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Biological Antioxidants [and Discussion]

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Biological antioxidants

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The mechanism of lipid peroxidation and the ways in which the rate of this reaction can be reduced by small quantities of certain specific chemicals, called antioxidants, are described. The types and roles of the different antioxidants found in living systems are considered. Vitamin E (α-tocopherol) has long been recognized as an important lipid-soluble, chain-breaking antioxidant. It has an unexpectedly high reactivity towards peroxyl radicals, which can be understood only after detailed consideration of its structure. It is the major antioxidant of its class in human blood and its effectiveness in plasma is greatly improved by a synergistic interaction with water-soluble reducing agents such as ascorbic acid. Experiments designed to locate vitamin E within phospholipid bilayers and to discover the origin of the different biopotencies of stereoisomers of α-tocopherol are also described.

Introduction

Many foods, even when stored in a refrigerator, will eventually become rancid, which is a sign that the lipid material (i.e. the fats) in the food have undergone a chemical reaction with atmospheric oxygen. Such more or less spontaneous oxidation processes, which occur under mild conditions at ambient and sub-ambient temperatures are called autoxidations or, more commonly in biological circles, lipid peroxidations, which may be non-enzymic or enzyme-catalysed reactions. The process is a free-radical chain reaction which can be represented by four elementary reactions:

initiation: production of $R^{\cdot}$ (rate $= R_i$); (1)

propagation: $R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$; (2)

$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$; (3)

termination $ROO^{\cdot} + ROO^{\cdot} \rightarrow$ molecular products. (4)

In this scheme, RH represents the lipidic material and $R^{\cdot}$ the carbon-centred radical derived from it by removal of a hydrogen atom. The radical $R^{\cdot}$ reacts very rapidly with oxygen to form the peroxyl radical $ROO^{\cdot}$ which, in a subsequent much slower step, attacks the lipid to form a molecule of hydroperoxide, ROOH, and a new $R^{\cdot}$ radical. The propagation sequence of reactions 2 and 3 is eventually broken when two of the chain-carrying $ROO^{\cdot}$ radicals react together to give molecular products. For every radical that initiates a new chain there are

therefore many molecules of RH that are oxidized to ROOH. The overall rate of autoxidation can be represented by

$$-\mathrm{d}[\mathrm{O}_2]/\mathrm{d}t = k_3[[\mathrm{RH}]R_\mathrm{i}^{\frac{1}{2}}/(2k_4)^{\frac{1}{2}}, \quad (5)$$

where R_i is the rate of chain initiation and the ks are the rate constants for the indicated reactions.

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 and this can have disastrous consequences for the organism, because one of the principal functions of a membrane is to act as a dividing wall that compartmentalizes biochemical processes into specific cells and into specific regions within an individual cell.

Antioxidants

How does Nature protect cell membranes against peroxidation?

To answer this question it is helpful to consider the ways in which man protects certain products of his technology such as lubricating oils, rubber, and plastics, against oxidative degradation. The protection is provided by the addition of fairly small quantities of certain specific compounds called antioxidants. Such compounds are divided into two broad classes, referred to as preventive antioxidants and chain-breaking antioxidants.

Preventive antioxidants reduce the rate of chain initiation. The molecular precursor for the initiation process is generally the hydroperoxidic product, ROOH, of the oxidation. Most commercial preventive antioxidants function by converting hydroperoxides to molecular products that are not potential sources of free radicals. Similarly, most biological preventive antioxidants are also peroxide decomposers. The most important are certain enzymes that are capable of reducing hydroperoxides: for example, catalase reduces H_2O_2 to H_2O and glutathione peroxidase can reduce H_2O_2 to H_2O and also lipid hydroperoxides to the corresponding alcohol, i.e.

$$\mathrm{ROOH} \xrightarrow[\text{glutathione peroxidase}]{[2\mathrm{H}]} \mathrm{ROH} + \mathrm{H_2O}. \quad (6)$$

A second class of preventive antioxidants are those which inhibit photo-induced autoxidations. One such process involves the dye-sensitized formation of singlet oxygen by visible light and its 'ene' reaction with unsaturated fatty acids to form hydroperoxide.

$$\mathrm{dye} \xrightarrow[\text{light}]{\text{visible}} \mathrm{dye}^* \xrightarrow{^3\mathrm{O}_2} \mathrm{dye} + {}^1\mathrm{O}_2, \quad (7)$$

$$^1\mathrm{O}_2 + \mathrm{RCH{=}CHCH_2R'} \rightarrow \mathrm{RCH(OOH)CH{=}CHR'}. \quad (8)$$

The compound β-carotene is an extremely efficient quencher of singlet oxygen. It can therefore also be classified as a preventive antioxidant.

$$^1\mathrm{O}_2 + \beta\text{-carotene} \rightarrow {}^3\mathrm{O}_2 + \beta\text{-carotene} + \text{heat}. \quad (9)$$

