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Review

Chemistry and biology of vitamin E

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Our understanding of the role of vitamin E in human nutrition, health, and disease has broadened and changed over the past two decades. Viewed initially as nature's most potent lipid-soluble antioxidant (and discovered for its crucial role in mammalian reproduction) we have now come to realize that vitamin E action has many more facets, depending on the physiological context. Although mainly acting as an antioxidant, vitamin E can also be a pro-oxidant; it can even have nonantioxidant functions: as a signaling molecule, as a regulator of gene expression, and, possibly, in the prevention of cancer and atherosclerosis. Since the term vitamin E encompasses a group of eight structurally related tocopherols and tocotrienols, individual isomers have different propensities with respect to these novel, nontraditional roles. The particular beneficial effects of the individual isomers have to be considered when dissecting the physiological impact of dietary vitamin E or supplements (mainly containing only the α-tocopherol isomer) in clinical trials. These considerations are also relevant for the design of transgenic crop plants with the goal of enhancing vitamin E content because an engineered biosynthetic pathway may be biased toward formation of one isomer. In contrast to the tremendous recent advances in knowledge of vitamin E chemistry and biology, there is little hard evidence from clinical and epidemiologic studies on the beneficial effects of supplementation with vitamin E beyond the essential requirement.

Keywords: Antioxidant / *Arabidopsis* / Atherosclerosis / Autoxidation / Review / Tocopherol-mediated peroxidation

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Abbreviations: GGPP, geranylgeranyl pyrophosphate; **HPD**, *p*-hydroxyphenylpyruvate dioxygenase; **HPT**, homogentisate prenyltransferase; **IPP**, isopentenyl pyrophosphate; **LDL**, low-density lipoprotein; **PKC**, protein kinase C; **SPF**, supernatant protein factor; **TAP**, tocopherol-associated protein; **TMP**, tocopherol-mediated peroxidation; **γ-TMT**, γ-tocopherol methyltransferase; **αTTP**, α-tocopherol transfer protein; **VLDL**, very-low-density lipoprotein

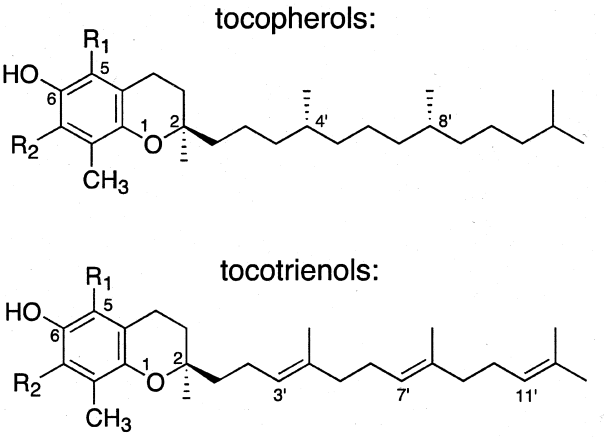
1 Introduction

Research over the past decades has led to many new insights into molecular details of vitamin E action but also to controversies about its usefulness as a dietary supplement. The aim of this review is to give a comprehensive overview of the current state of the discussion. Novel findings on the chemistry, biochemistry, and physiology of vitamin E will be discussed. The opening section covers some of the essential mechanisms of fatty acid autoxidation, which will provide a basis for discussing the antioxidant and pro-oxidant chemistry of α-tocopherol. In human physiology, α-tocopherol and its isomers may be involved in the progression of low-density lipoprotein (LDL) oxidation and atherosclerosis, not only through their antioxidant or pro-oxidant properties but also through their ability to regulate gene expression and functional activities of proteins that are critically involved in atherogenesis. Dietary uptake of vitamin E through the intestine is nonspecific, but its transport into very-low-density lipoprotein (VLDL) and ensuing lipoproteins is regulated by cytosolic proteins that are involved in intracellular trafficking of the water-insoluble tocopherols. Polymorphisms and mutations of the genes encoding the trafficking proteins result in more or less severe neurologic abnormalities. Whether vitamin E has a protective role in human health beyond its essential function in mammalian reproduction is the subject of large prospective clinical trials. By and large, these trials proved disappointingly negative in showing a beneficial role in cancer prevention and in the attenuation of heart or cardiovascular diseases. Finally, the biosynthesis of vitamin E in plants and recent success in cloning of the pertinent genes will be reviewed. These advances have marked impact on attempts to enhance vitamin E levels in transgenic plants.

2 The chemistry of vitamin E

2.1 Composition of vitamin E

The term vitamin E is used for a family of eight molecules of related structure. The four tocopherols consist of a chromanol ring with different substitution pattern of methyl groups at positions 5, 7, and 8 of the head group (α-, β-, δ-, and γ-) and a 16-carbon saturated phytyl rest as a side chain (Fig. 1). Tocopherols have three chiral centers at carbons 2, 4′, and 8′, and the naturally occurring isomers have the *R*-configuration at all three positions. The tocotrienols have the same substitution pattern on the chromanol ring but an unsaturated C_{16} isoprenoid side chain with double bonds in the positions 3′, 7′, and 11′ (Fig. 1). All of these molecules possess antioxidant activity, although α-tocopherol is chemically and biologically the most active. The other naturally occurring forms of vitamin E (β-, γ-, and δ-tocopherols and the tocotrienols) do not contribute toward meeting the vitamin E requirement because (although absorbed) they are not converted to α-tocopherol by humans, and they are recognized poorly by the α-tocopherol transfer protein in the liver (see Section 3).



tocopherol/ tocotrienol	R_1	R_2	relative antioxidant activity (%) in vitro
α-	CH_3	CH_3	100
β-	CH_3	H	71
γ-	H	CH_3	68
δ-	H	H	28

Figure 1. The structures of tocopherols and tocotrienols. Vitamin E is the common name for the eight related tocopherols and tocotrienols that have the capacity to compensate the symptoms of vitamin E deficiency in the rat fetal resorption test. The natural configuration of α-tocopherol is 2*R*,4′*R*,8′*R*. Important for the antioxidant activity is the hydroxy group at C-6 of the chromanol ring which can donate its hydrogen atom to terminate the radical chain in the autoxidation reaction (Fig. 2). The *in vitro* antioxidant activity is dependent on the substitution pattern of methyl groups at the aromatic ring. The side chain has no influence on the antioxidant activity but contributes to the anchoring in liposomes and suppresses the transfer of vitamin E between liposomal membranes [13]. The biological activity of each isomer is mainly determined by its binding specificity to the α-tocopherol transfer protein (see Section 3). Note that α- and β-tocopherol are formed by methyl transfer to the 5-position of the chromanol ring from the precursors γ- and δ-tocopherol, respectively. The relative *in vitro* antioxidant activities are taken from [24] for the rates of reaction (4) in Fig. 2.

2.2 Biopotency, biokinetics, and biophysical properties

The IU equivalents for vitamin E according to the U.S. Pharmacopoeia USP XXI (1985) are defined as follows: for

the racemic forms of α-tocopherol acetate 1.00 IU/mg, α-tocopherol 1.10 IU/mg, and α-tocopheryl succinate 0.89 IU/mg. The natural *R*,*R*,*R*-isomers are for α-tocopherol acetate 1.36 IU/mg, α-tocopherol 1.49 IU/mg, and α-tocopheryl succinate 1.21 I.U./mg. There are, however, some concerns in the literature whether these numbers appropriately reflect the bioavailability [1], and whether the available data justify to change the biopotency factors [2]. The recent increase in dietary reference intake for vitamin E from 10 mg/d to 15 mg/d by the Food and Nutrition Board of the Institute of Medicine was disputed as not supported by new data [3, 4]. The tolerable upper intake level of vitamin E is reported to be 1000 mg/d [3].

The serum concentrations of α- and γ-tocopherol are about 25 μM and between 1.5 and 3 μM, respectively [5]. The elimination of *R*,*R*,*R*-α-tocopherol occurs with a half life of about 48 h, and is about 4 h for α- and γ-tocotrienol [1, 6, 7]. α-Tocopherol is practically insoluble in aqueous solutions, but freely soluble in oils, acetone, ethanol, ether, and other organic solvents. This poses the obvious question of how to get α-tocopherol into an aqueous solution when studying its cellular effects. One successful strategy is the formulation into liposomes by excessive sonication in the presence of phosphatidylcholine [8]. Alternatively, α-tocopherol is dissolved in ethanol and added to the assays using a relatively high final concentration of the vehicle (0.1 – 4%) [9]. For incubation of cultured cells, α-tocopherol from an ethanolic stock solution can be preincubated with fetal bovine serum and then added to the culture medium [10].

2.3 The chemistry of fatty acid autoxidation

In order to understand the chemistry behind the antioxidant activity of vitamin E, a brief background on the mechanism of autoxidation of polyunsaturated fatty acids will be given here. Fatty acid autoxidation is a process of radical reactions. Once an initial fatty acid radical is formed, it immediately reacts with oxygen; the resulting peroxyl radical abstracts a hydrogen from another fatty acid giving the hydroperoxide product and a new fatty acid radical, resulting in propagation of the chain. The antioxidant's task and capability is to efficiently interrupt this chain process.

