VITAMIN E IN RUMINANT: THE IMPACT OF FORM AND DOSING SITE ON METABOLISM BY STEERS

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BY STEERS

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Format of Dissertation

This dissertation is prepared in the Journal of Animal Science format, as followed by the Oklahoma State University Graduate College style manual. This format allows to be suitable for submission to scientific journals for independent chapters. These papers are prepared to partly fulfill the requirement for the Ph. D. degree. Each chapter is composed of abstracts, introduction, materials and methods, results and discussion, implication, and literature cited.

CHAPTER I

GENERAL INTRODUCTION

Certain nutrients are essential for preventing deficiencies as well as for optimizing animal health and animal product quality. Antioxidants are one class of nutrients that includes vitamin E, vitamin C and beta carotene. These antioxidants not only helps to prevent cardiovascular diseases and cancer in human but also can enhance immune system, activity and improve quality of animal products when fed at levels above the NRC requirement for preventing deficiencies. As an antioxidant, protecting cell membranes and lipoproteins from peroxidative damage. Current study suggests that adequate amount of vitamin E intake prevents or minimizes the free radical production which is responsible for damages associated with specific diseases and life style, including cancer, aging, circulatory condition, arthritis, cataract, and strenuous exercise.

Vitamin E includes a group of fat soluble substances, i.e., tocopherols and tocotrienols. The most biologically active form is α-tocopherol. Many studies have examined the bioavailability of various form of vitamin E including as α-tocopheryl acetate, α-tocopheryl succinate, and α-tocopherol polyethylene glycol. Different stereo-isomer of α-tocopherol also have been compared. Most of these study have used non-ruminants. In ruminant, vitamin E response measurements have included feeding trials

and blood and tissue α-tocopherol concentrations. Microorganisms in the rumen degrade or destroy vitamin E is debated.

The objective of my research was to characterize the bioavailability of several different forms of vitamin E and to measure destruction of vitamin E in the digestive tract of steers. For my study, α-tocopherol and α-tocopheryl acetate were compared in bioavailability when administer into either the rumen or duodenum of cattle. Results may prove useful to enhance our understanding of metabolism of vitamin E by ruminants.

CHAPTER II

LITERATURE REVIEW

Introduction

Beef production has evolved from grazing native grass toward feeding confined diet containing various amounts of concentrates and forages. That changes in feeds, in feeding conditions, and rates of production theoretically have altered the dietary requirement of the vitamins and other nutrients. For vitamin E specially, requirements for beef cattle can be altered by several factors such as stresses caused by diseases, or weaning. Vitamin E requirements presumably also depend on levels of dietary antioxidants, sulfur-containing amino acids, polyunsaturated fatty acids, and selenium. High intakes of polyunsaturated fatty acids in unsaturated oil such as corn oil, linseed oil, and soybean oil markedly increase the vitamin E requirement.

The vitamin E requirement for beef cattle is not clearly defined numerically. Between 15 and 60 IU/kg dry diet for young calves is suggested (NRC, 1996). Results of one depletion and refeeding study suggested that the requirement for optimum growth by growing finishing steers was 50 to 100 IU vitamin E daily or 10 IU/kg dry feed (Hutcheson and Cole, 1985). The bioavailability, absorption mechanisms, and effects of ruminal microorganism on vitamin E status are not well understood. This review will summarize information about vitamin E function, bioavailability of different forms, and its metabolism in the rumen and small intestine.

Chemistry of vitamin E

Vitamin E is a generic term that includes group of tocopherol and tocotrienol derivatives with vitamin E activity. In the early 1920s, Herbert Evans and his colleague, Katherine Bishop, studied influence of nutrition on reproductivity in the rat. They discovered that rats fed rancid fat failed to recover when fed lettuce or whole wheat. It was found that wheat germ oil contained the vitamin. In 1925, Herbert Evans adopted the E following the vitamin D. Tocopherol was coined from the Greek *tocos*, meaning childbirth or offspring, and the *pherin*, meaning to bring birth, and *ol*, indicates an alcohol. Until 1920s, vitamin E was believed to be required only for reproduction.

Eight different active compounds have been found from plant that has vitamin E activity (Table 1). That includes four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ). All tocols have a chromanol ring structure and side chain. Tocopherols have phytol side chain, and tocotrienols have similar structure with double bonds at 3, 7, and 11 positions at the side chain. α-Tocopherol has the highest activity. Biological activity of vitamin E is expressed in International Units (IU). An IU is equivalent to 1 mg of dl-α-tocopheryl acetate. Figure 1 shows the form of α-tocopherol and α-tocopheryl acetate.

d-α-Tocopherol and dl-α-tocopherol is two sources of vitamin E in commercial. These forms are unstable and available in the form of esterified as acetate esters. dl- Form is synthetic and d-form is found from natural sources. dl-α-Tocopheryl acetate is widely used as vitamin E on market.

Effects of vitamin E on ruminant

The absolute requirement of vitamin E in ruminant has not been established. Newborn animals have low blood α-tocopherol concentration, presumably caused by low placenta transfer of vitamin E (Paulson et al., 1968). Vitamin E appears to be needed for rapid growth of animals; and limited amount of dietary vitamin E may cause the damage of newly formed lipid membrane due to the oxidation damage by free radical (McCay and King, 1980).

Effects of vitamin E on performance of steers

Numerous studies have examined effects of vitamin E on cattle performance. Vitamin E has been reported to enhance the daily weight gain and feed efficiency of young cattle (Lee et al., 1985; Gill et al., 1986; Hays et al., 1987). However, other studies have detected no beneficial effects of vitamin E supplementation on growth and performance (Garber et al., 1996; Pehrson et al., 1991).

Effects of feeding diets containing various amounts of vitamin E on ruminants have been reported elsewhere. When vitamin E was fed to heifers finished on low and high energy rations with10 or 20 IU vitamin E added /day, added vitamin E for cattle fed the higher corn silage diet had a greater increased daily gain and improved feed efficiency (Stob et al., 1974).

Gill et al. (1986) tested effects of vitamin E supplementation at 800 IU/lb feed on health and performance of newly arrived stocker cattle. In their study, vitamin E supplementation improved weight gain, and gain to feed ratios, but feed intake was not

changed. Number of sick pen days and morbidity was reduced. In concurrent study, Hays et al.(1987) reported similar results, but they also compared method of vitamin E supplementation (injected 3,000 IU/head of DL-α-tocopherol/animal at the time of arrival, and supplemented 400 IU DL-α-tocopheryl acetate/lb supplement for 28 day). Daily gain, feed intake was increased, and sick day and morbidity were decreased with vitamin E added to the feed but there was no change in gain to feed ratios. No responses to injections were detected.

Garber et al. (1996) detected no increase in performance from adding 0, 250, 500, 1,000, or 2,000 IU dl-α-tocopheryl acetate/day/head to seventy-five yearling crossbred beef steers averaging 361 kg. However, supplementing vitamin E improved immune response and increased serum and muscle α-tocopherol levels, this delayed metmyoglobin formation and lipid oxidation and prolonged shelf life of beef. Supplemental vitamin E proved more effective with meat from beef steers than meat from dairy cows. Asghar et al. (1991) reported improved growth rates in swine fed DL-α-tocopheryl acetate at 100 IU/kg diet compared to pigs fed 10 IU/kg diet.

Effect of vitamin E on immune system

Vitamin E, as a biological antioxidant, is a free radical scavenger in the immune system (Tappel, 1972; Hoekstra, 1975; McCay and King, 1980). In normal marketing procedures, cattle are subjected to various stresses including deprivation of feed and water, crowding, weaning, castration, dehorning, transportation, and changes in environment (Hutcheson and Cole, 1986). Stress increases release of norephinephrine and epinephrine from the adrenal cortex into the circulation (Stephens, 1980). Cattle,

whether stressed or receiving epinephrine injections have reduced α-tocopherol concentration in plasma, neutrophils, and red blood cells (Sconberg et al., 1993).

Vitamin E, a free radical scavenger, plays a critical role in maintaining optimal immune function (Tengerdy, 1990). Several studies have evaluated the effect of vitamin E on disease resistance and immune function. Humoral and cellular immune response were enhanced by dietary supplementation with vitamin E and selenium both in small animals (Nockels, 1979; Sheffy and Schults, 1979) and in steers (Droke and Loerch, 1989). In contrast, Nemec et al.(1990) detected no added response to *Brucella abortus* in IgG1, IgG2, and IgM antibody level from supplementation with 1,400 IU dl-α-tocopherol and 3 g of elemental selenium in two 30 g iron boluses in 7 mo. old 213 kg in twenty-four crossbred heifers. However, total IgM natural antibody was greater in these heifers receiving the vitamin E supplement.

Reddy et al.(1987) used 32 Holstein heifer calves from birth to 24 weeks of age fed 0, 125, 250, or 500 IU DL-α-tocopherol/day till 8 weeks of age then fed DL-α-tocopheryl acetate in dry feed to investigate the function of vitamin E on immune response. They reported that bovine herpes virus antibody titers were greater in calves supplemented with 125 IU of vitamin E/day than control calves. When lambs were received 60 mg vitamin E/kg diet, it had higher antibody titers 14 day after secondary inoculation with parainfluenza3 virus than did control lambs (Reffett et al., 1988). An increased antibody production may reflect a higher degree of lymphocyte activation (Stabel et al., 1992). Tengerdy et al. (1973) observed that supplementation of the diet with 60 to 180 mg dl-α-tocopheryl acetate /kg increased antibody production by 30 to 40 % in mice.

Effects of vitamin E on immune function may relate to as free radical scavenging (Corwin and Gordon, 1982). As an antioxidant, vitamin E possibly maintains cell membranes in a reduced state (Campbell et al., 1974) that facilitate more active response to antigenic or mitogenic stimulation by improving the binding ability of membrane receptors (Stabel et al., 1992).

Pherson et al. (1991) investigated the effects of vitamin E on weight gain, immune response, and disease incidence. They administered 200 mg for first 2 mo., 400 mg next 2 mo., and 600 mg during the rest 2 mo. of dl-α-tocopheryl acetate/head to twenty-six Swedish Red and White and twelve Swedish Friesian male calves averaging body weight of 54 kg. They detected no difference between control and vitamin E supplemented cattle. However, the initial serum α-tocopherol level in all cattle before treatment was remarkably low (high of only 0.67 mg/l) during vitamin E supplementation (ARC recommended level). This may be the reason why no response to vitamin E supplementation was detected. They attributed low biological availability of supplemental vitamin E to degradation of vitamin E by microorganisms in the rumen of animals fed high grain diet as suggested by Alderson et al. (1971) or to poor digestibility of vitamin E by ruminants (Hidiroglou and Karpinski, 1987). Based on these studies, vitamin E may enhance performance and immune system activity, but response to vitamin E probably varies with condition, age, breed, and initial vitamin E status of the animal.

Nutritional Deficiency of vitamin E

The most common symptom of vitamin E deficiency in calves is muscular dystrophy (MD). Animals with less than 1.0 to 1.5 mg vitamin E /L serum may exhibit the muscular dystrophy (Arthur, 1982; McMurray and Rice, 1982). External symptoms of MD are limited. A creatine kinase concentration in serum can be used as a diagnostic tool. Creatine kinase activity is increased due to muscle damage (Crookshank et al., 1979; Mitchell et al., 1988; Sconberg et al., 1993). An increased α-tocopherol concentration in tissue, by preventing lipid oxidation of muscle cells, avoids this problem.

Reddy et al. (1985) provided twenty-eight Holstein heifer calves at birth with either 0, 1,400, or 2,800 mg dl-α-tocopheryl acetate dosed orally at weekly interval or with 1,400 mg dl-α-tocopherol weekly given by intramuscular injection. They detected no significant response in gain, but the creatine kinase was higher for unsupplemented calves and negatively related to serum α-tocopherol concentrations. Similarly, 1,000 IU of α-tocopheryl acetate daily to 16 crossbred heifers average body weight of 380 kg fed a corn/corn silage diet for 28 days reduced creatine kinase ($P < .13$) in heifers (Nockels et al., 1996). Following stress, plasma α-tocopherol levels of control heifers increased (1.67 to 2.3 μg/ml vs 3.28 to 2.84 μg/ml in plasma, respectively). In contrast, stress of the vitamin E supplemented cattle decreased plasma α-tocopherol level into marginally adequate range (2.0-3.0 μg/ml). Similar observations were reported by Sconberg et al. (1993). They observed that plasma α-tocopherol level decreased with stress despite feeding 1,000 IU dl-α-tocopheryl acetate to 15 beef cattle daily. Liver α-tocopherol concentration also decreased with stress. This indicates that the plasma concentration of

vitamin E alone may not directly reflect vitamin E status of the body (Nockels et al., 1996).

Vitamin E as an antioxidant

As an antioxidant, vitamin E prevents polyunsaturated fatty acids in cell membranes from oxidation by free radicals. The most prevalent free radicals in aerobic environments are oxygen radicals (including superoxide anion O_2•), conjugated acid HOO•, peroxyl radicals derived from polyunsaturated fatty acids, and hydroxyl radical HO•. From these free radicals, the peroxyl radical is involved most closely in lipid peroxidation. These reactive molecules with unpaired electron are formed during aerobic metabolism of cells as follows:

In the initiation stage, carbon-centered lipid radicals, R•, are produced from polyunsaturated fatty acids:

PUFA:H + R• → PUFA• + RH

In propagation stage, removal of hydrogen from PUFA by free radical R• initiates a chain reaction:

PUFA• + O2 → PUFAOO•

PUFAOO• + PUFA:H → PUFAOOH + PUFA•

This chain reaction does not stop until ROO• (PUFAOO•) meets another ROO• and is inactivated:

PUFAOO• + PUFA• → PUFA-OO-PUFA

This undesirable autocatalytic process can be prevented in two ways. First, antioxidants such as glutathione peroxidase convert free radical products into harmless

products. When free radicals are formed, chain-breaking antioxidants can stop the propagation step of lipid peroxidation. α-Tocopherol has a chain-breaking function. From its phenol ring, α-tocopherol donates a hydrogen from its hydroxyl group to either PUFAOO• or alkoxyl radicals generated during lipid peroxidation:

α-tocopherol-OH + PUFAOO• → α-tocopherol-O• + PUFAOOH

α-Tocopherol competes much better than polyunsaturated fatty acids for free radicals. This reaction effectively terminates a chain reaction in lipid peroxidation. After donating its phenolic hydrogen atom to a free radical, α-tocopherol is oxidized to its quinone form, tocopherylquinone. The oxidized quinone is reduced to hydroquinone in rat liver. Most of the tocopherol is excreted through feces.

The presence of hydroxyl and methyl groups in chromanol ring and the side chain of the hetero ring play an important role in its biological activity. When the hydroxyl group was masked with ether or other substance, complete inactivity resulted. Esterification of hydroxyl group did not alter or slightly altered its activity. However, replacement of hydroxyl with amine or N-methylamine did not change the activity. The presence of methyl group in benzene ring is also important for biological activity. Replacement of methyl group with other substrates (hydrogen) dramatically reduces activity. Shortening or elimination of isoprenoid side chain resulted in decrease in activity. Only less than 1 % of tocopherol is excreted into urine and most of tocopherol is excreted in feces.

Blood α-tocopherol level as an index of vitamin E status

Vitamin E is distributed throughout body tissues, and most is stored in cell membranes, i.e., microsomes and mitochondria (Machlin, 1984). Based on several

studies in different species, biological activity of vitamin E compounds appears to be related to the α-tocopherol concentrations in blood (Horwitt et al., 1984). For evaluation of vitamin E status in animals, α-tocopherol concentrations in various samples has been measured. These include red blood cells (Hassan and Hakkarainen, 1990), platelets (Lehman et al., 1988; Njeru et al., 1994;Njeru et al., 1995), and various tissues (Molenaar et al., 1973); all respond to vitamin E intake.

Recently, Charmley et al. (1992) suggested that the circulating level of tocopherol is influenced by blood lipid concentration. This would make the serum tocopherol level unreliable as an index of vitamin E status. Instead of serum α-tocopherol alone, the ratio of serum α-tocopherol:blood lipids ratio was suggested as being more reliable as an indicator of vitamin E nutritional status (Horwitt et al., 1972; Lehmann, 1981; Lehmann et al., 1988). Horowitt et al.(1972) further suggested that any α-tocopherol index must be sensitive to dietary intake of the vitamin as well as consistent with blood lipids to be useful as an valid indicator of vitamin E nutritional status. However, no difference between serum α-tocopherol response as μg/ml of serum or mg/g blood lipids has been detected (Chung et al., 1992; Njeru et al., 1994).

