The European perspective on vitamin E: current knowledge and future research1,2

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ABSTRACT
Vitamin E is indispensable for reproduction in female rats. In humans, vitamin E deficiency primarily causes neurologic dysfunctions, but the underlying molecular mechanisms are unclear. Because of its antioxidative properties, vitamin E is believed to help prevent diseases associated with oxidative stress, such as cardiovascular disease, cancer, chronic inflammation, and neurologic disorders. However, recent clinical trials undertaken to prove this hypothesis failed to verify a consistent benefit. Given these findings, a group of European scientists met to analyze the most recent knowledge of vitamin E function and metabolism. An overview of their discussions is presented in this article, which includes considerations of the mechanisms of absorption, distribution, and metabolism of different forms of vitamin E, including the α-tocopherol transfer protein and α-tocopherol–associated proteins; the mechanism of tocopherol side-chain degradation and its putative interaction with drug metabolism; the usefulness of tocopherol metabolites as biomarkers; and the novel mechanisms of the antiatherosclerotic and anticancerogenic properties of vitamin E, which involve modulation of cellular signaling, transcriptional regulation, and induction of apoptosis. Clinical trials were analyzed on the basis of the selection of subjects, the stage of disease, and the mode of intake, dosage, and chemical form of vitamin E. In addition, the scarce knowledge on the role of vitamin E in reproduction was summarized. In conclusion, the scientists agreed that the functions of vitamin E were underestimated if one considered only its antioxidative properties. Future research on this essential vitamin should focus on what makes it essential for humans, why the body apparently utilizes α-tocopherol preferentially, and what functions other forms of vitamin E have.

KEY WORDS Vitamin E, metabolism, binding proteins, novel functions, chemical forms

INTRODUCTION
Eighty years ago vitamin E was discovered as a micronutrient that was indispensable for reproduction in female rats (1). Its essential functions in humans, however, are still not understood. Symptoms of vitamin E deficiency have been produced experimentally in different animal species, e.g., neurologic lesions in rats and monkeys and cerebellar ataxia in chickens. With the possible exception of hemolytic anemia and retinopathy in premature newborns, vitamin E deficiency due to dietary limitations has not been observed in humans. However, genetic deficiencies in apolipoprotein B (apo B) or the α-tocopherol transfer protein (α-TTP) lead to severe vitamin E deficiency syndromes. The symptoms are primarily neurologic and include loss of deep tendon reflexes, cerebellar ataxia, dysarthria, and mental retardation. Furthermore, skeletal myopathy can occur and retinitis pigmentosa is often observed (2–6).

The role of vitamin E in the interplay between nerves and muscles remains elusive. In addition, its role in human reproduction has not been investigated in any detail. Instead, most investigators have focused on the antioxidant potential of vitamin E and have tried to explain its various actions based on this property. Accordingly, it is widely promoted to be helpful in preventing or modulating diseases that are supposedly associated with oxidative stress. Beneficial effects of supranutritional dosages of vitamin E have been reported for cardiovascular diseases, cancer, chronic inflammation, Alzheimer disease, and Parkinson disease. Although the scientific rationale, epidemiologic data, and retrospective studies have been persuasive, prospective, randomized, placebo-controlled trials have so far failed to verify a consistent benefit. Indeed, the data were not convincing enough for the Panel on Dietary Antioxidants of the US Food and Nutrition Board to recommend more than a 50% increase in the daily allowances (from 10 to 15 mg/dl) (7) or for some European institutions to increase the dietary reference intake by more than 3 mg/d (now 15 mg/dl) (8). The primary reason given for each of these decisions is a lack of information regarding the mode of action of vitamin E at the tissue level.

In response to this situation, a group of European scientists met recently to review the present knowledge on vitamin E and to discuss promising lines of future research. The scientists particularly emphasized the novel functions and metabolic fate of vitamin E, about which most information was discovered in the past decade. These topics include the role of vitamin E in the regulation of cellular signaling and gene activity, the role of proteins that specifically bind and guide α-tocopherol to cellular and subcellular

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VITAMIN E

ABSORPTION, DISTRIBUTION, AND METABOLISM OF VITAMIN E

Chemical forms of vitamin E

The term vitamin E covers 8 different forms that are produced by plants alone: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol (9). Tocotrienols have an unsaturated side chain, whereas tocopherols contain a phytol tail with 3 chiral centers that naturally occur in the RRR configuration. Commercially available vitamin E consists of either a mixture of naturally occurring tocopherols and tocotrienols; RRR-α-tocopherol (formerly called d-α-tocopherol); synthetic α-tocopherol, which consists of the 8 possible stereoisomers in equal amounts (all rac-α-tocopherol, formerly called dl-α-tocopherol); or their esters. The bioavailability and bioequivalence of the different forms of vitamin E differ. For example, although the amount of γ-tocopherol in the diet is higher than that of α-tocopherol, the plasma γ-tocopherol concentration is only ≈10% of that of α-tocopherol, which is the most abundant form in plasma.

Absorption

The mechanism of vitamin E absorption is surprisingly unclear. All forms of vitamin E are taken up by intestinal cells and released into the circulation with chylomicrons. At this step there is probably no discrimination between the different forms. The tocopherols reach the liver via chylomicron remnants. In the liver, a specific protein, α-TTP, selectively sorts out α-tocopherol from all incoming tocopherols for incorporation into VLDL. Other forms are much less well retained and are excreted via the bile, the urine [as carboxymethyl hydroxychromans (CEHCs)], or unknown routes. In addition, the capacity of the plasma to increase α-tocopherol concentrations is limited. In subjects with a normal α-tocopherol concentration of ≈25 μmol/L, the concentration cannot be increased > 2–3 fold, irrespective of the amount or duration of supplementation (10–13). This is apparently not due to limited absorption, because α-tocopherol is absorbed at a constant fractional rate with increasing doses (up to 150 mg) (14). Moreover, newly absorbed α-tocopherol replaces old α-tocopherol in plasma lipoproteins, which may be the limiting step in the overall incorporation (14, 15).

Healthy subjects supplemented with α-tocopherol do not have equal increases in plasma α-tocopherol concentrations. In one study, the increase in plasma α-tocopherol concentration 12 h after an intake of 75 mg dl-RRR-α-tocopherol ranged from 0.3 to 12.4 μmol/L (16). A 6-fold variation in α-tocopherol uptake by red blood cells was also seen. The underlying reasons are unknown but may include variations in α-TTP activity, metabolic rate, lipid content and composition, the status of other micronutrients that recycle α-tocopherol, and environmental conditions. Thus, it becomes clear that an adequate or optimal supply of vitamin E can differ tremendously between individuals.