The initiating reaction of the autoxidation process is the abstraction of a hydrogen atom from the fatty acid "substrate" to yield a carbon-centered radical (Fig. 2) [11]. Almost invariably, the hydrogen atom is abstracted from a carbon that is located in between two double bonds (*bis*-allylic methylene) of a polyunsaturated fatty acid, for example, linoleic acid (C18:2) or arachidonic acid (C20:4). This carbon-hydrogen bond is preferred because it is the weakest in the molecule, and therefore, considerably less energy is necessary for abstraction of a *bis*-allylic hydrogen compared to a hydrogen that is only allylic, as, for example,

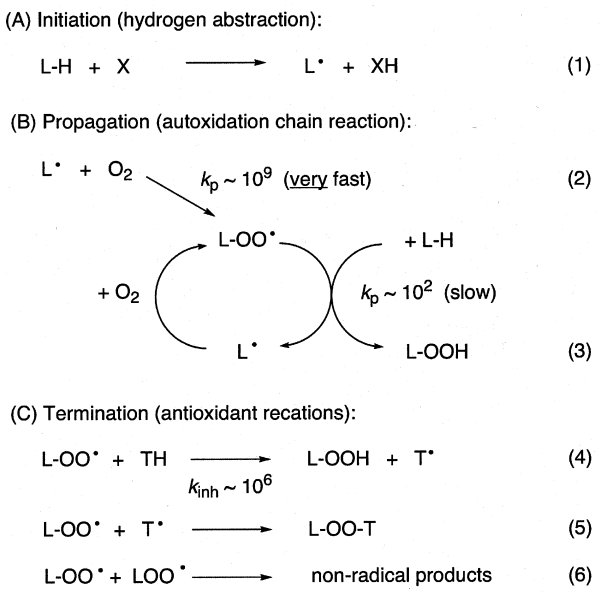


Figure 2. Initiation, propagation, and termination of the autoxidation chain reaction. (A) The initial hydrogen abstraction from the *bis*-allylic methylene of a polyunsaturated fatty acid (L-H) can be accomplished by various agents (X), including metal ions, UV and γ-radiation, superoxide, and synthetic radical starters. (B) The reaction of the fatty acid radical (L•) with molecular oxygen (O_2) is exceedingly fast. The actual chain propagation is a hydrogen abstraction by the fatty acid peroxyl radical from another fatty acid. This is the slowest of the radical reactions, and consequently, the peroxyl radical is the primary target for the antioxidant reaction. (C) α-Tocopherol (TH) donates the hydrogen from the 6-OH group to the fatty acid peroxyl radical to form a (stable) hydroperoxide and a tocopheroxyl radical. The rate of hydrogen transfer from α-tocopherol to the peroxyl radical is about 4 orders of magnitude faster than the rate for the peroxyl radical to abstract a hydrogen form another fatty acid. Other reactions resulting in chain termination are the reaction of the tocopheroxyl radical with a fatty acid peroxyl radical or the dimerization of two peroxyl radicals. Both reactions yield nonradical products. L-H, fatty acid (lipid); L•, fatty acid radical; LOO•, fatty acid peroxyl radical; X, radical initiator; TH, tocopherol; T, tocopheroxyl radical.

in oleic acid (C18:1) [11]. This makes polyunsaturated fatty acids the prime targets for autoxidation. Several natural and synthetic "reagents" are capable of starting the autoxidation radical chain by performing the initial hydrogen abstraction. These include metal ions (Cu^{2+} and Fe^{2+}), UV and γ-radiation, cells that form active oxygen species, and, of course, fatty acid peroxyl radicals leading to the elongation of the chain reaction [12]. Furthermore, in order to study the mechanisms of initiation of autoxidation, specific radical initiators have been designed, and these molecules come as lipophilic, hydrophilic, or amphiphilic variants [13 – 15].

Once the carbon radical is formed by hydrogen abstraction it immediately reacts with molecular oxygen to form a fatty

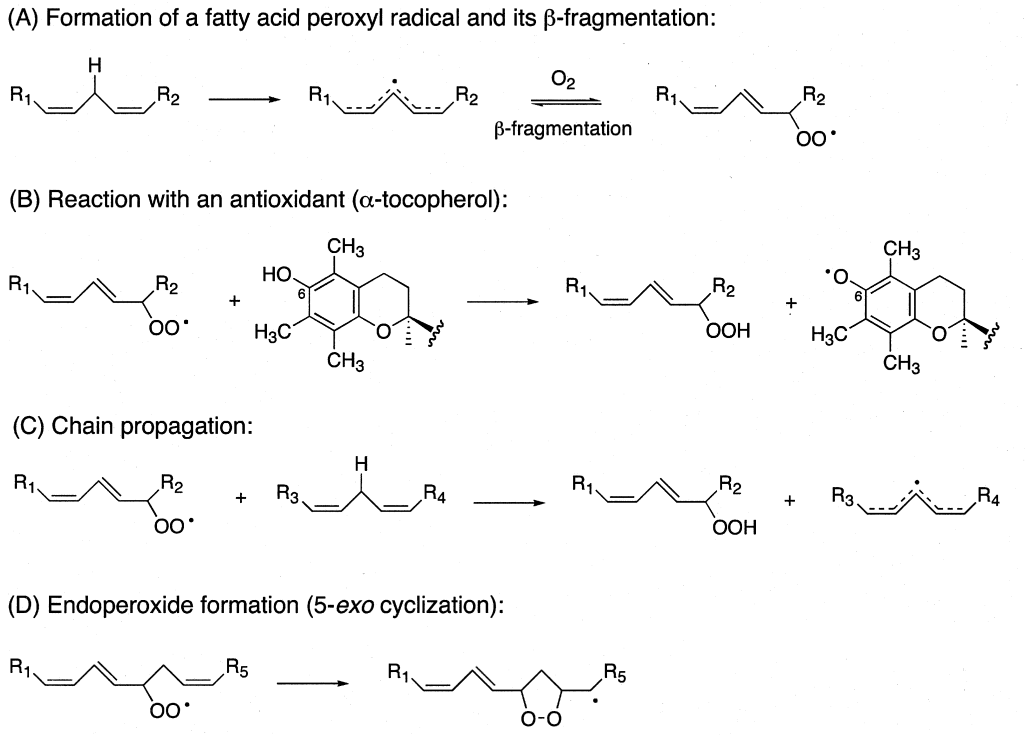


Figure 3. Reactions of fatty acid peroxyl radicals. (A) Hydrogen abstraction from a *bis*-allylic methylene carbon leads to a delocalized radical that immediately reacts with oxygen to form the fatty acid peroxyl radical. β-Fragmentation of the peroxyl radical is the loss of molecular oxygen and its reversal back to the pentadienyl radical. (B) In the antioxidant reaction, the peroxyl radical abstracts a hydrogen from the 6-OH group of α-tocopherol to form a fatty acid hydroperoxide and the stable tocopheroxyl radical. (C) The chain propagation reaction is the abstraction of *bis*-allylic hydrogen from a new fatty acid molecule. (D) If a β,γ-double bond is present in the fatty acid peroxyl radical, the peroxyl radical can cyclize in a 5-exo cyclization to form a cyclic peroxide (endoperoxide).

acid peroxyl radical. Oxygen itself does not need to be activated because in its ground state it is already a radical suitable for this reaction ("triplet oxygen"). The reaction of oxygen with the fatty acid radical is extremely fast ($>10^9$ M^{-1} s^{-1}), in fact, the rate of this reaction is only limited by the diffusion of oxygen toward the fatty acid radical [11]. Therefore, fatty acid radicals cannot be effectively targeted by an antioxidant molecule. The fate of the peroxyl radical itself is diverse because it has a considerable lifetime, and chemically several reactions are possible which will briefly be discussed here (summarized in Fig. 3) [11].

(i) Encounter with an antioxidant (chain termination). The peroxyl radical abstracts a hydrogen atom from the antioxidant forming a fatty acid hydroperoxide as a rather stable product. This reaction is the "antioxidant" reaction because it leads to the termination of the autoxidation chain reaction. The higher the ability of the antioxidant to donate a hydrogen atom to the peroxyl radical, the better it fulfills its function as an antioxidant. One implicit condition is, however, that the resulting antioxidant radical by itself refrains from starting a radical reaction. This possibility exists and will be discussed below.

(ii) Encounter with a pentadiene moiety of another fatty acid (chain propagation). The peroxyl radical itself is strong enough as a reagent (oxidant) to perform a hydrogen atom abstraction from a *bis*-allylic methylene of another fatty acid molecule. This reaction propagates the chain by transferring the radical to a new fatty acid molecule. It is the slowest reaction during autoxidation, and therefore the rate-limiting step. As a consequence, a molecule that can transfer a hydrogen atom faster to the peroxyl radical than the peroxyl radical does a hydrogen abstraction, acts as an antioxidant.

(iii) Encounter with a double bond in the same fatty acid (endoperoxide formation). This reaction is possible for fatty acids that have a double bond in addition to the 1,4-pentadiene (*i.e.*, at least three; for example, linolenic acid, C18:3, and arachidonic acid, C20:4) [16]. The peroxyl radical can react in a 5-*exo*-cyclization with the additional double bond to yield a 5-membered cyclic peroxide (endoperoxide) and a carbon radical outside the endoperoxide ring ("exo"). This reaction has high importance in fatty acid biochemistry. If it is catalyzed by the enzyme cyclooxygenase using arachidonic acid as substrate, it leads to formation

of the prostaglandin hormones (for recent reviews, see [17, 18]). If it occurs nonenzymatically in biological systems (*e.g.*, during conditions of oxidative stress) it leads to the formation of isoprostanes (iso-prostaglandins) [19]. Isoprostanes are stable end products of the autoxidation of arachidonic acid, and although they are not formed in high abundance, their quantification in biological tissues and fluids offers the most accurate and reliable index of fatty acid oxidation associated with oxidative stress [20]. Analogous products are formed in plant tissues from the prevalent C-18 unsaturated fatty acids, α- and γ-linolenic acid, and are called phytoprostanes [21].

(iv) Loss of oxygen from the peroxyl radical (β-fragmentation). This is the reverse reaction to the formation of the peroxyl radical yielding molecular oxygen and the fatty acid radical. It is important to note that this reaction will not lead to the reversal of autoxidation, because the committing step is the initial hydrogen abstraction, and the on-rate of oxygen, as described above, is exceedingly fast. However, β-fragmentation is part of a reaction sequence that leads to the rearrangement of the carbon chain configuration during prolonged autoxidation, *i.e.*, the transformation of *cis*, *trans*-hydroperoxides into *trans*,*trans*-hydroperoxides [22].

