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I am submitting herewith a dissertation written by John R. Moore entitled "Effects of Pharmacological Concentrations of Alpha-Tocopherol, Ascorbic Acid, and Iron on Lipid Peroxidation, Tissue Iron Distribution, Hematological, Immunological, Performance, and Tissue Parameters of Young Rats and Pigs." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

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EFFECTS OF PHARMACOLOGICAL CONCENTRATIONS OF ALPHA-TOCOPHEROL, ASCORBIC ACID AND IRON ON LIPID PEROXIDATION, TISSUE IRON DISTRIBUTION, HEMATOLOGICAL, IMMUNOLOGICAL, PERFORMANCE, AND TISSUE PARAMETERS OF YOUNG RATS AND PIGS.

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ABSTRACT

Four experiments were conducted to assess the physiological effects of alpha-tocopherol, ascorbic acid, and iron on lipid peroxidation, tissue iron distribution, blood platelet aggregation and concentration, polymorphonuclear leukocyte concentration, hematological and performance parameters utilizing rats and pigs. The primary emphasis was placed on alpha-tocopherol and its interactive effects with ascorbic acid and iron.

In the first experiment 96 male Sprague-Dawley rats weighing between 51 and 75 grams were utilized in a 4 x 2 factorial arrangement with six animals per treatment. The treatments consisted of four added dietary levels of alpha-tocopheryl acetate: 7.5, 25, 37.5, and 75 IU/kg and two dietary concentrations of ascorbic acid: 0 and 0.5%.

The second experiment utilized 48 Sprague-Dawley rats weighing between 175 and 200 grams in a 2 x 2 factorial arrangement with eight animals per treatment. The treatments consisted of two added dietary levels of alpha-tocopheryl acetate: 30 and 300 IU/kg and two dietary levels of ascorbic acid: 0 and 0.5%. The third study utilized 48 three-day-old Yorkshire x Duroc x Hampshire pigs in a 3 x 2 factorial arrangement with eight pigs per treatment. The treatments consisted of 3 levels of iron injection: 0, 100, or 400 mg and two levels of alpha-

tocopherol injection: 0 or 900 IU. The fourth experiment utilized 48 weaned Yorkshire x Duroc x Hampshire pigs from 6 litters in a 4 x 2 factorial arrangement with four dietary additions of alpha-tocopheryl acetate: 0, 100, 300, and 1000 IU/kg and two dietary levels of ascorbic acid: 0 and 0.2%.

The results obtained indicated that added dietary concentrations of alpha-tocopheryl acetate significantly decreased lipid peroxidation in the tissues of rats and pigs regardless of the age and size of the animal involved. Injection of alpha-tocopherol also significantly decreased lipid peroxidation in the tissues of baby pigs. The decrease in lipid peroxidation appeared to be linear with lipid peroxidation decreasing inversely as biological concentrations of alpha-tocopherol increased.

The results from experiment two provided evidence that dietary supplementation with alpha-tocopheryl acetate at 3000 IU/kg significantly decreased blood platelet aggregation on day 21 and 28 of the experiment in 200 and 250 gram rats. Blood platelet concentration was not affected by dietary alpha-tocopheryl acetate or ascorbic acid.

Experiment two appeared to indicate that added dietary additions of alpha-tocopheryl acetate significantly increased the percentage of polymorphonuclear leukocytes

(PMN) in white blood cells during the last week of the trial. Ascorbic acid did not appear to significantly increase PMN concentrations in white blood cells.

The results obtained from these experiments indicated that added dietary concentrations of alpha-tocopheryl acetate significantly increased plasma iron concentration, decreased total iron binding capacity in plasma and decreased tissue concentrations of iron.

Injected iron at 400 mg significantly increased lipid peroxidation in the plasma and tissues of baby and weaned pigs.

Added dietary levels of ascorbic acid did not appear to physiologically interact with pharmacological concentrations of alpha-tocopherol based on the variables measured except for the reduction of lipid peroxidation in plasma and the tissues analyzed.

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Chapter I

INTRODUCTION

Vitamins are usually present in animal diets at levels that will prevent nutritional deficiency symptoms. If the vitamin is supplemented at much higher dosages, at some level it may be proper to talk about the pharmacological action of the vitamin. This distinction is important until the recommended daily allowance of a vitamin is defined for maximal metabolic function rather than for the absence of deficiency symptoms. Further complicating the picture of maximal vitamin function are the possible synergistic or antagonistic relationships between specific vitamins and between certain vitamins and trace metals in physiological systems.

The production of free radicals by chemical and enzymatic processes in biological systems is a normal and continuing process. Free radicals are produced in many subcellular membranes during the enzymatically controlled production of prostaglandins, prostacyclins and the leukotrienes. They are produced by neutrophils during phagocytosis and the destruction of foreign pathogens. Free radicals are also produced during electron transfer in the mitochondria of cells as a means of energy production. It is evident that the production of free radicals is a necessary and normal condition of subcellular function.

Damage from free radicals occurs when the rate of production or the specific type of radicals produced is greater than the body's ability to control them within the limits of the cell's ability to absorb damage. Under specific circumstances the body's normal defense mechanisms cannot prevent highly active free radicals from damaging inter- and intra-cellular structures. Cell function, genetic mutation, and cellular death may then occur.

To control or minimize cellular damage from free radicals the body has developed a number of defense mechanisms. Most of these systems are enzymatic and many are under nutritional control. Vitamin E is a nutritionally controlled lipophilic free radical scavenger which minimizes free radically induced damage in the lipid environment of the cell. Although vitamin E is not the only mechanism available to the body for control of free radicals, it is the only significant free radical scavenger in the lipid environment of the cell. In this manner vitamin E may quench the potential formation of lipid peroxides at their source.

It has been proposed that high dietary levels of vitamin E and vitamin C could possibly lead to a redistribution of iron in tissues (Chen and Thacker, 1988). Chou *et al.* (1978) reported abnormalities of iron metabolism in vitamin E deficient rabbits. The total body

iron content was not altered but the sequestration of iron in muscle tissue was increased.

Vitamin E has been demonstrated to be an inhibitor of the platelet-release reaction. Studies have shown that platelet secretory release is inhibited in a dose-dependent relationship by alpha-tocopherol. This effect was not found to be as potent as that of aspirin but a definite arachidonic acid-induced reduction in platelet aggregation was observed *in vivo* and *in vitro* (Steiner and Mower, 1982).

Polymorphonuclear leukocytes (PMN's) appear to undergo functional alterations with the administration of pharmacological doses of vitamin E. There appears to be an increased ability of PMN's to ingest foreign particles but a slightly decreased bactericidal potency of the PMN.

Vitamins E and C have been shown to be synergistic in their antioxidant properties *in vivo* and *in vitro* (Bendich *et al.*, 1986). Ascorbic acid can reduce the tocopheroxy radical with the formation of an ascorbate radical which can be enzymatically reduced back to ascorbic acid by an NADH-dependent system. This is the only known method by which tocopherol may be continuously regenerated (Niki, 1987).

When diets deficient in Vitamin C were fed to guinea pigs, decreased concentrations of Vitamin E were observed in the adrenals and lungs. This would suggest that Vitamin

C can regenerate Vitamin E *in vivo*. Ascorbic acid's sparing effect on Vitamin E can be demonstrated when the ascorbate is in the liquid phase and the tocopherol is in the lipid phase. It would appear that they can interact at the interface of the aqueous and lipid phases (Machlin, 1991).

Iron is present in a number of enzyme systems that are responsible for electron transport (cytochromes), it is necessary for the transport of oxygen when part of the hemoglobin molecule in myoglobin molecules, and is required for the activation of oxygen in conjunction with oxidases and oxygenases. The hydroxyl radical is a product of a Haber-Weiss-Fenton reaction involving an iron catalyst. The administration of iron to baby pigs has become necessary in modern swine production due to total confinement practices. While iron is necessary to the health of the young pig, a number of negative physiological manifestations occur. Vitamin E protects against the organ tissue, skeletal and cardiac damage that can occur following the administration of iron to piglets (Tollerz, 1973). Vitamin E reduced hemolysis in mice and rats when those animals were injected with iron dextran (Machlin, 1991). Vitamin E reduced ethane production in rats (Tappel and Daillard, 1981). Iron overload is associated with the peroxidations of lipids and reduced vitamin E levels in plasma and tissues (Kato *et al.*, 1987).

The objective of the experiments reported in this dissertation was to evaluate the physiological effects of pharmacological concentrations of alpha-tocopherol, ascorbic acid and iron on lipid peroxidation, performance, hematological, immunological, and tissue mineral parameters of young rats and pigs.

Chapter II

LITERATURE REVIEW

Vitamin E

General

There is considerable controversy concerning the physiological role and function of vitamin E. While vitamin E is certainly one of the most studied and researched nutrients, its function is still largely a mystery. There are a number of facets of this vitamin which are not well understood and are probably indicative of its subtle mode of action.

There is considerable disagreement as to how the vitamin functions at the cellular and molecular level. Its exact nutritional role and the possible therapeutic usefulness of the vitamin are of great interest throughout the nutritional community.

Osborne and Mendel (1919) recognized that semipurified diets that were adequate in the nutritional constituents known at that time, failed to support normal reproduction in the laboratory rat. Evans and Bishop (1922) were the first to demonstrate the existence of an unknown dietary factor, given the name factor X, which, when deficient, caused fetal death and resorption in the laboratory rat. They had ascertained that wheat germ and wheat germ oil were sources of this fat soluble factor. In 1924, Sure

(1924) named the unknown factor vitamin E as the next serial alphabetical designation after vitamin D.

Vitamin E was given the name tocopherol from the Greek *tokos*, which means childbirth, and the verb *pherin*, to bring forth. The *ol* was added to signify the alcohol nature of the compound. Vitamin E is the generic descriptor for the toco and tocotrienal derivatives which exhibit quantitatively some degree of the biological activity of alpha tocopherol.

Mason (1980) reported that in 1938, Fernholz determined the chemical structure of alpha-tocopherol and Kaurer provided the first synthesis soon afterward. During the two decades following the discovery of vitamin E a great deal of progress was made in determining its biological properties. Mason (1980) reported that male rats that were on vitamin E deficient diets developed testis degeneration. Olcott and Matill (1941) found that muscle degeneration was also a common deficiency symptom. A number of other vitamin E deficiency symptoms were discovered in the 1940's and 1950's; these included encephalomalacia and exudative diathesis in chickens, anemia in primates, liver necrosis and tooth enamel depigmentation in rats, and steatitis or "yellow fat" disease in farm animals (Mason, 1980).

Many of the early investigators recognized the antioxidant properties of vitamin E. Dam (1957) recognized

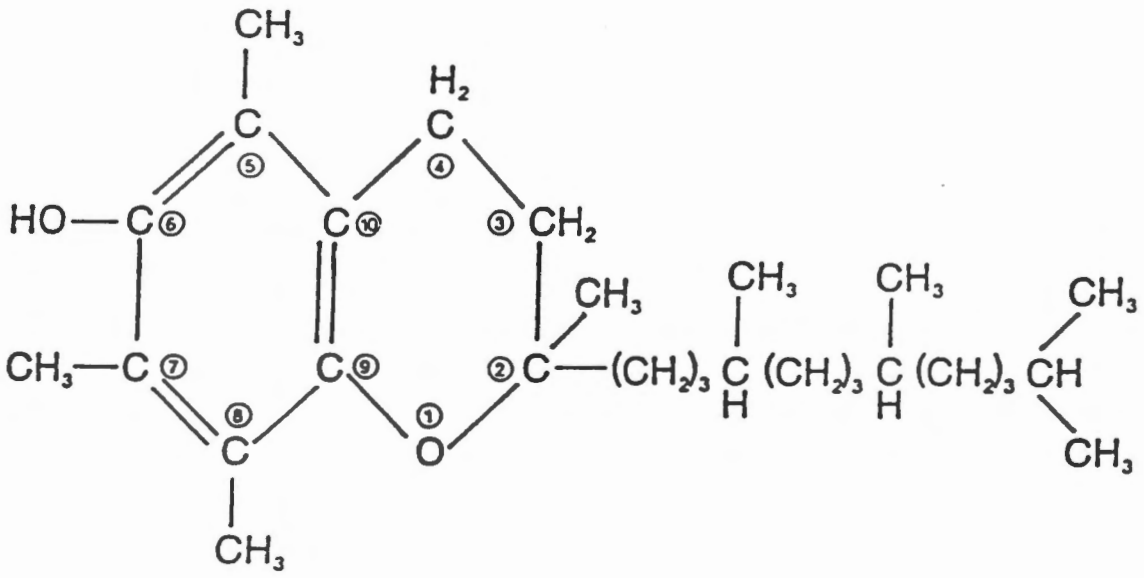
the presence of peroxides in the adipose tissue of animals fed vitamin E deficient diets and demonstrated that a number of synthetic antioxidants prevented vitamin E deficiency signs in animals. This work and the work of others provided the basis for the "antioxidant" theory of vitamin E function. The most accepted theory for vitamin E function is that it acts together with a number of small molecules, such as ascorbic acid and glutathione along with the enzymes, glutathione peroxidase, superoxide dismutase and catalase, to protect the cell against the damaging effects of free radicals. In the absence of vitamin E there is a great deal of damage to many cells, especially red blood cells, nerve cells, and muscle cells (Burton *et al.*, 1983).

It is only within the last 15 years that a specific deficiency disease in humans has been established for vitamin E. A debilitating neuropathy has been discovered in adults and children with severe fat malabsorption. The misinformation and exaggerated claims of the past have given way in the last 15 years to the development of solid scientific evidence that vitamin E plays a unique role against free-radical injury. The evidence is accumulating that vitamin E enhances the immune response, and that it may play a role in the prevention of cataracts, Parkinson's disease, cardiovascular disease, the prevention of several

types of cancer, and the prevention of blood platelet hyperaggregation (Machlin, 1991).

Chemistry

Until recently, vitamin E has been defined and evaluated in terms of international units. One international unit has been defined as 1 mg of dl-α-tocopheryl acetate. Due to its relatively stable nature, this form is the preferred form for use in animal and human diets; however, it does not occur in nature and is considerably less active than the physiologically active form which is d-α-tocopherol (Machlin, 1991). There has been a great deal of confusion among scientists in expressing dietary vitamin E values. The National Research Council's Committee on vitamin E recommended daily allowances (RDA's) decided in 1980 to express the total activity of diets as the equivalents of 2R, 4'R, 8'R-α-tocopherol, formerly called d-α-tocopherol in milligrams (Recommended Dietary Allowances, 1980). The structure and various names of d-α-tocopherol are illustrated in Figure 1. It must be remembered that vitamin E is a generic term that includes all of the compounds that have been found to have vitamin E activity; these are d-alpha, d-beta, d-gamma, and d-delta-tocopherol, and d-alpha, d-beta, d-gamma, and d-delta-tocotrienol. All of the acetate and



Chemical Name:	2,5,7,8-Tetramethyl-2-(4',8',12-trimethyl-tridecyl)-6-chromanol
IUPAC Name:	2R, 4'R, 8'R-alpha tocopherol
Trivial Name:	RRR-alpha tocopherol
Common Names:	d-alpha tocopherol, natural vitamin E
Molecular Weight:	430.69
Empirical formula:	$C_{29}H_{50}O_2$

Figure 1. d-Alpha tocopherol

succinate derivatives of the natural tocopherols have vitamin E activity as do the synthetic tocopherols and their derivatives (Machlin, 1991).

It is extremely important that the form of vitamin E being used in experimental work be accurately defined due to the different biopotencies exhibited by the various forms of the vitamin. The eight compounds found in nature with vitamin E activity are given in Table 1. It has been found that d-α-tocopherol has the highest biopotency, and its activity is used as the standard for determining the activity of all other forms. The natural forms of vitamin E are all d-stereoisomers. The tocotrienols are found in much smaller quantities in animal foods, and have the same chemical structure as the tocopherols except that there are three double bonds, one for each isoprene unit, in the side chain.

Synthetic vitamin E is a mixture of stereoisomers that is prepared by coupling isophytol with trimethylhydroquinone (TMHQ). When using natural isophytol, a single stereoisomer, the product is a 50/50 mixture of d- and L-α-tocopherol. The eight isomeric forms that comprise synthetic vitamin E are given in Table 2. Only 2R,4'R,8'R-d-α-tocopherol has the same activity as natural d-α-tocopherol. The remaining stereoisomers are a mixture with biological activities from 21 to 90% of natural vitamin E (Machlin, 1991).

Table 1. The eight naturally occurring vitamin E compounds

Structure	Common Name	Biological Activity Compared to IU/mg	d-Alpha
	d-alpha tocopherol	1.49	100%
	d-beta tocopherol	0.75	50%
	d-gamma tocopherol	0.15	10%
	d-delta tocopherol	0.05	3%
	d-alpha tocotrienol	0.75	30%
	d-beta tocotrienol	0.08	5%
	d-gamma tocotrienol	unknown	
	d-delta tocotrineol	unknown	

From: Bunyan, J., D. McHale, J. Green and S. Marcinkeiwicz. (1961.).

Table 2. The eight isomeric forms of synthetic vitamin E

Name	Configuration	Biological Activity IU/mg	Biological Activity Compared to d-Alpha
d-α-tocopherol	2R4'R8'R	1.49	100%
l-α-tocopherol	2S4'R8'R	0.45	31%
α-tocopherol	2R4'R8'S	1.34	90%
α-tocopherol	2S4'R8'S	0.55	37%
α-tocopherol	2R4'S8'S	1.09	73%
α-tocopherol	2S4'S8'R	0.31	21%
α-tocopherol	2R4'S8'R	0.85	57%
α-tocopherol	2S4'S8'S	1.10	60%

Weiser and Vecchi, 1982.

The differences in biological activity between synthetic and natural tocopherols and tocotrienols is dependent upon the position and presence of methyl groups on the aromatic ring of the compound and the asymmetric carbons of the side chain. The configuration at the number two carbon is generally considered to be most important in terms of the level of vitamin E activity. The natural tocopherols have an R-configuration at the second carbon, while d-α-tocopherol has an additional R-configuration at the 4′ carbon and the 8′ carbon, and has the greatest vitamin E activity in animals.

The free-alcohol form of vitamin E may lose bioactivity in the presence of air, heat and light. For this reason, vitamin E acetate and succinate derivatives are often used when formulating diets. Vitamin E acetate and succinate have no antioxidant activity but are readily hydrolyzed to the biologically active free alcohol *in vivo*. The synthetic esters of α-tocopherol are compared in terms of their activities in Table 3. Table 4 contains a list of chemical properties for the most important commercially available forms of vitamin E.

Assessment of Vitamin E

The assessment of vitamin E nutritional status in animals is generally based on the plasma α-tocopherol concentration. Within the last five years, a significant

Table 3. The vitamin E acetate and succinate derivatives of alpha-tocopherol

Common Name	Trivial Name	Biological Activity Compared to IU/mg	d-Alpha
d-α-tocopherol	RRR-α-tocopherol	1.49	100%
d-α-tocopherol acetate	RRR-α-tocopherol acetate	1.36	91%
d-α-tocopherol acid succinate	RRR-α-tocopherol acid succinate	1.21	81%
dl-α-tocopherol	All-rac-α-tocopherol (a mixture of 8 stereoisomers)	1.10	74%
dl-α-tocopheryl acetate	All-rac-α tocopherol acetate (a mixture of 8 stereoisomers)	1.00	67%
dl-α-tocopherol acid succinate	All-rac-α-tocopherol acid succinate (a mixture of 8 stereoisomers)	0.89	60%

Table 4. Chemical properties

Item	dl-α-tocohperol	d-α-tocopherol	dl-α-tocopheryl acetate	d-α-tocopheryl acetate
Color	Colorless to pale yellow, viscous oil	Colorless to pale yellow, viscous oil	Colorless to pale yellow, viscous oil	Colorless to pale yellow, viscous oil
Boiling point (°C)	200-220	200-220	224	224
Molecular weight	430.69	430.69	472.73	472.73
Spectrophometric data				
Absorption Maxima (nm)	292-294	292-294	285.5	285.5
$E^{1\%}_{1cm}$ (ethanol)	71-76	72-76	40-44	40-44
Spectrofluormetric data				
Excitation (nm)	295	295	360	360
Emission (nm)	330	330	415	415

Machlin (1991)

improvement in vitamin E analysis has been realized with the application of high performance liquid chromatography (HPLC). HPLC is relatively simple, non-destructive and rapid. Due to the small volume of plasma required, 25 to 150 μl, HPLC has become a universal procedure for vitamin E determination.

There has been considerable disagreement regarding the relative biological potencies of dl-α-tocopherol as compared to that of d-α-tocopherol. The disagreement centers around the blood serum concentrations of humans after ingesting the two compounds. However, Knight and Roberts (1985), after studying supplementation of baby rabbits with relatively large doses of both the synthetic and natural tocopherols, concluded that the vitamin E level of blood may not be the best criteria of availability since such concentrations depend upon rates of absorption which may not give comparable information on the transfer and tissue retention of the vitamin, and concluded that serum vitamin levels were not a good criterion of vitamin E potency. Ogihara *et al.* (1980) have shown much higher levels of tocopherol in the red blood cells after oral administration of d-alpha-tocopherol than after dl-alpha-tocopherol acetate. The concentration of the tocopherols in the red blood cell, which was used as a criterion for vitamin E in biological membranes, was four times greater, 6 to 24 h after administration of alpha-tocopherol than

after dl-alpha-tocopheryl acetate. Machlin and Gabriel (1982) reported that the tocopherol concentrations in spleen, heart, liver and kidney were six to eighteen times greater in the first four hours and two to three times greater between 8 to 16 h after oral dosing with d-alpha-tocopherol than with dl-alpha-tocopheryl acetate. An investigation by Burton and Ingold (1984) showed that labeled d-alpha-tocopherol remained in the tissues significantly longer than did labeled dl-alpha-tocopherol. Kitabchi *et al.* (1980) has determined that the lower retention of the dl-alpha-tocopherol product by the tissues was due to specific protein receptors for d-alpha-tocopherol which make the transport and retention of the natural product more efficient than the dl-form of the vitamin.

The succinate and acetate esters are generally used as vitamin E sources in commercial supplements because of their greater stability in the presence of light and oxygen than are the alcohol forms. The succinate ester is a powder at room temperature, whereas the acetate ester is an oil.

Relatively all of the vitamin E in the blood and tissue is in the unesterified tocopherol form. Prior to absorption, pancreatic enzymes participate in the removal of the acetate or succinate moiety. Only the free tocopherol is absorbed. Bile and a healthy, small

intestine are necessary for normal absorption of vitamin E (Machlin and Gabriel, 1982).

Metabolism

A. Absorption

The term absorption will be used to define the entire sequence of chemical and physical events which take place between the introduction of tocopherol or its various esters into the gastrointestinal tract and its subsequent appearnace in the lymphatic system or portal blood.

The absorption of vitamin E takes place primarily at the junction of the upper and middle thirds of the small intestine. Most findings suggest that the medial small intestine of mammals has a greater functional absorptive surface than does the proximal or distal segments of the small intestine (Gallo-Torres, 1980).

Absorption of the tocopherols takes place principally via the lymphatic system where they are transported as part of a lipoprotein complex. Intestinal absorption is dependent on a number of other processes such as pancreatic function, micellar formation, biliary secretion and movement across the intestinal mucosa. In mammals the absorption of tocopherol is incomplete. In one study 20-30% of labeled tocopherol was absorbed while in another, 35-50% was absorbed. It appears that as the dose

increases, the percentages of absorption decreases (Bjorneboe *et al.*, 1990).

Vitamin E absorption efficiency is greatly enhanced by the concurrent digestion and absorption of dietary lipids. Medium-chain triglycerides have been shown to increase absorption, while polyunsaturated fatty acids are inhibitory. In animal experiments, vitamin E, when given orally, was absorbed twice as rapidly when given as an aqueous-miscible emulsion as when given as an oil solution (Machlin, 1991).

A variety of methods have been employed in order to study the absorption of α-tocopherol after oral feeding or slow infusion into the gut. Among the first methods used was measuring the amount of fecal unabsorbed α-tocopherol after oral feeding and gut perfusion experiments. The problems associated with these methods were that they were nonspecific, and the quantitative interpretation of the experimental results was difficult because of the large number of experimental variables used by the various authors (Hollander *et al.*, 1985). A more precise measurement of the α-tocopherol absorbed may be obtained by cannulating the main mesenteric lymph duct or the thoracic lymph duct. One problem with cannulating the main mesenteric lymph duct is that it is also drained by accessory ducts. But an advantage is that hepatic and

peripheral lymph interference may be avoided (Bjorneboe *et al.*, 1986).

When evaluating the results of absorption experiments the method of administering vitamin E must also be considered. Traber *et al.* (1986) has demonstrated, in studies on absorption of α-tocopherol, that 50% of the amount administered was absorbed when given as a bolus. This was in contrast to over 97% absorption when given as a slow continuous infusion at a rate which was similar to dietary input.

Tocopheryl acetate is almost completely hydrolyzed in the gut lumen under physiological conditions prior to absorption, but Mathias *et al.* (1981) has identified a mucosal esterase in the endoplasmic reticulum of the enterocyte. Pancreatic juice and bile are required for maximal absorption of vitamin E. Apparently vitamin E is absorbed as a bile-lipid micelle, along with other fat-soluble vitamins, free fatty acids and monoglycerides. The epithelial cells are penetrated through the apical plasma membrane of the cells in the brush border by a passive diffusion process from the small intestine to the enterocyte (Hollander *et al.*, 1985).

Weiser *et al.* (1986) suggested that α-tocopherol acetate may be more efficient in alleviating vitamin E deficiency signs than free α-tocopherol. However, studies which compared the two forms at high levels of intake (>400

IU/d), revealed that higher levels of tocopherol were obtained with free tocopherol than with the ester. This could suggest that at high does rates there may be a limitation in hydrolysis of the ester to the alcohol form (Baker *et al.*, 1980). The difference in observed uptake of α-tocopherol between the alcohol and the ester forms may be due to autoxidation of the free form.

It has been suggested that medium-chain triglycerides increase the absorption of vitamin E, while long-chain polyunsaturated fatty acids may reduce absorption. The reduction in absorption could be the result of increased breakdown of α-tocopherol due to oxidation (Horwitt, 1962).

Alpha-tocopherol is transported in the lymph bound to chylomicrons. Unlike both retinol and cholesterol, α-tocopherol does not undergo re-esterification during the absorption process. The portal vein does not appear to make a significant contribution to the absorption of α-tocopherol in mammals but does play an absorptive role in birds. In chickens vitamin E appears to be absorbed almost entirely by the portal vein and transported directly to the liver. There seems to be no compartmentalization in the chicken with regard to absorption of lipid material between portal blood vessls and lymphatic vessels (Gallo-Torres, 1980).

Absorption studies of tocpoherols other than α-tocopherol have not been very extensive. The intake of α-

tocopherol has been found to be two to four times that of γ-tocopherol due to its dietary predominance. Several studies have indicated that the level of γ-tocopherol averages only 10% of the concentration of α-tocopherol in most mammalian plasma (Behrens and Madera, 1985). Traber *et al.* (1986) found no significant difference in the rate of absorption between γ- and α-tocopherol in cannulation studies with rats. Baker *et al.* (1986) observed a reduction in plasma γ-tocopherol when high dietary levels of α-tocopherol or RRR-γ-tocopherol were given either orally or by intramuscular injection to weanling pigs. There was no significant difference in the maximum increase above initial concentration or the time of peak concentration. However, α-tocopherol was retained significantly longer in plasma than was γ-tocopherol. Both vitamins appear to be absorbed by pigs equally but γ-tocopherol is eliminated much more rapidly than is α-tocopherol.

B. Transport and Distribution

Vitamin E is transported in the lymph and blood bound to chylomicrons and lipoproteins and is distributed in each fraction according to the fat composition of that fraction (Gallo-Torres, 1980). Chylomicrons are formed when free fatty acids and monoglycerides are taken up by the smooth endoplasmic reticulum, after entering the epithelial cell

from the gastrointestinal tract, and reconstituted into triglycerides. The triglycerides then join with absorbed cholesterol and phospholipids to form globules. The phospholipids arrange themselves so that the lipophilic portion is toward the center of the globule and the lipophobic or polar portions are toward the surface. Small quantities of ß-lipoprotein, synthesized in the smooth endoplasmic reticulum, coat part of the surface of the globule. This provides an electrically charged surface that helps to make the globule miscible with the aqueous environment of the cell. The globule then diffuses to the side of the epithelial cell and is excreted by a process called exocytosis to the space between cells where it passes into the lymph via the central lacteal of the villus. Once in the central lacteal of the villus the chylomicron is propelled through the lymph by the lymphatic pump through the thoracic duct where it is emptied into the veins of the neck (Guyton, 1991a). In this manner, α-tocopherol is transported by the chylomicrons, attached to an apolipoprotein, and moved into the blood via the lymphatic system.

