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Interrelationships Between Sulfate Metabolism and Tocopherols in the Weanling Rat

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To the Graduate Council:

I am submitting herewith a thesis written by Young Ping Chin entitled "Interrelationships Between Sulfate Metabolism and Tocopherols in the Weanling Rat." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

John T. Smith, Major Professor

We have read this thesis and recommend its acceptance:

Florence L. MacLeod, Ruth Buckley

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

Nutrition Dept

THE UNIVERSITY OF TENNESSEE

THE GRADUATE SCHOOL

ABSTRACT OF EDUCATIONAL RESEARCH STUDY COMPLETED

Author of Study Hung Ping Chin Date November 30, 1961

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Abstract approved by John T. Smith
(signature of major professor)

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There have been numerous investigations in which reported observations point toward the existence of metabolic interrelationships between tocopherols, the sulfur-containing amino acids, and inorganic sulfur. In previous work, this laboratory has used mixtures partially composed of oxidized casein and casein to produce a protein source having a low methionine content and lending variation with respect to inorganic sulfate. Alpha protein, a purified soybean protein, is characteristically similar to oxidized casein in that both substances have a low sulfur amino acid content. Due to the expense involved in the laboratory use of oxidized casein, the present investigation was undertaken to determine if alpha protein rather than a casein-oxidized casein mixture could be used advantageously in studying the interrelationships of sulfur-containing amino acids, tocopherols, and sulfate. In addition, it would be of interest to study growth effects of young animals receiving diets containing varied amounts and forms of sulfur both with and without sufficient vitamin E.

Four groups of diets were used. Although two diets were basically the same with regard to level of sulfur, there was some variation in the ratio of inorganic: organic sulfur. Eight matched groups of five rats each were maintained on the experimental diets for 70 days, with each group receiving diets containing sufficient amounts of vitamin E while the corresponding littermates consumed the same diet which had been depleted with respect to alpha-tocopherol. Food consumption for each group was determined and recorded daily. Weekly recordings of weights were made to observe growth.

None of the rats attained the weight normally observed for ninety-day-old rats in this colony. In contrast, eight of the animals on the vitamin-E deficient diets died. Supplementation of the diets with both vitamin E and methionine resulted in improved growth and greater food efficiency. Supplementation with cysteine and vitamin E did not effect improved growth or food efficiency. A sex difference was observed in the effect of vitamin E supplementation on weight change in all diets showing any weight gain. The female rats responded most favorably to vitamin E supplementation. The data obtained from this investigation confirm a previous proposal that sufficient dietary vitamin E is essential for optimal utilization of sulfur-containing amino acids. In addition, alpha protein does not appear to be a satisfactory protein source, low in sulfur-containing amino acids, for investigational purposes when weanling rats are employed in studying interrelationships between sulfur metabolism and vitamin E.

Form G-20

December 6, 1961

To the Graduate Council:

I am submitting herewith a thesis written by Young Ping Chin entitled "Interrelationships Between Sulfate Metabolism and Tocopherols in the Weanling Rat." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

John T. Smith
Major Professor

We have read this thesis and recommend its acceptance:

Florence Fluor Sod

Ruth Buckley

Accepted for the Council:

Dean of the Graduate School

INTERRELATIONSHIPS BETWEEN SULFATE METABOLISM AND TOCOPHEROLS IN THE WEANLING RAT

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Young Ping Chin

December 1961

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INTRODUCTION

There have been numerous investigations in which reported observations point toward the existence of metabolic interrelationships between tocopherols, the sulfur-containing amino acids, and inorganic sulfur. However, the exact nature of these interrelationships has not been elucidated. In previous work, this laboratory has used mixtures partially composed of oxidized casein and casein to produce a protein source having a low methionine content and lending variation with respect to inorganic sulfate. Adult rats were used to study the interrelationships of tocopherols and sulfur metabolism through analysis of blood samples for circulating sulfhydryl, per cent chemical hemolysis, and incorporation of $S^{35}O_4^=$ into erythrocyte stroma. From this study with adult rats, it was concluded that dietary vitamin E affected the conversion of methionine to sulfate.

Alpha protein, a purified soybean protein, is characteristically similar to oxidized casein in that both substances have a low sulfur amino acid content. Due to the expense involved in the laboratory use of oxidized casein, the present investigation was undertaken to determine if alpha protein rather than a casein-oxidized casein mixture could be used advantageously in studying the interrelationships of sulfur-containing amino acids, tocopherols, and sulfate.

Although adult rats served as experimental animals in the previous study, it was felt that the same information could be obtained using weanling rats. In addition, it would be of interest to study

growth effects on young animals receiving diets containing various amounts and forms of sulfur both with and without sufficient vitamin E. Therefore, in the present investigation, weanling rats were selected for the study.

REVIEW OF LITERATURE

The original suggestion of a requirement for vitamin E in animals was based on the protection it offered against sterility (Evans and Bishop, '23; Sure, '24; Mattill et al., '24) and the development of muscular dystrophy. Although these phenomena have not been associated with vitamin E deficiency in man, a tocopherol requirement has been generally accepted as essential for human nutrition.

A variety of pathologic states has been produced from animals fed on diets deficient in vitamin E. Differences in lesions have varied from one species to another and even differ with ages in the same species. According to Gordon et al. ('58), some of the conditions which have been found are: fetal resorption, encephalomalacia, acute hemorrhagic necrosis of the liver, degeneration of renal tubules, necrosis of cardiac muscle, nutritional muscular dystrophy, testicular degeneration, and generalized edema. Also, there have been clinical findings associated with vitamin E deficiency in the young of various species. These findings include: hemorrhagic manifestations in rat fetuses and chick embryos (Adamson, '47), hemorrhages in the lungs, visceral and cranial cavities in puppies (Elvehjem et al., '44), subcutaneous pulmonary and cerebral edema in young chickens (Dam and Glavind, '39; Bird and Culton, '40), anemia in monkeys (Dinning and Day, '57), and hemolysis after administration of large doses of vitamin K to rats (Allison et al., '56). However, nutritional muscular dystrophy appears to be the lesion occurring in the greatest number of species.

