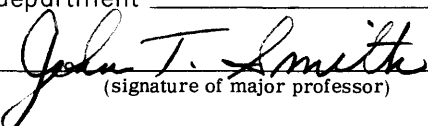


THE UNIVERSITY OF TENNESSEE
THE GRADUATE SCHOOL

ABSTRACT OF EDUCATIONAL RESEARCH STUDY COMPLETED

Author of Study Helen Raisty Rutledge Date December 27, 1966
Title of Study "Inhibition of Cysteine Oxidation in Vitamin E Deficiency"
Course Number 600
Under direction of what department Nutrition Degree Ph. D.
Abstract approved by  Quarter Graduating March 1967
(signature of major professor)

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 8 1/2 x 11 inches and of quality equivalent to that required in the case of the thesis. (Master's thesis - submit **one** single-spaced typed copy; doctoral dissertation - submit **two** double-spaced typed copies.)

Since previous results in this laboratory had indicated that vitamin E was necessary in vivo not only for the optimal sulfation of mucopolysaccharides, but also for the oxidation of neutral sulfur to sulfate, the ability of whole liver homogenates from vitamin E-sufficient and -deficient rats to convert cysteine to sulfate was investigated. Then an attempt was made to determine the specific site of the decreased oxidation of cysteine or its intermediate oxidation products. Since several alternative pathways have been proposed in animals for the ultimate oxidation of the sulfur of cysteine to sulfate and the use of the sulfate formed, it was necessary to limit this investigation to the most widely accepted pathway, that of the oxidation of cysteine to sulfate through cysteinesulfinic acid and sulfite. Low sulfate diets were employed to force the animals to satisfy their inorganic sulfate needs by the oxidation of the sulfur amino acids.

A statistically significant decrease in the conversion of ³⁵S-cysteine to ³⁵S-sulfate was found in whole liver homogenates from the

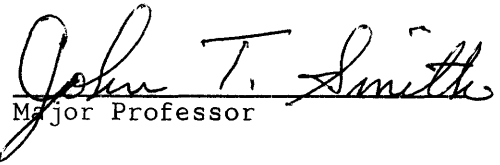
vitamin E-deficient male, but not the female, rats fed the low sulfate diets when these were compared to their littermate controls. When male and female rats fed normal sulfate vitamin E-sufficient diets were compared, there was no statistically significant difference in the conversion of ^{35}S -cysteine to ^{35}S -sulfate. There was a statistically significant decrease in the ability of mitochondrial homogenates from the vitamin E-deficient "prepped" female rats to convert ^{35}S -cysteine to ^{35}S -sulfate when these were compared with their littermate controls. No difference could be detected in the ability of acetone powder extracts from vitamin E-deficient and -sufficient male rats to oxidize sulfite to sulfate, as indicated by ferricyanide reduction. Tests were made to determine the presence of cysteinesulfinic acid and sulfite after incubation of cysteine with denucleated homogenates, but neither substance could be detected. The conversion of cysteine to a ninhydrin-positive material thought to be cysteinesulfinic acid by supernatant preparations of liver from vitamin E-deficient and -sufficient male and female rats was also studied in the presence of an inhibitor. There was no detectable difference in the ability of these preparations to oxidize cysteine.

A mechanism is proposed for the involvement of ATP in the oxidation of the sulfur group of cysteine to sulfate, since ATP, as well as vitamin E, is implicated in this conversion by results found during the course of this research.

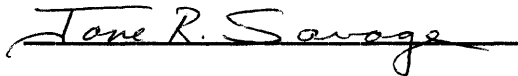
January 31, 1966

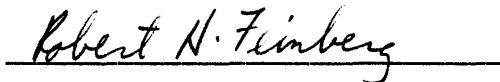
To the Graduate Council:

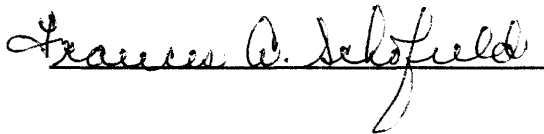
I am submitting herewith a dissertation written by Helen Raisty Rutledge entitled "Inhibition of Cysteine Oxidation in Vitamin E Deficiency." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.


Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the Council:

Dean of the Graduate School

INHIBITION OF CYSTEINE OXIDATION
IN VITAMIN E DEFICIENCY

A Dissertation
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Helen Raisty Rutledge
March 1967

ACKNOWLEDGEMENT

The author wishes to express her sincere appreciation to Dr. John T. Smith for his suggestion of this problem and for his help and criticism throughout its course, and to Dr. Frances A. Schofield, Dr. Mary Rose Gram, and Dr. Jane R. Savage for reviewing the manuscript.

The author also wants to thank the National Science Foundation for its support of the project throughout most of its course.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION.	1
II. REVIEW OF THE LITERATURE.	3
Vitamin E	3
Metabolites of vitamin E.	3
Interrelationships between vitamin E and other factors. . .	9
Ascorbic acid	9
Nucleic acid, nitrogen, and phosphorus metabolism . . .	9
Hemolysis of erythrocytes	13
Erythrocyte survival.	15
Growth in the rat	15
Reproduction in the rat	16
Other conditions in the rat	17
Sulfur and selenium metabolism and peroxidation	18
Membrane permeability	26
Poultry nutrition	28
Respiratory chain-electron transport.	31
Oxygen consumption.	33
Xanthine oxidase and coenzyme A levels.	36
Determination of vitamin E.	37
Evaluation of vitamin E status.	37
Transport and storage of vitamin E.	38

CHAPTER	PAGE
II. (CONTINUED)	
Sulfur metabolism	38
Absorption.	39
In vivo studies	39
Balance experiments	39
Feeding and injection studies	41
In vitro studies.	53
Cystathionine as substrate.	53
Cysteine as substrate	56
Cysteinesulfinic and cysteic acids as substrates.	60
Sulfite as substrate.	62
Sulfate and PAPS as substrates.	63
Non-enzymic reactions	65
Methods	67
III. EXPERIMENTAL.	69
General Plan.	69
Diets	69
Animals	73
Methods	75
Preparation of enzyme solutions	75
The whole liver homogenates	75
The liver fractions	75
The denucleated homogenates	77

CHAPTER	PAGE
III. (CONTINUED)	
The supernatant preparations.	77
The acetone powder extracts	77
The preliminary purification of the enzyme.	78
Enzyme assays	79
Oxidation of cysteine to sulfate.	79
Oxidation of sulfite to sulfate	79
Oxidation of cysteine to sulfite.	80
Oxidation of cysteine to cysteinesulfinic acid.	81
Oxygen uptake of preparations incubated with cysteine .	86
Measurement of ATP effect	86
Nitrogen or protein content of enzyme solutions	86
The micro-Kjeldahl method	87
The Lowry method.	88
Assay of erythrocyte sensitivity to dialuric acid	88
Statistics.	90
IV. RESULTS	91
Effect on Animals	91
Effect on Sulfur Metabolism	92
V. DISCUSSION.	107
VI. SUMMARY	118
LITERATURE CITED.	120

LIST OF TABLES

TABLE	PAGE
1. Composition of Experimental Diets.	70
2. Composition of Salt Mixture.	71
3. Growth and Feed Efficiency of Young Adult Male Rats Fed The Diet Without Cod-Liver Oil	93
4. Conversion of Cysteine to Sulfate by Liver Homogenates from Vitamin E-Sufficient and -Deficient Young Adult Male Rats. .	94
5. Conversion of Cysteine to Sulfate by Liver Homogenates from Vitamin E-Sufficient and -Deficient Adult Male Rats.	95
6. Conversion of Cysteine to Sulfate by Liver Homogenates from Vitamin E-Sufficient and -Deficient Adult Female Rats.	96
7. Conversion of Cysteine to Sulfate by Liver Preparations from Vitamin E-Sufficient and -Deficient Female Rats.	97
8. Conversion of Cysteine to Sulfate by Liver Homogenates from Vitamin E-Sufficient and -Defficient Male Rats Fed Normal Sulfate Diets with 18 Per Cent Casein.	99
9. Conversion of Cysteine to Sulfate by Liver Homogenates from Vitamin E-Sufficient Animals Fed a Normal Sulfate Diet . . .	100
10. Conversion of Cysteine to Cysteinesulfinic Acid by Supernatant Fractions from Vitamin E-Sufficient and -Deficient Rats. . .	102
11. Oxygen Uptake of Partially Purified Fractions.	104
12. Sample Data Suggesting An ATP Requirement for the Conversion of ³⁵ S-Cysteine to ³⁵ S-Sulfate	105

LIST OF FIGURES

FIGURE	PAGE
1. Interconversions of Tocopherol Metabolites	5
2. Possible Formation of ATP by Oxidation of Substituted Hydroquinones.	8
3. A Possible Relationship among Lipid Peroxidation, Tocopherol, Ascorbic Acid, Sulfhydryl Compounds, and NAD	20
4. Possible Mechanism for Respiratory Control by Oxidative Phosphorylation.	34
5. Summary of Possible Pathways for the Oxidation of the Sulfur of Cysteine to Sulfate	54
6. Non-Enzymic Cleavage of Cystine.	66
7. Conversion of Methionine to Cysteine	109
8. Conversion of the Sulfur of Cysteine to Sulfate.	110
9. Proposed Scheme of Conversion of Methionine to Cysteine. . . .	111
10. Proposed Conversion of the Sulfur of Cysteine to Sulfate . . .	112

CHAPTER I

INTRODUCTION

Many of the in vivo and in vitro functions of vitamin E can be influenced by selenium, the sulfur amino acids, other antioxidants, or prooxidants. Whether the entire function of vitamin E is that of an antioxidant is still the subject of some debate, but there are increasing reports of other biochemical and physiological functions for the vitamin. Many of the changes involved in a deficiency of the vitamin have been attributed to lipid peroxidation, but there is a growing indication that peroxidation may be only a result of a far more complicated process.

A previous report from this laboratory has shown a decreased fixation of injected $^{35}\text{SO}_4^-$ by the mucopolysaccharides of vitamin E-deficient rats maintained on a typical, with respect to inorganic sulfur, diet. However, the combination of a drastic restriction of the dietary inorganic sulfur with avitaminosis E resulted in a stimulatory effect on sulfate fixation by cartilage, a decrease in the per cent sulfur in the cellular lipoproteins, and an increase in the blood sulfhydryl level. These results were interpreted as an indication that vitamin E was necessary not only for the optimal sulfation of mucopolysaccharides, but also for the oxidation of neutral sulfur to sulfate (1).

Because of the in vivo effect of vitamin E deficiency on the metabolism of sulfur, a study of the in vitro oxidation of cysteine to sulfate was undertaken to try to determine the site of the decreased production of sulfate. Since several alternative pathways have been proposed in animals for the ultimate oxidation of the sulfur of cysteine to sulfate and the use of the sulfate formed, it was necessary to limit this investigation to the most widely accepted pathway, that of the oxidation of cysteine to sulfate through cysteinesulfinic acid and sulfite.

CHAPTER II

REVIEW OF THE LITERATURE

I. VITAMIN E

The primary activities in which a vitamin may engage are that the vitamin is a part of an enzyme molecule or that it cooperates with- in an enzymic reaction. A secondary activity is that the vitamin pre- serves another vitamin or enzyme against destruction by some toxic agent. It may replace or be replaced by another vitamin or anti- vitamin. The requirement for the vitamin may be altered by nutritional factors not themselves vitamins (2).

Vitamin E definitely has a role as an antioxidant, but its role also seems more specific (3). α -Tocopherol is probably a cofactor in the synthesis of ascorbic acid and may be concerned with the biogenesis of ubiquinone or may function in connection with its similarity to ubiquinone U₍₅₀₎ (4, 5). α -Tocopherol or a metabolite is necessary for the maintenance of α -ketoglutarate and succinate oxidation by liver homogenates (4). The specific structure of vitamin E may be to increase the fat solubility of the vitamin or to adapt it to function as a coenzyme (6).

Metabolites of Vitamin E

To understand the role of α -tocopherol one needs to isolate, separate, and identify the metabolites (7). There is still some

disagreement as to what these metabolites are. Incubation of tocopherol with rat liver homogenates under conditions which have been shown to prevent an in vitro metabolic lesion did not lead to the formation of new active metabolites of the vitamin. Other than appearing to be bound to the protein, α -tocopherol seemed to be its own active metabolite in the in vitro system (8).

The interconversions of α -tocopherol and its oxidation products have been discussed (Figure 1). α -Tocopheroxide was active but less potent than α -tocopherol in rat fertility. α -Tocopherylquinone and α -tocopherylhydroquinone were inactive in high doses, but both were active if given frequently (9, 10). α -Tocopherylhydroquinone disuccinate was active against dystrophy in rats but was not active against sterility (11). Nutritional muscular dystrophy in the rabbit could be prevented or cured by α -tocopherylquinone, α -tocopherylhydroquinone, or α -tocopheroxide, as well as by α -tocopherol (9). Some vitamin E compounds helped reverse respiratory decline when injected into the animal (12).

Selenium and vitamin E increased ubiquinone in heart, kidney, and liver, but only vitamin E increased the level in the uterus of the rat (13). Pantothenic acid deficiency also increased liver ubiquinone. Vitamin A deficiency had no effect on ubiquinone or ubichromenol except in the liver, but vitamin E deficiency depressed ubiquinone levels in thirteen tissues and ubichromenol in most tissues. With vitamin E supplementation after a deficiency, ubiquinone increased while ubichrom-

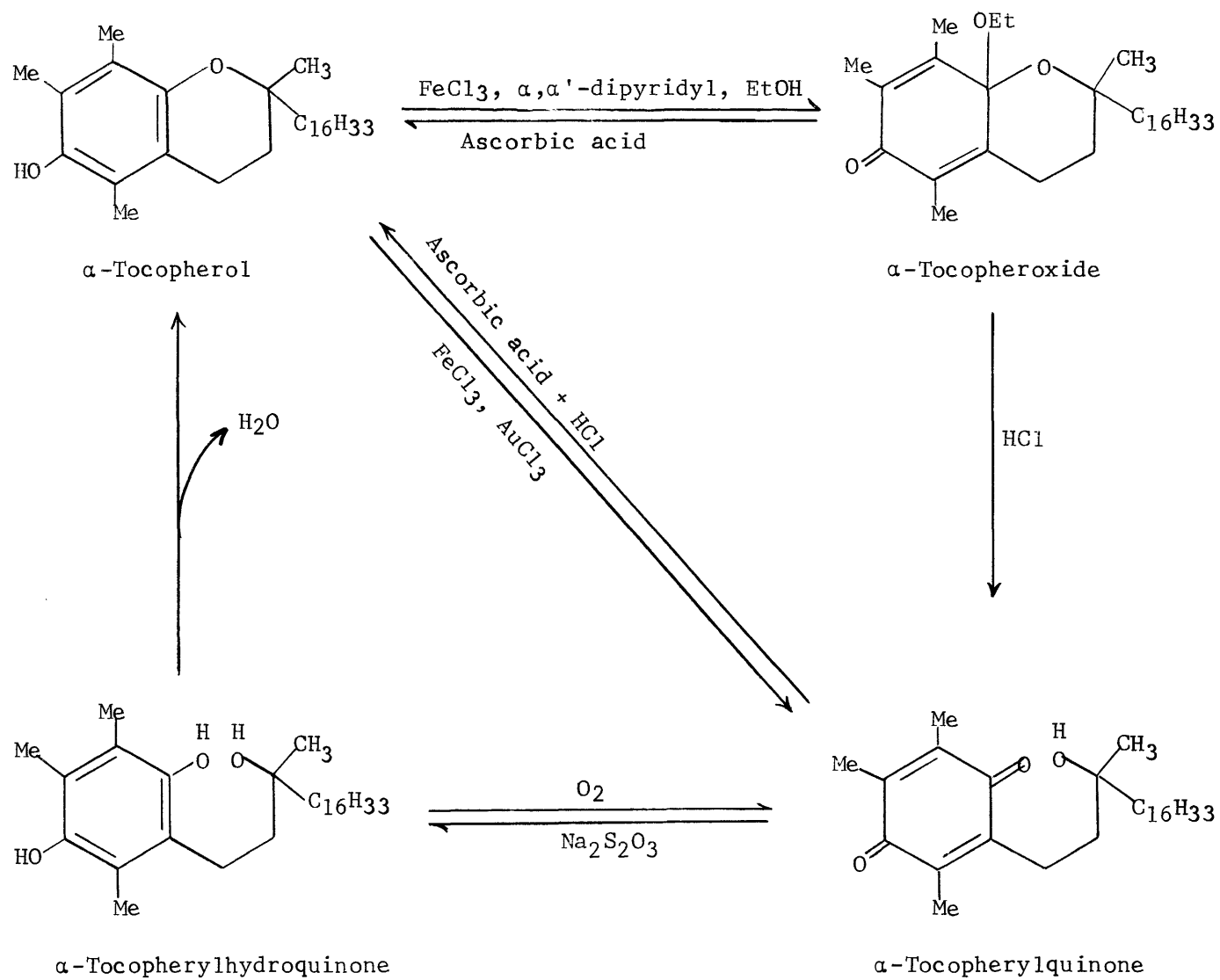


Figure 1. Interconversions of tocopherol metabolites (9).

enol decreased. Sodium selenite, when added to a vitamin E-deficient diet, increased ubiquinone in the liver. The level of ubiquinone was higher in the female than in the male and varied with the age of the animal (14). DPPD (N,N'-diphenyl-p-phenylenediamine) and Santoquin had no effect on ubiquinone or ubichromenol levels. These results suggest no antioxidant or protective effect for vitamin E under these conditions (15).

The chromanol of hexahydrocoenzyme Q₄ prevented encephalomalacia in the chick, resorption in the rat, and cured muscular dystrophy in the rabbit. The chromanol of vitamin K₁(20) also protected against encephalomalacia in the chick (16).

α -Tocopherol maintained a high level of ubiquinone in rat tissues. A large dose of α -tocopherol elevated ubiquinone in the heart without elevating tocopherol. Tocopherylquinone also increased ubiquinone concentrations. These data provided further evidence that the main biological function of tocopherol is unrelated to its lipid antioxidant property. Simon's quinone was checked to see if it was a tocopherol metabolite. It caused a greater and more rapid increase in ubiquinone concentration on a molar basis than α -tocopherol. This water soluble metabolite of vitamin E was suggested as the active biological form. The lactone form of Simon's quinone perhaps should be called tocopheronolactone and the acid form, the tocopheronic acid (17).

The tocopheronolactone increased ubiquinone and protected in vitro against lipid peroxidation in the vitamin E-deficient rat liver homogenate. It also prevented respiratory decline and restored oxidation of α -ketoglutarate. The biochemical role may be in oxidation-reduction. A soluble enzyme was found that coupled the reduction of tocopheronolactone to the oxidation of reduced pyridine nucleotides (18).

It has also been said that α -tocopherol is neither a precursor of ubiquinone nor necessary for the biosynthesis of ubiquinone (19) and that there is no evidence for a metabolic role of vitamin A in ubiquinone synthesis (20), but the tissue levels of ubiquinone and tocopherol in birds tend to support the concept that there is a relationship between tocopherol, ubiquinone, and oxidative processes in animals (21).

α -Tocopherol phosphate lowered the oxygen consumption of dystrophic rabbit muscle to normal in a short time. The creatine levels returned to normal. α -Tocopherol phosphate decreased the high succinoxidase activity in vitamin E-deficient hamster muscle (22). The oxygen uptake of dystrophic rabbit muscle was reduced in less than ten hours after the administration of α -tocopherol acetate (23). The phosphate ester of α -tocopherol inhibited most enzymes (24). The administration of α -tocopheryl phosphate to rats led to muscular weakness, depression of basal oxygen consumption, and depression of endogenous respiration in skeletal muscle. This suggested that glycogen phosphorylase might be impaired (25). A mechanism for the possible formation of ATP (adenosine triphosphate) by oxidation of substituted hydroquinones is given in Figure 2 (26).

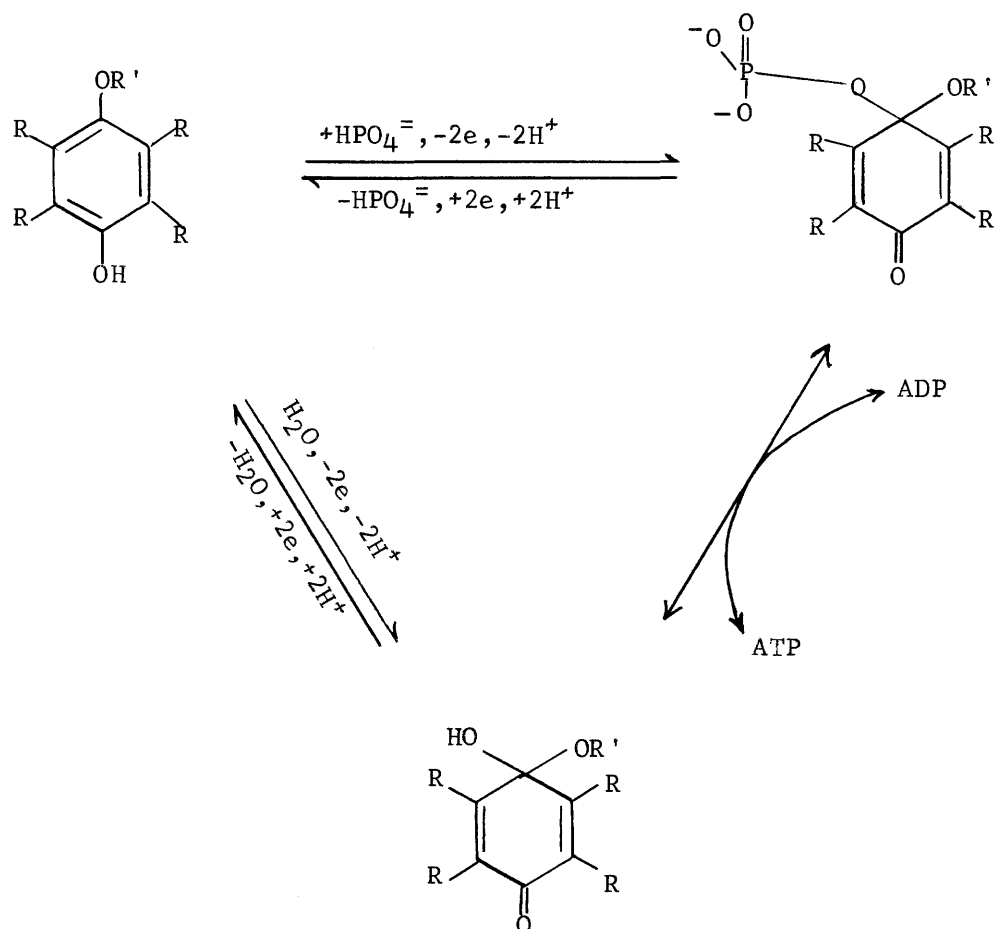


Figure 2. Possible formation of ATP by oxidation of substituted hydroquinones (26).

The main source of energy for the synthesis of high energy phosphate is coupled with oxidation in the electron transport system. Ubiquinone is known to be an essential component of the electron transport chain and is probably concerned with the flow of electrons from substrate to the cytochromes (4).

Interrelationships Between Vitamin E and Other Factors

Ascorbic acid. Ascorbic acid in diets low in vitamin E delayed the onset of dystrophy in rabbits. Ascorbic acid synthesis seemed to depend on coenzymic factors altered by a deficiency of vitamin E. Control and deficient animals showed no difference in the destruction of vitamin C by enzyme preparations (27). The inhibitor produced by gulonolactone oxidase in the presence of its substrate accelerated the decay of succinate oxidation in mitochondria from vitamin E-deficient animals (28).

Nucleic acid, nitrogen, and phosphorus metabolism. The relationship of vitamin E to nucleic acid, nitrogen, and phosphorus metabolism has been investigated. Vitamin E-deficient rabbits excreted large quantities of allantoin compared to control rabbits. This suggested deranged nucleic acid metabolism, which could be either accelerated turnover of nucleic acids or accelerated purine synthesis with failure to incorporate the product formed. Because of the increased rate of incorporation of formate into tissue nucleic acids, vitamin E was thought

to regulate the turnover of nucleic acids (29). Since vitamin E deficiency did not significantly influence the oxidation of formate to carbon dioxide in monkeys and since there was increased incorporation of formate into tissue nucleic acids in skeletal muscle and bone marrow in vitamin E deficiency, the conclusion was that this deficiency affected mainly DNA (deoxyribonucleic acid). Other tissues were unaffected (30). Vitamin E deficiency in rabbits led to an increase in the incorporation of ^{32}P into tissue nucleic acids (31). The DNA per gram of liver, coenzyme A, ATP, and ADP (adenosine diphosphate) levels increased when additional vitamin E was fed to rats (32).

In vitamin E deficiency in rabbits phosphate left the serum and entered the muscle cell faster than in normal rabbits, but it was not incorporated into organic phosphorus compounds so well. When the utilization of creatine phosphate by muscle extracts from vitamin E-deficient rabbits was investigated, there seemed to be a specific impairment of the system that phosphorylated creatine; however, tocopherol was doubted as a cofactor (33). Dystrophy; increased urinary creatine, allantoin, and free amino acids; decreased urinary creatinine; anemia; and granulocytosis are symptoms of vitamin E deficiency in the monkey (34).

The specific activity of plasma inorganic phosphate was unchanged in vitamin E deficiency and decreased in hyperthyroidism. The skeletal muscle inorganic phosphate specific activity was increased in vitamin E deficiency. The specific activity of creatine phosphate and ATP was

high in vitamin E-deficient rabbits and hyperthyroid rats. This indicated an elevated turnover of skeletal muscle intracellular inorganic phosphate and a normal or decreased turnover of skeletal muscle creatine phosphate and ATP in vitamin E deficiency. There was no decrease in creatine phosphate or ATP in hyperthyroid rats. Hyperthyroidism may affect mitochondrial membranes (35).

The creatine pool would increase in vitamin E deficiency in the rat because the uptake of creatine by muscle decreased. This decreased uptake would account for the lowered creatine content of the muscle and the lowered creatinine excretion. There may be a defect in the creatine transport across the cell membrane (36).

Pyridoxine deprivation hastened the appearance of dystrophy when vitamin E was absent in the rat (34). Rats deficient in both vitamin E and vitamin B₆ had increased excretion of allantoin and creatine, and adding either vitamin prevented this increase. Vitamin B₆ deficiency, but not vitamin E deficiency, decreased growth and excretion of xanthurenic acid after a tryptophan load (37).

Decreased phosphorylase activity in vitamin E-deficient chicks has been reported (38). The increase in total muscle phosphorus in the dystrophic chick was due to an increase in the inorganic phosphate fraction, while ATP and creatine phosphate decreased. There was greater permeability of phosphorus, potassium, and cesium and an increased potassium turnover in the dystrophic chick muscles than in the control (39). This indicated phosphorus metabolism might be specifically affected during vitamin E deficiency in the chick (40).

The free amino acids in muscle extracts from vitamin E-deficient rabbits were elevated when compared to normal rabbits. The muscle glycine in the deficient animal was decreased (41, 42). Taurine decreased in the muscle of the vitamin E-deficient rabbit and climbed slightly or did not change in the other tissues checked (42).

A deficiency of vitamin E increased the recovery of respiratory ^{14}C from injected $1\text{-}^{14}\text{C}$ -glycine, $1\text{-}^{14}\text{C}$ -leucine, and $1\text{-}^{14}\text{C}$ -lysine in the rabbit. Avitaminosis E, uncomplicated by inanition, had no effect on the oxidation of glucose or palmitate. The rate of oxidation of amino acids seemed to be increased in vitamin E-deficiency (43). The specific activity of protein increased in vitamin E-deficient rabbits, when compared to controls, after administration of ^{14}C -glycine. The specific activity of methionine was also increased after administration of ^{14}C -formate on the basis of protein content, but formate incorporation was increased only on a μmole basis. Removal of formate for synthesis of the creatine and allantoin lost in excess would account for the increased specific activity of the compounds requiring 1-carbon fragments (44).

An increase in the glutathione content in the liver and muscle of rabbits with avitaminosis E has been reported (45). Erythrocyte glutathione was slightly elevated, and glycine was incorporated into the glutathione of muscles at an increased rate. The metabolism of glutathione in tissues of rabbits was felt to be, to some degree, controlled by vitamin E (46).

Since glutathione might play an important part in the maintenance of the integrity of the structure and function of the erythrocyte and, perhaps, of all tissue cells (47), the glutathione content of erythrocytes from tocopherol-deficient and tocopherol-supplemented rats was measured and compared to the extent of hemolysis by various compounds. There was no difference in the glutathione content of the red blood cells of the supplemented and deficient animals. Hemolysis was independent of glutathione content when the cells were exposed to oxygen, selenite, or heavy metal sulfhydryl reagents. Susceptibility to hemolysis in the vitamin E-deficient animal was found not to be related to any alteration in the glutathione concentration (48).

Hemolysis of erythrocytes. Rat blood cells were hemolyzed in the presence of hydrogen peroxide even when the animals were fed stock diet or rations containing vitamin E, but cells from the vitamin E-deficient rats were hemolyzed to a greater extent than those from vitamin E-sufficient animals. Catalase should prevent hemolysis if hydrogen peroxide production were the basic cause for hemolysis by dialuric acid. The more rapid hemolysis with dialuric acid than with hydrogen peroxide and the lower concentration required suggested that hydrogen peroxide per se was not the hemolyzing agent (49).

Hydrogen peroxide-induced hemolysis of red blood cells from vitamin E-deficient rats is said to be closely associated with lipid peroxidation of the cell stroma (50). However, red blood cells from tocopherol-deficient rats did not have increased levels of lipid peroxides until after treatment with an oxidizing agent. Therefore, there was no evidence for

alteration in the red cell membrane in avitaminosis E (51). Large oral doses of methylene blue had no effect on the prevention of hemolysis (52, 53). Thyroxine, Co^{+2} , Mn^{+2} , Cr_2O_7^- , and CrO_4^- protected in vitro against hemolysis and peroxidation. Hemoglobin, Fe^{+3} , ascorbic acid, cysteine, glutathione, and selenite increased hemolysis of red blood cells from vitamin E-deficient animals (54, 55). Attempts have been made to explain the mechanism of hemolysis (56). Susceptibility of erythrocytes to hemolysis is also an indication of vitamin E deficiency in chicks (57, 58).

Hemolysis of rat erythrocytes by dialuric acid could be prevented by 0.4 mg. of DL- α -tocopherol per kg. of body weight per day. Several times this dose of the vitamin were required to prevent sterility and dystrophy (59). Males required 3.5 mg. of vitamin E per kg. per day and females only 2.5 mg. per kg. per day (60).

Dialuric acid hemolysis in the premature infant was not effective in evaluating the vitamin E status (61). Vitamin E was effective in vivo or in vitro for the prevention of hydrogen peroxide-induced hemolysis, but administration of the vitamin to the mother before delivery was not effective (62). In the adult recovery from the susceptibility of erythrocytes to hemolysis required a long time after resupplementation with the vitamin (63).

