

**Cytochrome P450  $\omega$ -hydroxylase pathway of tocopherol catabolism:  
Novel mechanism of regulation of vitamin E status**

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## **Summary**

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  $\omega$ -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  $\gamma$ -tocopherol metabolism via this pathway were identified in hepatocyte cultures using gas chromatography-mass spectrometry. NADPH-dependent synthesis of the initial  $\gamma$ - and  $\alpha$ -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- $\omega$ -hydroxylase activity was associated only with CYP4F2, which also catalyzes  $\omega$ -hydroxylation of leukotriene B<sub>4</sub> and arachidonic acid. Tocopherol- $\omega$ -hydroxylase exhibited similar binding affinities but markedly higher catalytic activities for  $\gamma$ -tocopherol than  $\alpha$ -tocopherol, suggesting a role for this pathway in the preferential physiological retention of  $\alpha$ -tocopherol and elimination of  $\gamma$ -tocopherol. Sesamin potently inhibited tocopherol- $\omega$ -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 peroxy 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  $\alpha$ -tocopherol ( $\alpha$ -TOH)<sup>1</sup> recent studies indicate several of these important roles may be specific to  $\gamma$ -tocopherol ( $\gamma$ -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  $\alpha$ -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- $\alpha$ -TOH even when other tocopherols are consumed in greater proportions.  $\gamma$ -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  $\alpha$ -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,  $\alpha$ -tocopherol transfer protein (TTP), has been characterized as playing a role in vitamin E status. This protein selectively facilitates hepatic secretion of  $\alpha$ -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.  $\gamma$ -TOH) has not been characterized.

We postulated the existence of an enzyme-mediated mechanism that results in the preferential elimination of  $\gamma$ -TOH relative to  $\alpha$ -TOH. Water-soluble metabolites of the three major dietary tocopherols,  $\alpha$ -,  $\gamma$ -, and  $\delta$ -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  $\gamma$ -TOH is excreted in human urine as its 3'-carboxychromanol metabolite, 2,7,8-trimethyl-2-( $\beta$ -carboxyethyl)-6-hydroxychroman ( $\gamma$ -CEHC) (13), but a much smaller proportion of  $\alpha$ -TOH intake was excreted as  $\alpha$ -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.

## **Experimental Procedures**

Tocopherols were purchased from Fluka Biochemicals, Milwaukee, WI (RRR- $\gamma$ -TOH)

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or ACROS Organics, Fisher Scientific, Pittsburgh, PA (RRR- $\alpha$ -TOH).  $\gamma$ -Tocotrienol was a gift from Rex Parker, Bristol-Meyers Squibb, Wallingford, CT.  $\beta$ -NADPH,  $\beta$ -NAD<sup>+</sup>, 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<sub>3</sub> 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- $\gamma$ -TOH, or RRR- $\alpha$ -TOH (12 mM solutions in ethanol), was added dropwise to FBS while mixing gently; the FBS was stored at 4°C for a minimum of 4 hours, then diluted 1:10 with MEM. Final tocopherol concentrations were 25-100  $\mu$ M, 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-

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wise to complete media to a concentration of 1.0  $\mu$ M. 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°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-Elvehjem 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  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4) and frozen at -80°C until assayed for activity.

The standard 1ml reaction system consisted of 100 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4) with 0.05-0.2 mg cell fraction protein, 0.5 mM NADPH, and with or without 0.5 mM  $\text{NAD}^+$ . Tocopherols were added as a complex with 1% BSA (Fraction V, Sigma) passed through a 0.22 $\mu$

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mixed cellulose ester filter. Cytochrome P450 substrates or inhibitors were added as solutions in EtOH. The reactions were pre-incubated at 37°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  $\mu$ l 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 6 $\beta$ -hydroxylation (CYP3A), 12- and 11-hydroxylation of lauric acid (CYP2E1, 4A), 7-ethoxycoumarin de-ethylation (CYP2E1, 2B, 1A), and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) 20- $\omega$ -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<sub>2</sub>- $\gamma$ -CEHC (13) or d<sub>9</sub>-

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$\alpha$ -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  $d_9\alpha$ -TOH (22) added as an internal standard for TOH reactions and  $17\alpha$ -CH<sub>3</sub>-testosterone as an internal standard for testosterone reactions. Solvents were removed under a stream of N<sub>2</sub> and the residue silylated with pyridine and N,O-bis-[trimethylsilyl]-trifluoroacetamide + 1% trimethylchlorosilane (BSTFA/ 1% TMCS; Pierce Chemical Co., Rockford, IL) under nitrogen at 70°C for 30 min. LTB<sub>4</sub> 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°C in 1ml KH<sub>2</sub>PO<sub>4</sub> buffer with various concentrations of an equimolar mixture of  $\gamma$ - and  $\alpha$ -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  $d_9\alpha$ -TOH as internal standard. Extracts were silylated and analyzed by gas chromatography-mass spectrometry.

