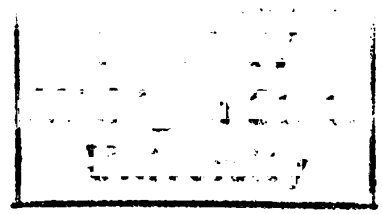

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Polymorphonuclear Neutrophils
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THE EFFECTS OF VITAMIN E AND SELENIUM ON THE RESPIRATORY BURST OF SWINE POLYMORPHONUCLEAR NEUTROPHILS AS MEASURED BY CHEMILUMINESCENCE

By

Andrea Denise Wastell

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ABSTRACT

THE EFFECTS OF VITAMIN E AND SELENIUM ON THE RESPIRATORY BURST OF SWINE POLYMORPHONUCLEAR NEUTROPHILS AS MEASURED BY CHEMILUMINESCENCE

By

Andrea Denise Wastell

The effects of supplemental vitamin E and/or selenium on the phagocytic and killing abilities of growing swine neutrophils were evaluated by chemiluminescence in three trials. Twelve pigs in Trial 1 and 20 pigs in Trial 2 were randomly assigned to one of four diets containing either no supplemental vitamin E or selenium, 100 IU vitamin E/kg diet, 1.0 ppm selenium, or both vitamin E and selenium in a 2 x 2 factorial split plot design. In Trial 3, 18 pigs were randomly assigned to one of three diets containing 0.1 ppm selenium and no supplemental vitamin E, 100 IU or 1000 IU vitamin E/kg diet in split plot design. Supplementation of vitamin E increased plasma tocopherol concentrations. Selenium supplementation increased plasma selenium concentrations, plasma and whole blood glutathione peroxidase activities, and the white blood cell count in Trial 1. Dietary supplementation of vitamin E and selenium did not result in any apparent treatment diffenences in the chemiluminescence of neutrophils.

To my father,

Marvin Eugene Wastell

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My education means a great deal to me and Dr. Elwyn R. Miller has given me the chance to broaden my educational experiences. His patience, understanding, and wisdom has taught me far more than could ever be learned from a text book.

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I have appreciated the love and support of my family throughout my years of education. My parents have inspired and encouraged me to pursue my goals. I would also like to mention that I enjoy my two brothers' company and I would not trade them.

Finally, I like to express my gratitude to Brad who has encouraged, inspired, and loved me during the past year. His inspiration will be my motivating force for many years to come.

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INTRODUCTION

The need for vitamin E and selenium in swine diets has been well established, but field reports of problems in swine fed legally-permitted levels of selenium have led to proposals that higher dietary concentrations of vitamin E and/or selenium be used. Evidence from other species in addition to swine indicates that a deficiency of vitamin E and/or selenium may reduce immune responses. Moreover, there are some reports that suggest pharmacological doses of these two nutrients may enhance immunity.

Leukocytes have the responsibility to defend the host body against foreign invaders such as viruses and bacteria. In particular, phagocytes engulf and kill foreign antigens. During the phagocytic ingestion of bacteria, there is a 10 to 15 fold increase in oxygen consumption, known as a respiratory burst. This increase is not due to mitochondrial metabolism but does involve generation of large amounts of superoxide. The release of superoxide or other oxidants into the ingested bacteria is considered to be the killing factor. Potentially reactive oxidants, such as singlet oxygen, hydroxyl radical, and hydrogen peroxide, escape the phagosome into the surrounding milieu during the microbicidal process. These oxygen species are extremely toxic to the host cells.

A system of enzymes involving superoxide dismutases, catalases, and peroxidases have evolved to avoid the catastrophic damage that could be done to biologically important molecules. These enzymes reduce superoxide and hydrogen peroxide to form water. Glutathione peroxidase (GSH-Px), a selenium-dependent enzyme, can also destroy organic hydroperoxides within the cytosol and mitochondrial membranes. Additionally, alpha-tocopherol has antioxidant properties that are capable of neutralizing toxic oxygen metabolites in cellular membranes.

Measurement of the oxygen species released during the phagocytic process could be useful in assessing the functional abilities of neutrophils associated with the respiratory burst. Chemiluminescence (CL) is a method which nonspecifically measures the oxygen species released during phagocytosis and the respiratory burst. This procedure has been used in the medical field to assess the killing function of neutrophils in the rare inherited disorder, chronic granulomatous disease, in which neutrophils are deficient in myeloperoxidase. To amplify the chemiluminescent response from the oxygen burst, luminol is added in an in vitro system and reacts with the toxic oxygen species to produce photons that can be measured.

To determine the effects of vitamin E and/or selenium on the phagocytic and killing abilities of swine neutrophils, 3 trials were designed. The first 2 trials were 2 x 2 factorials in a split plot design in which either a basal

diet or diets supplemented with 100 IU vitamin E/kg diet or 1.0 ppm selenium or both vitamin E and selenium were fed to pigs. In the third trial, either a basal diet or 100 IU vitamin E/kg diet or 1000 IU vitamin E/kg diet were fed to pigs. Chemiluminescence was measured from the neutrophils. In addition, the vitamin E and selenium status of the animals were assessed. An increase in CL reflects an increase in the amount of oxygen species released during phagocytosis associated with the respiratory burst. Animals deficient in vitamin E and selenium would be expected to have an increase in CL as compared to animals adequate in these nutrients. Vitamin E and selenium have antioxidant properties which would neutralize oxygen species produced during the respiratory burst.

REVIEW OF THE LITERATURE

Vitamin E and Selenium Metabolism

I. History

In 1920, rats fed a semi-purified diet with all known vitamins at that time, were not able to successfully reproduce. Evans and Bishop (1922) identified a factor then called factor X in lettuce and wheat germ which protected the animals from fetal reabsorption. Factor X soon became known as vitamin E and was shown to prevent sterility in female rats, fetal reabsorption, and degeneration of germinal epithelium of the testis in male rats (Mason, 1925; Evans and Burr, 1927). Hence, the name tocopherol from the Greek tokos (offspring) and pherein (to bear) and ol for the alcohol form was proposed by Evans et al. (1936). The Greek letters alpha, beta, gamma, and delta were used to designate the different forms found in vegetable oil.

Chicks, guinea pigs, rabbits and ducklings fed semi-purified diets in the 1920s and 1930s showed signs of reproductive degeneration, nutritional encephalomalacia, and nutritional muscular dystrophy (Pappenheimer and Goettsch, 1934). However, it was not until 1940 that vitamin E

deficiency was specifically linked to these pathologies (Pappenheimer, 1940). Finally in 1961, Grant established the relationship between vitamin E and mulberry heart in swine.

Schwarz and Foltz (1957) were the first to demonstrate that sodium selenite would prevent liver necrosis in rats fed torula yeast diets. Subsequently, selenium has been shown to alleviate a number of deficiency signs including exudative diathesis in chicks, mulberry heart in swine and muscular dystrophy in lambs. The following year, Grant and Thafvelin (1958) found a relationship between hepatosis dietetica and selenium deficiency in swine. In 1957, an enzyme, glutathione peroxidase (Mills, 1957), was discovered in the presence of reduced glutathione to protect erythrocytes from hemoglobin oxidation and hemolysis induced by hydrogen peroxide and ascorbate.

Vitamin E, either dietary or added in vitro, also protected erythrocytes against hemolysis (Dam, 1957); however, early reports indicated selenium did not have the same protecting effects as vitamin E (Christensen et al., 1958; Gitler et al., 1958). Glucose was shown to protect erythrocytes by maintaining glutathione levels within the cell which was the source of reducing substrate for glutathione peroxidase (Mills and Randall, 1958; Cohen and Hochstein, 1963). Rotruck et al., 1971, 1972, 1973) assimilated these facts and demonstrated that dietary selenium would protect erythrocytes from hemolysis if

glucose was also added to the system. Protection by vitamin E was not dependent on glucose. Consequently, Rotruck et al. (1973) focused on GSH-Px and discovered that it was a selenium-dependent enzyme.

II. Vitamin E

A. Sources

Tocopherols and tocotrienols are synthesized by many plants and occur mainly in green leaves and seeds. Alpha-tocopherol has the highest biological activity of all eight isomers. Animal tissues are not a good source of vitamin E. Animals do not synthesize tocopherols; consequently, tissue levels tend to be low and are a reflection of dietary intake. Vitamin E activity levels are related to plant species, stage of maturity, harvesting, storage and processing. Artificial drying of grain is unlikely to greatly affect tocopherol loss. Forage crops exposed to sunlight on the other hand, rapidly lose their tocopherol content. Light is the major destructive force and can initiate lipid peroxidation. The most commonly fed forms of vitamin E are all-rac-alpha-tocopheryl and RRR-alpha-tocopherol commercially available in liquid or dry form.

B. Absorption

Measurement of tocopherol levels in blood, plasma, or serum reflect the influx and efflux between gut tissue

concentration and various tissue concentrations. Twenty to 40 percent of tocopherols and/or their esters are absorbed in the gut (Gallo-Torres, 1980). Bile and pancreatic lipase and the rate of fat digestion can affect the absorption of alpha-tocopherol (Wiss et al., 1962). The nutritional status of the animal should be noted. Deficient animals will more rapidly absorb vitamin E than repleted animals. As oral doses of tocopherol increase, they are absorbed less efficiently (Schmandke and Schmidt, 1965). Serum concentrations of lipids and lipoproteins, tissue tocopherol levels and gut-motility are other factors affecting vitamin E absorption. Polyunsaturated fatty acids are inhibitory to absorption, whereas, medium-chain triglycerides enhance absorption.

Tocopherols are absorbed primarily through the lymphatic system and are transported complexed to lipoproteins, mostly of the very low density lipoprotein (VLDL) fraction. Gallo-Torres and colleagues (1970, 1971, 1974) have established that esterified tocopherol is cleaved to the free phenol before absorption. Investigation with monogastrics and ruminants indicates that absorption takes place in the medial small intestine (Gallo-Torres, 1980). Hollander et al. (1975) found that the rate of absorption was not affected by inhibitors, suggesting alpha-tocopherols are absorbed by a non-saturable passive diffusion process.

Plasma transport of tocopherols is similar to lymphatic transport (Chow, 1975). Low density lipoproteins (LDL)

carry the majority of tocopherol although high density lipoproteins also transport tocopherols (Davies et al., 1969). There is a high correlation between serum lipid levels and tocopherol levels. Thus, disorders affecting serum lipids also affect circulating tocopherol levels.

C. Retention

Erythrocytes also transport tocopherols (Kayden et al., 1973). Vitamin E is largely localized in the erythrocyte cell membrane where it is rapidly exchanged with the plasma. In erythrocyte membranes, the molar ratio of alpha-tocopherol to PUFA was reported to be approximately 1:850 (Diplock, 1985).

Tissue uptake varies logarithmically with tocopherol intake (Behrens et al., 1982) and varies considerably in concentration. Adrenal and pituitary glands, testis, and platelets have the highest concentrations of vitamin E. Cell fractions with high concentrations of membranes, such as mitochondria and microsomes contain most of the vitamin. Adipose tissue, liver and muscle represent storage deposits of vitamin E. Dietary intakes affect plasma and liver concentrations most readily followed by a slower turnover in skeletal and heart muscle and a very slow exchange in adipose tissue (Machlin and Gabriel, 1982).

D. Metabolism and Excretion

Current understanding of in vivo metabolism of alpha-tocopherol is contradictory and controversial. Difficulties arise in differentiating between metabolites which are genuine and those which are artifacts induced by the isolation process. Antioxidants are typically used in analytical procedures, perhaps reducing true metabolites. Vitamin E undergoes very little metabolism in the tissues (Gallo-Torres, 1980). Alpha-tocopherol is deposited mainly unmodified in its unesterified form in tissues. Small amounts of water-soluble metabolites have been found in urine by Simon and coworkers (1956) and confirmed by other investigators (Bunyan et al., 1961). These compounds referred to as Simon's metabolites, appear in urine as less than one percent of alpha-tocopherol excreted. Fecal excretion via bile is the major route of alpha-tocopherol elimination.

E. Toxicity

Vitamin E is relatively nontoxic as shown by animal studies with both acute and chronic doses (Food and Drug Administration, 1975). Yasunaga et al. (1982) studied optimal and toxic doses of vitamin E in mice. They reported that all the mice died within 3 days after daily intraperitoneal (ip) injections of 400 IU all-rac-alpha-tocopherol/kg. The immune response as measured by

lymphoproliferation assays with phytohemagglutinin (PHA), concanavalin A (Con A) and lipopolysaccharide (LPS) was enhanced with injections between 5 and 20 IU/kg per day but inhibited at 80 IU/kg per day. Serum tocopherol levels were 5.39, 7.29, and 21.91 µg/ml for daily ip injections of 5, 20, and 80 IU/kg, respectively. Twenty-eight human volunteers were orally supplemented with 100 to 800 IU vitamin E/day over a 3 year period with no apparent affect on the liver, kidney, muscle, thyroid, erythrocytes, and leukocytes (Farrell and Bieri, 1975). Bendich et al. (1986) concluded that 50 mg/kg daily (at least 3 times nutritional levels) are necessary for optimum immune response in rats. Other investigators are in agreement with these findings (Tengerdy and Brown, 1977; Tengerdy, 1980).

III. Selenium

A. Sources

Selenium found in feedstuffs varies with plant species and geographical area. The most common organic forms of selenium in plant sources are selenocystine, selenocysteine, selenomethionine, and methylselenomethionine (Shrift, 1969; Olson et al., 1970). However, many areas in the United States have selenium poor soil resulting in selenium-deficient feeds and forages. Deficient areas with approximately 80% of all forages and grains containing less than 0.10 ppm selenium include the Northeastern states, the

Atlantic coastal areas, Florida, the Northwest and many states east of the Mississippi River (particularly the Great Lakes states including MI, IL, WI, IN, and OH). To compensate for selenium deficiency in some feedstuffs, sodium selenite or sodium selenate are added to animal feeds.

B. Absorption

Wright and Bell (1966) found retention of selenium taken orally to be 66 percent in swine. The greatest absorption occurred in the last part of the small intestine, cecum and colon. Different forms of selenium influence the transport routes. McConnell and Cho (1965) reported selenomethionine was transported against a concentration gradient and was inhibited by methionine. By contrast, selenite and selenocystine were not transported against a gradient nor were they inhibited by their respective sulfur analogues.

Selenium associated with GSH-Px in erythrocytes is species dependent. In sheep 75 to 85 percent of selenium is associated with erythrocyte GSH-Px compared to less than 10 percent for primates (Oh et al., 1974; Behne and Wolters, 1979). In the plasma, the binding of selenium in the selenite form to plasma proteins is not energy dependent nor is protein synthesis required (Porter et al., 1979). However, selenium binding to protein is dependent on the presence of erythrocytes (Sandholm, 1975). Rather, the

uptake and incorporation of selenium is dependent on reduced glutathione concentrations in erythrocytes (Gasiewicz and Smith, 1978). The plasma carrier of selenium seems to be species dependent. Selenium is mainly transported by albumin in mice (Sandholm, 1974) whereas, lipoproteins seems to be the selenium-binding protein in humans.

C. Retention of Selenium

Kidney has the highest selenium concentration followed by liver, spleen and pancreas. Intestinal and lung tissues have relatively high amounts followed by cardiac muscle and then skeletal muscle. The selenium status of the animal affects tissue content. Chemical form also affects deposition. Organic forms, in general, are deposited in higher concentrations. Diets containing seleno-methionine compared to selenite or selenocystine result in higher selenium muscle concentrations (Osman and Latshaw, 1976).

D. Metabolism and Excretion

Many factors affect selenium metabolism such as chemical form of selenium, sulfur, arsenic, metals microorganisms, vitamin E, and previous selenium intake. Animal tissues are able to convert inorganic forms to organic forms. Organic forms have different biopotency in various tissues. Data from a number of investigators would indicate that selenium compounds are not metabolized to

common intermediates. The primary route of excretion of selenium in monogastrics is urine (Burk et al., 1972).

E. Glutathione peroxidase

Selenium is essential for the synthesis of GSH-Px and for the enzymatic activity. Sixty percent of GSH-Px activity in rat livers is cytosolic and 30 percent mitochondrial (Green and O'Brien, 1970; Flohe and Schlegel, 1971) with at least 60 percent of rat liver mitochondrial selenium being associated with GSH-Px (Levander et al. 1974). Animals maintained on a selenium deficient diet exhibit a rapid decline in tissue GSH-Px activity that is correlated with selenium deficiency signs (Hafeman et al., 1974); Cantor et al., 1975). Upon repletion of selenium, tissue GSH-Px activity is restored (Chow and Tappel, 1974). Glutathione peroxidase activities increase and decrease most rapidly in liver and plasma in response to dietary levels of selenium (Chow and Tappel, 1974; Lawrence et al., 1974).

