# ENZYMATIC REGULATION OF VITAMIN E STATUS: IDENTIFICATION AND CHARACTERIZATION OF THE NOVEL TOCOPHEROL-OMEGA-HYDROXYLASE PATHWAY OF VITAMIN E CATABOLISM

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# ENZYMATIC REGULATION OF VITAMIN E STATUS: IDENTIFICATION AND CHARACTERIZATION OF THE NOVEL TOCOPHEROL-OMEGA-HYDROXYLASE PATHWAY OF VITAMIN E CATABOLISM

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Tocopherols and tocotrienols all possess to varying levels the vitamin E activity of α-tocopherol, the most bioactive form of the vitamin in vivo. α-Tocopherol is the most abundant vitamer in vivo, potentially explaining its higher bioactivity. This is despite higher dietary levels of other vitamers. The preferential retention of α-tocopherol appears to be at the level of elimination. Urinary metabolites of tocopherols, with the phytyl tail truncated to a three-carbon carboxylated moiety, were reported previously. The goal of this work was to elucidate the pathway(s) by which these metabolites are formed. Incubations of tocopherols in hepatocyte culture produced all expected intermediates in the predicted pathway of catabolism of vitamin E. This pathway involves ω-hydroxylation of a terminal methyl group of the phytyl tail, followed by step-wise removal of two or three carbon units by a β-oxidation mechanism. Analysis of microsomal enzyme activity led to the elucidation of CYP4F2 as the major P450 involved in the initial ω-hydroxylation reaction. Substrate-specificity of this enzyme was high, with activity toward γ-tocopherol being 10-fold greater than toward α-tocopherol. Sesamin, known to raise plasma γ-tocopherol to levels near those of α-tocopherol in vivo, was a potent

inhibitor of this enzyme. Together, these data supported the hypothesis that the ω-oxidation pathway is an important regulator of vitamin E status.

Kinetic analyses revealed key features of the tocopherol molecule which govern the affinity and activity of the enzyme toward its substrates. These studies also revealed an allosteric nature of CYP4F2. The finding that α-tocopherol is a positive effector of γ-tocopherol metabolism may explain why supplementation with α-tocopherol decreases plasma concentrations of non-α-tocopherols.

Tocotrienols differed from tocopherols in their effects on lateral and rotational mobility of the microsomal membrane components, and inhibited the activity of membrane-bound P450 enzymes, possibly through inhibition of interaction of components of the cytochrome P450 multi-enzyme complex. These effects may play a role in the cytotoxic nature of the tocotrienols and highlight the importance of the tocopherol-ω-oxidation pathway in minimizing their concentration in biomembranes.

# BIOGRAPHICAL SKETCH

Timothy J. Sontag was born in Englewood, New Jersey, and at the age of ten made the decision to accept Jesus Christ as being the Son of God and his Lord. Everything else in his life was affected by that decision. It gave him someone to look to for direction throughout his youth. As he got older, it made him want to use the blessings God had given him for whatever purposes God had for him.

From the time he was young Tim loved science and learning how things worked. In 1993, he entered the Massachusetts Institute of Technology where he was a Course 7 (Biology) major. After finishing his Bachelor's degree in 1997, Tim stayed on as an undergraduate for one extra year to learn a new language, studying Spanish at the Universidad de Granada in Spain. During this year, he became interested in a more applied approach to biological science and entered the graduate program in Nutritional Sciences at Cornell University the following year. After a month-long rotation in the laboratory of Robert Parker, Tim felt that this was the right place to carry out his doctoral research studying lipid metabolism. Obtaining his Ph.D. in 2004, Tim is moving on to a post-doctoral position at the University of Chicago to continue learning about lipids. After that, where the next step will lead, only God knows.

To God,

who brought me this far

and who is always there to guide my way

and light my path.

Psalm 119:105; Matthew 28:18-20

To Mom, Jess, Hanna, and Stine,

for your love, hope and faith.

1 Corinthians 13: 13

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# TABLE OF CONTENTS

# LIST OF FIGURES

**CHAPTER IV**

# LIST OF TABLES

# CHAPTER I

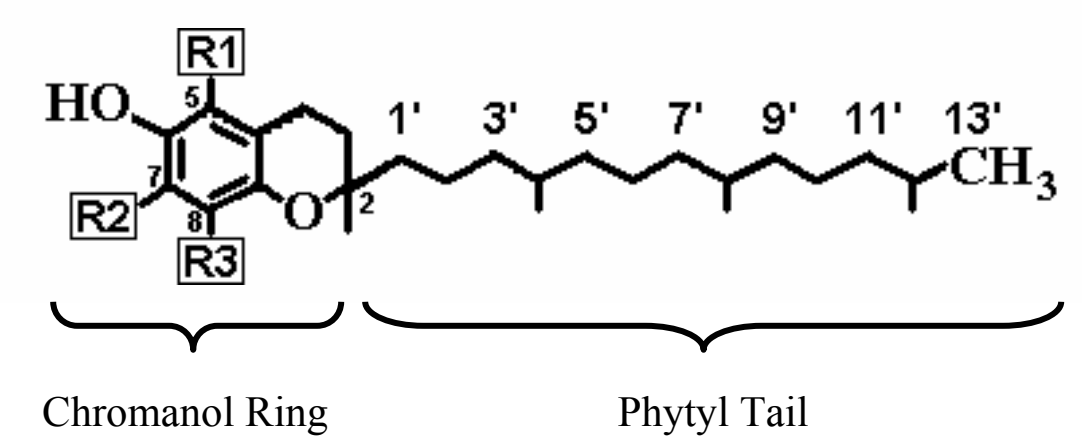
# LITERATURE REVIEW

**Vitamin E History**

The dietary substance termed vitamin E was discovered in 1922 as a component of the lipid-soluble fraction of the diet which prevented fetal death and resorption in the rat (1). Several years later work by Olcott and Mattill demonstrated the newly discovered vitamin possessed antioxidant activity, although the potency of this activity was not always well correlated with the vitamin activity (2). The structure of the vitamin was discovered over the course of several years as it was realized that the vitamin was actually a group of molecules possessing the same generic structure but differing in vitamin potency based on minor variations in their molecular structure. Work by Evans et al demonstrated the vitamin extract from wheat germ contained three different molecules, each possessing an alcohol moiety, but with the molecule designated "α" much more potent than the "γ" or "β" molecules in the vitamin activity assays (2,3). Meanwhile, Fernholz proposed the full structure of the vitamin after identifying the presence of a hydroquinone and phytyl tail within the vitamin structure (4). The structure was designated "tocopherol" by Evans and GM Calhoun to designate its function in childbirth (greek "tokos-pherein") and its alcohol structure (-ol). One more tocopherol, designated "δ" was isolated from soybean oil (5). Years later, the tocotrienols were discovered in wheat germ oil and bran as the unsaturated counterparts of the tocopherols (6). As with the tocopherols, the tocotrienols too possess vitamin E activity, and as such the term "vitamin E" designates both the tocotrienols and tocopherols. Thus, it is erroneous to use this term synonymously with only α-TOH.

**Vitamin E Structure**

The generic structure of vitamin E is now known to be made up of a heterocyclic chromanol ring substituted at the 2-carbon with a phytyl tail (**Figure 1.1**).




| | | R1 | R2 | R3 |
|---|---|---|---|---|
| Tri-methyl | α | **$CH_3$** | **$CH_3$** | **$CH_3$** |
| Di-methyl | γ | H | **$CH_3$** | **$CH_3$** |
| Di-methyl | β | **$CH_3$** | H | **$CH_3$** |
| Di-methyl | ε | **$CH_3$** | **$CH_3$** | H |
| Mono-methyl | δ | H | H | **$CH_3$** |
| No-methyl | **tocol** | H | H | H |

Tocopherol = no double bonds on phytyl tail
All rac = racemization at carbons 2, 4', and 8'
Tocotrienol = double bonds at carbons 3', 7', and 11'

**Figure 1.1**

Vitamin E structure depicting variations in vitamer structural features.

The greek letter designations α-, γ-, β-, and δ- indicate tri-, di-, di-, and mono-methylation variations respectively around the phenol moiety of the chromanol ring at carbons 5, 7, and 8 for both the tocopherols and tocotrienols. The unsaturated tocotrienols possess three unconjugated double bonds at the 3', 7', and 11' carbons of the phytyl tail. The chiral carbons of the phytyl tail at the 2, 4' and 8' positions exist in the synthetic forms of the vitamin in both the R or S positions, resulting in eight potential stereoisomers of the all racemic (all-rac) vitamin E. The hydroxyl group on the chromanol ring may be synthetically esterified with substituents such as an acetate or succinate moiety.

**Activity as a Vitamin**

As mentioned, the various vitamers of vitamin E differ in their vitamin E activity based on a variety of activity assays. Over the years since the first identification of vitamin E activity there has been much debate as to the true vitamin function of E and therefore the best assays to measure deficiency and to compare the activities of the vitamers. The original rat fetal resorption assay continues to be the most commonly accepted measure of vitamin activity, although several other methods exist, including prevention of red blood cell hemolysis in the rat, myopathy prevention in the chick, and myopathy cure in the rat. Official bioactivity data for vitamin E are currently based on the rat fetal resorption assay. By this assay, 1 mg all-rac-α-tocopherol acetate is defined to be equal to 1 international unit (IU) or 1 US Pharmacopeia unit (USP) to which all other vitamers are compared. Accordingly, the natural RRR-α-tocopherol possesses 1.36 times the activity of the standard and is the most bioactive of the vitamers. The bioactivity values of each of the vitamers are shown in **Table 1.1** (7). Current Daily Recommended Intake guidelines ignore the non-α forms of the vitamin and are based only on α-tocopherol intake (8). The quantitative human requirements are based largely on data from studies using the ex

**Table 1.1**

Bioactivity of the vitamin E vitamers as determined by the rat fetal resorption assay (Beiri and Mckenna, 1981). One α-TOH equivalent is equal to the activity of 1 mg α-TOH and the activity of 1 mg of each of the other vitamers is determined by comparison with 1 mg α-TOH.

| **Vitamer (1 mg)** | **α-TOH equivalents** |
|---|---|
| α-TOH | 1.0 |
| all rac-α-TOH | 0.74 |
| γ-TOH | 0.1 |
| β-TOH | 0.5 |
| δ-TOH | 0.03 |
| α-T3 | 0.3 |
| β-T3 | 0.05 |
| γ-T3 | n.d. |
| δ-T3 | n.d. |

vivo hydrogen peroxide-induced erythrocyte lysis (9-14). From these data, a plasma concentration of 12μM was chosen as adequate (Estimated Adequate Requirement, EAR) to prevent in vitro hydrogen peroxide-induced hemolysis, by which the NHANES III study indicates greater than 95% of the US population is not vitamin E deficient (15). By vitamin E repletion studies (9) this plasma level can be achieved in adults by an intake of 12 mg/day α-tocopherol. Based on an RDA of 120% the EAR, the current Recommended Daily Allowance of vitamin E is set at 15 mg/day of α-tocopherol (8). There is little evidence of toxicity of supplemental doses of α-tocopherol at levels as high as 2,100 mg/day.

**Vitamin E function**

While there are several measures of vitamin E deficiency, no one molecular function has been clearly identified as responsible for deficiency symptoms. The most well accepted function of vitamin E is as an antioxidant in lipid membranes that prevents the propagation of free-radical reactions, especially in the context of polyunsaturated fatty acids (PUFAs) found in membranes and lipoproteins (16,17). The vitamin E molecule is able to donate an electron to a peroxyl radical, effectively quenching the free radical chain according to the following scenario where RH indicates a PUFA, ROO* indicates a peroxyl radical, and VitE-O* indicates a tocopheroxyl radical (18):

(1) ROO* + R'H → ROOH + R'*

(2) R'* + $O_2$ → R'OO* …and so on…

In the presence of vitamin E the reaction becomes:

(1) $R_1OO^*$ + $R_2H$ + VitE-OH→ $R_1OOH$ + $R_2H$ + VitE-O*

This radical-quenching reaction is made favorable by the fact that the peroxyl radical reacts with the vitamin E molecule several orders of magnitude faster than with another PUFA (19). Once the tocopheroxyl radical has been generated, it can

potentially act as a prooxidant to oxidize other lipids; however, other fates may be more favorable, including recycling back to the tocopherol molecule by other antioxidants or further oxidation to the stable tocopherol quinone.

While vitamin E is an extremely potent antioxidant, this capability does not explain the various biopotencies noted among the vitamers. Chemical analysis of the antioxidant activity in vivo of the tocopherols and tocotrienols reveals all have similar antioxidant activity to the most biopotent vitamer, α-tocopherol (20). Some reports have even demonstrated a greater antioxidant effect of tocotrienols when examined in lipid membranes (21). Clearly, the bioactivity differences among the vitamers observed in vivo are not due to differences in radical-trapping ability.

In recent years numerous non-antioxidant functions have been discovered for vitamin E, many of which are specific to certain vitamers. α-Tocopherol has been shown to function as an inhibitor of protein kinase C and smooth muscle cell proliferation (22,23), 5-lipoxygenase ((24), and phospholipase $A_2$ (25). It has also been demonstrated to activate protein phosphatase 2A and diacylglycerol kinase (26), to be a regulator of gene expression of numerous proteins including scavenger receptors (27), α-tropomyosin (28), and α-tocopherol transfer protein (29,30), and an inhibitor of platelet aggregation (31), and monocyte adhesion (32). γ-Tocopherol, lacking a methyl group at the carbon 5 position is able to scavenge peroxynitrite radicals at this nucleophilic position (33). This effect is not seen for α-tocopherol which is methylated at carbon 5. Brillant et al. have demonstrated that inhibition of $O_2^-$ production by THP-1 promonocytes is essentially limited to δ-tocopherol (34). γ-Tocopherol is additionally a much greater inhibitor of PGE2 mediated inflammatory response than α-tocopherol (35). Urinary metabolites of γ-TOH show this same anti-inflammatory effect and were originally discovered as natriuretic factors (36). Several studies have indicated a role for γ-TOH in the inhibition of prostate and colon cancer

cell growth (37-39). The tocotrienols regulate the expression of HMG-CoA Reductase by increasing the degradation rate of the reductase, thereby decreasing cholesterol synthesis (40). The tocotrienols induce apoptosis and inhibit proliferation in a wide variety of human and murine cell types (41-44). They also are more effective than the tocopherols in reducing endothelial adhesion to monocytes by reducing expression of adhesion molecules (45) and in preventing glutamate-induced neuronal cell death (46). Despite this wide variety of vitamin E activities, no activity has been clearly elucidated as the one giving the tocopherols or tocotrienols their vitamer function. Still, it is clear that there are numerous potential beneficial effects of all the vitamers beyond that of α-tocopherol.

**Sources of Vitamin E and average intake**

As vitamin E are lipid soluble substances synthesized only by plants, its main source is from plant oils. While all the tocopherols and tocotrienols are found at various levels in the plant seed, the leaves and other chloroplast-rich parts of the plant predominantly contain α-tocopherol. The distribution of the vitamers in plant oils can vary substantially, depending on the plant from which the oil is derived (**Figure 1.2**). In many cases, α-tocopherol is only a minor vitamer found in the oil. Animal sources of vitamin E in the diet are derived wholly from feeds as no animals can synthesize the tocochromanol molecule. However, the vitamer composition of all animal-derived food products is >90% α-tocopherol (47).

Reports on average intakes of vitamin E vary greatly due to the difficulty in obtaining accurate food intake data. The intake of α-tocopherol has been estimated between 4 and 13 mg/day (48). Given the main sources of dietary lipids in the U.S. diet being soy and corn oils, the average intake of γ-tocopherol is about 2.5 times that of α-tocopherol (49). Very little data is available concerning the average daily intake of the other vitamers. Dietary supplemental forms of vitamin E are mainly limited to

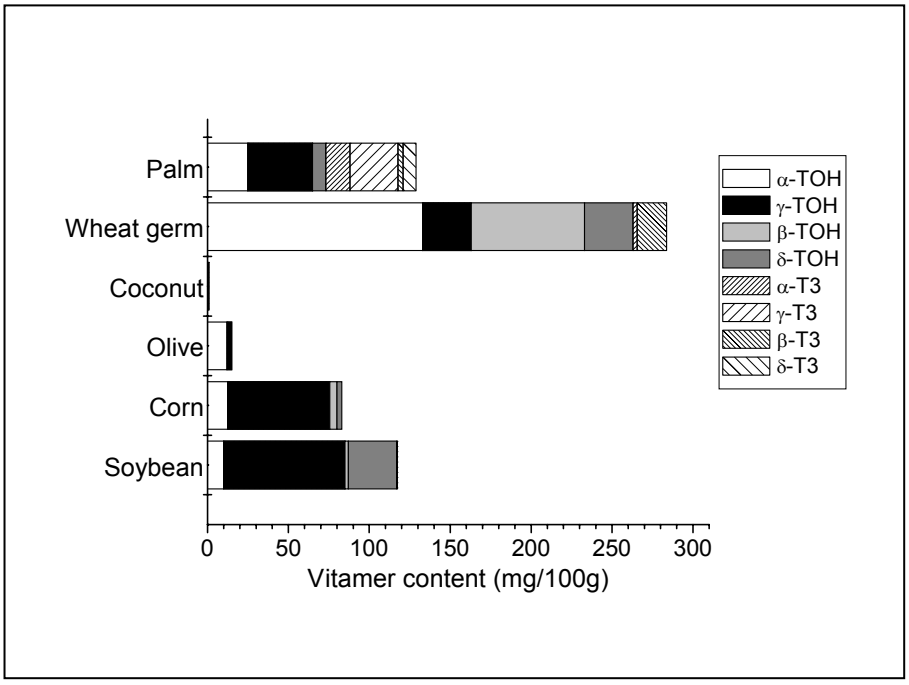



**Figure 1.2**

Distribution of vitamin E vitamers in plant oils (mg/100g oil). Tocotrienols were not analyzed in corn oil. γ- and δ-T3 were not analyzed in wheat germ.
(Data taken from Dial and Eitenmiller, 1995; Sheppard et al, 1993; Ong, 1993)
α-tocopherol, with a large proportion of the supplements consisting of synthetic, and therefore all-racemic, α-tocopherol rather than the natural RRR form.

**Vitamin E Plasma and Tissue Concentrations**

Despite the predominance of γ-tocopherol in the diet over α-tocopherol, data of plasma and tissue tocopherol concentrations reveal that the relative ratios of the vitamin E vitamers in the body do not reflect those in the diet. **Table 1.2** shows the relative concentrations of tocopherols in the plasma and tissues, demonstrating α-tocopherol plasma concentrations are greater than 10 times the concentration of the other vitamers (50,51).

The concentration of α-tocopherol in tissues is also greater than that of the other vitamers, although the ratio of α-TOH:γ-TOH in adipose tissue is reported to be half that in plasma, suggesting the tissues are more enriched in the other vitamers (or more depleted of α-tocopherol) compared to plasma (52). In fact, there are reports of near equal, or higher levels of γ-tocopherol in the skin and muscle when compared with α-tocopherol (53). A high correlation was shown between dietary intake and adipose tissue concentration for γ-tocopherol, yet this correlation did not hold for α-tocopherol (54). Supplementation of the tocopherols appears to have some effect on plasma levels of the other vitamers. It has been shown that supplementation of α-tocopherol depresses plasma and tissue γ- levels (55). In another study, that effect was seen, but supplementation with γ-tocopherol had no effect on α-tocopherol concentrations (56). On the other hand, Cement and Bourre fed vitamin E deficient rats a diet of a constant α-tocopherol concentration, but graded levels of γ-tocopherol (57). The rats fed the γ-diets showed much higher tissue concentrations of α-tocopherol than the rats fed α-tocopherol alone and the higher the γ:α ratio, the higher the tissue α concentration. In other studies, γ-tocopherol-deficient weanling rats deprived of vitamin E for 13-15 weeks showed tissue α- levels 10% lower than those of γ-tocopherol sufficient rats (58). These studies suggest the ability of α-tocopherol to increase the elimination of γ-tocopherol and the ability of γ- to increase the

**Table 1.2**

Plasma concentrations of vitamin E vitamers (Lee et al, 2003; Chow, 1975).

| Vitamer (μmol/L) | Average | +/- Standard dev. |
|---|---|---|
| α-TOH | 27.3 | 4.7 |
| γ-TOH | 1.3 | 0.5 |
| β-TOH | <0.7 | - |
| δ-TOH | 0.1 | 0.0 |
| α-T3 | 0.1 | 0.1 |
| γ-T3 | 0.1 | 0.1 |
| δ-T3 | 0.0 | 0.0 |

retention of α-tocopherol. One other possibility that Emmel and Celle suggested is the conversion of γ-tocopherol to α-tocopherol by the intestinal microflora, although this does not explain the effect of α-TOH on γ-TOH plasma and tissue levels (58). Elmadfa et al supported this argument by a generational study done in Wistar rats, in which the first 3 generations were supplemented with synthetic γ-tocopherol (59). In the fourth generation, one group was fed a vitamin E free diet and subcutaneously injected with γ-tocopherol so as to avoid intestinal dietary tocopherol. The other groups were fed γ-tocopherol as with the first three generations along with additional methionine or choline as methyl donors. The ratio of α-/γ- was calculated as transformation rate and it was found that those supplemented with additional methyl donors displayed a higher ratio of α-/γ-. The rats injected with γ-TOH showed a much lower ratio of α-/γ-, which the authors said indicated a conversion of γ- to α-TOH by the intestinal flora which could then be absorbed through the gut. Still, no data currently exists demonstrating the actual conversion of one dietary form of tocopherol to another. In all cases the body appears to selectively enrich plasma and tissues with α-tocopherol while eliminating the non-α forms.

The fact that the plasma and tissue levels of the tocopherols and tocotrienols do not reflect the dietary ratios of these vitamers is a central vitamin E paradox that has driven much of the research in vitamin absorption, distribution and elimination until now.

**Vitamin E Absorption and Distribution**

Dietary vitamin E is absorbed mainly in the small intestine in a manner similar to other lipid food components. Esterified (synthetic) forms of the vitamin are hydrolyzed prior to absorption. The free tocopherol is packaged into micelles with bile acids and dietary lipids where it is then absorbed with other lipids by means of passive diffusion (60). Studies with thoracic-duct cannulated rats suggested an

absorption efficiency of 65% for α- and γ-tocopherol (61). Fecal recovery of a radioactive dose of α-tocopherol in humans has indicated about 70% absorption (62). Other studies suggest the tocopherols do not compete for absorption (61). Cystic fibrosis patients displaying impaired lipid absorption likewise display impaired tocopherol absorption (63). Once incorporated into the enterocytes, the vitamers are packaged into chylomicrons for secretion into the lymph (64). The importance of this step in regulation of vitamin E status is highlighted by the fact that patients with familial abetalipoproteinemia display impaired vitamin E absorption and low plasma tocopherol levels (65). Secretion of tocopherols into chylomicrons peaks in humans between 6 and 12 hours after vitamin E administration (66), however the half life of tocopherols in these chylomicrons is only 10 to 15 minutes (67). The newly secreted chylomicrons move from the lymph to the thoracic ducts where they enter the blood circulation. Once in the circulation, the core triglycerides of the chylomicrons are hydrolyzed by lipoprotein lipase, resulting in the smaller chylomicron remnant particles. During this time the vitamers may be taken up along with free fatty acids into peripheral cells as the chylomicrons are hydrolyzed (68), or also transferred to HDL. HDL is able to then transfer its vitamin E contents to other lipoproteins (69,70). From here, the vitamin E remaining in the chylomicron remnants are taken into the liver. Investigation of the chylomicron-rich fraction of vitamin E supplemented individuals reveals there is no discrimination between the tocopherols prior to uptake by the liver (71). Additionally, the exchange of vitamin E between the lipoproteins is non-discriminatory, and therefore equimolar doses of tocopherols result in equimolar concentrations in the lipoproteins during the first 6-9 hours after dosing (66).

Vitamin E was originally shown to be secreted from the liver with other lipids packaged into nascent triglyceride-rich VLDL (72). The VLDL particles are hydrolyzed in the circulation to LDL and the tocopherol is again distributed between

all the circulating lipoproteins (73). However, Arita et al have demonstrated in cultured hepatocytes that an inhibitor of Golgi function, Brefeldin A, inhibits VLDL secretion but does not affect secretion of α-tocopherol (74). It is possible that the VLDL secretory pathway is functional but not necessary for hepatic secretion of vitamin E. Another mechanism must exist for export of the vitamin from the liver. Oram et al have recently demonstrated the ability of the ABC-A1 transporter to facilitate the transfer of α-tocopherol from human fibroblasts and murine macrophages to plasma Apo-A1 (75). As the ABC-A1 transporter is expressed on the hepatocyte cell membrane, it is postulated that ABC-A1 transport is the mechanism for tocopherol secretion from the liver. This is supported by evidence from mice which lack a functional ABC-A1 transporter and are models for Tangiers disease. This defect results in a general deficiency of fat soluble vitamins including vitamin E (76). Additionally, fibroblasts from Tangiers patients lack the ability to deliver α -tocopherol to Apo-A1 (75). A phospholipid transfer protein in the plasma appears to facilitate the transfer of vitamin E between the lipoproteins and cells (77,78). The LDL receptor appears to play a role in vitamin E uptake into cells as radiolabeled α-tocopherol is incorporated into LDL receptor expressing cells at a higher rate than cells lacking the receptor (79). However, rabbits with a defect in LDL receptor expression showed no particular defect in delivery of α-tocopherol to tissues (80), suggesting this mechanism of tocopherol delivery is present, but not essential. α-Tocopherol is also taken up into hepatocytes by means of selective uptake from HDL, possibly by means of the SR-B1 scavenger receptor (81). The importance of this mechanism of cellular tocopherol uptake is indicated by the demonstration that α-tocopherol uptake from HDL is 5 times higher than from LDL in porcine primary endothelial cells (82).

While there appears to be no discrimination between the vitamers from absorption through hepatic clearance of chylomicron remnants, the hepatic secretion of the vitamers into the plasma is highly discriminatory. Monkeys fed equimolar amounts of d6-RRR-α-tocopherol, d3-rac-α-tocopherol, and d2-RRR-γ-tocopherol showed a VLDL fraction containing 80% d6-RRR-α-tocopherol after 24 hours, demonstrating post hepatic discrimination of non-α vitamers as well as synthetic forms (83). The subsequent distribution of the vitamers between lipoproteins and into tissues therefore reflects this discrimination. **Figure 1.3** depicts this pathway of tocopherol uptake and distribution. It is now clear that the liver is the main site of Vitamin E vitamer discrimination.

The post-absorptive selectivity for α-tocopherol compared with the other vitamers has been attempted to be explained by various mechanisms. It has been suggested that biliary excretion of the tocopherols favors the non α- forms resulting in preferential α-tocopherol retention. Non-supplemented patients with an indwelling t-tube in the bile ducts displayed no appreciable differences in the α/γ ratio in the bile when compared with plasma samples (84). In a patient supplemented with large equivalent doses of all-rac-α- and RRR-γ-tocopherol, biliary α-TOH excretion decreased while the γ-TOH excretion increased over the first 8 hours. After that, the tocopherols were found in the bile in ratios similar to that of the plasma, leading the authors to conclude that while γ- is secreted indiscriminately into the bile, α-tocopherol is preferentially secreted from the liver into lipoproteins until the mechanism for recognition is saturated, after which both tocopherols are nonspecifically secreted into the bile (84). The biliary route of tocopherol elimination was explored as a means of vitamer discrimination by Yamashita et al in which rats were fed diets containing either 300 mg/kg α-tocopherol, γ-tocopherol, a mix of the two, or γ-tocopherol + sesame seed (85). The plasma, liver, and bile concentrations of

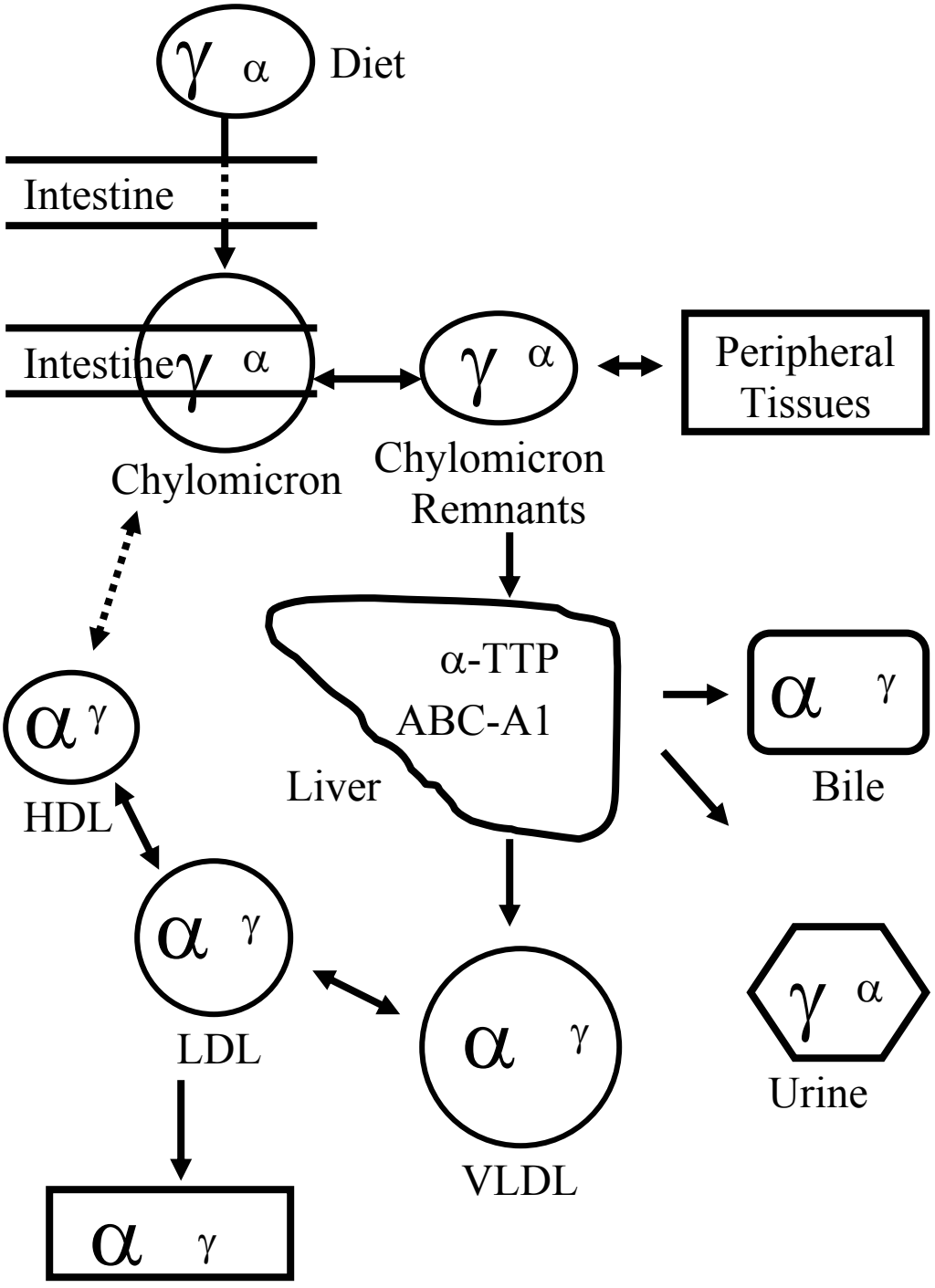



Figure 1.3
Vitamin E absorption and distribution. Relative amounts of α-TOH (α) or other vitamers (γ) distributed in the diet and body are depicted by character size.

tocopherol were measured. The plasma, liver, and bile ratios of α- and γ- were very closely correlated in all groups. This included the γ + sesame group which showed a marked increase in γ-tocopherol concentrations in liver, plasma and bile. Under the conditions of biliary excretion being the main route of non-α-tocopherol elimination, one would expect an increase in plasma/tissue γ-tocopherol concentrations to be marked by a correlating decrease in biliary excretion. The opposite effect was seen, suggesting biliary tocopherol excretion is rather a marker for tocopherol liver concentrations.

**Sesame Oil and Vitamin E Plasma and Tissue Concentrations**

The effect of sesame dietary sesame components on plasma and tissue tocopherol components has been explored in several other studies, especially the effect of the lignan components of sesame oil, sesamin and sesaminol (**Figure 1.4**). Yamashita et al first noted this effect while studying the effects of a mix of tocopherol and sesame lignans on indices of oxidative stress in rats (86). Rats were fed 4 diets: vitamin E-free control, α-tocopherol, γ-tocopherol, γ + sesaminol, and γ + sesamin. All tocopherols were at 50 mg/kg diet. Neither sesaminol nor sesamin affected plasma or liver concentrations of α-tocopherol to a significant level when compared with control. γ-Tocopherol on the other hand was elevated between 2-4 times the concentration of α-tocopherol in both plasma and liver. This was still only half the concentration of α-tocopherol in the α-supplemented group, but these levels of γ-tocopherol were able to increase the bioactivity of γ-tocopherol to levels matching those of α-tocopherol in plasma and liver lipid peroxide, RBC hemolysis, and pyruvate kinase measures of oxidative stress. This in turn suggests that it is the greater plasma and tissue concentrations that dictate the superiority of α-tocopherol in bioactivity measurements over the other vitamers, rather than intrinsic differences in chemical reactivity. Diets containing both α- and γ-tocopherol with sesame lignans

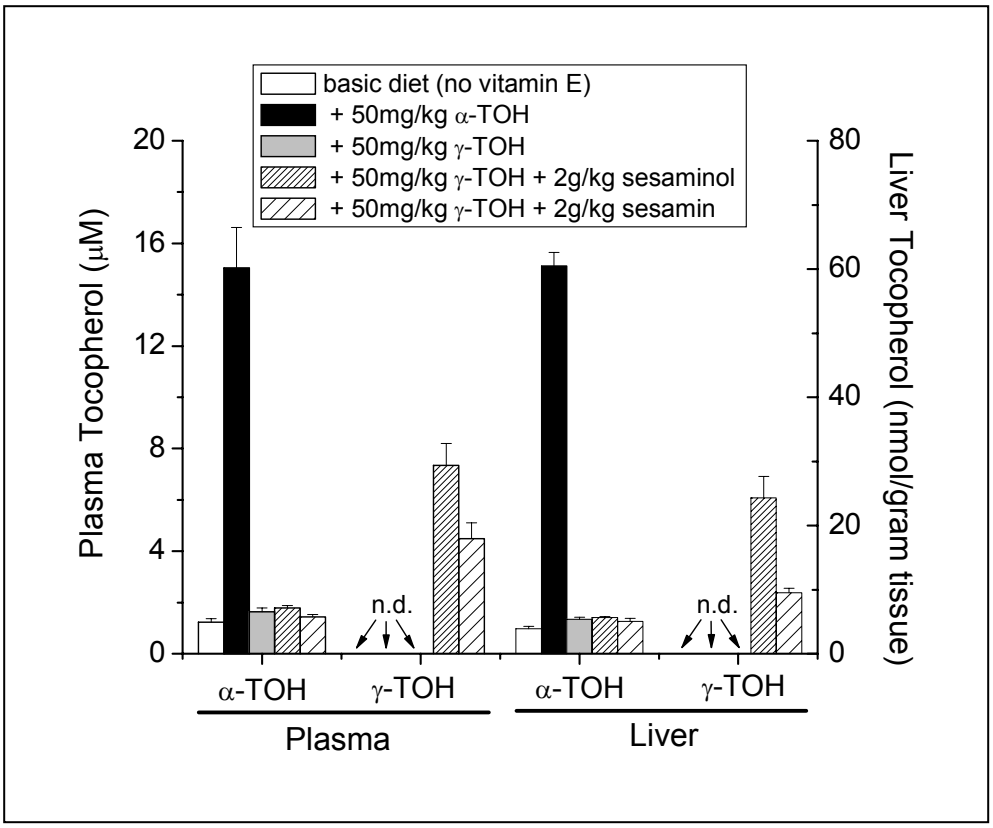



**Figure 1.4**

The effects of sesame lignans on plasma and liver concentrations of α- and γ-TOH in rats fed experimental diets for 8 weeks. Data is shown as means +/- standard error, n = 6. n.d. = not detected. (Yamashita et al, 1992).

were also shown to raise γ-tocopherol plasma levels with minimal effect on α levels in rats (87). Similar effects are seen in humans fed diets containing sesame seed (88). It appears that components of sesame are able to affect the mechanism(s) that regulate elimination of the non-α vitamers, although the mechanism was not identified in these studies.

**Tocopherol Transfer Protein**

In 1975 a cytosolic protein was identified in rat liver which bound α-tocopherol. This protein was later found to transfer tocopherol between membranes and it is therefore designated α-Tocopherol Transfer Protein or α-TTP (89). This protein along with the so called tocopherol binding protein (TBP) and tocopherol associated protein (TAP) are members of the Sec 14 family of lipid binding and transfer proteins (90). While the liver appears to be the main site of expression of α-TTP, messenger RNA has been detected in brain, spleen, lung, kidney, and placenta (91-93).

