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An Effect of Dietary Sulfate in Rats Fed Diets Low in Sulfur Containing Amino Acids

Helen T. Pfuderer
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Helen T. Pfuderer entitled "An Effect of Dietary Sulfate in Rats Fed Diets Low in Sulfur Containing Amino Acids." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

John T. Smith, Major Professor

We have read this thesis and recommend its acceptance:

Ada Marie Campbell, Mary Rose Gram

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

December 13, 1968

To the Graduate Council:

I am submitting herewith a thesis written by Helen T. Pfuderer entitled "An Effect of Dietary Sulfate in Rats Fed Diets Low in Sulfur Containing Amino Acids." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

John T. Smith
Major Professor

We have read this thesis and recommend its acceptance:

Ada Marie Campbell

Mary Rose Sam

Accepted for the Council:

Hilton A. Smith
Vice Chancellor for
~~Graduate~~ Studies and Research

AN EFFECT OF DIETARY SULFATE IN RATS FED DIETS LOW IN SULFUR CONTAINING AMINO ACIDS

A Thesis

Submitted to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Helen T. Pfuderer

March 1969

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ABSTRACT

Seven diets which were low in sulfur containing amino acids but with inorganic sulfate from 0.0002 percent to 3.130 percent in systematic increases were fed to rats. The sulfur as sulfate in freeze-dried and combusted lungs and livers of these rats was determined.

The data obtained showed a significant difference in both the amount of total sulfur in the lung as sulfate and the sulfur per mg. of dried lung. However, no significant difference was found in the total sulfur as sulfate in the liver or sulfur as sulfate per mg. of dried liver. Since there is a high percentage of cartilaginous material in the lungs, it is reasonable to assume there is more mucopolysaccharide sulfur in the lung than in the liver and that the effect of increased mucopolysaccharide sulfation could be masked when the total liver sulfate was measured, but not in the lung. Based on previous data obtained from this laboratory, the increase in lung sulfate was interpreted as a reflection of increased synthesis of mucopolysaccharides.

The sulfate content of the lungs obtained in this investigation shows quite conclusively that before dietary sulfate additions will give a measurable effect on retention of sulfate by the tissues, greater than approximately 0.1 percent sulfate should be added to the diet.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Previous work in this laboratory has shown that there is alteration of collagen and mucopolysaccharide metabolism if a rat is forced to satisfy its sulfate requirements by the oxidation of sulfur from the sulfur containing amino acids (1). In addition there is a greater potential for avitaminoses E symptoms, an apparent requirement for cod liver oil (1) and an increase of sulfate mobilization in malathion toxicity (2). Michels (3) fed sulfur to rats as $^{35}SO_4^=$ and ^{35}S-methionine in diets in which the total sulfur was kept constant but the ratio of inorganic to organic was changed. This investigation demonstrated that the rat could use dietary sulfate and endogenous sulfate from the sulfur amino acids equally well for the sulfation of cartilage mucopolysaccharides. Based on the uptake of $^{35}SO_4^=$ by cartilage mucopolysaccharides, those diets which were highest in inorganic sulfate promoted the greatest incorporation of $^{35}SO_4^=$ by tissue.

The results which have been described above have shown an apparent requirement for dietary sulfate in addition to sulfur containing amino acids but since the uptake experiments have been carried out with radioactive tracers no net increase in tissue sulfur has been demonstrated. The present investigation was designed to measure tissue sulfur which might change as a result of feeding diets low in sulfur amino acids but with increasing amounts of sulfate in an attempt to determine the optimum level of dietary sulfate.

CHAPTER II

REVIEW OF LITERATURE

The major source of dietary sulfur in most animals is methionine and cystine. Methionine is an essential amino acid for growth and maintenance in humans, rats and chicks but not in the cat, and cystine spares methionine. Any need for sulfur by an animal which can be met by inorganic sulfate could be of real importance in places in the world where methionine and cystine may become limited.

Until 1961, it was believed by some researchers that sulfate was not absorbed or utilized; however, Anast and others (4) found 10.3 percent absorption in rats and work is continuing on the manner and extent of sulfate absorption. Incorporation of $^{35}SO_4^{=}$ into mucopolysaccharides presents a criterion for measurement of sulfate utilization following absorption.

Absorption of sulfur amino acids and inorganic sulfate

Serial perfusion of the jejunum of normal subjects with equimolar mixtures of eighteen L-amino acids showed a preferential and characteristic rate of absorption for each amino acid (5,6). Methionine, isoleucine, leucine and valine were the most rapidly absorbed. At perfusion concentrations of 2, 4 and 8 mM, the absorption of methionine and branched chain amino acids was approximately 90 percent. D-, L-, and DL-methionine were fed to fasted white rats by stomach tube. The rates of absorption

were calculated by determining the methionine in the gastrointestinal tract after 1, 2, and 3 hours. The rates of absorption for D-, L-, and DL-methionine as the sodium salts were 35.6, 35.7, and 38.2 mg. per 100 gms. per hr. respectively (7).

Edwards and others (8,9) found the absorption of L-methionine is 1.1 times that of its DL-isomer. They also found that after 30 minutes the plasma and plasma proteins of rats fed L-methionine-methyl-^{14}C contained greater activity than the corresponding components of rats fed the DL form. Significantly greater concentrations of ^{14}C were present in brain, lungs, liver and hearts of rats fed L methionine-methyl-^{14}C than in tissues of those fed the DL form. Absorption of L-methionine was measured in all parts of the human small intestine using transintestinal intubation and perfusion (10) in four normal subjects. Absorption was higher in the proximal than in the distal intestine. In two patients with nontropical sprue in relapse, there was a proximal zone of low absorption with higher absorption distally. In normal subjects the proximal K_m was three-fold greater than in the distal which suggests a difference in transport mechanisms between the 2 segments.

