Abstract. The regeneration of alpha-tocopherol (vitamin E; VE) by coantioxidants such as phenolics and ascorbate has been studied in homogeneous hydrocarbon solution and in biological systems. However, VE phenoxyl radicals (VE•) may be sufficiently reactive to cooxidize phenolic compounds and ascorbates. The coantioxidant behavior of some relevant phenols such as eugenol (EUG), isoeugenol (IsoEUG), 2,6-di-tert-butyl-4-methoxyphenol (DTBMP), trans-resveratrol (RES) and L-ascorbyl-2,6-dibutyrate (ASDB; an ascorbate derivative) with the antioxidant VE at a molar ratio of 1:1 was investigated by the induction period (IP) method in the kinetics of polymerization of methyl methacrylate (MMA) initiated by the thermal decomposition of 2,2'-azobis(isobutyronitrile) (AIBN; a source of alkyl radicals, R•) or benzoyl peroxide (BPO; a source of peroxy radicals, PhCOO•) under nearly anaerobic conditions. Synergism, implying regeneration of VE by the coantioxidant, was observed with only two of these combinations, VE/EUG with PhCOO• and VE/DTBMP with R•. For other mixtures of VE with a phenolic coantioxidant, VE was able to cooxidize the phenolic. Regeneration can only be observed if the bond dissociation energy (BDE) of the coantioxidant is lower than, or at least close to, that of VE. The driving force for regeneration of VE by EUG may be removal of the semiquinone radical of EUG by VE, leading to the formation of VE and EUG-quinonemethide, even though the BDE value of EUG is greater by 5.8 kcal/mol than that of VE. Further evidence for this mechanism of regeneration is provided by the value of approximately 2 for the stoichiometric factor (n) of EUG induced by PhCOO•, but not by R•, again implying the formation of EUG-quinonemethide. The regeneration of VE by DTBMP in the R• system may result from their much smaller difference in BDE (0.1-1.3 kcal/mol). Since VE is rapidly oxidized by PhCOO•, regeneration of VE by DTBMP was not found in this system. The observed IP for the VE/ASDB mixture in the R• system was much lower than that for VE alone, whereas the IP for VE/ASDB in the PhCOO• system was similar to that of VE. In the R• system, VE• was sufficiently reactive to cooxidize ASDB and, in addition, the reproxidation of VE may be promoted by the catalytic action of the ascorbate derivative. The present system, under nearly anaerobic conditions, is relatively biomimetic, since oxygen in living cells is sparse. Such studies could help to explain the mechanism of regeneration of VE by coantioxidants such as phenolic compounds and vitamin C in vivo.

A free radical interaction occurs between alpha-tocopherol (VE) and vitamin C, resulting in enhancement of the antioxidant activity of VE in the presence of vitamin C (1-3). There are also a large number of experimental reports on the mechanisms of regeneration of VE by other coantioxidants such as glutathione (GSH) (4) and poly- and mono-functional phenolics (3, 5). However, the regeneration mechanism of VE (Figure 1, I) by coantioxidants has not yet been fully elucidated, since the efficiency of regeneration has been found to be dependent not only on the structure of the coantioxidant, but also on its relative quantity and on the microenvironment of the reaction medium (3). For maximum biological relevance, studies on the regeneration of VE by coantioxidants such as trans-resveratrol and ascorbate should be performed under anaerobic conditions, because biological systems have a low oxygen tension. Resveratrol is the main contributor to the total antioxidant activity of red wine and has been reported to have not only chemopreventive effects, but also therapeutic effects on
cancer (6). Cancer cells are anaerobic in their metabolism and generally have very poor mechanisms for absorbing antioxidants. Alone among the antioxidants, ascorbate, which is remarkably similar in chemical structure to glucose, is absorbed to a much greater extent by cancer cells than by normal cells (7,8). A recent review of clinical studies has concluded that there is little evidence that supplementation with VE reduces the risk of cancer (9).

With this background, a systematic investigation of the free radical interaction between VE and the coantioxidants eugenol (EUG, compound 2), isoeugenol (IsoEUG, compound 3), 2,6-di-tert-butyl-4-methoxyphenol (DTBMP, compound 4), L-ascorbyl-2,6-dibutyrate (ASDB, compound 6) and trans-resveratrol (RES, compound 5) in order to clarify the synergistic behavior of combinations of antioxidants under anaerobic conditions.