The membrane transfer function of α-TTP was used to test its activity toward various forms of vitamin E (94). The ability of the vitamers to compete with α-tocopherol for transfer between mitochondrial membranes and liposomes demonstrated the specificity of α-TTP for transferring α-tocopherol (**Table 1.3**). α-TTP stimulated α-tocopherol secretion from α-TTP-expressing McARH7777 hepatic cells (74). The function of α-TTP in hepatocytes is therefore presumed to be in selectively regulating the transport of α-tocopherol between membranes, resulting in the enhanced secretion of α-TOH into the plasma. The lower binding affinity of the other vitamers would then result in less secretion of these non-α- forms into the plasma. The same study by Arita et al indicated that this TTP-facilitated secretion did not occur via enhanced packaging of α-tocopherol into nascent VLDL as previously

**Table 1.3**

Relative affinities of vitamin E vitamers for the α-tocopherol transfer protein. The rate of transfer of radiolabeled RRR-α-TOH between membranes was calculated. A 50 fold excess of each vitamer was tested for competition with the radiolabeled RRR-α-TOH for transfer. Relative affinities for each vitamer are expressed as a percentage of RRR-α-TOH value. Data are expressed as mean +/- standard error. (Hosomi et al, 1998)

| Vitamer | Relative Affinity (%) | Standard error |
|---|---|---|
| RRR-α-TOH | 100 | - |
| SRR-α-TOH | 10.5 | 0.4 |
| γ-TOH | 8.9 | 0.6 |
| β-TOH | 38.1 | 9.3 |
| δ-TOH | 1.6 | 0.3 |
| α-T3 | 12.4 | 2.3 |

assumed, as TTP-facilitated secretion occurred when VLDL synthesis was chemically blocked.

The importance of α-TTP in vitamin E status is highlighted by a vitamin E deficiency syndrome termed AVED (ataxia with vitamin E deficiency). Mutations in the α-TTP gene were found to result in this syndrome, and patients had to be highly supplemented with α-tocopherol to overcome the nervous system disorder symptoms (95,96). α-TTP knockout mice display the symptoms of AVED patients and extremely low plasma and tissue tocopherol levels (97,98). As α-TTP does show some binding affinity for non-α-tocopherols, it may also aid in the secretion (albeit low secretion) of non-α forms of the vitamin, as evidenced by the fact that in α-TTP compromised mice, plasma γ-tocopherol levels also decrease although not to the same extent as α-tocopherol (98). Additionally, tissue ratios of RRR-α- to rac-α-tocopherol levels were around 2 in control mice, but only 1 in TTP knockouts, suggesting TTP preferentially secretes the 2R diastereomers over the 2S diastereomers (97). There is some evidence of up-regulation of α-TTP by vitamin E deficiency although the literature is equivocal on this subject (29,30). In all cases, the data clearly indicates this protein is an important regulator of plasma and tissue tocopherol status with a selective preference toward α-tocopherol. However, α-TTP alone is not sufficient to explain the lower levels of non-α-tocopherols found in plasma and virtually all tissues despite their predominance in the diet. Based on the current theory of α-TTP-mediated transfer of α-tocopherol out of the liver, the body must possess another mechanism for eliminating the remaining tocopherols, as there is no evidence of a build-up of non-α vitamers in the liver or other tissues. Additionally, there is no increase of α-tocopherol excretion in the bile of α-TTP knockout mice (97). Another route must therefore exist to explain tocopherol elimination.

**Vitamin E Catabolism and Excretion**

Simon and colleagues described the presence of an apparent metabolite of α-tocopherol in human urine (99,100). These metabolites, tocopheronic acid and its tocopheronolactone, commonly referred to as the Simon metabolites, were the side-chain shortened carboxyl derivatives of α-tocopherol, whose chromanol head group had been oxidized to the corresponding quinone (**Figure 1.5**). Nearly half a century later, Schonfeld et al identified another metabolite of α-tocopherol, the 2,5,7,8-2(2'-carboxyethyl)-6-hydroxychroman α-CEHC (**Figure 1.5**) (101). This metabolite differed from the Simon metabolites in that it possessed an intact (non-oxidized) chromanol head group. All of the metabolites were present in urine as glucuronide conjugates. It is in question whether the Simon metabolites are truly metabolic results of enzymatic activity, or simply artifacts of the extraction and analytical procedures used by Simon's group (102). The analogous γ-CEHC and δ-CEHC metabolites have also been identified, each being found in the conjugated form in urine (**Figure 1.5**) (36,103). The tocotrienols also are metabolized to the corresponding CEHC metabolite (104,105). As the side chain is truncated to the 3' carbon of the tocotrienols, all double bonds are removed and the CEHC metabolites of the tocotrienols are therefore identical to the CEHC metabolites of the corresponding tocopherol (**Figure 1.5**). Interestingly, the γ-CEHC metabolite was originally identified as a natriuretic factor, while this activity was not noted for the α- form (36).

While the CEHC metabolites were discovered in the urine, their presence has also been detected in both the plasma and bile (106,107). Supplementation of the diet with α- or γ-TOH increases the concentration of their CEHC metabolites in the plasma, raising the question as to whether the plasma concentration of the α-CEHC may be used as a measure of an adequate vitamin E supply (102,106). Patients with end-stage renal disease show accumulation of CEHC metabolites in the plasma, which

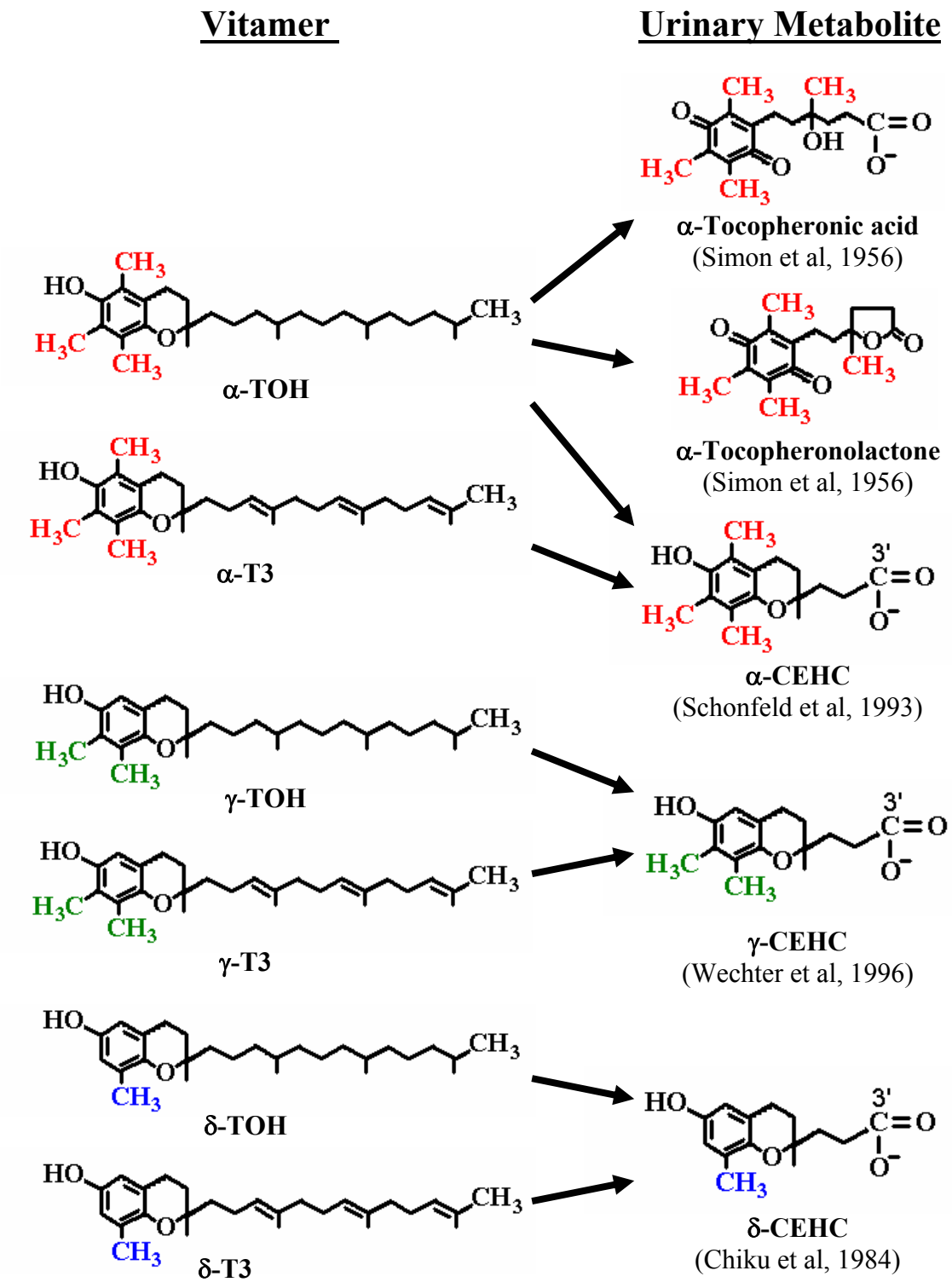



**Figure 1.5**

Vitamin E Vitamers and their metabolites identified in urine

may suggest that renal filtration plays a role in determining plasma CEHC concentrations (108,109).

Given the natriuretic activity of γ-CEHC, there has been considerable interest in the role of tocopherols as precursors to active metabolites. The intact chromanol ring of the CEHC metabolites suggested they might possess antioxidant activity as is the case for their tocopherol and tocotrienol precursors. Indeed the CEHC metabolites posses the ability to scavenge radicals, but this activity seems to be mainly relegated to the aqueous environment, whereas tocopherol antioxidant activity lies mainly in the hydrophobic environment of membranes (20). γ-CEHC is able to inhibit cyclooxygenase-2-mediated synthesis of PGE2, resulting in an anti-inflammatory activity greater than γ-TOH, α-TOH or α-CEHC (110).

**Metabolism as a Potential Mechanism of Regulation of Vitamin E Status**

The identification of urinary metabolites of vitamin E suggested a potential role of metabolism in regulating plasma and tissue vitamin E status. As described, the tissue and circulating ratio of α-/γ-tocopherol strongly favors α-tocopherol, under virtually all normal dietary conditions. Quantification of plasma α- and γ-CEHC metabolites revealed a 10-fold difference in baseline levels, favoring γ-CEHC (106). Similarly, while studies using deuterium-labeled RRR- and rac-α-tocopherol reveal a preferential retention of the natural RRR form, the synthetic form is preferentially excreted as α-CEHC in human urine (111). Swanson et al used deuterium labeled γ-CEHC to accurately determine the concentration of γ-CEHC in human urine (112). Based on estimates of typical daily γ-tocopherol intake, the authors concluded that urinary excretion of the CEHC metabolite is the major route of elimination of γ-tocopherol in humans. This identified a route for elimination of the vitamers not retained after post-absorptive uptake into the liver. Further evidence of the importance of this route in regulation of tocopherol (vitamin E) status is given by the comparison

of α-CEHC excretion by patients with defects in α-TTP and control patients (113). The α-TTP defective patients showed excretion of α-CEHC significantly above that of controls, suggesting α-TTP is protecting α-tocopherol from enzymatic elimination. The enzymatic elimination of tocopherols may additionally explain the effects of α-tocopherol supplementation on plasma and tissue γ-tocopherol levels. It has recently been demonstrated that vitamin E-deficient rats fed 10 mg each α- and γ-tocopherol showed a greater increase in γ-CEHC urinary excretion than rats fed 10 mg γ-tocopherol alone (114).

The mechanism behind the metabolism of vitamin E to their CEHC metabolites was speculated to be based on an ω-oxidation of the phytyl tail, followed by sequential removal of 2-3 carbons by a mechanism similar to the β-oxidation of fatty acids (103). This speculation was based solely on comparison of the molecular structures of δ-tocopherol with its CEHC metabolite. Additional evidence in support of such a mechanism came with the identification by Parker and Swanson of a 5'-carboxychromanol analog of the γ-CEHC metabolite in HepG2 hepatoblastoma cell culture incubated with γ-tocopherol and in human urine (115). This molecule differed from CEHC by the addition of 3 carbon units on the phytyl tail, making it the potential β-oxidation precursor of the γ-CEHC molecule. Additionally, this was the first report of a cell culture model that would carry out catabolism of tocopherols to their urinary metabolites. The analogous α-5'-carboxychromanol was identified in the urine of patients with a defect in α-TTP (113). The development of a cell culture model of vitamin E catabolism provided a powerful tool for characterizing this catabolic pathway. The putative ω-oxidation step in the metabolism of tocopherols, as proposed by Chiku et al, suggested the involvement of a cytochrome P450 enzyme, as several such enzymes are known to catalyze the hydroxylation of hydrophobic molecules

**The Cytochrome P450 Family**

Cytochrome P450 (CYP) enzymes (isozymes) are the name for a superfamily of thiolate hemeproteins that, when complexed with carbon monoxide, give a maximum absorbance at 450 nm. The many P450 isozymes are divided into different subfamilies based on sequence homology of the genes that encode them. Since their discovery over half a century ago, more than five hundred P450 enzymes have been identified, cloned and sequenced (116) and are divided into families by having > 40% gene sequence homology (e.g. CYP1) and into subfamilies based on > 55% sequence homology (e.g.CYP1A). Each enzyme within the subfamily is numbered as specific isoforms according to the order in which they were discovered (e.g. CYP1A1).

The P450s display a wide variety of catalytic functions. The main function appears to be in the transfer of one oxygen atom of $O_2$ to a substrate as in monooxygenation reactions:

$$RH + O_2 + 2e^- + 2H \rightarrow ROH + H_2O$$

These enzymes may also catalyze C-C or C=N bond cleavage, reductions, dehydrations dehydrogenations, or isomerizations (117). There are two main roles that these functions play. The first is in catalyzing the reactions needed for the biosynthesis or degradation of endogenous compounds such as steroids. The other role is in the biotransformation of exogenous compounds to forms that are useful to the organism, forms that are readily excreted from the organism, or in a negative fashion, forms that can be potentially harmful to the organism. This first catabolic process is known as phase 1 metabolism. Phase II metabolism often accompanies this in which the newly modified compound is conjugated with a glucuronide, sulfate or other such moiety, usually rendering the molecule substantially more water soluble, and readily able to be excreted in the urine or bile. Many individual P450 enzymes are able to metabolize several substrates, often of remarkably different chemical structure.

The CYP1, 2, and 3 families appear to be mainly responsible for the metabolism of xenobiotics, while the others are more specific to transformation of endogenous substances (118).

On a tissue basis, there are varying P450 isozymes expressed in all tissues, however the liver is the main site of expression (119). The expression of enzymes within a subfamily may differ from tissue to tissue as is the case for such P450's as CYP1A1 and 1A2 which are found at their highest levels in the lung and liver, respectively (120). The expression of P450 isoforms may also change depending on age, with the CYP3A7 isoform abundantly expressed in fetal liver, yet after birth, levels of this isoform decrease while expression of the CYP3A4 (adult) isoform increases (121). The expression levels of individual P450 enzymes varies greatly, with the CYP3A4 isoform being the most highly expressed, making up nearly 30% of the P450 found in the liver (**Table 1.4**) (122). Other P450s are expressed at lower levels, some representing less than 1% of the total hepatic P450. Caution must be taken in the estimates of hepatic P450 content when the concentrations of individual P450 enzymes are measured using immunoblot analysis and the total P450 content is measured using CO-reduced difference spectra, as this may lead to over-estimations of the percentage of individual P450 content (123).

The abundance of the P450 enzymes in the liver makes this tissue the main site of drug metabolism and results in what is called the "first pass effect". Orally administered drugs or components of food that are absorbed through the gut may be partially metabolized by intestinal P450 enzymes, but quickly make their way to the liver before distribution throughout the body. Before leaving the liver, some fraction of the dose is metabolized by hepatic P450s, often leading to a greatly diminished plasma concentration of the active compound. This means the effective administered dose of a drug may be much greater than the actual amount needed post-hepatically.

**Table 1.4**

Major Human Cytochrome P450 enzymes in the liver as a percentage of total hepatic P450 and the extent to which the levels may vary by subject (Rendic and DiCarlo, 1997; Powell et al, 1998 (CYP4A); Jin et al, 1998 (CYP4F)).
* Specific P450 measured by immunoblot, total P450 measured by CO-reduced difference spectrum.

| CYP Enzyme | Level of Enzyme (% of total) | Extent of Variability in Level |
|---|---|---|
| 1A2 | 13 | 40 fold |
| 1B1 | < 1 | |
| 2A6 | 4 | 30-100 fold |
| 2B6 | < 1 | 50 fold |
| 2C | 18 | 25-100 fold |
| 2D6 | Up to 2.5 | >1000 fold |
| 2E1 | Up to 7 | 20 fold |
| 2F1 | No data | No data |
| 2J2 | No data | No data |
| 3A4 | Up to 28 | 20 fold |
| 4[a] | Up to 20 * | No data |
| 4F | Up to 40 * | No data |

While the P450 enzymes are protective against harmful xenobiotics, they also decrease the effectivity of desired compounds. On the downside, the metabolism of drugs by these enzymes often results in metabolites which are more toxic than their parent compound. As a result, drug dosage and P450 induction and inhibition must be carefully monitored so as to avoid drug toxicity.

**P450 Induction**

On an individual enzyme basis many of the P450s are very sensitive to induction or down-regulation by inducers or inhibitors (124). Endogenous compounds, as well as those from the environment or diet, can increase gene expression of individual cytochrome P450s by affecting nuclear receptor binding regions or other factors in the promoter region of the P450 gene. These mechanisms of induction have been thoroughly characterized for P450s such as the highly inducible CYP1A1 (124). The induction of a P450 enzyme can therefore have dramatic effects on the clearance of a substrate, as is the case for the anticoagulant warfarin, whose treatment dose must be increased up to 10 fold during simultaneous treatment with the P450 inducer phenobarbital (125).

**P450 Inhibition**

Inhibitors of cytochrome P450s have proven a powerful tool in the identification of the individual P450 isozymes that are involved in specific reactions. Potent inhibitors of certain P450 enzymes often have no effect on others. However, in many cases, the specificity of an inhibitor for the P450 is highly dependent on the inhibitor being used. The antifungal ketoconazole has been commonly reported as a very specific inhibitor of CYP3A4 at low concentrations (<10 μM) but a non-specific P450 inhibitor at high (>100 μM) concentrations (126). The role of CYP3A in the metabolism of many substrates has been assumed based on the inhibition of substrate metabolism by low concentrations of ketoconazole. In the case of ketoconazole, it is

able to act as a competitive inhibitor of CYP3A, as evidenced by kinetic inhibition analysis (127). Other forms of inhibition of P450s include non-competitive inhibition, in which increasing substrate concentration does not alter inhibition, and also mechanism based inhibition, in which a metabolite of the P450 substrate is what inhibits the enzyme. Suicide inhibition occurs when the inhibitor is permanently complexed with the enzyme and the protein is unalterably non-functional.

**P450 Polymorphisms**

Activity of the P450s can additionally vary greatly on an interindividual basis. While some of this is driven by P450 induction or inhibition by environmental factors such as diet, much of individual variation in P450 expression and activity can result from the polymorphisms known to occur within a P450 gene sequence. One of the best described examples of this is in the polymorphic CYP2D6 gene. This polymorphism occurs in about 7% of Caucasians and leads to an absence of a functional CYP2D6 protein (128,129). Individuals with this variation are known as "poor metabolizers" versus those who have the active protein, "extensive metabolizers" and are identified by their ability to metabolize key compounds such as bufuralol or dextromethorphan (130,131). The opposing polymorphism has also been identified in which individuals carry multiple copies of the CYP2D6 gene and therefore display even greater substrate metabolism than the general population (132).

**P450s are Organized in a Membrane-Associated Electron Transfer Complex**

On a cellular level, human cytochrome P450s are found in membranes, mainly in the endoplasmic reticulum of cells, although they also may exist in mitochondria and other organelles (133). As membrane bound proteins, the crystal structures of the various isoforms have not been easily elucidated. X-ray crystallography, along with other methods, has provided evidence of a conformation for the enzyme which places the substrate access channel facing the membrane (134,135). This is logical, as many

of the substrates of this enzyme family are hydrophobic molecules which would favor their incorporation into the membrane. Studies using liposome-bound (reconstituted) cytochrome P450 has supported the idea that at least some P450 enzymes may bind their substrates directly from the membrane (136).

Within the membrane, the cytochrome P450 exists as part of a multi-protein complex. In order to carry out the oxidative reactions they are best known for, the enzymes must have a source of electrons. The cofactors NADPH or NADH provide the electrons for the reaction, making P450 activity completely dependent on the presence of these cofactors. The electrons are transferred to the enzyme through the action of the FAD- and FMN-containing cytochrome P450 reductase in the ER (**Figure 1.6**). Mitochondrial P450s receive their electrons in a two-step process, first from ferredoxin reductase to ferredoxin, which then transfers the electrons to the P450 enzyme. Often, cytochrome b5 is associated with the P450-reductase complex, acting as a positive modifier of the P450 monooxygenase reaction (137).

**Cooperativity in P450 Kinetic Activity**

The kinetics of most P450 enzymes toward their substrates can be described with simple hyperbolic Michaelis-Menten kinetics. However, recent work in P450 biochemistry has identified an allosteric effect for certain enzyme-substrate interactions. The CYP3A4, 1A2, and 2C9 isoforms have been characterized as displaying positive or negative cooperativity when challenged with certain substrates, resulting in sigmoidal or biphasic kinetics, respectively (138-140). These allosteric effects can take place by two different binding scenarios. In the first, the binding sites on the enzyme are in two distinct sites. The cooperative effect occurs when either one molecule of the substrate (homotropic) or a molecule different than the substrate (heterotropic) binds one site, changing the affinity of the enzyme for its substrate and/or increase the rate of product formation. In the second, the binding pocket of the

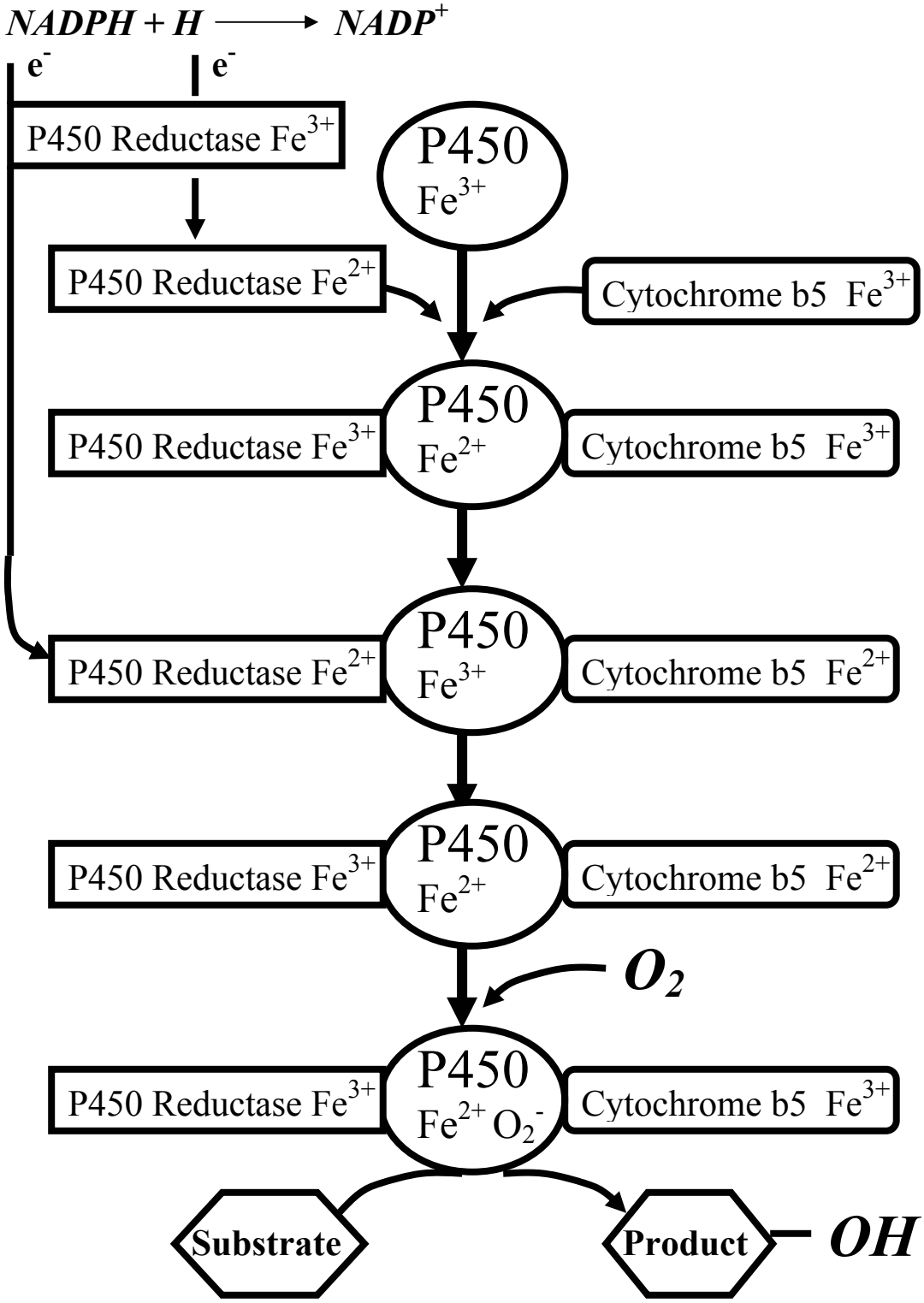



**Figure 1.6**

Electron Transfer from NADPH to Substrate by the cytochrome P450 multi-protein complex (Two-electron acceptor complex model, Shenkman and Jansson, 2003).

enzyme is large enough for entry of the substrate and the effector molecule which then results in the cooperativity. Kinetic studies as well as molecular modeling seem to support the binding of two molecules within one binding site (139,141,142).

**Identification of a Potential Tocopherol-ω-hydroxylase P450**

The identification of the 3'- and 5' carboxychromanol metabolites in the media of HepG2 hepatoblastoma cells incubated with γ-tocopherol provided a model for identifying the P450 enzyme(s) involved in the catabolism of the tocopherols (115). Taking advantage of the use of the reported specificity of inhibitors of individual P450 isoforms, our lab reported >90% inhibition of γ- and α-tocopherol metabolism to their 3'- and 5'-carboxychromanols by 1μM ketoconazole (143). This effect was not seen with inhibitors of several other P450s. This result strongly implicated CYP3A4 as the human P450 responsible for the ω-hydroxylation of the tocopherols, as ketoconazole is reportedly specific for CYP3A4 at 1 μM (144). The identification of P450-based inhibition of tocopherol metabolism suggested the possibility of alterations in plasma and tissue tocopherol status being regulated by inhibition of the P450 involved. The aforementioned influence of sesame lignans such as sesamin on tocopherol status was suspected to be an example of this inhibition. Sesamin was originally reported to be an insecticide synergist (145). These synergists often function by inhibiting insect P450 enzymes which detoxify insecticides. This increases the insecticide half-life, rendering it more potent at lower concentrations (146,147). We demonstrated that 1μM sesamin is as effective as ketoconazole at inhibiting tocopherol metabolism in HepG2 cultures, indicating that inhibition of tocopherol catabolism is indeed the mechanism by which sesame oil or its lignans modify plasma and tissue tocopherol status in vivo (143). This reported effect led to a follow-up study by Ikeda et al which demonstrated that dietary sesame seed, sesame lignans, and ketoconazole inhibit the excretion of γ-CEHC into urine of rats fed γ-tocopherol. This represented an in vivo

conformation of our studies, clearly demonstrating the physiological importance of this catabolic pathway to vitamin E status in vivo.

The inhibition of tocopherol metabolism by ketoconazole suggested the role of CYP3A4 in the catabolic pathway, based on the reported specificity of ketoconazole for this isozyme. Birringer et al reported a 5-fold induction of rac-α-tocopherol metabolism by pre-treatment with rifampicin and suggested it confirmed the involvement of the CYP3A4 enzyme (148). Rifampicin has been characterized as an inducer of CYP3A. It is however by no means specific to this isoform, being also an inducer of numerous other P450 enzymes, especially of the CYP2C family (124). The possibility that other cytochrome P450 enzymes could be involved in the metabolism of the tocopherols could not be discounted.

**Cytochrome P450 4F2**

The presence of the phytyl tail as a major structural feature of vitamin E led us to explore the involvement of P450 enzymes known to ω-hydroxylate lipids possessing a carbon tail analogous to vitamin E. While the CYP1, CYP2 and CYP3 family of P450 enzymes play a large role in the metabolism of xenobiotics, the CYP4 family is mainly involved in the ω-hydroxylation of fatty acids and eicosanoids, both possessing long chain carbon tails as is the case for vitamin E. The hepatic CYP4F enzyme, CYP4F2 was identified by Kikuta et al as an $LTB_4$ ω-hydroxylase enzyme in the liver (149). This was not the first example of $LTB_4$ ω-hydroxylation by a member of the CYP4F family. It was earlier known that $LTB_4$ ω-hydroxylation was catalyzed by a P450 in human neutrophils (150). Kikuta's group then identified cDNA from neutrophils that gave rise to a protein possessing $LTB_4$-ω-hydroxylase activity. Analysis of the DNA sequence demonstrated this gene to be part of a new P450 subfamily, the CYP4F family and was designated CYP4F3 (151). Analysis of the cDNA of CYP4F2 revealed it possessed over 87% sequence homology with CYP4F3,

although CYP4F2 is not expressed in neutrophils, nor is neutrophil CYP4F3 expressed in the liver (149). The main differences in the two proteins are at positions 66-114 which only share 27% homology, whereas the rest of the proteins sequence is 93% homologous. This low homology region corresponds to the open end of the substrate binding pocket when compared with the primary sequence of CYP102, whose 3D structure was previously determined (152). This region is found in exon 3 of the CYP4F2 gene and exon 4 of the neutrophil CYP4F3 gene (153). It now is evident that neutrophil CYP4F3 is an alternative splice variant, as a hepatic isoform from the same gene has been described which undergoes the same splicing as CYP4F2 in which exon 3 is included and exon 4 removed and was designated CYP4F3B (153). The importance of this region in substrate specificity is indicated by the low Km of CYP4F3A for $LTB_4$, in contrast to both 4F2 and 4F3B which have high Km values for $LTB_4$ (153). Additionally, the Km of 4F3A for conversion of arachidonic acid to it's vasoactive metabolite 20-HETE is high, while it is low for both CYP4F2 and 4F3B ((154,155). This is likely explained again by the high degree of sequence homology (80%) of exon 3 between CYP4F2 and 4F3B (154). Unlike the neutrophil CYP4F3A, both CYP4F2 and CYP4F3B have a wide tissue distribution. The liver is their principle location, but they are also found in kidney, prostate, small intestine, and trachea and in each case mRNA levels of CYP4F3B are 2-5-fold higher than that of CYP4F2 (154).

$LTB_4$ normally functions as a chemotactic and chemokinetic agent. It is able to recruit and activate neutrophils, leading to an inflammatory response. CYP4F enzymes in neutrophils and the liver play a large role in regulating this inflammatory response. Upon recruitment of neutrophils to a site of inflammation, CYP4F3A converts $LTB_4$ to the inactive 20-OH-$LTB_4$, leading to less inflammatory activity.

Likewise, the liver is the major organ for elimination of $LTB_4$ and thus the presence of CYP4F2 and 3B may regulate the overall levels of $LTB_4$ in the body (156,157).

The other major metabolite of the CYP4F family is 20-Hydroxyeicosatetraenoic Acid (20-HETE), derived from 20-hydroxylation of arachidonic acid. This metabolite inhibits $Na^+/K^+$-ATPase, is a potent vasoconstrictor by means of $Ca^{2+}$-activated potassium channels, and induces hypertension in rats (158-161). The presence of CYP4F2 and 3B in the kidney and lungs appear to play a large role in the production of this metabolite (155). Other functions of CYP4F2 include ω-oxidation of 6-*trans*-$LTB_4$, Lipoxin $A_4$, other HETEs, and fatty acid epoxides, as well as deethylase and demethylase activities toward ethoxycoumarin and p-nitroanisole, respectively (162,163).

Inhibitors of CYP4Fs have only recently been characterized. Inhibitors of CYP4A ω-hydroxylation of arachidonic acid such as 10-Undecynyl Sulfate and N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanoic acid showed weak effects on CYP4F activity, however, 17-Octadecynoic acid was very potent, with an IC50 of 1-5 μM (164). Ketoconazole, which has long been considered a specific inhibitor of CYP3A at low concentrations, inhibited 50% of LTB4 20-hydroxylation by CYP4F at a concentration of only 10 μM (165).

As LTB4 is a ligand for the Peroxisome Proliferator Activated Receptor (PPAR) orphan nuclear receptor (166), it was suspected that this receptor may be involved in CYP4F gene expression. While the CYP4A family is upregulated by the activity of PPAR, there is no evidence for an effect on CYP4F expression (167). On the other hand, the CYP4F gene promoter region contains many nuclear regulatory element sequences, including an interferon regulator factor, NF-kβ nuclear factor, retinoic acid receptor, retinoid X receptor, and a thyroid hormone receptor sequence (168). The promoter region of CYP4F2 shows a strong response to the presence of

retinoic acid and lauric acid, but not peroxisome proliferators, suggesting these response elements may play an important role in the regulation of CYP4F2 gene expression (167).

Several cell culture models for CYP4F2 activity have been developed. The hepatoblastoma HepG2 and lung adenocarcinoma A549 cell lines both exhibit constitutive expression of CYP4F2, making these cell lines useful tools in the study of 4F2 activities (169,170). Additionally, the expression of CYP4F2 in yeast, bacteria, and insect cells have shown it to be active for in vitro analysis (149,162) [BD Gentest].

**Objectives and Specific Aims**

The overall objective of the work presented here was to characterize the pathway by which vitamin E is metabolized to its water soluble urinary metabolites. Specific aims of the study included the following:

1. To identify the reactions and intermediates in the pathway involving the ω-oxidation of tocopherols to their CEHC metabolites.
2. To verify the involvement of CYP3A in tocopherol ω-oxidation.
3. To determine if other Cytochrome P450 enzymes are involved in tocopherol metabolism.
4. To develop an in vitro microsomal system for characterizing the enzymology of vitamin E metabolism by cytochrome P450 isozymes.
5. To verify that sesamin acts specifically at an initial ω-hydroxylation step of tocopherol metabolism through the inhibition of a cytochrome P450 enzyme.
6. To assess any identified tocopherol-ω-hydroxylation enzymes for their levels of activity toward α- and γ-TOH, in order to determine if differences in

substrate specificity of the enzyme are able to explain the differences in retention of α over γ-tocopherol in vivo.

7. To compare the importance of the specific structural features of the various isoforms of vitamin E in determining substrate affinity and activity of the tocopherol-ω-hydroxylase enzyme.
8. To determine if α-tocopherol enhances microsomal metabolism of the other tocopherols or tocotrienols, and thus provide a mechanism of how supplementation with α-tocopherol depresses plasma levels of other tocopherols.

