Moderate Antioxidative Efficiencies of Flavonoids During Peroxidation of Methyl Linoleate in Homogeneous and Micellar Solutions

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ABSTRACT: The relative reactivities as well as the stoichiometric coefficients for a number of flavonoids, catechols, and—for comparison—standard phenolic antioxidants were determined by analyzing the kinetics of oxygen consumption in organic and micellar systems, with peroxidation initiated by lipid- and water-soluble azo initiators. The results demonstrated that the flavonoids did not behave as classic phenolic antioxidants such as α-tocopherol, but showed only moderate chain-breaking activities. The results were in line with other structure-activity relationship studies on the importance of the B-ring catechol structure, the 2,3-double bond, and the 3,5-hydroxy groups. The data are discussed in view of possible explanations of the deviations flavonoids reveal in their behavior compared with regular phenolic antioxidants.
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KEY WORDS: Antioxidants, azoinitiators, catechol compounds, flavonoids, kinetic study, methyl linoleate, oxygen consumption, peroxidation, phenols, relative rate constants.

Flavonoids, natural polyphenolic compounds, are widely distributed in the plant kingdom, including fruits and vegetables. It is estimated that humans consume on the average 1 g/d of flavonoids (1). Flavonoids display pronounced biological effects, especially pharmacological and vitamin-like activities (2–6). The latter effect is commonly associated with their ability to scavenge reactive free radicals (7–9) and/or to deactivate catalytic metals due to complexation (10–12).

A distinguishing feature of flavonoids is their high reactivity toward active free radicals, such as hydroxyl, alkoxyl, peroxyl, and superoxide anions, (7,9,13,14). As for reactions with peroxyl radicals, the few known rate constants are all in excess of $1 \cdot 10^7$ $M^{-1}s^{-1}$ (9,15–17). These values are among the highest for reactions between peroxyl radicals and oxyaromatic compounds (18–20). This observation, as well as the fact that flavonoids are natural and rather accessible substances of low-toxicity, makes the idea intriguing to apply flavonoids as stabilizers with antioxidative action for food, cosmetics, and other lipid-containing products.

The ability of flavonoids to inhibit lipid peroxidation is well documented, both for natural lipid products and for model lipids (6,21–27). Nevertheless, for a number of reasons, a shortage exists of reliable quantitative kinetic information on the chain-breaking ability of flavonoids. In most studies, complex experimental models were used, which estimated total rather than chain-breaking antioxidative activity. Almost as a rule, experiments were performed under poorly reproducible conditions because the rate of free-radical generation (the rate of initiation) was not under control. This resulted in a situation in which it became difficult to compare data obtained in different studies. Furthermore, antioxidative abilities of flavonoids were seldom compared with those of standard chain-breaking antioxidants, such as α-tocopherol, or butylated hydroxytoluene (BHT).

In this work, the chain-breaking antioxidative abilities were determined for flavonoids and related compounds with a catechol moiety as a structural feature (Fig. 1) by applying the kinetics of oxygen consumption in three kinetic models: model 1—the autoxidation of cumene in chlorobenzene (28), model 2—oxidation of methyl linoleate (ML) in chlorobenzene (29); and model 3—the oxidation of ML in sodium dodecyl sulfate (SDS) micelles (30). In all instances, the oxidation was carried out at a constant and well-controlled rate of free-radical generation. The latter was achieved by the use of free-radical initiators, the lipid-soluble azo compound 2,2′-azo*bis*isobutyronitrile (ABN) for models 1 and 2 and the water-soluble 2,2′-azo*bis*(4-carboxyisovalero)-nitrile (ACVN) for micellar model 3. Because we are dealing with the so-called mode of initiated chain oxidation (19), this drastically simplifies the kinetic treatment of experimental data and permits collection of well-defined and reproducible kinetic information. This work also compares flavonoid antioxidative capabilities with those of standard lipid antioxidants α-tocopherol (*XIV*) and BHT (*XV*), as well as with that of an idealized, classic antioxidant functioning without undesirable side reactions and providing the best possible inhibition. By using these models, we determined only the chain-breaking activity rather than effects due to chelating transition metals, which might activate peroxides accumulated during oxidation.

EXPERIMENTAL PROCEDURES

Quercetin, dihydroquercetin, naringenin (Serva, Heidelberg, Germany), kaempferol, catechin, rutin, chlorogenic acid, el-

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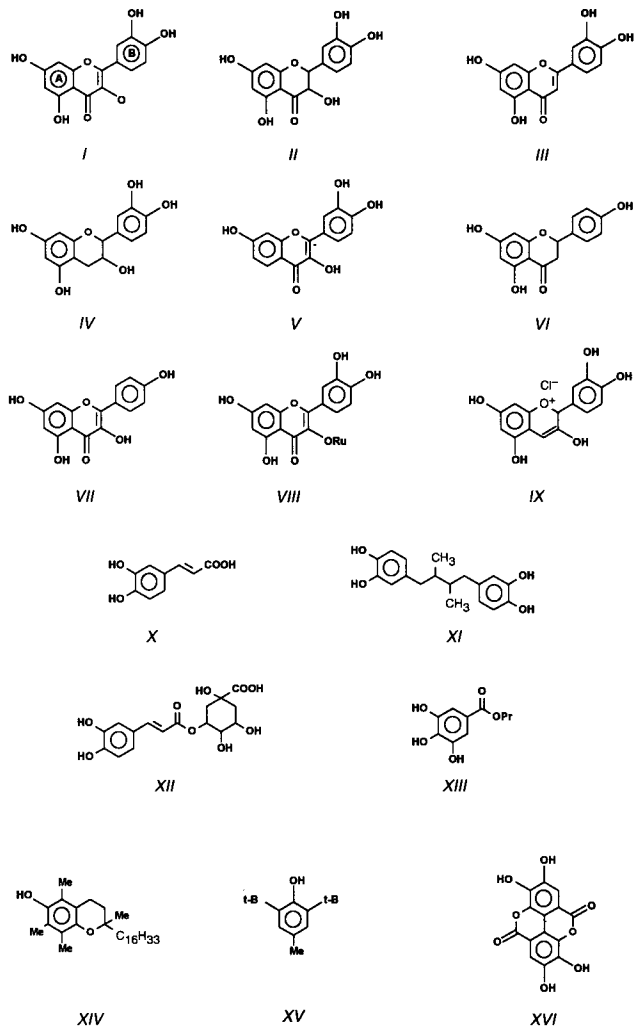


