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A Comparison of the Utilization of Organic and Inorganic Sulfur by the Rat

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To the Graduate Council:

I am submitting herewith a thesis written by Mary Frances Gilmore entitled "A Comparison of the Utilization of Organic and Inorganic Sulfur by the Rat." 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, Jeannette Biggs

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

Mary Frances Gilmore May 14, 1963

A Comparison of the Utilization of Organic and Inorganic Sulfur by the Rat

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Nutrition May, 1963

Previous work in this laboratory has shown the response of rats to avitaminosis E to be related to the ratio of inorganic to neutral sulfur. Since literature reports state that sulfate from inorganic sources is poorly utilized by animals, an investigation of the utilization of sulfur from inorganic and organic dietary sources was made. Cartilage mucopolysaccharides were the natural product that was isolated and analyzed.

Six adult, litter-mate, female, albino rats from the Wistar strain were maintained for a period of ten days on diets containing S-35-sulfur as S-35-methionine, $CaS^{35}O_4$, and $Na_2S^{35}O_4$ following a seven day preliminary period on respective non-radioactive diets. The sulfur content of all diets was the same, while the ratio of organic to inorganic sulfur was varied by adjustments made in the salt mixtures. The 14 per cent, 3.34 per cent, and .007 per cent sulfate salt mixtures used gave 0.42 per cent, 0.10 per cent and 0.00 per cent dietary inorganic sulfate respectively.

Collections of urine, feces, blood, and cartilage from each animal were, in each case, prepared in a way so as to make it possible to collect data representing S^{35}-sulfate incorporation as a percentage of ingested S^{35} activity. In the latter case, mucopolysaccharides were isolated and analyzed for S^{35} activity. The data were treated statistically by comparison of sample means of paired observations.

The data presented indicate that the sulfate ion from inorganic sources contributes as much to the metabolic pool as when supplied from organic sources. Also, that the availability of the $SO_4^=$ ion is related to the ratio of organic to inorganic sulfur in the diet is shown by the data showing methionine better absorbed from the diet with no inorganic $SO_4^=$ and the data showing significantly greater $S^{35}O_4^=$ incorporation into cartilage mucopolysaccharides when the diet contained an inorganic sulfur source.

The literature indicates that the amount of methionine at the high level used in this investigation is in excess of that required by the rat and, therefore, should give rise to high activity in the excreta. Since this was not found in this study, further investigation to determine the optimum amount of the $SO_4^=$ ion required by the rat would be interesting and possibly would give greater significance to the relationship of inorganic and organic sulfur supplied in the diet.

May 15, 1963

To the Graduate Council:

I am submitting herewith a thesis written by Mary Frances Gilmore entitled "A Comparison of the Utilization of Organic and Inorganic Sulfur by the Rat." 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

Jeannette Biggs

Accepted for the Council:

Dean of the Graduate School

A COMPARISON OF THE UTILIZATION OF ORGANIC AND INORGANIC SULFUR BY THE RAT

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Mary Frances Gilmore

June 1963

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INTRODUCTION

Previous work in this laboratory (Pendergrass, '61; Smith and Pendergrass, '62) has shown that the response of rats to avitaminosis E was related not only to the total sulfur in the diet but also to the ratio of inorganic to neutral sulfur. When rats were forced to satisfy their sulfate requirements by oxidation of organic sulfur, a decreased rate of sulfation and decreased sulfur content in the cellular lipoproteins was observed. Since the sulfur of the sulfur-containing amino acids is rapidly oxidized to sulfate, it was assumed that vitamin E was necessary for the optimal conversion of amino acid sulfur to sulfate in these animals. In confirmation, preliminary work in this laboratory has shown that fortified liver homogenates from vitamin E deficient rats were less effective in converting the sulfur of cysteine-S^{35} to sulfate-S^{35} than those from their E sufficient litter mates.

However, certain literature reports (Kun, '61) have indicated that sulfate from inorganic sources was poorly utilized by animals. Our findings seem contradictory to literature reports since the response of the rat should be independent of the level of dietary sulfate if inorganic sulfate is not utilized by animals, and our observations would be difficult to explain.