There are four primary classes of lipoproteins. These have been divided based on their densities. They are, in increasing density, chylomicrons, very-low-density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Lipoproteins are not

static; there is a great deal of movement of molecules between the surface and the core, between different lipoproteins and between lipoproteins and the various tissues (Norum *et al.*, 1983). The apolipoproteins are synthesized in the liver and small intestine. Lipoproteins are synthesized in the intestine (chylomicrons and HDL), liver (VLDL and HDL) or the plasma (HDL and LDL) (Mahley *et al.*, 1984).

Vitamin E is transported in the blood bound to lipoproteins. Although no specific transport protein has been identified there is evidence that vitamin E may selectively bind to the HDL at a higher rate than to the other lipoproteins (Behrens *et al.*, 1982). Bjorneboe *et al.* (1986) reported that in fasting rats and humans α-tocopherol is evenly distributed between VLDL and HDL but is highest in LDL. A very high relationship exists between total plasma lipids and tocopherol concentration in plasma (Gallo-Torres, 1980).

Bjornson *et al.* (1976) reported that when vitamin E was dietarily supplemented to humans, a rapid and parallel increase of the vitamin in plasma and red blood cells occurred. Most of the α-tocopherol in the erythrocytes was located in the cellular membranes.

The sequestering of α-tocopherol in peripheral tissues occurs during the catabolism of triacylglycerol rich lipoproteins by the activity of lipoprotein lipase, by the

LDL receptor, or by nonreceptor uptake (Traber and Kayden, 1984).

Bjorneboe *et al.* (1987) found that pharmacological doses of α-tocopherol are primarily cleaved from the blood along with chylomicron remnants and that the α-tocopherol was redistributed from VLDL and LDL to HDL. After hepatectomy Bjorneboe *et al.* (1987) also found that the redistribution was delayed, indicating that the transfer process may depend on the processing of α-tocopherol containing chylomicrons in the liver.

Because of the high correlation between total plasma lipids and α-tocopherol certain diseases may alter the normal α-tocopherol profile in plasma or serum. Disorders such as diabetes mellitus, hypothyroidism and hypercholesterolemia in which total plasma lipids are high will also exhibit higher vitamin E concentrations than normal. While in diseases which cause lower total plasma lipids such as protein malnutrition, beta-lipoproteinemia, cystic fibrosis and liver disease, α-tocopherol will also be lower than normal in plasma and serum (Behrens *et al.*, 1982).

C. Tissue Deposition

The concentration of α-tocopherol in tissues is directly correlated with tocopherol intake. This relationship may explain some of the pharmacological effects of vitamin E since most other vitamins have

distinct concentration thresholds in tissues other than the liver. Alpha-tocopherol is concentrated in intracellular structures that are rich in membrane such as the mitochondria, microsomes and nucleus (Machlin, 1991).

The importance of the methyl groups on the chromanol ring is illustrated by the fact that while γ-tocopherol is absorbed and deposited in tissues, it is retained for only a short time when compared with α-tocopherol. The phytyl side chain of α-tocopherol has been shown to interact with the acyl chain of polyunsaturated phospholipids. Changes in the side chain of α-tocopherol drastically alter biological activity of the molecule (Lucy, 1978). It appears that both the ring structure and the side chain of the compound are important in determining the specific inclusion site in the cell membrane. Burton and Ingold (1988) demonstrated that in biological membranes, α-tocopherol is oriented with the chromanol group toward the surface of the membrane along with the phosphate region of the phospholipid molecules and the hydrophobic phytyl tail is embedded in the hydrocarbon region of the membrane. Alpha-tocopherol is probably not immobile in the cell membrane, but may move vertically or laterally within the membrane.

There appears to be a specific binding protein for tocopherol in the cytosol, the nucleus, the mitochondria, in tumor cells and in the cellular membrane of the adrenals

and in the erythrocytes (Machlin, 1991). The exact mechanism of the binding of α-tocopherol in the cell membrane and the specific composition of the binding protein is unknown.

The liver, adipose tissue and muscle tissue are the major storage sites for vitamin E. The liver consists of a number of different cell types with the larger parenchymal cells accounting for 85 to 90% of total liver mass and the smaller nonparenchymal liver cells accounting for approximately 35% of the total number of cells. The nonparenchymal liver cells may be further divided into stellate cells, Kupffer cells and endothelial cells (Wisse and Knook, 1979). Bjorneboe *et al.* (1987) reported that rats intravenously injected with labeled α-tocopherol had a maximum liver concentration after about 45 minutes when over 50% of the injected tocopherol was recovered. After 24 hours only 11% of the injected amount was retained in the liver. When nonparenchymal and parenchymal cells were separated by differential centrifugation, it was found that α-tocopherol uptake was significantly greater in the parenchymal cells.

Bjorneboe *et al.* (1988) found that in rats a significantly higher concentration of α-tocopherol was located in the peroxidase-positive Kupffer cells than in peroxidase negative endothelial cells. The parenchymal cells contained over 90% of the hepatic α-tocopherol.

Although this is higher than that reported in most papers it was noted that the rats were on 100 mg/kg dietary vitamin E prior to the experiment which was higher than those in previous experiments. After supplementation with higher amounts of dietary α-tocopherol, Bjorneboe *et al.* (1988) found that the α-tocopherol concentration of the parenchymal cells was increased six times, but the α-tocopherol content of the nonparenchymal cells remained at presupplemented levels. This would indicate that the parenchymal liver cells are the chief storage cells for large dietary levels of α-tocopherol. When rats were placed on a low α-tocopherol diet for 8 wk the parenchymal cells had the least amount of reduction of the vitamin, but the nonparenchymal cells lost 95% of their predepleted levels. Parenchymal cells may have the ability to conserve α-tocopherol when dietary levels are low by mobilization from other tissues.

The mitochondria, microsomes, lysosomes and the Golgi apparatus have been found to have the highest concentration of α-tocopherol in the subcellular compartment with the cytosol and peroxisomes having the lowest concentrations (Hagen *et al.*, 1989).

Alpha-tocopheryl quinone is the primary hepatic oxidation product of α-tocopherol. Tocopheryl quinone is further reduced to hydroquinone which, after conjugation with glucuronic acid, is excreted in the bile or converted

to tocopheronic acid and eliminated in the urine (Bjorneboe *et al.*, 1987).

The liver, skeletal muscle and adipose tissue have the ability to store excess α-tocopherol. These three tissues accounted for over 90% of the total quantity of α-tocopherol recovered in 10 separate organs in a study with rats (Traber and Kayden, 1987). In the adipose tissue α-tocopherol is localized in the lipid droplet, and mobilization from adipose tissue is extremely slow. The adrenals had the highest levels of α-tocopherol per gram of tissue because of the specific binding sites for HDL in rat adrenal glands. Bjorneboe *et al.* (1986) found the lungs and spleen to have high concentrations of α-tocopherol. This may reflect on the importance of α-tocopherol in protecting the lungs from injury due to hyperoxia and in helping to maintain the immune response. The brain and the testes contained less α-tocopherol per gram of tissue than any other organ. This may be the reason that two of the major vitamin E deficiency signs are neurological dysfunction and reproductive failure (Kayden and Traber, 1986). The cerebellum has the lowest concentration of α-tocopherol in the rat brain, and the cerebral cortex has the highest concentration of α-tocopherol; this is an inverse relationship between tocopherol and the activity of glutathione peroxidase in the brain (Goss-Sampson and Muller, 1987).

Biochemical Function

A. Membrane Antioxidant

Vitamin E is a natural ingredient in most animal feeds and since eicosanoid synthesis is a normal body process, it would be natural to assume that there would be enough vitamin E in the diet to prevent the leaking of polyunsaturated fatty acid radicals and cellular damage would not occur. During the normal evolutionary process this would probably have been true as animals adapted to their normal diets. Unfortunately, modern technology, environmental pollution, and modern farming methods have caused the vitamin E requirements of animals and man to exceed the concentrations found in most diets (Rice and Kennedy, 1988). Animals which have evolved on grass diets, which are relatively high in vitamin E, are now required to consume cereal based diets which are relatively low in this vitamin. If a high oxygen radical attack initiates a lipid peroxidation challenge, then one would expect a greatly increased requirement for vitamin E to prevent autoxidation of membrane polyunsaturated fatty acids that would normally be required to maintain an optimum polyunsaturated fatty acid, cyclo-oxygenase, vitamin E ratio in the eicosanoid producing cell. The principal fatty acids required in the diet by mammals are the polyunsaturated fatty acids (PUFA), linoleic, linolenic and arachidonic. These fatty acids are essential due to their requirement in cellular and

subcellular membranes such as the mitochondria, lysosomes and nucleus. In the cellular membrane they have two primary functions. They, along with cholesterol, help to ensure that the cell membrane will be fluid and elastic; and they (especially arachidonic acid) are essential for eicosanoid synthesis (Horwitt, 1962).

Autoxidation, which is a free radical chain reaction, occurs when many different types of organic compounds come into contact with dioxygen *in vitro* and *in vivo*. The speed at which autoxidation progresses can be greatly reduced or controlled by specific compounds known as antioxidants. We may divide antioxidants into two classes. Antioxidants which control the initiation of free radical production are known as primary or preventive antioxidants. Examples of primary antioxidants are catalase and glutathione peroxidase which can convert hydrogen peroxide (H_2O_2), a free radical precursor, and the hydroperoxides (ROOH) of fatty acids into harmless products. Another type of primary antioxidant is ceruloplasmin. Ceruloplasmin prevents transition metal ion-catalyzed production of radicals from hydroperoxides by binding the metal ion (Burton and Traber, 1990). Antioxidants which trap free radicals such as the peroxyl radicals (ROO·) and the hydroxyl radical (HO·) reduce the actual number of existing radicals which may cause a further production of free radicals and are called secondary or chain-breaking

antioxidants (AH). Most of the chain breaking antioxidants are phenols or diarylamines. Each molecule of the chain breaking antioxidant quenches two free radicals by the following reactions:

$$ROO\cdot + AH \longrightarrow ROOH + A\cdot$$

$$ROO\cdot + A\cdot \longrightarrow ROOA$$

Vitamin E is a hydrophobic, free radical-trapping, chain breaking antioxidant found in the lipid membranes of most living organisms (Burton *et al.*, 1983).

Early investigations recognized that α-tocopherol could function as an *in vivo* antioxidant (Olcott and Matill, 1941). Polyunsaturated fatty acids are extremely sensitive to attack by free radicals. The more double bonds the greater is the susceptibility to oxidative free radical damage. Dam (1957) proposed that vitamin E acted as an *in vivo* antioxidant, which was reinforced by his ability to ascertain the existence of higher concentrations of peroxides in the tissues of vitamin E deficient animals. The phenol hydroxyl group at position 6, of the vitamin E molecule, can donate a hydrogen to the lipid free radical. The hydrogen then reacts with the free electron, quenching the free radical and preventing a chain reaction. Table 5 gives a summary of autoxidation and antioxidant reactions which involve vitamin E.

At first it was thought that dietary vitamin E was destroyed *in vitro* when polyunsaturated fatty acids

Table 5. Autoxidation and chain-breaking reactions and vitamin E

I. Initiation

$LH + O_2 \cdot^- \longrightarrow L\cdot$

2. Reaction of radical with oxygen

$L\cdot + O_2 \longrightarrow OO\cdot$

3. Propagation

$LOO\cdot + LH \longrightarrow L\cdot + ROOH$

$L\cdot + ROOH \longrightarrow ROO\cdot + LH$

4. Chain breaking reactions

$LOO\cdot + EH \longrightarrow LOOH + E\cdot$

$ROO\cdot + E\cdot \longrightarrow ROOE$

Abbreviations: LH, fatty acid; $O_2 \cdot^-$, superoxide; L·, fatty acid radical; LOO·, peroxy radical; E, α-tocopherol; E·, tocopheroxy radical; LOOH, hydroperoxide.

underwent peroxidation in the diet. When vitamin E was given separately from the dietary PUFA, the PUFA still brought on symptoms of vitamin E deficiency (Machlin, 1991). Dam (1957) detected peroxides in the adipose tissue of chicks and rats which were fed a diet that was rich in PUFA but deficient in vitamin E. These studies led Tappel (1962) to propose that vitamin E functions as an *in vivo* antioxidant that protects tissues from attack by free radicals.

Alpha-tocopherol intercepts the peroxy radical more rapidly than can the polyunsaturated fatty acids located in the cellular membranes. The α-tocopherol then donates its phenolic hydrogen at position 6 to the free radical and converts it to hydrogen peroxide. The tocopheroxyl radical that is formed is too stable to continue the radical chain reaction and is removed from the system by reaction with another peroxyl radical, converted into inactive products, and eliminated via the liver (Burton and Ingold, 1989).

The reaction rate at which an antioxidant reacts with free radicals is a measure of its antioxidant efficiency (Burton, *et al.*, 1985). It has been determined that α-tocopherol is one of the most efficient chain-breaking antioxidants known. It reacts 200 times faster than does butylated hydroxytoluene (BHT), a well known commercial antioxidant, with the peroxyl radical (Burton and Ingold, 1989).

Because of the speed with which α-tocopherol can compete for peroxyl and hydroxyl radicals, compared to polyunsaturated fats in the lipid membrane, a very small quantity of the vitamin is able to protect relatively large amounts of polyunsaturated fat. Protective concentrations of α-tocopherol in biological membranes are one molecule of tocopherol per 1000 lipid molecules. In protecting the polyunsaturated lipids of the membrane vitamin E must be either regenerated from the tocopheroxyl radical or replaced through body reserves or the diet (Scholich *et al.*, 1989).

The relative importance of vitamin E for protecting the integrity of lipid membranes is enhanced by the fact that it is the only major lipid soluble, chain-breaking free radical scavenger that has been found in plasma, red cells, platelets and tissues (Burton and Ingold, 1989; Burton *et al.*, 1983). Beta-carotene is a lipid soluble chain-breaking antioxidant, but it is far less efficient than α-tocopherol and is important only in biological regions of very low oxygen partial pressure (Burton and Ingold, 1984).

Tocopherol is unique compared with synthetic antioxidants *in vivo* because of its more specific localization in membranes and in the persistency with which it remains in biological tissues. Most synthetic antioxidants must be administered on a continuous basis, in

relatively large quantities in order to be effective, compared to vitamin E (Machlin, 1991).

Another unique feature of vitamin E is its ability to be biologically regenerated from the tocopheroxy radical back to α-tocopherol by vitamin C and reduced glutathione *in vivo*. When ascorbic acid reduces the tocopheroxy radical it is transformed into an ascorbate radical. The ascorbate radical can be reduced back to ascorbate by an enzymatically controlled NADH-dependent system (Doba *et al.*, 1985).

From the information presented it is evident that α-tocopherol has a unique role as the major lipid soluble antioxidant protecting cellular membranes from damage by free radicals which result from lipid peroxidation.

B. Nucleic Acid and Protein

Catigani (1980) found increased rates of DNA synthesis in muscles in vitamin E deficient rabbits and in the bone marrow of vitamin E deficient monkeys. Rather than reflecting a direct influence in DNA synthesis the author felt that this phenomenon was probably due to increased erythropoiesis in response to a decrease in red blood cell survival time and an increase in muscle cell turnover rate caused by necrotizing myopathy due to vitamin E deficiency.

A number of enzyme levels have been found to be altered in vitamin E deficient animals. Creatine kinase

and xanthine oxidase levels were elevated in vitamin E deficient animals representing actual increases in apoenzyme synthesis and not simply changes in enzyme activity (Fraga *et al.*, 1989). It has also been suggested that α-tocopherol plays a specific role in heme synthesis but this has not been supported by a preponderance of the evidence (Machlin, 1991).

The mitochondria contains very high concentrations of α-tocopherol, most of which is in the inner membrane. Microscopic examination of liver, muscle and heart tissues demonstrated that the disruption of the mitochondria is one of the first pathologies observed in animals that are vitamin E deficient. There was a decline in the oxidation of succinate in the livers of deficient animals that seems to indicate that the effect of α-tocopherol may be to protect the dehydrogenases in the early part of the electron transport chain (Packer, *et al.*, 1989). The actual role of α-tocopherol may be to protect dehydrogenase sulfhydryl groups from oxidation or from reactions with metal ions. It has been demonstrated that the mitochondria from the muscle and liver of vitamin E deficient rats are subject to damage by ultraviolet light or contact with dioxygen (Quintanhila *et al.*, 1982).

Alpha-tocopherol and Platelet Function

Blood platelets are small oval or round discs that are between 2 and 4 microns in diameter and are formed in the bone marrow from megakaryocytes. Megakaryocytes are large hemopoietic cells in the bone marrow which differentiate into blood platelets either in the bone marrow or soon after entering the circulatory system. The normal concentration of blood platelets is between 150,000 and 500,000 per microliter (μl) except in rodents where average platelet concentration is at least 900,000/μl (Dodds, 1989). Blood platelets do not have nuclei but still have a number of functional characteristics of whole cells. In the platelet cytoplasm are active substances such as thrombosthenin, a contractile protein that can cause the platelet to contract, myocin and actin like those found in muscle cells, parts of the endoplasmic reticulum and the Golgi apparatus that have the ability to synthesize a number of enzymes and have the capacity to store large concentrations of calcium ions. The blood platelet also has mitochondria and the enzyme systems capable of producing adenosine triphosphate (ATP) and adenosine diphosphate (ADP), and specific enzyme systems that utilize arachidonic acid to synthesize prostaglandins, which are hormones capable of causing different types of vascular and localized tissue reactions. Fibrin-stabilizing factor, another protein produced in blood platelets, helps to

strengthen the fibrin reticulum during the first few minutes of blood clot formation. Finally a growth factor is found in blood platelets that causes growth in the vascular endothelial cells and vascular smooth muscle cells that causes the cells to multiply and helps to repair damaged vascular cells (Dodds, 1989).

The cellular membrane of the blood platelet is very important to cell function. A coating of glycoproteins cover the cell surface and prevents the platelet from adhering to normal endothelium while allowing it to adhere to injured or damaged endothelium cells in the blood vessel wall. This cellular adherence is especially active when there is exposed collagen from deeper within the vessel wall (Dodds, 1989).

Blood platelets are part of the hemostasis mechanism of blood loss prevention in the animal system. Platelets perform the action by forming a platelet plug soon after injury to a blood vessel wall occurs. When blood platelets come into direct contact with damaged endothelial cells or collagen fibers in the vascular wall, they begin to change their physiological characteristics. They increase in size and become irregular in shape with a number of protrusions sticking out from the cell membrane. Contractile proteins, within the cell, cause the platelet to contract with the release of granules that cause the platelet cell to stick to the collagen fibers. The platelet then secretes large

quantities of ADP and thromboxane A_2. The thromboxane and the ADP then act on nearby platelets to activate them, a cycle of activation begins with the marshalling of increasing numbers of platelets that attract more platelets (aggreation) until a platelet plug is formed. This plug is generally successful in stopping blood loss if the damaged opening is small. If the damaged area is larger, other clotting mechanisms come into action until a number of fibrin threads are formed which form a tight clot (Guyton, 1991b).

Blood platelets have a very short lifespan (9 to 11 days) compared to most hemopoietic cells found in domestic animals. Approximately two-thirds of all available platelets in an animal are in the circulation with the remaining one-third stored in the spleen (Steiner and Mower, 1982).

After the normal lifespan of the platelet is concluded it is eliminated from circulation by the macrophage system with over 50% being removed in the spleen (Dodds, 1989).

A number of specific hemorrhagic problems can be caused by either a deficiency of blood platelets (thrombocytopenia) or by an excess of platelets (thrombocytosis). When the numbers of blood platelets are adequate but biochemical function is impaired, thrombasthenia occurs (Benson *et al.*, 1986). Platelet concentrations may increase dramatically under certain

pathological conditions such as traumatic injury, iron deficiency anemia, infections, cancer and removal of the spleen (Benson *et al.*, 1986). Thrombopoietin appears to be the regulating hormone for thrombocytopoiesis in mammals. Evidence has been presented that appears to indicate that poorly functioning platelets cause an increase in the production and release of thrombopoietin causing a concurrent increase in thrombocytopoiesis (Clift *et al.*, 1991).

The arachidonic acid cascade involves both the cyclooxygenase and the lipoxygenase pathways. These pathways involve reactions which produce free radicals. The cyclooxygenase pathway is also responsible for the production of thromboxane and prostacyclin, two extremely important compounds necessary for the normal function of blood platelets (Rice and Kennedy, 1988). A scheme for the synthesis of thromboxane and prostacyclin is given in Figure 2.

Since both the cyclooxygenase and the lipoxygenase pathways contain free radical producing reactions, it has been proposed that vitamin E might inhibit some of the reactions involving thromboxane, prostacyclin or the prostaglandins (Machlin, 1991).

Aspirin is a cyclooxygenase inhibitor. Aspirin decreases the production of eicosanoids with the added

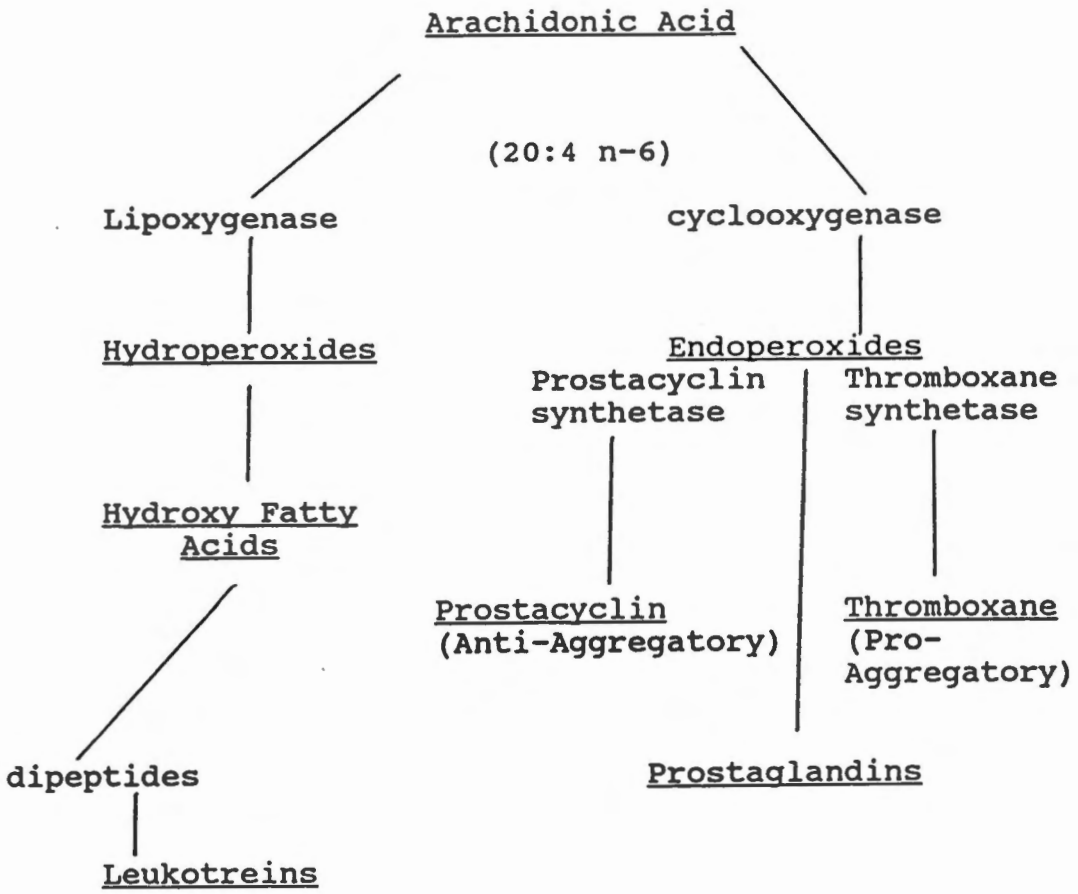


Figure 2. Arachidonic acid metabolism

beneficial effect of reducing headaches in humans, and also decreases the prostacyclin/thromboxane ratio (PGI_2/TXA_2) which in doing so, minimizes the risk of clotting and thrombosis. Many millions of dollars are spent yearly on a search for lipoxygenase inhibitors suitable for pharmacological use.

Toivanen (1987) has demonstrated beneficial roles for vitamin E and vitamin C in preventing blood clots by decreasing the production of thromboxane (TXA_2) which promotes clotting and increases the production of PGI_2, which is anti-aggregatory.

While a normally functioning blood clotting mechanism is necessary to protect against blood loss following injury and capillary hemorrhage, increased platelet aggregation isa factor in the development of atherosclerosis and other vascular diseases.

Steiner (1978) and Srivastava (1986) have demonstrated that vitamin E appears to inhibit platelet aggregation and the production of prostaglandins, which also stimulate platelet clumping. Research on marmoset monkeys demonstrated an increase in platelet aggregation in vitamin E deficient animals, but vitamin E supplemented marmosets exhibited resistance to collagen-induced platelet aggregation (McIntosh, 1987). Vitamin E supplementation depressed the enhanced platelet aggregation associated with estrogen therapy in female rats (Ciavatti, 1984).

It is believed that vitamin E stimulates the production of prostacyclin, a powerful antiplatelet aggregation substance. Szczeklik (1985) found that an atherosclerosis-promoting diet almost completely suppressed prostacyclin production in rabbits, but supplementation with vitamin E appeared to protect the prostacyclin-generating system in arteries. Vitamin E deficient rats have exhibited diminished prostacyclin production when compared to vitamin E supplemented animals (Karpen *et al.*, 1981). In a study utilizing human volunteers Steiner (1978) found that platelet aggregation by ADP, epinephrine and collagen was inhibited between 30-50% on daily intakes of 800-1600 IU of vitamin E, but found that arachidonic acid-induced aggregation was almost completely inhibited. Szczeklik (1985) observed a mild suppressant effect on induced platelet aggregation in human subjects supplemented with 600 ml vitamin E per day, but 300 mg vitamin E per day had no suppressant effect. Diez Marques *et al.* (1987) reported that rats receiving daily injections of dl-α-tocopheryl acetate (50 mg/kg) had only slightly higher plasma α-tocopherol concentrations but platelet aggregation was significantly reduced when compared to control rats.

Silbert *et al.* (1990) found no significant changes in the aggregation of platelet rich plasma to ADP or collagen during a short term (14 days) vitamin E study with human males who were given 1500 IU/day of dietary dl-α-tocopheryl

acetate. But Kakishita *et al.* (1990) found that d-α-tocopherol inhibited human platelet aggregation in whole blood. A synergistic effect was reported when aspirin was added to the tocopherol treatments. Jandak *et al.* (1989) tested platelet adhesion after 14 days of supplementing vitamin E in human subjects. The average decrease in adhesion after 14 days of 200 IU/day of tocopherol was 75%; after 14 days of 400 IU/day, platelet adhesion decreased by 82%.

Vitamin E and Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PMN's) comprise the blood phagocytes. These cells are unique in that they are able to leave the circulation and migrate to extravascular sites of infection and inflammation where they recognize and ingest microbes and other particulate material. Similar to platelets and lymphocytes, but distinct from red cells, PMN's are capable of achieving an increased state of functional and metabolic activation in response to immunochemical and particulate signals. Metabolic activation of the PMN includes the activation of one or more cyanide-insensitive oxidases and related enzyme systems, which oxidize substrate NADH and NADPH leading to increased oxygen utilization and the subsequent reduction of oxygen to superoxide anion, hydrogen peroxide, hydroxyl radical and other free radicals (McCay and King, 1980).

Baehner *et al.* (1982a) have demonstrated that the reduced oxygen by-products and free radicals, especially hydrogen peroxide, are utilized for microbicidal reactions linked to the myeloperoxidase halide system present in PMN's.

Polymorphonuclear leukocytes (PMN's) appear to undergo functional alterations with the administration of pharmacological doses of vitamin E. There appears to be an increased ability to ingest particles, but also a mild decrease in the bactericidal potency of the PMN. Superoxide anion release is slightly enhanced, but H_2O_2 release from the PMN appears to be markedly impaired.

Shohet *et al.* (1974) suggested that vitamin E itself could not increase phagocytosis. During phagocytosis the killing of bacteria may be partially dependent on peroxides generated via bacterial lipid peroxidation. This peroxidation may be promoted by ubiquinone, a prooxidant but not by alpha tocopherol, an antioxidant. In this manner, the phagocytosis-promoting effects of vitamin E may then be compatible with the concept that vitamin E helps maintain high ubiquinone levels in phagocytic cells and the ubiquinones directly promote phagocytosis.

Harris *et al.* (1980) reported that daily administration of large amounts of vitamin E to rats resulted in alterations of the ability of human PMN's to ingest and kill bacteria, to utilize oxygen and release the oxygen by-products, superoxide anion and hydrogen peroxide.