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Commercial chain-breaking antioxidants are generally phenols or aromatic amines. They owe their antioxidant activity to their ability to trap peroxyl radicals. For a phenol, such as one of the four tocopherols (α-(see figure 1), β-, γ- and δ-) that together constitute vitamin E, the initial step involves a very rapid transfer of the phenolic hydrogen atom (Burton & Ingold 1981).

$$ROO^{\cdot} + ArOH \rightarrow ROOH + ArO^{\cdot}. \quad (10)$$

The phenoxyl radical is resonance-stabilized and is relatively unreactive toward RH and O_2, and therefore it does not continue the chain. It is eventually either destroyed by reaction with a second peroxyl radical,

$$ROO^{\cdot} + ArO^{\cdot} \rightarrow \text{molecular products} \quad (11)$$

or, in certain systems, it may be 'repaired', that is, reduced to the starting phenol by reaction with a water-soluble reducing agent such as the ascorbate anion, AH^-, vitamin C (Packer *et al.* 1979).

$$ArO^{\cdot} + AH^- \rightarrow ArOH + A^{-\cdot}. \quad (12)$$

Such water-soluble reducing agents may also trap any chain-carrying peroxyl radicals that are present in the aqueous phase (Doba *et al.* 1984), whereas vitamin E, being lipid-soluble, traps $ROO^{\cdot}$ radicals in the lipid phase.

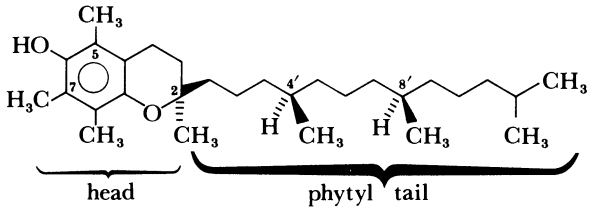

FIGURE 1. Natural (2*R*, 4′*R*, 8′*R*) α-tocopherol. Only those positions that are specifically referred to in the text have been numbered.

The main radical species that is likely to be present in the aqueous phase of aerobically respiring cells is the superoxide anion. Though not itself particularly reactive, O_2^- is in equilibrium with $HOO^{\cdot}$, its conjugate acid.

$$O_2^- + H^+ \rightleftharpoons HOO^{\cdot}. \quad (13)$$

The $HOO^{\cdot}$ radical is generally more reactive than O_2^-. However, at physiological pH this equilibrium favours O_2^- by a factor of about 100. The superoxide dismutase enzymes are the biological chain-breaking antioxidants that control the O_2^- threat to the organism.

Finally, let us reconsider the roles of β-carotene. Epidemiological studies have sparked a growing interest in this compound because they have indicated a lower incidence of certain types of cancer among individuals with an above-average intake of β-carotene and other carotenoids (Peto *et al.* 1980; Shekelle *et al.* 1981). These studies led us to investigate the chain-breaking antioxidant activity of β-carotene. We found that in addition to its efficient quenching of singlet oxygen, $^1O_2$, it belongs to a previously unknown class of biological antioxidants that exhibit good radical-trapping behaviour only at partial pressures of oxygen significantly less than the 150 Torr† of normal air (Burton & Ingold 1984). Such low O_2

† 1 Torr = 101325/760 Pa.

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pressures are found in most tissues under physiological conditions (e.g. 15 Torr in the capillaries of actively working muscles). The antioxidant activity of β-carotene arises because this compound is highly reactive towards $ROO^{\cdot}$ radicals yielding a resonance-stabilized carbon-centred radical. The reaction of this carbon-centred radical with oxygen is reversible at ambient temperatures.

$$ROO^{\cdot} + \beta\text{-carotene} \rightarrow \beta\text{-carotenyl radical}, \tag{14}$$

$$\beta\text{-carotenyl}^{\cdot} + O_2 \rightleftharpoons \beta\text{-caroteneperoxyl radical}. \tag{15}$$

At low O_2 partial pressures the β-carotenyl radical acts as an efficient trap for a second peroxyl radical.

$$ROO^{\cdot} + \beta\text{-carotenyl}^{\cdot} \rightarrow \text{molecular products}. \tag{16}$$

