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Free Radicals in the 1900's: from in Vitro to in Vivo

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Remarkable progress has been achieved in the past 100 years in the field of free radical chemistry, biology and medicine since the discovery of free radicals in 1900. Free radical-mediated processes play a major role in the present industrial chemistry, but they also cause deleterious effects on rubber, plastics, oil products and foods. The importance of free radicals in vivo has been recognized increasingly from both positive and negative sides. Free radicals play an important role in phagocytosis, the production of some biologically essential compounds and possibly cell signaling. At the same time, they may cause oxidative modification of biological molecules, which leads to oxidative damage and eventually to various diseases, cancer and aging. The role and beneficial effects of antioxidants against such oxidative stress support this view. Furthermore, novel issues have been continuously found in this fascinating and yet controversial field of free radicals in biology. In this short article, the past work, present problems and future perspectives of free radicals in life science will be briefly discussed.

Keywords: free radical, autoxidation, oxidative stress, antioxidant

FREE RADICALS IN VITRO

In 1900, Gomberg [1] published a paper entitled "An Instance of Trivalent Carbon: Triphenylmethyl." This is accepted as the first scientific evidence which shows the presence of a free radical having an unpaired odd electron. The triphenylmethyl radical is stable and persistent due to its high resonance and steric stabilization. Remarkable advancements occurred in the 1930's in the scientific and applied fields of free radical chemistry. Mayo and Kharasch [2] discovered that the addition of hydrogen bromide to a double bond in the presence of benzoyl peroxide gave products which were not expected from the Markovnikov theory. For example, the reaction of 3-bromopropylene and hydrogen bromide gave 1,3-dibromopropane rather than 1,2-dibromopropane which is selectively formed in ionic reactions. They called such observation as a peroxide effect, since the formation of unexpected products were attributed to benzoyl peroxide. Later, together with Walling, they explained the reason for such an anti-Markovnikov addition by the free radical-mediated addition reaction which proceeds by a chain mechanism. They showed that such an unexpected addition reaction was not specific for peroxide-induced reaction but, rather, was typical of free radical reactions. Hey and Waters also suggested the involvement of neutral radicals in a few chemi-

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cal reactions in the liquid phase. In 1934, Haber and Weiss [3] reported free radical mechanisms in the iron-catalyzed decomposition of hydrogen peroxide. This explained the strong oxidizing capacity of the combination of iron and hydrogen peroxide which Fenton first discovered in 1894. [4] The Imperial Chemical Industries of UK succeeded in the synthesis of polyethylene from ethylene in the 1930's. The low density polyethylene prepared by this free radical addition reaction under high pressure is one of the most important and widely-used synthetic polymers even today. In the early 1940's, the production of synthetic rubber became practically important in the USA because the import of natural rubber from south east asia was stopped due to an outbreak of the World War II.

Thus, scientific, industrial and political events all acted synergistically to promote free radical chemistry. Advancements in our understanding of free radical reactions such as hydrogen atom abstraction, addition, polymerization, aromatic substitution, dissociation, and rearrangements have been summarized by Walling in a milestone book entitled Free Radicals in Solution. [5] The free radical-mediated oxidation of hydrocarbons by atmospheric oxygen as an oxidant, often referred to as autoxidation, becomes important industrial processes; for example, oxidation of cyclohexane and para-xylene is still important for synthesis of raw materials for synthetic fiber and plastics.

As the fundamental science and industrial application of oxidation proceed, the oxidative degradation and deterioration of rubber, plastics and foods and their inhibition became the subject of extensive studies. The kinetics of oxidation of olefinic compounds were studied quite extensively by a group of British Rubber Producers' Research Association. [6] The role of vitamin C and vitamin E as an antioxidant was known and the combination of these two vitamins was proposed for synergistic antioxidant effect in the 1940's. [7]

FREE RADICALS IN VIVO

The role and importance of free radicals in biological systems initially received less attention, but the involvement of free radicals has been implicated in oxygen poisoning and X-ray irradiation. Gershman et al. [8] reported in 1954 that irradiation and oxygen poisoning might produce some of their lethal effects through at least one common mechanism, that is, the formation of oxidizing free radicals. In 1956, Harman [9] proposed a free radical theory of aging in which he proposed that human aging may be caused by the sum of free radical reactions which proceed continuously throughout the cells and tissues. The discovery by Fridovich and McCord [10] in 1969 of superoxide dismutase (SOD) created a burst of studies on negative role of free radicals and active oxygen species in biological systems. The involvement of free radicals in liver damage as induced by carbon tetrachloride was also studied in 1970. Numerous monographs and scientific meetings on free radicals in biology have been published and held in the 1970's. Since then, the physical properties and reactions of free radicals and active oxygen species, free radical-induced oxidative damage, and role and action of antioxidants both in vitro and in vivo have been the subjects of extensive studies. However, a number of issues that need to be clarified or demonstrated remain.

