Assessment of Potential Prooxidant and Antioxidant Actions[1]

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ABSTRACT: Suggestions that oxidative stress plays a role in human diseases have led to the proposal that health might be improved by increased dietary intake of antioxidants. Plant-derived antioxidants, such as flavonoids or rosemary extracts, are increasingly proposed as important dietary antioxidant factors, and foods rich in antioxidants are also receiving attention. Before widescale usage of natural and synthetic antioxidants can be suggested, it is necessary to establish the properties of such molecules. Assays for characterizing the potential prooxidant/antioxidant actions of food additives, antioxidant supplements, antioxidant drug molecules, and nutrient components have been developed for this purpose.
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KEY WORDS: Antioxidants, DNA damage, flavonoids, free radicals, lipid peroxidation, oxidative stress, prooxidants, vanillin, vitamin C, vitamin E.

For the health-conscious consumer, the words "free radicals" and "antioxidants" have become fashionable. A free radical is any chemical species capable of independent existence that possesses one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. Conventionally, radicals are denoted by the insertion of a dot(·). The term "reactive oxygen species" (ROS) is often used to denote all oxygen-derived species. Table 1 presents examples of ROS and their characteristics (1). Excessive production of these species, beyond the antioxidant defense capacity of the body, can cause oxidative stress (2). In principle, oxidative stress also may be mediated by activation of phospholipases, increased activity of the radical-generating enzymes (e.g., xanthine oxidase) and/or their substrates (e.g., hypoxanthine), disruption of electron transport chains and increased electron leakage for $O_2^{\cdot -}$, release of free metal ions from sequestered sites and/or muscle, activation of cyclooxygenase and lipoxygenase, and release of heme proteins (hemoglobin, myoglobin). Free-radical oxidation in foods and in living organisms may be considered from the standpoint of four distinctive interfacial groups: bulk food lipids (e.g., oils), dispersed food lipids (e.g., membranes and emulsions such as salad dressing), dispersed lipids in living organisms (membranes and organelles), and free-radical reactions in watery fluids in organisms (e.g., cytoplasm, plasma). Prooxidant considerations may have different implications for each of the groups, e.g., they may be totally irrelevant for the bulk food lipids but highly relevant for the dispersed food lipids and biological fluids in organisms.

Free radical oxidation of the lipid components in foods by the chain reaction of lipid peroxidation is a major problem for food manufacturers (3–8). The extent to which oxidation of fatty acids and their esters occurs in foods depends on the chemical structure of the fatty acid, the nature of food processing and the temperature at which the foods are stored and/or cooked, and the minor constituent antioxidants. An antioxidant may be defined as a substance that, when present at low concentrations compared with those of an oxidizable substrate such as fats, proteins, carbohydrates or DNA, significantly delays or prevents oxidation of the substrate. Other definitions abound. For example, Porter (9) considered an antioxidant as "any acidic compound (including phenols) usable in foods which can readily donate an electron or a hydrogen atom to a peroxyl or alkoxy radical to terminate a lipid peroxidation chain reaction or to regenerate a phenolic compound, or which can effectively chelate a prooxidant transition metal." Food manufacturers continue to add antioxidants during food processing to minimize lipid oxidation (3,4,8,9). Antioxidants can act at different levels in the oxidative sequence that involves lipids. For example, they may act by decreasing localized oxygen concentrations; preventing first-chain initiation by scavenging initiating radicals, such as hydroxyl radicals; binding metal ions in forms that will not generate the lipid peroxidation-initiating species, such as hydroxyl radical, ferryl radical, or $Fe^{2+}/Fe^{3+}/O_2$ complexes, and/or will not decompose lipid peroxides to peroxyl or alkoxyl radicals; decomposing peroxides by converting them to nonradical products, such as alcohols; and/or by chain-breaking whereby intermediate radicals, such as peroxyl and alkoxyl radicals, are scavenged to prevent continued hydrogen abstraction. Porter *et al.* (10) recently discussed the current status of the mechanism of free-radical oxidation of unsaturated lipids.