Cystic fibrosis could make man appear to have nutritional muscular dystrophy (64). This condition was induced because of the poor absorption of tocopherol (65). In normal adults 7 per cent of the people studied had blood tocopherol values that were considered

too low. The cause could be either low intake of vitamin E or high intake of unsaturated fatty acids (66). There also seemed to be a relationship between sex and blood tocopherol levels in man (67). The tocopherol requirement was a function of the amounts of certain peroxidizable lipids in the diet and tissues. Linoleic acid appeared to be the most significant oxidizable lipid in the diet, but other unsaturated fatty acids might also be important. When the peroxidizable fatty acids were low, the tocopherol need decreased. The peroxide hemolysis test was felt to indicate only the rate at which erythrocyte fatty acids could be oxidized (68).

Erythrocyte survival. In vivo hemolysis after vitamin K administration to vitamin E-deficient animals suggested that the vitamin E status might have a bearing on the in vivo red cell survival in certain circumstances (69). The survival of erythrocytes from monkeys and rats was shortened in vitamin E deficiency (70, 71). The vitamin may be considered a hematopoietic agent in monkeys and in malnourished human infants with macrocytic anemia (72).

Growth in the rat. Vitamin E has some effect on growth in rats. The females responded less, if at all, to lack of vitamin E. The males decreased in growth on the vitamin E-deficient diet (73). The failure in growth due to vitamin E deficiency was characterized only in the last phase, that after attainment of maturity, normally viewed as a plateau in the growth curve, although all animals showed a slow ascent. The

plateau occurred after about 100 days of life. These diets contained more than adequate protein (74). Increased growth when vitamin E was added to an otherwise adequate diet lacking in the vitamin has also been observed (75). On the other hand, vitamin E is reported to have an effect on growth only when the protein level is below 10 per cent (76). The tocopherol requirement for growth in the rat could be related to dietary or tissue lipid fatty acids when methionine and the environment were optimal. If the vitamin E level in the diet was low, growth could be related to the level of selenium (77). The adult rat suffering from chronic avitaminosis E developed paresis after the nineteenth experimental week (77).

Reproduction in the rat. There has been some disagreement about the absolute necessity of vitamin E for the prevention of sterility in the rat. DPPD replaced vitamin E in the prevention of sterility in females in some studies (79). This antioxidant was toxic, and the amount needed to prevent stillbirths was only slightly below the toxic level (80). The role of vitamin E could, therefore, be only in the preservation of a non-vitamin E compound (81). Factor 3 did not prevent resorption-gestation in the rat. Vitamin free casein is generally a good source of Factor 3 (82).

If the vitamin E-deficient female rat received supplementary vitamin E before the eighth day of gestation, the pups were normal. Supplementation between the ninth and twelfth day resulted in deformed young. If supplementation was delayed until after the twelfth day,

resorption of the fetuses occurred (83).

Other conditions in the rat. Other symptoms of vitamin E deficiency in the rat are a brownish discoloration of adipose tissue, depigmentation of incisor enamel, and renal histolysis. The yellow-brown discoloration of adipose tissue depended on the presence of dietary cod-liver oil and the depigmentation of the incisor enamel upon the dietary fats (84). If 0.9 p.p.m. selenium was fed to rats whose teeth were white from a vitamin E-deficient diet, the pigment returned to the teeth, but growth was retarded. A lower level of selenium reversed the tooth depigmentation only temporarily (85). Dietary fat and vitamin E influenced renal histolysis, with the time elapsed between death of the animal and examination of the kidney also having some effect (86, 87). This phenomenon may be related to an altered state of unsaturated fatty acid incorporation into phospholipids and lipoproteins of membranes (87). Methylene blue, an antioxidant, has been found to delay incisor depigmentation and prevent uterine pigmentation, but its effect on the prevention of sterility and erythrocyte hemolysis is in doubt. It also increased the vitamin A stored in the rat (88).

The testes of the vitamin E-deficient rat have increased proportions of 18:2 ω 6, 20:4 ω 6, and 22:4 ω 6 fatty acids and decreased amounts of 22:5 ω 6, but no change in 20:3 ω 6. This suggested that inhibition in the conversion of 20:4 to 22:5 fatty acids occurred (89).

Sulfur and selenium metabolism and peroxidation. Tocopherol acts in some way to maintain the proper functioning of enzyme sulfhydryl groups (90). The protective effect could be by oxidation-reduction reactions of the quinone with sulfhydryl groups at the active sites, which would force equilibrium to the disulfide form, thus inhibiting the point of attack by heavy metals. Reduced tocopherol is reoxidized by Fe^{+3} . A vitamin E metabolite might serve as an intermediate carrier for electron transfer from reduced mono- or dithiol sites of enzymes or as a cofactor to the iron-containing catalysts. Or the quinoid structure might react with the sulfhydryl groups by condensation. Tocopherol metabolites might have a shielding effect on labile sulfhydryl groups because of this. These products could be the real electron transfer configurations and should be sensitive to heavy metals (91). Loss of free sulfhydryl groups occurs concurrently with oxidative decline (90). There has been a suggestion that protein carriers are involved in the transport of vitamin E in the blood (69).

The term "antioxidant" designates compounds which quench electron mobility, scavenge free radicals, break free radical chain reactions, or chelate trace metals (92). Vitamin E is an antioxidant. Sulfur amino acids could also act as antioxidants somewhat similar to vitamin E by reacting with free radical intermediates of lipid peroxidation, thereby breaking the chain reaction, or by decomposing lipid peroxides. Cysteine, glutathione, and sulfhydryl proteins are a large and reactive pool of redox compounds for reduction of vitamin E. If free radical

lipid peroxidation did proceed, the sulfur amino acids would protect cellular constituents. Further, the amino acids could react to repair the damage (93). The soluble thiols in the aqueous phase and vitamin E in the lipid phase might inactivate damaging free radicals. The cerebella in chicks with encephalomalacia showed damage expected from formation of free radicals (94). A scheme for the relationship between lipid peroxidation, vitamin E, ascorbic acid, and sulfhydryl compounds was proposed (Figure 3) (93).

Vitamin E and selenium may act independently in alternative pathways of energy metabolism and both pathways would have to be impaired before illness occurred (95). Selenium will not prevent all aspects of vitamin E deficiency in animals. It is apparently incorporated into some cellular component. Since a general rule should apply to a variety of species, vitamin E and selenium cannot act exclusively as antioxidants because rat and guinea pig tissue do not respond to both in the same way (96). There are several other objections to interpreting the biological activity of selenium in terms of an antioxidant. Sulfur compounds with antioxidant properties are present in much greater concentrations than any selenium compounds. The selenium antioxidant has been proposed to be a selenium amino acid. However, the amount of selenium in the tissues is much greater than can be accounted for by known selenium amino acids. Inhibition of lipid peroxidation with selenium reaches a maximum (77).

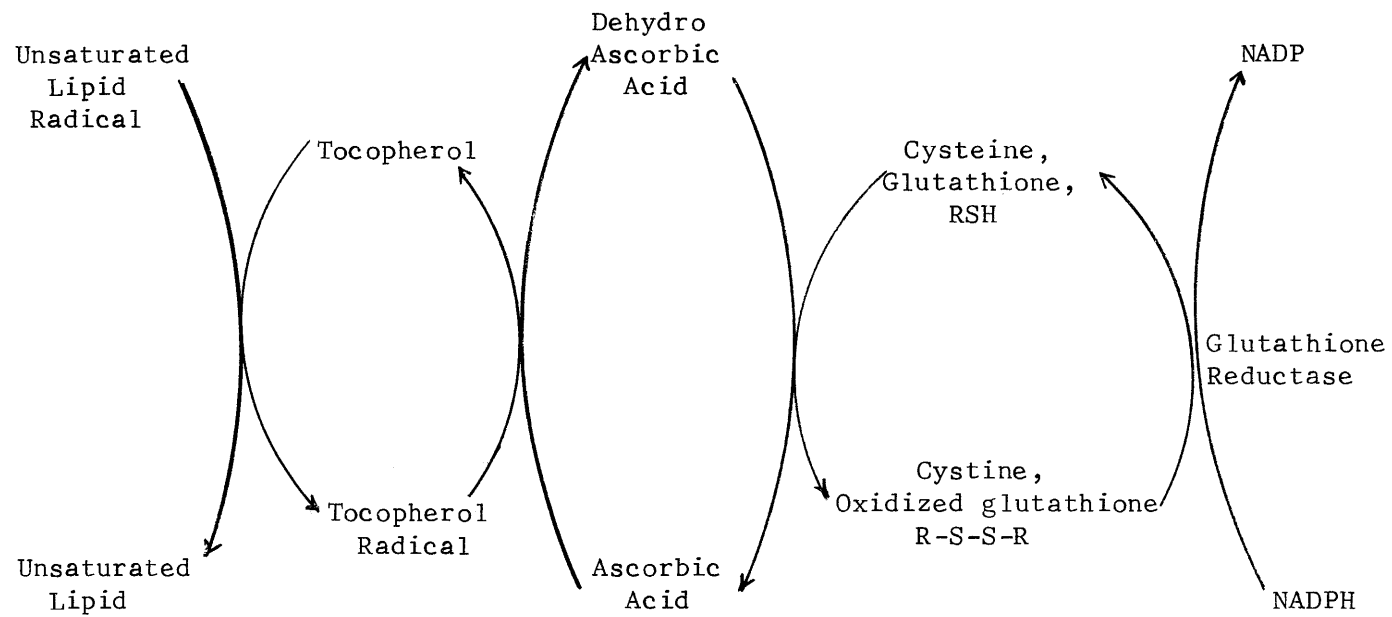


Figure 3. A possible relationship among lipid peroxidation, tocopherol, ascorbic acid, sulfhydryl compounds, and NAD (93).

Three factors, cystine, vitamin E, and Factor 3 (selenium), separately protected livers from necrosis. Cystine was more effective than cysteine (98). The development of liver necrosis depended on the composition of the diet, the endocrine status, the daily food allowance, and the environmental temperature (99). Methionine and cysteine could not replace selenium as one of the three factors preventing necrosis if they did not contain traces of selenium (100). The female rat was not so affected by a necrogenic diet as the male. Thyroid removal served to prevent necrosis, possibly through its adverse effect on appetite (101). Liability to necrosis decreased with age (102). Diets adequate in protein protected against liver necrosis (103).

Increased cystine in the diet protected against necrosis in rats, but not against hemolysis (104). The potency of cystine is largely due to contamination with Factor 3 (105). A contamination in sulfur compounds of one selenium atom for 350,000 sulfur atoms per 100 g. of diet would be sufficient to prevent necrosis (106). Factor 3 is water soluble and stable to acid hydrolysis (107).

The latent phase of necrotic liver degeneration is characterized by respiratory decline in homogenates from rat liver (108). In rats fed *Torula* yeast and deficient in vitamin E and Factor 3 respiratory decline occurred 1-2 weeks before liver degeneration. Added vitamin E completely prevented the decline, while additional amounts of Factor 3 and selenite only helped prevent this decline. A tocopherol metabolite and DPPD prevented in vitro respiratory failure of rat liver slices.

Fresh homogenate had to be used because aging for several hours would nullify the protective effect of tocopherol (109).

The decline in rate of oxidation with incubation time differed depending on substrate employed, its concentration, and whether or not vitamin E was present in the diet. The concentration of α -ketoglutarate, β -hydroxybutyrate, pyruvate, and malate affected the vitamin E-deficient homogenates differently from the controls with respect to respiratory decline. There was no difference in decline between the vitamin E-sufficient and -deficient homogenates with respect to succinate or NADH (reduced nicotinamide adenine dinucleotide) concentration. NADH seemed to prevent decline. This indicated that the metabolic lesion was in the Kreb's cycle and the electron transport chain was unaffected (110).

Oxidation of certain substrates by liver homogenates from vitamin E-deficient rats declined after a short time when compared to the rate exhibited by livers from vitamin E-sufficient animals, but no such decline was exhibited by the mitochondria from the deficient animals. If the microsomes were added to the mitochondria or supernatant fluid, there was an increased rate of decline, but boiled microsomes under the same conditions produced only a slight decline. The microsomes were therefore felt to be the site of decline in oxidation (111). Dietary selenite largely prevented a fall in mitochondrial NADH-cytochrome c reductase and pyruvate oxidation rates and in the P:O ratio in the rat (112).

The agent in microsomes which caused respiratory decline by its effect on the mitochondria was reported to be a heavy metal which reacted

with sensitive sulfhydryl sites or similar points of attack. The block in the system was not in oxidative phosphorylation or in the mitochondrial electron transport system but at certain dehydrogenase systems which connect the tricarboxylic acid cycle to the cytochrome chain. With α -keto-glutarate as the substrate lipoyl dehydrogenase was found to be an enzyme involved. This enzyme is sensitive to trace metals and arsenite even in the presence of reducing substrates (113).

In the absence of dietary vitamin E and selenite swelling of axons in sensory relay nuclei or spinal cord and medulla of rats developed. Succinic dehydrogenase was the only enzyme measured that was low in the swollen axons of the vitamin E-deficient animals. Other enzymes tested were NAD-(nicotinamide adenine dinucleotide) or NADP-(nicotinamide adenine dinucleotide phosphate) diaphorase, NAD- and NADP-dependent isocitric dehydrogenase, glutamic dehydrogenase, and glucose-6-phosphate dehydrogenase (114).

Isocitric acid dehydrogenase in the skeletal muscle of the vitamin E-deficient rabbit was decreased. The serum isocitric acid dehydrogenase decreased in moderate dystrophy but increased when dystrophy was more pronounced. The serum citrate levels decreased with an increase in isocitric acid dehydrogenase (115).

Glutamic-pyruvic transaminase increased and glutamic-oxaloacetic transaminase decreased in rabbit liver in the early stages of dystrophy. Glutamic-pyruvic transaminase also increased in the muscle and serum of the rabbit at this time. As the disease progressed, this enzyme decreased in muscle but was still elevated in the serum. This suggested leakage

through deteriorated cell membranes of muscle. But glutamic-oxaloacetic transaminase decreased in muscle and did not increase in serum in advanced dystrophy (116).

The level of different keto acids in the tissues from rats and chicks was higher in vitamin E deficiency than in controls. The glutamic-oxaloacetic transaminase from chick serum and rat liver was higher in vitamin E deficiency than in control animals (117).

Of thirteen antioxidants which prevented liver necrosis most also prevented respiratory decline. Vitamin E was inactive in vitro and methylene blue was inactive in vivo, but methylene blue is in the leuco state in vivo and is oxidized in vitro (118). There was a lack of correlation between the TBA (thiobarbituric acid) reaction and the decline of succinate and α -ketoglutarate oxidation in the vitamin E-deficient homogenate (119).

The TBA test for peroxidation is a measure of the amount of malonaldehyde formed (120). Cysteine and glutathione catalyzed lipid peroxidation, causing malonaldehyde formation. Tissue aldehydes and sugars heated with hydrochloric acid also developed the color with TBA incubation. Linolenic acid would produce a positive test, but arachidonic acid gave negative results (121). In vitro tocopherol increased synthesis of ascorbic acid, which catalyzes peroxidation of fatty acids and inhibits its own synthesis. Vitamin E also inhibited and ascorbic acid increased malonaldehyde production. Both prevent hematin-catalyzed peroxidation of unsaturated fatty acids (122). Due

to the colors given by heme-containing systems in the TBA test it is necessary to apply the test to erythrocyte membrane components in the absence of heme in order to obtain reliable data on the oxidation of these components (123).

Detection of peroxidation by TBA in liver homogenates from rats fed vitamin E-deficient diets was increased by the presence of NAD-linked substrates, but not by succinate. The peroxidation was prevented by respiratory inhibitors. Evidence suggested that damage to cell fractions might result in endogenous peroxide formation rather than the reverse (124). Phenergan and Versene prevented respiratory decline and liver necrosis in vitamin E deficiency. These must block a chain of reactions in which trauma leads to a release of toxic breakdown products causing further damage (125).

The microsomes from vitamin E-deficient rabbits were susceptible to lipid peroxidation (126). When homogenates from liver and muscle of vitamin E-deficient animals were combined with homogenates from vitamin E-sufficient animals, inhibition of peroxidation occurred only in the liver. The boiled homogenate from the liver of the vitamin E-sufficient animal had no protective effect (127).

The effects of various dietary levels of selenium, vitamin E, and DPPD on liver necrosis and lipid peroxidation were investigated. Selenium prevented necrosis, but not peroxidation. Vitamin E prevented, at the levels administered, peroxidation, but not necrosis. The effect of vitamin E and selenium may therefore be on some other biochemical

or biophysical system concerned with the maintenance of cell integrity (128). Selenium and cystine in some way alter the composition of tissues (129). In vitamin E deficiency in chicks a high capillary permeability, associated with edema, was suggested as the cause of exudative diathesis (40, 130).

Membrane permeability. In skeletal muscle of vitamin E-deficient rabbits aminoisobutyric acid rose faster and fell earlier than in controls. This was not caused by changes in water content or intracellular space. It was suggested that vitamin E deficiency causes an increased loss of amino acids from muscle cells due to increased membrane permeability and that the increased uptake is a compensatory mechanism (131). Vitamin E deficiency caused an increase in erythrocyte permeability in the rat (132).

A comparison of vitamin A-deficient rats fed high or low levels of vitamin E demonstrated an increased storage of vitamin A with the higher level of vitamin E. Vitamin A-deficient animals, when compared to their vitamin A-sufficient controls, showed increased levels of vitamin E in liver and decreased serum albumin but increased globulin levels. The in vitro incorporation of ^{14}C -amino acids into diaphragm protein was higher in vitamin A-deficient low vitamin E rats than in the controls, but the incorporation of ^{14}C -amino acids into protein from diaphragm of the vitamin A-deficient rats with the higher vitamin E level was lower than in the pair fed control. These changes were

explained as being from an effect of vitamin E and vitamin A on membrane properties. There was an increased permeability of cell membranes in vitamin A deficiency. This changed the amino acid pool inside the cell. Vitamin E reversed the effect of vitamin A on the cell membrane. This would lower the loss of amino acids from the cellular pool (133). The action of the fat soluble vitamins on sulfomucopolysaccharides associated with the cell membrane might cause these changes in permeability (134).

The increased permeability of various cells in vitamin E-deficient animals may be due to release of lysosomal enzymes following peroxidation (40). Aryl sulfatase, a lysosomal enzyme, was absent in control animals but present in high concentration in vitamin E-deficiency (93). Hamster skeletal muscle possessed proteolytic and autolytic activity similar to that of rat skeletal muscle, and the enzyme activity of muscles from both species was increased when made dystrophic by vitamin E deficiency (135). Increased proteolytic activity in the muscle of the vitamin E-deficient rabbit has also been reported (136).

In an attempt to determine whether peroxidizability and increased lysosomal enzymes occurred before or with dystrophy or with the regression of dystrophy, the methionine added to a vitamin E-deficient chick diet was removed. Dystrophy developed within 48 hours. After resupplementation with methionine the lysosomes stayed high until almost complete recovery. Peroxidizability also remained high on the vitamin E-deficient diet with methionine repletion and returned to normal only at the end. The addition of vitamin E resulted in a slower return to

normal than added methionine for both lysosomal activity and dystrophy. Since there was no induction of lysosomal enzyme activity before the symptoms of dystrophy developed, the increased lysosomal activity could not have been the cause of muscular dystrophy in the chick (137).

The effect of the antioxidant DPPD in the prevention of muscular dystrophy was not due to conservation of tocopherol in the tissues of the rabbit. DPPD caused remission of muscular dystrophy on a fat free diet. This was in accord with the view that muscular dystrophy is associated with antioxygenic potency and also agreed with the activity of α -tocopherylhydroquinone and α -tocopherylquinone. DPPD, however, did not prevent hypercholesterolemia and muscular degeneration in guinea pigs fed a diet low in vitamin E (138). Factor 3 did not influence muscular dystrophy in rabbits, but vitamin E did (139).

Poultry nutrition. In chicks exudative diathesis, encephalomalacia, and muscular dystrophy have been related to vitamin E-deficiency. Exudative diathesis was most sensitive to selenium supplementation. Each could be produced uncomplicated by the others, on the basis of diet, and none of these was an uncomplicated vitamin E deficiency. In exudative diathesis the plasma protein was low and the albumin fraction was particularly depleted. Muscular dystrophy could be produced by diets low in selenium and sulfur amino acids. Encephalomalacia was not prevented by selenium, but was prevented by most antioxidants (140).

Dystrophy in chicks due to deficiencies of vitamin E and sulfur was associated with an elevation in the myocardial catecholamines (141).

No muscular dystrophy occurred when chicks fed diets deficient in vitamin E and sulfur amino acids were also deficient in arginine (142). As the level of arginine in the diet was increased, the level of methionine necessary for the prevention of muscular dystrophy was also increased. The increased growth resulting from the amino acid supplementation might be responsible for the increased methionine requirement. Cystine and methionine seemed to have a metabolic importance more specific than that of supplying sulfur. Vitamin E appeared to be interrelated with sulfur amino acid metabolism (143). Antioxidants and sodium sulfate added to a low sulfur diet helped reduce muscular dystrophy in the chick (144).

Vitamin E was necessary for the development of chick embryos (145). In turkeys unsaturated oil in the diet accentuated a deficiency of vitamin E. Eggs from turkeys fed diets high in unsaturated oils failed to hatch unless DPPD or vitamin E was added. DPPD did not increase tocopherol deposition in the egg yolk, but tocopherol did. Pollack-liver oil caused a reduction in the tocopherol content of the yolk (146). High levels of vitamin E produced maximum hatchability. Natural vitamin E was destroyed by fish liver oil in the diet, but this oil did not increase the metabolic requirement for vitamin E (147).

Exudative diathesis and encephalomalacia rarely occurred on low fat diets and could be altered by dietary changes not related to the vitamin E status (130). Selenium as selenite prevented diathesis at 0.3 p.p.m. (148). In chicks with exudative diathesis fed diets deficient only in vitamin E and selenium it was not possible to correlate

the extent of in vitro lipid peroxidation with the severity of the symptoms. Selenium did not modify the amount or type of polyunsaturated fatty acids. Those antioxidants which went into the tissues were effective in preventing peroxidation (149). The major portion of the requirement of the chick for vitamin E could be satisfied by a biologically active antioxidant such as DPPD or ethoxyquin. The antioxidant either protected vitamin E carried over from the egg or replaced the vitamin in its functions. Less ethoxyquin was required to prevent encephalomalacia than to prevent muscular degeneration. Vitamin E may function as a prooxidant (150).

The activity of antioxidants was in vivo and was not only protection of the diet against autoxidation. These compounds might substitute for vitamin E, but might also protect the vitamin that is there (105). When negative results occurred with an antioxidant, it was felt that the wrong antioxidant had been used or that the level was too low. The antioxidant might also be toxic. The hydroxyanalog of methionine remedied deficiencies of the sulfur amino acids. The vitamin E deficiency symptoms could be brought about by low sulfur, low antioxidant diets which produce dystrophy, low selenium, low antioxidant, high linolenic acid diets which produce exudative diathesis, and high linoleic acid, low antioxidant diets which produce encephalomalacia (151).

Linoleic acid added to chick diets low in vitamin E resulted in encephalomalacia. This was uncomplicated by selenium deficiency or

possible oxidation of fatty acids. Cod-liver oil in chick diets caused encephalomalacia. Linoleic acid might destroy vitamin E in the diet and gut during fatty acid oxidation or increase the destruction of vitamin E in the tissues because of high levels of fatty acids and subsequent autoxidation, but antioxidants added to the diet would have prevented this. The linoleic acid might also disrupt some cell structure or metabolic process because of autoxidation of linoleic or arachidonic acid in the tissues. This is the supposed cause of the influence of linoleic acid on the development of encephalomalacia (152). When hog liver fractions were fed to chicks receiving a vitamin E-deficient diet, the most unsaturated fraction produced the most symptoms of encephalomalacia and killed some of the animals before they could develop exudative diathesis. Otherwise, the less unsaturated the fatty acid fraction, the less sick the animals became from exudative diathesis (153). Encephalomalacia in the chick could be accelerated by feeding oxidized fats and fatty acid oxidation products (154). Injections of linoleate hydroperoxide also accelerated the development of encephalomalacia. Vitamin E-deficient diets with accompanying high fat and protein were more severely damaging (155).

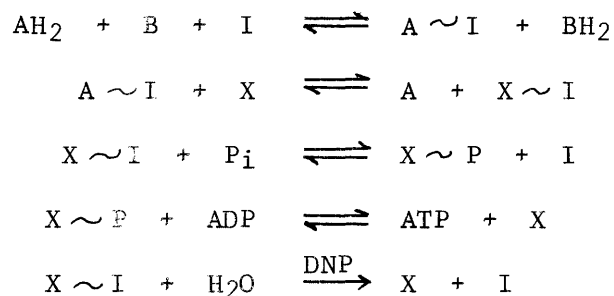
Respiratory chain-electron transport. Vitamin E has been suggested as having a function as a cofactor in the terminal respiratory chain just before cytochrome c (156). Tocopherol could reverse digitonin inhibition but no other substance commonly used could.

Digitonin is used to solubilize mitochondrial enzymes and is usually added to concentrated preparations which have to be diluted before assay. The inhibition could be reversed by dilution. Digitonin also inhibited succinate-cytochrome c reductase, but other factors could reverse this inhibition. However, NAD-cytochrome c reductase inhibition responded only to tocopherol (157). In the NADH-cytochrome c reductase system tocopherol was not oxidized or reduced. The reaction might involve free radicals. The tocopherol bound to the enzyme might function in electron transport, but it might be part of the lipid sheath that maintains proper orientation for electron transport (158). It may function indirectly as a binding or cementing substance for other components of the cytochrome c reductase system (159). The NADH-cytochrome c reductase of particulate preparations was inactivated by isooctane extraction but reactivated by α -tocopherol. Several other antioxidants did not replace the activity that vitamin E restored (160).

Others have said that tocopherol is not a cofactor in the cytochrome c reductase system. They reasoned that the inhibitory products came from aged enzyme preparations and tocopherol removed these products (161). DL- α -tocopherol and D- α -tocopherol seemed to behave the same in restoring the activity of isooctane-extracted heart muscle, suggesting that isooctane inhibited rather than extracted a component of the enzyme system (162). The solvent might act as a physical inhibitor when some enzymes were extracted with organic

solvents. Tocopherol and other solvents were non-specific in reversing inhibition (163, 164).

Slater has suggested that the structure of the mitochondrion is altered in vitamin E deficiency to promote hydrolysis of $X \sim I$. This compound arises as follows:



He suggested that X is vitamin E (165). He later altered this scheme slightly and applied it specifically to vitamin E metabolism (Figure 4) (166). The horse heart muscle preparation generally used contains both α -tocopherol and ubiquinone, which are localized mainly in the mitochondria (167).

Oxygen consumption. Oxygen consumption in the skeletal muscle of young rats born to mothers fed vitamin E-deficient diets was increased even in the absence of microscopic alteration in the muscle. There was no effect in the liver (168). Muscle from vitamin E-deficient rats and rabbits showed increased oxygen consumption (169). The oxygen consumption of hamsters fed vitamin E-deficient diets returned to normal after the administration of vitamin E, but the muscles still did not work well (170). Dystrophic muscles from vitamin E-deficient animals

had higher oxygen consumption, lower creatine content, and higher chloride content. Oral administration of α -tocopherol lowered oxygen consumption toward normal within 27 hours (171).

Erythrocytes from vitamin E-deficient rats underwent in vivo hemolysis in high oxygen pressures. Conditions which increased the rate of oxygen consumption increased sensitivity to oxygen pressure. The red blood cells from the vitamin E-deficient animals took up oxygen at twice the rate as the controls (172). Thyroxine protected weanling vitamin E-deficient rats against hemolysis, but older animals were not protected except in vitro (173). When the effects of tocopherols, methylene blue, and glutathione on the manifestations of oxygen poisoning in the vitamin E-deficient rat were measured, the methylene blue protected against nervous system damage and tocopherol against hemolysis, but neither protected against lung damage. Glutathione had some effect on the lung damage. Sulfhydryl enzymes were those that were damaged (174).

Oxygen at one atmosphere pressure inhibited brain respiration in normal animals. The respiration of minced brain decreased in oxygen more than in air, particularly in the presence of glucose, sodium lactate, or sodium pyruvate. In the presence of sodium succinate the tissue was not so sensitive to oxygen. Lactic dehydrogenase and the diaphorase of brain were not affected. D-amino acid oxidase and xanthine oxidase were also poisoned. These data suggested that pyruvate oxidase, a thiol enzyme, was affected (175). Thyroxine, dinitrophenol,

tetrahydronaphthylamine, adrenaline, pituitary extract, insulin, and eserine enhanced oxygen poisoning. The damage was from excessive and rapid oxidation in nerve cells and was attributed to carbon dioxide (176).

Vitamin E also helped guinea pigs to withstand high altitudes. Thyroidectomy increased and injections of thyroxine or dinitrophenol decreased resistance to anoxia. Thiourea and thiouracil increased survival time of rats in acute anoxia. These are not only basal metabolism depressants but are also water soluble antioxidants. Presumably, the higher the fat in the diet, the greater oxygen requirement the animal should have, since the respiratory quotient would be lower. The shorter survival time under conditions of anoxia of rats fed high fat diets, when compared to controls fed less fat, is apparently a reflection of this increased oxygen requirement. Higher than physiological doses of tocopherol increased susceptibility to anoxia (177). The stress of vitamin E deficiency increased the iodine requirement (178), but there was no evidence that the thyroid changed (179).

Xanthine oxidase and coenzyme A levels. Vitamin E depressed liver xanthine oxidase levels in rats fed low protein diets. Methionine countered the effect, but cystine had little value. The coenzyme A level in livers from necrotic rats was also depressed. Dietary methionine seemed to be diverted by vitamin E into other channels than the synthesis of xanthine oxidase. Low levels of coenzyme A in liver necrosis might be due to a defect in the utilization of cystine for formation of coenzyme A (180). The rate of incorporation of ^{35}S -cystine into liver coenzyme A was also depressed. The diet used also caused

decreased oxidative activity of the Kreb's cycle and decreased lipogenesis. The incorporation of cystine into liver protein proceeded at a high rate (181). The suggestion was made that vitamin E might regulate sulfur amino acid metabolism (182). Dietary selenium as selenite increased incorporation of ^{35}S -cystine into coenzyme A and liver protein in the rat (112).

In contrast, other workers believed that the coenzyme A level was related to growth. If the animal was growing, there was no difference in the coenzyme A level (183).

Determination of Vitamin E

Analysis for vitamin E is complicated because most of the analytical methods measure total reducing materials present and are not specific for tocopherol. Such substances are chromenols, reduced ubiquinones, and other antioxidants. Not all tocopherols are equally active biologically (184).

Evaluation of Vitamin E Status

The vitamin E status in the rat cannot be simply defined. Levels of vitamin E in serum and fat do not change enough to be meaningful. Other tissues must be assayed. Heart muscle analysis has been suggested. The level of the vitamin in the adrenal glands is high even in vitamin E deficiency. This is another factor indicating that the function of the vitamin is not simply that of an antioxidant, because tissues most subject to peroxidation do not have the highest levels of the vitamin

(185). Dietary selenite had no effect in changing tocopherol content of rat liver (112).