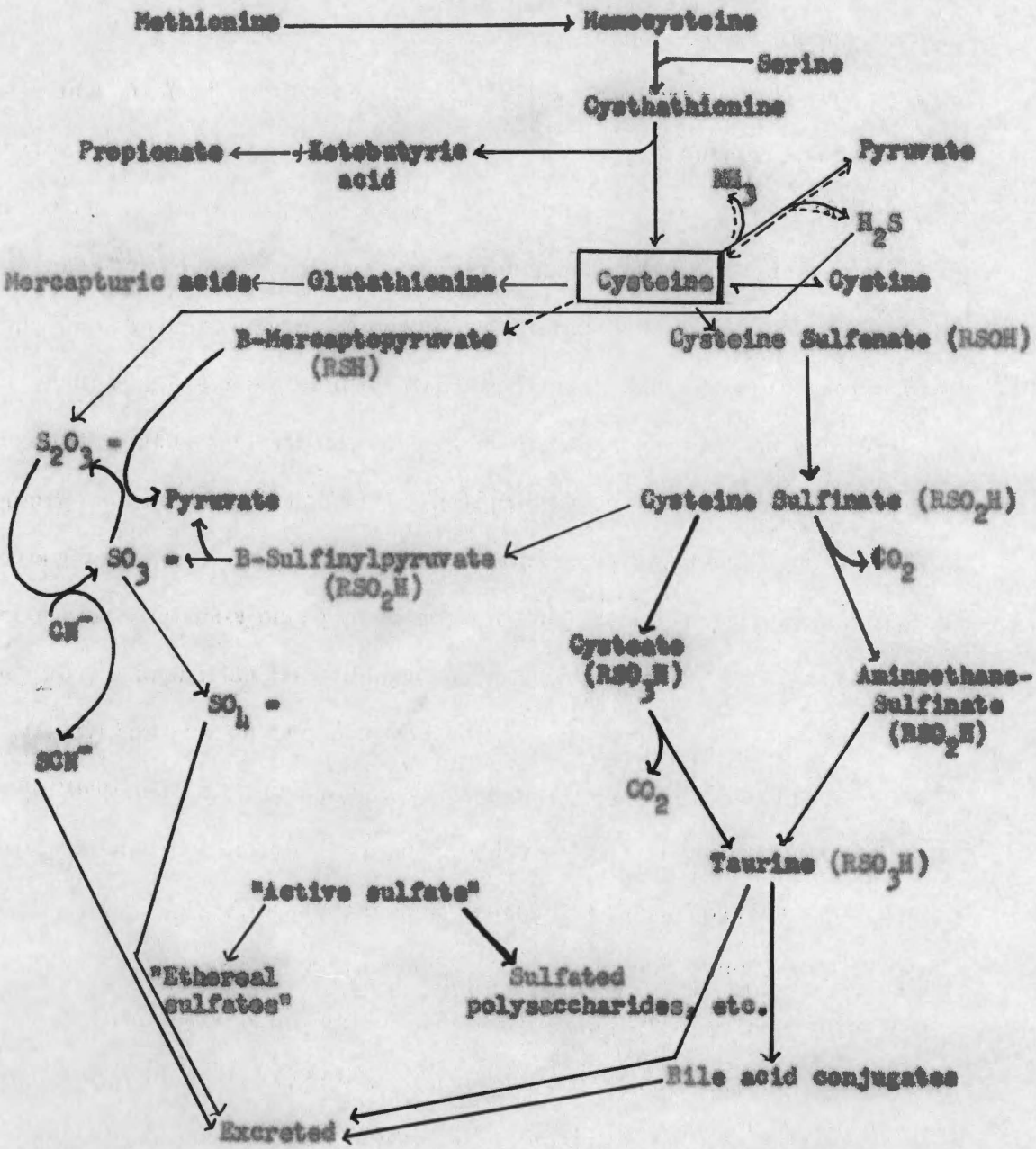
The present investigation, therefore, was designed to compare the extent to which inorganic sulfur and neutral sulfur sources could be used for sulfation of natural products. Cartilage mucopolysaccharides, which are more easily isolated than other naturally occurring sulfate compounds, were chosen as the natural product to be isolated and analyzed.

REVIEW OF LITERATURE

All living organisms require sulfur as an elemental constituent of amino acids, peptides, proteins, coenzymes, bile acids, mercapturic acids, mucopolysaccharides, hormones, and vitamins.

Dietary sulfur is composed of organic and inorganic sulfur. The organic sulfur fraction is made up primarily of the sulfur-containing amino acids L-cystine, L-cysteine, and L-methionine, together with biotin and thiamine (Young and Ness, '58), chondroitin sulfuric acid, ergothioneine, and lipoic acid (Kleiner and Orten, '62).

A discussion of the metabolism of the sulfur-containing amino acids represents the metabolism of the organic sulfur fraction of the diet. A certain amount of the amino acids cysteine and methionine is used for the synthesis of protein, peptides, and regulatory substances. The dietary sulfur amino acids in excess of these required for these functions are further metabolized. Methionine is first converted to cysteine in the body by demethylation to homocysteine; the combination of homocysteine and L-serine forms cystathionine. The cystathionine undergoes a hydrolytic cleavage to give homoserine and cysteine. The cysteine then undergoes a series of oxidative steps leading eventually to either taurine or inorganic sulfate. The taurine may be used for conjugation with cholic acid to form taurocholic acid or excreted in the urine as taurine (Figure 1). The sulfate fraction forms active sulfate and represents the inorganic and ethereal sulfur of the urine (Frusen and Simmonds, '60).



Source: Cantarow and Scheparts, '62.

FIGURE 1

CYSTEINE METABOLISM

The vitamins biotin and thiamine are important as coenzymes. A biotin enzyme appears to be necessary for carbon dioxide fixation (Lardy and Peanasky, '53) and extra mitochondrial synthesis of fatty acids. Thiamine pyrophosphate is the coenzyme necessary for decarboxylation of alpha-keto acids, such as pyruvic acid (Read, '53). Oxidative decarboxylation requires not only thiamine pyrophosphate but another sulfur compound, 6-8 thioctic acid (lipoic acid). Ergothioneine, a naturally occurring histidine derivative formed by microorganisms, is present in mammalian blood and tissues and is of dietary origin (Melville et al., '57).

The inorganic sulfate is present in the diet in small amounts and is, according to Kun ('61) and Galambos and Cornell ('62), poorly absorbed from the intestinal tract. Although inorganic sulfate is required for the synthesis of sulfomucopolysaccharides (Dziewiatkowski, (51a) and other sulfuric acid esters, the body apparently has no mechanism for conserving it; since, whether formed by oxidative metabolism of the sulfur-containing amino acids or absorption from the gastrointestinal tract, it is readily excreted by the kidneys.

Three sulfur fractions have been identified in urine: inorganic sulfur, ethereal (or ester) sulfate, and neutral sulfur, each contributing 79 to 85 per cent, 4 to 7 per cent, and 16 to 21 per cent respectively (Kleiner and Orten, '62; Lewis, '24: Laidlaw and Young, '48).