2.4 α-Tocopherol as an antioxidant

It is important to keep in mind the general features that make up an antioxidant. The antioxidant reaction of α-tocopherol is not a reaction with oxygen. Many molecules react with oxygen, but they do so without being antioxidants. β-Carotene, for example, readily reacts with oxygen, but it is by no means an efficient antioxidant [23]. The basis of an antioxidant reaction is not the removal of oxygen but the interception of the autoxidation radical chain process which is not perpetuated by oxygen but by the fatty acid. α-Tocopherol reacts with fatty acid peroxyl radicals, the primary products of lipid peroxidation, and intercepts the chain reaction [24]. What makes α-tocopherol such a highly efficient antioxidant is (i) that it reacts with the peroxyl radical extremely fast, much faster than to allow for the peroxyl radical to do any other reactions; (ii) it takes away the radical character from the oxidizing fatty acid and prevents it from further radical reactions; (iii) in the antioxidant reaction, α-tocopherol is turned into a fairly stable radical. Under normal circumstances, it will only react with another radical (either a tocopheroxyl radical or a fatty acid peroxyl radical) to form stable, nonradical products. In this setting, α-tocopherol is the most powerful lipid soluble antioxidant known, and only recently novel synthetic antioxidants have been developed that surpass α-tocopherol's antioxidant capacity [25].

Chemically α-tocopherol is the most active form of vitamin E due to the substitution pattern of methyl groups on the chromanol ring making the hydrogen of the C-6 hydroxy group especially active, *i.e.*, facilitating the transfer of the hydrogen to a peroxyl radical [24]. Under the conditions of a homogeneous lipid phase α-tocopherol has a greater rate of hydrogen atom transfer to a fatty acid peroxyl radical than any other lipid-soluble antioxidant (3×10^6 M^{-1} s^{-1}) [24]. Biologically it is the most active form because of the preference of the transport mechanism in the liver toward α-tocopherol (see Section 3). One of the main forms of vitamin E present in dietary supplements, α-tocopheryl acetate, carries an acetyl group esterified at the C-6 hydroxyl to increase its stability, but this also blocks the antioxidant properties. Hydrolysis of the ester is achieved by pancreatic esterases which release the free alcohol form that is subsequently absorbed in the small intestine [26, 27].

Abstraction of the 6-OH hydrogen yields a tocopheroxyl radical. Tocopherol can be restored by reduction of the tocopheroxyl radical with redox-active reagents like vitamin C (ascorbate) or ubiquinol [28 – 32]. In homogeneous solution phase autoxidation, the tocopheroxyl radical will react with a second peroxyl radical to give nonradical products. This second reaction leads to the destruction of α-tocopherol as an antioxidant. Thus, one molecule of α-tocopherol can terminate two autoxidation chains.

The antioxidant activity of α-tocopherol has led to the expectation that dietary supplementation with vitamin E could prove a beneficial measure against the major pathological autoxidation reaction in Western societies, the oxidation of LDL and the pathogenesis of atherosclerosis [33, 34]. Although this hypothesis appears compelling and straightforward, data from large clinical trials so far have provided little supporting evidence (see below) [35]. A part of the answer to this dilemma might be provided by the discovery of the so-called "tocopherol-mediated peroxidation" exemplifying the paradoxical role of α-tocopherol in the autoxidation of LDL [12, 36 – 38].

2.5 A special case: the oxidation of LDL and the role of α-tocopherol as a pro-oxidant

Why should the autoxidation of an LDL lipid particle and the mechanism of action of α-tocopherol be different from the *in vitro* solution phase autoxidation? The answer to this question lies in the remarkable physical and chemical features of the LDL particle [12]. The LDL particle has a lipid core consisting of neutral lipids (cholesteryl and triglyceride esters of polyunsaturated fatty acids, and free cholesterol) and a coat of polar lipids (mainly phosphatidylcholine esters of C18:2 and C20:4), and it also contains one molecule of apolipoprotein B-100. With relevance to autoxidation, per particle there are ≈1450 *bis*-allylic methylene groups, between 6 and 12 molecules of α-tocopherol, less

than one molecule of ubiquinol-10, and 4 – 5 molecules of oxygen [12, 37]. What makes LDL so uniquely different in its mechanism of autoxidation is its lipid-filled interior core and its particle size: it is too small to contain more than one radical at any given time [39].

What does this mean? Assuming a peroxyl radical hits an LDL particle and is being incorporated, it will likely react with α-tocopherol to give a hydroperoxide and the tocopheroxyl radical. Now the tocopheroxyl radical is the sole radical, and it will spend a considerable fraction of its lifetime in the lipid core region of LDL, protected from encounter with a peroxyl radical in the outer aqueous phase [39]. This lifetime (estimated at about 12.5 s) is sufficiently long that it will eventually abstract a hydrogen from a *bis*-allylic methylene from linoleic or arachidonic acid, creating a fatty acid radical and subsequently a peroxyl radical that will start a new autoxidation chain reaction [12]. This phenomenon is known as α-tocopherol-mediated peroxidation (TMP). TMP does not occur in normal solution phase autoxidation because the tocopheroxyl radical will react with any other radical before it will perform a hydrogen abstraction. TMP has been studied in great detail because understanding the chemistry of LDL oxidation has important implications for the potential benefits of α-tocopherol in the treatment or prevention of atherosclerosis [35, 40].

Vitamin C (ascorbate) has proven to be highly protective against TMP [12]. Interestingly, this protective effect is not simply due to its ability to reduce (and restore) the tocopheroxyl radical back to α-tocopherol, but mainly due to its ability to export the radical out of the LDL particle. The reaction between ascorbate and the tocopheroxyl radical is so facile that it can occur across the phase boundary of the water-lipid system, *i.e.*, it is not necessary for vitamin C to enter the LDL particle to react with the tocopheroxyl radical [41]. The ascorbyl radical can be restored to ascorbate by reaction with the ubiquitous selenoenzyme thioredoxin reductase and other NADPH-dependent reductases [42]. Radical export is the irreversible transfer of the radical into the outer aqueous phase, and an LDL particle that does not contain a radical is protected from further autoxidation. With relevance to atherogenesis, this also implies that as soon as the lipoprotein leaves the plasma (containing vitamin C) the protective mechanism of the vitamin E/vitamin C pair is interrupted, and LDL becomes much more prone to oxidation. This, for example, is the case after LDL enters the intimal space of the arterial wall. Radical export can also be achieved by the minor antioxidant in the LDL particle, ubiquinol-10, albeit by a more indirect mechanism. The tocopheroxyl radical readily reacts with ubiquinol-10 but ubiquinol-10 and its radical are too lipophilic to leave the LDL particle. Instead, the ubiquinol-10 radical is assumed to react with molecular oxygen to give a water-soluble superoxide radical and the restored ubiquinol-10 [12]. The water-soluble superoxide radical then can leave the lipid particle. Radical export demonstrates the significance of co-antioxidants in maintaining the antioxidant properties of α-tocopherol [43, 44].

Discovery of the phenomenon of TMP has led researchers to hypothesize that the LDL particle would actually be perfectly protected from autoxidation if it did not contain the "antioxidant" α-tocopherol [12]. In line with this thinking, it has been shown that the rate of autoxidation of LDL decreases once all of the α-tocopherol has been depleted [15]. Furthermore, *in vitro* and *in vivo* α-tocopherol-depleted LDL was highly resistant to peroxidation initiation under mild radical flux conditions [40]. But α-tocopherol has even a second role to act as a villain in LDL oxidation: due to its phase transfer character it can transport a radical into the lipoprotein, and therefore act as an initiator of an autoxidation chain [15].

The rescue for α-tocopherol as an antioxidant in LDL came when a careful examination of the nature of the peroxyl radical initiator in TMP was conducted [45]. In this study, the effect of two different types of peroxyl radicals was studied: the conventionally used tertiary alkylperoxyl radicals and the superoxide anion radical. Only the superoxide anion radical is physiologically relevant as a peroxyl radical (it is supposed to be produced in an amount of ≈10 kg/year per adult [45]) while the alkylperoxyls used previously have no equivalent in human physiology. Both were generated from synthetic precursors that decay into the radicals at a defined rate of reaction. Once again, the results were unexpected: only a small fraction of the superoxide radicals reacted with LDL and the rate of peroxidation in the presence of α-tocopherol was slower than with an equivalent amount of alkyl peroxyls which showed the expected behavior in initiating TMP [45]. In other words, when the oxidation of LDL was initiated by the physiological superoxide radical, α-tocopherol acted as an antioxidant, and also the rate of peroxidation of LDL increased after α-tocopherol was consumed. These findings were explained by the lower efficiency of the superoxide radical in initiating TMP, and its higher efficiency in terminating TMP [45].

There is also a second possibility by which α-tocopherol can exert a pro-oxidant activity. This comes from its ability to reduce transition metal ions to their lower oxidation states, *e.g.*, Fe^{2+} and Cu^{+}. The reduced metal ions have a higher rate of reaction with hydroperoxide and hydrogen peroxide than the oxidized forms [38]. The reactions can result in homolytic cleavage of the hydroperoxides and lead to the generation of reactive alkoxyl radicals.

Is TMP a relevant factor for the oxidation of LDL in atherogenesis, in other words, does α-tocopherol act as an anti- or pro-oxidant *in vivo*? This question is difficult to answer because even in the *in vitro* settings a slight shift in the

experimental conditions can cause α-tocopherol to change from anti- to pro-oxidant activity [40]. The attempts to study TMP *in vivo* are necessarily of indirect nature. For example, intact α-tocopherol was isolated from advanced atherosclerotic plaques that contained large amounts of oxidized lipids implying lipid oxidation in the presence of α-tocopherol [46, 47]. On the other hand, preferential isolation from advanced carotid lesions of hydroperoxides with *cis*,*trans*-configuration of the double bonds rather than *trans*,*trans* [35, 46] implies that α-tocopherol is functioning as an antioxidant because *trans*,*trans*-configurated hydroperoxides are typical products of un-inhibited fatty acid autoxidation (*cf.* Section 2.3 and [11]). This result, however, again suggests that lipid peroxidation can occur in the presence of α-tocopherol [35]. At this point, there is no conclusive answer to the role of α-tocopherol in the oxidation of LDL *in vivo*. The potential relevance of TMP in atherosclerosis and for the outcome of clinical trials has been discussed in great detail in two recent reviews by Stocker and colleagues [35, 48].

