ROLES OF OXYGEN-DERIVED ACTIVE SPECIES AND TRANSITION METAL IONS IN DNA BREAKAGE BY PHENOLIC PRO-OXIDANTS

by

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Roles of oxygen-derived active species and transition metal ions in DNA breakage by phenolic pro-oxidants

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ABSTRACT

Phenolic compounds are genotoxic and antigenotoxic agents, widely distributed in the environment. In this study, we report the DNA breaking activities of four phenolic compounds, namely, 1,2,4-benzenetriol, caffeic acid (3,4-dihydroxycinnamic acid), gallic acid (3,4,5-trihydroxybenzoic acid) and gossypol (1,1',6,6',7,7'-hexahydroxy-3,3'-dimethyl-5,5'-bis(1-methylethyl)[2,2'-binaphthalene] -8,8',-dicarboxaldehyde). Caffeic acid, gallic acid and gossypol are antioxidants and anticarcinogens *in vitro*. However, their biological effects are paradoxical because they also are mutagens and cancer promoters.

When they were the only active species in the reaction system, these phenolic compounds (except gossypol) acted as pro-oxidants and cleaved DNA strands. The order of DNA nicking activity is 1,2,4-benzenetriol > gallic acid > caffeic acid > gossypol. The transition metal ions, copper and iron, enhanced DNA breakage induced by these phenolic compounds. Copper was more effective than iron. In contrast, when more active species are generated by the addition of ascorbate/Fe(III) to the reaction system, caffeic acid and gossypol protected DNA against breakage. However, caffeic acid decreased oxygen consumption by ascorbate/Fe (III), and gossypol increased it.

1,2,4-Benzenetriol reacts with oxygen and generates active species. Therefore, it is a pro-oxidant. By determining effects of antioxidants and metal chelators, we studied the roles of active oxygen species and metal ions in DNA breakage induced by 1,2,4-benzenetriol.

1,2,4-Benzenetriol induced supercoiled DNA breakage at pH 7.4 and 37°C. Within the first 5 minutes 1,2,4-benzenetriol (20 μM) induced extensive BPV-1 DNA

strand scission. The concentration of 1,2,4-benzenetriol required to cleave 50% supercoiled PM2 DNA (ED_{50}) was 6.7×10^{-6} M.

Catalase (20 U/ml), superoxide dismutase (20 U/ml), 25 mM formate and 50 mM mannitol inhibited 18 μM 1,2,4-benzenetriol-induced DNA degradation by 80.5, 30.1, 40.8 and 28.4 percent respectively. On this basis, hydrogen peroxide, superoxide, and hydroxyl radicals all mediate DNA breakage induced by 1,2,4-benzenetriol.

A metal chelator (0.02 mM desferrioxamine) inhibited DNA breakage induced by 18 μM 1,2,4-benzenetriol by up to 80 percent. However, the inhibition can be overwhelmed by a high concentration of 1,2,4-benzenetriol (210 μM). Addition of metal ions such as copper and iron enhanced DNA breakage. These findings confirm the requirement for transition metal ions in DNA breakage.

In view of the almost complete inhibition by either catalase or a metal chelator, and the weaker effects by hydroxyl radical scavengers in DNA breakage, it is concluded that 1,2,4-benzenetriol induces DNA breakage via a metal-mediated site-specific mechanism.

DEDICATION

This thesis is dedicated to my parents and relatives. Their love and encouragement enabled me to succeed in my studies.

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PREFACE

This thesis consists of three sections and ends with a final general discussion.

The first section is a general introduction that outlines the formation and chemistry of active oxygen species, oxidative damage in biological systems and protective roles of antioxidants and metal chelators, and defines the objectives of this study.

The second section is a manuscript that describes genotoxic and antigenotoxic actions of the plant phenolic compounds, caffeic acid, gallic acid and gossypol, compared to the action of a metabolite of benzene (1,2,4-benzenetriol).

The third section is a manuscript presenting a study of DNA breakage induced by 1,2,4-benzenetriol and the effects of antioxidants and metal chelators. The data are interpreted as evidence that 1,2,4-benzenetriol induces DNA breakage by a metal-mediated site-specific mechanism.

The last part is a general discussion of the findings and mechanisms by which active species are produced in autoxidation of 1,2,4-benzenetriol under specified circumstances. Finally, we attempt to frame our findings in a broader perspective.

CHAPTER I

Literature review

INTRODUCTION

Oxidative stress induces DNA damage and mutations in plant seeds (1), bacteria (2), yeast (3), bacterial viruses (4) and mammalian cells (5). Oxygen acts as a mutagen, clastogen and teratogen. The genotoxicity of oxygen is mediated by formation of free radicals and other reactive species (6). Thus antioxidants and metal chelators are appropriate tools to study the roles of active oxygen species and metals in DNA breakage.

FORMATION AND CHEMISTRY OF OXYGEN-DERIVED ACTIVE SPECIES

Molecular oxygen contains two unpaired electrons that have the same spin quantum number. The spin restrictions and orbital restrictions protect oxygen from reaction with biological molecules (7). Many reagents increase the levels of active oxygen and lead to oxidative damage. These reagents include radiation, xenobiotic metabolites, transition metals, modulators of the cytochrome P-450 electron transport chain, peroxisome proliferators, inhibitors of the antioxidant defenses, and membrane-active agents (8).

Molecular oxygen usually carries out one-electron transfer reactions. Univalent reduction of oxygen leads to the formation of superoxide ($O_2 \cdot^-$) and water (H_2O).

$$O_2 + e^- \text{---> } O_2^{\cdot -}$$

$$2O_2^{\cdot -} + 2H^+ \text{---> } H_2O_2 + O_2$$

$$H_2O_2 + e^- \text{---> } {\cdot}OH + OH^-$$

$${\cdot}OH + H^+ + e^- \text{---> } H_2O \qquad (9)$$

Formation and chemistry of superoxide

Superoxide is generated from the electron transport chain as well as non-electron transport mechanisms. The major source of superoxide is from the electron transport chain. Electrons generated in the oxidation of carbohydrates, proteins and lipids reduce 1 to 4 percent of oxygen to superoxide within mitochondria (10). The leakage of electrons to oxygen also takes place in chloroplasts and endoplasmic reticulum.

Superoxide is also produced during the respiratory burst of phagocytic cells (e.g., neutrophils, monocytes, macrophages and eosinophils), as a side product in prostaglandin synthesis, and in the oxidation of hypoxanthine or xanthine by xanthine oxidase (11, 12).

In aqueous solution, superoxide acts as a reducing agent compared to cytochrome *c*, or a weak oxidizing agent compared to ascorbic acid. It also dismutates to H_2O_2 through disproportionation at physiological pH.

$$O_2^{\cdot -} + H^+ \text{---> } HO_2^{\cdot}$$

$$HO_2^{\cdot} + O_2^{\cdot -} + H^+ \text{---> } H_2O_2 + O_2$$

The rate constant at pH 7 is $4.5 \times 10^5\ M^{-1}S^{-1}$ (7).

In non-aqueous solution, superoxide acts as a strong base and nucleophile. It undergoes four reactions: electron transfer, nucleophilic substitution, deprotonation and

H-atom abstraction (13). In biological systems, environments are both aqueous and non-aqueous, and the effects of superoxide in these environments must be different.

Chemicals such as bleomycin (14), paraquat (15), phorbol esters (16) and potassium superoxide (17) induce the production of superoxide anion. Protection of biological macromolecules against the oxidative damage by superoxide dismutase has shown superoxide as an important reaction intermediate. Because of its weak reactivity, superoxide can diffuse a long way from its site of production, and then damage macromolecules. However, due to its negative charge, superoxide crosses biological membranes slowly unless there is an anion channel such as that in the erythrocyte membrane (18). This limits its reactivity in biological systems.

Formation and chemistry of hydrogen peroxide

Hydrogen peroxide is not an oxygen free radical because it has no unpaired electrons. Hydrogen peroxide is produced mainly by non-enzymatic or enzymatic dismutation of $O_2^{\cdot -}$. Such H_2O_2 production has been observed in bacteria, phagocytic cells, spermatozoa, mitochondria and chloroplasts (7). Another source is enzymes that generate H_2O_2 without superoxide as an intermediate. The enzymes include glycollate oxidase, D-amino acid oxidase and urate oxidase (12).

Hydrogen peroxide is not highly reactive toward organic molecules. Heat, light or transition metals can increase the reactivity of hydrogen peroxide (19, 20, 21). Hydrogen peroxide can undergo nucleophilic reactions. An important reaction is the formation of hypochlorous acid from the reaction of H_2O_2 and hydrochloride. This reaction is catalyzed by myeloperoxidase in neutrophils and monocytes (22). The decomposition of hydrogen peroxide yields hydroxyl radicals.

Although hydrogen peroxide is not a free radical, it is an important genotoxic agent. It has no charge and can cross biological membranes easily, and either H_2O_2 or

the hydroxyl radical can damage macromolecules. Many studies have reported the deleterious effects of H_2O_2 in biological systems (23, 24, 25, 26). Besides damaging biological macromolecules (e.g., DNA, chromosomes), H_2O_2 impairs Ca^{2+} homeostasis and causes ATP depletion (27), and increases poly(ADP-ribose)polymerase activity (28). H_2O_2 may be a factor in aging since production of H_2O_2 in the flight muscle mitochondria of the housefly increases during aging (29). Seven different human tumor cell lines of four histological types also generate H_2O_2 at rates of 0.5 to 2.0 nmol/10^4 cells/h (30). Probably, H_2O_2 plays a role in mutagenesis.

Formation and chemistry of the hydroxyl radical

The hydroxyl radical is among the most reactive free radicals formed in biological systems (12). *In vivo* most hydroxyl radicals are generated from the metal-dependent breakdown of H_2O_2 in the Fenton reaction as follows:

$$M^{(n-1)+} + H_2O_2 \text{---} > M^{n+} + \cdot OH + OH^-.$$

Hydroxyl radicals are also generated by ionizing radiation (12).

The hydroxyl radical has a very short half-life (<1 ns) (31). However, it has a high second-order rate constant of 10^9-10^{10} $M^{-1}S^{-1}$ with biomolecules (32). Thus the hydroxyl radical is highly reactive compared with superoxide. The reactions which hydroxyl radicals are involved in, however, are limited by diffusion. The hydroxyl radical can only diffuse 5 to 10 molecular diameters from its site of formation before it reacts with another molecule (33).

The damage elicited by hydroxyl radicals depends on several factors. First, damage depends on the site of hydroxyl radical formation. When the formation of hydroxyl radicals is close to DNA, DNA strand scission takes place (34, 35). Otherwise the chance of DNA damage will be random. Second, the reaction of the hydroxyl radical with a biomolecule will generate radicals with low activity. This type

of radical can diffuse away from the site of formation, attack and further damage biomolecules (12).

OXIDATIVE DAMAGE IN BIOLOGICAL SYSTEMS

Free radical reactions play important roles in human diseases such as cancer, aging and disorders of immune systems (12). The variety of oxidative damage to macromolecules includes: (a) nucleic acid damage which results in mutation and carcinogenesis; (b) lipid peroxidation which causes membrane damage; (c) protein damage which affects activities of enzymes and disturbs transport systems; (d) damage to polysaccharide which alters their functions (36).

DNA is an important cellular target subject to attack by oxygen radicals. The degradation of DNA can be induced by radiation (e.g., X, alpha, beta, gamma rays) (37), oxygen radicals (e.g., H_2O_2 and potassium superoxide) (17, 38), or oxidizing reagents (e.g., bleomycin, paraquat) (39, 40). Oxygen radicals can attack either at the bases or the sugars of the DNA molecule. Damage to the sugar residues results in sugar fragmentation, base loss and strand breaks (41). The base alterations often are at guanine (forming 8-OH guanosine) and thymidine (forming thymidine glycol). The biological consequences include cell death, reproductive death, mutation and carcinogenic transformation.

Transition metals have important effects in oxidative damage

Transition metals in the first row of the d-block in the Periodic Table (except zinc) contain unpaired electrons (12). The variable valences of transition metals allow them to accept and donate a single electron. Iron and copper are the most interesting metals in studies of free radicals.

1. Storage and mobilization of iron and copper

Two-thirds of iron *in situ* is bound to hemoglobin. A small amount of iron is associated with myoglobin, enzymes and the iron-transport protein, transferrin. The remaining iron is stored in ferritin. Albumin and amino acids bind 5% of the human plasma copper, and the remaining copper is stored in ceruloplasmin (7). The binding of copper and iron with proteins is an important protective mechanism that minimizes the size of the intracellular metal pool.

Ferritin releases iron when it encounters certain reducing agents, including dithionite, thioglycolate, dihydroriboflavin 5'-phosphate ($FMNH_2$) (42), adrenaline (43), *o*-diphenols (44), electrons (45), superoxide (46), semiquinone, anthracycline, bipyridyl and nitroaromatic radicals (47). Ceruloplasmin can transfer copper to proteins which contain copper such as superoxide dismutase. Because ceruloplasmin inhibits Fe^{2+}-dependent radical reactions, it is an important extracellular antioxidant. Copper that binds to albumin and amino acids also can still react with superoxide and H_2O_2 (7).

2. Iron and copper catalyze the Haber-Weiss reaction

In the absence of metals, the Haber-Weiss reaction

$$O_2{\cdot}^- + H_2O_2 \text{ ---> } {\cdot}OH + OH^- + O_2$$

is too slow to be biologically significant, having a rate constant of only 0.13 $M^{-1}S^{-1}$ (48). Because iron and copper can accept and donate electrons, they can catalyze the Haber-Weiss reaction. The reaction steps include: (a) reduction of a metal chelated by $O_2{\cdot}^-$; (b) dismutation of $O_2{\cdot}^-$; (c) reduction of H_2O_2 to ·OH.

$O_2^{\cdot -} + Fe^{3+}/Cu^{2+} \rightarrow Fe^{2+}/Cu^{+} + O_2$ (a)

$O_2^{\cdot -} + HO_2^{\cdot} + H^{+} \rightarrow O_2 + H_2O_2$ (b)

$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + {\cdot}OH + OH^{-}$ (c)

Iron and copper catalyze reaction (c) at different rates. The second-order rate constant for Fe(II) is 76 $M^{-1}S^{-1}$, and for Cu(I) is 4.7 x 10^3 $M^{-1}S^{-1}$. Thus, by generating ·OH faster, copper induces greater damage than iron.

3. Site-specific mechanism of metal-mediated biological damage

In a site-specific mechanism, iron or copper binds to biological molecules and undergoes cyclic reduction and oxidation. Iron or copper reduced by reductants (e.g., $O_2^{\cdot -}$, ascorbate) reacts with H_2O_2 and generates hydroxyl radicals that damage macromolecules at the site of their production (49). By this mechanism, metals induce the production of oxygen-derived active species that damage DNA, enzymes, albumin and carbohydrates (50, 51, 52, 53).

4. Metals play dual roles in $O_2^{\cdot -}$ mediated damage

Goldstein and Czapski [1986] have proposed that metals play dual roles in $O_2^{\cdot -}$ mediated damages. Metals can potentiate the toxic effects of $O_2^{\cdot -}$ by catalyzing the production of ·OH in the Fenton reaction.

Biol-Cu(II) + $O_2^{\cdot -} \rightarrow$ Biol-Cu(I) + O_2 (a)

Biol-Cu(I) + $H_2O_2 \rightarrow$ Biol-Cu(II) + ·OH + OH^{-} (b)

Metals also protect against damage by dismutation of $O_2^{\cdot -}$ in a "ping-pong" mechanism.

Biol-Cu(II) + $O_2^{\cdot -} \rightarrow$ Biol-Cu(I) + O_2 (c)

Biol-Cu(I) + $O_2^{\cdot -}$ + $2H^{+} \rightarrow$ Biol-Cu(II) + H_2O_2 (d) (54, 13)

Whether metals exert protection or deleterious effects depends on the rate constants and the concentrations of biomolecules and metals. If rate constants for reactions (c) and (d) are greater than that of reactions (a) and (b), and concentrations of metals are higher than that of biomolecules, the protection will predominate (55).

Metabolites of benzene induce oxidative damage

1. Bioactive routes of benzene *in situ*

Benzene is one of the most widely distributed environmental pollutants. Repeated exposure to benzene is reported to cause leukopenia, aplastic anemia and leukemia (56).

Benzene metabolites are more genotoxic than benzene (57). After benzene enters body, it is converted to benzeneoxide by monoxygenase in liver, and then to phenol, a major metabolite of benzene *in vivo*. Cytochrome P-450 catalyzes the hydroxylation of phenol to hydroquinone and catechol. Catechol is oxidized to 1,2,4-benzenetriol (58, 59, 60). Accumulation of phenol, hydroquinone and catechol in bone marrow poses the toxic effect (61, 62).

2. Benzene alters iron metabolism

Exposure to benzene disturbs iron metabolism and releases iron ions. Benzene inhibits iron uptake by haemoglobin (63) and decreases the amount of heme regulated by liver (64). Iron accumulates in tissues such as bone marrow. Studies of bone marrow fraction have shown that the maximal accumulation of iron is in the mitochondrial fraction (65). These iron ions play a role in benzene toxicity via Haber-Weiss-like reaction (66).

3. 1,2,4-Benzenetriol is an active genotoxic metabolite of benzene

1,2,4-Benzenetriol is the most active metabolite of benzene in oxidative damage, although 1,2,4-benzenetriol accounts for only 0.3% of urinary benzene metabolites (58).

1,2,4-Benzenetriol induces DNA breakage in mouse lymphoma cell line (L5178YS) and isolated human DNA fragments (67, 68). It induces the formation of deoxyguanosine adducts in isolated rabbit bone marrow cells and deoxyguanosine (69, 70). Exposure of bone marrow cells and isolated DNA to 1,2,4-benzenetriol inhibits DNA synthesis by interfering with the activity of DNA polymerase α (71).

4. Autoxidation of 1,2,4-benzenetriol induces DNA strand breakage

1,2,4-Benzenetriol has a low reduction potential. It rapidly autoxidizes to 2-hydroxybenzoquinone under physiological conditions (pH 7.4 and 37°C) (72). The reaction involves a semiquinone radical intermediate, and two molecules of superoxide are released. Scavengers of superoxide, hydrogen peroxide and hydroxyl radicals inhibit the breakage of DNA by 1,2,4-benzenetriol to varying extents (68, 72). Thus superoxide, hydrogen peroxide and hydroxyl radical mediate DNA strand scission by 1,2,4-benzenetriol. The effects of scavengers of active oxygen species from different studies, however, are not concordant. The inhibition of DNA cleavage by superoxide dismutase differs in different reaction systems. Scavengers of the hydroxyl radical such as methional and sodium formate have different effects even under similar reaction conditions (68, 72).

5. The roles of transition metals in oxidative damage induced by 1,2,4-benzenetriol

In the presence of Cu(II), 1,2,4-benzenetriol releases 2-thiobarbituric acid reactive products (TBAR) from DNA, and thiourea and catalase completely inhibit the formation of TBAR. Thus hydroxyl radicals formed by a Fenton-type reaction play an important role (73). Addition of Cu(II) accelerates DNA breakage, but Fe(III) does not have a significant effect on DNA damage. Fe(III), however, increases the production of hydroxyl radicals by 1,2,4-benzenetriol but Cu(II) does not. A chelator of Cu(I), bathocuproine, inhibits DNA damage induced by 1,2,4-benzenetriol. The evidence suggests that trace metals are essential for autoxidation of 1,2,4-benzenetriol.

Phenolic compounds induce and paradoxically prevent mutagenesis

Phenolic compounds, caffeic acid, gallic acid and gossypol, are widely distributed in plants. They are sometimes carcinogenic, and sometimes chemopreventive. Usually they inhibit the genotoxicity of carcinogens. These phenolics inhibit lipoxidase activity (74), and induce chromosomal aberrations (75), chromosomal breakage (76), and sister-chromatid exchanges (77). Less often these compounds are genotoxic. They induce DNA breakage (78, 79), which is oxygen-dependent (80). A wide variety of biological effects are attributable to either antioxidant or oxidant activities of these compounds.

ANTIOXIDANTS AND SCAVENGERS OF TRANSITION METALS PLAY IMPORTANT ROLES IN OXIDATIVE DAMAGE

Classification and defense mechanism of antioxidants

The primary antioxidant defense consists of superoxide dismutase, catalase, glutathione peroxidase, DT-diaphorase and small molecules (e.g., ascorbic acid, alpha-tocopherol, GSH, beta-carotene and uric acid). The secondary defenses are proteolytic and lipolytic enzymes and the DNA repair systems (13). The mechanisms of inhibition of radical formation by antioxidants include (a) decrease of localized O_2 concentration; (b) termination of free radical chain reactions (e.g., phenolic antioxidants, tertiary amines, flavonoids, alpha-tocopherol); (c) decomposition of peroxides or radicals (e.g., sulfur compounds, selenium, enzymic antioxidants), (d) chelation of metals (e.g., ferritin, ferrioxamines, citric acid, phytic acid), and (e) synergism with other antioxidants (e.g., regeneration of alpha-tocopherol by ascorbate) (81, 12).

Superoxide dismutase protects against oxidative stress

There are three types of superoxide dismutase associated with specific metal ions. They are copper-zinc, manganese and iron superoxide dismutases. All these enzymes catalyze the reaction $2O_2^{\cdot -} + 2H^+ \text{---} > H_2O_2 + O_2$, removing the superoxide and diminishing oxidative stress. However, H_2O_2, inactivates both copper-zinc superoxide dismutase and iron superoxide dismutase. H_2O_2 modifies the imidazole moiety of a histidine residue at the active site of both superoxide dismutases (82). This inactivation is slower at physiological pH because the affinity between superoxide dismutase and H_2O_2 decreases with pH. At 25°C, the half-saturation constant for

peroxide decreased from 15.7 to 3.2 mM and a maximal pseudo-first-order rate constant for inactivation increased from 0.83 to 2.43 per minute as pH increased from 9.0 to 11.5 (83). Copper-zinc superoxide dismutase also catalyzes the formation of "free " hydroxyl radicals from H_2O_2 (84).

Superoxide dismutase has multiple effects on the autoxidation of quinoid compounds. Superoxide dismutase inhibits the autoxidation of 1,2,4-benzenetriol by terminating superoxide-propagated reaction chains and by metal chelation. In autoxidation of 1,2-naphthohydroquinone and 5-hydroxy-1,4-naphthohydroquinone, superoxide is primarily a chain reaction terminator and slows formation of semiquinone and quinone. Superoxide dismutase stimulates the autoxidation of both quinones by removing superoxide (85). Thus superoxide dismutase can protect selectively against oxidative damage induced by quinoid compounds.

Hydroxyl radical scavengers may not strongly decrease oxidative damage

Hydroxyl radical scavengers, formate and mannitol, remove hydroxyl radicals, and diminish oxidative damage in ·OH-generating systems. Formate and mannitol, however, double the yield of $O_2{\cdot}^-$ while they remove ·OH in reaction systems. In the presence of O_2, formate converts ·OH to $O_2{\cdot}^-$ as follows:

$$\cdot OH + HCOO^- \text{---}> H_2O + CO_2{\cdot}^-$$

$$CO_2{\cdot}^- + O_2 \text{---}> CO_2 + O_2{\cdot}^- \quad (86)$$

Attack of ·OH on mannitol produces a carbon radical. The carbon radical reacts with oxygen and yields a hydroxylalkylperoxyl radical that can decompose and generate superoxide.

$$R\text{-}CH_2OH + \cdot OH \text{---}> R\text{-}\cdot CHOH + H_2O$$

$$R\text{-}\cdot CHOH + O_2 \text{---}> \cdot O_2CHOH$$

$$\cdot O_2CHOH \text{---}> R\text{-}CHO + H^+ + O_2{\cdot}^- \quad (12)$$

In the presence of metals, $O_2^{\cdot -}$ contributes to the production of ·OH by the Haber-Weiss reaction, and aggravates oxidative damage. Thus a combination of superoxide dismutase and mannitol or formate is required to fully protect against hydroxyl radical mediated oxidative damage.

Desferrioxamine and EDTA affect oxidative damage to different extents

Desferrioxamine is a chelator which binds iron through six oxygen atoms as ligands (12). It chelates Fe(III) more strongly than Fe(II). Desferrioxamine chelates ferric iron, blocks redox cycling and thereby diminishes damage induced by iron (87, 88). For this reason, desferrioxamine inhibits the iron-dependent ·OH radical production in $O_2^{\cdot -}$ generating systems (89). Desferrioxamine also is a powerful scavenger of hydroxyl radicals [K_2- 10^{10} $M^{-1}S^{-1}$] and a weak scavenger of superoxide anion [K_2- 9 x 10^2 $M\text{-}^1S^{-1}$] (90, 89). The trihydroxamate moiety of desferrioxamine plays a role in electron transfer reactions that involve the superoxide radicals. Desferrioxamine acts as a lipid chain-breaking antioxidant by donating an electron or hydrogen atom from the hydroxamate center to alkoxyl and peroxyl radicals in lipid peroxidation of membranes. This ability is independent of its iron chelating properties (91). Thus desferrioxamine will decrease oxidative damage.

EDTA is a chelating agent and suppresses redox cycling of copper ions in its reaction with $O_2^{\cdot -}$ and H_2O_2 (51, 26). Thus EDTA decreases the oxidative damage catalyzed by copper ions. EDTA, however, has dual effects in reactions involving iron ions. EDTA enhances the redox cycling of iron ions when the ratio of [EDTA]/[iron] is low and inhibits the redox cycling when the ratio is high. At low concentrations, EDTA prevents the precipitation of iron, and favors the reduction of iron by $O_2^{\cdot -}$ and oxidation by H_2O_2. The second-order rate constant for the reduction of $O_2^{\cdot -}$ with

Fe(III)-EDTA complex is 1.9 x 10^6 $M^{-1}S^{-1}$ at pH 7.0 (92). At critical ratios of [EDTA]/[iron], EDTA changes from being a pro-oxidant to an anti-oxidant (93, 87). EDTA decreases the rate of the reduction of Fe(III) by superoxide and slows the Fenton reaction. At high concentration EDTA inhibits DNA damage since it traps iron.

SUMMARY

Oxygen derived active species are involved in oxidative damage. Superoxide, hydrogen peroxide and the hydroxyl radical are the most studied species. Their chemical properties dictate their roles in oxidative damage. Transition metals may aggravate oxidative damage by accelerating the Haber-Weiss reaction and the production of the oxygen derived species. Thus scavengers of oxygen active species and metals can decrease the extent of oxidative damage. Study of genetic damage induced by phenolics will help to reveal the roles of oxygen-derived active species, transition metals or scavengers.

OBJECTIVES OF THIS STUDY

DNA damage induced by 1,2,4-benzenetriol and the effects of antioxidants has been previously studied (68, 72). The relative contribution of different active oxygen species to DNA damage induced by 1,2,4-benzenetriol, the mechanisms of genotoxicity of 1,2,4-benzenetriol, and the effects of selective antioxidants and metal scavengers remain to be unambiguously determined. The paradoxical biological actions of phenolic compounds need to be clarified under defined circumstances.

Specific questions addressed in this study are summarized as follows:

1. Do plant phenolic compounds (caffeic acid, gallic acid and gossypol) protect against or induce DNA damage?
2. Under what conditions does each of these phenolic compounds act as pro-oxidants or anti-oxidants ?
3. What are the effects of transition metal ions in DNA cleavage induced by these phenolic compounds?
4. To what extent does DNA breakage induced by 1,2,4-benzenetriol depend on pro-oxidant activity?
5. What are the efficacies of antioxidants and scavenger of transition metal ions individually or in combination in protecting DNA against breakage?
6. Under what specific conditions do active oxygen species play important roles in DNA breakage by 1,2,4-benzenetriol?