Baehner *et al.* (1982b) demonstrated that the administration of 1,600 IU of vitamin E to humans resulted in functional alterations of their PMN with an increased ability to ingest particles and an enhanced respiratory burst with an increase in superoxide anion release. A slight decrease in bactericidal potency of the PMN was observed along with a marked decrease in H_2O_2 release from the PMN. The hexose monophosphate shunt activity, which is dependent on intracellular H_2O_2, was also decreased during phagocytosis.

Prasad (1980) found a decreased leukocyte bactericidal capacity in an uncontrolled study of thirteen men and boys supplemented with 300 mg of vitamin E daily for three weeks. The decreased bactericidal activity of the leukocytes was a result of decreased free radical production and increased membrane stability. In a similar study, daily supplementation of 1,600 IU vitamin E for 7 d markedly increased the neutrophil phagocytic rate, but the bactericidal capacity of neutrophils was decreased (Baehner *et al.*, 1977).

Chirico *et al.* (1983) in a study of premature infants found that vitamin E supplementation resulted in increased phagocytosis at 5 d of age as compared to nonsupplemented infants. By 2 wk of age phagocytosis appeared to be normal in both untreated and treated infants suggesting that

vitamin E may be of benefit in accelerating normal phagocytic function in premature infants.

Ingraham *et al.* (1982) reported the release of arachidonic acid from membranes of vitamin E supplemented PMN's was slightly enhanced during phagocytosis. NADH oxidase-derived H_2O_2 was not impaired within phagocytic vesicles of vitamin E supplemented PMN's. Vitamin E appeared to selectively deplete H_2O_2 generated by NADPH oxidase in phagocyte vesicles, accounting for the impairment of bactericidal activity in these cells.

Wastell *et al.* (1985) observed a tendency for slower earlier functional response to an antigen by PMN's from pigs supplemented with 100 IU vitamin E per kg of diet. There was also an increase in total WBC count for vitamin E PMN's over that of controls fed 7 IU/kg diet. Wastell *et al.* (1987) reported that high dietary levels of vitamin E did not appear to pose a significant threat to the disease resistance of the young pig because of reduced H_2O_2 production by these cells.

Deficiency

Vitamin E deficiency affects a very large number of cellular and tissue systems. The extent of the effect and the specific tissue affected depends on the effects of interacting nutrients such as ascorbic acid, selenium and polyunsaturated fatty acids and the particular species.

Necrotizing myopathy is one of the most frequently encountered deficiency signs that is common to a large number of species (Machlin, 1991). This problem occurs primarily in the skeletal muscles (muscular dystrophy) but is also frequently observed in the heart (mulberry heart disease) and in the liver (liver necrosis). Additional problems found in swine include yellow fat disease, acute circulatory failure, infertility, reproduction and lactation problems (Whitehair and Miller, 1986). There are instances when vitamin E deficiency and that of selenium are hard to separate. In calves myopathy results from a selenium deficiency while in rabbits, swine, and other species vitamin E status is as important as that of selenium, or is the main determinant (Machlin, 1991). A number of changes over the last twenty years have contributed to increased incidences of vitamin E deficiency among production animals. These include: the use of polyunsaturated fats and oils in diets, the very fine grinding, pelleting and restructuring of feed which causes more surface area to come into contact with oxygen, and higher concentrations of trace metals such as iron and copper which increase the production of free radicals. Longer storage periods for feeds and contamination with molds and the increasing lipid peroxidation of the natural lipid content in feeds also causes low vitamin E concentrations. The use of high moisture grains, total and

partial animal confinement, feeding a high grain content in feeds for increased growth as opposed to green forages which are higher in vitamin E, increased animal productivity with early weaning and rebreeding, which can deplete maternal tissue concentrations of vitamin E and selenium are additional factors which may contribute to vitamin E deficiency (Mahan, 1991). Table 6 shows a number of the effects of vitamin E deficiency in animals.

There are a number of possible explanations that may account for the species differences and the variety of symptoms that affect the development of vitamin E deficiency signs. Species differ in their ability to deposit vitamin E in various tissues. Chickens deposit a great deal more vitamin E in muscle than do turkeys. The concentrations of polyunsaturated fatty acids, such as 20:4 and 22:6, vary a great deal from tissue to tissue and from species to species (Nelson, 1980). The efficiency withwhich dietary unsaturated fatty acids are deposited within the cellular membranes varies with tissue, species, and stage of growth (Machlin, 1991). Selenium is a constituent of glutathione peroxidase and is found in different concentrations in the various tissues and species. Since a large number of deficiency signs and manifestations are shared between vitamin E and selenium, this could have a direct influence on the variety of symptoms and species differences.

Table 6. Effects of vitamin E deficiency

Pathology	Effected Tissue	Species
Necrotizing myopathy	Skeletal muscle	Pig, rabbit, horse, rat, chicken, lamb, catfish, duck
	Heart	Pig, rat, calf, goat, deer
Necrosis	Liver	Pig, rat, chick
Fibrosis	Pancreas	Chick, mouse
Sterility (male)	Testes	Pig, rat, chicken, rabbit, monkey
Fetal death and resorption	Placenta	Pig, rat, chicken, cow, mouse
Exudative diathesis	Vascular system	Chicken, turkey, catfish
Anemia	Erythrocytes	Pig, rat, catfish
Hyperthrombopoiesis	Platelets	Rat
Cataracts	Eyes	Turkey, rabbit
Hemolysis	Erythrocytes	Chick, rat, premature human infant
Encephalomalacia	Cerebellum	Chick
Axonal dystrophy	Nerves	Rat, dog, duck, mouse, monkey
Gastric ulceration	Stomach	Pig
Steatitis (Lipofuscin accumulation)	Adipose tissue	Pig, rat, mouse cat, mink
Depigmentation	Incisors	Rat

(Newberne and Conner, 1989; Machlin, 1991)

Nutritional and Physiological Assessment

Plasma and serum tocopherol concentrations have been the most useful and easily performed index of vitamin E status (Farrel, 1980). An erythrocyte hemolysis test has been used but is less precise than blood tocopherol levels and is useless in certain species such as the pig and the cow. There can be a wide variation in plasma lipid concentration between individuals within a particular species. There has been a correlation between plasma lipid level and α-tocopherol concentration reported (Horwitt *et al.*, 1972). Individuals with a low plasma lipid content will have a low plasma vitamin E level and individuals with a high plasma lipid content will have a correspondingly high plasma vitamin E level if all other factors are equal. The ratio of plasma tocopherol to total plasma lipid is considered to be a more accurate criterion of tocopherol adequacy than plasma α-tocopherol alone. The ratio of tocopherol to cholesterol has been found to be a more convenient criterion of tocopherol adequacy than total plasma lipid, but does not achieve the same level of accuracy (Horwitt *et al.*, 1972).

Lehman *et al.* (1988) have suggested using platelet tocopherol as an index of α-tocopherol status. Red blood cells have also been used to determine the α-tocopherol status of an animal (Mino *et al.*, 1988). The increase in the α-tocopherol concentration of plasma, platelets and red

blood cells following supplementation parallel one another, but blood platelets and red blood cells may reflect a more accurate level of tocopherol in tissues with a very slow turnover such as brain and adipose tissue (Machlin, 1991). Breath pentane analysis, which is a measurement of *in vivo* lipid peroxidation has also been suggested as a test for tocopherol adequacy (Horwitt, 1974).

Nutritional Requirements

Since a number of dietary and physiological factors influence the nutrient requirement of vitamin E, it has been difficult to accurately define the precise requirement for the vitamin. Polyunsaturated fatty acids, selenium, high trace metal concentrations, such as iron and copper, and environmental conditions have all been shown to influence the vitamin E requirement. Vitamin E requirement may differ between species as well as between strains within the same species. Gabriel *et al.* (1984) reported that the spontaneously hypertensive rat is more sensitive to vitamin E deficieny and has a higher requirement for the vitamin than the Wistar-Kyoto or Sprague-Dawley strains.

Low dietary selenium levels or high unsaturated fatty acid, such as linoleic acid, levels have been shown to increase the requirements for vitamin E. The vitamin E requirements have been established by determining that dietary level which will prevent deficiency symptoms. The

dietary requirement for vitamin E that is necessary for optimum response might be higher than that necessary to prevent deficiency (Bendich *et al.*, 1986a). Table 7 lists the vitamin E requirements for some animals based on the recommendations of the National Research Council of the National Academy of Sciences.

Interactions with Unsaturated Fatty Acids

A. Unsaturated fatty acids

In animals, the dietary requirements for vitamin E has been shown to increase as the intake of polyunsaturated fatty acids increases (Drapier, 1980). High levels of linoleic acid increase the requirement to prevent encephalomalacia in the chicken. Increased levels of fish oil in animal diets may amplify skeletal and cardiac myopathy (Drapier, 1980). When plasma concentration of vitamin E has been used as the basis, a vitamin E to PUFA ratio of .4 (mg vitamin E per g PUFA) has been found to prevent deficiency symptoms (Drapier, 1980). With the increasing use of soybean meal in animal diets the concentrations of vitamin E may have to be increased in some species since the vitamin E to PUFA in soybean oil is only .2. As the consumption of polyunsaturated fatty acids increases in both animal and human diets a reevaluation of the interrelationship between vitmain E and PUFA may be necessary.

Table 7. Vitamin E requirements for animals.

Species	Requirements (IU/kg diet)
Swine	
Prewean (1-5 kg)	16
Early Weaner (5-10 kg)	16
Starter (10-20 kg)	11
Grower (20-50 kg)	11
Finisher (50-110 kg)	11
Breeding gilts, sows, and boars	11
Turkeys	
Starting and growing	30
Breeding	40
Rat	30
Mouse	30
Chickens	
Starting	30
Growing	10
Laying	10
Breeding	30
Cattle	
Young	20
Finishing	10
Breeding	20
Horses	
Young	20
Breeding	20
Fish	
Catfish	100
Trout	30

Machlin (1991)

B. Selenium

Glutathione peroxidase is a cytosolic enzyme that can reduce fatty hydroperoxides to fatty alcohols and H_2O_2 to H_2O (Ursini and Bindoli, 1987). The reducing of hydroperoxides has been suggested as the activity which facilitates the physiological action of α-tocopherol in the reduction of peroxy and hydroxy radicals.

In a number of animals such as chickens and pigs, liver necrosis, pancreatic atrophy, and heart muscle myopathy can be prevented or cured by either vitamin E or selenium. A severe selenium deficiency in chickens impairs vitamin E absorption. The selenium appears to alleviate vitamin E deficiencies by increasing the quantity of vitamin E absorbed and by serving in glutathione peroxidase (Draper, 1980).

Vitamin E Content in Food

All eight of the isomers of tocopherol are found abundantly in nature with α-tocopherol having the highest *in vivo* activity. In foods from animal origin, alpha-tocopherol accounts for almost all of the vitamin E activity. In plants, especially those with a high oil content, the other isomers of vitamin E are found in larger concentrations. In corn oil the α-tocopherol content is approximately 159 μg/g while the γ-tocopherol content is approximately 600 μg/g. Soybean oil contains an average of

79 μg/g of α-tocopherol but has 593 μg/g of γ-tocopherol. As more data is obtained on the distribution of tocopherol isomers in food, a more accurate determination of the vitamin E activity of all isomers in foods may be possible (Bauernfeind, 1980).

There will always be problems when using calculated data in estimating the vitamin E contents of various diets, or when trying to determine the vitamin E intakes of individuals or of a group of animals. Bauernfeind (1980) has demonstrated that there is no correlation between the vitamin E intake estimated by analysis and that estimated by calculation from preexisting data. The reason for this difference is that there are a great number of factors that may influence the vitamin E concentration in foods. These factors result in a very wide variation in the vitamin level of a particular dietary constituent. For most foods there is a three- to ten-fold range in the reported vitamin E values (Bauernfeind, 1980).

Tocopherol is extremely unstable, and significant losses often occur during storage and processing of feedstuffs. This is particularly true when the diets have had significant exposure to heat, oxygen and sun light. In order to obtain precise values for vitamin E intake an analytical analysis of the diets must be made. When α-tocopherol acetate is used in diets a more accurate determination of vitamin E concentration in diets may be

obtained because the acetate form is very stable in foods and diets.

Vitamin C

General

A wide variety of plant and animal life can synthesize ascorbic acid from carbohydrate precursors which include galactose and glucose. Primates, guinea pigs, insects, invertebrates, fishes and some species of birds lack the ability to synthesize ascorbic acid due to the absence of the microsomal enzyme L-gulonolactone oxidase (Sato *et al.*, 1976; Sato and Udenfriend, 1978).

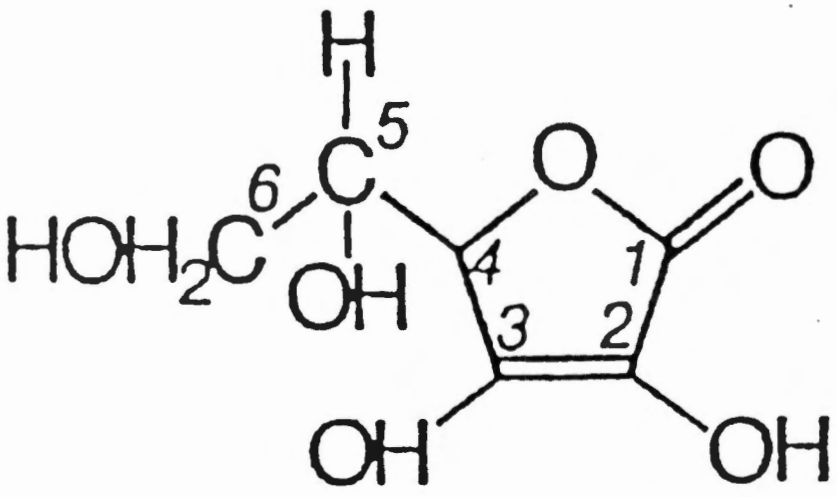
The disease scurvy has been known since ancient times, and was prevalent in Northern Europe during the Middle Ages. It was a major cause of death for explorers and sailors on long trips and voyages. Early observations were reported in the 16th century about the scurvy-preventive action of lemon juice (Nobile and Woodhill, 1981). Lind, during the 18th century, described in great detail the symptoms of scurvy and its possible prevention by including fresh fruit in the diet (Nobile and Woodhille, 1981).

Szent-Gyrgy isolated a water-soluble, strongly reducing, optically active, white crystalline compound from cabbage and the adrenal glands of several animals. He called the new compound hexuronic acid. During the same year, it was determined that hexuronic acid was identical

to the antiscorbutic factor which had been isolated from lemon juice by Zilva in 1923 (Nobile and Woodhille, 1981). The substance was then named ascorbic acid. Zilva (1927) and Tillmans (1930) discovered the existance of an equally active but oxidized compound, called dehydroascorbic acid, and established that the two substances were interconvertible and formed a redox-system. The chemical structure and names for vitamin C are found in Figure 3. L-ascorbic acid forms a redox-system with its first oxidation product the dehydro-L-ascorbic acid, and its chemical formula is $C_6H_6O_6$ (Figure 4). The monodehydro-L-ascorbic acid-free radical, an intermediate product of the oxidation reaction, has the chemical formula $C_6H_7O_6$ (Figure 4) (Nobile and Woodhill, 1981).

Function

The primary function of vitamin C revolves around its redox potential (+ 0.08 mV) which allows the compound to reduce transition metals permitting the vitamin to participate in metabolically important hydroxylation reactions (Stevenson, 1974). Thus, the biologically activesites of vitamin C are at carbons two and three. It has been indicated that the function of ascorbic acid in domestic animals that produce their own vitamin C is the same as for those animals which require an exogenous source of the vitamin (Stevenson, 1974). It would also appear



Chemical Name: L-threo-2,3,4,5,6 pentahydroxy-1-hexenoic acid-4-lactone

IUPAC Name:

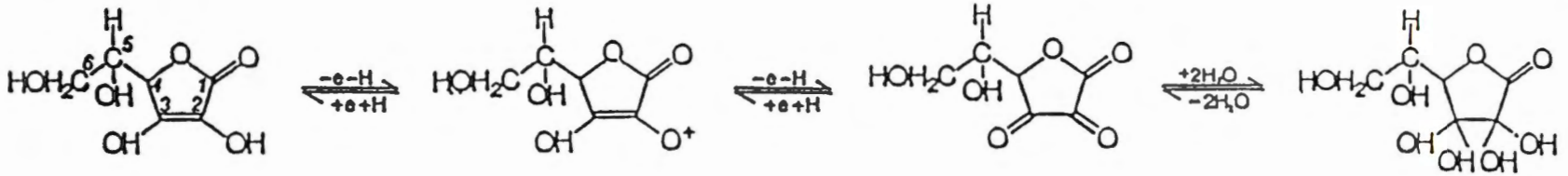
Trivial Name: L-Ascorbic Acid

Common Name: Ascorbic Acid

Molecular Weight: 176

Empirical formula: $C_6H_8O_6$

Figure 3 - L-ascorbic acid



L-Ascorbic acid (reduced)

Monodehydro-L-ascorbic acid free radical (intermediate product)

Dehydro-L-ascorbic acid (oxidized)

Dehydro-L-ascorbic acid (hydrated)

Structural formulae of L-ascorbic acid, dehydro-L-ascorbic acid and the intermediate product monodehydro-L-ascorbic acid free radical.

Figure 4. Vitamin C redox-system

appear that there are specific circumstances in which the biosynthesis of ascorbic acid in domestic animals may not be sufficient to meet metabolic requirements. Exogenous supplementation of vitamin C has been a benefit in cases of disease, exposure to environmental pollutants, high environmental temperature, extremely rapid growth, periods of extreme stress and in humans, those individuals who smoke or drink to excess (Bauernfeind, 1982; Cameron and Pauling, 1981; Duthie *et al.*, 1991; Irwin and Hutchins, 1976; Pauling, 1979; Teare *et al.*, 1979).

Absorption

Hornig (1975) and Kallner *et al.* (1977) found that the site and mechanism of absorption may differ between those animals that are capable of synthesizing the vitamin and those animals requiring a dietary source. In guinea pigs and humans, maximum absorption occurs in the duodenum by a Na^+ dependent, active, carrier-mediated system (Stevenson, 1974). Ascorbic acid absorption in the rat, which is able to synthesize vitamin C, is a passive process that occurs in the ileum (Kallner *et al.*, 1977; Hornig *et al.*, 1973).

Metabolism

At the molecular level, ascorbic acid, like vitamin E, is a very powerful reducing agent, and has a general

importance as an antioxidant. Ascorbic acid appears to function as a general source of reducing equivalents throughout the animals system. Only a few enzyme reactions have been shown to specifically require vitamin C. Ascorbic acid is involved in hydroxylations using molecular oxygen and often use Fe^{+2} or Cu^{+2} as a cofactor. Ascorbate is thought to play either of two roles: (1) as a source of electrons for reduction of O_2, or (2) as a protective agent for maintaining iron and copper in a reduced state (Lewis, 1976; Linder, 1985).

Vitamin C at low concentrations initiates lipid peroxidation and at high concentrations quenches it, probably by recycling the vitamin E radical. This would allow a single vitamin E molecule to scavenge many free radicals (Packer *et al.*, 1979). A possible mechanism for the recycling of vitamin E by ascorbic acid and the reduction of the ascorbate radical is as follows:

$$E\cdot + C \longrightarrow E + C\cdot$$

$$C\cdot + NADPH \longrightarrow C + NADP\cdot$$

Packer *et al.* (1979) have demonstrated that the tocopherol molecule is apparently continuously regenerated at the expense of vitamin C:

LOO· α-tocopherol OH Vitamin C·

LOOH α-tocopherol O· Vitamin C

The strong possibility of an interactive relationship between iron and its participation in free radical

formation, via the Haber-Weiss-Fenton reaction, vitamin E with its free radical quenching ability, and vitamin C with its tocopherol regenerating capacity exists.

Vitamin C and Polymorphonuclear Leukocytes

Vitamin C is distributed in all body tissues with polymorphonuclear leukocytes considered as a labile storage pool. Normal plasma values range from 5-20 μg/ml in humans. Vitamin C concentrations in leukocytes may reach 60-70 μg per 10^8 cells (Tolbert, 1979). Vitamin C affects polymorphonuclear cells *in vitro* and *in vivo*. DeChatelet *et al.* (1972) found that ascorbate or dehydroascorbic acid reduced both glutathione and nicotinamide adenine dinucleotide phosphate. High concentrations of ascorbic acid in phagocytic PMN's in human subjects was also noted.

Olson and Polk (1977) found that ascorbic acid reversed the inhibitory effect of corticosteroids on PMN phagocytosis *in vitro*. Boxer *et al.* (1979) noted that ascorbic acid appeared to promote microtubule assembly, normalized chemotaxis and degranulation, and corrected adherence of granulocytes in neutrophils from patients with Chediak-Higashi syndrome.

The locomoter functions (random motor and chemotactic migration) of neutrophils and macrophages seem to be impaired in the absence of vitamin C.

Okano, *et al.* (1990) found that the ascorbic acid content of guinea pig leukocytes was reduced by a factor of 16:1 between normal and scorbutic animals. Leukocyte phagocytic activity did not appear to be affected but leukocyte morphology was affected. A deficiency of the vitamin appeared to significantly interfere with the effectiveness of circulating leukocytes against ingested, cell-associated and extracellular bacterial pathogens. Leukocytes from water supplemented with ascorbic acid killed approximately 83% of ingested pathogens, while nonsupplemented leukocytes were able to phagocytize approximately 13% of ingested pathogens.

Wilson (1983) reported a synergistic effect between vitamin E and vitamin C in leukocyte formation and phagocytosis of bacteria in guinea pigs.

Vitamin E, Vitamin C and PMN

It would appear, from the limited number of investigations conducted on the possible interaction of vitamin E and C, that there may be a synergistic relationship between the two vitamins in terms of their ability to influence leukocyte concentration, function and humoral immune response.

Leukocyte and immune response depends on many factors, some that have to do with the host and some related to the infectious agent. The so-called general health of the host

is as important as the specific immune state of the animal. Disease resistance also depends on environmental effects such as nutrition, behavioral and mental stress. These factors must be taken into consideration when evaluating the effect of vitamin E and C on leukocyte numbers and function and disease resistance, because vitamin E and C may also affect some of these factors. Even though experimental data may appear to clearly demonstrate a point, we must exercise great caution in extrapolating the findings to animals, including man, other than the specific test subjects.

Iron

General

Iron has been associated with life for several thousand years, and especially with blood, possibly because of the color of blood and that of rust. Iron preparations were used to cure a variety of disorders as long ago as 2735 B.C., when the Chinese Emperor Shen Nung listed iron as a cure for "anemia" (Cook and Finch, 1975). Iron therapy was used for many centuries as a remedy by the ancient Greeks, Romans, Arabs, and Europeans. Iron is found in all cells of the animal and human body and is the most abundant trace element. Iron has an atomic number of 26 with an atomic weight of 55.85. It is the 4th most abundant element, comprising approximately 5% of the

composition of the earth's crust (Cook and Finch, 1975). It is present in a number of enzyme systems responsible for electron transport (cytochromes), for the transport of oxygen and CO_2, when part of the hemoglobin and myoglobin molecules, and for the activation of oxygen in conjunction with oxidases and oxygenases. Approximately 10-15% of dietary iron is absorbed. The comparatively low rates of absorption may be due in large part to endogenous control of the rate of uptake. The primary regulatory mechanism of the absorptive capacity of the intestinal mucosa is still unknown. In a condition of iron deficiency a larger portion of dietary iron is absorbed, and absorption is greatly reduced when the body has relatively large stores. Tannins, phytates, oxalates and phosphates tend to form insoluble iron precipitates that cause the iron to be unavailable for absorption (Balcerzak *et al.*, 1967). Iron-chelating agents such as fructose, fumarate, certain amino acids, and ascorbic acid retain iron in a soluble form and make it available for absorption. Iron in the form of heme groups, found in meat, is naturally chelated and is absorbed more easily than iron found in plants (Balcerzak *et al.*, 1967).

Iron metabolism is composed of two loops: one internal and one external. The internal loop continuously reutilizes iron from cells that have been catabolized in the body. Iron released from hemoglobin in the

reticuloendothelial system is taken up by transferrin and transported to the bone marrow where it becomes a component of hemoglobin in newly developing red cells. Although iron is utilized in other cells in the body, internal iron metabolism is primarily concerned with the recycling of iron in the red blood cell component of the body. The external loop is represented by the losses of iron from the body and its concomitant absorption from the diet (Balcerzak *et al.*, 1967).

Metabolism

Iron in the animal is found in ferritin and hemosiderin for storage, in transferrin for transport in plasma, in uteroferrin for iron transport in the uterus, and in lactoferrin and transferrin in milk for iron transfer to the nursing animal (Miller, 1991).

The total quantity of iron stored in the body will average 4 grams. The largest proportion of iron is present in hemoglobin at approximately 65%, with 4% found in the form of myoglobin (Balcerzak *et al.*, 1967). Protein transferrin accounts for 0.1 percent in blood plasma, and 15 to 30 percent of iron is stored in the reticuloendothelial system and in liver cells as ferritin (Balcerzak *et al.*, 1967).

Transport and Storage

When dietary iron is absorbed from the small intestine, it is combined in blood plasma with a beta globulin, apotransferrin, which then forms transferrin, the protein that transports iron in the plasma. Transferrin binds iron very loosely so that it can be released to all cells of the animal's body. The chief storage site of iron in the animal body is the liver hepatocytes and the reticuloendothelial cells (Guyton, 1991b). In the cellular cytoplasm iron combines with apoferritin to form ferritin, the principal storage protein for iron. Since ferritin is a very large molecule, different quantities of iron may combine in small clusters so that each molecule of ferritin may contain a variable amount of iron. When very large quantities of iron are present so that the apoferritin pool is overloaded, then the excess iron is stored in very large clusters in an extremely insoluble form known as hemosidern (Guyton, 1991b). When plasma iron levels fall very low then ferritin releases iron to transferrin and iron levels are restored. Iron stored in hemosidern is only very slowly released regardless of iron tissue needs. This mechanism may lead to iron overload over a period of time (Furugouri, 1974).

Absorption

Iron is absorbed from all sections of the small intestine. The absorption mechanism begins when the liver secretes apotransferrin into bile which flows into the small intestine and binds with free iron to become transferrin. The transferrin binds with receptors located in the outer membranes of the intestinal epithelial cells and is carried into the epithelial cells by the process of pinocytosis and then released on the blood side of the cells as plasma transferrin (Guyton, 1991b).

When all of the apoferritin in the body has become saturated the absorption of iron from the intestinal tract decreases. At this point the transferrin, which is normally 30 percent saturated, becomes almost completely saturated. The liver decreases its rate of production of apotransferrin and mucosal iron transport ceases (Guyton, 1991b). This mucosal blockage of iron has a very limited capacity, because high doses of dietary iron will cause excess iron to enter the blood with a large deposition of hemosiderin in the reticuloendothelial cells.

Free Radicals

The production of free radicals by enzymatic and chemical processes in animal biological systems is a part of normal body function. An example of this process is the production of free radicals by neutrophils during the

process of phagocytosis and the subsequent destruction of pathogens. The free radicals are produced in subcellular membranes during the enzymatic production of prostaglandins, prostacyclins and leukotrienes. The mitochondria of cells is also a source of free radical production in the course of electron transfer (Rice and Kennedy, 1988).

There are instances where the type and rate of free radical production exceeds the body's ability to control them or limit their concentration within the physiological limits of the cell where their effect is necessary. The normal body defense mechanisms are unable to prevent these highly reactive free radicals from reacting with other cellular molecules which may then be destroyed or damaged. Loss of cellular function, cell death and/or genetic mutation may then occur.

Nature has provided for a variety of natural defense mechanisms to attempt to limit the cellular damage from uncontrolled free radical production. Most of these are under nutritional control and many are enzymatic in nature. One of the primary nutritional defense mechanisms is vitamin E, which acts like a lipophilic free radical scavenger protecting the bi-lipid layer of subcellular membranes. While reaction with vitamin E is not the only mechanism available for detoxifying free radicals, vitamin E has the unique ability to scavenge free radicals in a

subcellular lipid environment. This ability allows vitamin E to halt the potential formation of lipid peroxides at their source (Burton *et al.*, 1983).

A free radical is a molecule or part of a molecule which contains a single unpaired electron. This unpaired electron gives the molecule a high degree of chemical activity. Free radicals vary in their degree of reactivity, with some being relatively unreactive and others being extremely reactive and therefore potentially dangerous. Depending on the biological conditions free radicals may have either reducing or oxidizing properties. Those radicals with oxidizing ability are the most important, in terms of vitamin E, because they can remove a hydrogen from polyunsaturated fatty acids initiating lipid peroxidation.

In biological systems free radicals are produced by: (1) the action of metal ions, (2) by enzymatic action, (3) by ionizing radiation, and (4) by the action of xenobiotics. Some oxygen species that can initiate lipid peroxidation are given in Table 8 (Pryor, 1986). These oxygen free radicals can initiate self-propagating lipid peroxidation.

