A Rapid Screening Test To Determine the Antioxidant Potencies of Natural and Synthetic Antioxidants

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This paper presents a simple and convenient method for measuring antioxidant efficiencies in a model system consisting of micelles of sodium dodecyl sulfate (SDS) with added linoleic acid. The analytical method involves following the development of absorption at 234 nm due to the conjugated diene hydroperoxide of linoleic acid; 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) is used as the initiator. The antioxidant efficiency of an antioxidant is defined as AE = k_{inh}/k_p, where k_{inh} is the rate constant for reaction of the peroxyl radical from linoleic acid with the antioxidant (eq 7 in the text) and k_p is the propagation rate constant for autoxidation of linoleic acid (eq 5). The relative antioxidant efficiency (RAE) is defined as the AE of the test compound divided by that for α-tocopherol; thus, RAE values are equal to $k_{inh}/k_{inh,\alpha\text{-tocopherol}}$. Since the absolute value of the rate constant for reaction of α-tocopherol with peroxyl radicals is known in micellar systems, the absolute value of k_{inh} for a given antioxidant can be calculated if required. To establish the reliability of the method we report the AE of 17 synthetic and naturally occurring antioxidants and compare values obtained by this method to others in the literature. For neutral antioxidants, the same AE values are obtained in both negatively-charged micelles of SDS and positively-charged micelles of hexadecyl trimethylammonium bromide (HDTBr). The relative antioxidant efficiencies are reported for more than 40 synthetic compounds that are potential antioxidants or anti-inflammatory compounds; most of these compounds are tocopherol, ascorbate, or 2-hydroxytetronic acid analogues. Although the charge type of the micelle has no effect on the RAE of neutral antioxidants, micelles of different charge types do have an effect on the RAE value of charged antioxidants such as ascorbic acid, other 2-hydroxytetronic acids, and Trolox C. In 0.10 M SDS micelles, ascorbic acid and α-tocopherol are shown to have an additive effect, not a synergistic effect, as antioxidants.

Introduction

This paper reports a rapid and convenient method for determining antioxidant efficiencies (AE) based on the rate of oxidation of linoleic acid to its conjugated diene hydroperoxide (CD) in aqueous micelles. Studies of inhibitors have been reported in organic solvents, micelles, and liposomes,[1-15] often using the rate of oxygen uptake measured by an oxygen electrode[5-7] or a pressure transducer.[1-3,8] The method presented here, based on conjugated diene formation, has higher sensitivity than does oxygen uptake methods and allows the study of autoxidation processes at lower conversions where the steady state kinetic analysis is likely to apply.

The initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) was used to provide a constant rate of radical production.[16,17] While ABAP is often regarded as water soluble, in SDS micelles it partitions about 91% into the micellar phase and has a distribution not very different from the 95% observed for linoleic acid.[13] Thus, most of the primary radicals from ABAP probably are produced either within or at the surface of the micelle. Oil-soluble inhibitors, therefore, would be expected to behave normally, scavenging lipid peroxyl radicals. Water-

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‖ List of abbreviations: ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; AE, antioxidant efficiency(-ies); BHT, 2,6-di-*tert*-butyl-4-methylphenol; CD, conjugated diene hydroperoxide; DMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); L-DOPA, L-3,4-dihydroxyphenylalanine; DPPD, *N,N′*-diphenyl-1,4-phenylenediamine; HDTBr, hexadecyl trimethylammonium bromide; InH, an inhibitor; *K*, a proportionality constant relating oxygen uptake and conjugated diene formation; LH, linoleic acid; *n*, the stoichiometric factor; oxidizability, $k_p/(2k_t)^{1/2}$; PMHC, 2,2,5,7,8-pentamethyl-6-hydroxychroman; RAE, relative antioxidant efficiency; SDS, sodium dodecyl sulfate; SF, stoichiometric factor; *T*, the induction time.

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soluble inhibitors could react either with the small fraction of radicals produced in the water phase from ABAP or at the surface of the micelle with either primary radicals or (more likely) the more numerous lipid peroxyl radicals. In order to evaluate the effect of micellar charge on these kinds of reactions, data were obtained using both the anionic surfactant SDS and the cationic surfactant, hexadecyltrimethylammonium bromide (HDTBr). We studied only a few water-soluble inhibitors (Trolox C, DOPA, and ascorbate) and, as will be seen, these are sensitive to the nature of the surfactant used.

The antioxidants used in this study include the four tocopherols;[18,19] the water-soluble tocopherol analogue Trolox C; an extensive series of synthetic analogues of 2-hydroxytetronic acids and tocopherol; ascorbate and ascorbyl palmitate; β-carotene[20,21] and 13-*cis*-retinoic acid;[22] BHT; L-β-3,4-dihydroxyphenylalanine (L-DOPA); DPPD; probucol and a probucol analogue, MDL 29311.

Materials and Methods

Chemicals. Linoleic acid (99%) was purchased from Nu-Chek-Prep, Inc., Elysian, MN; 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) and 2,2′-azobis(2,4-dimethylvaleronitrile) (DMVN) from Wako Chemicals USA, Inc., Dallas, TX; hexane (HPLC grade) and sodium phosphate, dibasic heptahydrate (99.49%, AR) from Mallinckrodt, Paris, KY; sodium phosphate, monobasic, monohydrate (100%, AR) from J. T. Baker Chemical Co., Phillipsburg, NJ; and Chelex-100 chelating resin, 200–400 mesh, sodium form (analytical grade) from Bio-Rad Laboratories, South Richmond, CA. Trolox C (97%), L-ascorbyl palmitate (97%), BHT (99%), *N,N′*-diphenyl-1,4-phenylenediamine (DPPD; 98%), quercitin dihydrate (98%), and 1,3-dimethyl-2-thiourea (99%) were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Natural *d*-isomers of α-, β-, γ-, and δ-tocopherols (98.5%) and 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMHC; 98.5%) were gifts from Eisai Co., Ltd., Tokyo, Japan. Probucol and the probucol analog, 4′,4-[methylenebis(thio)]bis[2,6-bis(1,1-dimethylethyl)]phenol were gifts from Marion Merrell Dow Research Institute, Marion Merrell Dow Inc., Cincinnati, OH. The α-tocopherol analog (Upjohn U-78517F) was a gift from the Upjohn Co., Kalamazoo, MI. All the following chemicals were purchased from Sigma Chemical Co., St. Louis, MO: SDS (99%), HDTBr (99%), L-ascorbic acid (99%, ACS grade), L-DOPA (97%), β-carotene (95%), folic acid (98%), and 13-*cis*-retinoic acid (96%). All chemicals were used as received. The remaining compounds were synthesized as described below.

Preparation of Compounds. NMR spectra were determined on a 250-MHz instrument. Elemental analysis for carbon, hydrogen, and nitrogen were within 0.4% of the theoretical values unless otherwise reported. Materials obtained from commercial suppliers were used without further purification. The concentration of compounds or intermediates and the removal of volatiles was carried out under reduced pressure. Solvents and solutions were dried over $MgSO_4$.

The following preparation of *N*-[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-yl)methoxy]phenyl]-*N′*-(2-methoxyphenyl)urea (compound **16**) represents a general method for the synthesis of the various urea derivatives utilized in these studies, including compounds **17** and **21–23**. A reaction mixture of 6-(benzyloxy)-2-[(4-aminophenoxy)methyl]-2,5,7,8-tetramethylchroman[23] (2.5 g; 0.006 mol) and 2-methoxyphenyl isocyanate (0.89 g; 0.006 mol) in 50 mL of ethyl acetate was stirred at room temperature for 20 h. Volatiles were removed under reduced pressure, and the residue was taken up in 75 mL of ether. The precipitated solid was filtered, washed with ether, and dried yielding 3.0 g (88.5%) of the benzyl ether derivative (mp 172–174 °C). Anal. Calcd for $C_{35}H_{38}N_2O_5$; C = 74.18; H = 6.75; N = 4.94; found C = 73.95; H = 6.75; N = 4.76. The benzyl ether derivative (2 g) upon hydrogenolysis using 20% Pd/C in methanol–THF (1:1) gave the required phenol **16** in 99% (1.65 g) yield (mp 82–92 °C). Anal. Calcd for $C_{28}H_{32}N_2O_5 \cdot 0.5H_2O$; C = 69.26; H = 6.85; N = 5.77; found C = 69.30; H = 7.06; N = 5.43.

