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Kinetic Studies of the Radical-scavenging Activity of Estrogens and Antiestrogens

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Abstract. *Quinoids, quinoid radicals and phenoxyl radicals formed from estrogens (estrone; diethylstilbestrol, DES) and antiestrogens (tamoxifen; toremifene) may be responsible for adverse effects such as carcinogenesis. The radical-scavenging activity of estrogens and antiestrogens was determined quantitatively by the induction period method for the polymerization of methyl methacrylate initiated by thermal decomposition of 2,2'-azobisisobutyronitrile (AIBN) or benzoyl peroxide (BPO) under nearly anaerobic conditions. The inhibition rate constant (k_{inh}, $x10^{-3}$ $M^{-1}s^{-1}$) for estrone, DES, tamoxifen, toremifene and 2,6-di-t-butyl-4-methyphenol (BHT) was 1-3, 2-4, 6-12, 6-13 and 1-2, respectively. The k_{inh} for antiestrogens was two-fold greater than that for estrogens or BHT. In contrast, the stoichiometric factor (n, number of free radicals trapped by one mole of antioxidant moiety) for estrone, DES, tamoxifen, toremifene and BHT was 1.2-1.5, 1.8-2.4, 0.5-0.9, 0.4-0.5 and 1.5-1.9, respectively. The fully oxidized n values for estrone, DES and BHT would be 2, whereas that for antiestrogens would be 1. However, the n values for estrone and antiestrogens were markedly less than 2 and 1, respectively, suggesting a complex oxidation process resulting in the formation of quinoids, quinoid radicals and phenoxyl radicals during the induction period.*

Estrogens such as estrones and DES 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 (1-4). Similarly, antiestrogens such as tamoxifen and toremifene exhibit effectiveness both in treating breast cancers as well as in preventing this disease (5,6). Studies using models for active oxygen radicals ($ROO^{\cdot}$, $RO^{\cdot}$ and $HO^{\cdot}$) in biological systems have reported that estrogens are effective antioxidants (7,8). As for the antioxidant activity of antiestrogens, the interaction between tamoxifen and peroxy radicals ($ROO^{\cdot}$) generated by water-soluble and lipid-soluble azo-initiators has been investigated, indicating that tamoxifen is a scavenger of $ROO^{\cdot}$ radicals (8), but the kinetics of this reaction were not studied. Previous quantitative *in vitro* studies of the radical-scavenging activity of phenolic compounds (10, 11) and estrogens (9) were performed by the induction period method under aerobic conditions. The oxygen tension under a 15 torr oxygen atmosphere is similar to that in many tissues (12, 13), suggesting that oxygen is scarce in living cells and that the radical-scavenging activity of estrogens and antiestrogens *in vivo* may differ considerably from that under aerobic conditions.

We have previously reported the use of differential scanning calorimetry (DSC) to evaluate the radical-scavenging activity of ferulic acids (14), curcumins (15), flavonoids (16), polyenes (17) and BHT metabolites (18) by induction period methods under nearly anaerobic conditions, and the antioxidant activity of these compounds was predicted well by a model based on kinetic and thermodynamic data. In the present study, we used this previously reported (14-18) induction period method with DSC to investigate the radical-scavenging activity of estrogen, DES, tamoxifen, toremifene and BHT by determining the stoichiometric factor (*n*, the number of free radicals trapped by 1 mole of antioxidant) and the inhibition rate constant of polymerization (k_{inh}).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated sources: estrone [3-hydroxyestra-1,3,5(10)-trien-17-one] (Wako Pure Chemical Ind., Ltd., Osaka, Japan), DES (Tokyo Kasei Chemical Co., Tokyo, Japan), tamoxifen [(Z)-1-[4-[2-dimethyl-amino]ethoxy]phenyl]-1,2-diphenyl-1-butene] (ICN Biomedicals Inc., Ohio, USA) and toremifene [(Z)-4-chloro-1,2-diphenyl-1-[4-[2-(*N,N*-dimethylamino)ethoxy]-phenyl]-1-butene] (LKT Laboratories, Inc., Minnesota, USA). The chemical structures of these compounds are