OXIDATION OF BIOLOGICAL MOLECULES

Lipids, proteins and DNA are the major target of free radicals and active oxygen species. Above all, the polyunsaturated fatty acids and their esters are quite susceptible to radical attack and oxidation. Their oxidation, often referred to as lipid peroxidation, has been studied extensively since the 1970's and the mechanisms and kinetics are now fairly well understood especially in homogeneous solution. [11]

The oxidation of linoleic acid and arachidonic acid, and their esters, the major polyunsaturated lipids in vivo, have been studied in detail. The initial site being attacked depends on the type of free radicals. For example, a hydroxyl radical is capable of abstracting hydrogen atom from both bisallylic and other hydrogens but it adds to a double bond more favorably. [12] In any event, the resulting carbon centered radical reacts rapidly with oxygen to give peroxyl radical, which reacts with bisallylic hydrogen selectively. Thus, peroxyl radicals act as a chain propagating species to carry chain reaction of lipid peroxidation. [13] Consequently, an H atom from C11 is abstracted to give pentadienyl radical, to which oxygen molecule adds rapidly, not to the C11 position, but to mesomeric C9 and C13 radical sites because of the resonant nature of the bisallylic radicals and stabilization by conjugate diene formed. [14]The resultant C9 and C13 peroxyl radicals react with another polyunsaturated lipid to give the corresponding hydroperoxides. Similarly, arachidonic acid gives peroxyl radicals of C5, C8, C9, C11, C12 and C15 positions. It is now well understood from an elegant study of Porter how *cis, trans* and *trans, trans* hydroperoxides are formed from *cis, cis* polyunsaturated lipids. [11]

Lipoxygenase is another important oxidant in vivo. [15] It is known that the enzymatic oxidation of arachidonic acid and linoleic acid gives regio-, stereo-, and enantio-specific hydroperoxides. For example, 15-lipoxygenase oxidizes linoleic acid and its esters to give 13(S)-9Z,11E-hydroperoxyoctadecadienoic acid and its esters (HPODE) predominantly. 15-Lipoxygenase oxidizes even cholesteryl ester as well as phospholipids in low density lipoprotein(LDL) and human plasma in the presence of ample endogenous antioxidants such as vitamin C and vitamin E. [16]

The different product specificity of the free radical and lipoxygenase-mediated oxidation has been used to identify the responsible oxidant. For example, the enantioisomer ratio S/R larger than 1 suggests the involvement of 15-lipoxygenase in vivo. An analysis of enantio isomers by a chiral-phase high performance liquid chromatography(HPLC) is quite useful for idetifying the enzymatic oxidation. As shown in Fig. 1, mammalian 15-lipoxygenase oxidizes linoleic acid esters in human plasma to give 13–9Z,11E-hydroperoxyoctadecadienoates selectively and it is largely the S-form, while the free radical-mediated oxidation of human plasma gives mixtures of four isomers of HPODE and 13–9Z,11E-HPODE is a racemic product, that is, both the S and R forms are formed in equal amounts. [16] It is noteworthy that the specificity of lipid peroxidation induced by lipoxygenase depends on lipoxygenase, the substrates and the milieu.

The issue of whether lipid peroxidation is a cause or consequence of oxidative damage, diseases and aging has often been argued. Although there is still no unequivocal evidence, the circumstantial evidence suggests that lipid peroxidation may play a causative role in oxidative damage. Firstly, lipid peroxidation of *cis,cis*-lipids to *cis,trans*- and *trans,trans*-lipid hydroperoxides may disturb the fine structure of biological membranes and may thus affect the permeability and functions of membranes. The mechanism by which lipid hydroperoxides exert toxicity directly is not known, although they may oxidize thiols, which may result in protein modification and/or enzyme inactivation. It has been reported that free cholesterol hydroperoxides are especially toxic and the toxicity has been interpreted to be caused by free radicals generated by the decomposition of cholesterol hydroperoxides. [17,18] However, free cholesterol is much less reactive toward oxygen radicals than polyunsaturated lipids and in fact it has been observed to be oxidized at the latest stage after most of the unsaturated lipids have been oxidized in the oxidation of human LDL. [19] If the toxicity is exerted by the radicals generated in the decomposition of hydroperoxides, it is difficult to explain why free cholesterol hydroper-