Njeru et al. (1995) fed four different dietary level of dl-α-tocopheryl acetate (0, 500, 1,500, and 3,000 IU/animal/day) and measured α-tocopherol concentrations in serum, red blood cell, and muscle biceps femoris and liver tissue of 32 yearling beef heifers (average body weight of 256 kg) during 84 day trial. Supplemental vitamin E was fed for 28 day, it was withdrawn for 28 day, and then feeding was resumed for another 28 day. Serum α-tocopherol level increased during feeding, declined during withdrawal, and increased rapidly and linearly when supplementation was resumed. They observed no response to

vitamin E treatment in blood cholesterol or triglycerides; responses in serum α-tocopherol concentration or the serum tocopherol:cholesterol plus triglycerides ratio to vitamin E intake was linear. They suggested that serum α-tocopherol concentrations reflect vitamin E status. Anderson et al. (1995a) also reported that serum α-tocopherol level was increased linearly with 0, 15, or 150 IU DL-α-tocopheryl acetate /kg diet fed to 84 crossbred pigs (26 kg initial body weight) for 83 day or 90 day. Consequently vitamin E status still is estimated by serum α-tocopherol concentration in blood. Only if plasma lipids change dramatically would the ratio to blood lipids become necessary.

Adams (1982) first reported the level of α-tocopherol in plasma in cattle. In his report, a deficient level has suggested to be less than 2.0 μg/ml, marginal level is between 2.0 to 3.0 μg/ml, and the minimal but adequate level is between 3.0 and 4.0 μg/ml. McMurray et al. (1983) reported that plasma α-tocopherol level ranged from 3.0 to 5.0 μg/ml in normal young cattle fed grass silage or hay. Caravaggi (1969) reported that plasma α-tocopherol level was greater in grazing cattle than in cattle fed grain (15.9 vs 2.1 μg/ml). This difference may reflect dietary intake. Class, breed, age, disease, pregnancy, and physiological status also may affect the response to supplemental vitamin E (Charmley et al., 1992; Horwitt et al., 1972). These factors must be considered when using α-tocopherol as an index of vitamin E status.

Effects of dietary vitamin E on the serum and tissue cholesterol was evaluated by Agboola et al. (1988). When 30 nonruminating Holstein bull calves weighing average 46 kg received 18 g/d monosodium phosphate and 100 IU/day of DL-α-tocopheryl acetate in a milk replacer diet for 2 week, serum cholesterol level was 6.4% lower (142.3 vs 152.0 μg/100 ml sera in control) for those receiving these two compounds than for control

calves. They suggested that cholesterol 7 α-hydroxylase, enzyme for cholesterol degradation, is increased by dietary vitamin E. However, cholesterol 7 α-hydroxylase enzyme activity was not measured in this study.

Dietary supplementation to 180 crossbred weanling 5.5 kg pig with 220 IU α-tocopheryl acetate/kg diet until fed finisher diets failed to change blood or tissue cholesterol levels (Lepine et al., 1990). They suggested that serum and tissue cholesterol concentrations of the pigs are less responsive in pigs than in veal calves to dietary intervention.

In horses, the production of potentially harmful free radicals during exercise has been proposed to cause exercise-induced muscle disease (Sjodin et al., 1990) and to increase the vitamin E requirement (Quantanilha, 1984; Bendich, 1991). Effects of vitamin E intake on indicators of skeletal muscle integrity were studied on exercised horses by Siciliano et al. (1997). Vitamin E was supplemented in the diet (0 or 300 IU dl-α-tocopherol acetate /kg diet) to average 532 and 539 kg horses. After exercise, α-tocopherol level in serum was maintained and had increased in muscle of supplemented horses while in horses receiving no supplemental vitamin E, α-tocopherol had decreased in serum but maintained in muscle.

Quantifying value of antioxidants for preventing oxidation of lipids has become of interest recently. Relative effects of an antioxidant are dependent on the condition of lipid substrates, the test system, oxidation time, and method of lipid oxidation evaluation (Huang et al., 1996). Several variables such as emulsifier, pH, and buffer system can influences in antioxidant's behavior in emulsion systems or bulk oil systems. Comparison of antioxidant activity of α-tocopherol and Trolox (a water-soluble analogue

of α-tocopherol) with different lipid forms suggest that antioxidant activity is affected by the physical state of different lipid systems and by the concentration and location of the antioxidants.

Bioavailability of tocopherol and tocopheryl acetate

To evaluate the vitamin E status in an animal, one must understand dynamics of this vitamin in the body. Various methods have been undertaken to appraise vitamin E status of humans and of animals. These methods include the measurement of 1) α-tocopherol level in a) plasma, b) red blood cells, c) platelets, and d) adipose tissue and 2) the vitamin E/lipid ratio.

Biological availability of various form of vitamin E has been evaluated using rat fetal fetus assay. The bioavailability of vitamin E was as follows: 1mg of dl-α-tocopheryl acetate = 1.00 IU; 1 mg of d-α-tocopheryl acetate = 1.36 IU; 1 mg of d-α-tocopheryl succinate = 1.21 IU, and 1 mg of dl-α-tocopheryl succinate = 0.89 IU. This indicates that the l form has 47% of the activity of the d form and that the difference between succinate and acetate forms can be attributed simply to dilution of the vitamin E.

The predominant form of vitamin E that is supplemented to animal feeds is DL-α-tocopheryl acetate. This is synthesized chemically. The natural D-α-tocopherol and synthetic DL-α-tocopherol differ in bioavailability.

D-α-tocopherol has higher bioavailability than DL-α-tocopherol (Hidiroglou and McDowell, 1987). For humans, D-α-tocopheryl acetate was 2.16 times more potent for elevating plasma α-tocopherol levels than DL-α-tocopheryl acetate after single oral dose (Horwitt, 1980; Horwitt et al., 1984). Using the deuterium-labeled vitamin E acetate

forms, the plasma α-tocopherol also was greater from D-α-tocopherol than DL-α-tocopherol (Acuff et al., 1991).

Burton et al. (1988) compared the bioavailability of free α-tocopherol and α-tocopheryl acetate in rats and humans. In rat study, the net uptake of α-tocopherol free form was half of that from the acetate. In human, however, the amount of free α-tocopherol in blood from the free α-tocopherol form in diet was equal to that from acetate. They explained that destruction of free α-tocopherol in prepared rat diet would be the reason of low bioavailability. They also pointed out the difference of bioavailability of free α-tocopherol in rat and human.

Chung et al. (1992) compared the efficiency of D-α-tocopherol with DL-α-tocopheryl acetate with weanling pigs; D-α-tocopherol was more effectively absorbed and retained than DL-α-tocopheryl acetate. However, for finishing pigs, Anderson et al. (1995) concluded that D-α-tocopheryl acetate showed greater bioavailability and deposition in tissues than D-α-tocopherol, DL-α-tocopherol, and DL-α-tocopheryl acetate. They suggested that the lower bioavailability of D-α-tocopherol than D-α-tocopheryl acetate was due to oxidation in the feed over the time prior to consumption.

Hidiroglou et al. (1988 a, b) studied bioavailability of D- and DL- forms of tocopheryl acetate for ruminants. Serum α-tocopherol levels were greater from D-α-tocopheryl acetate than from DL-α-tocopheryl acetate.

Using lambs, Hidiroglou et al. (1992) compared DL-α-tocopheryl acetate, D-α-tocopheryl acetate, D-α-tocopheryl succinate, D-α-tocopheryl polyethylene glycol 1,000 succinate (TPGS), DL-α-tocopheryl nicotinate, and combinations of DL-α-tocopheryl

nicotinate with TPGS or D-α-tocopheryl acetate with TPGS. Doses equivalent to 300 mg of DL, D-α-tocopheryl acetate was added to the daily diet and effects of serum α-tocopherol concentration were measured. D-α-tocopheryl acetate resulted in highest serum α-tocopherol than the other forms of vitamin E except for a combination of D-α-tocopheryl acetate and TPGS, suggesting a synergism between these forms. In conclusion, studies with humans, swine, and ruminants, D-α-tocopherol appears to have greater than other DL-α-tocopherol.

D-α-tocopheryl polyethylene glycol, a water soluble form of vitamin E, has been used as a therapeutic agent for preventing vitamin E deficiency in humans with fat malabsorption that reduces absorption and induces the vitamin E deficiency (Traber et al., 1986, 1994; Sokol et al., 1987). Long-term oral administration of TPGS (4,000 IU/day) maintained normal plasma α-tocopherol concentrations, increased adipose tissue α-tocopherol concentrations, and prevented further progression of the neurological abnormalities associated with vitamin E deficiency. The degree of vitamin E absorption correlates with total fat absorption in malabsorption disorders. The water soluble vitamin E (TPGS) study showed that TPGS was absorbed and that α-tocopherol released was transported normally by lipoproteins, suggesting that metabolic turnover of α-tocopherol was normal (Traber et al., 1994).

When biopotency of D- and DL-α-tocopheryl acetate esters were compared, D-α-tocopheryl acetate resulted in a higher blood response then DL form (Hidiroglou et al., 1992, 1988a,b, Horowit et al., 1980). Bioavailability of several forms of vitamin E compounds were compared in lambs (Hidiroglou et al., 1992); D-α-tocopheryl acetate

outranked DL-α-tocopheryl acetate, D-α-tocopheryl succinate, DL-α-tocopheryl nicotinate and D-α-tocopheryl polyethylene glycol.

Corn is used as good energy source in ruminant as well as in swine diet. The α-tocopherol content in artificially dried corn has been reported as 1.28 to 1.85 μg/g (Pond et al., 1971), 8.99μg/g (Young et al., 1975), and 11.4 to 19.9 μg/g (Bunnell et al., 1968). α-Tocopherol in the corn can be changed by processing. Artificial drying did not decrease the α-tocopherol level of corn but α-tocopherol content of high moisture ensiled corn declined from 9.3 to 0.98 μg/g after 230 days of storage (Young et al., 1975). Level of free fatty acids in high moisture corn increased from 5.3 to 36 μmole during storage whereas there was no increase of free fatty acids in artificially or naturally dried corn. Moisture content was suggested earlier to the main contributor to the loss of α-tocopherol (Chow and Draper, 1969; Madsen et al., 1971). Young et al. (1975) proposed that this loss of α-tocopherol is due to acid independent of moisture content. Young et al. (1975) detected no change of α-tocopheryl acetate concentration in dried or ensiled corn when α-tocopheryl acetate was added. Because α-tocopheryl acetate needs to be hydrolyzed to act as an antioxidant, α-tocopheryl acetate was stable during fermentation even though free fatty acids consumed α-tocopherol as antioxidant.

Degradation of vitamin E in the rumen

Metabolism of vitamin E in the rumen has been proposed. Alderson et al. (1971) reported that a large amount of vitamin E was lost before it reached the intestines of adult

ruminants. They found that vitamin E disappearance increased from 8.4 to 22.2, 25.0, and 42.4% as the corn content in the diet increased from 20 to 40, 60, and 80 %, respectively.

Shin and Owens (1990) measured ruminal and postruminal disappearance of different sources of vitamin E. Ruminal disappearance, from greater to least, was for free DL-α-tocopherol at 52.1 %; followed by DL-α-tocopheryl acetate in the liquid (50.3 %), absorbate (39.9 %), and the spray form (35.6 %). Postruminal disappearance from highest to lowest was for absorbate 44.3 %, free 37.5 %, spray 34.5 %, and liquid 15.6 %. From this study, the free form had the highest availability in small intestine but also was readily available for ruminal destruction; the absorbate form was resistant to ruminal destruction but yet available in small intestine. From these two studies, extensive microbial degradation of vitamin E in the rumen was implicated.

In vitro incubation of vitamin E with ruminal contents should be useful to test degradation of vitamin E in the rumen. Astrup et al.(1974) incubated DL-α-tocopherol in the ruminal fluid for 25 hour from sheep fed alfalfa and oats. Following incubation, recovery of the vitamin E was complete; this suggests that vitamin E is not destroyed in the rumen.

Leedle et al. (1993) incubated DL-α-tocopheryl acetate with undiluted rumen fluid from steer fed high-concentrate diet. They used a high concentrate diet because Alderson et al. (1971) had reported that disappearance was greater with high concentrate diets. They also detected no destruction of DL-α-tocopheryl acetate after 24 hours of incubation. Leedle et al. (1993) proposed that apparent loss of vitamin E in the rumen was a result of the incomplete extraction. However, they studied only DL-α-tocopheryl acetate, not the free form of vitamin E.

Roquet et al. (1992) compared different routes of administration of DL-α-tocopheryl acetate. They administered DL-α-tocopheryl acetate either into the rumen or the duodenum. The plasma α-tocopherol level was increased by ruminal administration; duodenal administration resulted minimal changes in blood concentrations. Roquet et al.(1992) proposed that the low plasma response in α-tocopherol due to insufficient time in the intestine for emulsification. Hidiroglou et al. (1990) also observed the similar results with 24 sheep (weighing 45 to 50 kg) administered D-α-tocopherol (60 mg), D-α-tocopheryl acetate (70 mg), and DL-α-tocopheryl acetate (80 mg). They detected that α-tocopherol increases in plasma were greater after ruminal supplementation than after duodenal supplementation.

Based on these studies, *in vivo* results suggest that vitamin E (free or ester form) is partially destroyed in the rumen. In contrast, *in vitro* studies indicate that vitamin E is not destroyed. However, the only form of vitamin E evaluated *in vitro* was ester; no report on destruction of free form of vitamin E is available.

Absorption of vitamin E

Factors influencing the intestinal absorption of vitamin E is still poorly understood. The efficiency of vitamin E absorption varies with animal species; only 20-40% of orally ingested tocopherol or its esters were absorbed (Gallo-Torres, 1980).

Information about the site of absorption of tocopherol is limited. In a study using rats, the greatest uptake region was near the junction of the upper and middle thirds of the small intestine (Gallo-Torres, 1980). Hidiroglou and Jenkins (1974) compared rates of tissue retention and radioactive urine excretion by administration of radiolabeled DL-α-

tocopherol in the rumen, abomasum, and duodenum using 62 sheep. They found that the jejunum is the main site of absorption of tocopherol. In nonruminant, the primary site of absorption is believed to be the medial small intestine (Thompson and Dietschy, 1981). Following a jejunoileal bypass operation, vitamin E level in plasma of rat decreased from 700 μl/dl (ad libitum fed) to 50 μl/dl (Baker et al., 1992). This suggests that most of the vitamin E absorption occurs in the jejunum and ileum. However, site of absorption of vitamin E may depend on several factors such as administered form and dose, and amount and composition of gut contents (Gallo-Torres, 1980).

Dietary lipids may alter efficiency of vitamin E absorption. Medium chain triglycerides enhanced the absorption of vitamin E whereas polyunsaturated fatty acids and long chain triglycerides decreased its absorption (Gallo-Torres, 1978). Gallo-Torres et al. (1971) proposed earlier that increasing the percentage of long chain triglyceride should increase absorption of tocopherol. However, when radiolabeled vitamin E was emulsified in either medium chain triglycerides or long chain triglycerides, absorption by the rat, as measured by levels of radioactivity in the small intestinal wall, liver, plasma, skeletal muscle, and adipose tissue were higher with medium chain triglyceride than with the long chain triglyceride (Gallo-Torres et al., 1978). This study suggests that intestinal absorption of vitamin E was enhanced by solubilization in medium chain triglyceride. Medium chain triglycerides have 6-12 carbon in their side chain and differ from long chain triglycerides in their absorption. Being more soluble in water than long-chain fatty acids, fatty acids from medium chain triglycerides can be absorbed through portal venous system whereas long chain fatty acids are absorbed in chylomicrons and carried by the lymphatic stream. Generally, aqueous-miscible emulsion is absorbed twice as well than

an oil solution when both are dosed orally. Emulsions are absorbed even in the absence of pancreatic lipase and bile salts. Therefore, vitamin E should be absorbed more efficiently with medium chain triglycerides even without pancreatic enzyme or bile salts. However, Gallo-Torres et al. (1978) compared the medium chain fatty acids and long chain fatty acids on effects of vitamin E absorption in overnight fasted rats fed radiolabeled D-α-tocopherol. They observed that ratio of portal/systemic radioactivity was less than 1. This indicated that vitamin E absorption still occurred through lymph even in the presence of medium chain triglycerides.

While chroman group of α-tocopherol is responsible for the antioxidant activity, the phytyl group largely controls the kinetics of transport, and retention in cell membranes (Burton and Traber, 1990). Using different deuterated forms of stereoisomers that differ only at the position where the chroman ring and the phytil tail are joined, competitive uptake study provided the importance of ring-tail junction for their bioavailability. This study supports that the tail (phytyl group) is responsible for the absorption.

Additional factors that alter fat digestion and fatty acids absorption may be important in vitamin E absorption. These factors include rate of gastric emptying, motility of the small intestine, triglyceride hydrolysis, and lymph flow. Since it is well known that free medium chain fatty acids are 100 times more soluble in water than ordinary long chain fatty acids (Ralston and Hoerr. 1942), products of hydrolysis of medium chain triglycerides are disperse easily; this speeds uptake in small intestine (Gallo-Torres et al., 1978). Similarly, solubilities of various forms of vitamin E may alter dispersibility and potential for absorption.

