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Title A METABOLIC FUNCTION OF SELENIUM, ASSESSED WITH RAT LIVER HOMOGENATES

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In three separate trials rats were fed either a basal diet deficient in selenium and vitamin E or the same diet supplemented with one or both of these factors. In addition to these treatments in two of the trials, ethoxyquin was included to provide adequate lipid antioxidant protection and to alleviate the primary need for selenium and vitamin E in the role of a lipid antioxidant. On termination of either five- or eleven-week feeding trials, the rats were killed, their livers removed and prepared for in vitro studies. These liver preparations (either homogenates or mitochondria preparations) were used to study the metabolic activities of selenium and vitamin E in a NAD- and flavoprotein-dependent system, using pyruvate or succinate as the substrate.

Results showed that selenium function was associated with the oxidation of pyruvate by the liver preparations, but not with oxidation of succinate. The inclusion of vitamin E along with selenium in

the diet did not significantly increase the oxygen utilization of the pyruvate by animals which were on the test diets for only five weeks. However, by extending the feeding period to eleven weeks and including ethoxyquin in the diets, the combination of both selenium and vitamin E was required to increase the rate of pyruvate oxidation. This suggests that both selenium and vitamin E are biologically necessary in the oxidation of pyruvate and have a function other than that of a lipid antioxidant.

This interaction between selenium and vitamin E could not be explained by the lipid antioxidant properties of vitamin E. The presence of ethoxyquin in these diets would be expected to alleviate further requirements of a lipid antioxidant by the animal tissue. In addition, dietary supplementation with vitamin E did not have the same response on pyruvate oxidation as noted by the combination of vitamin E and selenium since the oxygen utilization values with vitamin E alone were not significantly different from the deficient group. If further antioxidants were required for the body tissue, supplementation with vitamin E would be expected to reflect this in the oxidation values.

For further proof of selenium function in the oxidative pathways involving pyruvate, sodium malonate was included in the incubation medium. This inhibited the flavoprotein-dependent system and made it possible to observe only the influence of selenium on the

NAD-dependent oxidative system. The results from animals receiving ethoxyquin in their diet indicate that supplementation with selenium did not significantly increase the oxidative rate. A slight increase in pyruvate oxidation was noted following the combination of selenium and vitamin E in the diet, but this was not significantly different from the groups not supplemented with both.

An investigation into the cause of the non-significant difference due to the inclusion of sodium malonate in the incubation medium revealed that oxaloacetic acid was not in sufficient supply. This probably limited the oxidation of pyruvate to the availability of oxaloacetic acid to combine with the acetyl CoA to form citric acid and did not reflect the extent of the response of selenium on the oxidation of pyruvate.

As noted in the first trial, vitamin E exhibited a significant increase in the oxygen uptake values with succinate as the substrate for the liver homogenates. In later trials this influence was suggested to be not a direct one on the oxidative process involving succinate oxidation since through the inclusion of amytal to isolate the flavoprotein-dependent oxidative system, no difference in the succinate oxidation was noted. The suggestion that certain end-products may have masked the response of vitamin E was discounted since addition of sodium fumarate to the medium containing the amytal did not decrease the oxidation of succinate.

A METABOLIC FUNCTION OF SELENIUM, ASSESSED WITH RAT LIVER HOMOGENATES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF FIGURES

A METABOLIC FUNCTION OF SELENIUM, ASSESSED WITH RAT LIVER HOMOGENATES

INTRODUCTION

Early studies with selenium were oriented toward the problem of toxicity due to excess in the diet of animals, and it was not until 1957 that Schwarz and Foltz discovered that minute quantities of this element could play a useful role in preventing liver necrosis in the rat. Shortly thereafter, selenium was also found to prevent disorders in other species including exudative diathesis in the chick, white muscle disease in the lamb and calf and certain myopathies in other animals. With these evidences of some useful function for selenium, further interest has developed in the site of its biochemical activity. Such demonstration would provide conclusive proof that selenium is an essential nutrient in the diet of animals.

Several factors have hindered universal acceptance of the concept of selenium as an essential nutrient. One of the major difficulties lies in the extremely small quantity of selenium which appears to be required. Under practical conditions a 0.1 ppm of selenium is sufficient in the diet dry matter to prevent the symptoms of deficiency, with actual requirement probably somewhat less than this amount. With the requirement of such small quantities, a suitable diet sufficiently free of the element for comparative studies has not always been available. Selenium and sulfur are closely related

chemically, and in certain protein molecules the sulfur atom may be substituted for selenium. Thus the likelihood of selenium contamination in sufficient quantity in an experimental diet is ever present and is likely to lead to improper interpretation of some results. Until the development of a method by Cousins (1960), later modified by Allaway and Cary (1964), the determination of submicrogram amounts of selenium in biological materials was only possible through neutron activation analysis which was quite expensive and not without procedural difficulties. Thus previous to this time, dietary content of selenium was usually not measured, and it was assumed to be low if symptoms of selenium deficiency were observed in the animals.

Selenium deficiency symptoms may be masked due to interrelationships with other dietary constituents. Some symptoms commonly associated with vitamin E-deficient diets may be prevented by either vitamin E or selenium. However, if the diet contains relatively large amounts of unsaturated fat, only vitamin E or a synthetic lipid antioxidant appears effective in the prevention of the symptoms. Certain sulfur amino acids tend to postpone the onset of selenium deficiency, at least for a short period of time.

The establishment of selenium as an essential nutrient is therefore dependent upon the establishment of a specific essential function within the metabolic schemes of the animal. The research

embodied in this thesis investigates whether selenium may be involved in the metabolism of some intermediates in the citric acid cycle and also the possible implications of an antioxidant function. Through the use of different dietary and in vitro treatments with regard to selenium, vitamin E and a synthetic lipid antioxidant, the location of selenium activity has been investigated with rat liver preparations through the use of the Warburg manometric technique.

REVIEW OF LITERATURE

The nutritional implications of selenium as an essential nutrient in the diet of animals was almost simultaneously reported by Schwarz and Foltz (1957), using rats, and by Stokstad et al. (1957), using chicks. Prior to this time there were several evidences that selenium might, in minute quantities, play a beneficial role in nutrition. Munsell et al. (1936), while studying selenium toxicity, provided data showing that selenium might have a beneficial effect upon reproduction in the rat. Somewhat later Poley et al. (1941), also conducting a toxicity trial, showed an indication of improved growth in chicks when 2 ppm of selenium were added to their diet. For the most part these results attracted little attention and went unheralded in the extensive investigations of the toxic aspect of selenium.

It was not until the subsequent work in the laboratories of Schwarz and Stokstad that a useful function for selenium was noted. Schwarz (1951) described a condition in rats called "nutritional liver necrosis" which could be prevented by: (1) vitamin E, (2) cystine and (3) an unidentified material designated as "Factor 3". Subsequently, as a part of their extensive investigations in the area of dietary liver necrosis, Schwarz and Foltz (1957) identified selenium as an integral part of Factor 3.

This recognition of selenium being required by the animal as

well as exhibiting toxic properties when present in larger amounts in the diet marked a dichotomy in the nutritional status of the element. Its status could then be defined in quantitative terms as resulting in a deficiency state when the diet contains less than 0.06 ppm of selenium (Oldfield, Muth and Schubert, 1960) and reaching toxicity status when the diet contains more than 8.5 ppm (Moxon and Rhian, 1943). A wide application of selenium was found in the treatment and prevention of several metabolic disorders which may be defined as selenium-responsive diseases. These include certain myopathies such as white muscle disease (WMD) in lambs and calves (Muth et al., 1958; Proctor et al., 1958; Grant et al., 1960; Jolly, 1960a & b; Young et al., 1961a, b, & c; and others); exudative diathesis of chicks (Nesheim and Scott, 1958); and certain liver diseases such as necrotic liver degeneration in the mouse and rat (Schwarz and Foltz, 1957).

There are two main approaches which investigators have used to determine whether selenium should be classified as an essential nutrient or only as a non-nutrient additive having certain growth promoting properties. The first approach was the establishment of deficiency symptoms in animals when the diet was relatively free of the element. Reports of selenium administration preventing certain animal disorders are numerous throughout the world. These studies involved a large number of different species.

The second approach, which is a basic continuation of the first, has been the establishment of a specific function for selenium within the metabolic systems of the animal. Under the conditions of selenium deficiency the metabolic scheme of the cell would be disrupted and thus produce the biochemical and visible symptoms in the animal.

The biological function of selenium is often dependent on other dietary factors which include the level of unsaturated lipids, the presence of vitamin E and synthetic lipid antioxidants, and the level of certain sulfur amino acids in the diet. These factors when administered in various proportions may alter the visible symptoms in the animal so as to mask the condition or possibly exert a sparing effect upon the selenium in the body which alleviates the deficiency signs. One of the biological functions of selenium has been postulated to be that of an in vivo lipid antioxidant which protects certain vital processes of the cell altered by lipid oxidation. Most dietary factors which exhibit some influence upon the incidence of selenium deficiency are related to the stability of lipids, thus suggesting that selenium might function as a lipid antioxidant.

Through investigating the biological function of vitamin E, a factor protecting against muscular dystrophy in the rabbit, liver necrosis in the rat and others; some leads into the biological site of function for selenium could be gained. Vitamin E (tocopherol)

possesses the properties of an effective lipid antioxidant and possibly other essential properties.

Antioxidant Implications of Selenium and Vitamin E

The principal effect of an antioxidant is the neutralization of a free radical representing the initial stage in the autoxidative chain reaction, the following stages of which are the formation of peroxidic free radical, hydro-peroxides, keto and hydroxy compounds, and polymerization products (Lea, 1962). The reaction between the antioxidant and the free radical involves the transformation of the antioxidant into an unstable free radical form which easily undergoes irreversible changes, whereby the antioxidant is destroyed. In the case of vitamin E the free radical is the semiquinone. Replacement of dietary tocopherol with structurally dissimilar lipid antioxidants shows that the major quantitative requirement is for a biologically active lipid antioxidant (Draper et al., 1958; Draper, 1960).

Tocopherol requirements in animals appear to be related to the amount of peroxidizable fatty acids ingested and/or stored in the tissue as demonstrated by Century and Horwitt (1960). Through the technique of feeding different tocopherol-free oils to rats, an oil relatively high in unsaturated fatty acids like corn oil readily caused creatinuria (a symptom of nutritional muscular dystrophy in

the rat) while an oil low in unsaturated fatty acids like coconut oil fed at the same level did not. Decreasing the level of corn oil so that it was equal in unsaturation to the coconut oil also prevented the onset of creatinuria. Horwitt (1965) relates the relative peroxidizability of a fatty acid mixture by multiplying the percentages of dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic fatty acids by 1, 2, 4, 6, and 8, respectively. This gives some basis for the animal's requirements for vitamin E or other lipid antioxidants due to the potential peroxidizability of the oil. Dam and Søndergaard (1964) based the vitamin E requirement on the dietary linoleic acid content with a value of 0.8 mg d-α-tocopherol/gm of linoleic acid or 0.63 mg d-α-tocopherol/gm of total polyunsaturated fatty acids.

With the unsaturation of the fatty acid content being an important factor in determining the desired level of vitamin E required in the diet of the animal, a large part of the studies with vitamin E and selenium have been concerned with the relationship of different oxidative products of lipid rancidity on the development of selenium and vitamin E deficiencies in animals. Many experiments have been conducted on oils which were artificially altered to promote the oxidative rancidity processes through either heating or aerating, and the incidences of vitamin E deficiency symptoms developed by the animals consuming the diets were observed. Under such conditions 44 to 88 International Units of vitamin E per kg were effective in

preventing the symptoms as were certain other synthetic lipid antioxidants like 0.025 percent ethoxyquin (1, 2-dihydro-6-ethoxy-2, 2, 4-trimethylquinoline), and DPPD (N, N'-diphenyl-p-phenylenediamine) (Machlin and Gordon, 1960; Oldfield, Sinnhuber, and Rasheed, 1963; and others).

In an attempt to further demonstrate that lipid peroxides are involved in the condition of encephalomalacia, Kakatnur et al. (1960) fed chicks 0.25 percent long chain keto acids in a vitamin E deficient diet and induced symptoms of encephalomalacia after seven days of feeding. 12-oxo-cis-9-octadecenoic acid or its methyl ester caused the highest rate of incidence. 12-oxo-trans-10-octadecenoic or 12-oxo-octadecenoic acid were not as potent as 12-oxo-cis-9-octadecenoic acid. Furthermore, the need for unsaturated fatty acids in the diet for the development of symptoms was evident, as a higher rate of incidence was noted at a 10 percent rather than at a 5 or 2.5 percent level of corn oil. These results of feeding 12-oxo-cis-9-octadecenoic (keto) acid producing encephalomalacia were confirmed by Sugai et al. (1960). However, α-tocopherol was able to protect chicks against the symptoms of encephalomalacia with methyl linoleate, but it provided no protection in the presence of the keto acid. This keto acid also caused intestinal hemorrhage in both weanling and adult rats.