The inorganic sulfur fraction of normal urine is composed of the following sulfur compounds or ions: thiosulfate, thiocyanate, sulfide, sulfite, and sulfate (Young and Mau, '58); Lewis, '24). The small

thiosulfate content of urine is a consequence of enzymic action between B-mercaptopyruvate and inorganic sulfite, metabolism products of L-cysteine. Recent work (Dimet and Wellers, '62) has revealed extremely high levels of urinary sulfate following dietaries of sodium thiosulfate--thiosulfate being a normal intermediary in the metabolism of cystine to SO_4--(Weber et al., '61). In experiments with rats Dziewiatkowski ('49) found a high level of inorganic sulfate in the urine after dosing with cystine or methionine.

The neutral urinary sulfur includes compounds having the -SH, -S-, and -SCN groups. For example, the sulfur-containing amino acids and peptides, thiols, taurine, ergothioneine, urochrome and the thiazole part of thiamine are included in this group (Cavalline et al., '60).

The ethereal sulfates are formed in the liver by conjugation of aromatic hydroxy compounds with sulfate. Aromatic hydroxy compounds are produced in large part by the bacterial degradation of aromatic amino acids in the intestinal tract (Wood et al., '47; Gender and Happold, '54). The conjugation involves the initial "activation" of inorganic sulfate in an enzymic reaction with ATP; and the resulting "active sulfate," PAPS (Robbins and Lipmann, '57), reacts with a phenol in the presence of a second enzyme (Dekhis et al., '55; DeKhis and Wisenkaniuk, '56).

Filogin ('61) found that inadequate protein in the diet of rats made no change in the percentage distribution of S^{35} between inorganic sulfur, ester sulfate, and neutral sulfur. Wellers et al. ('60) found that sulfate sulfur could supply about one-third of the total sulfur

requirement and all of the sulfur required beyond that of amino acids. Generally, most investigators have failed to find inorganic sulfate incorporated into amino acids used for protein synthesis (Fromageot and Chaperville, '55; Hunderickx, '61; Waldschmidt, '62). However, Waldschmidt ('62) after feeding trials with sulfate and sulfide found significant incorporation of radioactive S^{35}-sulfide into tissue proteins. It has been assumed, therefore, that the body's inability to reduce sulfate to sulfite is responsible for the lack of utilization of sulfate for protein synthesis. The trace amounts of radioactivity observed in tissue proteins by Chredovic and Hansen ('60) were presumably due to reduction of sulfate to sulfite by bacteria of the intestinal flora.

It is well known that some autotropic bacteria can utilize inorganic sulfur as a primary source of energy. Plants also obtain their sulfur from inorganic sulfate present in the soil, and it has been observed that the quality of plants as a food was related to the sulfur content of the soil on which they were grown (Cairns and Carson, '61). Ruminant animals, presumably by virtue of the rumen bacteria, are able to use inorganic sulfur for the synthesis of tissues, especially hair. For example, Gahite'kii and Makar ('61) and Soljanskij ('58) have demonstrated an improvement in the growth and quality of wool by including sodium sulfate in the diets of sheep and lambs.

Connective tissue in dermis, tendons, ligaments, cartilage, and bone matrix acts as a supportive by means of the fibrils of insoluble proteins, collagen or elastin, embedded in the ground substances.

The ground substance of connective tissue is composed largely of mucopolysaccharides and mucoproteins. The mucoproteins are easily hydrolyzed to mucopolysaccharides and protein (Shatton and Schubert, '54). The mucopolysaccharides present in connective tissue are polymers of hyaluronic acid and the chondroitins A, B, and C (Kleiner and Ortom, '62), with cartilage primarily containing chondroitin A and some of the C form (Mest and Todd, '59; Meyer et al, '56).

The biosynthesis of mucopolysaccharides occurs by polymerization of uridine diphosphohexuronates and uridine diphospho-N-acetylhexosamine, to form a high molecular weight polymer. The high molecular weight polymer is esterified with sulfate from active sulfate (Gregory and Robbins, '60; Balasubramanian and Bachhawat, '62). Active sulfate, formed from inorganic sulfate, ATP, and Mg^{++} by interaction of ATP-sulfurylase and APS-kinase is 3'-phosphoadenosine-5'-phosphosulfate (Lipmann, '58).

Böstren and Gardell ('53) demonstrated that mucopolysaccharides could be easily labelled by a subcutaneous injection of S^{35}-sulfate. They determined the time required for maximum labeling of mucopolysaccharides from skin following injection of a test dose of radioactive sulfur as S^{35}-sodium sulfate and found maximal incorporation into the mucopolysaccharides 24 hours following injection. These observations were confirmed for the mucopolysaccharides of rib cartilage by Dziewiatkowski ('51b), Böstren and Aqvist ('52), and Valdiguie et al. ('61). Recently Picard et al. ('62) investigating incorporation of sulfur into the aorta found maximal incorporation from 6 to 8 hours. However, the incorporating substance was found to be identical with uridine

diphosphate acetylgalactosamine sulfate and even in this system, maximum incorporation into mucopolysaccharides was observed after 24 hours.

METHODS

I. PREPARATION OF DIETS

This study was designed to compare the effects of organic and inorganic sources of dietary sulfur on the availability of the sulfate ion for utilization by the rat. Six diets (Table I), modifications of those used by Pendergrass ('61) as patterned after that of Caputto et al. ('58), equal in sulfur content and differing in ratio of organic to inorganic sulfur were selected. These modifications were obtained by varying the Hubbell, Mendel, and Wakeman ('37) salt mixtures; and the resulting 14 per cent, 3.34 per cent, and 0.007 per cent salt mixtures gave 0.42 per cent, 0.10 per cent, and 0.00 per cent dietary inorganic sulfate respectively (Table II). In previous work in this laboratory calcium sulfate, as opposed to the commonly used sodium sulfate, has been employed for altering the level of sulfate in the diet. In order to compare the utilization of the sulfate ion from these two inorganic sources with that from an organic dietary sulfur source, as well as comparing utilization from the two inorganic sources, six diets were used. The composition of the salt mixtures is given in detail in Table III.