Compound **18** was synthesized as described by Yoshioka et al.[23] The related compounds **19** and **25** were prepared according to the methods of Mukai et al.[24] Compound **26** was synthesized according to the procedure of Burton et al.[14]

The preparation of 2*H*-1-benzopyran-2-carboxamide, *N*-[4-(cyanomethyl)phenyl]-3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl- (compound **24**) and 2*H*-benzopyran-2-carboxamide, 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-*N*-(2,4,6-trimethoxyphenyl)- (compound **20**) were achieved by the following route. To a solution of Trolox C (compound **6**) (2.5 g; 0.010 mol) in 50 mL THF was added CDI (1.62 g; 0.010 mol) and the reaction was stirred at room temperature for 2 h. (*p*-Aminophenyl)acetonitrile (1.32 g; 0.010 mol) was added to the reaction with continued stirring at room temperature for 20 h. Volatiles were removed and the residue was dissolved in 150 mL of chloroform. The organic solution was twice extracted with 0.5 N HCl (50 mL) followed by two extractions with saturated $NaHCO_3$ (50 mL) and a single extraction with 100 mL of brine. The washed organic layer was dried, filtered, and evaporated. The residue was taken up in ether. The precipitated solid was filtered and dried. Preparation of **24** yielded 2.8 g (76.9%), (mp 177–179 °C).

The synthesis of 4-[(dimethylamino)methyl]-2,3-dihydro-2,2,6,7-tetramethyl-5-benzofuranol (**27**) is described below. 2,3-Dimethylquinone (12.5 g; 90.5 mmol), acetone (300 mL), 3-chloro-2-methylpropene (29.65 g; 327 mmol), potassium carbonate (135 g; 981 mmol), and sodium iodide (4.9 g; 33 mmol) were refluxed under N_2 for three days. After cooling to room temperature, the mixture was poured into 300 mL of CH_2Cl_2 and 300 mL water. The aqueous layer was extracted with 200 mL of CH_2Cl_2 twice. The organic extracts were combined and washed with 100 mL of saturated $Na_2S_2O_4$. After it was dried and concentrated, the resulting material was chromatographed on silica using hexane/ethyl acetate (96/4 to 90/10) as eluants, affording 5.0 g of 2,3-dimethyl-4-[(2-methyl-2-propenyl)oxy]phenol as a white solid: mp 85–87 °C; ^{1}H-NMR (250 MHz, DMSO) δ 1.76 (3H,s), 2.03 (3H,s), 2.08 (3H,s), 4.30 (2H,s), 4.92 (1H,s), 5.05 (1H,s), 6.54 (d, J = 8.8 Hz, 1H), 6.57 (d, J = 8.8 Hz, 1H0, 8.71 (1H,s); MS (EI+) 192.2, 137.1. Anal. Calcd for $C_{12}H_{16}O_2$; C = 74.97; H = 8.39; found C = 75.05; H = 8.43.

2,3-Dimethyl-4-[(2-methyl-2-propenyl)oxy]phenol (2.5 g, 13 mmol) in 100 mL of 1,2-dichloromethane was heated at reflux for 16 h. The reaction was cooled to room temperature and chromatographed on silica using hexane/ethyl acetate (95/5 to 90/10) as eluant giving 2.14 g (85%) of 2,3-dimethyl-5-[(2-methyl)-2-propenyl]hydroquinone. 2,3-Dimethyl-5-[(2-methyl)-2-propenyl)]hydroquinone (2.14 g; 11.1 mmol) in 50 mL of CH_2Cl_2 and five drops of methanesulfonic acid were stirred at room temperature. Additional quantities of methanesulfonic acid were added after 2 (5 drops), 4 (5 drops), and 21 h (0.1 mL). After stirring an additional 2 h at room temperature, the reaction mixture was poured into 100 mL of saturated aqueous $NaHCO_3$ and 200 mL of CH_2Cl_2. The aqueous layer was extracted with 100 mL of CH_2Cl_2 three times. The combined CH_2Cl_2 extracts were dried and concentrated. Purification was effected by flash chromatography on silica (hexane/ethyl acetate; 95/5) and trituration from hexane. This yielded 1.95 g (91%) of 2,3-dihydro-2,2,6,7-tetramethyl-5-benzofuranol as a white solid: mp 105–106.5 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.44 (6H, s), 2.11 (6H, s), 2.92 (2H, s), 4.49 (1H, s), 6.47 (1H, s); IR (cm^{-1}) 3607, 3007, 2977, 2948, 1481, 1460, 1383, 1168; MS (EI) 192.1, 177.0. Anal. Calcd for $C_{12}H_{16}O_2$; C = 74.97; H = 8.39; found C = 74.58; H = 8.46.

2,3-Dihydro-2,2,6,7-tetramethyl-5-benzofuranol (0.25 g; 1.3 mmol), 4 mL of ethanol, 37% aqueous formaldehyde (0.29 mL;

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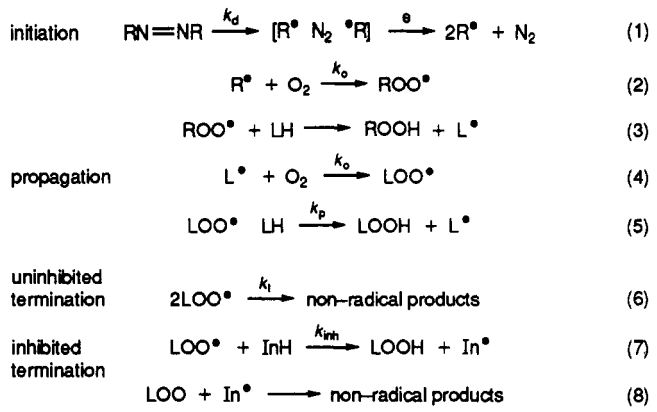
Scheme I. The Mechanism of Autoxidation of Linoleic Acid



3.9 mmoles), and 40% aqueous dimethylamine (0.30 mL; 2.6 mmol) were stirred for 2.5 h at room temperature, followed by heating to reflux for 3 h and stirring overnight at room temperature. The reaction was poured into diethyl ether (200 mL) and H_2O (100 mL). The aqueous layer was extracted with 200 mL of diethyl ether twice. The combined organic extracts were washed with saturated aqueous NaCl (100 mL), dried, and concentrated. Flash chromatography on silica (ethyl acetate/hexane/Et_3N; 75/25/1 to 50/50/5) and recrystallization from hexane gave 0.17 g (52%) of 4-[(dimethylamino)methyl]-2,3-dihydro-2,2,6,7-tetramethyl-5-benzofuranol (**27**) as a white solid: mp 81.5–82.5 °C; ^{1}H NMR (250 MHz, DMSO) δ 1.35 (6H, s), 1.96 (3H, s), 1.98 (3H, s), 2.22 (6H, s), 2.85 (2H, s), 3.44 (2H, s), 10.5 (1H, s); MS (EI) 249.1, 204.0, 189.0. Anal. Calcd for $C_{15}H_{23}NO_2$; C = 72.25; H = 9.30; N = 5.61; found C = 71.85; H = 9.43; N = 5.94.

Compounds **31**, **33**, **34**, **36**, and **37** were prepared according to the method of Romstedt, Witiak et al.[25] Witiak and Tehim have described the preparation of compound **30**.[26] Compound **32** has been prepared according to the method of Terao.[27] The procedure of Witiak et al.[28] was used to prepare compound **35**, and the hydroxytetronic acids **38** and **39** were prepared according to the method of Witiak and Tehim.[26] The synthesis of compounds **40–43** have also been described by Witiak et al.[29] Compound **44** was prepared according to the method of Unangst et al.[30]

Preparation of Solutions. Stock solutions of 0.30 M phosphate buffer (pH 7.4) were treated overnight with Chelex-100 at room temperature and stored in a plastic bottle for no more than 2 weeks. Stock solutions (0.12 M) of SDS and HDTBr were prepared and used within 2 weeks. Solutions of 0.5 M ABAP were freshly prepared in 0.05 M phosphate buffer (pH 7.4); DMVN was prepared in the hexane reaction solvent. Stock solutions of antioxidants, except L-ascorbic acid, L-DOPA, and Trolox C, were freshly prepared in methanol or absolute ethanol at concentrations of 10^{-4} M; the three water-soluble antioxidants were prepared in 0.05 M phosphate buffer (pH 7.4) as 10^{-4} M stock solutions. Deionized water of very high purity (resistance >15 MΩ) was used in all experiments.