Erwin (1961) suggests that the deposition of linoleic acid in

tissues, and consequently the need for an antioxidant, can be affected by one or all of the following factors: (1) level of dietary linoleic acid; (2) proportion of saturated fatty acids to linoleic acid in the diet; (3) the proportion of linoleic acid to the level of dietary fatty acids that are deposited or incorporated per se intracellularly.

Abnormalities in Linoleic Acid Utilization

Century and Horwitt (1964a & b), working on the premise that since fish liver oils contain low levels of essential fatty acids which vary, in contrast, with high levels found in seed oils and mammalian liver lipids, the ability of some cod liver oils to inhibit the incidence of encephalomalacia in chicks might be related to a specific ability of the polyunsaturated fatty acids of the linolenic series to lower the content of higher essential fatty acids in tissues. Adding 8 percent reconstituted cod liver oil to tocopherol-free diets with 8 percent reconstituted corn oil essentially prevented the appearance of encephalomalacia, whereas adding 8 percent reconstituted cod liver oil to a diet with 1 percent ethyl arachidonate resulted in a 50 percent incidence. Encephalomalacia did not occur with 1 percent ethyl arachidonate alone, but with 2 percent ethyl arachidonate all of the birds rapidly developed encephalomalacia. It would appear that cod liver oil inhibits the conversion of linoleate to arachidonate and higher essential polyunsaturated fatty acids and results in the

substitution of higher polyunsaturated fatty acids of the linolenic acid series.

In each of four experiments, when a tocopherol-deficient diet containing 4 percent corn oil was fed to chicks, addition of 0.13 ppm of selenium resulted in a lower incidence of encephalomalacia. Addition of 0.26 ppm of selenium to the 4 percent corn oil diet instead of the 0.13 ppm had no further effect upon the incidence. When, instead, a diet containing 8 percent corn oil was fed, the added selenium had no significant effect upon the incidence of encephalomalacia, even when included in levels up to 1.56 ppm (Century and Horwitt, 1964a).

Calvert, Desai and Scott (1964), working with chicks, found that either 0.5 percent linoleic acid alone or 1.95 percent oleic acid plus 0.5 percent linoleic acid produced the same degree of incidence and severity of muscular dystrophy as rated visually and the same as that observed with 4 percent lard, indicating that linoleic acid is the only fatty acid in lard which influences the production of muscular dystrophy. Addition of vitamin E to the diet containing 1.0 percent dietary linoleic acid prevented muscular dystrophy, but did not affect the level of linoleic acid incorporated into muscle lipids.

Experiments testing whether the vitamin E requirement should be based directly upon the dietary level of linoleic acid showed that the effect of linoleic acid upon the vitamin E requirement was highly

significant between the levels of zero and 0.5 percent. However, statistical analysis of the data by a chi-square test for interaction revealed no effect of linoleic acid on the vitamin E requirement for the prevention of muscular dystrophy when the dietary level of this fatty acid was increased from 0.5 to 2.5 percent. Therefore, the effect of linoleic acid on the vitamin E requirement for the prevention of nutritional muscular dystrophy is evident only in diets containing less than 0.5 percent linoleic acid. Selenium at 1.0 mg/kg of diet (as sodium selenite) was added to reduce the vitamin E requirement below 10 mg of d-α-tocopheryl acetate/kg of diet.

Witting and Horwitt (1964) found that the fats that produce the earliest creatinuria (5 to 7 weeks) were those in which a given amount of unsaturation was highly concentrated in a relatively small percentage of the fatty acid chains, rather than being distributed evenly as in the case of monoenoic fat, where the production of creatinuria required 17 to 18 weeks. Addition of selenium and methionine to the ration increased the time required for the development of creatinuria by approximately 40 percent with any given synthetic fat. When the graded levels of tocopherol (0 to 2.4 mg/kg rat/week) used in this study were administered, little protective effect was noted (1 mg of d-α-tocopheryl acetate/kg rat/week). When selenium and methionine were added, the onset of creatinuria was delayed by approximately 17 to 20 weeks per mg of tocopherol when dienoic, trienoic, and

polyenoic fats were fed, and approximately 45 weeks when monoenoic fat was fed.

Composition Studies of Deposited Fat

The content of polyunsaturated fatty acids in heart, liver, adrenals and plasma, but not in brain, is decreased in animals with vitamin E deficiency. No significant differences in the lipid peroxide content of the tissue *in vivo* was found between normal and deficient rats by Pritchard (1960). However, the tissues of heart, liver and adrenals, but not of brain, showed a much greater tendency to lipid peroxide formation in vitamin E-deficient animals. In brain and heart the most active sub-cellular locus of lipid peroxide formation was located in a microsomal supernatant.

An investigation by El-Khatib *et al.* (1964) on the production of peroxides from tocopherol-deficient animals showed that in every case in which the determination of peroxides was made anaerobically, no difference was found between the animals which received tocopherol and those deprived of it. By contrast, if the organ was reduced to small fragments under aerobic conditions, lipid peroxides accumulated in the preparations from tocopherol-deficient animals. This was determined by the use of three different methods: (1) the oxidation of brilliant cresyl blue, (2) the oxidation of Fe^{++} to Fe^{+++} and (3) the oxidation of I^{-} to I.

The phospholipid studies by Witting (1964) show that the muscle phospholipid fatty acids from the tocopherol-deficient group are significantly lower in polyunsaturated fatty acids and the difference occurs mainly in the most highly unsaturated fatty acids (C 22:5 and higher). Some observations of adipose tissue by Mock and Emmel (1963) indicated that vitamin E-deficient and control rats showed qualitatively the same lipid components, although there appeared to be a reduction in the free fatty acid and tri-glyceride fractions in the E-deficient animals and an increase in phosphatides in these same animals. In incorporation studies with P^{32} labeled orthophosphate the specific activity of the adipose tissue in relation to that of the liver from deficient animals was slightly greater than the relative specific activity of adipose tissue from the controls. There was, however, a much greater amount of labeled phospholipid per unit weight of adipose tissue in the deficient animals since relatively more total phospholipid was present. On the basis of inorganic P^{32} uptake, no significant distinction can be made between the phospholipid extracted from the adipose tissue of deficient and control animals, despite the increased amount of phospholipid in the former.

Distribution of Lipids and Antioxidants in Animal Tissue

The distribution of vitamin E and other biological antioxidants

within the intracellular components of a cell was determined by Csallary and Draper (1960). When they replaced the vitamin E in the diet with DPPD, over half of the DPPD was recovered in the soluble fraction of liver cells, one-quarter was associated with microsomes, and a small fraction recovered from mitochondria. This distribution pattern is similar to the pattern reported for total lipids, and indicates that the intracellular location of DPPD may be passively determined by its lipid solubility. On the other hand, α-tocopherol was found predominantly in mitochondria.

Tappel and Zalkin (1959) suggest that the free-radical intermediates of lipid peroxidation damage the mitochondria structurally and functionally, thus bringing about disarranged metabolism. Isolated liver mitochondria from vitamin E-deficient rabbits had undergone lipid peroxidation in vivo as measured by the thiobarbituric acid reaction. When isolated these mitochondria were more labile to lipid peroxidation in vitro than were the controls.

Microsomes should be labile to lipid peroxidation for the same reason as mitochondria. The microsomal fraction contains 30 to 40 percent of the total lipid, mainly phospholipids, and contains twice the amount of polyunsaturated lipids, when expressed in relation to nitrogen content, as do mitochondria. Tappel and Zalkin (1960) found that the microsome fraction from rat livers deteriorated by lipid peroxidation at a rate of 0.1 ml oxygen reacted/mg nitrogen/hr

at 37° C. Increase in thiobarbituric acid reactants paralleled oxygen absorption during the first two hours. Added α-tocopherol at 0.25 mg/mg nitrogen gave 72 percent inhibition as measured by oxygen absorption or thiobarbituric acid reactants. Since 0.01 M cyanide and 0.1 M methylene blue gave 71 to 94 percent inhibition as measured by oxygen absorption or thiobarbituric acid reactants, the lipid peroxidation appears to be catalyzed by cytochrome b_5 and other hemochromes present. In vitro studies indicate that microsomes from vitamin E-deficient rabbits had five to seven times as much thiobarbituric acid reactants per ml as the adequate controls.

Implications of Selenium as an Antioxidant

Since selenium and vitamin E are effective in preventing the symptoms of exudative diathesis in the chick, white muscle disease in lambs and calves, and liver necrosis in rats, several attempts have been made to show that selenium is functioning in the body as a biological antioxidant. Zalkin and Tappel (1960) found selenomethionine was as effective as α-tocopherol in reducing the initial rates of a hemoglobin-catalyzed linoleate oxidation. However, tocopherol was more effective in inhibiting the steady-state rate of oxidation. They indicate that selenomethionine, phenylselenoglycine, selenocystine, and cystine are capable of synergizing with α-tocopherol to a small extent. At high levels the organic selenium

compounds are much less effective relative to their sulfur analogues or to α-tocopherol. Although Bunyan, Green and Diplock (1963) found 0.05 ppm selenium clearly protected against liver necrosis, livers and kidneys of rats receiving this amount of selenium developed about the same amount of malonaldehyde as deficient rat tissues whether tested before or after incubation at 37° C. Selenium at 0.50 ppm resulted in significantly less malonaldehyde formation in kidney but not in liver.

These results agree with work of Bieri et al. (1961a) and Bieri (1961b) involving incubated tissue homogenate from chicks fed a soybean protein diet containing sufficient α-tocopherol or other antioxidant (ethoxyquin). Little or no peroxide formation occurred in the tissue lipids under such conditions. When 0.33 ppm selenium was in the diet, a significant reduction ($P < 0.01$) in TBA values was observed in the liver, kidney and heart. It is thus apparent that the antioxidant action of dietary selenium is not restricted to the liver as was previously stated (Bieri, 1961b). There was no effect of cystine in this diet on the TBA values.

It is interesting that a tenfold increase in the dietary level of selenium produced no additional reduction in the amount of peroxidation. This suggests that the observed effect of selenium is not due to the total selenium content of the tissue but probably is due to a functional form which is present in optimum amount even at a

very low dietary level.

It was noted by Carpenter et al. (1959)--and confirmed by Krishnamurthy and Bieri (1962)--that if tissues from vitamin E-deficient rats were homogenized in trichloroacetic acid, rather than in water or buffer, no evidence of preformed malonic dialdehyde was seen. In addition, Krishnamurthy and Bieri (1962) also found that when the homogenizing medium was buffered and contained small amounts of an antioxidant, no malonic dialdehyde was detectable in vitamin E-deficient tissues.

Corwin (1962) found that higher quantities of tocopherol, but not dietary selenite, will prevent enzymic peroxide formation. Tocopherol can be added in vitro in quantities sufficient to prevent the decline in α-ketoglutarate oxidation, but not enough to to prevent peroxide formation.

Cellular Damage Due to Lipid Peroxides

The action of peroxides formed in vitamin E deficiency is not readily understood although certain metabolic dysfunctions give a possible indication as to their action. Tappel et al. (1962) discuss the comparative effects of vitamin E deficiency and those of ionizing radiation. At least part of the toxicity of such radiation may depend upon production of lipid peroxides. De Duve (1959) has summarized the evidence linking lysosomal hydrolases to various processes of

pathological autolysis and necrosis following the effect of total body X-irradiation. After radiation the spleen decreased in size and lysosomal enzymes ribonuclease, β-glucuronidase and acid phosphatase were released from the bound state. Tappel et al. (1962) and Zalkin et al. (1962) indicate that vitamin E deficiency is another case of pathological autolysis involving hydrolysis by lysosomal enzymes. Since lysosomal enzymes are specific for hydrolysis of muscle components, they could account for the biochemical changes characteristic of increased catabolism. The definition of the mechanisms responsible for initiating these increases in lysosomal enzymes has been attempted by Tappel. The probability of cellular death immediately preceding the increase in lysosomal enzymes is suggested where the sequence of events is: lipid peroxidation gives free radical intermediates, free radicals cause widespread cellular damage, and cellular damage leads to increased lysosomal enzymes. Such increase in lysosomes can be related to the increases in the following: creatinuria and turnover of muscle creatine, proteolytic and autolytic activity, free amino acids in muscle, catabolism of protein, excretion of amino acids, turnover and catabolism of nucleic acids and excretion of allantoin.

Desai et al. (1964) found that the lysosome enzymes of acid phosphatase, cathepsin, ribonuclease, β-glucuronidase, and β-galactosidase were significantly increased in the pectoral

muscle of nutritionally dystrophic chicks as compared with the normal controls. The increase in lysosomal enzymes in the dystrophic tissue ranged from about 2 X for acid phosphatase and cathepsin to about 5 to 6 X for ribonuclease, β-glucuronidase and β-galactosidase. The results show that a correlation was obtained between the levels of linoleic acid in the diet and incidence and severity of muscular dystrophy up to a level of about one percent linoleic acid. Correlations also existed between incidence of dystrophy, thiobarbituric acid index and activity of the indicator lysosomal enzyme, β-glucuronidase. Supplementation of the diet with 5 to 10 mg d-α -tocopheryl acetate per kg in the presence of linoleic acid prevented the muscular dystrophy and reduced TBA index and lysosomal enzyme activity accordingly.

