increased spermine concentrations in the tissue. This would be consistent with the observation that spermine inhibits SAMD activity (17).

The effect of sulfur nutrition upon the formation of the polyamines

The results for the formation of tissue putrescine are variable in both male and female animals and seem to follow no dietary pattern (Table 9, 12, 15, pages 41, 45, 49). Because ODC activity was stimulated in rats fed cysteine supplemented diets containing 0.02% of inorganic sulfate, this increase in tissue ODC activity might have been expected to yield a statistically significant increase in putrescine concentrations in the tissues of these animals. This significant increase in putrescine was not obtained in liver or brain tissue, but did occur in the kidneys of female animals fed the optimal percentage of inorganic sulfate with added cysteine. In the liver tissues from male and female animals fed the optimal level of inorganic sulfate and supplemented with cysteine, there was a trend in male liver tissue and female brain tissue to have increased concentrations of putrescine in response to the added cysteine versus methionine diet.

Although spermidine synthase was not measured in these animals, it could be postulated that putrescine was converted to spermidine rapidly as a result of a possible stimulatory effect by cysteine upon this enzyme. This may be the case because there was an increase in spermidine in several tissues (liver and brain) examined from animals fed the optimal level of inorganic sulfate and supplemented with equal molar amounts of cysteine when compared to decreased spermidine levels in methionine supplemented rats (Tables 10, 13, 17, pages 42, 46, 52).

It has been reported (29) that following partial hepatectomy, increases in ODC activity and increases in putrescine activate SAMD

which, in turn, increase the formation of spermidine. However, increases in SAMD activity were not observed in the animals receiving added cysteine at the 0.02% level of inorganic sulfate. Maudsley (17) has summarized that there is a wide variation between the activity of ODC and SAMD, and "that a coordinated response of the two enzymes is a comparative rarity." This variation between the activity of ODC and SAMD demonstrated with these data, underscores the above statement.

A sulfate effect upon the formation of putrescine in male rat tissues is consistent with the reported data by Smith (75) which has been collected over a period of several years. The observation that the optimal level of dietary inorganic sulfate (68) is necessary for the avoidance of metabolic alterations in an animal is strengthened when the data relating dietary sulfate and putrescine are considered. Because putrescine is a powerful physiological manipulator, the increased accumulation of this amine in tissues of rats fed diets high in sulfate could have profound metabolic implications.

Liver spermidine concentrations showed an increase in male animals supplemented with cysteine at the optimal level of inorganic sulfate when compared to animals supplemented with methionine at the 0.02% level of inorganic sulfate. Spermidine synthase was not measured in liver tissues from these animals, but it is possible that this enzyme was stimulated by dietary cysteine resulting in an increase in the accumulation of spermidine in the liver of those animals fed the 0.02% level of inorganic sulfate supplemented with cysteine. Because there is a decrease in the SAMD activity in liver tissues from animals fed cysteine supplemented diets containing the optimal percentage of

inorganic sulfate when compared to animals fed methionine supplemented diets, there may be an increase in the propylamine group available for the formation of tissue spermidine in animals fed the cysteine supplemented diet (35); therefore increasing the formation of spermidine.

The data collected seem to indicate that SAM is available for decarboxylation by SAMD in the liver of cysteine supplemented animals, but because of decreased SAMD activity in these animals this indicates that increased tissue spermidine levels inhibited SAMD activity in the cysteine supplemented animals.

The increased spermine concentrations in the liver and kidney tissues from male rats supplemented with equal molar cysteine versus methionine at the optimal level of inorganic sulfate, indicates that cysteine stimulates the formation of spermine. This may be the result of a stimulatory action upon spermine synthase. The observed increase in spermine tissue concentrations would be consistent with the observed decreases in SAMD tissue activity because increased spermine concentrations in tissue have been shown to inhibit SAMD activity (35).

Methylation of tRNA

The decrease in methylated tRNA in female rats and statistically non-significant average decrease in male rats fed diets supplemented with cysteine at the optimal level of inorganic sulfate when compared to methionine supplemented animals indicated that methylation can be altered by diet (Table 19, page 55). Further, the average decrease in tRNA methylation seen in the liver from cysteine supplemented animals might be attributed to the increases in liver spermidine levels as a result of feeding animals the dietary regimen of added cysteine at the

optimal percentage of inorganic sulfate. Increases in tissue spermidine and spermine concentrations may inhibit protein biosynthesis (4). The data collected here indicate that increases in these two amines may be the contributing factors for average decreases observed in the methylation of tRNA in animals fed cysteine supplemented diets at the optimal level of inorganic sulfate. This is consistent with the increased SAM concentrations in the liver tissue from these animals indicating that the methyl-groups are available to the methyltransferases for methyl-group transfer to tRNA. Spermine and spermidine have been shown to modify both the extent and the pattern of tRNA methylation by specific methylases (35); an observation which supports the average results obtained in this investigation.