Kinetic Analysis. The mechanism of autoxidation of linoleic acid in homogeneous solutions under sufficient oxygen pressure can be represented by eqs 1–8 in Scheme I.[17,31] In these equations, R′N=NR is the azo initiator, LH is linoleic acid with its allylic hydrogen, and InH is the test antioxidant. The classic rate laws for both uninhibited (R_{uninh}) and inhibited (R_{inh}) autoxidations of linoleic acid in Scheme I, assuming the long-chain approximation, are given in eqs 9 and 10, respectively. We[4,17] and others[31] have derived these equations, and they have been shown applicable to micellar systems.[3,5,7,12–15] In eqs 9 and 10, $-d[O_2]_{uninh}/dt$ and $-d[O_2]_{inh}/dt$ are the rates of oxygen uptake for the

$$R_{uninh} = -\frac{d[O_2]_{uninh}}{dt} = \frac{k_p}{(2k_t)^{1/2}}[LH]R_i^{1/2} \quad (9)$$

$$R_{inh} = -\frac{d[O_2]_{inh}}{dt} = \frac{k_p[LH]R_i}{nk_{inh}[InH]} \quad (10)$$

uninhibited and inhibited autoxidations, respectively; n is the number of radicals trapped per inhibitor molecule (the stoichiometric factor, SF); square brackets represent molar concentrations; R_i is the rate of initiation; k_p, k_t, and k_{inh} are rate constants of the propagation, termination, and inhibition steps, respectively; and $k_p(2k_t)^{1/2}$ is the oxidizability of linoleic acid.[32]

Since all of the oxygen consumed is not utilized in the production of conjugated diene hydroperoxides, and since some conjugated diene hydroperoxide may be destroyed during subsequent autoxidation processes, we introduce a proportionality constant, K, to correlate the rate of oxygen uptake to that of conjugated diene formation. We assume the same value of K applies to both uninhibited and inhibited autoxidations (eqs 11 and 12). The ratio of k_{inh} to k_p is defined as the antioxidant

$$-\frac{d[O_2]_{uninh}}{dt} = K\frac{d[CD]_{uninh}}{dt} \quad (11)$$

$$-\frac{d[O_2]_{inh}}{dt} = K\frac{d[CD]_{inh}}{dt} \quad (12)$$

efficiency, AE, as shown in eq 13, and RAE as the antioxidant efficiency of an inhibitor relative to α-tocopherol, as defined in eq 14.

$$AE = k_{inh}/k_p \quad (13)$$

$$RAE = \frac{AE(InH)}{AE(\alpha\text{-tocopherol})} = \frac{k_{inhib}}{k_{inhib,\alpha\text{-tocopherol}}} \quad (14)$$

Autoxidation Procedure. **Method A.** This is the method used at LSU.[33] The sample UV cuvette containing 2.4 mL of 2.6 mM linoleic acid in a surfactant–phosphate solution was thermostated at 37 ± 0.2 °C and stirred continuously in the sample compartment of the spectrophotometer. The reference cell contained the same solution except for linoleic acid. After about 20 min, when the thermal equilibration in the spectrophotometer was completed, aliquots (5 ± 0.1 μL each) of 0.5 M ABAP were added to both the sample and reference cuvettes and incubated for an additional 15 min in order to allow the rate of autoxidation to become constant. An aliquot (5 ± 0.1 μL) of an antioxidant phosphate buffer solution was then added, and the changes in absorbance monitored with time at 234 nm.

Method B. This is a more rapid screening method that gives RAE values and was used at Parke-Davis. A multiport cell holder was used that allowed a control value for α-tocopherol to be determined each time an unknown antioxidant was studied. The antioxidant measurements were carried out as in method A, with these minor modifications. A solution of 2.5 mM linoleic acid was prepared in 0.1 M SDS and 0.05 M sodium phosphate buffer (pH 7.4) and thermostated at 40 °C. One milliliter of this solution was added to each cuvette and the reaction initiated by the addition of 10 μL of a standard solution containing 0.4 M ABAP. The absorption at 234 nm was followed for several minutes to

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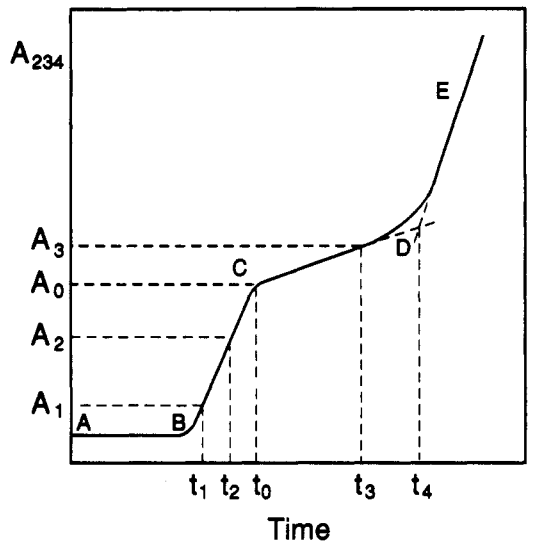

Figure 1. A representative UV trace of the inhibited autoxidation of linoleic acid measured at 234 nm in 0.10 M SDS/0.05 M phosphate buffer (pH 7.4). Line AB: spontaneous autoxidation without initiator; B: initiator (ABAP) added; C: α-tocopherol (at 10^{-4} M) added; D: a cross-point for the inhibited and uninhibited lines; t_0, the time when the antioxidant is added; and t_4, the time corresponding to the point D. The data of $d[CD]_{uninh}/dt$, $d[CD]_{inh}/dt$, and T are derived from this trace and refer to eqs 18–20.

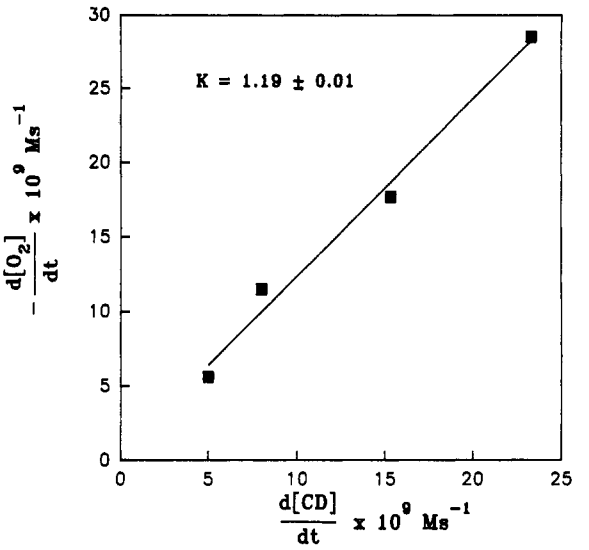

Figure 2. The proportionality factor, K, obtained from the slope of plots of the rates of oxygen uptake measured by an oxygen electrode apparatus and conjugated diene formation. Data are from Table I.

establish the uninhibited rate of autoxidation. The test antioxidants were then added to cuvettes 2 through 4 as ethanolic solutions to a final concentration of 2.5 μM. The inhibited reactions were followed until the antioxidants were consumed and the rate of change of absorption at 234 nm returned to that observed at the outset. Control experiments in which ABAP was deleted showed no changes in absorption at 234 nm. A control was used in which ethanol alone was added to a cuvette at a concentration equal to that which would be present in the ethanol–antioxidant solutions; no change in the rate of autoxidation was observed. The uninhibited and inhibited rates of autoxidation were quite constant, varying by less than 1%.

The stoichiometric factor, SF, was calculated by dividing the induction period, T, of the test compound by that for α-tocopherol and multiplying by the SF factor for α-tocopherol, which was taken as 2. The RAE was calculated as the ratio of the slope of the rate of change of absorption of the test compound relative to that of the standard, α-tocopherol.

Measurement of Average Molar Extinction Coefficients (ϵ) of Conjugated Diene(s). Linoleic acid containing about 1% conjugated dienes (as judged by the absorbance at 234 nm) was prepared at the same concentration in methanol, hexane, and micelles. These solutions were used to determine the molar extinction coefficient of linoleic acid conjugated diene hydroperoxides in different solvents, using the known value of ϵ (234 nm) in methanol, 27000 M^{-1} cm^{-1}.[34,35] Values of ϵ (234 nm) in hexane, 0.1 M SDS, and 0.1 M HDTBr were found to be 25500 ± 500, 26100 ± 400, and 26600 ● 1500, respectively, with the surfactant solutions also being 0.1 M phosphate buffer pH 7.4.

Calculation of the Rates of Conjugated Diene Appearance and the Induction Time (T). Figure 1 shows a typical UV absorption trace for the autoxidation of linoleic acid with α-tocopherol as the antioxidant. The rates of conjugated diene formation and the induction time of T can be obtained from that trace (eqs 15–17). In eqs 15–17, ϵ is the molar extinction coefficient of the conjugated dienes at 234 nm; L = 1 cm, the light path for the cell; A_1 and A_2, the absorbance at times t_1 and t_2 in the uninhibited period, respectively; A_0, the absorbance at the time t_0 when an antioxidant is added; A_3, the absorbance at the time t_3 in the inhibited period; and the induction time, T, is the difference between t_4 and t_0 where t_4 is the time corresponding to the cross-point D for the inhibited and uninhibited lines (see Figure 1).

$$\frac{d[CD]_{uninh}}{dt} = \frac{-(A_2 - A_1)}{(\epsilon L)(t_2 - t_1)} \quad (M^{-1}\,s^{-1}) \tag{15}$$

$$\frac{d[CD]_{inh}}{dt}(t_0) = \frac{-(A_3 - A_0)}{(\epsilon L)(t_3 - t_0)} \quad (M^{-1}\,s^{-1}) \tag{16}$$

$$T = t_4 - t_0 \quad (s) \tag{17}$$

Determination of the Proportionality Factor (K), the Rate of Initiation, the Oxidizability of Linoleic Acid ($k_p/(2k_t)^{1/2}$), and the Efficiency of Radical formation (e). Aliquots from the same autoxidation solution (in 0.10 M SDS containing 0.05 M phosphate, pH 7.4) at 37 °C were followed using both an oxygen electrode apparatus and a UV spectrophotometer.[33] By varying the concentrations of ABAP and linoleic acid, both $-d[O_2]/dt$ and $d[CD]/dt$ were obtained for the same reaction solution. The value of K was calculated from the slope obtained from the plot of $-d[O_2]/dt$ versus $d[CD]/dt$ (see Figure 2). (This correction factor cancels out and is not required if only relative antioxidant values are desired.)

