November 29, 1966

To the Graduate Council:

I am submitting a thesis written by Sara Frierson Fulton entitled "The Effect of Avitaminosis E on the Incorporation of ^{35}S-Sulfate by Rat Tissue Components." I recommend that it be accepted for nine quarter hours 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:

Frances A. Schofield

Ada Marie Campbell

Vice President for
Graduate Studies and Research

THE EFFECT OF AVITAMINOSIS E ON THE INCORPORATION OF ^{35}S-SULFATE BY RAT TISSUE COMPONENTS

A Thesis

Presented 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

Sara Frierson Fulton

December 1966

ACKNOWLEDGEMENT

The author wishes to express her deepest appreciation to Dr. John T. Smith for his guidance while doing this work and for his patience and timely sense of humor, and to Dr. Frances A. Schofield and Dr. Ada Marie Campbell for their aid in preparing the manuscript.

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CHAPTER I

INTRODUCTION

Previous work in this laboratory has shown that there is a decrease in sulfate production when ^{35}S-cysteine is incubated with liver homogenates prepared from vitamin E-deficient rats as compared with the sulfate produced by homogenates from vitamin E-sufficient rats. In avitaminosis E the lowered incorporation of a test dose of $^{35}SO_4^{=}$ into cellular lipoprotein and the mucopolysaccharides isolated from rib cartilage and lung has been shown. A decrease in the breaking strength of rats' aorta, presumably the result of impaired collagen production, was obtained when the aortas from vitamin E-deficient rats were compared with those from vitamin E-sufficient rats. The effects were more pronounced if the rats were also fed diets low in inorganic sulfur.

This study was undertaken to determine which of the lipoprotein fractions are affected by the lowered sulfate production apparent in avitaminosis E.

CHAPTER II

REVIEW OF LITERATURE

Vitamin E was first discovered as a factor present in the non-saponifiable fraction of oil from wheat germ and several other products. The factor was essential for the prevention of resorption in gestation of female rats and of sterility in male rats and for optimal growth. The factor was finally isolated and identified as one of the tocopherols (1), which have since been synthesized. Vitamin E has been shown to follow the same pattern of absorption and storage as the other fat-soluble vitamins. There appears to be no selectivity in the absorption of the vitamin (2). Most of the vitamin E absorbed is found in the mitochrondria and submicroscopic particles with about one-fourth to one-fifth of it in the nuclear fraction (3).

Although the tocopherols are powerful antioxidants and can function in this role in the animal, it has become evident from deficiency studies that the vitamin has one or several other roles in the body (4).

The increased rate of incorporation of formate-^{14}C into nucleic acids along with an increased allantoin excretion in avitaminosis E may suggest an increased rate of turnover for nucleic acids in an E-deficiency (5). The increased incorporation also may suggest an increase in nucleic acid synthesis. The increase was found to be in

the acid-soluble nucleotides in both bone marrow and skeletal muscle where there was a larger increase in desoxyribonucleic acid (DNA) than in ribonucleic acid (RNA). By using thymidine-^{14}C Dinning (6) has found that DNA synthesis is greatly increased in the skeletal muscle of vitamin E-deficient monkeys. The bone marrow RNA/DNA ratio also has been found to be elevated in an E-deficiency (7). Sodium formate-^{14}C also shows greater incorporation into muscle creatine. This may suggest an inability of the skeletal muscle to retain creatine and thereby be an explanation for the creatinuria found in E-deficient monkeys (8). With glycine-1-^{14}C the increased incorporation into skeletal muscle and bone marrow was not shown as has been demonstated with sodium formate-^{14}C. The difference in incorporation suggests that there may be an exchange of the number 2 carbon atom on the purine ring without degradation of the entire molecule and that vitamin E may be a coenzyme to control the reaction (9). Although the nucleic acid phosphorus present in tissues does not appear to be changed (5), a statistically significant increase in the incorporation of ^{32}P into nucleic acids, especially RNA, has been demonstrated in an E-deficiency (10).

Erythrocytes from animals deficient in vitamin E have been shown to be susceptible to hemolysis by dialuric acid in a given concentration but with higher concentrations of dialuric acid the erythrocytes appear to be protected against hemolysis (11). The susceptibility to dialuric acid has been used in the development

of a bioassay method for vitamin E potency which takes only two days (12). Supplementation with vitamin E but raising the level of cod-liver oil in the diet to 20 per cent affords no protection against the hemolysis indicating an increased requirement for vitamin E in this instance (13). Although it had previously been hard to determine the susceptibility of chicken erythrocytes from E-deficient animals to hemolysis, it now has been reported that the erythrocytes are susceptible to hemolysis by dialuric acid but not to hemolysis by heating in physiological saline as has been reported for erythrocytes from E-deficient animals of other species (14).

Dystrophic muscles from E-deficient animals have shown a loss of amino acids from the cells possibly due to increased membrane permeability (15). Rat liver slices, after losing potassium in a cold saline solution, are unable to reaccumulate the potassium when placed in warm, oxygenated Ringer solution if from E-deficient animals. The same is not true of E-sufficient animals (16). Saline washed erythrocytes from E-deficient animals have shown a statistically significant higher uptake of ^{22}Na for the entire incubation period when compared with E-sufficient animals. This indicates possible changes in the lipoprotein membrane (17).

