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The Influence of Dietary Sulfate on the Excretion of ^{35}S-Cysteine Sulfur as ^{35}S-Taurine Sulfur by the Rat

Betty Ann Whittle
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To the Graduate Council:

I am submitting herewith a dissertation written by Betty Ann Whittle entitled "The Influence of Dietary Sulfate on the Excretion of ^{35}S-Cysteine Sulfur as ^{35}S-Taurine Sulfur by the Rat." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Frances A. Schofield, Mary Rose Gram, Bernadine Meyer

Accepted for the Council:
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Vice Provost and Dean of the Graduate School

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

July 28, 1970

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THE INFLUENCE OF DIETARY SULFATE ON THE EXCRETION OF ^{35}S-CYSTEINE SULFUR AS ^{35}S-TAURINE SULFUR BY THE RAT

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Betty Ann Whittle

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ABSTRACT

The relationship of dietary sulfate to the efficiency of feed utilization, excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur, and sulfation of lung tissue by the rat was investigated.

The feed efficiencies of animals fed diets from weaning that contained 0.10 per cent of inorganic sulfate and 0.47 per cent of organic sulfur as sulfate were significantly higher at the end of a six-week feeding period than were those of littermates fed diets that contained higher or lower levels of inorganic sulfate but comparable levels of total sulfur as sulfate. These findings showed that equivalent levels of sulfur as sulfate supplied by amino acids did not compensate for the omission of sulfate from the diet of the rat.

Results of a subsequent experiment revealed that the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur at the end of a 17-day feeding period was 58 and 82 per cent lower, respectively, among adult rats fed normal and high levels of inorganic sulfate than among those fed low levels of inorganic sulfate in diets that contained equal levels of total sulfur as sulfate. Reductions of 50 and 45 per cent, respectively, from the level of ^{35}S-taurine excreted

by the animals fed the low sulfate diet were observed when normal and high levels of sulfate were fed in diets that contained equal levels of ^{35}S-cysteine.

When 18 groups of rats were fed different levels of inorganic sulfate in diets supplemented with 0.40 per cent of cysteine, differences could be detected neither in ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur excreted in the urine nor in the total sulfur as sulfate in the lungs at the end of the six-week feeding period.

After the experimental diets had been consumed for one week, the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur was significantly higher among weanling rats fed a low sulfate diet than among those fed a normal sulfate diet that contained the same level of cysteine, but no differences could be detected by the end of week 2. Adult rats fed the low sulfate diet excreted significantly higher levels of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur at the end of weeks 1 and 2 of the study than did the animals fed the normal sulfate diet, but not at the end of week 3.

Dietary adaptation, which results in reduction in the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur to the level excreted by the animal fed the normal sulfate diet, occurs when low sulfate diets are fed to rats for extended periods. The initial rise in cysteine sulfur as

taurine sulfur in the urine illustrates an inefficiency in the oxidation of amino acid sulfur to sulfate. The significance of the adaptation to the low sulfate diet, demonstrated by decreased ^{35}S-taurine excretion, as a means of conserving the sulfur-containing amino acids is not revealed by the present findings.

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INTRODUCTION

Considerable evidence has been obtained that the nutritional status of the rat may be improved by inclusion of sulfate in its diet. It has been demonstrated in the laboratories of the Nutrition Department, the University of Tennessee, Knoxville, that, although the sulfur-containing amino acids are supplied at a level reported to be adequate for maintenance and growth, avitaminosis E is enhanced and collagen metabolism impaired when sulfate intake of the rat is restricted. In addition, the degree of incorporation of ^{35}S-sulfate into rib cartilage mucopolysaccharides has been found to change when the ratio of organic to total sulfur fed to adult rats in diets with constant total sulfur as sulfate is varied. Maximum uptake of a test dose of ^{35}S-sulfate does not occur when so-called "adequate" levels of organic sulfur are supplied if inorganic sulfate is not included in the diet. Rather, the requirement for the sulfur-containing amino acids, as indicated by the uptake of ^{35}S-sulfate, is increased to a level greater than that theoretically necessary to produce endogenous sulfate equivalent to the sulfate that has been omitted from the diet. Not only do these findings refute

reports that the level of inorganic sulfur in the diet of the non-ruminant mammal is inconsequential, but they also indicate that the obligatory oxidation of amino acid sulfur to sulfate is not an efficient process and that additional oxidation products are formed.

Taurine, which generally is considered an organic end product of the metabolism of the sulfur-containing amino acids, is excreted in the urine in varying amounts. Since cysteinesulfinic acid may be a common intermediate in the formation of sulfate and taurine from the sulfur-containing amino acids, it seemed reasonable to assume that increased demands for sulfate synthesis would result in increased production of cysteinesulfinic acid and, without metabolic control, could lead to increased taurine synthesis.

In view of these considerations, the present study was designed to compare the cysteine sulfur excreted as taurine sulfur in the urine of rats fed varying levels of inorganic sulfur as sulfate.

CHAPTER II

REVIEW OF THE LITERATURE

Present knowledge of the chemistry and metabolism of the sulfur-containing compounds has been likened to an "unfinished mosaic" (1). While it long has been recognized that sulfur is required by all living organisms, definitive knowledge of many pathways and enzymes involved in its utilization has not been obtained.

Sulfur is ingested by higher animals as a constituent of organic and inorganic compounds. The organic fraction normally predominates with the amino acids cysteine, cystine, and methionine furnishing the greatest portion of the animal's sulfur. The importance of these compounds as well as the sulfur-containing vitamins thiamine, biotin, and lipoic acid has been reviewed extensively (1-5). Dietary inorganic sulfur exists primarily as sulfate and sulfide (1). While its role traditionally has been discounted (2, 3), evidence is mounting that sulfate sulfur is important in the nutrition of the non-ruminant mammal.

Evidence for the conversion of cystine to taurine in the dog was presented in 1904 (6) and 30 years later the oxidation of cysteine to sulfate in the presence of rat tissue preparations was demonstrated (7). Today, both

taurine and sulfate are recognized as quantitatively important products of sulfur metabolism, but many questions concerning their production remain unanswered. The metabolic relationship of neither the sulfur-containing amino acids nor the inorganic sulfur-containing compounds to the production of taurine and sulfate has been elucidated fully.

Von Bergmann (6) and Wohlgemuth (8) are credited with having established the in vivo conversion of cystine to taurine (9, 10). After feeding cystine and sodium cholate, von Bergmann (6) observed an increase in the excretion of taurocholic acid by bile fistula dogs. The increase diminished when sodium cholate alone was administered and disappeared when only cystine was fed. These findings were interpreted to indicate that cystine could serve as a precursor of taurine and that repeated feeding of cholic acid depleted the taurine supply. Wohlgemuth (8) found that the amount of neutral sulfur excreted by cats was much greater after cystine had been administered and, since he could detect no cystine in the urine, concluded that it had been converted to taurine.

Results of subsequent investigations (9-13) supported the ideas that had been advanced and demonstrated that the administration of methionine (10) or cysteine (13) with cholic acid to the bile fistula dog yielded results similar to those obtained with cystine. The effects

could not be attributed to simple amino acid stimulation since addition of alanine to the diet failed to change taurocholic acid excretion (10).

White (14) used weight gain rather than taurocholic acid excretion as a criterion for evaluating taurine production. Based on his observation that the administration of cystine or methionine resulted in resumption of growth in young rats fed a low casein diet with high levels of cholate, this worker also concluded that cystine was needed for taurine synthesis and suggested that a metabolic interrelationship existed among the sulfur-containing amino acids. Since taurine was incapable of relieving the deficiency imposed on the animals by cholic acid, he concluded that cystine and methionine must have a metabolic role in addition to the one in taurine formation.

These findings plus the report by White and Fishman (15) that the reaction could occur in vitro left little doubt concerning the conversion of cystine to taurine in higher animals. The in vitro process, which involved the oxidation of cystine to cysteic acid with subsequent transformation to taurine, was reported in 1903 (16), but other workers had been unable to repeat the second step of the reaction.

Possible intermediates in the synthesis of taurine were suggested by Virtue and Doster-Virtue (12, 13, 17)

who studied the effects of various sulfur-containing compounds administered with cholic acid to fasted bile fistula dogs. An increase in the excretion of taurine as taurocholic acid occurred when cystine disulfoxide, cysteinesulfinic acid, cysteic acid, homocysteine, cysteine, cystine, methionine, or methionine sulfoxide was administered but the effect was not observed when homocystine or thioglycolic acid was given. The urinary sulfur excretion patterns indicated that the more highly the sulfur was oxidized in the compound administered, the more difficult became the oxidation to sulfate (17). From these studies, it was suggested that one pathway of methionine catabolism might be the series methionine, homocysteine, cysteine, and taurine (13).

Evidence for additional pathways was furnished by Pirie (7), Medes (18), and Medes and Floyd (19, 20). The oxidation of the sulfur of methionine, cystine, and cysteine to inorganic sulfate by preparations of rat liver and kidney was demonstrated by Pirie (7) who concluded that sulfate formation proceeded through the oxidation of sulfhydryl groups, to which methionine and cystine were reduced enzymatically. He suggested that cysteinesulfinic acid was an intermediate which could be converted to sulfite and finally to sulfate. This was supported by the observation of Medes (18) that cysteine in vivo gave rise to sulfate

more rapidly than did cystine and that the most rapid conversion was from cysteinesulfinic acid. Rat tissue enzymes were discovered which could oxidize cysteinesulfinic acid to sulfate, cysteine to cysteic acid, cysteine to cystine, and cystine to sulfate (18). Medes and Floyd (19) found no evidence that the conversion of cystine to cysteine was enzymatic and they suggested, in contrast to Pirie (7), that the reaction involved non-enzymatic hydrolysis to cysteine and cysteinesulfenic acid, followed by spontaneous dismutation to cysteinesulfinic acid. They also suggested that cysteine might be converted to the sulfenic acid by an enzyme active in the conversion of cysteine to cysteic acid. They found no evidence that cysteic acid could give rise to sulfate (19, 20), but observed that it could be decarboxylated by rat kidney and intestine preparations to form taurine (20).

Two enzymes systems capable of acting on cysteinesulfinic acid were extracted from rabbit liver by Bergeret and Chatagner (21). One of the systems transformed the sulfinic acid into alanine and sulfite while the other yielded 2-aminoethanesulfinic acid and carbon dioxide. This was the first indication that 2-aminoethanesulfinic acid, which these workers named hypotaurine, was an intermediate in cysteine metabolism. They concluded that both systems must play an active role in the living organism

since the intravenous injection of cysteinesulfinic acid in rats was followed by formation of alanine and hypotaurine in the liver (22).

Awapara (23) reported that the intravenous injection of cysteine in rats resulted in increased alanine and taurine in the liver, but later reported (24) that the fraction originally identified as alanine could be separated into two components. One he identified as alanine and the other corresponded to Bergeret and Chatagner's (21) hypotaurine. He concluded that cysteine probably was oxidized to cysteinesulfinic acid, which was subsequently decarboxylated to hypotaurine (24).

Since the reports of their activities by Medes and Floyd (20) and Bergeret and Chatagner (21), the decarboxylases that act on cysteic and cysteinesulfinic acids have been studied extensively. A soluble enzyme, L-cysteine sulfinate carboxy-lyase (EC 4.1.1.29), has been partially purified from rat tissues (25, 26) and, while its substrate specificity is not known precisely, evidence derived from inhibition experiments indicated that it decarboxylated both cysteic and cysteinesulfinic acids (25-28). Support was given by observations that male rats yielded liver extracts with higher activity than did females toward both substrates (27, 29, 30), competitive inhibition occurred when both amino acids were added to liver preparations in

equal concentrations (26), and pyridoxine deficiency resulted in loss of activity toward both substrates (30-35).

Numerous differences between reactions observed in brain and liver preparations led to the conclusion that cysteic and cysteinesulfinic acid decarboxylases in those two tissues are isozymes (25, 26, 36). It was found that after feeding rats a diet deficient in vitamin B_6, brain cysteinesulfinic acid decarboxylase activity disappeared slowly and could be reactivated in vitro by addition of pyridoxal phosphate, whereas in liver the activity disappeared rapidly and could not be reactivated in vitro (25, 32). Also, the rates of decarboxylation of cysteinesulfinic and cysteic acids were observed to be more rapid with brain than with liver preparations (25). Next, L-glutamic acid was found to interfere with the decarboxylation of cysteinesulfinic acid by brain enzyme but to have no effect on liver enzyme activity (25, 26). Finally, it was observed that dialyzed liver preparations, incubated with glutathione alone, did not require the addition of pyridoxal phosphate in order for enzymic activity to be restored, whereas dialyzed brain preparations did (26).

That pyridoxal phosphate has a role in the decarboxylation of cysteinesulfinic and cysteic acids is well

established, but the exact nature of its involvement has not been determined (30-35, 37). Inability to reactivate in vitro the cysteinesulfinic acid decarboxylase activity in livers from rats fed vitamin B_6-deficient diets was interpreted to indicate loss of apoenzyme in the absence of cofactor (30, 32). Bergeret and coworkers (32) suggested that pyridoxal phosphate had a stabilizing influence on the enzyme. Greengard and Gordon (35) observed an increase in liver cysteinesulfinic acid decarboxylase activity when pyridoxine was administered to vitamin B_6-deficient rats over a six-hour period before they were sacrificed, but the increase was not observed when puromycin also was administered. These findings indicated that, in addition to regulating the activity of the existing enzyme, pyridoxal phosphate might influence de novo enzyme synthesis (35, 38).