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### *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°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. Media concentrations of tocopherol metabolites were determined using the appropriate deuterated internal standards. 6 $\beta$ -hydroxy-testosterone, 12-hydroxy-lauric acid, and 7-hydroxycoumarin were analyzed as above with minor changes in the oven temperature program. LTB<sub>4</sub> 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<sub>2</sub> gas and the residue reduced with palladium on carbon catalyst under H<sub>2</sub> gas at 65°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 $\gamma$ -tocopherol metabolism in HepG2 cultures*

Previously published mass spectra of  $\gamma$ -TOH and its 3'-carboxychromanol ( $\gamma$ -CEHC) and 5'-carboxychromanol ( $\gamma$ -CMBHC) metabolites all exhibit a base peak at  $m/z$  223, reflecting a common fragmentation pattern of the  $\gamma$ -chroman-O-trimethylsilyl (TMS) ring moiety (11, 14). GC-MS analyses of extracts of media from HepG2 cells incubated in the presence of 50  $\mu$ M  $\gamma$ -TOH revealed several substances not present in extracts of control cultures and which exhibited a base peak at  $m/z$  223. Figure 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  $\gamma$ -TOH. Peaks I, II, and V correspond to the di-TMS derivatives of the 3'-carboxychromanol ( $\gamma$ -CEHC) and 5'-carboxychromanol ( $\gamma$ -CMBHC) metabolites of  $\gamma$ -TOH, and to the TMS derivative of  $\gamma$ -TOH respectively, as evidenced by their mass spectra and by comparison of retention times to synthetic di-TMS- $\gamma$ -CEHC or TMS- $\gamma$ -TOH.

#### **Insert Figure 1**

The mass spectra of peaks III, IV, and VI of Figure 1 are shown in Figure 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  $\gamma$ -TOH, respectively, as illustrated in Figure 5.

#### **Insert Figure 2**

Peaks III and VI, identified as 7'- and 11'- $\gamma$ -carboxychromanols respectively, were each consistently accompanied by two minor peaks exhibiting base ions at  $m/z$  223 but molecular ions two mass units less than their respective major peak (peaks III\* and VI\*, Figure 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 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  $\beta$ -oxidation, the putative unsaturated analogs were assigned the structures of III\* and VI\* in Figure 5.

### **Insert Figure 3**

The mass spectra of peaks VII and VIII, eluting at 22.7 min and 24.5 min respectively (Figure 1), are presented in Figure 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 ( $-\text{CH}_2\text{-O-TMS}$ ), consistent with a metabolite possessing an intact  $\gamma$ -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  $\gamma$ -TOH possessing an intact  $\gamma$ -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  $\gamma$ -TOH, and as illustrated in Figure 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

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normally attenuated metabolism of  $\alpha$ -TOH by HepG2 cells (14), the terminal hydroxy and carboxy metabolites of  $\alpha$ -TOH were not detected in these cultures but were consistently present in rat liver subcellular fractions incubated with  $\alpha$ -TOH. The expected unsaturated metabolites of  $\gamma$ -tocotrienol were observed in the hepatocyte cultures (data not shown), suggesting that tocopherols and tocotrienols are metabolized via this pathway.

#### **Insert Figure 4 and Figure 5**

To test the hypothesis that the initial steps in tocopherol side-chain metabolism consist of a cytochrome P450 (CYP)-mediated  $\omega$ -hydroxylation followed by dehydrogenation to the carboxylic acid, time course reactions with either  $\gamma$ - or  $\alpha$ -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  $\gamma$ -TOH, suggestive of a precursor-product relationship (Figure 6). Additionally, when 0.5 mM  $\text{NAD}^+$  was also included, the hydroxylated metabolite accumulated only during the initial stage of the reaction, but was relatively suppressed thereafter. Conversely,  $\text{NAD}^+$  stimulated accumulation of the carboxylated metabolite to levels above those observed for the hydroxylated metabolite in the absence of  $\text{NAD}^+$ . Throughout the course of the reaction (80 minutes) metabolism of  $\gamma$ -TOH (figure 6, panel A) in the rat liver microsomes was between five- and ten-fold greater than that of  $\alpha$ -TOH (figure 6, panel B).

#### **Insert Figure 6**

*Involvement of Cytochrome P450 4F2 in the  $\omega$ -hydroxylation of tocopherols*

The identification of a terminally hydroxylated metabolite of  $\gamma$ -TOH and  $\alpha$ -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  $\gamma$ -TOH metabolism by 1  $\mu$ M ketoconazole in both HepG2 cells and rat primary hepatocytes and by 1  $\mu$ M sesamin, a sesame seed lignan, in HepG2 cells (15). More recent findings have shown that ketoconazole and sesamin (1  $\mu$ M) both potently inhibit (>80%)  $\gamma$ - and  $\alpha$ -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-6 $\beta$ -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- $\omega$ -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  $\gamma$ - or  $\alpha$ -TOH. Additionally,

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GM-2E1 fibroblasts stably expressing recombinant human CYP2E1 (21), while actively carrying out O-de-ethylation of 7-ethoxycoumarin, did not metabolize  $\gamma$ -TOH to any identified metabolite (not shown). In contrast, insect microsomes expressing recombinant human liver CYP4F2 exhibited clear NADPH-dependent  $\omega$ -oxidation of  $\gamma$ - and  $\alpha$ -TOH to their terminally hydroxylated and carboxylated metabolites. Insect microsomes expressing recombinant human liver CYP4F3B also contained tocopherol- $\omega$ -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- $\omega$ -hydroxylation of LTB<sub>4</sub> (data not shown). Tocopherol- $\omega$ -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  $\gamma$ - and  $\alpha$ -TOH  $\omega$ -hydroxylation demonstrated in rat liver was compared to that in human liver microsomes and insect microsomes expressing recombinant human CYP4F2. As illustrated in Figure 7, all three microsomal systems exhibited marked substrate preference for  $\gamma$ -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  $\gamma$ - and  $\alpha$ -TOH was significantly inhibited (80-100%) by 1  $\mu$ M sesamin (Figure 7).