There was no significant difference between irradiated and control rats in the liver fatty acid ester content (about 105 μM/gm); in the liver lipid phosphorus (40 μM/gm) or in the iodine values, peroxide content, or proportion of glycerides, cephalin and lecithin fractions. The later results of Desai, Calvert and Scott (1964) indicated that the lysosomal enzymes are not directly implicated in the case of nutritional muscular dystrophy; their increase apparently is involved in the hydrolytic breakdown and removal of

degradative products from tissues.

Specific Metabolic Functions for Vitamin E and Selenium

Other workers indicate that a deficiency of vitamin E results in a metabolic disturbance within the mitochondrial fraction of the cell. Corwin and Schwarz (1960) incubated liver homogenates from rats fed a Torula yeast diet deficient in vitamin E and found them unable to maintain α-ketoglutarate or succinate oxidation over a period of 1.5 hours. The respiratory decline--percent decline = 100 × [(0-30 min) - (60-90 min)/(0-30 min)] --of α-ketoglutarate oxidation is completely prevented by dietary α-tocopherol and DPPD or by in vitro addition of α-tocopherol, the Simon tocopherol metabolite, menadione, DPPD, or methylene blue. After rehomogenizing the tocopherol with the liver homogenate, nearly physiological quantities (3 μg/3 ml medium, equivalent to 50 mg tissue) of the vitamin were found sufficient to prevent decline of α-ketoglutarate oxidation.

Similar results were obtained by Rosenkrantz and Laferte (1960), studying the various dehydrogenase systems by following the quantitative reduction of triphenyl-tetrazolium chloride to form formazan. Tissues of vitamin E-deficient rabbits and mice were incubated with triphenyl-tetrazolium chloride with either isocitric

or α-ketoglutaric acids as substrate. Vitamin E-deficient muscle produced less formazan in the presence of isocitric acid and α-ketoglutaric acid than did control muscle. Dystrophic muscle from mice gave an increase in formazan in the presence of glutamic acid. This suggests that the histological similarities of vitamin E deficiency and inherited myopathy of the mouse result from a biochemical lesion at a different site in the same biochemical pathway or cycle. McCaman (1960) determined the levels of activity of glucose-6-phosphate, glutathione reductase, lactic dehydrogenase and α-glycerophosphate dehydrogenase in muscle tissue of mice with dystrophia muscularis. The activity of the enzyme requiring triphosphopyridine nucleotide (NADP) as a cofactor is increased relative to the control group, whereas the activity of those enzymes requiring diphosphopyridine nucleotide (NAD) is decreased.

Data presented by Corwin and Schwarz (1963) show that tocopherol supplementation of vitamin E-deficient liver homogenates does not affect the cytochrome oxidase activity, and that there is no difference between deficient and vitamin E-supplemented homogenates in their reaction against antimycin A, using the succinate-cytochrome c reductase system of mitochondria. The notion that tocopherol functions between cytochromes b and c, the site of antimycin A block, does not appear to have validity in explaining the action of the vitamin in preventing oxidative decline. They concluded

that tocopherol acts in some way to maintain the proper functioning of enzyme sulfhydryl groups. This was supported by the data which showed that the decline of α-ketoglutarate oxidation by α-tocopherol-deficient rat liver homogenates could be prevented by glutathione and BAL which protect the sulfhydryl sites. A concomitant decline of free sulfhydryl groups in the homogenate with time can be prevented by all the reagents which protect against oxidative decline.

Investigations by Kimura and Kummerow (1963) indicated that significant differences in the inhibition rates existed with antimycin A and potassium cyanide which inhibited cytochrome c and cytochrome a systems, respectively. The in vitro addition of α-tocopherol to the Warburg medium affected these two inhibitors less than the in vivo injection of α-tocopherol. The in vitro addition of 11.6 μM α-tocopherol/mg mitochondria completely prevented lipid peroxidation in the isolated liver mitochondria obtained from vitamin E-deficient chicks. However, it is possible that the cytochrome system is more sensitive than other enzyme systems to damage caused by lipid peroxidation, and that dietary or intramuscularly injected α-tocopherol has a protective effect.

The addition of NAD to rat liver mitochondria deficient in vitamin E causes an initial lowering of succinate oxidation followed by subsequent decline of residual oxidation. Vitamin E added in vitro prevents much of the initial lowering of oxygen uptake, and the

subsequent decline is almost completely halted (Corwin, 1965). Since tocopherol has no effect on the removal of oxaloacetate or the accumulation of pyruvate and citrate from succinate oxidation, and since in the absence of tocopherol three times as much CO_2 is formed, it can be concluded that tocopherol deficiency inhibits the formation of oxaloacetate.

The activating effect of tocopherol on aged preparations of chick heart cytochrome c reductase was examined by Pollard and Bieri (1960). Non-linearity of activity with increasing amounts of aged preparations suggested the presence of inhibitors. The inhibitory action of dibenzoyl peroxide on the enzyme from the respiratory chain was reversed by the addition of tocopherol and other antioxidants. Banks, Eddie and Smith (1961) also worked on the effects of lipid peroxides on the cytochrome system, and found that cytochrome c could act as a lipid antioxidant. The effect of cytochrome c on pure trans-trans methyl linoleate was studied. Working with a 5×10^{-3} M suspension of the pure hydroperoxide in a very weak solution of sodium dodecyl sulfate, it was observed that cytochrome c at a concentration of 1.6×10^{-4} will cause almost instantaneous destruction of 80 to 90 percent of the peroxide, and that about a third of the cytochrome is 'denatured' at the same time. Oxygen is not liberated during the reaction, but there is a loss of conjugated di-ene unsaturation and just a slight loss of peroxide. The reaction is not

affected in any way by diluting the peroxide with an equal quantity of pure methyl linoleate or by a change in pH over the range 5.8 to 8.0.

Many workers, e.g. Nason and Lehman (1955) and Deul, Stater and Valdstra (1958), have shown that extraction of skeletal muscle with a non-polar solvent like isooctane inactivates the NADH-cytochrome c reductase activity of isooctane-extracted particles which were reactivated by α-tocopherol. The mechanism of inactivation of respiratory enzyme systems in mitochondrial preparation on treatment with organic solvents was studied by Redfearn, Pumphrey and Fynn (1960). The inactivation of the enzyme system could be attributed mainly to the presence of residual solvent which acted as a physical inhibitor. This inhibition could be reversed by any one of a number of different treatments which removed the solvent. No evidence was obtained for the presence of tocopheryl-quinone in tissue. Similar results were obtained by Pollard and Bieri (1960) which point out the inhibitory effect was due to isooctane rather than to the lipid fractions being removed.

Digitonin inhibits 80 percent of the activity of a 100-fold purified NADH-cytochrome c reductase from bovine heart muscle. This inhibition could be largely or entirely prevented by the addition of either α-, β-, γ-, or δ-tocopherol (Nason et al., 1964). The restored activity of NADH-cytochrome c reductase, like that of the

uninhibited enzyme, is extremely sensitive to antimycin A (about 0.004 μg, of the antibiotic per ml reaction mixture causing 80 percent or more inhibition). None of the tocopherol derivatives tested, which include the esters α-tocopheryl succinate, α-tocopheryl phosphate, α-tocopheryl polyethylene glycol 1000 succinate, and α-tocopheryl acetate as well as tocopheryl quinone and its corresponding hydroquinone, were effective. Coenzyme Q_{10}, plastoquinone and vitamin K_1 also failed to restore activity. Some of the other substances tested include selenite, selenate, lipoic acid, the synthetic antioxidants nordihydroguaiaretic acid (NDGA), ethoxyquin, dibutyl p-cresol, "santoflex", amyl hydroquinone, DPPD and propyl-p-hydroxybenzoate, and were ineffective against digitonin inhibition. Digitonin is also inhibitory to the succinate-cytochrome c reductase system of bovine heart muscle (and of rat muscle). In contrast to the effect on NADH-cytochrome c reductase, inhibition can be prevented or reversed not only by α-, β-, γ-, and δ-tocopherols, but also by α-tocopheryl succinate, α-tocopheryl phosphate, α-tocopheryl polyethylene glycol 1000 succinate, lecithin, and tween 80 (but not α-tocopheryl acetate). All other substances tested including selenate, selenite, several metal ions (manganous and cadmium), the synthetic antioxidants previously listed and steroids (cholesterol and estradiol) failed to activate the inhibited enzyme.

Caldwell and Tappel (1965) and Tappel (1965) found that alcohol dehydrogenase and creatine kinase, both sulfhydryl enzymes, are partially protected from oxidative inactivation in the presence of selenocystine. The oxidations of cysteine, glutathione, and homocysteine by hydrogen peroxide, organic peroxides, and molecular oxygen were accelerated by selenocystine but not by cystine. This finding that selenocystine stimulates oxidations of low molecular weight thiols while protecting sensitive sulfhydryl enzymes could mean that some selenium compounds function in the maintenance of sensitive sulfhydryl groups.

The research of Desai and Scott (1965) indicates that selenium improves the effectiveness of vitamin E by increasing the retention of α-tocopherols, especially d-α-tocopherol. They suggest that vitamin E may be carried by a selenolipoprotein fraction associated with serum γ-globulin.

EXPERIMENTAL PROCEDURE

These investigations were designed to study the metabolic functions of selenium and vitamin E in rat liver tissue and distinguish any differences in their biological function. Through the feeding of different dietary combinations of selenium, vitamin E and a synthetic lipid antioxidant (ethoxyquin) to rats, some insight into the role of these metabolites was achieved by measuring oxygen uptake of rat liver homogenates with different substrates of intermediary metabolism.

Trial 1

Male rats from the Wistar strain weighing approximately 100 gm were purchased from Northwest Rodent Company, Pullman, Washington, in groups of eight for five consecutive weeks. Each week the rats were randomly allotted to one of four dietary treatments until a total of ten rats had been assigned to each treatment. The diets were based upon various modifications of a 30 percent Torula yeast diet previously shown to produce dystrophy (Corwin and Schwarz, 1960), and their composition is shown in Table 1. The dietary treatments are defined as follows:

1. Basal (dystrophogenic--deficient in Se and vitamin E)
2. Basal + vitamin E

Table 1. Composition of Rat Diet--Trial 1.

Ingredients	Basal	+Vitamin E	+Selenium	+Vitamin E + Se
Torula yeast	300 gm	300 gm	300 gm	300 gm
Corn starch	624	624	624	624
Stripped lard	50	50	50	50
Solka-Floc	20	20	20	20
Salts[1]	40	40	40	40
Glucose	6	6	6	6
B-vitamin mix[2]	10	10	10	10
Vitamin A	2223 IU	2223 IU	2223 IU	2223 IU
Vitamin D	555	555	555	555
Vitamin E[3]	0	500	0	500
Na_2SeO_3[4]	0	0	0.00213 gm	0.00213 gm

[1] Jones and Foster (1942).

[2] B-vitamin mix consists of 0.6 mg thiamine, 1.2 mg riboflavin, 0.4 mg pyridoxine, 5.0 mg niacin, 4.0 mg calcium pantothenate, 100.0 mg inositol, 200.0 mg choline Cl, 2.5 mg p-aminobenzoic acid, 1.0 mg biotin, 1.0 mg folic acid and 1.0 mg cyanocobalamin in each gram of starch mixture.

[3] Vitamin E added as dl-α-tocopheryl succinate.

[4] Equivalent to 1 ppm Se.

3. Basal + Se

4. Basal + vitamin E + Se

The diets were mixed in large plastic bags in quantities sufficient for two weeks feeding and stored in a refrigerator to reduce the chances of oxidation. Feed consumption and body weights were measured weekly.

Each rat was housed in an individual galvanized iron cage in a temperature-controlled room from 74° to 80° F. The diet was provided ad. lib. in a feeder consisting of a stainless steel cup with screen and cover to reduce feed wastage. Distilled water was used to avoid contamination of selenium from the water source. It was provided by waterers constructed from 250 ml wide mouth bottles with rubber stoppers fitted with glass drinking tubes.

At the end of the five week feeding period the rats were removed for in vitro metabolic studies. The animals were killed by a hard blow on the head, and their livers were immediately removed and placed in ice cold saline solution. After the livers were chilled and excess saline solution removed by the use of blotting paper, a 4 gm sample of tissue was weighed and placed in 40 ml of cold 0.25 M sucrose containing 0.001 M EDTA and 100 ml of 0.1 M Krebs-Ringer phosphate buffer solution previously adjusted to pH 7.4. The liver tissue was homogenized in a Potter-Elvehjem homogenizer equipped with a teflon plunger and powered by a Power Stirring

Apparatus (Thomas 9230-T). The homogenizing procedure was carried out using an ice bath wherever possible.