Degradation of vitamin E

Vitamin E is extensively metabolized before excretion. In the 1950s 2 major urinary metabolites of α-tocopherol, tocopheronic acid and the tocopheronolactone derived therefrom, were described (17, 18). Both metabolites are excreted in the urine as glucuronides or sulfates and have a shortened side chain and an opened chroman structure, indicating that they were formed from α-tocopherol that had reacted as an antioxidant. Accordingly, these so-called Simon metabolites are often mentioned as proof of the antioxidant function of α-tocopherol in vivo. They increased markedly in the urine of healthy volunteers after a daily intake of 2–3 g all rac-α-tocopherol (17, 18). However, why a high α-tocopherol intake in unstressed volunteers led to oxidative stress with excretion of oxidized α-tocopherol metabolites is not easily understood.

More than 40 y later, vitamin E metabolism in humans was reanalyzed. Instead of Simon metabolites, a compound with a shortened side chain but an intact chroman structure, α-CEHC, was identified after supplementation with RRR-α-tocopherol (13). This metabolite is analogous to the metabolite of δ-tocopherol found previously in rats (19) and to the metabolite of γ-tocopherol identified in human urine and proposed as a natriuretic factor (20). The proposed pathway of side-chain degradation via ω- and β-oxidation (19; Figure 1) was confirmed by the identification of the immediate precursors of CEHCs, the carboxymethylbutyl...
hydroxymans (CMBHCs) α-CMBHC (21, 22), γ-CMBHC (23), and δ-CMBHC (22, 24). The precursor of α-CMBHC in the presumed sequence of β-oxidation steps, α-carboxymethylhexyl hydroxymans, was also detected (24). The initial step, the α-hydroxylation of the side chain by the action of cytochrome P450 (CYP)–dependent hydroxylases, was indirectly proven by the inhibition of γ-CEHC formation by sesamin and ketoconazole, both of which are inhibitors of the CYP3A family (25); by the increase in α-tocopherol concentrations after dietary intervention with sesame oils in humans (26) and in rats (27); and by the increase in α-tocopherol metabolites after induction of CYP3A by rifampicin (24) in HepG2 cells.

Tocopherol metabolites as biomarkers?

The intact chroman structure of CEHCs suggests that they are derived from tocopherols that have not reacted as antioxidants. Because Schultz et al (13) did not find Simon metabolites and because α-CEHC is easily converted to α-tocopheronolactone by oxygenation, they considered the Simon metabolites to be artifacts produced during sample preparation. However, low amounts of α-tocopheronolactone were consistently found by Pope et al (22), although with poor reproducibility. Optimization of the sample preparation and detection procedures is therefore considered desirable, because if analyzed reliably, α-tocopheronolactone could provide a suitable biomarker of oxidative stress. Appearance of the metabolite would indicate that the α-tocopherol had been confronted with oxidative stress.

Schultz et al (13) also reported that α-CEHC excretion increased with increasing vitamin E intake after a threshold concentration of plasma α-tocopherol had been exceeded. Therefore, they concluded that α-CEHC excretion might be a suitable biomarker for vitamin E status. This proposal, however, has its limitations. First, in patients with a defect in the gene for α-TTP (see below), high amounts of α-CEHC are excreted even when the α-tocopherol plasma concentration is close to zero. This means that in these patients, and probably also in healthy subjects, the capacity of α-TTP rather than the plasma α-tocopherol concentration determines α-tocopherol degradation (21). Second, in both healthy volunteers and α-TTP–deficient patients, the excreted α-CEHC did not exceed 5% of the supplemented α-tocopherol (13, 21). Thus, α-CEHC is not the major urinary metabolite of α-tocopherol. Accumulation of precursors might occur if the metabolic pathway is overloaded. Longer side-chain metabolites, however, may not be excreted in the urine because of their greater hydrophobicity. Accordingly, the amount of α-CMBHC in urine is only ≈10% that of α-CEHC (21). Accumulation of CEHC precursors in plasma or bile has not yet been investigated. In contrast to α-CEHC, γ-CEHC appears to be the major metabolite by which γ-tocopherol is eliminated. About 50%, if not all, of γ-tocopherol is degraded and excreted in the urine (28).

Interference of vitamin E with drug metabolism?

The involvement of CYP3A in the degradation of tocopherols is surprising. CYP3A is a drug-metabolizing system with broad substrate specificity that is induced by steroids, antibiotics like rifampicin, and other pharmacologic agents (29). In HepG2 cells rifampicin stimulates α-CEHC production from all rac-α-tocopherol but not from RRR-α-tocopherol (24). This observation supports the view that part of the synthetic racemic α-tocopherol is identified as foreign and therefore degraded, whereas natural α-tocopherol is retained as long as the organism’s transport capacity is not overwhelmed. Accordingly, α-CEHC excretion from all rac-α-tocopherol is 3–4 times that from RRR-α-tocopherol in humans (30). Parker and Swanson (23) found that γ- but not α-CEHC was released from HepG2 cells, whereas α-tocopherol was metabolized by primary hepatocytes. They suggested that mature isoforms of CYP3A, which are present in primary cells, oxidize all tocopherols, whereas the fetal form, which is expressed in HepG2 cells, exhibits a preference for the non–α-tocopherols (25), which may include synthetic α-tocopherol (24). However, although the involvement of CYP3A has not been shown directly, a non-CYP3A cytochrome P450 that is rifampicin-inducible and ketoconazole- and sesamin-sensitive may actually be responsible for tocopherol metabolism. In view of the multiple roles of the CYP system, drug interactions have to be taken into account when patients are supplemented with supranutritional dosages of vitamin E.

The side effects of supranutritional dosages of vitamin E should also be considered. Tocopherols (31) and especially their oxidation product tocopheryl quinone (32) inhibit platelet aggregation. It has therefore been recommended that anticoagulants and vitamin E supplements not be provided simultaneously (33).