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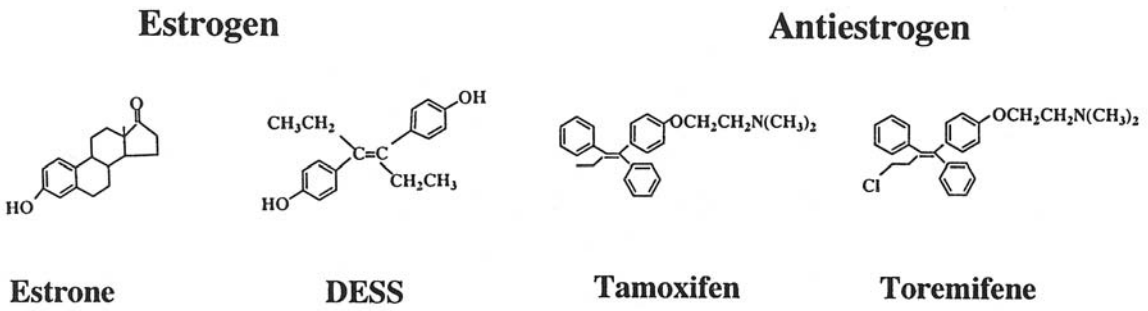

Figure 1. *Chemical structures of estrogen and antiestrogen compounds.*

shown in Figure 1. 2,6-Di-*t*-butyl-4-methoxyphenol (DTBMP) and 2,6-di-*t*-butyl-4-methylphenol (BHT) were obtained from Tokyo Kasei Chemical Co. MMA (Tokyo Kasei Chemical Co.) was purified by distillation. AIBN and BPO were recrystallized from methanol and chloroform/methanol (1:1 v/v), respectively.

Induction period and initial rate of polymerization. The induction period and initial rate of polymerization were determined by the method recently reported in this journal (12,13). 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 0.001, 0.01, 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 92-96%. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by the polymerization of MMA. Time-exotherm and time-conversion curves for estrogens and antiestrogens are shown in Figure 2 and Figure 3. Polymerization curves break when an inhibitor is consumed. These breaks are sharp and provide a reliable measure of the induction period of the inhibitor. The presence of oxygen retards polymerization because oxygen reacts with MMA radicals activated by the initiator and then subsequently produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen since it had been sealed in air. Tangents were drawn to polymerization curves at an early stage in the run. The induction period of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the early stage of polymerization. The induction period (IP) was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence (Rp_{con}) and presence (Rp_{inh}) of estrogens and antiestrogens were calculated from the slope of the plots of the first linear portion of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage).

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

$$n = R_i[IP]/[IH] \quad (1)$$

where [IP] is the induction period in the presence of an inhibitor. The number of moles of peroxy radicals trapped by the relevant phenol is calculated with respect to 1 mole of inhibitor moiety unit. The Ri values of AIBN and BPO using DTBMP were 5.66 x 10^{-6} and 2.28 x 10^{-6} Ms^{-1}, respectively (17,18).

Measurement of the inhibition rate constant, k_{inh}. When Ri 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) (17,18):

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

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 both AIBN (1 mol%) and BPO (1 mol%) at 70˚C was 9.86 x 10^{-2} $M^{-1/2}$ $s^{-1/2}$ (17,18).

When polymerization of MMA is suppressed and retarded by an antioxidant, the rate can be expressed by Eq. (3):

$$Rp_{inh} /Rp_{con} = (2k_tR_i)^{1/2}/\{n\ k_{inh}\ [IH]\} \quad (3)$$

The rate constant k_{inh} is given by Eq. (4):