Hydrolysis of vitamin E esters

Presumably α-tocopheryl acetate must be hydrolyzed prior to absorption from the small intestine. However, when an equimolar mixture of deuterated tocopherol (free and acetate form) was administered to five adult humans with an evening meal, the average α-tocopherol/α-tocopheryl acetate ratio in plasma after two days was close to 1.0 (Burton et al., 1988). This result implies that both the acetate ester and free forms of vitamin E were equally absorbed and transported into plasma (Burton and Traber, 1990).

In contrast, Blomstrand and Forsgren (1968) reported that radiolabeled DL-α-tocopheryl acetate given orally to cannulated thoratic duct in man was hydrolyzed before being transported to lymph as free α-tocopherol. Gallo-Torres (1970) reported similar results with radiolabeled α-tocopheryl nicotinate in rat. Most of free unesterified α-tocopherol was detected in lymph with almost no labeled α-tocopheryl nicotinate being detected. Both bile and pancreatic juice were found to be requisite for intestinal absorption of vitamin E.

Nakamura et al. (1975) examined *in vitro* hydrolysis and *in vivo* absorption of different tocopheryl esters including acetate, propionate, butyrate, isobutyrate, caprylate, palmitate, acid succinate, benzoate, nicotinate, o-hydroxybenzoate, o-acetoxybenzoate, and pivalate by rat. More easily hydrolyzed esters were more absorbed and recovered free form in lymph. However, esters that resisted hydrolysis were absorbed and recovered in lymph unchanged. This suggested that hydrolysis of α-tocopheryl eaters is not necessarily prerequisite for intestinal absorption. To investigate the function of pancreatic esterase and bile salts in hydrolysis, Mathias et al. (1981) measured absorption of free α-tocopherol and α-tocopheryl acetate from mixed micellar solutions using isolated jejunal

closed loops in rat *in vivo*. Pancreatic esterase and bile salts were removed by washing with saline solution. They detected absorption of both free and ester forms of α-tocopherol in absence of added pancreatic esterase and bile salts. However, they also detected esterase activity in the closed jejunal loops, presumably being released from jejunal enterocyte even after through washing. These results support the contention that hydrolysis of vitamin E esters by pancreatic esterase is important, though not essential for absorption. They suggested that micellar solubilization of α-tocopheryl acetate is sufficient for the uptake by jejunal enterocyte without luminal hydrolysis. Burton et al. (1988) also detected small amounts of both free and ester forms of α-tocopherol in plasma after oral administration to humans. For absorption of vitamin E under normal conditions, pancreatic esterase and bile salts can account for hydrolysis of the ester form of vitamin E. However, small amounts of micellar solubilized α-tocopherol esters should be hydrolyzed by mucosal esterase although some may escape hydrolysis and be absorbed intact (Mathias et al., 1981).

Vitamin E and other nutrients

Studies of the relationship of vitamin E to vitamin A have given inconsistent results. High dietary levels of vitamin A may interfere with vitamin E absorption and decrease level of α-tocopherol in blood. Skalan and Donoghue (1982) and Abawi and Sullivan (1989) observed reduced absorption of dietary α-tocopherol level with high dietary levels of vitamin A in chicks. With rats, Blakely et al. (1991) reported that plasma vitamin E was reduced by 77 % when vitamin A was fed at 100 times of its requirement. These negative effects of high vitamin A have been attributed to competition for absorption

sites in the small intestine or to increased oxidation of vitamin E before it reaches the small intestine (Sklan and Donoghue, 1982).

With pigs, no effect of high dietary vitamin A supplementation on α-tocopherol status has been detected (Hoppe et al., 1992; Anderson et al., 1995b). Anderson et al. (1995b) evaluated effects of high dietary vitamin A (2,000 or 20,000 IU of retinyl acetate / kg diet) on vitamin E status and performance with various levels of vitamin E (0, 15, or 150 IU of DL-α-tocopheryl acetate / kg diet) supplementation for 90 days in 84 crossbred growing-finishing pigs. They absorbed that serum α-tocopherol increased with increased dietary vitamin E supplementation with no effect of high vitamin A intake on serum and tissue α-tocopherol concentrations.

Effect of tocopherol in stability of color and oxidation in meat

Meat products supply high quality protein, and essential minerals and vitamins to the human diet. Meat quality is appraised by tenderness, color, and flavor. Most nutritionists favor unsaturated to saturated dietary fats. However, such foods containing unsaturated fatty acids are susceptible to lipid oxidation and develop off-flavor. An increased level of antioxidants in meat tissues can reduce these problems and delay discoloration of meat. Discoloration causes meat products to be discount and discard which in turn decreases economic return. Therefore, prolonging the cherry-red color is desired by fresh meat merchants (Schaefer et al., 1991).

Supplementation of diets of feedlot steers with 500 to 1,000 IU vitamin E daily prolongs the stability of color and lipids in displayed fresh beef (Arnold et al., 1992; Faustman et al., 1989 a,b) and pork (Asghar et al., 1991; Buckley et al., 1989).

Accumulation of a sufficient amount of α-tocopherol in muscle seems critical for this response (Arnold et al., 1993b). Long-term (50 to 100 days) supplementation of vitamin E to feedlot cattle has improved the stability of fresh, frozen, and cooked beef (Liu et al., 1995; Sherbeck et al., 1995), probably due to the deposition of α-tocopherol in subcellular membranes (Gray and Pearson, 1987; Schaefer et al., 1995). In contrast, effects of vitamin E supplementation on cattle performance have been more evident early in the confinement period than over the total finishing period (Hill and Williams, 1993). Supplementation with 486 mg of α-tocopheryl acetate/steer/day for 126 day increased overall display life of meat (Liu et al., 1996). However, on even higher level of supplementation (over 2,000 IU/day) was required to improve color stability of meat stored for 56 days under vacuum.

Three pigments in beef are responsible for its color (Livingston and Brown, 1981). Deoxymyoglobin is a purple pigment. After several minutes of exposure to oxygen, deoxymyoglobin becomes to oxygenated oxymyoglobin, this has bright cherry-red color. Then after prolonged exposure to oxygen, oxymyoglobin changes to metmyoglobin that makes beef brown. Both deoxy- and oxymyoglobin have ferrous (Fe^{+2}) whereas metmyoglobin has the ferric (Fe^{+3}). Form of iron and with oxidation, ferrous ion is changed to ferric ion. Several oxidizing agents such as superoxide, hydrogen peroxide, and hydroxyl radicals can react with ferrous ion and cause the lipid oxidation. Lipid oxidation results in short chain length fatty acids that cause rancidity in uncooked meat and the warmed-over flavor of cooked meat (Kanner, 1994).

Chan et al. (1995) reported that vitamin E supplementation (500 and 2,000 IU/head daily for 126 days) did not change total bacterial numbers on beef during 12 days of

storage at 4°C. Asghar et al. (1991) also reported that bacterial load on pork chops was higher for those pigs that received vitamin E supplement when chops were stored at 4°C for 10 days.

Overall, α-tocopheryl acetate supplements prolong the stability of beef color and delay lipid oxidation. However, the increased α-tocopherol concentration in meat does not reduce the total bacterial load on the meat.

Table 1. Commercially available forms of vitamin E

Form	IU/mg
dl-α-tocopheryl acetate (all-rac)	1.00
dl-α-tocopherol (RRR)	1.10
d-α-tocopheryl acetate	1.36
d-α-tocopherol	1.49
dl-α-tocopheryl acid succinate	0.89
d-α-tocopheryl acid succinate	1.21

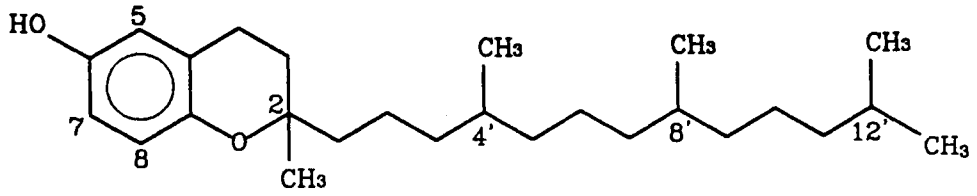
Cited from Machlin (1984)

Table 2. Relative biopotency of vitamin E forms.

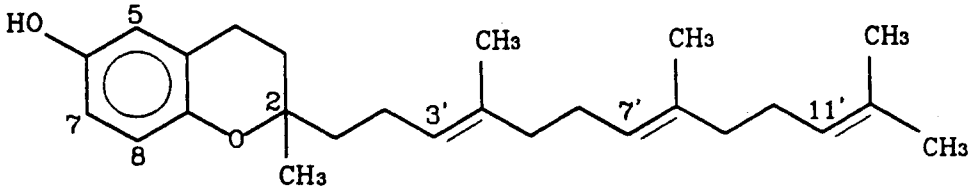
	structure	rat antisterility	rat weight gain	rabbit, cure of muscular dystrophy	Hemolysis of erythrocytes in vivo	in vitro
Tocopherols						
d-α	5,7,8 trimethyl	135	-	100	130	100
dl-α	5,7,8 trimethyl	100	100	90	100	100
d-β	5,8 dimethyl	54	-	30	30	40
dl-β	5,8 dimethyl	27	25	-	25	54
d-γ	7,8 dimethyl	1	-	20	4-22	30
dl-γ	7,8 dimethyl	1-11	19	6	18	67
d-δ	8 methyl	1	-	-	3	20
Tocotrienols						
d-β	5,8 dimethyl	5	-	-	1-5	133
d-α	5,7,8 trimethyl	29	-	-	23	106
d-γ	8 methyl	3	-	-	-	88

Cited from McDowell (1989)

Figure 1. Structure of tocopherol and tocotrienol.

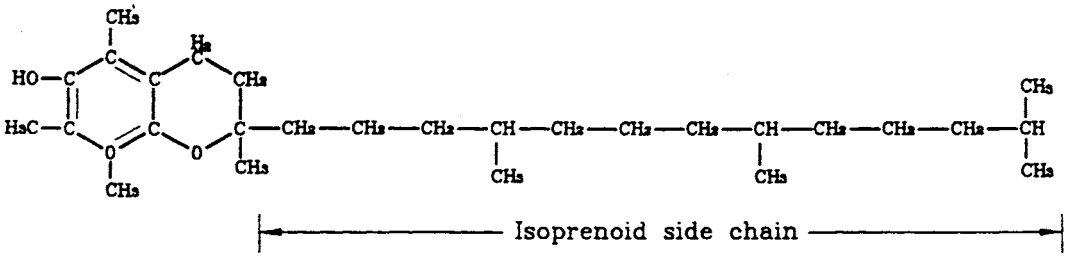


Tocol Structure

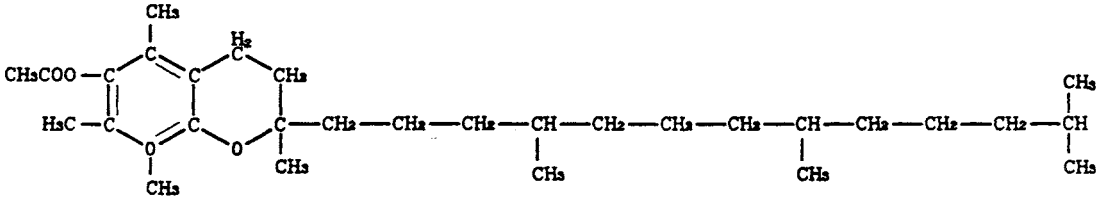


Trienol Structure

Figure 2. Structure of tocopherol and tocopheryl acetate.

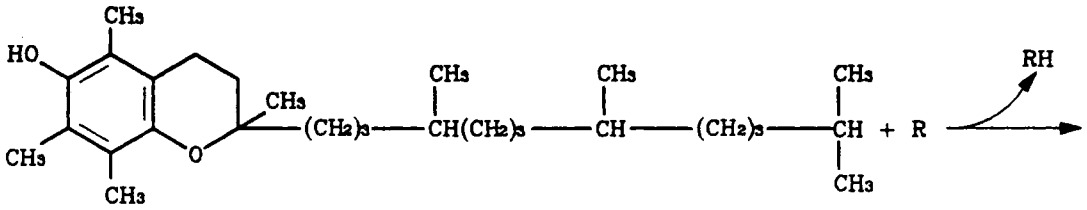


Alpha – Tocopherol

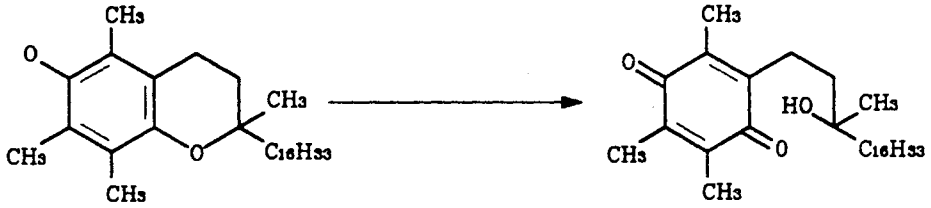


Alpha – Tocopheryl Acetate

Figure 3. Metabolites of tocopherol.



Alpha – Tocopherol



Tocopheryl semiquinone

Tocopheryl quinone

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CHAPTER III

IMPACT OF TOCOPHEROL FORM AND DOSING SITE ON BLOOD SERUM RESPONSES BY STEERS

ABSTRACT: Eight mature ruminally and duodenally cannulated beef steers (510 kg) were used to determine the relative bioavailability of α-tocopherol from ruminally and duodenally dosed α-tocopherol or α-tocopheryl acetate. Relative availability was calculated as the increase in serum concentration from pre-dosing and two weeks after daily dosing. Rest periods of at least 3 weeks followed each experiment. In experiment 1, six steers were dosed ruminally or duodenally with 500 IU α-tocopheryl acetate (dry form) daily for 14 days. Surprisingly, serum α-tocopherol concentrations were higher (1.33 vs .40 μg/ml) with ruminally than duodenally dosed acetate, opposite what one would expect if ruminal destruction of α-tocopherol is extensive. In experiment 2, 4 steers received 3,000 IU of either α-tocopherol or α-tocopheryl acetate dosed directly into the omasum of steers. Serum α-tocopherol concentration reached 8.53 μg/ml 24 hour after dosing. Serum concentration of steers with α-tocopheryl acetate increased only slightly and reached a mean of 4.3 μg/ml at 24 hour. In experiment 3, eight steers were dosed either ruminally or duodenally with 2,000 IU of either α-tocopherol or α-tocopheryl acetate (both liquids) daily for 14 days. With ruminal dosing, serum response tended to be greater (2.80 vs 2.29 ug/ml) from the acetate than from the free alcohol form of vitamin E. However, with duodenal dosing, the serum response was opposite, tending to be greater from the free alcohol than from the acetate form (.79 vs - .43ug/ml). Serum responses were less from duodenally than ruminally dosed α-tocopherol. Indeed, serum responses to duodenally infused α-tocopherol acetate were nil. In conclusion, serum responses indicate that postruminal availability of the acetate form of α-tocopherol is very low. However, availability appears to be increased by exposure of the acetate form to ruminal contents. Whether this enhancement in availability of α-tocopherol acetate in

the rumen is due to hydrolysis of the ester, attenuated passage to the small intestine or other factors is not yet clear.

(Key Words; α-tocopherol acetate, Rumen, Duodenum)

Introduction

The α-tocopherol found in nature is quite unstable and subject to destruction in the digestive tract (Alderson et al., 1974; Shin and Owens, 1990). Therefore the acetate ester, DL-α-tocopheryl acetate, is widely used as a vitamin E supplement. Presumably much of the acetate is removed during digestion or absorption by the intestinal mucosa so that the alcohol form is formed and this is the form that enters blood stream. In the free form, α-tocopherol acts as a biological antioxidant.

In ruminants, dietary vitamin E, is exposed to rumen microorganisms before it reaches to small intestine for absorption. Several studies have examined destruction of vitamin E by rumen microorganisms. Some workers have concluded that the α-tocopherol disappears from the rumen of cattle (Alderson et al., 1971; Shin and Owens, 1990) implying that ruminal microbes degrade vitamin E. In contrast, based on i*n vitro* incubation studies, loss of vitamin E has been very small (Astrup et al., 1974; Leedle et al., 1993). In these *in vitro* studies, neither α-tocopheryl acetate (Leedle et al., 1993) or α-tocopherol (Astrup et al., 1974) disappeared. Leedle et al. (1993) suggested that inadequate extraction resulted in failure to detect all of the α-tocopherol.