FIG. 1. The structures of flavonoids and other phenolic antioxidants studied in this work: *I*, quercetin; *II*, dihydroquercetin; *III*, luteolin; *IV*, catechin; *V*, fisetin; *VI*, naringenin; *VII*, kaempferol; *VIII*, rutin (rut = rutoside); *IX*, cyanidine chloride; *X*, caffeic acid; *XI*, nordihydroguaiaretic acid; *XII*, chlorogenic acid; *XIII*, propyl gallate; *XIV*, α-tocopherol; *XV*, 2,6-di-*tert*-butyl-4-methylphenol (BHT); and *XVI*, ellagic acid.

lagic acid (Fluka, Neu-Ulm, Germany), luteolin, fisetin, cyanidine chloride (Roth, Karlsruhe, Germany), caffeic acid, nordihydroguaiaretic acid (Aldrich, Steinheim, Germany), propyl gallate, α-tocopherol, ML, ACVN, BHT (Sigma, Deisenhofen, Germany), and 6-hydroxy-2,2,5,7,8-pentamethylchromane (HPMC; a gift by N.G. Chrapova) were used as received. Anhydrous sodium phosphates, Na_2HPO_4 and NaH_2PO_4 of highest quality, were purchased from Merck (Darmstadt, Germany). Cumene and chlorobenzene were purified by standard methods (31). ABN (Merck) was purified by repeated recrystallization from benzene and acetone. Flavonoids and other phenols were used as stock solutions, depending on solubility, in dimethyl sulfoxide, chlorobenzene, water, or in mixtures of these solvents. Solutions of Na_2HPO_4 and NaH_2PO_4 in double-distilled water, used for the preparation of buffer solutions, were purged from traces of transition metals by the batch method (32) with Chelex-100 resin (Bio-Rad Laboratories, Munich, Germany).

The kinetics of oxygen consumption during the oxidation of chlorobenzene solutions of cumene and ML (50.0 ± 0.1°C, air, 10 mM ABN as a free-radical initiator, volume of sample tested of 0.1–0.3 mL) was studied by using a homemade glass-capillary microvolumometer of high sensitivity with a cell construction that allowed addition of the required components without opening the cell (29). A kinetic run was started by measuring the rate of oxidation in the absence of antioxidants (noninhibited oxidation, R_0) and a rate of initiation (R_{in}). The latter was determined by the inhibitor method with HPMC as inhibitor (29). After the determination of R_0 and R_{in}, flavonoids or other phenols at various concentrations were added. Oxygen consumption monitoring was started 3–5 min after adding the antioxidant.

The oxidation of 0.02 M ML in 0.2 M SDS micelles was studied in 0.05 M Na phosphate buffer, pH 7.40 ± 0.02, at 37.0 ± 0.2°C in the presence of 50 mM ACVN as an initiator. Under these conditions, ML peroxidation was found to be a chain process with a kinetic chainlength of about 40. The kinetics of oxygen consumption was studied with a Yellow Springs Instrument Co. (Yellow Springs, OH) Model 5300 Biological Oxygen Monitor with a Clark electrode as sensor. Similar to the study of the oxidation of homogeneous solutions of ML, a run was started with the determination of R_0 and R_{in}. Then, aliquots of a stock solution of phenol were added without suspending $[O_2]$ monitoring by using a microsyringe with a long needle (see Ref. 28 for more detail). Kinetic simulations were performed with the "Kinetics-2" program (33).

RESULTS

Kinetic theory of lipid peroxidation inhibited by a "classic" chain-breaking antioxidant. The basic kinetic scheme of LH oxidation under conditions of a constant rate of free-radical generation due to the thermodecomposition of a free-radical initiator, *Y*, in the presence of a classic phenolic antioxidant is as follows:

$Y (+ LH, + O_2)$	$\rightarrow$	$LO_2^{\cdot}$	[0]
$LO_2^{\cdot} + LH + O_2$	$\rightarrow$	$LOOH + LO_2^{\cdot}$	[1]
$LO_2^{\cdot} + LO_2^{\cdot}$	$\rightarrow$	products	[2]
$LO_2^{\cdot} + PhOH$	$\rightarrow$	$LOOH + PhO^{\cdot}$	[3]
$PhO^{\cdot} + LO_2^{\cdot}$	$\rightarrow$	products	[4]
$PhO^{\cdot} + PhO^{\cdot}$	$\rightarrow$	$PhOH + Q$	[5]

where *Q* is a quinone or quinone methide, depending on the structure of the phenoxyl radical, $PhO^{\cdot}$ (19). The classical behavior means that PhOH is consumed only in Reaction 3 and $PhO^{\cdot}$ only in Reactions 4 and/or 5. The contribution of Reaction 4, α, is given by the ratio:

$$\alpha = \frac{R_4}{R_4 + R_5} \quad [6]$$

respectively:

$$\frac{1}{\alpha} = 1 + \frac{k_5[\mathrm{PhO^{\bullet}}]}{k_4[\mathrm{LOO^{\bullet}}]} \quad [7]$$

in which k_3 and k_4 denote the rate constants for the respective reactions. In the absence of phenols, the rate of chain oxidation:

$$R_0 = \frac{d[O_2]}{dt} = \frac{d[\mathrm{LOOH}]}{dt} = \frac{k_1}{\sqrt{k_2}}[\mathrm{LH}]\sqrt{R_{in}} \quad [8]$$

In the presence of phenols when k_3[PhOH] is high enough so that $R << R_0$, and, hence, the rate of Reaction 2 << R_{in}, the system is in the induction period. The duration of the induction period, t_{ind}, and the rate of chain inhibited oxidation are given by:

$$t_{ind} = \frac{f[\mathrm{PhOH}]_0}{R_{in}} \quad [9]$$

$$R = \frac{k_1 R_{in}[\mathrm{LH}]}{2\,k_3[\mathrm{PhOH}]} \quad [10]$$

where $[\mathrm{PhOH}]_0$ is a starting concentration of phenol and f is the stoichiometric coefficient of inhibition that represents the number of LOO· molecules deactivated by one molecule of phenol. In the classic variant under consideration, a value of f for phenols containing only one active OH group is generally equal to two without reference to whether PhO· decays in Reaction 4 or 5 (19). Equation 10 is given here for the case in which PhO· decays in Reaction 4. When PhO· decays by Reaction 5, Equation 10 remains valid, but the constant in the denominator changes from 2 to 1. In conclusion, for a classic phenol, t_{ind} increases directly with $[\mathrm{PhOH}]_0$ and R decreases inversely with [PhOH]. Equations 9 and 10 have been confirmed experimentally many times (19). They are also expected to be a valid principle for a phenol with two ortho-OH groups, such as many flavonoids and other catechol derivatives (discussed later).

