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Dr. P. R. Cheeke

A two-part study was conducted to investigate some of the nutritional relationships between dietary tri-o-cresylphosphate (TOCP), a toxic stress material, and dietary vitamin E and selenium. In part one, growth rate of male weanling Long-Evans rats and day-old Japanese quail (Coturnix coturnix japonica) was used to determine the protective action of various levels of selenium and/or vitamin E in overcoming the growth depressant effect of dietary TOCP. Also, lipase activity was measured in pancreatic homogenates and intestinal contents of rats. In part two, experiments were conducted to determine the influence of TOCP on the capacity of dietary selenium (with glucose added in vitro) and dietary vitamin E to prevent in vitro red blood cell hemolysis.

The addition of 0.2% TOCP to the torula yeast based diet low in vitamin E and selenium resulted in growth cessation of rats 12 to 20 days (depending on initial weight) before the control. Addition of 0.5,

1.0, 2.0 or 5.0 ppm selenium ($Na_2SeO_3$) resulted in a positive growth response; one ppm selenium was the most effective level. With supplemental vitamin E, 50, 100, 250, 500 or 1000 IU (dl-α-tocopherol) per kg of diet, positive growth responses resulted whereas the 10 IU level was ineffective. Supplementation of 0.0125% ethoxyquin (a synthetic antioxidant) produced no favorable growth response; dietary cystine (1%) initially improved the growth of TOCP-fed rats but did not prevent death. One ppm selenium with and without 500 IU vitamin E were the most effective treatments. Also, rats maintained on the basal diet plus 0.2% TOCP for 33 days (cessation of growth occurred at 20 days) were supplemented with 1 ppm selenium + 100 IU dl-α tocopherol; the subsequent response in growth to selenium was immediate and marked in contrast to a lesser response to dl-α-tocopherol.

In day-old Japanese quail fed a torula yeast diet low in vitamin E and selenium, growth ceased at nine days; 90% mortality had occurred by 17 days. Selenium (0.5 ppm) supplementation prevented the cessation of growth and mortality. Quail supplemented with vitamin E (20 IU dl-α-tocopherol/kg of diet) grew poorly and 50% mortality occurred indicating a specific need for selenium. Quail fed 0.10% TOCP responded poorly in growth when neither selenium nor vitamin E were supplemented to the diet. The addition of 0.5 ppm selenium greatly improved growth; supplementation of vitamin E alone was much less effective.

Dietary TOCP did not appear to inhibit pancreatic lipase activity in rats *in vivo* or *in vitro*. An increase in activity was observed in rats supplemented with 0.5 and 1.0 ppm selenium. In contrast, activity was slightly depressed in pancreatic homogenates obtained from rats supplemented with dietary vitamin E. Activity of intestinal lipase followed the same apparent trend.

Addition of TOCP to the diet of growing rats supplemented with 10 IU of dl-α-tocopherol/kg of diet increased the extent of dialuric acid-induced hemolysis of erythrocytes. Only an initial difference was evident with the higher level of vitamin E (50 IU dl-α-tocopherol/kg of diet) which was totally protective after 16 days. An adaptation to the low vitamin E intake apparently occurred because the percent hemolysis decreased following an initial rapid rise.

Red cells from animals supplemented with selenium and low vitamin E autohemolyzed less when glucose was added to the incubation medium. Dietary TOCP (0.2%) completely blocked the effect of glucose in the 1 ppm selenium-supplemented cells; higher dietary selenium (5 ppm) only slightly reduced the inhibitory affect of TOCP. In contrast, TOCP interfered less with utilization of glucose in vitamin E-supplemented erythrocytes.

The results of this study indicate that TOCP is an antagonist of both selenium and vitamin E. The growth depression resulting from TOCP consumption is primarily the result of selenium antagonism.

# Vitamin E, Selenium and Tri-o-cresylphosphate Interrelationships

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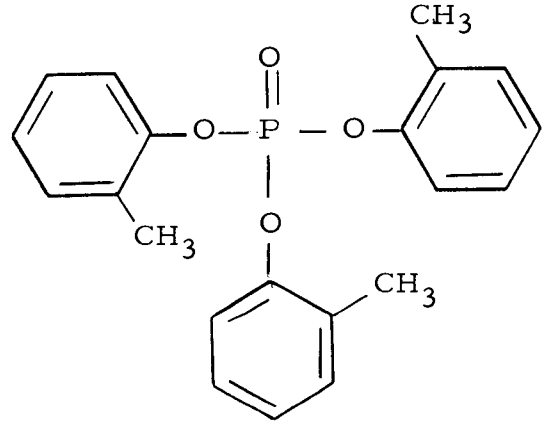
### INTRODUCTION

Certain compounds classified as "antivitamin E stress factors" interfere with normal vitamin E utilization and generally accelerate the onset of various deficiency symptoms. Green and Bunyan (1969) have enumerated 18 such vitamin E antagonists; the most widely studied is unsaturated fat. The organophosphate, tri-o-cresyl phosphate (TOCP), has been reported to induce creatinurea and muscular




dystrophy in rabbits (Bloch and Hottinger, 1943), leg weakness in lambs (Draper et al., 1952), liver necrosis and lung hemorrhage in rats (Hove, 1955), testicular atrophy, bronchopneumonia, and muscular disorders in dogs (Carpenter et al., 1959); the administration of vitamin E effectively prevented these symptoms. As with the

unsaturated fats, TOCP may catalyze the formation of an oxidative state in body lipids creating a greater need for vitamin E (Hove, 1953; Hove, 1955; Seward et al., 1966). However, vitamin E does not completely correct the severely depressed growth rate resulting from the administration of TOCP (Draper et al., 1952; Hove, 1953; Hove, 1955). Because of the well established interrelationship between vitamin E and selenium, it was of interest to investigate the possible interaction between TOCP and these two nutrients.

# LITERATURE REVIEW

## A Brief Review of the Vitamin E-Selenium Interrelationship

Since it's discovery in 1922 by Evans and Bishop, a requirement for vitamin E in many biological systems has been demonstrated. Briefly, the most frequently occurring symptoms which have been found to respond to vitamin E in mammals include liver necrosis, kidney damage, heart muscle degeneration, muscular atrophy, pancreatic atrophy, lung hemorrhage, spinal cord degeneration, testicular atrophy, resorption sterility, calcifications, anemia, and exudative diathesis. In avian species, anemia, encephalomalacia, and exudative diathesis have been implicated (Schwartz, 1969). Since the discovery of the nutritional significance of selenium by Schwartz and Foltz (1957), study of the biological role of vitamin E has been complicated. Selenium protects against some vitamin E deficiency conditions but not others.

It is well established that a vitamin E deficiency state can be accelerated by including high polyunsaturated fatty acids (PUFA) in the diet (Green and Bunyan, 1969). It is especially significant to note that selenium does not substitute for vitamin E in preventing the disorders induced by PUFA; selenium has been demonstrated to prevent numerous other vitamin E deficiency conditions (Sondergaard, 1967, p. 365). The evidence suggests then that selenium is not an *in vivo*

antioxidant as is vitamin E and also that vitamin E functions other than just as an in vivo antioxidant. Most workers involved in this area of nutrition agree that vitamin E and selenium are separate, essential, dietary agents and that much concerning their interrelationship is yet to be learned.

## Toxic Organophosphate Compounds in Nutritional Studies

Dietary stress has played a significant role in the study of vitamin E. The inclusion of PUFA in a vitamin E deficient diet accelerates the onset of a vitamin E deficiency (Harris and Embree, 1963). Green and Bunyan (1969) have enumerated 18 such vitamin E antagonists, one being tri-o-cresyl phosphate (TOCP). Like the unsaturated fats, TOCP is thought to enhance in vivo lipid peroxidation resulting in vitamin E depletion. Green and Bunyan (1969) have criticized this theory and suggest that insufficient evidence is available to link dietary stress with the catabolism of vitamin E.

Little information is available concerning the relationship between TOCP and vitamin E. The first hint of such an interrelationship was reported in 1943 by Bloch and Hottinger. They observed that TOCP administered to rats and rabbits resulted in muscular dystrophy, creatinurea, and other symptoms of an acute vitamin E deficiency. Later, Meunier et al. (1947, p. 424) demonstrated the protective effect of vitamin E on TOCP-induced muscular dystrophy in the rabbit.

Draper et al. (1952) showed that the addition of 0.2% TOCP to a vitamin E-low ration induced earlier development of a vitamin E deficency, as assessed by growth depression and increased erythrocyte hemolysis. Their research also demonstrated in lambs a TOCP-induced condition similar to "stiff lamb disease." They also confirmed the earlier report (Bloch and Hottinger, 1943) implicating TOCP with the occurrence of muscular dystrophy, creatinurea, and lowered plasma tocopherol.

In 1953, two groups of workers suggested a biological function for TOCP and it's apparent relationship to vitamin E. Hove (1953) demonstrated that a substantial growth inhibition in rats fed 0.1% TOCP could be partially prevented by high levels of vitamin E. (However, a greater response was observed when high dietary casein levels even without vitamin E were fed.) Hove (1953) proposed pro-oxidation as an explanation for the activity of TOCP. Evidence for this included; 1) increased conjugated dienes (fatty peroxides); 2) decreased tissue arachidonic acid concentrations; 3) decreased tocopherol levels in tissue lipids, and 4) decreased liver storage of vitamin A. Based on these findings, it was suggested that the main effect of TOCP on vitamin E was destruction by oxidation.

Myers et al. (1953) observed that following the injection of rats with TOCP the serum vitamin E levels dropped appreciably. TOCP also prevented an increase in serum vitamin E after feeding

α-tocopherol. However, TOCP did not affect the rate at which vitamin E was removed from the blood nor did it inhibit the penetration of vitamin E into tissues. This evidence suggested to these workers that the vitamin E deficiency induced by TOCP was a result of poor absorption of vitamin E. Large doses of α-tocopherol overcame the vitamin E deficiency, a result in support of the contention of Hove (1953).

Later work by Cowlishaw and Blaxter (1955) supported the contention of Myers et al. (1953). It was found that calves fed a vitamin E-low diet with up to 4 g TOCP/day displayed signs of a severe neurological disorder within seven days. Muscles appeared normal and no reduction in creatine content or increase in Na:K ratio, characteristic of dystrophy, was noted. However, increased excretion of vitamin E and it's quinones and lowered serum vitamin E was observed. Massive doses of vitamin E were ineffective against the neurological complications; however, the vitamin E levels in the blood returned to normal. This suggested also that TOCP reduced the absorption of vitamin E from the intestine.

Hove (1955) demonstrated that TOCP-poisoned rats died sooner of liver necrosis and lung hemorrhage; TOCP increased the incidence and decreased the time of fatalities in rats. Vitamin E was completely protective. It was suggested that these effects were due to increased lipid peroxidation occurring in the absence adequate dietary vitamin E.

An effort to demonstrate an antagonism by TOCP on vitamin E in poultry has been complicated by the occurrence of neurological disorders before the appearance of symptoms of a vitamin E deficiency. Consequently, Myers et al. (1953) and Casida et al. (1961) have reported no beneficial effect of α-tocopherol on paralysis induced by TOCP in chickens; chicks under 8 weeks of age are apparently insensitive to TOCP-induced neurotoxia (Barnes and Denz, 1953; Bondy et al., 1960).

In contrast, TOCP does not cause paralysis in rats at any age (Smith et al., 1932). This characteristic makes rats especially useful for the study of TOCP as a vitamin E antagonist.

Probably the most recent work directly involving TOCP and vitamin E was reported by Carpenter et al. (1959). TOCP administered to dogs resulted in testicular atrophy, hemorrhagic bronchopneumonia, and muscular disorders, all vitamin E deficiency symptoms. It was suggested that the interference with vitamin E metabolism by TOCP was due to the relative structural similarity between TOCP and vitamin E e.g. both contain phenolic ring structures with methyl groups in the ortho position and both are fat soluble.

Several compounds closely related to TOCP have been reported to show similar antivitamin E activity. Tedeschi and de Cicco (1952) demonstrated an antagonism toward vitamin E by o-cresol succinate or acetate. m-Cresol acetate was without effect. In a later report (1954)

the same group demonstrated that gauicol acetate, a trisubstituted aromatic compound, given to female rats prior to mating resulted in high embryo mortality. Low levels of α-tocopherol were found to completely prevent this disorder.

Recently, Goyer et al. (1970) have investigated the effect of parathion and paraoxon on the in vivo status of vitamin E. Parathion (an alkylorganophosphate) is classed with TOCP (a triarylorganophosphate) as a "systemic insecticide" e.g. a compound stable to hydrolysis at neutral pH having no in vivo activity (insecticidal) prior to metabolic transformation (Jandorf, 1956). Goyer et al (1970) showed that feeding chronic levels (30 ppm) of parathion or paraoxon (the active insecticidal metabolite of parathion) did not interfere with plasma or liver levels of vitamin E.

No reports have appeared relating TOCP and selenium. However, Hove (1953) demonstrated that high levels of casein would almost entirely prevent depression of growth caused by feeding TOCP. It was suggested that the observed protective effect of casein could be Schwartz's Factor 3 (Se). However, no experimental evidence has been reported to either confirm or deny this suggestion.

Some reports indicating an effect of TOCP on vitamin A have appeared. Hove (1953) observed that 0.1% TOCP in the diet depressed vitamin A utilization and storage. This effect was believed due to pro-oxidation of vitamin A by TOCP. Later, Seward et al. (1966)

demonstrated that liver storage of vitamin A decreased in the presence of TOCP but the authors suggested that the reduction may reflect only a change in liver size or food consumption. No vitamin A deficiency was evident. Similarly, Phillips (1964) reported that dietary parathion (10 ppm) did not decrease the utilization of vitamin A nor the rate of vitamin A metabolism in the liver.

A direct influence of TOCP on the function of vitamin K has not been reported; however, hypothrombinemia has been observed as a result of salicyclic acid administration (Hochster and Quastel, 1963, p. 433). Salicyclic acid has been suggested also to be major metabolic product of TOCP metabolism (Eto et al., 1962a). Mounter et al. (1957) reported an inhibition of thrombin by several organophosphate poisons; however, TOCP was not included. These bits of evidence suggest that the metabolism of TOCP might affect the clotting process in some way. However, as mentioned above, this has not been demonstrated.

