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INHIBITION OF PEROXIDATIONS OF LIPIDS AND MEMBRANES BY ESTROGENS

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The antioxidant activity of 2-hydroxyestradiol was measured quantitatively and compared with those of other estrogens in oxidations of methyl linoleate in homogeneous solution and in aqueous dispersions, oxidations of soybean phosphatidylcholine liposomal membranes and oxidative haemolysis of rabbit erythrocytes, all induced by free radicals generated from a radical initiator. Similar antioxidant activities of estrogens were observed, irrespective of the oxidation system. 2-Hydroxyestradiol was found to be a potent radical scavenger and its antioxidant activity was close to that of α-tocopherol, the strongest natural antioxidant. Other phenolic estrogens such as estrone, estriol and β-estradiol showed a modest antioxidant activity and testosterone did not act as an antioxidant.

INTRODUCTION

There is increasing evidence to suggest the involvement of free radical-mediated oxidations of lipids, membranes and tissues in biological systems in a variety of pathological events such as arteriosclerosis, rheumatoid arthritis, post-ischaemic injury, cancer and even the degenerative processes associated with ageing, and many reviews, books and proceedings have been published (e.g. Ref. 1). Aerobic organisms are protected by an array of defence systems against oxygen toxicity and free radical attack on various membranes and tissues.[2–4] Preventive antioxidants suppress the generation of free radicals by deactivating the active species and possible precursors of free radicals; for example, catalase and glutathione peroxidase decompose (without generation of free radicals) hydrogen peroxide, which is a precursor of the hydroxyl radical. Chain-breaking antioxidants such as vitamins E and C scavenge the oxygen radicals rapidly and terminate the free-radical chain reactions. There have also been a number of reports on the protective action of superoxide dismutase (SOD), which specifically and efficiently catalyses the dismutation of superoxide to hydrogen peroxide and oxygen.[5,6]

Recently, it has been found qualitatively[7–10] that estrogens, especially catechol estrogens, can function as radical scavengers and suppress peroxidation both *in vitro* and *in vivo*. This study was undertaken to measure quantitatively the antioxidant activity of 2-hydroxyestradiol and other estrogens in the oxidations of lipids and membranes.

EXPERIMENTAL

Methyl linoleate was chosen as a representative of polyunsaturated fatty acids, since its oxidation mechanism is well known to give four conjugated diene hydroperoxides quantitatively.[11–16] Commercial methyl linoleate obtained from Tokyo Kasei Kogyo (Tokyo, Japan) was purified on a silica gel column. Soybean phosphatidylcholine (PC) was purchased from Daigo Chemical (Osaka, Japan) and purified on silica gel and alumina columns before use. Fresh, heparinized rabbit blood was centrifuged and plasma and buffy coat were removed to obtain erythrocytes,[17] which were washed three times with physiological saline.

2-Hydroxyestradiol, estrone, estriol, β-estradiol and testosterone were purchased from Sigma (St Louis, MO, USA) and used as received. (*R,R,R*)-α-tocopherol was kindly supplied by Eisai (Tokyo, Japan). Both water-soluble and lipid-soluble azo compounds [2,2′-azobis

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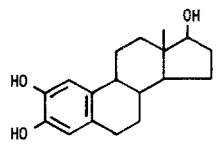
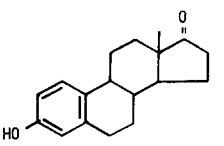
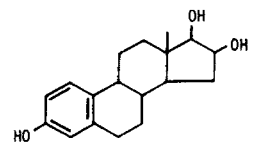
(2-amidinopropane dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile (AMVN), respectively] were used as radical initiators to generate free radicals at a constant rate and at a specific site as previously.[18,19] AAPH and AMVN were obtained from Wako (Osaka, Japan) and used without further purification. Other chemicals were of analytical-reagent grade.