Because the extent of degradation of α-tocopherol and α-tocopheryl acetate in the rumen is not yet clear, this study was designed to investigate the bioavailability, based on

blood α-tocopherol concentrations, of different forms of vitamin E administered into the rumen or into the duodenum.

Materials and methods

Eight mature ruminally and duodenally cannulated beef steers (510 kg) fed 40 % rolled corn and 35 % alfalfa hay (Table 3) were used to determine relative bioavailability of α-tocopherol from ruminally or duodenally dosed α-tocopherol or α-tocopheryl acetate. Relative bioavailability of vitamin E was calculated as the change in serum α-tocopherol concentration between pre-dosing and two weeks after daily doses of the two materials. In each experiment, a rest period of at least 3 weeks followed each dosing period.

In experiment 1, six steers in a 4x6 square were dosed ruminally or duodenally with 500 or 1,000 IU of α-tocopheryl acetate (vitamin E, dry form, Roche Chemical Division, Nutley, NJ) daily for 14 days. Each animal received its diet once daily 0730 and its dose of vitamin E at 0800 each day for 14 days. Blood was drawn each morning at 0730.

In experiment 2, four steers were received 3,000 IU of either α-tocopherol or α-tocopheryl acetate (both liquids) dosed directly into omasum; blood was drawn 2, 4, 6, 12, and 24 hours after dosing.

In experiment 3, 2,000 IU of either α-tocopherol or α-tocopheryl acetate (both liquids, Vitamin E, USP-FCC, Roche Chemical Division, Nutley, NJ) was infused into rumen or duodenum daily for 14 days. Blood was drawn from jugular or coccygeal vein each morning (0730).

Blood was centrifuged immediately for 15 minutes at 1,000 x g. The supernatant fluid was pipetted into tube and stored at –20°C for α-tocopherol analysis by HPLC methods.

α-Tocopherol was extracted with an acetonitrile:methanol:chloroform (60:25:15, v:v:v) solution containing 0.1 % ascorbic acid as an antioxidant (Craig et al., 1992). A 200 µl serum sample was added to 1.8 ml of the extraction solution in a glass test tube and mixed using a vortex mixer for 30 seconds. Then the sample was centrifuged for 5 minutes at 1,000 x g. The supernatant fluid was transferred into 4 ml amber autosampler vial. Standards also were mixed with extraction solution and transferred into vials. An autosampler (WISP 710B, Waters, Milford, MA) was used to inject the sample into the HPLC. The mobile phase consisted of 100 % methanol at a flow rate of 1.0ml/min pumped through a C-18 column (25 cm, 4.6 mm id, Supelco, Bellefonte, PA) equipped with precolumn. A fluorescence detector (Waters 470, Waters, Milford, MA) was used for quantification of α-tocopherol with excitation at 295 nm and emission meaured at 330 nm with 10 nm of slit width. The average retention time of α-tocopherol was 11.5 minutes. Quantification was measured by comparing the integrated area under the curve of α-tocopherol peaks against standards. The dl-α-tocopherol standards (Vitamin E, USP-FCC, Roche Chemical Division, Nutley, NJ) at 1.25, 2.5, and 5 µg/ml were analyzed at the beginning and end of sample analysis.

Data analysis

Means and standard deviations for each sample were calculated. Initial to final and maximum serum α-tocopherol concentrations then were compared. Treatment means were calculated by using PROC GLM (SAS, 1988).

Results and discussion

Experiment 1.

Surprisingly, after 14 days of dosing with 500 IU DL-α-tocopheryl acetate per day, α-tocopherol concentrations in serum were elevated more by ruminal than duodenal doses (1.33 vs 0.40 μg/ml; $P < .01$) (Table 4). Serum α-tocopherol level had increased after only 7 days and increased only slightly after day 7. The initial serum α-tocopherol concentrations were 3.63 and 4.10 μg/ml for duodenal administration and ruminal administration, respectively with the lowest animal having 3.22 μg/ml and highest 4.34μg/ml. When steers received 1000 IU of α-tocopheryl acetate into rumen or duodenum, again blood α-tocopherol concentrations were elevated by ruminal dosing but there was virtually no response in serum α-tocopherol to duodenal doses. The difference between day 0 and day 14 was greater from 1,000 IU than from the 500 IU dose (1.68 vs 1.33 μg/ml).

Each steer in this experiment had initial serum α-tocopherol levels similar to those reported in several studies. Adams (1982) reported that a value below 2.0 μg/ml represents a deficiency, values from 2.0 to 3.0 μg/ml reflect marginal status, while values between 3.0 - 4.0 μg/ml imply adequate status. Caravaggi (1969) reported values up to 15.9 μg/ml in plasma of grazing cattle. α-Tocopherol levels may be affected by diet, age and health condition.

The fact that serum α-tocopherol level was higher for the ruminally supplemented group than the duodenally supplemented group after 14 days implies that ruminal

destruction of α-tocopherol as proposed by Alderson et al. (1971) and Shin and Owens (1990) is not reducing bioavailability. Alderson et al. (1971) fed diets containing 20, 40, 60 , and 80 % corn to steers. After an adaptation period, they dosed 20,000 IU of α-tocopherol on day 14 and 21; they observed that destruction of vitamin E in the rumen was proportional to the amount of corn in the diet. Shin and Owens (1990) conducted a study with several sources of vitamin E (DL-α-tocopherol, DL-α-tocopheryl acetate). In their study, ruminal destruction, from highest to lowest, was for α-tocopherol, α-tocopheryl acetate (liquid), α-tocopheryl acetate (absorbate), and α-tocopheryl acetate (spray). Incomplete duodenal recovery led to the conclusion that vitamin E was destroyed in the rumen.

Hidiroglou (1990) compared bioavailability of three different forms (D-α-tocopherol, D-α-tocopheryl acetate, and DL-α-tocopherol) of vitamin E administered to sheep either into rumen or duodenum. They also observed that the serum concentration of α-tocopherol was higher following intraruminal than following intraduodenal administration.

Njeru et al. (1994) conducted a study to assess the vitamin E status in sheep. When they increase the dietary dosage (0, 15, 30, and 60 IU/day), they observed that serum vitamin E level after 60 day had increased linearly with dosage rate. Njeru et al. (1995) reported similar results using yearling beef heifers feeding four different levels of dietary vitamin E (0, 500, 1,500, and 3,000 IU/animal/day) for 28 day with vitamin E, withdrawn for 28 day, and then resumed for another 28 day. They reported that the relationship between serum α-tocopherol concentration and vitamin E intake was linear.

These result imply that the serum vitamin E to an increased dose level should be linear and that serum α-tocopherol may reflect vitamin E status or at least vitamin E intake.

Potential reasons for greater response from ruminally than duodenally dosed α-tocopheryl acetate include 1) hydrolysis of the ester in the rumen, 2) bulk effect of excessive duodenal load reducing esterification and absorption, 3) absorption through the ruminal wall and 4) absorption of the esterified form.

Experiment 2.

To test whether ruminal hydrolysis of α-tocopheryl acetate to α-tocopherol was increasing bioavailability and responsible for higher serum values following ruminal than duodenal dosing, 3,000 IU of two different forms (DL-α-tocopherol or DL-α-tocopheryl acetate) of vitamin E was administered directly into omasum of steers and blood response was measured for 24 hours (Figure 4). Blood response was greater following dosage of the free form than acetate form after 24 hour. The blood response to omasal administration of free form was greatest at 12 and 24 hours after dosing. However, serum α-tocopherol also increased following DL-α-tocopheryl acetate administration, albeit, a smaller increase than from the free form. This result supports the concepts that ruminal exposure, if hydrolyzing the ester to the free form of α-tocopherol, should enhance the bioavailability of vitamin E.

Experiment 3.

Effects of different dosing site of α-tocopherol or α-tocopheryl acetate on serum α-tocopherol level were then compared (Table 5). When either tocopherol or tocopheryl

acetate was dosed into rumen, serum response tended to be slightly higher (2.80 vs 2.29 μg/ml, P=.42) from α-tocopheryl acetate than from the free alcohol form. However, with duodenal infusion, serum response was opposite, tending to be greater from the free alcohol than from the acetate form (0.79 vs -0.43□μg/ml, P=.24). Serum α-tocopherol responses were less when either forms of vitamin E were dosed into duodenum than when they were dosed into rumen. This difference concurs with results of experiment 1. However, the initial level of serum α-tocopherol in this trial was lower in ruminally dosed steers than in steers with duodenal dose (1.06 and 3.36 μg/ml for ruminal and duodenal dose, respectively). In this experiment, average age of steers that received vitamin E through rumen was 5 years old while others were only 15 month old. This age difference may be responsible for the difference in initial serum α-tocopherol levels.

Hidiroglou et al. (1988a, b) compared different form of vitamin E in cattle (1988a) and in sheep(1988b). They administered various racemic forms of α-tocopherol (dl- and d-) and α-tocopheryl acetate (dl- and d-). In their studies, d- form was more bioavailable and among the d- forms, free α-tocopherol produced highest serum response.

Results of this experiment agree with other studies in calves (Roquet et al., 1992) and in sheep (Hidiroglou et al., 1990). Hidiroglou et al.(1990) dosed only once with a large amount of either D-α-tocopherol (60 mg/kg body weight) or D-α-tocopheryl acetate (70 mg/kg body weight) to 24 sheep weighing 45 to 50 kg. No difference was found between these different chemical forms when dosed into rumen. However, when dosed into duodenum, blood response was higher with α-tocopherol than α-tocopheryl acetate, matching our observations.

The ester form of α-tocopheryl acetate presumably has to be converted to the free form by pancreatic lipase before it can be absorbed through the small intestine (Burton and Traber, 1990). Pancreatic lipase acts only on emulsified fats. Thereby, Roquet et al. (1992) proposed that the low blood response to duodenally dosed α-tocopheryl acetate may be a result of incomplete emulsification. Roquet et al. (1992) suggested that the poor response of α-tocopheryl acetate in duodenum supplement may be attributed to insufficient time for emulsification. In the rumen, hydrolysis as well as the emulsification would occur to enhance the absorption. This would suggest that rumen environment could have a beneficial effect on bioavailability of α-tocopheryl acetate.

Availability of various forms also may differ in other animal species. When free α-tocopherol or α-tocopheryl acetate form was administered to humans (Baker et al., 1980; Horwitt et al., 1984), swine (Chung et al., 1992), and rainbow trout (Hung et al., 1982), the serum α-tocopherol level increased more from a dose of free α-tocopherol than from α-tocopheryl acetate. These studies imply that the free form α-tocopherol is absorbed more efficiently than α-tocopheryl acetate as suggested from studies with ruminants. In the study of Hidiroglou and Jenkins (1974), administration of radio labeled α-tocopherol resulted in higher radioactivity in the blood and in tissues when dosed into the duodenum than into rumen in sheep.

Figure 5 presents the daily values for each steer for serum α-tocopherol during 14 days when 1,000 IU of free or ester form of vitamin E was administered into duodenum. When the free form was dosed into duodenum, serum α-tocopherol level increased in all three steers. However, when the ester form was dosed into duodenum, serum α-

tocopherol only increased in one steer. This steer had higher initial serum α-tocopherol level.

Figure 6 shows the change of vitamin E concentration during 5 days of tocopherol or tocopheryl acetate infusion to rumen. There was no difference in serum tocopherol concentration between tocopherol and tocopheryl acetate treatment. These two results concur with the results in experiment 1.

In conclusion, serum responses indicate that postruminal availability of the acetate form of α-tocopherol is very low and that availability is increased by exposure of the acetate form to ruminal contents. Whether this enhancement in availability of α-tocopheryl acetate in the rumen is due to hydrolysis of the ester, attenuated passage to the small intestine or other factors is not yet clear.

Implications

The free or non-esterified form of vitamin E was more readily absorbed and had higher bioavailability when dosed into the duodenum. However, the ester form of vitamin E had similar or higher bioavailability than the free form when it was administered into rumen. When administered into duodenum, bioavailability of the ester form was very low. These results illustrate that exposure to the rumen environment enhances the capacity of ester form of vitamin E to be hydrolyzed in and(or) absorbed from the small intestine.

Table 3. Composition of supplement.

Ingredient	%
Corn, rolled	40.0
Alfalfa hay, ground	35
Cottonseed hulls	21.75
Cane Molasses	3
Salt	0.25

Table 4. Change of serum α-tocopherol concentration after 14 days of α-tocopheryl acetate administration into rumen or duodenum.

	day 0	day 7	day 14	day 14-day 0	S. E.
		μg/ml			
500 D	3.676	3.966	4.073	0.397	0.1224
1,000 D	2.855	-	2.915	0.060	0.0947
500 R	4.047	5.083	5.377	1.330	0.1224
1,000 R	2.767	-	4.449	1.681	0.0947

Table 5. Change of serum α-tocopherol concentration after 14 days of administration of DL-α-tocopherol or DL-α-tocopheryl acetate into rumen or duodenum.

	day 0	day 14	day 14 – day 0	S. E.
		μg/ml		
AC (R)	0.97	3.78	2.80	0.3882
OH (R)	1.15	3.44	2.29	0.4482
AC (D)	3.13	2.70	-0.43	0.7043
OH (D)	3.59	4.38	0.79	0.6099

Figure 4. Serum tocopherol concentration after infusion of 3,000 IU tocopherol or tocopheryl acetate into omasum.

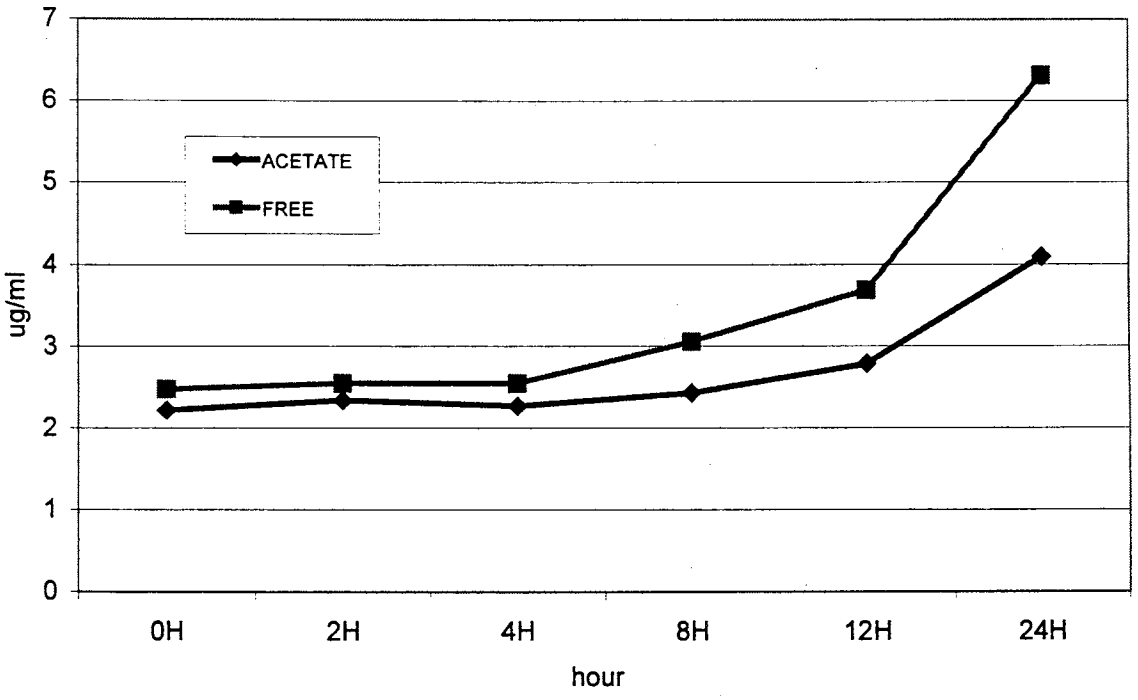


Figure 5. Serum tocopherol concentration before (day 0) and during duodenal dosing with tocopherol or tocopheryl acetate.