Considerable information has been obtained concerning the distribution of cysteinesulfinic and cysteic acid decarboxylase activities in mammalian tissues. Activity has been found in brain tissue from every species studied (37) and in liver extracts from dogs (26, 27, 30, 36, 39), mice (27, 30), pigs (39), cows (37), guinea pigs (27, 30, 38), and rats (25-27, 30, 32, 36, 39). The enzyme has not been found in liver extracts from cats or man (30, 37, 39), two species known to excrete taurine, and it also is absent

from mammalian heart tissue where taurine is found in high concentrations (26). These observations appear to support the idea that taurine synthesis can occur by pathways other than those involving cysteic or cysteinesulfinic acid (30, 37).

In all tissues and species in which decarboxylase activity has been demonstrated, cysteinesulfinic acid has been decarboxylated much more rapidly than cysteic acid, indicating that cysteinesulfinic acid is the preferred substrate (26). This is consistent with the detection by Awapara and Wingo (28) of significant amounts of ^{35}S-hypotaurine and ^{35}S-taurine but no labeled cysteic acid in livers from rats injected with a small dose of ^{35}S-cysteine. Although the injection of large quantities of cysteic acid resulted in increased taurine production, the absence of labeled cysteic acid following the administration of the small amount of ^{35}S-cysteine indicated that cysteic acid was not the most likely precursor of taurine (28).

The pathway from cysteinesulfinic acid via hypotaurine is accepted generally as a significant means of taurine synthesis, but very little is known about the biochemistry of the step from hypotaurine to taurine (37). Cavallini et al. (40) were unable to demonstrate the in vitro conversion of hypotaurine to taurine by rat tissue homogenates, although taurine excretion was higher among rats given

hypotaurine. Eldjarn et al. (41) found that, 30 minutes after injecting mice with ^{35}S-hypotaurine, the amount of labeled taurine exceeded that of labeled hypotaurine in the serum. In 1962, Sumizu (42) reported the presence of hypotaurine dehydrogenase in the soluble fraction of rat liver. The enzyme was very unstable and required NAD as cofactor. No other reports directly related to this reaction were found in the literature.

Another route of cysteinesulfinic acid metabolism was shown by the work of Singer and Kearney (43-45). They demonstrated that mitochondrial acetone powder of rat liver homogenates catalyzed the coupled oxidation of cysteinesulfinic acid and fumarate or malate to yield pyruvate, aspartate, and inorganic sulfate. Analogous reactions between cysteinesulfinic acid and α-ketoglutarate or oxaloacetate to yield pyruvate, sulfate, and glutamate or aspartate, respectively, were catalyzed by the same preparations. The reactions involved transamination of cysteinesulfinic acid with α-ketoglutarate or oxaloacetate to form β-sulfinylpyruvate which was rapidly cleaved to pyruvate and sulfite. Sulfite oxidase, an enzyme present in liver but not in heart mitochondria, oxidized sulfite to sulfate. They observed that glutamic-oxalacetic transaminase catalyzed the reaction of cysteinesulfinic acid and α-ketoglutarate, and suggested that it might be the enzyme

responsible for the transamination of cysteinesulfinic acid. A non-transaminative oxidation of cysteinesulfinic acid to pyruvate, sulfate and ammonia in fresh preparations of rat liver mitochondrial acetone powder with a requirement for NAD, an autooxidizable dye, and diaphorase also was observed. Since the amounts of cysteic acid detected in reaction mixtures with cysteinesulfinic acid and the mitochondrial preparations never represented more than a few per cent of the cysteinesulfinic acid utilized, Singer and Kearney concluded that direct oxidation of the sulfur moiety of cysteinesulfinic acid to cysteic acid did not represent a quantitatively important reaction. They stated that the main products of cysteinesulfinic acid metabolism, pyruvate and sulfate, also were the main products of cysteine metabolism in mammals and that cysteinesulfinic acid was part of the main pathway of cysteine metabolism (43-45).

Evidence for the link between the sulfur-containing amino acids and cysteinesulfinic acid was furnished by Chapeville and Fromageot (46) who demonstrated the presence of ^{35}S-cysteinesulfinic acid in the livers of rats injected with ^{35}S-cystine. Similarly, Peck and Awapara (47) demonstrated the conversion of ^{35}S-methionine to cysteine and cysteinesulfinic acid in rat brain slices and homogenates. According to Jacobsen and Smith (37), rat brain is the only mammalian tissue in which cysteinesulfinic acid has been

detected without preloading the animal with cysteinesulfinic acid precursors (48). Wainer (49) attributed failure to detect this intermediate in other tissues to the presence of cysteinesulfinic acid decarboxylase activity.

More recently, the presence of an enzyme that catalyzed the oxidation of cysteine to cysteinesulfinic acid was demonstrated in 105,000 X g supernatant of rat liver (49-51). Its activity was stimulated by reduced NADP, ferrous ions, and mitochondria or microsomes (51). Very little activity was detected when D-cysteine, glutathione, or cysteamine served as substrate, and activity toward L-cystine was only 12 per cent of that toward L-cysteine (49). Wainer (52) previously had demonstrated the production of significant amounts of sulfate from cysteine in rat liver mitochondria without the formation of cysteinesulfinic acid. This, coupled with his observation that the enzymes for the production of hypotaurine from cysteine were prsent in the supernatant with the enzyme for cysteinesulfinic acid synthesis, led him to conclude that the major role of cysteinesulfinic acid probably was related to the synthesis of taurine (49).

Wainer (53) has pointed out that, quantitatively, the most important pathway for the metabolism of cysteine sulfur in mammals involves the formation of inorganic sulfate. In light of this, his conclusion that the major

role of cysteinesulfinic acid relates to taurine synthesis (49) appears to be in conflict with the earlier concept of cysteinesulfinic acid as a key intermediate in the formation of inorganic sulfate (43-45, 53). Based on his observation that the mitochondrial fraction of rat liver was more active than microsomes or 105,000 X g supernatant fraction in the production of ^{35}S-sulfate from ^{35}S-cysteine, Wainer (53) suggested that the mitochondrial process was of greatest quantitative significance. Pyruvate, sulfate, acetate, and alanine were identified as the products when cysteine was added to the mitochondrial incubation mixture, and neither cysteinesulfinic acid, β-mercaptopyruvate (BMP), nor free hydrogen sulfide was detected as an intermediate in the reaction sequence. It was suggested, however, that hydrogen sulfide might be an intermediate bound to an enzyme, and then exchanged.

BMP was suggested as a possible intermediate in cysteine degradation by Meister et al. (54) who observed that it was converted to pyruvate by rat tissue preparations. When certain thiol compounds were present in the preparation, hydrogen sulfide was formed. Otherwise, free sulfur was released (54). Other workers observed that the addition of cyanide to liver preparations with BMP resulted in the formation of thiocyanate (55, 56), while the addition of sulfite or sulfinates yielded thiosulfate or thiosulfonates, respectively (56). Demonstration of the enzymic

formation of thiosulfate from BMP and sulfite led Sorbo (56) to propose a sequence of reactions through which one molecule of thiosulfate could be produced from two of cysteine. According to his scheme, one cysteine molecule is transaminated to form BMP. A second cysteine molecule is converted to sulfite and pyruvate via cysteinesulfinic acid (43-45). Finally, sulfur is transferred from BMP to sulfite, yielding thiosulfate (56). In 1967 Szczepkowski and Wood (57) stated that, while deamination of cystine to form BMP had been observed and a mercaptopyruvate transsulfuration enzyme had been found, the concerted action of the two enzymes to transfer cystine sulfur to an ultimate acceptor had not been demonstrated.

The conversion of thiosulfate to sulfate has been demonstrated in rat tissue preparations (58-60) and in the intact rat (61, 62). Sulfite formed from cysteinesulfinic acid (43-45) generally has been considered the most important precursor of sulfate (2, 58), but observations of Szczepkowski et al. (61) were not interpreted as supporting this theory. When rats were injected with ^{35}S-cystine, most of the radioactivity appeared in the urine as sulfate. Injection of unlabeled thiosulfate with the ^{35}S-cystine resulted in the excretion of much more radioactivity as thiosulfate and less as sulfate. It was concluded that the large external dose of thiosulfate

inhibited the oxidation of endogenous thiosulfate to sulfate (61). The mechanism of the transformation was postulated by Sorbo (59) to involve reduction of thiosulfate to sulfite with dihydrolipoate or glutathione, followed by oxidation to sulfate. Rhodanese was shown to catalyze the thiosulfate reduction with dihydrolipoate, as well as the reaction between thiotaurine and cysteinesulfinic acid to give hypotaurine and thiocysteate (58). Thiosulfate reductase catalyzed the reduction of thiosulfate to sulfite with glutathione (59), and it appeared that this glutathione-dependent enzyme was more important than rhodanese in the oxidation of thiosulfate. The presence of both enzymes has been demonstrated in rat liver homogenates (58-61).

The relative velocities of sulfate formation from the two atoms of thiosulfate by rat liver mitochondria in the presence of glutathione were studied by Koj et al. (60). Observations that ^{35}S-sulfate accumulated more rapidly when inner-labeled $(S\cdot{}^{35}SO_3)^=$ rather than outer-labeled $(^{35}S\cdot SO_3)^=$ thiosulfate was added to the medium led them to conclude that sulfate was formed preferentially from the inner sulfur atom. This appeared consistent with reports by Skarzynski et al. (62) that 82 per cent of the inner sulfur atom of thiosulfate compared with 15 per cent of the outer sulfur atom administered to rats was detected

in the urine within the first six hours following administration. Koj et al. (60) also observed that thiosulfate labeled in both sulfur atoms was formed during $^{35}S \cdot SO_3^=$ oxidation, which indicated to them that the outer sulfur atom was incorporated into the inner position before oxidation to sulfate. Although their data appeared to suggest that this was the primary fate of the outer sulfur atom, the existence of other intermediates could not be discounted since the amount of sulfate produced did not account for the amount of sulfur in the thiosulfate utilized (60).

More recently, Schneider and Westley (63) reported that the intraperitoneal injection of tracer quantities of $^{35}S \cdot SO_3^=$ into weanling rats resulted in oxidation and excretion of 40±10 per cent of the labeled sulfur atoms as sulfate. Some of the retained outer sulfur atoms were incorporated into cystine while others were detected in polythionates and as elemental sulfur associated with serum proteins. Absence of incorporation of radioactivity into cysteine when the administered thiosulfate was labeled in the inner sulfur atom was interpreted by these investigators to indicate that sulfur in vivo is irreversibly lost when it is highly oxidized. They postulated that the outer sulfur atom of thiosulfate is at a metabolic branch point from which it can be oxidized to sulfate or retained in a

pool of active sulfur (63). Koj et al. (60) suggested that the thiosulfate oxidation system may be a physiologically significant means of slowing down the irreversible oxidation of sulfide and sulfite to sulfate.

Thiosulfate was reported by Szczepkowski and Wood (57) to be used less efficiently than thiocystine as a substrate for rhodanese in an isolated system. These workers observed the appearance of thiocystine following the action of cystathionase on cystine, and suggested that the importance of rhodanese may relate to its participation in a transsulfuration system with cystathionase (57).

The action on cystine ascribed by Szczepkowski and Wood (57) to cystathionase originally was attributed to cysteine desulfhydrase (4, 57). As early as 1950, however, it was suggested that the two enzymes were identical (64), and several subsequent reports have substantiated the suggestion (4, 57, 65, 66). Cavallini et al. (65, 66) concluded that cystine, but not cysteine, was a substrate for the pyridoxal phosphate-dependent enzyme and that pyruvate, ammonia, sulfur, and hydrogen sulfide were the products of the reaction. From the α-β elimination mechanism that had been proposed for other cystathionase reactions, these workers speculated that the disulfide analog of cystine, alanine hydrogen disulfide, was formed

as an intermediate in the reaction (65, 66). Although this substance, to which the name thiocysteine was given (67), was not isolated, its presence was indicated by the ability of the system to transsulfurate hypotaurine and to form a cyanolyzable compound (65). Spontaneous decomposition of thiocysteine was said to account for the formation of pyruvate, ammonia, sulfur, and hydrogen sulfide. A cycle was proposed whereby cysteine would react non-enzymically with the intermediate thiocysteine to yield cystine and hydrogen sulfide. In that way, pyruvate, ammonia, and hydrogen sulfide would be formed, and the substrate for cystathionase would be reproduced while one molecule of cysteine was utilized (66).

Szczepkowski and Wood (57) demonstrated that thiocysteine was converted to the trisulfide, thiocystine, in a dilute solution and in the presence of an excess of cystine. This more stable substance was active as a substrate for rhodanese, an enzyme that had been found previously to correlate in presence and activity with cystathionase. As mentioned earlier, these findings suggested to them that cystathionase and rhodanese may form a coupled enzyme system capable of utilizing cysteine sulfur for transsulfuration (57).

Evidence for the existence of still another pathway by which the sulfur-containing amino acids can be metabolized

has been furnished primarily by the work of Cavallini and his coworkers (68-79). The pathway which involves the conversion of cystamine, the expected product of cystine decarboxylation, to taurine was proposed by Schöberl (81) in 1933 following his in vitro demonstration of the reaction. According to Jacobsen and Smith (37), experimental evidence obtained during the next 20 years indicated that several mammals, including man, were capable of effecting the in vivo conversion of cystamine and cysteamine to taurine with hypotaurine as an intermediate, but information concerning the mechanism and importance of these reactions remained scarce. Medes and Floyd (19) suggested that the pathway involved enzymic oxidation of cystine to cystine disulfoxide, followed by a decarboxylation reaction to yield cystamine disulfoxide. Experimental evidence in support of their proposal is lacking.