A close similarity between the disorders induced by TOCP and a thiamine deficiency have been reported (Barnes and Denz, 1955; Swank, 1940; Aring et al., 1941; Cavanaugh, 1964). Casida et al. (1961) suggested that TOCP metabolites may inhibit pseudocholinesterases to the extent that no hydrolysis of thiamine esters would occur. As a result, the availability of free thiamine would be critically impaired. Since thiamine is required by nervous tissue, a paralytic

state characteristic of TOCP poisoning would occur. However, Casida et al. (1961) could not demonstrate a protective effect by feeding free thiamine. Cavanaugh (1964) has confirmed these results by demonstrating that the B-group vitamins and cofactors have no protective action against organophosphorous-induced lesions.

## Metabolism of Tri-o-cresylphosphate

Organophosphates esters are degraded by 2 major enzyme groups (Miyata et al., 1972): the microsomal oxidative system and the non-oxidative enzymes such as esterases. With respect to the latter, Mounter (1955) has described a DFPase, Hodgson and Casida (1962) a malathion hydrolyzing aliesterase, and Yang et al. (1971) a NADH requiring diazinon degrading enzyme. Kojima and O'Brien (1968) have also described an NADH requiring detoxifying enzyme for paraoxon. TOCP is metabolized partially by the microsomal oxidative system (Casida et al., 1961); however, degradation of some of it's metabolites is unclear. A more detailed discussion of this subject will follow.

An early report by Gross and Grosse (1932) showed that TOCP administered intraperitoneally was absorbed readily across the peritoneal membrane. This was based on the amount of cresol ester measured in liver, intestine, and other body tissues. However, Nelson

(1950) suggested that the degree of serum pseudocholinesterase inhibition is a good indication of TOCP absorption.

For most species, utilization of this method has been sufficient for instance in hens (Earl and Thompson, 1952a, b) and rats (Mendel and Rudney, 1944). In man, TOCP is a very selective inhibitor of pseudocholinesterase (Earl and Thompson, 1952a) and even blood cell cholinesterases are affected (Geoffry et al., 1960). Other primates, monkeys for example, have been found to be very resistant to TOCP poisoning (Smith and Elvove, 1930); however, Hern (1967) observed that enzymatic inhibition of emulsified TOCP was greater than that of pure dietary TOCP alone. This suggested the lack of TOCP absorption, apparently a discrete characteristic of this particular species.

Study on the absorption of TOCP from a different perspective has appeared in recent literature especially in the area of industrial health. Increasing use of TOCP in lubricants, hydraulic fluids, as a lead scavenger in gasolines, plasticizers, solvents, flame retardants, etc. (Carpenter et al., 1959; Casida et al., 1961) has prompted study to determine the toxicity of TOCP in these forms. Balbridge et al. (1959) reported that men working in the manufacture of TOCP for nine years showed no other clinical disorders than reduced cholinesterase levels. Likewise, Carpenter et al. (1959) is in agreement with Hodges et al. (1943) in reporting that TOCP can be absorbed through the skin

and lungs. Smith (1934) reported also that TOCP diffuses slowly from the lungs after intravenous injection.

Glees et al. (1960) reported that 0.1 ml TOCP/kg (b.w.) painted on the combs of hens was absorbed in sufficient amounts to cause paralysis within 8 days. Carpenter et al. (1959) has demonstrated also that an oral administration of TOCP is 5-10 times as toxic (based on esterase inhibition) as the same dose administered by subcutaneous injection indicating rapid absorption.

Based on the evidence presented above, TOCP appears to traverse most biological membranes easily. Evidence presented below, however, indicates that even though TOCP is absorbed readily from the gastrointestinal tract, a certain amount of it is not metabolized but excreted back into the tract unchanged.

The earliest report on TOCP metabolism was presented by Gross and Grosse (1932). They observed that when TOCP was injected into rabbits, after 2 hrs., an amount of ortho-cresol equivalent to 70% of the administered dose could be recovered from the intestinal tract and 50% from the liver. Little o-cresol was found in other tissues. Smith (1934) carried this aspect one step further by ellucidating the time required for TOCP to metabolize to o-cresol. It was found that the mono-ester of o-cresol phosphate hydrolyzed within 1 hour, the diester required 3.5 hrs., and the triester had not begun to hydrolyze within 5 hrs.

Myers et al. (1955) suggested a recycling process for TOCP metabolites. He observed that 80% of the total antiesterase activity exerting 6,000 times the inhibition of the original dose was recovered from the liver and 80% from the remainder of the body. This work suggested hepatic conversion of TOCP to metabolites excreted in the bile and reabsorbed in the intestine.

Casida et al. (1961) showed that the administration of $^{32}$P-labelled TOCP resulted in rapid hydrolysis and excretion of diarylphosphates, monoaryl phosphates, and phosphoric acid. It was also observed that incubation of TOCP with rat liver microsomes fortified with DPNH yielded three esterase inhibitors.

An intensive study by Eto et al. (1962a) ellucidated the respective structures, quantities, and antiesterase activity of the three metabolites. The structure of metabolite-1 (M-1) was based on isolation via column chromatography and synthesis; for M-2 and M-3[d], infrared spectra and paper chromatography of degradation products; for M-3, derivatives formed on spontaneous degradation. Proportional antiesterase activity for 1, 2 and 3 respectively was 100:1-1.5:4. From 44.6 ml TOCP administered to 140 rats (44.6 kg total body weight) the respective quantities of the metabolites recovered was from liver: M-1, 1.2 g; M-2, 0.17 g; M-3, 0.22 g and from intestinal tissues: M-1, 18.2 g; M-2, 2.8 g; M-3, 1.7 g. Greater antiesterase specific activity was observed with all metabolites from liver

than intestine. This most likely agrees with the recycling theory of Myers et al. (1955) due to the fact that actually more total activity was present in intestinal homogenates but the greater specific activity of liver tissue suggests perhaps that a greater amount of TOCP metabolism occurs in the liver than in the intestine.

The suggested metabolism of TOCP in the liver or intestinal wall firstly involves reduction in the presence NADH to the theoretical intermediate hydroxymethyl-TOCP. This highly unstable compound immediately undergoes ring cyclization resulting in M-1 or is further reduced to M-3 which cyclizes although not as rapidly to M-2. M-1 (2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one) is the principal cyclic phosphate formed and it would be sufficient to follow it's catabolic route; M-2, M-3 are eventually catabolized to identical products.

o-Cresol is a by-product of the cyclization reaction and it's metabolism has been mentioned previously (Gross and Grosse, 1932). Work with rabbits, dogs, and rats in the metabolism of o-cresol has been reported (Bray et al., 1950). A major proportion of o-cresol (60-80%) becomes conjugated as a o-cresol glucuronide and is immediately excreted. A minor amount (15%) is excreted after combining with a sulfate group. In any case, o-cresol free or conjugated is excreted from the body rapidly (Smith, 1934).

In the presence of hydroxyl ions ($OH^-$), M-1 or M-2 may be connected to o-hydroxybenzyl alcohol e.g. salicyl alcohol (Eto et al.,

1962a, b; Eto and Oshima, 1962). This reaction involves the removal of a mono-o-cresol phosphoric acid molecule which can be further degraded to o-cresol and phosphoric acid both of which are excreted; mono-o-cresol phosphoric acid can be excreted prior to further catabolism (Casida et al., 1961). o-Hydroxybenzyl alcohol is oxidized mostly to salicylic acid (Eto, 1962; Williams, 1959, p. 320).

Salicylic acid is mostly excreted in the free form; however, Williams (1938) reported that o-hydroxybenzyl alcohol can become conjugated to an ethereal sulfate prior to oxidation. A small amount has been isolated from urine. Alpen et al. (1951) reported that 50% salicylic acid is excreted unchanged, 25% as an ether glucuronide, 10% as salicyluric acid, and 4-5% as gentisic acid. An injection of salicylic acid was totally removed by 24 hours.

The combination of M-1 with chymotrypsin results in total inhibition of this enzyme by phosphoryllation (Eto et al., 1962a). A product of this reaction, phenol, is probably conjugated to a glucuronide or sulfate and excreted (Williams, 1959, p. 279).

Since Casida et al. (1961) reported the appearance of di-aryl phosphates in urine of TOCP-treated rats, it would be logical to assume that TOCP could simply lose an o-cresol spontaneously and that the diaryl phosphate is excreted as such; the extent of this is not known.

Several other o-cresol phosphate esters have been reported to produce strong antiesterase metabolites in vivo and in vitro (Eto et al.,

1962b). Similarly, such highly active inhibitors have been found to be derived from tri-phenyl phosphate (Myers et al., 1955) and even from tri-o-chlorophenyl phosphate (Bloch and Hottinger, 1943). The similar activity of metabolites of these compounds are most likely all acting by phosphorylation of the enzyme active site and the concomitant opening of the cyclic phosphate at the P-O-C (aryl) bond (Eto et al., 1962a). Examples of this class of compounds include phosphofluoridate, sarin, TEPP, and paraoxon (Jandorf, 1956). In contrast, the para- and meta-isomers of TOCP do not produce paralysis or reduce serum cholinesterase activity in chick serum (Alderidge, 1954a) thus exemplifying the apparent specificity of the metabolic pathway of TOCP.

A summary representing the reports of several workers is compiled into a metabolic scheme (Figure 5) presented in the general discussion of part I given below.

# MATERIALS AND METHODS

Male rats of the Long-Evans strain averaging 70 80 grams of body weight were used in each of four experiments.[1] Rats were housed in individual wire-bottom cages. Temperature was maintained at 22° C. Food and distilled water were available ad libitum. In each trial, five rats were randomly allotted to each treatment group. The basal vitamin E-selenium deficient Torula yeast diet used in all the rat studies was previously reported (Cheeke, 1972).

## Rat Trial 1

The objective of this experiment was to compare the effects of selenium, vitamin E and ethoxyquin (a synthetic antioxidant) in overcoming the growth depression resulting from TOCP ingestion. Forty rats were arranged in a 2 x 4 experimental design. One-half received 0.2% TOCP. The four treatments were: basal (negative control); basal + 250 IU vitamin E (dl-α-tocopherol)/kg of diet; basal + 2 ppm selenium ($Na_2SeO_3$); and basal + 0.0125% ethoxyquin[2] (santoquin). Growth and food consumption were observed over a 45-day period.

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[1] In experiments 1, 2, and 3 the rats were obtained from Simonsen Laboratories, Gilroy, California. Rats in experiment 4 were reared (from Simonsen stock) at the Small Animal Laboratory, Oregon State University.

[2] Monsanto Co., St. Louis.

Rat Trial 2

The objective was to determine the effective levels of vitamin E or selenium required to overcome TOCP-induced growth depression. One hundred rats were randomly allotted to 20 treatment groups in a 2 x 10 arrangement. The following supplements were tested with and without 0.2% TOCP: 0.5, 2.0, and 5.0 ppm selenium ($Na_2SeO_3$); 10, 50, 100, 500, and 1000 IU vitamin E (dl-α-tocopherol)/kg of diet. The basal group served as a negative control and a 1.0 ppm selenium + 500 IU vitamin E group was a positive control. Growth and feed consumption were measured in all groups for at least 8 weeks.

Pancreatic tissue and intestinal content homogenates were prepared using the procedure of Reddy et al. (1969). Supernatants were sealed and immediately frozen. Lipase activity was measured using the electrometric titration procedure of Roe and Byler (1963) with modifications. An expanded scale Fischer pH meter was calibrated to 37° C., the desired incubation temperature. Twenty ml. of buffered substrate (olive-oil) was volumetrically pipetted into a 50 ml. beaker, placed in a 37° C. water bath, and allowed to equilibrate for five minutes before the initial pH was recorded. Two hundred μl of supernatant was then pipetted into the substrate, mixed, and incubated at 37° C. for eight minutes. During the last two minutes of incubation, titration with 0.01 N NaOH was carried out so that the solution was

returned to the original pH by ten minutes. Spontaneous hydrolysis of substrate was checked periodically. The volume of NaOH used was plotted against time over a ten minute period. Enzyme activity was calculated from micromoles of fatty acid liberated per minute. Pancreatic lipase activity was expressed as specific activity (units of activity/mg of protein); protein content of homogenates was determined using the method of Lowry et al. (1951). Intestinal lipase activity was measured from pooled supernatants using an appropriate dilution; activity was expressed as micromoles of fatty acid/gram of intestinal contents. The in vitro procedure involved the blending of TOCP into the buffered substrate to a desired concentration of $10^{-2}$ M.

Rat Trial 3

The objective was to determine the effectiveness of selenium and vitamin E when administered to rats which had ceased growing as a result of TOCP ingestion. Four groups of rats were fed the basal diet for 33 days at which time supplements of either 1.0 ppm selenium, 100 IU α-tocopherol/kg diet, or the combination was added to the diet. The resultant growth response was observed.

Rat Trial 4

Several compounds closely related to selenium were tested in a further attempt to overcome the growth depressing effect of TOCP.

Thirty rats were allotted to six treatments. Supplements to the 0.2% TOCP treated diet were: 1 ppm selenium ($Na_2SeO_3$); 10 ppm arsenic ($NaHAsO_4 \cdot 12H_2O$); 1% $Na_2SO_4$; 1% methionine; and 1% cystine. The basal group served as a negative control. Growth was observed over a 44 day period.

Quail Trial 1

The effect of selenium and vitamin E supplementation on TOCP toxicity in Japanese quail (Coturnix coturnix japonica) was examined. The quail chicks were housed in wire-bottom cages. Heat, via overhead infrared heat lamps and floor heaters, was maintained at 35° C. for the first week and reduced to 28° C. thereafter. Continuous lighting was in effect. Quail were given free access to food and tap water. The basal diet was the vitamin E-selenium deficient 60% Torula yeast diet described by Bieri et al. (1957).

Eighty day-old quail chicks without regard to sex were randomly allotted to eight treatment groups in a 2 x 4 design. Treatments included the following supplements with and without 0.1% TOCP: 0.5 ppm selenium ($Na_2SeO_3$); 20 IU dl-α-tocopherol/kg of diet; 0.5 ppm selenium + 20 IU dl-α-tocopherol/kg of diet (positive control). Basal groups served as negative controls. Growth was observed over a 17 day period. Post mortem observations were made.