The rate of initiation, R_i, and the efficiency of radical formation, e, were calculated from the measured rates of decomposition of the azo initiator, eq 18,[17] where $[\alpha\text{-Toc}]_0$ is the initial molarity

$$R_i = \frac{2[\alpha\text{-Toc}]_0}{T} = 2ek_d[R\text{-}N{=}N\text{-}R] \tag{18}$$

of α-tocopherol and T is the induction period. The rate constant for decomposition of the initiator, k_d, was taken to be 2.43×10^{-6} s^{-1} for DMVN in hexane from the value in chlorobenzene at 37 °C.[4] The value of k_d for ABAP in SDS micelles at 37 °C was previously determined as 3.72×10^{-7} s^{-1}.[3] The stoichiometric factor (SF) was calculated as equal to the ratio of the lag times for the test antioxidant and the standard solution containing α-tocopherol, the stoichiometric factor of which was taken to be 2.

The oxidizability of linoleic acid, $k_p/(2k_t)^{1/2}$, was measured in hexane, varying the concentration of linoleic acid from 0.66 to 2.65×10^{-2} M and the concentration of DMVN from 0.22 to 1.0 $\times 10^{-3}$ M. A similar treatment was used for micelles except ABAP was used.

(34) Chan, H. W.-S.; Levett, G. *Lipids* **1976**, *12*, 99–104.
(35) Pryor, W. A. and Castle, L. *Methods in Enzymology, Volume 105. Oxygen Radicals in Biological Systems*; Packer, L., Ed.; Academic Press: New York, 1984; p 293–299.

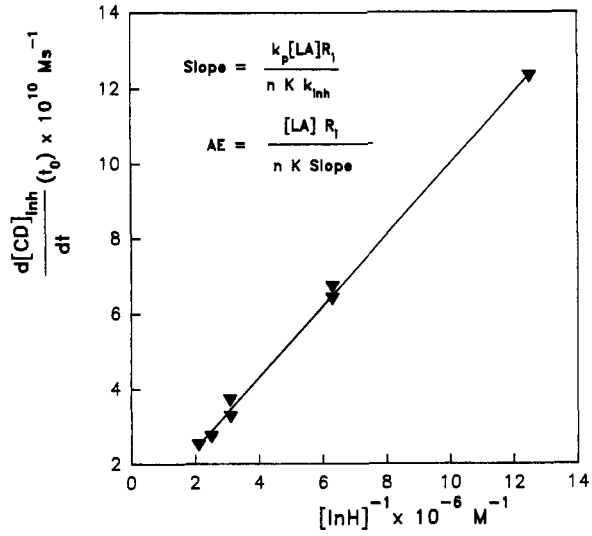

Figure 3. Calculation of the AE value for α-tocopherol in 0.10 M SDS/0.05 M phosphate buffer (pH 7.4); $d[CD]_{inh}/dt(t_0)$ is the rate of conjugated diene formation at the time ($t = t_0$) when an antioxidant is added.

Measurement of the Antioxidant Efficiency (AE) and Relative Antioxidant Efficiency (RAE). From eqs 10 and 12, a plot of $d[CD]_{inh}/dt(t_0)$ *versus* $[InH]_0^{-1}$ yields a line with the slope, S, where S is defined as in eq 19. The AE values can be calculated from S, as illustrated in Figure 3 using data for α-tocopherol in 0.10 M SDS/0.05 M phosphate buffer pH 7.4. RAE were obtained either from individually-determined AE values as in method A (eq 20) or directly from the ratios of the slopes of the plots for the simultaneous runs involving the inhibitor and α-tocopherol (eq 21), as described in method B above.

$$S = \frac{k_p[LH]R_i}{nKk_{inh}} \tag{19}$$

$$AE = \frac{k_{inh}}{k_p} = \frac{[LH]R_i}{nKS} \tag{20}$$

$$RAE = \frac{S(\alpha\text{-tocopherol})}{S(InH)} = \frac{k_{inhib}}{k_{inhib,\alpha\text{-tocopherol}}} \tag{21}$$

Results

Proportionality Factor (K), Efficiencies of the Initiators (e), and Oxidizability Values. Values of K are required only if the rates of oxygen utilization and connjugated diene formation are calculated, as shown in eqs 11 and 12. The RAE values, as determined in method B, do not depend on K since the same value is assumed to apply to both the unknown inhibitor and to α-tocopherol; however, comparison of our K value with the literature serves to establish the conjugated diene method.

The rates of autoxidation of linoleic acid used for calculation of K together with the conditions used are listed in Table I. The data are plotted in Figure 2 and K is found to be 1.19; i.e., there is 19% less conjugated diene than the amount of oxygen used would indicate. The efficiencies of radical formation (e) and the oxidizability values of linoleic acid in hexane and in micelles of 0.10 M SDS and 0.10 M HDTBr surfactants are listed in Table II.

Values of AE and RAE and Induction Times. Table III gives the RAE values of α-, β-, γ- and δ-tocopherols and BHT determined using the conjugated diene method

Table I. Data for the Measurement of the Proportionality Factor K[a]

		$R_p \times 10^9$ ($M^{-1}\ s^{-1}$)[b]	
[ABAP] $\times 10^2$ M	[LH] $\times 10^3$ M	OE[c]	UV[d]
2.08	2.65	5.6 ± 0.4	5.0 ± 0.2
4.16	2.65	11.5 ± 1.0	8.0 ± 0.3
8.33	2.65	17.7 ± 1.6	15.3 ± 0.6
4.16	6.63	28.5 ● 2.4	23.3 ± 1.0

[a] The data on R_p values using both an oxygen electrode and UV spectrophotometer method are plotted in Figure 2 for calculation of K. [b] R_p is the rate of propagation. [c] OE: using an oxygen electrode apparatus. [d] UV: using a UV spectrophotometer.

(both methods A and B) are compared with those measured by ourselves and other workers using a variety of methods and in several solvent systems. The agreement seems reasonable. Table IV gives AE values at 37 °C and pH 7.4 for all of the compounds studied by the conjugated diene method (method A) in both 0.10 M SDS and 0.10 M HDTBr and using a pressure transducer in 0.5 M SDS,[3] showing the effects of changing surfactant concentration and charge type, and Table V presents these same data in terms of RAE values. While there are differences when the AE values are compared, the RAE values show reasonable agreement.

Table VI reports the induction times (T) for ascorbic acid and α-tocopherol and for combinations of these two antioxidants. These data demonstrate that the lag time is additive and synergistic effects are not observed.

The RAE values for all of the compounds studied in SDS micelles are listed in Table VII. The agreement of RAE values between methods A and B appears to be satisfactory. A plot of the RAE values determined by method A versus that determined by method B shows a high degree of correlation ($r = 0.9286$) which is highly significant ($p < 0.001$). In general, the values obtained by method A are approximately 60% of those determined by method B, probably because of unidentified variations in methodologies in the LSU and Parke-Davis laboratories.