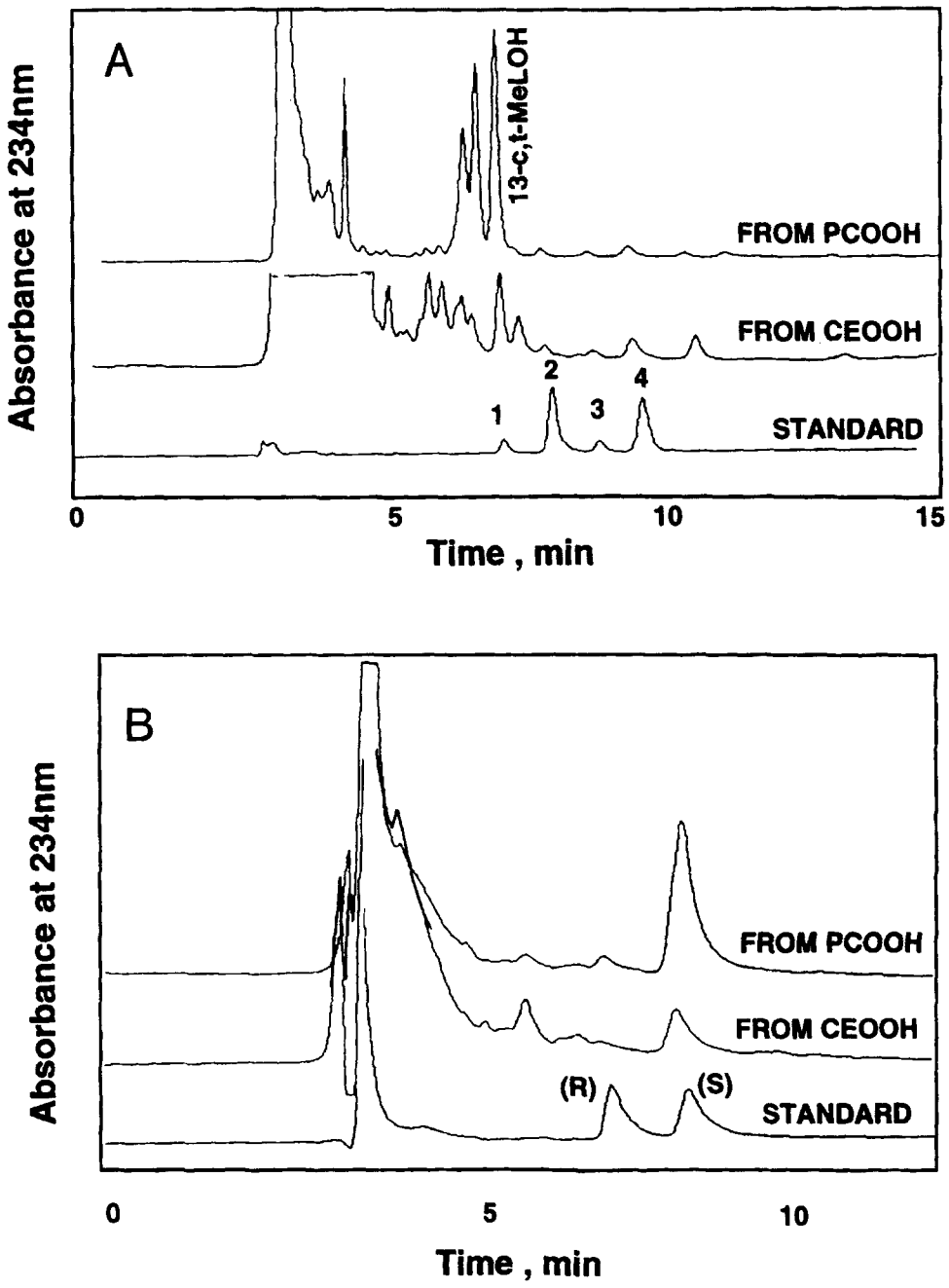

FIGURE 1 Geometrical and optical specificities in the oxidation of plasma by 15-lipoxygenase. Human plasma was incubated with rabbit reticulocyte 15-lipoxygenase (49 nkat/ml) at room temperature in air for 20 min. A: Phosphatidylcholine and cholesteryl ester hydroperoxides (PCOOH and CEOOH respectively) were collected and converted into methyl hydroxylinoleate (HODE) and analyzed with an HPLC. 1: 13–9Z,11-EHODE; 2: 13–9E,11E-HODE; 3: 9–10E,12Z-HODE; 4: 9–10E,12E-HODE. B: 13–9Z,11E-HODE from PCOOH and CEOOH were collected and analyzed with chiral phase HPCL. The standard products were obtained from the oxidation of methyl linoleate with AMVN and analyzed similarly

oxide is particularly toxic. It should be noted that free cholesterol could be an important substrate torward singlet oxygen since singlet oxygen oxidizes unsaturated compounds almost equally and free cholesterol is one of the major unsaturated compounds in the LDL surface layer.

