

August 8, 1961

To the Graduate Council:

I am submitting herewith a thesis written by Betty June Pendergrass entitled "The Interrelationship of Tocopherols and Sulfur Metabolism." 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:

Helen Placard
Margaret S. Milliken

Accepted for the Council:

Dean of the Graduate School

THE INTERRELATIONSHIP OF TOCOPHEROLS
AND SULFUR METABOLISM

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
Betty June Pendergrass
August 1961

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ABSTRACT OF EDUCATIONAL RESEARCH STUDY COMPLETED

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Note: The student should consult with his major professor and follow his advice concerning the general format of the abstract. Additional pages, if required, should be 8½ x 11 inches and of quality equivalent to that required in the case of the thesis.

The metabolic effects of the tocopherols and of sulfur have been inadvertently linked through research findings. Although not initially postulated, a relationship between vitamin E and sulfur has turned up in such phenomena as prevention of dietary hepatic necrosis, muscular dystrophy, erythrocyte hemolysis; and structural alteration of mucopolysaccharides, proteins, and lipoproteins. The relationship between vitamin E and sulfur remains ill-defined at present; the available information has been obtained as a by-product of research efforts oriented toward other goals. However, an increasing body of evidence indicates that significant behavioral interactions may occur. The present investigation was designed to evaluate possible interrelationships between tocopherol and sulfur metabolism by observing S^{35} incorporation into erythrocyte stroma, erythrocyte hemolysis, and circulating sulfhydryl levels of the blood in animals receiving diets varied in amounts and kinds of sulfur and with and without sufficient vitamin E.

Four groups of diets were used; three of these contained essentially the same level of sulfur, calculated as sulfate, but the ratio of inorganic to organic sulfur was varied. The fourth diet contained virtually no inorganic sulfate and was low in organic sulfate. Each diet was tested both vitamin E sufficient and deficient. These diets were fed to eight matched groups of five rats each for a total of 65 days. Blood analyses were made throughout the experiment period for per cent hemolysis and circulating sulfhydryl. At the end of the investigation, the animals were injected with $Na_2S^{35}O_4$, fasted twenty-four hours, and terminated by withdrawing the blood by cardiac puncture. The erythrocyte stroma was collected and its incorporation of radioactive sulfur determined.

No statistically significant differences were observed with respect to dialytic acid hemolysis between groups. The data on the circulating sulfhydryl and the S^{35} incorporation into erythrocytes were complementary. Animals receiving no vitamin E and only methionine as the source of sulfur exhibited increased sulfhydryl values and decreased erythrocyte incorporation of S^{35} at highly significant levels. The converse was true for those animals receiving both methionine and vitamin E. Animals receiving no methionine, but extremely high levels of inorganic sulfate also exhibited a significantly increased erythrocyte incorporation of S^{35} . The sulfhydryl levels of this latter group of animals were observed to be higher than that of any other dietary group. The data obtained from this investigation suggest that adequate vitamin E plays a part in accelerating the conversion of cysteine to sulfate in the rat.

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INTRODUCTION

The metabolic effects of the tocopherols and of sulfur have been inadvertently linked through research findings. These two substances have little in common physically. The tocopherols are a class of physiologically active, fat-soluble compounds with alpha-tocopherol possessing the highest vitamin E activity, and delta-tocopherol, the highest antioxidant activity. Sulfur is an elemental constituent of amino acids, coenzymes, mercapturic acids, mucopolysaccharides, peptides, proteins, and vitamins, and in addition is nutritionally necessary in its inorganic form. Although not initially postulated, a relationship between vitamin E and sulfur has turned up in such phenomena as prevention of dietary hepatic necrosis, muscular dystrophy, erythrocyte hemolysis; and structural alteration of mucopolysaccharides, lipoproteins, and of proteins, as evidenced by rate changes in enzymatic reactions.

The relationship between vitamin E and sulfur remains ill-defined at present; the available information has been obtained as a by-product of research efforts oriented toward other goals. However, an increasing body of evidence indicates that significant behavioral interactions may occur. For this reason, it appeared feasible to investigate possible interrelationships more directly by observing the erythrocyte hemolysis and circulating sulfhydryl behavior of the blood in animals receiving diets varied in amounts and kinds of sulfur and with and without sufficient vitamin E.

REVIEW OF LITERATURE

In spite of the differences between tocopherol and sulfur, it has been well-established that these two substances exert a similar protective action against dietary hepatic necrosis (Himsworth and Lindan, '46; György and Goldblatt, '49; György et al., '50; Schwarz, '54; Chernick et al., '55; Olson et al., '55) and against muscular degeneration (Tallan, '55; Machlin and Shalkop, '56; Oppenheimer et al., '58) resulting from a dietary deficiency of vitamin E. György et al. ('49) have shown that the presence of tocopherol in the diet may compensate for the lack of sulfur amino acids, and vice versa, in preventing dietary hepatic necrosis. Their theory is that the protection afforded by sulfur and tocopherol is due primarily to a detoxifying mechanism rather than to the correction of a deficiency.

Machlin and Shalkop's work ('56) indicates that a diet containing 0.3 per cent L-cystine and adequate amounts of tocopherol will prevent muscular degeneration in chickens. Each of the substances also afford complete protection against the muscular lesion when used separately. They found that methionine and cystine were approximately equal in their protective effect. In addition, 0.5 per cent sodium sulfate in the diet resulted in a slight reduction in the incidence of muscular dystrophy. This they felt was due to the action of the sulfate in sparing the requirement of the chicken for sulfur amino acids.

Alfin-Slater ('60) suggests that vitamin E may be tied to lipid metabolism both enzymatically and as an in vivo antioxidant. The extensive Elgin project on vitamin E indicates that the feeding of

increased quantities of unsaturated fats raises the vitamin requirement of the human (Horwitt, '59). Although these lipids, especially of high linoleic acid content, are frequently high in tocopherol, Horwitt ('60) postulates that linoleic acid may be stored to the detriment of tocopherol storage, a phenomenon which is emphasized with increased lipid consumption.