The k_3/k_1 ratio, which determines actually the inhibiting ability of classic phenols during the oxidation of a certain hydrocarbon, may be calculated from the kinetics of oxygen consumption with the help of Equation 10 or Equation 11, or 12 (29,30):

$$F_1 = \frac{R_0}{R} - \frac{R}{R_0} = \frac{2\,k_3 R_0}{k_1[\mathrm{LH}]R_{in}}[\mathrm{PhOH}] \quad [11]$$

$$F_2 = ln\frac{1+R/R_0}{1-R/R_0} - \frac{R_0}{R} = \frac{k_3 R_0}{k_1[LH]}t + const \quad [12]$$

which are valid over the whole region of R variation, not only where $R << R_0$. Equation 11 may be used if a value for [PhOH] is known, for instance, just after adding the phenol. Equation 12 reflects the change in R with time as the phenol is consumed due to Reaction 3. In this case, a value of [PhOH] is not required to calculate k_3/k_1 from the plot of O_2 consumed vs. time.

Stoichiometric coefficients of inhibition during the oxidation of cumene inhibited by flavonoids (model 1). Lipid-soluble flavonoids *I–V*, and *VII*, as well as the catechol derivatives *X* and *XI* and the standard antioxidants *XIV* and *XV*, showed a pronounced inhibiting capability during cumene oxidation. The exceptions were *VI* and *XVI*, which did not inhibit cumene oxidation, even at a concentration of 1 mM (data not shown).

An example of the kinetics of oxygen consumption accompanying cumene oxidation in the presence of *I* is shown in Figure 2 (plot 2). It shows the sharply defined induction period; furthermore, t_{ind} was found to be proportional to $[\mathrm{PhOH}]_0$ in agreement with Equation 9 (data not shown). Similar dependencies were observed with *II–V, VII, X, XI, XIV* and *XV*. Values of f, calculated from t_{ind} with Equation 9 are listed in the second column of Table 1. For most phenols, the f values are close to two. For flavonoids and catechol derivatives, this corresponds to a decay of PhO· predominantly by Reaction 4 in a reaction with $LO_2^{\bullet}$ that occurs as a second oxidation step (Scheme 1).

This pathway may be controlled thermodynamically by the low strength of the O-H bond in the B ring of PhO· (34). Also feasible is a recombination of both types of radicals with the formation of unstable peroxides (Scheme 2), which may occur most readily with phenols having only one active OH group, such as *XIV* and *XV*. Both pathways of reaction between PhO· and $LO_2^{\bullet}$ correspond kinetically to Reaction 4.

The fact that f for *XI* is close to 4 likely reflects that both catechol moieties in its structure react independently with

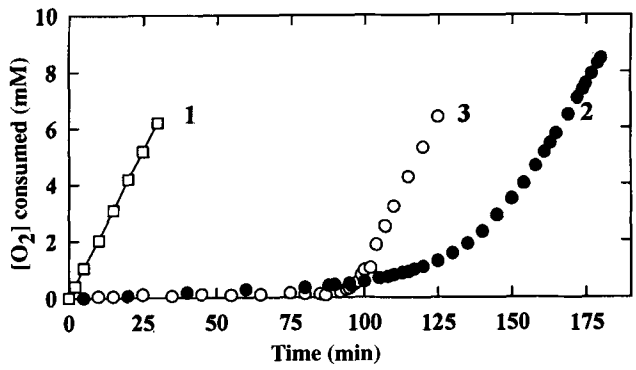


FIG. 2. Kinetics of oxygen consumption during the oxidation of 6.5 M cumene in chlorobenzene at 50°C (R_{in} = 3.45 • 10^{-8} Ms^{-1}): 1, without antioxidant; 2, in the presence of 0.11 mM *I*; 3, in the presence of 0.11 mM *XIV*.

TABLE 1
Antioxidative Capability of Flavonoids and Other Phenolic Antioxidants[a]

PhOH	f	t_3 (min)/k_3/k_1* [PhOH](mM) 0.03	0.09	0.15	0.30	0.60	0.90	3.0	k_3/k_1 in SDS
Flavonoids:									
I	2.6	33/590	48/250	59/150	68/75	59/30	42/20	nd	110
II	2.0	22/160	nd	34/77	39/37	nd	29/7.8	nd	23
III	2.8	34/250	38/120	36/81	50/47	90/55	99/24	117/9.4	31
IV	1.7	28/390	33/150	45/100	35/33	nd	22/5.5	nd	52
V	2.4	28/730	54/210	63/170	64/90	nd	74/39	nd	105
VI	no inh	no inh	no inh	nd	nd	nd	no inh	no inh	0.5
VII	1.5	7/nd	nd	12/36	26/21	nd	27/15	nd	50
VIII	not sol	not sol	not sol	not sol	not sol	not sol	not sol	not sol	31
IX	not sol	not sol	not sol	not sol	not sol	not sol	not sol	not sol	310
Catechols:									
X	2.4	30/550	59/340	84/190	100/91	108/84	100/49	112/13	23
XI[b]	4.1	71/nd	150/760	240/810	200/270	150/150	nd	nd	250
XII	not sol	not sol	not sol	not sol	not sol	not sol	not sol	not sol	150
XIII	nd	nd	nd	nd	110/160	nd	130/54	nd	160
XVI	no inh	no inh	nd	no inh	nd	no inh	nd	no inh	no inh
Phenols:									
XIV[c]	2.0	nd	155	nd	>500	nd	>500	nd	290[e]
XV[d]	2.0	nd	55	96	170	nd	>500	nd	240[e]
A[f]	—	61	173	284	561	1120	1670	5560	—

[a]See Figure 1 for abbreviations of antioxidants structures: f is a stoichiometric coefficient of inhibiting determined during the oxidation of 6.5 M cumene in chlorobenzene at 50°C (model 1) ($R_{in} = 3.45 \cdot 10^{-8}$ Ms^{-1}); t_3 is the time required to consume 3 mM oxygen and k_3/k_1* is an effective parameter characterizing chain-breaking activity of phenols (see text) during the oxidation of 2.48 M methyl linoleate (ML) in chlorobenzene at 50°C (model 2) ($R_{in} = (1.6 \pm 0.1) \cdot 10^{-8}$ Ms^{-1}); k_3/k_1 in sodium dodecyls sulfate (SDS) was determined during the oxidation of 0.02 M ML in 0.2 M SDS micellar solutions in 0.1 M sodium phosphate buffer, pH 7.40, at 37°C (model 3) ($R_{in} = 1.5 \cdot 10^{-9}$ Ms^{-1}). Notes: no inh, no inhibition; not sol, not soluble in cumeme or ML; nd, not determined.