Free radicals are produced in animals as represented by the following equations:

$$O + e^{-} \longrightarrow O_2\cdot \text{ (superoxide radical)}$$

TABLE 8. Examples of oxygen free radicals and oxygen species important in free radical production.

Symbol	Description of Radical
O_2	Singlet oxygen
NO_2	Nitrogen dioxide
HO·	Hydroxyl radical
O_2·	Superoxide
HOO·	Hydroperoxyl radical
H_2O_2	Hydrogen peroxide
RO·	Aloxyl radical
ROO·	Peroxyl radical

Pryor (1986)

Superoxide dismutase (a copper dependent enzyme) converts superoxide to hydrogen peroxide:

$2)_2 \cdot^- + 2\ H^+ \longrightarrow H_2O_2 + O_2$ (hydrogen peroxide)

Superoxide is not a particularly active species but it can rapidly be converted *in vivo* to more active radicals such as its protonated form ($HO_2\cdot$) and the hydroxyl radical ($OH\cdot$). The hydroxyl radical is a product of a Haber-Weiss-Fenton reaction involving an iron catalyst as shown:

$$H_2O_2 + FE^{+2} \longrightarrow OH\cdot + FE^{+3} + OH^-$$

$$O_2\cdot^- + FE^{+3} \longrightarrow FE^{+2} + O_2$$

The net effect of these reactions is:

$$O_2\cdot^- + H_2O_2Fe^{+3} \longrightarrow OH^- + O\cdot + O_2$$

These oxygen radicals can initiate lipid peroxidation by removing a hydrogen atom from a specific methylinic carbon of a polyunsaturated fatty acid. The hydroxyl radical is the principal instigator of the peroxidation shown by the following:

$$LH - H \longrightarrow L\cdot$$

<u>initiation</u>

$$L\cdot + O_2 \longrightarrow LOO\cdot$$

(lipid peroxy free radical)

The lipid peroxidation chain reaction is self-perpetuating until there is a chemical termination:

$$LOO\cdot + LH \dashrightarrow LOOH + L\cdot$$

lipid <u>Propagation</u>

The termination of this self-perpetuating reaction can occur by the reaction of the lipid radical with other cell components or by interaction with vitamin E which is a free radical scavenger (Rice and Kennedy, 1988).

Vitamin E, C, and Iron Interactions

Oski and Barnes (1967) reported the occurrence of mild hemolytic anemia during the second month of life in vitamin E-deficient infants. Further studies have subsequently indicated abnormal *in vitro* sensitivity of erythrocytes to hydrogen peroxide exposure. One of the known biological activities of vitamin E is that of an inhibitor of lipid peroxidation at the cellular and subcellular level. A lack of vitamin E may be manifested by an accelerated red cell lysis as a direct result of an increased rate of peroxidation of the lipid bilayer of the red blood cell (RBC) membrane (Binder *et al.*, 1965). Abnormal *in vitro* red cell H_2O_2 fragility in combination with vitamin E deficiency has been described in a number of diseases, including cystic fibrosis and a number of other malabsorptive disorders without an associated anemia (Binder *et al.*, 1965). It would seem reasonable to assume that other factors coexist with deficiency of tocopherol as mediators of vitamin E-dependent anemia.

Exogenous iron is often given in relatively large amounts to premature infants and occasionally in infant

formulas to prevent the late anemia associated with rapid growth and the depletion of body iron stores during the latter part of the first year of life (Hammond and Murphy, 1960). Melborn and Gross (1971) found significantly low hemoglobin and serum vitamin E concentrations with higher reticulocyte counts and increased red blood cell hydrogen peroxide fragility in infants who received supplemental iron and no supplemental vitamin E. They suggested that therapeutic doses of iron increased red cell hemolysis.

Chen and Thacker (1986) found that vitamin E deficiency in rats resulted in significantly decreased plasma iron concentrations and total iron binding capacity. The total iron contents of the liver and spleen were higher in vitamin E-deficient groups compared to control groups, but supplemented vitamin E seemed to reverse this trend. It appeared that vitamin E and C interactively affected the iron tissue distribution in rats. The sparing effect of ascorbic acid on vitamin E metabolism in vitamin E-deficient rats as well as an apparent sparing effect of vitamin E on plasma in rats has been demonstrated (Chin *et al.*, 1980).

Iron dextran is widely used as an intramuscular injection for the prevention of iron deficiency anemia in newborn pigs. Tollerz (1965) found that sows fed on rations deficient in vitamin E produced pigs which were more susceptible to the toxic effects of oral or injected

iron. The clinical symptoms of high iron dosing with low dietary vitamin E levels include prostration, muscular weakness, spasm and skeletal muscle which histologically exhibits signs of coagulative degeneration. Occasionally some pigs die within a few hours of dosing with iron, with the actual cause of death obscure.

Patterson *et al.* (1967) reported deaths from iron dextran injections as a result of cardiac arrest and hyperkalaemia. Plasma levels as high as 13 mEq/L of iron were reported from an injection of iron equivalent to 47 mg of iron per kilogram of body weight.

Krider *et al.* (1982) reported that baby pig losses as a direct or indirect result of iron injection were approximately 1.4% of all pigs produced. If these numbers are correct then over one million pigs died as a result of iron injections from the over 85 million pigs produced in 1990.

The initial effect of iron on muscle is probably to increase the rate of lipid peroxidation. Even in non-fatal cases, the levels of muscle-tissue peroxides have been observed to increase within approximately 2 h after iron injection (Patterson *et al.*, 1967).

Lannek *et al.* (1962) postulated that iron (Fe^{+3}) accelerated the formation of lipoperoxides and the destruction of tocopherols in autoxidizing fats with the resultant hydroperoxide-metal complexes being extremely

reactive. Their results supported the assumption that large amounts of iron destroy vitamin E *in vivo*.

Vitamin E exerts a free radical scavenging effect on the lipid environment of cells and the introduction of iron into tissues apparently speeds up the oxidation of polyunsaturated fatty acids in the cell membrane. Since vitamin C appears to have a regenerating effect on the tocopherol molecule, more study into the possible interactive effects of vitamin E, vitamin C and iron and their physiological interrelationships is needed and is an extremely interesting line of investigation.

CHAPTER III

EXPERIMENT 1

Introduction

Vitamins are generally supplemented to animal diets at concentrations that will prevent deficiency symptoms. When the vitamin is supplemented at higher concentrations, at some elevated level it may be appropriate to discuss the pharmacological response of the vitamin.

It has been proposed that high dietary levels of vitamin E and vitamin C could possibly lead to a redistribution of iron in tissues (Chen and Thacker, 1988). Chou *et al.* (1978) reported abnormalities of iron metabolism in vitamin E deficient rabbits. The total body iron content was not altered but the sequestration of iron in muscle tissue was affected.

The production of free radicals in biological systems is a continuing process. They are produced in cellular membranes during the production of prostaglandins, leukotrienes, thromboxane, and prostacyclines. Free radicals are produced in the inner membrane of the mitochondria of cells during the utilization of electron transfer as a means of energy conversion. They are produced by neutrophils during phagocytosis and during the destruction of pathogens. When the rate of production of free radicals exceeds the physiological system's capacity

to control them, the body's normal defense mechanisms cannot prevent the destruction or damage of intra-cellular components or the cell membranes. This may result in cell and tissue death, loss of function or genetic mutation (Rice and Kennedy, 1988).

To control or prevent free radical damage a variety of defense systems have been developed. A number of these systems are under nutritional control. One of the most powerful lipophilic free radical scavengers is α-tocopherol. Packer *et al.* (1979) demonstrated that ascorbic acid may continuously regenerate α-tocopherol by its strong reducing ability. There appeared to be a physiological interaction between the two vitamins.

Iron has been demonstrated to be the central metal ion catalyst in the Haber-Weiss-Fenton reaction (Pryor, 1988). The primary product of this reaction is the highly reactive hydroxyl radical. Iron concentration in tissues has been linked to increased lipid peroxidation initiated by the hydroxyl radical (Rice & Kennedy, 1986).

Since little is known concerning the interaction of α-tocopherol and ascorbic acid on tissue iron distribution and because their interactive effects on lipid peroxidation are not well defined, the objective of this study was to determine the effect of α-tocopherol and ascorbic acid on iron tissue distribution, tissue lipid peroxidation, and

hematological parameters of young rats weighing between 50 and 75 grams.

Materials and Methods

Animals and Treatments

This experiment utilized 96 male weanling (murine pathogen free) Sprague-Dawley rats purchased from Taconic Laboratory Animals (TAC:N(SD)fBR) weighing between 51-75 grams. The rats were allotted to treatment by weight and placed in stainless steel cages measuring 36 cm x 30 cm x 26 cm with four animals per cage. Water and experimental diets were provided *ad libitum*. The rats were ear notched for identification purposes. The litter trays beneath each cage were cleaned daily with waterers and feeders emptied, washed and refilled with fresh water and diet. Waterers had a stainless steel curved drinking tube with a 500 ml capacity glass body. The feeders were stainless steel with a feed saver ring and were anchored to the side of the cage with a stainless steel tension spring.

The rats were fed a vitamin E-free purified basal diet (Table 9) for five days prior to the beginning of the experiment. After five days the rats were allocated to experimental diets. All diets were mixed within 24 hours of the beginning of the experiment and stored in a refrigerated room at 4°C for the duration of the experiment. Eight experimental diets were fed in this

Table 9. Composition of basal diet - experiment 1

Ingredient	Percent
Glucose	63.8
Vitamin free casein	20.0
Corn oil-tocopherol stripped	10.0
Vitamin mixture[a]	2.2
Salt mixture[b]	4.0
	100

[a]Vitamin mixture (gm/kg): Vitamin A acetate (500,000 IU/gm), 180,000; Vitamin D_2 (850,000 IU/gm), 0.12500; Ascorbic acid, 4,500,000; Inositol, 500,000; Choline chloride, 7,500,000; Menadione, 225,000; p-Aminobenzoic acid, 500,000; Niacin, 425,000; Riboflavin, 100,000; Pyridoxine hydrochloride, 100,000; Thiamine hydrochloride, 100,000; Calcium pantothenate, 300,000; Biotin, 0.02; Folic acid, 0.09; Vitamin B_{12}, 0.00135.

[b]Salt mixture: Sodium chloride, 11.88%; Potassium phosphate dibasic, 8.5%; Potassium carbonate, 8.75%; Calcium phosphate dibasic, 39.11%; Calcium carbonate, 318.5524%; Magnesium carbonate, 5.89%; Ferric citrate (16-17% Fe), 1.7555%; Manganese sulfate·H_2O, 0.41%; Zinc carbonate, 0.1%; Copper sulfate·$5H_2O$, 0.06%; Sodium selenite, 0.000055%; Potassium iodate, 0.0021%; Chromium potassium sulfate $12\cdot H_2O$, 0.01%

experiment. The eight diets utilized the same basal diet, Table 9, with two concentrations of ascorbic acid and four concentrations of α-tocopheryl acetate. Experimental treatments and diet descriptions are given in Table 10. Treatment diets consisted of two added levels of ascorbic acid: 0 and 0.5% and four added levels of α-tocopheryl acetate: 7.5, 25, 37.5, and 75 IU/kg. The experiment was a 2 x 4 factorial arrangement with six animals per treatment.

Procedures and Analysis of Samples

At the beginning of the experiment a 20 gram sample of each experimental diet was taken for analysis. Samples were analyzed for tocopheryl acetate, ascorbic acid, crude protein, percent dry matter, percent crude fiber, percent ash, percent ether extract, gross energy, calcium, phosphorus, iron, zinc and copper (Table 11).

Three rats from each treatment were sacrificed weekly with blood samples and tissue samples (liver, muscle, spleen) taken on days 7, 14, 21, and 28 of the trial. The rats were anesthetized with a lethal dose of metofane (inhalation). A midline abdominal incision was performed, and blood was collected with a 20 gauge needle and 6 ml syringe from the abdominal aorta into heparinized (sodium heparin) 4 ml polypropylene tubes with plastic snap caps. After heart and respiration ceased the liver, spleen, and kidney were removed for further analysis. The blood and

Table 10. Experimental treatments - experiment 1

Treatment	Ascorbic acid[a] (%)	α-tocopheryl acetate[b] (IU/kg)
1	0	7.5
2	0	25
3	0	37.5
4	0	75
5	.5	7.5
6	.5	25
7	.5	37.5
8	.5	75

[a]Dietary addition (ICN Biochemicals)

[b]Dietary addition (Henkel Corp.)

Table 11. Analyzed composition of experimental diets - experiment 1

	Experimental diets							
	1	2	3	4	5	6	7	8
α-tocopheryl acetate IU/kg	7.5	25	37.5	75	7.5	25	37.5	75
Ascorbic Acid (%)	0	0	0	0	.5	.5	.5	.5
Analyzed Components:								
Dry matter (%)	98.77	98.77	98.77	98.76	98.72	98.74	98.75	98.78
Ash (%)	3.44	3.55	3.41	3.49	3.37	3.40	3.60	3.35
Crude protein (%)	18.19	18.48	18.44	18.30	18.54	18.10	18.36	18.55
Crude fiber (%)	1.35	1.10	1.12	1.50	1.28	1.30	1.08	1.47
Ether extract (%)	9.69	9.58	9.64	9.58	10.35	11.01	9.76	10.00
Gross energy Kcal/g	4.55	4.55	4.52	4.54	4.52	4.57	4.53	4.52
α-tocopherol (μg/g)	5.25	18.10	27.30	48.50	5.39	18.42	27.89	48.92
Ascorbic acid (%)	.003	.003	.002	.003	.43	.42	.43	.43
Calcium (mg/g)	8.51	8.51	8.61	8.27	7.95	8.07	7.94	8.81
Phosphorus (mg/g)	5.50	5.40	5.51	5.41	5.35	5.34	5.34	5.31
Iron (μg/g)	131.6	125.4	123.1	123.4	125.7	120.2	125.8	120.9
Copper (μg/g)	10.4	9.2	8.7	9.5	8.9	9.3	8.8	8.9
Zinc (μg/g)	31.0	31.5	32.4	32.3	31.6	33.9	31.6	31.2

tissue removal was carried out under a safelight (non-UV) and immediately placed on ice to prevent loss of ascorbic acid and α-tocopherol. The blood samples were split into two sets; one set was left at room temperature for analysis of red and white blood cells and hematocrit and hemoglobin concentrations, and the second set was used to obtain plasma by centrifugation of the whole blood at an RCF (relative centrifugal force) of 3090 g's for 10 min, with removal of the plasma into a separate polypropylene tube. The plasma was then flushed with nitrogen and stored at -45°C until analysis. All subsequent handling and analysis of plasma and tissues were carried out under a safelight (non-UV) in order to prevent loss of α-tocopherol.

Blood hemoglobin was determined by the method of Crosby *et al.* (1954) and hematocrits were determined by the microhematocrit method of McGovern *et al.* (1955). Red blood cell and white blood cell concentrations were determined by the method of Helleman (1972) on heparinized whole blood within four hours after each bleeding.

Lipid peroxidation analysis was carried out utilizing a modified procedure by Yaggi (1987) (Appendix A1) for blood plasma, and a modified procedure by Ohkawa *et al.* (1979) (Appendix A2) for tissue.

Alpha-tocopherol analysis of plasma and tissues was carried out utilizing a general lipid extraction procedure developed in our laboratory (Appendix A3). Analysis of

alpha-tocopherol in feed samples was carried out utilizing the method of McMurray and W. J. Blanchflower (1979). Vitamin C (ascorbic acid) analysis was carried out utilizing the procedure of Markku *et al.* (1986) for plasma and the method of Watada (1982) for feed samples. Alpha-tocopherol and ascorbic acid for developing standards were obtained from Eastman Kodak, Inc. Total iron binding capacity analysis was carried out utilizing the method of Olson and Hamlin (1969). Plasma samples to be analyzed for iron concentration were first diluted with MilliPore Corp. Milli-Q ultrafiltered water and analyzed on an Instrumentation Laboratory aa/ae spectrophotometer using the methods described by Instrumentation Laboratory (1978). Tissues were analyzed for iron concentration by homogenizing the tissues 1:3 with Milli-Q water and then wet ashing the samples by weighing 5 grams of tissue homogenate into a 125 ml Phillips beaker and adding 30 ml of reagent grade nitric acid. The solution was heated on a hotplate until near dryness was achieved. The flask was allowed to cool and 3.5 ml of reagent grade perchloric acid (70%) (G. Frederick Smith Co.) was added and evaporated until approximately 1 ml remained. This material was diluted with Milli-Q water and analyzed spectrophotometrically as previously described for plasma.

Total plasma lipid analysis utilized the procedure of Zoellner and Kirsch (1962) with all necessary reagents and

instructions contained in Kit form produced by Boehringer Mannheim Diagnostics, cat. no. 124303.

Statistical Analysis of Data

The data were analyzed by the General Linear Model (GLM) procedures of the Statistical Analysis System (SAS) (Barr *et al.*, 1979). The GLM procedure is a multivariate regression analysis, determining differences by significant F tests. The Student-Neuman-Keuls procedure (SNK) was used to separate means when significant F values indicated differences among means (Steel and Torrie, 1960).

The independent variables used in the multivariate regression analysis were dietary concentration of ascorbic acid and α-tocopheryl acetate, and ascorbic acid concentration x α-tocopheryl acetate concentration interaction. The dependent variables were red blood cell concentration, white blood cell concentration, total plasma lipids, hemoglobin, hematocrit, total iron binding capacity, lipid peroxidation in plasma and tissue, plasma and tissue α-tocopherol, iron, and ascorbic acid concentrations.

Results and Discussion

The α-tocopherol concentrations in plasma are presented in Table 12. There was a linear increase in plasma concentration of α-tocopherol as dietary α-

Table 12. Effect of α-tocopheryl acetate and ascorbic acid on plasma α-tocopherol concentration

No. of rats	Ascorbic acid (%) 0: 12	Ascorbic acid (%) .5: 12		α-tocopheryl acetate (IU/kg) 7.5: 6	25: 6	37.5: 6	75: 6		
Day	α-tocopherol (μg/ml)		P value	α-tocopherol (μg/ml)				P value	SEM
7	5.71	5.21	.150	2.34^{d}	4.26^{c}	5.54^{b}	9.69^{a}	.0001	.168
14	3.68	4.49	.117	1.78^{c}	3.10^{c}	4.88^{b}	6.63^{a}	.0001	.271
21	4.36	4.31	.882	1.59^{d}	2.97^{c}	4.76^{b}	8.02^{a}	.0001	.153
28	4.02	3.36	.092	1.05^{d}	2.64^{c}	4.92^{b}	6.15^{a}	.0001	.183

^{a,b,c,d}Means in the same row under a common heading with different superscripts differ significantly.

tocopheryl acetate increased. At days 7, 14, 21, and 28, a significant ($P<.0001$) increase in α-tocopherol concentration of plasma was observed. This was in apparent agreement with results obtained by Machlin (1991), Bjorneboe *et al.* (1988), and Traber and Kayden (1987) who demonstrated that concentration of α-tocopherol in plasma and tissues was directly correlated with tocopherol intake in rats. Plasma α-tocopherol was not affected by dietary ascorbic acid concentration. This would appear to be in disagreement with Chin *et al.* (1980) who reported that plasma α-tocopherol was spared in rats when ascorbic acid was added to the diet. In the study by Chin *et al.* (1980), the rats were challenged by a large dose of iron so that lipid peroxidation was greatly enhanced in blood plasma over what might be expected in animals receiving a more normal dietary concentration of iron. Also, vitamin E supplementation was at deficiency or near deficiency levels. Since a lipid peroxidation challenge was not used one might hypothesize that a comparatively low rate of lipid peroxidation would occur. Alpha-tocopherol in the diets of the rats of this experiment may have been adequate to control lipid peroxidation without becoming depleted in the plasma.

Plasma ascorbic acid concentrations are presented in Table 13. Plasma ascorbic acid concentration increased significantly ($P<.0001$) as dietary ascorbic acid increased

Table 13. Effect of α-tocopheryl acetate and ascorbic acid on plasma ascorbic acid concentration

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 12	.5 12		7.5 6	25 6	37.5 6	75 6		
Day	ascorbic acid (μg/ml)		P value	ascorbic acid (μg/ml)				P value	SEM
7	1.14[b]	4.16[a]	.0001	2.62	2.59	2.76	2.64	.727	.054
14	1.16[b]	4.70[a]	.0001	2.95	2.95	2.99	2.84	.684	.044
21	1.17[a]	4.83[a]	.0001	2.91	3.01	3.09	3.00	.382	.171
28	1.42[b]	4.63[a]	.0001	2.92	2.77	2.88	3.07	.579	.059

[a,b]Means in the same row under a common heading with different superscripts differ significantly.

on days 7, 14, 21, and 28. This is in agreement with Lowik *et al*. (1990) who reported an increase in plasma ascorbic acid concentration in rats as dietary levels increased. Dietary α-tocopherol acetate concentration did not significantly affect plasma ascorbic acid levels. This was expected as there was no specific lipid peroxidative challenge that would have rapidly depleted α-tocopherol and so ascorbic acid since rats have the ability to synthesize ascorbic acid under normal conditions.

Data concerning plasma iron concentrations are presented in Table 14. Dietary ascorbic acid level did not have a significant effect on plasma iron concentration. This disagrees with the work of Chen and Thacker (1986) who reported that dietary ascorbic acid (.2%) decreased plasma iron concentration in rats. On days 14, 21, and 28, dietary ascorbic acid had a tendency to increase plasma iron as ascorbic acid concentration increased, although the effects were not significant. This is in apparent agreement with Monsen (1982) who concluded that ascorbic acid increased plasma iron concentration in adult males. Dietary α-tocopheryl acetate at 75 IU/kg significantly increased plasma iron concentration on days 7, 21, and 28. While plasma iron was not significantly affected by α-tocopheryl acetate on day 14, there was an increase in plasma iron concentration at the 75 IU/kg α-tocopheryl supplementation. Chen and Thacker (1986) reported that

Table 14. Effect of α-tocopheryl acetate and ascorbic acid on plasma iron concentration

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 12	.5 12		7.5 6	25 6	37.5 6	75 6		
Day	iron (μg/ml)		P value	iron (μg/ml)				P value	SEM
7	3.57	3.50	.841	3.08^{b}	2.76^{b}	3.88^{ab}	4.42^{a}	.018	.179
14	3.49	3.56	.808	3.11	3.51	3.38	4.12	.092	.135
21	3.39	3.96	.067	2.78^{b}	3.57^{ab}	3.81^{ab}	4.57^{a}	.005	.145
28	3.32	3.68	.278	2.71^{b}	2.97^{b}	3.70^{ab}	4.62^{a}	.004	.163

^{a,b}Means in the same row under a common heading with different superscripts differ significantly.

rats whose diets were supplemented with α-tocopheryl acetate increased plasma iron concentration. This could have been due to the decreased sequestering of iron in various tissues in the body.

The effects of ascorbic acid and α-tocopheryl acetate on plasma total iron binding capacity are presented in Table 15. There was a significant decrease in plasma total iron binding capacity as dietary α-tocopheryl acetate concentration increased on days 7, 14, 21, and 28. The effect was inverse with the lowest total iron binding capacity at the highest α-tocopheryl acetate supplementation. This would be expected as total iron binding capacity and plasma α-tocopherol concentration would be expected to have an inverse relationship. As plasma iron concentration increased the total available capacity for iron to be bound would decrease. Conversely, as plasma iron concentration decreased we would expect that the total available capacity for iron to be carried in the plasma would increase. Ascorbic acid supplementation at .5% in the diet significantly increased total iron binding capacity at days 7, 14, and 21, and significantly decreased total iron binding capacity at day 28. Plasma iron concentration increased on days 14, 21, and 28 with ascorbic acid supplementation. It is possible that the large dose of ascorbic acid caused the release of iron from ferritin.

Table 15. Effect of α-tocopheryl acetate and ascorbic acid on plasma total iron binding capacity (TIBC)

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 12	.5 12		7.5 6	25 6	37.5 6	75 6		
Day	TIBC (μg/dl)		P value	TIBC (μg/dl)				P value	SEM
7	411.1^{b}	431.2^{a}	.0005	455.1^{a}	432.2^{b}	419.0^{c}	378.4^{d}	.027	7.321
14	447.7^{b}	504.3^{a}	.004	524.9^{a}	452.6^{b}	475.4^{b}	451.2^{b}	.0008	4.467
21	476.4^{b}	528.1^{a}	.0001	534.2^{a}	520.8^{b}	482.9^{c}	471.2^{d}	.0001	1.711
28	487.5^{a}	450.5^{b}	.001	485.6^{a}	487.6^{a}	457.9^{b}	444.9^{b}	.0001	2.700

^{a,b,c,d}Means in the same row under a common heading with different superscripts differ significantly.

Data concerning the effect of α-tocopheryl acetate and ascorbic acid on tissue iron concentration are presented in Table 16. Tissue iron concentration was not significantly affected by ascorbic acid supplementation. Liver iron concentration was significantly decreased by α-tocopheryl acetate at dietary supplementation of 75 IU/kg as compared to 7.5, 25, and 37.5 IU/kg of supplementation. As α-tocopheryl acetate concentration increased there was a linear decrease in liver iron concentration. Muscle iron concentration was significantly decreased at 75 IU/kg of dietary α-tocopheryl acetate supplementation. While not significantly different, there was a linear trend toward decreasing muscle iron concentrations as dietary α-tocopheryl acetate levels increased. Iron concentration of spleen tissue was significantly decreased by α-tocopheryl acetate at dietary supplementations of 37.5 and 75 IU/kg. Although not significant, dietary supplementation of α-tocopheryl acetate at 7.5 and 25 IU/kg linearly decreased spleen tissue iron concentration. This is in apparent agreement with Chou *et al.* (1978) and Bjorneboe *et al.* (1990) who reported a decrease in tissue concentration of iron as dietary concentrations of α-tocopheryl acetate increased.

Data concerning lipid peroxidation in tissue by main effect is presented in Table 17. Lipid peroxidation in liver, muscle and spleen were significantly decreased by

Table 16. Effect of α-tocopheryl acetate and ascorbic acid on tissue iron concentration

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 48	.5 48		7.5 24	25 24	37.5 24	75 24		
Tissue	iron (μg/g)		P value	iron (μg/g)				P value	SEM
Liver (wet)	63.7	63.5	.941	72.2^{a}	67.2^{ab}	64.1^{b}	50.9^{c}	.0001	1.124
Liver (dry)	278.3	277.4	.941	315.4^{a}	293.6^{ab}	280.0^{b}	222.4^{c}	.0001	1.124
Muscle (wet)	21.5	21.3	.902	26.15^{a}	24.8^{a}	21.9^{a}	12.7^{b}	.0001	.701
Muscle (dry)	98.2	97.3	.902	119.5^{a}	113.3^{a}	100.0^{a}	58.0^{b}	.0001	.701
Spleen (wet)	147.0	148.7	.611	162.9^{a}	154.8^{a}	143.6^{b}	130.1^{c}	.0001	1.634
Spleen (dry)	758.9	767.7	.611	840.9^{a}	799.2^{a}	741.4^{b}	671.7^{c}	.0001	1.634

^{a,b,c}Means in the same row under a common heading with different superscripts differ significantly.

Table 17. Effect of α-tocopheryl acetate and ascorbic acid on tissue lipid peroxidation (thiobarbituric acid-reactive substances)

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 48	.5 48		7.5 24	25 24	37.5 24	75 24		
Tissue	malonaldehyde (nmol/g)		P value	malonaldehyde (nmol/g)				P value	SEM
Liver (wet)	300.4[b]	282.4[a]	.038	332.7[a]	317.6[a]	291.1[b]	224.4[c]	.0001	3.973
Liver (dry)	1,312.4[b]	1,233.7[a]	.038	1,453.5[a]	1,387.5[a]	1,271.7[b]	990.3[c]	.0001	3.973
Muscle (wet)	241.7[b]	221.0[a]	.009	259.2[a]	257.3[a]	240.6[a]	170.1[b]	.0001	3.347
Muscle (dry)	1,104.2[b]	1,009.6[a]	.009	1,184.1[a]	1,175.4[a]	1,099.1[a]	777.1[b]	.0001	3.347
Spleen (wet)	350.1[b]	315.5[a]	.001	344.9[a]	357.8[a]	337.7[a]	291.1[b]	.0001	2.875
Spleen (dry)	1,807.4[b]	1,628.8[a]	.001	1,780.6[a]	1,847.2[a]	1,743.4[a]	1,502.8[b]	.0001	2.875

[a,b,c]Means in the same row under a common heading with different superscripts differ significantly.

added dietary levels (.5%) of ascorbic acid. Lipid peroxidation was significantly decreased by added dietary concentrations of α-tocopheryl acetate at 37.5 and 75 IU/kg in liver and by α-tocopheryl acetate at the 75 IU/kg level in muscle and spleen. Tissue lipid peroxidation by treatment is given in Table 18. The dietary treatment consisting of 75 IU/kg of α-tocopheryl acetate and .5% ascorbic acid demonstrated a significant decrease in tissue lipid peroxidation in muscle and spleen over dietary addition of α-tocopheryl acetate at 75 IU/kg alone. While not significant, treatments of dietary supplementation of α-tocopheryl acetate and ascorbic acid demonstrated a linear decrease in tissue lipid peroxidation of liver, muscle, and spleen over those treatments consisting of α-tocopheryl acetate supplementation without ascorbic acid. There appears to be an interactive effect by high dietary concentrations of α-tocopheryl acetate and ascorbic acid to lower the incidence of lipid peroxidation in rat tissues on this study. This is in apparent agreement with Bitner (1989), Chin *et al.* (1980), and Doba *et al.* (1985) whose work demonstrated in rats a synergistic and interactive effect between high dietary contrasts of α-tocopherol and ascorbic acid to decrease lipid peroxidation in tissues.