This was produced exclusively by dietary means in 1939 (MacKenzie, '53). If the deficiency is prolonged, the lesions found in animals may eventually resemble those found in human progressive muscular dystrophy.

Reported observations from various laboratories have pointed toward the existence of metabolic interrelationships between sulfur-containing amino acids and vitamin E. Machlin and Shalhop ('56) in studies with chickens up to four weeks of age were able to demonstrate the development of muscular degeneration from feeding diets low in vitamin E and sulfur. Upon the addition of alpha-tocopherol acetate, methionine, cystine, or a high level of diphenyl-p-phenylenadiamine (0.25 per cent) to the diets, it was possible to completely prevent muscular degeneration. Addition of 0.5 per cent Na_2SO_4 afforded slight protection.

Diets deficient in vitamin E and sulfur amino acids have been shown to cause a well-defined syndrome of massive hepatic necrosis in weanling rats (Olson et al., '55; Gitler, '58; Chernick et al., '55; Schwarz, '54, '58; György et al., '50). Chernick et al. ('55) and Schwarz, ('54) have shown that either vitamin E or cystine alone affords protection against the necrotic liver degeneration produced in animals by diets deficient in sulfur-containing amino acids and vitamin E. Studies conducted by Gitler et al. ('58) on the induction of liver necrosis by diets low in vitamin E showed the syndrome was prevented by additions of cysteine, methionine, some antioxidants, brewer's yeast, or selenium as well as by tocopherol. The results shown by the above studies contribute to the growing body of evidence that several

symptoms of vitamin E deficiency may develop independently and that agents substituting for vitamin E are capable of correcting only certain of these symptoms while tocopherol itself corrects all of them.

Some detailed studies by Schwarz and Foltz ('60) concerning the interrelationship between sulfur amino acid supplementation and tocopherol requirement in protecting against liver necrosis have revealed that sulfur amino acids decrease the requirement for alpha-tocopherol to approximately one-tenth of that normally required.

Necrogenic diets used in work done by Olson et al. ('55) contained washed alpha protein as a source of nitrogen and vitamin E-free lard as a source of fat. Vitamin E was one of the supplements found to be partially or wholly protective against hepatic necrosis. Supplements of cystine exerted paradoxical effects. At a level of 0.2 per cent, the onset of necrosis was hastened; whereas, at 1.0 per cent, appreciable protection was afforded.

In an attempt to further clarify some of the interrelationships involved in the protection offered against hepatic necrosis, the fates of S^{35}-labeled cystine and C^{14}-methyl-labeled methionine were investigated in deficient and control animals. In earlier studies (Weinstock et al., '54), it was shown that S^{35}-labeled cystine is incorporated into liver coenzyme A in normal rats; however, in deficient animals, the rate of incorporation is greatly depressed despite the high rate of incorporation into liver protein. Incorporation of C^{14}-methyl-labeled methionine into liver protein and liver choline appeared similar in both deficient and vitamin E-fed control animals. Tallan ('55)

was able to show elevated glutathione levels in muscle extracts from vitamin E-deficient rabbits. In contrast, Tsen and Collier ('60) found no difference in the glutathione content of erythrocytes from either vitamin E-sufficient or deficient rats but were able to observe a non-specific hemolysis by sulfhydryl reagents. Previous work in this laboratory (Pendergrass, '61) has shown a decrease in both circulating free sulfhydryl levels and erythrocyte stroma incorporation of $S^{35}O_4^{--}$ in vitamin E-sufficient rats maintained on diets low in inorganic sulfate. These data have been interpreted to indicate a requirement of vitamin E for optimal use of the sulfur-containing amino acids.

Reports concerning relations between vitamin E and various phases of cholesterol metabolism are limited and are controversial in nature. Century and Horwitt ('58) and Dam et al. ('58) found vitamin E had virtually no effect on cholesterol levels in the liver. In studying the effects of sulfur compounds on hypercholesteremia, Mann et al. ('60) made the observation that marked lipidemia was prevented when monkeys fed diets rich in cholesterol, choline, and neutral fats, but low in organic sulfur compounds, were placed on identical diets supplemented with L-cystine or DL-methionine. If supplementation was made following the appearance of serum change, there was a large but incomplete reversal of the abnormality. Similar observations have been made with mice, rats (Fillos and Mann, '54) and chickens (Kokatnur et al., '58). Jones et al. ('57) found that both methionine and alpha-tocopherol in serum cholesterol were effective in preventing hyper-cholesterolemia; whereas, when used singly, neither offered protection

against the malady.

The extended Elgin study of human tocopherol needs (Horwitt, '59) has resulted in an indication that tocopherol levels of tissues can be related to the amounts of oxidizable lipid consumed. In addition, it was concluded that the feeding of unsaturated lipids, which had been slightly oxidized to remove tocopherols, produced gastrointestinal lesions diagnosed as peptic ulcers. In other studies, Horwitt ('60) found that lipids high in linoleic acid increased the need for tocopherol. This evidence is an indication that tissue tocopherol may be stored less efficiently than tissue linoleic acid, leaving a tocopherol deficiency where linoleic consumption has been abnormally high.

The role of tocopherols as an intracellular antioxidant (Dam, '57) may be the cause of many of its effects. Some types of metabolic reactions occur in which tocopherol can be replaced by other antioxidants. However, in some instances, the requirement for tocopherol appears to be specific; therefore, it is credited with being a principal antioxidant in the body. The action of the vitamin as an in vivo antioxidant is probably responsible for regulating cholesterol levels in various body tissues but is probably also due to the role vitamin E plays in certain enzymatic processes (Alfin-Slater, '60).