While it has been suggested frequently that cystamine might arise from the degradation of coenzyme A (2, 4, 82), other possibilities such as direct decarboxylation of the parent amino acid and transsulfuration between cysteine and aminoethanol also have been mentioned (4).

Delineation of the mechanism by which cystamine is converted to taurine has occurred chiefly through the partial purification from pig and horse kidney of three enzymes active in the process. The conversion is thought to

proceed through the oxidative deamination of cystamine to the corresponding cyclized amino aldehyde, cystaldimine, catalyzed by diamine:0_2-oxidoreductase (EC 1.4.3.6) (37, 68-70). Cystaldimine undergoes further enzymic degradation giving rise to a two-carbon fragment and to thiocysteamine which spontaneously decomposes to cysteamine and sulfur (70-72). Cysteamine then may be enzymically oxidized to hypotaurine or non-enzymically converted to cystamine in a reaction catalyzed by sulfur or sulfide (72-76).

Persulfurase, the enzyme shown to catalyze hypotaurine formation from cysteamine, contains one atom of non-heme iron per molecule and has a molecular weight of 83,000 (75). Catalytic amounts of sulfur, sulfide, thiotaurine, tetrathionate, thiosulfate, thioacetic acid, or methylene blue can serve as cofactors, but a high concentration of any of these substances depresses the final amounts of hypotaurine produced (74-76). Cavallini et al. (74) proposed that high levels of cofactor with limited amounts of enzyme would be likely to favor the non-enzymic oxidation of cystamine.

Only indirect evidence has been offered for the in vivo operation of the cystine-cystamine-taurine pathway. Thiotaurine, a by-product formed in this path by spontaneous transsulfuration of hypotaurine (73), has been

identified in rat tissue and urine following the administration of cysteine, cystine, or cystamine (4, 37, 78), and persulfurase has been found in a wide variety of mammalian tissues (37, 79). Jacobsen and Smith (37) pointed out that heart tissue from several species contains persulfurase but not cysteinesulfinic acid decarboxylase. The high concentration of taurine found in the mammalian heart and the ability of dog heart slices to convert cystine to taurine (80) indicated to them that the cystamine-related reactions might operate in that tissue. Much more information will have to be obtained before the significance of the pathway can be fairly assessed.

Based on the information presented up to this point, it scarcely can be doubted that numerous pathways constitute a system by which the sulfur-containing amino acids are metabolized in higher animals. Pathways for which experimental evidence has been given are outlined in Figure 1. It can be seen that, while the suggested pathways are interrelated, the forms in which the sulfur ultimately can appear are different. Several in vivo functions have been assigned to sulfate, while most of those for taurine have been proposed only tentatively. Since the two products appear to differ vastly in function, the

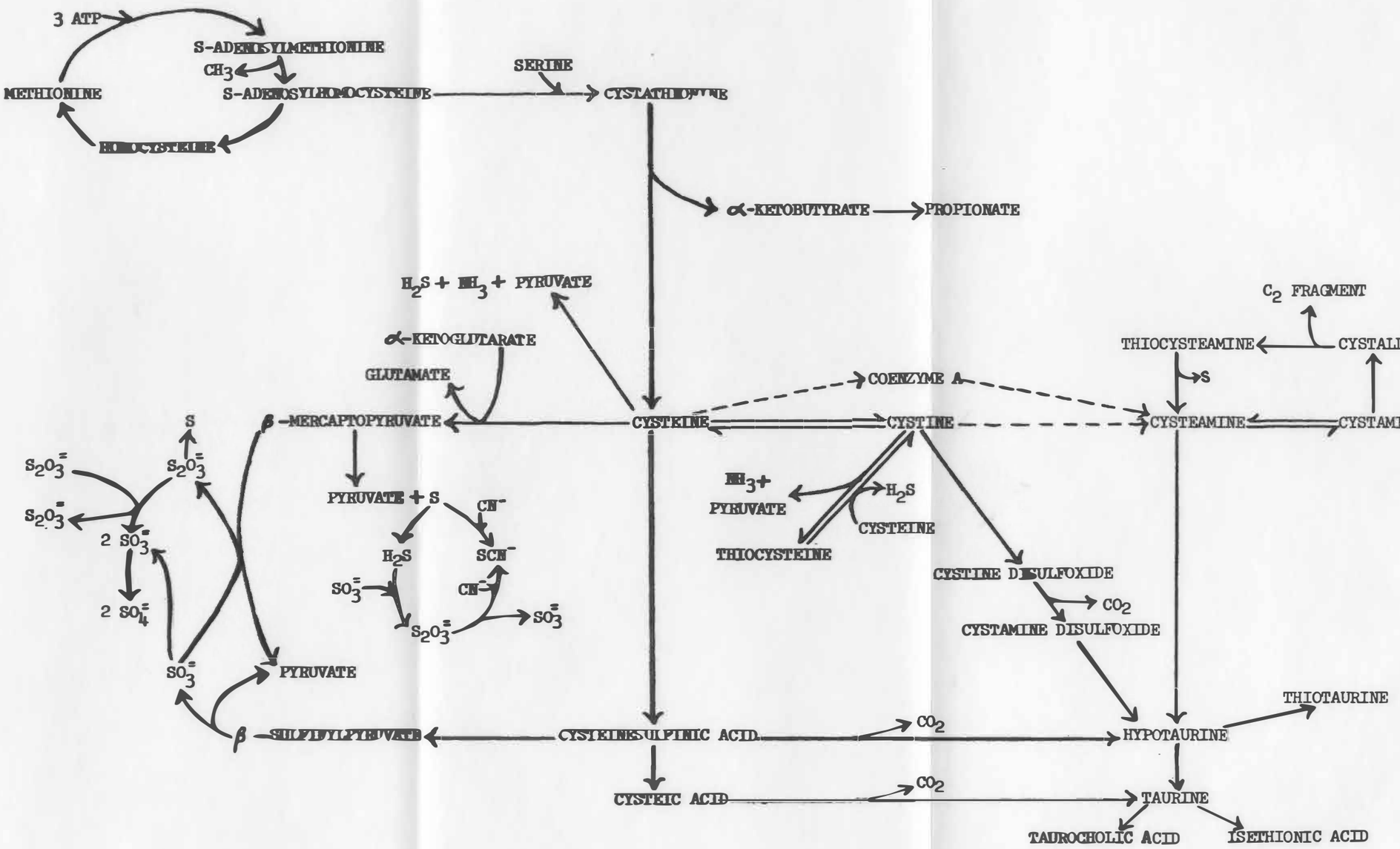

Figure 1. Suggested pathways of sulfate and taurine production in mammals.

route by which methionine, cysteine, or cystine is metabolized must be of biological significance.

Sulfate is found in mammalian tissues as a constituent of a variety of metabolically-formed compounds, including the sulfolipids of brain and liver and the sulfomucopolysaccharides, chondroitin sulfate, mucoitin sulfate, and heparin. Prerequisite to the synthesis of these compounds, sulfate is activated in a two-step reaction which utilizes two separate enzymes (83-85) and energy expenditure equivalent to three molecules of adenosine triphosphate (ATP) (83-86). The first step of the activation process as described by Bandurski et al. (83) and Robbins and Lipmann (84), involves the ATP-sulfurylase-catalyzed attack by one of the oxygens of sulfate on ATP to produce adenosine-5'-phosphosulfate (APS). Phosphorylation in the 3'-position by another molecule of ATP is then catalyzed by APS-kinase. The active compound formed in the reaction, identified by Robbins and Lipmann (87) as adenosine-3'-phosphate-5' phosphosulfate (PAPS), appears to be a general sulfate donor from which acceptor sulfokinases transfer the sulfate to other molecules. Work leading to the elucidation of sulfate activation and transfer was reviewed by Lipmann (88) in 1958.

Numerous authors have suggested that the sulfate supplied by in vivo oxidation of the sulfur-containing amino acids is the only significant source of utilizable sulfate for the non-ruminant mammal (2, 3). According to their reports, inorganic sulfate present in the diet is of little or no physiological importance. Recent studies have demonstrated, however, that not only can dietary inorganic sulfate be used, but also that it has a definite role in the nutrition of the animal.

With the everted gut sac technique, Anast et al. (89) demonstrated the transport of inorganic sulfate from the mucosal to the serosal surface of the distal segment of the small intestine of the rat, rabbit, and hampster. Movement occurred against electrochemical gradients and required the presence of sodium. Gut segments from hypophysectomized animals exhibited a markedly lower sulfate transport, which was restored to nearly normal when the animals were given bovine growth hormone. Batt (90) observed that active transport of inorganic sulfate was restricted to the distal segment of the small intestine in the adult mouse, whereas the entire length of the small intestine and colon accumulated sulfate in the infant. Accumulation gradually declined in all segments during the first two weeks postpartum. During the third week, it increased to

adult levels in the terminal ileum and disappeared from the rest of the gut. Administration of adrenocortical steroids to infant mice resulted in the appearance of adult patterns of sulfate transport (90).

According to Dziewiatkowski (91), most of the sulfate retained by the rat following the administration of inorganic sulfate appears as ester sulfate in mucopolysaccharides. Little evidence has been presented in support of significant non-bacterial incorporation of sulfate into sulfur-containing amino acids. Boström and Åqvist (92) detected the incorporation of small amounts of ^{35}S into taurine in the livers of rats injected with labeled sodium sulfate, but they were unable to demonstrate the presence of labeled cysteine or cystine. Small quantities of labeled cystine were observed, however, by Dziewiatkowski (91) in various tissues of rats injected with ^{35}S-sodium sulfate. Observations that labeled cystine was most abundant in the internal organs supported his proposition that microorganisms were responsible for the synthesis (91). In 1967, Huovinen and Gustafsson (93) reported the incorporation of ^{35}S from sulfide into cystine in the germ-free rat and, more recently, Schneider and Westley (63) reported the isolation of ^{35}S-cystine from the hair of rats injected with outer-labeled thiosulfate. Since incorporation of

radioactivity into cystine was greater in animals treated with neomycin, it could not be ascribed to bacterial synthesis. The failure of unlabeled sulfide to decrease the formation of labeled cystine suggested that the synthetic pathway from thiosulfate did not include sulfide as an intermediate. Incorporation of the inner sulfur atom of thiosulfate into cystine could not be detected (63). Although details concerning the synthesis of cystine and taurine from inorganic sulfate are not available yet, it appears that the reactions are of little physiological significance in the rat (37).

Pendergrass (94) found that the response of rats to avitaminosis E was related both to the total sulfur in the diet and to the ratio of neutral to inorganic sulfur. Vitamin E-deficient rats that were forced to satisfy their sulfate requirements by oxidation of neutral sulfur exhibited a decreased rate of mucopolysaccharide sulfation and decreased content of sulfur in cellular lipoproteins. Observations in the same animals of increased circulating sulfhydryl groups and decreased dilution of injected ^{35}S-sulfate led Pendergrass to assume that vitamin E was essential for optimal conversion of amino acid sulfur to sulfate (94). As Michels and Smith (95) have pointed out, the effect of avitaminosis E should be independent of the

level of dietary sulfate if inorganic sulfate were not a factor in the nutrition of the rat.

Button et al. (96) subsequently reported that rats absorbed a major portion of radioactive sulfur from dietary calcium or sodium sulfate and incorporated it into rib cartilage mucopolysaccharides. Animals fed diets low in organic sulfate absorbed significantly more of the inorganic sulfate than did those fed diets comparable with respect to total sulfur as sulfate but differing in the ratios of the two fractions. Furthermore, feed efficiency was significantly higher when inorganic sulfate was included in the diet. It was suggested that omission of inorganic sulfate from the diet forced the rat to oxidize amino acid sulfur to sulfate and, consequently, limited the supply of those amino acids available for other purposes (96).

Numerous changes in collagen metabolism in the rat were attributed by Brown et al. (97) to the consumption of diets low in inorganic sulfate. Significantly less neutral salt-soluble and total collagen was found in skins from rats fed diets that contained 0.0002 per cent of sulfate than from those fed diets that contained 0.02 per cent. Soluble collagen isolated from the skins of the "low-sulfate" animals failed to form normal collagen gels (97), and aortas from those animals exhibited a decreased

breaking strength per unit of collagen (98-99). Button et al. (100) reported that cellular lipoproteins isolated from rats fed diets low in inorganic sulfate contained significantly less hexosamine than did their normal controls, which indicated a failure in sulfomucopolysaccharide metabolism. A relationship between that finding and the changes in collagen metabolism was postulated (97).

Additional information concerning the utilization of organic and inorganic sulfate was obtained by Gilmore (101) who measured the distribution of ^{35}S-sulfate from dietary methionine or calcium sulfate among blood, urine, feces, and rib cartilage mucopolysaccharides from rats fed diets that contained 0.0002, 0.10, or 0.42 per cent of inorganic sulfate. Supplements of the sulfur-containing amino acids were adjusted so that the level of total sulfur as sulfate in each diet was similar. From the percentages of total ingested radioactivity recovered in the feces, it appeared that the absorption of inorganic sulfur was influenced by the level of organic sulfur in the diet. While the absorption of radioactivity supplied as inorganic sulfate was significantly higher from those diets not supplemented with methionine, the greatest absorption of radioactivity occurred from the diet containing no added inorganic sulfate but supplemented with ^{35}S-methionine. The blood radioactivity levels were 10 to 20 times higher

when the radioactivity was fed in the form of methionine rather than calcium sulfate, and were significantly higher when 0.1 per cent of inorganic sulfate was added to the diet than when it was omitted. These findings, coupled with the observation that the specific activity of the urine was lower when the rats received no dietary inorganic sulfate than when they received 0.1 per cent, indicated to Gilmore (101) that dietary inorganic sulfate could fill some of the demands that otherwise were imposed upon organic sulfur.