# RESULTS AND DISCUSSION

## Rat Trial 1

With no supplements (negative control) and no dietary TOCP, growth ceased at 27 days (Figure 1). The animals remained in weight stasis for the remaining 18 days. Of the supplements tested, 0.0125% ethoxyquin had no apparent effect on growth; vitamin E supplemented rats grew normally, and selenium-treated animals grew linearly even though apparently suffering from slight selenium toxicity (Figure 1). The data indicate that either selenium or vitamin E initially supplemented to the necrogenic diet is adequate for normal growth as previously reported in rats (Cheeke and Shull, 1972) and in swine (Ewan et al., 1969). It has been shown (Cheeke and Shull, 1972) that the characteristic growth cessation occurring at 4-5 weeks in rats fed Torula yeast is a manifestation of inadequate vitamin E, not selenium.

The addition of 0.2% TOCP reduced growth in all groups (Table 1). The appearance of rats fed the TOCP-containing diets was characterized by roughness and unkempt appearance of the fur. No neurotoxic symptoms appeared; it has previously been suggested (Smith et al., 1932) that the rat does not display neurotoxicity symptoms when exposed to TOCP. Diarrhea, a characteristic of TOCP poisoning (Draper et al., 1952) occurred in some animals but was not correlated with growth rate. Even though TOCP is an odorless and tasteless

Figure 1. Growth Response to 250 IU Vitamin E/kg of Diet, 1 ppm Selenium, and 0.0125% Ethoxyquin All ± 0.2% Tri-o-cresyl Phosphate

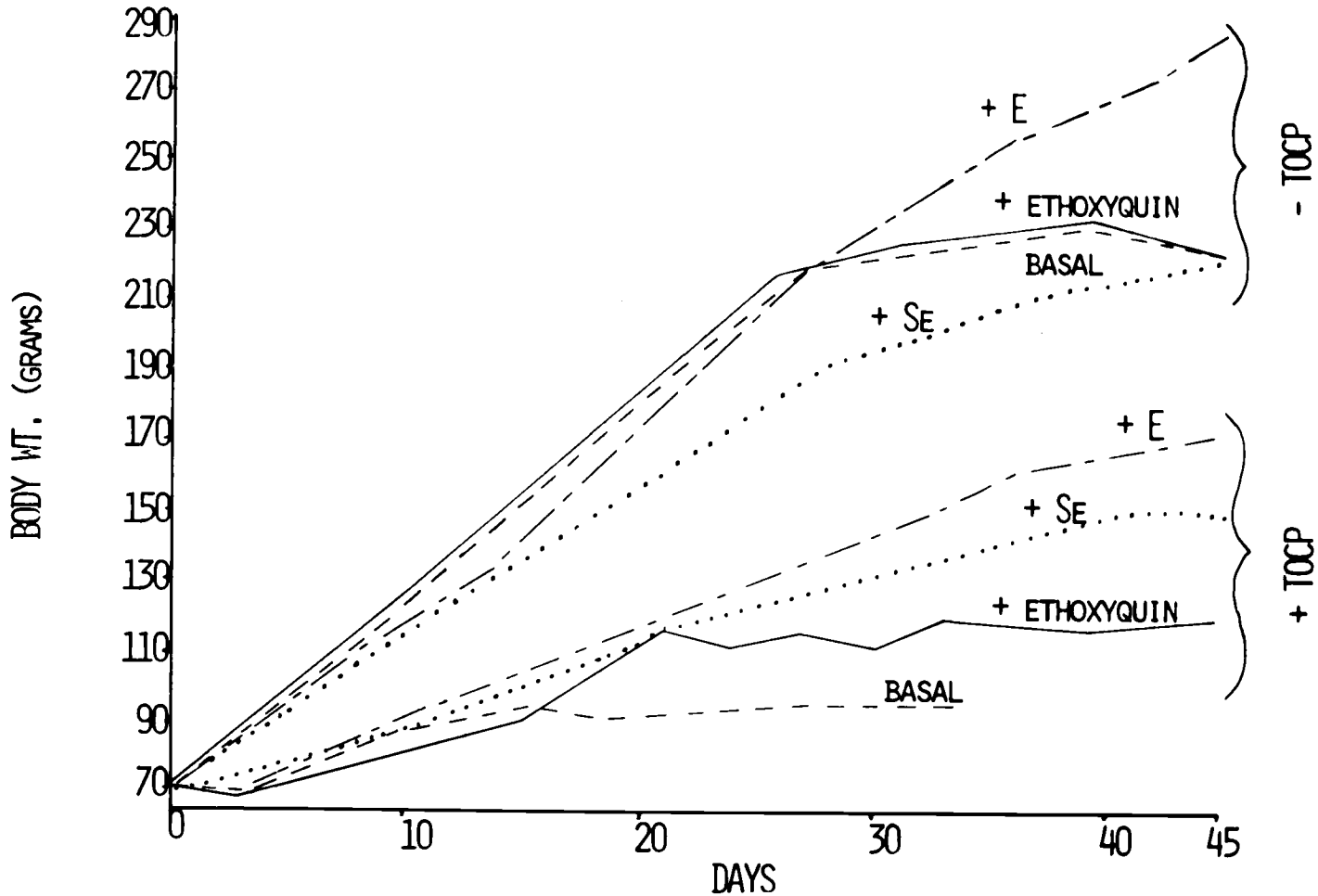

290
270
250
230
210
190
170
150
130
110
90
70
BODY WT. (GRAMS)
0
10
20
30
40
45
DAYS
+ E
+ ETHOXYQUIN
BASAL
+ SE
+ E
+ SE
+ ETHOXYQUIN
BASAL
- TOCP
+ TOCP

Table 1. Effect of Dietary Vitamin E, Selenium, or Etyoxyquin on Weight Gain and Food Intake of Rats Fed a Diet Low in Vitamin E and Selenium Either With or Without 0.2%, TOCP, Rat Trial 1.

| | | Dietary Treatments | | | |
|---|---|---|---|---|---|
| | | Basal | Vitamin E | Selenium | Ethoxyquin |
| Body wt. at 33 days (g) | - TOCP | $222^{a} \pm 12$[1] | $242^{b} \pm 8$ | $200^{c} \pm 10$ | $221^{a} \pm 19$ |
| | + TOCP | $95^{a} \pm 8$ | $151^{b} \pm 12$ | $136^{b} \pm 14$ | $117^{a} \pm 7$ |
| Body wt. at 45 days (g) | - TOCP | $219 \pm 29$ | $285 \pm 7$ | $218 \pm 14$ | $210 \pm 43$ |
| | + TOCP | ---[2] | $167 \pm 13$ | $146 \pm 18$ | $120 \pm 12$ |
| Average daily food consumed (g) | - TOCP | $14.8 \pm 1.7$ | $16.6 \pm 0.6$ | $13.4 \pm 0.5$ | $14.5 \pm 2.1$ |
| | + TOCP | $8.8 \pm 0.9$ | $12.1 \pm 1.3$ | $9.8 \pm 0.9$ | $10.4 \pm 0.6$ |

[1] Mean ± SD. Means followed by different superscripts (rows) are significantly ($P < .05$) different. Statistical comparisons were made using the l.s.d. test (Steele and Torrie, 1960).

[2] Terminated at 33 days.

compound, food consumption was severely affected as evidenced by the loss of weight in the first three days (Figure 1). The effect of TOCP was most severe in the basal rats; growth ceased at 15 days (Figure 1). Compared to the same treatments without TOCP, the terminal weights for the TOCP-fed animals were 43%, 62%, and 68% for the basal, vitamin E, and selenium-supplemented groups, respectively.

Rat Trial 2

Without TOCP, the lowest level of selenium tested, 0.5 ppm, gave maximal growth (Table 2). The highest level tested 5 ppm, was obviously toxic (Table 2), as indicated by the reduced growth. With vitamin E, a response to both 10 and 50 IU/kg of diet was apparent and the optimal level for growth probably lies between 50 and 100 IU.

With TOCP, the dietary requirement for selenium was increased (Table 2); higher dietary selenium (1.0 ppm) improved growth slightly more than 0.5 ppm, the level producing maximal growth without TOCP. In contrast, the dietary requirement for vitamin E did not appear to be significantly affected by TOCP. No improvement in growth resulted from levels beyond 50 IU. However, TOCP almost totally inhibited a response to the lowest vitamin E level (10 IU) indicating interference possibly from reduced absorption (Myers and Mulder, 1953). Comparatively, 1.0 ppm selenium improved growth

Table 2. Comparative Effects of Graded Levels of Vitamin E and Selenium on Weight Gain and Food Intake of Rats Fed a Diet Low in Vitamin E and Selenium Either With or Without 0.2% TOCP for 54 Days, Rat Trial 2.

| Treatment | - TOCP | | + TOCP | |
|---|---|---|---|---|
| | Body weight (g) | Ave. daily feed consumption (g) | Body weight (g) | Ave. daily feed consumption (g) |
| Basal | $249^{a} \pm 24$[1] | 14.3 ± 0.6 | $136^{a} \pm 10$ | 10.2 ± 0.7 |
| Basal + selenium (ppm) | | | | |
| 0.5 | $342^{b} \pm 26$ | 18.2 ± 1.4 | $195^{b} \pm 10$ | 13.1 ± 1.5 |
| 1.0 | $330^{b} \pm 43$ | 17.5 ± 1.0 | $203^{b} \pm 39$ | 11.8 ± 1.7 |
| 5.0 | $252^{a} \pm 11$ | 14.0 ± 1.4 | $150^{a} \pm 18$ | 10.1 ± 0.9 |
| Basal + vitamin E (IU/kg diet) | | | | |
| 10 | $310^{b} \pm 10$ | 18.8 ± 1.9 | $149^{a} \pm 31$ | 11.3 ± 1.7 |
| 50 | $312^{b} \pm 11$ | 19.0 ± 0.8 | $184^{b} \pm 27$ | 12.2 ± 1.4 |
| 100 | $326^{b} \pm 8$ | 17.7 ± 0.7 | $185^{b} \pm 23$ | 12.3 ± 1.6 |
| 500 | $335^{b} \pm 42$ | 18.8 ± 1.9 | $185^{b} \pm 37$ | 11.8 ± 1.4 |
| 1000 | $329^{b} \pm 8$ | 19.1 ± 1.0 | $195^{b} \pm 20$ | 12.5 ± 1.3 |
| Basal + selenium (1 ppm) + vitamin E (500 IU/kg diet) | 342 ± 18 | 18.8 ± 0.8 | $208^{b} \pm 26$ | 13.1 ± 1.3 |

[1] Mean ± SD. Means followed by different superscripts (vertically) are significantly ($P < .05$) different. Statistical comparisons were made using the l. s. d. test (Steele and Torrie, 1960).

more than any other treatment except the positive control (Figure 2). It appeared that vitamin E delayed the onset of weight loss until 60 days, whereas growth was continuous with added selenium. The effect of vitamin E may have been to "spare" selenium in some of its metabolic functions, thus delaying the onset of TOCP-induced selenium deficiency.

Compared to the same treatments without 0.2% TOCP, lipase activity was increased in all groups not receiving vitamin E except the positive control (Table 3). The largest and most significant increase in pancreatic lipase activity occurred in TOCP-treated rats fed 0.5 ppm selenium. In contrast, high vitamin E (500 IU) depressed lipase activity in the presence of TOCP. Intestinal lipase activity appeared to indicate the same basic trend; increased activity occurred in groups receiving the TOCP-selenium combinations whereas activity was generally reduced in TOCP-fed rats supplemented with graded levels of vitamin E. Generally, TOCP appeared to depress lipase activity only when rats were fed vitamin E; the opposite trend occurred when selenium was fed. TOCP added *in vitro* (emulsified with the buffered substrate) did not inhibit lipase. The data confirm earlier reports (Alderidge, 1953; 1954) that pancreatic lipase is an A-type esterase not susceptible to organophosphate inhibition either *in vivo* as reported earlier in humans and rabbits (Bloch and Hottinger, 1943), or *in vitro*.