In reviewing past studies, Rose and György ('52) brought attention to reports (György and Rose, '48; Rose and György, '50) which show the dialuric acid hemolysis of red blood cells from vitamin E-deficient rats. Treatment by the use of fat soluble antioxidants: alpha-, beta-, gamma-, and delta-tocopherols inhibited the hemolysis of erythrocytes

of rats deficient in vitamin E by dialuric acid. This activity was not demonstrated by ethinyl estradiol, diethylsilbesterol, or four substituted hydroquinones. Effectiveness of the tocopherols decreased in the order alpha-, beta-, gamma-, and delta-. A similar series may be obtained with respect to the effectiveness in the prevention of fetal reabsorption by the various tocopherols.

György et al. ('52) have offered further evidence for a physiological deficiency of vitamin E in newborn infants. They demonstrated that vitamin E administration blocks in vitro hydrogen peroxide hemolysis of erythrocytes from the newborn.

In observing effects of certain necrosis-preventing factors on hemolysis in vitamin E-deficient rats and chickens, Gitler ('58) felt hemolysis may also serve as an indication of vitamin E depletion in chicks. In other work related to this area (Bieri et al., '60), it was demonstrated that under certain dietary conditions, young chicks grew and developed normally without dietary vitamin E and other antioxidants.

Another investigation reported by Bieri ('61) indicated that selenium and cystine altered the composition of chick tissues so that the capacity to peroxidize lipides was reduced. It appears to be an indirect action since the addition of selenium and cystine in vitro to homogenates did not reduce peroxidation. It is probable that selenium was acting by sparing tocopherol since experience has indicated that the rate of vitamin E depletion from tissues is not influenced by biologically active selenium.

It is now well established that in the absence of vitamin E, the production of various vitamin E deficiency symptoms in chicks can be controlled at will not only by alterations in type and amount of polyunsaturated fatty acids (Dam et al., '58) but also by inclusion or omission of dietary selenium (Patterson et al., '57; Schwarz et al., '57) or cystine (Dam et al., '52). Likewise, it has been established that all of the vitamin E deficiency syndromes in the chick can be prevented by antioxidants chemically unrelated to alpha-tocopherol (Dam, '57; Machlin et al., '59); therefore, the sole biochemical function of vitamin E appears to be that of a cellular antioxidant. From his studies, Bieri ('61) suggested that selenium and cystine perform as antioxidants by a mechanism similar to alpha-tocopherol. In view of the intimate association of selenium with sulfur amino acids (McConnell and Wabnitz, '57), it may be hypothesized that an alteration of normal proteins by their selenium content in some manner increases the antioxigenic potential of the cell. Despite the observations mentioned, Schwarz ('61) feels that tocopherol, in its active form, has a distinct catalytic role in intermediary metabolism, and the antioxidant function of vitamin E may be strictly coincidental to its true metabolic function.

From work done by Nitowsky and Tildon ('56), there seems to be a correlation between the ability of various antioxidants to inhibit both hemolysis and catalysis of unsaturated fatty acid oxidation by lipoxidase or hematin compounds. These observations lend support to the hypothesis that vitamin E may play a significant role in maintaining

the integrity of the erythrocyte stroma by inhibition of certain oxidases which act upon the unsaturated fatty acids of the cell membrane.

Documentary evidence (du Vigneaud et al., '42; Binkley et al., '42; Binkley and du Vigneaud, '42; Stetten, '42; Binkley, '44; du Vigneaud et al., '44) has been shown for the mechanism by which cysteine and cystine may be formed rapidly from methionine. This transformation has been shown to proceed stepwise in the following manner: first, a demethylation of methionine to form homocysteine; a reaction of homocysteine with serine to form cystathionine, and finally, hydrolytic cleavage of cystathionine to yield cysteine and homoserine.

Upon the formation of cysteine, a series of oxidation reactions occur in which either sulfate or taurine are formed and are either utilized to form bile salts, mucopolysaccharides, etc. or excreted as urinary constituents. A prompt excretion of excess urinary sulfur results from the consumption of excess methionine, cysteine and/or cystine. Of the sulfur present in the urine, 80 per cent occurs as sulfate.

There have been observations that the biological value of proteins in which methionine is the limiting amino acid is lower for the adult rat than either the growing rat or adult human (Albanese, '59). The most probable explanation given for these age and species differences in requirements may rest on the high cystine content of hair and other keratins (Block and Bolling, '51) as well as on the probability that the sulfur-containing amino acid requirements of the adult rat are dominated by the requirement for hair growth rather than tissue growth.

It was shown by Heard and Lewis ('38) that dietary sulfur levels influence the sulfur content and distribution of hair in growing rats. Mueller and Cox ('47) and Cox et al. ('47) have proposed the following concept explaining the difference between the adult rat and man with respect to protein nutrition. They believe hair growth in the adult human is a minor factor in determining requirements for amino acids since the human body is only sparsely covered with hair.

In studies of sulfate requirements for adult rats, Wellers ('59) using diets consisting of essential amino acids plus glutamic acid and ammonium carbonate as a source of nitrogen found the minimum requirement in methionine sulfur to be 30 mg/day/kg. When methionine was omitted from the diet, an equal quantity of glutamic acid was substituted. When methionine was given in adequate quantity and was the only source of sulfur available in the diet, it secured a positive sulfur balance and a weight increase. In the same investigation, it was concluded that when methionine or homocysteine were given, neutral sulfur represented the most important fraction of sulfur urinary excretion. Another study of sulfur excretion in the adult rat (Wellers and Chevan, '59) indicated that a third of the endogenic sulfur excretion came from the metabolism of sulfur-containing compounds other than proteins, with the remaining two-thirds coming from the metabolism of proteins. Wellers and Chevan, in addition, found that urinary neutral sulfur always represented an important fraction of the urinary sulfur in endogenic sulfur excretion.