Transport and Storage of Vitamin E

Most of the ingested vitamin E goes to the liver, but transfer to other tissues is rapid. In man most of the vitamin is in adipose tissue and only a little is in the liver. Generally, it increases with age, but there is a decrease in old age. In rats removed from their vitamin E-deficient and -sufficient mothers on the 21st day of gestation the tocopherol levels were almost the same. The vitamin E-sufficient mothers had lower levels of the vitamin in sera, but liver and muscle stores were higher (186).

There is a high concentration of vitamin E in adrenals, nerves, heart, and uterus (187). In chicken liver on the basis of nitrogen content 20-23 per cent of the vitamin was in the nuclei, 44-45 per cent in the mitochondria, and 33-35 per cent in the supernatant (188). DPPD was found mainly in the soluble fraction of liver cells, mainly in the microsomes. Its distribution pattern was similar to that for total lipids and not for vitamin E (189).

There is a lipid present in yeast with certain properties similar to vitamin E (190). This lipid is probably ubichromenol (191).

II. SULFUR METABOLISM

Sulfur metabolism has been studied in animals both in vivo and in vitro. Many of the suggested mechanisms have been based on feeding

experiments, with accompanying measurements of varying metabolites in urine and feces or in blood or other body tissues. Similar measurements have been carried out after injection of radioactive isotopes. Another approach to the problem has been measurement or identification of products produced by incubation of tissue preparations and attempts to relate the results to in vivo work.

Absorption

Several studies have been carried out on the absorption of sulfur compounds. L-, DL-, and meso-cystine were fed to fasted white rats by stomach tube. The rates of absorption were calculated by determination of the cystine in the gastrointestinal tract after 1, 2, and 3 hours. For L- the rate was 49.9; D-, 45.6; and DL-, 53.6 mg. per 100 g. per hr. But the rate for meso-cystine was 41.3 mg. per 100 g. per hr. Cysteine hydrochloride was slowly absorbed, 25.7 mg. per 100 g., per hr., but the sodium salt was absorbed faster, 41.4 mg. per 100 g. per hr. The rates of absorption for D-, L-, and DL-methionine as the sodium salts were 35.6, 35.7, and 38.2 mg. per 100 g. per hr. respectively (192). There was evidence of cystine malabsorption in the small intestine in cystinuria (193).

In Vivo Studies

Balance experiments. Sulfur balance studies have been conducted in rats to determine the requirements for dietary sulfur compounds. In

starvation the sulfur loss was approximately 36 mg. per kg. per day. The deficit dropped with the length of time the diet was fed. The decrease was in fecal sulfur, but there was more sulfur per gram of feces than when the animal was eating. The neutral sulfur in urine increased, but free and conjugated sulfur decreased. When the animal was fed a sulfur-free diet, the loss was about 26 mg. per kg. per day. There was weight loss in spite of good eating. Free sulfate disappeared entirely in both urine and feces, and only neutral sulfur appeared in feces (194). On similar diets to which methionine was added at the level of 0.053 to 0.15 parts per 100 parts sulfur or homocysteine at 0.094 parts per 100 parts sulfur, only the rats receiving the most sulfur had free sulfate in the urine. The conjugated urinary sulfate was constant and independent of the diet. The neutral sulfur was high. At least half of the sulfur excreted was in the feces and this was independent of ingestion. The requirement for methionine in the adult rat was determined to be 30 mg. per kg. per day (195).

In other sulfur balance experiments in rats cystine and cysteine were employed as sulfur sources. Cystine was less beneficial than cysteine, although the fecal excretion of cysteine was higher. Conclusions were that cysteine could replace 87 per cent of the methionine and cystine, 66 per cent (196).

In rats fed an otherwise sulfur-free diet sodium sulfate was completely absorbed. Sulfate would cover all a rat's needs for sulfur compounds except that necessary for protein metabolism (197).

When cats were fed a 3 per cent nitrogen, artificial amino acid diet, removal of the sulfur amino acids had no effect on the rate of nitrogen loss. The cat apparently made methionine and cystine from inorganic sulfate and has no exogenous requirement for the sulfur amino acids (198).

If there was no other sulfate in the diet, sodium sulfate could increase the growth and feed efficiency of chickens. The chick could satisfy part of its total sulfur requirement with inorganic sulfate. Large amounts of methionine were incapable of satisfying the total sulfur requirement when fed to chicks on low sulfate-low cystine diets (199).

Feeding and injection studies. When ^{35}S -methionine was fed to rats, ^{35}S -cystine was produced, but when ^{35}S -sulfate was fed to rats, no cystine was produced. This indicated that the animal could not produce cystine from sulfate. Cysteine did not exchange sulfur with hydrogen sulfide (200). Methionine also produced taurine in the dog (201). ^{35}S -Methionine could be incorporated into the tissue protein of the dog even after hepatectomy. Methionine could be converted to cystine in such tissues as the kidney, pancreas, and intestinal mucosa (202).

Most of the extra sulfur in urine after the administration of DL-methionine and L-cystine, either oral or injected, was excreted as sulfate. Disulfide compounds were found in the urine of the rabbit

after the administration of methionine. When the α -amino group of methionine was blocked, the sulfur excreted in the urine was in the organic sulfur fraction. The mechanism suggested is that the primary reaction in the catabolism of methionine is demethylation (203).

In cystinuria in man homocystine, like cystine, could be completely oxidized. Homocysteine was excreted as cystine and homocystine. Not much was oxidized to sulfate. Homocysteine is apparently the first major intermediate in the conversion of methionine to cysteine (204). In the conversion of methionine to cysteine in vivo the sulfur of cysteine, but not the carbon chain, came from methionine (205).

When the cystine excretion of normal humans was measured, there was a positive correlation between cystine and neutral sulfur and between urinary cystine and creatinine, but there was no correlation between total nitrogen or body weight and cystine excretion. Man excretes a fairly constant amount of cystine regardless of diet (206). When L-cystine, L-cysteine hydrochloride, L-methionine, L-cystine disulfoxide, L-cysteinesulfinic acid, L-cysteic acid, and DL-methionine were administered, cysteinesulfinic acid was poorly oxidized to sulfate, cysteic acid oxidation was negligible, and cysteine was not metabolized in the disulfide form. Since the oxidation of cysteine was greater than that of cystine, which in turn exceeded cystine disulfoxide, cysteinesulfinic acid, and cysteic acid, cysteinesulfinic acid was thought not to be an intermediate in the oxidation of cysteine to inorganic sulfate in man. Deamination must have occurred early in the process (207).

Hypotaurine was isolated from extracts of urine from rats fed a diet of 6 per cent cystine (208). After administration of ^{35}S -DL-cystine, taurine, hypotaurine, thiotaurine, thiazolidine carboxylic acid, and cysteinesulfonate were isolated from a kidney extract and from urine (209).

The inner sulfur atom of thiosulfate was eliminated rapidly in rats as sulfate, while the outer was transformed into sulfate much more slowly. Low sulfate and thiosulfate in the urine did not necessarily mean low thiosulfate in the animal, because the thiosulfate pool might be large. When ^{35}S -DL-cystine and thiosulfate, or the cystine alone, were injected and the urine collected, cystine without thiosulfate produced almost no thiosulfate in the urine, but with thiosulfate 33 per cent of the sulfur of cysteine was excreted in the form of thiosulfate in 24 hours. This was interpreted as meaning that the large dose of thiosulfate inhibited the oxidation of endogenous thiosulfate (210).

Rhodanese catalyzed exchange of ^{35}S between sulfite and thiosulfate. When ^{35}S -cystine was injected into rats along with thiosulfate, more ^{35}S appeared in the urine as thiosulfate and less as sulfate. This indicated that thiosulfate is an important metabolic precursor for sulfate. Sulfinates also participated in rhodanese-catalyzed transulfuration reactions (211).

When cystine was fed to a rabbit, the oxidation to sulfate began within three hours after absorption from the intestine and reached a

maximum at eight hours. Most of the excess sulfate was not conjugated (212). There was an increased excretion of sulfate in urine from rabbits fed β -mercaptopyruvate (213).

In rats fed a pyridoxine-deficient diet there was no increase in taurine excretion after a cysteine load or after a cysteine and vitamin B₆ load. In a normal animal there was an increase in taurine excretion after both treatments (214). When cysteinesulfinic acid was administered to pyridoxine-deficient rats, measurement of the urinary products indicated that the desulfination reaction still was operating normally (215). In man vitamin B₆ depletion resulted in failure to respond to load doses of cysteine by increased taurine excretion, but when both cysteine and vitamin B₆ were administered at the same time, increased taurine excretion occurred. The level of vitamin B₆ did not affect sulfate excretion or nitrogen balance (216). Injection of cysteinesulfinic acid into sheep caused an increase in the thiosulfate excreted in the urine (217).

Hypotaurine administered to rats was metabolized to sulfate and taurine in the urine (218). When taurine was administered to man, the increase in sulfur excretion in urine occurred in the neutral sulfur fraction (219).

When ³⁵S-N-acetyl-D-glucosamine-6-O-sulfate and other esters were injected and urine and feces collected, most of the radioactivity appeared in the inorganic and ester sulfate fraction within 24 hours. Since some of the sulfate ester is metabolized to sulfate, the excre-

tory ester sulfate could represent direct metabolism of the ester or reincorporation of the sulfate formed (220).

Vitamin A has some influence on sulfur metabolism in the rat. Excess vitamin A did not inhibit the oxidation to sulfate of ^{35}S -methionine or of ^{35}S -cystine, since the same proportion of total ^{35}S excreted in the urine of both control and hypervitaminotic rats occurred as $^{35}\text{SO}_4$. When single or repeated vitamin A injections were given to rats treated previously with ^{35}S -methionine, a larger amount of inorganic $^{35}\text{SO}_4$ was excreted than in the controls. After injection of $\text{Na}_2^{35}\text{SO}_4$, vitamin A-treated rats showed a more rapid decrease in the specific activity of urinary inorganic sulfate than control rats (221).

Low protein diets caused reduced urinary excretion of injected ^{35}S -taurine. The finding that the urinary excretion of taurine is dependent upon dosage indicated a renal threshold for taurine or a limited pool size for taurine or its metabolic products (222).

Tissue and excretory taurine increased in rats during fasting. Tissue cystine and methionine also increased, but there was no correlation between urinary excretion of cystine and taurine. No cysteic acid could be detected in the tissues or urine of fasted rats. Taurine in zein-fed rats remained essentially constant when compared to the levels in fasted rats. This indicated that the normal course of oxidative processes of the sulfur-containing amino acids might be impaired in fasting and that the conversion of cysteine to taurine became predominant (223).

Data on urinary sulfate excretion indicated that dogs oxidized L- and DL-cystine, L- and DL-cysteine, and glutathione to the same degree

(224). After oral administration of cholic acid to fasted bile fistula dogs for several days, the oral administration of methionine, homocysteine, cysteine, cystine, or cysteic acid with the cholic acid resulted in an increased production of taurocholic acid in the bile while no extra taurocholic acid was produced from homocystine. Injection of methionine sulfoxide, homocysteine, cysteine, cystine disulfoxide, cysteic acid, cysteine-sulfinic acid, cystamine dihydrochloride, and thioglycolic acid after cholic acid feeding caused no increase in taurocholic acid formation with the thioglycolic acid or cystamine dihydrochloride, but some increase with the others. These results indicated that dogs could not make taurine from thioglycolic acid, cystamine dihydrochloride, or homocystine, but could from the other compounds tested. Most of the excess sulfur excreted in the urine after injection of homocysteine, cystine disulfoxide, cysteine, cystine, cysteinesulfinic acid, cystamine dihydrochloride, and thioglycolic acid was as sulfate (225-229). The urinary sulfur excretion patterns indicated that the sulfur of homocystine and homocysteine was oxidized to approximately the same extent as the sulfur of cystine or methionine (225). With methionine sulfoxide much of the extra urinary sulfur appeared as organic sulfur, indicating that the oxidation of this compound to sulfate was more difficult than the oxidation of methionine to sulfate (226). Very little of the sulfur of cysteic acid was oxidized to sulfate (229).

Bile loss aggravated a deficiency of organic sulfur in the rat. ^{35}S -taurocholate feeding led to ^{35}S excretion in the urine of rats. In rats fed a chow diet and treated with ^{35}S -taurocholate, 17 per cent of

the sulfur administered was found in the bile and 45 per cent in the urine. The animals fed a low organic sulfur diet had an equivalent amount of sulfur in the bile, but less in the urine, and the specific activity of the bile sulfur was higher (230).

The feeding of α -protein instead of casein led to hypercholesterolemia. This was partially prevented by methionine supplementation. There was little correlation between liver cholesterol and serum cholesterol in rats, and no atherosclerosis (231). Diets high in cholesterol and low in sulfur amino acids produced atherosclerosis in Cebus monkeys (232).

Since transport systems of sulfate, phosphate, amino acids, and glucose are functionally interrelated, the same anatomical site is probably common for all (233). Sulfate and thiosulfate mutually and strongly inhibited each other's transport through the renal tubules by a competitive mechanism and share the same absorption mechanism (234).

With toxic levels of selenite there was abnormal bone and cartilage development in chick embryos. This suggested disturbed sulfate metabolism. There was a significant increase in the μ moles of ^{35}S -sulfate incorporated per mole of glucuronic acid and in the taurine content in the animals fed excess selenium, but the μ mole of ^{35}S -sulfate incorporated per mole of taurine was the same in both sets of animals (235).

When gypsum was used as fertilizer on pastures, it appeared that excess sulfur in the herbage might be involved in the etiology of muscular dystrophy. There was a high incidence of muscular dystrophy with high inorganic sulfate in basal rations. Excess sulfur must interfere

with the metabolism of selenium. Adding sulfate to a dystrophogenic diet containing selenium reduced the prophylactic efficacy of selenium (236).

The effects of sulfate on the toxicity of selenate, selenite, and seleniferous wheat were measured, as well as the effect of sulfite and thiosulfate on selenium toxicity. Addition of sulfate to the rations did not relieve the toxicity of the wheat. Only when the toxic selenium compound was selenate did administration of sulfate help (237). Sulfate seemed to affect urinary selenium excretion (238).

The quokka, a marsupial with digestion similar to ruminants, eats plants deficient in copper and cobalt, high in sulfate, and moderate in molybdenum. When half the animals fed such plants were given sulfate after all had been pretreated with molybdenum in addition to the normal feed, there was a decrease in whole blood molybdenum and increased molybdenum excretion in urine (239). In general, with a constant molybdenum intake, blood levels of molybdenum decreased with increasing sulfate. Supplementation of the diet with methionine or sodium sulfide was not so effective as giving sulfate. Tissue retention of sulfur was depressed by dietary molybdenum, but the total sulfur of bones in molybdenum toxicity was increased. This toxicity was more critical on low protein diets. High dietary molybdenum induced copper deficiency, and copper toxicity was cured by molybdenum. There was a similarity of behavior of molybdenum to phosphorus (240). Molybdenum may exert an in vivo hydrolytic effect on phosphate esters. Molybdenum led to an increase in urinary and fecal phosphate in the rat (241).

Copper deficiency during molybdate toxicity is reported to be caused by an accumulation of sulfide produced by sulfide oxidase. Excess cystine aggravated the symptoms. An increase in dietary copper or inclusion of dietary sulfate ameliorated the symptoms (242).

Sulfate may affect selenate transfer across membranes as postulated for molybdenum. There should be a common mechanism of alleviation by sulfate of selenate and molybdate toxicities (243).

Cystathionine has been identified by paper chromatography as a constituent of the liver following feeding of both L-methionine and L-serine to a fasting rat. L-methionine alone was ineffective in cystathionine production (244). When cysteine was injected into the rat, there was increased alanine in the liver. The taurine-glycine concentration also increased. This warranted the assumption that taurine and alanine were formed in the cysteine-injected animal (245). After injection of ^{35}S -cysteine into a rat, sulfate incorporation was found in the liver before hypotaurine and this before taurine (246). Hypotaurine was also identified as an intermediate (247). Injection of cysteine-sulfinic acid led to the production of alanine and hypotaurine in rat liver (248). Cysteic acid and hypotaurine have been identified in rat brain (249).

Exclusive of synthesis in the digestive tract and exchange between the sulfur of cysteinesulfinic acid and sulfite, the sulfur of sulfite, but not sulfate, was utilized by the rabbit to make cystine, cysteine-sulfinic acid, and taurine. Cysteinesulfinic acid was apparently the precursor for cystine and taurine. After administration of $^{35}\text{SO}_3^-$,

^{35}S -cysteinesulfinic acid was identified in extracts of organs from bacterially sterilized or eviscerated animals (250).

Taurine is a major component of the amino acid fraction of most organs. The level is highest in the heart and varies in the liver (251). In the rat the liver taurine level is 0.1 to 0.6 mg. per g. of liver for the male and 0.7 to 1.1 mg. per g. for the female. Since these values do not correspond to the cysteic acid decarboxylase activity, there may be other pathways for the formation of taurine in the rat (252).

When ^{35}S -taurine was administered to chick embryos, day-old chicks, and rats, there was some incorporation into tissues. This does not fit with the classical description of taurine as a biologically inactive end-product of cystine metabolism (222).

When the laying hen was injected with ^{35}S -sulfate, ^{35}S was present in the albumen of the egg as dialyzable and non-dialyzable sulfate and organic sulfur. ^{35}S was present in the yolk primarily as dialyzable sulfate and non-dialyzable organic sulfur. Eighty per cent of the non-dialyzable sulfate in the egg yolk was attached to the yolk membrane. Some radiation was present in cystine, but none was found in methionine. The hen could incorporate sulfate into cysteine (253). The chick embryo, however, incorporated sulfate into taurine rather than into cystine. Over 65 per cent of the sulfate administered to a 24 hour embryo was recovered as taurine in the day-old chick, presumably in the absence of microorganisms. Taurine was free in tissues and could not be considered primarily as a part of taurocholic acid (254).

In studies of the incorporation of uniformly labeled ^{14}C -glucose into the hexosamine part of cartilage mucopolysaccharides, the isotope appeared in the galactosamine part of the light mucoprotein fraction and then in the insoluble residue. The label went into chondroitin sulfate first before it entered keratosulfate, suggesting that different compounds were precursors. Cortisone inhibited sulfate fixation. It may be that cortisone had no direct effect on sulfate fixation or chain synthesis but influenced the availability of simple precursors for the formation of the polysaccharide chain. This may be related to capillary permeability or diffusion of nutrients to the cell responsible for synthesis (255).

The presence of an active sulfate-synthesizing enzyme system in cultivated connective tissue was determined. Granulomata activity of the PAPS- (3'phosphoadenosine-5'-phosphosulfate) synthesizing system was at a maximum 96 hours after implantation of a cotton pellet and 48 hours after implantation of a cotton pellet with carrageenin. High doses of cortisone inhibited the PAPS-synthesizing system in both the ordinary granulomata and in those induced by carrageenin. Sulfate was transferred from PAPS to the mucopolysaccharide at the mucopolysaccharide level. The rate of mucopolysaccharide synthesis was sex dependent, but there was no difference in the sulfate-activating enzyme system in either male or female rats. The rate of mucopolysaccharide synthesis for the female was half that of the male (256).

After intraperitoneal injections of ^{35}S in the rat, of the tissues measured the bone marrow had the most ^{35}S regardless of whether the source was heptylaldehyde bisulfite, cinnamaldehyde bisulfite, or sodium sul-

fate (257). After administration of ^{35}S -sodium sulfate, most of the radiation was found in chondroitin sulfate and some in taurine, with the maximum radiation occurring in chondroitin sulfate at 24 hours and in taurine at 8 hours (258). Rats could convert 0.02 per cent of a dose of ^{35}S -sodium sulfate to cystine (259). Most of the ^{35}S -sulfate was concentrated in the suckling rat in the knee joint (260). Sulfate increased in the bone up to 8 hours and in the bone marrow up to 24 hours after intraperitoneal administration of ^{35}S -sodium sulfate. The administration of 1 mg. of ^{35}S -sodium sulfate led to the excretion of 95 per cent of the dose in the urine after 5 days (261). In the rat fetus the level of ^{35}S in the kidneys, liver, and lungs corresponded to the level in the pregnant rat. The level was lower in the fetal gastrointestinal tract. The level of radiation in the fetal humerus was three times that in the maternal sternum, fifteen times greater per gram of embryonic biceps femoris than in the pregnant rat, and more per gram in embryonic brain and skin. The older embryos incorporated more radiation than the younger in the same length of time. There was definite transfer of sulfate to the fetus (262).

In the detoxication of phenols the first sulfur to be used was probably the sulfate in the cell fluids. When this was removed, it was replaced by oxidation products of metabolism. When the fluids were depleted by administration of phenols, sulfate accumulated slowly over a series of days as it arose from the metabolism or diet until excretion became normal. The dog could utilize oral or injected sodium sulfate to make phenyl or indoxyl sulfate from phenol or indole (263).

Sulfate did not help in growth resumption after growth was stopped by the administration of p-bromophenol, but cystine did restore growth. This resumption correlated with increased excretion of ethereal sulfates (264).

In Vitro Studies

A summary of the possible pathways for the oxidation of the sulfur of cysteine to sulfate in animals is given in Figure 5.

Cystathionine as substrate. In vitro metabolism of the sulfur compounds has been studied extensively. An enzyme for the cleavage of cystathionine has been isolated. It did not produce ammonia from DL-serine or homoserine, but did produce hydrogen sulfide from cystine. It seemed to be cysteine desulfurase (265). A dark red enzyme from liver catalyzed the deamination of homoserine and cleavage of cystathionine. Cysteine, a product, inhibited (266). It is unlikely that homoserine was derived metabolically from cystathionine in the liver of the rat. No radioactivity could be found in homoserine following cleavage of ^{14}C -cystathionine by a crystalline enzyme of rat liver, but ^{14}C -cystathionine could be produced from ^{14}C -homoserine and cysteine (267). The cystathionine synthetase-serine dehydrase activity was repressed when rats were fed low methionine-cystine diets for a week. Cysteine added to the incubation mixture did not inhibit the enzyme (268).

Cystathionase is probably identical with soluble cysteine desulfurase and perhaps the same as serine dehydrase and threonine

dehydrase (269). Cysteine desulfhydrase activity was lower in rats treated with thyroxine and greater in thyroidectomized rats (270-272). The enzyme activity was not changed after adrenalectomy but increased after the administration of hydrocortisone. When rats were injected with cysteine, L-methionine, or DL-homoserine and the hydrogen sulfide formation measured after sacrifice of the animal, cysteine administration had no effect on hydrogen sulfide formation, but homoserine and methionine increased it. The enzyme was apparently adaptive (271).

Cystathionase in rat liver acted upon cystine and hypotaaurine to make thiotaurine. A similar reaction took place with cysteine. This reaction was apparently due to the oxidation of cysteine to cystine, because no pyruvic acid was produced when cysteine was used in an anaerobic system, but pyruvic acid was produced under similar circumstances from cystine. The proposed mechanism is that two cysteines combine to form cystine, which then forms thiocysteine and pyruvate. The thiocysteine reacts with a compound such as cysteinesulfinic acid to form cysteine and thiocysteinesulfinic acid (273). Sulfite can react with cystine and cystamine to form S-sulfocysteine and S-sulfocysteamine (274). It has been reported with another incubation system, that cystathionase acts on cysteine, not cystine (275).

In rat liver there is an enzyme which converts sulfocysteine to thiosulfate, ammonia, and pyruvate. No thiosulfate was produced from aminoethylthiosulfuric acid. Sulfite was formed from both thiosulfate esters under anaerobic conditions, and the production was augmented by

heat denaturation. Sulfite could be detected under anaerobic conditions (276). Rhodanese could be reactivated by sulfhydryl compounds such as cysteamine, mercaptoethanol, or thioglycolate (277).

D-amine oxidase from hog kidney acted upon cystamine to form cystaldimine and other products. With labeled compounds thiotaurine was produced and thiocysteamine was suggested as the intermediate. The pathway may involve cystamine to cystaldimine to thiocysteamine to thiotaurine and hypotaurine (278). Thiocysteamine could be decomposed to cysteamine and sulfur (279). There is also an enzyme that catalyzes the oxidation of cysteamine to hypotaurine in the presence of sulfide or sulfur. The sulfur may function as a hydrogen carrier to oxygen (280, 281). Certain reducible dyes may be used as cofactors for the enzymic oxidation of cysteamine to hypotaurine (280). Alanine thio-sulfonic acid reacts with α -ketoglutaric acid in the presence of rat liver mitochondria to form thiosulfate. This reaction took place without the consumption of oxygen and suggested elimination of sulfite and sulfur rather than reaction to give thiosulfate (282). The oxidation of hypotaurine required oxygen (283). The oxidation of hypotaurine has also been studied by Sumizu (284).

Cysteine as substrate. Cysteine can be split in vitro in many ways. One of these is a direct desulfurization producing pyruvate, ammonia, and hydrogen sulfide (285). In rat liver the enzyme for the direct desulfhydration is in the supernatant fraction (286). Cystine is thought by some to be the true substrate for desulfhydration. β -

Mercaptopyruvate was used as an artificial substrate because it and hypotaurine should react to form thiotaurine, and this should be an indication of cystine transamination. If hypotaurine could derive its sulfur from both β -mercaptopyruvate and thiocysteine, then hypotaurine should be derivable from cysteine by two pathways. Since no α -ketoglutarate was required, the conversion must involve cystathionase (285).

A transaminative mechanism of degradation of cysteine has also been reported. The transaminase is in the particulate fraction (286). Cysteine and α -ketoglutarate were converted to glutamate and what should be β -mercaptopyruvate in heart and liver preparations. The β -mercaptopyruvate should decompose to sulfur or sulfide and pyruvic acid (287). β -Mercaptopyruvate desulfurase has been found in the particulate fraction (288).

Both ammonia and hydrogen sulfide were formed during incubation of L-cysteine with liver extracts, but hydrogen sulfide production was always greater than the production of ammonia. α -Ketoglutarate had no effect, but pyridoxal phosphate caused a rise. Glutamic acid and alanine were produced without cysteine in the presence of α -ketoglutarate. The suggestion was made that both transamination and desulfuration reactions operate (289). Dog and rat liver extracts could remove hydrogen sulfide from cysteine anaerobically without the production of ammonia. There was greater activity with L-cysteine than with D-cysteine (290). No hydrogen sulfide could be isolated from a living animal; so the place of this in vitro enzyme was questioned (291).

Two soluble rat liver enzymes, one heat stable and the other heat labile, were required to catalyze the aerobic oxidation of sulfide to thiosulfate (292). No polythionate was formed from sulfide in liver. Free heme was an active catalyst for the oxidation of sulfide. Sulfide oxidase activity could be demonstrated only under rather unphysiological conditions, and thiosulfate probably arose from β -mercaptopyruvate and sulfite. The sulfide oxidase activity of liver homogenates was probably due to the presence of hemoglobin in these preparations (293). If sulfite was replaced by sulfinic acid, thiosulfinates could be formed (294).

Serine sulfhydrylase in chick liver will degrade cysteine to serine and hydrogen sulfide (295). The enzyme could be reduced by low protein or protein-free diets and increased by high protein diets rich in gelatin. L-cysteine, L-histidine, cortisone, histamine, fasting, and thiouracil increased activity. Methionine, glutamic acid, arginine and thyroprotein decreased activity (296).

Mn^{+2} was found to inhibit cystine oxidase, as measured by oxygen uptake in rat liver, but there was no inhibition in kidney and brain (297). In addition to the formation of cystine from the reaction of cysteine with oxygen in tissues, sulfate, carbon dioxide, and sulfite were also formed. Cysteic acid did not produce sulfate. There was a suggestion of a feed-back mechanism with excess taurine or cysteic acid leading to sulfate production (298). In rat liver 3 per cent of the cysteine given formed sulfate and 65-89 per cent formed cystine

(299). Liver and kidney had about the same activity toward cysteine, but blood, testis, spleen, heart, and lung of the rat were inactive. Cysteine, rather than cystine, was the preferred substrate. Liver and kidney could also oxidize methionine (300).

In an aerobic reaction cysteine could be converted to cysteinesulfinic acid by enzymes from rat liver. Hydroxylamine was used to inhibit the conversion of cysteinesulfinic acid to other compounds. Fe^{+2} and NADPH were cofactors. ATP, hypoxanthine, cytochrome c, hydrogen peroxide, Mg^{+2} , Mn^{+2} , and Cu^{+2} had no effect on stimulating cysteinesulfinic acid formation (301). The role of cysteinesulfinic acid in the production of sulfate from cysteine has been questioned as a pathway for rat liver mitochondria (302). However, a supernatant preparation from liver did produce cysteinesulfinic acid from cysteine. Less cysteinesulfinic acid was produced when ^{35}S -cystine was incubated with the enzyme preparation. Both ^{35}S -glutathione and ^{35}S -cysteine produced cysteinesulfinic acid (303). Cysteinesulfinic acid has also been isolated from an incubation medium after incubation of a rat liver homogenate with cysteine (304).

When radioactive cystine was administered to the rat, 25 per cent of the radiation was found in hypotaurine and only 1 per cent in cysteinesulfinic acid. This indicated to the experimenters that there was a rapid turnover for cysteinesulfinic acid and that hypotaurine was derived metabolically from cysteinesulfinic acid and not from cysteic acid (305).

Cysteinesulfinic and cysteic acids as substrates. The two main in vivo and in vitro reactions in rats for cysteinesulfinic acid are desulfinating transamination to produce sulfite, which oxidizes to sulfate, and decarboxylation to form hypotaurine, which oxidizes to taurine. Both reactions should require pyridoxal phosphate. In the vitamin B₆-deficient rat taurine and hypotaurine disappeared from the urine, and the rat excreted cystathionine. Enzymes prepared from pyridoxine-deficient rat liver did not decarboxylate cysteinesulfinic acid, while transamination with α -ketoglutarate was not inhibited. Pyruvic acid transamination was inhibited, however. Decarboxylation of cysteinesulfinic acid was two to three times greater in male than in female rat liver. Removal of the ovaries in the female enhanced decarboxylation, while estradiol given to ovariectomized females made decarboxylation return to normal levels (306).

In fresh rat liver mitochondria there may be an NAD-linked enzyme which anaerobically converts cysteinesulfinic acid to β -sulfinyl pyruvate and ammonia (307, 308). In heart mitochondria sulfur accumulated as sulfite. In the opinion of the authors the accumulation of pyruvate and sulfate as the end-products of the aerobic metabolism of cysteine in animal tissues lent further support to the view that cysteinesulfinic acid is on the major pathway of the metabolism of cysteine (307). Alanine is also reportedly formed in rat liver from cysteinesulfinic acid. The pyruvate normally considered to arise from cysteinesulfinic acid by transamination should lead to the produc-

tion of alanine, but evidence indicates that alanine is not produced from pyruvate. This suggests that there is direct cleavage of the cysteinesulfinic acid to alanine and sulfite in rat liver (309, 310). The liver made more decarboxylated product than it did alanine and sulfite (311). Cysteinesulfinic acid led to increased carbon dioxide production by livers from the rabbit, rat, and dog, but not from the horse and cat. Taurine in man might be formed by a different pathway (312).

