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Effects of fat saturation and vitamin E on fatty acid and vitamin E status in platelets and prostaglandins and cholesterol concentrations in serum

Haw-Wen Chen
Iowa State University

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Chen, Haw-Wen, Ph.D.

Iowa State University, 1992

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

**Effects of fat saturation and vitamin E on fatty acid and vitamin E status in
platelets and prostaglandins and cholesterol concentrations in serum**

by

Haw-Wen Chen

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Food Science and Human Nutrition
Major: Nutrition**

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**Iowa State University
Ames, Iowa**

1992

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GENERAL INTRODUCTION

Vitamin E Supplementation and Plasma Cholesterol

Elevated plasma cholesterol is well established as a factor in the development of atherosclerosis. Clinical trials have shown an association between reduced total plasma cholesterol and reduced coronary heart disease (CHD) in humans (1,2) and a 1% reduction in plasma cholesterol was associated with an approximate 2% reduction in CHD.

Brown and Goldstein (3) and Mahley and Innerarity (4) have shown that cholesterol homeostasis is closely related to plasma lipoprotein metabolism. Plasma high-density lipoprotein cholesterol (HDL-C) has an inverse relation with the risk of CHD (5,6). There is persuasive evidence that this protective association is causal because the role of HDL is to transport cholesterol from peripheral tissues to the liver where it is catabolized and excreted. In contrast, the role of low-density lipoprotein (LDL) is to transport cholesterol from liver to peripheral tissues and regulate de novo cholesterol synthesis at these sites. Therefore, there is great interest in factors that alter these lipoprotein concentrations.

Studies of the relationships between vitamin E and cholesterol distribution and concentration in plasma lipoproteins have given equivocal data. Sundaram et al. (7), Behrens et al. (8) and Takahashi et al. (9) have proposed that vitamin E is transported by the blood lipoproteins. Vitamin E has been reported by several laboratories to affect cholesterol distribution in lipoproteins. For 21 days, Sundaram et al. (7) studied 26 patients with clinically confirmed mammary dysplasia and five age-matched controls. The volunteers took 600 mg d,l- α -tocopherol per day, and serum samples were

collected before and after the treatment. As a result of therapy, HDL-cholesterol increased and LDL-cholesterol decreased. Chupukcharoen et al. (10) determined the effect of vitamin E deficiency on lipoprotein cholesterol distribution. An increase in plasma cholesterol was detected in LDL and very low density lipoprotein (VLDL), but not in the HDL fraction of plasma lipoproteins. Phonpanichrasamee et al. (11) reported that an oral supplementation of 600 mg d,l- α -tocopherol acetate every other day exerted a hypocholesterolemic effect on LDL-cholesterol and VLDL-cholesterol in rabbits fed an atherosclerosis-promoting diet containing 0.25% or 0.5% cholesterol. W'ojciaki et al. (12) found that selenium and/or vitamin E remediation of deficiency had an elevating effect on HDL-cholesterol in rabbits.

However, in another study vitamin E had little effect on plasma lipoproteins. Hatam and Kayden (13) studied 11 subjects, 6 subjects with normal lipid values, 4 subjects with hypercholesterolemia and 1 subject with hypertriglyceridemia for six weeks. Subjects were supplemented with 800 mg of vitamin E daily. Fasting blood samples were analyzed before vitamin E supplementation and after approximately three and six weeks of daily supplementation. Although the serum cholesterol concentration did decrease slightly in some subjects with vitamin E supplementation, the percentage of cholesterol in the HDL fraction was not significantly altered. Thus it appeared from this study that α -tocopherol did not influence the distribution of cholesterol in the lipoproteins of normal or hyperlipoproteinemic patients. Howard et al. (14) determined the effect of vitamin E supplementation on HDL-cholesterol level. Thirty-nine adult volunteers were involved in this

study and 600 mg of oral d,1- α -tocopherol acetate were given daily for 30 days. No significant change in HDL-cholesterol was noted after 30 days of oral intake of vitamin E. Jacques et al. (15) showed that vitamin E supplementation did not affect HDL-cholesterol in the elderly who consumed greater than or equal to 10 times the recommended dietary allowance (RDA). Stampfer et al. (16) conducted a randomized, double-blind, placebo-controlled clinical trial of vitamin E administration (800 mg/day) for 16 weeks. Thirty volunteers aged 30-60 years were involved in this study. Fasting HDL-cholesterol was measured at 8 and 16 weeks, and vitamin E had no effect on HDL-cholesterol. Also, in other human studies conducted by Kalbfleisch et al. (17) and Kesaniemi and co-workers (18), vitamin E did not show any effect on HDL-cholesterol concentrations.

Hepatic cholesterol 7 α -hydroxylase is the rate-limiting enzyme in the degradation of cholesterol through bile acid synthesis (Fig. 1) (19). Chupukcharoen et al. (10) studied hepatic cholesterol 7 α -hydroxylase activity in control and vitamin E-deficient rabbits. The activity of cholesterol 7 α -hydroxylase in vitamin E-deficient rabbits was approximately one-fifth that of control rabbits. Also, Phonpanichrasamee et al. (11) reported that rabbits with oral supplementation of 600 mg d,1- α -tocopherol acetate every other day for 8 weeks showed a 4-5 fold increase in hepatic cholesterol 7 α -hydroxylase activity compared with the control rabbits. Because greater plasma cholesterol results in greater incidence of atherosclerosis, it is important to determine the role of vitamin E in cholesterol metabolism.

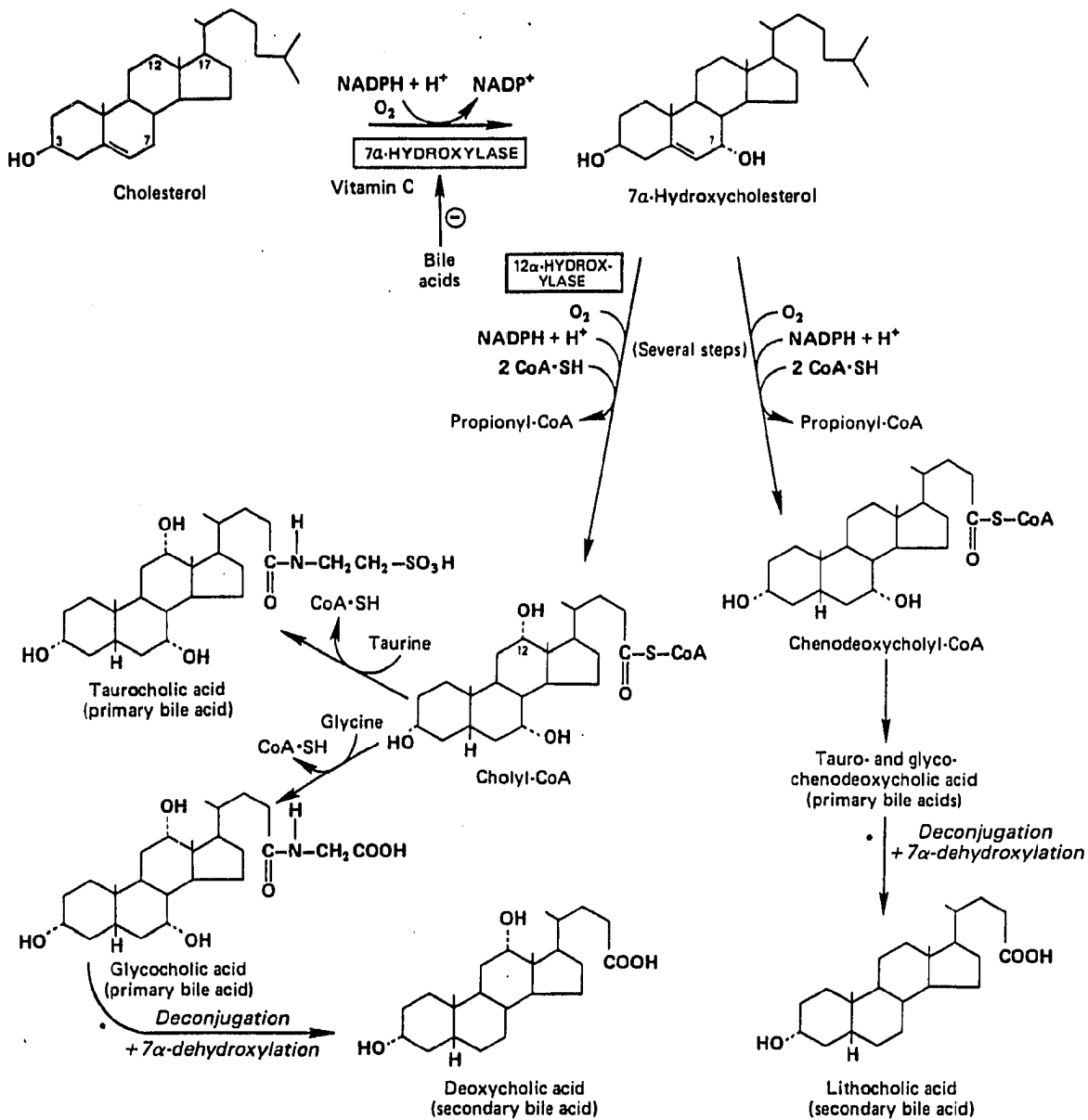


Figure 1. Biosynthesis and degradation of bile acids. *Catalyzed by microbial enzymes (19)

Vitamin E and Lipid Peroxidation

Dietary antioxidants have attracted increasing attention in the past few years because of their suspected role in health and disease. Numerous trials are currently in progress to demonstrate the potential effects of antioxidant supplementation in humans with a variety of diseases, such as cancer (20). Consumption of marine oils has been linked to a low incidence of coronary heart disease (21,22). However, fish oils are rich in unsaturated n-3 fatty acids that are very susceptible to lipid peroxidation. A high dietary intake of n-3 fatty acids might overwhelm the normal antioxidant defenses, thus potentially increasing the requirement for dietary antioxidants.

The basic process of lipid peroxidation can be described in several stages (Fig. 2). Vitamin E (α -TH) is believed to inhibit lipid peroxidation via donation of a hydrogen atom to a lipid peroxy radical ($\text{ROO}\cdot$) forming a lipid hydroperoxide (ROOH) and reversibly oxidized vitamin E (α -T \cdot) (23). Oxidized vitamin E is reduced to vitamin E by glutathione (GSH) as suggested by Reddy et al. (24). Reddy and coworkers (24) also provided preliminary evidence for the involvement of a membrane protein in this mechanism. The membrane protein was shown to be specific for GSH and relatively unstable. It will be referred to as GSH-dependent vitamin E reductase.

Vitamin E has a consistently inhibitory effect on lipid peroxidation regardless of the lipid peroxidation products measured. For example, the production of pentane (25), ethane (26), thiobarbituric acid-reactive substances (TBARS), conjugated dienes (CD), headspace hexanal and total volatiles (TOV) (27) in rats is reduced by vitamin E supplementation within

the ranges of 35 to 200 mg vitamin E/kg diet.

Macrophage-derived foam cells are an important indicator of the early atherosclerotic lesion (28,29). Oxidatively modified LDL (30) can cause foam cell formation via uptake through the macrophage acetyl-LDL receptor. Sparrow et al. (31) speculated that the introduction of lipid hydroperoxides into an LDL particle can initiate biological modification of the lipoprotein. The protective role of vitamin E in atherosclerosis could be via its inhibition of lipid peroxidation therefore permitting less oxidatively modified LDL and foam cell formation.

1. Initiation: $RH \rightarrow R\cdot$
2. Oxidation: $R\cdot + O_2 \rightarrow ROO\cdot$
3. Propagation: $ROO\cdot + RH \rightarrow R\cdot + ROOH$
4. $ROO\cdot + \alpha\text{-TH} \rightarrow ROOH + \alpha\text{-T}\cdot$
5. $\alpha\text{-T}\cdot + GSH \rightarrow \alpha\text{-TH} + 1/2GSSG$

Figure 2. Stages of autoxidation and inhibition of autoxidation by vitamin E

Biochemistry of Prostanoids

"Prostanoids" are the collective name for unsaturated lipids derived from arachidonic acid (C₂₀:_{4n-6}) or similar polyunsaturated fatty acid precursors, via the cyclo-oxygenase metabolic pathway (Fig. 3) (32). Arachidonic acid is always derived from the 2-position of phospholipids in the plasma membrane as a result of phospholipase A₂ activity (33,34,35). Prostanoids have 20 carbon atoms and include a five-member ring. Their three precursor fatty acids contain at least three double bonds and give rise to

3 groups of eicosanoids characterized by the number of double bonds in the side chains. This group of compounds consists of prostaglandins and thromboxanes. Variations in the substituent groups attached to the rings give rise to different types in each series of prostaglandins and thromboxanes, labeled A, B, etc.

Thromboxanes are synthesized in platelets and cause vasoconstriction and platelet aggregation upon release. Prostacyclins (PGI_2) are produced by blood vessel walls and inhibit platelet aggregation (36). Thus, thromboxanes and prostacyclins are antagonistic. The balance between thromboxanes and prostacyclins is important, because vasoconstriction and platelet aggregation are prothrombogenic and can be important to acute atherosclerosis-related diseases such as myocardial infarction and stroke (37,38,39).

Measurement of prostacyclin in a system generating the compound *in vitro* is often done by immediate assay of its biologic activity, such as measurement of induced relaxation of vessels. Because its half-life is only about 2 min, this approach is generally impossible for measurement of the generation of prostacyclin *in vivo* and the immediate hydrolysis product, 6-keto- $\text{PGF}_{1\alpha}$, has been used for this purpose (40). To assay thromboxane production *in vivo*, the most common approach is to measure the stable hydrolysis product, TXB_2 , in plasma (40).

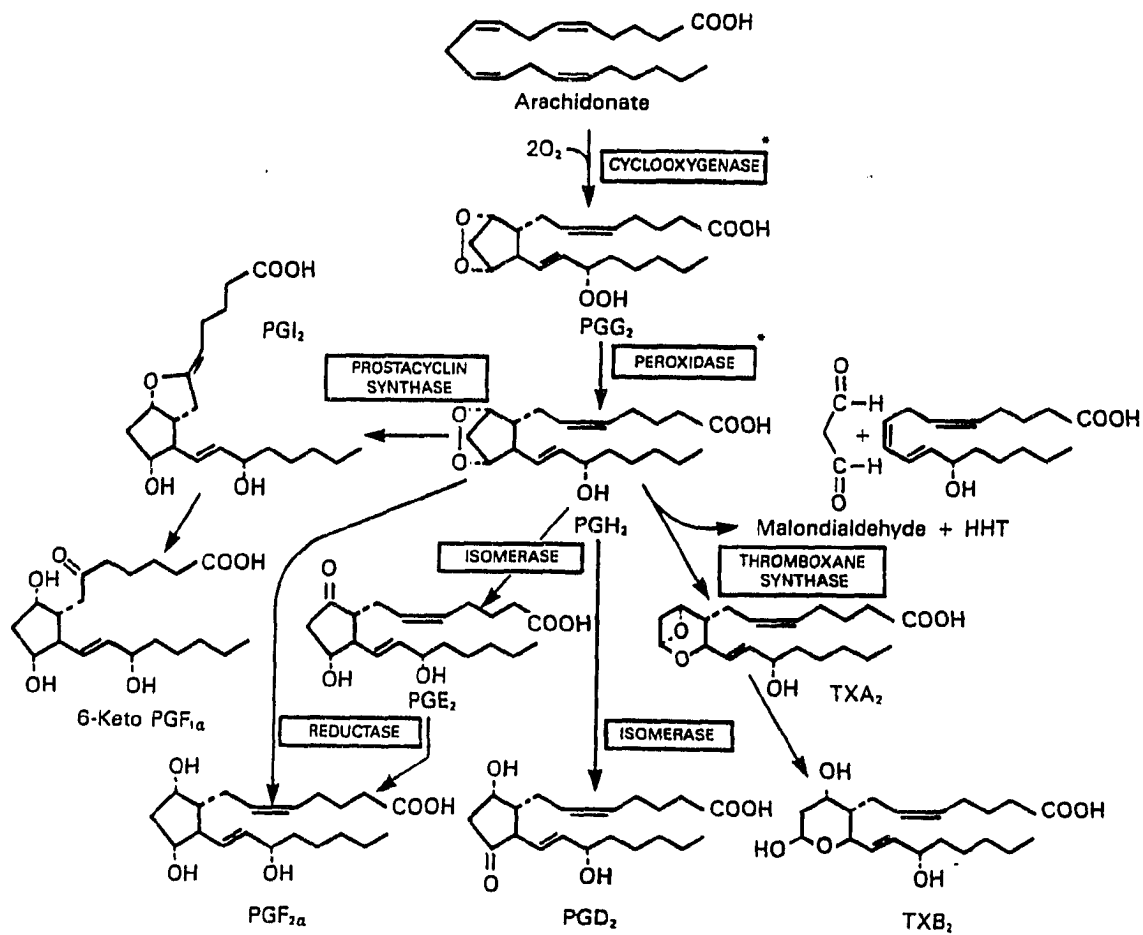


Figure 3. Synthesis of Prostaglandins and Thromboxanes (32)

Vitamin E and Prostanoid Synthesis

In addition to its role as a lipid antioxidant, vitamin E also plays a significant role in maintenance of normal function of the blood and vascular system (41,42,43). Vitamin E exerts its effect on prostacyclin and thromboxane production via its influence on phospholipase and the oxygenases acting on arachidonic acid. The basic process of vitamin E's effect on prostanoid synthesis as proposed by Panganamala and Cornwell (41) can be described in several stages (Fig. 4). Panganamala and Cornwell found that vitamin E can inhibit phospholipase A₂ activity and thereby cause the release of less arachidonic acid. On the other hand, vitamin E enhances cyclooxygenase activity. Furthermore, vitamin E can enhance prostacyclin synthetase by blocking the inhibitory effect of hydroperoxy fatty acids on prostacyclin synthetase.

The role of vitamin E in prostanoid synthesis is controversial. Prostacyclin generation is inhibited by hydroperoxy arachidonic acid (44), raising the possibility that vitamin E might enhance prostacyclin production by blocking the inhibitory effects of lipid peroxides on prostacyclin synthetase (41). In rat testes (45), vitamin E deficiency diminished 6-keto-PGF_{1α} production. However, in human studies, Stampfer et al. (46) have shown no effect of vitamin E on 6-keto-PGF_{1α} production, while, Swartz et al. (47) have reported decreased plasma PGI₂ in healthy adult subjects supplemented with vitamin E.

Studies on the effect of dietary vitamin E on thromboxane production have not resulted in a consistent picture. For example, Karpen et al. (48) found that platelets from vitamin E-deficient rats produced more thromboxane

A₂ than platelets from vitamin E-supplemented rats when the platelets were challenged with collagen. Stampfer et al. found no effect of vitamin E supplementation on thromboxane A₂ production (46). Hwang and Donovan found increased concentrations of thromboxane B₂ in collagen-stimulated whole blood but not platelet-rich plasma of vitamin E-deficient rats (49). Contradictory results obtained by different laboratories could be due to different vitamin E dosage used in the ranges of 0 to 1000 mg/kg diet, differences in feeding period from 8 to 23 weeks, species variation, for example rats, rabbits and humans and techniques of tissues sampling and preparation.

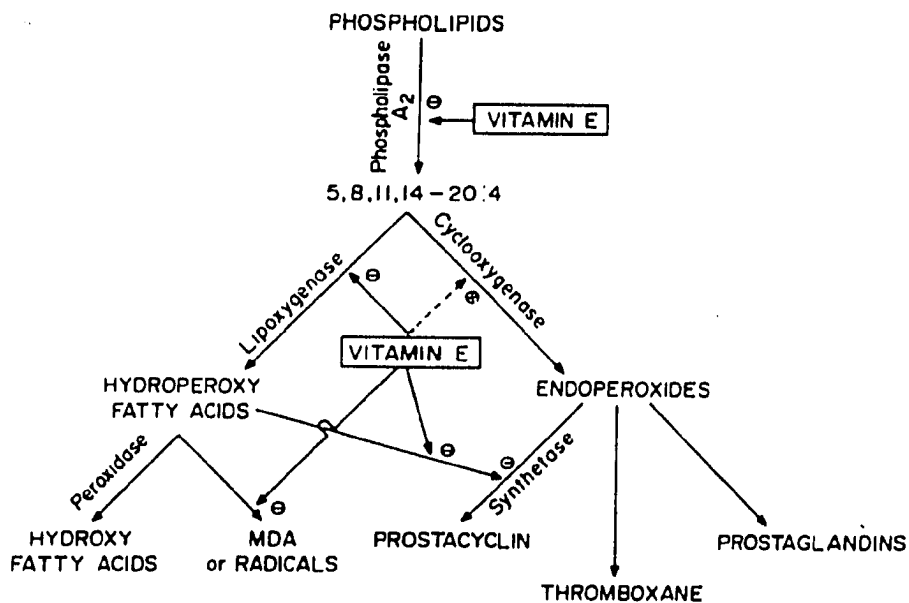


Figure 4. A model for the effects of vitamin E on the biosynthesis of prostanoids from arachidonic acid (41)

Dietary Fat and Platelet Function

It is well established that dietary lipids induce extensive modifications in the fatty acid composition of platelets (50,51,52). Such modifications can cause a variety of functional changes, influencing, for example, membrane fluidity (53), platelet aggregability (54) and the biosynthesis of eicosanoids (55,56,57). Piche and Mahadevappa (50) demonstrated that platelets of rats fed animal fat (lard or cod liver oil) contained significantly lower levels of linoleic acid, 18:2 (n-6), than did platelets from rats fed fats of vegetable origin (corn oil, soybean oil or canola oil) for 7 weeks. When rats were fed diets composed of corn oil, olive oil, hydrogenated coconut oil and hydrogenated palm oil which supplied 1.3 (group 1) or 13.2 (group 2) en% from polyunsaturated fatty acids (18:2 +18:3), differences in dietary fat changed the fatty acid composition (%) of the platelet lipids (51). Diets high in saturated fatty acids markedly increased rat platelet sensitivity to thrombin (51). Rats fed the saturated fats (group 1) as compared with those fed the polyunsaturated fats (group 2), had a significantly greater, relative content of 18:1n-9, 20:3n-9, 20:3n-6 and 22:3n-6 at the expense mostly of 18:2n-6, 20:2n-6 and 22:4n-6. Platelet phospholipid fatty acid profile can be manipulated by dietary fat (52). When rats were fed a fat-free diet, endogenous n-9 eicosatrienoic acid was the major polyunsaturated fatty acid in liver, plasma and platelets while a high 20:3 (n-9)/20:4 (n-6) ratio was found, a sign of essential fatty acid deficiency (58). After fat (corn oil or fish oil) was substituted for 5% sucrose in the diet, the platelet phospholipid fatty acids, 20:4 (n-6), 20:5 (n-3) and 22:6 (n-3), increased and reached a peak at day 12.