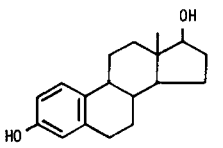
2-Hydroxyestradiol



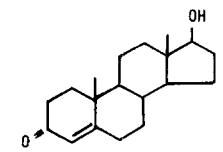
Estrone



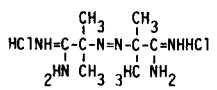
Estriol



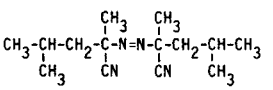
β-Estradiol



Testosterone



AAPH



AMVN

Methyl linoleate was oxidized either in an organic solvent or in aqueous dispersions. Methanol was used as a solvent. Methyl linoleate emulsions were prepared by mixing appropriate amounts of methyl linoleate and 10 mM Triton X-100 aqueous solution containing, when necessary, water-soluble additives with a vortex mixer for 2 min. Soybean PC liposomes were prepared as described previously.[19,20] Soybean PC liposomes was composed of about 70% linoleic acid with small amounts of linolenic acid and saturated fatty acids. PC and lipid-soluble additives, when necessary, were dissolved in chloroform and placed in a pear-shaped flask. Chloroform was removed at reduced pressure to obtain a thin film on the glass wall. An appropriate amount of 0·1 M sodium chloride solution was added and the PC film was slowly peeled off by shaking to obtain white, milky liposome suspensions. When required, it was then sonicated with a Branson Model 185 Sonifier to obtain unilamellar liposomal membranes.

The oxidation was carried out aerobically and under constant shaking in a water-bath maintained at 37 °C. The rate of oxygen uptake was measured with either a pressure transducer as described previously[15] or an oxygen electrode, Biological Oxygen Monitor, Model YSI 53 and Model 5300 (Yellow Springs Instrument Co., Yellow Springs, OH, USA). As mentioned above, the rate of oxidation of methyl linoleate and soybean PC can be measured quantitatively by following the rate of formation of conjugated diene hydroperoxides from the increase in absorption at 234 nm by using high-performance liquid chromatography (HPLC). For this analysis, an aliquot of the sample was injected directly into the HPLC system. A JASCO LC-18 column was used and methanol–water (90 : 10 v/v) was used as the eluent at 1·20 ml min^{-1}.

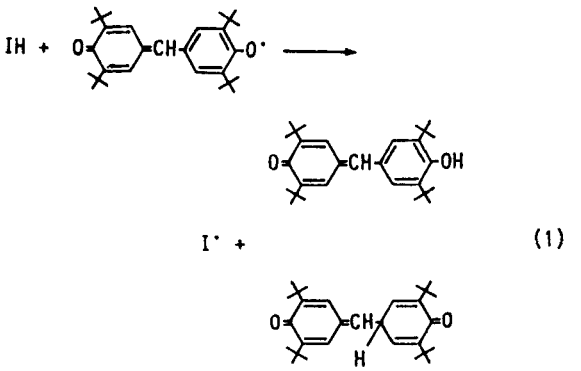
The erythrocytes were also oxidized at 37 °C under air. The incubation mixture consisted of suspensions of rabbit erythrocytes and soybean PC liposomes containing appropriate amount of lipid-soluble additives in physiological saline (pH 7.4). Samples were withdrawn from the incubation mixture at specific time intervals to follow the time course of a haemolysis spectrometrically as reported previously.[17]

The ESR spectra of galvinoxyl radical and phenoxyl radicals from estrogens were recorded in methanol on a JEOL FE1X X-band spectrometer. Estrogen taken into the side-arm of the quartz ESR tube was mixed under vacuum with galvinoxyl dissolved in methanol. In several experiments, either magnesium(II) acetylacetonate or zinc(II) acetylacetonate was also dissolved in methanol.

RESULTS

Interaction of 2-hydroxyestradiol with galvinoxyl

The reactivity of a chain-breaking antioxidant (IH) towards oxygen radicals can be easily and qualitatively estimated by measuring the interaction with galvinoxyl, a stable free radical [reaction (1)]. A strong antioxidant such as α-tocopherol reacts with galvinoxyl instantaneously.[21] The consumption of galvinoxyl can be observed by the disappearance of its ESR spectrum or its absorption at 428 nm. It was found that 2-hydroxyestradiol reacted with galvinoxyl very rapidly when they were mixed in an equimolar ratio (data not shown). On the other hand, β-estradiol and testosterone did not react at an appreciable rate even in the presence of excess estrogens. Figure 1 shows the ESR spectrum observed when 2-hydroxyestradiol was added to the solution of galvinoxyl in methanol. Figure 2 shows the ESR spectra of 2-hydroxyestradiol in the presence of magnesium(II) or zinc(II) acetylacetonate.