We have previously proposed a quantitative model rationalizing the radical-scavenging activity of various antioxidants (10,11). In the present study, the radical-scavenging activity of mixtures of VE and coantioxidants were investigated by the induction period method in the polymerization of methyl methacrylate (MMA) initiated by the thermal decomposition of azobisisobutyronitrile (AIBN) or benzoyl peroxide (BPO). The reaction was monitored by the sensitive method of differential scanning calorimetry (DSC) and the model was well able to explain the mechanism of the radical-scavenging activity of these antioxidants. ASDB was used as a representative ascorbate derivative instead of vitamin C, which has only limited solubility in MMA.

Materials and Methods

The following chemicals and reagents were obtained from the indicated companies: EUG (2-methoxy-4-allylphenol), isoEUG (4-propenyl-2-methoxyphenol), DTBMP (2,6-di-tert-butyl-4-methoxyphenol), ASDB, MMA, AIBN and BPO (Tokyo Kasei Chem. Co., Japan); RES (trans-3,4',5-trihydroxystilbene) (Sigma Chemical Co., St. Louis, MO, USA). MMA was purified by distillation. AIBN and BPO were recrystallized from methanol and methanol/chloroform (1:1 v/v), respectively. The chemical structures of the compounds tested are shown in Figure 1.

Experimental procedures. The induction period (IP) and initial rates of polymerization in the presence (Rp inh) or absence (Rp con) of an antioxidant were determined by the method previously reported. In brief, the experimental resin consisted of MMA and AIBN (or BPO) in the presence of VE, EUG, IsoEUG, DTBMP, RES or ASDB and mixtures of VE and the coantioxidants EUG, IsoEUG, DTBMP, RES or ASDB at a molar ratio of 1:1. AIBN (or BPO) was added at 1.0 mol%, and the additives were used at 0.001, 0.01, or 0.05 mol%. Approximately 10 μl of the experimental resin (MMA: 9.12-9.96 mg) were loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70°C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of all samples, as calculated from DSC thermograms, was 92-97%. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by the polymerization of MMA. Time-conversion curves for the monofunctional phenols EUG, IsoEUG and DTBMP and the VE/EUG, VE/IsoEUG and VE/DTBMP mixtures for the BPO system are shown in Figure 2. Polymerization curves break when an inhibitor is consumed. These breaks are sharp and provide a reliable measure of the IP of the inhibitor. The presence of oxygen retards polymerization because oxygen reacts with MMA radicals activated by the initiator and then subsequently produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen since it had been sealed in air. Tangents were drawn to polymerization curves at an early stage in the run. The IP of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence (Rp con) and presence (Rp inh) of natural and synthetic antioxidants were calculated from the slopes of the plots of the first linear line of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage). Values for the rate of initiation (Ri) by AIBN and BPO were those previously reported (10, 11): 5.66x10⁻⁶ Ms⁻¹ for AIBN and 2.28x10⁻⁶ Ms⁻¹ for BPO.

Computational details. Theoretical calculations of heats of formation were carried out by the semi-empirical molecular orbital (MO) method PM5, as implemented in the MOPAC program (Fujitsu CaChe 5.0).

Results and Discussion

Vitamin E (alpha-tocopherol). The radical-scavenging activity of VE for the AIBN and BPO systems was...
determined from the findings shown in Figure 2. The radical-scavenging activity of VE is characterized by two independent parameters (11); one is the stoichiometric factor ($n$) and the other is $R_{inh}$. When the initiating radicals are generated at a constant rate by AIBN or BPO, then $n$, IP and $R_{pinh}$ are given by:

$$ [IP] = n [IH] R_i $$

$$ R_{pinh} = \frac{k_p [MMA] R_i}{n k_{inh} [IH]} $$

where $R_i$ is the rate of chain initiation.

Equations 1 and 2 can be combined to give Equation 3. The ratio of rate constants, $k_{inh}/k_p$, determines the ratio of the rate of inhibition to the rate of propagation (11):