The data for those animals receiving the optimal level of inorganic sulfate and supplemented with cysteine or methionine prove further the metabolic inequivalency of these two sulfur-containing amino acids in the biosynthesis and formation of the polyamines. Further, the differences Smith (75) observed between cysteine and methionine supplementation in the rat at the optimal level of dietary inorganic sulfate can be explained by the data collected during this investigation and from existing research data that have appeared in the literature.

Jamdar (51) reported changes in lipid formation as a result of spermine and spermidine alterations in the microsomal fractions from the epididymal fat pads of male Sprague-Dawley rats. However, the reported responses (1) of the polyamines upon the metabolism of carbohydrates and lipids have not agreed with the results of Jamdar. The

findings of Smith (75) indicate that cysteine (0.505%) added to the diet of a rat consuming the optimal level of inorganic sulfate forces the animal to store excess energy in the form of glycogen when compared to those animals receiving a similar diet but supplemented with an equal molar amount of methionine (0.620%). In the methionine supplemented animals, excess energy was shown to be stored as lipid. If increases in spermine and spermidine levels increase lipolysis (1) then the observations reported by Smith may be explained with the data presented in this investigation. Decreases in spermine and spermidine in the tissues of methionine supplemented animals, could increase the lipid content of the body. The increase in glycogen storage reported in cysteine supplemented animals may be a result of the increases in spermidine and spermine, thereby decreasing the animal's ability to store excess energy as lipid. This alteration in metabolism may be influenced by the polyamines' ability to alter the enzymatic machinery of carbohydrate and lipid biosynthesis (1,50,51).

The increase in the glucoronide-salicylamide : sulfate-salicylamide (GS : SS) ratio in the urine of rats fed diets containing 15% of casein and supplemented with cysteine at the optimal percentage of dietary inorganic sulfate when compared to methionine supplemented rats (74), may be a result of the increase in spermidine and spermine levels in those rats supplemented with cysteine and fed the 0.02% level of inorganic sulfate. Although the action of the polyamines upon the activity of uridinephosphoglucuronate transferase has not been reported, the increase in this enzyme by spermidine and/or spermine

would explain the results reported by these investigators. Several reports have appeared implicating the polyamines in the detoxication of drugs and xenobiotics (1,5,55).

CHAPTER VI

SUMMARY

The purpose of this study was to investigate the effects of sulfur nutrition upon the formation and biosynthesis of the polyamines in the rat. In this investigation further documentation was obtained to support the concept that variation from the established, optimal dietary inorganic sulfate level in the monogastric animal could lead to metabolic alterations. The change in tissue ODC activity, SAMD activity, concentrations of tissue polyamines, SAM concentrations in liver tissue, and the alteration in the methylation of tRNA are further evidence for the necessity of a dietary inorganic sulfate level that will meet the required sulfate needs of the monogastric animal and avoid the alterations in metabolism that have been documented in this laboratory.

The supplementation of cysteine or methionine in equal molar amounts to the diet containing the optimal percentage (0.02%) of inorganic sulfate provides further evidence that cysteine and methionine are inequivalent as dietary constituents. Cysteine supplementation (0.505%) to the optimal level of dietary inorganic sulfate (0.02%) had a stimulatory effect upon the activity of L-ornithine decarboxylase in liver, kidney and brain tissues from these animals. Further, cysteine supplementation at the optimal level of inorganic sulfate had a suppressive effect upon the activity of S-adenosyl-L-methionine decarboxylase in the liver from both male and female animals and the brain

tissues from male animals. Formation and/or accumulation of the polyamines, spermidine and spermine, was stimulated in the liver tissues of male animals receiving the cysteine supplemented diet at the optimal level of inorganic sulfate. Putrescine formation in liver, kidney, and brain tissues was not significantly affected at the 0.02% level of dietary inorganic sulfate when cysteine was added to the diet of animals at the optimal level. Increased S-adenosyl-L-methionine concentrations in the livers of cysteine supplemented animals indicated a decreased SAMD activity in these animals. Decreased methylation of tRNA in female animals given a pulse dose of (methyl-¹⁴C) methionine and receiving a diet supplemented with cysteine at the optimal level of dietary inorganic sulfate, may indicate a change in protein biosynthesis when compared to animals receiving a diet supplemented with methionine at the 0.02% level of inorganic sulfate.

The significance of this study is that it provides evidence that dietary factors, and in particular sulfur nutrition, affect the formation and/or the accumulation of the polyamines. Because the optimal level of dietary inorganic sulfate has been firmly established, it is suggested that further research in this area concentrate upon the effect of supplementing cysteine or methionine in graded amounts to diets containing the optimal percentage (0.02) of inorganic sulfate.

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