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Vitamin E: Application of the Principles of Physical Organic Chemistry to the Exploration of Its Structure and Function[1]

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Even when stored in a refrigerator many foods will eventually become rancid, a sign that the lipid material, i.e., the fats, in the food have undergone a chemical reaction with atmospheric oxygen. Such oxidations that occur under mild conditions are called *autoxidations*[2] or, in biological circles, *lipid peroxidations*. The process involves a free radical chain, which can be represented by

initiation

$$\text{production of } R^{\bullet} \text{ (Rate} = R_i) \quad (1)$$

propagation

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (2)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (3)$$

termination

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow \text{molecular products} \quad (4)$$

In this scheme, RH represents a lipid molecule and $R^{\bullet}$ the carbon-centered radical derived from it by removal of a hydrogen atom.

The first step for each chain involves the production of a radical from some molecular precursor. Such chain initiation may be nonenzymic, being caused by heat, or light, or by a single electron transfer (SET) from a reducing agent such as Fe^{2+} to an acceptor such as the hydroperoxide, ROOH, or it may be an enzyme-catalyzed SET reaction. The radical $R^{\bullet}$ reacts extremely rapidly[3] with oxygen to form the peroxyl radical which, in a subsequent much slower step,[4] attacks RH to form ROOH and a new $R^{\bullet}$. The propagation sequence of reactions 2 and 3 is eventually broken when two chain-carrying $ROO^{\bullet}$ radicals react together to give molecular products. For every initiation event, therefore, many molecules of RH may be oxidized to ROOH.

Living organisms are exposed to much more severe oxidative stress than is food in a refrigerator. Nevertheless, they do not become rancid until they, in their turn, become food. What this means is that living organisms have some mechanism or mechanisms by which they protect themselves against autoxidation. The materials that are most readily autoxidized and hence are in most need of protection are the polyunsaturated fatty acids. Like other fatty acids, these form a part of various lipid materials within the organism including, in particular, biomembranes. Autoxidation of a biological membrane will breach its integrity, which can have disastrous consequences for the organism since one of the principal functions of a membrane is to act as a dividing wall which compartmentalizes biochemical processes into specific cells and into specific regions within an individual cell.

The protection of organic materials, including living organisms, against oxidative degradation is provided by fairly small quantities of certain specific compounds called *antioxidants*.[5] Such compounds can be divided into two broad classes, referred to as *preventive* antioxidants, which reduce the rate of chain initiation, and *chain-breaking* antioxidants, which interfere with one or more of the propagation steps. Vitamin E is a chain-breaking antioxidant and the term vitamin E[6] refers to one or more of four structurally related phenolic compounds called tocopherols. The tocopherols differ from one another only in the number and position of the methyl groups on the aromatic ring. α-Tocopherol, (α-T), which is fully methylated, is the most

VITAMIN E

Natural (2R, 4'R, 8'R) Tocopherol

"Head" Phytyl "Tail"

	r_7	r_5
α-T	CH_3	CH_3
β-T	H	CH_3
γ-T	CH_3	H
δ-T	H	H

plentiful and the most biologically active of these four compounds.

Most phenols are chain-breaking antioxidants.[7] The chain-carrying peroxyl radicals are "trapped"

$$ROO^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet} \quad (5)$$

Since the phenoxyl radicals produced are resonance stabilized, they do not, in general, continue the chain but are eventually destroyed by reaction with a second peroxyl radical:

$$ROO^{\bullet} + ArO^{\bullet} \rightarrow \text{molecular products} \quad (6)$$

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Table I.
Values of k_5 at 30 °C for Tocopherols and 2,6-Di-*tert*-butyl-4-methylphenol (BMP)

phenol	$10^4 k_5$, $M^{-1} s^{-1}$ [a]	phenol	$10^4 k_5$, $M^{-1} s^{-1}$ [a]
α-T	320	γ-T	140
β-T	130	δ-T	44
		BMP	1.4

[a] Values are from ref 16.

In certain systems the phenoxyls may be "repaired", that is, reduced to the starting phenol by water-soluble reducing agents (vide infra).

The effectiveness of a chain-breaking antioxidant, particularly one that must function in living organisms, depends on a number of factors including its reactivity towards peroxyl radicals. That is, if a chain-breaking antioxidant is to be effective, a relatively small quantity must protect a much greater quantity of RH. The rate constant for reaction 5 must therefore be much greater than that for reaction 3, i.e., $k_5 \gg k_3$. To compare different chain-breaking antioxidants *quantitatively* it is essential to measure their k_5 values under comparable conditions.