Discussion

Comparison of the Conjugated Diene and Oxygen Uptake Methods. Theoretically, 1 mol of oxygen consumed should produce 1 mol of linoleic acid hydroperoxide (see Scheme I). However, the ratio of oxygen consumed to conjugated dienes formed is consistently found to be greater than 1 by most workers, perhaps because some conjugated diene hydroperoxides are destroyed in subsequent autoxidation reactions.[36] (Conjugated dienes are more reactive toward radicals than are nonconjugated olefins such as linoleic acid.[32]) The value of K for the ratio of the rate of oxygen uptake to conjugated diene formation in the autoxidation of linoleic acid in SDS was found to be 1.19 (Figure 2), in good agreement with previous values.[36]

The efficiency of radical formation by ABAP in SDS micelles determined here is close to the literature value (Table II). [The efficiency of DMVN in organic solvents, on the other hand, is found to be lower in hexane than the reported value in chlorobenzene (Table II).] Both the oxidizability of linoleic acid and efficiency of radical formation from ABAP are lower in 0.10 M HDTBr than in 0.10 M SDS micelles, possibly because of the higher

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Table II. Comparison of Conjugated Diene (CD) Formation with Oxygen Uptake Methods[a]

	method				
	CD formation			oxygen uptake	
	hexane[f]	SDS[b,f]	HDTBr[b,f]	chlorobenzene[f]	SDS[b,f]
relative autoxidation rate	–	1.0	–	–	1.19 ± 0.01
$k_p/(2k_t)^{1/2}$ [c]	0.016 ± 0.003	0.039 ± 0.002	0.022 ± 0.002	0.020 ± 0.0004[d]	0.049 ± 0.004[e]
e	0.51 ± 0.01	0.49 ± 0.03	0.22 ± 0.02	0.75 ± 0.14[d]	0.49[g,h]

[a] Linoleic acid was used as a substrate for the autoxidation studies; the concentration of linoleic acid was varied from 6.6 to 26.5 mM in hexane, 1.3 to 6.6 mM in SDS micelles, and 2.6 to 10.6 mM in HDTBr micelles. The micelles of 0.10 M SDS and 0.10 M HDTBr contained 0.05 M phosphate buffer (pH 7.4) in a total volume of 2.4 mL. All reactions were conducted at 37 °C. [b] Each detergent was 0.10 M in 0.05 M phosphate buffer, pH 7.4. [c] Oxidizability, $k_p/(2k_t)^{1/2}$, in units of $M^{-1/2}\,s^{-1/2}$. [d] See ref 4. [e] In 0.5 M SDS containing 0.05 M phosphate buffer, pH 7.4, ref 2. [f] The efficiency of radical production from DMVN in hexane and chlorobenzene and ABAP in the aqueous buffers. [g] See ref 3. [h] In 0.5 M SDS.

Table III. Antioxidant Efficiencies (AE), k_{inh}/k_p, for Tocopherols and BHT Obtained Using the Conjugated Diene Method Compared to Other Methods

	solvent system and temperature					
antioxidants	0.10 M SDS[a] at 40 °C	0.10 M SDS[b] at 37 °C	0.50 M SDS[c] at 37 °C	liposomes[d] at 30 °C	ethanol[e] at 25 °C	styrene[f] at 30 °C
α-tocopherol	[1.00]	[1.00]	[1.00]	[1.00]	[1.00]	[1.00]
β-tocopherol	0.87	0.52	0.59	0.54	0.44	0.41
γ-tocopherol	0.89	0.53	0.59	0.50	0.47	0.44
δ-tocopherol	0.83	0.31	0.24	0.47	0.10	0.14
BHT	0.04	0.26	0.30			0.0043

[a] Measured at Parke-Davis by method B. [b] Measured at LSU by method A. [c] Measured using a pressure transducer.[3] [d] Measured using a ESR spectrometer and the galvinoxyl radical as the substrate.[37] [e] Measured using a stopped-flow technique and 2,6-di-*tert*-butyl-4-(4-methoxyphenyl)phenol as the substrate.[38] [f] Measured using a pressure transducer and styrene as the substrate.[8,39]

Table IV. The Antioxidant Efficiencies Determined in Aqueous Micelles of 0.10 M SDS and 0.10 M HDTBr (pH 7.4 and 37 °C) Using the CD Method

antioxidants	0.10 M HDTBr	0.10 M SDS	0.5 M SDS[a]
α-tocopherol	1770 ± 90	1830 ● 50	1000
β-tocopherol	970 ± 140	950 ± 30	590
γ-tocopherol	1020 ± 40	980 ± 60	590
δ-tocopherol	530 ± 30	570 ± 50	240
PMHC	2850 ± 100	2880 ● 120	4050
Trolox C	8300 ± 360	1320 ± 90	2970
BHT	450 ± 20	480 ± 20	300
ascorbyl palmitate	6930 ● 530	2000 ± 130	1750[b]
ascorbic acid	22800 ± 1620	26 ± 1.5	27[b]
L-DOPA	3710 ± 250	260 ± 10	–
β-carotene	210 ± 4	5.7 ± 0.6	ND[c]
13-*cis*-retinoic acid	100 ● 10	4.7 ± 0.3	–
DPPD	–	6740 ● 460	4050
quercetin	–	340 ± 50	–
Probucol	–	300 ± 14	140
MDL 29311	–	450 ± 20	–
U78517F	–	1860 ± 140	–

[a] Our previous results[3] obtained from the oxygen uptake method using a pressure transducer. [b] See ref 7. [c] ND, not detected.

Table V. Comparison of Relative Antioxidant Efficiencies, AE(InH)/AE(αTocopherol), in SDS Micelles (pH 7.4 and 37 °C) Using ABAP and Methods A and B

antioxidants	0.5 M SDS[a]	0.10 M SDS[b]	0.10 M SDS[c]
α-tocopherol	1.00	1.00	1.00
β-tocopherol	0.59	0.89 ± 0.01	0.52 ● 0.01
γ-tocopherol	0.59	0.84 ± 0.04	0.53 ± 0.03
δ-tocopherol	0.24	0.74 ± 0.03	0.31 ± 0.03
PMHC	4.05	2.50	1.57 ± 0.07
Trolox C	2.97	0.60 ± 0.08	0.72 ± 0.05
BHT	0.30	0.03 ● 0.03	0.26 ● 0.01
ascorbic acid	0.03[d]	–	0.014 ± 0.001
ascorbyl palmitate	1.75[d]	–	1.09 ± 0.07
β-carotene	ND[e]	ND	0.0031 ± 0.0003
13-*cis*-retinoic acid	–	–	0.0026 ± 0.0002
Probucol	0.14	0.21 ± 0.03[f]	0.16 ± 0.01[f]
MDL 29311	–	–	0.25 ● 0.01[f]
DPPD	4.05	–	3.68 ± 0.25
L-DOPA	–	–	0.14 ● 0.01
quercitin	–	–	0.19 ± 0.03
U78517	–	–	1.02 ● 0.08

[a] Obtained using an oxygen uptake method and a transducer apparatus.[3] [b] These compounds were studied at Parke-Davis using method B. [c] These compounds were studied at LSU using method A. [d] Obtained using an oxygen electrode in 0.05 M SDS at 40 °C.[7] [e] ND, the value is too small to determine using this method. [f] The stoichiometric factors for these compounds are assumed to be 4 and all others are assumed to be 2.

microviscosity of HDTBr (η = 47 cP at 27 °C) compared to SDS (η = 12 cP at 25 °C) micelles.[37] The higher oxidizability we obtain for linoleic acid in micelles relative to organic solvents (Table II) is in agreement with the literature[3,4,9] and may result from some ability of micelles to sequester radicals and retard termination reactions.

The UV spectrophotometric technique used here is more sensitive than oxygen uptake methods and a 10-fold lower rate of initiation, R_i, and a 10-fold lower concentration of linoleic acid were used in this study as compared to studies using the pressure transducer method.[3,13] This has the advantages of using low conversions, where the steady state kinetic treatment is more likely to apply, and requiring less material, which is desirable when scarce synthetic antioxidants are tested. Changes in absorbance at 234 nm, calculated as concentration changes of conjugated dienes, could be determined as low as 1×10^{-6} M and the method is applicable to a conversion of linoleic acid of less than 1%.

Table III compiles relative antioxidant efficiency values of α-, β-, γ-, and δ-tocopherol and BHT in SDS micelles and compares these values with those reported in previous studies that used different methodologies and oxidizable substrates.[3,8,38-40] Agreement is reasonable.

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(38) Niki, E.; Tsuchiya, J.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 497–501.

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Table VI. The Induction Time (T) from the Combination of Ascorbic Acid and α-Tocopherol

[AA] × 10^7 M	[E] × 10^7 M	induction period (min) T_{AA}	T_E	sum[c]	$T_{AA} + T_E$[d]
2.25	1.22	13 ± 0.1	25 ± 0.7	38	39 ± 0.7
4.30	1.22	28 ± 0.7	25 ± 0.7	53	53 ± 1.0

[a] The system used for these experiments consisted of 2.6 mM linoleic acid, 0.10 M SDS, 0.05 M phosphate buffer (pH 7.4), 0.1 mM DTPA, and 0.5 mM ABAP. The reaction was followed by measuring the formation of conjugated dienes at 37 °C. [b] AA stands for ascorbic acid; E for α-tocopherol [c] This is the predicted induction period, calculated as the sum of that due to AA and E separately. [d] This is the measured induction period when both ascorbic acid and α-tocopherol are both present.

Effect of SDS Concentration. As shown in Table V, the relative antioxidant effectiveness (RAE) values for 17 antioxidants are within a factor of 2 in both 0.1 M SDS and 0.5 M SDS. All AE values except PMHC and Trolox C are larger in 0.1 M SDS using the conjugated diene method than we had reported previously using 0.5 M SDS and an oxygen uptake method.[3] The reason for this difference is not apparent, but it may be related to the fact that higher conversions are required for the oxygen uptake relative to the conjugated diene method.

