Full Research Paper

Radical-scavenging Activity of Estrogen and Estrogen-like Compounds Using the Induction Period Method

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Abstract: The radical-scavenging activity of estrogens (estrone, 2-hydroxyestradiol), estrogen-like compounds (diethylstilbestrol, DES; bisphenol A, BPA) and the mono-phenolic compound 2,6-di-*t*-butyl-4-methoxyphenol (BMP) was investigated using the method of measuring the induction period for polymerization of methyl methacrylate (MMA) initiated by thermal decomposition of 2,2'-azobisisobutyronitrile (AIBN) and benzoyl peroxide (BPO) at 70°C using differential scanning calorimetry (DSC). The stoichiometric factor (*n*, number of free radicals trapped by one mole of antioxidant moiety) for the AIBN system declined in the order BMP (2.0), 2-hydroxyestradiol (2.0)> DES (1.3) > BPA (1.2) > estrone (0.9), whereas that for the BPO system declined in the order BMP (2.0) >DES (1.9), BPA (1.9) > estrone (1.3) > 2-hydroxyestradiol (0.7). The inhibition rate constant (k_{inh} x 10^{-3} $M^{-1}s^{-1}$) for the AIBN system declined in the order estrone (2.2) > BPA (2.0) > DES (1.9) > 2-hydroxyestradiol (1.2) > BMP (1.1), whereas that for the BPO system declined in the order 2-hydroxyestradiol (3.2) > estrone (1.4) > DES (1.2) > BPA (1.0) > BMP (0.9). The radical-scavenging activity for bioactive compounds such as estrogens should be evaluated using these two methods (the *n* and k_{inh}) to elucidate the mechanism of a particular reaction. The great difference of the *n* and k_{inh} for estrogens between the AIBN and BPO system suggested that their oxidation process is complex.

Introduction

Estrogens such as estrones and 2-hydroxyestradiols possess a phenolic hydroxy group and have a variety of beneficial effects *in vivo*, including protection against osteoporosis, coronary heart disease, Alzheimer's disease and stroke [1], and hormone replacement therapy appears to have many unexpected beneficial effects in the treatment of these diseases [2]. In contrast quinoids, quinoid radicals and phenoxy radicals formed from estrogens may be responsible for adverse effects such as carcinogenesis [3]. The biological effects of estrogens involve their dual role as prooxidants and antioxidants. Studies using models for active oxygen radicals ($ROO^{\bullet}$, $RO^{\bullet}$ and $HO^{\bullet}$) in biological systems have reported that estrogens are effective antioxidants [4,5]. Previously quantitative *in vitro* studies of the radical-scavenging activity of phenolic compounds [6,7] were performed by the induction period method under aerobic conditions. The oxygen tension under a 15 torr oxygen atomsphere is similar to that in many tissues [8, 9], suggesting that oxygen is scarce in living cells and that the radical-scavenging activity of estrogens *in vivo* may differ considerably from that observed under aerobic conditions.

We have previously reported the use of differential scanning calorimetry (DSC) to evaluate the radical-scavenging activity of estrones and antiestrogenes (tamoxifen, toremifene) [10] by the induction period method [10]. In the present study, we used this previously reported method induction with DSC to investigate the radical scavenging activity of estrogens (estrone, 2-hydroxyestradiol) and estrogen-like compounds (diethylstilbestrol, DES; bisphenol A, BPA) by determining the corresponding stoichiometric factors (*n*) and the inhibition rate constants of polymerization (k_{inh}).

Results and Discussion

The chemical structures of estrone [3-hydroxyesta-1,3,5 (10)-tren-17-one] and 2-hydroxyestradiol [estra-1,3,5 (10)-triene-2,3,17β-triol], respectively, are shown in Figure 1.

Figure 1. Chemical structures of estrone and 2-hydroxyestradiol.