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

Results and Discussion

Stoichiometric factor (n). Time-exotherm (top) and time-conversion (bottom) curves of estrogens (estrone, DES) and antiestrogens (tamoxifen, toremifene) for AIBN and BPO are shown in Figures 2 and 3, respectively. The induction periods for each antioxidant were estimated from the time-conversion curves. Plots of induction period *vs* concentration of antioxidants for AIBN (A) and BPO (B) are shown in Figure 4. A linear relationship between the induction period and the concentration of antioxidants was observed. Relative *n* values for antioxidants calculated from Eq. (1) are summarized in Table I. The *n* value for the AIBN system declined in the order DES (1.8) > BHT (1.5) > estrone (1.2) > tamoxifen (0.5) > toremifene (0.4). In contrast, that for the BPO system declined in the order DES (2.4) > BHT (1.9) > estrone (1.5) > tamoxifen, toremifene (0.5). In both systems, the *n* values

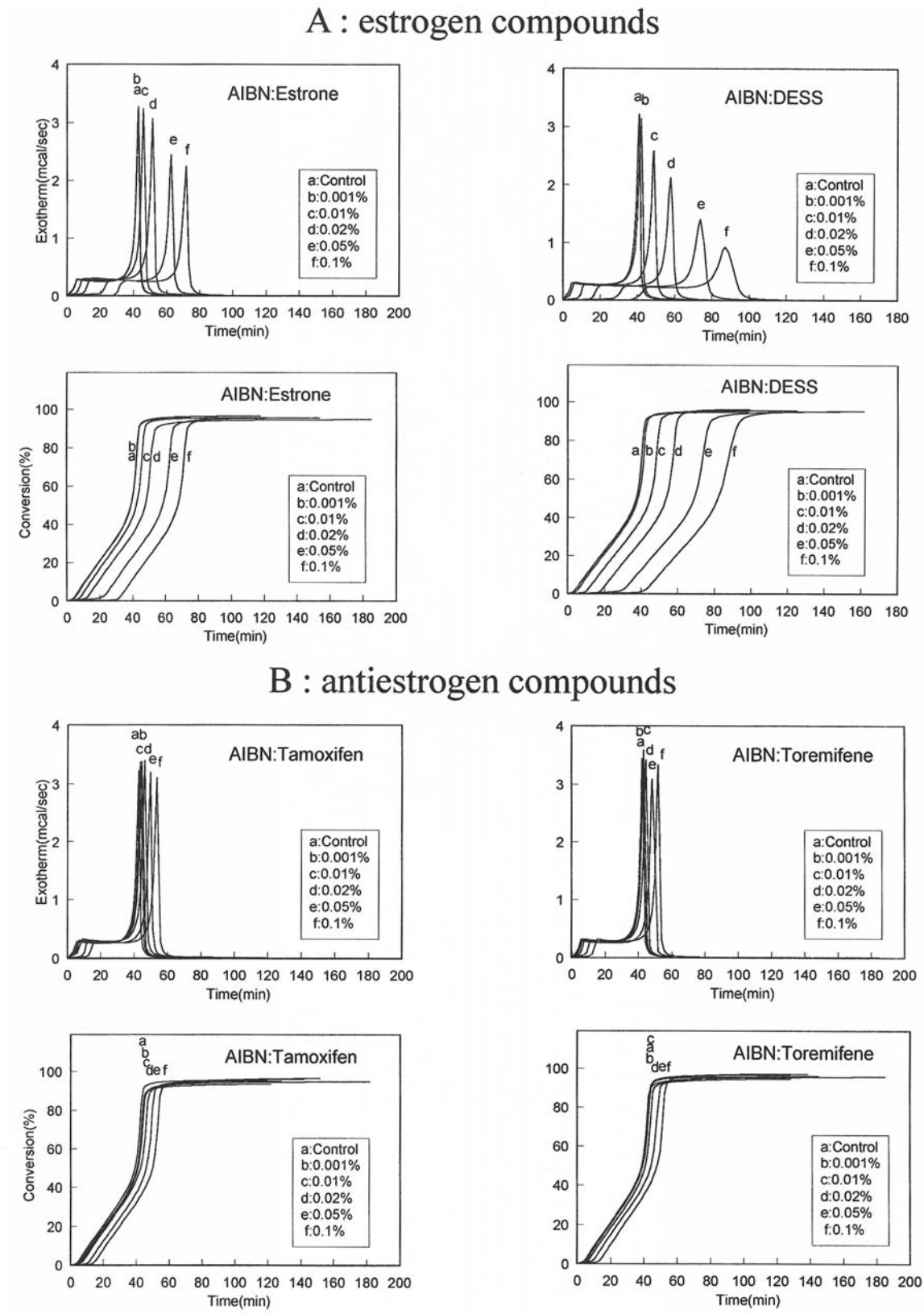

Figure 2. *Exothermic (top) and time-conversion (bottom) curves for the polymerization of MMA with AIBN in the presence of estrogen or antiestrogen compounds.*