Lerner and Wright (11) report the intestinal absorption of D- and L-methionine in the chicken occurs by attachment to a common L-preferring site, presumably on the mucosal epithelial membrane. Neutral amino acids of the L-configuration had a high affinity for the methionine transport site, while neutral D-amino acids except for D-methionine had a very low affinity. Methionine enhanced the transport of tryptophan but large concentrations inhibited tryptophan transport. This was

thought to be a counterflow effect of methionine on the transport of tryptophan by the di-amino acid carrier (12). Hart and Nissim (13) believe the mechanism of intestinal absorption may involve active transport by a spectrum of mobile intracellular proteins. They explain this with the inhibition of the absorption of D-glucose, DL-methionine and sodium butyrate by a concentration of centramide of 10^{-3} (weight per volume) while at lower concentrations (2.5×10^{-5} weight per volume), it stimulates the absorption of these compounds by the small intestine of the rat. Centramide acts on a mobile receptor which is concerned in active transport. The association-dissociation constants for nutrients are controlled by a pH or electrochemical gradient which is maintained by the metabolic activities of the cell and requires expenditure of energy. Hart and Nissim conclude this mobile receptor is located within the cell and not on the cell surface or within the cell membrane.

Binder et al. (14) demonstrated species differences in the response of amino acid transport to ouabain and to a sodium-free medium. Addition of ouabain, an inhibitor of the sodium dependent ATPase, failed to inhibit amino acid absorption by the hamster and mouse but completely inhibited transport in the guinea pig. Complete inhibition in the sodium-free medium was demonstrated in the hamster and guinea pig but was minimal in the mouse. Enthacrynic acid (5mM) and probenecid (5mM) both of which are inhibitors of renal transport also inhibited L-methionine intestinal absorption (15). Transferable hexoses have an inhibitory effect on the transport of L-methionine which is probably due to competition for some common requirement involved in active transfer

(16,17). L-methionine as well as D-, L-histidine considerably inhibited the active transport mechanism of D glucose and diminished other sugars. D-methionine is believed to interfere with the process by which epithelial cells capture and concentrate sugar at the luminal border (18). Kemeny and others (19) reported there was an insignificant decrease in methionine absorption in rats fed protein-deficient diets for 24 days or more. It was also found that free methionine was absorbed much faster than methionine in proteins (20).

Cystine and cysteine absorption are of particular interest as there is evidence of cystine malabsorption in the small intestine in cystinuria (21). Cysteine uptake is not defective in the gut of cystinuric patients and thus it was concluded that cystine and cysteine are transported by different mechanisms (22). These facts suggested to Spencer et al. a role for the disulfide bond found in the normal transport of cystine (23,24). They found that transport of cystine was abolished by replacing the disulfide bond by other linkage groups. Schwatzman and others (25) think this is also the defect in renal transport of cystine in cystinuria. D-, L-, DL-, and meso cystine were fed to fasted white rats by stomach tube (7). For L- the rate was 49.9; D-, 45.6 and DL-, 53.6 mg. per 100 gms. per hr. The rate for meso cystine was 41.3 mg. per 100 gms. per hr. Cysteine hydrochloride was absorbed slowly, 25.7 mg. per 100 gms. per hr. The sodium salt was absorbed faster, 41.4 mg. per 100 gms. per hr.

The site of sulfate absorption has been studied by the ability of intestinal mucosa to hydrolyze p-nitro catechol sulfate or p-nitro

phenyl sulfate and by the measurement of ratio of sulfate in serosal and mucosal fluid. Sulfate metabolism in homogenates of rat intestinal mucosa was investigated in jejunal and ileal segments from adult (43 day old) and suckling (13 day old) rats (26). Hydrolysis of p-nitro catechol sulfate or p-nitro phenyl sulfate proceeded most rapidly in incubation mixtures containing homogenates of ileal mucosa from 13 day old rats. However, differences were marked with age in the rat and suggested important developmental changes in the physiological function of these 2 segments of the gut.

Anast and others (4) compared sulfate transport in the small intestine of rats, rabbits and hamsters. The transport of $^{35}SO_4^{=}$ from $Na_2{}^{35}SO_4$ was expressed as the ratio of counts per minute per ml. in the serosal fluid to counts per minute per ml. in the mucosal fluid (S/M). The greatest concentration of transport occurred in the distal segment of the intestine where the S/M was 6.1, 10.3 and 15.7 in the hamster, rat and rabbit, respectively. The midgut and proximal gut showed little transport. Puromycin at 5×10^{-5} M caused no inhibition of $SO_4^{=}$ transport, but incubation in nitrogen markedly reduced transport as did 2, 4-dinitrophenol and ouabain. Sulfate transport against an electrochemical gradient fulfilled the criteria for active transport of an ionized substance. Michels (3) found that in rats fed a normal diet there is a significantly higher percentage absorption of radioactivity ingested as ^{35}S-methionine when compared to that ingested as inorganic salts. It was also observed that the percentage absorption of the radioactivity supplied as inorganic salts was significantly higher from those diets

not supplemented with methionine than from those supplemented with methionine. Michels also found Ca $^{35}SO_4$ was more readily absorbed than Na_2 $^{35}SO_4$. In rats fed an otherwise sulfur-free diet sodium sulfate was completely absorbed (27).

From these studies it is evident methionine is actively absorbed in the proximal intestine. Cystine and cysteine are actively absorbed but by different mechanisms. Sulfate is also actively absorbed. Sulfate absorption may be depressed when adequate or excess methionine is present in the diet.