A water soluble, acid hydrolysis resistant factor has been isolated from American yeasts and vitamin free casein which will prevent the necrotic liver degeneration sometimes associated with E-deficiency. It was designated factor 3 (18) and has been shown

to contain selenium. The three factors that must be absent to cause liver necrosis are cystine, vitamin E and factor 3 (selenium). Cysteine and methionine do not give as much protection as cystine does (19). There is an apparent decrease in the oxidative activity of the citric acid cycle in dietary necrosis as suggested by the accumulation of pyruvic and lactic acids in the terminal stages of the disease (19, 20), a decrease in lipogenesis and in the coenzyme A levels in the liver and an increase in the xanthine oxidase. With ^{14}C-methyl-methionine the utilization was found to be similar in both E-sufficient and E-deficient rats. The rate of incorporation of ^{35}S-cystine into liver protein was found to be high but with necrosis-developing animals the incorporation into coenzyme A was decreased (20). By activation analysis, cystine was found to contain 1.9 μg. selenium per gram of cystine. Although the selenium could possibly account for the protective effect of cystine in liver necrosis, it does not explain the difference in activity between cystine and cysteine and methionine (21).

When increased amounts of bakers' yeast or its alcohol or ether extract of the unsaponifiable fraction are used in a necrogenic diet, they are capable of preventing necrosis. The extracts are also capable of preventing erythrocyte hemolysis in vitro and have been shown to contain an Emmerie-Engel positive material which is not α, β, γ or δ tocopherol (22, 23).

On a vitamin E-deficient diet the incisors of rats lose their color and the enamel organs begin to deteriorate. Some workers have reported a reversal effect of 0.9 parts per million (p.p.m.) of selenium in the diet. With only 0.3 p.p.m. the deterioration was reversed for awhile but then returned (24).

If selenium or factor 3 can replace vitamin E in some functions, it might also relieve symptoms in other instances. All levels of selenium in the diet of the female rat were found to be ineffective in preventing resorption in gestation (25).

The chicken maintained on a 40 per cent Torula yeast diet deficient in vitamin E usually develops exudative diathesis. Factor 3 and selenium have been found capable of preventing the diathesis (26, 27).

Muscular dystrophy has been found in the chicken, rabbit and monkey on a vitamin E-deficient diet (28, 29, 30). Methionine and cystine have been found capable of preventing the degeneration in chickens (28, 31) but 0.5 per cent Na_2SO_4 only slightly reduces the degeneration (28). Factor 3 active compounds, even when added to a diet capable of preventing necrosis in the rat, appear to be ineffective in preventing muscular dystrophy in the rabbit (29).

Chickens on a diet low in arginine as well as the sulfur-containing amino acids and vitamin E have not developed muscular degeneration. When the arginine was added to the diet, the dystrophy developed unless the sulfur-containing amino acids were also added to the diet (31, 32).

By studying the relationship between vitamin E and the sulfur-containing amino acids, Olson (33) has found that on an E-deficient, low organic sulfur diet there is no difference in liver coenzyme A levels. However, when ^{35}S-cystine was administered, he found that only about 33 per cent of that incorporated into coenzyme A in sufficient animals was incorporated into coenzyme A by the E-deficient animals. By the inclusion of 5 µg per cent selenium in the diet the incorporation of ^{35}S-cystine into coenzyme A was found to increase 7-fold per unit enzyme and 14-fold per gram liver (34).

Due to a decreased incorporation of $^{35}SO_4^{=}$ into mucopolysaccharides and cellular lipoprotein isolated from vitamin E-deficient rats, a relationship has been suggested between vitamin E and the metabolism of sulfur-containing amino acids (35).

A cellular lipoprotein was first isolated from rat liver nuclei by extraction with NaOH and then precipitation with acetic acid. It was soluble in alkali but insoluble in water and neutral salts. Electrophoretic analysis showed one component to be present (36) even when different types of nuclei were used. The isoelectric point of the compound ranged between 4.65 and 4.85 (37). All soluble cellular components were then extracted from the whole liver cells by repeated extractions with 0.16 and 1.0M KCl solutions at 3 to 5°C. The residue was divided into three fractions which contained the same amount of lipid, phosphorus and hexosamine and approximately the same amount of nitrogen, thereby indicating that cellular residue is essentially

a homogeneous lipoprotein (38). When liver cells were extracted intact so as to leave ghost cells, the ghost cell lipoprotein was found to have the same crude lipid and almost the same nitrogen content as broken cell lipoprotein (39). When the liver cells were divided into the nuclear, mitochondrial and microsomal fractions and a 0.4M KCl solution was added to the extraction procedure, a characteristic and reproducible nitrogen, phosphorus, lipid, lipid phosphorus and hexosamine content was found for each fraction with the microsomal constituents differing significantly from the components of the other two fractions (40). By isolating cellular lipoprotein from various tissues and different species, Thomas and Levin (41) found that the lipoprotein differs between tissues and between species for the same tissue.