Measurement of k_5 for Tocopherols and Other Phenols in Vitro

In a homogeneous, nonpolar organic solvent, which may or may not serve also as the oxidizable substrate, a phenolic antioxidant traps two peroxyl radicals,[7,8] thereby breaking two oxidation chains and so reducing the chain length and the rate of oxidation. The initial rate of a phenol inhibited oxidation is given by

$$-d[O_2]/dt = k_3[RH]R_i/2k_5[ArOH] \qquad (I)$$

We have utilized this equation to obtain absolute values of k_5 for a large number of phenols. Some of these data are listed in Tables I and II. The phenols were used to inhibit the azobisisobutyronitrile (AIBN) thermally initiated autoxidation of styrene at 30 °C and initial rates of autoxidation were measured. The use of AIBN as the initiator ensures that R_i remains constant throughout an experiment. The precise value of R_i is determined by measuring the length of time the rate of oxidation is suppressed by a known concentration of ArOH.[8,9] This is known as the *induction period* and its duration, τ, is very simply related to R_i and the phenol concentration by

$$\tau = 2[ArOH]/R_i \qquad (II)$$

Styrene possesses a number of advantages over other potential oxidizable substrates. These have been enumerated[10,11] and we note here only the fact that the rate constant for chain propagation, k_3, is comparatively large (41 $M^{-1} s^{-1}$ at 30 °C).[12] This means that even with a very good antioxidant, oxidation can occur at a measurable rate and with a chain length greater than one. That is, the inhibited autoxidation is still a *chain* reaction (which is a necessary condition if k_5 is to be derived from eq I). All our measurements of k_5 were carried out at chain lengths > 4.

Table II.
Values of k_5 at 30 °C and Some Other Properties of Certain Phenols Which Show a Dependence on θ'

phenol	$10^4 k_5$, $M^{-1} s^{-1}$ [a]	θ' [b]	ΔV_2,[c] kcal/mol	Σa, $(o\text{-}CH_3)$ [a] gauss
2	39	89°	0.45	12.36
3	250	38° [d]	0.92	n.d.[e]
1	380	17°	1.04	10.45
4	570	6° [d]	1.13	10.55
5	130	8° [f]	n.d.[e]	n.d.[e]

[a] Values from ref 16. [b] Values from ref 10 and 16. [c] Reference 18. Values are given as the difference $\Delta V_2 = V_2$ (2,3,5,6-tetramethylphenol) − V_2 (ArOH) because differences are more precise than absolute values. [d] Angle for a structurally related phenol, ref 16. [e] Not determined. [f] Angle for 4-methoxyphenol, ref 10.

The results in Table I show that the order of antioxidant activity of the tocopherols is $\alpha > \beta \approx \gamma > \delta$. This is more or less the same order as that of their in vivo activities in the rat fetal resorption bioassay.[6,13] However, these relative bioactivities (viz. α-T ~ 2β-T ~ 10γ-T ~ 30δ-T) show a larger variation than do the k_5 values.

Our kinetic results[10] helped to resolve a long-standing problem. Qualitative measurements of antioxidant activity of the tocopherols in vitro under conditions where R_i was not controlled had led to the general view that γ-T was a better antioxidant than α-T and, furthermore, that the tocopherols had only a rather modest antioxidant activity in comparison with many synthetic phenolic antioxidants used in commerce.[14] The apparent "discrepancy" between the high in vivo vitamin E activity of α-T and its apparently low in vitro antioxidant activity had even been used to argue against the antioxidant theory for the role of vitamin E in living organisms.[15] Our own results[10] showed not only that k_5 (α-T) > k_5 (γ-T) but also that α-T and the structurally related model compound, 2,2,5,7,8-pentamethyl-6-hydroxychroman, 1 (see Table II), were the best phenolic antioxidants known at that time and, in particular, that they were very much better antioxidants than the major commercial antioxidant, 2,6-di-*tert*-bu-

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tyl-4-methylphenol (see Table I).

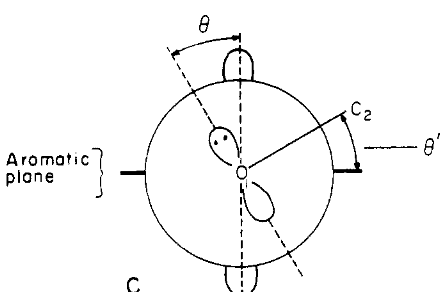
Why Is α-T Such a Good Chain-Breaking Antioxidant?

Over 20 years ago Howard and Ingold[11] explored the effect of various ring substituents on the k_5 values for simple phenols. They showed that to maximize k_5 the best 4-substituent was a methoxy group and that the best pattern of substitution at the other four positions was achieved with four methyl groups. However, the phenol that logically should have been "the best" antioxidant, i.e., 4-methoxy-2,3,5,6-tetramethylphenol (**2**), was not examined. Many years later this oversight was rectified.[10] We assumed that **2** would show an antioxidant activity similar to that of α-T and **1** and were therefore quite surprised when it was found to be very much less active (see Table II).