3 Uptake and transport of vitamin E

3.1 Uptake of vitamin E

Vitamin E is taken up as the free alcohol form (*i.e.*, 6-hydroxyl) by the intestine without discrimination of the individual isomers. No significant difference in the absorption kinetics between free α-tocopherol, and its acetate or succinate esters was observed following oral administration [49]. The esterified forms of α-tocopheryl (as the acetate, succinate, nicotinate, or phosphate) commonly present in dietary supplements are hydrolyzed by the pancreatic carboxyl ester hydrolase in the intestine in a bile acid-dependent reaction [26, 27, 50].

Studies on the uptake and transport of vitamin E were greatly facilitated by the availability of deuterated analogs of natural *R*,*R*,*R*- and racemic α-tocopherol, and other isomers [51]. Direct comparison of the distribution of particular tocopherol isomers with different degrees of deuterium content enabled parallel monitoring of their uptake and transport using GC-MS. This methodology was initially used to determine the biokinetics of *R*,*R*,*R*- and *S*,*R*,*R*-α-tocopherol acetates in rats showing the preferential uptake of the natural diastereomer by all tissues with the greatest discrimination occurring in the brain [51]. There was no chiral discrimination for the transport of α-tocopherol from plasma into red blood cells, but preferential retention of the natural *R*,*R*,*R*-isomer [52]. Using single doses of tri- and hexa-deuterated *R*,*R*,*R*-α-tocopherol it was then shown that newly absorbed α-tocopherol is secreted by the intestine into chylomicrons, and chylomicron remnants are then taken up by the liver from which α-tocopherol is secreted into VLDL [53]. Metabolism of VLDL results in the delivery of α-tocopherol into LDL and high-density lipoprotein (HDL) [53, 54]. Assembly of VLDL in the liver appeared to be the step of chiral discrimination between *R*,*R*,*R*- and *S*,*R*,*R*-α-tocopherol implicating an active transport mechanism [55] (*cf.* Section 3.4 below for further discussion). These early studies on the absorption, transport, and lipoprotein secretion of α-tocopherol have been discussed in an excellent review (see [56]).

3.2 Isolation and cloning of an α-tocopherol transfer protein

A protein with the ability to bind α-tocopherol was first identified in rat liver and subsequently purified from the same source [57 – 60]. The rat α-tocopherol transfer protein (αTTP) has a molecular mass of 31 kDa; the corresponding cDNA was isolated from a rat liver cDNA library, and the open reading frame encodes a protein of 278 amino acids with a calculated molecular mass of 31 845 kDa [61]. By Northern and Western analyses αTTP was initially thought to be exclusively expressed in the liver, but low levels of the mRNA were later also detected in brain, spleen, lung, and kidney of the rat [62]. An *in vitro* assay for the transfer of tocopherols by αTTP between membranes gave for the degree of competition (*R*,*R*,*R*-α-tocopherol = 100%): β-tocopherol, 38%; α-tocotrienol, 12%; γ-tocopherol, 9%; *S*,*R*,*R*-α-tocopherol, 11%; δ-tocopherol, 2%; and α-tocopherol acetate, 2% [63]. It was noted in this study that there was a strong correlation between the affinity of individual tocopherol isomers and their activity in the rat resorption-gestation assay, revealing binding to αTTP as a critical determinant for the biological activity of vitamin E isomers [63]. Thus, αTTP is the major responsible protein involved in the transfer of α-tocopherol into nascent VLDL in the liver [64].

3.3 Structure of αTTP

The human αTTP is also a cytosolic protein of 278 amino acids, and, as expected, it is mainly expressed in the liver [65]. Based on sequence analysis αTTP is a member of the SEC14-like protein family comprising cellular retinaldehyde binding protein (CRALBP) [66], supernatant protein factor (SPF) [67], and the phosphatidylinositol/phosphatidylcholine transfer protein (SEC14p) [68]. All of these proteins exhibit one or more so-called CRAL-TRIO lipid-binding domains (named after the two prototypical examples) which are generally involved in the intracellular trafficking of hydrophobic molecules [69]. The crystal structure of human αTTP reveals a globular protein with an *N*-terminal three-helix coil and the *C*-terminal CRAL-TRIO lipid-binding domain [70, 71]. Interestingly, two conforma-

tions of the protein were observed, an open form probably representing a membrane-bound conformation, and a closed tocopherol-transport form, in which a mobile helical surface segment seals the hydrophobic binding pocket [70]. The ligand-binding pocket provides an extensive hydrogen-bonding network to snugly fit the chromanol ring of α-tocopherol while the phytyl side chain is acquiring a U-shape within the pocket [71]. A substitution pattern at the chromanol ring different from α-tocopherol will reduce the number of methyl groups and therefore diminish binding affinity through the decrease of hydrophobic van der Waals interactions. Likewise, a change in the C-2 stereocenter from the natural 2*R* configuration to 2*S* would require a major rearrangement of the binding pocket and therefore diminish binding affinity [71].

3.4 Mechanism of α-tocopherol secretion into VLDL

How does αTTP mediate the secretion of α-tocopherol into VLDL? Early studies had suggested that α-tocopherol secretion is coupled to VLDL secretion, implying that α-tocopherol may be packaged into nascent VLDL within the liver cell, and VLDL subsequently is exocytosed *via* the Golgi apparatus [72, 73]. Using the inhibitor brefeldin A that disrupts intracellular transfer and secretion of proteins *via* the Golgi apparatus, it was recently shown that secretion of α-tocopherol was not affected by the inhibitor [74]. The authors concluded that αTTP-mediated secretion of α-tocopherol was not coupled to VLDL secretion but used a novel, non-Golgi mediated pathway [74]. In line with this hypothesis, it was shown that the ATP-binding cassette transporter ABCA1 mediates cellular excretion of α-tocopherol in hepatocytes [75]. It has now been hypothesized that the role of αTTP is not in the secretion, but in the cytosolic transport of α-tocopherol from late endosomes (where the circulating chylomicron remnants and lipoproteins containing α-tocopherol are deposited) to the plasma membrane where α-tocopherol is then exocytosed by the ABCA1 transporter [76]. This assumption was prompted by the identification of a liver-specific target protein on the late endosome surface that facilitated transient binding of αTTP to the late endosome [76]. Binding specifically occurred in hepatoma cell lines and cultured hepatocytes, and it was specific for αTTP since other members of the SEC14p family did not bind to late endosomes. A unique stretch of 30 amino acids near the *N*-terminus of αTTP was found to be responsible for binding [76].

3.5 αTTP in reproduction

By virtue of its discovery as the agent that prevented resorption of gestation in female rats, vitamin E has been linked to fertility [77]. The consensus understanding since has been that vitamin E acts as an antioxidant for protection of the fetus during a time when it is most vulnerable by oxidative stress. Consequently, αTTP expression in pregnant mice has been localized by immunohistochemistry to the secretory columnar epithelium, the glandular epithelial cells, and the inner decidual reaction zone surrounding the implantation site [78]. In female αTTP -/- mice the placentas of pregnant animals were severely impaired with marked reduction of labyrinthine trophoblasts; the embryos of αTTP -/- females died at mid-gestation [79]. Excess use of dietary α-tocopherol or of a synthetic antioxidant could rescue placental failure and allowed full-term pregnancies [79]. Male αTTP -/- mice were found to be fertile [79]. In human placenta, αTTP is expressed in syncytiotrophoblasts, in villous and invading extravillous cytotrophoblasts, in the glandular epithelium of the deciduas, and in the mesothelial layer of the secondary yolk sac [80, 81]. αTTP also showed nuclear staining in trophoblasts and in amnion cells, possibly indicating a function of αTTP beyond the expected transfer of α-tocopherol to the fetus [82].

3.6 α-Tocopherol-associated protein and SPF

SPF is a member of the SEC14-like protein family assisting in the epoxidation of squalene by squalene monooxygenase through an incompletely understood mechanism [83]. Epoxidation of squalene is the first oxidative step in cholesterol biosynthesis. SPF has been shown recently to bind *R*,*R*,*R*-α-tocopherylquinone [84], the main oxidation product of α-tocopherol [85], thus providing a possible link of the antioxidant activity of α-tocopherol and cellular cholesterol synthesis. The binding affinity of SPF for α-tocopherylquinone was eightfold higher than for α-tocopherol while binding attempts with squalene were unsuccessful [84]. SPF has independently been identified and cloned as the 46-kDa tocopherol-associated protein (TAP) [86]. Recent results from competition binding assays, however, revealed that SPF has highest affinity (K_d) for phosphatidylinositol (216 nM) and γ-tocopherol (268 nM) while α-tocopherol (615 nM) and squalene (879 nM) bound significantly weaker [69]. These findings have since raised some concern whether binding of α-tocopherol by SPF was specific enough to call it a true ligand, or whether binding was merely due to association of the lipophilic molecule with the somewhat promiscuous CRAL-TRIO lipid binding domain of SPF [87].

3.7 Other tocopherol-binding proteins

Several other proteins with binding capacity for α-tocopherol have been described. The 15 kDa tocopherol-binding protein has a preference for α-tocopherol over δ- and

γ-tocopherol and may be involve in intracellular transport of α-tocopherol [88, 89]). The scavenger receptor class B type 1 transfers vitamin E into the cell [90 – 92]. Finally, the human plasma protein afamin, the fourth member of the albumin gene family, was shown to be a specific binding protein both for α- and γ-tocopherol (K_d = 18 μM) with multiple binding sites [93, 94]. It was speculated that afamin may take over the role of vitamin E transport under conditions where the lipoprotein system is insufficient [94].