Decarboxylation of cysteic acid by livers from male rats was greater than by livers from female rats. The activity was equal in the male and female rat brain. Ovariectomy made the decarboxylase activity in the female approach that in the male, but there was more taurine in the female than in the male. This suggested that taurine formation in liver is independent of the decarboxylation of the sulfur amino acids (313). The enzyme for the decarboxylation of cysteinesulfinic acid and cysteic acid was thought by some to be the same in liver but not in brain (314-316), but not by others (317). Both the enzyme in liver and that in brain required free thiol groups and pyridoxal phosphate. The decarboxylase is a supernatant enzyme for liver, but the brain enzyme may be in the particulate fraction (315). There was no cysteinesulfinic or cysteic acid decarboxylase in mammalian heart tissue, but there was a high concentration of taurine in this tissue (316). The cysteic acid transaminase in heart muscle is probably a different enzyme from cysteine transaminase (287).

Cysteine was an inhibitor for the decarboxylation of cysteinesulfinic and cysteic acids in the rabbit. The brain enzyme also was active with glutamic acid, but the liver enzyme was active only with the sulfur acids (318). Isoniazide inhibited cysteinesulfinic acid decarboxylase. Hydroxylamine inhibition was reversible (319). Brain tissue from cat and man can decarboxylate cysteinesulfinic acid. The decarboxylation of cysteic acid and cysteinesulfinic acid was mutually inhibitory (316).

Cysteic acid may be on the pathway of taurine synthesis in the cat (320). Dog heart slices could make taurine from cystine. Isethionic acid (2-hydroxyethanesulfonic acid) was also produced. This acid may dominate the cell membrane potential by accumulating cations (321). Taurine, by serving as a precursor of isethionic acid, may have a role in regulating the irritability of cardiac tissue (322).

Sulfite as substrate. Ground embryonic calf liver could produce hypotaurine and cysteinesulfinic acid from sulfite, but not from taurine or cysteine. Cysteinesulfinic acid formation decreased in the presence of serine. Cysteinesulfinic acid could not be reduced to cystine under the conditions employed, but the reaction does take place in vivo (323).

A hemoprotein enzyme which oxidizes sulfite to sulfate has been partially purified from liver preparations. The enzyme is localized largely in the microsomal fraction of liver, heart, and kidney (324). Rat liver mitochondria could couple the oxidation of sulfite with the synthesis of ATP (325).

Sulfate and PAPS as substrates. There was a high level of sulfate incorporation into the colonic mucosa of sheep (326). PAPS was degraded by an enzyme from sheep brain. ADP inhibited the degradation, and Co^{+2} and Mn^{+2} activated it. The products of the degradation were ADP and sulfate (327). In the small intestine of the rat PAPS will form PAP-SO_3^- with NADPH in the presence of ATP. For the production of S-sulfogluthathione, $\text{PAPS} + \text{GS}^- (\text{glutathione}) \longrightarrow \text{GSSO}_3^- + \text{PAP}$ (3'-phosphoadenosine-5'-phosphate). Or $\text{PAPS} + \text{a reducing agent}$ will make $\text{PAP} + \text{SO}_3^-$ and $\text{SO}_3^- + \text{GSSG} \longrightarrow \text{GS}^- + \text{GSSO}_3^-$. The second reaction was considered to be the most likely. This reaction took place in the intestinal mucosa. There was no activity in rat liver (328). S-sulfogluthathione might be formed in vivo by the addition of a sulfate to a cysteinyl residue or of sulfite to cystinyl. Sulfate could be reduced in the rat intestine (329).

A rat liver homogenate had 20-50 times higher ^{35}S activity in the protein after incubation with H_2^{35}S than it did after incubation with $\text{H}_2^{35}\text{SO}_4$. After intracardial injection of either compound the protein radioactivity was 8 times higher with H_2^{35}S . The conclusion was that the rat was not able to reduce sulfate to sulfide (330).

The sulfokinase present in a tissue is related to the mucopolysaccharide component of the tissue. Chondroitin sulfate B could not replace chondroitin sulfate A or C as a sulfate acceptor, and chondroitin sulfate B was not in cartilage (331). The transfer of sulfate to chondroitin sulfate from PAPS was inhibited by sulfate for chondroitin

sulfate A, but not for chondroitin sulfate B (332). When a particle-free high-speed supernatant fraction from the rat liver was incubated with ATP, sulfate, and Mg^{+2} , the rate of formation of PAPS decreased with increasing length of incubation time and eventually the amount of PAPS actually decreased. This could be because sulfate was being transferred to various acceptors such as phenols and steroids. Versene suppressed the formation of PAPS and inhibited the 3'-nucleotidase of rat liver which degrades PAPS to APS (adenosine phosphosulfate). In the absence of ATP and Mg^{+2} PAPS produced sulfate (333).

PAPS served as a sulfate donor to heparin in the mouse mast cell tumor (334). PAPS with a cell-free extract of human adrenals led to the sulfation of testosterone and some other steroids, but not to sulfation of cortisone, hydrocortisone, or aldosterone (335). Chondroitin did not act as a sulfate acceptor nor influence the incorporation of glucose into chondroitin sulfate (336). A priming mechanism for the synthesis of isomeric chondroitin sulfate is proposed (337).

The inner egg-shell membrane, which contains a sulfated mucopolysaccharide, is secreted by the isthmus of the hen oviduct. Many mucopolysaccharides accept sulfate from PAPS, but the rate varies for each acceptor (338). PAPS was a precursor for the sulfated polysaccharides in the isthmus of the hen oviduct (339).

Phenol conjugation almost disappeared in the absence of sulfate in one strain of rats (340). A 10 per cent fresh liver homogenate from rat bound sulfate to phenol (341). PAP was a product rather than a par-

ticipant in the enzymic conjugation of phenols with sulfate. The PAPS available for sulfation might be regulated by a nucleotidase capable of degrading PAPS (342).

Non-Enzymic Reactions

Many non-enzymic reactions led to the production of the various metabolites proposed for cysteine. Excess thiols reduce disulfides by thiol-disulfide exchange (343). Cysteine, alanine thiosulfonic acid, S-sulfocysteine, cysteic acid, ammonia, and thiosulfate were formed from the oxidative degradation of cystine in alkaline medium with copper (344). Pyridoxal and Cu^{+2} at pH 8.5 converted cystine to pyruvate, ammonia, carbon dioxide, sulfur, thiosulfonate, cysteinesulfinic acid, and alanine thiosulfonate. Sulfonates make more thiosulfonates. Thiopyruvic acid transulfurates sulfonates. The apparent pathway is given in Figure 6. The transulfuration of cysteinesulfuric acid leads to alanine thiosulfonate. Cysteinesulfinic acid and thiosulfate come from the hydrolytic cleavage of thiocystine (345).

The detachment of the thiol of cysteine by pyridoxal and metals such as aluminum or vanadium is reversible. Cysteic acid is synthesized in the presence of sulfite (346).

Cysteamine will react with oxygen and sulfur or sulfide to make cystamine. Methylene blue is also active in place of oxygen. The sulfide can also react with oxygen to produce sulfur and polysulfide (280).

The dismutation of cystine disulfoxide produces the sulfinic acid, which is the most stable oxidation product. Cystine and water

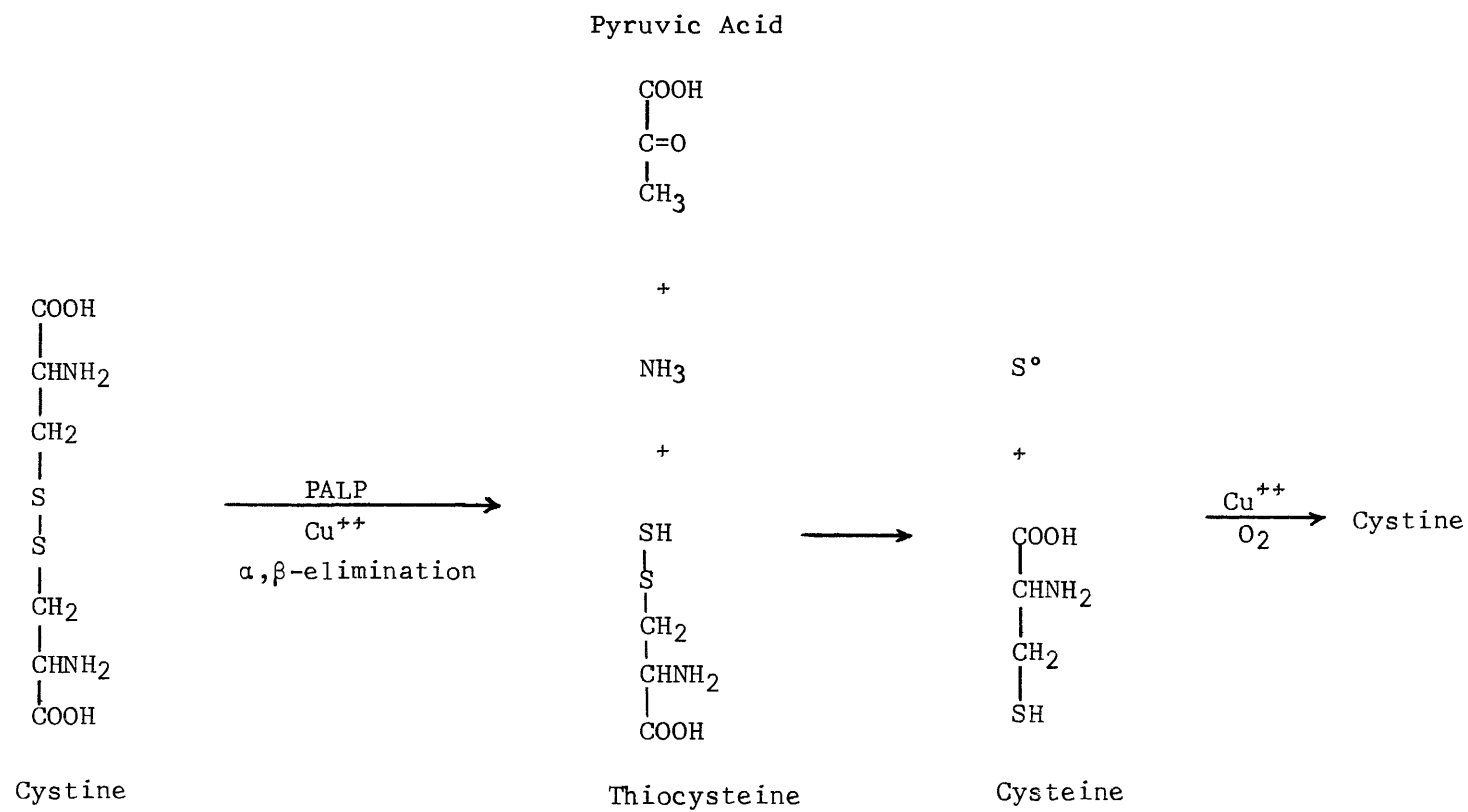


Figure 6. Non-enzymic cleavage of cystine (345).

will make cysteine and cysteinesulfinic acid. The sulfenic acid produces cystine and cysteinesulfinic acid (347). Cysteic acid can also be produced from β -substituted amino acids, pyruvate, and copper ions. Cysteinesulfinic acid and sulfite make cysteic acid. Cysteic acid can come from serine, phosphoserine, or cysteinesulfinic acid in the presence of Cu^{+2} and pyruvate or pyridoxal. With pyridoxal other metals can be used to replace copper, but copper is specific for pyruvate (348).

Many of the inorganic sulfur compounds available are either not so pure as the label would indicate or decompose rapidly in solution. When metabolites are measured, the lack of purity can lead to false conclusions (349).

The catalytic oxidation of glutathione and other sulfhydryl compounds by various inorganic compounds has been studied. Selenate and selenite were equally active catalysts for glutathione oxidation. Tellurite inhibited selenite oxidation but did oxidize glutathione. Sulfite did not inhibit selenite catalysis or catalyze glutathione oxidation. Arsenite inhibited glutathione oxidation when added 30 minutes before selenite, but not when added simultaneously. Selenite is a better catalyst for cysteine oxidation than copper. It is also a good catalyst for dihydrolipoic acid and coenzyme A oxidation (350).

Methods

Several methods have been used for the separation of various sulfur metabolites by passage through ion exchange resins (351-358).

When acid paper chromatography solutions were used with taurine and related compounds, streaking occurred in the chromatograms. Urea will pass through resins (351). In a phenol-ammonia solvent the $\underline{R_f}$ values for taurine and glycine and for cysteinesulfinic acid and aspartic acid were similar (359). Iodine, potassium iodide in hydrochloric acid, and potassium iodoplatinate sprays, as well as ninhydrin, have been used to develop the chromatograms (360-362).

CHAPTER III

EXPERIMENTAL

I. GENERAL PLAN

Since previous results in this laboratory had indicated that vitamin E was necessary in vivo not only for the optimal sulfation of mucopolysaccharides, but also for the oxidation of neutral sulfur to sulfate (1), the ability of whole liver homogenates from vitamin E-sufficient and -deficient rats to convert cysteine to sulfate was investigated. Then an attempt was made to determine the specific site of the decreased oxidation of cysteine or its intermediate oxidation products. Low sulfate diets were employed to force the animals to satisfy their inorganic sulfate needs by the oxidation of the sulfur amino acids.

Diets

The composition of the basic diets appears in Table 1. These diets were modifications of that of Pendergrass (363) originally modified from Caputto et al. (27). Similar diets have also been used in previous studies in this laboratory (364-367). The salt mixture was a modification of that of Hubbell et al. (368) to give 0.0002 per cent inorganic sulfate in the diet. The composition of the modified salt mixture appears in Table 2.

The basal diet was generally mixed in 4 to 6 kg. lots, the quantity prepared being determined by the amount of diet expected to

TABLE 1
COMPOSITION OF EXPERIMENTAL DIETS

Component	$\bar{s}CLO$ g./100 g.	+CLO g./100 g.	Brown ^a g./100 g.
Casein, vitamin free ^b	15.00	15.00	18.00
DL-Methionine ^b	0.35	0.35	--
Sucrose	30.35	30.35	32.00
Cornstarch	32.30	32.30	34.00
Stripped lard ^c	8.00	6.00	5.00
"Alphacel," non-nutritive bulk ^b	10.00	10.00	6.00
Cod-liver oil	--	2.00	1.00
Salt mixture	3.00 ^d	3.00 ^d	3.00 ^e
Vitamin mixture ^f	1.00	1.00	1.00

^aUnpublished.

^bNutritional Biochemicals Corp., Cleveland, Ohio.

^cDistillation Products Industries, Inc., Rochester, New York.

^dSee Table 2.

^eHubbell, Mendel and Wakeman Salt Mixture, Nutritional Biochemicals Corp., Cleveland, Ohio.

^fSynthetic vitamins added as supplement to each 100 g. of diet: (in mg.) 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-methylnaphthoquinone, 0.025; vitamin B₁₂, 0.005; choline chloride, 100.0; inositol, 100.0; p-aminobenzoic acid, 7.5. The vitamins were triturated with sufficient sucrose to make 1.0 g. DL- α -tocopherol acetate was added to the sufficient diets at the level of 28 mg. per 100 g. diet. Vitamin A acetate and vitamin D (Viosterol) were added to the diets $\bar{s}CLO$ at the level of 400 and 200 I. U. per 100 g. of diet, respectively.

TABLE 2
COMPOSITION OF SALT MIXTURE^a

Composition	Grams per 100 Grams
CaCO_3	44.750
MgCO_3	3.060
NaCl	6.900
KCl	11.200
KH_2PO_4	21.200
$\text{FePO}_4 \cdot 2\text{H}_2\text{O}$	2.050
KI	0.008
NaF	0.010
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.040
$\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$	0.017 ^b
$\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$	0.072
Cornstarch	10.693

^aHubbell et al. (368), as modified by Pendergrass (363).

^bCorrect weight only if the anhydrous salt is used.

be consumed completely in from 10 days to 2 weeks. At the time of preparation the diet was mixed thoroughly by hand and then sieved through household strainers several times to insure uniformity. The appropriate quantity of vitamin E (28 mg. per 100 g. diet) was added to half the quantity of diet at this time. This is the +E diet. The sieving process was repeated on this portion. The feed was stored in clean, dark-colored jars at refrigerator temperature until used. As precautions against autocatalytic rancidity, clean jars were used for each new diet preparation, and all remaining old diet was fed to the animals rather than being mixed with the new preparation. Feed and distilled water were given ad libitum until sacrifice. For the prevention of deterioration of feed in the cages the animals were generally fed daily only slightly more than the amount they were expected to consume. The cage papers were changed and any feed remaining in the feed cup was cleared of debris three times a week. If appreciable amounts of feed had been thrown from the feed cups to the papers below, this was sifted and re-fed. Distilled water was given fresh three times a week. Clean water bottles were used as needed. Stoppers for the bottles were kept clean by rinsing thoroughly in hot water each time the water was changed and then transferring them to new bottles. Feed cups were changed only on rare occasions. These precautions were necessitated by the fact that feed residue from the stock diet often became deposited on the inside of the stoppers and spouts and was incompletely washed out of the feed cups also used by the stock colony. Even though these appeared to be clean, the contamination from the high vitamin E content of the whole wheat,

whole milk stock diet seemed to prevent hemolysis on the vitamin E deficient diet. At no time did the diet smell rancid.

Animals

Selected littermate albino rats of the Wistar strain from the University of Tennessee stock colony were used as tissue donors. Both male and female rats were used, but each test group consisted of only one sex. The young rats were placed on vitamin E-sufficient and -deficient diets containing 0.0002 per cent sulfate (Table 1). Generally, the animals were weaned at 21 days of age and fed the experimental diets. Exceptions will be mentioned. At some time prior to sacrifice blood was withdrawn from tail cuts to establish vitamin E deficiency by dialuric acid hemolysis. Generally, except for one animal in the $\overline{\text{SCL0}}$ (without cod-liver oil) study, the animals were considered to be vitamin E deficient only when the hemolysis values from the animals fed the diet unsupplemented with vitamin E were greater than 90 per cent. The erythrocytes from the $\overline{\text{SE}}$ animals usually exhibited hemolysis after four weeks of feeding the experimental diet. No animals were tested earlier. Both the vitamin E-deficient animal and the -sufficient littermate were then killed on the same day, and all samples treated in parallel.

The term "prepped" refers to animals whose mothers had been fed the $\overline{\text{SE}}$ $\overline{\text{SCL0}}$ diet (Table 1) two weeks before the litters were weaned. These "prepped" animals were littermates to those used by Button in her Experiment 7 (364).

The animals were generally weighed weekly and checked for gross defects. The only defect generally noted was the reddish discharge on the whiskers noted by Button (364), which developed after about the fourth week of feeding the vitamin E-deficient diet.

Animals were housed in galvanized metal cages according to diet. When only a few animals were available at the start of an experiment, all, regardless of sex, were housed in the same cage. Generally, however, separation was also made by sex. Except for the first few weeks of the $\bar{s}$ CLO experiment, the animals fed the vitamin E-deficient diet were always housed above the animals fed the other diet.

For all but the experiment measuring cysteinesulfinic acid formation by the quantity of ninhydrin-positive material formed, the animals were decapitated after light ether anesthesia. In this experiment the animals were stunned by a blow to the head before decapitation.

In a supplementary study to measure the effect of increased organic and inorganic sulfur in the diet upon the ability of liver homogenates to oxidize the sulfur of cysteine to sulfate, livers were obtained from four pairs of animals raised and used as sources of tissue by Brown.* The animals had been fed the 18 per cent casein normal sulfate diet included in Table 1.

In another supplementary study to measure the effect of increased inorganic sulfate in the diet upon the ability of liver homogenates to oxidize the sulfur of cysteine to sulfate, livers were obtained at the

*R. G. Brown, unpublished.

termination of the experiment from animals fed the diet described as the normal sulfate (0.02 per cent sulfate) diet used for a collagen study in this laboratory (369). This diet was essentially the +E+CLO diet (Table 1) with an equal weight of the Hubbell, Mendel, and Wakeman salt mixture* substituted for the low sulfate salt mixture normally used.

II. METHODS

Preparation of Enzyme Solutions

Several methods of preparing the enzyme were used. All homogenizations were performed in motor-driven glass homogenizers placed in ice baths. Unless otherwise specified, not more than 90 minutes elapsed between killing and the start of the incubation.

The whole liver homogenates. The whole liver homogenates were prepared in 0.067 M. sodium phosphate, buffered at pH 7.4; the same buffer containing 0.002 per cent Versene (disodium ethylenediaminetetraacetic acid); the same buffer with 0.005 per cent Versent-Fe-3;** or 0.05 M. potassium phosphate, buffered at pH 7.8 and containing 0.005 per cent Versene-Fe-3. Specific buffers used for each experiment are indicated in Tables 4-9 in the results. All solutions used were cold.

The liver fractions. The liver homogenates were fractionated into mitochondria (370) and supernatant fluid. Ammonium sulfate was added

*Nutritional Biochemicals Corp., Cleveland, Ohio.

**Fisher Scientific Company, Fairlawn, New Jersey.

to the supernatant fluid to 0-50 per cent and 50-70 per cent saturation. The mitochondrial and ammonium sulfate fractions were then rehomogenized with buffer and the ammonium sulfate fractions dialyzed against the buffer at refrigerator temperature until no sulfate could be detected in the surrounding media. Since the phosphate in the buffer was insoluble when tested with barium chloride, it was necessary to acidify the samples from the surrounding media before testing for sulfate. The mitochondrial suspension was frozen until used. Before assay the ammonium sulfate fractions were recentrifuged to remove protein denatured in the process of dialysis. The whole liver homogenate was assayed the day after preparation, the mitochondria were thawed and assayed the day after that, the 0-50 per cent fraction on the next day, and the 50-70 per cent fraction on the next. Two pairs of experimental animals were processed at a time.

Specifically, a small portion of the liver was homogenized in buffer (Table 7) and stored until use at refrigerator temperature. The remainder of the liver was then homogenized in a cold solution containing 0.35 M. sucrose, 0.035 M. potassium bicarbonate, 0.004 M. magnesium chloride, and 0.025 M. potassium chloride. Three milliliters of this solution were used per gram of liver. The homogenate was then centrifuged at $700 \times g$. for 10 minutes at 0° in a Lourdes refrigerated centrifuge. The supernatant fluid was decanted and the residue (the nuclei) discarded. The supernatant fluid was then centrifuged at $14,000 \times g$. for 10 minutes. The particulate fraction containing the mitochondria was then homogenized in 5 ml. of buffer and frozen. To the supernatant fluid remaining after removal of the mitochondria 0.35 g. of

$(\text{NH}_4)_2\text{SO}_4$ was added per ml. of liquid. The system was allowed to equilibrate for about an hour. The fraction was then centrifuged for 10 minutes at 14,000 x g. The residue (the 0-50 per cent fraction) was homogenized with 10 ml. of buffer and dialyzed against this buffer until free of sulfate.

To the supernatant fluid remaining after the above centrifugation 0.15 g. of $(\text{NH}_4)_2\text{SO}_4$ was added per ml. of liquid. The system was allowed to equilibrate for about an hour and was then centrifuged for 10 minutes at 14,000 x g. The residue (the 50-70 per cent fraction) was then homogenized with 25 ml. of buffer and the resulting solution dialyzed until free of sulfate.

The denucleated homogenates. The fresh liver was homogenized with 0.067 M. sodium phosphate buffer, pH 7.4, containing 0.005 per cent Versene-Fe-3 and centrifuged at 1,200 x g. for 10 minutes in a Lourdes refrigerated centrifuge at 0° to remove the nuclei.

The supernatant preparations. The fresh liver was homogenized with buffer (Table 10 in the results) and centrifuged at 10,000 x g. for 15 minutes in a Lourdes refrigerated centrifuge at 0° to remove the nuclei and mitochondria. Similar preparations have been used by Sorbo and Ewetz (301).

The acetone powder extracts. A 2.5 per cent homogenate of acetone powder in 0.05 M. potassium phosphate, buffered at pH 7.8 and containing 0.005 per cent Versene-Fe-3, was shaken for two hours in the cold and centrifuged at 1,200 x g. at 0° to remove the non-extracted

material. This is essentially the beginning step in the fractionation procedure used by MacLeod et al. (324).

The preliminary purification of the enzyme. Rat liver acetone powder was homogenized with ten volumes of 0.05 M. potassium phosphate, buffered at pH 7.8 and containing 0.005 per cent Versene-Fe-3. The homogenate was heated for 5 minutes at 56°, cooled for 10 minutes in an ice bath, and centrifuged at 3,200 x g. for 10 minutes at 0°. One volume of cold 95 per cent ethanol was added slowly to the supernatant fluid. The mixture was stored in the cold for from one to two hours and the precipitate removed by centrifugation as before. The crude enzyme was removed from the supernatant fluid by the addition of 1.5 volumes of additional cold ethanol and collected by centrifugation after one hour. The precipitate was extracted with a volume of buffer equivalent to the volume of the supernatant fluid remaining after the first centrifugation. The undissolved material was removed by centrifugation and the supernatant fluid chromatographed on a 2.5 x 25 cm. column of DEAE-cellulose,* which had been prepared as detailed by MacLeod et al. (324). The reddish-orange fluid was allowed to pass almost entirely into the column and the effluent was discarded. Then 100 ml. portions of 0.05 M., 0.10 M., 0.15 M., 0.20 M., and 0.25 M. potassium phosphate, each buffered at pH 7.8 and containing 0.005 per cent Versene-Fe-3, were added after the less concentrated eluant had passed almost entirely into the column. Fractions of 20 ml. each were collected.

*Cellulose, diethylaminoethyl ether, Matheson Coleman and Bell, Norwood, Ohio.

Enzyme Assays

Several methods were used to assay the enzymes involved in the oxidation of cysteine.

Oxidation of cysteine to sulfate. The assay resembles that employed by Patrick (371). Experimental details are given in Tables 4-9 in the results. After addition of the trichloroacetic acid the protein precipitates were removed by filtration and washed. The filtrates were then concentrated and acidified. Sulfate was precipitated with barium chloride. Sufficient carrier sulfuric acid was added to insure 12 mg. of barium sulfate. The precipitate was collected on glass fiber filter papers, placed on planchets, and counted. This is essentially the method of Katz and Golden (372) without previous combustion. The specific activity of the cysteine used was determined by the method of Katz and Golden (372). A Nuclear-Chicago windowless gas-flow counter was used for all measurements of radioactivity. The period of incubation was chosen because it gave a maximal difference between autoxidation and formation of sulfate due to enzyme activity, and the net sulfate formed was proportional to enzyme concentration.*

Oxidation of sulfite to sulfate. Oxidation of sulfite to sulfate was measured by an adaptation of the ferricyanide assay method (324, 373). All reagents used in the assay were prepared in 0.05 M. potassium phosphate buffer, pH 7.8, containing 0.005 per cent Versene-Fe-3. The

*J. T. Smith, unpublished results.

incubation system consisted of 0.1 ml. of the acetone powder extract; 3 μ moles of $K_3Fe(CN)_6$, prepared in buffer; 10 μ moles of Na_2SO_3 ; and sufficient buffer to make a final volume of 6 ml. All tubes were prepared with buffer and the potassium ferricyanide. The sodium sulfite was added and mixed. The enzyme solution was added, mixed, and the optical density of the reaction mixture determined immediately with a Bausch and Lomb Spectronic 20 colorimeter at 420 m μ . The sample was allowed to stand for exactly ten minutes at room temperature, and the optical density was measured a second time. Controls without sulfite were also employed to measure the reduction of the ferricyanide by the enzyme preparations. These values were subtracted from those obtained for the enzymic reduction of ferricyanide.

The 2,6-dichlorophenolindophenol assay of MacLeod et al. (324) was also tried on similar extracts. This time a boiled enzyme solution was used to measure the non-enzymic reduction of the dye.

Oxidation of cysteine to sulfite. Sulfite production from cysteine by denucleated homogenates was measured by the method of Grant (374) as modified by Leinweber and Monty (375). The basic incubation mixture consisted of 1.0 ml. of denucleated 10 per cent liver homogenate in 0.067 M. sodium phosphate buffer, pH 7.4, containing 0.005 per cent Versene-Fe-3; 200 μ moles of ^{35}S -cysteine-HCl, neutralized and diluted in this same buffer; 100 μ moles of $MgCl_2$; 10 μ moles of ATP, prepared in buffer; and sufficient buffer to make a total of 5 ml. The mixture was incubated in air for 1.5 hours at 37°. The enzymic activity was destroyed by adding 0.5 ml. of a 1 per cent solution of KOH in 95 per cent ethanol

and 1.0 ml. of a saturated solution of HgCl_2 . The samples were then centrifuged. One milliliter samples of the resultant supernatant fluid were then placed in test tubes and 4 ml. of a color reagent consisting of 11 ml. of concentrated sulfuric acid, 234 ml. of distilled water, 4 ml. of a 1 per cent solution of basic fuchsin in ethanol, and 1 ml. of 40 per cent formaldehyde. This reagent had been decolorized with Norit A as suggested by Leinweber and Monty (375), and the Norit A had been removed by gravity filtration. After the color had developed for 15 minutes, the optical density of the samples was determined in a Klett-Summerson photometer with a green (520 m μ) filter. The instrument was set to zero with a reagent blank. The color reagent was prepared fresh daily, and, because it was occasionally defective, it was always tested against a known sulfite solution to make certain that the color would develop before the reagent was used with the samples resulting from the incubation procedure. The sulfite standards were prepared in the Versene-Fe-3 buffer, since this seemed to increase the stability of the sulfite in the presence of mercuric chloride, and, as a result, increase the sensitivity of the method. An amount of mercuric chloride equivalent to that in the samples was added to each standard.

Oxidation of cysteine to cysteinesulfinic acid. The formation of cysteinesulfinic acid from cysteine was assayed in two ways. De-nucleated homogenates from female rats were used without added inhibitor, and supernatant preparations from both male and female rats were used with hydroxylamine added as an inhibitor.

The basic incubation mixture and reaction conditions for the formation of cysteinesulfinic acid from the denucleated homogenates were identical to that used for the oxidation of cysteine to sulfite. The enzyme was denatured by placing the tubes in boiling water for 10 minutes. The tubes were then cooled and centrifuged at 2000 rev./min. for 10 minutes in an International Model SBV, Size 1, centrifuge to remove the precipitates. Any cysteinesulfinic acid formed should be converted to sulfite by incubation with α -ketoglutaric acid and glutamic-oxaloacetic transaminase (375). Therefore, a measured sample of the supernatant fluid was incubated for 15 minutes with 0.1 ml. of a solution of glutamic-oxaloacetic transaminase prepared by dialyzing a combination of 1 ml. of the commercial enzyme preparation* and 2 ml. of 0.05 M. potassium phosphate, buffered at pH 6.8, against this same buffer for about 16 hours. An additional factor in the incubation mixture was 0.2 ml. of a solution of α -ketoglutaric acid, prepared by dissolving 275 mg. of the acid in 4 ml. of 0.5 M. K_2HPO_4 and 10 ml. of 0.2 M. K_3PO_4 , adjusting the pH to 8.0, and diluting to 25 ml. with water. Enough 0.05 M. potassium phosphate, buffered at pH 7.8 and containing 0.005 per cent Versene-Fe-3 was also added to the incubation mixture to make a total volume of 2.5 ml. The activity of the glutamic-oxaloacetic transaminase was then destroyed by the addition of the same quantities of alcoholic potassium hydroxide and mercuric chloride used for the destruction of the enzymic activity in the oxidation of cysteine to sulfite, and the analysis for the sulfite

*Sigma Chemical Company, St. Louis, Missouri.

formed proceeded as before. Commercial cysteinesulfinic acid** was used as a standard to check reagents for the entire procedure.