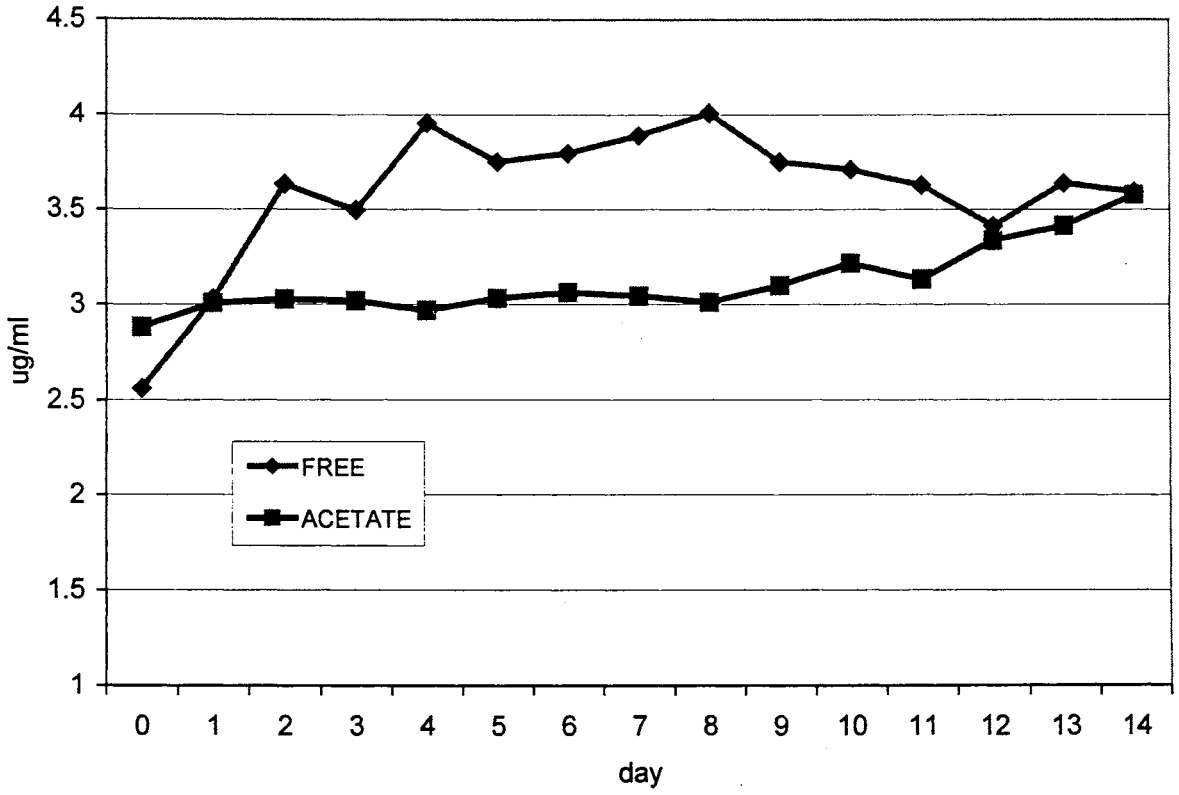
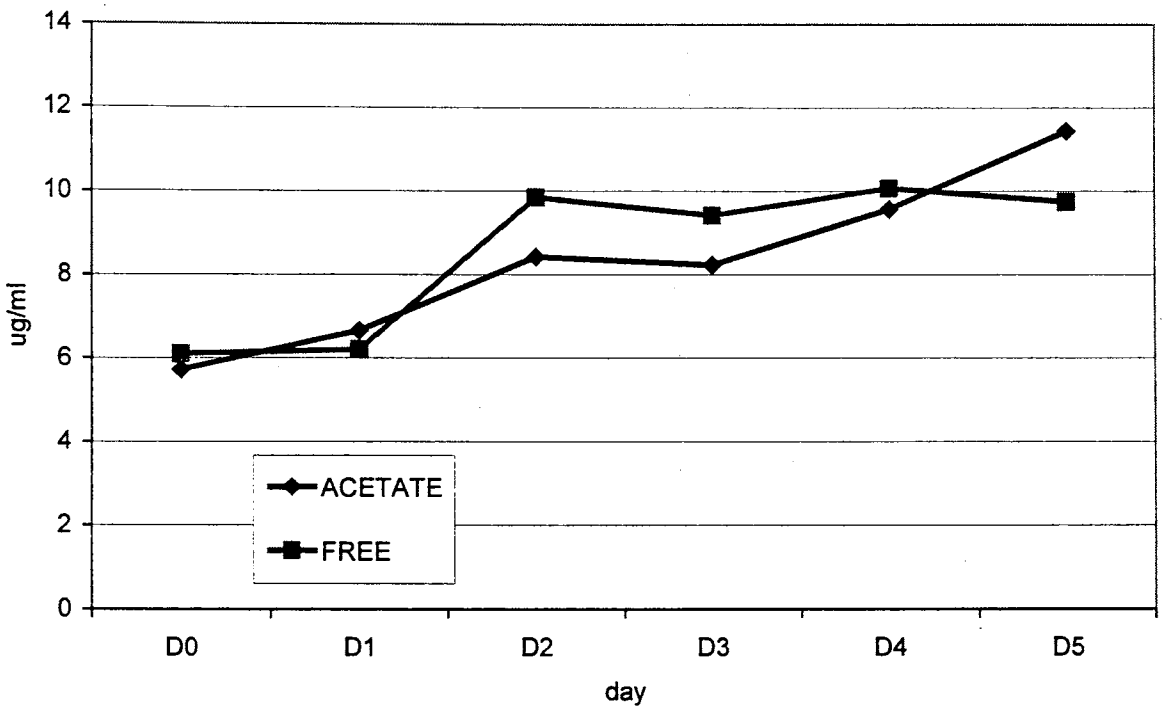


Figure 6. Serum tocopherol concentration before (day 0) and during ruminal dosing with tocopherol or tocopheryl acetate.



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CHAPTER IV

DEGRADATION OF TOCOPHEROL AND TOCOPHERYL ACETATE *IN VITRO*

ABSTRACT: Ruminal fluid from ruminally canulated beef steers (300 kg) fed a concentrate or a high roughage diet were used to determine disappearance of α-tocopherol and hydrolysis of α-tocopheryl acetate to α-tocopherol *in vitro*. Undiluted ruminal fluid was used in experiment 1, while McDougal's buffer was added in experiment 2. Degradation of vitamin E was examined by incubation of vitamin E with duodenal fluid in experiment 3. Degradation of α-tocopherol or conversion of α-tocopheryl acetate into α-tocopherol alcohol was estimated by 12 and 24 hr incubation of each α-tocopherol in rumen fluid. Ferric iron reducing ability (FRA) also was measured to estimate total antioxidant capacity of ruminal fluid. After 24 hour incubation of α-tocopherol or tocopheryl acetate in rumen fluid, the α-tocopherol concentration in rumen fluid was not changed in either experiment 1 and 2. Incubation of α-tocopherol or α-tocopheryl acetate with duodenal fluid for 4 h decreased α-tocopherol concentration. No relationship between α-tocopherol concentration and FRA value was detected. These results suggest that α-tocopherol is not destroyed in the rumen and that α-tocopheryl acetate was not extensively changed into α-tocopherol in the rumen.
(Key Words: In vitro, rumen, vitamin E, destruction.)

Introduction

Most studies of bioavailability of vitamin E have monitored responses in the blood or tissue α-tocopherol concentration. Such studies have been reported for ruminants (Njeru et al., 1992; Hidiroglou et al., 1992; Hidiroglou et al., 1988; Rocquet et al., 1992), swine (Chung et al., 1992) and horses (Siciliano et al., 1997). Based on these studies, d-α-tocopherol has a higher bioavailability than other form of vitamin E do.

However, because it is less susceptible to oxidative destruction, α-tocopheryl acetate, the acetate ester, is used widely in the feed industry as a source of vitamin E. Prior to absorption, α-tocopheryl acetate ester presumably needs to be hydrolyzed prior to absorption from the small intestine in nonruminants (Burton and Traber, 1990).

Availability of vitamin E for ruminants may be very low depending on composition of diet. Requirements for vitamin E for ruminant have not been clearly established although recommendations for ruminant range from 50 IU/day (NRC, 1996), 250 to 350 IU/day for light weight-finishing cattle (Coelho, 1991), and 200 to 500 IU/day for growing and finishing cattle (Williams et al., 1993).

Because the digestive tract of ruminants differ from that of the nonruminant, especially in lipid metabolism, vitamin E metabolism has been of interest. Failure to recover dosed vitamin E at the duodenum led to the conclusion that destruction of vitamin E in the rumen is extensive (Alderson et al., 1971; Shin and Owens, 1990). However, *in vitro* data on microbial destruction of vitamin E using rumen fluid from fasted sheep incubated by Astrup et al. (1974) detected no significant microbial destruction of DL-α-tocopherol after 24 hours of incubation. In another *in vitro* study,

ruminal fluid from a steer fed high-concentrate diet was used for *in vitro* incubation (Leedle et al., 1993). In their study, they again detected no disappearance of DL-α-tocopheryl acetate. However, the destruction of free DL-α-tocopherol in the *in vitro* incubation is not clear. The purpose of this study is to monitor the disappearance of free α-tocopherol and hydrolysis of α-tocopheryl acetate to α-tocopherol during incubation with rumen fluid. Both a high concentrate and a high roughage diet were used in this experiment because the number of *in vitro* studies about vitamin E degradation are limited (Astrup et al., 1974;Leedle et al., 1993). Astrup et al. (1974) used ruminal fluid from fasted sheep fed high forage diet whereas Leedle et al. (1993) incubated vitamin E with undiluted rumen fluid from steers fed 82 % corn diet. Therefore, in our study, we used two different diets and two different *in vitro* methods was used to investigate the destruction and hydrolysis of vitamin E.

Materials and Methods

Six beef steers (300 kg) were used to obtain rumen fluid. Each steer had a ruminal and duodenal fistula. The high roughage group received a diet of 30 % alfalfa hay, 45 % of cottonseed hulls. The high concentrate group was fed 75 % corn grain, 7 % cottonseed hulls, and 5% alfalfa hay (Table 6). The high concentrate group was adapted to its diet for 7 days. On day 8, ruminal contents were collected and squeezed with 4 layers of cheese cloth. Ruminal fluid was transferred into lab, flushed with CO_2 and held in a water bath (39°C) until it was dispensed in 50 ml aliquots into incubation tubes. No buffer or mineral was added in the ruminal fluid. Tocopherol and tocopheryl acetate (USP-FCC, Roche

Chemical Division, NJ) stock solutions were made by dissolving them in 95 % ethanol and storing them at 4°C in the dark.

Tocopherol or tocopheryl acetate solution (100 or 200 µl of 2,500 ppm) was dispensed into each incubation tube to achieve final concentration of 6 and 12 ppm of tocopherol in ruminal fluid. Each incubation bottle was set in water bath (39°C) and incubated for 24 hours. Another bottle, stored in the freezer without incubation served as a control. After 24 hours, bottle was frozen and stored at –20°C until being analyzed for tocopherol.

In experiment 2, ruminal fluid was obtained from steers fed high roughage diet. McDougal's buffer was added to rumen fluid by 50:50 and followed the same incubation procedure as experiment 1.

Duodenal fluid was obtained from steers fed high concentrate diet in experiment 3. Same method in experiment 1 was used in incubation of tocopherol or tocopheryl acetate.

α-Tocopherol was extracted from ruminal fluid with chloroform and methanol (Folch et al., 1957). For extraction, 10 ml of the rumen fluid sample was transferred into centrifuge tube and centrifuged (5,000 X g, 15 min). The supernatant fluid was transferred and retained. Then 50 ml of chloroform was added, the mixture was shaken vigorously, and 25 ml of methanol was added. The mixture again was shaken and held in a water bath at 50°C for 15 min. The mixture was filtered through Whatman No. 1 filter paper using a Buchner funnel under vacuum. The pellet was extracted a second time with premixed chloroform/methanol (2:1). Extracts were combined and evaporated until approximately 10 ml remained. The supernatant then was mixed and extracted twice with 90 ml of petroleum ether. The residue remaining after evaporating the petroleum ether

was dissolved into 20 ml of methanol and transferred into an amber vial. A 10 μl sample then was injected into the HPLC for tocopherol analysis.

α-Tocopherol was analyzed by HPLC (Waters, Milford, MA) using reverse phase chromatography at ambient temperature. A C-18 column (5 um, 25cm X 4.6 mm i.d.; Supelco, Bellefonte, PA) was used with 100 % methanol serving as the mobile phase at a flow rate of 1 ml/min with a Waters 470 Fluorescence detector. The instrument was set at absorbance with excitation of 295 nm and emission of 330 nm and slit width of 1.5 nm. Concentration of α-tocopherol was determined by comparing integrated peak areas of the sample and standards at an average retention time of 11 min. Ferric reducing ability of ruminal fluid was measured using a Cobas Fara 2 (Cobas Fara 2 Clinical Analyzer, Roche, Montclair, NJ) based on the procedure of Benzie and Strain (1996).

Mean value and standard deviations were calculated. Data were analyzed by PROG GLM (SAS, 1988).

Results and Discussion

Experiment 1. Undiluted rumen fluid incubations.

Initial pH was in the normal range but decreased during the 24 hour incubation period (Table 7). This pH drop agrees with the result of Leedle et al. (1993) who noted a drop 5.7 to 4.9 after 24 hour. They also measured the gas production every 4 hours during 24 hour incubation and observed that gas production was correlated negatively with pH, possibly reflecting lower CO_2 solubility at a low pH. During incubation with ruminal fluid from steers fed a high roughage diet, pH was dropped from 7.0 to 5.9. Leedle et al. (1993) used strained rumen fluid without buffers. For a 24 hour incubation, they

suggested that it was not necessary to buffer. Although they implied that use of buffers or added energy may not be required for a 24 hour incubation, final pH in their study and in ours was lower than typically formed in the rumen.

The average initial concentration of α-tocopherol in undiluted rumen fluid was 0.95 μg/ml (Table 8). α-Tocopherol and α-tocopheryl acetate dissolved in ethanol were added to achieve final concentrations of 6 and 12 μg/ml. In rumen fluid from steers fed the concentrate diet, α-tocopherol concentration increased significantly ($P<.05$) during 24 hr incubation with α-tocopheryl acetate in both 6 and 12 ppm and free α-tocopherol at 6 ppm while there was no significant change after incubation in free α-tocopherol treatment. However, α-tocopherol concentration in control fluid also was greater ($P<.05$) after 24 hour incubation (1.32 vs 1.03 μg/ml). With high roughage diet, concentration also increased with higher initial concentrations of either α-tocopheryl acetate or α-tocopherol. However, no change was detected with lower levels or the control. Although α-tocopherol concentration increased slightly during incubation of α-tocopheryl acetate, this small increase cannot explain the similar blood responses from α-tocopherol and α-tocopheryl acetate if the acetate were unavailable postruminally, yet, the change of α-tocopheryl acetate into α-tocopherol was a maximum of only about 47 % of the dose. No loss of α-tocopherol during incubation was detected. This supports observations by Astrup et al. (1974) and Leedle et al. (1993).

During incubation of diluted ruminal fluid from steers fed concentrates with 10 ppm α-tocopheryl acetate, α-tocopherol concentration had increased ($P< .05$) after 24 hours of incubation (Table 9). This suggests that some hydrolysis of α-tocopheryl acetate occur in

the rumen. No change in α-tocopherol concentration was detected with free α-tocopherol as a substrate. These results agree with results of Experiment 1. Concentration of α-tocopherol increased in control tubes. Because rumen microbes are not likely to synthesize α-tocopherol, this could be due to problem in the α-tocopherol extraction procedure.

The slight increases in α-tocopherol concentration after incubation of α-tocopheryl acetate suggests that limited hydrolysis of α-tocopheryl acetate to α-tocopherol may occur in the rumen. Because Leedle et al. (1993) used only α-tocopheryl acetate in their study to investigate disappearance, one cannot compare with their study directly with ours on hydrolysis of α-tocopheryl acetate. However, they reported that they did not detect any free α-tocopherol concentration after incubation. Astrup et al. (1974) also reported no destruction of vitamin E after 24 hour *in vitro* incubation with radiolabeled dl-α-tocopherol.

Tocopherol is stable to heat and alkali in the absence of oxygen. It is not affected by acids up to 100°C (Machlin, 1984). However, it is readily oxidized in the presence of oxygen. The ester form of tocopherol is used widely as an antioxidant to prevent the oxidation of free tocopherol. Whether α-tocopherol is destroyed in the digestive tract prior to absorption is not clear. Tucker et al. (1971) conducted a study to investigate activities of ethoxyquin, an antioxidant, and sodium nitrate, an oxidizing agent, on the destruction of vitamin E in the rumen of steers. When only α-tocopherol was administered, the recovery at abomasum was 73%, suggesting that 28 % was destroyed in the rumen. With 4.2g ethoxyquin added to the 7.2 kg of 60 % corn, 30.8 % hay, and 2.4 % soybean meal ration, recovery was 75%, with no saving effect of ethoxyquin.

However, recovery of α-tocopherol in the control and the sodium nitrate treatment groups were 39 and 36 %. They suggested that ruminal loss of vitamin E was not increased by adding the oxidant although they could not explain why recovery was lower in their second trial than their first trial. Shin and Owens (1990) also measured the ruminal disappearance of α-tocopherol and α-tocopheryl acetate in young steers. When vitamin E was fed with marker, they reported that disappearance of vitamin E in the rumen, from highest to least, ranked α-tocopherol, α-tocopheryl acetate (liquid form), α-tocopheryl acetate (absorbate), and α-tocopheryl acetate (spray dried) at 52, 50, 40, and 36 %, respectively.