Mann et al. ('60) in their work with the development of experimental atherosclerosis noted that L-cystine, DL-methionine, L-cysteine hydrochloride, cystamine, taurine, and reduced glutathione were effective in reducing hypercholesteremia in sulfur deficient monkeys. They have suggested that this behavior could be due to cysteine or metabolites of cysteine which are not essential or effective in more general anabolic processes. In addition, it has been reported that a vitamin E deficiency in rabbits and guinea pigs results in elevated plasma cholesterol levels (Dam, '44; Deuel et al., '55; Oppenheimer et al., '58; Shull et al., '58). It has been postulated that the relationship of tocopherol to lipid metabolism may be explained by its function as an antioxidant. The integrity of polyunsaturated fatty acids which have been demonstrated to be important regulators of certain aspects of cholesterol metabolism would be thus preserved (Alfin-Slater et al., '54). However, this phase of vitamin E and lipid metabolism has not been clearly elucidated and is still controversial in nature.

Shinowara ('57) has observed that the fatty acids in erythrocyte stroma lipoprotein are highly unsaturated. Oppenheimer et al. ('58) revealed that lipoprotein concentrations of both cholesterol and

phospholipids vary with intake of vitamin E. The lipoproteins of the erythrocyte stroma (Shinowara, '57) appear to be somewhat similar to the lipoproteins prepared from the tissue cells of calf liver (Bruemmer and Thomas, '57; Smith et al., '57). From this latter class of lipoproteins, a sulfur-containing mucopolysaccharide has been isolated (Smith and Thomas, '57). Böstrom and Åqvist ('52) and Dziewiatkowski ('54) present evidence indicating that the main uptake of sulfate occurs in mucopolysaccharides containing ester sulfate. Most recent evidence indicates enzymatic transfer of sulfate to the high molecular weight polymer (Gregory and Robbins, '60). In addition, it has been shown that vitamin E reduces the hyaluronidase skin diffusion area in young people but not in the aged, which suggests that mucopolysaccharides may also be affected by tocopherol (Bottiglionni and Carinci, '56).

Erythrocyte hemolysis is one lesion resulting from a tocopherol deficiency which is common to all investigated species, including man. This probably occurs as a result of an alteration in the lipoproteins of the erythrocyte membrane (Horwitt et al., '56). With the absence of tocopherol in the cell membrane, an increased peroxidation of the unsaturated lipid portion is thought to occur (Hove, '55; Horwitt et al., '56). Such a protective action on the part of tocopherol presumably results from the uptake of tocopherol by the lipoproteins in the red cell surface as a shield against peroxidation of the unsaturated fatty acids. Tsen and Collier ('60a) support this with the observation that the rate of hemolysis parallels the formation of lipid peroxides.

Erythrocytes from tocopherol deficient animals have been shown

to be subject to chemical hemolysis by dialuric acid, a reduction product of alloxan (György and Rose, '49; Rose and György, '50; Heard et al., '52; Rose and György, '52; Muyltjens, '56; Friedman et al., '58; Gitler et al., '58; Tsen and Collier, 60a) and/or hydrogen peroxide (Rose and György, '52; György and Rose, '48). The latter compound has been proposed as a common intermediate (György and Rose, '49); however, much larger concentrations of hydrogen peroxide are required (Rose and György, '52). In addition, species differences do exist. Christensen et al. ('56) have shown that the erythrocytes from vitamin E deficient chickens hemolyze with difficulty with dialuric acid but are easily hemolyzed by hydrogen peroxide. Rose and György ('49) have also added the autoxidizable compounds of cysteine, glutathione, ascorbic acid, and a cysteine-alloxan combination to the list of chemicals which will cause erythrocyte hemolysis in tocopherol deficient animals. This supports their belief that the reduction of alloxan to dialuric acid is due to the action of sulfhydryl compounds (Rose and György, '50).

Tsen and Collier (60b) noted the hemolytic action of various organic and heavy-metal sulfhydryl reagents. P-chloromercuribenzoate, mercuric chloride and silver nitrate caused virtually complete hemolysis without affecting the level of free glutathione in the cells. On the other hand, iodoacetate and N-ethylmaleimide caused little or no hemolysis, but reduced the concentration of glutathione to low levels. Since hemolysis was not related to the level of reduced glutathione, they have postulated that a reaction between the sulfhydryl of the stroma protein and the heavy-metal reagents was responsible for the hemolysis.

Horwitt et al. ('56) in applying the 2-thiobarbituric acid color test for lipid peroxides to the stroma of human erythrocytes found that on treatment with hydrogen peroxide, the stroma from individuals on tocopherol deficient diets had a much higher level of lipid peroxides than those from normal people. This was found to be true when dialuric acid was substituted for hydrogen peroxide in rats in vivo and in vitro (György and Rose, '48; Rose and György, '52). Nitowsky and Tilden ('56) noticed that various antioxidants, including tocopherol, inhibited both hemolysis and lipoxidase activity in vitamin deficient infants and children. On the basis of this, they suggest that tocopherol may play a role in maintaining erythrocyte integrity.

It has been postulated that these various compounds hemolyze erythrocytes through a catalytic mechanism whereby peroxidation of the unsaturated lipid portion of the cell membrane is accelerated. Supposedly, free radicals are formed during the initial stages of the autoxidation of these compounds, and the free radicals then initiate the peroxidation. Tsen and Collier's ('60a) experimental data show that erythrocytes from tocopherol deficient rats do not show increased levels of lipid peroxides until they have been treated with an oxidizing agent. This indicates that it is a deficiency of tocopherol as an antioxidant rather than as a vitamin, which is responsible for the hemolytic lesion.

Hove ('55) gives a more detailed outline for this behavior. His theory is that the tissue unsaturated fats, which may be oxidized by metal-porphyrin catalysts such as myoglobin or hemoglobin, pass through

various intermediate peroxides to the relatively stable hydroperoxide (Dubouloz et al., '51). Further catalytic disruption occurs to form active oxygen and other miscellaneous products. A heme-pigment (Haurowitz et al., '41) isolated from liver will catalyze the breakdown of lipid peroxides. Both of these heme-catalyzed steps are capable of being inhibited by vitamin E (Hove, '43; Hove and Hove, '44; Chang and Watts, '49; Tappel, '53). Therefore, any substance which promotes the heme-catalyzed reactions may be considered as a vitamin E antagonist. Obviously an increase in dietary unsaturated fat would do this unless a counteracting increase in vitamin E consumption occurred. Dubouloz and Fondarai ('53) believe that the pathological disturbances in animals deprived of vitamin E are due to the lipid peroxides acting directly to oxidize sulfhydryl groups which in turn disrupt various enzyme systems. The work of Hove ('55), Tsen and Collier ('60a), and Alfin-Slater ('60) tend to support the theory that this action may be enzymatic.