For the supernatant preparations with an inhibitor the incubation procedure is described in Table 10. The procedure is essentially that of Sorbo and Ewetz (301) for the isolation of cysteinesulfinic acid. The supernatant fluid resulting from the centrifugation of the tubes after destroying the enzymic activity with hydrochloric acid and trichloroacetic acid was placed on a Dowex 50 x 8 column (1 x 5 cm.) in the hydrogen form. The precipitate was washed twice with 5 ml. of a 0.01 N. HCl solution and the precipitate separated from the supernatant solution each time by centrifugation. The supernatant solutions were added to the appropriate column after the previous addition had passed into the column. The effluents from the original solution and the two washings were combined. Then 50 ml. volumetric flasks were placed under the columns, and sufficient 0.01 N. HCl was added to the columns to bring the effluent volume to 50 ml. Both portions of the effluent were quantitatively transferred to separate 50 ml. beakers and placed in an air-convection oven at 67° until they reached dryness, usually overnight. Two milliliters of water were added to each beaker to dissolve the material. Aliquots of the resulting solutions were then analyzed for ninhydrin-positive materials. A maximum of 0.5 ml. of the solution to be analyzed was pipetted into a test tube, and 0.5 ml. of the buffer solution and 5 ml. of the phenol reagent were added to each tube and

**Calbiochem, Los Angeles, California.

the contents mixed thoroughly with a Vortex Jr. mixer. Then 0.5 ml. of the ninhydrin reagent was added. The tubes were again mixed and placed in a boiling water bath for 5 minutes, removed, and placed in cool tap water for a few minutes. Three milliliters of 60 per cent ethanol were added to the samples and the standards during cooling if the salt concentration was such that the phenol-water mixture in any of the samples formed an emulsion. This was usually the case with all samples of the first effluent. All samples were again mixed thoroughly with the Vortex Jr. mixer. The experiment was performed so that the determination of the optical density of the samples in the Klett-Summerson photometer could begin about ten minutes after the samples were removed from the boiling water. A green filter (520 m μ) was used. The instrument was set to zero with a reagent blank. The usual standards used were 1.5 μ moles of cysteinesulfinic acid per ml. and 3 μ moles of taurine, which had been recrystallized from water, per ml. The buffer solution (pH 4.3) was prepared by dissolving 5 g. of Versene and 240 mg. of KCN in 300 ml. of 2 N. sodium acetate and 700 ml. of 2 N. acetic acid. The phenol reagent was prepared by dissolving 400 g. of melted phenol in 100 ml. of absolute ethanol. Five grams of permutit were added and the solution was stirred for ten minutes. The permutit was removed by gravity filtration. For the ninhydrin reagent 6 g. of ninhydrin were dissolved in 100 ml. of absolute ethanol. Two grams of permutit were added, and the solution was stirred with a magnetic stirrer for ten minutes. The solution was filtered by gravity filtration, and the clear yellow solution was stored

in a brown bottle. This procedure is essentially that given by Fisher et al. (376). The reason that the effluents were separated was that checks of known solutions indicated that taurine was eluted in the first fraction and cysteinesulfinic acid in the last fraction. This is in essential agreement with results obtained by Wainer (303). Samples from both fractions, prepared from the same solutions used for the ninhydrin determination, were also analyzed for cysteinesulfinic acid as described above. Since it was not known what material would be present in the liver that might produce ninhydrin-positive material from the incubation mixture, identical incubation mixtures had been prepared with boiled enzyme preparations being substituted for the undenatured preparations. The values obtained for ninhydrin-positive material formed in these reaction mixtures were subtracted from the values obtained for the undenatured preparations.

In another attempt to isolate cysteinesulfinic acid duplicate samples to those reported in Table 9 in the results were prepared. The enzymic activity was destroyed by the addition of 20 ml. of 95 per cent ethanol. The tubes were centrifuged at 2000 rev./min. for 10 minutes in an International Model SBV, size 1, centrifuge. The supernatant fluid was decanted into a separatory funnel, and 75 ml. of chloroform were added and mixed. The layers were allowed to separate and the chloroform layer was removed and discarded. This extraction procedure was used by Awapara (377). The aqueous layer was tested for the presence of cysteinesulfinic acid by the method of Leinweber and Monty (375). The $\overline{SE}$ extracts and the $+E$ extracts were combined and concen-

trated. They were subjected to paper chromatography with 1-butanol--1-propanol-0.1 N. HCl (1:1:1, v/v) and water-saturated phenol +0.5 per cent ammonia. A ninhydrin spray was used to aid in identification of the samples. This is essentially the procedure used by Wainer (303).

Oxygen uptake of preparations incubated with cysteine. The oxygen uptake of various enzyme preparations was measured in a Warburg respirometer at 37° in an air atmosphere with cysteine as the substrate.

Measurement of ATP effect. The conversion of cysteine to sulfate by whole liver homogenates was measured in the presence and absence of ATP.

Nitrogen or Protein Content of Enzyme Solutions

Nitrogen content of enzyme solutions was measured by the micro-Kjeldahl method (378) for whole liver homogenates, denucleated homogenates, mitochondrial homogenates, and supernatant preparations. Protein content of the ammonium sulfate fractions and acetone powder extracts was measured by the method of Lowry et al. (379) with casein as the standard. The protein content was converted to nitrogen content with the casein factor.

The micro-Kjeldahl method. A measured volume of the solution to be analyzed was pipetted into a 30-ml. Kjeldahl digestion flask containing 1.25-1.35 g. of K_2SO_4 and 35-45 mg. of HgO . Two milliliters of

concentrated sulfuric acid were added and the flask contents digested until the solution became clear. About 5 ml. of water were added to dissolve the material, and, after the flask had cooled, the contents were quantitatively transferred to a steam distillation apparatus. Eight milliliters of the sodium hydroxide-sodium thiosulfate solution were then added and distillation begun. Approximately 45 ml. were distilled into 5 ml. of a saturated boric acid solution containing 4 drops of either the methyl red-bromcresol green or methyl red-methylene blue indicator. The amount of nitrogen was determined by titration with 0.02 N. HCl. The milligrams of nitrogen were calculated by the following formula:

$$(\text{Ml. acid}) \cdot (\text{N. acid}) \cdot (\text{Meq. wt. nitrogen}) = \text{mg. nitrogen.}$$

The sodium hydroxide-sodium thiosulfate solution was prepared by dissolving 500 g. of NaOH and 50 g. of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in sufficient water to make one liter. The saturated boric acid solution was prepared by dissolving 40 g. of H_3BO_3 in about 900 ml. of distilled water with heating, cooling the solution, and diluting to a final volume of one liter. The methyl red-bromcresol green indicator was prepared by mixing 5 parts of 0.2 per cent bromcresol green with 1 part of 0.2 per cent methyl red, both in 95 per cent ethanol. The methyl red-methylene blue indicator was prepared by mixing 2 parts of 0.2 per cent methyl red with 1 part of 0.2 per cent methylene blue, both in 95 per cent alcohol.

The Lowry method. A measured quantity of the solution to be assayed was dissolved in 0.5 N. NaOH and diluted to a known volume with the base. Five milliliters of reagent A were mixed with one milliliter samples of the protein solution. After the samples had stood for at least 10 minutes at room temperature, 0.5 ml. of reagent B was added rapidly and mixed. After at least 30 minutes the absorption was measured in a Klett-Summerson photometer with a red (770 m μ) filter. A casein standard was measured in a similar way to obtain a standard curve. Reagent A was made by adding 1 ml. of 2.7 per cent sodium potassium tartrate tetrahydrate and 1 ml. of 1 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to 100 ml. of 2 per cent Na_2CO_3 . For reagent B commercial Folin-Ciocalteu reagent* was diluted with enough water so that it contained 1 N. acid.

Assay of Erythrocyte Sensitivity to Dialuric Acid

The susceptibility of erythrocytes to chemical hemolysis by the procedure of Friedman et al. (52) was selected for the determination of the vitamin E status of the animals. About 30 μl . of blood were withdrawn with a hemoglobin pipette from a transverse cut in a tail vein of the rat and added to 5 ml. of a saline-phosphate buffer solution in a 15 ml. conical centrifuge tube. The cells were suspended by tapping the tube gently and then collected by centrifugation at 2000 rev./min. for 10 minutes in an International Model SBV, size 1, centrifuge. The supernatant fluid was withdrawn with suction, and the cells were resuspended in approximately 3.5 ml. of saline-phosphate buffer. One

*"Banco" Phenol Reagent, distributed by E. H. Sargent and Company.

milliliter of this cell suspension was pipetted into each of three test tubes. One milliliter of dialuric acid solution was added to each of tubes 1 and 2. One milliliter of saline-phosphate buffer was added to tube 3. The tubes were then incubated in a water bath at 37° for one hour and left standing at room temperature for another hour. At the end of this period, 5 ml. of the saline-phosphate buffer were added to tubes 1 and 3 and 5 ml. of water to tube 2. The contents were mixed and then centrifuged as before. The optical density of the supernatant fluid was determined in a Klett-Summerson photometer with a blue (420 mμ) filter. The instrument was set to zero with a distilled water blank. Per cent hemolysis is calculated with the following formula:

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

The saline-phosphate buffer was prepared by combining equal parts of 0.1 M. phosphate buffer, pH 7.4, and isotonic saline solution. The phosphate buffer was made by dissolving 14.2 g. of anhydrous dibasic sodium phosphate in distilled water, adding 20 ml. of 1.0 N. hydrochloric acid, and diluting to one liter. Isotonic saline was 0.89 per cent sodium chloride in distilled water. The dialuric acid solution was prepared by dissolving 10 mg. of dialuric acid in 50 ml. of saline-phosphate buffer immediately before use. This test was performed on all deficient animals before sacrifice and on a few of the sufficient animals as controls.

Statistics

Statistical analysis was done by the method of paired comparisons or by analysis of variance (380). The validity of these methods may be questioned because some of the data tested may appear to have come from a skewed population; however, the samples involved (4 to 12 animals) are too few to establish that the underlying population is skewed.

CHAPTER IV

RESULTS

The effects of the dietary treatments upon the animals and upon the oxidation of cysteine to cysteinesulfinic acid, sulfite, and sulfate and of sulfite to sulfate are given below.

I. EFFECT ON ANIMALS

The "prepped" vitamin E-deficient females used in the fractionation studies (Table 7) were the only animals that appeared to be abnormal. These exhibited the arching of the back reported in the early literature on vitamin E (381). The weight gains of the +E rats from weaning to 12 weeks on the diet were very highly significantly greater ($\underline{P} < 0.005$) than their $\overline{SE}$ littermates by analysis of variance, and there was a significant interaction ($\underline{P} < 0.05$) between the effect of "prepping" and the effect of vitamin E supplementation when these animals were compared to "non-prepped" animals fed the same experimental diets from weaning.

For the other females only after the experimental diets were fed for over five months was there a significant difference in the weight gains of the animals, with the deficient animals gaining significantly ($\underline{P} < 0.05$) less weight than their littermate controls. This is in agreement with a previous report in the literature (381).

Only in the male animals fed the experimental diets for nine weeks from weaning or for more than three months if placed on the diets after only a few days on the stock diet was there a significant ($\underline{P} < 0.05$) difference in weight gain in vitamin E deficiency. All these animals were still gaining weight, but the sufficient animals were gaining more. Growth and feed efficiencies of young adult male rats fed the $\overline{\text{S}}\text{CLO}$ diets are given in Table 3. Additional information on growth is given in the tables accompanying the metabolism studies.

II. EFFECT ON SULFUR METABOLISM

The results presented in Table 4 show a decrease in the conversion of the sulfur of cysteine to sulfate by whole liver homogenates from vitamin E-deficient young adult male rats compared to their vitamin E-sufficient controls. Table 5 reports the decreased conversion of cysteine to sulfate by whole liver homogenates from vitamin E-deficient adult male rats compared to their vitamin E-sufficient controls. These differences were statistically significant ($\underline{P} < 0.05$).

Table 6 shows the conversion of cysteine to sulfate by liver homogenates from vitamin E-sufficient and -deficient adult female rats. There was no statistically significant difference in the ability of the two sets of animals to convert cysteine to sulfate.

Table 7 shows the ability of various liver fractions from "prepped" female rats to convert cysteine to sulfate. All the fractions investigated exhibited increased sulfate formation from cysteine when

TABLE 3
GROWTH AND FEED EFFICIENCY OF YOUNG ADULT MALE RATS FED
THE DIET WITHOUT COD-LIVER OIL

Animal	Weaning Weight (Grams)	Death Weight (Grams)	Weight Gain (Grams)	Feed Efficiency	Days on Diet
			SE		
1	32	225	193	0.44 ^a	34
2	36	213	177	0.38 ^a	36
3	34	209	175	0.40	41
4	30	206	176	0.37	43
5	<u>38</u>	<u>233</u>	<u>195</u>	<u>0.36</u>	48 ^b
Mean	34	217	183	0.39	
			+E		
1	34	197	163	0.45	34
2	38	213	175	0.40 ^a	36
3	36	211	175	0.39	41
4	34	228	194	0.38	43
5	<u>40</u>	<u>283</u>	<u>243</u>	<u>0.38^a</u>	48
Mean	36	226	190	0.40	

^aShared cage.

^bLess than 90 per cent hemolysis.

TABLE 4

CONVERSION OF CYSTEINE TO SULFATE BY LIVER HOMOGENATES FROM VITAMIN E-SUFFICIENT AND -DEFICIENT YOUNG ADULT MALE RATS^a

Pair Number	Vitamin E	
	Deficient	Sufficient
	$\mu\text{moles sulfate formed per mg, nitrogen}$	
1	0.20	0.39
2	0.71	1.13
3	0.64	1.41
4	0.35	0.66
	$0.48 \pm 0.12^{b,c}$	0.90 ± 0.23

^aThe basic incubation mixture contained 0.2-0.6 ml. of a 20 per cent whole liver homogenate in 0.067 M. sodium phosphate, buffered at pH 7.4; 125 μmoles of ³⁵S-cysteine-HCl in buffer; 100 μmoles of MgCl₂; 10 μmoles of ATP in buffer; 0.18 μmoles of NAD in buffer; and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 2 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. The sources of tissue were male rats raised from weaning for between 5 and 7 weeks on the $\bar{s}$ CLO diet given in Table 1. The $\bar{s}$ E animals weighed from 206 to 233 g. at death while the +E animals weighed from 197 to 283 g.

^bMean $\pm$ standard error of the mean.

^cStatistically significant by the method of paired comparisons (380) ($\underline{P} < 0.05$).

TABLE 5

CONVERSION OF CYSTEINE TO SULFATE BY LIVER HOMOGENATES FROM VITAMIN E-SUFFICIENT AND -DEFICIENT ADULT MALE RATS^a

Pair Number	Vitamin E	
	Deficient	Sufficient
	$\mu\text{moles SO}_4^{=}$ formed per mg. nitrogen	
1	0.22	1.27
2	0.49	0.82
3	0.47	0.85
4	1.34	1.62
5	1.29	1.18
6	2.03	2.95
	$0.97 \pm 0.28^{b,c}$	1.45 ± 0.32

^aThe basic incubation mixture contained 0.5 ml. of a 10 per cent whole liver homogenate in 0.067 M. sodium phosphate buffered at pH 7.4; 125 μmoles of ^{35}S -cysteine-HCl in buffer; 100 μmoles of MgCl_2 ; 10 μmoles of ATP in buffer; and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 1.5 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. The sources of tissue were male rats fed the +CLO diet given in Table 1 for 13+ weeks from 11 to 16 days after weaning. The $\overline{\text{SE}}$ animals weighed 300-363 g. at death, while the +E animals weighed 337-400 g. at death.

^bMean $\pm$ standard error of the mean.

^cStatistically significant by the method of paired comparisons (380) ($P < 0.05$).

TABLE 6

CONVERSION OF CYSTEINE TO SULFATE BY LIVER HOMOGENATES FROM VITAMIN E-SUFFICIENT AND -DEFICIENT ADULT FEMALE RATS^a

Pair Number	Vitamin E	
	Deficient	Sufficient
	$\mu\text{moles SO}_4^-$ formed per mg. nitrogen	
1	2.67	1.15
2	1.87	1.83
3	2.37	3.27
4	1.96	2.13
5	1.71	2.35
6	2.19	1.73
	2.13 ± 0.14^b	2.07 ± 0.29

^aThe basic incubation mixture consisted of 0.5 ml. of a 5 per cent whole liver homogenate in 0.067 M. sodium phosphate, buffered at pH 7.4 and containing 0.002 per cent Versene; 125 μmoles of ^{35}S -cysteine-HCl in buffer; 100 μmoles of MgCl_2 ; 10 μmoles of ATP in buffer; and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 1.5 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. The sources of tissue were female rats fed the +CLO diets given in Table 1 for 5+ months from 11-12 days after weaning. The $\bar{\text{SE}}$ animals weighed 196-251 g. at death, while the +E animals weighed 212-262 g.

^bMean $\pm$ standard error of the mean.

TABLE 7

CONVERSION OF CYSTEINE TO SULFATE BY LIVER PREPARATIONS FROM VITAMIN E-
SUFFICIENT AND -DEFICIENT FEMALE RATS^a

Pair Number	Whole Liver		Mitochondria		0-50 Per Cent		50-70 Per Cent	
	<u>sE</u>	+E	<u>sE</u>	+E	<u>sE</u>	+E	<u>sE</u>	+E
$\mu\text{moles SO}_4^{=}$ formed per mg. nitrogen								
1	1.70	2.33	0.84	0.91	1.28	1.34	1.74	1.49
2	2.15	2.02	0.70	0.91	0.99	1.60	1.70	5.65
3	1.54	3.13	1.18	1.42	0.79	1.17	1.08	8.60
4	<u>1.67</u>	<u>2.01</u>	<u>1.09</u>	<u>1.23</u>	<u>0.64</u>	<u>0.76</u>	<u>1.20</u>	<u>1.18</u>
Mean	1.76	2.37	0.95 ^b	1.12	0.92	1.22	1.43	4.23
Standard error of the mean	± 0.13	± 0.26	± 0.11	± 0.13	± 0.14	± 0.18	± 0.17	± 1.78

^aThe basic incubation mixture contained 0.5 ml. of a 5 per cent whole liver homogenate, 0.5 ml. of the mitochondrial preparation, 0.5 ml. of the 0-50 per cent fraction, or 1.0 ml. of the 50-70 per cent fraction, all made in 0.067 M. sodium phosphate buffered at pH 7.4 and containing 0.005 per cent Versene; 125 μmoles of ³⁵S-cysteine-HCl in buffer; 100 μmoles of MgCl₂; 10 μmoles of ATP in buffer; and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 1.5 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. The sources of tissue were the female rats that had been prepped, as described in the text, for 2 weeks prior to weaning and then transferred to the +CLO diet in Table 1. The sE animals weighed from 133 to 163 g. at death, while the +E animals weighed from 214 to 234 g. at death after being fed the experimental diets for 12+ weeks. Methods of preparation for the various fractions are given in the text.

^bStatistically significant by the method of paired comparisons (380) ($P < 0.05$).

fractions from vitamin E-sufficient liver were compared with those from vitamin E-deficient liver, but only the mitochondrial fraction displayed a significantly greater ($P < 0.05$) sulfate production for the supplemented animals. The 50-70 per cent fraction had the highest activity; however, the high standard error obtained leaves these data open to question.

Table 8 reports the ability of liver homogenates from vitamin E-sufficient and -deficient male rats fed 18 per cent casein, normal sulfate diets to convert cysteine to sulfate. Unfortunately, no specific activity measurements were made on the ^{35}S -cysteine used, but the results indicate that there is a definite decrease in the ability of the deficient animals to convert cysteine to sulfate. Although the results are not significant, only a few animals were available, and the difference definitely approaches significance ($0.1 > P > 0.05$). Table 9 indicates that there is no sex difference in the ability of vitamin E-sufficient animals fed normal sulfate diets to convert cysteine to sulfate.

Since the results presented in Tables 4-9 indicated a significant reduction in sulfate production from cysteine by preparations from vitamin E-deficient male rats and from mitochondria of vitamin E-deficient "prepped" female rats, the oxidation of sulfite to sulfate by sulfite oxidase with ferricyanide (324, 373) was investigated in an attempt to determine the enzyme system responsible for the decreased sulfate production. Twenty-six acetone powder extracts of livers from 10 vitamin E-deficient male rats produced an average change in optical

TABLE 8
CONVERSION OF ^{35}S -CYSTEINE TO ^{35}S -SULFATE BY LIVER HOMOGENATES FROM
VITAMIN E-SUFFICIENT AND -DEFICIENT MALE RATS FED
NORMAL SULFATE DIETS WITH 18 PER CENT CASEIN^a

Pair Number	Vitamin E		Per cent Difference
	Deficient	Sufficient	
	Counts per minute per mg. nitrogen		
1	33.7	50.7	-33.5
2	55.8	53.1	+ 5.1
3	35.6	53.7	-33.8
4	42.5	57.8	-26.5

^aThe basic incubation mixture contained 0.2 or 0.6 ml. of a 20 per cent whole liver homogenate in 0.067 M. sodium phosphate, buffered at pH 7.4; 125 μ moles of ^{35}S -cysteine-HCl; 10 μ moles of ATP; 0.18 μ moles of NAD, and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 2 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. The livers came from adult animals raised by Brown from weaning (unpublished) and fed an 18 per cent casein diet (Table 1). $0.1 > \underline{P} > 0.05$ by the method of paired comparisons (380).

TABLE 9

CONVERSION OF CYSTEINE TO SULFATE BY LIVER HOMOGENATES FROM VITAMIN E-SUFFICIENT ANIMALS FED A NORMAL SULFATE DIET^a

Males	Females
$\mu\text{moles SO}_4^{=}$ formed per mg. nitrogen	
1.47	2.37
1.67	1.67
1.27	0.51
1.06	2.39
0.46	2.77
1.19	1.79
2.67 ^b	1.22 ^b
0.74 ^b	2.68 ^b
2.02 ^b	7.06 ^b
1.94 ^b	0.51 ^b
7.05 ^b	0.76 ^b
2.71 ^b	0.61 ^b
2.02 $\pm$ 0.53 ^c	2.02 $\pm$ 0.52

^aThe basic incubation mixture contained 0.5 ml. of a 10 per cent whole liver homogenate in 0.067 M. sodium phosphate, buffered at pH 7.4; 125 μmoles of ^{35}S -cysteine-HCl; 10 μmoles of ATP; 100 μmoles of MgCl_2 ; and sufficient buffer to make 5 ml. The samples were incubated at 37° for 1.5 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. The livers came from animals raised by Button for 12+ weeks and fed the +E +CLO diet with the Hubbell, Mendel and Wakeman salt mixture substituted on a weight basis for the low sulfate salt mixture normally used (Table 1). The males ranged in weight from 301 to 406 g. and the females from 202 to 258 g. at death.

^b0.002 per cent Versene in buffer, 5 per cent homogenates.

^cMean $\pm$ standard error of the mean.

density of 0.070 ± 0.004 per mg. of protein in 10 minutes, while the extracts from their vitamin E-sufficient littermates produced a change of 0.074 ± 0.004 mg. of protein in 10 minutes. There was no statistically significant difference in the ability of these acetone powder extracts to oxidize sulfite to sulfate, nor was there any measurable sulfite accumulation in either case when denculeated homogenates were incubated with cysteine. No measurable cysteinesulfinic acid could be detected after incubation of the denucleated homogenates with cysteine. The 2,6-dichlorophenolindophenol assay for sulfite oxidase (324) produced such high blanks that the data were uninterpretable.

Since both commonly proposed pathways for the removal of cysteinesulfinic acid involve enzymes which should require pyridoxal phosphate, hydroxylamine was employed with the hope that cysteinesulfinic acid would accumulate and that this accumulation could be measured. Since possible destruction of the acid could take place during the isolation procedure, a determination of the ninhydrin-positive material produced was employed, as suggested by Sorbo and Ewetz (301). Results of this study are reported in Table 10. There was no statistically significant difference in the ability of livers from either sex or dietary treatment to convert cysteine to a ninhydrin-positive material presumed to be cysteinesulfinic acid, but the high standard error and a disagreement between duplicate samples would prevent any real conclusions.

In an attempt to identify cysteinesulfinic acid without the chemical degradation possible in the above isolation procedure the incubation mixture (Table 10) was denatured with 95 per cent ethanol and the material

TABLE 10

CONVERSION OF CYSTEINE TO A NINHYDRIN-POSITIVE METABOLITE
BY SUPERNATANT FRACTIONS FROM VITAMIN E-SUFFICIENT
AND -DEFICIENT RATS^a

Pair Number	Males		Females	
	<u>SE</u>	+E	<u>SE</u>	+E
	<u>µmoles ninhydrin-positive material per mg. nitrogen</u>			
1	0.207	0.254	0.345	0.297
2	0.172	0.139	0.378	0.294
3	1.138	-0.184	0.640	0.230
4	0.530	0.086	0.340	0.348
5	-0.032	0.448	2.130	-1.010
6	<u>0.173</u>	<u>1.030</u>	<u>--</u>	<u>--</u>
	0.364 <u>±</u> 0.172 ^b	0.295 <u>±</u> 0.170	0.766 <u>±</u> 0.345	0.031 <u>±</u> 0.261

^aThe basic incubation mixture contained 1.0 ml. of the supernatant fraction resulting from centrifuging a 10 per cent whole liver homogenate prepared in 0.05 M. potassium phosphate, buffered at pH 7.8 and containing 0.005 per cent Versene-Fe-3; 4.5 µmoles of NAD in buffer; 1 µmole of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$; 4 µmoles of nicotinic acid neutralized with sodium hydroxide and diluted with buffer; 37 µmoles of hydroxylamine hydrochloride and 80 µmoles of NaOH, mixed before use; 10 µmoles of cysteine in buffer; and sufficient buffer to make 5 ml. The samples were incubated at 37° for 1.5 hours. The enzymic activity was destroyed by the addition of 0.1 ml. of 1:3 HCl and 1.0 ml. of 10 per cent trichloroacetic acid. Isolation and measurement of the metabolite is detailed in the text. The animals were raised from weaning to 6+ weeks on the +CLO diet (Table 1). The males ranged in weight at death from 193 to 275 g. and the females from 148 to 179 g.

^bMean ± standard error of the mean.

was extracted with chloroform. While ninhydrin-positive spots could be seen on paper chromatograms after chromatography of the concentrated solutions, satisfactory identification of cysteinesulfinic acid could not be made. If the chromatograms were developed with alkaline solvents best for the prevention of streaking of taurine, cysteinesulfinic acid, and cysteic acid, the spots from the material isolated were streaked. In an acid solvent poor separation of taurine and cysteinesulfinic acid standards prevented identification.

Results of oxygen uptake by liver preparations when incubated with cysteine were inconclusive. Generally, with the impure fractions, the enzymic oxygen uptake was small. With purified fractions denaturation of the enzyme frequently occurred before oxygen uptake could be measured. Data from a successful experiment are presented in Table 11.

Sample data suggesting an influence of ATP upon the oxidation of cysteine to sulfate are presented in Table 12.

This research was hampered by the failure of the active enzyme solutions to retain activity after freezing or lyophilization or upon storage at refrigerator temperatures for longer than 24 hours. For the partially purified preparations activity could not be retained consistently after storage for 16 hours at refrigerator temperatures. The partially purified preparation, in its active form, had about the same color as a solution of cytochrome c. If this color had

TABLE 11

OXYGEN UPTAKE OF PARTIALLY PURIFIED FRACTIONS^a

Fraction	ul. change
20-40 ml. of 0.05 M. eluant	20.3
	19.1
Same boiled 10 minutes	9.3
80-100 ml. of 0.10 M. eluant	4.8
	4.8
Same boiled 10 minutes	3.9
80-100 ml. of 0.15 M. eluant	4.8
	4.6
Same boiled 10 minutes	5.6
80-100 ml. of 0.20 M. eluant	4.2
	3.9
Same boiled 10 minutes	4.4
80-100 ml. of 0.25 M. eluant	4.6
	4.5
Same boiled 10 minutes	2.0

^aThe basic incubation mixture contained 0.4 ml. of the indicated fraction (page 78), 125 μ moles of cysteine-HCl, neutralized in 0.05 M. potassium phosphate with NaOH, buffered at pH 7.4 and containing 0.005 per cent Versene-Fe-3; 10 μ moles of ATP in buffer; 10 μ moles of MgCl₂; 0.2 ml. of 1 per cent Versene in buffer, 0.1 ml. of cytochrome c (65 mg. in 25 ml. of buffer); and sufficient buffer to make 3 ml. The samples were incubated in air at 37.5° for 40 minutes after a 10 minute equilibration period. Stock colony rats fed the whole wheat-whole milk stock diet were used as sources of tissue. Fractions were selected for testing on the basis of visual color intensity.

TABLE 12

SAMPLE DATA SUGGESTING AN ATP REQUIREMENT FOR THE CONVERSION
OF ^{35}S -CYSTEINE TO ^{35}S -SULFATE^a

ml. homogenate	with ATP	without ATP
	counts per minute	
0.2	50	17
0.6	89	40
0.1	96	86

^aThe basic incubation mixture contained the indicated quantity of a 20 per cent whole liver homogenate in 0.067 M. sodium phosphate, buffered at pH 7.4; 125 μmoles of ^{35}S -cysteine-HCl in buffer; 10 μmoles of ATP in buffer, where indicated; and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 2 hours. Enzymic activity was destroyed by the addition of 5 ml. of 10 per cent trichloroacetic acid. A stock colony rat fed the whole wheat-whole milk stock diet was used as the source of tissue.

changed to a muddy orange, the preparation was no longer active.

Cytochrome c did not restore activity.

CHAPTER V

DISCUSSION

The results reported in Tables 4-9, together with the in vivo results from this laboratory reported elsewhere (1), indicate that vitamin E definitely has a role in the oxidation of the sulfur amino acids to sulfate and may have a role in the direct sulfation of the mucopolysaccharides. The exact site of the function of vitamin E in the metabolism of the sulfur amino acids could not be determined, both because of difficulties in methods of analysis and the failure of the assay techniques to confirm the generally accepted pathway for the oxidation of the sulfur of cysteine to sulfate. The pathway studied, that of oxidation of cysteine to sulfate through cysteinesulfinic acid and sulfite, could, however, not be discarded on the basis of the experimental results. The ability of rat liver preparations to react with the proposed intermediates, together with a general failure to isolate any of the intermediate reaction products was confirmed.