Since steric hindrance to abstraction of the phenolic hydrogen by ROO• must be very similar in α-T and in **2**, it was clear that the difference in k_5 values for these two phenols must be due mainly to differences in the exothermicities of reaction 5. That is, the O–H bond in α-T must be weaker than that in **2** and hence the phenoxyl radical derived from α-T must be more stabilized than that derived from **2**. This focused our attention on the oxygen atom that is para to the OH group. This atom can stabilize the phenoxyl radical by conjugative electron delocalization, A ↔ B, *provided*

A ⟷ B

its p-type lone-pair orbital overlaps with the semioccupied molecular orbital (SOMO) in the radical. The extent of such overlap will depend on the dihedral angle, θ between the p-type orbital on O_1 and a perpendicular to the aromatic plane (see C), and this angle



should be equal to the dihedral angle, θ′, between the O_1–C_2 bond and the aromatic plane. Stabilization will be maximized when $\theta = 0°$ and will be at a minimum when $\theta = 90°$. X-ray values of θ′ for various phenols are given in Table II.[10,16] It can be seen that our data supports this stereoelectronic explanation for the high reactivity of α-T. That is, k_5 increases as θ′ decreases along the series **2**, **3**, **1**, **4**, the last compound being the most active simple phenolic antioxidant that we have discovered.[16,17]

The influence of stereoelectronics can be seen in other properties of the phenols and their radicals. For example,[18] the potential barriers to rotation about the HO–Ar bond, V_2, decrease by ca. 0.68 kcal/mol on going from **2** ($\theta \sim 90°$) to **4** ($\theta \sim 6°$); see Table II. In the phenoxyl radicals, the increase in delocalization of the unpaired electron onto the para oxygen which occurs as θ′ decreases is accompanied by a decrease in spin density in the aromatic ring and hence in the hyperfine splittings by the two methyl groups that are ortho to the phenoxyl oxygen.[16,19] The magnitude of the sum of these two splittings is significantly greater for **2** ($\theta \sim 90°$) than for **1** or **4** (see Table II).

Proof that Vitamin E Is the Major Lipid Soluble, Peroxyl Radical-Trapping, Chain-Breaking Antioxidant in Human Blood

Although α-T is a highly effective antioxidant in vitro, the possibility that other lipophilic compounds which act in the same manner are present in vivo had not been tested experimentally. This was surprising to us, particularly because during the past 20 years several compounds which are (or could be) present in the lipids of biological systems had been suggested to be chain-breaking antioxidants. To detect and measure the concentration of lipid soluble, chain-breaking antioxidants we made use of eq II—a procedure first utilized by Mahoney and co-workers[20] for the measurement of chain-breaking antioxidants in automobile engine lubricating oils! Human blood plasma[21] and red blood cells (RBC) were chosen for this study since blood is the vehicle by which vitamin E and, presumably, any other exogeneous, lipid-soluble antioxidants are transported to the tissues.

For plasma the experiment was straightforward.[22,23] The lipids were extracted from fresh plasma using equal volumes of ethanol and *n*-octane. The lipid extracts were divided into two portions. One portion was analyzed for α, β, γ, and δ-T by HPLC, with α-T being found to be the predominant tocopherol.[23] The other portion was "titrated" with peroxyl radicals at 30 °C in autoxidizing styrene (1 mL): chlorobenzene (1 mL) initiated by the thermal decomposition of 14 mmol AIBN. The rate of chain initiation was determined both before and after the addition of a sample of lipid extract by measurement of the induction period produced by injection of 50 μL of a standard 5.2×10^{-5} M solution of α-T in chlorobenzene (eq II). The total concentration of chain-breaking antioxidant in the lipid extract, [AH], was calculated from

$$[\mathrm{AH}] = \frac{\tau_{\mathrm{lipid}} V_{\alpha\text{-T}} [\alpha\text{-T}]}{\tau_{\alpha\text{-T}} V_{\mathrm{lipid}}} \qquad \text{(III)}$$

In this equation τ_{lipid} and $\tau_{\alpha\text{-T}}$ are the induction periods produced by the addition of volumes V_{lipid} and $V_{\alpha\text{-T}}$ of the lipid extract and α-T standard solutions respec-

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tively. It is assumed in eq III that each molecule of antioxidant in the lipid extract traps two peroxyl radicals as is the case for the tocopherols[10] and most other phenols.[8,9]

For the RBC the experiment was more complex because the measurement of [AH] could not be undertaken in the presence of hemoglobin, which is itself an autoxidation catalyst. The membranes of the RBC, which are commonly referred to as "erythrocyte ghost membranes", were separated from the hemoglobin[24,25] and their lipids were extracted by the Folch method[26] using chloroform/methanol and after some workup[23] were dissolved in *n*-octane and treated the same way as the extract of plasma lipids.

