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**LA THÈSE A ÉTÉ
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Modulation of Rat Platelet Phospholipase A₂ and 12-Lipoxygenase
Activities by Dietary Vitamin E

by

Cheryl E. Douglas

A Thesis submitted to the school of Graduate Studies and Research of the
University of Ottawa in partial fulfillment of the requirement for the
degree of Master of Sciences.

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SUMMARY

Eicosanoids, derived from oxygenation of arachidonic acid in platelets have potent biological activities and influence such pathological processes as platelet aggregation and inflammatory responses. In this study, we demonstrated that vitamin E, a membrane-bound antioxidant, modulates eicosanoid biosynthesis in rat platelets. Specifically, rats supplemented with three different levels of D- α -tocopheryl acetate (0, 100, 1000 ppm) in their diet were used as models to examine the effect of vitamin E on arachidonate release by phospholipase A₂ and on arachidonate oxygenation by 12-lipoxygenase. After approximately one year on their respective diet, the platelet phospholipase A₂ activity and 12-lipoxygenase activity was determined for each group of rats.

Platelets from rats fed 100 or 1000 ppm vitamin E exhibited diminished phospholipase A₂ activity compared to those from rats fed a vitamin E-deficient diet. Addition of vitamin E to sonicated platelet suspensions resulted in further suppression of the phospholipase A₂ activity in all three groups of rats. In order to gain further insight into the possible mechanism of vitamin E action on platelet phospholipase A₂ activity, this enzyme was partially purified by gel filtration chromatography and the effect of various tocopherol analogues on this form of the enzyme was examined. Enzyme activity was localized in the soluble supernatant fraction of a high speed spin, had an absolute requirement for Ca⁺², and was inhibited by various analogues of tocopherol. Tocol inhibited this partially purified phospholipase A₂ to a greater extent than either D or DL- α -tocopherol, while DL- α -tocopheryl acetate had little effect on enzyme activity. These results stress the importance of the hydroxyl group on the chromanol ring for the inhibitory action of vitamin E on platelet phospholipase A₂

compared to the chromanol ring methyl groups, which are absent in tocopherol. Inhibition of platelet phospholipase A₂ by vitamin E suggests a crucial role for this vitamin in regulating arachidonate release from membrane phospholipids and its subsequent metabolism to the biologically active eicosanoids.

12-lipoxygenase, responsible for the production of 12-hydroperoxy-eicosatetraenoic acid (12-HPETE) and 12-hydroxyeicosatetraenoic acid (12-HETE) in platelets, was also affected by the dietary level of vitamin E in rats. Platelet lipoxygenase activity from rats fed a vitamin E-free diet and rats fed a vitamin E-excess diet (1000 ppm) was significantly enhanced compared to rats fed an adequate vitamin E diet (100 ppm). Furthermore, a stimulation of lipoxygenase activity was noted in platelets from deficient rats that were refed a diet containing 100 ppm vitamin E for several days. Both 12-HPETE and 12-HETE mediate inflammatory reactions via their effects on leukocyte lipoxygenase activity and on neutrophil chemotaxis, respectively. Since vitamin E is capable of modulating the activity of these key enzymes in arachidonate metabolism, it should have potential in regulating platelet-leukocyte interactions.

TABLE OF CONTENTS

	PAGE
Acknowledgement	i
Summary	ii
List of Figures	vi
List of Tables	vii
INTRODUCTION	1
The Biological Roles of Vitamin E	2
Platelet Eicosanoid Biosynthesis	8
Platelet Phospholipase A ₂ Activity	12
Platelet 12-Lipoxygenase Activity	15
Vitamin E, Eicosanoid Biosynthesis and Platelet Function	16
EXPERIMENTAL DESIGN	19
MATERIALS AND METHODS	20
Materials	20
Animal Care and Diet	20
Determination of Vitamin E Status of rats	22
Platelet Isolation	24
Platelet Phospholipase A ₂ Assay	27
Platelet Phospholipase A ₂ Purification	28
Platelet 12-Lipoxygenase Assay	28
Statistical Analysis	30

	PAGE
RESULTS	31
Vitamin E Status of Rats	31
Effect of Dietary Vitamin E on Platelet Phospholipase A ₂ Activity	31
Partially Purified Phospholipase A ₂ and Vitamin E	35
Rat Platelet 12-Lipoxygenase Activity	39
Effect of Vitamin E on Platelet Lipoxygenase Activity	39
Vitamin E Refeeding and Platelet Lipoxygenase Activity	43
DISCUSSION	45
Vitamin E and Platelet Phospholipase A ₂ Activity	46
Vitamin E and Platelet Lipoxygenase Activity	49
CONCLUSION	53
REFERENCES	54

LIST OF FIGURES

FIGURE		PAGE
1	Initiation and Propagation Reactions of Lipid Peroxidation	4
2	Enzyme-catalyzed Peroxidation and its control by Antioxidants	6
3	Structural Interaction of Vitamin E with Membrane Components	7
4	Pathway of Arachidonic Acid Metabolism in Platelets	10
5	HPLC Chromatogram of α -tocopherol in Rat Plasma	25
6	Effect of D- α -tocopherol on Phospholipase A ₂ Activity	33
7	Effect of Tocol on Phospholipase A ₂ Activity	34
8	Elution Profile of Platelet Phospholipase A ₂	36
9	Ca ⁺² Curve of Partially Purified Platelet Phospholipase A ₂	37
10	Protein Curve of Partially Purified Platelet Phospholipase A ₂	37
11	Inhibition of Partially Purified Phospholipase A ₂ by Tocopherol and some of its Analogues	38
12	Radio-TLC Scan of Arachidonate Metabolites via Lipoxygenase	40
13	Time Course of Arachidonate Conversion by 12-Lipoxygenase	41
14	Dietary Vitamin E and 12-Lipoxygenase Activity	42
15	Effect of Vitamin E Refeeding on 12-Lipoxygenase Activity	44
16	Structure of some Tocopherol Analogues	48
17	Interaction of 12-Lipoxygenase Products with other Enzymes of the Arachidonate Cascade	52

LIST OF TABLES

TABLE		PAGE
1	Composition of Rat Diet	21
2	Pyruvate Kinase Assay	23
3	Sulfo-phosphovanillin assay for determination of total Plasma Lipids	26
4	Purification table of Rat Platelet Phospholipase A ₂	29
5	Plasma Pyruvate Kinase Activity, Tocopherol Concentration and Lipid Levels from Rats fed different levels of Vitamin E	32

INTRODUCTION

Once released from platelet membrane phospholipids, arachidonate undergoes a complex series of enzymatic transformations into biologically active eicosanoids. These substances play specific roles in mediating blood clotting, inflammation, and may influence the development of pathological disorders such as atherosclerosis. Vitamin E, a biological antioxidant, has been implicated in the eicosanoid biosynthetic pathway since the enzymatic reactions yielding eicosanoids proceed via free radical initiated mechanisms. Because the level of this vitamin in tissues can be manipulated by dietary means, it is of particular importance to study its role in arachidonate metabolism. In fact, earlier studies in this laboratory have shown that dietary vitamin E inhibits the formation of thromboxane B₂, the main cyclo-oxygenase product, in rat platelets (St. Jacques-Hamelin and Chan, 1983). An alternate pathway for arachidonate metabolism in platelets is catalyzed by 12-lipoxygenase, resulting in the formation of 12-hydroperoxyeicosatetraenoic acid (12-HPETE). In this study, the effect of vitamin E on 12-lipoxygenase activity in rat platelets was examined. In addition, the influence of tocopherol and some of its analogues on platelet phospholipase A₂, one of the enzymes responsible for supplying free arachidonate for both cyclo-oxygenase and 12-lipoxygenase activity, was studied. The ability of vitamin E to influence specific biochemical processes through its dual function as membrane stabilizer and antioxidant in biological systems will be discussed throughout this thesis.

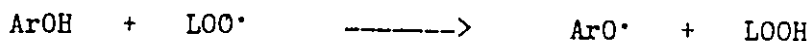
The Biological Roles of Vitamin E

Vitamin E is a generic description for a mixture of eight naturally occurring isomers of tocopherol, of which α -tocopherol is the most predominant and most biologically active in animal tissues. The tocopherol molecule consists of a polar chromanol ring to which is attached a nonpolar hydrocarbon tail, referred to as a phytyl chain. Since its discovery as an essential nutrient in 1922 (Evans and Bishop, 1922), a lack of vitamin E has been associated with pathological disorders of the vertebrate reproductive system, hematopoietic tissue, nervous system, cardiovascular system, skeletal muscle, and liver (Mason, 1954; Follis, 1958). The precise mechanism of action of vitamin E in these pathological processes, however, remains to be elucidated. Although tocopherol is a known biological antioxidant (Tappel, 1962; Tappel, 1980), it is unclear as to whether all of its actions can be accounted for in molecular terms solely on the basis of its antioxidant property. It has been hypothesized that, in addition to its antioxidant function, vitamin E plays a structural role in membrane stabilization by virtue of specific physical and chemical interactions between the tocopherol molecule and membrane phospholipids (Lucy, 1972; Diplock and Lucy, 1973). This dual role of vitamin E in biological systems is the subject of this section.

Auto-oxidation of biological membranes is potentially disastrous to organisms since many biochemical reactions required for cell function are altered. Since the lipids that are most susceptible to peroxidation are located in the membrane and form non-polar regions within the cell, a fat-soluble antioxidant which terminates free radical reactions is important for membrane stability. Tocopherol fulfills this requirement and, in addition, is relatively non-toxic at levels several times higher than

requirement.

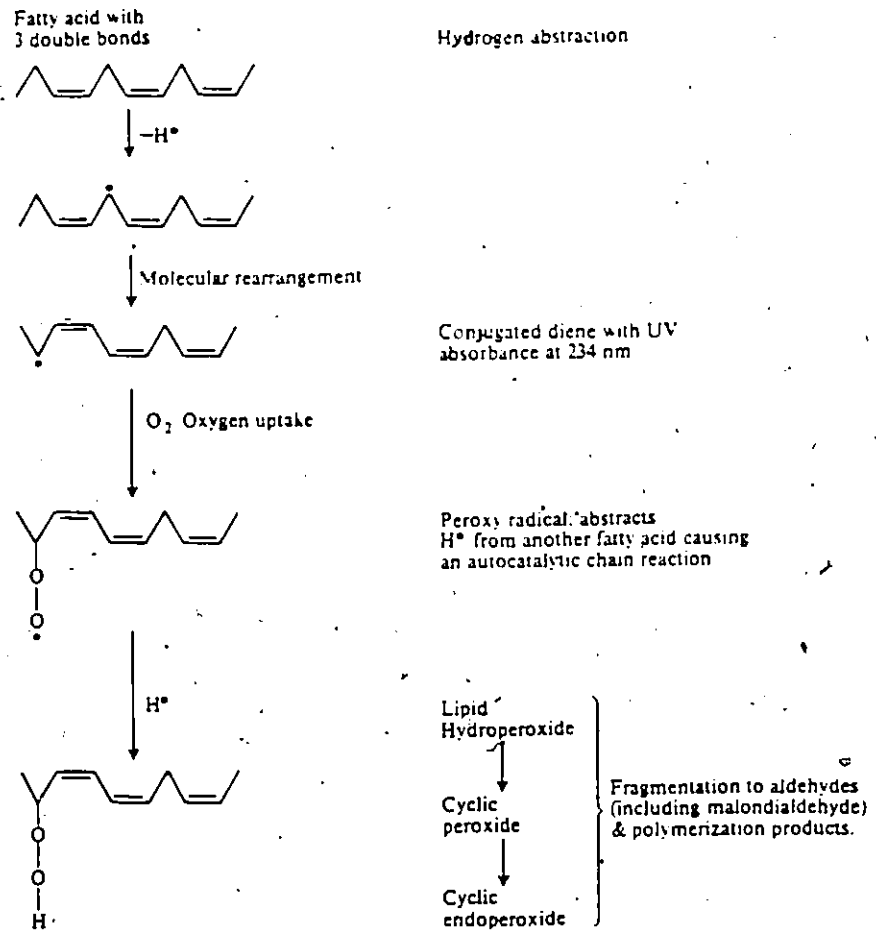
Autocatalytic lipid peroxidation proceeds via the non-enzymatic, free radical-generated mechanism outlined in figure 1 (Halliwell and Gutteridge, 1985). Highly reactive species capable of abstracting a hydrogen atom from a methylene group ($-\text{CH}_2-$) can attack unsaturated fatty acids to form lipid radicals. These reactive species include lipid free radicals (L^\cdot), lipid peroxy radicals (LOO^\cdot), protonated superoxide (HO_2^\cdot) ozone (O_3), nitrogen dioxide (NO_2^\cdot) or, more commonly, hydroxyl radical ($\cdot\text{OH}$) generated in vivo from hydrogen peroxide (H_2O_2). The lipid radical, after undergoing a molecular rearrangement, reacts with molecular oxygen to form a lipid peroxy radical which can abstract a hydrogen atom from another unsaturated fatty acid, and convert itself to a lipid hydroperoxide. Pure hydroperoxides are relatively stable in vivo, but in the presence of transition metal complexes, they can be decomposed into hydrocarbon gases and malonaldehyde which is found in the age pigment, lipofuscin. Free radical scavengers such as tocopherol protect against the damaging effects of peroxidation by interrupting the autocatalytic chain reaction. Tocopherol (ArOH) is capable of donating a hydrogen atom to the lipid peroxy radical (LOO^\cdot), giving rise to a relatively stable tocopheryl radical (ArO^\cdot) as shown in the following scheme:



The tocopheryl radical is too unreactive to propagate the chain reaction, (Burton and Ingold, 1981; Witting, 1980) but is degraded to tocopherylquinone and other products or is reduced back to tocopherol by ascorbate (Slater, 1984). Because tocopherol breaks the chain of auto-oxidation, the overall rate of lipid peroxidation decreases in the presence of this antioxidant. Evidence for the antioxidant behavior of vitamin E is

Figure 1: Initiation and Propagation of lipid peroxidation. The peroxidation of a fatty acid with three conjugated double bonds is shown (Halliwell and Gutteridge, 1985).

FIGURE 1



provided by studies which show 1) the ability of synthetic antioxidants such as diphenyl-p-phenylenediamine (DPPD), otherwise unrelated to vitamin E, to substitute for the vitamin in preventing certain deficiency symptoms (Draper and Csallany, 1958) and 2) the inhibition by vitamin E of the auto-oxidation of lipids, in vitro (Lucy, 1969).

A unified hypothesis relating the antioxidant behavior of vitamin E with membrane-bound enzyme systems such as NADPH oxidase, capable of initiating lipid peroxidation by generating superoxide and hydroperoxide, has been proposed (McKay and King, 1980) and is outlined in figure 2. It should be noted that, in this scheme, glutathione peroxidase, a selenium-containing enzyme, plays an important role in reducing peroxide formation. It is not surprising, then, that selenium provides a "sparing" effect on the vitamin E requirement (Chow, Reddy and Tappel, 1973) by playing a role, in eliminating hydroperoxide accumulation, and interrupting the sequence of events leading to hydroxyl and lipid free radical formation. Although the soluble enzymes, catalase and glutathione peroxidase, control the level of hydroperoxide in the cytosol, it is expected that some of these destructive molecules, generated in the membrane, may remain in the non-aqueous phase of the cell to form highly reactive hydroxyl radicals. According to this proposed scheme, the presence of the lipid-soluble free radical scavengers, such as tocopherol, are necessary to quench hydroxyl and lipid radical formation.