After homogenization of the liver tissue, the homogenate was transferred to a 40 ml polyethylene centrifuge tube and centrifuged in a Serval refrigerated centrifuge at 600 X gravity for ten minutes at a temperature of 1° to 3° C. The supernate was place in an ice bath until it was used in the oxygen uptake studies on the Warburg apparatus. The procedures used in this technique were similar to those described by Umbreit, Burris and Stauffer (1957). The incubation medium was similar to that reported by Corwin and Schwarz (1963).

The salt solution consisted of the following:

300 μM NaCl/flask

12 μM KCl/flask

40 μM Na phosphate buffer at pH 7.4/flask

4 μM $MgSO_4 \cdot H_2O$/flask

All of the salts except the sodium phosphate buffer solution were combined into a solution which was five times the final concentration used in the incubation medium. This concentrated solution was also adjusted to a pH of 7.4 and diluted to the proper concentration prior to being put into the reaction vessel. The sodium phosphate buffer solution was mixed separately and pH adjusted to 7.4 with a Beckman pH meter. The phosphate buffer and other salts were then mixed

together, placed in an ice bath and gassed with O_2 for approximately 20 minutes.

Sixty μM of either pyruvate or succinate, purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, were added to each flask. The substrates were prepared fresh each day and kept in a refrigerator when not in use so as to reduce destruction during storage. In addition to the substrate, 3 μM of oxidized NAD (Cozymase or Coenzyme I, Nutritional Biochemical Corporation) were added to each reaction vessel after adjusting the pH to 7.4.

In the center well of the reaction vessels 0.2 ml of a 20 percent KOH solution and a piece of folded filter paper (one inch square) were placed. A thin layer of petroleum jelly (Vaseline) was spread around the top of the cylinder to prevent KOH from reaching the homogenate medium.

The liver homogenate as previously described was added just prior to putting the filter paper in the center well. The in vitro additions of α-tocopherol and selenium were prepared by rehomogenizing with the liver homogenate so each flask would receive either 0 or 20 μg dl-α-tocopherol or 0 or 0.3 μg selenium, or both for each 0.5 ml of liver homogenate.

The reaction flasks were attached to the manometers with a small application of Vaseline on the connection joints to establish a complete seal. Rubber bands were used to hold the flasks tight

to the manometers in order to prevent possible movement while in the water bath.

The manometers with their attached reaction flasks were placed on the Warburg shaker with the flasks in the constant temperature bath at 37° C., and each flask was allowed to equilibrate with the bath temperature for ten minutes. The external valve was closed after adjusting the manometer fluid to the 250 mm mark. Every ten minutes for a total of 90 minutes, manometer readings were recorded and later oxygen consumption was calculated.

The flask-manometer volume and flask constants were calculated, using the methods described by Scholander, Niemeyer and Claff (1950) and by Umbreit, Burris and Stauffer (1957), respectively.

Trial 2

The object of this trial was to determine whether the lipid antioxidant property of vitamin E and selenium was their sole metabolic function. This was accomplished by feeding a synthetic lipid antioxidant (ethoxyquin) in addition to the dietary supplementation of vitamin E and selenium and observing the substrate utilization by the liver mitochondria. By providing the animal with an adequate supply of antioxidant protection through the inclusion of the synthetic antioxidant, any response to vitamin E and selenium could be attributed to a biological function other than that of a lipid antioxidant.

Male Wistar rats weighing approximately 50 gm each were purchased from the same source as in Trial 1 in groups of 15 for three consecutive weeks. Ten rats were randomly assigned to each of the four dietary treatments after a two week pretrial on a basal diet lacking vitamin E, selenium and ethoxyquin to help deplete the body stores. The dietary treatments were as follows:

1. Basal + ethoxyquin
2. Basal + ethoxyquin + vitamin E
3. Basal + ethoxyquin + selenium
4. Basal + ethoxyquin + vitamin E + selenium

(The diet composition is shown in Table 2.)

The animal care and the mixing of the diets were the same as described in Trial 1. The trial feeding period was for a total of nine weeks.

After completing the feeding trial the rats were removed for in vitro metabolic studies. The animals were killed and livers prepared for homogenizing as described in Trial 1. Mitochondria were prepared using the method of Remmert, Johnston and Steele.[1] The liver was homogenized in a Potter-Elvehjem homogenizer with enough Steele's homogenizing medium to make a 10 percent solution.

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[1] Remmert, L. F., W. K. Johnston and Wilbert Steele. Unpublished research on mitochondria preparation. Corvallis, Oregon. Agricultural Experiment Station, Dept. of Agricultural Chemistry, 1965.

Table 2. Composition of Rat Diet--Trials 2 and 3.

Ingredients	Basal	+Vitamin E	+Selenium	+Vitamin E + Se
Torula yeast	300 gm	300 gm	300 gm	300 gm
Corn starch	574	574	574	574
Glucose	6	6	6	6
Salts[1]	40	40	40	40
B-vitamin mix[2]	10	10	10	10
Alphacel	20	20	20	20
Stripped lard	50	50	50	50
Methionine	1.9	1.9	1.9	1.9
Ethoxyquin[3]	0.125	0.125	0.125	0.125
Na_2SeO_3[4]	0	0	0.00213	0.00213
Vitamin A	2000 IU	2000 IU	2000 IU	2000 IU
Vitamin E[5]	0	500	0	500
Vitamin D	555.5	555.5	555.5	555.5

[1] Jones and Foster (1942).

[2] B-vitamin mix composition is shown in Table 1.

[3] Ethoxyquin was not included in the diet of the control group in Trial 3.

[4] Equivalent to 1 ppm Se.

[5] Vitamin E added as dl-α-tocopheryl succinate.

Two or three full strokes of the homogenizer were sufficient for complete homogenization of the liver sample. The slurry was transferred to a chilled, round-bottom polyethylene centrifuge tube and centrifuged for ten minutes at 400 X gravity at 0° to 3° C. The supernate was carefully decanted into a fresh (chilled) centrifuge tube and centrifuged for ten minutes at 9000 X gravity. The supernate was discarded and the sides of the centrifuge tube were carefully wiped with a clean tissue to remove the lipid material which adhered to the side. The mitochondria "button" was resuspended in Steele's suspension medium by "muddling" the mitochondria with a round-bottom test tube and slowly blending the suspension medium with the mitochondria. This resuspended preparation was again centrifuged at 9000 X gravity for ten minutes. After the final centrifugation the supernate was discarded and the mitochondria were carefully transferred to a Potter-Elvehjem homogenizer and gently resuspended by hand in fresh Steele's suspension medium.

Steele's homogenizing medium

Part A	
Sucrose	3×10^{-1} M
Tris	5×10^{-2} M
EDTA·Na_2	1×10^{-4} M
$MgCl_2 \cdot H_2O$	1×10^{-4} M
Part B	
Na pyruvate	5×10^{-3} M
Na fumarate	3×10^{-5} M
Albumin	0.3 gm/250 ml final volume

Steele's suspension medium

Part A	
Sucrose	3×10^{-1} M
Tris	3×10^{-2} M
EDTA·Na_2	3.1×10^{-3} M
$MgCl_2 \cdot 6H_2O$	1×10^{-4} M
Part B	
Na pyruvate	5×10^{-3} M
Na fumarate	3×10^{-5} M
Albumin	0.3 gm/250 ml final volume

Part A was mixed, the pH adjusted to 7.4 with HCl and frozen in polyethylene bottles in measured aliquots. Part B was weighed out and mixed with Part A just prior to use.

A one ml aliquot of the mitochondria preparation was added to each flask containing the incubation medium. The composition of this medium and the in vitro treatment are shown in Table 3.

Table 3. Incubation Treatments for Liver Mitochondria--Trial 2.

Reagent	In Vitro Treatment							
	1	2	3	4	5	6	7	8
Basal medium[1]	x	x	x	x	x	x	x	x
18.0 μM Na succinate	x	x	x	x	x			
18.0 μM Na pyruvate						x	x	x
1.0 μM NAD	x		x	x	x	x	x	x
1.8 μM Amytal		x	x	x	x			
20.0 μM Na malonate							x	x
2.0 μM Na fumarate				x		x	x	x
40.0 μg dl-α-tocopherol				x				x

[1] Basal medium contains 30 mg bovine serum albumin (crystallized and lyophilized), 100 KM type II hexokinase (yeast), 10 μM adenosine 5'-triphosphate (ATP), 60 μM Tris buffer (Tris, hydroxymethyl, aminomethane), 300 μM sucrose, 20 μM glucose, 20 μM $MgCl_2 \cdot 6H_2O$, and 100 μM potassium phosphate buffer.

All reagents were adjusted to pH 7.4 prior to incubation with the liver mitochondria preparations. The in vitro incubation method and oxygen uptake measurements were the same as outlined in Trial 1.

Trial 3

The antioxidant properties of vitamin E and selenium were studied in this trial to establish their importance in the in vitro oxidation of pyruvate and succinate. This was accomplished by providing the animal with a non-related synthetic lipid antioxidant in addition to the various dietary treatments of selenium and vitamin E. The resulting data were compared to those from animals receiving the various dietary treatments of selenium and vitamin E without the dietary addition of ethoxyquin.

The composition of the diets was similar to those described in Trial 2: all 80 rats were fed a basal diet which was deficient in vitamin E and selenium during a two-week pretrial. Then half the rats were divided into four groups of ten and fed the diets described in Table 2. The other 40 rats, which served as the controls, were similarly divided and the same diets were administered with the exclusion of the 0.0125 percent ethoxyquin. The animal care and mixing of the diets were the same as described in Trial 2. The feeding trial period was a total of nine weeks.

After completion of the feeding trial, the rats were removed

for in vitro metabolic studies. The procedures were the same as described for Trial 2 for the isolation of mitochondria and the preparation of the Warburg incubation medium. The incubation treatments are shown in Table 4.

Table 4. Incubation Treatments for Liver Mitochondria--Trial 3.

Reagent	In Vitro Treatment					
	1	2	3	4	5	6
Basal medium[1]	x	x	x	x	x	x
18.0 μM Na succinate				x	x	x
18.0 μM Na pyruvate	x	x	x			
1.0 μM NAD	x	x	x	x	x	x
1.8 μM amytal				x	x	x
20.0 μM Na malonate	x	x	x			
40.0 μg dl-α-tocopherol		x			x	
40.0 μg ethoxyquin			x	x		

[1] Basal medium composition same as in Table 3.

A supplement to this trial was conducted with four normal female rats to determine the effect of sodium oxaloacetate on the utilization of pyruvate by liver mitochondria when sodium malonate was included in the incubation medium. The preparation of the mitochondria and basal incubation medium was the same as described in Trial 3. The in vitro treatments are shown in Table 5.

Table 5. Incubation Treatments for Liver Mitochondria--Trial 3 Supplement.

Reagent	In Vitro Treatment			
	1	2	3	4
Basal medium[1]	x	x	x	x
18.0 μM Na pyruvate	x	x	x	x
1.0 μM NAD	x	x	x	x
20.0 μM Na malonate			x	x
18.0 μM Na oxaloacetate		x	x	

[1]Basal medium composition same as in Table 3.

Statistical Analysis

The statistical analysis of the data was conducted by the Department of Statistics, Oregon State University, on a randomized split-split plot design analysis of variance. The design identifies the dietary treatment as the whole plot; the in vitro treatments and interaction between in vitro and dietary treatments as the sub-plot; and the period and the interaction between periods and diet as the sub-sub-plot.

RESULTS AND DISCUSSION

Trial 1

The *in vitro* incubation of liver homogenates with either pyruvate or succinate separated the oxidative processes into NAD-(pyruvate) or flavoprotein-(succinate) dependent systems. After completion of the five week feeding trial the liver from each rat was subjected to *in vitro* incubation to determine the response of selenium and vitamin E on these specific oxidative pathways.

Utilization of Pyruvate--Trial 1

The dietary administration of either selenium or vitamin E significantly increased ($P<0.01$) the utilization of pyruvate by the liver preparations as compared to the non-supplemented basal group. It can be seen in Table 6 and by the statistical analysis in Appendix, Table 1 that both supplements increased the rate of oxidation of pyruvate over a 90 minute incubation period. Although there was an increase in the oxygen consumption by the liver preparations from the combined dietary treatment with selenium and vitamin E over each singly administered supplement, the statistical treatment indicates a non-significant interaction between selenium and vitamin E. This compares the values of 32.5 and 34.5 μl of oxygen uptake

for the vitamin E and selenium-supplemented groups, respectively, to 37.0 μl of oxygen uptake for the combined supplement. Without such interaction the utilization of pyruvate by the liver preparations was presumed to be equally influenced by the presence of either selenium or vitamin E without any additive response. While this suggests a similar role for the function of vitamin E and selenium, a marked difference in pyruvate utilization is evident for the individual 30 minute incubation periods. A significant decrease ($P<0.01$) in pyruvate oxidation during the individual time periods was noted with dietary supplementation of vitamin E. A possible reason for this change might be the exhaustion of the tocopherol's antioxidant properties during the 90 minute incubation period. Selenium function could have a more direct involvement in the utilization of pyruvate since its presence in the dietary supplement helped maintain substrate utilization by the liver homogenate. This does not exclude the possibility of it acting as an *in vivo* antioxidant closely associated with pyruvate utilization.