Figure 2. Growth of TOCP-Fed Rats Receiving Supplements of Vitamin E and/or Selenium

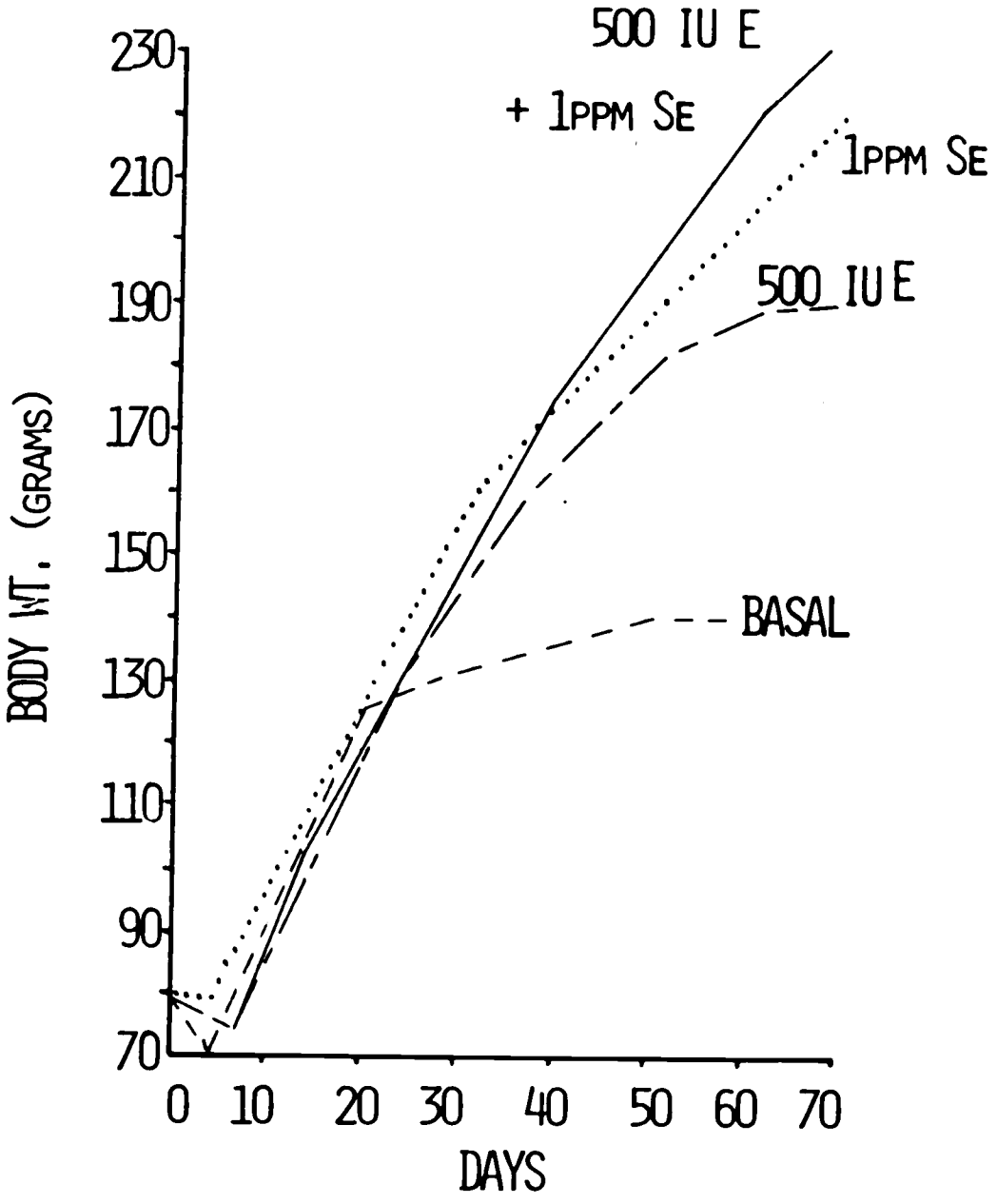

500 IU E
+ 1PPM SE
1PPM SE
500 IU E
BASAL
BODY WT. (GRAMS)
230
210
190
170
150
130
110
90
70
0
10
20
30
40
50
60
70
DAYS

Table 3. Effect of Vitamin E and/or Selenium on Lipase Activity of Pancreatic Tissue Homogenates and Intestinal Contents From Rats Treated With or Without 0.2%, Rat Trial 2.

| Treatment | Days of treatment | Pancreas (Specific activity)[1] -TOCP | p[2] | Pancreas (Specific activity)[1] +TOCP | Intestinal contents (units[1]/g contents) -TOCP | Intestinal contents (units[1]/g contents) +TOCP |
|---|---|---|---|---|---|---|
| Basal | 56 | 7.55 ± 1.56[3] | <0.1 | 10.08 ± 0.74 | 13.50[4] | 44.30 |
| Basal + selenium (ppm) | | | | | | |
| 0.5 | 69 | 11.19 ± 3.17 | <.005 | 22.91 ± 2.96 | 83.25 | 85.00 |
| 1.0 | 73 | 8.25 ± 0.85 | <.01 | 12.66 ± 2.33 | 34.90 | 108.00 |
| 5.0 | 56 | 8.57 ± 2.81 | n.s. | 12.13 ± 2.75 | 31.50 | 79.00 |
| Basal + vitamin E (IU/kg diet) | | | | | | |
| 10 | 58 | 10.67 ± 1.11 | n.s. | 10.06 ± 2.75 | 55.50 | 21.90 |
| 50 | 61 | 10.24 ± 1.09 | <0.1 | 7.37 ± 2.47 | 65.40 | 43.00 |
| 500 | 66 | 10.54 ± 1.61 | <0.1 | 6.87 ± 1.35 | 59.00 | 64.30 |
| Basal + selenium (1 ppm) + vitamin E (500 IU/kg diet) | 76 | 11.77 ± 1.92 | n.s. | 12.37 ± 3.76 | 78.30 | 62.30 |

[1] Specific activity = units/mg protein. 1 unit = micromoles of fatty acid liberated/minute.

[2] Students "t" (Steele and Torrie, 1960).

[3] Mean ± SD.

[4] Pooled supernatants.

That TOCP appeared to increase lipase activity in rats supplemented with selenium indicates that the differences are probably a reflection of effects of TOCP on the synthesis of the enzyme. Paraoxon (a trialkyl organophosphate) has been recently reported to increase protein synthesis in certain cases (Welsch and Dettbarn, 1971; Cehovic et al., 1972). Reduced pancreatic lipase activity in selenium deficient chicks has been reported (Thompson and Scott, 1970); the low selenium status in rats induced by TOCP administration in this study did not result in reduced lipase activity (Table 3). However, it would appear that the severely reduced carcass fat content characteristic of TOCP poisoning (Hove, 1953) is not a result of impaired lipase function.

Rat Trial 3

Growth of rats fed the basal necrogenic diet supplemented with 0.2% TOCP was depressed after 20 days (Figure 3). After 13 days of almost no weight gain, a positive response resulted from each of the three supplements (vitamin E, selenium, or both). However, the response to added selenium was significantly greater ($P < 0.05$) than the response to vitamin E (Table 4). Also, growth rate of both selenium supplemented groups (± vitamin E) was linear, whereas the vitamin E growth curve was curvilinear with no weight gain after 60 days (Figure 3). In a similar experiment (Cheeke and Shull, 1972), it was shown that rats fed an identical diet (without TOCP) responded only to vitamin

Figure 3. Response to Supplements of 100 IU of Vitamin E and/or 1 ppm Selenium Plus TOCP at 33 Days

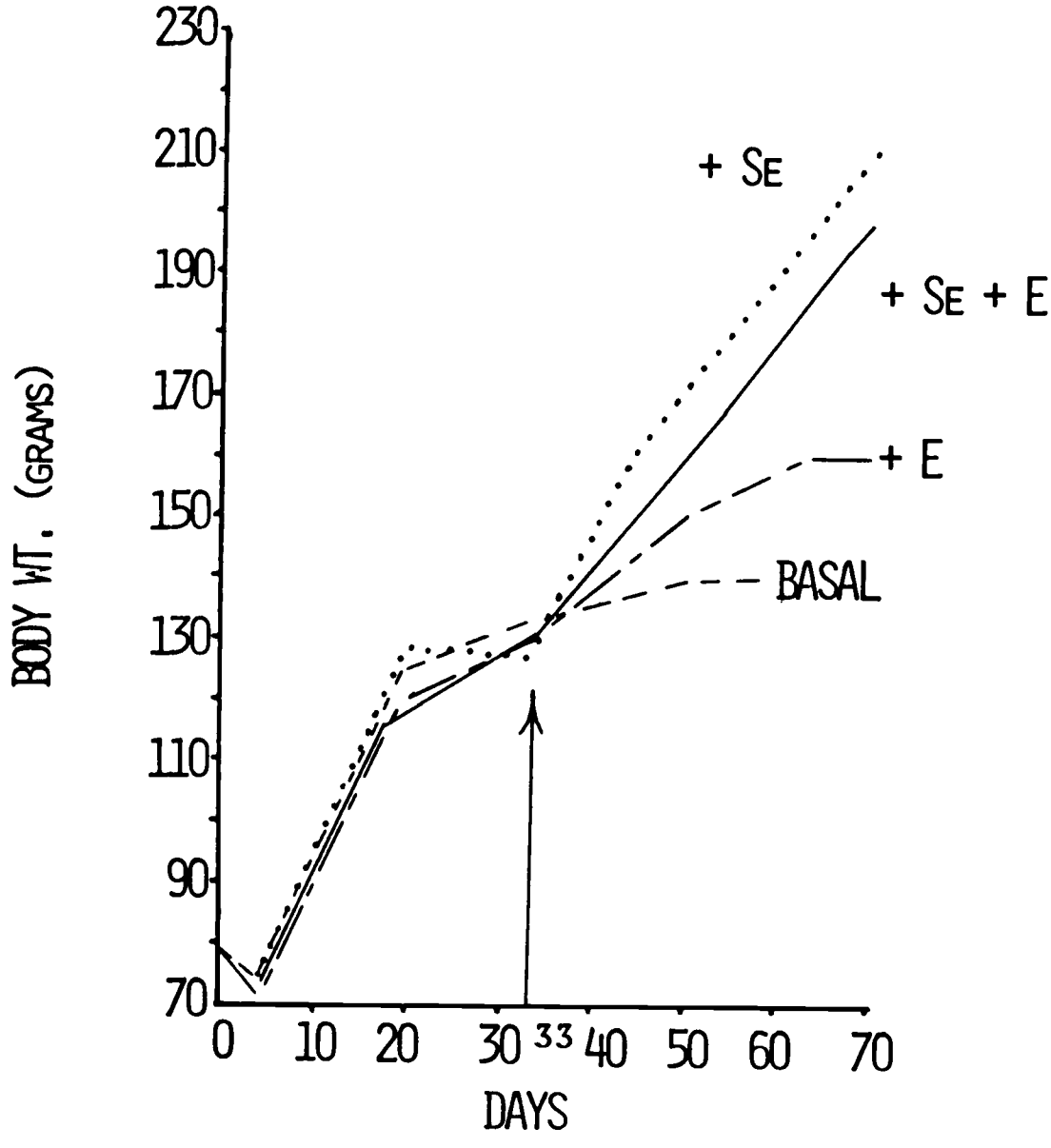

230
210
190
170
150
130
110
90
70
BODY WT. (GRAMS)
+ SE
+ SE + E
+ E
BASAL
0
10
20
30
33
40
50
60
70
DAYS

Table 4. Effect of Vitamin E and/or Selenium on Weight Gain and Food Consumption of Rats Fed the Basal Diet with 0.2% TOCP for 33 Days, Rat Trial 3.

| Group | Gain (g)/ 33 days | Average daily feed consumption (g) (33 days) | Dietary Supplement at 33 days | Gain (g)/ 37 days | Average daily feed consumption (g) (37 days) |
|---|---|---|---|---|---|
| 1 | 55.8 ± 5.4[1] | 11.1 ± 1.1 | control | -1.0 (21 days)[2] | 10.7 ± 2.0 |
| 2 | 45.0 ± 18.4 | 10.0 ± 1.0 | selenium | 87.3 ± 18.2[a] | 15.0 ± 1.3 |
| 3 | 49.0 ± 8.9 | 10.2 ± 0.4 | vitamin E | 32.0 ± 20.1[b] | 13.0 ± 0.7 |
| 4 | 49.8 ± 11.7 | 10.0 ± 0.5 | selenium + vitamin E | 69.5 ± 25.2[a] | 14.6 ± 1.8 |

[1] Mean ± SD. Means followed by different superscripts are significantly ($P < .05$) different.

[2] The negative control was not included in the statistical analysis (l. s. d. test; Steele and Torrie, 1960) due to earlier termination.

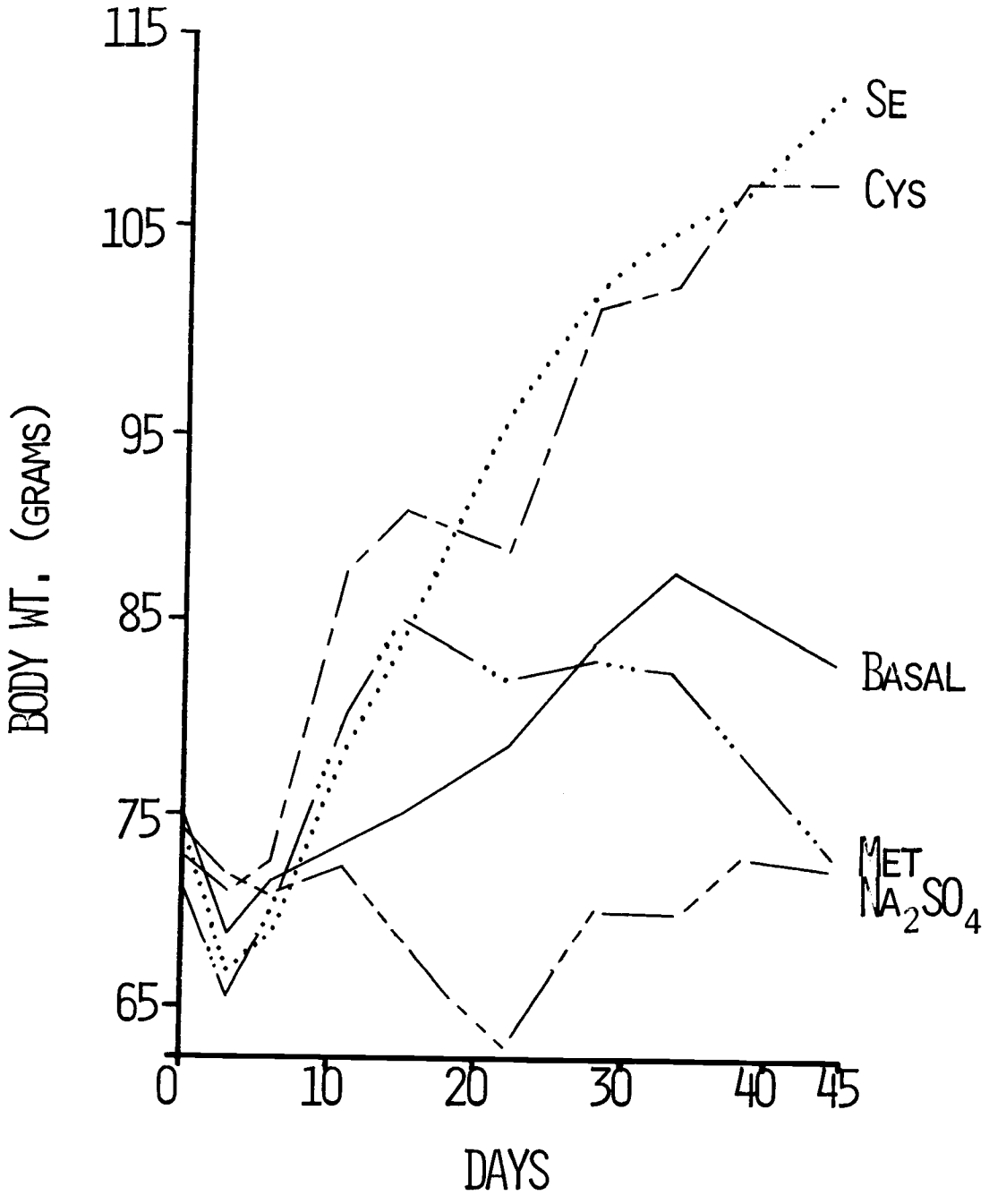
E, not selenium, once growth had ceased indicating a more critical need for vitamin E. The need for selenium in a diet containing adequate vitamin E has not been shown until the second generation (McCoy and Weswig, 1969) at which time a growth response to seleniumwas noted (Whanger and Weswig, 1970; Hurt et al., 1971). Similarly, low weight gain in cattle and sheep fed low-selenium forages is improved only with selenium, not vitamin E (Hartley, 1967, p. 79). Therefore, the findings of this experiment suggest that TOCP rapidly depletes body selenium stores normally used to maintain growth.