Food manufacturers strive to produce products that maintain their shelf life and nutritional quality over a specific period under given conditions. For the consumer, the considera-

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TABLE 1
Examples of Reactive Oxygen Species (ROS)[a]

		Characteristics
Radicals		
Superoxide	$O_2^{\cdot -}$	Oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems, by autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. In aqueous solution, $O_2^{\cdot -}$ can oxidize ascorbic acid. It can also reduce certain iron complexes, such as cytochrome c and ferric-ethylenediaminetetraacetic acid (Fe^{3+} EDTA). Superoxide dismutase (SOD) accelerates the dismutation of $O_2^{\cdot -}$, converting it to hydrogen peroxide (H_2O_2) and oxygen (O_2).
Hydroxyl	$OH^{\cdot}$	A highly reactive oxygen-centered radical, which attacks all molecules in the human body, including tissues that may occur in the myocardium.
Peroxyl, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Typically organic radicals often encountered as intermediates during the breakdown of peroxides of lipids in the free-radical reaction of peroxidation. $CCl_3O_2^{\cdot}$ has been used extensively to study potential antioxidant action of biomolecules.
Oxides of nitrogen	$NO^{\cdot}$, $NO_2^{\cdot}$	Nitric oxide is formed *in vivo* from the amino acid L-arginine. Nitrogen dioxide is formed when NO reacts with O_2 and is found in polluted air and smoke, such as from cigarettes.
Nonradicals		
Hydrogen peroxide	H_2O_2	Formed *in vivo* when $O_2^{\cdot -}$ dismutates and also by many oxidase enzymes. H_2O_2 at low (micromolar) levels also appears poorly reactive. However, higher levels of H_2O_2 can attack several cellular energy-producing systems; for example, it inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. H_2O_2 also forms $OH^{\cdot}$ in the presence of transition metal ions and $O_2^{\cdot -}$ can facilitate this reaction.
Hypochlorous acid	HOCl	A powerful oxidant often present in household bleaches but formed in the human neutrophils at sites of inflammation by action of the enzyme myeloperoxidase. May also react with $O_2^{\cdot -}$ to generate $OH^{\cdot}$ in neutrophils.
Ozone	O_3	Formed in the environment inhabited by humans. This noxious gas has been shown to deplete plasma antioxidants vitamin D, vitamin E, and uric acid.
Singlet oxygen	$^1O_2$	The spin of one of the electrons of the two outer orbitals is inverted, removing the quantum-mechanical spin restrictions of molecular oxygen.

[a]Adapted from Reference 1.

tion is somewhat different. Almost all foreign materials ingested undergo biochemical transformation and are processed such that the body derives, where applicable, benefits from the ingested food. The chemical nature of the food components, effects of components within the food matrix, the diet under consideration, and the health status of the consumer all combine to affect the bioavailability of the nutritive component (11). Products from oxidation of lipid components (12–15) or of other substrates in the food, which may accumulate in deteriorated foods, can become available when consumed. They may present pharmacological problems to the consumer if such products are absorbed in high enough quan-

tities. The effect of cholesterol oxidation products in cholesterol-containing foods in the human diet is illustrative. There is compelling evidence that several cholesterol oxides are cytotoxic, atherogenic, mutagenic, and carcinogenic (14,16). Cholesterol molecules are closely associated with membrane phospholipids and contain a Δ^5-double bond, which is prone to oxidation (17). Antioxidants indigenous to foods (3,4,18,19) and oil-soluble antioxidants in foods, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), esters of 3,4,5-trihydroxybenzoic acid (propyl, octyl, and dodecyl esters), ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethyl quinoline) (used mostly in animal feeds), and dl-α-tocopherols, continue to be used commercially (4,20). However, there have been numerous concerns (although no data exist for humans) about the toxicity of synthetic antioxidants (21–28). Nevertheless, there is an interest in the use of natural antioxidants, such as those from rosemary extracts, in the preservation of food materials. Plant extracts that also have been proposed to contain antioxidant capabilities include cocoa shells, oats, tea, olives, garlic, ginger, red onion skin, grapes, apple cuticle, wheat gliadin, korum rind, licorice, nutmeg, clove, oregano, thyme (*Thymus vulgaris*), mustard leaf seed, chia seed, peanut seed coat, birch bark, carob pod, tempeli, yam, mango, mangostum, and *Vanilla planifolia*. Flavonoids and other polyphenols found in some of these extracts are widely discussed as potential antioxidant prophylactics (1,29,30). Flavonoids are naturally occurring benzo-γ-pyrone derivatives, which are ubiquitous in plant cells and are therefore accessible to consumers through diets of plant origin (31,32). Ramanathan and Das (33) have showed that addition of polyphenols and antioxidants reduced the extent of lipid peroxidation, measured by thiobarbituric acid (TBA) number, in raw fish stored at 4°C and in steam-cooked fish stored for 1 wk at 4°C and −20°C. Lipids in fish are more prone to lipid peroxidation than the lipid components in meat due to the high degree of unsaturation in fish lipids and to high concentrations of metal ions, especially in seafoods (34). Flavonoids are used commercially and are often found in milk, lard, and butter. They are combined with synergists, such as citric acid, ascorbic acid or phosphoric acid (4), and are components of many folk medicines (35,36).