Although many enzymes are found to be inactivated when their sulfhydryl groups are blocked or oxidized, the sulfhydryl groups show varying degrees of reactivity (Neurath and Bailey, '53). Glutathione, cysteine, H_2S , and HCN are known to activate these enzymes, but the mechanism of the reaction is still obscure (West and Todd, '59). These activating agents may remove metal inhibitors (West and Todd, '59) or reduce $-S-S-$ to $-SH$ (Hellerman, '37). As a result of their varying degrees of reactivity, the sulfhydryl groups of any one protein can often be divided into different categories according to the reactivity

toward one reagent or another; and in different proteins, the sulfhydryl groups essential for enzyme activity may also belong to different categories (Neurath and Bailey, '53).

It has been noted that under proper conditions, inactivation of an enzyme system by oxidation of sulfhydryl groups can be overcome by reduction (Neurath and Bailey, '53). The oxidation-reduction status of sulfur amino acids can be determined by measuring the level of free sulfhydryl found in the blood (West and Todd, '59).

As a constituent of blood proteins, the sulfur amino acids do not fit into a definite pattern. There are considerable differences in the absolute quantities and relative proportions of sulfur, cystine, and methionine in globins and hemoglobins of different species. This is distinctly different from the relatively small species differences noted in other amino acids. However, the proteins of the erythrocyte stroma do not appear to exhibit any distinct species specificity with respect to sulfur and cystine (Black and Bolling, '51). By measuring the circulating sulfhydryl in the blood to determine the oxidation-reduction status of the sulfur amino acids as they are metabolized, it may be possible to demonstrate the influence of organic and inorganic sulfur on the blood picture.

METHODS

PREPARATION OF DIETS

The present investigation was designed to evaluate possible interrelationships between tocopherol and sulfur metabolism. Primarily the problem was to establish if tocopherol influenced the metabolism of neutral and/or inorganic sulfur. Therefore, four groups of diets patterned after that of Caputto et al. ('58) were used; three of these contained essentially the same level of sulfur, calculated as sulfate, but the ratio of inorganic or organic sulfur was varied. Reference to Table I will give the composition of the diets in detail; it may be observed (Table II) that one of these diets, group C, composed of diets C and C_E, contained virtually no inorganic sulfate and was low in organic sulfate.

In order to vary the inorganic sulfate, modifications of the Hubbell-Mendel-Wakeman ('37) salt mixture were devised using $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ as a partial source of calcium and a primary source of sulfur. The three mixtures developed are listed in Table III. Because there was some question as to the toxicity of the mixtures due to the partial substitution of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ for CaCO_3 , a preliminary test was run on weanling rats. Animals on Hubbell-Mendel-Wakeman ('37) and Osborne and Mendel ('19) salt mixtures were used as controls. The data which are presented in Table IV show no difference in growth weight or food efficiency regardless of salt mixture incorporated into the diets.

Preliminary investigations have indicated that the laboratory

TABLE I
COMPOSITION OF THE DIETS

Component	Quantity per 100 grams			
	A & A _E	B & B _E	C & C _E	D & D _E
	gms	gms	gms	gms
Casein (vitamin free)	10	10	10	10
Oxidized casein ¹	5	5	5	5
DL Tryptophan	0.160	0.160	0.160	0.160
DL Methionine	0	0.500	0	0.500
Sucrose	32	32	32	32
Cornstarch	34	34	34	34
Stripped lard	4	4	4	4
Cod liver oil	2	2	2	2
Alphacel	10	10	10	10
Vitamin mixture ²	1	1	1	1
Salt mixture ³	No. 1	3	-	-
	2	-	3	-
	3	-	-	3

¹Toennies, '47.

²Synthetic vitamins added as supplement to each 100 grams of diet: (in milligrams) alpha-tocopherol acetate, 28.0; nicotinic acid, 20.0; pyridoxine-HCl, 0.5; thiamine-HCl, 0.5; riboflavin, 0.5; calcium pantothenate, 1.0; folic acid, 0.5; biotin, 0.005; 2-methylnapthoquinone, 0.025; vitamin B₁₂, 0.0045; choline chloride, 100.0; inositol, 100.0; PABA, 7.5; vitamin A, 400 I. U.; calciferol, 200, I. U. The vitamins were triturated with sufficient sucrose to make 1 gram. Vitamin E was omitted from one mixture. When the vitamin E deficient vitamin mixture was used, the diets were designated A_E, B_E, C_E, and D_E.

³See Table III.

TABLE II
CALCULATED LEVELS OF DIETARY SULFATE

Diets	Quantity of inorganic SO_4 per cent	Quantity of organic SO_4 per cent
A and A_E	0.42	0.25
B and B_E	0.10	0.57
C and C_E	0.00	0.25
D and D_E	0.00	0.57

TABLE III
SALT MIXTURES

Component	#1 14.00% SO_4 gm	#2 3.34% SO_4 gm	#3 0.007% SO_4 gm
CaCO_3	30.346	41.250	44.750
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	25.097	6.000	0.000
Cornstarch	0.000	8.193	10.693
MgCO_3	3.060	3.060	3.060
NaCl	6.900	6.900	6.900
KCl	11.200	11.200	11.200
KH_2PO_4	21.200	21.200	21.200
$\text{FePO}_4(2\text{H}_2\text{O})$	2.050	2.050	2.050
KI	0.008	0.008	0.008
NaF	0.010	0.010	0.010
$\text{MnCl}_2(4\text{H}_2\text{O})$	0.040	0.040	0.040
$\text{AlK}(\text{SO}_4)_2$	0.017	0.017	0.017
$\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$	0.072	0.072	0.072

TABLE IV
GROWTH RECORD AND ESTIMATED FOOD EFFICIENCY RATIOS
USING MODIFIED SALT MIXTURES

	O & M		H M W			14.7% SO ₄			3.34% SO ₄		
	♀	♂	♀	♂	♀	♀	♂	♀	♀	♂	♀
Initial weight grams	43	33	40	50	38	42	50	42	48	53	38
Final weight grams	145	182	143	167	203	140	176	198	162	171	205
Weight gain grams	102	149	103	114	165	90	126	156	114	118	167
Food consumed grams	359	416	350	368	434	396	375	417	357	373	445
Food efficiency ratios	0.32		0.38			0.32			0.33		

stock diet is high in its tocopherol content. As an economy measure, the animals were placed on a vitamin E restricted diet approximately one month prior to the beginning of the experiment. It was hoped that this would lower the animals' vitamin E stores and result in a more rapid onset of avitaminosis E during the experimental period. The detailed composition of this diet is listed in Table V.