Estrone   **2-Hydroxyestradiol**
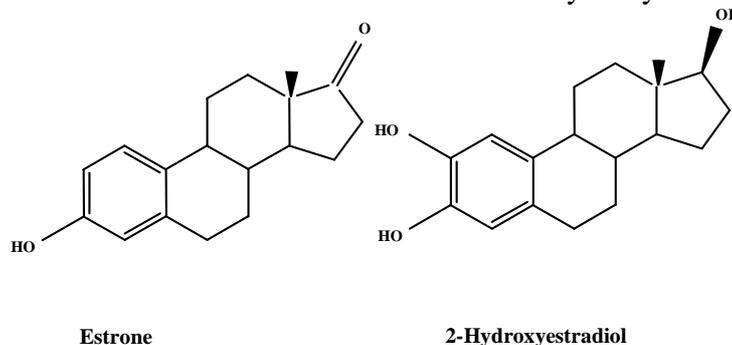
Stoichiometric factors (n).

Typical time-exotherm and time-conversion curves for estrogens and estrogen-like compounds are shown in Figures 2 and 3, respectively.

Figure 2. Time-exothermic curves for the polymerization of MMA with 1.0 mol% AIBN in the presence of 0.1 mol% additives. MMA, 9.4 mol/L; AIBN, 1.0 mol%; at 70 °C. A, control; DES, diethylstilbestrol; BPA, bisphenol A; HED, 2-hydroxyestradiol; EST, estrone.
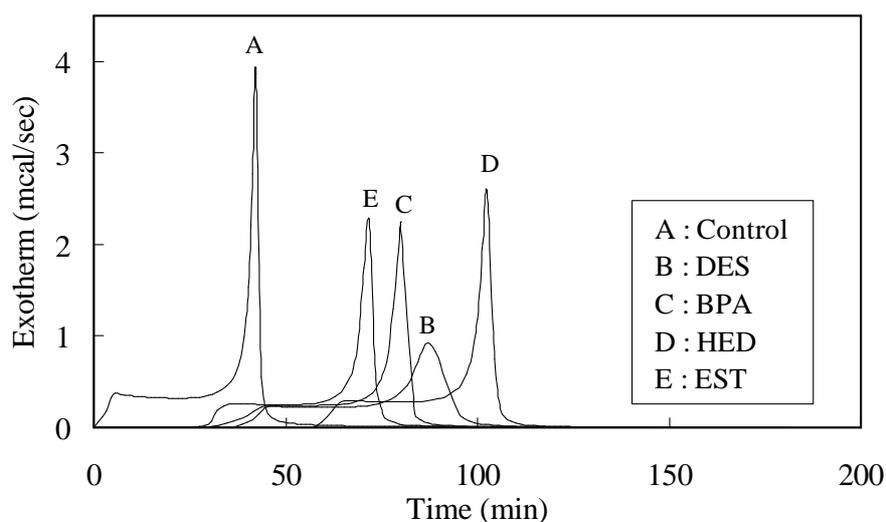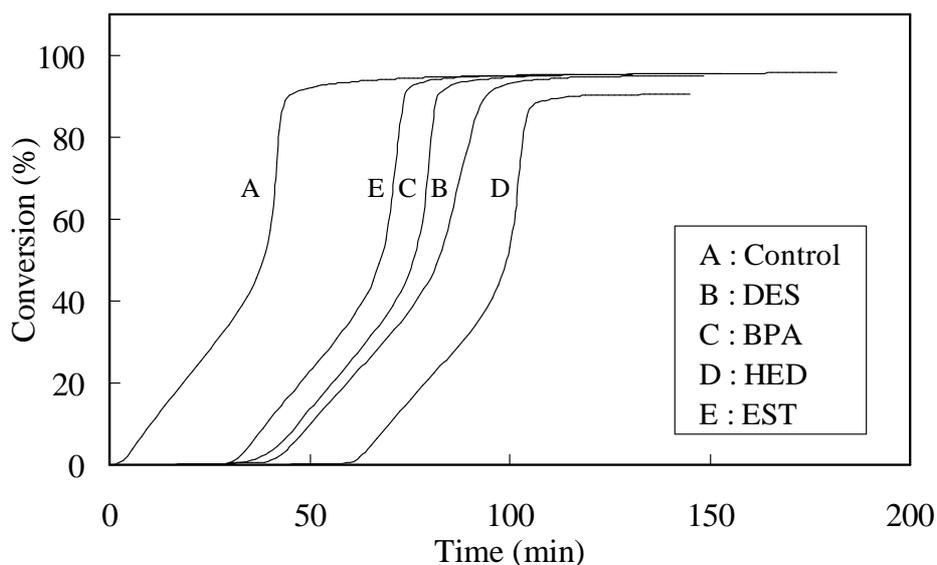

Figure 3. Time-conversion curves for the polymerization of MMA with 1.0 mol% AIBN in the presence of 0.1 mol% additives.