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DNA breaking activity of phenolic compounds *in vitro* as a function of pro-oxidant and anti-oxidant activity

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CHAPTER II

DNA breaking activity of phenolic compounds *in vitro* as a function of pro-oxidant and anti-oxidant activity

ABSTRACT

The four phenolic compounds tested, caffeic acid, gallic acid, gossypol and 1,2,4-benzenetriol, vary dramatically in their ability to nick purified supercoiled DNA [pdBPV-1 (142-6)], with nicking ranging from 20 to 100% at 0.02 mM. In the concentration range of 0.02 mM to 0.2 mM the phenolic compounds were effective in the order: 1,2,4-benzenetriol > gallic acid > caffeic acid > gossypol. Oxygen-derived active species participated in DNA breakage induced by these phenolic compounds, with the exception of gossypol. Transition metal ions at 0.025 mM, accelerated DNA nicking, copper being more effective than iron. Caffeic acid and gossypol, but not 1,2,4-benzenetriol nor gallic acid, inhibited DNA breakage induced by ascorbate/Fe. The observation is discordant with effects of these compounds on oxygen consumption by ascorbate/Fe. 1,2,4-Benzenetriol and gossypol increased oxygen consumption by ascorbate/Fe, but gallic acid and caffeic acid decreased it.

Keywords: phenolic compounds, DNA breakage, copper, iron, ascorbate/Fe system, Haber-Weiss reaction, prooxidant, antioxidant, free radical

INTRODUCTION

Phenolic compounds such as caffeic acid, gallic acid and gossypol are widely distributed in plants. They are present in many foods and beverages. Many of these compounds have substantial biological effects, but it is still not known to what extent a given phenolic is harmful or beneficial. The inability to come to clear conclusions reflects their paradoxical actions in diverse test systems or reaction conditions.

The harmful effects of phenolic compounds, including their genotoxicity are related to their pro-oxidant properties. Caffeic acid and gallic acid can induce DNA breakage in mammalian cells (1). Gossypol increases the frequency of DNA-strand breaks in human leukocytes (2), catalyzes oxygen-dependent DNA degradation in the presence of 2-mercaptoethanol and metal ions (3), and generates superoxide radicals in the presence of liver microsomes and NADPH (4) or Fe^{3+}-EDTA (5). Metals accelerate the reduction of oxygen by redox-active phenolic compounds and increase their deleterious effects, perhaps by catalyzing Haber-Weiss type reactions (6). Copper and iron enhance formation of hydroxyl radicals, chromatid exchange and breakage by caffeic acid and gallic acid (7, 8), and DNA degradation by gossypol (3).

Paradoxically, the beneficial effects of phenolic compounds include the ability to *protect* against genotoxins. Caffeic acid and gallic acid suppress chromosomal breakage and sister-chromatid exchange induced by carcinogens [e.g., aflatoxin-B_1 (9), N-metyl-N'-nitro-N-nitrosoguanidine (10), benzo[a]pyrene (11) and 7,12-dimethylbenz[a]anthracene (12)], or uv-light (13), in mammalian cells and bacteria. Such protection may be related to their antioxidant properties since *in vitro* these phenolic compounds, in some circumstances, can decrease the formation of hydroxyl radicals (14) and inhibit lipoxidase activity *in vitro* (15). Consistent with

antioxidant activity, caffeic acid suppresses the formation of superoxide generated by phenazine methosulfate in the presence of NADH, and decreases production of lipid peroxides in mouse liver microsomes (16). Gossypol inhibits microsomal lipid peroxidation and lipoxygenase activity induced by a NADPH generating system *in vivo* (17, 18).

The aim of the present work is to investigate the extent to which DNA nicking by above-mentioned plant phenolic compounds reflects their primary prooxidant activity. We report effects of these phenolic compounds on DNA strand breakage induced by a hydroxyl radical generator (the ascorbate/Fe couple). We will also describe the effects of transition metal ions (copper and iron), a metal chelator (desferrioxamine) and enzyme scavengers of oxygen-derived active species on DNA breakage induced by these phenolic compounds.

MATERIALS AND METHODS

Reagents

1,2,4-Benzenetriol and gallic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Caffeic acid and L-ascorbate were purchased from Sigma Chemical Co. (St. Louis, MO). Cupric sulfate, cuprous chloride, ferric chloride and ferrous sulfate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Gossypol was supplied by Peking Union Medical College, China. Superoxide dismutase (4050 U/mg) was from DDI Pharmaceutical Inc., (Mountain View, CA). Catalase (65,000 U/mg) was from Boehringer Mannheim Co. (Dorval, PQ). Desferrioxamine was a gift from Ciba-Geigy Pharmaceutic Co. (Summit, NJ).

Preparation of plasmid pdBPV-1 (142-6) DNA

Supercoiled plasmid pdBPV-1 DNA was prepared according to Maniatis et al. (19). Briefly, E. coli containing the plasmid was grown overnight in standard LB medium (10 gm bacto-tryptone, 5 gm bacto-yeast extract and 10 gm NaCl per litre of deionized H_2O), supplemented with ampicillin (40 mg per litre of LB medium). Bacteria were recovered by centrifugation. The bacteria were lysed by digestion with lysozyme and treatment with alkali. The lysate was precipitated by isopropanol and recovered by centrifugation. The plasmid DNA was selectively precipitated with polyethylene glycol (PEG). The supercoiled plasmid DNA and linear DNA (including the chromosomal DNA from bacteria) were separated by centrifugation in a CsCl-ethidium bromide gradient, and the purified supercoiled plasmid DNA was recovered by ethanol precipitation.

DNA concentration was determined by measuring the absorbance at 260 nM using a Lambda 3 UV/VIS spectrophotometer. On this basis, unit absorbance corresponds to 50 μg/ml double-stranded DNA. DNA was stored in 10 mM Tris-HCl/1mM EDTA buffer, pH 8.0, at -20°C.

Preparation of ^{3}H-labelled PM2 DNA

^{3}H-labelled supercoiled bacteriophage PM2 DNA was prepared as described by the method of Espejo and Canelo (20, 21) as modified by Tsang (22). Pseudomonas Bal-31 was grown at 28-30°C in 500 ml Bal-broth [10 ml of 10 mM Tris-HCl (pH 7.5), 12 gm magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 26 gm sodium chloride, 8 gm bacto-nutrient broth, 10 ml of 1 M calcium chloride and 3.5 ml of 20% potassium chloride made up to one litre with deionized water]. When the bacteria reached a density of 3 x 10^8/ml, 50 mg 2'-deoxyadenosine (Sigma) was added. Five minutes later, the bacteria were infected with 1-2 x 10^{12} bacteriophage PM2. After five minutes, 0.5 mCi of methyl-^{3}H thymidine (25 Ci/mmol, Amersham International) was added. The culture was incubated overnight. Phage particles were purified by cesium chloride density equilibrium centrifugation. The phage particles were dialyzed against 1.2 litre buffer (0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA) at 4°C and lysed by 10% sodium dodecyl sulfate. Phage DNA was extracted from the aqueous phase by phenol.

Agarose gel electrophoresis analysis of DNA cleavage

Supercoiled plasmid BPV-1 DNA was incubated with the reagents for the defined period, and the reaction was terminated by diluting the samples in loading buffer (0.05% bromophenol blue, and 6% glycerol). These samples were fractioned by agarose gel electrophoresis at 60 volts for 90 minutes. Each gel contained 0.7% (W/V)

agarose gel prepared with a buffer that consisted of 0.04 M Tris-acetate, 0.002 M EDTA. The gels were stained with 0.5 μg/ml ethidium bromide for 30 minutes, destained in water for 2-3 minutes, and photographed under ultraviolet illumination. A Polaroid MP-4 camera and Polaroid 57 or 55 professional films were used for the photography.

Supercoiled circular (form I), nicked circular (form II), and linear (form III) forms of plasmid DNA migrated through 0.7% (W/V) agarose gels at different rates. Form I migrated fastest, and form III migrated between form I and II.

Analysis of DNA damage by filter-binding assay using nitrocellulose filters

The ^{3}H-labeled supercoiled PM2 DNA was incubated with test reagents. The incubation was terminated via precipitation of PM2 DNA by ethanol. PM2 DNA was resuspended in 25 or 50 μl of 1 mM sodium phosphate buffer (pH 7.4). The samples were then treated as follows, to allow strand separation of the nicked DNA molecules. A 10 μl aliquot of the DNA reaction mixture was diluted with 40 μl of 10 mM Tris-HCl/1 mM EDTA buffer, pH 8.0. To this solution, 5 μl of 100 mM Tris-HCl (pH 7.5) and 0.15 ml SE buffer (0.01% sodium dodecyl sulfate, 2.5 mM EDTA-NaOH, pH 7.0) were added. The DNA was then denatured by the addition of 0.2 ml of 0.3 M K_2HPO_4-KOH (pH 12.4) for 2 minutes at room temperature, and neutralized with 0.1 ml of 1 M KH_2PO_4-HCl (pH 4.0). The solution was diluted with 0.2 ml of 5 M NaCl and 5 ml of NT buffer (1 M NaCl, 50 mM Tris-HCl, pH 8.0), and filtered through nitrocellulose filters presoaked in NT buffer. The reaction tube was rinsed with 2 ml NT buffer, and the rinse was filtered. The filters were then washed with another 2 ml NT buffer, dried, and the radioactivity was determined by liquid scintillation counting. Total DNA was determined by measuring the radioactivity of 10 μl of reaction mixture spotted on a blank filter. By this method, the average number

of nicks (N) per DNA molecule can be calculated by equation $N = -\ln P$; where P was the fraction of DNA that was not retained on the nitrocellulose filter (23).

Densitometric analysis

The relative amounts of supercoiled, nicked circular and linear DNA were estimated by scanning the photographic negatives with a densitometer. This analysis was performed with a SP4100 computing integrator and Soft Laser Scanning Densitometer from Hoefer Scientific instruments, San Francisco, California.

Measurement of oxygen consumption

Deionized distilled water, 50 mM sodium phosphate buffer (pH 7.4), 50 mM Na_2HPO_4 buffer (pH 8.7) or 100 mM Na_2CO_3 buffer (pH 10.0) were bubbled with argon in sealed vials for at least 30 minutes. Vials containing solid reagents were filled with argon. 1,2,4-Benzenetriol was dissolved in anaerobic 50 mM sodium phosphate buffer (pH 7.4). Gallic acid and caffeic acid were dissolved in anaerobic 50 mM Na_2HPO_4 (pH 8.7). Gossypol and ferric chloride were dissolved in anaerobic 100 mM Na_2CO_3 buffer (pH 10.0) or deionized distilled water, respectively. Ascorbate was dissolved in anaerobic alkalinized deionized distilled water. The final pH of the ascorbate solution was at 7.4. Sodium phosphate buffer (50 mM), pH 7.4, was saturated by air at 25°C. Aliquots of the reagent solutions were added to 3 ml of air-saturated 50 mM sodium phosphate buffer to initiate the reactions. Final pH in the reaction solutions was between 7.4 to 7.44. Oxygen consumption was monitored by a Clark-type oxygen electrode at 25°C. Data were collected on a linear strip chart recorder. The calculated rates represented the maximal rates of oxygen consumption.

Statistical analysis

Standard statistical methods were used to calculate the standard errors of the means. All reactions were performed in duplicate and usually triplicate. Since variance depended on the magnitude of the fraction of DNA remaining intact, in determining standard deviation for the procedure, we used relative rather than absolute variances in Fig. 3 a-d. The standard deviation for a population was divided by the square root of the number of replicates to estimate the standard error of the means shown in the figures. Significance of differences between means was examined by student's t-test. The level of confidence required for significance was set in advance to $p < 0.05$.

RESULTS

Phenolic compounds alone induce DNA breakage

Phenolic compounds induce rapid DNA breakage at low concentrations

All four phenolic compounds tested cleaved supercoiled plasmid pdBPV-1 DNA [form I] to the nicked circular DNA [form II] or linear DNA [form III]. The phenolic compounds differ in ability to nick DNA with damage ranging from 20 to 100% at 0.02 mM (Fig. 1). Over the concentration range 0.02 mM to 0.2 mM, the DNA breaking activity was 1,2,4-benzenetriol > gallic acid > caffeic acid > gossypol. Treatment with 0.01 mM 1,2,4-benzenetriol rapidly converted supercoiled DNA to nicked circular DNA. Linear DNA was observed at 0.05 mM, and smaller heterogeneous DNA fragments appeared at 0.2 mM. At all concentrations tested, gallic acid converted supercoiled DNA to nicked circular DNA, but not to linear DNA nor DNA fragments. Caffeic acid induced less DNA breakage than gallic acid, and was effective only at concentrations between 0.02 mM and 0.2 mM. Gossypol was the least effective in inducing DNA breakage, only partly converting supercoiled DNA to nicked circular DNA, and that only at concentrations above 0.1 mM.

DNA nicking ability correlates with ability to reduce oxygen, except gossypol

On the assumption that DNA-breaking activity of a phenolic and its ability to reduce oxygen are both determined in part by its reduction potential, we compared the rates of reduction of oxygen by the phenolic compounds. Rates of oxygen consumption were in the order 1,2,4-benzenetriol > gallic acid > gossypol > caffeic acid, with 1,2,4-benzenetriol being over 70 times as reactive as gallic acid (Fig. 2). DNA-breaking ability was in the order 1,2,4-benzenetriol > gallic acid > caffeic acid

> gossypol (Fig. 1). In comparison with caffeic acid, gossypol's oxygen consumption was unexpectedly high given that it has the lowest DNA nicking activity. However, as we shall see, gossypol seems to damage DNA by mechanisms which are independent of oxygen-derived active species.

Oxygen derived active species mediate DNA nicking by phenolic compounds, expect gossypol

To clarify the roles of oxygen-derived active species in DNA breakage induced by these phenolic compounds, we examined the effects of catalase and superoxide dismutase in presence and absence of desferrioxamine (Fig. 3 a-d).

Catalase inhibited DNA breakage by 1,2,4-benzenetriol, gallic acid and caffeic acid almost completely. Superoxide dismutase did not significantly decrease DNA breakage by 1,2,4-benzenetriol, or enhance DNA breakage by caffeic acid and gossypol. However, it did enhance DNA breakage by gallic acid. Summing up these actions, hydrogen peroxide was the common active species in DNA breakage induced by these phenolic compounds. Superoxide played ambiguous roles. Gossypol, however, was anomalous, in that neither of the scavengers protected.

Transition metals potentiate DNA breakage induced by phenolic compounds, except gossypol

Transition metal ions, including copper and iron, potentiate biological actions of many pro-oxidants (23-28). Figures 3 (a-d) showed that desferrioxamine significantly diminished DNA nicking induced by gallic acid, but not 1,2,4-benzenetriol, caffeic acid or gossypol. Traces of metal ions in solution are evidently only crucial to DNA breakage by gallic acid. In the presence of 210 μM 1,2,4-benzenetriol (Fig. 3a), desferrioxamine enhanced inhibition by superoxide dismutase and catalase ($p < 0.05$).

Nicking of DNA by each of the phenolic compounds was enhanced by the presence of 0.025 mM copper ions, Cu(II) and Cu(I), or iron ions, Fe(II) and Fe(III) (Fig. 4 a-d). Copper ions were more effective than iron ions in potentiating DNA-breaking activity of the phenolic compounds. Added alone, however, only Fe(II) induced massive DNA nicking. Cu(I), Cu(II), or Fe(III) alone were relatively ineffective.

Phenolic compounds can inhibit or enhance DNA breakage by ascorbate/Fe(III)

Ascorbate and Fe(III) together comprise a hydroxyl radical generating system which readily induces DNA strand breaks (29). Fig. 5 a-d shows the effects of phenolic compounds on DNA breakage induced by the 0.2 mM ascorbate/0.02 mM iron couple. Only caffeic acid and gossypol inhibited DNA breakage induced by ascorbate in presence of Fe. This inhibition was concentration dependent over the concentration range 0.1 mM to 2 mM, i.e. 0.5 to 10 times the concentration of ascorbate. DNA nicking was decreased about one third ($p < 0.05$), even when these two phenolic compounds were present only at half of the ascorbate concentration.

We concomitantly determined the actions of these phenolic compounds on oxygen consumption by 1.5 mM ascorbate in the presence of 0.4 mM Fe (III). Caffeic acid (0.75 mM) and gallic acid (0.75 mM) decreased the oxygen consumption by ascorbate/Fe(III) by 56% and 22%. 1,2,4-Benzenetriol (0.75 mM) increased the oxygen consumption in the presence of ascorbate/Fe(III) by 62%. Gossypol (0.5 mM) was even more effective as a pro-oxidant, increasing oxygen consumption by 459% (Fig. 6).

DISCUSSION

Phenolic compounds act as pro-oxidants inducing DNA breakage, except gossypol

Oxygen-derived active species mediate DNA breakage induced by caffeic acid, gallic acid and 1,2,4-benzenetriol. In contrast, DNA breakage by gossypol was apparently not mediated by oxygen-derived active species, since none of the scavengers tested decreased gossypol-induced DNA breakage. A role for semiquinone-type species remains possible, but the site-specific Fenton type reaction is excluded by the lack of effect of catalase or desferrioxamine.

Since superoxide dismutase did not significantly decrease or increase DNA breakage induced by 1,2,4-benzenetriol, caffeic acid, or gossypol, the role of superoxide was unclear. Ambiguously, superoxide was predominantly a generator of radicals in the autoxidation of 1,2,4-benzenetriol (31), and a remover of radicals (by chain termination) in autoxidation of gallic acid. Hydrogen peroxide was the pre-eminent intermediate in DNA breakage by these phenolic compounds, except for gossypol, based on inhibition of DNA breakage by catalase.

Rates of reduction of oxygen by phenolic compounds correlate imperfectly with DNA breakage

The ability of a phenolic compound to induce DNA breakage depends (i.a.) on the number and position of phenolic hydroxyl groups and other substituents on the benzene ring. Thus, *o*- and *p*-dihydroxyphenols (catechols) readily induce DNA nicking, but *m*-dihydroxyphenols do not (1). The replacement of a hydroxyl group by an amino group also decreases the DNA breaking activity (1). Increased genotoxicity is associated with increased stability of intermediates in the reduction of oxygen,

presumably reflecting increased ability to generate active species. The rapid reductions of oxygen by 1,2,4-benzenetriol and gallic acid are consistent with their strong DNA nicking ability.

The inability of caffeic acid to induce measurable oxygen consumption was consistent with its weak ability to nick DNA and its high reduction potential resulting from ring deactivation by alkenyl and carboxyl groups (Fig. 7). Since catalase, and catalase plus desferrioxamine inhibited caffeic acid-induced DNA breakage by over 80%, one can nevertheless infer that in this case the DNA breakage occurs by a site-specific the Fenton type reaction. Therefore ability to donate electrons in Fenton reaction does not necessarily reflect ability to reduce molecular oxygen. Evidently, even the slow autoxidation allows caffeic acid to generate sufficient hydrogen peroxide to nick DNA. Failure of superoxide dismutase to inhibit suggests that caffeic acid itself, or its semiquinone, rather than superoxide, serve as Fenton donors. The oxygen consumption of gossypol was inconsistent with its relatively low DNA nicking ability. Clearly gossypol produces oxygen-derived active species, but perhaps its molecular size and architecture prohibit site-specific radical generation and active oxygen species are produced too far from the DNA strands to induce nicking.

Fe(II) is more effective than Cu(I) or Cu(II) added alone, but less effective in the presence of phenolic compounds

The observation that, added alone, reduced iron was more genotoxic than reduced copper is contrary to thermodynamic considerations. It also contrasts with the commonly observed greater genotoxicity of copper in DNA nicking (32), and base damage (33). In the current studies, copper was more effective when the presence of reductants allowed redox cycling (Fig. 4 a-d). On this basis, copper may be more effective than iron when ability to both accept and donate electrons is important.

Phenolic compounds are anti-oxidants in relation to more reactive species and pro-oxidants in relation to less reactive species

The actions of the phenolic compounds on ascorbate/Fe-induced DNA nicking (Figure 5 a-b) reflect metal binding, ability to generate active oxygen species, and impact on site-specific attack on DNA by ascorbate/Fe. Since all four phenolic compounds tested include the o-diphenol residue, they readily bind metals. In comparison with ascorbate, 1,2,4-benzenetriol is a strong reducing agent, as reflected in their respective rates of autoxidation [64.1 μM O_2/min for 1,2,4-benzenetriol, versus 1.68 μM/min for ascorbate in presence of Fe(III)]. Gallic acid, gossypol and caffeic acid are weaker reductants, as reflected in much lower rates of oxygen consumption (Fig. 6). Addition of 1,2,4-benzenetriol increased oxygen consumption when added to ascorbate in the presence of Fe, whereas caffeic acid and gallic acid decreased oxygen consumption. On this basis, with respect to ascorbate, 1,2,4-benzenetriol is a pro-oxidant, while gallic acid and caffeic acid are antioxidants. It is readily understandable that a radical generator is an anti-oxidant in the presence of more reactive species, and pro-oxidant in the presence of less reactive species.

The ability of caffeic acid to inhibit DNA breakage by ascorbate/Fe(III) paralleled its ability to attenuate oxygen consumption by ascorbate/Fe(III). However, the actions of gallic acid and gossypol on DNA breakage and oxygen consumption by ascorbate/Fe were discordant. Thus, gallic acid which slowed oxygen consumption by ascorbate in the presence of Fe, nevertheless accelerated DNA breakage. In contrast, gossypol which increased oxygen consumption by ascorbate/Fe, protected superhelical DNA against nicking induced by ascorbate/Fe. Further studies are needed to clarify these observations.

CONCLUSION

1,2,4-Benzenetriol, caffeic acid, gallic acid and gossypol, are pro-oxidants which cleave DNA strands when they are the only active species in the reaction system. Unlike damage induced by other phenolic compounds, gossypol-induced DNA nicking is not mediated by hydrogen peroxide, superoxide, or metal ions. Transition metal ions aggravate DNA damage by these phenolic compounds. 1,2,4-Benzenetriol and gallic acid are damaging under all circumstances tested, but caffeic acid and gossypol can protect DNA against cleavage by ascorbate in the presence of Fe.

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LEGENDS TO FIGURES

Figure 1. **DNA breakage induced by phenolic compounds.** The photograph shows fluorescence of gels stained with ethidium bromide. DNA (0.02 μg/10 μl) was exposed to phenolic compounds in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 minutes. Control means no treatment by phenolic compounds. No control was shown in the gallic acid group, since both 1,2,4-benzenetriol and gallic acid groups ran on a single gel and thus only a single control was needed. Similarly gossypol and caffeic acid shared the control in the caffeic acid group. Notations: I SC, supercoiled DNA; II NI, nicked circular DNA; III LI, linear DNA.

Figure 2. **Comparison of oxygen consumption by phenolic compounds: Velocity/concentration relationship.** The graph shows rates of oxygen consumption measured polarographically over a range of concentrations of each phenolic compound. Reactions were carried out in air-saturated (246 $\pm$ 6 μM O_2) 50 mM sodium phosphate buffer, pH 7.4 at 25°C. The reaction was initiated by injection of the phenolic compound into the polarography cuvet. Squares represent 1,2,4-benzenetriol (BT); diamonds are gallic acid (GAL); triangles are gossypol (GOS); circles are caffeic acid (CAF). Lines are drawn smoothly through the points by an arbitrary least squares spline function having no mechanistic significance.

Figure 3. **Effects of antioxidants on DNA breakage induced by phenolic compounds with or without desferrioxamine.** ^{3}H-labelled DNA (0.12 μg/50 μl reaction mixture) was exposed to phenolic compounds in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. The X axis represents reagents, and the Y axis represents the percentage of supercoiled DNA breakage. Abbreviations are as follows: DES, desferrioxamine (20 μM); SOD, superoxide dismutase and inactivatedSOD, inactivated superoxide dismutase (20 U/ml); CAT, catalase and inactivatedCAT, inactivated catalase (20 U/ml). Inactivated superoxide dismutase and catalase were boiled for 40 minutes prior to addition to the reaction mixtures. Antioxidants and desferrioxamine were added prior to addition of phenolic compounds. Desferrioxamine was not tested in the presence of inactive enzymes. Error bars represent standard errors of mean. Graphs represent: (a) 1,2,4-Benzenetriol (BT) 210 μM; (b) Caffeic acid (Caf) 200 μM; (c) Gallic acid (Gal) 247 μM; (d) Gossypol (Gos) 600 μM.

Figure 4. **Effects of copper and iron on DNA breakage induced by phenolic compounds.** DNA (0.02 μg/10 μl) was exposed to appropriate phenolic compound, copper and iron (25 μM) in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 minutes. DNA breakage was evaluated by agarose gel electrophoresis and densitometer as described in Material and Methods. The X axis represents reagents, and the Y axis represents percentage of total DNA. Abbreviations are as follows: Fe(II), ferrous sulfate; Fe(III), ferric chloride; Cu(I), cuprous chloride; Cu(II), cupric sulfate. Graphs represent: (a)

1,2,4-Benzenetriol (BT) 10 μM; (b) Caffeic acid (Caf) 15 μM; (c) Gallic acid (Gal) 2 μM; (d) Gossypol (Gos) 50 μM.

Figure 5. **Effects of phenolic compounds on DNA breakage induced by ascorbate/Fe system.** DNA (0.046 μg/25 μl reaction mixture) was exposed to different combinations of 0.02 mM ferric chloride (Fe), 0.2 mM ascorbate (Asc) and graded concentrations of appropriate phenolic compound in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 minutes. DNA cleavage was evaluated by agarose gel electrophoresis and densitometer as described in Materials and Methods. The X axis represents reagents, and the Y axis represents percentage of total DNA. Error bars represent the standard error of mean. Graphs represent: (a) Caffeic acid (Caf); (b) Gossypol (Gos); (c) Gallic acid (Gal); (d) 1,2,4-Benzenetriol (BT).

Figure 6. Oxygen consumption of ascorbate/Fe(III): Effects of phenolic compounds. Experimental conditions are as stated in the legend to Fig. 2. The X axis represents reagents, and the Y axis represents logarithm of oxygen consumption. Abbreviations are as follows: Asc, ascorbate (1.5 mM); Fe, ferric chloride (0.4 mM); BT, 1,2,4-benzenetriol (0.75 mM); GAL, gallic acid (0.75 mM); CAF, caffeic acid (0.75 mM); GOS, gossypol (0.5 mM).