It is generally known that the secondary oxidation products of lipid hydroperoxides exert more toxic effects than hydroperoxides. In particular, unsaturated aldehydes such as 4-hydroxynonenal and acrolein have been shown to be quite bioactive. [20] The presence of LDL modified by such aldehydes has been demonstrated in human atherosclerotic plaques. It was recently found by Takabe and Noguchi in this laboratory that acrolein acts as a promoter in the benzopyrene-initiated transformation of cells.

Another interesting issue is the biological effects of oxidized lipids on cellular signal transduction. [21] For example, oxidized LDL enhances the proliferation of smooth muscle cells and induces the release of various cytokines such as adhesion molecules and this may be the important reason for the pro-atherogenic effect of oxidized LDL. However, the physiological importance of such effects remain to be elucidated.

ROLE AND ACTION OF ANTIOXIDANTS

Antioxidants were first studied against oxidative deterioration and the degradation of foods and rubber. Various hindered phenolic and amine compounds have been synthesized and used as antioxidants for plastics, rubber, oils and food. With our increasing knowledge of oxidative stress in vivo, the role of antioxidants in biological systems has received considerable attention. It is now known that aerobic organisms are protected against oxidative stress by a well constructed defense system in which various antioxidants with different functions play pivotal roles (Table I). It is generally accepted that glutathione peroxidases play an important role in the defense system. [22]In addition to the classic, cytosolic glutathione peroxidase and gastric intestinal glutathione peroxidase, plasma glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase have been discovered. Phospholipid hydroperoxides and cholesteryl ester hydroperoxides are reduced directly by these enzymes. The dynamics of reduction of such lipophilic hydroperoxides by glutathione peroxidases have been often studied by using isolated hydroperoxides, but intact cell membrances or lipoproteins should be used as a substrate.

TABLE I Defense systems *in vivo* against oxidative damage

1. Preventive antioxidants: suppress the formation of free radicals	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide	
catalase	decomposition of hydrogen peroxide $2H_2O_2 \rightarrow 2H_2O+O_2$
glutathione peroxidase (cellular)	decomposition of hydrogen peroxide and free fatty acid hydroperoxides $H_2O_2+2GSH \rightarrow 2H_2O+GSSG$ $LOOH+2GSH \rightarrow LOH+H_2O+GSSG$
glutathione peroxidase (plasma)	decomposition of hydrogen peroxide and phospholipid hydroperoxides $PLOOH+2GSH \rightarrow PLOH+H_2O+GSSG$
phospholipid hydroperoxide glutathione peroxidase	decomposition of phospholipid hydroperoxides
peroxidase	decomposition of hydrogen peroxide and lipid hydroperoxides $LOOH+AH_2 \rightarrow LOH+H_2O+A$ $H_2O_2+AH_2 \rightarrow 2H_2O+A$

glutathione-S-transferase	decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
transferrin, lactoferrin	sequestration of iron
haptoglobin	sequestration of hemoglobin
hemopexin	stabilization of heme
ceruloplasmin, albumin	sequestration of copper
(c) Quenching of active oxygens	
superoxide dismutase (SOD)	disproportionation of superoxide $2O_2^{\cdot-}+2H^+ \rightarrow H_2O_2+O_2$
carotenoids, vitamin E	quenching of singlet oxygen
2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation	
hydrophilic: vitamin C, uric acid, bilirubin, albumin	
lipophilic: vitamin E, ubiquinol, carotenoids, flavonoids	
3. Repair and *de novo* enzymes: repair the damage and reconstitute membranes	
lipase, protease, DNA repair enzymes, transferase	
4. Adaptation: generate appropriate antioxidant enzymes and transfer them to the light site at the right time and in the right concentration	

The discovery of SOD was quite important in attracting attention of the role of free radicals in biology. However, it was not always clear why SOD has to dismutate superoxide which is not so active per se. However, the discovery by Beckman and his colleagues [23] that superoxide reacts rapidly with nitric oxide (NO) to form peroxynitrite which is capable of inducing oxidative damage suggests the underlying mechanism of the action of SOD as an antioxidant. In fact, it has been observed that SOD inhibits oxidative damage induced by NO quite effectively.