Phenolic antioxidants, such as carnosic acid or carnosol from rosemary, have been shown to stimulate free-radical damage to nonlipid components, such as DNA, proteins, and carbohydrates, by *in vitro* assays (37–39). This has led to the development of experimental tools for characterizing potential prooxidant and/or antioxidant actions (1,38–42). The emerging *in vitro* data would help delineate the *in vivo* contribution of antioxidants to modulation of the pathological consequences of free radicals in the human body as well as evaluation of the potential uses of natural antioxidants during food processing.

The need for the food manufacturer to produce quality products that have reasonable shelf lives by use of food-grade antioxidants during food processing must be balanced by the increasing interest in the use of antioxidants as prophylactics in human degenerative diseases.

PROOXIDANT ACTION AND ITS MEASUREMENT *IN VITRO*

Table 1 highlights some of the reactive oxygen species often discussed in the literature and their biological characteristics. In Figure 1, some current tools for characterizing antioxidant/prooxidant actions (reviewed in Refs. 1, 42, and 43) are summarized. The remainder of this paper will discuss the assays for prooxidant action.

Hydroxyl radical ($OH^{\cdot}$) is often generated in the test tube by means of a reaction mixture that contains ascorbate, H_2O_2, and Fe^{3+}-EDTA at pH 7.4 (Eqs. 1 and 2) (44,45). Under the assay conditions, addition of ascorbic acid greatly increases the rate of $OH^{\cdot}$ generation by reducing iron and maintaining a supply of Fe^{2+}:

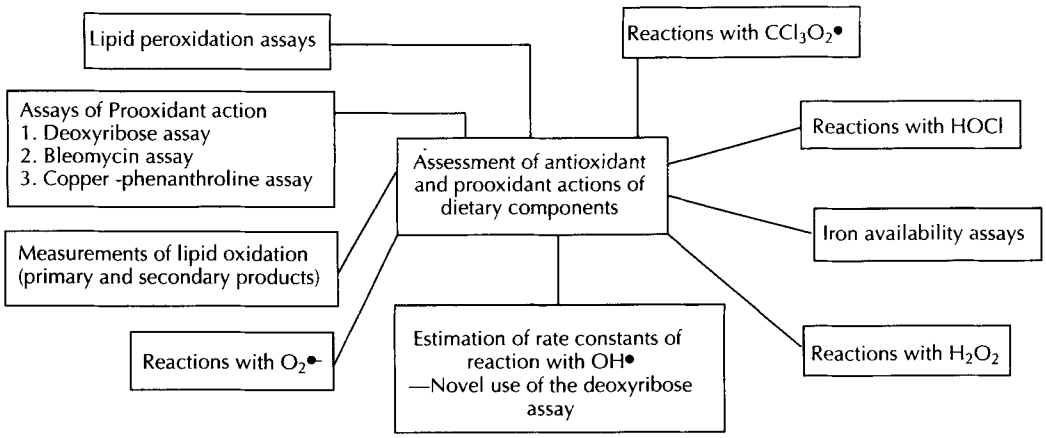

FIG. 1. Experimental approaches for the characterization of potential antioxidant and prooxidant actions.