Platelet membrane fluidity also can be affected by dietary lipid (53). Membrane fluidity has been defined by Van Blitterswijk et al. (59) as the reciprocal of the lipid structural order parameter (r_S). Lower values of r_S measured by fluorescence polarization, indicate decreased structural order parameters and increased membrane fluidity. The administration of a diet rich in sunflower seed oil, 50 en% vs 5 en%, significantly enhanced the overall fluidity of platelet membranes, measured with the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (53).

It also has been found that platelet response to aggregators was affected by dietary fat. In a rat model, using a loop-shaped polyethylene cannula in the abdominal aorta to study arterial thrombosis, it appeared that sunflower seed oil, and its linoleic acid fraction in particular, lowered the tendency toward arterial thrombosis in a dose-dependent manner (60). In a further series of experiments using rats (61), the anti-thrombotic effect of linoleic acid was confirmed. Moreover, long-chain saturated fatty acids containing fourteen or more carbons increased arterial thrombosis in a dose-dependent manner. In rabbits fed corn oil, the sensitivity toward collagen and arachidonic acid-induced platelet aggregation was lowered when compared to aggregation in butter-fed animals (62). In a human study, platelet aggregation in response to thrombin and adenosine diphosphate was increased in a group of French farmers eating a high saturated fat diet, compared with a second group eating a diet containing less saturated fat (63). However, Winocour et al. (64) could not find an effect of dietary fat on platelet function in rats. Diets containing 1.5% safflower or corn oil or 16% milk fat were used in this study. Dietary fat did affect platelet phospholipid fatty acid composition. But no effect on in

vitro platelet function or in vivo platelet survival in response to indwelling aortic catheters was observed.

Dietary Fat and Eicosanoid Synthesis

In 1968, Lands and Samuelsson (65) proposed that eicosanoid synthesis required the free, nonesterified form of arachidonate, because the oxygenase will not act on substrate bound to phospholipid. Neufeld et al. (66) and Wilson et al. (67) demonstrated that the resting platelet contained no free arachidonate, perhaps related to the activity of an arachidonoyl coenzyme A synthetase which mediates the transfer of free arachidonate into phospholipid. Platelets contain little or no free substrate and eicosanoid synthesis requires the release of free substrates from phospholipids. Arachidonic acid is formed in the liver and released into the plasma. Many tissues lack desaturase enzymes and cannot convert dietary linoleic acid into arachidonic acid. Both platelets and endothelial cells lack desaturase enzymes, and so take up arachidonic acid from plasma (68,69). Because arachidonic acid is the precursor of many eicosanoids, and since platelets actively incorporate arachidonic acid into their phospholipid, the platelet is a good model in which to study the effects of dietary fat on eicosanoid synthesis.

It has been estimated that the American diet (70) contains less than 0.2 en% arachidonic acid and approximately 6 en% linoleate; thus one can conclude that most tissue arachidonic acid is derived from dietary linoleate. Because arachidonic acid is the direct precursor of many eicosanoids, dietary linoleate plays an important role in eicosanoid synthesis. Dupont et al. (55) studied the effect of dietary linoleate on thromboxane B₂ (TXB₂) synthesis in

incubated blood from female Sprague-Dawley rats. Zero to 27 en% linoleate and 40% of energy (en%) from fat was fed to rats for 6 months. The various en% from linoleate were obtained by the combination of different ratios of beef tallow, soybean oil and safflower oil. TXB₂ synthesis increased as linoleate increased from 0 to 2% of kcal, however, between 2 and 8 en%, reduced eicosanoid synthesis was observed. Between 10 and 27 en% linoleate, TXB₂ synthesis returned to the level at 2 en% linoleate. The conclusion reached by Dupont et al. (55) was that prostaglandin synthesis appeared to be abnormally regulated with less than 8 en% linoleate in a 40% fat calorie diet. Sullivan and Mathias (56) determined the effect of dietary fat on platelet PG-producing activity. Beef tallow, corn oil and safflower oil were used to supply 40% fat calories and 0, 0.4, 14, 22 and 29 en% linoleate. Diets enriched with larger amounts of linoleate did not produce further increases in platelet PG synthesis but remained unchanged after reaching peak production at 0.4 en% linoleate. On the other hand, serum TXB₂ levels declined to levels found during essential fatty acid deficiency when linoleate was increased to 14 en% and stayed depressed as linoleate increased to 29 en%. Apparently, excess dietary linoleate did not increase eicosanoid synthesis, probably because eicosanoid precursors did not increase in the platelet. Although linoleate is the precursor of arachidonic acid, it is also a potent inhibitor of TXA₂ synthesis in the platelet. The relationship between dietary linoleate and TX synthesis in cardiac tissue found by Mathias and Dupont (57) was similar to the results of Sullivan and Mathias (56). Based on the study of Mathias and Dupont (57), the relationship between dietary linoleate and 6-keto-PGF_{1α} synthesis appears to be tissue-specific, because

the same results were not produced in different tissues. Perhaps the rate of incorporation of linoleate into phospholipid differs among various tissues, or the inhibitory effect of linoleate on PG synthetases is different among various tissues.

Steel et al. (71) conducted a short-term feeding study using 10, 30 and 50 en% fat from beef tallow, olive oil, peanut oil and butter. Butter- and beef tallow-feeding reduced aortic PGI₂ production and collagen-induced TXA₂ production in a dose-dependent manner as the level of fat in the diet increased. Decreases in aortic PGI₂ and collagen-induced TXA₂ production were paralleled by similar decreases in aortic and plasma phospholipid arachidonic acid content. As a result of dietary treatments, only the most saturated fats, butter and beef tallow, had significant inhibitory effects on eicosanoid synthesis. Similar effects of saturated fat on eicosanoid synthesis were observed by O'Dea et al. (72) who fed rats with 10%, 30% or 50% energy as fat predominantly from butter or lard. Prostacyclin production in the abdominal aorta and the mesenteric artery fell in a concentration-dependent manner in butter-supplemented rats. However, lard did not affect PG production. These results indicated that butter had a greater inhibitory effect on eicosanoid synthesis than did lard, perhaps reflecting a difference in their P/S ratios.

Steinberg et al. (73) determined the effect of dietary lipids on serum and urine prostaglandin concentrations. The diets provided 40 en% as fat, and 0.4, 14, 21, 22 and 29 en% linoleate. The results showed that the 6-keto-PGF_{1α} and TXB₂ levels in serum and urine did not differ significantly with dietary treatment. Also, Fine et al. (74) demonstrated that dietary linoleate level did

not significantly affect serum PG levels. The results were not consistent with those of Dupont et al. (55), Sullivan and Mathias (56) and Mathias and Dupont (57).

Although the effect of dietary fat on eicosanoid synthesis has not been consistent, this observation can be attributed to a variety of factors. The amount of dietary fat and duration of the experiments were not identical. Additionally, the mechanisms of conversion of linoleate to arachidonate, arachidonic acid deposition in tissues, long chain polyunsaturated fatty acid turnover and release of arachidonic acid may differ due to diets (75,76,77). Other factors such as competition between cyclooxygenase and lipoxygenase and kinetic properties of eicosanoid synthetases may also be affected by dietary factors. In addition, other nutrients such as vitamin E may also play a role in PG synthesis.

Dietary Fat and Phospholipase A₂ Activity

Lagarde et al. demonstrated that platelet phospholipase A₂ was located in the inner membranes of the platelets and the inner membranes had a higher content of phosphatidylcholine (PC) and phosphatidylinositol (PI) (78,79). Bills et al. (80) reported that radioactive arachidonate was preferentially incorporated into platelet PI and PC in platelet-rich plasma through arachidonoyl coenzyme A synthetase. Bills et al. (81) also showed that in platelets upon stimulation by thrombin, labeled arachidonate was lost from platelet PI and PC. So the fatty acid composition of PI and PC can be a determinant of arachidonic acid metabolism. Ballou and Cheung (82) described an inhibitor of phospholipase A₂ activity in platelet lysates. The

inhibitor appeared to be a lipid, because it was heat-stable, insensitive to trypsin and extractable in chloroform-methanol. Later, the inhibitor was shown to be a mixture of unsaturated fatty acids, palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) and arachidonic acid (20:4) (83). Momchilova et al. (84) conducted a study to determine the effects of a highly unsaturated diet (HUD) (15% w/w sunflower oil) and a highly saturated diet (HSD) (15% w/w hydrogenated sunflower oil) on phospholipase A₂ activity. The substrate used for phospholipase A₂ activity of liver plasma membranes of rats was 1-acyl-2-[¹⁴C]linoleoyl-sn-glycerophosphocholine. Hepatic phospholipase A₂ activity was significantly higher in rats fed HUD than in rats fed HSD. However, the degree of saturation was not shown and the presence of trans fatty acids was not determined in diets, so it is possible that more than one dietary factor caused the results. There were significant differences in the fatty acid composition of plasma membrane PC of HUD or HSD fed rats. Palmitoleic acid (16:1) and oleic acid (18:1) were significantly greater in plasma membrane PC of HSD fed rats, but linoleic acid (18:2) and arachidonic acid (20:4) were significantly greater in plasma membrane PC of HUD fed rats. Dietary fat could affect phospholipase A₂ activity via its effect on fatty acid composition of membranes where phospholipase A₂ resides. Fatty acid effects on membrane-bound proteins could be due to three mechanisms (1) direct effects on protein structure and/or mobility; (2) modification of protein function through posttranscriptional, covalent binding of fatty acids, or complex lipids; and (3) the potential of the fatty acids (e.g., arachidonic acid) to be substrates for synthesis of bioactive metabolites (85).

Dietary Fat and Cholesterol

It is well established that polyunsaturated fatty acids are hypocholesterolemic in humans and animals. Ibrahim and McNamara (86) conducted a study in guinea pigs to investigate the effects of saturated fat (lard) and unsaturated fat (corn oil) on plasma cholesterol level when diets supplied 20 en% as fat. In comparison to the lard diet, the corn oil diet produced a 34% reduction in plasma total cholesterol level ($p < 0.02$) and a 40% lower triacylglycerol level ($p < 0.02$). Also, feeding the corn oil diet decreased the percent cholesterol ester in both LDL and VLDL compared to feeding the lard diet. The results of this study were consistent with that of Shepherd et al. (87) who determined the effects of saturated and polyunsaturated fats on the chemical composition of lipoproteins. The fats supplied 40% energy. The polyunsaturated fat lowered both plasma cholesterol (23%, $p < 0.001$) and triglyceride (14%, $p < 0.001$) levels. The reduction in plasma cholesterol primarily resulted from a fall in LDL cholesterol (23%, $p < 0.001$), although decreases also were detected in VLDL and HDL cholesterol.

We hypothesized that increasing dietary linoleate content could increase TXA₂ and PGI₂ synthesis, while dietary vitamin E supplementation could inhibit TXA₂ synthesis and stimulate PGI₂ synthesis, and that increasing dietary linoleate could lower serum cholesterol and triglyceride levels and dietary vitamin E supplementation could increase serum HDL-cholesterol. TXA₂ and PGI₂ are important to platelet and vascular functions which are related to the development of heart disease. Cholesterol and triglyceride have important roles in the pathogenesis of heart diseases. Heart disease is

responsible for the majority of deaths in the United States and most industrialized countries. It is important to study dietary factors such as fat and vitamin E to determine the extent to which these nutrients can affect the risk of heart disease.

Explanation of Dissertation Format

This dissertation is composed of two papers, each of which has its own abstract, introduction, materials and methods, results, discussion, and literature cited. The first paper reports the influences of increasing dietary linoleate and vitamin E supplementation on platelet fatty acid composition, platelet vitamin E, lipid peroxidation, phospholipase A₂ activity and TXA₂/PGI₂ status. The second paper is a report of the influences of increasing dietary linoleate and vitamin E supplementation on serum vitamin E and lipid concentrations. These papers will be submitted to scientific journals for publication, with modifications to meet the journal's format requirements. Following these papers are the general summary of this dissertation and following the general summary is the literature cited in the general introduction and general summary. For both papers, the first author will be the Ph.D. candidate, Haw-Wen Chen.

**PAPER I. INFLUENCE OF INCREASING DIETARY LINOLEATE
AND VITAMIN E SUPPLEMENTATION ON
THROMBOXANE AND PROSTACYCLIN STATUS IN
MALE SPRAGUE-DAWLEY RATS**

ABSTRACT

The present study was conducted to determine whether increasing dietary linoleate and vitamin E supplementation interact to maintain a relatively antithrombogenic ratio of thromboxane : prostacyclin. The influence of increasing dietary linoleate and d,1- α -tocopheryl acetate (vitamin E) on platelet fatty acid composition, phospholipase A₂ activity, lipid peroxidation, serum thromboxane B₂ (TXB₂), 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) and aortic 6-keto-PGF_{1 α} concentrations was studied in 72 weanling male Sprague-Dawley rats. Rats were fed semipurified diets containing 11 or 18% of energy from linoleate (en% linoleate) and graded levels (0, 100 or 5000 ppm) of vitamin E for 10 weeks. Vitamin E status was confirmed by platelet vitamin E level. Rats fed no vitamin E had the lowest platelet vitamin E and rats fed 5000 ppm vitamin E had the greatest platelet vitamin E ($p < 0.05$). Lipid status was verified by platelet fatty acid composition. Platelet C18:1n-9 (oleic acid) was significantly greater in the 11 en% linoleate group than in the 18 en% linoleate group, and platelet C18:2n-6 (linoleic acid) was significantly greater in the 18 en% linoleate group than in the 11 en% linoleate group ($p < 0.05$). Increasing dietary linoleate from 11 to 18 en% had no adverse effect on thrombogenic potential as reflected in serum TXB₂/6-keto-PGF_{1 α} ratio. Serum TXB₂ level and platelet lipid peroxidation (TBARS) were significantly greater in the vitamin E-deficient group than in other groups ($p < 0.05$). Vitamin E supplementation had no protective effect on thrombosis based on serum TXB₂/6-keto-PGF_{1 α} ratio. There was no interaction between increasing dietary linoleate and vitamin E supplementation on thrombogenic potential, as reflected by eicosanoid status,

however, vitamin E deficiency may be an important stimulator of thrombogenesis. The treatments had no effect on food intake, body weight gain, platelet phospholipase A₂ activity, serum 6-keto-PGF_{1α} and aortic 6-keto-PGF_{1α} concentrations.

INTRODUCTION

In addition to its role as a lipid antioxidant, vitamin E also plays a significant role in maintaining the normal function of the blood and vascular system (1,2,3). Vitamin E affects prostacyclin and thromboxane synthesis via its influence on phospholipase and the prostaglandin synthetase acting on arachidonic acid. Thromboxanes are synthesized in platelets and cause vasoconstriction and platelet aggregation upon release. Prostacyclins (PGI₂) are produced by blood vessel walls and inhibit platelet aggregation. These prostaglandins could have an important role in the pathogenesis of cardiovascular disease (4,5,6) via their effect on the platelet and vascular system.

The role of vitamin E in prostaglandin synthesis is controversial. Prostacyclin generation is inhibited by hydroperoxy arachidonic acid (7). Vitamin E might enhance prostacyclin production by blocking the inhibitory effect of lipid peroxides on prostacyclin synthetase (1). In rat testes (8), vitamin E deficiency caused decreased 6-keto-PGF_{1α} production. However, in human studies, Stampfer et al. (9) showed no effect of vitamin E on 6-keto-PGF_{1α} production, whereas Swartz et al. (10) reported decreased plasma PGI₂ levels in healthy adult subjects supplemented with vitamin E. Although the effect of vitamin E on thromboxane production has been studied by many laboratories, the effect has not been consistent. Karpen et al. (11) found that platelets from vitamin E-deficient rats, when challenged with collagen, produced more thromboxane A₂ than platelets from vitamin E-supplemented rats. Stampfer (9) found no effect of vitamin E on thromboxane A₂ production. Hwang and Donovan (12) found increased concentrations of

thromboxane B₂ in collagen-stimulated whole blood but not in platelet-rich plasma of vitamin E-deficient rats.

It has been estimated that the American diet (13) contains less than 0.2 en% arachidonic acid and approximately 6 en% linoleate suggesting that most of the tissue arachidonic acid is derived from dietary linoleate. Because arachidonic acid is the direct precursor of many prostaglandins, dietary linoleate must play an important role in prostaglandin synthesis. The role of linoleate in prostaglandin synthesis is complicated. It is not only the precursor for prostaglandins but it decreases arachidonic acid in the phospholipid fraction (14,15) and inhibits phospholipase A₂ activity (16). The effects of dietary linoleate on prostaglandin synthesis have been studied by many laboratories (17,18) but results have been not consistent. Dupont et al. (17) fed rats with 0 to 27 en% linoleate and 40 en% fat. TXB₂ synthesis increased as linoleate increased from 0 to 2 en% linoleate, however, between 2 and 8 en% linoleate, reduced eicosanoid was observed. Between 10 and 27 en% linoleate, TXB₂ synthesis returned to the level at 2 en% linoleate. Sullivan and Mathias (18) used 0, 0.4, 14, 22 and 29 en% linoleate and 40 en% fat. Platelet PG synthesis reached peak production with 0.4 en% linoleate. Serum TXB₂ levels declined to levels found during essential fatty acid deficiency when linoleate was increased to 14 en% and stayed depressed as linoleate increased to 29 en%.

We hypothesized that increasing dietary linoleate could increase TXA₂ and PGI₂ synthesis because of the precursor role of linoleate for arachidonate, and vitamin E supplementation could inhibit TXA₂ synthesis and stimulate PGI₂ synthesis. This present study reports the involvement and interactions of

increasing dietary linoleate and vitamin E supplementation in thromboxane and prostacyclin status, and therefore, in thrombogenic potential.

MATERIALS AND METHODS

Animals and diets

Seventy-two weanling male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 50-60 g were individually housed in stainless cages on a 12-h light-dark cycle. The room was maintained at 22°C and approximately 50% humidity. Rats were randomly assigned to experimental diets in a randomized block experimental design, and the blocks represented the days when the rats were sacrificed and samples were processed.

The experimental diets were nutritionally complete and supplied 30% of energy (en%) as fat (Table 1). Diets supplied 0, 100 or 5000 mg d,l- α -tocopheryl acetate/kg of diet. Dietary fat was composed of different proportions of tocopherol-stripped corn oil and tocopherol-stripped lard to provide 11 or 18 en% linoleate, with cholesterol added as required to provide equal amount of cholesterol in all diets. Fatty acid composition of fat sources was analyzed by gas chromatography (20) (Table 2). Six groups of rats were fed ad libitum with 12 rats/diet for 10 weeks. Tap water was available ad libitum. Food consumption and body weight were measured weekly. After 10 weeks of feeding, rats were fasted overnight and anesthetized with ether, blood was drawn from exposed jugular veins, and rats were killed by ether overdose. Eight milliliters of blood was drawn from exposed jugular veins into a syringe containing 1 ml acid citrate dextrose anticoagulant (4.5 g% sodium citrate, 2.7 g% citric acid (monohydrate) and 3.6 g% dextrose) for platelet preparation. Two milliliters of blood was drawn from exposed jugular veins for serum preparation.

Preparation of platelets

Platelets were isolated from the uncoagulated blood by centrifugation using a modification of the procedure of Chan et al. (21). Blood samples from two animals in the same dietary group were pooled to obtain enough blood from which to prepare platelets. Blood was centrifuged at 100x g for 15 min at room temperature, and the supernatant platelet rich plasma (PRP) was recentrifuged at 150x g for 15 min to remove contaminating red and white blood cells. Finally, the PRP was centrifuged at 1000x g for 15 min to sediment the platelets. The platelet poor plasma (PPP) was removed, 1 ml of Dulbecco's phosphate buffered saline (PBS), 2.7 mM KCl, 1.5 mM KH₂PO₄, 138 mM NaCl, 8.1 mM Na₂HPO₄, pH 7.5, (Sigma Chemical Co., St. Louis, MO) was added, and the platelets were resuspended by gentle vortexing. The platelets were resedimented by centrifugation at 1000x g for 15 min. The Dulbecco's PBS was discarded and 3 ml of fresh Dulbecco's PBS was added and, again, the platelets were resuspended by gentle vortexing. The purity of platelet suspensions was determined by Coulter Counter (Counter Electronics, Inc., Hialeah, FL). The purity was at least 95%. The platelet suspensions were stored at -80°C.

Aortic 6-keto-PGF_{1α} production

Segments of aorta were removed carefully, cleaned of connective tissue and incubated in Dulbecco's PBS (pH 7.5) at 37°C for 30 min in a total volume of 1 ml. At the end of the incubation, aorta was removed, blotted on filter paper and weighed to obtain the "wet weight". The incubation media were stored at -80°C for radioimmunoassay (RIA) which was done later.

Platelet fatty acid analyses

Fatty acid analyses were performed by methods described by Krumhardt (20) and Morrison and Smith (22). Analyses were done under nitrogen. Fatty acid methyl esters were quantified by gas chromatography on a Varian 330 gas chromatograph (Walnut Creek, CA) equipped with a 3390A Hewlett Packard Integrator (Avondale, PA). An Alltech (Deerfield, IL) 183x0.32 cm column loaded with a 10% 100/120 mesh Silan 10C stationary phase was used. Nitrogen carrier gas flow rate was 30 ml/min. Column temperature was 180°C, injection port temperature was 220°C and flame ionization detector temperature was 250°C. Peaks were identified by comparison of retention times with those of authentic fatty acid methyl ester standards (Alltech, Deerfield, IL). The percentage of each fatty acid was determined by integration of peak areas.

Vitamin E analysis

Platelet α -tocopherol content was determined using a modification of the procedure of Catignani and Bieri (23). All samples were processed in a room illuminated by yellow light. One hundred microliter internal standard (α -tocopheryl acetate in ethanol, 62.2 mg/l) and 500 μ l platelet suspension were mixed by vortexing for 1 min. HPLC grade hexane was added and mixed for another 1 min. Phases were separated by centrifugation at 2000 rpm for 10 min. The hexane layer was withdrawn and evaporated under nitrogen. The residue was redissolved in 100 μ l filtered HPLC grade methanol by mixing and 30 μ l was injected. Chromatography was performed at ambient temperature. Reversed-phase HPLC was carried out on a Waters 'Resolve' 5 μ Spherical C18 column. The detector wavelength was 290 nm,

sensitivity 0.01 absorbance units full scale. Flow rate of 100% HPLC grade methanol was 1.5 ml/min and, recorder chart speed, 1 cm/min. Peak-area ratios of samples were converted to ng's α -tocopherol by a standard curve prepared with samples containing a constant amount of α -tocopherol acetate combined with different amounts of α -tocopherol standard.

Thromboxane B₂ and 6-keto-PGF_{1 α} assays

Thromboxane B₂ and 6-keto-PGF_{1 α} levels were determined by double antibody radioimmunoassay as described by McCosh et al. (24) and Steinberg et al (25).