That point was strengthened by the observation that the specific activity of rib cartilage mucopolysaccharides from animals fed the highest level of inorganic sulfate was approximately twice that from animals fed the highest level of organic sulfate. Specific activity of mucopolysaccharides was lowest when inorganic sulfate was omitted from the diet, even thought methionine sulfur as sulfate had been substituted in equal amounts. While methionine was supplied at a level greater than that previously believed to be adequate for maintenance and growth, it did not appear adequate when dietary sulfate was omitted. Gilmore (101) suggested that competition among metabolic acceptors for the sulfur of the sulfur-containing amino acids resulted in the diversion of part of the sulfur to other products when rats were forced to produce sulfate in vivo.

Evidence, albeit indirect, for a feedback mechanism between the level of inorganic sulfate and cysteine metabolism was presented by Tigert (102). This investigator observed that more ^{35}S-cysteine was utilized by fortified rat liver homogenates when BMP was added to the system than when it was omitted. In addition, the synthesis of sulfate decreased significantly and the production of taurine and thiosulfate increased in those systems containing BMP. She interpreted the findings to indicate that the decrease in inorganic sulfate which accompanied the addition of BMP signaled increased oxidation of cysteine through some intermediate common also to taurine (102).

Except for its well-established role in bile conjugation, taurine generally has been regarded as an end product of sulfur metabolism (1, 2, 37). Its conversion to other compounds has been detected, however, and observations of its ubiquitous occurrence among members of the animal kingdom have led to suggestions that it must have additional physiological functions (1, 37, 103-107). Information concerning its biochemistry and physiology was reviewed extensively by Jacobsen and Smith (37) in 1968.

Taurine is distributed widely among animal tissues, with the highest concentrations appearing in the heart and striated muscles (108-110). According to Stern and Stim

(111), over 75 per cent of the total body taurine is contained in striated muscles. It is confined primarily to the intracellular fluid, where it often is found at a level considerably higher than that of any other amino acid (108, 109). Boquet and Fromageot (112) have estimated that taurine concentration in the rat is approximately 663 μM per 100 gm of body weight. The average half-life of tissue taurine in the rat is 12 to 13 days, with that in muscle showing the slowest rate of turnover (112).

Taurine in tissues is maintained at fairly constant levels, but the amount excreted in the urine may fluctuate greatly. Urinary taurine levels are influenced by the amounts of pre-formed taurine, other amino acids, and protein in the diet (104, 113-117), whereas Awapara (117) observed no change in taurine concentration in liver, kidney, heart, lung, spleen, or muscle of rats fed protein-free diets for 10 days. Wu (118), however, reported that urine and tissue levels of taurine increased steadily in rats during a fasting period of nine days. He suggested that, as fasting progressed, certain oxidative processes of the sulfur-containing amino acids became impaired and the conversion to taurine became predominant. The retention of ingested taurine is greater in animals fed diets low in protein or sulfur-containing amino acids

than in those fed normal or high-protein diets (104, 115, 119). Differences exist among tissues as to the amount of the taurine absorbed and the length of time it is retained (120). Irradiation has been reported to produce species-related effects on taurine excretion. Numerous workers have reported that rats excreted significantly more taurine after irradiation than before (121-126), but the change was not detected in guinea pigs (122). No consistent pattern was observed in dogs or rabbits (122). Kay and Entenman (121) found that kidney and liver taurine levels were lower in irradiated rats, while Stern and Stim (111) detected no change in the levels in liver, muscle, spleen, or thymus. Decreased taurine excretion accompanies vitamin B_6 deficiency in rats (127-129), but the effect has not been observed consistently in humans (38, 130, 131). Park and Linkswiler (131) stated that taurine excretion is a poor criterion for assessing vitamin B_6 nutriture of man.

The so-called "normal" levels of urinary taurine reported for the rat vary from the 0.8 to 1.5 mg per 24 hours reported by McAfee and Williams (128) to the 18.6 mg per 24 hours reported by Hope (129). Evered (113) found values ranging between 120 and 160 mg of taurine per 24 hours in urine from adult humans fed a "normal" diet, while McCoy and Wehrle (130) reported that 64 mg per 24

hours was an average value for humans. According to Boquet and Fromageot (112), very little taurine is excreted in the feces.

A relationship between the two has not been authenticated, but researchers have observed that hypotaurinuria frequently accompanies Down's syndrome (130, 132-134). Fifty-seven per cent of the mongoloid individuals observed by Wainer (133) excreted less than 20 mg of taurine per gram of creatinine, while only 10 per cent of the normal individuals exhibited values in that range. Administration of vitamin B_6 to the "low excretors" for six weeks yielded no response (134), and administration of ^{35}S-taurine resulted in the excretion of taurine metabolites in amounts greater than taurine itself (133). Goodman et al. (135) and King et al. (134) suggested that one of the products may by isethionic acid (ISA). Greater renal tubular reabsorption of taurine by mongoloid than by normal subjects was observed by King et al. (134) who offered the finding as partial explanation of the similarity in serum taurine values of the two groups of individuals.

ISA, the deaminated analog of taurine, was identified by Koechlin (136) in 1954 as the major anion in the axoplasm of the giant squid. His findings led to speculation that taurine and ISA constituted a functional complex in nervous tissue, but conflicting views have appeared since that time

(37, 105, 107). The presence of ISA in mammalian tissues was demonstrated in 1962 by Welty et al. (107) who found that it could be synthesized from taurine in dog heart (80). Peck and Awapara (45) demonstrated its synthesis in rat brain homogenates, and Jacobsen et al. (107) reported its presence in human urine. It has been proposed that ISA, by altering membrane potential, plays a role in regulating excitability of various tissues (106), but little information related to that suggestion was found in the literature.

The formation of inorganic sulfate from taurine in mammals has been noted, but several observations appear to indicate that the activity is attributable to intestinal microflora. Roe (115) reported that rats injected intraperitoneally with ^{35}S-taurine excreted small portions of labeled sulfate in the urine. The greatest portion of the radioactivity was retained, however, and most of that excreted was in the form of taurine. The quantity recovered as sulfate was greater from protein-deficient rats than from rats fed a 30 per cent casein diet. Schram and Crokaert (137) also observed the excretion of labeled sulfate by rats injected with ^{35}S-taurine, but they found that the administration of antibiotics to the rats resulted in significant reduction of ^{35}S-sulfate excretion. Similar effects of antibiotics were seen by Boquet and Fromageot

(112). The report that inorganic sulfate was not produced when taurine was added to homogenates of rat liver or brain (138) lends further support to the idea that microorganisms are responsible for the conversion of taurine to inorganic sulfate in mammals.

From the information available at this time, few unequivocal statements can be made concerning the physiological significance of taurine. Participation in bile salt formation is its only clearly established function in mammals, but logic suggests that other functions also must be performed by the widely-distributed compound.

Regardless of the physiological function of taurine, in vivo synthesis and excretion of the compound represents a drain on the sulfur-containing amino acid supply of an animal, and conditions forcing the animal to increase its taurine production without metabolic benefit would result in wasting of the sulfur-containing amino acids. Since factors influencing taurine synthesis also could influence the availability of substrate for sulfate synthesis, a relationship between the synthesis of taurine and sulfate could explain the findings reported by Gilmore (101) of decreased ^{35}S-sulfate in rib cartilage mucopolysaccharides when theoretically-equivalent amounts of sulfur as sulfate in sulfur-containing amino acids were substituted for ^{35}S-calcium or -sodium sulfate in the diet of the rat.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

The effects of varying levels of dietary sulfate on the feed efficiency, excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur, and sulfur content of lung tissue were examined in an attempt to elucidate earlier reports from this laboratory that the obligatory oxidation of amino acid sulfur to sulfate is an inefficient process in the rat (101). The investigation proceeded as four separate experiments.

Experiment 1

Feed efficiency was used as a criterion for evaluating the ability of a theoretically-equivalent amount of potential sulfate supplied in the form of methionine to compensate for the omission of inorganic sulfate from the diet of the rat. The experimental diets A, B, and C, which are shown in Table 1, were modified from those used by Pendergrass (94) originally patterned after that of Caputto et al. (139). In an attempt to remove undetermined quantities of inorganic sulfate, casein, cornstarch, and non-nutritive bulk were washed with distilled water and brought to dryness before being incorporated into the diets.

TABLE 1

Composition of diets fed in experiment 1

Component	A 0.0002% Dietary SO_4	B 0.10% Dietary SO_4	C 0.42% Dietary SO_4
	g/ 100 g diet	g/ 100 g diet	g/ 100 g diet
Casein (Vitamin Free)[a]	15	15	15
Cornstarch[a]	60	60	60
Non-nutritive Bulk[a,b]	11.72	11.90	12.00
Vegetable Shortening[c]	6	6	6
Vegetable Oil[d]	2	2	2
DL-Methionine	0.60	0.35	0
Vitamin Mixture[e]	2	2	2
Basal Salt Mixture	1.34	1.34	1.34
$CaCO_3$	1.34	1.23	0.91
$CaSO_4 \cdot 2H_2O$	0	0.18	0.75

[a]Washed with distilled water.

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

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

[d]Wesson Oil, Hunt Wesson Foods, Fullerton, California

[e]Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

Sucrose was omitted and cornstarch additions were increased to compensate for the omission. Since cod-liver oil contains an unidentified factor that influences the utilization of sulfate (5), vegetable oil was substituted as a source of fatty acids. The 15 per cent of casein supplied each diet with the sulfur-containing amino acids at a level of 0.5 per cent, whereas the requirement of the rat for those amino acids has been reported to be 0.6 per cent (140). Additions of calcium sulfate, methionine, calcium carbonate, and non-nutritive bulk were adjusted so that three levels of inorganic sulfate were achieved in diets equivalent in calcium and similar in total sulfur as sulfate. The term "total sulfur as sulfate" is used to refer to the level of dietary sulfate plus the level of sulfate that theoretically would be produced if all the amino acid sulfur were oxidized to sulfate. Calculated levels of inorganic, organic, and total sulfur as sulfate of diets A, B, and C are shown in Table 2. The composition of the salt mixture, presented in Table 3, was modified from that designed by Hubbell et al. (141), and it supplied 0.0002 per cent of inorganic sulfate to the diet when added at the level shown in Table 1.

Seven sets of three littermate weanling male albino rats of the Wistar strain from the stock colony maintained in the Nutrition Department of The University of Tennessee,

TABLE 2

Calculated levels of inorganic, organic, and total sulfur as sulfate of diets fed in experiment 1

Diet	Inorganic SO_4	Organic S as SO_4	Total S as SO_4
	% of diet	% of diet	% of diet
A	0.00	0.64	0.64
B	0.10	0.47	0.57
C	0.42	0.25	0.67

TABLE 3

Composition of basal salt mixture[a]

Component	g
$CaCO_3$	44.750
$MgCO_3$	3.060
NaCl	6.900
KCl	11.200
KH_2PO_4	21.200
$FePO_4 \cdot 2H_2O$	2.050
KI	0.008
NaF	0.010
$AlK(SO_4)$	0.017
$Cu(C_2H_3O_2)_2 \cdot H_2O$	0.072
$MnCl_2 \cdot 4H_2O$	0.040
Cornstarch	10.693

[a]Source: Pendergrass, B. J. 1961 The interrelationship of tocopherol and sulfur metabolism. Unpublished Master's Thesis, The University of Tennessee, Knoxville.

Knoxville, were employed for this study. For a period of six weeks, each littermate was fed a different one of the three diets outlined in Table 1, page 39. The animals were housed in individual wire mesh, galvanized cages grouped so as to avoid contamination of feed in one cage with spillage of a different diet. Feed and distilled water were fed ad libitum. Collection trays beneath the cages were lined with waxed paper from which spillage was recovered daily, sifted, and the feed returned to the cups on the floors of the cages. Individual feed consumption was recorded on a one- or two-day basis and animals were weighed weekly. Feed efficiency ratios were calculated as g of weight gained per g of feed consumed.

Experiment 2

Because cysteinsulfinic acid has been considered a key intermediate in the in vivo formation of both sulfate and taurine, it seemed reasonable to hypothesize the existence of a metabolic interrelationship between sulfate and taurine. Therefore, the effects of varying levels of dietary sulfate on the urinary excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur by the rat were studied.

Three lots of the basal mixture shown in Table 4 were supplemented with calcium sulfate and cysteine in inverse amounts, as shown in Table 5, to produce diets

TABLE 4

Composition of basal mixture for diets fed in experiments 2, 3, and 4

Component	g/100 g Diet
Casein (Vitamin Free)	15.00
Sucrose	30.00
Cornstarch	30.00
Cod-liver Oil	2.00
Vegetable Shortening[a]	6.00
Vitamin Mixture[b]	2.00
Basal Salt Mixture[c]	1.34

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

[b]Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[c]See Table 3, page 42.