The specific activity of the S^{35} was calculated each time the diet was prepared, and correction was made for radioactive decay according to the formula:

$$\text{Log } \frac{N_0}{N} = 3.47 \times 10^{-3} \text{ t}$$

TABLE I

COMPOSITION OF THE DIETS

Component		Quantity per 100 grams					
		A	B	C	D	E	F
		gms	gms	gms	gms	gms	gms
Casein		15	15	15	15	15	15
DL Methionine		0	.35	.35	.60	0	.35
Sucrose		31	31	31	31	31	31
Cornstarch		32	32	32	32	32	32
Alphacel		10	10	10	10	10	10
Lard		6	6	6	6	6	6
Cod liver oil		2	2	2	2	2	2
Vitamin mixture[a]		1	1	1	1	1	1
Vitamin K		.02	.02	.02	.02	.02	.02
Salt mixture[b]	No. 1	3	-	-	-	-	-
	2	-	3	3	-	-	-
	3	-	-	-	3	-	-
	4	-	-	-	-	3	-
	5	-	-	-	-	-	3

[a]Synthetic vitamins added as supplement to each 100 grams of diet: (in milligrams) vitamin K, 0.025; thiamine-HCl, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium pantothenate, 1.0; nicotinic acid, 20.0; vitamin B_{12}, 0.005; choline, 100.0; pteroylglutamic acid, 0.50; biotin, 0.005; inositol, 100.0; PABA, 7.5. The vitamins were triturated with sufficient sucrose to make 1 gram.

[b]See Table III.

TABLE II

CALCULATED LEVELS OF DIETARY SULFATE

Diets	Quantity of inorganic SO_4 per unit	Quantity of organic SO_4 per unit	Total
A	0.42 ($CaS^{35}O_4$)[a]	0.25	0.67
B	0.10	0.57 (S^{35}-methionine)[b]	0.67
C	0.10 ($CaS^{35}O_4$)	0.57	0.67
D	0.00	0.67 (S^{35}-methionine)	0.67
E	0.42 ($Na_2S^{35}O_4$)	0.25	0.67
F	0.10 ($Na_2S^{35}O_4$)	0.57	0.67

[a]Carrier free $MgS^{35}O_4$ was obtained from Oak Ridge National Laboratories, Oak Ridge, Tennessee.

[b]Obtained from Schwarz BioResearch Inc., Mount Vernon, New York.

TABLE III

SALT MIXTURES

Component	#1 14.00% SO_4 gm	#2 3.34% SO_4 gm	#3 0.007% SO_4 gm	#4[a] 14.00% SO_4 gm	#5 3.34% SO_4 gm
$CaCO_3$	30.346	41.250	44.750	34.750	44.750
$CaSO_4 \cdot 2H_2O$	25.097	6.000	0.000	0.000	0.000
Na_2SO_4	0.000	0.000	0.000	20.720	4.943
Cornstarch	0.000	8.193	10.693	0.000	5.750
$MgCO_3$	3.060	3.060	3.060	3.060	3.060
NaCl	6.900	6.900	6.900	6.900	6.900
KCl	11.200	11.200	11.200	11.200	11.200
KH_2PO_4	21.200	21.200	21.200	21.200	21.200
$FePO_4(2H_2O)$	2.050	2.050	2.050	2.050	2.050
KI	0.008	0.008	0.008	0.008	0.008
NaF	0.010	0.010	0.010	0.010	0.010
$AlK(SO_4)$	0.017	0.017	0.017	0.017	0.017
$Cu(C_2H_3O_2)_2H_2O$	0.072	0.072	0.072	0.072	0.072
$MnCl_2(4H_2O)$	0.040	0.040	0.040	0.040	0.040

[a]Added to Diet E at a 10 per cent higher level to keep the calcium content of all diets constant.

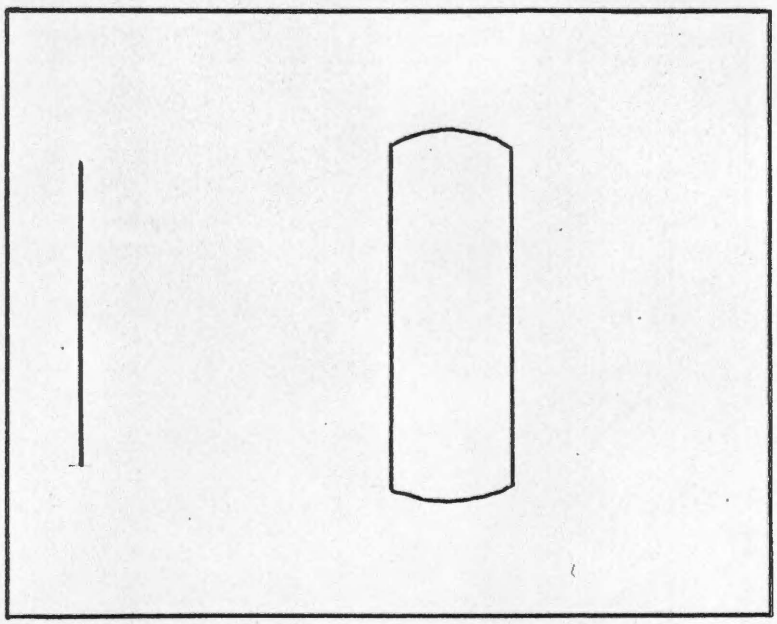
where N_0 is activity at zero time, N is activity after time t, and t represents time in days. The diet was calculated to contain .02 microcuries S^{35} per gram. The radioactivity was incorperated into the salt mixtures of the diets as the S^{35}-sulfate of $CaS^{35}O_4$ or $Na_2S^{35}O_4$ and as S^{35}-L-methionine. The purity of the S^{35}-methionine used was tested by paper chromatography. The chromatogram was cut into strips which were combusted and assayed for S^{35} activity. The Rf values as related to the activity expressed as counts per minute of the S^{35}-methionine are shown in Figure 2.