Because of its amphipathic nature, with a polar head group and an apolar tail, tocopherol can easily partition into the lipid bilayer structures of biological membranes (figure 3). Lucy hypothesized that the methyl groups on the tocopherol phytyl chain fits into "pockets" created by the cis double bonds of the unsaturated fatty acyl chains, and the chromanol ring is positioned in the polar region of the membrane, adjacent to the phospholipid head groups

Figure 2: Diagrammatic representation of the events which appear to be involved in enzyme-catalyzed lipid peroxidation and its control by antioxidants (McKay and King, 1980).

FIGURE 2

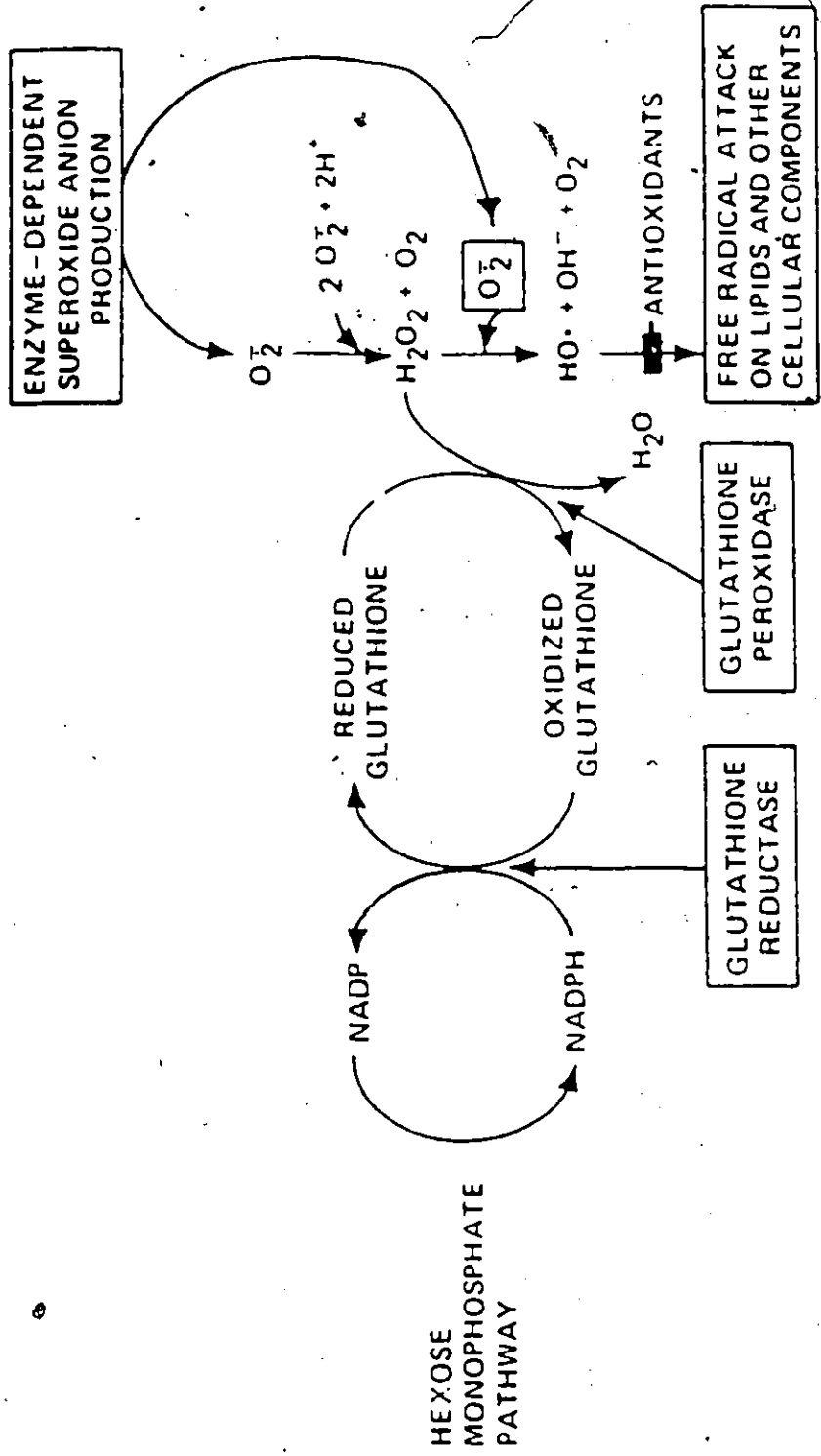


Figure 3: Diagrammatic representation of the proposed interactions between vitamin E, polyunsaturated phospholipids, and proteins in a biological membrane. For simplicity, the phospholipids are shown as rigid structures in a bilayer membrane, but this does not imply that the membrane has a "crystalline" rather than a partially fluid character (Diplock and Lucy, 1973).

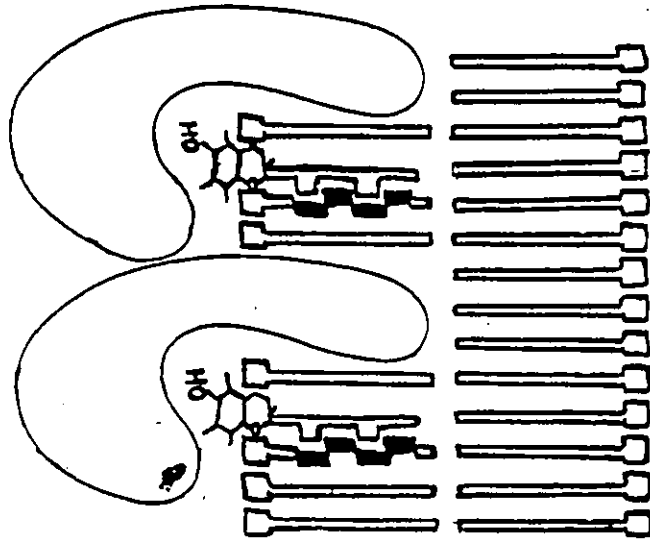


FIGURE 3

-8-

(Lucy, 1972; Diplock and Lucy, 1973). This model is not dissimilar to that proposed for the interaction of cholesterol with phospholipids in lipid bilayers (Huang, 1977). The close association of tocopherol with phospholipid constituents maximizes both polar and nonpolar interactions in the membrane, and has been shown to render greater stability to the membrane structure (Fukuzawa, Hayashi and Suzuki, 1977; Erin, Spirin, Fabidze and Kagan, 1984). Furthermore, vitamin E has been reported to decrease the permeability of unsaturated lipid bilayers (Diplock, Lucy, Verrinder and Zieleniewski, 1977; Diplock and Lucy, 1973; Stillwell and Bryant, 1983; Srivastava, Phadke, Govil and Rao, 1983), and to decrease platelet membrane fluidity by restricting the motion of the hydrocarbon chains of the phospholipids (Steiner, 1981). It has been proposed, as a result, that it is the structural interaction of vitamin E with membrane components that affords protection against tissue damage such as in the case of erythrocyte hemolysis (Lucy and Dingle, 1969) and lysosomal membrane leakage (Fukuzawa et.al., 1977) resulting from vitamin E deficiency.

Therefore, the unique physical and chemical properties of tocopherol enables structural interactions with membrane components which not only facilitates the antioxidant behavior of vitamin E, but directly influences certain membrane functions such as transport, permeability, and the activity of membrane-associated enzymes.

Platelet Eicosanoid Biosynthesis

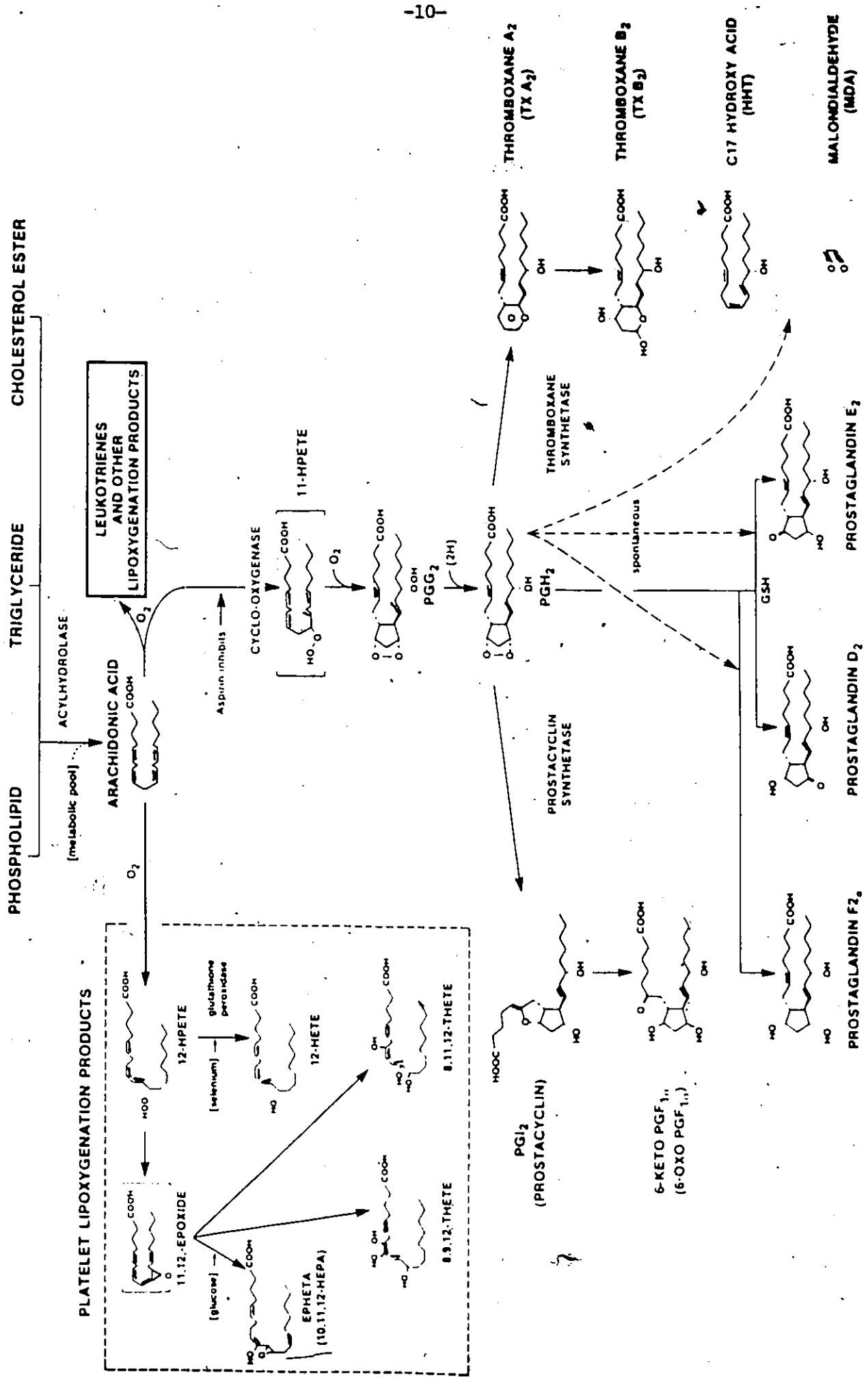
Platelets are anucleated cells derived from the fragmentation of megakaryocytes in bone marrow (Schick, Schick and Chase, 1981), and are considered as "the most sensitive of all cells in circulating blood to

chemical and physical agents" (White, 1968). Most platelet interactions are membrane-associated phenomena, such as their ability to respond to blood vessel damage and, in the process, release biologically active substances. A large body of evidence indicates that arachidonic acid, released from membrane phospholipids after platelet activation, is converted to a group of compounds, collectively termed eicosanoids, which play a significant role in platelet physiology (Smith and Willis, 1971; Smith, Ingeman, Kocsis and Silver, 1974; Malmsten, Hamberg, Svensson and Samuelsson, 1975). Although these compounds are "hormone-like" in action they differ from hormones in that their sites of synthesis are in the cells upon which they act, and they are not stored in tissue but are synthesized as a result of membrane perturbations that cause arachidonate release.

Following their release from esterified lipid by acyl hydrolases, arachidonic acid is oxygenated and converted to various compounds as depicted in figure 4 (Willis and Smith, 1981). The precise nature of these compounds depend on the tissue from which the fatty acid is released. For example, leukotrienes are synthesized predominantly in polymorphonuclear leukocytes (PMNLs) via 5 and 15-lipoxygenases, and play a major role in inflammatory reactions by inducing leukocyte recruitment (Ford-Hutchinson, Bray, Doig, Shipley and Smith, 1980). Elevated levels of leukotrienes are found in various human disease states such as asthma, psoriasis, and rheumatoid arthritis (Samuelsson, 1983). In platelets, arachidonic acid is converted via two major pathways to form active oxygenated metabolites. One of these pathways is catalyzed by cyclo-oxygenase which transforms arachidonic acid to prostaglandin endoperoxides (PGH₂) which are then converted predominantly to thromboxane A₂ (TXA₂) via thromboxane synthetase, malonaldehyde (MDA), and hydroxyheptadecatrienoic acid (HHT), with minor formation of prostaglandins (PGE₂, PGF_{2α}, PGD₂). In aortic

Figure 4: Metabolic Pathways of Arachidonic Acid metabolism in platelets (Willis and Smith, 1981).

Figure 4



tissue, PGH_2 is transformed into prostacyclin (PGI_2) via prostacyclin synthetase. These cyclo-oxygenase products of arachidonate play a major role in mediating platelet aggregation (Hamberg, Svensson and Samuelsson, 1975).

Of particular interest are PGI_2 and TXA_2 which have opposing effects; that is, while TXA_2 is a vasoconstrictor and stimulates platelet aggregation, PGI_2 relaxes aortic tissue and prevents platelet aggregation (Moncada, 1976).

Because of the vasoactive characteristics of these compounds, they are thought to be instrumental in maintaining hemostasis and to control the development of thrombotic disorders. The alternate metabolic route for arachidonic acid in platelets is catalyzed by 12-lipoxygenase, yielding 12-HPETE which is rapidly reduced to 12-HETE, a compound that is chemotactic in nature and, therefore, mediates inflammatory reactions (Hamberg and Samuelsson, 1974). The precise nature of this reaction and the products will be discussed in greater detail in an ensuing section. It should be noted that, while the 12-lipoxygenase pathway operates in unstimulated platelets, the cyclo-oxygenase pathway occurs only in stimulated platelets.