$$ k_{inh}/k_p = \frac{[MMA]}{[IP] \ R_{pinh}} $$

The $n$ value of VE for BPO and AIBN at 0.05 mol% (5 mM) was calculated from Equation 1, yielding values of 0.14 and 1.9, respectively. In general, the $n$ for VE is 2, but it can be 3 in certain circumstances, such as high temperatures (12). The IP of VE for BPO was also examined at low concentrations, 0.001 mol% and 0.01 mol%, because of the small $n$ value at high concentrations. The $n$ values for 0.001 mol% and 0.01 mol% VE were 2.7 and 0.3, respectively (Table I), indicating that the reactivity of VE with PhCOO$^*$ depends on concentration. VE acted as an antioxidant for peroxyl radicals at lower concentrations, whereas it acted as a prooxidant at higher concentrations. In contrast, the $n$ for AIBN was about 2 even at lower concentrations. The reactivity of VE for PhCOO$^*$ derived from BPO was greater than that for $R^*$ from AIBN. Two-electron oxidation of alpha-tocopherol ($n=2$) produces alpha-tocopherolquinone. One-electron oxidation ($n=1$) produces the dihydroxydimer. Loss of a
proton and electron from both hydroxy groups of this compound has been reported to result in its rapid cyclization, generating the same oxa-spiro dimer molecule (13). To clarify the mechanism of oxidation of alpha-tocopherol, the heats of formation of alpha-tocopherol model compounds and their oxidized derivatives were determined by semi-empirical MO calculations using PM5 (Figure 3). The results indicated that formation of the dihydroxy dimer may be much more energetically favorable than the formation of tocopherolquinone or the oxa-spiro dimer.

Previous studies suggest that a coantioxidant may be effective in regenerating VE when the O-H bond dissociation enthalpy (BDE) of the coantioxidant is lower than, or at least similar to, that of the antioxidant (VE) itself (3). Previously reported values of BDE for the phenols investigated (14-17) are presented in Table II, together with their k_{inh}/k_p values, calculated using Equation 3 from the results in Table I. The k_{inh}/k_p of VE for the BPO system was about 7-fold greater than that for the AIBN system, but in general the values for the other compounds were relatively similar and fell within the range 2-5.

Mixtures of VE with the monofunctional phenols EUG, IsoEUG or DTBMP. Typical time-conversion curves for VE/EUG, VE/IsoEUG and VE/DTBMP mixtures at molar...
ratios of 1:1 in the BPO systems are shown in Figure 2. The $n$ values for the BPO system declined in the order DTBMP (2.0) $>$ EUG (1.6) $>$ IsoEUG (1.1) $>$ VE (0.14), whereas the $n$ values in the AIBN system declined in the order DTBMP (2.4) $>$ VE (1.9) $>$ IsoEUG (1.7) $>$ EUG (1.0) (Table I). Fully oxidized phenolic compounds show $n=2$. The $n$ for DTBMP was 2. The $n$ value for the o-methoxyphenols EUG and IsoEUG was less than 2, and the $n$ value of 1.0 for EUG induced by $R^*$ suggests that this compound undergoes dimerization.

The observed IPs and calculated IPs for mixtures of VE and phenolic coantioxidants are shown in Table I. In the BPO system, the observed IP of the VE/EUG mixture was extended by approx. 4.5 min compared with the calculated IP, whereas the observed IPs of the VE/IsoEUG and VE/DTBMP mixtures were shortened by 2-3 min compared with the corresponding calculated IPs. In the AIBN system, the observed IP of the VE/DTBMP mixture was extended by approx. 3.1 min, whereas the observed IPs of the VE/EUG, VE/IsoEUG and VE/RES mixtures were shortened by approx. 3.4, 8.0 and 5.8 min, respectively, compared with the corresponding calculated IPs. These findings suggest synergism in the VE/EUG mixture in the BPO system and in the VE/DTBMP mixture in the AIBN system, namely a partial regeneration of VE by EUG and DTBMP induced by $PhCOO^*$ and by $R^*$, respectively. In contrast, in the other mixtures the observed IP of the VE/coantioxidant mixture was significantly less than the corresponding calculated value, suggesting the occurrence of prooxidation activity in the VE/coantioxidant mixtures induced by $PhCOO^*$ or by $R^*$ and implying that VE was sufficiently reactive to cooxidize the phenolic coantioxidants.