Rat Trial 4

Of the two sulfur-containing amino acids tested, 1% cystine was comparable to 1 ppm selenium in improving growth (Figure 4). However, two of the five rats fed cystine died of lung hemorrhage whereas no deaths occurred in the selenium-fed group. Growth was depressed with added methionine after 15 days (Figure 4). Four rats of this group died of liver necrosis between 33 and 42 days. Growth of the negative control group was generally retarded after 12 days; two of the five rats died, one of liver necrosis at 19 days and the other of both liver necrosis and lung hemorrhage at 33 days. All groups suffered from diarrhea induced by TOCP.

When rats were fed either 10 ppm arsenic or 1% $Na_2SO_4$, no improvement in growth resulted (Figure 4). In the case of $Na_2SO_4$,

Figure 4. Growth of TOCP-Fed Rats Supplemented With 1% Cystine, 1% Methionine, 1 ppm Selenium, or 1% $Na_2SO_4$


115
105
95
85
75
65
BODY WT. (GRAMS)
0
10
20
30
40
45
DAYS
SE
CYS
BASAL
MET
NA2SO4

growth was severely depressed and 80% mortality had occurred by 28 days.

Quail Trial 1

Quail fed the basal diet (low vitamin E and selenium) without 0.1% TOCP showed reduced growth by nine days (Table 5) and between nine and 17 days, 80% mortality occurred. Similarly, 80-100% mortality (exudative diathesis) occurred in chicks fed an identical diet between 14 and 18 days (Scott et al., 1955; Bieri et al., 1957). In the quail, small petechial hemorrhages located in the region of the ventral neck and anterior portion of the breast muscle were observed in some birds post mortem. This form of exudative diathesis has been described in quail (Scott and Thompson, 1968, p. 1). An, as yet, unidentified antioxidant in Torula yeast (Cheeke, 1972) which has been reported to protect chicks from encephalomalacia (Sondergaard et al., 1962) apparently was effective in the quail since no encephalomalacia was observed. Of the treatments tested, the addition of selenium and vitamin E together was more effective than either alone (Table 5). In comparison, selenium alone was significantly more effective than vitamin E alone as evidenced by improved growth and reduced mortality (Table 5) in accordance with other reports on chicks (Thompson and Scott, 1967a, p. 130; 1967b) and quail (Thompson and Scott, 1967a, p. 130). Some growth improvement and reduced mortality was attributed

Table 5. Effect of Vitamin E and/or Selenium on Weight Gain and Mortality of Japanese Quail Fed a 60% Torula Yeast Diet With and Without 0.1% TOCP.

| Treatment | Weight gain,[1] g | | | | Final body weight,[2] g | Percent mortality |
|---|---|---|---|---|---|---|
| | 0-5 days | 5-9 days | 9-13 days | 13-17 days | | |
| Basal | 7.0 (1) | 5.5 (0) | 3.5 (5) | ... (3) | 24.5 (15 days) | 90 |
| Basal + Se | 6.9 (0) | 6.3 (0) | 10.3 (0) | 7.9 (0) | $37.5 \pm 5.2^{ab}$ | 0 |
| Basal + vit. E | 7.1 (1) | 5.4 (0) | 7.9 (0) | 6.8 (4) | $32.6 \pm 5.6^{a}$ | 50 |
| Basal + Se + vit. E | 7.3 (2) | 6.7 (0) | 9.1 (0) | 11.5 (0) | $40.6 \pm 4.3^{b}$ | 20 |
| Basal + TOCP | 4.5 (1) | 1.8 (2) | 1.1 (5) | ... (1) | 13.0 (13 days) | 90 |
| Basal + Se + TOCP | 4.9 (0) | 4.4 (2) | 5.4 (0) | 7.5 (1) | $28.4 \pm 4.7^{a}$ | 30 |
| Basal + vit. E + TOCP | 4.9 (3) | 2.8 (1) | 2.5 (0) | 4.6 (1) | $21.0 \pm 3.3^{b}$ | 50 |
| Basal + vit. E + Se + TOCP | 4.4 (1) | 3.6 (0) | 4.3 (0) | 6.8 (0) | $24.9 \pm 6.2^{ab}$ | 10 |

[1] Number of mortalities in parentheses.

[2] Mean $\pm$ SD. Means followed (vertically) by different superscripts are significantly ($P < 0.05$) different; statistical comparisons were made using the l.s.d. test (Steele and Torrie, 1960).

to the presence of vitamin E. It has been proposed that vitamin E merely spares the selenium requirements in chicks for prevention of exudative diathesis (Scott et al., 1969, p. 321) and for growth (Underwood, 1871, p. 345).

The addition of 0.1% TOCP resulted in reduced growth in all treatment groups (Table 5). No neurotoxic symptoms appeared; chicks under eight weeks of age are insensitive to the neurotoxic action of organophosphorus compounds (Bondy et al., 1950; Barnes and Denz, 1953). Diarrhea occurred shortly after the trials had begun and the birds generally appeared ungroomed throughout the experiment. The basal group receiving TOCP exhibited almost complete growth cessation by five days. Between five and 13 days, 70% mortality occurred; subcutaneous hemorrhages were visible as in the basal group without TOCP. The addition of 0.5 ppm selenium and/or 20 IU α-tocopherol/kg diet greatly improved growth and reduced mortality (Table 5). However, the growth response to selenium alone was much greater than to vitamin E alone indicating as in rats an antagonistic effect to selenium by TOCP.

# GENERAL DISCUSSION

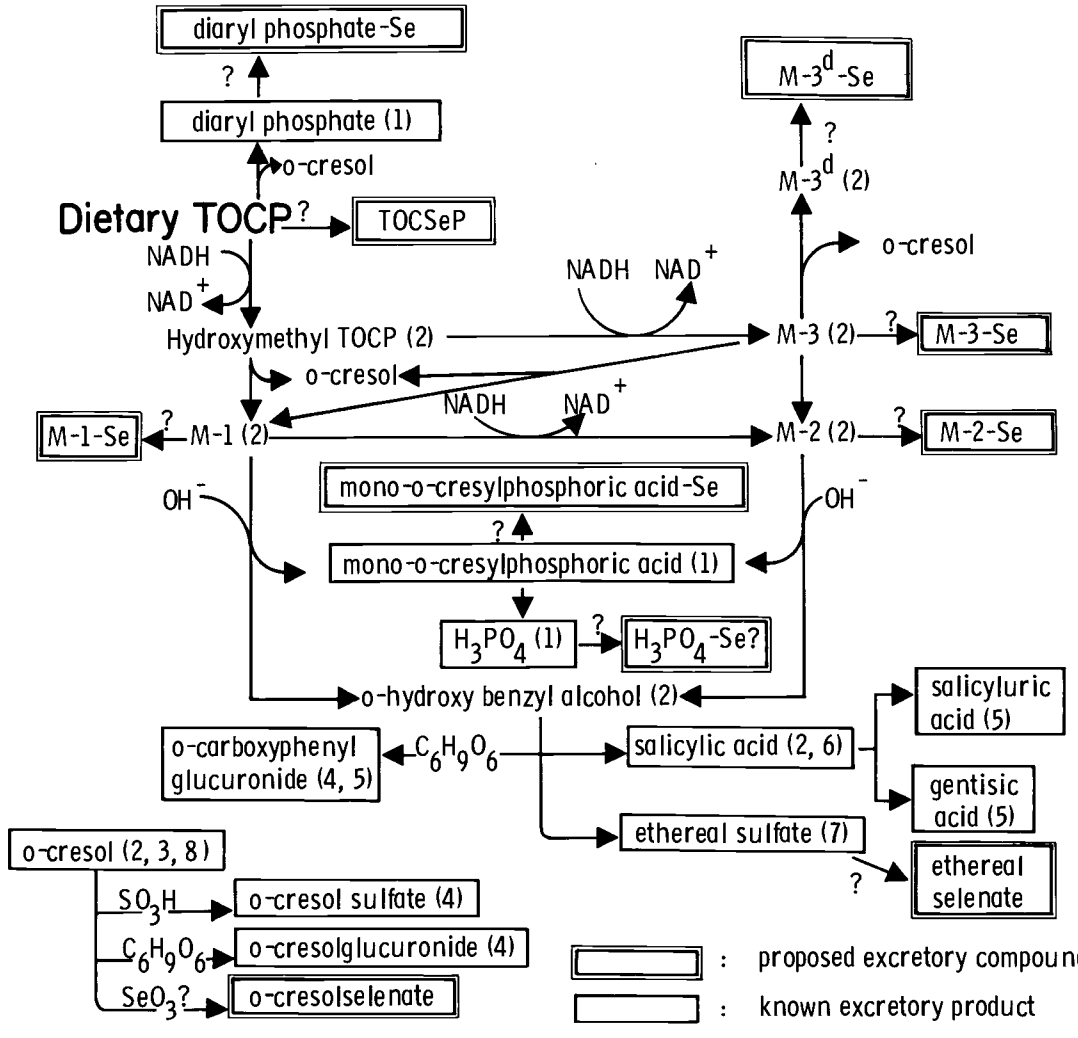
The antivitamin E activity of organophosphates has been questioned (Myers and Mulder, 1953; Cowlishaw and Blaxter, 1955; Ferrando, 1956; Green and Bunyan, 1969; Goyer et al., 1970). Some have proposed that TOCP merely reduces the absorption of vitamin E from the intestinal tract thereby accounting for the lowered blood level of vitamin E characteristic of TOCP poisoning (Myers and Mulder, 1953; Cowlishaw and Blaxter, 1955). Our findings indicate that the administration of TOCP significantly increases the selenium requirement for growth whereas the requirement for vitamin E is affected to a much lesser degree. In TOCP-treated rats, growth was improved with a level of selenium of about ten times the normal requirement. In contrast, increased dietary vitamin E as high as about 20 times the normal level (50 IU/kg of diet) produced no additional benefit, thus confirming other reports (Draper et al., 1952; Hove, 1953; Hove, 1955). However, vitamin E alone did appear to delay the onset of weight loss suggesting a sparing of selenium. Similarly, in TOCP-treated quail receiving no dietary vitamin E or selenium, growth was immediately impaired; supplementation with selenium improved growth and reduced mortality much more effectively that did vitamin E. Finally, in rats, growth cessation induced by feeding TOCP was reversed by supplemental selenium; vitamin E was much less effective.

These data are interpreted to indicate that an antagonism of selenium by TOCP occurs.

To account for a TOCP-Se interaction, one might postulate the formation of one or more compounds composed of selenium and a metabolic product of TOCP (Figure 5). The phosphorus-containing excretory fraction is reportedly composed of diaryl phosphates, monoaryl phosphates, and phosphoric acid (Casida et al., 1961) and the major non-phosphoric excretory products are o-cresol (Gross and Grosse, 1932; Smith, 1934; Eto et al., 1962a) and salicylic acid (Eto et al., 1962a). With the possible exception of salicylic acid (Williams 1959, p. 360), the existence of three selenium-containing compounds is possible. First, the formation of a seleno-phosphorus compound is suggested. Theoretically, formation could occur at any point along the known (Figure 5) catabolic pathway. Because TOCP is absorbed readily (Gross and Grosse, 1932; Mendel and Rudney, 1944; Casida et al., 1961), metabolized rapidly (Gross and Grosse, 1932; Smith, 1934; Casida et al., 1961; Eto et al., 1962a) and presumably taken up by most body tissues with ease, a selenophosphorus excretory compound eventually would deplete body selenium stores. TOCSP, a thiophosphate, has been reported to contain no neurotoxic properties (Smith et al., 1932), and is not metabolized to TOCP (Buttar et al., 1968). Similarly, the formation of a thiophosphate may account for the positive response to cystine observed in the present study.

Figure 5. Metabolism of TOCP and Possible Sites of Selenium Interaction

[1]Casida et al., 1961; [2]Eto et al., 1962a; [3]Gross and Grosse, 1932; [4]Bray et al., 1950; [5]Alpen et al., 1951; [6]Williams, 1959, p 320; Williams, 1938; [8]Smith et al., 1934.


diaryl phosphate-Se
?
diaryl phosphate (1)
o-cresol
Dietary TOCP ?
TOCSeP
NADH
NAD+
Hydroxymethyl TOCP (2)
o-cresol
M-1-Se
?
M-1 (2)
OH-
mono-o-cresylphosphoric acid-Se
?
mono-o-cresylphosphoric acid (1)
H3PO4 (1)
?
H3PO4-Se?
o-hydroxy benzyl alcohol (2)
o-carboxyphenyl glucuronide (4, 5)
C6H9O6
salicylic acid (2, 6)
salicyluric acid (5)
gentisic acid (5)
ethereal sulfate (7)
ethereal selenate
o-cresol (2, 3, 8)
SO3H
o-cresol sulfate (4)
C6H9O6
o-cresolglucuronide (4)
SeO3?
o-cresolselenate
NADH NAD+
M-3d-Se
M-3d (2)
o-cresol
M-3 (2)
M-3-Se
NADH NAD+
M-2 (2)
M-2-Se
OH-
: proposed excretory compound
: known excretory product

Secondly, within the non-phosphoric fraction, selenium might conceivably be excreted combined to o-cresol. Bray et al. (1950) showed that o-cresol is mostly excreted conjugated with a glucuronide (72%) and a sulfate (15%) in rabbits (Figure 5). Even if a very small amount of selenium became conjugated to o-cresol, the result of continual TOCP consumption and o-cresol excretion might eventually create a selenium deficient state. o-Cresol traverses biological membranes readily and is excreted rapidly (Smith et al., 1932). Thirdly, from o-hydroxybenzyl alcohol (Figure 5) is formed primarily salicylic acid (Eto et al., 1962a) but also an ethereal sulfate has been identified (Williams, 1938). Westfall and Smith (1941) have demonstrated that rabbits fed seleniferous wheat excreted selenium in combination with ethereal sulfate and the neutral sulfur fraction of urine.