Several stages in the eicosanoid biosynthetic pathway involve the formation of hydroperoxide intermediates and, in addition, low concentrations of hydroperoxides are required for the initiation and continuation of both cyclo-oxygenase and lipoxygenase activities (Lands and Hemler, 1979). Antioxidants, capable of scavenging free radicals, have the potential to alter cellular peroxide level and, therefore, alter the activities of both of these enzymes. When examining the control of eicosanoid biosynthesis in platelets, the availability of non-esterified fatty acid substrate must also be considered. Platelets contain only trace amounts of free fatty acids, although esterified arachidonate is one of the most abundant fatty acid in platelet membrane constituents (Marcus, Ullman and Safier, 1969; Cohen and

Derksen, 1969). Although several pathways exist for the release of eicosanoid precursors, phospholipase A₂ has been recognized as an important factor in fatty acid release in platelets. Therefore, agents capable of influencing phospholipase A₂ activity will also affect platelet function.

Platelet Phospholipase A₂ Activity

Platelet cyclo-oxygenase and lipoxygenase act only on free fatty acids (Lands and Samuelsson, 1968; Samuelsson, 1972) and, since platelets contain very little free arachidonate, control of eicosanoid biosynthesis must involve the availability of substrate for these enzymes. Two major pathways exist for the release of arachidonate from membrane phospholipids: one is direct hydrolysis by phospholipase A₂ (Derksen and Cohen, 1975; Jesse and Cohen, 1976; Blackwell, Duncombe, Flower, Parsons and Vane, 1977), and the other is the sequential action of a phosphatidylinositol-specific phospholipase C and diglyceride lipase (Rittenhouse-Simmons, 1979; Billah, Lapetina and Cuatrecasa, 1979; Bell, Kennerly, Stanford and Majerus, 1979). Although the relative contribution of these two pathways in platelets is unclear, the importance of the direct hydrolytic action of phospholipase A₂ has been reported (McKean, Smith and Silver, 1981; Imai, Yano, Kameyama and Nozawa, 1982).

Phospholipase A₂ activity, which catalyzes the hydrolysis of fatty acid ester bonds at position 2 of glycerophospholipids, has been demonstrated in platelets to act on both exogenous and endogenous substrate (Derksen and Cohen, 1975; Trugman, Bereziat, Manier and Polonovski, 1979). Phosphatidylcholine (PC) is the most predominant phospholipid in platelets (Marcus et. al., 1969; Blackwell et. al., 1977; Broekman, Handin, Derksen

and Cohen, 1976) and radiolabelling studies have shown that platelet phospholipase A₂ releases arachidonate predominantly from PC and phosphatidylinositol (PI) (Bills, Smith and Silver, 1976; Bills, Smith and Silver, 1977). As a result, the majority of experimental procedures studying phospholipase A₂ activity involve using 2-arachidonyl PC as substrate.

Most of the information on the structure, chemistry, and mechanism of action of phospholipase A₂ has been derived from studies with venom and pancreatic enzymes (Van Deenen and de Haas, 1966; Slotboom, van Darn-Mieras, Jansen, Pattus, Verhey and de Haas, 1978), although the occurrence of this enzyme in several animal tissues has long been apparent. (Van den Bosch, 1980). In fact, phospholipase A₂ from human platelets has been purified to near homogeneity and has been found to be a membrane-bound, calcium-requiring enzyme (Apriz-Castro, Maz, Cruz and Jain, 1979; Franson, Eisen, Jesse and Lanni, 1980).

Studies have shown that platelet phospholipase A₂ is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (Franson, Eisen, Jesse and Lanni, 1979). Since phospholipase A₂ is extremely sensitive and dependent on Ca⁺² for reactivity (Franson and Waite, 1978; Jesse and Franson, 1979) and, since previous reports showed that NSAIDs function as Ca⁺² agonists (Northover, 1977), it has been suggested that the sensitivity of phospholipase A₂ to these agents is modulated by Ca⁺². Calcium has been reported to regulate platelet prostaglandin biosynthesis at a step preceding the formation of endoperoxides; that is, at the stage of arachidonate cleavage from membrane phospholipids by phospholipase A₂ (Pickett, Jesse and Cohen, 1977; Rittenhouse-Simmons and Deykin, 1977). As a result, inhibition of Ca⁺² release by vitamin E was thought to be the cause of enhanced phospholipase A₂ activity in platelet membrane vesicles,

as reported by Butler et. al. (Butler, Gerrard, Peller, Stoddard, Rao and White, 1979). This suggestion was based on the premise that, since vitamin E functions in membrane stabilization, it will also affect membrane permeability to Ca^{+2} ions. However, these results are in direct conflict with others which showed that vitamin E inhibits platelet prostaglandin biosynthesis (Steiner and Anastasi, 1976; Hope, Dalton, Machlin, Filipski and Vane, 1975), and the discrepancy is probably due to the high level of vitamin E used in the study as well as the fact that the platelets were stimulated by thrombin or Ca^{+2} ionophore.

Since vitamin E structurally interacts with the fatty acyl groups of phospholipids, its regulation of phospholipase A_2 activity may be achieved through changes in the structural arrangement of membrane constituents. Considering Lucy's model of the interaction of tocopherol with the fatty acyl residues of membrane phospholipids (Lucy, 1972), vitamin E may interfere with phospholipase A_2 activity by binding to arachidonyl residues of the membrane phospholipids. Stable complexes have been demonstrated to form between the PUFAs of PC and tocopherol (Porter, Levasseur and Henick, 1971). In addition, Steiner reported a modification of platelet membrane fluidity in tocopherol-loaded platelets, which suggests an alteration in the mobility of surface enzymes (Steiner, 1978). Although vitamin E has been reported to modulate phospholipase A_2 activity in mammalian tissue (Pappu, Fatterpaker and Sreenivasan, 1978; Butler et. al., 1979), the mechanism of vitamin E action on the enzyme remains to be elucidated.

Platelet 12-Lipoxygenase Activity

Platelet lipoxygenase catalyzes the oxidative conversion of arachidonic acid to 12-HPETE, an unstable metabolite that is rapidly reduced to 12-HETE by the action of platelet peroxidases (Hamberg and Samuelsson, 1974; Nugteren, 1975). The stable product of these reactions, 12-HETE, has been thought to act solely as a stimulator of neutrophil chemotaxis (Goetzl, Woods and Gorman, 1977; Turner, Trainer and Lynn, 1975); however, it has recently been reported to be a potent inhibitor of platelet phospholipase A₂ (Chang, Blazek, Drefst, and Lewis, 1985) and may, therefore, regulate arachidonate metabolism at the level of its release from phospholipids. In addition, the action of a leukocyte ω-hydroxylase on platelet-derived 12-HETE results in the formation of a novel metabolite, 12,20-diHETE (Wong, Westlund, Hamberg, Granstrom, Chao and Samuelsson, 1984), the biological activity of which is under investigation. 12-HPETE is a potent inhibitor of vascular PGI synthesis (Moncada et. al., 1976) and, as a result, its release is thought to be deleterious to vascular tissue. Recent reports indicate that 12-HPETE not only activates its own production (Seigel, McConnell and Cuatrecasas, 1979) but also activates leukocyte 5-lipoxygenase activity, increasing the formation of 5-HETE and LTB₄ (Maclouf, DeLaclos and Borgeat, 1982), which are potent mediators of hypersensitivity and inflammatory responses. Since both 12-HPETE and 12-HETE can mediate platelet and leukocyte function, factors influencing their synthesis are of major physiological significance and have recently been the subject of intense research.

The initial step in the conversion of arachidonic acid to 12-HPETE by lipoxygenase is removal of a hydrogen atom from C-10 of the fatty acid, followed by insertion of molecular oxygen at position 12 (Hamberg and Hamberg,

1980). The overall mechanism by which this reaction proceeds requires peroxides for initiation of the radical chain reaction (Lands and Hemler, 1979). Alterations in the cellular peroxide concentration or "peroxide tone" may, therefore, have profound effects on lipoxygenase activity. Endogenous compounds with pro or antioxidant properties can influence the rate of peroxide formation and, hence, the rate of arachidonate lipoxygenation. Lipoxygenase activity has been reported to be altered by several antioxidants, including vitamin E (VanWauwe, 1983). Although vitamin E has been reported to be an effective inhibitor of plant lipoxygenase, *in vitro*, (Panganamala, Miller, Gwebu, Sharma and Cornwell, 1977), there have been conflicting reports of its action on mammalian lipoxygenase. For example, platelets from vitamin E deficient rabbits have been shown to form greater amounts of 12-HETE from endogenous arachidonate than controls (Gwebu, Trewyn, Cornwell and Panganamala, 1980), whereas other studies indicate that vitamin E has no effect on 12-HETE formation in human platelets (Butler et. al., 1980). As a result, further study is warranted in order to obtain more interpretive results on the action of vitamin E on platelet lipoxygenase activity.

Vitamin E, Eicosanoid Biosynthesis, and Platelet Function

Antioxidants, including tocopherol, have been demonstrated to influence eicosanoid biosynthesis, *in vitro* (Lands, LeTellier, Rome and Vanderhoeck, 1973). Since these compounds are potent mediators of the platelet response (Hamberg et. al., 1974; Smith et. al, 1973, 1974), it was expected that vitamin E may play a role in mediating platelet aggregation. In fact, vitamin E has been reported to inhibit platelet aggregation

and , an inverse relationship between serum prostaglandin levels and vitamin E was observed (Hope et. al. 1975). Furthermore, the platelet release reaction is associated with a sudden rise in intracellular lipid peroxidation (Okuma, Steiner and Baldwin, 1971) required for the formation of endoperoxide intermediates. An inhibition of the platelet release reaction by vitamin E has been reported (Steiner and Anastasi, 1976). From these observations, it was thought that the effect of vitamin E on platelet function was due solely to its antioxidant effect in inhibiting the formation of peroxide intermediates in eicosanoid biosynthesis. Indeed, as early as 1966, Nugteren reported that α -tocopherol inhibits eicosanoid biosynthesis in sheep vesicular gland preparations (Nugteren, Beerthuis and Van Dorp, 1966). Their results were later confirmed by Lands who showed that several antioxidants, including tocopherol, inhibited arachidonate oxygenation in these preparations (Lands et. al., 1973). On the other hand, Chan demonstrated that vitamin E deficiency decreased cyclo-oxygenase activity in rabbit muscle (Chan, Allen and Hegarty, 1980), whereas Panganamala's group found that tocopherol had no effect on prostaglandin biosynthesis in bovine vesicular gland microsomal preparations (Panganamala et. al., 1977).

A major role for vitamin E in platelet physiology is not unexpected since it has been shown that, compared to plasma and red cells, platelets are more sensitive to changes in dietary tocopherol levels (Lehmann, 1981 and 1984), probably due to their high PUFA content and high turnover rate. Although a definite link exists between platelet eicosanoids and the platelet response, the exact relationship between vitamin E and eicosanoid biosynthesis is less clear. The antioxidant property of vitamin E implies that the vitamin exerts its action on the peroxide-requiring reactions catalyzed by cyclo-oxygenase and lipoxygenase in platelets. However, since

arachidonate release from membrane phospholipids by phospholipase A₂ is a rate-limiting step in eicosanoid biosynthesis, the platelet response can also be influenced at this stage. In fact, it has been reported that phospholipase A₂ is capable of triggering platelet aggregation (Chap, Zwaal and VanDeenen, 1977; Iatridis, Iatridis, Tsiala-Taraschou, Markidou and Ragatz, 1976). It is not unreasonable to suggest that the effect of vitamin E on platelet response may not only be due to its action on platelet arachidonic acid oxygenation, but also to its action of platelet arachidonic acid release by phospholipase A₂.

Vitamin E-deficient rats and rats fed an excess of vitamin E provide excellent models for assessing the physiological effects of vitamin E on platelet eicosanoid biosynthesis. The dual function of vitamin E in platelets, as antioxidant and membrane stabilizer, is demonstrated by its actions on the membrane-bound platelet phospholipase A₂ and the soluble platelet 12-lipoxygenase activities.

EXPERIMENTAL DESIGN

Male, weanling (3 week old), Sprague Dawley rats were obtained, separated into three groups, and maintained on a purified diet containing either 0, 100, or 1000 ppm vitamin E in the form of DL- α -tocopheryl acetate for approximately one year. Following verification of vitamin E status, blood was drawn from each rat and platelets were isolated for study. In each case, plasma tocopherol concentration, pyruvate kinase activity, and plasma lipid concentration were determined, in addition to phospholipase A₂ and 12-lipoxygenase activities. In the phospholipase A₂ study, several levels of D- α -tocopherol and tocol were added to the assay mixtures in order to study the dose-dependent behavior of phospholipase A₂ to added vitamin E. In addition, the rat platelet phospholipase A₂ was partially purified by gel filtration chromatography to examine the effect of tocopherol and several of its analogues on a more homogeneous preparation. The lipoxygenase study was complemented by a refeeding experiment in which deficient rats were fed a vitamin E sufficient diet for up to two weeks, and the change in 12-lipoxygenase activity determined with the change in vitamin E status of the rat.

MATERIALS AND METHODS

Materials

All solvents were redistilled and all glassware was siliconized before use. All reagents and solvents were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (New Jersey, NY). Dietary ingredients were purchased from ICN Nutritional Biochemicals (Cleveland, OH) with the exception of "tocopherol stripped" corn oil which was obtained from Eastman Kodak Chemicals (Rochester, NY). The synthetic vitamin E (DL- α -tocopherol) and its acetate were from Sigma Chemical Co. (St. Louis, MO). The natural vitamin E (D- α -tocopherol) and tocol were gifts from Henkel Co. (Minneapolis, MN) and Eisai Co. Ltd. (Tokyo, Japan), respectively. Sn-1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine (54.5 mCi/mmol) and [1- ^{14}C]-arachidonic acid (52.0 mCi/mmol) were from New England Nuclear (NEN) Canada Ltd. (Montreal, Canada). Sepharose 6B was from Pharmacia (Sweden), and ACS II was from Amersham (Oakville, Canada).

Animal Care and Diet

Weanling male Sprague Dawley rats, weighing 45-50 grams, were obtained and housed in individual metal cages at the University of Ottawa animal care facilities. The rats were fed ad libitum on a purified diet, the composition of which is outlined in table 1, and had a constant supply of drinking water. Sanitary conditions were maintained by the animal care staff, and lighting and temperature were rigidly controlled. Rats were separated into three groups and fed diets containing 0, 100, or 1000 parts per million (ppm) vitamin E as DL- α -tocopheryl acetate. Diets were

TABLE 1: Composition of purified rat diet¹

Ingredient	Weight %
Vitamin-free casein	20.0
DL-methionine	0.3
Corn starch	10.2
Dextrose	50.0
Alphacel fibre	5.0
AIN salt mix	3.5
Tocopherol-stripped vitamin mix	1.0
Tocopherol-stripped corn oil	10.0

¹Vitamin E supplemented diets contained 100 or 1000 mg DL-tocopheryl acetate per kg diet mixture. Male weanling (3 week old) rats, weighing 45-50 grams, were maintained on this diet for approximately one year.

prepared. in one kilogram batches by thoroughly mixing the dry ingredients in a Cuisinart food processor, adding the corn oil, and further mixing until well blended. For the vitamin E supplemented diets, the tocopherol was first dissolved in the corn oil before its addition to the dry ingredients. After its preparation, the diet was stored in a cold room at approximately 10°C.