Curves were calculated from the findings shown in Figure 2. Abbreviations used are shown in Figure 2. Polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen as it had been sealed in air.

Tangents were drawn to polymerization curves at an early stage in the run. The induction period (IP) of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of the tangents drawn to the curves at the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens and that of controls. The *n* values can be calculated from Equation 1. We also examined the relationships between induction period [IP] and $[IH]/R_i$ for estrogens (Figure 4). The IP was linearly related to the $[IH]/R_i$. The *n* value was determined from each slope.

Figure 4. Plot of the induction period *vs* $[IH]/R_i$ for estrone and 2-hydroxyestradiol for the AIBN and BPO system. [IH], concentration of estrogen inhibitor; R_i, initiation rate of initiators. The slope for the induction period *vs* $[IH]/R_i$ corresponds to *n* value. Data are expressed as the mean of three independent experiments.
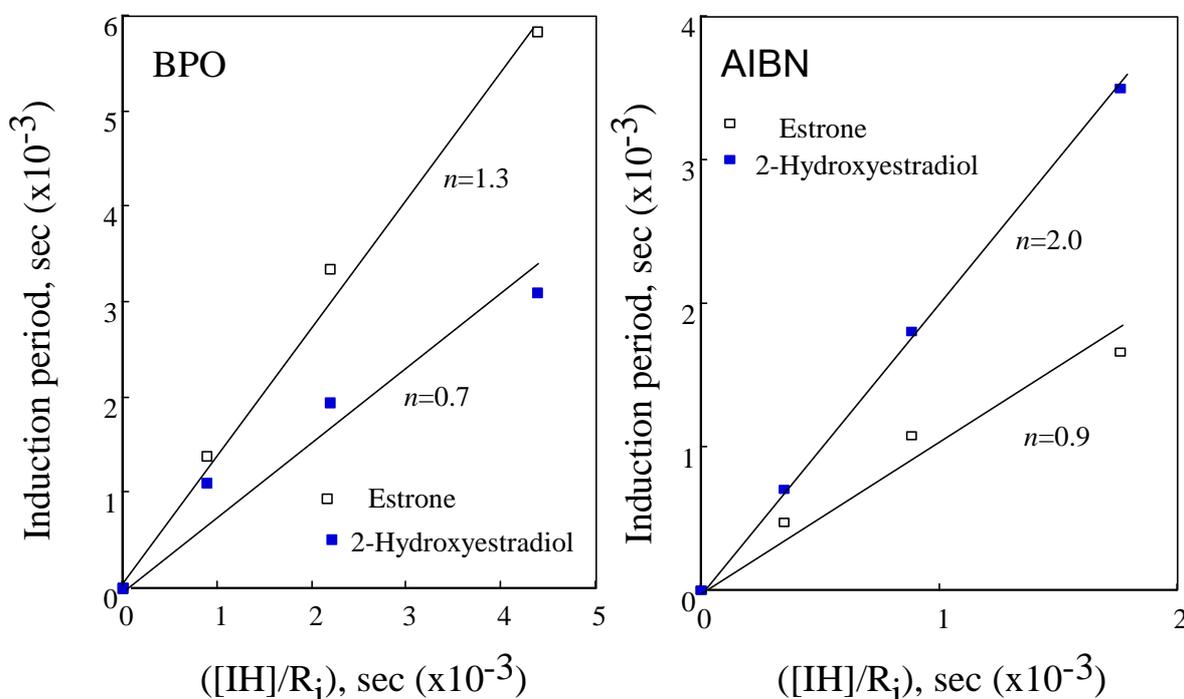
The initial rates (%/sec) of polymerization in the absence (Rp_{con}) and presence (Rp_{inh}) of estrogens were calculated from the slope of the plots of the first linear line of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage, Figure 3). The relationships between Rp_{inh}/Rp_{con} and concentration for estrogens are shown in Figure 5. As concentrations increased, Rp_{inh}/Rp_{con} values linearly decreased. For the AIBN system, but not for the BPO one, 2-hydroxy-estradiol showed the greatest suppression of MMA radical growth, whereas estrone showed the greater suppression for the BPO system.