In both the plasma and the ghosts the concentration of vitamin E was approximately equal to the total concentration of all chain-breaking antioxidants, leading inevitably to the conclusion that *vitamin E is the major (and possibly only) lipid-soluble, chain-breaking antioxidant in human blood.*

Vitamin E and Cell Division

The rate of lipid peroxidation is often low in tumor tissue as compared to the corresponding normal tissue.[27,28] Tumor cells are therefore relieved of the effects of the oxidation products of fatty acids which would otherwise inhibit cell division.[28] Tumor cells and organelles also often have an abnormal lipid composition and contain low, often undetectable, levels of the enzymes of the cytochrome P-450 system which can initiate (via an SET process) lipid peroxidation.[29] Furthermore, tumor tissue has been reported to contain elevated levels of antioxidants that are usually assayed by the ability of tumor tissue extracts to inhibit lipid peroxidation in normal tissue preparations.[29] Related observations have been made in dividing normal cells.[30] Generally, a low rate of lipid peroxidation is associated with an increased rate of cell division. In the liver, normally high in lipid peroxidation activity, the rate is much lower at times of high cell division, e.g., in fetal and neonatal liver.[31] A large amount of work stemming from Shuster's paper[28] has established that the products of lipid peroxidation can inhibit both the synthesis of DNA and cell division.

Drawing these aspects together, it has been proposed, notably by Burlakova et al.,[30] that lipid peroxidation occurs in normal cells under physiological conditions and that it acts as a "coarse" regulator of cell division—possibly via the effects of the products of lipid peroxidation.[32,33] According to this hypothesis, the increased rate of cell division in cancerous tissue may be due to a decreased level of lipid peroxidation activity. To test this hypothesis we have begun a collaborative program to study an extreme example of an experimentally induced liver tumor, the Novikoff ascites hepatoma.[34,35]

We have used various methods to initiate and monitor the progress of lipid peroxidation in microsomes, in lipids extracted from microsomes, and in lipids extracts from whole cells.[34,35] Under all conditions the samples from the Novikoff cells peroxidize more slowly than the samples from the normal liver cells. For certain methods of initiation of peroxidation of microsomal suspensions which rely on SET processes by microsomal enzymes, the small extent (or absence) of peroxidation in the Novikoff samples can be partially attributed to the reduced level (NADPH: cytochrome c reductase, 10% of normal) or absence (cytochrome P-450) of these enzymes. However, increased induction periods and reduced rates of oxidation after the end of the induction period are also observed using lipids extracted from Novikoff microsomes and whole cells with initiation by the thermal decomposition of AIBN. Under these conditions the induction period for Novikoff derived lipids is ca. 3 times longer than for normal cell lipids under the same conditions, while the rate of oxidation after the induction period is about half that for the normal lipids. Comparison of total chain-breaking antioxidant levels by the induction period method (vide supra) with vitamin E levels measured by HPLC shows that vitamin E is once again the major lipid-soluble, chain-breaking antioxidant in both types of cells, there being ca. 3 times as much in the Novikoff lipids. The lower rate of oxidation of the Novikoff lipids after the induction period is over is simply due to the fact that the Novikoff lipids contain a relatively lower concentration of highly unsaturated fatty acids, i.e., those fatty acids which contain one or more of the readily oxidizable bis-allylic methylene units, $-CH{=}CHCH_2CH{=}CH-$.

These differences between Novikoff and normal rat liver cells might reflect the fact that the former are rapidly proliferating rather than being differences directly linked to the malignant transformation. We have investigated this possibility using the normal regenerating rat liver (following partial hepatectomy) as a model.[36] The regenerating liver showed marked differences in lipid peroxidation and antioxidant content relative to "sham-operated" rats and these changes showed diurnal cyclic variations that were in phase with DNA synthesis. ("Sham-operated" rats had been operated upon and had their liver handled but not partially excised.)

What are the possible biological significances of low rates of lipid peroxidation in rapidly dividing cells? As already mentioned, some of the products of lipid peroxidation can inhibit cell division, while others are

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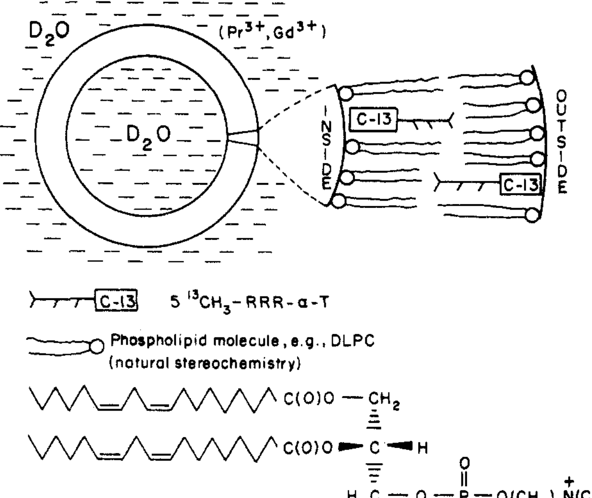