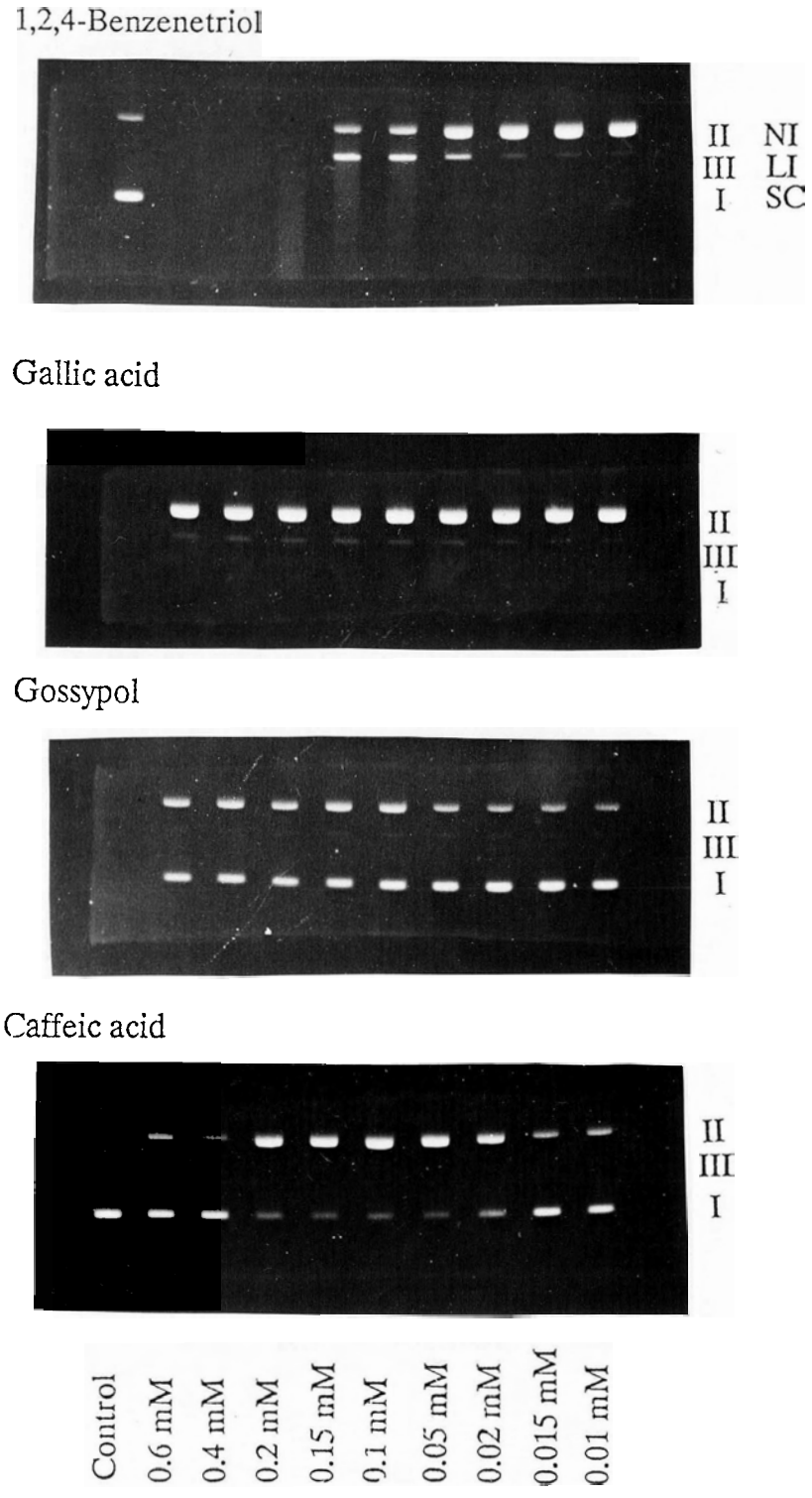


Figure 1. DNA breakage induced by phenolic compounds

Figure 2. Comparison of oxygen consumption by phenolic compounds: Velocity/concentration relationship

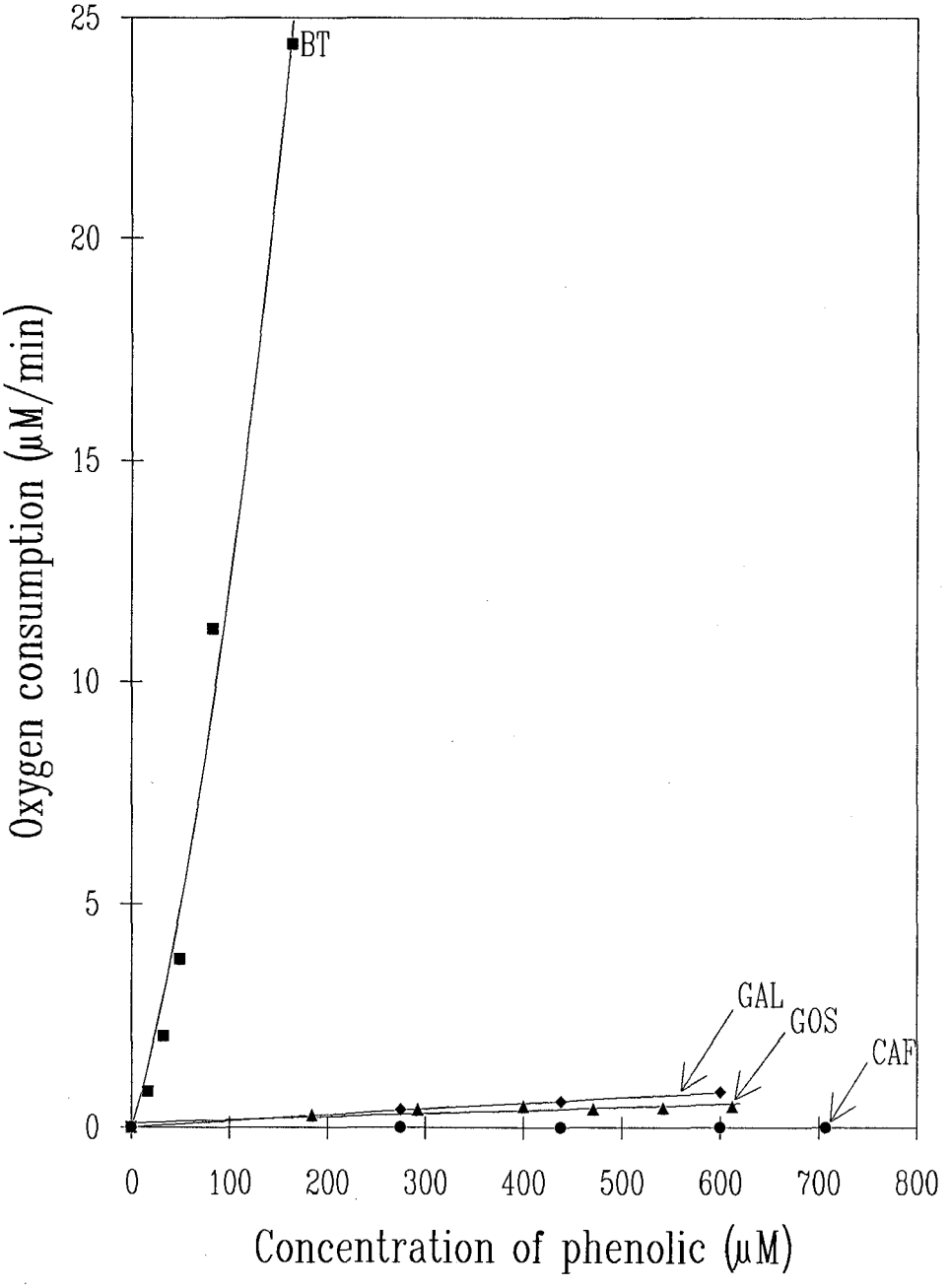


FIGURE 3

Effects of antioxidants on DNA breakage induced by phenolic compounds with or without desferrioxamine (a) 1,2,4-benzenetriol