In contrast, Astrup et al. (1974) detected no destruction of vitamin E when incubated with ruminal contents from fasted sheep fed a high forage diet. Leedle et al. (1993) who incubated α-tocopheryl acetate with undiluted strained rumen fluid, detected no ruminal degradation of α-tocopherol. In their study, to verify the degradation of tocopherol in the rumen, three different extraction procedures for α-tocopherol have been used. These included hot ethanol in a Soxhlet apparatus (Cort et.al., 1983), methanol/hexane extraction method (Schuep and Steiner, 1988), and the chloroform/methanol extraction (Folch et al., 1957) method. These three different extraction methods resulted in different recoveries. The chloroform/methanol extraction method yielded over 96% recovery. However, they found that vitamin E disappeared when spiked ruminal contents held at 39°C for a few minutes. However, it seems questionable that vitamin E is unstable when warmed up to 39°C because vitamin E is very stable against heat (Machlin, 1980). They suggested that ruminal disappearance of vitamin E *in vitro* might be an artifact of inadequate extraction rather than destruction of vitamin E in the rumen.

Total antioxidant capacity was measured using Cobas Fara 2 by methods of Benzie and Strain (1996). No treatment effect on FRA value was detected although average FRA value was higher with rumen fluid from steer fed high roughage diet than from steer fed the high concentrate diet (Table 11). FRA value decreased during 24 hour incubation. Spicer et al. (1998) detected no relationship between α-tocopherol level and FRA value in rat serum and testes. Because this procedure was developed to measure the total antioxidant capacity in plasma, it may not be proper to apply this method to samples other than plasma.

Even though our results indicates that α-tocopheryl acetate was partially hydrolyzed after 24 hour incubation with rumen fluid, the increase was not large enough to explain the high bioavailability of α-tocopheryl acetate *in vivo*. No destruction of α-tocopherol in the rumen fluid was detected at 24 hour incubation.

Implications

It has been postulated previously that α-tocopherol and α-tocopheryl acetate are degraded or destroyed in the rumen. However, α-tocopherol concentrations remained during a 24 hour incubation with rumen fluid. Some rumen hydrolysis of α-tocopheryl acetate was detected but amounts were small. This *in vitro* study supports the concept that neither α-tocopheryl acetate nor α-tocopherol are destroyed.

Table 6. Composition of diet.

Ingredients	Roughage diet, %	Concentrate diet, %
Corn, ground	15	74.3
Soybean meal	4	8
Cottonseed hulls	45	7
Alfalfa hay	30	5
Cane molasses	4.84	4.54
Limestone, 38%	0.83	0.83
Salt	0.3	0.3
Rumensin 80	0.0175	0.0175
Tylan 40	0.0115	0.0115

Table 7. Sample pH during *in vitro* incubation.

Item	0 hr	24 hr
High concentrate ruminal fluid		
Control	5.8	4.9
Acetate, 5ppm	5.9	5.0
Acetate, 10ppm	5.9	5.0
Free, 5ppm	6.0	5.1
Free, 10ppm	5.9	5.0
High roughage ruminal fluid		
Control	6.5	5.9
Acetate, 5ppm	6.8	5.8
Acetate, 10ppm	7.0	5.8
Free, 5ppm	7.0	5.8
Free, 10ppm	6.8	5.8

Table 8. α-Tocopherol concentration (μg/ml) in rumen fluid after 24 hour incubation *in vitro*.

Item	0 Hr	S. E.	24 Hr	S. E.	*P* =
Concentrate rumen fluid					
Control	1.03	0.0897	1.32	0.0518	0.0298
Acetate, 5ppm	0.96	0.0871	2.28	0.0617	0.0004
Acetate, 10ppm	1.32	0.1955	2.23	0.1382	0.0537
Free, 5ppm	4.30	0.5532	9.40	0.3194	0.0002
Free, 10ppm	12.90	1.6975	12.81	0.9800	0.9652
Roughage rumen fluid					
Control	1.05	0.0830	1.03	0.0479	0.8475
Acetate, 5ppm	0.73	0.0946	0.78	0.0546	0.6101
Acetate, 10ppm	0.73	0.1225	1.11	0.0707	0.0381
Free, 5ppm	7.95	0.6049	7.96	0.3492	0.9834
Free, 10ppm	14.73	0.2350	16.80	0.1357	0.0003

Table 9. α-Tocopherol concentrations (μg/ml) during incubation with diluted, buffered rumen fluid from fed a concentrate diet.

Item	0hr	12hr	24hr
Control	0.14	0.14	0.47
Control +buffer +hay	0.32	0.35	0.46
Acetate, 5ppm	0.32	0.34	0.38
Acetate, 10ppm	0.19	0.32	0.42
Free, 5ppm	5.98	5.81	5.25
Free, 10ppm	12.14	9.97	12.26

Table 10. α-Tocopherol concentrations (μg/ml) in duodenal fluid during 4 hour incubation.

Item	0hr	4hr	S. E.	*P* =
α-Tocopheryl Acetate	0.450	0.308	0.0084	0.0003
α-Tocopherol	2.264	1.689	0.0569	0.0020

Table 11. Total antioxidant assay during 24 incubation.

Item	0 hr	24 hr	S. E.	*P* =
Concentrate rumen fluid				
Control	3344	2992	51.52	0.0085
Acetate, 5ppm	3209	2958	71.94	0.0694
Acetate, 10ppm	3144	2887	46.27	0.017
Free, 5ppm	3111	2880	36.73	0.0113
Free, 10ppm	3252	2888	24.03	0.0004
Roughage rumen fluid				
Control	4276	3345	33.31	0.0001
Acetate, 5ppm	4065	3422	44.73	0.0005
Acetate, 10ppm	4140	3739	302.17	0.4015
Free, 5ppm	4004	3367	45.53	0.0006
Free, 10ppm	4247	3300	88.34	0.0016

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CHAPTER V

IMPACT OF FORM OF TOCOPHEROL ON BLOOD SERUM AND DIGESTIVE TRACT TOCOPHEROL RESPONSES BY STEERS TO RUMINAL DOSING

ABSTRACT: Bioavailability of two different forms of vitamin E was studied using beef steers. Six Hereford-crossbred steers (500 kg) equipped with fistula in rumen and the duodenum received 2,000 IU of α-tocopherol or α-tocopheryl acetate through their ruminal cannula daily for 6 days. α-Tocopherol concentration in blood, ruminal fluid, duodenal fluid and feces were measured. In daily, sample was taken before and during treatment and collected every 3 hours for 24 hours on the final day of vitamin E treatment dosing. The α-tocopherol level in blood was increased ($P<.01$) by dosing with either α-tocopherol or α-tocopheryl acetate with a slightly higher responses from α-tocopherol than α-tocopheryl acetate. In the ruminal fluid, the α-tocopherol concentration was increased with α-tocopherol dosing, as expected. But it also increased during α-tocopheryl acetate dosing. Similarly, α-tocopherol in the duodenum was increased by both forms of vitamin E tested although at both sites, levels were lower from α-tocopheryl acetate than from α-tocopherol. Digestibility of α-tocopherol in total digestive tract averaged 42 %. Based on ruminal and duodenal α-tocopherol analysis, α-tocopheryl acetate presumably was hydrolyzed to form free α-tocopherol by ruminal microorganism. Such hydrolysis would decrease the need for pancreatic or intestinal esterase to liberate α-tocopherol for intestinal absorption.

(Key Words; α-tocopherol acetate, Rumen, Duodenum)

Introduction

Based on research with cannulated ruminants, vitamin E partly disappears (42.4 % to 52 %) during passage through the rumen (Alderson et al., 1971; Shin and Owens, 1990). However, *in vitro* studies reveal little if any microbial destruction of α-tocopherol (Leedle et al., 1993). In contrast to this, vitamin E contents of high moisture corn decrease during storage whereas dried corn maintains vitamin E activity (Young et al., 1975). The loss of vitamin E during corn fermentation was related negatively to the free fatty acids concentration of high moisture corn. These discrepancies need further research attention.

Gallo-Torres (1970) reported that bile and pancreatic secretions are requisites for absorption of vitamin E from the small intestine. Muller et al. (1976) showed that pancreatic esterase rather than pancreatic lipase was the principal hydrolytic enzyme for α-tocopheryl acetate in human duodenal juice. Gallo-Torres (1970) found that hydrolysis of α-tocopheryl acetate is required for the intestinal absorption and the lymphatic transport of vitamin E. However, in rat study, Mathias et al. (1981) later demonstrated that the hydrolysis of vitamin E esters by pancreatic esterase in small intestine is not obligatory for vitamin E absorption. They suggested that even without prior luminal hydrolysis, micellar solubilization is sufficient for uptake of vitamin E by the mucosa. Within the mucosa, mucosal esterase hydrolyzes the solubilized esters intraluminally in endoplasmic reticulum of enterocyte. Similar studies have not been conducted with ruminants. One cannot exclude the rumen as a site of hydrolysis of α-tocopheryl acetate.

Most studies of bioavailability of α-tocopherol have been based on relative α-tocopherol concentrations in serum (Horwitt et al., 1982; Baker et al., 1986; Roquet et al., 1992; Hidiroglou et al., 1992; Njeru et al., 1992) or in various tissues (Hidiroglou et al., 1988; Chung et al., 1992). Thereby the site and extent of metabolism have been ignored. Determination of vitamin E in concentrations at various points in the digestive tract may prove useful for enhancing vitamin E bioavailability. The purpose of this study was to examine kinetics of different forms of vitamin E in the ruminant digestive tract.

Materials and Methods

Six Hereford crossbred beef steers (500 kg) equipped with ruminal and duodenal fistula were used in this experiment. A diet of rolled corn with ground alfalfa hay (Table 12) was fed throughout this study at a rate of 7 kg /day. α-Tocopherol and α-tocopheryl acetate were purchased from Hoffman Ra Loche company (Nutley, New Jersey). Each animal received 2,000 IU of free vitamin E or vitamin E acetate via the rumen cannula at 0800 for 6 days. Based on our previous studies, α-tocopherol level in the serum reached a plateau in about five days. Therefore, vitamin E was dosed for 6 consecutive days in this experiment. Chromium oxide mixed with cottonseed hulls and fed as a pellet was used as a solid marker and cobalt EDTA was used as a liquid marker to correct for dilution of α-tocopherol in ruminal and duodenal fluids. Blood, rumen fluid, duodenal fluid, and feces were collected every day for 6 days. Collected blood was allowed to clot and then centrifuged at 1,000 x g for 15 minutes. The supernatant serum was transferred into tubes and stored at –20°C for α-tocopherol and ferric reducing ability (FRA)

analysis. On day 6 after the final day dose, 500 ml of ruminal and duodenal fluid were collected every 3 hours for 24 hour period and stored at –20°C for α-tocopherol and FRA analysis. Cobalt EDTA was dosed daily into the rumen at a rate of 100 ml each day. Samples were analyzed for cobalt using an Atomic Absorption Analyzer (Perkins-Elmer, Norwalk, CT).

Ferric reducing ability, a measure of total antioxidant power, was measured by the method described by Benzie and Strain (1996) using a Cobas Fara 2 (Cobas Fara 2 Clinical Analyzer, Roche, Montclair, NJ). Several methods (Wayner et al., 1986; Whitehead et al., 1992) can measure the ability of plasma to withstand the oxidative effects of a reactive species generated in the reaction mixture. These methods require specialized equipment, and are quite time-consuming. Ferric reducing ability of plasma (FRAP) method has been developed as a similar alternative to assess the total antioxidant power (or reducing ability) of blood or tissues. When ferric tripyridyltriazine complex is reduced to the ferrous (Fe^{2+}) form, a blue color develops at low pH. This color represents the reducing ability of the sample (Benzie and Strain, 1996). We used this method to examine reducing capacity of serum, ruminal or duodenal contents.

α-Tocopherol was extracted from samples using a mixture of acetonitrile:methanol :chloroform (60:25:15, v:v:v) with 0.1 % of ascorbic acid included as an antioxidant (Craig et al., 1992). For extraction of serum, 200 µl samples were added to 1.8 ml of extraction solution in a glass test tube and mixed for 30 seconds using a vortex mixer. Then, the sample was centrifuged for 5 minutes at 1,000 x g. The supernatant fluid was transferred into 4 ml amber autosampler vials. Similar amounts of standards were mixed with extraction solution and transferred into vials. The dl-α-tocopherol standards

(Vitamin E, USP-FCC, Roche Chemical Division, Nutley, NJ) at 1.25, 2.5, and 5 μg/ml were analyzed at the beginning and end of sample analysis.

For ruminal or duodenal samples, 10 ml samples were centrifuged at 5000 x g for 15 minutes and the supernatant was saved. The pellet then was extracted by adding 50 ml chloroform and 25 ml methanol. The mixture was shaken vigorously and heated in a water bath at 50°C for 15 minutes. The mixture then was filtered through Whatman No 1 filter paper under vacuum. The residue was extracted a second time with 15 ml of a premixed chloroform:methanol (2:1) solution. These liquid extracts were evaporated with a rotary evaporator until only 10 ml of liquid remained. This residue was combined with the initial supernatant fluid and extracted with 100 ml of petroleum ether in a separatory funnel. Petroleum ether was evaporated and the sample was dissolved in 20 ml of methanol. A 3 ml aliquot was pipetted into an amber autosampler vial.

An autosampler (WISP 710B, Waters, Milford, MA) was used to inject the sample into the HPLC. A mobile phase of 100 % methanol at flow rate of 1.0ml/min. was pumped through a C-18 column (25cm, 4.6 mm id, Supelco, Bellefonte, PA) equipped with precolumn filter. A fluorescence detector (Waters 470, Waters, Milford, MA) was used to quantify of α-tocopherol using excitation of 295 nm and detecting emission at 330 nm with a 10 nm of slit width. Mean retention time of α-tocopherol was 11.5 minutes. Integrated area under the curve of the α-tocopherol peak for standards and samples were used to calculate concentrations in various samples.

Data analysis

The design of this experiment is repeated measures design. The design of the experiment consists of a one-way whole plot treatment structure in a completely

randomized whole plot design structure and a one-way subplot treatment structure in a randomized complete block design structure. The model and the analysis consists two parts, one for steer and one for day or time intervals. Treatment mean values were compared by PORC GLM (SAS, 1988) procedure.

Results and discussion

Concentrations of cobalt in duodenal fluid are presented in Figure 7. The concentration of cobalt in duodenal fluid remained relatively constant on day 2 through 5. Marker concentration in ruminal and duodenal samples tended to be lowest in the morning after feeding; this may be caused by dilution with consumed water.

Ruminal α-tocopherol concentration was greater ($P<.01$) on day 6 than day 0 before infusions began (10 vs 2 μg α-tocopherol per ml) (Figure 8). There was also difference between treatment ($P<.05$). On day 6, animals that received α-tocopheryl acetate had ruminal α-tocopherol concentrations ranging from 2 mg/l to 9 mg/l. Subsequent samples up to 24 hours contained only slightly lower α-tocopherol concentrations. After dosing with free α-tocopherol (day 6), α-tocopherol concentration in the rumen was increased relative to day 0. On day 6, ruminal concentrations ranged from 5 to 19 mg/l, considerably higher than from α-tocopheryl acetate. However, the fact that ruminal α-tocopherol concentrations in the rumen increased after α-tocopheryl acetate was administered in the rumen indicates that α-tocopheryl acetate must have been hydrolyzed into free form in the rumen. Presumably, it was believed that ester form of vitamin E was hydrolyzed only in the lumen of small intestine (Gallo-Torres, 1970) due to action of bile

and gastric secretions and (or) intracellular esterases of the endoplasmic reticulum (Mathias et al., 1981) in human and rat. However, these results support the contention that partial hydrolysis of α-tocopheryl acetate occurs in the rumen. Why this has not been observed *in vitro* may be a result of 1) inadequate culture conditions, 2) adaptation of ruminal microbes to hydrolyze this substrate.

Baker et al. (1980) compared the bioavailability of free or acetylated tocopherol in humans. Plasma α-tocopherol concentration levels were greater after ingestion of free α-tocopherol than of the esterified form. Blood response also was more rapid from free α-tocopherol. Absorption of free α-tocopherol is facilitated by bile salts that emulsify tocopherol for absorption, whereas the added step of esterification by pancreatic secretions is needed to hydrolyze the esterified tocopherol in preparation for absorption (Gallo-Torres, 1970). Muller et al. (1976) proposed that the principal hydrolytic enzyme for tocopheryl acetate is not pancreatic lipase but pancreatic esterase. Absorption of free α-tocopherol is reduced in the absence of bile, whereas absorption of esterified α-tocopherol is impeded by pancreatic insufficiency (Baker et al., 1980). Although, all these studies were conducted with rats or humans, a parallel in absorption of this fat soluble vitamin E absorption mechanism may apply directly to ruminants. Unfortunately we did not collect the ruminal samples during the vitamin E adaptation period but only on the final day of dosing.