Effect of Micellar Charges on Relative Antioxidant Efficiencies. The data on the effect of micellar charge type on AE values, taken from Table IV, are shown in bar graph form in Figure 4. For the uncharged antioxidants such as α-, β-, γ-, and δ-tocopherol, PMHC, and BHT, the relative antioxidant efficiencies values are about the same in both negatively-charged and positively-charged micelles. However, micellar charges play an important role in relative antioxidant efficiencies for charged antioxidants. The relative antioxidant efficiency of Trolox C in SDS (about 1) is 4-fold smaller than in the positively charged HDTBr (RAE 4.7), perhaps because its ionized carboxylate group causes it to be repelled from the negatively-charged SDS micellar sphere. Ascorbic acid shows this effect even more strongly, with an RAE of about 0.01 in SDS and about 13 in HDTBr, suggesting that ascorbate is strongly attracted to the positively-charged HDTBr micellar sphere where autoxidation is occurring.[41,42] Ascorbyl palmitate shows this effect to a much smaller degree, with an RAE value of about 1 in SDS and 3.9 in HDTBr. Even in negatively charged micelles, ascorbyl palmitate may be able to partition appreciably into SDS micelles due to a balancing effect caused by electrostatic and lipophilic affinities.

Thus, we conclude that the method presented here is relatively independent of the surfactant concentration and charge type for uncharged antioxidants. However, charged antioxidants will give values using this method that must be viewed with caution, since they will vary with the charge type of the surfactant.

Possible Synergy between Ascorbic Acid and α-Tocopherol. The effect of combinations of ascorbic acid and α-tocopherol are compared individually and in combinations in SDS micelles in Table VI. The length of the induction periods due to ascorbic acid and α-tocopherol are found to be additive and no synergistic effect on the length of the induction period is observed in this SDS micellar system.

There are a number of reports on the interactions of ascorbate and tocopherol. In 1968, Tappel suggested that ascorbate might be able to reduce the α-tocopheryloxyl radical to "spare" α-tocopherol.[43] This prediction was first tested in mixed solvents in which both tocopherol and ascorbate would be soluble. In 1979, Packer et al.[44] used pulse radiolysis to shown that ascorbate reduces the α-tocopheryloxyl radical with a very fast rate constant ($k = 10^6$ M^{-1} s^{-1}) in a solvent consisting of 50% 2-propanol, 10% acetone, and 0.04 M carbon tetrachloride, and with the two antioxidants present at about 10^{-3} M. In 1987, Mukai et al.[45] studied a solvent of 2:1 benzene–ethanol and report that ascorbate, 6-*O*-ascorbyl stearate, and 2,6-*O*,*O*-ascorbyl dipalmitate react with the tocopheryloxyl radical with rate constants of 549, 626, and 4.8 M^{-1} s^{-1}, respectively. The smaller values obtained here relative to the value given above for a solvent consisting mainly of alcohol–acetone probably are due to the reduced ion-supporting power of the benzene-containing solvent and consequent reduced rates of electron transfer. The explanation for the 100-fold smaller rate constant for the dipalmitate is not obvious.

In 1991, in a discussion of ascorbate as an antioxidant, Niki[46,47] reviewed evidence for reduction of the tocopheryloxl radical by ascorbate in a number of test systems. In 3:1 (by volume) *tert*-butyl alcohol–methanol as solvent, the induction period for the autoxidation of methyl linoleate in the presence of both tocopherol and ascorbate is the sum of the individual induction periods. This lack of synergy in the length of the induction period agrees with our results in a micellar system. Despite this lack of synergy in induction period length, the rate constants for reaction of the linolate peroxyl radical observed using the mixture of both antioxidants is 4.0×10^5 M^{-1} s^{-1}, close to the value of 5.1×10^5 M^{-1} s^{-1} observed for α-tocopherol alone and larger than the value of 7.5×10^4 M^{-1} s^{-1} observed for ascorbate alone. Furthermore, when both antioxidants are present, ascorbate disappears first and only when it is gone does tocopherol disappear. The same is also true when 5,6-*O*,*O*-ascorbyl distearate is used. These results suggest that peroxyl radicals react virtually exclusively with tocopherol and that the reducing equivalents of ascorbate are used virtually exclusively to rereduce the tocopheryloxyl radical back to tocopherol, rather than reacting with peroxyl radicals directly. This "Tinkers-to-Evers-to-Chance" relay of reducing equivalents takes advantage of the more rapid reaction of tocopherol with peroxyl radicals and the fact that ascorbate, which often is present in larger concentrations in biological systems, can act as a reservoir of reducing power.

Niki and his collaborators[46,48] also have studied the interaction of ascorbate and tocopherol in a soy phosphatidylcholine liposome system. When a water-soluble initiator was used, both compounds act as antioxidants, and the inhibition period when both are present is the

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Table VII. The Relative Antioxidant Efficiencies and the Stoichiometric Factor of Natural and Synthetic Antioxidants Determined by Methods A and B[a]

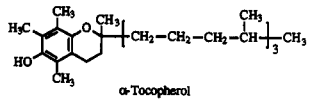
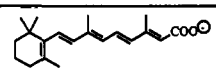
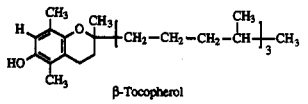
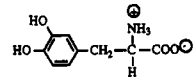
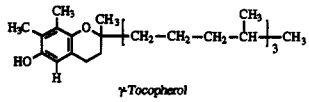
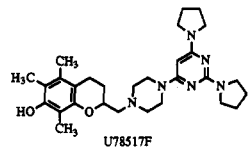
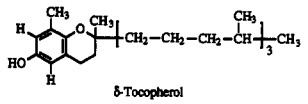
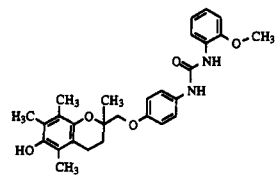
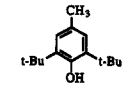
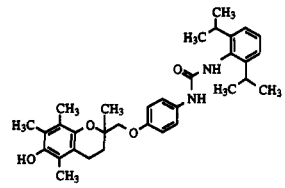
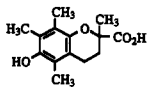
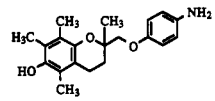
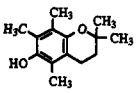
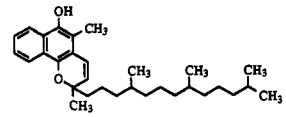
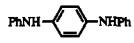
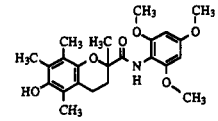
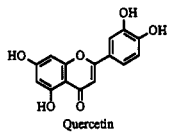
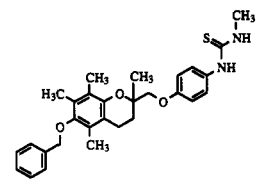
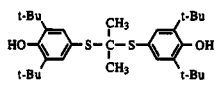
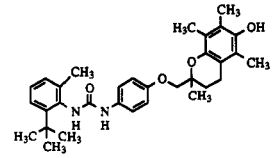
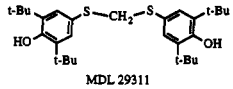
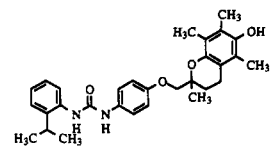
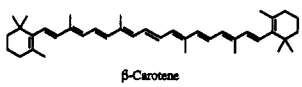
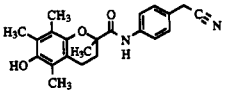
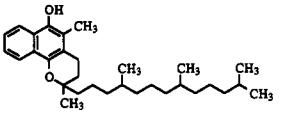
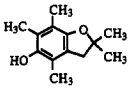
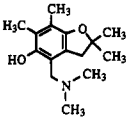
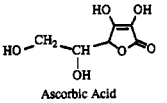
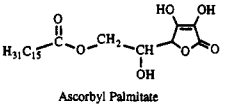
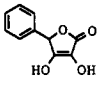
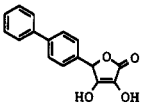
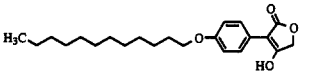
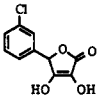
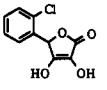
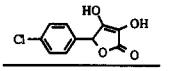
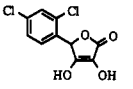
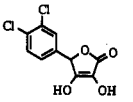
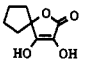
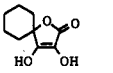
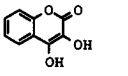
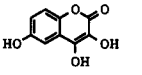
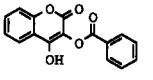
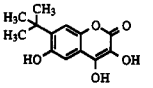
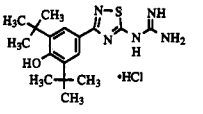
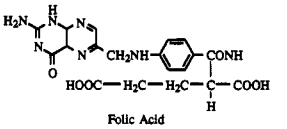
serial no.[b]	compound name, structure, and abbreviation	RAE method A[c]	RAE method B[d]	SF[e]	serial no.[b]	compound name, structure, and abbreviation	RAE method A[c]	RAE method B[d]	SF[e]
1	 α-Tocopherol	[1]	[1]	[2]	13	 13-*cis*-RA	.002	ND	–
2	 β-Tocopherol	0.52	0.89	2.04	14	 DOPA	0.14	ND	–
3	 γ-Tocopherol	0.53	0.84	1.89	15	 U78517F	1.02	ND	–
4	 δ-Tocopherol	0.31	0.74	1.78	16		ND	0.72	2
5	 Butylated Hydroxy Toluene; BHT	0.26	0.03	1.4	17		ND	0.90	2.1
6	 Trolox	0.72	0.55	2.45	18		ND	0.86	2.0
7	 PMHC	1.57	2.50	2.11	19		ND	0.16	0.2
8	 DPPD	3.68	ND	–	20		ND	0.32	0.9
9	 Quercetin	0.19	ND	–	21		ND	NA	–
10	 Probucol	0.16	0.50	2.79	22		ND	0.96	2.2
11	 MDL 29311	0.25	ND	–	23		ND	0.90	2.0
12	 β-Carotene	0.003	NA	–					