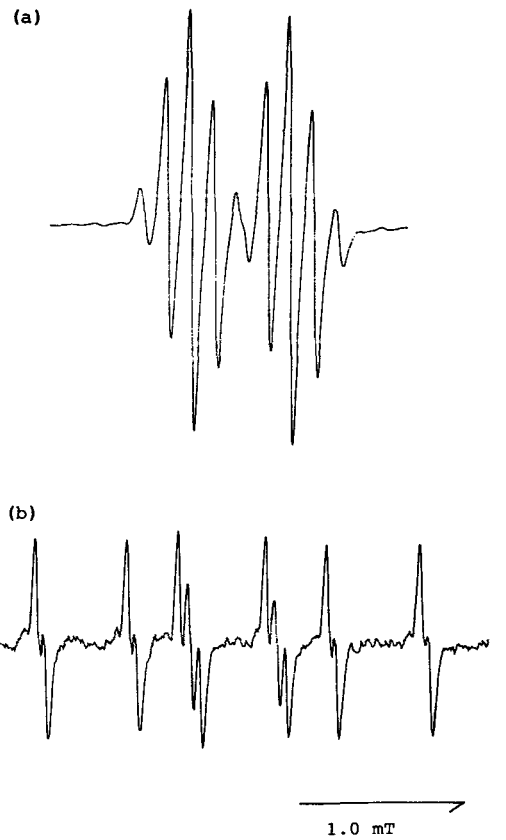


Figure 1. ESR spectra observed when 1·16 mM 2-hydroxyestradiol was added to 1·19 mM galvinoxyl in methanol. (a) ESR spectrum of galvinoxyl in methanol; (b) ESR spectrum observed immediately after the addition of 2-hydroxyestradiol to galvinoxyl in methanol. The ESR spectra were recorded under vacuum at 13 °C; modulation, 0·05 mT; amplitude, (a) 25 and (b) 10^4

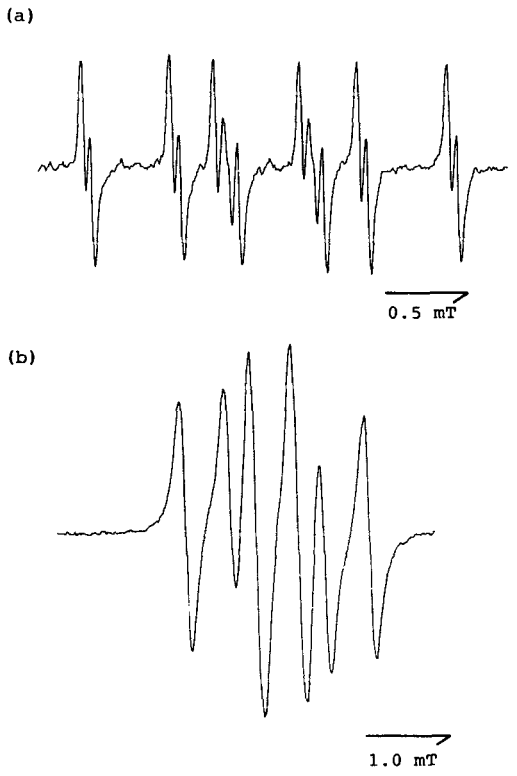


Figure 2. ESR spectra observed when 1·2 mM 2-hydroxyestradiol was mixed with 1·2 mM galvinoxyl in methanol containing (a) magnesium (II) acetylacetonate or (b) zinc(II) acetylacetonate.The ESR spectra were recorded under vacuum at 37 °C; modulation, (a) 0·02 mT and (b) 0·1 mT; amplitude, (a) $7·9 \times 10^3$ and (b) $2·5 \times 10^3$

Inhibition of oxidation of methyl linoleate in solution

The antioxidant activity of estrogens was first studied in the oxidation of methyl linoleate in a simple homogeneous solution of methanol. The spontaneous oxidation of methyl linoleate at 37 °C was small, but it was oxidized at a constant rate in the presence of a radical initiator. Figure 3 shows the accumulation of conjugated diene hydroperoxides during the AMVN-initiated oxidation of methyl linoleate in the absence and presence of antioxidant. Similar antioxidant effects were observed when the rate of oxidation was measured by following the oxygen uptake using a pressure transducer (data not shown). As shown in Figure 3, the addition of 2-hydroxyestradiol suppressed the oxidation markedly and produced a clear inhibition period, after which the oxidation proceeded at a similar rate to that without antioxidant. β-Estradiol retarded the oxidation and acted as a modest antioxidant. Estrone and estriol were weak antioxidants, whereas testosterone did not retard