HEMOLYSIS OF ERYTHROCYTES

The procedure of Friedman et al. ('58) was chosen for estimating the susceptibility of erythrocytes to chemical hemolysis. Briefly, the erythrocytes were washed with an isotonic saline solution, resuspended in an isotonic saline-phosphate buffer solution composed of equal parts isotonic saline and 0.1 M phosphate buffer (pH 7.4), and incubated with dialuric acid. The dialuric acid solution was prepared by dissolving 10 mg of finely ground dialuric acid in 50 ml of the saline-phosphate buffer solution immediately before use.

Thirty to forty lambda of blood was drawn from a cut across the tail vein of the rat with a hemoglobin pipette. This was added directly to 5 ml of the saline-phosphate buffer solution in a 15 ml glass centrifuge tube. The blood was mixed with the solution by twirling gently, followed by centrifugation at 2,000 rpm. The supernatant liquid was drawn off, and the cells resuspended in approximately 4.5 ml of saline-phosphate buffer solution. One ml of the suspension was pipetted into each of three test tubes. To tubes one and two, 1 ml of dialuric acid solution was added; to tube three, 1 ml of saline-phosphate buffer

TABLE V
RESTRICTED VITAMIN E DIET

Component	Grams/100 grams of diet
Casein	15
Sucrose	28
Cornstarch	28
Alphacel	10
Brewer's yeast	10
Lard	3
Cottonseed oil ¹	3
Salt mixture ²	3

¹Contained 16,000 I. U. vitamin A and 8,000 I. U. vitamin D per 120 grams.

²Hubbell et al., '37.

solution. The tubes were incubated in a 37° C. water bath for one hour; then removed and left standing at room temperature for another hour. At the end of this time, 5 ml of saline-phosphate buffer solution was added to tubes one and three, and 5 ml distilled water, to tube two. The tube contents were mixed gently by twirling, and centrifuged for five minutes at 2,000 rpm on an International No. 3. The supernatant liquid was poured into cuvettes for reading in the Klett-Summerson Photometer. A blue filter, No. 42, was used, and optical density was set at zero with a distilled water blank. Per cent hemolysis was calculated with the following formula using the Klett readings obtained for each tube.

$$\text{Per cent hemolysis} = \frac{\text{Tube 1} - \text{Tube 3}}{\text{Tube 2} - \text{Tube 3}} \times 100$$

CIRCULATING SULFHYDRYL

Ellman's method ('58) for the determination of circulating sulfhydryl was followed with slight modification. The mechanism of this method depends on the reaction of sulfhydryl with bis-(p-nitrophenyl)-disulfide to form a mixed disulfide and release a molecule of p-nitrophenylthiol. This compound is measured at pH 8.0 with a blue filter (No. 42) in the Klett-Summerson Photometer. It is necessary to maintain the pH at 8.0, because at higher pH's the bis-(p-nitrophenyl)-disulfide is dissociated spontaneously, while at lower pH's, the p-nitrophenylthiol formed will recombine.

The modification employed used a 0.1 M Tris buffer of pH 8.0 to control the pH, rather than a phosphate buffer, the latter being less stable in the acetone solutions.

Twenty lambda of blood drawn from a cut across the tail vein of the rat with a hemoglobin pipette was immediately placed in a cuvette containing 4 ml distilled water. Earlier a concentration of ten lambda of blood to 4 ml of distilled water had been used; however, the higher concentration seemed to be better suited to analysis with the Klett-Summerson Photometer. Two ml of the Tris buffer, 3 ml acetone, and 1 ml of the bis-(p-nitrophenyl)-disulfide solution were added in that order. The tubes were then centrifuged at 2,000 rpm for approximately five minutes to remove the precipitated hemoglobin, wiped clean, and read in the Klett-Summerson Photometer. Optical density was set at zero with a distilled water blank.

With the measurement of sulfhydryl being performed on blood, a standard curve would be of little value since the nature of the mercapto groups in blood is not definitely known. Therefore, all values were calculated from the extinction coefficient given by Ellman ('58). According to Ellman, the concentration of sulfhydryl in moles per liter of the solution in the cuvette may be calculated from the formula

$$C = A/13,600$$

where C equals moles per liter and A equals optical density. Using this formula to measure the sulfhydryl in human blood, Ellman obtained values for different subjects ranging from 10 to 19 meq per liter, with the average value being 14.5 meq per liter. The formula given in the handbook for the Klett-Summerson Photometer to convert from optical density to Klett readings is

$$\frac{1000 \times A}{2} = \text{Klett reading.}$$

Therefore, $A = 2 \text{ KR}/1000 = \text{KR}/500.$

Substituting this value of A into Ellman's equation, one obtains

$$C = \text{KR}/500/13,600 = \text{KR}/6.5 \times 10^6.$$

Since the concentration of sulfhydryl is to be expressed in millimoles per liter, this expression can be further reduced to

$$C = \text{KR}/6.5 \times 10^3 = \text{Conc. millimoles per liter.}$$

However, since 10 ml of solution equals only 0.02 ml of blood in the first case or 0.01 ml in the latter, the equation becomes

$$C = \text{KR}/13 = \text{meq/liter with 0.02 ml blood}$$

$$C = \text{KR}/6.5 = \text{meq/liter with 0.01 ml blood.}$$

ERYTHROCYTE INCORPORATION OF S^{35}

The procedure of Ågren and Engström ('56) was followed for collecting the erythrocyte membrane. The blood was withdrawn from the animals by cardiac puncture into 10 ml of a 2.9 per cent, isotonic, sodium citrate solution. This was centrifuged ten minutes at 2,000 rpm and the supernatant drawn off, followed by two washings with isotonic saline. The cells were hemolyzed with distilled water, and the stroma collected by centrifugation at $15,000 \times G$ for 20 minutes. After two washings with distilled water, the samples were treated according to the method of Katz and Golden ('59) for the analysis of radioactive sulfur in biological materials. A combustion mixture, composed of two volumes concentrated nitric acid added to one volume 70 per cent perchloric acid and containing 10 mg copper nitrate per ml of total mixture, was added to the extracted material at a level of 8 to 10 ml.