Tocopherol transfer and binding proteins

As a lipophilic molecule, vitamin E is poorly soluble in the hydrophilic milieu of plasma, extracellular fluids, and cytosol. Like other lipophilic vitamins, it may be bound to specific proteins or lipoproteins during absorption, transportation, and distribution. Release of absorbed vitamin E into the circulation occurs via chylomicrons. Although the mechanism of chylomycin assembly and lipid loading has been intensively investigated (for review see reference 34), it is not known how vitamin E is packed into chylomicrons. Transport of vitamin E in the plasma occurs via binding to lipoproteins (for review see reference 35), and intracellular transport may be facilitated by the newly discovered tocopherol-associated proteins (see below). Thus, vitamin E may resemble vitamin A, which requires extracellular and intracellular binding proteins for its different forms, retinol, retinal, and retinoic acid. These proteins include plasma retinol binding protein and the cellular proteins cellular retinaldehyde binding protein, cellular retinoic acid binding protein, and cellular retinol binding protein (36). Nuclear retinoic acid receptors are transcription factors that change their DNA binding activity when retinoic acid is bound (37). Binding proteins also exist for vitamin D. These proteins regulate the transport and cellular distribution of vitamin D (38), and nuclear receptors regulate gene activities when vitamin D becomes bound. In contrast to the relatively well investigated binding proteins for vitamins A and D, proteins that bind and transport vitamin E have only been identified in the past decade (39–42), and many of their specific biological roles remain elusive. The general term “tocopherol binding proteins” is used in this review to define several proteins that have not been well characterized except for their property of binding tocopherol. The term “tocopherol-associated proteins” (TAPs) has been used to distinguish a molecularly defined family of proteins that are capable of binding α-tocopherol (42) with a higher affinity than other tocopherols (43) and are also capable of binding phospholipids. TAP1 is identical to the supernatant protein factor, which stimulates the conversion of squalene to lanosterol. Despite this property of the protein factor, squalene does not seem to bind to it (44).
α-Tocopherol transfer protein

α-TTP is a 32-kilodalton (kDa) protein that acts in the liver to specifically select α-tocopherol with the side chain linked to the chroman ring in the R configuration at position 2 for incorporation into nascent VLDL (45). However, α-TTP facilitation of α-tocopherol secretion in hepatocytes is not dependent on VLDL secretion only. In cultured cells, a lipoprotein assembly–independent but α-TTP–dependent process has been reported (46). This process appears to be mediated by the ABCA1 transporter (47). These observations were made either in hepatocytes (46) or in cells transfected with the ABC transporter (47). Thus, a differentiation between release via the sinusoidal or the canicular membrane is not possible. At this stage in our knowledge, we can only speculate that the transport systems involved may be different but that both require the delivery of α-tocopherol from α-TTP. Clearly, there are unanswered questions regarding the functional role of α-TTP in the absorption and distribution of different forms of vitamin E. Such a conclusion is supported by the unexpected finding that α- and γ-tocopherol are equally deficient in α-TTP knockout mice (48).

However, the crucial role of α-TTP in the maintenance of normal plasma tocopherol concentrations was recognized when mutations in the gene for α-TTP were found in patients with familial isolated vitamin E deficiency (5), also called ataxia with vitamin E deficiency (3, 49). Various mutations have been analyzed, and the severity of the disease depends on the kind of mutation (50). α-TTP–deficient patients have extremely low plasma α-tocopherol concentrations and suffer from neurologic symptoms typical of vitamin E deficiency, including cerebellar ataxia (4, 6). These patients have to be supplemented with large amounts of vitamin E (up to 2 g/d) to normalize plasma concentrations (3, 5, 21, 51–53).

α-TTP is predominantly expressed in liver, and its expression appears to depend on dietary tocopherols themselves. When vitamin E–depleted rats were fed a diet containing α-tocopherol or δ-tocopherol or both, α-TTP messenger RNA increased (54). This was not expected, because the biological activity of δ-tocopherol, which does not bind α-TTP (45), differs strongly from that of α-tocopherol. This shows that α-tocopherols and other tocopherols may have similar effects on gene expression that still await detection. However, Kim et al (55) did not observe an increase in α-TTP messenger RNA after refeeding of α-tocopherol. These authors found instead that vitamin E depletion strongly increased α-TTP messenger RNA concentrations, which then decreased after refeeding of α-tocopherol to below the concentrations in the control rats. The reason for this discrepancy is not clear. Nevertheless, these studies show that dietary vitamin E influences hepatic α-TTP concentrations.

Low concentrations of α-TTP have also been detected in brain, spleen, lung, and kidney (56). In rat brains, α-TTP transcripts were found in the cerebellar cortex in association with the Purkinje cell layer. α-TTP was expressed in the Bergmann glial cells surrounding the Purkinje cells rather than in the Purkinje cells themselves. Because glial cells generally supply neurons with essential nutrients, it has been suggested that vitamin E is supplied to Purkinje cells by Bergmann glial cells with the help of α-TTP (56). In a control patient, α-TTP was not detected in Purkinje cells with the use of immunocytochemical methods. However, it was detected in its mutated form in a patient with ataxia with vitamin E deficiency, and it was detected in patients with marked vitamin E deficiency (cholestatic liver disease and abetalipoproteinemia) (57). Positive α-TTP signals in Purkinje cells were also found in patients with Alzheimer disease or Down syndrome. The failure to find α-TTP in the control patients may thus be due to a physiologically low expression, whereas the data in patients with ataxia with vitamin E deficiency may indicate an up-regulation of α-TTP to compensate for the limited supply of vitamin E. Although the observations described support the essentialness of vitamin E for the function of specific neurons, the underlying mechanisms remain elusive.

Tocopherol-associated proteins

How many other α-tocopherol binding proteins exist apart from α-TTP is still unclear, as is the mechanism that regulates the transfer of α-tocopherol and its concentration in peripheral cells. Early work suggested the existence of several additional tocopherol binding proteins (58, 59); however, their identity and function have never been clarified, and their genes have not been cloned. Recently, a novel tocopherol binding protein, the 46-kDa TAP, was identified (42). It appears to be ubiquitously expressed, although initial data indicated a preferential expression in brain, liver, and prostate. TAP may be involved specifically in the intracellular transport of tocopherol, eg, between membrane compartments. TAP may also act as a molecular chaperone that protects tocopherol from tocopherol-metabolizing enzymes. Recently, human TAP (hTAP) was shown to be identical to the previously described supernatant protein factor, which stimulates the synthesis of cholesterol by mediating the transport of squalene to the squalene epoxidase (44). The similarity of TAP to the yeast secretory protein (Sec14) indicates that TAP may trigger Sec14-like functions, such as phospholipid or tocopherol transport and signaling, phospholipid or tocopherol secretion, or adjustment of the tocopherol composition of membranes. The crystal structure of Saccharomyces cerevisiae Sec14p is known and shows a relatively large hydrophobic pocket, which may be able to accommodate several different hydrophobic ligands (60). Molecular modeling of the hTAP domain that is similar to Sec14p showed that tocopherol molecules can occupy this pocket (Figure 2), although the molecular details of binding await the crystallization of hTAP with its natural ligand.