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \quad [1]$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + OH^{\cdot} + OH^{-} \quad [2]$$

THE DEOXYRIBOSE ASSAY AND ITS APPLICATION

When the sugar deoxyribose (DR) is added to the system discussed above, the $OH^{\cdot}$-dependent oxidation is directed to DR to yield products (Eqs. 3, 4) that can be quantitatively measured as TBA–malondialdehyde (MDA) adducts (44,45). The DR assay has become a useful experimental tool for investigating the ability of molecules to react with $OH^{\cdot}$:

$$OH^{\cdot} + DR \rightarrow \text{fragment} \rightarrow MDA \quad [3]$$

$$2\ TBA + MDA \rightarrow \text{chromogen} \quad [4]$$

The extent of inhibition by antioxidants in the DR assay is dependent on the concentration of the molecule compared with DR and on its rate constant for reaction with $OH^{\cdot}$ (45).

Studies in which the DR assay is used can provide useful information on the likelihood that molecules could chelate iron ions in a way that prevents them from catalyzing $OH^{\cdot}$ formation. Thus, when iron is added to the assay mixture as ferric chloride instead of as ferric-EDTA, some of the Fe^{3+} ions bind to DR, and damage to the sugar becomes site-specific such that the $OH^{\cdot}$ formed by bound iron ions immediately attacks the DR (46). The ability of a substance to inhibit DR degradation under these reaction conditions is a measure of its ability to interfere with site-specific Fenton chemistry (47,48).

When ascorbate is omitted from the DR reaction mixture, the ability of added compounds to reduce the Fe^{3+}-EDTA complex (reaction 2) can be tested. This has been developed into an index for prooxidant action, allowing assessment of antioxidant molecules in nonlipid systems (1,46). It is the ability of the added compound to mediate reaction similar to that of ascorbate that constitutes the basis of the prooxidant test. "The Fe^{3+} -EDTA complex has a tested propensity to be reduced by the prooxidant (if, indeed, it is able to do so). It follows that the redox potentials of other metal complexes (which may be physiologically relevant) would vary" (1). This simple idea led to the proposal to use assays that involve DNA damage to specifically test for the abilities of dietary antioxidants to exert prooxidant actions, different from their intended abilities to minimize oxidation of lipids. The tests involve measurement of DNA damage in the presence of bleomycin–iron and copper–1,10-phenanthroline complexes.

OXIDATIVE DNA DAMAGE AND MEASUREMENT OF PROOXIDANT ACTIONS

Aside from lipids, DNA is also a major cellular component and is prone to oxidative attack. DNA damage is often measured as single-strand breaks, double-strand breaks, or chromosomal aberrations (49). Mechanisms involving the Fenton system, ionizing radiation, and nuclease activation have been suggested to account for much of the DNA damage that occurs in biological systems (50–58). In the Fenton mechanism, oxidative stress could cause release of catalytic copper or iron within cells, which could bind to DNA. Metal ions are among the naturally occurring metal constituents of the cell's nucleus (59,60). Generation of $OH^{\cdot}$ by reaction of H_2O_2 with the transition metal ions already bound onto the DNA would lead to strand breakage, base modification, and DR fragmentation. In the nuclease activation mechanism, oxidative stress leads to inactivation of Ca^{2+}-binding by endoplasmic reticulum, inhibition of plasma membrane Ca^{2+}-extrusion systems, and the release of Ca^{2+} from mitochondria. This sequence of events leads to increases in the levels of intracellular free calcium ions. The resulting endonuclease activation leads to DNA fragmentation without the base modification observed in the Fenton mechanism.

Hydroxyl radicals ($OH^{\cdot}$) induce extensive damage to all four bases in DNA to yield a variety of products (Fig. 2) (51,53,54,56,61,62). Thus, $OH^{\cdot}$ can add to guanine residues at C_4, C_5, and C_8 positions to give hydroxyguanine radicals that can have various fates. For example, addition of $OH^{\cdot}$ to C_8 of guanine produces a radical that can be reduced to 8-hydroxy-7,8-dihydroguanine, oxidized to 8-hydroxyguanine (8-OH-Gua) or can undergo ring opening, followed by one-electron reduction and protonation, to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FapyGua. $OH^{\cdot}$ can add on to C_4, C_5, or C_8 of adenine. Among other fates, the C_8 $OH^{\cdot}$ adenine radical can be converted into 8-hydroxyadenine (8-OH-Ade) by oxidation or can undergo ring opening, followed by one-electron reduction, to give 5-formamido-4,6-diaminopyrimidine (FapyAde). Pyrimidines are also attacked by $OH^{\cdot}$ to give multiple products. Thus,