4 Vitamin E deficiency

4.1 Animal models of dietary vitamin E deficiency

Experimental vitamin E deficiency in laboratory animals led to muscular dystrophy and encephalomalaecia in chickens [95, 96] and neurologic lesions in rats [97 – 100]. Recently, Burk and colleagues [101] have shown that combined dietary deficiency of selenium and vitamin E caused fatal myopathy in Guinea pigs. About half of the animals on the vitamin E and selenium double-deficient diet had to be euthanized after one month due to severe myopathy. Muscle necrosis was severe in these animals while it was minimal in the animals on the only vitamin E-deficient diet, and absent in controls [101]. When dietary vitamin E deficiency was combined with vitamin C deficiency in Guinea pigs, a distinct clinical syndrome was observed [102]. While about one-third of the animals on the double-deficient diet died within the first nine days of the study, others became paralyzed in the hind limbs and within a few hours showed progression of paralysis over all limbs and difficulty in breathing requiring euthanasia. Lesions in muscles or the nervous system of these animals were not observed. An increase in oxidative injury in the central nervous system was suggested to cause the syndrome [102]. In both cases the clinical effect was much more severe when vitamin E deficiency was combined with absence of either selenium or vitamin C implying a synergistic antioxidant effect between vitamin E and selenium and/or vitamin C *in vivo* [102].

4.2 αTTP-deficient mice

Lack of a functional αTTP gene in mice caused a delayed onset of ataxia and retinal degeneration after one year of age [103]. The brains of αTTP -/- animals were depleted of α-tocopherol, and lipid peroxidation was increased, especially in degenerating neurons. Although the neurological phenotype was more severe than that caused by an α-tocopherol-deficient diet, it was overcome almost completely by dietary supplementation with α-tocopherol [103]. In addition, αTTP -/- mice showed enhanced inflammatory response upon stimulation with lipopolysaccharide [104]. As expected from the lack of a functional αTTP in the liver, αTTP -/- animals accumulated dietary α-tocopherol in the liver but were depleted of α-tocopherol in peripheral tissue [105].

In order to study the influence of vitamin E deficiency on the progression and severity of atherosclerosis, αTTP -/- mice were crossed with apoE -/- mice [106]. The double knockouts showed increased levels of isoprostanes as markers of lipid peroxidation and also increased severity of atherosclerotic lesions in the proximal aorta [106]. These findings implied a protective for vitamin E in an animal model that is susceptible to atherosclerosis [106].

4.3 Ataxia with isolated vitamin E deficiency (AVED)

Dietary deficiency of vitamin E almost never occurs in humans. Only certain diseases of fat malabsorption are connected to vitamin E deficiency [107]. Deficiency in vitamin E causes severe, debilitating spinocerebellar lesions. A very rare form of vitamin E deficiency, however, that is not caused by fat malabsorption is an autosomal recessive neurodegenerative disease called ataxia with isolated vitamin E deficiency (AVED). Patients with isolated vitamin E deficiency have an impaired ability to incorporate α-tocopherol into lipoproteins in the liver, and usually show symptoms of spinocerebellar dysfunction before adolescence [108].

The first evidence that AVED was connected to dysfunctional incorporation of α-tocopherol into nascent VLDL in the liver came in 1990 when Traber and co-workers [73] showed that deuterated α-tocopherol was taken up normally by the patients but could not be retained in the plasma. This finding was a major step forward in identifying the functional significance of a putative αTTP, and it also laid the basis for studying the molecular mechanisms of AVED.

In 1995, two groups reported the identification of mutations in the gene coding for αTTP [108, 109]. One mutation identified in a 60-year old Japanese patient changed a histidine to a glutamine at position 101 resulting in a protein that had only about 11% of the wild-type transfer activity [108]. The second group identified three frame-shift mutations in the αTTP gene in families in North Africa and Italy [109]. Since 1995, many more mutations have been described [110]. Morley and co-workers [111] studied six missense mutations with regard to their affinity for *R*,*R*,*R*-α-tocopherol and their ability to catalyze tocopherol transfer between membranes. Although three of the mutations were associated with a severe clinical phenotype, the *in vitro* tocopherol transfer activity of the recombinant purified mutant proteins was reduced only 2- to 3-fold. Furthermore, three mutations causing milder forms of AVED, gave mutant αTTP proteins that were remarkably similar to the wild-type protein in the *in vitro* assays [111]. These find-

ings led the authors to suggest that the AVED syndrome may not arise from an inability of αTTP to bind or transfer α-tocopherol, but rather from defects in other activities of the protein [111].

Patients with AVED have extremely low plasma levels of α-tocopherol which can be elevated by dietary vitamin E supplementation [73]. In some patients this led to a stabilization of the neurologic functions and even to an improvement in others [112, 113]. The focus of clinical trials on the beneficial effects of vitamin E supplementation on chronic diseases like atherosclerosis sometimes overlooks the fact that vitamin E is an essential nutrient. Its requirement for human health is clearly demonstrated by the severe pathologies caused by vitamin E deficiency disorders in humans.

5 Vitamin E: more than an antioxidant

There is now emerging evidence that individual tocopherols have physiological activity beyond their action as biological chain breaking antioxidants, and some of these roles will be discussed in the following.

5.1 Direct effects of α-tocopherol on enzymatic activities

In 1991 it was discovered that α-tocopherol could inhibit the action of protein kinase C (PKC) in vascular smooth muscle cells leading to growth arrest [9]. This finding appeared to be among the first experimental setting where vitamin E did not exhibit a function as a biological antioxidant but rather in modifying the enzymatic activity of an important player in cellular signaling. Two findings made this a compelling discovery: (i) the effect was specific for α-tocopherol whereas β-tocopherol, the synthetic analog trolox, and α-tocopherol esters had no such effect, and (ii) the effect occurred at concentrations of α-tocopherol that are physiological in healthy adults (25 μM) [9, 114]. It was shown in the following that inhibition of smooth muscle cell proliferation was not a direct effect on PKC but a stimulatory effect on protein phosphatase 2A (an enzyme that cleaves phosphate groups from proteins) which led to increased dephosphorylation of PKC and therefore to its inactivation [115].

α-Tocopherol (≈50 μM) also inhibited the respiratory burst (formation of superoxide) by 40% in human monocytes by impairing the assembly of the NADPH-oxidase enzyme complex at the membrane. Translocation and phosphorylation of the cytosolic factor $p47^{phox}$ were reduced in monocytes preincubated with α-tocopherol but not with β-tocopherol [8]. Reduced phosphorylation of $p47^{phox}$ was suggested to result from a decrease in PKC activity [8].

5.2 Nonantioxidant functions of α-tocopherol in the prevention of atherosclerosis

An early step in the development of atherosclerosis is the accumulation of oxidized LDL in the arterial wall [116]. Oxidized LDL is recognized and scavenged by specific scavenger receptors on macrophages. Uptake of oxidized LDL transforms macrophages into lipid-laden foam cells that deposit as fatty streaks on the artery wall [117, 118]. The traditional view of the possible role of α-tocopherol in the prevention of atherosclerosis is to act as a biological antioxidant and thus inhibit the initial oxidation of the LDL particle [33, 34]. The protective role as an antioxidant has been challenged by the discovery of the phenomenon of TMP [35]. The discovery of additional, nonantioxidative functions of α-tocopherol have emerged into a branch of research that is focused on defining a role for vitamin E in the prevention of atherosclerosis that is independent of its role as an antioxidant. This research is focusing on at least four events in early atherogenesis: (i) inhibition of monocyte-endothelial cell adhesion, (ii) inhibition of platelet adhesion and aggregation, (iii) inhibition of formation of inflammatory mediators by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), and (iv) the inhibition of the scavenger receptors SR-A and CD36.

(i) Inhibition of monocyte-endothelial cell adhesion. Adhesion of monocytes (macrophages) to aortic endothelial cells is one of the earliest events in the development of atherosclerosis [119]. Monocyte recruitment is regulated by two types of endothelial cell-derived adhesion molecules, soluble intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [119]. Presupplementation of endothelial cells with physiological concentrations of α-tocopherol (19 μM, [10]) led to a significant reduction of monocyte adhesion (42%), which was at least in part due to downregulation of ICAM-1 [10] and VCAM-1 [120, 121].

(ii) Inhibition of platelet adhesion and aggregation. Several studies have indicated that vitamin E may have a positive effect on reducing platelet adhesion and aggregation which contribute to intra-arterial thrombus formation. A clinical trial with 400 IU vitamin E per day led to a significant increase in the EC50 for thrombin-induced platelet aggregation following six weeks of oral supplementation compared to placebo [122]. It was shown later that lower intake (75 IU/day) was sufficient to achieve a maximal beneficial effect on platelet aggregation [123]. In a study monitoring induced lipid peroxidation in Sprague-Dawley rats, γ-tocopherol was significantly more potent than α-tocopherol in decreasing platelet aggregation [124]. Similar results were observed in a small study with 46 subjects where the group receiving a mixture of α- and γ-tocopherol showed a significant increase in NO release and decrease in ADP-induced

platelet aggregation compared to controls receiving placebo or the group receiving only α-tocopherol [125]. A likely mechanism for the antiplatelet action of vitamin E is increase of nitric oxide release *via* the inhibition of PKC-dependent phosphorylation of endothelial nitric oxide synthase [126, 127].

(iii) Inhibition of COX-2 and 5-LOX. Atherosclerosis is an inflammatory disease, and expression of COX-2 and 5-LOX in activated macrophages may contribute to the progression of the disease by synthesis of inflammatory mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4). The expression of 5-LOX during various stages of atherosclerotic lesions has been demonstrated in macrophages, dendritic cells, foam cells, mast cells, and neutrophilic granulocytes using immunohistochemistry and real-time PCR [128]. Whether COX-2 plays a harmful role or maybe no role at all in the progression of atherosclerosis is currently matter of intense debate (for a recent review, see [129]). The available evidence from studies using either LDL-receptor deficient or ApoE-deficient mice as models of atherosclerosis and selective or nonselective COX-2 inhibitors points toward an involvement of COX-2 in promoting early atherosclerosis [130]. In advanced lesions COX-2 expression in macrophages is downregulated [131], and it appears that the effect of COX-2 on atherogenesis might be attenuated as shown by the lack of a beneficial effect of treatment with selective COX-2 inhibitors in advanced stages of the disease [132]. These studies imply that inhibition of either enzyme could have an attenuating effect on the inflammatory component of atherosclerosis.