[b]k_3/k_1 (*XI*) was calculated taking into consideration that this antioxidant has two catechol moieties.

[c]$k_3/k_1 = 9500$ [Ref. 29].

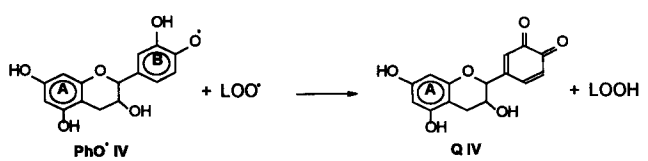
[d]$k_3/k_1 = 290$ [Ref. 29].

[e]From Reference 30, the value of k_3/k_1 for 6-hydroxy-2,2,5,7,8-pentamethylchromane, an analog of *XIV* without long-chain substituent in position 2 is 4400 M^{-1}s^{-1}.

[f]t_3 Values calculated for classic inhibitors with $k_3 = 1 \times 10^6$ M^{-1}s^{-1} by using the kinetic scheme given in text. Kinetic parameters were: $R_{in} = 1.6 \cdot 10^{-8}$ Ms^{-1}; [LH] = 2.48 M; $k_1 = 120$ M^{-1}s^{-1} (see Ref. 29 for references); $k_2 = 1.5 \cdot 10^7$ M^{-1}s^{-1} (see Ref. 29 for references); $k_4 = 3 \cdot 10^8$ M^{-1}s^{-1} (19); $k_5 = 1 \cdot 10^8$ M^{-1}s^{-1}.

$LO_2^{\cdot}$. In all probability, other OH groups of flavonoids associated with the A-ring or the pyran ring (see Fig. 1) do not contribute significantly to f values because of their poor reactivities toward $LO_2^{\cdot}$. Yet, some participation of OH groups linked to the A-ring manifests itself in the fact that the rate of oxidation upon completion of the induction period appeared to be somewhat lower than R_0 (Fig. 2, plot 2). This is also typical for other flavonoids (data not shown). For phenols with only one OH group, for example *XIV* and *XV*, the rate of oxidation upon completion of the induction period is actually coincident with R_0 (Fig. 2, plot 3).

The kinetics of oxygen consumption during oxidation of chlorobenzene solutions of ML in the presence of flavonoids (model 2). The kinetics of oxygen consumption during the oxidation of ML in the presence of various concentrations of *I* is depicted in Figure 3. Although *I* showed a pronounced inhibiting activity, R was not proportional to $[PhOH]_0$; rather, it decreased to a minimum level and then increased again. For instance, R at a concentration of 0.9 mM was evidently higher than at 0.3 mM. Except for runs for which the concentration of *I* was relatively low, it was difficult to observe any pronounced induction period. Similar situations were more or less typical for most flavonoids and the catechol derivatives *X* and *XI* (see below). Under these circumstances, parameters f and k_3, which commonly characterize the inhibiting capability of classic chain-breaking antioxidants, are hardly applicable for flavonoids. To account for this deviation of flavonoids and related compounds from classic behavior, the following two parameters were used: the time required to consume 3 mM oxygen, t_3 and the k_3/k_1* ratio. The first parameter is more or less analogous to t_{ind}; the second should be read in this instance as an effective value rather than a ratio of rate constants for elementary Reactions 3 and 1. A value of t_3 was



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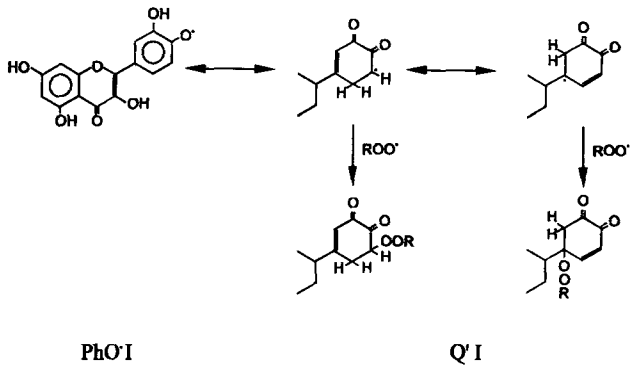

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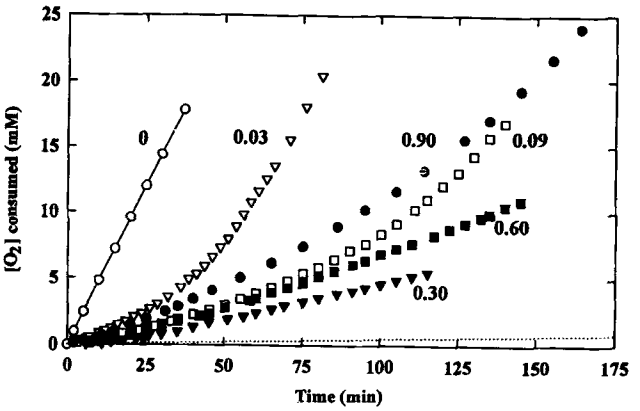


FIG. 3. Kinetics of oxygen consumption during the oxidation of 2.48 M methyl linoleate (ML) in chlorobenzene at 50°C (R_{in} = 1.60•10^{-8} Ms^{-1}) in the presence of various concentrations of *I* (indicated in mM next to the kinetic curves).