TABLE 5

Variations of basal mixture fed in experiment 2

Diet	Additions to Basal Mixture				Levels of Dietary Sulfur		
	$CaSO_4 \cdot 2H_2O$	$CaCO_3$	Cysteine	Non-nutritive Bulk[a]	Inorganic SO_4	Organic S as SO_4	Total S as SO_4
	g/100 g diet	g/100 g diet	g/100 g diet	g/100 g diet	%	%	%
1	0	1.34	0.53	11.79	0.0002	0.67	0.67
2	0.18	1.23	0.40	11.85	0.10	0.57	0.67
3	0.75	0.91	0	12.00	0.42	0.25	0.67
4	0	1.34	0.40	11.92	0.0002	0.57	0.57
5	0.18	1.23	0.40	11.85	0.10	0.57	0.67
6	0.75	0.91	0.40	11.60	0.42	0.57	0.99

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

with inorganic sulfate levels of 0.0002 per cent (diet 1), 0.10 per cent (diet 2), and 0.42 per cent (diet 3) while maintaining the total sulfur as sulfate level of each at 0.67 per cent. The pattern of supplementation of the basal mixture with calcium sulfate was repeated in diets 4, 5, and 6, each of which contained 0.40 per cent of added cysteine, so that the levels of total sulfur as sulfate increased as inorganic sulfate additions increased. Diets 1 and 2 in the first series and all of the diets in the second series were designed to supply the sulfur-containing amino acids at a level slightly above that reported to be required by the rat (140).

Duplicates of the six diets also were prepared, except that 0.05 per cent of ^{35}S-cysteine was added at the expense of that quantity of non-radioactive cysteine in each diet. The only non-radioactive diet that differed from its radioactive counterpart in sulfate content was diet 3, the one that contained 0.42 per cent of inorganic sulfate and no cysteine supplement. The 0.05 per cent of ^{35}S-cysteine added about 0.02 per cent of sulfate to the radioactive diet. The calculated levels of organic, inorganic, and total sulfur as sulfate, as well as the amounts of calcium sulfate and cysteine which were added to achieve those levels in the six diets, are shown in Table 5. The amounts of calcium carbonate added to keep

the calcium content constant and non-nutritive bulk added to adjust the weight of the diets are given in the same table. These diets, like the ones used in experiment 1, contained the low sulfate adaptation of the salt mixture of Hubbell et al. (141) (Table 3, page 42) and were modifications of the diets used by Pendergrass (94).

In the preparation of the radioactive diets, ^{35}S-cysteine obtained from Schwartz Bioresearch, Orangeburg, New York, was diluted with sufficient non-radioactive amino acid to yield a preparation with a specific activity of about 25,000 counts per minute (cpm) per mg. A 0.50-g portion of the preparation then was added to a kg of each of the three diets that contained varying levels of inorganic and organic sulfate (diets 1, 2, and 3). Each diet, which already had been sifted two times through a fine mesh household strainer, was stirred thoroughly as the cysteine was being added and was sifted again two times to insure uniform distribution of the labeled compound. About 10 weeks later, the procedure was repeated with diets 4, 5, and 6, the three diets that contained varying levels of inorganic and of total sulfate. Since 0.50 g of ^{35}S-cysteine could be incorporated evenly into 1000 g of diet with greater accuracy than might be indicated if the specific activity were determined from randomly selected 1-g samples of the diets, diets prepared at the same time

with ^{35}S-cysteine from the same dilution were assumed to have equal specific activities. In order to decrease chances of error, results of feeding a particular radioactive diet were analyzed on a relative basis and were compared only with the results obtained from other animals fed diets that contained ^{35}S-cysteine from the same dilution.

Six groups of randomly-selected adult male albino rats of approximately the same age and weight were used in experiment 2. They were of the Sprague-Dawley strain and previously had been fed Purina Laboratory Chow. Each group of five rats were fed a different one of the diets outlined in Tables 4 and 5, pages 44 and 45, for 17 days, the length of time Gilmore (101) had fed similar diets when she observed differences in sulfation reactions in her rats. For 10 days the animals were individually housed in wire cages racked above waxed paper-lined spillage collection trays from which debris was removed on alternate days and feed recovered. Distilled water and non-radioactive feed were fed ad libitum, and records were kept of feed consumption. As in the other experiments, caution was exercised to prevent contamination of one diet with another.

At the end of 10 days, the animals were transferred to individual metabolism cages and diets that contained ^{35}S-cysteine were substituted for their non-radioactive

counterparts. Fresh distilled water and feed were fed ad libitum throughout the experiment. When the animals had been fed the radioactive diets for five days, vials that contained 1 ml of 0.1 N hydrochloric acid were placed at the tips of clean collection funnels under the cages and two separate 24-hour urine specimens were obtained from each rat. The vials were labeled and stored at -20°C until analyses for ^{35}S-taurine could be carried out. No gross differences among the animals could be detected either from weights of each animal recorded at the beginning and end of the experiment or from feed consumption records kept during the study.

Experiment 3

When the results of experiment 2 revealed that the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur increased when the rat was forced to oxidize amino acid sulfur to sulfate, an experiment was designed to determine the limit to which inorganic sulfate additions to a 15 per cent casein diet supplemented with 0.40 per cent of cysteine could be lowered before an increase in the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur by the rat could be detected. Because data obtained by another investigator in this laboratory had shown a relationship between inorganic sulfate in the diet of the rat and total sulfur as

sulfate in its lungs,[1] total sulfur as sulfate in the lungs of each animal used in this experiment also was determined.

Eighteen variations of the basal mixture employed in experiment 2 (Table 4, page 44) were prepared by adding calcium sulfate, calcium carbonate, and non-nutritive bulk at the levels shown in Table 6 while cysteine was added to each diet at the level of 0.40 per cent. The calculated levels of inorganic and total sulfur as sulfate, which are shown in Table 7, ranged between the extremes of those in the diets used in the preceding experiment and, as in experiment 2, non-labeled cysteine was used in one set of diets and a second set contained ^{35}S-cysteine added at the expense of the non-radioactive substance. The 18 diets were prepared with equal amounts of ^{35}S-cysteine from the same dilution so that their specific activities would be the same.

To determine whether differences due to diet might be enhanced by longer feeding periods, the Sprague-Dawley male albino rats used in this experiment were fed the diets from weaning and for a period of six weeks. Metabolism cage limitations made it necessary to begin the experiment with 18 rats, the number used to test three

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[1]Smith, J. T. Unpublished observations.

TABLE 6

Diets fed in experiment 3: additions to basal mixture[a]

Diet	$CaSO_4 \cdot 2H_2O$	$CaCO_3$	Non-nutritive Bulk[b]
	g/100 g	g/100 g	g/100 g
1	0.00	1.34	11.92
2	0.02	1.33	11.91
3	0.04	1.32	11.90
4	0.07	1.30	11.89
5	0.09	1.29	11.88
6	0.11	1.28	11.87
7	0.13	1.26	11.87
8	0.15	1.25	11.86
9	0.18	1.24	11.85
10	0.20	1.23	11.84
11	0.22	1.21	11.83
12	0.27	1.18	11.81
13	0.31	1.16	11.79
14	0.36	1.13	11.77
15	0.45	1.08	11.73
16	0.54	1.03	11.69
17	0.63	0.97	11.66
18	0.72	0.92	11.62

[a]Each diet was supplemented with 0.40% of cysteine.

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

TABLE 7

Calculated sulfate levels of diets fed in experiment 3[a]

Diet	Inorganic SO_4	Total S as SO_4
	%	%
1	0.00	0.57
2	0.01	0.58
3	0.02	0.59
4	0.04	0.61
5	0.05	0.62
6	0.06	0.63
7	0.08	0.65
8	0.09	0.66
9	0.10	0.67
10	0.11	0.68
11	0.13	0.70
12	0.15	0.72
13	0.18	0.75
14	0.20	0.77
15	0.25	0.82
16	0.30	0.87
17	0.35	0.92
18	0.40	0.97

[a]Each diet contained 0.57% of amino acid sulfur as sulfate.

diets, and to add 18 animals weekly until the desired number had been placed under test. For five weeks the animals were housed in group cages, six per cage, where fresh distilled water and a different one of the non-radioactive diets were available to each group. Weight changes of each animal were recorded weekly and feed consumption of each group was recorded two or three times weekly.

The five animals from each experimental group which were most similar in body weight at the end of the five-week period were moved to individual metabolism cages where they were fed their respective ^{35}S-cysteine-containing diets for one week. During days six and seven of that week, urine was collected as described under experiment 2. The animals then were decapitated and their lungs transferred to individual labeled vials. Urine and lungs were stored at -20°C.

Experiment 4

Upon evaluation of the results of experiment 3, a study was designed to determine if adaptation to diets low in inorganic sulfate, as evidenced by ^{35}S-taurine excretion patterns, could be associated with either the age of the rat or the length of time it was fed the diet.

Twenty-four weanling male albino rats of the Sprague-Dawley strain were purchased together and divided randomly

into four equal groups. For six weeks, two groups were fed Purina Laboratory Chow while the others were fed ^{35}S-cysteine-containing diets of equal specific activities and with inorganic sulfate added at the levels of 0.0002 and 0.10 per cent, respectively. The experimental diets, each with 0.40 per cent of added cysteine, had the same composition as diets 2, 4, and 5 used in experiment 2 (Table 4, page 44; Table 5, page 45) and diets 1 and 9 used in experiment 3 (Table 6, page 51).

For the last two days of each week, the rats fed the experimental diets were transferred from their group cages to individual metabolism cages where two 24-hour urine specimens were collected from each animal in the manner described previously. After six weeks, the animals that had been fed laboratory chow were switched to the experimental diets and urine samples were obtained weekly for three weeks. During the week following their collection, samples were analyzed for ^{35}S-taurine.

II. METHODS

Determination of ^{35}S-taurine in Urine

Numerous methods for the determination of taurine have appeared in the literature (142-146). Most of them have involved paper chromatography or chromatography on ion exchange resins, followed by a colorimetric determination with ninhydrin. The methods generally have been

developed for measuring taurine content of tissues or of human urine, and have been criticized for lacking the accuracy and precision needed for comparative studies and for being unadaptable to large numbers of determinations (144-146). Whether the methods are insensitive in the range of concentrations found in rat urine or whether some interfering substance is present, numerous trials in this laboratory of the methods of Garvin (145) and of Sorbo (146) have failed to provide a satisfactory colorimetric procedure for the quantitative determination of taurine in rat urine. A method for use in this study was developed which employed the same principles for isolating taurine as those employed by other investigators but eliminated the use of colorimetry. Instead, taurine was isolated from the urine of rats fed ^{35}S-cysteine-containing diets, the specific activity of the isolated taurine was determined by liquid scintillation counting, and its concentration expressed as cpm per 24-hour urine sample X 10^{-3}. The determinations were carried out in the manner described below.

After its volume had been determined, the 24-hour urine sample was deproteinized according to the method of Somogyi (147) as described by Sorbo (146). The urine was adjusted to pH$\simeq$8 as indicated by phenolphthalein and quantitatively transferred to a 50-ml centrifuge tube.

One ml of 10 per cent zinc sulfate·$7H_2O$ (w/v) in water and 2.5 ml of 0.2M sodium hydroxide were added per 5 ml of urine. The sample was centrifuged at 2000 X g for 10 minutes, after which the pH was readjusted, when necessary, to $\simeq$8 and the supernatant was brought to volume with distilled water. Since the deproteinization process approximately doubled the volume, quantitation of the sample generally was to 25 ml.

A quantity of the deproteinized sample representing no more than 1 ml of urine was transferred to a small mixed-bed ion exchange resin column. The column was 1 cm in diameter and contained 3 cm of Dowex 1-X8 (200-400 mesh) in the chloride form over 5 cm of Dowex 50W-X8 (200-400 mesh) in the hydrogen form. A new column was packed for each determination. When the sample had passed into the resin, the column was washed two times with 5-ml portions of distilled water and the effluent was collected in a small beaker. A total of 1/5 of each urine sample was treated in the above manner. When the volume of urine required that more than 1 column be used, the effluents were collected in the same beaker. The effluent was evaporated to a volume of less than 0.5 ml, but not to dryness, and transferred to a liquid scintillation counting vial with 6 ml of ethanol and 8.4 ml of scintillator in toluene (148). The scintillator was a preparation from

Picker Nuclear which contained 98 per cent of PPO(2,5-diphenyloxazole) and 2 per cent of bis-MSB [p-bis-(0-methylstyryl)-benzene] and was packaged so that one envelope, 5 g, was mixed with 1 liter of toluene. The radioactivity of the samples was determined using a Picker Nuclear Liquimat 220 Liquid Scintillation Counter preset for 8,192 counts for each sample in channel A with window settings of 50 and 600. Channel B, with window settings of 350 and 550, facilitated the determination of the degree of quenching of each sample.

The method was tested in the following way. A large quantity of unseparated excreta from rats fed ^{35}S-cysteine-containing diets was extracted with water. The extract was deproteinized by the method already described, and the protein-free supernatant fluids were placed on a column 2 cm in diameter which contained 8 cm of Dowex 1-X8 over 8 cm of Dowex 50W-X8. The column was washed with distilled water, and the effluent was concentrated and transferred to a 10-ml volumetric flask. One ml of the substance was placed in a counting vial and evaporated to a volume of less than 0.5 ml, after which 6 ml of ethanol and 8.4 ml of scintillator in toluene were added. The 1-ml sample was found to have an activity of 100 cpm. Paper chromatography of the material in a solvent system of isopropanol, water, ethanol, pyridine, and formic acid

revealed only one ninhydrin-positive substance, and the R_f value of that substance was identical with the R_f value for taurine. When the chromatogram was stripped and counted, counts were found to be associated only with the ninhydrin-positive area. Therefore, the substance was used as a ^{35}S-taurine standard. Columns with a diameter of 1 cm were packed with 3 cm of Dowex 1-X8 over 5 cm of Dowex 50W-X8, as described earlier, and the recovery of 1-ml portions of the standard was tested. The data are shown in Table 8.