The samples were digested in 50 ml Erlenmeyer flasks on a hot plate until they were evaporated down to 2 to 3 ml. One ml of a sulfate standard equivalent to 12 mg BaSO_4 , 10 ml distilled water, and 1 ml of 10 per cent BaCl_2 were added to the digestion flasks. The resultant precipitate of BaSO_4 was filtered by vacuum onto weighed glass fiber filter paper disks, 24 mm in diameter, held between a perforated hard rubber disk and a glass funnel made from the neck of a catsup bottle. The precipitates were washed successively with about 10 ml distilled water, 0.5 N HCl, 95 per cent ethanol, and acetone, and allowed to air dry. The plated filter papers were weighed and counted with a thin-window G.M. tube and a Nuclear 64 Scaler.

PROCEDURE

Forty adult albino rats of the Wistar strain were used in the experiment. The males were housed in individual wire-bottom cages; the two females on each diet were caged together. Distilled water and food were provided ad libitum. At the beginning of the experimental period the animals were placed in metabolism cages, and forty-eight hour urine samples collected, acidified, and frozen for future analyses.

Ten litter mates were placed on each experimental diet. Five of them received adequate amounts of tocopherol while the remaining five were depleted of vitamin E.

The entire experiment ran for sixty-five days. Nine days after the beginning of the dietary regimen, sulfhydryl and hemolysis readings were taken. Thereafter, readings were recorded three days weekly. A

total of thirty-four analyses per group was made for circulating sulfhydryl, and thirty-three for hemolysis.

At the end of the first month, the stripped lard was increased to six per cent in all the diets in an effort to bring about hemolysis. Ten days later the cod liver oil was increased to four per cent in the A and the B diets, again in an attempt to promote hemolysis. The corn-starch and sucrose were adjusted accordingly. The cod liver oil in the C and the D diets was not increased since the data from the sulfhydryl readings had become sufficiently interesting that it did not appear necessary to force the lesion.

At the end of the experimental period, the animals were again placed in metabolism cages, and forty-eight hour urine samples collected, acidified, and frozen for future analyses. Following the urine collection, the rats were injected with four microcuries of S^{35} as $Na_2S^{35}O_4$ in 0.6 ml isotonic saline solution and fasted twenty-four hours. Sulfhydryl and hemolysis data were collected on the injected rats before they were sacrificed by cardiac puncture. Eight rats at a time, litter mates from each group, were successively put through this procedure. In addition, the organs, skin, and rib cartilage were collected and frozen for future analyses.

RESULTS AND DISCUSSION

As was stated earlier, the purpose of this investigation was to evaluate the interrelationship of tocopherols and sulfur metabolism. Although urine samples were collected, both at the beginning and end of the experimental period for determination of taurine, sulfate, and urea, time available for this investigation would only permit analysis of the blood for sulfhydryl level, per cent chemical hemolysis, and incorporation of $S^{35}O_4^{--}$ into erythrocyte stroma. Therefore, these are the data presented.

Since the hemolysis determinations were quite variable, it did not seem reasonable to average them. The highest values obtained for each animal are listed in Table VI. It should also be pointed out that these values may have occurred early in the experiment. As may be observed in Table VI, the extreme values are quite wide, with the most consistent data being obtained for those rats consuming dietary groups C and D. Although the data cannot be treated statistically, there is a suggestion that the erythrocytes of the animals consuming diets C and D may be more susceptible to hemolysis by dialuric acid than those of animals consuming diets adequate in inorganic sulfate. This suggestion is given even stronger support by the fact that the cod liver oil level of these diets was not increased as it was in diet groups A and B. An explanation for the failure of the erythrocytes to hemolyze consistently is not immediately apparent. The fluctuation in per cent hemolysis with each animal indicates either accidental addition of vitamin E to the diet or contamination of the blood during

TABLE VI
HIGHEST PER CENT HEMOLYSIS OBTAINED PER ANIMAL

Sex	Diet groups							
	A _E	A	B _E	B	C _E	C	D _E	D
♂	18.0	29.7	29.1	24.5	6.4	6.7	37.3	5.4
♂	42.0	6.4	8.3	11.7	31.2	22.8	49.4	31.7
♂	14.5	14.9	19.9	25.5	32.9	15.0	16.2	13.5
♀	9.5	27.6	24.4	25.7	40.5	6.5	34.6	23.0
♀	22.3	10.6	15.9	17.5	10.2	7.5	17.4	19.2
Average ♂	24.8	17.0	19.1	20.7	23.5	14.8	34.3	16.7
Average ♀	15.9	19.1	20.2	21.6	25.4	7.0	26.0	21.1
Total average	21.3	17.8	19.5	21.0	28.2	11.7	31.0	18.7

analysis. Friedman et al. ('58) have observed that contact of the blood with anything other than glass would prevent hemolysis. However, since elaborate precautions were taken both with diet preparation and blood handling technique, these thoughts do not explain the discrepancies satisfactorily. On the contrary, other investigations have shown that erythrocytes from the rats of this particular colony hemolyze with difficulty. Vitamin E deficient rats of other colonies consuming similar diets have exhibited 98 per cent erythrocyte hemolysis in six weeks (Tsen and Collier, '60a).

The sulfhydryl data are presented in Table VII. The significant deviations of the D diets from the pattern observed for the other dietary groups gives cause for interesting speculations. The author is cognizant of the fact that this method of analysis may reflect only the oxidation-reduction status of the blood; however, such a consideration should not invalidate any between- or within-group comparisons. It was also felt that these values might merely reflect changes in blood hemoglobin level of rats consuming different diets; therefore, this was checked by concomitant hemoglobin-sulfhydryl measurements during the initial stages of the experiment. When it became apparent that there was no correlation between hemoglobin measurements and sulfhydryl levels, the hemoglobin determinations were discontinued.