Hematocrit data are presented in Table 19. Hematocrit was not significantly affected by ascorbic acid supplementation. Dietary supplementation of α-tocopheryl

Table 18. Effect of experimental treatment on tissue lipid peroxidation (thiobarbituric acid-reactive substances)

	Treatment								
Ascorbic acid (%)	0	0	0	0	.5	.5	.5	.5	
α-tocopheryl acetate (IU/kg)	7.5	25	37.5	75	7.5	25	37.5	75	
No. of rats	12	12	12	12	12	12	12	12	
Tissue	malonaldehyde (nmol/g)								SEM
Liver (wet)	344.0^{a}	326.9^{ab}	298.5^{ab}	232.2^{c}	321.4^{ab}	308.2^{ab}	283.6^{b}	216.6^{c}	3.965
Liver (dry)	1502.8^{a}	$1428.a^{ab}$	$1304.a^{ab}$	1014.4^{c}	1404.1^{ab}	1346.4^{ab}	1238.9^{b}	946.3^{c}	3.965
Muscle (wet)	269.6^{a}	264.8^{a}	246.5^{a}	185.7^{b}	248.7^{a}	249.7^{a}	234.7^{a}	154.6^{c}	3.352
Muscle (dry)	1231.6^{a}	1209.7^{a}	1126.1^{a}	848.3^{b}	1136.1^{a}	1140.7^{a}	1072.2^{a}	706.3^{c}	3.352
Spleen (wet)	363.8^{ab}	369.5^{a}	355.9^{ab}	311.3^{c}	311.7^{c}	346.1^{ab}	333.9^{bc}	270.8^{d}	2.875
Spleen (wet)	1878.2^{ab}	1907.6^{a}	1837.4^{ab}	1607.1^{c}	1609.2^{c}	1786.8^{ab}	1723.8^{bc}	1398.0^{d}	2.875

^{a,b,c,d}Means in the same row with different superscripts differ significantly (P<.05).

Table 19. Effect of α-tocopheryl acetate and ascorbic acid on hematocrit (% PCV)

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 48	.5 48		7.5 24	25 24	37.5 24	75 24		
Day	% PCV		P value	% PCV				P value	SEM
7	35.5	35.6	.962	33.8	35.5	36.3	36.5	.690	.866
14	39.5	38.3	.298	39.8	39.3	39.7	36.7	.219	.580
21	37.8	38.1	.759	39.3^{a}	38.5^{ab}	38.0^{ab}	36.0^{b}	.054	.402
28	38.8	39.5	.445	39.0^{b}	40.3^{b}	40.2^{b}	36.6^{a}	.029	.398

^{a,b}Means in the same row under a common heading with different superscripts differ significantly.

acetate at 75 IU/kg significantly decreased hematocrit over supplementation at 7.5 IU/kg on day 21. On day 28, α-tocopheryl acetate supplemented at 75 IU/kg significantly decreased hematocrit over supplementation of 7.5, 25, and 75 IU/kg of diet.

Hemoglobin data are presented in Table 20. Ascorbic acid supplementation (.5%) significantly increased hemoglobin concentration at day 21. Although not significant, there was a trend toward higher hemoglobin concentration with dietary addition of ascorbic acid (.5%) at days 7, 14, and 28. Hemoglobin concentration was not significantly affected by dietary supplementation of α-tocopheryl acetate. Since it appears that higher plasma concentrations of iron may remove some iron from the spleen and bone marrow iron pool, the chief sites of erythropoiesis in very young rats, we would expect hemoglobin concentration to either be unaffected or to be slightly decreased. Since iron tissue levels were decreased, perhaps by release of iron from ferritin by ascorbic acid, more iron may actually be available for use by these erythropoietic tissues.

Data concerning red blood cell count are presented in Table 21. Red blood cell concentration was not significantly affected by dietary α-tocopheryl acetate supplementation across all treatments. Ascorbic acid

Table 20. Effect of α-tocopheryl acetate and ascorbic acid on hemoglobin (Hb)

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 48	.5 48		7.5 24	25 24	37.5 24	75 24		
Day	Hb (g/dl)		P value	Hb (g/dl)				P value	SEM
7	10.64	10.99	.302	10.45	10.85	10.99	10.98	.632	.165
14	12.07	12.26	.570	12.23	12.03	12.24	12.16	.966	.161
21	11.96[b]	13.51[a]	.0003	12.26	12.31	13.13	13.24	.102	.116
28	12.96	13.16	.573	12.65	13.36	13.60	12.54	.068	.144

[a,b]Means in the same row under a common heading with different superscripts differ signifcantly

Table 21. Effect of α-tocopheryl acetate and ascorbic acid on red blood cell count (RBC)

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 48	.5 48		7.5 24	25 24	37.5 24	75 24		
Day	RBC (10^6/mm^3)		P value	RBC (10^6/mm^3)				P value	SEM
7	4.42[b]	4.76[a]	.002	4.46	4.57	4.58	4.70	.359	.041
14	5.18	5.27	.426	5.37	5.26	5.21	5.07	.369	.041
21	5.18	5.12	.581	5.12	5.16	4.96	5.36	.083	.051
28	5.61	5.71	.386	5.63	5.67	5.79	5.51	.285	.049

[a,b]Means in the same row under a common heading with different superscripts differ signifcantly

supplementation (.5%) significantly increased red blood cell count on day 7, but not on days 14, 21, and 28.

White blood cell data are presented in Table 22. Alpha-tocopheryl acetate at 75 IU/kg significantly decreased white blood count on day 28, but not on days 7 and 21. On day 14, white blood cell count was higher for dietary α-tocopheryl acetate at 37.5 IU/kg but not for dietary additions at 7.5, 25, and 75 IU/kg. White blood cell count was significantly higher on day 28 with dietary ascorbic acid added at .5%, but not on days 7, 14, and 21. This is in apparent agreement with the work of Wastell *et al.* (1985) where increased dietary concentrations of α-tocopherol had no effect on white blood cell concentration in weanling pigs.

Data for total plasma lipid concentration are given in Table 23. Total plasma lipid concentration was significantly decreased by dietary supplementation of ascorbic acid (.5%) on day 14, but significantly increased on day 28. Total plasma lipid concentration was not significantly affected by ascorbic acid supplementation on days 7 and 21.

Total plasma lipids were significantly decreased by dietary supplementation of α-tocopheryl acetate at 75 IU/kg on days 14, 21, and 28, but not on day 7. This is in apparent agreement with Herman *et al.* (1979), Serfontein *et al.* (1983), and Szczeklik (1985) who demonstrated that α-

Table 22. Effect of α-tocopheryl acetate and ascorbic acid on white blood cell count (WBC)

No. of rats	Ascorbic acid (%) 0 48	.5 48		α-tocopheryl acetate (IU/kg) 7.5 24	25 24	37.5 24	75 24		
Day	WBC ($10^3/mm^3$)		P value	WBC ($10^3/mm^3$)				P value	SEM
7	5.87	6.76	.135	6.95	5.91	5.82	6.59	.451	.272
14	6.30	5.74	.310	5.47^{b}	4.99^{b}	7.41^{a}	6.21^{ab}	.027	.264
21	5.04	5.40	.996	5.92	4.88	4.04	5.34	.105	.256
28	4.47^{b}	5.72^{a}	.011	5.35^{ab}	6.42^{a}	4.82^{b}	3.42^{c}	.0009	.189

^{a,b,c}Means in the same row under a common heading with different superscripts differ signifcantly

Table 23. Effect of α-tocopheryl acetate and ascorbic acid on total plasma lipids

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)					
No. of rats	0 12	.5 12		7.5 6	25 6	37.5 6	75 6		
Day	total lipids (mg/dl)		P value	total lipids (mg/dl)				P value	SEM
7	645.9	655.7	.213	631.2^{a}	661.9^{b}	639.8^{a}	670.1^{b}	.017	3.635
14	762.4^{a}	725.8^{b}	.0002	776.5^{a}	741.1^{b}	748.4^{b}	720.4^{c}	.0007	2.906
21	691.3	703.1	.072	751.3^{b}	801.9^{b}	654.5^{c}	581.0^{d}	.0001	2.851
28	639.1^{b}	674.9^{a}	.0002	671.6^{b}	652.3^{c}	705.2^{a}	599.0^{d}	.0001	2.842

^{a,b,c,d}Means in the same row under a common heading with different superscripts differ significantly.

tocopherol decreased total serum lipids in man. Kalbfleisch and Barboriak (1989), Ehnholm *et al.* (1982), and Kesaniemi and Grundy (1982) concluded that α-tocopherol does not effect plasma lipids and lipoprotein in man. The apparent contradiction between these studies within the same animal species (man) points out the extreme difficulty in clearly defining the relationship of one lipid insoluble material with another. One must be very cautious when extrapolating findings in this area due to the difficulty in controlling the numerous factors and variables which may influence the lipid concentration of plasma.

CHAPTER IV

EXPERIMENT 2

Introduction

Experiment 2 was designed to follow up on the results of Experiment 1. Experiment 2 utilized very young rats weighing between 50 and 75 grams. Experiment 2 utilized older rats weighing between 175 and 200 grams. Older rats have a slower metabolic rate and decreased growth rate. Experiment 2 was set up to ascertain if vitamin E and vitamin C would have the same effect on lipid peroxidation, iron tissue distribution and hematology with older slower growing rats as with very fast growing weanling rats.

Toivenen (1987) has demonstrated beneficial roles for α-tocopherol and ascorbic acid in the prevention of blood clots by decreasing the production of thromboxane (TXA_2) which promotes clotting and increases the production of prostacyclin (PGI_2), which is anti-aggregatory. Steiner (1987) and Srivastava (1986) have shown that vitamin E appears to inhibit platelet aggregation and prostaglandins, which also stimulate platelet clumping. Increased platelet aggregation is a factor in the development of atherosclerosis and other vascular diseases.

Polymorphonuclear leukocytes (PMN's) and monocytes comprise the blood phagocytes. These cells are unique in that they can migrate to extravascular sites of infection

where they recognize the ingest microbes and other particulate material. Baehner *et al.* (1977) reported that daily supplementation of 1600 IU of vitamin E markedly increased the neutrophil phagocytic rate, but the bactericidal capacity of neutrophils was decreased. Wastell *et al.* (1985) observed an increase in total WBC count and PMN's in vitamin E supplemented pigs. Wilson (1983) reported a synergistic effect between vitamin E and vitamin C in PMN formation.

The objective of this experiment was to determine the action of α-tocopherol and ascorbic acid on tissue iron distribution, lipid peroxidation, PMN concentration and platelet aggregation and concentration. The following study was undertaken to determine the physiological effects of α-tocopherol and ascorbic acid on the above mentioned functions.

Materials and Methods

Animals and Treatments

This experiment utilized 48 male (murine pathogen free) Sprague-Dawley rats obtained from Taconic Laboratory Animals (TAC:N(SD)fBR) weighing between 175 and 200 grams. The rats were allotted to treatment by weight and placed in individual stainless steel cages measuring 36 cm x 30 cm x 26 cm. Water and experimental diets were provided *ad*

libitum. The rats were identified and managed as described in chapter III.

The rats were fed a vitamin E-free purified basal diet (Table 24) for five days prior to the beginning of the experiment. The rats were then allocated to the experimental diets. All diets were mixed within 24 hours of the beginning of the experiment and stored in a refrigerated room at 4° C for the duration of the experiment. Four experimental diets were fed in this experiment. The four diets utilized the same basal diet (Table 24) with two concentrations of α-tocopheryl acetate and two concentrations of ascorbic acid (Table 25). Experimental treatments consisted of two added levels of ascorbic acid: 0 and 0.5%, and two added levels of α-tocopheryl acetate: 30 and 300 IU/kg. The experiment was a 2 x 2 factorial arrangement with 12 animals per treatment. Three animals from each treatment were sacrificed weekly with weight, feed intake, blood, and tissues collected from each animal.

Procedures and Analysis of Samples

At the beginning of the experiment a 25 gram sample of each experimental diet was obtained for analysis. Samples were analyzed as explained in Chapter III. The values for the analyzed diets are presented in Table 26.

Table 24. Composition of basal diet - experiment 2

Ingredient	Percent
Glucose	63.8
Vitamin free casein	20.0
Corn oil-tocopherol stripped	10.0
Vitamin mixture[a]	2.2
Salt mixture[b]	4.0
	100

[a]Vitamin mixture (gm/kg): Vitamin A acetate (500,000 IU/gm), 180,000; Vitamin D_2 (850,000 IU/gm), 0.12500; Ascorbic acid, 4,500,000; Inositol, 500,000; Choline chloride, 7,500,000; Menadione, 225,000; p-Aminobenzoic acid, 500,000; Niacin, 425,000; Riboflavin, 100,000; Pyridoxine hydrochloride, 100,000; Thiamine hydrochloride, 100,000; Calcium pantothenate, 300,000; Biotin, 0.02; Folic acid, 0.09; Vitamin B_{12}, 0.00135.

[b]Salt mixture: Sodium chloride, 11.88%; Potassium phosphate dibasic, 8.5%; Potassium carbonate, 8.75%; Calcium phosphate dibasic, 39.11%; Calcium carbonate, 318.5524%; Magnesium carbonate, 5.89%; Ferric citrate (16-17% Fe), 1.7555%; Manganese sulfate·H_2O, 0.41%; Zinc carbonate, 0.1%; Copper sulfate·$5H_2O$, 0.06%; Sodium selenite, 0.000055%; Potassium iodate, 0.0021%; Chromium potassium sulfate $12\cdot H_2O$, 0.01%

Table 25. Experimental treatments - experiment 2

Treatment	Ascorbic Acid[a] (%)	α-Tocopheryl[b] Acetate (IU/kg)
1	0	30
2	0	300
3	.5	30
4	.5	300

[a]Dietary addition (ICN Biomedical)

[b]Dietary addition (Henkel Corp.)

Table 26. Analyzed composition of experimental diets - experiment 2

Analyzed Components	α-Tocopheryl Acetate (IU/kg) / Ascorbic Acid	Experimental Diets 1	2	3	4
		30 / 0	300 / 0	30 / .5	300 / .5
Dry matter (%)		93.7	89.2	93.9	92.6
Ash (%)		3.5	3.5	3.4	3.4
Crude protein (%)		17.8	17.8	17.5	17.4
Crude fiber (%)		1.7	2.3	1.8	1.5
Ether extract (%)		9.2	8.5	10.0	10.1
Energy Kcal/g		4.4	4.1	4.5	4.5
α-tocopherol (μg/g)		16.21	182.57	17.33	188.41
Ascorbic acid (%)		.003	.002	.43	.43
Calcium (mg/g)		7.93	7.46	7.67	7.61
Phosphorus (mg/g)		5.30	4.98	5.28	5.16
Iron (μg/g)		126.49	120.6	127.67	122.52
Copper (μg/g)		9.35	7.41	8.55	10.23
Zinc (μg/g)		31.18	30.81	32.08	30.72

Three rats from each treatment were sacrificed weekly with physiological samples taken on days 7, 14, 21, and 28 of the trial. The methodology used to obtain, handle, and store the samples is the same as described in Chapter III. Analysis of hematocrit, hemoglobin, red and white blood cell counts, total plasma lipids, lipid peroxidation in plasma and tissues, plasma and tissue concentrations of α-tocopherol, iron, and ascorbic acid are the same as those described in Chapter III.

Platelet counting utilized the procedure of Brecher and Cronkite (1950). Platelet aggregation was ascertained using the method of DiezMarques *et al.* (1987) utilizing a two-channel aggregometer and was quantified by measuring the maximum increase in light transmittance. Results were expressed as a percent of light transmission through platelet poor plasma.

Polymorphonuclear leukocytes (PMN's) were ascertained by utilizing the procedure of Thompson and Hunt (1966) in which a blood smear is stained so that a differentiation may be made between the various blood cells. The stained slide is placed under a microscope and 100 white blood cells are counted (leukocytes and lymphocytes). The number of PMN's is then calculated and expressed as the percentage of polymorphonuclear leukocytes in white blood cells.

Animal weights were determined by weighing animals at the beginning of the experiment and thereafter every 7

days. Animals were weighed on a Mettler 1200 digital balance.

Feed intake was determined by weighing feed containers at the inception of the experiment and then at 7 day intervals. Weigh backs were obtained from wasted diet and from the diet that was still in the feeders on weigh dates.

Statistical Analysis of Data

The data were analyzed by the General Linear Model (GLM) procedures of the Statistical Analysis System (SAS) (Barr *et al.*, 1979). The GLM procedure is a multivariate regression analysis, determining differences by significant F tests. The Student-Neuman-Keuls procedure (SMK) was used to separate means when significant F values indicated differences among means (Steel and Torrie, 1960).

The independent variables used in the multivariate regression analysis were dietary concentration of ascorbic acid and α-tocopheryl acetate, and ascorbic acid concentration x α-tocopheryl acetate concentration interaction. The dependent variables were red blood cell concentration, white blood cell concentration, total plasma lipids, hemoglobin, hematocrit, total iron binding capacity, lipid peroxidation in plasma and tissue, plasma and tissue α-tocopherol, iron, and ascorbic acid concentrations, blood platelet concentration and

aggregation, PMN concentration, average daily gain, feed intake, and feed efficiency.

Results and Discussion

Plasma α-tocopherol concentrations are presented in Table 27. Plasma α-tocopherol concentration was significantly increased by dietary α-tocopheryl acetate supplementation of 300 IU/kg as compared to 30 IU/kg at d 7, 14, 21, and 28. This is in apparent agreement with results obtained by Machlin (1991), Bjorneboe *et al.* (1988), and Traber and Kayden (1987) who demonstrated that concentration of α-tocopherol in plasma and tissues was directly correlated with tocopherol intake in rats. Ascorbic acid supplementation (.5%) to the diet significantly decreased α-tocopherol plasma levels on d 21 and 28. On d 7 and 14 plasma α-tocopherol levels were lower when ascorbic acid was supplemented (.5%) than non-supplemented, but not significantly lower. This would appear to be in disagreement with Chin *et al.* (1980) who reported that plasma α-tocopherol was spared in rats when levels of ascorbic acid was added to the diet. In the study of Chin *et al.* (1980) the rats were challenged by a large dose of iron so that lipid peroxidation was greatly enhanced in blood plasma over what might be expected in animals receiving a more normal dietary concentration of iron. Also, vitamin E supplementation was at deficiency or

Table 27. Effect of α-tocopheryl acetate and ascorbic acid on plasma α-tocopherol concentrations

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6		α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6		
Day	α-tocopherol (μg/ml)		P value	α-tocopherol (μg/ml)		P value	SEM
7	2.74	2.51	.541	1.23[b]	4.03[a]	.0001	.178
14	2.44	2.01	.062	1.02[b]	3.43[a]	.0001	.097
21	2.84[b]	1.97[a]	.001	.81[b]	4.00[a]	.0001	.086
28	2.06[b]	1.77[a]	.008	.69[b]	3.14[a]	.0001	.041

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

near deficiency levels. Since a lipid peroxidation challenge was not used one might hypothesize that a comparatively low rate of lipid peroxidation would occur. Alpha-tocopherol in the diets of the rats on this experiment may have been adequate to control lipid peroxidation without becoming depleted in the plasma.

Plasma ascorbic acid data are presented in Table 28. Plasma ascorbic acid concentration was not significantly affected by dietary supplementation of α-tocopheryl acetate. Plasma ascorbic acid concentration was significantly increased by dietary supplementation of ascorbic acid on d 7, 14, 21, and 28. This is in agreement with Lowik *et al.* (1990) who reported an increase in plasma ascorbic acid concentration in rats as dietary levels increased.

Data for plasma iron concentration by main effect can be found in Table 29. Plasma iron concentration was significantly increased by dietary supplementation of α-tocopheryl acetate of 300 IU/kg as compared to 30 IU/kg on d 7, 14, 21, and 28. Plasma iron concentration was significantly increased by dietary supplementation of ascorbic acid (.5%) on d 7, 14, 21, and 28. Plasma iron concentrations by experimental treatment are found in Table 30. The experimental treatment consisting of dietary supplementation of α-tocopheryl acetate and ascorbic acid

Table 28. Effect of α-tocopheryl acetate and ascorbic acid on plasma ascorbic acid concentration (AA)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	AA (μg/ml)			AA (μg/ml)			
7	1.75[b]	4.19[a]	.0001	3.05	2.89	.250	.059
14	1.83[b]	4.11[a]	.0001	2.99	2.94	.799	.095
21	1.72[b]	3.97[a]	.0001	2.72	2.97	.312	.117
28	1.82[b]	3.76[a]	.009	2.58	3.00	.481	.286

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

Table 29. Effect of α-tocopheryl acetate and ascorbic acid on plasma iron concentration

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	Iron (μg/ml)			Iron (μg/ml)			
7	1.85[b]	2.29[a]	.0006	1.18[b]	2.96[a]	.0001	.040
14	1.59[b]	2.11[a]	.013	1.22[b]	2.48[a]	.0001	.081
21	1.50[b]	2.38[a]	.0001	1.29[b]	2.59[a]	.0001	.052
28	1.40[b]	2.43[a]	.0001	1.36[b]	2.47[a]	.0001	.063

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

Table 30. Effect of experimental treatment on plasma iron concentration

	Treatment				
Ascorbic Acid (%)	0	0	.5	.5	
α-Tocopheryl Acetate (IU/kg)	30	300	30	300	
No. of rats	3	3	3	3	
Day	Iron (μg/ml)				SEM
7	.90[d]	2.80[b]	1.46[c]	3.12[a]	.040
14	.867[c]	2.32[a]	1.58[b]	2.65[a]	.081
21	.84[d]	2.17[b]	1.74[c]	3.02[a]	.052
28	.79[c]	2.01[b]	1.92[b]	2.94[a]	.063

[a,b,c,d]Means in the same row with different superscripts are significantly different (P<.05).

at .5% and 300 IU/kg exhibited significantly higher plasma iron concentrations than those treatments with dietary supplementation of α-tocopheryl acetate alone except for day 14, which was not significantly different. This is in apparent agreement with Monsen (1982) who concluded that ascorbic acid increased plasma iron concentration in adult males. Chen and Thacker (1986) reported that rats whose diets were supplemented with α-tocopheryl acetate increased plasma iron concentration. They hypothezied that this could have been due to the decreased sequestering of iron in various tissues in the body.

Plasma total iron binding capacity data are presented in Table 31. Total iron binding capacity was significantly decreased by dietary addition of ascorbic acid (.5%) on d 7, but not on d 14, 21, and 28. Dietary supplementation of α-tocopheryl acetate at 300 IU/kg significantly decreased total iron binding capacity on d 7, 14, 21, and 28. This would be expected as total iron binding capacity and plasma α-tocopherol concentration would be expected to have an inverse relationship. As plasma iron concentration increased, the total available capacity for iron to be bound would decrease. Conversely as plasma iron concentration decreased we would expect that the total available capacity for iron to be carried in the plasma would increase.

Table 31. Effect of α-tocopheryl acetate and ascorbic acid on plasma total iron binding capacity (TIBC)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	TIBC (μg/dl)			TIBC (μg/dl)			
7	499.1[b]	434.1[a]	.0001	490.6[b]	442.5[a]	.0001	1.532
14	441.4	436.4	.237	450.0[b]	427.8[a]	.0005	1.981
21	463.8	469.1	.177	490.9[b]	442.1[a]	.0001	1.780
28	437.1	432.1	.489	449.5[b]	419.6[a]	.003	3.459

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

Data for tissue iron concentration are presented in Table 32. Tissue iron concentration was not significantly affected by dietary supplementation of ascorbic acid. Although not significant, dietary supplementation of ascorbic acid (.5%) did decrease iron concentration in liver, muscle, and spleen. Iron concentration in liver, muscle and spleen was significantly decreased by dietary supplementation of 300 IU/kg of α-tocopheryl acetate. This is in apparent agreement with Chou *et al.* (1978) and Bjorneboe *et al.* (1990) who reported a decrease in tissue concentration of iron as dietary concentrations of α-tocopheryl acetate increased.

Data on lipid peroxidation in tissues are presented in Table 33. Lipid peroxidation was significantly increased by dietary supplementation of ascorbic acid (.5%) in liver, but not in spleen, muscle, or kidney. Lipid peroxidation in liver, spleen, muscle, and kidney was significantly decreased by dietary supplementation of α-tocopheryl acetate. It would appear that α-tocopherol decreases lipid peroxidation in tissues with the highest concentration of α-tocopherol associated with the lowest rates of lipid peroxidation.

Hematocrit means are presented in Table 34. Hematocrit was significantly decreased by dietary supplementation of ascorbic acid (.5%) for d 7, but not for d 14, 21, and 28. Hematocrit was significantly decreased

Table 32. Effect of α-tocopheryl acetate and ascorbic acid on tissue iron concentration

	Ascorbic Acid (%)			α-Tocopheryl Acetate (IU/kg)			
No. of rats	0 24	.5 24		30 24	300 24		
Tissue	Iron (μg/g)		P value	Iron (μg/g)		P value	SEM
Liver (wet)	73.03	69.43	.332	80.83^{b}	61.63^{a}	.0001	1.813
Liver (dry)	319.0	303.3	.332	353.1^{b}	269.2^{a}	.0001	1.813
Muscle (wet)	17.96	15.27	.192	20.12^{b}	13.12^{a}	.002	.998
Muscle (dry)	82.0	69.8	.192	91.9^{b}	59.9^{a}	.002	.998
Spleen (wet)	129.88	124.87	.332	135.52^{b}	119.23^{a}	.004	2.519
Spleen (dry)	670.5	644.7	.332	699.6^{b}	615.5^{a}	.004	2.519

^{a,b}Means in the same row under a common heading with different superscripts are significantly different.

Table 33. Effect of α-tocopheryl acetate and ascorbic acid on lipid peroxidation in tissues (thiobarbituric acid-reactive substances)

	Ascorbic acid (%)			α-tocopheryl acetate (IU/kg)			
No. of rats	0 24	.5 24		30 24	300 24		
Tissue	Malonaldehyde (nmol/g)		P value	Malonaldehyde (nmol/g)		P value	SEM
Liver (wet)	208.9^{b}	221.5^{a}	.006	239.6^{b}	190.8^{a}	.0001	2.163
Liver (dry)	912.6^{b}	967.7^{a}	.006	1046.7^{b}	833.6^{a}	.0001	2.163
Spleen (wet)	186.1	192.8	.389	222.5^{b}	156.4^{a}	.0001	3.888
Spleen (dry)	960.8	995.4	.389	1148.7^{b}	807.4^{a}	.0001	3.888
Muscle (wet)	161.1	150.6	.992	169.2^{b}	142.5^{a}	.0001	3.092
Muscle (dry)	735.9	687.9	.992	772.9^{b}	651.0^{a}	.0001	3.092
Kidney (wet)	221.8	208.9	.091	227.7^{b}	203.1^{a}	.002	3.713
Kidney (dry)	1186.1	1117.1	.091	1217.6^{b}	1086.1^{a}	.002	3.713

^{a,b}Means in the same row under a common heading with different superscripts are significantly different.

Table 34. Effect of α-tocopheryl acetate and ascorbic acid on hematocrit (% PCV)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6		α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6		
Day	% PCV		P value	% PCV		P value	SEM
7	38.50[b]	35.06[a]	.011	38.17[b]	36.00[a]	.037	.433
14	38.17	37.50	.622	37.50	38.16	.622	.651
21	40.50	40.83	.857	41.33	40.00	.457	.894
28	41.60	42.00	.690	42.20	41.50	.433	.414

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

by supplementation of α-tocopheryl acetate at 300 IU/kg on d 7, but not on d 14, 21, and 28.

Hemoglobin data are presented in Table 35. Hemoglobin concentration was significantly increased by dietary supplementation of ascorbic acid (.5%) on d 28, but not on d 7, 14, and 21. Hemoglobin was not significantly affected by dietary supplementation of α-tocopheryl acetate. There was a trend toward lower hemoglobin values for d 7, 14, 21, and 28 when α-tocopheryl acetate was supplemented at 300 IU/kg of diet.