**References**


1. Evans, H. M., and Bishop, K. S. (1922) *Science* **56**, 650-651
2. Olcott, H. S., and Mattill, H. A. (1931) *J. Biol. Chem.* **93**, 65-70
3. Evans, H. M., Emerson, O. H., and Emerson, G. A. (1936) *J. Biol. Chem.* **113**, 319-332
4. Fernholz, E. (1938) *J Am Chem Soc* **60**, 700
5. Stern, M. H., Robeson, C. D., Weisler, L., and Blaxter, J. G. (1947) *J. Am. Chem. Soc.* **69**, 869–874
6. Eggitt, P. W. R., and Ward, L. D. (1953) *Journal Of The Science Of Food And Agriculture* **4**, 569
7. Bieri, J. G., and McKenna, M. C. (1981) *Am. J. Clin. Nutr.* **34**, 289-295
8. Institute of Medicine (2000) in *Dietary Reference Intakes*, pp. 186-263, National Academy Press, Washington, DC
9. Horwitt, M. K. (1960) *Am J Clin Nutr* **8**, 451-461
10. Horwitt, M. K. (1962) *Vitam Horm* **20**, 541-558
11. Horwitt, M. K. (1974) *Am J Clin Nutr* **27**, 1182-1193
12. Horwitt, M. K., Century, B., and Zeman, A. A. (1963) *Am J Clin Nutr* **12**, 99-106
13. Horwitt, M. K., Harvey, C. C., Dahm, C. H., and Searcy, M. T. (1972) *Ann NY Acad Sci* **203**, 223-236
14. Horwitt, M. K., Harvey, C. C., Duncan, G. D., and Wilson, W. C. (1956) *Am J Clin Nutr* **4**, 408-419
15. Alaimo, K., McDowell, M. A., Briefel, R. R., Bischof, A. M., Caughman, C. R., Loria, C. M., and Johnson, C. L. (1994) *Adv Data*, 1-28
16. Burton, G. W., and Ingold, K. U. (1986) *Acc Chem Res* **19**, 194-201

17. Burton, G. W., Joyce, A., and Ingold, K. U. (1983) *Arch Biochem Biophys* **221**, 281-290

18. Burton, G. W., Doba, T., Gabe, E. J., Hughes, L., Lee, F. L., Prasad, L., and Ingold, K. U. (1985) *J Am Chem Soc* **107**, 7053-7065

19. Packer, L. (1994) *Sci Am Sci Med* **1**, 54-63

20. Yoshida, Y., and Niki, E. (2002) *Biofactors* **16**, 93-103

21. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) *Free Radic Biol Med* **10**, 263-275

22. Boscoboinik, D., Szewczyk, A., Hensey, C., and Azzi, A. (1991) *J Biol Chem* **266**, 6188-6194

23. Tasinato, A., Boscoboinik, D., Bartoli, G. M., Maroni, P., and Azzi, A. (1995) *Proc Natl Acad Sci U S A* **92**, 12190-12194

24. Reddanna, P., Rao, M. K., and Reddy, C. C. (1985) *FEBS Lett* **193**, 39-43

25. Grau, A., and Ortiz, A. (1998) *Chem Phys Lipids* **91**, 109-118

26. Lee, I. K., Koya, D., Ishi, H., Kanoh, H., and King, G. L. (1999) *Diabetes Res Clin Pract* **45**, 183-190

27. Ricciarelli, R., Zingg, J. M., and Azzi, A. (2000) *Circulation* **102**, 82-87

28. Aratri, E., Spycher, S. E., Breyer, I., and Azzi, A. (1999) *FEBS Lett* **447**, 91-94

29. Fechner, H., Schlame, M., Guthmann, F., Stevens, P. A., and Rustow, B. (1998) *Biochem J* **331 ( Pt 2)**, 577-581

30. Kim, H. S., Arai, H., Arita, M., Sato, Y., Ogihara, T., Inoue, K., Mino, M., and Tamai, H. (1998) *Free Radic Res* **28**, 87-92

31. Wu, H. P., Tai, T. Y., Chuang, L. M., Lin, B. J., Wang, J. D., and Teng, C. M. (1992) *J Formos Med Assoc* **91**, 270-275

32. Wu, D., Koga, T., Martin, K. R., and Meydani, M. (1999) *Atherosclerosis* **147**, 297-307

33. Hoglen, N. C., Waller, S. C., Sipes, I. G., and Liebler, D. C. (1997) *Chem Res Toxicol* **10**, 401-407

34. Brillant, L., Leger, C. L., and Descomps, B. (1999) *Lipids* **34 Suppl**, S293

35. Jiang, Q., Elson-Schwab, I., Courtemanche, C., and Ames, B. N. (2000) *Proc Natl Acad Sci U S A* **97**, 11494-11499

36. Wechter, W. J., Kantoci, D., Murray, E. D., Jr., D'Amico, D. C., Jung, M. E., and Wang, W. H. (1996) *Proc Natl Acad Sci U S A* **93**, 6002-6007

37. Moyad, M. A., Brumfield, S. K., and Pienta, K. J. (1999) *Semin Urol Oncol* **17**, 85-90

38. Giovannucci, E. (2000) *J Natl Cancer Inst* **92**, 1966-1967

39. Campbell, S., Stone, W., Whaley, S., and Krishnan, K. (2003) *Crit Rev Oncol Hematol* **47**, 249-259

40. Parker, R. A., Pearce, B. C., Clark, R. W., Gordon, D. A., and Wright, J. J. (1993) *J Biol Chem* **268**, 11230-11238

41. Yu, W., Simmons-Menchaca, M., Gapor, A., Sanders, B. G., and Kline, K. (1999) *Nutr Cancer* **33**, 26-32

42. He, L., Mo, H., Hadisusilo, S., Qureshi, A. A., and Elson, C. E. (1997) *J Nutr* **127**, 668-674

43. Guthrie, N., Gapor, A., Chambers, A. F., and Carroll, K. K. (1997) *J Nutr* **127**, 544S-548S

44. Qureshi, A. A., Mo, H., Packer, L., and Peterson, D. M. (2000) *J Agric Food Chem* **48**, 3130-3140

45. Theriault, A., Chao, J. T., and Gapor, A. (2002) *Atherosclerosis* **160**, 21-30

46. Sen, C. K., Khanna, S., Roy, S., and Packer, L. (2000) *J Biol Chem* **275**, 13049-13055

47. Chow, C. K. (1985) *World Rev Nutr Diet* **45**, 133

48. Bieri, J. G., and Evarts, R. P. (1973) *J Am Diet Assoc* **62**, 147-151

49. Bieri, J. G., and Evarts, R. P. (1974) *Am J Clin Nutr* **27**, 980-986

50. Lee, B. L., New, A. L., and Ong, C. N. (2003) *Clin Chem*

51. Chow, C. K. (1975) *Am J Clin Nutr* **28**, 756-760

52. Ohrvall, M., Tengblad, S., and Vessby, B. (1994) *Eur J Clin Nutr* **48**, 212-218

53. Jiang, Q., Christen, S., Shigenaga, M. K., and Ames, B. N. (2001) *Am J Clin Nutr* **74**, 714-722

54. El-Sohemy, A., Baylin, A., Ascherio, A., Kabagambe, E., Spiegelman, D., and Campos, H. (2001) *Am J Clin Nutr* **74**, 356-363

55. Handelman, G. J., Machlin, L. J., Fitch, K., Weiter, J. J., and Dratz, E. A. (1985) *J Nutr* **115**, 807-813

56. Lee, R., Galli, F., and Kelly, F. J. (2003) in *The Antioxidant Vitamins C and E* (Packer, L., Traber, M. G., Kreaemer, K., and Frei, B., eds), pp. 180-194, AOCS Press, Champaign, IL

57. Clement, M., and Bourre, J. M. (1997) *Biochim Biophys Acta* **1334**, 173-181

58. Emmel, V. M., and Celle, P. L. (1959) *Anat Rec* **133**, 376

59. Elmadfa, I., Kim, S. W., Reutlinger, M., and Siewert, R. (1989) *Z Ernahrungswiss* **28**, 36-48

60. Gallo-Torres, H. E. (1970) *Lipids* **5**, 379-384

61. Traber, M. G., Kayden, H. J., Green, J. B., and Green, M. H. (1986) *Am J Clin Nutr* **44**, 914-923

62. Kelleher, J., and Losowsky, M. S. (1970) *Br J Nutr* **24**, 1033-1047

63. Sokol, R. J., Reardon, M. C., Accurso, F. J., Stall, C., Narkewicz, M., Abman, S. H., and Hammond, K. B. (1989) *Am J Clin Nutr* **50**, 1064-1071

64. Bjorneboe, A., Bjorneboe, G. E., Bodd, E., Hagen, B. F., Kveseth, N., and Drevon, C. A. (1986) *Biochim Biophys Acta* **889**, 310-315

65. Traber, M. G., Rader, D., Acuff, R. V., Brewer, H. B., Jr., and Kayden, H. J. (1994) *Atherosclerosis* **108**, 27-37

66. Traber, M. G., Ingold, K. U., Burton, G. W., and Kayden, H. J. (1988) *Lipids* **23**, 791-797

67. Bjorneboe, A., Bjorneboe, G. E., and Drevon, C. A. (1987) *Biochim Biophys Acta* **921**, 175-181

68. Traber, M. G., Olivecrona, T., and Kayden, H. J. (1985) *J Clin Invest* **75**, 1729-1734

69. Granot, E., Tamir, I., and Deckelbaum, R. J. (1988) *Lipids* **23**, 17-21

70. Traber, M. G., Lane, J. C., Lagmay, N. R., and Kayden, H. J. (1992) *Lipids* **27**, 657-663

71. Traber, M. G., Burton, G. W., Ingold, K. U., and Kayden, H. J. (1990) *J Lipid Res* **31**, 675-685

72. Cohn, W., Loechleiter, F., and Weber, F. (1988) *J Lipid Res* **29**, 1359-1366

73. Parhofer, K. G., Hugh, P., Barrett, R., Bier, D. M., and Schonfeld, G. (1991) *J Lipid Res* **32**, 1311-1323

74. Arita, M., Nomura, K., Arai, H., and Inoue, K. (1997) *Proc Natl Acad Sci U S A* **94**, 12437-12441

75. Oram, J. F., Vaughan, A. M., and Stocker, R. (2001) *J Biol Chem* **276**, 39898-39902

76. Orso, E., Broccardo, C., Kaminski, W. E., Bottcher, A., Liebisch, G., Drobnik, W., Gotz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M. F., Rothe, G., Lackner, K. J., Chimini, G., and Schmitz, G. (2000) *Nat Genet* **24**, 192-196

77. Kostner, G. M., Oettl, K., Jauhiainen, M., Ehnholm, C., Esterbauer, H., and Dieplinger, H. (1995) *Biochem J* **305 ( Pt 2)**, 659-667

78. Desrumaux, C., Deckert, V., Athias, A., Masson, D., Lizard, G., Palleau, V., Gambert, P., and Lagrost, L. (1999) *Faseb J* **13**, 883-892

79. Traber, M. G., and Kayden, H. J. (1984) *Am J Clin Nutr* **40**, 747-751

80. Cohn, W., Goss-Sampson, M. A., Grun, H., and Muller, D. P. (1992) *Biochem J* **287 ( Pt 1)**, 247-254

81. Goti, D., Reicher, H., Malle, E., Kostner, G. M., Panzenboeck, U., and Sattler, W. (1998) *Biochem J* **332 ( Pt 1)**, 57-65

82. Goti, D., Hammer, A., Galla, H. J., Malle, E., and Sattler, W. (2000) *J Neurochem* **74**, 1374-1383

83. Traber, M. G., Rudel, L. L., Burton, G. W., Hughes, L., Ingold, K. U., and Kayden, H. J. (1990) *J Lipid Res* **31**, 687-694

84. Traber, M. G., and Kayden, H. J. (1989) *Am J Clin Nutr* **49**, 517-526

85. Yamashita, K., Takeda, N., and Ikeda, S. (2000) *Lipids* **35**, 163-170

86. Yamashita, K., Nohara, Y., Katayama, K., and Namiki, M. (1992) *J Nutr* **122**, 2440-2446

87. Kamal-Eldin, A., Pettersson, D., and Appelqvist, L. A. (1995) *Lipids* **30**, 499-505

88. Cooney, R. V., Custer, L. J., Okinaka, L., and Franke, A. A. (2001) *Nutr Cancer* **39**, 66-71

89. Murphy, D. J., and Mavis, R. D. (1981) *J Biol Chem* **256**, 10464-10468

90. Kaempf-Rotzoll, D. E., Traber, M. G., and Arai, H. (2003) *Curr Opin Lipidol* **14**, 249-254

91. Hosomi, A., Goto, K., Kondo, H., Iwatsubo, T., Yokota, T., Ogawa, M., Arita, M., Aoki, J., Arai, H., and Inoue, K. (1998) *Neurosci Lett* **256**, 159-162

92. Copp, R. P., Wisniewski, T., Hentati, F., Larnaout, A., Ben Hamida, M., and Kayden, H. J. (1999) *Brain Res* **822**, 80-87

93. Kaempf-Rotzoll, D. E., Horiguchi, M., Hashiguchi, K., Aoki, J., Tamai, H., Linderkamp, O., and Arai, H. (2003) *Placenta* **24**, 439-444

94. Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H., and Inoue, K. (1997) *FEBS Lett* **409**, 105-108

95. Cavalier, L., Ouahchi, K., Kayden, H. J., Di Donato, S., Reutenauer, L., Mandel, J. L., and Koenig, M. (1998) *Am J Hum Genet* **62**, 301-310

96. Traber, M. G., Sokol, R. J., Burton, G. W., Ingold, K. U., Papas, A. M., Huffaker, J. E., and Kayden, H. J. (1990) *J Clin Invest* **85**, 397-407

97. Leonard, S. W., Terasawa, Y., Farese, R. V., Jr., and Traber, M. G. (2002) *Am J Clin Nutr* **75**, 555-560

98. Terasawa, Y., Ladha, Z., Leonard, S. W., Morrow, J. D., Newland, D., Sanan, D., Packer, L., Traber, M. G., and Farese, R. V., Jr. (2000) *Proc Natl Acad Sci U S A* **97**, 13830-13834

99. Simon, E. J., Eisengart, A., Sundheim, L., and Milhorat, A. T. (1956) *J Biol Chem* **221**, 807-817

100. Simon, E. J., Gross, C. S., and Milhorat, A. T. (1956) *J Biol Chem* **221**, 707-805

101. Schonfeld, A., Schultz, M., Petrizka, M., and Gassmann, B. (1993) *Nahrung* **37**, 498-500

102. Schultz, M., Leist, M., Petrzika, M., Gassmann, B., and Brigelius-Flohe, R. (1995) *Am J Clin Nutr* **62**, 1527S-1534S

103. Chiku, S., Hamamura, K., and Nakamura, T. (1984) *J Lipid Res* **25**, 40-48

104. Hattori, A., Fukushima, T., Yoshimura, H., Abe, K., and Imai, K. (2000) *Biol Pharm Bull* **23**, 1395-1397

105. Lodge, J. K., Ridlington, J., Leonard, S., Vaule, H., and Traber, M. G. (2001) *Lipids* **36**, 43-48

106. Stahl, W., Graf, P., Brigelius-Flohe, R., Wechter, W., and Sies, H. (1999) *Anal Biochem* **275**, 254-259

107. Hattori, A., Fukushima, T., and Imai, K. (2000) *Anal Biochem* **281**, 209-215

108. Smith, K. S., Lee, C. L., Ridlington, J. W., Leonard, S. W., Devaraj, S., and Traber, M. G. (2003) *Lipids* **38**, 813-819

109. Himmelfarb, J., Kane, J., McMonagle, E., Zaltas, E., Bobzin, S., Boddupalli, S., Phinney, S., and Miller, G. (2003) *Kidney Int* **64**, 978-991

110. Jiang, Q., and Ames, B. N. (2003) *Faseb J* **17**, 816-822

111. Traber, M. G., Elsner, A., and Brigelius-Flohe, R. (1998) *FEBS Lett* **437**, 145-148

112. Swanson, J. E., Ben, R. N., Burton, G. W., and Parker, R. S. (1999) *J Lipid Res* **40**, 665-671

113. Schuelke, M., Elsner, A., Finckh, B., Kohlschutter, A., Hubner, C., and Brigelius-Flohe, R. (2000) *J Lipid Res* **41**, 1543-1551

114. Kiyose, C., Saito, H., Kaneko, K., Hamamura, K., Tomioka, M., Ueda, T., and Igarashi, O. (2001) *Lipids* **36**, 467-472

115. Parker, R. S., and Swanson, J. E. (2000) *Biochem Biophys Res Commun* **269**, 580-583

116. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenetics* **6**, 1-42

117. Mansuy, D. (1998) *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **121**, 5-14

118. Gonzalez, F. J. (1992) *Trends Pharmacol Sci* **13**, 346-352

119. Hakkola, J., Tanaka, E., and Pelkonen, O. (1998) *Pharmacol Toxicol* **82**, 209-217

120. Nishimura, M., Yaguti, H., Yoshitsugu, H., Naito, S., and Satoh, T. (2003) *Yakugaku Zasshi* **123**, 369-375

121. Lacroix, D., Sonnier, M., Moncion, A., Cheron, G., and Cresteil, T. (1997) *Eur J Biochem* **247**, 625-634

122. Snawder, J. E., and Lipscomb, J. C. (2000) *Regul Toxicol Pharmacol* **32**, 200-209

123. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) *J Pharmacol Exp Ther* **270**, 414-423

124. Pelkonen, O., Maenpaa, J., Taavitsainen, P., Rautio, A., and Raunio, H. (1998) *Xenobiotica* **28**, 1203-1253

125. Patsalos, P. N., and Duncan, J. S. (1993) *Drug Safety* **3**, 156-184

126. Newton, D. J., Wang, R. W., and Lu, A. Y. (1995) *Drug Metab Dispos* **23**, 154-158

127. Boxenbaum, H. (1999) *J Pharm Pharm Sci* **2**, 47-52

128. Gonzalez, F. J. (1990) *Birth Defects Orig Artic Ser* **26**, 17-42

129. Meyer, U. A. (1994) *Proc Natl Acad Sci* **91**, 1984-1985

130. Mahgoub, A., Idle, J. R., Dring, L. G., Lancaster, R., and Smith, R. L. (1977) *Lancet* **2**, 584-586

131. Eichelbaum, M., Spannbrucker, N., Steincke, B., and Dengler, H. J. (1979) *Eur J Clin Pharmacol* **16**, 183-187

132. Johansson, I., Lundqvist, E., Bertilsson, L., Dahl, M. L., Sjoqvist, F., and Ingelman-Sundberg, M. (1993) *Proc Natl Acad Sci U S A* **90**, 11825-11829

133. Lewis, D. F., and Hlavica, P. (2000) *Biochim Biophys Acta* **1460**, 353-374

134. Nelson, D. R., and Strobel, H. W. (1988) *J Biol Chem* **263**, 6038-6050

135. Ruan, K. H., So, S. P., Zheng, W., Wu, J., Li, D., and Kung, J. (2002) *Biochem J* **368**, 721-728

136. Taniguchi, H., Imai, Y., and Sato, R. (1984) *Biochem Biophys Res Commun* **118**, 916-922

137. Schenkman, J. B., and Jansson, I. (2003) *Pharmacol Ther* **97**, 139-152

138. Inouye, K., Mizokawa, T., Saito, A., Tonomura, B., and Ohkawa, H. (2000) *Biochim Biophys Acta* **1481**, 265-272

139. Korzekwa, K. R., Krishnamachary, N., Shou, M., Ogai, A., Parise, R. A., Rettie, A. E., Gonzalez, F. J., and Tracy, T. S. (1998) *Biochemistry* **37**, 4137-4147

140. Shou, M., Mei, Q., Ettore, M. W., Jr., Dai, R., Baillie, T. A., and Rushmore, T. H. (1999) *Biochem J* **340 ( Pt 3)**, 845-853

141. Shou, M., Grogan, J., Mancewicz, J. A., Krausz, K. W., Gonzalez, F. J., Gelboin, H. V., and Korzekwa, K. R. (1994) *Biochemistry* **33**, 6450-6455

142. Lewis, D. F., Eddershaw, P. J., Goldfarb, P. S., and Tarbit, M. H. (1996) *Xenobiotica* **26**, 1067-1086

143. Parker, R. S., Sontag, T. J., and Swanson, J. E. (2000) *Biochem Biophys Res Commun* **277**, 531-534

144. Bourrie, M., Meunier, V., Berger, Y., and Fabre, G. (1996) *J Pharmacol Exp Ther* **277**, 321-332

145. Windholz, M. (ed) (1983) *Merck Index*, 10 Ed., Merck & CO., Inc., Rahway, NJ

146. Casida, J. E. (1970) *J Agric Food Chem* **18**, 753-772

147. MacRae, W. D., and Towers, G. H. (1984) *Phytochem* **23**, 1207–1220

148. Birringer, M., Drogan, D., and Brigelius-Flohe, R. (2001) *Free Radic Biol Med* **31**, 226-232

149. Kikuta, Y., Kusunose, E., Kondo, T., Yamamoto, S., Kinoshita, H., and Kusunose, M. (1994) *FEBS Lett* **348**, 70-74

150. Shak, S., and Goldstein, I. M. (1985) *J Clin Invest* **76**, 1218-1228

151. Kikuta, Y., Kusunose, E., Endo, K., Yamamoto, S., Sogawa, K., Fujii-Kuriyama, Y., and Kusunose, M. (1993) *J Biol Chem* **268**, 9376-9380

152. Ravichandran, K. G., Boddupalli, S. S., Hasermann, C. A., Peterson, J. A., and Deisenhofer, J. (1993) *Science* **261**, 731-736

153. Christmas, P., Ursino, S. R., Fox, J. W., and Soberman, R. J. (1999) *J Biol Chem* **274**, 21191-21199

154. Christmas, P., Jones, J. P., Patten, C. J., Rock, D. A., Zheng, Y., Cheng, S. M., Weber, B. M., Carlesso, N., Scadden, D. T., Rettie, A. E., and Soberman, R. J. (2001) *J Biol Chem* **276**, 38166-38172

155. Powell, P. K., Wolf, I., Jin, R., and Lasker, J. M. (1998) *J Pharmacol Exp Ther* **285**, 1327-1336

156. Hagmann, W., and Korte, M. (1990) *Biochem J* **267**, 467-470

157. Leier, I., Muller, M., Jedlitschky, G., and Keppler, D. (1992) *Eur J Biochem* **209**, 281-289

158. Escalante, B., Sessa, W. C., Falck, J. R., Yadagiri, P., and Schwartzman, M. L. (1989) *J Pharmacol Exp Ther* **248**, 229-232

159. Harder, D. R., Lange, A. R., Gebremedhin, D., Birks, E. K., and Roman, R. J. (1997) *J Vasc Res* **34**, 237-243

160. Stec, D. E., Mattson, D. L., and Roman, R. J. (1997) *Hypertension* **29**, 315-319

161. Schwartzman, M. L., da Silva, J. L., Lin, F., Nishimura, M., and Abraham, N. G. (1996) *Nephron* **73**, 652-663

162. Kikuta, Y., Kusunose, E., and Kusunose, M. (2000) *J Biochem (Tokyo)* **127**, 1047-1052

163. Le Quere, V., Plee-Gautier, E., Potin, P., Madec, S., and Salaun, J. P. (2004) *J Lipid Res* **45**, 1446-1458

164. Xu, F., Falck, J. R., Ortiz de Montellano, P. R., and Kroetz, D. L. (2004) *J Pharmacol Exp Ther* **308**, 887-895

165. Jin, R., Koop, D. R., Raucy, J. L., and Lasker, J. M. (1998) *Arch Biochem Biophys* **359**, 89-98

166. Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., and Wahli, W. (1996) *Nature* **384**, 39-43

167. Zhang, X., and Hardwick, J. P. (2000) *Biochem Biophys Res Commun* **279**, 864-871

168. Zhang, X., Chen, L., and Hardwick, J. P. (2000) *Arch Biochem Biophys* **378**, 364-376

169. Kikuta, Y., Miyauchi, Y., Kusunose, E., and Kusunose, M. (1999) *DNA Cell Biol* **18**, 723-730

170. Yamane, M., and Abe, A. (2000) *J Biochem (Tokyo)* **128**, 827-835

# CHAPTER II

# CYTOCHROME P450 ω-HYDROXYLASE PATHWAY OF TOCOPHEROL CATABOLISM: NOVEL MECHANISM OF REGULATION OF VITAMIN E STATUS*

*Sontag, T. J., and Parker, R. S. (2002) *J Biol Chem* **277**, 25290-25296

**Abstract**

Post-absorptive elimination of the various forms of vitamin E appears to play a key role in regulation of tissue tocopherol concentrations, but mechanisms of tocopherol metabolism have not been elucidated. Here we describe a pathway involving cytochrome P450-mediated ω-hydroxylation of the tocopherol phytyl side chain, followed by stepwise removal of two- or three-carbon moieties, ultimately yielding the 3'-carboxychromanol metabolite that is excreted in urine. All key intermediates of γ-tocopherol metabolism via this pathway were identified in hepatocyte cultures using gas chromatography-mass spectrometry. NADPH-dependent synthesis of the initial γ- and α-tocopherol 13'-hydroxy- and carboxy-metabolites was demonstrated in rat and human liver microsomes. Functional analysis of several recombinant human liver P450 enzymes revealed that tocopherol-ω-hydroxylase activity was associated only with CYP4F2, which also catalyzes ω-hydroxylation of leukotriene $B_4$ and arachidonic acid. Tocopherol-ω-hydroxylase exhibited similar binding affinities but markedly higher catalytic activities for γ-tocopherol than α-tocopherol, suggesting a role for this pathway in the preferential physiological retention of α-tocopherol and elimination of γ-tocopherol. Sesamin potently inhibited tocopherol-ω-hydroxylase activity exhibited by CYP4F2 and rat or human liver microsomes. Since dietary sesamin also results in elevated tocopherol levels in vivo, this pathway appears to represent a functionally significant means of regulating vitamin E status.

**Introduction**

The tocopherol and tocotrienol vitamers that comprise the vitamin E family are considered the most important lipophilic radical-quenching antioxidants in cell membranes. While their function is most often associated with reduction of peroxyl radicals, novel vitamer-specific roles for tocopherols in signal transduction, and in the quenching of other reactive chemical species such as nitrogen dioxide and peroxynitrite are now being investigated (1). While much attention has been devoted to α-tocopherol (α-TOH) recent studies indicate several of these important roles may be specific to γ-tocopherol (γ-TOH) (2). The mechanisms that regulate tissue concentrations and relative proportions of these tocopherols (vitamin E status) are not well understood. Two lines of evidence suggest that vitamin E status is regulated. First, large increases in intake of α-TOH result in only small increases in its plasma concentration (3). Secondly, the relative proportions of tocopherols in plasma and tissues do not reflect those of the diet. Tissues selectively incorporate RRR-α-TOH even when other tocopherols are consumed in greater proportions. γ-TOH is the major form of vitamin E in the North American diet yet this vitamer occurs in blood and tissues at much lower concentrations than that of α-TOH (4, 5). Since tocopherol absorption apparently occurs via passive diffusion with similar efficiency among the vitamers (6, 7), there clearly exist post-absorptive, vitamer-selective processes that ultimately determine vitamin E status. To date only one protein with vitamer-selective properties, α-tocopherol transfer protein (TTP), has been characterized as playing a role in vitamin E status. This protein selectively facilitates hepatic secretion of α-TOH into the bloodstream relative to other tocopherols, and its absence precipitates vitamin E deficiency in humans and mice despite adequate dietary vitamin E (8, 9). The metabolic fate of tocopherols that are poorly retained (e.g. γ-TOH) has not been characterized.

We postulated the existence of an enzyme-mediated mechanism that results in the preferential elimination of γ-TOH relative to α-TOH. Water-soluble metabolites of the three major dietary tocopherols, α-, γ-, and δ-TOH in which the phytyl tail is truncated to the 3' carbon without modification of the chromanol head group, have been reported to occur in urine (10, 11, 12). Building on these findings, we reported that in non-supplemented individuals a substantial proportion of estimated daily intake of γ-TOH is excreted in human urine as its 3'-carboxychromanol metabolite, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) (13), but a much smaller proportion of α-TOH intake was excreted as α-CEHC, implicating this pathway in the differential retention of tocopherols. We further reported that HepG2 cells, a human hepatoblastoma cell line, and rat primary hepatocytes, are capable of synthesizing the carboxychromanol metabolites excreted in human urine (14, 15). Here, using cell culture models, liver subcellular fractions, and a variety of cytochrome P450 (CYP) expression systems, we characterized an enzymatic pathway of tocopherol catabolism to their carboxychromanol metabolites. This pathway involves the initial hydroxylation, catalyzed by CYP4F2, of a terminal methyl group of the phytyl tail, followed by stepwise removal of two- or three-carbon moieties, ultimately yielding the 3'-carboxychromanol metabolite of the parent tocopherol. Substrate specificity and inhibition studies suggest the physiological significance of this pathway in the regulation of tissue tocopherol status.

**Materials and Methods**

Tocopherols were purchased from Fluka Biochemicals, Milwaukee, WI (RRR-γ-TOH) or ACROS Organics, Fisher Scientific, Pittsburgh, PA (RRR-α-TOH). γ-Tocotrienol was a gift from Rex Parker, Bristol-Meyers Squibb, Wallingford, CT. β-NADPH, β-$NAD^+$, and cytochrome P450 substrates and inhibitors were purchased

from Sigma Chemical Co, St. Louis, MO. Sesamin was purchased from Cayman Chemical, Ann Arbor, MI. Human liver microsomes, control insect cell microsomes, and insect cell microsomes expressing various human liver recombinant cytochrome P450 enzymes in combination with human recombinant cytochrome P450 reductase were purchased from BD-Gentest, Woburn, MA. SV40 transformed human skin fibroblasts (GM0637) stably expressing CYP2E1, and sham-transfected control cells, were kindly provided by Paul Hollenberg, University of Michigan, Ann Arbor, MI.

*Cell Culture*

HepG2 cells (C3A subclone CRL-10741, American Type Culture Collection, Manassas, VA) were maintained in Minimal Essential Media (MEM; Atlanta Biologicals, Atlanta, GA) containing $NaHCO_3$ and 10% fetal bovine serum (FBS-Premium; Atlanta Biologicals, Atlanta, GA) without antibiotics under standard cell culture conditions.

To prepare TOH-enriched media, an appropriate volume of RRR-γ-TOH, or RRR-α-TOH (12 mM solutions in ethanol), was added dropwise to FBS while mixing gently; the FBS was stored at 4$^{o}$C for a minimum of 4 hours, then diluted 1:10 with MEM. Final tocopherol concentrations were 25-100 μM, and EtOH concentrations less than 0.85%.

Experiments involving cytochrome P450 inhibitors were performed as described above with the following changes. Stock solutions of various inhibitors in EtOH, were added drop-wise to complete media to a concentration of 1.0 μM. Media was removed from confluent monolayers and replaced with inhibitor-containing media. After a 4 hr preincubation period, this media was replaced with tocopherol-enriched media containing the inhibitor, and then media and cells were collected after 48 hr.

Suspensions of saline-washed cells were disrupted by sonication on ice, and an aliquot taken for protein quantification. The remaining sample was stored at -20$^{o}$C under argon until analysis. Protein was determined by the Bio-Rad method (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as the standard.

*Subcellular fraction preparation and incubation*

Subcellular fractions were prepared by differential centrifugation from the liver of male CD rats sacrificed 3-5 hours after their last feeding (16). Livers were minced in four volumes TES buffer (50 mM Tris/HCl, 5 mM EDTA, 0.25 M sucrose, pH 7.4) and homogenized with a Potter-Elveheim apparatus with teflon pestle. The 800 x g supernatant was centrifuged to obtain the 6,000 x g, 20,000 x g, and 100,000 x g pellets, representing the heavy mitochondrial, light mitochondrial-peroxisomal, and microsomal fractions, respectively. Confluent HepG2/C3A cultures were processed into similar fractions. The fractions were subdivided in 100mM $KH_2PO_4$ buffer (pH 7.4) and frozen at -80$^{o}$C until assayed for activity.

The standard 1ml reaction system consisted of 100 mM $KH_2PO_4$ buffer (pH 7.4) with 0.05-0.2 mg cell fraction protein, 0.5 mM NADPH, and with or without 0.5 mM $NAD^{+}$. Tocopherols were added as a complex with 1% BSA (Fraction V, Sigma) passed through a 0.22μ mixed cellulose ester filter. Cytochrome P450 substrates or inhibitors were added as solutions in EtOH. The reactions were pre-incubated at 37$^{o}$C for 10 min with vehicle or inhibitor and initiated by the addition of substrate or NADPH. Reactions were terminated by the addition of 100 μl 3N HCl and 1 volume cold absolute ethanol.

*Cytochrome P450 inhibition and expression systems*

Inhibition of tocopherol metabolism in HepG2 cell cultures and rat or human liver microsomal fractions was investigated using a variety of characterized P450 inhibitors. Positive controls for characterized P450 activities included testosterone 6β-

hydroxylation (CYP3A), 12- and 11-hydroxylation of lauric acid (CYP2E1, 4A), 7-ethoxycoumarin de-ethylation (CYP2E1, 2B, 1A), and leukotriene $B_4$ ($LTB_4$) 20-ω-hydroxylation (CYP4F2, 4F3A, 4F3B) (17-20). Tocopherol metabolism was also investigated in fibroblasts stably expressing human liver CYP2E1 (21), and in insect microsomes selectively expressing various recombinant human CYP enzymes (CYP3A4, 3A7, 1A1, 2A6, 2B6, 2C19, 4A11, 4F2, 4F3A, 4F3B) or a combination of 1A2, 2C8/9/19, 2D6, and 3A4 (Gentest, Woburn, MA), using reaction conditions as described above with modifications according to supplier recommendations.

*Metabolite Analyses*

For analysis of tocopherols and their metabolites in cell culture, media samples (3-10 ml) were acidified to pH 1.5 with 3N HCl and extracted with methyl-tert-butyl-ether (MTBE). As appropriate, custom-synthesized deuterium-labeled internal standards, $d_2$-γ-CEHC (13) or $d_9$-α-CEHC (the synthesis of which will be published separately) were added prior to acidification. Sonicated cell pellet suspensions were acidified to pH 1.5 with 3N HCl, one volume cold absolute EtOH added, and the sample extracted twice with 8 ml hexanes. Acidified subcellular fraction reaction samples were extracted with 9:1 hexanes:MTBE (TOH or lauric acid metabolites) or ethyl acetate (testosterone or 7-ethoxycoumarin metabolites) with $d_9$α-TOH (22) added as an internal standard for TOH reactions and 17α-$CH_3$-testosterone as an internal standard for testosterone reactions. Solvents were removed under a stream of $N_2$ and the residue silylated with pyridine and N,O-bis-[trimethylsilyl]-trifluoroacetamide + 1%trimethylchrorosilane (BSTFA/ 1%TMCS; Pierce Chemical Co., Rockford, IL) under nitrogen at 70$^o$C for 30 min. $LTB_4$ reactions were stopped with 0.5 volumes of acetonitrile + 1% glacial acetic acid and centrifuged (10, 000 x g) for 3 minutes.

*Preparation of tocopherol-loaded microsomes*

Isolated rat microsomes (0.05 mg protein) were incubated 30 min at 37$^{o}$C in 1ml $KH_2PO_4$ buffer with various concentrations of an equimolar mixture of γ- and α-TOH complexed with BSA. Microsomes were re-isolated by centrifugation (100,000 x g, 1 hour), washed with buffer and again re-isolated. The microsomal pellet was resuspended in 1 mL buffer and extracted using a cold ETOH/Hexane extraction similar to the extractions described above, using $d_9$α-TOH as internal standard. Extracts were silylated and analyzed by gas chromatography-mass spectrometry.

*Gas Chromatography-Mass Spectrometry (GC-MS) and HPLC*

A Hewlett Packard 6890 gas chromatograph, coupled to a Hewlett Packard 5872 mass selective detector operated in either selected ion (SIM) or scan mode, was used for all analyses. The GC was fitted with a Hewlett Packard HP-1 methylsiloxane capillary column (30 m X 0.25 mm) and operated in split injection mode using helium as the carrier gas. For tocopherol metabolite analyses the oven was programmed to ramp from 200$^{o}$C (2 min hold) to 250$^{o}$C at 7$^{o}$C/min, followed by a 6 min hold at 250$^{o}$C, then ramped to 280$^{o}$C at 25$^{o}$C/min, with a final hold at 280$^{o}$C for 9 min. Media concentrations of tocopherol metabolites were determined using the appropriate deuterated internal standards. 6β-hydroxy-testosterone, 12-hydroxy-lauric acid, and 7-hydoxycoumarin were analyzed as above with minor changes in the oven temperature program. $LTB_4$ samples were assessed using the gradient reverse phase HPLC method of Shak (23).

*Catalytic Hydrogenation*

To ascertain the presence of double bonds in the metabolic intermediates, silylated media extracts from HepG2 cultures were dried under $N_2$ gas and the residue reduced with palladium on carbon catalyst under $H_2$ gas at 65$^{o}$C. Samples were compared by GC-MS with and without hydrogenation.

*Statistical Analysis*

Statistical analyses of enzyme activity data were performed using Microcal Origin 4.1 statistical software.

## Results

*Identification of intermediates of γ-tocopherol metabolism in HepG2 cultures*

Previously published mass spectra of γ-TOH and it's 3'-carboxychromanol (γ-CEHC) and 5'-carboxychromanol (γ-CMBHC) metabolites all exhibit a base peak at m/z 223, reflecting a common fragmentation pattern of the γ-chroman-O-trimethylsilyl (TMS) ring moiety (11, 14). GC-MS analyses of extracts of media from HepG2 cells incubated in the presence of 50 μM γ-TOH revealed several substances not present in extracts of control cultures and which exhibited a base peak at m/z 223. **Figure 2.1** illustrates a typical ion chromatogram of a media extract using the selected ion monitoring (SIM) mode monitoring m/z 223. Peaks labeled with roman numerals occurred only in samples from cells incubated with γ-TOH. Peaks I, II, and V correspond to the di-TMS derivatives of the 3'-carboxychromanol (γ-CEHC) and 5'-carboxychromanol (γ-CMBHC) metabolites of γ-TOH, and to the TMS derivative of γ-TOH respectively, as evidenced by their mass spectra and by comparison of retention times to synthetic di-TMS-γ-CEHC or TMS-γ-TOH.

The mass spectra of peaks III, IV, and VI of **Figure 2.1** are shown in **Figure 2.2**. These spectra all exhibited strong base peaks at m/z 223, the expected molecular ions, and other characteristics consistent with the structures of the di-TMS derivatives of the 7'-, 9'-, and 11'-carboxychromanol metabolites of γ-TOH, respectively, as illustrated in **Figure 2.5**.