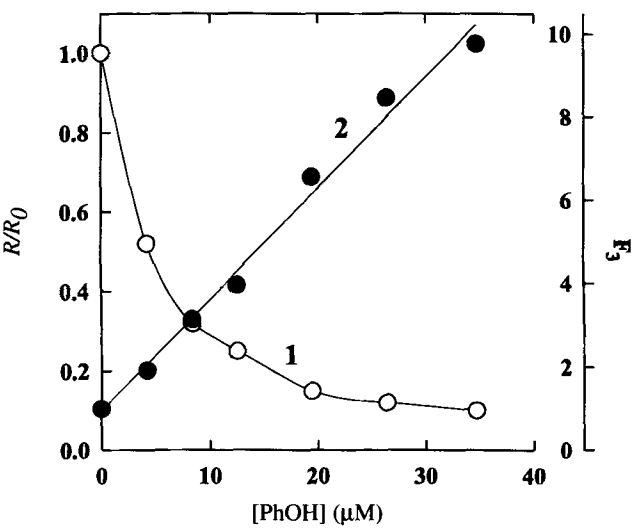
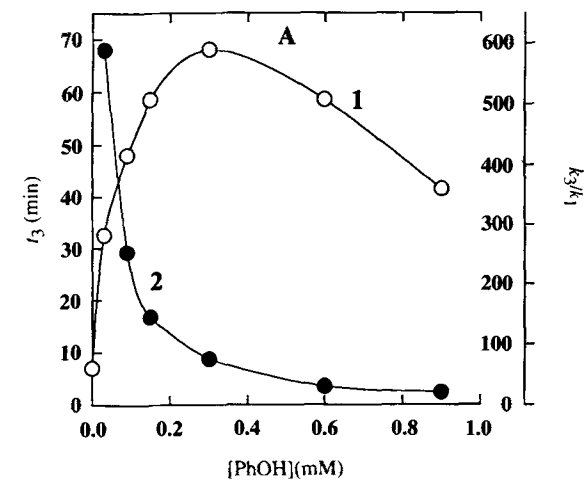


FIG. 4. Kinetics of oxygen consumption during the oxidation of 2.48 M methyl linoleate (ML) in chlorobenzene at 50°C (R_{in} = 1.60•10^{-8} Ms^{-1}) in the presence of 0.03 mM *I* in the plot of Equation 12. Original data were taken from Figure 3.



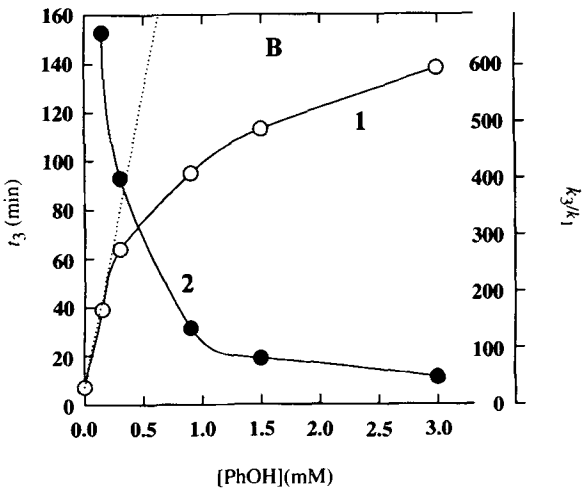


FIG. 5. Concentration dependence of t_3 (1) and k_3/k_1 (2) during the oxidation of 2.48 M methyl linoleate in chlorobenzene at 50°C (R_{in} = 1.60•10^{-8} Ms^{-1}) (A) and that of 0.60 M in chlorobenzene at 50°C (R_{in} = 1.7•10^{-7} Ms^{-1}) (B). The dotted line in Figure 5B shows the plot of t_3 computer-simulated on the basis of the classic kinetic scheme given in the text. Parameters used were R_{in} = 1.7•10^{-7} Ms^{-1}; k_1 = 120 M^{-1}s^{-1}; k_2 = 1.5•10^7 M^{-1}s^{-1}; k_3 = 1•10^5 M^{-1}s^{-1}; k_4 = 3•10^8 M^{-1}s^{-1}; k_5 = 1•10^8 M^{-1}s^{-1}.

determined directly from plots of [O_2] consumed vs. time (Fig. 3). At the lowest concentration of the phenol (0.03 mM), k_3/k_1 could be determined from the kinetics of oxygen uptake by using Equation 12 (Fig. 4). Because Equation 12 did not work at higher concentrations of PhOH, k_3/k_1* was determined from the starting value of *R* with the help of Equation 11.

Values of t_3 and k_3/k_1* determined in this way are given in Table 1. Figure 5A depicts the concentration dependence of both parameters for the oxidation of 2.48 M ML inhibited by *I*. One can see that the plot of t_3 shows a distinct maximum at [PhOH] = 0.3 mM. The k_3/k_1* value decreases hyperbolically with [PhOH]. Similar concentration dependencies were observed for *II, IV,* and *XI* (Table 1). For *III, V, VII,* and *X*, the value of t_3 approached a certain limit, whereas k_3/k_1* values

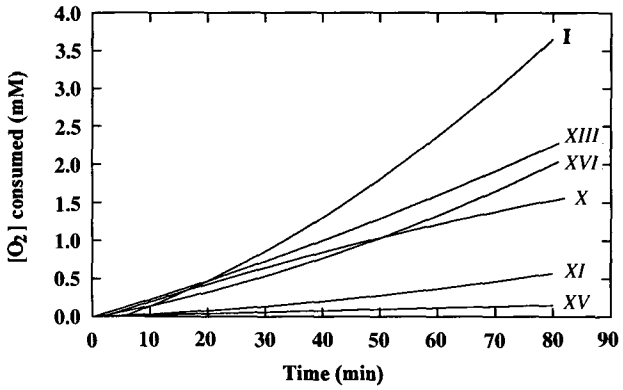


FIG. 6. Comparison of traces of oxygen consumption during the oxidation of 2.48 M methyl linoleate in chlorobenzene at 50°C (R_{in} = 1.6•10^{-8} Ms^{-1}) in the presence of 0.3 mM of various phenolic antioxidants (see Fig. 1 for the abbreviations used).

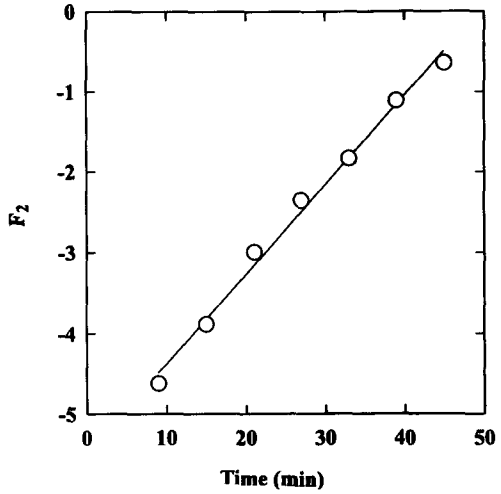


FIG. 7. The concentration dependence of the relative rate of chain oxidation of 0.02 M methyl linoleate in 0.2 M sodium dodecyl sulfate micellar solution in 0.05 M Na phosphate buffer, pH 7.40, at 37°C (R_{in} = 1.65•10^{-9} Ms^{-1}) in the presence of *V* - 1; 2 - the same as a plot of Equation 14.

dropped progressively with [PhOH]. The phenols *VI* and *XVI* did not display any inhibiting activity in line with the negative results during cumene oxidation (model 1). Figure 5B depicts the concentration dependencies of t_3 and k_3/k_1* when the ML concentration was reduced to 0.60 M. It is shown that t_3 continues to increase with [PhOH], even at a concentration of *I* of 3 mM, although this occurs more slowly than predicted by the theory of classic antioxidants. At the same time, k_3/k_1* drops progressively with [PhOH], as was observed during the oxidation of more concentrated ML solutions (Fig. 5A).