Further explanation of vitamin E and selenium function was gained from the studies of their *in vitro* addition. When vitamin E was added to the liver homogenates, a significant increase ($P<0.01$) in the oxidation of pyruvate was noted. More important, however, is the significant interaction ($P<0.05$) between dietary supplementation of selenium and *in vitro* supplementation of vitamin E. The

Table 6. Oxygen Uptake (μl) of Liver Homogenates at Intervals During Incubation with Sodium Pyruvate Substrate--Trial 1.

In vitro Supplementation	Dietary Supplementation			
	Basal	+Vitamin E	+Selenium	+Vitamin E + Se
None				
0-30 min.	12.6	14.9	14.7	16.8
30-60	8.2	10.1	10.9	11.4
60-90	6.1	7.5	8.9	8.8
Total	26.9	32.5	34.5	37.0
+Vitamin E				
0-30 min.	11.3	15.6	15.4	17.5
30-60	8.7	11.6	11.2	13.2
60-90	7.3	8.0	9.8	9.7
Total	27.3	35.2	36.4	40.4
+Se				
0-30 min.	8.6	12.6	10.2	14.5
30-60	6.9	8.0	7.8	10.5
60-90	6.9	6.5	5.3	7.1
Total	22.4	27.1	23.3	32.1
+Vitamin E + Se				
0-30 min.	10.1	15.7	13.6	16.4
30-60	6.1	9.0	9.5	11.2
60-90	6.6	6.3	8.0	9.0
Total	22.8	31.0	31.1	38.6

greatest response of vitamin E to pyruvate oxidation was thus in the presence of selenium in the liver homogenates by way of the dietary supplementation. With this increase in oxygen uptake with in vitro administration of vitamin E, the level of it in the liver tissues apparently was not sufficient to maintain maximum utilization of the pyruvate under the in vitro conditions. The non-significant interaction between the dietary supplementation with selenium and vitamin E in their effect on pyruvate oxidation could be due to insufficient vitamin E in the tissue to maintain the increased metabolic rate.

The in vitro addition of Na_2SeO_3 to the homogenate preparations caused a significant decrease ($P<0.01$) in the oxidation of pyruvate. On the other hand, in vitro administration of vitamin E tended to alleviate the toxicity of this selenium administration. This may be a reflection of either the balanced effect of in vitro stimulation by vitamin E against the toxicity of selenium or the protection of certain vital processes by vitamin E from the toxic properties of selenium. The in vitro selenium apparently cannot be synthesized by the liver homogenate into a metabolically active form to increase the utilization of pyruvate. However, this does not exclude the possibility that the selenium administered to the homogenate was at such a high level that the toxicity dominated over any possible beneficial response.

Utilization of Succinate--Trial 1

The utilization of succinate by the liver homogenates was strongly influenced by the presence of dietary vitamin E. A highly significant increase ($P < 0.01$) in oxidation of succinate (see Table 7) was noted in the animals receiving the dietary supplementation of vitamin E. In contrast to the results obtained with pyruvate, dietary supplementation with selenium had little or no influence on the oxidation of succinate. The slight increase observed, both when the selenium-supplemented group was compared to the basal group and when the combination of selenium and vitamin E was compared to the vitamin E group, was not significantly different. This indicated that vitamin E was the main factor in the proper utilization of succinate in these *in vitro* studies.

As was suggested in the case of pyruvate oxidation, the function of vitamin E cannot be fully explained by its lipid antioxidant properties. *In vitro* supplementation with vitamin E did not result in significant increase in the succinate oxidation. This addition would appear to have some effect upon the maintenance of the oxidation rate, however, since the decline rate was somewhat reduced by the inclusion of *in vitro* vitamin E. The initial 30 minute incubation period was somewhat lower than the same period for the non-*in vitro* vitamin E-supplemented group, suggesting an adjustment period was

Table 7. Oxygen Uptake (μl) of Liver Homogenates at Intervals During Incubation with Sodium Succinate Substrate--Trial 1.

In vitro Supplementation	Dietary Supplementation			
	Basal	+Vitamin E	+Selenium	+Vitamin E + Se
None				
0-30 min.	50.5	57.2	55.6	61.8
30-60	37.1	49.5	41.5	45.7
60-90	34.8	37.9	34.2	41.6
Total	122.4	144.6	131.3	149.1
+Vitamin E				
0-30 min.	47.1	53.6	54.2	59.8
30-60	36.9	48.9	44.5	47.7
60-90	35.7	39.9	37.0	46.4
Total	119.7	142.4	135.7	153.9
+Se				
0-30 min.	47.9	54.5	51.8	59.4
30-60	37.3	45.8	37.7	45.3
60-90	31.1	38.0	34.2	39.8
Total	116.4	138.4	123.7	144.5
+Vitamin E + Se				
0-30 min.	44.1	52.4	51.7	55.5
30-60	34.7	45.2	39.1	43.8
60-90	33.8	35.0	35.5	35.4
Total	112.6	132.6	126.4	134.7

necessary before vitamin E could act on the system. After this initial time period the rate of oxidation was greater in the vitamin E-supplemented than the non-supplemented group, suggesting that in vitro vitamin E could have a direct role in the oxidation of succinate.

In vitro additions of selenium to the liver homogenates also resulted in a highly significant decrease ($P<0.01$) in the utilization of succinate. In this case the inclusion of vitamin E alone with the addition of selenium did not result in any protection from the toxic properties of the selenium.

Trial 2

Some further insight into the metabolic function of selenium and vitamin E was accomplished by inclusion of a synthetic lipid antioxidant (ethoxyquin) in the diet of the rats. By providing the rats with this antioxidant, any response of liver mitochondria to the dietary supplementation with selenium and vitamin E would be a reflection of a metabolic function other than those of a general lipid antioxidant. These in vitro incubation studies included the substrates pyruvate and succinate; and to further isolate the flavoprotein- or NAD-dependent systems, either sodium malonate or amytal was administered.

Utilization of Pyruvate--Trial 2

The data from this trial indicate that oxidation of pyruvate by the liver mitochondria is significantly increased by the inclusion of selenium and vitamin E in the diet of the rat. As shown in Table 8 a significant increase ($P<0.01$) was noted in oxygen uptake of the group receiving selenium and vitamin E in addition to their basal diet. The supplementation with either selenium or vitamin E alone in the diet did not increase the oxidation of pyruvate over the group receiving the basal (deficient) diet. Thus these data suggest an interdependence upon the presence of both selenium and vitamin E for any increased utilization of pyruvate.

With the inclusion of presumably adequate synthetic lipid antioxidant (ethoxyquin) in the diets, any further antioxidant role of selenium and vitamin E would be expected to be a very minor one. Therefore, any increase in oxidation could be attributed to properties other than that of a general lipid antioxidant. As observed in Table 8 the increase due to dietary supplementation with selenium and vitamin E was evident during the first 30 minute incubation period and continued throughout the total 90 minute period. The maintenance of the oxidative rate by all dietary treatments through the first 60 minutes, only decreasing slightly during the final 30 minute period, also suggests that the increase is not related to the lipid antioxidant

Table 8. Oxygen Uptake (μl) of Liver Mitochondria at Intervals During Incubation with Sodium Pyruvate Substrate--Trial 2.

In vitro Supplementation	Dietary Supplementation			
	Basal	+Vitamin E	+Selenium	+Vitamin E + Se
None				
0-30 min.	25.9	22.6	23.2	27.9
30-60	24.3	23.1	23.7	27.0
60-90	21.0	20.1	21.6	24.7
Total	71.2	65.8	68.5	79.6
+Na malonate				
0-30 min.	17.8	15.8	17.9	18.0
30-60	13.7	13.9	14.6	15.8
60-90	10.5	10.0	12.0	12.1
Total	42.0	39.7	44.5	45.9
+Na malonate + vitamin E				
0-30 min.	15.5	14.3	15.7	15.8
30-60	10.2	10.6	12.6	11.9
60-90	9.1	7.7	9.1	9.8
Total	34.8	32.6	37.4	37.5

properties. Different rates of respiratory decline would be expected between the different dietary groups if selenium and vitamin E were functioning as lipid antioxidants. The inclusion of the succinic dehydrogenase inhibitor (sodium malonate) along with the pyruvate to isolate the NAD-dependent oxidative system decreased the rate of oxidation and also eliminated the effect of dietary supplementation with selenium and vitamin E on pyruvate oxidation that was noted without the inhibitor. As shown in Table 8 a trend was exhibited with the selenium administration to increase the oxidation uptake, but was not significantly different from the non-selenium treated groups. This suggests that vitamin E and possibly selenium are not directly involved in an NAD-dependent system. However, this lack of response to selenium and vitamin E supplementation may be a reflection of other factors not directly related to the NAD-dependent system which overshadowed any beneficial response due to their supplementation. By inhibiting the succinic dehydrogenase system with malonate, the citric acid cycle would not be a functional unit. This could limit the oxidation of pyruvate by either supplying an inadequate amount of oxaloacetic acid to combine with acetyl CoA to form citric acid or by causing a product buildup prior to the inhibitor site, thus reducing pyruvate oxidation. An inadequate supply of oxaloacetic acid is questionable since the incubation medium also contained 2 μM of fumarate which can be converted to oxaloacetic acid. The

data clearly show a larger decline in pyruvate oxidation with the addition of the inhibitor to the incubation medium than was noted without the inhibitor over the 90 minute incubation period. This would indicate some limitation to the system not directly involved in the utilization of pyruvate.

The addition of in vitro vitamin E to the incubation medium containing pyruvate and malonate resulted in a further decrease in oxygen uptake. The reason for this cannot be fully explained except that in the previous trial the in vitro addition of vitamin E caused an initial decrease in the oxidation of pyruvate during the first 30 minute incubation period. However, the oxidation rate increased during the final 60 minutes of incubation to give a net increase in oxygen consumption in that trial. The initial 30 minute incubation period for Trial 2 was also less, but the recovery noted previously did not occur. The decline in the oxygen consumption was similar to that seen with the malonate group and notably higher than was observed with the group receiving no vitamin E or inhibitor. As was noted with the inclusion of malonate, the different dietary treatments did not significantly alter the oxidation of pyruvate.

Utilization of Succinate--Trial 2

Through the use of succinate as the substrate for the liver mitochondria, the oxidative system dependent upon a flavoprotein

could be evaluated. As shown in Table 9 the dietary supplementation with selenium and vitamin E did not significantly affect the utilization of succinate. When the incubation medium included in addition to the basic incubation medium, succinate and NAD, a slight, but non-significant, increase was noted apparently due to interaction of selenium and vitamin E in the diet. By including amytal along with the succinate and NAD, the oxygen consumption values were essentially equal for all dietary treatments. By isolating the succinic dehydrogenase system from other oxidative pathways through the use of amytal, any differences noted because of the inclusion of selenium and vitamin E in the diet would not be a direct result of the oxidation of succinate.

Other oxidative processes dependent upon NAD apparently still contribute to the oxidation values even in the presence of amytal. An increase of approximately 10 to 15 μl of oxygen was noted when NAD was added to a system containing amytal in the medium. This suggests that amytal at the concentration used in these studies was not in sufficient quantity to inhibit the additional 3 μM of NAD.

The effect of end-products inhibiting succinate oxidation was determined by incubating 2 μM of sodium fumarate in the presence of amytal and NAD and observing the oxygen consumption of the mitochondrial preparations. Table 9 shows that the inclusion of fumarate did not significantly alter the consumption of succinate.

Table 9. Oxygen Uptake (μl) of Liver Mitochondria at Intervals During Incubation with Sodium Succinate Substrate--Trial 2.