The assays were performed over a 3-day period. On the first day, diluted primary antibody and diluted ARGG (1:1) were mixed and refrigerated at 4°C overnight. Also, dilutions of NRS and ARGG (1:1) were mixed and refrigerated at 4°C overnight. The overnight incubation allowed binding of ARGG to the antibody. Rabbit antibodies to TXB₂ and 6-keto-PGF_{1 α} (primary antibodies) were gifts from Dr. Melvin M. Mathias at USDA/CSRS and Dr. Jacqueline Dupont at USDA/ARS, Washington, D. C. Primary antibodies were diluted to 1/400 in PBS-EDTA and further diluted to 1/1500 for TXB₂ and 1/3000 for 6-keto-PGF_{1 α} with 1/400 NRS to give 30% binding to ³H-labeled TXB₂ and 6-keto-PGF_{1 α} in the assay system and stored at -20°C. Anti-Rabbit Gamma Globulin (ARGG) (goat) (secondary antibody) was purchased from Western Chemical Research Corp., Fort Collins, CO; and was diluted to 1/60 with PBS-EDTA to give 30% binding to ³H-labeled TXB₂ and 6-keto-PGF_{1 α} in the assay system and stored at -20°C. PBS-EDTA was 1.8612 g % Na₂EDTA in PBS and adjusted to pH 7 with NaOH. Phosphate buffered saline (0.01 M) contained 0.82 g % NaCl, 0.0394 g %

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.185 g % $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.00952 g % ethyl mercurithiosalicylic acid (sodium salt) and was adjusted to pH 7 with HCl. Normal Rabbit Serum (NRS) was obtained from unimmunized rabbits and used to determine background binding of ^3H -labeled TXB_2 and 6-keto- $\text{PGF}_{1\alpha}$ in the assay system. NRS was diluted to 1/400 in PBS-EDTA for use.

On the second day, samples were diluted 1/50 with PBS-gel which contained 0.1 g % Knox gelatin in PBS, and pipetted into 4 ml polypropylene tubes. One milliliter reaction mixture containing 200 μl platelet suspension (diluted 1/50 in PBS-gel), 300 μl PBS-gel, 400 μl Ab:ARGG, and 100 μl ^3H - TXB_2 or ^3H -6-keto- $\text{PGF}_{1\alpha}$ were incubated overnight at 4°C . Labeled ^3H - TXB_2 (specific activity 110.6 Ci/mmol) and ^3H -6-keto- $\text{PGF}_{1\alpha}$ (specific activity 151 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA and stored at -80°C . Tubes for background binding contained 500 μl PBS-gel, 400 μl NRS:ARGG, and 100 μl ^3H - TXB_2 or ^3H -6-keto- $\text{PGF}_{1\alpha}$. Total binding tubes contained 500 μl PBS-gel, 400 μl Ab:ARGG, and 100 μl ^3H - TXB_2 or ^3H -6-keto- $\text{PGF}_{1\alpha}$. ^3H - TXB_2 and ^3H -6-keto- $\text{PGF}_{1\alpha}$ were diluted with PBS-gel to give approximately 10,000 counts per min (cpm) in a 100 μl aliquot. One hundred μl ^3H - TXB_2 or ^3H -6-keto- $\text{PGF}_{1\alpha}$ was added to total count tubes to determine total counts per min added to each tube. All samples were run in duplicate. All background and total count determinations were done in triplicate, and total binding determinations were done with six replicates. A standard curve was prepared and run along with the samples. Duplicates were run with one set of standards at the beginning of the sample run and one set at the end. Means of measurements were used to calculate the concentration of samples. Standard TXB_2 and 6-keto- $\text{PGF}_{1\alpha}$ were gifts from

Upjohn (Kalamazoo MI), stored at -20°C in ethanol ($2\ \mu\text{g}/\text{ml}$) and diluted to $20\ \text{ng}/\text{ml}$ in PBS-gel for use. Components used to determine the standard curve are shown in Table 3. All tubes were covered with parafilm, gently mixed in racks, and refrigerated overnight.

On the third day, 3 ml of PBS was added to all tubes except the total count tubes. All tubes except the total count tubes were centrifuged at 3000 rpm for 30 min (Beckman J-6B centrifuge, JR-3.2 rotor). The supernatant was poured off and the tubes were blotted on absorbent paper. To each tube, including the total count tubes, 0.3 ml water and 3.5 ml Scintiverse BD (Fisher) were added. Tubes were capped and vortexed until clear. Radioactivity as cpm was determined using a Beta Trac Liquid Scintillation Counter (Model 6895) (TM Analytic Inc., Elk Grove Village, IL), counting for either 10 min or until 10,000 counts were measured.

The assay was based on competition between ^3H -labeled prostaglandin and cold prostaglandin for binding sites on antibody. ARGG served to precipitate the antibody so that it was pelleted by the centrifugation step. When more standard prostaglandin or sample prostaglandin was present, less ^3H -labeled prostaglandin was bound, and vice versa. A computer program developed by Duddleson et al. (26) was used to determine the prostaglandin concentration by comparison with the standard curve.

Lipid peroxidation analysis

Platelet lipid peroxidation was measured by assaying thiobarbituric acid-reactive substances (TBARS) using a modification of the procedure described by Fraga et al. (27). Platelet suspension was incubated with 1 mM t-butyl hydroperoxide (t-BuOOH) for 30 min. To the incubation mixture, 0.5

ml of 3% sodium dodecyl sulfate was added. After mixing, 2 ml of 0.1 N HCl, 0.3 ml of 10% phosphotungstic acid, and 1 ml of 0.7% 2-thiobarbituric acid were added. The mixture was then heated for 30 min in boiling water, followed by TBARS extraction into 5 ml 1-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission. in a Fluorolog 2 spectrometer (Spex Industries, Inc., Metuchen, NJ). The values were expressed as nanomoles of TBARS per mg of protein. Malondialdehyde standard curve was prepared by using 1,1,3,3-tetramethoxypropane.

Platelet phospholipase A₂ activity assay

Platelet phospholipase A₂ activity was measured by a modification of the procedure of Steinbrecher and Pritchard (28). Platelet suspension was incubated with 25 nmol of substrate (1-palmitoyl 2-[12-(13-nitrobenzoxadiazolyl)-amino]caproyl phosphatidylcholine (C₆NBD PC) in 3 ml Dulbecco's PBS containing 50 mM taurocholic acid, 20 mM calcium chloride, and 0.05 g% fatty acid free bovine serum albumin at 37°C for 40 min. Taurocholic acid was used as a biological amphipath in the assay to aid in access of PLA₂ enzyme to the substrate. The concentration used was the same as found in human serum by Dupont et al. (29). Reactions were stopped by the addition of 1.2 ml methanol and 1.2 ml chloroform followed by mixing for 1 min. The mixtures were centrifuged at 2000 g for 15 min to separate the phases and the fluorescence of the aqueous layer was measured with a Fluorolog 2 Spectrometer (Spex Industries, Inc., Metuchen, NJ) at 470 nm excitation and 533 nm emission. The mass of fluorescent substrate hydrolyzed was determined by comparison with a standard curve generated by

serial dilutions of 6(7-nitrobenzoxadiazolyl) aminocaproic acid (Avanti Polar Lipids, Birmingham, AL).

Protein analysis

Protein was measured by the method of Lowry et al. (30) using bovine serum albumin as the standard.

Statistical analysis

All analyses were done in duplicate for each sample. Data were analyzed by using General Linear Model (SAS Institute, Cary, NC). Least Square Difference of the Statistical Analysis System (SAS Institute, Cary, NC) was used to test the significance between means of treatments. Two-way ANOVA analysis was used to determine the interaction between increasing dietary linoleate and vitamin E supplementation. Pearson correlation coefficients also were calculated. A $p < 0.05$ was taken to be statistically significant.

RESULTS

Animals and diets

There was no difference in food intake, liver weight or body weight gain of rats fed 11 or 18 en% linoleate (Fig. 1). The liver weight as a percentage of body weight was not different due to en% linoleate (Fig. 2). There was no difference in food intake and body weight gain of rats fed 0, 100 or 5000 ppm vitamin E, however, liver weight was significantly greater in the group fed 5000 ppm vitamin E than in the group fed 100 ppm vitamin E (10.4 ± 0.8 vs 9.7 ± 0.7 g) (Fig. 3). The liver weight as a percentage of body weight was significantly greater in the 5000 ppm vitamin E group than in the 100 ppm vitamin E group (2.6 ± 0.1 vs 2.5 ± 0.1 %) (Fig. 4).

Platelet fatty acid composition

There was no difference in platelet fatty acids, C12:0, C14:0, C16:0, C18:0, C20:1n-9 and C20:4n-6, in rats fed different levels of en% linoleate (Fig. 5). But platelet C18:1n-9 (oleic acid) was significantly greater in the 11 en% linoleate group (4.8 ± 1.2 vs 3.3 ± 1.7 %) and C18:2n-6 (linoleic acid) was significantly greater in the 18 en% linoleate group (7.8 ± 2.3 vs 4.7 ± 1.2 %) (Fig. 5). There was no difference in platelet fatty acids, C12:0, C14:0, C16:0, C18:0, C18:1n-9, C18:2n-6 and C20:1n-9, in rats fed different levels of vitamin E (Fig. 6). However, platelet C20:4n-6 (arachidonic acid) was significantly greater in the vitamin E-deficient group than in the 5000 ppm vitamin E group (28.4 ± 4.6 vs 23.8 ± 6.3 %) (Fig. 6). Platelet C18:0 (stearic acid) was decreased with increasing vitamin E supplementation in the 11 en% linoleate group, but it was increased with 5000 ppm vitamin E supplementation in the 18 en% linoleate group (Fig. 7). There was an

interaction of increasing dietary linoleate and vitamin E supplementation on platelet C18:0 fatty acid.

Platelet vitamin E level

Effects of increasing dietary linoleate on platelet vitamin E concentration was examined. There was no difference in platelet vitamin E of rats fed 11 or 18 en% linoleate (Fig. 8). There were significant differences in platelet vitamin E of rats fed 0, 100 or 5000 ppm vitamin E (0.2 ± 0.4 , 2.7 ± 0.7 , 4.2 ± 1.0 ng/ μ g protein, respectively) (Fig. 9).

Prostaglandins

There were no differences in serum TXB₂, 6-keto-PGF_{1 α} and aortic 6-keto-PGF_{1 α} concentrations of rats fed 11 or 18 en% linoleate (Fig. 10). The serum TXB₂/6-keto-PGF_{1 α} ratio of rats fed 11 or 18 en% linoleate also did not differ (Fig. 11). There was no difference in serum and aortic 6-keto-PGF_{1 α} concentrations due to vitamin E supplementation (Fig. 12). But, 100 or 5000 ppm vitamin E significantly reduced serum TXB₂ production compared with rats receiving no vitamin E (215.5 ± 71.1 , 170.5 ± 66.4 , 399.6 ± 108.9 ng/ml, respectively) (Fig. 12). The serum TXB₂/6-keto-PGF_{1 α} ratio of rats was significantly greater in the vitamin E-deficient group than in the 5000 ppm vitamin E group (49.7 ± 13.4 vs 35.1 ± 17.2) (Fig. 13).

Platelet lipid peroxidation

Platelet lipid peroxidation was measured by assaying t-BuOOH-induced TBARS. Platelet TBARS were not significantly different between rats fed 11 or 18 en% linoleate (5.1 ± 0.6 vs 5.4 ± 0.8 pmol/ μ g protein) (Fig. 14). Rats fed no vitamin E had significantly greater platelet TBARS than rats fed 100 or 5000 ppm vitamin E (5.9 ± 0.7 , 5.0 ± 0.4 , 4.7 ± 0.2 pmol/ μ g protein, respectively)

(Fig. 15).

Platelet phospholipase A₂ activity

Platelet phospholipase A₂ activity did not differ with increasing dietary linoleate or vitamin E status (Fig. 16,17).

DISCUSSION

McIntosh et al. (31) investigated the effect of vitamin E deficiency on platelet function and prostaglandin production in marmoset monkeys and found during their 9 month study that vitamin E deficiency reduced body weight gain and resulted in lower skeletal muscle weights. Vitamin E deficiency did not affect weight gain in our study which could be due to the difference in animal species or feeding period (Fig. 3). The length of our study, 2.5 months, probably was not long enough to cause myopathies and decrease muscle weights (29), therefore, differences in body weight gain were not found. Rice and Kennedy (32) showed that vitamin E deficiency would lead to skeletal and cardiac myopathies in a variety of animals and poultry. They also indicated that vitamin E deficiency can cause hepatic necrosis, telangiectasis and lipid degeneration which could lead to lower liver weights. Mean liver weight of rats fed 5000 ppm vitamin E in our study was significantly greater than the mean liver weight of rats fed 100 ppm vitamin E (Fig. 3). However, a diet containing 100 ppm vitamin E is adequate for rats. The mechanism for reduced liver weight in the group receiving 100 ppm vitamin E compared with the group fed 5000 ppm vitamin E is unknown.

The changes in platelet fatty acid composition, increasing 18:2n-6 with increasing dietary linoleate (Fig 5), were consistent with results of Piche and Mahadevappa (33) and Iritani and Narita (34). Platelet arachidonic acid level was greatest in the vitamin E-deficient group (Fig. 6). This suggests that vitamin E or oxidative stress may have a role in fatty acid desaturation and elongation. McIntosh et al. (31) observed that vitamin E increased or decreased platelet phospholipid arachidonic acid depending on dietary

treatments. In a basal diet which supplied 1.2% (w/w) sunflower seed oil, vitamin E supplementation decreased platelet phospholipid arachidonic acid. But, after increasing sunflower seed oil to 10% (w/w), vitamin E supplementation increased platelet phospholipid arachidonic acid. The role of vitamin E in fatty acid desaturation and elongation needs further study.

The measurement of platelet vitamin E was chosen to reflect dietary vitamin E intake based on the suggestion of Lehmann (35) who showed that platelet vitamin E was more sensitive than red blood cell or plasma in reflecting vitamin E intake. The amount of vitamin E required to prevent lipid peroxidation can depend on the dietary intake of fat, particularly the intake of polyunsaturated fatty acids (36). In this study, linoleate contributed 11 and 18 en%. Even though there were differences in platelet linoleate (Fig. 5), platelet vitamin E concentration (Fig. 8) was not altered by dietary linoleate. Chan et al. (37) indicated that the retention of tocopherol by rat platelets appeared to be logarithmically related to increasing vitamin E concentration in the diets. Results in this study (Fig. 9) tended to be logarithmic in agreement with the experiment of Chan et al.

Effects of dietary fat on prostaglandin biosynthesis have been studied by Dupont et al. (17), Sullivan and Mathias (18) and Mathias and Dupont (38). In these studies, 2 en% linoleate was found to be optimal for prostaglandin production. Greater en% linoleate was not correlated with greater prostaglandin production and varying the dietary P/S ratios has also been found to affect prostaglandin synthesis (38). Dietary P/S ratios between 0.4 and 5.5 did not significantly affect serum prostaglandin levels while a dietary P/S ratio of 9 was found to increase serum prostaglandin levels

markedly (39). The proportion of total energy supplied by linoleate in the present study was 11 and 18% and the dietary P/S ratios of 1.6 and 5.4 were well within the ranges previously described (39) not to affect prostaglandin status. Neither change in en% linoleate nor P/S ratio caused change in prostaglandin status (Fig. 10). These results were consistent with observations of other laboratories (17,18,38,39).

Studies on the effect of dietary vitamin E on prostaglandin synthesis have not been consistent. In several species, vitamin E supplementation has been demonstrated to depress platelet TXA₂ synthesis and usually to enhance aortic PGI₂ synthesis (2,11,12). However, Stampfer et al. (9) found no effect of vitamin E on TXA₂ production or PGI₂ production. Swartz et al. (10) have reported decreased plasma PGI₂ levels in healthy adult subjects supplemented with vitamin E. Falanga et al. (40) indicated increased serum PGI₂ in vitamin E deficient rats. In the present study, vitamin E did not affect prostacyclin synthesis (Fig. 12). These results were in agreement with the work of Stampfer et al. (9). Thromboxane A₂ production, measured as TXB₂, was inhibited by vitamin E adequacy and supplementation in this study (Fig. 12). These results support the work of Karpen et al. (11). Contradictory results obtained by different laboratories could be due to different vitamin E dosages used in the ranges of 0 to 1000 mg/kg diet, differences in feeding period from 8 to 23 weeks, species variation, for example rats, rabbits and humans and different techniques of tissue sampling and preparation.

TXA₂ is a platelet aggregator and vasoconstrictor, and PGI₂ is a platelet antiaggregator and vasodilator. The balance between TXA₂ and PGI₂ is important, because platelet aggregation and vasoconstriction may precipitate

acute atherosclerotic incidents such as infarcts. An altered balance of TXA₂/PGI₂ in streptozotocin-induced diabetic rats was reported by Karpen et al. (41). TXA₂/PGI₂ balance was restored to normal when diabetic rats were supplemented with vitamin E (3). The serum TXA₂/PGI₂ ratio of rats in the present study was significantly greater in the vitamin E-deficient group compared with the ratio of rats in the 5000 ppm vitamin E group (Fig. 13). Therefore, results of the present study indicate that vitamin E supplementation may have an antithrombotic effect or that vitamin E deficiency is prothrombogenic.

There was a significant correlation between platelet TXB₂ and t-BuOOH-induced TBARS ($r=0.66$, $p<0.0001$). This suggests that increased lipid peroxidation due to vitamin E deficiency can stimulate TXB₂ synthesis. However, vitamin E supplementation did not inhibit TXB₂ synthesis compared with vitamin E adequacy. (Fig. 12). It is known that oxidative stress can cause lipid peroxidation (42). Other conditions associated with oxidative stress, such as dietary copper deficiency can increase hepatic PGE₂ and PGF_{2 α} production (43) and depress aortic PGI₂ synthesis rates (44) and both of these changes were associated with reduced superoxide dismutase (SOD) activity which is one of the major intracellular antioxidant defenses of mammalian cells (45). Reduced SOD activity implies increased oxidative stress. Oxidative stress, such as occurs in vitamin E deficiency, might increase synthesis of TXB₂, it may just as enhance PGE₂ and PGF_{2 α} production (43,46,47). The effect of vitamin E deficiency on TXA₂ production may be mediated by oxidative stress which has been shown to stimulate production of other PGs as well (50). In the present study, vitamin

E supplementation did not affect PGI₂ status (Fig. 12). The effects of vitamin E on TXA₂ and PGI₂ production differ, and TXA₂ and PGI₂ do not seem to respond in the same way during oxidative stress because increased TXA₂ synthesis during oxidative stress but no alteration in PGI₂ synthesis during oxidative stress. The mechanisms of oxidative stress-induced PG production are not understood.

Vitamin E did not show an effect on endogenous platelet phospholipase A₂ activity in the present study (Fig. 17), although vitamin E supplementation is suggested to inhibit phospholipase A₂ activity (1). The mechanism by which vitamin E may influence platelet phospholipase activity is not clear. Sulfhydryl cross-linking with the oxidizing agent diazenedicarboxylic acid(bis)dimethylamide enhanced arachidonic acid release from platelet membranes (48) and lipid peroxides could enhance sulfhydryl cross-linking. Furthermore, Yasuda and Fujita (49) showed that lipid peroxidation enhanced fatty acid release from mitochondria. Since vitamin E inhibits lipid peroxidation, this inhibition could diminish the sulfhydryl cross-linking that promotes phospholipase A₂ activity. Because vitamin E deficiency only stimulated lipid peroxidation when an oxidative stressor, t-BuOOH, was introduced, perhaps only more extreme oxidative stress than vitamin E deficiency activates phospholipase A₂.

Results from platelet lipid peroxidation measurements support the role of vitamin E as an antioxidant which inhibits peroxidation of lipids containing polyunsaturated fatty acids (PUFA). Krumhardt (20) has shown that en% linoleate greater than 4.5 can inhibit platelet phospholipase A₂ activity. There was no difference in phospholipase A₂ activity of rats fed 11 or 18 en%

linoleate (Fig. 16). The en% linoleate in this study was different from that of Krumhardt who fed 3.0, 4.5, 6.0, 7.5 or 9.0 en% linoleate, making it difficult to compare the effect of en% linoleate on phospholipase A₂ activity. Tangney and Driskell (50) demonstrated that vitamin E inhibited platelet phospholipase A₂ activity. They found an enhancement of thrombin-induced phospholipase A₂ activity in platelets of vitamin E deficient rabbits that had been deprived of vitamin E for 7 months. Perhaps the present results showing no effect of vitamin E status on PLA₂ activity (Fig. 17) differed from those of Tangney and Driskell (50) because of the absence of thrombin-induction.

C18:2n-6 was negatively correlated with C20:4n-6 in platelets ($r=-0.37$, $p<0.05$), and this finding was in agreement with Spector et al. (14,15). These authors demonstrated an increase in the percentage of C18:2n-6 associated with a decrease in C20:4n-6 in the phospholipid fraction. This suggests that high concentrations of linoleate reduce the incorporation of arachidonic acid into cellular phospholipids. C18:2n-6 was negatively correlated with phospholipase A₂ activity ($r=-0.41$, $p<0.05$). An inhibitory effect of unsaturated fatty acids on phospholipase A₂ activity was shown by Ballou and Cheung (16). In the present study, because C20:4n-6 was positively correlated with TXB₂ concentration ($r=0.34$, $p<0.05$), an increased arachidonic acid pool may be necessary for increased TXB₂ production. That vitamin E deficiency causes lipid peroxidation was demonstrated in the present study (Fig. 15). Elevated C20:4n-6 was also associated with increased TBARS ($r=0.44$, $p<0.02$). Increased arachidonate may have permitted the increased TX production in vitamin E deficiency. In some way, vitamin E deficiency causes greater C20:4n-6 (arachidonate) and increased TXA₂, but

not PGI₂ status. Perhaps, other oxidative stressors can also stimulate PG production by increasing arachidonate. How oxidative stress might increase tissue arachidonate is unclear.

The present study suggests that oxidative stress produced by vitamin E deficiency can be prothrombogenic by virtue of its stimulatory effect on TXA₂ synthesis. Although the mechanism is unknown, perhaps it is mediated by an effect on the PG precursor, arachidonate.

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Table 1. Composition of experimental diets

Ingredient	Energy Percentage Linoleate^a	
	11	18
	----- g/100 g -----	
Lard, tocopherol-stripped ^b	7.2	—
Corn Oil, tocopherol-stripped ^b	7.2	14.3
Casein "Vitamin Free" Test ^b	22.4	22.4
Sucrose ^b	26.4	26.4
Corn Starch ^b	26.4	26.4
Cellulose ^b	5.0	5.0
AIN Mineral Mixture 76 ^{bc}	3.9	3.9
Vitamin Mixture (α-tocopherol devoid) ^{bd}	1.1	1.1
Tocopheryl Acetate ^b	0, 0.01, 0.5	0, 0.01, 0.5
L-Methionine ^b	0.3	0.3
Choline Chloride ^b	0.08	0.08
Ascorbic Acid (antioxidant) ^b	0.01	0.01
Cholesterol (mg/100 g) ^b	0	6.8

^aEach level of linoleate was fed with 0, 100 or 5000 ppm α-tocopheryl acetate.