Jiang and co-workers [133] reported that γ-tocopherol reduced the synthesis of PGE_2 in lipopolysaccharide-treated RAW264.7 macrophages and in IL-1β-treated A549 lung epithelial cells with an apparent IC50 of 7.5 and 4 μM, respectively. Treatment with lipopolysaccharide or IL-1β leads to the induction of COX-2 expression in these cells. In contrast, only high concentrations of α-tocopherol (50 μM) reduced the formation of PGE_2 slightly in the macrophages (by 25%) but not in the epithelial cells. This effect was found to be due to inhibition of the activity of COX-2 by γ-tocopherol because expression levels and substrate availability were unchanged [133]. It was then shown that vitamin E could also reduce COX-2 activity in aged mice, and this effect was attributed to the scavenging by vitamin E of peroxynitrite, an endogenous mechanistic activator of COX-2 [134]. This is compatible with findings by Christen and co-workers [135] showing that γ-tocopherol is a better scavenger for peroxynitrite than α-tocopherol.

There have also been a few reports on the inhibition of 5-LOX activity in monocytes [136, 137]. Activated human monocytes showed a significant reduction in the levels of the 5-LOX metabolite LTB_4 and subsequent reduction of IL-1β (45% and 66% inhibition) upon treatment with α-tocopherol (50 and 100 μM, respectively), and this effect could be reversed by the addition of exogenous LTB_4 (100 nM) [136]. *In vivo* formation of LTB_4 in carrageenan-induced inflammation in male Wistar rats was reduced significantly (70%) by γ-tocopherol (33 mg/kg) [137]; administration of equivalent doses of α-tocopherol showed no significant effect. In the same model, γ-tocopherol also reduced the levels of PGE_2, total nitrate/nitrite, TNFα, and attenuated the inflammation-mediated damage [137]. The mechanism of inhibition of 5-LOX activity has not yet been elucidated.

Taken together there are potential roles for γ-tocopherol in the attenuation of atherosclerosis [138]. When considering these possibilities, it has to be kept in mind for clinical trials that common vitamin E supplements consist either of synthetic (a mixture of all eight stereoisomers) or natural α-tocopherol, in free form or esterified, but usually do not contain any the other tocopherol isomers. Furthermore, supplementation of the diet with α-tocopherol reduces serum concentrations of γ- and δ-tocopherol [139] which partially explains why vitamin E taken up through the diet may be more beneficial than supplementation with a pill [140].

(iv) Inhibition of SR-A and CD36. α-Tocopherol inhibits the uptake of oxidized LDL into monocyte-derived macrophages by a mechanism that involves downregulation of mRNA and protein expression of the scavenger receptors SR-A (at 10–50 μM α-tocopherol) [141] and CD36 (at 50 μM) [142, 143]. CD36 is expressed on the surface of adipocytes, microvascular endothelial cells, and macrophages. The receptor functions in recognition and scavenging of oxidized lipoproteins, and therefore participates in the transformation of macrophages into foam cells [144]. Mice deficient in CD36 showed a 70–80% reduction in size of aortic lesions proving the important role CD36 plays in the etiology of atherosclerosis [145].

5.3 Regulation of gene transcription

In addition to the downregulation of transcription of the scavenger receptors SR-A and CD36 α-tocopherol (and not β-tocopherol) also weakly induced expression of connective tissue growth factor (CTGF) [146] and α-tropomyosin [147]. The two targets were identified using gene expression arrays or a differential display technique, respectively, in the mRNA of treated *versus* untreated cells. Recently, TAP was identified as a ligand-dependent transcription factor [86, 148]. Binding of α-tocopherol (50 μM) led to nuclear translocation of TAP. Again, only α-tocopherol was an active ligand, while the β-, γ-, and δ-isomers were found to be less active in competition for binding and in reporter

gene assays [148]. Whether TAP is involved in the transcriptional regulation of SR-A, CD36, CTGF, or α-tropomyosin has not yet been established.

5.4 Roles in cancer

In 1982, α-tocopheryl succinate (α-TS) was the first vitamin E analogue for which a role in growth inhibition of cancer cells was demonstrated [149]. To date this effect has been shown for numerous additional cancer cell lines [150]. The growth inhibitory effect appears to be specific for cancer cells while the proliferation of nonmalignant cells is apparently not inhibited [151, 152]. The molecular action of α-TS includes the induction of apoptosis by inhibition of PKC *via* increasing PP2A activity [153]. In the PC-3 human prostate cancer cell line α-TS was more efficient than racemic α-tocopherol and α-tocopheryl acetate in growth inhibition. In these cells, induction of apoptosis through the mitochondrial pathway was shown by poly(ADP-ribose) polymerase cleavage and activation of pro-apoptotic caspases [154]. Additional treatment with the selenium agent, methylseleninic acid, resulted in a synergistic effect [154]. A similar apoptotic response *via* activation of the mitochondrial pathway was observed in MDA-MB-435 human breast cancer cells upon treatment with α-TS [155, 156]. Other groups have reported that α-TS induced apoptosis through destabilization of lysosomal membranes [157], and through restoring TGF-β and Fas signaling pathways [158].

α-TS acted as a apoptogenic compound in malignant mesothelioma, a fatal type of neoplasia that is resistant to apoptosis [159]. The cells are also resistant to the immunological apoptogen TRAIL (TNF-related apoptosis-inducing ligand). Treatment of malignant mesothelioma cells with α-TS induced apoptosis, and also induced sensitivity to TRAIL in the presence of α-TS at concentrations that were subapoptotic [159]. It was concluded that α-TS and TRAIL act synergistically to kill malignant mesothelioma cells via the mitochondrial pathway [160], while nonmalignant mesothelioma cells were not affected [159]. A synergistic effect of α-TS and TRAIL in the suppression of tumor growth was also shown *in vivo* using mouse HCT116 colon cancer cell xenografts [161].

Using three different prostate cancer cell lines, Zhang and co-workers [162] showed that α-TS inhibited cell invasiveness at 20 μM concentration without altering cell survival, cell cycle, adhesion, or motility. The inhibition of invasiveness was attributed to the reduction of levels of matrix metalloproteinases, key enzymes in the proteolysis of the basement membrane during invasion. Interestingly, this effect was only observed in PC-3 and DU-145 cells while LNCaP cells which are poorly invasive and do not express matrix metalloproteinases were not affected [162].

It appears that the succinate ester moiety is required for the proapoptotic effect since the two hydrolysis products α-tocopherol and succinic acid are inactive in the assays [163 – 165]. It has been shown that also the malonate and oxalate esters of α-tocopherol are inducers of apoptosis in vascular smooth muscle cells and in the mouse breast cancer cell line C127I [165]. In a very detailed structure-function analysis a variety of further α-TS analogues were tested as proapoptotic agents in malignant cell lines [166]. For example, methylation of the free carboxylate of the succinyl group abolished apoptogenic activity; maleate esters were more active while glutarate esters had reduced activity, and neither phytyl nor oleyl succinate caused apoptosis [166]. A nonhydrolyzable ether acid analogue of α-tocopheryl succinate has been synthesized and tested, and it was found to be pro-apoptotic, providing another line of evidence that the nonhydrolyzed tocopherol derivative is the active agent [163, 167]. This analogue, α-TEA (2,5,7,8-tetramethyl-(2*R*-4′*R*,8′*R*,12-trimethyltridecyl)chroman-6-yloxy acetic acid) was about three times more effective in inducing apoptosis in human ovarian and cervical cancer cell line, and it was not hydrolyzed by endogenous esterases [168].

Like with α-tocopheryl acetate, hydrolysis of α-TS is catalyzed by the bile acid-dependent pancreatic carboxyl ester hydrolase in the intestine [50]. The maximum rate for hydrolysis of α-tocopheryl acetate was more than 20 times higher than for hydrolysis of α-TS [50], and it appears also that intact α-TS can be absorbed by the intestine [26, 169]. Since comparative studies on the absorption of free and esterified α-tocopherol in humans, however, showed no discrimination between these forms, it was implied that vitamin E esters are completely hydrolyzed prior to intestinal absorption [49]. Whether dietary α-TS could actually reach peripheral cancerous tissue intact and in levels necessary for the anti-cancer effect, appears to be an open question. A definite answer to resolve this issue will require further investigation.

The anti-cancer effects of γ-tocopherol include the inhibition of cell cycle progression of DU-145 prostate carcinoma cells *via* downregulation of cyclins D1 and E [170]. A similar effect of downregulation of cyclin-related signaling by γ-tocopherol (1 – 10 μM), and to a lesser extent by α-tocopherol (50 μM), was shown in the PC-3 prostate cancer cell line [171]. A second anti-cancer role for γ-tocopherol is its ability to scavenge the mutagenic oxidant peroxynitrite by forming stable carbon-centered adducts through the nucleophilic 5-position, which is blocked by a methyl group in α-tocopherol [135].

In summary, there is much stronger *in vitro* evidence for α-TS and γ-tocopherol than for α-tocopherol in the prevention and treatment of cancer [172, 173]. However, there are also confounding reports in the literature, for example, the sig-

nificant inhibition of mammary tumor growth and metastasis in a transgenic mouse model upon depletion of dietary vitamin E [174]. The reduction in the number of large primary mammary tumors and lung metastases was attributed to the increase in reactive oxygen species (caused by the lack of vitamin E) and subsequent apoptosis [174]. A very recent report describes the unexplained increase of 13 times of the standardized incidence ratio for renal cell carcinoma in workers in a vitamin A and E synthesis plant in France [175]. However, more workers in vitamin A than in vitamin E production were affected, and tumor development may be due to occupational exposure to any of the chemicals used in synthesis (more than 60), although none of these had previously been related to renal cell carcinoma [175].

A careful and detailed recent evaluation of available clinical trials testing the role of vitamin E in cancer prevention conducted over the past decades was reported by Sung and coworkers [176]. The evidence for a protective role of vitamin E is most convincing in carcinomas of the prostate and gastrointestinal tract [177 – 179]. These results, however, were not confirmed in a second large cohort study of 72 704 men, and there is now some notion that the protective role of vitamin E may be limited to smokers [180]. There was less supportive evidence for a beneficial role in breast, ovarian, lung, pancreatic, or urinary tract cancer; and there is also a large number of studies that failed to support a protective effect of vitamin E against cancer [176, 181 – 184]. A main corollary for most prospective and cohort trials was seen in the problem of attributing the observed effects to vitamin E supplementation or to confounding factors such as a generally healthier diet and lifestyle among participants taking vitamins [176]. Given the *in vitro* evidence accumulated for α-tocopheryl succinate and γ-tocopherol it is surprising to see that to date clinical trials have not addressed the efficacy of these compounds.