The effect of TOCP on the apparent depletion of selenium as suggested by the present findings may involve any one or all of the above mentioned possibilities. More work is needed to identify the mechanism by which TOCP antagonized selenium.

# PART II: THE AFFECT OF TRI-O-CRESYL PHOSPHATE ON THE ANTI-HEMOLYTIC ACTIVITY OF DIETARY VITAMIN E AND SELENIUM

## INTRODUCTION

Until recently, selenium (Se) has not been considered to be effective in preventing in vitro erythrocyte hemolysis. Rotruck et al. (1971, 1972) has demonstrated a glucose-dependent protective action of dietary selenium against autohemolysis and oxidation of hemoglobin. They have suggested that selenium is involved with the function of reduced glutathione in the maintenance of cell integrity. This finding explained the results of an earlier study (Krishnamurthy and Bieri, 1961) in which protection against dialuric acid-induced hemolysis of vitamin E-deficient red blood cells from selenium-supplemented rats within the first 15 minutes of a one-hour incubation period was observed. The fact that protection lasted no longer than 15 minutes was apparently due to the exhaustion of the cellular glucose supply.

Tri-o-cresyl phosphate (TOCP) has been shown to increase in vitro hemolysis in vitamin E-deficient rats (Draper et al., 1952), suggested to reduce the intestinal absorption of vitamin E (Myers and Mulder, 1953; Cowlishaw and Blaxter, 1955), and believed to interfere with the utilization of selenium (see Part I). The present study was concerned with the interaction of selenium and glucose in the

prevention of hemolysis *in vitro* and the influence of TOCP on the anti-hemolytic function of dietary selenium and vitamin E.

# LITERATURE REVIEW

## Vitamin E and Red Blood Cell Hemolysis

Gyorgy and Rose (1948) first reported that rats fed a vitamin E deficient diet following an injection of alloxan underwent *in vivo* erythrocyte hemolysis, resulting in death. However, when dietary α-tocopherol was included there was complete protection. Later, it was demonstrated that injection of the reduction products of alloxan or dialuric acid) gave the same result (Gyŏrgy and Rose, 1949). *In vitro*, a similar reaction could be produced with washed red cells incubated with dialuric acid or alloxantin but not alloxan (Gyŏrgy and Rose, 1949; Rose and Gyorgy, 1950a). α-Tocopherol again was completely protective.

Reports on the occurrence of hemolysis with respect to species are few. Rose and Gyŏrgy (1952a) showed that rats are susceptible to not only alloxantin and dialuric acid but also to dilute solutions of hydrogen peroxide. Rose and Gyŏrgy (1952a) also demonstrated that dialuric acid was only partially effective as a hemolytic agent in human erythrocytes; hydrogen peroxide was more effective.

Christensen *et al.* (1956) reported no apparent correlation between the degree of hemolysis and the length of time chickens were fed a vitamin E-deficient diet. Also, no correlation between hemolysis and the incidence of encephalomalacia was noted. It was suggested

that hemolysis could not be used as an indication of a vitamin E deficiency in chickens.

The apparent correlation between dietary tocopherol and the susceptibility of cells to hemolyze has led to the development of a bioassay for vitamin E. Rose and György (1950b, 1952a) first suggested qualitative and quantitative procedures for determining an animals requirement based on hemolysis. Friedman et al. (1958) later refined the method and increased it's quantitative capacity by adapting the procedure to a spectrophotometer. Bunnel (1969) found a good correlation between the hemolysis test and the gestation resorption bioassay. The comparatively simple procedure and earlier detection of a vitamin E-deficiency renders the hemolysis test more practical.

Draper and Csallany (1969) have proposed a bioassay procedure based on the susceptibility of vitamin E-deficiency cells to hemolyze spontaneously at 37° C. This procedure utilizes no hemolytic agent such as dialuric acid but relies on the oxidizing capacity of atmospheric oxygen. Christensen et al. (1956) noted that by increasing the temperature to 37° C. spontaneous hemolysis was accelerated.

Draper and Csallamy (1969) compared the spontaneous test to the dialuric acid procedure of Friedman et al. (1958) and suggested that the former contained such advantages as sensitivity, simplicity, and reproduceability. One obvious disadvantage of the spontaneous test is the rather long incubation time required (4-24 hrs.).

The hemolysis bioassay has been used to determine the minimal vitamin E requirement. The criterion for this analysis has been the least amount of α-tocopherol required to prevent hemolysis (Rose and György, 1950b; Ward, 1963, Jager, 1968). Ward (1963) also used the hemolysis test to establish that male rats have a higher vitamin E requirement than females.

Ahmed (1957) and Ward (1963) have compared the levels of -tocopherol required to prevent in vitro hemolysis with the levels needed to prevent other vitamin E-deficiency manifestations. They reported that, compared to all the other symptoms tested e.g. feotal resorption, reduction of hepatic vitamin A, kidney histolysis, uterine pigmentation, testicular degeneration, and hemolysis, only from one sixth to one-third of the amount of α-tocopherol was needed to prevent in vitro hemolysis.

Other workers have used the test to determine the relative anti-hemolytic activity of various compounds (Rose and György, 1952a; Moore and Sharman, 1959; Bunyan et al., 1960) as well as the hemolytic effects of others (Rose and György, 1950a; 1952a; Allen and Jandl, 1961; Cohen and Hochstein, 1964; Jacob and Jandl, 1966). The test has also been used to demonstrate the susceptibility of human infant erythrocytes to hemolyze (Rose et al., 1962b) and the influence of various dietary components such as PUFA (Bieri and Poukka, 1970). Similarly, the pathology of various diseases has been demonstrated

using the hemolysis assay e.g. hereditary spherocytosis (Jacob and Jandl, 1964), acanthocytosis (Silber and Kayden, 1965), G-6-P dehydrogenase deficiency (Cohen and Hochstein, 1961), and hereditary acatalasia (Jacob et al., 1965).

Hemolysis per se has been known for a long time. It is simple to evoke complete red cell destruction by changing the isotonicity of the bathing medium e.g. adding water with erythrocytes. However, the hemolysis reported by Györgyi and Rose (1948) that occurs with vitamin E deficient erythrocytes in the presence of an oxidizing agent is not simply due to osmotic effects.

The most widely accepted theory on the occurrence of hemolysis relates the generation of lipid peroxides to the destruction of the erythrocyte membrane. Blum (1930a) demonstrated the effect of eosine and other fluorescent dyes and first suggested the involvement of an oxidative process. Blum (1930b) also reported that hydrogen peroxide contained hemolytic activity. Although the different studies were made on normal blood cells (not vitamin E-deficient) the effect and suggestion were apparently accurate.

Rose and Györgyi (1952a) showed that a low level of hydrogen peroxide would hemolyze vitamin E-deficient cells but not vitamin E supplemented cells. With dialuric acid, added catalase was found to reduce the degree of hemolysis suggesting dialuric acid produces hydrogen peroxide even in vitro. Hydrogen peroxide was not implicated

as the hemolyzing agent because of its slower effect compared to dialuric acid; however, it was suggested to be closely related to the hemolytic mechanism.

Dam (1949) first reported that lipid peroxides increased in body lipids of vitamin E deficient animals. Later, Hove (1955) proposed that all vitamin E deficiency complications could be related to the destructive nature of lipid peroxides formed freely without adequate vitamin E. With respect to the red cell, Horwitt et al. (1956) confirmed this theory by showing that vitamin E-deficient cells treated with hydrogen peroxide contained a much higher level of lipid peroxides in the stromata than vitamin E-supplemented cells; vitamin E added in vitro to the deficient cells markedly reduced the levels of lipid peroxides. Similarly, Tsen and Collier (1960) demonstrated that dialuric acid acted by catalyzing the formation of lipid peroxides (measured by TBA) in the unsaturated lipid fraction of the membrane. Added α-tocopherol inhibited their formation and no hemolysis was observed. Tappel and Zalkins (1959, 1960) showed that vitamin E directly inhibited the formation of lipid peroxides and suggested a mechanism for this function.

Further confirming the role of oxidation in hemolysis was the finding that oxygen gas could induce hemolysis. Raiha (1955) first noted the protective nature of α-tocopherol against hemolysis of human erythrocytes incubated with oxygen. Christensen et al. (1956)

demonstrated that $O_2$ (instead of an oxidizing agent such as dialuric acid) caused spontaneous hemolysis since none occurred when $N_2$ was bubbled into the red blood cell suspension. By increasing the $O_2$ pressure, Taylor et al. (1956, 1958) found rats fed a vitamin E-deficient diet were even susceptible to in vivo hemolysis; vitamin E supplementation was protective. Later, Taylor and Wiseman (1962) reported that vitamin E deficient cells consumed twice the oxygen as normal cells. It was concluded that this phenomenon represented uninhibited peroxide formation. Tsen and Collier (1960) earlier had demonstrated that the degree of hemolysis of vitamin E deficient cells in an atmosphere of pure oxygen paralleled the formation of lipid peroxides. In humans, Mengel et al. (1965) reported a case of hemolytic anemia induced by "hyperbaric oxygenation."

Vitamin E is thought to control the incidence of lipid peroxides by acting as an antioxidant. Tappel (1962b, p. 367) suggested that the free radicals, including the lipid radical R', lipid peroxy radical ROO', lipid-oxy radical RO', hydroxyl radical OH' and others, result from the cleavage of unsaturated fatty acid chains. The damaging effect of such free radicals would go unchecked without the scavenging ability of vitamin E. Tappel (1962a) also postulated that the antioxidant capacity of tocopherol could be greatly enhanced by the formation of redox couples involving ascorbate and glutathione.

Other forms of tocopherol have been tested against hemolysis. Their potencies differ depending on administration in vivo or in vitro. In vivo, alpha-tocopherol is the most effective followed by beta, gamma, etc. However, in vitro, the opposite occurs; gamma, beta, then alpha, is the order of potency (Ross and Györgyy, 1952a; Bunyan et al., 1960; Bunnel, 1969; Peake and Bieri, 1971). The reduced potency of the dimethylated forms (Beta, Gamma) in vivo has been associated with reduced intestinal absorption of these forms (Scott and Desai, 1964). Peake and Bieri (1971) showed that red blood cells prefer the gamma- form; however, it's disappearance from body tissues is more rapid compared to the alpha form. One theory proposed by Voth and Miller (1958) as to the in vivo effect relates potency to the degree of methylation and the subsequent ability of each tocopherol type to form hydrogen bonds with membrane proteins. Bunyan et al. (1960) demonstrated more specifically the depressive effect of the $C_8$ methyl group and concluded that the explanation proposed by Voth and Miller (1958) was insufficient.

Various synthetic antioxidants have been shown to substitute for vitamin E in preventing specific vitamin E deficiency symptoms. With respect to hemolysis, Dam et al. (1957) and Christensen et al. (1956) showed that dietary methylene blue (MB) partially prevented in vitro dialuric acid induced hemolysis of vitamin E-deficient cells. In contrast, Moore (1956) reported no response to MB. Similarly,

Friedman et al. (1958) demonstrated no response to MB (10 mg) when given orally and Bunyan et al. (1960) could show no response to 50 mg MB orally; only larger doses of MB were effective in reducing the degree of hemolysis.

NN'-diphenyl-p-phenylenediamine (DPPD) probably the most potent synthetic antioxidant available (Green and Bunyan, 1969) has been generally effective against hemolysis (Moore and Sharman, 1959; Draper and Csallany, 1958). However, much larger doses compared to the effective dose of α-tocopherol are apparently required. In fact, Bunyan et al. (1960) determined that DPPD is only 3% as potent as α-tocopherol. Similarly, Friedman et al. (1958) reported no effect of DPPD using a dose equivalent to tocopherol in weight, not potency.

As previously mentioned, oxidizing agents e.g. dialuric acid, hydrogen peroxide, or atmospheric oxygen, promote the formation of lipid peroxides or free radicals that inflict membrane damage to the point of total cell disruption. Actually, the membrane is normally doubly protected. Firstly, hydrogen peroxide is generated endogenously but it's cellular concentration is controlled by two detoxifying enzyme systems, catalase and glutathione peroxidase. Secondly, hydrogen peroxide not detoxified is kept from attacking unsaturated bonds and forming lipid peroxides by vitamin E. Thus, overloading the enzyme system in the absence of vitamin E should and does result in membrane destruction.

Different ways of overloading the detoxifying system have been reported. Besides adding dialuric acid or hydrogen peroxide directly, Cohen and Hochstein (1964) and Jacob et al. (1965) demonstrated that hydrogen peroxide was generated in vivo by various "oxidant drugs." Jacob and Jandl (1966) reported the ability of various "endogenous substrates" such as thyroxine, ascorbic acid, and uric acid to produce hydrogen peroxide intracellularly. Rose and Györgi (1950a) had earlier reported on the hemolytic effect of cysteine, glutathione, and ascorbic acid in vitro when added with vitamin E-deficient cells.

The hemolytic process was associated with membrane deterioration since no intracellular abnormalities prior to or during hemolysis could be shown (Jacob et al., 1966). Membrane destruction and hemolysis is now associated with the depletion of two membrane components, phosphatidyl ethanolamine (PE) and various sulfhydryl compounds.

Jacob and Lux (1968) first demonstrated the loss of PE in the membranes of vitamin E-deficient cells hemolyzed by $H_2O_2$. Ways and Hanahan (1964) had earlier reported that the unsaturated lipid fraction of erythrocytes was mostly phosphotidyl ethanolamine and DeGier and VanDeenen (1961) reported that PE madeup a large percentage of the total phospholipid fraction. However, Dodge et al. (1967) demonstrated the destruction of another phospholipid by hydrogen peroxide in vitamin E-deficient cells, phosphotidyl serine.