Glutathione peroxidase (80,000 MW) has four identical subunits. Erythrocytes from ovine and bovine contain 4 g-atom of selenium per mole of GSH-Px (Flohe et al., 1973; Oh et al., 1974). The molecular weight can vary from species to species and from tissue to tissue (Flohe et al., 1971; Nakamura et al., 1974; Sunde et al., 1978; and Awasthi et al., 1979). Rat liver GSH-Px has 153 amino acids per subunits compared to bovine GSH-Px with 178 per subunit. In contrast to many other peroxidases, GSH-Px has a high

specificity for its substrate, glutathione (Mills, 1959). Moreover, no other thiol substrate has been found to have more than 30 percent of the activity of glutathione (Flohe et al., 1971). Unlike catalase, GSH-Px will destroy a number of hydroperoxides at a similar rate as hydrogen peroxide destruction (Little and O'Brien, 1968). Forstrom et al. (1978) have suggested selenocyteine is the active site for GSH-Px.

IV. Requirements for Vitamin E and Selenium

The National Research Council (1979) recommends 11 IU vitamin E/kg diet for swine. The requirement for vitamin E is influenced by other dietary factors such as PUFA, selenium and sulfur amino acids. Molds in feed and feed processing may also affect the requirement for vitamin E. Corn-soybean meal diets grown in the Midwest probably contain inadequate amounts of vitamin E and selenium to meet the needs of confined pigs. Ullrey (1981) proposed that vitamin E concentration should be at least 10 to 20 IU/kg for corn-soybean type swine diets when supplemental selenium is limited to 0.1 ppm. He further recommended that 30 IU vitamin E/kg diet may be beneficial for the breeding herd and young pigs.

Approved selenium supplementation levels for swine are upto 0.3 ppm in prestarter and starter diets and 0.1 ppm in all other swine diets. Groce et al. (1973) demonstrated

that supplementation of 0.1 ppm selenium as sodium selenite to growing-finishing swine diets prevented death losses, gross pathology and histopathological lesions of nutritional muscular dystrophy, and dietary hepatic necrosis.

Trapp et al. (1970) thoroughly described the lesions seen in vitamin E deficiency. The signs were described as sudden death in feeder pigs (20-40 kg bodyweight), lesions of hepatic necrosis, icterus, edema, ulcers, hyalinization of walls of arterioles, and skeletal and cardiac muscular degeneration. Ullrey et al. (1971) demonstrated a reduced incidence of mastitis-metritis-agalactia complex (MMA) involving 191 farrowings when 0.2 ppm selenium, 22 IU vitamin E/kg, and 880 mg choline chloride/kg were added to a corn-soybean meal diet. These investigators also noted an increase in number of live pigs born per litter of sows fed the supplemented diet.

Anemia also has been associated with vitamin E deficiency. Nafstad (1965) reported hematological changes as anemia, leukocytosis, multinucleation of erythrocyte precursors and increased numbers of megakaryocytes. These observations were supported by the work of others (Obel, 1953; Grant, 1961; Baustad and Nafstad, 1972). However, other researchers have found no hematological changes associated with vitamin E deficiency in swine (Michel et al., 1969; Fontaine et al., 1977a, 1977b). Niyo et al. (1980) supported the conclusions of the latter investigators and found no anemia or morphological changes in circulating

erythrocytes or leukocytes of vitamin E- and selenium-deficient pigs. However, in bone smears, multinucleated erythroblasts were observed.

Vitamin E and Selenium Functions

I. Vitamin E

It seems most probable that vitamin E has functions besides its role as an antioxidant. Diplock and Lucy (1973) suggested that alpha-tocopherol may stabilize biological membranes by binding its side chains with the membranes of PUFA. As an antioxidant, vitamin E may affect arachidonic acid metabolism by inhibiting the formation of hydroxy-eicosatetraenoic acid, thromboxane A_2, or prostaglandins (Stuart, 1982; Chan and Leith, 1981; Goetzl, 1980), thereby affecting platelet aggregation, blood clotting, the immune system and inflammation. Mitochondria is rich in alpha-tocopherol. Vitamin E may protect the membranes from oxidative damage induced by the electron transport chain. Although reproduction is impaired in vitamin E deficiency, there is no clear evidence for vitamin E being involved with hormone production. Many enzymes are affected by vitamin E deficiency but no direct role for vitamin E in RNA or protein synthesis has been established.

II. Selenium

Glutathione peroxidase, a selenium-dependent enzyme has many intercellular and intracellular functions. This enzyme is the key to modulating the GSH/GSSH ratio and indirectly the NADP/NADPH of the cell. Therefore, GSH-Px may regulate a number of multiple cellular functions including cell division (Kosower and Kosower, 1974), pentose-phosphate shunt (Flohe, 1976), gluconeogenesis (Sies et al., 1974), and mitochondrial oxidation of alpha-oxo-acids (Sies and Moss, 1978). Other functions important to the biomedical field include protection of unsaturated lipids in cell membranes (Little and O'Brien, 1968; Flohe and Zimmermann, 1974), prevention of chemical mutagenesis (Schwarz, 1976; Shamberger, 1976), and interaction with the arachidonic acid cascade (Nugteren and Hazelhof, 1973; Gryglewski et al., 1976).

III. Lipid peroxidation

Lipid peroxidation is the reaction of polyunsaturated fatty acids (PUFA) with oxygen or derived free radicals. When this reaction occurs in membranes (endoplasmic reticulum, mitochondrial membranes, or plasma membrane) deleterious effects occur to the structural organization (Slater, 1972) and to the associated enzymatic function (Lewis and Willis, 1962). There are numerous examples where lipid peroxidation causes irreversible damage that results

in cell death. Some of these are from high-energy irradiation (Desai et al., 1964), photosensitization (Slater and Riley, 1966), exposure to ozone (Goldstein et al., 1969), administration of CCl_4 (Slater, 1972) and exposure to paraquat (Bus et al., 1975).

Initiation of lipid peroxidation involves the reaction between a PUFA and an oxidizing radical (R.). A proton is extracted from the PUFA to form a PUFA radical. Chain-

$$R\bullet + PUFAH \rightarrow RH + PUFA\bullet \quad (1)$$

propagation steps follow to form a fatty acid peroxy-radical.

$$PUFA\bullet + O_2 \rightarrow PUFAO_2\bullet \quad (2)$$

The first two steps involve oxygen-derived radicals such as superoxide, singlet oxygen, hydroxyl radical, CCl_3O_2., and $PUFAO_2$. (Baird et al., 1977; Fong et al., 1973). The latter stages (3-5) involve forming a variety of low-molecular-weight water soluble products.

$$PUFAO_2\bullet \rightarrow \text{bond rearrangement, diene formation} \quad (3)$$

$$PUFAO_2\bullet + PUFAH \rightarrow PUFAO_2H + PUFA\bullet \quad (4)$$

$$PUFAO_2\bullet,\ PUFAO_2H \rightarrow \text{degradation products; malondialdehyde, ethane, etc.} \quad (5)$$

Malondialdehyde production is not equivalent to the amounts of fatty acid oxidized, nevertheless, it is a measure of lipid peroxidation.

IV. Protective Mechanism

The antioxidant effects of vitamin E have generally been regarded as protection against lipid peroxidation of cell membranes (Fahrenholtz et al., 1974). In vitamin E-deficient animals, lipid peroxidation of all tissues is likely to occur. The autooxidation of lipids may begin with a free radical or by singlet oxygen. As free radical scavenger and antioxidant, alpha-tocopherol is capable of terminating chain reactions of PUFA (Figure 1). The protective characteristics of tocopherols are derived from its primary location in the cell membrane. This means it is adjacent to membrane-bound enzymes, such as NADPH oxidase, which generates free-radicals (Molenaar et al., 1980).

McCay and King (1980) proposed a mechanism by which vitamin E and selenium work together. Hydrogen peroxide is generated from superoxide which is distributed both in the cytosol and membrane portions of the cell. Glutathione peroxidase (a selenium-dependent enzyme) destroys hydrogen peroxide in the inside aqueous portion leaving the remainder of hydrogen peroxide in the membrane. Hydrogen peroxide and superoxide react to form hydroxyl radical in the membrane which is trapped by tocopherol. A shortage of tocopherol may lead to lipid peroxidation of membrane PUFAs.

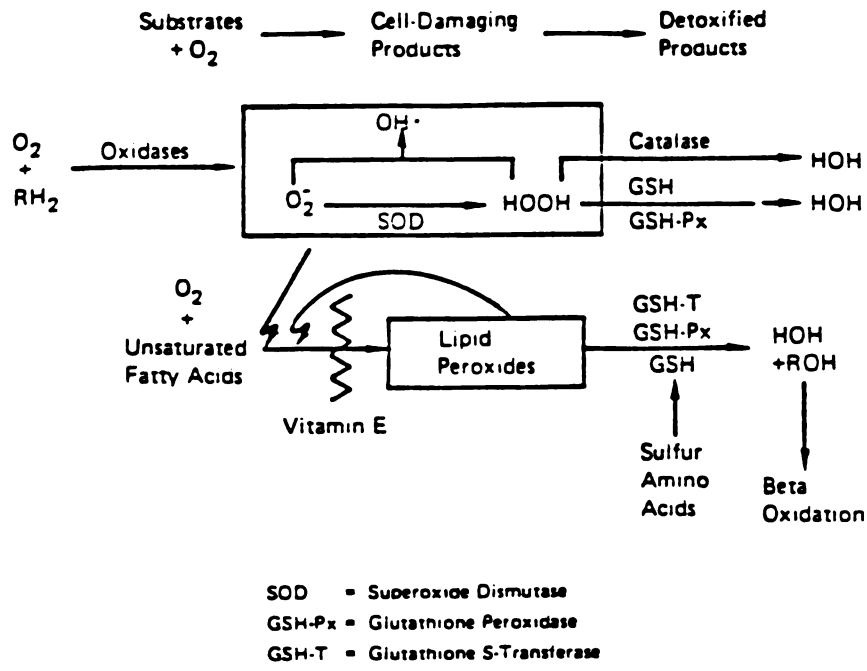


Figure 1. Interrelationships of selenium, vitamin E, and sulfur amino acids in oxidative metabolism (From Ganther, 1983).

V. Influence on Immunity

Vitamin E and selenium have been shown to improve humoral immunity response of mice, chicks, turkeys, guinea pigs, and sheep (Nockels, 1980; Stephens et al., 1979; Marsh et al.; 1981). Passively transferred antibodies in chicks from hens fed 150 or 450 ppm vitamin E were significantly higher than chicks from hens fed 0, 90, 300, or 900 ppm (Jackson et al., 1978). Tengerdy et al. (1973) conducted a series of experiments involving mice inoculated with sheep red blood cells (SRBC) or tetanus toxoid and fed a vitamin E- deficient diet supplemented with either vitamin E or the antioxidant N,N-diphenyl-p-phenylene diamine (DPPD). Sixty IU vitamin E/kg significantly increased spleen weight, plaque forming colonies, and hemagglutinin titers. The investigators also concluded that vitamin E , and not an antioxidant, was necessary for IgG antibody production and that vitamin E enhanced the primary response more than the secondary response.

A two to three fold increase in antibody (Ab) titers in 6 to 8 week old pigs fed supplemental vitamin E was noted by Ellis and Vorhies (1976). The pigs were supplemented with 110 IU vitamin E/kg diet two weeks before innoculation with killed Escherichia coli. Peplowski et al. (1981) also found increased Ab titers with vitamin E and selenium supplementation. In two 2 x 2 factorial designs, these investigators fed a diet or injected weaned pigs with 0 or

220 IU vitamin E/kg diet and 0 or 0.5 ppm selenium. In both experiments, 1 x 10^8 SRBC were administered ip weekly. The results indicated higher hemagglutination titers with either vitamin E or selenium supplementation by either administration route.

Combination of both nutrients from either diet or injection further enhanced hemagglutinin titers. Possibly vitamin E and selenium have an additive effect on humoral response. Dietary supplementation of 300 mg vitamin E/kg diet reduced E. coli induced mortality (Tengerdy and Brown, 1977). These investigators attributed the protection to increased antibody production and increased phagocytosis. Marsh et al. (1981) concluded that both vitamin E (100 IU all-rac-alpha-tocopheryl acetate/kg diet) and selenium (0.1 ppm of selenium Na_2SeO_3) are required for optimum immune function in 2 week old chicks. However, at 3 weeks, either vitamin E or selenium was adequate for optimum immune response.

Vitamin E also affects other aspects of the immune system including antibody-dependent cell cytotoxicity (ADCC), delayed hypersensitivity and mitogenic responsiveness. Leb et al. (1985) studied the effect of alpha-tocopherol on phorbol myristate acetate (PMA)-induced monocyte cytotoxicity and on ADCC. They observed a decrease in hydrogen peroxide release by monocytes preincubated with alpha-tocopherol. Ultimately this led to a decrease in PMA-induced monocyte cytotoxicity and to some extent inhibition

of ADCC. Seemingly, PMA-induced monocyte cytotoxicity depended on hydrogen peroxide release whereas ADCC was less dependent on oxygen metabolism. Vitamin E may be an important mediator in chronic infections or inflammations where hydrogen peroxide is likely to cause damage to host tissue.

Other investigators have found the macrophage to be the cell most affected by vitamin E deficiency. Gebremichael et al. (1984) found that vitamin E-deficient mice expressed less Ia+ macrophages and were less able to present antigen to nonadherent cells. The defect appears to be with the accessory cell function since Ia+ macrophages are required for antigen presentation and for initiation of lymphokine production (Larsson et al., 1980).

Vitamin E is also required for optimal lymphocyte mitogen response (Bendich et al., 1983). Spontaneously hypertensive rats fed a vitamin E-deficient diet for 17 weeks as compared to rats of the same strain supplemented with vitamin E had depressed T- and B-cell splenic mitogen responses to Con A, PHA, and LPS. Other investigators have also reported that high levels of vitamin E are immunostimulatory toward pathogens and mitogens (Sheffy and Schultz, 1978; Corwin and Schloss, 1980a; Tengerdy et al., 1984). Plasma vitamin E levels over a range of 0.04 μg/ml are correlated with T- and B-cell responses to mitogens (Bendich et al., 1986).

The mechanism by which vitamin E stimulates blastogenesis in deficient animals has not been elucidated. Vitamin E may act similarly to antioxidants. Ethoxyquin and 2-mercapto-ethanol but not ascorbic acid stimulated lymphocyte proliferation (Langweiler et al., 1983). Fanger et al. (1970) proposed PMA-induced lymphocyte blastogenesis was enhanced by supplementation with L-cysteine, glutathione, and sulfite. They suggested that the reducing agents made cells more sensitive to mitogenic agents by cleaving disulfide bonds on the cell membrane. Alternatively, Corwin and Schloss (1980) reported that vitamin E has a stimulating effect for mitogenic response of murine spleen cells, but the response was not related to antioxidant properties (Corwin and Schloss, 1980b).

Arachidonic acid (AA) metabolism may be affected by vitamin E. Vitamin E alleviates the immunodepressive effect of prostaglandins (Machlin, 1978). As an antioxidant, vitamin E inhibits prostaglandin synthesis by preventing the oxidation of arachidonic acid. Tengerdy and Brown (1977) demonstrated that vitamin E supplemented to E. coli-infected chicks reduced the production of prostaglandin E_2 and prostaglandin E_1 in bursa homogenates. Likoff et al. (1981) fed diets supplemented with 6 times normal levels to chicks and found prostaglandin E_1, prostaglandin E_2, and prostaglandin $F_{2\alpha}$ levels decreased in the bursa and spleen. Moreover, Ab titers to E. coli and phagocytosis increased at the same time.

Physiology of the Neutrophil

I. Functions

Neutrophils (polymorphonuclear leukocytes) are the primary line of host defense against microbial invasion. These cells have the ability to recognize and ingest foreign particles, and thus are termed phagocytes. Once inside the phagocyte, the foreign object is subject to chemical and enzymatic attack. During the phagocytic process, the neutrophil undergoes a respiratory burst which is comprised of increased oxygen consumption, increased production of hydrogen peroxide, production of superoxide (Babior, 1978), and increased oxidation of glucose by the hexose monophosphate shunt (Stahelin et al., 1956; Stahelin et al., 1957). The respiratory burst is not due to oxygen consumption by the mitochondria, but rather oxygen metabolites are needed for killing bacteria.