Concentrations of α-tocopherol in duodenal contents during adaptation periods are presented in Figure 9. Dosing increased the α-tocopherol concentration above day 0 for all animals ($P<.01$). Surprisingly, the α-tocopherol concentration in duodenal fluid was increased nearly as much in steers receiving α-tocopheryl acetate ($P<.01$) as those

receiving free α-tocopherol. All these samples were obtained from proximal duodenum, which should preclude hydrolysis. Indeed, incubation of the ester with duodenal contents in a previous trial (Chapter 4) failed to release α-tocopherol. There was no treatment effect between tocopherol and tocopheryl acetate that implies the hydrolysis of tocopheryl acetate to tocopherol in the rumen. Variation implies that hydrolysis of α-tocopheryl acetate in the rumen though present, differs over time and from animal to animal.

As expected, the α-tocopherol concentration was greater for α-tocopherol than α-tocopheryl acetate. This presumably reflects partial hydrolysis of α-tocopheryl acetate. If α-tocopheryl acetate were not partly hydrolyzed in the rumen, no increase of α-tocopherol level in duodenal contents beyond the baseline would be expected. Results indicate that partial hydrolysis of α-tocopheryl acetate occurred before α-tocopheryl acetate entered the small intestine indicating that α-tocopheryl acetate was being hydrolyzed without bile and pancreatic secretions.

When D-α-tocopheryl acetate was supplied through duodenum, it failed to increase circulation levels of α-tocopherol in the serum of steer (Rocquet et al., 1991). This concurs with our previous study (Chapter 3). Hidiroglou et al. (1990) also observed the response to duodenal dosing was less for the acetate form (D-α-tocopheryl acetate) than free forms (D-α-tocopherol or DL-α-tocopherol) of vitamin E in sheep. This implies that vitamin E esters are digested by ruminants in a manner similar to dietary triglycerides.

Esterified lipids are extensively hydrolyzed in the rumen by microbial lipase (Byers and Schelling, 1988). In small intestine, action of bile salts and pancreatic juice results in formation of micelles are absorbed; and fatty acids are re-esterified and packaged as

chylomicrons for transport by lymph from the small intestine. Administration of vitamin E acetate through duodenum forces the animal to rely on intestinal esterases, which in turn decreases absorption (Hidiroglou et al., 1990). Inadequate intestinal esterase may explain partially the low postruminal bioavailability of α-tocopheryl acetate. However, if α-tocopheryl acetate already is partially hydrolyzed to free α-tocopherol before it enters the proximal duodenum, bioavailability is less dependent on bile salts and pancreatic juice.

Anderson et al. (1995) evaluated the relative bioavailability of various vitamin E compounds for finishing swine. In their study, the highest serum α-tocopherol resulted from dietary supplementation of D-α-tocopheryl acetate rather than D-α-tocopherol. They suggested that instability of free α-tocopherol during feed storage may have decreased vitamin E intake. This means that they were evaluating both feed stability and bioavailability in their trial. Albeit, in practice, both factors must be considered.

Gallo-Torres (1970) studied the role of bile and pancreatic juice in absorption of vitamin E in the rat by cannulating in the thoratic duct. They suggested that the presence of bile is a requisite for hydrolysis of α-tocopheryl acetate prior to absorption. By cannulating the thoratic duct in man, Blomstrand and Forsgren (1968) also reported that DL-α-tocopheryl acetate must be hydrolyzed before absorption into lymph as free α-tocopherol. These results demonstrate that hydrolysis of α-tocopheryl acetate must precede absorption from the small intestine in the human and the rat. Muller et al. (1976) reported that pancreatic esterase is primarily responsible for the hydrolysis of α-tocopheryl acetate based on a study with human duodenal juice.

On the final day of vitamin E supplementation, α-tocopherol concentration in the duodenum did not changed substantially except for one steer that received free α-tocopherol (Figure 10). This stability may represents the gradual metering of ruminal contents to the duodenum. However, the mean concentration was higher from dosed free α-tocopherol than from dosed α-tocopheryl acetate.

Presumably, levels of α-tocopherol in duodenum reflect hydrolysis of α-tocopheryl acetate in the rumen. In our previous study, we detected no difference in serum α-tocopherol response from the two forms when both were administered into rumen. But when administered into duodenum, serum response from α-tocopheryl acetate treatment was nil. Probably, absorption of esterified vitamin E is affected by the route of administration while absorption of free α-tocopherol is not due to partial hydrolysis of the esterified form of vitamin E in the rumen that compensates for incomplete hydrolysis in the small intestine of ruminants.

The concentration of free α-tocopherol in serum was significantly ($P<.01$) increased during dosing with either free or acetylated α-tocopherol. These match with increased α-tocopherol concentrations in duodenum during dosing (Figure 9)

The steer with the lowest serum response had the least increase in α-tocopherol in the duodenum. Generally, serum α-tocopherol concentrations were higher when concentrations in the proximal duodenum were higher. This suggests that ruminal hydrolysis is the rate-limiting step for α-tocopherol absorption. This might explain failure of oral doses of α-tocopheryl acetate to increase serum concentrations in pre-ruminant calves and in shipping stressed calves with depressed ruminal function.

On the final day of dosing, serum α-tocopherol concentration tended to decrease among steers receiving α-tocopherol, while those receiving α-tocopheryl acetate, though lower, remained more constant (Figure 12). However, all steers had higher levels before dosing on day 0 (Figure 11). Serum α-tocopherol concentration has been reported to drop to normal level within a week after dosing is stopped.

Ferric reducing ability, a measure of antioxidant capacity, for ruminal fluid, duodenal fluid, and serum are presented in Figures 13 through 15. No change in FRA values with administration of vitamin E was detected. The FRA values in ruminal fluid (Figure 13) and in serum (Figure 15) tended to be lower during the period of vitamin E dosing. No response in FRA of duodenal fluid was apparent. Because antioxidant capacity is determined by development of a blue color, this method may not be accurate with either ruminal or duodenal contents. Such samples need to be discolored before testing the reaction to reagents. However, no change in the serum to vitamin E supplementation was detected and the correlation between serum α-tocopherol and FRA was low. Spicer et al. (1998) used this method to determine the antioxidant capacity in rat serum and testes. They found positive relationship between testes FRA and serum α-tocopherol concentration although the relationship was not significant. This suggests that compounds other than α-tocopherol are more important determinants of serum reducing power.

Digestibility for α-tocopherol in total digestive tract is presented in Table 13. Digestibility of α-tocopherol averaged 41 %. Shin and Owens (1990) reported that availability of vitamin E in small intestine averaged 33 % while NRC (1989) reported a 30 % availability.

Further studies could examine the impact of various ruminal microbes on metabolism and absorption of α-tocopheryl acetate.

Direct analysis of α-tocopheryl acetate, not just α-tocopherol, would help to ascertain the degree to which α-tocopherol is hydrolyzed in the rumen. Also postruminal administration of α-tocopheryl acetate with various emulsifying agent or esterase would help to detect the specific factor limiting postruminal bioavailability of duodenally dosed α-tocopheryl acetate. Depending on which factor is limiting, other sources of vitamin E , including the succinate, the nicotinate, the benzoate, the palmitate, the pivalate, might be more suited for ruminants.

Implication

α-Tocopherol concentration in both ruminal and duodenal fluids increased during supplementation of α-tocopheryl acetate in rumen. Blood α-tocopherol concentration increased when either α-tocopherol or α-tocopheryl acetate were infused into the rumen indicating that ruminal bioavailability of the free and the acetate forms were equal. Because duodenal α-tocopherol concentration increased during ruminal dosing of α-tocopheryl acetate, this compound must be partially hydrolyzed before it reaches small intestine. This suggests that when ruminal function is compromised, e.g. in pre-ruminants or dietary stress, bioavailability of α-tocopheryl acetate will be reduced.

Table 12. Composition of diet, as fed

Ingredients	%
Corn, rolled	40.0
Alfalfa hay, ground	35
Cottonseed hulls	21.75
Cane Molasses	3
Salt	0.25

Figure 7. Cobalt concentration in duodenal fluid before (day 0) and during daily ruminal dosing of cobalt EDTA.

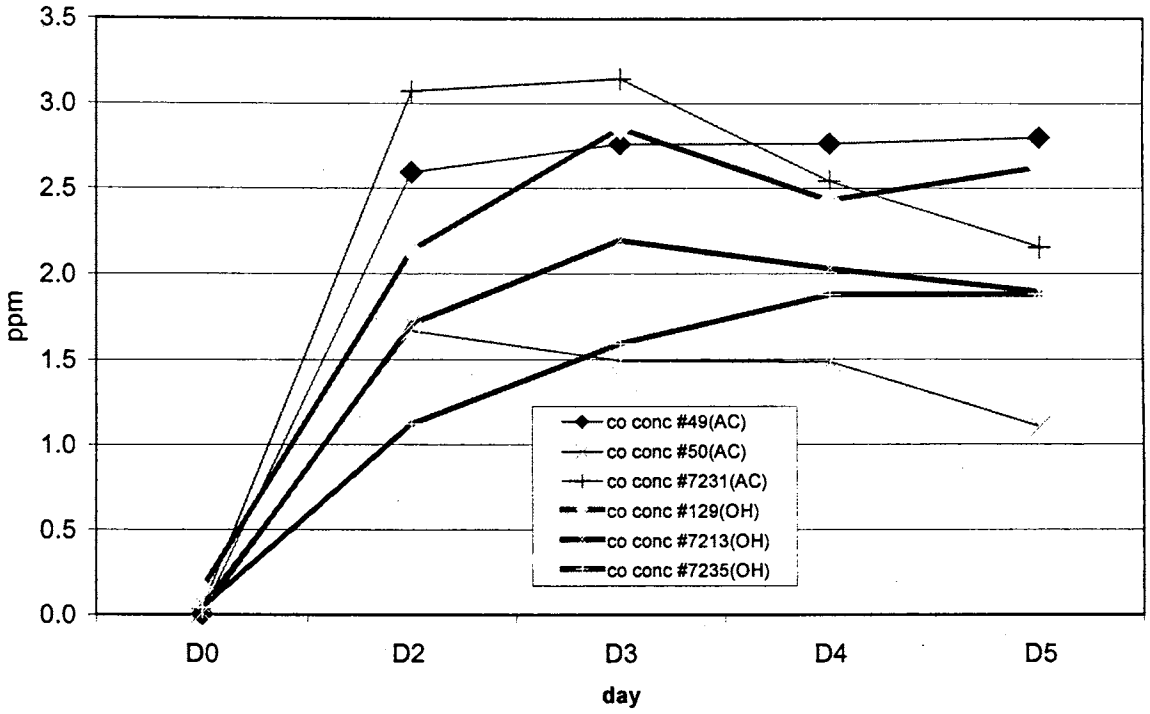


Figure 8. Ruminal tocopherol concentration during final day with ruminal dosing of tocopherol or tocopheryl acetate.

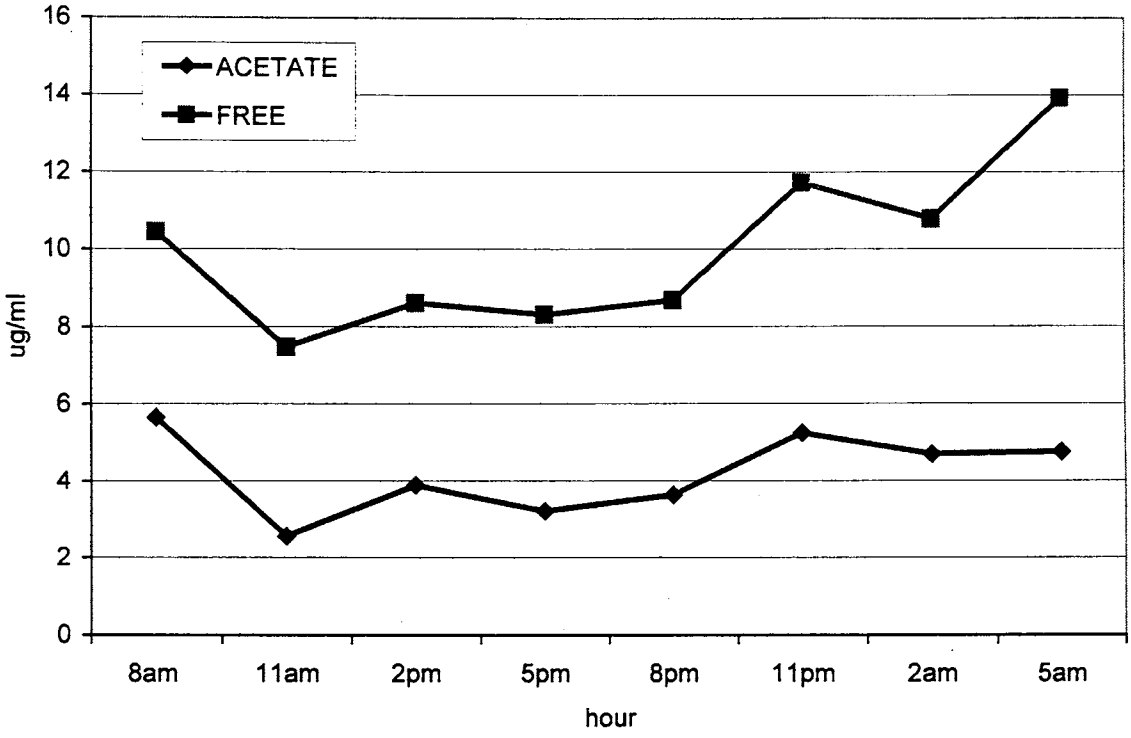


Figure 9. Duodenal tocopherol concentrations before (day 0) and during ruminal dosing with tocopherol or tocopheryl acetate.

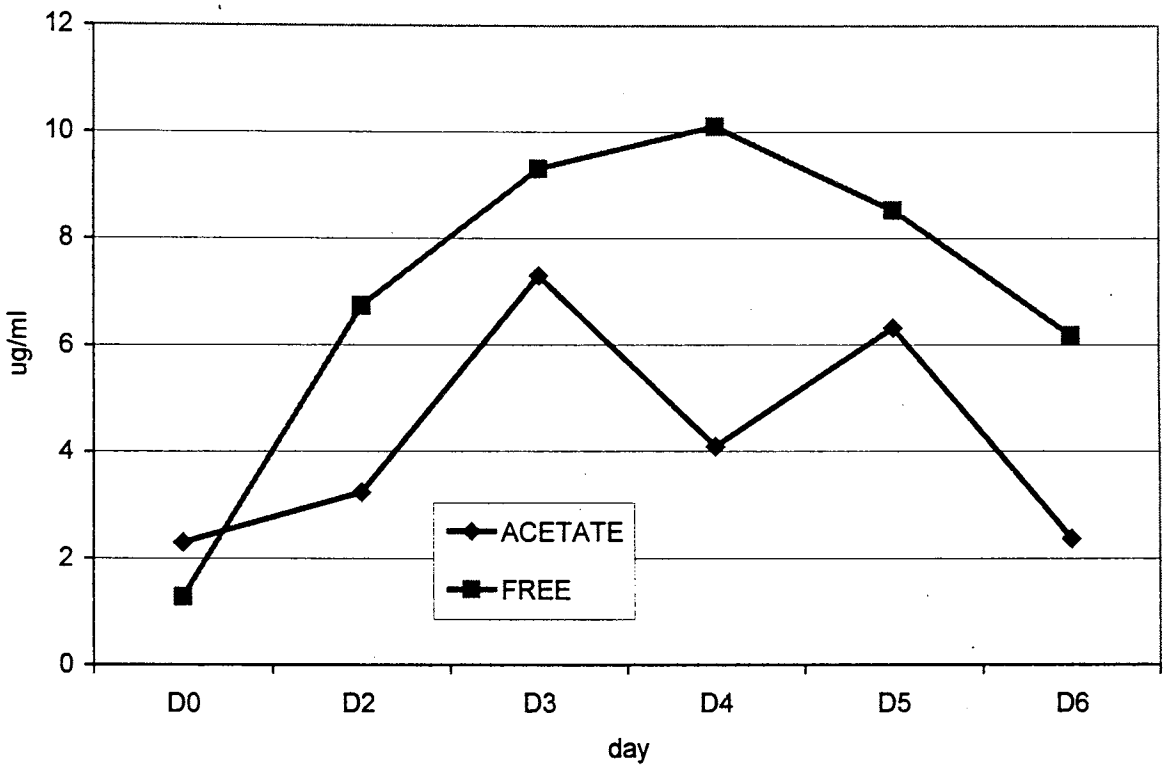


Figure 10. Duodenal tocopherol concentration during final day with ruminal dosing of tocopherol or tocopheryl acetate.