Determination of Vitamin E status of Rats

Elevated plasma pyruvate kinase activity has been noted in vitamin E deficient animals and is thought to indicate muscular damage and leakage of the enzyme from the damaged muscle into the blood (Chow, 1975). It is, therefore, routinely used as an index of the vitamin E-deficiency status of experimental animals (Machlin, Gabriel, Spiegel, Horn, Brin and Velson, 1978). Pyruvate kinase activity was determined to verify the deficiency state of each rat prior to each experiment. The pyruvate kinase reaction is coupled with that of lactic dehydrogenase and assayed spectrophotometrically at 340 nm (Worthington, 1972). In each case, a small volume of blood was obtained from the rat's tail and centrifuged at 1000 rpm for 15 min to separate the plasma from the red blood cells. Details of this assay are on table 2.

The plasma tocopherol level of each rat was determined by high pressure liquid chromatography (HPLC) as outlined by Bieri (Bieri, Tolliver and Catignani, 1979). Briefly, tocopherol compounds were extracted from plasma samples and mixed with the internal standard, α -tocopheryl acetate. These compounds, dissolved in heptane, were injected into the HPLC equipped with a Bondapak C-18 column and eluted with MeOH:H₂O (95:5).

TABLE 2: Pyruvate Kinase Assay.¹

Reagent	Volume (ml)
Imidazole buffer, 0.5M, pH 7.5	2.6
Adenosine Diphosphate, 20 mg/ml	0.1
Phospho-enol pyruvate, 5 mg/ml	0.1
NADH, 5 mg/ml ^a	0.1
Lactate dehydrogenase, 0.3-0.5 units/assay	0.01

^aNicotinamide adenine dinucleotide phosphate (Sodium salt).

¹At time 0, 10ul plasma (deficient rats) or 50ul plasma (sufficient rats) were added to cuvettes containing the reagents outlined above, and the decrease in absorbance at 340 mu was measured.

$$\text{Units/ml} = \frac{\text{Absorbance}_{340}/\text{min}}{6.2 \times \text{ml serum added}}$$

Tocopherol peaks were detected using a ultraviolet (UV) detector absorbing at 280 nm. Figure 5 is a typical chromatograph of one plasma sample; α -tocopherol was quantitated by peak height ratios.

To establish whether plasma lipid levels were altered by the level of dietary vitamin E, plasma lipid concentration was determined using a colorimetric assay based on the sulfo-phospho vanillin reaction (Fringe and Dunn, 1970). The assay is described on table 3. As this method detects compounds with carbon-carbon double bonds in serum, there are essentially no interfering substances since naturally occurring compounds with carbon-carbon double bonds other than lipids are found in very small amounts (i.e. at levels too low to cause colour formation in this assay system).

Platelet Isolation

Blood was obtained by abdominal aortic puncture from rats under light ether anesthesia and collected into siliconized citrate-containing tubes to a final concentration of 0.38% trisodium citrate. Platelets were isolated by differential centrifugation; that is, the blood was first spun at 900 rpm for 20 minutes in a Beckman tabletop centrifuge to separate the platelet rich plasma (PRP) from the red cells. The PRP was then centrifuged at 2000 rpm for a further 20 minutes to separate platelets from platelet poor plasma (PPP). Finally, the platelets were washed and resuspended in the appropriate buffer. For the lipoxygenase assay, the isolated platelets were resuspended in Ca^{+2} -free Ringer Tyrodes buffer at pH 7.4 containing 2mM EDTA. For the phospholipase A_2 assay, platelets were resuspended in Tris-HCl buffer at pH 9.0 with 2 mM EDTA and 2 mM EGTA. Platelet protein concentration was estimated by the procedure of Lowry et. al., using bovine serum albumin as the standard (Lowry, Rosebrough, Farr and Randall, 1951).

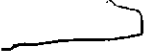


Figure 5: HPLC Chromatogram of α -tocopherol in one rat plasma sample, with α -tocopheryl acetate as internal standard. These compounds were eluted with methanol:water (95:5) at a flow rate of 2.5 ml/min, and detected with a UV detector at 280 nm.

FIGURE 5

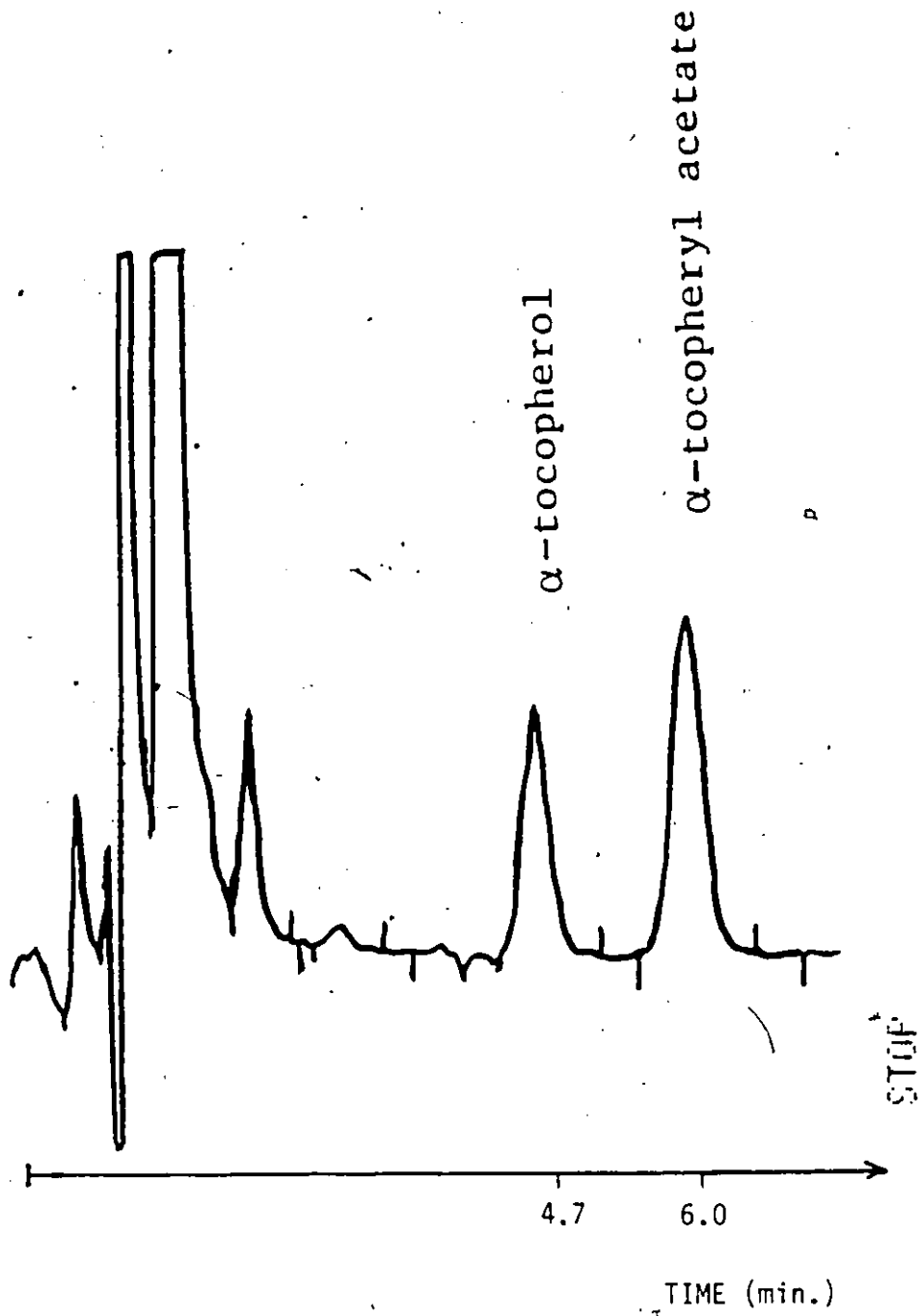


TABLE 3: Sulfo-phosphovanillin Assay for determination of total Plasma Lipid.¹

<u>TUBE 1</u>	
2 ml concentrated H ₂ SO ₄	
0.1 ml plasma or standard ^a	
<hr/>	
Boil 10 min. and Cool 5 min.	
<hr/>	
<u>TUBE 2</u>	
0.1 ml Tube 1	
5 ml phosphovanillin reagent ^b	
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Incubate 15 min. at 37°C	
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^aStandards were prepared, containing between 200 and 1000 mg/dl corn oil, to produce a linear standard curve.

^b200 ml 0.6% (w/v) vanillin + 800 ml phosphoric acid.

¹The Absorbance at 540 mμ was read for each assay tube against a blank tube containing 0.1 ml H₂SO₄ and phosphovanillin reagent. This colorimetric reaction follows Beer's Law up to 1000 mg/dl (Frings and Dunn, 1970).

Platelet Phospholipase A₂ Assay

Enzyme activity was estimated by measuring the rate of ¹⁴C-arachidonic acid release from sn-1-palmitoyl-2-[1-¹⁴C]-arachidonyl phosphatidylcholine. Optimal assay conditions were pre-determined and each assay mixture contained 12 mM Ca⁺², 0.1 M Tris-HCl (pH 9.0) with 2 mM EDTA and 2 mM EGTA, 1 nmole labelled and 9.0 nmole unlabelled PC sonicated (4 times 15 seconds, alternating 15 seconds on ice) in water and 30-40 ug protein from sonicated platelets (2 times 15 sec.), in a final volume of 170 ul. Tocopherol and its analogues (D and DL- α -tocopherol, DL- α -tocopheryl acetate, and tocol) were dissolved in dimethyl sulfoxide (DMSO) and 2 ul of the appropriate form were added to each assay tube. 2 ul of DMSO were added to control assays. Assay mixtures were pre-incubated for 10 minutes at 37°C in a shaking water bath, after which the reaction was initiated by the addition of Ca⁺². The reaction was terminated after incubating the mixture for a further 10 minutes, by the addition of 20 ul CHCl₃. Released arachidonate was extracted from the incubation medium by the method of Doles (Doles and Meinertz, 1960). That is, to the terminated reaction mixture was added 1 ml Dole's reagent (isopropanol:heptane:1N sulfuric acid (4: 1: 0.1, v/v/v)), the tubes were vortexed, 0.5 ml water and 0.75 ml heptane added, and the tubes centrifuged at 3000 rpm for 5 min. 0.8 ml of the upper phase was mixed with 1 ml heptane and 100 mg silicic acid powder, to adsorb the unreacted phospholipid, and the tubes again centrifuged briefly. 1.2 ml of the heptane layer, containing the hydrolyzed arachidonic acid, was transferred to scintillation vials, containing 10 ml ACS II cocktail, and counted on a Beckman Scintillation counter. All assays were performed in duplicate.

Platelet Phospholipase A₂ Purification

Isolated rat platelets were sonicated four times 15 seconds (alternating 15 seconds on ice after each sonication) with a Branson sonifier, and the resulting homogenate centrifuged at 9000 x g for 20 minutes at 4°C. The supernatant fraction was further centrifuged at 105,000 x g for 90 minutes. The supernatant fraction from this spin, containing the majority of the phospholipase A₂ activity, was concentrated by an amicon ultrafiltration device. The concentrated fraction, containing molecular sizes over 10,000 daltons was mixed with sucrose, loaded onto a sepharose 6B column (58 cm in height and 1 cm in diameter, total volume 45.5 cm³) and eluted with Tris-HCl buffer (pH 9.0, 2 mM EDTA and 2 mM EGTA) at a flow rate of 4 ml per hour. After fraction collection, the absorbance at 280 nm, representative of protein concentration, and phospholipase A₂ activity in the fractions were measured. The void volume of the column was determined to be 10 ml by loading and eluting a sample of the high molecular weight Blue Dextran from the column. Refer to table 4 for platelet protein yield and phospholipase A₂ activity during the purification procedure.

Platelet Lipoxygenase Assay

Enzyme activity was determined by measuring the conversion of labelled, exogenously added, arachidonate to 12-HPETE and 12-HETE in intact platelet suspensions. 12-lipoxygenase activity was linear up to 10 minutes of incubation and 400 ug intact platelet protein. Each assay mixture contained 33 uM arachidonate (4.2 uM ¹⁴C-arachidonate and 28.8 uM unlabelled arachidonate) in 5 ul ethanol, and 200 ug platelet in a total volume of 500

TABLE 4: Purification Table of Rat Platelet Phospholipase A₂.¹

PURIFICATION STEP	PROTEIN RECOVERY (%)	PURIFICATION FOLD
Platelet Homogenate	100	---
High Speed Supernatant ^a	52.1	1.99
High Speed Supernatant concentrate ^b	20.6	1.85
Fractions 21-25	4.6	3.03

^a100,000xg for 90 minutes.

^bMolecular sizes greater than 10,000 daltons.

¹Details of each purification step are outlined in the text.

ul. The reaction was initiated by the addition of arachidonate substrate to platelet suspensions, incubated for 5 minutes at 37°C with constant stirring, and terminated by acidification with 10% formate to pH 3 - 3.5. Arachidonic acid and its metabolites were extracted with 6 times the assay volume of cold diethyl ether and separated by Thin Layer Chromatography (TLC), using 250 uM silica gel G coated plates, in the solvent system: Chloroform:Methanol:Acetic acid: Water (90:8:1:0.8, v/v/v/v). Products were detected and quantitated by radio TLC-scanning, and verified by autoradiography and scintillation counting, using appropriate standards.

Statistical Analysis

Results are expressed as mean \pm standard error of the mean (S.E.M). Students' t tests were used to determine statistical significance.

RESULTS

Vitamin E Status of Rats

Both pyruvate kinase (PK) activity, an indication of tissue myopathy, and plasma tocopherol concentration, reflective of dietary levels of vitamin E, were determined to verify the vitamin E status of the rats. In this study, the PK activity was markedly elevated ($P < 0.01$) in the deficient rats compared to that in the 100 and 1000 ppm vitamin E supplemented rats (table 5). There was no significant difference in PK activities between the 100 and 1000 ppm vitamin E supplemented groups. Plasma tocopherol levels, quantitated by HPLC, was significantly different in all three groups of rats (table 5). There was no significant difference in plasma lipid concentration in the three groups of rats (table 5). At the end of the dietary treatment period, the vitamin E sufficient rats weighed approximately 700 ± 50 g and were in apparent good shape. The deficient rats, however, showed obvious signs of degeneration such as edema of the testes, discoloration of fur which could easily be pulled off, and staggering gait. The average weight of this group was not significantly different from the sufficient rats. The most noticeable difference in these rats was the increased platelet number in the deficient rats (approximately 30% more platelet protein) compared to the vitamin E sufficient rats.