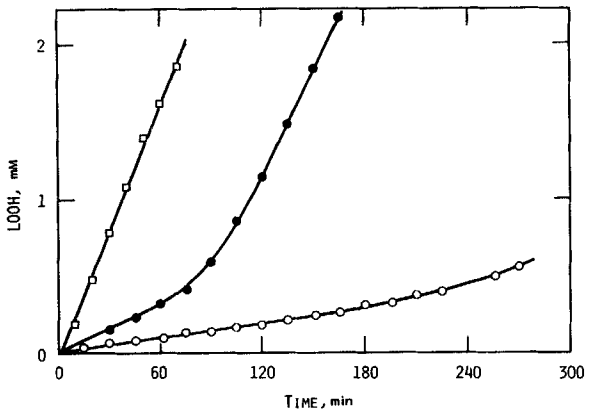


Figure 3. Rate of accumulation of conjugated diene hydroperoxides in the oxidation of 435 mM methyl linoleate in methanol at 37 °C under air induced by 0·2 mM AMVN in the absence (□) and presence (•, 5·0 μM; ○, 40μM) of 2-hydroxyestradiol

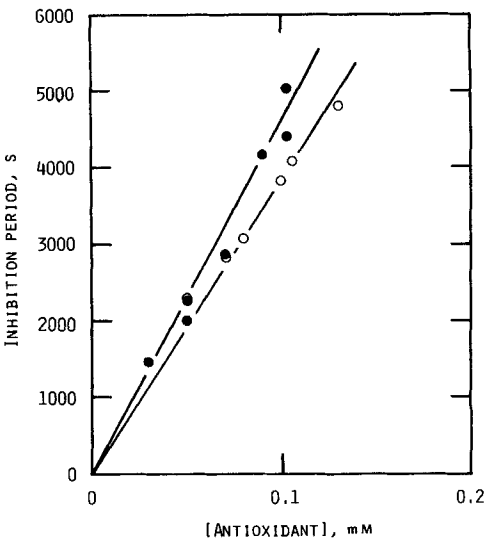


Figure 4. Plot of inhibition period as a function of antioxidant concentration observed in the oxidation of 0·30 M methyl linoleate in methanol induced by 15 mM AMVN at 37 °C. •, 2-Hydroxyestradiol; ○, α-tocopherol

the oxidation appreciably. As shown in Figure 4, the length of the inhibition period produced by 2-hydroxyestradiol was proportional to its concentration and 2-hydroxyestradiol and α-tocopherol gave similar inhibition periods.

Inhibition of oxidation of methyl linoleate emulsions in aqueous dispersions.

Antioxidant activities of estrogens were then studied in the oxidation of methyl linoleate emulsions in 10 mM Triton X-100 aqueous dispersions. Methyl linoleate emulsions were not oxidized appreciably by themselves at 37 °C, but the addition of a water-soluble radical initiator, AAPH, to the aqueous phase induced the oxidation without any induction peroid and a constant rate of oxygen uptake was observed. The lipid-soluble AMVN incorporated into methyl linoleate emulsions also induced chain oxidation. Similar results were obtained for the antioxidant activities of estrogens in the oxidation of methyl linoleate emulsions in aqueous dispersions to those for the oxidation of methyl linoleate in methanol. 2-Hydroxyestradiol functioned as a potent antioxidant, whereas estrone, estriol and β-estradiol showed only modest antioxidant activities and testosterone did not show any activity (data not shown). The pertinent results are summarized in Table 1.