Peaks III and VI, identified as 7'- and 11'-γ-carboxychromanols respectively, were each consistently accompanied by two minor peaks exhibiting base ions at m/z

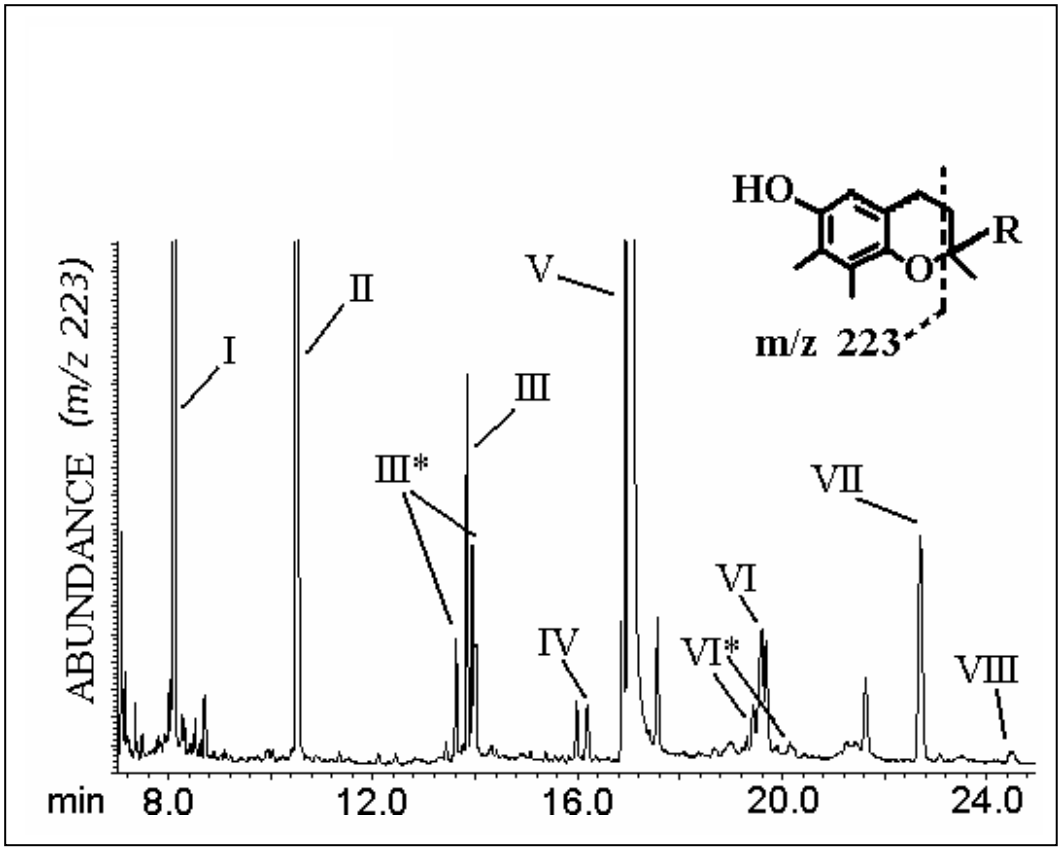



**Figure 2.1**

GC-MS (EI) chromatogram of extracts of HepG2 cultures incubated with 50 μM γ-TOH, obtained using selected ion (SIM) analysis of the major γ-chroman ring fragment, m/z 223. Peaks corresponding to γ-TOH and its metabolites are labeled with roman numerals I-VIII.

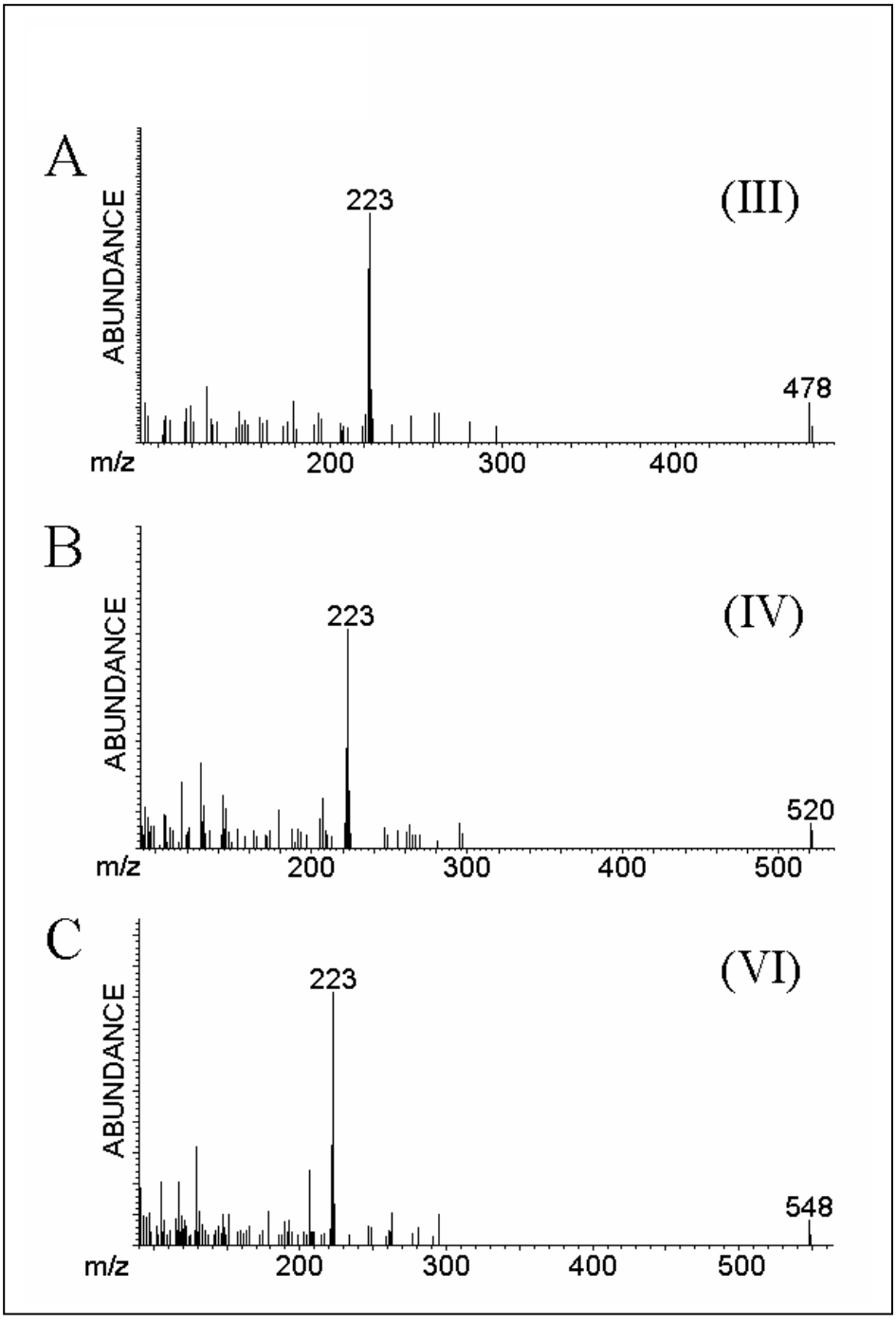



**Figure 2.2**

Mass spectra (EI) of peaks III, IV, and VI of the chromatogram shown in **Figure 2.1**, interpreted as the TMS derivatives of the 7'-, 9'-, and 11'-γ-carboxychromanol metabolites of γ-TOH, respectively.

223 but molecular ions two mass units less than their respective major peak (peaks III* and VI*, **Figure 2.3A, B**). Extracts were compared before and after catalytic hydrogenation. Hydrogenation was accompanied by the disappearance of peaks III* and VI*, with a corresponding increase in the relative abundance of Peaks III and VI (**Figure 2.3C, D**). While the position of the double bond along the phytyl tail is yet to be determined, based on the analogy to fatty acid β-oxidation, the putative unsaturated analogs were assigned the structures of III* and VI* in **Figure 2.5**.

The mass spectra of peaks VII and VIII, eluting at 22.7 min and 24.5 min respectively (**Figure 2.1**), are presented in **Figure 2.4**. These were the only metabolites common to both HepG2 cell cultures and rat liver subcellular reaction systems. Peak VII exhibited a molecular ion at m/z 576 and m/z 103 (-$CH_2$-O-TMS), consistent with a metabolite possessing an intact γ-chromanol ring and a hydroxylated, but otherwise full-length, phytyl side chain. Peak VIII exhibited a molecular ion at m/z 590 and other features consistent with a di-TMS derivative of γ-TOH possessing an intact γ-chromanol ring, a carboxylic acid moiety and a full-length phytyl side chain. Consistent with the presence of the 11'-carboxychromanol intermediate (VI) and the absence of other hydroxylated intermediates, peaks VII and VIII were assigned the structures of the terminal hydroxy and carboxy analogs of γ-TOH, and as illustrated in **Figure 2.5**, and designated 13'-hydroxytocopherol (13'-OH-TOH) and 13'-carboxytocopherol (13'-COOH-TOH). Relative to media extracts, cell extracts were consistently enriched in the longer, more hydrophobic metabolites, particularly the hydroxychromanol metabolite (VII). Due to the normally attenuated metabolism of α-TOH by HepG2 cells (14), the terminal hydroxy and carboxy metabolites of α-TOH were not detected in these cultures but were consistently present in rat liver subcellular fractions incubated with α-TOH. The expected unsaturated metabolites of

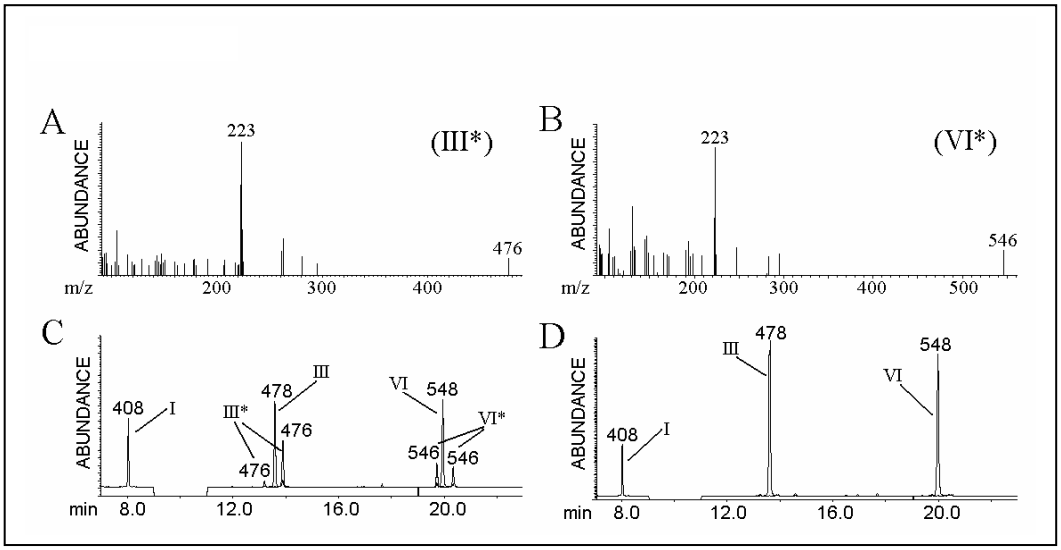



**Figure 2.3**

Mass spectra (EI) of peaks labeled as III* (panel A) and VI* (panel B) of the chromatogram shown in **Figure 2.1**. Panel C: SIM chromatogram, monitoring the indicated ions, of one half of a 50 μM γ-TOH HepG2 culture extract prior to catalytic hydrogenation. Panel D: SIM chromatogram of the remainder of the extract following catalytic hydrogenation, illustrating the absence of peaks III* and VI*, interpreted to represent TMS derivatives of unsaturated β-oxidation intermediates of the 7'- and 11'-γ-carboxychromanol metabolites of γ-TOH.

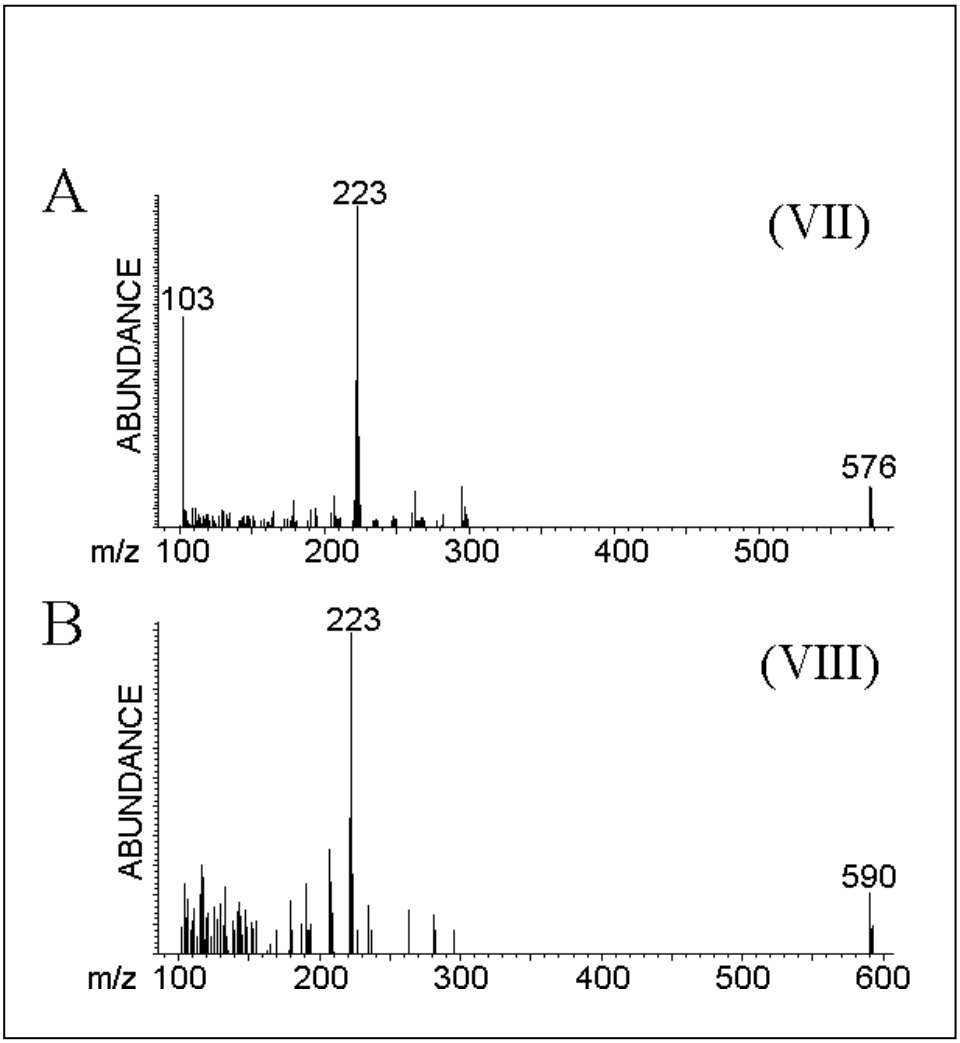



**Figure 2.4**

Mass spectra (EI) of peaks VII and VIII of the chromatogram shown in **Figure 2.1**, interpreted as the TMS derivatives of the terminal 13'-hydroxy (13'-OH-TOH) and 13'-carboxy (13'-COOH-TOH) metabolites of γ-TOH, respectively.

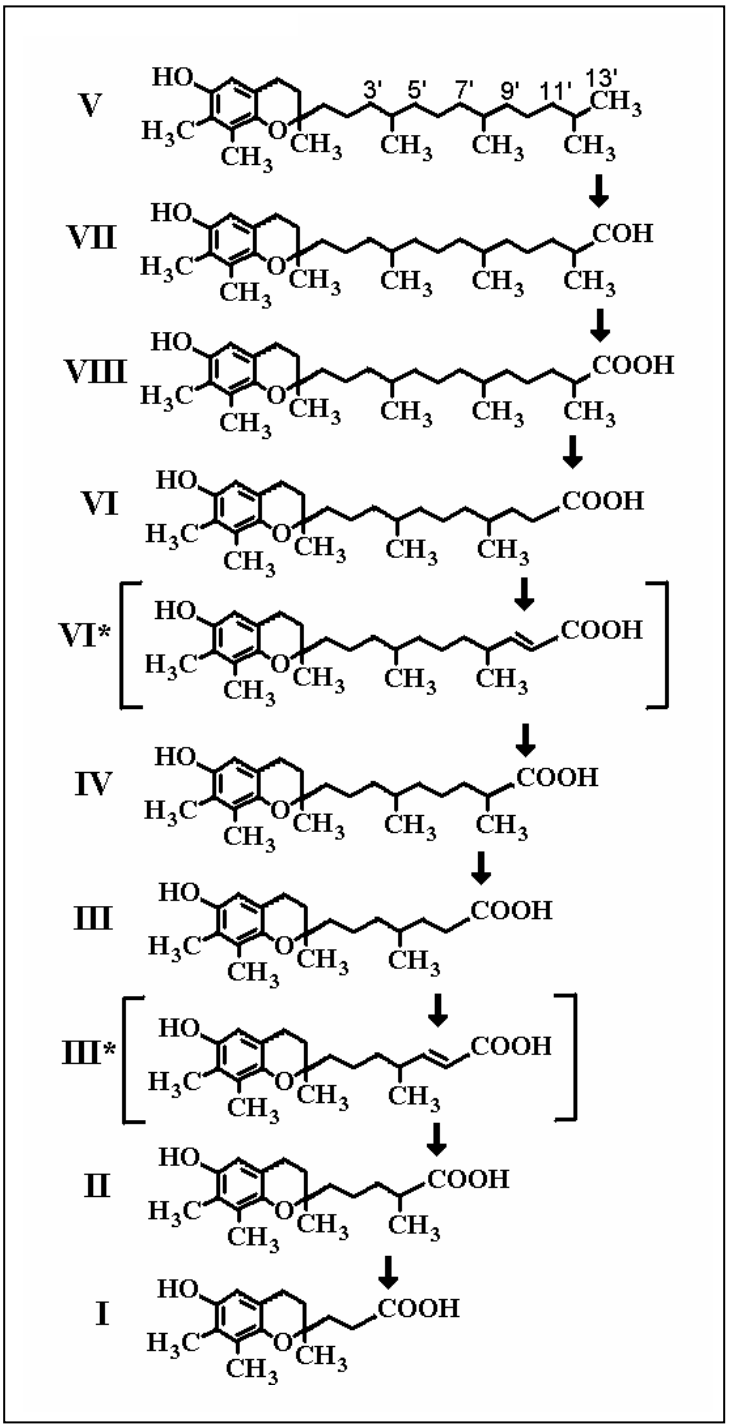



**Figure 2.5**

Pathway of metabolism of γ-TOH to its 3'-γ-carboxychromanol metabolite, based on identification of intermediates from HepG2 cultures incubated with 50μM γ-TOH. Roman numerals correspond to those of **Figures 2.1-2.4**.

γ-tocotrienol were observed in the hepatocyte cultures (data not shown), suggesting that tocopherols and tocotrienols are metabolized via this pathway.

To test the hypothesis that the initial steps in tocopherol side-chain metabolism consist of a cytochrome P450 (CYP)-mediated ω-hydroxylation followed by dehydrogenation to the carboxylic acid, time course reactions with either γ- or α-TOH as substrates were carried out in rat liver microsomes. Synthesis of the 13'-OH-TOH and 13'-COOH-TOH metabolites was observed for both tocopherols in the presence of NADPH but not in its absence. With 0.5 mM NADPH as the only cofactor added, accumulation of the carboxylated metabolite occurred subsequent to that of the hydroxylated metabolite, particularly for γ-TOH, suggestive of a precursor-product relationship (**Figure 2.6**). Additionally, when 0.5 mM $NAD^+$ was also included, the hydroxylated metabolite accumulated only during the initial stage of the reaction, but was relatively suppressed thereafter. Conversely, $NAD^+$ stimulated accumulation of the carboxylated metabolite to levels above those observed for the hydroxylated metabolite in the absence of $NAD^+$. Throughout the course of the reaction (80 minutes) metabolism of γ-TOH (**Figure 2.6**, panel A) in the rat liver microsomes was between five- and ten-fold greater than that of α-TOH (**Figure 2.6**, panel B).

*Involvement of Cytochrome P450 4F2 in the ω-hydroxylation of tocopherols*

The identification of a terminally hydroxylated metabolite of γ-TOH and α-TOH upon the incubation of rat liver microsomes with NADPH suggested a role for one or more P450 mono-oxygenases in the initiation of side chain truncation of tocopherols. In an effort to determine which CYP isoform(s) might be involved, a variety of CYP expression and inhibition systems were employed. We earlier reported a striking inhibition of γ-TOH metabolism by 1 μM ketoconazole in both HepG2 cells and rat primary hepatocytes and by 1 μM sesamin, a sesame seed lignan, in HepG2 cells (15). More recent findings have shown that ketoconazole and sesamin (1 μM)

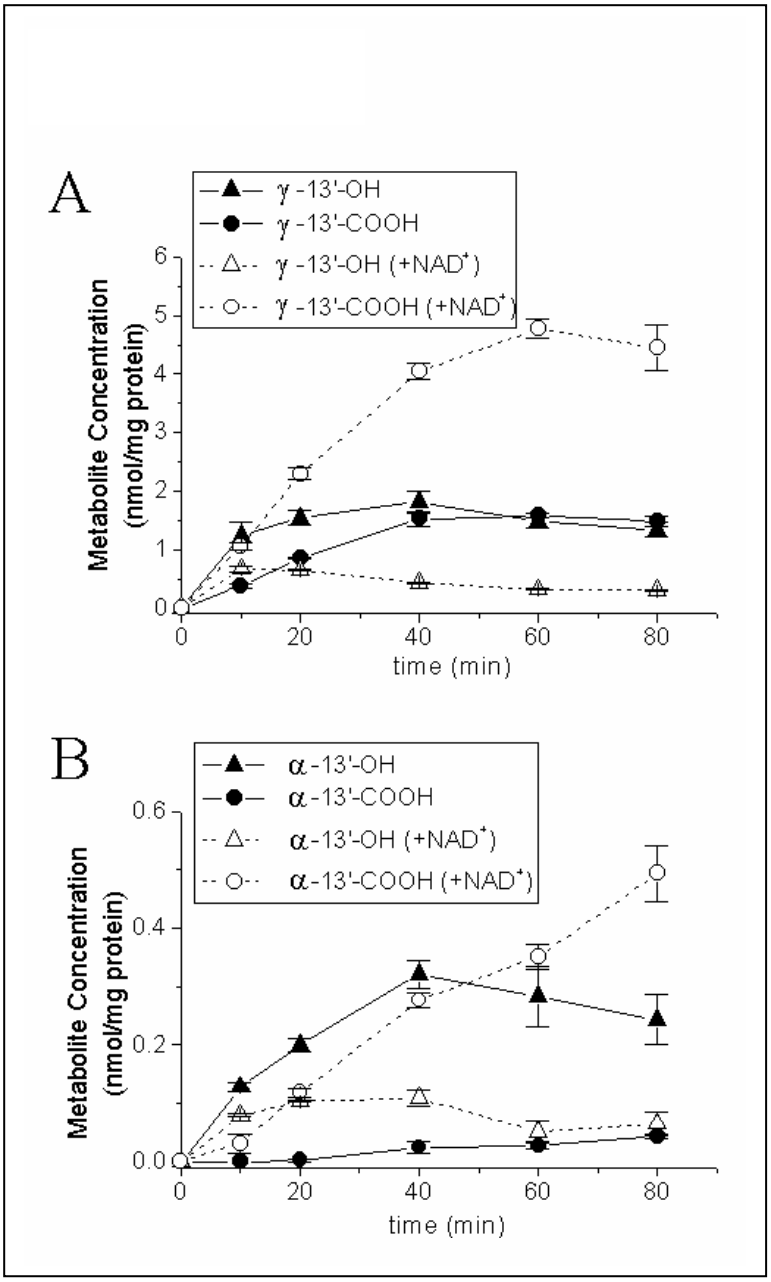



**Figure 2.6**

Time course of synthesis of the 13'-OH-TOH and 13'-COOH-TOH metabolites of γ-TOH (panel A) and α-TOH (panel B) in rat liver microsomes incubated with 25 μM tocopherol as a BSA complex, 0.5 mM NADPH and with or without the addition of 0.5 mM $NAD^+$. Note the difference (10X) in scale of the Y-axis between panels A and B. Data (representative experiment) are means and standard deviations of triplicate analyses at each time point.

both potently inhibit (>80%) γ- and α-TOH metabolism in hepatocyte cell culture (data not shown). Inhibition by either substance was not accompanied by increases in any intermediate, indicative of inhibition at the initial oxidation step of the pathway. Based on the reported specificity of ketoconazole for CYP3A at this low concentration (24), we originally proposed a role for CYP3A in tocopherol catabolism (15). However, in the present study both control insect microsomes expressing no human P450 enzymes and insect microsomes expressing active recombinant human CYP3A4 or CYP3A7 failed to produce any of the tocopherol metabolites identified from HepG2 cultures or rat liver subcellular fractions. Furthermore, testosterone-6β-hydroxylase activity in these microsomes or in rat liver microsomes, while strongly inhibitable by ketoconazole, was not inhibitable by sesamin, a potent inhibitor of tocopherol metabolism. These findings demonstrate that CYP3A does not possess tocopherol-ω-hydroxylase activity. Subsequent investigation showed that insect microsomes expressing other major human liver CYP enzymes (CYP1A1/2, CYP2C8/9/19, 2A6, 2B6, 2D6, 4A11) likewise exhibited no appreciable activity toward either γ- or α-TOH. Additionally, GM-2E1 fibroblasts stably expressing recombinant human CYP2E1 (21), while actively carrying out O-de-ethylation of 7-ethoxycoumarin, did not metabolize γ-TOH to any identified metabolite (not shown). In contrast, insect microsomes expressing recombinant human liver CYP4F2 exhibited clear NADPH-dependent ω-oxidation of γ- and α-TOH to their terminally hydroxylated and carboxylated metabolites. Insect microsomes expressing recombinant human liver CYP4F3B also contained tocopherol-ω-hydroxylase activity, but at levels less than 1% that of CYP4F2. Those expressing human neutrophil CYP4F3A exhibited no activity toward the tocopherols. All three CYP4F isoforms actively catalyzed the 20-ω-hydroxylation of $LTB_4$ (data not shown). Tocopherol-ω-

hydroxylase activity was also observed in rat kidney homogenates and microsomes (data not shown), consistent with the expression of CYP4F2 in kidney tissue (25).

The extent of discrimination between γ- and α-TOH ω-hydroxylation demonstrated in rat liver was compared to that in human liver microsomes and insect microsomes expressing recombinant human CYP4F2. As illustrated in **Figure 2.7**, all three microsomal systems exhibited marked substrate preference for γ-TOH when incubated with both tocopherols under initial velocity conditions. Rat liver microsomes, which contain CYP4F1, a P450 isoform closely related to human CYP4F2 (26), exhibited over four-fold greater activity toward both tocopherols when compared with the human microsomal preparation. Rat and human liver microsomes showed greater discrimination between the two tocopherols than the insect microsomes containing expressed CYP4F2. In all cases metabolism of both γ- and α-TOH was significantly inhibited (80-100%) by 1 μM sesamin (**Figure 2.7**).

The observed difference in tocopherol-ω-hydroxylase activity toward γ- and α-TOH in both separate (**Figure 2.6**) and mixed (**Figure 2.7**) substrate incubation conditions was further investigated through the determination of the kinetic constants for the rat liver microsomal reaction and the recombinant human CYP4F2 reaction. Under initial velocity conditions, rat liver microsomes (**Figure 2.8**, left panel) exhibited roughly similar Km values (68 and 42 μM) for γ- and α-TOH, respectively, but a nearly 6-fold greater Vmax for γ-TOH vs. α-TOH (0.73 vs. 0.13 nmol/mg protein/min, respectively). Recombinant human CYP4F2 (**Figure 2.8**, right panel) likewise exhibited similar Km values of 37 and 21 μM for γ- and α-TOH, respectively, while having a Vmax for γ-TOH much greater than that for α-TOH (1.99 vs. 0.16 nmol/nmol P450/min, respectively). Hyperbolic regression analysis revealed simple Michaelis-Menten kinetics for the microsomal systems with both tocopherols regardless of whether they were presented singly or combined.

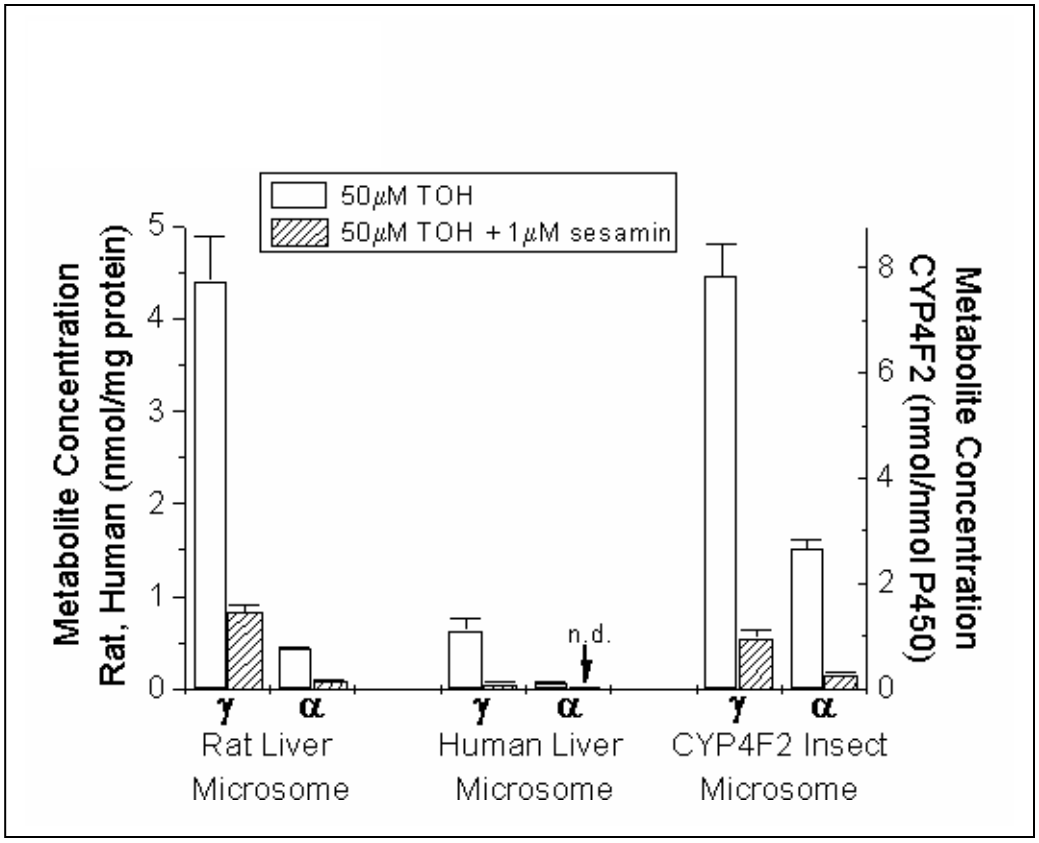



**Figure 2.7**
Substrate discrimination in synthesis of metabolites of γ- and α-TOH by rat or human liver microsomes (0.01 mg protein/reaction; left Y-axis), and insect cell microsomes expressing recombinant human liver CYP4F2 (15 pmol P450/reaction; right Y-axis). Also shown is the inhibitory effect of 1 μM sesamin on synthesis of the metabolites of γ- and α-TOH in each microsomal system. Bars represent the sum of concentrations of the 13'-OH-TOH and 13'-COOH-TOH metabolites of each tocopherol after 20 minute incubation with an equimolar BSA complex mixture of 50 μM γ-TOH plus 50 μM α-TOH, along with 0.5 mM NADPH, and 0.5 mM $NAD^+$. "n.d.", not detected. Data (representative experiment) are means and standard deviations of triplicate analyses.

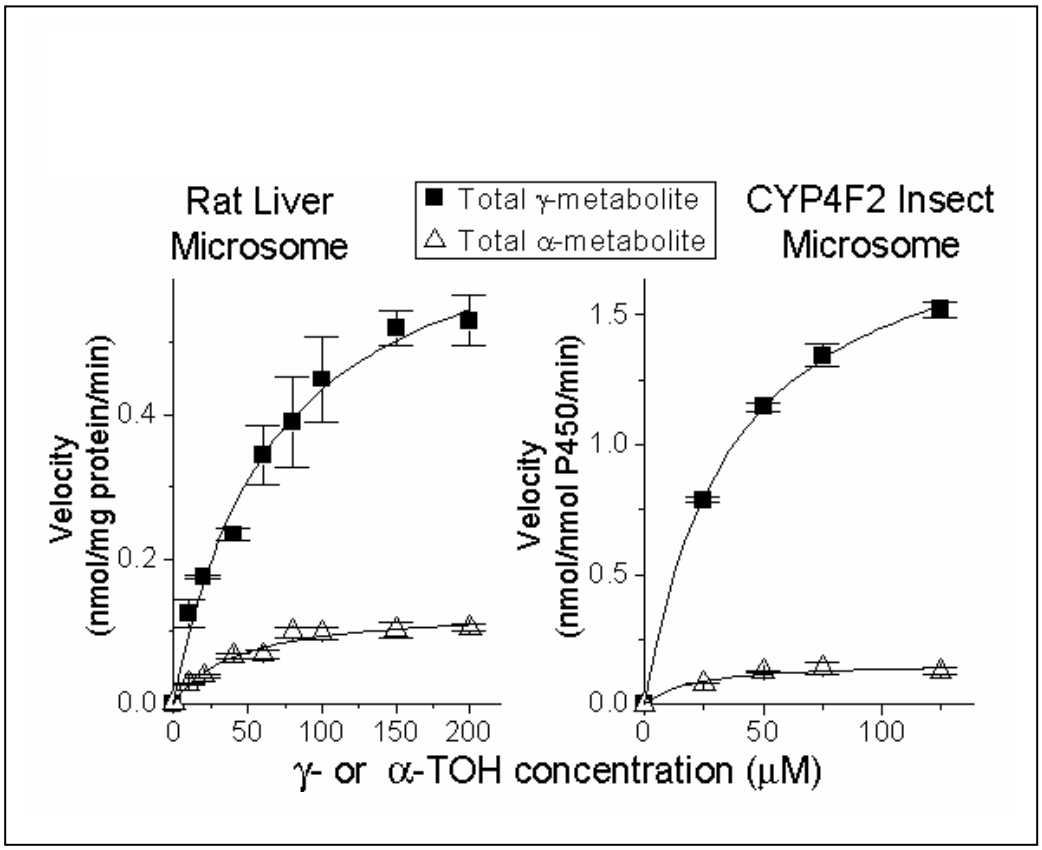



**Figure 2.8**

Kinetic analysis of formation of metabolites of γ- and α-TOH by rat liver microsomes (0.05 mg protein/reaction; panel A) and by insect microsomes expressing recombinant human CYP4F2 (0.015 nmol P450/reaction; panel B) over a range (10-200 μM, rat or 25-125 μM, CYP4F2) of tocopherol concentrations during a 20 minute incubation with 0.5 mM NADPH and 0.5 mM $NAD^+$. Tocopherols were added to separate reactions in a BSA complex as described in Experimental Procedures. Total metabolite represents the sum of 13'-OH-TOH and 13'-COOH-TOH metabolites. Data (representative experiment) presented as scatter plot of means and standard deviations of triplicate analyses at each TOH concentration overlayed with best fit hyperbolic curve as determined by non-linear regression analysis and defined by goodness-of-fit $\chi^2$ minimization. Apparent Km and Vmax values were determined from resulting hyperbolic equation. Rat liver microsomes: γ-TOH; Km = 68 μM, Vmax = 0.73 nmol/mg/min, $\chi^2$ = 0.00128. α-TOH; Km = 42 μM, Vmax = 0.13 nmol/mg/min, $\chi^2$ = 0.00006. CYP4F2 microsomes: γ-TOH; Km = 37 μM, Vmax = 1.99 nmol/nmol P450/min, $\chi^2$ = 0.00061. α-TOH; Km = 21 μM, Vmax = 0.16 nmol/nmol P450/min, $\chi^2$ = 0.00021.

To assess the extent of association of the tocopherols with the microsomes during a typical reaction, rat liver microsomes were incubated with varying concentrations of an equimolar mixture of γ- and α-TOH (BSA complex) as described in the Experimental Procedures. Membrane tocopherol association was similar for both tocopherols and increased linearly throughout the substrate concentrations tested (25-200 μM each TOH). Baseline (endogenous) concentrations of α-TOH were nearly 3-fold higher than those of γ-TOH (0.29 +/- 0.01 vs. 0.11 +/- 0.08 nmol/mg protein, respectively), and both increased markedly to 219 +/- 9 nmol/mg protein after a 30 min incubation with 25μM tocopherol-BSA complex.

**Discussion**

The objective of this study was to elucidate the pathway by which tocopherols are metabolized to their side chain truncated, water-soluble carboxychromanol metabolites excreted in human urine, and to determine whether such a pathway exhibits specificity among the common tocopherol vitamers. Here we present direct evidence from several experimental systems for the expected intermediates in a pathway involving terminal ω-hydroxylation of the tocopherol phytyl side chain, oxidation to the corresponding terminal carboxylic acid, and sequential removal of three- or two-carbon moieties by β-oxidation ultimately yielding a water-soluble 3'-carboxychromanol. This represents the first characterized enzymatic pathway of tocopherol biotransformation in mammalian tissues.

We additionally provide evidence for the involvement of the cytochrome P450 isoform 4F2 in the initial ω-hydroxylation of both γ- and α- tocopherol and for its catabolic discrimination between these two tocopherols. This isoform was the only major human liver P450 isoform tested which exhibited appreciable tocopherol-ω-hydroxylase activity. This finding does not exclude the possibility that other minor

P450 enzymes may exhibit such activity. Human liver microsomes exhibited a higher degree of discrimination between the two tocopherols than insect microsomes expressing only recombinant human CYP4F2 (**Figure 2.7**). This may indicate the presence of other P450 enzymes in human liver that contribute to the observed discrimination. However, a considerable specificity of the activity was indicated by the fact that two other recombinant human CYP4F isoforms closely related to CYP4F2, namely 4F3A and 4F3B (20), exhibited little or no tocopherol-ω-hydroxylase activity while all three enzymes catalyzed the 20-ω-hydroxylation of $LTB_4$. Kinetic analyses of the tocopherol-ω-hydroxylase activity in the rat liver microsomal system and recombinant human CYP4F2 microsomal system revealed similar Km but notably different Vmax for γ- and α-TOH, with the catalytic activity several fold higher for γ-TOH, regardless of whether the substrates were presented singly or in combination. Comparison of the determined kinetic constants with hepatic tocopherol concentrations is not straightforward as the latter is dynamic and exists in several pools. These include tocopherols associated with membranes, lipid droplets or vesicles, and with cytosolic proteins such as tocopherol transfer protein (TTP). The relevance of each to the enzyme activity characterized here is not yet clear. Hepatic cytosolic and membrane concentrations have been reported at 0.005 and 0.2-0.4 nmol/mg protein, respectively (27, 28), the latter of which agrees with the endogenous microsomal tocopherol concentrations reported here. Incubation of microsomes with 25 μM tocopherol-BSA, i.e. near the apparent Km, yielded microsomal tocopherol levels of approximately 219 nmol/mg protein, or three orders of magnitude above the endogenous level. Thus, although in vivo hepatic tocopherol concentrations probably fluctuate considerably with feeding state, membrane concentrations are most likely well below the Km for the tocopherol-ω-hydroxylase which is therefore never saturated.