Sulfur requirement studies

Sulfur balance studies have been conducted in rats to determine the requirements for dietary sulfur compounds. In starvation neutral sulfur in urine increased but free and conjugated sulfur decreased (28). When methionine was added to diets at the level of 0.053 to 0.15 parts per 100 parts sulfur or homocysteine at 0.094 parts per 100 parts sulfur only the rats receiving the most sulfur had free sulfur in the urine. The conjugated urinary sulfate was constant and independent of the diet. The neutral sulfur was high. The requirement for methionine in the adult rat was determined to be 30 mg. per kg. per day (29). It was shown that neutral sulfur of the urine was directly related to methionine metabolism and unrelated to total sulfur or nitrogen balances (30).

It was found that cysteine could replace 87 percent of the methionine and cystine, 66 percent (31).

Weller et al. reported that sulfate could supply about one-third of the total sulfur requirement in rats and sulfur containing amino acids were necessary for the remainder (27).

Huovinen and Gustafsson found after injection of labeled sulfate or sulfite, both cysteine and methionine were labeled slightly in conventional rats whereas no labeling of amino acid was detectable in germ-free animals. After injection of sulfide, cysteine was labeled to approximately the same extent in both conventional and germ-free rats. Labeling of methionine in conventional rats was much less and in germ-free rats was not detectable (32). These authors and Waldschmidt (33) agree the rat cannot use inorganic sulfate for protein synthesis. Robinson (34) found inorganic sulfate-^{35}S was incorporated in ^{35}S-sulfoglutathione by the rat mucosa, but found no ^{35}S-sulfoglutathione in rat liver or colon.

The cat apparently can synthesize methionine and cystine from inorganic sulfate and has no exogenous requirement for the sulfur amino acids (35).

In an otherwise sulfur-free diet sodium sulfate could increase the growth and feed efficiency of chickens. The chick could satisfy part of its total sulfur requirement with inorganic sulfate (36). The presence of inorganic sulfate in the diet appeared to enhance the formation of taurine more than did organic sulfate. Dietary cysteine repressed the formation of taurine in the liver and taurocholate in the bile fluid (37). Large amounts of methionine were incapable of satisfying the total sulfur requirement when fed to chicks on low sulfate-low cystine diets (36).

It seems apparent that rats and chickens can utilize sulfate. The cat can synthesize methionine and cystine from dietary inorganic sulfate. The fate of the sulfur from the sulfur amino acids and inorganic sulfate in the body has been reviewed in detail by Rutledge (38). Incorporation of sulfate into mucopolysaccharides presents a criterion for measurement of sulfate utilization.

Mucopolysaccharide sulfate acceptors

In general it has been found that extracts from various tissues such as embryonic cartilage (39-42), hen oviduct (43), serum (44), skin (40,45), cornea (46), chondrosarcoma (30,31,38), carcinoma, leiomyosarcoma (47), and mast cell tumors (48) catalyze the direct sulfation by 3'-phosphoadenosine-5'-phosphosulfate (PAPS) of a wide range of mucopolysaccharides. The formation of PAPS has been reviewed in detail by Disney (2).

Sulfates are transferred to acetylhexosamine residues with the formation of acetylhexosamine monosulfate residues or they are transferred to hexosamine monosulfate residues with the formation of acetylhexosamine disulfate residues. In the reports mentioned above the sulfate transferring enzymes were generally described to be in high speed supernatant fractions of the tissue homogenates. Enzyme fractionation and the study of acceptor specificity have shown separate enzymes called sulfokinases are involved in the addition of sulfate to chondroitin sulfate A, B, or C, heparitin sulfate and heparin.

The cellular location and mechanism of sulfation of mucopolysaccharides has been studied in rats. Microsomes from embryonic cartilage were subfractionated to yield smooth microsomes and rough microsomes. All the activities necessary for linkage to protein as well as for completion of the polysaccharide chain, in chondroitin sulfate biosynthesis, were present in both the smooth and rough fraction (50).

After the administration of $Na_2\ ^{35}SO_4$ the different cell types of the connective tissue showed the following radiosulfate incorporation. The loss of radiosulfate in the fibroblasts and other cells coincided with the gain in the intercellular substance. The sulfomucopolysaccharides in the intercellular substance of the subcutaneous connective tissue were produced mainly by the fibroblasts. Fatty cells seemed to incorporate sulfate by sulfonating their already existing mucopolysaccharide and the macrophagepolyblasts seemed to undergo a metamorphosis into fatty cells by incorporating higher amounts of sulfate (51).

Serine-^{14}C, acetate-^{14}C, and inorganic sulfate-^{35}S were incorporated into polysaccharides by a suspension of minced embryonic cartilage. Puromycin, and 6-diazo-5-oxonorleucine inhibited the incorporation of all 3 compounds but the inhibition involving ^{35}S was reduced by addition of glucosamine. A biosynthetic mechanism that involves addition of carbohydrate units to preformed protein to yield a protein-polysaccharide complex is indicated (52).

The activity of cartilage and spinal discs to incorporate sulfate into polymeric material as early as 4 hours after administration of the isotope strongly suggests that sites for sulfation pre-exist in the

tissue. This implies that the addition of sulfate to form the ester sulfate group of the polysaccharide is the terminal step in the biosynthesis of protein-polysaccharide complexes found in the extracellular matrix of connective tissues. It seems quite likely that the bulk of fixed sulfate rapidly appearing in cartilage may represent inorganic sulfate chelated to calcium by a bridge type arrangement whereby the calcium is bound to ester sulfate pre-existing in the tissue but is still able to bind inorganic sulfate from the environment (52).