^bTeklad, Madison, WI.

^cComposition described by the AIN Ad Hoc Committee on Standards for Nutritional Studies, 1977 (19).

^dComposition described by the AIN Ad Hoc Committee on Standards for Nutritional Studies, 1977 (19), except that vitamin E acetate was substituted by sucrose.

Table 2. Fatty acids composition of experimental diets

	11 en% linoleate	18 en% linoleate
	----- % -----	-----
14	1.0	—
15	0.1	—
16	18.1	10.2
16:1	2.1	—
18	5.5	1.8
18:1	33.8	23.9
18:2	37.5	62.0
18:3	1.1	2.1
20:1	0.6	—
20:2	0.2	—
P/S ratio	1.6	5.4
P/M ratio	1.1	2.7

Table 3. Preparation of standard curve for TXB₂ and 6-keto-PGF_{1α}

Tube No.	PBS-Gel	Standard (20 ng /ml) TXB₂ or 6- keto-PGF_{1α}	Ab:ARGG	³H-TXB₂ or ³H-6-keto- PGF_{1α}
		-----	μl	-----
1	0	500.0	400	100
2	250.0	250.0	400	100
3	375.0	125.0	400	100
4	437.5	62.5	400	100
5	468.8	31.2	400	100
6	484.4	15.6	400	100
7	492.2	7.8	400	100
8	496.1	3.9	400	100
9	498.0	2.0	400	100
10	499.0	1.0	400	100

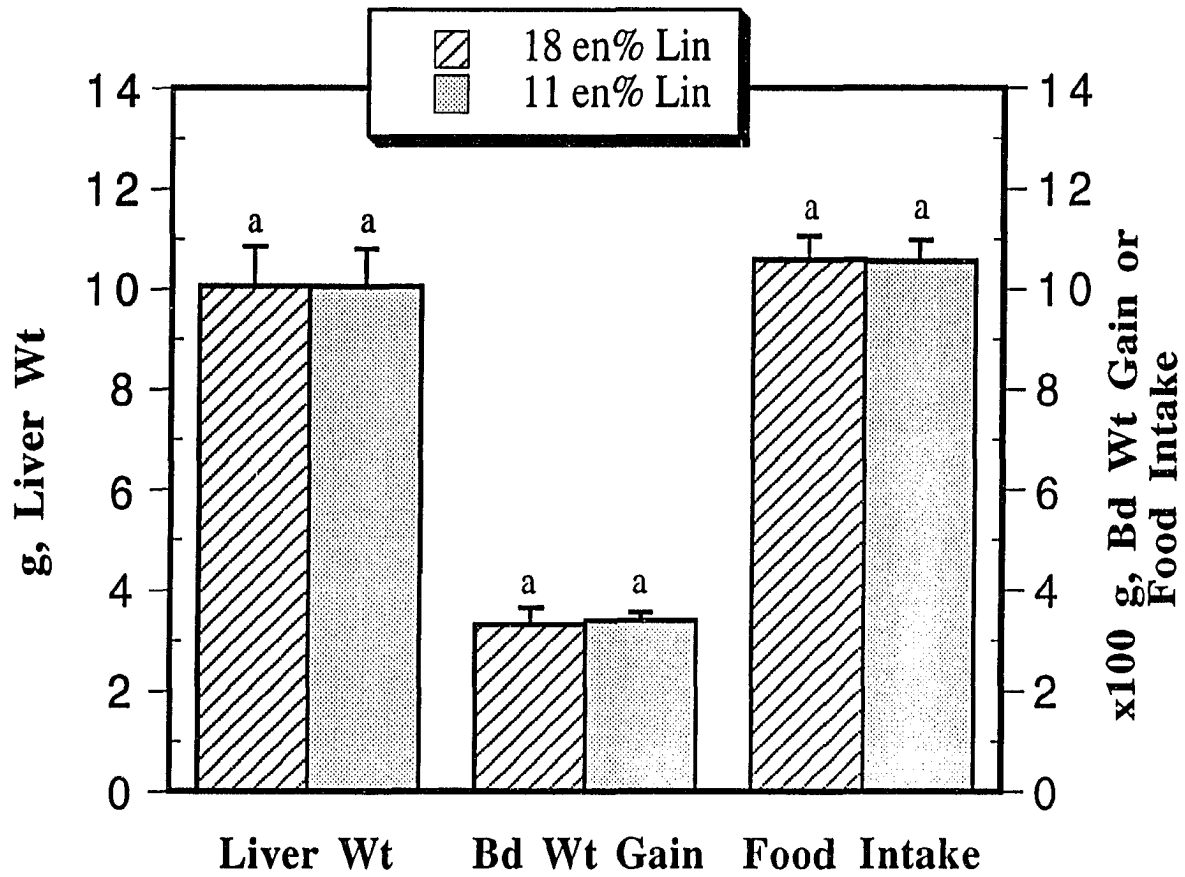


Figure 1. Effect of en% linoleate on liver weight, body weight gain and food intake. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

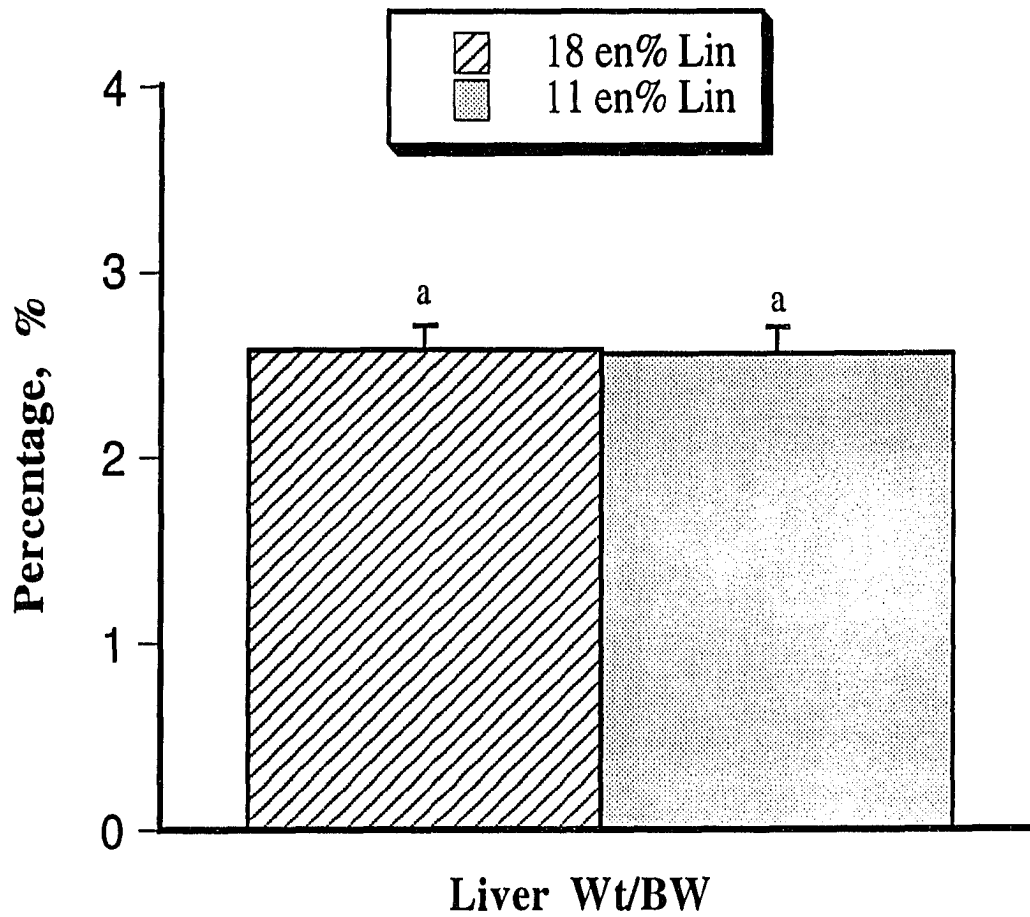


Figure 2. Effect of en% linoleate on liver weight as percentage of body weight . Groups that do not share the same letter are significantly different from one another, $p < 0.05$

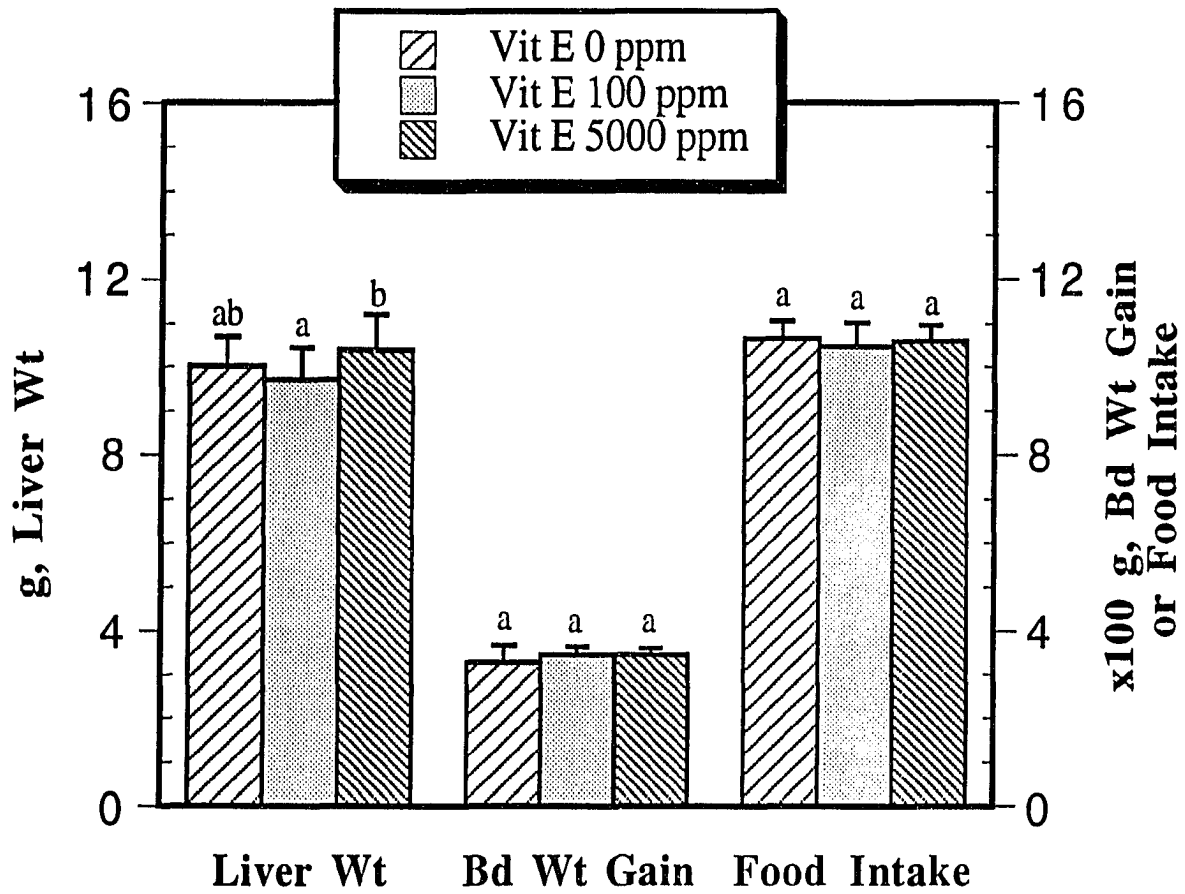


Figure 3. Effect of vitamin E status on liver weight, body weight gain and food intake. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

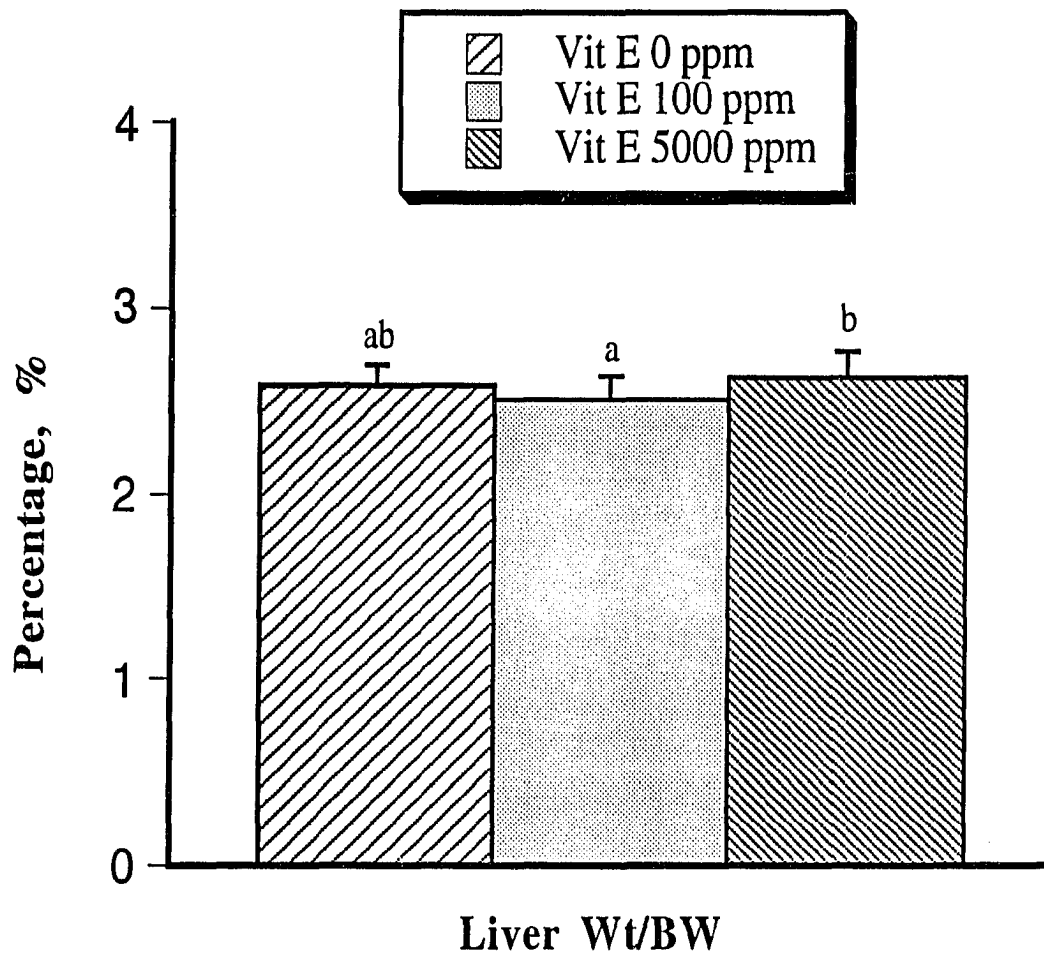


Figure 4. Effect of vitamin E status on liver weight as percentage of body weight. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

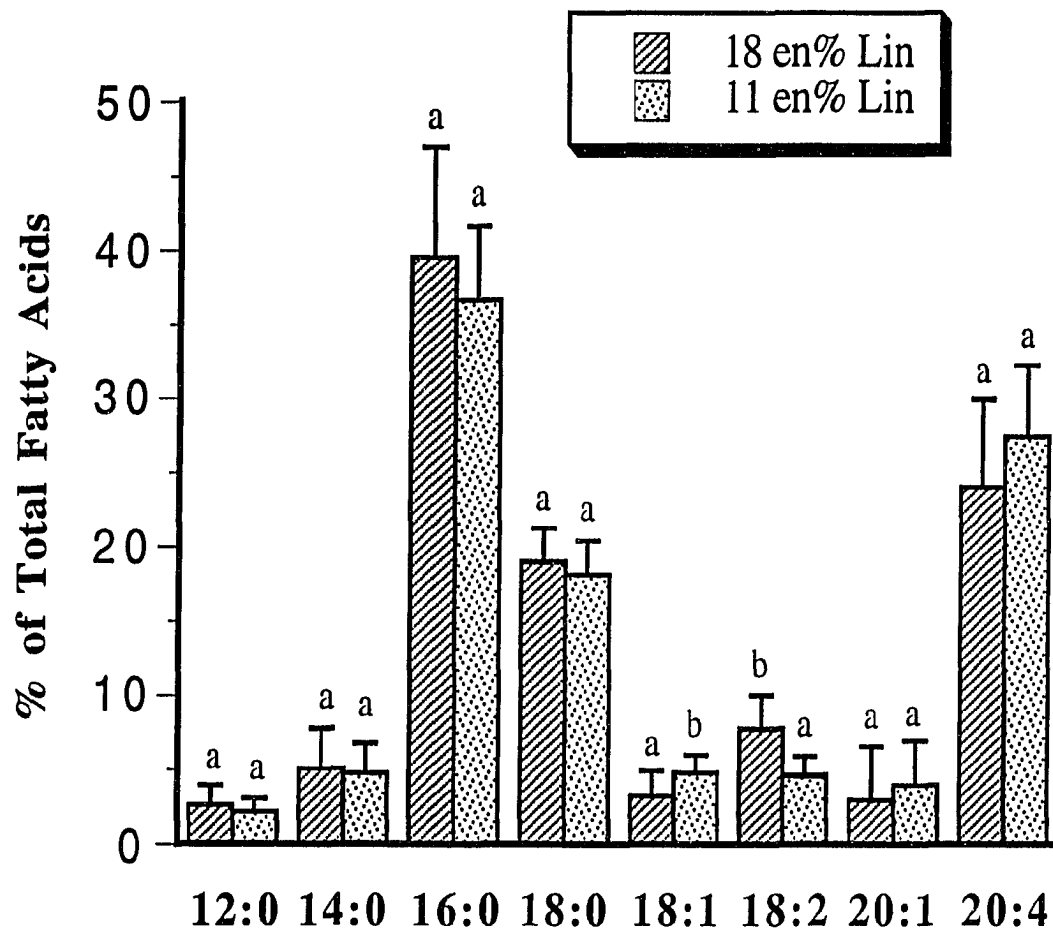


Figure 5. Effect of en% linoleate on platelet fatty acid composition. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

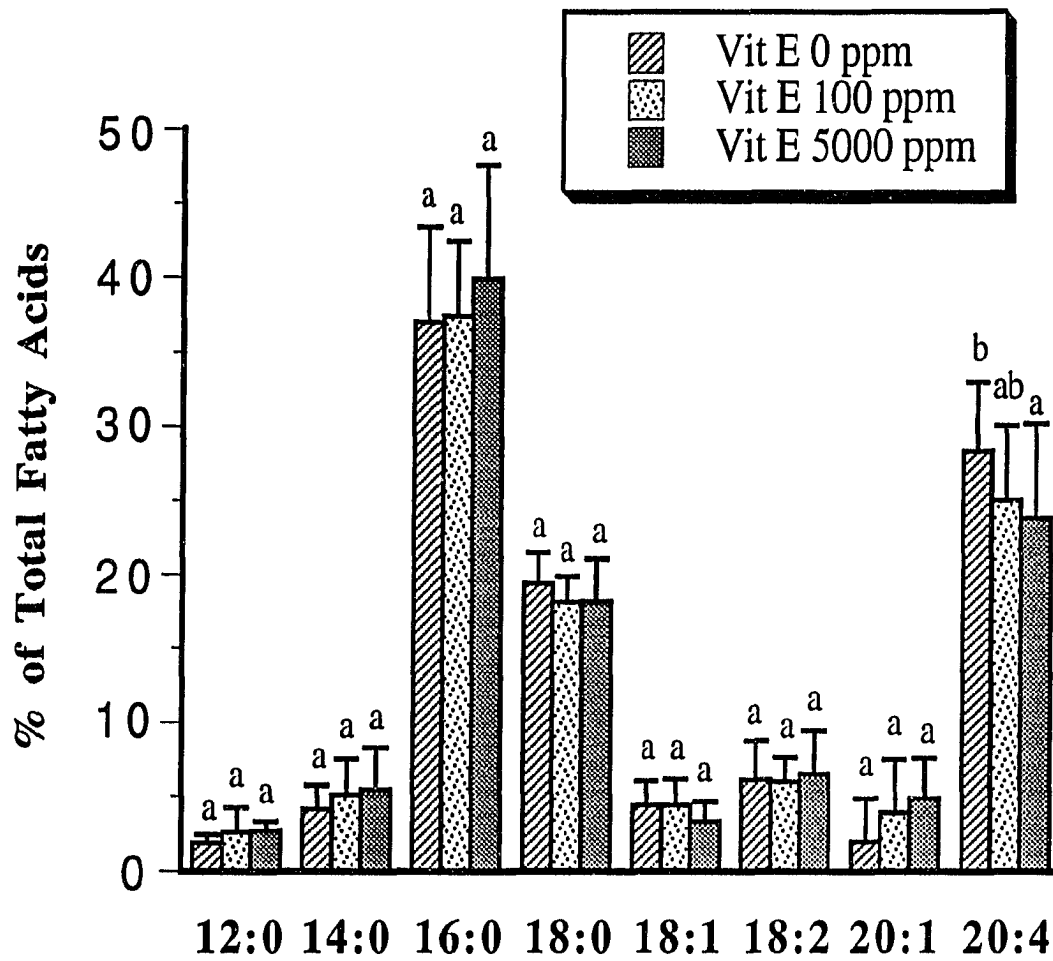


Figure 6. Effect of vitamin E status on platelet fatty acid composition. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