Wilgus ('36) in his growth studies reported that the use of raw

soybean oil meal in the diet of chicks and other monogastric animals had long been known to result in detrimental effects due to the presence of growth-inhibiting materials which were heat labile. Moist heat treatment was found effective in bringing about an improvement in the nutritive value of extracted soybeans for these animals. Bowman ('44) and Ham and Sandstedt ('44) were among the first investigators to discover the presence of potent trypsin inhibitors in raw soybean meal. Extensive investigations carried out by Kunitz ('45, '46, '47), Ham et al. ('45), and Borchers et al. ('48) reported the isolation, crystallization, and characterization of a soybean trypsin inhibitor. These studies demonstrated an inhibition of growth in rats and chicks fed diets supplemented with a crude preparation of a trypsin inhibitor.

Protein efficiency of soybean flour for the mouse was found to be inversely proportional to the trypsin inhibitor content of the flour (Westfall and Hague, '48). Westfall et al. ('48) observed a decreased protein efficiency and growth rate when mice were fed protein hydrolysates supplemented with a crude preparation of soybean trypsin inhibitor. Several attempts have been made by workers (Brochers and Ackerson, '51; Almquist and Merritt, '52, '53) in this area. They have reported that small amounts of crude or crystalline trypsin were effective in overcoming the growth inhibition of rats and chickens receiving raw soybean oil meal. Some divergence from this point of view was reported by Brambila et al. ('61) who studied chicks fed diets containing raw soybean oil meal. These workers indicated that trypsin supplementation would not overcome the growth-depressing properties of

raw soybean oil meal. Supplementation with a crude or crystalline preparation failed to improve the metabolizable energy of nonfat components of these diets. Trypsin inhibitor preparations were more detrimental in diets containing heated soybean oil meal than those with raw soybean oil meal. Trypsin supplementation improved the digestibility of dietary soybean oil. The effect of raw soybeans on fat digestion appeared dependent upon the age of the bird.

METHODS

I. PREPARATION OF DIETS

The present investigation was designed to evaluate alpha protein rather than a casein-oxidized casein mixture as the principal nitrogen source in studying the metabolic interrelationships between the sulfur-containing amino acids, inorganic sulfur, and vitamin E in weanling rats. Since the study was fundamentally designed to examine the role of tocopherol as related to sulfur metabolism, diets were selected with regard for the inorganic and/or organic sulfur content. Consequently, four groups of diets which were modifications of those used by Pendergrass ('61) as modeled after that of Capputo et al. ('58) were selected. Although two diets were basically the same with regard to level of sulfur, there was some variation in the ratio of inorganic to organic sulfur. Composition of the diets used are presented in further detail in Table I. The essential amino acid composition of alpha protein may be obtained by reference to Table II.

Inorganic sulfur was varied by using modifications of the Hubbell-Mendel-Wakeman ('37) salt mixture. These modifications were made and tested in this laboratory (Pendergrass, '61). In order to vary the sulfate content of the salt mixtures (Table III), $CaSO_4 \cdot 2H_2O$ was used as a partial substitute for $CaCO_3$. Because of molecular weight differences, this resulted in a slight reduction in the total calcium in the salt mixture; however, the modification has been shown to support

TABLE I

COMPOSITION OF THE DIETS

Component	Grams / 100 grams			
	A & A_E	B & B_E	C & C_E	D & D_E
Alpha protein	15	15	15	15
DL Threonine	0.100	0.100	0.100	0.100
DL Methionine	0	0.500	0	0.500
Sucrose	39	39	39	39
Cornstarch	34	34	34	34
Stripped lard	6	6	6	6
Cod liver oil	2	2	2	2
Vitamin mixture[a]	1	1	1	1
Salt mixture[b]	3 (#1)	3 (#2)	3 (#3)	3 (#3)

[a]Synthetic vitamins added as supplement to each 100 grams of diet: (in milligrams) alpha-tocopherol acetate, 28.0; nicotinic acid, 20.0; pyridoxine-HCl, 0.5; thiamine-HCl, 0.5; riboflavin, 0.5; calcium pantothenate, 1.0; folic acid, 0.5; biotin, 0.005; 2-methylnapthoquinone, 0.025; vitamin B_{12}, 0.0045; choline chloride, 100.0; inositol, 100.0; PABA, 7.5; vitamin A, 400 I. U.; calciferol, 200 I. U. The vitamins were triturated with sufficient sucrose to make 1 gram. Vitamin E was omitted from one mixture. When the vitamin E deficient vitamin mixture was used, the diets were designated A, B, C, and D.

[b]See Table III.

TABLE II

ESSENTIAL AMINO ACID COMPOSITION OF ALPHA PROTEIN[a]

Essential amino acids	Grams / 100 grams protein (16 grams nitrogen)
Arginine	7.6
Histidine	2.9
Lysine	6.7
Tyrosine	3.8
Tryptophan	1.5
Phenylalanine	6.3
Cystine	1.3
Methionine	1.7
Threonine	3.2
Leucine	7.3
Isoleucine	6.2
Valine	5.1

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

TABLE III

SALT MIXTURES

Component	Grams / 100 grams		
	#1 14.00 per cent SO_4	#2 3.34 per cent SO_4	#3 0.007 per cent SO_4
$CaCO_3$	30.346	41.250	44.750
$CaSO_4 \cdot H_2O$	25.097	6.000	0.000
Cornstarch	0.000	8.193	10.693
$MgCO_3$	3.060	3.060	3.060
NaCl	6.900	6.900	6.900
KCl	11.200	11.200	11.200
KH_2PO_4	21.200	21.200	21.200
$FePO_4(2H_2O)$	2.050	2.050	2.050
KI	0.008	0.008	0.008
NaF	0.010	0.010	0.010
$MnCl_2(4H_2O)$	0.040	0.040	0.040
$AlK(SO_4)_2$	0.017	0.017	0.017
$Cu(C_2H_3O_2)_2H_2O$	0.072	0.072	0.072

growth of weanling rats as well as the Osborne-Mendel ('19) salt mix (Pendergrass, '61). For the tests described by Pendergrass, the Osborne-Mendel salt mixture was used at the 5 per cent level, and the modified Hubbell-Mendel-Wakeman salt mixture at the 3 per cent level; therefore, the modified mixture was incorporated into these diets at the 3 per cent level.