Absolute reactivities of lipid-soluble phenolic antioxidants *in vitro*

In model systems a phenolic antioxidant traps two peroxyl radicals, thereby breaking two oxidation chains and so reducing the chain length and the rate of oxidation. Naturally, the phenol is being continuously consumed and when it has all been used up the rate will return to the value it would have had if no antioxidant had been added (see figure 2). The time during which the rate of oxidation is suppressed is known as the induction period and its duration, τ, is very simply related to the concentration of phenol and rate of chain initiation by the equation

$$\tau = 2[\mathrm{ArOH}]/R_i. \tag{17}$$

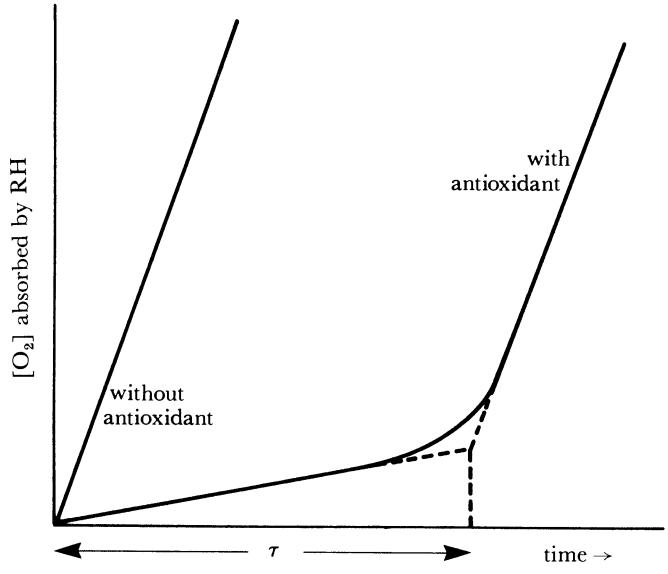

Figure 2. Effect of a chain-breaking antioxidant.

The initial rate of a phenol-inhibited oxidation in a model system can be represented by

$$-\mathrm{d}[O_2]/\mathrm{d}t = k_3[\mathrm{RH}]\,R_i/2k_{10}[\mathrm{ArOH}]. \tag{18}$$

The activity of the phenolic antioxidant is directly related to the magnitude of k_{10}. This quantity is determined by measuring the inhibited rate of autoxidation of some substrate RH, for which

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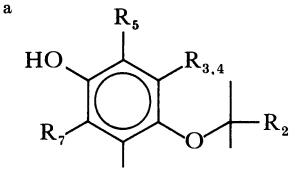
k_3 has been measured, at a constant and known rate of chain initiation. We have used this technique to measure k_{10} values for a variety of phenols at 30 °C (Burton & Ingold 1981; Burton *et al.* 1983, 1984). Our results, some of which are summarized in table 1, show that the order of antioxidant activity of the tocopherols (α- > β- ⩾ γ- > δ-) is the same as that of their biological activities (Century & Horwitt 1965). Furthermore, α-tocopherol (α-T) and the structurally related model compound, pentamethylhydroxychroman (**3**), are considerably better traps for peroxyl radicals than the major phenolic antioxidants used in commerce, 2,6-di-t-butyl-4-methyl (**5**) and 4-methoxyphenol (**6**).

TABLE 1. VALUES OF k_{10} AT 30 °C AND SOME θ VALUES FOR CERTAIN PHENOLIC ANTIOXIDANTS

phenol	$10^{-4}\,k_{10}/(M^{-1}\,s^{-1})$	θ
δ-T[a]	65	—
γ-T[a]	130	—
α-T[a]	320	—
(**1**)[b]	39	90°
(**2**)[a]	250	38°
(**3**)[a]	380	17°
(**4**)[a]	570	⩽ 5°
(**5**)[c]	1.4	—
(**6**)[c]	11	—

	R_2	$R_{3,4}$	R_5	R_7
δ-T	$C_{16}H_{33}$	CH_2CH_2	H	H
γ-T	$C_{16}H_{33}$	CH_2CH_2	H	CH_3
α-T	$C_{16}H_{33}$	CH_2CH_2	CH_3	CH_3
(**2**)	CH_3	CH=CH	CH_3	CH_3
(**3**)	CH_3	CH_2CH_2	CH_3	CH_3
(**4**)	CH_3	CH_2	CH_3	CH_3

a



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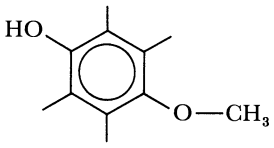
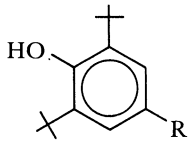


c



(**5**) R = CH_3
(**6**) R = CH_3O

We have invoked stereoelectronic factors to explain the fact that 4-methoxytetramethylphenol (**1**) has only about 10% of the reactivity of α-T or **3** (Burton & Ingold 1981). In (**1**) the methoxy group is perpendicular to the plane of the aromatic ring (i.e. $\theta = 90°$; see figure 3). In this position, the p-type lone pair on the ethereal oxygen atom lies in the plane of the aromatic ring and so cannot stabilize the corresponding phenoxyl radical. However, in α-T and in (**3**) the second ring adopts a half-chair conformation and holds the ethereal oxygen in such a position that $\theta \approx 17°$. The lone pair orbital can therefore overlap with the orbital containing the unpaired electron and stabilize the phenoxyl radical. The more stable the phenoxyl radical, the weaker will be the O—H bond in the parent phenol, and the weaker this bond, the more readily it will be cleaved by an attacking ROO•, i.e. the more effective it will be as an antioxidant.