In analogy with retinoic acid–mediated transcriptional regulation, hTAP may either transport α-tocopherol to a bona fide nuclear receptor or act by itself as a tocopherol-dependent receptor. In agreement with this, hTAP has been shown to modulate transcriptional activity in response to the binding of tocopherol, but thus far this has been shown only by linking an artificial DNA binding domain to hTAP (43). However, modulation of transcription by a tocopherol receptor may explain some of the effects of α-tocopherol on gene expression (see below).

A 15-kDa tocopherol binding protein, which preferentially binds α-tocopherol, may be responsible for intracellular distribution of α-tocopherol (61). In addition, the presence of a membrane tocopherol binding protein in tissues may regulate their α-tocopherol concentrations (40). The 75-kDa plasma phospholipid transfer protein, which is known to catalyze the exchange of phospholipids and other amphiphatic compounds between lipid structures, also binds α-tocopherol and facilitates the exchange of α-tocopherol between HDL and LDL (62).

VITAMIN E AND REPRODUCTION

Vitamin E and fetal development

Vitamin E was detected as a micronutrient that was indispensable for proper fetal development in rats. Vitamin E supplementation
lar modeling of the glycoside molecules bound by the crystal structure of Sec14p (60). Tocopherol molecules have been inserted only by analogy with the 2 octyl-calculation was based on the sequence homology of the 2 proteins. The 2 develop to build up the nutritional transport unit. In 9.5. At that time the labyrinth region of the placenta has to

whether vitamin E deficiency causes or is merely associated with these developmental defects is not clear. However, the phenotype in apo B knockout mice, α-tocopherol was undetectable in embryonic tissue (63). There was an apparent defect in lipid export from the visceral endodermal cells in the yolk sac, which may have caused vitamin E deficiency in the embryos. Whether vitamin E deficiency causes or is merely associated with these developmental defects is not clear. However, the phenotype in apo B−/− mice and vitamin E-deficient rats is similar. In pregnant α-TTP knockout mice, embryos develop normally until day 9.5. At that time the labyrinth region of the placenta has to develop to build up the nutritional transport unit. In α-TTP−/− mice, however, the labyrinth region is abnormally small. Embryonic blood vessels are virtually absent in the trophoblast. Furthermore, embryos show neural tube malformation and die between 11.5 and 14.5 d after coitus (64). Targeted disruption of both apo B and α-TTP, i.e., apo B−/− and α-TTP−/−, affects absorptive surfaces that provide nutrients to the embryo before the chorionallantoic placental circulation is established. In contrast with rodents, humans do not transport nutrients via the yolk sac placenta but instead use chorionallantoic placentaion from the beginning. Whether embryogenesis is impaired in humans also by vitamin E, α-TTP, or apo B deficiency has not been reported.

Vitamin E in human pregnancy

During pregnancy, the blood α-tocopherol concentration increases in association with the increase in blood lipid concentration (65). In abnormal pregnancies, blood α-tocopherol concentrations are lower than those in normal pregnancies at a corresponding gestational age (66). It is assumed, though unproven, that vitamin E requirements increase during pregnancy. For this reason, vitamin supplements designed for pregnancy usually contain small doses (50 mg) of vitamin E, but adverse effects have not been observed with higher levels of supplementation.

It is well established that there is no clear relation between maternal and fetal blood concentrations of vitamin E. Newborns have significantly lower plasma vitamin E concentrations than their mothers (67). However, when plasma vitamin E concentrations were standardized for phospholipids or total lipids, significant differences were not seen (68, 69). Short-term supplementation of pregnant women before delivery significantly enhanced the vitamin E status of the mother only (70), suggesting that vitamin E does not pass efficiently through the placenta to the newborn circulation. What regulates the placental transfer remains unclear. An α-tocopherol binding protein was isolated from human placenta (71), whereas the presence of α-TTP in human placenta has not been firmly established. RRR-α-tocopherol, however, is preferentially transferred to the cord blood (72). This observation points to a role for α-TTP in regulating the transfer of tocopherols through the placental barrier.

VITAMIN E AND OXIDATIVE STRESS DISEASES

Cardiovascular disease

Of the many ideas on the etiology of cardiovascular disease (CVD), 2 theories related to antioxidant defense have been generated to explain the initiation of the atherogenic process: the oxidation theory (73, 74) and the response-to-injury theory (75). In both theories the oxidative modification of LDL is considered to be a key step in the initiation and progression of the disease. The ability of α-tocopherol to inhibit the oxidation of LDL in vitro has been shown unequivocally. This observation is the basis for the assumption that vitamin E is also able to prevent atherosclerosis, because inhibition of LDL oxidation by the antioxidant should lead to an inhibition of early atherogenic events. To date, the clinical trials undertaken have mostly enrolled patients with established atherosclerosis or at high risk of developing the disease, and these studies failed to unequivocally show that vitamin E reduces the progression of the disease.

The ability of α-tocopherol supplementation to prevent cardiovascular events in different populations was tested in four larger prospective clinical trials: the α-Tocopherol β-Carotene (ATBC) study (76), the Cambridge Heart Antioxidant Study (CHAOS) (77), the Gruppo Italiano per lo Studio della Supervivenza nell’Infarto miocardico (GISSI) study (78), and the Heart Outcomes Prevention Evaluation (HOPE) study (79). In addition, at least 2 smaller prospective clinical trials have been completed: the Secondary Prevention with Antioxidants of Cardiovascular disease in Endstage renal disease (SPACE) study (80) and the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (81). The first 4 studies have been reviewed and discussed several times (82–85) and are therefore only briefly summarized here.