Figure 1.

mutagenic. Thus decreased peroxidation can remove a potential source of inhibition of DNA synthesis and ensure that the genome "exposed" during cell division is not subjected to the hazardous effects of peroxidative reactions (including attack by free radicals). Other features may also be of importance including, in particular, the interactions of lipid peroxides with the prostaglandin cascade. We believe that further investigations currently underway will aid in interpreting the relative importance of the changes associated with the expression of the malignant transformation and of cell division.

Where Is the Vitamin E Molecule?

Biological membranes, which are composed largely of phospholipid molecules, cholesterol, and the membrane-bound proteins[37] must be protected against peroxidation. Vitamin E appears to play a vital role in this protection. As in any organized assembly of molecules, the chemistry that can occur depends on the average positions of the various reactants and their mobilities, as well as on their reactivities, concentrations, etc. Phospholipids spontaneously form bilayers when dispersed in water (see Figure 1), and these can serve as excellent models for real biological membranes. The autoxidation chain, reactions 2 and 3, involves the polyunsaturated fatty acid moieties and must occur well inside the bilayer. Model studies show that the kinetic behavior for the autoxidation of bilayers is essentially the same as that for the autoxidation of the same phospholipids dispersed in organic solvents, or for the free fatty acids or their methyl esters.[38-43] It has generally been assumed that in lipid bilayers (and biomembranes) α-T is oriented with the chromanol "head" group towards the surface and with the hydrophobic phytyl "tail" buried within the hydrocarbon region (see Figure 1).

To provide unequivocal proof that this general assumption is correct, we synthesized [5-$^{13}CH_3$]-2*R*,4′*R*,8′*R*-α-T, i.e., "natural" α-T with a ^{13}C adjacent to the OH group.[44] This compound (5 mol %) was then codissolved with egg lecithin phosphatidylcholine (egg PC, obtained from hen yolks) in $CHCl_3$/MeOH. After evaporation to dryness the lipid film was hydrated with D_2O and converted into small unilamellar vesicles by sonication (see Figure 1). The ^{13}C resonance due to the label (12.5 ppm) was easily identified by comparison of lipid dispersions with and without the label. Addition of the shift reagent Pr^{3+} to the vesicle suspension produced a splitting of this resonance, one line remaining unchanged at 12.5 ppm but the other moving progressively to lower fields as $[Pr^{3+}]$ was increased.[44] Obviously, the high-field resonance is due to ^{13}C-α-T in the inner monolayer where it is uninfluenced by the added shift reagent and the low field resonance is due to ^{13}C-α-T in the outer monolayer. This assignment was confirmed by the simultaneous addition of the resonance broadening reagent, Gd^{3+}. The value of T_1 for the inner ^{13}C resonance was essentially unchanged but for the outer resonance T_1 decreased dramatically, and the line disappeared completely at $[Gd^{3+}] \geq 10^{-3}$ M.[44] (Bilayers of the type used in these experiments are impermeable to lanthanide ions on the time scale of the experiment.)

These results indicate that the aromatic ring is in the proximity of the carbonyl groups of the fatty acyl chains. The insensitivity of the T_1 value for the ^{13}C label in the inner monolayer species implies that it is $\geq \sim$40–50 Å from the site of lanthanide binding on the outer monolayer. Since the bilayer is ~60 Å in thickness the only arrangement possible places the outer and inner α-T in a phytyl tail-to-phytyl tail arrangement, with the phenolic hydroxyl group located near the phosphate region of the bilayer. We are currently undertaking ^2H NMR experiments and neutron diffraction experiments on deuterium labelled α-T in bilayers which should define its conformation and position more precisely.

If the tocopheroxyl radical, ArO• resides in essentially the same position as α-T it is easy to understand the "regeneration" of α-T by water-soluble reducing agents (vide infra). It is less easy to see how the ROO• radicals can "reach" the phenolic hydroxyl group. However, for other reasons,[38,45] we have hypothesized that the peroxyl radical, which has a large dipole moment,[38,46] must be relatively hydrophilic and so may tend to "float" towards the bilayer surface. Quite probably α-T meets it "halfway", being able to "bob up and down" around its average position.

"Regeneration" of Vitamin E

Because of their importance and fragility, biomembranes must be protected against oxidation at all costs.