II. DETERMINATION OF TOTAL SULFATE

The method selected for determination of total sulfate was that of Häkkinen and Häkkinen ('59) with modifications. Their efforts to develop more rapid methods suitable for routine determination of sulfate in serum and urine resulted in a relatively simple and easy method for spectrophotometric or colorimetric determinations. Due to the simplicity and sensitivity of their procedure, it was chosen and followed with some degree of modification.

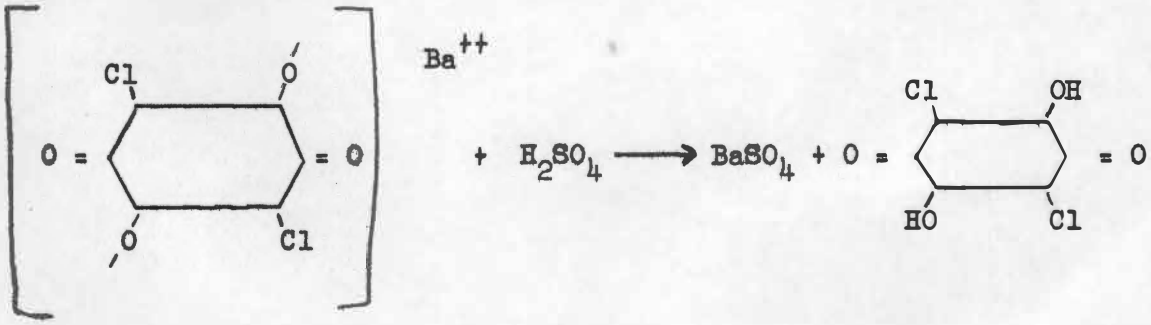
After diet samples were dried to constant weights and placed on a hot plate, 10 ml portions of concentrated HNO_3 were added to digest the samples in 50 ml Erlenmeyer flasks. When the mixtures evaporated to an ash, an additional 5 ml of the acid was added to each flask. Following evaporation of the liquid, additional acid was added in 1 ml portions until the digested samples resulted in a clear liquid.

Following complete evaporation in the digestion flasks, 10 ml water and 5 ml 0.5 per cent oxalic acid were added to each flask. As a result, upon boiling and allowing to cool for one-half hour, the oxalic acid precipitated the calcium that was present. The calcium

oxalate was then filtered on to #42 filter paper into 25 ml beakers. The Erlenmeyer flasks were rinsed with 5 ml portions of 1 M acetate buffer (ph 4). Following the transfer of the original filtrate into 25 ml volumetric flasks, successive rinsings with 5 cc portions of the acetate buffer were continued until the filtrate was diluted to the 25 ml mark on the flasks. Use of the buffer allowed the removal of larger samples for determinations.

Three ml portions of solution were removed from each volumetric flask representing a different diet sample. These were placed into 5 x 125 mm test tubes. To each tube, 3 ml 95 per cent ethanol and approximately 50 mg barium chloranilate were added. A blank containing approximately 50 mg barium chloranilate, 1 ml 1 M acetate buffer, and 2 ml distilled water was prepared in order to subtract the absorption of color of the samples from the result. In addition, a standard was made with 50 mg barium chloranilate, 1 ml 1 M acetate buffer, 1 ml distilled water, and 1 ml 240 ug $SO_4^=$ / ml of $SO_4^=$ standard solution.

The tubes were agitated by a twirling technique for 15 minutes. During this period, the sulfate reacted with barium chloranilate to form barium sulfate and chloranilic acid. The following equation illustrates the reaction which occurred:



Because chloranilic acid is a purple-colored compound, a purple color resulted.

The tubes were centrifuged approximately 5 minutes at 2000 rpm with a rheostat setting of 30 in order to remove the precipitate. After the liquid was decanted into Klett tubes, the tubes were wiped clean. Measurements of the purple color were made with a Klett-Summerson Photometer, using the No. 52 (green) filter. Optical density was set to zero with the prepared blank. Finally, the analyzed values of total sulfate were determined by the following equations:

1. $\frac{RU}{RS} \times \frac{240}{1} = \mu g\ SO_4^{=}$ in 3 ml

Where RU = Reading of the Unknown

RS = Klett Reading of the Standard

2. $\frac{\mu g\ SO_4^{=} \text{ in 3 ml} \times 8.34}{\text{Wt. sample (gms)} \times 1 \times 10^4} = \%\ SO_4^{=}$ in the Sample

III. PROCEDURE

Forty weanling albino rats of the Wistar strain and of the same age were placed on eight different diets. The animals were divided into eight groups; 3 males and 2 females to each group. The males were caged individually in wire-bottomed cages while the pair of females on each diet was housed in the same cage. Both food and distilled water were offered ad libitum. The designated diet for each group was supplied daily in amounts of 20 gms for each animal. Food consumption for each of the eight groups was determined and recorded

daily. Weekly recordings of weights were made in order to observe growth. The animals were maintained on the experimental diets for a period of 70 days. With the exception of one case, littermates were selected with ten rats used on each of the four diets. Five rats, comprising a group, received diets containing sufficient amounts of vitamin E while the corresponding littermates consumed diets which had been depleted with respect to alpha-tocopherol.

In the third week of the experimental period, 0.2 per cent cysteine HCl was added to the diets for groups C and C_E. After five weeks on their dietary regime, groups A and A_E began receiving 20 per cent alpha protein. Diet adjustment for this alteration was made at the expense of sucrose.