In the ATBC study, the subjects who were supplemented with 50 mg all rac-α-tocopheryl acetate/d for 5–8 y had only a modestly lower incidence of angina pectoris than did the control subjects, and among male smokers, cardiovascular mortality...
did not differ significantly between those who received supplementation and those who did not. However, the subjects who received supplementation had a significantly higher incidence of hemorrhagic strokes than did the control subjects. Note that the ATBC study was not designed to investigate cardiovascular disease development but was rather a primary cancer prevention study.

In the CHAOS, 2002 individuals with overt clinical and angiographic evidence of CVD received RRR-α-tocopherol (natural source vitamin E); the first 546 subjects received 800 IU/d, and the remaining subjects received 400 IU/d. However, the 2 groups were combined for statistical analyses. The subjects who received α-tocopherol supplementation had significantly (77%) fewer nonfatal myocardial infarctions than did the control subjects, but early deaths from CVD and total mortality were nonsignificantly higher in the subjects who received supplementation. An increased risk of hemorrhagic stroke was not observed.

In the GISSI trial, 11 324 patients with a previous myocardial infarction were given a daily dose of 300 mg all rac-α-tocopherol alone or in combination with 1 g polysaturated fatty acids. Examination of the preselected combined primary endpoints, which consisted of all-cause death, cardiovascular death, nonfatal myocardial infarction, and nonfatal stroke, indicated that α-tocopherol did not yield any beneficial effect. In a later 4-way reanalysis in which each individual variable was considered as an endpoint, there were significantly fewer (20%) cardiovascular deaths in the α-tocopherol group than in the control group (86).

In the HOPE study, 2545 women and 6996 men at high risk of CVD were randomly assigned to receive either 400 IU vitamin E from natural sources/d (RRR-α-tocopheryl acetate; J Clark, personal communication, 2002), ramipril (an angiotensin-converting enzyme inhibitor), or placebo. The primary endpoints were nonfatal myocardial infarction, stroke, and cardiovascular death; the secondary endpoints were unstable angina, congestive heart failure, revascularization or amputation, death from any cause, and complications of diabetes and cancer. No effect of vitamin E on any of the variables related to atherosclerosis was observed.

In the recently completed SPACE study, 196 hemodialysis patients with preexisting cardiovascular disease received 800 IU RRR-α-tocopherol or placebo/d. A composite variable consisting of fatal and nonfatal myocardial infarction, ischemic stroke, peripheral vascular disease, and unstable angina was defined as the primary endpoint. The secondary endpoints were total mortality and CVD mortality. The subjects who received RRR-α-tocopherol had significantly (54%) fewer composite CVD endpoints than did the control subjects.

In the ASAP study, 225 men and 233 women were given RRR-α-tocopheryl acetate (136 IU twice daily), slow-release vitamin C (250 mg twice daily), a combination of both, or placebo for 3 y. Atherosclerotic progression was followed by measuring the mean intima-media thickness of the common carotid artery with an ultrasonograph. The progression in intima-media thickness was significantly retarded only in the men who smoked and took both of the vitamins.

The short description of the trials shows that each one has its inherent problems and that the trials are not easily compared. Taken together, the results are inconsistent. To properly interpret the results, the major differences between the studies must be considered. These differences involve the selection of subjects; the geographic location and hence the dietary preferences of the subjects; the stage of the disease; the selected endpoints; and the dosage, mode of intake, and chemical form of vitamin E.

Selection of individuals

In a study to test the efficacy of supplementation with vitamin E, the vitamin E status of enrolled subjects should be known from the beginning to be able to monitor sufficient absorption and increases in plasma concentrations. However, baseline concentrations of plasma α-tocopherol were not measured in all of the trials. Consequently, there was no way to confirm successful vitamin E absorption and an amelioration of the vitamin E status (the GISSI and HOPE studies). If measured, the plasma α-tocopherol concentrations of the subjects were approximately equal to normal values: 26.7 μmol/L (the ATBC study), 22 μmol/L (the SPACE study), 31.7 μmol/L (the ASAP study), and 34.2 μmol/L (the CHAOS). Thus, with the possible exception of the SPACE study, the studies did not include subjects who were vitamin E deficient or otherwise include populations that would benefit the most from supplementation.

About 20% of subjects do not respond to supplementation with an increased blood α-tocopherol concentration (JT Salonen, personal communication, 2001) (also see above). The percentage of such nonresponders in clinical trials could have a major influence on the outcome but has not been considered thus far.

Women generally develop fewer cardiovascular diseases than do men. Thus, women profit less from CVD prevention treatment than do men. The ASAP study monitored men and women, and a positive effect of vitamins E and C was observed, almost expectedly, in the men only. In the SPACE, HOPE, GISSI, and CHAOS studies, men and women were included but were not analyzed separately. The low incidence of CVD in women may weaken the statistical power of the trials in which many women were enrolled.

The genetic background of subjects is not generally considered. There are polymorphisms in the genes for Apo A IV (87, 88), inducible nitric-oxide synthase (EC 1.14.13.39) (89), and CD36 (90, 91), for example, that might facilitate atherosclerosis. In addition, mutations in proteins directly involved in cellular α-tocopherol metabolism, α-TTP and TAP (see above), influence the bioavailability of α-tocopherol.

The stage of the disease

The development of atherosclerosis begins in childhood and proceeds continuously with age. Eight stages of the disease have been defined on the basis of histologic classification of lesions (92). Lesions of types I and II are small lipid deposits that do not deform artery walls. These lesions occur at a young age in almost everyone. Type III lesions are called preatheroma and signal the probability of future clinical disease. Lipid accumulation is highest during the development of these first 3 types of lesions. Lesions of types IV–VIII are advanced atherosclerotic lesions in which structural disorganizations in the intima become manifest. Type IV lesions, called atheroma, begin to appear in the third decade of life; the arterial lumen starts to narrow, but this may not yet be visible by angiography. However, the lesion may quickly manifest itself clinically by a change in the surface caused by a fissure or thrombus. Type V lesions, called fibroatheroma, are characterized by the proliferation of smooth muscle cells, which synthesize collagen. This occurs in the fourth
decade of life. When type VI lesions develop, morbidity and mortality occurs. Type VI lesions are characterized by surface disruptions and ulcerations or hemorrhage and thrombotic deposits. Type VII lesions are predominantly characterized by mineralization, whereas type VIII (fibrotic) lesions are predominantly characterized by collagen (92). Evidently, not all lesion types are initiated and promoted by oxidative stress. Consequently, an antioxidant cannot be expected to be equally effective at all stages of atherosclerosis. If it were, it would have pharmacodynamic actions beyond any antioxidative function.