Red blood cell data are presented in Table 36. Red blood cell concentration was significantly higher for d 7 with dietary supplemented ascorbic acid (.5%), but not for d 14, 21, and 28. Although not significant, red blood cell concentration was higher for all 3 d when ascorbic acid was supplemented. Red blood cell concentration was significantly increased by α-tocopheryl acetate supplemented at 300 IU/kg on d 7, but was significantly decreased on d 21 and 28.

White blood cell data are presented in Table 37. White blood cell concentration was not significantly affected by dietary supplementation of ascorbic acid or α-tocopheryl acetate.

Data for blood platelet aggregation are presented in Table 38. Blood platelet aggregation was not significantly

Table 35. Effect of α-tocopheryl acetate and ascorbic acid on hemoglobin (Hb)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	Hb (mg/dl)			Hb (mg/dl)			
7	12.93	12.55	.418	13.08	12.40	.162	.221
14	12.42	12.56	.675	12.71	12.27	.215	.163
21	13.26	12.88	.530	13.02	13.12	.868	.296
28	13.57[b]	14.18[a]	.054	14.19	13.65	.071	.116

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

Table 36. Effect of α-tocopheryl acetate and ascorbic acid on white blood cell count (WBC)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	WBC ($10^3/mm^3$)			WBC ($10^3/mm^3$)			
7	4.13	4.13	.988	4.13	4.12	.942	.055
14	4.33	4.33	.929	4.32	4.35	.837	.055
21	4.49	4.50	.953	4.56	4.44	.055	.027
28	4.80	4.85	.739	4.77	4.88	.407	.063

Table 37. Effect of α-tocopheryl acetate and ascorbic acid on red blood cell count (RBC)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	RBC (10^6/mm^3)			RBC (10^6/mm^3)			
7	5.54[b]	5.89[a]	.0001	5.64[b]	5.79[a]	.007	.021
14	5.48	5.53	.744	5.56	5.45	.352	.067
21	5.41	5.47	.352	5.57[a]	5.32[b]	.004	.031
28	5.44	5.59	.127	5.71[a]	5.33[b]	.002	.044

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

Table 38. Effect of α-tocopheryl acetate and ascorbic acid on blood platelet aggregation[c,d]

No. of rats	Ascorbic Acid (%) 0 6	.5 6	P value	α-Tocopheryl Acetate (IU/kg) 30 6	300 6	P value	SEM
Day	% transmittance			% transmittance			
7	30.59	30.10	.563	30.82	29.87	.270	.399
14	29.81	30.42	.508	30.06	30.22	.810	.443
21	30.31	29.62	.280	31.30[b]	28.62[a]	.002	.299
28	27.83	27.22	.473	30.56[b]	24.49[a]	.0001	.401

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

[c]Platelet aggregation is measured in percent transmittance of light; less light transmitted demonstrates less platelet aggregation.

[d]Aggregation was induced by adenosine diphosphate (ADP).

affected by dietary supplementation of ascorbic acid. There was a general trend for decreased platelet aggregation for d 7, 21, and 28 when ascorbic acid was supplemented (.5%). Blood platelet aggregation was significantly decreased by dietary supplementation of α-tocopheryl acetate on d 21 and 28, but not d 7 and 14. This is in apparent agreement with Steiner (1978), Srivastava (1986), Diez Marques *et al.* (1987), and McIntosh (1987) for man, rats, rats, and marmoset monkeys, respectively. That platelet aggregation was inhibited at d 21 and 28 is consistent with the work by the investigators previously mentioned who demonstrated that the longer the supplementation of α-tocopheryl acetate or α-tocopherol the more pronounced was the inhibition of platelet aggregation.

Blood platelet concentration data are presented in Table 39. Blood platelet concentration was not significantly affected by dietary supplementation of α-tocopheryl acetate or ascorbic acid. This is consistent with the investigations of Steiner (1987) and Srivastava (1986) that inhibition of platelet aggregation by α-tocopherol is due to a decrease in the concentration of thromboxane or to a disruption of the normal arachidonic acid cascade, and not to a decrease in blood platelet concentration.

The percentage of polymorphonuclear leukocytes (PMN's) in white blood cells are presented in Table 40. PMN

Table 39. Effect of α-tocopheryl acetate and ascorbic acid on blood platelet concentration (PLT)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-tocopheryl Acetate (IU/kg) 30: 6	α-tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	PLT (x $10^{-5}/mm^3$)			PLT (x $10^{-5}/mm^3$)			
7	10.92	10.47	.427	10.66	10.73	.901	.271
14	10.75	10.91	.705	10.79	10.86	.871	.208
21	11.29	10.73	.222	11.02	10.99	.941	.210
28	10.37	10.78	.416	10.47	10.70	.619	.240

Table 40. Effect of α-tocopheryl acetate and ascorbic acid on the percentage of polymorphonuclear leukocyte (PMN) in white blood cells

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6	P value	α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6	P value	SEM
Day	% PMN			% PMN			
7	62.86	66.69	.275	64.82	64.71	.974	1.634
14	65.08	66.98	.376	64.77	67.30	.249	1.016
21	70.42	72.42	.500	71.55	71.57	.919	1.386
28	68.17	72.34	.188	65.84[b]	74.68[a]	.016	1.449

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

percentage was not significantly affected by dietary supplementation of ascorbic acid. There was a trend toward higher percentages of PMN's with supplementation of ascorbic acid (.5%) on d 7, 14, 21, and 28. At d 28, rats fed α-tocopheryl acetate supplemented at 300 IU/kg had a significantly higher PMN percentage than those supplemented at 30 IU/kg. This response was similar to that observed by Bendich (1989) where high dietary concentrations of α-tocopheryl acetate increased the percentage of polymorphonuclear leukocytes (PMN) in white blood cells. This may imply that the increase in PMN phagocytosis may be due to increased numbers of PMN's. Okano *et al.* (1990) demonstrated that α-tocopherol suppressed superoxide production in individual PMN's but that overall PMN function was not impaired.

Total plasma lipid concentrations are found in Table 41. Total plasma lipid concentration was not significantly affected by dietary supplementation of α-tocopheryl acetate or by supplementation of ascorbic acid. Dietary supplementation of ascorbic acid (.5%) did tend to decrease total plasma lipids for d 7, 14, 21, and 28. This is in apparent disagreement with Herman *et al.* (1979), Serfontein *et al.* (1983), and Szczeklik (1985) who demonstrated that α-tocopherol decreased total serum lipids in man. Kalbfleisch and Barboriak (1989), Ehnholm *et al.* (1982), and Kesaniemi and Grundy (1982) concluded that α-tocopherol

Table 41. Effect of α-tocopheryl acetate and ascorbic acid on total plasma lipids (TPL)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6		α-Tocopheryl acetate (IU/kg) 30: 6	α-Tocopheryl acetate (IU/kg) 300: 6		
Day	TPL (mg/dl)		P value	TPL (mg/dl)		P value	SEM
7	755.6	750.7	.800	753.2	758.0	.995	9.495
14	750.3	750.3	.995	750.4	750.1	.997	9.399
21	747.0	739.0	.696	739.4	746.7	.721	9.84
28	755.4	743.1	.739	747.9	753.3	.883	14.34

does not effect plasma lipids and lipoprotein in man. The apparent contradiction between these studies within the same animal species (man) points out the extreme difficulty in clearly defining the relationship of one lipid insoluble material with another. One must be very cautions when extrapolating findings in this area due to the difficulty in controlling the numerous factors and variables which may influence the lipid concentration of plasma.

Data for average daily gain and feed efficiency are presented in Tables 42 and 43 respectively. Average daily gain and feed efficiency were not significantly affected by dietary supplementation of α-tocopheryl acetate or by supplementation of ascorbic acid. There was a trend toward decreased average daily gain and decreased gain per feed with dietary supplementation of both ascorbic acid (.5%) and α-tocopheryl acetate at 300 IU/kg.

Average daily feed intakes are presented in Table 44. Average daily feed intake was not significantly affected by dietary supplementation of α-tocopheryl acetate. Average daily feed intake was significantly decreased by dietary supplementation of ascorbic acid (.5%) only on d 21. Average daily feed intake tended to be lower on d 14, 28, and for total average daily feed when ascorbic acid was supplemented (.5%). This may have been due to the highly acidic taste of the ascorbic acid in the supplemented diet.

Table 42. Effect of α-tocopheryl acetate and ascorbic acid on average daily gain (ADG)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6		α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6		
Day	ADG (g)		P value	ADG (g)		P value	SEM
7	6.83	6.85	.929	6.80	6.89	.749	.129
14	6.95	6.11	.106	6.50	6.56	.893	.231
21	6.12	5.79	.666	6.19	5.71	.539	.372
28	4.45	3.50	.085	4.14	3.81	.511	.243
Total ADG	6.09	5.56	.135	5.91	5.74	.616	.158

Table 43. Effect of α-tocopheryl acetate and ascorbic acid on gain per feed (G/F)

No. of rats	Ascorbic Acid (%) 0: 6	Ascorbic Acid (%) .5: 6		α-Tocopheryl Acetate (IU/kg) 30: 6	α-Tocopheryl Acetate (IU/kg) 300: 6		
Day	G/F		P value	G/F		P value	SEM
7	.43	.42	.887	.42	.43	.851	.078
14	.36	.32	.105	.34	.34	.941	.093
21	.34	.34	.891	.35	.33	.677	.135
28	.23	.19	.113	.22	.20	.454	.275
Total G/F	.34	.32	.165	.33	.32	.565	.066

Table 44. Effect of α-tocopheryl acetate and ascorbic acid on average daily feed intake (ADF)

	Ascorbic Acid (%)			α-Tocopheryl Acetate (IU/kg)			
No. of rats	0 6	.5 6		30 6	300 6		
Day	ADF (g)		P value	ADF (g)		P value	SEM
7	15.97	16.21	.834	16.06	16.13	.951	.544
14	19.15	18.64	.403	18.77	19.03	.669	.293
21	17.82[b]	16.28[a]	.003	17.18	16.93	.514	.183
28	18.92	18.45	.691	18.59	18.78	.873	.577
Total ADF	17.97	17.40	.103	17.65	17.72	.835	.156

[a,b]Means in the same row under a common heading with different superscripts are significantly different.

CHAPTER V

EXPERIMENT 3

Introduction

The sow exhibits a very low rate of placental iron transfer to the unborn pig. As a result, pigs are born with a very low body store of iron. Sow milk contains an average of 1.25 mg of iron per liter of milk. The baby pig obtains less than 0.8 liters of milk per day (<2 mg iron) while needing approximately 7 grams of iron per day. The iron needs of newborn pigs, in modern confinement systems, are met by iron injections at 1 to 5 days of age.

It has been proposed that high dietary concentrations of vitamin E and vitamin C could possibly lead to a redistribution of iron in tissues (Chen and Thacker, 1988). Chou *et al.* (1978) reported abnormalities of iron metabolism in vitamin E deficient rabbits. The total body iron content was not altered but the sequestration of iron in muscle tissue was affected.

The production of free radicals in biological systems is a continuing process. They are produced in cellular membranes during the production of prostaglandins, leukotrienes, thromboxane, and prostacyclines. Free radicals are produced in the inner membrane of the mitochondria of cells during the utilization of electron transfer as a means of energy conversion. They are

produced by neutrophils during phagocytosis and the destruction of pathogens. When the rate of production of free radicals exceeds the physiological system's capacity to control them, the body's normal defense mechanisms cannot prevent the destruction or damage of intra-cellular components or the cell membranes. This may result in cell and tissue death, loss of function or genetic mutation (Rice and Kennedy, 1988).

To control or prevent free radical damage a variety of defense systems have been developed. A number of these systems are under nutritional control. One of the most powerful lipophilic free radical scavengers is α-tocopherol.

Iron has been demonstrated to be the central metal ion catalyst in the Haber-Weiss-Fenton reaction (Pryor, 1988). The primary product of this reaction is the highly reactive hydroxyl radical. Iron concentration in tissues has been linked to increased lipid peroxidation initiated by the hydroxyl radical (Rice & Kennedy, 1986).

The purpose of this experiment was to assess if large doses of α-tocopherol and iron, given as single injections, would affect tissue lipid peroxidation, iron and α-tocopherol tissue concentrations and distribution and hematological parameters in baby pigs.

Materials and Methods

Animals and Treatments

The experiment was conducted utilizing forty-eight three-day-old Yorkshire x Duroc x Hampshire pigs with 6 pigs coming from each of eight litters. The pigs were allotted within litter blocks by weight and with an equal number of males and females assigned to each treatment. Baby pigs were ear tagged at 3 days of age and a blood sample was taken to analyze for various hematological parameters. The baby pigs were housed in farrowing crates with their mothers for the duration of the experiment.

Sows were fed a corn soybean meal based lactation diet which met or exceeded NRC recommendations. Sow diets were analyzed periodically during the experiment to assure that vitamin E and iron concentrations remained constant. Sows were milked within 12 hrs of farrowing, twelve hours after, and on days 4, 8, 12, and 16 following farrowing. The milk was analyzed for α-tocopherol and iron.

Procedures and Analysis of Samples

Baby pigs on the experiment had blood samples taken three days from birth and were given treatment injections in the left ham. The treatments consisted of 3 iron levels of 0, 100, and 400 mg of iron as gleptoferrin (200 mg/ml) and 2 vitamin E (D-α-tocopherol) levels: 0 and 900 IU (Table 45). The experiment was a 3 x 2 factorial

Table 45. Experimental treatments - experiment 3

Treatment	Iron (mg)[a]	Vitamin E(IU)[b]
1	0	0
2	100	0
3	400	0
4	0	900
5	100	900
6	400	900

[a]Iron as gleptoferron, 200 mg/ml. Single injection.
[b]Vitamin E as D-α-tocopherol. 300 IU/ml. Single injection.

arrangement with eight animals per treatment. Pigs were weighed at the beginning of the experiment and thereafter on the same days as blood samples were taken. The animals were bled via anterior vena cava puncture at the initiation of the experiment and at 1, 4, 8, 12, and 16 days thereafter. The whole blood samples collected from the pigs were heparinized with sodium heparin to prevent coagulation and subjected to further analysis. The blood was collected and placed on ice in low light conditions in order to prevent loss of vitamin E activity due to UV electromagnetic radiation. Blood hemoglobin was determined by the method of Crosby *et al.* (1954) and hematocrits were determined by the microhematocrit method of McGovern *et al.* (1955). Plasma was obtained by centrifugation of the whole blood at an RCF (relative centrifugal force) of 3090 g's for 10 minutes and removal of the plasma to a separate tube. The plasma was then stored at -45°C until analyses were conducted. All handling and analysis of plasma and tissues were carried out under a safelight (non-UV) in order to prevent loss of α-tocopherol. In addition, all samples to be utilized for α-tocopherol analysis were flushed with nitrogen gas prior to freezing. On completion of the experiment, all pigs were sacrificed and tissues taken for further study. All dissections were carried out under a safelight.

Analysis of α-tocopherol in plasma and tissue utilized the procedures described in chapter III.

Red and white blood cell counts were ascertained utilizing the procedures described in chapter III.

Lipid peroxidation analysis was carried out utilizing a modified procedure by Yaggi (1987) for blood plasma and a modified procedure by Ohkawa *et al.* (1979) for tissue. All α-tocopherol analyses were conducted utilizing a Waters HPLC system including a Waters 490 multiwavelength detector, 712 WISP autosampler, Waters 510 and 6000A HPLC pumps, Waters 470 scanning fluorescence detector, 745B Waters data module and a μbonda-pak C_{18} 10μ column. Iron in plasma and tissues was analyzed with an Instrumentation Lab aa/ae spectrophotometer. All tissue samples were homogenized and wet ashed prior to mineral analysis.

Statistical Analysis of Data

The data were analyzed by the general linear model (GLM) procedure of the Statistical Analysis System (SAS) (Barr *et al.*, 1979). The GLM procedure is a multivariate regression analysis, determining differences by significant F tests. The Student-Neuman-Keuls procedure (SNK) was used to separate means (Steel and Torrie, 1960).

The independent variables used in the multivariate regression analysis were iron injection and α-tocopherol concentration and iron x α-tocopherol interaction. The

dependent variables were hematocrit, hemoglobin, red and white blood cell concentration, lipid peroxidation of plasma and tissue, plasma and tissue concentrations of both iron and α-tocopherol, iron and α-tocopherol concentration of sow's milk.

Results and Discussion

The concentrations of α-tocopherol in plasma are presented in Table 46. Alpha-tocopherol injected at 900 IU significantly increased plasma α-tocopherol concentration on d 1, 4, 8, 12, and 16. This is in apparent agreement with results obtained by Machlin (1991), Bjorneboe *et al.* (1988), and Traber and Kayden (1987) who demonstrated that concentration of α-tocopherol in plasma and tissues was directly correlated with tocopherol intake in rats. An iron injection of 400 mg significantly decreased plasma α-tocopherol concentration when compared to iron injection at 100 mg on d 1, 4, 8, 12, and 16. This appears to agree with Dallman (1974) who reported that iron decreased plasma vitamin E concentration in preterm infants.

Plasma iron concentration data are presented in Table 47. Plasma iron concentration was significantly increased by iron injections of 100 and 400 mg on d 1, 4, 8, 12, and 16. The plasma iron concentration increase was linear, as iron injection amount increased. Plasma iron concentration was significantly increased by α-tocopherol injection

Table 46. Effect of iron and vitamin E on plasma α-tocopherol concentration

		Iron (mg)					α-tocopherol (IU)			
		0	100	400			0	900		
No. of pigs		16	16	16			24	24		
Day	(Age Day)	α-tocopherol (μg/ml)			P value	CV	α-tocopherol (μg/ml)		P value	CV
0	(3)	5.01	4.49	3.99	.066	18.01	4.65	4.22	.068	18.01
1	(4)	207.92^{c}	266.61^{a}	251.51^{b}	.0001	5.83	4.29^{b}	479.74^{a}	.0001	5.83
4	(7)	20.89^{c}	28.74^{a}	27.03^{b}	.0001	8.01	6.03^{b}	45.08^{a}	.0001	8.01
8	(11)	16.00^{a}	17.16^{a}	12.72^{b}	.0001	7.85	2.62^{b}	19.96^{a}	.0001	7.85
12	(15)	4.43^{b}	5.12^{a}	4.94^{b}	.0001	7.52	2.46^{b}	7.20^{a}	.0001	7.52
16	(19)	4.78^{b}	5.35^{a}	4.84^{b}	.038	13.35	3.04^{b}	6.95^{a}	.0001	13.35

^{a,b,c}Means in the same row under a common heading with different superscripts differ significantly.

Table 47. Effect of iron and α-tocopherol on plasma iron concentration

	Iron (mg)					α-tocopherol (IU)			
	0	100	400			0	900		
No. of pigs	16	16	16	P		24	24	P	
Day (Age Day)	Iron (μg/ml)			value	CV	Iron (μg/ml)		value	CV
0 (3)	.65	.84	.91	.443	25.37	.86	.75	.463	25.37
1 (4)	3.35^{c}	59.73^{b}	528.81^{a}	.0001	5.08	173.53^{b}	212.63^{a}	.0001	5.08
4 (7)	.74^{c}	1.79^{b}	2.42^{a}	.0001	16.03	1.42^{b}	1.85^{a}	.0001	16.03
8 (11)	.59^{c}	1.36^{b}	2.46a	.0001	30.68	1.44	1.46	.483	30.68
12 (15)	.54^{c}	.96^{b}	2.36^{a}	.0001	26.66	1.21	1.28	.368	26.66
16 (19)	.62^{c}	1.34^{b}	2.96^{a}	.0001	21.96	1.51^{b}	1.78^{a}	.013	21.96

^{a,b,c}Means in the same row under a common heading with different superscripts differ significantly.

concentration of 900 IU on d 1, 4, and 16. While not significant, plasma iron concentration was numerically higher with α-tocopherol injections of 900 IU at d 8 and 12. Plasma iron concentration data, by treatment effect, are given in Table 48. Plasma iron concentration was significantly higher with treatment injections of iron at 400 mg and α-tocopherol of 900 IU as compared to treatment injections containing 400 mg of iron with no α-tocopherol at d 1 and 16. There was a tendency for plasma iron concentration to be increased with treatment injections of 400 mg iron and 900 IU of α-tocopherol on d 4, 8, and 12 when compared to pigs which received 400 mg of iron without α-tocopherol. Chen and Thacker (1986) reported that rats whose diets were supplemented with α-tocopheryl acetate increased plasma iron concentration. This could have been due to the decreased sequestering of iron in various tissues in the body.

Tissue α-tocopherol concentration are presented in Table 49. Liver, spleen, kidney, muscle, and heart tissue α-tocopherol concentrations were significantly decreased by iron injections of 400 mg. Alpha-tocopherol injection of 900 IU significantly increased plasma α-tocopherol concentration in liver, spleen, kidney, muscle, and heart tissue. It is possible that as physiological levels of iron in the animal system increased one would expect an increase in tissue lipid peroxidation with the subsequent

Table 48. Effect of experimental treatment on plasma iron concentration[e]

	Treatments						
Iron (mg)	0	100	400	0	100	400	
α-tocopherol (IU)	0	0	0	900	900	900	
Day (Age day)	IRON (μg/ml)						CV
0 (3)	.76	.87	.94	.64	.80	.88	25.37
1 (4)	.56[d]	55.80[c]	449.53[b]	6.15[d]	63.17[c]	619.41[a]	5.07
4 (7)	.94[c]	2.313[a]	2.43[a]	.54[d]	1.33[b]	2.47[a]	16.03
8 (11)	.72[c]	1.36[b]	2.41[a]	.46[c]	1.40[b]	2.51[a]	19.02
12 (15)	.55[b]	.98[b]	2.26[a]	.53[b]	.93[b]	2.46[a]	26.65
16 (19)	.63[d]	1.34[c]	2.55[b]	.63[d]	1.35[c]	3.36[a]	21.96

[a,b,c,d]Means in the same row with different superscripts differ significantly (P<.05).

[e]Values represent the average of 8 pigs per treatment.

Table 49. Effect of iron and α-tocopherol on tissue α-tocopherol concentration

	Iron (mg)					α-Tocopherol (IU)			
	0	100	400			0	900		
No. of pigs	16	16	16			24	24		
Tissue[d]	α-Tocopherol (μg/g)			P value	CV	α-Tocopherol (μg/g)		P value	CV
Liver (wet)	8.93[b]	8.06[b]	5.18[a]	.042	48.12	3.59[b]	11.48[a]	.0001	48.12
Liver (dry)	39.01[b]	35.21[b]	22.63[a]	.042	48.12	15.68[b]	50.15[a]	.0001	48.12
Spleen (wet)	4.67[b]	5.14[b]	2.82[a]	.003	41.36	2.01[b]	6.49[a]	.0001	41.36
Spleen (dry)	24.11[b]	26.54[b]	14.55[a]	.003	41.36	10.38[b]	33.51[a]	.0001	41.36
Kidney (wet)	9.06[b]	9.26[b]	6.66[a]	.019	36.19	2.78[b]	14.52[a]	.0001	36.19
Kidney (dry)	48.45[b]	49.52[b]	35.62[a]	.019	36.19	14.87[b]	77.65[a]	.0001	36.19
Muscle (wet)	6.25[c]	5.25[b]	4.00[a]	.0002	33.27	1.17[b]	9.66[a]	.0001	33.27
Muscle (dry)	28.55[c]	23.98[b]	18.27[a]	.0002	33.27	5.34[b]	44.13[a]	.0001	33.27
Heart (wet)	13.33[b]	11.74[b]	9.21[a]	.017	38.18	4.85[b]	16.97[a]	.0001	38.18
Heart (dry)	67.63[b]	59.56[b]	46.73[a]	.017	38.18	24.61[b]	86.10[a]	.0001	38.18

[a,b,c]Means in the same row under a common heading with different superscripts differ significantly.
[d]Tissues taken at day 16 of experiment.

decrease in tissue α-tocopherol concentration as the tocopherol molecules were oxidized in the free radical quenching process.

Tissue iron concentrations are presented in Table 50. Iron injections of 400 mg significantly increased tissue iron concentration in liver, spleen, kidney, muscle, heart, and lung. The increase in tissue iron concentration was linear as iron injection increased from 0, 100, to 400 mg. Tissue iron concentration was significantly increased with α-tocopherol injection of 900 IU for liver and lung, but not for spleen, kidney, muscle, or heart.

Lipid peroxidation values in tissue are presented in Table 51. Iron injections of 100 and 400 mg significantly increased lipid peroxidation in spleen, kidney, heart, and lung. There was a tendency for iron injection of 100 and 400 mg to increase lipid peroxidation in liver as iron injection amount increased. This is consistent with reports by Patterson *et al.* (1967) and Tollerz (1965) who asserted that iron injections in baby pigs increased the rate of lipid peroxidation in plasma tissues. Tissue lipid peroxidation was significantly decreased by α-tocopherol injection of 900 IU in spleen, kidney, muscle, heart, and lung. Although not significant, lipid peroxidation decreased in liver with α-tocopherol injection of 900 IU. This information is consistent with studies by Burton *et al.* (1983), Lucy (1978), and Nelson (1980) that high

Table 50. Effect of iron and α-tocopherol on tissue iron concentration

	Iron (mg)					α-Tocopherol (IU)			
	0	100	400			0	900		
No. of pigs	16	16	16	P		24	24	P	
Tissue[d]	Iron (μg/g)			value	CV	Iron (μg/g)		value	CV
Liver (wet)	12.89[b]	28.87[b]	251.81[a]	.0001	35.07	80.73[b]	122.82[a]	.002	35.07
Liver (dry)	56.31[b]	126.12[b]	1,100.09[a]	.0001	35.07	352.69[b]	586.57[a]	.002	35.07
Spleen (wet)	79.48[c]	120.20[b]	248.09[a]	.0001	33.93	148.58	143.09	.905	33.93
Spleen (dry)	410.32[c]	620.55[b]	1,258.70[a]	.0001	33.93	767.06	738.72	.905	33.93
Kidney (wet)	14.85[c]	27.79[b]	82.13[a]	.0001	36.07	38.05	41.60	.396	36.07
Kidney (dry)	79.41[c]	148.61[b]	439.20[a]	.0001	36.07	203.48	222.45	.396	36.07
Muscle (wet)	5.86[c]	8.81[b]	13.61[a]	.0001	18.67	9.40	9.45	.921	18.67
Muscle (dry)	26.77[c]	40.25[b]	62.17[a]	.0001	18.67	42.94	43.17	.921	18.67
Heart (wet)	23.06[c]	34.72[b]	61.08[a]	.0001	45.76	34.48	44.11	.083	45.76
Heart (dry)	116.99[c]	176.15[b]	309.89[a]	.0001	45.76	174.94	223.80	.083	45.76
Lung (wet)	29.16[c]	59.89[b]	147.21[a]	.0001	34.13	71.48[b]	92.09[a]	.027	34.13
Lung (dry)	165.49[c]	339.90[b]	835.47[a]	.001	34.13	405.68[b]	522.64[a]	.027	34.13

[a,b,c]Means in the same row under a common heading with different superscripts differ significantly.
[d]Tissues taken at day 16 of experiment.

Table 51. Effect of iron and α-tocopherol on tissue lipid peroxidation (thiobarbituric acid-reactive substances)[e]

	Iron (mg)					α-Tocopherol (IU)			
	0	100	400			0	900		
No. of pigs	16	16	16			24	24		
Tissue[d]	malonaldehyde (nmol/g)			P value	CV	malonaldehyde (nmol/g)		P value	CV
Liver (wet)	364.8	399.9	412.5	.112	16.60	401.6	383.2	.334	16.60
Liver (dry)	1,593.7	1,747.1	1,802.1	.112	16.60	1,754.5	1,674.1	.334	16.60
Spleen (wet	224.6^{c}	300.8^{b}	373.9^{a}	.0001	12.38	334.7^{b}	264.9^{a}	.0001	12.36
Spleen (dry)	$1,159.5^{c}$	$1,552.9^{b}$	193.03^{a}	.0001	12.38	$1,727.9^{b}$	$1,367.6^{a}$	.0001	12.36
Kidney (wet)	198.6^{c}	243.8^{b}	287.9^{a}	.0001	13.19	267.7^{b}	219.2^{a}	.0001	13.19
Kidney (dry)	1062.0^{c}	1303.7^{b}	1539.6^{a}	.0001	13.19	1431.6^{b}	1172.2^{a}	.0001	13.19
Muscle (wet)	215.4^{b}	236.5^{ab}	249.6^{a}	.047	16.28	244.9^{b}	222.9^{a}	.051	16.28
Muscle (dry)	984.0^{b}	1080.4^{ab}	1140.2^{a}	.047	16.28	1118.8^{b}	1018.3^{a}	.051	16.28
Heart (wet)	347.1^{c}	392.6^{b}	442.5^{a}	.0003	15.23	415.0^{b}	373.1^{a}	.019	15.23
Heart (dry)	1761.0^{c}	1991.9^{b}	2245.1^{a}	.0003	15.23	2105.5^{b}	1892.9^{a}	.019	15.23
Lung (wet)	249.6^{c}	286.2^{b}	320.2^{a}	.0001	14.22	301.9^{b}	268.8^{a}	.007	14.22
Lung (dry)	1416.6^{c}	1624.3^{b}	1817.3^{a}	.0001	14.22	1713.4^{b}	1525.5^{a}	.007	14.22

[a,b,c]Means in the same row under a common heading with different superscripts differ significantly.
[d]Tissues taken at day 16 of experiment.
[e]Expressed as nmol of malonaldehyde.

concentrations of α-tocopherol may lower lipid peroxidation in tissues and plasma in animals and man.