Two lines of evidence indicate that the tocopherol-ω-hydroxylase pathway described here is of physiological importance in the post-absorptive regulation of tocopherol status in vivo, in particular the preferential retention of α-TOH relative to other tocopherols. First, in humans a substantial proportion of estimated daily intake of γ-TOH, but not of α-TOH, undergoes urinary excretion as its 3'-carboxychromanol (13), the major product of this catabolic pathway. This observation is consistent with the greater tocopherol-ω-hydroxylase activity exhibited toward γ-TOH than α-TOH reported here. Secondly, administration of sesame oil or purified sesamin results in elevated tocopherol concentrations in rats and humans, with the effect greater toward γ-TOH (29-31). We have demonstrated here and in a previous report (15) that sesamin is a potent inhibitor of tocopherol-ω-hydroxylase activity exhibited by hepatocyte cultures, rat and human liver microsomes, and recombinantly expressed human liver CYP4F2. Taken together, the in vivo and in vitro evidence strongly indicate that the tocopherol-ω-hydroxylase pathway is a physiologically important mechanism in the regulation of vitamin E status.

To date, only one other protein, the hepatic tocopherol transfer protein (TTP), has been implicated in the regulation of vitamin E status in vivo, and to exhibit selectivity toward α-TOH (8, 9, 32). TTP has been proposed to facilitate the selective secretion of α-TOH from liver into the bloodstream via very low-density lipoproteins (8), and may modulate intracellular tocopherol-ω-hydroxylase substrate concentrations in the liver, but such an interaction remains to be demonstrated.

The involvement of CYP4F2 in tocopherol catabolism is of potential physiological significance. As mentioned, CYP4F2 catalyzes the ω-hydroxylation of leukotriene $B_4$ ($LTB_4$) to 20-OH-$LTB_4$, a metabolite with considerably less chemotactic activity (19). In addition, CYP4F2 ω-hydroxylates arachidonic acid to 20-OH-arachidonic acid, a metabolite proposed to play critical roles in kidney

function, including vascular tone and natriuresis (33). The reported Km values for arachidonic acid (24 μM) and $LTB_4$ (45 μM) are similar to those reported here for γ- and α-TOH (25, 34). Whether some or all tocopherols, from dietary sources or supplements, can influence physiological phenomena involving CYP4F2-dependent leukotriene or arachidonic acid metabolism clearly merits investigation.

The extent to which carboxychromanol metabolites of tocopherols exhibit important biological effects in vivo remains uncertain. With an intact chromanol moiety, these metabolites could in principle participate in radical trapping reactions in the aqueous milieu of tissues and plasma. However, these metabolites are excreted in urine largely, if not entirely, as glucuronide conjugates (13), and a large proportion of the plasma pool of these metabolites likewise appears to be conjugated (35). While the nature of the conjugated forms of these metabolites has not been characterized, conjugation at the phenolic hydroxyl group would effectively abolish antioxidant activity. Tocopherol metabolites may also exhibit biological activities apart from their radical quenching abilities. The 3'-γ-carboxychromanol metabolite of γ-TOH was first reported as a natriuretic factor (11), and more recently as an inhibitor of prostaglandin $E_2$ synthesis (36).

In summary, we describe a novel CYP4F2-mediated tocopherol-ω-hydroxylase pathway of metabolism of tocopherols to water-soluble carboxychromans that are excreted in urine. This pathway preferentially metabolizes γ-TOH over α-TOH, and inhibition studies, both in vitro and in vivo, indicate its importance in the regulation of tissue tocopherol concentrations. Differential rates of catabolism of tocopherols via this pathway may well prove to underlie the large differences in their bioactivity that do not appear to be explained by their intrinsic radical trapping properties (37-39).

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**References**


1. Brigelius-Flohe, R. and Traber, M.G. (1999) *FASEB J.* **13**, 1145-1155.
2. Jiang, Q., Christen, S., Shigenaga, M.K., and Ames, B.N. (2001) *Am. J. Clin. Nutr.* **74**, 714-722.
3. Princen, H.M., van Duyvenvoorde, W., Buytenhek, R., van der Laarse, A., van Poppel, G. Gevers, L.J.A., and van Hinsbergh, V.W. (1995) *Arterioscler. Thromb. Vasc. Biol*. **15**, 325-333.
4. Vatassery, G.T., Johnson, G.J., and Krezowski, A.M. (1983) *J. Am. Coll. Nutr.* **2**, 369-375.
5. Behrens, W.A. and Madere, R. (1986) *J. Am. Coll. Nutr.* **5**, 91-96.
6. Kayden, H.J. and Traber, M.G. (1993) *J.Lipid Res.* **34**, 343-358.
7. Traber, M.G., Burton, G.W., and Hughes, L. (1992) *J.Lipid Res.* **33**, 1171-1182.
8. Traber, M.G. and Arai, H. (1999) *Annu. Rev. Nutr.* **19**, 343-355.
9. Terasawa, Y., Ladha, Z., Leonard, S.W., Morrow, J.D., Newland, D., Sanan, D., Packer, L., Traber, M.G., and Farese, R.V. Jr. (2000) Proc. Natl. Acad. Sci. U S A. **97**, 13830-13834.
10. Schonfeld, A., Schultz, M., Petrzika, M., and Gassman, B. (1993) *Die. Nahrung.* **37**, 498-500.
11. Wechter, W.J., Kantoci, D., Murray, E.D. Jr., D'Amico, D.C., Jung, M.E., and Wang, W.H. (1996) *Proc. Natl. Acad. Sci.* USA. **93**, 6002-6007.
12. Chiku, S., Hamamura, K., and Nakamura, T. (1984) *J. Lipid. Res*. **25**, 40-48.
13. Swanson, J.E., Ben, R.E., Burton, G.W., and Parker, R.S. (1999) *J. Lipid Res*. **40**, 665-671.
14. Parker, R.S. and Swanson, J.E. (2000) *Biochem. Biophys. Res. Com.* **269**, 580-583.
15. Parker, R.S., Sontag, T.J., and Swanson, J.E. (2000) *Biochem. Biophys. Res. Comm*. **277**, 531-534.

16. Watkins, P.A, Ferrell, E.V. Jr., Pedersen, J.I., and Hoefler, G. (1991) *Arch. Biochem. Biophys.* **289**, 329-36.

17. Powell, P.K., Wolf, I., and Lasker, J.M. (1996) *Arch. Biochem. Biophys*. **335**, 219-226.

18. Sai, Y., Dai, R., Yang, T.J., Krausz, K.W., Gonzalez, F.J., Gelboin, H.V., and Shou, M. (2000) *Xenobiotica* **30**, 327-343.

19. Jin, R., Koop, D.R., Raucy, J.L., and Lasker, J.M. (1998) *Arch. Biochem. Biophys.* **359**, 89-98.

20. Christmas, P., Ursino, S.R., Fox, J.W., and Soberman, R.J. (1999) *J. Biol. Chem.* **274**, 21191-21199.

21. Lin H.L., Roberts, E.S., and Hollenberg, P.F. (1998) *Carcinogenesis* **19**, 321-329.

22. Hughes, L., Slaby, M., Burton, G.W., and Ingold, K.U. (1990) *J. Labelled Comp. Radiopharm.* **28**, 1049-1057.

23. Shak, S. (1987) *Meth. Enzymol.* **141**, 355-371.

24. Pelkonen, O., Maenpaa, J., Taavitsainen, P., Rautio, A., and Raunio, H. (1998) *Xenobiotica.* **28**, 1203-1253.

25. Powell, P.K. Wolf, I., Jin, R., and Lasker, J.M. (1998) *J. Pharmacol. Exp. Ther.* **285**, 1327-1336.

26. Kikuta, Y., Kusunose, E., Ito, M., and Kusunose, M. (1999) *Arch. Biochem. Biophys*. **15**, 193-196.

27. Kornbrust, D.J. and Mavis, R.D. (1980) *Lipids* **15**, 315-322.

28. Taylor, S.L., Lamden, M.P., and Tappel, A.L. (1976) *Lipids* **11**, 530-538

29. Yamashita, K., Iizuka, Y., Imai, T., and Namiki, M. (1995) *Lipids* **30**, 1019-1028.

30. Kamal-Eldin, A., Frank, J., Razdan, A., Tengblad, S., Basu, S., and Vessby, B. (2000) *Lipids* **35**, 427-35.

31. Cooney, R.V., Custer, L.J., Okinaka, L., and Franke, A.A. (2001) *Nutr. Cancer.* **39**, 66-71.

32. Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H., and Inoue, K. (1997) *FEBS Lett.* **409**, 105-108.

33. Lasker, J.M., Chen, W.B., Wolf, I., Bloswick, B.P., Wilson, P.D., and Powell, P.K. (2000) *J. Biol. Chem.* **275**, 4118-4126.

34. Kikuta, Y., Kusunose, E., Kondo, T., Yamamoto, S., Kinoshita, H., and Kusonose, M. (1994) *FEBS Lett.* **348**, 70-74.

35. Stahl, W., Graf, P., Brigelius-Flohe, R., Wechter, W., and Sies, H. (1999) *Anal. Biochem.* **275**, 254-259.

36. Jiang, Q., Elson-Schwab, I., Courtemanche, C., and Ames, B.N. (2000) *Proc. Natl. Acad. Sci. USA*. **97**, 11494-11499.

37. Bieri, J. and Poukka Evarts, R. (1974) *J. Nutr.* **104**, 850-857.

38. Bieri, J. and Poukka Evarts, R. (1974) *Am. J. Clin. Nutr.* **27**, 980-986.

39. Burton, G.W. and Ingold, K. (1981) *J. Am. Chem. Soc.* **103**, 6472-6477.

# CHAPTER III

# COMPARATIVE INFLUENCE OF MAJOR STRUCTURAL FEATURES OF TOCOPHEROLS AND TOCOTRIENOLS IN THE KINETICS OF ω-OXIDATION BY CELLULAR AND MICROSOMAL TOCOPHEROL-ω-HYDROXYLASE

**Abstract**

Differential catabolism (ω-hydroxylation/β-oxidation) of the tocochromanol vitamers making up the vitamin E family of molecules is one of the major factors determining vitamin E status, as evidenced by studies using inhibitors of this catabolic pathway. We previously demonstrated that tocopherol-ω-hydroxylase (cytochrome P450 CYP4F2) is responsible for the initiation of this pathway. Here, we characterize the activity of this enzyme and the influence of key structural features of the vitamin E substrates on its activity. The influence of extent and position of methylation around the chromanol ring, and of stereochemistry and degree of saturation of the phytyl side-chain, were explored using hepatocytes and various microsomal enzyme systems. In HepG2 cells methylation at carbon 5 of the chromanol ring was inhibitory toward ω-oxidation, while methylation at carbons 7 or 8 was less influential. There was no significant effect of side-chain stereochemistry on metabolism of tocopherols to their 3'and 5'-carboxychromanols. The tocotrienols were metabolized to a much greater extent than their corresponding tocopherols. Structure-specific effects similar to those seen in cell culture were observed for tocopherol-ω-hydroxylase activity by rat and human liver microsomes, as well as in microsomes from insect cells selectively expressing CYP4F2. Substrate access to the enzyme was a strong determinant of metabolic rates, as the tocotrienols were incorporated into cell and microsomal membrane to a greater extent than the tocopherols. Kinetic parameters Vmax and apparent Km were determined for the tocopherol-ω-hydroxylase toward each substrate, revealing allosterism by the enzyme toward most vitamers, especially the tocotrienols, but normal Michaelis-Menten kinetics when α-TOH was the substrate. Substrate competition experiments revealed α-tocopherol acted as a positive effector toward the substrates displaying allosteric metabolism, increasing the Vmax of the enzyme toward these substrates. In vivo supplementation of α-tocopherol is

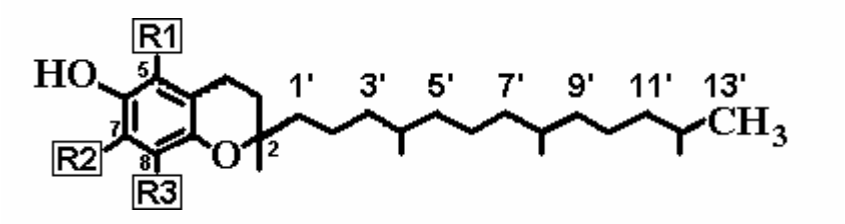
suppresses the plasma levels of the other vitamers, and our data suggest for the first time an *enzymatic* mechanism explaining this phenomenon. Taken together, these results support the contention that CYP4F2-mediated tocopherol-ω-hydroxylation is a highly specific mechanism underlying the different biological half-lives, and therefore biopotency, of the vitamin E vitamers.

**Introduction**

Vitamin E is the generic name for a group of lipid soluble molecules made up of the tocopherols and tocotrienols, some of which are more biologically active than others (1). These molecules differ in the number and position of methyl groups around the chromanol ring, as well as in the saturation and stereochemistry of the phytyl tail (**Figure 3.1**). These slight structural variations appear to play a strong role in the biological activity of the different vitamers.

Until recently, the term Vitamin E was generally associated with α-tocopherol, the most abundant form of vitamin E found in human plasma and tissues, which has led to this vitamer being the focus of many E-related studies. Current research is pointing toward many important roles for the non-α vitamers of vitamin E, roles distinct from those of α-tocopherol and based on the minor structural differences between the various vitamers.

Vitamin E function has been most closely associated with its ability to act as a lipid-soluble anti-oxidant. The antioxidant activity of individual vitamers appears to be highly dependent on their structural features with α-TOH being the most effective of the tocopherols in vivo (2,3), a feature which largely disappears under conditions where plasma and tissue levels of other vitamers are similar to those of α-TOH (4). The tocotrienols appear to posses even greater antioxidant activity in lipid membranes than their corresponding tocopherols (5). However, similar antioxidant activity has




| | | R1 | R2 | R3 |
|---|---|---|---|---|
| Tri-methyl | α | **$CH_3$** | **$CH_3$** | **$CH_3$** |
| Di-methyl | γ | H | **$CH_3$** | **$CH_3$** |
| Di-methyl | β | **$CH_3$** | H | **$CH_3$** |
| Di-methyl | ε | **$CH_3$** | **$CH_3$** | H |
| Mono-methyl | δ | H | H | **$CH_3$** |
| No-methyl | **tocol** | H | H | H |

**Tocopherol** = no double bonds on phytyl tail

**All rac** = racemization at carbons 2, 4', and 8'

**Tocotrienol** = double bonds at carbons 3'-4', 7'-8', and 11'-12'

**Figure 3.1**

Vitamin E structure depicting variations in vitamer structural features.

been found in selenium-deficient cells, once differences in vitamer uptake into the cells had been accounted for (6). Vitamer specific functions have been demonstrated apart from their radical trapping ability. α-TOH has been shown to be an effective inhibitor of PKC activity, smooth muscle cell growth, and nitric oxide production, as well as being implicated in the regulation of gene transcription (7-10). γ-TOH, due to its lack of a methyl group in the C-5 position, is better than α-TOH at trapping reactive nitrogen oxide species (11,12). Additionally, γ-TOH possesses anti-inflammatory activity which is not found for α-TOH (13). Several studies have reported an inverse correlation between plasma γ-TOH levels and cardiovascular disease while this same correlation was not observed for α-TOH (14). The tocotrienols appear to act as inducers of apoptosis in certain cell types to a far greater degree than their tocopherol counterparts (15-17). The tocotrienols also have demonstrated hypocholesterolemic properties in rats which is attributed their vitamer-specific ability to act as inhibitors of HMG-CoA Reductase (18).

The superior activity of α-TOH in vivo appears to result from its superior retention in the body relative to other vitamers [i.e. the body selectively retains only the natural RRR form of α-TOH while eliminating the other vitamers] (19). There appear to be several mechanisms underlying this selectivity. One mechanism involves the tocopherol transfer protein α-TTP. This protein displays a strong binding affinity for α-TOH which aids in the hepatic packaging of α-TOH for distribution to extrahepatic tissues (20,21). Non-α vitamers as well as the synthetic racemic α-TOH show poor affinity for α-TTP and are rapidly metabolized and excreted into the urine (22,23).

In addition to the preferential retention of α-TOH relative to the other vitamers, α-TOH itself appears to facilitate the elimination of the non-α vitamers. Several in vivo studies have reported a decrease in plasma concentrations of the non-α

tocopherols upon supplementation with α-TOH, although the mechanism behind this phenomenon has remained largely unknown (24,25).

The pathway of metabolism of the tocopherols to water soluble metabolites appears itself to be a second important mechanism of discrimination between the vitamers. The tocopherols and tocotrienols are metabolized to water soluble urinary metabolites with the side chain shortened to the 3'-carbon (26-30). Our lab has shown that this takes place by means of an initial ω-hydroxylation at a terminal methyl group of the phytyl tail, followed by sequential β-oxidation to the 3' carboxychromanol metabolite (CEHC). The intermediates in this pathway have been identified for both the tocopherols and tocotrienols (31,32). The initial ω-hydroxylation step in this pathway has been demonstrated by our lab to be catalyzed by a member of the cytochrome P450 family of enzymes, CYP4F2, and is an important regulator of vitamer status in vivo. The inhibition of tocopherol-ω-hydroxylation by sesame lignans in rats has been shown to increase the plasma levels of γ-TOH to levels approaching those of α-TOH (4). CYP4F2 is a member of the CYP4F family of P450 enzymes which were originally found to carry out the ω-oxidation of leukotriene $B_4$ and arachidonic acid (33,34). Kinetic analysis of the activity of the major isoforms of CYP4F, hepatic 4F2 and 4F3A, and neutrophil 4F3B, reveals CYP4F2 has the lowest activity toward $LTB_4$ (35,36) while activity of the CYP4F toward vitamin E is only present in appreciable amounts in the 4F2 isoform (31). This activity is several fold greater toward γ-TOH than α-TOH. Given the wide range of structural variations among the substrates that individual P450 enzymes can metabolize, it is surprising to find the presence of one methyl group on α-TOH can so greatly attenuate the activity of the CYP4F2 enzyme when compared to γ-TOH. These findings suggest not only substrate specificity for vitamin E relative to the other CYP4F2 substrates, but also a

strong vitamer preference by the enzyme based on intramolecular variations within the vitamin structure.

We here investigated the effects of structural variations among the vitamers of vitamin E on the activity of the tocopherol-ω-hydroxylase enzyme. The effects of methylation, stereochemistry, and phytyl tail unsaturation on vitamin E catabolism are demonstrated in both cell culture and microsomal models. Kinetic analysis of the tocopherol-ω-hydroxylase activity revealed allosteric effects within the enzyme-substrate interactions. α-Tocopherol acted as a positive effector, increasing the ω-hydroxylation of the non-α vitamers. Lastly, we propose a role for tocopherol-ω-hydroxylase in mediating the suppression effect of α-TOH on levels of other tocopherols in vivo.

**Materials and Methods**

Tocopherols were purchased from Fluka Biochemicals, Milwaukee, WI (RRR-γ-TOH), ACROS Organics, Fisher Scientific, Pittsburgh, PA (RRR-α-TOH), or Matreya Inc. (Rac-α-, γ-, β-, ε-TOH, and tocol (τ). The tocotrienols were a gift from Volker Berl, BASF, Ludvigshafen, Germany. β-NADPH and NAD were purchased from Sigma Chemical Co, St. Louis, MO. Pooled human liver microsomes and insect microsomes expressing various human liver cDNA Cytochrome P450s in combination with human cDNA P450 Reductase were purchased from Gentest, Inc., Woburn, MA. Rat liver microsomes were prepared as described previously (31).

*Cell Culture*

HepG2 cells (C3A subclone CRL-10741, American Type Culture Collection, Manassas, VA) were maintained in Debubelco's Minimal Essential Media (DMEM) containing $NaHCO_3$ and 10% fetal bovine serum (FBS, USDA Certified; Mediatech, Inc, Herndon, VA) and without antibiotics under standard cell culture conditions.

TOH-enriched media was prepared using an appropriate volume of each TOH (5-30 mM solution in ethanol) added drop-wise to FBS while mixing gently. The FBS was stored at 4°C for a minimum of 4 hours, then diluted 1:10 with DMEM. Final tocopherol concentrations were 25 μM, and EtOH concentrations were less than 0.85%. After 0-48 hour incubations with cells, the media was collected and cells were washed and scraped into 0.9% NaCl. Media and cells were stored at -20°C under argon until analyzed.

*Microsomal Metabolism System*

The microsomal reaction system consisted of 100 mM $KH_2PO_4$ buffer (pH 7.4) with 100 mg/ml pooled human or rat liver microsomes, or 20 pmol/ml CYP4F2 insect cell microsomes, and 0.5-1.0 mM NADPH + NAD. Tocopherols were added as a complex with 1% (w/v) Fraction V bovine serum albumin (BSA) as described previously (31). Microsomes were pre-incubated with the TOH-BSA complex at 37°C for 60 min to allow substrate to associate with membrane. TOH metabolite reactions were initiated with the addition of NADPH + NAD either in the presence of any remaining exogenous TOH-BSA immediately following the pre-incubations, or after centrifugal isolation of the TOH-loaded membranes to eliminate any unbound TOH-BSA. TOH-loaded membranes were isolated by centrifugation for 1 hr at 100,000 x g, washed and resuspended in reaction buffer. Reactions were carried out for 0-40min at 37°C and terminated by the addition of 100 μl 3N HCl and 2 volumes cold absolute ethanol.

*Metabolite Analyses*

For the analysis of short chain metabolites in cell culture, custom-synthesized deuterium labeled d9-α-CEHC was added to media samples (3 ml) which were then acidified to pH 1.5 with 3N HCl and extracted with ethyl acetate. Cell pellet suspensions were sonicated, acidified to pH 1.5 with 3N HCl, and precipitated with 2

volumes of cold absolute ethanol. Long chain metabolites and the parent tocopherol or tocotrienol were extracted from both cell suspensions and metabolite reaction with 1 volume methyl-tert-butyl-ether and 8 volumes hexane following the addition of deuterium labeled d9α-TOH as internal standard. Solvents were removed under a stream of $N_2$ and the residue silylated with pyridine and N,O-bis-[Trimethylsilyl]-trifluoroacetamide containing 1%Trimethylchrorosilane (BSTFA + 1%TMCS) (Pierce Chemical Co., Rockford, IL) under $N_2$ at 70°C for 30 min.

*Gas Chromatography-Mass Spectrometry (GC-MS)*

A Hewlett Packard 6890 gas chromatograph, coupled to a Hewlett Packard 5872 mass selective detector operated in either selected ion (SIM) or scan mode, was used for all analyses. The GC was fitted with a Hewlett Packard HP-1 methylsiloxane capillary column (30 m X 0.25 mm) and operated in split injection mode using helium as the carrier gas. For short-chain tocopherol metabolite analyses the oven was programmed to ramp from 200°C (2 min hold) to 250°C at 7°C/min, followed by a 6 min hold at 250°C, then ramped to 280°C at 25°C/min, with a final hold at 280°C for 9 min. Long chain metabolite and parent tocopherols were analyzed with the oven set at 280°C for 10 min. Tocopherol and metabolite concentrations were determined using the appropriate deuterated internal standards. Cell- and microsome-associated vitamer are expressed on a per cholesterol basis, as cholesterol content is a good standard for total membrane mass.

*Kinetic analysis*

Kinetic constants were obtained using OriginLab Origin 7 software. Kinetic data were analyzed using a non-linear curve fit method included in the software to yield a best fit line and error values.

**Results**

The comparative metabolism of the various forms of vitamin E were examined in hepatocyte culture and in various microsomal systems, including human liver microsomes and microsomes from insect cells in which recombinant human cytochrome P450 tocopherol-ω-hydroxylase (CYP4F2) had been selectively expressed. Total flux through the ω-hydroxylation/β-oxidation pathway was expressed as the sum of the metabolites produced over the course of the experiment. Rates of metabolism of the vitamers were compared based on four features of the tocochromanol molecule depicted in **Figure 3.1**: (a) extent of methylation of the chromanol ring (α-, γ-, δ-TOH, and Tocol, which contain 3, 2, 1, and 0 methyl groups respectively), (b) position of these methyl groups, focusing on the dimethyl tocopherols (γ-, β-, ε-TOH, which lack a methyl group at positions 5, 7, and 8 respectively), (c) racemerization of the phytyl tail (RRR- vs. all-rac α- and γ-TOH), and (d) saturation of the phytyl tail (α-, γ-, δ-TOH vs. α-, γ-, δ-T3).

*Vitamer Metabolism in Hepatocyte Cell Culture*

HepG2/C3A hepatocytes were incubated with equimolar amounts of each of the 11 structurally different vitamers for 48 hours, and the generation of total metabolite was compared (**Figure 3.2**). The metabolites generated were consistent with all forms of vitamin E being metabolized through the same ω-hydroxylation/β-oxidation pathway we previously characterized for α- and γ-TOH (31). The hepatocytes displayed substrate discrimination based on the structural features described. The degree to which the chromanol ring is methylated appeared to be a factor in enzymatic activity toward the vitamers. Non-methylated and mono-methylated Tocol and δ-TOH were most effectively converted to the water-soluble urinary metabolites, followed by the di-methyl γ-TOH, while the tri-methyl α-TOH was poorly metabolized. The comparison of metabolism of the di-methyl tocopherols

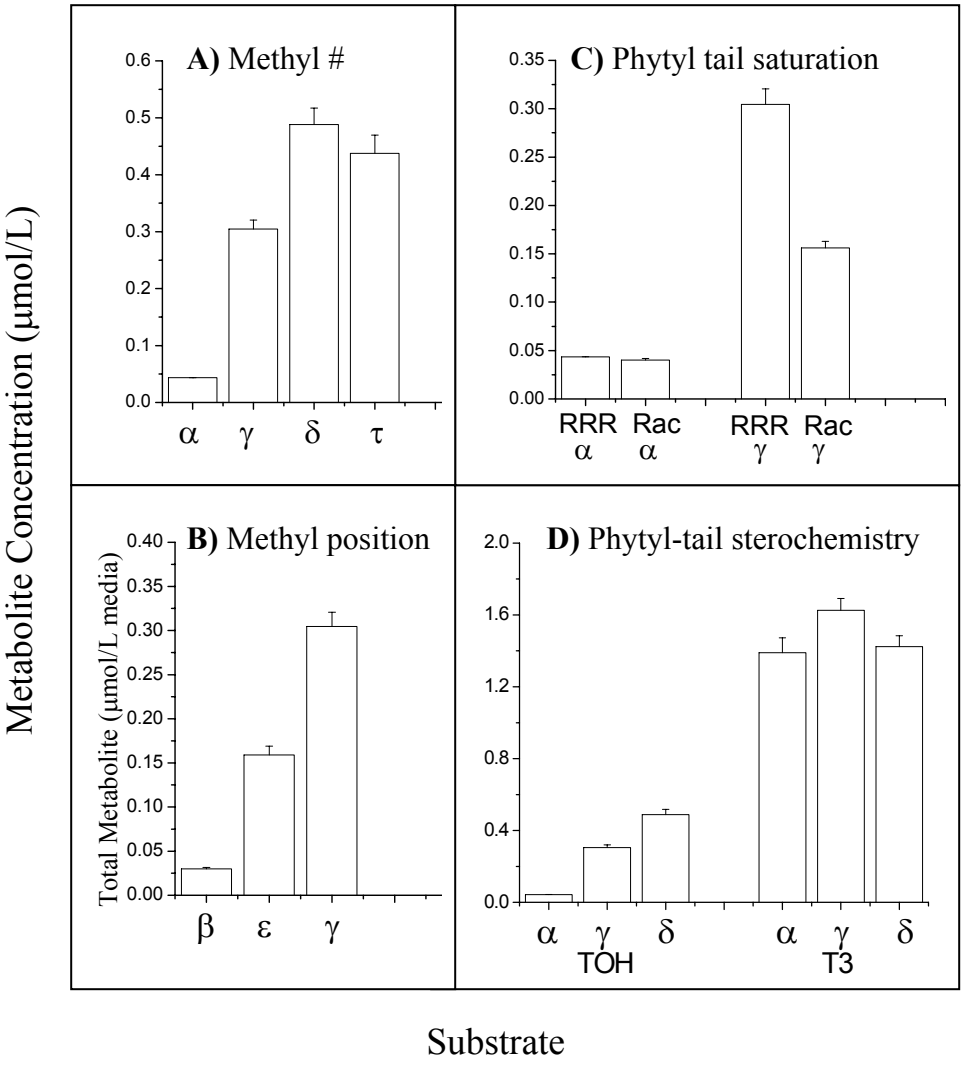



**Figure 3.2**

Influence of vitamer structural variations on Vitamin E metabolism in HepG2 hepatocytes. Confluent cells were incubated 48hours with 25μM substrate and the total metabolite in the media was quantified as described in Materials and Methods.

revealed a strong inhibitory effect of a methyl group at carbon 5 (β-TOH), while methylation at other positions (γ- and ε-TOH) were not as influential. No significant differences in metabolism were noted between the RRR and all-rac isomers of either α-TOH or γ-TOH.

The structural feature contributing greatest to flux through the tocopherol-ω-oxidation pathway was unsaturation of the phytyl tail. Each of the three tocotrienols were metabolized to a greater extent than their corresponding tocopherols.

Comparison of uptake of the vitamers by HepG2 cells over time revealed large variations in uptake of the various substrates (**Figure 3.3**). Similar results were obtained when tested in bovine aortal endothelial (BAE) cells which do not metabolize vitamin E (data not shown). These data were consistent with reports of significant variation of vitamer uptake in a variety of other cell types (6,37). As demonstrated in **Figure 3.3**, decreased ring methylation increased the rate of tocopherol incorporation into cells. Tocol displayed the highest rate of cell incorporation. Tocotrienols were more rapidly taken up by cells than their corresponding tocopherols. There were no significant effects of methyl position or phytyl tail racemerization on the rate of cell uptake.

Organelle isolation assays revealed rapid distribution of vitamin E from the extracellular media into mitochondria, here used as an indicator of rate of vitamer movement to inner cellular membranes where the P450 ω-hydroxylation of the vitamers likely occurs. After 2 hours of incubation with 25μM substrate, the tocotrienols were present in the mitochondrial fraction at levels more than 3 fold higher than that of the corresponding tocopherols (data not shown).

*Microsomal Metabolism of Tocochromanols*

The culture data strongly suggested structural discrimination by the enzyme(s) involved in the ω-oxidation/β-oxidation of the tocochromanol substrates. The level of

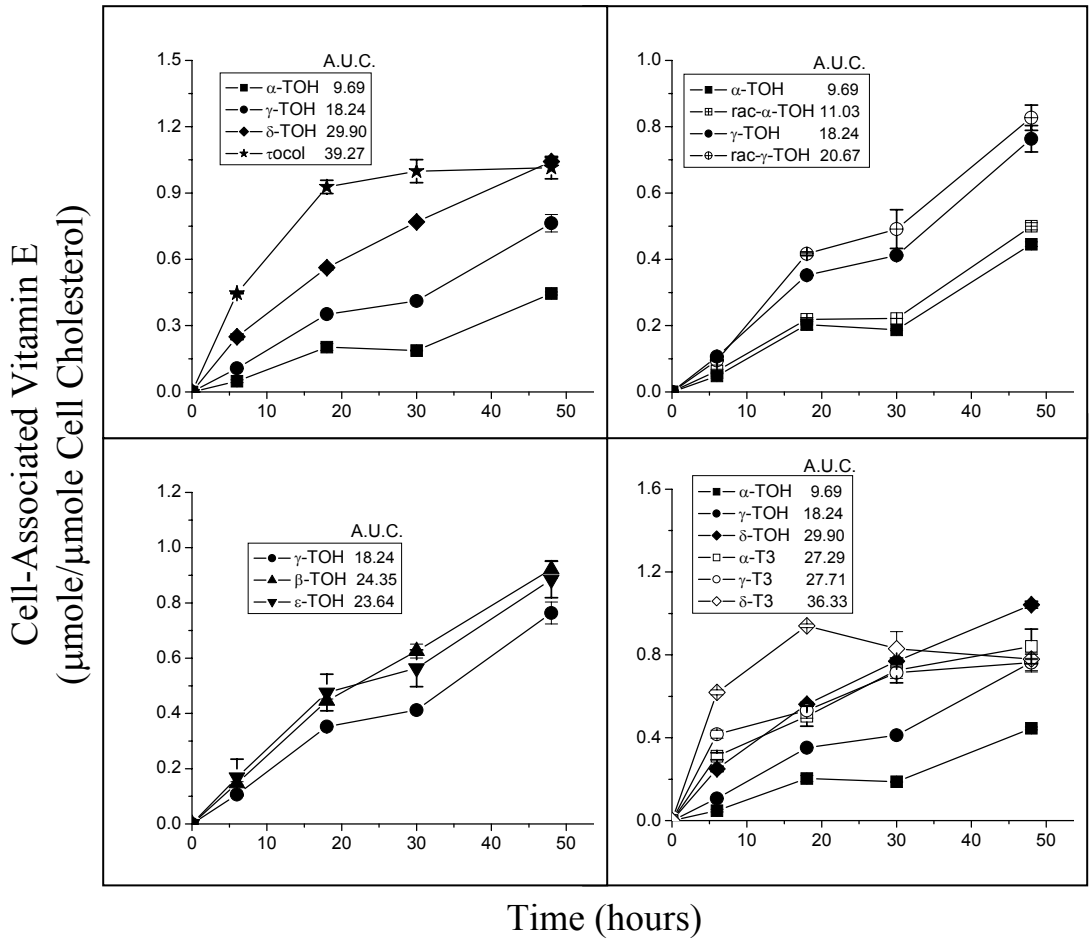



**Figure 3.3**

Variations in HepG2 cell uptake of vitamin E over 48hours. Confluent cells were incubated with 25µM vitamer and harvested at 6, 18, 30, and 48 hours and the amount of vitamer associated with the cell was measured. The area under the curve (A.U.C) was determined using Origin 7.0 software.

metabolism is heavily influenced by not only the enzymatic activity, but also the actual substrate concentrations presented to the enzyme. As such, the variation in cellular uptake and distribution of the vitamers over time obscured the degree to which the actual enzymatic activity was controlling metabolic discrimination. We have developed a microsomal assay in which the substrates are presented to the P450 enzyme through incorporation of the vitamin directly into the microsomal membrane. Assays were conducted for each of the substrates using microsomes isolated from human liver or from insect cells selectively expressing recombinant human liver CYP4F2.

First, a comparison of the rate of incorporation of the various tocochromanols into human liver microsomal membranes was undertaken. The results in **Figure 3.4** show not only wide differences in the rates of incorporation, but a large range in membrane substrate capacity. As seen in HepG2 cells, both the rate of incorporation and the overall capacity of the membrane for the tocotrienols exceeds that of the tocopherols. Loading was linear with added substrate throughout the concentration range used for this and all subsequent experiments (data not shown).

The variation in membrane loading efficiency (incorporated substrate per added substrate) between the vitamers results in two pools of substrate during the tocopherol-ω-hydroxylase reaction: the membrane associated pool, and the pool of substrate remaining in the aqueous phase (complexed with BSA). Pre-incubation of substrate with the microsomes consistently yielded higher levels of metabolite production, suggesting that the membrane-associated substrate is the pool upon which tocopherol-ω-hydroxylase acts (data not shown).