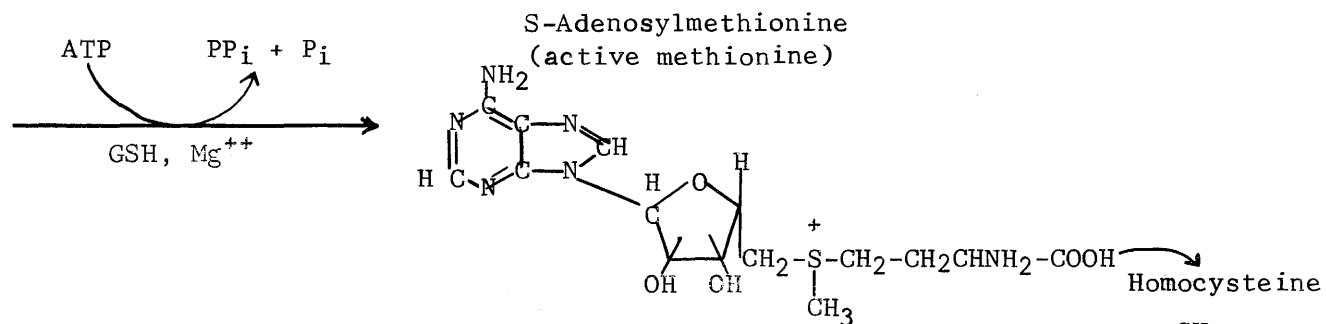
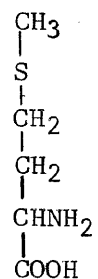
The need for Fe^{+2} and NADPH (301) in the oxidation of cysteine to a ninhydrin-positive substance thought to be cysteinesulfinic acid suggests that cysteine is the true substrate for the reaction, although why two reducing agents should be needed for an oxidation is interesting, and NADH did not have the same effect as NADPH in increasing "cysteinesulfinic acid" formation. This would suggest that cysteine desulfhydrase is not the enzyme involved in this reaction. Since no

influence of dietary levels of vitamin E could be found upon the ability of the enzyme assayed to produce "cysteinesulfinic acid," the acid may not be an intermediate in the conversion of cysteine to sulfate by vitamin E. The most important indication that the decreased oxidation of cysteine to sulfate is due to a decreased oxidation of cysteine to cysteinesulfinic acid is that sulfate added to the incubation mixture in increasing amounts led to increased production of taurine by the homogenates from the vitamin E-deficient rats but not by homogenates from the rats supplemented with the vitamin, while sulfate production decreased in both groups.* Since cysteinesulfinic acid is thought to be the last common precursor for the formation of both taurine and sulfate, a defect in the formation of the cysteinesulfinic acid is indicated. If sulfate is formed until the sulfate needs of the animal are met, when no sulfate is present in the diet, then, and only then, is taurine formed by the liver in normal amounts. A feedback mechanism involving taurine and sulfate has been suggested previously (298).

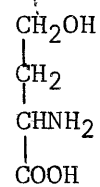
The conversion of methionine to cysteine has been outlined in Figure 7 and the conversion of cysteine to sulfate through cysteinesulfinic acid in Figure 8. A slight alteration in the production of cysteine from methionine is proposed (Figure 9), in which the sulfur atom remains bound to adenosine in the course of the reaction. The sulfhydryl group of cysteine should also be able to react with ATP to form the same compound (Figure 10). This type of reaction could prevent the oxidation

*J. T. Smith, Unpublished results.

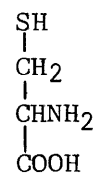
Methionine



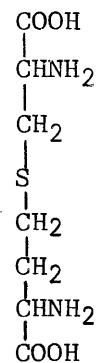
Homoserine



+



Cysteine



Cystathionine

Serine

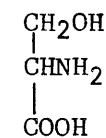


Figure 7. Conversion of methionine to cysteine.

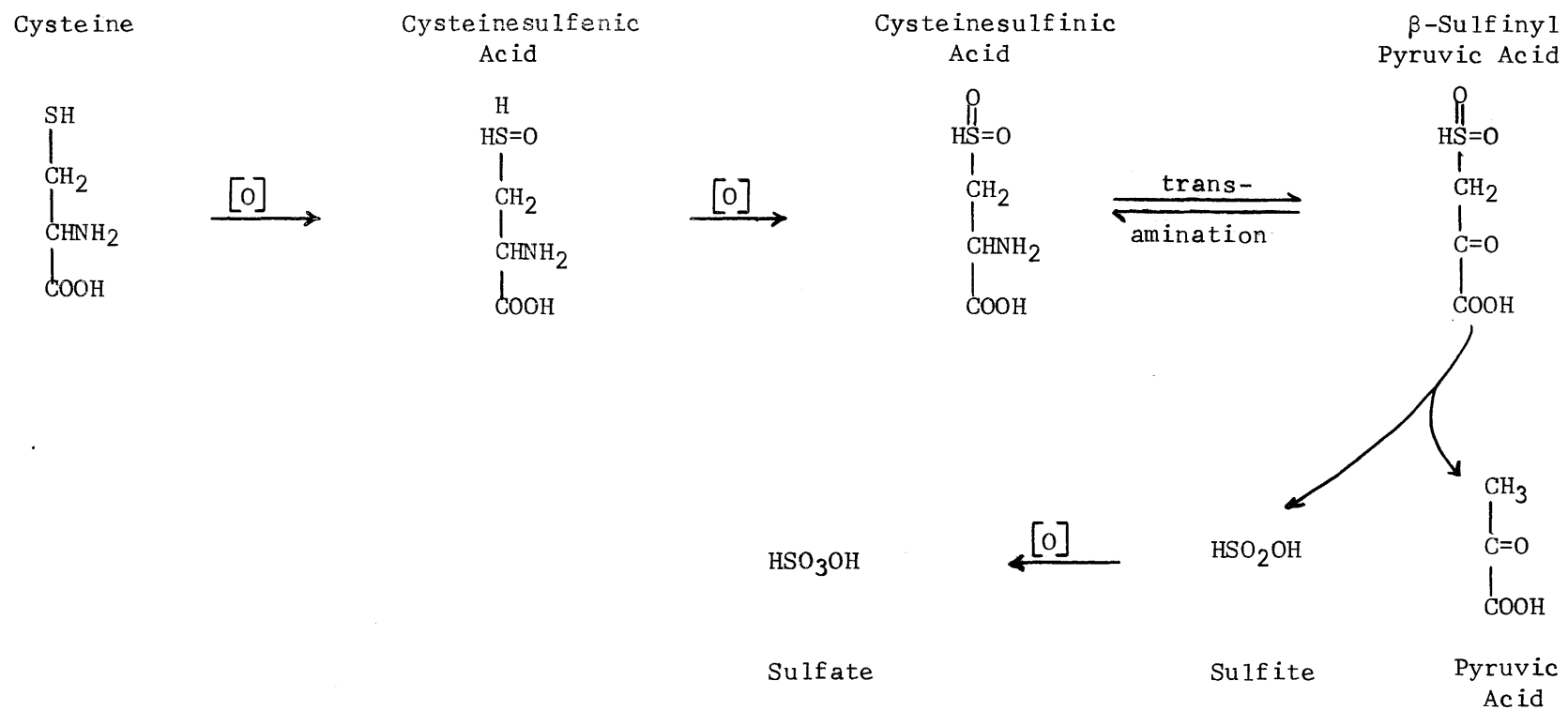


Figure 8. Conversion of the sulfur of cysteine to sulfate.

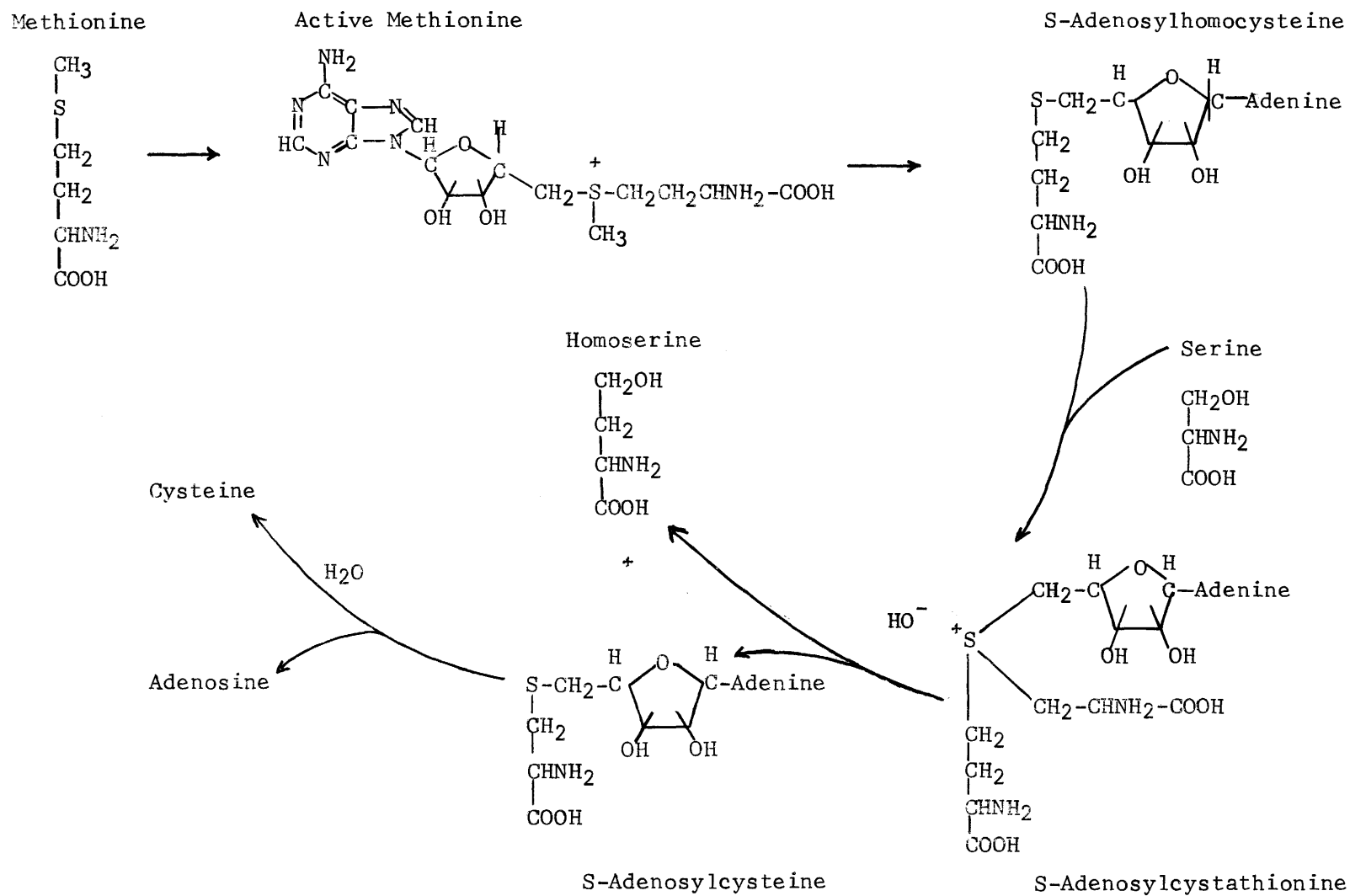


Figure 9. Proposed scheme of conversion of methionine to cysteine.

of the sulfur amino acid to cysteine. Then the sulfur of the S-adenosylcysteine could be oxidized to S-adenosylcysteinesulfinic acid, S-adenosylsulfite, and S-adenosylsulfate by molecular oxygen or some other electron acceptor. These compounds have not been reported in the literature searched, but ease of hydrolysis would prevent identification by many methods. PAPS and APS are generally considered, at the present time, to be products of the reaction of sulfate with ATP, and not intermediates in the oxidation of cysteine to sulfate. The ATP reacts with sulfate to form APS and pyrophosphate. The APS then reacts with ATP to form PAPS, which is used in sulfation of various compounds, including the phenols, which are common inhibitors for oxidative phosphorylation. There is evidence in microorganisms that PAPS is an intermediate in the reduction of sulfate to sulfite (382). Another possible intermediate in this conversion is $X-SO_3^-$, where X is thought to be a protein thiol group (383). In the oxidation of sulfite to sulfate by ferricyanide by bacteria in the presence of AMP, which produces APS instead of PAPS for the particular organisms involved, $AMP-SO_3^-$ is suggested as a possible, but unlikely intermediate (384). In animals, even if ATP were not required for reaction with cysteine, it could serve to increase sulfate production by binding some of the sulfate formed during the incubation period and subsequently releasing the sulfate during the analytical procedure. A requirement for ATP is suggested by the data in Table 12.

Another interesting factor in the reactivity of ATP with various anions is that selenate, sulfite, chromate, tungstate, and molybdate

lead to increasingly active enzymic cleavage by yeast of pyrophosphate from ATP, which should again influence oxidative phosphorylation. Sulfate inhibits this liberation (385). The enzyme acts upon ATP to transfer AMP to sulfate to selenate with the formation of APS and traces of APSe, but forms free AMP from ATP and sulfite, chromate, tungstate, or molybdate. The presence of sulfate will prevent the formation of pyrophosphate in the reactions where free AMP is liberated (386). The order of reactivity in the first paper suggests that very minute traces of AP-SO_3^- could exist. The existence of APSe, possible existence of AP-SO_3^- in microorganisms, the role of selenium in the prevention of respiratory decline in rats, and the ability of these compounds, plus sulfite and molybdate, to hydrolyze ATP to pyrophosphate and AMP to various degrees in yeast suggests several ways in which the metabolism of these compounds is interrelated. The decreased absorption and increased renal excretion of molybdate with increasing dietary levels of sulfate fed to animals suffering from molybdate toxicity (239) suggests that sulfate and molybdate are absorbed and excreted by the same mechanism, but that sulfate is the preferred anion. Since the process is one of active transport involving ATP (387), much more stable binding of sulfate to ATP should increase sulfate transport through the walls of the digestive tract and increase sulfate resorption through the renal tubules, while decreasing molybdate absorption and increasing excretion. Selenate and possibly selenite toxicity should be influenced in a similar way and selenium deficiency in an inverse way. Appreciable molybdate in the tissues would interfere with sulfur and copper meta-

bolism by the chemical reactions involved. Any alteration in ATP formation or removal should affect phosphorylation, active transport, etc.

The role of vitamin E could be to enable the introduction of the high-energy phosphate bonds, thus coupling the oxidation of the sulfur amino acids to phosphorylation. An explanation for the failure of animals fed a vitamin E-deficient high sulfate, low sulfur amino acid diet to incorporate as much $^{35}\text{SO}_4^-$ after injection as their vitamin E-sufficient counterparts is that the formation of PAPS or APS from sulfate or the transfer of the sulfate from PAPS to the acceptor compounds could involve vitamin E or a metabolite.

The ability of both vitamin E and selenium compounds to protect against lipid peroxidation has been attributed by some to the ability of both to act as antioxidants. Vitamin E and selenium may function in the metabolism of sulfur amino acids in alternative pathways of the oxidation of these acids. Cysteine has also been suggested as an antioxidant. If the role of vitamin E and selenium is only to prevent lipid peroxidation or to protect labile sulfhydryl groups, it is hard to visualize why two antioxidants should influence the oxidation of a third. This is the anomalous situation suggested by the importance of vitamin E in the oxidation of cysteine to sulfate and the proposed importance of selenium in this oxidation. Both vitamin E and vitamin B₆ have the ability to relieve some of the symptoms of vitamin E deficiency. Since vitamin B₆ is implicated in transaminase reactions in the various schemes for the oxidation of the sulfur of cysteine to sulfate, taurine, and various other intermediates (388), this suggests

the possibilities that alternative pathways for taurine formation exist which spare vitamin E for use in the formation of sulfate, that oxidizing cysteinesulfinic acid to sulfate does not require vitamin B₆, or that cysteinesulfinic acid is not on the direct pathway for sulfate formation. Some available experimental evidence suggests that vitamin B₆ is not necessary for sulfate formation, but is for the other proposed deaminations in sulfur amino acid metabolism.

If the role of vitamin E in the prevention of lipid peroxidation is not simply one of an antioxidant, another function needs to be proposed. Sulfation of olefins is a common organic chemistry reaction which is fairly easily reversible. If such an enzymic reaction could exist in tissue lipids, this would certainly prevent peroxidation of lipids because the site of attack would be removed. This sulfation would also affect cell permeability through alteration in the structure of the lipid in the cell membrane. Since sulfation, or at least the formation of sulfate, has been shown to be under the influence of vitamin E, this could account partially for the increased peroxidizability of tissue lipids from vitamin E-deficient animals. The changed membrane structure could also account for the increased susceptibility of tissues to lysosomal activity. Since sulfuric acid is commonly employed in lipid work, the small amount of sulfate bound to the cell membrane could be masked.

It has been reported that Weil-Malherbe suggested a selective inhibition of the energy-coupling mechanism (i.e. oxidative phosphorylation) might account for the increased respiration of dystrophic muscle

and the increased excretion of creatine. In the absence of oxidative phosphorylation creatine would not be phosphorylated, and the unphosphorylated creatine, which the muscle is unable to retain, would be excreted in greatly increased amounts (165). A defect in oxidative phosphorylation would also affect oxidation of Kreb's cycle intermediates. Influence of thyroxine could be directly on oxidative phosphorylation. Again, the thyroid function affects both symptoms of vitamin E deficiency and sulfur metabolism.

Since fasted animals were not used for the current experiments and since inanition has been shown on similar diets, (Table 3 and reference 364), not to be a factor in the slightly decreased size of many of the vitamin E-deficient animals, comparison between this study and those of previous workers involving inanition or fasting has not been made. The comparisons made have dealt mainly with rats and rat liver, since tissue and species differences have indicated alterations in both the apparent mechanism for the oxidation of the sulfur of the sulfur amino acids and in the response to the effects of vitamin E deficiency.

While there is very little experimental evidence to support many of the mechanisms suggested in this discussion, there is also little to contradict the possible existence of the mechanisms. The precise location of the defect in in vitro sulfur metabolism in vitamin E deficiency must await elucidation of the exact pathway for the oxidation of cysteine to sulfate in rat liver and the development and thorough testing of quantitative analytical procedures for these methods.

CHAPTER VI

SUMMARY

The conversion of ^{35}S -cysteine to ^{35}S -sulfate has been measured with liver preparations from adult male and female rats. A statistically significant decrease in the conversion of cysteine to sulfate was found in whole liver homogenates from the vitamin E-deficient male, but not the female, rats fed the low sulfate diets when these were compared with their littermate controls. When male and female rats fed normal sulfate vitamin E-sufficient diets were compared, there was no statistically significant difference in the conversion of cysteine to sulfate. There was also a statistically significant decrease in the ability of mitochondrial homogenates from the vitamin E-deficient "prepped" female rats to convert cysteine to sulfate when these were compared with their littermate controls.

No difference could be detected in the ability of acetone powder extracts from vitamin E-deficient and -sufficient male rats to oxidize sulfite to sulfate.

Tests were made to determine the presence of cysteinesulfinic acid and sulfite after incubation of cysteine with denucleated homogenates, but neither substance could be detected.

The conversion of cysteine to a ninhydrin-positive material thought to be cysteinesulfinic acid by supernatant preparations of liver from vitamin E-deficient and -sufficient male and female rats was

also studied in the presence of an inhibitor. There was no detectable difference in the ability of these preparations to oxidize cysteine.

A mechanism is proposed for the involvement of ATP in the oxidation of the sulfur group of cysteine to sulfate.

LITERATURE CITED

LITERATURE CITED

1. Smith, J. T., and B. J. Pendergrass 1962 A relationship between vitamin E and the sulfation of mucopolysaccharides and cellular lipoproteins. Federation Proc., 21: 170 (abstract).
2. Hickman, K. C. D., and P. L. Harris 1946 Tocopherol interrelationships. Advances in Enzymol., 6: 469.
3. Alfin-Slater, R. B. 1960 Relation of vitamin E to lipid metabolism. Am. J. Clin. Nutrition, 8: 445.
4. Marks, J. 1962 Critical appraisal of the therapeutic value of α -tocopherol. Vitamins and Hormones, 20: 573.
5. Martius, C. 1962 Metabolism of vitamin E based on tissue analysis. Vitamins and Hormones, 20: 457.
6. Michaelis, L., and S. H. Wollman 1950 Free radicals derived from tocopherol and related substances. Biochim. Biophys. Acta, 4: 156.
7. Alaupovic, P., B. C. Johnson, Q. Crider, H. N. Bhagavan and B. J. Johnson 1961 Metabolism of alpha-tocopherol and the isolation of a nontocopherol reducing substance from animal tissues. Am. J. Clin. Nutrition, 9, part II: 76.
8. Seward, C. R., and L. M. Corwin 1963 In vitro studies on the fate of α -tocopherol in rat-liver homogenates. Arch. Biochem. Biophys., 101: 71.
9. Harrison, W. H., J. E. Gander, E. R. Blakly and P. D. Boyer 1956 Interconversion of α -tocopherol and its oxidation products. Biochim. Biophys. Acta, 21: 150.
10. Mackenzie, J. B., and C. G. Mackenzie 1960 The antisterility activity of alpha-tocohydroquinone in the female rat. J. Nutrition, 72: 322.
11. Mackenzie, J. B., and C. G. Mackenzie 1953 Vitamin E activity of alpha-tocopherylhydroquinone and muscular dystrophy. Proc. Soc. Exp. Biol. Med., 84: 388.
12. Rodnan, G. P., S. S. Chernick and K. Schwarz 1956 Reversal of respiratory decline in necrotic liver degeneration by intraportal tocopherols. J. Biol. Chem., 21: 231.

13. Green, J., E. E. Edwin, A. T. Diplock and J. Bunyan 1961 Role of selenium in relation to ubiquinone in the rat. *Nature*, 189: 748.
14. Diplock, A. T., J. Bunyan, E. E. Edwin and J. Green 1962 Tocopherol, selenium and ubiquinone in the turkey and pigeon. *Brit. J. Nutrition*, 16: 109.
15. Green, J., E. E. Edwin, A. T. Diplock and J. Bunyan 1960 The effect of vitamin E, vitamin A, and some other dietary factors on ubiquinone and ubichromenols in the rat. *Biochem. Biophys. Res. Comms.*, 2: 388.
16. Smith, J. L., H. N. Bhagavan, R. L. Hill, S. Gaetani, P. B. Rama Rao, Q. E. Crider, B. C. Johnson, C. H. Shunk, A. F. Wagner and K. Folkers 1963 Biological activities of compounds in the vitamin E, vitamin K, and Coenzyme Q groups in chicks, rabbits, and rats. *Arch. Biochem. Biophys.*, 101: 388.
17. Green, J., E. E. Edwin, A. T. Diplock and J. Bunyan 1961 The effect of a water-soluble metabolite of α -tocopherol on ubiquinone in the rat. *Biochim. Biophys. Acta*, 49: 417.
18. Bunyan, J., J. Green, A. T. Diplock and E. E. Edwin 1961 Pyridine nucleotide-tocopheronolactone reductase. *Biochim. Biophys. Acta*, 49: 420.
19. Morton, R. A., and W. E. J. Phillips 1959 Unsaponifiable constituents of liver, kidney and heart tissues from vitamin E deficient rats compared with α -tocopherol supplemented rats. *Biochem. J.*, 73: 427.
20. Edwin, E. E., J. Bunyan, J. Green, and A. T. Diplock 1962 The effect of vitamin A on ubiquinone and ubichromenol in the rat, and its relation to the effect of vitamin E. *Brit. J. Nutrition*, 16: 135.
21. Bunyan, J., E. E. Edwin, A. T. Diplock and J. Green 1961 Ubiquinone and tocopherol in birds. *Nature*, 190: 637.
22. Houchin, O. B. 1942 The in vitro effect of α -tocopherol and its phosphate derivative on oxidation in muscle tissue. *J. Biol. Chem.*, 146: 313.
23. Houchin, O. B., and H. A. Mattill 1942 Influence of parenteral administration of α -tocopherol phosphate on the metabolic processes of dystrophic muscle. *J. Biol. Chem.*, 146: 309.
24. Nason, A., K. O. Donaldson, and I. A. Lehman 1957 The role of vitamin E at the enzymatic level. *Trans. N. Y. Acad. Sci.*, 20: 27.

25. Zierler, K. L., B. P. Folk, C. Eyzaguirre, L. W. Jaicho, D. Grob and J. Lilienthal 1949 On certain effects of alpha-tocopheryl phosphate on oxidative mechanism of striated muscle. *Ann. N. Y. Acad. Sci.*, 52: 180.
26. Boyer, P. D. 1960 Vitamin E. In: *The Enzymes*, ed. 2, eds. P. D. Boyer, H. Lardy and K. Myrback, Vol. 3, Academic Press, Inc., New York, p. 353.
27. Caputto, R., R. B. McCay and M. P. Carpenter 1958 Requirements for Mn^{++} and Co^{++} for synthesis of ascorbic acid by liver extracts of animals deprived of tocopherol. *J. Biol. Chem.*, 233: 1025.
28. McCay, P. B., A. E. Kitabchi, R. Caputto and R. E. Trucco 1961 Inhibition of succinoxidase by L-gulonolactone oxidase in liver preparations from tocopherol-deficient rats. *Biochem. Biophys. Res. Comm.*, 4: 469.
29. Dinning, J. S. 1955 The role of vitamin E in regulating the turnover rate of nucleic acids. *J. Biol. Chem.*, 212: 735.
30. Dinning, J. S., and P. L. Day 1958 Vitamin E deficiency in the monkey. III. The metabolism of sodium formate- C^{14} . *J. Biol. Chem.*, 233: 240.
31. Dinning, J. S., J. T. Sime and P. L. Day 1956 An increased incorporation of P^{32} into nucleic acids by vitamin E-deficient rabbits. *J. Biol. Chem.*, 222: 215.
32. Gray, D. E., J. Chisholm and C. H. Lee Peng 1960 Metabolic effects of alpha-tocopherol acetate. IV. Deoxyribonucleic acid of the rat liver cell, following daily dietary supplements of α -tocopherol acetate. *J. Vitaminology*, 6: 291.
33. Masters, Y. F., P. C. Johnson, W. H. Mosley, P. B. McCay and R. Caputto 1960 Incorporation of radioactive phosphate into organic phosphates of tocopherol-deficient and control rabbit muscles. *Am. J. Physiol.*, 199: 295.
34. Dinning, J. S., and P. L. Day 1957 Vitamin E deficiency in the monkey. I. Muscular dystrophy, hematologic changes, and the excretion of urinary nitrogenous constituents. *J. Exp. Med.*, 105: 395.
35. Fitch, C. D., and J. S. Dinning 1959 Phosphate metabolism in nutritional muscular dystrophy and hyperthyroidism. *Proc. Soc. Exp. Biol. Med.*, 100: 201.
36. Gerber, G. B., G. Gerber, T. R. Koszalka and V. M. Emmel 1962 Creatine metabolism in vitamin E deficiency in the rat. *Am. J. Physiol.*, 202: 453.

37. Young, J. M., J. S. Dinning and P. L. Day 1955 Metabolic inter-relationship of vitamins E and B₆. Proc. Soc. Exp. Biol. Med., 89: 216.
38. Nesheim, M. C., S. L. Leonard and M. L. Scott 1959 Alterations in some biochemical constituents of skeletal muscle of vitamin E deficient chicks. J. Nutrition, 68: 359.
39. Calvert, C. C., R. A. Monroe and M. L. Scott 1961 Studies on phosphorus metabolism in dystrophic chicks. J. Nutrition, 73: 355.
40. Scott, M. L. 1962 Vitamin E in health and disease of poultry. Vitamins and Hormones, 20: 621.
41. Tallan, H. H. 1955 Free amino acids of muscle of normal and vitamin E-deficient rabbits. Proc. Soc. Exp. Biol. Med., 89: 553.
42. Smith, L. C., and S. R. Nelson 1957 Effect of vitamin E-deficiency on free amino acids of various rabbit tissues. Proc. Soc. Exp. Biol. Med., 94: 644.
43. Diehl, J. F. 1962 Effect of vitamin E deficiency and fasting on in vivo oxidation of C¹⁴ labeled compounds in rabbits. Arch. Biochem. Biophys., 99: 148.
44. Sime, J. T., D. E. Young, J. S. Dinning and P. L. Day 1956 Vitamin E deficiency and biosynthesis of glycine, serine and methionine in rabbits. Proc. Soc. Exp. Biol. Med., 92: 556.
45. Azzone, G. F., E. Carofoli and G. Barbolini 1957 Glutathione in muscle and liver of rabbits with E avitaminosis. Atti. Accad. Naz. Lincei Rend., Classe Sci. fis, mat. e nat., 22: 763 (cited in Chem. Abstr., 52: 6519).
46. Ryerson, S. J., P. J. McMillan and R. A. Mortensen 1958 Effects of vitamin E deficiency on tissue and erythrocyte glutathione. J. Biol. Chem., 233: 1172.
47. Fegler, G. 1952 Relationship between reduced glutathione content and spontaneous hemolysis in shed blood. Nature, 170: 624.
48. Tsen, C. C., and H. B. Collier 1960 The relationship between the glutathione content of rat erythrocytes and their hemolysis by various agents in vitro. Canad. J. Biochem. Physiol., 38: 981.
49. Rose, C. S., and P. Gyorgy 1952 Specificity of hemolytic reaction in vitamin E deficient erythrocytes. Am. J. Physiol., 168: 414.
50. Horwitt, M. K. 1962 Interrelations between vitamin E and polyunsaturated fatty acids in adult men. Vitamins and Hormones, 20: 541.

51. Tsen, C. C., and H. B. Collier 1960 The protective action of tocopherol against hemolysis of rat erythrocytes by dialuric acid. *Canad. J. Biochem. Physiol.*, 38: 957.
52. Friedman, L., W. Weiss, F. Wherry, and O. L. Kline 1958 Bioassay of vitamin E by the dialuric acid hemolysis method. *J. Nutrition*, 65: 143.
53. Heard, D. H., T. Moore and I. M. Sharman 1952 The dialuric acid haemolysis test for vitamin E deficiency in rats. *Biochem. J.*, 52: i.
54. Bunyan, J., J. Green, E. E. Edwin and A. T. Diplock 1960 Studies on vitamin E. 5. Lipid peroxidation in dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes. *Biochem. J.*, 77: 47.
55. Rose, C. S., and P. Gyorgy 1950 Hemolysis with alloxan and alloxan-like compounds and the protective action of tocopherol. *Blood*, 5: 1062.
56. György, P., and C. S. Rose 1949 Tocopherol and hemolysis in vivo and in vitro. *Ann. N. Y. Acad. Sci.*, 52: 231.
57. Gitler, C., M. L. Sunde and C. A. Baumann 1958 Effect of certain necrosis-preventing factors on hemolysis in vitamin E deficient rats and chickens. *J. Nutrition*, 65: 397.
58. Muytjens, E. E. 1956 Haemolysis of erythrocytes from vitamin E-deficient chickens. *Biochim. Biophys. Acta*, 20: 553.
59. Rose, C. S., and P. György 1950 Tocopherol requirement of rats by means of the hemolysis test. *Proc. Soc. Exp. Biol. Med.*, 74: 411.
60. Ward, R. J. 1963 Sex differences in the α -tocopherol requirement of rats as shown by the hemolysis test. *Brit. J. Nutrition*, 17: 135.
61. Gordon, H. H., and J. P. DeMetry 1952 Hemolysis in hydrogen peroxide of erythrocytes of premature infants. Effect of alpha-tocopherol. *Proc. Soc. Exp. Biol. Med.*, 79: 446.
62. György, P., G. Cogan and C. S. Rose 1952 Availability of vitamin E in the newborn infant. *Proc. Soc. Exp. Biol. Med.*, 81: 536.
63. Horwitt, M. K., C. C. Harvey, G. D. Duncan, and W. C. Wilson 1956 Effects of limited tocopherol intake in man with relationships to erythrocyte hemolysis and lipid oxidations. *Am. J. Clin. Nutrition*, 4: 408.