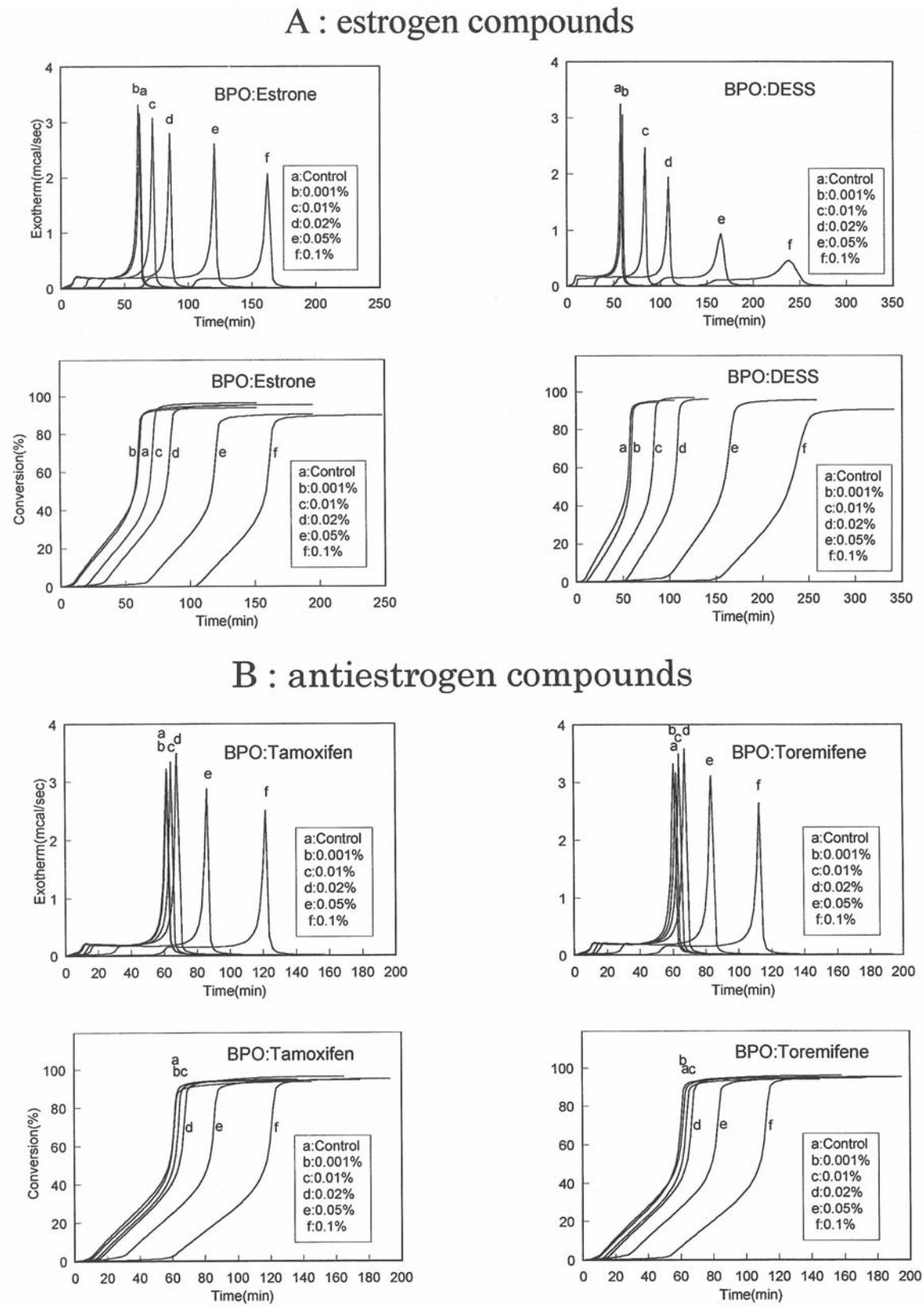

Figure 3. *Exothermic (top) and time-conversion (bottom) curves for the polymerization of MMA with BPO in the presence of estrogen or antiestrogen compounds.*

for antiestrogens were markedly less than those for estrogens. The *n* value for BHT in the BPO system was approximately 2. In general, phenolic compounds show *n* values of 2 (19). Phenolic compounds with *n* values less than 2 undergo dimerization or other reactions. Dimerization of phenolic compounds is due to their radical-radical coupling reaction. The kinetics of the reaction of peroxy radicals with BHT, with a *p*-methyl group, have previously been intensively investigated, showing that with *n* values less than 2 BHTs give stilbenequinones as a result of the *p-p* coupling reaction (19). In contrast, fully oxidized DES gives an *n* value of 2; DES may produce diethylstilbestrol-4,4-quinone. In the BPO system, the *n* value for DES was 2.4, suggesting the further oxidation of quinone compounds of DES.

The estrone quinol 10β-hydroxyestra-1,4-dien-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 (20). Therefore, the fully oxidized *n* for estrone should be 2 because of the formation of estrone quinol, but estrone gave $n=1.2$-1.5 in the present study. Oxidation of estrone produces steroidal quinols and *syn*-epoxyquinols (21). The *n* value for estrone found in the present study suggests that oxidation of estrogens is complex, and that quinoids, quinoid radicals and phenoxyl radicals may be formed from oxidation of estrones and may be responsible for carcinogenicity and/or inflammatory effects in biological systems (22). The free radical-scavenging activity of estrones is a result of their A-ring phenolic hydroxy group, but the mechanism of the reaction is complicated and the chemical nature of the products derived from the radical-scavenging reaction remains essentially unknown.