RESULTS AND DISCUSSION

The data which are reported in Table IV compare the analyzed values for the per cent sulfur in the various diets with the calculated per cent sulfur expressed as sulfate. These data indicate that the calculated amounts of sulfate and those actually found are in good agreement. As was stated previously, these diets were varied with respect to inorganic and neutral sulfur. For example, the sulfate in diet groups C and D is all furnished by neutral sulfur; whereas, in diet group B, .58 per cent is contributed by neutral sulfur and .10 per cent by inorganic sulfate, and in diet group A, .32 per cent comes from neutral sulfur and .42 per cent from inorganic sulfate.

The nomogram shown in Figure 1 illustrates a comparison between the percentage essential amino acid composition of a diet prepared with 15 per cent alpha protein and the essential amino acid requirements reported by Rose ('37). In addition, this nomogram depicts the essential amino acid pattern following supplementation with 100 mg threonine and 500 mg of methionine per 100 gms of diet. As stated previously, threonine supplementation was made in all diets; however, only diet groups B and D were supplemented with methionine. As one may see from the nomogram, supplementation with methionine and threonine appears to give an adequate amino acid pattern for growth.

The data which are shown in Tables V and VI represent the response of littermate weanling rats to a ten-week feeding of these diets. The data which are shown in these tables represent the initial

TABLE IV

SULFUR IN DIETS AS SULFATE

Diets	Per cent sulfate	
	Calculated	Analyzed
A and A_E	.74	.71
B and B_E	.68	.68
C and C_E	.44	.47
D and D_E	.58	.58

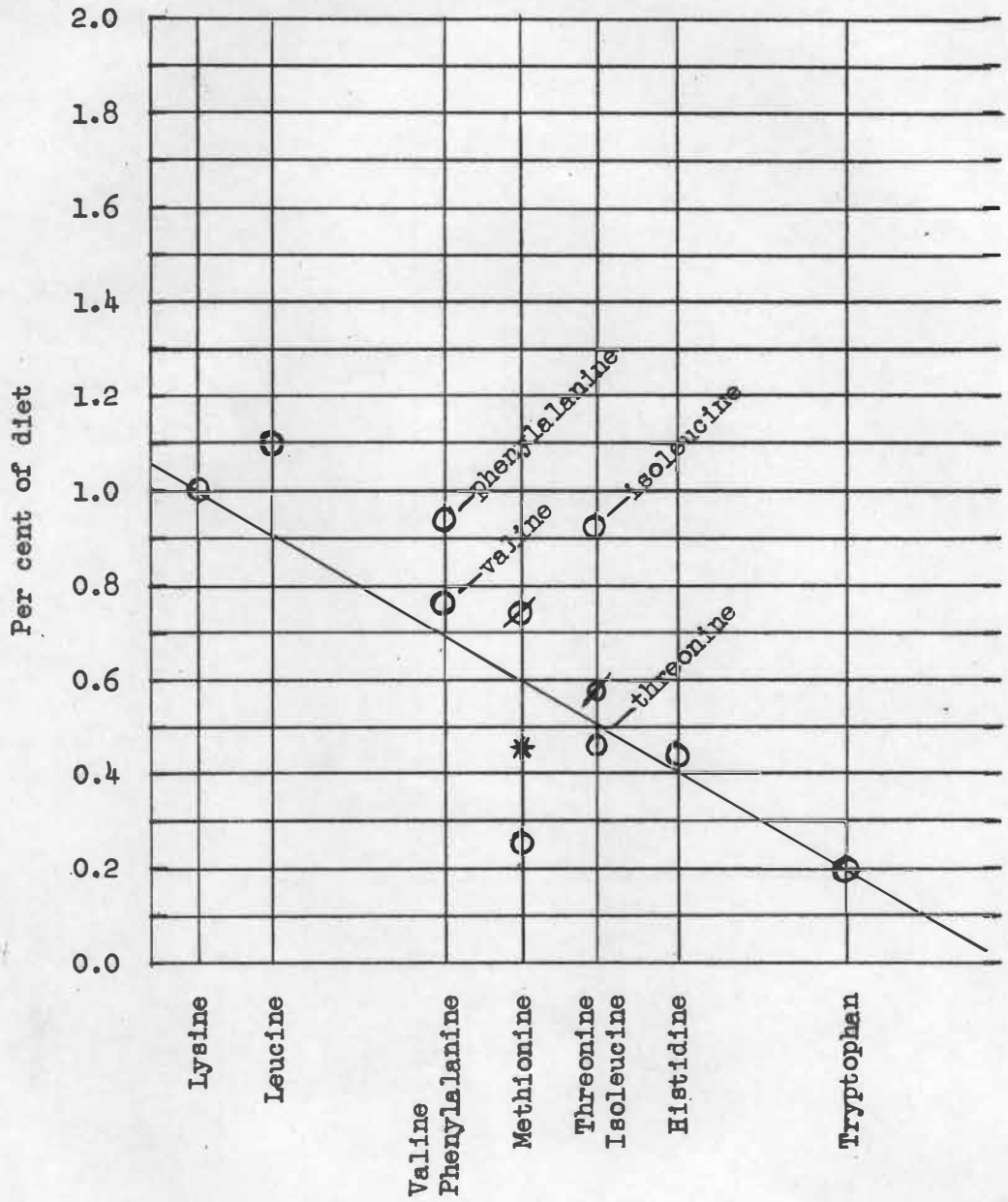


FIGURE 1

NOMOGRAM COMPARING ESSENTIAL AMINO ACID CONTENT OF ALPHA PROTEIN WITH GROWTH REQUIREMENTS OF WEANLING RATS[a]

○ = level for 15 per cent alpha protein

⊘ = level of alpha protein following supplementation

* = methionine + cysteine

[a]Rose, '37.