**Insert Figure 7**

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The observed difference in tocopherol- $\omega$ -hydroxylase activity toward  $\gamma$ - and  $\alpha$ -TOH in both separate (Figure 6) and mixed (Figure 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 8, left panel) exhibited roughly similar  $K_m$  values (68 and 42  $\mu$ M) for  $\gamma$ - and  $\alpha$ -TOH, respectively, but a nearly 6-fold greater  $V_{max}$  for  $\gamma$ -TOH vs.  $\alpha$ -TOH (0.73 vs. 0.13 nmol/mg protein/min, respectively). Recombinant human CYP4F2 (Figure 8, right panel) likewise exhibited similar  $K_m$  values of 37 and 21  $\mu$ M for  $\gamma$ - and  $\alpha$ -TOH, respectively, while having a  $V_{max}$  for  $\gamma$ -TOH much greater than that for  $\alpha$ -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.

### **Insert Figure 8**

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  $\gamma$ - and  $\alpha$ -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  $\mu$ M each TOH). Baseline (endogenous) concentrations of  $\alpha$ -TOH were nearly 3-fold higher than those of  $\gamma$ -TOH (0.29  $\pm$  0.01 vs. 0.11  $\pm$  0.08 nmol/mg protein, respectively), and both increased markedly to 219  $\pm$  9 nmol/mg protein after a 30 min incubation with 25 $\mu$ M 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  $\omega$ -hydroxylation of the tocopherol phytyl side chain, oxidation to the corresponding terminal carboxylic acid, and sequential removal of three- or two-carbon moieties by  $\beta$ -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  $\omega$ -hydroxylation of both  $\gamma$ - and  $\alpha$ - 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- $\omega$ -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 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- $\omega$ -hydroxylase activity while all three enzymes catalyzed the 20- $\omega$ -hydroxylation of LTB<sub>4</sub>. Kinetic analyses of the tocopherol- $\omega$ -

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hydroxylase activity in the rat liver microsomal system and recombinant human CYP4F2 microsomal system revealed similar  $K_m$  but notably different  $V_{max}$  for  $\gamma$ - and  $\alpha$ -TOH, with the catalytic activity several fold higher for  $\gamma$ -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  $\mu$ M tocopherol-BSA, i.e. near the apparent  $K_m$ , 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  $K_m$  for the tocopherol- $\omega$ -hydroxylase which is therefore never saturated.

Two lines of evidence indicate that the tocopherol- $\omega$ -hydroxylase pathway described here is of physiological importance in the post-absorptive regulation of tocopherol status in vivo, in particular the preferential retention of  $\alpha$ -TOH relative to other tocopherols. First, in humans a substantial proportion of estimated daily intake of  $\gamma$ -TOH, but not of  $\alpha$ -TOH, undergoes urinary excretion as its 3-carboxychromanol (13), the major product of this catabolic pathway. This observation is consistent with the greater tocopherol- $\omega$ -hydroxylase activity exhibited toward

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$\gamma$ -TOH than  $\alpha$ -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  $\gamma$ -TOH (29-31). We have demonstrated here and in a previous report (15) that sesamin is a potent inhibitor of tocopherol- $\omega$ -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- $\omega$ -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  $\alpha$ -TOH (8, 9, 32). TTP has been proposed to facilitate the selective secretion of  $\alpha$ -TOH from liver into the bloodstream via very low-density lipoproteins (8), and may modulate intracellular tocopherol- $\omega$ -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  $\omega$ -hydroxylation of leukotriene B<sub>4</sub> (LTB<sub>4</sub>) to 20-OH-LTB<sub>4</sub>, a metabolite with considerably less chemotactic activity (19). In addition, CYP4F2  $\omega$ -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 K<sub>m</sub> values for arachidonic acid (24  $\mu$ M) and LTB<sub>4</sub> (45  $\mu$ M) are similar to those reported here for  $\gamma$ - and  $\alpha$ -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'- $\gamma$ -carboxychromanol metabolite of  $\gamma$ -TOH was first reported as a natriuretic factor (11), and more recently as an inhibitor of prostaglandin E<sub>2</sub> synthesis (36).

In summary, we describe a novel CYP4F2-mediated tocopherol- $\omega$ -hydroxylase pathway of metabolism of tocopherols to water-soluble carboxychromans that are excreted in urine. This pathway preferentially metabolizes  $\gamma$ -TOH over  $\alpha$ -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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#### Footnotes

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<sup>1</sup>The abbreviations used are:  $\alpha$ -TOH,  $\alpha$ -tocopherol;  $\gamma$ -TOH,  $\gamma$ -tocopherol; CYP, cytochrome P450;  $\gamma$ -CEHC, 2,7,8-trimethyl-2-( $\beta$ -carboxyethyl)-6-hydroxychroman; MEM, Minimal Essential Media; BSA, Bovine Serum Albumin; FBS, Fetal Bovine Serum; MTBE, methyl-tert-butyl-ether; BSTFA/ 1% TMCS, N,O-bis-[trimethylsilyl]-trifluoroacetamide + 1% trimethylchlorosilane; GC-MS, gas chromatography-mass spectrometry; SIM, selected ion monitoring; EI, electron impact; TMS, trimethylsilyl;  $\gamma$ -CMBHC, 2,7,8-trimethyl-2-( $\beta$ -carboxymethylbutyl)-6-hydroxychroman; 13'-OH-TOH, 13'-hydroxy-tocopherol metabolite; 13'-COOH-TOH, 13-carboxy-tocopherol metabolite; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; TTP,  $\alpha$ -tocopherol transfer protein.