Figure 5. Plot of Rp_{inh}/Rp_{con} *vs* concentration for estrone and 2-hydroxyestradiol for the AIBN and BPO system. Rp_{inh} and Rp_{con} are initial rate of polymerization in the absence and presence of an inhibitor. MMA, 9.4 mol/L. AIBN or BPO, 0.1 mol/L. Data are expressed as the mean of three independent experiments.
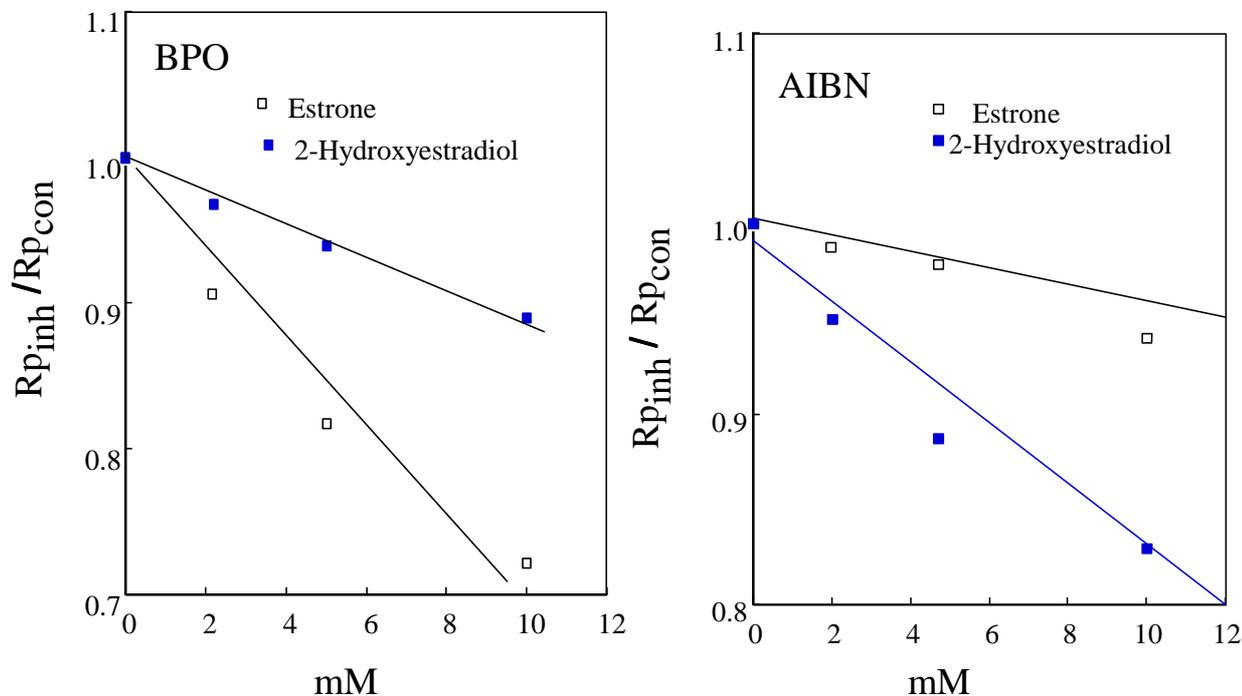

Table 1. Stoichiometric factors (n) and inhibition rate constants (k_{inh}) for estrogens and estrogen-like compounds in the AIBN and BPO system

	AIBN system		BPO system	
	n	k_{inh} x 10^{-3}	n	k_{inh} x 10^{-3}
Estrone	0.93	2.18	1.30	1.35
2-Hydroxyestradiol	1.99	1.23	0.68	3.22
Diethylstilbestrol (DES)	1.30	1.86	1.91	1.19
Bisphenol A (BPA)	1.20	2.01	1.92	1.03
2,6-di-*t*-butyl-4-methoxyphenol (BMP)	2.00	1.14	2.00	0.86

The measurement for antioxidant activities is described in the text. Values are the mean of three independent experiments. Standard error <10%. The determination of n and k_{inh} is described in the text. The k_t is approximately 3.7 x $10^7 M^{-1}s^{-1}$ [18].