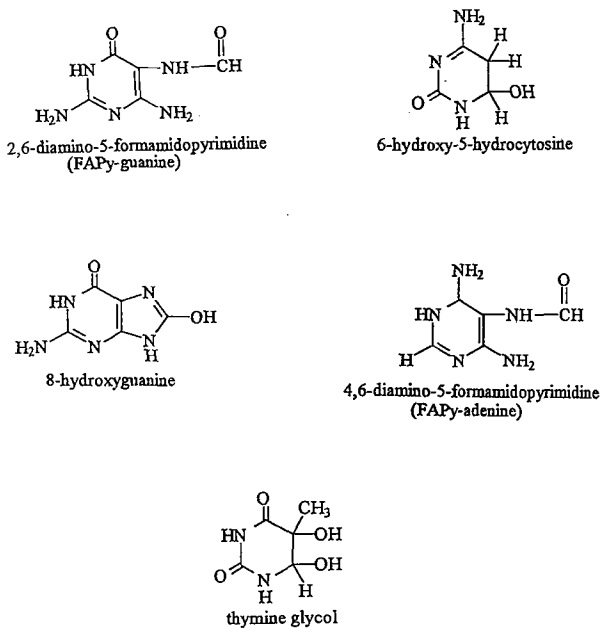

FIG. 2. Structures of modified bases in DNA induced by $OH^{\cdot}$ and identified by gas chromatography–mass spectrometry with selected ion monitoring (Refs. 61,62).

thymine can form *cis*- and *trans*-thymine glycols (5,6-dihydroxy-6-hydrothymines), 5-hydroxy-5-methylhydantoin, 5,6-dihydrothymine and 5-hydroxymethyluracil. Cytosine can form several products, including cytosine glycol and 5,6-dihydroxycytosine.

$OH^{\cdot}$ generation within whole cells or isolated chromatin can result in the formation of cross-links between DNA bases and amino-acid residues in nuclear proteins. Thus, thymine–tyrosine, thymine–aliphatic amino acid, and cytosine–tyrosine links have been identified in isolated calf thymus chromatin that was subjected to γ-irradiation or treated with metal ions and H_2O_2 (61). The ability of antioxidants to induce $OH^{\cdot}$-dependent base modification may therefore be used as a tool for assessing prooxidant potentials. It also follows that antioxidants may be assessed for their ability to inhibit DNA base modifications *in vitro*. The following example is illustrative of the rationale advocated.

The classic flavor of vanilla, prominent in certain foods, is attributed to vanillin and vanillic acid that occur in the fruit of *V. planifolia*, a tropical plant. Vanillin and vanillic acid protect lipid substrates from peroxidation (38,63,64). Incubation of calf-thymus DNA with a system that produces $OH^{\cdot}$ radicals gives rise to extensive chemical modification of the DNA bases in a way that appears to be diagnostic for $OH^{\cdot}$ radical. Thymine glycol, 8-hydroxyguanine, dihydroxycytosine, and formamidopyrimidines (Fig. 2) were measured by gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring (61,65).

Table 2 shows that incubation of DNA with Fe(III)-EDTA, ascorbate, and H_2O_2 led to significant rises in the amounts of several oxidatively-modified bases: this is characteristic of attack by $OH^{\cdot}$. Modification was inhibited by $OH^{\cdot}$ scavengers, such as mannitol, dimethylsulfoxide, and hypotaurine (Table 2). Trolox C, an effective scavenger of $OH^{\cdot}$, also inhibited the base modification (Table 2). Omission of ascorbate from the reaction mixture greatly decreased the DNA base modification (Table 2, first line), but vanillin restored some of it (Table 2, last line).