6 Clinical trials: a protective role of vitamin E in disease?

Clinical trials with large study populations have focused on assessing the effect of vitamin E on the prevention of diseases associated with oxidative stress (cardiovascular disease, atherosclerosis, Alzheimer's disease) and cancer (see above). These studies were prompted by epidemiologic evidence that implicates high dietary uptake of fruits and vegetables with a reduced risk of these diseases (despite the fact that fruits and vegetables by and large are not rich sources of vitamin E).

Several large randomized and placebo-controlled trials with populations between 2000 and ≈30 000 subjects have been reported: the ATBC study investigated 29 133 male Finnish smokers (50 mg α-tocopherol daily, or 20 mg β-carotene, or both) [185]; the CHAOS trial in the UK investigated 2002 patients (400 – 800 IU daily) [186]; the GISSI trial in Italy investigated 11 324 survivors of recent myocardial infarction (300 mg vitamin E daily) [187]; the HOPE trial investigated 9541 patients with known cardiovascular disease or diabetes (400 IU daily) [188]; and the HPS study in the UK investigated 20 536 subjects with known vascular disease (600 mg vitamin E, 250 mg vitamin C, and 20 mg β-carotene daily) [189]. All of these five studies were secondary prevention trials, *i.e.*, the enrolled patients were already at high-risk for recurring cardiovascular events. Primary prevention trials were the AREDS study (4757 subjects; 400 IU daily) [190], and the PPP study (4495 subjects; 300 mg synthetic α-tocopherol daily) [191].

The outcome of these studies and implications for vitamin E supplementation have since been discussed extensively and with controversy in a number of articles [35, 44, 192 – 199]. An overall impression of all the clinical trials and intervention studies is the apparent discrepancy between how straightforward the hypothesis is (vitamin E has a beneficial role as an antioxidant in preventing LDL oxidation, and therefore in cardiovascular disease) and how difficult it is to prove this hypothesis. Taken all the trials together, it appears that dietary supplementation with vitamin E as an intervention did not significantly decrease mortality, the risk of cardiovascular death, or cerebrovascular accident in the study populations [198].

7 Biosynthesis of vitamin E and its production in transgenic plants

The main source for dietary uptake of vitamin E is plant food (vegetables, fruits, seeds, and seed oils). Sunflower seeds, olive oil, and almonds are rich sources of α-tocopherol. While other seeds and seed oils generally contain more γ-tocopherol than α-tocopherol, the opposite is true for green leaves. β-Tocopherol and δ-tocopherol are the least abundant, and so, in general, are the different tocotrienols [200]. A web-page hosted by the US Department of Agriculture gives a detailed listing of the vitamin E content of various foods (http://www.nal.usda.gov/fnic/foodcomp/). A clear difference in the dietary habits is responsible for the higher consumption of α-tocopherol in the European diet while the American diet is richer in γ-tocopherol, due to the greater usage of plant oils in food preparation [201].

7.1 Strategies for enhancing the vitamin E content in plants

Advances in molecular biology and in the tools for generation of transgenic plants have fostered the idea of generating genetically modified plants with the goal of either

achieving high-level production for extraction of desired molecules or to improve the nutritional content of crop plants. The most publicized example for the latter efforts is the creation of the so-called "golden rice" by engineering the pathway for biosynthesis of provitamin A into the rice endosperm [202].

Like with other vitamins and dietary supplements, the worldwide market for vitamin E is large and steadily increasing. The market value for vitamin E-containing products was estimated at about one billion USD in 1995 [203]. More than 80% of the α-tocopherol production is by chemical synthesis (usually racemic; eight stereoisomers) and less than 20% are extracted as a mixture of γ-, δ-, and α-tocopherols from natural sources (mostly soybean oil) followed by methylation to yield the natural *R*,*R*,*R*-α-tocopherol. There is no doubt that the possibility of enrichment of crop plants with α-tocopherol or the other vitamin E isomers offers a huge prospect, not only with regard to the potential benefits for human health but also in a financial respect.

Enhancement of vitamin E levels in plants was approached by modern methods of plant breeding in corn [204], as well as by direct engineering of crucial enzymes of the biosynthetic pathway into transgenic plants [205 – 207]. A key prerequisite for metabolic engineering is the identification of the pertinent genes. Although the enzymatic reactions involved in the biosynthesis of tocopherols and tocotrienols were discovered in the 1970s using radiotracer methods [208, 209], the corresponding genes were cloned only within the past few years. Most of the enzymes are localized on the chloroplast membranes and therefore proved difficult to isolate and purify [210]. Some of the genes involved were initially identified using genomics-based approaches owing to the fact that they were part of an operon for biosynthesis of α-tocopherol in the blue-green algae (cyanobacterium) *Synechocystis* [205]. These sequences were subsequently used to identify the homologous sequences in *Arabidopsis*.

7.2 Biosynthesis of tocopherols and tocotrienols

Tocopherols and tocotrienols are synthesized *via* two converging pathways, fusing the head group and the side chain building blocks together (Fig. 4). All enzymes involved have now been cloned from *Arabidopsis* and the cyanobacterium *Synechocystis* (sp. PCC6803). The aromatic part of the chromanol ring is derived from the precursor homogentisic acid, formed by oxygenation of the tyrosine metabolite, *p*-hydroxyphenylpyruvate, by the enzyme *p*-hydroxyphenylpyruvate dioxygenase (HPD) (Fig. 4). HPD is a member of the large family of non-heme iron α-ketoglutarate-dependent dioxygenases [211]. In plants, the enzyme is involved in the biosynthesis of plastoquinone and tocopherols [212, 213], whereas in mammals it is involved in tyrosine and phenylalanine catabolism [214, 215]. HPD catalyzes a highly complex reaction, the oxidative decarboxylation of the 2-keto acid side chain of 4-hydroxyphenylpyruvate, the hydroxylation of the 1-position of the aromatic ring under 1,2-migration of the carboxymethyl group, and the overall consumption of one molecule of oxygen: one atom of oxygen is added as the hydroxyl group to the aromatic ring, the second atom becomes part of the carboxylic acid of the acetate side chain following decarboxylation [211]. The overall reaction yielding homogentisic acid shortens the pyruvate side chain to acetate, moves the acetate side chain into the *meta*-position relative to the original hydroxyl group, and adds the novel hydroxyl group at the *para*-position, the former position of the pyruvate side group. The HPDs from *Arabidopsis* and corn have recently been crystallized [216].

The second ring of the chromanol head group is partly derived from the side chain (Fig. 4). The phytyl side chain is an isoprenoid synthesized through the non-mevalonate pathway of isopentenyl pyrophosphate (IPP) formation in plastids (chloroplasts). IPP and its isomerization products dimethylallyl pyrophosphate are condensed to the C_{10} unit geranyl pyrophosphate; two more cycles of addition of IPP yield the C_{20} unit geranylgeranyl pyrophosphate (GGPP). GGPP is either used directly to synthesize the tocotrienols or, for formation of the tocopherols, it is reduced (hydrogenated) in the 6-, 10-, and 14-positions to the saturated phytyl pyrophosphate by GGPP reductase [217, 218]. Phytylpyrophosphate is also used for the biosynthesis of phylloquinone (vitamin K_1) in chloroplasts of higher plants; GGPP is the precursor for synthesis of the carotenes [209].

In the first committed step of tocopherol biosynthesis homogentisate and phytyl pyrophosphate are fused together by homogentisate prenyltransferase (HPT) to yield 2-methyl-6-phytylplastoquinone, the first tocopherol intermediate and common precursor to all tocopherols (Fig. 4) [206, 219, 220]. Tocotrienols result from the condensation of homentisate with geranylgeranyl pyrophosphate catalyzed by the enzyme homogentisate geranylgeranyl transferase (HGGT) [207]. Plant HPT and HGGT are specific for either phytyl pyrophosphate or geranylgeranyl pyrophosphate, respectively, while HPT from the cyanobacterium *Synechocystis* will utilize both substrates [219]. The prenyltransferases catalyze not only the fusion of the prenyl chain to the aromatic ring in the 6-position but also the decarboxylation of the acetate group, thus yielding a methyl group in the 2-position of the aromatic ring.

Following condensation with the prenyl side chain, transfer of a methyl group from *S*-adenosylmethionine to the 3-position of the aromatic ring may take place. A methyl group at