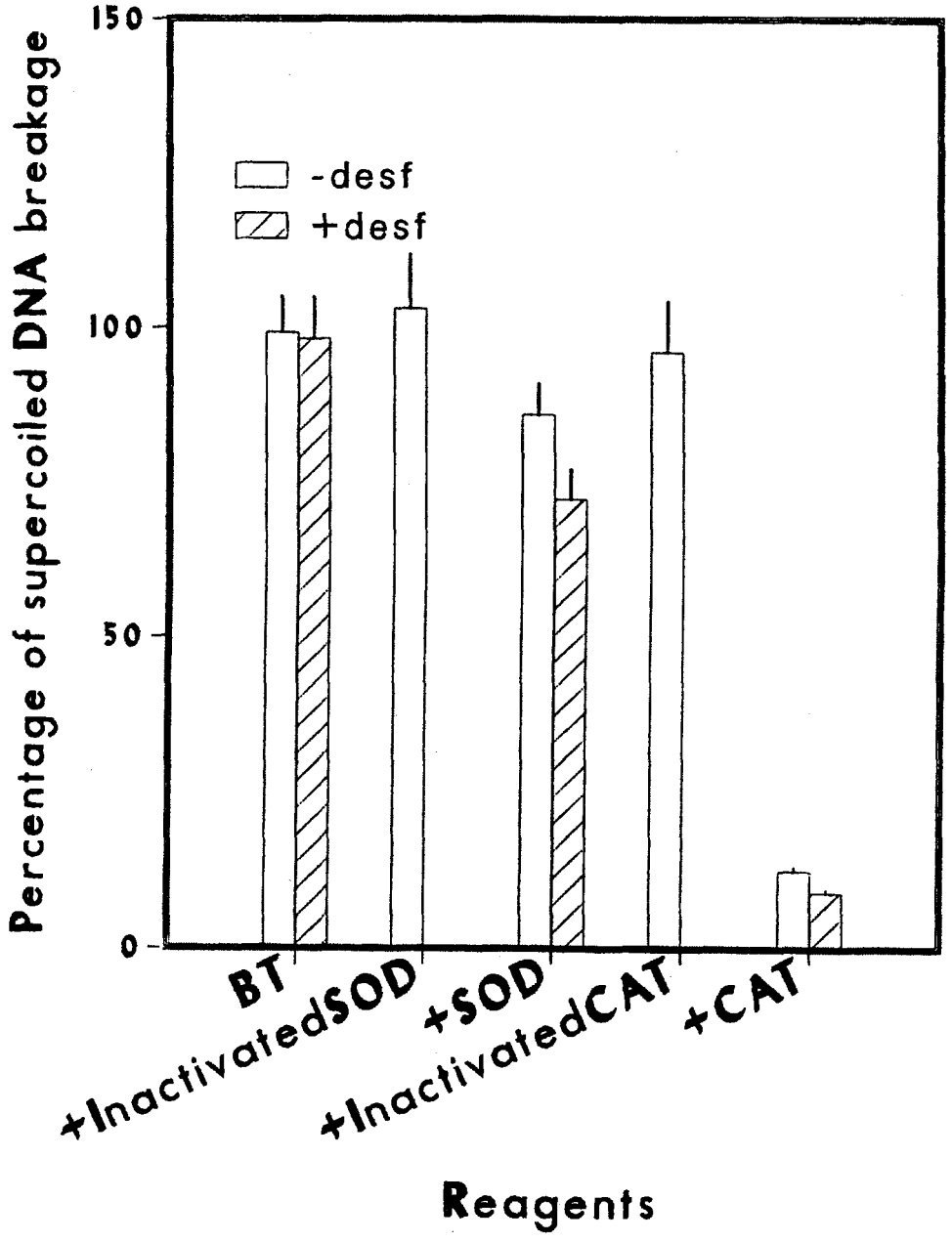


FIGURE 3 (b) caffeic acid

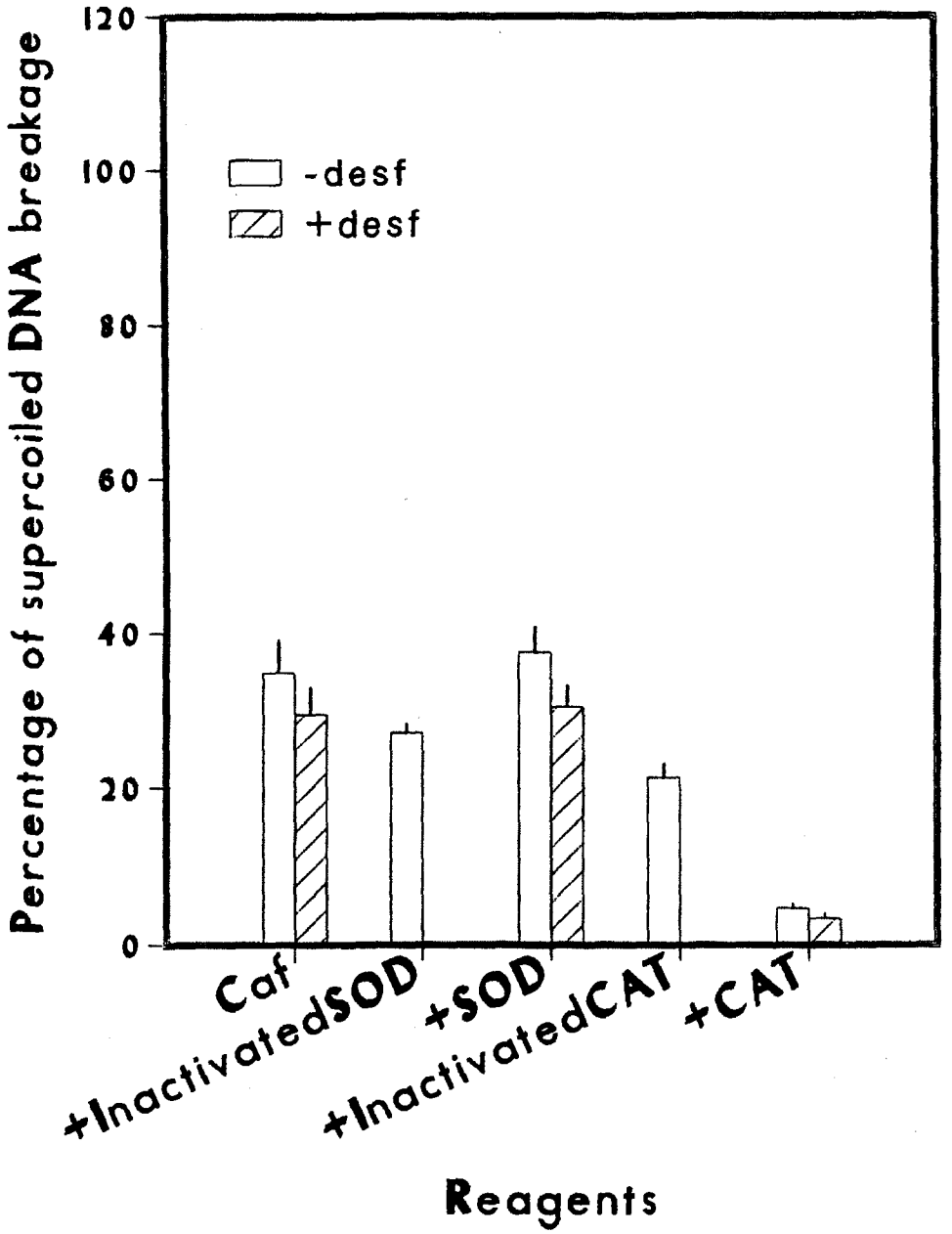


FIGURE 3 (c) gallic acid

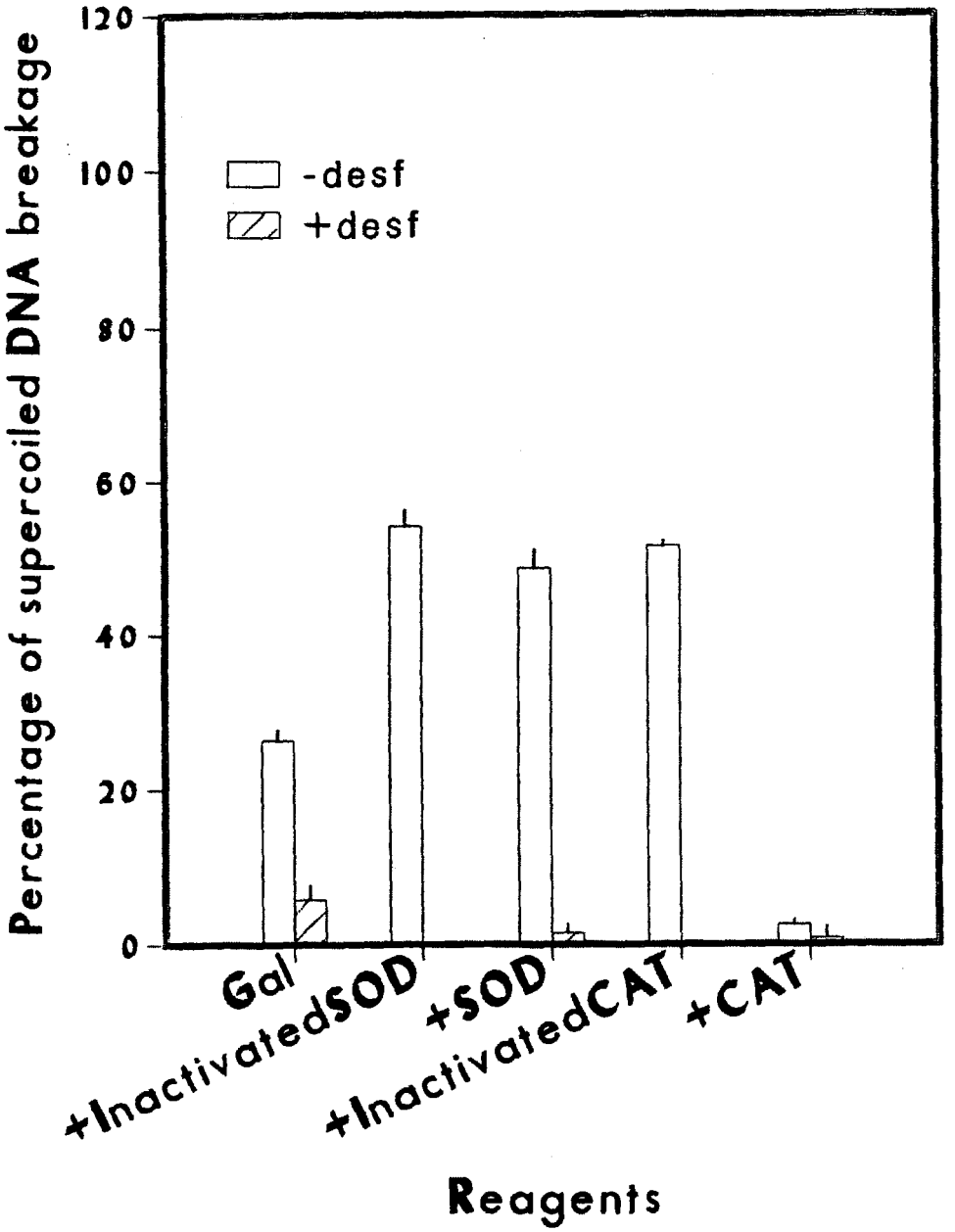


FIGURE 3 (d) gossypol

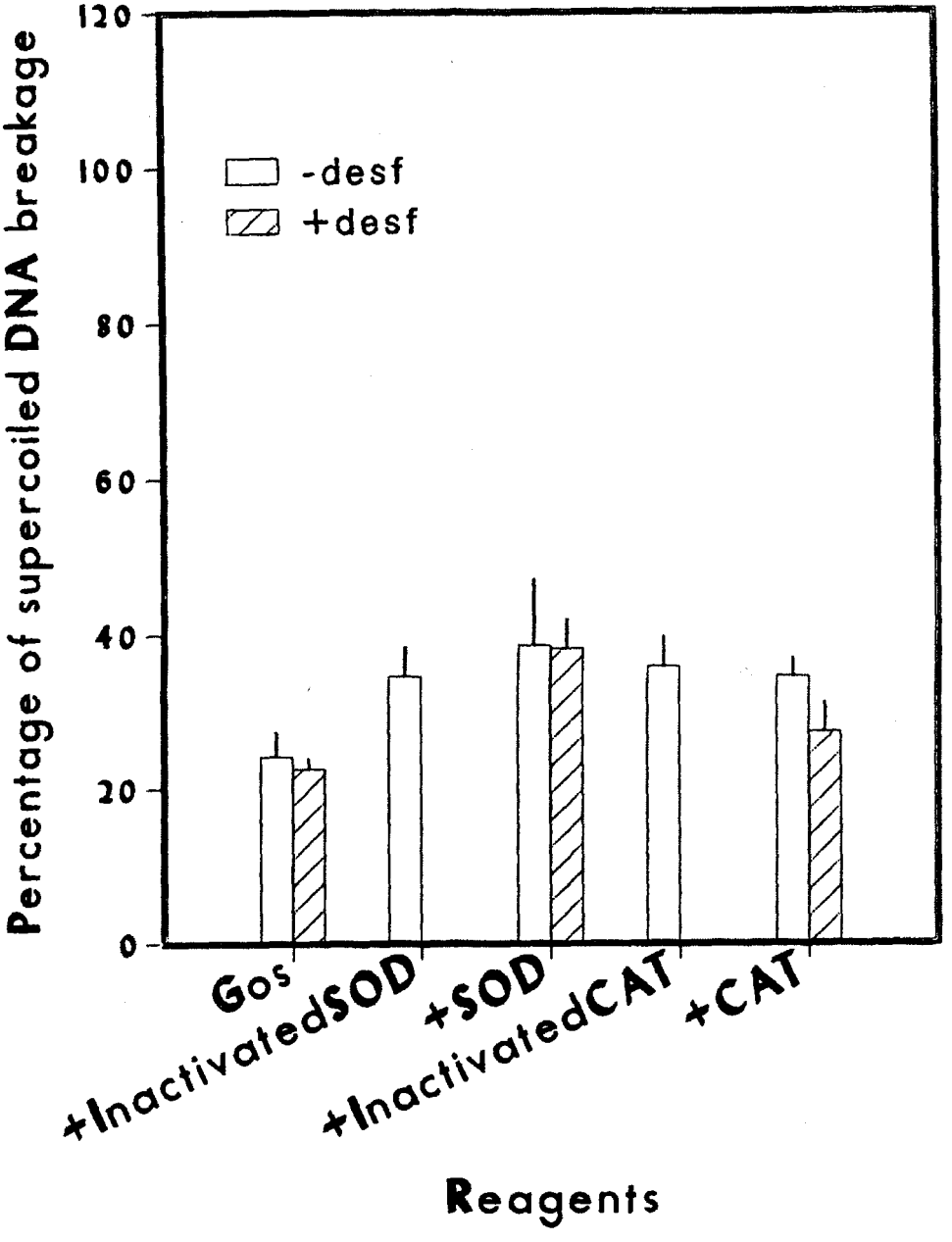


FIGURE 4

Effects of copper and iron on DNA breakage induced by phenolic compounds

(a) 1,2,4-benzenetriol

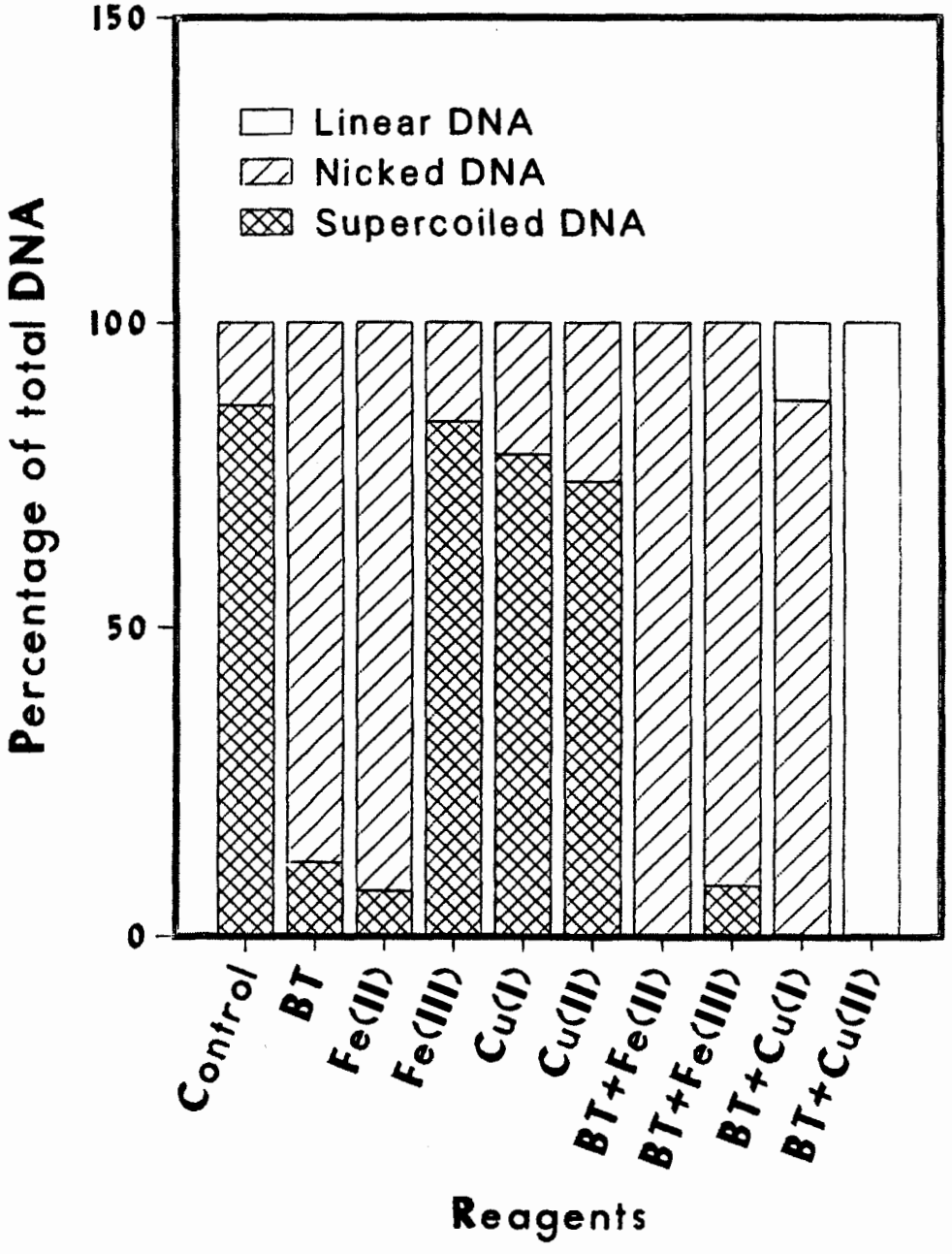


FIGURE 4 (b) caffeic acid

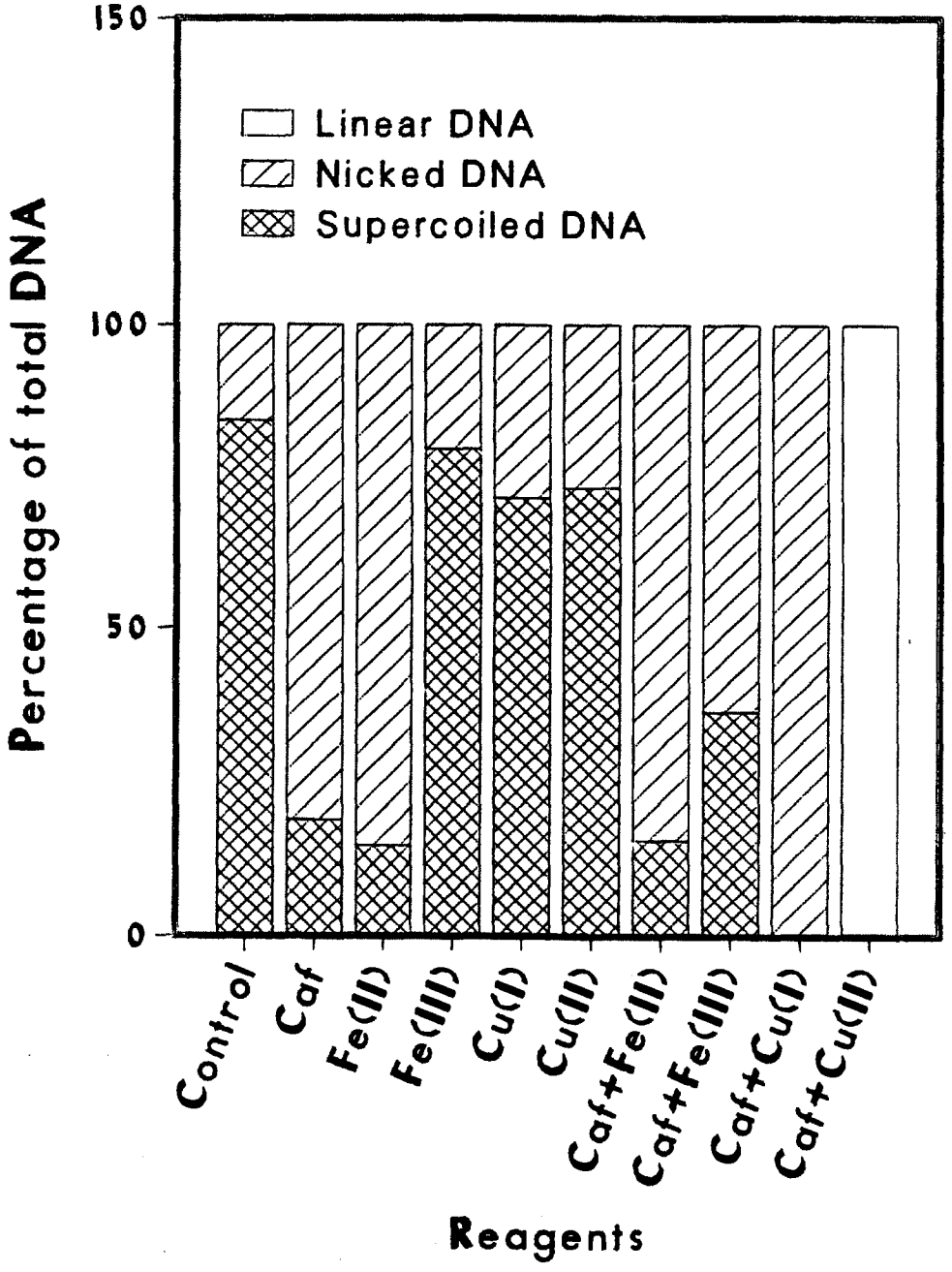


FIGURE 4 (c) gallic acid

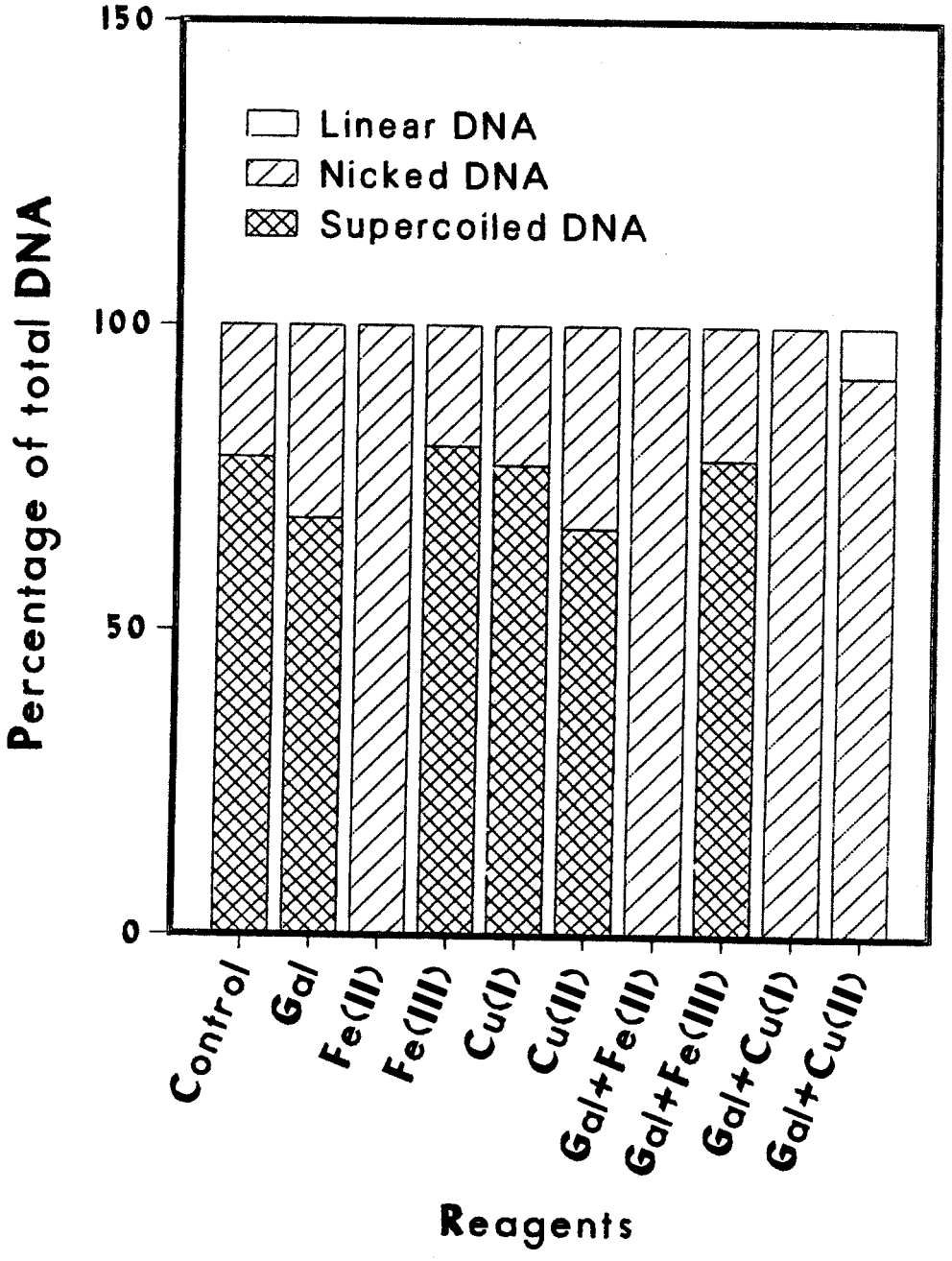


FIGURE 4 (d) gossypol

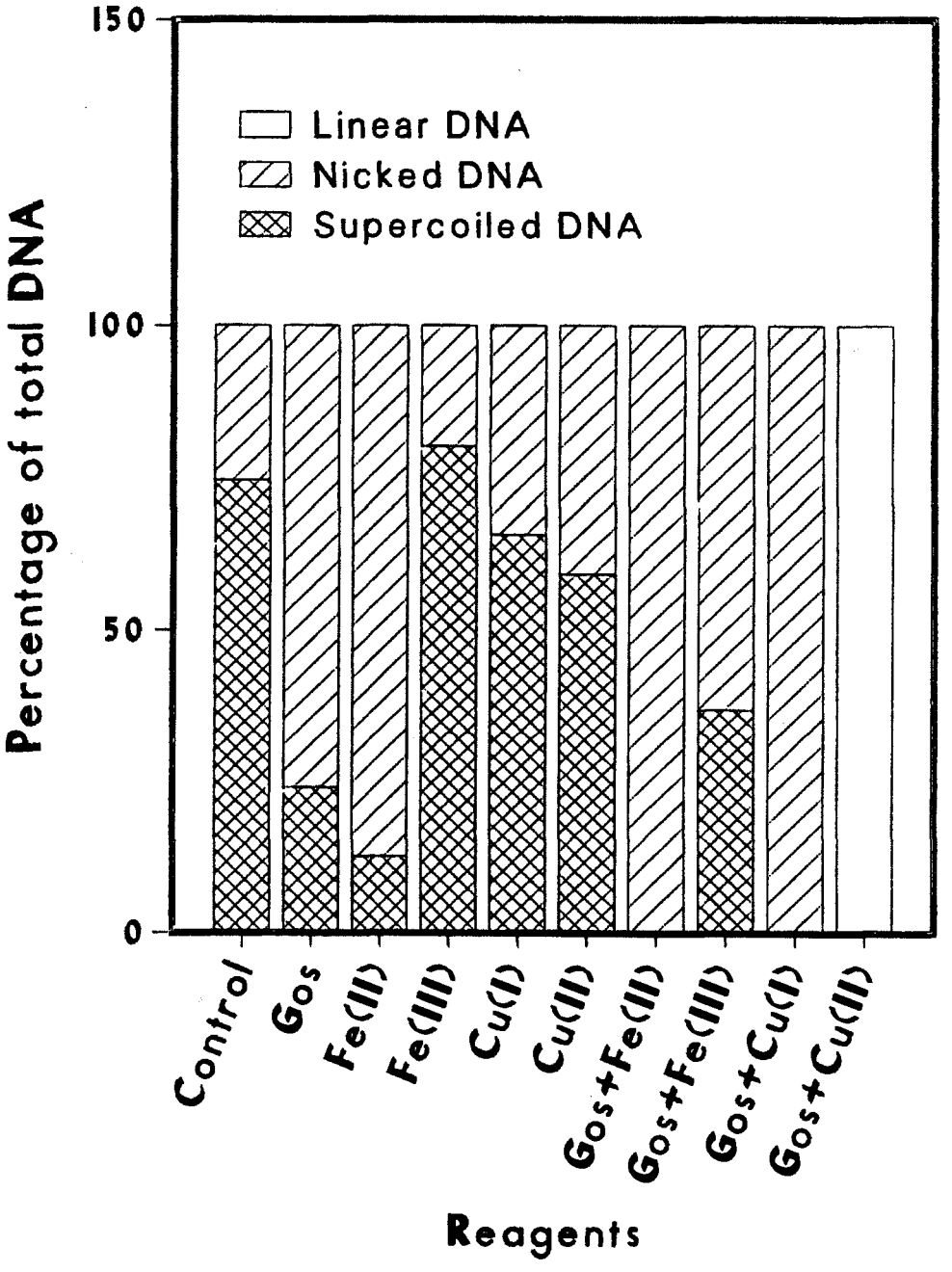


FIGURE 5
Effects of phenolic compounds on **DNA** breakage induced by ascorbate/Fe system
(a) caffeic acid

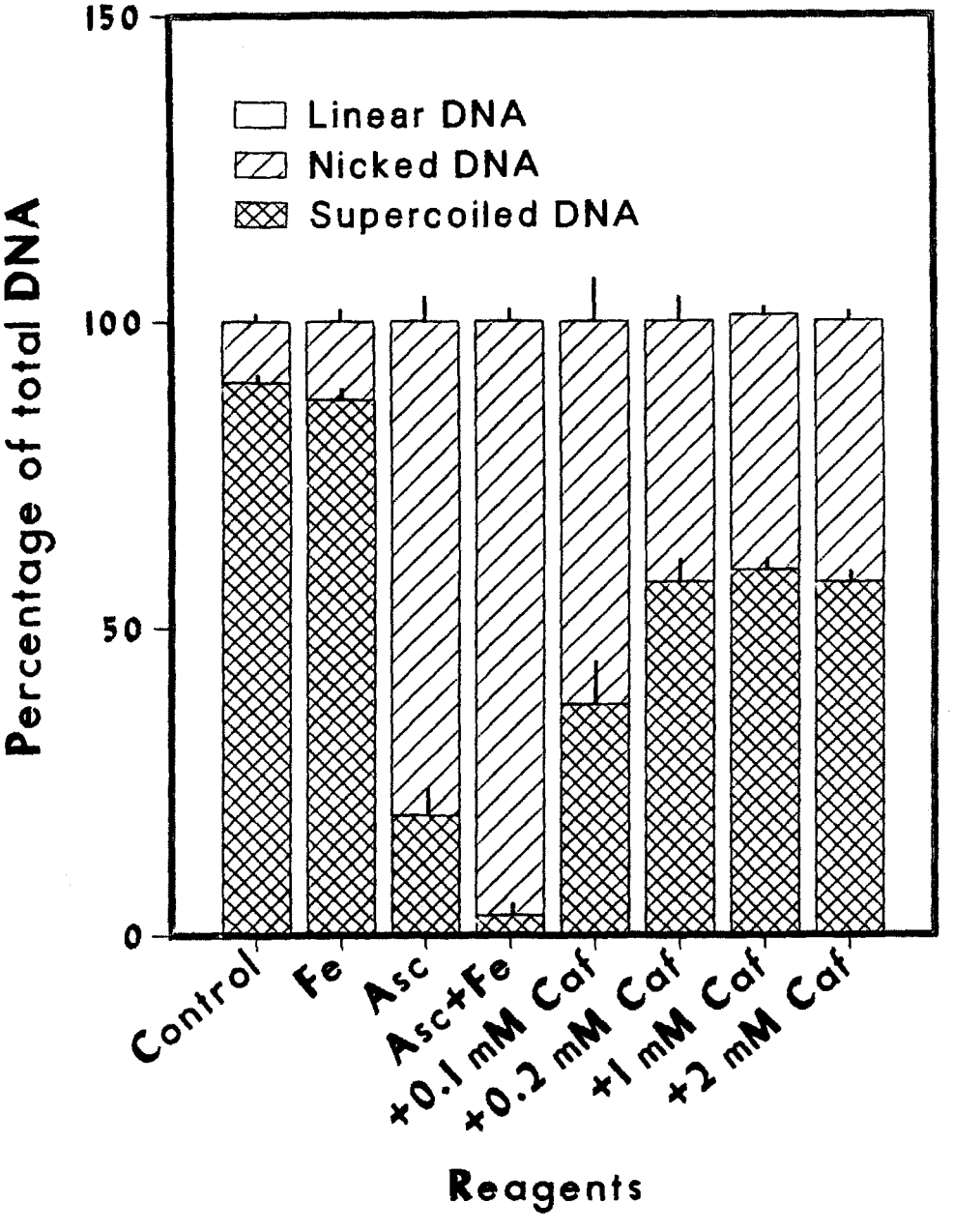


FIGURE 5 (b) gossypol

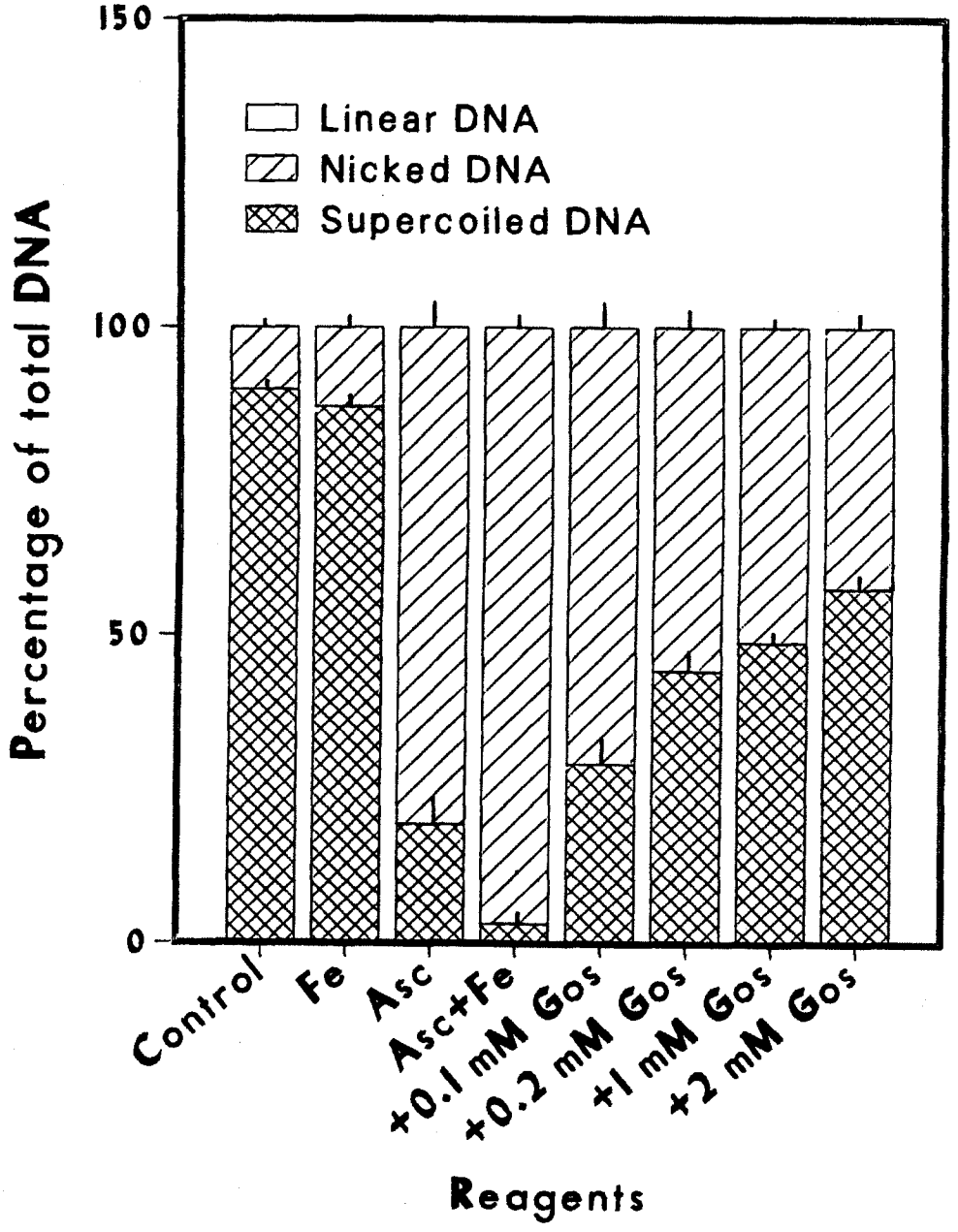


FIGURE 5 (c) gallic acid

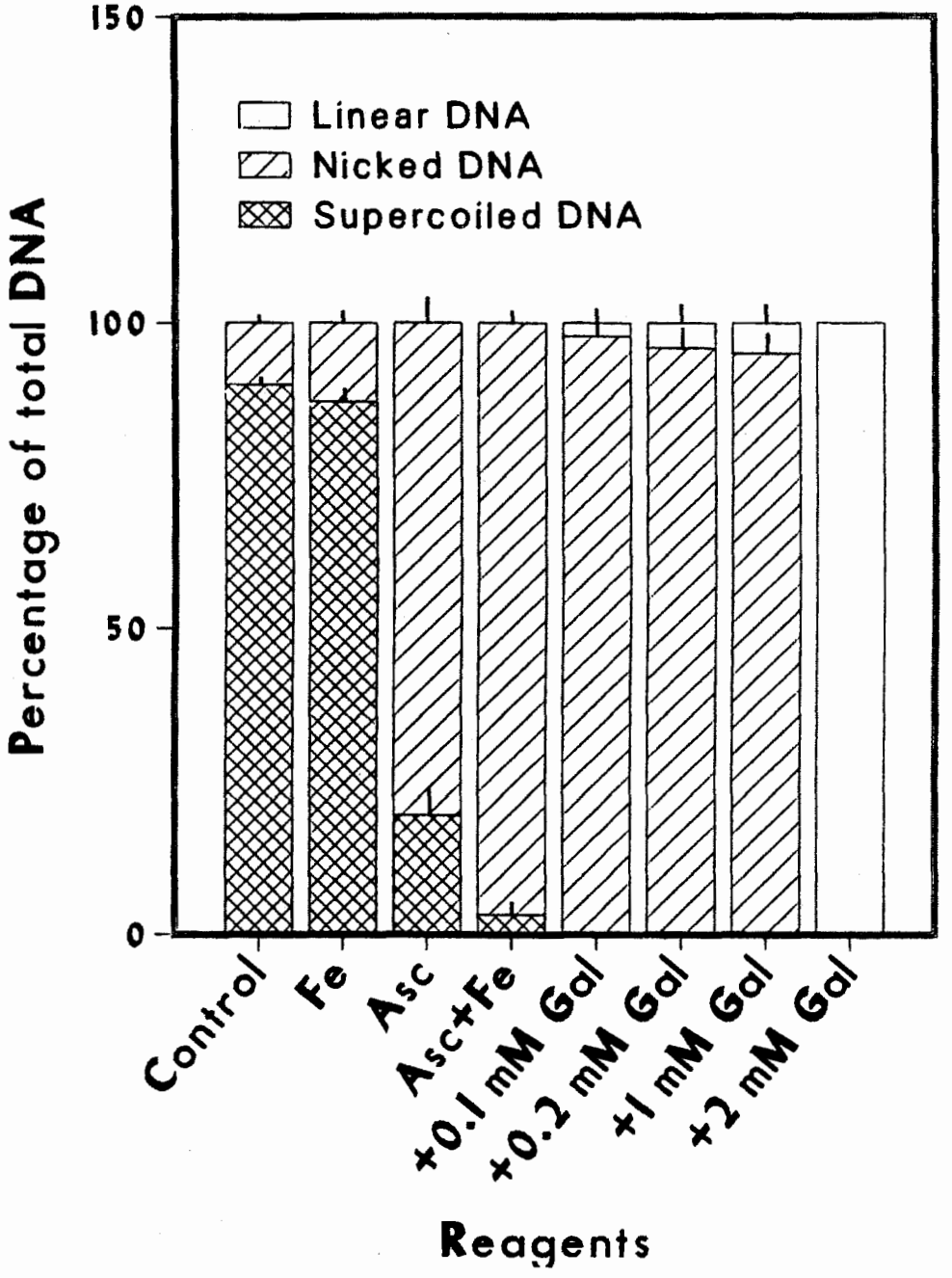


FIGURE 5 (d) 1,2,4-benzenetriol

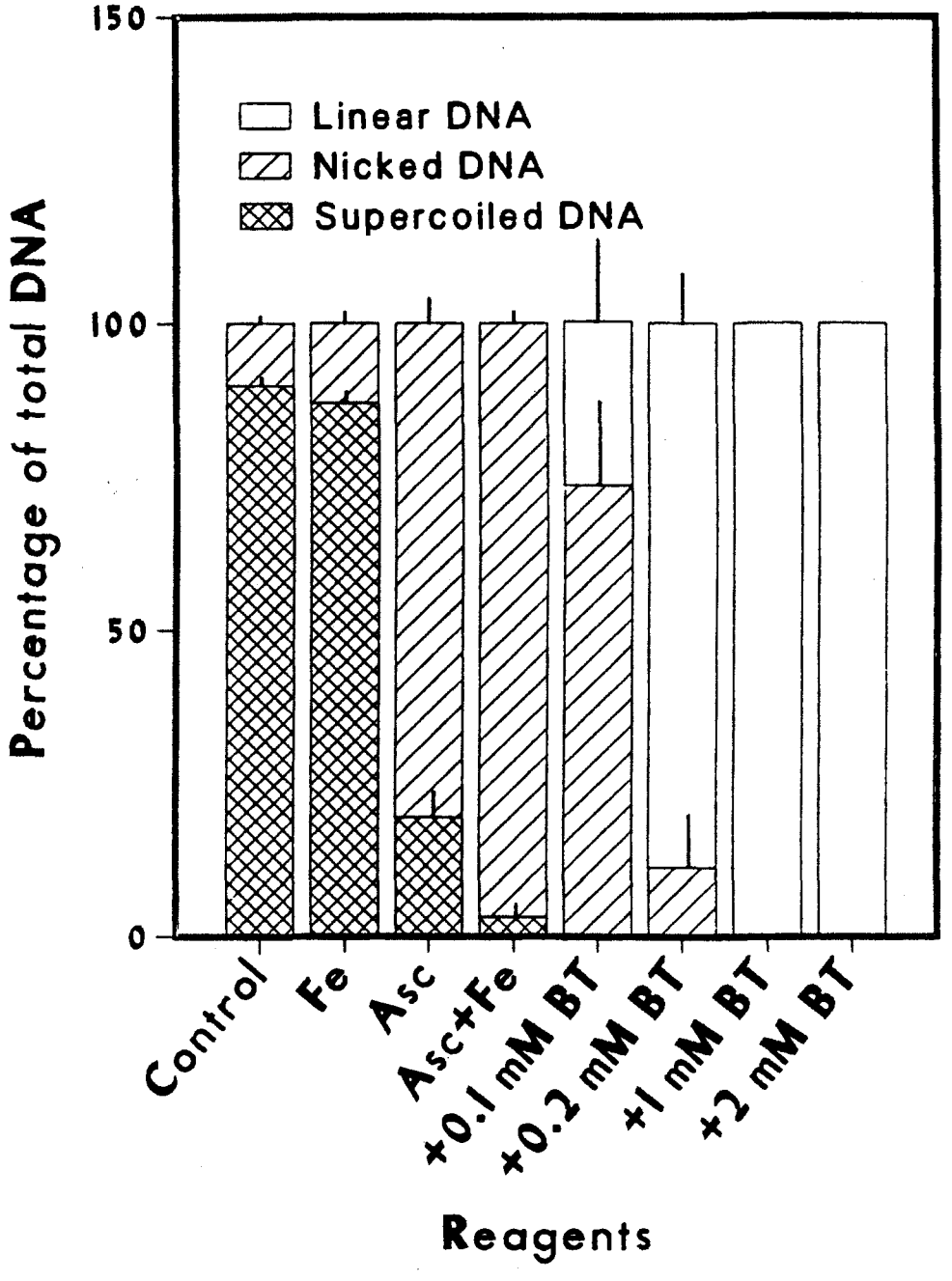
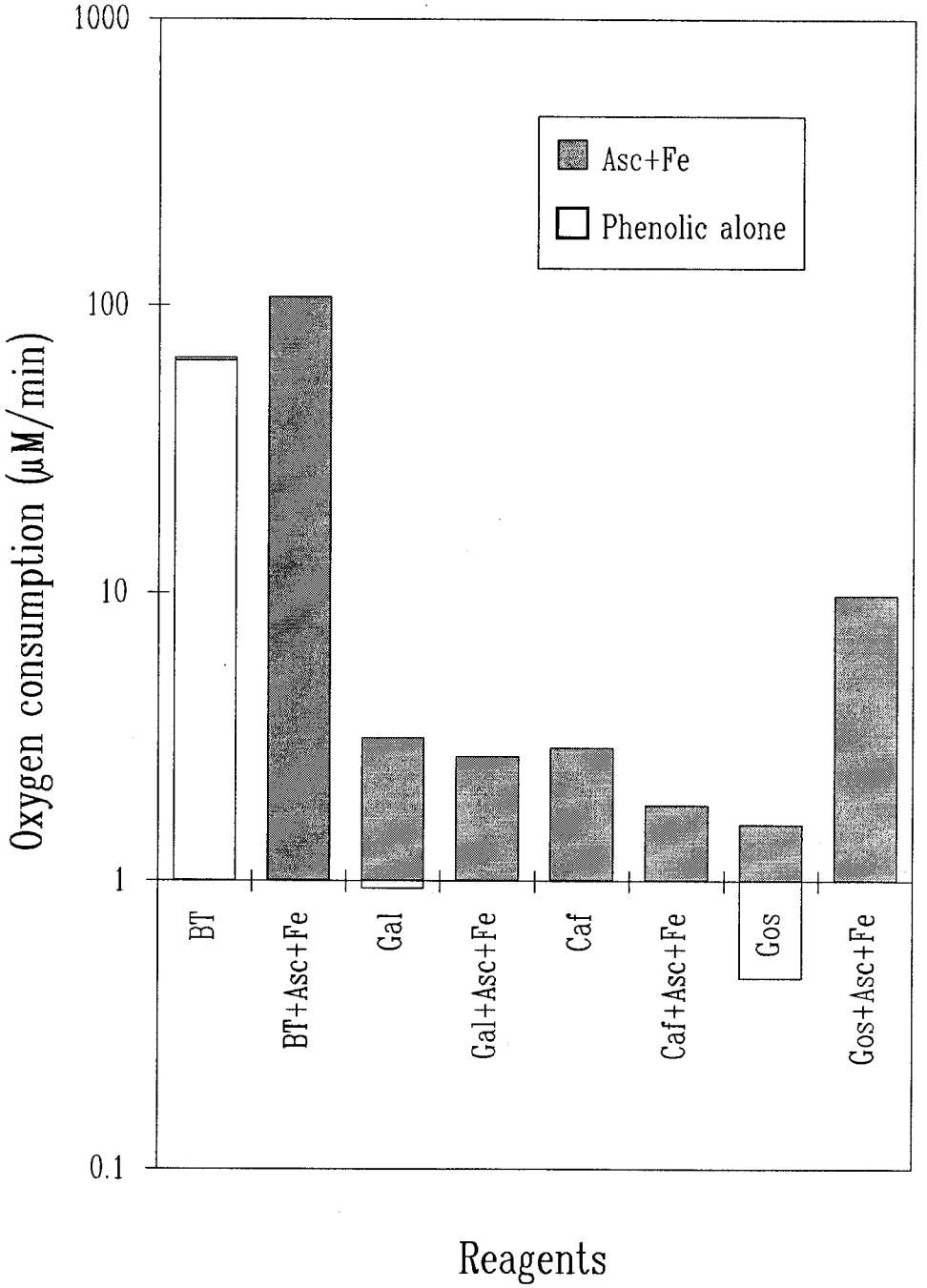
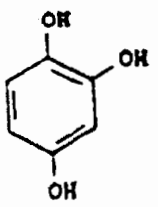
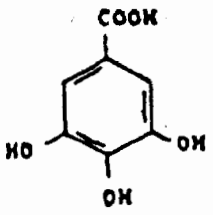


Figure 6. Oxygen consumption of ascorbate/Fe(III): Effects of phenolics

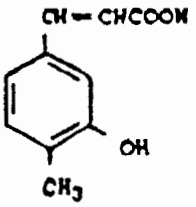




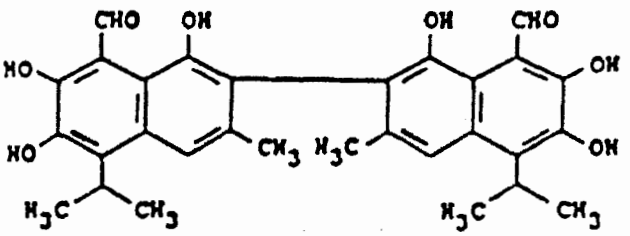
1,2,4-Benzenetriol



Gallic acid



Caffeic acid



Gossypol

Figure 7. Structures of phenolic compounds

DNA breakage induced by 1,2,4-benzenetriol: Roles of oxygen-derived active species and transition metal ions

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CHAPTER III

DNA breakage induced by 1,2,4-benzenetriol: Roles of oxygen-derived active species and transition metal ions

ABSTRACT

The extent of supercoiled DNA breakage induced by 1,2,4-benzenetriol was influenced by incubation time, buffer systems and concentration of 1,2,4-benzenetriol. At pH 7.4 and 37°C, the concentration of 1,2,4-benzenetriol which induced cleavage of 50% supercoiled PM2 DNA in 15 minutes (ED_{50}) was 6.7 μM.