The age of the subjects in the studies differed: 50–69 y in the ATBC study, a mean ± SD age of 61.8 ± 9.3 y in the CHAOS, < 50 to > 80 y in the GISSI study, ≥ 55 y in the HOPE study, 40–75 y in the SPACE study, and 45–69 y in the ASAP study. Accordingly, the subjects were probably at different stages of atherosclerosis although all were probably at a reasonably advanced stage. The CHAOS, HOPE, GISSI, and SPACE trials were secondary prevention studies, which implies that CVD had already been established for a considerable period. If the antioxidant hypothesis is correct, vitamin E acts to prevent oxidative damage of the endothelium and oxidation of LDL, ie, early events in the atherosclerotic process. The beneficial effects observed in the CHAOS and in the SPACE study must therefore have been due to alternative mechanisms. In other words, if current hypotheses on the relation between oxidative stress and CVD were approached seriously, prevention of CVD would have to start at an early age, eg, childhood. Thus, patients with established CVD are clearly not the ideal target population for introducing vitamin E supplementation to regulate antioxidant concentrations. On the other hand, endpoints like death, stroke, or myocardial infarction would not be abundant enough at earlier stages of disease development to provide statistically meaningful data. Approaches such as following intima-media thickness as used in the ASAP trial would be more appropriate. Clearly, many discrepancies between theory and the design of clinical trials still exist.

Mode of intake, dosage, and chemical form of vitamin E

Vitamin E is absorbed together with lipids and thus has to be taken with a meal containing a sufficient amount of fat to guarantee optimal bioavailability. This requirement was not consistently considered in the various clinical trials, and this fact may have contributed to the conflicting results. Although it is difficult to control dietary habits in trials that last for years, the subjects enrolled should at least be informed about this problem.

More importantly, the amount and form of the supplemented all-rac-α-tocopherol differed between the studies: 50 mg all rac-α-tocopherol acetate/d in the ATBC study, 800 IU (400 IU later) RRR-α-tocopherol from natural sources in soy oil/d in the CHAOS; 400 IU vitamin E from natural sources/d in the HOPE trial; 300 mg all rac-α-tocopherol acetate/d in the GISSI trial; 800 IU vitamin E/d in the SPACE study; and 136 IU (100 mg RRR-α-tocopherol acetate) twice daily (corresponding to 91 mg RRR-α-tocopherol twice daily) in the ASAP study. Thus, neither the dosage nor the chemical form of vitamin E was comparable, and both were sometimes hard to figure out from the specifications given in the literature. Natural α-tocopherol is RRR-α-tocopherol, a pure stereoisomer. What “vitamin E from natural sources” means is unclear; it may refer to a tocopherol mixture or to the chemically methylated tocopherol mixture resulting in RRR-α-tocopherol. The conventional synthetic form, all rac-α-tocopherol, consists of equal amounts of 8 different stereoisomers. Synthetic all rac-α-tocopherol increases plasma α-tocopherol concentrations only half as much as RRR-α-tocopherol does (15, 30, 93). Furthermore, the degradation of synthetic all rac-α-tocopherol to α-CEHC is 3–4 times that of RRR-α-tocopherol (30). The efficacy and side effects of the remaining synthetic stereoisomers are unknown. Whether the efficacy of all rac-α-tocopherol is only one-half that of RRR-α-tocopherol is still a matter of debate. In view of the different incorporation of the individual tocopherols into plasma lipoproteins, of the different metabolism of the individual tocopherols, and of the different effects that the metabolites may have, it is not surprising when positive effects of “vitamin E” are observed in some studies and not in others.

When α-tocopherol is administered as an acetate, it must be hydrolyzed by esterases in the intestine. The activity of esterases may vary between individuals and may further complicate the complex bioavailability issue.

If α-tocopherol reacts as an antioxidant in vivo, it is converted to the tocopheroyl radical during the chain-breaking reaction. If not reduced by a coantioxidant, the tocopheroyl radical can react with lipids to generate lipid radicals, thereby promoting the chain instead of breaking it (85, 94–96). α-Tocopherol probably requires coantioxidants to have a beneficial effect. This was one of the reasons for the decision to administer α-tocopherol together with vitamin C in the ASAP study. With this approach, the progression of atherosclerosis was retarded in men who smoked.

Cancer

Attempts to prevent cancer by vitamin E are based on the rationale that oncogenesis results from free radicals attacking DNA. As an antioxidant, vitamin E may inhibit cancer formation by scavenging reactive oxygen or nitrogen species. However, the epidemiologic evidence supporting a link between vitamin E and cancer is limited, and intervention studies are scarce. The few completed studies mostly provided equivocal results. A reduced cancer incidence with vitamin E supplementation was reported in 2 studies.

In a study in Linxian, China, ≈ 15 000 of 29 584 Chinese adults received a mixture of 30 mg α-tocopherol/d, 50 mg selenium yeast/d, and 15 mg β-carotene/d for 5.5 y. The subjects who received this mixture had a 13% lower incidence of cancer and a 10% lower mortality from stomach and esophageal cancer than did the subjects who did not receive the mixture (97).

Prostate cancer was the only cancer that was suppressed by vitamin E in large intervention trials. In the ATBC study, male smokers who took vitamin E supplements had a 34% lower incidence of prostate cancer and 41% lower mortality from prostate cancer than did those who did not take the supplements (98, 99). In contrast, cancer of other organs was not affected in the ATBC study (98) or in the other trials. A lower incidence of clinically overt cancers in the supplemented group appeared soon after the onset of supplementation, suggesting that α-tocopherol slows down the transformation of latent preneoplastic cancer to clinically manifest cancer. α-Tocopherol had no effect on advanced prostate cancer.

A large increase in knowledge is expected from the SELECT (SELeNium and vitamin E Cancer prevention Trial), in which 32 400 men will be followed up for 12 y to monitor cases of prostate, lung, and colon cancers. Specific variables, including polymorphisms of genes that encode proteins involved in metabolizing steroid hormones, DNA repair, and metabolizing carcinogens, will
provide information on factors that are hypothesized to contribute to the risk of prostate cancer (100).