Determination of Total Sulfur as Sulfate in Lungs

The lungs of each animal described under experiment 3 were lyophilized, weighed, and combusted in a Parr Bomb. The residue from each set of lungs was diluted to 25 ml with distilled water, and portions of the dilutions were analyzed in duplicate for total sulfur as sulfate by a modification used by Pfuderer (149) of the method described by Roe et al. (150).

One ml of 5 per cent lanthanum chloride and 2 ml of 15 per cent barium chloride were added to 2 ml of the lung dilution in a 50-ml centrifuge tube. The tube was shaken well and centrifuged at 2000 X g for 10 minutes. The supernatant was discarded and the precipitate was washed with 5 ml of distilled water to remove excess

TABLE 8

Recovery of ^{35}S-taurine from rat urine

Substance	cpm[a]
Standard (Before Column)	98 ± 2[b]
Standard (After Column)	97 ± 2[c]
Urine	78 ± 2[d]
Urine + Standard	177 ± 3[b]

[a] cpm ± standard error of the mean.

[b] Based on two determinations.

[c] Based on three determinations.

[d] Based on four determinations.

barium from the tube. The sample was centrifuged again, the supernatant discarded, and the washing repeated. After the supernatant from the third centrifugation had been removed, the precipitate was dissolved in 10 ml of an alkaline disodium ethylenediamine tetracetate (EDTA) solution which was prepared by dissolving 10 g EDTA in 500 ml of distilled water, adding 20 g of sodium hydroxide, and diluting to 2000 ml.

A stock sulfate solution was made by dissolving 1.479 g of sodium sulfate in 500 ml of distilled water to yield a concentration of 2000 ppm of sulfate. Duplicate working standards, which contained 0.00, 0.10, 0.25, 0.40 and 0.50 ml, respectively, of the stock sulfate solution plus enough distilled water to bring the volume of each one up to 5 ml, were treated in the manner described for the sample. The standards contained 0, 20, 50, 80, and 100 ppm of sulfate. A set of standards was run with each group of samples, and 36 samples (18 pair of duplicates) generally were run at the same time.

Samples and standards were aspirated into the flame of a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer and the per cent absorption of each was recorded. Per cent absorption, an exponential function, was converted to absorbance, a linear function, so that the following equation could be used for determining the absolute values of sulfate:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \frac{\text{Conc of standard } (\mu g)}{1}$$

$$\times \frac{\text{Dilution}}{\text{Dry weight of tissue (mg)}} = \mu g \text{ S as } SO_4/\text{mg of tissue}$$

III. STATISTICAL METHODS

The Olivetti-Underwood Programma 101 was used for the statistical treatment of all data. Data from experiment 1 were analyzed by the method of paired comparisons (151). Those from the other three experiments were analyzed by the method of unpaired comparisons as described by Steel and Torrie (151).

CHAPTER IV

RESULTS

Experiment 1

Feed efficiencies frequently are employed as indicators for comparing the adequacies with which dietary treatments meet the nutritional needs of animals. Individual efficiencies of feed utilization in progressions of 1 week were calculated for the animals in experiment 1, and the findings are summarized in Table 9. Analysis of those data revealed that by the end of the experimental period the diet supplemented with 0.35 per cent of methionine and 0.10 per cent of sulfate, referred to as a "normal sulfate" diet, was being utilized most efficiently. Average feed efficiency values of the animals fed the high sulfate, low methionine diet generally were lower than those of the other 2 groups, but the values never differed significantly from those of the animals fed the low sulfate diet. The data in Table 9 show that significant differences in feed efficiency between the animals fed the high sulfate diet and those fed the normal sulfate diet were detected at several times during the 6-week study. Weight gain, as shown in Table 10, consistently was lower among the animals in the high sulfate group than among

TABLE 9

Feed efficiency ratios of rats fed three levels of dietary sulfate for six weeks

Weeks Diet Consumed	Feed Efficiency Ratio[a]			Statistical Comparisons[e]		
	Diet A[b] Low SO_4	Diet B Normal SO_4[c]	Diet C High SO_4[d]	Low[b] to Normal[c]	Normal[c] to High[d]	Low[b] to High[d]
1	0.38 ± 0.02	0.36 ± 0.01	0.35 ± 0.03	$P < 0.6$	$P < 0.8$	$P < 0.5$
2	0.37 ± 0.01	0.39 ± 0.01	0.36 ± 0.01	$P < 0.4$	$P < 0.01$	$P < 0.6$
3	0.38 ± 0.01	0.38 ± 0.01	0.36 ± 0.01	$P < 0.9$	$P < 0.3$	$P < 0.1$
4	0.35 ± 0.01	0.36 ± 0.00	0.33 ± 0.01	$P < 0.6$	$P < 0.05$	$P < 0.1$
5	0.34 ± 0.01	0.35 ± 0.00	0.33 ± 0.01	$P < 0.05$	$P < 0.1$	$P < 0.6$
6	0.32 ± 0.01	0.35 ± 0.00	0.32 ± 0.01	$P < 0.001$	$P < 0.01$	$P < 0.5$

[a]Results are the average of seven animals ± standard error of the mean.

[b]Diet contained 0.0002% of inorganic sulfate, 0.64% of organic sulfur as sulfate.

[c]Diet contained 0.10% of inorganic sulfate, 0.47% of organic sulfur as sulfate.

[d]Diet contained 0.42% of inorganic sulfate, 0.25% of organic sulfur as sulfate.

[e]Probability that differences were due to chance, as determined by the method of paired comparisons (151).

TABLE 10

Weight gain of rats fed three levels of dietary sulfate for six weeks

Weeks Diet Consumed	Weight Gain[a]			Statistical Comparisons[e]		
	Diet A Low SO_4[b]	Diet B Normal SO_4[c]	Diet C High SO_4[d]	Low[b] to Normal[c]	Normal[c] to High[d]	Low[b] to High[d]
	g	g	g			
1	15 ± 1	15 ± 1	14 ± 1	$P < 0.8$	$P < 0.4$	$P < 0.8$
2	40 ± 1	42 ± 2	37 ± 2	$P < 0.4$	$P < 0.005$	$P < 0.6$
3	75 ± 1	75 ± 3	69 ± 3	$P < 1$	$P < 0.05$	$P < 0.4$
4	111 ± 3	113 ± 4	102 ± 4	$P < 0.6$	$P < 0.005$	$P < 0.05$
5	150 ± 4	150 ± 5	140 ± 6	$P < 1$	$P < 0.05$	$P < 0.1$
6	187 ± 4	192 ± 5	172 ± 9	$P < 0.4$	$P < 0.05$	$P < 0.2$

[a]Results are the average of seven animals ± standard error of the mean.

[b]Diet contained 0.0002% of inorganic sulfate, 0.64% of organic sulfur as sulfate.

[c]Diet contained 0.10% of inorganic sulfate, 0.4% of organic sulfur as sulfate.

[d]Diet contained 0.42% of inorganic sulfate, 0.25% of organic sulfur as sulfate.

[e]Probability that differences were due to chance, as determined by the method of paired comparisons.

those in the normal sulfate group, and the differences were significant from week 2 until the experiment was terminated. Only at the end of week 4 did weight gain differ significantly between the low and high sulfate groups ($P < 0.05$), and at no time did it differ significantly between the low and normal sulfate groups. Although the diet that contained the lowest level of added sulfate also contained the highest level of methionine, the animals fed that diet exhibited significantly lower feed efficiency values at the end of weeks 5 ($P < 0.05$) and 6 ($P < 0.001$) than did the animals fed the normal sulfate diet.

Experiment 2

When adult rats were fed the ^{35}S-cysteine-containing diets 1, 2, and 3, which furnished inorganic, organic, and total sulfur as sulfate in ratios and amounts similar to those furnished by the diets used in the first experiment, the animals fed diet 1, which contained the smallest quantity of added inorganic sulfate were found to excrete the greatest amount of urinary ^{35}S-taurine at the end of the 17-day feeding period. The data presented in Table 11 indicate that 58 per cent less ^{35}S-taurine was excreted by the animals fed diet 2 to which 0.10 per cent of inorganic sulfate had been added to the expense of that quantity

TABLE 11

Urinary excretion of ^{35}S-taurine by rats fed three levels of sulfate in diets that contained equivalent levels of total sulfur as sulfate[a]

Diet Number	Level of Dietary SO_4	Level of Organic S as SO_4 in Diet	Level of Total S as SO_4 in Diet	Total ^{35}S-Taurine Excreted/ 24 Hours
	%	%	%	cpm X 10^{-3}
1	0.0002	0.67	0.67	3.3 ± 0.7
2	0.10	0.57	0.67	1.4 ± 0.1
3	0.42	0.25	0.67	0.6 ± 0.1
		Statistical Comparisons[b]		
1 to 2				P < 0.02
2 to 3				P < 0.001
1 to 3				P < 0.01

[a]Values represent averages of 10 values obtained from determinations on two 24-hour urine collections from each of five rats ± standard error of mean.

[b]Probability that differences due to chance, as determined by the method of unpaired comparison.

of cysteine sulfur as sulfate ($P < 0.02$). The amount excreted by the animals fed diet 3, the diet that furnished the greater portion of the total sulfur as calcium sulfate was 82 per cent less than the amount excreted by those fed the low sulfate diet (diet 1) ($P < 0.01$) and 42 per cent less than the amount excreted by the animals fed diet 2, which was supplemented with cysteine and sulfate ($P < 0.001$).

When ^{35}S-taurine was measured in urine from the adult rats whose diets each had contained 0.40 per cent of added cysteine but had contained three different levels of calcium sulfate, the data presented in Table 12 were obtained. The animals fed the low sulfate diet (diet 4) again excreted significantly more ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur than did either of the other groups. Reductions of approximately 50 ($P < 0.001$) and 45 ($P < 0.01$) per cent, respectively, were detected in the excretion of ^{35}S-taurine among those animals whose diets were supplemented with 0.10 or 0.42 per cent of inorganic sulfate (diets 5 and 6). Unlike the results obtained when decreases in dietary cysteine accompanied additions of calcium sulfate, however, diminution in ^{35}S-taurine excretion greater than that produced by the addition of 0.10 per cent of inorganic sulfate did not accompany the addition of 0.42 per cent of sulfate to the diet supplemented with 0.40 per cent of cysteine.

TABLE 12

Urinary excretion of ^{35}S-taurine by rats fed three levels of sulfate in diets that contained different levels of total sulfur as sulfate[a]

Diet Number	Level of Dietary SO_4	Level of Organic S as SO_4 in Diet	Level of Total S as SO_4 in Diet	Total ^{35}S-Taurine Excreted/ 24 Hours
	%	%	%	cpm X 10^{-3}
4	0.0002	0.57	0.57	7.3 ± 0.5
5	0.10	0.57	0.67	3.5 ± 0.4
6	0.42	0.57	0.99	4.7 ± 0.6
		Statistical Comparisons[b]		
4 to 5				$P < 0.001$
5 to 6				$P < 0.01$
4 to 6				$P < 0.001$

[a]Values represent averages of 10 values obtained from determinations on two 24-hour urine collections from each of five rats ± standard error of the mean.

[b]Probability that differences due to chance, as determined by the method of unpaired comparison.

Experiment 3

Although the 18 diets fed in experiment 3, each supplemented with 0.40 per cent of ^{35}S-cysteine, ranged in inorganic sulfate content between the extremes of those fed in the other experiments, the criteria used for evaluating the effects of feeding the diets to rats from weaning for six weeks did not reveal significant differences between animals fed high and low levels of sulfate.

Growth data from the animals used in experiment 3 are summarized in Table 13. Since the animals were housed in group cages, feed efficiencies had to be calculated as g of weight gained by a group of animals per g of feed consumed by the group. Individual records of weight gain were kept, but the values shown in Table 13 are expressed as the average weight gain per group $\pm$ the standard error of the mean. Differences in neither feed efficiencies nor weight gains were detected between the animals fed diets supplemented with more or less than 0.10 per cent of inorganic sulfate.

Data presented in Table 14 show that the animals fed the diet supplemented with the lowest level of sulfate in experiment 3 excreted one of the lower levels of ^{35}S-taurine. A plot of cpm of ^{35}S-taurine excreted by each of the 18 experimental groups versus per cent of sulfate

TABLE 13

Weight gain and feed efficiency ratios of rats fed 18 levels of dietary sulfate for six weeks

Level of Dietary SO_4	Weight Gain[a]	Feed Efficiency Ratio[b]
%	g	
0.00	218 ± 6	0.31
0.01	237 ± 13	0.31
0.02	241 ± 7	0.32
0.04	223 ± 6	0.28
0.05	224 ± 4	0.31
0.06	227 ± 13	0.30
0.08	218 ± 13	0.34
0.09	224 ± 22	0.32
0.10	235 ± 12	0.34
0.11	216 ± 12	0.32
0.13	198 ± 14	0.33
0.15	239 ± 4	0.33
0.18	242 ± 5	0.36
0.20	239 ± 3	0.34
0.25	229 ± 8	0.33
0.30	239 ± 18	0.33
0.35	220 ± 11	0.32
0.40	218 ± 6	0.32

[a]Average of six animals ± standard error of mean.