Finally, Figure 6 compares the kinetics of oxygen consumption accompanying the oxidation of ML in the presence of 0.3 mM of *I*, the best flavonoid antioxidant, with that of standard phenolic inhibitors at the same concentration. A concentration of 0.3 mM has been chosen because *I* shows the highest capability at that concentration. One can see that *I* ranks not only below *XIV*, one of the most powerful natural chain-breaking antioxidants (20), but even below the rather moderate synthetic antioxidant *XV*. The capabilities of *XI* and *XII*, nonflavonoid catechol derivatives, seem to be somewhat better.

Inhibiting activities of flavonoids during the oxidation of ML in SDS micelles (model 3). This model imitates peroxidation in heterogeneous lipid systems and under *in vivo* conditions. With this model, it is possible to determine an inhibiting activity of both lipid- and water-soluble chain-breaking antioxidants (30). In contrast to ML oxidation in chlorobenzene, where R is proportional to $\sqrt{R_{in}}$, reflecting the bimolecular chain termination (Reaction 2), in SDS micellar solutions, R is nearly proportional to R_{in} (30). From the point of view of formal kinetics, the latter means that chain termination occurs by a first-order reaction with respect to [$LO_2^{\bullet}$]:

$$LO_2^{\bullet} \rightarrow \text{products} \qquad [13]$$

For this reason, Equations 11 and 12, which were used to determine k_3/k_1 from the kinetics of oxygen uptake during inhibited oxidation, must be somewhat changed. In particular, Equation 11 should be changed to Equation 14:

$$F_3 = \frac{R_0}{R} - 1 = \frac{2\,k_3 R_0}{k_1 R_{in}[\text{LH}]}\,[\text{PhOH}] + const \qquad [14]$$

All tested flavonoid and catechol derivatives, excluding *VI* and *XVI*, displayed a pronounced ability to depress ML chain oxidation in the system under consideration. Figure 7 shows a presentation of this effect with *V*. It shows that, in accord with Equation 14, the plot of F_3 vs. [PhOH] is a straight line. The value of k_3/k_1, calculated from the slope of the line by using Equation 14, was found to be 103 ± 9. The behavior of other antioxidants, both lipid- and water-soluble, was qualitatively the same. Values of k_3/k_1 are listed in the last column of Table 1. Comparison of these data with those of antioxidants that have been tested previously with this model (30,35,36) shows that the chain-breaking antioxidative activities of the best flavonoids and related compounds (*I, V, IX, XII,* and *XII*) are comparable with those of *XIV* and *XV* (Table 1) and some other synthetic phenolic antioxidants (30). Meanwhile, all flavonoids and catechol derivatives studied in this work rank below some synthetic derivatives of vitamin E, Trolox, and HPMC (30).

DISCUSSION

Flavonoids as polyhydroxylated aromatic compounds are obvious candidates to act as phenolic antioxidants. A number of investigations indeed verified their high efficiencies as radical scavengers, with most rate constants determined in aqueous solutions approaching diffusion-controlled limits (7–9). In contrast, the majority of studies take these antioxidative capabilities for granted and merely extend the observations from one system to others. However, in view of the preva-

lence of flavonoids in food, a more quantitative correlation of their inhibition of lipid peroxidation with their function as chain-breaking antioxidants is clearly required. With peroxyl radicals as the principal chain-propagating species, the few rate constants determined thus far (9,15–17) illuminate only part of the picture. What is lacking are, first, hard data from systems that at least resemble biological environments and, second, information on subsequent reactions of the flavonoid radicals formed during the chain-breaking step.

The present study is an attempt to bridge this information gap by determining the ratio of chain-terminating to chain-propagating reactivities (k_3/k_1) and of individual stoichiometric factors (f) for a number of flavonoids, catechols, and some known phenolic antioxidants. The systems investigated, initiated autoxidation of cumene or of ML in chlorobenzene and initiated oxidation of ML in SDS micelles, were chosen as a gradual approach to *in vivo* conditions. The monitoring technique used is similar to the methods used by Niki (37), Burton and Ingold (20), and Barclay (38).

Pulse radiolytic studies of peroxyl radical reactions with flavonoids were limited (due mainly to their technical complexities) to linoleic acid peroxyl radicals at pH 11.5 (15) and to isopropyl peroxyl radicals at pH 8.5 (9) with the flavonols quercetin (*I*) and kaempferol (*VII*). Thus, only the previous investigation on the inhibited chemiluminescence in chlorobenzene, containing oxidizing diphenylmethane (17), and the present one include a sufficient number of flavonoids for the detection of potential structure-activity relationships (SAR).

What is, most surprising perhaps, in view of the previously determined high rate constants with various types of peroxyl radicals (9,15–17), is the fact that in this study only moderate antioxidative efficiencies were obtained. First, the behavior of all lipid-soluble flavonoid and catechol derivatives studied differed considerably from that predicted by the theory of a classical chain-breaking antioxidant. Recall that for a classic compound, a value of t_3, which is more or less equivalent to the induction period, is expected to be correlated directly with [PhOH], and k_3/k_1 is kept constant at any [PhOH]. The comparison of t_3 values, determined experimentally for flavonoids and related compounds, with that calculated for a classic antioxidant with moderate reactivity toward $LO_2^{\cdot}$ (last line in Table 1) shows that, starting at 0.09 mM PhOH, flavonoids and catechol derivatives possess much less antioxidative capability than predicted on the base of the theory of a classic antioxidant. This is despite the fact that compounds *I–V, X,* and *XI* display the highest reactivity toward $LO_2^{\cdot}$ among phenolic antioxidants (7,16,17).