Tocopherol-ω-hydroxylase activity of human liver microsomes was compared under two conditions. In both, microsomes were pre-incubated with concentrations of substrate that had been previously determined to yield 100 nmol/mg protein in the

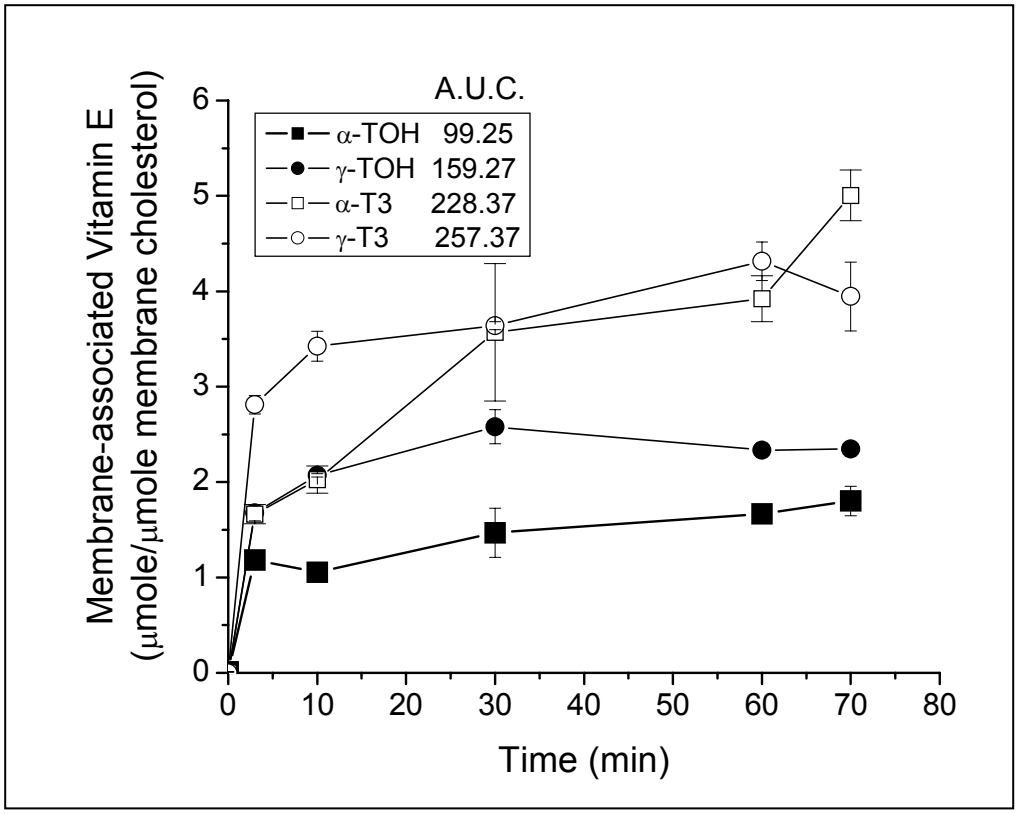



**Figure 3.4**

Incorporation of Vitamin E into microsomal membranes over time. 100μg/ml human liver microsomes were incubated with 25μM vitamer-BSA complex over a 70 min time period. The membrane associated vitamer was separated from the remaining aqueous vitamer by 100,000 x g centrifugation and the amount of vitamer associated with the membrane was measured. The area under the curve (A.U.C) was determined using Origin 7.0 software.

membrane (10 μM in the total reaction volume) at the end of the pre-incubation period. As a result the remaining unbound substrate concentrations varied greatly depending on the substrate used (**Figure 3.5A, inset**). In the first condition, the microsomal reaction was carried out in the presence of both membrane-associated and unbound substrate. In the second condition, microsomes were re-isolated by ultracentrifugation and the reaction carried out in the presence of membrane-associated substrate only. The results showed a similar pattern of vitamer metabolism under both conditions (**Figure 3.5A**). This occurred despite the wide range of total vitamer concentration present due to unbound substrate in the first condition (**Figure 3.5A, inset**). The overall activity under the first condition was higher, probably because of the continued uptake of aqueous vitamer into the membrane during the reaction, or from a partial loss of activity during membrane re-isolation under the second condition.

A time course of metabolite production in human liver microsomes was conducted. When the linear production of metabolite was extrapolated to the earliest time points of the reaction, it indicated a rapid initial burst of metabolite production, followed by a slower linear increase in metabolite (**Figure 3.5B**). This phenomenon occurs often in reactions in which the enzyme and substrate are components of the same microsomal membrane. The substrate in close proximity to the enzyme is rapidly catabolized before additional substrate can move into, or through the membrane to access the enzyme (38). This then results in a slow phase of metabolism, whose rate is limited by the rate of substrate access to the enzyme. Taken together, the results of Figure 3.5 show the tocopherol-ω-hydroxylase acts on the membrane vitamer pool. Additionally, the rate of catabolism may be influenced by both incorporation of tocopherol into the membrane, and the rate at which the vitamers move through the membrane to access the enzyme.

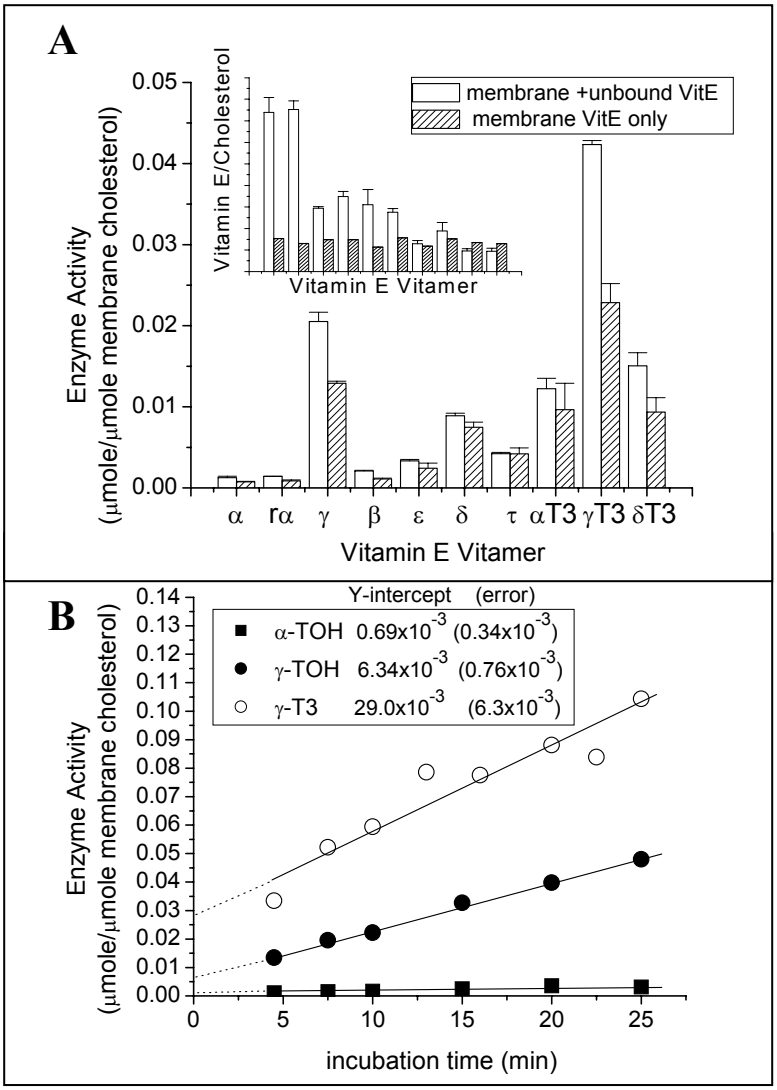



**Figure 3.5**

**(A)** Comparison of 13'OH + 13'COOH metabolite formation of Vitamin E vitamers loaded to equimolar concentrations in the membrane of human liver microsomes. 100ug/ml human liver microsome was pre-incubated 60min with the various concentrations of vitamer-BSA complex pre-determined to reach 100 nmol VitE/mg protein in the membrane. A 20 min reaction was carried out with or without the removal of the remaining aqueous vitamin E, which was removed by 100,000 x g centrifugation (See Material and Methods). The reaction was initiated by the addition of 0.5mM NADPH + NAD. Inset shows the total amount of vitamin E remaining in the reaction with or without the removal of the aqueous vitamin pool. **(B)** Time course of 13'OH + 13'COOH metabolite formation in human liver microsomes pre-incubated 60 min with 25μM vitamer. Reaction initiated by the addition of 1mM NADPH + NAD. Amount of initial formation of metabolite indicated by the y-intercept of the extrapolated dotted line.

*Kinetics of vitamer metabolism*

Kinetic analyses of microsomal tocochromanol metabolism were carried out with each individual substrate. While the majority of tocopherols tested displayed typical Michaelis-Menten kinetics, the tocotrienols, and to a lesser extent δ-TOH and tocol demonstrated concentration-dependent inhibition of their own metabolism at high substrate concentrations as illustrated in **Figure 3.6A** for γ-T3. Kinetic analyses of data from both human liver and CYP4F2 microsomes were carried out over a range of concentrations for the vitamers below those resulting in the inhibition (**Figure 3.6**). α-TOH and rac-α-TOH displayed simple hyperbolic kinetics, while the other vitamers displayed sigmoidal kinetics typical of allosteric enzyme reactions. As such, kinetic constants were determined using Origin software to fit the data to both Michaelis-Menten (hyperbolic) and Hill (sigmoidal) curves as shown below:

Michaelis-Menten: $V = Vmax[S]/(km + [S])$

Hill: $V = Vmax[S]^n/(k^n + [S]^n)$

The Hill equation differs from the Michaelis-Menten equation only in the variable "n", the Hill coefficient, which is a measure of the positive (n>1) or negative (n<1) cooperativity of the enzyme with its substrate. A value of n = 1 indicates no cooperativity. Tocopherol-ω-hydroxylase substrates could be categorized into three groups: those fitting only Michaelis-Menten kinetics (α- and rac-α-TOH), those fitting both Michaelis-Menten or Hill kinetics (γ-, rac-γ-, β-, ε-, δ-TOH, and tocol), and those fitting Hill kinetics only (all three tocotrienols). The results indicate positive cooperativity for all three tocotrienols, and either some or no cooperativity for the tocopherols (**Table 3.1**). Determination of Vmax and Km demonstrated a remarkably

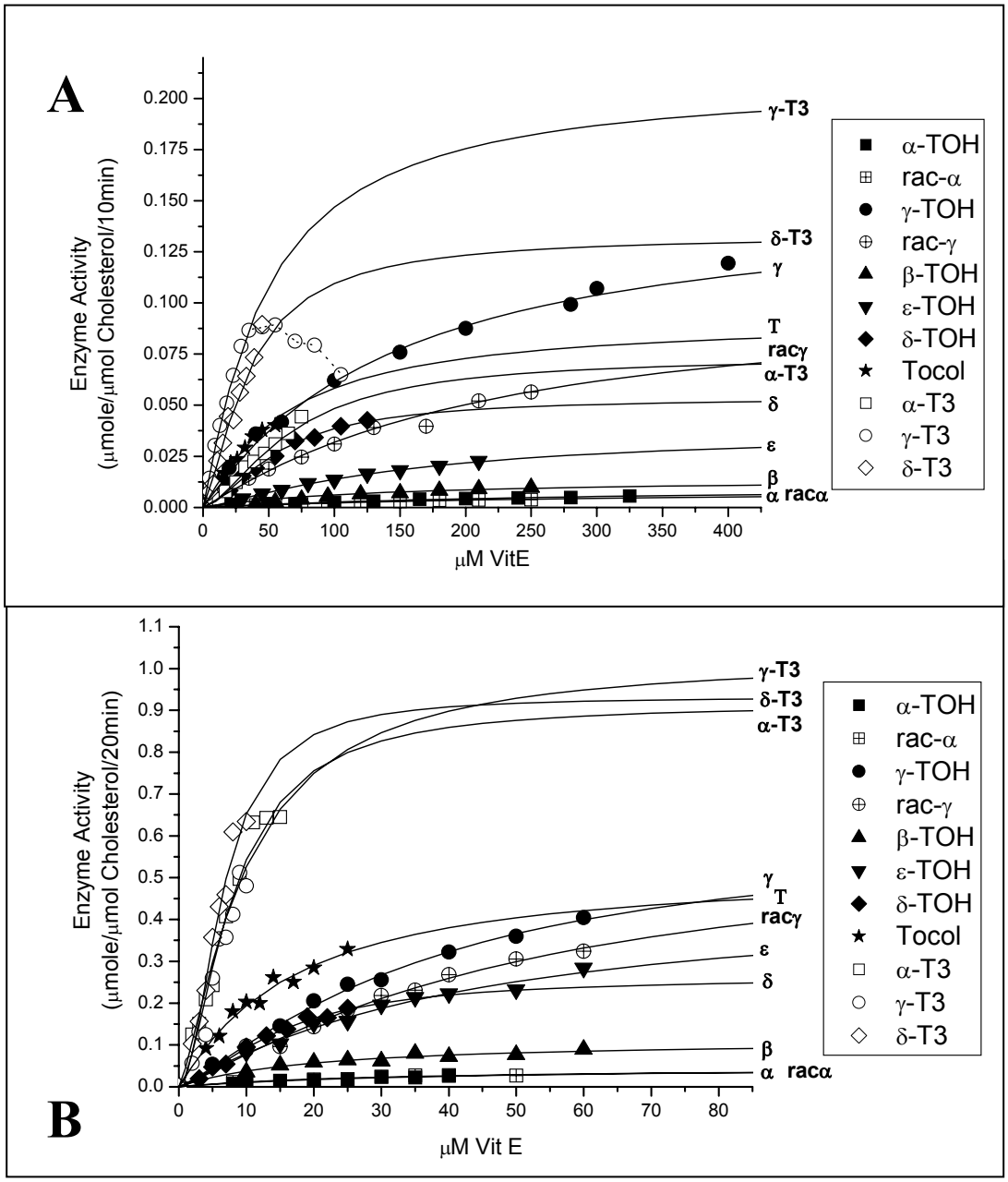



**Figure 3.6**

Kinetic analysis of vitamin E metabolism in Human Liver Microsome (HLM) & CYP4F2 microsome. **(A)** 100ug/ml human liver microsome or **(B)** 20pmol/ml CYP4F2 microsome was pre-incubated with vitamer-BSA complex for 60 min. 10 or 20 min reaction was initiated by the addition of 1mM NADPH + NAD.

**Table 3.1**

Kinetic parameters for human liver microsome (HLM) or CYP4F2 microsome. Kinetic fit to Michaelis-Menten or Hill equation was done using Origin 7.0 curve-fitting software.

| HLM | Vmax (E-03) Metab/Chol/10min | Error | Km (μM) | Error | n | Error | $R^2$ |
|---|---|---|---|---|---|---|---|
| α-TOH | 10.72 | +/-1.45 | 306.2 | +/-69.8 | 1.00 | +/-0.00 | 0.977 |
| rac-α | 9.22 | +/-1.42 | 318.3 | +/-74.1 | 1.00 | +/-0.00 | 0.982 |
| γ-TOH | 152.51 | +/-20.72 | 145.6 | +/-42.4 | 1.05 | +/-0.12 | 0.994 |
| rac-γ | 105.41 | +/-14.50 | 217.7 | +/-54.9 | 1.07 | +/-0.08 | 0.998 |
| β-TOH | 14.39 | +/-2.31 | 134.2 | +/-44.6 | 1.00 | +/-0.11 | 0.994 |
| ε-TOH | 37.72 | +/-3.55 | 155.5 | +/-23.8 | 1.25 | +/-0.07 | 0.998 |
| δ-TOH | 53.69 | +/-5.43 | 56.1 | +/-8.0 | 1.61 | +/-0.15 | 0.995 |
| Tocol | 92.27 | +/-43.07 | 61.8 | +/-45.1 | 1.13 | +/-0.19 | 0.992 |
| α-T3 | 73.81 | +/-8.14 | 66.8 | +/-8.6 | 1.60 | +/-0.08 | 0.999 |
| γ-T3 | 210.29 | +/-70.21 | 47.3 | +/-25.1 | 1.12 | +/-0.14 | 0.996 |
| δ-T3 | 133.24 | +/-46.06 | 34.5 | +/-16.3 | 1.44 | +/-0.27 | 0.986 |
| | | | | | | | |
| CYP4F2 | Vmax (E-02) Metab/Chol/20min | Error | Km (μM) | Error | n | Error | $R^2$ |
| α-TOH | 4.82 | +/-0.95 | 36.7 | +/-12.5 | 1.00 | +/-0.00 | 0.965 |
| rac-α | 4.59 | +/-0.63 | 29.6 | +/-8.1 | 1.00 | +/-0.00 | 0.963 |
| γ-TOH | 62.56 | +/-9.93 | 38.0 | +/-10.2 | 1.25 | +/-0.15 | 0.994 |
| rac-γ | 60.31 | +/-23.63 | 50.6 | +/-33.2 | 1.18 | +/-0.28 | 0.981 |
| β-TOH | 11.29 | +/-4.22 | 19.8 | +/-15.7 | 1.01 | +/-0.53 | 0.912 |
| ε-TOH | 46.38 | +/-18.63 | 42.9 | +/-32.4 | 1.08 | +/-0.31 | 0.973 |
| δ-TOH | 26.31 | +/-4.07 | 14.6 | +/-3.2 | 1.60 | +/-0.22 | 0.991 |
| Tocol | 52.34 | +/-21.95 | 16.3 | +/-13.0 | 1.09 | +/-0.35 | 0.966 |
| α-T3 | 91.59 | +/-18.11 | 8.0 | +/-2.2 | 1.69 | +/-0.34 | 0.979 |
| γ-T3 | 102.80 | +/-46.48 | 9.7 | +/-5.5 | 1.36 | +/-0.20 | 0.993 |
| δ-T3 | 93.28 | +/-21.49 | 6.6 | +/-1.7 | 2.00 | +/-0.41 | 0.979 |

large variation in enzyme activity for the vitamers based on the aforementioned structural features, disentangled from the confounding effects of cell or membrane substrate incorporation. The pattern of substrate structural effects on enzyme activity was similar between human liver microsomes and CYP4F2 microsomes. Apparent Km, a measure of affinity of enzyme for substrate, increased with number of methyl groups around the chromanol ring, with α-TOH showing the least affinity for the enzyme compared with the unmethylated tocol and monomethylated δ-TOH which exhibited the highest enzyme affinities. There were no significant effects of methyl position (γ- vs. β- vs. ε-TOH) or phytyl tail stereochemistry (RRR- vs. Rac-α- or γ-TOH) on enzyme affinity. Unsaturation of the phytyl tail proved to exert the strongest effect on enzyme affinity, with all three tocotrienols displaying low Km values compared with their tocopherol counterparts.

Vmax, a measure of maximal specific activity of the enzyme toward a substrate, did not show a pattern based on number of methyls substituted around the chromanol ring, as the dimethyl γ-TOH shows the highest activity. The position of the methyl group appears to play a greater role, with the 5-position methyl group most greatly attenuating the activity as evidenced by the low activities of the α- and β-TOH. The presence of the 7-position methyl seems to have the greatest positive effect on activity, given the activities of γ- and ε-TOH. That the enzyme does not possess as high activity toward ε-TOH as toward γ-TOH may again be due to the presence of the addition inhibitory 5-position methyl on the ε-TOH molecule. Stereochemistry of the phytyl tail does not influence the specific activity of the enzyme toward vitamin E (RRR- vs. Rac). The tocotrienols display a very high Vmax value compared with their analogous tocopherols. Taken together with the low Km values of the tocotrienols, the tocopherol-ω-hydroxylase appears to display the greatest overall preference for the unsaturated vitamin E molecule above other structural features.

*α-Tocopherol-Mediated Stimulation of Vitamer Metabolism*

The effect of the vitamers on the metabolism of each other was investigated in Rat liver microsomes. γ-TOH was incubated with several concentrations of α-, rac-α-, β-, or δ-TOH and γ-T3 (**Figure 3.7**). γ-T3, which is highly metabolized, acted as a good inhibitor of γ-TOH even at low concentrations. δ-TOH, which is metabolized at rates more comparable to γ-TOH, was a moderate inhibitor of γ-metabolism. Based on these results, α-, rac-α, and β-TOH were all expected to show little inhibition, given the fact that they are poorly metabolized. Surprisingly, no inhibition was observed. Instead, these tocopherols were found to increase the metabolism of γ-TOH, with the effects greatest for the α- vitamers irregardless of stereochemistry of their phytyl tail. Subsequent experiments revealed this stimulative effect of α-TOH toward all tocopherols and tocotrienols tested (data not shown). This stimulatory effect was observed in both human liver and CYP4F2 microsomes. α-TOH did not affect the membrane uptake of the other vitamers tested. At high concentrations of α-TOH, i.e. 500 μM, the stimulation disappeared and α-TOH became inhibitory. To test whether α-TOH was stimulating its own metabolism, RRR-$d_6$α-TOH was used as a substrate to distinguish it from the unlabeled α-TOH. Unlike its effects on the non-α vitamers, α-tocopherol is unable to stimulate its own metabolism (data not shown).

We suspected α-TOH was influencing the allosteric interaction between tocopherol-ω-hydroxylase and its substrates. Kinetic analyses of γ-TOH or d6α-TOH metabolism were carried out in the presence or absence of 50 μM α-TOH (**Figure 3.8**). In the absence of α-TOH, γ-TOH displayed the expected sigmoidal kinetics and non-linear Lineweaver-Burk kinetics typical of homotropic cooperativity. In the presence of α-TOH, the kinetics become hyperbolic and the Lineweaver-Burk analysis reverted to linearity (not shown). Additionally, the Vmax of the reaction increased in the presence of α-TOH while the Km was unaffected. In the presence of unlabeled α-

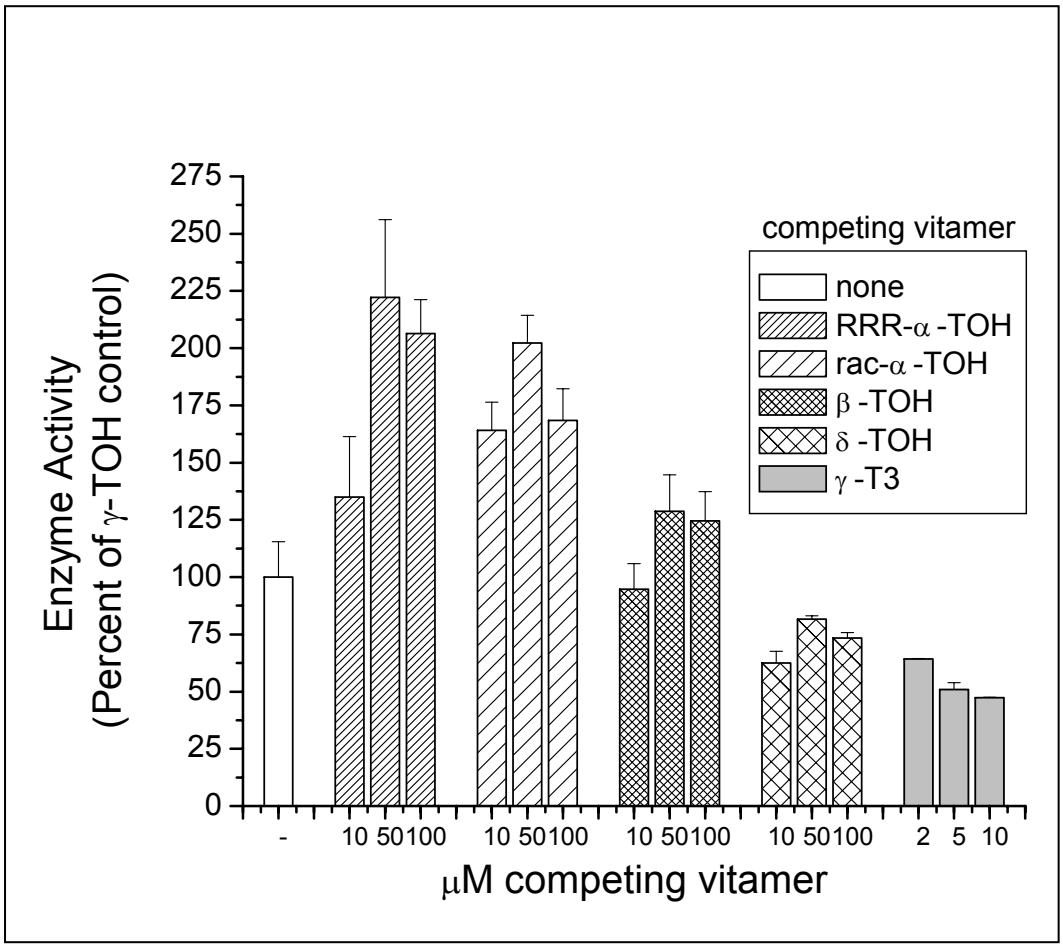



**Figure 3.7**

Influence of various vitamers on metabolism of 10μM γ-TOH in Rat liver microsomes. 100μg/ml rat liver microsome was pre-incubated with 10μM γ-TOH-BSA complex with or without the addition of several concentrations of various competing vitamers. A 20min reaction was then initiated by the addition of 0.5 mM NADPH + NAD and the combined levels of the 13'OH- and 13'COOH- γ-TOH metabolites generated was measured per microsomal cholesterol. Data is expressed as a percent of the γ-TOH-only (control) reaction.

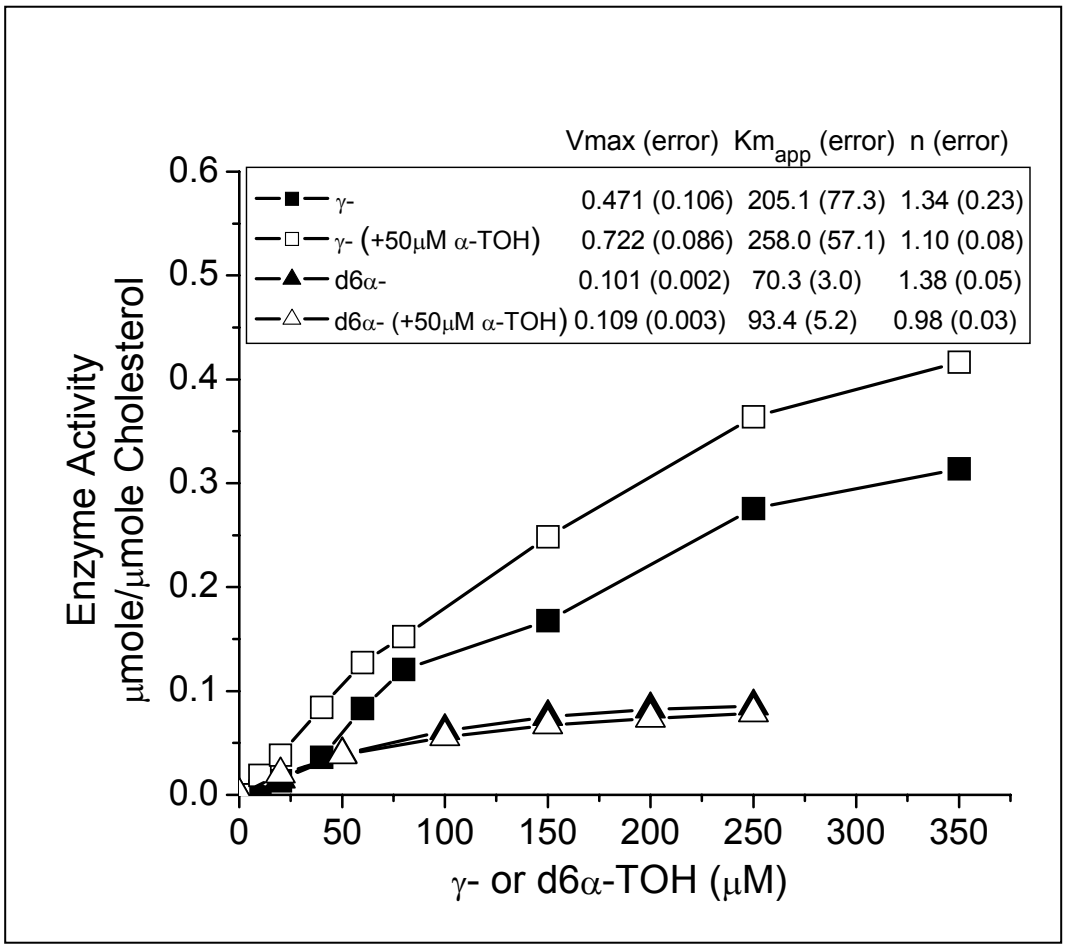



**Figure 3.8**

Kinetics of α-TOH stimulation of γ-TOH or d6α-TOH metabolism on Rat Liver Microsomes. 100μg/ml microsomal protein was preincubated with substrate + 50μM α-TOH for 60 min, followed by a 20 min reaction initiated by the addition of 0.5mM NADPH + NAD. Kinetic parameters are given (+/-error) based on best fit to the Hill equation.

TOH, metabolism of d6α-TOH showed no increase in Vmax, while the increase in Km was not significant.

**Discussion**

Despite the high intake of vitamers such as γ-TOH the body is very efficient in retaining mainly α-TOH while eliminating the non α-forms. The mechanisms underlying this apparent paradox in vitamin E bioavailability are just recently becoming clear. Several mechanisms have been proposed to explain the preferential retention of α-TOH, including loss of other forms in the bile, conversion of γ-TOH to α-TOH by microflora in the gut, and the preferential secretion of α-TOH into the plasma by the hepatic α-TTP (19,20,39). We have previously described the first enzyme shown to be involved in regulation of vitamin E status and demonstrated its preferential metabolism of γ-TOH over α-TOH (31). This enzyme, cytochrome P450 CYP4F2, is expressed in liver, kidney, and intestinal cells, and is constitutively expressed in several cell lines including the hepatoblastoma HepG2 and the lung adenocarcinoma A549 (40,41). Here, we further characterize this enzyme and demonstrate the importance of key structural features of its many tocochromanol substrates.

In HepG2 cell culture, human liver microsomes, and CYP4F2 microsomes, the two structural features most determining tocopherol-ω-hydroxylase activity toward each substrate were (a) the presence of the three double bonds along the phytyl tail and (b) the position of the methyl groups around the chromanol ring. The degree to which the α-T3 vitamer was metabolized compared with α-TOH indicates the dominance of phytyl tail unsaturation over the inhibitory effect of the methyl group at the 5 carbon position of the chromanol ring. While the number of methyl groups initially appeared to play a role in the differences in activity, these differences most likely indirectly

reflected the effects of methyl position and rates of vitamer incorporation into the cells or microsomal membranes. Racemerization around the chiral carbons of the tail did not significantly affect the rate of metabolism of α-TOH indicating the enzyme has no preference between the natural and synthetic forms of the vitamin. The differences in the metabolism of RRR-γ-TOH and all-rac γ-TOH suggest the stereochemistry of the phytyl tail may only affect metabolism when the enzyme activity toward the substrate is high.

The membrane environment appears to play a major role in the apparent activity of tocopherol-ω-hydroxylase toward its substrates. The evidence presented here indicates the membrane is the true environment of the entire reaction, with Vitamin E-P450 interaction occurring within the confines of the membrane. In whole cells, the substrates are not only incorporated into the whole cells at varying rates, but the access to the inner organelle membranes also varies greatly among the substrates, resulting in different substrate concentrations at the putative intracellular site of enzyme activity. Similarly, microsomal data reveal the strong influence of substrate access to the membrane-associated enzyme. As such, the kinetics of metabolism are not equivalent to those of an aqueous system (38). Differences in rates of membrane association of the vitamer as well as differences in rates of lateral motion through the membrane may influence the apparent affinity of the enzyme for vitamers possessing different structural features. The apparent Km may then better be a measure of affinity of substrate for the combined enzyme-membrane system. Vmax, which is not influenced by substrate access to the enzyme, may be a better indicator of substrate specificity of the actual enzyme.

The allosteric nature of the tocopherol-ω-hydroxylase was an unexpected finding, as no indication of allostery has been associated for this enzyme with its previously characterized substrates, $LTB_4$ or arachidonic acid (34,36,42). The best

studied case of cooperativity in a cytochrome P450 system is that of CYP3A4, which displays both homotropic and heterotropic cooperativity (43,44). CYP3A4 possesses a large active site, able to bind both small molecules such as naphthalene and large substrates such as dibenzopyrenes. Such a large active site may accommodate the simultaneous binding of two molecules. Shou et al (44) described the simultaneous binding of two substrates to CYP3A4 with no effect on Km, and surmised that the binding of both substrates (or substrate + effector) occur within the same active site, and later (45) that this active site possesses two distinct substrate-binding sites. The results of Harlow and Halbert (46) supported this theory. Reduction in the size of the proposed effector site of the CYP3A4 enzyme by site-directed mutagenesis mimicked the stimulation produced by α-naphthoflavone, a known effector of CYP3A4. Based on this model, positive cooperativity can occur during enzyme-substrate interactions by increasing Vmax, decreasing Km, or both. The binding of the effector molecule may alter the size and shape of the active site to allow for increased affinity of enzyme for substrate, thus lowering the Km. Kcat of substrate + effector may also be higher than Kcat of substrate alone, thereby increasing Vmax (46). The second scenario is consistent with our findings for tocopherol-ω-hydroxylase in the presence of α-TOH, where the substrate activity of the enzyme increased toward the non-α vitamers with no significant change in Vmax (**Figure 3.8**). This may reflect several scenarios including (a) one large active site containing two substrates, (b) a large "active site" containing two binding sites, or (c) two geographically separated binding sites. These possibilities merit further study.

There is a large body of evidence showing supplementation of α-TOH decreases plasma levels of γ-TOH (24,25). Additionally, supplementation of rats with equivalent amounts of α- and γ-TOH causes a greater increase in urinary γ-CEHC excretion than when γ-TOH is fed alone (47). Our finding here of the stimulatory

effect of α-TOH on the enzymatic metabolism of non-α-TOH vitamers presents a mechanism by which these phenomena may occur. The development of this heterotropic cooperativity may be an enzymatic means by which the body can decrease the elimination of non-α-TOH vitamers under conditions where tissue α-TOH is limited, while at the same time enhancing their elimination when α-TOH status is sufficient. This effector activity is observed for the synthetic racemic-α-TOH as well, highlighting the role of tocopherol-ω-hydroxylase in the influence of vitamin E supplementation on plasma and tissue tocochromanol status.

We previously reported that other members of the CYP4F family, 4F3A and 4F3B, show very little activity toward tocopherols despite having a high degree of sequence homology with CYP4F2. The possibility that other P450 enzymes exist in the liver which are able to metabolize vitamin E seems unlikely based on the evidence presented here. The kinetics of metabolism in human liver microsomes showed similar profiles to that of CYP4F2 microsomes for the vitamers, with no evidence of the biphasic kinetics normally associated with two enzymes acting on the same substrates. Any additional hepatic enzyme possessing significant tocopherol-ω-hydroxylase activity would need to also possess similar kinetics and substrate specificity as that of CYP4F2. Additionally, the comparative flux of the vitamers through the rest of the pathway (β-oxidation) has not been determined. However, when differences in cell vitamer uptake are taken into account, the pattern of the complete ω/β-oxidation in the hepatocyte system and of ω-hydroxylation in the single enzyme system show overall similarities, indicating the tocopherol-ω-hydroxylase mediated hydroxylation may be the major discriminatory step within the pathway.

The effects of substrate structure on tocopherol-ω-hydroxylase activity are very interesting in light of the relative affinities of the various tocochromanols for the hepatic α-tocopherol transfer protein as determined by Hosomi et al (20). RRR-α-

TOH exhibited the highest affinity for the protein (assigned 100%), followed by β-TOH (38%), α-T3 (12%), SRR-α-TOH (11%), γ-TOH (9%), and δ-TOH (2%). Unfortunately, the affinities of the more highly metabolized γ- and δ-tocotrienols were not determined. These affinities are for the most part the inverse to their ability to act as ω-hydroxylase substrates, suggesting a collaborative role for these two proteins in producing and maintaining the α-TOH enriched phenotype. The fact that TTP shows a high degree of specificity for the RRR stereochemistry of the phytyl tail, while the tocopherol-ω-hydroxylase does not, suggests that α-TTP may be the main regulatory feature of the preference of natural vitamin E over synthetic. However, data from TTP knockout mice shows the plasma of these mice lacking the transfer protein is still enriched in α-TOH over γ-TOH, although total tocopherol levels are greatly reduced relative to controls (48). Additionally, rats fed an inhibitor of the tocopherol-ω-hydroxylase, sesamin, have plasma γ-TOH levels nearing those of α-TOH (4). Taken together, these data support the following model. Dietary tocochromanols are taken into the liver without pre-hepatic discrimination (49). In the absence of α-TTP, there is no preference for α-TOH being removed from the liver. All the vitamers may leave the liver at equal rates. However, due to the specificity of the tocopherol-ω-hydroxylase, less α-TOH is metabolized and more will subsequently be available to leave the liver even by non-selective export, albeit in lower amounts than in the presence of TTP. In the absence (i.e. inhibition) of tocopherol-ω-hydroxylase, other non-α-TOH vitamers are not catabolized. The lower rate of transfer of non-α-TOH out of the liver by a α-TTP mediated mechanism not as influential in regulating vitamin E status, since the non-α-TOH is present long enough to be exported out of the liver.

The reasons behind the structural selectivity that both α-TTP and the tocopherol-ω-hydroxylase exhibit are not clear. This function may be desirable to rid

the system of potentially toxic or less active vitamers (1,15,17), or to generate bioactive metabolites of these vitamers (13,27). On the other hand the numerous potential benefits of the non-α forms of vitamin E may be lost upon their rapid catabolism and elimination. The fact that many organisms throughout nature exhibit selective retention of α-TOH above the other vitamers may indicate a selective advantage to this trait.

The activities of the tocopherol transfer protein and the tocopherol-ω-hydroxylase enzyme are only in recent years being clarified. As we continue to learn more about the roles of specific proteins in the regulation of tocopherol status, we find an increasingly complex means by which the body appears to select among vitamers differing in minor structural features for retention or elimination. Elucidation of these mechanisms opens new doors for enhancing the plasma levels of vitamers (e.g. γ-TOH) which would otherwise be eliminated. Given the potential therapeutic effects of forms of vitamin E other than α-TOH, new strategies may emerge to impact the ability of these tocochromanols to be used in the prevention or treatment of a variety of conditions.