TABLE V

WEIGHT CHANGE OF RATS ON DIET GROUPS A AND B

Rat number		Diet	Initial weight (grams)	Final		Weight change (grams) (final-initial wt.)
				Weight (grams)	Date	
32992	♂	A	56	46	8-24	-10
32973	♂	A	40	32	8-24	- 8
33012	♂	A	30	24	8-11[a]	- 6
32977	♀	A	43	35	8-24	- 8
33013	♀	A	38	29	8-20[a]	- 9
32996	♂	A_E	47	45	8-24	- 2
32974	♂	A_E	46	41	8-24	- 5
33011	♂	A_E	38	30	8-24	- 8
32978	♀	A_E	40	35	8-24	- 5
33014	♀	A_E	36	30	8-24	- 6
32994	♂	B	50	88	8-24	+38
32975	♂	B	44	102	8-24	+58
33010	♂	B	38	93	8-24	+55
32979	♀	B	40	88	8-15[a]	+48
33015	♀	B	34	63	8-10[a]	+29
32993	♂	B_E	52	132	8-24	+80
32976	♂	B_E	46	123	8-24	+77
33009	♂	B_E	35	98	8-24	+63
32980	♀	B_E	44	124	8-24	+80
33016	♀	B_E	34	101	8-24	+67

[a]Died before experiment terminated.

TABLE VI

WEIGHT CHANGE OF RATS ON DIET GROUPS C AND D

Rat number	Diet	Initial weight (grams)	Final		Weight change (grams) (final-initial wt.)
			Weight (grams)	Date	
33000 ♂	C	50	73	8-24	+23
32986 ♂	C	56	68	8-24	+12
32990 ♂	C	54	69	8-24	+15
32981 ♀	C	40	44	8-20[a]	+ 4
33004 ♀	C	50	54	8-24	+ 4
33001 ♂	C_E	48	55	8-24	+ 7
32987 ♂	C_E	54	55	8-24	+ 1
32995 ♂	C_E	54	58	8-24	+ 4
32982 ♀	C_E	44	57	8-24	+13
33005 ♀	C_E	48	51	8-24	+ 3
33002 ♂	D	47	93	8-24	+46
32988 ♂	D	54	95	7-28[a]	+41
32967 ♂	D	60	94	8-24	+34
32983 ♀	D	40	84	8-01[a]	+44
33006 ♀	D	45	72	8-21[a]	+27
33003 ♂	D_E	50	113	8-24	+63
32989 ♂	D_E	58	90	8-24	+32
32968 ♂	D_E	48	103	8-24	+55
32984 ♀	D_E	38	102	8-24	+64
33007 ♀	D_E	49	117	8-24	+68

[a]Died before experiment terminated.

and final weights as well as the weight change in grams. In addition, the date of the final weight is shown; this date was considered necessary since eight of the animals died previous to termination of the experiment. It is remarkable that the animals consuming diet group A lost weight throughout the experimental period since the per cent sulfur amino acids is only 0.15 per cent below optimal values. It is also striking that none of the animals attained the weight normally expected of rats of this age. Nevertheless, certain differences were noted within this group. It may be observed that none of the rats consuming diets supplemented with vitamin E died before termination of the experiment. Another interesting point of observation is that two of the animals consuming diet D died at least nine days before the animals fed any of the other diets.

The data arranged with respect to group weight change, food consumption, and food efficiency are shown in Table VII. These data are of interest, since the three groups showing positive food efficiency, diet groups B, C, and D, demonstrated a difference with respect to vitamin E supplementation. The two diets supplemented with methionine, diet groups B and D, show an increased food efficiency in animals receiving E-sufficient diets. In contrast, a decreased food efficiency with vitamin E supplementation was observed in the animals of diet group C, not supplemented with methionine but supplemented with 0.1 per cent cysteine.

Since the data which were presented earlier indicated a sex difference with respect to vitamin E supplementation in the various

TABLE VII

WEIGHT CHANGE, FOOD CONSUMPTION, AND FOOD EFFICIENCY FOR ALL GROUPS

Group	Weight change (grams)	Food consumption (grams)	Food efficiency
A	- 41	1290	-.03178
A_E	- 26	1569	-.01657
B	+228	2122	+.10745
B_E	+367	2542	+.14437
C	+ 58	2158	+.02688
C_E	+ 28	2094	+.01337
D	+192	2047	+.09380
D_E	+282	2664	+.10586

dietary groups, the mean weight changes have been arranged according to sex and are presented in Table VIII. These data show, for all dietary groups, that the female rats respond better to vitamin E supplementation than do the males. Although these data are clear with respect to the changes in weights of the rats on dietary groups B and D, they are not as clear with respect to diet group C. Since the male animals consuming diet C without vitamin E supplementation gained more than those with supplementation, and the females gained more with vitamin E supplementation, the net change with respect to females versus males following vitamin E supplementation was 17 grams for group C, 13 grams for group B, and 20 grams for group D.

The data which have been presented demonstrate that those diets supplemented with methionine and vitamin E support growth the best. This is particularly true with respect to female rats. In all of these diets, vitamin E appears to be necessary in preventing death, particularly in the case of the D diets since three-fifths of the animals on the vitamin E-free diet died.

Since the original purpose of this investigation was to compare data obtained with diets composed of alpha protein as a source of protein, low in sulfur-containing amino acids, with results from diets partially composed of oxidized casein, these data should be interpreted with respect to findings reported with the use of oxidized casein. As stated earlier, one major difference in this experiment and the previous one (Pendergrass, '61) was the use of adult rats in the oxidized casein experiments and weanling rats in the present

TABLE VIII

AVERAGE WEIGHT CHANGE ACCORDING TO SEX AND GROUP

Group	Sex	
	♂	♀
A	- 8	- 8
A_E	- 5	- 5
Mean difference	- 3	- 3
B	+50	+38
B_E	+73	+74
Mean difference	-23	-36
C	+17	+ 4
C_E	+ 4	+ 8
Mean difference	+13	- 4
D	+40	+36
D_E	+50	+66
Mean difference	-10	-30

investigation. Therefore, since the conclusions reached in the previous experiment (Pendergrass, '61) were based on certain analytical determinations, the small size of the rats and their mortality rates in the present investigation necessitate the interpretation of these data on the basis of growth and food efficiency. The conclusion which was reached in the previous investigation indicated that vitamin E was necessary for proper utilization of the sulfur-containing amino acids, perhaps in the conversion of cysteine to sulfate. Thus, it seems proper to examine the above data in view of this conclusion.