64. Nitowsky, H. M., J. T. Tildon, S. Levin and H. H. Gordon 1962 Studies of tocopherol deficiency in infants and children. VII. Effect of tocopherol on urinary, plasma, and muscle creatine. *Am. J. Clin. Nutrition*, 10: 368.
65. Nitowsky, H. M., H. H. Gordon and J. T. Tildon 1956 Studies of tocopherol deficiency in patients with cystic fibrosis of the pancreas and biliary atresia. *A. M. A. J. Diseases Children*, 92: 491.
66. Harris, P. L., E. G. Hardenbrook, F. P. Dean, E. R. Cusack and J. L. Jensen 1961 Blood tocopherol values in normal human adults and incidence of vitamin E deficiency. *Proc. Soc. Exp. Biol. Med.*, 107: 381.
67. Leitner, Z. A., T. Moore and I. M. Sharman 1960 Vitamin A and vitamin E in human blood. 2. Levels of vitamin E in the blood of British men and women, 1952-7. *Brit. J. Nutrition*, 14: 281.
68. Horwitt, M. K. 1960 Vitamin E and lipid metabolism in man. *Am. J. Clin. Nutrition*, 8: 451.
69. Nitowsky, H. M., K. S. Hsu and H. H. Gordon 1962 Vitamin E requirements of human infants. *Vitamins and Hormones*, 20: 559.
70. Marvin, H. N., J. S. Dinning and P. L. Day 1960 Erythrocyte survival in vitamin E deficient monkeys. *Proc. Soc. Exp. Biol. Med.*, 105: 473.
71. Marvin, H. N. 1963 Erythrocyte survival of rats deficient in vitamin E or vitamin B₆. *J. Nutrition*, 80: 185.
72. Majaj, A. S., J. S. Dinning, S. A. Azzan and W. J. Darby 1963 Vitamin E responsive megaloblastic anemia in infants with protein-calorie malnutrition. *Am. J. Clin. Nutrition*, 12: 374.
73. Olcott, H. S., and H. A. Mattill 1937 Vitamin E and growth. *J. Nutrition*, 14: 305.
74. Emerson, G. A., and H. M. Evans 1937 The effect of vitamin E deficiency upon growth. *J. Nutrition*, 14: 169.
75. Reber, E. F., C. C. Morrell, H. W. Norton and H. E. Rhoades 1956 Studies of the effects of chlortetracycline, vitamin E and vitamin K in the nutrition of the rat. I. Growth. *J. Nutrition*, 58: 19.
76. Shirley, R. L., and G. K. Davis 1959 Effect of dietary protein, vitamin E and age on the lactic dehydrogenase and succinoxidase of the hearts of rats. *J. Nutrition*, 67: 635.

77. Witting, L. A., and M. K. Horwitt 1964 Effects of dietary selenium, methionine, fat level and tocopherol on rat growth. J. Nutrition, 84: 351.
78. Ringsted, A. 1935 A preliminary note on the appearance of paresis in adult rats suffering from chronic avitaminosis E. Biochem. J., 29: 788.
79. Draper, H. H., J. G. Bergan, M. Chiu, A. S. Csallany and A. V. Boaro 1964 A further study of the specificity of the vitamin E requirements for reproduction. J. Nutrition, 84: 395.
80. Draper, H. H., S. Goodyear, K. D. Barbee and B. C. Johnson 1956 Tolerance of the rat for N,N'-diphenyl-p-phenylenediamine. Proc. Soc. Exp. Biol. Med., 93: 186.
81. Crider, Q., A. T. Alaupovic and B. C. Johnson 1960 On the function and metabolism of vitamin E. 11. The effect of vitamin E on the level of non-vitamin E reducing compounds present in animal tissue. Biochem. Biophys. Res. Comm., 2: 293.
82. Harris, P. L., M. I. Ludwig and K. Schwarz 1958 Ineffectiveness of Factor 3 active selenium compounds in resorption-gestation bio-assay for vitamin E. Proc. Soc. Exp. Biol. Med., 97: 686.
83. Cheng, D. W., T. A. Bairnson, A. N. Rao and S. Subbammal 1960 Effect of variation of rations on the incidence of teratogeny in vitamin E-deficient rats. J. Nutrition, 71: 54.
84. Dam, H., and H. Granados 1945 Role of unsaturated fatty acids in changes of adipose and dental tissues in vitamin E deficiency. Science, 102: 327.
85. Irving, J. T. 1959 Curative effect of selenium upon the incisor teeth of rats deficient in vitamin E. Nature, 184: 645.
86. Moore, T., I. M. Sharman and K. R. Symonds 1958 Kidney changes in vitamin E-deficient rats. J. Nutrition, 65: 183.
87. Emmel, V. M., and P. L. LaCelle 1961 Studies on the kidney in vitamin E deficiency. II. Renal tocopherol content in relation to vitamin E deficiency changes in the kidney. J. Nutrition, 75: 335.
88. Moore, T., I. M. Sharman and R. J. Ward 1953 The vitamin E activity of methylene blue. Biochem. J., 53: xxxi.
89. Bieri, J. G., and E. L. Andrews 1964 Fatty acids in rat testes as affected by vitamin E. Biochem. Biophys. Res. Comm., 17: 115.

90. Corwin, L. M., and K. Schwarz 1963 Relation of tocopherol to enzyme sulfhydryl sites. Arch. Biochem. Biophys., 100: 385.
91. Schwarz, K. 1960 A possible site of action for vitamin E in intermediary metabolism. Am. J. Clin. Nutrition, 9, Part II: 94.
92. Schwarz, K. 1962 Vitamin E, trace elements and sulfhydryl groups in respiratory decline (an approach to the mode of action of tocopherol and related compounds). Vitamins and Hormones, 20: 463.
93. Tappel, A. L. 1962 Vitamin E as the biological lipid antioxidant. Vitamins and Hormones, 20: 493.
94. Budowski, P., and S. Mokadi 1961 Detection of free-radical damage in the vitamin E deficient chick. Biochim. Biophys. Acta, 52: 609.
95. Schwarz, K. 1961 (Note to symposia). Am. J. Clin. Nutrition, 9: 94.
96. Bieri, J. G., and E. L. Andrews 1963 Metabolic effects of selenium as related to vitamin E. J. Am. Oil Chemists Soc., 40: 365.
97. Bunyan, J., A. T. Diplock, E. E. Edwin and J. Green 1962 Exudative diathesis and lipid peroxidation in the chick. Brit. J. Nutrition, 16: 519.
98. Chernick, S. S., G. G. Moe, G. P. Rodnan and K. Schwarz 1955 A metabolic lesion in dietary necrotic liver degeneration. J. Biol. Chem., 217: 829.
99. Schwarz, K. 1954 Factors protecting against dietary necrotic liver degeneration. Ann. N. Y. Acad. Sci., 57: 878.
100. Schwarz, K. 1960 Factor 3, seleneium, and vitamin E. Nutrition Rev., 19: 193.
101. Beveridge, J. M. R. 1954 The influence of the endocrine glands on the development of acute massive liver necrosis. Ann. N. Y. Acad. Sci., 57: 873.
102. Lindan, O., and H. P. Himsworth 1950 Tocopherol and protein deficiency in relation to the development of dietetic massive necrosis of the liver in rats. Brit. J. Exp. Path., 31: 651.
103. Hove, E. L., D. H. Copeland and W. D. Salmon 1949 A fatal vitamin E deficiency disease in rats characterized by massive lung hemorrhage and liver necrosis. J. Nutrition, 39: 397.

104. Forbes, M., and P. Gyorgy 1957 Effect of yeast and of yeast extracts on liver necrosis and hemolysis by dialuric acid on red blood cells of rats on a necrogenic diet. *J. Nutrition*, 63: 461.
105. Mertz, W., and K. Schwarz 1958 Reversal of respiratory decline in necrotic liver degeneration by intraportal antioxidants. *Proc. Soc. Exp. Biol. Med.*, 98: 808.
106. Schwarz, K., J. A. Stesney and C. M. Foltz 1957 Relation between selenium traces in L-cystine and protection against dietary liver necrosis. *Metabolism*, 8: 88.
107. Schwarz, K. 1951 A hitherto unrecognized factor against dietary necrotic liver degeneration in American yeast (Factor 3). *Proc. Soc. Exp. Biol. Med.*, 78: 852.
108. Schwarz, K., W. Mertz and E. J. Simon 1959 In vitro effect of tocopherol metabolites on respiratory decline in dietary necrotic liver degeneration. *Biochim. Biophys. Acta*, 32: 484.
109. Corwin, L. M., and K. Schwarz 1960 Maintenance of α -ketoglutarate and succinate oxidation in E-deficient liver homogenates by α -tocopherol, a tocopherol metabolite, menadione and diphenylphenylenediamine. *Nature*, 186: 1048.
110. Grove, J. A., R. M. Johnson and J. H. Cline 1965 Vitamin E deficiency in the rat. I. Effect of substrate concentration on respiratory decline in liver homogenates from rats fed a vitamin E-deficient diet. *Arch. Biochem. Biophys.*, 110: 357.
111. Corwin, L. M. 1961 Role of microsomes in decline of oxidation in vitamin E-deficient rat liver homogenates. *Federation Proc.*, 20: 145 (abstract).
112. Yang, C. S., G. H. Dialameh, and R. E. Olson 1959 Selenium-sulfur interrelationships in the vitamin E-deficient weanling rat. *Federation Proc.*, 18: 553 (abstract).
113. Schwarz, K., C. E. Lee and J. A. P. Stesney 1962 Lipoyl dehydrogenase as a site of enzymic breakdown in respiratory decline. *Ztschr. Naturforsch.*, 176: 788.
114. Carpenter, S. 1965 A histochemical study of oxidative enzymes in the nervous system of vitamin E-deficient rats. *Neurology*, 15: 328.
115. Barry, T. A., and H. Rosenkrantz 1962 The level of isocitric acid dehydrogenase in tissues of vitamin E-deficient rabbits. *J. Nutrition*, 76: 447.

116. Laferte, R. O., H. Rosenkrantz and L. Berlinquet 1963 Trans-amination in muscular dystrophy and the effect of exogenous glutamate: A study on vitamin E-deficient rabbits, and mice with hereditary dystrophy. *Canad. J. Biochem. Physiol.*, 41: 1423.
117. Biswas, D. K., H. N. Bhagavan and B. C. Johnson 1963 Effect of vitamin E deficiency on the α -keto acid content and certain enzyme levels in rats and chicks. *Federation Proc.*, 22: 319 (abstract).
118. Mertz, W., and K. Schwarz 1959 Prevention of respiratory decline in necrotic liver degeneration by antioxidants in vitro. *Proc. Soc. Exp. Biol. Med.*, 102: 561.
119. Corwin, L. M. 1960 Thiobarbituric acid reaction in incubated rat liver homogenates. *Federation Proc.*, 19: 419 (abstract).
120. Zalkin, H., and A. L. Tappel 1960 Studies of the mechanism of vitamin E action. IV. Lipid peroxidation in the vitamin E-deficient rabbit. *Arch. Biochem. Biophys.*, 88: 113.
121. Wilbur, K. M., F. Bernheim and O. W. Shapiro 1949 The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. *Arch. Biochem.*, 24: 305.
122. Green, J., A. T. Diplock, J. Bunyan, E. E. Edwin, and P. McHale 1961 Ubiquinone (Coenzyme Q) and the function of vitamin E. *Nature*, 190: 318.
123. Cowlshaw, B. 1961 Interference by haem in the application of the thiobarbituric acid test. *Biochim. Biophys. Acta*, 49: 583.
124. Corwin, L. M. 1962 Studies on peroxidation in vitamin E-deficient rat liver homogenates. *Arch. Biochem. Biophys.*, 97: 51.
125. McLean, A. E. M. 1960 "Phenergan" and Versene in dietary liver necrosis. *Nature*, 185: 191.
126. Tappel, A. L., and H. Zalkin 1960 Inhibition of lipid peroxidation in microsomes by vitamin E. *Nature*, 185: 35.
127. Bieri, J. G., and A. A. Anderson 1960 Peroxidation of lipids in tissue homogenates as related to vitamin E. *Arch. Biochem. Biophys.*, 90: 105.
128. Bunyan, J., J. Green and A. T. Diplock 1963 Liver necrosis in lipid peroxidation in the rat. *Brit. J. Nutrition*, 17: 117.
129. Bieri, J. G. 1959 An effect of selenium and cystine on lipid peroxidation in tissues deficient in vitamin E. *Nature*, 184: 1148.

130. Dam, H. 1944 Studies on vitamin E deficiency in chicks. *J. Nutrition*, 27: 193.
131. Diehl, J. F. 1963 Effect of nutritional muscular dystrophy and of starvation on amino acid penetration in rabbit tissues. *Biochem. Ztschr.*, 337: 333.
132. Brown, R. G., G. M. Button and J. T. Smith 1963 Permeability changes induced in erythrocytes by vitamin E deficiency. *Federation Proc.*, 22: 318 (abstract).
133. Roels, O. A., A. Guha, M. Trout, U. Vakil and K. Joseph 1964 Effect of dietary α -tocopherol on protein metabolism in vitamin A-deficient rats. *J. Nutrition*, 84: 161.
134. Brown, R. G., G. M. Button and J. T. Smith 1963 A hypothesis for a common mode of action of the fat soluble vitamins A, D, and E. *J. Theoret. Biol.*, 5: 489.
135. Koszalka, T. R., K. E. Mason and G. Krol 1961 Relation of vitamin E to proteolytic and autolytic activity of skeletal muscle. *J. Nutrition*, 73: 78.
136. Weinstock, I. M., A. D. Goldrich and A. T. Milhorat 1955 Enzyme studies in muscular dystrophy. I. Muscle proteolytic activity and vitamin E-deficiency. *Proc. Soc. Exp. Biol. Med.*, 88: 257.
137. Desai, I. D., C. C. Calvert and M. L. Scott 1964 A time sequence study of the relationship of peroxidation, lysosomal enzymes, and nutritional muscular dystrophy. *Arch. Biochem. Biophys.*, 108: 60.
138. Draper, H. H., and A. S. Csallany 1958 Action of N,N'-diphenyl-p-phenylenediamine in tocopherol deficiency diseases. *Proc. Soc. Exp. Biol. Med.*, 99: 739.
139. Hove, E. L., G. S. Fry and K. Schwarz 1958 Ineffectiveness of Factor 3-active selenium compounds in muscular dystrophy of rabbits on vitamin E-free diets. *Proc. Soc. Exp. Biol. Med.*, 98: 27.
140. Nesheim, M. C., and M. L. Scott 1961 Nutritional effects of selenium compounds in chicks and turkeys. *Federation Proc.*, 20: 674.
141. Jellenek, M., L. L. Tureen and T. Cooper 1963 Myocardial catechol amine content in vitamin E- and sulfur-deficient chickens. *Proc. Soc. Exp. Biol. Med.*, 113: 524.

142. Nesheim, M. C., C. C. Calvert and M. L. Scott 1960 Effects of arginine deficiency on nutritional muscular dystrophy in the chick. *Proc. Soc. Exp. Biol. Med.*, 104: 783.
143. Scott, M. L., and C. C. Calvert 1962 Evidence of a specific effect of cystine in the prevention of nutritional muscular dystrophy in vitamin E-deficient chicks. *J. Nutrition*, 77: 105.
144. Machlin, L. J., and W. T. Shalkop 1956 Muscular degeneration in chickens fed diets low in vitamin E and sulfur. *J. Nutrition*, 60: 87.
145. Jensen, L. S., and J. McGinnis 1960 Influence of selenium, antioxidants and type of yeast on vitamin E deficiency in the adult chicken. *J. Nutrition*, 72: 23.
146. Jensen, L. S., J. S. Carver and J. McGinnis 1956 Effects of vitamin E, N,N'-diphenyl-para-phenylene diamine and fish liver oil on reproduction in turkeys. *Proc. Soc. Exp. Biol. Med.*, 91: 386.
147. Jensen, L. S., and J. McGinnis 1957 Studies on the vitamin E requirement of turkeys for reproduction. *Poultry Sci.*, 36: 1344.
148. Patterson, E. L., R. Mitstrey and E. L. R. Stokstad 1957 Effect of selenium in preventing exudative diathesis in chicks. *Proc. Soc. Exp. Biol. Med.*, 95: 617.
149. Krishnamurthy, S., and J. G. Bieri 1962 Dietary antioxidants as related to vitamin E function. *J. Nutrition*, 77: 245.
150. Machlin, L. J., R. S. Gordon and K. H. Meisky 1959 The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken. *J. Nutrition*, 67: 333.
151. Machlin, L. J., and R. S. Gordon 1960 (Note) *Nutrition Rev.*, 18: 95.
152. Machlin, L. J., and R. S. Gordon 1960 Linoleic acid as a causative agent of encephalomalacia in chickens fed oxidized fat. *Proc. Soc. Exp. Biol. Med.*, 103: 659.
153. Dam, H. 1944 Toxicity of fractions of hog liver fatty acids to chicks fed a vitamin E deficient diet. *J. Nutrition*, 28: 297.
154. Kokatnur, M. G., S. Akui, F. A. Kummerow and H. M. Scott. 1960 Effects of long chain keto acids on encephalomalacia in chicks. *Proc. Soc. Exp. Biol. Med.*, 104: 170.

155. Nishida, T., H. Tsuchiyama, M. Inoue and F. A. Kummerow 1960 Effect of intravenous injections of oxidized methyl esters of unsaturated fatty acids on chick encephalomalacia. *Proc. Soc. Exp. Biol. Med.*, 105: 508.
156. Donaldson, K. O., A. Nason, K. B. Moore and R. H. Garrett 1957 Specific restoration of mammalian cytochrome c reductase by tocopherol. *Biochim. Biophys. Acta*, 26: 665.
157. Nason, A., R. H. Garrett, P. P. Nair, F. D. Vasington and T. C. Detwiler 1964 Inhibition by digitonin of bovine heart muscle DPNH-cytochrome c reductase and its specific reversal by the tocopherols. *Biochem. Biophys. Res. Comm.*, 14: 220.
158. Donaldson, K. O., A. Nason and R. H. Garrett 1958 The role of lipids in electron transport. 10. Tocopherol as a specific co-factor of mammalian cytochrome c reductase. *J. Biol. Chem.*, 233: 572.
159. Donaldson, K. O., and A. Nason 1957 Interrelationship between vitamin E and lipid cofactor in the cytochrome c reductase system. *Proc. Natl. Acad. Sci.*, 43: 364.
160. Nason, A., and I. R. Lehman 1956 The role of lipide in electron transport. II. Lipide cofactor replaceable by tocopherol for the enzymatic reduction of cytochrome c. *J. Biol. Chem.*, 222: 511.
161. Pollard, C. J., and J. G. Bieri 1960 Studies of the biological function of vitamin E. II. The nature of the specific activating effect of tocopherol in aged preparations of cytochrome reductases. *J. Biol. Chem.*, 235: 1178.
162. Berne, R. M. 1960 Comparison of DL- and D- α -tocopherol in the reactivation of the DPNH-cytochrome c reductase of isooctane treated heart muscle preparations. *Biochim. Biophys. Acta*, 41: 527.
163. Redfearn, E. R., A. M. Pumphrey and G. H. Fynn 1960 The mechanism of reactivation of enzyme systems in mitochondrial preparations treated with organic solvents. *Biochim. Biophys. Acta*, 44: 404.
164. Pollard, C. J., and J. G. Bieri 1959 Studies of the biological function of vitamin E. I. Tocopherol and reduced diphosphopyridine nucleotide-cytochrome c reductase. *Biochim. Biophys. Acta*, 34: 420.
165. Slater, E. C. 1960 The possible role of vitamin E in respiratory enzymes. In: *Proceedings of the Fourth International Congress of Biochemistry*, Vol. XI, Pergamon Press, New York, p. 316.

166. Slater, E. C. 1961 The possible function of quinols, chromanols and chromenols in intracellular respiration. *Am. J. Clin. Nutrition*, 9, Part II: 50.
167. Slater, E. C., H. Rudney, J. Bouman and J. Links 1961 The possible role of α -tocopherol in the respiratory chain. III. The tocopherol and ubiquinone content of heart muscle preparations. *Biochim. Biophys. Acta*, 47: 497.
168. Kaunitz, H., and A. M. Pappenheimer 1942 Oxygen consumption in vitamin E deficiency. *Am. J. Physiol.*, 138: 328.
169. Friedman, I., and H. A. Matill 1941 The oxygen consumption of skeletal muscle from animals deprived of vitamin E. *Am. J. Physiol.*, 131: 595.
170. Houchin, O. B., and H. A. Matill 1942 In vitro effect of α -tocopherol phosphate on oxygen consumption of muscle from vitamin E-deficient animals. *Proc. Soc. Exp. Biol. Med.*, 50: 216.
171. Houchin, O. B., and H. A. Matill 1942 The oxygen consumption, creatine, and chloride content of muscles from vitamin E-deficient animals as influenced by feeding α -tocopherol. *J. Biol. Chem.*, 146: 301.
172. Taylor, D. W., and R. Wiseman 1962 Rate of oxygen uptake of erythrocytes of vitamin E-deficient rats. *Nature*, 196: 1102.
173. Bunyan, J., J. Green, E. E. Edwin and A. T. Diplock 1961 Some antioxidant properties of L-thyroxine in biological systems. *Biochim. Biophys. Acta*, 47: 401.
174. Taylor, D. W. 1958 Effects of tocopherols, methylene blue and glutathione on the manifestations of oxygen poisoning in vitamin E-deficient rats. *J. Physiol.*, 140: 37.
175. Mann, P. J. G., and J. H. Quaster 1946 Toxic effects of oxygen and hydrogen peroxide on brain metabolism. *Biochem. J.*, 40: 139.
176. Campbell, J. A. 1937 Oxygen poisoning and the thyroid gland. *J. Physiol.*, 90: 91P.
177. Hove, E. L., K. Hickman and P. L. Harris 1955 The effect of tocopherol and of fat on the resistance of rats to anoxic anoxia. *Arch. Biochem.*, 8: 395.
178. Biddulph, C., and R. K. Meyer 1942 The influence of vitamin E deficiency on the thyroid and basal metabolism of male rats. *Endocrinology*, 30: 551.

179. Telford, I. R., G. A. Emerson and H. M. Evans 1936 Claim for thyroid subnormality in vitamin E-low rats. Proc. Soc. Exp. Biol. Med., 38: 623.
180. Olson, R. E., and J. S. Dinning 1954 Enzyme abnormalities associated with dietary necrotic liver degeneration in rats. Ann. N. Y. Acad. Sci., 57: 889.
181. Olson, R. E., C. S. Yang, M. Riegl and B. Stewart 1955 Sulfur amino acid metabolism in dietary hepatic necrosis in rats. Federation Proc., 14: 447.
182. Olson, R. E. 1956 (discussion following paper). Am. J. Clin. Nutrition, 4: 389.
183. Chernick, S. S., J. G. Moe and K. Schwarz 1955 Dietary necrotic liver degeneration and Coenzyme A. Proc. Soc. Exp. Biol. Med., 89: 520.
184. Edwin, E. E., A. T. Diplock, J. Bunyan and J. Green 1960 Studies on vitamin E. 1. The determination of tocopherols in animal tissues. Biochem. J., 75: 450.
185. Edwin, E. E., A. T. Diplock, J. Bunyan and J. Green 1961 Studies on vitamin E. 6. Distribution. Biochem. J., 79: 91.
186. Cheng, D. W., K. G. Braun, B. J. Braun and K. H. Udani 1961 Tocopherol content of maternal and fetal rat tissues as related to vitamin E intake during gestation. J. Nutrition, 74: 111.
187. Wiss, O., R. H. Bunnell and U. Gloor 1962 Absorption and distribution of vitamin E in the tissues. Vitamins and Hormones, 20: 441.
188. Cowlshaw, B., E. Sondergaard, I. Prang and H. Dam 1957 Intracellular distribution of vitamin E and vitamin A in chicken liver. Biochim. Biophys. Acta, 25: 644.
189. Csallany, A. S., and H. H. Draper 1960 Determination of N,N'-diphenyl-p-phenylenediamine in animal tissues. Proc. Soc. Exp. Biol. Med., 104: 739.
190. Cowlshaw, B., and I. Prange 1957 A lipid present in yeast with certain properties similar to those of tocopherol. Biochim. Biophys. Acta, 23: 663.
191. Diplock, A. T., J. Green, E. E. Edwin and J. Bunyan 1961 Tocopherol, ubiquinones and ubichromenols in yeasts and mushrooms. Nature 189: 749.

192. Hess, W. C. 1949 The rates of absorption and the formation of liver glycogen by methionine, cystine, and cysteine. *J. Biol. Chem.*, 181: 23.
193. London, D. R., and T. H. Foley 1965 Cystine and cysteine metabolism in cystinuria. *Biochem. J.*, 94: 33P.
194. Wellers, G., and J. Chevan 1959 The metabolism of sulfur. I. Replacement of endogenous sulfur by adult rats during water starvation and nitrogen starvation and sulfur starvation. *J. Physiol. (Paris)*, 51: 723.
195. Wellers, G. 1959 Metabolism of sulfur. II. Replacement of endogenous loss in the adult rat by methionine and homocysteine. The minimum sulfur requirement. *J. Physiol. (Paris)*, 51: 999.
196. Wellers, G. 1962 Recherches sur le métabolisme du soufre. X. La non-équivalence de la cystine et de la cystéine dans la couverture des besoins soufres du rat adulte. *J. Physiol. (Paris)*, 54: 677.
197. Wellers, G., G. Boelle and J. Chevan 1960 Studies of sulfur metabolism. 4. Partial replacement of endogenous loss of sulfur by sodium sulfate in the adult white rat. *J. Physiol. (Paris)*, 52: 903.
198. Rambaut, P. C., and S. A. Miller 1965 Studies of sulfur amino acid nutrition in the adult cat. *Federation Proc.*, 24: 373 (abstract).
199. Gordon, R. S., and I. W. Sizer 1955 Ability of sodium sulfate to stimulate growth of the chicken, *Science*, 122: 1270.
200. Tarver, H., and C. L. A. Schmidt 1939 The conversion of methionine to cystine: experiments with radioactive sulfur. *J. Biol. Chem.*, 130: 67.
201. Tarver, H., and C. L. A. Schmidt 1942 Radioactive sulfur studies. I. Synthesis of methionine. II. Conversion of methionine sulfur to taurine sulfur in dogs and rats. III. Distribution of sulfur* in the proteins of animals fed sulfur* or methionine.* IV. Experiment in vitro with sulfur* and hydrogen sulfide.* *J. Biol. Chem.*, 146: 69.
202. Tarver, H., and W. O. Reinhardt 1947 Methionine labeled with radioactive sulfur as an indicator of protein formation in the hepatectomized dog. *J. Biol. Chem.*, 167: 395.

203. Virtue, R. W., and H. B. Lewis 1934 The metabolism of sulfur. XXI. Comparative studies of the metabolism of L-cystine and DL-methionine in the rabbit. J. Biol. Chem., 104: 59.
204. Brand, E., G. F. Cahill and R. J. Block 1935 Cystinuria. IV. The metabolism of homocysteine and homocystine. J. Biol. Chem., 110: 399.
205. DuVigneaud, V., G. W. Kilmer, J. R. Rachele and M. Cohn 1944 On the mechanism of the conversion in vivo of methionine to cystine. J. Biol. Chem., 155: 645.
206. Medes, G. 1937 Metabolism of sulphur. III. The excretion of cystine by normal individuals. Biochem. J., 31: 12.
207. Medes, G. 1937 Metabolism of sulphur. VI. Oxidation in the body of the sulfur-containing amino acids and some of their partially oxidized derivatives. Biochem. J., 31: 1330.
208. Cavallini, D., B. Mondovi and C. DeMarco 1955 The isolation of pure hypotaurine from the urine of rats fed cystine. J. Biol. Chem., 216: 577.
209. Cavallini, D., C. DeMarco, B. Mondovi and L. Tentori 1960 Radioautographic detection of metabolites of S³⁵-DL-cystine. J. Chromat., 3: 20.
210. Szczepkowski, T. W., B. Skarzynski and M. Weber 1961 The metabolic state of thiosulphate. Nature, 189: 1007.
211. Sorbo, B. 1962 On the acceptor specificity of rhodanese. Acta Chem. Scand., 16: 243.
212. Stearns, G., and H. B. Lewis 1930 The metabolism of sulfur. XVIII. The rate of oxidation of ingested cystine in the organism of the rabbit. J. Biol. Chem., 86: 93.
213. Meister, A., P. E. Fraser and S. V. Tice 1954 Enzymatic desulfuration of β -mercaptopyruvate to pyruvate. J. Biol. Chem., 206: 561.
214. McAfee, J. W., and M. A. Williams 1962 Effect of cysteine and pyridoxine on taurine excretion of male rats. Proc. Soc. Exp. Biol. Med., 109: 102.
215. Chatagner, F., H. Tabechian and B. Bergeret 1954 Repercussion d'une carence en vitamine B₆ sur le metabolisme de l'acide L-cysteine-sulfinique in vitro et in vivo, chez le rat. Biochim. Biophys. Acta, 13: 313.