**References**


1. Bieri, J. G., and McKenna, M. C. (1981) *Am. J. Clin. Nutr.* **34**, 289-295
2. Kamal-Eldin, A., and Appelqvist, L. A. (1996) *Lipids* **31**, 671-701
3. Burton, G. W., and Ingold, K. U. (1986) *Acc Chem Res* **19**, 194-201
4. Yamashita, K., Nohara, Y., Katayama, K., and Namiki, M. (1992) *J Nutr* **122**, 2440-2446
5. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) *Free Radic Biol Med* **10**, 263-275
6. Saito, Y., Yoshida, Y., Akazawa, T., Takahashi, K., and Niki, E. (2003) *J Biol Chem* **278**, 39428-39434
7. Boscoboinik, D., Szewczyk, A., Hensey, C., and Azzi, A. (1991) *J Biol Chem* **266**, 6188-6194
8. Tasinato, A., Boscoboinik, D., Bartoli, G. M., Maroni, P., and Azzi, A. (1995) *Proc Natl Acad Sci U S A* **92**, 12190-12194
9. Ricciarelli, R., Zingg, J. M., and Azzi, A. (2000) *Circulation* **102**, 82-87
10. Keaney, J. F., Jr., Simon, D. I., and Freedman, J. E. (1999) *Faseb J* **13**, 965-975
11. Hoglen, N. C., Waller, S. C., Sipes, I. G., and Liebler, D. C. (1997) *Chem Res Toxicol* **10**, 401-407
12. Williamson, K. S., Gabbita, S. P., Mou, S., West, M., Pye, Q. N., Markesbery, W. R., Cooney, R. V., Grammas, P., Reimann-Philipp, U., Floyd, R. A., and Hensley, K. (2002) *Nitric Oxide* **6**, 221-227
13. Jiang, Q., Elson-Schwab, I., Courtemanche, C., and Ames, B. N. (2000) *Proc Natl Acad Sci U S A* **97**, 11494-11499

14. Kushi, L. H., Folsom, A. R., Prineas, R. J., Mink, P. J., Wu, Y., and Bostick, R. M. (1996) *N Engl J Med* **334**, 1156-1162

15. Yu, W., Simmons-Menchaca, M., Gapor, A., Sanders, B. G., and Kline, K. (1999) *Nutr Cancer* **33**, 26-32

16. Qureshi, A. A., Mo, H., Packer, L., and Peterson, D. M. (2000) *J Agric Food Chem* **48**, 3130-3140

17. McIntyre, B. S., Briski, K. P., Tirmenstein, M. A., Fariss, M. W., Gapor, A., and Sylvester, P. W. (2000) *Lipids* **35**, 171-180

18. Parker, R. A., Pearce, B. C., Clark, R. W., Gordon, D. A., and Wright, J. J. (1993) *J Biol Chem* **268**, 11230-11238

19. Traber, M. G., and Kayden, H. J. (1989) *Am J Clin Nutr* **49**, 517-526

20. Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H., and Inoue, K. (1997) *FEBS Lett* **409**, 105-108

21. Arita, M., Nomura, K., Arai, H., and Inoue, K. (1997) *Proc Natl Acad Sci U S A* **94**, 12437-12441

22. Morinobu, T., Yoshikawa, S., Hamamura, K., and Tamai, H. (2003) *Eur J Clin Nutr* **57**, 410-414

23. Kaneko, K., Kiyose, C., Ueda, T., Ichikawa, H., and Igarashi, O. (2000) *J Lipid Res* **41**, 357-367

24. Handelman, G. J., Machlin, L. J., Fitch, K., Weiter, J. J., and Dratz, E. A. (1985) *J Nutr* **115**, 807-813

25. Burton, G. W., Traber, M. G., Acuff, R. V., Walters, D. N., Kayden, H., Hughes, L., and Ingold, K. U. (1998) *Am J Clin Nutr* **67**, 669-684

26. Schonfeld, A., Schultz, M., Petrizka, M., and Gassmann, B. (1993) *Nahrung* **37**, 498-500

27. Wechter, W. J., Kantoci, D., Murray, E. D., Jr., D'Amico, D. C., Jung, M. E., and Wang, W. H. (1996) *Proc Natl Acad Sci U S A* **93**, 6002-6007

28. Chiku, S., Hamamura, K., and Nakamura, T. (1984) *J Lipid Res* **25**, 40-48

29. Hattori, A., Fukushima, T., Yoshimura, H., Abe, K., and Imai, K. (2000) *Biol Pharm Bull* **23**, 1395-1397

30. Lodge, J. K., Ridlington, J., Leonard, S., Vaule, H., and Traber, M. G. (2001) *Lipids* **36**, 43-48

31. Sontag, T. J., and Parker, R. S. (2002) *J Biol Chem* **277**, 25290-25296

32. Birringer, M., Drogan, D., and Brigelius-Flohe, R. (2001) *Free Radic Biol Med* **31**, 226-232

33. Kikuta, Y., Kusunose, E., Kondo, T., Yamamoto, S., Kinoshita, H., and Kusunose, M. (1994) *FEBS Lett* **348**, 70-74

34. Powell, P. K., Wolf, I., Jin, R., and Lasker, J. M. (1998) *J Pharmacol Exp Ther* **285**, 1327-1336

35. Christmas, P., Ursino, S. R., Fox, J. W., and Soberman, R. J. (1999) *J Biol Chem* **274**, 21191-21199

36. Kikuta, Y., Kusunose, E., and Kusunose, M. (2000) *J Biochem (Tokyo)* **127**, 1047-1052

37. Sen, C. K., Khanna, S., Roy, S., and Packer, L. (2000) *J Biol Chem* **275**, 13049-13055

38. Takakuwa, Y., Nishino, H., Ishibe, Y., and Ishibashi, T. (1994) *J Biol Chem* **269**, 27889-27893

39. Elmadfa, I., Kim, S. W., Reutlinger, M., and Siewert, R. (1989) *Z Ernahrungswiss* **28**, 36-48

40. Kikuta, Y., Miyauchi, Y., Kusunose, E., and Kusunose, M. (1999) *DNA Cell Biol* **18**, 723-730

41. Yamane, M., and Abe, A. (2000) *J Biochem (Tokyo)* **128**, 827-835

42. Jin, R., Koop, D. R., Raucy, J. L., and Lasker, J. M. (1998) *Arch Biochem Biophys* **359**, 89-98

43. Houston, J. B., and Kenworthy, K. E. (2000) *Drug Metab Dispos* **28**, 246-254

44. Shou, M., Grogan, J., Mancewicz, J. A., Krausz, K. W., Gonzalez, F. J., Gelboin, H. V., and Korzekwa, K. R. (1994) *Biochemistry* **33**, 6450-6455

45. Shou, M., Mei, Q., Ettore, M. W., Jr., Dai, R., Baillie, T. A., and Rushmore, T. H. (1999) *Biochem J* **340 ( Pt 3)**, 845-853

46. Harlow, G. R., and Halpert, J. R. (1998) *Proc Natl Acad Sci U S A* **95**, 6636-6641

47. Kiyose, C., Saito, H., Kaneko, K., Hamamura, K., Tomioka, M., Ueda, T., and Igarashi, O. (2001) *Lipids* **36**, 467-472

48. Terasawa, Y., Ladha, Z., Leonard, S. W., Morrow, J. D., Newland, D., Sanan, D., Packer, L., Traber, M. G., and Farese, R. V., Jr. (2000) *Proc Natl Acad Sci U S A* **97**, 13830-13834

49. Traber, M. G., Burton, G. W., Ingold, K. U., and Kayden, H. J. (1990) *J Lipid Res* **31**, 675-685

# CHAPTER IV

# TOCOTRIENOLS BUT NOT TOCOPHEROLS REDUCE THE MOBILITY OF LIPID MEMBRANE COMPONENTS AND INHIBIT MICROSOMAL CYTOCHROME P450 ACTIVITY

**Abstract**

The tocopherol and tocotrienol vitamers of the vitamin E family of tocochromanols have recently been demonstrated by our lab to be degraded to water soluble metabolites through the action of the microsomal cytochrome P450 (CYP4F2) tocopherol-ω-hydroxylase enzyme and subsequent β-oxidation of the phytyl side chain. Tocotrienols differ from tocopherols only in the presence of three double bonds along the phytyl tail. Kinetic analysis of activity of tocopherol-ω-hydroxylase toward these substrates reveals a substantial concentration-dependent impairment of enzyme activity by tocotrienols incorporated into the microsomal membrane, an effect not shared by the tocopherols. We tested the effect of tocopherols and tocotrienols on other microsomal cytochrome P450 enzymes to test if this was a generalized effect on P450 enzymes. The metabolism of testosterone (CYP3A, 2C), 7-ethoxycoumarin (CYP1A, 2B, 2E), lauric acid (CYP4A), and p-nitrophenol (CYP2E) were inhibited to varying degrees by tocotrienols, but not tocopherols. This inhibition was non-competitive. Similar effects on enzyme activity were seen by enriching microsomal membranes with cholesterol or unsaturated fatty acids in the membrane, but not with saturated fatty acids. Analysis of membrane physical characteristics by diphenyl-hexatriene fluorescence anisotropy and pyrene eximerization revealed that γ-tocotrienol decreased the rotational mobility of membrane lipids more than α-tocopherol, while both tocopherols and tocotrienols decreased the lateral diffusivity within the membrane. This decrease in membrane fluidity is similar to cholesterol which also decreases the activity of the electron transport partners involved in reduction of P450 enzymes. In the absence of microsomal cytochrome b5, γ-tocotrienol was not as inhibitory toward P450 activity as when b5 was present. We suggest the incorporation of tocotrienols into microsomal membranes disturbs the interactions between cytochrome b5 and cytochrome P450, resulting in inhibition of

those P450 enzymes whose activity is strongly modified by cytochrome b5 activity. This effect may play a role in the reported apoptotic effects of tocotrienols in various cell lines and highlights the importance of the tocopherol-ω-hydroxylase pathway in minimizing membrane tocotrienol concentration.

**Introduction**

Vitamin E exists in nature as two classes of tocols, the tocopherols (TOH) and the tocotrienols (T3). The tocotrienols differ from the tocopherols only in the presence of three double bonds along the phytyl tail of the vitamin molecule at positions 3', 7', and 11'. α-Tocopherol is the major form of vitamin E found in plasma and tissues despite the high intake of other forms of the vitamin (1). Normal plasma levels of α-tocopherol range from 15-35 μM, while the tocotrienols are normally found in only trace amounts (2). Even when supplemented at 250 mg/day, plasma levels of α-T3 reached only about 1 μM (3).

The structural differences between the tocopherols and tocotrienols result in large differences in bioactivities. α-TOH is the most bioactive form of the vitamin as determined by the rat fetal resorption assay, while α-T3 possessed only 0.3% of the activity of α-TOH (4). The bioactivities of γ- and δ-T3 were below detection. These differences in activity may be largely explained by the very low levels of tocotrienols achieved in tissues relative to α-TOH.

While the tocopherols and tocotrienols both possess vitamin E activity in this animal model, the mechanism behind this activity has not been elucidated. The classically accepted function of these molecules has been as antioxidants, in which both forms of vitamin E are able to scavenge the chain-propagating peroxyl radical (5). Since these molecules are lipophilic, this antioxidant function occurs in the lipid environment of membranes. As such, the mobility and location of these molecules

within the membrane will affect their interaction in with radicals, factors which are partially dependent on the structure of the phytyl tail (6). α-T3 and α-TOH were reported to possess equivalent peroxyl radical scavenging capabilities in hexane solution, but the protection by α-T3 against lipid peroxidation was greater than by α-TOH in liposomes (1.5-fold) and in rat liver microsomes (40-fold) (7). In the same study, when P450 oxidative damage was assessed in the presence of added vitamin E, α-T3 was over 6 times more protective than α-TOH. These effects may be dependent on the experimental conditions, as Yoshida et al found similar reactivities of α-TOH and α-T3 in liposomal membranes (8).

Other functions of the tocotrienols have been described for which the tocopherols show less activity. α-Tocotrienol was reported to be an effective inhibitor of cholesterol biosynthesis in vitro and in vivo by means of inhibition of HMG CoA reductase activity (9). This inhibition was found to occur by means of post-transcriptional regulation of enzyme levels (10). Tocotrienols were also much more effective than tocopherols at inhibiting glutamate-induced neuronal cell death and 12-lipoxygenase activity (11,12). Additionally, while α-TOH appears to reduce endothelial expression of adhesion molecules, α-T3 has been found to be more potent (13). These effects suggest an anti-atherogenic potential of the tocotrienols. Other studies have implicated the tocotrienols in the prevention/treatment of cancer (14). Several studies have shown that tocotrienols cause the induction of apoptosis and inhibition of cell proliferation in cancer cells, whereas α-TOH does not (15,16).

Tocotrienols are more rapidly taken up into cells than tocopherols, including being readily absorbed through the skin (11,17,18). A greater membrane affinity is seen for the tocotrienols compared with the tocopherols in vitro in the rapid transfer of tocotrienols between membranes (11,17,18). This effect is seen in vitro by the rapid transfer of tocotrienols between membranes (8). The tocotrienols have been shown to

be more uniformly distributed throughout the lipid membrane bilayer and show a stronger disordering of membrane lipids (7). Electron spin resonance studies have indicated that tocotrienols are located closer to the surface of liposomal lipid membrane bilayers than the tocopherols. This same methodology was used to conclude that effects of tocopherols and tocotrienols on membrane fluidity, and molecular motion throughout the membrane are similar (6,8). Other lipid components of the membrane have been shown to affect the fluidity of the membrane, as well as the activity of membrane-associated enzymes. Incorporation of cholesterol into microsomal membranes decreases both rotational and translational mobilities of the membranes. This in turn affects the activities of membrane-bound enzymes (19), including that of cytochrome b5, a component of the cytochrome P450 monooxygenase system (20). Fluidizing effects are produced when isoamyl alcohols are incorporated into the membrane (21).

The metabolism of tocopherols and tocotrienols to water-soluble metabolites followed by their excretion in the urine occurs via an initial ω-hydroxylation of the phytyl side-chain by the tocopherol-ω-hydroxylase enzyme, cytochrome P450 CYP4F2 (22). This process plays a key role in the regulation of vitamin E status due to the preferential metabolism of the non-α isomers of vitamin E. This may be a means by which the organism is able to minimize the concentration of vitamers which potentially have the greatest deleterious effects. Here we investigated the effects of tocopherols and tocotrienols on microsomal membranes and the activity of enzymes within these membranes, and demonstrate tocotrienols-specific effects on these parameters.

**Materials and Methods**

Most chemicals were purchased from Sigma with the exception of the tocotrienols, which were a gift from Volker Berl (BASF, Ludvigshafen, Germany). Pyridine and N,O-bis-[Trimethylsilyl]-trifluoroacetamide containing 1%Trimethylchrorosilane (BSTFA + 1%TMCS) were from Pierce Chemical Co., Rockford, IL. Pooled human liver microsomes, purified cytochrome P450 reductase, and specific recombinant human cytochrome P450 enzymes expressed in insect cell microsomes with human cytochrome P450 reductase, and with or without recombinant human cytochrome B5, were purchased from BD Gentest (Woburn, MA).

*Microsome preparation*

Microsomal fractions from fresh rat liver were prepared by differential centrifugation as described previously (22) and resuspended in 100mM $KH_2PO_4$ buffer, pH 7.4 and stored at -80$^{o}$C until use. Microsomal protein content was determined using the Biorad protein kit (kit # 500-0002; Biorad, Hercules, CA).

*Microsomal metabolism*

Microsomal metabolism of vitamin E vitamers to their corresponding 13'OH or 13'COOH metabolites was carried out using 0.1 mg/ml rat or human liver microsomal protein, or 10-20 pmol/ml insect microsome-expressed P450. Microsomes were pre-incubated 0-60 min with substrate vitamers complexed with 1% bovine serum albumin (BSA fraction IV) which allowed for incorporation of the vitamer into the membrane. A 0 - 30 min reaction was initiated with the addition of 0.5 - 1.0 mM NADPH, with or without 0.5 - 1.0 mM NAD. The reaction was terminated with the addition of 0.1 volume 3N HCl, and metabolites, substrate, and membrane cholesterol extracted with ice cold ethanol, methyl-tert-butyl-ether, and hexane in a 1: 0.5: 8 volume ratio. Extracts were evaporated under $N_2$ to dryness and derivatized in Pyridine using BSTFA + 1%TMCS. Derivatized samples were

analyzed by gas chromatography-mass spectrometry (GC-MS) as described previously (22). Unless noted, all microsomal activity is expressed as μmoles of metabolite per membrane cholesterol, as endogenous membrane cholesterol is a good internal standard for microsomal mass.

Metabolism of other substrates was carried out in a similar manner to vitamin E with the following differences. Testosterone, 7-ethoxycoumarin, and lauric acid were added to the reaction mixture in ethanol vehicle and the reaction was terminated by the addition of 4 volumes of ethyl acetate and products were extracted into this solvent. p-Nitrophenol metabolism was terminated with the addition of 0.2 volumes of trichloroacetic acid and the protein was pelleted at 10,000 x g for 8 min. The resulting supernatant was mixed with 0.5 volumes of 2N NaOH and p-nitrocatechol production was assessed by spectrophotometry (535 nm) using a 0 min incubation preparation as the blank. Glucose-6-phosphate metabolism to glucose was measured using a modification of the glucose dehydrogenase coupled assay described by Alegre et al (23). Briefly, 12.5 mM glucose-6-phosphate was incubated with 0.2 mg/ml rat liver microsome and 1.8 mM EDTA in $KH_2PO_4$ buffer for 1 hour at 37$^{o}$C. The glucose dehydrogenase reaction was initiated by adding 12 IU/ml glucose dehydrogenase (Sigma) and 2 mM NAD for 15min at 37$^{o}$C. Production of NADH was measured by spectrophotometry (340 nm) using a 0 min incubation preparation as the blank.

*Microsomal enzyme activity and inhibition*

Inhibition of activities of several microsomal enzymes by tocopherols and tocotrienols, or other membrane lipids, was assessed using activities of these enzymes toward model substrates of each enzyme as shown in **Table 4.1** in combination with the test substances. The test substance was pre-incubated with the microsomal membrane for 0 – 60 min in the presence or absence of NADPH. The enzymatic

**Table 4.1**

Reactions occurring in microsomal membranes and the enzymes carrying out each reaction.

| Substrate | Metabolite | Enzyme | Reference |
|---|---|---|---|
| Vitamin E (VitE) | 13'OH+13'COOH-VitE | CYP4F | (22) |
| Testosterone (TST) | 6β-OH-TST | CYP3A | (24) |
| Testosterone (TST) | 16α-OH-TST | CYP2C | (25) |
| 7-Ethoxycoumarin (7-EC) | 7-Hydroxycoumarin (7-HC) | CYP1A, CYP2B | (26,27) |
| Lauric Acid (LA) | 12-OH-LA | CYP4A | (28) |
| Lauric Acid (LA) | 11-OH-LA | CYP4A | (29) |
| p-nitrophenol (p-NP) | p-nitrocatechol (p-NC) | CYP2E | (30) |
| Glucose-6-Phosphate | Glucose | Glucose-6-Phosphatase | (23) |
| Cytochrome C | Reduced Cytochrome C | CYP Reductase | (31) |

reaction was initiated by the addition of substrate plus additional NADPH. In the case of glucose-6-phosphatase activity, the loaded microsomes were re-isolated after the pre-incubation by ultracentrifugation for 1 hour at 100,000 x g to remove remaining NADPH.

*Pyrene eximerization*

Lateral diffusivity of the microsomal membrane was assessed by pyrene eximerization. 1 mg/ml rat liver microsome was incubated for 1 hour with 6 μM pyrene (added in 10μl acetone) at 25°C. α-TOH (150 nmol per 0.1mg protein) or γ-T3 (60 nmol per 0.1mg protein), as their BSA complex, plus 0.5 mM NADPH were then added, and the incubation continued for 1hr at 37°C. These were vitamin E concentrations which resulted in equivalent membrane vitamer concentrations. Microsomes were re-isolated by ultracentrifugation for 1 hr at 100,000 x g and resuspended at 1 mg/ml in $KH_2PO_4$ buffer. Pyrene eximerization was measured by fluorometry with an exitation wavelength of 334 nm. Monomer emission was measured at 391 nm and eximer emission at 470. The fluorescence intensity of minus-pyrene controls was subtracted from that of samples. Efficiency of pyrene eximer formation was expressed as the ratio of fluorescence intensity of eximer ($I_e$) to monomer ($I_m$).

*Diphenyl-hexatriene fluorescence anisotropy*

The rotational mobility of the microsomal membrane was evaluated using 1,6-diphenyl-1,3,5-hexatriene as a probe. 0.1 mg/ml rat liver microsome was incubated for 1 hour with 0.25 μM DPH (added in 10 μl dimethylformamide). α-TOH or γ-T3 loading and membrane re-isolation was done as for pyrene eximerization experiments. Fluorescence anisotropy was measured and expressed as $r_s$ values as described by Garda and Brenner (21).

*Microsomal Cytochrome P450 reductase activity*

The reduction of exogenous cytochrome c was used to measure the effect of γ-T3 on microsomal cytochrome P450 reductase activity. 0.2 mg/ml rat liver microsomes were pre-incubated with 75 nmol γ-T3 per mg protein and 1mM NADPH. Loaded microsomes were re-isolated by ultracentrifugation at 100,000 x g for 1 hour and resuspended (0.2 mg/ml protein) in 50 mM $KH_2PO_4$ containing 0.1 mM EDTA (pH 7.7). Two mg cytochrome c was added and the reaction initiated with 0.8 mM NADPH. Cytochrome P450 reductase activity was calculated by measuring the rate of change in absorption at 550 nm.

*NADPH reduction of Microsomal Cytochrome P450 and b5*

Reduction of microsomal cytochrome P450 was determined using a modification of the method of Omura and Sato (32). 0.25 mg/ml rat liver microsomes were incubated 1 hour with or without 1.5 μmol/mg protein α-TOH or 0.6 μmol/mg protein γ-T3 (as their BSA complexes) and 0.5 mM NADPH at 37°C. The microsomes were re-isolated by centrifugation at 100,000 x g for 1 hr and resuspended in $KH_2PO_4$ buffer. The microsomes were again treated for 5 min with 0.5 mM NADPH. Samples were then bubbled with CO for 1 min and the absorbance from 400-500 nm was measured and compared with controls to which no NADPH had been added during the pre-incubation. Reduced cytochrome P450 was assessed by spectrophotometry at 450 nm and reduced cytochrome b5 at 426 nm.

*Microsomal depletion or enrichment of cholesterol*

Rat liver microsomes were depleted of cholesterol by incubating 2 mg/ml microsomes with 50% hydroxypropyl-β-cyclodextrin in $KH_2PO_4$ buffer for 1.5 hr at 37°C. Rat liver microsomes were enriched in cholesterol by a 1 hr incubation of 0.8 mg/ml microsome with cholesterol-loaded hydroxypropyl-β-cyclodextrin at 37°C. Cholesterol depleted or enriched microsomes were isolated from buffer by

centrifugation at 100,000 x g for 1hr, and resuspended in $KH_2PO_4$ buffer. Membrane cholesterol content was determined by GC-MS (22).

*Statistical Analysis*

All statistical analysis was done using the statistical program included in the OriginLab Origin 7 graphing software. Where applicable, data represent mean and standard deviation of duplicate or triplicate determinations. Comparison of differences between treatments was done using a two-sample t-test. Enzyme kinetics data were fit to the best curve using Origin 7 non-linear curve fit software.

**Results**

The metabolite production from added α-TOH, γ-TOH and γ-T3 substrates was determined as a function of increasing tocopherol concentration in microsomes prepared from rat liver (**Figure 4.1**). The metabolism of the tocopherols increased until plateauing at their respective Vmax values. γ-T3 metabolism increased with substrate concentration through 60-70 μM, after which metabolite production is increasingly inhibited. The same result was obtained for α- and δ-T3 and to a lesser extent with δ-TOH and with the non-methylated substrate tocol (data not shown). It was possible that this was due to substrate inhibition of the tocopherol-ω-hydroxylase enzyme which metabolizes vitamin E (22). However, the vitamers which exhibited this substrate inhibition were also those having the greatest affinity for the lipid membrane, as determined by the differences in efficiency of membrane association when the vitamers were added to the microsomal suspensions (data not shown).

A second possible explanation for the observed inhibition was substrate-induced alteration of the membrane environment, which may have affected membrane-associated enzyme activity. To test this hypothesis, we measured the activity of several microsomal enzymes which do not metabolize vitamin E, after

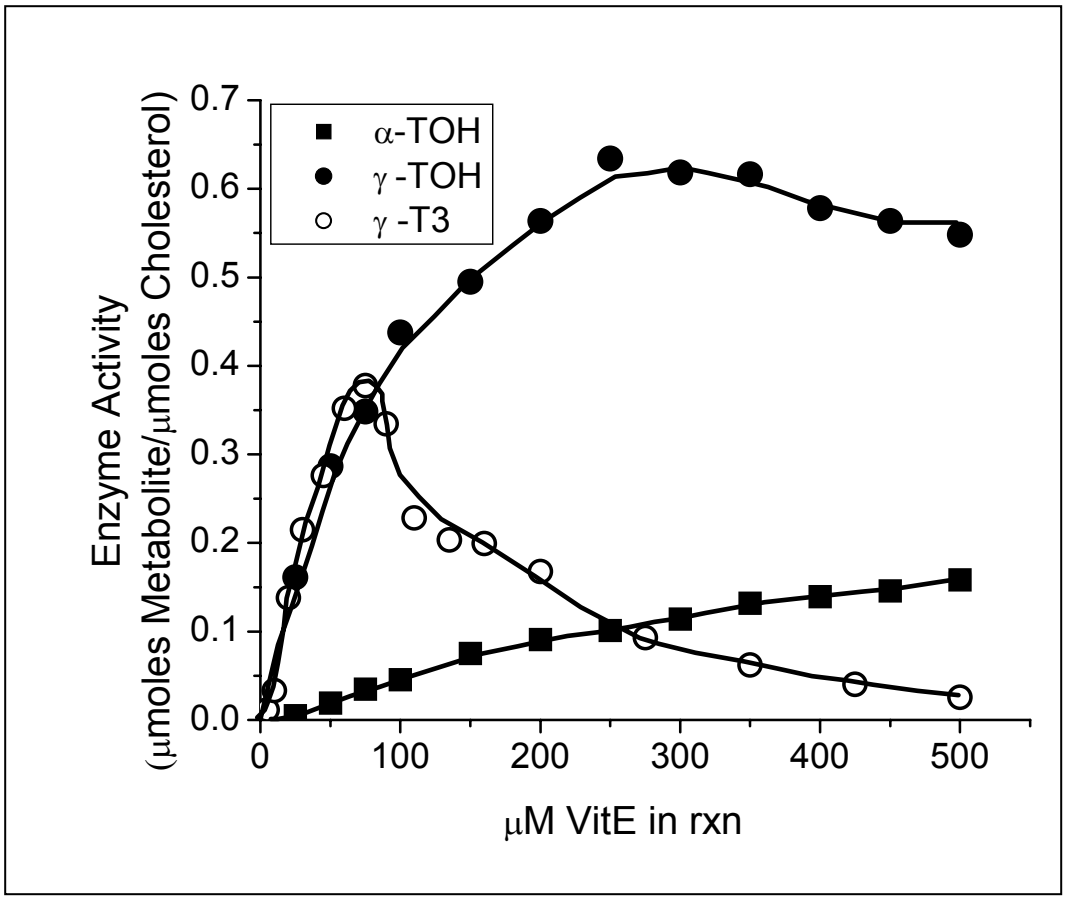



**Figure 4.1**

Kinetics of metabolism of α-TOH, γ-TOH and γ-T3 on 100 μg/ml rat liver microsome demonstrating self-inhibition of metabolism by γ-T3. Increasing concentrations of vitamer-BSA complex were pre-incubated 60 min with microsomes. Reaction was initiated by the addition of 0.5 mM NADPH. Data points represent sum of 13'OH and 13'COOH metabolites (μmoles per μmoles membrane cholesterol). Curves were fit using Origin 7 FFT curve smoothing function.

loading the microsomes with α-TOH or γ-T3 to equal extents. The metabolism by rat liver microsomes of model substrates to their known metabolites was used as a measure of activity of the microsomal enzymes which carry out these reactions as shown in **Table 4.1**. The microsomes were pre-loaded with the vitamers at concentrations determined to yield equivalent membrane vitamer concentrations during the reaction. The results shown in **Figure 4.2A** indicate α-TOH, at 0.6 μmol/mg protein in the membrane (150 μM in reaction) did not affect the metabolism of any enzyme tested. γ-T3 at this same membrane concentration (60 μM in reaction medium) strongly inhibited the metabolism of testosterone (TST) to its CYP3A metabolite, 6β-OH-TST, and moderately inhibited its metabolism to the CYP2C metabolite, 16α-OH-TST. Similar effects were seen in the metabolism of lauric acid to the 11-OH and 12-OH metabolites, respectively, suggesting the effect of γ-T3 was not by modifying substrate access to the enzyme. Glucose-6-phosphatase activity was not inhibited by γ-T3, indicating the effect may be specific to P450 enzymes. **Figure 4.2B** illustrates that the effect of γ-T3 concentration on enzyme activity is more pronounced for some microsomal P450 enzymes (e.g. CYP3A) than others (e.g. CYP2C).

A P450 inhibitor may often alter the activity of P450 enzymes by competitive inhibition. The kinetics of testosterone metabolism in the absence or presence of γ-T3 was used to determine the mechanism of inhibition of the tocotrienol. The kinetic curves in **Figure 4.3** for 6β-OH-TST and 16α-OH-TST synthesis were fit to the Michaelis-Menten equation and demonstrated a decrease in Vmax in the presence of 60 μM γ-T3 for both CYP3A and CYP2C-mediated testosterone metabolism. There was no significant effect on Km, indicating γ-T3 acted as a non-competitive inhibitor of these enzymes.

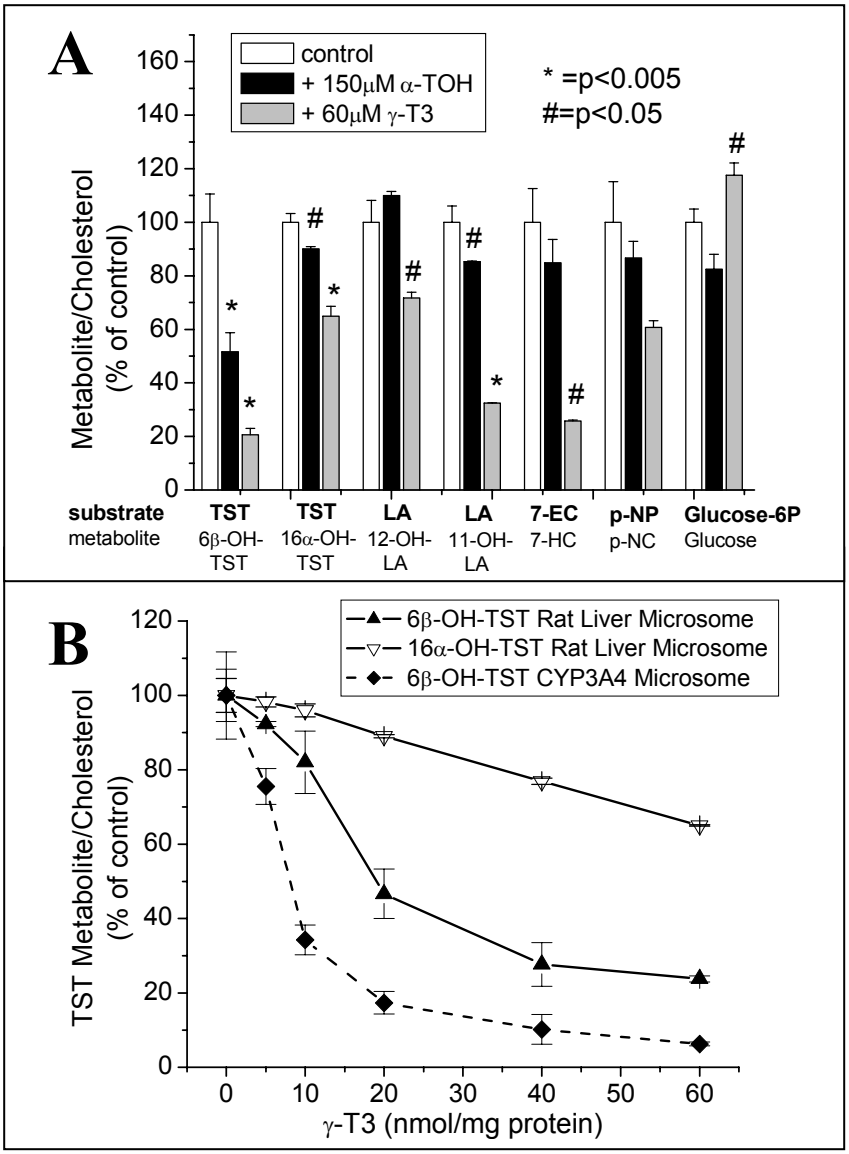



**Figure 4.2**

Effects of α-TOH and γ-T3 on microsomal membrane-associated enzymes. **(A)** Reactions were carried with in 0.1 mg/ml rat liver microsomal membranes loaded 60 min with α-TOH or γ-T3 in the presence of 0.5 mM NADPH as described in Materials and Methods. 200 μM testosterone (TST), 200 μM lauric acid (LA), 100 μM 7-ethoxycoumarin (7-EC), and 500 μM p-nitrophenol were added to individual samples at time 60 min along with 0.5 mM NADPH and incubated 20-30 min. Glucose-6-phosphatase assay was carried out as described in Materials and Methods. **(B)** Reactions were carried out for testosterone as in (A) but with increasing concentrations of γ-T3 during the pre-incubation on either 0.1 mg/ml rat liver microsome or 4 pmol CYP3A4 microsome. Results are presented as percentage of control where control = 100%. Data points represent mean +/- std dev. Statistical differences from controls: * = $p<0.005$, # = $p<0.05$

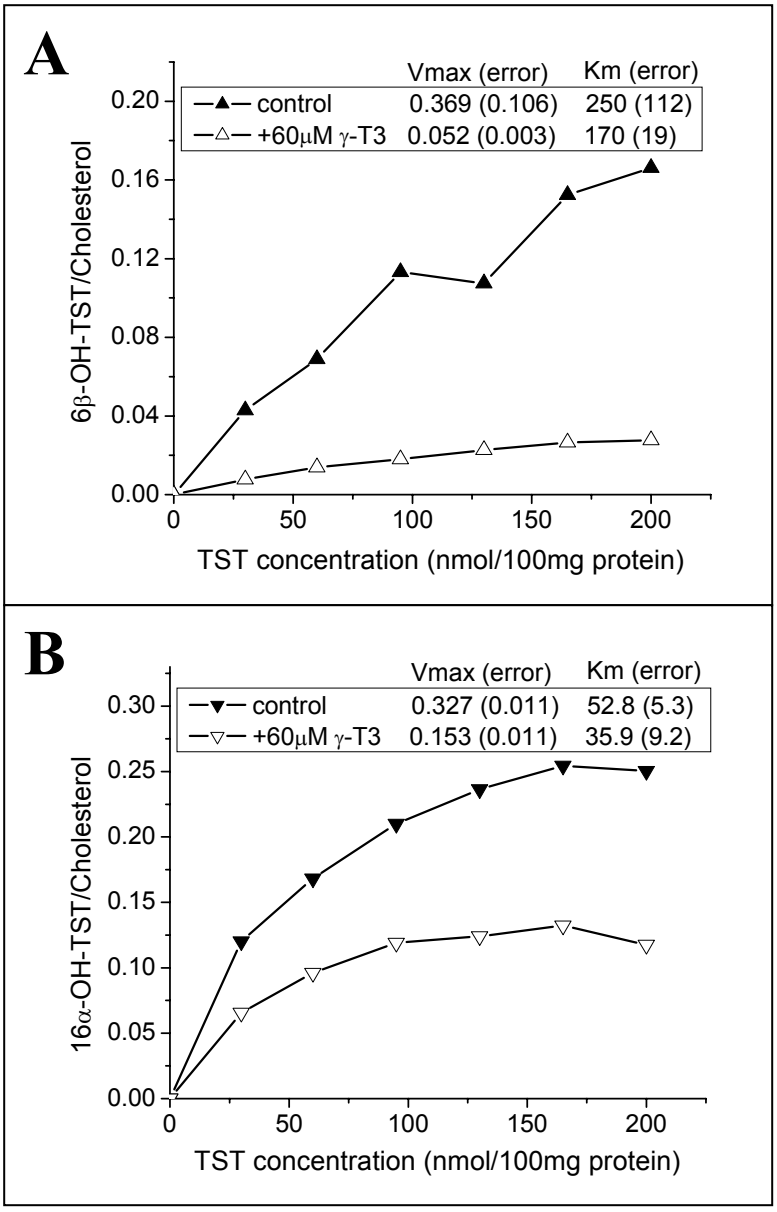



**Figure 4.3**

Kinetics of testosterone metabolism by rat liver microsomes in the presence or absence of γ-T3 (60 nmol/100 μg protein (60μM)). 100 μg/ml microsome was pre-incubated 60 min in the presence or absence of γ-T3 and 0.5 mM NADPH. A 20 min reaction was initiated by the addition of testosterone and 0.5 mM NADPH. A) CYP3A mediated 6β-OH-TST production. B) CYP2C-mediated 16α-OH-TST production. Product formation is expressed relative to microsomal cholesterol content. Vmax and Km values were derived by a hyperbolic fit to the Michaelis-Menten equation using Origin 7 software.