Several very potent inhibitors of the incorporation of sulfate into mucopolysaccharides have been found among metal ion complexing compounds particularly the mercaptoamines. A close correlation between percent inhibition of sulfation and metal binding strength was observed which establishes that the mode of inhibition is one of metal binding of metalloenzymes involved in mucopolysaccharide sulfation (54).

A 2 hr. incubation with cortisone, hydrocortisone, predisolone, dexamethasone, predytidine, or deoxycorticosterone (all 0.2-0.8 mM) gave a dose-related inhibition of $^{35}SO_4^=$ incorporation into calf costal cartilage *in vitro*. Of these steroids, deoxycorticosterone had the greatest inhibitory effect. No relation was found between the anti-inflammatory and antirheumatic effect of the steroids and their ability to inhibit $^{35}SO_4^=$ uptake into cartilage (55).

Insulin stimulated incorporation of sulfate into protein polysaccharide complexes. Both puromycin and actinomycin inhibited incorporation of sulfate in both the presence and absence of insulin (56).

When the serum from hypophysectomized rats which were injected with growth hormone was incubated with sulfate and cartilage in vitro, the growth hormone led to a marked increase in sulfate uptake of costal cartilage. The direct addition of growth hormone to the incubation media even in high nonphysiological concentrations had little effect on sulfate uptake. Additions of thyroxine, adrenal corticosteroid or insulin to hypophysectomized rat serum were without effect on the system. The factor in the plasma for growth hormone-treated animals which was capable of stimulating uptake of sulfate was removed from the serum by dialysis (57).

Cortisone, injected at the level of 1.25 mg. per 20 gms. of maternal body weight, resulted in decreased ^{35}S uptake by 10-14 day old mouse embryos, all of which exhibited cleft palates. Vitamin A, at a level of 10,000-40,000 I.U. per 20 gms. maternal weight, increased embryonic sulfate 1.8 times over the level of untreated control embryos. Trypan blue, injected at 1 mg. per 20 gms. maternal weight also stimulated ^{35}S uptake and resulted in deformed embryos. Congenital malformations of mesodermally derived tissues may be related to abnormal embryonic sulfate metabolism (58).

Research on the sulfokinases has been reviewed by Disney (2). Spolter and Marx (49) showed that labeled inorganic sulfate was incorporated into heparin by mouse mast cell tumor homogenates thus proving that PAPS is involved in sulfation of heparin. Research by Sawicki (59) pointed to a definite site of mast cell cytoplasm for synthesis, or at least sulfation of heparin. Silbert (60) describes a mouse mast cell

tumor microsomal preparation which catalyzes the incorporation of $^{35}SO_4^=$ from PAPS into microsomal heparin. This enzyme preparation is the same preparation which had been shown to catalyze the incorporation of sugars into a microsomal glycosaminoglycan related to heparin. It was suggested that polysaccharide polymerization and sulfation take place in close proximity in the cell.

A possible role of several nucleotides in sulfation has been suggested in two papers. Picard and Gardaus (61) found that injected $Na_2\ ^{35}SO_4$ was incorporated more rapidly into nucleotide sulfates than into the mucopolysaccharide sulfates in the same tissue. In aorta and oviduct cartilage Harada and others (62) purified an enzyme which catalyzes the transfer of sulfate from PAPS to the acetylgalactosamine moiety of uridine diphosphate N-acetylgalactosamine 4-sulfate of hen oviduct.

There appears to be universal agreement that inorganic sulfate is coupled to the formation of ester sulfates by energy from ATP (2).

Methionine, cystine, cysteine and inorganic sulfate are actively absorbed. Research on mechanisms and enzymes of mucopolysaccharide sulfation is continuing. The research which is the subject of this manuscript examines 2 tissues of the rat for sulfate which might increase as a result of increased dietary sulfate.

CHAPTER III

EXPERIMENTAL

I. GENERAL PLAN

Previous work in this laboratory has compared the utilization of organic and inorganic sulfate by the rat fed diets adequate in sulfur amino acids. This investigation was designed to study the sparing effect of inorganic sulfate in rats fed diets in which the sulfur containing amino acids were limiting. This study was divided into 2 experiments in which the diets and methods were similar so that they may be discussed simultaneously.

Diets

Alpha protein, a purified soybean protein, was chosen as the protein source for these experiments because of its low sulfur amino acid content. The essential amino acid content of alpha protein may be obtained by reference to Table I and Figure 1. A level of 15 percent alpha protein in the diet was chosen which covers all reported amino acid needs for the rat for growth (63) except for threonine, which was supplemented to the required level, and the sulfur amino acids which were left limiting.

The diets were modifications of those used by Pendergrass (64) and Chin (65). Composition of the diets is presented in further detail in Tables II and III. Inorganic sulfate was varied using modifications

TABLE I

ESSENTIAL AMINO ACID COMPOSITION OF ALPHA PROTEIN[a]

Essential amino acids	Grams/100 grams protein (16 grams nitrogen)
Arginine	7.6
Histidine	2.9
Lysine	6.7
Tryptophan + Tyrosine	5.3
Phenylalanine	6.3
Methionine + Cystine	3.0
Threonine	3.2
Leucine	7.3
Isoleucine	6.2
Valine	5.1

[a]As assayed by Nutritional Biochemicals Corporation, Cleveland, Ohio.