The radical-scavenging and antioxidant activity of the antiestrogens tamoxifen and toremifene has previously been reported (9), but their kinetics have not been sufficiently elucidated. Their *n* values in the present study, less than 1, are similar to those of polyenes such as β-carotene (17). The interaction between polyenes and free radicals at low oxygen tension is predominantly due to addition of the radicals to conjugation-activated unsaturated bonds (13). Tamoxifen and toremifene possess a triphenyl ethylene moiety, and, at less than ambient O_2 tension, they functioned as effective antioxidants with activity similar to that of polyenes; conversely, under higher oxygen tension, they would probably show less inhibitory activity. Also, tamoxifen and toremifene have a dimethylamino group, a tertiary amine having high reactivity for free radicals. However, aminopyrine, a tertiary amine, although referred to as an antioxidant, is not expected to scavenge peroxy radicals under aerobic conditions (23). In the present study, tamoxifen and toremifene scavenged radicals. The major oxidation products for these compounds may be dimethylation products derived from the dimethylamine group. The radical-scavenging mechanism of tamoxifen and toremifene could not be sufficiently elucidated in the present study.

The *n* values of these antioxidants in the BPO system were considerably greater than those in the AIBN system, suggesting that oxidation of estrogens and antiestrogens occurs preferentially by $PhCOO^{\cdot}$ radicals derived from $BPO^{\cdot}$. Benzoyl peroxide is a much more effective oxidant of estrogen than is AIBN (21).