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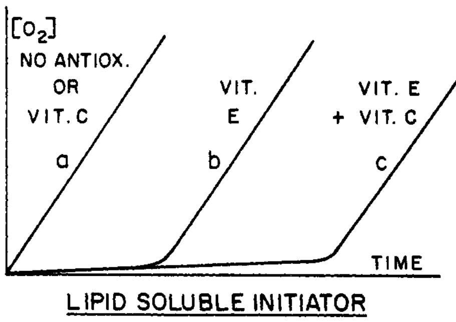

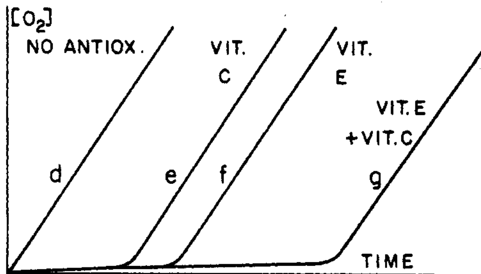

Figure 2.

Since vitamin E is essentially the only protective agent present within the membrane, we have been led to the conclusion that vitamin E must itself be protected in vivo because it is the "last-line" of defense against membrane peroxidation. The question is: how is this protection provided?

Over 40 years ago Golumbic and Mattill[47] reported that although ascorbic acid (vitamin C) was a very poor antioxidant for lard and hydrogenated cottonseed oil it enhanced the antioxidant activity of tocopherols, i.e., the induction period produced by a vitamin C and E mixture was much greater than the sum of their individual induction periods. Later, Tappel[48] made the important suggestion that vitamin C could reduce oxidized E in vivo. Subsequent in vitro experiments showed that the ascorbate anion could indeed reduce the α-tocopheroxyl radical,[49,50] and there have been a number of studies on the effects of vitamins C and E on autoxidation induction periods both in homogeneous solution[51] and in aqueous micellar dispersions.[40,52] However, the relevance of such experiments to the situation in vivo is questionable since vitamin C is water-soluble and resides largely in the cytosol, while vitamin E is lipid-soluble and resides largely within biomembranes (vide supra).

We have studied the autoxidation of dilinoleoylphophatidylcholine (DLPC) (see Figure 1) dispersed in water as multilamellar liposomes at 37 °C.[53] By using lipid- and water-soluble azo-initiators we have been able to compare situations in which the initiating radicals originate at a steady and known rate either within the DLPC bilayer or in the aqueous phase.[41] Our results are shown pictorially in Figure 2. Using a lipid-soluble initiator causes autoxidation of the DLPC bilayer and this oxidation is virtually unaffected by the addition of vitamin C to the aqueous phase (curve a). The oxidation is readily inhibited by α-T (curve b) and the length of the induction period can be increased by the presence of vitamin C (curve c). We interpret these results as indicating that lipid peroxyl radicals in the bilayer cannot be "trapped" by the ascorbate anion in the aqueous phase, $(AH^-)_{aq}$, but that the tocopheroxyl radical can be reduced to α-T by this anion, i.e., the partially oxidized α-T in the bilayer is "regenerated" by ascorbate in the aqueous phase. Using a water-soluble

$$(ROO^\bullet)_{lipid} + (AH^-)_{aq} \not\rightarrow \text{no reaction}$$

$$(ArO^\bullet)_{lipid} + (AH^-)_{aq} \rightarrow (ArOH)_{lipid} + (A^-)_{aq}$$

initiator causes oxidation of the bilayer to occur (curve d) but this oxidation can be prevented by vitamin C (curve e) or α-T (curve f). The effect of vitamins C and E together is purely additive under these conditions (curve g). Obviously peroxyl radicals formed in the aqueous phase are trapped by $(AH^-)_{aq}$ more readily than they enter the bilayer to initiate autoxidation. Results that are essentially identical have been obtained by Niki et al.[54] using soybean phosphatidylcholine liposomes in aqueous dispersion.

Ames et al.[55] have suggested that uric acid, which is present in human plasma at much higher concentrations than vitamin C, can also act as an antioxidant in vivo. It has been found[56,57] that urate behaves somewhat like ascorbate. Thus, it will inhibit the oxidation of a phospholipid bilayer that has been initiated in the aqueous phase but not one that has been initiated in the lipid phase.[56,57] However, in contrast to ascorbate, urate will not extend the induction period produced by α-T for reactions that are initiated in the lipid phase.[56b,57]

We have examined human plasma for other water-soluble, chain-breaking antioxidants by essentially the same ROO$^\bullet$ "titration" technique described above.[22,23] Whole plasma was studied, the plasma lipids serving as the oxidizable substrate, RH, and their oxidation being initiated by a water-soluble azo compound.[58,59] Vitamin E, ascorbate and urate were analyzed by HPLC and the stoichiometric factors, n, i.e., the number of ROO$^\bullet$ trapped per antioxidant molecule, were determined for ascorbate and urate under realistic conditions by measuring the increase in the induction period