Sialic acid has been found in the membranous material of all fractions of the cells except the nuclear fraction. N-acetylneuraminic acid has been suggested as the main component as isolated (42). By further separation of lipoprotein from the mitochondria and microsomes of rat liver and heart, a family of lipoproteins with a density of 1.14 to 1.16 has been determined (43).

As can be seen from the previous statements, although much work has been done on vitamin E and some on cellular lipoproteins, little is actually understood about vitamin E and its relationship to the sulfur-containing amino acids and thereby its influence on cellular lipoproteins.

CHAPTER III

METHODS

I. GENERAL PLAN

Forty-eight male, albino, weanling rats were fed a low inorganic sulfate (0.0002 per cent), 15 per cent casein, methionine supplemented diet with stripped lard and cod-liver oil as the fat source and without added vitamin E (Table 1). Forty-eight additional males were fed the same diet supplemented with 28 mg.DL-α-tocopheryl acetate per 100 g. diet. The rats were housed in group wire mesh cages and received food and distilled water ad libitum. There was no apparent difference in the growth rate of the rats and all rats appeared to be healthy. After the rats had been on the experimental diet for at least six weeks and weighed 250 g. or more, the erythrocyte hemolysis test was used to determine whether or not those rats on the deficient diet were vitamin E-deficient by about 90 per cent hemolysis with dialuric acid as compared to 0 per cent hemolysis with sufficient E. After the vitamin E-deficiency of the rats had been established, they were injected subcutaneously with 10 to 25 μc.$Na_2{}^{35}SO_4$ as carrier free Na_2SO_4 in isotonic saline and killed 8 and 24 hr. later. The rats were randomly divided into the 8 and 24 hr. groups. The rats were decapitated and the livers were removed, put into labeled jars in groups of six and stored at -20°C until the analysis could be run.

TABLE 1

COMPOSITION OF EXPERIMENTAL DIETS

	g./100 g.
Vitamin free casein	15.00
Methionine	0.35
Sucrose	31.00
Cornstarch	32.00
Non-nutritive bulk[a]	10.00
Stripped lard[b]	6.00
Cod-liver oil	2.00
Vitamin mixture[c]	1.00
Salt mixture[d]	3.00

[a]Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[b]Distillation Products Industries, Inc., Rochester, New York.

[c]Synthetic vitamins added per 100 g. diet were: (in mg.) nicotinic acid, 20.0; pyridoxine·HCl, 0.5; thiamine·HCl, 0.5; riboflavin, 0.5; Ca pantothenate, 1.0; folic acid, 0.5; biotin, 0.005; 2-methylnapthoquinone, 0.025; vitamin B_{12}, 0.0045; choline chloride, 100.0; i-inositol, 100.0; p-aminobenzoic acid, 7.5. The vitamins were tritiated with sufficient sucrose to make 1.0 g. DL-α-tocopheryl acetate was added to the E-sufficient diet at the level of 28 mg./100 g. diet.

[d]See Table 2.

TABLE 2

COMPOSITION OF SALT MIXTURE[a]

	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
$AlK(SO_4)_2$	0.017
$Cu(C_2H_3O_2)_2 \cdot H_2O$	0.072
$MnCl_2 \cdot 4H_2O$	0.040
Cornstarch	10.693

[a]Modification of Hubbell, et al. (44).

The livers were removed from the freezer, allowed to partially thaw and the lipoprotein was isolated and then fractionated into lipid, nucleosides, nucleotides, mucopolysaccharides and an unidentified fraction extracted with NaOH and precipitated with ethanol (EtOH). The lipoprotein and each fraction were combusted, precipitated, collected and counted to determine the $^{35}SO_4^{=}$ distribution.

II. ERYTHROCYTE HEMOLYSIS

The method of Friedman, et al. (12) was followed to determine the susceptibility of the erythrocytes of the E-deficient rats to hemolysis by dialuric acid. About 40 λ blood were collected by a blood pipette from an incision in the rat's tail and put into 5 ml. saline-phosphate buffer, containing equal quantities of a 0.9 per cent saline solution and a 0.1M Na_2HPO_4 solution with 20 ml. 1.0N HCl per liter and a pH of 7.4, in 15 ml. centrifuge tubes. Clotting was prevented by gentle twirling of the tubes to thoroughly mix the blood with the buffer solution. The suspension was centrifuged at 1000 x g. for 10 min. The supernatant fluid was sucked off and the cells were resuspended in 3.5 ml. of the buffer. A 1 ml. aliquot of the erythrocyte suspension was placed in each of three test tubes. To tubes 1 and 2 1 ml. dialuric acid solution, prepared by adding 10 g. dialuric acid to 50 ml. of the saline-phosphate buffer, was added and to tube 3 1 ml. saline-phosphate buffer was added. The tubes were incubated in a 37°C water bath for an hour. After cooling for an hour, 5 ml.

distilled water were added to tube 2 for complete hemolysis of the erythrocytes and 5 ml. saline-phosphate buffer were added to tubes 1 and 3. The tubes were centrifuged at 1000 x g for 5 min. The supernatant fluid was poured into cuvettes and read in the Klett-Summerson colorimeter, which was zeroed with distilled water, with a blue filter. Per cent hemolysis was calculated by the formula

$$\frac{\text{reading tube 1 - reading tube 3}}{\text{reading tube 2 - reading tube 3}} \times 100 = \text{per cent hemolysis}$$