Table VII. (Continued)

serial no.[b]	compound name, structure, and abbreviation	RAE method A[c]	RAE method B[d]	SF[e]
24		ND	1.0	2.0
25		ND	0.49	0.3
26		ND	1.2	2.2
27		ND	0.64	2.4
28	 Ascorbic Acid	.014	NA	-
29	 Ascorbyl Palmitate	1.09	ND	-
30		ND	0.26	0.30
31		ND	0.62	0.70
32		ND	0.52	1.5
33		ND	0.59	0.6
34		ND	0.40	0.6
35		ND	0.40	0.40
36		ND	0.80	1.07
37		ND	0.40	0.71
38		ND	0.15	-
39		ND	0.36	0.40
40		ND	0.29	0.40
41		ND	0.63	1.23
42		ND	0.60	0.20
43		ND	0.90	0.41
44		ND	0.23	-
45	 Folic Acid	ND	NA	-

[a] See the Experimental Section for the experimental protocols for methods A and B. NA = not active; ND = not determined with this method. [b] See Table VIII for purity data on compounds not given in the text. [c] Method A was used at LSU (25). [d] Method B was used at Parke-Davis. [e] The stoichiometric factor (SF) is the moles of radicals captured by each mole of inhibitor. It is taken as 2 for α-tocopherol, and the other values of the SF are calculated relative to this value.

Table VIII. Analytical Purity Data for Various PD Compounds

	C		H		N	
serial no.	calcd	found	calcd	found	calcd	found
17	74.18	74.00	7.99	7.95	5.24	5.09
20	66.49	66.59	7.03	7.21	3.37	3.08
21	70.34	70.47	7.02	6.81	5.25	5.23
22	73.74	73.64	7.83	7.85	5.37	5.44
23	72.41	72.22	7.49	7.58	5.63	5.39
24	71.62	71.45	6.69	6.52	7.59	7.69

sum of that observed for the individual compounds. When a lipid-soluble initiator was used, 2,2′-azobis(2,4-dimethylvaleronitrile), tocopherol suppressed the autoxidation but ascorbate did not; apparently the lipid radicals in the oil phase do not diffuse into the bulk aqueous medium. Despite this, the inhibition period observed for tocopherol was increased when ascorbate also was present; this suggests that, for liposomes at least, reducing equivalents can be passed from ascorbate in the aqueous phase to the tocopheryloxyl radical in the oil phase.

Barclay et al.[9] have reported the autoxidation of linoleic acid initiated by the oil-soluble initiator di-*tert*-butyl hyponitrite in 0.5 M SDS solutions at 30 °C and pH 7.0. Ascorbate alone is found to be an inefficient inhibitor, but in the presence of α-tocopherol it extends the inhibition period beyond that represented by the sum of the inhibition periods observed for ascorbate and tocopherol in individual experiments, but only by about 10%. The reason for the discrepancy of this result with the data

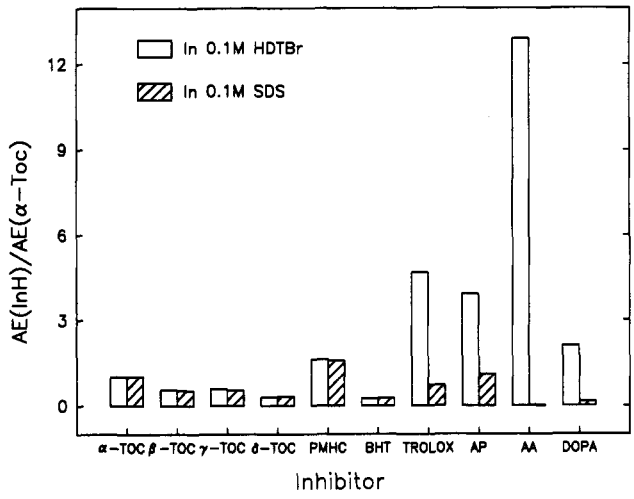


Figure 4. A bar graph of the relative antioxidant efficiencies of α-, β-, γ-, and δ-tocopherols (TOC), PMHC, BHT, Trolox C (TROLOX), ascorbyl palmitate (AP), ascorbic acid (AA), and L-DOPA (DOPA) in aqueous micelles of either 0.10 M SDS or 0.10 M HDTBr surfactants (both at pH 7.4).

shown in Table VI is not clear, but it may be connected with the fact that Barclay et al. used a pressure transducer apparatus, which requires higher conversions than does the spectrophotometric method reported here. Barclay et al. report that in their system the stoichiometric factor for solutions containing both ascorbate and tocopherol are about 1 rather than the expected value of 2 and comment that "a larger value might be applicable at lower rates of initiation and higher ratios of tocopherol to ascorbate". Barclay et al. find that tocopherol is used up only after all of the ascorbate has disappeared, a clear indication that ascorbate is reducing the vitamin E radical in their system.

Recently, Burton, Wronska, Stone, Foster, and Ingold[49] have studied the possible synergy between vitamins C and E *in vivo*. Using the guinea pig, they find that dietary vitamin C does not appear to "spare" vitamin E. Their experiments involved feeding deuterated RRR-α-tocopherol ("natural" vitamin E) for 8 weeks followed by unlabeled vitamin E with or without either 50 or 5000 mg of vitamin C/kg of diet. They found no significant differences in the concentrations of either labeled or unlabeled vitamin E in any of the nine tissues studied between the animals fed 0, 50, or 5000 mg of vitamin C. Thus, Burton et al. conclude that the "sparing action of vitamin C on vitamin E, which is well documented *in vitro*, is of negligible importance *in vivo* in guinea pigs that are not oxidatively stressed in comparison with the normal metabolic processes which consume vitamin E." There are at least two possible explanations for this important *in vivo* result. The first is that reducing equivalents cannot be exchanged between the oil-soluble vitamin E and the water-soluble vitamin C in biological membranes *in vivo*. While the data support that explanation (and it is the one favored by Burton et al.) we would prefer to believe that reducing equivalents between vitamins C and E, as well as glutathione and even NADH, can find channels for exchange.

It also is possible that the lack of oxidative stress in these experiments is important, as hinted by the passage quoted above. Barclay et al.[9] discuss the importance of the rate of radical production on synergy between vitamins C and E in their *in vitro* system. It might be expected that the rates of radical production *in vivo* in nonstressed animals would be considerably lower than the relatively rapid rates of autoxidation that are used *in vitro* when autoxidations are studied. Under the slow rates of radical production that occur *in vivo*, normal repair and catabolism of vitamin E and its radical may dominate and the sparing effect of vitamin C may not be apparent on the turnover time for vitamin E. Unfortunately, the direct kinetic experiment in which ascorbate reduces tocopheryloxyl radicals, cannot be done *in vivo*.

Water-versus Oil-Soluble Initiators; Micelles, Liposomes, and the LDL Particle. We have used the water-soluble initiator ABAP in these studies. A number of authors have compared and discussed the effect of the use of water-soluble and oil-soluble initiators (such as DMVN) on the effectiveness of antioxidants.[50-54] In general, the pattern that is observed is that both types of initiators lead to similar RAE values for oil-soluble inhibitors. *Oil-soluble initiators* produce radicals directly in the oil phase where the oil-soluble inhibitor resides, whereas radicals from a water-soluble initiator must diffuse to the oil phase before initiating the peroxidation chain that the inhibitor blocks; however, once in the oil phase, radicals from either type of initiator appear to have the same effect. However, oil-soluble initiators such as DMVN generally have a lower efficiency than do water-soluble initiators such as ABAP; the primary cause of this is probably that oil-soluble initiators produce their radicals as a geminate pair in an oil droplet and one member of this pair must diffuse from the oil droplet to reduce termination rates. This is consistent with the data shown in Table II. (The smaller efficiencies also may be due, in part, to the higher viscosity of the oil phase relative to water.)