II. DETERMINATION OF S^{35}-SULFATE ACTIVITY

A 0.5 gram sample of each diet was treated according to the method of Kats and Golden ('59) for the analysis of radioactive sulfur in biological materials as described by Pendergrass ('61). Sample activity was determined by counting in an automatic Nuclear-Chicago gas flow counter. A total of 4,000 counts were taken. Since the activity of the diet as determined by analysis was different for each period, all data collected and presented in this thesis were related to the diet activity and dietary intake for the respective period.

For each rat total fecal samples of the period were collected and ground by mortar and pestle to obtain a homogeneous mixture. Duplicate 0.3 gram samples were digested, collected, and specific activity determined by the previously described method (Kats and Golden, '59).

Urine samples were collected by placing Whatman No. 40 filter paper in the individual cages, removing sections of the paper spotted



Chromatogram

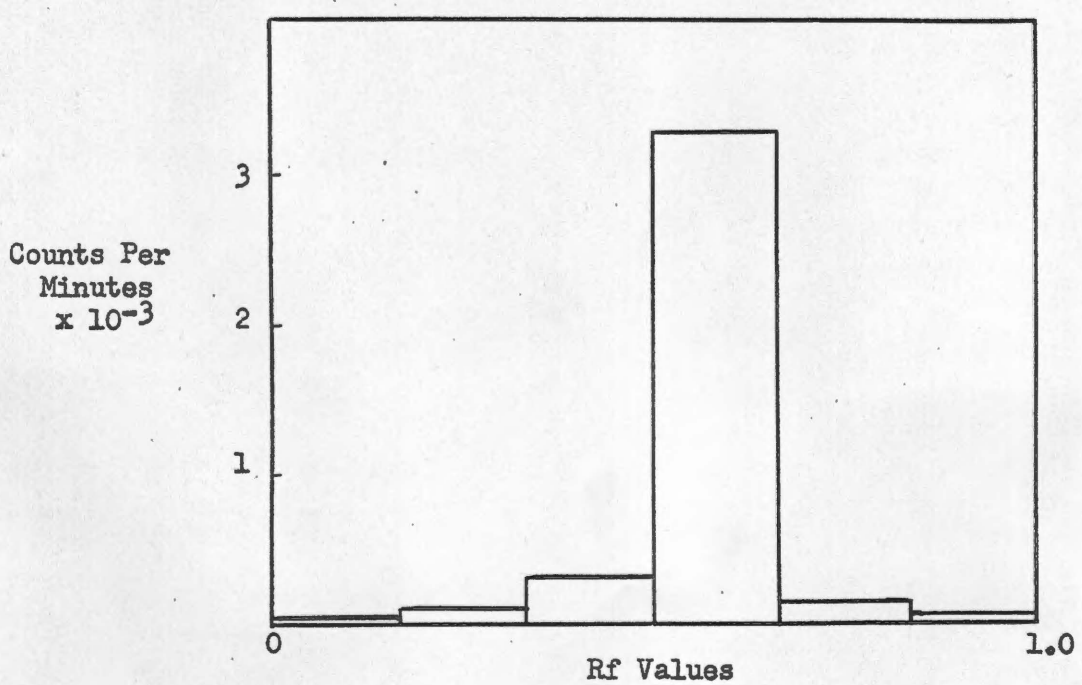


FIGURE 2

PAPER CHROMATOGRAM OF S^{35}-METHIONINE

with urine, and combusting in the manner used for the diet. Because the amount of sulfate in the sample was small, 1 ml of a sulfate standard equivalent to 10 mg $BaSO_4$ was added to each flask before precipitation with $BaCl_2$. The procedure used with the diet and fecal samples was again followed in handling the precipitate and counting for S^{35}-sulfate activity.

Blood from each animal was collected in a 50 ml centrifuge tube containing 10 ml of a 2.9 per cent, isotonic, sodium citrate solution. Duplicate analyses were made using 5 ml portions from each tube. Samples were precombusted with 5 ml of 50 per cent nitric acid to prevent violent reaction between the citrate and the combustion mixture. The samples were digested in 25 ml Erlenmeyer flasks on a hot plate until the mixture was evaporated down to about 2 to 3 ml. Five to 7 ml of a combustion mixture were then added to each flask. The samples again were heated until evaporated to a volume of 1 to 2 ml. The procedure of Kats and Golden ('59) was then used for collecting and assaying the sulfate content.