#### Figure Legends

Figure 1. GC-MS (EI) chromatogram of extracts of HepG2 cultures incubated with 50  $\mu$ M  $\gamma$ -TOH, obtained using selected ion (SIM) analysis of the major  $\gamma$ -chroman ring fragment, m/z 223. Peaks corresponding to  $\gamma$ -TOH and its metabolites are labeled with roman numerals I-VIII.

**Figure 2.** Mass spectra (EI) of peaks III, IV, and VI of the chromatogram shown in Figure 1, interpreted as the TMS derivatives of the 7'-, 9'-, and 11'- $\gamma$ -carboxychromanol metabolites of

$\gamma$ -TOH, respectively.

**Figure 3.** Mass spectra (EI) of peaks labeled as III\* (panel A) and VI\* (panel B) of the chromatogram shown in Figure 1. Panel C: SIM chromatogram, monitoring the indicated ions, of one half of a 50  $\mu$ M  $\gamma$ -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  $\beta$ -oxidation intermediates of the 7'- and 11'- $\gamma$ -carboxychromanol metabolites of  $\gamma$ -TOH.

**Figure 4.** Mass spectra (EI) of peaks VII and VIII of the chromatogram shown in Figure 1, interpreted as the TMS derivatives of the terminal 13'-hydroxy (13'-OH-TOH) and 13'-carboxy (13'-COOH-TOH) metabolites of  $\gamma$ -TOH, respectively.

**Figure 5.** Pathway of metabolism of  $\gamma$ -TOH to its 3'- $\gamma$ -carboxychromanol metabolite, based on identification of intermediates from HepG2 cultures incubated with 50 $\mu$ M  $\gamma$ -TOH. Roman numerals correspond to those of Figures 1-4.

**Figure 6.** Time course of synthesis of the 13'-OH-TOH and 13'-COOH-TOH metabolites of  $\gamma$ -TOH (panel A) and  $\alpha$ -TOH (panel B) in rat liver microsomes incubated with 25  $\mu$ M tocopherol as a BSA complex, 0.5 mM NADPH and with or without the addition of 0.5 mM NAD<sup>+</sup>. 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.

**Figure 7.** Substrate discrimination in synthesis of metabolites of  $\gamma$ - and  $\alpha$ -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  $\mu$ M sesamin on synthesis of the metabolites of  $\gamma$ - and  $\alpha$ -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  $\mu$ M  $\gamma$ -TOH plus 50  $\mu$ M  $\alpha$ -TOH, along with 0.5 mM NADPH, and 0.5 mM NAD<sup>+</sup>. n.d., not detected. Data (representative experiment) are means and standard deviations of triplicate analyses.

**Figure 8.** Kinetic analysis of formation of metabolites of  $\gamma$ - and  $\alpha$ -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  $\mu$ M, rat or 25-125  $\mu$ M, CYP4F2) of tocopherol concentrations during a 20 minute incubation with 0.5 mM NADPH and 0.5 mM NAD<sup>+</sup>. 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 overlaid with best fit hyperbolic curve as determined by non-linear regression analysis and defined by goodness-of-fit  $\chi^2$  minimization. Apparent  $K_m$  and  $V_{max}$  values were determined from resulting hyperbolic equation. Rat liver microsomes:  $\gamma$ -TOH;  $K_m$  = 68  $\mu$ M,  $V_{max}$  = 0.73 nmol/mg/min,  $\chi^2$  =

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0.00128.  $\alpha$ -TOH;  $K_m = 42 \mu\text{M}$ ,  $V_{\text{max}} = 0.13 \text{ nmol/mg/min}$ ,  $\zeta^2 = 0.00006$ . CYP4F2

microsomes:  $\gamma$ -TOH;  $K_m = 37 \mu\text{M}$ ,  $V_{\text{max}} = 1.99 \text{ nmol/nmol P450/min}$ ,  $\zeta^2 = 0.00061$ .  $\alpha$ -TOH;

$K_m = 21 \mu\text{M}$ ,  $V_{\text{max}} = 0.16 \text{ nmol/nmol P450/min}$ ,  $\zeta^2 = 0.00021$ .