Oxygen metabolites produced during the respiratory burst also can damage the surrounding host cells and tissue. Superoxide, hydroxyl radical, and singlet oxygen have been implicated as initiating agents in lipid peroxidation (Fong et al., 1973; Kellogg and Fridovich, 1975; Lynch and Fridovich, 1978) which results in severe damage to cellular organelles and membranes (Tappel, 1973). Hydroxyl radical attacked neutrophils after phagocytosis resulting in death

of the cell and the subsequent release of hydrolytic enzymes into the surrounding milieu (Salin and McCord, 1977).

The cell has mechanisms to protect itself from oxidant activity both inside and outside of the cells, glutathione and vitamin E, respectively. Intracellular mechanisms for detoxification of oxygen species include glutathione and the hexose monophosphate shunt. Vitamin E acts as the reducer for lipid peroxidation. Catalase also can destroy peroxides but is not known to decompose lipid hydroperoxides, and unlike GSH-Px is not usually predominant in the cytosol (O'Brien, 1969). Superoxide dismutase is able to reduce superoxide to prevent lipid hydroperoxidation.

II. Phagocytosis

Phagocytosis is the process by which cells recognize particles on their cell-membrane and surround those objects with plasma membrane (the phagosome). Some of the most important phagocytes include neutrophils, macrophages, and eosinophils. Throughout phagocytosis, there is an increase in production of hydrogen peroxide, superoxide, and singlet oxygen which is known as the respiratory burst. These toxic oxygen metabolites and the lysosomal enzymes released into the phagosome are the killing mechanisms of neutrophils.

DeChatelet (1978) has proposed the following hypothesis of the sequence of events during phagocytosis. First, the neutrophil encounters and recognizes a microbe or

particulant through a cell membrane receptor (Figure 2a). Upon attachment to the receptor, the membrane is perturbed (Figure 2b). A signal from the altered membrane is transduced to a granule which contains oxidase. Microtubules and/or microfilaments may be the key communication between the vacuole and the granule. Pseudopodia form and begin to surround the object (Figure 2c). Finally, the pseudopodia fuse completely enclosing the particle with extracellular membrane. The granule-bound oxidase has been activated through the transduction of the original membrane perturbation (Figure 2d). The phagosome is now completely in the center of the cell and fuses with the cytoplasmic granules which release their contents within the vacuole. This process is accompanied by production of hydrogen peroxide, superoxide, and singlet oxygen. It is not known with certainty as to the exact initiation of the respiratory burst (Figure 2e). Degranulation has occurred, the bacterium has been killed and is being digested within the phagosome (Figure 2f).

Most neutrophils from different species contain two major types of granules, azurophilic (primary) and specific (secondary). The two can be distinguished by morphogenesis and cytochemistry (Bainton and Farquhar, 1968) and by biochemical analysis of cell fractionation (Baggiolini et al., 1970a and 1970b). The two types are chemically differentiated by their associated proteins: myeloperoxidase and lactoferrin for azurophilic and specific

Figure 2. Hypothetical scheme outlining possible sequences of events during phagocytosis. a.) Neutrophil encounters phagocytizable particle. b.) Particle attaches to one of the receptors on cell membrane, resulting in perturbed membrane. c.) A signal from perturbed membrane is transduced to a granule containing oxidase. Pseudopodia of the cell surround the particle in formation of the phagocytic vacuole. d.) The pseudopodia fuse and phagosome is formed from the plasma membrane. Membrane perturbation has been transduced into activation of granule bound oxidase. e.) Phagosome containing the bacterium is completely separated from plasma membrane. Granules fuse with the membrane of phagosome and deliver their contents into the phagosome. f.) Few granules left as most have undergone degranulation. Bacterium is killed and partially digested within phagosome. (Adapted from DeChatelet, 1978).

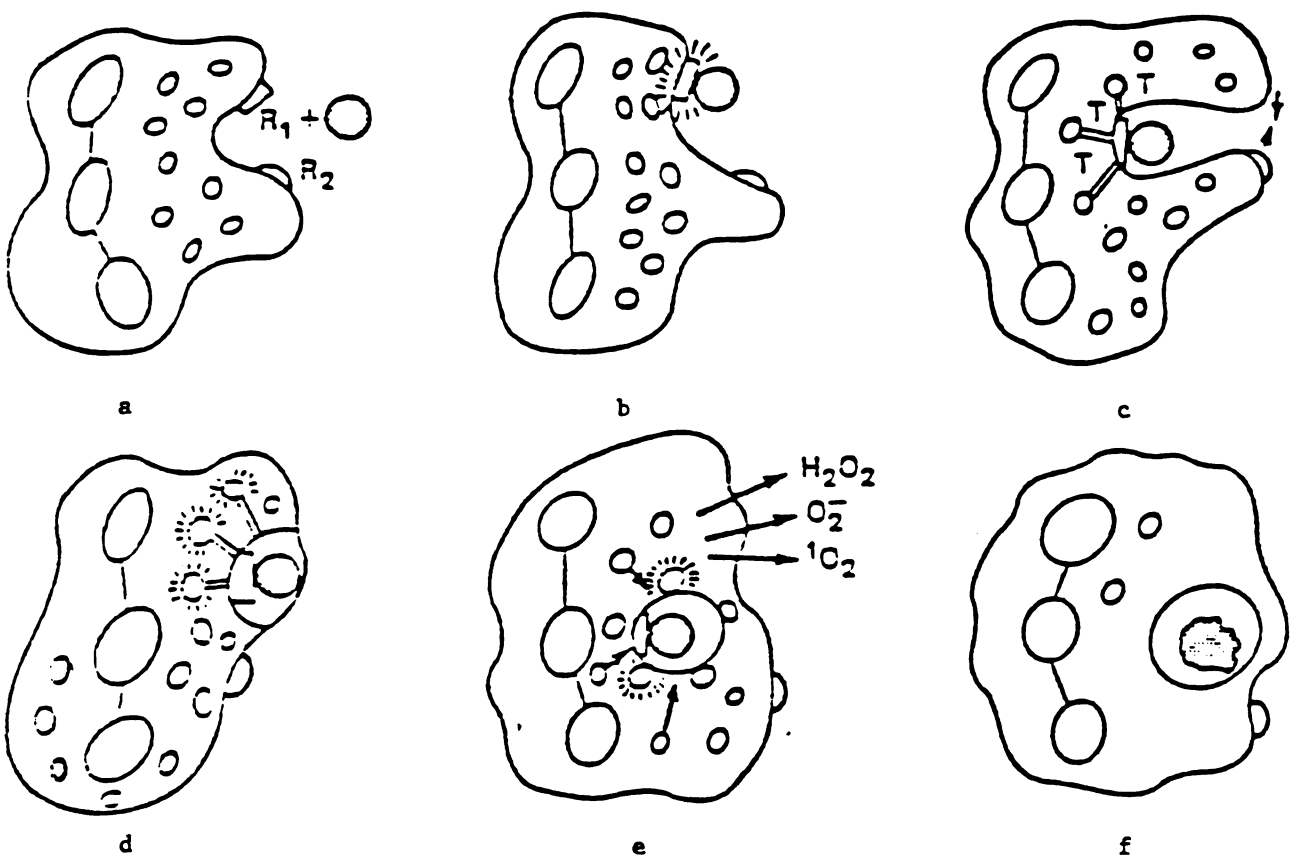


Figure 2.

granules, respectively. The azurophilic granules stain purplish with Wright's stain and constitute 10-20% of the granules. These granules are typical lysosomes which are responsible for digesting the carcasses of dead bacteria. The hydrolytic enzymes, β-glucuronidase, β-galactosidase, and acid cathepsin (Bainton et al., 1976), have optimal activity at pH 5 and collectively breakdown organic material into amino acids, sugars, and nucleotides. Moreover, two neutral proteases in these granules, myeloperoxidase and lysozyme may actually kill bacteria. Specific granules in neutrophils stain a pinkish color and consist of 80-90% of the total granules. Like the azurophilic granules, the specific granules also contain lysozyme, collagenase, alkaline phosphatase, and lactoferrin (Baggiolini et al., 1970a and 1970b).

Bainton (1970) demonstrated that specific granules fuse with the phagosome before azurophilic granules. Surface-bound aggregated gamma-globulin on rabbit neutrophils stimulated specific granule release first (Henson, 1971). Bentwood and Henson (1980) concluded that mobilization and release of specific and azurophilic granules is controlled by separate intracellular mechanisms. Enzymes associated with specific granules are released selectively upon induction of PMA (Goldstein et al., 1975a), Con A (Hawkins, 1974) and calcium ionophor A23187 or Ca alone (Goldstein et al., 1974; Hoffstein and Weissmann, 1978). These observations imply specific granule degranulation is an

independent process from phagocytosis and can occur without the release of hydrolytic enzymes from azurophilic granules (Bentwood and Hensen, 1980).

An alternative hypothesis would be that neutrophils may respond to various stimuli through a modulated sequence of responses. The multistep response would include depolarization (Korchak and Weissmann, 1978), respiratory burst (Henson and Oades, 1975), intracellular Ca distribution (Hoffstein, 1977), cytoskeletal changes (Hoffstein et al., 1977), and the release of varying amounts of specific and/or azurophilic granules. Other factors influencing the neutrophils response may be concentration of foreign object, particle size, and degree of valency of soluble and membrane-bound components.

III. Respiratory Burst

The respiratory burst represents changes in oxidative metabolism that occur during phagocytosis. When phagocytes are exposed to certain stimuli, their oxygen consumption increases up to 50 fold, they produce large quantities of superoxide and hydrogen peroxide, and they increase the oxidation of glucose by the hexose monophosphate shunt. The purpose of the respiratory burst is to generate toxic oxidants which are powerful microbicidal agents. Neither phagocytosis nor degranulation are required to initiate the respiratory burst (Curnutte et al., 1979). Simply,

initiation requires contact between the foreign object and the phagocyte.

The enzyme that apparently initiates the respiratory burst upon neutrophil stimulation is an NADPH oxidase (Babior, 1978). This enzyme system is dormant in resting cells, but in activated cells, catalyzes the reduction of oxygen to superoxide. The reaction can be summarized as follows:

$$2O_2 + NADPH + H+ \rightarrow NADP+ + 2H+ + 2O_2- \quad (6)$$

The physiological electron donor is NADPH with a Km 0.025-0.05 mM, but the enzyme can also use NADH Km 0.5-1.0 mM (Kakinuma and Kaneda, 1982). For maximal activity, the enzyme is FAD dependent and is pH dependent with a broad optimum near neutrality (Gabig and Babior, 1979). A cytochrome b which is uniquely found in phagocytes (Segal et al., 1983) has been implicated in the NADPH-dependent superoxide generating system, although the mechanism has not been worked out (Gabig et al., 1982). NADPH oxidase is located in the plasmalemma (DeWald et al., 1979). This location optimizes the delivery of oxidants onto the microorganisms.

McPhail and coworkers (1984) demonstrated that NADPH oxidase can be regulated by at least three different messengers. The priming (signal 1) does not cause activation but rather forms an intermediate that is required for oxidase activation by signal 2. Activation (signal 2) of the oxidase is induced by the concomitant priming of

signal 1 and increased concentration of stimuli. Inactivation (signal 3) results in the inactivation of oxidase. Some stimuli (A23187 and fMet-Leu-Phe) result in cessation of oxidase activity whereas PMA does not. It is possible that more than one intracellular signal determines the type of effect elicited and/or that an enzyme cascade could activate/inactivate the oxidase (Chock and Stadtman, 1977; Rasmussen, 1982).

IV. Bactericidal Effects

The products of the respiratory burst, superoxide and hydrogen peroxide are not bactericidal agents used by phagocytes. However, superoxide reacts with itself in a dismutation reaction to form hydrogen peroxide and oxygen. The rate of the reaction occurs faster at lower pHs, which means the dismutation reaction is favored in the phagosome. Hydrogen peroxide has weak antimicrobial properties and superoxide is innocuous (Babior, 1978). Instead, these products are intermediate for the true microbicidal products, oxidized halogens and oxidizing radicals. Phagocytes have a number of killing functions and back up systems which can incapacitate and eliminate a wide variety of microbes (Klebanoff and Clark, 1978).

Myeloperoxidase is the most important enzyme involved in producing oxidized halogens (Klebanoff and Clark, 1978). The reaction that produces hypochlorite is catalyzed by

myeloperoxidase as follows:

$$Cl^- + H_2O_2 \rightarrow OCl^- + H_2O \quad (7)$$

Myeloperoxidase is stored in the azurophilic granules of neutrophils and is released into the phagosome during the course of phagocytosis. It reacts with hydrogen peroxide produced during the respiratory burst to form hypochlorite (OCl^-). Physiologically, I^- and Br^- can be substituted into this system, however Cl^- is probably the major halide source utilized. Hypochlorite is an extremely effective microbicidal agent in killing bacteria, yeast, mycoplasma, viruses, and tumor cells (Klebanoff and Clark, 1978). Another oxidized halogen, chloramine, is formed from hypochlorite and ammonia or amines.

Among the class of oxidizing radicals is the hydroxyl radical. Habor and Weiss (1934) proposed that the radical could be formed by the reduction of hydrogen peroxide by superoxide.

$$O_2^- + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2 \quad (8)$$

However, while this reaction is thermodynamically possible, it probably occurs too slowly in biological systems to be significant (McCord and Day, 1978). When Fe serves as a redox catalyst, the rate constant is increased from 10^{-4} to 3.4 $M^{-1}sec^{-1}$ to 1,000 $M^{-1}sec^{-1}$ (McCord and Day, 1978). This reaction is now known as the Fenton reaction. Evidence that supports the hydroxyl radical production by neutrophils during phagocytosis comes from studies which indicate that bactericidal activities are inhibited by superoxide

dismutase, catalase, and scavengers of hydroxyl radical (mannitol, benzoate, and ethanol) (Johnson et al., 1975; Tauber and Babior, 1977; Weiss et al., 1977).

Singlet oxygen has been reported to be produced by the myeloperoxidase-hydrogen peroxide-halide antimicrobial system (Piatt et al., 1977; and Rosen and Klebanoff, 1977). The formation of singlet oxygen results from the interaction of hypochlorite and hydrogen peroxide (Kearns, 1971). Much criticism has been raised against the formation of singlet oxygen in the phagocytic cell (Harrison et al., 1978; and Held and Hurst, 1978).

Two oxygen metabolites, singlet oxygen and hydroxyl radical have been implicated in the microbial killing mechanisms of lipid peroxidation (Lynch and Fridovich, 1978). The hydroxyl radical can initiate lipid peroxidation of unsaturated fatty acids by removing hydrogen atoms from the allylic position to yield hydroperoxides (Pryor and Tang, 1978). Singlet oxygen directly attacks PUFA to form hydroperoxide (Kellogg and Fridovich, 1975). Lipid peroxidation can result in severe damage to both the host and the microorganism. Cellular membranes and organelles as well as associated enzymes become oxidized in lipid peroxidation (Tappel, 1973).

Chemiluminescence

The oxygen metabolites produced during the phagocytic process of neutrophils emit a burst of light or chemiluminescence (CL) as first described by Allen et al. (1972). The reactive oxygen species include superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Cheson et al., 1976; Klebanoff and Clark, 1978) which are believed to either directly contribute to light emission (as reactive species go to ground state as in the case of singlet oxygen) or indirectly by the oxidation of constituents of ingested opsonized particles (Cheung et al., 1983). Luminol (5-amino-2,3-dehydro-1,4 phthalazinedione) is a compound that can be used to amplify the CL response (Allen and Loose, 1976; Prendergast and Proctor, 1981); therefore, it lowers the the number of cells required for a response (Stevens et al., 1978), obviates the need for dark-adapted conditions (Prendergast and Proctor, 1981) and allows for whole blood samples to be used (Faden and Maciejewski, 1981).

The question to be answered is "What causes the CL of phagocytizing cells?" Hydrogen peroxide and superoxide play a part in killing of bacteria and so do hydroxyl radical and singlet oxygen. But questions remain, "Which of these oxidants if any are involved in the CL system and what reaction(s) cause CL?" To answer such questions,

investigators have employed the use of inhibitors of a particular species.