Vitamin E and vitamin C are major lipophilic and hydrophilic radical-scavenging antioxidants in vivo respectively. The physico-chemical properties of vitamin E and its radical and the mechanisms and kinetics of antioxidant action of vitamin E, especially in homogeneous solution, have been studied extensively in the 1980's and are now well understood. [24,25] Vitamin E is present in vivo in the lipophilic domain of cell membranses and lipoproteins where lipids and proteins are neatly packed. Under such circumstances, the antioxidant action and efficacy of vitamin E might be different from those in solution such as frying oil. It has been shown experimentally that the antioxidant activities are determined by the local concentration and mobility within the lipophilic compartment and the efficacy of the interaction with other antioxidants, as well as the inherent chemical reactivity of vitamin E. The type of active radicals and the sites of their generation are also important. Vitamin E and ubiquinol, a reduced form of coenzyme Q which acts as another important lipophilic radical-scavenging antroxidant, have a long side chain which, like an anchor, facilitates their incorporation and retainment in membranes and lipoproteins. On the other hand, such long side chain decreases the mobility of antioxidants within and between the membranes and lipoproteins and the antioxidant activities decrease with increasing side chain length. [26] The length of the side chains appears to be determined by nature for specific antioxidants and animal species.

Thus, the antioxidant efficacy of vitamin E was found to be considerably smaller in the membranes and LDL. [27,28] Hydrogen bonding between phenolic hydrogen and a hydrogen bond accepting solvent such as water has also been proposed to be responsible for the decreased antioxidant activity in the membrane. [29,30] The fact that the antioxidant activities of vitamin E analogues decrease with increasing

side chain length at the 2-position suggests that the mobility within the lipophilic compartment is also an important factor. It has been also reported that vitamin E analogue with shorter side chain escapes from LDL particles into aqueous phase more easily, which increases the apparent antioxidant activity by exporting radicals out of the oxidation site. [31]It has been experimentally shown that the antioxidant activities of vitamin E analogues and ubiquinols with side chains of different length incorporated into the membranes against the oxidation of different membranes decrease with increasing side chain length. [28,32] Furthermore, a study using stearic acid which contained nitroxide radical along the chain as a spin probe showed that the efficacy of radical scavenging by vitamin E decreased as the radical penetrated deeper into the interior of the membranes [27] and LDL [28] and that such an effect was not observed for 2,2,5,7,8-pentamethyl-6-chromanol (PMC).

Such physical effects are easily observed in the experiments shown in Figs. 2 and 3. In an organic homogeneous solution, α-tocophenol, PMC, and 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox) exert similar antioxidant activities, while 2,6-di-tert-butyl-4-methylphenol (BHT) is a much weaker antioxidant. In homogeneous solution, the antioxidant activity is determined primarily by its chemical reactivity toward the radical. In membranes, however, the relative reactivities are determined by other factors as mentioned above such as location of the antioxidant and radical and antioxidant mobility. Another example is shown in Fig. 3. β-Carotene is chemically about 30 times less reactive toward peroxyl radicals than α-tocopherol. When both α-tocopherol and β-carotene are present in equal concentrations in homogeneous solution, α-tocopherol is preferentially consumed and little consumption of β-carotene is observed. In the membranes, the active site of α-tocopherol is located at the membrane surface, while β-carotene which is lacking in hydrophilic group is assumed to be localized in the interior of the membrane. α-Tocopherol scavenges aqueous peroxyl radicals faster than β-carotene, but β-carotene scavenges peroxyl radicals within the membranes faster than α-tocopherol. These results clearly show that physical factors are also important in determining antioxidant activity against the oxidation of membranes and lipoproteins..

. The above system is convenient for evaluating antioxidant action. However, the fact that β-carotene scavenges faster than α-tocopherol does not necessarily mean that β-carotene is superior to α-tocopherol as an antioxidant in this system. The fate of antioxidant-derived radicals is another factor that should be considered. β-Carotene scavenges peroxyl radicals via an addition reaction, not by the donation of hydrogen atom to a radical. [33] The resulting β-carotene radical is highly resonance-stabilized, but it reacts with oxygen to give peroxyl radical and may continue the chain oxidation. Thus, the total antioxidant activity is determined by the fate of the β-carotene radical, that is, the relative importance of the following competing reactions. The antioxidant efficacy of β-carotene is another interesting issue and has been the subject of lively argument. The oxidation products or metabolites may be also important.