**Figure 1**



Figure 2

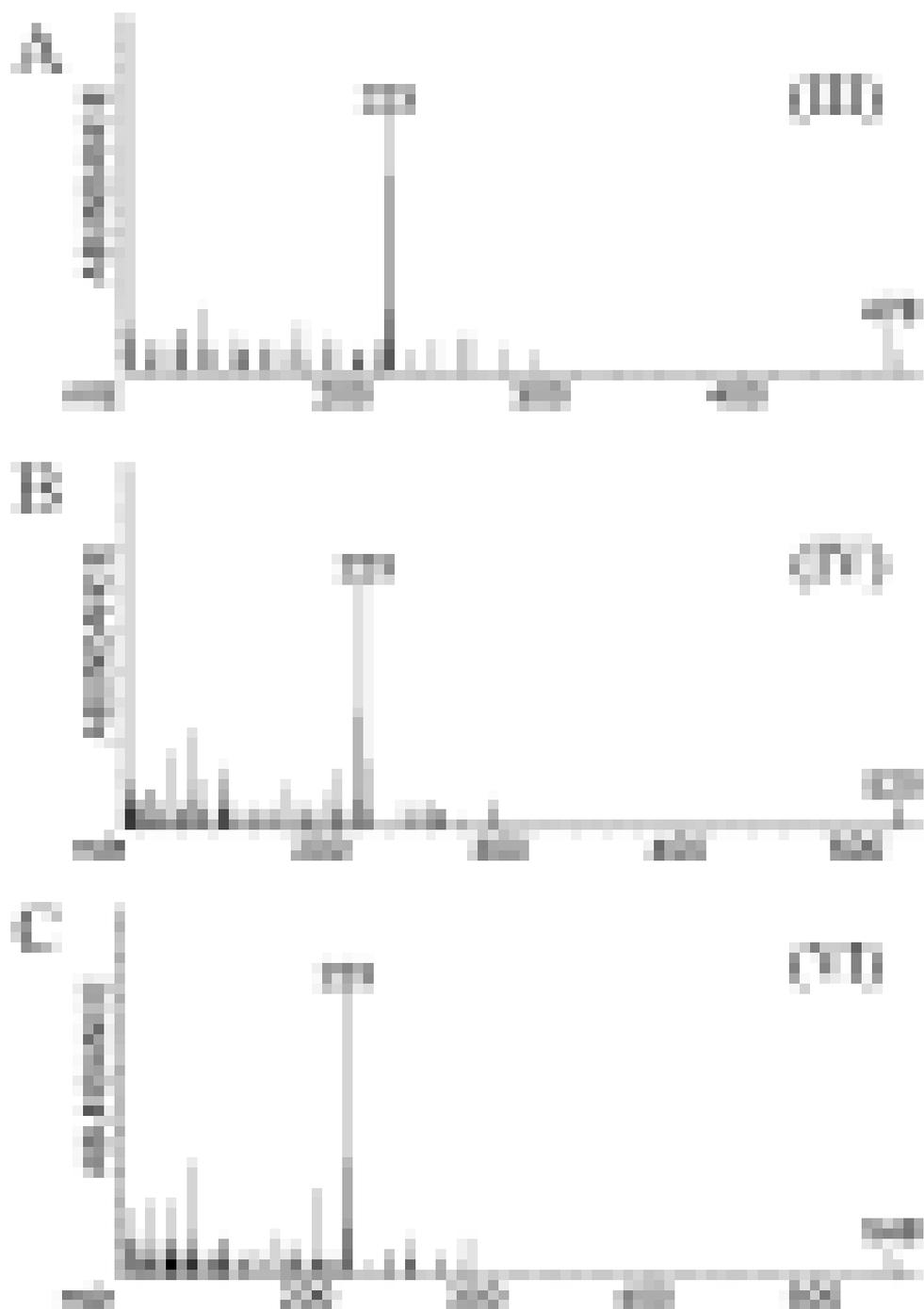


Figure 3