Effect of Dietary Vitamin E on Platelet Phospholipase A₂ Activity

Platelet phospholipase A₂ activity from rats fed a vitamin E deficient diet was significantly greater ($P < 0.05$) than that from rats fed the 100 or 1000 ppm vitamin E (figures 6, 7). There was no significant

TABLE 5: Plasma Pyruvate Kinase (PK) activity, Tocopherol concentration, and Lipid levels from rats fed different levels of Vitamin E. ¹

DIETARY VITAMIN E (DL- α -Tocopheryl acetate) (ppm)	P.K. ACTIVITY (U/ml)	PLASMA LIPID (mg/dl)	PLASMA TOCOPHEROL (mg/dl)
0	0.87 \pm 0.12 ^a	300 \pm 54	0.23 \pm 0.06 ^a
100	0.05 \pm 0.01 ^b	321 \pm 1.0	2.36 \pm 0.02 ^b
1000	0.04 \pm 0.02 ^b	314 \pm 3.5	3.34 \pm 0.12 ^c

¹Values are mean \pm S.E.M. of 3 rats per group. Columns sharing different supercripts are significantly different ($P < 0.02$).

Figure 6: Effect of in vitro addition of D- α -tocopherol on platelet phospholipase A₂ activity (pmole labelled arachidonate released per ug platelet protein) from rats fed vitamin E deficient (□), 100 ppm (▨), and 1000 ppm (⊠) DL- α -tocopheryl acetate. Platelets were preincubated with D- α -tocopherol in 2 ul DMSO (or DMSO alone) for 10 min. at 37°C prior to addition of substrate. Values are means \pm S.E.M. of duplicate determinations from 3 animals per group. Phospholipase A₂ activity from vitamin E deficient platelets was significantly higher (P < 0.001) than from vitamin E supplemented platelets. Within each group, in vitro addition of D- α -tocopherol, in 2 ul DMSO, causes significantly different activities (** P < 0.05, *** P < 0.01) from controls with DMSO alone.

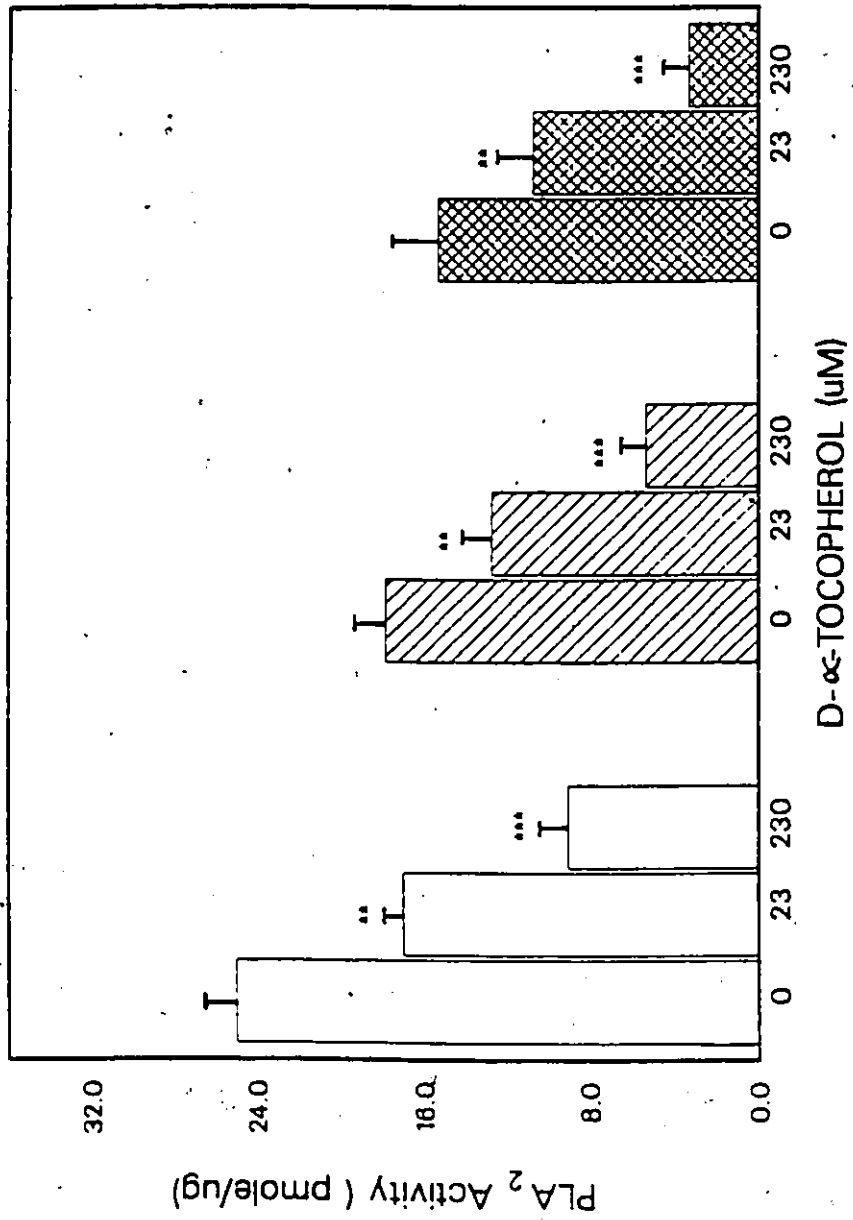


FIGURE 6

Figure 7: Effect of in vitro addition of tocol on platelet phospholipase.A² activity (pmole labelled arachidonate released per ug platelet protein) from rats fed vitamin E deficient (□), 100 ppm (▨) and 1000 ppm vitamin E (▩) diets. Values are means ± S.E.M. of duplicate determinations from 3 animals per group. Within each group, in vitro addition of tocol in 2 ul DMSO causes significantly different activities (* P < 0.001, ** P < 0.05, *** P < 0.01) from controls with DMSO (without tocol).

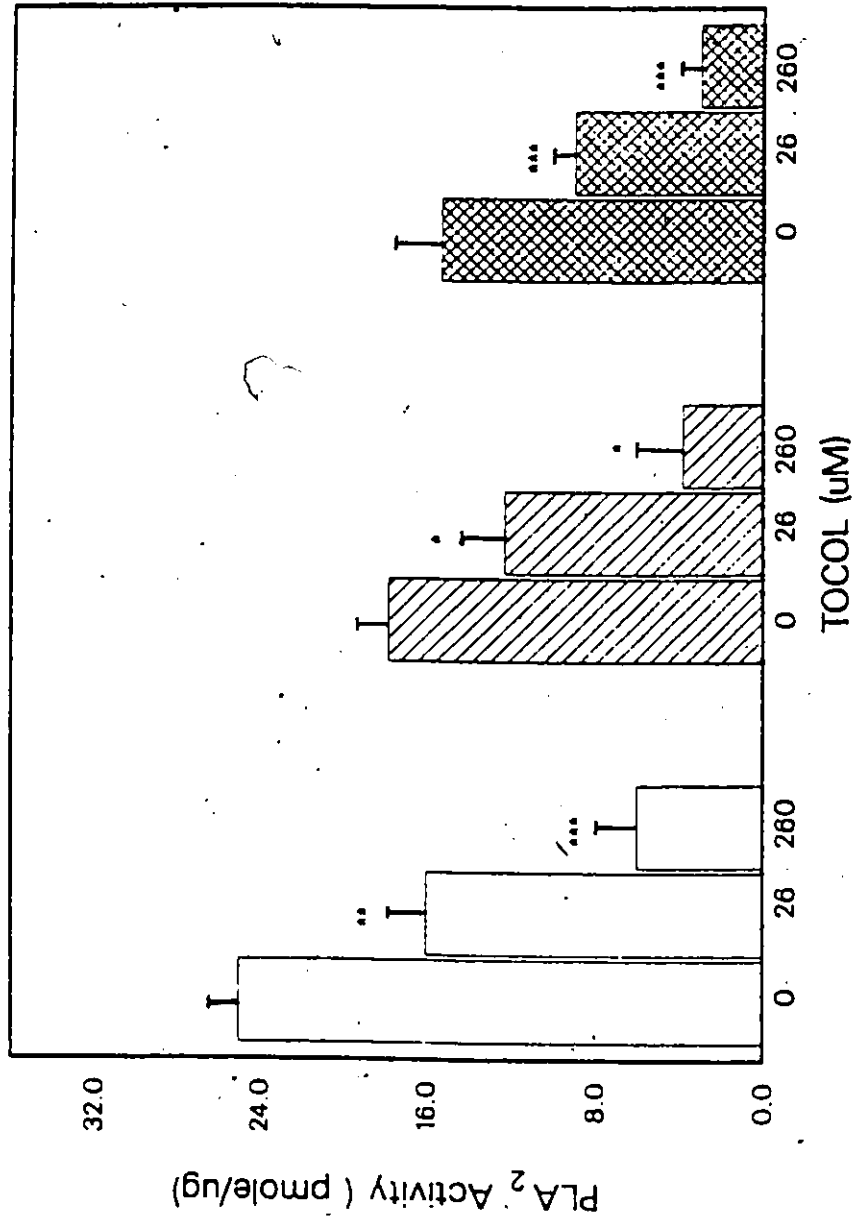


FIGURE 7

difference in activities between the 100 and 1000 ppm vitamin E groups.

The addition of tocopherol to sonicated platelet suspensions decreased the phospholipase A₂ activity in all three groups of rats. The degree of inhibition by added tocopherol was greater in the vitamin E deficient platelets compared to vitamin E supplemented platelets. It was also noted that tocol, an analogue of tocopherol in which the methyl groups on the chromanol ring are absent (figure 7), inhibited phospholipase A₂ activity to a greater degree than the natural α -tocopherol (figure 6).

Partially Purified Phospholipase A₂ and Vitamin E

In the experiments mentioned above, crude platelet suspensions were used as the enzyme source. In an attempt to examine the inhibitory action of vitamin E on platelet phospholipase A₂, this enzyme was partially purified from rat platelets. This was accomplished by solubilization of the enzyme by sonication, subjecting the homogenate to a high speed centrifugation, and separation of the soluble fraction by gel filtration chromatography. The partially purified form of the enzyme had a molecular weight of approximately 250,000 daltons, and was associated with some phospholipid components as evidenced by a positive phosphorus analysis. The elution profile (figure 8) shows peak phospholipase A₂ activity between fractions 22 and 25. These fractions were combined and utilized for further characterization. The activity of this partially purified phospholipase A₂ was found to be absolutely dependent on Ca⁺² concentration (figure 9), and to be linearly dependent on protein concentration (figure 10). The activity of this form of the enzyme was inhibited by tocol, D- α -tocopherol, DL- α -tocopherol and to, a much lesser extent, by DL- α -tocopheryl acetate (figure 11).

Figure 8: Purification profile of rat platelet phospholipase A₂ from the high speed supernatant.

Pattern of proteins eluted from column indicated by O.D. at 280 nm (●-----●) and

phospholipase A₂ activity indicated by the release of labelled arachidonate (●-----●).

Fractions 22-25 (—) were combined and further characterized. For details see methods section.

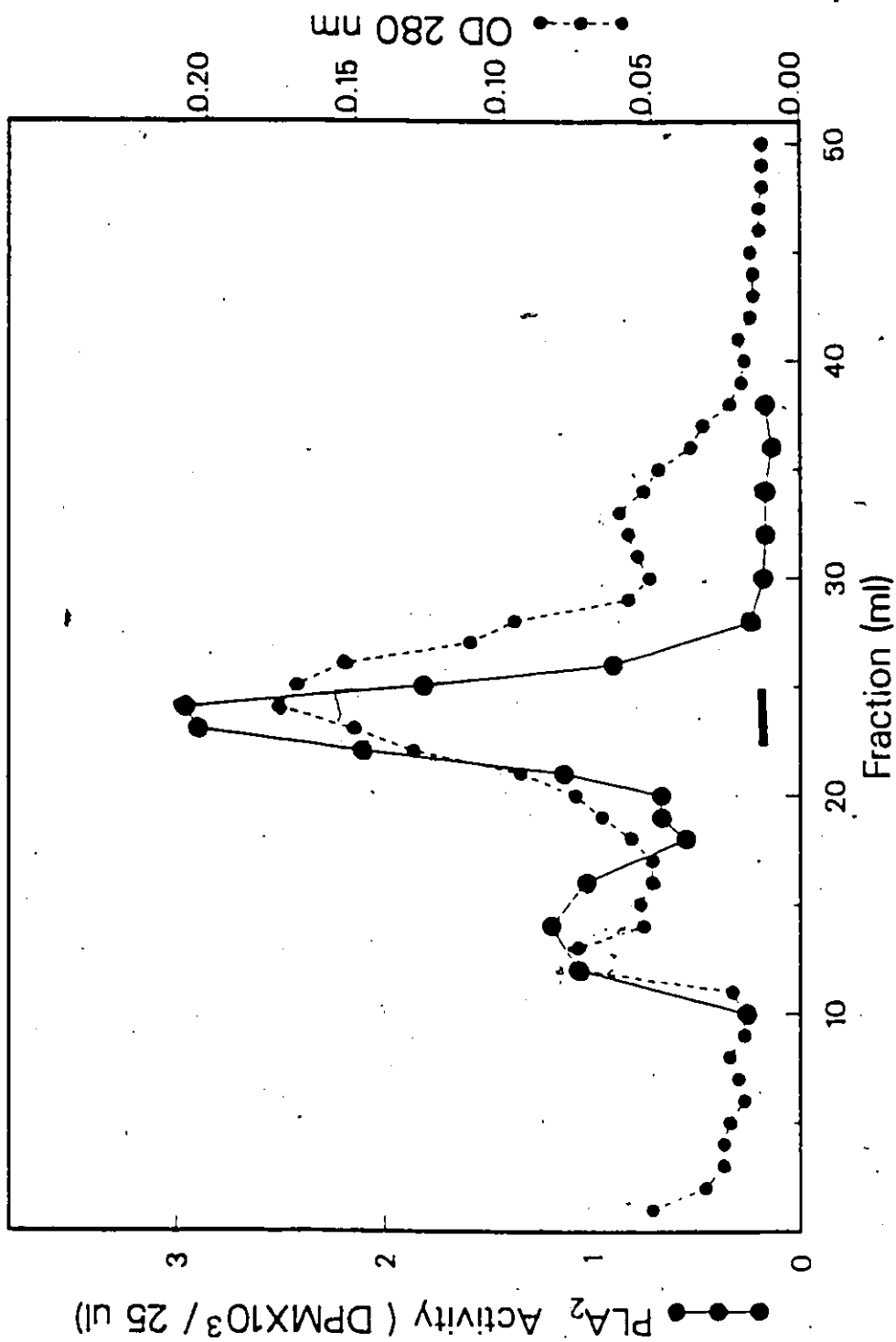


FIGURE 8

Figure 9: Linear relationship of phospholipase A₂ activity with platelet protein eluted from fractions 22-25. Assay conditions are as described in the text.

Figure 10: Dependency for Ca⁺² of partially purified phospholipase A₂ (fractions 22-25 from figure 8). Assay conditions are as described in the text.

