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On the Antioxidant Mechanism of Curcumin: Classical Methods Are Needed To Determine Antioxidant Mechanism and Activity

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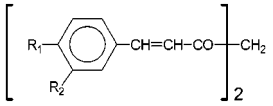
ABSTRACT



R_1 = OH, R_2 = OCH_3 : Antioxidant
R_1, R_2 = OCH_3 : No Activity

The antioxidant activity of curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was determined by inhibition of controlled initiation of styrene oxidation. Synthetic nonphenolic curcuminoids exhibited *no* antioxidant activity; therefore, curcumin is a classical *phenolic* chain-breaking antioxidant, donating H atoms from the phenolic groups *not* the CH_2 group as has been suggested (Jovanovic et al. *J. Am. Chem. Soc.* 1999, *121*, 9677). The antioxidant activities of *o*-methoxyphenols are decreased in hydrogen bond accepting media.

The antioxidant activities of *o*-methoxyphenols attract remarkable current interest because this structure is important in biological systems, such as the ubiquinols,[1a] and such phenols are the subject of recent experimental and theoretical studies.[1] Natural *o*-methoxyphenols of the curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dion, **1a**) class now receive a lot of attention due to evidence that they not only are antioxidants[2a−e] but also act to inhibit oxidative stress in red blood cell membranes,[3a,b] induce detoxification enzymes,[4] and appear to provide protection against degenerative diseases.[5a,b] During our investigation of the antioxidant activities of various *o*-methoxyphenols employing classical inhibition of oxygen uptake (IOU) methods, an interesting report appeared on a new antioxidant mechanism for curcumin and related compounds.[2a] Reaction of curcumin with

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reactive radicals, such as *tert*-butoxyl or carbon-centered radicals generated by pulse radiolysis or laser flash photolysis, produced "curcumin transients", which were interpreted as originating by H atom abstraction from the central active -CH_2- group rather than the phenolic group, and it was concluded that it was H atom donation from this CH_2 group which was responsible for the "superb antioxidant" properties of curcumin.[2a] If true, this conclusion has important implications for the mechanism of antioxidants of the curcuminoid class and possibly others bearing active -CH_2- groups. Therefore, we decided to test this by syntheses and determinations of antioxidant activity and stoichiometric factors for compounds that have the basic curcumin skeleton but do not possess phenolic groups, such as compounds **1b**, **1c**, and **1d**.[6] In addition, we report on these properties for other *o*-methoxyphenols (Figure 1, **2a**−**c**) for comparison purposes.

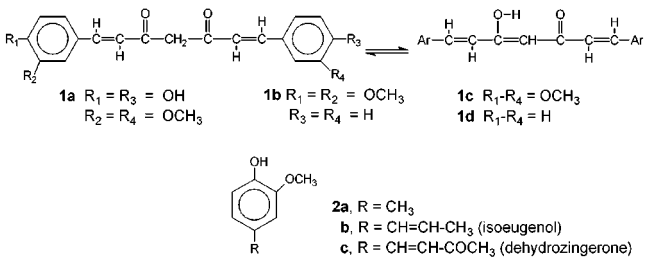

Figure 1. Structures of antioxidants.

Our method employed peroxyl radicals, ROO•, because these radicals, formed by the very rapid reaction of oxygen with initial carbon-centered radicals,[7] are the main (or only) chain-carrying radicals in lipid peroxidation.[8] Chain-breaking phenolic antioxidants trap peroxyl radicals by H atom transfer from the phenolic group, according to considerable experimental evidence[9a,b] and a number of reviews.[9c−e] In our experiments peroxyl radicals were generated under conditions which controlled the rate of free radical initiation, R_i, by employing an azo initiator, azo-bis-isobutyrylnitrile (AIBN). We measured antioxidant activities using the inhibition of styrene oxidation in chlorobenzene, conditions whereby a wide variety of phenolic antioxidant activities have been determined.[9a] In styrene, the absolute rate constants for antioxidant activity, k_{inh}[10] (from eq 1), and the stoichiometric

$$R{-}O{-}O^{\bullet} + ArOH \xrightarrow{k_{inh}} R{-}O{-}OH + Ar{-}O^{\bullet} \qquad (1)$$

factors, n (the number of peroxyl radicals trapped per mole of antioxidant), are as follows: **1a**, $k_{inh} = 34 \times 10^4$ M^{-1} s^{-1}, $n = 4$; **2a**, $k_{inh} = 14 \times 10^4$ M^{-1} s^{-1}, $n = 2$; **2b**, $k_{inh} = 14 \times 10^4$ M^{-1} s^{-1}, $n = 2$; and **2c**, $k_{inh} = 17 \times 10^4$ M^{-1} s^{-1}, $n = 2$. Sample inhibition profiles in styrene are illustrated in Figure 2.