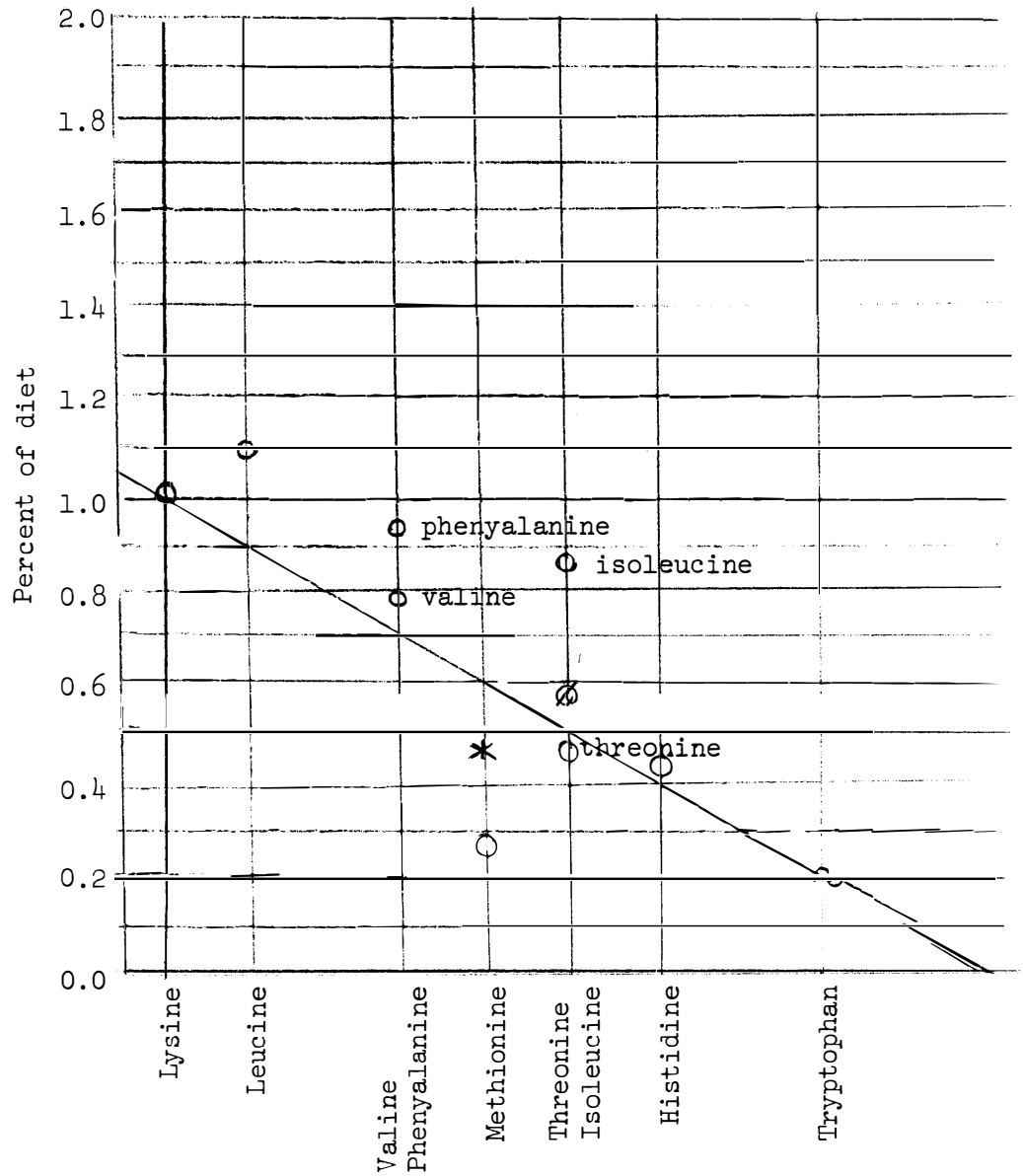


Figure 1. Nomogram comparing essential amino acid content of 15 percent alpha protein diets with amino acids required for growth of weanling rats.[a]

0 - level for 15 percent alpha protein
Ø - level of threonine following supplementation
* - methionine and cystine

[a]Rose, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, 86:198.

TABLE II

COMPOSITION OF BASIC DIETS

Component	Quantity per 100 grams diet	
	Diets 1-7	Diet 8
	g	g
Alpha Protein	15	15
DL Threonine	0.100	0.100
Sucrose	31.1	31.1
Cornstarch	31.1	31.1
Crisco	6	6
Wesson Oil	2	2
Vitamin Mix[a]	2	2
Basic Salt Mix[b]	1.33	1.33
$CaCO_3$	--[b]	0
$CaSO_4 \cdot 2H_2O$	--[b]	5.60
Non-nutritive Bulk[a]	10	4.1

[a]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[b]See Table IV, page 20.

TABLE III

SALT MIXTURE VARIATIONS USED IN THE DIET

Sulfate Diet	Diet Number	Components	
		$CaCO_3$	$CaSO_4 \cdot 2H_2O$
%		gms./100 gms. diet	
0.0002	1	1.34	0
0.001	2	1.34	0.002
0.005	3	1.34	0.009
0.025	4 and 5	1.31	0.045
0.125	6	1.21	0.224
0.625	7	0.068	1.12
3.130	8	0	5.60

of the salt mixture of Hubbell et al. (66). These modifications were made and tested in this laboratory (2,3,64). In order to vary the sulfate content of the salt mixtures $CaSO_4 \cdot 2H_2O$ was used as a partial substitute for $CaCO_3$. The lowest level of inorganic sulfate in the diets was 0.0002 percent and the level was increased systematically in each diet to the level of 3.130 percent inorganic sulfate. The basic salt mix is shown in Table IV.