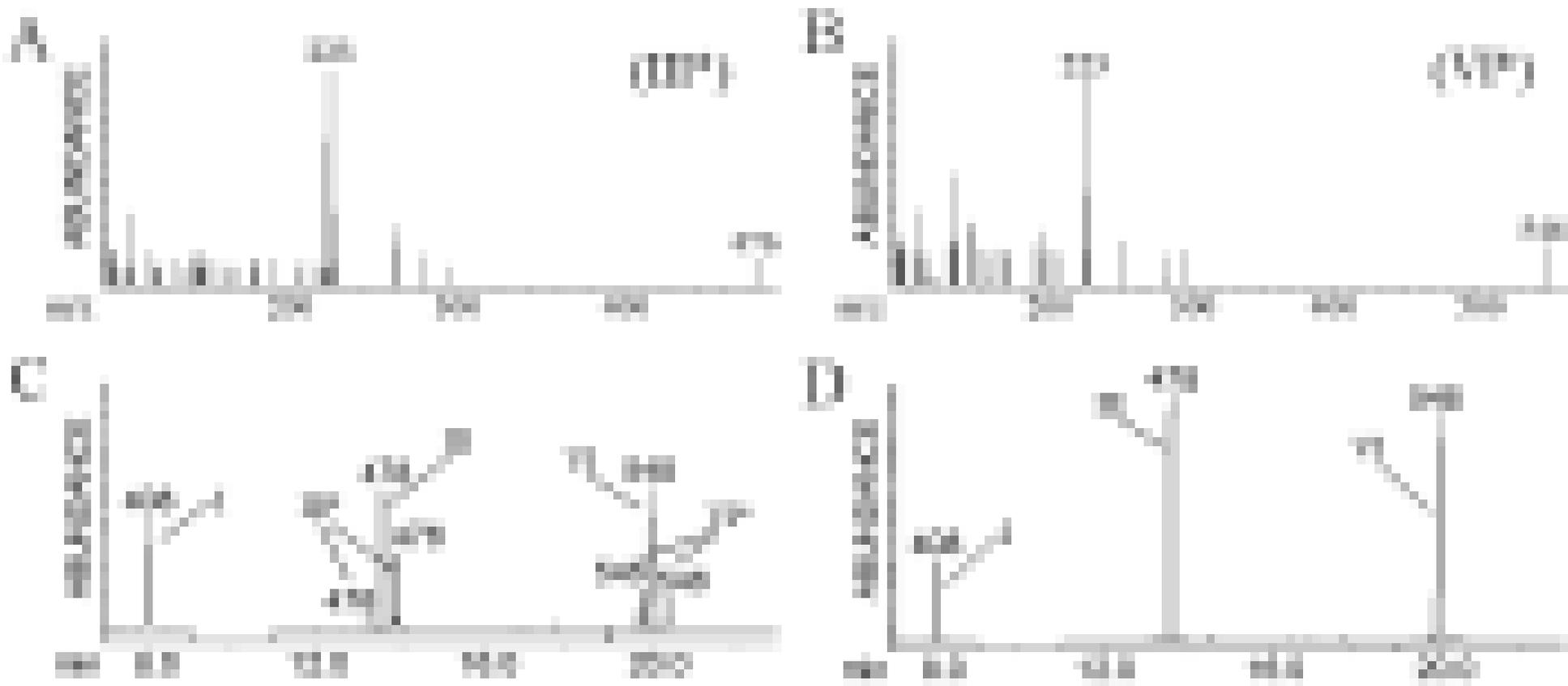


Figure 3

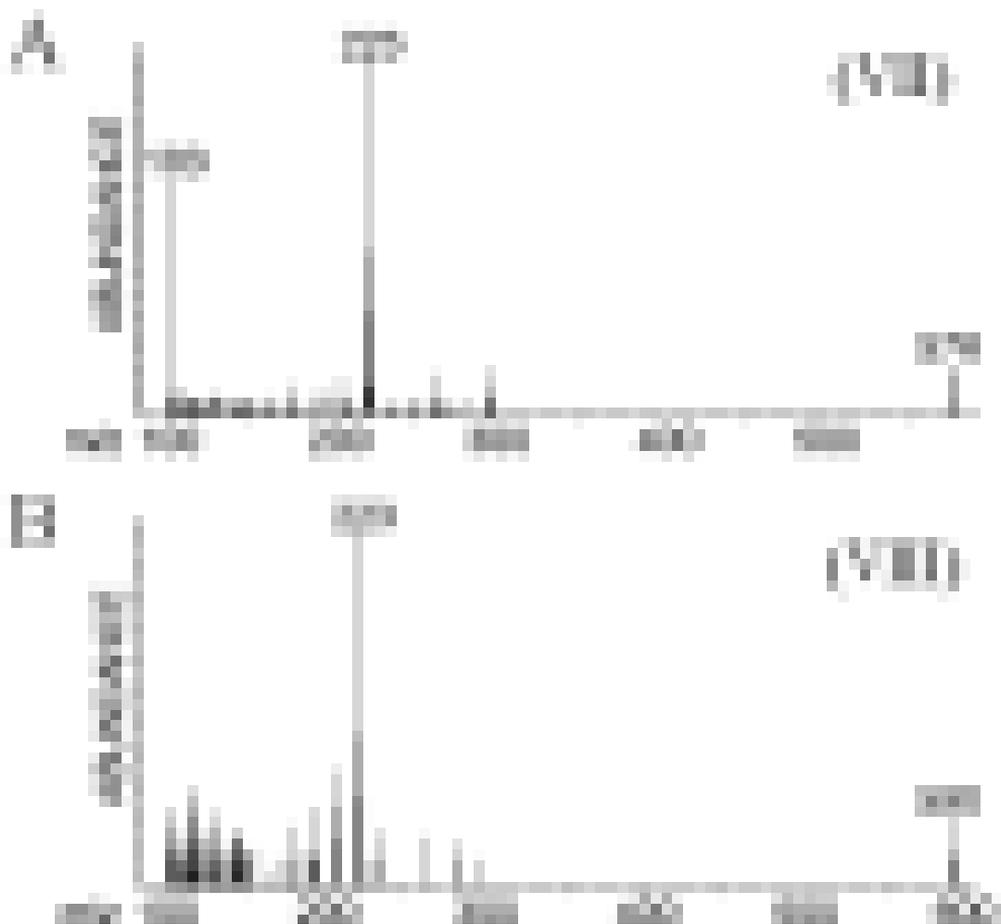
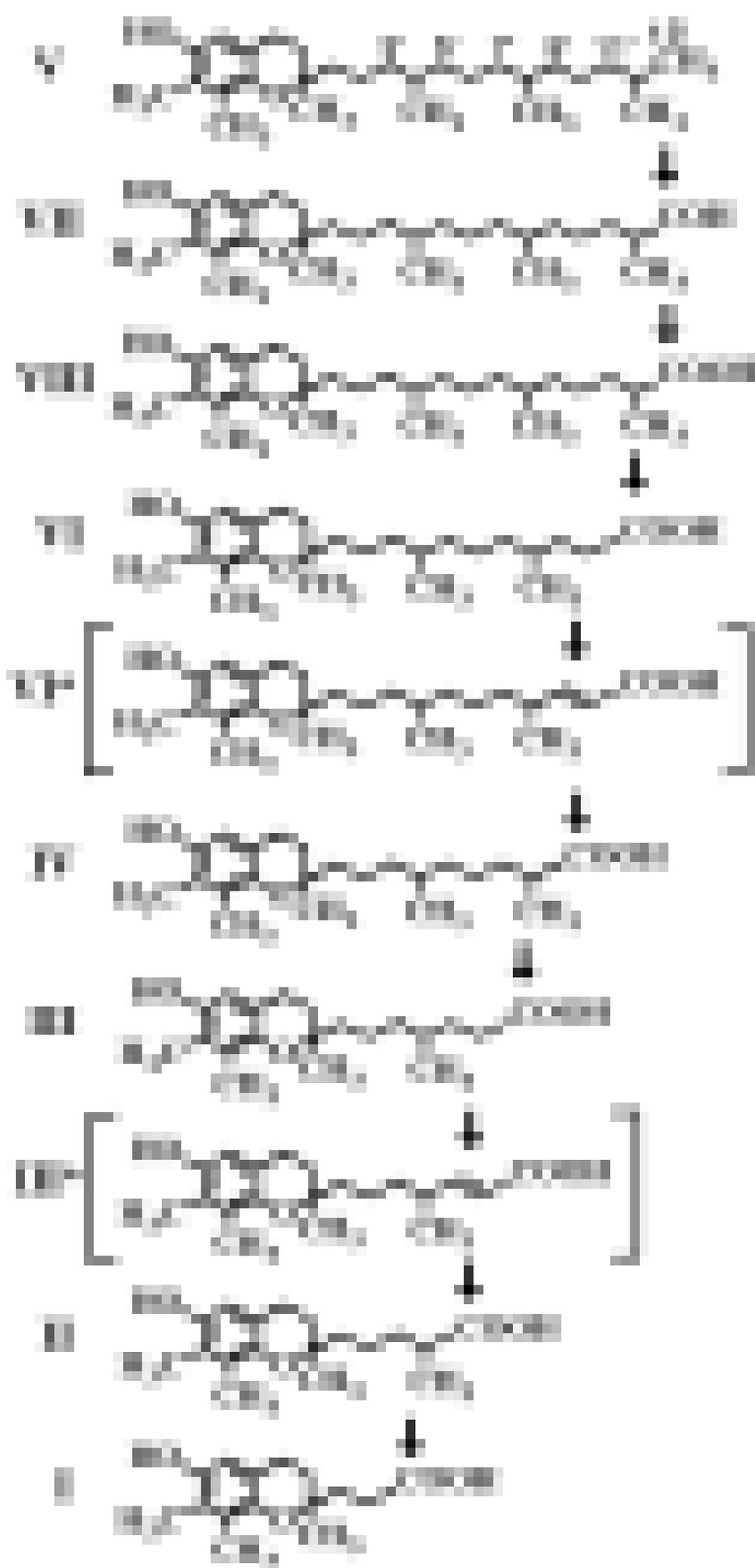