Jacob and Lux (1968) listed four points of evidence that hemolysis could result from PE depletion: 1) a large loss of PE is noted just prior to hemolysis; 2) by enhancing the loss of PE with fluorides or sulfhydryl inhibitors, hemolysis is accelerated; 3) young red cells which are shown to maintain more phospholipids are less susceptible to $H_2O_2$ than mature cells and 4) in vitamin E-deficient cells, phospholipase A which deacylates phosphatides will accelerate $H_2O_2$-hemolysis. From this evidence, it was suggested that the actual hemolytic mechanism was the initial degradation of PE sequences leaving large gaps in the membrane with no lipid fraction; the membrane disorganization results in the outward diffusion of hemoglobin and eventually to total cell disruption.

Hove (1955) first suggested that the various vitamin E-deficiency symptoms might be due to the destructive action of peroxides on sulfhydryl sensitive enzyme systems. Jacob and Jandl (1962) associated two conditions both susceptible to oxidative hemolysis e.g. Heinz-body anemia and cell aging, to a loss of membrane sulfhydryl activity. It was shown that by inhibiting membrane sulfhydryl activity *in vitro*, osmotic hemolysis resulted.

The protective nature of thiol compounds against a number of vitamin E-deficiency conditions has been reported. For instance, cystine and methionine exert a strong beneficial effect on liver necrosis in rats (Daft *et al.*, 1942) and muscular dystrophy in chickens

(Nesheim et al., 1960). Similarly, glutathione (GSH) protects chickens from muscular dystrophy (Hatchcock and Scott, 1967) and hyperoxygenated rats from hemolytic anemia (Taylor, 1958). The chemical nature of the sulfhydryl compounds apparently accounts for their protective abilities e.g. by adding across the double bonds of unsaturated lipids in an "unstable chemical union" (Robinson, 1965).

Peroxidized lipids have been shown to oxidize sulfhydryl compounds in vitro including GSH (Lewis and Wills, 1962; O'Brien and Little, 1967). The role of GSH in protecting cellular enzyme systems is well known (Knox, 1959, p. 253). Finally, Jacob and Lux (1968) quantitatively demonstrated the destruction of membrane sulfhydryls in vitamin E-deficient red blood cells exposed to hydrogen peroxide; with supplemented vitamin E, no destruction was observed. It was suggested that the depletion of sulfhydryls may lead to either increased permeability and ultimately to "colloid osmotic hemolysis" or to peroxidative cleavage resulting in membrane destruction and hemolysis.

At present, it is not known whether it is the depletion of PE or sulfhydryl compounds which actually causes hemolysis to occur. Jacob and Lux (1968) in their report suggested it was a combination of the two, but tended to believe the depletion of membrane PE was the more critical.

Probably the most recent demonstration of a need for vitamin E in the life of the red blood cell involves erythrocyte synthesis or

erythropoesis. Porter and Fitch (1966) reported that heme synthesis was significantly impaired in vitamin E deficient monkeys. Apparently, delta-aminolaevulinic acid was inadequately synthesized; administration of vitamin E or coenzyme Q-10 corrected the insufficiency. Ludwig et al. (1967) observed a similar response to vitamin E in vitamin E-deficient rabbits. Carpenter (1967) demonstrated reduced porphyrin synthesis in vitamin E deficient rats by showing reduced activity of the porphyrin containing $P_{450}$ drug metabolizing system e.g. demethylation of codine was significantly reduced.

Murty et al. (1970) described the actual point of the defect by demonstrating the loss of enzymic activity of delta-aminolaevulinic acid synthetase and dehydratase; both enzymes are critical to porphyrin synthesis.

Several reports have appeared relating vitamin E with dietary protein in erythropoesis. Whitaker et al. (1967) reported that anemia in Thai children suffering from protein-calorie malnutrition was cured by vitamin E and protein, but not protein alone. Earlier, Moore (1949) observed anemia in rats receiving low casein, low vitamin E diets but anemia was not observed in rats receiving vitamin E or adequate casein; although the RBC count was low, hemoglobin level was not abnormal. Aterman et al. (1963) observed that vitamin E would prevent anemia induced by an unspecified yeast diet. Lazier and Beveridge (1964) reported that 12-20% dietary Torula yeast was

comparable to a combination of supplements including selenium, vitamin E, methionine, and cystine. Anemia was not found to occur in vitamin E-deficient rats given a normal level of protein (Dining et al., 1954). Bencze et al. (1966) reported that vitamin E raised the very low hemoglobin (70% loss) levels of rats given a vitamin E-deficient, low protein diet with no apparent effect on erythrocyte count. Bunyan et al. (1968) could not confirm the work of Bencze et al. (1966) and suggested that the severe anemia observed may have been due to factors other than the depletion of protein and vitamin E.

Recent work by Nair et al. (1970) has suggested a role for vitamin E in regulating protein synthesis. Vitamin E was implicated in preventing the induction of two enzymes when experimental porphyria was chemically induced. It was suggested that vitamin E plays a regulatory role in protein synthesis during porphyrin production. To date, this phenomenon has not been completely substantiated. However, it becomes apparent that vitamin E may not only function in maintenance and integrity of red cell membranes but also specifically in their genesis.

Selenium and Red Blood Cell Hemolysis

As previously indicated, selenium is not thought to substitute for vitamin E in preventing conditions induced by dietary stress e.g.

PUFA. However, recent work has suggested that selenium may perform a role in the erythrocyte membrane.

Several groups have tested the ability of selenium administered *in vivo* to protect against hemolysis mostly in rats on vitamin E-deficient diets; no positive response has appeared (Christensen *et al.*, 1958; Gitler, 1958; and Friedmann *et al.*, 1958). Krishnamurthy *et al.* (1961) did, however, demonstrate that dietary 0.5 ppm selenium does exert a protective action but only during the first 15 minutes of the dialuric acid test; longer incubation destroyed the effect. In conjunction with the initial depressed hemolysis was the observed lower level of malonic dialdehyde (indicative of lipid peroxide formation). Exactly how selenium is involved in this protective mechanism is not exactly clear.

Bunyan *et al.* (1960) demonstrated that the reducing anion $SeO_3^{-2}$ added *in vitro* to the incubation medium containing vitamin E-deficient red blood cells accelerated hemolysis. The anion ($SeO_3^{-2}$) exerted a strong enough action to completely oppose the effect of an otherwise protective level of α-tocopherol. In contrast, $SeO_4^{-2}$ was without effect.

Bunyan *et al.* (1960) also demonstrated on antagonistic action against vitamin E by other metal ions or compounds e.g. $Zn^{+2}$, $Cu^{+2}$, Cd, $AuCl_4^-$. Vincent and Blackburn (1958) reported earlier that these same metals caused $K^+$ loss from the red blood cells of humans.

Similarly, $K^+$ loss has been associated with $H_2O_2$-hemolysis (Jacob and Lux, 1968). The evidence then is that $SeO_3^{-2}$ and other metals oppose vitamin E *in vitro* and cause $K^+$ loss from within the cell concurrent with increased hemolysis.

In contrast, Bunyan *et al.* (1960) found some other metals to increase the effectiveness of vitamin E in preventing hemolysis. $Co^{++}$ and $Mn^{++}$ apparently act synergistically with vitamin E since the effective dose of vitamin E was lower. $SeO_3^{-2}$ also opposed the action of $Co^{++}$ and $Mn^{++}$. Vincent and Blackburn (1958) reported these two metals do not result in $K^+$ loss from erythrocytes.

That $Mn^{++}$ and $Co^{++}$ have vitamin E-like activity has been reported previously. Green *et al.* (1960) showed that $Co^{++}$ was effective in reversing respiratory decline in liver slices from rats on a vitamin E-selenium deficient diet. Corwin and Schwartz (1959) reported $Mn^{++}$ to have a vitamin E like effect in restoring succinate oxidation in mitochondria from vitamin E-deficient rat livers. Caputto *et al.* (1958) found $Co^{++}$ and $Mn^{++}$ were capable of restoring enzymic synthesis of ascorbic acid in livers of vitamin E-deficient rats and rabbits. The evidence suggests then that $Co^{++}$ and $Mn^{++}$ may inhibit to a certain degree the formation of lipid peroxides or function in some type of enzymatic or catalytic process in the cell; the same does not appear to apply to selenium.

With the exception of the report by Krishnamurthy and Bieri (1961), selenium has been found to be without effect in vivo and even accelerates hemolysis in vitro. However, Rotruck et al. (1971) demonstrated that dietary selenium does prevent spontaneous hemolysis but only if glucose is added to the incubation medium. The need for glucose had previously been shown; Cohen and Hochstein (1961) found that a G-6-P dehydrogenase deficiency resulted in hemolysis because of the apparent inability of the cells to generate reduced glutathione (GSH) from glucose. Later, the same workers demonstrated more specifically that it was the lack of NADPH generated from glucose metabolism which caused the GSH deficiency (Cohen and Hochstein, 1964).

Prior also to the finding of Rotruck and others was the work of Jacob and Lux (1968). They reported that by suspending vitamin E-deficient red cells in high molecular weight dextrans, the degree of $H_2O_2$-hemolysis was reduced. Since $K^+$ loss did continue unabated, it was concluded that the dextrans did not prevent membrane damage induced by $H_2O_2$ but rather balanced the osmotic pressure. In the same report, Jacob and Lux (1968) showed that spontaneous hemolysis occurring because of a build up of endogenous hydrogen peroxide in vitamin E-deficient cells could be inhibited by suspending the cells in their own plasma with added glucose. Without both conditions satisfied no lowering of hemolysis was observed. It was suggested that some factor (Se?) in plasma was utilized. Similarly, Rotruck et al. (1971)

demonstrated that glucose exerted no effect in cells deficient in selenium. A more recent report by Rotruck et al. (1972) indicates selenium is directly involved with the enzyme glutathione peroxidase. This enzyme is responsible for the metabolism of hydrogen peroxide, a reaction which directly requires GSH; the synthesis of GSH requires NADPH produced from glucose metabolism. This recent work also shows in vitro protection against oxidative damage of hemoglobin and lower Heinz body formation in rats fed selenium.

## Tri-o-cresylphosphate and Red Blood Cell Hemolysis

Only one report has appeared relating TOCP directly with erythrocyte hemolysis. Draper et al. (1952) demonstrated that 0.2% dietary TOCP administered to rats fed a vitamin E-deficient diet accelerated the onset of hemolysis. This effect was attributed to an antagonistic action of TOCP on vitamin E reserves. Some believe that TOCP functions strictly as a pro-oxidant (Seward et al., 1966; Hove, 1953) in unsaturated lipid systems e.g. the red cell membrane. If so, TOCP then should lead to the formation of more lipid peroxides by acting as an oxidizing agent. However, studies conducted in vivo are too inconclusive. Subsequently, the label of pro-oxidant has been questioned (Green and Bunyan, 1969).

TOCP is also a very potent esterase inhibitor (Heath, 1961, p. 337). Causey et al. (1957) demonstrated that repeated intraperitoneal

injections of a sublethal dose of TOCP (0.1 mg/kg) resulted in 73% inhibition of erythrocyte cholinesterases. The significance of cholinesterases in regulating $Na^+$-$K^+$ concentrations in the red cell has also been reported (Holland and Grieg, 1950). The actual physiological role of these esterases in this regard has not been substantiated. Frawley et al. (1952) showed an increased fragility of erythrocytes from animals subjected to several different organophosphorus poisons; however, TOCP was not tested.

The $Na^+$-$K^+$ equilibration resulting from organophosphorous poisoning might also be due to ATPase inhibition. The role of this enzyme in maintaining red blood cells in a polarized state is fairly well established (Davson, 1970, p. 621). That ATPase is susceptible to inhibition by compounds with the ability to phosphoryllate the active site (TOCP; Heath, 1961, p. 337) has been reported (Hokin et al. 1966;; Sachs et al., 1969). The metabolites of TOCP, specifically M-1, possess potent phosphoryllative capabilities (Eto et al., 1962a). However, no reports are available implicating TOCP or its metabolites in ATPase inhibition.

Since the availability of glucose for production of NADPH is apparently crucial to the synthesis of GSH, the effect of TOCP on glucose oxidation is significant. Earl et al. (1953) reported that 167 μg of TOCP/ml added to the medium of a warburg flask had no effect on the

$O_2$ uptake of human brain tissue. Similarly, hens fed TOCP showed no change in the rate of glucose oxidation in brain slices (Earl et al., 1953).

Seward et al. (1966) demonstrated that the addition of TOCP to the essential fatty acid-deficient diet further depressed $P_i$ uptake with little or no change in $O_2$ consumption. A depressed P/O ratio coincided with increasing levels of dietary TOCP. However, when 0.1% TOCP was added to the control diet (EFA deficient) containing 2% corn oil, the P/O ratio did not change but $P_i$ as well as $O_2$ uptake decreased slightly. However, in nutritionally balanced diets, it has been shown that TOCP can cause arrestment of glucose oxidation at high concentrations in vitro but these concentrations are about 500 times greater than those likely to be present in vivo (Heath, 1961, p. 338). Thus, it would appear based on published data, that TOCP should not interfere with the synthesis of NADPH in the red blood cell.

# MATERIALS AND METHODS

Male rats[3] of the Long-Evans strain averaging 80 grams in body weight were housed individually in wire-bottom metal cages and maintained at 22.2° C. All diets and distilled water were provided ad libitum. The composition of the selenium and vitamin E deficient diet has been described previously (Cheeke, 1972). Fifty animals were randomly assigned to 10 treatment groups of 5 rats per group in a 2 x 5 design. The five treatments with and without 0.2% dietary tri-o-cresyl phosphate[4] consisted of basal (negative control), 1.0 and 5.0 ppm selenium supplied as sodium selenite, and 10 and 50 IU of dl-α-tocopherol/kilogram of diet.

The dialuric acid procedure of Friedman et al. (1958) was used to periodically assess the degree of hemolysis over a 39 day period. Five drops of tail blood (1 drop/animal/treatment group) were pooled in 10 mls of cold phosphate-saline buffer at pH 7.4. Dialuric acid was prepared immediately before use at a concentration of 5 mg/100 ml of buffer.