III. PREPARATION OF CHONDROITIN SULFATE

Boetrom's method ('52) for the preparation of chondroitin sulfate from the costal cartilage was followed with slight modification. The excised rib cartilage was boiled for 5 minutes, freed of muscle and connective tissue, dried with acetone, ground with a mortar and pestle to a fine powder, and then weighed. Distilled water, 12 ml water per gram powder, was added to the flask, and the contents were

boiled on a hot plate for 15 minutes. The flask was cooled to 5° C and 10 per cent NaOH added to a 2 per cent final concentration. After shaking for 16 hours at 5 degrees C, 10 per cent acetic acid was used to bring the solution to a pH of 6.0, and the contents of the flask were transferred to a centrifuge tube and spun at 2,000 rpm for 20 minutes in an International No. 3 centrifuge. The supernatant was saved and the precipitate was washed with 10 ml distilled water and recentrifuged. The combined supernatants were centrifuged in a Loudes LRA-1 refrigerated centrifuge at 12,000 rpm for 30 minutes. This supernatant was collected, measured, and precipitated with three volumes 95 per cent ethanol. The precipitate, collected by centrifugation, was dissolved in distilled water and reprecipitated with eight volumes acetic acid. After two hours this sediment was collected by centrifugation, washed with glacial acetic acid and then with absolute ethanol until free of acetic acid. Chondroitin sulfuric acid was then precipitated with three volumes of ethanol. The ester sulfate of the mucopolysaccharide was released by hydrolysing with 6 N HCl for three hours (Dodgson and Rice, '62). One ml of a sulfate standard equivalent to 10 mg $BaSO_4$ was added to each tube before precipitating the freed sulfate with $BaCl_2$. Collection of the precipitate then proceeded by the method of Kats and Golden ('50) as described by Pendergrass ('61), and the samples were counted in a Nuclear-Chicago gas flow counter.

The activity of the feces, urine, blood, and cartilage was related to the sulfate content of the sample and the specific activity

was expressed as counts per minute per millimole of sulfate. The net activity of the diet was expressed as counts per minute.

IV. PROCEDURE

Thirty adult, female, albino rats of the Wistar strain were used during the five replications of this experiment. Each animal was housed in an individual wire-bottomed cage. Distilled water and food were provided ad libitum.

In a preliminary period, one litter mate was sacrificed on the second, fourth, sixth, eighth, tenth, and twelfth day of receiving a radioactive diet. The greatest S^{35} activity in the costal cartilage appeared after the eighth day. Therefore, animals were kept on the radioactive diets ten days in subsequent periods.

Six litter-mated animals were placed on the six test diets for an experimental period of 17 days--the first seven days the diet was non-radioactive. During the last 10 days of the period, when the animals received the radioactive diets, food intake measurements and fecal collections were made daily. Urine was collected on the sixteenth day. At the end of each dietary period the animals were sacrificed by decapitation while under light ether anesthesia. Rib cartilage and blood samples were saved from each animal. The livers and brains were collected and frozen for future analyses.

RESULTS AND DISCUSSION

The data which are presented in Table IV show the specific activity of the fecal sulfate as a percentage of the total ingested radioactivity. These data show that on a normal diet there is a significantly (Table VIII) higher percentage absorption of radioactivity ingested as S^{35}-methionine (diet B) when compared to that ingested as inorganic salts ($CaS^{35}O_4 \cdot 2H_2O$, diet C, and $Na_2S^{35}O_4$, diet F). It was surprising to observe also that the absorption of the radioactivity supplied as inorganic sulfate was significantly higher (Table VIII) from those diets not supplemented with methionine (diets A and E) than from those supplemented with methionine (diets C and F). Conversely, it was observed that the radioactivity from diet D, a diet containing no added inorganic sulfate but supplemented with 0.60 grams of methionine-S^{35} was, of all diets, most readily absorbed. The data for diets B and D suggest that the level of inorganic sulfur in the diet may influence the absorption of organic sulfur. A comparison of data for diets A and E with those for diets C and F suggests that the absorption of inorganic sulfur may be influenced by the level of organic sulfur in the diet. The author is unaware of any reports in the literature which relate the absorption of organic and inorganic sulfur; however, if the interpretation of the data presented is correct, the implication for future investigation is exciting.

One other problem in our laboratory has been to determine if $CaSO_4 \cdot 2H_2O$ as a sulfate source was superior to Na_2SO_4. The data shown

TABLE IV

S^{35} RADIOACTIVITY OF THE FECES FROM EACH ANIMAL IN CPM/MILLIMOLE SO_4--AS A PERCENTAGE OF INGESTED ACTIVITY

Period	Diet Groups					
	A	B	C	D	E	F
I	6.38	6.82	6.48	5.49	4.74	9.94
II	2.78	3.77	4.60	2.63	4.95	5.27
III	6.15	3.50	6.88	4.82	7.37	7.10
IV	6.72	4.77	10.16	4.39	14.74	13.05
V	8.72	13.50	13.70	8.57	10.14	15.98
Average	6.15	6.35	8.24	5.06	8.38	10.26

in Table IV for the relative absorption of the radioactivity from $CaS^{35}O_4$ containing diets (diets A and C) compared to $Na_2S^{35}O_4$ diets (diets E and F) show that the radioactivity was absorbed better from the $CaSO_4 \cdot 2H_2O$ containing diets. Any interpretation of this increased absorption with calcium sulfate is difficult because it is assumed that minerals are absorbed as ions from the intestinal tract, and sodium sulfate is better ionized than calcium sulfate.

The data with respect to the relative blood levels of sulfur radioactivity are presented in Table V. It is interesting to observe that, although the data for the relative absorption of radioactivity from the diet only slightly favored the absorption of organic sulfur (Table IV), the blood radioactivity levels are from 10 to 20 times as high in those rats fed the diets containing radioactive methionine (diets B and D). Perhaps of more interest are the data which show the difference in blood levels of radioactivity from rats fed these two diets (diets B and D). The blood from those rats fed diet D, a diet which contains no inorganic sulfate, contains less relative radioactivity than does the blood from those rats consuming a diet with a comparatively normal ratio of organic to inorganic sulfur. These data strengthen the interpretation placed on the fecal absorption data; the level of inorganic sulfur in the diet influenced the absorption of organic sulfur. They are not surprising in view of the dual role of methionine, being utilized not only for the synthesis of protein, peptides, and regulatory substances, but also as a source of inorganic sulfate.