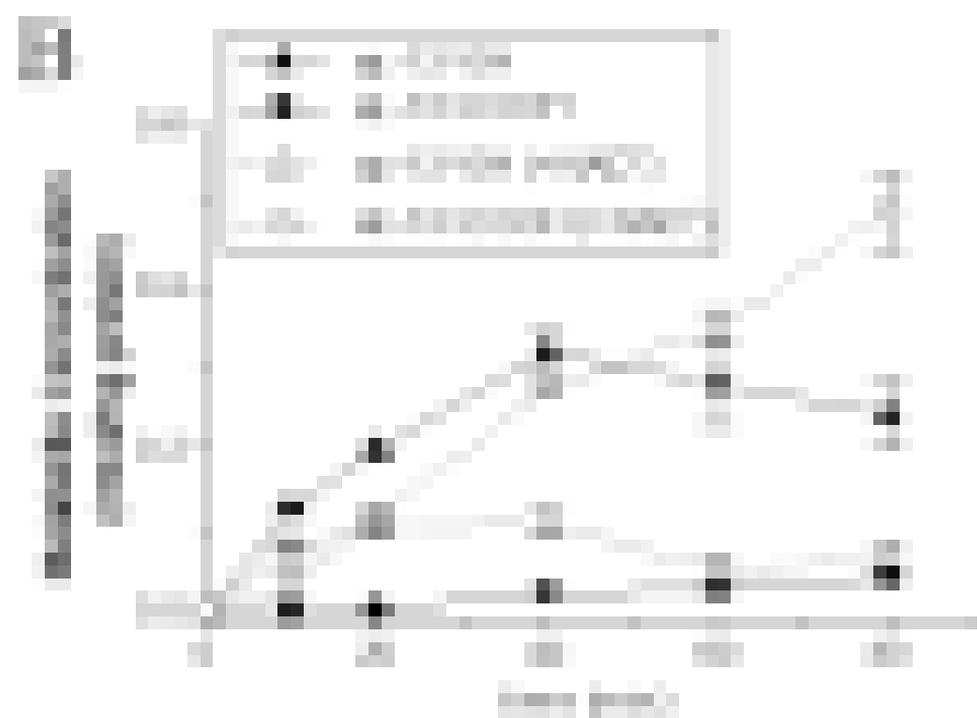
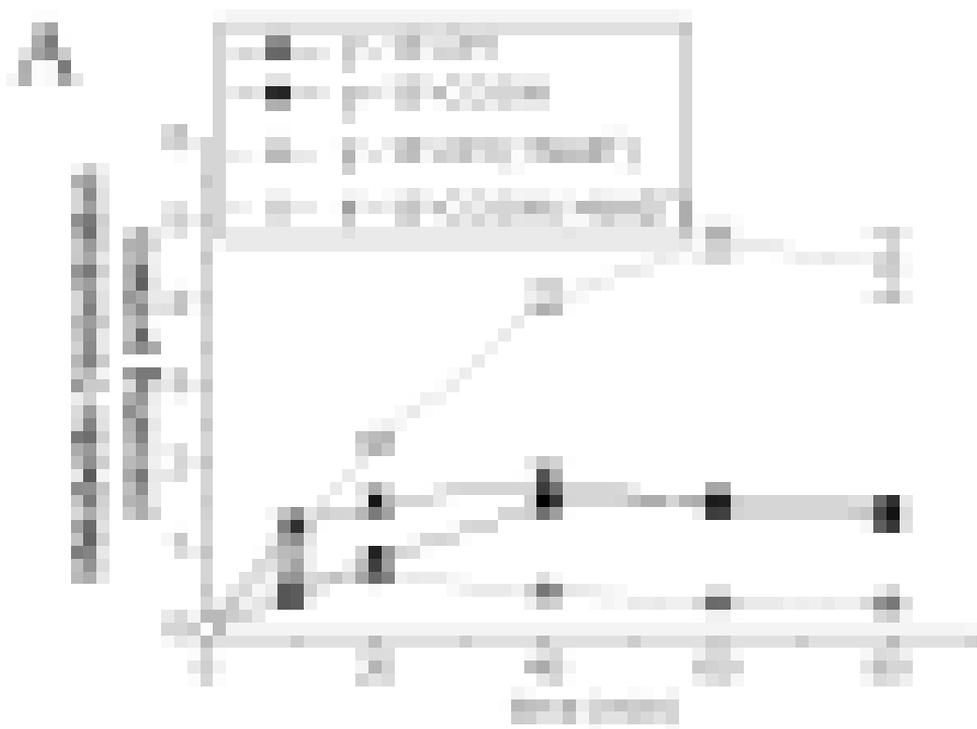