Catalase (20 U/ml), formate (25 mM), superoxide dismutase (20 U/ml) and mannitol (50 mM) inhibited DNA degradation induced by 1,2,4-benzenetriol (18 μM) from 31 to 85%. The efficacy of inhibition was in the order: catalase > formate > superoxide dismutase > mannitol. We conclude that H_2O_2 is the dominant active species with $O_2\cdot^-$ and $\cdot OH$ playing subordinate roles. Added together, with hydroxyl radical scavengers, superoxide dismutase augmented their protective effects. However, superoxide dismutase and hydroxyl radical scavengers alone or in combination showed no synergism with catalase.

Desferrioxamine (0.02 mM) inhibited DNA breakage induced by 18 μM 1,2,4-benzenetriol, by about 85%, indicating that DNA breakage depends almost completely on adventitious transition metal ions. Further addition of cupric, cuprous, ferric or ferrous ions enhanced DNA breakage. Thus, 1,2,4-benzenetriol likely induces DNA breakage by a metal-mediated site-specific mechanism. However, parallel, less potent, metal-independent pathways were detectable at high concentrations of 1,2,4-benzenetriol. Thus, at 210 μM 1,2,4-benzenetriol, desferrioxamine failed to protect DNA significantly, but catalase continued to protect by over 80%.

Keywords: DNA breakage, 1,2,4-benzenetriol, oxygen-derived active species, iron, copper, Fenton reaction, antioxidant, desferrioxamine

INTRODUCTION

Benzene is a widely distributed environmental carcinogen, but the mechanisms by which it increases the risk of leukemia are poorly understood. Benzene itself is relatively non-toxic, but it is metabolized to toxic phenolics, among which 1,2,4-benzenetriol is the most reactive.[1] Although a minor product, 1,2,4-benzenetriol mediates a number of genotoxic actions. 1,2,4-Benzenetriol breaks ΦX-174 supercoiled DNA[2] and is clastogenic in L5178YS cells.[3] In addition, 1,2,4-benzenetriol inhibits DNA synthesis in L5178YS cells, bone marrow cells and isolated calf thymus DNA.[4,5] Benzene metabolites induce genotoxicity by: (1) forming DNA adducts;[5] (2) inhibiting DNA polymerase α;[6] (3) inducing micronuclei;[7] (4) inhibiting polymerization of tubulin;[8] and (5) generating active oxygen species that cleave the DNA backbone.[2]

In comparison with benzene and its other metabolites, the greater genotoxicity of 1,2,4-benzenetriol is thought to result from its low reduction potential. As a strong reductant, it readily participates in co-oxidation and autoxidation. The reactive oxygen intermediates generated in the reduction of oxygen by 1,2,4-benzenetriol include $O_2\cdot^-$, H_2O_2, $\cdot OH$ and semiquinone.[2,9] Scavengers of oxygen-derived active species or chain-breaking antioxidants can block DNA degradation by 1,2,4-benzenetriol.

There has been little extrapolation from the action of scavengers *in vitro* to cellular implications, because so little is known regarding scavengers and metal chelating agents acting in concert. The spectrum of propagating intermediates will clearly be different in the presence of a scavenger. Consequently the protective role of each scavenger will be changed by the presence of the others. Only a minority of studies have considered the actions of scavengers, and even the actions of single scavengers on 1,2,4-benzenetriol genotoxicity are contradictory. In one study,

superoxide dismutase, catalase or methional inhibited DNA damage by 1,2,4-benzenetriol completely, but sodium formate did not.[10] In a contrasting study, catalase and benzoate decreased DNA damage by 1,2,4-benzenetriol while superoxide dismutase failed to prevent DNA breakage.[2] To resolve such inconsistencies, it is important to define the factors in a given experimental situation which determine the relative contributions of active oxygen species to the genotoxicity of 1,2,4-benzenetriol.

In general, redox active transition metal ions enhance free radical-induced biological damage, in most cases by site-specific mechanisms.[11] Iron ions may be cytotoxic *in vivo* if excess iron absorption leads to accumulation of iron in such organs as liver, pancreas or bone marrow. After exposure to 1,2,4-benzenetriol, iron accumulates mainly in the mitochondrial fraction of bone marrow cells[12] and participates in Haber-Weiss-like reactions.[13] In the presence of Cu(II), 1,2,4-benzenetriol-generated hydroxyl radicals react with deoxyribose, releasing 2-thiobarbituric acid reactive products.[14] Transition metals differ widely in their abilities to accelerate DNA damage induced by 1,2,4-benzenetriol. Copper is more effective than iron. Addition of Cu(II) enhanced the DNA damage induced by 1,2,4-benzenetriol, but did not increase the production of hydroxyl radicals as measured by a spin trapping method.[10] Addition of Fe(III) did not significantly alter DNA damage, although it accelerated the production of ·OH by 1,2,4-benzenetriol.[10] The Cu(I)-specific chelator, bathocuproine, inhibited DNA damage, but desferrioxamine failed to inhibit.[10] The availability of decompartmentlized metals intracellularly requires special circumstances (see discussion).

In the current study, we wished to resolve contradictory reports of superoxide dismutase and hydroxyl radical scavengers in DNA breakage induced by 1,2,4-benzenetriol *in vitro*.[2,10] Moreover, we wished to clarify the roles of metals in 1,2,4-benzenetriol-induced DNA damage. We, therefore, undertook: (1) to investigate

the participation of active oxygen species and transition metal ions in DNA breakage by 1,2,4-benzenetriol, (2) to explore the interactions between antioxidants and a metal chelator, and (3) to assess the physiological importance of antioxidants and metal chelators in cellular resistance to 1,2,4-benzenetriol-induced genotoxicity. The current results suggest a metal-mediated site-specific mechanism for DNA breakage by 1,2,4-benzenetriol at micromolar concentrations. At ten-fold higher 1,2,4-benzenetriol concentrations, another mechanism can be detected, which nicks DNA in the presence of desferrioxamine. This metal-independent mechanism is mediated by hydrogen peroxide, but superoxide is relatively unimportant.

MATERIALS AND METHODS

Reagents

1,2,4-Benzenetriol was purchased from Aldrich Chemical Co. (Milwaukee, WI). Cupric sulfate, cuprous chloride, ferric chloride, ferrous sulfate and disodium ethylenediamine-tetraacetate (EDTA) were from Fisher Scientific Co. (Fair Lawn, NJ). Superoxide dismutase (4050 U/mg) was from DDI Pharmaceutical Inc., (Mountain View, CA). Catalase (65,000 U/mg) was from Boehringer Mannheim Co. (Dorval, PQ). Desferrioxamine was a gift from Ciba-Geigy Pharmaceutical Co. (Summit, NJ). Formic acid (sodium salt) and D-mannitol were from Sigma Chemical Co. (St. Louis, MO).

Preparation of 1,2,4-benzenetriol under anaerobic condition

Argon-saturated 1,2,4-benzenetriol solution was prepared fresh each day, by flushing deionized distilled water with argon for at least 30 minutes, and sealing the vial to maintain a positive pressure of argon.

Preparation of plasmid pdBPV-1 (142-6) DNA

The supercoiled plasmid pdBPV-1 DNA was prepared as previously described.[15] Briefly, extraction and purification of plasmid DNA consisted of three steps: (1) growth of *Escherichia coli* (E. coli) strain containing the plasmid; (2) harvesting and lysis of the E coli; (3) purification of the plasmid DNA.

Preparation of ^3H-labelled PM2 DNA

^3H-labelled supercoiled bacteriophage PM2 DNA was prepared as described by method of Espejo and Canelo[16,17] as modified by Tsang.[18]

The titer of a PM2 phage stock was estimated by counting the number of plaques on a pseudomonas culture plate. Pseudomonas Bal-31 was grown at 28-30°C in 500 ml Bal-broth [10 ml of 10 mM Tris-HCl (pH 7.5), 12 gm magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 26 gm sodium chloride, 8 gm bacto-nutrient broth, 10 ml 1 M calcium chloride, and 3.5 ml 20% potassium chloride per liter of deionized water]. When the bacteria reached a density of 3 x 10^8/ml, 50 mg 2'-deoxyadenosine (Sigma) was added. Five minutes later, the bacteria were infected with 1-2 x 10^{12} bacteriophage PM2. After five minutes, 0.5 mCi of methyl-^3H thymidine (25 Ci/mmol, Amersham International) was added. The culture was incubated overnight. Phage particles were separated by centrifugation and purified by cesium chloride density equilibrium centrifugation. Phage particles were collected, dialyzed extensively against 1.2 litre of buffer [0.02 M Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA] at 4°C, and then lysed by adding 10% sodium dodecyl sulfate. The phage DNA was extracted in the aqueous phase by phenol.

Detection of DNA cleavage by agarose gel electrophoresis

In pilot studies we decided on 15 minutes for the standard incubation period. We selected phosphate as the buffer medium because of its presence intracellularly. Supercoiled plasmid BPV-1 DNA was dissolved in 1 mM sodium phosphate buffer (pH 7.4) and incubated with test reagents under various conditions in a final volume of 10 μl. The detection of DNA strand breaks by agarose gel electrophoresis has been previously described.[19] After incubation with test reagents, samples were diluted in buffer containing 0.05% bromophenol blue and 6% glycerol and fractioned by

electrophoresis at 60 volts in 0.7% (weight/volume) agarose gels containing 0.04 M Tris-acetate and 0.002 M EDTA. The gel was stained with 0.5 μg/ml ethidium bromide for 30 minutes and destained in water for 2-3 minutes. The DNA bands were visualized under illumination by UV-light.

Analysis of DNA damage by filter-binding assay using nitrocellulose filters

The ^{3}H-labelled supercoiled PM2 DNA was incubated with the test reagents in 0.5 ml tubules in a water bath at 37°C. After treatment, PM2 DNA was precipitated by ethanol and resuspended in 25 or 50 μl 1 mM sodium phosphate buffer (pH 7.4). A 10 μl aliquot of the DNA reaction mixture was diluted with 40 μl 10 mM Tris-HCl/1 mM EDTA (pH 8.0). 5 μl of 100 mM Tris-HCl (pH 7.5) and 0.15 ml SE buffer (0.01% sodium dodecyl sulfate, 2.5 mM EDTA-NaOH, pH 7.0) were added. The DNA was then denatured with 0.2 ml of 0.3 M K_2HPO_4-KOH (pH 12.4) for 2 minutes at room temperature. The solution was neutralized with 0.1 ml of 1 M KH_2PO_4-HCl (pH 4.0) to induce strand separation of the nicked DNA molecules. The solution was diluted with 0.2 ml of 5 M NaCl and 5 ml of NT buffer (1 M NaCl, 50 mM Tris-HCl, pH 8.0), and filtered through nitrocellulose filters that had been presoaked in NT buffer. The reaction tube was rinsed with 2 ml NT buffer. The rinse was filtered. The filters were then washed with another 2 ml NT buffer, dried, and the radioactivity was determined by liquid scintillation counting. Total DNA was determined by measuring the radioactivity of 10 μl of reaction mixture spotted on a blank filter. The average number of nicks (N) per DNA molecule was calculated using the equation $N = -\ln P$; where P was the fraction of DNA that was not retained on the nitrocellulose filter.[20]

Statistical analysis

All reactions were performed at least in duplicate and usually in triplicate. Standard statistical methods were used to calculate means and their standard errors. Significance of differences between means was examined by student's t-test and confirmed by one-way analysis of variance. The level of confidence required for each selected comparison was set in advance to $p < 0.05$. Prior to analysis of variance, the data were logarithmically transformed to minimize heteroscedasticity. In the analysis of variance it was established that it was inappropriate to pool the variances of the logarithmically transformed data and consequently, unpooled variances were used.

RESULTS

1,2,4-Benzenetriol cleaved supercoiled DNA (form I) to nicked circular DNA (form II), linear DNA (form III) and eventually to small fragments. In this section, we will (1) establish the effects of reaction conditions, (2) explore the roles of the transition metal ions, copper and iron, and finally (3) report the actions of selective scavengers of oxygen-derived active species. The data will be interpreted in terms of the contribution of oxygen-derived active species to 1,2,4-benzenetriol-induced DNA breakage, individually and in combination.

DNA cleavage mediated by 1,2,4-benzenetriol depended on incubation time, dosage and incubating medium

Using 20 μM 1,2,4-benzenetriol, in 1 mM sodium phosphate buffer (pH 7.4), most of the supercoiled BPV-1 DNA was converted to nicked circular DNA (form II) within the first 5 minutes at 37°C (Fig. 1). Double stranded DNA breakage, however, continued for over 60 minutes.

We examined DNA breakage by 1,2,4-benzenetriol in three media (Fig. 2). Initially we used the standard medium in this laboratory, namely 10 mM Tris-HCl/ 1 mM EDTA buffer (pH 8.0). However, due to the presence of EDTA, 1,2,4-benzenetriol (0.5 to 1 mM) failed to break DNA. In 1 mM sodium phosphate buffer (pH 7.4), 1,2,4-benzenetriol was moderately effective, breaking single DNA strands. 10 mM Tris-HCl buffer (pH 7.4) was the most effective medium. In this medium 1,2,4-benzenetriol induced DNA double-strand breaks.

Supercoiled PM2 DNA breakage increased with increasing concentration of 1,2,4-benzenetriol (Fig. 3). After incubation for 15 minutes, DNA nicking was

detectable at 0.5 μM 1,2,4-benzenetriol, which nicked 6% of supercoiled DNA, i.e., an average of one nick per ten DNA molecules. 1,2,4-Benzenetriol at 20 μM converted 70% of supercoiled DNA to nicked circular DNA, inducing an average of 2.1 nicks per DNA molecule. The concentration of 1,2,4-benzenetriol which cleaved 50% supercoiled PM2 DNA (ED_{50}) was 6.7 μM.

Metal chelators strongly inhibited DNA breakage

Either EDTA or desferrioxamine protected against DNA breakage by 20 μM 1,2,4-benzenetriol (Fig. 4). Protection by EDTA was concentration dependent. 0.5 mM EDTA inhibited DNA breakage by 81% while 0.1 mM EDTA was only half as effective. Desferrioxamine was more potent at lower concentrations, so that even 0.02 mM protected almost completely.

To model intracellular toxicity of 1,2,4-benzenetriol, we assessed the extent and mechanism of the genotoxicity of 1,2,4-benzenetriol under conditions where a site specific mechanism was precluded. Protection by desferrioxamine could be overpowered by raising the 1,2,4-benzenetriol concentration sufficiently (Fig. 5). Thus, at the high concentrations of 1,2,4-benzenetriol (210 to 270 μM), there were sufficient DNA nicking in the presence of desferrioxamine to assess the further protective action of additional scavengers.

Transition metal ions enhanced DNA breakage induced by 1,2,4-benzenetriol

Added alone, 25 μM Fe(II) nicked 90% of the supercoiled DNA within 30 minutes. Cu(I), Cu(II) or Fe(III) alone, were virtually ineffective in inducing DNA breakage. However, when allowed to redox cycle by the presence of 10 μM 1,2,4-benzenetriol, Cu(II), Cu(I) and Fe(II) were more effective than Fe(III)

(Fig. 6 a-b). In the presence of 1,2,4-benzenetriol, 10 or 25 μM Cu(II) converted supercoiled DNA to small DNA fragments.

Catalase strongly protected DNA

Among the scavengers of oxygen-derived active species, catalase was the most potent and consistent inhibitor of 1,2,4-benzenetriol-induced DNA nicking. Surprisingly, protection by catalase was dose-dependent from insignificant at 2 U/ml to 90% at the highest catalase concentration (30 U/ml) (Fig. 7). Heat-inactivated catalase was ineffective (Fig. 8). Protection by catalase, unlike protection by desferrioxamine, was not overcome by raising 1,2,4-benzenetriol concentration to 210 μM (Fig. 8).

Hydroxyl radical scavengers moderately inhibited DNA breakage

Formate inhibited DNA cleavage more than did mannitol, at concentrations of 5 to 50 mM (Fig. 9). However, protection by formate levelled off over 30 mM, while protection by mannitol continued to increase throughout the range tested. Hydroxyl radicals or related species clearly mediate DNA breakage induced by 1,2,4-benzenetriol.

Superoxide dismutase slightly protected DNA

Inhibition of 1,2,4-benzenetriol-induced DNA breakage by superoxide dismutase averaged 30% (Table), and was unaffected by superoxide dismutase concentration in the range 5 to 30 U/ml (data not shown). Heat-inactivated superoxide dismutase failed to protect (Fig. 8). On the basis of the result superoxide protected minimally, but significantly against 1,2,4-benzenetriol-induced DNA breakage.

Comparison of the effects of scavengers and a metal chelator added individually

Added individually, scavengers protected DNA against 18 μM 1,2,4-benzenetriol in the order: desferrioxamine (85%) = catalase (81%) > formate (41%) > superoxide dismutase (30%) = mannitol (28%) (Table). Thus H_2O_2 is a more important species than $O_2^{\cdot -}$ or hydroxyl radicals in DNA breakage mediated by 18 μM 1,2,4-benzenetriol.

Actions of scavengers when present in combination

In the living cells, antioxidants are always present in combination, and act in a coordinated manner. To assess the mediators in the presence of each of the scavengers, we determined protection by the scavengers in various combinations against 1,2,4-benzenetriol-induced DNA breakage (Table). The proportion of protection afforded by adding a scavenger in the presence of others was calculated as follows:

protection = 100 x [(damage before adding scavenger - damage after adding scavenger)/damage before adding scavenger].

For each scavenger, conditions could be found under which it completely failed to protect. Moreover the actions of every scavenger except formate could be augmented by the presence of other synergistic scavengers.

Protection by desferrioxamine is enhanced by catalase and diminished by increased 1,2,4-benzenetriol concentration. When 1,2,4-benzenetriol was 18 μM, desferrioxamine inhibited by 70 to 90% regardless of the presence of other scavengers (Fig. 10). The greatest protection by desferrioxamine against 18 μM 1,2,4-benzenetriol was in the presence of other scavengers, added in the presence of superoxide dismutase

and/or catalase, or superoxide dismutase plus formate. Desferrioxamine protected by 90% or more, leading to virtually complete inhibition (> 96%).

Under these conditions it was not possible to detect further inhibition by adding further scavengers. Only when 1,2,4-benzenetriol concentrations were increased to 210 μM was there sufficient residual damage to detect additional protection by the further addition of scavengers (Fig. 8). At 210 μM 1,2,4-benzenetriol desferrioxamine protected by only 25%, and superoxide dismutase failed to protect. The decrease in protection by desferrioxamine at high 1,2,4-benzenetriol concentration (210 μM) could be accounted for by the presence of contaminant metals in the 1,2,4-benzenetriol, sufficient to eventually saturate the desferrioxamine. A simpler explanation, however, is that a background level of desferrioxamine-independent damage becomes more evident as 1,2,4-benzenetriol concentration increases, eventually reaching levels at which nearly all DNA becomes nicked.

Protection by catalase is enhanced by desferrioxamine and diminished by formate plus superoxide dismutase. Catalase protected by about 80%, and this was not significantly increased by the presence of other scavengers except desferrioxamine (Fig. 10). Protection by catalase was, however, diminished by the presence of formate or superoxide dismutase. In the simultaneous presence of formate plus superoxide dismutase (which already protected by 65%), the further addition of catalase failed to provide significant additional protection, whether or not desferrioxamine was present.

Protection by superoxide dismutase is increased by desferrioxamine or mannitol and abolished by catalase. The minor protection by superoxide dismutase was almost doubled by the presence of desferrioxamine or mannitol (Fig. 11). In contrast,

superoxide dismutase failed to provide any additional protection in the presence of catalase (whether or not formate or formate plus desferrioxamine was also present).

Protection by formate or mannitol is increased by superoxide dismutase and abolished by desferrioxamine. Although protection by mannitol was doubled by the prior presence of superoxide dismutase (with or without catalase), protection by formate was not increased substantially by any other scavenger (Fig. 12). Thus formate protected by 40-50% in the presence of superoxide dismutase with or without desferrioxamine. Protection by either mannitol or formate was abolished by desferrioxamine plus either catalase or superoxide dismutase (Fig. 13).

DISCUSSION

The current study confirmed that oxygen-derived active species generated in the autoxidation of 1,2,4-benzenetriol mediated DNA cleavage *in vitro*. The process is dominated by hydrogen peroxide and requires traces of transition metal ions. However, no single scavenger inhibits completely, suggesting that there are multiple pathways for DNA damage. This conclusion is confirmed by the observation that every scavenger protected under some circumstances, and failed to protect under others.

Properties of DNA breakage mediated by 1,2,4-benzenetriol

DNA breakage continued after virtual completion of the 1,2,4-benzenetriol/O_2 reaction. 1,2,4-Benzenetriol at 20 μM should be virtually completely oxidized in 18 minutes or less in the absence of DNA, since the time constant ($t_{1/2}$) for the autoxidation of 1,2,4-benzenetriol at 250 μM was 2.45 minutes, and the reaction was essentially complete in less than 18 minutes.[21]

Double stranded DNA breakage, however, continued for over 60 minutes, by which time the 1,2,4-benzenetriol should be virtually exhausted (Fig. 1). This phenomenon raises the question: which species induces DNA breakage after oxidation of 1,2,4-benzenetriol is complete? The possibility that benzoquinone oxidation products induce delayed DNA breakage is negated by the observation that benzoquinone itself caused very little or no DNA strand breakage *in vitro*.[2,10] However, the semiquinone present in the final equilibrium mixture is a potential Fenton donor.[22] The presence of hydrogen peroxide as a long lived reaction product provides further mechanisms for semiquinone-mediated delayed DNA breakage. An alternative explanation is long-lived thymine and guanine-derived radicals generated by the attack of ·OH on DNA.[23] Such

radicals could continue to cleave DNA even after the initiating pro-oxidant has disappeared.

Relatively low concentrations of 1,2,4-benzenetriol induced DNA breakage especially in the presence of iron or copper. In agreement with a previous report,[2] concentrations of 1,2,4-benzenetriol as low as 0.5 μM produced measurable DNA damage. Such concentrations are low enough to be significant for the toxicity of 1,2,4-benzenetriol *in vivo*. In animal toxicity studies doses of benzene which induced chromosomal abnormalities were 200 mg/kg.[24] Pharmacokinetic studies predict that such a dose exposes the bone marrow to prolonged concentrations of 1,2,4-benzenetriol (around 3 μM) over several days.[24] The simultaneous presence of iron or copper ions, as low as 5 μM amplified the genotoxicity of 1,2,4-benzenetriol.

Roles of active oxygen species and transition metals in 1,2,4-benzenetriol-induced DNA breakage

H_2O_2 is a major contributor to 1,2,4-benzenetriol-induced DNA strand scission. Because catalase inhibits DNA breakage so strongly, H_2O_2 is clearly a major species in DNA breakage by 1,2,4-benzenetriol (Table). The increasing protection afforded by increasing concentrations of catalase (Fig. 7) requires comment, since even the lowest concentrations of catalase tested should remove almost all H_2O_2. One unit of catalase decomposes 1.0 μmol of H_2O_2 per minute.[2] Even if we assume that autoxidation of 18 μM 1,2,4-benzenetriol yields 18 μM H_2O_2 in one minute, 20-30 units of catalase per ml can convert 2500 μM H_2O_2 to H_2O per minute. On this basis, the failure of catalase (20-30 U/ml) to inhibit DNA breakage completely is not easily explicable, except on the basis that some of the H_2O_2 is not free in solution, but is complexed (Fig. 7).