The results of n and k_{inh} values for the indicated compounds are summarized in Table 1. As shown, the n value of the indicated compounds for the AIBN system declined in the order BMP (2.0), 2-hydroxyestradiol (2.0)> DES (1.3) > BPA (1.2) > estrone (0.9), whereas that for the BPO system declined in the order BMP (2.0) > DES (1.9), BPA (1.9) > estrone (1.3) > 2-hydroxyestradiol (0.7).

The *n* value for estrone for both AIBN and BPO was about 1. This value was similar to that mentioned in a previous report [10]. The estrone quinol 10β-hydroxyesta-1,4-diene-3,7-dione has been reported to be a product of the oxidation of estrone with 3-chloroperbenzoic acid in the presence of BPO as a radical initiator and under light irradiation in refluxing dry dichloromethane [11]. Oxidation of estrone produces steroidal quinols and *syn*-epoxyquinols [12]. Therefore, the fully oxidized *n* for estrone should be 2, due to the formation of estrone quinol, but estrone gave approximately *n*=1 in the present study. The *n* value found for estrone in the present study suggests that oxidation of estrogen is complex, and that quinoids, quinoid radicals and phenoxyl radicals may be formed from oxidation of estrones and may be responsible for carcinogenecity and/or inflammatory effects in biological systems [3]. The free radical-scavenging activity of estrones is a result of their A-ring phenolic hydroxy group, but the oxidation of estrones by hydrogen peroxide has shown to occur at the carbonyl group, with a cleavage of D-ring and production of a hydroxy acid; this reaction is essentially the oxidation of a cyclic ketone to a lactone [13]. The mechanism of the radical reaction is complicated and the chemical nature of the products derived from the radical-scavenging reaction remains unknown.

For AIBN, the *n* value for 2-hydroxyestradiol, a catechol estrogen, was approximately 2, whereas that for BPO was approximately 1. Cytochrome P450 oxidizes 2-hydroxyestradiol to 2,3-estradiol quinone [14]. This suggested that oxidation of 2-hydroxyestradiol may produce catechol quinones. Oxidation of catechols to semiquinones and quinones is a mechanism of tumor initiation, not only for endogenous estrogens, but also for synthetic estrogens such as DES and BPA, a human carcinogen, because quinone reacts with DNA [15]. The *n* values for DES and BPA for the AIBN and BPO system were about 1 and 2, respectively. These compounds preferentially scavenged PhCOO˙ radicals derived from BPO, which may result in the formation of quinone.

Rp_{inh}/Rp_{con} and k_{inh}.

Figure 5 shows the relation between Rp_{inh}/Rp_{con} and concentration of estrone and 2-hydroxy-estradiol for both the AIBN and BPO system. In the both systems, Rp_{inh}/Rp_{con} for each estrogen decreased linearly with increasing concentration. 2-Hydroxyestradiol showed much stronger enhancement of the rate in the AIBN system. In contrast, estrone showed much more enhancement in the BPO system. The reduction of Rp_{inh}/Rp_{con} implies the retardation of polymerization. The oxidized products of estrogens produced by initiator radicals during the induction period could interact strongly with growing MMA radicals. The k_{inh} values for estrogens, estrogen-like compounds and monophenol, BMP calculated from Eq. (5) are shown also in Table 1. The inhibition rate constant ($k_{inh} \times 10^{-3} M^{-1}s^{-1}$) for the AIBN system declined in the order estrone (2.2) > BPA (2.0) > DES (1.9) > 2-hydroxyestradiol (1.2) > BMP (1.1), whereas that for the BPO system declined in the order 2-hydroxyestradiol (3.2) > estrone (1.4) > DES (1.2) > BPA (1.0) > BMP (0.9).

Several studies are available in which each of these two methods (the *n* and k_{inh}) was applied to elucidate the mechanism of a particular reaction, but there is no comparative study with respect to the scope and limitation of these methods [16]. In the present study, we examined the radical-scavenging activity using both methods under nearly anaerobic conditions. The compounds having both large *n* and k_{inh} values are good radical-scavengers.