In vitro Supplementation	Dietary Supplementation			
	Basal	+Vitamin E	+Selenium	+Vitamin E+Se
+NAD				
0-30 min.	90.2	83.0	84.2	88.7
30-60	74.0	72.5	71.4	78.8
60-90	60.9	61.9	56.9	64.4
Total	225.1	217.4	212.5	231.9
+Amytal				
0-30 min.	62.4	58.1	57.4	57.9
30-60	30.2	30.8	30.2	31.2
60-90	16.6	16.3	18.3	15.8
Total	109.2	105.2	105.9	104.9
+NAD + amytal				
0-30 min.	69.3	67.1	67.2	65.9
30-60	34.4	34.9	32.8	33.9
60-90	17.8	18.4	20.5	19.9
Total	121.5	120.4	120.5	119.7
+NAD + amytal + fumarate				
0-30 min.	67.8	63.8	62.4	63.9
30-60	34.8	35.7	35.4	36.0
60-90	19.0	19.3	20.1	21.1
Total	121.6	118.8	117.9	121.0
+Vitamin E + NAD + amytal + fumarate				
0-30 min.	62.5	59.5	56.7	57.2
30-60	32.5	35.1	32.5	32.9
60-90	20.2	19.9	20.5	22.4
Total	115.2	114.5	109.7	112.5

By including vitamin E along with the fumarate, a reduction in the total oxygen consumption was noted, and there was a decreased rate of oxidation during the first 30 minutes of incubation. This was also noted in the previous trial: the in vitro addition of vitamin E resulted in an initial decrease in oxygen consumption.

Trial 3

Through the use of dietary treatments similar to those used in the previous two trials, a direct comparison was made to further clarify the true function of selenium and vitamin E under the same conditions. As noted in Trial 2, the development of liver necrosis was not evident, suggesting that the synthetic lipid antioxidant (ethoxyquin) was effective in its prevention. However, another dietary difference between the first trials was the inclusion of methionine in all diets in Trial 2 to meet the recommended level prescribed by the U. S. National Academy of Sciences--National Research Council (1962). In vitro studies were used in this third trial to evaluate the effect of ethoxyquin on the dietary treatments which included the inhibitors to isolate the NAD-(pyruvate) and flavoprotein-(succinate) dependent systems.

Utilization of Pyruvate--Trial 3

The results obtained when using pyruvate as the substrate in

the incubation medium indicated non-significant differences among all dietary treatments. The medium in this trial included the succinic dehydrogenase inhibitor (sodium malonate) to isolate the NAD-dependent oxidative system. With the inclusion of this inhibitor the rate of pyruvate oxidation was greatly reduced when compared to the previous trials. As noted in Table 10 the rate of oxidation did not decline as it did previously, and in certain instances an increase in the oxygen uptake was found during the final 30 minute incubation period.

The cause for this lower rate of substrate oxidation was apparently some factor not related to the dietary treatments. A blockage of the succinic dehydrogenase system by sodium malonate would alter the normal function of the citric acid cycle which provided oxaloacetic acid to combine with the acetyl CoA to form citric acid. Alternate pathways are known through which the cell may provide oxaloacetic acid from pyruvate, but the conditions in this experiment may not have been adequate to utilize this system properly.

A supplement to Trial 3 was conducted to determine whether an inadequate supply of oxaloacetic acid was the possible cause for the lower values of oxygen consumption with the pyruvate substrate. Different combinations of oxaloacetic acid and sodium malonate were used in the *in vitro* incubation medium, and the results of this study are shown in Table 11. A significant increase ($P < 0.01$) was observed

Table 10. Oxygen uptake (μl) of Liver Mitochondria at Intervals During Incubation with Sodium Pyruvate Substrate--Trial 3.

In vitro Supplementation	Dietary Supplementation			
	Basal	+Vitamin E	+Selenium	+Vitamin E + Se
	Without Ethoxyquin			
+Na malonate				
0-30 min.	6.9	7.8	6.6	6.1
30-60	6.9	6.9	6.3	6.5
60-90	5.7	6.5	6.3	7.3
Total	19.5	21.2	19.2	19.9
+Na malonate + vitamin E				
0-30 min.	6.7	8.5	6.8	7.3
30-60	4.3	7.2	5.6	6.1
60-90	2.9	6.6	5.4	6.3
Total	13.9	22.3	17.8	19.7
+ Na malonate + ethoxyquin				
0-30 min.	6.5	7.9	7.4	6.5
30-60	4.5	6.6	5.6	6.2
60-90	3.5	5.5	5.1	6.7
Total	14.5	20.0	18.1	19.4
	With Ethoxyquin			
+Na malonate				
0-30 min.	7.2	5.1	6.7	6.8
30-60	7.3	6.3	5.9	7.3
60-90	5.9	6.5	6.6	6.9
Total	20.4	17.9	19.2	21.0
+Na malonate + vitamin E				
0-30 min.	8.2	6.9	6.8	6.7
30-60	6.9	6.4	6.6	5.7
60-90	5.1	5.9	5.9	5.9
Total	20.2	19.2	19.3	18.3
+Na malonate + ethoxyquin				
0-30 min.	6.9	6.6	7.6	6.8
30-60	6.6	6.1	5.9	5.8
60-90	4.8	5.7	6.0	5.9
Total	18.3	18.4	19.5	18.5

in the utilization of pyruvate by the in vitro addition of oxaloacetic acid. The inclusion of sodium malonate along with the oxaloacetic acid did not significantly decrease the utilization of pyruvate. When sodium malonate was administered without the oxaloacetic acid, a significant decrease ($P<0.01$) in the oxygen consumption was observed. Through the inclusion of the oxaloacetic acid along with the sodium malonate, the depression in oxygen consumption due to the inhibitor was alleviated.

Table 11. The Influence of Oxaloacetic Acid and Sodium Malonate on Oxygen Uptake (μl) with Sodium Pyruvate by Liver Mitochondria from Normal Rats.

Incubation Time	In Vitro Addition			
	None	+Oxaloacetic acid	+Oxaloacetic +malonate	+Malonate
0-30 min.	14.1	14.5	13.3	9.3
30-60	7.8	13.9	13.2	6.1
60-90	5.5	11.3	9.7	5.1
Total	27.4	39.7[1]	36.2[1]	20.5

[1] Significantly ($P<0.01$) different from treatments not marked within the same category.

Relating these data to the experimental groups of Trial 3 with pyruvate as the substrate, a possible explanation for the reduced utilization of pyruvate could be that the supply of oxaloacetic acid in the medium was insufficient. Approximately 20.0 μl of oxygen

were consumed in 90 minutes for all groups in both Trial 3 and the supplemental trial which included the sodium malonate. As can be seen in Table 11 the true potential for this mitochondria preparation was much larger than the 20.5 μl of oxygen indicated since the addition of oxaloacetic acid to the medium caused an increase in the oxygen consumption to 36.2 μl.

Utilization of Succinate--Trial 3

In vitro studies using succinate as the substrate were carried out on the eight dietary treatments along with the three in vitro treatments. As shown in Table 12 the oxygen consumption values for all dietary and in vitro treatments were not significantly different. This incubation medium included amytal to inhibit the oxidation of the NAD-dependent oxidative system. Amytal limits the oxygen consumption to the succinic dehydrogenase system (Corwin, 1965), and the values obtained would thus be a reflection of succinate oxidation.

These data indicate that the oxidation of succinate by mitochondria is not dependent upon the presence of selenium or vitamin E in the diet of the rat and, moreover, the influence of a lipid antioxidant would not be of importance in succinate utilization. Antioxidant addition, either in the form of vitamin E or ethoxyquin, to the diet or to the incubation medium did not alter the oxygen consumption of the liver mitochondria.

Table 12. Oxygen Uptake (μl) of Liver Mitochondria at Intervals During Incubation with Sodium Succinate Substrate--Trial 3.

In vitro Supplementation (includes amytal)	Dietary Supplementation			
	Basal	+Vitamin E	+Selenium	+Vitamin E+Se
	Without Ethoxyquin			
+Succinate				
0-30 min.	55.3	56.4	53.9	52.6
30-60	37.7	35.0	34.0	36.5
60-90	30.1	28.5	28.4	28.9
Total	123.1	119.9	116.3	118.0
+Succinate + vitamin E				
0-30 min.	41.7	53.9	51.2	49.3
30-60	26.2	34.7	32.8	33.5
60-90	19.4	26.7	27.0	26.9
Total	87.3	115.3	111.0	109.7
+Succinate + ethoxyquin				
0-30 min.	52.2	56.2	53.4	49.5
30-60	36.3	34.6	33.9	34.7
60-90	27.5	27.1	27.4	27.8
Total	116.0	117.9	114.7	112.0
	With Ethoxyquin			
+Succinate				
0-30 min.	54.0	47.8	52.9	48.9
30-60	35.4	34.7	34.2	33.3
60-90	29.1	28.0	29.3	26.5
Total	118.5	110.5	116.4	108.7
+Succinate + vitamin E				
0-30 min.	52.7	47.7	50.6	50.2
30-60	34.1	34.5	32.8	32.1
60-90	27.3	27.4	27.7	27.0
Total	114.1	109.6	111.1	108.3
+Succinate + ethoxyquin				
0-30 min.	55.1	49.2	50.2	48.5
30-60	34.8	35.6	34.3	32.9
60-90	28.8	28.0	28.4	26.9
Total	118.6	112.8	112.9	108.3

The effect of the amytal on the system could be implicated as a factor which overshadows the effect of dietary and in vitro supplementation with selenium, vitamin E and the ethoxyquin. However, in Trial 2 the supplementation with vitamin E and selenium did not influence the succinate oxidation whether amytal was present in the incubation medium or not. An accumulation of an end-product was suggested by Corwin (1965) as a possible cause for a reduction of succinate oxidation by rats deficient in vitamin E. He implicated oxaloacetic acid as the more likely item involved since it accumulates twice as rapidly when mitochondria were from vitamin E-deficient rats. By blocking the NAD oxidation with amytal, one can expect an increased amount of end-products like fumarate, malate, etc., but the effect on succinate oxidation is questionable. In Trial 2 the addition of 2 μM of fumarate to a medium containing amytal did not alter the oxygen consumption values as compared to the treatment without fumarate. The data of Grove, Johnson and Cline (1965) indicate that accumulation of oxaloacetic acid did not inhibit succinate oxidation.

Theory of Selenium Function

From these studies, the biological function of selenium was associated within the mitochondria of the liver with pyruvate in its oxidation scheme. Selenium proved to be the dominant factor in

both Trial 1 and 2 for pyruvate oxidation, but only when vitamin E was also present was selenium effective in Trial 2. This would indicate that through the longer feeding period used in the second trial, the body stores of vitamin E were depleted sufficiently to develop a vitamin E deficiency. The five week feeding period of the first trial probably was not adequate to deplete the body sufficiently to illustrate this interaction of selenium and vitamin E on the utilization of pyruvate.

The metabolic role of selenium, based on the data from these experiments, would constitute more than that of a general in vivo lipid antioxidant. As noted by Zalkin and Tappel (1960), Bieri et al. (1961a), Bieri (1961b) and Olcott, Brown and Van der Veen (1961), selenium possesses the properties of a lipid antioxidant by reducing the initial stages of free radical formation. Under the condition in Trial 2 an effective in vivo lipid antioxidant (ethoxyquin) was included in the diets of the rats which would presumably alleviate the need for selenium to act in this capacity. Thus an increase in metabolic rate due to selenium added, over and above a presumably adequate supply of antioxidant (either with vitamin E or ethoxyquin), it seems, should be attributed to properties other than those of a lipid antioxidant.

Selenium had little or no influence upon the utilization of succinate, yet a marked influence on pyruvate breakdown. A general lipid antioxidant effect would be expected to influence the rate of

substrate breakdown with either pyruvate or succinate. More direct involvement of selenium in energy metabolism appears likely, either in conversion of pyruvate to acetyl CoA, in the electron transport system dependent upon NAD as the electron donor, or in the subsequent metabolism of acetyl CoA. The concept of involvement of selenium in these processes is in accord with the work of Connolly and Schwarz (1965) who found a 25 percent increase in $C^{14}O_2$ release from α-ketoglutarate by rats receiving selenium in their diets as compared to a non-supplemented group. The breakdown of pyruvate and α-ketoglutarate involves similar enzyme systems which require the same cofactors for activity.

The hypothesis that selenium is essential in certain oxidative pathways implies that when the selenium supply is inadequate, the metabolic scheme is altered, producing deficiency symptoms in the animal. If a metabolic pathway is dependent upon selenium (i. e., it involves pyruvate and α-ketoglutarate), but is limited by a selenium deficiency, the tissue must utilize alternate schemes to provide energy and intermediates for synthesis of tissue. The biochemical symptom of an elevation of serum glutamic-oxaloacetic transaminase (SGOT) value in lambs and calves suffering from white muscle disease illustrates this. A change in the metabolic scheme takes place whereby α-ketoglutarate bypasses the conversion to succinate and instead forms glutamate. At the same time aspartate continues

to be converted to oxaloacetate through the transaminase reaction. A limited supply of aspartate for protein synthesis and for conversion to oxaloacetate may result in protein breakdown. This is shown in tissue necrosis in the rat and myopathies of other animals.

A contributing factor in the development of the symptoms of myopathy would be the stress of growth, muscular activity and environment. The demand for energy to meet these stresses would promote alternate means for providing this energy. In lambs and calves the normal energy-supply pattern changes from one dependent on glucose to one dependent on the volatile fatty acids (VFA) when a functional rumen develops. These VFA's might alleviate some problems of selenium deficiency since propionic acid is utilized by being converted to succinic acid which may later be used as the source of oxaloacetate. This may be the reason why the young but not older ruminants are susceptible to a deficiency of selenium.