The basal diets were mixed in 2 kg. lots. At the time of preparation the diets were thoroughly mixed, sieved through a household strainer several times to insure uniformity and then stored in the refrigerator. Feed and distilled water were given ad libitum until sacrifice. Feed consumption of each cage of the rats was determined and recorded when additional feed was necessary. Fresh distilled water and clean water bottles were given as needed, generally once a week. Weekly recordings of weights were made in order to observe growth.

Animals

In both experiments weanling male albino rats of the Wistar strain of the Nutrition Department of the University of Tennessee were used. Litter mates were used on each of the diets as much as possible. The weanling weights of the rats varied from 25 to 40 gms.

In the first experiment five rats were placed on each of four diets. The animals were offered one of diets containing 0.0002 percent sulfate to 0.025 percent sulfate. These diets are listed in Tables II and III.

TABLE IV

COMPOSITION OF SALT MIXTURE[a]

Component	Grams per 100 grams salt mixture
$MgCO_3$	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
$MnCl_2 \cdot 4H_2O$	0.040
$AlK(SO_4)_2 \cdot 12H_2O$	0.017
$Cu(C_2H_3O_2)_2 \cdot H_2O$	0.072

[a]Hubbell, R. G., L. B. Mendel, and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, 14:273, as modified by Pendergrass, B. S. 1961 The interrelationships between sulfate metabolism and the tocopherols in the weanling rat. Unpublished Master's Thesis. The University of Tennessee.

Ten rats were offered each of four diets from 0.025 percent to 3.130 percent sulfate in the second experiment. The vitamin mix was inadvertently left out of the rats' diets for a period of a few days to three weeks and as a result growth was very small. It was decided to sacrifice the rats after they reached 100 gms. in weight. Therefore, their terminal age varied from 42 to 86 days.

II. METHODS

Preparation of liver and lungs

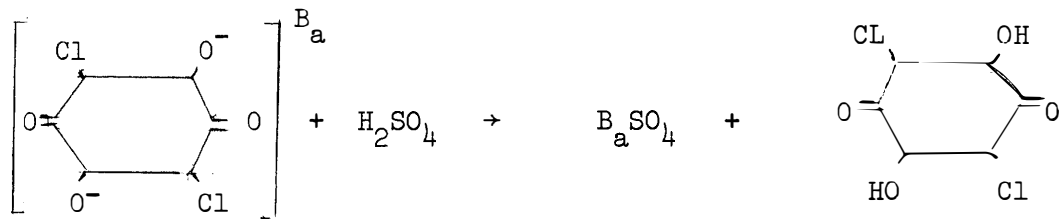
The lungs and livers were taken from each rat at the time of sacrifice and the lungs and livers were frozen. These lungs and livers were freeze-dried, weighed, and combusted in a Parr Bomb. The residue was diluted to 25 ml. with distilled water.

Analysis for total sulfate

In the first experiment the method selected for determination of total sulfate was that of Hakkinen and Hakkinen (67). Thirty mgs. of barium chloranilate were added to 2 ml. portions of the combusted samples in a centrifuge tube. To this were added 1 ml. of 1 M acetate buffer (pH 4) and 3 ml. ethyl alcohol. The centifuge tube was agitated for 15 minutes and centrifuged for 10 minutes at 2000 rpm in the International Centrifuge Model SBV.

The liquid was decanted into Klett tubes and the tubes were wiped clean. The density of the purple color released by the chloranilic acid was measured in the Klett-Summerson Photometer using a No. 52 (green)

filter. The barium combined with the $SO_4^{=}$ ion and this precipitate was removed by centrifuging. The following equation illustrates the reaction which occurred.



The scale was set to zero with a reagent blank. A standard solution containing 480 µg. $SO_4^{=}$ per ml. was also treated like the combusted samples. The sulfate contained in the 25 ml. volumetric flask as represented by total sulfur in the tissues was determined by the following equation.

$$\frac{RU}{R} \times \frac{480}{1} \times \frac{25}{2} = \text{total µg. S as sulfate/lung or liver.}$$

$$\frac{\text{Reading of Unknown}}{\text{Reading of Standard}} \times \text{Concentration of the standard in µg.} \times \text{Dilution} = \text{Total µg. S as sulfate/lung or liver}$$

A graph was prepared from a series of standards which showed the direct relationship of concentration to optical density.

In the second experiment the Perkin Elmer Model 303 Atomic Absorption Spectrophotometer was used to determine the sulfate using a modification of the method of Roe *et al.* (68). In this experiment nitrous oxide gas was used in place of the air used by Roe *et al.* To a 5 ml. aliquot of the sample in a centrifuge tube, 1 ml. of 5 percent

barium chloride and 2 ml. of 15 percent lanthanum chloride was added and thoroughly mixed.

The mixture was centrifuged at 2600 rpm for 10 minutes in the International Centrifuge Model SBV. The supernatant was discarded, 10 ml. of distilled water were added to the centrifuge tube and the precipitate was dispersed in the water. This washing of the precipitate was to remove excess Ba^{++} which had not complexed to $SO_4^{=}$. The sample was centrifuged again at 2000 rpm for 10 minutes. The precipitate was dissolved in 10 ml. of disodium ethylenediaminetetraacetate (EDTA) solution. One ml. of the barium sulfate, lanthanum chloride and disodium EDTA mixture was added to 4 ml. of distilled water. This solution was aspirated into the flame of the atomic absorption spectrophotometer.