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Table III.
α-T Biokinetics in the Rat

tissue	expt 1. uptake of d_3-RRR-α-T		expt 2. competitive uptake of d_6-RRR-α-T and d_3-SRR-α-T			
	days until $d_3/d_0 = 1.0$	d_3/d_0, after 65 days	d_6/d_3 at day			
			2	8	32	154
lung	8	8.6	1.3	1.2	1.9	2.6
liver	10	6.8	0.5	0.7	1.2	1.2
plasma	11	6.1	1.4	1.6	2.3	2.4
RBC	13	5.2	2.1	2.0	3.1	3.6
brain	29	2.0		1.4	3.2	5.3

produced by incremental additions of these compounds to a standard plasma sample. Vitamin E ascorbate and urate all have antioxidant activity but together they account for only 50–70% of the total radical-trapping antioxidant capacity (TRAC) of human plasma.[58,59] The plasma proteins account for the balance of the TRAC. Their antioxidant activity is a nonspecific property since it was found in all plasma protein fractions separated by gel filtration.[58,59] This nonspecificity also manifested itself as an increase in the induction period of a standard plasma sample that could be produced by the addition of various proteins, e.g., bovine serum albumin, transferrin, ceruloplasmin, and papain. Studies with specific amino acids suggest that this antioxidant activity is largely confined to cysteine residues bearing free thiol groups.[59]

Although vitamin E represents only a small fraction of the plasma's TRAC we believe it plays a vital role in maintaining *efficient* inhibition of peroxidation through its interaction with water-soluble antioxidants (especially ascorbate). The latter should cause the vitamin E to be "regenerated" continuously until they themselves have been consumed. Consistent with this view is our observation that during a controlled (azo-initiated) plasma autoxidation the protein SH groups start to deplete immediately.[59] When they have decreased to ca. 50% of their initial value the urate starts to deplete, and only when essentially all the SH groups and >80% of the urate have gone does the vitamin E level drop below 50% of its initial value. The induction period ends when all the vitamin E has been consumed.

The Phytyl Tail of α-T and Its Stereochemistry

Although 1 is an excellent antioxidant it has no vitamin E activity. The phytyl "tail" of tocopherols serves to hold the chemically reactive "head" in biomembranes. Thus, model studies by Niki et al.[60] have shown that 1 incorporated into one type of PC liposome can inhibit the oxidation of a second type of PC liposome whereas α-T cannot do this. It is clear that the smaller molecule (1) is rapidly exchanged between the liposomes but that this is not the case for α-T.

The stereochemistry of the phytyl tail is known to affect the bioactivity of α-T. Of the eight stereoisomers of α-T, the natural isomer, *RRR*-α-T has been shown to be the most bioactive.[61] The isomer, with inverted stereochemistry at position 2, i.e., *SRR*-α-T has about 30% of the activity of *RRR*-α-T. There appear to have been no real attempts to explain or explore the origin of the different bioactivities of α-T stereoisomers. To us the magnitude of the *RRR*/*SRR* difference appeared far too small to be due to some enzyme or protein mediated chemical or physical (e.g., transport) process. We therefore undertook two biokinetic experiments.[62,63]

Experiment 1: Three-week old male rats were raised on a standard vitamin E-free diet to which we had added 36 mg of *RRR*-α-T acetate/kg chow. After 4 weeks the diet was changed to one based on the same chow but in which the natural α-T acetate was replaced by an equal quantity of 5CD$_3$-*RRR*-α-T acetate. Blood and tissue samples were taken at various times and their lipids were extracted by a new procedure involving sodium dodecylsulfate which we were forced to develop[64] because traditional extraction methods[26,65] were found to be very slow, inefficient, and cumbersome. The α-T fraction was separated by HPLC, silylated, and analyzed by GC/MS. The ratio d_3-α-T/α-T increases, with time, but at quite different rates in different tissues (see Table III). This ratio gives for the first time a measure of the rate at which natural α-T is "consumed" by different tissues under steady-state conditions, i.e., $t_{1/2}$, corresponding to 50% consumption of *RRR*-α-T is ca. 1 week for the lung but is ca. 1 month for the brain.

Experiment 2: The change in the rats' diet after 4 weeks involved the addition of 18 mg 5,7-(CD$_3$)$_2$-*RRR*-α-T plus 18 mg 5-CD$_3$-*SRR*-α-T acetates/kg chow. Analysis of the ratio d_6-α-T/d_3-α-T gives a measure of the discrimination in favor of the natural isomer. The degree of discrimination depends not only on the tissue but also on the duration of the experiment (see Table III). To us these results suggest that there can be no simple comparative tests of the biopotency of synthetic antioxidants vs. natural α-T. The results obtained in any test must depend on the nature of the test (e.g., the tissue examined) and the duration of the test. The general assumption[66] that *RRR*-α-T has 1.36 times the activity of synthetic *all-rac*-α-T (which is an equimolar mixture of all eight stereoisomers) must be questioned. Thus, if the brain needs extra protection against radical damage (because of radiation therapy following surgery, for example) massive oral doses of synthetic α-T will not only be absorbed very slowly by the brain but also there will be some discrimination against at least some of the α-T that is consumed.