III. LIPOPROTEIN ISOLATION

The partially thawed and weighed livers were homogenized in solution I, a 0.16M KCl solution containing 186 mg. iodoacetic acid and 5 g. sodium citrate per liter. After the connective tissue was removed, the homogenate was diluted to 100 ml. in a ground glass stoppered 100 ml. mixing cylinder. Thorough mixing was assured by gentle inversion of the mixing cylinder. The homogenate was prepared with a motor driven Potter-Elvehjem homogenizer which contained a Teflon pestle. In order to keep the homogenate cold the homogenizer was immersed in an ice water bath during the homogenization. The solutions were kept at about 4°C throughout the entire isolation procedure. The homogeneous mass was centrifuged at 26,000 x g in a refrigerated centrifuge, with the Lourdes 9RA head being used, for 1 hr. at 4°C. The supernatant fluid was discarded and the homogenization and centrifugation were repeated with solution I. The centrifuge cake was then homogenized with solution II, a 1.0M KCl solution

containing 186 mg. iodoacetic acid and 10 g. sodium citrate per liter and adjusted to a pH of 4.7 with concentrated HCl, to a final volume of 80 ml. The procedure was repeated, alternating between solution II and solution III, a 0.4M KCl solution containing 186 mg. iodoacetic acid and 10 g. sodium citrate per liter, until the supernatant fluid gave a negative protein test with 6 per cent trichloroacetic acid (TCA). The centrifuge cake was then washed with solution I to lower the salt concentration. The resulting centrifuge cake of insoluble cellular lipoprotein was quick-frozen over an acetone-dry ice mixture and lyophilized. Duplicate 50 mg. samples of the lipoprotein were combusted with 1 ml. carrier sulfate, precipitated, collected and counted according to the method of Katz and Golden (45). The carrier sulfate is a dilute H_2SO_4 solution which yields approximately 12 mg. $BaSO_4$ per milliliter when precipitated with 10 per cent $BaCl_2$. The sample was placed in a 25 ml. Erlenmeyer flask with 1 ml. carrier sulfate and 4 to 5 ml. of the combustion mixture and a drop or two of a 1 per cent ammonium vanadate solution in 20 per cent HNO_3 as a catalyst were added. The mixture was heated on a hot plate at about 300°F until all brown gaseous fumes had been driven off. The temperature was raised to about 450°F and the heating was continued until all the moisture was evaporated and a yellow-green residue remained. To the residue 10 ml. distilled water were added and if all of the residue was not dissolved, the solution was heated to boiling to dissolve the remaining residue. The sulfate in the solution was precipitated

by the addition of 1 ml. 10 per cent $BaCl_2$. The $BaSO_4$ precipitate was collected on preweighed glass filter paper by suction and then washed with 0.5N. HCl, distilled water, ethanol and acetone, which also dries the precipitate. The filter paper with the precipitate was then weighed, glued to an aluminum planchet and counted in a Nuclear Chicago, windowless, gas flow counter for 4000 counts and the time automatically recorded. The proper correction factor for absorption was applied to the calculated counts per minute so that all counts were expressed on the basis of 12 mg. $BaSO_4$. For each group of animals the proper factor for decay was used so that all results could be expressed without involving the time taken for analysis and the decay involved since the half-life of ^{35}S is 87 days.

IV. LIPOPROTEIN FRACTIONATION

Lipid

The lipoprotein was heated with 20 ml. 2:1 $CHCl_3$:CH_3OH per gram in a 60°C water bath for 2 hr. The resulting mixture was filtered by suction and washed with 2:1 $CHCl_3$:CH_3OH. The residue was allowed to air dry. The lipid fraction in 2:1 $CHCl_3$:CH_3OH was made to 100 ml. in a volumetric flask and duplicate 25 ml. aliquots were taken for combustion with 1 ml. carrier sulfate, precipitation, collection and counting.

Mucopolysaccharide

The air dried residue was stirred with 10 ml. 0.5M KSCN per gram for 3 hr. For each gram of sample 5 ml. 80 per cent phenol in distilled water were added and the stirring was continued for an additional 4 hr. The mixture was allowed to stand overnight, centrifuged at 5000 x g. for 10 min., the supernatant fluid saved and the residue washed once with the 0.5M KSCN. The supernatant fluids were combined, three or four drops 20 per cent sodium acetate and 3 vol. EtOH were added, and the solution was allowed to precipitate overnight at -20°C. The precipitate was collected by centrifugation at 5000 x g. for 10 min. and washed three times with EtOH and dried with ether. The precipitate, mucopolysaccharide, was weighed and 5 mg. samples, in duplicate, were combusted with 1 ml. carrier sulfate, precipitated, collected and counted. The residue remaining after the extraction was dried with acetone. The KSCN in the EtOH, ether and acetone gave such a large amount of precipitate when combusted that it was impossible to determine the amount of $^{35}SO_4^{=}$ removed by the isolation and drying procedure.