This stereoelectronic description of antioxidant activity leads to the prediction that for structurally related 4-alkoxyphenols, k_{10} will increase as θ decreases from 90° (**1**) towards 0°.

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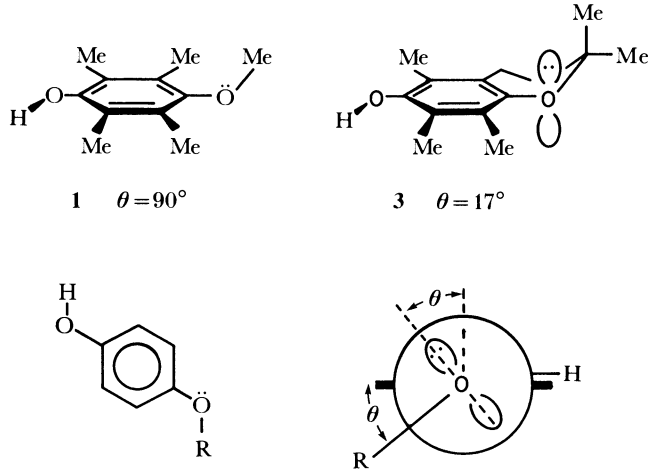

FIGURE 3. Structures of **1** and **3** and their θ values. The definition of θ is shown at the lower right for the 4-alkoxyphenol shown at the lower left.

This prediction was confirmed by showing that the pentamethylhydroxychromenol (**2**), with $\theta = 38°$, is less reactive than **3**, while the pentamethylhydroxydihydrobenzofuran (**4**), with $\theta \leqslant 5°$, is more reactive, see table 1 (Burton *et al.* 1983, 1984).

PROOF THAT VITAMIN E IS THE MAJOR LIPID-SOLUBLE, CHAIN-BREAKING ANTIOXIDANT IN HUMAN BLOOD

Vitamin E functions as a protective agent against lipid peroxidation *in vivo* (Machlin 1980; Tappel 1980). As we have demonstrated, it is also an extremely potent (though not the most potent) chain-breaking phenolic antioxidant *in vitro*. We have performed a series of experiments (Burton *et al.* 1983) in which high pressure liquid chromatography (h.p.l.c.) was used to measure the concentrations of the four tocopherols (mainly α-T) in lipid extracts of human blood plasma and human erythrocyte ghost membranes. We also added a small portion of each of these lipid extracts to styrene that was undergoing autoxidation at 30 °C with a constant and known rate of chain initiation. The lipid extracts produced induction periods and from the length of these induction periods we could calculate, by using (17), the total concentration of all chain-breaking antioxidants present in the lipid extract. Comparison with the total concentration of tocopherols, measured by h.p.l.c. (that equals, of course, the total concentration of vitamin E), showed that most, if not all, of the chain-breaking antioxidant in the plasma and ghost membrane extracts was vitamin E. We have therefore concluded that vitamin E is the major lipid-soluble, chain-breaking antioxidant in human blood (Burton *et al.* 1983).

A tetramethylhydroxydihydrobenzofuran having a $C_{16}H_{33}$ phytyl 'tail' attached at position 2 (i.e. **4** but with $R_2 = C_{16}H_{33}$) has recently been synthesized in our laboratory (L. Hughes 1984, unpublished results). We hope that the phytyl tail will give this compound appropriate lipophilicity and other desirable characteristics for its incorporation into biomembranes *in vivo*. We have recently started to measure the vitamin E activity of this compound on vitamin-E-depleted rats (C. D. O. Foster and coworkers 1984, unpublished results) by using one of the standard bioassay procedures (Machlin *et al.* 1982).

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Kinetic studies on the autoxidation of phospholipid bilayers

The kinetic rate law for uninhibited, liquid-phase, autoxidation (5) has frequently been shown to apply to homogeneous organic systems. However, it is not self-evident that it should also apply to the heterogeneous types of systems that would be involved in lipid peroxidation *in vivo*, nor even for the autoxidation of phospholipid bilayers dispersed in water. The first quantitative kinetic studies on the latter type of system used egg lecithin phosphatidylcholine bilayers dispersed as multilamellar vesicles (Barclay & Ingold 1981). Oxidation was initiated by the thermal decomposition of a lipid-soluble azo compound, and the rate of chain initiation that it produced was determined (by means of (17)) by using α-T as the lipid-soluble, chain-breaking antioxidant. Both the azo initiator and the α-T had to be added to a methylene chloride solution of the egg lecithin. The CH_2Cl_2 was then removed by evaporation and this was followed by the dispersion of the residue in water. Despite the somewhat inelegant experimental approach, (5) was shown to describe the oxidation kinetics of this system.