Using the autohemolysis procedure of Draper and Csallany (1969) as applied by Rotruck et al. (1972) with minor modifications, red blood cells were tested after 31, 33 and 35 days. Two drops of tail blood

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[3] Simonsen Laboratory Inc.; Gilroy, California.

[4] Eastman Kodak Company.

were taken from each animal and pooled in phosphate-saline buffer (pH 7.4) according to treatment. The cells were washed, resuspended, and divided into two different buffered incubation mediums; one contained 200 mg % glucose and the other had no added glucose. The cells were then incubated for 11 hrs. (incubation time based on the time at which the negative control cells reached > 90% hemolysis) at 37° C and shaken by hand every 2-3 hrs. The percent hemolysis in both tests was determined spectrophotometrically.[5] To assure reproductibility, glassware was cleaned according to the suggestions of Friedman et al. (1958) for both the dialuric and spontaneous tests.

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[5] Baucsh and Lomb Spectronic 20.

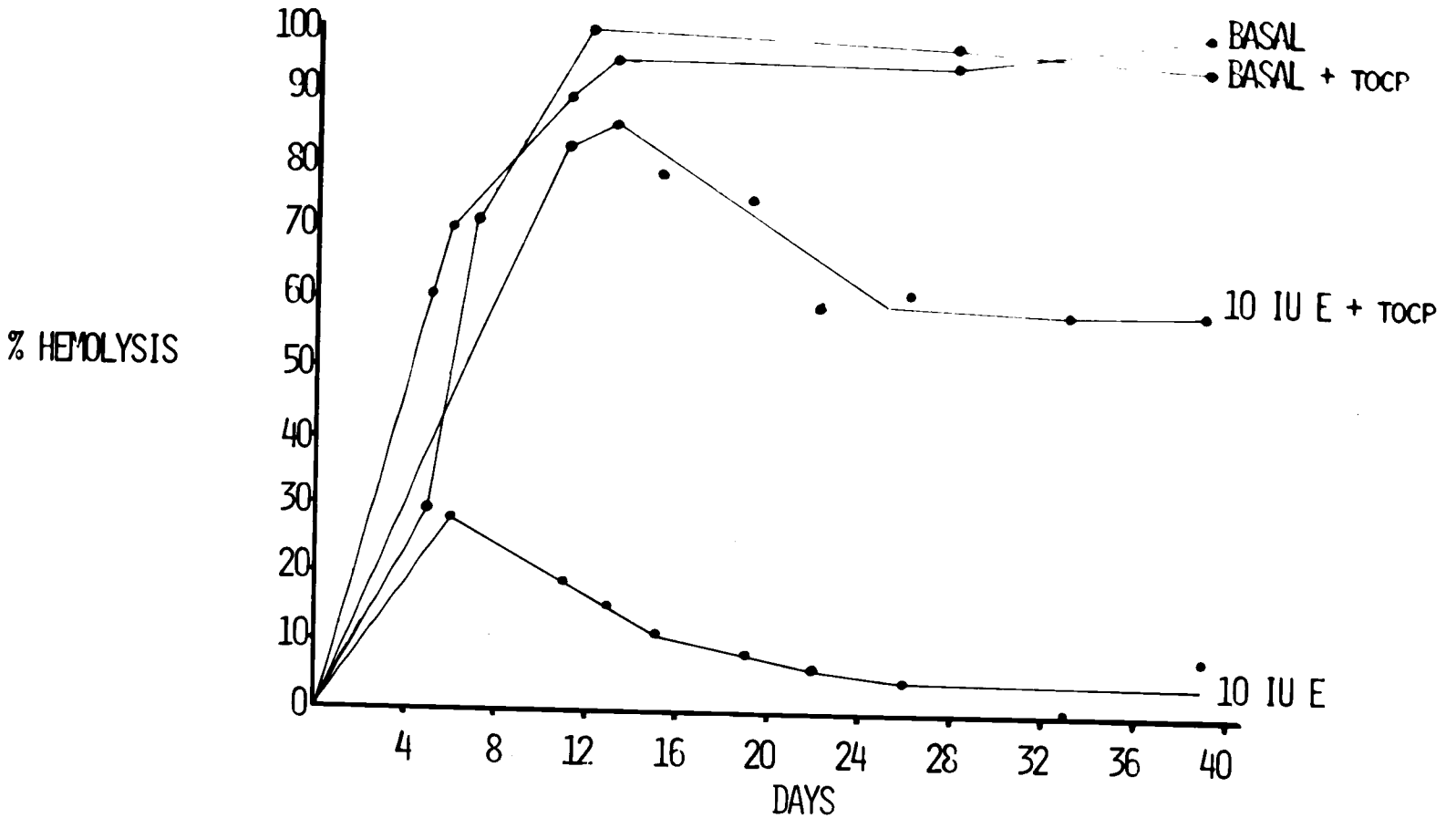
# RESULTS AND DISCUSSION

Growing rats deprived of vitamin E and selenium exhibited an immediate susceptibility of erythrocytes to hemolyze in dilute dialuric acid (Figure 6). Within 12 days an almost maximal degree of hemolysis had been reached, as has been observed by others (Rose and György, 1950a). Dietary selenium in the absence of dietary vitamin E had no apparent influence in delaying the onset of hemolysis thus confirming other reports (Friedman, et al., 1958; Christensen, et al., 1958; Gitler et al., 1958).

Rats ingested the vitamin E-supplemented (10 IU dl-α-tocopherol/kg of diet) diet without TOCP at an average rate of 2.42 mg of vitamin E/day/kg of body weight during the trial; less than 10% hemolysis occurred after 20 days (Figure 6). This data is in close agreement with an earlier report (Rose and György, 1950a) that 3 mg/day/kg of body weight is the minimal level of vitamin E required to prevent hemolysis.

The addition of 0.2% TOCP to the diet did not appear to accelerate the time taken to reach maximum hemolysis in the negative control groups. This is interpreted to indicate that the antagonist similarly did not accentuate the depletion of vitamin E from the blood as previously suggested (Myers and Mulder, 1953). However, TOCP did appear to interfere with the prophylactic activity of dietary vitamin E; whereas 10 IU/kg of diet (2.42 mg/day/kg b.w.) had been adequate to

Figure 6. Influence of TOCP on the Availability of Dietary Vitamin E to Prevent Dialuric Acid-Induced Hemolysis


% HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
0
4
8
12
16
20
24
28
32
36
40
DAYS
BASAL
BASAL + TOCP
10 IU E + TOCP
10 IU E

protect against hemolysis, the same level ingested at a rate of 2.83 mg/day/kg of b.w. was only partially protective (Figure 6). The higher level (50 IU) was completely protective after 16 days. These data support the contention of others (Myers and Mulder, 1953; Cowlishaw and Blaxter, 1955) that TOCP does in fact reduce the absorption of α-tocopherol, but probably does not influence its metabolism in the tissues.

Between ten and 24 days a substantial decline in percent hemolysis was observed with the low vitamin E groups with and without TOCP (Figure 6). The phenomenon does not appear to reflect a consequence of change of diet because of the fact that the 10 IU group with TOCP gained normally the first five days. Jager (1968) observed a similar result in which weanling rats demonstrated a reduced requirement for vitamin E needed to prevent hemolysis within the first 100 days on trial. However, the present findings indicate a much sooner stabilization (24 days) suggesting more likely an adaptation to a low vitamin E intake.

Table 6 shows the effects of selenium, vitamin E, TOCP, and glucose on in vitro autohemolysis. With no dietary TOCP, and no glucose added to the incubation medium, all treatments showed hemolysis. In contrast to the results of the dialuric acid test, both 1 ppm and 5 ppm selenium significantly ($P < .1$) reduced the extent of hemolysis, while vitamin E (10 IU/kg diet) appeared to be less effective.

Table 6. Effects of Dietary Selenium and Vitamin E ± 0.2% TOCP on *In Vitro* Hemolysis in the Presence and Absence of Glucose in the Incubation Medium.

| Dietary Treatment | - Dietary TOCP | | | | + Dietary TOCP | | |
|---|---|---|---|---|---|---|---|
| | - Glucose | P[1] | + Glucose | P | - Glucose | P | + Glucose |
| Basal (negative control) | $90.0^{a} \pm 3.1$[2] | <.005 | $35.8^{a} \pm 10.5$ | <.005 | $91.4^{a} \pm 1.4$ | N.S. | $88.3^{a} \pm 3.0$ |
| Basal + 1 ppm Se | $71.8^{a} \pm 5.1$ | <.005 | $12.5^{b} \pm 1.3$ | <.005 | $85.2^{a} \pm 3.0$ | N.S. | $90.2^{a} \pm 2.0$ |
| Basal + 5 ppm Se | $76.8^{a} \pm 2.4$ | <.005 | $15.8^{b} \pm 3.7$ | <.005 | $77.4^{a} \pm 5.4$ | N.S. | $72.6^{a} \pm 7.5$ |
| Basal + 10 IU vit. E | $51.9^{b} \pm 23.4$ | <.005 | $2.5^{c} \pm 0.8$ | <.005 | $72.5^{a} \pm 8.9$ | <.01 | $41.4^{b} \pm 24.5$ |

[1] Statistically analyzed as a 2 x 4 x 2 factorial experiment (Steele and Torrie, 1960).

[2] Mean ± S.D. for 3 replications; each replicate is composed of pooled blood equally representing all animals within each treatment group. Means followed by different superscripts (vertically) are significantly ($P < .05$) different.

Comparisons of the two tests have been made (Draper and Csallany, 1969) and our findings agree that the midrange values for the spontaneous test are consistently higher compared to the dialuric acid test. With glucose added to the medium, and no dietary TOCP, considerable protection (significant $P < .05$) against hemolysis was observed in all treatments (Table 6). The greatest effect was with the vitamin E treatment (95.2% protection), followed by 1 and 5 ppm selenium (82.6% and 79.2% protection respectively), and the negative control (62.2% protection). Rotruck et al. (1971, 1972) has proposed that the effect of glucose depends specifically on the presence of dietary selenium. The apparent response to glucose noted in the basal and vitamin E groups could be explained as due to either the lack of a selenium deficiency or protection afforded by GSH not exhausted in the allotted incubation time of 11 hrs. In support of the latter, (Rotruck et al. (1971, 1972) used 24 hours of incubation at 37° C) 18 hours of incubation at 37° C was tested and glucose had no effect in reducing hemolysis with the basal group whereas it gave 30% protection with the 1 ppm selenium group. Similarly, utilization of intracellular glucose stores could serve to explain the slightly reduced hemolysis arising from dietary selenium when no glucose was added; the protective effect was also reduced with longer incubation at 37° C.

The major effect of TOCP was to drastically reduce the effect of glucose. With no added glucose, TOCP had a significant effect on

hemolysis only with the vitamin E and 1 ppm selenium groups; in both cases, the extent of hemolysis was significantly increased ($P < 0.05$ and $P < 0.10$, respectively). TOCP virtually eliminated the protective effect of glucose in the basal and selenium groups; increased selenium (5 ppm) had very little influence on the apparent inhibited utilization of glucose. Also, TOCP significantly ($P < 0.05$) reduced the capacity of vitamin E to protect against hemolysis as was also indicated by the dialuric acid tests. With added glucose, hemolysis was not eliminated but unlike the other three treatments, there was definitely a response to glucose in the presence of TOCP.

# GENERAL DISCUSSION

It has been proposed (Rotruck et al., 1972) that selenium is required for the activity of glutathione peroxidase, an enzyme that converts hydrogen peroxide to water using GSH as the main source of hydrogen atoms. The hemolytic effect of hydrogen peroxide in the absence of vitamin E has been thoroughly studied (Rose and Györgyi, 1952a; Jacob and Lux, 1968). Glucose is apparently utilized for the production of GSH mediated through the cytoplasmic phosphogluconate pathway (Rotruck et al., 1971). The present findings are consistent with those of Rotruck et al. (1971, 1972) in that, dietary selenium does prevent in vitro hemolysis when glucose is available. The data also indicates that an insufficient level of vitamin E becomes sufficient in the presence of glucose. This pronounced response to added glucose may result from a recycling of α-tocopherol consistent with its redox properties. Such a system has been proposed (Tappel, 1962a) in which NADPH from glucose metabolism is used via glutathione and ascorbic acid to reduce oxidized α-tocopherol thereby theoretically allowing it's reutilization.

The effect of TOCP is unclear. It has been reported (Hove, 1953) that TOCP leads to the production of lipid peroxides in vitamin E deficient rat carcass fat. Assuming a similar action in red blood cells, the rate of hydrogen peroxide production being greater than the ability

of the glucose-dependent glutathione system to detoxify the peroxides, could account for the increased erythrocyte destruction. However, it has been demonstrated (Rotruck et al., 1972) that in vitro hemolysis of vitamin E-deficient cells induced by ascorbic acid (an oxidizing agent) was effectively reduced by the glucose-dependent selenium system. One might postulate also that TOCP simply depletes selenium as previously suggested (Part I) from the red blood cell thereby causing deactivation of the glucose-dependent system. The fact that higher dietary selenium (5 ppm) significantly ($P < .1$) reduced the extent of hemolysis would support this hypothesis. In contrast, unlike the apparent incapacitation of the GSH system by TOCP, the synergistic action of glucose with vitamin E was not so affected. Assuming recyclization of α-tocopherol as mentioned above, it would appear that TOCP does not inhibit the synthesis of NADPH from glucose metabolism; TOCP has in fact been reported to have no effect on the oxidation of glucose (Heath, 1961, p. 337; Seward et al., 1966).

Aside from its oxidizing capacity, TOCP also is a very potent antiesterase (Heath, 1961, p. 337). The 0.2% dietary level of TOCP used in this study effectively inhibits several serum cholinesterases (unpublished data). The role of cholinesterases in regulating $Na^+$-$K^+$ concentrations in the red cell (Holland and Grieg, 1950) may be responsible for the increased fragility of erythrocytes reported in

animals subjected to organophosphorus poisoning (Frawley et al., 1952). Thus, cholinesterase inhibition of vitamin E and/or selenium depleted erythrocytes may accentuate the susceptibility of erythrocytes to hemolyze.

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