Nucleosides and Nucleotides

The acetone dried residue was extracted with 10 ml. cold 10 per cent TCA per 200 mg. sample to remove the nucleosides. The nucleotides were then extracted by heating the residue with 10 ml. 5 per cent TCA per 200 mg. in a 90°C water bath for 30 min. The residue

was dried with 50 ml. acetone. Aliquots of the nucleoside and nucleotide fractions and the acetone were combusted with 1 ml. carrier sulfate, precipitated, collected and counted.

Non-carbohydrate Alcohol Precipitate

The residue remaining after the nucleotide extraction was ground with a mortar and pestle and weighed. After the addition of 20 ml. distilled water per gram residue, the mixture was boiled for 15 min. and cooled to 5°C. Ten per cent NaOH was added to a final concentration of 2 per cent NaOH (1 ml. 10 per cent NaOH for each 4 ml. water used) and the mixture shaken for 16 hr. at 0 to 5°C. The pH was adjusted to 6.0 with 10 per cent acetic acid and the residue removed by centrifuging at 5000 x g. for 10 min. The residue was washed with distilled water. The supernatant fluids were combined with 3 vol. EtOH and allowed to precipitate overnight at -20°C with 2 to 3 drops of 20 per cent sodium acetate added. The precipitate was collected by centrifuging at 5000 x g. for 10 min., washed three times with EtOH and dried with ether. The residue was dried with 25 ml. acetone. Aliquots in duplicate of the EtOH and ether used to precipitate, wash and dry the precipitate and the acetone used to dry the residue were combusted with 1 ml. carrier sulfate, precipitated, collected and counted. The precipitate was weighed and 20 mg. samples in duplicate were combusted with 1 ml. carrier sulfate, precipitated, collected and counted. The remaining residue was weighed

and 200 mg. samples, in duplicate, were combusted with 1 ml. carrier sulfate, precipitated, collected and counted.

V. RNA

The amount of ribonucleic acids (RNA) present in the nucleotide fraction was determined by the Bial's reaction. The nucleotide fraction was evaporated to 50 ml. on a flash evaporator. Into each of two test tubes 1 ml. of the nucleotide solution was pipetted, and 1 ml. of the orcinol reagent (prepared by adding 100 mg. orcinol to 10 ml. of a 0.1 per cent $FeCl_3$ solution in concentrated HCl within an hour of its use) was added to each tube. The tubes were immersed in a boiling water bath for exactly 20 min., removed, made to 4 ml. with distilled water, allowed to cool and the optical density was read with a Klett-Summerson colorimeter which was zeroed with distilled water, with the red filter being used. The ribose standard solution was prepared by dissolving 100 mg. adenosine triphosphate (ATP) in 5 ml. 0.1N. NaOH and making to 250 ml. with 5 per cent TCA (contained 0.4 mg. nucleotide per milliliter). Since the Bial's reaction is used to measure the amount of ribose present, ATP was suitable for a standard. Although the molecular weight of RNA is slightly smaller than that of ATP due to the presence of both purines and pyrimidines in RNA and only the purine adenosine in ATP, this difference would make the amount of RNA present really slightly more than calculated from the determination. This difference is only slight and the same

error is included in all the results since the same ATP solution was used in each determination. A standard curve was prepared by using varied amounts of the stock solution and following the same procedure as that for determining the RNA of the samples. The milligrams RNA in the nucleotide fraction were determined by readings from the standard curve.

VI. STATISTICS

The method of paired comparisons and the student's t test as described by Steel and Torrie (46) were used to determine statistical significance of the results. The differences between results being compared were found and the standard error of the differences ($s_{\bar{d}}$) was calculated by the formula,

$$s_{\bar{d}} = \sqrt{\frac{\Sigma d^2 - (\Sigma d)^2/n}{n(n-1)}}$$

where d represents differences and n the number of experiments or animals in a group. The t value was determined by $t = \frac{d}{s_{\bar{d}}}$ where d is the difference in arithmetic means and the $s_{\bar{d}}$ is the one calculated above. The t value was compared with tables for the degrees of freedom (n - 1) allowed in each case to determine the p value. A p value of 0.05 or less (happens at least 95 per cent of the time) was used to denote statistical significance. The standard error ($s_{\bar{x}}$) of the mean for each set of figures was calculated by the

formula,

$$s_{\bar{x}} = \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n(n-1)}}$$

where x equals the numbers and n the number of experiments or animals in a group.

CHAPTER IV

RESULTS AND DISCUSSION

The relationship between sulfur metabolism and vitamin E has been investigated through the use of a diet deficient in vitamin E and low in inorganic sulfate but sufficient in organic sulfur. With this diet the rat is forced to metabolize the sulfur-containing amino acids to obtain the sulfate needed for the sulfation of various ester sulfate containing compounds in the body.

Much of the work with vitamin E-deficient animals has been criticized because the drastic diets which have been used resulted in inanition. The rats in this experiment were not sick as can be seen from a comparison of the average difference in weanling and final weights of the rats fed the two diets (Table 3). The weights show that there is little difference between the weights of the rats fed the vitamin E-sufficient and deficient diets. The weights are not shown separately for 8 and 24 hr. animals since they were randomly divided and were not identified as to which ones went into each group.