Cystic fibrosis

Cystic fibrosis is an autosomal inherited genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (101). The disease is not rare. In Europe, 1 of every 20 persons is a carrier, and 1 of every 2500 persons is affected. Among cystic fibrosis patients, 85–90% have exocrine pancreatic insufficiency. In contrast to CVD and cancer, cystic fibrosis is not a disease that can be prevented or even treated with vitamin E. However, because of the resulting malabsorption of lipids, cystic fibrosis patients frequently exhibit vitamin E deficiency. In addition, cystic fibrosis patients experience subacute inflammatory processes almost continuously, and these processes probably place an increased burden on, and may lead to the exhaustion of, the patients’ antioxidant defenses. Vitamin E supplementation has therefore become part of the routine therapy (102). The recommended dose for adults is 200–400 IU/d without any specification of the form. In a study with 31 patients and 29 control subjects, a single dose of 400 IU α-tocopherol was administered either as RRR-α-tocopherol (268 mg) or as all rac-α-tocopherol acetate (400 mg) (103). Both forms normalized plasma α-tocopherol concentrations in cystic fibrosis patients.

After long-term supplementation, a decrease in the prothrombin time was observed. This observation underscores the need for a reliable biomarker of the individual vitamin E status so that a supplementation regime that includes the optimal dosage and form of vitamin E can be obtained.

Vitamin E in preeclampsia

Preeclampsia is an important cause of maternal morbidity and mortality and accounts for most preterm deliveries. The incidence of preeclampsia is between 3% and 10% and appears to be on the increase. The condition is characterized by vasospasm, increased vascular resistance, and reduced organ perfusion. Symptoms develop rapidly and include hypertension and proteinuria. The major cause of fetal difficulties is reduced utero-placental perfusion, and the only successful treatment is delivery of the fetus. Women with preeclampsia have elevated blood pressure and total urinary protein, with no evidence of preeclampsia in the women who received vitamin E supplements, provide a sound biochemical basis for understanding the improved clinical symptoms.

NONANTIOXIDANT MECHANISMS OF α-TOCOPHEROL

If vitamin E merely acted as an antioxidant, α-tocopherol would inhibit and prevent all diseases linked to oxidative stress and all types of cancer. Moreover, when other antioxidants are adapted to have an antioxidant capacity equivalent to that of vitamin E, they should be equally efficacious. This definitely is not the case. Thus, it is time to consider that vitamin E, especially α-tocopherol, may have alternative functions.

Antiatherosclerotic mechanisms

Modulation of cellular signaling by α-tocopherol

Leukocyte rolling and adherence at the vascular endothelium are early events in the process of atherogenesis. Endothelial selectins mediate rolling, and integrins on leukocytes and cellular adhesion molecules (CAMs) on endothelial cells mediate firm adhesion of leukocytes to the endothelium. Subsequently, leukocytes enter the subendothelial space by diapedesis. In the subendothelial layer, monocytes differentiate into macrophages, which are able to oxidize LDL, probably via the production of superoxide by activated NADPH oxidase (EC 1.6.99.6), and to ingest oxidized LDL (oxLDL) by means of the scavenger receptor CD36, which in turn is upregulated by oxLDL. Macrophages become foam cells and fatty streaks develop. Activated endothelial cells secrete various cytokines, eg, interleukin 1β (IL-1β), and chemokines, like IL-8 and monocyte chemotactic protein 1, which recruit more monocytes and smooth muscle cells and further activate endothelial cells. Smooth muscle cell proliferation, production of collagen, and aggregation of platelets finally lead to fibrous plaque formation. Expression of CAMs and leukocyte adhesion are equally relevant in the onset of inflammation, supporting the response-to-injury theory, in which the response is an inflammatory reaction (109). α-Tocopherol has been shown to inhibit many key events in inflammation. These events include the following: 1) the release of IL-1β from lipopolysaccharide-activated monocytes (110), 2) monocyte adhesion to endothelial cells (111, 112), 3) the production of monocyte chemotactic protein 1 and IL-8 in human aortic endothelial cells (112), 4) LDL-induced proliferation of smooth muscle cells (113), 5) the aggregation of platelets (114–117) (the α-tocopherol quinone is even more effective than is authentic α-tocopherol; 118), 6) the activation of NADPH oxidase by the prevention of p47(phox) membrane translocation and phosphorylation (119), 7) the production of collagen α1(I) in human fibroblasts (120) and in the livers of C57BL/6 mice (121), and 8) the age-dependent increase in collagenase expression in human skin fibroblasts (122).

All of these effects are predominantly exerted by α-tocopherol. The other tocophers (β, γ, δ) with equivalent antioxidant efficacy either do not exert these effects or if they do, they do so to a much lesser extent. Therefore, the efficacy of α-tocopherol does not depend on its antioxidative capability. Moreover, it cannot be mimicked by other antioxidants. The link between these multiple
functions of α-tocopherol appears to be protein kinase C (PKC), a key player in the signaling of cytokines, growth factors, and hormones. PKC is a family of several isoenzymes including PKCα, which is inhibited by α-tocopherol (Figure 3; 123–125). Inhibition occurs either by the prevention of the autophosphorylation of the enzyme, which is necessary for activity, or by the dephosphorylation of PKC by protein phosphatase PP2A, which is activated by α-tocopherol (125, 126). Most of the effects of α-tocopherol on cellular signaling that have been described can be explained by the inhibition of PKC.

**Transcriptional regulation by α-tocopherol**

The influence of α-tocopherol on the activity of certain genes is one of the most striking observations made in the field of vitamin E research (127). With the use of the differential display technique, a transient up-regulation of genes is one of the most striking observations made in the field (132). α-Tocopherol also decreases 1) cytokine-induced adhesion molecule expression in human vascular endothelial cells (133, 134), 2) vascular cell adhesion molecule 1 expression in lung type II cells and macrophages (135), 3) L-selectin expression in lung macrophages (135), 4) oxLDL-induced Mac-1 (CD11/CD18) expression in monocytes (136), and 5) oxLDL-induced vascular cell adhesion molecule 1 expression in human umbilical cord endothelial cells (137). Furthermore, α-TTP concentrations may be modulated, as discussed above, by tocopherols (54, 55).

If α-tocopherol’s effect on gene activity were a direct one, α-tocopherol would need to be able to move into the nucleus. Using an α-TTP-green fluorescent protein fusion protein expression system, Yamauchi et al (43) observed that TAP translocates from the cytosol to the nucleus of COS cells in an α-tocopherol–dependent fashion. Transient transfection experiments also indicated that TAP activates transcription of the reporter gene in an α-tocopherol–dependent manner. These results support earlier proposals that α-tocopherol functions not only as an antioxidant but also as a transcriptional regulator of gene expression (127).