SUMMARY

A study of selenium was conducted to clarify its biological function in the prevention of certain metabolic disorders in animal tissue. Conditions such as white muscle disease in lambs and calves, exudative diathesis in chicks, liver necrosis in the rat and certain myopathies in other species have been shown to respond to selenium treatment. The NAD - (pyruvate) and flavoprotein- (succinate) dependent oxidative pathways were investigated in liver tissue preparations from rats which were fed diets containing various combinations of selenium, vitamin E and a synthetic antioxidant (ethoxyquin) to determine the effect of these different supplements on substrate utilization. Through the inclusion of presumably adequate amounts of vitamin E and ethoxyquin, it could be postulated whether selenium performed a specific metabolic function or acted more indirectly as a lipid antioxidant.

Results showed that selenium function was associated with the oxidation of pyruvate by the liver preparations, but not with oxidation of succinate. The inclusion of vitamin E along with selenium in the diet did not significantly increase the oxygen utilization of the pyruvate by animals which were on the test diets for only five weeks. However, by extending the feeding period to eleven weeks and including ethoxyquin in the diets, the combination of both selenium and

vitamin E was required to increase the rate of pyruvate oxidation. This suggests that both selenium and vitamin E are biologically necessary in the oxidation of pyruvate and have a function other than that of a lipid antioxidant.

This interaction between selenium and vitamin E could not be explained by the lipid antioxidant properties of vitamin E. The presence of ethoxyquin in these diets would be expected to alleviate further requirements of a lipid antioxidant by the animal tissue. In addition, dietary supplementation with vitamin E did not have the same response on pyruvate oxidation as noted by the combination of vitamin E and selenium since the oxygen utilization values with vitamin E alone were not significantly different from the deficient group. If further antioxidants were required for the body tissue, supplementation with vitamin E would be expected to reflect this in the oxidation values.

For further proof of selenium function in the oxidative pathways involving pyruvate, sodium malonate was included in the incubation medium. This inhibited the flavoprotein-dependent system and made it possible to observe only the influence of selenium on the NAD-dependent oxidative system. The results from animals receiving ethoxyquin in their diet indicate that supplementation with selenium did not significantly increase the oxidative rate. A slight increase in pyruvate oxidation was noted by the combination of selenium and

vitamin E in the diet, but this was not significantly different from the groups not supplemented with both.

An investigation into the cause of the non-significant difference due to the inclusion of sodium malonate in the incubation medium revealed that oxaloacetic acid was not in sufficient supply. This probably limited the oxidation of pyruvate to the availability of oxaloacetic acid to combine with the acetyl CoA to form citric acid, and did not reflect the extent of the response of selenium on the oxidation of pyruvate.

As noted in the first trial, vitamin E exhibited a significant increase in the oxygen uptake values with succinate as the substrate for the liver homogenates. In later trials this influence was suggested to be not a direct one on the oxidative process involving succinate oxidation since through the inclusion of amytal to isolate the flavoprotein-dependent oxidative system, no difference in the succinate oxidation was noted. The suggestion that certain end-products may have masked the response of vitamin E was discounted since addition of sodium fumarate to the medium containing the amytal did not decrease the oxidation of succinate.

In conclusion, these results suggest a specific metabolic function for selenium in the oxilation of pyruvate, that apparently requires the presence of vitamin E. The other metabolic site investigated--that of succinate oxidation, was apparently unaffected by either selenium or vitamin E.

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APPENDIX

Appendix, Table 1. Analysis of Variance of Oxygen Uptake by Liver Homogenates from Rats on Various Dietary Treatments of Vitamin E and Selenium--Trial 1.

Source of Variation	d. f.	Pyruvate Mean Square	Pyruvate F	Succinate Mean Square	Succinate F
Whole Plot					
Diet (D)	3	305.704	5.500**	1974.267	3.28*
Vitamin E(E)	1	463.072	8.333**	4803.458	7.975**
Se	1	447.567	8.054**	1019.550	1.693
ExSe	1	6.473	0.116	99.791	0.166
Error	36	55.573		602.315	
Sub-plot					
In vitro (I)	3	182.254	34.45**	380.769	9.62**
Vitamin E(E)	1	131.482	24.851**	32.375	0.818
Se	1	401.502	75.885**	1017.162	25.686**
ExSe	1	13.777	2.604	92.770	2.343
Diet x In Vitro	9	9.6824	1.83	29.850	0.75
DE x IE	1	3.581	0.677	39.308	0.993
DE x ISe	1	10.549	1.994	19.104	0.482
DE x I(ExSe)	1	2.617	0.495	49.588	1.252
DSe x IE	1	21.599	4.082*	55.788	1.409
DSe x ISe	1	8.480	1.603	27.094	0.684
DSe x I(ExSe)	1	7.105	1.343	28.871	0.729
D(ExSe) x IE	1	11.788	2.228	23.719	0.599
D(ExSe) x ISe	1	13.913	2.630	3.478	0.088
D(ExSe) x I(ExSe)	1	7.510	1.419	21.701	0.548
Error	108	5.291		39.600	
Sub-Sub-plot					
Periods (P)	2	1586.665	237.51**	11539.541	290.44**
P x D	6	33.599	5.072**	144.121	3.627**
P x Se	2	7.034	1.062	180.287	4.538*
P x E	2	87.780	13.251**	101.379	2.552
P x Se x E	2	5.983	0.903	150.697	3.793
D x I x P	18	3.9631	0.600	20.013	0.500
Error	288	6.6346		39.731	

*$P < 0.05$

**$P < 0.01$

Appendix, Table 2. Split-Split-Plot Analysis of Variance for *In Vitro* Treatments of Pyruvate and Succinate--Trial 2.

Source of Variation	Pyruvate			Succinate		
	d.f.	Mean Square	F	d.f.	Mean Square	F
Whole Plot						
Diet (D)	3	179.57	1.570	3	91.16	0.726
Error (a)	35	114.67		35	125.51	
Sub-plot						
In vitro (I)	2	9376.18	862.57**	4	30334.01	465.03**
D x I	6	31.26	2.88*	12	44.22	0.68
Error (b)	70	10.87		140	65.23	
Sub-sub-plot						
Periods (P)	2	1729.75	171.09**	2	80023.65	3034.65**
P x D	6	16.94	1.68	6	112.19	4.25**
P x I	4	95.34	9.43**	8	933.20	35.39**
P x D x I	12	2.96	0.29	24	11.89	0.45
Error (c)	210	10.11		350	26.37	

*$P < 0.05$

**$P < 0.01$

Appendix, Table 3. Analysis of Variance for In Vitro Treatment of Pyruvate with NAD--Trial 2.

Source of Variation	d. f.	Mean Square	F
		In vitro treatment--pyruvate + NAD	
Diet (D)	3	171.8345	2.988*
Vitamin E (E)	1	3.6177	0.063
Se	1	71.4904	1.243
E x Se	1	428.9773	7.461*
Error	35	57.4921	
Periods (P)	2	207.4492	19.149**
D x P	6	13.1855	1.2[illegible]
Error	70	10.9378	
		In vitro treatment--pyruvate + NAD + malonate	
Diet (D)	3	37.8695	1.260
E	1	0.5993	0.020
Se	1	75.1838	2.502
E x Se	1	29.7685	0.991
Error	35	30.0477	
Periods (P)	2	347.5129	70.32**
D x P	6	4.6039	0.382
Error	70	12.0520	
		In vitro treatment--pyruvate + NAD + malonate + vitamin E	
Diet (D)	3	32.4098	0.663
E	1	14.7418	0.302
Se	1	70.0804	1.434
E x Se	1	12.6253	0.258
Error	35	48.8797	
Periods (P)	2	863.4780	117.88**
D x P	6	5.0822	0.694
Error	70	7.3249	