Since the rats were not suffering from inanition and showed no outward signs of a vitamin E-deficiency, the question naturally arises as to how it was shown that the animals were really vitamin E-deficient with such a mild deficiency state. To determine the E-deficiency status of the animals, erythrocyte hemolysis by dialuric

TABLE 3

WEIGHT OF RATS WHEN PLACED ON DIETS AND WHEN KILLED

Experiment[a]	Sufficient E				Deficient E			
	Weanling[b]		Killed[b]		Weanling[b]		Killed[b]	
	g.		g.		g.		g.	
I (12)	39	± 1.8	249	± 4.2	35	± 1.7	229	± 5.1
II (12)	39	± 2.5	251	± 3.8	45	± 2.1	252	± 3.6
III (12)	36	± 2.5	258	± 5.8	48	± 2.2	257	± 6.7
IV (12)	36	± 3.1	300	± 12.5	35	± 2.7	263	± 11.4
AM	37	± 1.2	265	± 4.7	41	± 1.4	250	± 4.0

[a]Numbers in parentheses refer to the number of animals per group.

[b]All figures are averages ± standard error of the mean.

acid was used as a criterion. One of the tubes was used as a check on the amount of hemolysis in the buffer solution alone, one had complete hemolysis by the addition of distilled water, and the other one showed the susceptibility of the erythrocytes to dialuric acid. In each set of rats checked, one of the E-sufficient animals was also used to show its susceptibility to the dialuric acid. All of the E-deficient animals showed hemolysis of 85 per cent or more with most running 90 to 95 per cent. The erythrocytes from the E-sufficient animals showed 0 per cent hemolysis consistently.

The two times chosen for the study of incorporation were 8 and 24 hr. after the subcutaneous injection of the carrier free $Na_2{}^{35}SO_4$ in isotonic saline. When the mucopolysaccharides isolated from costal cartilage were studied, the time for maximum incorporation was 24 hr. after the injection. However, with the cellular lipoprotein 8 hr. was found to be the time for maximum incorporation of the sulfate. Therefore, 8 hr. and 24 hr. were used to determine the time for maximum incorporation of sulfate and effect of time on each fraction of the lipoprotein. The 8:24 hr. ratios (Table 4) show that the lipoprotein, mucopolysaccharides and non-carbohydrate alcohol precipitate (NCAP) are more active at 8 hr.; the nucleotides with sufficient E are more active at 24 hr.; and the lipid fraction, residue, mg RNA and nucleotides with deficient E have about a 1:1 ratio showing no difference for the two times.

TABLE 4

8:24 HOUR RATIO OF THE LIPOPROTEIN AND ITS FRACTIONS

Sample	Sufficient E	Deficient E
Lipoprotein	1.5:1	1.4:1
Lipid	1.1:1	1.2:1
Mucopolysaccharide	1.3:1	1.6:1
RNA (mg.)	1.1:1	1.0:1
Nucleotides (total counts/min.)	0.6:1	0.8:1
Nucleotides (counts/min./mg. RNA)	0.2:1	0.9:1
NCAP	2.8:1	3.8:1
Residue	0.9:1	0.9:1

With about the same amount of cellular lipoprotein isolated from the livers of the rats with both sufficient and deficient vitamin E, there was less incorporation of ^{35}S-sulfate in the lipoprotein isolated from the vitamin E-deficient animal as compared to that from the vitamin E-sufficient rat when the animal was killed 8 hr. after the subcutaneous injection of $Na_2{}^{35}SO_4$ (Table 5). The difference in incorporation was statistically significant ($.02 < p < .05$). Although the same trend was observed in the 24 hr. lipoprotein samples, the difference was not statistically significant. Since the lipoprotein was the insoluble cellular lipoprotein from the membranes of the cells, the structure of the cell may be affected in an E-deficiency state.

When it was found that the lipoprotein could be separated into several fractions, the fractions were isolated and the incorporation of ^{35}S-sulfate by each one was studied in relation to an E-deficiency.

Thirty to 40 per cent of the radioactivity in the isolated lipid fraction has been recovered as a sulfolipid.[1] Although this is all that was recovered, there was only one radioactive band in thin-layer chromatograms indicating that the rest may have been lost in the isolation process. The lipid fraction showed a statistically

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[1]Smith, J. T., unpublished observation. By thin-layer chromatography and column chromatography this fraction behaves similarly to the sulfolipid isolated from brains.

TABLE 5

SPECIFIC ACTIVITY OF SULFUR IN CELLULAR LIPOPROTEIN

	Specific Activity (counts/min./mg. sample)			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	3.4	1.9	1.6	2.0
II (6)	3.6	3.5	2.9	2.0
III (6)	4.8	3.9	3.0	1.9
IV (6)	10.9	9.6	8.2	7.7
AM[b]	5.7 ± 1.77*	4.7 ± 1.68*	3.9 ± 1.46	3.4 ± 1.43

[a]Numbers in parentheses indicate number of animals per group.

[b]Average of the experiments ± standard error of the mean.