The following possible reasons for nonclassic behavior and relatively low antioxidative capability of flavonoid and catechol derivatives as inhibitors of lipid peroxidation could be considered: (i) the decrease of the effective values of k_3 for the reaction of $LO_2^{\cdot}$ with phenols in ML medium as compared with that of hydrocarbons due to formation of H-bonded complexes of phenols with ML and other fatty acid derivatives (29); (ii) monomolecular transformation of $PhO^{\cdot}$ formed from the phenol compounds under study, resulting in the formation of a reactive free radical capable of propagating chain reactions (17); (iii) the regeneration of the principal chain-carrying radical $LO_2^{\cdot}$ by the reaction of $PhO^{\cdot}$ with LH:

$$PhO^{\cdot} + LH + (O_2) \rightarrow PhOH + LO_2^{\cdot} \quad [15]$$

Other pertinent properties, e.g., lipophilicity or univalent redox potentials, may be crucial.

Considering each argument individually, (i) the decrease in the reactivity of phenol compounds that have no bulky *ortho*-substituents toward $LO_2^{\cdot}$ if one goes from the oxidation of hydrocarbons to fatty esters, has been reported (29). It has been shown that the most likely reason for the effect is the supposition that such phenols form H-bonded complexes with lipid carboxyl group in which the phenol has little if any reactivity toward $LO_2^{\cdot}$. A maximally tenfold reduction of k_3 was reported for phenols without *ortho*-substituents (for instance, 4-methoxyphenol). It is unlikely that this effect could exceed that of common phenols in the case of flavonoid and catechol derivatives. At the same time, the decrease of k_3 as one moves from the oxidation of hydrocarbons (diphenylmethane, Ref. 17) to that of ML is at least two orders of magnitude, estimated in view of the mechanism under consideration from a value of k_3/k_1^* (see Table 1) and corresponding to $k_1 = 120$ $M^{-1}s^{-1}$ (29). Furthermore, the effect increases with [PhOH], which is not predicted by this mechanism. (ii) A monomolecular transformation (with unknown mechanism) of the aroxyl radicals of flavonoid and catechol derivatives, resulting in the formation of a more reactive radical

$$PhO^{\cdot} + (O_2) \rightarrow rO_2^{\cdot} + \text{products} \quad [16]$$

that is capable of propagating the chain reaction leading to chemiluminescence, was proposed after studies in our laboratory (17). In principle, Reaction 16 could explain the unexpectedly low antioxidative capability of flavonoid and catechol derivatives. To exemplify this effect, we carried out a computer simulation on the basis of the kinetic scheme that included Reactions 0–5, Reaction 6, and the following reactions with participation of $rO_2^{\cdot}$:

$$rO_2^{\cdot} + LH + (O_2) \rightarrow rOOH + LO_2^{\cdot} \quad [17]$$

$$rO_2^{\cdot} + PhOH \rightarrow rOOH + PhO^{\cdot} \quad [18]$$

$$rO_2^{\cdot} + PhO^{\cdot} \rightarrow rOOH + Q \quad [19]$$

$$Q + PhOH \rightarrow 2\,PhO^{\cdot} \quad [20]$$

The latter reaction, the reversal of Reaction 5, was found to be required to simulate an extreme [PhOH] dependence of t_3. The result of the simulation is given in Figure 8. The comparison of this plot with plot 1 of Figure 5 shows that, despite

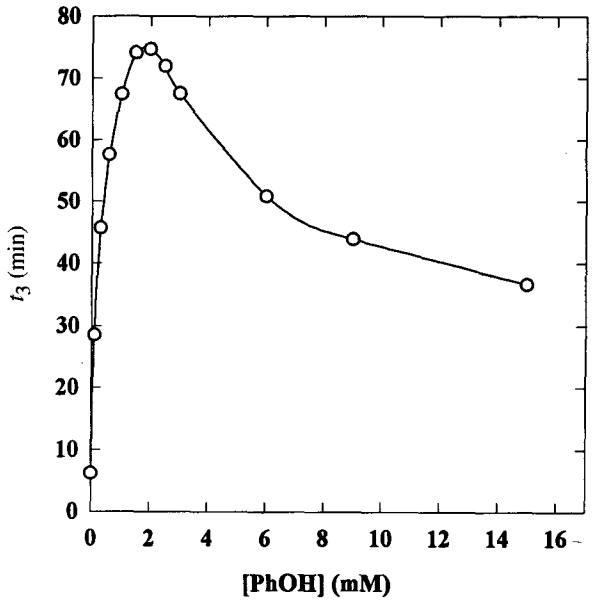


FIG. 8. Computer simulation of the concentration plot of t_3 during the oxidation of 2.48 M methyl linoleate. The kinetic scheme used for the simulation is given in the text. The kinetic parameters used were: R_{in} = 1.6 •10^{-9} Ms^{-1}; $k_1 = k_{1'}$ = 120 $M^{-1}s^{-1}$: k_2 = 1.5•10^7 $M^{-1}s^{-1}$; $k_3 = k_{18'}$ = 1•10^6 $M^{-1}s^{-1}$; $k_4 = k_{19'}$ = 5•10^8 $M^{-1}s^{-1}$; k_5 = 3•10^8 $M^{-1}s^{-1}$; k_{16} =50 $M^{-1}s^{-1}$; k_{20} = 10 s^{-1}.

the qualitative similarity of experimental and simulated plots, the positions of the maxima of t_3 differ from each other by one order of magnitude (in fact, the maximum of t_3 is barely sensitive to the values of the parameters used for the simulations). (iii) Reaction 15 has been recognized for quite some time as one of the main reasons for the diminishing of antioxidative capabilities of phenol antioxidants (19). In particular, this was reported as a reason for the relatively low effectiveness of α-tocopherol as an antioxidant during lipid peroxidation (39,40). In all data collected in the current work, primarily the effect of [ML] on the k_3/k_1^* parameter (Fig. 5) is in qualitative agreement with this mechanism. (iv) The fact that lipophilicity may control the antioxidative behavior, especially in the systems studied, is already apparent in Table 1, where the most hydrophilic substances, the glycoside rutin (*VIII*) and the flavylium salt (anthocyanidin) cyanidine chloride (*IX*), are not even soluble in chlorobenzene. No further conclusions can be drawn at this moment, because the partition coefficients of most flavonoids are not known. Another property of flavonoids, potentially affecting the SAR of antioxidative efficiency, is the respective redox potential. Adding to the few data on univalent redox potentials of flavonoids (13,41), we were able to expand this list recently by applying a novel kinetic method for their determination (42).