[b]Total g of weight gained by six rats divided by total g of feed consumed.

TABLE 14

Urinary excretion of ^{35}S-taurine by rats fed 18 levels of dietary sulfate for six weeks[a]

Level of Dietary SO_4	Total ^{35}S-Taurine Excreted/ 24 Hours
%	cpm X 10^{-3}
0.00	5.8 ± 0.9
0.01	9.4 ± 2.0
0.02	7.2 ± 1.3
0.04	5.3 ± 1.3
0.05	7.8 ± 1.5
0.06	7.4 ± 2.5
0.08	6.2 ± 1.4
0.09	8.2 ± 2.0
0.10	6.8 ± 0.8
0.11	7.9 ± 1.6
0.13	5.4 ± 1.0
0.15	6.0 ± 1.6
0.18	5.8 ± 1.3
0.20	5.7 ± 0.4
0.25	6.1 ± 1.3
0.30	10.0 ± 2.9
0.35	5.9 ± 1.0
0.40	7.4 ± 1.4

[a]Each value represents average of 10 values obtained from determinations on two 24-hour urine collections from each of five rats ± standard error of mean.

added to the diets is shown in Figure 2. While a trend toward lower ^{35}S-taurine excretion appeared among the rats whose diets were supplemented with more than 0.10 per cent of sulfate, the difference was not clear-cut and was not statistically significant.

The average μg of sulfur as sulfate per mg of dry lung tissue from the rats fed the 18 levels of sulfate are shown in Table 15 and in its companion figure, Figure 3. These data, like the ^{35}S-taurine excretion data, indicated no significant differences among the animals fed the different levels of sulfate.

Experiment 4

Data obtained from experiment 4 show that the age of the rat and the length of the feeding period can influence the effect of dietary sulfate on the excretion of ^{35}S-taurine.

When diets supplemented with 0.40 per cent of ^{35}S-cysteine and either 0.0002 or 0.10 per cent of sulfate were fed to weanling rats, the ^{35}S-taurine excretion values shown in Table 16 were detected. After one week of eating the diets, the animals whose diet contained 0.10 per cent of sulfate excreted significantly less ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur than did the other group ($P < 0.01$). While the averages remained lower at the end of weeks 2

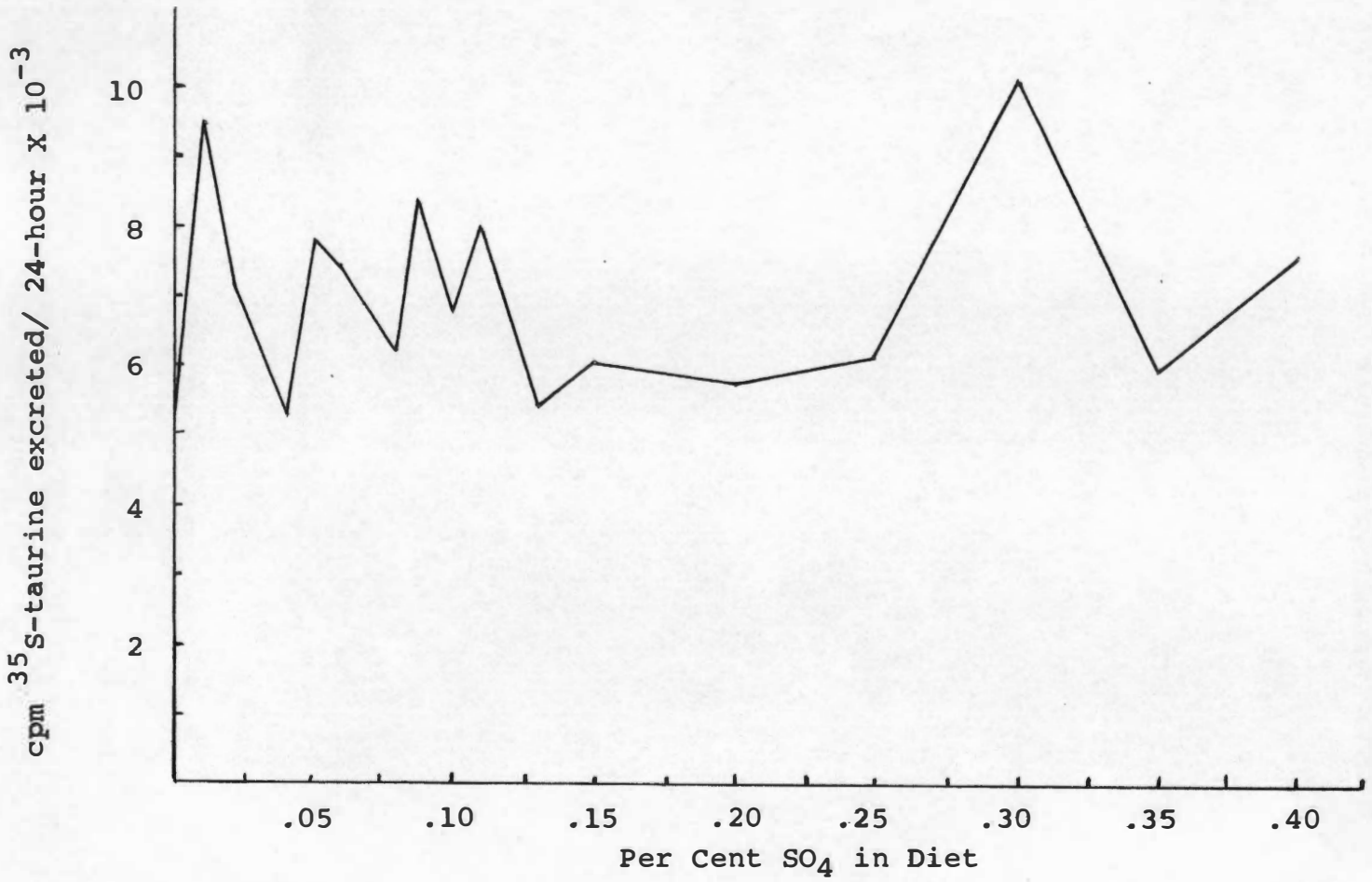

Figure 2. 24-hour excretion of ^{35}S-taurine by rats maintained on 18 levels of dietary sulfate for six weeks. Each point represents the average of 10 values obtained from determinations on two 24-hour urine samples from each of five rats.

TABLE 15

Sulfur as sulfate in lungs of rats fed 18 levels of dietary sulfate

Levels of Dietary SO_4	Average S as SO_4/ mg Dry Lung[a]
%	μg
0.00	24.95 ± 1.25
0.01	27.15 ± 1.55
0.02	23.22 ± 1.70
0.04	25.39 ± 0.80
0.05	24.99 ± 1.29
0.06	21.30 ± 1.46
0.08	24.28 ± 1.24
0.09	25.03 ± 0.44
0.10	25.85 ± 1.30
0.11	24.52 ± 1.26
0.13	26.33 ± 1.49
0.15	24.53 ± 1.27
0.18	22.34 ± 1.70
0.20	25.94 ± 1.28
0.25	24.05 ± 2.33
0.30	22.54 ± 2.19
0.35	25.94 ± 0.21
0.40	25.35 ± 1.24

[a]Each value represents average of 12 values obtained from duplicate analyses of lungs from six rats ± standard error of the mean.

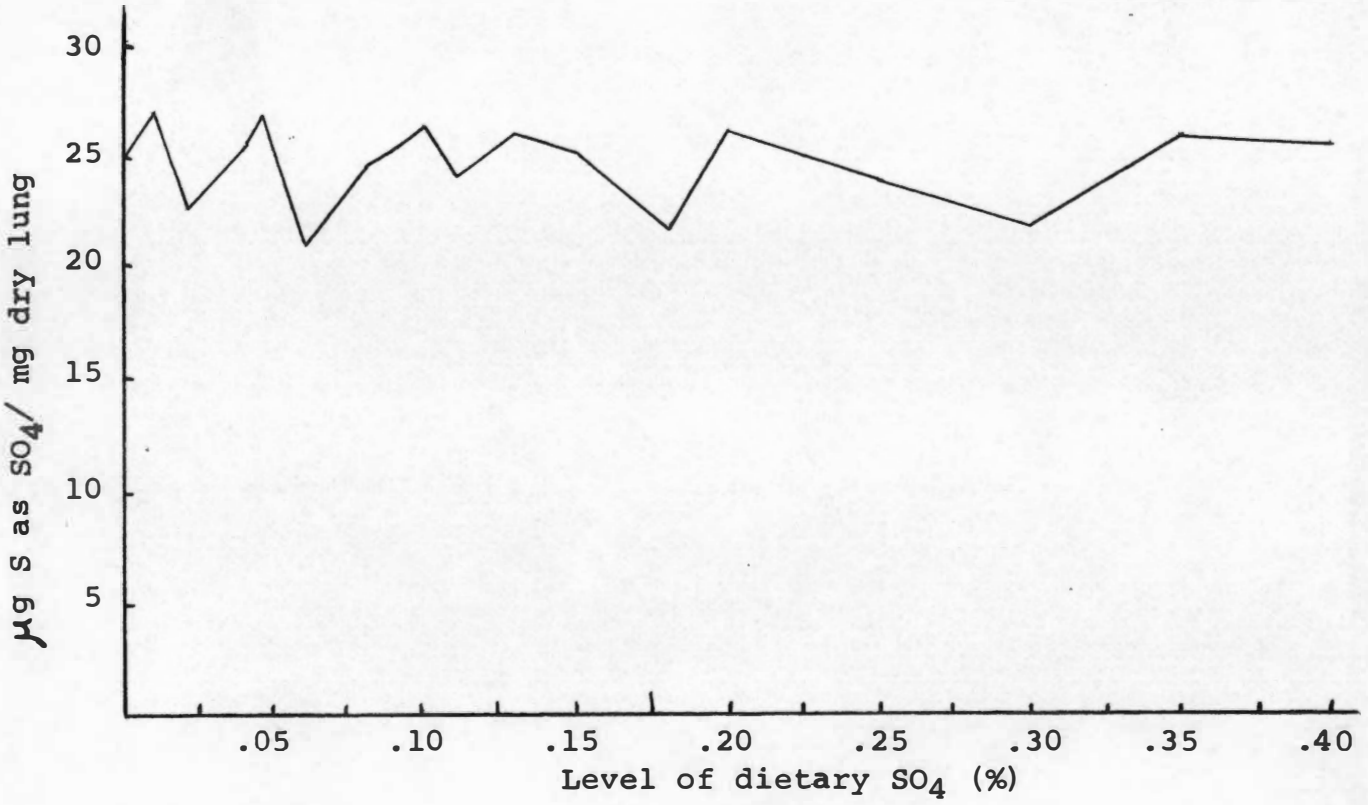

Figure 3. Total sulfur as sulfate in lungs of 18 groups of rats fed different levels of sulfate for six weeks. Each point represents average of six animals.

TABLE 16

Weekly determinations of ^{35}S-taurine excreted by rats fed two levels of dietary sulfate from weaning[a]

Weeks Diet Consumed	Total ^{35}S-Taurine Excreted/24 Hours		P Value[b]
	0.0002% Dietary SO_4	0.10% Dietary SO_4	
	cpm X 10^{-3}	cpm X 10^{-3}	
1	4.6 ± 0.8	1.6 ± 0.9	<0.01
2	3.3 ± 0.9	2.6 ± 0.4	<0.5
3	6.4 ± 0.9	5.3 ± 0.8	<0.3
4	5.4 ± 0.6	7.4 ± 1.9	<0.01
5	7.2 ± 1.5	6.3 ± 1.3	<0.5
6	9.4 ± 2.0	12.1 ± 1.3	<0.2

[a]Average of six animals ± standard error of mean.

[b]Probability that difference due to chance, as determined by method of unpaired comparison.

and 3, the differences were not statistically significant. At no time after the first week of the experiment was the level of ^{35}S-taurine excreted by the young rats fed the 0.0002 per cent sulfate diet significantly higher than that of the rats fed the diet to which 0.10 per cent of sulfate had been added. In fact, the ^{35}S-taurine excretion of the animals fed the low sulfate diet was significantly lower ($P < 0.01$) than that of the other group at the end of week 4.

A similar pattern of adaptation was observed when adult rats were fed the same two radioactive diets. Data presented in Table 17 show that rats fed the diet that contained 0.10 per cent of sulfate excreted significantly less ($P < 0.02$) ^{35}S-taurine than did the other group at the end of weeks 1 and 2, but not at the end of week 3.

TABLE 17

Weekly determinations of ^{35}S-taurine excreted by adult rats fed two levels of dietary sulfate[a]

Weeks Diet Consumed	Total ^{35}S-Taurine Excreted/24 Hours		
	0.0002% Dietary SO_4	0.10% Dietary SO_4	P Value[b]
	cpm X 10^{-3}	cpm X 10^{-3}	
1	6.2 $\pm$ 0.6	4.6 $\pm$ 0.7	<0.02
2	4.0 $\pm$ 0.3	3.2 $\pm$ 0.3	<0.02
3	2.8 $\pm$ 0.4	4.7 $\pm$ 1.2	<0.01

[a]Average of six animals $\pm$ standard error of mean.