Polyphenolic compounds such as flavonoids and the catechins of green tea, fruits, spice, and wines have also received much attention. In vitro structure-activity relationship and in vivo biological activities have been studied. In order to evaluate the antioxidant activity of polyphenols, the reactivity toward radicals and antioxidant activities in various model systems have been often measured. It should be clearly appreciated what specific activity is being measured in the particular system, the method employed and that the biological antioxidant potency in vivo is determined not simply by the reactivity toward radical alone, but by the many factors mentioned above. It must be also borne in mind that polyphenols are present, not in their free form, but as metabolites such as glucuronides. The reactivities toward radicals and localizations could be different between the free form and metabolites.

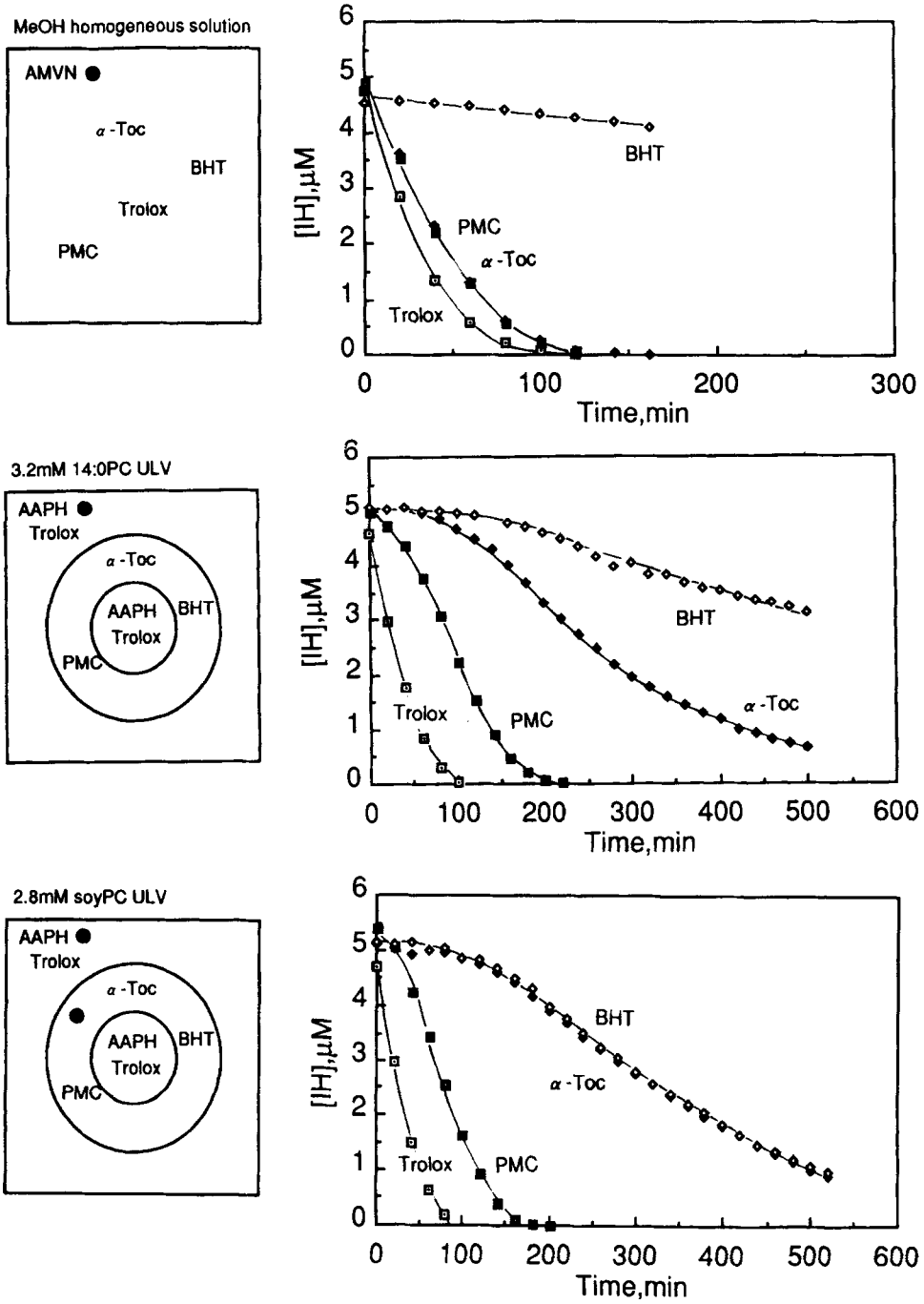

FIGURE 2 Rates of consumption of antioxidants. Equal concentrations of α-tocopherol (α-Toc), 2,2,5,7,8-pentamethyl-6-chromanol (PMC), 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox) and 2,6-di-tert-butyl-4-methylphenol (BHT) were treated with AMVN or AAPH in different media and their consumption was followed with an HPLC. (1) In methanol; (2) dimyristoyl phosphatidylcholine liposomal membranes aqueous suspension, and (3) soybean phosphatidylcholine liposome aqueous suspensions

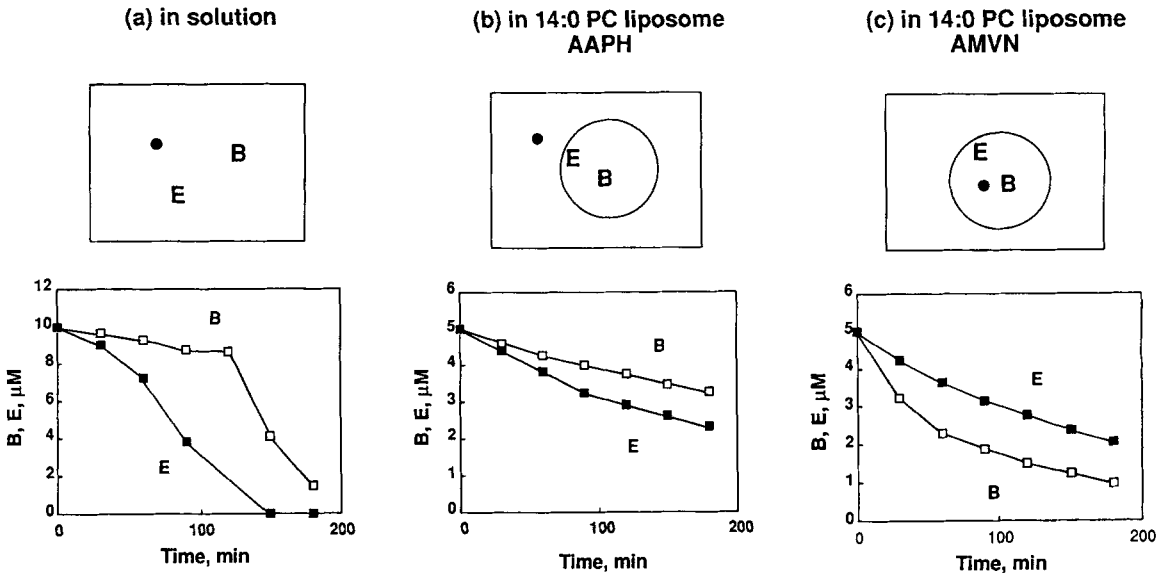

FIGURE 3 Rates of consumption of α-tocopherol (E) and β-carotene (B) induced by AMVN (a and c) or AAPH (b). (a): in acetonitrile. (b) and (c): in dimyristoyl phosphatidylcholine liposomes