FIGURE 9

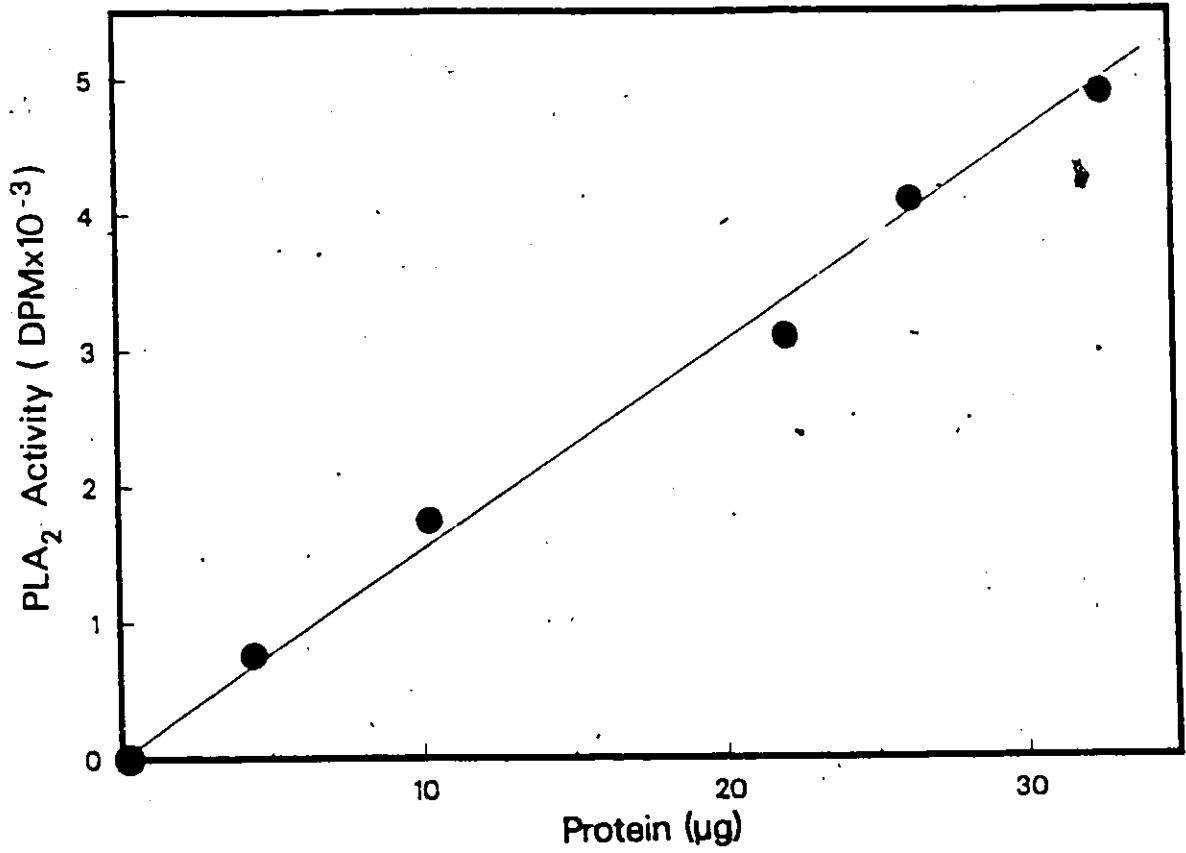


FIGURE 10

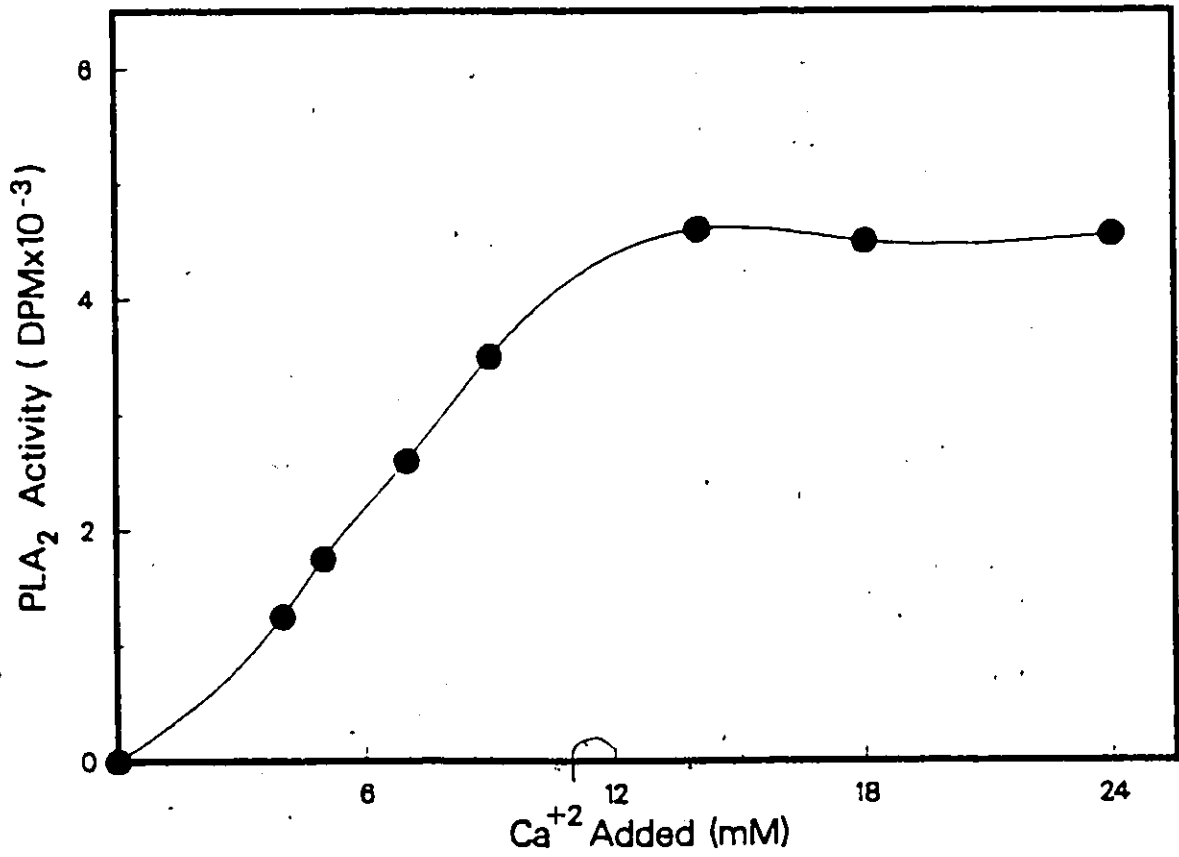
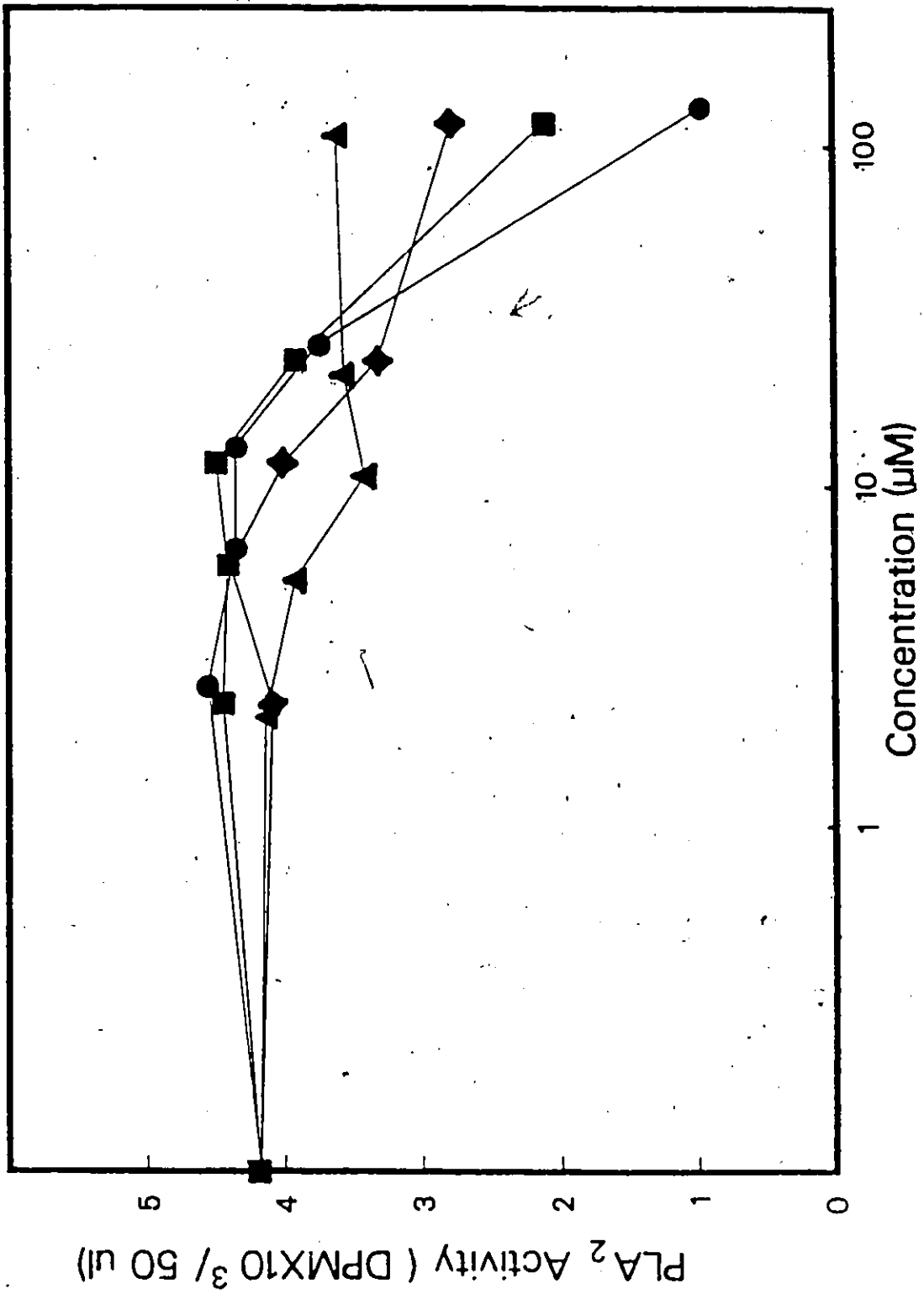


Figure 11: Inhibition of partially purified phospholipase A₂ activity (fractions 22-25) by added tocopherol and its analogues. Effect of varying concentrations of D- α -tocopherol (■), DL- α -tocopherol (◆), Tocol (●), and DL- α -tocopheryl acetate (▲) on phospholipase A₂ activity. Assay conditions are as described in the text.

FIGURE 11



Rat Platelet 12-Lipoxygenase Activity

Lipoxygenase activity in rat platelet suspensions was determined by quantifying the conversion of exogenously added labelled arachidonic acid to 12-HPETE and 12-HETE. Figure 12 is a typical radio-TLC scan of arachidonic acid and its metabolites under the conditions of our assay system. In a preliminary experiment, the time course of 12-HPETE and 12-HETE formation, and arachidonic acid disappearance was followed. At short incubation times periods (0.5 to 2 minutes) there were equivalent amounts of each product whereas, after a 5 minute incubation, the predominant product was 12-HETE although detectable amounts of 12-HPETE remained (figure 13). In subsequent experiments, 12-lipoxygenase activity was expressed as the sum of 12-HPETE and 12-HETE converted from arachidonic acid.

Effect of Dietary Vitamin E on Platelet Lipoxygenase Activity

Lipoxygenase activity in platelets from rats fed 100 ppm vitamin E was significantly lower ($P < 0.05$) than that from rats fed a vitamin E deficient or vitamin E excess (1000 ppm) diet (figure 14). There was no significant difference in 12-lipoxygenase activities between the vitamin E deficient rats and the 1000 ppm vitamin E supplemented rats. Differences in lipoxygenase activity among the three groups of rats were largely due to changes in 12-HETE formation; that is, the amount of 12-HPETE remained essentially unchanged among the different groups of rats.




Figure 12: Radio-TLC scan of arachidonic acid and its 12-lipoxygenase metabolites, with accompanying autoradiogram strip showing the location of compounds of interest. ¹⁴C-arachidonate was incubated with 200 ug intact platelet protein suspensions for 5 minutes at 37 °C.

FIGURE 12

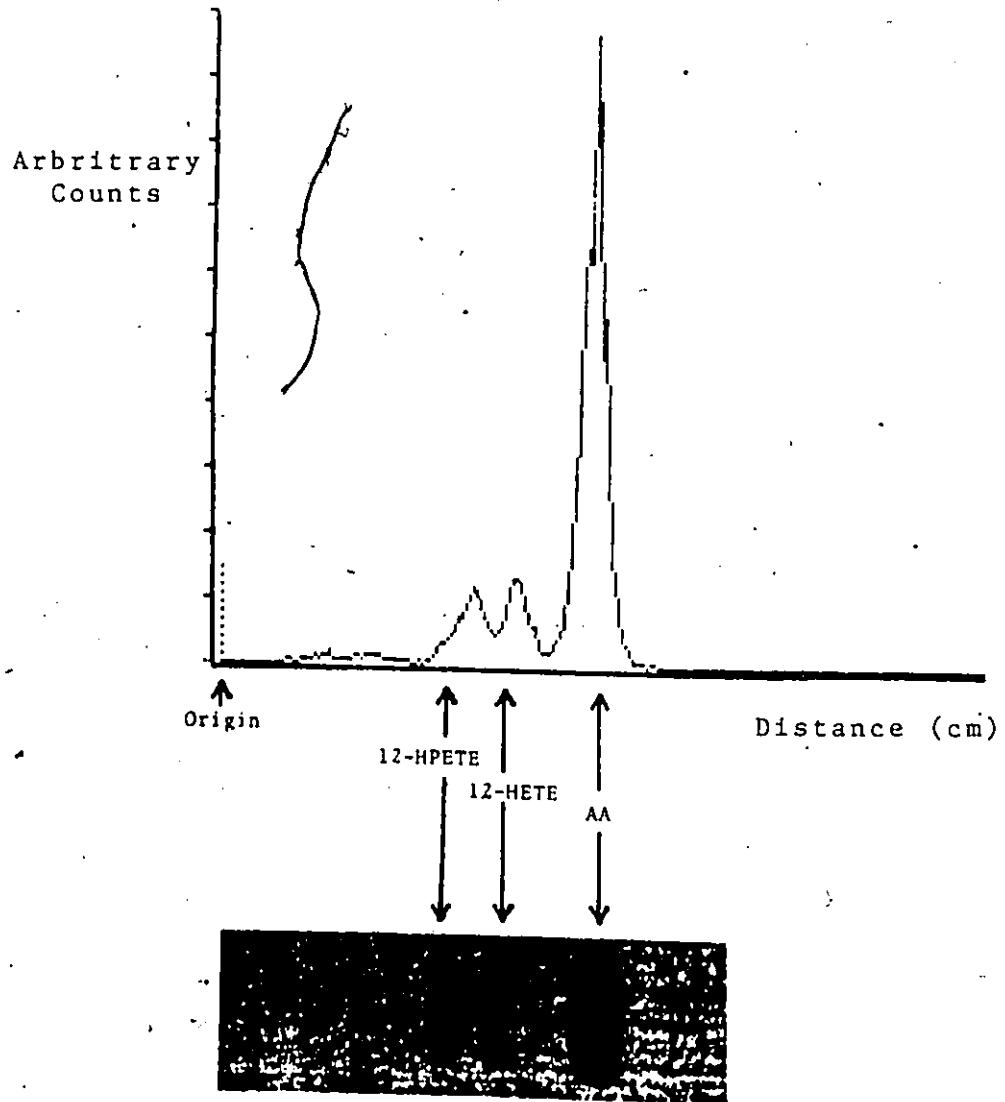


Figure 13: Time course of arachidonate conversion via rat platelet 12-lipoxygenase. The disappearance of exogenously added ¹⁴C-arachidonic acid (○---○), the appearance of 12-HPETE (●.....●), 12-HETE (▲---▲), and 12-HPETE + 12-HETE (■---■) were determined by the procedure described in the text. 33 μM C-arachidonate was incubated with 600 μg intact platelet protein suspension for 5 min. at 37 °C. In subsequent experiments, 12-lipoxygenase activity is expressed as a sum of 12-HPETE and 12-HETE formation.