Standards were prepared in a like manner to the sample. A stock sulfate solution was made by dissolving 1.479 gms. sodium sulfate in 500 ml. of distilled water. Triplicate standards containing 0.0, 0.1, 0.25, 0.4 and 0.5 ml. of stock sulfate solution were pipetted into centrifuge tubes and distilled water to bring the volume to 5 ml. was added. One ml. of 5 percent lanthanum chloride and 2 ml. of 15 percent barium chloride solution was added to each tube. The standards were centrifuged at 2600 rpm in the International Centrifuge Model SBV for 10 minutes. The supernatant was discarded and the barium sulfate was washed with 10 ml. of distilled water. The precipitate was dissolved in 10 ml. of disodium EDTA solution. This provides solutions of 0, 20, 50, 80 and 100 ppm sulfate for aspiration into the flame.

Measurements of absorption were converted to absorbance. A graph was prepared showing the direct relationship of the absorbance values to $SO_4^{=}$ in solution. The sulfate contained in the 25 ml. volumetric flask as represented by the total sulfur in the tissues was calculated as before by the following equation:

$$\frac{\text{Reading of the Unknown}}{\text{Reading of the Standard}} \times \text{Concentration of X the standard in µg.} \times \text{Dilution} = \text{Total µg. S as sulfate/lung or liver}$$

Statistics

The student's t test as described by Steel and Torrie (69) was used to determine the statistical significance of the results. The t value was computed using a method for unpaired observations and unequal numbers.

CHAPTER IV

RESULTS AND DISCUSSION

Although there is no statistically significant difference in either weight gain or feed consumption in Table V in either of the two experiments when figured separately, these data show that the rats did not reject the diets. In fact they consumed more feed than would be eaten by rats of this weight (64) fed a normal diet. The data demonstrated by Figure 1, page 16, show that in the alpha protein, methionine was the first limiting amino acid. Since no methionine was added, the diets would be expected to be poorly utilized by the rats, an expectation which was confirmed by the feed efficiencies obtained, Table V.

Since the data which are presented in Table VI show a difference both in the amount of total sulfur in the lung as sulfate and the sulfur as sulfate per mg. of dried lung it may be surprising that no difference was obtained in the feed efficiencies. However, the data which are presented in Table VII show little difference in the total sulfur as sulfate or the sulfur as sulfate per mg. in the liver. The sulfur in the liver and lung is associated with the tissue proteins and the mucopolysaccharides. Since there is a high percentage of cartilaginous material in the lungs, it is reasonable to assume that there is more mucopolysaccharide sulfur in the lungs than in the liver and that the effect of increased mucopolysaccharide sulfation could be masked in the liver when total sulfate was measured. The significance of the data is

TABLE V

FEED EFFICIENCY

Dietary Sulfate	Diet Number	Number of Rats[a]	Terminal Age	Average Weight Gain	Average Feed Consumption	Average Feed Efficiency[c]
%			days after weaning	g	g	
0.0002	1	5	46	44 ± 6[b]	324 ± 26	.13 ± .020
0.001	2	4	46	42 ± 5	324 ± 11	.13 ± .023
0.005	3	5	46	43 ± 6	334 ± 13	.13 ± .021
0.025	4	5	46	44 ± 6	332 ± 20	.13 ± .019
0.025	5	10	61 ± 5	55 ± 6	400 ± 32	.13 ± .019
0.125	6	9	67 ± 3	60 ± 4	400 ± 36	.15 ± .010
0.625	7	9	61 ± 4	64 ± 4	399 ± 20	.16 ± .009
3.130	8	9	75 ± 2	60 ± 2	497 ± 18	.13 ± .004

[a] A rat from each of diet numbers 2, 6, 7 and 8 died.

[b] Standard error of the mean.

[c] $\frac{\text{Weight gained}}{\text{Feed consumed}}$ = feed efficiency.

TABLE VI

SULFATE IN LUNG

Dietary Sulfate	Diet Number	Average Weight of Dry Lung	Average Total S as Sulfate	Average µg. S as Sulfate/mg.
%		mg.	µg.	
0.0002	1	170.4 ± 4.5[a]	9452 ± 40	50.79 ± 2.34
0.001	2	175.4 ± 5.1	8942 ± 430	48.63 ± 3.52
0.005	3	160.0 ± 8.0	7362 ± 440	47.48 ± 4.43
0.025	4	189.2 ± 9.5	9032 ± 598	48.53 ± 4.75
0.025	5	194.1 ± 11.3	10060 ± 1130	50.33 ± 6.12
0.125	6	186.4 ± 23.1	9550 ± 700	52.80 ± 6.23
0.625	7	230.6 ± 26.9	14340 ± 2570	55.88 ± 4.16
3.130	8	188.3 ± 28.3	16090 ± 2680	86.46 ± 19.34

[a]Standard error of the mean.

TABLE VII

SULFATE IN LIVER

Dietary Sulfate	Diet Number	Average Weight of Dry Liver	Average Total S As Sulfate	Average µg S As Sulfate/mg.
%		mg.	µg.	
0.0002	1	824.3 ± 5.2	76662 ± 610	82.75 ± 2.10[a]
0.001	2	594.0 ± 8.3	53082 ± 213	74.94 ± 4.33
0.005	3	665.3 ± 7.4	58832 ± 218	72.49 ± 5.14
0.025	4	987.8 ± 10.8	63822 ± 211	58.76 ± 8.43
0.025	5	1060.9 ± 67.0	66110 ± 821	62.36 ± 9.32
0.125	6	1163.3 ± 60.3	67500 ± 871	67.14 ± 7.32
0.625	7	1283.0 ± 85.5	64400 ± 979	60.23 ± 9.21
3.130	8	1028.6 ± 35.9	58410 ± 820	66.81 ± 6.42

[a]Standard error of the mean.

presented in Table VIII. There is a significant difference both in the amount of total sulfur in the lung as sulfate and the sulfur as sulfate per mg. of dried lung. No significant difference was found in the liver in total sulfur as sulfate or in the sulfur as sulfate per mg. liver tissue.