The interaction between antioxidants which was referred to by Packer as the antioxidant network [34] is another important issue. An antioxidant does not act alone in vivo but functions in combination and in competition with other antioxidants. The synergistic antioxidant effect by a combination of vitamins E and C has, in particular, received much attention. The physiological significance of such a combination effect remains to be proved in the future.

As described above, NO might act as a strong oxidant in vivo along with superoxide. It was shown that NO can also act as a strong radical-scavenging antioxidant. [35]This is a radical-radical coupling reaction and proceeds quite rapidly. The stability of the adducts affect the antioxidant potency of NO.

ANTIOXIDANT DRUGS

With increasing experimental, clinical and epidimological evidence which suggests the involvement of oxidative damage induced by free radicals and active oxygen and nitrogen species in a variety of diseases, cancer and aging, the role of antioxidants has received much attention. For example, antioxidant drugs have been examined for preventive and therapeutic purposes. Natural antioxidants and extracts from fruits, plants, spices and vegetables have been used as health supplements. A number of synthetic antioxidant drugs have been developed. [36] Modified SOD, SOD mimics, glutathione peroxidase mimics, NOS inhibitors, lipoxygenase inhibitors, and phenolic antioxidants are examples. An understanding of the basic science of oxidative damage in vivo is essential for the development of proper and potent antioxidant drug.