Rp_{con}/Rp_{inh} and k_{inh}. Figure 4(C) shows the relationship between Rp_{con}/Rp_{inh} and concentrations of estrogens and antiestrogens for both the AIBN and BPO systems. In the BPO system, Rp_{con}/Rp_{inh} for each compound increased linearly with increasing concentration, indicating much stronger enhancement of the rate in the BPO system in the increasing order toremifene < estrone < tamoxifen < DES. DES showed the highest value of Rp_{con}/Rp_{inh} in both the AIBN and BPO systems, possibly due to the formation of diethylstilbestrol-4,4-quinone after the complete oxidation of DES, as indicated by the *n* value of approximately 2.

The k_{inh} values calculated from Eq. (5) are shown in Table I. The k_{inh} increased in the order BHT, DES < estrone < toremifene < tamoxifen. The k_{inh} value for the AIBN system was approximately two-fold greater than that for the BPO system. This may be related to the R_i value of the initiator, because the R_i for AIBN was approximately two-fold greater than that for BPO in the present study. Under aerobic conditions at 25˚C, k_{inh} values for estrone, estradiol, 2-hydroxyestrone and BHT against 2,6-di-t-butyl-4-(4-methoxyphenyl)phenoxyl radical (PhO.) have previously been reported by a stopped flow technique to be 84 $M^{-1}s^{-1}$, 138 $M^{-1}s^{-1}$, 2.6 x 10^5 $M^{-1}s^{-1}$ and 84 $M^{-1}s^{-1}$, respectively (7). In the present study, k_{inh} values for estrone (2-4 x 10^3 $M^{-1}s^{-1}$) and BHT (1-3 x 10^3 $M^{-1}s^{-1}$) were approximately one order of magnitude greater. This considerable difference in the absolute values of inhibition rate constant is probably caused by differences in methodology between the two studies (scavenging of $PhO^{\cdot}$ radical and induction period method, respectively). The ratio k_{inh}/k_p for 2-hydroxyestradiol has previously been reported by the induction period method to be 2.4 x 10^3 for oxidation of methyl linoleate micelles under aerobic conditions (8), 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 37˚C (11). This value is in agreement with that mentioned above (7), although 2-hydroxyestrone differs from 2-hydroxyestradiol. On the other hand, the real chain-breaking antioxidant activity of polyphenols is probably determined not so much by the reactivity of the original polyphenol as by the probability of the formation of active products (*i.e.* quinones) and their antioxidant activities (24). We previously reported that quinones derived from BHT suppressed growing MMA radicals (18). These findings