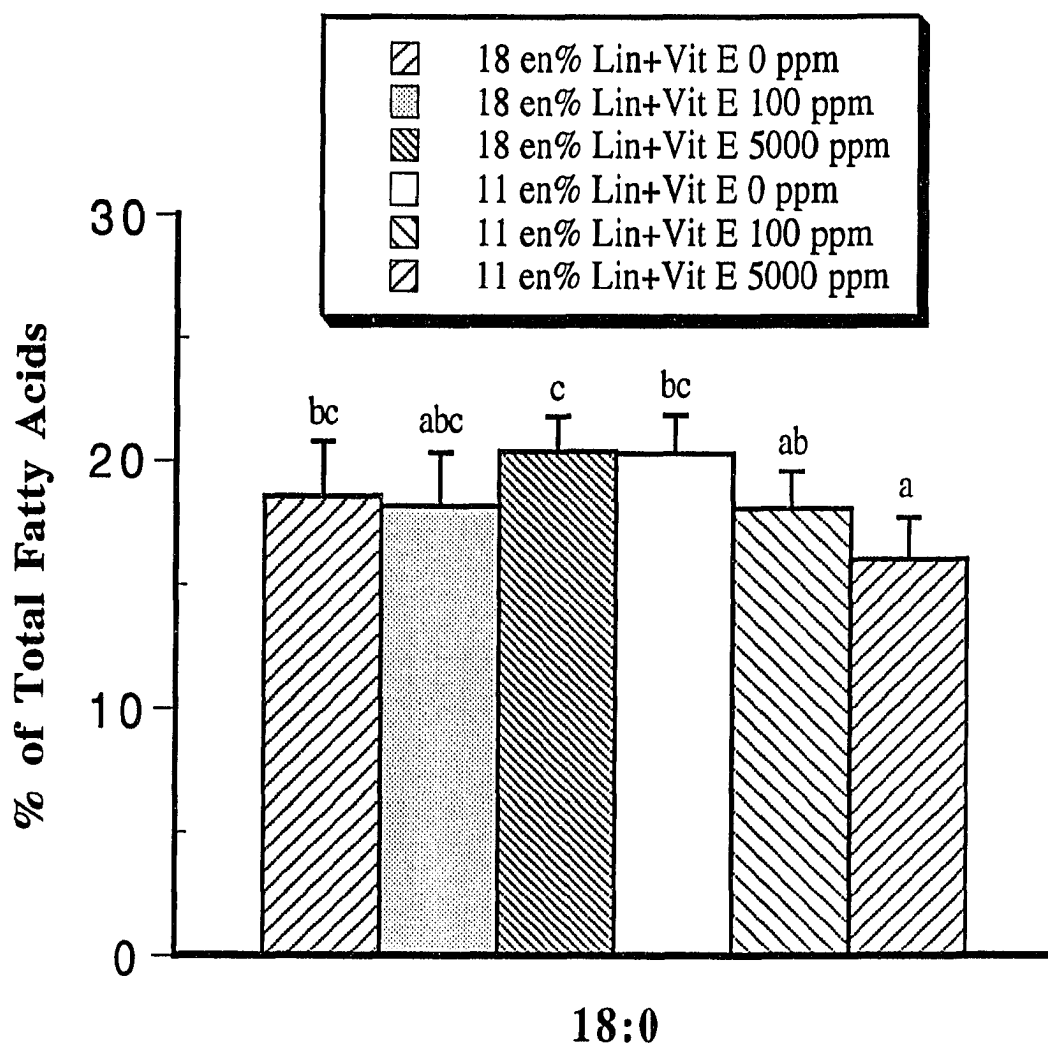


Figure 7. Interaction of en% linoleate and vitamin E status on platelet C18:0. Groups that do not share the same letter are significantly different, $p < 0.05$

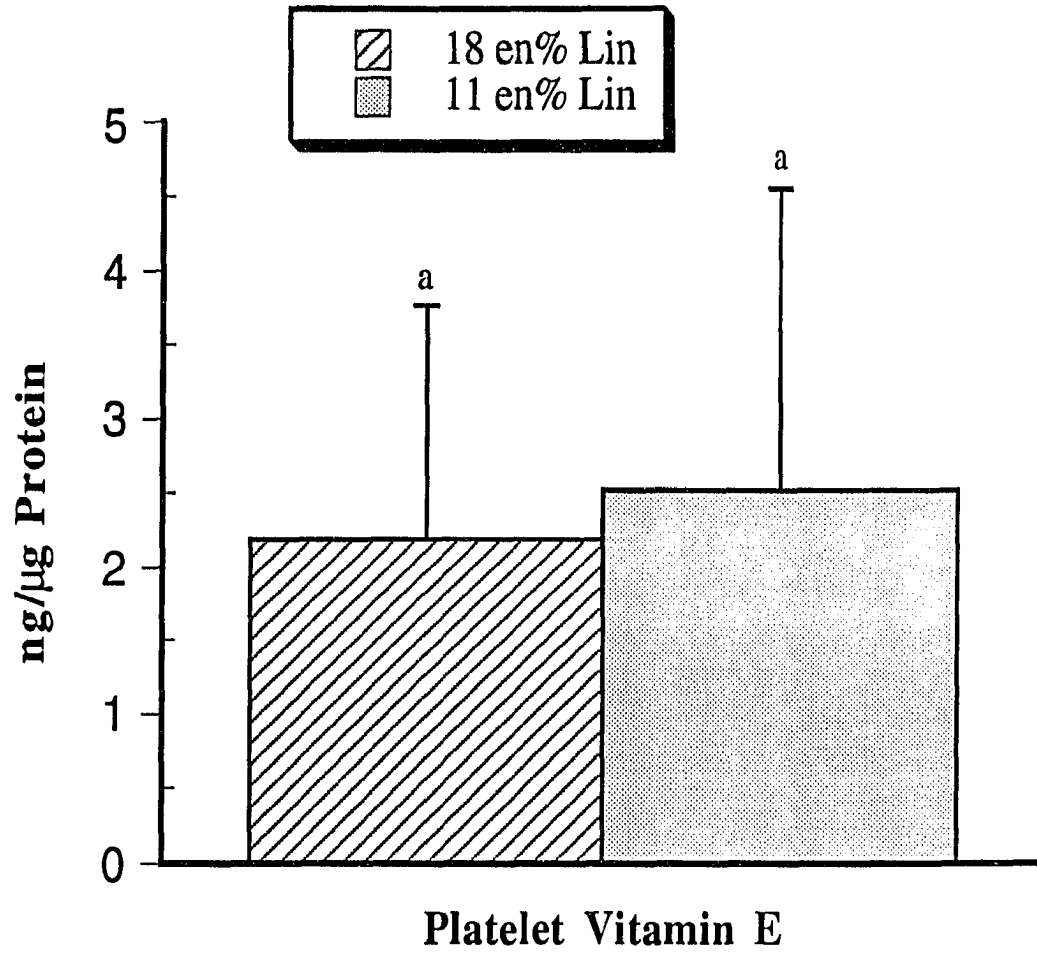


Figure 8. Effect of en% linoleate on platelet vitamin E level. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

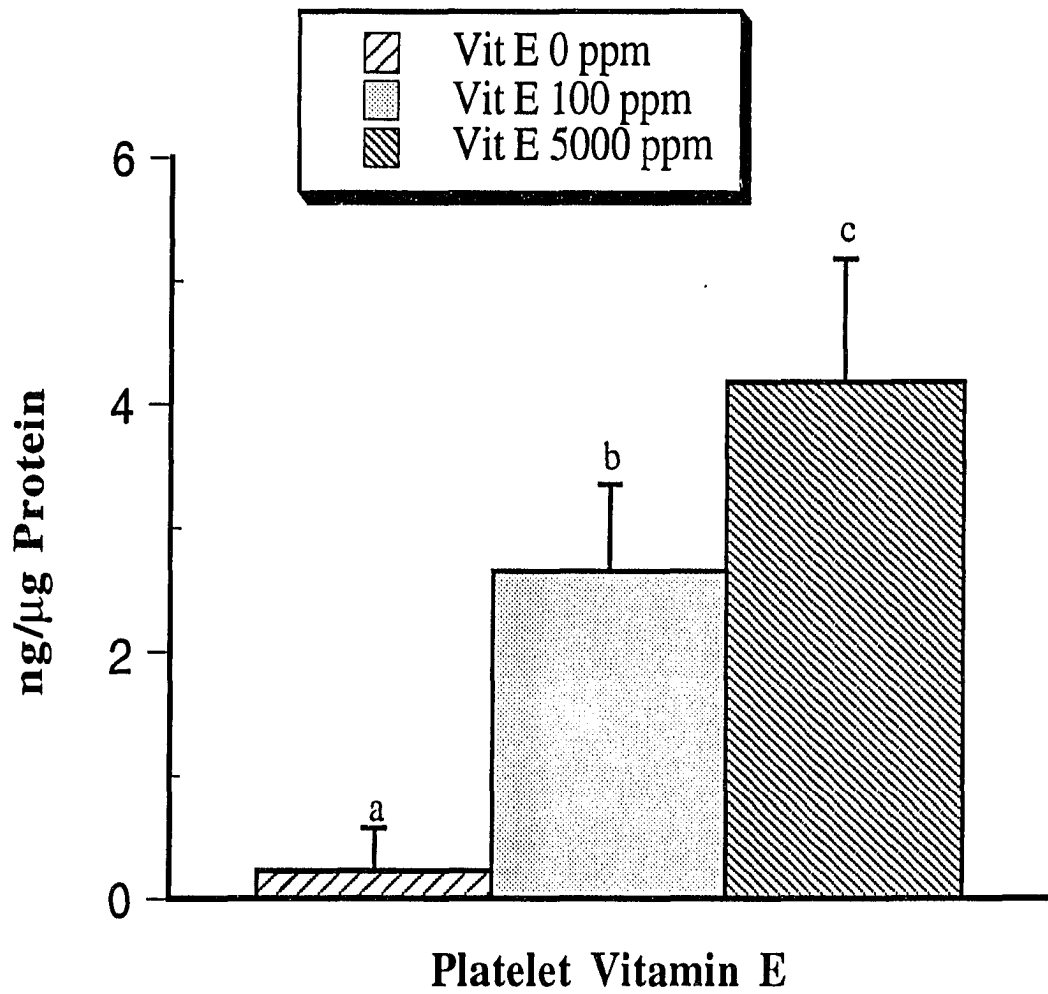


Figure 9. Effect of vitamin E supplementation on platelet vitamin E level. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

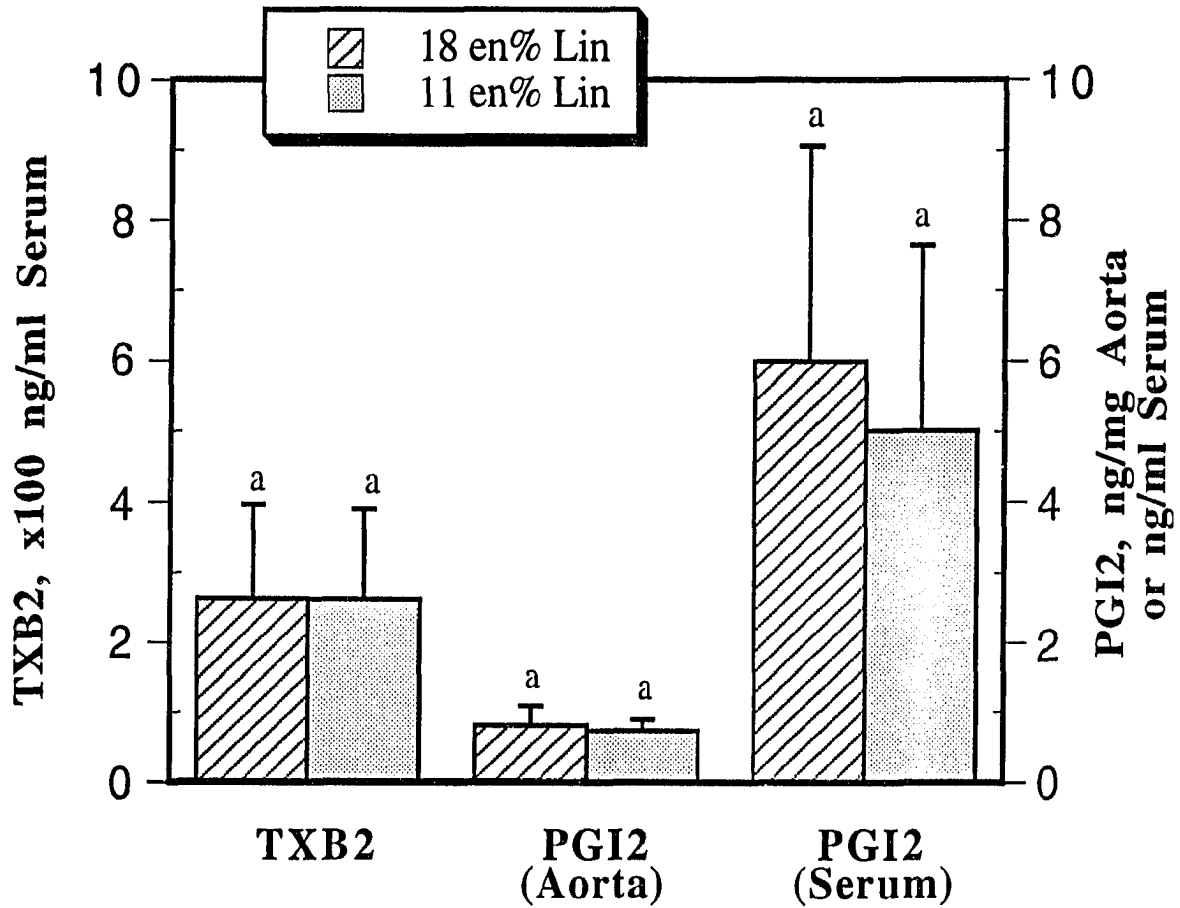


Figure 10. Effect of en% linoleate on prostaglandin status. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

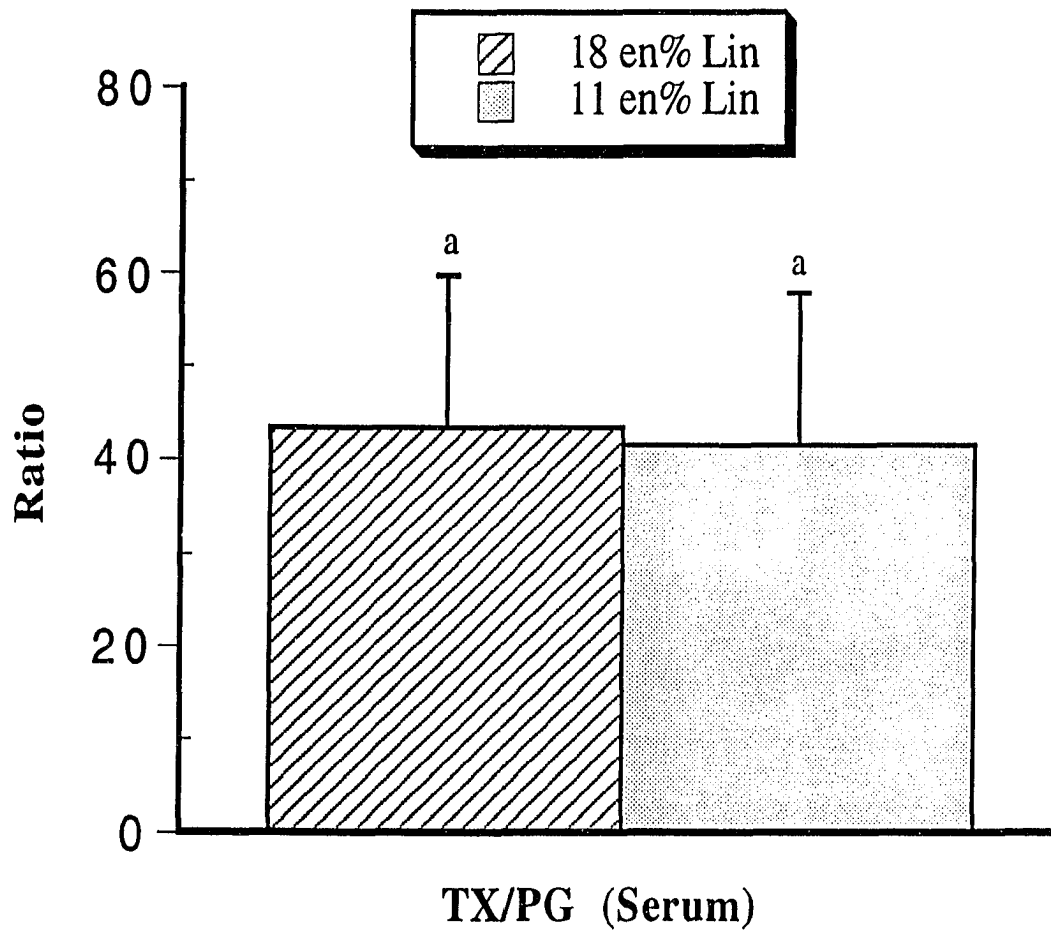


Figure 11. Effect of en% linoleate on serum $\text{TXB}_2/6\text{-keto-PGF}_{1\alpha}$ (TX/PG) ratio. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

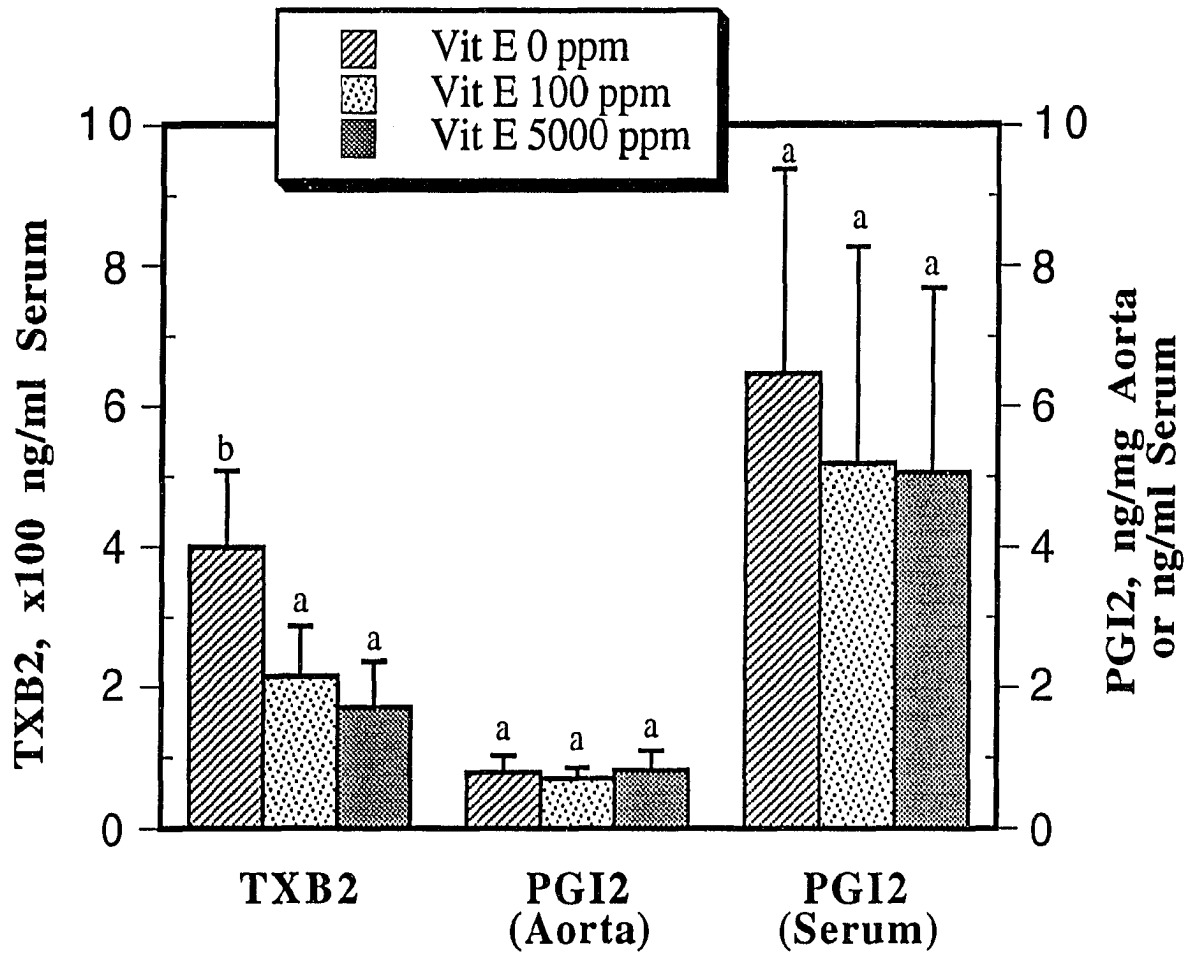


Figure 12. Effect of vitamin E status on prostaglandin status. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

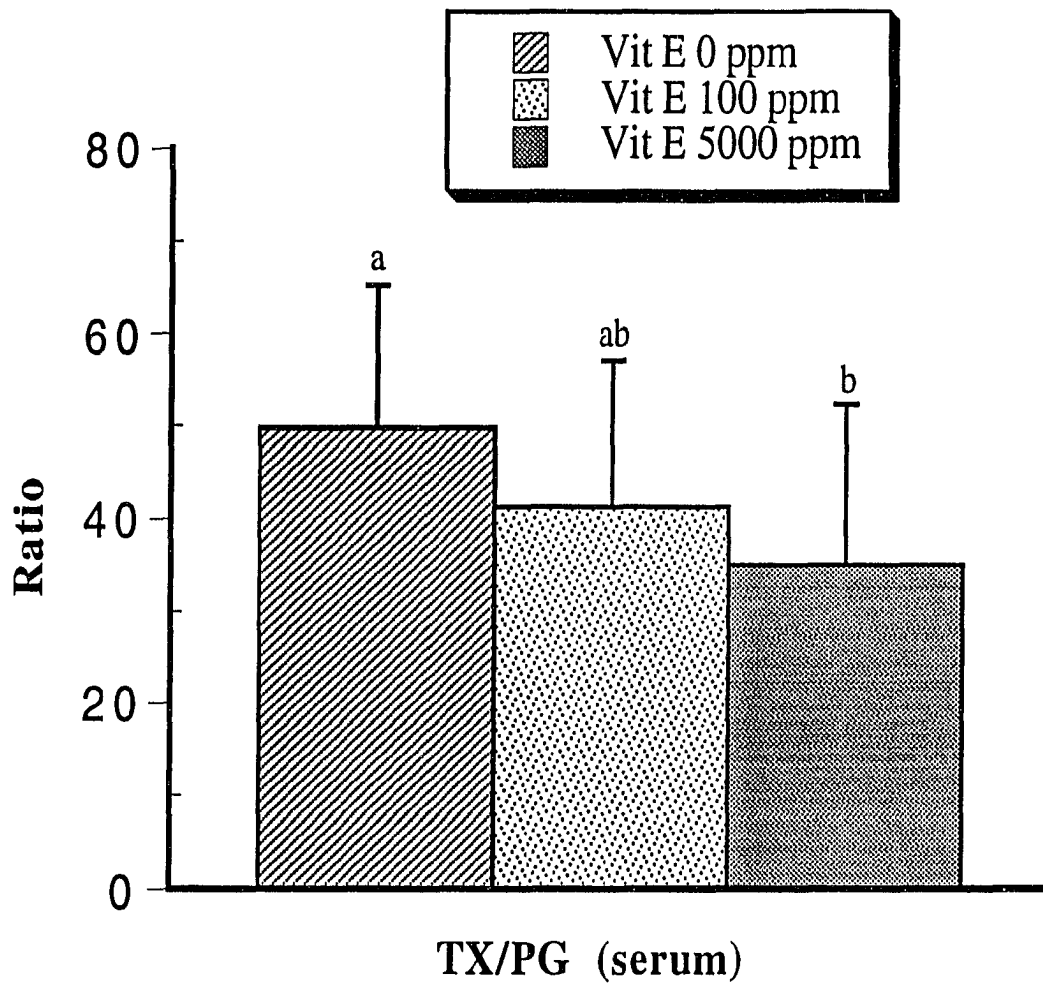


Figure 13. Effect of vitamin E status on serum $\text{TXB}_2/6\text{-keto-PGF}_{1\alpha}$ (TX/PG) ratio. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