[b]Probability that differences due to chance, as determined by method of unpaired comparison.

CHAPTER V

DISCUSSION

The literature contains little information concerning the significance of sulfate in the diet of the non-ruminant mammal. Reports generally have stated that requirements for inorganic sulfate are met by the in vivo oxidation of organic sulfur, and have minimized or disregarded the contributions of dietary sulfate. If inorganic sulfate were of no nutritional import, then omission of the substance from the diet would be inconsequential to the animal. Results of the present study and of earlier studies in this laboratory show, however, that dietary sulfate is utilized by the rat and that its consumption increases the efficiency with which the sulfur-containing amino acids are utilized.

A sparing effect of dietary sulfate toward the sulfur-containing amino acids is evidenced by the results of experiment 1. The data presented in Table 9, page 63, show that feed efficiencies were significantly higher among the animals fed the diet supplemented with 0.10 per cent of sulfate and 0.35 per cent of methionine (diet B) than among those fed either the high sulfate ($P < 0.01$) or the low sulfate ($P < 0.001$) diet. Because

the experiment was designed to supply the three groups of animals with similar levels of total sulfur as sulfate but with different levels of dietary sulfate, the high sulfate diet contained less than the 0.6 per cent of sulfur-containing amino acids recommended for the rat (140). It contained only 0.5 per cent, which was supplied by the 15 per cent of casein, while the normal sulfate diet contained 0.8 per cent and the low sulfate diet contained 1.1 per cent of the sulfur-containing amino acids.

The significantly lower feed efficiency ratios of the animals fed the low sulfate diet (diet A) compared with those of the animals fed the normal sulfate diet (diet B) appears to indicate that the sulfur-containing amino acids were limiting, although they were present in the low sulfate diet at the level of 1.1 per cent. In 1965, Button et al. (96) reported that rats fed 15 per cent casein diets supplemented with 0.35 per cent of methionine exhibited significantly higher feed efficiencies when the diets also were supplemented with 0.42 or 0.10 per cent of inorganic sulfate than when they were supplemented with 0.02 per cent. Those investigators suggested that, since part of the sulfur requirement of the rat could be filled by dietary inorganic sulfate, a reduction

of the level of inorganic sulfate in the diet increased the quantity of the sulfur-containing amino acids needed by the animal (100).

Accordant with the findings of Gilmore (101), the results of the present investigation show that the omission of inorganic sulfate from the diet is not compensated by the addition of a theoretically-slightly excessive amount of potential sulfate as methionine above the level of that amino acid reported to be adequate for the rat (140). Gilmore (101) observed the distribution of ^{35}S from methionine and from dietary sulfate among feces, urine, blood, and rib cartilage mucopolysaccharides of rats fed diets similar in their contents of inorganic, organic, and total sulfur as sulfate to the ones employed in the present study. Her results suggested that oxidation products, which were excreted and of little use to the animal, were synthesized concurrently with the obligatory oxidation of amino acid sulfur to sulfate. The significantly higher ($P < 0.001$) feed efficiencies of the animals fed the normal sulfate diet (diet B) compared with those of the animals fed the low sulfate diet (diet A) in the present study are compatible with the findings of Gilmore (101); the 0.25 per cent of sulfur-containing amino acids furnished by diet A above the level in diet B theoretically could supply 0.16 per cent of sulfate, but did

not compensate for the omission of 0.10 per cent of dietary sulfate. If sulfur-containing by-products were formed in the amino acid sulfur-to-sulfate oxidation, sulfur-containing amino acids above the theoretical amount would be needed for sulfate synthesis and the level of those amino acids in the diet could become limiting. The failure of the animals fed diet A, the low sulfate diet, to exhibit feed efficiencies or weight changes that differed significantly from those of the animals fed diet C, the high sulfate diet, despite the difference in the level of sulfur-containing amino acids received by the two groups suggests that the efficiency of the amino acid sulfur-to-sulfate conversion could be as low as 50 per cent.

Additional evidence for the inefficiency of the in vivo conversion is furnished by the results of experiment 2. The excretion of higher levels of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur by the animals fed the low sulfate diets, irrespective of the level of total sulfur as sulfate, compared with the levels excreted by the animals fed the normal sulfate diets indicated that increased taurine synthesis is signaled concurrently with the signal for increased in vivo synthesis of sulfate. Results of an earlier study conducted by Tigert (102) showed that taurine synthesis by rat liver homogenates was increased when the system was blocked so that thiosulfate rather

than sulfate was produced from ^{35}S-cysteine. The in vitro findings and the present in vivo observations appear to suggest that the oxidation of amino acid sulfur to sulfate and to taurine is induced by the absence of sulfate.

If dietary sulfate and the sulfur-containing amino acids can fill some of the same metabolic functions, then a level of the sulfur-containing amino acids that is inadequate in the absence of dietary sulfate could be in excess of that needed by the animal when other sources of sulfate also are available. Data presented in Table 12, page 68, may be interpreted as indicating that these conditions existed when the animals were fed diets 4, 5, and 6, each supplemented with 0.40 per cent of cysteine but with different levels of dietary sulfate. The highest level of ^{35}S-taurine, which was excreted by the animals fed the diet not supplemented with inorganic sulfate (diet 4), parallels the ^{35}S-taurine excretion pattern observed when the three levels of dietary sulfate were fed in diets supplemented with different levels of organic sulfur as sulfate (Table 11, page 66) and reflects the in vivo synthesis of inorganic sulfate. That the excretion of ^{35}S-taurine was significantly higher ($P < 0.01$) among rats fed 0.42 per cent of dietary sulfate (diet 6) than among those fed 0.10 per cent (diet 5) when the sulfur-containing amino acids comprised 0.9 per cent of the diets,

but was 42 per cent lower among those fed the high sulfate diets in which a reduction in amino acid sulfur accompanied the addition of sulfate (diet 3), suggests, however, that 0.9 per cent of sulfur-containing amino acids was more than adequate for the rat when supplemented with 0.42 per cent of dietary sulfate. The higher level of ^{35}S-taurine excreted by the rats fed diet 6 reflects a metabolic loss attributable to an excessive supply of the sulfur-containing amino acids.

Rather than minimizing the role of either, the present data show that both dietary sulfate and the sulfur-containing amino acids are important in maintaining the nutritional status of the rat. The significantly lower weight gains (Table 10, page 64) and feed efficiencies (Table 9, page 63) of the rats fed the high sulfate diet compared with those of the rats fed the normal sulfate diet show that certain needs of the animal can be met only by the sulfur-containing amino acids and that a dietary level of 0.5 per cent of those amino acids, as supplied by the 15 per cent casein diet, does not meet the requirements for maintenance and growth even at the highest levels of inorganic sulfate fed in this study. The significantly lower excretion ($P < 0.001$) of ^{35}S-taurine by the animals in experiment 2 fed the high sulfate diet that contained no sulfur-containing amino acids beyond

those furnished by casein (diet 3) compared with the level excreted by the animals fed the normal sulfate diet (diet 2) probably is attributable also to the deficiency of the sulfur-containing amino acids. However, the effects of feeding a diet low in inorganic sulfate and low in the sulfur-containing amino acids were not determined in the present study and should be tested to verify this suggestion.

Neither the maximum levels of dietary sulfate and organic sulfur as sulfate that can be utilized by the rat nor the ratio of the two fractions that brings about most efficient utilization has been determined. The findings do appear to indicate, however, that 0.10 per cent of dietary sulfate may be adequate when total sulfur as sulfate comprises 0.67 per cent of the diet. As mentioned previously, the data presented in Table 12, page 68, suggest that a 15 per cent casein diet supplemented with 0.42 per cent of sulfate and 0.40 per cent of cysteine may furnish the rat with more than adequate amounts of the sulfur-containing amino acids. Results of an earlier investigation in this laboratory showed that the total sulfur as sulfate was no higher in lungs from animals fed diets for six weeks that contained 0.42 per cent of inorganic sulfate than in those from animals fed 0.10 per cent of sulfate, but both groups of animals exhibited significantly

higher levels of lung sulfur as sulfate than did a group fed a diet to which no inorganic sulfate had been added.[1] Gilmore (101) detected greater incorporation of ^{35}S into rib cartilage mucopolysaccharides from rats whose diets contained 0.42 per cent of ^{35}S-calcium sulfate than from those whose diets contained 0.10 per cent of ^{35}S-calcium sulfate and equal levels of total sulfur as sulfate. Since her findings were based on the uptake of ^{35}S, they do not necessarily indicate a difference in actual levels of total sulfur as sulfate in the mucopolysaccharides. Determination of feed utilization, tissue sulfur as sulfate, and urinary taurine levels of rats fed diets similar to those fed in experiment 3, but for a shorter period, should provide information concerning the actual sulfate needs of the animal.

The ^{35}S-taurine excretion data obtained from experiments 3 and 4 show that when the rat is forced to satisfy its sulfate requirements by oxidation of amino acid sulfur to sulfate for an extended period adaptation occurs which reduces the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur to the level excreted by the animal fed the normal sulfate diet. These data do not reveal the mechanism of the adaptation, nor do they reveal the

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[1]Smith, J. T. Unpublished observations.

effect of the decreased cysteine-to-taurine conversion on the synthesis of sulfate or other sulfur-containing products.

No differences were detected in the average μg of sulfur as sulfate per mg of dry lung tissue from the 18 groups of animals fed different levels of inorganic sulfate, but this observation does not exclude the possibility that differences in sulfate production existed among the groups. Diets with a lower level of sulfur-containing amino acids than the 0.9 per cent contained in each of the diets fed in experiment 3 were employed in the study mentioned previously in which differences in total sulfur as sulfate in lungs from two groups of rats fed different levels of sulfate were detected,[2] and that factor should be taken into account if the results of the two studies are compared. Further investigation is needed to determine whether the in vivo synthesis of sulfate is affected by the adaptation to the low sulfate diet evidenced by decreased excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur.

The currently-available information does not elucidate the manner in which the metabolism of amino acid sulfur is regulated, but several mechanisms of

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[2] Smith, J. T. Unpublished observations.

action are indicated. While the substrates logically must be present in order for the enzymes to function, the pattern of sulfate synthesis which has been observed cannot be explained by the influence of the substrates. Rather, the mechanism suggested by the observations is that activity of the enzymes involved in the production of endogenous sulfate are induced by the absence of dietary sulfate. Evidence obtained from this and earlier in vivo studies (95, 101) and from in vitro studies (102) suggests that increased taurine synthesis is signaled concurrently with increased sulfate synthesis, thus resulting in increased excretion of amino acid sulfur in the urine.

The mechanism of adaptation to diets low in inorganic sulfate, as demonstrated by decreased excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur, is less clearly defined. The change could be effected by the end product, i.e. repression of the enzyme(s) controlling taurine synthesis by the level of taurine, or it could be effected by induction of some enzyme(s) involved in the synthesis of sulfate by pathway(s) other than via cysteinesulfinic acid. Enzymes of pathways leading to the production of other sulfur-containing products also could be induced. The significance of the adaptation as a mechanism of conserving the sulfur-containing amino acids for other

functions is not revealed by the present findings, but the results of this study are the first to suggest that the rat is forced to alter its normal pattern of sulfur metabolism when dietary sulfate is not supplied.

CHAPTER VI

SUMMARY

The relationship of dietary sulfate to the efficiency of feed utilization, excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur, and sulfation of lung tissue by the rat was investigated.

The feed efficiencies of animals fed diets from weaning that contained 0.10 per cent of inorganic sulfate and 0.47 per cent of organic sulfur as sulfate were significantly higher at the end of a six-week feeding period than were those of littermates fed diets that contained higher or lower levels of inorganic sulfate but comparable levels of total sulfur as sulfate. These findings showed that equivalent levels of sulfur as sulfate supplied by amino acids did not compensate for the omission of sulfate from the diet, and they were interpreted as indicating that the obligatory oxidation of amino acid sulfur to sulfate is not an efficient process.

Results of a subsequent experiment revealed that the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur at the end of a 17-day feeding period was 58 and 82 per cent lower, respectively, among adult rats fed normal and high levels of inorganic sulfate than among those fed

low levels of inorganic sulfate in diets that contained equal levels of total sulfur as sulfate. Reductions of 50 and 45 per cent, respectively, from the level of ^{35}S-taurine excreted by the animals fed the low sulfate diet were observed when normal and high levels of sulfate were fed in diets that contained equal levels of cysteine.

When 18 groups of rats were fed different levels of inorganic sulfate in diets supplemented with 0.40 per cent of cysteine, differences could be detected neither in ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur excreted in the urine nor in the total sulfur as sulfate in the lungs at the end of the six-week feeding period.

After the experimental diets had been consumed for one week, the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur was significantly higher among weanling rats fed a low sulfate diet than among those fed a normal sulfate diet that contained the same level of cysteine, but no difference could be detected by the end of week 2. Adult rats fed the low sulfate diet excreted significantly higher levels of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur at the end of weeks 1 and 2 of the study than did the animals fed the normal sulfate diet, but not at the end of week 3.

Dietary adaptation, which results in reduction in the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur

to the level excreted by the animal fed the normal sulfate diet, occurs when low sulfate diets are fed to rats for extended periods. The initial rise in ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur in the urine illustrates an inefficiency in the oxidation of amino acid sulfur to sulfate. The significance of the adaptation to the low sulfate diet, demonstrated by decreased ^{35}S-taurine excretion, as a means of conserving the sulfur-containing amino acids is not revealed by the present findings.

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