Inhibition of CL of phagocytic cells has been demonstrated with SOD, catalase and benzoate. This led to the proposal that CL is a result of the generation of hydrogen peroxide, superoxide, hydroxyl radical and singlet oxygen from the activated membrane-bound NADPH system (Cheson et al., 1976; Klebanoff and Clark, 1978). Nevertheless, these oxidizing agents themselves do not luminesce as demonstrated by their production in the xanthine oxidase-purine system or in fluoride-stimulated neutrophils (Cheson et al., 1976). The oxidation of zymosan by oxidizing agents may account for light emisson (Cheson et al., 1976). But the inability of SOD and catalase to completely inhibit CL has not been elucidated (Allen et al., 1974). In addition, the CL produced by stimulated neutrophils from nonparticulated, nonphagocytosable agents (soluble immune complexes, Con A, PMA, and Ca ionophor A23187) has not been explained.

Arachidonic acid metabolism in neutrophils has been described as having CL potential (Smith and Weidemann, 1980; Van Dyke et al., 1981). Hume et al. (1981) reported that the luminol-dependent CL of rat thymocytes stimulated with Con A can be divided into glucose-dependent and glucose-independent segments. The glucose-dependent branch was inhibited by catalase, amobarbital, and hexose analogues implying that Con A may activate the NADPH oxidase system.

The reducing equivalents produced by the oxidation of glucose result in the dismutation of superoxide. On the other hand, the glucose-independent mechanism may be involved with AA metabolism in the conversion of hydroperoxy intermediates to hydroxy products by the lipoxygenase pathway. This is supported by the inhibition of CL by eicosa 5,8,11,14-tetraynoic acid but not indomethacin.

Cheung and coworkers (1983) used AA metabolism inhibitors including nordihydroguaiaretic acid, 5,8,11,14-eicosatetraynoic acid, quinacrine, indomethacin, and aspirin to demonstrate the importance of AA metabolism on zymosan-induced CL. Their data concur with Smith and Weidemann (1980) suggesting that the glucose-independent component of CL is linked to AA metabolism via lipooxygenase (and cyclooxygenase) pathway(s). These investigators proposed a hypothesis linking the NADPH oxidase system and AA metabolism in the generation of luminescence.

The proposed mechanism is outlined in Figure 3 and is as follows. Zymosan binds to cell surface receptors activating the neutrophil via complement (Goldstein et al., 1975b). The neutrophil membrane is perturbed resulting in the release of AA from membrane phospholipids (Walsh et al., 1981). Membrane phospholipase A_2 is the key enzyme which releases AA from membrane phospholipids (Franson et al., 1980). The oxidation of AA via lipoxygenase (and cyclooxygenase) produces CL. Concurrently, the membrane perturbation has resulted in the activation of NADPH oxidase

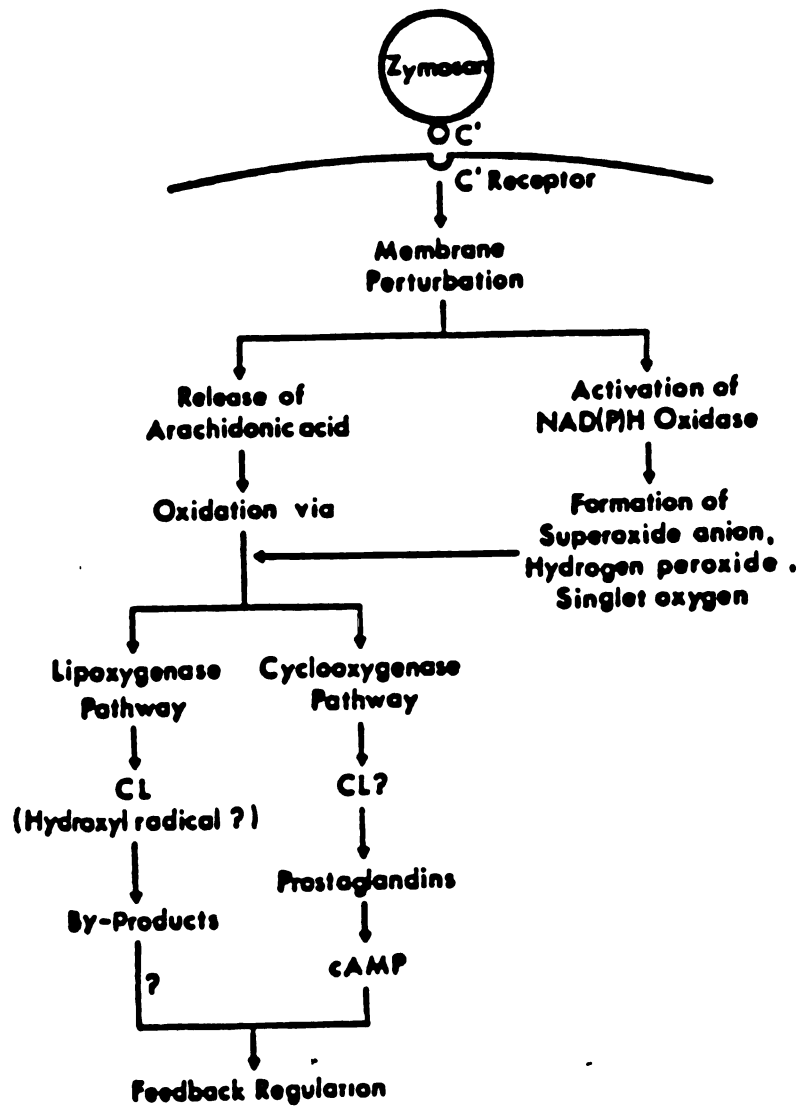


Figure 3. Schematic of a proposed mechanism to describe the origin and regulation of CL in neutrophils phagocytosing zymosan. (From Cheung et al., 1983).

and the generation of superoxide, hydrogen peroxide, and singlet oxygen. To tie the two systems together, the researchers proposed that oxygen metabolites act as oxidants of AA and its derivatives (Van Dyke et al., 1979). The two systems in combination amplify the magnitude of CL produced. Arachidonic acid metabolites, metabolized via the cyclooxygenase pathway, result in the production of prostaglandins. Prostaglandins are known to stimulate the production of cAMP (Weissmann et al., 1980). Cheung et al. (1983) have proposed cAMP to be the feedback regulator of CL via either AA metabolism or NADPH oxidase system. If this hypothesis is true, it would explain the inhibitory effect NaF has on zymosan-induced CL. NaF is a known stimulator of cAMP (White et al., 1973).

Luminol has been demonstrated to chemiluminesce upon reaction with the oxygen species generated during the respiratory burst (Allen and Loose, 1976). This compound was originally used to detect free-radical production by xanthine oxidase (Totter et al., 1960) because of its high quantum efficiency (Isacsson and Wettermark, 1974). In the presence of oxidizing species, luminol is converted to an excited aminophthalate anion that relaxes to ground state with the production of light (Allen and Loose, 1976). This reaction emits blue light (425 nm) as illustrated in Figure 4 (White et al., 1964). The advantage of the luminol-enhanced CL system is that small amounts of blood can be used.

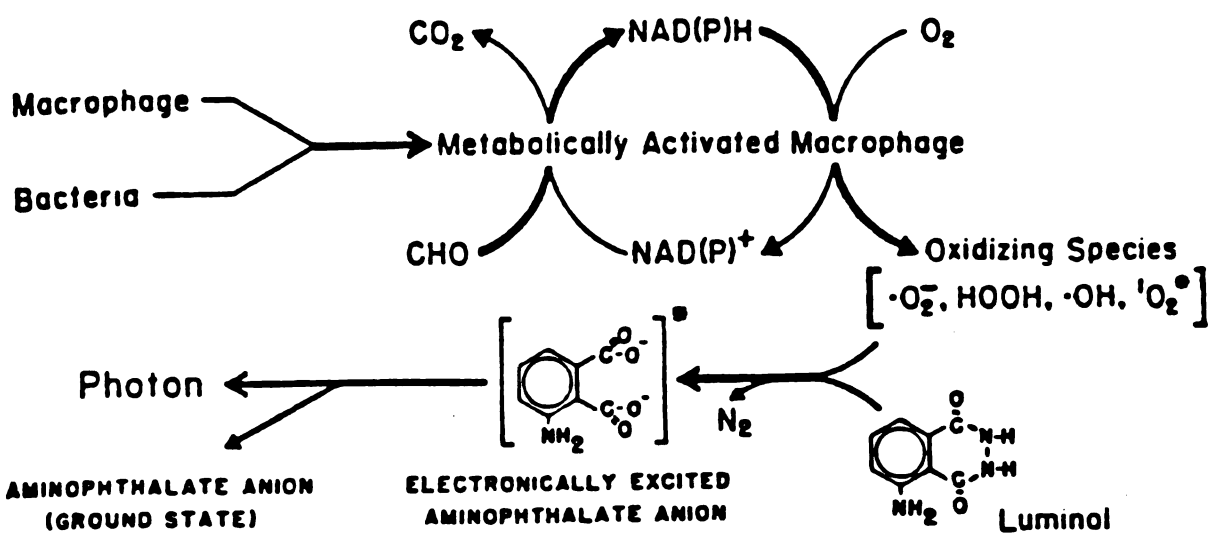


Figure 4. Schematic description of the proposed mechanism to describe luminol-mediated chemiluminescence from the various phagocytes (From Allen and Loose, 1976).

DeChatelet and coworkers (1982) investigated the mechanism of the luminol-dependent CL response in human neutrophils. They hypothesized from their data and data of others (Faden and Maciejewski, 1981; Rosen and Klebanoff, 1976; Stevens et al., 1978) that nonenhanced CL and luminol-enhanced CL response were produced by different mechanisms. The nonenhanced system was dependent equally upon myeloperoxidase (MPO) and superoxide (Cheson et al., 1976). By contrast, the enhanced system relies entirely on the MPO reaction, probably due to the low number of cells producing measurable amounts of superoxide. DeChatelet et al. (1982) concluded that HOCl, which is produced in the MPO-hydrogen peroxide-Cl- system, is responsible for the oxidation of luminol at physiological pH. They suggest HOCl diffuses outside of the cell to react with luminol.

METHODS

Three trials were designed to evaluate swine neutrophils phagocytic and killing abilities in relation to their vitamin E and selenium status. Trials 1 and 2 were 2 x 2 factorials in a split plot design where either a basal diet or diets supplemented with 100 IU vitamin E/kg diet or 1.0 ppm selenium or 100 IU vitamin E/kg diet and 1.0 ppm selenium were fed. Trial 3 was 3 treatments in a split plot design where either a basal diet or diets supplemented with 100 IU vitamin E/kg diet or 1000 IU vitamin E/kg diet were fed. In this trial, 0.1 ppm selenium was supplemented to all the diets. Chemiluminescence was preformed in each trial to determine the phagocytic and killing abilities of the neutrophils from each treatment. In addition, the tocopherol and selenium status were assessed.

I. Animals

A. Trial I

Twelve pigs from two litters were used from sows fed gestation and lactation diets which were not supplemented with vitamin E and selenium. The pigs averaging 18.9 kg were randomly assigned from litters to one of four treatments in a 2 x 2 factorial repeated measures design.

Experimental diets included a basal diet with no supplemental vitamin E or selenium, basal + 100 IU vitamin E/kg diet, basal + 1.0 ppm selenium, and basal + 100 IU vitamin E/kg + 1.0 ppm selenium. See Table 1 for diet composition and Table 3 for analyzed values of vitamin E and selenium.

Because it was difficult to perform the chemiluminescence (CL) procedure on more than 4 pigs daily, one pig from each of 4 treatments formed a quadruplet which was bled on the same day in subsequent bleedings. Blood samples were collected in 10 ml monovettes (Sarstedt) precoated with EDTA anticoagulant using an 18 gauge, 1 1/2 inch needle from three sets of quadruplets at 0, 2, 4, and 10 weeks on experiment. In the first three collections, pigs were inverted in a wooden trough and bled from the anterior vena cava. Pigs were snared in the last collection because they were too large to place on their backs. Whole blood was used for CL in the first three collections, while separated neutrophils (PMNs) were used for CL at 10 weeks. This was done because potentially there is less day to day variation with separated cells than with whole blood.

Plasma was harvested from each sample and stored at -20 ^{o}C for vitamin E and selenium analysis at a later date. White blood cell counts and glutathione peroxidase activities for both whole blood and plasma were performed the day of collection.

Table 1. Composition of Diets for Trials 1 and 2

Ingredient	Basal	+100 IU Vit E/kg	+1.0 ppm Se	+Vit E & Se
Corn	780	780	780	780
Soybean meal (44% CP)	190	190	190	190
Salt	5	5	5	5
Limestone (38% Ca)	10	10	10	10
Dicalcium phosphate	10	10	10	10
VTM premix[a]	5	5	5	5
Se 90 premix (200 mg Se/kg)	--	--	5	5
Vit E (50% E)	--	0.2	--	0.2

[a]Supplied per kg of diet:

Vitamin A	3300 IU
Vitamin D	660 IU
Menadione Na bisulfite	2 mg
Riboflavin	3 mg
Nicotinic acid	18 mg
D-pantothenic acid	13 mg
Choline	100 mg
Cyanocobalamin	12 µg
Zinc	75 mg
Manganese	37 mg
Iodine	0.5 mg
Copper	10 mg
Iron	60 mg

B. Trial 2

Trial 2 was conducted similarly to Trial 1 with the following exceptions. Twenty pigs averaging 14.5 kg from three litters were randomly assigned to the same four treatment groups resulting in five quadruplets. Twenty ml of blood were collected in a glass syringe and transferred to 50 ml polypropylene centrifuge tubes with 0.8 ml EDTA anticoagulant. Blood was collected at 0, 2, and 4 weeks on trial and the PMNs were separated for CL at each collection. Plasma was harvested and stored for analysis of vitamin E and selenium concentrations and glutathione peroxidase activity. A white blood cell count was performed on fresh blood. See Table 1 for diet composition and Table 3 for analyzed values of vitamin E and selenium.

C. Trial 3

Eighteen pigs from two litters averaging 21.6 kg were randomly assigned from litters to one of three treatments. One litter was on a vitamin E- and selenium-deficient diet from birth and their dam also had been on a deficient diet throughout gestation and lactation. The other litter and their dam had been on diets with adequate levels of vitamin E and selenium until the time of the experiment. The pigs were fed either a basal diet with no supplemental vitamin E, a basal plus 100 IU vitamin E/kg diet, or a basal plus 1000 IU vitamin E/kg diet. See Table 2 for diet composition and Table 3 for analyzed values of vitamin E.

Table 2. Composition of Diets for Trial 3

Ingredient	Basal	+100 IU Vit E/kg	+1000 IU Vit E/kg
Corn	780	780	780
Soybean meal (44% CP)	190	190	190
Salt	5	5	5
Limestone (38% Ca)	10	10	10
Dicalcium phosphate	10	10	10
VTM premix[a]	5	5	5
Se 90 premix (200 mg Se/kg)	0.5	0.5	0.5
Vit E (50% E)	--	0.2	2

[a]Supplied per kg of diet:

Vitamin A	3300 IU
Vitamin D	660 IU
Menadione Na bisulfite	2 mg
Riboflavin	3 mg
Nicotinic acid	18 mg
D-pantothenic acid	13 mg
Choline	100 mg
Cyanocobalamin	12 µg
Zinc	75 mg
Manganese	37 mg
Iodine	0.5 mg
Copper	10 mg
Iron	60 mg

Table 3. Selenium and Vitamin E Analysis of the Experimental Diets

	Level Supplemented, air dry basis		Level Analyzed, dry matter basis	
	Vit E IU/kg diet	Se ppm	Vit E IU/kg diet	Se ppm
Trial 1				
Basal	0	0	4.78	0.06
+Vit E	100	0	70.86	0.06
+Se	0	1.0	38.56	0.78
+Vit E +Se	100	1.0	53.07	0.76
Trial 2				
Basal	0	0	11.56	0.09
+Vit E	100	0	560.38	0.06
+Se	0	1.0	10.10	0.89
+Vit E +Se	100	1.0	42.47	0.84
Trial 3				
Basal	0	0.1	2.09	--
+Vit E	100	0.1	44.62	--
+Vit E	1000	0.1	948.09	--

Animals were grouped by six, two on each treatment and of those, one from each litter was to be bled on a common day. Blood samples were drawn from each group of six pigs at 3, 6, and 9 weeks on trial and handled in the same manner as in Trial 2. The pigs were held in a trough for the first two samplings and snared for the last sampling. CL was performed on separated PMNs, white blood cells were counted in fresh blood samples, and plasma was stored for later analysis of alpha-tocopherol.