Partial regeneration of VE by EUG in the reaction initiated by $PhCOO^*$ is feasible even though the BDE value of EUG is significantly higher than that of VE (ABDE = 5.8 kcal/mol; Table I). In general, when the BDE value of a coantioxidant was significantly higher than that of VE, regeneration should not occur. However, a possible regeneration mechanism has previously been proposed for the mixture of VE and 4-tert-butylcatechol (3), namely the removal of the semiquinone radical from 4-tert-butylcatechol by VE. In the case of EUG, partial regeneration of VE by EUG may be due to the removal of the semiquinone radical from VE$^*$ and the formation of VE and EUG-quinonemethide (2-methoxy-4-allylidene-2,5-cyclohexadien-1-one). However, regeneration of VE was not observed in the

Figure 4. Time-exotherm and/or time-conversion curves for ASDB, VE and VE/ASDB mixture at a molar ratio of 1:1 for the AIBN and BPO systems at 70°C. BPO (or AIBN), 1.0 mol%; MMA, 9.4 mol/l; ASDB (or TOC), 0.05 mol%.
case of induction by R*: The n value for 0.05 mol% VE in the R* system was about 1 (Table I), suggesting that the oxidation of VE produced a dimer rather than EUG-quinonemethide (Figure 3). Thus, regeneration of VE by EUG could not occur in the R* system because no quinonemethide was formed. On the other hand, regeneration of VE by DTBMP, a hindered phenol with bulky tert-butyl substituents, has been reported to occur in the autoxidation of styrene initiated by an azo-initiator in chlorobenzene (5). The driving force for this reaction may be the small difference between the BDEs of DTBMP and VE (ΔBDE = 0.1-1.32 kcal/mol; Table II).

In general, regeneration of VE will be more easily observed at high coantioxidant concentrations or at low rates of initiation and high VE content. The lack of regeneration of VE by DTBMP in the reaction induced by PhCOO* may be attributed to the rapid oxidation and consumption of VE by BPO; consequently, PhCOO* will be scavenged by the residual coantioxidant, DTBMP.

Mixture of VE and RES. The observed and calculated IP values are also shown in Table I. In the BPO system, the observed IP of the VE/RES mixture was extended by approx. 0.7 min compared with the calculated value, but the difference was not significant. Conversely, in the AIBN system, the observed IP of the VE/RES mixture was shortened by approx. 5.8 min compared with the calculated value. trans-Resveratrol (compound 5) was previously reported to be unable to recycle VE (compound 1) in an experimental system of autoxidation of styrene initiated by ROO* from azo-initiators in chlorobenzene solution in the presence of oxygen (5). Similarly, regeneration of VE by RES did not occur in the present study.

Mixture of VE and ASDB, an ascorbate derivative. Time-exothermic and time-conversion curves for these reactions are shown in Figure 4, and the results are summarized in Table I. There are a number of reports of regeneration of VE by vitamin C (1-3). On the basis of these data, it would be expected that, when VE and vitamin C are present in solution in similar amounts, peroxy radicals will react first with VE to yield VE* radicals that will immediately react with vitamin C to regenerate VE (2). In the present study, the observed IP of the VE/ASDB mixture in the AIBN system was less than that of VE alone, whereas the observed IP in the BPO system was similar to that of VE alone. No regeneration of VE by ASDB was observed, and ASDB tended to cause prooxidation of VE. It was previously reported that VE is an ineffective, and ascorbate is a poor, scavenger of carbon radicals derived from azo-initiators under anaerobic conditions, but that ascorbate in aerobic plasma can reduce VE radicals with subsequent recycling of the oxidized ascorbate by a thiol antioxidant network (18). These findings support our observation that regeneration of VE by ascorbate did not occur under anaerobic conditions. However, VE is a good antioxidant against R* with a calculated n value for fully oxidized VE of 2 (Figure 3), suggesting the formation of tocopherolquinone. The n value of VE in mixtures with ASDB (nv,E) can be calculated from Equation 4:

\[ n_{VE} = R_1 (IP_{VE+ASDB} – IP_{ASDB})/[VE] \]  

(4)

The calculated nVE for the AIBN system was about 1, suggesting that VE undergoes dimerization as a result of a radical-radical coupling reaction. This finding also suggests that the catalytic activity of ascorbate may promote the dimerization of VE because the formation of the dimer is energetically favorable (Figure 3).

Since biological systems tend to have low oxygen tensions, the effectiveness of antioxidants in vivo may be considerably different from that in aerobic systems in vitro. However, in vivo experiments are too complex to be amenable to simple interpretation and, therefore, we undertook physicochemical studies using the IP method in the radical polymerization of MMA in the presence of antioxidants as a biomimetic model for radical-scavenging activity in vivo. Such studies can help explain the mechanisms of regeneration of VE by coantioxidants, such as phenolic compounds and ascorbate derivatives under anaerobic conditions, and may contribute to our knowledge of the potential of these compounds in chemoprevention by induction of cancer cell apoptosis.

References


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