Plasma lipid peroxidation values are presented in Table 52. Iron injection of 400 mg significantly increased plasma lipid peroxidation on days 1 and 12. Plasma lipid peroxidation was significantly decreased by α-tocopherol injection of 900 IU on d 1, 4, 8, and 12. While not significant, plasma lipid peroxidation was lower on d 16 for those pigs that received 900 IU of α-tocopherol.

Hematocrit data are presented in Table 53. Hematocrit was not significantly affected by α-tocopherol injection of 900 IU. There was a tendency for hematocrit to be higher when α-tocopherol was injected at 900 IU. Hematocrit was significantly increased by iron injection of 100 and 400 mg on d 4, 8, 12, and 16. Iron injections of 100 and 400 mg resulted in increased hematocrit on day 1.

Hemoglobin data are presented in Table 54. Hemoglobin concentration was significantly increased by iron injections of 100 and 400 mg on d 4, 8, 12, and 16. The response was linear on d 8, 12, and 16. Hemaglobin was not significantly affected by α-tocopherol injection.

Data for red blood cell concentrations are presented in Table 55. Red blood cell concentration was not significantly affected by α-tocopherol injection. However, iron injections of 100 and 400 mg significantly increased red blood cell concentration on d 1, 4, 8, 12, and 16.

Table 52. Effect of iron and α-tocopherol on plasma lipid peroxidation (thiobarbituric acid-reactive substances)[c]

	Iron (mg)					α-tocopherol (IU)			
	0	100	400			0	900		
No. of Pigs	16	16	16			24	24		
Day (Age days)	malonaldehyde (nmol/g)			P value	CV	malonaldehyde (nmol/g)		P value	CV
0 (3)	13.8	12.7	13.3	.514	20.8	13.9	12.7	.123	20.8
1 (4)	13.2^{b}	14.2^{b}	16.1^{a}	.008	16.9	16.2^{b}	12.9^{a}	.0001	16.9
4 (7)	14.8	13.0	14.4	.172	21.8	16.8^{b}	11.1^{a}	.0001	21.8
8 (11)	14.4	12.9	14.9	.072	18.1	15.3^{b}	12.8^{a}	.002	18.1
12 (15)	15.1^{b}	13.3^{b}	19.0^{a}	.0005	24.8	19.8^{b}	14.5^{a}	.0001	24.8
16 (19)	8.9	9.0	9.8	.245	16.60	9.7	8.8	.061	16.60

[a,b] Means in the same row under a common heading with different superscripts differ signifcantly.
[c] Expressed as nmol of malonaldehyde.

Table 53. Effect of iron and α-tocopherol on hematocrit (%PCV)

	Iron (mg)					α-tocopherol (IU)			
	0	100	400			0	900		
No. of Pigs Day (Age Day)	16	16 % PCV	16	P value	CV	24	24 %PCV	P value	CV
0 (3)	27.4	27.0	28.3	.498	15.8	26.9	28.7	.191	15.8
1 (4)	23.6	25.6	27.4	.073	17.9	24.4	26.8	.097	17.9
4 (7)	23.3^{b}	29.0^{a}	29.4^{a}	.0002	16.2	27.3	27.3	.771	16.2
8 (11)	21.5^{c}	31.4^{b}	34.8^{a}	.0001	12.6	28.6	30.0	.518	12.6
12 (15)	22.4^{c}	32.1^{a}	37.1^{a}	.0001	11.5	30.0	31.2	.637	11.5
16 (19)	22.6^{c}	32.8^{b}	39.4^{a}	.0001	16.4	31.0	31.5	.692	16.4

^{a,b,c}Means in the same row under a common heading with different superscripts differ significantly.

Table 54. Effect of iron and α-tocopherol on hemoglobin

		Iron (mg)					α-tocopherol (IU)			
		0	100	400			0	900		
No. of pigs Day	(Age day)	16	16 g/dl	16	P value	CV	24	24 g/dl	P value	CV
0	(3)	7.16	7.47	7.89	.208	13.72	7.27	7.76	.124	13.72
1	(4)	6.36	6.87	7.19	.121	17.66	6.70	6.92	.606	17.66
4	(7)	6.17^{b}	7.93^{a}	8.19^{a}	.0001	14.15	7.30	7.56	.626	14.15
8	(11)	5.81^{c}	8.77^{b}	9.82^{a}	.0001	12.81	7.93	8.34	.487	12.81
12	(15)	6.16^{c}	8.97^{b}	10.53^{a}	.0001	11.23	8.42	8.70	.755	11.23
16	(19)	6.26^{c}	9.18^{b}	11.25^{a}	.0001	16.33	8.85	8.85	.811	16.33

^{a,b,c}Means in the same row with different superscripts differ significantly.

Table 55. Effect of iron and α-tocopherol on red blood cell count

		Iron (mg)					α-tocopherol (IU)			
		0	100	400			0	900		
No. of pigs Day	(Age day)	16	16	16	P value	CV	24	24	P value	CV
		RBC (10^6/mm^3)					RBC (10^6/mm^3)			
0	(3)	4.48	4.45	4.33	.570	9.91	4.36	4.48	.331	9.91
1	(4)	4.08[b]	4.59[a]	4.64[a]	.001	9.93	4.54	4.33	.108	9.93
4	(7)	3.85[b]	4.68[a]	4.86[a]	.0001	12.35	4.50	4.42	.627	12.35
8	(11)	3.83[b]	4.74[a]	5.13[a]	.0001	15.14	4.62	4.51	.587	15.14
12	(15)	4.00[b]	4.84[a]	5.40[a]	.0001	17.24	4.84	4.63	.481	17.24
16	(19)	4.02[b]	4.96[a]	5.42[a]	.0001	17.12	4.83	4.73	.789	17.12

[a,b]Means in the same row under a common heading with different superscripts differ significantly.

There was a tendency for red blood cell concentration to be decreased with α-tocopherol injection of 900 IU.

White blood cell concentration data are presented in Table 56. Iron injections of 100 and 400 mg significantly increased white blood cell concentration at d 8, 12, and 16. White blood cell concentration was not affected by α-tocopherol injections across all levels of iron injection. This is in apparent agreement with the work of Wastell *et al.* (1985) where increased dietary concentrations of α-tocopherol had no effect on white blood cell concentration in weanling pigs.

Sow colostrum and milk data are presented in Tables 57 and 58. Those sows with the highest concentration of iron had the lowest concentrations of α-tocopherol at d colost, 0, 1, 4, 8, 12, and 16. The values for iron concentration presented in Table 58 are similar to those reported by Pond *et al.* (1965). Loudenslager *et al.* (1986) cited α-tocopherol concentrations in milk that were comparable to those given in Table 57.

Average pig weights are presented in Table 59. Iron injections of 400 mg significantly increased pig weights at d 12 and 16. Pig weights were not significantly affected by α-tocopherol injection of 900 IU.

Table 56. Effect of iron and α-tocopherol on white blood cell count

	Iron (mg)					α-tocopherol (IU)			
	0	100	400			0	900		
No. of Pigs Day (Age Day)	16 WBC ($10^3/mm^3$)	16	16	P value	CV	24 WBC ($10^3/mm^3$)	24	P value	CV
0 (3)	8.14	8.18	7.86	.643	12.17	7.89	8.23	.600	12.17
1 (4)	7.49	8.10	8.07	.120	11.71	8.03	7.75	.276	11.71
4 (7)	6.42	7.43	7.40	.089	20.47	7.26	6.91	.198	20.47
8 (11)	5.81^b	7.41^a	7.64^a	.004	23.19	7.02	6.88	.489	23.19
12 (15)	5.68^b	7.36^a	7.63^a	.014	27.93	6.98	6.76	.344	27.93
16 (19)	5.59^b	7.70^a	7.97^a	.0003	23.40	6.82	7.32	.879	23.40

^{a,b}Means in the same row under a common heading with different superscripts differ signifcantly.

Table 57. α-tocopherol concentration in sow colostrum and milk

		Sow No.								
		1	2	3	4	5	6	7	8	
Day[a]	(Age days)	α-tocopherol (μg/ml)								Mean
Colost[b]	(0)[c]	5.13	4.76	3.01	2.62	3.16	4.49	3.21	3.22	3.70
0	(3)	2.37	2.00	1.87	1.66	1.89	2.10	1.79	1.63	1.91
1	(4)	2.01	1.79	1.52	1.33	1.41	1.62	1.44	1.33	1.56
4	(7)	1.88	1.70	1.31	1.17	1.21	1.44	1.22	1.20	1.39
8	(11)	1.69	1.61	1.07	.96	.98	1.26	1.11	1.00	1.21
12	(15)	1.61	1.43	.92	.77	.86	1.12	.94	.88	1.07
16	(19)	1.43	1.20	.79	.68	.83	.99	.86	.79	.95
MEAN		2.30	2.07	1.49	1.31	1.47	1.86	1.51	1.43	

[a]Day refers to days on experiment
[b]Colost equals sow colostrum.
[c]Refers to first 12 hours from birth.

Table 58. Iron concentration in sow colostrum and milk

		Sow No.								
		1	2	3	4	5	6	7	8	
Day[a]	(Age days)	Iron (μg/ml)								Mean
Colost[b]	(0)[c]	1.16	1.30	1.34	1.38	1.32	1.31	1.32	1.33	1.31
0	(3)	1.03	1.13	1.28	1.35	1.26	1.25	1.27	1.27	1.23
1	(4)	.99	1.17	1.28	1.34	1.26	1.23	1.24	1.27	1.22
4	(7)	.96	1.11	1.27	1.34	1.27	1.23	1.24	1.26	1.21
8	(11)	.97	1.14	1.27	1.33	1.24	1.23	1.24	1.28	1.21
12	(15)	.97	1.16	1.28	1.34	1.25	1.21	1.23	1.28	1.22
16	(19)	.95	1.18	1.28	1.35	1.25	1.21	1.23	1.28	1.22
MEAN		1.00	1.17	1.29	1.35	1.26	1.24	1.25	1.28	

[a]Day refers to days the litter was on experiment
[b]Colost equals sow colostrum.
[c]Refers to first 12 hours from birth.

Table 59. Effect of iron and α-tocopherol on pig weights

	Iron (mg)					α-Tocopherol			
	0	100	400			0	900		
No. of pigs	16	16	16			24	24		
Day (Age days)	Weight (kg)			P value	CV	Weight (kg)		P value	CV
8 (11)	3.68	3.90	4.05	.104	12.37	3.82	3.93	.432	12.37
12 (15)	4.36^{b}	4.54^{ab}	5.01^{a}	.023	14.17	4.59	4.67	.596	14.17
16 (19)	5.37^{b}	5.53^{b}	6.08^{a}	.032	13.50	5.64	5.67	.822	13.50

^{a,b}Means in the same row under a common heading with different superscripts differ signifcantly.

CHAPTER VI

EXPERIMENT 4

Introduction

Experiment 4 was set up in order to investigate the results of Experiment 3 with dietary additions of vitamin E and vitamin C. Since vitamin E and iron had significant effects on lipid peroxidation, iron tissue distribution, and hematology in baby pigs, their effects on the same criteria in older weanling pigs was determined. Weanling pigs are under unique oxidative stress because of the very high levels of iron supplemented in the diet. Concentrations of 80 to 100 ppm iron are usually supplemented in the diet in order to achieve maximum growth. This growth may be at the expense of cellular and tissue damage brought about by the comparatively high concentrations of iron in the diet.

Since little is known concerning the interaction of α-tocopherol and ascorbic acid on tissue iron distribution and because their interactive effects on lipid peroxidation are not well defined, the following study was undertaken to determine the effect of α-tocopherol and ascorbic acid on iron tissue distribution, tissue lipid peroxidation, and hematological parameters of weaned pigs.

Materials and Methods

Animals and Treatments

The experiment utilized 48 Yorkshire x Duroc x Hampshire pigs from 6 litters. The 48 crossbred pigs were allotted within litter blocks by weight and with an equal number of males and females assigned to each treatment. The experiment was a 2 x 4 factorial arrangement with 6 animals per treatment. The pigs were housed individually in expanded metal pens measuring 76 centimeters (cm) in length x 60 cm in width x 57 cm in height. Pigs were given water and feed *ad libitum*. The pigs were 6 to 7 weeks of age and treatment groups had averages between 9.8 and 10.0 kilograms (kg) body weight at the initiation of the experiment. A period of 5 days was used to allow animals to acclimate to the pens, basal diets, and feeding regimen.

The 48 pigs were fed corn soybean meal based diets with a single basal diet, being mixed at one time, divided into eight equal portions, with treatment concentrations of ascorbic acid and α-tocopheryl acetate added at the initiation of the experiment (Table 60). The eight experimental diets utilized the same basal diet (Table 61), with four concentrations of α-tocopheryl acetate: 0, 100, 300, and 1,000 IU/kg; and two concentrations of ascorbic acid: 0 and .2%. Conformation of diets to calculated

Table 60. Composition of basal diet - experiment 4

Ingredient	International Reference Number	Percent
No. 2 yellow corn (9% crude protein)	4-02-931	72.6
Soybean meal (48% crude protein)	5-04-604	24.0
Dicalcium phosphate Reagent grade		1.5
Calcium carbonate Reagent grade		1.0
Salt		.3
Vitamin/mineral premix[a]		.5
Antibiotic[b]		.1
		100%

[a]Provided the following per kg of premix: Vitamin A, IU, 1,210,000; vitamin D_3, IU, 220,000; vitamin E, IU, 2,200; riboflavin, mg, 880; d-pantothenic acid, mg, 2,640; vitamin B_{12}, mg, 3.3; niacin, mg, 4,400; vitamin K activity, mg, 440; choline chloride, mg, 44,000; manganese (Mn), 17,600 ppm; zinc (Zn), 33,000 ppm; iron (Fe), 26,400 ppm; copper (Cu), 2,640 ppm; selenium (Se), 66 ppm; iodine (I), 176 ppm.

[b]Tylan 10 (tylosin phosphate) 22 ppm.

Table 61. Experimental treatments - experiment 4

Treatment	Ascorbic Acid (%)[a]	Alpha-Tocopheryl Acetate (IU)[b]
1	0	0
2	0	100
3	0	300
4	0	1000
5	.2	0
6	.2	100
7	.2	300
8	.2	1000

[a]Dietary addition.
[b]Dietary addition per kg of diet.

nutrient and treatment parameters was confirmed by chemical analyses (Table 62).

On day 21 of the experiment, a single injection of 400 mg of iron was given to half of the pigs on each treatment in order to provide a lipid peroxidation challenge and to assess its effects on the various dependent variables to be analyzed.

Procedures and Analysis of Samples

Pigs were weighed at the beginning of the experiment and thereafter on the same day as blood samples were taken. The animals were bled via anterior vena cava puncture at the initiation of the experiment and at 7, 14, 21, and 28 days. The whole blood samples collected from the pigs were heparinized with sodium heparin to prevent coagulation and subjected to further analysis. The handling of blood, tissues, and all physiologicals was the same as that presented in chapter III. Procedures for analyzing hematocrit, hemoglobin concentration, red and white blood cell counts, lipid peroxidation in plasma, total plasma lipids, plasma and tissue iron, ascorbic acid, and α-tocopherol concentrations, average daily gain, and feed efficiency were described in chapters III and IV.

Table 62. Analyzed composition of experimental diets - experiment 4

	Experimental diet							
	1	2	3	4	5	6	7	8
α-Tocopheryl Acetate IU/kg	0	100	300	1000	0	100	300	1000
Ascorbic Acid (%)	0	0	0	0	.2	.2	.2	.2
Analyzed Components:								
Dry matter (%)	89.4	89.3	89.2	89.3	89.3	89.2	89.2	89.3
Ash (%)	5.1	5.5	5.4	5.9	5.7	5.1	5.6	5.3
Crude protein (%)	17.8	18.9	19.0	18.7	18.9	19.1	18.9	18.7
Crude fiber (%)	3.0	3.0	3.3	3.0	3.2	3.0	3.2	3.0
Ether extract (%)	1.6	1.6	1.9	2.0	1.9	1.9	1.8	2.4
α-tocopherol (μg/g)	32.9	94.1	224.4	695.2	36.3	97.6	230.6	707.9
Ascorbic acid (%)	.0002	.0002	.0001	.0002	.17	.17	.17	.18
Calcium (mg/g)	9.38	8.57	8.86	9.53	8.41	8.87	8.87	7.87
Phosphorus (mg/g)	5.81	5.36	5.81	6.47	5.99	6.40	5.72	5.75
Iron (μg/g)	186.5	187.2	193.7	193.8	187.2	201.5	207.2	183.9
Copper (μg/g)	8.7	9.3	8.2	9.5	8.0	7.9	8.4	7.9
Zinc (μg/g)	97.5	86.9	93.6	95.2	82.2	93.0	109.9	95.3

Statistical Analysis of Data

The data were analyzed by the general linear model (GLM) procedure of the Statistical Analysis System (SAS) (Baar *et al.*, 1979). The GLM procedure is a multivariate regression analysis, determining differences by significant F tests. The Student-Neuman-Keuls procedure (SNK) was used to separate means (Steel and Torrie, 1960).

The independent variables used in the multivariate regression analysis were: dietary α-tocopheryl acetate; ascorbic acid and iron injection and α-tocopheryl acetate x ascorbic acid; iron x α-tocopheryl acetate; iron x ascorbic acid; and iron x α-tocopheryl acetate x ascorbic acid interaction. The dependent variables were hematocrit, hemoglobin, red and white blood cell counts, lipid peroxidation of plasma, total plasma lipids, plasma and tissue concentrations of α-tocopherol, ascorbic acid and iron, feed efficiency, and average daily gain.

Results and Discussion

Alpha-tocopherol concentration in plasma are presented in Table 63. There was a significant linear increase in plasma α-tocopherol concentration as dietary α-tocopheryl acetate concentration increased on d 7, 14, 21, and 28. This is in apparent agreement with results obtained by Machlin (1991), Bjorneboe *et al.* (1988), and Traber and Kayden (1987) who demonstrated that concentration of α-

Table 63. Effect of ascorbic acid, α-tocopheryl acetate, and iron on plasma α-tocopherol concentration

	Ascorbic Acid			α-Tocopheryl Acetate (IU)/kg					Iron[e] (mg)			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24	P	12	12	12	12	P	24	24	P	
Day	α-tocopherol (μg/ml)		value	α-tocopherol (μg/ml)				value	α-tocopherol (μg/ml)		value	SEM
0	.40	.34	.087	.40	.36	.37	.33	.602	.38	.34	.269	.018
7	1.95	1.95	.908	.58[d]	1.42[c]	2.29[b]	3.46[a]	.0001	2.05	1.85	.287	.341
14	1.95	2.31	.264	.54[d]	1.70[c]	2.49[b]	3.71[a]	.0001	2.03	2.23	.649	.579
21	3.83	3.73	.987	1.55[c]	3.70[b]	4.72[a]	4.95[a]	.0001	3.91	3.65	.190	.811
28	3.16	3.13	.510	1.11[d]	2.97[c]	3.93[b]	4.71[a]	.0001	3.12	3.18	.406	.316

[a,b,c,d]Means in the same row under a common heading with different superscripts differ significantly.

[c]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.

tocopherol in plasma and tissues was directly correlated with tocopherol intake in rats. Plasma α-tocopherol concentration was not significantly affected by dietary ascorbic acid concentration. Iron injection did not significantly effect plasma α-tocopherol concentration. Plasma α-tocopherol concentrations data by treatment combination are presented in Table 64. The dietary addition of ascorbic acid (.2%) and α-tocopheryl acetate at 100, 300, or 1000 IU did not affect plasma α-tocopherol concentration over α-tocopherol alone except on d 28 where ascorbic acid at .2 and α-tocopheryl acetate at 300 IU/kg significantly decreased plasma α-tocopherol. This would appear to be in disagreement with Chin *et al.* (1980) who reported that plasma α-tocopherol was spared in rats when levels of ascorbic acid were added to the diet. In the study of Chin *et al.* (1980) the rats were challenged by a large dose of iron so that lipid peroxidation was greatly enhanced in blood plasma over what might be expected in animals receiving a more normal dietary concentration of iron. Also, vitamin E supplementation was at deficiency or near deficiency levels. Since a lipid peroxidation challenge was not used one might hypothesize that a comparatively low rate of lipid peroxidation would occur. Alpha-tocopherol in the diets of the rats on this experiment may have been adequate to control lipid peroxidation without becoming depleted in the plasma.

Table 64. Effect of experimental treatment on plasma α-tocopherol concentration

	Treatments[f]								
Ascorbic Acid (%)	0	0	0	0	.2	.2	.2	.2	
α-Tocopheryl Acetate (IU/kg)	0	100	300	1000	0	100	300	1000	SEM
Day	α-tocopherol (μg/ml)								
0	.43	.42	.42	.34	.38	.29	.32	.33	.016
7	.58[e]	1.54[cd]	2.44[b]	3.23[a]	.56[e]	1.27[d]	2.14[bc]	3.70[a]	.330
14	.42[e]	1.51[cd]	2.60[bc]	3.40[ab]	.68[d]	1.89[c]	2.39[c]	4.03[a]	.485
21	1.26[b]	3.78[a]	5.01[a]	4.83[a]	1.79[b]	3.62[a]	4.44[a]	5.07[a]	.865
28	1.05[c]	3.02[b]	4.35[a]	4.44[a]	1.16[c]	2.91[b]	3.52[b]	4.94[a]	.294

[a,b,c,d,e]Means in the same row with different superscripts differ (P<.05).
[f]Values represent the average of 6 pigs per treatment.

Tissue α-tocopherol concentrations are presented in Table 65. Dietary α-tocopheryl acetate at 100, 300, and 1000 IU/kg significantly increased tissue α-tocopherol concentration in liver, spleen, kidney, heart, muscle, and lung. The effect was linear with tissue α-tocopherol concentration significantly increasing as dietary α-tocopheryl acetate concentration increased. Tissue α-tocopherol concentration was not significantly affected by iron injection. This is in contrast to Dallman (1974) who suggested that an increase in physiological iron would deplete tissue α-tocopherol due to an increase in tissue lipid peroxidation. Dietary supplementation of ascorbic acid (.2%) significantly increased tissue α-tocopherol concentration in heart and liver, but not in spleen, kidney, muscle, and lung. There was a trend toward increased α-tocopherol concentration in spleen, muscle, and lung with the dietary addition of ascorbic acid.

Tissue iron concentrations are presented in Table 66. Tissue iron concentration was not significantly affected by dietary α-tocopheryl acetate concentration. There was a tendency for tissue iron concentration to be lower in liver, spleen, kidney, heart, and muscle from pigs fed α-tocopheryl acetate added at 1000 IU/kg. Iron injection significantly increased tissue iron concentration in liver, spleen, kidney, heart, muscle, and lung. Tissue iron concentration was significantly decreased by the addition of ascorbic acid

Table 65. Effect of ascorbic acid, α-tocopheryl acetate, and iron on tissue α-tocopherol concentration[f]

	Ascorbic Acid			α-Tocopheryl Acetate (IU)/kg					Iron[e] (mg)			
	0	.2%		0	15	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Tissue	α-tocopherol (μg/g)		P value	α-tocopherol (μg/g)				P value	α-tocopherol (μg/g)		P value	SEM
Liver (wet)	12.93[b]	14.30[a]	.046	2.30[d]	11.71[c]	19.13[b]	22.63[a]	.0001	13.53	13.74	.954	5.841
Liver (dry)	56.49[b]	62.47[a]	.046	10.05[d]	51.16[c]	83.57[b]	98.86[a]	.0001	59.11	60.03	.954	5.841
Spleen (wet)	9.68	9.91	.284	2.57[d]	9.50[c]	11.88[b]	14.77[a]	.0001	9.57	10.03	.656	4.332
Spleen (dry)	49.97	51.16	.284	13.27[d]	49.04[c]	61.33[b]	76.25[a]	.0001	49.41	51.78	.656	4.332
Kidney (wet)	7.92	7.89	.883	2.08[d]	7.37[c]	9.91[b]	12.42[a]	.0001	7.87	7.94	.683	1.663
Kidney (dry)	42.35	42.19	.883	11.12[d]	39.41[c]	52.99[b]	66.42[a]	.0001	42.09	42.46	.683	1.663
Heart (wet)	4.40[b]	5.73[a]	.021	.86[d]	3.47[c]	6.36[b]	9.62[a]	.0001	4.80	5.29	.413	3.529
Heart (dry)	22.32[b]	29.07[a]	.021	4.36[d]	17.61[c]	32.27[b]	48.81[a]	.0001	24.35	26.84	.413	3.529
Muscle (wet)	3.71	3.92	.374	1.22[d]	3.52[c]	4.66[b]	5.86[a]	.0001	3.78	3.85	.731	.657
Muscle (dry)	16.95	17.91	.374	5.57[d]	16.08[c]	21.29[b]	26.77[a]	.0001	17.27	17.59	.731	.657
Lung (wet)	6.39	6.65	.904	1.83[d]	6.12[c]	7.73[b]	9.79[a]	.0001	6.56	6.18	.267	1.341
Lung (dry)	36.27	37.74	.904	10.39[d]	34.73[c]	43.87[b]	55.56[a]	.0001	37.23	35.07	.267	1.341

[a,b,c,d]Means in the same row under a common heading with different superscripts differ significantly.
[e]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.
[f]Tissue taken at day 28 of experiment.

Table 66. Effect of ascorbic acid, α-tocopheryl acetate, and iron on tissue iron concentration[f]

	Ascorbic Acid			α-Tocopheryl Acetate (IU/kg)					Iron[e] (mg)			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24	P	12	12	12	12	P	24	24	P	
Tissue	Iron (μg/g)		value	Iron (μg/g)				value	Iron (μg/g)		value	SEM
Liver (wet)	226.8	224.9	.903	222.4	226.7	255.9	198.2	.093	113.0^{b}	338.6^{a}	.0001	23.7
Liver (dry)	990.8	982.5	.903	971.6	990.4	1,118.0	865.9	.093	593.7^{b}	1,479.3^{a}	.0001	23.7
Spleen (wet)	248.3^{b}	220.7^{a}	.039	227.3	261.3	227.2	222.1	.139	195.9^{a}	273.0^{a}	.0001	19.01
Spleen (dry)	1,281.9^{b}	1,139.4^{a}	.039	1,173.46	1,349.0	1,173.0	1,146.6	.139	1,011.4^{b}	1,409.4^{a}	.0001	19.01
Kidney (wet)	52.9^{b}	46.8^{a}	.007	51.8	48.9	52.1	47.1	.287	42.0^{b}	58.3^{a}	.0001	13.9
Kidney (dry)	282.9^{b}	250.3^{a}	.007	277.0	261.5	278.6	251.9	.287	224.6^{b}	311.8^{a}	.0001	13.9
Heart (wet)	44.4^{b}	40.9^{a}	.014	44.9	43.1	41.9	40.8	.150	39.6^{b}	45.7^{a}	.0001	10.7
Heart (dry)	225.3^{b}	207.5^{a}	.014	227.8	218.7	212.6	207.0	.150	200.9^{b}	231.9^{a}	.0001	10.7
Muscle (wet)	10.8	9.5	.058	10.7	10.1	10.0	8.7	.091	8.9^{b}	11.2^{a}	.003	22.5
Muscle (dry)	49.3	43.4	.058	48.9	46.1	45.7	39.7	.091	40.7^{b}	51.2^{a}	.003	22.5
Lung (wet)	78.2^{b}	99.4^{a}	.002	76.3	93.5	91.2	94.8	.171	60.7^{b}	119.5^{a}	.0001	25.7
Lung (dry)	443.8^{b}	564.1^{a}	.002	433.0	530.7	517.6	538.0	.171	344.5^{b}	678.2^{a}	.0001	25.7

^{a,b,c,d}Means in the same row under a common heading with different superscripts differ significantly.
^{e}Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.
^{f}Tissues taken at day 28 of experiment.

in spleen, kidney, and heart. The tissue iron concentration of lung tissue was significantly increased by the addition of ascorbic acid.