*Denotes a statistically significant difference.

significant difference in the 8 hr. samples with more ^{35}S-sulfate incorporated with sufficient E than with deficient E (Table 6). The same trend was observed for the lipid fraction of the 24 hr. sample but these values were not statistically significant. The time for maximum incorporation of ^{35}S-sulfate into brain sulfolipid has been shown to be two days. The soluble sulfolipid from the liver (not investigated here) has been shown to have maximum incorporation at 12 hr. (47). These differences in time have suggested a difference in the method of sulfation with the liver's soluble sulfolipid being sulfated by exchange and the brain's sulfolipid, a cerebroside, sulfated by synthesis (48). The fact that the sulfolipid isolated from the cellular lipoprotein behaves similarly when subjected to chromatography to that from brains suggests that it is a cerebroside, although the cerebrosides are believed to occur only in myelinated cells. The best way to answer this question would be to purify the isolated sulfolipid and analyze it for galactose. However, such a small amount was isolated from the animals that all of the sample would have to be pooled to have sufficient material to work with and this would not leave any samples for a statistical comparison.

The difference in activity of the mucopolysaccharides has been observed in the chondroitin sulfate from the costal cartilage (Table 7). With this set of experiments the mucopolysaccharides isolated from the deficient E lipoprotein tended to contain less ^{35}S-sulfate than the mucopolysaccharides from E-sufficient animals (Table 8).

TABLE 6

SPECIFIC ACTIVITY OF SULFUR IN LIPID FRACTION

	Specific Activity (counts/min. x 10^{-4}/mM $SO_4^=$)			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	4.0	2.5	2.4	2.1
II (6)	2.9	1.7	3.9	2.5
III (6)	3.9	3.2	4.5	3.0
IV (6)	10.6	8.1	8.8	5.7
AM[b]	5.4 ± 1.77*	3.9 ± 1.44*	5.0 ± 1.36	3.3 ± 0.81

[a]Numbers in parentheses indicate the number of animals per group.

[b]Average ± standard error of the mean.

*Denotes a statistically significant difference.

TABLE 7

SPECIFIC ACTIVITY OF THE SULFUR IN TISSUE COMPONENTS

Component	Specific Activity (counts/min. x 10^{-3}/mM $SO_4^=$) Vitamin E	
	Sufficient	Deficient
Lipoprotein	2.6 ± 0.05	2.1 ± 0.04
Erythrocyte membrane	2.7 ± 0.20	2.3 ± 0.01
Mucopolysaccharides		
Cartilage	10.4 ± 2.1	6.3 ± 1.9
Lung	26.0 ± 6.9	10.8 ± 3.4

TABLE 8

SPECIFIC ACTIVITY OF SULFUR IN MUCOPOLYSACCHARIDES

	Specific Activity (counts/min./mg.)			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	57	29	34	19
II (6)	16	9	7	5
III (6)	53	17	59	10
AM[b]	42 ± 13.1	18 ± 6.0	33 ± 15.0	11 ± 4.1

[a]Numbers in parentheses indicate number of animals per group.

[b]Average ± standard error of the mean.

This is the same trend that had been observed in other tissues (Table 7, page 29) but the results were not statistically significant for either the 8 hr. or the 24 hr. samples. Even with the pooled samples used, there really was not sufficient mucopolysaccharide to analyze. With such a small amount isolated two samples were lost and with only three sets of experiments for statistical analysis, the resulting decrease in the degrees of freedom prevented any statistical significance, although the t values were large enough to give statistical significance with five samples. The sulfomucopolysaccharides have been suggested as being important to the integrity of the cell (49). The hexosamine content of cellular lipoprotein is 0.20 ± 0.01 per cent. The isolation of sialic acid from cellular fractions accounts for 80 per cent[2] of the hexosamine of the lipoprotein and leaves very little to be accounted for as a mucopolysaccharide. Therefore, it is understandable that such difficulty was experienced in isolating the small amount of mucopolysaccharides present and in having enough for statistical analysis.

Several other workers have reported an increase in nucleic acids in vitamin E-deficiency (5-10). Although there was a trend for more RNA to be isolated from the lipoprotein of the deficient rats at both 8 and 24 hr. (nucleotide fraction) than from the sufficient animals, this difference was not statistically significant (Table 9). The total activity of the nucleotides was about the same with sufficient E and deficient E (Table 10). When the results are expressed in terms

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[2]Smith, J. T., unpublished observation.

TABLE 9

AMOUNT RNA ISOLATED FROM LIPOPROTEIN

	mg. RNA			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	29.7	33.0	28.3	38.0
II (6)	28.9	40.5	40.5	46.9
III (6)	25.6	33.8	25.3	40.0
IV (6)	23.5	64.0	3.8	47.0
AM[b]	26.9 ± 1.45	42.8 ± 7.25	24.5 ± 7.63	43.0 ± 2.33

[a]Numbers in parentheses indicate number of animals per group.

[b]Average ± standard error of the mean.

TABLE 10

TOTAL ACTIVITY OF SULFUR IN NUCLEOTIDES

	Specific Activity (total counts/min. x 10^{-3})			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	1.0	1.1	1.0	0.8
II (6)	0.9	1.1	0.6	0.7
III (6)	1.3	1.4	4.0	2.9
IV (6)	2.8	1.8	2.9	3.0
AM[b]	1.2 ± 0.20	1.6 ± 0.40	2.1 ± 0.80	1.9 ± 0.64

[a]Numbers in parentheses indicate number of animals per group.