Table 1. Inhibition by 2-hydroxyestradiol (IH) of oxidation of methyl linoleate emulsions in 10 mM Triton X-100 aqueous dispersions induced by AAPH or AMVN in air 37 °C

MeLH (mM)	[AAPH] (mM)	[AMVN] (mM)	[IH] (μM)	t_{inh}[a] (s)	$10^8 R_{inh}$[b] ($mol\,l^{-1}\,s^{-1}$)	$10^8 R_p$[c] ($mol\,l^{-1}\,s^{-1}$)	$10^{-3} k_{inh}/k_p$[d]
72·9	2·02		0	0		7·26	
72·9	2·02		3·03	1220	2·28	5·34	2·62
72·9	2·02		4·03	1700	2·11	4·65	2·04
72·9	2·02		5·02	2170	1·66	2·87	2·02
211		1·01	2·04	1420	5·41	7·90	2·74
211		0·99	3·15	1480	5·92	8·16	2·40
211		0·99	4·08	2240	4·07	5·28	2·31
211		1·01	5·03	2890	2·81	5·21	2·60

[a] Inhibition period.
[b] Rate of oxidation during the inhibition period.
[c] Rate of oxidation without antioxidant or after the inhibition period.
[d] See Discussion.

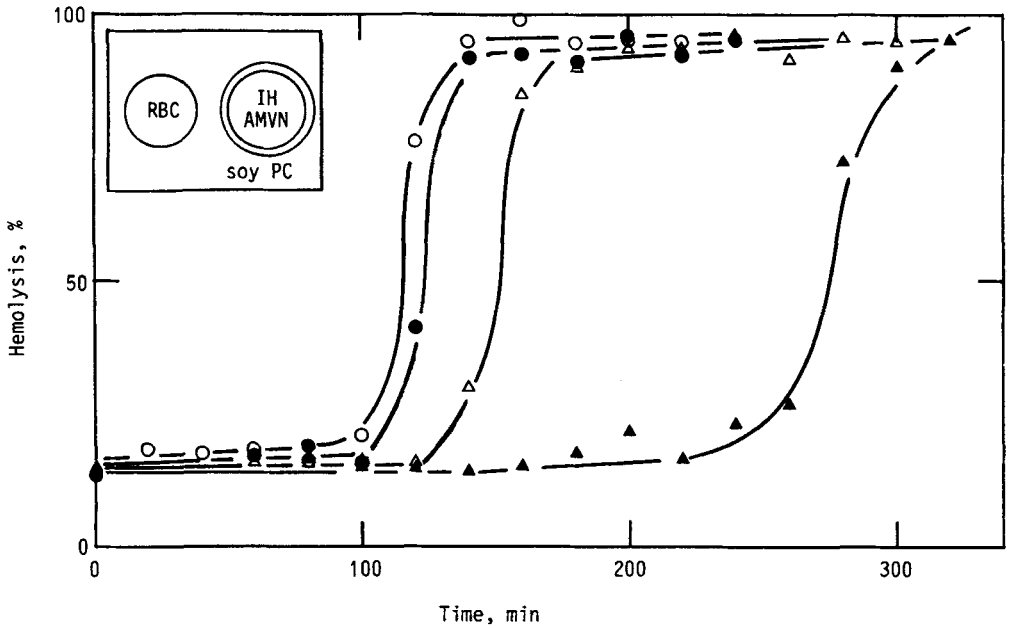


Figure 5. Inhibition by 2-hydroxyestradiol of haemolysis of rabbit erythrocytes induced by the addition of 1·61 mM soybean PC liposomes containing 0·25 mM AMVN at 37 °C in 10 mM phosphate buffer (pH 7·4) in air. The experiments were carried out as described under Experimental. The concentrations of 2-hydroxyestradiol were (○) 0, (●) 5, (△) 10 and (▲) 20 μM

Inhibition of oxidation of soybean PC liposomes

The soybean PC liposomal membranes were also oxidized by a free-radical chain mechanism in the presence of a radical initiator. Estrogens were incorporated into soybean PC liposomal membranes and their antioxidant activities were studied. AMVN was used as a radical initiator and it was also incorporated into soybean PC liposomes simultaneously. β-Estradiol, estriol and testosterone were poor antioxidants and β-estradiol retarded the oxidation appreciably only at high concentations. 2-Hydroxyestradiol suppressed the oxidations and, as shown in Table 2, the inhibition period increased and the rate of oxidation during the inhibition period decreased with an increase in 2-hydroxyestradiol concentration.

Table 2. Inhibition by 2-hydroxyestradiol of oxidation of 10·3 mM soybean PC liposomes induced by 2·00 mM AMVN at 37 °C in air

[IH] [a] (μM)	t_{inh} [b] (s)	$10^8 R_{inh}$ [c] ($mol l^{-1} s^{-1}$)	k_{inh}/k_p [d]
1·00	2330	2·25	196
1·01	2400	1·77	242
1·53	3140	1·37	239
2·00	5110	1·21	167

[a] Concentration of 2-hydroxyestradiol.
[b] Inhibition period.
[c] Rate of oxidation during the inhibition period.
[d] See Discussion.