It was clear that this experimental approach would have to be modified if we were to study the autoxidation kinetics of biologically interesting systems. The breakthrough came when we discovered that not only could the oxidation of phospholipid bilayers be initiated with a lipid-soluble initiator, but also with a water-soluble initiator (Barclay *et al.* 1984). Furthermore, whichever initiator was used, the reaction could be inhibited by both α-T and a water-soluble analogue of α-T (Trolox(−), which has a modified structure **3** with R_2 as CO_2^-). The rate constant ratio, $k_3/(2k_4)^{\frac{1}{2}}$ of (5) (which is generally referred to as the oxidizability of RH) had, as it should, essentially the same value irrespective of the phase in which the initiator and inhibitor were present. Clearly peroxyl radicals generated in the aqueous phase can diffuse into the bilayer to start oxidation chains, while Trolox(−) can get sufficiently close to the peroxyl radicals that carry the chain within the bilayer to intercept them (via reaction 10).

Vitamin E–vitamin C interaction

It has been known for many years that vitamin C is, by itself, an ineffective antioxidant but that in many systems it will greatly extend the induction period produced by vitamin E (Golumbic & Mattill 1941). That is to say, vitamin E–vitamin C mixtures are synergistic because their joint effect is greater than the sum of their individual effects. As Tappel (1968) originally suggested, vitamin C may, in living organisms, 'repair' (i.e. regenerate) vitamin E by reducing the tocopheroxyl radicals formed in reaction (10). Pulse radiolytic experiments have subsequently shown that in homogeneous solution the ascorbate anion can indeed reduce the α-tocopheroxyl radical, reaction (12) (Packer *et al.* 1979).

Although there have been several studies of vitamin E–vitamin C synergistic antioxidant effects in, for example, micellar oxidations (Barclay *et al.* 1983; Yamamoto *et al.* 1984) only some of our own recent measurements appear to be relevant to the probable situation in living organisms (Doba *et al.* 1985). By using multilamellar vesicles of dilinoleoylphosphatidylcholine (DLPC) as the oxidizable bilayer we have found that for oxidations initiated by the thermal decomposition of a lipid-soluble azo compound, vitamin C produces no induction period, though it will extend the induction period due to α-T. (Based on the fact that α-T traps two peroxyl radicals per molecule α-T, the increase in τ produced by the addition of ascorbate indicates an effective trapping of *ca.* 0.4 peroxyl radicals per molecule ascorbate in the vitamin E–vitamin C system). However, in reactions initiated by thermal decomposition of a

water-soluble azo-compound, vitamin C produces an excellent induction period, the length of which implies that each molecule of ascorbate can trap 0.6 peroxyl radicals. Therefore, although the ascorbate anion (unlike Trolox(−)) cannot penetrate sufficiently deeply into the phospholipid bilayer (see also Schreier-Mucillo *et al.* 1976) to intercept the chain-carrying $ROO^{\cdot}$ radicals, it can and does trap peroxyl radicals that are formed in the aqueous phase before they can diffuse into the bilayer. It is therefore highly probable that in living organisms vitamin C functions both as a direct chain-breaking antioxidant for water-soluble peroxyl radicals (e.g. $HOO^{\cdot}$) and as an indirect chain-breaking antioxidant by regenerating vitamin E.

Kinetic studies on the autoxidation of blood plasma

The discovery that water-soluble azo compounds can be used in heterogeneous systems to initiate lipid peroxidation has allowed us to commence investigation of the autoxidation of human blood plasma. We have already shown (vide supra) that vitamin E is essentially the only lipid-soluble antioxidant in plasma. However, preliminary comparison of vitamin E concentration measured by h.p.l.c. with the length of the induction period indicate that the 'effective' antioxidant level in plasma is approximately 15–20 times greater than can be accounted for by the vitamin E alone (Wayner *et al.* 1986). The relative contributions to this extended induction period of ascorbate, urate (see Ames *et al.* 1981) and superoxide dismutase are currently being explored (D. Wayner 1984, unpublished results).

Comparison of natural *RRR*-α-T with unnatural *SRR*-α-T

Tocopherols have three chiral carbon atoms and the natural materials have the 2*R*, 4′*R*, 8′*R*-configuration (see figure 1). Natural *RRR*-α-T is the most bioactive form of vitamin E having, for example, about three times the bioactivity of the synthetic isomer with inverted stereochemistry at position 2, i.e. *SRR*-α-T (Weiser & Vecchi 1982). The magnitude of this difference in bioactivity between stereoisomers would appear to be far too small to be a result of some enzyme-mediated chemical or physical (such as transport) process. There appears to have been no real attempt to explain and few attempts to explore the origin of the difference in bioactivity of these two stereoisomers (Weber *et al.* 1964*a*, *b*). We therefore undertook the following experiment.