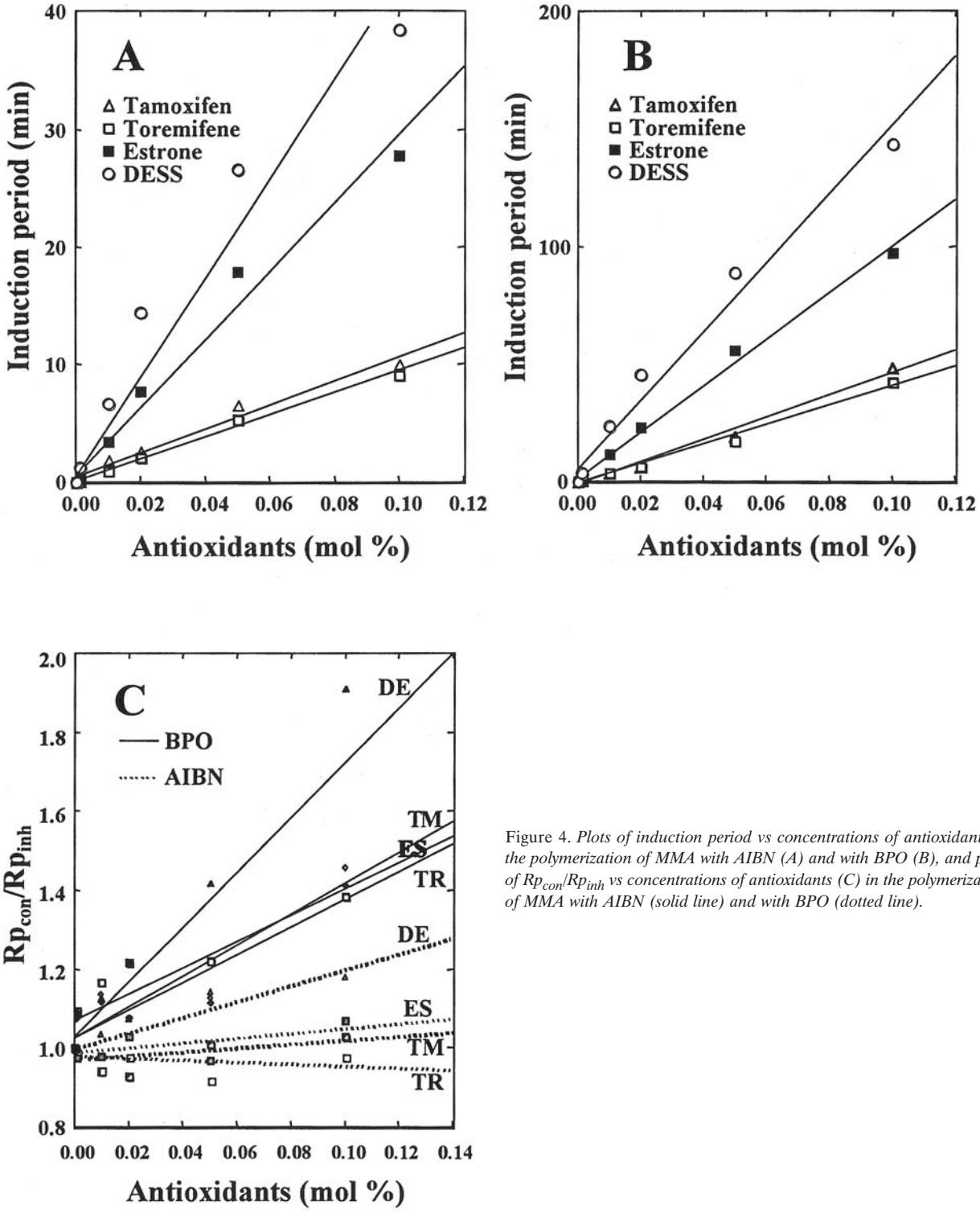

Figure 4. *Plots of induction period vs concentrations of antioxidants in the polymerization of MMA with AIBN (A) and with BPO (B), and plots of Rp_{con}/Rp_{inh} vs concentrations of antioxidants (C) in the polymerization of MMA with AIBN (solid line) and with BPO (dotted line).*

suggest that the formation of active products (*i.e.* quinoids) and their antioxidant activities may predominantly govern the k_{inh} values of estrogens and antiestrogens during the induction period. An *n* value of 1.2-1.5 for estrone strongly suggests that the process of oxidation of estrone is complex. The k_{inh} values for estrogens and antiestrogens determined in the present study will be relevant for the development of compounds that mimic their biological activity.

Table I. *Stoichiometric factors (n), inhibition rate constants (k_{inh}), the ratio of the initial rate of polymerization with antioxidants to that without antioxidants (Rp_{inh}/Rp_{con}) in the AIBN/MMA and BPO/MMA system.*

	AIBN/MMA system			BPO/MMA-system		
Antioxidants	n	k_{inh} x10^{-3}	Rp_{inh}/Rp_{con}	n	k_{inh} x10^{-3}	Rp_{inh}/Rp_{con}
Tamoxifen	0.49	11.58	0.99	0.53	5.53	0.89
Toremifene	0.36	13.29	1.01	0.47	6.46	0.90
Estrone	1.21	4.13	1.00	1.53	2.07	0.82
DESS	1.80	3.15	0.87	2.43	1.42	0.75
BHT	1.50	2.73	0.99	1.91	1.32	0.98

The measurement for antioxidant activities is described in the text. Values were the mean of two or three independent experiments. Standard error <10%. MMA, 9.4 mole/l; AIBN (or BPO), 1 mol %; 70°C. The determination of k_{inh} is described in the text. The k_t is about $3.7x10^7$ $M^{-1}s^{-1}$.

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