Inhibition of oxidative haemolysis of rabbit erythrocytes

Erythrocyte membranes are also attacked by free radicals and both lipids and proteins are oxidized, eventually causing haemolysis.[17,22-25] For example, it has been found that the addition of AAPH to aqueous suspensions of rabbit erythrocytes induces the oxidation of membrane lipids and proteins and causes haemolysis, and that it can be suppressed by radical scavengers such as α-tocopherol, ascorbic acid and uric acid.[17,23,25] As Figure 5 shows, the addition of soybean PC liposomes containing AMVN also induced haemolyis and 2-hydroxyestradiol incorporated simultaneously with AMVN into the soybean PC liposomes suppressed the haemolysis in a dose-dependent manner.

DISCUSSION

The results show clearly that, as observed previously,[8] 2-hydroxyestradiol acts as a potent radical scavenger and can suppress the free radical-mediated peroxidations of lipids and membranes. Yoshino *et al.*[10] also

observed that the intraperitoneal administration of 2-hydroxyestradiol brought about a decrease in liver lipid peroxide levels in both male and female mice. Estrone, estriol and β-estradiol acted only as weak antioxidants, and testosterone did not act as an antioxidant. Obviously the phenolic hydrogens at the 2- and 3-positions in estrogens are important in scavenging the oxygen radicals. The results of interactions of estrogens with galvinoxyl suggest that the phenoxyl radicals derived from estrone, estriol and β-estradiol are less stable than galvinoxyl radical. The phenoxyl radical derived from 2-hydroxyestradiol may be more resonance-stabilized than galvinoxyl. The ESR signal intensity in Figure 1 shows that only a small fraction of 2-hydroxyestradiol that reacted with galvinoxyl is observed as a radical by ESR. Probably the semiquinone radical reacts with another galvinoxyl radical to give a stable quinone. As reported by Kalyanaraman *et al.*,[26] the semiquinone radical is stabilized by magnesium(II) and zinc(II) ions, as shown in Figure 2.

The oxidation of methyl linoleate (LH) in homogeneous solution induced by an azo radical initiator proceeds by the well known free radical-mediated chain mechanism.[15,16,27-30] Under these conditions, the rate of oxidation in the absence of an antioxidant (R_p) is given by

$$R_p = k_p[\mathrm{LH}](R_i/2k_t)^{1/2} \quad (2)$$

where R_i is the rate of chain initiation and k_p and k_t are the rate constants for the chain propagation and termination reactions, respectively.

In the presence of a chain-breaking antioxidant (IH), the chain termination proceeds by reactions (3) and (4) instead of the bimolecular interaction of peroxyl radicals:

$$\mathrm{LO_2}^{\cdot} + \mathrm{IH} \xrightarrow{k_{inh}} \mathrm{LOOH} + \mathrm{I}^{\cdot} \quad (3)$$

$$(n-1)\mathrm{LO2}^{\cdot} + \mathrm{I}^{\cdot} \longrightarrow \text{stable products} \quad (4)$$

where $\mathrm{LO_2}^{\cdot}$ is lipid peroxyl radical, k_{inh} is the rate constant for scavenging of peroxyl radical by an antioxidant and n is the stoichiometric number of peroxyl radicals trapped by each antioxidant.

The rate of inhibited oxidation (R_{inh}) in the presence of antioxidant is given by the equation (5), and the length of the inhibition period (t_{inh}) is given by equation (6).[27-30] Figure 4 shows that, as expected from equation (6), the length of inhibition period is proportional to the antioxidant concentration.[27-30]

$$R_{inh} = \frac{k_p[\mathrm{LH}]R_i}{nk_{inh}[\mathrm{IH}]} \quad (5)$$

$$t_{inh} = \frac{n[\mathrm{IH}]}{R_i} \quad (6)$$

The antioxidant activity is determined by the competition between the inhibition reaction (3) and the chain propagation reaction (7). The faster the antioxidant scavenges the peroxyl radical before the peroxyl radical attacks the lipid, the higher is the antioxidant activity. The ratio of the rates of reactions (3) and (7) is given by equation (8).