In site-specific reactions involving macromolecules (e.g., protein, DNA), oxygen can be reduced to H_2O_2 while remaining complexed by metals, and further reduced to produce site-specific radicals without free H_2O_2 ever leaving the solvent cage. The increased protection at the highest catalase concentrations carries two implications. First, DNA breakage can be induced by H_2O_2 even at an extremely low concentration of free H_2O_2. Second, H_2O_2 molecules at the fringe of the solvent cage have a "choice" between reacting with catalase and returning to the site-specific transition metal. Since they are less accessible than H_2O_2 molecules in free solution, higher concentrations of catalase may be required to produce maximal inhibition.

Superoxide is not the major intermediate in 1,2,4-benzenetriol-induced DNA breakage. Although not the main damaging species, superoxide was an active species in DNA breakage. Superoxide can participate in DNA breakage in two ways. First, superoxide propagates one of the radical mediated chain reactions which mediates autoxidation of 1,2,4-benzenetriol.[25] Second, superoxide can contribute as a "non-classical" Fenton-type reductant of metal ions bound to DNA.[26] Thus, superoxide dismutase can decrease DNA breakage both by slowing production of active species and by blocking their actions.

Because one unit of superoxide dismutase per ml can scavenge approximately 0.6 nM of superoxide per min,[27] 20-30 U/ml of superoxide dismutase should readily remove the total predicted yield of $O_2\cdot^-$ from 20 μM 1,2,4-benzenetriol (less than 0.1 nmol/ml/min). Since even this excess of superoxide dismutase protected only moderately, superoxide was not a major intermediate (Table). Other reductants such as 1,2,4-benzenetriol itself or its semiquinone likely compete more effectively than $O_2\cdot^-$ in the reduction of metals. Moreover, one-electron transfer to oxygen is not a major route in the metal catalyzed oxidation of 1,2,4-benzenetriol.[28] Our results, therefore, are

more consistent with those showing marginal or no protection by superoxide dismutase[2] than with those showing complete protection.[10] Only in the presence of other scavengers was protection by superoxide dismutase substantial.

To what extent are hydroxyl radicals active in breaking DNA strands? Formate and mannitol did not strongly inhibit DNA breakage by 1,2,4-benzenetriol (Fig. 9), and this was consistent with site-specific hydroxyl radicals as the main species breaking DNA. Such hydroxyl radicals attack DNA close to the sites where they are formed, without becoming accessible to hydroxyl radical scavengers. In addition, to the extent that these scavengers remove ·OH, they concomitantly regenerate superoxide.[23,29] In presence of metals, any $O_2\cdot^-$ so generated may be converted to site-specific hydroxyl radicals which further damage the DNA. Finally, damage to DNA by H_2O_2 in the presence of iron or copper may be mediated by higher oxidation states of copper or iron (ferryl species), without intervention by hydroxyl radicals.[30] Once again our results are more consistent with those showing marginal or low protection by hydroxyl radical scavengers[10] than with those showing complete protection.[2]

Metals are important participants in DNA strand scission. The current data confirm dramatic enhancement of DNA breakage by copper or iron,[10] and substantial protection by desferrioxamine (Fig. 4, 6 a-b). Metals reportedly have two effects in DNA breakage. First, traces of iron and copper in solution are required for Fenton type reactions. When reduced, these metal ions convert superoxide radicals to hydroxyl radicals by catalyzing Haber-Weiss-like reactions. Second, binding these metals by DNA provides centers for repeated generation of ferryl species or hydroxyl radicals close to sites which lead to breakage. Addition of desferrioxamine or EDTA removes the metal ions from DNA molecules and force the release of one-electron and two-

electron propagators into free solution where they are accessible to self-termination reactions or any scavengers present.

Interactions of oxygen-derived active species in DNA damage in the presence of scavengers and desferrioxamine

Active species in the presence of desferrioxamine. In the presence of desferrioxamine, hydrogen peroxide remained the most important active oxygen species mediating DNA nicking because catalase remained about as effective as when added alone. Superoxide dismutase doubled its relative effectiveness, inhibiting over half of the 1,2,4-benzenetriol-induced damage which persisted in the presence of desferrioxamine. Formate in contrast lost its protective effect when desferrioxamine was present. These findings lead to two inferences which merit investigation *in vivo*. First, protection by superoxide dismutase is synergistic with the chelation of redox active transition metal ions. Second, the decompartmentalization of metals can be predicted to increase the fraction of damage mediated by formate-accessible hydroxyl radicals.

Active species in the presence of catalase. In the presence of catalase, the further addition of desferrioxamine protected strongly against 18 μM 1,2,4-benzenetriol, blocking 80 to 90% of the catalase-resistant damage to DNA. On this basis, intracellular availability of H_2O_2 makes available pathways for other 1,2,4-benzenetriol-derived species to nick DNA, presumably by Fenton type mechanisms (Fig. 10).

Active species in the presence of superoxide dismutase. In the presence of superoxide dismutase, desferrioxamine and catalase retained their protective effects. Protection by

formate remained at about 50% protection, but mannitol was twice as effective as when added alone (Fig. 11). In the absence of superoxide, mannitol-accessible hydroxyl radicals become more significant.

Active species in the presence of formate or mannitol. The presence of formate or mannitol increased the fraction of the residual damage which was mediated by superoxide. However, catalase or desferrioxamine were rendered less protective (Fig. 12, 13), presumably because their scavenging actions diminished the contribution of Fenton-type reactions. The importance of superoxide was further increased if hydroxyl scavengers plus desferrioxamine were simultaneously present. Desferrioxamine increases the fraction of damage proceeding through one-electron pathways, and formate converts a variety of other radicals to superoxide. Thus, these agents cooperate to increase the fraction of the reaction which is mediated by superoxide.

Characterization of active species and metals in autoxidation of 1,2,4-benzenetriol. Summing the protection by desferrioxamine and catalase gave a total of well over 100%. This implies a synergism between metals and H_2O_2, readily explicable on the basis that most damage occurs via a Fenton mechanism which simultaneously requires both H_2O_2 and reduced metals on the DNA. As another example of synergistic interactions, superoxide dismutase is more than twice as effective in the presence of desferrioxamine as when added alone. This may reflect the almost complete inhibition of autoxidation of 1,2,4-benzenetriol by the simultaneous presence of these scavengers and consequent cessation of production of oxygen-derived active species.[21,22]

What are the mechanisms of DNA damage in the presence of combinations of metal chelators and scavengers? Based on above discussions, a few inferences can be drawn from the ways in which cellular scavengers combine to protect against the genotoxic actions of 1,2,4-benzenetriol. Two generalizations are helpful. First, one scavenger can decrease the susceptibility (of the remaining damage) to a second scavenger. For example, the addition of formate decreased the protection by catalase. This implies synergism between the *radicals*. In this case, accumulation of the first radical scavenged opens up pathways for damage involving the second radical. Second, addition of a scavenger can increase the protection by a second scavenger. For example, the protection by superoxide dismutase was enhanced in the presence of mannitol. This implies synergism between the *scavengers*. In this case, accumulation of the radical scavenged by the first opens up pathways which are independent of the radical scavenged by the second.

Importance *in vivo* of interactions among antioxidants and metal chelators in combination

The current data confirm that intracellular antioxidants and metal chelators act in concert to prevent 1,2,4-benzenetriol-induced DNA breakage. They act at three different levels. When superoxide dismutase or hydroxyl radical scavengers were added individually, protection was 29 to 41%. In combination they protected 65 to 75%. If catalase was simultaneously present, protection increased to 89%, and desferrioxamine further enhanced the protection to 98%.

Substantial *in vivo* genotoxicity of 1,2,4-benzenetriol requires that more than one of the protective systems is inhibited or overwhelmed by the production of oxygen-derived active species. Consider the almost complete protection by mannitol, catalase,

desferrioxamine plus superoxide dismutase. The synergism between these scavengers provided sufficient redundancy that any one could be removed, without significantly decreasing protection. Even the removal of both superoxide dismutase and mannitol failed to diminish protection appreciably.

The protection by catalase plus desferrioxamine was profound, at close to 100%. Therefore, to induce substantial genotoxicity in a living cell containing superoxide dismutase and hydroxyl radical scavengers, 1,2,4-benzenetriol must diminish the actions of both catalase and metal chelators. Both catalase inactivation and iron decompartmentalization are in fact predictable from the known actions of 1,2,4-benzenetriol. One electron donors such as ascorbate can inhibit the activity of catalase (31) and release iron from ferritin (23). These capabilities suggest potentially fruitful pathways for further experiments *in vivo*.

Implications of 1,2,4-benzenetriol induced DNA breakage by metal-mediated site-specific mechanisms

The current results support, in large measure, two widely held beliefs regarding oxygen mediated cytotoxicity "without metals there is no damage", and "without H_2O_2 there is no damage". We propose, therefore, that the site-specific mechanisms for DNA damage by other pro-oxidants should be extended to 1,2,4-benzenetriol. This contention rests on the following characteristics of DNA breakage:

(1) Inhibition of DNA breakage by desferrioxamine or EDTA. These chelators stimulate the reaction of 1,2,4-benzenetriol with oxygen, increasing the production of hydroxyl radicals.[25] Opposing this, desferrioxamine and EDTA compete with DNA to bind the metals ions, removing the metal from DNA.

(2) The progressive inhibition of DNA breakage by increasing concentrations of catalase. Additional evidence for a site-specific mechanism is the partial protection by relatively high concentrations of formate and mannitol, suggesting that relatively inaccessible hydroxyl-type radicals nick DNA strands at or near the site of their formation.

Taking the current data with previous results for oxidation of triphenols, a plausible mechanism for 1,2,4-benzenetriol induced cleavage of supercoiled DNA can be proposed as follows:

Initiation

$$DNA + M^{n+} \text{---} > DNA\text{-}M^{n+}$$

$$DNA\text{-}M^{n+} + QH_2 \text{---} > DNA\text{-}M^{(n-1)+} + \cdot SQ$$

Propagation

$$\cdot SQ + O_2 \text{---} > O_2\cdot^- + O_2$$

$$QH_2 + O_2\cdot^- \text{---} > \cdot SQ + H_2O_2$$

Termination

$$2O_2\cdot^- \text{---} > H_2O_2 + O_2$$

Chain scission

$$DNA\text{-}M^{(n-1)+} + H_2O_2 \text{---} > DNA\text{-}M^{(n-1)+}\text{-}H_2O_2$$

$$DNA\text{-}M^{(n-1)+}\text{-}H_2O_2 \text{---} > DNA\cdot\text{-}M^{n+} + H_2O + OH^-$$

$$DNA\cdot \text{---} > \text{scission products}$$

(Note that propagation and termination reactions may be metal mediated even though this is not indicated in the reactions)

CONCLUSION

We conclude that each of the oxygen-derived active species tested for participates in DNA breakage by 1,2,4-benzenetriol. Metal ions and H_2O_2 mediate most if not all of the observed 1,2,4-benzenetriol-induced DNA strand-breakage. Metal ions may act at the levels of: (1) propagation of chain interactions, (2) site-specific production of active species, and (3) the direct attack of active species on the DNA. Metal independent mechanisms exist, but they require such high 1,2,4-benzenetriol concentrations that they are unlikely to be biologically significant. The cellular genotoxicity of 1,2,4-benzenetriol is therefore contingent upon its abilities to inactivate catalase and to decompartmentalize metals.

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Figure 1. **1,2,4-Benzenetriol-induced breakage of superhelical DNA: Time-dependence.** This photograph shows fluorescence of gel stained with ethidium bromide. DNA (0.021 μg) was exposed to 20 μM 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for the stated incubation period. The notations: I SC, supercoiled DNA; II NI, nicked circular DNA; III LI, linear DNA.

Figure 2. **1,2,4-Benzenetriol-induced breakage of superhelical DNA: Influence of the buffer and EDTA.** The photographs show fluorescence of gel stained with ethidium bromide. DNA (0.021 μg) was exposed to 1,2,4-benzenetriol at 37°C for 30 minutes in 1 mM sodium phosphate buffer (pH 7.4), 10 mM Tris-HCl buffer (pH 7.4) and 10 mM Tris-HCl/ 1 mM EDTA buffer (pH 8.0), respectively. Control was no treatment of 1,2,4-benzenetriol. The notations: I SC, supercoiled DNA; II NI, nicked circular DNA; III LI, linear DNA.

Figure 3. **Damage to superhelical DNA induced by 1,2,4-benzenetriol: dose/response relationship.** ^{3}H-labelled DNA (0.1025 μg/50 μl reaction mixture) was exposed to 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. The number of nicks per DNA molecule and DNA breakage as percent of total DNA are plotted separately as a function of concentration of 1,2,4-benzenetriol. Bars represent DNA

breakage as percent of total DNA. The curve represents nicks per DNA molecule.

Figure 4. **1,2,4-Benzenetriol-induced breakage of superhelical DNA: Protection by desferrioxamine and EDTA.** Nicked DNA as fraction of total DNA is plotted as a function of 1,2,4-benzenetriol concentration. ^{3}H-labelled DNA (0.070 μg/25 μl reaction mixture) was exposed to 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. EDTA and desferrioxamine (DES) were added prior to addition of 1,2,4-benzenetriol. The X axis represents concentrations of 1,2,4-benzenetriol, and the Y axis represents the fraction of nicked DNA. Control was no addition of desferrioxamine or EDTA.

Figure 5. **Effect of 1,2,4-benzenetriol concentration on protection by desferrioxamine.** ^{3}H-labelled DNA (0.12 μg/50 μl reaction mixture) was exposed to 1,2,4-benzenetriol, in the presence of desferrioxamine (20 μM), in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. The concentration of 1,2,4-benzenetriol/percent nicked DNA is plotted as a function of concentration of 1,2,4-benzenetriol.

Figure 6 (a). **Nicking of superhelical DNA by 1,2,4-benzenetriol in the presence of iron and copper.** DNA (0.021 μg) was exposed for 30 minutes to 10 μM 1,2,4-benzenetriol and graded concentrations of copper and iron in 10 μl reaction mixtures containing 1 mM sodium phosphate buffer (pH 7.4) at 37°C. DNA breakage was evaluated by agarose electrophoresis and densitometry. The X axis represents reagents, and the Y axis represents

percentage of superhelical DNA. Abbreviations are as follows: BT, 1,2,4-benzenetriol; Fe(II), ferrous sulfate; Fe(III), ferric chloride; Cu(I), cuprous chloride; Cu(II), cupric sulfate.

Figure 6 (b). **Cleavage of nicked DNA by 1,2,4-benzenetriol in the presence of iron and copper.** Experimental conditions are as stated in the legend to Fig. 6 (a). The X axis represents reagents, and the Y axis represents percentage of linear DNA.

Figure 7. **1,2,4-Benzenetriol-induced breakage of superhelical DNA: Protection by catalase.** Nicks per DNA molecule are plotted as a function of the concentration of catalase. ^{3}H-labelled DNA (0.070 μg/25 μl reaction mixture) was exposed to 18 μM 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 5 minutes. Catalase was added prior to addition of 1,2,4-benzenetriol.

Figure 8. **Effects of antioxidants on DNA breakage induced by 1,2,4-benzenetriol (210 μM) with or without desferrioxamine.** ^{3}H-labelled DNA (0.12 μg/50 μl reaction mixture) was exposed to 210 μM 1,2,4-benzenetriol (BT) in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. The X axis represents reagents, and the Y axis represents the percentage of supercoiled DNA breakage. Abbreviations are as follows: DES, desferrioxamine (20 μM); SOD, superoxide dismutase and inactivated SOD, inactivated superoxide dismutase (20 U/ml); CAT, catalase and inactivated CAT, inactivated catalase (20 U/ml). Inactivated superoxide dismutase and catalase were boiled for 40 minutes prior to addition to the

reaction mixtures. Antioxidants and desferrioxamine were added prior to addition of phenolics. Desferrioxamine was not tested in the presence of inactive enzymes. Error bars represent standard errors of mean.

Figure 9. **1,2,4-Benzenetriol-induced breakage of superhelical DNA: Protection by formate and mannitol.** Nicked DNA (fraction of total DNA) is plotted as a function of concentration of the appropriate scavenger. ^{3}H-labelled DNA (0.070 μg/25 μl reaction mixture) was exposed to 18 μM 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. Scavengers were added prior to addition of 1,2,4-benzenetriol.

Figure 10. **DNA breakage in the presence of combinations of scavengers: Effects of desferrioxamine and catalase.** The X axis represents scavengers, and the Y axis represents nicks per DNA molecule. ^{3}H-labelled DNA (0.1025 μg/50 μl reaction mixture) was exposed to 18 μM 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. All antioxidants and desferrioxamine were added prior to addition of 1,2,4 benzenetriol. Abbreviations: SOD, superoxide dismutase (20 U/ml); CAT, catalase (20 U/ml); FOR, formate (25 mM); MAN, mannitol (50 mM); DES, desferrioxamine (0.02 mM). Error bars represent standard errors of mean.

Figure 11. **DNA breakage in the presence of combinations of scavengers: Effect of superoxide dismutase.** Experimental conditions are as stated in the legend to Fig. 10.

Figure 12. **DNA breakage in the presence of combinations of scavengers: Effect of formate.** Experimental conditions are as stated in the legend to Fig. 10.

Figure 13. **DNA breakage in the presence of combinations of scavengers: Effect of mannitol.** Experimental conditions are as stated in the legend to Fig. 10.

LEGEND OF TABLE

Table. **Breakage of superhelical DNA induced by 1,2,4-benzenetriol: Protection by scavengers in the presence and absence of desferrioxamine.** ^{3}H-labelled DNA (0.1025 μg/50 μl reaction mixture) was exposed to 18 μM 1,2,4-benzenetriol in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes. All antioxidants and desferrioxamine were added prior to addition of 1,2,4-benzenetriol. The effects of scavengers were represented as nicks per DNA molecule and percentage of protection. Abbreviations: SOD, superoxide dismutase (20 U/ml); CAT, catalase (20 U/ml); FOR, formate (25 mM); MAN, mannitol (50 mM); DES, desferrioxamine (0.02 mM). Standard error of mean was calculated from a minimum of 3 replicates on separate days.

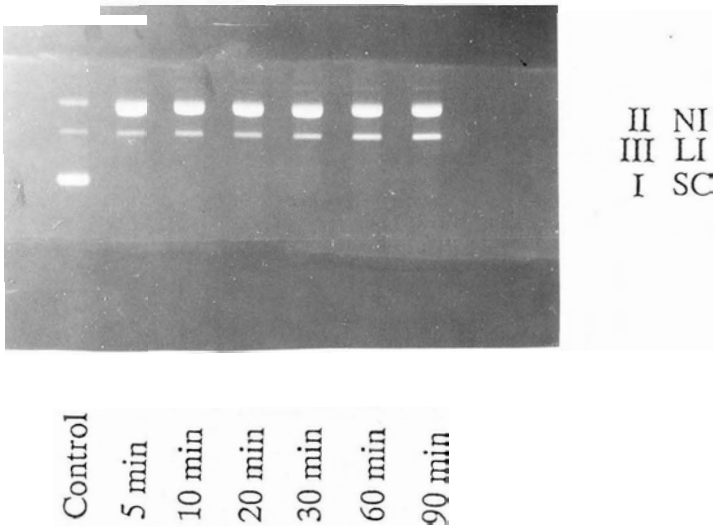

Figure 1. 1,2,4-Benzenetriol-induced breakage of superhelical DNA: Time-dependence

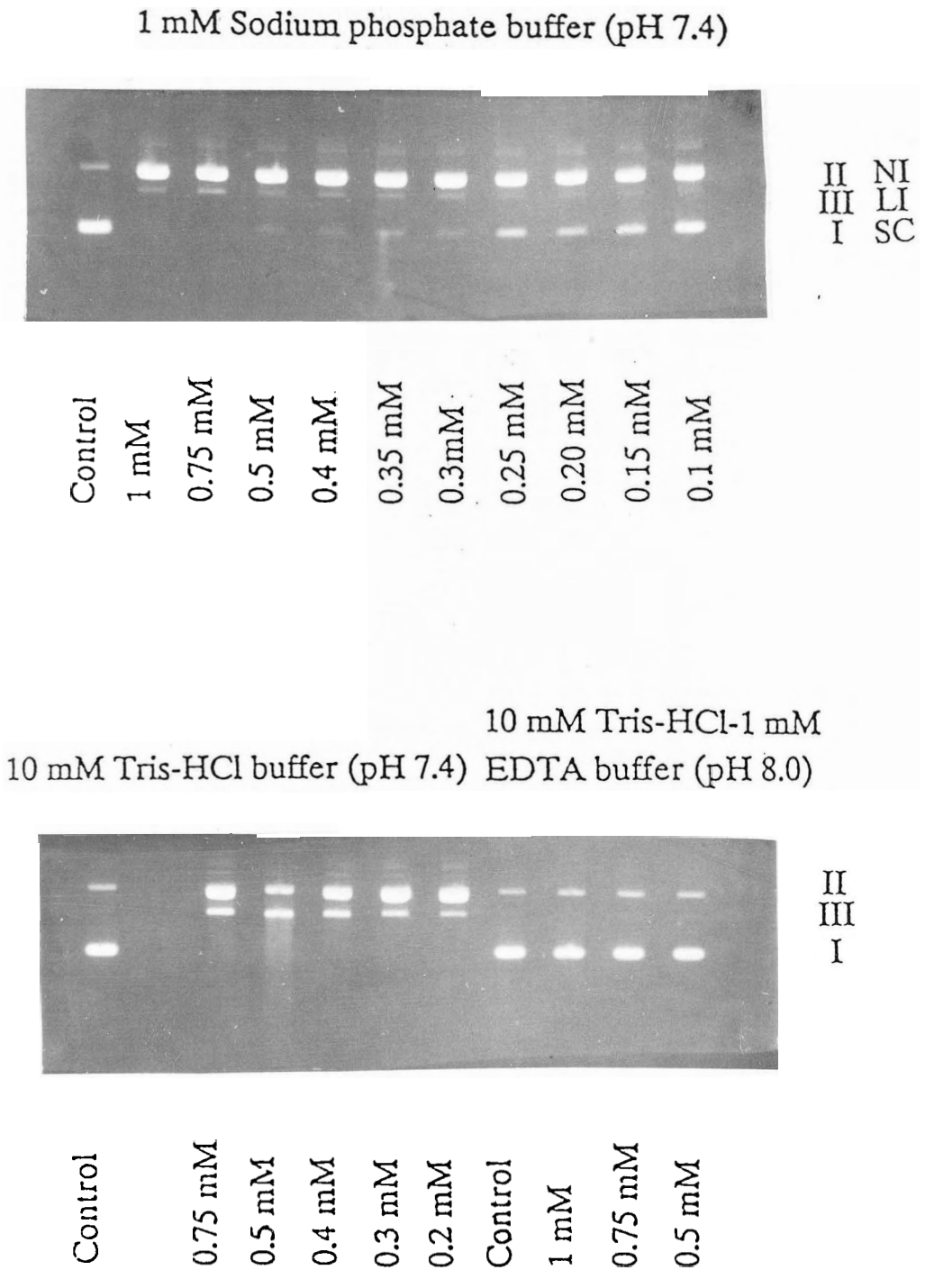

Figure 2. 1,2,4-Benzenetriol-introduced breakage of superhelical DNA: Influnce of the buffer and EDTA

Figure 3. Damage to superhelical DNA induced by 1,2,4-benzenetriol: Dose/response relationship

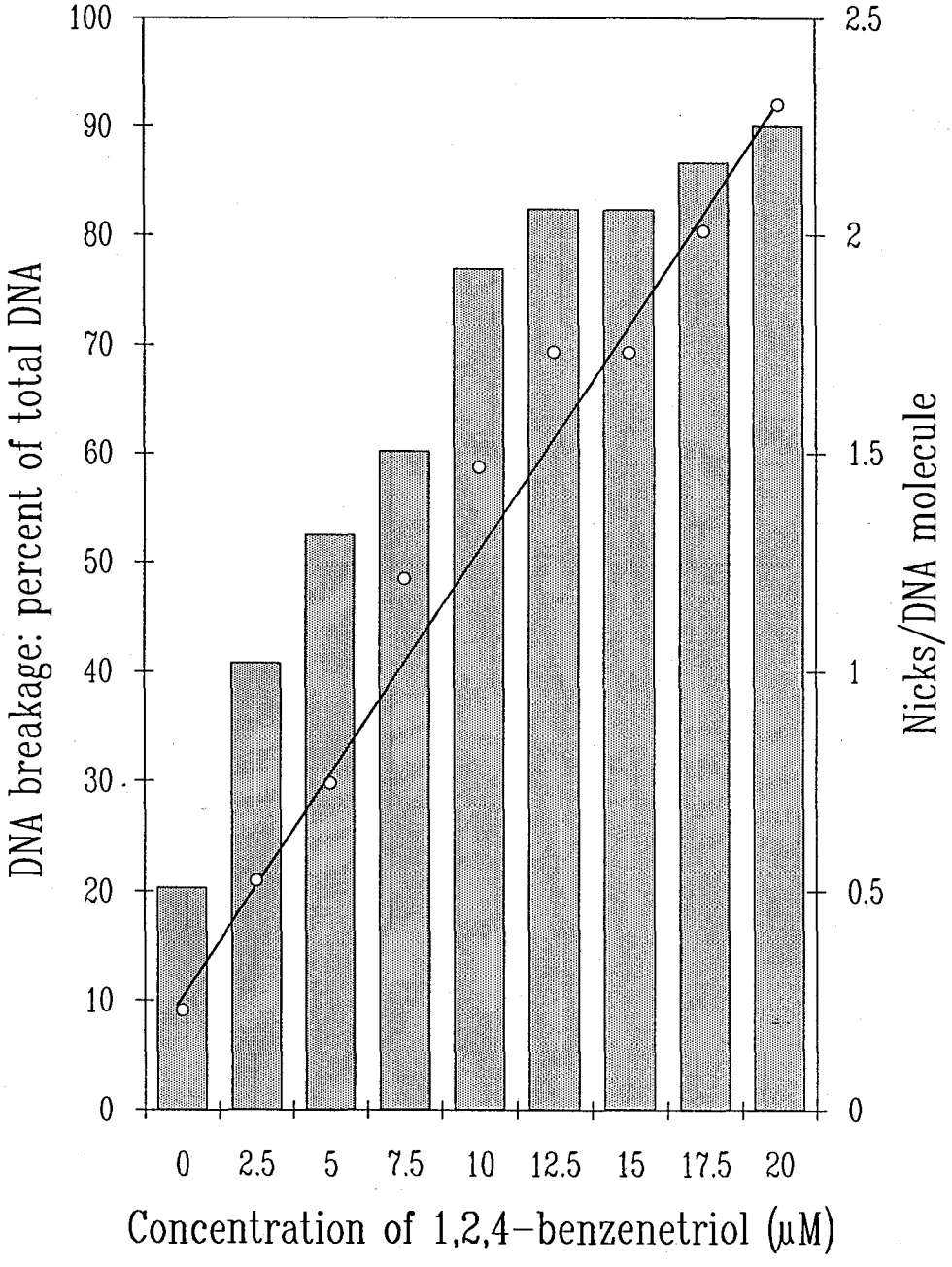

Figure 4. 1,2,4-Benzenetriol-induced breakage of superhelical DNA: Protection by desferrioxamine and EDTA