Under aerobic conditions at 25 °C, k_{inh} values for estrone and estradiol against 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl radical (PhO·) measured by the stopped flow technique have previously been reported by to be 84 $M^{-1}s^{-1}$ and 138 $M^{-1}s^{-1}$, respectively [4]. In the present study, the k_{inh} value for estrone was approximately one order of magnitude greater. The ratio k_{inh}/k_p for 2-hydroxyestradiol has previously been determined using the induction period method to be 2.4 x 10^3 for oxidation of methyl linoleate micells under aerobic conditions [5], from which a k_{inh} value of approximately 2.4 x 10^5 $M^{-1}s^{-1}$ can be calculated by estimating a k_p value of about 100 $M^{-1}s^{-1}$ at 30°C [7]. This value was approximately two-fold order of greater than that obtained in the present study. This considerable difference in the absolute values of k_{inh} is probably caused by difference in methodology between the studies (PhO· radical, linoleate radical LOO· or growing MMA radical; stopped flow technique or induction period method, respectively). *In vivo* experiments are too complex to amenable to simple interpretation and, hence, we employed physical-chemical studies using the induction period method in the radical polymerization of MMA under nearly anaerobic conditions. We expect that the *n* and k_{inh} values for estrogens and estrogen-like compounds determined in the present study will be relevant for the development of compounds that mimic their biological activity.

Conclusions

The *n* and k_{inh} for estrone, 2-hydroxyestradiol, DES, BPA and BMP were determined using the induction period method under nearly anaerobic conditions at 70°C. The *n* values for these compounds were 1-2 and their k_{inh} were 0.9 x 10^3 - 3.2 x $10^3 M^{-1}s^{-1}$. 2-Hydroxyestradiol scavenged R· radicals preferentially, whereas DES and BPA preferentially scavenged PhCOO· radicals. The k_{inh} of 2-hydroxyestradiol for the BPO system was the greatest, whereas that of estrone for the AIBN system was the greatest among the indicated compounds.

Experimental

General

DSC (differential scanning calorimeter) measurements were similar to those in our previous publication [17]. In brief, the experimental resin consisted of MMA and AIBN (or BPO) with or without additives. AIBN (or BPO) were added at 1.0 mol%, and the additives were used at 0, 0.02, 0.05 and 0.1 mol%. Approximately 10 μL of the experimental resin (MMA: 9.12-9.96 mg) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70°C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of all samples, as calculated from DSC thermograms, was 91-96%.

Measurement of stoichiometric factor (n)

The relative *n* value in Eq. (1) can be calculated from the induction period in the presence of inhibitors:

$$n = R_i[IP]/[IH] \tag{1}$$

where [IP] is the induction period in the presence of an inhibitor. The number of moles of peroxy radicals trapped by the antioxidant is calculated with respect to 1 mole of inhibitor moiety unit.

The R_i values for AIBN and BPO were $5.66 \times 10^{-6}\,Ms^{-1}$ and $2.28 \times 10^{-6}\,Ms^{-1}$, respectively [17].

Measurement of the inhibition rate constant (k_{inh})

When R_i is constant, i.e. when new chains are started at a constant rate, a steady-state treatment can be applied and the initial rate of polymerization of MMA is given by Eq. (2) [17]:

$$Rp_{con} = \{k_p\,[MMA]\,R_i^{1/2}\}/(2k_t)^{1/2} \tag{2}$$

where MMA represents methyl methacrylate and k_p and k_t are the rate constants for chain propagation and termination, respectively.

The $k_p/(2k_t)^{1/2}$ rate of polymerization of MMA (9.4 M) by AIBN (1 mol%) and BPO (1 mol%) at 70°C was a constant value, $9.86 \times 10^{-2}\,M^{-1/2}\,s^{-1/2}$ [18]. The Rp_{inh} rates are determined by Eq. (3):

$$Rp_{inh} = \{k_p\,[MMA]\,R_i\}/\{n\,k_{inh}\,[IH]\} \tag{3}$$

in which Rp_{inh} is the initial rate of inhibited polymerization, [MMA], *n*, [IH] and k_p are defined above, and k_{inh} is the rate constant for scavenging (inhibiting) of MMA radicals by an antioxidant. From Eq. (**2**) and Eq. (**3**), the rate can be expressed by Eq. (4).

$$Rp_{inh}/Rp_{con} = (2k_tR_i)^{1/2}/\{n\,k_{inh}\,[IH]\} \tag{4}$$

The rate constant is given by Eq. (5).

$$k_{inh} = \{Rp_{con}\,(2k_tR_i)^{1/2}\}/\{n\,[IH]\,Rp_{inh}\} \tag{5}$$

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Sample Availability: Available from the authors.