$$\mathrm{LO_2}^{\cdot} + \mathrm{LH} \longrightarrow \mathrm{LOOH} + \mathrm{L}^{\cdot} \quad (7)$$

$$\frac{\text{Rate (3)}}{\text{Rate (7)}} = \frac{k_{inh}[\mathrm{LO_2}^{\cdot}][\mathrm{IH}]}{k_p[\mathrm{LO_2}^{\cdot}][\mathrm{LH}]} = \frac{k_{inh}[\mathrm{IH}]}{k_p[\mathrm{LH}]} \quad (8)$$

Thus, the ratio k_{inh}/k_p is important in determining antioxidant activity and gives its quantitative measure. Equations (5) and (6) give

$$\frac{k_{inh}}{k_p} = \frac{[\mathrm{LH}]}{R_{inh}t_{inh}} \quad (9)$$

The length of the inhibition period and the rate of oxidation during the inhibition period can be measured experimentally. Since the concentration of the substrate is known, the ratio k_{inh}/k_p can be calculated from equation (9).

Obviously, for the measurement of k_{inh}/k_p it is required that equations (5) and (6) hold, the rate of chain initiation is constant throughout the experiment and the kinetic chain length is long even during the inhibition period. The data in Tables 1 and 2 and in Figure 4 and also those reported in the literature[27-30] show that equations (5) and (6) hold. One of the advantages of using an azo compound as a radical initiator is that the rate of chain initiation is constant and can be measured from equation (6). The half-lives of AAPH and AMVN at 37 °C are about 1 week and hence the consumption of these initiators in the first few hours is negligibly small and the rate of chain initiation is substantially constant. Admittedly, these azo initiators are not biologically relevant, but the experiment using these compounds can be a model for biological oxidations and a useful tool for their elucidation. It may be worth noting that the kinetic chain length, and the importance of the chain reaction, can be estimated only when the rate of chain initiation is known and also that the rate and products of oxidation of lipids after the chain initiation are not dependent on the nature of the initial attacking species.

The data in Figure 3 give $t_{inh} = 5{\cdot}1 \times 10^3$ s and $R_{inh} = 7{\cdot}75 \times 10^{-8}$ mol l^{-1} s^{-1} and since [LH] = 453 mM, k_{inh}/k_p is obtained as $1{\cdot}1 \times 10^3$ for 2-hydroxyestradiol at 37 °C. Tables 1 and 2 show that the ratio k_{inh}/k_p for 2-hydroxyestradiol was $1{\cdot}9 \times 10^3$ and $2{\cdot}1 \times 10^2$ for methyl linoleate in aqueous dispersions and liposomal membranes, respectively. These values are close to those for α-tocopherol under similar conditions.[31] It has bee shown that the high reactivity of α-tocopherol toward peroxyl radical is due to stereoelectronic effects, that is, α-tocopherol has a structure such that the α-tocopheroxyl radical is highly resonance stabilized.[32] The high reactivity of 2-hydroxyestradiol, much higher than that of β-estradiol, must stem from

the polar effect of the hydroxyl group at the 2-position and the stabilization of the phenoxyl radical derived from it. As observed for α-tocopherol,[31] k_{inh}/k_p in liposomal membranes is smaller than that in homogeneous solution or in aqueous emulsions.

The above results and discussion show that 2-hydroxyestradiol is a strong radical scavenger and can function as a potent antioxidant. However, this does not mean that 2-hydroxyestradiol and other phenolic estrogens do in fact act as antioxidants *in vivo*. The real biological systems are heterogeneous in nature and the site of radical generation and the location of antioxidant as well as the inherent reactivity and total concentration of the antioxidant are important in determining the efficacy of antioxidants.[2] Relatively high concentrations of catechol estrogens have been found in pituitary, hypothalamus and cerebral cortex,[33,34] but it is not known if the estrogens or catechol estrogens really function as antioxidants *in vivo*, nor is it certain whether estrogens, which are female hormones, have any effect on the longer average life span of women than men. It may be also worth noting that the free radicals from 2-hydroxyestradiol have been implicated in their toxicity.[35-37]

In any event, it may be concluded that 2-hydroxyestradiol, a major metabolite of 17 β-estradiol, is a strong antioxidant having a similar reactivity to α-tocopherol, that is, similar n and k_{inh}.

ACKNOWLEDGEMENT

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