Male rats were raised from weaning on a standard vitamin-E-free diet to which we had added 36 mg of natural *RRR*-α-T acetate per kilogram of chow. After four weeks the diet was changed to one based on the same chow but in which the natural α-T acetate was replaced by an equimolar mixture of *RRR*-α-T 5,7-$(CD_3)_2$ acetate and *SRR*-α-T 5-CD_3 acetate, in a total quantity identical to that of the *RRR*-α-T acetate present in the initial diet. Blood samples were taken at various times by heart puncture under anaesthetic and tissue samples after sacrifice. Lipids were extracted and the α-tocopherol-containing fraction was separated by h.p.l.c. Subsequentl analysis by g.c.–m.s. allowed the direct measurement of the *RRR*:*SRR* ratio of the two deuterated isomers without interference by the natural (i.e. unlabelled) *RRR*-α-T. Figure 4 shows this ratio as a function of time for blood plasma and red blood cell membranes. Measurements of this ratio at times less than one day are clearly desirable (see Weber *et al.* 1964*b*) and will soon be undertaken. However, in the meantime we tentatively

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suggest that the initial (time$\rightarrow$0) ratio of 1.35 in plasma is a result of chiral discrimination (favouring the natural stereochemistry) on the transport from gut to plasma. The initial ratio of 1.8 in the red blood cell membranes suggests a second chiral discrimination favouring the absorption into this membrane of the natural stereoisomer, again by a factor of *ca.* 1.35 (i.e. $(1.35)^2 \approx 1.8$). If we assume that α-T in the lipoproteins of the plasma and in the red blood cell membranes are in equilibrium on the timescale of our experiment, as seems highly probable (Silber *et al.* 1969; Poukka & Bieri 1970; Bjornson *et al.* 1975), then the rate of transfer of *RRR*-α-T from plasma to membrane is larger than that for *SRR*-α-T, or the transfer rate of *RRR*-α-T in the reverse direction is smaller than for *SRR*-α-T, or both. We believe that this rather small chiral discrimination by the red blood cell membrane simply reflects the fact that membranes are themselves composed of chiral molecules that 'recognize' (i.e. absorb) one stereoisomer of vitamin E preferentially. Passage through a series of membranes could lead to a fairly high discrimination in favour of the *RRR*-isomer in certain tissues, which could explain the origin of the observed differences in bioactivity. Experiments are currently underway to measure the magnitude of this chiral discrimination, by using *in vitro* (rather than *in vivo*) model systems and both natural and synthetic membranes.

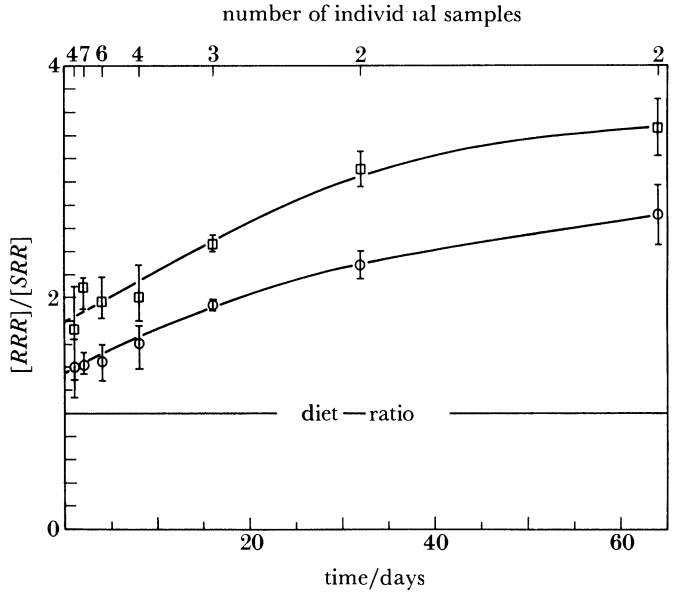

FIGURE 4. Ratio of [*RRR*]/[*SRR*]α-tocoperhol of rats receiving a 1:1 ratio of these stereoisomers in their diet; ○, plasma; □, red blood cell membranes. Number of samples refers to the number of separate rats. Error bars represent the total spread in the ratio.