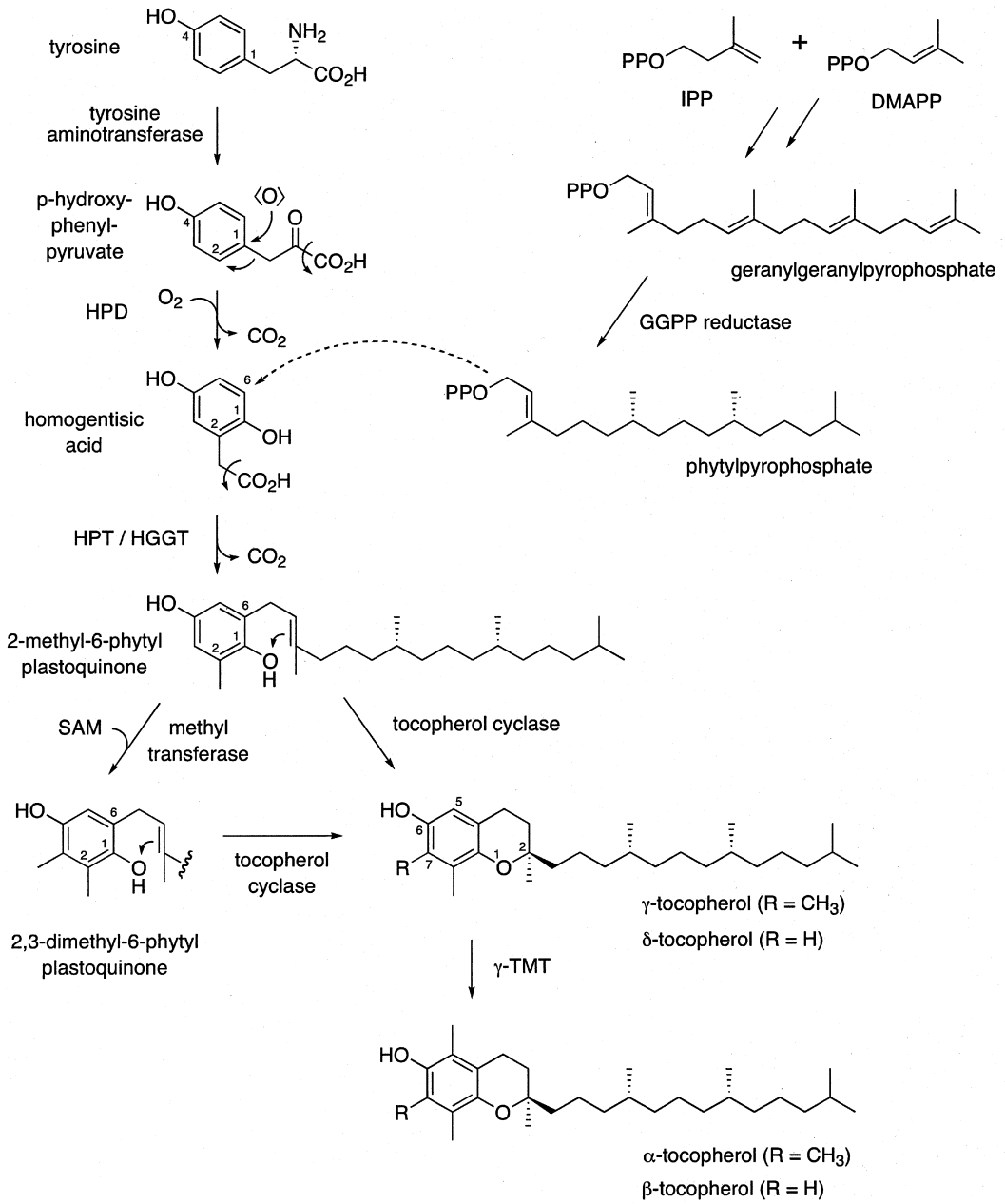


Figure 4. Biosynthesis of tocopherols and tocotrienols. Tocopherols and tocotrienols are biosynthesized in plants from two converging pathways, the catabolism of the amino acid tyrosine and isoprenoid biosynthesis *via* the non-mevalonate pathway in plastids. Metabolism of tyrosine by an amino transferase yields *p*-hydroxyphenylpyruvate. HPD catalyzes the most complex reaction in this pathway: (i) oxidative carboxylation of the pyruvate side chain to acetate, (ii) 1,2-migration of the acetate chain, (iii) hydroxylation of C-1 of the aromatic ring to yield the final product homogentisic acid. The prenyl side chain is formed from the condensation of dimethylallylpyrophosphate (DMAPP) and three units of IPP to form geranylgeranylpyrophosphate (GGPP). GGPP is reduced to phytylpyrophosphate by GGPP reductase. The first commited step toward tocopherol biosynthesis is the alkylation of the 6-position of the aromatic ring of homogentisic acid. Two transferases, homogentisate prenyltransferase (HPT) and homogentisate geranylgeranyl transferase (HGGT), catalyze the transfer of phytylpyrophosphate or GGPP, respectively, and also the decarboxylation of the 2-acetate group to a methyl group. Addition of the saturated phytyl chain leads to tocopherols, addition of the unsaturated chain to tocotrienols (not shown). The product 2-methyl-6-phytylplastoquinone can directly be cyclized by tocopherol cyclase to yield δ-tocopherol. Alternatively, prior methyl transfer to the 3-position yields 2,3-dimethyl-6-plastoquinone, and subsequently γ-tocopherol. Note that after the cyclase reaction the numbering system in the chromanol ring has changed compared to the aromatic ring. Final transfer of a methyl group to the 5-position of the chromanol ring by γ-TMT converts γ- and δ-tocopherol into α- and β-tocopherol, respectively. The four tocotrienols are formed by a corresponding pathway. SAM, *S*-adenosylmethionine.

the 3-position will ultimately yield γ- and α-tocopherol (γ- and α-tocotrienol, respectively); lack of this methyl group yields δ- and β-tocopherol (tocotrienol) (Fig. 1). This methylation reaction is catalyzed by 2-methyl-6-phytylbenzoquinone methyltransferase and yields 2,3-dimethyl-6-phytylplastoquinone [221, 222]. Regardless whether the 3-position of the aromatic ring is being methylated or not, the next step is the cyclization of the 2′,3′-*trans* double bond in the prenyl side chain with the 4-hydroxy group of the aromatic ring catalyzed by the enzyme tocopherol cyclase [223]. Tocopherol cyclase from *Arabidopsis* was found to be active with 2-methyl-6-phytylplastoquinone and with 2,3-dimethyl-6-phytylplastoquinone as well as with the (saturated) phytyl or (unsaturated) geranylgeranyl side chain making this a common enzyme in the biosynthesis of all tocopherols and tocotrienols [223]. The last step on the pathway to formation of α- and β-tocopherol is a final transfer of a methyl group to the 5-position of the aromatic ring of γ- and δ-tocopherol, respectively, catalyzed by the enzyme γ-tocopherol methyltransferase (γ-TMT) [205, 224, 225]. (The reader should keep in mind that after the cyclase reaction forming the chromanol ring the numbering system of the carbon atoms has to be changed, see Fig. 4.) γ-Tocopherol methyltransferase from *Arabidopsis* was found to be specific for methyl transfer to the 5-position only of the aromatic ring [205].

7.3 Development of transgenic plants

Several successful transgenic strategies have been reported for the enhancement of vitamin E levels by genetic engineering of plants. Since the first tocopherol-specific biosynthetic enzyme cloned was γ-tocopherol methyltransferase, Shintani and DellaPenna [205] overexpressed the gene for A.t. γ-TMT in *Arabidopsis* using a seed specific promoter and found that the transgenic seeds had a >80-fold increase in α-tocopherol levels at the expense of the γ-TMT substrate, γ-tocopherol. In control *Arabidopsis* seeds γ-tocopherol is the most abundant tocopherol present. Transgene expression had no influence on the total content of tocopherols in the seeds. Crossing the γ-TMT overexpressing line with a line overexpressing *Arabidopsis* HPT lead to a 12-fold increase in vitamin E activity relative to wild-type [226]. In the seeds of the doubly transgenic *Arabidopsis* lines, nearly all γ- and δ-tocopherol was methylated to α- and β-tocopherol [226].

Savidge and co-workers [206] cloned the gene for homogentisate phytyltransferase from *Arabidopsis*, and seed-specific overexpression led to a 2-fold increase in total tocopherol content. More recently, Cahoon and co-workers [207] overexpressed barley homogentisic acid geranylgeranyl transferase (which is involved in tocotrienol synthesis) in *Arabidopsis* and corn seeds and found a 10- and 6-fold increases in total vitamin E content, respectively, notably without reducing the amount of tocopherols in the transgenic seeds. Although all genes involved in vitamin E biosynthesis have been identified in the past years, transgenic strategies are still hampered by the lack of knowledge of regulation of these pathways [203].

Design of transgenic plants to enhance levels of vitamin E should be guided by the consideration that mainly α-tocopherol is biologically active as a vitamin E compound. Individual tocopherol isomers, however, may have particular physiological effects in human health and disease (see Section 5), and there is still only an incomplete understanding of all aspects of the actions of vitamin E; new effects may still be discovered. Both of these issues warrant that limitation or bias to enhancing levels of one isomer may limit the beneficial outcome. In any case, these limitations will be less dramatic than those that may result from consumption of dietary vitamin E supplements which are almost exclusively restricted to α-tocopherol, either as the natural isomer or the synthetic racemic mixture.

7.4 The role of vitamin E in plants

Finally, it is interesting to note that the physiological roles of vitamin E in plants are also far from being clear. As an antioxidant biosynthesized in the chloroplast envelope, an obvious function for α-tocopherol is the protection from lipid peroxidation of the photosynthetic complex or of seeds during storage, germination, and early development [209, 227, 228]. The knock-out mutation of tocopherol cyclase in maize (*sxd1*, *sucrose export defective 1*), however, caused malfunctioning of a specific set of plasmodesmata, gap junctions in plants that connect the cytoplasm of neighboring cells for the exchange of chemicals, proteins, and RNAs [229, 230]. This mutant was originally described without knowledge of its connection to vitamin E biosynthesis (the mutant lacks proper transport and accumulates anthocyanins, sugars, and starch in leaves) and SXD1 was thought to be involved in chloroplast-to-nucleus signaling in the bundle sheath essential for plasmodesmata formation [231]. In which way α-tocopherol and/or tocopherol cyclase could be connected to plasmodesmata functioning is completely unknown [223, 232].

8 Conclusion

Vitamins are essential nutrients required for human health, and prolonged deficiency in any vitamin will result in disease. To date, the only proven benefit of additional vitamin supplementation beyond satisfying this basic requirement is the reduction of the incidence of recurrent neural-tube defects by folic acid in pregnant women [195, 233].

Whether dietary supplementation with vitamin E has an additional beneficial outcome on human health is still an open debate. Results from intervention trials relying on relatively high doses of a single isomer of vitamin E have been inconsistent. Most of these supplements lack the potentially beneficial contribution of the other minor vitamin E isomers, and they lack the protective contribution of other micronutrients generally included in plant foods. Two statements from recent reviews exemplify this dilemma by reminding us that the risk of cancer associated with low fruit or vegetable consumption may only be exceeded by that of smoking [140], and that the most prudent public health advice remains to increase the consumption of plant foods, as such dietary patterns are associated with reduced risk of chronic disease [234].

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