Inspection of Table VII will show that although the average total sulfate in each sulfate group is not greatly different, the average µg. sulfur as sulfate per mg. of liver is highest in the three lowest dietary sulfate groups. This is accounted for by the fact that these were the rats in the first experiment and they were sacrificed at an earlier age and with smaller livers than the rats in the second experiment. The higher average µg. sulfur as sulfate per µg. liver is thought to be a function of liver weight and age in this instance and unrelated to sulfate intake.

Previous data have shown that the collagen synthesized by a rat fed a diet low in sulfate was altered as reflected by a decrease in the breaking strength of aortas and by changes in the ratio of soluble to insoluble collagen (70). It was suggested that this alteration in collagen metabolism might be a reflection of decreased mucopolysaccharide synthesis. This suggestion was supported by the observation that the percentage of hexosamine in cellular lipoprotein prepared from the livers of rats fed diets low in sulfate was decreased.

Although the data presented in the present investigation do not indicate a gross sparing effect of sulfate on methionine since there was no difference in feed efficiencies or total sulfur in an organ which was

TABLE VIII

SIGNIFICANCE OF DATA ON LUNG SULFATE

Dietary Sulfate Groups Compared		Average Total Lung S As Sulfate	Average µg S As Sulfate/mg.
	%		
5 to 6	0.025 to 0.125	$P < 0.5$	$0.2 > P > 0.1$
5 to 7	0.025 to 0.625	$0.01 > P > 0.001$	$0.5 > P > 0.4$
5 to 8	0.025 to 3.130	$P > 0.001$	$P > 0.001$
6 to 7	0.125 to 0.625	$P > 0.001$	$0.2 > P > 0.1$
6 to 8	0.125 to 3.130	$P > 0.001$	$P > 0.001$
7 to 8	0.625 to 3.130	$P < 0.5$	$0.05 > P > 0.02$

predominantly protein, they do show an increase in sulfur retention by lung tissue in those rats fed the two highest sulfate diets as shown in Table VI, page 27. Since the lung contains more cartilage and more mucopolysaccharides than the liver (71), it is reasonable to assume that these data represent either increased sulfation and/or increased synthesis of the mucopolysaccharides by rats fed diets with increasing levels of inorganic sulfate. In view of the hexosamine data mentioned earlier the latter possibility, increased synthesis, seems most likely.

As stated previously although a requirement for inorganic sulfate in the diet if the apparent requirement for sulfur containing amino acids is not to be raised has been demonstrated (3) no information was obtained to indicate the optimum level of sulfate in the diet. Michels (3) fed diets which contained up to 0.42 percent sulfate. Based on the uptake of radioactive $^{35}SO_4^{=}$ she obtained the greatest percentage utilization of the sulfate for sulfation of mucopolysaccharides when she fed the diets containing the higher level (0.42 percent) of sulfate.

If the sulfate content of the lungs obtained in this investigation is used to predict the optimal amount of dietary sulfate, one conclusion is obvious. It is that until more than approximately 0.1 percent of sulfate is added to the diets little improvement in sulfate utilization is obtained when the data are expressed as total sulfate per lung. There is a large increase in sulfate utilization between 0.1 and 0.6 percent of dietary sulfate. Little further improvement is obtained by raising the sulfate content to 3.130 percent. On the other hand, if the data are expressed as µg. S as sulfate per mg. of tissue, an increase in

sulfate utilization is obtained for the levels of 0.625 percent and 3.130 percent. This apparent divergence in the data is a reflection of the increased lung weights obtained in those rats fed the diets containing 0.625 percent of sulfate. It is tempting to postulate that these increases in total µg. sulfur as sulfate per mg. dry lung weight reflect the ultimate in a rat's well being, however, such a proposal would be difficult to document at this time, since these rats also had the heaviest dry liver weight. Therefore, although these data are difficult to interpret with respect to both the upper and lower limits for dietary sulfate, they do show that before sulfate additions will give a measurable effect on the retention of sulfate by the tissues more than approximately 0.1 percent sulfate should be added to the diet.

CHAPTER V

SUMMARY

The change in tissue sulfate which resulted from diets which were low in sulfur amino acids but with increasing amounts of sulfate was investigated in rats. The lungs and livers were freeze-dried, weighed and combusted in a Parr bomb. The sulfate was determined by using a barium chloranilate precipitation and the Klett-Summerson Photometer and by using the Perkin and Elmer Model 303 Atomic Absorption Spectrophotometer.

The data obtained showed a significant difference in both the amount of total sulfur as sulfate in the lung and the sulfur as sulfate per mg. of dried lung. However, no significant difference was found in the sulfur as sulfate in liver or sulfur as sulfate per mg. of dried liver. Since there is a high percentage of cartilaginous material in the lungs, it is reasonable to assume that there is more mucopolysaccharide sulfur in the lung than in the liver and that the effect of increased mucopolysaccharide sulfation could be masked when total liver sulfate was measured, but not in the lung when total lung sulfate was measured. Based on previous data obtained from this laboratory, the increase in lung sulfate was interpreted as a reflection of increased synthesis of mucopolysaccharides.

The sulfate content of the lungs obtained in this investigation shows quite conclusively that before dietary sulfate additions will give

a measurable effect on retention of sulfate by the tissues, greater than approximately 0.1 percent sulfate should be added to the diet.

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VITA

The author was born in Iowa in 1939 and was educated there. She went to Ames Public Schools and was graduated with a Bachelor of Science degree in Home Economics for a General Education from Iowa State University in Ames in 1961. She received an Alumni Achievement Award Scholarship to attend Iowa State University. She is married and has two children.