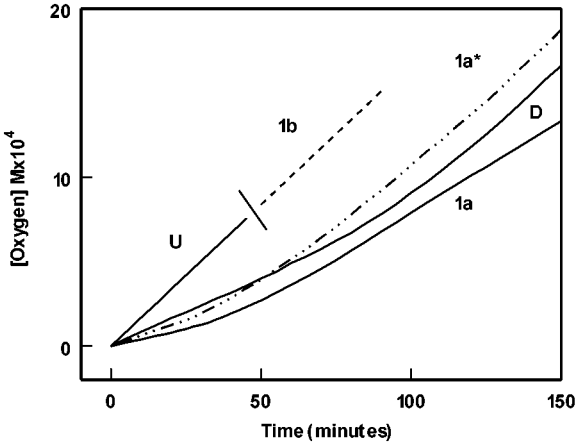

Figure 2. Profiles of inhibition during AIBN (0.02 M) initiated oxidation of 1.1 M styrene in chlorobenzene; solution studies at 30 °C under oxygen. U = uninhibited rate of oxidation; **1a** = 3.2 μM curcumin, $\tau = 51$ min, $R_i = 3.0 \times 10^{-9}$ M s^{-1}, $n = 4$, $k_{inh} = 35 \times 10^4$ M^{-1} s^{-1}, rate eventually returns to uninhibited rate (not shown); **1a*** = 4.2 μM curcumin, 0.73 M methyl stearate present, $\tau = 87$ min, $R_i = 2.8 \times 10^{-9}$ M s^{-1}, $n = 4$, $k_{inh} = 10 \times 10^4$ M^{-1} s^{-1}; **1b** = 7.3 μM compound **1b**; D = 7.1 μM DBHA, $\tau = 60$ min, $R_i = 2.0 \times 10^{-9}$ M s^{-1}, $n = 2.0$, $k_{inh} = 11 \times 10^4$ M^{-1} s^{-1}.

Methyl linoleate was also used as a substrate in experiments for inhibition of lipid peroxidation (Figure 3). Studies on the behavior of the nonphenolic curcuminoids **1b**, **1c**, and **1d** in our two systems, styrene or methyl linoleate, revealed that these compounds do *not* suppress oxygen uptake. Results for **1b** are illustrated in Figures 2 and 3. These synthetic analogues do *not* possess any antioxidant activity,[11] contrary to what would be expected if the -CH_2- is the antioxidant site.[2a]

We conclude that curcumin is a *phenolic* chain-breaking antioxidant. This is contrary to the conclusion, based on

(5) For reviews see:(a) Conney, A. H.; Lou, Y.-R.; Xie, J.-G.; Osawa, T.; Newmark, H. L.; Liu, L.; Chanj, R. L.; Huang, M.-T. *Proc. Soc. Exp. Biol. Med*. **1997**, *216*, 234−245. (b) Nakatani, N. In *Natural Antioxidants, Chemistry, Health Effects, and Applications*; Shahidi, F., Ed.; AOCS Press: Champaign, IL, 1997; Chapter 4, pp 64−75.

(6) Compounds **1b**, **1c**, and **1d** were synthesized by modification of the reported method of Pabon, H. *J. Recuel* **1964**, *83*, 379−386. These compounds gave the expected ^1H and ^{13}C NMR spectra, and these spectra as well as the infrared spectra indicated that they exist entirely in the enol form in solution.

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(8) Kinetic models (Antunes, F.; Salvador, A.; Marinho, H. S.; Alves, R.; Pinto, R. E. *Free Radical Biol. Med.* **1996**, *21*, 917−943) and some evidence (Dix, A. T.; Aikens. J. *Chem. Res. Toxicol.* **1993**, *6*, 2−18) indicate that peroxyl radicals are actually more efficient at initiating (and propagating) lipid peroxidation than the more reactive hydroxyl radical.

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(10) The k_{inh} values were calculated from linear plots of the integrated rate expression during suppressed oxygen uptake, $\Delta[O_2]_t = -k_p/k_{inh}[RH] \ln(1 - t/\tau)$, where τ is the inhibition period (seconds), using known values for the k_p of styrene, 41 M^{-1} s^{-1},[9a] and of methyl linoleate, 62 M^{-1} s^{-1} (Howard, J. A. *Adv. Free Radical Chem.* **1972**, *4*, 49). Details of these experiments are available in the Supporting Information.