LOCATION OF VITAMIN E IN PHOSPHOLIPID BILAYERS

Knowledge of the preferred location of vitamin E within a biomembrane would aid in our understanding of its interaction with the membrane derived peroxyl radicals, of the synergistic interaction with vitamin C and urate, and even of the origin of the chiral discrimination described above. A relatively simple ^{13}C-n.m.r. technique that can yield information on the position of α-T in a phospholipid bilayer has been developed by using *RRR*-α-T [5-^{13}C]H_3

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(B. Perly 1984, unpublished results). Unilamellar egg lecithin phosphatidylcholine vesicles containing relatively high concentrations of this labelled compound show a single peak from the ^{13}C label. Addition of the shift reagent, Pr^{3+}, to the liposome suspension causes this signal to split into two distinct peaks. The position of one peak remains unchanged, while the other is shifted downfield by an amount dependent on the Pr^{3+} concentration. We interpret this result as indicating that the α-T cannot be randomly distributed throughout the bilayer but is, instead, located in two definite and distinct regions in the liposome bilayer with slow exchange (on the n.m.r. timescale) between the regions. The unshifted signal must be due to α-T in the inner monolayer of the bilayer, no Pr^{3+} penetrating into the aqueous centre of the liposome. The other signal must be due to α-T in the outer monolayer. Because it is fairly sharp and is also well shifted, the hydroxyl group of α-T must be fairly close to the surface of the bilayer.

In a second experiment, varying concentrations of Gd^{3+} were added to the *RRR*-α-T [5-^{13}C]H_3-containing liposomes dispersed with sufficient Pr^{3+} to split the ^{13}C line. Values for T_1 of the two ^{13}C peaks were measured at varying concentrations of Gd^{3+}. (For a given Gd^{3+} concentration, T_1 will vary as the sixth power of the distance between the Gd and ^{13}C nuclei.) For the 'inner' ^{13}C signal, T_1 is unchanged up to the maximum Gd^{3+} concentration that could be used (10^{-2} M). For the outer signal, however, T_1 had decreased by more than 90% at the maximum Gd^{3+} concentration. These results confirm that the 5-CH_3 group of *RRR*-α-T is close to the surface of the bilayer and that the separation between the 'inner' and 'outer' 5-CH_3 groups is greater than 40–50 Å† (Perly 1984). (The overall thickness of the bilayer should be *ca.* 60 Å.)

Deuterium n.m.r. experiments aimed at determining both the precise shape of the heterocyclic ring of α-T and the orientation of its phytyl tail when the α-T is present in a phospholipid bilayer (I. C. P. Smith & I. H. Ekiel 1984, unpublished results) are being performed. Numerous specifically deuterated *RRR*-α-tocopherols have been prepared for this purpose (L. Hughes 1984, unpublished results). These experiments will also yield information about the mobility of various portions of the α-T molecule within the bilayer.

Conclusion

Our overall defences against free-radical damage have been represented in figure 5. It can be seen that α-tocopherol plays a vital role in these defences. Application of the techniques and, more particularly, the powerful methodology of physical organic chemistry have revealed many facts about α-tocopherol. The most important are (i) its high antioxidant activity and the stereoelectronic explanation for this activity; (ii) its presence as the major (or only) lipid-soluble, chain-breaking antioxidant in human blood and the 'extension' of its antioxidant activity by other water-soluble compounds that are present in the plasma (e.g. ascorbate) and (iii) possible thermodynamic reasons (absorption by chiral membranes) for the varying bioactivities of its different stereoisomers. Similarly, the techniques of biophysics are now providing preliminary information about the location, conformation, and mobility of α-T in phospholipid bilayers and eventually biomembranes. Because radical damage to living organisms has been implicated in many pathological processes (such as heart disease, cancer and ageing), a better understanding of the role of vitamin E should have both scientific and practical benefits. As an example, our

† 1 Å = 10^{-10} m = 10^{-1} nm.

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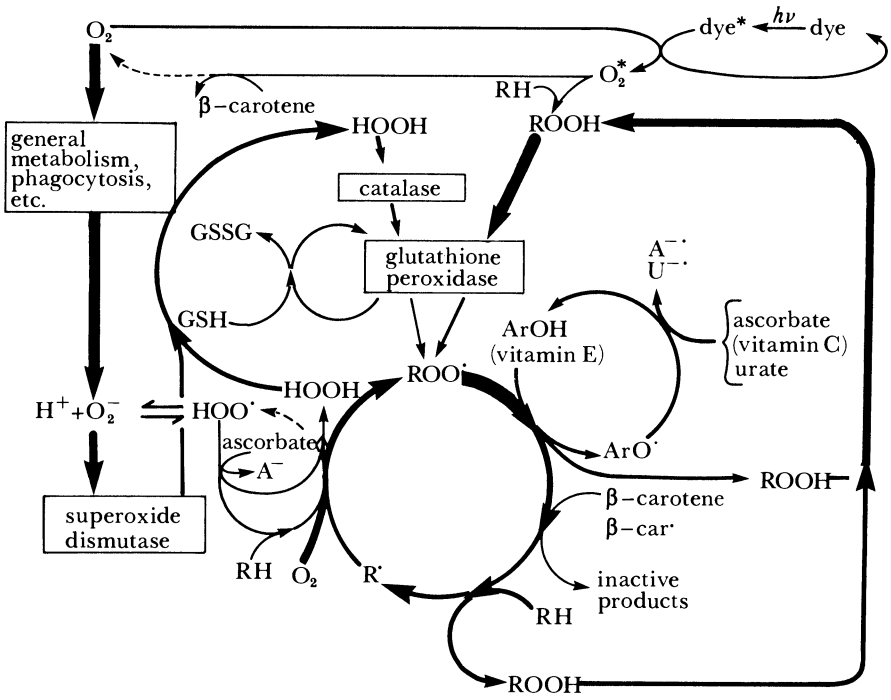