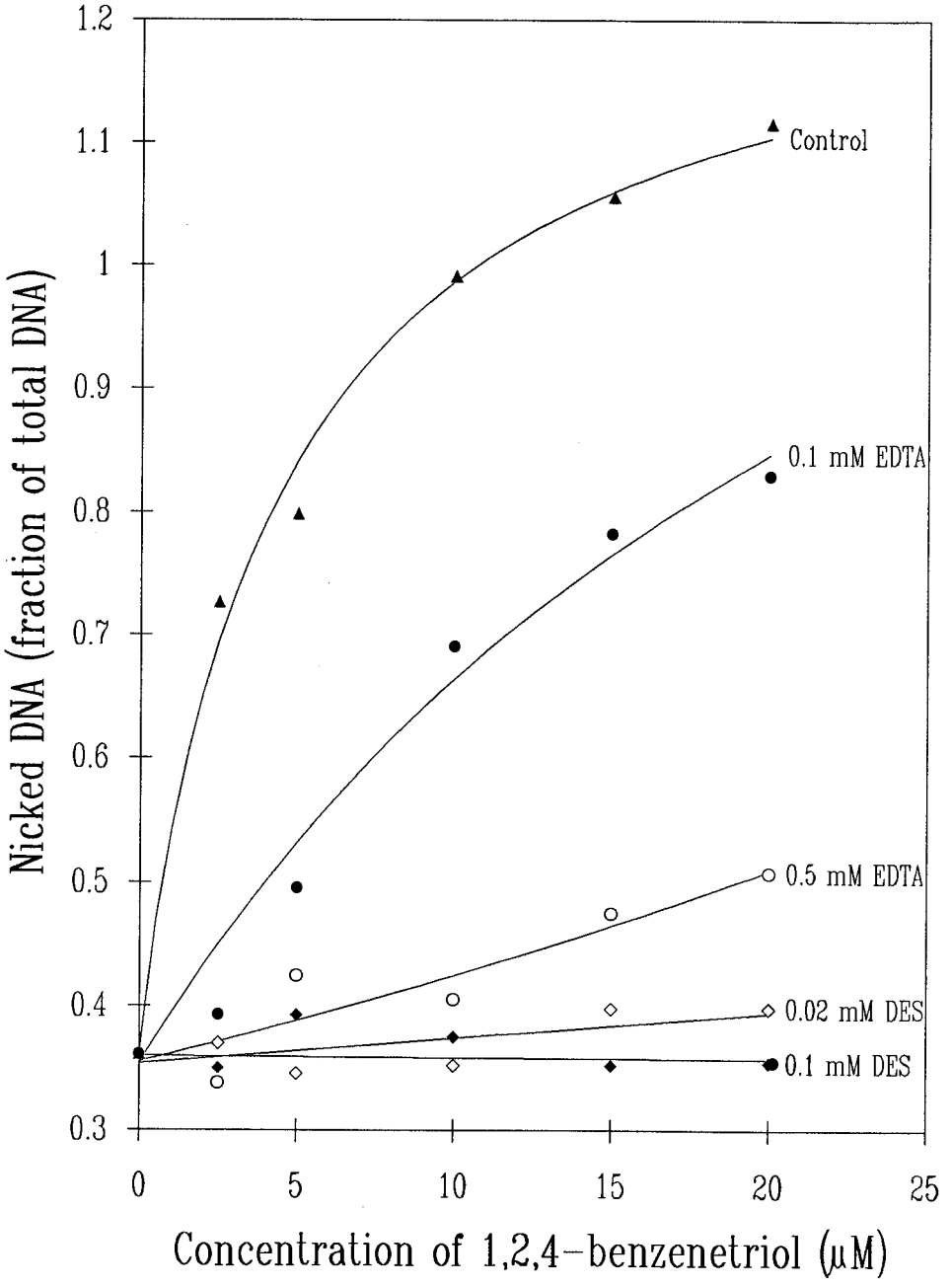

Figure 5. Effect of 1,2,4-benzenetriol concentration on protection by desferrioxamine

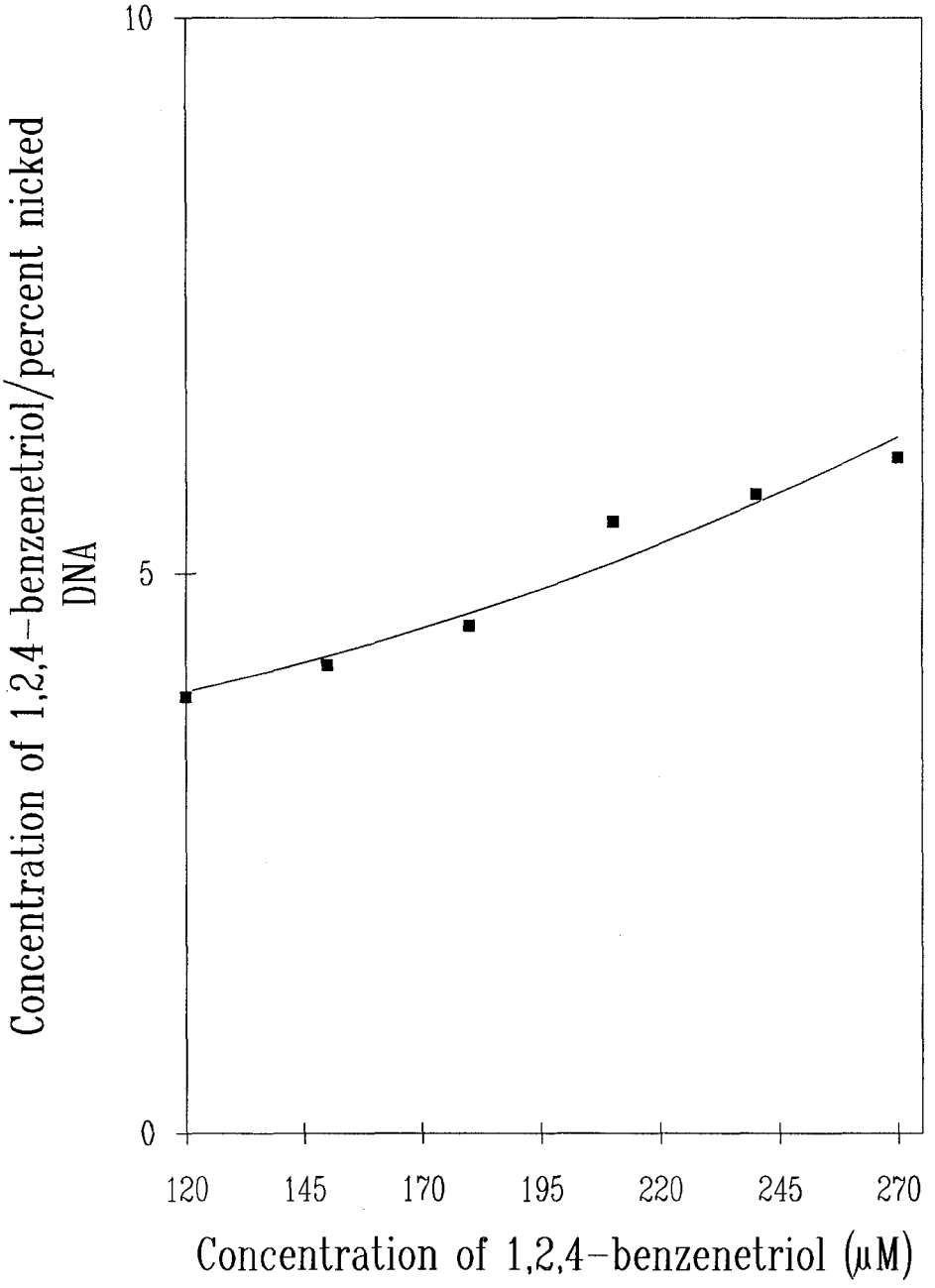

Figure 6(a). Nicking of superhelical DNA by 1,2,4-benzenetriol in the presence of iron and copper

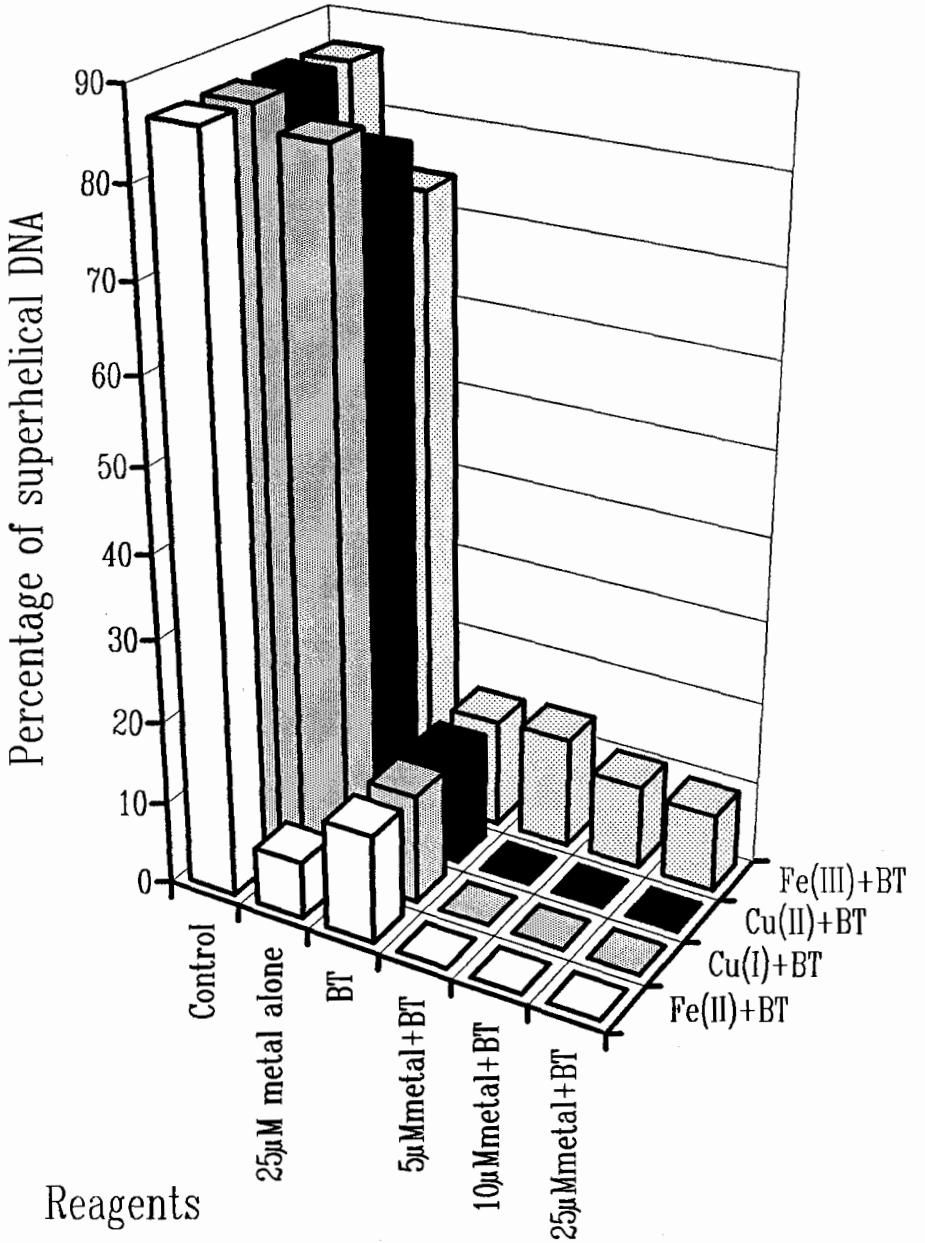

Figure 6(b). Cleavage of nicked DNA by 1,2,4-benzenetriol in the presence of iron and copper

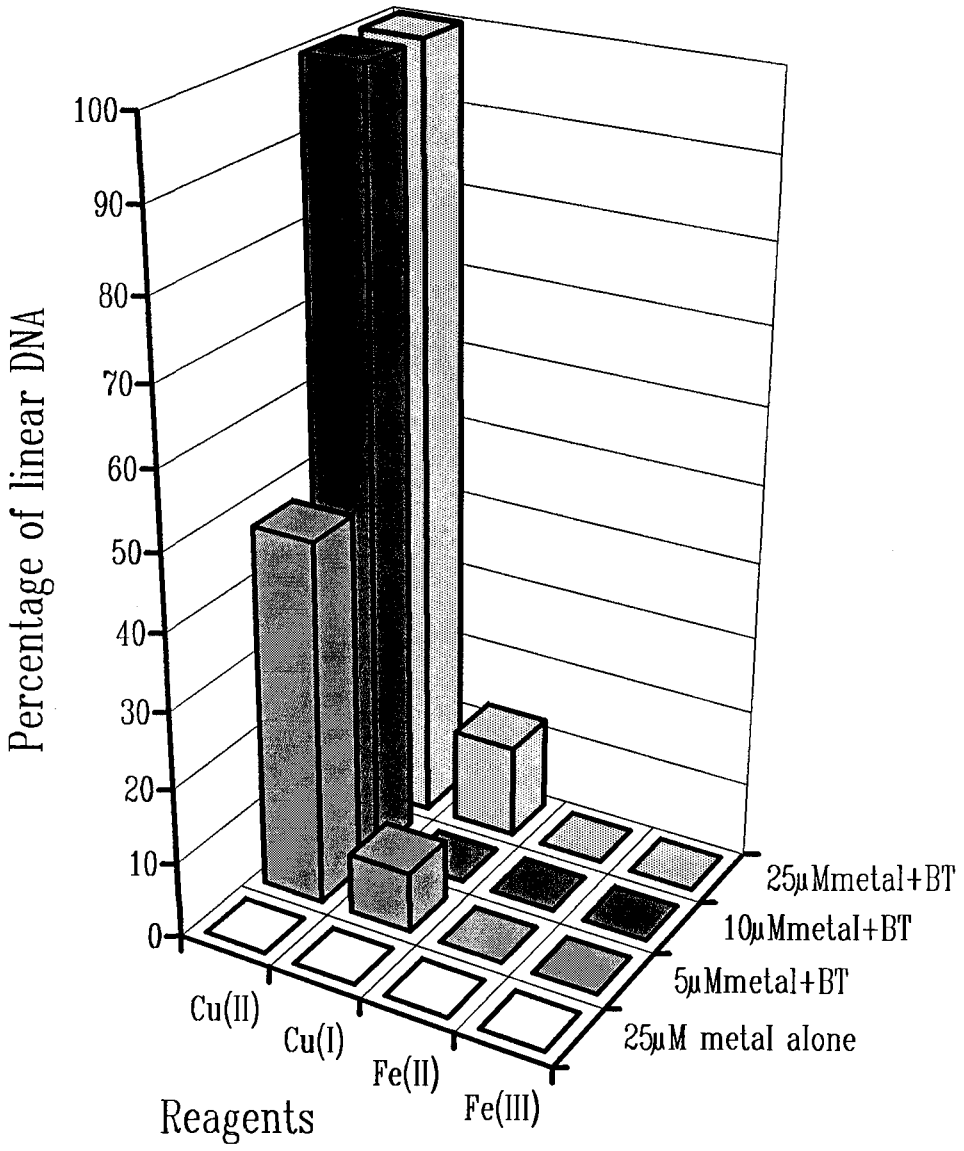

Figure 7. 1,2,4-Benzenetriol-induced breakage of superhelical DNA: Protection by catalase

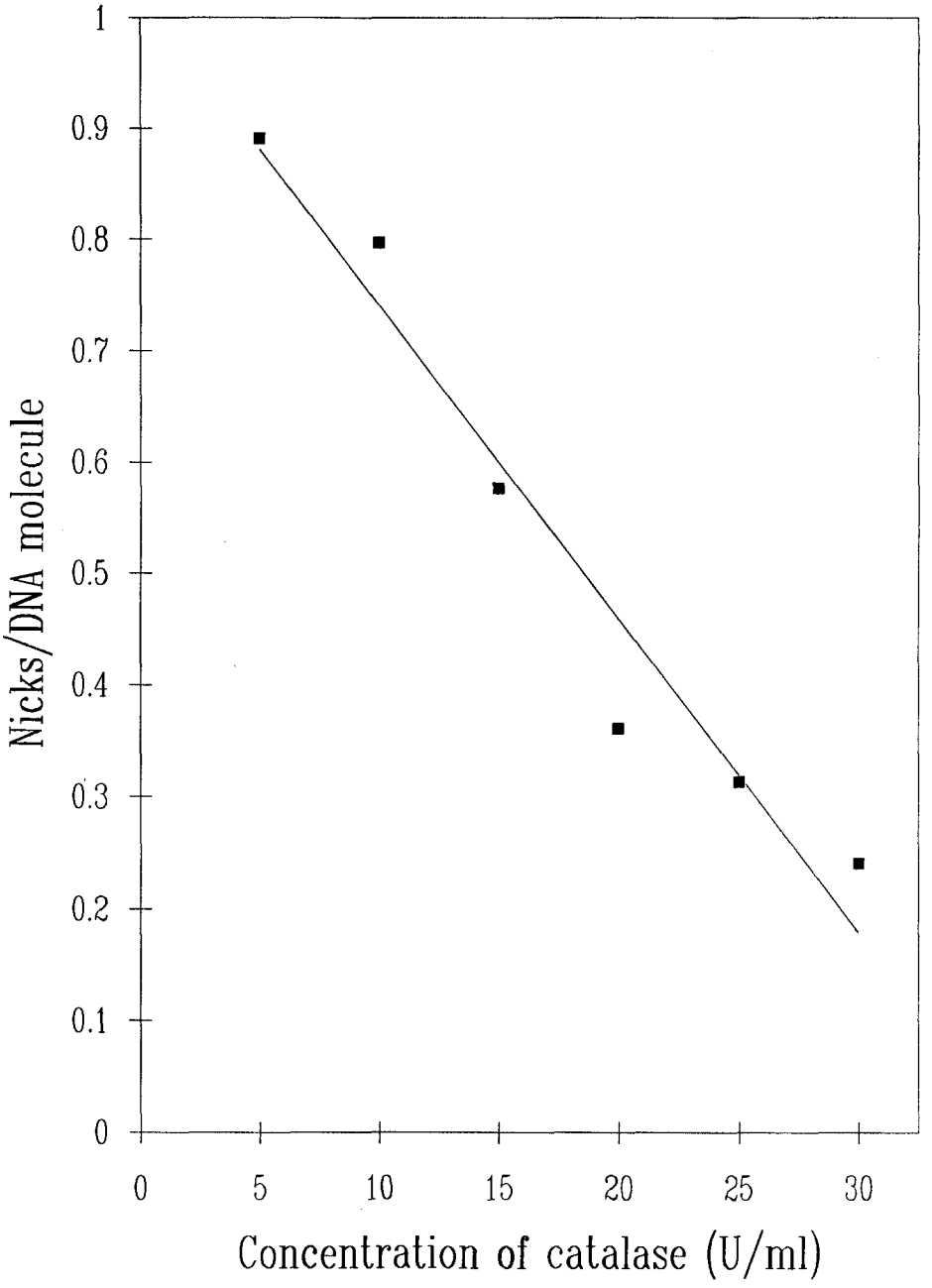

Figure 8. Effects of antioxidants on DNA breakage induced by 1,2,4-benzenetriol (210 μM) with or without desferrioxamine

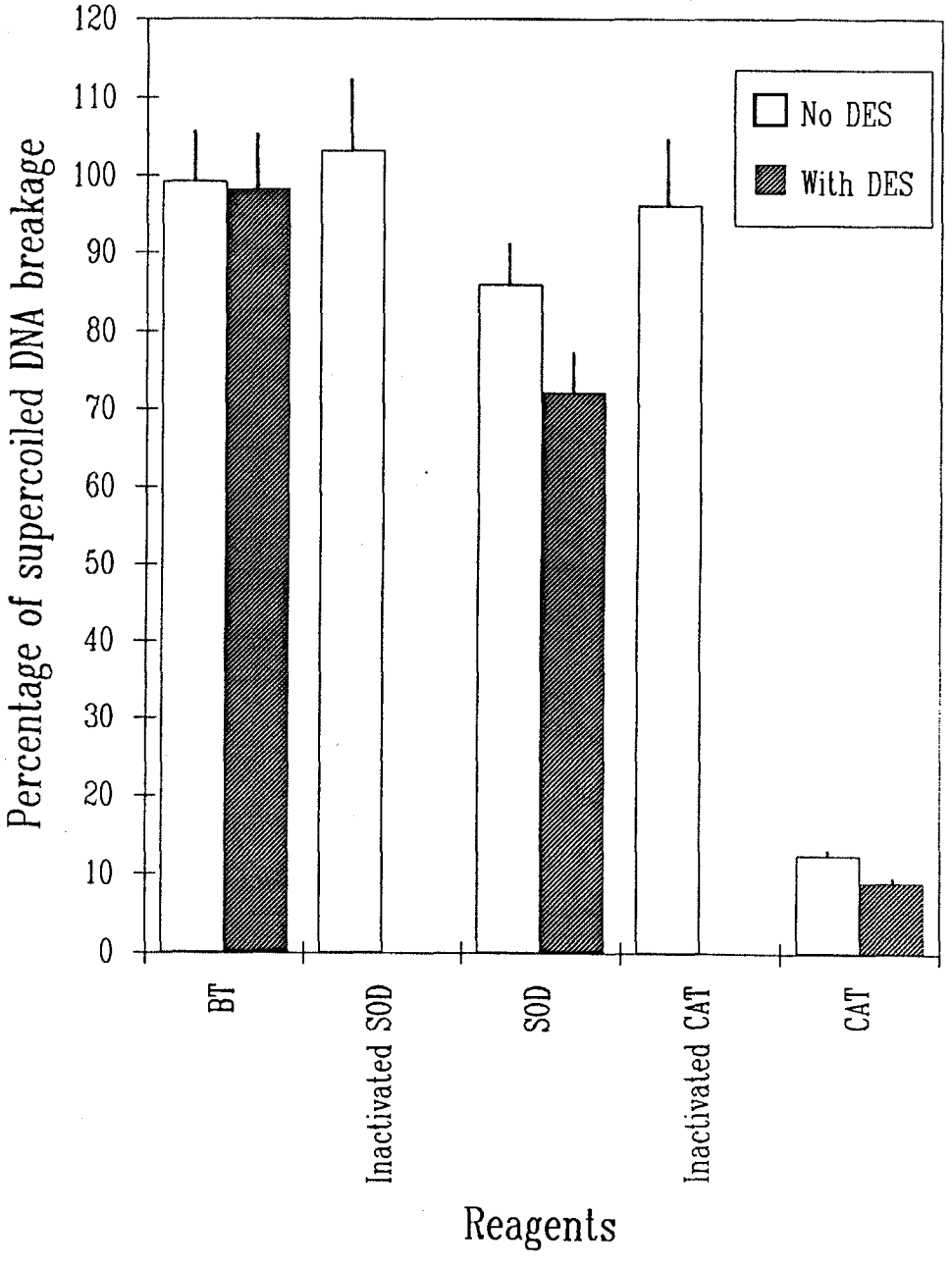

Figure 9. 1,2,4-Benzenetriol-induced breakage of superhelical DNA: Protection by formate and mannitol

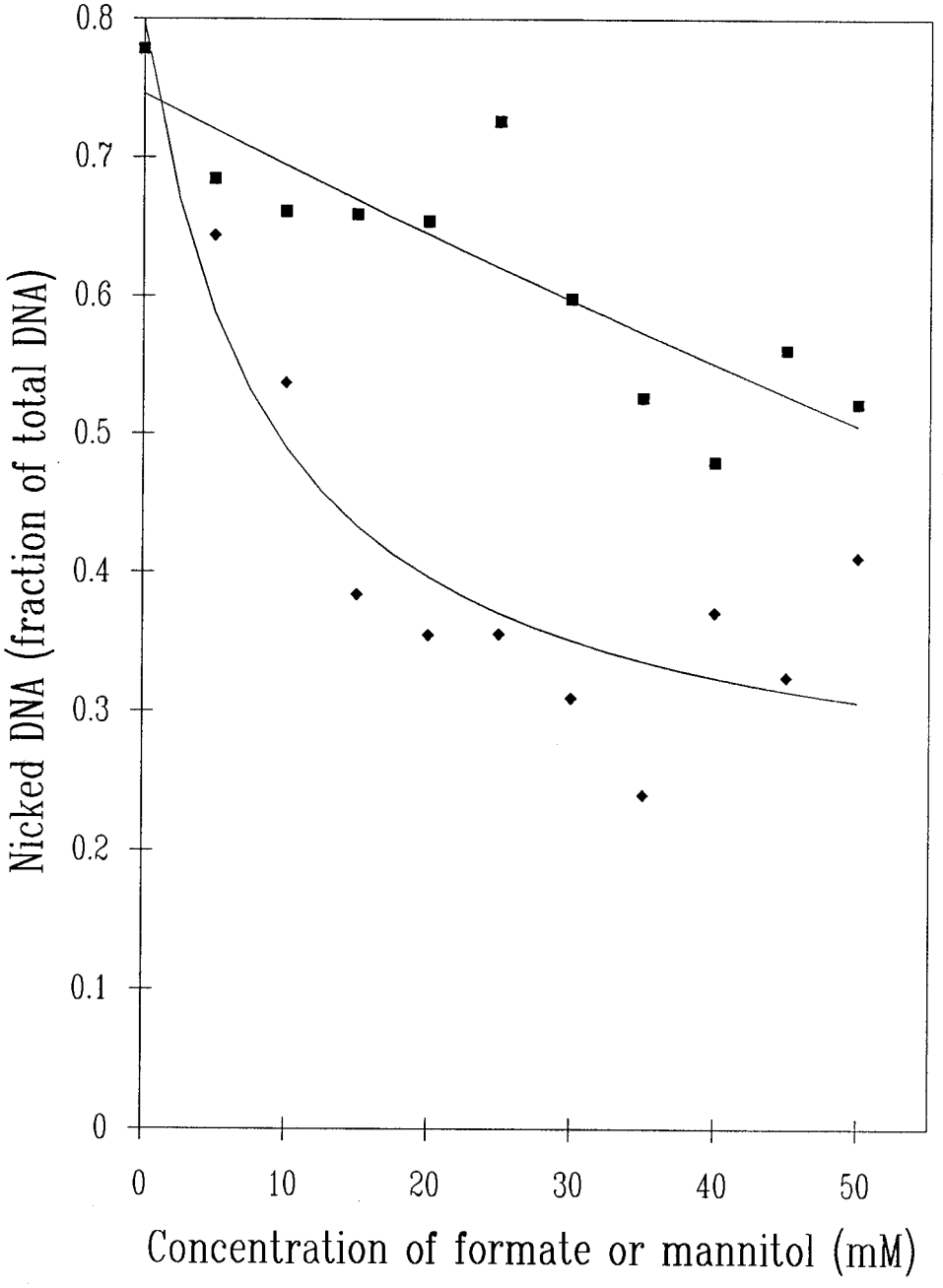

Figure 10. DNA breakage in the presence of combinations of scavengers: Effects of desferrioxamine and catalase