II. Chemiluminescence

A. Isolation of Neutrophils

The isolation procedure is one adapted from that of Boyum (1968). Live cells need to be handled gently and therefore all mixing must be done with care. Mixing was achieved by carefully submerging a transfer pipet without introducing air bubbles into the mixture and squeezing the bulb several times without lifting the tip. Five ml of blood were diluted with an equal volume of Dulbecco's phosphated buffered saline (PBS). The mixture was then layered over 4 ml of Ficoll-Hypaque (5 g Ficoll and 2.7 g sodium diatrizoate in 100 ml DD water) (Ficoll MW 400,000; Sigma, St. Louis, MO 63178; Sodium Diatrizoate, Sigma) and centrifuged at 400 x g for 30 minutes at 5 ^{o}C. The supernate was discarded and the cells were resuspended with approximately 10 ml PBS. Four ml of 3% dextran (500,000 MW;

Sigma) was added to sediment the erythrocytes at room temperature for 30 minutes. The supernate was washed with PBS and centrifuged at 200 x g for 10 minutes. Five ml of 0.83% NH_4Cl were used to resuspend the pellet, and the mixture was allowed to sit for 15 minutes to lyse the red blood cells. The PMNs were washed twice with PBS and checked for viability with the trypan blue exclusion test.

B. Preparation of Phosphated Buffered Saline

Phosphated buffered saline was prepared fresh daily from autoclaved deionized distilled water and the following recipe:

$CaCl_2$	0.1 g
KCl	0.2
KH_2PO_4	0.2
$MgCl_2 \cdot 6H_2O$	0.1
NaCl	8.0
Glucose	1.0
Na_2HPO_4	1.145

All chemicals except Na_2HPO_4 were dissolved using room temperature autoclaved water before adding the final ingredient to avoid precipitation. Water was added to bring the volume to one liter.

C. Chemiluminescence

Chemiluminescence was performed on a Isocap/300 Liquid Scintillation Counter, Model 6870 (Searle Analytic Inc., Des Paines, IL 60018) in the out-of-coincidence mode. Each

sample was run in triplicate with a total of four samples being run according to their assigned quadruplet. For trial one in week 0, 2, and 4, 0.9 ml PBS and 0.1 ml blood was run for 2 cycles for background counts. For week 10, 0.1 ml neutrophils (20,000/µl) were substituted for blood. Zymosan A particles opsanized with luminol (ZAP, United Technologies Packard, Downers Grove, IL 60519) (0.5 ml) were added to each vial and the luminescence of each vial was measured every 6 minutes for 10 seconds at least ten times. Vials were dark adapted and work was done in a darkened room with the aid of a red light to avoid excessive light contamination. The counts were adjusted according to the number of cells, and background luminescence was subtracted.

Trial 2 was conducted in a similar manner. Separated neutrophils were used for all the CL determinations. The amount of PBS was changed to 1.15 ml and zymosan A was changed to 0.25 ml. The volume of cells remained at 0.1 ml.

Trial 3 consisted of six samples on each day of drawing blood counted for 10 seconds every 6 minutes. Luminol (3-aminophthalhydrazide; Aldrich Chemical Co., Milwaukee, WI 53233). was dissolved in dimethyl sulfoxide and diluted with PBS for a final concentration of 2 µg luminol/ml. Zymosan A (Sigma) was opsanized with fresh swine plasma (deficient in vitamin E and selenium) at 37 ^{o}C for 30 minutes and washed with PBS. The final concentration was 3 mg zymosan/ml. One ml PBS, 0.1 ml luminol, and 0.3 ml opsanized zymosan were checked for background luminescence

counts. The PMN concentration was adjusted to approximately 20,000 cells/ml and 0.1 ml cells were added to the vials. The reaction was allowed to procede for 90 minutes.

Each sample was averaged for CL response at each data point and plotted against time. The area under the curve was calculated using an electronic board and a digitizing program written by William Enslin from the Remote Sensing Center at Michigan State University. Each point was registered on the electronic board and the coordinates were used to calculate the area under the curve.

III. Plasma Analysis

The whole blood was centrifuged at 2050 x g for 15 minutes. The plasma was harvested into labeled, 5 ml polystyrene tubes, and the air space was displaced with nitrogen. The samples were frozen and stored at -20 ^{o}C for later analysis.

A. Alpha-tocopherol

Plasma alpha-tocopherol concentration was determined either by a fluorometric procedure (Trials 1 and 2) developed by Whetter and Ku (1982) from a tissue alpha-tocopherol procedure (Taylor, 1976) or by high pressure liquid chromotography (Trial 3). The fluorometric procedure was done in the following manner. Standards (Eastman Kodak Co., Rochester, NY 14650) were prepared from a stock standard solution in absolute ethanol and diluted with

absolute ethanol to obtain concentrations of 0, 1, 2, and 4 µg alpha-tocopherol/ml. Distilled deionized water was added for a final volume of 1 ml and the standards were then processed in the manner as the plasmas. Duplicate samples were prepared by the addition of 2 ml of absolute ethanol to each test tube followed by the addition of 1 ml plasma and purging with nitrogen. Tubes were vortexed for 5 seconds to precipitate the proteins and were then purged with nitrogen.

To extract the alpha-tocopherol, 2 ml cyclohexane (Eastman Kodak, AR grade) was added to each tube, and the tube was purged with nitrogen and vortexed for 20 seconds. Samples were centrifuged at 2070 x g for 15 minutes in a Damon/IEC model PR-6000 refrigerated centrifuge. The top layer containing cyclohexane and alpha-tocopherol was removed to a dram vial for spectrophotofluorometric reading. Fluorescence of alpha-tocopherol was determined by excitation at 296 nm and emission at 330 nm in an Aminco-Bowman spectrophotofluorometer SPF-125 (American Instrument Co., Urbana, IL 61801).

Tocopherol concentrations were determined by regressing the transmission (%T) of the standards against the known µg of alpha-tocopherol in the standards in a curvilinear program. The %T of the unknowns was used to find the concentration of alpha-tocopherol in µg/ml plasma.

In trial 3 alpha-tocopherol was determined by reverse phase HPLC. Standards were prepared from a stock standard of alpha-tocopherol (Eastman Kodak) for a final

concentration of 10 µg/ml in methanol (HPLC grade). The internal standard, apocarotenal (courtesy of Dr. H. N. Bhagavan, Hoffmann-La Roche Inc.), was prepared from a stock solution for a final concentration of 0.1 µg/ml. One ml methanol (HPLC grade) was added to each tube to be used for the unknowns and the appropriate amount of methanol was added to the standards for a final volume of 1 ml. Two hundred µl of saturated ascorbate solution and 100 µl of apocarotenal solution were added to all tubes. Zero, 100, 200, or 400 µl of working standard was added to the standard tubes. Deionized distilled water was added to the standards (0.5 ml) and 0.5 ml of plasma in duplicate was added to the unknown tubes.

Care was used not to oxidize the tocopherol. Each tube was purged with nitrogen and capped. The tubes were vortexed for 10 seconds, 3 ml 0.05% BHT hexane added, purged with nitrogen, and vortexed again for 1 minute. Complete separation of the layers was accomplished by centrifugation at 2070 x g for 10 minutes. The hexane layer was transferred to 25 ml Erlenmeyer flasks and evaporated in a cold vacuum oven leaving only the tocopherols in the flasks. The tocopherols were picked up in 1 ml of methanol and filtered through Millex-HV filter units (0.45 µ) (Millipore) into 2 dram vials, purged with nitrogen, and capped.

The samples were read on a Waters and Associates, Inc. (Milford, MA) HPLC instrument. The system included a Model

45-M solvent delivery system, a Model U6K universal liquid chromatograph injector, and a Model 440 absorbance detector. The recorder (Servogor 120) was set at 0.25 cm/min. The column was a Bondapak C_{18} reverse phase, 3.9 mm x 15 cm column and the precolumn was a RCSS Guard-Pak (C_{18}). The mobile phase, 95:5 methanol:water, was pumped at the rate of 1.5 ml/min. A curvilinear standard regression line was used to calculate the alpha-tocopherol concentration as described for the fluorometric determination. The alpha-tocopherol peak height, as determined by retention time compared to the standard, was divided by the internal standard peak height to adjust for experimental error.

B. Selenium

Plasma selenium concentrations were determined by a spectrofluorometric procedure (Whetter and Ullrey, 1978). Duplicate standards of 0, 0.025, 0.05, 0.1, 0.2, and 0.3 µg selenium/ml and duplicate samples of 1 ml of plasma were digested in 2 ml nitric acid and 2 ml 70% perchloric acid. The nitric acid was driven off and 9 ml ethylene diamino tetraacetic acid (EDTA) (14.2 g/l) was washed down the sides of the flasks to chelate the other metals. Approximately 0.8 ml sodium hydroxide was used to neutralize the perchloric acid. Cresol red was added as an indicator and pH was adjusted with drops of concentrated sodium hydroxide or 1.2 N hydrochloric acid to obtain an orange color.

Selenium was complexed by the addition of 5 ml 2, 3-diaminonaphthalene (1 mg/ml 0.12 N HCl) to form diazoselenol, a light sensitive complex. This complex was extracted with 5 ml cyclohexane and transferred to a test tube for reading in the Aminco-Bowman spectrophotofluorometer at 376 nm excitation and 510 nm emission. Selenium concentration was calculated using a curvilinear standard regression line.

C. Glutathione Peroxidase

Plasma glutathione peroxidase (GSH-Px) activity was determined by the coupled method of Paglia and Valentine (1967) as revised by Lawrence et al. (1974). This assay is based on the spectrophotometrically measured oxidation of a known amount of nicotinamide adenine dinucleotide phosphate (NADPH). Stoichiometric amounts of NADPH, reduced glutathione and glutathione reductase along with an unknown amount of GSH-Px were allowed to react in a phosphate buffer with hydrogen peroxide as substrate for initating the reaction. Glutathione peroxidase activity was expressed as EU/g protein.

The mixture included 0.05 ml reduced glutathione (0.05 ml; 12.3 mg/ml), 0.925 ml phosphate buffer, 0.025 ml plasma and 0.01 hydrogen peroxide (0.124 ml 30% hydrogen peroxide) and was incubated at 25 ^{o}C. The reaction was allowed to proceed for 3 min at A_{340} nm on a Beckman DU-Gilford spectrophotometer (Gilford Instrument Laboratories Inc.,

Oberlin, OH 44074) and recorded on a Varian Model 9176 chart recorder. Enzyme units were calculated as umoles glutathione oxidized per minute according to the molar extinction coefficient for NADPH of 6.22×10^3 and the stoichiometry of the reaction of 2 moles glutathione formed per mole NADPH oxidized. Triplicates change in A_{340}/min were averaged and the change in A_{340}/min for the blank was subtracted and the sum was multiplied by 12.86 (a factor derived from the sample amount and the stoichiometric amounts of the reactants) to obtain the EU/ml plasma.

IV. Whole Blood

A. Glutathione Peroxidase

Whole blood samples were basically analyzed the same way with a few modifications. Triplicates of 0.025 ml whole blood were each diluted in 1 ml distilled deionized H_2O. Diluted blood (0.05 ml) was used in place of the plasma. The factor used to obtain the EU was 263.67 and was related to hemoglobin (HB) concentration yielding EU/g HB for final units.

B. White Blood Cell Count

In trial one, the white blood cells were counted with a microscope in an improved Neubauer hemacytometer. Whole blood was diluted 1:20 dilution using Unopettes (Becton-Dickinson, Rutherford, NJ 07070). Duplicates were averaged and the number of cells counted was multiplied by 50 to

account for the volume counted and expressed in total leukocyte counts per µl.

In trials two and three, the white blood cells were counted on a Coulter Counter Model Z_{B1} (Coulter Electronics, Inc.). Blood was first diluted in isotonic buffered saline (American Scientific Products, McGraw Park, IL 60085) with a Coulter Diluter II (Coulter Electronics, Inc.). Red blood cells were lysed with lysing and hemoglobin reagent (American Scientific Products) and then counted in the Coulter Counter. The total count was corrected by a coincidence factor obtained from a correction chart.

IV. Chemiluminescence Precision Experiment

The day to day and within day precision was measured. One pig was bled on four different days and its blood was aliquotted into four subsamples. Chemiluminescence was performed in the same manner as for Trial three except the final concentration of luminol was 1 µg/ml.

V. Statistics

Trials 1 and 2 were 2 x 2 factorial split plot design. Trial 3 was a three treatment split plot design. Multianalysis of variance was performed on each measure using SAS (1982).

RESULTS

I. Trial 1

Pigs on diets not supplemented with vitamin E had lower ($P < 0.0001$) plasma tocopherol levels (Table 4). Plasma tocopherol concentrations generally increased over time ($P < 0.0001$) and there was an interaction between time and dietary vitamin E ($P < 0.0001$). In diets supplemented with vitamin E, plasma tocopherol concentrations increased over time; whereas, in the unsupplemented diets, plasma tocopherol concentrations changed very little (Figure 5).

Selenium supplementation resulted in increased ($P < 0.0001$) plasma selenium concentrations in Trial 1 (Table 4). Vitamin E supplementation also increased ($P < 0.14$) plasma selenium concentrations. There was an interaction between dietary selenium and dietary vitamin E ($P < 0.05$). All treatment groups had similar initial plasma selenium values while those pigs receiving selenium-supplemented diets had higher increases in plasma selenium over time ($P < 0.0001$). Also, there was an interaction between dietary selenium and time on plasma selenium levels ($P < 0.0001$). In addition, there was an interaction of time, dietary selenium, and dietary vitamin E on plasma selenium concentrations ($P < 0.02$).

Table 4. Effects of Dietary Vitamin E and Selenium Supplementation on Plasma Measures over Time for Trial 1

	Time						
Diet	Initial	Week 2	Week 4	Week 10	AVG	MSE	P
			Plasma Tocopherol, µg/ml				
						0.33	0.0001
Basal	0.42	0.57	0.34	2.18	0.88		
B + Vit E	0.97	1.69	3.90	4.77	2.83		
B + Se	0.56	1.34	0.46	1.20	0.89		
B+Vit E+Se	0.87	2.53	3.40	4.89	2.92		
AVG	0.70	1.53	2.02	3.26	1.88		
			Plasma Selenium, ng/ml				
						221.4	0.0001
Basal	53.5	57.0	70.5	103.0	71.0		
B + Vit E	51.9	42.5	71.2	107.6	68.3		
B + Se	51.0	117.5	181.0	194.0	135.9		
B+Vit E+Se	46.8	188.6	176.7	187.8	150.0		
AVG	50.8	101.4	124.8	147.9	106.2		
			Plasma GSH-Px, EU/g protein				
						4.65	0.05
Basal	7.53	6.21	5.98	12.68	8.10		
B + Vit E	7.12	4.49	6.84	10.44	7.22		
B + Se	8.01	10.72	9.18	12.29	10.05		
B+Vit E+Se	5.77	10.41	9.11	13.54	9.71		
AVG	7.11	7.96	7.78	12.24	8.77		
			Whole Blood GSH-Px, EU/g HB				
						278.2	0.02
Basal	38.2	38.1	41.9	43.8	40.5		
B + Vit E	40.9	37.7	34.9	35.8	37.3		
B + Se	32.2	46.1	64.2	60.8	50.8		
B+Vit E+Se	46.2	64.4	85.5	73.5	67.4		
AVG	39.4	46.6	56.6	53.5	49.0		