FIGURE 5. Biological autoxidation and antioxidants: the global picture (1984). For simplicity, thin arrows leading from an enzyme are meant to represent that portion of the substrate that is not destroyed by the enzyme.

discovery that the lipids present in various cancerous tissues (Slater *et al.* 1984) and in other rapidly multiplying cells (Cheeseman *et al.* 1985) contain significantly enhanced levels of vitamin E can be cited.

Thanks are due to all those colleagues and collaborators whose names are in the references. Without their unstinting efforts and imagination such a complete investigation of vitamin E would never have been possible. 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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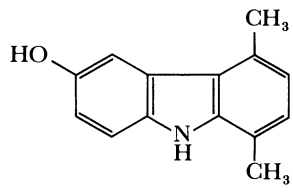
Discussion

R. L. Willson (*Department of Biochemistry, Brunel University, Uxbridge, Middlesex*). We have actually found a compound that has very much the structure Professor Ingold is suggesting; 6-hydroxy-1,4-dimethylcarbazole (HDC).



HDC

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In our *in vitro* microsomal lipid peroxidation system this is a much better antioxidant than vitamin E; indeed, it is the most potent antioxidant we have yet found. Would not its structure be in agreement with Professor Ingold's electron orbital requirements? (See Willson 1983.)

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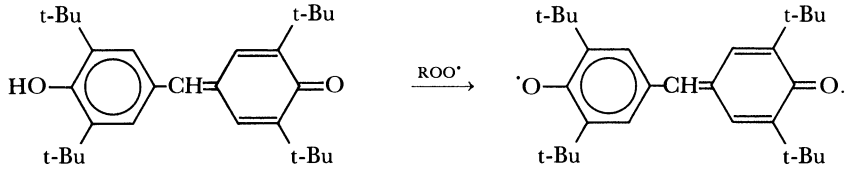
Willson, R. L. 1983 In *Ciba Symposium: Biology of vitamin E*, pp. 19–44. London: Pitman.

K. U. Ingold. Yes.

G. Scott (*Department of Molecular Sciences, Aston University, Gosta Green, Birmingham B4 7ET*). I have a comment and a question. The comment is that BHT is by no means the most active of the synthetic antioxidants, and I wonder whether Professor Ingold has done full justice to the products that man has already produced. Chain-breaking antioxidants are known that are very much more effective than BHT because they give rise to much more stable aryloxyls on reaction with alkylperoxyl. Perhaps the most studied is the reduced form of the stable radical galvinoxyl,



hydrogalvinoxyl galvinoxyl

This system is particularly potent because galvinoxyl is itself an effective antioxidant (chain-breaking electron acceptor) under conditions of limited oxygen availability. This is because it can oxidize an alkyl radical by hydrogen abstraction from the α-position with the regeneration of hydrogalvinoxyl. This amounts to a catalytic antioxidant process in which one molecule of antioxidant can knock out many chain-propagating radicals (Scott 1984).

My question is whether Professor Ingold has any explanation for the fact reported by several workers that, *in vivo*, the concentration of α-tocopherol does not reduce to zero as required by the mechanism he has outlined under conditions of oxidative stress? Indeed there is some evidence that in iron overload, the α-tocopherol concentration is actually higher than it is normally (Green *et al.* 1967). We wonder whether the α-tocopheroloxyl radical may not be reduced by the substrate radicals as in the case of galvinoxyl.

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K. U. Ingold. In biological systems there may be some 'regeneration' of the α-tocopheroxyl radicals by the mechanism Professor Scott suggests under certain conditions. However, Nature appears to have arranged things so that the α-tocopheroxyl formed in the biomembranes is generally reduced back to α-tocopherol by reducing agents that are present in the surrounding aqueous medium (urate, ascorbate, etc.).

There is no requirement in our mechanism that α-tocopherol levels should fall to zero under

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conditions of *in vivo* 'oxidative stress'. One would probably have to reduce the concentration of all the aqueous reducing agents (some of which were referred to above) to zero before the α-tocopherol level would reach zero. I am confident that the *in vivo* experimental object would be dead long before this limit was reached. The possibility that *in vivo* 'oxidative stress' may induce certain biological antioxidants must not be ignored.

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