216. Swan, P., J. Wentworth and H. Linkswiller 1964 Vitamin B₆ depletion in man. I. Cysteine metabolism. *Federation Proc.*, 23: 136 (abstract).
217. Fromageot, C., and M. A. Royané 1946 Quelques aperçus concernant l'oxydation du soufre organique chez les animaux supérieurs. *Helv. Chim. Acta*, 29: 1279.
218. Eldjarn, L., and A. Sverdrup 1955 The metabolism of cysteamine sulphinic acid (hypotaurine) in rats, investigated by means of radioactive sulphur (³⁵S). *Acta Chem. Scand.*, 9: 1037.
219. Schmidt, C. L. A., E. von Adelung and T. Watson 1918 On the elimination of taurine administered to man. *J. Biol. Chem.*, 33: 501.
220. Lloyd, A. G. 1961 In vivo metabolism of N-acetyl glucosamine ³⁵S-sulfate esters. *Nature*, 190: 915.
221. Rodahl, K., B. Issekutz, Jr., and D. M. Shumen 1965 Effect of excess vitamin A on sulfur metabolism in the rat. *J. Nutrition*, 85: 174.
222. Portman, O. W., and G. V. Mann 1956 Further studies of the metabolism of taurine S³⁵ by the rat. *J. Biol. Chem.*, 220: 105.
223. Wu, C. 1954 Metabolism of free amino acids in fasted and zein-fed rats. *J. Biol. Chem.*, 207: 775.
224. Hele, T. S., and N. W. Pirie 1931 Studies on the sulphur metabolism of the dog. VIII. The metabolism of glutathione compared with that of other cystine derivatives. *Biochem. J.*, 25: 1095.
225. Virtue, R. W., and M. E. Doster-Virtue 1937 Studies on the production of taurocholic acid in the dog. *J. Biol. Chem.*, 119: 697.
226. Virtue, R. W., and M. E. Doster-Virtue 1941 Studies on the production of taurocholic acid in the dog. V. Methionine sulfoxide. *J. Biol. Chem.*, 137: 227.
227. Virtue, R. W., and M. E. Doster-Virtue 1939 Studies on the production of taurocholic acid in the dog. IV. Cysteine, homocysteine, and thioglycolic acid. *J. Biol. Chem.*, 128: 665.
228. Virtue, R. W., and M. E. Doster-Virtue 1938 Studies on the production of taurocholic acid in the dog. II. Cystamine. *J. Biol. Chem.*, 126: 141.

229. Virtue, R. W., and M. E. Doster-Virtue 1939 Studies on the production of taurocholic acid in the dog. III. Cystine disulfoxide, cysteine sulfinic acid, and cysteic acid. J. Biol. Chem., 127: 431.
230. Portman, O. W., and G. V. Mann 1955 The disposition of taurine S³⁵ and taurocholate-S³⁵ in the rat: dietary influences. J. Biol. Chem., 213: 733.
231. Fillios, L. L., and G. V. Mann 1954 Influence of sulfur amino acid deficiency on cholesterol metabolism. Metabolism 3: 16.
232. Mann, G. V., S. B. Andrus, A. McNally and F. J. Stare 1953 Experimental atherosclerosis in Cebus monkeys. J. Exp. Med., 98: 195.
233. Hierholzer, K., R. Cade, R. Gurd, R. H. Kissler and R. Pitts 1960 Stop-flow analysis of renal reabsorption and excretion of sulfate in the dog. Am. J. Physiol., 198: 833.
234. Berglund, F., C. G. Helander and R. B. Howe 1960 Inorganic sulfate and thiosulfate transport and competition in renal tubules of the dog. Am. J. Physiol., 198: 586.
235. Halverson, A. W., C. L. Hills and E. I. Whitehead 1964 Studies on selenium toxicity and chondroitin sulfate and taurine biosynthesis in the chick embryo. Arch. Biochem. Biophys., 107: 88.
236. Blaxter, K. L. 1962 Vitamin E in health and disease of cattle and sheep. Vitamins and Hormones, 20: 633.
237. Halverson, A. W., P. L. Guss, and O. E. Olson 1962 Effect of sulfur salts on selenium poisoning in the rat. J. Nutrition, 77: 459.
238. Ganther, H. E., and C. A. Baumann 1962 Selenium metabolism. II. Modifying effects of sulfate. J. Nutrition, 77: 408.
239. Barker, S. 1960 Effect of inorganic sulphate on molybdenum excretion by a marsupial. Nature, 185: 41.
240. Miller, R. F., and R. W. Engel 1960 Interrelationships of copper, molybdenum and sulfate sulfur in nutrition. Federation Proc., 19: 666.
241. Nair, P. P., and N. G. Magar 1958 Studies in vitamin E-molybdenum interrelationships. Metabolism of phosphorus, creatine-creatinine, molybdenum and vitamin E in the albino rat. Ind. J. Med. Res., 46: 561.

242. Siegal, L. M., and K. J. Monty 1961 A mechanism for the copper-molybdenum interrelation. II. Response of liver sulfide oxidase activity to nutritional factors. *J. Nutrition*, 74: 167.
243. Halverson, A. W., and K. J. Monty 1960 An effect of dietary sulfate on selenium poisoning in the rat. *J. Nutrition*, 70: 100.
244. Hess, W. C. 1952 Cystathionine in the liver following the feeding of methionine and serine. *Arch. Biochem. Biophys.*, 40: 127.
245. Awapara, J. 1950 Alanine and taurine formation from injected cysteine in the rat. *Nature*, 165: 76.
246. Awapara, J., and W. J. Wingo 1953 On the mechanism of taurine formation from cysteine in the rat. *J. Biol. Chem.*, 203: 189.
247. Awapara, J. 1953 2-Aminoethanesulfinic acid: an intermediate in the oxidation of cysteine in vivo. *J. Biol. Chem.*, 203: 183.
248. Bergeret, B., and F. Chatagner 1952 Désulfinication et décarboxylation de l'acide L-cystéine sulfinique chez l'animal vivant. *Biochim. Biophys. Acta*, 9: 147.
249. Bergeret, B., and F. Chatagner 1954 Sur la présence d'acide cystéinesulfinique dans le cerveau du rat normal. *Biochim. Biophys. Acta*, 14: 297.
250. Chapeville, F., P. Fromageot, A. Brigelhuber and M. Henry 1956 Utilisation des sulfites par l'animal supérieur. *Biochim. Biophys. Acta*, 20: 351.
251. Awapara, J., A. J. Landua and R. Fuerst 1950 Distribution of free amino acids and related substances in organs of the rat. *Biochim. Biophys. Acta*, 5: 457.
252. Bremer, J. 1955 A method for the estimation of the taurine contents and its conjugation with cholic acid in rat liver. *Acta Chem. Scand.*, 9: 683.
253. Machlin, L. J., P. B. Pearson, C. A. Denton and H. R. Bird 1953 The utilization of sulfate sulfur by the laying hen and its incorporation into cystine. *J. Biol. Chem.*, 205: 213.
254. Machlin, L. J., P. B. Pearson and C. A. Denton 1955 The utilization of sulfate sulfur for the synthesis of taurine in the developing chick embryo. *J. Biol. Chem.*, 212: 469.

255. Davidson, E. A., and W. Small 1963 Metabolism in vivo of connective tissue mucopolysaccharides. III. Chondroitin sulfate and keratosulfate of cartilage. *Biochim. Biophys. Acta*, 69: 459.
256. Balasubramanian, A. S., and B. K. Bachhawat 1961 Studies on the formation of active sulfate during connective tissue growth. *Biochim. Biophys. Acta*, 54: 266.
257. Singher, H. O., and L. Marinelle 1945 Distribution of radioactive sulfur in the rat. *Science*, 101: 414.
258. Boström, H., and S. Aqvist 1952 Utilization of S³⁵-labelled sodium sulphate in the synthesis of chondroitin sulphuric acid, taurine, methionine and cystine. *Acta Chem. Scand.*, 6: 1557.
259. Dziewiatkowski, D. D. 1954 Utilization of sulfate sulfur in the rat for the synthesis of cystine. *J. Biol. Chem.*, 207: 181.
260. Dziewiatkowski, D. D., R. E. Benesch and R. Benesch 1949 On the possible utilization of sulfate sulfur by the suckling rat for the synthesis of chondroitin sulfate as indicated by the use of radioactive sulfur. *J. Biol. Chem.*, 178: 931.
261. Dziewiatkowski, D. D. 1949 Rate of excretion of radioactive sulfur and its concentration in some tissues of the rat after intraperitoneal administration of labeled sodium sulfate. *J. Biol. Chem.*, 178: 197.
262. Dziewiatkowski, D. D. 1953 Sulfate-sulfur metabolism in the rat fetus as indicated by sulfur-35. *J. Exp. Med.*, 98: 119.
263. Hele, T. S. 1936 Studies in the sulphur metabolism of the dog. IX. The synthesis of phenyl sulphate and indoxyl sulphate. *Biochem. J.*, 25: 1736.
264. Binkley, F. 1949 The source of sulfate in the formation of ethereal sulfates. *J. Biol. Chem.*, 178: 821.
265. Binkley, F., and D. Okeson 1950 Purification of the enzyme responsible for the cleavage of cystathionine. *J. Biol. Chem.*, 182: 273.
266. Matsuo, Y., and D. M. Greenberg 1958 A crystalline enzyme that cleaves homoserine and cystathionine. I. Isolation procedures and some physicochemical properties. *J. Biol. Chem.*, 230: 545.
267. Matsuo, Y., and D. M. Greenberg 1959 A crystalline enzyme that cleaves homoserine and cystathionine. IV. Mechanism of action, reversability and substrate specificity. *J. Biol. Chem.*, 234: 516.

268. Kato, A., T. Matsuzawa, M. Suda, H. Nakagawa and J. Ishizuka 1964 Control mechanism in the rat liver enzyme system converting L-methionine to L-cystine. I. Enzymatic basis for the methionine-sparing action of L-cystine. *J. Biochem.*, 55: 401.
269. Chatagner, F., J. Labouesse, O. Trautmann and B. Jolles-Bergeret 1961 Identite probable de la cystéine désulfurase "soluble" et de la cystathionase du foie du rat (note). *C. R. Acad. Sci. (Paris)*, 253: 742.
270. Jolles-Bergeret, B., J. Labouesse and F. Chatagner 1960 Action of thyroid hormones on desulfuration of cysteine by rat liver injections. Comparison of desulfuration with other enzymic reactions which necessitate pyridoxal phosphate. *Bull. Soc. Chim. Biol.*, 42: 51.
271. Chatagner, F., and O. Trautmann 1962 "Soluble" cysteine desulphurase of rat liver as an adaptive enzyme. *Nature*, 194: 1281.
272. Bergeret, B., D. Brun, J. Labouesse and F. Chatagner 1963 Degradation of L-cysteine by a purified enzyme from rat liver, soluble cysteine desulfurase or cystathionase. *Bull. Soc. Chem. Biol.*, 45: 397.
273. Mondovi, B., and C. DeMarco 1961 The mechanism of cysteine-hypotaurine trans-sulphuration in the presence of cystathionase. *Enzymologia*, 23: 156.
274. DeMarco, C., and M. Colletta 1961 Production of S-sulfocysteine and S-sulfocysteamine from cystine and cystamine, coupled to enzymic transamination and deamination of cysteinesulfinic acid. *Biochim. Biophys. Acta*, 47: 262.
275. Jolles-Bergeret, B., and F. Chatagner 1964 Effect of 2-mercaptoethanol on the deamination of L-cysteine by cystathionase of rat liver (letter). *Arch. Biochem. Biophys.*, 105: 640.
276. Sorbo, B. H. 1958 On the metabolism of thiosulfate esters. *Acta Chem. Scand.*, 12: 1990.
277. Sorbo, B. 1962 Mechanism of rhodanese inhibition of sulfite and cyanide. *Acta Chem. Scand.*, 16: 2455.
278. Cavallini, D., C. DeMarco and B. Mondovi 1961 The enzymic conversion of cystamine and thiocysteamine into thiotaurine and hypotaurine. *Enzymologia*, 23: 101.

279. DeMarco, C., G. Bombardieri, F. Riva, S. Dupei and D. Cavallini 1965 Degradation of cystaldimine, the product of oxidative deamination of cystamine. *Biochim. Biophys. Acta*, 100: 89.
280. Cavallini, D., R. Scandurra and C. DeMarco 1965 The role of sulphur, sulphide and reducible dyes in the enzymic oxidation of cysteamine to hypotaurine. *Biochem. J.*, 96: 781.
281. Cavallini, D., R. Scandurra and C. DeMarco 1963 The enzymic oxidation of cysteamine to hypotaurine in the presence of sulfide. *J. Biol. Chem.*, 238: 2999.
282. DeMarco, C., and M. Colletta 1961 Thiosulfate production during transamination of alanine-thiosulfonic acid. *Biochim. Biophys. Acta*, 47: 257.
283. Cavallini, D., C. DeMarco, B. Mondovi and F. Stirpe 1954 The biological oxidation of hypotaurine. *Biochim. Biophys. Acta*, 15: 301.
284. Sumizu, K. 1962 Oxidation of hypotaurine in rat liver. *Biochim. Biophys. Acta*, 63: 210.
285. Mondovi, B., A. Scioscia-Santoro and M. T. Costa 1962 Distribution of some enzymes concerned with cleavage of cysteine in animal organs. *Enzymologia*, 25: 167.
286. Chatagner, F., B. Jolles-Bergeret and J. Labouesse 1960 Different cellular localization of the two enzyme systems producing hydrogen sulfide from cysteine in rat liver. *C. R. Acad. Sci. (Paris)*, 251: 3097.
287. Meister, A. 1955 Transamination. *Advances in Enzymology*, 16: 185.
288. Bergeret, B., D. Brun, J. Labouesse and F. Chatagner 1963 Étude de la dégradation de la L-cystéine par un enzyme purifié isolé du foie de rat (cystéine désulfurase "soluble" ou cystathionase). *Bull. Soc. Chim. Biol.*, 45: 397.
289. Goryachenkova, E. V. 1961 Pathways of enzymic formation of hydrogen sulfide from L-cysteine in the liver. *Biokhimiya*, 26: 480.
290. Fromageot, C., E. Wookey and P. Chaix 1940 Sur la dégradation anaérobie de la cystéine par le désulfurase du foie. *Enzymologia*, 9: 198.
291. Fromageot, C. 1955 The metabolism of sulfur and its relations to general metabolism. *Harvey Lectures*, 1953-4 series, p. 1.

292. Baxter, C. F., R. Van Reen and P. B. Pearson 1956 Oxidation of sulfide by an adaptive system in rat liver. *Federation Proc.*, 15: 215 (abstract).
293. Sörbo, B. 1958 On the formation of thiosulfate from inorganic sulfide by liver tissue and heme compounds. *Biochim. Biophys. Acta*, 27: 324.
294. Sörbo, B. 1957 Enzymic transfer of sulfur from mercaptopyruvate to sulfite or sulfinates. *Biochim. Biophys. Acta*, 24: 324.
295. Brüggemann, J., and M. Waldschmidt 1962 Die Serensulfhydrase aus Hühnerleber: Rückreaktion und Vergleich mit die Cysteinde-sulfhydrase. *Biochem. Ztschr.*, 335: 408.
296. Mahendra, N. D., A. Goswami, R. Robblee and L. W. McElroy 1959 Further observations on factors affecting L-cysteine desulfhydrase activity in chick liver. *J. Nutrition*, 68: 671.
297. Bernheim, F., and M. L. C. Bernheim 1939 Note on the action of manganese and some other metals on the oxidation of certain substances by liver. *J. Biol. Chem.*, 128: 79.
298. Medes, G., and N. Floyd 1942 Metabolism of sulphur. XI. Further investigation of the enzymic oxidation of sulfur-containing amino acids. *Biochem. J.*, 36: 259.
299. Medes, G. 1939 Metabolism of sulphur. VIII. Oxidation of the sulfur-containing amino acids by enzymes from the liver of the albino rat. *Biochem. J.*, 33: 1559.
300. Pirie, N. W. 1934 The formation of sulphate from cysteine and methionine by tissues in vitro. *Biochem. J.*, 28: 305.
301. Sörbo, B., and L. Ewet 1965 The enzymatic oxidation of cysteine to cysteine sulfinic acid in rat liver. *Biochem. Biophys. Res. Comm.*, 18: 359.
302. Wainer, A. 1964 The production of sulfate from cysteine without the formation of free cysteinesulfinic acid. *Biochem. Biophys. Res. Comm.*, 16: 141.
303. Wainer, A. 1965 The production of cysteinesulfinic acid from cysteine in vitro. *Biochem. Biophys. Acta*, 104: 405.
304. Awapara, J., and V. M. Doctor 1955 Enzymatic oxidation of cysteine-S³⁵ to cysteine sulfinic acid. *Arch. Biochem. Biophys.*, 58: 506 (Note).

305. Chapeville, F., and F. Chatagner 1955 La formation de l'acide cystéinesulfinique à partir de la cystine chez le rat. *Biochim. Biophys. Acta*, 17: 275.
306. Chatagner, F. 1961 The contributions of Professor Claude Fromageot and his co-workers to the study of enzymic reactions of sulfur compounds. In *Organic Sulfur Compounds*, ed., N. Kharasch. Pergamon Press, New York, p. 399.
307. Singer, T. P., and E. B. Kearney 1956 Intermediary metabolism of L-cysteinesulfinic acid in animal tissues. *Arch. Biochem. Biophys.*, 61: 397.
308. Singer, T. P., and E. B. Kearney 1954 Pathways of L-cysteinesulfinate metabolism in animal tissues. *Biochim. Biophys. Acta*, 14: 570.
309. Chatagner, F., B. Bergeret, T. Sejourné and C. Fromageot 1952 Transamination et desulfination de l'acide L-cysteinesulfinique. *Biochim. Biophys. Acta*, 9: 340.
310. Sumizu, K. 1961 Cysteinesulfinic desulfinase in rat liver. *Biochim. Biophys. Acta*, 53: 435.
311. Chatagner, F., and B. Bergeret 1951 Décarboxylation enzymatique, in vitro et in vivo, de l'acide-L-cystéinesulfinique dans le foie des animaux supérieurs. *C. R. Acad. Sci. (Paris)*, 232: 448.
312. Jacobsen, J., and L. H. Smith, Jr. 1963 Comparison of decarboxylation of cysteine sulphinic acid-1-C¹⁴ and cysteic acid 1-C¹⁴ by human, dog, and rat liver and brain. *Nature*, 200: 575.
313. Chatagner, F., and B. Bergeret 1956 Décarboxylation de l'acide cystéinesulfinique par le foie et le cerveau du rat mâle, du rat femelle et du rat femelle ovariectomisé, *Bull. Soc. Chim. Biol.*, 38: 1159.
314. Hope, D. B. 1955 Pyridoxal phosphate as the coenzyme of the mammalian decarboxylase for L-cysteine sulphinic and L-cysteic acids. *Biochem. J.*, 59: 497.
315. Davison, A. N. 1956 Amino acid decarboxylases in rat brain and liver. *Biochim. Biophys. Acta*, 19: 66.
316. Jacobsen, J. G., L. L. Thomas and L. H. Smith 1964 Properties and distribution of mammalian L-cysteine sulfinic acid carboxylases. *Biochim. Biophys. Acta*, 85: 103.

317. Sorbo, B., and T. Heyman 1957 On the purification of cysteine-sulfinic acid decarboxylase and its substrate specificity. *Biochim. Biophys. Acta*, 23: 624.
318. Bergeret, B., F. Chatagner and C. Fromageot 1956 Study of the decarboxylation of cysteinesulfinic acid, cysteic acid and glutamic acid by different organs of the rabbit. Influence of pyridoxal phosphate and thiol groups. *Biochim. Biophys. Acta*, 22: 329.
319. Davison, A. N. 1956 The mechanism of the inhibition of decarboxylases by isonicotinyl hydrazide. *Biochim. Biophys. Acta*, 19: 131.
320. Blaschko, H. 1945 The amino acid decarboxylases of mammalian tissue. *Advances in Enzymology*, 5: 67.
321. Read, W. O., and J. D. Welty 1962 Synthesis of taurine and isethionic acid by dog heart slices. *J. Biol. Chem.*, 237: 1521.
322. Welty, J. D., W. O. Read and E. H. Shaw 1962 Isolation of 2-hydroxyethanesulfonic acid (Isethionic acid) from dog heart. *J. Biol. Chem.*, 237: 1160.
323. Fromageot, P., F. Chapeville and L. Petit 1957 Formation enzymatique de l'acide cysteinesulfinique a partir de sulfite par l'embryon de veau. *Biochim. Biophys. Acta*, 23: 12.
324. MacLeod, R. M., W. Farkas, I. Fridovich and P. Handler 1961 Purification and properties of hepatic sulfite oxidase. *J. Biol. Chem.*, 236: 1841.
325. Hunter, F. E., Jr., and L. Ford 1954 Phosphorylation coupled with sulfite oxidation in liver mitochondria. *Federation Proc.*, 13: 234 (abstract).
326. Kent, P. W., and C. A. Pasternak 1958 Biosynthesis of intestinal mucins. 3. Formation of "active sulphate" by cell-free extracts of sheep colonic mucosa. *Biochem. J.*, 69: 453.
327. Balasubramanian, A. S., and B. K. Bachhawat 1962 Enzymic degradation of active sulfate. *Biochim. Biophys. Acta*, 59: 389.
328. Robinson, H. C. 1965 The reduction of inorganic sulphate to inorganic sulphite in the small intestine of the rat. *Biochem. J.*, 94: 687.
329. Robinson, H. C., and C. A. Pasternak 1964 The isolation of S-sulphoglutathione from the small intestine of the rat. *Biochem. J.*, 93: 487.

330. Waldschmidt, M. 1962 Vergleich des Einbaues von ^{35}S -Sulfid und ^{35}S -Sulfat in das Korpereiweiss von Ratten. Biochem. Ztschr., 335: 400.
331. Adams, J. B., and M. F. Meaney 1961 Specificity of sulfate transfer to chondroitin sulfate in human tumor extracts. Biochim. Biophys. Acta, 54: 592.
332. Hasegawa, E., A. Delbruck and F. Lipmann 1961 Sulfate transfer specificity for chondroitin sulfates in tissue preparations. Federation Proc., 20: 86.
333. Spencer, B. 1960 Endogenous sulfate acceptors in the rat. Biochem. J., 77: 294.
334. Spolter, L., and W. Marx 1959 Transfer of radioactive sulfate from phosphoadenosine phosphosulfate to heparin. Biochim. Biophys. Acta, 32: 291.
335. Adams, J. B. 1963 Formation of steroid sulfates by extracts of human adrenals. Biochim. Biophys. Acta, 71: 243.
336. Adams, J. B. 1959 Effect of chondroitin on the uptake of radioactive sulfate into chondroitin sulfate. Biochim. Biophys. Acta, 32: 559.
337. Adams, J. B. 1960 The biosynthesis of chondroitin sulfate. Incorporation of sulphate into chondroitin sulfate in embryonic chick cartilage. Biochem. J., 76: 520.
338. Suzuki, S., R. H. Trenn and J. L. Strominger 1961 Separation of specific mucopolysaccharide sulfotransferases. Biochim. Biophys. Acta, 50: 169.
339. Suzuki, S., and J. L. Strominger 1959 Enzymic synthesis of sulfated mucopolysaccharides in hen oviduct. Biochim. Biophys. Acta, 31: 283.
340. DeMeio, R. H., and R. I. Arnolt 1944 Phenol conjugation. II. The conjugation by rat and cat tissues in vitro. J. Biol. Chem., 156: 577.
341. DeMeio, R. H., and L. Tkacz 1950 Conjugation of phenol by rat liver homogenate. Arch. Biochem., 27: 242.
342. Brunngraber, E. G. 1958 Nucleotides involved in the enzymatic conjugation of phenols with sulfate. J. Biol. Chem., 233: 472.

343. Cavallini, D., B. Mondovi, C. DeMarco and S. Scioscia-Santoro 1962 Inhibitory effect of mercaptoethanol and hypotaurine on the desulfhydration of cysteine by cystathionase. Arch. Biochem. Biophys., 96: 456.
344. DeMarco, C., M. Coletta and D. Cavallini 1963 Cystine cleavage in alkaline medium. Arch. Biochem. Biophys., 100: 51.
345. Cavallini, D., C. DeMarco and B. Mondovi 1960 Cleavage of cystine by a pyridoxal model. Arch. Biochem. Biophys., 87: 281.
346. Ratsisalovanina, O., F. Chapeville and P. Fromageot 1961 Synthesis of sulfur amino acids from cysteine, serine and phosphoserine catalyzed by pyridoxal and a metal. Biochim. Biophys. Acta, 49: 322.
347. Lavine, T. F. 1937 The action of mercuric sulfate and chloride on cysteine, cystine, cysteine sulfinic acid ($R-SO_2H$) and cysteic acid with reference to the dismutation of cystine. J. Biol. Chem., 117: 309.
348. Fromageot, P., V. R. Roderick and J. Pougat 1963 Synthèse non-enzymatique de l'acide cystéique à partir de quelques acides amines β -substitués, de pyruvate, de sulfite et d'ions cuivre. Biochim. Biophys. Acta, 78: 126.
349. Postgate, J. R. 1963 The examination of sulfur auxotrophs: a warning. J. Gen. Microbiol., 30: 481.
350. Tsen, C. C., and A. L. Tappel 1958 Catalytic oxidation of glutathione and other sulfhydryl compounds by selenate. J. Biol. Chem., 233: 1230.
351. Garvin, J. E. 1960 A new method for the determination of taurine in tissues. Arch. Biochem. Biophys., 91: 219.
352. Schram, E., and R. Crokaut 1957 Radiochromatographic study of substances formed from S^{35} -taurine in the rat. Biochem. J., 66: 20P.
353. Bergeret, B., and F. Chatagner 1954 Utilisation d'échangeurs d'ions pour la séparation de quelques acides aminés formés au cours de la dégradation enzymatique de l'acide cystéinesulfinique. Application à l'isolement de l'hypotaurine. Biochim. Biophys. Acta, 14: 543.
354. Pentz, E. I., C. H. Davenport, W. Glover and D. D. Smith 1957 A test for the determination of taurine in urine. J. Biol. Chem., 228: 433.

355. Tallan, H. H., S. Moore and W. H. Stein 1954 Studies on the free amino acids and related compounds in the tissues of the cat. *J. Biol. Chem.*, 211: 927.
356. Bricas, E., F. Kieffer and C. Fromageot 1955 Nouvelle synthese de l'acide amino-2 ethanesulfinique (hypotaurine). *Biochim. Biophys. Acta*, 18: 358.
357. Hope, D. B. 1957 Cystathionine in the urine of pyridoxine-deficient rats. *Biochem. J.*, 66: 486.
358. Awapara, J. 1957 Absorption of injected taurine-S³⁵ by rat organs. *J. Biol. Chem.*, 225: 877.
359. Bergeret, B., and F. Chatagner 1952 Désulfinication et décarboxylation enzymatiques de l'acide L-cystéine-sulfinique: sa transformation quantitative en alanine et en hypotaurine. *Biochim. Biophys. Acta*, 9: 141.
360. Fromageot, C. 1955 Sulfatases and desulfinases. In *Methods in Enzymology*, Vol. 2, eds., S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York, p. 324.
361. DeMarco, C. 1963 Detection of sulphur compounds on paper chromatograms. *Nature*, 198: 683.
362. Winegard, H. M., and G. Toennies 1948 Detection of sulfur-containing amino acids on paper chromatograms. *Science*, 108: 506.
363. Pendergrass, B. J. 1961 The Interrelationships Between Sulfate Metabolism and the Tocopherols in the Weanling Rat. Unpublished Master's Thesis. The University of Tennessee.
364. Button, G. M. 1965 The Influence of Cod-liver Oil Upon the Metabolism of Sulfur. Unpublished Ph. D. Thesis. The University of Tennessee.
365. Brown, R. G. 1964 Some Aspects of Vitamin E and Collagen Relationships. Unpublished Ph. D. Thesis. The University of Tennessee.
366. Gilmore, M. F. 1963 A Comparison of the Utilization of Organic and Inorganic Sulfur by the Rat. Unpublished Master's Thesis. The University of Tennessee.
367. Michels, F. G., and J. T. Smith 1965 A comparison of the utilization of organic and inorganic sulfur by the rat. *J. Nutrition*, 87: 217.

368. Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. *J. Nutrition*, 14: 273.
369. Brown, R. G., G. M. Button and J. T. Smith 1965 Changes in collagen metabolism caused by feeding diets low in inorganic sulfur. *J. Nutrition*, 87: 228.
370. Hogeboom, G. H. 1955 Fractionation of cell components of animal tissues. In *Methods in Enzymology*, Vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York, p. 16.
371. Patrick, A. D. 1962 The degradative metabolism of L-cysteine and L-cystine in vitro by liver in cystinosis. *Biochem. J.*, 83: 248.
372. Katz, J., and S. B. Golden 1959 A rapid method for S³⁵ radioassay and gravimetric sulfur determination in biologic material. *J. Lab. Clin. Med.*, 53: 658.
373. Peck, H. D., Jr. 1961 Reversibility of the reaction catalyzed by adenosine-5'-phosphosulfate reductase. *Biochem. Biophys. Acta*, 49: 621.
374. Grant, W. 1947 Colorimetric determination of sulfur dioxide. *Anal. Chem.*, 19: 345.
375. Leinweber, F. J., and K. J. Monty 1962 Microdetermination of cysteinesulfinic acid. *Anal. Biochem.*, 4: 252.
376. Fisher, L. J., S. L. Bunting and L. E. Rosenberg 1963 A modified ninhydrin colorimetric method for the determination of plasma-alpha-amino nitrogen. *Clin. Chem.*, 9: 573.
377. Awapara, J. 1948 Application of paper chromatography to the estimation of free amino acids in tissues. *Arch. Biochem.*, 19: 172.
378. Willets, C. O., and C. L. Ogg 1950 Report on standardization of microchemical methods. Micro-Kjeldahl nitrogen determination. *J. Assoc. Official Agric. Chem.*, 33: 179.
379. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265.
380. Steele, R. G. D., and J. H. Torrie 1960 *Principles and Procedures of Statistics*. McGraw-Hill Book Company, New York, p. 78.
381. Blumberg, H. 1935 A growth deficiency disease curable by wheat germ oil. *J. Biol. Chem.*, 108: 227.

382. Wilson, L. G., and R. S. Bandurski 1958 Enzymatic reduction of sulfate. J. Am. Chem. Soc., 80: 5576.
383. Torii, K., and R. S. Bandurski 1964 A possible intermediate in the reduction of 3' phosphoryl-5' adenosinephosphosulfate to sulfite. Biochem. Biophys. Res. Comm., 14: 537.
384. Peck, H. D., Jr., T. E. Deacon and J. T. Davidson 1965 Studies on adenosine-5'-phosphosulfate reductase from Desulfovibrio desulfuricans and Thiobacillus thioparus. I. The assay and purification. Biochim. Biophys. Acta, 96: 429.
385. Bandurski, R. S., L. G. Wilson and C. L. Squires 1956 The mechanism of "active sulfate" formation. J. Am. Chem. Soc., 78: 6408.
386. Wilson, L. G., and R. S. Bandurski 1958 Enzymatic reactions involving sulfate, sulfite, selenate, and molybdate. J. Biol. Chem., 233: 975.
387. Anast, C., R. Kennedy, G. Volk and L. Adamson 1965 In vitro studies of sulfate transport by the small intestine of the rat, rabbit, and hamster. J. Lab. Clin. Med., 65: 903.
388. Chatagner, F. 1959 Influence de quelques vitamines et hormones sur le metabolisme du soufre. Proceedings of the Fourth International Congress of Biochemistry, Vienna, 1958, Vol. XI. Pergamon Press, New York, p. 5.