The results of GC–MS analysis of modified bases in DNA are often expressed as nanomoles of modified bases per milligram of DNA (equivalent to pmol/μg DNA). However, it is easy to convert these data into the actual number of bases modified. Dividing the amount of nmol bases/mg DNA by 3.14 (or multiplying by 0.318) gives the number of modified bases per 10^3 bases in DNA, i.e., 1 nmol/mg DNA corresponds to about 318 modified bases per 10^6 DNA bases. The *in vivo* significance of this prooxidant characteristic for vanillin is not at all clear, and much more work is needed in this area, primarily to evaluate the contribution of phenolic antioxidant compounds to oxidative damage in humans (see later discussions).

MEASUREMENT OF PROOXIDATION ACTION BY USE OF THE BLEOMYCIN–IRON-DEPENDENT DNA DAMAGE

Bleomycin, an antitumor antibiotic, binds to DNA by using its bithiazole and terminal amine residues, and also complexes with metals (such as iron) by using the β-aminoalaninepyrimidine-β-hydroxy histidine portion of the molecule (Fig. 3). The bleomycin assay was first described to measure nontransferrin bound iron in biological samples (66). Bleomycin binds iron ions, and the bleomycin–iron complex will degrade DNA in the presence of O_2 and a reducing agent, such as ascorbic acid. The reaction occurs by attack of a ferric bleomycin peroxide (BLM-Fe(III)-O_2H^-) on the DNA. The ferric peroxide can be formed by direct reaction of ferric–bleomycin with hydrogen peroxide, or from a BLM-Fe(III)-O_2^-complex. It is possible that, under certain conditions, BLM-Fe(III)-O_2^- might decompose to yield O_2^-, and BLM-Fe(III)-O_2H^- to release $OH^{\cdot}$ (67,68). Other studies [e.g., Gajewski *et al.* (69)] have shown that the hydroxyl radical is not necessarily the major DNA-damaging species in the bleomycin system. Damage to DNA in the presence of a BLM-Fe complex has been adopted as one mechanism to examine the prooxidant actions of antioxidants (natural or synthetic) and for assessing proposed antioxidants (38,42,70).

The BLM-Fe (III) complex by itself is inactive in inducing damage in DNA. Oxygen and a reducing agent or hydrogen peroxide are required for the damage to DNA to occur. DNA cleavage by BLM releases some free bases and base propenals in amounts that are stoichiometric with strand cleavage (71). When heated with TBA at low pH, base prope-

TABLE 2
Damage to DNA Bases by Hydroxyl Radicals Generated from Fe(III)-EDTA, H_2O_2 and Ascorbate or Vanillin at pH 7.4[a]

Systems tested		Products monitored (nmol/mg DNA)					
		A	B	C	D	E	Total
DNA, Fe^{3+}-EDTA, H_2O_2 (RM)		0.08	0.02	0.06	0.10	0.43	0.69
RM + ascorbate	100 μM (RMA)	1.13	0.98	1.76	8.20	2.94	15.01
RMA + mannitol	100 mM	0.23	0.06	0.12	0.33	0.31	1.05
RMA + Trolox C	20 mM	0.31	0.11	0.17	0.88	0.21	1.68
RMA + DMSO	100 mM	0.14	0.01	0.08	0.19	0.15	0.57
RMA + hypotaurine	20 mM	0.19	0.04	0.18	0.41	0.42	1.24
RM + vanillin	20 mM	0.40	0.18	0.26	1.20	0.50	2.30

[a]A = thymine glycol; B = 5,6-dihydroxycytosine; C = 4,6-diamino-5-formamidopyrimidine; D = 2,6-diamino-4-hydroxy-5-formamidopyrimidine; E = 8-hydroxyguanine. Taken from Aruoma *et al.* (38). Values for the products formed are the means of three determinations that agreed to within 10%. Control experiments showed that Trolox C, mannitol, hypotaurine, vanillin, or dimethylsulfoxide (DMSO) did not themselves cause any base modification. RM, reaction mixture; RMA, reaction mixture plus ascorbate.