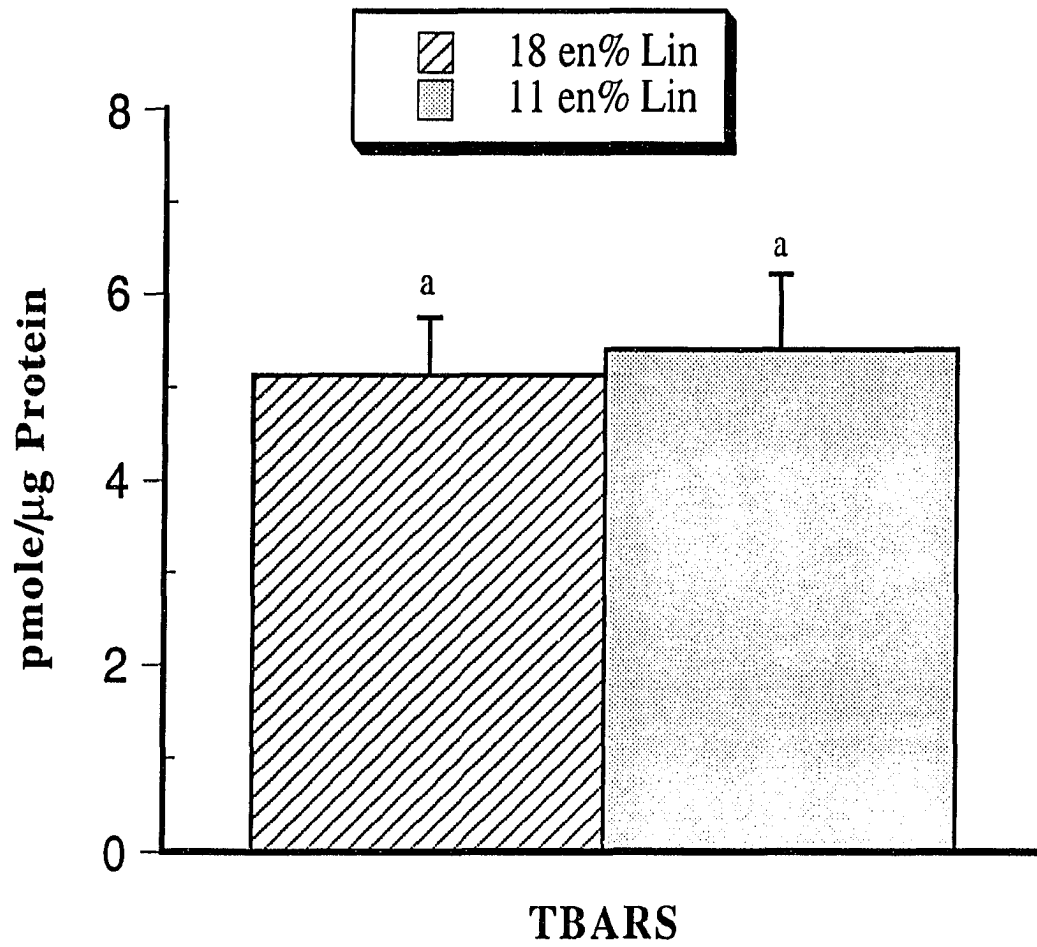


Figure 14. Effect of en% linoleate on t-BuOOH-generated platelet lipid peroxidation (as measuring thiobarbituric acid reactive substance). Groups that do not share the same letter are significantly different from one another, $p < 0.05$

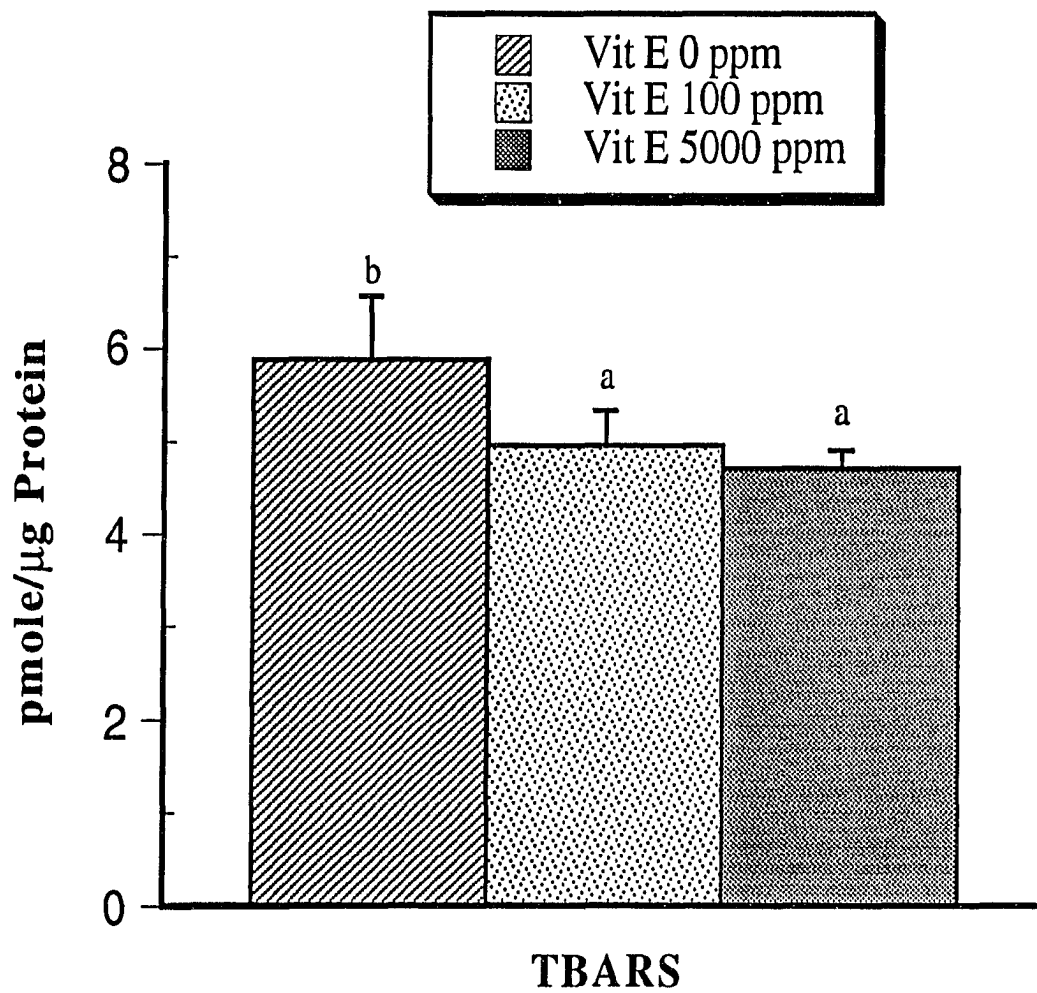


Figure 15. Effect of vitamin E status on t-BuOOH-generated platelet lipid peroxidation (as measuring thiobarbituric acid reactive substance). Groups that do not share the same letter are significantly different from one another, $p < 0.05$

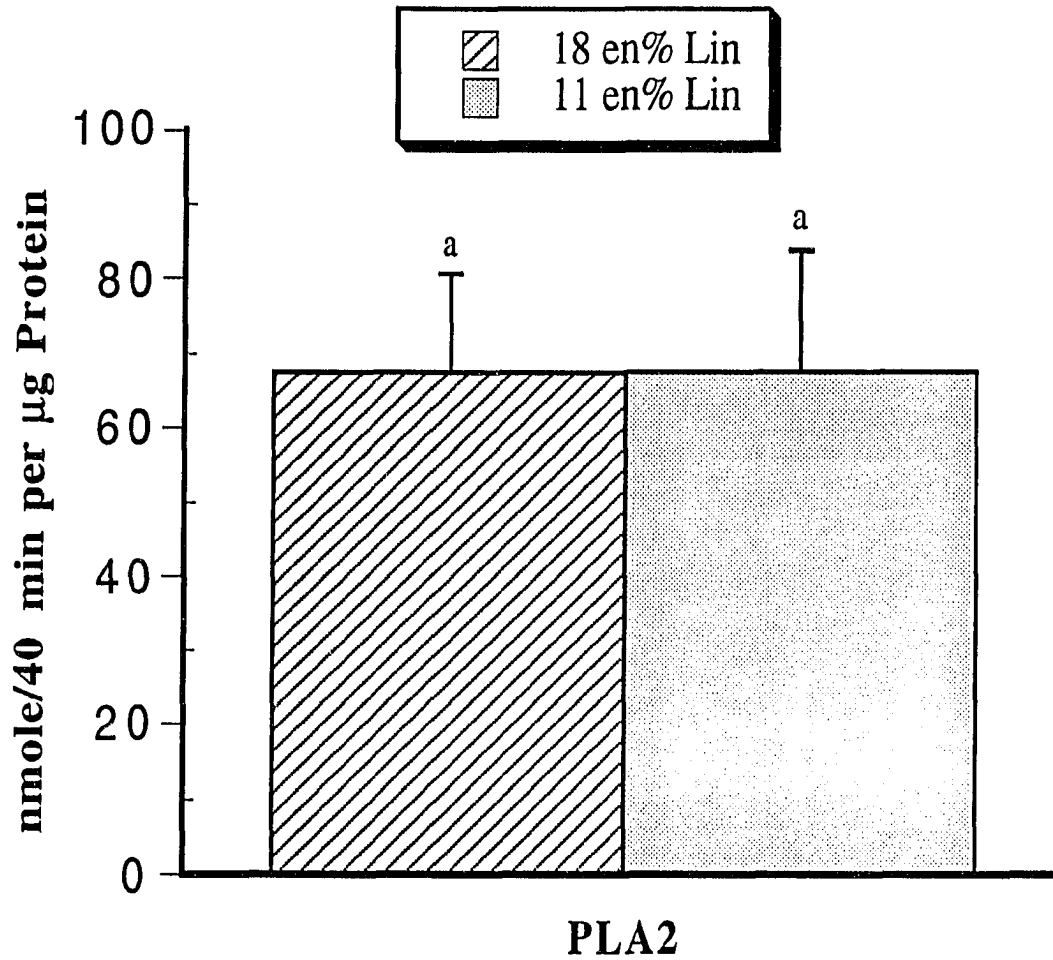


Figure 16. Effect of en% linoleate on platelet phospholipase A₂ activity (PLA₂). nmole 6(7-nitrobenzoxadiazolyl) aminocaproic acid released from 1-palmitoyl 2-[12-(13-nitrobenzoxadiazolyl)-amino] caproyl phosphatidylcholine in 40 min per μg platelet protein. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

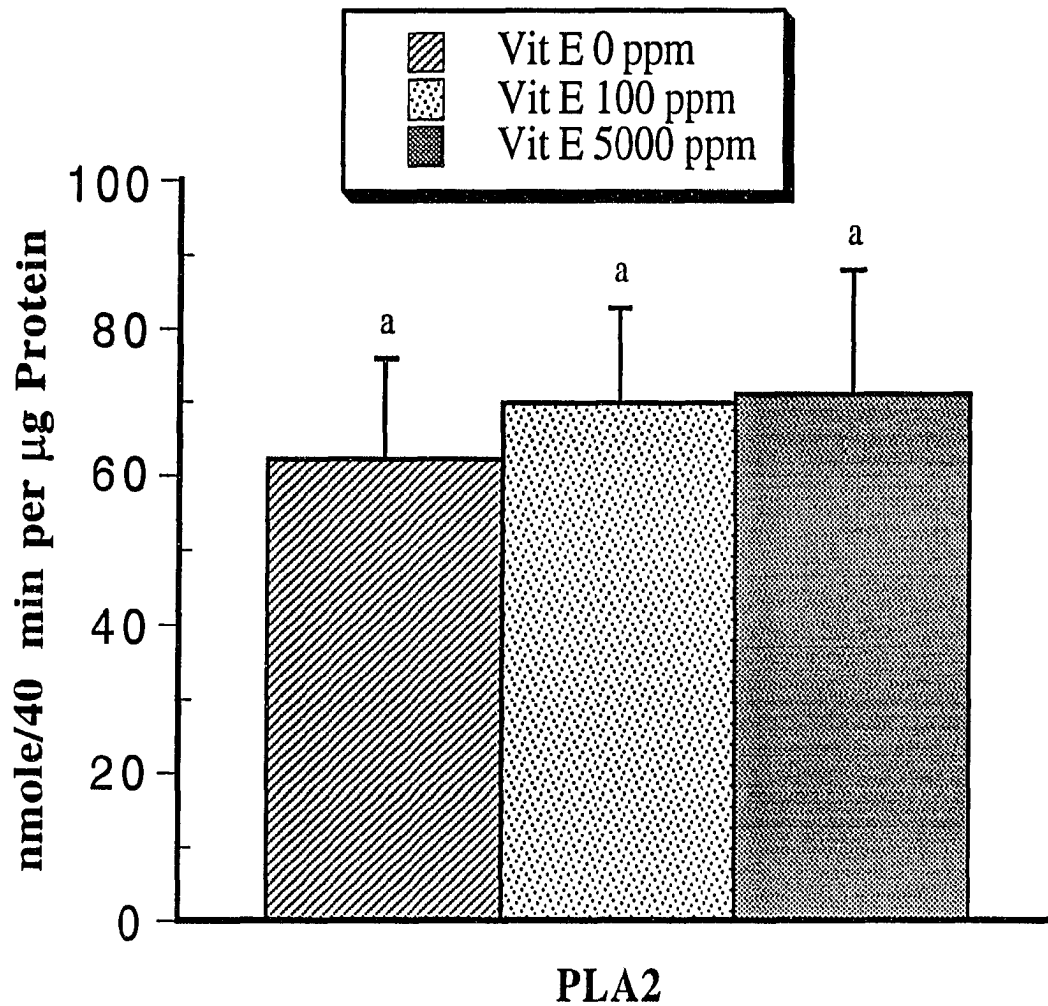


Figure 17. Effect of vitamin E status on platelet phospholipase A₂ activity (PLA₂). nmole 6(7-nitrobenzoxadiazolyl) aminocaproic acid released from 1-palmitoyl 2-[12-(13-nitrobenzoxadiazolyl)-amino] caproyl phosphatidylcholine in 40 min per µg platelet protein. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

**PAPER II. INFLUENCE OF INCREASING DIETARY LINOLEATE
AND VITAMIN E SUPPLEMENT ON SERUM LIPID
LEVELS IN MALE SPRAGUE-DAWLEY RATS**

ABSTRACT

The present study was conducted to determine whether increasing dietary linoleate and vitamin E supplementation interact to lower serum lipid levels. We hypothesized that increasing dietary linoleate could decrease serum lipid levels (e.g., total cholesterol, LDL-cholesterol and triglyceride) and vitamin E supplementation could increase serum HDL-cholesterol level. The influence of increasing dietary linoleate and d,l- α -tocopheryl acetate (vitamin E) supplementation on serum triglyceride, total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol was studied in 72 weanling male Sprague-Dawley rats. Rats were fed semipurified diets containing 11 or 18% of energy (en%) from linoleate and graded levels (0, 100 or 5000 ppm) of vitamin E for 10 weeks. Vitamin E status was confirmed by serum vitamin E level. Rats fed no vitamin E had the lowest serum vitamin E and rats fed 5000 ppm vitamin E had the greatest serum vitamin E ($p < 0.05$). Lipid status was verified by platelet fatty acid composition. Platelet C18:1n-9 (oleic acid) was significantly greater in the 11 en% linoleate group than in the 18 en% linoleate group, and platelet C18:2n-6 (linoleic acid) was significantly greater in the 18 en% linoleate group than in the 11 en% linoleate group ($p < 0.05$). The treatments had no effect on food intake, body weight gain, serum total cholesterol and LDL-cholesterol levels. Serum triglyceride level was significantly greater in the group fed 11 en% linoleate than in the group fed 18 en% linoleate ($p < 0.05$). Serum HDL-cholesterol concentration was significantly less in the vitamin E-deficient group than in the other two groups ($p < 0.05$). The results indicate that serum triglyceride levels are sensitive to dietary linoleate manipulation while HDL-cholesterol is sensitive

to vitamin E status. Prevention of vitamin E deficiency, but not vitamin E supplementation, seems to alter serum lipids in a manner which may protect from atherosclerosis.

INTRODUCTION

Elevated plasma cholesterol is well established as a factor in the development of atherosclerosis. Clinical trials have shown that reduced total plasma cholesterol is associated with reduced coronary heart disease in human, and that a 1% reduction in plasma cholesterol may result in a 2% reduction in coronary heart disease (CHD) (1,2). Brown and Goldstein (3) and Mahley and Innerarity (4) have shown that cholesterol homeostasis is closely related to plasma lipoprotein metabolism. Plasma HDL-cholesterol has an inverse relation with the risk of (CHD) (5,6). There is persuasive evidence that this protective association is causal because the role of HDL is to transport cholesterol from peripheral tissues to the liver where it is catabolized and excreted. In contrast, the role of LDL is to transport cholesterol from liver to peripheral tissues and regulate de novo cholesterol synthesis at these sites. Therefore, there is great interest in factors that alter the composition and concentrations of these lipoproteins.

Serum tocopherol is carried primarily by the lipoproteins (7,8,9). Studies of the relationships between vitamin E and cholesterol distribution and concentration in plasma lipoproteins give equivocal data. Sundaram et al. (7) studied 26 patients who received 600 mg α -tocopherol per day for 21 days. Serum samples were collected before and after the treatment and HDL-cholesterol increased and LDL-cholesterol decreased due to the therapy. Chupukcharoen et al. (10) determined the effect of vitamin E deficiency on lipoprotein cholesterol distribution. An increase in plasma cholesterol was detected in LDL and VLDL, but not in the HDL fraction of the plasma lipoproteins. However, Hatam and Kayden (11) studied 11 subjects

supplemented with 800 mg of vitamin E daily. Although the serum cholesterol concentration did vary slightly in some subjects, the percentage of cholesterol in the HDL fraction was not significantly altered. The mechanism of vitamin E effect on serum cholesterol distribution is not clear. Perhaps, oxidative stress could increase serum cholesterol level and suppression of oxidative stress could decrease serum cholesterol level. Vitamin E deficiency is known to induce oxidative stress (12). Copper deficiency can cause oxidative stress (13,14) and increase plasma cholesterol level in rats (15). Other oxidative stressors (e.g., polychlorinated biphenyls (PCB) and phenobarbital) were found to increase serum cholesterol level (16). It seems that vitamin E affects serum cholesterol level via its role in oxidative stress.

It is well established that polyunsaturated fatty acids have a hypocholesterolemic action in humans and animals. Ibrahim and McNamara (17) conducted a study in guinea pigs to investigate the effects of saturated fat (lard) and unsaturated fat (corn oil) on plasma cholesterol level when diets supplied 20 en% as fat. In comparison to the lard diet, the corn oil diet resulted in a 34% reduction in plasma total cholesterol level ($p < 0.02$) and a 40% lower triacylglycerol level ($p < 0.02$). Also, feeding the corn oil decreased the percent cholesterol ester in both LDL and VLDL. The results of this study were consistent with that of Shepherd et al. (18) who determined the effects of saturated and polyunsaturated fats on the chemical composition of lipoproteins in eight normal male subjects. The fats supplied 40% energy. The polyunsaturated fat lowered both plasma cholesterol (23%, $p < 0.0001$) and triglyceride (14%, $p < 0.0001$) levels.

The present study reports the involvement and interactions between

increasing dietary linoleate and vitamin E status in regulating serum lipid levels. We hypothesized that increasing dietary linoleate could decrease serum lipid levels (e.g., total cholesterol, LDL-cholesterol and triglyceride) and vitamin E supplementation could increase HDL-cholesterol level. Dietary linoleate supplied either 11 or 18 en% and vitamin E levels are 0, 100 or 5000 ppm. Diets were fed to rats for 10 weeks and serum lipids measured and correlated with serum vitamin E level.

MATERIALS AND METHODS

Animals and diets

Seventy-two weanling male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 50-60 g were individually housed in stainless cages on a 12-h light-dark cycle. The room was maintained at 22°C and approximately 50% humidity. Rats were randomly assigned to experimental diets in a randomized block experimental design, and the blocks represented the days when the rats were sacrificed and samples were processed.

The experimental diets were nutritionally complete and supplied 30% of energy (en%) as fat (Table 1). Diets supplied 0, 100 or 5000 mg d,l- α -tocopheryl acetate/kg of diet. Dietary fat was composed of different proportions of tocopherol-stripped corn oil and tocopherol-stripped lard to provide 11 or 18 en% linoleate, with cholesterol added as required to provide equal amount of cholesterol in all diets. Fatty acid composition of fat sources was analyzed by gas chromatography (20) (Table 2). Six groups of rats were fed ad libitum with 12 rats/diet for 10 weeks. Tap water was available ad libitum. Food consumption and body weight were measured weekly. After 10 weeks of feeding, rats were fasted overnight and anesthetized with ether, blood was drawn from exposed jugular veins, and then rats were killed by ether overdose. Eight milliliters of blood was drawn from exposed jugular veins into a syringe containing 1 ml acid citrate dextrose anticoagulant (4.5 g% sodium citrate, 2.7 g% citric acid (monohydrate) and 3.6 g% dextrose) for platelet preparation. Two milliliters of blood was drawn from exposed jugular veins for serum preparation.

Preparation of platelets

Platelets were isolated from the uncoagulated blood by centrifugation using a modification of the procedure of Chan et al. (21). Blood samples from two animals in the same dietary group were pooled to obtain enough blood from which to prepare platelets. Blood was first centrifuged at 100x g for 15 min at room temperature, and then the supernatant platelet rich plasma (PRP) was recentrifuged at 150x g for 15 min to remove contaminating red and white blood cells. Finally, the PRP was centrifuged at 1000x g for 15 min to sediment the platelets. The platelet poor plasma (PPP) was removed, 1 ml of Dulbecco's phosphate buffered saline (PBS), 2.7 mM KCl, 1.5 mM KH₂PO₄, 138 mM NaCl, 8.1 mM Na₂HPO₄, pH 7.5, (Sigma Chemical Co., St. Louis, MO) was added, and the platelets were resuspended by gentle vortexing. The platelets were resedimented by centrifugation at 1000x g for 15 min. The Dulbecco's PBS was discarded and 3 ml of fresh Dulbecco's PBS was added and , again, the platelets were resuspended by gentle vortexing. The purity of platelet suspensions was determined by Coulter Counter (Counter Electronics, Inc., Hialeah, FL). The purity was at least 95%. The platelet suspensions were stored at -80°C.

Preparation of serum

Following collection, blood samples from two animals in the same dietary group were pooled and allowed to clot by incubation for 1 hr at 37°C. Serum was removed after centrifugation and stored at -80°C.