TABLE V

S^{35} RADIOACTIVITY OF THE BLOOD FROM EACH ANIMAL IN CPM/MILLIMOLE SO_4--AS A PERCENTAGE OF INGESTED ACTIVITY

Period	Diet groups					
	A	B	C	D	E	F
I	.14	4.20	.12	2.85	.08	.19
II	.12	3.81	.14	2.24	.20	.10
III	.12	2.62	.13	1.61	.25	.15
IV	.17	3.53	.20	2.82	.26	.15
V	.14	6.28	.15	4.06	.14	.25
Average	.14	4.09	.15	2.73	.19	.17

Not quite so obvious and perhaps more surprising is the slight increase in specific activity of the blood sulfur when the animals which have ingested radioactive sodium sulfate are compared with those fed radioactive calcium sulfate. This has occurred, it must be recalled, even though there was relatively poor absorption of radioactivity from the sodium sulfate-containing diets. These data could be explained if one could visualize the sodium ion as interfering with kidney function.

The data representing urinary sulfur radioactivity (Table VI) include urine samples from the first four periods. Due to experimental error no collection was made during the fifth period. It should be noted that the high figure from the inorganic S^{35}-calcium sulfate source for period IV makes the average for that group appear higher than that resulting from the comparable S^{35}-sodium sulfate source. An examination of the data for periods I, II, and III will show the activity from the S^{35}-sodium sulfate sources to be higher. The specific activity of the urine of the rats receiving no inorganic sulfate (diet D, Table VI) was lower than that of rats receiving the normal distribution of inorganic to organic sulfate (diet B). However, the lowered specific activity of sulfate in both the blood and urine in those animals ingesting no inorganic sulfate would seemingly indicate a considerable demand on the organic sulfur of these animals to supply their sulfur needs.

This point is strengthened by the data shown in the next table (VII) for the specific activity of the sulfur in the cartilage mucopolysaccharides. These data show that mucopolysaccharides isolated from

TABLE VI

S^{35} RADIOACTIVITY OF THE URINE FROM EACH ANIMAL IN CPM/MILLIMOLE SO_4--AS A PERCENTAGE OF INGESTED ACTIVITY

Periods	Diet groups					
	A	B	C	D	E	F
I	2.34	2.68	2.61	2.04	1.12	3.46
II	1.02	1.75	2.02	1.40	3.89	1.74
III	1.69	1.68	1.80	1.53	2.74	3.42
IV	13.72	3.91	5.10	2.34	3.13	4.44
Average	4.69	2.50	2.88	1.82	2.72	3.86

TABLE VII

S^{35} RADIOACTIVITY OF THE CARTILAGE FROM EACH ANIMAL IN CPM/MILLIMOLE SO_4^{--} AS A PERCENTAGE OF INGESTED ACTIVITY

Period	Dietary group					
	A	B	C	D	E	F
I	.76	.90	.48	.52	.18	.56
II	.78	.72	.69	.42	.64	.78
III	.71	.76	.53	.26	.75	.75
IV	.72	.37	.55	.37	.24	1.02
V	.95	.46	.57	.36	.59	.62
Average	.78	.64	.56	.39	.50	.75

the rib cartilage of these rats fed a diet containing no inorganic sulfate but 0.60 grams of methionine, had the lowest specific activity. The low specific activity of both the blood and mucopolysaccharide sulfur in these animals, compared to their litter mates fed diets with a normal inorganic to organic sulfur ratio, seems to reflect the competition of all systems for the dietary sulfur supplied by methionine; and indicates that, if the inorganic sulfur of the diet is limiting, supplementation of a 15 per cent casein diet with 0.60 grams methionine may be insufficient to satisfy the inorganic and organic sulfur requirements of the rat.

An exhaustive search of the literature did not reveal a definite requirement of inorganic sulfate for any species. In general it is stated that the inorganic sulfate requirement of the animal can be met by *in vivo* oxidation of organic sulfur. Although the data which have been presented in this thesis confirm the *in vivo* conversion of organic sulfur to sulfate, they indicate that there is a competition among the various sulfate acceptors for sulfur which thus raises the sulfur amino acid requirement above the level supplied by 1.1 per cent of methionine in the diet (diet D). According to Rose (Womack *et al.*, '37) a dietary level of methionine of 0.60 per cent is adequate for growth and maintenance of the rat. Whether these observations reflect an actual lack of dietary methionine or an overloading of the available enzyme systems cannot be elucidated by these data. The literature confirms the competition of many compounds for the available sulfur, but gives no indication of the ratio of organic sulfur converted to sulfate or to taurine.

This latter compound, known to be excreted in rather sizable quantities under certain conditions, may represent a significant portion of the slight radioactivity observed in the urine in this investigation.

One may compare the radioactivity of the cartilage mucopolysaccharides isolated from rats fed radioactive sulfur as organic sulfur (diets B and D) with the radioactivity of those isolated from rats fed radioactive sulfur as inorganic sulfur (diets A, C, E, and F). A comparison of this nature should serve to answer the question: can inorganic sulfur be utilized to satisfy the sulfate requirements of the rat. It may be observed that the specific activity of the mucopolysaccharide sulfur isolated from animals ingesting a high inorganic S^{35}-sulfate source (diets A and/or E) is approximately twice the specific activity of that from animals ingesting a high organic S^{35}-sulfur source (diet B). These data are not surprising in view of the previous discussion with respect to competition for available sulfate.