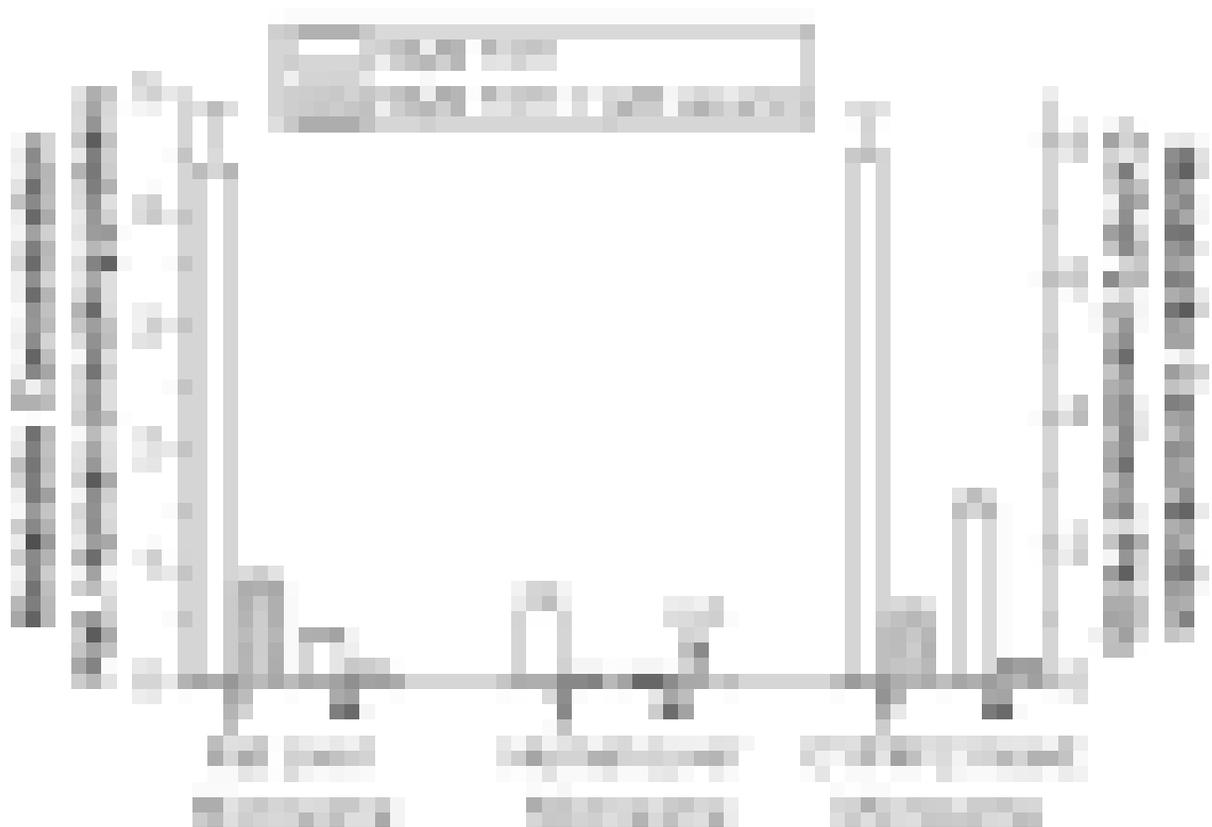
Figure 3



**Figure 1**



**Figure 7**  
**Figure 7**



**Figure 8**



**Cytochrome P450  $\omega$ -hydroxylase pathway of tocopherol catabolism: Novel mechanism of regulation of vitamin E status**

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