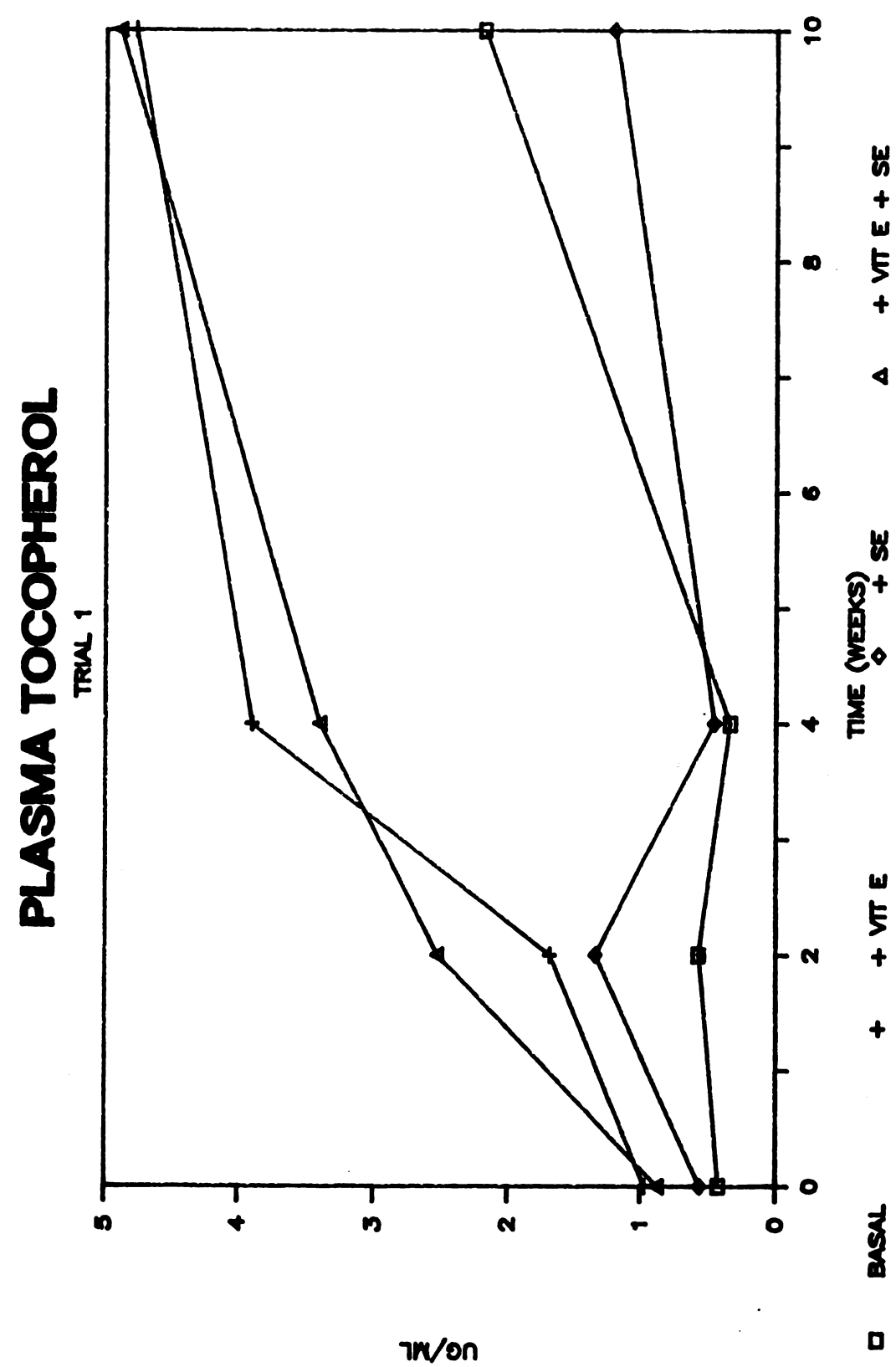


Figure 5. Comparison of plasma tocopherol concentration by treatment over time for Trial 1

Selenium supplementation resulted in increased ($P < 0.05$) plasma glutathione peroxidase (GSH-Px) activity (Table 4). Initial plasma GSH-Px activities were similar among treatment groups but selenium supplementation resulted in increased ($P < 0.0001$) plasma GSH-Px activities over time. One litter had higher ($P < 0.1$) enzyme activities than the other. There was an interaction ($P < 0.05$) between time and litter. Also there was an interaction ($P < 0.03$) between time and selenium influenced GSH-Px activities.

Selenium supplementation resulted in increased ($P < 0.02$) white blood cell counts (Table 5). Also, white blood cell counts increased ($P < 0.04$) over time for all treatments, but there was not a significant selenium by time interaction on leukocyte count.

Vitamin E and selenium supplementation did not result in a significant difference in chemiluminescence (CL) among treatments (Table 5 and Figure 6). The data from week 10 was not included in the analysis because a different methodology was used for this week. However, values for this week were analyzed within this time period and included for comparison purposes.

Without including week 10, there was a decrease in CL over time ($P < 0.01$) but dietary treatment did not interact with time. Bonferroni contrasts were made to test the effect of time on each separate treatment. The contrasts included a comparison between the initial week and week 2 and 4, between week 2 and 4, and between the initial week

Table 5. Effects of Dietary Vitamin E and Selenium Supplementation on Leukocyte Measures over Time for Trial 1

Diet	Time				AVG	MSE	P
	Initial	Week 2	Week 4	Week 10			
			White Blood Cell Count, WBC/µl				
						16302670	0.02
Basal	22000	19300	20935	19725	20490		
B + Vit E	16525	16890	18065	25165	19161		
B + Se	20968	23965	24443	28646	24506		
B+Vit E+Se	23104	20709	22017	29166	23749		
AVG	20649	20216	21365	25678	21976		
			CL, total cpm x 10^3/hour/2 x 10^6 PMN				
						3.66	0.006
Basal	6.656	2.472	3.905		4.344		
B + Vit E	6.349	3.172	2.975		4.165		
B + Se	4.796	3.241	2.599		3.545		
B+Vit E+Se	4.767	1.845	2.902		3.171		
AVG	5.642	2.682	3.095		3.806		
Basal				7.618			
B + Vit E				7.998			
B + Se				10.165			
B+Vit E+Se				9.662			
AVG				8.861			

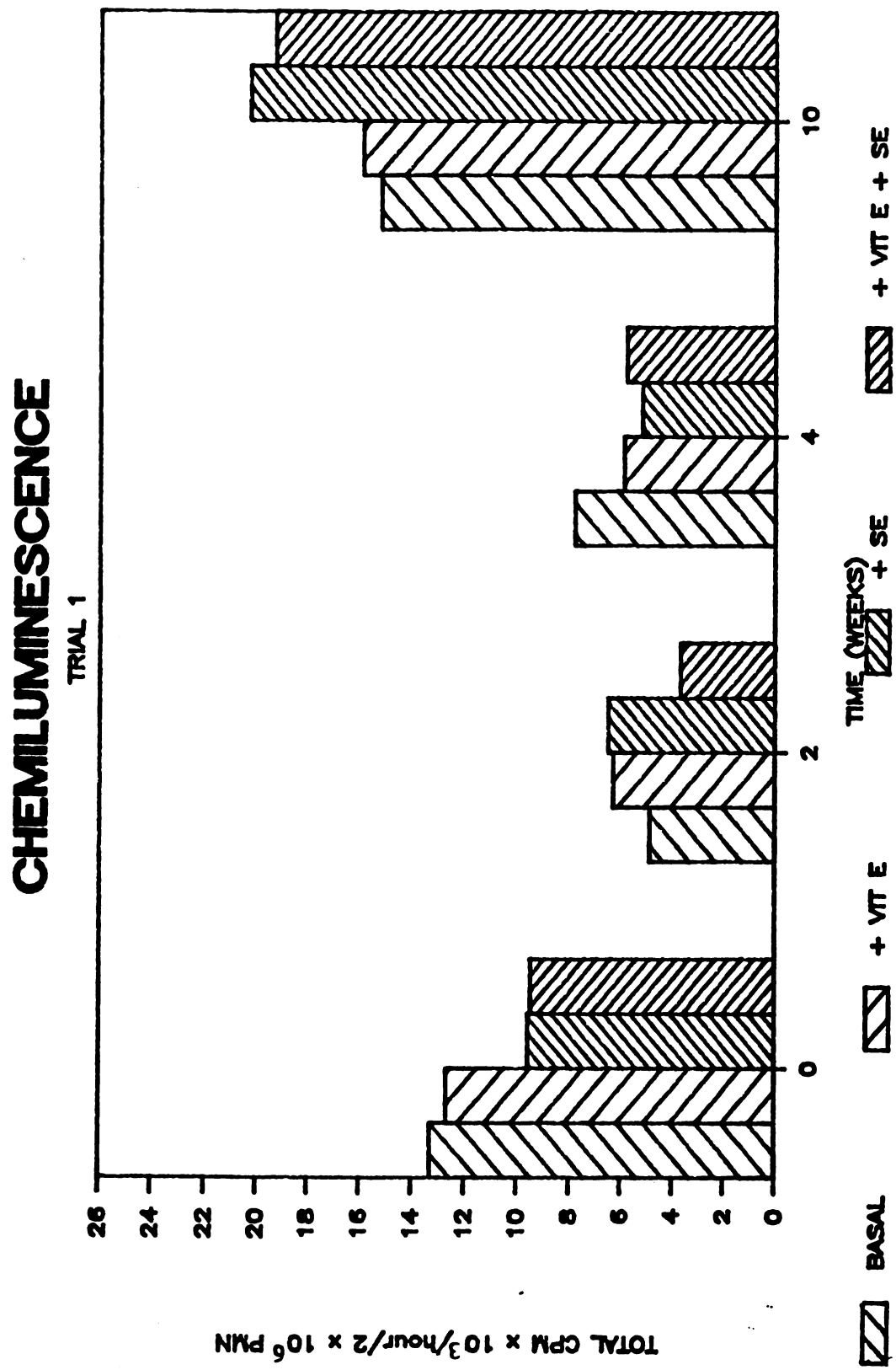


Figure 6. Comparison of CL by treatment over time for Trial 1

and week 4. For all dietary treatments except the diet supplemented with vitamin E, the initial time had higher ($P < 0.05$) CL than weeks 2 and 4. Dietary supplementations did not significantly influence CL between weeks 2 and 4. Dietary treatment indicated lower ($P < 0.05$) CL for week 4 than the initial week in the selenium supplemented group.

When week 10 (Table 5) was analyzed independently, there were no significant effects of dietary treatments on CL.

II. Trial 2

The plasma samples from week 2 were lost and therefore, could not be analyzed.

Plasma tocopherol concentrations were increased ($P < 0.001$) in pigs fed vitamin E-supplemented diets (Table 6 and Figure 7). Plasma tocopherol concentrations for all treatments increased ($P < 0.0001$) over time and there was a time by dietary vitamin E interaction ($P < 0.0001$) in that plasma tocopherol values of pigs fed diets not supplemented with vitamin E did not increase very much with time.

Supplementation of selenium increased ($P < 0.01$) plasma selenium concentrations (Table 6). There was a litter effect ($P < 0.05$) on plasma selenium concentrations and plasma selenium concentrations increased ($P < 0.0001$) over time for all treatment groups. The interaction of time and dietary selenium affected ($P < 0.05$) plasma selenium concentrations.

Table 6. Effects of Dietary Vitamin E and Selenium Supplementation on Plasma Measures over Time for Trial 2

	Time				
Diet	Initial	Week 4	AVG	MSE	P
	Plasma Tocopherol, µg/ml				
				0.34	0.0001
Basal	0.42	0.51	0.46		
B + Vit E	0.41	3.28	1.84		
B + Se	0.38	1.17	0.78		
B+Vit E+Se	0.25	3.59	1.92		
AVG	0.36	2.14	1.25		
	Plasma Selenium, ng/ml				
				462.4	0.01
Basal	60.2	112.4	86.3		
B + Vit E	61.1	106.6	83.8		
B + Se	59.6	137.5	98.6		
B+Vit E+Se	73.7	154.6	114.2		
AVG	63.6	127.8	95.7		
	Plasma GSH-Px, EU/g protein				
				4.93	0.01
Basal	9.05	10.12	9.58		
B + Vit E	7.25	10.77	9.01		
B + Se	7.24	11.97	9.60		
B+Vit E+Se	11.00	13.32	12.16		
AVG	8.64	11.54	10.09		

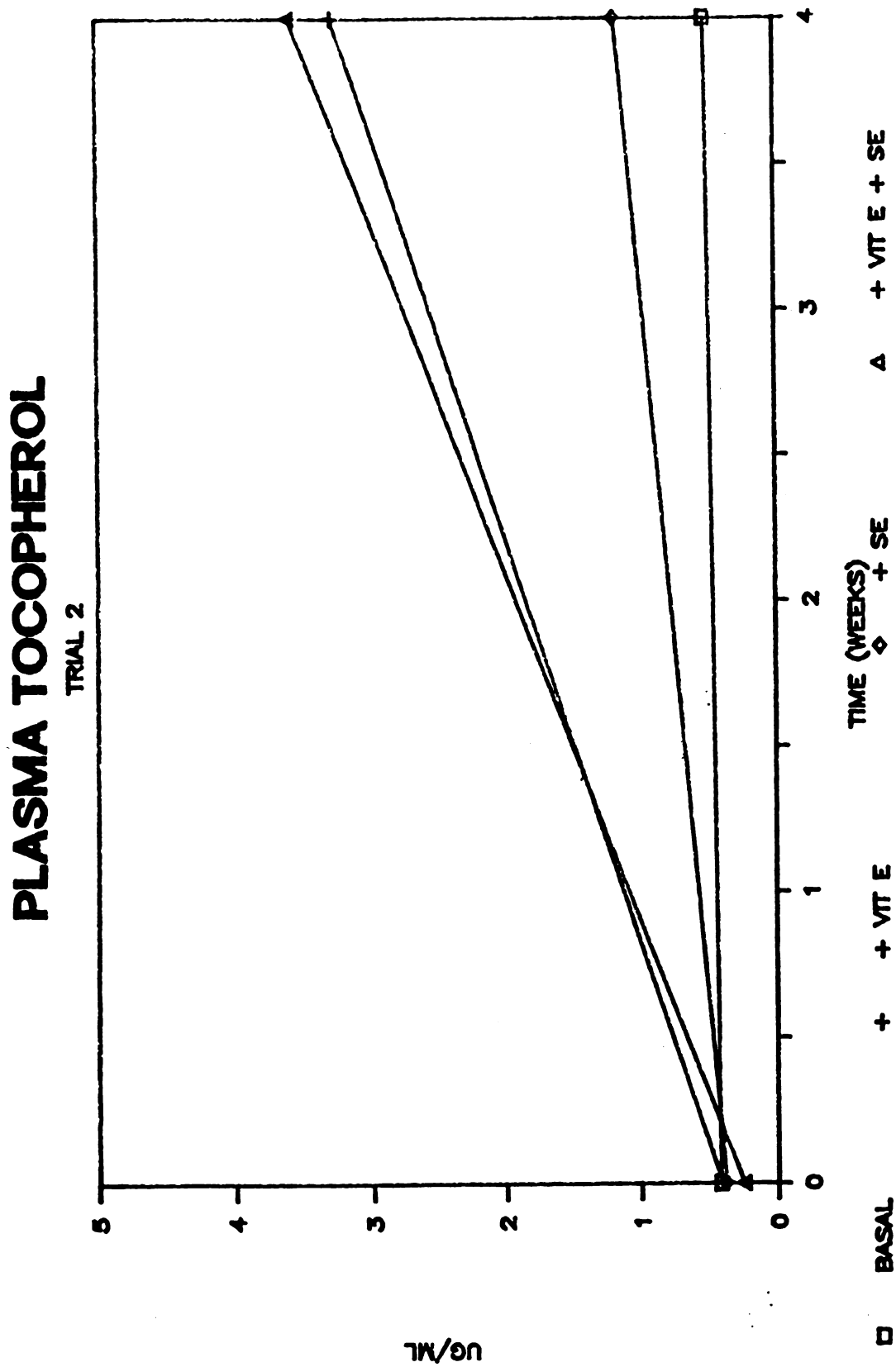


Figure 7. Comparison of plasma tocopherol concentration by treatment over time for Trial 2

Dietary supplementation of selenium increased ($P < 0.01$) plasma GSH-Px activities (Table 6). Dietary vitamin E increased ($P < 0.1$) plasma GSH-Px activities. Litters affected ($P < 0.001$) the plasma enzyme activity and there was an interaction ($P < 0.02$) between dietary vitamin E and dietary selenium on plasma GSH-Px activity. Plasma GSH-Px increased over time ($P < 0.001$). Consequently, there was an interaction between time and litter, and among time, dietary vitamin E, and dietary selenium ($P < 0.13$ and $P < 0.12$, respectively). Neither vitamin E nor selenium supplementation affected the white blood cell count (Table 7) or CL (Table 7 and Figure 8).

III. Trial 3

One of the pigs on the basal diet died after 5 weeks on trial and, unfortunately, the cause of death was not determined. Another pig in the 100 IU vitamin E supplemented group had a rectal prolapse after 6 weeks on trial and was removed from the study. These two pigs were excluded from the analysis.