Since inorganic sulfur cannot enter into many of the sulfur systems common to methionine, if only inorganic sulfur be supplied to an animal, sufficient sulfate should be available for sulfation of mucopolysaccharides. On the other hand, if primarily organic sulfur (methionine) be supplied to an animal, there may not be sufficient sulfate available for adequate sulfation of mucopolysaccharides after all other sulfur needs are met. From a practical standpoint, these conditions are met by diets A and E and diet D. Although diets A and E do contain some organic sulfur, that contained in 15 per cent casein, this is not considered to be sufficient to satisfy the sulfur needs of

TABLE VIII

SIGNIFICANCE OF DATA[a]

Diet groups compared	Feces	Blood	Urine	Cartilage
A to B	----	----	P< .5	P< .4
A to D	P< .1	----	P< .4	P< .02
C to B	P< .05	----	P< .2	----
C to D	P< .005	----	P< .2	P< .9
E to B	P< .05	----	P< .05	----
E to D	P< .02	----	P< .25	P< .25
F to B	P< .01	----	P< .1	P< .6
F to D	P< .005	----	P< .001	P< .005
A to C	----	----	P< .5	P< .005
A to F	----	----	P< .7	P< .8
E to C	P< .9	P< .1	----	----
E to F	----	P< .8	----	----
A to E	----	----	P< .6	P< .1
F to C	P< .01	P< .2	P< .5	P< .05
B to D	P< .25	P< .001	P< .1	P< .025
B to C	----	P< .001	----	P< .5

[a]Expressed as t values, Steel and Torrie, '60.

A.M.D. is Arithmetic Mean Difference

d is difference $\quad A.M.D. = \frac{\Sigma d}{n}$

$t = \frac{A.M.D.}{\sigma_M}$ $\quad \sigma_M = \sqrt{\frac{\Sigma d^2 - (\Sigma d)^2/n}{n(n-1)}}$

the rat; therefore, the rest must come from the 0.42 per cent dietary sulfate supplied as inorganic sulfate.

It is appropriate, in view of previous discussion, to compare the sulfation of mucopolysaccharides from rats which were on radioactive inorganic sulfate diets where the sulfate salt was calcium sulfate in one case and sodium sulfate in the other. The data which pertain to this are presented in Table VII. They show that, on the high inorganic sulfate diets (A and E), there is a significantly (Table VIII) higher rate of sulfation of mucopolysaccharides if the radioactivity be supplied as calcium sulfate. Since our original concern was with the high sulfate diets, it was gratifying to observe that at these high levels calcium sulfate appeared to be superior to sodium sulfate as a source of dietary inorganic sulfate. It may be recalled (Table IV) that calcium sulfate (diet A) was also better absorbed from the diet than was sodium sulfate (diet E). It should be pointed out that when the levels of inorganic sulfate were somewhat lower, a more normal diet, there seemed to be little difference in radioactivity whether calcium sulfate or sodium sulfate was used as the dietary source of inorganic sulfur (diets C and F). So far as absorption was concerned the calcium sulfate was better absorbed; however, the radioactivity of the cartilage mucopolysaccharides was slightly higher when isolated from the rats on the diet containing sodium sulfate (diet F) compared to calcium sulfate (diet C). These data cannot be explained at this time. It would appear, since a relatively large increase in the sodium content of the diet (diet E) was necessary in order to make the sulfate

level equal to that in the high calcium sulfate diet (diet A), that the sodium ion might be adversely affecting the sulfation of mucopolysaccharides.

SUMMARY

An investigation of the utilization of sulfur from inorganic and organic dietary sources for sulfation of mucopolysaccharides was made.

Six adult, litter-mate, female, albino rats from the Wister strain were maintained for a period of ten days on diets containing S^{35}-sulfur as S^{35}-methionine, $CaS^{35}O_4$, and $Na_2S^{35}O_4$ following a seven day preliminary period on respective non-radioactive diets. The sulfur content of all diets was the same, while the ratio of organic to inorganic sulfur was varied by adjustments made in the salt mixtures. The 1% per cent, 3.% per cent, and .007 per cent sulfate salt mixtures used gave 0.42 per cent, 0.10 per cent, and 0.00 per cent dietary inorganic sulfate respectively.

Collections of urine, feces, blood, and cartilage from each animal were, in each case, prepared in a way so as to make it possible to collect data representing S^{35}-sulfate incorporation as a percentage of ingested S^{35} activity, and the data were treated statistically by comparison of sample means of paired observations.

The data presented indicate that the sulfate ion from inorganic sources contributes as much to the metabolic pool as when supplied from organic sources. Also, that the availability of the SO_4-ion is related to the ratio of organic to inorganic sulfur in the diet is exemplified by the data showing methionine better absorbed from the diet with no inorganic SO_4--and the data showing significantly greater $S^{35}O_4$--

incorporation into cartilage mucopolysaccharides when the diet contained an inorganic sulfur source.

The literature indicates that the amount of methionine at the high level used in this investigation is in excess of that required by the rat and, therefore, should give rise to high activity in the excreta. Since this was not found in this study, further investigation to determine the optimum amount of the $SO_4^{=}$-ion required by the rat would be interesting and possibly would give greater significance to the relationship of inorganic and organic sulfur supplied in the diet.

CONCLUSIONS

The data obtained from this investigation show that the $SO_4^{=}$ ion from inorganic dietary sulfur sources is utilized by the rat. They also show that the level and ratio of organic and inorganic sulfur in the diet exert a profound effect upon sulfur metabolism.

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