Probucol, 4,4'-isopropylidenethio-bis-(2,6-di-tert-butylphenol), is a drug used for treatment of atherosclerosis. It is known to reduce cholesterol levels but its antioxidant capacity is also accepted to be one of the functions. It has been shown in many in vitro and in vivo studies that probucol suppresses the oxidative modification

of LDL and that it inhibits the development of atherosclerosis in model experiments using rabbits. However, important questions still remain. The first question is the effect of species. It has been shown by several groups that probucol inhibits the progression of atherosclerosis of rabbit but enhances it in mice. The reason for this is not known yet. Interestingly bisphenol, a reduced form of diphenoquinone which is an oxidation product of probucol, was observed in high concentration in rabbit plasma. Bisphenol is capable of acting as a radical-scavenging antioxidant and also reduces the α-tocopheroxyl radical, [37] although much more slowly than ascorbate. Bisphenol may play a role in the rabbit. α-Tocopherol is much more reactive toward peroxyl radical than probucol and α-tocopherol reduces the phenoxyl radical derived from probucol. In the in vitro oxidation of LDL separated from rabbits or patients taking probucol, probucol is not oxidized but it is spared quite effectively while α-tocopherol is present. It is not known why bisphenol is observed in such high concentration in the plasma of rabbit in spite of the presence of α-tocopherol. Neither bisphenol nor diphenoquinone, an oxidized form of bisphenol, was observed in appreciable amount in human plasma of patients receiving daily doses of probucol.

SIGNAL TRANSDUCTION BY OXIDANTS AND ANTIOXIDANTS

The emerging data suggest that free radicals, active oxygen species, and active nitrogen species can exert a diverse range of effects on cells ranging from proliferation to induction of necrosis or apoptosis. At the same time, interestingly, antioxidants can also activate the expression of a number of genes and signal transduction pathways. Thus, free radicals, oxidants and antioxidants can display more versatile and wider biological effects and functions than was first realized. Interestingly, cells must have evolved strategies to utilize these active species as biological stimuli. It is known that active species such as superoxide, hydrogen peroxide, hydroxyl radical and NO induce apoptosis, which is accepted as a form of programmed cell death. The question is how the type, amount, site and time of active species formation are regulated. It is noteworthy that AAPH, a synthetic water soluble azo compound which generates aqueous peroxyl radicals, can also induce apoptosis as well as necrosis. In other words, apoptosis can also be induced by non-physiological free radicals.

Lipid hydroperoxides may influence the cellular redox status and thus directly modulate the expression of redox-sensitive genes. It has been reported that lipid hydroperoxides induce the expression of c-fos, c-jun, and c-myc and activate mitogen-activated protein kinase.

The role of antioxidants beyond antioxidant function is also of interest and is now receiving much attention. [38] Azzi proposed that α-tocopherol acts as a sensor and an information transducer for the cells. [39] He found that α-tocopherol inhibited vascular smooth muscle cell proliferation at physiological concentrations by inhibiting protein kinase C activity not by directly binding to the enzyme but through changes in gene expression. Interestingly, β-tocopherol which also acts as a radical-scavenging antioxidant did not inhibit the proliferation of vascular smooth muscle cells but rather prevented the inhibition of cell growth and of PKC activity caused by α-tocopherol. Such an effect of β-tocopherol as an antagonist suggests that the above effect of α-tocopherol is independent of its radical-scavenging antioxidant properties.

There are increasing number of reports which show the role of α-tocopherol in cell regulation. Inhibition by α-tocopherol or its derivatives of nuclear factor kappa B (NF-κB) activation or of the expression of adhesion molecules on endothelial cells has been observed. A wealth of such data observed in vitro and vivo systems

strongly suggests the importance of the beyond-antioxidant function of antioxidant, but the physiological importance of such effect remains to be elucidated in the future work.

EPILOGUE

Free radicals, which were discovered in 1900 were important in the fields of chemistry and chemical industry. Later, free radicals and related oxidants and antioxidants in biological systems received more attention and undoubtedly will be one of the most important issues in the coming century. The role of free radicals in and on the environment, such as the toxicity of chemicals and diesel exhausts and the effect of ClOx radicals derived from chlorofluorocarbons on ozone depletion in the stratosphere, will also be important issues. Key scientific milestones have been witnessed in these past several decades and the next decade will surely provide new insight at an even greater pace.

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