The data which are reported in Table VIII give the specific activity of the sulfur of the erythrocyte membrane in counts per minute per millimole. All of the data which have been reported are summarized in Table IX. Since the animals on any dietary group were paired as to

TABLE VII
 MILLEQUIVALENTS OF SULFHYDRYL/LITER PER ANIMAL
 AVERAGES OF THIRTY-FOUR ANALYSES

Sex	Diet groups							
	A _E	A	B _E	B	C _E	C	D _E	D
♂	25.2	25.8	22.5	22.5	21.4	22.6	23.3	20.0
♂	27.5	25.8	25.6	26.2	23.3	24.6	22.8	20.8
♂	22.2	23.3	22.7	23.3	25.0	23.7	26.2	18.5
♀	23.5	25.3	25.2	24.5	24.0	25.6	22.8	20.8
♀	26.1	27.7	24.2	24.2	24.6	24.2	25.5	24.5
Average								
♂	25.0	25.0	23.6	24.0	23.2	23.6	24.1	19.8
Average								
♀	24.8	26.5	24.7	24.4	24.3	24.9	24.2	22.6
Total average	24.9	25.6	24.0	24.1	23.7	24.1	24.1	20.9

TABLE VIII
ERYTHROCYTE INCORPORATION OF S^{35} PER ANIMAL
IN CPM/MILLIMOLE OF SO_4

Sex	Diet groups							
	A_E	A	B_E	B	C_E	C	D_E	D
♂	259	653	213	312	184	854	536	197
♂	288	461	265	290	284	173	757	214
♂	354	460	204	244	318	472	132	178
♀	313	605	144	310	947	392	408	92
♀	327	254	462	250	455	670	323	147
Average								
♂	320	430	303	280	701	531	365	120
Average								
♀	300	524	227	282	262	500	475	196
Total average	308	486	257	281	437	512	431	165

TABLE IX
SIGNIFICANCE OF DATA¹
CIRCULATING SULHYDRYL - ERYTHROCYTE INCORPORATION OF S³⁵O₄--
ERYTHROCYTE HEMOLYSIS

Diet groups	Analyses					
	Average	P	Average S ³⁵	P	Average	P
	sulphydryl meq/l		incorporation cpm/millimole		hemolysis per cent	
A _E	24.9		308		21.3	
	± 0.0699*	>0.05	± 79.90*	<0.1>0.05		>0.05
A	25.6		486		17.8	
B _E	24.0		258		19.5	
	± 0.0644*	>0.05	± 63.36*	>0.05		>0.05
B	24.1		281		21.0	
C _E	23.7		438		28.2	
	± 0.0614*	>0.05	± 164.00*	>0.05		>0.05
C	24.1		512		11.7	
D _E	24.1		431		31.0	
	± 0.0685*	<0.01	± 16.78*	<0.05>0.02		>0.05
D	20.9		166		18.7	

¹Snedecor, '40.

$$\Sigma d \quad \Sigma d^2 \quad \text{A.M.D.}$$

$$\sigma M \sqrt{\frac{\Sigma d^2 - (\Sigma d)^2/N}{N(N-1)}}$$

$$t = \frac{\text{A.M.D.}}{\sigma M}$$

*Standard deviation of the mean

sex and litter mates, the data were subjected to statistical analysis based on significance of mean difference of values between animals in a group. These data show a highly significant difference between the sulfhydryl levels of the animals in the D dietary group receiving vitamin E supplementation and those not supplemented. Of interest is the observation that the highest sulfhydryl values are associated with those animals consuming group A diets high in inorganic sulfate. However, there is no within-group difference with respect to vitamin E supplementation in this or the B and C dietary groups.

Also of interest is the significant effect of vitamin E upon the sulfate incorporation into the erythrocyte membrane of those animals in the dietary groups A and D. In the first case, vitamin E is apparently necessary for the optimum incorporation of S^{35} into the erythrocyte membrane, while in the case of the D animals, it appears to be inhibitory. Sufficient, apparently contradictory, data have now been presented to make the picture appear hopelessly confusing. However, before discussing the data further, it may be helpful to review the metabolism of the sulfur-containing amino acids.

It has become well documented (du Vigneaud et al., '42; Binkley et al., '42; Binkley and du Vigneaud, '42; Stetten, '42; Binkley, '44; du Vigneaud et al., '44) that methionine, the one dietary difference between the C and D groups, is rapidly converted to cysteine in the body. According to research observations, the mechanism by which cysteine and cystine are formed from methionine is demethylation of methionine to homocysteine, reaction of homocysteine with serine to

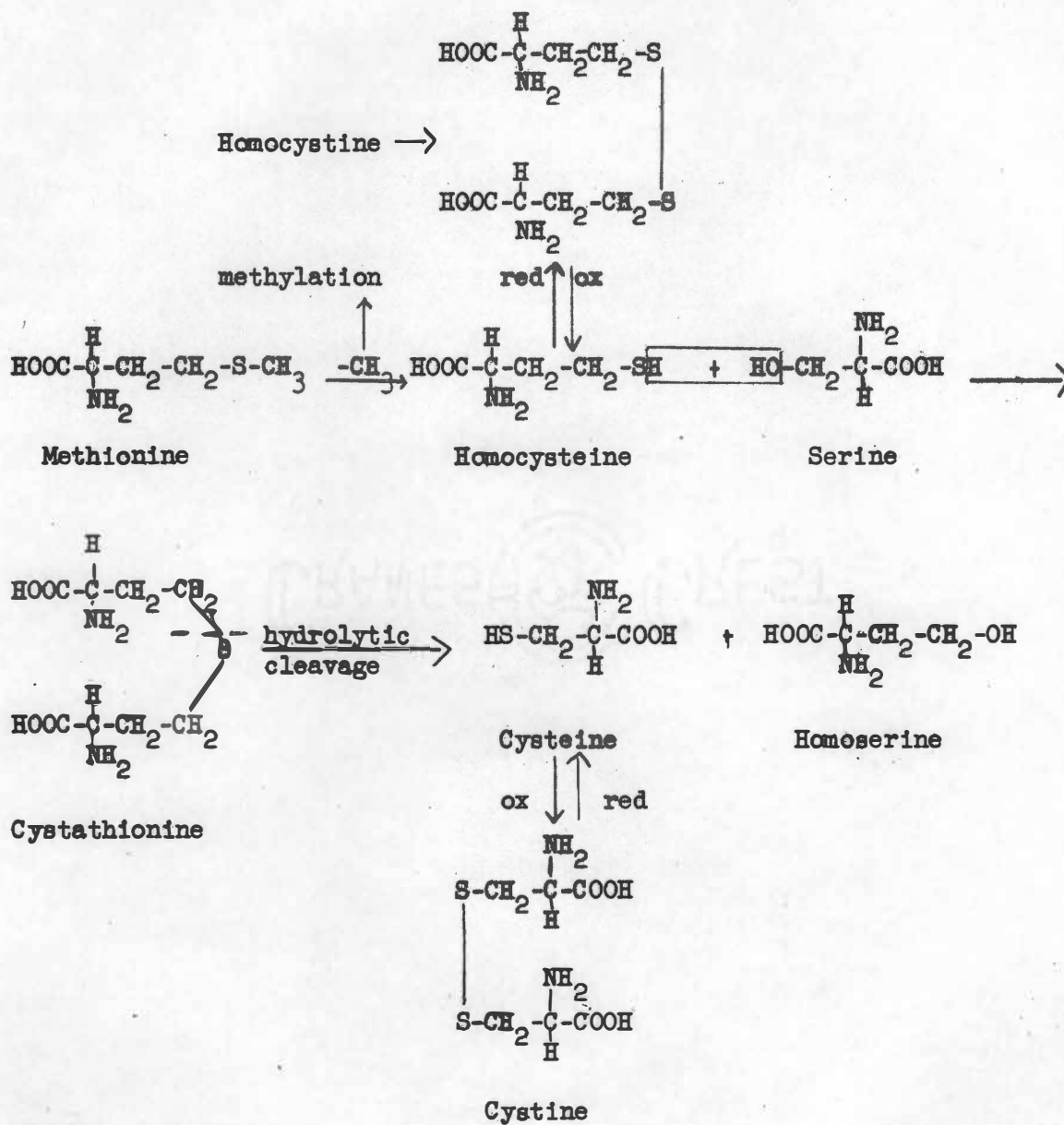
form cystathionine, and hydrolytic cleavage of cystathionine to yield cysteine and homoserine. The reactions shown in Figure I are representative of the process.