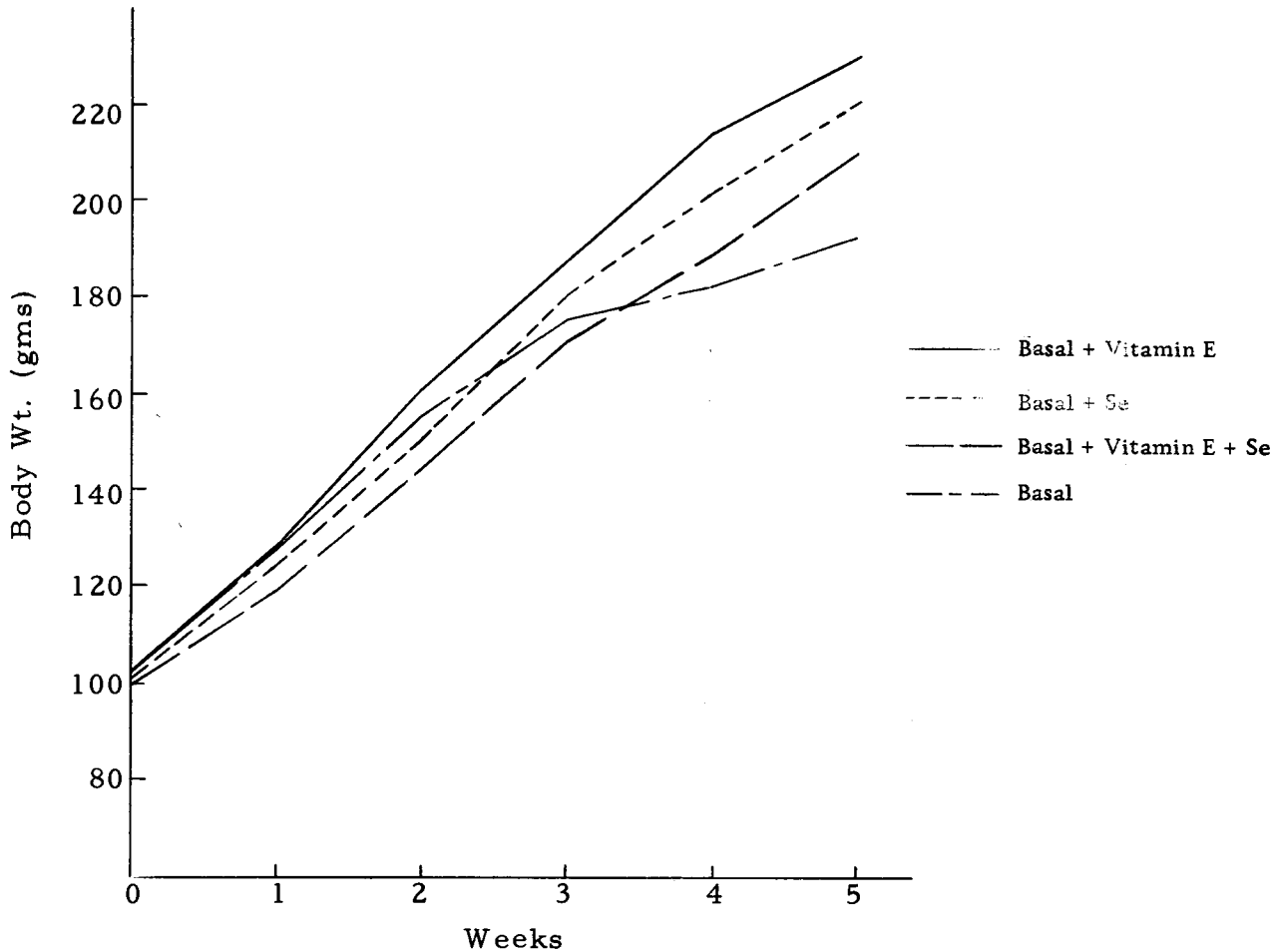
*P<0.05

**P<0.01

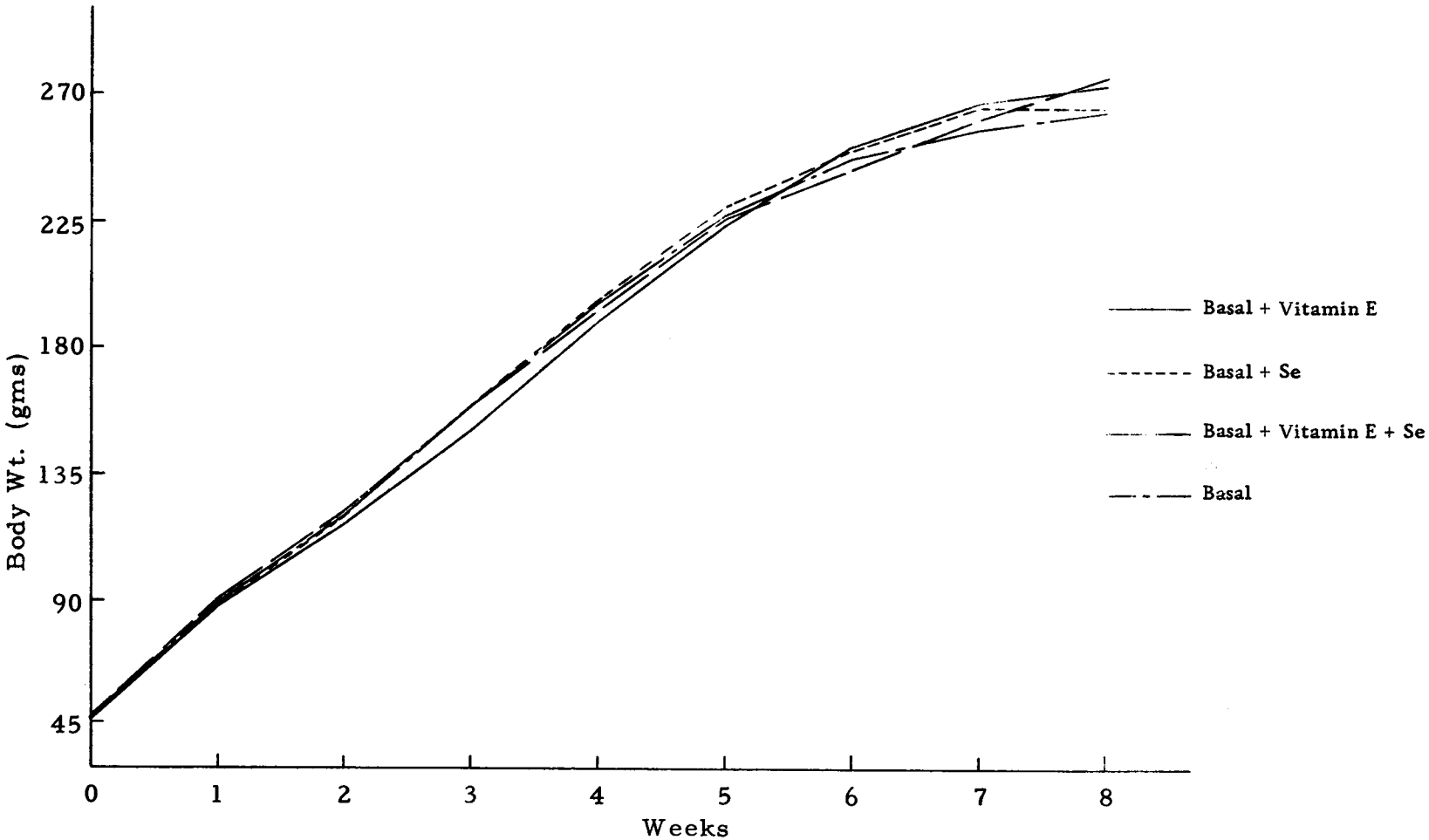
Appendix, Table 4. Analysis of Variance for In Vitro Treatment of Succinate with NAD--Trial 2.

Source of Variation	d. f.	Mean Square	F.
	In vitro treatment--succinate + NAD		
Diet (D)	3	224.4676	0.709
Vitamin E (E)	1	110.1113	0.348
Se	1	3.0974	0.010
E x Se	1	589.1934	1.860
Error	35	316.8136	
Periods (P)	2	6341.1378	108.58**
D x P	6	42.3850	0.726
Error	70	58.4015	
	In vitro treatment--succinate + amytal		
Diet (D)	3	13.6734	1.184
E	1	19.3200	1.673
Se	1	10.0709	0.872
E x Se	1	7.6286	0.660
Error	35	11.5508	
Periods (P)	2	18283.1284	1301.99**
D x P	6	27.3717	1.949
Error	70	14.0424	
	In vitro treatment--succinate + amytal + NAD		
Diet (D)	3	2.0664	0.157
E	1	3.0074	0.228
Se	1	2.6306	0.199
E x Se	1	0.0237	0.002
Error	35	13.1964	
Periods (P)	2	23926.5900	1634.27**
D x P	6	21.3053	1.455
Error	70	14.6405	
	In vitro treatment--succinate + amytal + NAD + Na fumarate		
Diet (D)	3	9.9045	0.481
E	1	0.0721	0.003
Se	1	1.6619	0.081
E x Se	1	28.4671	1.381
Error	35	20.6118	
Periods (P)	2	20208.1438	1296.00**
D x P	6	28.5034	1.828
Error	70	15.5927	
	In vitro treatment--succinate + NAD + Na fumarate + vitamin E + Amytal		
Diet (D)	3	17.9197	0.739
E	1	2.5331	0.104
Se	1	45.1686	1.862
E x Se	1	9.4137	0.388
Error	35	24.2608	
Periods (P)	2	14997.4396	514.40**
D x P	6	46.1961	1.379
Error	70	28.1554	

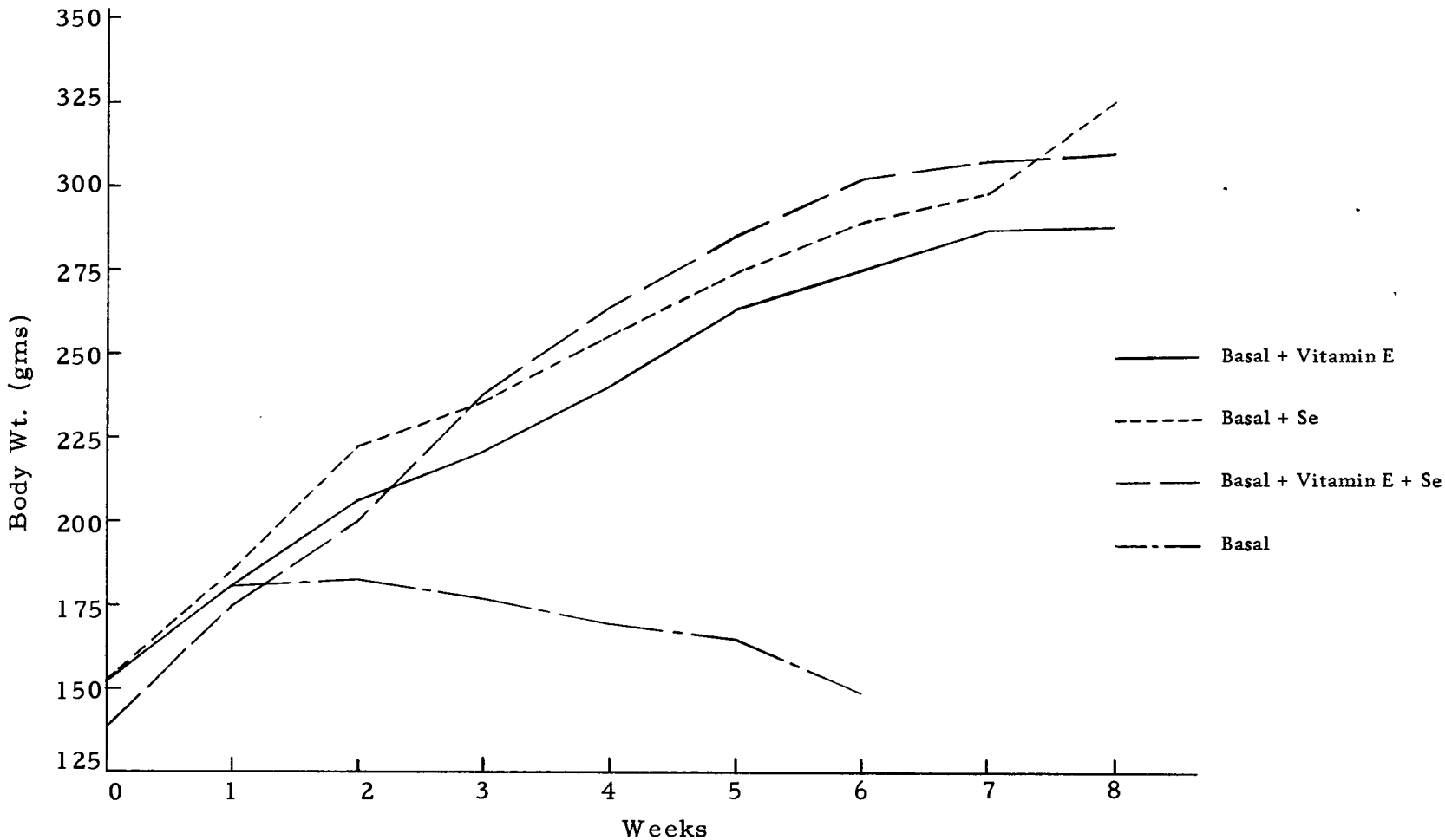
*$P < 0.05$
**$P < 0.01$



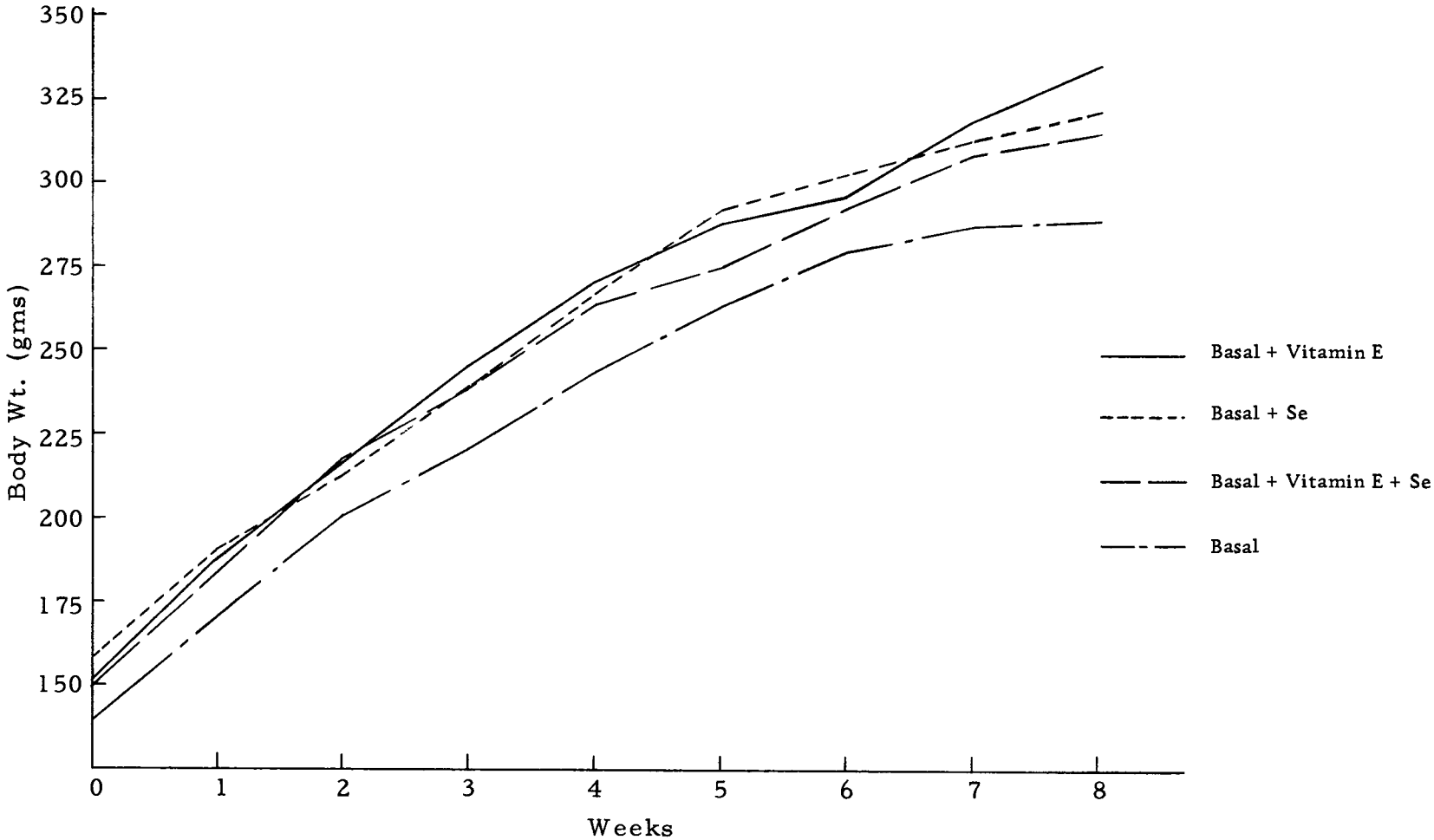
Appendix, Figure 1. Growth Rates of Rats Receiving Various Dietary Treatments of Vitamin E and Selenium--Trial 1.



Appendix, Figure 2. Growth Rates of Rats Receiving Ethoxyquin in Addition to Various Treatments of Vitamin E and Selenium--Trial 2.



Appendix, Figure 3. Growth Rates of Rats Receiving Various Dietary Treatments of Vitamin E and Selenium without Ethoxyquin--Trial 3.



Appendix, Figure 4. Growth Rates of Rats Receiving Various Dietary Treatments of Vitamin E and Selenium with Ethoxyquin--Trial 3.