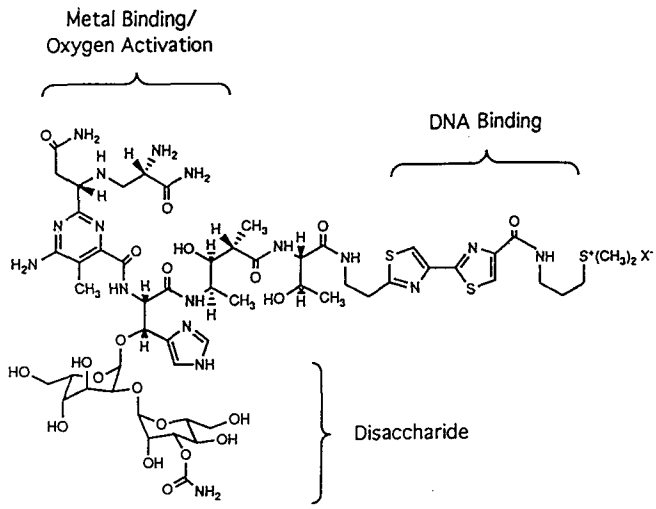

FIG. 3. Structure of antibiotic bleomycin A_2.

nals rapidly decompose to give MDA, which combines with TBA to form a pink $(TBA)_2MDA$ adduct (66,68,72). A positive test is obtained when the compound is able to reduce BLM-Fe^{3+}-DNA complex to the more active BLM-Fe^{2+}-DNA complex (in the presence of oxygen) in the absence of added ascorbate in the reaction mixture, resulting in DNA damage (42,70).

DNA DAMAGE IN THE PRESENCE OF A COPPER-1,10,PHENANTHROLINE COMPLEX

Another method for assessing iron-reducing prooxidant activity is copper-phenanthroline-mediated DNA damage. There is considerable interest in the use of copper ions, complexed to 1,10-phenanthroline (OP), to induce strand breakage in DNA. The complex has nuclease activity and has been used for structural studies upon DNA (73,74).

The original copper-phenanthroline assay was developed to measure copper ions in biological fluids (75) but has been adapted as a method for assessing the prooxidant action of food additives and/or nutrient components (42,70). Hydrogen peroxide is implicated in the mechanism of the DNA damage by the copper-phenanthroline system. Food additives and/or nutrient components that exert prooxidant action in the copper phenanthroline system do not react with hydrogen peroxide to the extent that would affect the outcome of the assay. Hydroxyl radicals are involved in the damage to DNA caused by the copper-phenanthroline system (76,77). Unlike the BLM-Fe-mediated damage to DNA, damage in the copper-phenanthroline system is confined mainly to the DNA bases. The small amount of DNA sugar damage is what the copper-phenanthroline assay measures. When a reducing agent is omitted from the reaction mixture, no damage to deoxysugar in DNA occurs in this system. Increasing the concentrations of the reducing agents, such as ascorbate and/or mercaptoethanol, leads to increased deoxysugar damage. This is in agreement with earlier observations that the nuclease activity of copper-phenanthroline complex is potentiated by thiols, a superoxide generating system, xanthine-xanthine oxidase, and NADH in the presence of hydrogen peroxide (78).

In summary, the deoxyribose assay allows determination of rate constants of reactions with $OH^{\cdot}$ radicals, assessment of abilities to exert prooxidant action, and assessment of abilities to chelate metal ions. The net outcome can be further investigated with assays that involve DNA damage to assess prooxidant actions. These assays have unique features. The positive prooxidant actions in the deoxyribose system rely on the ability of the compounds to promote reduction of Fe^{3+} to Fe^{2+} chelates and, hence, $OH^{\cdot}$ formation in the presence of H_2O_2. Assays that involve DNA rely on ability to reduce either the BLM-Fe-DNA or copper–1,10,phenanthroline–DNA complex. If the compound under test promotes the two reactions described, it possesses a prooxidant property and has to be subjected to a more careful evaluation. A compound might be prooxidant in the deoxyribose system and/or the DNA system but sometimes not in both. There are often solubility problems. Fortunately, organic solvents do not affect the outcome of DNA-dependent assays. Thus, where the deoxyribose assay cannot be performed due to solubility restriction, the copper–phenanthroline assay would suffice. Circumventing potential prooxidant action could contribute to increased protective ability of dietary antioxidants toward susceptible substrates. For example, proteins protect DNA against the prooxidant actions of some flavonoids and polyphenolic compounds in *in vitro* systems (79).