In conclusion, at the present time it is difficult to suggest either mechanism as the only reason for moderate antioxidative capability of flavonoid and catechol derivatives. Most likely, all arguments presented are valid, but their real contributions still have to be determined.

Concerning SAR, comparison of the reactivities (k_3/k_1 in SDS micelles, last column in Table 1) among the flavonoids (*IX* > *V* ≥ *I* > *IV* ≥ *VII* > *III* ≥ *VII* > *II* > *VI*) and with other catechols clearly marks the B-ring catechol moiety as the most important controlling feature. Following, or equally important, seems to be the ease by which the odd electron of the flavonoid radical can be delocalized over all three ring systems (7). This entails either the presence of a 2,3-double bond or the oxonium structure of *IX*. The 3-OH group seems also to have a more favorable effect than the 5-OH group. Apart from that, compounds *II*, *IV*, and *IX* obviously deviate from these predictions (*II* gives too low, *IV* and *IX* too high values), and thus, other functions may play a role as well.

Corroboration of the importance of the dominant structural criteria for antioxidative efficiency also comes from the stoichiometric factor (column 2 in Table 1)—the highest values were obtained for *I*, *III*, and *V*, all having a B-ring catechol, a 2,3-double bond and 3-OH and/or 5-OH groups. While the immense structural variations of the flavonoids (43,44) lend themselves naturally to the study of SAR, the major result of our study must again be emphasized. Aside from the fact that the flavonoids studied do not show the behavior of classic phenolic antioxidants, the fact that their inhibiting efficiencies are much lower than that of the standard antioxidants *XIV* and even *XV* (Table 1) is of considerable concern. This is especially significant from the point of view of potential application of flavonoids as antioxidants of practical importance for the stabilization of lipid products with a high content of polyunsaturated fatty acids.

APPENDIX

Determination of k_3 *from the kinetics of* O_2 *uptake during the oxidation of ML in homogeneous solution inhibited by PhOH (the deduction of Equations 11 and 12).* The kinetic scheme includes Reactions 0–4. The following system of differential equations written for the approximation of long kinetic chains ($R >> R_{in}$) corresponds to this scheme:

$$d[LO_2^{\cdot}]/dt = R_{in} - k_2[LO_2^{\cdot}]^2 - k_3[LO_2^{\cdot}][PhOH] - k_4[LO_2^{\cdot}][PhO^{\cdot}] \quad [21]$$

$$d[PhO^{\cdot}]/dt = k_3[LO_2^{\cdot}][PhOH] - k_4[LO_2^{\cdot}][PhO^{\cdot}] \quad [22]$$

$$-d[PhOH]/dt = k_3[LO_2^{\cdot}][PhOH] \quad [23]$$

The rate of oxidation in the absence of phenols equals

$$R_0 = \frac{k_1[LH]\sqrt{R_{in}}}{\sqrt{k_2}} \quad [24]$$

or, in terms of a current rate of both inhibited and noninhibited oxidation:

$$R = -d[O_2]/dt = k_1[LO_2^{\bullet}][LH] \quad [25]$$

The division of Equation 25 by Equation 24 results in a relative rate of oxidation:

$$R/R_0 = \frac{\sqrt{k_2}}{\sqrt{R_{in}}}[LO_2\bullet] \quad [26]$$

Hence, a certain concentration of $LO_2^{\bullet}$

$$[LO_2^{\bullet}] = \frac{R\sqrt{R_{in}}}{R_0\sqrt{k_2}} \quad [27]$$

The solution is given assuming a quasi-stationary state for all free radicals. Combining Equations 21 and 22 yields

$$d[LO_2^{\bullet}]/dt = R_{in} - k_2[LO_2^{\bullet}]^2 - 2\,k_3[LO_2^{\bullet}][PhOH] = 0 \quad [28]$$

Substituting Equation 27 into Equation 28 yields:

$$R_{in} - \frac{R^2}{R_0^2}R_{in} - \frac{2\,k_3R\sqrt{R_{in}}}{R_0\sqrt{k_2}}[PhOH] = 0 \quad [29]$$

After rearrangements of this relation with regard to Equation 24, Equation 11 may be obtained. The following protocol has to be applied to convert Equation 11 into the differential Equation 12—first, the differentiation of Equation 11 with respect to time, then the substitution of the relation obtained into Equation 23 with regard to Equations 25 and 26, followed by the integration of the final relation:

$$\int \frac{2\left(R/R_0\right)^2+1}{\left(1-\left(R/R_0\right)^2\right)(R/R_0)^2}dR = \int \frac{k_3R_0}{k_1[LH]}dt \quad [30]$$

Determination of k_3 *from the kinetics of* O_2 *uptake during the oxidation of LH in micellar solution inhibited by PhOH (the deduction of Equation 14).* The kinetic scheme under consideration includes Reactions 0, 1, 3, 4, and 17. The following system of differential equations corresponds to this scheme:

$$d[LO_2^{\bullet}]/dt = R_{in} - k_{na}[LO_2^{\bullet}] - k_3[LO_2^{\bullet}][PhOH] - k_4[LO_2^{\bullet}][PhO^{\bullet}] \quad [31]$$

$$d[PhO^{\bullet}]/dt = k_3[LO_2^{\bullet}][PhOH] - k_4[LO_2^{\bullet}][PhO^{\bullet}] \quad [32]$$

$$-d[PhOH]/dt = k_3[LO_2^{\bullet}][PhOH] \quad [33]$$

The rate of noninhibited oxidation equals

$$R_0 = \frac{k_1[LH]R_{in}}{k_{17}} \quad [34]$$

The rate of inhibited oxidation and the relative rate of oxidation are

$$R = k_1[LO_2^{\bullet}][LH] \quad [35]$$

$$\frac{R}{R_0} = \frac{k_{2a}}{R_{in}}[LO_2^{\bullet}] \quad [36]$$

The solution is again given for the quasi-stationary approximation for all free radicals. Combining Equations 31 and 32 results in:

$$R_{in} - k_{na}[LO_2] - 2\,k_3[LO_2^{\bullet}][PhOH] = 0 \quad [37]$$

Combinating Equation 37 with Equation 34 and Equation 36 yields

$$\frac{1}{R} - \frac{1}{R_0} = \frac{2\,k_3}{k_1[LH]R_{in}}[PhOH] \quad [38]$$

Simple rearrangement of Equation 38 results in Equation 14.

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