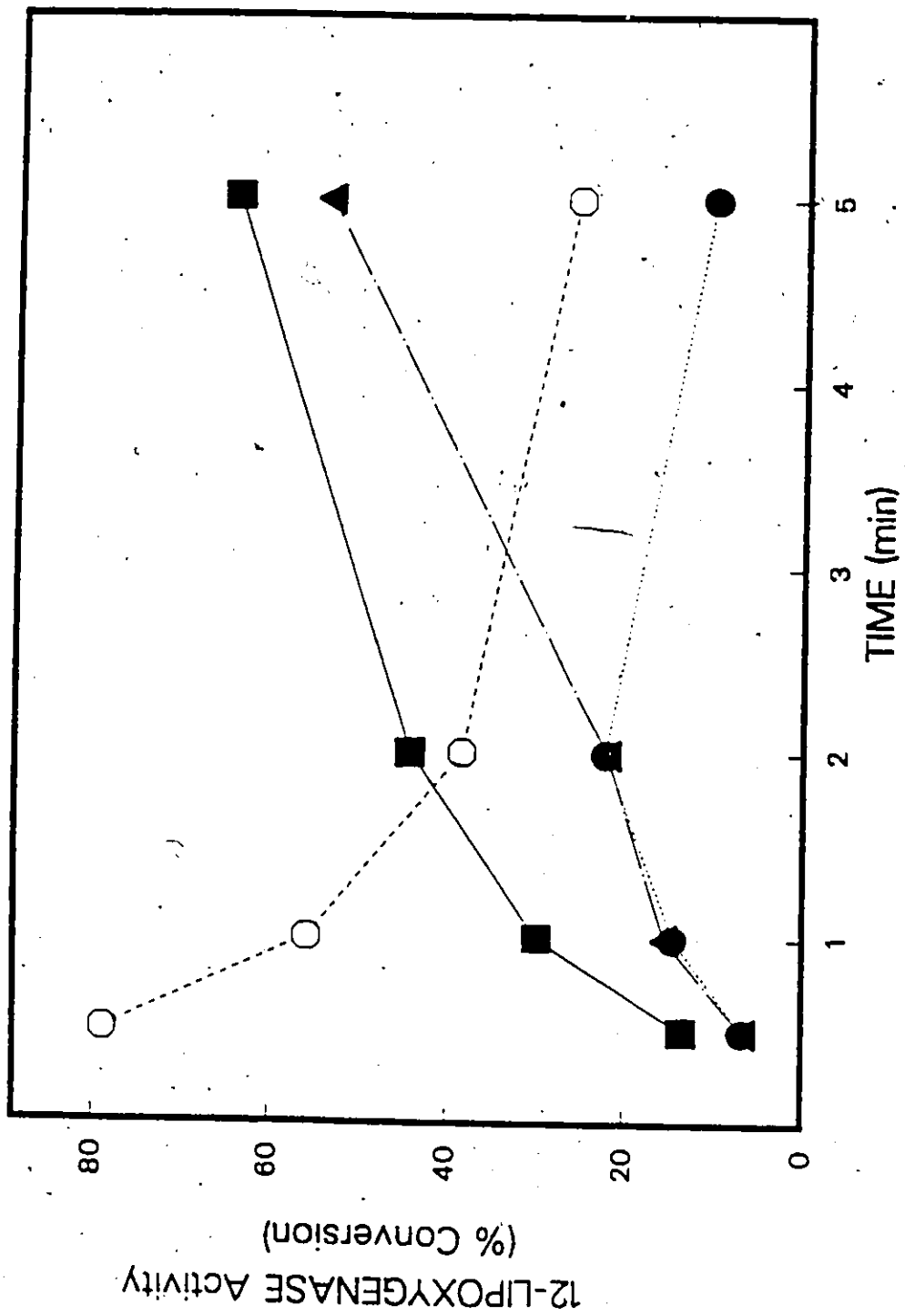


FIGURE 13

12-LIPOXYGENASE Activity

(% Conversion)

TIME (min)

Figure 14: Effect of dietary vitamin E on platelet lipoygenase activity, expressed as percentage conversion of ¹⁴C-arachidonic acid to labelled 12-HPETE and 12-HETE per ug platelet protein. Values are means \pm S.E.M. of duplicate determinations from 3 animals per group. Bars sharing different letters are significantly different ($P < 0.05$).

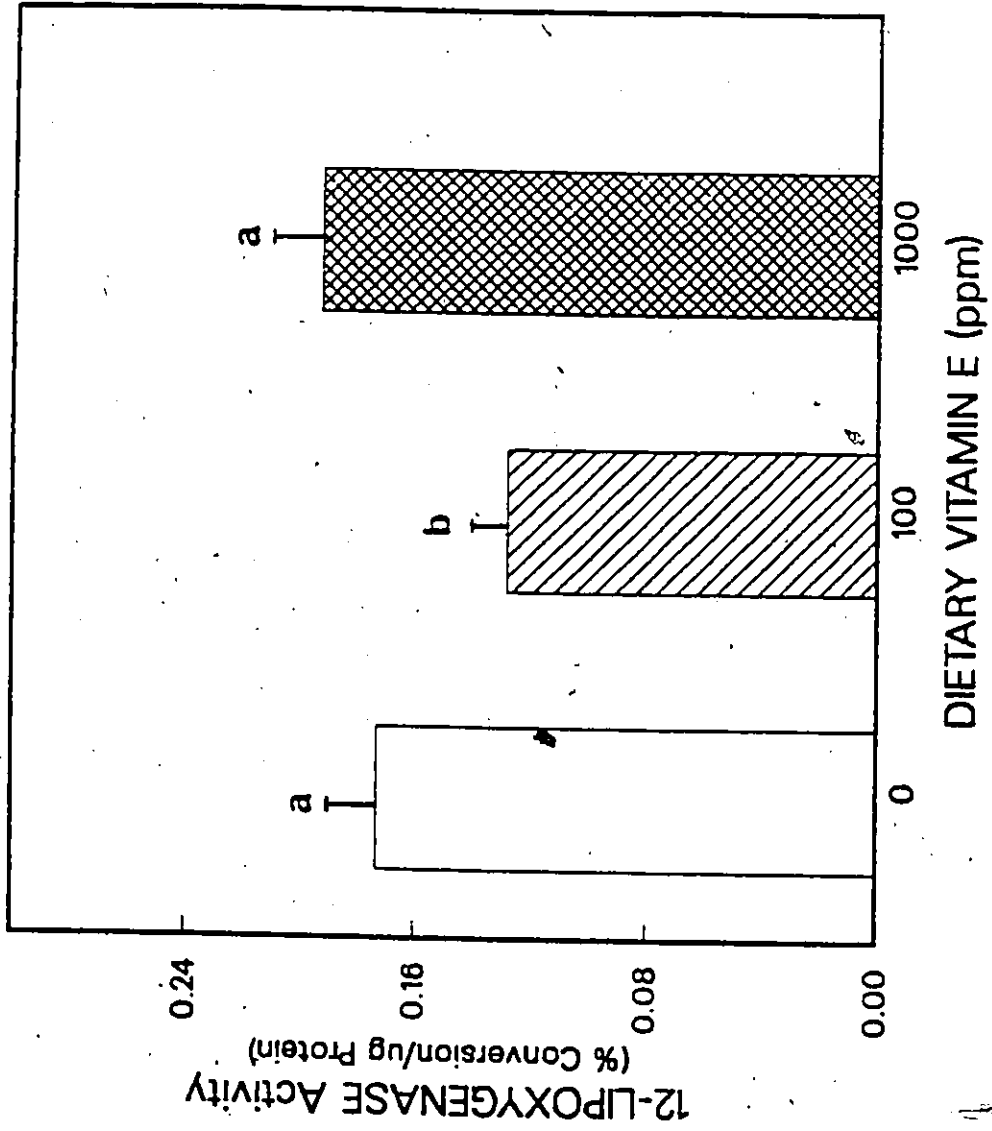


FIGURE 14.

Vitamin E Refeeding and platelet Lipoxygenase Activity.

Since platelet lipoxygenase activity was influenced by vitamin E deficiency and vitamin E excess in rats, it seemed appropriate to study the effect of vitamin E refeeding of deficient rats, on 12-HPETE and 12-HETE formation. Figure 15 shows the changes in plasma pyruvate kinase activity, plasma tocopherol concentration, and 12-lipoxygenase activity when deficient rats were supplemented with 100 ppm vitamin E for a varying number of days. Plasma pyruvate kinase activity remained high after 4 days of supplementation and reached 0.23 U/ml after 14 days of the refeeding period. This value is relatively high compared to values obtained for a typical 100 ppm vitamin E supplemented rat (table 5). Plasma tocopherol concentration after 7 days of refeeding was similar to that of the 100 ppm vitamin E supplemented rats (table 5). These changes in plasma pyruvate kinase activity and tocopherol concentration, induced by changes in the dietary level of vitamin E, are reflective of changes in the vitamin E status of the rat, which may influence platelet lipoxygenase activity. In fact, formation of 12-lipoxygenase products was markedly stimulated after 7 days of vitamin E supplementation, with no noticeable change after 2 and 4 days (figure 15).

Figure 15: Changes in plasma tocopherol concentration (▲), plasma pyruvate kinase activity (■), and platelet 12-lipoxygenase activity (●) as a result of vitamin E refeeding of deficient rats. Deficient rats were re-fed a diet containing 100 ppm DL- α -tocopheryl acetate for the number of days indicated. Each data point represents duplicate determinations of one animal.

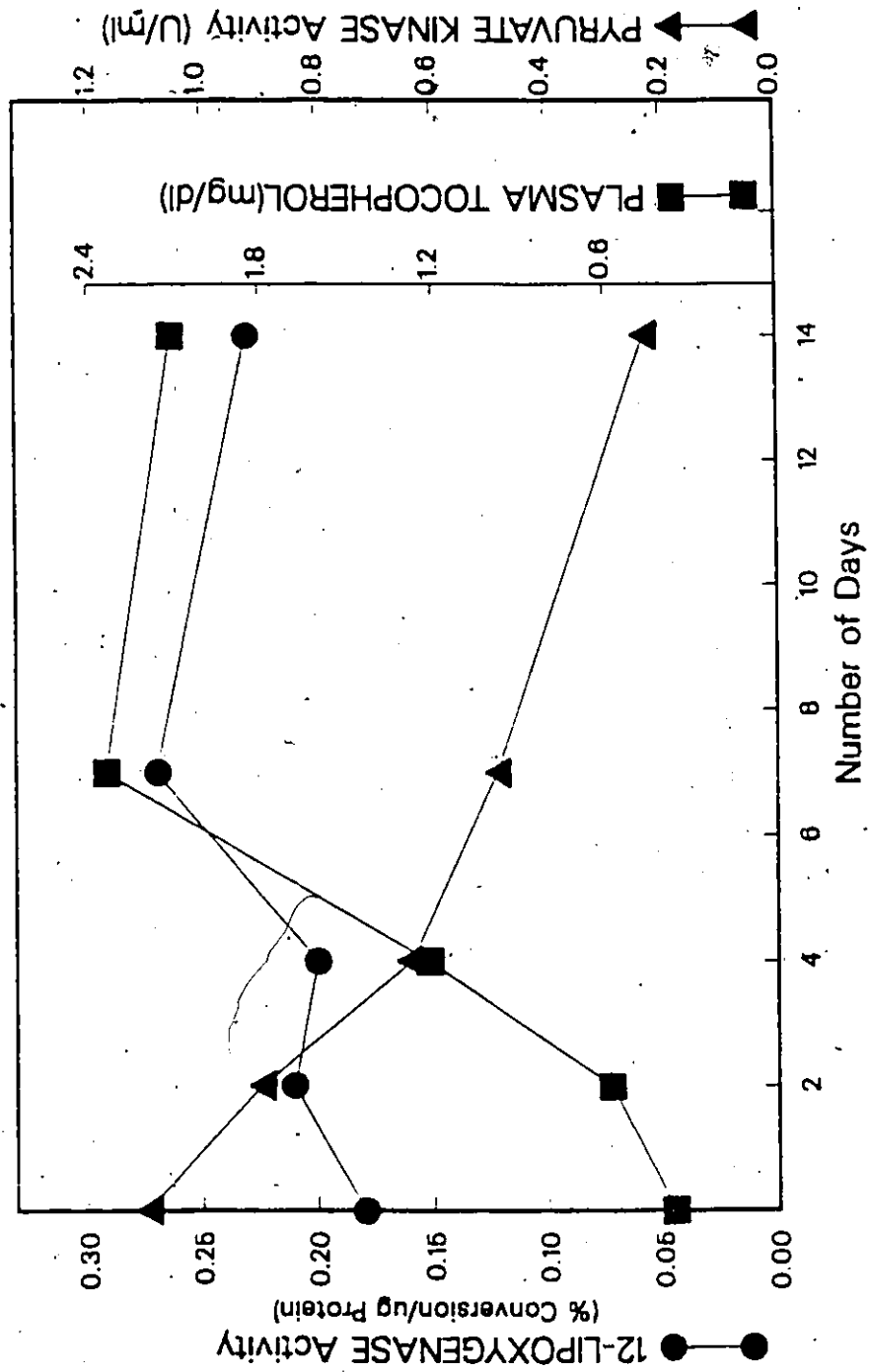


FIGURE 15

DISCUSSION

Vitamin E deficiency in the rats resulted in several apparent physiological changes as well as biochemical alterations. For example, at the end of the dietary study, the deficient rats were obviously showing signs of degeneration, as expected and confirmed by the elevated plasma pyruvate kinase activity. Rapid increases in PK activity due to leakage of this enzyme from damaged muscle in vitamin E deficient animals has been reported by others (Chow, 1975; Machlin et. al., 1978; Chan and Leith, 1981). The level of vitamin E supplementation (100 or 1000 ppm) did not alter the PK activity in the rats. Testicular degeneration, noted in the deficient rats, is a common pathology resulting from vitamin E deficiency (Mason, 1954). The increased platelet number in the deficient rats has previously been reported (St.Jaques-Hamelin and Chan, 1983; Machlin et. al, 1975), and may indicate an increased arachidonate conversion potential in deficient rat platelets. The lack of fluctuation in the plasma lipid levels among the three groups of rats is not unexpected since the level of corn oil in each diet was constant. It is possible, however, that certain sub-components of the plasma lipids such as cholesterol or lipoproteins may be altered by vitamin E status, since these compounds are important in absorption and transport of the vitamin. These small alterations may be masked by the major plasma lipid components and may not be detectable. Although the platelet tocopherol concentrations were not determined, it must be pointed out that the plasma tocopherol level does reflect the relative absorption and turnover of vitamin E in tissues. In fact, a positive correlation between platelet tocopherol and plasma tocopherol concentrations has been reported (Vatassery, Krezowski and Eckfeldt, 1983). It could be assumed, then, that the changes observed in platelet phospholipase A₂ and

12-lipoxygenase activity are a result of changes in platelet tocopherol concentration which were induced by the different dietary levels of vitamin E. These results clearly indicate that dietary vitamin E may play a major role in the release and subsequent metabolism of arachidonic acid in platelets.