As mentioned in the review of literature, either methionine and/or cysteine can contribute to body sulfate, glutathione, taurine, and other sulfur-containing compounds, and methionine can serve as a source of cysteine. Therefore, one would expect supplementation of a diet containing adequate vitamin E but low in sulfur amino acids with either methionine and/or cysteine to result in proper growth; however, this was not observed in the present investigation.

It has been well documented that crude soybean oil meal preparations contain both a trypsin inhibitor and a methionine antagonist. It was assumed that purified alpha protein would be low in both of these substances. Although it was stated previously that the essential amino acid pattern in these diets was sufficient to support growth, it now appears that this assumption was incorrect since none of the rats attained the weight normally expected.

It is not at all difficult to visualize addition of methionine, to a diet containing a methionine antagonist, as resulting in improved

growth. The author is unaware of any reports which implicate methionine as being antagonistic to trypsin inhibitor. Since cysteine has been shown to have a sparing action on methionine, the improvement in growth following addition of cysteine to diet group C might also be expected and explained on the basis of methionine antagonism. It is difficult to explain the growth and food efficiency differences observed between vitamin E supplementation of the diets also supplemented with methionine and with cysteine. In the first case, an improvement in growth and food efficiency was observed; in the latter case, little if any effect was observed except in the case of the female rats.

The difference in response of the female rats to vitamin E supplementation when compared to male rats on the same diet is interesting. Although not as definite, there was an indication of sex differences in response to the oxidized casein diet in the previous investigation (Pendergrass, '61). The most pronounced sex difference noted in that investigation was with respect to incorporation of $S^{35}O_4^=$ into the erythrocyte stroma. The female rats incorporated more than did the males.

These results would indicate that the sulfation of mucopolysaccharides is more susceptible to vitamin E deficiency in the female than in the male rat. Since the sulfated mucopolysaccharides are basic to the integrity of tissue, it is conceivable that a sex linked inhibition of the sulfation of mucopolysaccharides could explain the data obtained in the present investigation and establish some continuity between the two experiments.

With respect to pinpointing the lesion in sulfur amino acid metabolism which occurs in vitamin E deficiency, the two studies are not quite so compatible. The previous investigation indicated that the lesion occurred in the conversion of cysteine to sulfate. However, the present investigation seems to indicate that the vitamin E effect is at the level of methionine utilization. Supplementation of the diets with methionine and vitamin E resulted in improved growth and food efficiency, yet supplementation with cysteine did not show a vitamin E effect. It is realized that the cysteine supplementation was at a lower level than the methionine supplementation, a factor which may have resulted in this observation. However, the actual site of vitamin E action remains obscure.

It is obvious that even the high levels of inorganic sulfate used in this investigation were not beneficial in promotion of growth in animals maintained on diets containing 20 per cent alpha protein. If these animals had done better, they might have helped confirm or disprove the suggestion made earlier that the sex difference observed was one of sulfation of mucopolysaccharides. The B group diets were not considered low in inorganic sulfate, but they did exhibit the sex difference, an observation which strengthens the above suggestion.

It was mentioned earlier that purified alpha protein was assumed to have a low content of the trypsin inhibitor and a methionine antagonist which are known to be present in the crude soybean oil meal. Since the animals in this investigation did not attain weights normally expected when maintained on diets containing sufficient amounts of the

essential amino acids to promote growth, it might be recommended that further purification of alpha protein is necessary in order to render it free from the inhibitory and antagonistic substances which seem to prevent it from being a satisfactory protein source under the present experimental conditions.

Another suggestion that might add interest to further investigation in this area may be the possibility of increasing the amount of cysteine used in supplementing diet group C. In this study, cysteine, as a supplementary amino acid, was added to the diet at a lower level than was methionine. An increase in the level of cysteine may offer additional information of interest since cysteine has been shown to have a sparing action on methionine.

SUMMARY

This investigation was carried out to determine if alpha protein, which is low in sulfur amino acids, would serve as a satisfactory source of protein for use in investigating the interrelationships between sulfur metabolism and vitamin E.

Three groups of rats were maintained on diets composed of 15 per cent alpha protein, and varied both with respect to total sulfur and the neutral:inorganic sulfur ratio for a period of ten weeks. One group received a 20 per cent protein diet which had a high content of inorganic sulfate. Each group was subdivided into vitamin E-deficient and -sufficient subgroups. Records were kept for both weight change and group food consumption.

None of the rats attained the weight normally observed for ninety-day-old rats in this colony. In contrast, eight of the animals on the vitamin E-deficient diets died. Supplementation of the diets with both vitamin E and methionine resulted in improved growth and greater food efficiency. Supplementation with cysteine and vitamin E did not effect improved growth or food efficiency; in fact, food efficiency decreased for the vitamin E-sufficient animals. A sex difference was observed in the effect of vitamin E supplementation on weight change in all diets showing any weight gain. The female rats responded most favorably to vitamin E supplementation.

CONCLUSIONS

Consideration of the results obtained in this investigation allows the following conclusions. These data confirm a previous proposal that sufficient dietary vitamin E is essential for optimal utilization of sulfur-containing amino acids, although the site of action may not be as localized as was originally proposed.

Due to certain inconsistencies observed, alpha protein does not appear to be a satisfactory protein source, low in sulfur-containing amino acids, for investigational purposes when weanling rats are employed in studying the interrelationships between sulfur metabolism and vitamin E.

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