Vitamin E supplementation raised ($P < 0.0001$) the plasma alpha-tocopherol concentration (Table 8 and Figure 9). The litter prior to the commencement of the trial that had no vitamin E or selenium supplemenation had higher ($P < 0.05$) plasma alpha-tocopherol concentrations. There was an interaction ($P < 0.15$) between time and litter and between time and dietary vitamin E ($P < 0.0001$). The white blood

Table 7. Effects of Dietary Vitamin E and Selenium Supplementation on Leukocyte Measures over Time for Trial 2

	Time					
Diet	Initial	Week 2	Week 4	AVG	MSE	P
	White Blood Cell Count, WBC/µl					
					35149435	NS
Basal	21141	19886	24006	21678		
B + Vit E	22258	22848	18793	21300		
B + Se	20708	20738	18693	20046		
B+Vit E+Se	20132	22556	21106	21265		
AVG	21058	21507	20649	21072		
	CL, total cpm x 10^3/hour/2 x 10^6					
					48.77	NS
Basal	15.298	10.609	11.999	12.635		
B + Vit E	9.060	9.085	7.089	8.411		
B + Se	12.873	10.105	16.992	13.323		
B+Vit E+Se	15.284	8.998	10.974	11.752		
AVG	13.128	9.699	11.763	11.530		

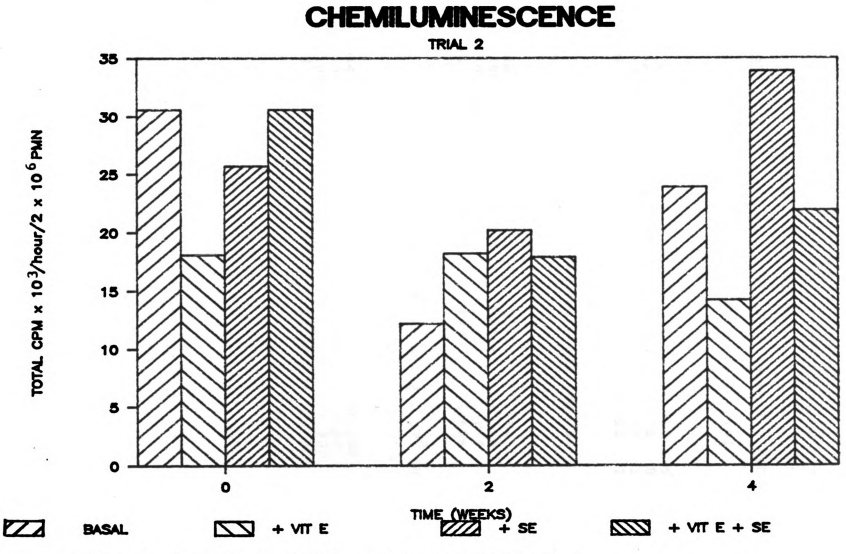


Figure 8. Comparison of CL by treatment over time for Trial 2

Table 8. Effects of Dietary Vitamin E Supplementation for Trial 3

Diet	Time: Week 3	Week 6	Week 9	AVG	MSE	P
Plasma Alpha-Tocopherol, µg/ml						
					0.027	0.0001
Basal	0.453	0.416	0.498	0.456		
B+100 IU Vit E	0.415	0.763	0.878	0.685		
B+1000 IU Vit E	2.876	2.664	2.530	2.690		
AVG	1.248	1.281	1.302	1.277		
White Blood Cell Count, WBC/µl						
					8130400	0.0001
Basal	24621	21992	20815	22476		
B+100 IU Vit E	25519	18468	18585	20857		
B+1000 IU Vit E	23615	19247	19658	20840		
AVG	24585	19902	19686	21391		
CL, total cpm x 10^3/90 min/2 x 10^6 PMN						
					357.1	NS
Basal	17.059	20.510	20.240	19.270		
B+100 IU Vit E	9.424	9.693	4.071	7.729		
B+1000 IU Vit E	14.698	17.327	25.907	19.311		
AVG	13.727	15.843	16.739	15.436		

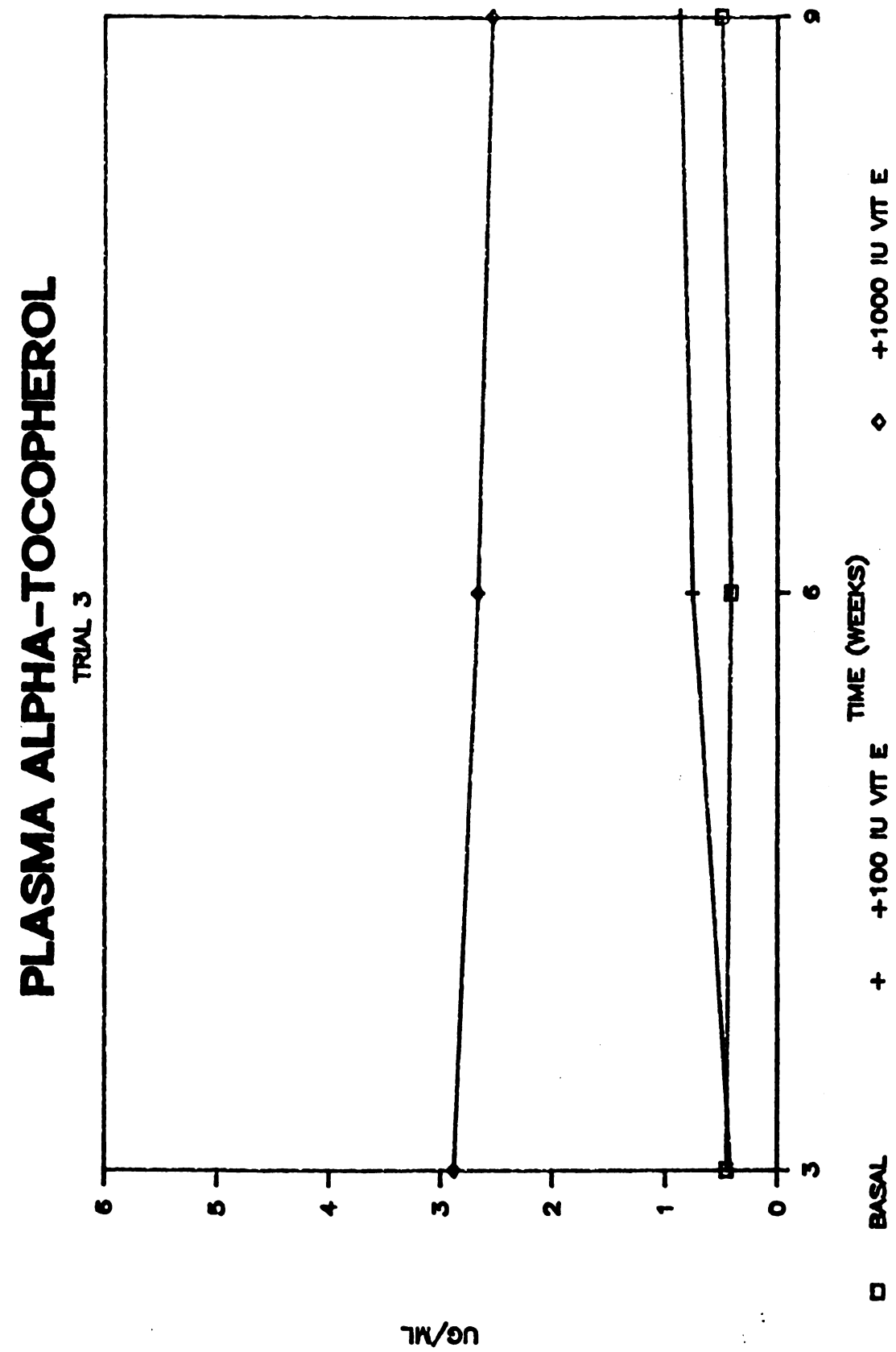


Figure 9. Comparison of plasma tocopherol concentration by treatment over time for Trial 3

cell count (Table 8) was influenced by litter and decreased over time ($P < 0.002$ and $P < 0.001$, respectively). Chemiluminescence was not affected by the supplementation of vitamin E (Table 8 and Figure 10).

IV. Chemiluminescence Precision Experiment

The variation within a sample and variation from day to day is shown in Figures 11 and 12.

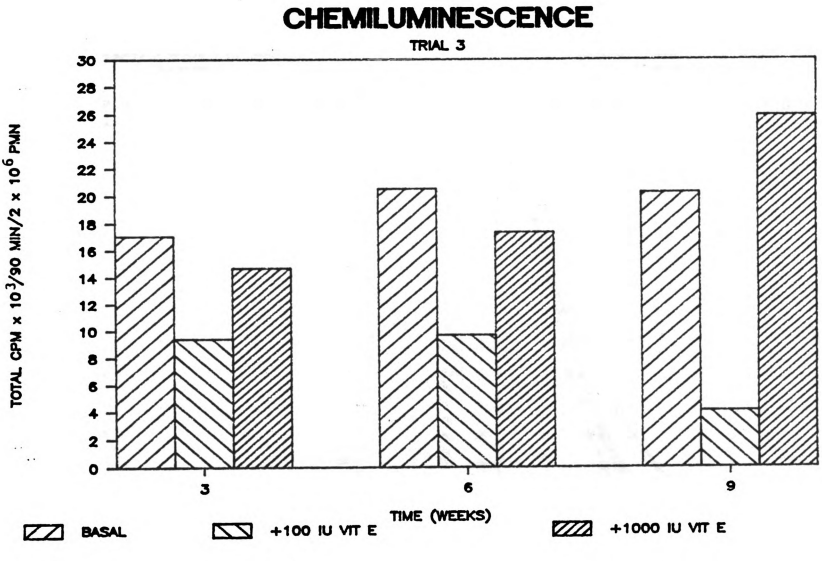
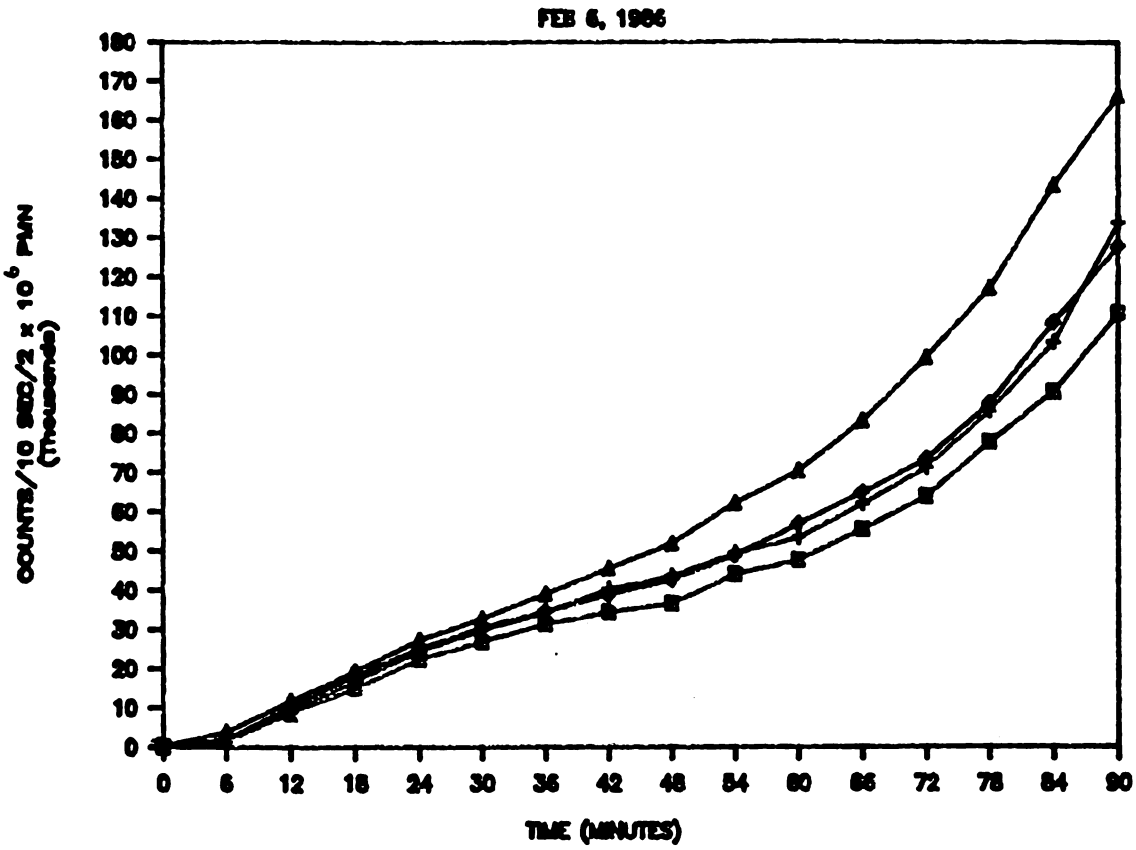


Figure 10. Comparison of CL by treatment over time for Trial 3



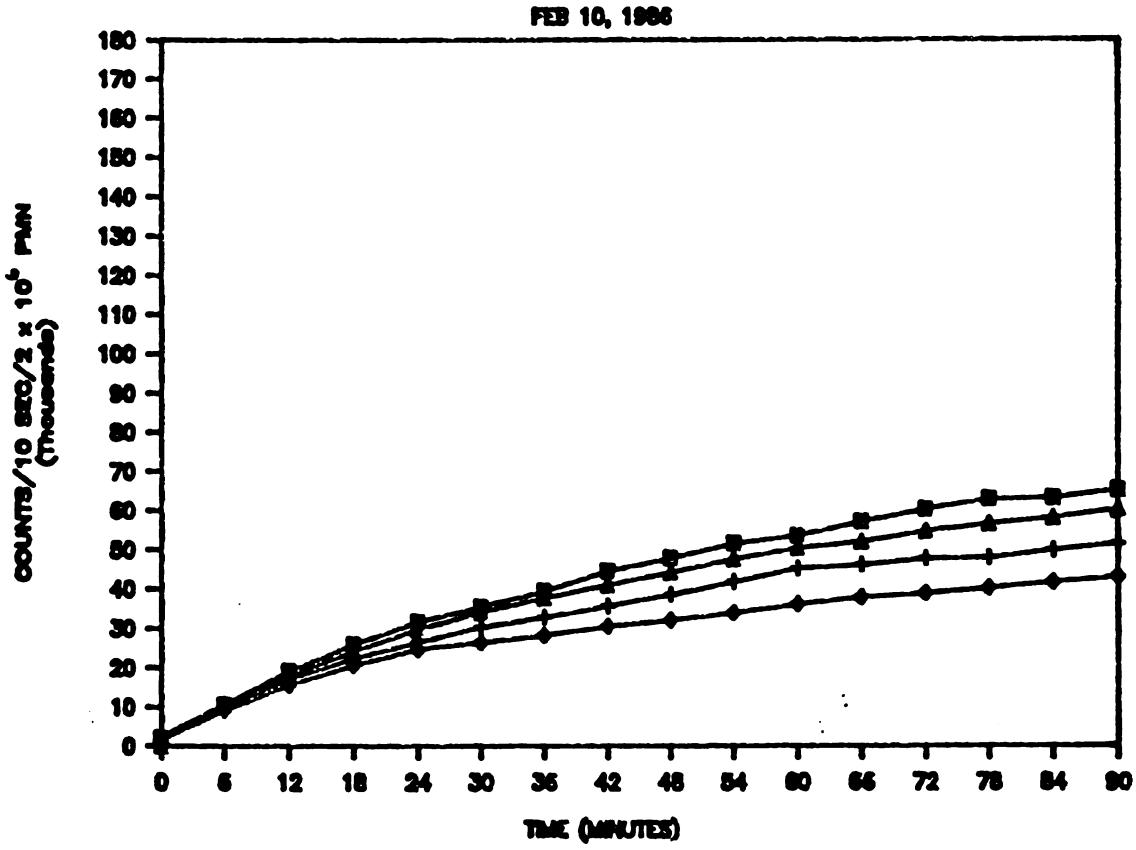
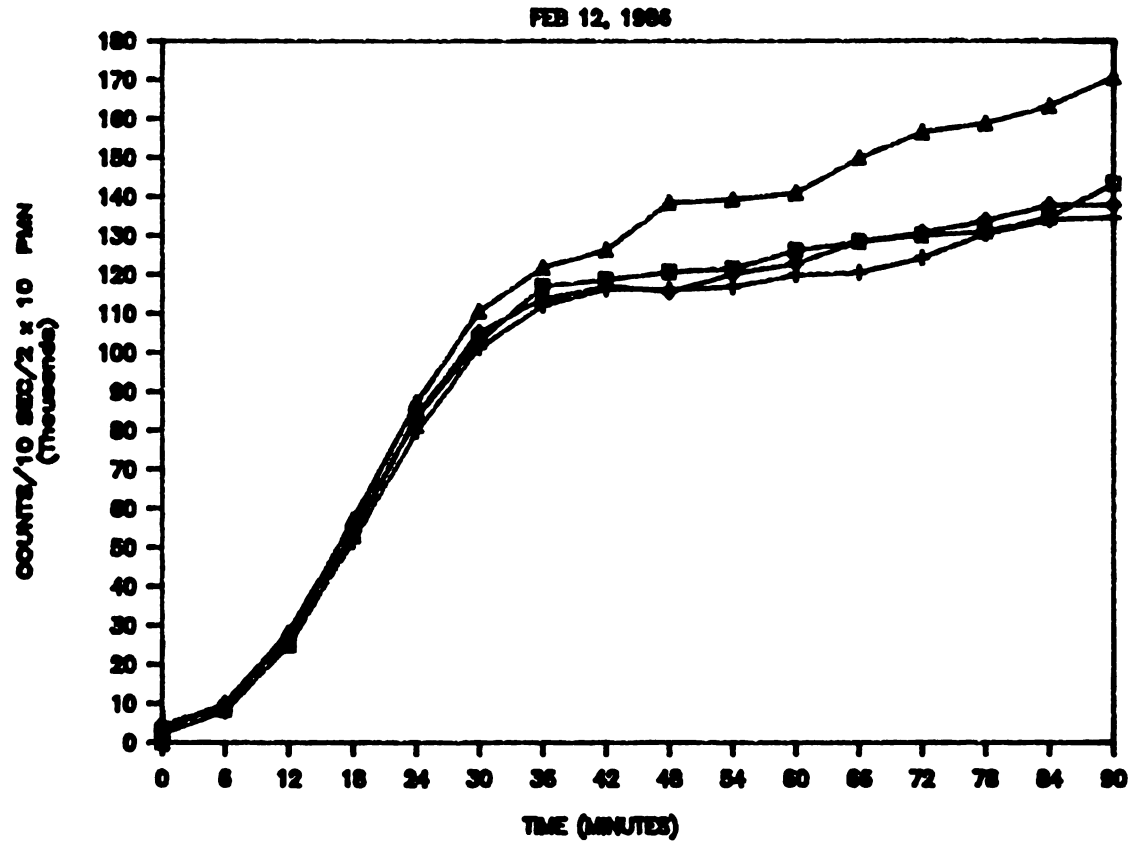