Certain questions remain unanswered, however. In some cases, differential effects of α-tocopherol and β-tocopherol were found, pointing to a nonantioxidant mechanism as the basis of gene regulation (128, 129). In other cases, however, only α-tocopherol was tested, thus leaving the mechanism of α-tocopherol action ambiguous. Furthermore, the involvement of PKC was not always assessed, and it remains to be established whether the transcriptional regulation of certain genes is a consequence of PKC inhibition or may involve some of the recently described novel tocopherol binding proteins.

**Anticarcinogenic mechanisms**

**Modulation of immune function**

Elimination of tumor cells by an increase in the efficacy of the immune system has been postulated as an anticarcinogenic mechanism (138). Such a mechanism suggests that vitamin E may have an anticarcinogenic action because vitamin E deficiency impairs immune function, including both T and B cell–mediated functions (139). Furthermore, vitamin E restores the age-related decrease in immune function (140, 141). Lipopolysaccharide-stimulated macrophages isolated from old mice produced significantly more prostaglandin E₂ than did those from young mice. Moreover, vitamin E supplementation of old mice shifted the response of their macrophages to one typical of the macrophages of young mice. Increased prostaglandin E₂ production correlated with an increased activity of cyclooxygenase (142). Although the chemical nature of vitamin E was not addressed in these studies, subsequent studies showed that cyclooxygenase activity was inhibited by α-, β-, γ-, and δ-tocopherol in isolated mouse peritoneal macrophages (143) and by γ-tocopherol and its metabolite, γ-CEHC, in macrophages and epithelial cells (144). These effects do not seem to depend solely on the antioxidative capacity of tocopherols. The latter 2 studies represent the first attempts to determine the precise mechanism by which tocopherols influence the immune system. Whether all tocopherols also exert these effects in vivo remains to be investigated. Differences in tocopherol accumulation and metabolism may limit such a generalization.

**Induction of apoptosis**

Programmed cell death is one of an organism’s main ways of getting rid of cells that are no longer needed, are abnormal, or are becoming dangerous. Elimination occurs without causing adventitious inflammatory reactions of the neighboring cells. Thus, induction of apoptosis may be an essential anticarcinogenic process. Among the vitamin E analogues, α-tocopherol succinate (α-TOS) has been shown to be a potent apoptogen in tumor cell lines but not in primary cells (145–148). It has been suggested that α-TOS uses the distal, mitochondrial pathway in
apoptosis signaling, involving activation of the caspase-3 cas-
cade (147, 149). Multiple signaling pathways were implicated in
alpha-TOS–mediated apoptosis; pathways mediated by trans-
forming growth factor-β, Fas, and mitogen-activated protein
kinase (150–152) and tumor necrosis factor–related apoptosis-
inducing ligand (153). Inhibition of PKCα by an increase in
PP2A activity again appeared to be the key event. Importantly,
alpha-tocopherol itself did not induce apoptosis but suppressed
the proapoptotic potential of alpha-TOS (154). Furthermore,
the moderate anticancer effect of alpha-tocopherol in immunocompromised
mice with colon cancer xenografts, which is probably due to its
antiproliferative activity, was significantly inferior to that of its
succinylated counterpart (148, 153). To exert an anticancer
effect, alpha-TOS had to be injected intraperitoneally, because after
oral application the ester would immediately be hydrolyzed to
the less effective alpha-tocopherol in the intestine (155). The need
to esterify alpha-tocopherol by succinate argues against an antioxidative
character of the proapoptotic and tumor-suppressing effect
of alpha-TOS. It is more likely that because of the presence of the
succinyl moiety, alpha-TOS has strong lysosomotrophic activity in
malignant cells (147, 156).

Interference with hormone production?

Men who received alpha-tocopherol had significantly lower serum
androstenedione and testosterone than did those who received a
placebo (157). This finding was regarded as an explanation of the
selective reduction in prostate cancer observed in the ATBC
study. Whether this finding represents a direct effect on the pro-
duction or metabolic turnover of the hormones themselves or on
the differentiation of hormone-producing cells remains to be
investigated.

CONCLUDING REMARKS

For long enough, vitamin E has been mislabeled simply as a
lipid-soluble antioxidant. Clearly, the tocopherols can act as chain-
breaking free radical scavengers, and alpha-tocopherol, in particular,
has in vivo potency in this respect. This does not, however, imply
that this potential reflects their primary physiologic role in vivo,
although their sensitivity to oxidants may lead to deficiency syn-
dromes in oxidative stress–related diseases. In addition, their ability
to undergo redox reactions is probably pivotal in any additional
mechanisms of action that they may have. In this sense, toco-
opherols may exert more specific functions as redox sensors or
redox regulators.

The strongest argument against a nonspecific antioxidant role
for the tocopherols is the specificity of RRR-alpha-tocopherol in a
growing list of biochemical events. For example, tocopherol bind-
ing and transport proteins have been discovered that select
RRR-alpha-tocopherol while leaving beta-, gamma-, and delta-
tocopherol and the stereoisomers of racemic alpha-tocopherol to degradation. In addition,
alpha-tocopherol specifically affects various signaling cascades, prob-
ably by influencing the phosphorylation state of PKCα. By this
mechanism or related mechanisms, alpha-tocopherol also specifically
modifies gene expression and has a specific function in cerebellar
cells involved in the regulation of coordinated movement. Finally,
alpha-tocopherol specifically contributes to a proper development of
labyrinthine trophoblasts in mice.

Even given these apparently specific alternative functions for
alpha-tocopherol, it would be wrong to conclude that beta-, gamma-, or
delta-tocopherol are unimportant physiologically because they may
also have distinct, but as yet unknown, functions. The natriuretic
potential of gamma-CEHC and selective cyclooxygenase inhibition by
gamma-tocopherol and alpha-CEHC may be the first hints of such properties.
The ability of the mammalian organism to retain and transport RRRAnti-tocopherol selectively, however, points to a prominent role for
this molecule. Therefore, we conclude that 80 y after the discovery of
RRR-alpha-tocopherol, it is time to take it more seriously and undertake
the research needed to determine what makes it a real vitamin.

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