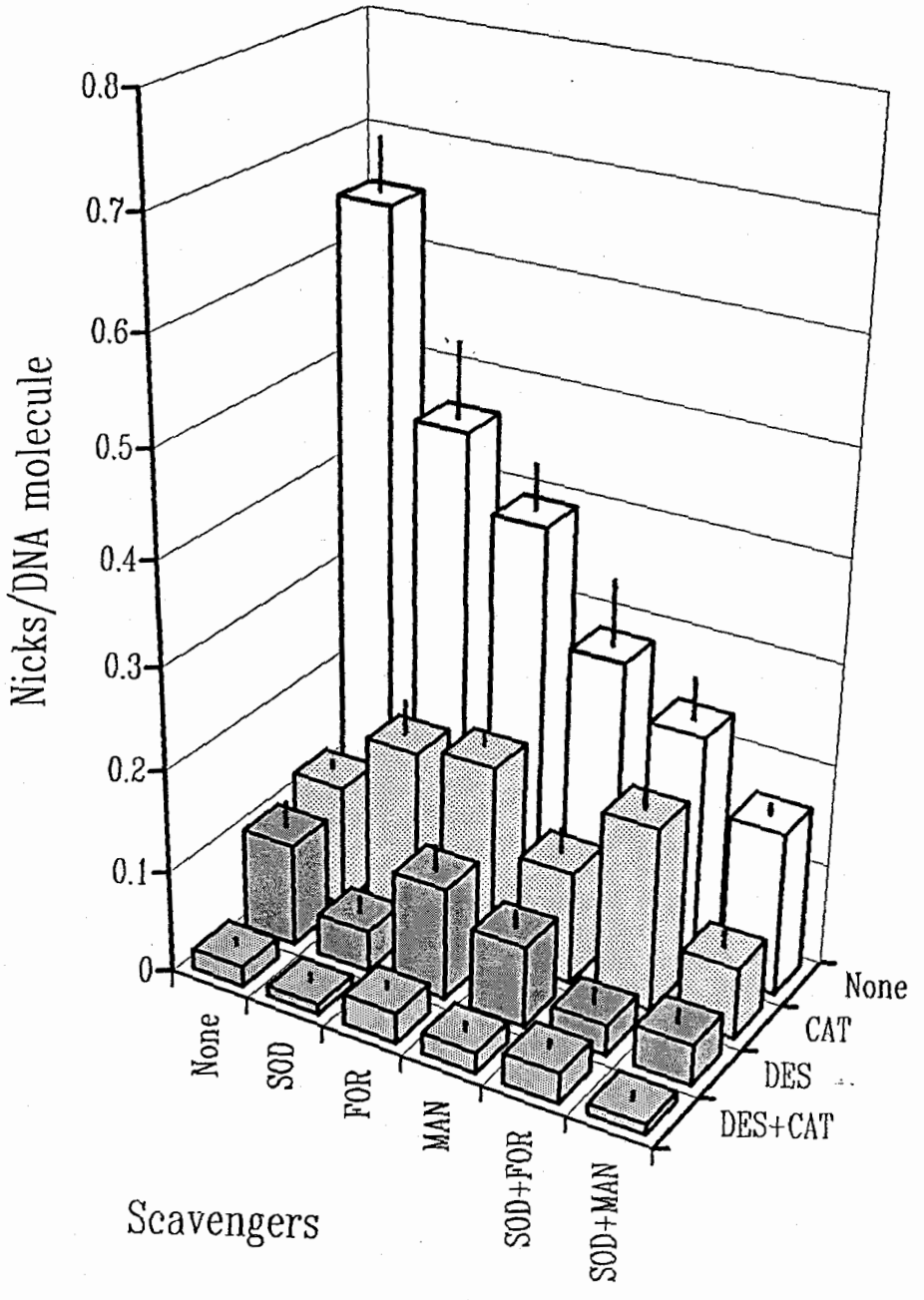

Figure 11. DNA breakage in the presence of combinations of scavengers: Effect of superoxide dismutase

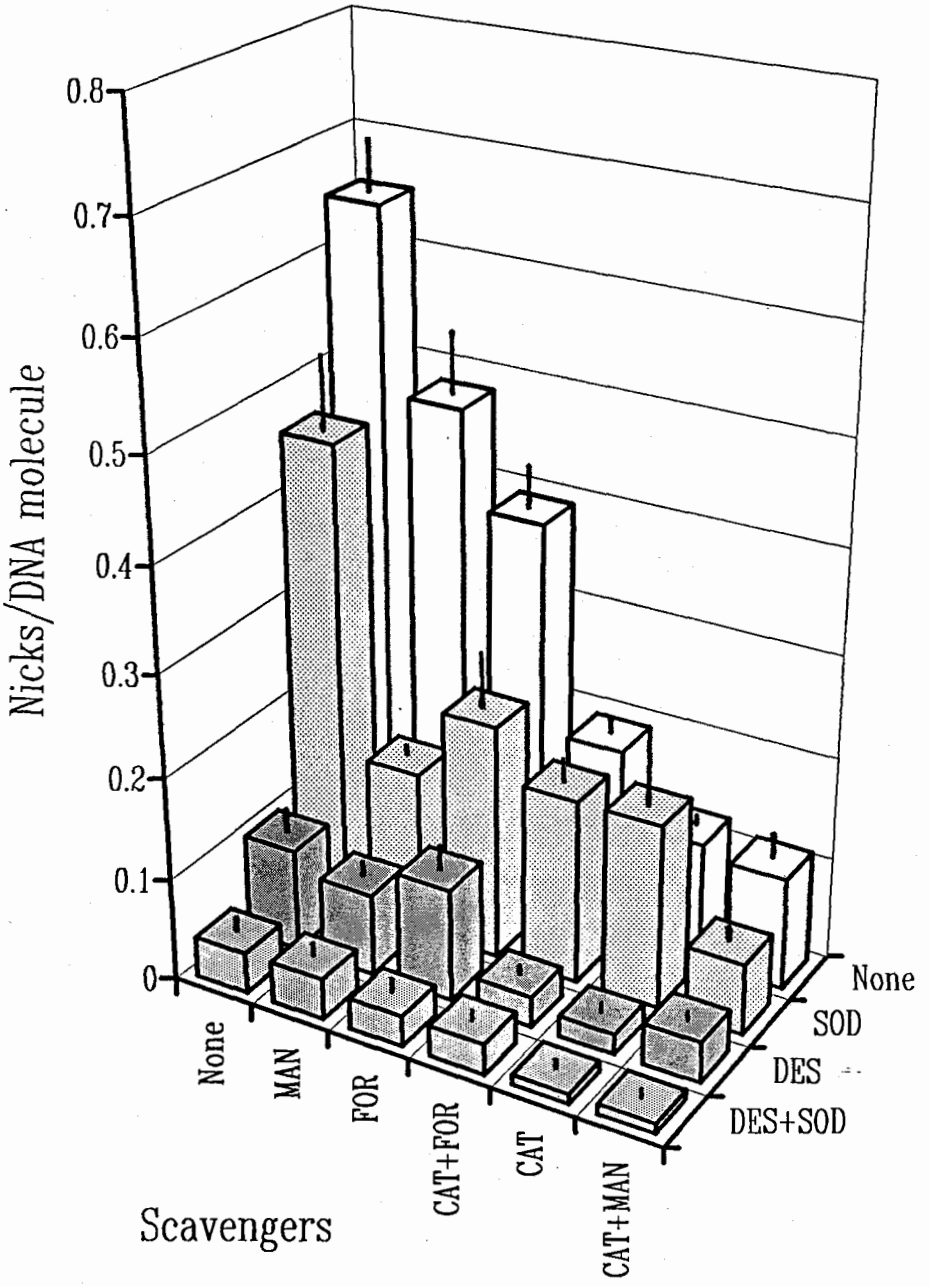

Figure 12. DNA breakage in the presence of combinations of scavengers: Effect of formate

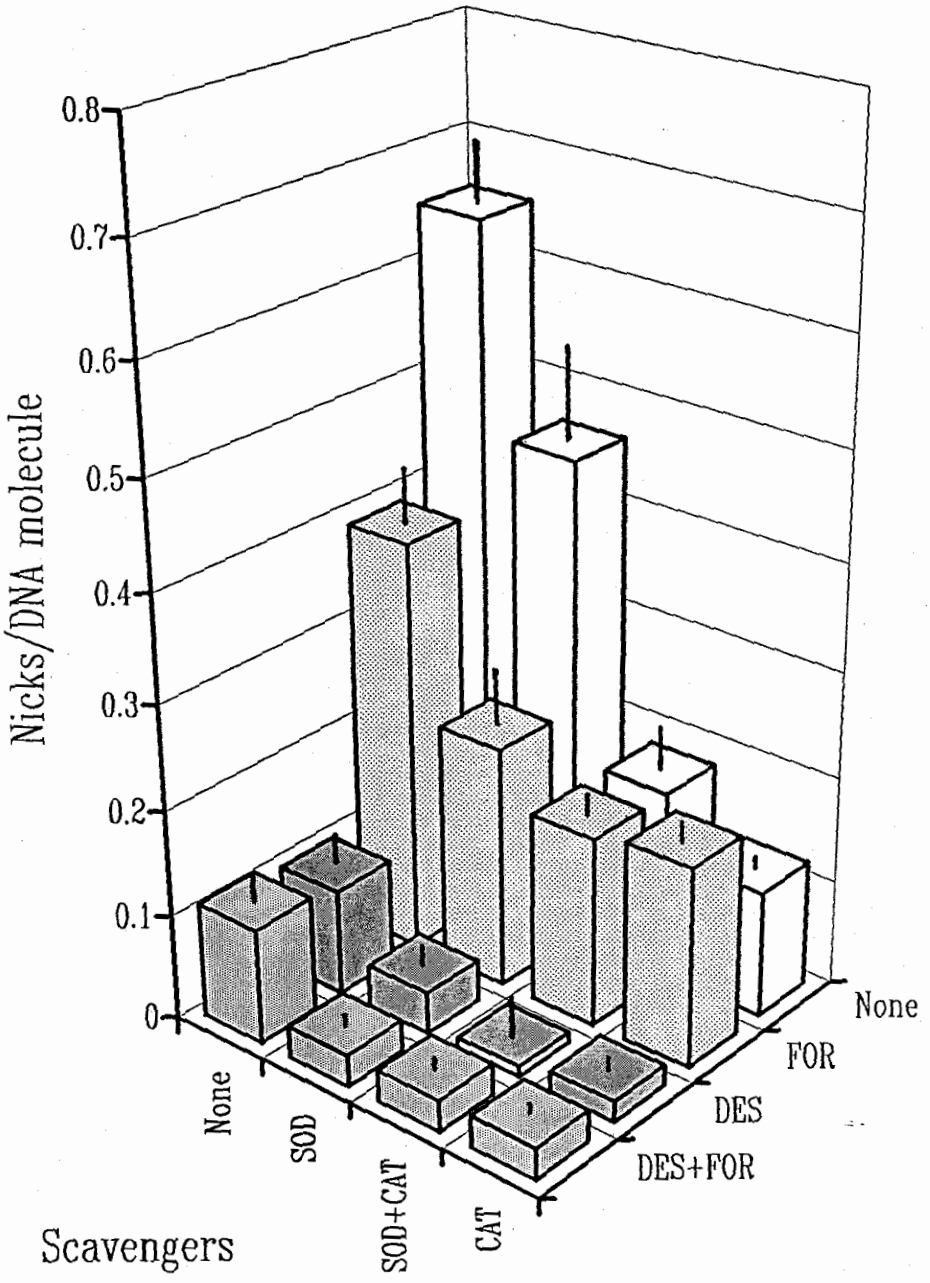

Figure 13. DNA breakage in the presence of combinations of scavengers: Effect of mannitol

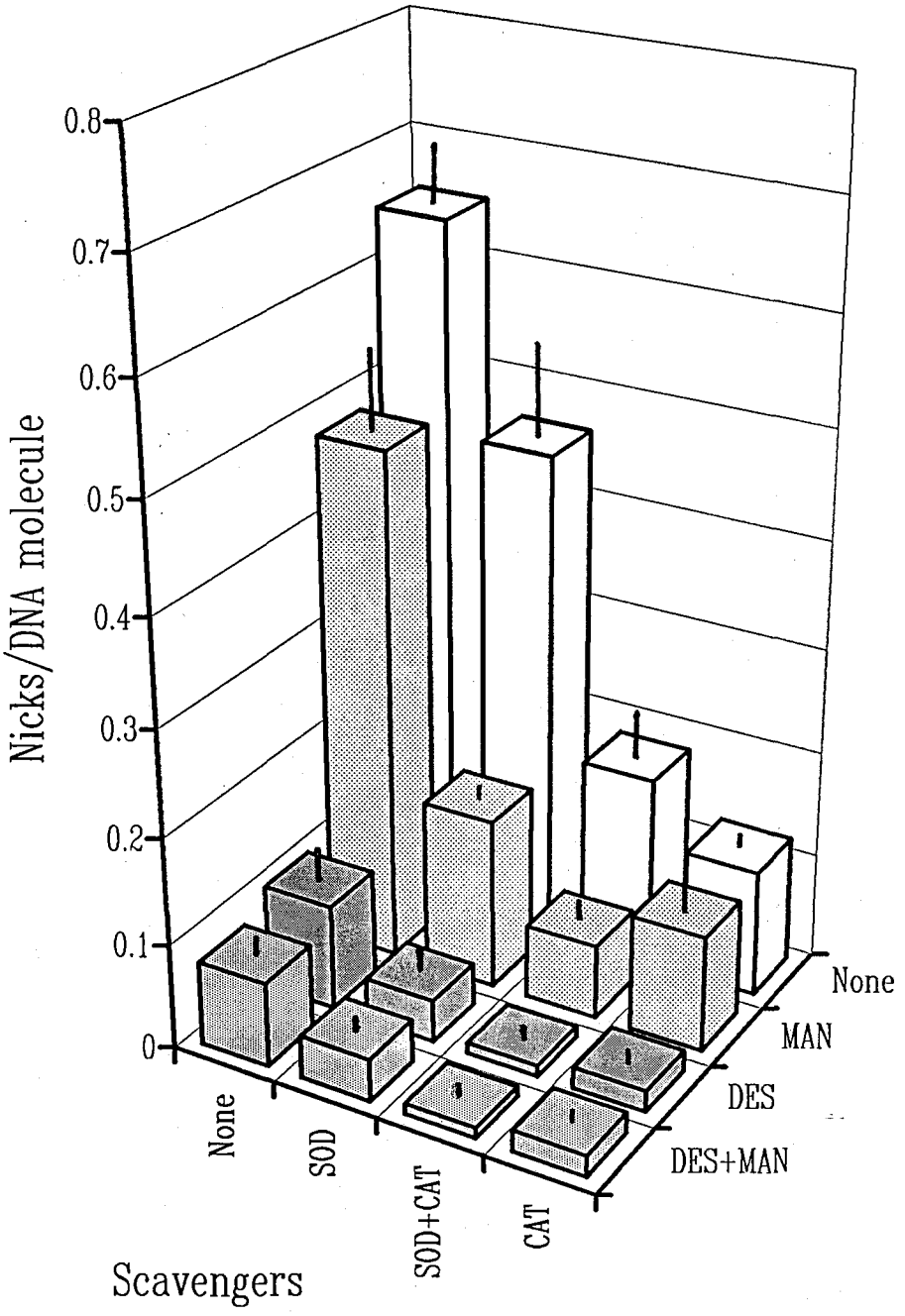

Table. Breakage of superhelical DNA induced by 1,2,4-benzenetriol: Protection by scavengers in the presence and absence of desferrioxamine

Reagent	No desferrioxamine		Plus desferrioxamine	
	Nicks	% protection	Nicks	% protection
Control	0.65±0.05		0.10±0.03	85.20±4.48
SOD	0.46±0.08	30.13±8.33	0.04±0.02	93.54±2.55
CAT	0.12±0.01	80.48±2.08	0.02±0.01	97.54±1.28
FOR	0.39±0.05	40.78±3.36	0.11±0.03	83.62±4.07
MAN	0.48±0.07	28.28±4.81	0.08±0.02	88.44±2.68
SOD+FOR	0.23±0.05	65.06±4.33	0.03±0.01	96.24±1.27
SOD+MAN	0.16±0.01	75.44±0.35	0.04±0.02	93.62±2.13
SOD+CAT	0.18±0.04	73.88±3.84	0.01±0.01	98.03±1.62
CAT+FOR	0.19±0.02	70.26±1.98	0.03±0.01	96.24±1.56
CAT+MAN	0.11±0.03	83.90±3.51	0.02±0.01	96.22±0.97
SOD+CAT+FOR	0.18±0.02	69.80±6.50	0.03±0.01	95.12±1.09
SOD+CAT+MAN	0.07±0.02	89.40±2.74	0.01±0.01	98.48±1.10

CHAPTER IV

General discussion

Using the techniques and reaction systems stated in chapter 2 and 3, we have addressed the questions posed in the introduction to the current study (chapter 1). We have studied the extent to which four phenolic compounds (1,2,4-benzenetriol, caffeic acid, gallic acid and gossypol) induce DNA breakage. We also have confirmed that these compounds can act as either pro-oxidants or anti-oxidants in DNA breakage, depending on reaction conditions. Through the addition of antioxidants and metal scavengers, as well as metal ions, we have assessed the contributions of oxygen-derived active species and transition metals in DNA breakage. With these data in mind we propose a metal-mediated site-specific mechanism for 1,2,4-benzenetriol-induced DNA breakage. This general discussion is an attempt to synthesize and frame a perspective on our findings.

PHENOLIC COMPOUNDS ACTED AS PRO-OXIDANTS IN DNA BREAKAGE UNDER MOST CIRCUMSTANCES

All four phenolics at low concentrations induced supercoiled DNA breakage when added alone in the reaction systems. This can be related to their genotoxicity *in vivo*. Protection by catalase revealed the involvement of oxygen-derived active species generated in the oxidation of these phenolic compounds. Thus, these phenolic compounds acted as pro-oxidants in DNA breakage. This generalization, however, is contingent upon these phenolic compounds being the sole active species in the reaction system. If more active species (e.g., ascorbate/Fe) are present in the reaction system, certain phenolic compounds including caffeic acid and gallic acid, can act as

antioxidants. Caffeic acid and gossypol decreased DNA damage. This finding is consistent with their antigenotoxicity *in vivo*.

CONTRIBUTION OF OXYGEN-DERIVED ACTIVE SPECIES IN DNA BREAKAGE BY PHENOLIC COMPOUNDS

The effects of antioxidants (superoxide dismutase, catalase) reveal the extent to which specific oxygen-derived active species mediate DNA breakage by these phenolic compounds. Superoxide dismutase inhibited DNA breakage by 1,2,4-benzenetriol, but enhanced that by the other three compounds. Catalase inhibited DNA breakage almost completely, except that induced by gossypol. Thus, hydrogen peroxide is the preeminent species in DNA breakage. Superoxide has two roles in autoxidation of these phenolic compounds: a chain propagator in autoxidation of 1,2,4-benzenetriol, and a chain terminator in autoxidation of the other three compounds. In DNA breakage by 1,2,4-benzenetriol, hydroxyl radical scavengers (formate and mannitol) weakly inhibited the DNA breakage. Thus, to the extent that hydroxyl radicals mediate DNA damage they are generated "site-specifically", in a way largely inaccessible to these scavengers.

TRANSITION METALS DIFFER IN THE MECHANISMS BY WHICH THEY GENERATE DNA BREAKING SPECIES

In the current study, both iron and copper enhanced DNA breakage induced by 1,2,4-benzenetriol, and both species also accelerated autoxidation of 1,2,4-benzenetriol (1). Copper, however, accelerated autoxidation of 1,2,4-benzenetriol and DNA breakage more strongly than iron. In seeking an explanation, the following considerations are relevant: (a) The initial rate of hydroxyl radical formation in the Cu(I)-catalyzed Fenton reaction

$$H_2O_2 + M^{(n-1)+} \text{---} > M^{n+} + {\cdot}OH + OH^-$$

is over 60 times faster than in the Fe(II) catalyzed reaction (2); (b) Iron and copper differ in the mechanisms by which they catalyze electron transfer between 1,2,4-benzenetriol and O_2, releasing dissimilar active oxygen species. Superoxide dismutase did not inhibit the copper-catalyzed oxidation of 1,2,4-benzenetriol, but slowed the iron-catalyzed oxidation. Desferrioxamine countered the effect of copper, but did not inhibit the acceleration by iron. Catalase inhibited the autoxidation of 1,2,4-benzenetriol in the presence of copper (1). These properties suggest that a ternary complex of 1,2,4-benzenetriol-copper-O_2 catalyzes two-electron transfer from 1,2,4-benzenetriol to O_2, releasing H_2O_2. In contrast, the 1,2,4-benzenetriol-iron complex prefers one-electron transfer from 1,2,4-benzenetriol to O_2, releasing $O_2{\cdot}^-$.

ALTERNATIVE MECHANISMS IN DNA BREAKAGE INDUCED BY 1,2,4-BENZENETRIOL

The effects of individual antioxidants and metal chelators clarify the roles of active oxygen species and transition metals in DNA breakage induced by 1,2,4-benzenetriol. 1,2,4-Benzenetriol, however, induced DNA breakage in the presence of antioxidants and a metal chelator simultaneously. Induction of DNA breakage by 1,2,4-benzenetriol occurs by multiple alternative pathways. The particular mechanisms operating under specific circumstances depend on which active species is most prevalent.

$O_2{\cdot}^-$ acts as a propagator

The combination of catalase, formate and mannitol excluded H_2O_2 and ·OH. The major intermediate in the 1,2,4-benzenetriol/O_2 reaction is $O_2{\cdot}^-$ which propagates the autoxidation of 1,2,4-benzenetriol.

$QH_2 + O_2 \text{---}> \cdot SQ + O_2^{\cdot -}$

$\cdot SQ + O_2 \text{---}> O_2^{\cdot -} + Q$

$DNA + M^{n+} \text{---}> DNA\text{-}M^{n+}$

$DNA\text{-}M^{n+} + QH_2 + O_2^{\cdot -} \text{---}> DNA\text{-}M^{n+}\text{-}QH_2\text{-}O_2^{\cdot -} \text{---}>$

$DNA\text{-}M^{(n-1)+}\text{-}H_2O_2 + Q \text{---}>$

$DNA\text{-}M^{n+} + \cdot OH + OH^-$

Metal ions are essential for production of autocatalytic species. Under these circumstances the ternary complex of 1,2,4-benzenetriol-metal-superoxide is involved in DNA breakage. The final products, site-specific hydroxyl radicals, being inaccessible to the hydroxyl radical scavengers present.

H_2O_2 acts as a propagator

The simultaneous presence of superoxide dismutase, formate and mannitol excludes $O_2^{\cdot -}$ and free $\cdot OH$. Here, H_2O_2 propagates the autoxidation of 1,2,4-benzenetriol. Thus H_2O_2 becomes the most important intermediate in DNA breakage by 1,2,4-benzenetriol.

$QH_2 + O_2 \text{---}> \cdot SQ + O_2^{\cdot -}$

$\cdot SQ + O_2 \text{---}> O_2^{\cdot -} + Q$

$2O_2^{\cdot -} \text{---}> H_2O_2 + O_2$

$DNA + M^{(n-1)+} \text{---}> DNA\text{-}M^{(n-1)+}$

$DNA\text{-}M^{(n-1)+} + H_2O_2 \text{---}> DNA\text{-}M^{(n-1)+}\text{-}H_2O_2$

$DNA\text{-}M^{(n-1)+}\text{-}H_2O_2 \text{---}> DNA\text{-}M^{n+} + \cdot OH + OH^-$

The high concentration of H_2O_2 in the reaction mixture, allows the reaction

$\cdot SQ + H_2O_2 \text{---}> \cdot OH + OH^- + Q.$

Under these circumstances, then, the semiquinone may participate in the DNA breakage as a Fenton donor.

Metal ions act as propagators

Metal ions play an important role in autoxidation of 1,2,4-benzenetriol. Gee et al. found that a metal-oxygen complex was an initiating and propagating species in oxidation of 6-hydroxydopamine (3). Addition of EDTA plus desferrioxamine decreased 6-hydroxydopamine-induced chromosomal aberrations by 90% (4). In the presence of superoxide dismutase and catalase, desferrioxamine completely inhibits autoxidation of 6-hydroxydopamine (5). In the present study, in the simultaneous presence of superoxide dismutase, catalase, formate and mannitol, metal ions act as primary propagators since $O_2\cdot^-$, H_2O_2 and $\cdot OH$ are excluded.

$$DNA + M^{n+} + QH_2 \text{---}> DNA\text{-}M^{(n-1)+} + \cdot SQ$$

$$DNA\text{-}M^{n+} + \cdot SQ \text{---}> DNA\text{-}M^{(n-1)+} + Q$$

$$DNA\text{-}M^{(n-1)+} + O_2 \text{---}> DNA\text{-}M^{(n-1)+}\text{-}O_2$$

$$DNA\text{-}M^{(n-1)+}\text{-}O_2 + QH_2 \text{---}> DNA\text{-}M^{(n-1)+}\text{-}O_2\cdot^- + \cdot SQ$$

$$DNA\text{-}M^{(n-1)+}\text{-}O_2\cdot^- + QH_2 \text{---}> DNA\text{-}M^{(n-1)+}\text{-}H_2O_2 + \cdot SQ$$

$$DNA\text{-}M^{(n-1)+}\text{-}H_2O_2 \text{---}> DNA\text{-}M^{n+} + \cdot OH + OH^-$$

Under these circumstances, then, site-specific hydroxyl radical or other species (e.g., ferryl radical) may participate in DNA breakage.

Role of the hydroxyl radical

Most of hydroxyl radicals are produced in the Fenton reaction in which H_2O_2 is required for reduction of $M^{(n-1)+}$ and formation of $\cdot OH$. In presence of superoxide

dismutase and catalase, $O_2\cdot^-$ is converted to H_2O_2. Hydrogen peroxide is in turn converted to H_2O. It is then impossible for the Fenton reaction to proceed, and ·OH can not be generated. Gee et al. found that inhibitions by formate and mannitol in the oxidation of 6-hydroxydopamine were the most significant in the presence of superoxide dismutase and catalase, and concluded that the hydroxyl radical can act as a propagator under these circumstances (5). In the present study DNA is nicked in the presence of superoxide dismutase and catalase. This observation raises the question of the production of ·OH in the absence of H_2O_2. Catalase has a relatively high K_m for H_2O_2 (6). At low concentrations of H_2O_2, side reactions can compete with catalase for H_2O_2. A metal-DNA complex could compete effectively with catalase. Therefore, hydroxyl radicals could be generated via the Fenton reaction, despite the presence of superoxide dismutase and catalase.

$$DNA + M^{n+} \text{---} > DNA\text{-}M^{n+}$$

$$DNA\text{-}M^{n+} + QH_2 \text{---} > DNA\text{-}M^{(n-1)+} + \cdot SQ$$

$$DNA\text{-}M^{n+} + \cdot SQ \text{---} > DNA\text{-}M^{(n-1)+} + Q$$

$$DNA\text{-}M^{(n-1)+} + QH_2 + O_2 \text{---} > DNA\text{-}M^{(n-1)+}\text{-}QH_2\text{-}O_2 \text{---} >$$

$$DNA\text{-}M^{(n-1)+}\text{-}H_2O_2 + Q \text{---} > DNA\text{-}M^{n+} + \cdot OH + OH^-$$

Since the major source of the hydroxyl radical is the Fenton reaction, the addition of desferrioxamine further decreases the DNA breakage because it removes metals from the DNA so that active species are generated further away from their targets.

FURTHER EXPERIMENTS

Our study has investigated the actions of several phenolic compounds in DNA breakage *in vitro*. Further studies should measure modification of bases of DNA, to

determine if these are consistent with a metal-mediate site-specific mechanism. Effects of DNA on autoxidation of the phenolic compounds should be determined, so as to learn how the electron transfer pathway is affected. Further research should examine mechanisms *in vivo* to clarify whether active oxygen species act similarly, and whether metal-mediated site-specific mechanisms are applicable.

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