Effect of ascorbic acid and α-tocopheryl acetate by treatment means are presented in Table 67. There was a tendency for tissue iron concentration to be decreased in liver, spleen, kidney, heart, and muscle with the treatment combination of ascorbic acid at .2% and α-tocopheryl acetate at 1000 IU/kg of diet when compared to the singular addition of ascorbic acid added at .2% or α-tocopheryl acetate supplemented at 1000 IU/kg diet.

Plasma lipid peroxidation (Table 68) was significantly decreased by α-tocopheryl acetate supplemented at 1000 IU/kg of diet for d 14, 21, and 28, but not on d 7. Ascorbic acid significantly decreased plasma lipid peroxidation on d 7 and 28, but not d 14 and 21. Iron injection (400 mg) significantly increased lipid peroxidation in plasma on d 28. As the length of the trial increased there appeared to be a synergistic effect between ascorbic acid and α-tocopherol to decrease lipid peroxidation. Iron injected on d 21 appeared to increase lipid peroxidation in blood plasma. This is in apparent agreement with Chin *et al.* (1980) and Doba *et al.* (1985) whose work demonstrated in rats a synergistic and interactive effect between high dietary concentrations of α-tocopherol and ascorbic acid to decrease lipid peroxidation in tissues.

Table 67. Effect of experimental treatment on tissue iron concentration[a,b]

	Treatments								
Ascorbic Acid (%)	0	0	0	0	.2	.2	.2	.2	
α-Tocopheryl acetate (IU/kg)	0	100	300	1000	0	100	300	1000	SEM
Tissue	Iron (μg/g)								
Liver(wet)	207.0	228.9	262.4	208.8	237.7	224.5	249.5	187.7	60.00
Liver (dry)	904.3	1,000.0	1,146.4	912.2	1038.4	980.8	1,090.0	820.0	60.00
Spleen (wet)	225.2	311.7	235.2	220.9	229.4	210.9	219.3	215.2	25.43
Spleen (dry)	1,162.6	1,609.2	1,214.3	1,140.4	1,184.3	1,088.8	1,132.2	1,110.0	25.43
Kidney (wet)	53.7	46.5	60.0	51.6	49.8	51.4	42.7	42.7	21.77
Kidney (dry)	287.2	248.7	320.9	275.9	266.3	275.0	228.3	228.3	21.77
Heart (wet)	45.5	42.5	46.7	43.2	44.4	43.8	37.0	38.5	13.25
Heart (dry)	230.9	215.6	237.0	219.2	225.3	222.2	187.7	195.3	13.25
Muscle (wet)	11.8	10.1	11.8	9.6	9.6	10.0	10.4	7.8	24.36
Muscle (dry)	53.9	46.1	53.9	43.9	43.9	45.7	45.9	35.6	24.36
Lung (wet)	65.9	92.1	59.0	92.5	86.7	95.1	118.1	97.0	45.81
Lung (dry)	374.0	522.7	334.9	525.0	492.1	539.7	670.3	550.5	45.81

[a]Values represent the average of 6 pigs per treatment.
[b]Tissues taken at day 28 of experiment.

Table 68. Effect of ascorbic acid, α-tocopheryl acetate, and iron on plasma lipid peroxidation (thiobarbituric acid-reactive substances)[e]

	Ascorbic acid			α-Tocopheryl Acetate (IU/kg)					Iron (mg)[d]			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Day	malonaldehyde (nmol/ml)		P value	malonaldehyde (nmol/ml)				P value	malonaldehyde (nmol/ml)		P	SEM
0	26.7	27.2	.452	25.6	25.8	27.9	28.6	.065	27.2	25.8	.071	.405
7	16.9[b]	16.0[a]	.039	16.3	16.1	16.5	16.1	.086	16.6	16.3	.662	.196
14	18.4	18.8	.486	19.1[b]	18.9[b]	18.8[b]	15.5[a]	.025	18.1	19.2	.088	.293
21	16.7	15.2	.0006	17.4[c]	16.6[b]	15.5[b]	14.2[a]	.0001	15.9	16.1	.698	.192
28	20.5[b]	18.6[a]	.0003	22.1[c]	20.1[b]	19.3[b]	16.4[a]	.0001	19.1[b]	21.3[a]	.038	.233

[a,b,c]Means in the same row under a common heading differ significantly.
[d]Elemental iron, from gleptoferrin, was given as a single intermuscular injection on day 21 to 50% of pigs on all treatments.
[e]Expressed as nmol of malonaldehyde.

Hematocrit data are presented in Table 69. Hematocrit was not significantly affected by dietary ascorbic acid concentration. Iron injection (400 mg) significantly increased hematocrit concentration on d 28. Dietary α-tocopheryl acetate concentration did not significantly effect hematocrit concentration.

Hemoglobin data are presented in Table 70. Ascorbic acid significantly increased hemoglobin concentration on d 28. Hemoglobin was not significantly affected by dietary α-tocopheryl acetate concentration. Iron injection (400 mg) significantly increased hemoglobin concentration on d 28.

Data for red blood cell concentration are presented in Table 71. Red blood cell concentration was not significantly affected by dietary additions of α-tocopheryl acetate and ascorbic acid or by iron injection.

White blood cell data are presented in Table 72. Dietary supplementation of α-tocopheryl acetate and ascorbic acid did not significantly affect white blood cell concentration. This is in apparent agreement with the work of Wastell *et al.* (1985) where increased dietary concentrations of α-tocopherol had no effect on white blood cell concentration in weanling pigs. Iron injection (400 mg) significantly increased white blood cell concentration on d 28.

Data for total plasma lipids are presented in Table 73. Dietary supplementation of α-tocopheryl acetate and ascorbic

Table 69. Effect of ascorbic acid, α-tocopheryl acetate, and iron on hematocrit (% PCV)

	Ascorbic Acid			α-Tocopheryl Acetate (IU)/kg					Iron[c] (mg)			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Day	% PCV		P value	% PCV				P value	% PCV		P value	SEM
0	34.5	36.4	.270	36.0	35.1	36.0	34.6	.545	34.8	36.0	.117	.412
7	35.8	35.3	.548	35.9	35.9	36.1	34.4	.451	34.5	36.7	.219	.411
14	35.2	35.7	.513	36.7	36.0	34.6	34.6	.147	35.8	35.1	.384	.378
21	36.3	36.5	.812	35.8	36.8	36.4	36.5	.867	36.4	36.3	.887	.435
28	35.9	36.2	.813	35.7	36.5	36.7	35.4	.690	34.9[b]	37.2[a]	.013	.437

[a,b]Means in the same row under a common heading with different superscripts differ significantly.

[c]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.

Table 70. Effect of ascorbic acid, α-tocopheryl acetate, and iron on hemoglobin

	Ascorbic Acid			α-Tocopheryl Acetate (IU)/kg					Iron[c] (mg)			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Day	g/dl		P value	g/dl				P value	g/dl		P value	SEM
0	10.4	10.7	.195	10.8	10.3	10.6	10.4	.572	10.7	10.8	.626	.131
7	10.3	10.1	.882	10.3	10.2	10.6	10.2	.675	10.6	10.6	.373	.131
14	11.1	11.4	.494	11.6	11.4	10.7	11.4	.733	11.0	11.5	.611	.280
21	10.4	10.7	.266	10.4	10.7	10.6	10.7	.762	10.5	10.6	.559	.114
28	11.0[b]	12.0[a]	.048	11.1	12.0	11.6	11.2	.524	10.8[b]	12.2[a]	.005	.234

[a,b]Means in the same row under a common heading with different superscripts differ significantly.

[c]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.

Table 71. Effect of ascorbic acid, α-tocopheryl acetate, and iron on red blood cell count (RBC)

	Ascorbic Acid			α-Tocopheryl Acetate (IU)/kg					Iron[a] (mg)			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Day	RBC (10^6/mm^3)		P value	RBC (10^6/mm^3)				P value	RBC (10^6/mm^3)		P value	SEM
0	5.80	5.53	.071	5.52	5.75	5.75	5.64	.659	5.57	5.76	.187	.074
7	5.94	5.56	.073	5.78	5.69	5.80	5.78	.978	5.54	5.94	.067	.102
14	6.00	5.69	.036	5.95	5.95	5.73	5.74	.496	5.83	5.86	.804	.071
21	5.68	5.59	.557	5.47	5.84	5.60	5.61	.385	5.60	5.66	.649	.075
28	5.70	5.64	.809	5.46	5.98	5.68	5.56	.123	5.65	5.70	.826	.081

[a]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.

Table 72. Effect of ascorbic acid, α-tocopheryl acetate, and iron on white blood cell count (WBC)

	Ascorbic Acid			α-Tocopheryl Acetate (IU0)/kg					Iron[e] (mg)			
	0	.2%		0	100	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Day	WBC ($10^3/mm^3$)		P value	WBC ($10^3/mm^3$)				P value	WBC ($10^3/mm^3$)		P value	SEM
0	15.42	15.94	.505	15.57	16.35	15.81	14.98	.654	15.73	15.63	.901	.383
7	15.84	16.22	.676	15.76	16.99	16.36	15.02	.463	16.13	15.93	.823	.450
14	15.96	16.31	.537	16.04	16.62	16.08	15.81	.751	15.74	16.53	.157	.273
21	15.46	15.86	.443	15.16	16.26	15.31	15.91	.383	15.13	16.19	.062	.252
28	15.19	15.02	.684	14.65	15.32	14.81	15.68	.636	14.12[b]	16.13[a]	.007	.359

[a,b]Means in the same row under a common heading with different superscripts differ significantly.

[c]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.

Table 73. Effect of ascorbic acid, α-tocopheryl acetate, and iron on total plasma lipids

	Ascorbic Acid			α-Tocopheryl Acetate (IU)/kg					Iron[a] (mg)			
	0	.2%		0	15	300	1000		0	400		
No. of pigs	24	24		12	12	12	12		24	24		
Day	total lipids (μg/dl)		P value	total lipids (μg/dl)				P value	total lipids (μg/dl)		P value	SEM
0	509.7	500.1	.652	499.1	507.6	509.3	503.7	.985	523.6	485.4	.066	13.44
7	510.9	506.3	.786	508.7	507.9	509.4	508.6	.999	523.4	493.9	.091	11.54
14	510.6	514.3	.781	511.9	512.0	520.9	504.3	.826	519.9	505.9	.737	11.38
21	514.2	513.4	.848	513.4	513.8	518.3	509.6	.981	524.5	503.5	.389	13.30
28	521.9	510.8	.599	509.4	515.6	515.3	525.0	.963	518.4	514.3	.848	14.10

[a]Injections of 400 mg of elemental iron from gleptoferrin, were given to 50% of pigs on all treatments on day 21.

acid did not significantly affect total plasma lipid concentration. Total plasma lipids concentration was not significantly affected by iron injection (400 mg). This is in apparent contrast with Herman *et al.* (1979), Serfontein *et al.* (1983), and Szczeklik *et al.* (1985) who demonstrated that α-tocopherol decreased total serum lipids in man. Kalbfleisch and Barboriak (1989), Ehnholm *et al.* (1982), and Kesaniemi and Grundy (1982) concluded that α-tocopherol does not effect plasma lipids and lipoprotein in man. The apparent contradiction between these studies within the same animal species (man) points out the extreme difficulty in clearly defining the relationship of one lipid soluble material with another. One must be very cautious when extrapolating findings in this area due to the difficulty in controlling the numerous factors and variables which may influence the lipid concentration of plasma.

Data for average daily gain and feed efficiency are presented in Tables 74 and 75, respectively. Dietary additions of α-tocopheryl acetate and ascorbic acid did not significantly affect average daily gain or feed efficiency.

Table 74. Effect of ascorbic acid and α-tocopheryl acetate on average daily gain (ADG)

	Ascorbic Acid			α-Tocopheryl Acetate (IU/kg)					
	0	.2%		0	100	300	1000		
No. of pigs	24	24		12	12	12	12		
Day	ADG (kg)		P value	ADG (kg)				P value	SEM
7	.45	.50	.077	.48	.44	.52	.47	.322	.033
14	.61	.59	.544	.55	.61	.62	.63	.308	.032
21	.67	.67	.892	.65	.66	.72	.64	.319	.037
Total ADG	.58	.59	.549	.56	.57	.62	.58	.098	.019

Table 75. Effect of ascorbic acid and α-tocopheryl acetate on feed efficiency (F/G)

	Ascorbic Acid			α-Tocopheryl Acetate (IU/kg)					
	0	.2%		0	100	300	1000		
No. of pigs	24	24		12	12	12	12		
Day	F/G		P value	F/G				P value	SEM
7	2.16	1.88	.051	2.00	2.19	1.96	1.95	.600	.071
14	2.19	2.23	.709	2.34	2.19	2.11	2.20	.519	.055
21	2.16	2.65	.784	2.83	2.61	2.34	2.73	.210	.085
Total F/G	2.30	2.25	.572	2.39	2.31	2.12	2.29	.244	.406

CHAPTER VII

GENERAL DISCUSSION

Four experiments were conducted during a 3-year period to assess the possible physiological effects of α-tocopherol, ascorbic acid, and iron on lipid peroxidation, tissue iron distribution, hematological, immunological, and performance parameters utilizing young rats and young pigs. Special emphasis was placed on α-tocopherol and its possible interactive effects with ascorbic acid and iron. In the first experiment 96 male Sprague-Dawley rats weighing between 51 and 75 grams were utilized in a 4 x 2 factorial arrangement with 12 animals per treatment. The treatments consisted of four added dietary levels of α-tocopheryl acetate: 7.5, 25, 37.5, and 75 IU/kg, and two dietary concentrations of ascorbic acid: 0 and 0.5%. The second experiment utilized 48 male Sprague-Dawley rats weighing between 175 and 200 grams in a 2 x 2 factorial arrangement with 12 animals per treatment. The treatments consisted of two added dietary concentrations of α-tocopherol acetate: 30 and 300 IU/kg and two dietary levels of ascorbic acid: 0 and 0.5%. The third study involved forty-eight three-day-old Yorkshire x Duroc x Hampshire pigs in a 3 x 2 factoral arrangement with eight pigs per treatment. The treatments consisted of three injections of iron: 0, 100, and 400 mg as gleptoferrin and

two α-tocopherol injections: 0 and 900 IU. The fourth experiment utilized 48 weaned Yorkshire x Duroc x Hampshire pigs from 6 litters in a 4 x 2 factorial arrangement with four dietary additions of α-tocopheryl acetate: 0, 100, 300, and 1,000 IU/kg and two dietary additions of ascorbic acid: 0 and 0.2%.

Results obtained indicate that added dietary concentrations of α-tocopheryl acetate decreased lipid peroxidation in the tissues of rats and pigs regardless of the age or size of the animals involved. The decrease in lipid peroxidation appeared to be linear with lipid peroxidation decreasing as concentration of α-tocopherol increased. Alpha-tocopheryl acetate is not a biologically active compound but is converted in the intestinal wall to the biologically active α-tocopherol during the absorption process. Alpha-tocopheryl acetate is generally used in animal and human diets because it is very stable and resistant to oxidation by dioxygen and electromagnetic energy. Alpha-tocopherol is very unstable in diets and foodstuffs. Animals under oxidative stress, very young rapidly growing animals, or those under a particular stress such as newly weaned animals may need higher than normal concentrations of α-tocopherol. The results of experiment three seemed to indicate that iron injections increased the rate of lipid peroxidation linearly, as the iron concentration increased, the rate of lipid peroxidation

increased. Newborn pigs are born with a very low body store of available iron due to the low rate of placental iron transfer from the sow. Newborn pigs need approximately seven grams of iron per day for normal growth. Sow milk, as demonstrated in this study, contains less than 1.25 mg of iron per liter. Baby pigs nurse 20 to 24 times a day for a total milk intake of from 600 to 800 ml. The baby pig gets less than 2 mg of iron from sow's milk per day. The baby pigs iron requirements, in modern total confinement operations, are met by injections of iron from iron dextran or gleptoferrin. Krider *et al.* (1982) has stated that iron toxicosis is responsible for the death of 1.4% of the baby pigs born each year in this country. If approximately 50 million pigs are born each year in the United States, then 700,000 are lost as a direct result of iron injections. Giving multiple injections of smaller quantities of iron during the first three weeks of life, before creep feeding begins is economically prohibitive. The results of the present study indicated that vitamin E injection may help decrease the cellular and tissue damage caused by iron injection in the baby pig. An avenue for further study may be to determine if a lower and more economical concentration of vitamin E would achieve comparable reductions in tissue lipid peroxidation. A study to determine the possible effect of α-tocopherol on baby pig mortality due to iron toxicosis is needed,

However, a very large number of pigs would be necessary since mortality due to this problem is only 1.4%.

The results from experiment two provide evidence that dietary supplementation with α-tocopheryl acetate decreased blood platelet aggregation in 200 to 250 gram rats. Platelet aggregation, in this study, was decreased at day 21 and 28 of the experiment. This is consistant with the work of McIntosh (1987), Okuma *et al.* (1970), and Karpen *et al.* (1981) who demonstrated that a period of from 2 to 4 wk were required for α-tocopherol concentrations to increase in the blood platelet to levels that would inhibit ADP mediated platelet aggregation, depending on dose or diet concentration of α-tocopherol or α-tocopheryl acetate. Alpha-tocopherol appears to inhibit blood platelet aggregation in the arachodonic acid cascade after the action of the enzyme cyclooxygenase, since thromboxane concentration is decreased and prostacyclin appears to be unaffected (Steiner, 1978). Aspirin is a very powerful inhibitor of blood platelet aggregation. It inhibits the enzyme cyclooxygenase and both thromboxane and prostacyclin concentrations are dramatically decreased. The various prostaglandins are also decreased which explains why aspirin is an anti-inflammatory agent. The inhibition of platelet aggregation has very significant implications in the reduction of coronary heart disease in man. While aspirin is widely proscribed for this purpose it has a

number of negative side effects. Alpha-tocopherol appears to decrease blood platelet aggregation without the negative side effects of aspirin. It is possible that α-tocopherol may be able to replace aspirin as a platelet aggregation inhibitor. Blood platelet concentration was not affected by dietary α-tocopherol or by ascorbic acid. This would appear to indicate that the inhibitory action of α-tocopherol is cellular and not caused by a total reduction in blood platelet numbers.

In experiment two, dietary additions of α-tocopherol significantly increased the percentage of polymorphonuclear leukocytes (PMN) in white blood cells during the last week of the trial. Alpha-tocopherol has been demonstrated to reduce the intracellular phagocytic capability of individual PMN's by controlling the free radicals produced by the PMN when it phagocytizes a foreign cell (Baehner *et al*, 1982b). It has been suggested that the incorporation of α-tocopherol into the PMN's is a slow process (Chirico *et al.*, 1983). Baehner *et al.* (1982a) has demonstrated that α-tocopherol increases overall PMN phagocytosis of foreign cells. Alpha-tocopherol may increase the membrane fluidity of PMN's allowing them to more easily ingest foreign cells (Ingraham *et al.*, 1982). Alpha-tocopherol may also protect or enhance membrane proteins on the outer cell membrane of the PMN allowing it to more easily recognize foreign material (Baehner *et al.*, 1982a). The

apparent increase in PMN phagocytosis may be due to increased numbers of PMN's in the white blood cell fraction of whole blood. The results of this study indicated that this may be a viable possibility. Ascorbic acid did not appear to significantly increase PMN concentration in white blood cells. Most studies which show an effect on PMN's by ascorbic acid deal with a deficiency state or a physiological state where ascorbic acid was being depleted over a relatively long period of time. In this experiment the basal diet had ascorbic acid and the added dietary level was used to bring about plasma and tissue saturation of the vitamin. Under these conditions additional ascorbic acid may not have elicited a response from PMN's.

The results obtained from these experiments indicate that added dietary concentrations of α-tocopherol increase plasma iron concentration, decrease total iron binding capacity in plasma, and decrease tissue concentrations of iron. Chen and Thacker (1986) reported that plasma iron was decreased and tissue iron increased in α-tocopherol deficiency. It is possible that α-tocopherol is affecting the primary iron storage pool (ferritin) or iron transport protein (transferrin) in such a way as to prevent normal storage and deposition in tissues. Alpha-tocopherol and ascorbic acid did not appear to consistently affect hematocrit, hemoglobin, red blood cell and white blood cell

concentrations, plasma lipids, or growth in these experiments.

Added dietary levels of ascorbic acid did not appear to physiologically interact with α-tocopherol in the dependent variables studied except for reduction of lipid peroxidation. Most studies that demonstrate an interaction between ascrobic acid and α-tocopherol deal with α-tocopherol in a deficiency state. Under those circumstances ascorbic acid appears to spare α-tocopherol. The results provided in these studies indicated that when α-tocopherol concentration is very high there may not be a need for the protective effects of tocopherol. It was noted that α-tocopherol concentrations in diets with added ascorbic acid tended to be higher than in diets that did not have added ascorbic acid. In experiment three, those sows whose milk had the highest concentration of iron appeared to have the lowest concentration of α-tocopherol. It is possible that iron mediated lipid peroxidation could be taking place in the milk and that tocopherol is being utilized to scavenge the free radicals that are produced. An alternate hypothesis may be that there is a difference in α-tocopherol absorption between sows on the same diet.

Chapter VIII

SUMMARY

Four experiments were conducted to assess the physiological effects of alpha-tocopherol, ascorbic acid, and iron on lipid peroxidation, tissue iron distribution, blood platelet aggregation and concentration, polymorphonuclear leukocyte concentration, hematological and performance parameters utilizing rats and pigs. The primary emphasis was placed on alpha-tocopherol and its interactive effects with ascorbic acid and iron.

In the first experiment 96 male Sprague-Dawley rats weighing between 51 and 75 grams were utilized in a 4 x 2 factorial arrangement with six animals per treatment. The treatments consisted of four added dietary levels of alpha-tocopheryl acetate: 7.5, 25, 37.5, and 75 IU/kg, and two dietary concentrations of ascorbic acid: 0 and 0.5%.

The second experiment utilized 48 Sprague-Dawley rats weighing between 175 and 200 grams in a 2 x 2 factorial arrangement with eight animals per treatment. The treatments consisted of two added dietary levels of alpha-tocopheryl acetate: 30 and 300 IU/kg, and two dietary levels of ascorbic acid: 0 and 0.5%. The third study utilized 48 three-day-old Yorkshire x Duroc x Hampshire pigs in a 3 x 2 factorial arrangement with eight pigs per treatment. The treatments consisted of 3 levels of iron

injection: 0, 100, or 400 mg, and two levels of alpha-tocopherol injection: 0 or 900 IU. The fourth experiment utilized 48 weaned Yorkshire x Duroc x Hampshire pigs from 6 liters in a 4 x 2 factorial arrangement with four dietary additions of alpha-tocopheryl acetate: 0, 100, 300, and 1000 IU/kg, and two dietary levels of ascorbic acid: 0 and .2%.

The results obtained indicated that added dietary concentrations of alpha-tocopheryl acetate significantly decreased lipid peroxidation in the tissues of rats and pigs regardless of the age or size of the animal involved. Injection of alpha-tocopherol also significantly decreased lipid peroxidation in the tissues of baby pigs. The decrease in lipid peroxidation appeared to be linear with lipid peroxidation decreasing inversely as biological concentrations of alpha-tocopherol increased.

The results from experiment two provided evidence that dietary supplementation with alpha-tocopheryl acetate at 300 IU/kg significantly decreased blood platelet aggregation on day 21 and 28 of the experiment in 200 to 250 gram rats. Blood platelet concentration was not affected by dietary alpha-tocopheryl acetate or ascorbic acid.

Experiment two appeared to indicate that added dietary additions of alpha-tocopheryl acetate significantly increased the percentage of polymorphonuclear leukocytes

(PMN) in white blood cells during the last week of the trial. Ascorbic acid did not appear to significantly increase PMN concentration in white blood cells. The results obtained from these experiments indicated that added dietary concentrations of alpha-tocopheryl acetate significantly increased plasma iron concentration, decreased total iron binding capacity in plasma and decreased tissue concentrations of iron.

Injected iron at 400 mg significantly increased lipid peroxidation in the plasma and tissues of baby and weaned pigs.

Added dietary levels of ascorbic acid did not appear to physiologically interact with pharmacological concentrations of alpha-tocopherol based on the variables measured except for the reduction of lipid peroxidation in plasma and the tissues analyzed.

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APPENDIX

A1. Vitamin E Extraction Method for Plasma

1. Place 0.5 ml plasma into 4 ml polypropylene tube.
2. Add 0.5 ml isopropyl alcohol (HPLC grade).
3. Vortex 15 sec. - allow to sit for 1 min.
4. Add 2.5 ml hexane (HPLC grade) - shake for 30 min.
5. Vortex 30 sec.
6. Allow to sit for 1 min.
7. Centrifuge 20 min @ 1000 g's.
8. Remove organic layer into 4 ml polypropylene tube.
9. Evaporate under N_2 - use hood.
10. Add 0.5 ml MEOH - shake for 5 min.
11. Allow to sit for 30 min.
12. Vortex for 10 sec.
13. Filter (.2 micron) into 1.5 ml capacity snap-cap polypropylene centrifuge vials.
14. Inject 10-50 μl into HPLC system.

Equipment

1. Polypropylene tubes with caps (4 ml)
2. Reagent grade hexane (HPLC grade)
3. Reagent grade isopropyl alcohol (HPLC grade)
4. Nitrogen gas (filtered)
5. Methyl alcohol (HPLC grade)
6. HPLC system
7. Mobile phase: 100% methyl alcohol
8. Column: Microbonda Pak C_{18} (Waters)
9. Fluorescence detection: 295 nm excitation - 330 emission

A2. Lipid Peroxidation

Thiobarbituric acid - tissue

Standard Procedure

1. Add 0.2 ml of tissue homogenate (1:3).
2. Add 0.2 ml of 8.1% SDS (sodium dodecyl sulfate).
3. Add 1.5 ml of 20% acetic acid.
4. Adjust solution to pH 3.5 with NaOH.
5. Add 1.5 ml of 0.8% aqueous solution of TBA (2-thiobarbituric acid).
6. Add distilled H_2O up to 4.0 ml.
7. Heat the mixture in a water bath at 95°C for 60 min. (Place cap loosely on a polypropylene tube.)
8. Cool with tap water.
9. Add 1.0 ml of distilled H_2O
10. Add 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v).
11. Shake vigorously for 5 min.
12. Centrifuge at 1000 g's for 10 min.
13. Remove the organic (top) layer.
14. Read on a spectrophotometer at between 530-537 nm. (Lambda max for the particular spectrophotometer.)

A set of standards using (TMP) 1,3,3,3,-tetramethoxypropane (Malonaldehyde tetramethyl acetal or tetraethoxypropane (TEP) are used as external standards and handled the same as the samples. A blank must also be run and the instrument zeroed on the blank before reading standards or samples. The standards are used to set up a standard curve against which the sample's values will be calculated.

Method of Calculation

$$\text{Tissue Lp} = 1.0 \times \frac{f}{F} \times \frac{1.9}{0.05} = \frac{f}{F} \times 20 = \text{nmol/ml} \times 3 = \text{nmol/g of tissue}$$

f = sample absorption
F = standard absorption

A3. Lipid Peroxidation

Thiobarbituric acid - plasma

1. Pipette 50 μl of plasma into a polypropylene tube (12 ml).
2. Add 4.0 ml of 12/N H_2SO_4.
3. Shake gently for 5 min.
4. Add 0.5 ml of 10% phosphotungstic acid and vortex for 5 sec.
5. Allow the mixture stand at room temperature for 10 min.
6. Centrifuge the mixture for 15 min. at 1000 g.
7. Discard the supernatant (pour off).
8. Add 4.0 ml of distilled water to the sediment.
9. To this mixture add 1.0 ml of TBA (2-thiobarbituric acid) reagent (a mixture of equal volumes of 0.67% TBA aqueous solution and glacial acetic acid).
10. Heat the mixture at 95°C for 60 min. in a water bath. Loosely cap the tube.
11. Cool the tube with cool tap water.
12. Add 5.0 ml of n-butanol and shake vigorously for 15 min.
13. Allow to stand at room temperature for 10 min.
14. Centrifuge the mixture at 1000 g for 30 min.
15. Remove the n-butanol layer and measure absorbance at 530-537 nm on a spectrophotometer.

Method of Calculation

$$\text{Tissue Lp} = 1.0 \times \frac{f}{F} \times \frac{1.9}{0.05} = \frac{f}{F} \times 20 = \text{nmol/ml}$$

f = sample absorption
F = standard absorption

VITA

John Rocco Moore was born July 31, 1950 in Jellico, Tennessee. He attended elementary and secondary schools in LaFollette and Jellico, Tennessee, and graduated from Jellico High School in June of 1970. In the fall of 1979 he enrolled in The University of Tennessee and graduated with a Bachelor of Science degree in Animal Science in December of 1983. His graduate studies were initiated in January of 1984 at The University of Tennessee, where he worked as a graduate research assistant. He received the Master of Science degree in Animal Science in December of 1987 and the Doctor of Philosophy degree in December, 1992.