Once cysteine has been formed, it goes through another series of reactions until it is converted into sulfate and excreted in the urine. Consumption of methionine and/or cystine is followed by a prompt excretion of excess sulfur in the urine. Eighty per cent of urinary sulfur occurs as sulfate. The mechanism of the further metabolism of the sulfur amino acids following the conversion to cysteine has been elucidated by a number of investigations.

Lewis ('24; '35) has demonstrated that cystine is reduced to cysteine preliminary to the deamination of the latter followed by oxidation of the sulfhydryl group to give cysteinesulfinic acid. That cysteinesulfinic acid is formed in vivo from cystine has been proven by Chapeville and Fromageot ('55). Awapara and Doctor ('55) present evidence, using cysteine- S^{35} , for the enzymatic oxidation of cysteine- S^{35} to cysteinesulfinic acid- S^{35} . Awapara ('50) has shown that alanine and taurine appear in rat livers following their injection with cysteine. After administering S^{35} -labeled cysteine to rats, Awapara and Wingo ('53) found S^{35} -labeled 2-aminoethanesulfinic acid, proving that a decarboxylation of cysteinesulfinic acid results in the formation of taurine rather than sulfate. The investigations of Singer and Kearney ('56) have revealed the presence of enzymes in animal tissues which act upon cysteinesulfinic acid through transamination to convert it to β -sulfinylpyruvate. This is then converted to pyruvate and sulfite. The conversion of sulfite to sulfate has remained rather obscure, but

FIGURE I¹

FORMATION OF CYSTEINE AND CYSTINE FROM METHIONINE

¹West and Todd, '59.

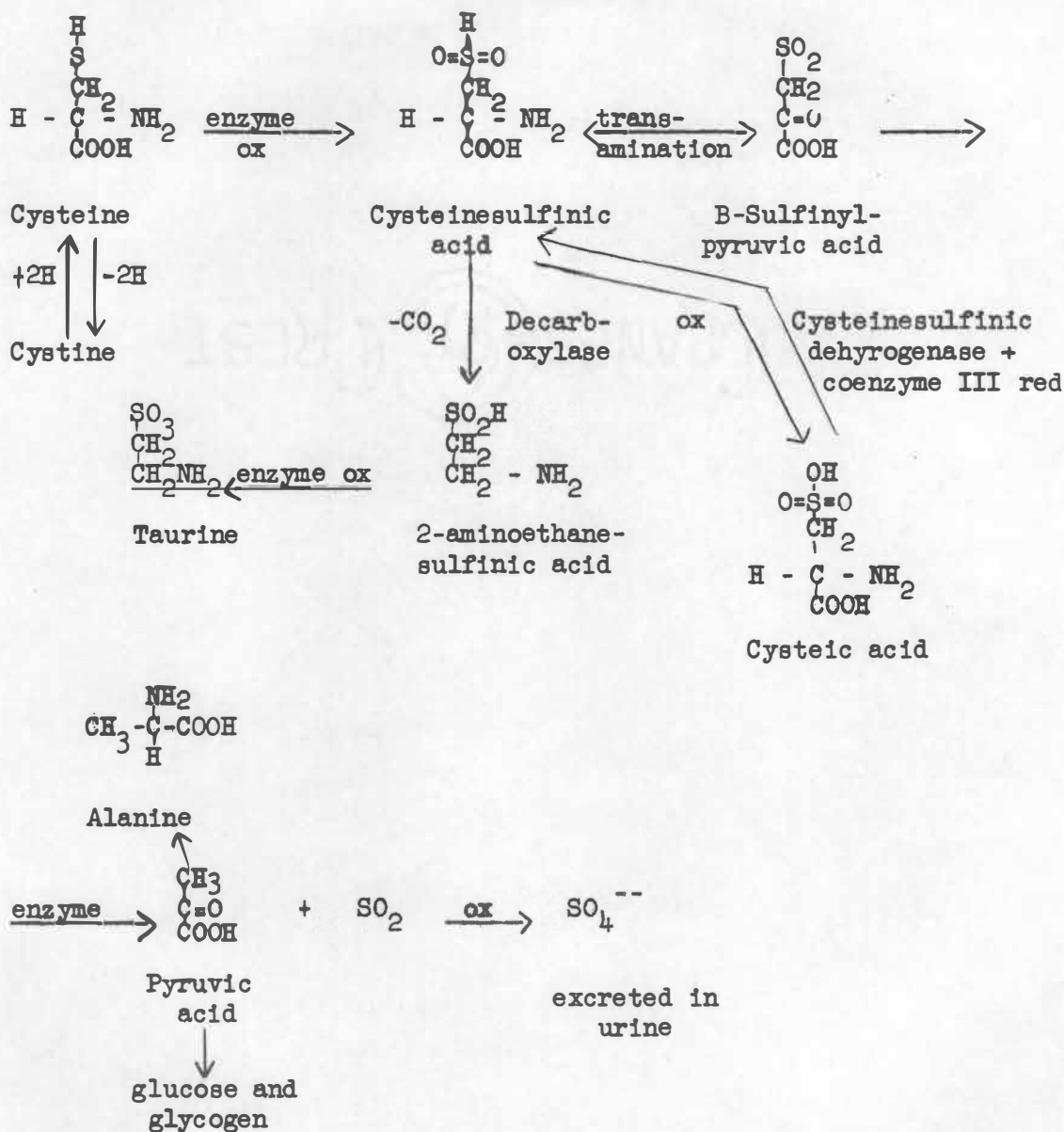
the recent work of MacLeod et al. ('61) has elucidated this somewhat. They have isolated a hepatic enzyme, a hemoprotein, which rapidly oxidizes sulfite to sulfate. The sequence of reactions shown in Figure II represents the metabolism of cysteine to sulfate.