In plasma the initial (time → 0) d_6/d_3 ratio is 1.35, presumably because of chiral discrimination favoring the natural stereoisomer in one or more steps during

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transport from gut to plasma. In the RBC membranes the initial ratio is 1.8 which suggests a second chiral discrimination step. Interestingly, the $(d_6/d_3)_{RBC}/(d_6/d_3)_{plasma}$ ratio is 1.35 ● 0.13 for the entire duration of the experiment (154 days). Since the α-T in the plasma lipoproteins and in the RBC membranes is in fairly rapid equilibrium,[67] our results imply that the rate of transfer of *RRR*-α-T from plasma to RBC is larger than that for *SRR*-α-T or/and the transfer rate of *RRR*-α-T in the reverse direction is smaller than for *SRR*-α-T. We believe this chiral discrimination by the RBC membrane simply reflects the fact that membranes are themselves composed of chiral molecules (including the phospholipids, see Figure 1) which "recognize", i.e., absorb, one stereoisomer of vitamin E preferentially. This provides a simple explanation for the differences in bioactivity of α-T stereoisomers, particularly since passage through a series of membranes could lead to a fairly high degree of discrimination.

An Attempt To Outdo Nature

Compound 4 (see Table II) was synthesized to verify the stereoelectronic explanation for the high k_5 values for α-T and 1. If vitamin E owes its bioactivity solely to the fact that it is a chain-breaking antioxidant with appropriate membrane- and lipid-philicity, then a derivative of 4 in which one of the methyl groups at the 2-position has been replaced by a phytyl tail *might* show more bioactivity than α-T. Whether it would actually exhibit more bioactivity than α-T would depend, of course, not only on the magnitude of k_5 but also on whether the transportation, positioning, *and* regeneration of such a compound were at least as efficient as is the case for α-T. We have synthesized 6 in the *all-rac*

HO, $[(CH_2)_3CHCH_3]_3CH_3$, CH_3, O

6

form and compared its bioactivity with that of *all-rac*-α-T using the curative myopathy bioassay.[68] In this test rats are depleted in vitamin E until their plasma pyruvate kinase (PK, an enzyme released by degenerating muscle) levels have risen by a factor of 10–100. The test compounds are then fed to groups of rats at three different dose levels for 4 days. If the compounds are bioactive the PK level will fall and a plot of the PK level after 4 days vs. log(dose) will have a negative slope indicating that bio-response increases with dose. A more bioactive compound causes a greater reduction in PK for a given dose than a less active compound. Our results[69] show that **6** has 1.5–1.9 times the bioactivity of *all-rac*-α-T. To our knowledge this represents the first time that a systematically designed, man-made compound has at least equaled, and perhaps even outperformed[70] the natural vitamin.

Concluding Remarks

Application of the principles of physical organic chemistry to the study of vitamin E has provided some rich rewards. These includes the stereoelectronic explanation for α-T's exceptional antioxidant activity and proof that vitamin E is the major chain-breaking, lipid-soluble antioxidant in mammalian tissues together with evidence that it may be involved in the division of normal and cancerous cells. The position of α-T in phospholipid bilayers has been defined and evidence obtained for α-T's "regeneration" by ascorbate and for the important role played in plasma by two other water-soluble, chain-breaking antioxidants, urate and protein SH groups. A simple explanation for the different bioactivities of two α-T stereoisomers has been provided. Finally, and we believe of most significance, a compound has been synthesized that, in one bioassay at least, equals or perhaps even outperforms natural vitamin E. We hope our work will add further impetus to the growing move to apply the principles of physical organic chemistry to problems of biological significance.

Thanks are due to all those colleagues and collaborators whose names are in the references. Without their unstinting efforts and imaginations such a complete investigation of vitamin E would never have been possible. As an encouragement to other scientists who attempt to cross traditional disciplinary lines we note that most of the more biologically oriented work described herein was rejected when first submitted for publication for reasons varying from editorial whim to the goring of oxen sacred to particular scientific establishments. That we persevered in our efforts to publish our work, generally without change, can be largely attributed to the kind and helpful advice of Professor T. F. Slater, so our special thanks go to him. We also thank the National Foundation for Cancer Research and the Association for International Cancer Research for their generous financial support for much of this work.

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(70) Assuming that *RRR*-α-T is 1.36 times as bioactive as all-*rac*-α-T.[66] We have now synthesized and are currently measuring the bioactivities of *RRR*-**6** and *SRR*-**6**.