There is a significant body of evidence suggesting the membrane lipid environment may affect the interaction and activity of membrane-bound multi-enzyme systems such as the cytochrome P450 reductase/cytochrome b5/cytochrome P450 electron transfer complex (19-21). Increases in membrane unsaturated fatty acids increases the molecular mobility of the membrane, enhancing electron transfer between membrane redox proteins such as cytochrome b5 reductase and cytochrome b5 (21,33). On the other hand, decreases in membrane fluidity (i.e. increased ordering), which occurs when membrane cholesterol content is increased, has the opposite effect, i.e. inhibition of cytochrome b5 reduction and NADH-cytochrome c reductase (19,20). We assessed rotational and lateral mobility of rat liver microsomal membranes in the presence of α-TOH or γ-T3 by diphenyl-hexatriene (DPH) fluorescence anisotropy and pyrene eximerization, respectively. The results (**Table 4.2)** revealed that both α-TOH and γ-T3 decreased the lateral mobility of the microsomal membrane (decreased pyrene eximer formation) with α-TOH displaying the greater ordering effect. However, γ-T3 also decreased the rotational mobility in the membrane (increased anisotropy) while α-TOH showed no significant effect on this parameter relative to vehicle control. γ-T3 thus appears to act in a manner similar to that of cholesterol, which decreases both the lateral and rotational mobility of membranes (19). The effect of membrane cholesterol concentration on cytochrome P450 activity was examined using testosterone metabolism as a marker for activity (**Figure 4.4A**). A four fold increase in cholesterol content dramatically reduced CYP3A activity (6β-OH-TST formation) and to a lesser extent, CYP2C activity (16α-OH-TST formation), similar to the effect produced by γ-T3. A five-fold decrease in membrane cholesterol concentration resulted in a more moderate effect on testosterone metabolism, and this treatment had similar effects on the two enzyme activities.

**Table 4.2**
Effects of α-TOH and γ-T3 on rotational and lateral mobility of rat liver microsomal membranes as determined by 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy and pyrene eximerization, respectively, as described in Materials and Methods. α-TOH and γ-T3 were incubated with rat liver microsome plus 1 mM NADPH at a concentration of 1.5 and 0.6 nmol/mg protein, respectively. * indicates significantly different from control ($p<0.05$).

| | **Fluorescence Anisotropy ($r_s$)** | **Pyrene Eximerization ($I_e/I_m$)** |
|---|---|---|
| **Control** | 0.298 (0.013) | 0.242 (0.029) |
| **α-TOH** | 0.322 (0.024) | 0.061 (0.016)* |
| **γ-T3** | 0.348 (0.024)* | 0.118 (0.023)* |

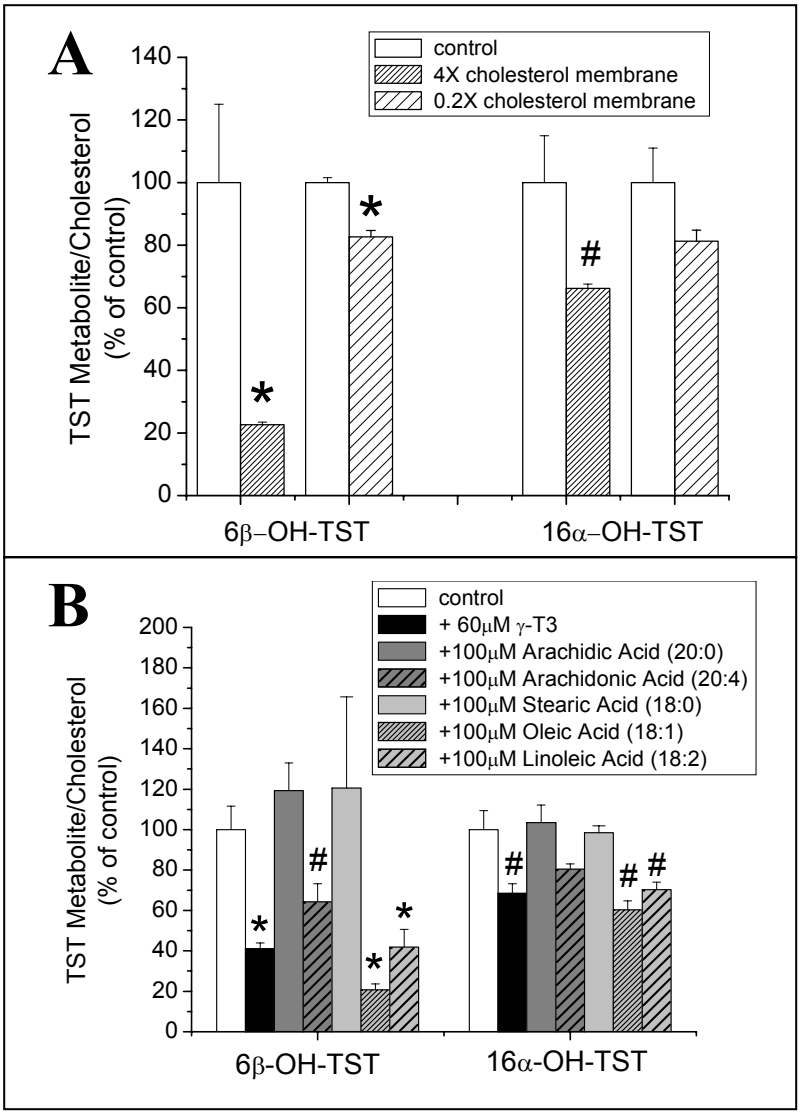



**Figure 4.4**

**(A)** Inhibition of testosterone metabolism by enrichment or removal of cholesterol from rat liver microsomes. Cholesterol was loaded or removed from rat liver microsome as described in Materials and Methods. 100 μg/ml microsome was pre-incubated 60 min in the presence or absence of γ-T3 and 0.5 mM NADPH. A 20 min reaction was initiated in the resuspended microsomes by the addition of 200 μM testosterone and 0.5 mM NADPH. **(B)** Inhibition of testosterone (TST) metabolism by unsaturated fatty acids in rat liver microsomes. 100 μg/ml microsome was pre-incubated 60 min in the presence or absence of γ-T3 or fatty acid and 0.5 mM NADPH. Lipid not incorporated into the membrane was removed from supernatant by ultracentrifugation. A 30 min reaction was initiated in the re-isolated microsomes by the addition of 200 μM testosterone and 0.5 mM NADPH. Results are presented as percentage of control where control equals 100% enzyme activity. Statistical differences from controls: * = $p<0.05$, # = $p<0.1$

The auto-inhibitory effect of γ-T3 on its own metabolism has also been observed in the ω-hydroxylation of fatty acids such as arachidonic acid, which is also catalyzed by the CYP4 family of P450 enzymes (34,35). The results in **Figure 4.4B** illustrate an inhibitory effect of the unsaturated fatty acids arachidonic acid, oleic acid, and linoleic acid on testosterone metabolism, while their saturated counterparts had no effect. This result correlates well with the observed P450 inhibition by the unsaturated tocotrienols but lack of effect of their saturated counterparts, the tocopherols. Additionally, the comparative degree to which CYP3A-mediated metabolism was inhibited compared with that of CYP2C was similar between γ-T3 and the unsaturated fatty acids, i.e. their effects being greater on CYP3A activity.

The activity of the individual enzyme components of the rat liver microsomal P450 electron transfer complex was examined in the presence or absence of γ-T3. There was no effect of γ-T3 on the reduction of cytochrome C by cytochrome P450 reductase (**Figure 4.5A**). Testosterone metabolism was also evaluated, with or without the addition of 5-10 μg/ml purified human cytochrome P450 reductase. The addition of exogenous reductase did not alter the inhibitory effects of γ-T3 on P450 activity (data not shown). The effects of γ-T3 on cytochrome b5 was analyzed using microsomes from insect cells expressing recombinant cytochrome P450 reductase, cytochrome P450 enzymes, and with or without recombinant cytochrome b5 (**Figure 4.5B**). In the case of both CYP3A4 and CYP2B6, the presence of cytochrome b5 increased the activity of the P450 enzyme. γ-T3 showed more of an inhibitory effect on substrate metabolism in the presence of b5 than in its absence, further suggesting the tocotrienols may be inhibiting membrane P450 activity by disrupting the electron flux between the electron transfer protein partners.

The effects of α-TOH and γ-T3 on the reduction of the microsomal cytochrome b5 and cytochrome P450 was assessed using spectrophotometric analysis

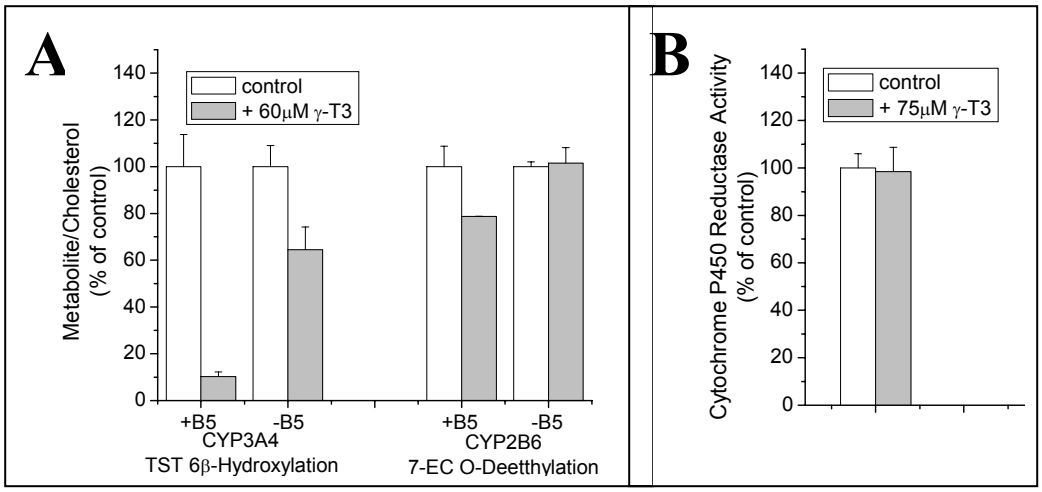



**Figure 4.5**

Effect of γ-T3 on microsomal cytochrome B5 and cytochrome P450 reductase activity. **(A)** 10 pmol insect microsomes expressing recombinant human CYP3A4 or CYP2B6, P450 reductase, and with or without cytochrome b5 were tested for inhibition of activity by γ-T3 using 200 μM testosterone (TST) or 7-ethoxycoumarin (7-EC) as substrates. Reactions were carried out as described in Materials and Methods, with a 60 min pre-inc containing microsome, with or without γ-T3, and 0.5 mM NADPH, and a 20 min incubation initiated by the addition of substrate plus 0.5 mM NADPH. **(B)** 0.1 mg/ml rat liver microsome was pre-incubated for 60 min with or without γ-T3 plus 0.5 mM NADPH. The resulting P450 reductase activity was measured as described in Materials and Methods. Data represent means +/- std dev.

of the two heme proteins. Reduced cytochrome b5 exhibits an absorbance peak at 426 nm only when b5 is in its reduced state, and is not affected by the presence of CO (32). Microsomal cytochrome P450 will not bind CO in its oxidized form, however upon reduction by the electron transport partners, P450 will bind CO, producing an absorbance peak at 450 nm. Rat liver microsomes loaded for an hour with 1.5 μmol α-TOH or 0.6 μmol γ-T3/mg protein, in the presence or absence of NADPH during the loading, were assessed for their level of reduced b5 and P450 compared with controls. The results in **Figure 4.6** illustrate the presence of the vitamers in the membrane did not affect the reduction of either cytochrome b5 or P450. Additionally, the presence of NADPH during the pre-incubation, which amplified the inhibitory effects of the tocotrienols, did not affect the reduction of either protein.

**Discussion**

Tocotrienols have attracted increasing attention in recent years due to their potential anti-atherogenic and anti-cancer effects. These unsaturated vitamers of vitamin E exhibit activities that their saturated tocopherol counterparts do not. However, plasma concentrations of the tocotrienols are usually far below that of α-TOH, due largely to their rapid catabolism and elimination via the tocopherol-ω-oxidation pathway. Here we report an inhibitory activity of the tocotrienols on certain microsomal enzymes that is not exhibited by the tocopherols when compared at similar membrane concentrations. Certain enzymes were more sensitive to the presence of tocotrienols than others. The inhibition was non-competitive, eliminating the possibility that the tocotrienols simply compete for binding sites on the P450 enzymes. The inhibition was greater when the microsomal reaction system was pre-incubated in the presence of NADPH, suggesting a mechanism involving a tocotrienol

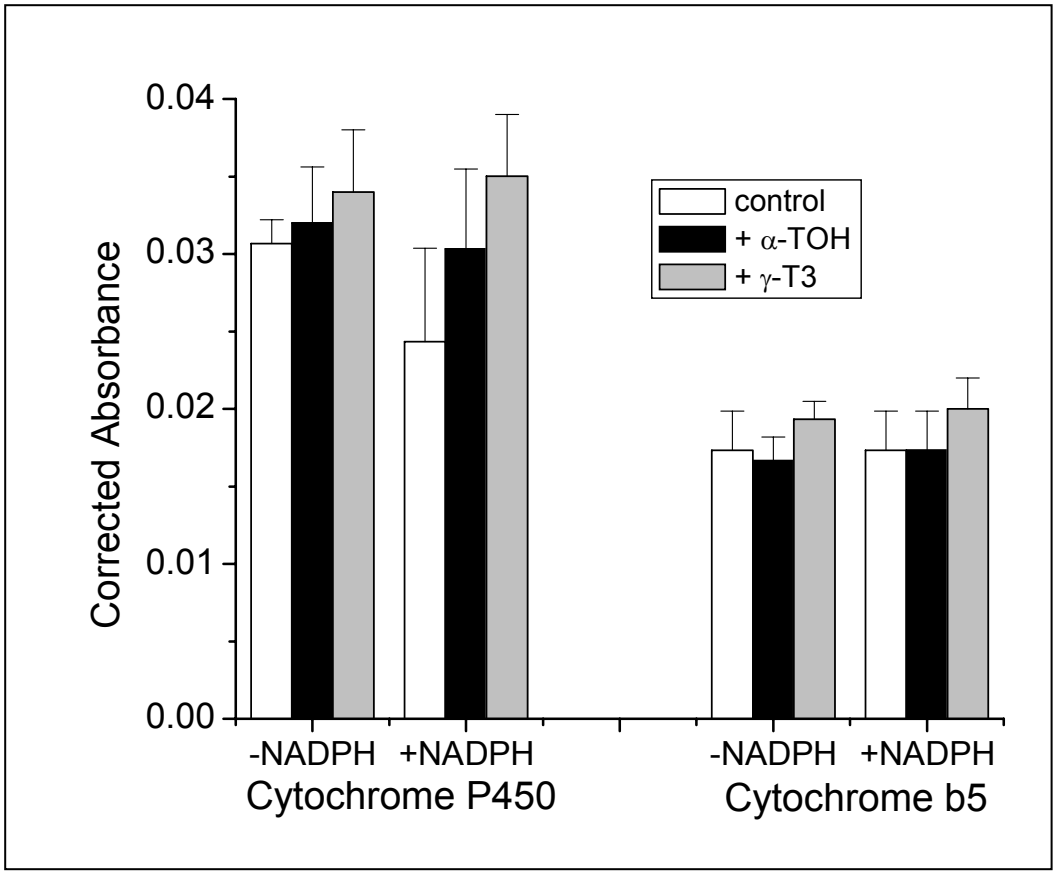



**Figure 4.6**

α-TOH and γ-T3 do not effect the reduction of cytochrome P450 or cytochrome b5 in rat liver microsomal membranes. Microsomes were loaded with 1.5 μmol α-TOH/mg protein or 0.6 μmol γ-T3/mg protein in the presence or absence of NADPH as described in Materials and Methods. A 5 min reaction was then carried out with loaded microsomes in the presence of 0.5 mM NADPH. Reduction of cytochromes P450 and b5 were measured by spectrophotometer after bubbling samples with CO for 1 min. All absorbances were read against blanks lacking NADPH. No significant differences were found between the controls and experimental samples.

metabolite. However, tocotrienol inhibition was also seen in microsomes expressing only P450 enzymes which are not involved in vitamin E metabolism.

We investigated a mechanism involving disruption by the tocotrienols of the transfer of electrons from NADPH to the cytochrome P450 resulting from alteration of membrane physical properties. Several studies have reported effects of membrane lipid composition on the electron transport chain (19-21). Lipids which decrease the fluidity of the membrane are postulated to affect the diffusion rate of the individual protein components of the electron transfer system, thereby limiting the interaction between these components (36-38). Such is the case for cholesterol, which decreases both lateral and rotational fluidity of the membrane (19). On the other hand unsaturated fatty acids, which increase the fluidity of the microsomal membrane, also inhibit the activity of P450s, an effect which has been proposed to be due to the detergent effects of the fatty acids on the electron transfer components within the membrane (34). Here we demonstrated that both cholesterol and unsaturated fatty acids inhibit P450 activity while the analogous saturated fatty acids had no effect. Additionally, these lipids inhibited CYP3A more strongly than CYP2C, a pattern identical to that of tocotrienol-mediated inhibition.

Both tocopherols and tocotrienols behave like cholesterol in that they decreased the lateral diffusivity of the microsomal membrane, in agreement with the electron spin resonance results of Yoshida et al (8). However, γ-T3, but not α-TOH, decreased the rotational mobility of the membrane, as does cholesterol. The unsaturation of the phytyl tail of the tocotrienols is clearly important to its inhibitory activity. Fatty acid inhibition of P450 activity is analogous, in that only the unsaturated fatty acids are inhibitory. The conformation that the unsaturated carbon tail assumes in the lipid bilayer may be the driving force of these inhibitory effects.

Structure-activity differences of tocopherols and tocotrienols on membrane enzyme activity may also arise due to the depth at which the vitamer resides in the membrane bilayer. Suzuki et al suggested that α-T3 sits closer to the membrane surface than α-TOH, based on small differences in ordering of the liquid crystalline state at carbons 10-13 of phospholipid fatty acid chains (6). On the other hand, Yoshida et al reported similar locations in the membrane based on similar electron spin resonance parameters, using similar spin labels (8). Grau and Ortiz found evidence in lipid vesicles that the α-, γ-, β-, and δ-TOHs are found at various depths within the membrane (39). These differences may potentially affect the function of membrane proteins according to their depth in the lipid bilayer.

The precise nature of the interactions between the electron transfer partners in the microsomal cytochrome P450 complex are not yet fully understood. In the simplest model, cytochrome P450 reductase accepts two electrons from NADPH, and transfers these electrons one at a time to cytochrome P450, without the need for cytochrome b5 (40,41). The cytochrome P450 then uses these reducing equivalents for the oxidation reactions specific to the individual P450. However, the transfer of the second electron is rate limiting (42). Several mechanisms have been proposed in which cytochrome b5 facilitates the input of the second electron into the P450 enzyme, thereby increasing the rate of the P450 reaction. These include (a) directly increasing the rate of input of the second electron, (b) enhancement of coupling of NADPH oxidation to substrate oxidation, (c) by forming a two electron acceptor complex with the P450, or (d) as an effector of the P450 enzyme, as reviewed by Schenkman and Jansson (43). In all cases there is postulated a direct interaction between the P450 reductase, the P450 enzyme, and the cytochrome b5 protein. There are several reports on the random distribution of the electron transfer components throughout the membrane (38,44,45). The rates of electron transfer and substrate

oxidation are therefore dependent on how these components move through and associate within the membrane. We demonstrated that γ-T3 in the membrane did not affect reduction of cytochrome C by P450 reductase. Since cytochrome C was added exogenously, the fluidity of the membrane would not affect its access to the membrane-bound reductase. Addition of exogenous P450 reductase has no protective effect against γ-T3 mediated P450 inhibition. Since the additional reductase is not associated with the membrane, it may not be able to complex with the P450 enzyme in such a way as to rescue it from the effects of γ-T3.

Cytochrome b5 does not play an obligate role in all CYP450 reactions (43). However, for many P450 reactions such as 3A4 mediated testosterone hydroxylation and CYP2E1 mediated 7-ethoxycoumarin de-ethylation, enhancement of activity by b5 is so great that it is considered an obligate partner (46-48). Disruption of interaction between b5 and the P450 enzymes would then be expected to have varied effects on activity measurements, dependent on both the type of P450 and the substrate in question (49). Such a P450 dependent effect was observed in the inhibition of P450 activity by γ-T3. A greater dependence of CYP3A4 on cytochrome b5 compared to other P450 enzymes may explain the greater effect of the presence of b5 on CYP3A4 inhibition compared with CYP2B6 (**Figure 4.5B**). No effect was observed for membrane tocotrienols on reduction of the cytochrome P450 or b5 in rat liver microsomes. This may be largely due to the presence of many different endogenous P450s in the rat microsomal membrane, many of which are not sensitive to the interaction with cytochrome b5. However, the possibility still exists that the tocotrienols are interacting directly with the P450 enzyme to inhibit its activity, in a site that is distinct from the catalytic site, resulting in the observed non-competitive inhibition.

The inhibitory effects described here of the tocotrienols on microsomal enzymes suggests a physiological role of the tocopherol-ω-hydroxylase pathway in the minimization of membrane tocotrienols concentration. The CYP4F2 enzyme responsible for this activity shows a large preference for the tocotrienols over their tocopherol counterparts (Sontag and Parker, unpublished results). This may be a mechanism by which cell membranes are protected from the adverse effects of membrane tocotrienols. Cytochrome P450 enzymes are involved in the elimination of a variety of toxic xenobiotics, as well as in the metabolism of numerous endogenous compounds (50). Alteration of the activity of these enzymes can dramatically alter physiological functions. The tocotrienols have been reported to cause growth inhibition and apoptotic cell death in a variety of cell types (16,51-54), but the mechanism has not been elucidated. The membrane effects of tocotrienols reported here may be relevant to these phenomena. The tocotrienols may mimic several of the membrane effects of free cholesterol, as shown here for membrane fluidity. Work by Feng et al showed that the endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages (55). Free cholesterol disrupts the membrane to a degree that generates a chain of responses, ultimately leading to apoptosis. Cells therefore tightly regulate the free cholesterol content of the ER relative to other membranes (56). Membrane effects of tocotrienols may generate a similar response, ultimately leading to the apoptotic cell death reported for these vitamers.

In summary, we describe an inhibitory effect of tocotrienols, but not tocopherols, on the activity of several cytochrome P450 enzymes found in the microsomal membrane. The inhibition is not generalized, but rather occurs toward the cytochrome P450 enzymes and takes place by a non-competitive mechanism. Both the α-TOH and γ-T3 decrease the lateral fluidity of the membrane, while only γ-T3 decreases the rotational fluidity in a manner similar to that of free cholesterol. Other

evidence presented here implicates a change in membrane environment induced by tocotrienols that in turn alter the interactions of the electron transfer partners involved in P450 activity. All together, the data presented here provide a further indication of the importance of the tocopherol-ω-hydroxylase mediated mechanisms that mammals have developed to retain α-TOH, while eliminating others, especially the tocotrienols, that may cause detrimental effects. While the mechanisms behind the observed vitamer-specific effects on membrane function have not been fully elucidated, they clearly merit further investigation.

**References**


1. Lee, B. L., New, A. L., and Ong, C. N. (2003) *Clin Chem*
2. Chow, C. K. (1975) *Am J Clin Nutr* **28**, 756-760
3. O'Byrne, D., Grundy, S., Packer, L., Devaraj, S., Baldenius, K., Hoppe, P. P., Kraemer, K., Jialal, I., and Traber, M. G. (2000) *Free Radic Biol Med* **29**, 834-845
4. NRC. (1989) *Recommended Dietary Allowances*, 10 Ed., National Academy Press, Washington, DC
5. Packer, L., Weber, S. U., and Rimbach, G. (2001) *J Nutr* **131**, 369S-373S
6. Suzuki, Y. J., Tsuchiya, M., Wassall, S. R., Choo, Y. M., Govil, G., Kagan, V. E., and Packer, L. (1993) *Biochemistry* **32**, 10692-10699
7. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) *Free Radic Biol Med* **10**, 263-275
8. Yoshida, Y., Niki, E., and Noguchi, N. (2003) *Chem Phys Lipids* **123**, 63-75
9. Qureshi, A. A., Burger, W. C., Peterson, D. M., and Elson, C. E. (1986) *J Biol Chem* **261**, 10544-10550
10. Parker, R. A., Pearce, B. C., Clark, R. W., Gordon, D. A., and Wright, J. J. (1993) *J Biol Chem* **268**, 11230-11238
11. Sen, C. K., Khanna, S., Roy, S., and Packer, L. (2000) *J Biol Chem* **275**, 13049-13055
12. Khanna, S., Roy, S., Ryu, H., Bahadduri, P., Swaan, P. W., Ratan, R. R., and Sen, C. K. (2003) *J Biol Chem* **278**, 43508-43515
13. Theriault, A., Chao, J. T., and Gapor, A. (2002) *Atherosclerosis* **160**, 21-30
14. Theriault, A., Chao, J. T., Wang, Q., Gapor, A., and Adeli, K. (1999) *Clin Biochem* **32**, 309-319

15. Nesaretnam, K., Guthrie, N., Chambers, A. F., and Carroll, K. K. (1995) *Lipids* **30**, 1139-1143

16. Yu, W., Simmons-Menchaca, M., Gapor, A., Sanders, B. G., and Kline, K. (1999) *Nutr Cancer* **33**, 26-32

17. Saito, Y., Yoshida, Y., Akazawa, T., Takahashi, K., and Niki, E. (2003) *J Biol Chem* **278**, 39428-39434

18. Traber, M. G., Rallis, M., Podda, M., Weber, C., Maibach, H. I., and Packer, L. (1998) *Lipids* **33**, 87-91

19. Garda, H. A., and Brenner, R. R. (1985) *Biochim Biophys Acta* **819**, 45-54

20. Archakov, A. I., Borodin, E. A., Dobretsov, G. E., Karasevich, E. I., and Karyakin, A. V. (1983) *Eur J Biochem* **134**, 89-95

21. Garda, H. A., and Brenner, R. R. (1984) *Biochim Biophys Acta* **769**, 160-170

22. Sontag, T. J., and Parker, R. S. (2002) *J Biol Chem* **277**, 25290-25296

23. Alegre, M., Ciudad, C. J., Fillat, C., and Guinovart, J. J. (1988) *Anal Biochem* **173**, 185-189

24. Waxman, D. J., Ko, A., and Walsh, C. (1983) *J Biol Chem* **258**, 11937-11947

25. Waxman, D. J. (1984) *J Biol Chem* **259**, 15481-15490

26. Yamazaki, H., Inoue, K., Mimura, M., Oda, Y., Guengerich, F. P., and Shimada, T. (1996) *Biochem Pharmacol* **51**, 313-319

27. Spatzenegger, M., Liu, H., Wang, Q., Debarber, A., Koop, D. R., and Halpert, J. R. (2003) *J Pharmacol Exp Ther* **304**, 477-487

28. Powell, P. K., Wolf, I., and Lasker, J. M. (1996) *Arch Biochem Biophys* **335**, 219-226

29. Hoch, U., Falck, J. R., and de Montellano, P. R. (2000) *J Biol Chem* **275**, 26952-26958

30. Peng, H. M., Porter, T. D., Ding, X. X., and Coon, M. J. (1991) *Mol Pharmacol* **40**, 58-62

31. Horecker, B. L. (1950) *J Biol Chem* **183**, 593-605

32. Omura, T., and Sato, R. (1964) *J Biol Chem* **239**, 2379-2385

33. Brenner, R. R. (1984) *Prog Lipid Res* **23**, 69-96

34. Hoch, U., Zhang, Z., Kroetz, D. L., and Ortiz de Montellano, P. R. (2000) *Arch Biochem Biophys* **373**, 63-71

35. Xu, F., Falck, J. R., Ortiz de Montellano, P. R., and Kroetz, D. L. (2004) *J Pharmacol Exp Ther* **308**, 887-895

36. Strittmatter, P., and Rogers, M. J. (1975) *Proc Natl Acad Sci U S A* **72**, 2658-2661

37. Rogers, M. J., and Strittmatter, P. (1974) *J Biol Chem* **249**, 5565-5569

38. Rogers, M. J., and Strittmatter, P. (1974) *J Biol Chem* **249**, 895-900

39. Grau, A., and Ortiz, A. (1998) *Chem Phys Lipids* **91**, 109-118

40. Peterson, J. A., White, R. E., Yasukochi, Y., Coomes, M. L., O'Keeffe, D. H., Ebel, R. E., Masters, B. S., Ballou, D. P., and Coon, M. J. (1977) *J Biol Chem* **252**, 4431-4434

41. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S., and Kim, J. J. (1997) *Proc Natl Acad Sci U S A* **94**, 8411-8416

42. Correia, M. A., and Mannering, G. J. (1973) *Mol Pharmacol* **9**, 455-469

43. Schenkman, J. B., and Jansson, I. (2003) *Pharmacol Ther* **97**, 139-152

44. Yang, C. S. (1975) *FEBS Lett* **54**, 61-64

45. Yang, C. S. (1977) *Life Sci* **21**, 1047-1057

46. Guengerich, F. P., Martin, M. V., Beaune, P. H., Kremers, P., Wolff, T., and Waxman, D. J. (1986) *J Biol Chem* **261**, 5051-5060

47. Yamazaki, H., Nakano, M., Gillam, E. M., Bell, L. C., Guengerich, F. P., and Shimada, T. (1996) *Biochem Pharmacol* **52**, 301-309

48. Yamazaki, H., Nakano, M., Imai, Y., Ueng, Y. F., Guengerich, F. P., and Shimada, T. (1996) *Arch Biochem Biophys* **325**, 174-182

49. Kuwahara, S., and Omura, T. (1980) *Biochem Biophys Res Commun* **96**, 1562-1568

50. Rendic, S., and Di Carlo, F. J. (1997) *Drug Metab Rev* **29**, 413-580

51. McIntyre, B. S., Briski, K. P., Tirmenstein, M. A., Fariss, M. W., Gapor, A., and Sylvester, P. W. (2000) *Lipids* **35**, 171-180

52. Mo, H., and Elson, C. E. (1999) *J Nutr* **129**, 804-813

53. Sakai, M., Okabe, M., Yamasaki, M., Tachibana, H., and Yamada, K. (2004) *Anticancer Res* **24**, 1683-1688

54. Galli, F., Stabile, A. M., Betti, M., Conte, C., Pistilli, A., Rende, M., Floridi, A., and Azzi, A. (2004) *Arch Biochem Biophys* **423**, 97-102

55. Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) *Nat Cell Biol* **5**, 781-792

56. Bretscher, M. S., and Munro, S. (1993) *Science* **261**, 1280-1281

# Chapter V
# Overall Conclusions

The main objective of this dissertation was to characterize the metabolic pathway by which the structural vitamers of vitamin E are metabolized to water soluble compounds for elimination in the urine. The presence of these metabolites in the urine has been known for half a century, although the mechanism(s) behind this transformation were unknown. Based on structural differences between vitamin and CEHC metabolite, it was speculated that these changes came about by means of an ω-hydroxylation of the terminal carbon of the phytyl tail of the vitamin, followed by sequential β-oxidation similar to that observed for fatty acids.

The experiments described here confirm the proposed ω-hydroxylation/β-oxidation pathway of vitamin E metabolism. All forms of vitamin E are metabolized through this pathway. Studies using individually expressed microsomal cytochrome P450 enzymes reveal the initial enzyme in this pathway is the membrane-bound tocopherol-ω-hydroxylase CYP4F2. This enzyme is highly substrate specific, demonstrating greater activity for certain forms of vitamin E based on minor variations in vitamer structure. This structural selectivity plays a major role in the high plasma levels of α-TOH and relatively low levels of the other vitamers. The α-tocopherol binding protein (α-TTP) appears to function together with tocopherol-ω-hydroxylase to promote the retention of α-TOH and the elimination of the other vitamer forms. Inhibition of this pathway changes plasma vitamin E status by decreasing the catabolism of the non-α- vitamers, allowing their plasma and tissue concentrations to increase. The work reported here has been followed up in vivo by Ikeda et al, in which the in vivo inhibition of this enzyme with sesame lignans inhibits the urinary excretion of the CEHC vitamin E metabolites (1).

The kinetics of metabolism of the vitamers is similar between human liver microsomes containing all hepatic P450 enzymes and microsomes containing only CYP4F2. Also, other CYP4F isoforms which have a high degree of sequence homology show little or no catabolic activity toward any of the vitamers. Taken together these results suggest CYP4F2 is the major human liver ω-hydroxylase of vitamin E.

CYP4F2 is also found in other tissues including the lungs, kidneys, and testes. The metabolism of vitamin E in these specific tissues may indicate the importance of maintaining low levels of non-α vitamers in certain cell types above others. On the other hand, given the natriuretic and anti-inflammatory nature of the most abundant metabolite, γ-CEHC, these tissues may express CYP4F2 as a means of local generation of an important physiological factor.

Kinetic analyses also revealed the allosteric nature of the tocopherol-ω-hydroxylase with its substrates. This cooperativity allows for low levels of metabolism when vitamin concentration around the enzyme is low, but a high rate of metabolism with increasing concentrations of the non-α forms of the vitamin. Additionally this cooperativity provides a mechanism by which α-TOH acts as a positive effector of metabolism of the other vitamers, increasing their rates of catabolism. This explains the phenomenon observed during supplementation with α-TOH, where α-TOH causes a decrease in the plasma levels of the other tocopherols, and an increase in their urinary excretion. By this mechanism, the other vitamers are available for physiological function when α-TOH concentration is limiting, but may be eliminated when α-TOH levels are such that the other forms are not necessary.

The rate of catabolism of the tocotrienols is greater than that of the tocopherols. The tocotrienols have been previously shown to be cytotoxic at concentrations at which the tocopherols show no deleterious effects. Here the

tocotrienols are shown to have effects on mobility of membrane constituents and on the activity of the P450 enzymes found in the membrane of the endoplasmic reticulum, acting to decrease membrane fluidity, and inhibit the activity of P450 enzymes. These effects are not observed for the tocopherols. The evidence presented here points toward an effect of the tocotrienols on the interactions of the electron transfer partners of the P450 enzyme complex, especially cytochrome b5. Some P450 enzymes are more sensitive to these effects than others, which is consistent with P450 enzymes varying in the degree to which they are dependent on interactions with cytochrome b5. The effects of the tocotrienols on the ER membrane may provide an explanation for their cytotoxicity. This additionally provides a motivation for the tocopherol-ω-hydroxylase enzyme to have developed a high affinity for molecules with the structural features of the tocotrienols, in order to minimize the membrane concentrations of these vitamers.

The full significance of the tocopherol-ω-hydroxylase pathway of vitamin E metabolism is still largely unknown. The generation of tocopherol-ω-hydroxylase knockout mice is a logical next step in understanding the relevance of this enzyme to physiological functions. These mice should allow for the achievement of plasma and tissue levels of the non-α vitamers that rival those of α-TOH, if this enzyme is truly the major source of selective elimination of these vitamers. Additionally, the interplay of tocopherol-ω-hydroxylase and the α-tocopherol transfer protein (α-TTP) will be better understood through various crosses of these mice with the previously generated TTP-null mouse. The potential anti-atherogenic and anti-carcinomic effects of the tocopherols and tocotrienols may be accentuated by an increase of the plasma levels of these vitamers, making inhibition of tocopherol-ω-hydroxylase a possible target of therapeutic approaches. This will of course require an understanding of the balance of positive outcomes of increased vitamer plasma and tissue concentrations with the toxic

effects which may result from increased concentrations of the tocotrienols in the membrane. Commonly used drugs such as the anti-fungal ketoconazole affect Vitamin E status through the inhibition of tocopherol-ω-hydroxylase (1). Likewise, hepatic diseases such as hepatitis may compromise the activity of the tocopherol-ω-hydroxylase enzyme, factors which must be considered in the use of drugs which affect cytochrome P450 activity, and the effects of disease states on vitamin E status.

Finally, while the ω-hydroxylation enzyme involved in the metabolism of vitamin E has been characterized, the remaining enzymes responsible for β-oxidation of the hydroxylated metabolite have not been determined. Unpublished results from our lab indicate a variety of cell types which do not metabolize vitamin E are able to carry out the subsequent phytyl tail truncation when presented with intermediates from this pathway. The enzymes involved in this β-oxidation may thus be found in a variety of tissues and may be involved in metabolizing any circulating intermediates generated by CYP4F2-expressing tissues.

Intracellularly, the tocopherol-ω-hydroxylase enzyme is resident in the endoplasmic reticulum. The β-oxidation enzymes may reside in other organelles, as is the case for the metabolism of $LTB_4$, whose metabolism originates in the ER but whose subsequent metabolism is carried out in the peroxisomes (2). If this is the case, the lipophilic intermediary metabolites require a means of reaching these other organelles, be it by non-specific transfer between membranes, or by means of specific transfer proteins yet to be discovered.

Overall, the discovery of the enzyme responsible for the selective elimination of vitamin E opens many new doors for understanding the reasons the α-TOH form of this vitamin is so favored in animals throughout nature. This will hopefully lead to new insights in the role of this vitamin in physiological function and disease prevention.

## References

1. Ikeda, S., Tohyama, T., and Yamashita, K. (2002) *J Nutr* **132**, 961-966

2. Ferdinandusse, S., Meissner, T., Wanders, R. J., and Mayatepek, E. (2002) *Biochem Biophys Res Commun* **293**, 269-273