One may now re-examine the data which have been presented. It seems that the data best fit the assumption that vitamin E is necessary for optimal conversion of cysteine to sulfate. If this assumption be true, one might find an increase in the sulfhydryl level of those animals on diets deficient in vitamin E and inorganic sulfate, but supplemented with high levels of methionine or cysteine. The diets of the D group are low in inorganic sulfate and are supplemented with 500 mg of methionine per 100 gms of diet. It is apparent from the data presented in Table IX that the vitamin E deficient animals in this group have a statistically significant higher level of sulfhydryl than their controls. It is further significant that this phenomenon is not observed in any of the rats consuming the other diets. As was pointed out earlier, metabolism of cysteine to sulfate is not necessarily an obligatory pathway since decarboxylation of cysteinesulfinic acid will yield taurine. Therefore, the effect of vitamin E on the conversion of cysteine to sulfate may be masked in the case of the animals in the B dietary group, the other diet with added methionine, since this diet contains adequate inorganic sulfate. Those animals consuming A and C group diets with no added methionine do not have an excess to convert, so would not be expected to show a vitamin E effect. It is of interest, however, that those animals in the A dietary group show a higher

FIGURE II¹

CONVERSION OF CYSTEINE TO TAURINE AND SULFATE

Methionine, Homocystine, Homocysteine

¹West and Todd, '59.

sulfhydryl level than animals in any of the other groups, perhaps the result of the displacement of the equilibrium away from the conversion to sulfate. If the above assumption were true in the case of those animals consuming the D diets, the sulfate pool in the vitamin E sufficient should be larger than that in the vitamin E deficient animals since they are consuming virtually no inorganic sulfate. If these animals were given radioactive sulfate of equal specific activity, the specific activity of the sulfate should decrease in the vitamin E sufficient animals as a result of dilution by the sulfate pool. Therefore, although the sulfate might be incorporated into the erythrocyte stroma at a more rapid rate in the vitamin E supplemented animals, as indicated by the data for the animals on all the other diets, the measured result, specific activity of sulfate, would be an apparent inhibition of incorporation due to vitamin E because of the incorporation of the sulfate of lowered specific activity in the vitamin E sufficient animals.

Tallan ('55), Azzone et al. ('57), and Ryerson et al. ('58) have shown that a deficiency of vitamin E causes an increase in the glutathione levels of the liver, muscle, and erythrocytes of rabbits. Since this tripeptide contains cysteine as an integral unit, cysteine, or methionine which is convertible to cysteine, is required for the synthesis of glutathione. This increased erythrocyte glutathione could be the result of a pile-up of sulfhydryl in the form of cysteine which was not being converted rapidly enough to sulfate due to a deficiency of vitamin E. Although Tsen and Collier ('60a) were unable to observe

this phenomenon in the erythrocytes from vitamin E deficient rats, their work further strengthens the observations reported in this investigation. It may be recalled that the vitamin E effect on the conversion of cysteine to sulfate was not demonstrable when the animals were consuming diets adequate in inorganic sulfate.

With the postulation of the hypothesis that a deficiency of vitamin E interferes with the conversion of cysteine to sulfate, it becomes necessary to pursue further investigations based on the information now at hand. If analysis of the urine samples from these animals for taurine and sulfate should reveal a lowered level of sulfate in the case of vitamin E deficient animals and an increased level of taurine, the hypothesis proposed in this thesis would assume even greater import. It might also be interesting to investigate the phenomenon in other species especially as it pertains to erythrocyte glutathione.

SUMMARY

This study entailed an investigation of the interrelationship of tocopherols and sulfur metabolism through the analysis of the blood for circulating sulfhydryl, per cent chemical hemolysis, and incorporation of $S^{35}O_4^{--}$ into erythrocyte stroma. Albino rats were maintained on diets varied both as to kinds and quantities of sulfur and presence or absence of dietary tocopherol. Blood analyses were made throughout the experimental period for per cent hemolysis and circulating sulfhydryl. At the end of the investigation, the animals were injected with $Na_2S^{35}O_4$, fasted twenty-four hours, and terminated by withdrawing the blood by cardiac puncture. The erythrocytes were then treated in such a way as to make it possible to count their stroma lipoprotein for incorporation of radioactive sulfur. Part of the data was treated statistically.

The data on hemolysis were such that it could not be analyzed statistically, but a trend suggesting an increased susceptibility to dialuric acid hemolysis with variations in the lower levels of inorganic dietary sulfur was observed.

The data on the circulating sulfhydryl and the S^{35} incorporation into erythrocytes were complementary. Animals receiving no vitamin E and only methionine as the source of sulfur exhibited increased sulfhydryl values and decreased erythrocyte incorporation of S^{35} at highly significant levels. The converse was true for those animals receiving both methionine and vitamin E. Animals receiving no methionine, but extremely high levels of inorganic sulfate also exhibited a

significantly increased erythrocyte incorporation of S^{35} . The sulfhydryl levels of this latter group of animals were observed to be higher than that of any other dietary group.

CONCLUSIONS

Based on the data obtained from this investigation, it is possible to suggest that adequate dietary vitamin E plays a part in accelerating the conversion of cysteine to sulfate in the rat. This hypothesis is substantiated by the findings that insufficient dietary vitamin E results in increased circulating sulphydryl levels and erythrocyte incorporation of $S^{35}_{O_4}$ -- when the animals are receiving methionine as the only added source of sulfur.

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