Figure 11. Day 1 and 2 of CL precision experiment



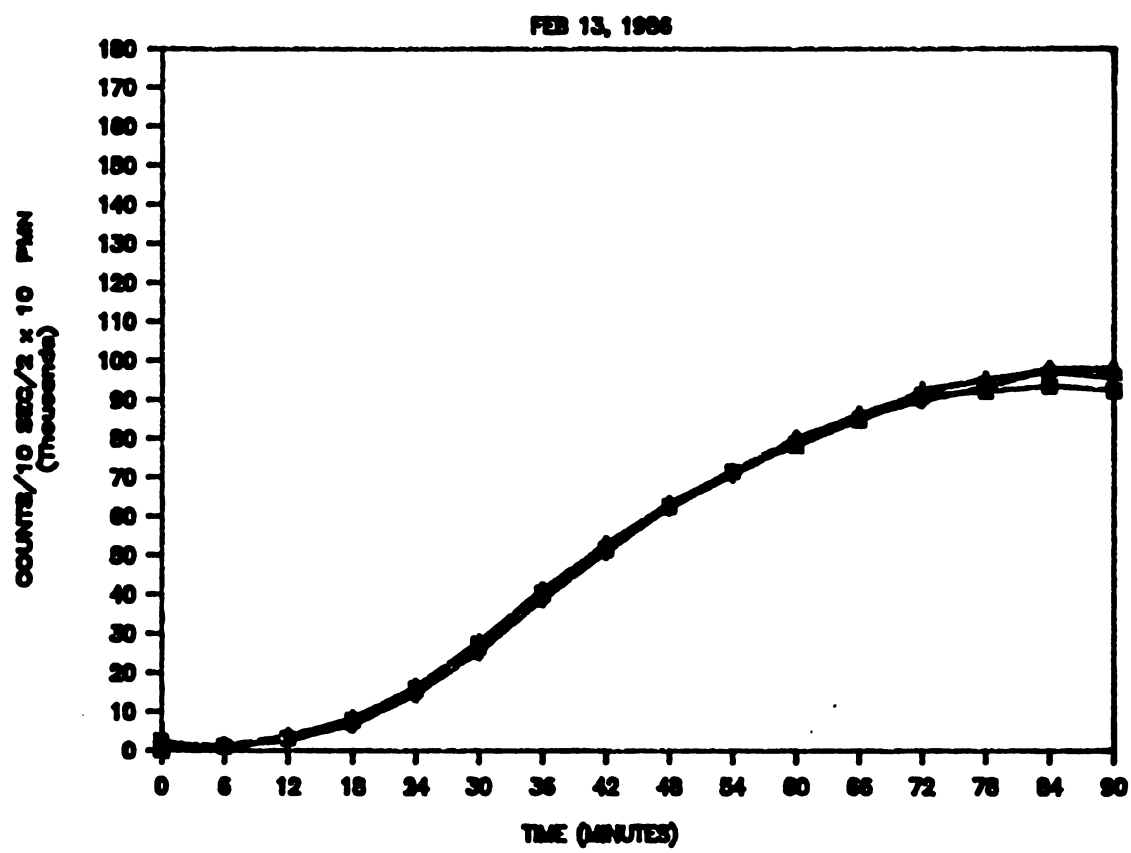


Figure 12. Day 3 and 4 of CL precision experiment

DISCUSSION

This series of experiments was designed to investigate the effect of vitamin E and selenium on the respiratory burst. The original hypothesis set forth was that CL produced by the neutrophils would increase in the vitamin E and/or selenium supplemented groups relative to the groups fed the basal diet. However, reexamination of the problem would indicate that vitamin E- and selenium-deficient pigs should, rather, increase CL compared to supplemented groups. The reason is that vitamin E and GSH-Px (a selenium-dependent enzyme) are both involved in the antioxidant process. Increased amounts of vitamin E and selenium would reduce oxygen metabolites, thus reducing the amount of respiratory burst and hence CL produced by phagocytic cells.

Young et al. (1977) reported serum tocopherol values for grower pigs on high moisture corn diets. Similar plasma tocopherol concentrations were reported after three weeks on supplemented diets as those reported here at approximately the same time for Trials 1 and 2. Although plasma tocopherol concentrations were different between treatments in Trial 3, the values were much lower than in other trials for the vitamin E-supplemented diets. Pigs fed the basal diets for all trials had similar plasma tocopherol

concentrations. In Trial 3, the 100 IU vitamin E/kg group had plasma tocopherol concentrations similar to those pigs unsupplemented with vitamin E in Trials 1 and 2. The 1000 IU vitamin E/kg group in Trial 3 had plasma tocopherol concentrations similar to those of pigs supplemented with 100 IU vitamin E/kg in Trials 1 and 2. Different methods of detection were used in Trials 1 and 2 than that used in Trial 3, but this should not have affected the plasma values of tocopherol as shown in a comparison test between the two methods (Table 9). The concentration of tocopherol used in the standards for Trials 1 and 2 was not verified so the values in these two trials are not absolute values because the extinction coefficient for tocopherol was not used to calculate the standard concentration. However, they still are a good estimate of the true values.

Animals fed a vitamin E-supplemented diet should have had and did have higher plasma tocopherol concentrations than depleted animals. Pudelkiewicz and Mary (1969) demonstrated that both plasma and liver tocopherol concentrations plateaued between 667 and 3,333 mg tocopheryl acetate/kg diet. In trial 3, there was also a litter effect and an interaction between time and treatment as well as an interaction between time and litter affecting plasma tocopherol concentration. The litter which prior to the trial had been on a vitamin E- and selenium-deficient diet, had increased plasma tocopherol concentrations relative to the other litter which had received vitamin E- and selenium-

Table 9. Comparison of Methods for Tocopherol Determination

Pig	Supplemental Vitamin E	Plasma Tocopherol, µg/ml			
		Original		Repeat	
		Fluorometric*	HPLC	Fluorometric	HPLC
		Trial 2, week 4			
168-1	0	0.497	--	0.594	0.459
173-1	100 IU	2.058	--	3.342	2.931
171-11	0	1.331	--	1.363	1.024
168-2	100 IU	4.886	--	5.156	5.262
		Trial 3, week 3			
171-5	0	--	0.299	0.319	0.141
171-6	100 IU	--	0.416	0.484	0.353
171-14	1000 IU	--	2.970	3.177	2.683

*The absolute value of the standard was not verified by the tocopherol extinction coefficient.

adequate diets. Possibly there is a genetic predisposition for vitamin E storage (Stowe and Miller, 1985), but more likely the effect was caused by repletion of the nutrient.

Selenium supplementation increased plasma selenium concentrations for both Trials 1 and 2. Young et al. (1977) found that feeding a basal diet of high moisture corn and soybean meal, basal plus 0.6 ppm selenium, and basal plus 1.2 ppm selenium resulted in plasma selenium levels of 0.027, 0.166, and 0.209 µg/ml, respectively. The pigs fed basal diets in our experiment had higher plasma selenium concentrations which can be explained by the fact that high moisture corn was not used in our experiments.

The supplemented groups in Trial 1 were very consistent with other reported values (Young et al., 1977; Stowe and Miller, 1985). However, in Trial 2, our values were not as high as those of Young and coworkers nor those of our own values in Trial 1. The unsupplemented diets in Trial 2 resulted in higher plasma selenium concentrations than the unsupplemented diets in Trial 1 at 4 weeks, but the initial plasma selenium values for Trial 2 were higher than initial plasma concentrations in Trial 1. In addition, the plasma selenium concentrations for Trial 2 were higher than Trial 1. Ullrey (1986) suggested that plasma selenium concentrations of 0.08 to 0.12 ppm are consistent with dietary adequacy. By the tenth week in Trial 1 and the fourth week in Trial 2, the pigs fed unsupplemented selenium diets had plasma selenium values that indicated dietary

adequacy. The litter effect found in Trial 2 can be explained by the findings of Stowe and Miller (1985) who reported a genetic influence on the selenium status of pigs.

Glutathione peroxidase is a selenium-dependent enzyme. Selenium supplementation increased the activity of this enzyme in plasma in both Trials 1 and 2. Whole blood GSH-Px in Trial 1 also increased in selenium supplemented groups (whole blood GSH-Px activity was not measured for Trial 2). The values reported are generally consistent with other values reported from our laboratory (Zhang et al., 1986; Brockway et al., 1985) and those reported by Stowe and Miller, 1985). The pigs fed diets not supplemented with selenium should have shown a decrease in GSH-Px activities over time instead of an increase. Furthermore, plasma selenium concentrations also increased over time which also was not expected in the unsupplemented pigs. Reports by others would indicate that both plasma selenium concentrations and GSH-Px activity should decrease in unsupplemented diets (Hakkarainen et al., 1978a; Sankari, 1985). There was a tendency for GSH-Px activity to be influenced by litters which is in agreement with the genetic influence reported by others (Atroshi et al., 1981; Sandholm et al., 1983; Stowe and Miller, 1985).

In Trial 1, selenium supplementation significantly increased the white blood cell count. The counts from all treatments also increased over time. The counts in Trials 2 and 3 were not affected by dietary treatments. However,

the counts significantly decreased over time and were significantly affected by litter in Trial 3. All the pigs from all the trials had white blood cell counts within normal ranges for pigs of their age. Serfass and Ganther (1977) found no apparent differences between selenium-deficient and control rats in leukocyte and differential counts after 17 weeks. Thus, the dietary effect in Trial 1 and time effect in Trials 1 and 3 do not necessarily indicate anything remarkable.

The results from the CL precision experiment would indicate that there is considerable variation from day to day. Most of the replicates within days were consistent with each other. Hopefully, the design of the CL procedure would nullify any variation due to day. However, treatment effects may be masked by the variation seen by day. The variation seen by day is probably attributable to either variation in concentration of opsonized zymosan or luminol (Trush et al, 1978). Cell concentration can also affect CL; however, cells were standardized to a specific concentration before analysis. Incubation time and temperature affect the opsonization of zymosan. Luminol (0.05-0.1 mg) is insoluble in PBS and must be dissolved in DSMO (0.05-0.1 ml) before it can be diluted with PBS. Fine dust particles of luminol can stick to the sides of the test tube making it difficult to dissolve all the luminol. DeChatelet and Shirley (1981) also report much variation among days. They found an independence of CL response with variation in cell

concentration using whole blood which they attributed to the quenching of fluorescence by hemoglobin. These investigators concluded that day to day variation (both in magnitude of response and shape of curve) was still useful for screening for chronic granulomatous disease in which an all or nothing response is observed. In our experiments, there may be a gradation of responses and great variation may obscure treatment results.

The CL experiments did not reveal any treatment differences. The decrease in CL seen within treatments in Trial 1, may be explained by increasing plasma concentrations of vitamin E and selenium. Chemiluminescence is produced by oxygen metabolites such as superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radical. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide and organic peroxides before they can react with superoxide. Vitamin E is a free radical scavenger in lipid membranes resulting in the prevention of lipid peroxidation of the membrane. Quenching these toxic oxygen metabolites would prevent host tissue damage during phagocytosis.

Baehner and coworkers (1977) investigated the effects of alpha-tocopherol on various human PMN functions. They found that human volunteers ingesting 1600 IU vitamin E daily had a significant increase in rate of release of superoxide but a decrease in hydrogen peroxide release from phagocytizing PMNs. During treatment, volunteers had faster rates of phagocytosis but diminished killing capabilities of

Staphylococcus aureus compared to controls. They hypothesized that vitamin supplementation decreased the availability of hydrogen peroxide in the phagosome containing the bacteria, but that the decrease in hydrogen peroxide protected the PMN from autotoxicity leading to improved phagocytosis. Harris et al. (1980) expanded on the findings of the above investigators with vitamin E-deficient rats. Polymorphonuclear neutrophils from vitamin E-deficient rats were unable to respond normally to both chemotactic and phagocytic stimuli. Although the deficient PMNs were compromised in their phagocytic ablities, hydrogen peroxide release was greater than in normal cells and killing of Staphylococcus aureus was not impaired when bacteria to cell ratio was no more than 10:1. Lipid peroxidation of PMNs altered the chemotaxis probably because the membranes had been damaged.

Biologically, CL may not be the best procedure to use to measure the killing capabilities of PMNs. Chemiluminescence measures the total oxygen metabolites produced during the respiratory burst. If superoxide production is increased and hydrogen peroxide is decreased with vitamin E supplementation as suggested by Baehner et al. (1977), it may be difficult to interpret the CL response. The findings of Serfass and Ganther (1977) further complicate matters. These reseachers found no apparent differences in ingestive abilities of PMNs from selenium-deficient and selenium-adequate rats. However, they reported an impairment in the

killing of ingested yeast cells from selenium-depleted rats. Serfass and Ganther (1977) suggest that GSH-Px protects cytosolic components and membranes from reactive metabolites diffusing from the phagosome and the reduced fungicidal capabilities in selenium-deficient rats and that production of killing metabolites is required before the effects of selenium are apparent. Other investigators confirmed the decreased bactericidal effects of selenium deficiency in cattle (Boyne and Arthur, 1979). Bass et al. (1977) did not observe an impairment in killing of Proteus mirabilis by selenium-deficient rat PMNs.

In the foregoing discussion, many cellular and biochemical events are occurring during phagocytosis and the subsequent killing of bacteria. Vitamin E and selenium play different roles at various stages of the whole event. Conflict arises when evidence indicates a decrease in phagocytosis in vitamin E-deficiency and no effect in selenium-deficiency. Killing functions do not seem to be affected by vitamin E-deficiency but by selenium deficiency. Chemiluminescence does not indicate what problem if any is occurring in the different cellular and biochemical events that are taking place during phagocytosis and the oxidative burst. Also, the small number of animals may have hindered our finding any treatment differences and the animals were not as deficient in either nutrient as we would have liked.

More reseach needs to be done to define the defects in the immune system caused by vitamin E and/or selenium deficiency.

Conclusions

Supplementation of vitamin E (either 100 IU/kg or 1000 IU/kg) increased plasma tocopherol concentrations in all trials and selenium supplementation (1.0 ppm) increased plasma selenium concentrations for Trials 1 and 2. Plasma and whole blood GSH-Px activities were increased with selenium supplementation in Trials 1 and 2.

Selenium supplementation increased white blood cell counts in Trial 1. Dietary supplementation of vitamin E and selenium did not result in any apparent treatment differences in CL. If CL can measure treatment differences, our results were masked by small animal numbers, animals not deficient enough in vitamin E and selenium, or problems in our CL technique.

We were not able to discriminate any differences among neutrophils of vitamin E and/or selenium deficient or supplemented swine with regards to phagocytic or killing abilities.

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