Radicals from a *water-soluble initiator*, as rationalized above, appear to have the same effect as do radicals from oil-soluble initiators on oil-soluble inhibitors. However, if water-soluble inhibitors are studied (e.g., ascorbate), the radicals from a water-soluble initiator may be trapped by the inhibitor before they reach the micelle or liposome, reducing radical efficiency. (This is a complication reminiscent of the process termed "primary radical termination" in the polymerization field.[55])

Micelles are very porous and rapidly exchange oil-soluble molecules or radicals between different micelles; this is the reason why oil-soluble inhibitors rapidly achieve a stable distribution in micelles, even if added to the bulk phase, and reproducible RAE values can be obtained without lengthy incubation times to allow for incorporation of the inhibitor within the oil phase. In contrast, liposomes, biological membranes, and the structured low-density lipoprotein (LDL) particle are much less porous, and this leads to a very significant difference in antioxidant behavior. For example, the LDL particle behaves much

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like the oil droplets encountered in the emulsion polymerization of styrene-containing rubber, in which a water-soluble inorganic initiator is used.[56] Radicals from water-soluble initiators can enter the LDL particle, become trapped there, and termination can not occur until a second radical enters the particle. In fact, radicals may only enter the LDL particle by being "imported" from the water to the oil phase by reaction of a water-soluble radical with the phenolic group of α-tocopherol at the interface of the particle. This reaction produces the tocopheryloxyl radical, which then abstracts a hydrogen atom from a polyunsaturated fatty acid to initiate lipid autoxidation. Thus α-tocopherol becomes a chain-carrying radical in the LDL, acting as a chain-transfer agent rather than an inhibitor.[57-60] An oil-soluble initiator would not be expected to show this effect, but would be expected to have a small efficiency factor since the geminate radical pair would terminate efficiently.

These considerations suggest that it is useful to employ water-soluble initiators in studies like those done here. However, the RAE values that are obtained must be applied with caution. For example, while α-tocopherol is a very effective initiator in this micellar system, it does not show inhibitor properties in the LDL particle.[57-60]

Compression of the Antioxidant Scale as Reflected in Table VII. Table VII presents the structures and RAE values of all of the compounds that we studied. Perhaps the most striking feature of the data that is recognized as Table VII is examined is the compression of the scale. Antioxidant compounds divide into three broad classes. (i) Some compounds are slightly better antioxidants than α-tocopherol; for example PMHC and DPPD are 2-fold to 4-fold more potent antioxidants in this SDS system (Table V). (ii) Most of the compounds we report here (which generally are analogues of vitamins E or C) are about as effective as is α-tocopherol; that is they have RAE values that lie between 1 and about 0.3. (iii) Some compounds are poorer antioxidants than is α-tocopherol. The better of these compounds have RAE values as small as 0.1 (e.g., compound number 38); RAE values any smaller than about 0.1 do not register as antioxidants in this test. This "not an antioxidant" status can apply to compounds of biological interest; for example menadione, aminopyrine, and ibuprofen,[3] and, in some systems, β-carotene.

Retarders and Antioxidants. A definite induction period (and, hence, a definite stoichiometric factor) could not be calculated for compounds 38 and 44. The spectrophotometric tracings for these compounds showed a definite, pronounced decrease in the rate of conjugated diene formation that failed to return to the initial rate. Compounds that behave in this way are generally classed as retardants rather than as antioxidants. Antioxidants, such as those of the tocopherol series, are able to quench lipid peroxidations by reacting with peroxyl radicals to form relatively stable tocopheroxyl radicals. Compounds of this type significantly slow the rate of lipid peroxidation (and conjugated diene formation) until all of the antioxidant has been consumed, at which time the rate of peroxidation returns to the uninhibited rates. Compounds that act as retardants react with peroxyl radicals to slow the propagation step of lipid oxidation but do not completely stop it.

Folate. There has been considerable interest in folate recently, since elevated intakes by pregnant women appear to reduce neural tube effects in newborn infants.[61] The structure of folid acid, **45**, suggests it might be an antioxidant. However, it does not show any antioxidant properties in this test system.

Calculation of Absolute Values for the Rate Constants for Reaction of Peroxyl Radicals with Inhibitors. Although values in Table VII are expressed as RAE, multiplication of any of these values by the absolute rate constant for reaction of peroxyl radicals with α-tocopherol converts the RAE value to the absolute value of k_{inhib} for that inhibitor (see eq 21). The value of k_{inhib} for α-tocopherol in these micellar systems has been measured by Barclay et al.[2,3] and found to be 4×10^4 M^{-1} s^{-1}; thus, within the accuracy of the method and assumptions involved, multiplying a given RAE value in Table VII by 4×10^4 converts it to the rate constant for that inhibitor reacting with a peroxyl radical in units of M^{-1} s^{-1}. For example, the rate constant for reaction of probucol with peroxyl radicals is obtained as about 4×10^3 M^{-1} s^{-1}, some 100-fold larger than the rate constant for propagation of the autoxidation of linoleate.

RAE Value for β-Carotene. The small RAE value observed for β-carotene in this study, which uses conjugated diene absorption, agrees with our previous study[3] that used oxygen pressure measurements. This suggests that even inhibitors like β-carotene, which might be expected to change absorption on oxidation,[62] do not produce products that interfere with the absorption at 234 nm attributed to conjugated lipid hydroperoxides in this assay. In addition, some of the compounds listed in Table VII, at the low concentrations used in the antioxidant assay, were oxidized, and the product mixture was scanned for absorption at 234 nm, and little or none was found.

Thus, in a SDS micelle system, β-carotene has a very small RAE value (Table V). Palozza, Moualla, and Krinsky[54] also found β-carotene to be a poor antioxidant in a rat liver microsomal system using either ABAP or DMVN as the initiator. Burton and Ingold[20] have suggested that β-carotene behaves as a strong antioxidant only in poorly oxygenated systems (as might be true, for example, in tumor cells). Since both our system and that of Palozza et al. was fully oxygenated, the relatively poor antioxidant effect observed for β-carotene supports this Burton–Ingold proposal. However, in some fully oxygenated systems, β-carotene may demonstrate some antioxidant properties; for example, β-carotene interacts with α-tocopherol in a synergistic way in microsomes.[63]

Burton and Ingold rationalized the fact that β-carotene is a better antioxidant in poorly oxygenated systems by proposing the β-carotene is a poor scavenger of peroxyl radicals but an excellent scavenger of carbon-centered radicals. Thus, carbon radicals add to the long, conjugated olefinic chain of β-carotene to produce a resonance-

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stabilized carbon radical, which would, in turn, react very slowly with polyunsaturated fatty acids to abstract hydrogen atoms and propagate the autoxidation chain. Thus, in poorly oxygenated systems, β-carotene traps the predominant carbon-centered radicals to stop chains. In well-oxygenated systems, the slow reaction of β-carotene with oxy-radicals makes it a poor antioxidant. [β-Carotene is converted (at least in part) to an epoxide during autoxidation.[64] When peroxyl radicals react with an olefin to form an epoxide, the peroxyl radical is converted to an alkoxyl radical, which is even more reactive than a peroxyl radical and would not slow or retard autoxidations; thus, the ineffectiveness of β-carotene must be due to a poor scavenging ability toward peroxyl radicals.]

It is possible that β-carotene behaves as a better antioxidant in liposomal and microsomal systems than in micellar systems.[63] This is not because of the lipid character of the mileau, since the original Burton–Ingold study[20] used the autoxidation of methyl linoleate in homogeneous solution in chorobenzene. It is possible that β-carotene behaves as a better antioxidant toward isolated lipid fractions that exchange organic species only slowly with the bulk aqueous solvent (such as microsomes and LDL) than toward the more "leaky" micellar system studied here. This is the reverse of the effect observed with α-tocopherol, discussed above. Firstly, β-carotene would not be expected to "import" radicals from the aqueous phase into LDL, as does α-tocopherol. In addition, β-carotene would react with carbon-centered radicals to produce a stable carbon-centered radical that might not abstract a hydrogen atom from a polyunsaturated fatty acid to propagate the oxidation chain, as does the α-tocopherol radical. In fully oxygenated systems, however, β-carotene would be expected to react with dioxygen to form a peroxyl radical that might continue the autoxidation chain.

Lack of a Unique Scale of Antioxidant Potencies. The difficulties in understanding the antioxidant properties of β-carotene discussed above, the different behavior of α-tocopherol in micelles and LDL, and the different values of antioxidant potencies observed for charged antioxidants in anionic and cationic micelles (Table IV), illustrate the fact that there is not a single, unique table of antioxidant efficiencies. Despite this inherent limitation, the assay described here is fast and easy to apply and is useful for routine screening of large numbers of synthetic compounds. This assay provides a screen to distinguish antioxidants that are roughly as good as α-tocopherol from compounds that demonstrate either only little or no antioxidant properties.

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