Platelet fatty acid analyses

Fatty acid analyses were performed by methods described by Krumhardt (20) and Morrison and Smith (22). Analyses were done under

nitrogen. Fatty acid methyl esters were quantified by gas chromatography on a Varian 330 gas chromatograph (Walnut Creek, CA) equipped with a 3390A Hewlett Packard Integrator (Avondale, PA). An Alltech (Deerfield, IL) 183x0.32 cm column loaded with a 10% 100/120 mesh Silan 10C stationary phase was used. Nitrogen carrier gas flow rate was 30 ml/min. Column temperature was 180°C, injection port temperature was 220°C and flame ionization detector temperature was 250°C. Peaks were identified by comparison of retention times with those of authentic fatty acid methyl ester standards (Alltech, Deerfield, IL). The percentage of each fatty acid was determined by integration of peak areas.

Vitamin E analysis

Serum α -tocopherol content was determined using a modification of the procedure of Catignani and Bieri (23). All samples were processed in a room illuminated by yellow light. Fifty μ l of internal standard (α -tocopheryl acetate in ethanol, 59.6 mg/l) and 100 μ l serum were mixed by vortexing for 1 min. For extraction of the lipid, 200 μ l HPLC grade hexane was added and mixed for another 1 min. Phases were separated by centrifugation at 2000 rpm for 2 min to separate phases. The hexane layer was withdrawn and evaporated under nitrogen. The residue was redissolved in 70 μ l filtered HPLC grade methanol by mixing and thirty μ l was injected. Chromatography was performed at ambient temperature. Reversed-phase HPLC was carried out on a 3.9 mm x 30 cm stainless steel packed with micro Bondapak C-18. The detector wavelength was 290 nm, sensitivity 0.01 absorbance units full scale. The solvent was HPLC grade methanol : water, 95 : 5, the flow rate was 2.0 ml/min, and recorder chart speed, 1 cm/min. Peak-area ratios of samples were

converted to μg 's α -tocopherol by a standard curve prepared with samples containing a constant amount of α -tococpherol acetate combined with different amounts of α -tocopherol standard. The least amount of α -tocopherol can be detected in serum is 4 $\mu\text{g}/\text{ml}$.

Serum cholesterol assays

Serum total cholesterol was determined enzymatically by using Sigma Diagnostics cholesterol reagent (Sigma, St. Louis, MO) in a modification of the method of Allain et al. (24). Serum HDL-cholesterol was determined by using Sigma Diagnostics HDL-cholesterol reagent. It is formulated according to the recommendations of Warnick et al. (25). Dextran sulfate and Mg ions were used to precipitate LDL and VLDL. Serum triglyceride was determined by using Sigma Diagnostics triglyceride reagent in a modification of the method of McGowan et al. (26). LDL-cholesterol was calculated according to the formula developed by Friedewald et al. (27): $\text{LDL-cholesterol} = \text{Total cholesterol} - \text{HDL-cholesterol} - \text{triglycerides}/5$.

Protein analysis

Protein was measured by the method of Lowry et al. (28) using bovine serum albumin as the standard.

Statistical analysis

All analyses were done in duplicate for each sample. Data were analyzed by using General Linear Model (SAS Institute, Cary, NC). Least Square Difference of the Statistical Analysis System (SAS Institute, Cary, NC) was used to test the significance between means of treatments. Two-way ANOVA analysis was used to determine the interaction between increasing dietary linoleate and vitamin E supplementation. A $p < 0.05$ was taken to be

statistically significant.

RESULTS

Animals and diets

There was no difference in food intake, liver weight, and body weight gain of rats fed 11 or 18 en% linoleate (Fig. 1). The liver weight as percentage of body weight was not different due to en% linoleate (Fig. 2). There was no difference in food intake and body weight gain of rats fed 0, 100 or 5000 ppm vitamin E, however, liver weight was significantly greater in the group receiving 5000 ppm vitamin E than in the group receiving 100 ppm vitamin E (10.4 ± 0.8 vs 9.7 ± 0.7 g) (Fig. 3). The liver weight as a percentage of body weight was significantly greater in the group receiving 5000 ppm vitamin E than in the group receiving 100 ppm vitamin E (2.6 ± 0.1 vs $2.5 \pm 0.1\%$) (Fig. 4).

Serum vitamin E

Vitamin E level was measured in serum. There was no difference in serum vitamin E of rats fed 11 or 18 en% linoleate (12.5 ± 12.2 vs 11.8 ± 12.2 $\mu\text{g/ml}$) (Fig. 5). There was significant difference in serum vitamin E of rats fed 0, 100 or 5000 ppm vitamin E (non detectable, 8.8 ± 2.0 , 27.1 ± 3.9 $\mu\text{g/ml}$, respectively) (Fig. 6).

Lipid status

Serum total cholesterol, HDL-cholesterol, LDL-cholesterol and HDL-cholesterol/LDL-cholesterol ratio (106.8 ± 5.9 vs 103.1 ± 7.9 mg/dl; 81.2 ± 8.9 vs 78.9 ± 8.0 mg/dl; 10.7 ± 3.2 vs 13.3 ± 6.0 mg/dl; 8.1 ± 3.1 vs 7.4 ± 3.3) were not different in rats fed 11 or 18 en% linoleate (Fig. 7). But, serum triglyceride level was significantly greater in the 11 en% linoleate group than the 18 en% linoleate group (76.6 ± 11.8 vs 67.4 ± 13.7 mg/dl) (Fig. 7). There were no

differences in total cholesterol, triglyceride and LDL-cholesterol concentrations of rats fed 0, 100 or 5000 ppm vitamin E (102.8 ± 8.5 , 104.6 ± 6.8 , 107.6 ± 5.5 mg/dl, respectively; 72.0 ± 10.9 , 69.3 ± 13.2 , 74.8 ± 16.3 mg/dl, respectively; 12.9 ± 5.0 , 12.2 ± 5.0 , 10.9 ± 5.0 mg/dl, respectively) (Fig. 8). But, HDL-cholesterol level was significantly greater in the rats fed 100 and 5000 ppm vitamin E than in the vitamin E-deficient rats (81.6 ± 5.7 , 84.9 ± 4.8 , 73.6 ± 9.8 mg/dl, respectively). The HDL-cholesterol/LDL-cholesterol ratio was significantly greater in the group fed 5000 ppm vitamin E than in the groups fed 100 and 0 ppm vitamin E (9.9 ± 3.9 , 7.1 ± 2.4 , 6.2 ± 1.8 , respectively) (Fig. 8).

DISCUSSION

Vitamin E concentration was determined in serum. Different en% linoleate did not affect serum vitamin E level, but different vitamin E treatments caused significantly different serum vitamin E levels (Fig. 5,6). These results were consistent with those of Lehmann and Dymysza and Park (29,30) who found that plasma α -tocopherol values progressively increased with larger doses of dietary α -tocopherol.

It is well established that cholesterol has an important role in atherosclerosis. Epidemiological studies have shown an inverse association of HDL-cholesterol with the incidence of coronary heart disease (CHD). Castelli et al. (31) conducted a study including 7000 men and women from five populations in the US. The mean HDL-cholesterol concentration was lower in subjects with CHD than in subjects without CHD. The difference was small (3-6 mg/dl) but statistically significant. In another portion of the Framingham study, nearly 3000 men and women 49-82 years old were followed for about four years (32). Plasma HDL-cholesterol level was shown to be the most potent lipid risk factor in both sexes ($p < 0.0001$). In Israel, Goldbourt and Medalie (33) conducted a five-year prospective study included approximately 10,000 men 40 years or older. Serum HDL-cholesterol level was measured in an apparently representative subsample of 6562 men. A significant inverse correlation between HDL-cholesterol and subsequent CHD was observed in men over 45 but not in those under 45 years old.

In the present study, vitamin E deficiency significantly diminishes serum HDL-cholesterol concentration. The mechanism of action of vitamin E deficiency to alter HDL-cholesterol is not clear, but vitamin E may regulate

HDL-cholesterol synthesis, transport and/or breakdown. Vitamin E deficiency causes oxidative stress (12). Katayama and coworkers (16) showed that xenobiotic oxidative stressors such as phenobarbital increase serum total cholesterol, HDL-cholesterol and LDL-cholesterol. However, under the oxidative stress of vitamin E deficiency, HDL-cholesterol decreases, whereas LDL-cholesterol and total cholesterol are not altered. Perhaps oxidative stress stimulates HDL-cholesterol synthesis and transport. In human studies, the effect of vitamin E on lipoprotein cholesterol distribution has been controversial (7,17,34,35,36). This could be due to difficulty in controlling other factors known to influence lipoprotein levels, such as diet, body weight, smoking, exercise and drug effects (37).

The effect of dietary fat manipulation on plasma triglyceride levels has been studied by many laboratories. Durrington et al. (38) conducted a human study, and demonstrated a 19.7% reduction in serum triglyceride levels when a high-polyunsaturated diet (18 en%) was fed. McIntosh et al. (39) used monkeys to study the effect of polyunsaturated fat on plasma triglyceride concentration, they detected a significant fall in triglycerides when animals were supplemented with linoleate as a source of sunflower seed oil. The serum triglyceride results were consistent with those found in previous studies. The mechanism of dietary polyunsaturates on serum triglyceride level could be due to action of polyunsaturates on lipoprotein lipase. This enzyme of lipoprotein metabolism hydrolyzes unsaturated triglycerides faster than saturated triglyceride (40). Kinsell et al. (41) in a human study showed that there was an association between atherosclerosis and plasma C18:2/C18:1 ratio. Greater plasma C18:2/C18:1 ratio was related to lower atherosclerosis

incidence. Low platelet C18:2/C18:1 ratio was affected by en% linoleate and vitamin E supplementation in the present study. Greater en% linoleate and vitamin E supplementation resulted in higher platelet C18:2/C18:1 ratio. This indicates that en% linoleate and vitamin E supplementation might play a role in preventing atherosclerosis based on the observation of Kinsell et al. (41).

In conclusion, with the exception of serum triglyceride, dietary linoleate composition had little effect on serum lipid levels. Increasing dietary linoleate decreased serum triglyceride concentration. Vitamin E supplementation did not affect serum lipid levels. Vitamin E deficiency decreased serum HDL-cholesterol level. There was no interaction between increasing dietary linoleate and vitamin E status in lipid levels. The present study suggests that vitamin E deficiency can be atherosclerotic by virtue of its inhibitory effect on serum HDL-cholesterol level by mechanisms which are as yet unclear, but may be due to oxidative stress.

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Table 1. Composition of experimental diets

Ingredient	Energy Percentage Linoleate ^a	
	11	18
	----- g/100 g -----	
Lard, tocopherol-stripped ^b	7.2	—
Corn Oil, tocopherol-stripped ^b	7.2	14.3
Casein "Vitamin Free" Test ^b	22.4	22.4
Sucrose ^b	26.4	26.4
Corn Starch ^b	26.4	26.4
Cellulose ^b	5.0	5.0
AIN Mineral Mixture 76 ^{bc}	3.9	3.9
Vitamin Mixture (a-tocopherol devoid) ^{bd}	1.1	1.1
Tocopheryl Acetate ^b	0, 0.01, 0.5	0, 0.01, 0.5
L-Methionine ^b	0.3	0.3
Choline Chloride ^b	0.08	0.08
Ascorbic Acid (antioxidant) ^b	0.01	0.01
Cholesterol (mg/100 g) ^b	0	6.8

^aEach level of linoleate was fed with 0, 100 or 5000 ppm α -tocopheryl acetate.

^bTeklad, Madison, WI.

^cComposition described by the AIN Ad Hoc Committee on Standards for Nutritional Studies, 1977 (19).

^dComposition described by the AIN Ad Hoc Committee on Standards for Nutritional Studies, 1977 (19), except that vitamin E acetate was substituted by sucrose.

Table 2. Fatty acids composition of experimental diets

	11 en% linoleate	18 en% linoleate
	----- % -----	-----
14	1.0	—
15	0.1	—
16	18.1	10.2
16:1	2.1	—
18	5.5	1.8
18:1	33.8	23.9
18:2	37.5	62.0
18:3	1.1	2.1
20:1	0.6	—
20:2	0.2	—
P/S ratio	1.6	5.4
P/M ratio	1.1	2.7

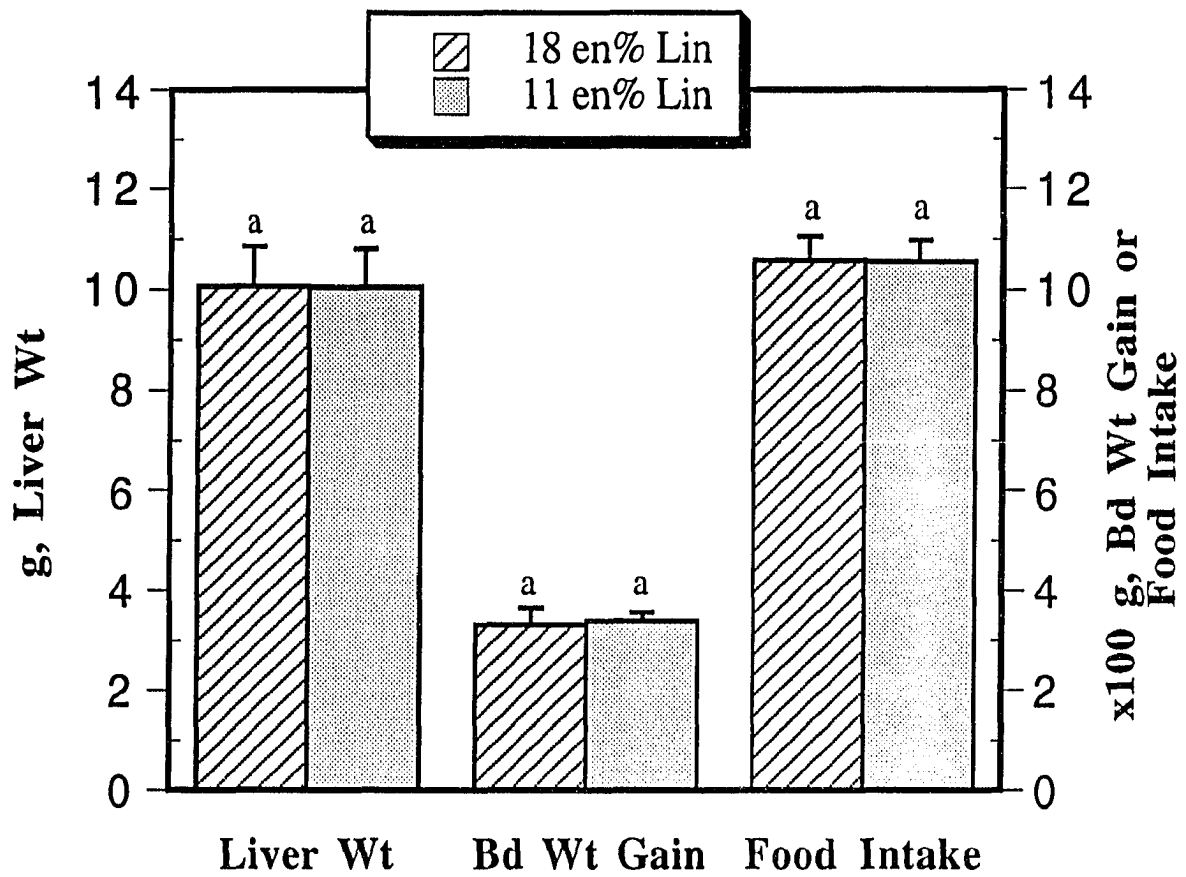


Figure 1. Effect of en% linoleate on liver weight, body weight gain and food intake. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

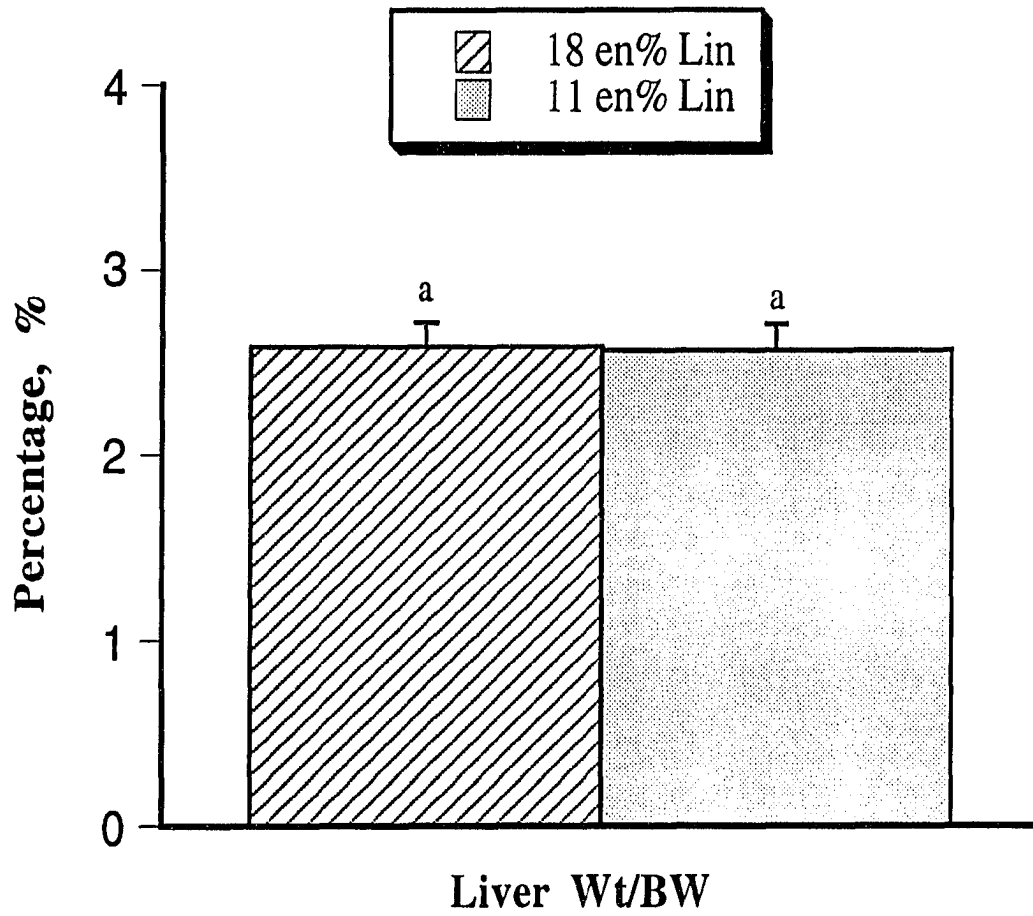


Figure 2. Effect of en% linoleate on liver weight as percentage of body weight . Groups that do not share the same letter are significantly different from one another, $p < 0.05$

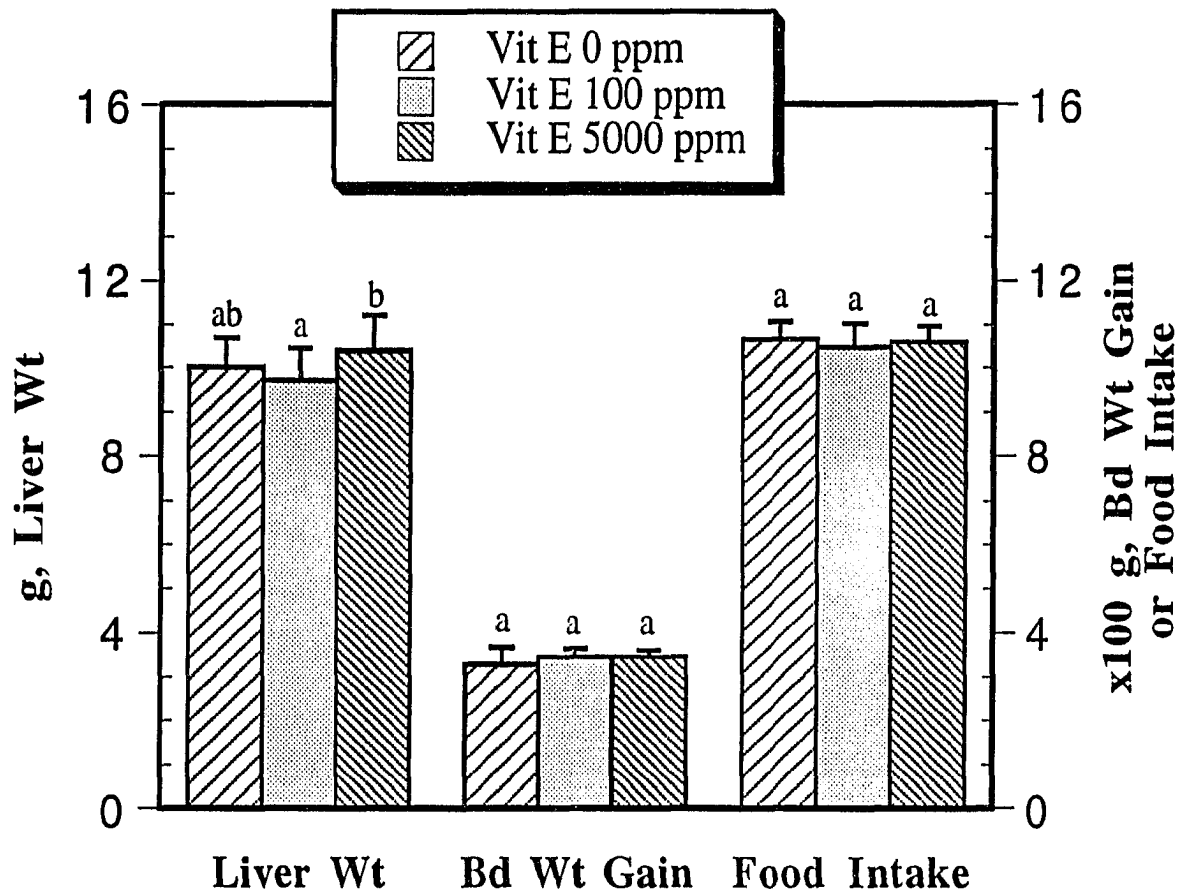


Figure 3. Effect of vitamin E status on liver weight, body weight gain and food intake. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