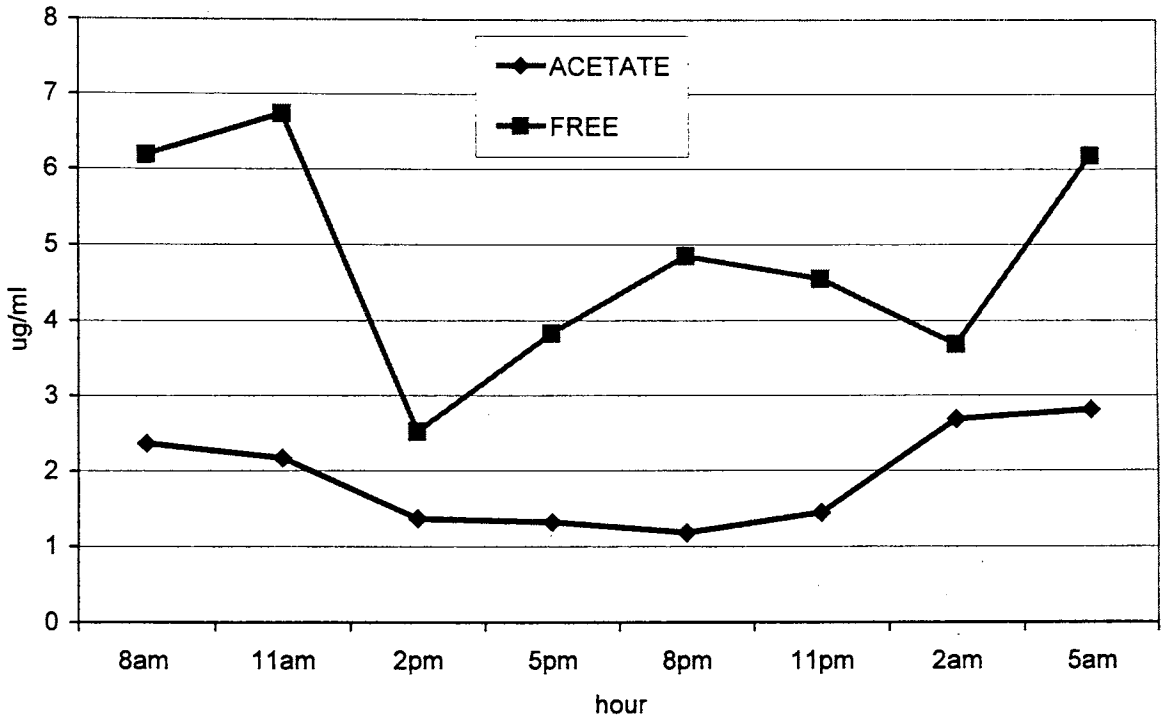


Figure 11. Serum tocopherol concentrations before (day 0) and during ruminal dosing with tocopherol or tocopheryl acetate.

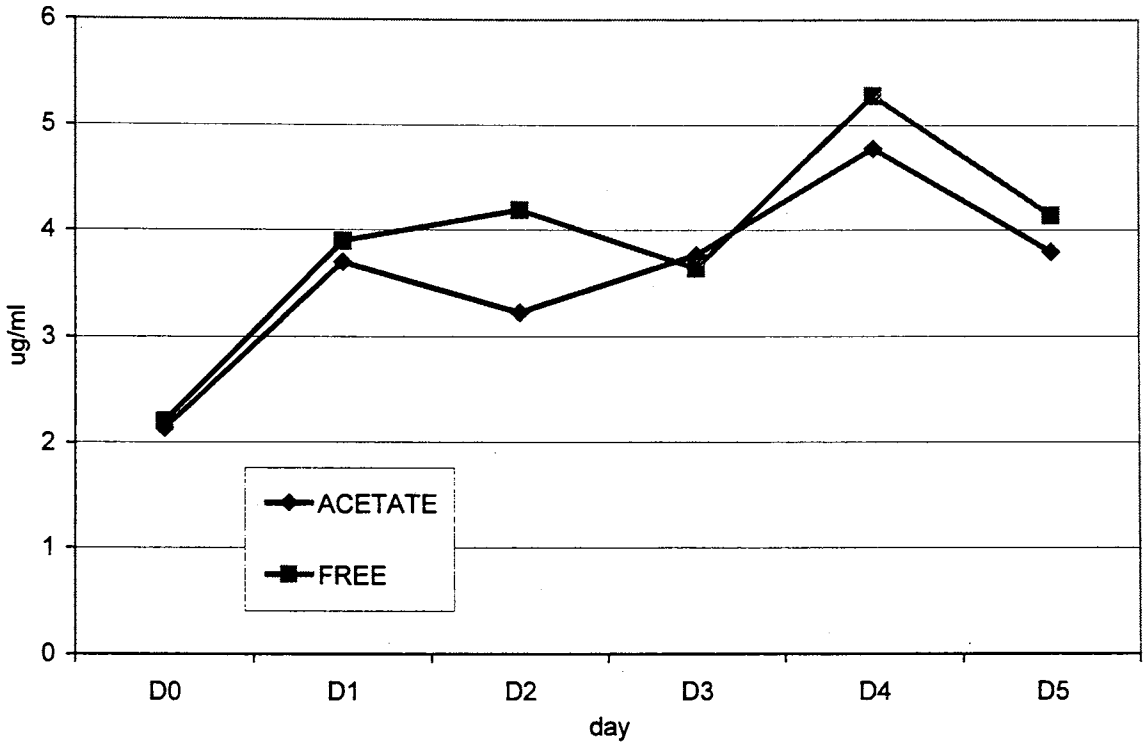


Figure 12. Serum tocopherol concentration during the final day of ruminal dosing with tocopherol or tocopheryl acetate.

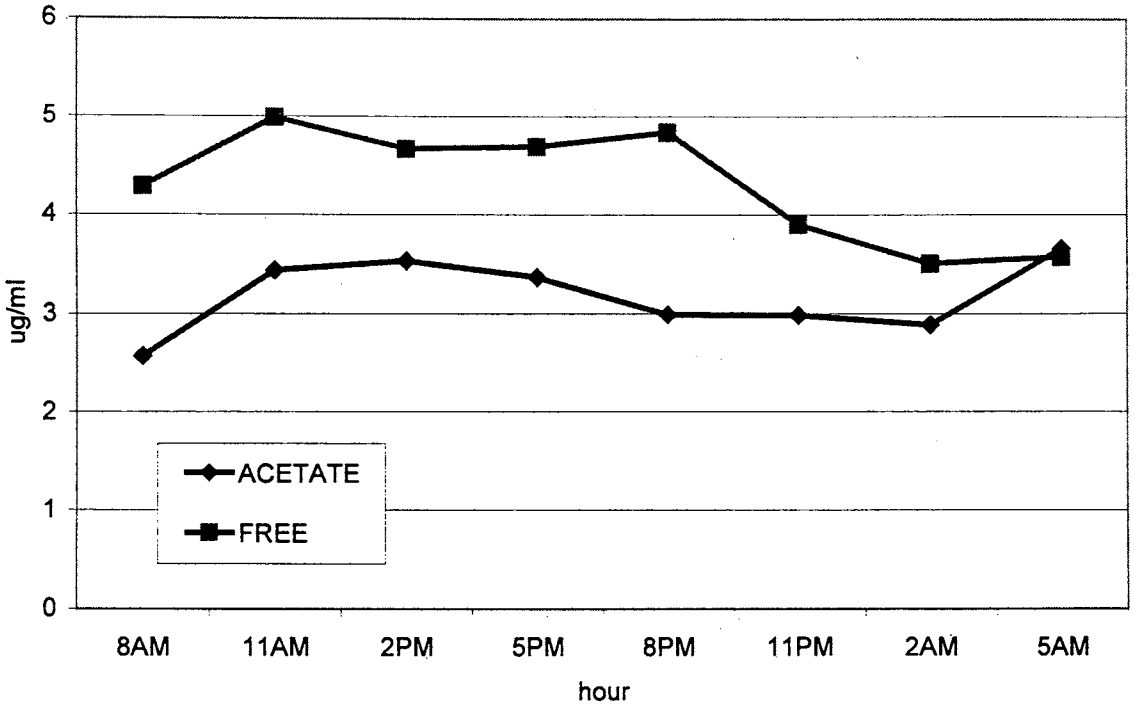


Figure 13. Ruminal Fluid FRA values on final day of ruminal dosing with tocopherol or tocopheryl acetate.

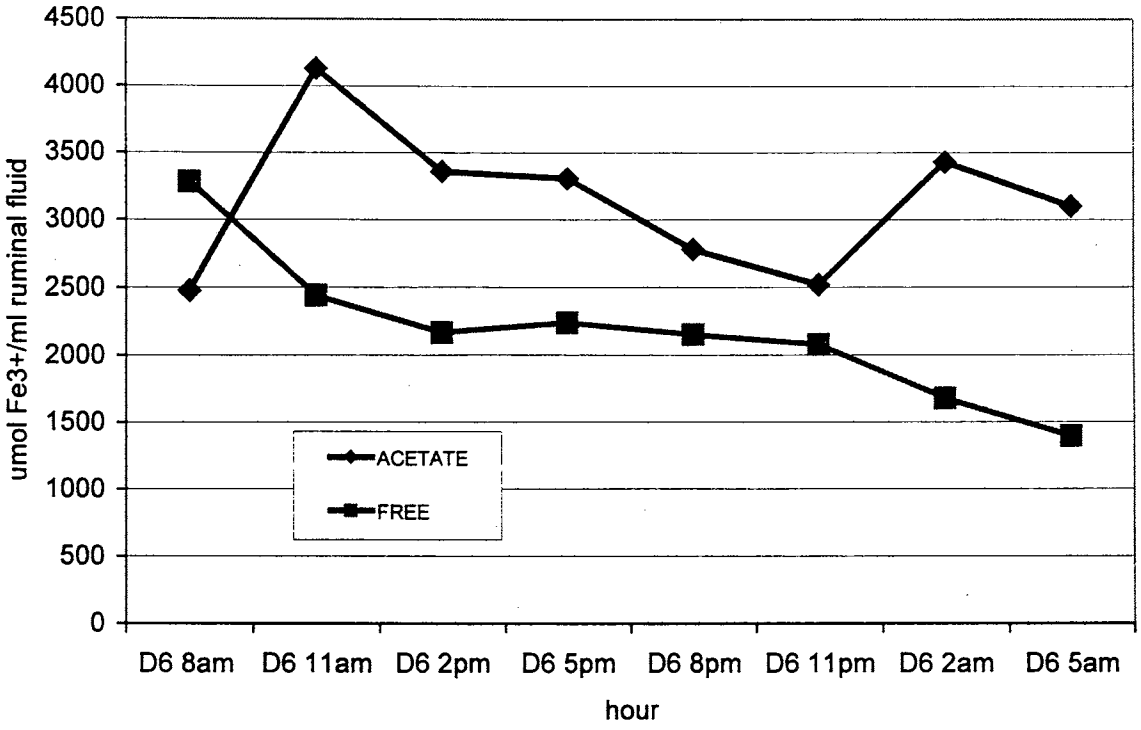


Figure 14. Duodenal FRA level before (day 0) and during ruminal dosing with tocopherol or tocopheryl acetate.

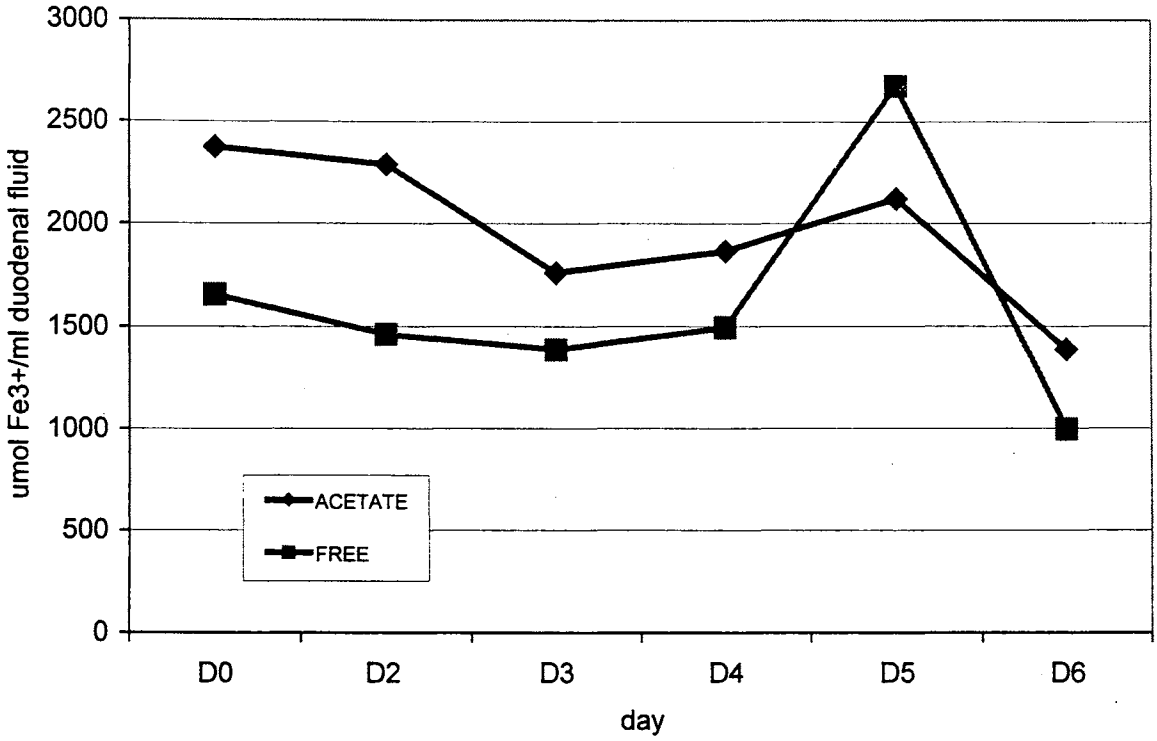
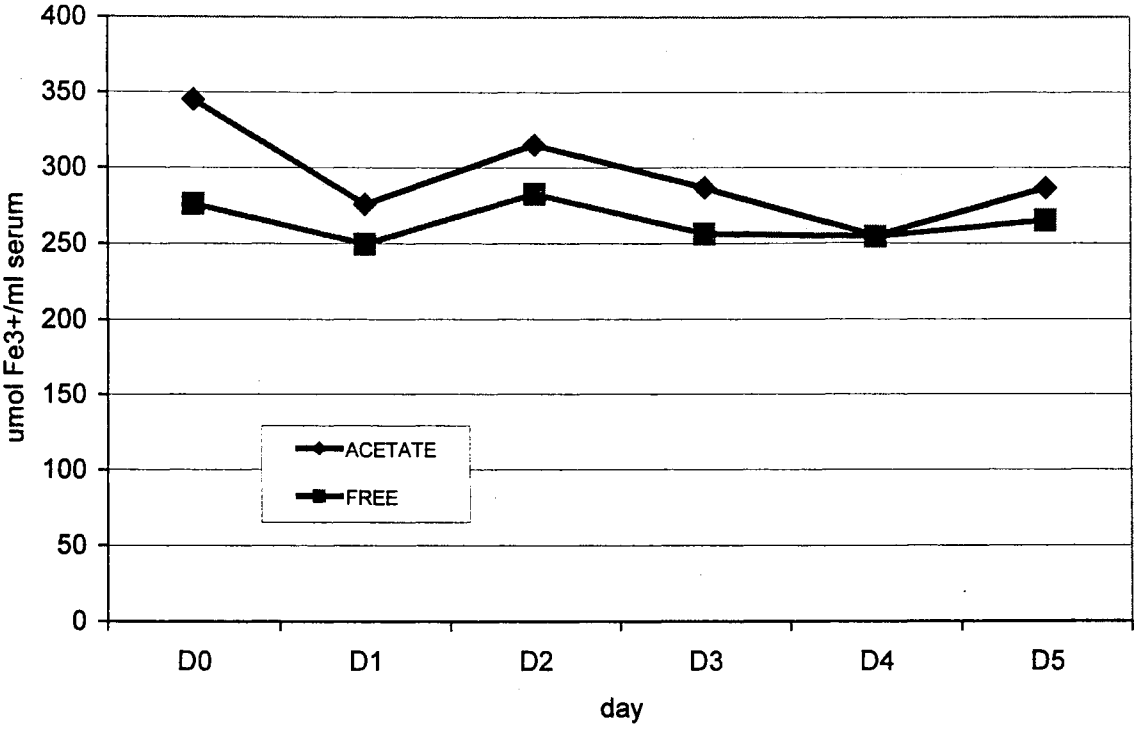


Figure 15. Serum FRA level before (day 0) and during ruminal dosing with tocopherol or tocopheryl acetate.



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