[b]Average ± standard error of the mean.

of the counts/min/mg RNA, it is shown that the specific activity tends to be higher with sufficient E; however, this difference is not statistically significant (Table 11). The lesion observed here seems to be bound to the synthesis of nucleotides rather than the utilization of sulfate. This fraction, which may contain "active sulfate," is one of the most active fractions in incorporating the $^{35}SO_4$. However, as can be observed from the data presented, nucleic acids are not affected in their ability to handle sulfate as most of the other fractions are. Any increase observed in the amount of incorporated ^{35}S-sulfate appears to be due to an increased RNA pool rather than increased utilization of sulfate by an animal which has been stressed in respect to the inorganic sulfate available along with a vitamin E-deficiency. This fraction behaves similarly to 3'-phosphoadenosine-5'phosphosulfate (PAPS) on a formic acid washed column but gives a second spot with paper chromatography.[3] If PAPS were the main compound being measured, it would be expected to be active in incorporation. Apparently there is no effect of vitamin E on "active sulfate" so the lesion must be one of either exchange or synthesis.

A very interesting fraction of the lipoprotein is an unidentified non-carbohydrate alcohol precipitate. It shows no effect of a vitamin E-deficiency, either 8 or 24 hr. after the subcutaneous

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[3]Button, G. M., unpublished observation.

TABLE 11

SPECIFIC ACTIVITY OF SULFUR IN NUCLEOTIDES

	Specific Activity (counts/min./mg. RNA)			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	33.7	33.4	35.5	20.2
II (6)	31.6	26.0	14.1	14.6
III (6)	51.3	41.5	157.0	72.2
IV (6)	75.0	44.0	766.0	63.0
AM[b]	47.9 ± 10.10	36.2 ± 4.09	243.1 ± 177.10	42.5 ± 14.66

[a]Number in parentheses indicates number of animals per group.

[b]Average ± standard error of the mean.

$Na_2{}^{35}SO_4$ injection (Table 12). The 8:24 hr. ratio indicates that this fraction is one of the most active ones in relation to its incorporation with much more incorporation of sulfate at 8 hr. than at 24 hr. The counts/min/mg. also show it to be the most active in sulfate incorporation. Since this fraction is uninfluenced by a vitamin E-deficiency, the lesion is apparently in the overall reduction of sulfate.

The final residue remaining after all the extractions shows no difference between sufficient E and deficient E (Table 13). Therefore, any compounds which have been affected by the E-deficiency and low inorganic sulfate diet have been removed during the fractionation of the lipoprotein.

TABLE 12

SPECIFIC ACTIVITY OF SULFUR IN NON-CARBOHYDRATE ALCOHOL PRECIPITATE

	Specific Activity (counts/min./mg. sample)			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	31	63	13	10
II (6)	78	68	32	20
III (6)	22	10	12	7
IV (6)	82	120	21	32
AM[b]	53 ± 15.6	65 ± 22.5	19 ± 4.6	17 ± 5.6

[a]Numbers in parentheses indicate number of animals per group.

[b]Average ± standard error of the mean.

TABLE 13

SPECIFIC ACTIVITY OF SULFUR IN RESIDUE

	Specific Activity (counts/min./mg. sample)			
	8 hr.		24 hr.	
Experiment[a]	Sufficient E	Deficient E	Sufficient E	Deficient E
I (6)	2.4	2.4	1.7	2.7
II (6)	2.4	2.0	2.4	2.0
III (6)	3.8	3.3	4.4	4.4
IV (6)	6.1	6.5	8.1	6.4
AM[b]	3.7 ± 0.87	3.6 ± 1.02	4.2 ± 1.43	3.9 ± 0.98

[a]Numbers in parentheses indicate number of animals per group.

[b]Average ± standard error of the mean.

CHAPTER V

SUMMARY

The relationship between vitamin E and sulfur metabolism in the rat has been investigated by feeding diets low in inorganic sulfur and deficient in vitamin E or supplemented with DL-α-tocopheryl acetate. The rate of incorporation of ^{35}S-sulfate into cellular lipoprotein and its various fractions 8 hr. and 24 hr. after a subcutaneous injection of $Na_2{}^{35}SO_4$ in isotonic saline was used as an index to sulfate utilization and metabolism by the rat.

The data presented above indicate a statistically significant decrease in ^{35}S-sulfate incorporation into the cellular lipoprotein and lipids 8 hr. after the injection and a trend in the same direction for the same components after 24 hr. and for the mucopolysaccharides at both 8 and 24 hr. in avitaminosis E.

Although the total incorporation of sulfate into the nucleotide fraction appears to be greater without vitamin E, this is found to be due to an apparent increase in total nucleotides, although it is not statistically significant, rather than an increase in the incorporation of ^{35}S-sulfate in a vitamin E-deficiency. The non-carbohydrate alcohol precipitate, although the most active fraction in incorporating sulfur, does not show any apparent effect from avitaminosis E. The final residue shows that the compounds influenced

by an E-deficiency have been removed during the fractionation of the lipoprotein.

Since these most active compounds do not show the effect of vitamin E while the cellular lipoprotein, lipids and mucopolysaccharides show a decreased sulfate incorporation in avitaminosis E, the lesion is an effect on the sulfation of ester sulfate compounds rather than a general overall effect on sulfur utilization.

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