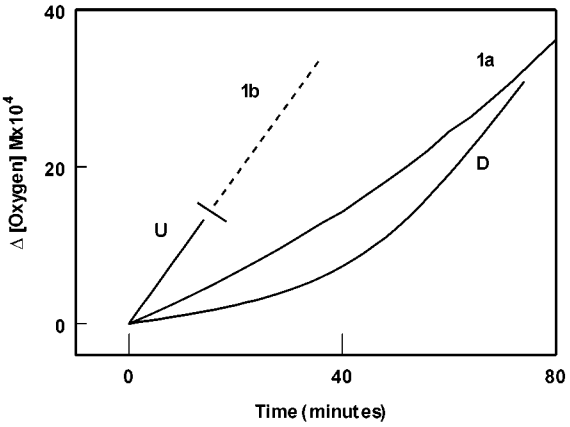
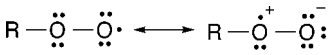


Figure 3. Profiles of inhibition during AIBN (0.04 M) initiated oxidation of 0.74 M methyl linoleate in chlorobenzene; solution studies at 30 °C under oxygen. U = uninhibited rate of oxidation; **1a** = 5.7 μM curcumin, τ = 64 min, $R_i = 5.6 \times 10^{-9}$ M s^{-1}, n = 4, $k_{inh} = 3.9 \times 10^4$ M^{-1} s^{-1}, rate eventually returns to uninhibited rate (not shown); **1b** = 19.3 μM compound **1b**; D = 5.9 μM DBHA, τ = 46 min, $R_i = 4.0 \times 10^{-9}$ M s^{-1}, n = 2.0, $k_{inh} = 11 \times 10^4$ M^{-1} s^{-1}.

results from laser flash photolysis or pulse radiolysis, that "the apparent site of reaction is the central CH_2 group of the heptadiene link".[2a] We offer two possible explanations for these different conclusions. First, as stated above, peroxyl radicals are the main chain-carrying radicals in autoxidation. They are selective in their reaction due to stabilization by electron delocalization:



Many organic compounds react readily with much more reactive radicals, such as hydroxyl or alkoxyl radicals, but, as pointed out before,[12] this does *not* mean that they are acting as classical chain-breaking antioxidants. Second, the main new experimental evidence for H atom abstraction from the -CH_2- group of curcumin by these reactive radicals appears to be the resulting absorption spectra, giving a characteristic band at around 500 nm.[2a] It is doubtful that this band can be specifically attributed to the radical derived by -C−H abstraction; indeed others have assigned such bands to phenoxyl radicals derived from curcumin[2b,c] or from dehydrozingerone.[13] The "danger" of drawing mechanistic conclusions based on the spectra of transients has been emphasized before.[14]

In styrene/chlorobenzene, compounds **2a**−**c** all inhibit styrene oxidation and have stoichiometric factors of approximately 2. Curcumin, **1a** (Figure 2), is considerably more active (higher k_{inh}) and the n actor doubles, which is expected since the two phenolic groups are isolated. Furthermore, the k_{inh} value for curcumin, **1a**, is double that of dehydrozingerone, **2c**, and isoeugenol, **2b**, consistent with curcumin donating H atoms from the two phenolic groups, not from the enol. Using methyl linoleate/chlorobenzene, the antioxidant activities of **2a**−**c** and curcumin (Figure 3) dropped to a range of 3 to 5 $\times$ 10^4 M^{-1} s^{-1}. This effect is attributed to the hydrogen bond acceptor group of the ester, which is known to reduce phenolic H atom transfer through H-bonding.[15] This effect was also observed, as expected, by the addition of a saturated ester (methyl stearate) to the styrene system, which caused the k_{inh} of curcumin to drop (see Figure 2, traces **1a** and **1a***). The k_{inh} of 2,6-di-*tert*-butyl-4-methoxyphenol (DBHA) was the same in both media, 11.1 $\times$ 10^4 M^{-1} s^{-1}. DBHA is not as susceptible to this solvent effect, probably because of steric effects provided by the two *ortho tert*-butyls.[16] The antioxidant efficiency of vitamin E, which has two *ortho* methyl groups, decreased by only 20% (not shown) when methyl stearate (0.74 M) was added to styrene. These results show that the reaction medium is a *very* important factor in determinations of antioxidant activities of phenols and that a relatively nonpolar medium such as styrene/chlorobenzene is a preferred one for the measurement of such antioxidant activities.

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Supporting Information Available: A table of experimental data giving the quantities of substrates, antioxidants, and initiator (AIBN) and methods of calculation. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Preliminary experiments to determine the H atom donating ability of these compounds using stopped flow methods and substituted phenoxyl radicals for H atom abstractions (Nagaoka, S.; Kuranaka, A.; Tsuboi. H.; Nagashima, U.; Mukai, K. *J. Phys. Chem.* **1992**, *96*, 2754−2761) showed that compound **1b** was not a H atom donor within the time frame of the slow loss of reagent, which places an upper limit of $<10^{-2}$ M^{-1} s^{-1} on the rate constant.

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(16) Steric effects of *o-tert*-butyl groups on antioxidant activities of phenols are well-known: Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1964**, *42*, 1044−1056. Barclay, L. R. C.; Edwards, C. E.; Vinqvist, M. R. *J. Am. Chem. Soc.* **1999**, *121*, 6226−6231.