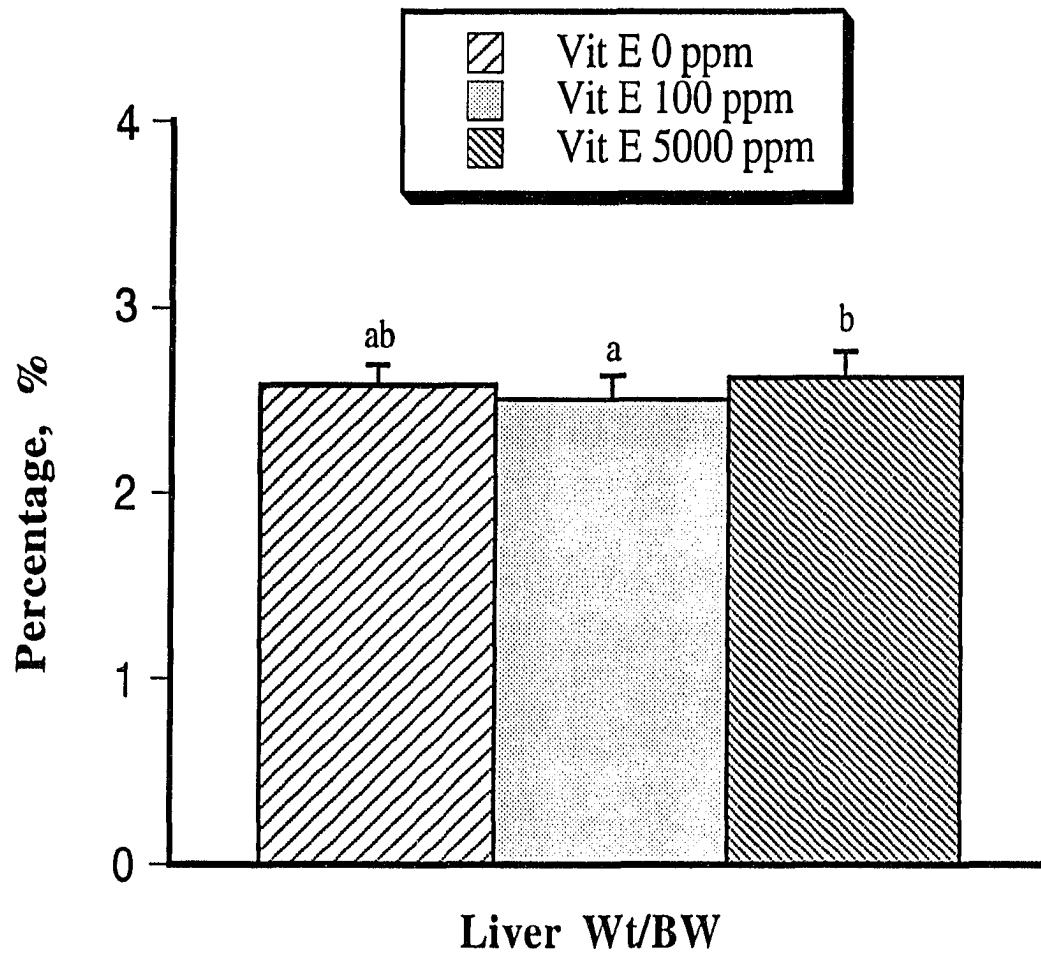


Figure 4. Effect of vitamin E status on liver weight as percentage of body weight. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

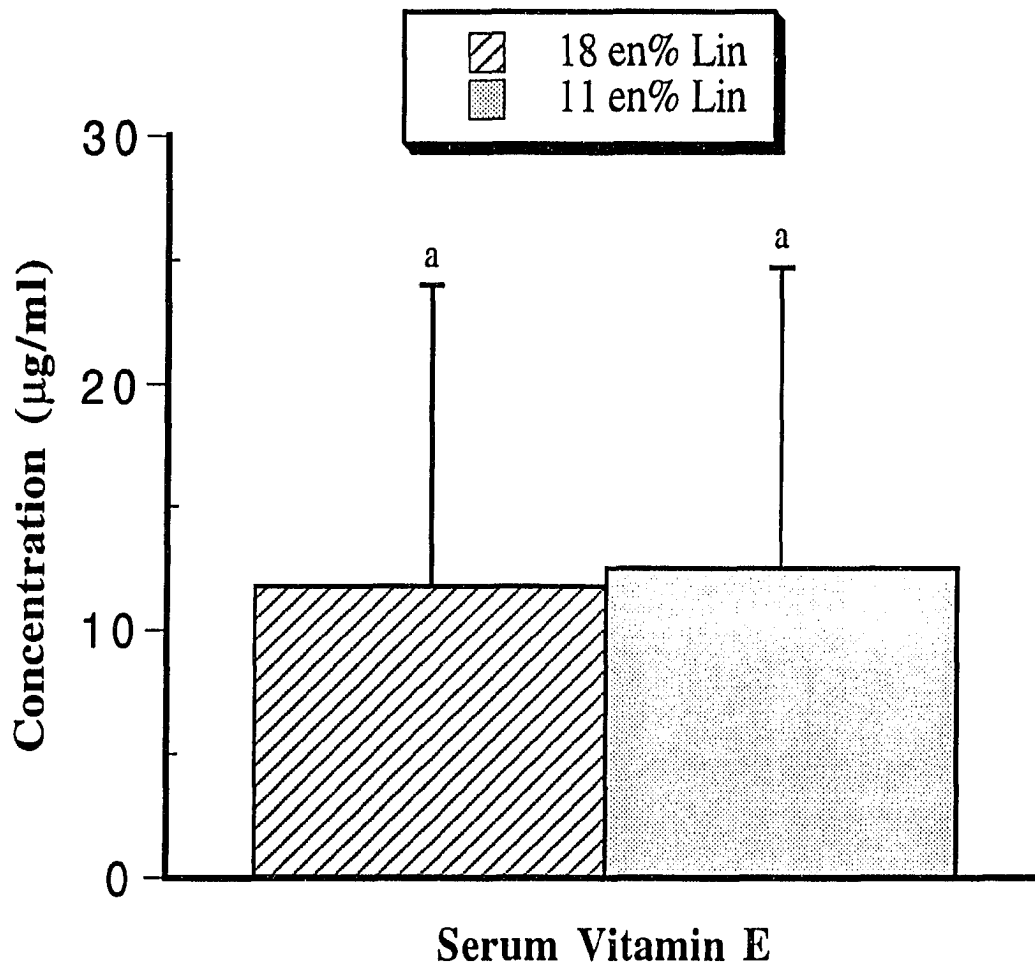


Figure 5. Effect of en% linoleate on serum vitamin E level. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

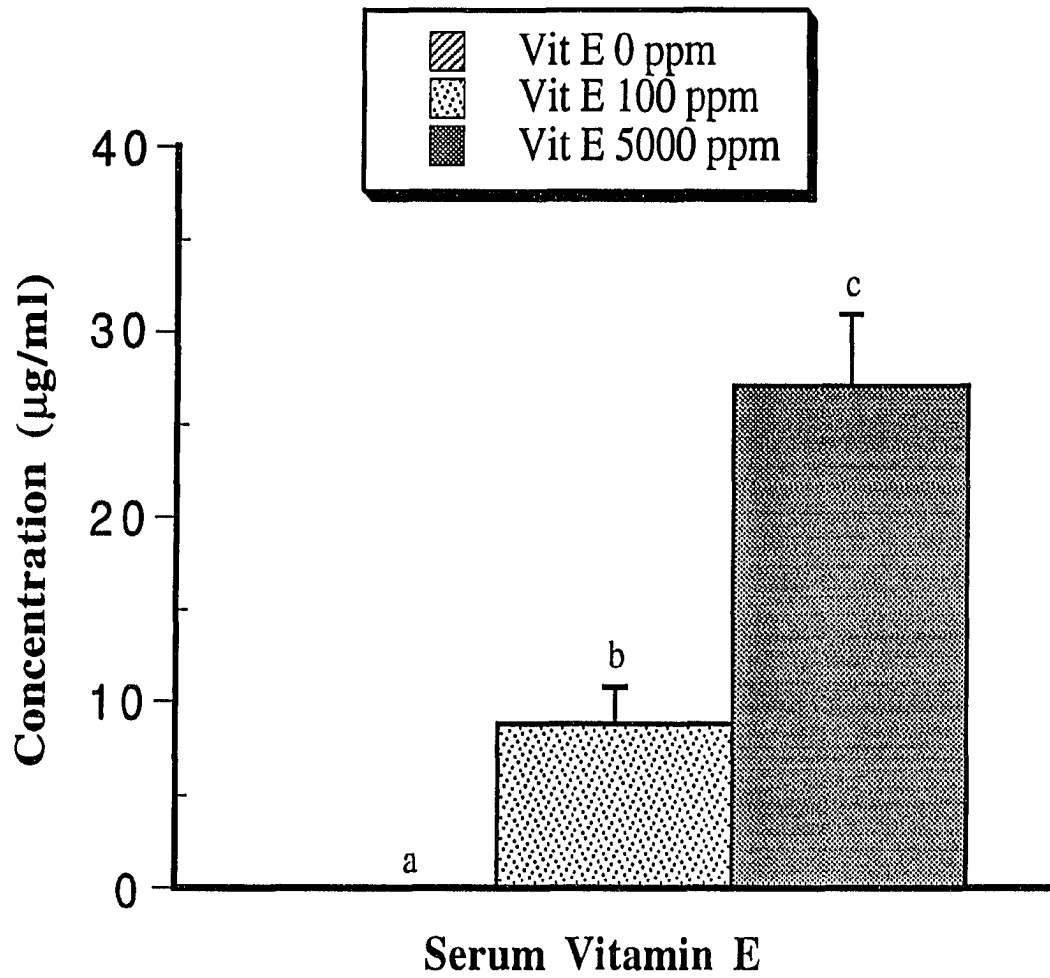


Figure 6. Effect of vitamin E supplementation on serum vitamin E level. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

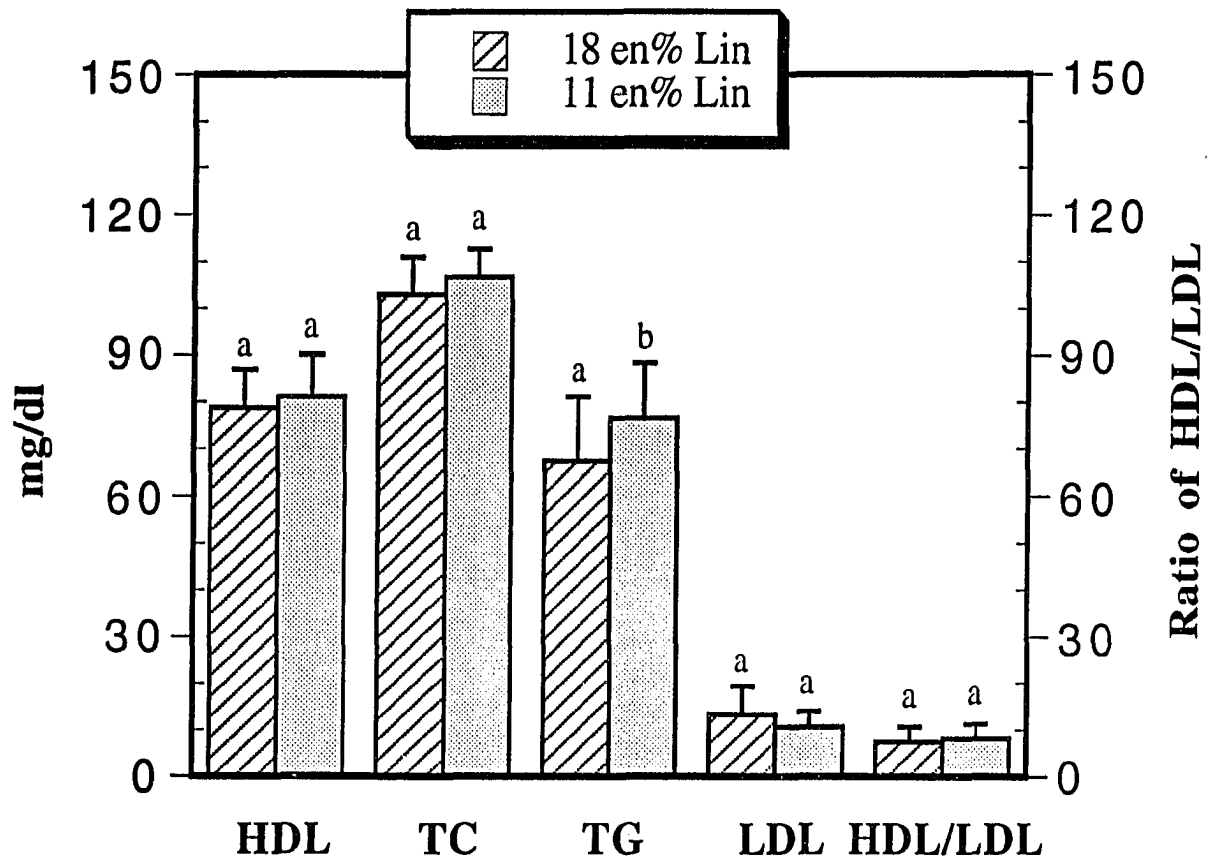


Figure 7. Effect of en% linoleate on serum lipid levels, and HDL-cholesterol/LDL-cholesterol ratio. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

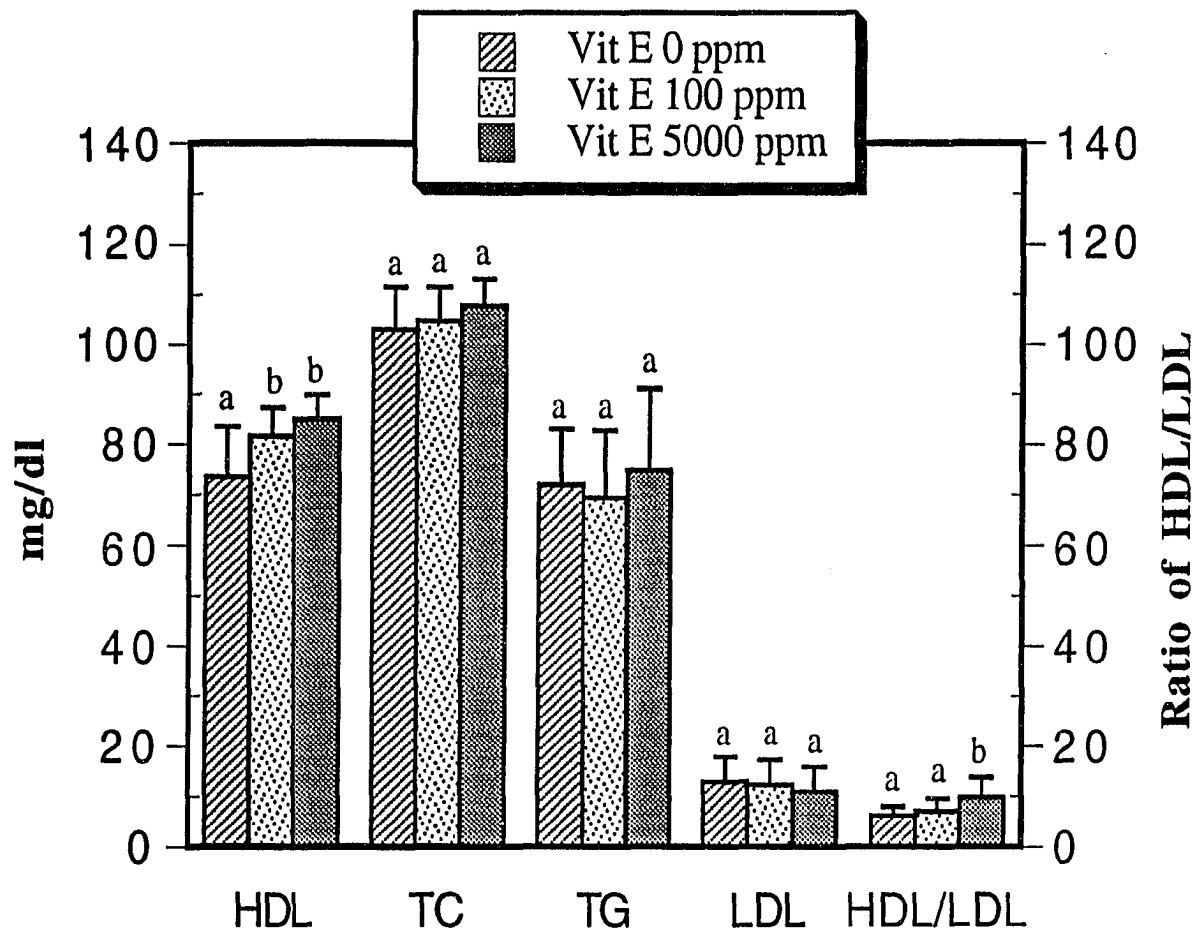


Figure 8. Effect of vitamin E status on serum lipid levels and HDL-cholesterol/LDL-cholesterol ratio. Groups that do not share the same letter are significantly different from one another, $p < 0.05$

GENERAL SUMMARY

Platelet Fatty Acids

The changes in platelet fatty acid composition due to different dietary treatments were consistent with results of Piche and Mahadevappa (50) and Iritani and Narita (52). Platelet arachidonic acid level was greatest in the vitamin E-deficient group. This suggests that vitamin E deficiency and other oxidative stressors could affect fatty acid desaturation and elongation. McIntosh et al. (88) observed that vitamin E increased or decreased platelet phospholipid arachidonic acid depending on dietary treatments. In a basal diet which supplied 1.2% (w/w) sunflower seed oil, vitamin E supplementation decreased platelet phospholipid arachidonic acid. But, after increasing sunflower seed oil to 10% (w/w), vitamin E supplementation increased platelet phospholipid arachidonic acid. However, in the present study, dietary linoleate ranging from 11 to 18% and vitamin E supplementation did not further stimulate platelet arachidonate. The role of vitamin E in fatty acid desaturation and elongation needs further study.

Prostaglandin Status

Effects of dietary fat on prostaglandin biosynthesis have been studied by Dupont et al. (55), Sullivan and Mathias (56) and Mathias and Dupont (57). Greater en% linoleate was not correlated with increased prostaglandin production in these studies, and 2 en% linoleate was found to be optimal for prostaglandin synthesis. Varying the dietary P/S ratios has also been found to affect prostaglandin synthesis (57). Dietary P/S ratios between 0.4 and 5.5 did not significantly affect serum prostaglandin levels while a dietary P/S ratio of

9 was found to increase serum prostaglandin levels markedly (89). In the present study, en% supplied by linoleate was in the range of 10 to 29 which had no effect on prostaglandin synthesis and the dietary P/S ratios, well within the ranges of 0.4 and 5.5, also did not affect prostaglandin production.

Studies examining the effect of dietary vitamin E on prostaglandin synthesis have not given a consistent picture. We found no effect of vitamin E on prostacyclin (PGI₂) synthesis in this study which was in agreement with the work of Stampfer et al. (46). Thromboxane A₂ production was increased by vitamin E deficiency. Karpen et al. reported the same results (48). Inconsistent results obtained by different laboratories could be due to different amount of vitamin E fed in the ranges of 0 to 1000 mg/kg diet, differences in feeding period from 8 to 23 weeks, species variation, for example rats, rabbits and humans and different tissue sampling and preparation. The balance between TXA₂ and PGI₂ is important, because platelet aggregation and vasoconstriction play important roles in atherosclerosis. The serum TXA₂/PGI₂ ratio was significantly greater in rats receiving no vitamin E compared with the ratio when 5000 ppm vitamin E was fed. Results of the present study indicates that vitamin E deficiency may have a prothrombotic effect through its role on TXA₂ status. Vitamin E deficiency can increase oxidative stress and platelet arachidonic acid concentration. Greater platelet arachidonic acid might increase TXA₂ production because arachidonate is the prostaglandin precursor. Therefore, vitamin E deficiency might increase TXA₂ in 2 ways by increasing TXA₂ precursor and by increasing oxidative stress-mediated TXA₂ production by as yet unknown mechanisms.

Platelet Phospholipase A₂ activity

Neither en% linoleate nor vitamin E supplementation affected platelet phospholipase A₂ activity. However, C18:2n-6 was negatively correlated with phospholipase A₂ activity ($r=-0.41$, $p<0.05$) which indicates an inhibition of phospholipase A₂ activity by polyunsaturated fatty acids. These results were observed in work by Ballou and Cheung (83). Tangney and Driskell (90) demonstrated that vitamin E inhibited platelet phospholipase A₂ activity. The mechanism by which vitamin E influences platelet phospholipase A₂ activity is not clear. In the present study, vitamin E did not affect endogenous platelet phospholipase A₂ activity. Perhaps these results differed from those of Tangney and Driskell because of the absence of thrombin-induction during the measurement of phospholipase A₂ activity.

Platelet and Serum Vitamin E Levels

Both platelet and serum vitamin E levels reflected dietary vitamin E. It has been long recognized that the amount of vitamin E required to prevent lipid peroxidation depends on the dietary intake of fat, particularly the intake of polyunsaturated fatty acids (91). In the present study, linoleate contributed 11 and 18 en%. Even though there was a difference in platelet linoleate levels, differences in platelet vitamin E concentration were not detected. It was indicated by Chan et al. (92) that the retention of tocopherol by rat platelets appeared to be logarithmically related to increasing vitamin E concentrations in the diets. The results in the present study tended to be logarithmic related to increasing dietary vitamin E concentration in agreement with the experiments of Chan et al.

Serum Lipid Levels

It is well established that cholesterol has an important role in atherosclerosis. HDL-cholesterol has an inverse relation with risk of coronary heart disease (5,6), while LDL has a positive relation with the incidence of coronary heart disease. In the present study, vitamin E deficiency caused significantly lower serum HDL-cholesterol concentrations. The mechanism by which vitamin E deficiency suppresses HDL-cholesterol is not clear but the lack of vitamin E stimulates oxidative stress, which might regulate HDL-cholesterol levels. Phenobarbital and Cu deficiency are oxidative stressors (95,96), both are found to increase serum and plasma total cholesterol concentrations. However, under the oxidative stress of vitamin E deficiency serum total cholesterol is not altered. The role of vitamin E and oxidative stress in the regulation of HDL-cholesterol needs further study.

Manipulations of dietary fat can affect plasma triglyceride levels. An increase in dietary PUFA will decrease triglyceride has been shown in many studies (88,93). McIntosh et al. (88) showed that the diets supplemented with linoleate produced a significant fall in plasma triglyceride levels. Durrington et al. (94) found that differences in en% from PUFA (4% vs 18%) produced significantly different serum triglyceride levels, and greater en% from PUFA resulted in less serum triglyceride. Serum triglyceride concentrations in our study were consistent with the results found by previous studies. The mechanism of the effect of dietary linoleate on serum triglyceride level could be due to the action of lipoprotein lipase on polyunsaturated fatty acids. This enzyme, involved in lipoprotein metabolism, hydrolyzes unsaturated triglycerides faster than

saturated triglycerides (94).

The present study suggests that vitamin E deficiency might be a cause of atherosclerosis because it can be prothrombogenic by virtue of its stimulatory effect on TXA₂ synthesis. Vitamin E deficiency can also suppress HDL-cholesterol. 11 en% linoleate between 11 and 18 en% did not affect prostaglandin status but it did decrease serum triglyceride level when 18 en% linoleate was substituted for 11 en% linoleate. Dietary linoleate and vitamin E status did not interact in altering atherosclerotic risk factors. Vitamin E supplementation did not seem to benefit any of the atherosclerotic risk factors studied.

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