SIGNIFICANCE OF THE CONCEPT OF PROOXIDANT ACTION OF ANTIOXIDANTS TO THE FOOD MATRIX AND *IN VIVO*

It is generally accepted that certain components in foods and some plant materials have beneficial antioxidant effects. For example, red wine contains beneficial flavonoids and polyphenolic compounds (80–82), but this is often complicated by the realization that some components in food and plant materials can have adverse effects. The interaction between food additives and nutrient components within the food matrix, coupled with other interactions when the food and plant materials are consumed, is an area of current interest. A majority of the antioxidants present in plants or added to foods [e.g., propyl gallate, flavonoids, α-tocopherols (vitamin E), gossypol, quercetin, carnosic acid, carnosol, catechins, epicatechins, vitamin C] are all capable of stimulating free-radical damage to nonlipid components, carbohydrates and DNA *in vitro*, and may therefore exact prooxidant actions in biological systems. Prooxidant action would have different implications for the food matrix and the biological system. For the food matrix, the emphasis is on minimizing oxidation to the lipid component of the food. For the human body, different factors have to be considered. For example, iron ions that are catalytic for free-radical reactions are safely sequestered in the human body. They can become available at sites of tissue injury, e.g., in advanced atherosclerotic lesions

(83). Swain and Gutteridge (84) showed that lesions can contain ferrioxidase I activity due to caeruloplasmin and immunologically detectable caeruloplasmin. Ehrenwald *et al.* (85) have demonstrated that caeruloplasmin, which contains one redox-active copper per protein molecule, can oxidize low-density lipoprotein (LDL). Indeed, increased serum copper levels are associated with accelerated progression of carotid atherosclerosis in humans. The body's iron storage protein ferritin has been reported to be associated with carotid atherosclerosis. That transition metal ions within human atherosclerotic lesions can stimulate LDL oxidation by macrophages (86,87) suggests that use of natural antioxidants needs to be approached with caution.

If the extent of prooxidant activity of compounds tested is limited, this could suggest that the effects are unlikely to present a problem in biological systems. Dietary antioxidants can exert a number of effects *in vivo*, such as promoting increased synthesis of endogenous antioxidant defenses by upregulation of their biosynthesis and/or increased gene expression (88). Ushakova *et al.* (88) suggested that "the elevated levels of glutathione in antioxidant-fed mice can be explained by the presence of vitamin E in the dietary supplement, which may preserve the pool of endogenous glutathione." The authors also demonstrated that dietary supplements can indeed modify gene expression induced by heat shock *in vivo* as well as protect animal tissues against oxidative stress by enhancing the level of endogenous antioxidants and inducing heat shock protein (hsp) –70 gene expression (88). Dietary antioxidants may act directly by scavenging reactive oxygen species *in vivo*. Cillard *et al.* (89), upon investigating the effects of experimental factors on the prooxidant behavior of α-tocopherol *in vitro,* found that the prooxidant effect was dependent on the concentration of the antioxidant, solvents, and hydroperoxides (or the oxidizable substrate) in the assay system. Ascorbic acid has the propensity to act as a prooxidant at low concentrations but act as an antioxidant at high concentrations in foods that contain autoxidizable lipids (90–96).

For a proposed antioxidant to have a physiologically meaningful effect *in vivo,* it must become absorbed and presented to the site of intended action at a concentration that actually exerts an antioxidant effect. However, the feasibility of a compound exerting a direct antioxidant effect can be evaluated by *in vitro* tests that investigate how the putative antioxidant can (or cannot) react with biologically relevant oxygen-derived species. This may then be extrapolated to *in vivo* situations (1,42).

This paper has discussed experimental tools that would facilitate the complete evaluation of the antioxidant actions of novel compounds that would complement data from bulk-phase lipid and lipid emulsion studies (e.g., 97–102).

As far as the ability of nutrients and drugs to act as antioxidants *in vivo* is concerned, specific assays are being developed to measure rates of oxidative damage to proteins, DNA, and lipids (1,103,104). Steady-state and total body oxidative damage to these molecular targets provide novel tools to examine the effects of antioxidants *in vivo*.

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