Vitamin E and Platelet Phospholipase A₂ Activity

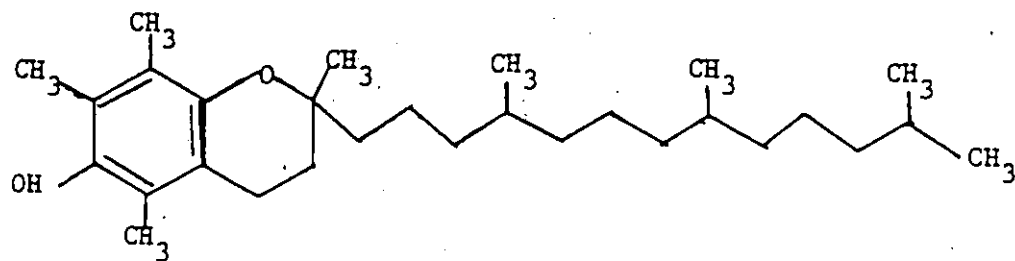
The results of these experiments clearly demonstrate that vitamin E is capable of attenuating the activity of platelet phospholipase A₂ and, hence it can control the release of free arachidonic acid. This effect of vitamin E could explain previous findings which showed that thrombin stimulated rat platelets produced significantly less thromboxane when vitamin E was increased in the diet (Karpen, Merola, Trewyn, Cornwell and Panganamala, 1981; St. Jacques-Hamelin and Chan, 1983). This behavior of phospholipase A₂ in response to vitamin E led to the following hypothesis: In normal platelets, phospholipase A₂ activity is partially suppressed by endogenous vitamin E while, in deficient rats, where endogenous vitamin E concentration is much lower (Lehmann, 1981), a higher enzyme activity is expected. In our study, not only is the phospholipase A₂ activity suppressed in vitamin E supplemented rat platelets, but addition of tocopherol to platelet suspensions further suppresses phospholipase A₂ activity. The fact that enzyme activity is inhibited in a dose-dependent manner by exogenously added vitamin E, lends support to this hypothesis. Examination of the behavior of phospholipase A₂ in response to various forms of tocopherol (figure 9) enables one to speculate on the possible mode of action of vitamin E on the enzyme. Two forms of tocopherol are normally used in

experimental studies; the natural vitamin (D- α -tocopherol) and the synthetic vitamin (DL- α -tocopherol) formed by a condensation reaction between trimethylhydroquinone and racemic isophytol. Experimental evidence indicates that the biological activity of the natural vitamin is much higher than that of the synthetic compound (Ames, 1979; Horwitt, 1980). In this study, there was little difference in the inhibitory effect of D- α -tocopherol and DL- α -tocopherol on phospholipase A₂ when they were incubated with the platelet preparation. It appears that the stereochemistry of the methyl groups in the tocopherol molecule is not a significant factor for its inhibitory effect on the enzyme. Tocol (figure 16), an analogue of tocopherol in which the methyl groups are absent from the chromanol ring, inhibited the activity of both the crude and the partially purified phospholipase A₂ to a greater extent than either the natural or the synthetic vitamin. On the other hand, DL- α -tocopheryl acetate (figure 16), in which the chromanol ring hydroxyl group is replaced by acetate, had little effect on enzyme activity, suggesting that the OH group on the tocopherol molecule plays a role in the inhibitory action of vitamin E on phospholipase A₂. Since tocol induced the greatest inhibitory effect on phospholipase A₂, one can conclude that the methyl groups on the chromanol ring are not crucial for inhibition, but their absence may potentiate this inhibitory action of vitamin E.

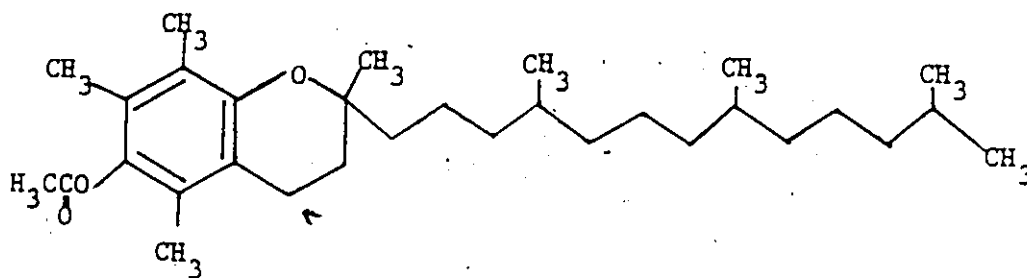
Since phospholipase A₂ is a membrane-bound enzyme exerting its action on membrane components, it is not unreasonable to speculate that the inhibitory action of vitamin E on the enzyme is due to its associations within the platelet membrane. According to Lucy's model, vitamin E is capable of partitioning in the lipid bilayer in such a manner that the hydroxyl moiety of the chromanol head group may form hydrogen bonds with the phosphate groups of the membrane phospholipids and the nonpolar phytol

Figure 16: Structure of tocopherol and its analogues used in the phospholipase A study.

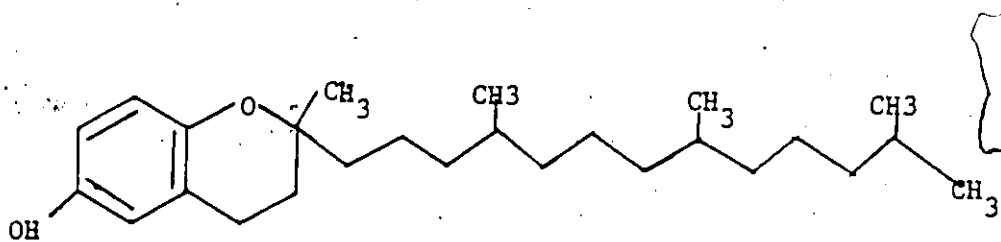
FIGURE 16



α - TOCOPHEROL



α - TOCOPHEROL ACETATE



TOCOL

chain may interact hydrophobically with the fatty acyl chains (Lucy, 1972). For such specific interactions to be possible, it is important that the amphipathic nature of the tocopherol molecule be maintained. The fact that tocopheryl acetate fails to inhibit phospholipase A₂ may be due to the absence of the hydroxyl group which alters the hydrogen-bonding capability of the molecule. In the case of tocol, the absence of the methyl groups on the chromanol ring is expected to alter the hydrophobicity of the molecule and, therefore, affect its interaction with membrane components.

Although the results of these experiments clearly demonstrate that dietary vitamin E alters the rat platelet phospholipase A₂ activity, the mechanism of this alteration remains unclear. Examination of the effect of several tocopherol analogues on a partially purified platelet phospholipase A₂ enabled us to speculate that the inhibitory action of vitamin E is most likely due to its effect on the platelet membrane. Whether vitamin E modulates platelet phospholipase A₂ through a direct interaction with the enzyme or with the phospholipid substrate, remains to be elucidated.

Vitamin E and Platelet Lipoxygenase Activity

Vitamin E deficiency as well as vitamin E excess was shown to enhance rat platelet 12-lipoxygenase activity. When deficient rats were refed with diets containing adequate vitamin E, a stimulation of enzyme activity was noted. Similar enhancement in lipoxygenase activity was observed in platelets from humans that were supplemented with high doses (400 IU/day) of vitamin E for several weeks (Chan, Patrick, Raynor, Boland and Douglas, 1985).

The mechanism by which vitamin E exerts its action on 12-

lipoygenase is unclear at present. Arachidonic acid oxygenation is a free radical-initiated reaction and requires a small but continuous presence of cellular peroxides for activation (Lands and Hemler, 1979). Antioxidants such as vitamin E are expected to reduce the rate of peroxide formation and, as a result, suppress lipoygenase activity. Although the free radical scavenging property of vitamin E can explain the enhanced lipoygenase activity in the deficient rat platelets, it cannot explain the enhanced activity in the 1000 ppm rat platelets or the stimulation of lipoygenase activity in platelets from deficient rats that were supplemented with adequate vitamin E. Membrane alterations induced by changes in vitamin E concentration are not expected to influence 12-lipoygenase activity since this enzyme is soluble.

The results of the lipoygenase study are interesting but perplexing. Since neither the free radical scavenging nor the membrane modifying property of vitamin E can fully explain the observed effects, the influence of vitamin E on platelet lipoygenase may be an indirect one. For example, excessive dietary vitamin E is known to suppress thromboxane synthesis in thrombin-stimulated platelets (St. Jacques-Hamelin and Chan, 1983); therefore, the increased lipoygenase activity in the platelets from rats fed excessive vitamin E may be due to a shift of arachidonate substrate from the cyclo-oxygenase to the lipoygenase pathway. Although both enzymes require the presence of peroxides for activation, the peroxide requirement for full cyclo-oxygenase and lipoygenase activities could be different. As a result, there must exist an optimal peroxide concentration, influenced by antioxidant level, that is required by each enzyme to permit initiation and continuation of enzyme activity.

The relationship of 12-lipoygenase and its products to other enzymes in the arachidonate cascade is not a simple one. A number of recent

studies indicate that a complex series of interactions exist among platelet and leukocyte enzymes in the regulation of arachidonate metabolism (figure 17) (Vanderhoek, Bryant and Bailey, 1980, 1982). For example, 12-HPETE is reported to stimulate 5-lipoxygenase activity in leukocytes, increasing the formation of 5-HETE and LTB₄ (Maclouf et. al, 1982). In addition, 12-HETE has been reported to be a potent inhibitor of platelet phospholipase A₂ activity (Chang et. al., 1985). In light of these reports it is not surprising that there is no clear-cut explanation for the modulatory effect of vitamin E on platelet lipoxygenase activity. Since the products of the lipoxygenase reaction, 12-HPETE and 12-HETE, both have profound influences on the reactions of platelets and leukocytes, it is likely the effects of vitamin E are mediated via some other part of the arachidonate pathway.

Figure 17: Interaction of the 12-lipoxygenase products, 12-HPEPE and 12-HETE, with other enzymes of the arachidonate cascade in platelets and leukocytes.

A

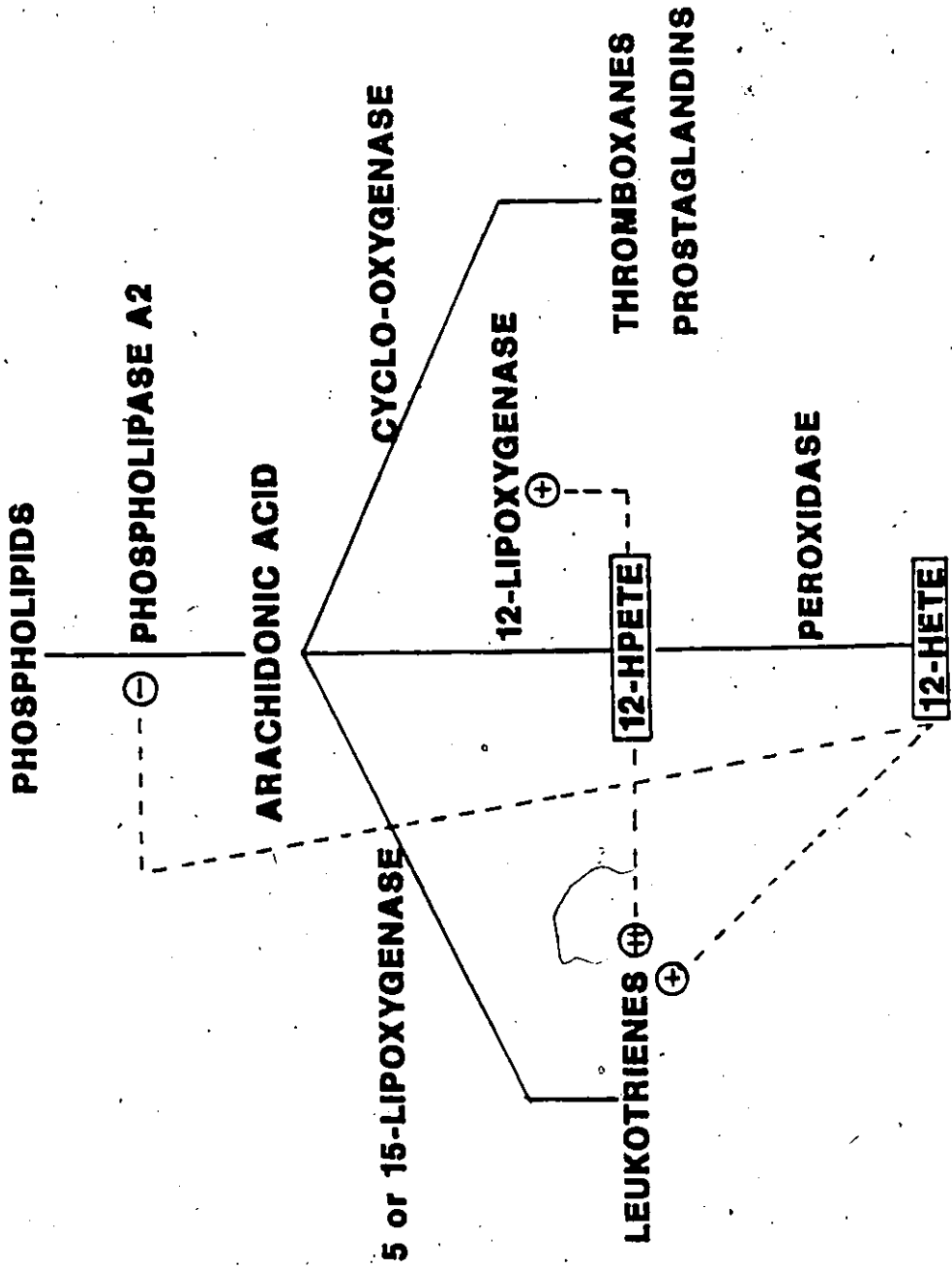


FIGURE 17

CONCLUSION

Although the results of these studies clearly demonstrate that vitamin E alters platelet phospholipase A₂ and 12-lipoxygenase activities, the exact mode of action of the vitamin on these enzymes remains unclear. Since arachidonate metabolites in platelets have profound influences in the cardiovascular system and in mediating inflammatory reactions, vitamin E may play a role in the treatment of thrombotic disorders and allergies.

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